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Influence of physiological hypoxia on whole blood (WB) from healthy donors and AML-patients: the (leukemic) antigen-presentation on blasts, on leukemia-derived dendritic cells (DC_{leu}) after stimulation with 'DC-generating cocktails' as well as the antileukemic functions of DC/ DC_{leu}-stimulated immune reactive cells

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II. Abstract

DC_{leu} generation *in vivo* and transferring to the patients could qualify as a novel treatment option. Furthermore, blasts modulated to leukemia-derived DC (DC_{leu}) *in vivo* could result in T-cell activation. We have already established a method using a combination of GM-CSF+ PICIBANIL (I), or +PGE2 (K) or + PGE1 (M) kits to convert leukemic blasts into DC_{leu} without induction of blasts' proliferation in a WB-culture model (in the presence of individual patients' soluble and cellular components). Such DC_{leu} cells can strongly induce antileukemic and memory T-cells.

To simulate physiological conditions, we compared Normoxic (No) standard- (21% O_2) vs. physiological Hypoxic (Hy) (10% O_2) culture conditions. In the first part of my work, DC-generation was done in AML cell lines to set up the physiological conditions. AML cell lines were cultured with immunomodulatory Kits (-I, -K, -M) under Normoxic condition (21% Oxygen) as well as under Hy (approx. 6% Oxygen). Our cell line results showed no significant changes in DC_{leu} (frequencies of DC_{leu}/Bla^{+}) or the proliferation of blasts in Hy condition under the stimulation of Kits compared to control and also as compared to No condition.

In the second part of my work, samples from AML-patients and healthy blood donors (peripheral blood mononuclear cells (PBMNC) and heparinized whole blood (WB)) were cultured in parallel under No (21% Oxygen) and Hy (10% Oxygen) using Pici-$_{PGE1}$, Pici-$_{PGE2}$ and Kit-I, Kit-K and Kit-M DC-generating media which followed by MLC (mixed lymphocyte culture) using patients' or healthy donors' T-cells and functional (blast-cytotoxicity- and cytokine secretion) assays in AML-samples. Compositions of DC-, blast-subtypes, the immune status, and results of functional assays were compared for all tested conditions. We found that DCs and their subtypes can be generated in comparable frequencies from AML and healthy PBMNCs samples using pici-$_{PGE1}$ and pici-$_{PGE2}$ under No and Hy conditions. Comparable frequencies of DC were generated with Kits (-I, -K, -M) from 35 AML and 16 healthy WB -samples. DC generation was comparable under No vs. Hy in AML and healthy samples.

Further, as control when there was no added response modifier (w/o), no or low production of DC/DC_{leu} was detected. Interestingly, no induction of blasts' proliferation was shown under the influence of kits under No or Hy conditions. After MLC of T-cells with kit-treated AML-WB under No vs. Hy (with Kit-I, -K, -M), we found a significant increase of activated T-cells, $CD4^{+}CD3^{+}/CD3^{+}$, T_{prol}, $T_{non\text{-}naive}$, $T_{eff\text{-}em}$ as well as of NK and iNKT cells and low frequencies of T_{reg}, T_{cm} DC, T-cells, NK, iNKT, and CIK cells after MLC under Hy as well as after No conditions.

Finally, we demonstrated an increase of antileukemic activity after the stimulation of patients' T-cells in MLC with (DC/DC_{leu} containing) Kit-I or -M treated (vs. untreated) WB in No as well as under Hy conditions. Average blast lysis was increased in $MLC^{WB\text{-}DC\ Kit\text{-}I}$ and $MLC^{WB\text{-}DC\ Kit\text{-}M}$ compared to MLC^{WB}, with equal average lysis in $MLC^{WB\text{-}DC\ Kit\text{-}I}$ and $MLC^{WB\text{-}DC\ Kit\text{-}M}$. We further found significantly higher amounts of INFγ secreting cells of the innate and adaptive immune system after $MLC^{WB\text{-}DC\ Kit\text{-}I}$ and $MLC^{WB\text{-}DC\ Kit\text{-}M}$ compared to MLC^{WB}. Results were comparable under No and Hy conditions. We found a significant positive correlation between the DC/WB produced with Kit-M and the antileukemic reactivity of T 'effector-cells' after $MLC^{WB\text{-}DC\ kit\text{-}M}$. Furthermore, we found a significant correlation between DC^{+}/WB and DC_{leu} /WB generated with kit-M and the best anti-leukemic reactivity of T-cells' $MLC^{WB\text{-}DC\ kit\text{-}M}$ in Hy conditions.

Conclusion: These results indicate comparable results under No and Hy conditions for the cultivation of WB-cells with kits (resulting in DC) as well as T-cell subtypes after MLC and induced antileukemic reactivity. These findings eliminate the necessity of using Hy (as physiological condition) for the production of cellular products (e.g for an adoptive cell transfer).. Our data further indicate that the treatment of patients with kit-M and kit-I could result in an improved or induced antileukemic reactivity *in vivo* by a DC_{leu}-mediated mechanism.

Für eine klinische Behandlungsstrategie könnten DC_{leu} *ex vivo* generiert und auf Patienten übertragen werden. Alternativ können Blasten zu Leukämie-abgeleiteten DC (DC_{leu}) in vivo moduliert werden, was zu einer T-Zell-Aktivierung führt. Wir haben bereits eine Methode etabliert, bei der eine Kombination aus GM-CSF+ PICIBANIL Kit (I), oder +PGE2 (K) oder + PGE1 (M) Blasten in eine 'DC_{leu}-Vakzine' umgewandelt und antileukämische T-Zellen induziert werden können, ohne dass eine Blastenproliferation in einem WB-Kulturmodell, in Anwesenheit von löslichen und zellulären Komponenten der Patienten, induziert wird. Um physiologische Bedingungen zu simulieren, verglichen wir normoxische (No) Standard- (21% O_2) vs. physiologische hypoxische (Hy) (10% O_2) Kulturbedingungen. Im ersten Teil meiner Arbeit wurde die DC-Generierung mit AML-Zelllinien durchgeführt, um die physiologischen Bedingungen einzustellen. AML-Zelllinien wurden mit immunmodulatorischen Kits (-I, -K, -M) sowohl unter No (21% O_2) als auch unter Hy (ca. 6% O_2) kultiviert. Unsere Ergebnisse zeigten keine signifikante unterschied für die DC_{leu} (Häufigkeit von DC_{leu}/Bla^+) und Blastenproliferation unter Hy-Bedingung unter dem Einfluss der Kits im Vergleich zur Kontrolle als im Vergleich zur No-Bedingung. Das wichtigste Ergebnis aus dem Zelllinien-Teil war, dass wir die DC-Generierung unter Hy-Bedingung auf einem vergleichbaren Niveau wie unter No-Bedingung bestätigen konnten.

Im zweiten Teil wurden AML (proben)- und gesunde Proben (PBMNC, WB) parallel unter No (21% O_2) und Hy (ca. 10% O_2) unter Verwendung von $Pici_{-PGE1}$, $Pici_{-PGE2}$ und Kit-I, Kit-K und Kit-M Behandlung kultiviert, gefolgt von DC-Generation (PBMNC, WB), MLC mit den T-Zellen der Probanden und Blasten-Zytotoxizitäts-Assays und Zytokinsekretions-Assay in AML-Proben (WB). Zelluläre Zusammensetzungen (DC-, Blasten-Subtypen und Immunstatus) und funktionelle Ergebnisse wurden verglichen. Wir fanden heraus, dass DCs und ihre Subtypen in vergleichbarer Menge aus gesunden und leukämischen PBMNCs mit $Pici_{-PGE1}$ und $Pici_{-PGE2}$ unter No- und Hy-Bedingungen generiert werden können. Vergleichbare Anteile an DC wurden mit Kit-I, -K, -M aus 16 Gesunden- und 35 AML-WB-Proben generiert. Die DC-Generierung war unter No vs. Hy in gesunden und AML-WB vergleichbar, außerdem produzierten die Kontrollen ohne zugesetzten Response Modifier (w/o) keine DC/DC_{leu}. Interessanterweise induzierten die Kits weder unter No- noch unter Hy-Bedingungen eine Proliferation der Blasten. Nach gemischter Lymphozytenkultur (MLC) von T-Zellen mit Kit-behandelten AML-WB proben unter No vs. Hy mit Kit I, K, M, fanden wir einen signifikanten Anstieg von aktivierten T-Zellen, $CD3^+CD4^+/CD3^+$, T_{prol}, $T_{non\text{-}naive}$, $T_{eff\text{-}em}$ sowie von NK- und iNKT-Zellen und T_{reg},T_{cm} unter No und Hy

Bedingungen. Die DC-, T-Zell- NK-, , iNKT und CIK-Zellwerte nach MLC waren unter physiologischen Hy- vs. No-Bedingungen vergleichbar.

Schließlich zeigten wir eine Zunahme der anti-leukämischen Aktivität von T-Zellen nach Stimulation in MLC mit DC/DC_{leu} enthaltenden Kit-I oder -M behandelten (vs. unbehandelten) WB sowohl unter No- als auch unter Hy-Bedingungen. Die durchschnittliche Lyse war in $MLC^{WB\text{-}DC\ Kit\text{-}I}$ und $MLC^{WB\text{-}DC\ Kit\text{-}M}$ höher als in MLC^{WB}, wobei die Durchschnittswerte der Lyse in $MLC^{WB\text{-}DC\ Kit\text{-}I}$ und $MLC^{WB\text{-}DC\ Kit\text{-}M}$ gleich waren. Weiterhin fanden wir signifikant höhere Mengen an INFγ sezernierenden Zellen des angeborenen und adaptiven Immunsystems nach $MLC^{WB\text{-}DC\ Kit\text{-}I}$ und $MLC^{WB\text{-}DC\ Kit\text{-}M}$ im Vergleich zu MLC^{WB}. Die Ergebnisse waren unter No- und Hy-Bedingungen vergleichbar. Wir fanden eine signifikante, positive Korrelation zwischen den mit Kit-M generierten DC^{+}/WB und der besten anti-leukämischen Reaktivität der T-'Effektorzellen' von $MLC^{WB\text{-}DC\ Kit\text{-}M}$ auch unter Hy-Bedingungen.

Schlussfolgerung: Diese Ergebnisse deuten auf vergleichbare Resultate für die Kultivierung von PB-Zellen mit Kits unter No- vs. Hy-Bedingungen hin (DC-Typen, T-Zell-Subtypen nach MLC, Vermittlung von antileukämischer Reaktivität). Diese Ergebnisse eliminieren die Notwendigkeit, Hy als physiologische Bedingungen für die Herstellung von zellulären Produkten (z. B. für den adoptiven Zelltransfer) bei der Kultivierung von PB-Zellen zu verwenden. Unsere Daten weisen ferner darauf hin, dass die Behandlung von Patienten mit Kit-M und Kit-I die antileukämische Reaktivität in vivo durch einen DC_{leu}-vermittelten Mechanismus verbessern könnte.

III. Abbreviations

AML	Acute myeloid leukemia
APC	Antigen presenting cells or allophycocyanin
Bla^{+}Annexin $V^{+}PI^{-}$	Apoptosis
$Bla_{apo\text{-}CD261}/Bla^{+}$	Blasts' apoptosis
$Bla_{apo\text{-}CD262}/Bla^{+}$	Blasts' apoptosis
$Bla_{CD274\ (PD1)}/Bla^{+}$	Checkpoints on blast
$Bla_{CD279\ (PDL1)}/Bla^{+}$	Checkpoints on blast
$Bla_{prol\text{-}Ipo\text{-}38}/Bla^{+}$	Blasts' proliferation
$Bla_{prol\text{-}CD71}/Bla^{+}$	Blasts' proliferation
BTLA	B and T-cell lymphocyte attenuator
CD	Cluster of differentiation
CIK cells	Cytokine induced killer cells
CR	Complete remission
CSA	Cytokine secretion assay
CTLA-4	Cytotoxic T-cell antigen-4
CTX	Cytotoxicity assay
DC	Dendritic cells
DCs subtypes	Dendritic cells subtypes
$DC_{apo\text{-}CD261}/DC^{+}$	Apoptosis marker expression on DC
$DC_{apo\text{-}CD262}/DC^{+}$	Apoptosis marker expression on DC
DC_{Leu}	Leukemia-derived dendritic cells
Dgn.	First diagnosis

DMSO	Dimethyl sulfoxide
ELN	European Leukemia Network
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward Scatter
GM-CSF	Granulocyte-macrophage-colony-stimulating factor
HSCT	Hematopoietic stem cell transplantation
Hy	Hypoxic condition
IFNγ	Interferon γ
IL-2/6	Interleukin 2/6
intCyt	Intracellular cytokine staining
iNKT cells	Invariant natural killer T-cells
LAA	Leukemia associated antigens
LAMP	Lysosomal associated membrane glycoprotein
MHC	Major histocompatibility complex
MLC	Mixed lymphocyte culture
MLC^{WB}	MLC using WB for T-cell stimulation
$MLC^{WB\text{-}DC\text{-}Kit\text{-}I\_or\_Kit\text{-}M}$	MLC using WB (pretreated with DC-generating protocol ‘Kit-I’ or ‘KitM’) for T-cell stimulation
moAb	Monoclonal antibody
NK cells	Natural killer cells
No	Normoxic condition
OK-432	Picibanil

PBMCs	Peripheral blood mononuclear cells
pAML	Primary AML
PBMNC	Peripheral blood mononuclear cell
PB	Peripheral blood
PBS	Phosphate-buffered saline
PC7	tandem Cy7-PE conjugation
PD-1 (CD274)	Programmed death-1 (PD-1)
PD-L1 (CD279)	Programmed death-Ligand1 (PD-1)
PE	phycoerythrin
PGE1	Prostaglandin
PGE2	Prostaglandin
pt	Patient
rel.	Relapse
rel. a. SCT	Relapse after stem cell transplantation
sAML	secondary AML
SEB	Staphylococcal Enterotoxin B
SSC	Side Scatter
TCR	T-cell receptor
T_h	T helper lymphocytes
TNFα	Tumor necrosis factor α
TRAIL	TNF-related apoptosis ligand
TRAILR	TNF-related apoptosis Receptor
TIM-3	T/cell immunoglobulin mucin-3 lymphocyte activation gene-3
vs.	versus

WB	Whole blood
Ø	on average

Chapter 1: Introduction

IV. Introduction

The most common acute leukemia found in adults is the Acute Myeloid leukemia (AML) (Khwaja, Bjorkholm et. al., 2016), with an overall five-year survival rate in elderly patients of about 28.3% (Yilmaz, Wang et. al., 2019; Döhner, Estey et. al., 2017). This type of cancer is characterized by excessive proliferation of immature and dysfunctional hematopoietic cells (blasts) that spread into the bone marrow (BM), Peripheral Blood (PB), and substitute healthy cells (Sawyers, Denny et. al., 1991; Dores, Devesa et. al., 2012; Azadniv, Myers et. al., 2020).

IV.1 Standard therapies

In general, more than 60% of all patients achieve a complete remission (CR), however, 70-80% of these patients relapse in the following 2 years (Heuser, Ofran et. al., 2020). New immunotherapeutic and immunomodulatory approaches are currently aiming to improve and stabilize the remission and prognosis of patients (Beyar-Katz and Gill 2018). These strategies include the transfer of manipulated immune reactive cells, such as T or NK-cell subsets, Chimeric-Antigen Receptor - T-cells (CAR-T) or of dendritic cells (DC) (Yuan, Song et. al., 2012, Christofi, Baritaki et. al., 2019; Van Acker, Versteven et. al., 2019). Another approach targets directly myeloid blasts by modulation and conversion of blasts to leukemia derived dendritic cells (DC_{leu}) (Schmetzer, Kremser et. al., 2007; Yuan, Song et. al., 2012; Van Acker, Versteven et. al., 2019) that specifically activate antileukemic (T) cells and induces an immunological memory (Kremser, Dressig et. al., 2010; Johnson and Jackson 2014; Amberger, Doraneh-Gard et. al., 2019). One of these options includes targeted therapies, e.g. antibodies directed against cellular targets like checkpoints (e.g. PD-1). The PD-1 receptor (CD279) belongs to the immunoglobulin superfamily, which binds to different ligands including PD-L2 (CD273) and PD-L1 (CD274) as two members from the B7 immunoglobulin superfamily.

IV.2 DC and DC stimulated immunoreactive cells

DCs are a portion of the innate immune system, specialized antigen-presenting cells (APCs), and most important potent stimulators of (T-cell) immunity (Beaulieu, Robbiani et. al., 2002; Wan and Dupasquier 2005; Amberger, Doraneh-Gard et. al., 2019). DC can be generated *ex vivo* from monocytes, a speciality of myeloid blasts (in Whole Blood (WB) or peripheral blood mononuclear cells (PBMNCs)) is that they can be converted into 'leukemia derived DCs' (DC_{leu}) *in vitro* using DC-generation methods. This approach includes the combination of different response modifiers including, myelopoiesis stimulating factors (e.g. Granulocyte-Macrophage-Colony Stimulating Factors (GM-CSF)) (Sabado, Balan et. al., 2017) and mediators for the maturation of "DC/DC_{leu} ((e.g. Prostaglandin (PGE) E_1 or E_2, Tumor-Necrosis-Factor alpha (TNF-α), Interleukin 4 (IL-4), and signals danger factors (e.g. bacterial or nucleic stimuli as Picibanil (OK432) from the Streptococcus Pyogenes which is a Lysis Product)) (Kremser, Dressig et. al., 2010; Johnson and Jackson 2014; Amberger, Doraneh-Gard et. al., 2019). These treatments convert myeloid progenitor cells to DC, presenting DCs' together with patients' blast antigens without induction of blasts' proliferation that resulting in an improved anti-leukemic T-cell reactivity in T-cell-enriched mixed lymphocyte culture (MLC) (Lanzavecchia and Sallusto 2001; Schmetzer, Kremser et. al., 2007; Kremser, Dressig et. al., 2010; Johnson and Jackson 2014; Grakoui, Bromley et. al., 2015).

Moreover, it has been shown that DC/DC_{leu} contribute to stimulation and activation of cells of the immune system (innate: Macrophages, DC, Natural killer cells (NK- cells: $CD56^+CD3^-$)). Fast response is shown by them to an immunologic menace (Robertson, Berzofsky et. al., 2014). The adaptive immune-system includes T-cells ($CD3^+$) and B cells that identify blast-(peptide)-antigens, presented by MHC-I- or MHC-II-(major histocompatibility complex) molecules via DC (Lanzavecchia and Sallusto 2001; Robertson, Berzofsky et. al., 2014; Grakoui, Bromley et. al., 2015).

DC/DC_{leu} mediated reactions lead to increased frequencies of non-Naïve T-cells ($T_{non\text{-}Naïve}$, $CD45RO^+$), proliferating $CD71^+$ or $CD69^+$ T-cells ($T_{prol\text{-}early}$, $T_{prol\text{-}late}$), central memory- T-cells (T_{cm}, $CD45RO^+$-$CCR7^+$), effector-memory T-cells ($T_{eff\text{-}em}$, $CD45RO^+CCR7^-$), and down-regulated frequencies of naïve-T cells (T_{naive}, $CD45RO^-$), and regulatory-T cells (T_{reg}, $CD25^{++}CD127^{low}$) (Schick, Vogt et. al., 2013; Vogt, Schick et. al., 2014; Golubovskaya and Wu 2016). Moreover, the activation of cells from the innate immune system was shown upon DC/DC_{leu} mediated reactions (e.g. increase of NK-, CIK, and iNKT- -cells) (Boeck, Amberger et. al., 2017). Invariant natural killer (iNKT-cells: $6B11^+CD161^+$ or $CD3^+6B11^+$) and cytokine-induced killer (CIK cells: $CD56^+CD3^+$) are characterized by phenotypic and functional structures of T- and NK-cells and thereby connect the innate and adaptive immune system (Wan and Dupasquier 2005; Montoya, Pollard et. al., 2007; Sun and Lanier 2009) but they do not show detection of cells via MHC/TCR mediated activities (Pittari, Filippini et. al., 2015; Boeck, Amberger et. al., 2017).

IV.3 Antileukemic reactions and their monitoring

Antileukemic (leukemia-specific) cytotoxic reactions (achieved after MLC or directly by peripheral blood cells) is exerted by various adaptive and innate immune cells via two principal mechanisms: (i) a direct pathway is mediated through the degranulation of the cytolytic-molecules perforin and granzyme or the interaction of Fas-ligand (FasL) or TNF-related apoptosis-including ligand (TRAIL). (ii) An indirect pathway is mediated through the secretion of TNFα and interferon-gamma (IFNγ) (Rauf, Khatri et. al., 2012; Martinez-Lostao, de Miguel et. al., 2015). The cytotoxicity can be quantified by a functional and non-radioactive cytotoxicity assay (CTX) or detection of leukemia specific T-cells (and subtypes) as well as NK, CIK, and iNKT cells, e.g by an Interferon-γ (IFN-γ) cytokine secretion assay (CSA). Especially IFN-γ is a cytokine produced by $CD4^+$ and $CD8^+$ T-cells as well as by NK cells and plays a critical role in tumour rejection (Hoekstra, Bornes et. al., 2020). Cytotoxic reactions can be quantified by functional non-radioactive cytotoxicity assays (CTX).

leukemia specific cells of the adaptive and innate cells can be detected by an Interferon-γ (IFN-γ) cytokine secretion assay (CSA)(Fousek, Watanabe et. al., 2020; Xu, Zhu et. al., 2020).

IV.4 Physiological Hypoxia and its relevance for immunoreactivity

Hematopoietic stem-cells and leukemic cells reside in the BM at hypoxic O_2 concentrations of about 0.1-1%, in the arterial blood of about 12% or of 4-15% in peripheral venous blood (Marenzana and Arnett 2013; Deynoux, Sunter et. al., 2016). That means that hematopoietic cells are exposed to changing O_2 concentrations during their differentiation (Rieger and Fiegl 2016). It was shown, that Hypoxia might change the reaction profiles of cells (e.g. it could have consequences on the NK-cells' generation) (Vasold, Wagner et. al., 2015). Other authors showed that the antileukemic effect might be suppressed under Hy condition (Kuett, Rieger et. al., 2015). In consequence, the course of leukemic disease could be influenced by Hy-influences in the BM or PB. Moreover, reactions of immune cells prepared *ex vivo* for adoptive transfer under Normoxic (No) conditions might be under/overestimated (Rieger and Fiegl 2016).

The aim of this study was:

1. To generate DC/DC_{leu} from leukemic WB/MNC using DC/DC_{leu} generating protocols and there with stimulating T-cell enriched immunoreactive cells in MLC under physiological (Hypoxic) and Normoxic conditions in MLC.
2. To analyze the effect of DC/DC_{leu} stimulation in MLC on the T-cell composition under physiological (Hypoxic) and Normoxic conditions.
3. To analyze the effect of DC/DC_{leu} stimulation on the antileukemic cytotoxicity via CTX under physiological (Hypoxic) and Normoxic conditions.

