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Role of Interferon (IFN)α in ‚Cocktails' for the generation of (leukemia-derived) dendritic cells (DC_{leu}) from blasts in blood from patients (pts) with acute myeloid leukemia (AML) and the induction of antileukemic reactions

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Content

1. Zusammenfassung

(Immuntherapeutische) Strategien zur Stabilisierung von Remissionen durch gezielte Eliminierung von im Körper verbliebenen AML-Blasten sind erforderlich. Dendritische Zellen (leukämischer Abstammung, DC_{leu}/DC), die aus myeloischen Blasten generiert werden können, verbessern die antileukämische T-Zell-Reaktivität und können zur "Installation" eines immunologischen Gedächtnisses führen. Es ist bekannt, dass mit IFNα DC-Methoden DC_{leu}/DC aus Blut von Patienten mit chronisch myeloischer Leukämie (CML) generiert werden können.

In dieser Studie wurde die Fähigkeit verschiedener Interferon Alpha (INFα) DC-Methoden im Vergleich zu anderen Standard DC-Methoden untersucht, DC_{leu} /DC aus Blasten in mononukleären (MNC) oder Vollblut (WB) - Fraktionen von AML Patienten (pts) herzustellen und diese durchflusszytometrisch zu quantifizieren. Nach DC_{leu}/DC-Stimulation in gemischten Lymphozytenkulturen (MLC) wurde das Potenzial von T-Zellen untersucht antileukämische Zytotoxizität hervorzubringen, die mit verschiedenen DC-Methoden erzielten Ergebnisse verglichen und mit DC_{leu}/DC-Anteilen korreliert.

1. **Generierung von DC_{leu}/DC**: a) "IFN-GIT" (enthält GM-CSF+IFNα+TNFα) produzierte DC erfolgreich (≥ 10% DC, ≥ 5% DC_{leu}/Zellsuspension) aus AML-MNC [WB] im Durchschnitt (ø) in 54 [56%], "MCM-Mimic" in ø76 [75%], "Picibanil" in ø83 [64%] und "Calcium-Ionophore" in ø42 [67%] der Fälle. Die Anteile der DC-Subtypen in MNC [WB] waren mit allen DC-Methoden vergleichbar.

b) IFNα-Kombinationen, die nur GM-CSF+IFNα oder nur IFNα enthielten, zeigten im Vergleich zu "IFN-GIT" eine geringe Effizienz DC_{leu}/DC aus MNC [WB] hervorzubringen.

2. Antileukämische Funktionalität: DC_{leu}/DC-stimulierte T-Zellen zeigten insgesamt eine verbesserte Leukämie-Zytotoxizität im Vergleich zu blastenstimulierten - oder unstimulierten T-Zellen. Die höchste Blastenvermehrung wurde nach Stimulation von T-Zellen mit "IFN-GIT" vorbehandelten MNC/WB-Zellen beobachtet. Insgesamt korrelierte die Wahrscheinlichkeit von Patienten in vivo auf eine Immuntherapie anzusprechen oder von DC_{leu}/DC-stimulierten T-Zellen in vitro eine Blastenlyse zu erzielen mit hohen Anteilen von DC_{leu}/DC nach DC-Kultur – unabhängig davon, welche DC-Generierungsmethode verwendet wurde.

3. Cytokin-Freisetzungsprofile: IL-6, IFN-γ und IL-2-Konzentrationen waren in DC-Kultur-Überständen (aus MNC/WB-Kulturen) mit "IFN-GIT" niedriger als in "MCM", "Pici" und "Ca" DC-Kulturüberständen.

Unsere Daten zeigen, dass 1) Kultur von WB die in-vivo-Situation von AML Patienten simuliert, 2) DC-Generation aus AML-MNC [WB] mit IFNα-haltigen und anderen DC-Methoden möglich ist, 3) erfolgreiche IFNα DC-Generation benötigt jedoch GM-CSF+IFNα+TNFα ("IFN-GIT"); "IFN-GIT" produziert allerdings weniger DC_{leu}/DC im Vergleich zu anderen (nicht IFNα) DC-Methoden, 4) T-Zellen, die mit "IFN-GIT"- (bzw anderen DC-Methoden) vorbehandelten (DC_{leu}/DC-haltigem) Blut stimuliert worden waren, erbrachten eine vergleichbare antileukämische Zytotoxizität, allerdings wurde bei Fällen ohne erreichte Blastenlyse nach "IFN-GIT"-Vorbehandlung eine stark erhöhte Blasten-Proliferation beobachtet.

Neue immuntherapeutische Strategien sind nötig um erreichte Remissionen in AML Patienten zu erhalten und residuale leukämische Blasten zu eliminieren. DC_{leu}, eventuell im Körper nach Gabe immunmodulatorisch wirksamen Substanzen generiert, könnten dazu geeignet sein T-Zellen zu reaktivieren, antileukämische Aktivität zu vermitteln und ein immunologisches Gedächtnis anzulegen.

2. Abstract

(Immunotherapeutic) strategies to stabilize remissions by specific elimination of residual AML-blasts are needed. DC_{leu}/DC generated from myeloid blasts improve antileukemic T-cell-reactivity and install T-cell memory. IFNα-DC-methods produce DC_{leu} from chronic myeloid leukemia patients' blood.

In this study various interferon alfa (INFα) containing DC-methods in comparison to other standard DC-methods were studied to produce DC_{leu} (evaluated by flowcytometry) from AML patients' blast-containing mononuclear (MNC) or whole blood (WB). After DC_{leu}/DC-stimulation in mixed lymphocyte cultures (MLC) T-cells' potential to gain antileukemic cytotoxicity was studied and correlated with different DC-methods and DC_{leu}/DC counts.

1. Generation of DC_{leu}/DC: a) "IFN-GIT" (containing GM-CSF+IFNα+TNFα) produced DC successfully (≥ 10% DC, ≥ 5% DC_{leu}/cells) from AML-MNC [WB] in ø54 [56%], "MCM-Mimic" in ø76 [75%], "Picibanil" in ø83 [64%] and "Calcium-ionophore" in ø42 [67%] of cases. Proportions of DC-subtypes in MNC [WB] were comparable with all DC-methods.

b) IFNα-combinations containing only GM-CSF+IFNα or only IFNα showed low efficiency to produce DC_{leu}/DC from MNC [WB] compared to "IFN-GIT".

2. Antileukemic functionality: DC_{leu}/DC-stimulated T-cells showed improved leukemia-cytotoxicity compared to blast- or unstimulated T-cells. The highest blast-proliferation (=insufficient T-cells) was seen with "IFN-GIT" DC-stimulated T-cells. Probability to respond to immunotherapy or to obtain blast-lysis of DC-stimulated T-cells correlated with high proportions of DC_{leu}/DC after DC-culture, independent of DC-generating methods.

3. Cytokine-release-profiles: Levels of IL-6, IFN-γ and IL-2 were lower in DC-culture-supernatants (from MNC/WB) with "IFN-GIT" compared to MCM", "Pici" and "Ca" DC-supernatants.

Our data shows that 1) **WB**-culture simulates AML patients' in-vivo-situation, 2) **DC-generation** is possible from AML-MNC [WB] with IFNα-containing and other DC-methods, 3) **successful** IFNα-DC-generation requires GM-CSF+IFNα+TNFα ("IFN-GIT"), however, "IFN-GIT" produces less DC_{leu}/DC compared to other (non IFNα) DC-methods, 4) **T-cells** stimulated with "IFN-GIT"- produced DC_{leu}/DC yielded comparable antileukemic cytotoxicity-however in cases without achieved blast-lysis an increased blast-proliferation was observed.

New immunotherapy strategies are needed to achieve remissions in AML patients and to eliminate residual leukemic blasts. DC_{leu}, possibly generated in the body after administration of immunomodulatory substances, may be able to reactivate T-cells, mediate antileukemic activity and establish an immunological memory.

3. Introduction

3.1. Leukemia

Leukemia diseases are divided into myeloid – and lymphocytic leukemia depending on the affected cells and their morphological and immunological properties. Both types of malignant diseases are further divided into acute or chronic subtypes.[1] Acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) are malignant diseases which appear in all age groups though ALL has a higher prevalence among the children. Chronic myeloid leukemia (CML) and chronic lymphocytic leukemia (CLL) can be seen as geriatric disorders.[1]

Secondary acute myeloid leukemia (sAML) is a term to antecedent hematological disorder (ADH) such as aplastic anemia (AA), myelodysplasia (MDS) and myeloproliferative disorder (MPN). It can also be therapy related occurrence after chemotherapy treatment on AML patients.[2]
The myelodysplastic syndrome (MDS) is a heterogeneous group of hematologic stem-cell malignancies. MDS-patients have a high tendency to develop AML. Even in 70% of high-risk and in 10% of low-risk MDS-patients AML occurs. In the past MDS was assumed to be a pre-level of AML.[3,4] Furthermore, chronic myelomonocytic leukemia (CMML) has many characteristics of MDS[5].

3.1.1. Acute myeloid leukemia (AML)

AML is a heterogenous neoplastic disorder arising from disturbed function of hematopoietic stem cells (HSC) or progenitor cells, which underlay chromosomal or molecular aberrations[6]. Abnormally accumulation of myeloid blast (≥ 20%) within bone marrow or peripheral blood and in other organs cause leukocytosis, anemia and thrombocytopenia in patients. Untreated patients die normally within a few months because of leading complications associated with bone mark failure.[7] AML can be seen as a malignant disease of elderly people. The average age of diagnosis is around 67 years. The high age of AML patients makes the therapy and prognose more challenging.[4,8] The patients can be divided into three risk groups (favourable – intermediate – adverse) concerning the analyzed cytogenetic profile[9]. The 5-years overall survival (OS) of patients' ≤ 60 years is less than 20%[8]. The etiology of AML is still not completely discovered and is still under the focus of leukemia research[6].

It is known that leukemogenesis and tumor progression are supported and mediated by vascular endothelial growth factors (VEGF), fibroblast growth factors and proteins of angiogenesis. Intensive expression of CXC chemokine ligand 4 (CXCR4) and activation of CXCR4-CXCR14 axis play an important role in hematopoiesis and involves development of AML.[10] Chromosomal aberrations are

found on average in 50-80% of AML patients. They are mostly detected by the elderly - and in secondary leukemia (sAML) patients. Chromosomal translocations are common cytogenetic aberrations. The deletion or loss of chromosomes mostly affects chromosomes 5, 7, Y and 9. Normally fusion of genes t (15;17), t (8; 21) or t (16;16) / inv (16) are involved.[6]

3.2. Immune system

The immune system is a complex machinery protecting the host from pathogenic microbes, toxic and allergenic substances and pre-cancerous and malignant cells. It is divided into the innate- and adaptive immune system.[11,12] The innate immune system comprises all the parts of immune reactivity encoded in germline genes. It includes complement system (protein molecule cascade), Toll-Like Receptors (TLRs) and phagocytic cells (DC, macrophages, natural killer (NK), mast cells, eosinophils, basophils and neutrophils). The innate immune system induces the immediate immune response against foreign invaders. It is the first line defense mechanism equipped with a short-term memory.[13] The adaptive immune system contains B- and T- lymphocytes which induce immune responses mediated by their antigen-specific receptors (TCR; T-cell receptors, Ig; B-lymphocyte immunoglobulin receptors) expressed on the surfaces of lymphocytes. B-cells mediate humoral functions of adaptive immune system. They produce antigen specific antibodies. Both B- and T-lymphocytes are responsible for long-term memory.[12,13]

Even though the immune system consists of two separate complexes – still they support each other and work together. Dendritic cells (DC) are antigen presenting cells (APC) and part of the innate immune system. They can be seen as a bridge in the function of immune system. DCs present processed foreign antigens on major histocompatibility complex (MHC I, MHC II) molecules to T-cells. Recognition of presented antigen by T-cell receptors (TCR) induce T-cell activation and elimination of foreign antigens.[12]

Not always the immune defense works properly and tumor cells are able to escape the host immune system which leads to relapse or progression of tumor disease. One explanation for escape mechanism could be defect in NK-cell cytotoxicity against tumor antigens.[14] The other possible reasons could be; impaired MHC-mediated antigen presentation or anergy function of T-cells through downregulation of stimulatory molecules of B-cell surface antigen (B7)[15] or increased secretion of immunoreceptor (Tim-3) which reduces T-cells IL-2 secretion. IL-2 is obligatory for further activation of NK- and T-cells.[16]

Cytokines play a central role in leukemogenesis, in persistence of AML-blasts and thus they have a central role in outcome of AML in patients[17]. Cytokines - released by blast cells or immunoreactive cells influence antileukemic reactions (Table 1).

Table 1. Cytokines and their functions

	Produced by -	Target	Induces -	References
1. Cytokines related to inflammations				
IL-6	TH2, phagocytes	B-cells, TH1 suppresses	BCDF	Sanches-Lopez, et al.[18]
IL-8 (CXCL)	monocytes, fibroblasts	neutrophils, T-cells	antitumor reaction of inflammatory cytokines, NAP-1, G-CSF	Schinke, et al.[19]
MCP-1 (CCL2)	macrophages, monocytes, DC	DC, monocytes, T-cells	chemoattractant, inflammation, infection	Driss, et al.[20]
2. Cytokines related to antitumor response				
IL-12p70	B-cells, DC, macrophages	CTL, NK, TH1	antitumor activity, IFNγ, proinflammatory cytokine	Curran, et al.[21]
TNF-α	TH1, TH2, CTL, Phagocytes	CTL, DC, T_{eff}, macrophages, monocytes, TH1	fever, inflammatory cytokines, sepsis	Lua, et al.[22]
IFN-γ	TH1, CTL, NK	B-cells, macrophages, NK, TH2	antitumor-,antiviral-, antitumor activity, MAF	Zhu, et al.[23]
IL-2	TH1, CTL	B-cells, CTL, NK	antitumor activity, TCGF, TNFα release	Zhu, et al.[23]
3. Cytokines related to antiinflammatory reaction				
IL-10	B-cells, CTL, TH1, TH2 macrophages,	B-cells, macrophages, T-cells, TH1	antiinflammatory cytokines, G-CSF, immunesuppression	Kozlowska, et al.[24]

DC dendritic cells; TH1 T-helper cells 1; TH2 T-helper cells 2; BCDF B-cell differentation factor; NAP-1 neutrophil-activating protein-1; G-CSF granulocyte-colony stimulating factor; CTL cytotoxic T-cells; T_{eff} effector T-cells; MAF macroprage-activating factor; TCGF T-cell growth factor

3.2.1. Dendritic cells

Dendritic cells (DCs) are well known antigen presenting cells (APC) of immune system which have a high capacity to activate adaptive immune system - induce T-cell stimulation and antitumor response against malignant diseases[25,26]. They are derived from hematopoietic precursor cell in bone marrow (BM) and are divided into tree subsets depending on their origin; plasmacytoid DC (pDC), conventional DC (cDC1/DC2) and monocyte derived DC (mDC). Conventional DCs have their origin from common myeloid progenitor (CMP) cells and plasmacytoid dendritic (pDC) cells from common lymphoid progenitors (CLP). For DC-differentiation and maturation DCs need growth

factors such as Fms-related tyrosine kinase-3 (Flt-3) and granulocyte macrophage colony stimulating factors (GM-CSF).[27] DC subsets have their own phenotype typical surface markers. Plasmacytoid DCs express markers such as; CD123, CD303, CD304 and NRP1/BDCA-1, conventional DC1 CD141 and BDCA-1, and conventional DC2 CD1c, CD11c and CD11b. After migration the periphery DCs have a lifespan of days to few months. DC subsets are specialized to respond to different pathogens and danger signals. Plasmacytoid DCs sense with their surface and endosomal tool-like-receptors 7/9 (TLR7/TLR9) and conventional DC1/DC2 with their tool-like-receptors 3/9 (TLR3/TLR9) antigen single-stand RNA and double-strand DNA and thus they response inducing release of interferon and other cytokines to activate immune system. DCs can be seen as a bridge between innate and adaptive immune system. After detection and procession of antigen DCs migrate to secondary lymphoid organs as mature-DC to present processed antigen to T-cells.[28]

3.3. DC-generation strategies

There are different ways to induce DC-generation. Firstly, DCs can be generated from monocytes ($CD14^+$) and progenitor cells ($CD34^+$). Those generated DCs need to be in lymphoid tissue while stimulation to gain strong capacity to activate T-cells. For generation and differentiation different settings of cytokines such as IL-3, GM-CSF, TNFα, and interferons are used. Secondly, DC-generation is possible by pulsing DCs with tumor specific antigens and tumor lysate. Thirdly, to use DCs antigen-processing and presenting strategies to process proteins with TAA (tumor associated antigen).[29,30] Advantage of this strategy is to induce antileukemic immune reactions against different antigens[30,31].