4. To investigate and compare the influence of Hy and No condition as well as the soluble and cellular composition of microenvironment on the antigen-expression profiles of AML-blasts, DC or immune reactive cells
5. To correlate functional findings with cellular subtypes and clinical parameters of patients.
6. To deduce protocols or recommendations to generate DC_{leu} from WB or MNC or immune reactive cells after DC_{leu} triggered MLC for adoptive transfer under Hy or No.
7. To deduce a protocol to simulate in vivo situations (using patients' WB and Hy) and to deduce a direct treatment with DC_{leu} generating Kits

Chapter 2: Material and Methods

V. Material and Methods

V.1 Sample Collection

After obtaining informed consent in accordance with the local Ethics Committee (Pettenkoferstr. 8a, 80336 Munich, Ludwig Maximilians University Hospital in Munich; Vote No 339-05), PB or BM samples were provided with the University of Oldenburg, Tuebingen and Augsburg (Table1B). Anticoagulation was performed with Lithium heparin-tubes (7.5 ml, Sarstedt, Nuernberg, Germany) containing standardized concentrations of Heparin. PBMNCs were separated by density gradient centrifugation (density gradient 1.077 g/ml) using the Ficoll Hypaque technique (Biocoll-Separating-solution, Biochrom, Berlin, Germany). PBMNCs were washed and suspended in phosphate-buffered saline (PBS, Biochrom, Berlin, Germany).

$CD3^+$T-cells were isolated by using the MACS technology (Milteney Biotech, Bergisch Gladbach, Germany) as described by manufacturer and us (Kremser, Dressig et. al. 2010). The purity of the resulting T-cells was on average 90.42 % (range 81.13-96.86 %). The quantification of viable cells was conducted via a Neubauer counting chamber by staining the cells with Trypan blue (Biochrom, Berlin, Germany). T-cells were frozen at -80 °C (using DMSO) and thawed according to standardized protocols.

V.2 Patients' characteristics and diagnostics

DC/DC_{leu} were generated from cell lines, PBMNC- and WB-samples obtained from AML patients (n=34) in acute phases of the disease and from healthy donors (n=16). Ø age of AML patients were 74 years (range 21 - 78) and of healthy volunteers 28.1 years (range 21 – 56). The female to male ratio of AML patients was 1:1.2 and of healthy 1:1.3.

The following cell-lines were included in analysis: Mono-Mac 6 (AML-M5), THP-1 (AML-M5), KG-1 (AML-M4) and NB-4 (M3). These cell lines were purchased from the DSMZ (German

collection of Microorganisms Cell Cultures, Braunschweig, Germany) and were cultured according to the manufacturer's instructions (Table 1A).

The classification and diagnosis of patients was based on the FAB ('French-American-British') Classification **(**Table 1B**)**, AML without maturation (M1: n=6), AML with granulocytic maturation (M2: n=7), acute myelomonocytic leukemia (M4: n=8), acute monocytic leukemia (M5: n=8). No FAB classification was available in 5AML cases. Patients presented with primary AML [pAML (n=24)] or with secondary AML [sAML (n=7)]. Patients' stages were: first diagnosis (n=29), relapse (n=4) or relapse after SCT (n=1). Patients' characteristics are given in (Table 1B**)**. Patients were further classified in cytogenetic risk groups based on the **European Leukemia Net Guidelines (ELN)** in 'favorable' (n=11), 'intermediate' (n=8) or 'adverse risk'groups (n=9) (Table 1B).

AML WB samples contained 32.1% (range 7.16 – 92.0%) leukemic 'blasts' ($CD15^+$, $CD33^+$, $CD34^+$, $CD65^+$ and/or $CD117^+$), 13.81% $CD3^+$T cells (range 3.5 - 68.9%), 2.3% $CD19^+$B cells (range 0.4 - 8.4%), 5.8% $CD56^+CD3^-$NK-cells (range 0.8 - 12.4%) and 3.1% $CD14^+$monocytes (range 0.5 - 15%) was obtained by flow cytometry.

AML PBMNCs samples contained Ø 51.3 % leukemic 'blasts' (range 21.0 – 62.0%), 4.2% $CD3^+$T- cells (range 0.3 - 17.3%), 0.9% $CD19^+$B cells (range 0.2– 1.9%), 1.4% $CD56^+CD3^-$NK-cells (range 0.1 - 3.0%) and 2.29% $CD14^+$monocytes (range 0.2 - 4.2%). In cases with aberrant expression of T-, B-, CD56 or monocytoid-antigens or on leukemic 'blasts' count could not be evaluated.

Healthy WB samples contained Ø 56% $CD14^+$monocytes (range 4.4 - 8.5%), 19.4% $CD3^+$T-cells (range 13.6 - 26.3%), 3.4% $CD56^+CD3^-$NK-cells (range 2.3 - 6.9%) and 2.3% $CD19^+$B cells (range 0.8 - 4.8%).

Healthy PBMNCs samples contained Ø $CD14^+$monocytes 6.6% (range 2.6 - 9.4%), 33.3% $CD3^+$T-cells (range 7.1 - 46.7%), 4.8% $CD56^+CD3^-$NK-cells (range 0.08 - 4.3%) and $CD19^+$B cells 21% (range 1.3 - 3.3%) (Table 1C).

Table 1A: Characteristics of AML Cell Lines

Name	Subtype AML	Original Source	Blast phenotype (CD)	Fusion gene
NB-4	M3	PB	13,**15**,**33**,38	PML-RARA
Mono-Mac-6	M5	PB	**13**,14,15,33,68	KMT2A-MLLT3
THP-1	M5	PB	**33**,13,14,15	KMT2A-MLLT4
KG	M4	PB	13,**15**,**33**,11	FGFR1OP2-FGFR1

Table 1B: Characteristics of AML Patients

	Pt #	Age at dgn.	Sex	Subtype FAB	Stage	Blast phenotype (CD)	Blasts in PB	ELN-Risk Stratification	Experiments Performed
AML	P1426	61	f	p/M5	dgn	13, 33, **34**, 64, **117**	40	adverse	DC^{WB}
	P1427	52	m	p/M2	dgn	13, 33, **117**	96	favorable	DC^{WB}, MLC, CTX
	P1434	79	m	p/M5	dgn	13, 33, **34**, **117**	70	favorable	DC^{WB}
	P1439	59	f	p/M5	rel.	33, **34**, **117**, 13, 64, 7, 56	58	n.d.	DC^{WB}
	P1441	59	f	s/M4	dgn	33, 13, 14, 65, **117**, **34**	15	favorable	DC^{WB}
	P1442	60	m	s/M4	dgn	33, 13, 14, 65, **117**, **34**	81	favorable	DC^{WB}
	P1443	73	f	P/M4	dgn	**117**, 33, 61	14	intermediate	DC^{WB}
	P1444*	64	m	p/ M1	dgn	13, 33, **34**, **117**	28	favorable	DC^{WB}, MLC, CTX
	P1447	36	f	p/M1	dgn	33, 65, 15, **34**, **117**	63	favorable	DC^{WB}, MLC, CTX
	P1452	21	m	p/M5	dgn	**34**, **117**, 33, 13	33	adverse	DC^{WB}
	P1459	44	m	p/M4	dgn	45, **34**, **117**, 33, 13	50	intermediate	DC^{WB}
	P1460	54	m	p/M4	dgn	33, 11b, 64, 38, 11, 4, **56**	7	favorable	DC^{WB}
	P1461	78	f	p/M2	dgn	**34**, 33, 15, **65**	61	adverse	DC^{WB}
	P1463/2	78	f	s/M	dgn	**34**, 64, 14, 33, 13	30	adverse	DC^{WB}
	P1471	60	f	p/M1	rel.a.SCT	13, 33, **34**, 56, 14	30	adverse	$DC^{WB, PBMNC}$, MLC, CTX
	P1472	39	m	p/M2	dgn	**15, 117**	30	favorable	$DC^{WB, PBMNC}$, MLC, CTX
	P1473	33	f	p/M2	dgn	33, 13, **34**, **117,** 56	83	favorable	$DC^{WB, PBMNC}$, MLC, CTX
	P1474	73	m	p/M2	dgn	33, 13, **34, 117**	84	adverse	$DC^{WB, PBMNC}$
	P1475	70	m	s/M	rel.	13, 33, **117**, **34**, 56, 64, 14	80	intermediate	$DC^{WB, PBMNC}$, MLC,CTX
	P1476	78	m	p/M1	rel.	13, 33, **34**, **117**	60	n.d.	DC^{PMNC}
	P1480	69	m	s/M	dgn	13, 33**, 117**	38	adverse	$DC^{WB, PBMNC}$
	P1481	66	m	n.d.	dgn	**34**, 13, 33**, 117**	12	intermediate	$DC^{WB, PBMNC}$, MLC, CTX
	P1482	63	f	p/M4	dgn	**117, 13**, 64, 11	12	intermediate	$DC^{WB, PBMNC}$, MLC
	P1483	75	m	p/M5	dgn	**117,** 33, 64	40	adverse	DC^{WB}, MLC, CTX
	P1484	77	m	p/M5	dgn	13**, 34**, 33, 64	55	n.d.	$DC^{WB, PBMNC}$, MLC
	P1486	44	m	p/M4	dgn	**34, 117,** 13, 33, 45, 11, 64	46	n.d.	DC^{WB}
	P1487	77	m	p/M1	dgn	13, 33**, 34, 117**	50	intermediate	$DC^{WB, PBMNC}$, MLC, CTX
	P1492	50	f	s/M2	dgn	13, 33**, 34, 117**	43	n.d.	DC^{WB}, MLC, CTX
	P1494	55	f	p/M5	dgn	13, **34,** 7**, 117**	87	adverse	DC^{WB}, MLC, CTX
	P1502	74	m	n.d.	dgn	13, 33, **117, 34**	53	n.d.	DC^{WB}
	P1521	56	m	p/M4	dgn.	13, 33, **34, 117**	66	intermediate	DC^{WB}, MLC
	P1522	47	m	p/M2	rel.	13, **34**, 38, 71**, 117**	56	Intermediate	DC^{WB}
	P1532	47	f	s /M	dgn.	**117, 33**, 38, 56, 4	61	favorable	DC^{WB}, MLC, CSA
	P1536	63	m	s/M5	dgn	4, 56**, 14, 34**	25	favorable	DC^{WB}, MLC, CTX, CSA

Legend: Pat.# Patient's number; f= female, m= male, dgn= first diagnosis, CD= cluster of differentiation, DC= DC culture of WB and/ or MNC, MLC^{WB} = mixed lymphocyte culture with WB, CTX= Cytotoxicity (fluorolysis) assay, rel.= relapse, a.SCT= relapse (after stem cell transplantation), s= secondary AML, p= primary AML, bold blasts markers used to detect DC_{leu} = antibody used for (co)expression analyses, n.d. = no data

Table 1C: Characteristics of Healthy samples

Sample type	Patient ID	Age	Sex	Sample used
Healthy	P1420	25	f	DC^{WB}
	P1425	37	m	DC^{WB}, MLC
	P1428	26	m	DC^{WB}, MLC
	P1429	21	m	DC^{WB}
	P1431	28	m	DC^{WB}
	P1436	56	f	DC^{WB}
	P1438	27	m	DC^{WB}
	P1440	26	f	DC^{WB}
	P1445	28	f	DC^{WB}, MLC
	P1478	32	m	DC^{WB}
	P1479	27	f	DC^{WB}
	P1484	24	m	$DC^{WB, PBMNC}$, MLC
	P1485	26	f	$DC^{WB, PBMNC}$, MLC
	P1486	21	m	DC^{WB}, MLC
	P1514	21	f	DC^{WB}, MLC
	P1519	25	m	$DC^{WB, PBMNC}$, MLC

Legend: P.# healthy's number; f= female, m= male, DC= DC culture of WB and/ or MNC, MLC^{WB} = mixed lymphocyte culture with WB

V.3 Incubation

To determine a difference between standard incubation conditions and physiological hypoxic conditions as would occur in the bone marrow and an effect on DC/DC_{leu} generation, T-cells, and their subpopulations, all DC cultures and MLC cultures were set up in parallel under standard conditions (37°C, 21% O_2 and 5% CO_2) and hypoxic conditions (37°C, 10% O_2 and 5% CO_2) (Sironi, Wagner et. al. 2015, Rieger and Fiegl 2016).

V.4 Generation of DC from AML cell lines

DC/DC_{leu} were generated from AML cells with the DC/DC_{leu} generating protocols Kit-I, Kit-K and Kit-M (Kugler, Deen et. al.). Therefore, 500µl cell suspensions were pipetted in 12-multiwell plates and diluted 1:2 in RPMI medium (Lonza, Basel, Swiss). Response modifiers and immune modulating factors were added to cultures as described below. A culture without added response modifiers served as a control. All response modifiers used for the DC/DC_{leu} generation are approved for human treatment. Compositions of DC/DC_{leu} generating protocols are given in (Table 2).

Table 2: Compositions of DC/DC_{leu} generating protocols

Cocktail	Component	Concentration	Sources of DC/DC_{leu}	Mode of action	Culture time	Reference
Picibanil$_{-PGE1}$ ($Pici_{-PGE1}$)	GM-CSF	500U/mL	PBMNC	GM-CSF: induction of myeloid (DC-) differentiation	7-10 Days	(Amberger et. al., 2019)
	IL-4	250U/mL				
	OK-432	10µg/mL				
	PGE1	1 µg/mL				
Picibanil$_{-PGE2}$ ($Pici_{-PGE2}$)	GM-CSF	500U/mL	PBMNC	IL-4: induction of DC-differentiation	7-10 Days	(Amberger et. al., 2019)
	IL-4	250U/mL				
	OK-432	10µg/mL				
	PGE2	1µg/mL				
Kit-I*	GM-CSF	800U/mL	WB, Cell lines	Picibanil (OK-432): lysis product from streptococcus pyogenes; stimulates DC-differentiation	7-10 Days	(Kugler et. al., 2019)
	OK-432	1 µg/mL				
Kit-K*	GM-CSF	800U/mL	WB, Cell lines	PGE2: increases CCR7-expression and enhances DC-migration	7-10 Days	(Kugler et. al., 2019)
	PGE2	1 µg/mL				
Kit-M*	GM-CSF	800U/mL	WB, Cell lines	PGE1: effects are comparable to PGE2	7-10 Days	(Kugler et. al., 2019)
	PGE2	10 µg/mL				

DC dendritic cells; DC_{leu} dendritic cells of leukemic origin; GM-CSF granulocyte macrophage colony stimulating factor; IL-4 interleukin 4; OK-432 Picibanil; PGE2 prostaglandin E2; PGE1 prostaglandin E1; PBMC peripheral blood mononuclear cells, WB whole blood. * 15 801 987.7-1118 European Patent.

V.5 Generation of DC from isolated MNC or WB

DC/DC_{leu} were generated from 3-4x10^6 isolated MNC from healthy donors or AML patients using Pici-$_{PGE1}$ and Pici-$_{PGE2}$ (Table 2) as described previously (Kremser, Dressig et. al. 2010, Deen, Hirn-Lopez et. al. 2013). MNC were diluted in 2ml serum-free X-Vivo15-medium (Lonza, Basel, Swiss) and were placed into 12-multiwell plates (Thermo Fisher Scientific, Darmstadt, Germany). Response modifier was added as described below. Half medium exchange was carried out after 3-4 cell culture days. A culture without added response modifiers served as a control.

DC/DC_{leu} were generated from healthy and leukemic WB (presenting the physiological cellular and soluble composition of the individual samples) with the DC/DC_{leu} generating protocols Kit- I, Kit-K, and Kit-M (Kugler, Deen et. al.). Therefore, 500µl WBs were pipetted in 12-multiwell-plates and diluted 1:2 in X-Vivo-15-medium (Lonza, Basel, Swiss) to imitate the physiological conditions. Response modifiers and immune-modulating factors were added to cultures as described below. A culture without added response modifiers served as a control. All response modifiers used for the DC/DC_{leu} generation are approved for human treatment. Compositions of DC/DC_{leu} generating protocols are given in (Table 2).

Picibanil-$_{PGE1}$ (Pici-$_{PGE1}$)

DC/DC_{leu} were generated from PBMNCs with the DC/DC_{leu} generating protocol Pici-$_{PGE1}$ -containing 500U/ml granulocyte-macrophage colony-stimulation factor (GM-CSF, Sanofi-Aventis, Frankfurt, Germany) and 250U/ml Interleukin-4 (IL-4) (PeproTech, Berlin, Germany). After 6-7 days, 10µg/ml Picibanil (OK 432) - a lysis product from Streptococcus pyogenes, which has unspecific immune modulatory effects (Chugai Pharmaceutical Co., Kajiwara, Japan) and 1µg/ml Prostaglandin E_1 (PGE_1) (PeproTech, Berlin, Germany) were added. After 7-10 days of incubation, cells were harvested and used for subsequent experiments.

Picibanil-$_{PGE2}$ (Pici-$_{PGE2}$)

DC/DC$_{leu}$ were generated from PBMNCs with the Pici-$_{PGE2}$ DC/DC$_{leu}$ generating protocol, with the same composition as given above for Pici-$_{PGE1}$, however substituting PGE_1 by PGE_2 (PeproTech, Berlin, Germany) (Sato, Takayama et. al. 2003, Kremser, Dressig et. al. 2010).

Kit-I

DC/DCleu were generated with Kit I from WB using 800 U/mL GM-CSF and 10 µg/mL Picibanil (Kugler, Deen et. al.). After 2–3 days the same amounts of cytokines were added and after in total 7–10 days of incubation cells were harvested and used for subsequent experiments.

Kit-K

Kit-K consisted of 800U/ml GM-CSF and 1µg/ml PGE_2 and was used to generate DC/DC$_{leu}$ from WB (Kugler, Deen et. al.). Incubations were performed in analogy to Kit-I.

Kit-M

The generation of DC/DC$_{leu}$ from WB with Kit-M was performed using 800U/ml GM-CSF and 1µg/ml PGE_1 (Kugler, Deen et. al.). Incubations were performed in analogy to Kit-I.

V.6 Cell-characterization by flow cytometry

Characterization of specific leukemic ,blasts, T-cell subsets, B cells, monocytes DC/DC$_{leu}$ subsets in the PBMNC- and WB-fractions before and after different cultures via a flow cytometric analyses and Panels with several monoclonal antibodies (moAbs) labeled with Fluorescein isothiocyanate (FITC), phycoerythrin (PE), tandem Cy7-PE conjugation (Cy7-PE), or allophycocyanin (APC) were used. Antibodies were provided by Beckman Coulter, Krefeld, Germany ([a]), Becton Dickinson, Heidelberg, Germany ([b]), Miltenyi Biotech, Bergisch Gladbach, Germany ([c]), Thermo Fisher, Darmstadt, Germany

([d]) and Santa Cruz Biotechnology, Heidelberg, Germany ([e]). FITC-conjugated moAbs against CD3[a], CD15[a], CD33[a], CD34[a], CD45RO[a], CD65[a], CD71[a], CD83[a], CD161[b], CD261[a] (TRAIL-R1), Annexin V Apoptosis Detection Kit and IPO-38[e] were used. To detect CD3[a], CD4[a], CD19[a], CD33[a], CD34[a], CD56[a], CD80[b], CD83[a], CD117[a] and CD206[a] PE-conjugated moAbs were used. MoAbs against CD3[a], CD4[a], CD14[b], CD15[b], CD19[a], CD33[a], CD34[a], CD56[a], CD65[c], CD80[b], CD117[a], 6B11[b], IFNy[d], CD274[a] and CD197[b] were labeled with Cy7-PE. APC-labeled moAbs against CD3[a], CD4[b], CD14[a], CD15[b], CD34[a], CD45RO[d], CD56[a], CD65[c], CD69[b], CD83[b], CD86[g], CD117[a], CD206[b], 6B11[d] and CD209[b], CD279[b], CD262[b] (TRAIL-R2) were used. 7AAD[b] was used to detect dead cells. To stain intracellular antigens (e.g., IPO-38, IFNy) the FIX & PERM® Cell Fixation and Cell Permeabilization Kit (Thermo Fisher Scientific, Darmstadt, Germany) were used.

Preparation of the cells for FACS measuring was as follows: The cells were resuspended in a solution of PBS (Biochrom, Berlin, Germany) and 10 % of fatal calf serum (FCS, Biochrom, Berlin, Germany) to inhibit unspecific binding. After the cells were added to the moABs they were incubated for 15 min in the dark at room temperature. The cells were then washed with PBS, centrifuged, and resuspended in 100-200 µL PBS. 5000 events were analyzed with a fluorescence-activated cell sorting Flow-Cytometer (FACSCalibur™) and Cell-Quest-data-acquisition and analysis software (Becton Dickson, Heidelberg, Germany). Isotype controls were conducted according to the manufacturer's instructions.

The quantification of DC_{leu} was made possible by staining the cells with patient's specific 'blast' staining antibodies (e.g., CD15, CD34, CD65, and CD117) according to diagnostic reports in combination with DC-staining antibodies (e.g., CD80, CD83, CD86, CD206, and CD209), which were not expressed on blasts before culture. For analysis and quantification of DCs and DC_{leu} in the total- or in the cell-subtype fractions after DC/DC_{leu} cultures we used a refined gating strategy (Schmetzer, Kremser et. al. 2007, Kremser, Dressig et. al. 2010). A prerequisite for the analysis was a percentage of

≥5% DC and ≥10 % blasts in the PBMNC/WB fractions. DC_{leu} were quantified in the total cell fraction (DC_{leu}/PBMNC or WB), in the DC-fraction (DC_{leu}/DC^+) or in the blast fraction, to quantify the amount of blasts converted to DC_{leu} (DC_{leu}/bla^+). The amount of mature DCs [DC co-expressing the migration marker CCR7 (CD197)] in the DC fraction after culture (DC_{mig}/DC^+) was quantified in cases with ≥5% DC^+/cells. A refined gating strategy was used to detect non-converted proliferating blasts in the PBMNC- or WB-fractions (Bla_{prol}/PBMNC or WB) after DC/DC_{leu} culture (Plett, Amberger et. al. 2017). Proliferating blasts were characterized by the co-expression of CD71 or IPO-38 without co-expression of DC-markers. Apoptosis-markers (apoptotic blasts or DC ($Bla_{apo\text{-}CD261}/Bla^+$, $Bla_{apo\text{-}CD262}/Bla^+$, $DC_{apo\text{-}CD261}/DC^+$, $DC_{apo\text{-}CD262}/DC^+$, $DC_{leu\ apo\text{-}CD261}/DC_{leu}^+$, $DC_{leu\ apo\text{-}CD262}/DC_{leu}^+$) and checkpoint markers (Bla^+CD279^+ or $CD274^+$) were also tested as mentioned in (Table 3A).