Fourthly, AML-blasts can be converted to leukemia-derived DC (DC_{leu}) via DC-generation process. In vitro based studies show that DC-generating methods based on selected combination of immunomodulatory substances can be used to generate functional leukemia-derived DCs (mature professional antigen presenting cells) from AML-blasts in mononuclear cell-fractions (MNC).[29,32-37] It is also approved that in vitro generation of leukemia-derived DC in whole blood (WB) medium is possible[38,39]. Whole blood as a DC-generation medium presents the whole cellular and soluble environment (Table 2) and thus it simulates the in vivo situation of body[38,39]. By using this DC-generation strategy all antigenic surface-markers of the myeloid blasts together with typical DC-antigen-markers are expressed on surface of generated leukemia-derived DCs[29,32,35,36,40,41].

Table 2. Advantages of DC-generation from whole blood and clinical relevance

Function	Comments on the clinical relevance / implementation in a clinical application
Simulation of physiological system	Transferability to the clinic
Capability to quantify and qualitatively characterize cells involved in responses: 1. proportions of AML-blasts 2. proportions of T-cell subtypes 3. proportions of DC_{leu}, DC-subtypes	Conclusion on biological mechanisms / reaction profiles: 1. (potentially) increased blast-proportions can be quantified 2. T_{reg}, CD4 / CD8 profiles 3. In vivo, DCs in the blood are difficult to quantify because of their migration into the tissue.
Analysis of the microenvironment on immunostimulating and -inhibiting influences possible	Detection of escape or antileukemic mechanisms and possible interactions, development of appropriate therapeutic strategies
Functional tests possible	Antileukemic responses quantifiable: decrease / increase of blasts, effector-cell profiles measurable: proliferation, subtypes

DC dendritic cell; DC_{leu} leukemia-derived DC; T_{reg} regulatory T-cell

3.4. Survey of interferon

Interferons are proteins belonging to group of cytokines which are further divided into three categories; interferon alfa (IFNα), interferon beta (IFNβ) and interferon gamma (IFNγ). They are separated into two types; interferon-type I (IFNα, IFNβ) and type II (IFNγ).[42-44] Several immune cells (NKs, fibroblasts, B- and T-cells) produce interferon-type I. Cytokines such as IL-1, IL-2, IL-3, TNFα and GM-CSF regulates production of interferons and they are tightly involved in immune reactions of immune system. Interferons are known to induce innate immune response against invaded virus or other foreign antigens. They can be seen as key players in regulation of major histocompatibility complexes (MHC-I/II). Upregulation of MHC-I induces T-cell cytotoxicity activity against viral antigens. Via MHC-II T-cell helper release higher levels of cytokines which attract other immune cells. Interferon release is triggered via antigen binding to membrane Toll-like receptors (TORs) or cytoplasmic receptors.[42]

By using the cloning technology production of high amounts of interferon-α has been made possible. For the clinical use interferon-α is produced using recombinant (r) DNA technology[43]. In this study for DC-generation interferon-α, IFN-α2b (Intron-A) was used.

Interferon-α type-I is used as therapeutic agent in many hematological diseases such as hairy cell leukemia, B- and T-cell lymphoma, chronic myeloid leukemia, multiple myeloma and in solid tumor diseases such as renal cell carcinoma, melanoma and kaposi's sarcoma. Before Imatinib (STI 571, Gleevec), a selective inhibitor of the BCR-ABL Tyrosin Kinase was available CML patients were treated with Interferon-α (IFNα).[45] Further it plays a central role in therapy of hepatitis-C and -B[42,44]. Therapeutic use of interferon-α is based on its' wide property to elicit antiviral activity, influence cellular metabolism and provoke antitumor activity[43]. It doesn't influence directly malignant cells. It influences immune reactive cells and thus regulates tumor cell proliferation, autophagy function and apoptosis.[44]

3.5. Aims of this study

Main intentions of this study were:

1) to culture blast-rich MNC-samples from AML patients (compared to some CML cases) with several DC-generating media MCM-Mimic ("MCM"), Picibanil ("Pici"), Calciumionophore ("Ca") and in addition with media containing IFNα ("IFN"), in order to study the influence of IFNα-containing methods on the generation of DC (-subtypes) compared to other DC-methods,

2) to culture blast-rich WB-samples (containing the patients' soluble microenvironment / cytokines) from AML patients with these methods,

3) to investigate the influence of different DC-methods (with a special focus on IFNα-methods) on DC-subtype compositions, on cytokine profiles (MNC/WB) and on T-cells' antileukemic activity after stimulation with DC-compared to blast-containing MNC,

4) to correlate findings before and after stimulation of T-cells with DC (generated from IFNα-methods compared to other methods) in different settings in a context of released cytokines with the antileukemic ex vivo or in vivo reactivity of stimulated T-cells.

4. Material and Methods

Some experiments (DC-culture, Cytokine analyses) were performed by cand. Dr. med. Dent. Yvonne Vokac and cand. Dr. med. Dent. Diana Deen, both members of our group.

Clinical data of the patients and diagnostic reports were provided by the leukemia laboratories of the Med III, University Hospital Großhadern, Munich, the Department for Hematology and Oncology, Municipal Hospital, Oldenburg and Department for Hematopoetic Transplantation, Municipal Hospital, Augsburg.

4.1. Sample Collection

Heparinized WB samples were taken from patients in acute phase of AML and CML. MNC were prepared from heparinized WB by density-gradient centrifugation (Ficoll-Hypaque, Biochrom, Berlin, Germany), washed and finally suspended in phosphate buffered saline (PBS, Biochrom, Berlin, Germany) without Ca^{2+} and Mg^{2+}.

4.2. Patients' characteristics

Samples for DC-generation (from MNC and WB) were collected in active stages of the disease from AML (n=56), one MDS-CMML (n=1) and from CML (n=3) patients after obtaining informed consent (Table 3). The average age of the patients was 49 years (range 21-85 y). The female to male ratio was 1:1.28.

Diagnosis of AML and CML cases was based on the French-American-British (FAB) classification[46]. AML patients presented with different FAB-subtypes: undifferentiated acute myeloblastic leukemia (M0: n=5), acute myeloblastic leukemia with minimal maturation (M1: n=11), immature granulocytic leukemia (M2: n=12), acute promyelocytic leukemia (M3: n=1), acute myelomonocytic leukemia (M4: n=7), acute monocytic leukemia (M5: n=7) and erythroid leukemia (M6: n=3). Three CML patients in blast-rich stages of the disease were included. As a control group served healthy probands (n=9).

"**Response"** to initiated immunotherapy was defined for pts who received a complete remission (CR) in the following 3 months after allogeneic SCT/DLI therapy that persisted for at least 9 months, remaining pts were "**non responders"** (Table 3).

AML and MDS-CMML patients presented with on average 7% B-cells, 14% T-cells, 8% NK-cells, 16% Monocytes, 56% blasts. CML patients in blast-crisis presented with on average 3% B-cells, 8% T-cells, 4% NK-cells, 21% Monocytes, 33% blasts as evaluated by flow cytometry (see below). In cases with aberrant expression of $CD19^+$, $CD56^+$, $CD14^+$ on blasts B-, NK-cells or monocytes could not be quantified.

Table 3. Patients' and samples' characteristics

Patient No.	Age/ Gender	Diagnosis AML/CML/ MDS-CMML	FAB-type	Stage	IC Bla %	Blast phenotype (CD)	Response to initiated immunotherapy	T-cell source and the stage of disease at T-cell harvest
763 *	38/f	AML	M0	rel.a.SCT	51	**34**,33,117,65	x	rel.a.SCT
1280 *	26/m	AML	M0	dgn.	88	**34**,13,117,33	NR	dgn.,autologous
569 M *	49/f	AML	M0	pers.	11	15,33,13,**34**,19	R	nd.
853 *	33/m	AML	M0	rel.a.SCT	50	34,**117**,33,56	NR	nd.
1172 *	24/m	AML	M0	dgn.	92	**34**,117,13,33	x	nd.
480 M *	39/f	AML	M1	rel.	80	34,**117**,13,33,56	x	nd.
761 M *	38/f	AML	M1	pers.	21	34,**117**,15,65,2,7	NR	nd.
824 *	70/m	AML	M1	dgn.	68	7,13,34,**117**	x	nd.
850 *	58/m	AML	M1	pers.	80	13,33,34,**117**	R	nd.
1011 **	57/f	AML	M1	rel.	88	117,33,**34**,13,65	R	nd.
1050 **	32/f	sAML	M1	dgn.	90	13,33,**117**,14	x	nd.
1127 **	61/f	AML	M1	rel.a.SCT	46	**34**,117,33,13	NR	nd.
1138 **	24/m	AML	M1	rel.a.SCT	50	33,**34**,117,4,56,65,14	NR	nd.
1144 **	32/m	AML	M1	dgn.	66	**34**,117,13,56,19	R	nd.
1265 **	47/f	AML	M1	rel.	45	33,**34**,117	x	nd.
1283 *	43/f	AML	M1	rel.a.SCT	24	117,33,15,64,34,**56**	x	nd.
1243 *	34/m	AML	M2	rel.	32	**34**,33,117,13,2	x	rel.,autologous
243 M *	61/m	sAML	M2	rel.	84	33, 34, **117**	x	nd.
427 M *	32/m	AML	M2	rel.	43	33,65,64,**117**	NR	nd.
820 *	45/m	AML	M2	rel.	50	33,34,13,**117**	R	nd.
821 *	56/f	AML	M2	rel.	88	33,13,15,65,**117**,34	NR	nd.
837 *	69/m	AML	M2	dgn.	39	13,34,**117**,33,15	x	nd.
914 *	66/m	sAML	M2	rel.	43	**34**,33,15,117,14,64	x	nd.
984 *	61/m	sAML	M2	rel.	42	13,33,34,**117**	x	nd.
1024 **	39/m	AML	M2	pers.rel.	80	33,**34**,117	NR	nd.
1080 **	37/f	AML	M2	rel.a.SCT	34	4,**34**,117,33,13	x	nd.
1123 **	70/m	AML	M2	rel.	47	**34**,117,33,13	x	nd.
1203 *	69/m	sAML	M2	dgn.	90	33,34,**117**	x	nd.
851 *	56/m	AML	M3	dgn.	73	34,**117**,33,64,2	x	nd.

1251 *	82/m	AML	M4	dgn.	67	**15**,13,33,64,7,1	x	dgn.,autologous
1285 *	56/f	AML	M4	dgn.	50	13,33,**34**,117	x	dgn.,autologous
1330 **	73/f	sAML	M4	rel.	79	33, **34**	x	rel.,autologous
458 *	47/f	AML	M4	rel.	15	13,34,**117**,33	R	nd.
855 *	68/m	sAML	M4	dgn.	37	13,33,34,65,**117**	x	nd.
1017 **	67/m	AML	M4	pers.rel.	34	33,**34**,117	x	nd.
755 *	38/f	AML	M4	rel.	44	**34**,117,33,13,15	R	nd.
799 *	49/f	AML	M4	dgn.	60	13,15,33,56,**65**	R	nd.
1201 *	60/f	AML	M5	dgn.	49	33,15,**65**,13,14,4,56	x	CR,autologous
1245 *	48/f	AML	M5	dgn.	57	56,**15**,65,33	x	dgn.,autologous
1263 *	40/m	AML	M5a	rel.a.SCT	18	117,33,19,**34**	NR	rel.a.SCT
1286 *	21/m	AML	M5	rel.a.SCT	35	34,**117**,33	x	CR,a.SCT
453 M *	36/m	sAML	M5	pers.a.SCT	70	13,33,**65**,4,14,15	x	nd.
793 *	46/f	AML	M5	pers.	9	15,33,56,65,**117**	x	nd.
948 *	42/f	AML	M5	dgn.	42	13,33,34,**65**,117	R	nd.
481 M *	39/f	AML	M6	pers.	50	33,**34**,13,15,117	x	nd.
748 M *	45/m	sAML	M6	pers.	50	33,**34**,65,117	x	nd.
1002 *	38/m	AML	M6	pers.rel.a.SCT	50	4,**117**,33,34,13,65	R	nd.
1226 *	69/m	sAML	nd	dgn.	65	**117**,34,33	x	dgn.,autologous
1277 *	45/f	sAML	nd	rel.a.SCT	46	**34**,117,65,15,13,56	x	rel.a.SCT
1344 **	60/m	sAML	nd	dgn.	80	13,**117**,33,34	x	dgn.,autologous
1345 **	85/m	AML	nd	dgn.	97	**34**,33,13,117	x	dgn.,autologous
887 *	59/f	MDS-CMML	nd	pers.	8	33,34,**117**,14,64	x	nd.
1056 **	27/m	sAML	nd	dgn.	42	13,33,**117**,34,15	x	nd.
1143 **	46/f	AML	nd	rel.a.SCT	75	**117**,34,33,13,15,56	x	nd.
1171 *	40/m	sAML	nd	pers.	90	**34**,117,33,13	R	nd.
935 *	45/f	sAML	nd	dgn.	40	**34**,117,65,13,15,56	NR	nd.
954 *	72/f	sAML	nd	pers.	43	**34**,33,117,15	NR	nd.
1228 *	67/m	CML	nd	dgn./BC	80	33**,34,**117,56	x	nd.
1267 *	46/m	CML	nd	dgn./AP	11	15,33,**34**,117,19,56	x	nd.
1275 *	61/f	CML	nd	dgn./CP	8	**34**, 117	R	nd.

AML acute myeloid leukemia; sAML secondary AML; CML chronic myeloid leukemia; MDS-CMML myelodysplastic syndrome-chronic myelomonocytic leukemia; CR complete remission; DC dendritic cells; dgn diagnosis; rel relapse; pers persisting diease; CP chronic phase CML; AP accelerated phase CML; BC blast crisis CML; SCT stem cell transplantation; IC Bla immunocytologically detected blasts; nd not done; x pts without immunotherapy or with criteria not fulfilled; R responder; NR non-responder; bold letters indicate blast markers usef for DC_{leu}-evaluation;* blast-containing MNC used as source for DC-generation, ** blast-containing MNC and /or WB used as source for DC-generation.

4.3. MNC-DC and WB-DC generation Strategy

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

DCs were cultured in 12-multiwell tissue culture plates from MNC and WB in X-vivo15 (Lonza, Belgum) media supplemented with different immunomodulatory factors (Table 4)[29,33-35.47]. MNC- and heparinized WB-samples were diluted to a total volume of 2ml with X-vivo15 fetal calf serum free medium (FCS). The cell counts were adjusted adequately to MNC and WB media and cultured accordingly. After culture supernatants were harvested and used for cytokine analyses. Part of the cells after culture were used for FACS-analyses to evaluate efficient DC-generation, the remaining cells were used for MLC.

Table 4. DC-generating Methods
DC-generating methods; medium, stimulating substances, mode of action and culture times are given

	DC-generating Method/Medium	DC differentiation-stimulating Substances	Mode of Action	Culture Time	References
Standard methods	**"MCM" ***	GM-CSF, IL-4, TNFα, IL-1ß, IL-6, PGE2, FL	Cytokine based DC differentiation, PGE2 increases CCR7-expression and improves migration	10-14 d	Kremser, et al.[35] Dreyßig, et al.[29]
	"Pici" *	GM-CSF, IL-4, lysate from Streptococcus pyogenes, PGE2	Bacterial lysat and PGE2 stimulate DC-differentiation	9-11 d	Kremser, et al.[35] Dreyßig, et al.[29]
	"Ca" *	IL-4, A23187	Bypass of cytokine-driven DC-differentiation	3-4 d	Kremser, et al.[35] Dreyßig, et al.[29]
	"IFN-GIT" *	GM-CSF, IFNα, TNFα	Cytokine based DC-differentiation	10-12 d	Chen, et al.[33] Ansprenger, et al.[47]
IFNα- containing methods	**"IFN-GI" ***	GM-CSF, IFNα	Cytokine based DC-differentiation	10-12 d	Cortes, et al.[34] Chen, et al.[33]
	"IFN-I" *	IFNα	Cytokine based DC-differentiation	10-12 d	Cortes, et al.[34]
	"IFN-GITZ" *	GM-CSF, IFNα, TNFα, Zylexis	Cytokine based DC-differentiation	10-12 d	Ansprenger, et al.[47]

DC dendritic cells; d Days; GM-CSF granulocyte macrophage-colony stimulating factor; IFNα interferon alfa; TNFα tumor necrosis factor alfa; FL FLT3 ligand; IL-4 interleukin 4; IL-β interleukin β, IL-6 interleukin 6; PGE_2 prostaglandin E2; * blast-containing MNC and/or WB used as source for DC-generation.

4.3.1. Standard – "MCM-Mimic" ("MCM")

The generation of DCs from 2.5 x 10^6 MNC/mL or 2-5 x 10^6 WB cells/mL was performed in "MCM" medium containing 800 U/mL granulocyte macrophage – colony stimulating factor (GM-CSF, Essex Pharma, Munich, Germany), 500 U/mL Interleukin IL-4 (Cell Concepts, Umkirch, Germany), and 40 ng/mL FLT3-Ligand (FL, PromoCell, Heidelberg, Germany). After 3 - 5 days the same amount of the cytokines was added into the culture. Half medium exchange was done on day 5 - 7. Concentrations were adjusted to 150 ng/mL IL-6 (Cell Concepts), 5 ng/mL (10 ng/mL WB) IL-1β (Cell Concepts), 1µg/mL Prostaglandin E2 (PGE_2, Pfizer, Vienna, Austria), and 200 U/mL (10 ng/mL WB) Tumor necrosis factor (TNF-α, Cell Concepts). Moreover 500 U/mL IL-4, 40 ng/mL FL and 800 U/mL GM-CSF were added. After 9 - 12 days, cells were harvested and used for subsequent experiments.[29,32,35]

4.3.2. Standard - "Picibanil" ("Pici")

The generation of DCs with "Pici" was performed from 1 - 1.25 x 10^6 MNC/mL or 2-5 x 10^6 WB cells/mL in the presence of 500 U/mL (400 U/mL WB) GM-CSF and 250 U/mL IL-4. After 3-4 days the same amount of same cytokines were added into WB-culture. After 5 - 7 days in culture, 10 µL/mL OK - 432 a lysis product of Streptococcus pyogenes, which has nonspecific immunomodulatory effect (Chugai Pharmaceuticals, Kamakura City, Japan) and 1µg/mL PGE_2 were added. Cells were harvested after 8 - 10 days in culture and used for subsequent experiments.[29,32,35]

4.3.3. Standard - "Calciumionophore" ("Ca")

DCs were generated from 7 x 10^5 MNC/mL or 2-5 x 10^6 WB cells/mL in the presence of 375 ng/mL "Ca" A23187 Calcimycin, a Calciumionophore and antibiotic (Sigma-Aldrich, Thum, Germany) and 250 U/mL IL-4. Cells were harvested after 3 - 4 days and used for subsequent experiments.[29,32,35]

4.3.4. Standard - Interferon-α ("IFN-GIT") - IFNα, GM-CSF and TNF-α

DCs were generated from 2-2.5 x 10^6 MNC/mL or 2-5 x 10^6 WB cells/mL in "IFN-GIT" medium in the presence of 800 U/mL GM-CSF and 500 U/mL IFNα (Essex Pharma, Munich, Germany). After 2 - 4 days of culture the same amount of cytokines was added. After 7 - 9 days the same procedure was repeated and in addition 10 ng/mL TNF-α added into the culture. Cells were harvested after 8 - 11 days and used for subsequent experiments.[33,47]

With the attempt to improve the T-cell stimulating capacity of DC we cocultured in some parallel experiments MNC-DC with inactivated Parapox ovis Virus (Pfizer, AH, Inc. formerly Baypamun from Bayer, Inc.) in addition to the "IFN-GIT" method. Lyophilized products were resolved in aqua ad injectionem as recommended, resulting in an end-concentration of 1.25 mg/ml (2.3 IFN-units) in culture.[47]

4.3.5. "IFN-GI" - IFNα and GM-CSF

DCs were generated from 2-2.5 x 10^6 MNC/mL or 2-5 x 10^6 WB cells/mL in "IFN-GI" medium in the presence of 800 U/mL (5µL/mL WB) GM-CSF and 500 U/mL (5µL/mL WB) IFNα. After 2 - 4 and 7 - 8 days of culture the same amount of cytokines was added. Cells were harvested after 8 - 11 days and used for subsequent experiments.[33,34]

4.3.6. "IFN" - only IFNα

DCs were generated from 2-2.5 x 10^6 MNC/mL or 2-5 x 10^6 WB cells/mL in "IFN-I" medium in the presence of 500 U/mL (5µL/mL WB) IFNα. After 2 - 4 and 7 - 8 days of culture the same amount of IFNα was added into the culture. Cells were harvested after 8 - 11 days and used for subsequent experiments.[34]

4.4. Cell characterization by Flow cytometry

With a panel of mouse monoclonal antibodies (moAbs) directly conjucated with fluorescein isothiocyanate (FITC[a]), phycoerythrin (PE[b]), tandem Cy7-PE conjugation (PC7[c]) or allophycocyanin (APC[d]) flow cytometric analyses were carried out to evaluate and quantify amounts and phenotypes of leukemic cells, B-, T- and natural killer (NK) cells and DC in the MNC- and WB-samples. Labeled antibodies were provided by Becton Dickinson (Heidelberg, Germany [CD1a[b], CD1b[a], CD14[c], CD15[d], CD71[c], CD206[d], 7AAD[c] and CCR7[c]]), Immunotec/Beckmann Coulter (Krefeld, Germany [CD1a[b], CD3[a], CD19[c], CD33[d], CD56[b], CD80[b], CD117[b,d], CD206[b], CD34[a,c],CD65[a] and CD83[a]]) and Caltag (Hamburg, Germany [CD1a[b],CD13[c],CD34[d]and CD86[a]]).

MNC, WB or cultured cells were suspended in phosphate buffered saline (PBS) with 20% fetal calf serum (Biochrome) and incubated with specific moAbs. Isotype controls according to manufacturers' instructions as described earlier using a Calibur Flow Cytometer (Becton Dickinson, BD) and Cell Quest data software (BD) were performed.[29,32,35,47] For analysis and quantification of lymphocytes, monocytes and leukemic cells before culture, the total MNC - and WB - fractions were gated. Composition of cells before or after conversion to DC was analyzed using patient-specific "**blast**"-staining antibodies in combination with "**DC**"-staining antibodies. After culture several DC-subtypes (Table 5) were quantified and calculated as described[32,36,48,49]: "migratory mature DC" (DC coexpressing the migration marker CCR7), viable DC (DC negative for 7AAD), leukemia-derived DC (DC coexpressing both blast and DC markers, DC_{leu}). DC_{leu} were quantified in the DC-fraction (DC_{leu}/DC), the blast-fraction (blasts converted to DC) as well as in the total MNC- or WB-fraction (DC_{leu}/cells [MNC or WB]). After culture only samples with ≥10% blasts in MNC or WB qualified for DC-subtype analysis. We defined DC- generation as successful if at least ≥10% DC and ≥5% DC_{leu}/cells (MNC or WB) suspension could be evaluated after culture[29,35]. For quantification of migratory mature $CCR7^+$ DC in the DC-fraction of at least 10% DC were postulated. We also studied induced blast-proliferation ($CD71^+$ blasts) in the blast population before and after culture in some (n=8) "IFN-I" WB-cultures and in "WB-controls" without any immunomodulatory substances. Only WB-samples with >10% $CD71^+$ proliferating blasts before culture were included in proliferation analysis.

Table 5. Cellular subtypes as evaluated by flow cytometry
Name of cellular subtypes, abbreviations, surface-marker-profiles, presuppositions and criteria for analysis are given.

Name of the subtype	Abbreviation	Surface - Marker - Profile	Presuppositions and criteria for analysis	Reference
Blasts in MNC or WB				
Blasts	Bla / cells	Bla^+(e.g. CD15, 33, 34, 65, 117)	≥ 10 % Bla / cells	Schmetzer, et al.[36]
DCs in MNC or WB				
DC	DC / cells	DC^+(e.g. CD1b, 80, 86, 206) *	≥ 10 % DC / cells	
leukemia-derived DC	DC_{leu} / cells	DC^+ Bla^+	≥ 5% DC_{leu} / cells, ≥10 % DC, ≥ 10 % Bla	Schmetzer, et al.[36]
blasts converted to DC in Blast-fraction	DC_{leu} / bla	DC^+ Bla^+	≥ 5% DC_{leu} / cells, ≥ 10 % DC, ≥ 10 % Bla	
DC_{leu} in DC-fraction	DC_{leu} / DC	DC^+ Bla^+	≥ 5% DC_{leu} / cells, ≥ 10 % DC, ≥ 10 % Bla	
migratory mature DC in DC-fraction	DC_{migr} / DC	$CCR7^+$,DC^+	≥ 10 % DC / cells	Grabrucker, et al.[32] Sanchez-Sanchez, et al.[48]
viable DC in DC-fraction	DC_{viable} / DC	$7AAD^-$, DC^+	≥ 10 % DC / cells	Grabrucker, et al.[32] Philpott, et al.[49]
T-cells in MNC or WB				
CD3+ T-cells	CD3+ T-cells	$CD3^+$	total T-cell population	Vogt, et al.[37] Sallusto, et al.[51]

* DC marker, which wasn´t expressed on naive blasts but which achieved the highest expression rate in the suspension after culture; DC_{leu} leukemia-derived DC in different cell-fractions (in blasts-, in DCs-, in MNC- or in WB-fractions) after culture.

Further we developed a ranking - strategy to subdivide and analyze the whole data obtained according to DC-subtype qualities. Two different approaches were applied: ranking 1) according to the best achieved DC-subtype values compared to at least two other parallelly tested standard DC-methods (“MCM”, ”Pici”, ”Ca”, ”IFN-GIT”), 2) applying an independent criteria-catalogue subdividing results in five different DC-generating categories (excellent, excellent-high, high, sufficient and not sufficient [Table 6]). In both ranking categories presuppositions as given above were applied.

Table 6. Criteria-catalogue to rank quantity and quality of DC-subtypes after DC-generation in AML-MNC or in AML-WB-samples according to different DC-generating categories (ranking2)

Ranking category	Presuppositions of cell-subtype-proportions for ranking (MNC or WB)			
	%DC/cells	$\%DC_{leu}$/cells	$\%DC_{leu}$/DC	$\%DC_{migr}$/DC *
excellent	> 25	> 15	> 25	> 30
excellent-high	25 - 20	15 - 12	25 - 16	30 - 21
high	19 - 14	11 - 8	15 - 11	20 - 15
sufficient	13 - 10	7 - 5	10 - 5	14 - 10
not sufficient	< 10	< 5	< 5	< 10

* migratory mature DCs were not available in all cell samples

Ranking of DC-subtype-qualities and -quantities as performed by defining "the best" and "second best" DC-generating method compared to at least two other parallelly tested DC-methods. Moreover a ranking-strategy was defined applying an independent criteria-catalogue based on proportions of DC-subtypes (DC/cells: DC generated, DC_{leu}/cells: leukemia-derived DC generated in cellsuspension, DC_{leu}/DC: DC_{leu} in DC-fraction, DC_{migr}:migratory mature DC in DC-fraction) found after culture.

Moreover three tested IFNα-containing WB-DC-Methods (“IFN-GIT”, ”IFN-GI”, ”IFN-I”) were compared and ranked as described above in order to evaluate categories and the best DC-subtype values gained.

4.5. Mixed lymphocyte cultures (MLC) from MNC and WB

$CD3^+$ T-cells (“effector” T-cells) were positively selected (Milteney Biotech, Bergisch-Gladbach, Germany) as described earlier[37,50,51] from AML-MNC from patients at first diagnosis (n=7), in remission (n=1), at relapse (n=2) or after allogenic SCT (n=4, Table 3). ”Èffector” T-cells ($1x10^6$ T-cells/mL) were cocultured with “stimulator cells”: irradiated (20 Gy) AML blast-containing MNC ($25x10^3$ “MNC”/mL) and in parallel with irradiated DC_{leu}-containing MNC-DC ($25x10^3$ “MNC-DC”/mL) as described earlier[29,32,35,40]. In analogy “effector“ T-cells were cocultured with AML-blast-containing WB ($25x10^3$ “WB” cells/mL) and DC_{leu}-containing WB-DC ($25x10^3$ “WB-DC”/mL). WB-samples were not irradiated. Stimulation conditions and culture times were comparable with AML-MNC-MLC. After MLC fluorolysis assays were carried out as described below.[29,32,35,40]

4.6. Cytotoxicity fluorolysis assay

Using a Fluorolysis Assay the antileukemic activity of “effector” T-cells was measured by counting viable (blast) target-cells labeled with specific fluorochrome conjugated antibodies before and after “effector” T-cell (E) contact. “MNC-DC” [”WB-DC”] or “MNC” [”WB”] stimulated T-cells from AML patients (=autologous) or from patients after SCT and unprimed T-cells as a control were cocultured in 1.5mL Eppendorf tubes with thawn target blasts (T) as described.[29,32,35,40]

4.7. Cytometric bead array – CBA

For evaluation of cytokines (IL-2, IL-6, IL-10, IL-12p70, TNF-α, IFN-γ)[18,21-24] and chemokines (CXCL8 (=IL-8), CCL2 (=MCP-1))[19,20] serum samples or supernatants collected from DC-cultures (“WB-DC”, “MNC-DC”) the BD TH1/TH2-CBA-kit II (Becton Dickson, BD, Biosciences) were used (Table 1). Detection limits were given by the company (BD) as follows: IL-2 5.49 pg/ml, IL-6 8.98 pg/ml, IL-8 9.03 pg/ml, IL-10 8.63 pg/ml, IL12p70 8.93 pg/ml, MCP-1 9.37 pg/ml, TNF-α 7.22 pg/ml and IFN-γ 8.47 pg/ml. Each 500 µl of serum and supernatants, were stored at -80°C until the day of analysis. Positive and negative controls of the standard serial dilutions of the respective cytokines and chemokines were used.

20 µl mixed human TH1/TH2 Cytokine capture beads, 20 µl PE Detection Reagent and 20 µl of each cytokine samples were incubated for three hours at room temperature in 96-well assay plates (BD Falcon). The samples were washed after incubation with 220 µl Wash Buffer, thereafter 96-well plates were centrifuged at 200g for five minutes. Before sample analysis with a LSRII Flow cytometer (BD Biosciences, Germany) the bead pellets were resuspended with Wash Buffer.

4.8. Statistical methods

Data was presented as mean, median, range and standard deviations. Two-tailed t-, Anova- or Chi-Quadrat-tests were conducted with a personal computer using Excel 2010/16 (Microsoft). For CBA data evaluation a special software (cytokine Array™ FCAP 3.0, BD) was used. Differences were considered as “highly significant” (***) in cases with p-values < 0.005, as “significant” (**) with p-values between 0.005 - 0.05 and as “tendentially significant” (*) in cases with p-values between 0.05 and 0.1.

5. Results

The focus of our work was to study the DC_{leu}-generation capability of various IFNα-containing - compared to other DC-generating media ("MCM", "Pici" or "Ca") from blast-rich MNC- (n=54) and WB-samples (n=15) from AML patients. We applied a ranking strategy to evaluate the quality and quantity of the generated DCs. We also studied DC-generation from blast-rich CML-MNC-samples (n=3). The WB-culture strategy was developed by us – thereby simulating the most physiological situation. Moreover, we investigated the influence of these DC_{leu} on T-cells' antileukemic activity and the soluble microenvironment after stimulation of T-cells in MLC and correlated findings with the antileukemic activity of T-cells.