Table 3A: Subtypes of monocytes, blasts and DCs as evaluated by Flow cytometry

	Name of Subgroup	Referred to	Surface marker	Abbreviation	Explanatory Note Premise for Analysis	Reference
Monocytes	$CD14^+$ monocytes	PBMNC, WB	$CD14^+$	Mono/PBMNC or WB		(Boeck, Amberger et. al. 2017)
Blasts and DC/DC_{leu}	Leukemic blasts	cells (PBMNC, WB)	Bla^+ (CD15, CD33, CD34, CD65, CD117)	Bla^+/ cells (PBMNC, WB		(Schmetzer, Kremser et. al. 2007)
	Dendritic cells	cells (PBMNC, WB)	DC^+(CD80, CD83, CD86, CD206, CD209)	DC^+/cells (PBMNC, WB)		(Schmetzer, Kremser et. al. 2007)
	Leukemia derived DC	cells (PBMNC, WB	DC^+Bla^+	DC_{leu}/ cells (PBMNC, WB)	≥ 5% DC^+ in cells	(Schmetzer, Kremser et. al. 2007)
	DC_{leu} in DC fraction	DC^+	DC^+Bla^+	DC_{leu}/DC^+	≥ 10% DC^+ in cells	(Schmetzer, Kremser et. al. 2007)
	DC_{leu} in leukemic blast fraction	Bla^+	DC^+Bla^+	DC_{leu}/Bla^+	≥ 10% DC^+ in cells	(Schmetzer, Kremser et. al. 2007)
	Mature DC in DC fraction	DC^+	DC^+CD197^+	DC_{mig}/DC^+	≥ 10% DC^+ in cells	(Kremser, Dressig et. al. 2010)
	Proliferating leukemic blasts	WB	$Bla^+DC^-CD71^+$	$Bla_{prol\text{-}CD71}/Bla^+$		(Plett et. al. 2017)
	Proliferating leukemic blasts	WB	Bla^+DC^- IPO-38^+	$Bla_{prol\text{-}IPO38}$/ Bla^+		(Plett et. al. 2017)
	Annexin V staining, / propidium iodide (PI)	Bla^+	Bla^+Annexin V^+PI^-	Bla^+Annexin V^+PI^- /Bla^+	Apoptotic	(Rieger et. al., 2011)
	Dendritic cells expressing CD261, CD261	DC^+	DC^+ $CD261^+$ DC^+ $CD262^+$	$DC_{apo\text{-}CD261}/DC^+$ $DC_{apo\text{-}CD262}/DC^+$	DC expressing apoptosis markers on DC^+	(Pathak, Sköld et. al. 2012)
	Leukemia derived DC expressing CD261, CD261	DC_{leu}^+	DC_{leu}^+ $CD261^+$ DC_{leu}^+ $CD262^+$	$DC_{leu\ apo\text{-}CD261}/DC_{leu}^+$ $DC_{leu\ apo\text{-}CD262}/DC_{leu}^+$	DC_{leu} expressing apoptosis markers on DC_{leu}^+	(Pathak et. al., 2012)
	Checkpoint markers CD279 or CD274 expressing blasts	Bla^+	Bla^+CD279^+ or $CD274^+$	$Bla_{CD274(PD1)}/Bla^+$ $Bla_{CD279(PDL1)}/Bla^+$	Blasts expressing checkpoint markers on blasts	(Sehgal, Whiteside et. al. 2015)

Surface marker combinations for the analysis of DC and DC_{leu} (including subsets) after flow cytometric staining with fluorochrome labelled antibodies are given. Cells were analysed before and after different cultures.

For the analysis and quantification of **T-, iNKT-, NK- and CIK-cells/-subtypes** before or after MLC cells were quantified in the total cell-fraction (e.g: $CD3^+$/cells) or in the subpopulations (e.g: $6B11^+CD3^+/CD3^+$). According to their expression-profiles we quantified proportions of immune-reactive cells as given in (Table 3B): **T-cells::** $CD3^+$, $CD8^+$, $CD4^+$(Montoya, Pollard et. al. 2007, Schick, Vogt et. al. 2013, Vogt, Schick et. al. 2014). **CIK-cells**: $CD3^+CD56^+$ or $CD3^+CD161^+$ cells. **NK-cells**: $CD3^-CD56^+$ or $CD3^-CD161^+$ cells (Montoya, Pollard et. al. 2007, Pittari, Filippini et. al. 2015). **iNKT-cells:** $6B11^+CD3^+$, $6B11^+CD161^+$. $\mathbf{T_{prol}}$ $CD3^+CD71^+$; $\mathbf{T_{naive}}$ $CD45RO^-CD3^+$; $\mathbf{T_{CD4^+\ naive}}$ $CD3^+CD4^+CD45RO^-$; $\mathbf{T_{CD8^+\ naive}}$ $CD3^+CD8^+CD45RO^-$; $\mathbf{T_{non\text{-}naive}}$ $CD45RO^+CD3^+$; $\mathbf{T_{CD8^+non\text{-}naive}}$ $CD3^+CD8^+CD45RO^+$; $\mathbf{T_{CD4^+non\text{-}naive}}$ $CD3^+CD4^+CD45RO^+$; $\mathbf{T_{eff\text{-}em}}$ $CD3^+CD45RO^+CCR7^-$; $\mathbf{T_{cm}}$ $CD3^+CD45RO^+CCR7^+$; $\mathbf{T_{prol}}$ $CD71^+CD3^+$; $\mathbf{T_{non\text{-}naive}}$ $CD45RO^+CD3^+$; $\mathbf{T_{CD8^+non\text{-}naive}}$ $CD3^+CD8^+CD45RO^+$; $\mathbf{T_{reg}}$ ($CD25^{++}CD127^{low}CD4^+/CD8^+$; PD-(L) 1^+ ($CD274^+$ /$CD279^+$ $CD3^+$) T-cells (Montoya, Pollard et. al. 2007) (Table 3B).

Table 3B: Subtypes of T-/iNKT-/NK-/CIK-cells as evaluated by flow cytometry

	Names of Subgroups	referred to	Surface Marker	Abbreviation	Explanatory Note	Reference
T-cells	$CD3^+$ pan-T cells	WB	$CD3^+$	$CD3^+$/cells	-	(Schick, Vogt et. al. 2013)
	$CD4^+$ co-expressing T-cells	$CD3^+$	$CD3^+CD4^+$	$CD3^+$ $CD4^+$/ $CD3^+$	$CD4^+$ T-cells	(Schick, Vogt et. al. 2013)
	$CD8^+$ co-expressing T-cells	$CD3^+$	$CD3^+CD8^+(=CD4^-)$	$CD3^+$ $CD8^+$ / $CD3^+$	$CD8^+$ T-cells	(Schick, Vogt et. al. 2013)
	Naive T-cells	$CD3^+$	$CD3^+CD45RO^-$	$T_{naive}/CD3^+$	Unprimed T-cells	(Vogt, Schick et. al. 2014)
	Non-naive T-cells	$CD3^+$	$CD3^+CD45RO^+$	$T_{non\text{-}naive}/CD3^+$	primed T-cells	(Vogt, Schick et. al. 2014)
	Central (memory) T-cells	$CD3^+$	$CD3^+CCR7^+CD45RO^+$	$T_{cm}/CD3^+$	Long-term immunity	(Vogt, Schick et. al. 2014)
	Effector (memory) T-cells	$CD3^+$	$CD3^+CCR7^+CD45RO^+$	$T_{eff\text{-}em}/CD3^+$	Memory + effector T-cells	(Vogt, Schick et. al. 2014)
	Early proliferating T-cells	$CD3^+$	$CD3^+CD69^+$	$T_{prol\text{-}early}/CD3^+$	Proliferating T-cells	(Vogt, Schick et. al. 2014)
	Late proliferating T-cells	$CD3^+$	$CD3^+CD71^+$	$T_{prol\text{-}late}/CD3^+$	Proliferating T-cells	(Vogt, Schick et. al. 2014)
	IL-2-R^+IL-7-R^{low} expressing $CD8^+$ T-cells	$CD3^+$	$CD3^+CD8^+CD25^{++}CD127^{low}$	$CD8^+$ $T_{reg}/CD8^+$	$CD8^+$ Regulatory T-cells	(Vogt, Schick et. al. 2014)
	IL-2-R^+IL-7-R^{low} expressing $CD4^+$ T-cells	$CD3^+$	$CD3^+CD4^+CD25^{++}CD127^{low}$	$CD4^+$ $T_{reg}/CD4^+$	$CD4^+$ Regulatory T-cells	(Vogt, Schick et. al. 2014)
	T cells expressing $CD274^+$ or $CD279^+$	$CD3^+$	$CD3^+$ $_{CD274}{}^+$ $CD3^+$ $_{CD279}{}^+$	$CD3^+$ $_{CD274}$ / $CD3^+$ $CD3^+$ $_{CD279}$ / $CD3^+$	checkpoint marker expressing T-cells	(Sag et. al., 2019)
CIK cells	$CD3^+CD56^+$ CIK cells	MLC	$CD3^+CD56^+$ $CD3^+CD161^+$	$CD3^+CD56^+$/ MLC $CD3^+CD161^+$/ MLC	Cells expressing T-cell (CD3) and NK cell (CD56) markers	(Boeck, Amberger et. al. 2017)
NK cells	$CD3^-CD56^+$ NK cells	MLC	$CD3^-CD56^+$ $CD3^-CD161^+$	$CD3^-CD56^+$/ MLC $CD3^-CD161^+$/ MLC	Cells expressing NK (CD56 or CD161), but not T-cell markers (CD3)	(Boeck, Amberger et. al. 2017)
iNKT cells	$6B11^+$ iNKT cells	MLC	$6B11^+$	$6B11^+$/ MLC	iNKT cells	(Boeck, Amberger et. al. 2017)
	$CD3^+$ co-expressing $6B11^+$ iNKT cells	MLC or $6B11^+$	$CD3^+6B11^+$	$CD3^+6B11^+/6B11^+$ $CD3^+6B11^+$/MLC	T cell like iNKT cells in iNKT cells	(Boeck, Amberger et. al. 2017)

Surface-marker-combinations as well as T-/iNKT-/NK-/CIK-cell-subtypes after flow cytometric staining with fluorochrome labelled-antibodies are given.

V.7 Mixed lymphocyte culture (MLC) of T-cell enriched immune reactive cells with DC/DC_{leu} generating from WB

During MLC, $1x10^6$ T-cells (effector T-cells) from AML patients (n=15) and healthy controls (n=8) were co-cultured with different DC/DC_{leu}-generating protocols in 12-multiwell-tissue-culture plates (Thermo Fisher Scientific, Darmstadt, Germany). A control without the addition of a DC/DC_{leu} generating protocol (MLC^{WB}) was also placed in the culture plate. The final volume of each culture adjusted to 1 mL with MLC-medium. After 2-3 days 50U/ml IL-2 was added to all cultures. Cells were harvested after 6-7 days the resulting cell suspensions were used for flow cytometric analysis. Before and after culture different T-cell subsets in MLC were quantified by flow cytometry (Table 2).

V.8 Cytotoxicity Fluorolysis Assay (CTX)

To analyze the blast lytic efficiency of T-cell enriched immunoreactive cells after MLC^{WB-DC} and MLC^{WB} a Fluorolysis assay was performed (Grabrucker et. al. 2010). Therefore, effector cells were co-cultured 1:1 with thawed blast-containing target cells. The lytic activity was analysed after 3 h and 24 h at 37 °C with 5 % CO_2 and 21 % O_2 and 10% O_2. As a control, effector- and target-cells were cultured separately under the same conditions. The cells were stained for 15 mins in the dark with FITC-, PE- and APC-conjugated ‘blast’ specific target cell antibodies, before culture. The effector- and target cells of the control sample were combined on ice shortly before flow cytometric analyses. To evaluate the number of viable blasts, cultures were harvested after 3 and 24 h. Subsequently, they were resuspended in PBS containing 7AAD (Becton, Dickson, Heidelberg, Germany) and a specific number of Fluorosphere beads (Beckman Coulter, Krefeld, Germany) were added. To analyze the cells a refined gating strategy was used, (Grabrucker, Liepert et. al. 2010) which meant that the viable target cells were gated in a Forward Scatter (FSC) $7AAD^-$ gate. The analysis was conducted via a fluorescence-activated cell sorting Flow-Cytometer (FACS Calibur™) and Cell-Quest-Data-acquisition and analysis software (Becton Dickson, Heidelberg, Germany). The lytic activity was determined by calculating the difference

in the percentage of viable target cells in the culture with co-cultured effector cells and in the control sample. The blast lysis of effector cells was determined by the percentage of viable target cells in the culture with co-cultured effector and target cells (after 3 and 24 h) and compared to the percentage of the control sample.

V.9 Cytokine secretion assay (CSA) - IFN-γ secretion assay

A cytokine secretion assay (CSA) was used to evaluate the amount of IFN-γ secreting cells in leukaemic WB after MLC. With the help of an IFNy Catch Reagent, connecting IFNy secretion to its producing cell, and an IFNy-specific PE-conjugated IFNy Detection Antibody, cells were primed to enable the flow cytometric analysis of IFNy secretion. Cells were counterstained with moAbs labelled with either FITC, Cy7-PE, and APC. A refined gating strategy was used to detect and quantify IFNy secreting cells (Table 3C).

Table 3C: IFNy secreting cells and subsets

	Name of Subgroups	**Referred To**	**Abbreviation**	**Surface Marker**	**Reference**
IFNy secreting cells	IFNy secreting $CD3^+$ pan T-cells	$CD3^+$	$CD3^+IFNy^+/CD3^+$	CD3+IFNy+	(Klauer et. al., 2020)
	IFNy secreting $CD4^+$-coexpressing T-cells	$CD3^+CD4^+$	$CD3^+CD4^+IFNy^+/CD3^+CD4^+$	$CD3^+CD4^+IFNy+$	(Klauer et. al., 2020)
	IFNy secreting $CD8^+$-coexpressing T-cells	$CD3^+CD8^+$	$CD3+CD8^+IFNy^+/CD3^+CD8^+$	$CD3^+CD8^+IFNy^+$	(Klauer et. al., 2020)
	IFNy secreting $CD3^-CD56^+$ NK cells	$CD3^-CD56^+$	$CD3^-CD56^+IFNy^+/CD3^-CD56^+$	$CD3^-CD56^+IFNy^+$	(Klauer et. al., 2020)
	IFNy secreting $CD3^-CD161^+$ NK cells	$CD3^-CD161^+$	$CD3^-CD161^+IFNy^+/CD3^-CD161^+$	$CD3^-CD161^+IFNy^+$	(Klauer et. al., 2020)
	IFNy secreting $6B11^+$ iNKT cells	$6B11^+$	$6B11^+IFNy^+/6B11^+$	$6B11^+IFNy^+$	(Klauer et. al., 2020)

V.10 Apoptosis

Apoptosis of cells (Table 3A) was analysed using the FITC-Annexin V Apoptosis Detection Kit I (BD Pharmingen, Heidelberg, Germany). Analysis of Annexin-V expression was performed using BD Cell Quest Pro software. After digest with 1µg/ml RNAse A (Sigma-Aldrich, St LouisMO, USA) cells were stained with 50µg/ml PI (Sigma-Aldrich, St Louis, MO, USA), and DNA content was quantified by flow cytometry. Flow cytometric data were acquired using a FACS Calibur cytometer (BD Biosciences, San Jose, USA).

V.11 Cell cycle

The cell cycle profile of cell line samples was determined by staining of DNA with PI- fluorescent dyes. PI intercalates into the groove of double-stranded DNA producing a highly fluorescent signal. As PI can also bind to double-stranded RNA, the cells must be treated with RNase for DNA resolution. We associated a fixation (paraformaldehyde) with PI staining. The PI staining of DNA, allowed the detection of cells in G0/G1, S phase, and G2/M.

V.12 Quantitative PCR (Real Time PCR)

Total RNA was isolated from 10^6 of each cell line using MagNA Pure LC mRNA HS Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. Then cDNA was synthesized from 1µg aliquots of total RNA in a 20 µL standard reaction mixture using SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen, Camarillo, CA, USA) according to manufacturer's instructions. Quantitative Real-time polymerase chain reaction (RT-PCR) was performed using the 7900HT Fast Real-Time PCR System (Applie Biosystems, Waltham, MA, USA) with 2 µL of cDNA, Fast SYBR® Green Master Mix (Applied Biosystems, Waltham, MA USA). We checked the expression of the fusion gene related to each cell line in Normoxic vs Hypoxic condition. Furthermore, β-actin (Eurofins

Scientific, Luxembourg, Luxembourg), was used as a reference for the normalization of ΔCt values. The PCR experiment was performed in the lab for leukaemia diagnostics Med III, university hospital Großhadern.

V.13 Statistical Methods

Mean ± standard derivations are given. Statistical comparisons of two groups were performed using the two-tailed t-test (in cases with data normally distributed) and the Mann-Whitney-Wilcoxon-Test (in cases with data not normally distributed). Statistical analyses were performed with Microsoft Excel 2010® (Microsoft, Redmond, Washington, USA). Differences were evaluated as 'not significant' in cases with p-values >0.1, as 'tendentially significant' (significant*) with p-values between 0.1 and 0.05, as 'significant' (significant**) with p-values between 0.05 and 0.005 and as 'highly significant' (significant***) with p-values <0.005. Correlation was defined as "negligible" with r values .00 to .30 (-.00 to -.30), as "low" with r values .30 to .50 (-.30 to -.50), as "moderate" with r values .50 to .70 (-.50 to -.70) and as "high correlation" with r values .70 to 1.00 (-.70 to -1.00). Figures were created with GraphPad Prism7© (GraphPad Software, California, USA).

Chapter 3: Results

VI. Results

VI.1 Generation of DC and DC_{leu} using blast modulatory Kits under Normoxic vs. Hypoxic conditions

We tested physiological cell culture conditions using AML cell lines under Normoxic (No, 21%) vs. Hypoxic (Hy, 6% O_2) conditions. Culturing healthy or AML PBMNCs (in Pici-$_{PGE1}$ / Pici-$_{PGE2}$) and WB (in Kit-I, -K, -M) we tested the generation of DC/DC_{leu} under No vs. Hy conditions. Finally, we assessed the impact of DC/DC_{leu} on the generation and activation of different T and of the immune reactive cell subtypes as well as anti- leukemia directed T-cells under subjected conditions.

VI.1.1 Generation of DC and DC_{leu} from AML Cell lines is comparable under Normoxic and Hypoxic conditions

VI.1.1.1 AML cell lines' phenotypic and genotypic profiles do not change during Hypoxic culture

We compared the mRNA expression levels of cell line-specific fusion genes (Table 1A) after several passages of AML cell lines (in RPMI media) in No vs. Hy Levels of mRNA expression were analysed by quantitative real-time RT-PCR, using GAPDH gene as housekeeping control. Our results indicated no significant differences for the expression of these fusion genes (No vs. Hy conditions**:** NB-4 **ΔΔ**CT 2.9 vs. 3.5; KG **ΔΔ**CT 27.6 vs. 23.1; Mono-mac-6 **ΔΔ**CT 20.3 vs. 19.6; THP-1 **ΔΔ**CT 114.5 vs. 101.2; Figure1). Furthermore, we did not detect any phenotypic changes of tested cell lines under No vs. Hy in RPMI and *ex vivo* media (see sections II.1.2 to II.1.3).

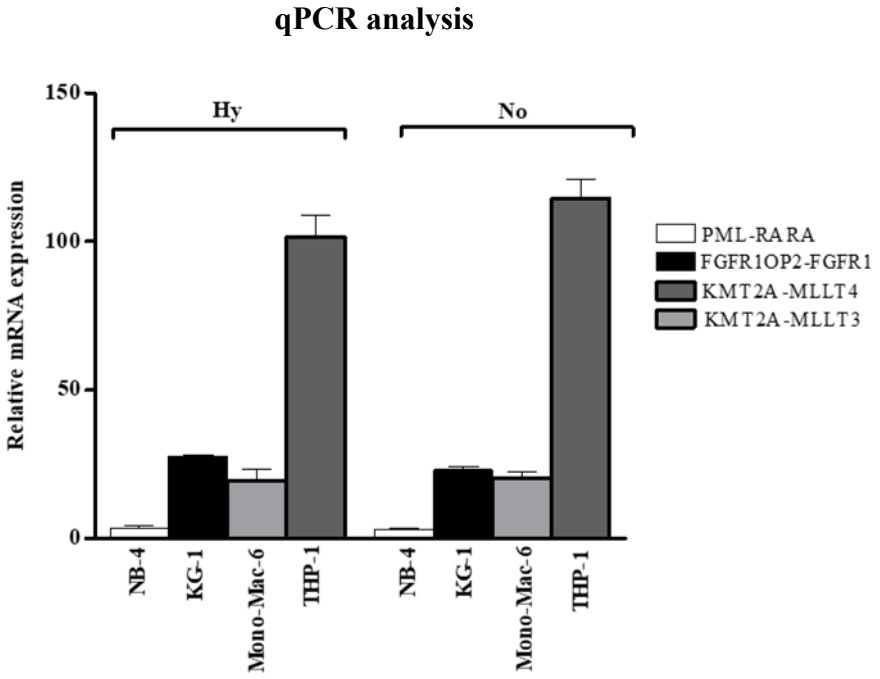

Figure1. qPCR analyses of fusion genes in AML cell lines: Four different AML cell lines (NB-4, KG-1, Mono-Mac-6, and THP-1) were cultured in RPMI medium - added Kits under Normoxic vs. Hypoxic conditions. Results show no differences between the expression of fusion genes (PML-PARA fusion gene in the NB-4 cell line, FGR1OPT-FGR1 fusion gene in the KG-1 cell lines, KMT2A-MLT4 fusion gene in the Mono-Mac-6 cell line, and KMT2A-MLT3 fusion gene in the THP-1 cell line) in the mRNA level induced by Hypoxic (Hy, left side) and Normoxic (No, right side) conditions. Relative expressions of fusion genes specific for each cell line were tested after five passages. GAPDH was used as the housekeeping control. The Y-axis shows the ΔΔCT differences between our genes of interest and housekeeping genes. Average frequencies ± standard deviation are given in all graphs. Statistical analyses were done using the student t-test. When the p values of differences in the statistical tests were between 0.1 and 0.05, we consider it as 'tendentially significant' and as 'significant'** with p values $0.005<x<0.05$ and as 'highly significant' *** with p-values <0.005.

VI.1.1.2 Successful DC/DC_{leu} generation from AML Cell lines with Kits (-I, -K, -M) is possible (compared to controls) under No vs. Hy conditions.

In the next step, the four cell lines were cultured with Kits in *ex vivo* and RPMI-media (Table 2, lower part) under No (21% O_2) vs. Hy (6% O_2) conditions. High frequencies of DC_{leu}/Bla^+ were found in No conditions in cell lines treated with Kits (in RPMI media) compared to controls (results with all Kits are pooled, Figure 2). Comparable results were obtained under Hypoxic conditions (Figure 2). There were no significant distinctions in frequencies of DC-subtypes cultured in *ex vivo* or RPMI media under No vs. Hy.

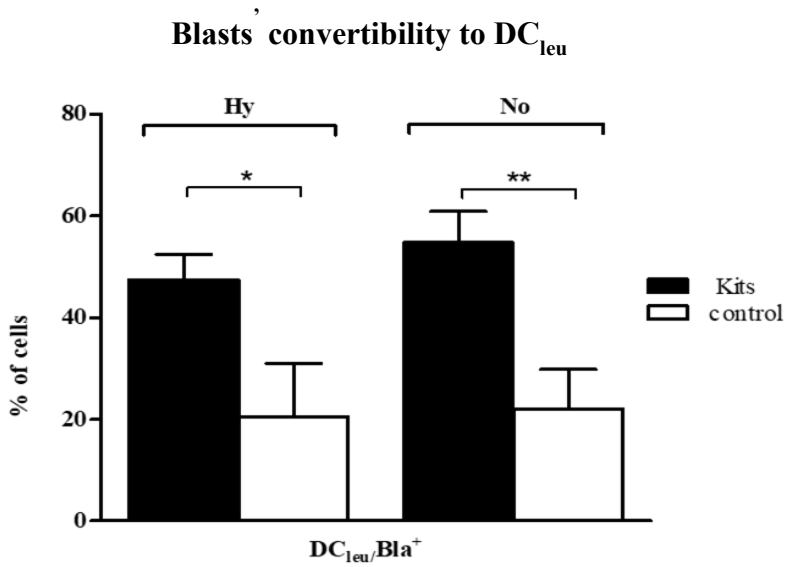

Figure 2. Blasts' convertibility to DC_{leu} is comparable under Normoxic vs. Hypoxic conditions. Results of all cell lines (NB-4, KG-1, Mono-Mac-6, and THP-1) with all kits (see section IV.5 for kit details) are pooled. Frequencies of DC_{leu}/Bla^+ were significantly increased in the presence of Kits compared to controls under Hypoxic vs. Normoxic conditions. Average frequencies ± standard deviation are given in all graphs. Statistical analyses were done using the student t-test. When the p values of differences in the statistical tests were between 0.1 and 0.05, we consider it as 'tendentially significant' and as 'significant'** with p values between 0.005<x <0.05 and as 'highly significant' *** with p-values <0.005.