Our first approach was to study DC-generation data from mononuclear cell samples and later WB from AML patients and healthy probands. We evaluated the whole DC-generation data by using crated DC-subtype proportions for successful DC-generation. After culture only samples with ≥10% blasts in MNC or WB qualified for DC-subtype analysis. We defined DC- generation as successful if at least ≥10% DC and ≥5% DC_{leu}/cells (MNC or WB) suspension could be evaluated after culture. In this part we used only data of successful DC-generation for further analysis (Figure 1).

5.1. DC-generation from leukemic AML- and healthy-samples

5.1.1. DC-generation from AML-MNC and healthy-MNC was less effective with "IFN-GIT" compared to standard DC-generating methods, however with comparable DC-subtype compositions found in "successful cases"'

We compared proportions of AML-MNC-DC-subtypes generated with "IFN-GIT" compared to three other standard DC-methods. "MCM" was successful in 41 of 54 (76%) cases, "Pici" in 43 of 52 (83%), "Ca" in 21 of 50 (42%) and "IFN-GIT" in 28 of 52 (54%) cases. A successful generation of DC_{leu}/DC was however possible in every given case with at least one of the DC-methods. In cases with successful DC-generation proportions of DC-subtypes obtained with "IFN-GIT" were comparable to the other three standard methods: proportion of DC/cells obtained with "IFN-GIT" was Ø 27% (range 10-69%) vs. "MCM" Ø 26% (range10-61%), vs. "Pici" Ø 24% (range 10-64%) and vs. "Ca" Ø 30% (range 10-74%). Moreover, no significant differences were found between the four methods with respect to DC-subtypes (e.g. DC_{leu}/ DC, DC_{viable}/DC, DC_{migr}/DC, DC_{leu}/bla, [Figure 1]).

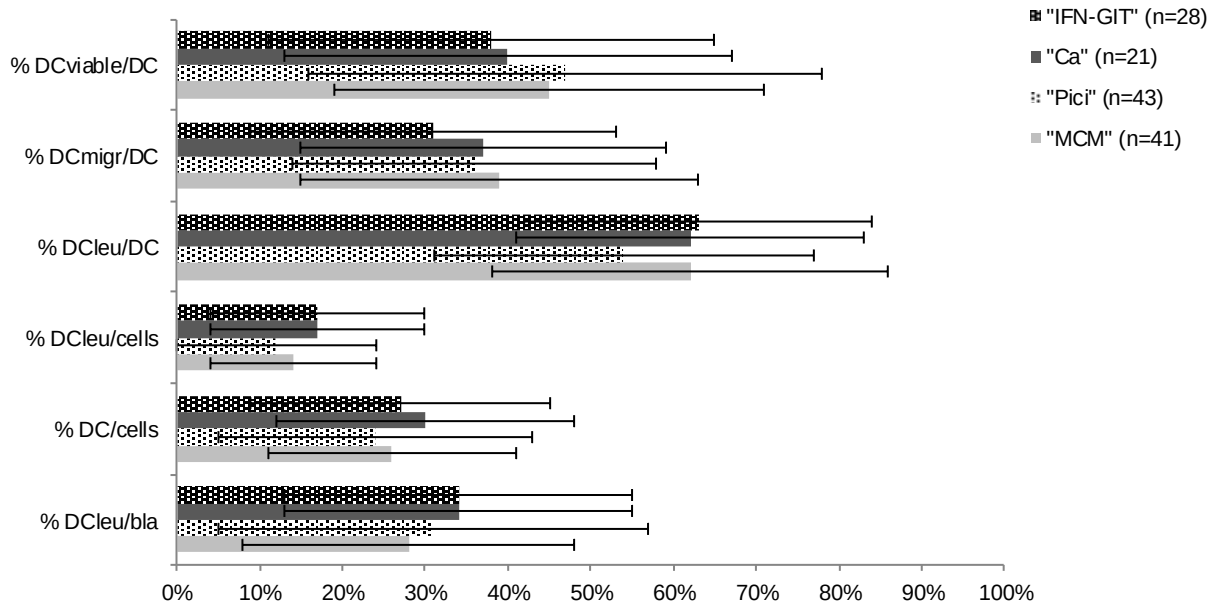


Figure 1. DC-subtypes generated from AML-MNC. Average proportions of DC-subtypes and standard-deviations are presented. Only cases with successful DC-generation (≥ 10% DC and ≥ 5% DC_{leu} in cell-suspensions) are given.

The generation of DC from healthy-MNC with “MCM” was successful in 9 of 9 (100%) cases, “Pici” in 7 of 9 (78%), “Ca” in 7 of 9 (78%) and “IFN-GIT” in 6 of 8 (75%) cases. The total amount of DC/cells in healthy-MNC-samples after generation with “IFN-GIT” was Ø 25% (range 14-40%) vs. “MCM” Ø 28% (range10-52%), vs. “Pici” Ø 25% (range 10-41%) and vs. “Ca” Ø 22% (range 13-70%). No significant differences were found either between the four standard methods with respect to DC-subtypes (e.g. DC_{viable}/DC, DC_{migr}/DC, monocytes converted to DC, data not shown).

A parallel comparison of various IFNα-containing methods (“IFN-GIT”, “IFN-GI”, ”IFN-I”) with AML-MNC-samples (n=3) showed, that DC-generation was successful only in 1 of 3 (33%) cases with “IFN-GI” or “IFN-I” cultures. “IFN-GIT” was successful in 2 of 3 (67%) cases. “MCM” in all three cases (data not shown).

A parallel comparison of “IFN-GIT” and “IFN-GITZ” with “MCM” of AML-MNC (n=3) showed that “IFN-GIT” and “IFN-GITZ” were successful in 1 of 3 (33%) cases, “MCM” in 3 of 3 (100%) cases (data not shown).

In summary, we show that “IFN-GIT” was a successful DC-generating method (≥10% DC/cells, ≥5% DC_{leu}/cells [MNC]) in ø 54% of AML cases, whereas “MCM” was successful in ø 76% and “Pici” in ø 83% of cases. ø amounts of DC-subtypes generated, however were comparable in cases of successful DC-generation with all methods. Other IFNα-containing DC-methods (“IFN-GI”, “IFN-I”, “IFN-GITZ”) decreased efficiency of DC-generation.

5.2. DC-generation from CML-MNC

5.2.1. DC-generation from CML-MNC with “IFN-GIT” compared to “MCM” was similar and DC-subtype composition comparable

We could show comparable (non significantly different) proportions of DC-subtypes (DC_{leu}/bla: Ø 48% vs. 45%, DC_{leu}/cells: Ø 15% vs.18%, DC/cells: Ø 22% vs. 31%, DC_{leu}/DC: Ø 56% vs. 54%, DC_{migr}/DC: Ø 45% vs. 26%, DC_{viable}/DC: Ø 16% vs.19%) after culture of CML-MNC with “IFN-GIT” vs. “MCM” (data not shown).

5.2.2. Comparable DC-subtype composition with “IFN-GIT” from AML-MNC and CML-MNC

DC-subtypes after DC-generation from AML- (n=28) and CML- (n=2) samples were similar (DC_{leu}/bla: Ø 34% vs. 48%, DC_{leu}/cells: Ø 17% vs.15%, DC/cells: Ø 27% vs. 22%, DC_{leu}/DC: Ø 63% vs. 56%, DC_{migr}/DC: Ø 31% vs. 45%, DC_{viable}/DC: Ø 38% vs. 16%), data not shown.

In summary, we showed a comparable efficiency of “IFN-GIT” vs. “MCM” to generate DC from CML-MNC and comparable DC-subtype compositions in AML vs. CML cases.

5.3. DC-generation from leukemic WB-samples

5.3.1. DC-generation from AML-WB-samples was less effective with “IFN-GIT” compared to standard methods, however with comparable DC-subtype compositions found in “successful cases”

“MCM” was successful to generate DC in 9 of 12 (75%), “Pici” in 7 of 11 (64%), “Ca” in 8 of 12 (67%) and “IFN-GIT” in 5 of 9 (56%) cases from AML-WB. No significant differences in DC-subtype compositions were found with four methods using “MNC” or “WB” as cell source (data not shown).

5.3.2. "IFN-GIT" was the most successful IFNα-containing DC-method

"IFN-GIT" was successful in 5 of 9 (56%), "IFN-GI" in 5 of 15 (33%), "IFN-I" in 1 of 10 (10%), "MCM" in 75%, "Pici" in 64% and "Ca" in 67% of cases. No significant differences were seen in DC-subtypes obtained with all methods (data not shown).

In summary, the success of "IFN-GIT" or other IFNα-containing methods to generate DC_{leu} from AML-WB was lower compared to all other cytokine-based DC-generating methods. However, proportions of DC-subtypes were comparable with all successful methods. Moreover "IFN-GIT" was the most effective IFNα-containing method to generate DCs.

5.4. Quality of DC-generation - "Ranking"

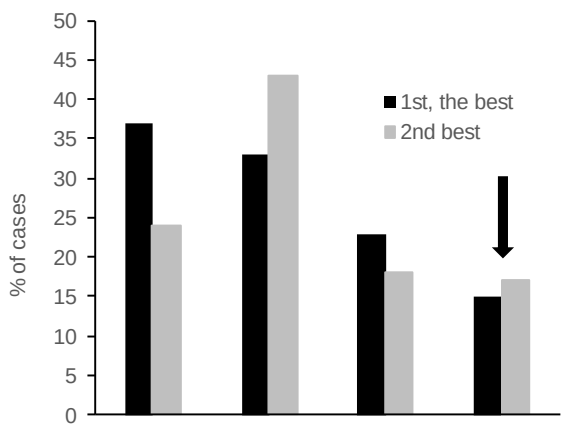
We quantified DC-subtypes after culture of AML-MNC [WB] with four standard-media and different IFNα-combinations (WB) and applied two ranking-categorizations to evaluate the quality of DC-generation (Figure 2). The applied ranking approaches were:

1) according to the best achieved DC-subtype values compared to at least two other parallelly tested standard DC-methods ("MCM", "Pici", "Ca", "IFN-GIT"),

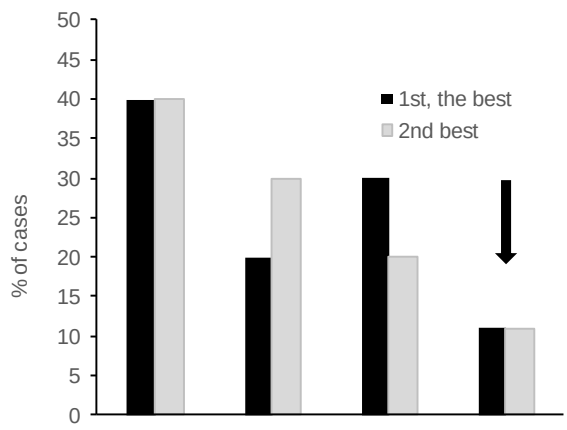
2) applying an independent criteria-catalogue subdividing results in five different DC-generating categories (excellent, excellent-high, high, sufficient and not sufficient [Table 6]).

a) Ranking 1

DC-cultures from MNC



DC-cultures from WB

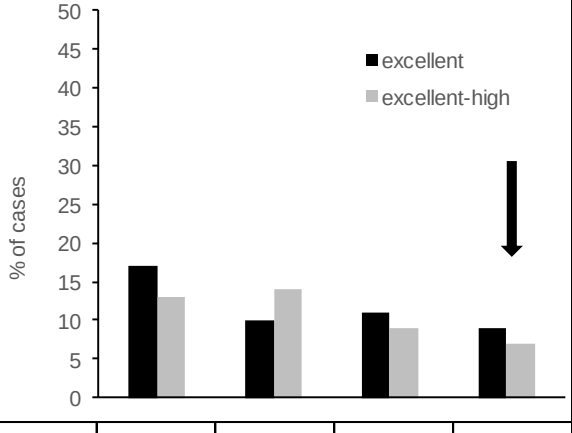


Ranking-criteria	"MCM" %	"Pici" %	"Ca" %	"IFN-GIT" %
1st, the best	**37**	33	23	15
2nd best	24	**43**	18	17
3rd best	35	16	16	**39**
4th best	4	8	**43**	29
Σ (n)	46	49	44	46

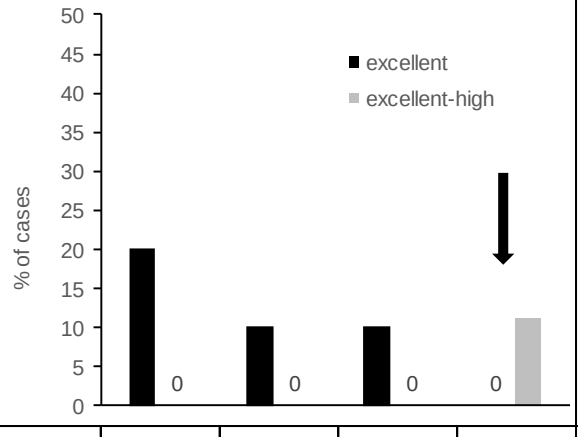
Ranking-criteria	"MCM" %	"Pici" %	"Ca" %	"IFN-GIT" %
1st, the best	**40**	20	30	11
2nd best	**40**	30	20	11
3rd best	20	30	10	**44**
4th best	0	20	**40**	33
Σ (n)	10	10	10	9

b) Ranking 2

DC-cultures from MNC



DC-cultures from WB



Ranking-criteria	"MCM" %	"Pici" %	"Ca" %	"IFN-GIT" %
excellent	**17**	10	11	9
excellent-high	13	**14**	9	7
high	**17**	10	14	11
sufficient	33	**47**	9	28
not sufficient	20	19	**57**	45
Σ (n)	46	49	44	46

Ranking-criteria	"MCM" %	"Pici" %	"Ca" %	"IFN-GIT" %
excellent	**20**	10	10	0
excellent-high	0	0	0	**11**
high	20	**30**	20	11
sufficient	**60**	30	30	22
not sufficient	0	30	40	**56**
Σ (n)	10	10	10	9

Figure 2. Quality of DC-generation from AML-MNC and AML-WB according to ranking-criteria.
Ranking 1 (a): the best DC-values compared to at least two other parallelly tested standard DC-methods, Ranking 2 (b): based on DC-subtype quality categories as given in Table 6. AML-MNC-DC ranking (left side), AML-WB-DC ranking (right side). The bold letters represent results obtained with the majority of cases with DC-method. The arrows point the results of IFNα-containing DC-method "IFN-GIT" .

5.4.1. Quality of DC-generation with different methods from AML-MNC

5.4.1.1. "MCM" and "Pici" were the "best" or "second" best DC-generating methods compared to at least two other standard methods

"MCM" was ranked as the "best method" in 17 of 46 (37%), "Pici" in 16 of 48 (33%), "Ca" in 10 of 43 (23%) cases, whereas "IFN-GIT" was ranked as the "best method" in 7 of 46 (15%) cases. However, compared to other standard methods "IFN-GIT" was ranked in 18 of 46 (39%) cases on the "third" and in 13 of 46 (29%) on the "fourth" position with respect to DC-generation criteria (Fig. 2a, left side).

5.4.1.2."MCM" was ranked as the DC-generating method yielding highest ("excellent") DC-subtype values

As shown in Figure 2b on the left side "MCM" was ranked as "excellent" DC-generating method in 8 of 46 (17%), "Pici" in 5 of 49 (10%), "Ca" in 5 of 44 (11%) cases, whereas "IFN-GIT" was ranked as "excellent" DC-method in 4 of 46 (9%) cases (Table 6). "IFN-GIT" was ranked as "excellent-high" in 3 of 46 (7%), "MCM" in 6 of 46 (13%), "Pici" in 7 of 49 (14%) and "Ca" in 4 of 44 (9%) cases. Furthermore, compared to other standard methods "IFN-GIT" was ranked in 21 of 46 (45%) cases as "not sufficient" (Fig. 2b, left side).

In summary, "IFN-GIT" did not qualify as method to generate regularly sufficient as well as high amounts of DC-subtypes from AML-MNC.

5.4.2. Quality of DC-generation with different methods from AML-WB

5.4.2.1."MCM" was ranked as the "best" and "second best" DC-generating methods compared to at least two other standard methods

"MCM" was ranked as the "best method" in 4 of 10 (40%), "Pici" in 2 of 10 (20%), "Ca" in 3 of 10 (30%) cases, whereas "IFN-GIT" was ranked as the "best method" in 1 of 9 (11%) cases. Results up to the best and including the fourth best are also given (Figure 2a, right side).

5.4.2.2. "MCM" was ranked as the DC-generating method yielding highest ("excellent") DC-subtype values

"MCM" was ranked as "excellent" in 2 of 10 (20%), "Ca" in 1 of 10 (10%), "Pici" in 1 of 10 (10%) cases, whereas "IFN-GIT" could not be ranked as "excellent" DC-method. "IFN-GIT" was ranked as "excellent-high" in 1 of 9 (11%). However, compared to other standard methods "IFN-GIT" was ranked in 5 of 9 (56%) cases as "not sufficient". "MCM" was not ranked as not sufficient DC-method (Figure 2b, left side).

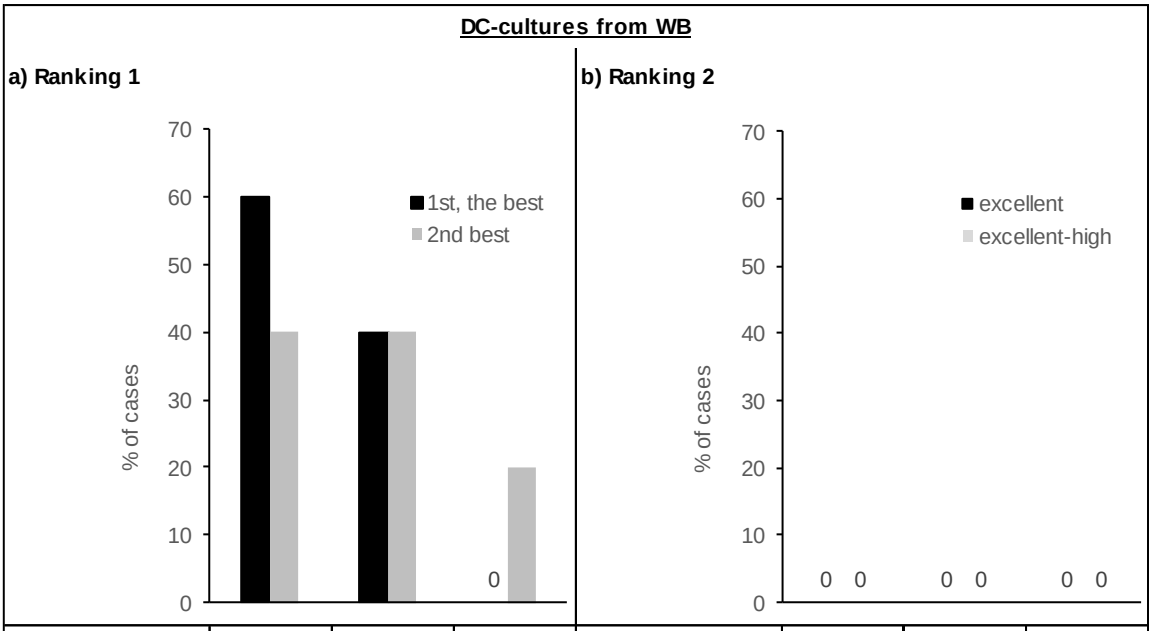
5.4.2.3. Compared to other IFNα-containing methods "IFN-GIT" was ranked as "the best" DC-generating method

"IFN-GIT" was "the best" IFNα-containing DC-method in 3 of 5 (60%) cases, followed by "IFN-GI" in 2 of 5 (40%) cases (Figure 3a).

5.4.2.4. None of the IFNα-containing DC-generating methods was ranked as DC-generating method yielding highest DC-subtype values

None of the three IFNα-containing DC-methods qualified as "excellent" or "excellent-high". "IFN-GIT" was "sufficient" in 2 of 5 (40%) cases, followed by "IFN-GI" in 1 of 5 (20%) cases. "IFN-I" was ranked as "not sufficient" in all cases (Figure 3b).

In summary, "IFN-GIT" is no preferential method to generate regularly sufficient and high amounts of DC-subtypes from AML-WB, although it is the most sufficient IFNα-containing method tested.



Ranking-criteria	"IFN-GIT" %	"IFN-GI" %	"IFN-I" %
1st, the best	**60**	40	0
2nd best	**40**	**40**	20
3th best	0	20	**80**
Σ (n)	5	5	5

Ranking-criteria	"IFN-GIT" %	"IFN-GI" %	"IFN-I" %
excellent	0	0	0
excellent-high	0	0	0
high	0	0	0
sufficient	**40**	20	0
not sufficient	60	80	**100**
Σ (n)	5	5	5

Figure 3. Quality of DC-generation from AML-WB-samples with IFNα-containing methods according to ranking criteria.
Ranking 1 (a): the best DC-values compared to at least two other parallelly tested IFNα-containing DC-methods, Ranking 2 (b): the best DC-value based on DC-subtype-quantity-categories as given in Table 6. Bold letters represent results obtained with the majority of cases with the respective DC-method.

5.5. Analysis of blast-proliferation in AML-WB-samples

Only WB-samples with >10% $CD71^+$ proliferating blasts before culture were included in proliferation analysis.

5.5.1. Addition of "IFN-GI" to AML-WB did not induce blast-proliferation

Proliferation ($CD71^+$ blasts) before and after DC-culture (n=8) from WB-samples in "IFN-GI" and "WB" (WB without any responsemodifiers as control) mediums were quantified and did not show induced blast-proliferation in "IFN-GI" compared to WB-control (data not shown).

In summary, addition of "IFN-GI" to WB DC-cultures did not induce blast-proliferation.

5.6. T-cells' antileukemic functionality after stimulation with "MNC", "DC" or without stimulation in AML-MNC- and AML-WB-samples

DC-stimulation in general improves the antileukemic (lytic) function of T-cells after MLC. "Effector" T-cells used were autologous patients' T-cells (n= 7) or T-cells from patients after allogenic SCT (n=4). Cases were subdivided in those with achieved blast-lytic activity (Lysis) or non-achieved blast-lysis (No-lysis) after 3h and/or 24h incubation of effector-cells with target-cells. For the following analyses (Figure 4a-c) cases with/without successful DC-generation were included.

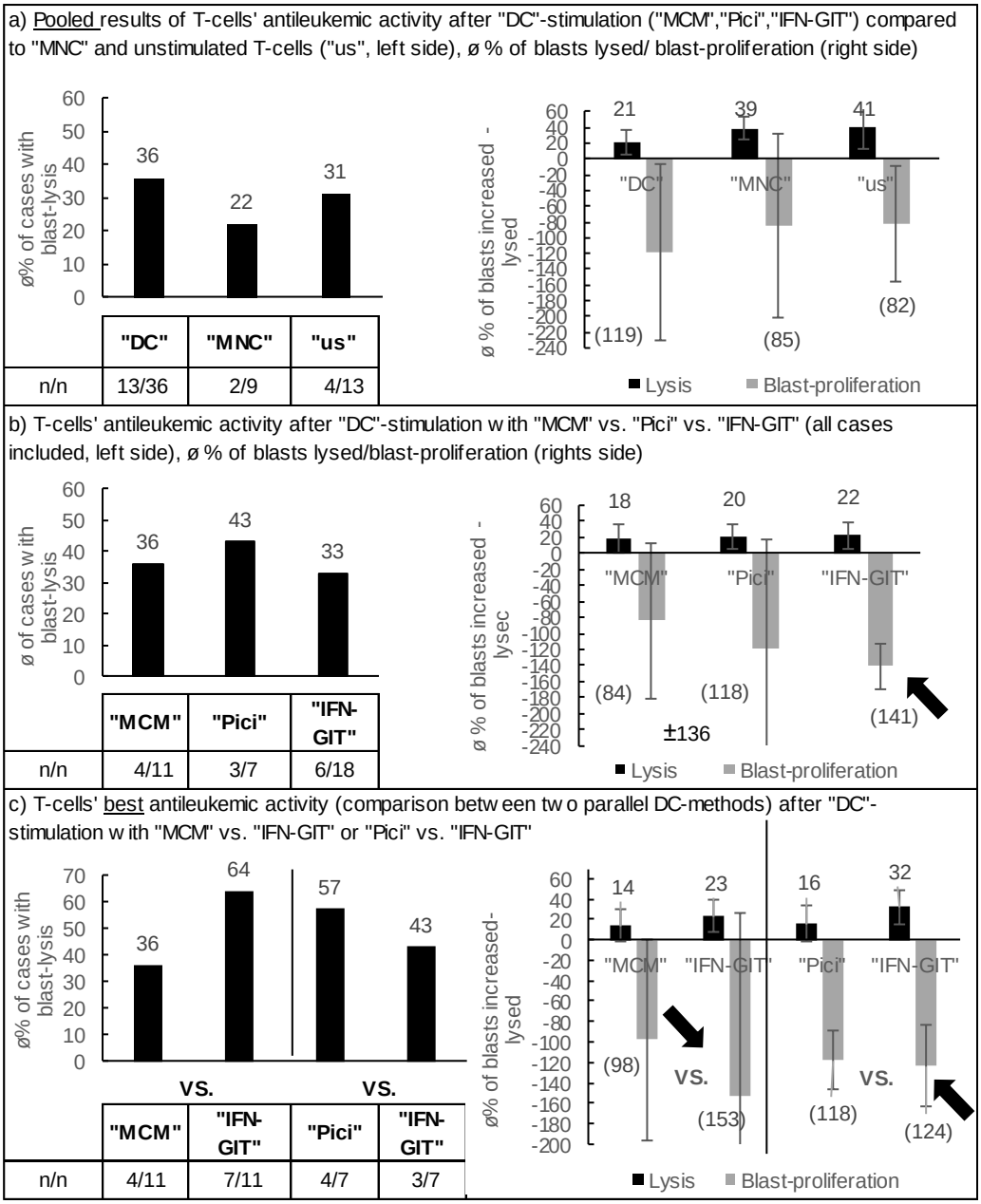


Figure 4. Potential of antileukemic activity of "IFN-GIT"-stimulated T-cells vs. other "DC"- ("MCM", "Pici"), "MNC"-stimulated or unstimulated T-cells. Antileukemic activity of effector T-cells was quantified after 3h or/and 24h incubation with "blast-target" cells. Arrows point to high proportions of blast in cases without achieved blast-lysis after "IFN-GIT" DC-stimulation.

5.6.1. In most cases antileukemic activity of "DC"-stimulation T-cells was improved compared to "MNC"- stimulated

The blast-lytic activity of "DC"- vs. "MNC"-stimulation was regularly improved (pooling all results obtained after "DC"-stimulation, Figure 4a, left side): 36% (13 of 36) vs. 22% (2 of 9) of cases

achieved antileukemic activity (p=0.05, t-test). Activity of unstimulated T-cells was comparable to "DC"-stimulated T-cells' activity 31% (4 of 13 cases).

Average proportions of lysed blasts were 21% vs. 39% vs. 41% after "DC"- vs. "MNC"- vs. unstimulated T-cells. Proportions of blasts were increased (in cases without lysis) ø by 119% vs. 85% vs. 82% (Figure 4a, right side).

Studying individual DC-methods' capacity to initiate T-cells' antileukemic activity showed, that "Pici" was with 43% (3 of 7 cases) the most effective method, followed by "MCM" with 36% (4 of 11) cases and "IFN-GIT" with 33% (6 of 18) cases (Figure 4b, left side). Average proportions of lysed blasts were 20% vs. 18% vs. 22% of blasts after "Pici"- vs. "MCM"- vs. "IFN-GIT" DC-stimulation. On average blast-proportions (in cases without lysis) increased by 118% vs. 84% vs. 141% (Fig. 4b, right side) after "Pici" vs. "MCM" vs. "IFN-GIT" DC-stimulation.

Parallel comparison of DC-methods' (Figure 4c, left side) capacity to initiate T-cells' antileukemic activity (comparison of "MCM" vs. "IFN-GIT" and "Pici" vs. "IFN-GIT") revealed that "IFN-GIT" was superior to "MCM" in 7 of 11 cases (64%), however compared to "Pici" only in 3 of 7 cases (43%, Figure 4c, left side). Average proportions of lysed blasts were 14% vs. 23% after "MCM"- vs. "IFN-GIT" DC-stimulation and 16% vs. 32% after "Pici"- vs. "IFN-GIT" DC-stimulation. Average increased blast-proportions (in cases without lysis) were 98% vs.153% of blasts after "MCM"- vs. "IFN-GIT" DC-stimulation and 118% vs. 124% after "Pici"- vs. "IFN-GIT" DC-stimulation (Figure 4c, right side).

The average DC-subtype proportions of DC_{leu}/blasts, DC/cells and DC_{leu}/DC in pooled "IFN-GIT", "MCM" and "Pici" DC-samples were comparable in cases without lysis/with lysis. Differences of DC_{leu}/cells between the cases without lysis and with lysis 19% vs. 31% were tendentially significant (p=0.05, data not shown). For DC-subtype analysis we used only cases with successful DC-generation.

In summary, we demonstrated a higher antileukemic lytic activity of "DC"-stimulated compared to "MNC"-stimulated or "us" T-cells. "IFN-GIT" DC-stimulated T-cells achieved in minority of cases antileukemic activity compared to "MCM" and "Pici". "IFN-GIT" DC-stimulated T-cells improved blast-lysis compared to "MCM" DC-stimulated, but not compared to "Pici" DC-stimulated.

5.6.2. Antileukemic activity of "WB-DC-IFN-GI"-stimulated T-cells was effective

After 3 and 24 hours of coculture of "WB-DC" ("IFN-GI") -stimulated T-cells with naïve AML-blasts showed in 3 of 6 cases (50%) blast-lysis with Ø18% (range 1-34%) of lysed blasts. In cases with no-lysis Ø190% (range 9-280%) increased blast-proportions were found. T-cells stimulated with "WB-control" resulted in 4 of 6 (67%) cases with blast-lysis with Ø44% (range 17-84%) of lysed blasts. In cases with no-lysis Ø140% (range 0-280%) increased blast-populations were found (data not shown).

In summary, T-cells' stimulation with "WB-DC-IFN-GI" showed lower antileukemic activity and higher blast-proportions after coculture with AML-blasts compared to "WB-control"-stimulated T-cells (Fluorolysis assay).

5.6.3. Proportions of leukemia-derived DC correlated ("highly significantly") with T-cells' antileukemic activity after DC-stimulation

Including cases with and without successful DC-generation we evaluated cut-off values for several DC-subtypes (generated with "IFN-GIT", "MCM" or "Pici"), that allowed the most predictive separation of cases in those with later on achieved blast-lysis and in those without achieved antileukemic lytic activity (Figure 5). In both groups ("IFN-GIT" vs. "MCM"/ "Pici") we could demonstrate, that high proportions of DC_{leu}/bla, DC/cells, DC_{leu}/cells and DC_{leu}/DC generated correlated with a later on achieved higher antileukemic lytic activity. Achieved lysis highly significantly correlated with proportions of blasts converted to DC_{leu} ($p=0.0004$ / Figure 5a). Comparable results were seen including only cases with successful DC-generation (data not shown).