VI.1.1.3 Adding Kits to AML cell lines does not induce blasts 'proliferation or influence blasts' apoptosis and Annexin V compared to controls under Normoxic and Hypoxic conditions

No significant increase of ($Bla_{prol\text{-}CD71}/Bla^{+}$) was found in No conditions under the influence of Kits compared to controls (Figure 3). Similar results were obtained in No vs. Hy. Comparable results were obtained for $Bla_{prol\text{-}IPO\text{-}38}/Bla^{+}$ (data not shown). No significant differences were found in frequencies of late ($Bla_{apo\text{-}CD261}/Bla^{+}$, $Bla_{apo\text{-}CD262}/Bla^{+}$) and early apoptotic blasts (Bla^{+}Annexin $V^{+}PI^{-}/Bla^{+}$) in Kit-treated cultures in No vs. Hy conditions compared to controls (Figure 3).

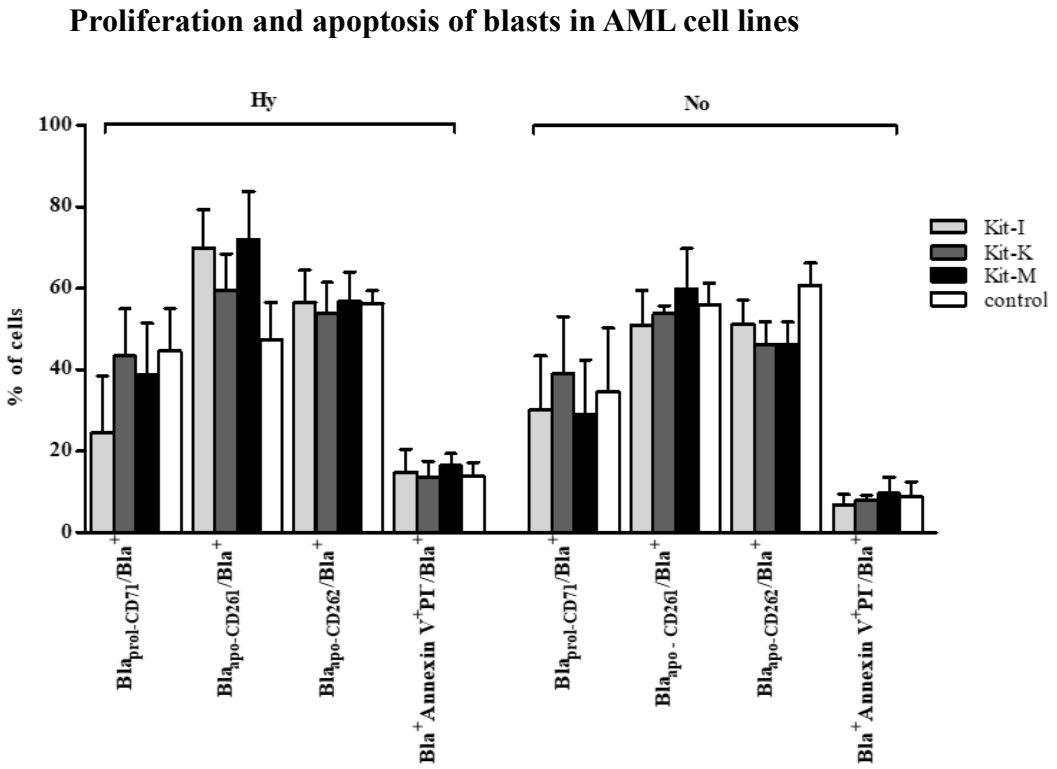

Figure 3. Results of proliferation and apoptosis of blasts in AML cell lines: Frequencies of ($Bla_{prol\text{-}CD71}/Bla^{+}$) or ($Bla_{apo\text{-}CD261}/Bla^{+}$, $Bla_{apo\text{-}CD262}/Bla^{+}$, Bla^{+}Annexin $V^{+}PI^{-}$ cells/Bla^{+}) were not influenced under the influence of kits in Normoxic vs. Hypoxic conditions. Results with all Kits (-I, -K, -M) with different cell lines are given. Average frequencies± standard deviations are given in all graphs. Statistical analyses were done using the student t-test. When the p values of differences in the statistical tests were between 0.1 and 0.05, we consider it as

'tendentially significant' and as 'significant'** with p values between 0.005<x <0.05 and as 'highly significant' *** with p-values <0.005.

VI.1.1.4 Significantly higher frequencies of cells found in S-phase of cell cycles under Normoxic compared to Hypoxic conditions in AML cell lines

We found significantly higher frequencies of cells in the S-phase for NB-4, Mono-mac-6, THP-1 cell lines but not of the KG line, under No vs. Hy conditions. Frequencies of cells in the other cell cycle phase were not different (Figure 4).

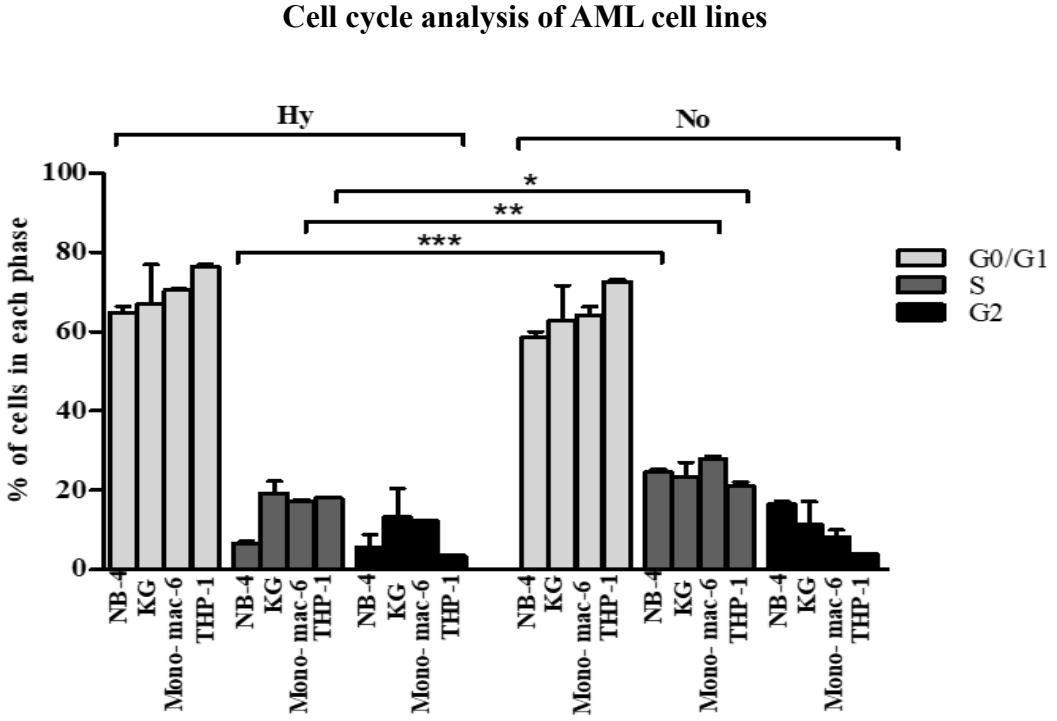

Figure 4. Cell cycle analysis of AML cell lines: Cell culture was done for 48h and cells were fixed in ethanol, stained with propidium iodide (PI) and analysed for DNA content to determine populations in G1 and S phases of the cell cycle. Significantly less cells were found in the S-phase under Normoxic vs. Hypoxic conditions for the NB-4, Mono-mac-6, and THP-1 cell lines. Average frequencies ± standard deviation is given in all graphs. Statistical analyses were done using the student t-test. When the p values of differences in the statistical tests were

between 0.1 and 0.05, we consider it as 'tendentially significant' and as 'significant'** with p values between 0.005<x <0.05 and as 'highly significant' *** with p-values <0.005.

VI.1.2 Generation of DC and DC_{leu} from leukemic PBMNCs and DC from healthy is comparable in Normoxic vs. Hypoxic conditions

Under No, we generated significantly higher frequencies of DCs and DC_{leu} from AML PBMNCs with Pici-$_{PGE1}$ or Pici-$_{PGE2}$ in comparison to controls (%DC^+/PBMNC: Pici-$_{PGE1}$:13.9±9.3, p<0.02; Pici-$_{PGE2}$:14.3±8.8, p<0.03; control: 5.8±2.6; %DC_{leu}/PBMNC: Pici-$_{PGE1}$:5.6±2.3, p<0.002; Pici-$_{PGE2}$:5.1±1.5, p<0.002; control: 1.4±0.6). Comparable frequencies of DC-subtypes were found in Pici-$_{PGE1}$ and Pici-$_{PGE2}$ treated leukemic PBMNC vs. control under Hy (Figure 5). Comparing results obtained in parallel under No vs. Hy conditions showed no significant differences in frequencies of DC-subtypes (data not shown).

DC subtypes from leukemic PBMNC

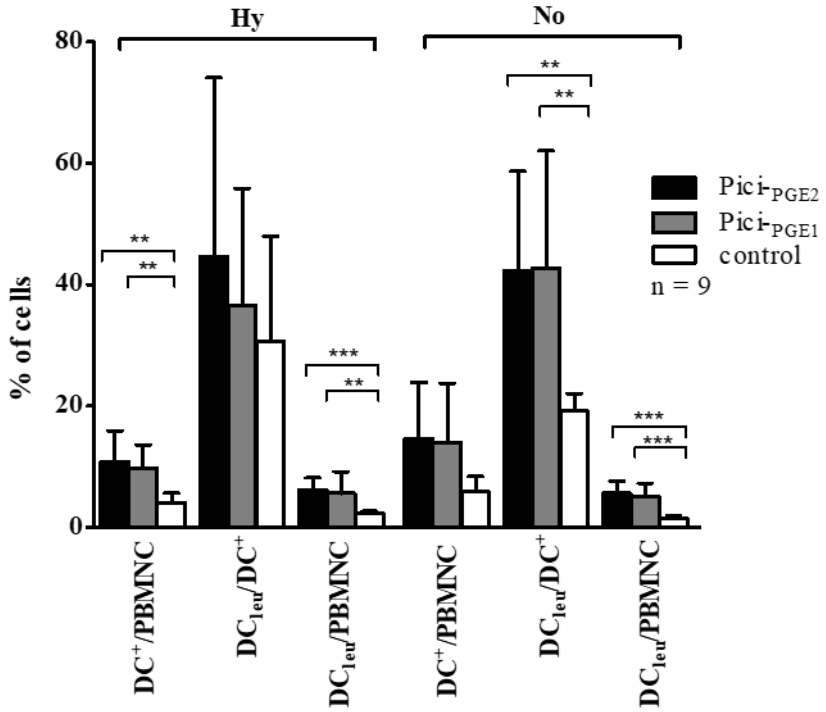

Figure 5. DC subtypes from leukemic PBMNC: Comparable frequencies of generated DCs with Pici-$_{PGE1}$ and Pici-$_{PGE2}$ from AML samples under Normoxic and Hypoxic conditions. Compositions of Pici-$_{\mathbf{PGE1}}$ and Pici-$_{\mathbf{PGE2}}$ protocols are given in Table 2. Hy: Hypoxic, No: Normoxic conditions. Average frequencies ± standard deviations of DCs and their subtypes under Hypoxic (Hy, left side) and Normoxic (No, right side) conditions are given. Statistical analyses were done using the student t-test. When the p values of differences in the statistical tests were between 0.1 and 0.05, we consider it as 'tendentially significant' and as 'significant'** with p values between $0.005<x<0.05$ and as 'highly significant' *** with p-values <0.005. Abbreviations for all cell subtypes are given in (Table 3A).

Using Pici-$_{PGE1}$ / Pici-$_{PGE2}$ protocols we generated significantly higher frequencies of DCs from healthy PBMNCs in No compared to controls (%DC^+/PBMNC: Pici-$_{PGE1}$:14.3±2.4, $p<0.01$; Pici-$_{PGE2}$:20.3±7.2, $p<0.07$; control: 6.1±3.2%). Under Hy, comparable frequencies of DC^+/PBMNC and DC_{mig}/DC^+ were found (Figure 2B, left side). Comparing results obtained in parallel under No vs. Hy showed no significant differences in frequencies of DC^+/PBMNC, DC_{mig}/DC^+ (Figure 6). We conclude that generation of DCs and their subtypes is possible in comparable frequencies from healthy and AML PBMNCs using Pici-$_{PGE1}$ and Pici-$_{PGE2}$ under No vs. Hy conditions.

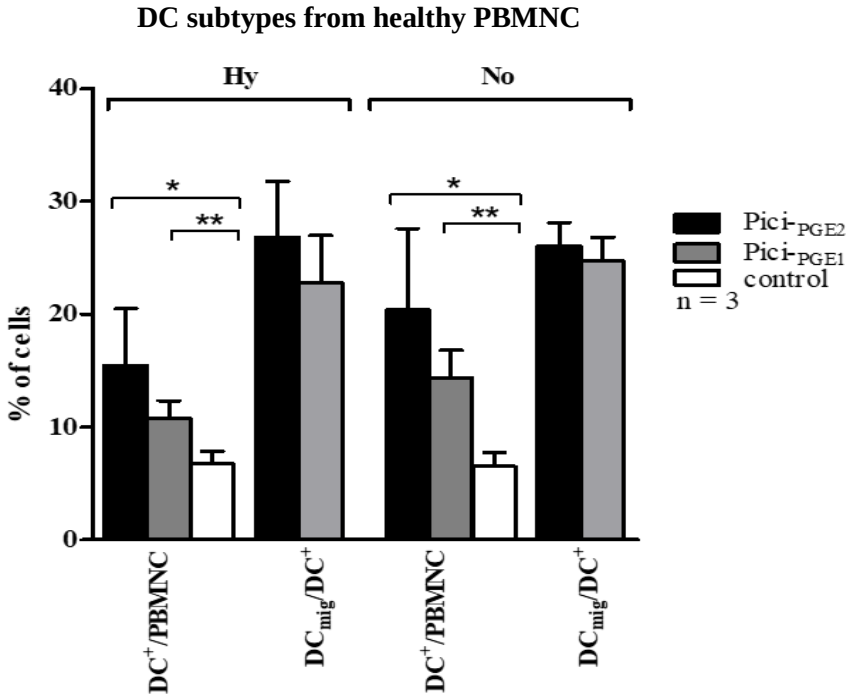

Figure 6. DC subtypes from healthy PBMNC: Healthy PBMNCs were cultured with DC generating methods Pici-$_{PGE1}$ and Pici-$_{PGE2}$ compared to controls under Normoxic and Hypoxic conditions. Compositions of Pici-**$_{PGE1}$** and Pici-**$_{PGE2}$** protocols are given in Table 2. Hy: Hypoxic, No: Normoxic conditions. Statistical analyses were done using the student t-test. When the p values of differences in the statistical tests were between 0.1 and 0.05, we consider it as 'tendentially significant' and as 'significant'** with p values between 0.005<x <0.05 and as 'highly significant' *** with p-values <0.005. Abbreviations for all cell subtypes are given in (Table 3A).

VI.1.3 Generation of DC and DC_{leu} using Kits (-I, -K, -M) from leukemic and healthy WB is comparable under Normoxic vs. Hypoxic conditions

VI.1.3.1 Comparable DC-generation from leukemic and healthy WB under Normoxic vs. Hypoxic conditions

A cut-off value was defined as of ≥5% DC as an ample DC-generation from leukemic or healthy WB. Under No, an ample DC-generation from leukemic WB was possible in 77-96% of cases cultured with Kits compared to only 25% of control cases (Figure 7A). Under Hy, a sufficient DC-

generation was found in 81-88% of cases and 18% of control cases (Figure 7B). Comparisons showed no significant differences between DC-generation in No vs. Hy conditions neither Kit treated nor control samples.

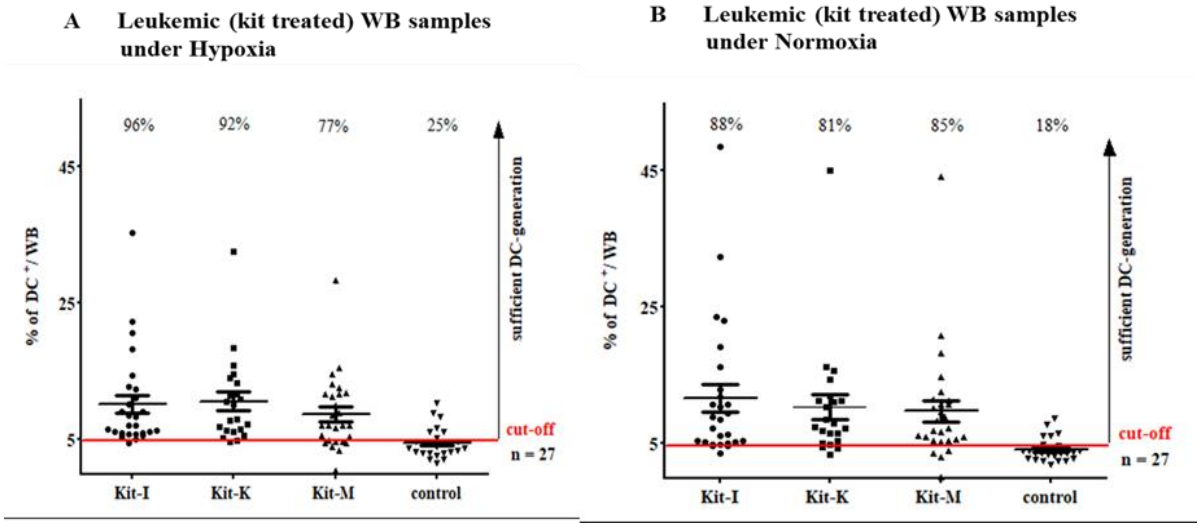

Figure 7. Comparable DC-generation from leukemic whole blood (WB) generated with Kit-I, -K and –M under Normoxic vs. Hypoxic conditions. Leukemic WB samples were cultured in parallel under Normoxic and Hypoxic conditions with Kit-I, -K and -M compared to controls without added response modifiers. Proportions of cases with 'sufficient DC-generation' (setting a cut-off-value at 5% DC^+/WB) from leukemic WB were not different under **(A)** Hypoxic vs **(B)** Normoxic conditions. Each dot (● ■ ● ▼) characterises DC-frequencies generated from each individual AML-patient. Graphs represent percentages of informative patients 'samples generating DC ≥5%. Statistical analyses were done using the student t-test. When the p values of differences in the statistical tests were between 0.1 and 0.05, we consider it as 'tendentially significant' and as 'significant'** with p values between 0.005<x <0.05 and as 'highly significant' *** with p-values <0.005. Abbreviations for all cell subtypes are given in (Table 3A).

VI.1.3.2 Significantly higher frequencies of DCs generated from AML and healthy WB with Kits compared to controls under Normoxic vs. Hypoxic conditions

In No, we generated significantly higher frequencies of DCs from AML WB with Kits compared to controls (%DC^+/WB: Kit-I: 10.2±7.0, $p<0.03$; Kit-K: 11.7±6.6, $p<0.009$; Kit-M: 9.6±5.3, $p<0.03$; control: 6.5±2.0, Figure 8B). Moreover, significantly higher frequencies of different DC-subtypes could be generated from WB: DC_{leu}/WB, DC_{leu}/DC, DC_{leu}/blasts as well as of mature DC (DC_{mig}/DC) compared to controls (Fig 8B, in cases with DC-counts <5% no DC-subtype-evaluations were possible). Similarly, in Hy comparable frequencies of DC-subtypes were found (Figure 8A). Comparing results obtained in parallel under No vs. Hy showed no significant differences in frequencies of DC-subtypes (Figure 8A, B).

In No, we generated significantly higher frequencies of DCs subtypes from healthy WB with Kits (results of all parallel analysed Kits are pooled, Figure 8C) compared to controls (%DC^+/WB: Kits: 9.5±5.6; control: 5.9±1.6, $p<0.001$; %DC_{mig}/DC^+: Kits: 22.6±5.6; control: 15.6±1.6, $p<0.003$). In Hy comparable frequencies of DC^+/WB, DC_{mig}/DC^+ were found (Figure 8C). Comparing results obtained in parallel under No vs. Hy revealed no significant change in frequencies of DC^+/WB, DC_{mig}/DC^+ (Figure 8C). We conclude that with Kit-I, -K, and -M it is possible to generate DC or DC_{leu} (subtypes) from healthy and leukemic WB in comparable amounts under No vs. Hy.

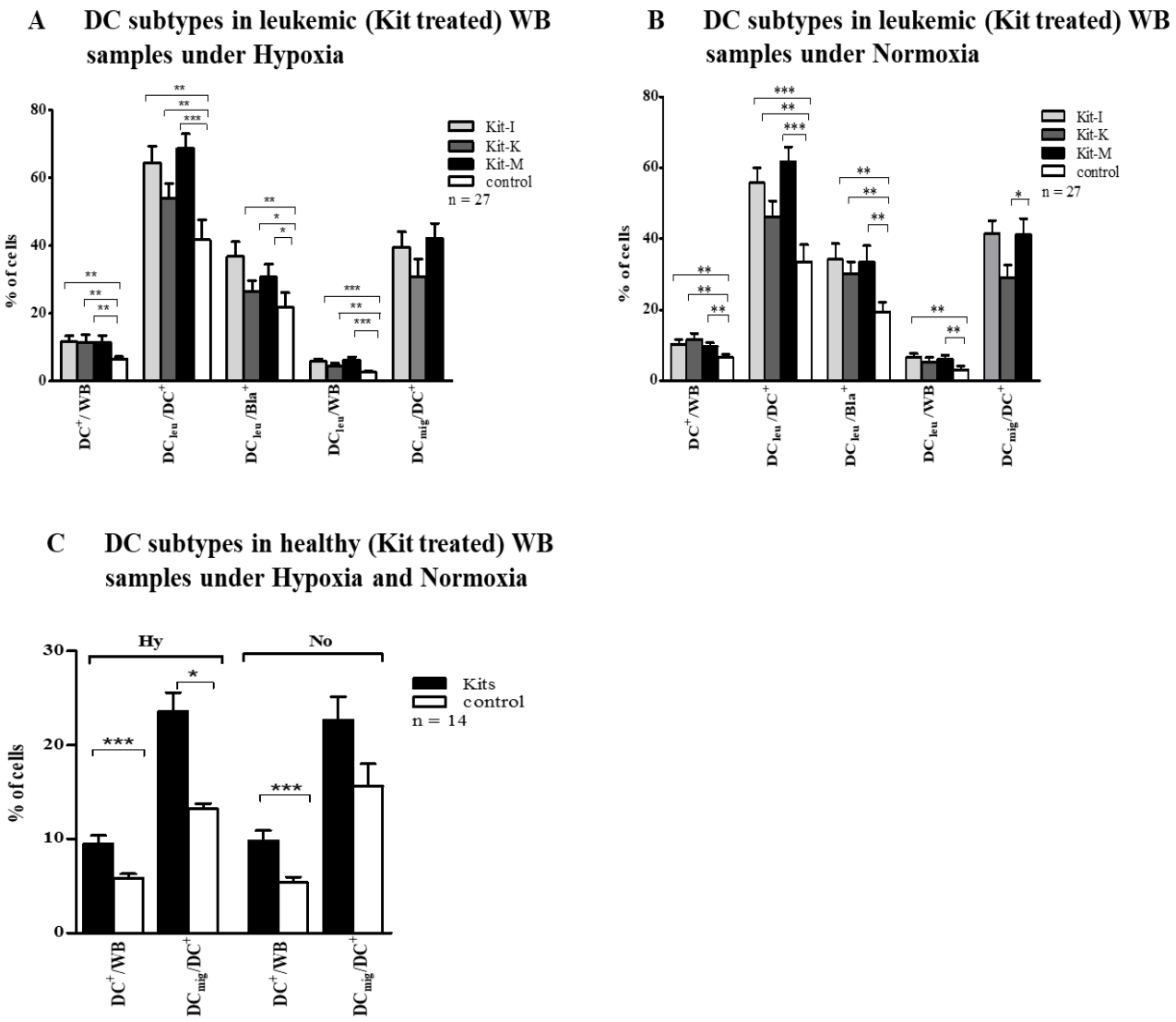

Figure 8. Comparable DC-generation from leukemic and healthy whole blood (WB) generated with Kit-I, -K and –M under Normoxic vs. Hypoxic conditions. (A) and **(B)** Leukemic WB samples **(C)** healthy were cultured in parallel under Normoxic and Hypoxic conditions with Kit-I, -K and -M compared to controls without added response modifiers under **(A)** Hypoxic vs **(B)** Normoxic conditions. Average frequencies ± standard deviation of DC and their subtypes generated from WB reaction are given Compositions of kits are given in (Table 2). Statistical analyses were done using the student t-test. When the p values of differences in the statistical tests were between 0.1 and 0.05, we consider it as 'tendentially significant' and as 'significant'** with p values between0.005<x <0.05 and as 'highly significant' *** with p-values <0.005. Abbreviations for all cell subtypes are given in (Table 3A).