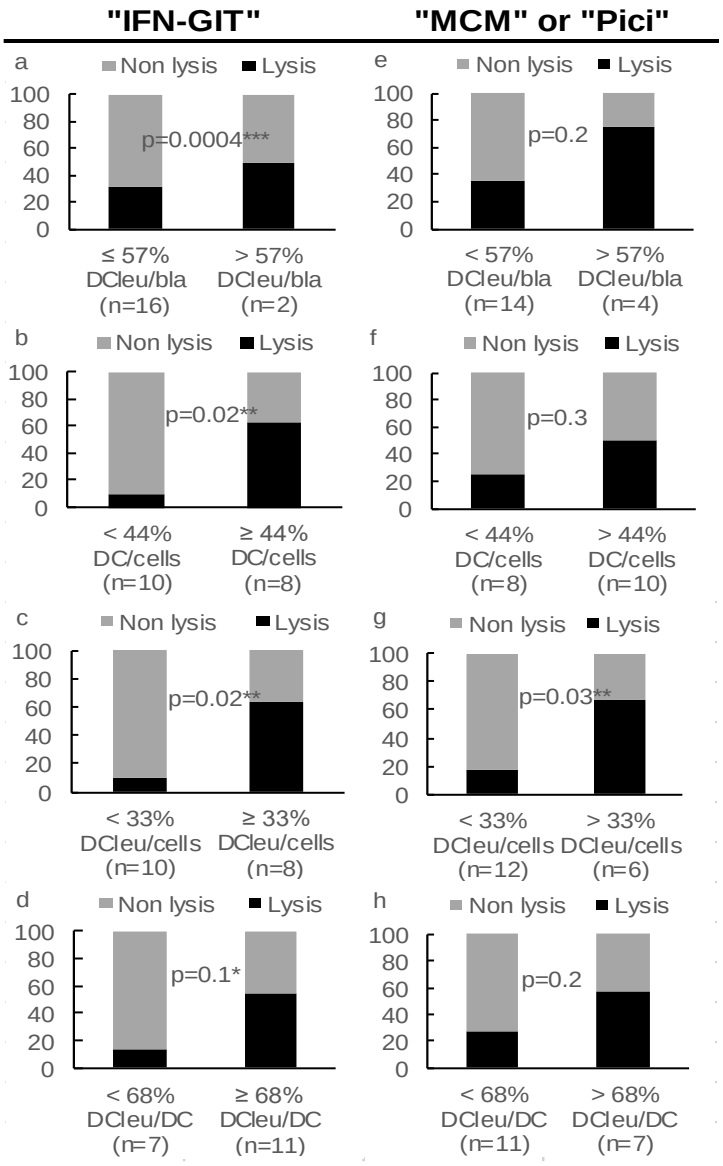


Figure 5. Proportions of DC-subtypes (DC_{leu}/bla: blast converted to DC in blast-fraction, DC/cells: DC total, DC_{leu}/DC: DC_{leu} in DC-fraction), which induced T-cell antileukemic activity (blast-lysis) or non-activity (non-lysis) after DC-stimulation in MLC. DCs were generated with IFNα-containing "IFN-GIT" (left side) and "MCM" or "Pici" (right side) media. Cut-off values could be found that predicted antileukemic T-cell activity after DC_{leu}/DC-stimulation: antileukemic activity correlated directly and in part significantly with generated DC- and DC_{leu}-counts. Cases with or without successful DC-generation were used for cut-off presentation.

In summary, we showed a direct (and in part highly significant) correlation of DC-subtype-proportions (generated with "IFN-GIT", "MCM" or "Pici") with T-cells' antileukemic lytic activity after "DC"-stimulation.

5.7. Clinical response to immunotherapy

5.7.1 Proportions of leukemia-derived DCs correlate with a higher response rate of patients to immunotherapy

We correlated proportions of DC-subtypes generated with “IFN-GIT” and “the best DC-method”. Evaluating cut-off values for several DC-subtypes, that allowed the most predictive separation of cases in “clinical responders” (n=11) and “non-responders” to immunotherapy (n=11) for both groups (“IFN-GIT” vs. “the best DC-method”) we could again correlate higher proportions of DCs (DC/cells, DC_{leu}/bla, DC_{leu}/cells, DC_{leu}/DC) with a higher rate (although non significant) of patients responding to immunotherapy (data not shown). Only cases with successful DC-generation were included (data not shown).

In summary, we demonstrated a direct (non significant) correlation of DC-subtype-proportions (generated with “IFN-GIT” and “the best DC-method”) with pts’ response to initiated immunotherapy.

5.8. Cytokine levels of DC-culture supernatants tested with CBA

We quantified cytokine (IL-2, IL-6, IL-8, IL-10, IL-12p70, TNF-α, IFN-γ, MCP-1) levels in DC-culture supernatants of AML-MNC- or WB- and healthy-MNC-samples after DC-generation with “IFN-GIT” or other standard DC-generating methods (“MCM”, “Pici”, “Ca”). Furthermore, we compared cytokine levels of different IFNα- (“IFN-GI”, “IFN-I”) containing DC-WB-culture supernatants. Only significant differences are given in the text.

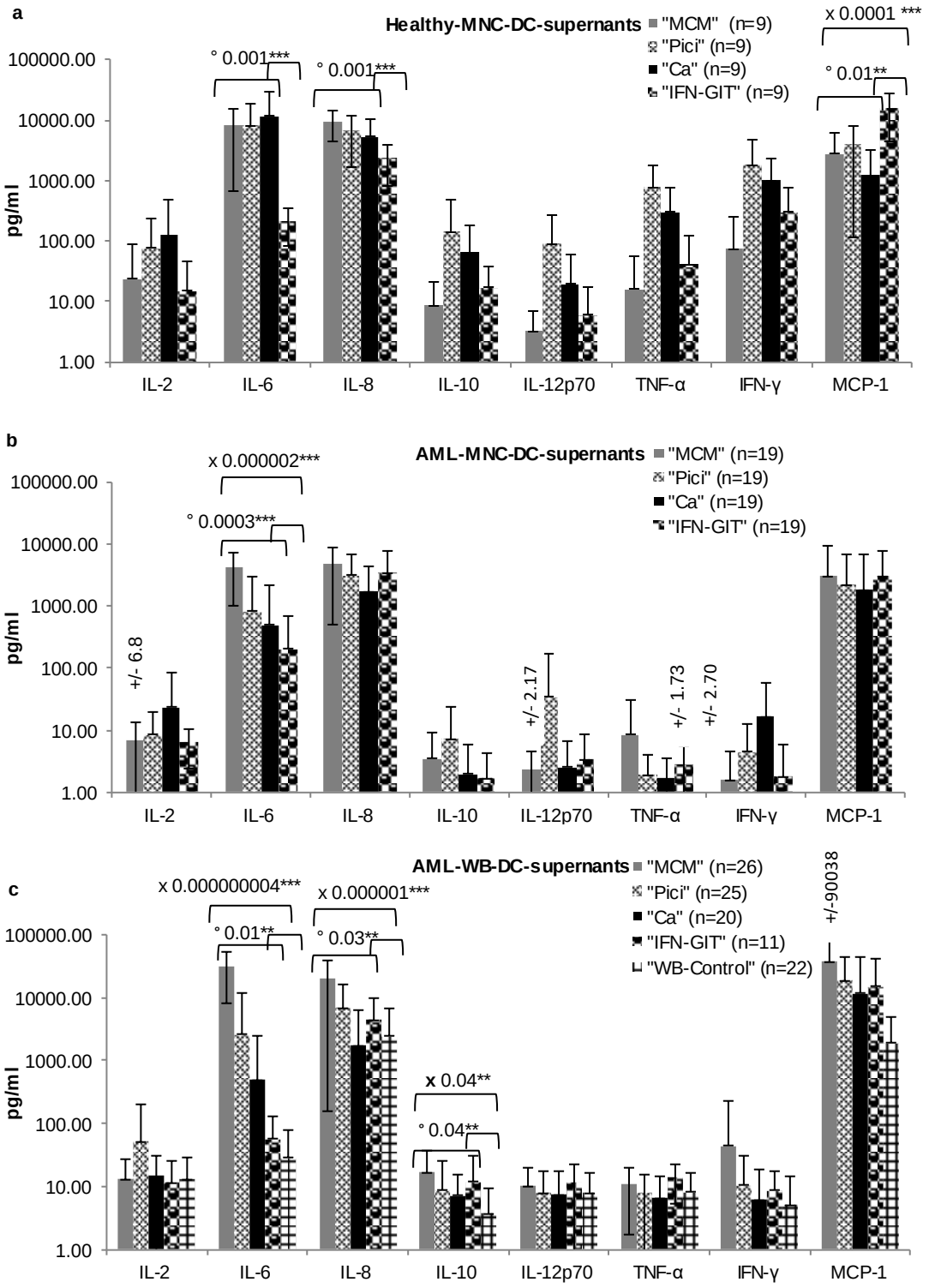


Figure 6. Cytokine levels obtained with CBA-assay in healthy-MNC-DC- and the p-values of pooled DC-supernants **x** and "MCM,Pici,Ca" vs."IFN-GIT" ° (a), in AML-MNC-DC- and the p-values of pooled DC-supernants **x** and "MCM,Pici,Ca" vs. "IFN-GIT" ° (b), and AML-WB-DC-culture supernants and the p-values of pooled DC-supernants **x** and "MCM,Pici,Ca,IFN-GIT" vs."WB-control" ° (c). DCs were generated from MNC (WB) in “IFN-GIT”, “MCM”, “Pici” and “Ca” media (and "WB-control" without added responsemodifiers). ** indicates p-values between 0.005-0.05 (significant); *** p-values < 0.005 (highly significant).

5.8.1. Higher release of anti-inflammatory cytokine IL-10, antitumor-response-related cytokines IL-2, IL-12p70, TNF-α, IFN-γ and inflammatory cytokines IL-6, IL-8, MCP-1 was found in healthy-MNC-DC-compared to AML-MNC-DC-cultures

Pooling all cytokine values (obtained with "MCM", "Pici", "Ca" and "IFN" from AML pts. vs. healthy-samples) is given in Figure 6: **IL-6, IL-8, TNF-α, IFN-γ** and **MCP-1** release profiles from AML-MNC-DC-supernatants vs. healthy-MNC-DC were "highly significantly" different or "significantly" different. **IL-6:** ø 1394 pg/ml vs. ø 7639 pg/ml (p=0.00005); **IL-8:** ø 3194 pg/ml vs. ø 6077 pg/ml (p=0.004); **TNF-α:** ø 4 pg/ml vs. ø 294 pg/ml (p=0.0005); **IFN-γ:** ø 6 pg/ml vs. ø 858 pg/ml (p=0.0002); **MCP-1:** ø 2542 pg/ml vs. ø 6205 pg/ml (p=0.006). **IL-10:** ø 4 pg/ml vs. ø 62 pg/ml (p=0.01) and **IL-2:** ø 11 pg/ml vs. ø 59 pg/ml (p=0.03) were "significantly" different. **IL-12p70** levels were not significantly different in AML-MNC-DC-supernatants vs. healthy-MNC-DC ø 11 pg/ml vs. ø 29 pg/ml (p=0.2).

"MCM","Pici" and "Ca" vs. "IFN-GIT" supernatants from healthy-MNC-DC showed "highly significantly" different **IL-6** (p=0.001) and **IL-8** (p=0.001) and "significantly" different **MCP-1** (p=0.01) levels. **MCP-1** level of "MCM","Pici", "Ca" and "IFN-GIT" was "highly significantly" (p=0.0001) different. In AML-MNC-DC-supernatants "MCM","Pici" and "Ca" vs. "IFN-GIT" showed "significantly" different **IL-6** (p=0.0003) levels. **IL-6** level of "MCM","Pici", "Ca" and "IFN-GIT" was "highly significantly" (p=0.000002) different (Figure 6a,b).

In summary, antitumor-response-related cytokines IFN-γ, TNF-α and MCP-1 were "highly significantly" or "significantly" higher and anti-inflammatory cytokine IL-10 "significantly" higher in culture supernatants of healthy-MNC-DC- compared to AML-MNC-DC-cultures.

5.8.2. Release of antitumor response, anti-inflammatory and inflammatory cytokines were lower in "IFN-GIT" DC-culture supernatants compared to "pooled" AML-DC-culture supernatants ("MCM", "Pici", "Ca")

Higher cytokine levels were found ("MCM"/"Pici"/"Ca") in AML-MNC-DC-supernatants (n=138, pooled data) compared to "IFN-GIT" AML-MNC-DC- (n=22) supernatants**.** Only levels of inflammatory **IL-6** were "significantly" higher in "pooled" vs. "IFN-GIT" DC-culture supernatants: ø 1669 pg/ml vs. ø 190 pg/ml, p=0.01. Levels of the remaining cytokines (IFN-γ, IL-2) were not significantly different in "IFN-GIT" vs. pooled AML-MNC-DC supernatants; **IFN-γ:** ø 2 pg/ml vs. ø 14 pg/ml; **IL-2:** ø 7 pg/ml vs. ø 13 pg/ml (data not shown).

In summary, results obtained with ”MCM”, “Pici” and “Ca” supernatants represented more the healthy cytokine profile than “IFN-GIT”.

5.8.3. Higher release of antitumor response, inflammatory and anti-inflammatory cytokines found in AML-WB-DC- vs. AML-MNC-DC-pooled-culture supernatants

Comparison of WB-DC- and MNC-DC**-**supernatants showed “highly significantly” higher cytokine levels of **IL-6**, **IL-8**, **IL-10**, **TNF-α** and **MCP-1** in WB-DC- vs. MNC-DC-supernatants (Figure 6); **IL-6:** ø 10447 pg/ml vs. ø 1414 pg/ml (p=0.0001); **IL-8:** ø 9311 pg/ml vs. ø 3193 pg/ml (p=0.002); **IL-10:** ø 11 pg/ml vs.4 pg/ml (p=0.001); **TNF-α:** ø 9 pg/ml vs. ø 4 pg/ml (p=0.002); **MCP-1:** ø 21990 pg/ml vs. ø 2541 pg/ml (p=0.003). Levels of the remaining cytokines (IFN-γ, IL-2) were (non significantly) higher in AML-WB-DC- vs. AML-MNC-DC-supernatants. **IFN-γ:** ø 20 pg/ml vs. ø 6 pg/ml (p=0.3); **IL-2:** ø 25 pg/ml vs. ø 11 pg/ml (p=0.2).

In summary, “(highly) significantly” higher release of inflammatory cytokines IL-6, IL-8 as well as IL-10, TNF-α and MCP-1 was found in AML-WB-DC- compared to AML-MNC-DC-culture supernatants.

5.8.4. Higher release of inflammatory and anti-inflammatory cytokines was found in supernatants from AML-WB-DC-cultures (pooled data; “MCM”, “Pici”, “Ca”, “IFN-GIT”) compared to AML ”WB-control”

Addition of DC-culture media to cultures significantly increased **IL-6**, **IL-8** and **IL-10** release levels compared to “WB-control” (Figure 6). **IL-6**: ø10447 pg/ml vs. ø 28 pg/ml (p=0.01); **IL-8**: ø 9311 pg/ml vs. ø 2385 pg/ml (p=0.03); **IL-10**: ø 11 pg/ml vs. ø 4 pg/ml (p=0.04). Levels of IFN-γ and IL-12 were not significantly different in pooled AML-WB-DC- vs. “WB-control”-cultures. Cytokine release levels of the **IFN-γ**: ø 19 pg/ml vs. ø 5 pg/ml; **IL-12**: ø 9 pg/ml vs. ø 8 pg/ml. Cytokine profiles of **IL-6** (p=0.000000004) and **IL-8** (p=0.000001) in “MCM”, “Pici”, “Ca”, “IFN-GIT” and “WB-control” supernatants showed “highly significantly” different levels (Figure 6c).

In summary, addition of DC-culture media to cells (results of all DC-methods pooled) significantly increased the release of inflammatory and anti-inflammatory cytokines compared with “WB-control”.

5.8.5. Higher release of antitumor response, inflammatory and anti-inflammatory cytokines was found in supernatants from AML-WB-DC "IFN-GIT" cultures compared to AML"WB-control"

"(Tendentially) significantly" higher release level of **IL-10**, **TNF-α** and **MCP-1** was measured in WB-DC "IFN-GIT" supernatants compared to "WB-control"; **IL-10**: ø 12 pg/ml vs. ø 4 pg/ml (p=0.07); **TNF-α**: ø 14 pg/ml vs. ø 8 pg/ml (p=0.07); **MCP-1:** ø 15019 pg/ml vs. ø 1883 pg/ml (p=0.02 [Figure 6]). Levels of the remaining cytokines (IFN-γ, IL-12) were not significantly different in "IFN-GIT" vs. "WB-control" cultures. **IFN-γ**: ø 9 pg/ml vs. ø 5 pg/ml; **IL-12**: ø 12 pg/ml vs. ø 8 pg/ml.

In summary, addition of "IFN-GIT" to WB increased (significantly) the release IL-10, TNF-α and MCP-1 compared to "WB-control".