VI.2 Adding Kits to leukemic WB does not induce blasts' proliferation nor influence blasts' or DCs' apoptosis or checkpoint marker expression under Normoxic vs. Hypoxic conditions

We found comparable frequencies of $Bla_{prol\text{-}CD71}/ Bla^{+}$ or $Bla_{prol\text{-}Ipo\text{-}38}/ Bla^{+}$ under the influence of Kits compared to controls (%$Bla_{prol\text{-}CD71}/Bla^{+}$: Kit-I: 10.5±11.7, $p<0.2$; Kit-K: 8.8±8.5, $p<0.5$; Kit-M: 10.6±11.4, $p<0.2$; control: 9.8±12.6). Similar results were obtained under Hy (Figure 9). Comparing results obtained in parallel under No and Hy revealed no significant differences in frequencies of $Bla_{prol\text{-}CD71}/Bla^{+}$ or $Bla_{prol\text{-}Ipo\text{-}38}/Bla^{+}$ (Figure 9, 10). No significant differences in frequencies of apoptotic blasts or DC ($Bla_{apo\text{-}CD261}/Bla^{+}$, $Bla_{apo\text{-}CD262}/Bla^{+}$, $DC_{apo\text{-}CD261}/DC^{+}$, $DC_{apo\text{-}CD262}/DC^{+}$, $DC_{leu\ apo\text{-}CD261}/DC_{leu}^{+}$, $DC_{leu\ apo\text{-}CD262}/DC_{leu}^{+}$) were found after Kit treatment of WB under No (Figure 9, 10). We found comparable frequencies of apoptotic blasts as well as apoptotic DC, DC_{leu} after DC generation under Hy (Figure 9).

Proliferation, checkpoint and apoptotic blasts or DC in WB under Hypoxia

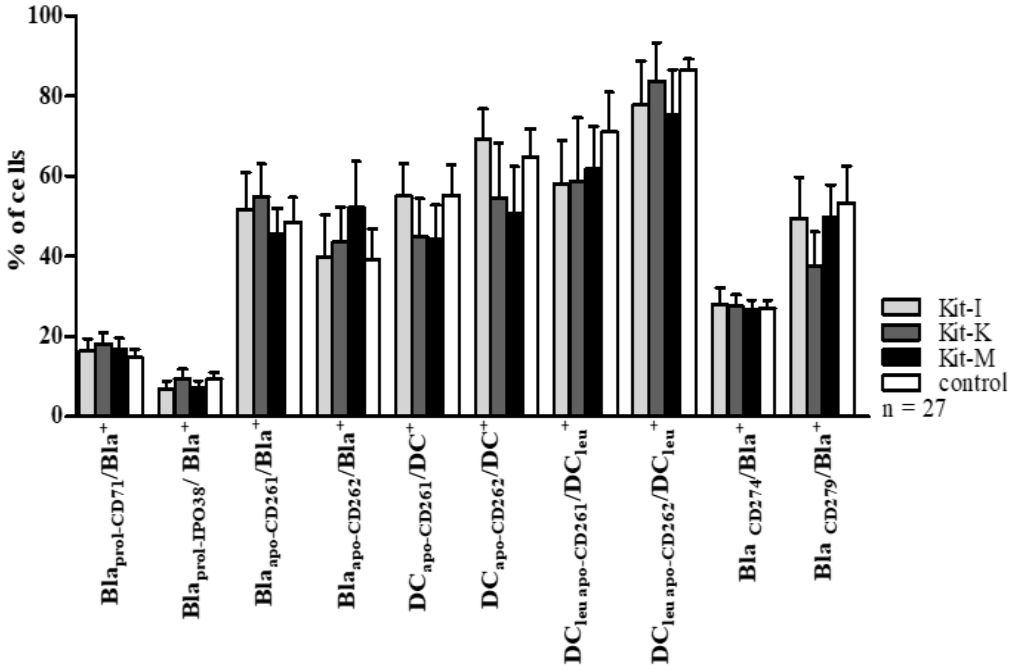

Figure 9. Comparable frequencies of blasts or DC expressing proliferation, apoptotic and checkpoint markers from leukemic WB generated with Kit-I, -K and –M under Hypoxic conditions. Leukemic WB samples were cultured in parallel under Normoxic and Hypoxic conditions with Kit-I, -K and -M compared to controls without added response modifiers. Frequencies of proliferating blasts (not converted to DC_{leu}), apoptotic blasts or DC and checkpoint expressing cells after DC generation from leukemic WB in AML patient under Hypoxic (Hy) condition. Average frequencies ± standard deviation of proliferating, apoptosis, and checkpoint marker expressing blasts WB with Kits (-I, -K, -M) are given. Statistical analyses were done using the student t-test. Abbreviations for all cell subtypes are given in (Table 3A).

Moreover, no significant differences in frequencies of checkpoint marker expressing blasts (Bla_{CD274}/Bla^+, Bla_{CD279}/Bla^+) in Kit treated WB under No compared to the controls were found. Comparing results obtained in parallel under No vs. Hy culture conditions revealed no significant differences in frequencies of checkpoint marker expressing blasts (Figure 9 and 10). We conclude that

Kits do neither induce blasts' proliferation nor influence apoptosis of blasts or DC or blasts' expression of checkpoint markers in leukemic WB under No and Hy.

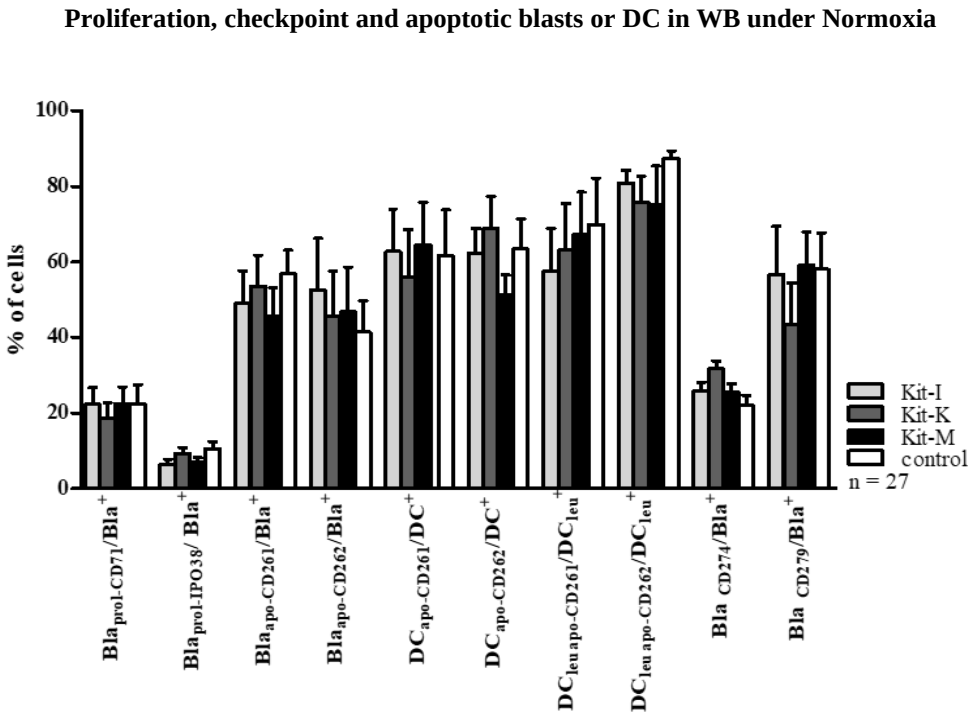

Figure 10. Comparable frequencies of blasts or DC expressing proliferation, apoptotic and checkpoint markers from leukemic WB generated with Kit-I, -K and –M under Normoxic conditions. Leukemic WB samples were cultured in parallel under Normoxic and Hypoxic conditions with Kit-I, -K and -M compared to controls without added response modifiers. Frequencies of proliferating blasts (not converted to DC_{leu}), apoptotic blasts, or DC and checkpoint expressing cells after DC generation from leukemic WB in AML patient under Normoxic (No) condition. Average frequencies ± standard deviation of proliferating, apoptosis, and checkpoint blasts WB with Kits (-I, -K, -M) are given. Statistical analyses were done using the student t-test. Abbreviations for all cell subtypes are given in (Table 3A).

VI.3 Influence of DC/DC_{leu} (produced with immunomodulatory Kits for leukemic and healthy WB) on T-cell enriched immunoreactive cells in MLC culture and the consequence blast-lysis activity is comparable under Normoxic and Hypoxic conditions

As our previous results have shown, the origination of "DC and DC_{leu} is possible from healthy and AML WB with different Kits under No vs. Hy conditions". Furthermore, we investigated the potentially stimulating impact of generated "DC and DC_{leu} on immunoreactive T-cells in MLC". Consequently, we evaluated and compared T-cell compositions of cells before ('Uncultured') and after stimulation with Kit treated "($MLC^{WB\text{-}DC\ Kit\text{-}I}$, $MLC^{WB\text{-}DC\ Kit\text{-}M}$) vs. without Kit treated WB (MLC^{WB}) as control under No vs. Hy conditions.

VI.3.1 Significantly increased frequencies of $CD3^+CD4^+/CD3^+$, T-cell, NK and iNKT cell subtypes found in AML samples after $MLC^{WB\text{-}DC\ Kits}$ vs. MLC^{WB} under Normoxic as well as Hypoxic conditions

In Uncultured cells, we found low frequencies of $CD3^+CD4^+/CD3^+$, $T_{prol\text{-}early}$, $T_{prol\text{-}late}$, T_{reg}, NK and iNKT cells and high frequencies of T_{naive} cells under No conditions (Figure 11, 13 right sides). Comparable frequencies were found under Hy conditions (Figure 11, 13 left side)**.** We found a significant shift of T-cell subsets and innate cells to a higher activation status after $MLC^{WB\text{-}DC\ Kits}$ compared to MLC^{WB} under No conditions (Figure 11 and 12 left side). In Hy higher frequencies of $CD3^+CD4^+/CD3^+$, NK, and iNKT cells after stimulation in $MLC^{WB\text{-}DC\ Kits}$ compared to MLC^{WB} were found (Figure 11, 13 right side). No significant differences in frequencies of T or innate cell subsets comparing No vs. Hy cultures were found (Figure 11 and 12 right side vs. left side). Since results obtained with Kit-M or Kit-I were comparable, we pooled results. After $MLC^{WB\text{-}DC\ Kits}$ and MLC^{WB} culture we found a significant increase of activated T-cells, $CD3^+CD4^+/CD3^+$, T_{prol}, $T_{non\text{-}naive}$, $T_{eff\text{-}em}$,

T_{reg},T_{cm} as well as of NK and iNKT cells what is probably due to IL-2 added to all MLC experiments. This was found under No as well as Hy conditions (Figure 11 and 12 left and right side).

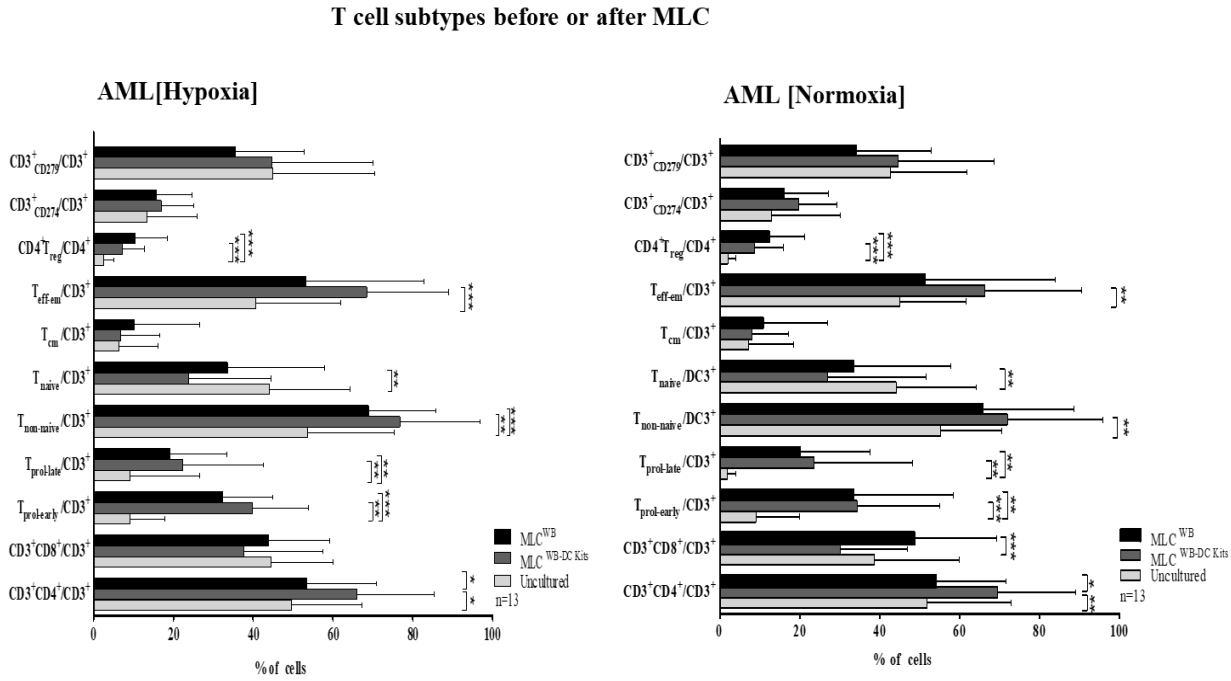

Figure 11. T-cell subtypes before or after MLC. Stimulatory effects of Kit treated (containing DC/DC_{leu}) vs. untreated WB from leukemic under Normoxic (right) vs. Hypoxic (left) conditions. Average frequencies ± standard deviations of different subsets of immunoreactive cells in MLC cultures before (Unculture) or after stimulation with Kit treated ($MLC^{WB\text{-}DC\ Kits}$) or untreated WB samples (MLC^{WB}) are given. Statistical analyses were done using the student t-test. When the p values of differences in the statistical tests were between 0.1 and 0.05, we consider it as 'tendentially significant' and as 'significant'** with p values between 0.005<x <0.05 and as 'highly significant' *** with p-values <0.005. Abbreviations for all cell subtypes are given in (Table 3B).

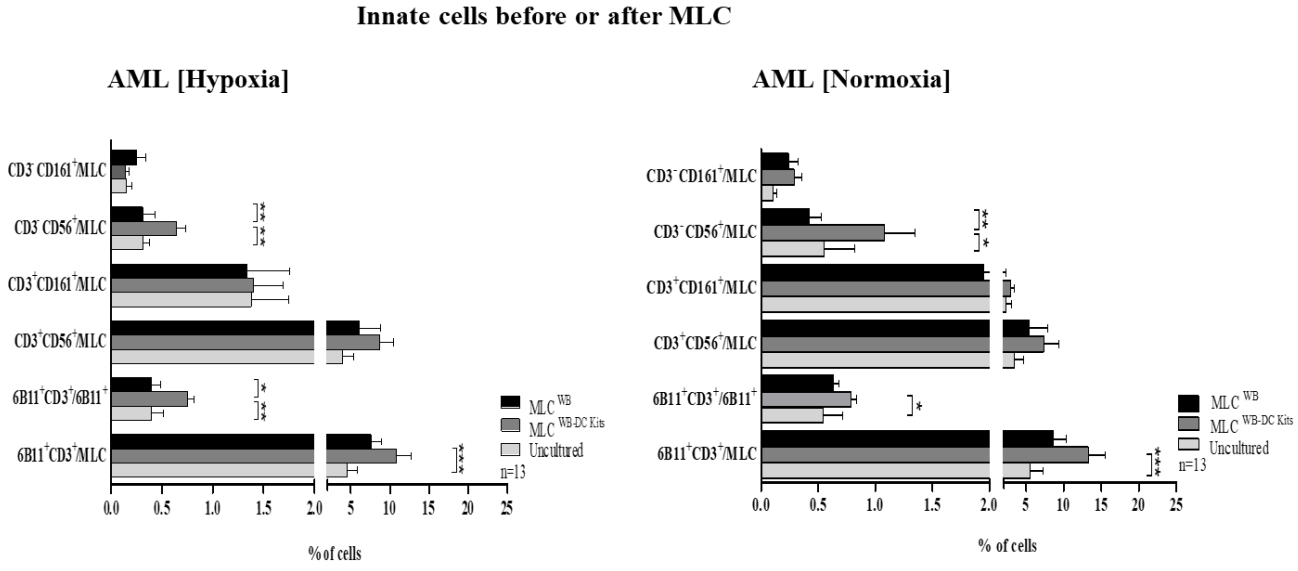

Figure 12. Innate cell subtypes before or after MLC for AML samples. iNKT-/NK-/CIK-cell subtypes in T-cell enriched MLC under Normoxic (right) vs. Hypoxic (left) conditions. Average frequencies ± standard deviations of different subsets of innate immunoreactive cells in MLC cultures before (Unculture) or after stimulation with Kit treated ($MLC^{WB\text{-}DC\ Kits}$) or untreated WB samples (MLC^{WB}) are given. Statistical analyses were done using the student t-test. When the p values of differences in the statistical tests were between 0.1 and 0.05, we consider it as 'tendentially significant' and as 'significant'** with p values between 0.005<x <0.05 and as 'highly significant' *** with p-values <0.005. Abbreviations for all cell subtypes are given in (Table 3B).

VI.3.2 Significantly increased frequencies of $CD3^+CD4^+/CD3^+$, T-cell, NK, and iNKT cell subtypes found in healthy samples after $MLC^{WB\text{-}DC}$ Kits vs. MLC^{WB} under Normoxic as well as Hypoxic conditions

We found almost similar frequencies of T and innate cell subtypes in healthy as in AML samples with concerning Uncultured, $MLC^{WB\text{-}DC\ Kits}$ or MLC^{WB} cultured cells. This was true for No and Hy conditions (Figure 13 and 14 right vs. left side). Moreover, the IL-2 effect on the Uncultured T-cells' activation vs. $MLC^{WB\text{-}DC\ Kits}$, or MLC^{WB} cultured T-cells was confirmed under No and Hy conditions (Figure 13 and 14).

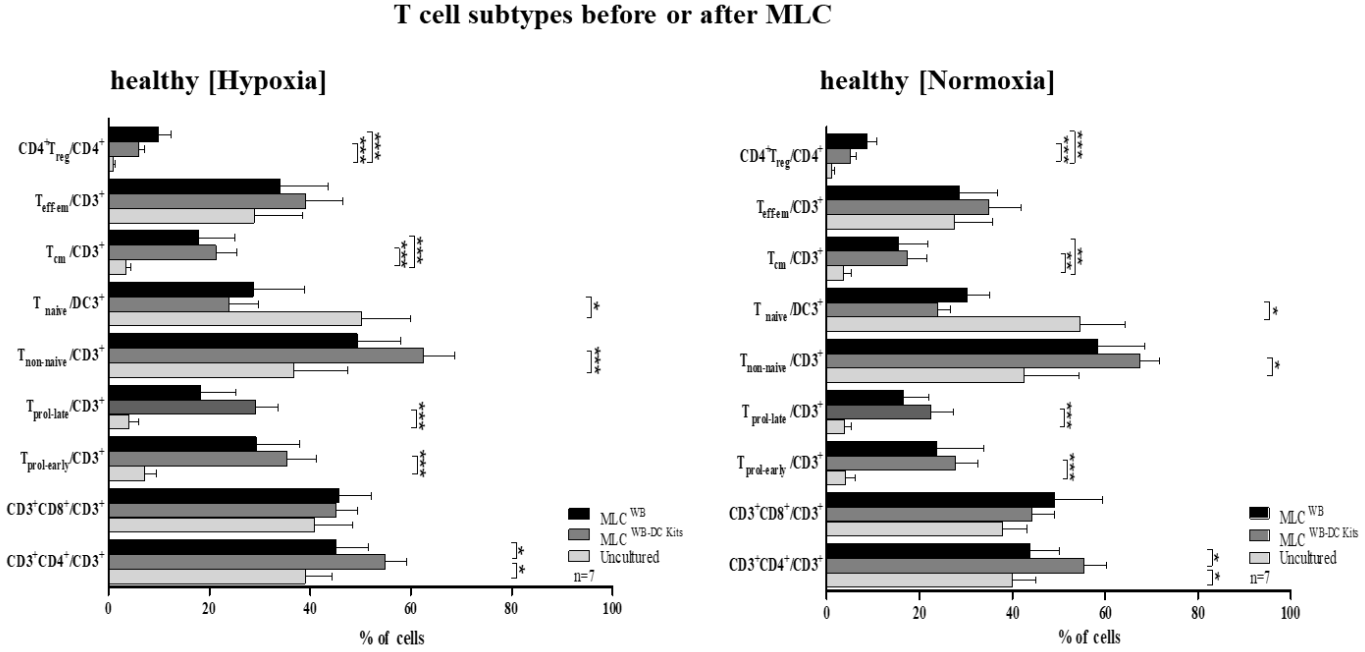

Figure 13. T-cell subtypes before or after MLC for healthy samples. Stimulatory effects of Kit treated (containing DC/DC_{leu}) vs. untreated WB from healthy under Normoxic (right) vs. Hypoxic (left) conditions. Average frequencies ± standard deviation of different subsets of immunoreactive cells in MLC cultures before (Unculture) or after stimulation with Kit treated ($MLC^{WB\text{-}DC\ Kits}$) or untreated WB samples (MLC^{WB}) are given in all graphs. Statistical analyses were done using the student t-test. When the p values of differences in the statistical tests were between 0.1 and 0.05, we consider it as 'tendentially significant' and as 'significant'** with p values between 0.005<x <0.05 and as 'highly significant' *** with p-values <0.005. Abbreviations for all cell subtypes are given in (Table 3B).

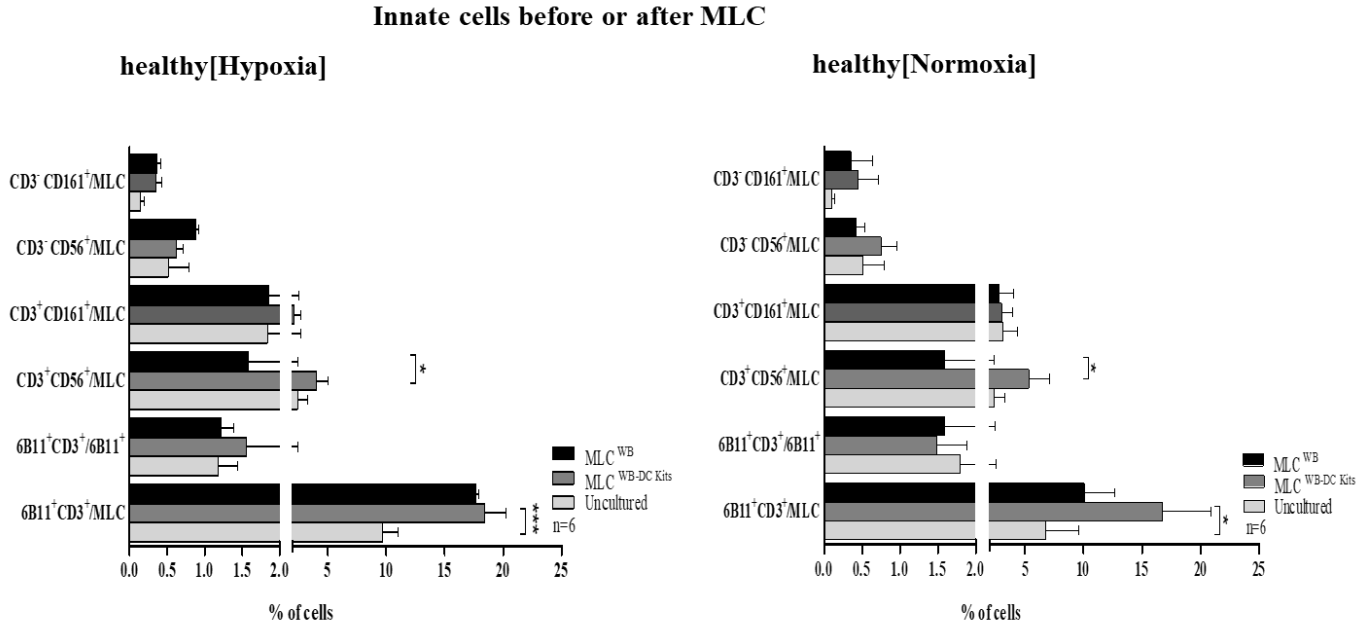

Figure 14. Innate cell subtypes before or after MLC for healthy samples. Stimulatory effects of Kit treated (containing DC/DC_{leu}) vs. untreated WB from leukemic sample under Normoxic (right) vs. Hypoxic (left) conditions. Average frequencies ± standard deviation of different subsets of innate immunoreactive cells in MLC cultures before (Unculture) or after stimulation with Kit treated ($MLC^{WB\text{-}DC\ Kits}$) or untreated WB samples (MLC^{WB}) are given in all graphs. Statistical analyses were done using the student t-test. When the p values of differences in the statistical tests were between 0.1 and 0.05, we consider it as 'tendentially significant' and as 'significant'** with p values between 0.005<x <0.05 and as 'highly significant' *** with p-values <0.005. Abbreviations for all cell subtypes are given in (Table 3B).

VI.3.3 Comparable cellular compositions (T and innate cells) from AML and healthy samples found before and after MLC culture under Normoxic vs. Hypoxic conditions

Results obtained with AML vs. healthy samples before and after MLC with Kit-treated or untreated WB did not show differences in T- and innate cell compositions under No and Hy conditions (Figure 11-14). In summary, we show that Kit-treated WB leads to a significant activation of

$CD3^+CD4^+/CD3^+$, T-cell, NK and iNKT cells and reduction of T_{reg} after MLC in AML and healthy samples in comparison to untreated (no kit treatment) under No as well as Hy conditions.

VI.3.4 Kit pre-treated WB leads to significantly improved anti-leukemic activation after MLC, especially in Hypoxic conditions

We tested the blast lysis effect of T-cell enriched immunoreactive MLC after stimulation with Kit treated (vs. untreated) WB under No and Hy conditions. Blast lysis was evaluated after **3h** and **24h** of incubation of blast target with (DC/DC_{leu}-stimulated) effector cells and the best-achieved lysis (after **3h** or **24h**) was evaluated (Figure 15-17). In No conditions, improved lysis with $MLC^{WB\text{-}DC\ Kit\text{-}I}$ in 60% (6 of 10) cases and in 67% (8 of 12) cases with $MLC^{WB\text{-}DC\ Kit\text{-}M}$ in comparison to controls (MLC^{WB}) after **3 h** of incubation of blast targets with effector cells were found. In Hy conditions, comparable results were seen. On average lysis could be improved in 64% of cases with $MLC^{WB\text{-}DC\ Kit\text{-}I}$ and in 69% of cases with $MLC^{WB\text{-}DC\ Kit\text{-}M}$ in comparison to controls (MLC^{WB}) after 3hours (Figure 15A, left side). Under No after **3h** compared to controls (MLC^{WB}) lysis was improved after stimulation with $MLC^{WB\text{-}DC\ Kit\text{-}I}$ by 43% and with $MLC^{WB\text{-}DC\ Kit\text{-}M}$ by 34%. Comparable distributions were found under Hy conditions (Figure 15A, right side).

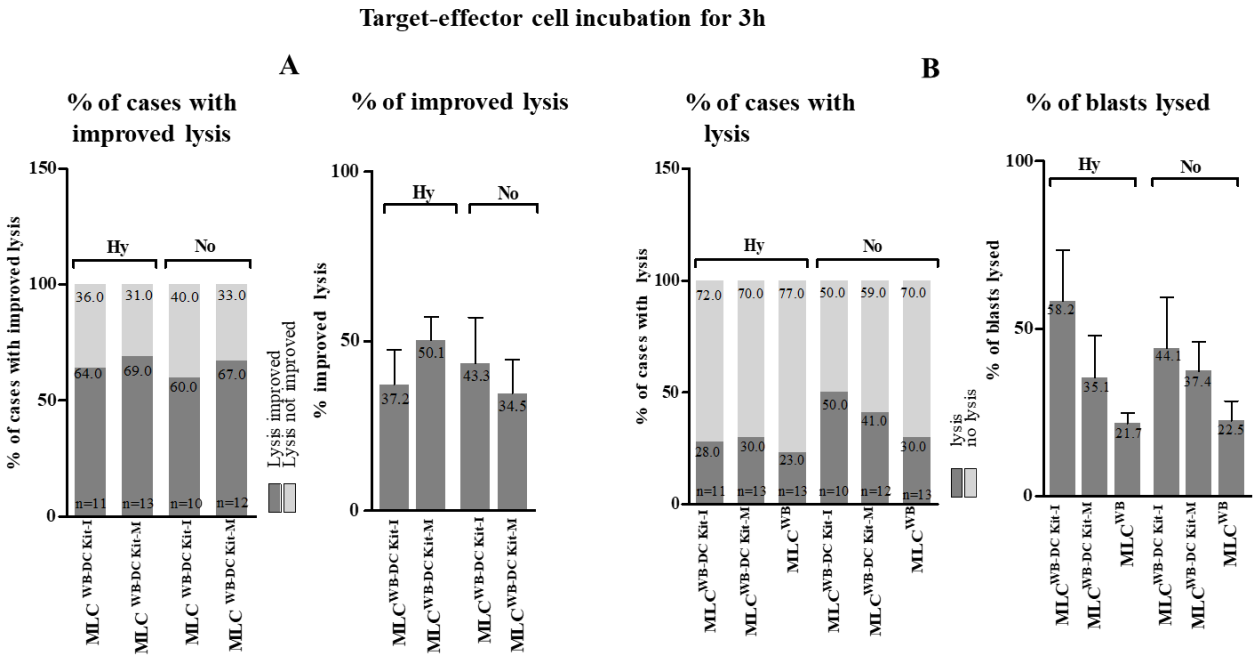

Figure 15. Blast lytic effects of immunoactive (T-cell enriched) MLC after 3h stimulation with Kit treated (vs. untreated) WB under No and Hy. A (significantly) improved blast lysis was seen especially with Kit M pre-treated WB compared to controls. The frequencies of cases with improved lysis and the mean ± range of improved lysis in $MLC^{WB\text{-}DC\ Kit\text{-}I}$, $MLC^{WB\text{-}DC\ Kit\text{-}M}$ compared to MLC^{WB} (control) shown after 3h of co-culture of target and effector cells. The frequencies of cases with lysis and the mean ± range of lysis $MLC^{WB\text{-}DC\ Kit\text{-}I}$, $MLC^{WB\text{-}DC\ Kit\text{-}M}$, MLC^{WB} are given. Improved blast-lysis-activity under No vs. Hy conditions was comparable. Statistical analyses were done using the chi-squared test. When the p values of differences in the statistical tests were between 0.1 and 0.05, we consider it as 'tendentially significant' and as 'significant'** with p values between $0.005<x<0.05$ and as 'highly significant' *** with p-values <0.005. Abbreviations are given in (Table 3B).

Referring to cases with lysis after **3h** of incubation of target with effector cells, we found more cases with lysis in No vs. Hy conditions in $MLC^{WB\text{-}DC\ Kit\text{-}I}$ and $MLC^{WB\text{-}DC\ Kit\text{-}M}$ compared to controls MLC^{WB} (Figure 15B, left side). Average blast lysis was non significantly more in $MLC^{WB\text{-}DC\ Kit\text{-}I}$ than in $MLC^{WB\text{-}DC\ Kit\text{-}M}$ and MLC^{WB} under No and Hy conditions (Figure 15B, right side). After **24h** of incubation of target with effector cells, more cases of $MLC^{WB\text{-}DC\ Kit\text{-}I}$, as well as $MLC^{WB\text{-}DC\ Kit\text{-}M}$,

attained an improvement in blast lysis. In No conditions an improvement of blast lysis with $MLC^{WB\text{-}DC\ Kit\text{-}I}$ in 70% (7 of 10) cases and in 75% (9 of 12) cases with $MLC^{WB\text{-}DC\ Kit\text{-}M}$ compared to MLC^{WB} was found. In Hy conditions, 72% (8 of 11) cases with $MLC^{WB\text{-}DC\ Kit\text{-}I}$ and 84% (11 of 13) cases with $MLC^{WB\text{-}DC\ Kit\text{-}M}$ compared to MLC^{WB} showed improved blasts lysis. On average lysis could be improved in $MLC^{WB\text{-}DC\ Kit\text{-}I}$ increased to levels of $MLC^{WB\text{-}DC\ Kit\text{-}M}$ under No conditions. Comparable results were found under Hy conditions (Figure 16A, left side). Under No after **24h** compared to controls (MLC^{WB}) lysis was improved after stimulation with $MLC^{WB\text{-}DC\ Kit\text{-}I}$ by 44% and with $MLC^{WB\text{-}DC\ Kit\text{-}M}$ by 39% (Figure 16A, right side). Comparable distributions were found under Hy conditions.

Referring to cases with lysis after **24h** of incubation of target with effector cells, in No conditions more cases of $MLC^{WB\text{-}DC\ Kit\text{-}I}$ and significantly more cases with Kit-M compared to controls MLC^{WB} attained lysis (Figure 16B, left side). On average, target cells' lysis increased in $MLC^{WB\text{-}DC\ Kit\text{-}I}$, $MLC^{WB\text{-}DC\ Kit\text{-}M}$ as well as MLC^{WB}. Results were comparable under Hy conditions (Figure 16B, right side).

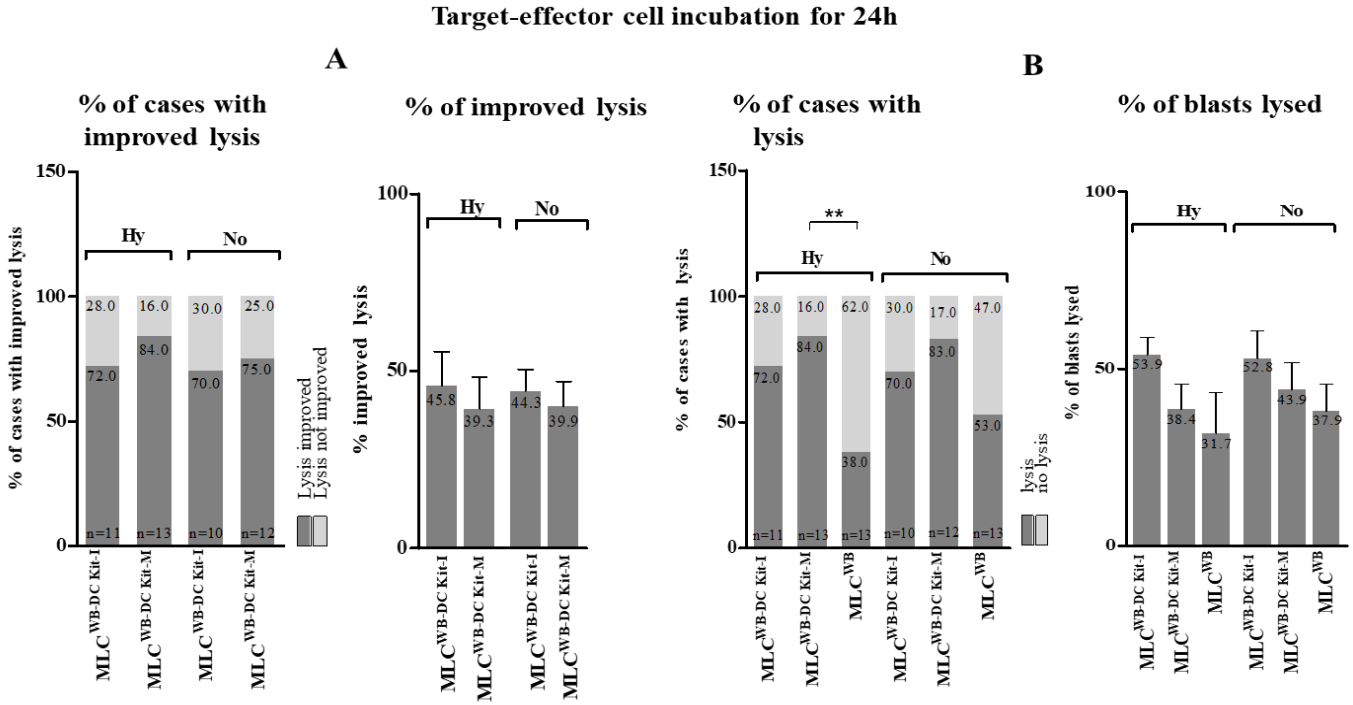

Figure 16. Blast lytic effects of immunoactive (T-cell enriched) MLC after 24h stimulation with Kit treated (vs. untreated) WB under No and Hy. A (significantly) improved blast lysis was seen specially with Kit M pre-treated WB compared to controls. The frequencies of cases with improved lysis and the mean ± range of improved lysis in $MLC^{WB\text{-}DC\ Kit\text{-}I}$, $MLC^{WB\text{-}DC\ Kit\text{-}M}$ compared to MLC^{WB} (control) shown after 24 h of co-culture of effector and target cells. The frequencies of cases associated with lysis and the mean ± range of lysis $MLC^{WB\text{-}DC\ Kit\text{-}I}$, $MLC^{WB\text{-}DC\ Kit\text{-}M}$, MLC^{WB} after are given. Improved blast-lysis-activity under No vs. Hy conditions was comparable. Statistical analyses were done using the chi-squared test. When the p values of differences in the statistical tests were between 0.1 and 0.05, we consider it as 'tendentially significant' and as 'significant'** with p values between $0.005<x<0.05$ and as 'highly significant' *** with p-values <0.005. Abbreviations are given in (Table 3B).

Choosing of the best anti-leukemic activity after **3** or **24h** hours of incubation of target with effector cells, $MLC^{WB\text{-}DC\ Kit\text{-}I}$ and $MLC^{WB\text{-}DC\ Kit\text{-}M}$ showed comparable numbers of cases with improved lysis as well as frequencies of improved lysis under No and Hy conditions (Figure 17A). In No conditions, we found more cases with lysis in $MLC^{WB\text{-}DC\ Kit\text{-}I}$ and significantly more cases in $MLC^{WB\text{-}DC\ Kit\text{-}M}$ than in MLC^{WB}. Average lysis was higher in $MLC^{WB\text{-}DC\ Kit\text{-}I}$ and $MLC^{WB\text{-}DC\ Kit\text{-}M}$ than in MLC^{WB}, with equal averages of lysis in $MLC^{WB\text{-}DC\ Kit\text{-}I}$ and $MLC^{WB\text{-}DC\ Kit\text{-}M}$. Similar results under conditions of Hy were found (Figure 17B).

Finally, we demonstrated anti-leukemic activity of T-cells after stimulation in MLC by DC/DC_{leu} containing Kit-I or -M treated has an increased (vs. untreated) WB in No as well as under Hy conditions after **3h** or **24h** of incubation of blast target with effector cells compared to controls. In general, results obtained in parallel in No vs. Hy cultures showed no significant differences, although in Hy significant differences in blast lysis after **24h** of target-effector cell incubation between Kit-M treated vs. untreated WB were seen.

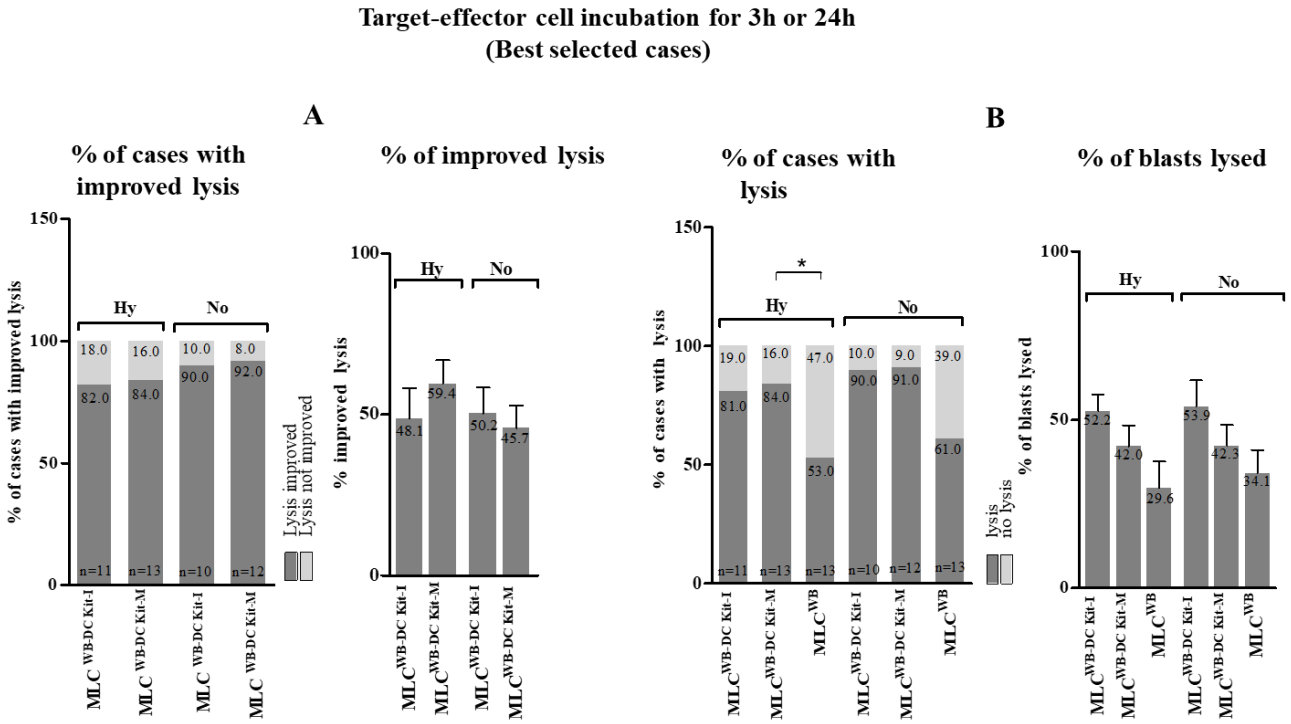

Figure 17. The best of blast lytic effects of immunoactive (T-cell enriched) MLC after 3 or 24h stimulation with Kit treated (vs. untreated) WB under No and Hy. A (significantly) improved blast lysis was seen specially with Kit M pre-treated WB compared to controls. The frequencies of cases with improved lysis and the mean ± range of improved lysis in $MLC^{WB\text{-}DC\ Kit\text{-}I}$, $MLC^{WB\text{-}DC\ Kit\text{-}M}$ compared to MLC^{WB} (control) shown the best of 3h or 24h of co-culture of target and effector cells. The frequencies of cases with lysis and the mean ± range of lysis $MLC^{WB\text{-}DC\ Kit\text{-}I}$, $MLC^{WB\text{-}DC\ Kit\text{-}M}$, MLC^{WB} are given. Improved blast-lysis-activity under No vs. Hy conditions was comparable. Statistical analyses were done using the chi-squared test. When the p values of differences in the statistical tests were between 0.1 and 0.05, we consider it as 'tendentially significant' and as 'significant'** with p values between $0.005<x<0.05$ and as 'highly significant' *** with p-values <0.005. Abbreviations are given in (Table 3B).

VI.4 Stimulatory effect of Kit pre-treated WB on the secretion of INFγ from T-cell enriched immunoreactive cells (detected by CSA) under Normoxic vs. Hypoxic conditions was comparable

We tested the stimulatory effect of DC/DC_{leu} in leukemic WB on the secretion of INFγ from immune cells (innate and adaptive) before (Uncultured WB) and after $MLC^{WB\text{-}DC}$ ($MLC^{WB\text{-}DC\ Kit\text{-}I}$, $MLC^{WB\text{-}DC\ Kit\text{-}M}$) stimulation vs. ML C^{WB} (n=3). We evaluated the frequency of INFγ secreting cells of the innate and adaptive immune system under NO vs. Hy conditions (Table 3C). Concerning cells of the adaptive immune system, under NO conditions we found significantly higher frequencies of $CD3^+INF\gamma^+/CD3^+$, $CD3^+CD4^+INF\gamma^+/CD3^+CD4^+$ and $CD3^+CD8^+INF\gamma^+/CD3^+CD8^+$ after MLC culture ($MLC^{WB\text{-}DC\ Kit\text{-}I}$ and $MLC^{WB\text{-}DC\ Kit\text{-}M}$) compared to control (MLC^{WB}), with $MLC^{WB\text{-}DC\ Kit\text{-}I}$ showing higher frequencies than $MLC^{WB\text{-}DC\ Kit\text{-}M}$. Comparable results were found in Hy conditions (data not shown).

Concerning cells of the innate immune system under NO conditions, we found significantly higher frequencies of $CD161^+INF\gamma^+/CD3^-CD161^+$ and $CD3^-CD56^+INF\gamma^+/CD3^-CD56^+$ in $MLC^{WB\text{-}DC\ Kit\text{-}I}$ and $MLC^{WB\text{-}DC\ Kit\text{-}M}$ compared to $MLC^{WB\text{-}DC}$ % $CD3^-CD56^+INF\gamma^+/CD3^-CD56^+$ [$MLC^{WB\text{-}DC\ Kit\text{-}I}$: 14.3±11.1, $p<0.007$; $MLC^{WB\text{-}DC\ Kit\text{-}M}$: 16.5±5.4, $p<0.008$; $MLC^{WB\text{-}DC}$: 1.3± 0.96] and % $CD3^-CD161^+INF\gamma^+/CD3^-CD161^+$ [$MLC^{WB\text{-}DC\ Kit\text{-}I}$: 11.4±3.2, $p<0.07$; $MLC^{WB\text{-}DC\ Kit\text{-}M}$: 14.8±1.5, $p<0.03$; $MLC^{WB\text{-}DC}$: 3.4±4.9]. Tendentially significant differences could be found in the frequencies of % $6B11^+INF\gamma^+/6B11^+$ [($MLC^{WB\text{-}DC\ Kit\text{-}I}$: 4.5±1.5, $p<0.1$; $MLC^{WB\text{-}DC\ Kit\text{-}M}$: 4.2±0.82, $p<0.1$) compared to ($MLC^{WB\text{-}DC}$: 3.1±0.1)]. Comparable results were found in Hy conditions (data not shown). Our results show that DC/DC_{leu} stimulation had similar (stimulating) effects on the INFγ secretion of immunoreactive cells in No vs. Hy conditions after MLC. Frequencies of INFγ secreting cells were not significantly different under No vs. Hy conditions (data not shown).