5.8.6. Addition of "IFN-GIT" to WB increased the release of inflammatory cytokines IL-8 and MCP-1 compared to other IFNα-containing Kits

Release of **IL-8**, **MCP-1** and **IFN-γ** was "tendentially significantly" higher in DC-culture supernatants of "IFN-GIT" vs. "IFN-GI" vs. "IFN-I"; **IL8**: ø 4305 pg/ml vs. ø 2736 pg/ml vs. ø 216 pg/ml (p=0.1); **MCP-1**: ø 15019 pg/ml vs. ø 6151 pg/ml vs. ø 1166 pg/ml (p=0.1); **IFN-γ:** ø 9 pg/ml vs. ø 7 pg/ml vs. ø 15 pg/ml (p=0.1). Levels of IL-12 were not significantly different in "IFN-GIT" vs. IFNα-containing Kits ("IFN-GI", "IFN-I"). **IL-12**: ø 12 pg/ml vs. ø 11 pg/ml vs. ø 14 pg/ml (data not shown).

In summary, "IFN-GIT" increased the release of inflammatory cytokines IL-8 and MCP-1 in DC-supernatants compared to other IFNα-containing media.

5.9. Conclusions from results

Our Data shows 1) that WB is a good tool to simulate AML patients' in vivo situation, 2) DC-generation is possible from AML-MNC (WB) with IFNα-containing methods and with other DC-methods without induction of blasts' proliferation, 3) successful DC-generation with IFNα needs the addition of GM-CSF and TNFα ("IFN-GIT"). However, "IFN-GIT" is inferior to produce quantitatively and qualitatively DC_{leu}/DC compared to "MCM" and "Pici", 4) "IFN-GIT" DC-stimulated T-cells yield comparable antileukemic cytotoxicity - however, in cases without achieved blast-lysis a very high increase of blasts was observed, 5) antileukemic activity goes along with high DC_{leu}/DC-counts in "IFN-GIT", "MCM", "Pici" and "Ca" cultures, 6) high DC_{leu}/DC-counts correlated with patients' achieved response to immunotherapy, 7) addition of any DC-media to WB produces more favorable cytokine release profile compared to "WB-control" and 8) addition of "IFN-GIT" produces a more favorable cytokine release profile compared to other IFNα methods.

6. Discussion

6.1. AML and treatment strategies

Only 20-30% of treated AML patients reach long term leukemia-free-survival[52]. SCT is the only curative treatment option with strong antileukemic-effect though it is associated with high risk of treatment related mortality[52-55].

With immunotherapeutic strategies such as DLI after SCT in combination with post remission chemotherapy remissions can be retained, restored and survival can be prolonged[54,56-58], although those patients also have a risk to relapse[59]. Recently promising (less aggressive) immunotherapeutic strategies have been developed increasing immunological effects or mechanisms or targeting directly tumor-cells e.g.: adoptive transfer of immune reactive T-, NK-cells or antibodies (targeting tumor cells or immunosuppressive cells), vaccinations with DCs pulsed with leukemic antigens, manipulated AML-blasts or tumor lysates[60.61]. Immunomodulatory strategies include e.g. the application of immune-attractants such as chemokines to attract T- and NK-cells, anti-immunosuppressants such as TGFβ-blockers and cyclooxygenase inhibitors, immune responsemodifiers (cytokines) and blast-modulatory substances[60.62]. These immunotherapeutic and immunomodulatory strategies are efficient tools to support and activate the patients' immune system. All of these strategies could result in enriched or specifically (re)activated immune effector-cells, a modified microenvironment, deactivated immune escape mechanisms in combination with established immunological memory[63,64]. Many of these strategies are mild therapies with low toxicity, and a potential of high efficiency, although often without creation of an immunological memory. Moreover, many immunotherapies need Gmp-facilities and infectiological testing which are technically demanding, expensive and lead to limited cellular products (e.g.: designed antibodies or T-cells).

A conversion of leukemic-blasts to leukemia-derived DCs by DC_{leu}/DC generating methods mildly modulate blasts to antigen presenting cells – presenting the whole leukemic antigen-repertoire of individual patients, leading to a (re)activation of the immune system and the installation of an immunological memory. Those DC_{leu} could either be generated from blast-containing MNC, prepared and adoptively transferred to patients. Alternatively, the generation of DC_{leu} could be induced after patients' treatment with approved drugs inducing DC_{leu}-production in vivo. Here we wanted to evaluate the role of IFNα-containing drug-combinations (for ex vivo and in vivo use) to generate DC_{leu} as effective mediators to activate antileukemic T-cells and to install an immunological memory against residual blasts.

The use of combined selected (approved) immunomodulatory substances in vivo to AML patients offers many advantages: the clinical use is easy, circumvention of technically demanding procedures and high costs, it can be repeated as required converting (residual) blasts to DC_{leu} with a potential to migrate to tissues and to induce a humoral and cellular immunological memory.

6.2. IFNα in different therapies

Recombinant human IFNα with/without pegylation increasing IFNα's half-life time[42-44] is already used in the therapy of hairy cell leukemia, CML, B- and T-cell lymphomas, melanoma, hepatitis-B (HBV) and -C (HCV), renal carcinoma and Karposi's sarcoma and has shown immunological effect[65,66]. In both acute HCV and HBV infections early IFNα treatment alone or (in case of HCV) combined with Ribavirin can eliminate the virus, induce remissions of the disease and reduce the risk of hepatocellular carcinoma[67,68]. In high-risk melanoma stage II and III a high-dose IFNα therapy has shown significant improvements of overall survival (OS) and RFS rates[69,70].

Before development of the tyrosinkinase-inhibitor Imatinib was discovered IFNα was used as a monotherapy or in combination with cytarabine in CML patients before or after SCT[45]. Therapy of early chronic CML with IFNα has been shown to induce hematological remissions in 60-80% of CML patients. Although with single IFNα-therapy major cytogenetic remissions could be achieved in 35-55% of CML patients[34] in an international randomized Phase III Study of 1106 CML patients a major cytogenetic response could be induced in 85% of the patients (n=553) with Imatinib but only in 22% of patients (n=553) using IFNα-Cytarabine[71]. In another Imatinib-IFNα combination trial of CML patients' molecular remissions could be achieved in some pts after withdrawal of Imatinib, but continuation of IFNα therapy. Interestingly higher levels of Proteinase-3 and PR-1-specific cytotoxic T-cells (PR1-CTL) were detected during the IFNα-therapy in those patients (pointing to a specific installation of immunological antileukemic response), but not during Imatinib therapy-although molecular remissions could be reinduced in patients with molecular relapse by further application of Imatinib.[72]

6.3. Dendritic cells – professional antigen presenting cells

DCs are well known APC with a high capacity to initiate T-cell immune responses against tumor cells[25,26]. DCs can be generated from monocytes ($CD14^+$) and loaded with tumor-specific leukemia associated antigens (LAA)[29,31]. DC-vaccinations of patients with (ex vivo generated, modified) monocyte derived DC have been approved to patients with melanoma, B-cell lymphoma and prostate cancer. They have been shown to be save, giving rise to antitumor immune responses and tumor regression.[73-76] Disadvantages of these DC-generation strategies are, limitations to particular antigens. Furthermore, it is an expensive and time-consuming methodological strategy. The advantage of a monocyte-derived process for a clinical use is its potential to be standardized and to install an immunological memory[77,78].

DC_{leu} are known to be generated efficiently in vitro by conversion of AML-blasts in MNC-fractions to mature APCs by using DC-generating methods, combinations of DC-inducing and maturating substances such as “MCM-mimic”, “Picibanil” or “Ca”[29,32,35,40] and cytokines like TNFα, GM-CSF or PGE_2 inducing hematopoetic myeloid cell differentiation, in combination with a danger signaling and DC_{leu}/DC maturation[79-84]. Here we can confirm, that in vitro a DC-generation from AML-MNC is possible (using “MCM”, “Pici” or “Ca”) in every given patient, thereby overcoming blasts’ resistance to be converted to DC_{leu}[29,30,32,35].We show in addition, that in vitro DC_{leu}/DC-generation using these methods is possible from WB, containing whole cellular and soluble environment of these blasts thereby simulating the situation in the body[85,86]. That means, that both AML-MNC- and WB-blasts are converted to DC_{leu} using these DC-methods and present the whole antigenic surface-markers of the individual patients’ myeloid blasts together with typical DC-antigen-markers. Without need of knowledge of distinct leukemic antigens[29,32,35,36,41]. In general DC obtained with both DC-generating strategies (using monocyte-derived LAA-loaded or leukemia-derived DC) are applicable for an adoptive transfer in patients to induce antileukemic DC-triggering effects against AML-blasts[32,37,87,88]. In addition in vitro DC stimulated T-cells could also be applied to AML patients. However, these strategies need Gmp-facilities, infectiological testings, yield limited cellular products and are expensive. Thus, an elegant and intelligent strategy could be an in vivo application of (preclinically tested) immunomodulatory substance combinations (Kits) to generate DC_{leu} from AML-blasts in vivo- after previous selection of the best Kit in a WB-model.

6.4. DC_{leu}-generation from blast-containing MNC and WB with IFNα-containing media

We wanted to find out, whether DC_{leu}/DC can be generated from blast-containing AML- (and from some CML-samples) MNC as well as from WB using IFNα-containing methods compared to other DC-methods. IFNα-containing DC-generating methods: "IFN-GIT" and "IFN-GI" contained GM-CSF, with /without TNFα.

GM-CSF is a glycoprotein stimulating proliferation and differentiation of myeloid stem cells to mature granulocytes as well as recruitment of dendritic competent cells[79]. Clinically GM-CSF is therefore applicated after chemotherapy (prophylactically or therapeutically) or after SCT to improve regeneration of hematopoetic cells in patients with leukemia or solid tumors, to shorten times of neutropenia (thereby reducing neutropenia-associated mortality, incidence of infections, administration rate of antibiotics and hospitalization times) and to activate monocytes and DC in vivo without inducing proliferation of myeloid blasts in patients with AML[79-83]. As an acute phase protein **TNFα** is involved in inflammatory processes, as a "regulatory cytokine" for cellular processes (e.g. cellular survival/apoptosis), as activator of the immune system and "inductor of DC-maturation"[84,89,90]. Clinically TNFα has been used for the treatment of soft tissue tumors (in high local concentrations) and systemically (in low concentrations) for the treatment of advanced neoplasia, e.g. metastatic melanoma[91.92]. Since **IFNα** has been used clinically in the treatment of melanoma, CML, hepatitis-B and -C[67-71], it could qualify as immunomodulatory substance to be applicated directly (together with GM-CSF +/- TNFα) to patients - resulting in an in vivo production of $DC_{leu.}$

We could show that a DC-generation with IFNα-containing method (especially "IFN-GIT") in general is possible from MNC- as well as WB-samples from healthy- as well as from AML-blood donors (giving rise to comparable amounts of DC_{leu}/DC in MNC compared to WB). In cases with successful DC-generation (as defined by us) proportions of viable, migratory, leukemia-derived DC were comparable - thereby confirming, that the generation of "leukemia-derived" DC is possible with IFNα-containing combinations of immunomodulators as already described by Chen et al[33]. However, comparing the efficiency of a DC-generation according to ranking criteria we could show, that "IFN-GIT" qualified less to produce (sufficient) DC_{leu}/DC from AML-samples compared to other DC-methods ("MCM", "Pici", "Ca") whereas DC_{leu}/DC from CML-samples could be produced in comparable amounts with "MCM" and "IFN-GIT". We conclude, that IFNα-containing methods might stimulate CML-blasts more efficiently than AML-blasts to produce DC_{leu}. This might explain the clinical observation, that IFNα treatment of CML patients results in complete (cytogenetic) remission in high proportions of patients[33,34,45].

Studying the effect of various IFNα-containing Kits ("IFN-GIT", "IFN-GI", "IFN-I") to generate DC_{leu}/DC from AML-WB we could show, that Kits containing only IFNα had a very low efficiency to generate DC_{leu}/DC at all, whereas the addition of GM-CSF (and even more together with TNFα) to these cocktails increased proportions of DC_{leu}/DC- thereby confirming data already published, that GM-CSF plays a central role in the ex vivo generation of DC_{leu}/DC[33,79].

We could already show, that the addition of Zylexis increased proportions of mature DC[47]. Therefore, we studied the role of Zylexis (a pox virus – product, known in veterinarian medicine to improve immunological reactions, especially of the innate immune system[93-95] in case of DC-generation with "IFN-GIT" and additionally added Zylexis "IFN-GITZ". We found, that IFNα-containing "IFN-GIT" and "IFN-GITZ" DC-generating methods induced comparable amounts of DC-subtypes. However, "MCM" was superior to those IFNα-containing DC-methods.

An important finding was, that neither the addition of any DC-method "Pici", "Ca" nor "IFN-GIT", "IFN-GI" or "IFN-GITZ" to leukemic WB-samples did induce a blast-proliferation as already shown by us[38]. That means, in general an application of "IFN-GI", -"GIT" or -"GITZ" to patients could be safe (with respect to the risk to induce a blast-proliferation in vivo) and since substances are approved for human treatment[79-84,89-92].

6.5. Improvement of T-cells' antileukemic reactivity after stimulation with "IFN-GIT", "MCM" or "Pici" pre-treated (DC_{leu}/DC-containing) blood

Our intention was to evaluate the potential of the IFNα-containing DC-generating method "IFN-GIT" compared to other DC-methods to give rise to antileukemic activity of T-cells after MLC. In the past we could already show, that a conversion of AML-blasts to DC_{leu} followed by MLC with T-cells improved T-cells' antileukemic reactivity in the majority of cases. Here we show, that in general (in most of the cases) "DC"-stimulated (either "IFN-GIT", "MCM", "Pici" pre-treated in MNC) T-cells' induced antileukemic activity compared to "MNC"-stimulated (control without added DC-media) and also to unstimulated T-cells'. Interestingly "IFN-GIT" DC-containing cultures improved T-cells' antileukemic activity and showed comparable antileukemic activity to "MCM" and "Pici" pre-treated T-cells. An important finding was, that cases without achieved blast-lysis by T-cells after stimulation with "IFN-GIT" pre-treated blasts (as demonstrated by cytotoxicity assays) were characterized by highest increase of blasts-proportions. This could be explained by (possible) IFNα-mediated influences on T-cells inhibiting their blast-attack (e.g. by inducing escape-mechanisms)[96]. Another important observation was, the finding of a direct correlation of DC_{leu}/DC-counts obtained by any DC-method with the achieved antileukemic function of T-cells after MLC with DC-method pre-treated blasts in

MLC. Average DC_{leu}/DC-counts obtained with “IFN-GIT”-, “MCM”- and “Pici”-samples after DC-culture were comparable pointing to the role of DC_{leu} (- counts) in the mediation of antileukemic reactions. This could mean, that 1) an adoptive DC_{leu}/DC-based immunotherapy of AML patients in general could contribute to improve antileukemic T-cells’ reactivity, that 2) ”MCM” and “Pici” are superior to “IFN-GIT” to generate DC_{leu}/DC-resulting in improved blast-lysis and 3) that “IFN-GIT” pre-treated blasts in MNC could only be used for adoptive transfer after preliminary confirmation of ex vivo induced T-cells’ blast-lysis after MLC.

We found reduced cytokine levels in AML-MNC-DC-supernatants of all DC-methods compared to supernatants of healthy DC-cultures. Compared to MNC/WB “MCM”-, “Pici”- and “Ca”- supernatants “IFN-GIT”- DC culture supernatants were characterized by lower release of IL-2, IL-6, IL-10 and also IL-12. Compared to “WB-control” the addition of any DC-generating method improved the average release of IFN-γ and MCP-1-pointing to an improved cytokine release profile. We assume, that patients’ treatment with immunomodulatory substances change and improve the cytokine release profile, which could contribute for patients’ improved response to immunotherapy as postulated by other authors[17,97,98]. Multifactorial analyses (including also cytokine - and chemokine - release - profiles, composition of cells of the adaptive and innate immune system) have to be performed to further illuminate these findings in correlation with antileukemic activity.