VI.5 Comparing cell subtypes (DC/DCleu subtypes, T-cell subtypes) in cases with improved vs. not improved anti-leukemic activity no significant changes after MLC WB-DC Kits under Normoxic vs. Hypoxic conditions were found

We further compared frequencies of DC and DC_{leu} subtypes (DC, DC_{leu}, DC_{mig}), of proliferating blasts ($Bla_{prol\text{-}CD71}$/ Bla^{+}) and of T-cell subtypes ($T_{non\text{-}naïve}$, T_{naive}, T_{prol}) in the groups with/without achieved improved anti-leukemic activity of effector cells (T-cell enriched WB not pre-treated or Kit pre-treated ($MLC^{WB\text{-}DC\ Kit\text{-}I}$, $MLC^{WB\text{-}DC\ Kit\text{-}M}$) against target cells. In No conditions we found (not significantly) higher frequencies of DC and DC_{leu} subtypes (DC, DC_{leu}, DC_{mig}), and of T-cell subtypes ($T_{non\text{-}naïve}$, T_{prol}) after MLC (as given above) in cases with (vs. not) achieved improved blast lysis ($MLC^{WB\text{-}DC\ Kit\text{-}M}$ (n=8) and $MLC^{WB\text{-}DC\ Kit\text{-}I}$ (n=5)). Comparable results were found in Hy conditions (data not shown). Cases with (vs. not) achieved improved (% DC:13.1±11.2 vs. % DC :12.8±10.8, p<0. 7 ; $\%DC_{leu}$: 6.3±3.2 vs. $\%DC_{leu}$; 5.8±2.8, p<0.8 ; DC_{mig}: 45.1±11.2 vs. DC_{mig}; 43.3±11.2, p<0.4 ; $T_{non\text{-}naïve}$: 71.1±24.3 vs. $T_{non\text{-}naïve}$: 66.2±22.1, p<0.3 , T_{prol} **:** 45.1±14.1 vs. T_{prol} **:**43.5±23.1, p<0.4).

VI.6 Achieved improved anti-leukemic activity of 'effector cells' (T-cell enriched Kit treated ($MLC^{WB\text{-}DC\ Kit\text{-}I}$, $MLC^{WB\text{-}DC\ Kit\text{-}M}$) or untreated WB showed no correlation with clinical subtypes of patients, but with DC(DC_{leu}-) frequencies and with cellular subtypes under No and Hy conditions

We correlated achieved anti-leukemic reactivity of effector cells (T-cell enriched WB treated (or not pre-treated) with Kits against 'target cells' with frequencies of several DC-, immune cell subtypes or patients 'clinical or diagnostic subtypes.

VI.6.1 No impact of age, blast frequencies and sex on achieved anti-leukemic reactivity

Pearson's Correlation analyses of cases (n=13) with (vs. without) achieved improved anti-leukemic reactivity of effector cells (enriched WB pretreated with Kit-I and Kit-M) showed no significant

correlations between patients' diagnostic or clinical parameters (e.g. patients' age, sex, blast frequencies) with achieved anti-leukemic reactions of T-cells after MLC (Figure 18).

Correlation of cases with achieved improved anti-leukemic activity Kits ($MLC^{WB\text{-}DC\ Kit\text{-}I}$, $MLC^{WB\text{-}DC\ Kit\text{-}M}$) with patients' characteristics under No vs. Hy conditions

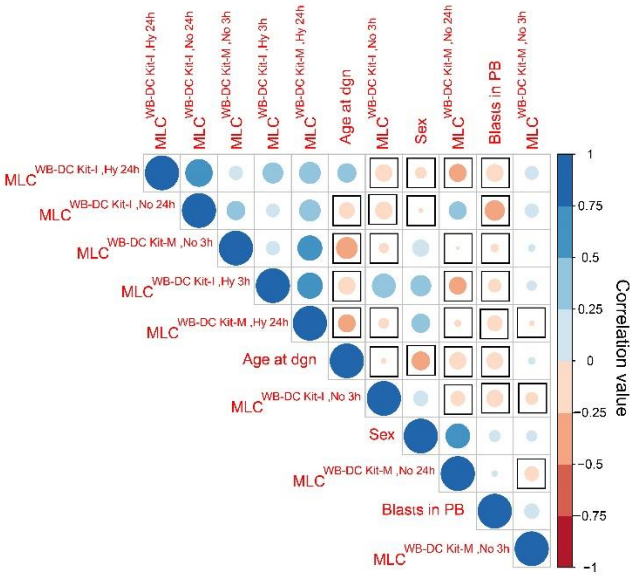

Figure 18. **Correlation analyses**. Correlation analyses (Pearson's correlation) of cases with achieved improved anti-leukemic activity of T-cell enriched MLC ($MLC^{WB\text{-}DC\ Kit\text{-}I}$, $MLC^{WB\text{-}DC\ Kit\text{-}M}$) obtained after 3h or 24h of culture of effector cells (T-cell enriched MLC with WB treated with Kits) against target cells) with patients' clinical or diagnostic parameters (Age, Sex, Blast counts) did not show significant (positive) correlations: cells with square represent negative correlation. Explanations of abbreviations are given in (Table 3A).

VI.6.2 Significant correlation of effector cells anti-leukemic reactivity and DC and DC_{leu} subtypes

We correlated achieved improved anti-leukemic reactivity of 'effector cells' (as given above) (n=13) with several DC subtypes (DC, DC_{leu}, DC_{mig}), T-cell subtypes ($T_{non\text{-}naïve}$, T_{naive}, $T_{prol\text{-}late,}$ T_{reg}) and innate immune cells. In No conditions, we found a significant positive correlation between the DC^{+}/WB (but not of DC_{leu}/WB) generated with Kit-M and the best anti-leukemic reactivity of T

'effector cells' after **3** or **24 h** of coculture with blast target cells. In Hy conditions, we found a significant correlation for both DC^+/WB and DC_{leu}/WB generated with Kit-M and the anti-leukemic activity of 'effector cells' (Figure 19, 20). This effect was not observed for DC^+/WB and DC_{leu}/WB generated with Kit-I.

No significant correlations between other cell subsets (DC_{mig}, $T_{naïve}$, T_{reg}, $T_{non\text{-}naïve}$, T_{cm}, T_{prol}, NK cells) and the anti-leukemic activity of 'effector cells' (T-cell enriched WB treated with Kit-M and Kit-I ($MLC^{WB\text{-}DC\ Kit\text{-}I}$, $MLC^{WB\text{-}DC\ Kit\text{-}M}$) under No vs. Hy conditions were found.

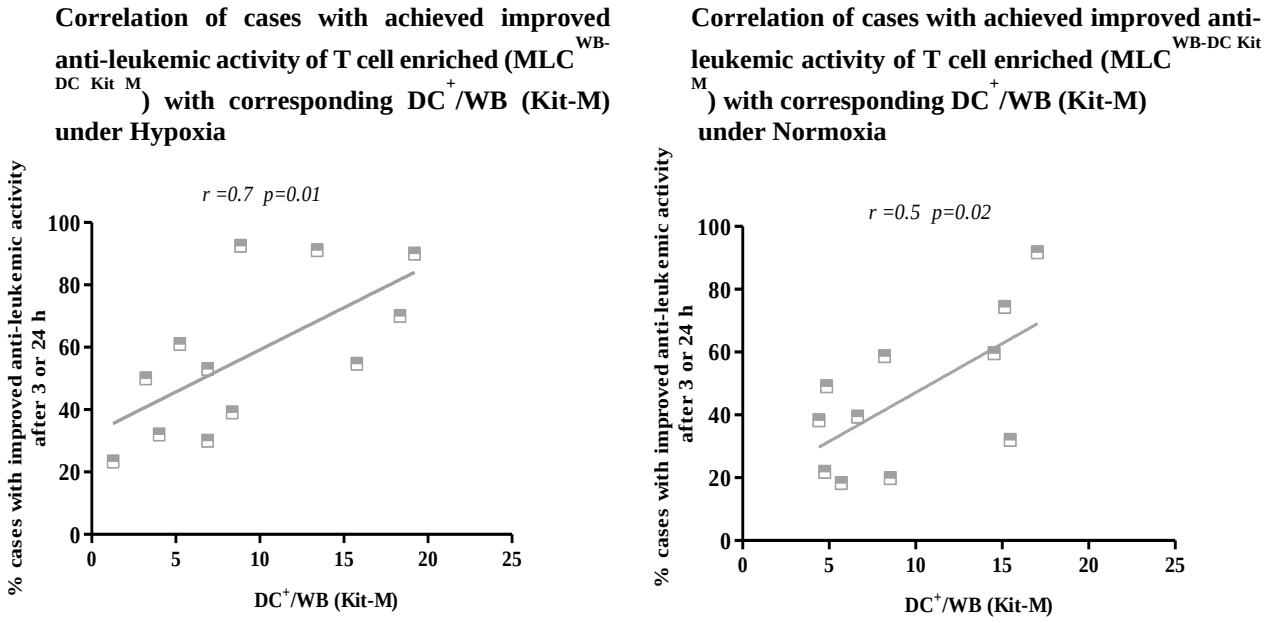

Figure 19. Correlation of cases with improved anti-leukemic reactivity and DC subtypes. Correlation of cases with achieved improved anti-leukemic activity of T-cell enriched MLC ($MLC^{WBDC\ Kit\ M}$) compared to MLC^{WB} (y axis) with DC^+ /WB in Hy (left) and No (right) condition corresponding Kit-M (x axis) treated WB. Normoxic (right) vs. Hypoxic (left) conditions. Correlation was definite as 'negligible' with r-values between .00 to .30 (-.00 to -.30), as 'low' with r-values .30 to .50 (-.30 to -.50), as 'moderate' with r-values .50 to .70 (-.50 to -.70) and as 'high correlation' with r values .70 to 1.00 (-.70 to -1.00). When the p values of differences in the statistical tests were between 0.1 and 0.05, we consider it as 'tendentially significant' and as 'significant'** with p values between 0.005<x <0.05 and as 'highly significant' *** with p-values <0.005.

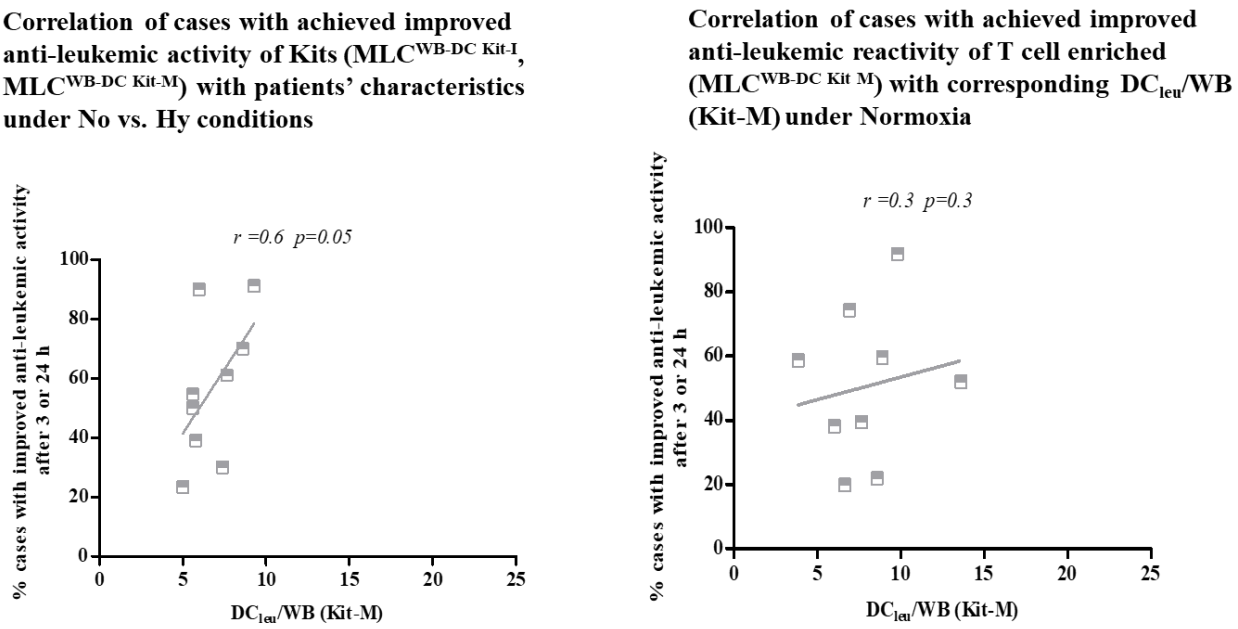

Figure 20. Correlation analyses of cases with improved anti-leukemic reactivity and DC_{leu} subtypes. Correlation of cases with achieved improved anti-leukemic activity of T-cell enriched MLC ($MLC^{WBDC\ Kit\ M}$) compared to MLC^{WB} (y axis) with DC_{leu}/WB in Hy (left) and No (right) condition corresponding Kit-M (x axis) treated WB. Normoxic (right) vs. Hypoxic (left) conditions. Correlation was defined as 'negligible' with r values between .00 to .30 (-.00 to -.30), as 'low' with r values .30 to .50 (-.30 to -.50), as 'moderate' with r values .50 to .70 (-.50 to -.70) and as 'high correlation' with r values .70 to 1.00 (-.70 to -1.00). When the p values of differences in the statistical tests were between 0.1 and 0.05, we considered them as 'tendentially significant' and as 'significant'** with p values between 0.005<x <0.05 and as 'highly significant' *** with p-values <0.005.

Chapter4: Discussion

VII. Discussion

VII.1 DC-based immunotherapy for AML

AML is a clonal disorder with a high risk of relapse. Anti-leukemic immune responses in the course of AML treatment might fail due to escaping mechanisms of the leukemic blasts such as impaired or mistaken antigen expressions, resistance to apoptosis, or other inhibitory mechanisms (Cassier, Castets et. al., 2017). Several immunotherapeutic strategies are investigating the re-activation of the antitumor immune response to overcome this problem (Geiger and Rubnitz 2015). One of the most convincing approaches, which has been evolved for such purposes, is the use of DC-based vaccines. Indeed, DCs generated *ex vivo* from monocytes can be equipped with different LAAs, tumor antigens. These DCs or leukemia derived DC, can be in practice applied as an effective and efficient treatment in the course of adoptive treatment (Amberger et. al., 2020). Such a vaccination has been reported to increase the frequencies of leukemia specific T-cells in AML-patients and achieved even complete remissions in elderly patients (Van Tendeloo, Van de Velde et. al., 2010; Rosenblatt, Stone et. al., 2016; Weinstock, Rosenblatt et. al., 2017).

Ex vivo generation of DCs and DC_{leu} has been reported from whole blood (WB) or peripheral blood mononuclear cells (PBMCs) for AML-patients, mostly without induction of proliferation of blasts (Kremser, Dressig et. al., 2010; Platt and Randolph 2013; Johnson and Jackson 2014). This goes along with the great potential to induce anti-leukemic T-cells in culture (Kremser, Dressig et. al., 2010; Grabrucker, Liepert et. al., 2010; Anguille, Van Tendeloo et. al., 2012; Nahas, Stroopinsky et. al., 2019; Ansprenger, Amberger et. al., 2020). Others and we already showed that DC_{leu} generation is possible with DC-generating protocols, independent from mutations, FAB classification, MHC and age factors (Kremser, Dressig et. al., 2010; Ansprenger, Amberger et. al., 2020).

DC-generating protocols are composed of a mixture of various response modifiers including Picibanil (OK432), Prostaglandin E2 (PGE2), granulocyte-macrophage colony-stimulating factor (GM-

CSF) and Tumor-necrosis-factor alpha (TNF-α) (Hirn Lopez, 2019). Moreover, some cytokines (e.g. Interleukin 1 beta (IL-1β), Interferon-gamma (IFN-γ)) have been additionally used in different protocols for the DCs / DC_{leu-} generation from leukemic or healthy PBMCs (Sato, Takayama et. al., 2003; Kremser, Dressig et. al., 2010; Hirn Lopez, Deen et. al., 2019).

An interesting approach could be, to induce the production of DC_{leu} from blasts *in vivo* after treatment of patients with DC_{leu}- inducing response modifiers. In the majority of existing protocols Normoxic (No) conditions have been applied for the *ex vivo* generation of DC_{leu} for adoptive transfer or to study DC_{leu}-generation from WB. However, this might not be equivalent to results after physiological Hypoxic (Hy) conditions (Rieger and Fiegl 2016). Therefore, we designed the current study to establish and compare a protocol under No and Hy conditions for the generation of DC/DC_{leu} from AML- WB or MNC using DC/DC_{leu} generating protocols. We further assessed the effect of DC/DC_{leu} stimulation on the T-cell stimulation, T-cell composition, and cytotoxicity under physiological (Hy) compared to No conditions. We speculated that Hy could be relevant in some clinical aspects as formulated in the following questions:

1. Would it be better to produce DC/DC_{leu} *ex vivo* under Hy similar to physiological conditions?
2. Can we deduce more relevant physiological data under Hy to predict *in vivo* reactions?
3. Are leukemic cell lines appropriate tools to test Hy condition on the generation of DC/DC_{leu}?

VII.2 Hypoxia, a condition with influence on haematological and immune reactions in the body

Hematopoietic and immunoreactive cells are exposed to changing O_2 in the BM at hypoxic O_2 concentrations of about 0.1-1%, in the arterial blood of about 12% or of 4-15% in peripheral venous blood (Marenzana and Arnett 2013; Deynoux, Sunter et. al., 2016). Several groups used Hy at 6% O_2 to

simulate physiological Hy conditions *ex vivo* (Fiegl, Samudio et. al., 2009; Deynoux, Sunter et. al., 2016). Some studies suggest that Hy might suppress the anti-leukemic effect of immune-cells that weakens the success of current anti-leukemic therapies (Lee, Mace et. al., 2010). Furthermore, physiological Hy could stimulate the proliferation (CD69 positivity) and activation of NK cells and thus contribute to anti-leukemic functions (Vasold, Wagner et. al., 2015). Presumably, however, there is no consistent effect of hypoxia on the immune system (Sitkovsky, Hatfield et. al., 2014). Furthermore, a correlation could be shown between the hypoxic tumor microenvironment with poor response to radiation/chemotherapy in patients (Lee, Mace et. al., 2010).

Different studies show that the effects of oxygen on immune cells are dependent on the tissue and the duration of hypoxia exposure. For example, the 6% O_2 may be routinely termed "Hypoxic" (Hy), however, this O_2 concentration is a normal physiological condition in the bone marrow and stem cell niche. (Abdollahi, Harris et. al., 2011; Carreau, El Hafny-Rahbi et. al., 2011). We used 10% O_2 as an average of venous arterial blood as described before (Marenzana and Arnett 2013; Deynoux, Sunter et. al., 2016). Moreover, intense blast-proliferation and O_2-consumption in leukemic-PB is known to cause a decrease of O_2 pressure (pO_2) (Drolle, Wagner et. al., 2015) which might affect the biology of blast cells (Deynoux, Sunter et. al., 2016) compared to standard Normoxic (No) condition (21% O_2).

In the low pO_2, the expression of membrane receptors (e.g. CXCR4) and the activation of intracellular signalling cascades (e.g. MAPK, HIF1a (as a pO_2-sensible-tumor-suppressor-genes)) change (Fiegl, Samudio et. al., 2009) and some pathophysiological mechanisms in AML including blast cell-cycle-arrest, blast-proliferation, expression of Hy induced factors (e.g.Hif-1α) were shown to be influenced. Physiological Hy induces arrest of cell cycle in the G0/G1-phase of the AML blasts (cell lines and primary AML samples) by increasing the expression of anti-apoptotic XIAP and activation of PI3K/Akt (Baharaghdam, Yousefi et. al., 2018). To answer the question of whether physiological Hy might influence on the behaviour and development of cells in culture (especially DC/DC_{leu} or MLC) we

cultured and analysed different apoptosis and proliferation aspects of leukemic cell lines under Hy vs. No.

VII.3 Generation of DC/DC_{leu} from leukemic cell lines

We used AML cell lines in the first step to test the impact of Hy, as it has been shown that AML cell lines are useful tools to study the influences of hypoxia on leukemic cell growth (Goto, Miwa et. al., 2014). Indeed, we showed that DC can be generated with DC-generating kits, independent from the cell line type under 6% O_2 (Hy) and 21% O_2 (No), independent from two different media we used.

Our results showed no significant decrease for DC_{leu} (frequencies of DC_{leu}/Bla^+) and no differences in blasts' proliferation in Hy compared to No condition under the influence of different Kits. HIF-1α might be the responsible factor for adaption to low oxygen levels in DCs' generation under Hy conditions. Therefore, it can be the reason for equal DC_{leu} generation under Hy compared to No conditions (Drolle, Wagner et. al., 2015). Interestingly, we found higher frequencies of cells in S-phase under No condition compared to Hy in the AML cell lines. Consistently, our apoptosis analyses for these cell lines under Hy and No conditions showed an (non-significant) increase of all blasts' apoptosis markers ($Bla_{apo\text{-}CD261}/Bla^+$, $Bla_{apo\text{-}CD262}/Bla^+$, $Bla^+AnnexinV^+PI^-$ cells/Bla^+) under Hy compared to No conditions. This can explain why we observed more S-phase committed cells in the No condition. The expression of apoptosis markers did not change under the influence of kits in No vs. Hy conditions. The most important result from the cell line part was, that we could confirm DC-generation under Hy condition at a comparable level as under No condition that encouraged us to perform further experiments with patients' materials.

VII.4 DCs / DC_{leu} generation from Healthy and AML PBMNCs

Generation of DC/DC_{leu} (including subgroups) with DC-generating protocols from healthy and AML PBMNCs using Pici-$_{PGE1}$/ Pici-$_{PGE2}$ was successful in comparison to controls. With this data, we confirm data already described by others and us (Sato, Takayama et. al., 2003; Grabrucker, Liepert et. al., 2010; Kremser, Dressig et. al., 2010; Amberger, Doraneh-Gard et. al., 2019). The frequencies of DC_{leu} in PBMNC from AML patients and healthy were shown to be higher in the Pici-$_{PGE1}$ and Pici-$_{PGE2}$ generating protocol compared to the control group. Interestingly all results obtained were similar with no significant differences between Hy and No conditions. These results prove that Hy, as a physiological condition, does not need to be implemented for the generation of DC/DC_{leu} and later adoptive transfer. In the context of clinical approaches, these results support the notion that AML patients could be treated directly by inducing DC/DC_{leu} generation *in vivo* without a need to further adoptively transfer of *ex vivo* generated DC/DC_{leu} under Hy condition (Köhler, Reizis et. al., 2012; Rein and Chao 2014).

VII.5 DCs/ DC_{leu} from Healthy and AML- WB Cultures

Whole blood contains all soluble and cellular (inhibitory and activating) factors present in the individual AML patient with potential activating or inhibitory influence on physiological immune reaction (Boeck, Amberger et. al., 2017). We wanted to know whether results under Hy and WB are comparable to No conditions.