6.6. IFNα-based immune modulating therapy of AML patients?

Using a culture model with blast-containing WB from AML patients, as developed by us, we could show, that the value and DC_{leu}/DC-generating efficiency of various immune modulating treatment approaches by using combinations of cytokines, danger-factors and/or DC-maturating substances can be tested very well. We found, that DC-generating methods (e.g, “MCM”, “Pici”, “Ca”, “IFN-GIT”) qualify to generate DC_{leu}/DC from blast-containing MNC- as well as WB-samples from AML patients and could therefore be transfused into patients. Moreover WB-cultures are a good tool to demonstrate whether Kits (containing only 1-3 approved drugs) qualify to generate (ex vivo) DC_{leu}/DC, that could be transferred to AML patients or whether that Kits could be directly used as immune modulatory drugs to treat AML patients inducing DC_{leu}/DC in vivo using blast-containing WB. We could show, that DC_{leu}/DC are not regularly produced using “IFN-I” or “IFN-GI” and in general in lower amounts using “IFN-GIT” compared to other DC-generating methods.

It was already shown, that after therapy of CML patients with GM-CSF+IFNα clinical and cytogenetic responses can be achieved, that might be due to direct antitumor IFNα effects and possibly in addition by transformation of myeloid CML-blasts - approach to treat AML patients with IFNα-containing

protocols has already been performed earlier with the aim to produce DC_{leu} in vivo that induce an antileukemic response by (specific) T-cell activation. Converted blasts to DC_{leu} – contribute to activate leukemia specific immunoreactive cells.[34,45]

The clinical use of IFNα for AML patients has been shown to be safe and well tolerated [65,66,99]. Moreover in 1979 Hill et al. described a 'modest antitumor effect' after (unpegylated) IFNα treatment[65]. These antitumor effects are known to be mediated by T-, NK-cells and DC[97]. For maintenance of these effects continuously high and stable IFNα-levels are necessary. This has been shown to be problematic to be induced in serum in AML patients – especially if unpegylated IFNα was used[65]. So far pegylated IFNα has been used in salvage therapies in several clinical AML-trials treating pts at relapse, relapse following SCT and also in CR after chemotherapy or after SCT[65]. It was also shown that remissions in AML patients with myelofibrosis can be achieved using PEG-IFNα[65,66]. Moreover the application of GM-CSF and IFNα was shown to induce remissions in 3 of 4 AML patients and in 2 of 3 ALL patients treated in a salvage therapy[81]. Although no detailed information about all pts were given there are hints, that some patients profited from this therapy (one of the patients still did not show any sign of the disease after 3.5 years, another was in CR with chronic GVHD after one year of initial relapse, the third showed persisting disease)[81].

Our data show, that IFNα-containing therapies have a potential to induce antileukemic reactivity of T-cells ex vivo, however only if GM-CSF and TNFα were combined with IFNα. We recommend a careful selection of patients in that antileukemic reactivity could be shown in simulated ex vivo settings to circumvent an explosion of blast-proliferation in vivo.

7. Conclusion

In conclusion, we could show that with the IFNα-containing "IFN-GIT" DC-method DC-generation is possible both from AML-MNC- and -WB-samples, although other standard DC-methods ("MCM" and "Pici") were (in quantity and quality) superior in the ex vivo production of DC_{leu}/DC. Blast-proliferation was not induced by any DC-generating method in WB.

We could show that successful antileukemic activity (induced after MLC of T-cells with DC-containing suspensions) correlated with high proportions of DC_{leu}/DC generated with "MCM", "Pici" and "IFN-GIT"- pointing to the central role of DC_{leu}/DC in the mediation of antileukemic reactions. Since "IFN-GIT" produced lower proportions of DC_{leu}/DC (compared to "MCM", "Pici" and "Ca") we conclude, that – although "IFN-GIT"– treated (blast-containing) MNC or WB can induce antileukemic activity after MLC – other DC-generating methods or Kits that result in higher DC_{leu}/DC counts and in consequence in superior antileukemic activity compared to "IFN-GIT". In cases without successful mediation of antileukemic reactivity after "IFN-GIT" pre-treated blast-containing "MNC" or "DC" we could even detect an increase of blasts - pointing to a possible severe adverse event (SAE) after "IFN-GIT"-treatment in vivo. In general, our data point to an important cytokine release profile of IFN-γ and MCP-1 under the influence of any DC-generating media compared to "WB-control".

There is a need to further develop, study and select combinations of immunomodulatory substances to generate leukemia-derived DC without inducing blasts' proliferation, but with a high potential to induce specific antileukemic T-cells, that could be used in AML-therapy e.g. to stabilize remissions by specific elimination of (residual) AML-blasts.

Using patients' WB to simulate the in vivo situation we could show, that DC_{leu}/DC can be generated with IFNα-containing Kits (especially "IFN-GIT"), although not on a regular basis. Probably a treatment approach using IFNα, TNFα and GM-CSF could give rise to DC_{leu}/DC without induction of blasts' proliferation in vivo followed by generation of antileukemic T-cells - in cases with proven DC_{leu}/DC-generation in WB. Our ongoing research focuses on the development of Kits, consisting of 2-3 blast modulatory substances, that induce DC_{leu}/DC-generation and T-cell activation in a WB model.

8. References

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9. List of tables

10. List of figures

The Figures 1,2,3,4,5 and 6 are already published in Hirn Lopez, et al., J Immunother, 2019[39].

Figure 1*:* DC-subtypes generated from AML-MNC.

Figure 2: Quality of DC-generation from AML-MNC and AML-WB according to ranking-criteria.

Figure 3: Quality of DC-generation from AML-WB-samples with IFNα-containing methods according to ranking criteria.

Figure 4: Potential of antileukemic activity of "IFN-GIT"-stimulated T-cells vs. other "DC"- ("MCM", "Pici"), "MNC"-stimulated or unstimulated T-cells.

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Figure 6: Cytokine levels obtained with CBA-assay in healthy-MNC-DC-, AML-MNC-DC- and AML-WB-DC-culture supernatants.

11. Abbreviations

ALL	acute lymphatic leukemia
AML	acute myeloid leukemia
AP	accelerated phase CML
APC	Allophycocyanin
APC	antigen presenting cell
BC	blast crisis CML
BCDF	B-cell differentiation factor
BCR-ABL	fusion protein BCR-ABL
CARs	chimeric antigen receptors
CBA	cytometric bead array
CCL2	chemokine MCP-1
CCXL	interleukin 8 (IL-8)
CD	differentiation antigen (cluster of differentiation)
CML	chronic myeloid leukemia
CMML	chronic myelomonocytic leukemia
CP	chronic phase CML
CR	complete remission
DC	dendritic cell
DC_{leu}	leukemia derived dendritic cell
DC_{leu}/bla	blast converted to dendritic cell in blast-fraction
DC_{leu}/DC	leukemia derived dendritic cell in dendritic cell-fraction

DC_{migr}/DC	migratory mature dendritic cell in dendritic cell-fraction
DC_{viable}/DC	viable dendritic cell in dendritic cell-fraction
dgn.	Diagnosis
DLI	donor lymphocyte infusion
FAB	French-American-British classification
FACS	fluorescence activated cell shorting
FCS	fetal calf serum-free medium
FITC	fluorescein isothiocyanate
FL	FLT3 ligand
GM-CSF	granulocyte macrophage - colony stimulating factor
GVHD	graft-versus-host disease
HBV	hepatitis-B
HCV	hepatitis-C
IC	Immunocytological
IFN	Interferon
IFN-I	DC-culture media (interferon alfa)
IFN-GI	DC-culture media (granulocyte macrophage - colony stimulating factor + interferon alfa)
IFN-GIT	DC-culture media (granulocyte macrophage - colony stimulating factor + interferon alfa + tumor necrosis factor alfa)
IFN-GITZ	DC-culture media (granulocyte macrophage - colony stimulating factor + interferon alfa + tumor necrosis factor alfa + Zylexis)
IL	Interleukin
LAA	leukemia associated antigen

M0	acute myeloblastic leukemia
M1	acute myeloblastic leukemia with minimal maturation
M2	immature granulocytic leukemia
M3	acute promyelocytic leukemia
M4	acute myelomonocytic leukemia
M5	acute monocytic leukemia
M6	erythroid leukemia
MAF	macrophage-activating factor
MCM	DC-culture media (MCM-Mimic)
MDS	myelodysplastic syndrome
MHC I	major histocompatibility complex I
MHC II	major histocompatibility complex II
MLC	mixed lymphocyte culture
MNC	mononuclear cell
mAbs	monoclonal antibodies
moAbs	mouse monoclonal antibodies
NAP-1	neutrophil-activating protein-1
NK-cell	natural killer cell
NR	non-responder
OS	overall survival
PBS	phosphate buffered saline
PC7	tandem Cy7-PE conjugation

PE	Phycoerythrin
Peg-IFNα2b	pegylated interferon alfa
PGE_2	Prostaglandin E2
Pici	DC-generation media (Picibanil)
PR1	proteinase-1
R	Responder
Rel	Relapse
Pers	persisting disease
RFS	relapse free survival
sAML	secondary AML
SCT	stem cell transplantation
SAE	severe adverse event
TCGF	T-cell growth factor
T_{eff}	effector T-cell
TGF	transforming growth factor
TNF	tumor necrosis factor
T_{reg}	regulatory T-cell
WB	whole blood

12. List of publications

12.1. Original studies

Ansprenger C, Vogt V, Schick J, Hirn Lopez A, Vokac Y, Harabacz I, Braeu M, Kroell T, Karenberg A, Kolb H.J., Schmetzer H.M.**. Paramunity-inducing Factors (PINDs) in dendritic cell (DC) cultures lead to impaired antileukemic functionality of DC-stimulated T-cells**. Cellular Immunology. 2018 June;328:33-48. ***Impact: 2.85***

Hirn Lopez A, Deen D, Fischer Z, Rabe A, Ansprenger C, Stein K, Vogt V, Schick J, Kroell T, Kraemer D, Kolb H-J, Tischer J, Schmid C, Schmetzer H: **Role of Interferon (IFN)α in ‚Cocktails' for the generation of (leukemia-derived) dendritic cells (DC_{leu}) from blasts in blood from patients (pts) with acute myeloid leukemia (AML) and the induction of antileukemic reactions**. Journal of Immunotherapy. 2019 June;42(5):143-161. ***Impact: 3.83***

Amberger D, Vokac Y, Hirn Lopez A, Deen D, Kroell T, Schmid C, Kolb H.J., Tischer J, Schmetzer H.M. **Released soluble factors in serum or supernatants of leukemia-derived dendritic cells or mixed lymphocyte cultures are predictive for T-cells' antileukemic functionality or clinical response to immunotherapy**. 2019 in preparation.

Plett C, Amberger D, Rabe A, Deen D, Fischer Z, Hirn Lopez A, Vokac Y, Werner J, Krämer D, Rank A, Schmid C, Schmetzer H.M.: **Immunomodulatory Kits do not induce AML-blats`proliferation ex vivo: IPO-38 is an appropriate and reliable marker to detect and quantify proliferating blasts**. 2019 in preparation.

Kugler C, Deen D, Fischer Z, Hirn Lopez A, Vokac Y, Rabe A, Kroell T, Krämer D, Kolb H.J., Tischer J, Schmetzer H.M.: **Immunomodulation of blasts in AML-patients (pts) with clinically approved response modifiers to improve antileukemic T-cell reactivity: an ex vivo simulation of the clinical situation**. 2019 in preparation.

Fischer Z, Hirn Lopez A, Schmetzer H.M.: **Compositions of T cells, DC and the cytokine release profile are predictive for the antileukemic response of DC-stimulated T cells**. 2019 in preparation.

12.2. Congress contributions

Deen D, Hirn-Lopez A, Vokac Y, Kroell T, Koehne C.H., Kolb H.J., Tischer J, Schmetzer H.M. **Generation of leukaemia-derived dendritic cells (DC/DCLEU) with clinically approved response modifiers from whole blood (WB) samples from AML-patients (PTS): an ex vivo simulation of the clinical situation**. EBMT, Bone Marrow Transplantation 48, suppl. 2, abstract 447 (2013).

Hirn-Lopez A, Deen D, Vokac Y, Kroell T, Kraemer D, Schuster F, Borkhardt A, Schmid C, Kolb H.J., Tischer J, Schmetzer H.M. **Role of IFN alpha in DC-cocktails for the generation of (leukemia-derived) dendritic cells from AML-blasts, the induction of antileukemic functionality of DC-stimulated T-cells and in correlation with clinical response to immunotherapy**. EBMT, Bone Marrow Transplantation 48, suppl. 2, abstract 456 (2013).

Vokac Y, Hirn-Lopez A, Deen D, Kroell T, Schmid C, Kolb H.J., Tischer J., Schmetzer H.M. **Released soluble factors in serum or supernatants of leukemia-derived dendritic cell or mixed lymphocyte cultures are predictive for T-cells' antileukemic functionality or clinical response to immunotherapy**. EBMT, Bone Marrow Transplantation 48, suppl. 21, abstract 458 (2013).

Deen D, Stankova Z, Hirn A, Vokac Y, Kroell T, Bund D, Buhmann R, Hausmann A, Schmid C, Schmetzer H.M. **Immunomodulation of Blasts in AML-Patients (pts) with Clinically Approved Response Modifiers to Improve Antileukemic T-cell Reactivity: an ex vivo Simulation of the Clinical Situation**. XVI Wissenschaftliches Symposium der Medizinischen Klinik III, Freising, poster 24 (2014).

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Inngjerdingen M, Rabe A, Deen D, Stankova Z, Hirn A, Vokac Y, Wendtner C, Tischer J, Schmid C, Schmetzer H.M. **Immunmodulation of AML-Blasts with clinically approved response modifiers improves Antileukemic T-Cell reactivity ex-vivo and leads to blast-reduction in-vivo (RAT-modell).** ITOC 2016, Eur J. Cancer 55, suppl. 1, 23, (2016).

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Plett C, Amberger D.C., Rabe A, Deen D, Stankova Z, Hirn A, Vokac Y, Werner J, Krämer D, Rank A, Schmid C, Schmetzer H.M., **Immunomodulatory Kits do not induce AML-blasts' proliferation ex vivo. IPO-38 is an appropriate and reliable marker to detect and qualify proliferating blasts**. ITOC 2017, Eur J Cancer, 5(1), 3-4, 2017.

Plett C, Amberger D.C., Rabe A, Deen D, Stankova Z, Hirn A, Vokac Y, Werner J, Krämer D, Rank A, Schmid C, Schmetzer H.M., **Immunomodulatory Kits do not induce AML-blasts' proliferation ex vivo. IPO-38 is an appropriate and reliable marker to detect and qualify proliferating blasts**. EBMT 2017, Bone Marrow Transplantation 43, suppl. 1, p 218, abstract s. 221 (2017).

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14. Eidesstaatliche Versicherung

Ich, Annika Maria Hirn Lopez, erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema

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

selbstständig verfasst, mich außer den angegebenen, keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich arbeitete Proben auf und führte Experimente (z.B: DC_{leu}/DCs, MLC, CTX und CBA) für den methodischen Teil der Studie durch. Ich war verantwortlich für die Datenerfassung, Auswertung und die statistische Aufarbeitung, war an der Entwicklung des Studiendesigns beteiligt, erstellte die gleichnamige Publikation (Hirn Lopez A. et al., J Immunotherapy 2019[39]) mit Hilfe der unten genannten Autorenbeiträgen und wurde dabei von Schmetzer H. betreut.

Von Deen D., Ansprenger C., Stein K., Vogt V., Schick J., Kroell T. und Rabe A. erhielt ich einige Daten (DC_{leu}/DC-, MLC- und CTX) zur Auswertung überlassen. Kroell T. unterstützte mit den Labor- und FACS-Einarbeitungen. Vokac Y. war beteiligt an CBA-Messungen und deren Auswertungen. Fischer Z. unterstützte die Studie mit Bereitstellung der Daten der (Nachweis proliferierender Blasten in Vollblutkulturen).

Kraemer D., Tischer J., Kolb H.-J., Schmid C. unterstützten das Projekt mit Patientenmaterial und –Befunden, Schmid C., und Kolb H.-J. waren an der Entwicklung des Studiendesigns und Diskussion der Daten beteiligt.

Schmetzer H. unterstützte, leitete und überwachte das gesamte Projekt und die Arbeit von Hirn Lopez A., sowohl die Publikation, als auch die Dissertation betreffend.

Ich erkläre des Weiteren, dass die hier vorliegende Dissertation nicht in gleicher oder ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

München, 16.12.2020 Annika Hirn Lopez

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