Our results showed that all DC/DC_{leu} -generating protocols which we applied in PBMNCs, (kit-I, K, and M) were promising in generating DCs and DC_{leu} from WB, AML samples as it has been also reported before (Kremser, Dressig et. al., 2010). GM-CSF can induce hematopoietic differentiation and/or Picibanil, PGE1 and PGE2 can mediate danger signalling and drive maturation of DC/DC_{leu}. Some response modifiers (e.g.IL-4 and other cytokines) are physiological components of WB compared to PBMNC-cultures (Fischbacher, Merle et. al., 2015; Subklewe, Geiger et. al., 2014). Here, we show

that we could generate significantly more DC/DC_{leu} from healthy or AML-WB using Kits (-I, -K, and -M) in comparison to the control group without added response modifiers.

DC- generating methods to produce (sufficient) DC/DC_{leu} work in physiological (Hy) conditions as well as in No conditions. Finally, we found that DC_{leu} subgroups do not differ significantly in No and Hy conditions. Importantly, (not to DC_{leu} converted) blasts' proliferation during DC/DC_{leu} -generation with immunomodulatory Kits was not induced under No and Hy conditions. Therefore, these immunomodulatory Kits could be used for patients' treatment. That means we can use the WB model under No to simulate *in vivo* reactions (and probably to predict *in vivo* reaction). Furthermore, we found higher amounts of DC_{leu} in both the DC- and Blast-fraction of Kit-I and Kit-M in comparison to controls. We also found (non-significantly) higher amounts of DC_{mig}/DC generated with Kit-I and Kit-M compared to controls with comparable amounts after Kit-I and Kit-M treatment. For the differentiation of DC_{mig} in its subgroups, we could not find significant differences between Kit-I and Kit-M and control in both Hy and No. The CCR7 (a maturation marker for DCs) expression is essential for DC and DC_{leu} migration to lymph nodes. CCR7 expression was induced through Kit-I and Kit-M and was shown to activate different T-cell subtypes and other immunoreactive cells (Ogihara, Iinuma et. al., 2004; Förster, Davalos-Misslitz et. al., 2008; Platt and Randolph 2013). In conclusion, we found that Kit-M and I are the best kits for generating mature DC/DC_{leu} *ex vivo* from healthy and AML- WB for both No and Hy conditions.

It appeared that Kit-I with the mixture of GM-CSF + OK-432, has a stronger stimulatory effect on the DC-differentiation than Kit-M with the combination of GM-CSF + PGE1, but a similar stimulatory effect on DC maturation. This difference can be due to OK-432 compared to PGE1 or could be due to a combination with GM-CSF in Kit-I compared to Kit-M, which has to be further investigated.

VII.6 Expression of checkpoint and apoptosis markers on Bla/DC in WB

PD-L1 is an immune checkpoint, which protects cells encountering the autoimmune responses with the help of the induction of apoptosis in antigen specific T-cells on one hand and the inhibition of apoptosis in regulatory T-cells on the other hand. PD-L2 is another player of this mechanism, which is abundantly expressed on dendritic cells and macrophages. When PD-L1/ PD-L2 show interactions with their receptors, a signalling cascade starts that impedes several downstream kinases pathways and shows involvement in the activation of T-cells (Parry, Chemnitz et. al., 2005).

We could already show that checkpoint markers are expressed on blasts in leukemic WB in AML patients after the generation of DCs. The average frequencies of generated PD-L1 and PD-1 expressing blast cells through the stimulation with DC/DCleu were comparable with Kit-I, Kit-K, and -Kit-M and control. We explored comparable results under No vs. Hy conditions. Our results for the stimulation of immunoreactive cells with DC/DCleu revealed an increase of PD-1 (CD279) compared to PD-L1 (CD274) on blasts. This finding might also be explained by the over expression of PD-1 which is necessary for the improvement of the immune response by the activation of T-cells (Chen, Jiang, et. al., 2016). Moreover, the hypoxia-inducible factor (HIF)-1 regulates PD-L1 gene expression through binding to a hypoxia response element of the PD-L1 promoter (Noman, Desantis, et. al., 2014). On this basis, we hypothesized that Hy might be enhancer of the checkpoint inhibitor PD-1 receptor and increased PD-L1 expression. However, our results could not confirm this hypothesis, and no significant change in frequencies of blasts expressing checkpoint markers (BlaCD274/Bla+, BlaCD279/Bla+) in Kit treated WB under No compared to Hy were found. This can also be due to different O_2 environments in the AML bone marrow niche (1%) compared to our Hy (10%) study conditions. This is contradictory to what is observed in solid tumor (melanoma, lung, breast, and prostate cancer) where Hy directly up-regulates the expression of PD-L1 via HIF-1α, that binds directly to the HRE (Hypoxia Response Elements) in the promoter of the PD-L1 gene (Noman, et. al., 2019).

Many studies reported that Hy facilitates cell death or injuries of cells in different ways including induction of pathways of oxidative stress, inflammation, acidosis, and apoptosis. Apoptosis, is the main mechanism for programmed cell death, acting as a very critical factor in hypoxia-induced cellular injury (Saraste and Pulkki 2000). Hy can induce apoptosis specifically by inducing of mitochondrial damages, calcium overload, increased oxygen free radicals, and the over-activation of hypoxia-inducible factor (HIF) (Guo, Tan, et. al., 2019).

Performing apoptosis analyses, we did not find an increase of expression of apoptosis markers (CD261/CD262) on blasts as well as DC, DC_{leu} from WB AMLs under No and Hy conditions. The average expression of apoptosis markers on Bla, DC/ DC_{leu} after influences of all Kits (-I, -K, and -M) were compared and there were no significant changes between No and Hy conditions.

VII.7 DC/DC_{leu} stimulation in T-cell enriched MLC results in activated cells of the innate and adaptive immune system

T-, iNKT-, NK- and CIK- cells and their subsets are important mediators of innate and adaptive immune responses: antigen-presenting DC can activate T-cells and their anti-tumor functionality can be further improved by DC_{leu} (Vogt, Schick et. al., 2014). DC/DC_{leu} -generated from healthy or leukemia WB using Kits were used to stimulate immune cells in T-cell enriched MLC. We could previously confirm that the T-cell enriched immunoreactive cells can be activated with DCs and DC_{leu} generated from leukemic WB. It could shift the T-cells composition in a positive way that induced antileukemic activity which is in line with previous studies (Grabrucker, Liepert et. al., 2010; Kremser, Dressig et. al., 2010; Schick, Vogt et. al., 2013; Vogt, Schick et. al., 2014). Results obtained with different Kits were pooled since results were comparable. The T-cells' composition could be changed after T-cell stimulation with DC/DC_{leu} generated from AML-WB and healthy under Hy and No conditions (Grabrucker, Liepert et. al., 2010; Kremser, Dressig et. al., 2010; Schick, Vogt et. al., 2013; Vogt, Schick et. al., 2014).

Consistently, in AML patients and healthy PB donors, we showed an increase in the frequencies of proliferating T-cells compared with Uncultured. We had a shift of cell subsets from T_{naive} to $T_{non\text{-}naïve}$ and an increase of $CD3^{+}CD4^{+}$ compared to Uncultured and MLC^{WB} in both No and Hy conditions. The higher frequencies of T-cell subtypes could be described by IL-2, which was added to the MLC culture (Brune, Castaigne et. al., 2006; Boyman and Sprent 2012). In AML patients we had a high frequency of $T_{eff\text{-}em}$ stimulated $MLC^{WB\ DC\text{-}Kits}$ as compared to Uncultured, but we had not a high frequency of T_{cm} compared to Uncultured.

T_{cm} cells showed comparable frequencies in $MLC^{WB\ DC\ -Kits}$, MLC^{WB}, and also in the uncultured cells. Therefore, only the $T_{eff\text{-}em}$ cells were produced. However, we had a high frequency of T_{cm} and a low level of $T_{eff\text{-}em}$ in healthy samples. $T_{eff\text{-}em}$ enable an immediate and fast immune-response, while T_{reg} cells can down regulate immune responses, what is necessary to inhibit auto aggressive immune reactions but also in a context to down-regulate anti-tumor-responses (Schick et. al., 2013; Vogt et. al., 2014). Our results indicated a specific and quick secondary immune response $T_{eff\text{-}em}$ after stimulation, which can cause cytotoxicity and inflammation in a variety of tissues (Sallusto, Lenig et. al., 1999; Zhang and Lakkis 2015). A critical role is played by memory T-cells in the complete remission of AML patients (Sallusto, Lenig et. al., 1999, Facciabene, Motz et. al., 2012). While T_{reg} cells have decreased (not significantly) in stimulated $MLC^{WB\ DC\text{-}Kits}$ compared to MLC^{WB} in AML and healthy under No and Hy, they were able to down regulate responses of the immune system. It could be critical to inhibit auto aggressive reactions of the immune system nevertheless to down regulate anti-tumor-responses (Facciabene, Motz et. al., 2012; Schick, Vogt et. al., 2013; Vogt, Schick et. al., 2014, Okeke and Uzonna 2019). The reaction of iNKT-, NK- and CIK-cells being fast in contrast to T-cells, by the production of chemokines and cytokines (Robertson et. al., 2014; Guo and Han, 2015) and without target-cells' recognition by a TCR or previous activation for the killing of tumour-cells (Pittari et. al., 2015).

Our data showed that stimulations with IL-2 can activate iNKT-cells, in AML-or healthy samples under No and Hy. Comparable results were found for NK- and CIK- cells. These results might point towards stimulation of these cell-subtypes by DC/DC_{leu}-. (Sato et. al., 2003; Kremser et. al., 2010; Boeck, Amberger et. al., 2017). iNKT cells serve as a bridge between the innate and adaptive immune system for the production of different chemokines and cytokines such as GM-CSF, IL-4/-2/-12 and TGF-ß. Consequently, iNKT can activate different kinds of cells such as NK-cells, DC, - T_{reg} and $CD4^+$/$CD8^+$ T-cells with a high potential which can directly and strongly induce the killing of the tumor cells (Matsuda, Takeda et. al., 2010; Van Kaer, Parekh et. al., 2011; Robertson, Berzofsky et. al., 2014). Our experimental data under No vs Hy conditions with WB cultured showed no significant differences in the generation of DC frequencies and compositions of T-, CIK- and NK- cells in MLC. However, we identified higher production of iNKT cells under Hy condition. This means that Hy could influence innate immunity in a positive manner.

VII.8 Expression of checkpoint markers on T-cells

Immune checkpoints are induced in the activated T-cells by some regulatory pathways and play an essential role to prevent autoimmunity and uncontrolled T-cell expansion. Many checkpoint molecules can regulate the activation of T-cells (e.g. programmed death-1 (PD-1), cytotoxic T-cell antigen-4 (CTLA-4)), B and T-cell lymphocyte attenuator (BTLA), lymphocyte activation gene-3 (LAG-3) (Sehgal, Whiteside et. al., 2015). The PD-1 receptor (CD279), as a transmembrane receptor can bind to two distinct ligands: PD-L1 (CD274) and PD-L2 (CD273). Both are members of the B7 immunoglobulin superfamily. PD-L1 is expressed on activated T-cells, B cells, and myeloid cells. PD-1 is considered to protect against autoimmune responses by mediating both induction of apoptosis in antigen-specific T-cells and inhibiting apoptosis in regulatory T-cells.

In general, T-cell activation upregulates PD-1 which is necessary for the regulation of the immune response. Expression of PD-L1 is under the influence of several transcriptional factors such as hypoxia-inducible factor (HIF)-1 that controls PD-L1 through binding to the hypoxia response elements in the PD-L1 promoter region. Therefore, we hypothesized that hypoxia, as our study model, might upregulate PD-L1 expression and increase the checkpoint inhibitor PD-1 receptor (Noman, Desantis et. al., 2014; Chen, Jiang et. al., 2016). Our results demonstrate that expression of PD-1 was higher than PD-L1 on activated T-cells, which is in conflict with previous observations (Noman, Desantis et. al., 2014), no differences were found in the expression of PD-1 and PD-L1 on activated T-cells in Hy compared to No conditions. It has been reported that exposure of AML cells to hypoxia caused the upregulation of both PD-L1 and Hif1α on activated T-cells (Noman, Desantis et. al., 2014). Indeed, PD-L1 is a downstream target of Hif1α (Hayashi, et. al., 2019). Thus, we speculate the discrepancy between our results and published data could be due to lack/low level of Hif1α expression in our Hy condition. Other more detailed experiments have to be performed in the future to further evaluate the influence of Hy or No on cellular expressions of checkpoint markers.

VII.9 DC/DC_{leu} stimulation in T-cell enriched MLC results in improved antileukemic (cytotoxicity/ leukemia specific) activity

The composition of Kits (-I, -K, -M) to DC/DC_{leu} generation, had no effect on the phenotypes and frequencies of T-cell subtypes after MLC culture, however, had some impact on T-cells' anti-leukemic activity. Most prominent findings of this study were that anti-leukemic activity of (T) cells after $MLC^{WB\text{-}DC\text{-}Kits}$ could be improved in comparison to controls (MLC^{WB}) in No as well as Hy. Interestingly, the induction of anti-leukemic activity was significantly stronger in Hy condition. Our results show that the DC/DC_{leu} generated from leukemic WB with modulatory Kits (-I,-K,-M) can activate the anti-leukemic activity of T-cells and the immune system against blast cells after MLC culture. Remarkably, some AML-samples improved or achieved lysis just after 3h and some cases only after 24h, while average

lysis was highest in $MLC^{WB\text{-}DC\ Kit\text{-}I}$ after 3h and in $MLC^{WB\text{-}DC\ Kit\text{-}M}$ after 24h. This could be explained by different killing mechanisms by immunoreactive cells: the slow pathway of Fas/FasL- or the fast pathway of perforin-granzyme-mediated killing . Both mechanisms can act synergistically or separately (Hassin, Garber et. al., 2011; Rauf, Khatri et. al., 2012). $MLC^{WB\text{-}DC\ Kit\text{-}I}$ hereby seems to act via the 'fast' pathway, while $MLC^{WB\text{-}DC\ Kit\text{-}M}$ appears to operate via the 'slow' pathway. Furthermore, the best anti-leukemic activity after 3 or 24h, $MLC^{WB\text{-}DC\ Kit\text{-}I}$ and - $MLC^{WB\text{-}DC\ Kit\text{-}M}$ appeared to be similarly efficient under No conditions. However, we found significantly more cases with lysis with $MLC^{WB\text{-}DC\ Kit\text{-}M}$ in comparison to controls (MLC^{WB}) after 3 or 24h in Hy compared to No. We speculate that this might be related to the higher production of iNKT cells under Hy condition. These results show that Hy as a physiological condition can improve better the anti-leukemic activity compared to No, especially with $MLC^{WB\text{-}DC\ Kit\text{-}M}$. Comprehensively, the cytotoxicity assay (CTX) provides us the opportunity to measure the acquired anti-leukemic cytotoxicity.

In addition, the same result was proven by CSA, as CSA is an efficient method to detect, enrich, and monitor IFN-γ secreting cells. Cells were stimulated *ex vivo* with a Kit containing GM-CSF and PGE_1, which resulted in increased anti-leukemic response and induction of IFN-γ secreting cells detected by CSA. This *ex vivo* induction of IFN-γ production could be induced by DC-mediated processes revealing that modulation of blasts (to DC_{leu}) could be a commendatory approach to treat (and cure) AML patients under Hy and No conditions. By stimulation of immunoreactive cells with $MLC^{WB\text{-}DC\ Kit\text{-}I}$ /$MLC^{WB\text{-}DC\ Kit\text{-}M}$, a significant increase of IFNy secreting cells of the adaptive immune system ($CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^+$) in $MLC^{WB\text{-}DC\ Kit\text{-}I}$ and $MLC^{WB\text{-}DC\ Kit\text{-}M}$ compared to uncultured WB was seen. This was also true for MLC^{WB} under Hy and No conditions. Moreover, frequencies of IFNy secreting cells of the innate immune system ($CD3^-CD56^+$) significantly increased after $MLC^{WB\text{-}DC\ Kit\text{-}I}$ and $MLC^{WB\text{-}DC\ Kit\text{-}M}$ compared to uncultured WB as well as after MLC^{WB} but not compared to MLC^{WB} under Hy and No conditions. Frequencies of $6B11^+$ cells did not show significant differences compared to Uncultured and MLC^{WB} neither under Hy and nor under No conditions. We have found an increased

innate and adaptive immunoreactivity after DC/DC_{leu} stimulation, directing the immune system to the induction of leukemia specific (T) cells. IL-2 is known to induce the expression of lectin-like transcript 1 (LLT1) of various cells, as cross-linking of LLT1 and CD161 could cause an inhibition of IFNy production of NK cells and eventually with their cytotoxicity (Llibre, Klenerman et. al., 2016). This can be the reason for decreased levels of IFNy secreting $CD3^-CD161^+$cells, as observed in our results after culture compared to Uncultured. Stimulation of NK cells with $MLC^{WB\text{-}DC\ Kit\text{-}I}$ and $MLC^{WB\text{-}DC\ Kit\text{-}}$ might at least in part compensate this effect.

VII.10 Correlation of anti-leukemic cytotoxicity of immunoreactive cells stimulated by DC/DC_{leu}

We correlated achieved anti-leukemic reactivity of effector cells (T-cell enriched WB treated (or not pre-treated) with Kits ($MLC^{WB\text{-}DC\ Kit\text{-}I}$, $MLC^{WB\text{-}DC\ Kit\text{-}M}$) against 'target cells' with frequencies of several DC-, immune cell subtypes. In No, we found a significant positive correlation between the DC^+/WB generated with immunomodulatory Kit-M and the best anti-leukemic activity of T 'effector cells' after 3 or 24 h of $MLC^{WB\text{-}DC\ Kit\text{-}M}$. In Hy conditions, we found a significant correlation in DC^+/WB and DC_{leu} /WB generated with Kit-M and the best anti-leukemic reactivity of T 'effector cells' $MLC^{WB\text{-}DC\ Kit\text{-}M}$ after 3 or 24 h. The other cell subtypes did not reveal a direct correlation with achieved cytotoxicity. Results presented here confirm results of previous studies where a crosstalk between DC-stimulation and anti-leukemic cytotoxicity of T-cells was described (Bakdash, Schreurs et. al., 2014). Moreover, the better anti-leukemic reactivity of $MLC^{WB\text{-}DC\ Kit\text{-}M}$ compared to MLC^{WB} could be a good explanation for the positive correlation between (DC_{leu}/WB) generated with Kit-M and the best anti-leukemic activity of T-cells ($MLC^{WB\text{-}DC\ Kit\text{-}M}$) in Hy. Thus, we conclude that culture in physiologic conditions (Hypoxia, WB) could have a positive impact on the DC generation, especially with Kit-M and anti-leukemia activity.

VIII. Conclusion

These results indicate that *ex vivo* Kit-I and Kit-M treatment of WB generates higher frequencies of DC subtypes (DC, DC_{leu}, DC_{mig}), activates T-cells' proliferation, $T_{non\text{-}naïve}$, differentiation (especially $T_{eff\text{-}em}$ cells) and decreases frequencies of T_{reg} ($CD3^+$, $CD4^+$) compared to controls after MLC under Hy that was similar to No condition. Furthermore, our results obtained from AML cell lines, MNC and WB from leukemic and healthy samples showed that (i) generation of DC, blast proliferation, DC apoptosis, and immune checkpoint marker expression, (ii) composition of different T-cell, NK-iNKT-and CIK-cell-(subtypes) after MLC, (iii) anti-leukemia activity of T-cells and cytokine secretion assay of T-cells under Hy/physiological condition is comparable to No condition. Interestingly, we found that the production of iNKT-cells after $MLC^{WB\ DC\text{-}Kits}$ in Hy condition is higher than No condition. Consistently, we found more lysis effect of T-cells under hypoxia condition. These results suggest that Hy condition has a greater influence on the generation of DC and the anti-leukemic activity of T-cells compared to No. However, additional studies should be done to find out the optimum Hy condition.

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X. List of publications

X.1 Original studies

F. Doraneh-Gard, D. C. Amberger, C. L. Boeck, C. Plett, C. Gunsilius, C.Kugler, J. O. Werner, J. Schmohl, D. Kraemer, B. Ismann, A. Rank, C. Schmid, H. Schmetzer: Influence of physiological hypoxia on whole blood (WB) from healthy donors and AML-pts: the (leukemic) antigen-presentation on blasts, on leukemia-derived dendritic cells (DC_{leu}) after stimulation with 'DC-generating cocktails' as well as the antileukemic functions of DC/ DC_{leu}-stimulated immune reative cells (in prep 2021).

L. Klauer1, O. Schutti1, S. Ugur1, **F. Doraneh-Gard**, Helga Maria Schmetzer. Cytokine secretion assay as a tool to monitor the production of antigenspecific IFN- γ secreting cells of the innate and adaptive immune system in culture systems of Tcells stimulated with leukemia-derived DC. functions of DC/ DC_{leu}-stimulated immune reative cells. Transfusion Medicine and Hemotherapy. **accepted, in press** (2021).

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X.2 Congress contributions

1) Standard-NORMOXIC versus physiological HYPOXIC culture of AML-patients' whole blood samples with immunomodulatory Kits yields comparable proportions of dendritic cells and functional results. Presentation; ITOC Conference. Berlin (2017).

2) Standard-NORMOXIC versus physiological HYPOXIC culture of AML-patients' whole blood samples with immunomodulatory Kits yields comparable proportions of dendritic cells and functional results. Poster; EBMT Conference. Lisbon (2017).

3) D. C. Amberger, **F. Doraneh-Gard**, C. L. Boeck, C. Plett, C. Gunsilius1, C. Kugler1, J. O. Werner, D. Kraemer, B. Ismann, A. Rank, C. Schmid, H. Schmetzer: A new method to generate mature (leukemia-derived) dendritic cells that improve antileukemic T-cell reactivity from mononuclear cells or whole blood from healthy volunteers or patients with AML. Abstract and poster presentation; ITOC 4 2017- Journal for Immunotherapy of Cancer 2017, 5 (1), 4-5 (2017).

4) D. C. Amberger, **F. Doraneh-Gard**, C. L. Boeck, C. Plett, C. Gunsilius1, C. Kugler1, J. O. Werner, D. Kraemer, B. Ismann, A. Rank, C. Schmid, H. Schmetzer: A new method to generate mature (leukemia-derived) dendritic cells that improve antileukemic T-cell reactivity from mononuclear cells or whole blood from healthy volunteers or patients with AML. Abstract and poster presentation; EBMT 2017: www.ebmt2017.org/poster-abstracts; Physician Poster Abstracts: A121.

5) C. L. Boeck, D. C. Amberger, **F. Doraneh-Gard**, W. Sutanto, T. Guenther, J. Schmohl, F. Schuster, H. Salih, F. Babor, A. Borkhardt, H. Schmetzer: Significance of frequencies, compositions and/ or antileukemic activity of (DC-stimulated) invariant NKT, NK and CIK cells is predictive for outcome of patients with AML, ALL and CLL. Poster and abstract in preparation for 19th scientific symposium of Med 3, University of Munich (2017).

XI. List of Tables

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XIII. Acknowledgments

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I hereby declare, that the submitted thesis entitled

Influence of physiological hypoxia on whole blood (WB) from healthy donors and AML-pts: the (leukemic) antigen-presentation on blasts, on leukemia-derived dendritic cells (DCleu) after stimulation with ‘DC-generating cocktails’ as well as the antileukemic functions ofDC/ DCleu-stimulated immune reactive cells

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I further declare that the submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

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