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Increased detection of (leukemia-specific) adaptive and innate immune-reactive cells under treatment of AML-diseased rats and therapy-refractory AML-Patients with clinically approved, blast modulating response modifiers

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Zusammenfassung

Hintergrund: Kits (Kombinationen von immunmodulatorischen Substanzen: GM-CSF + entweder Picibanil (Kit I), Prostaglandin E2 (PGE2, Kit K) oder Prostaglandin E1 (PGE1, Kit M, Patent DE102014014993) wandeln myeloische Blasten in dendritische Zellen leukämischen Ursprungs (DC_{leu}) um. Nach Stimulation mit DC_{leu} können ex vivo antileukämische (T-)Zellen gebildet werden. Die Wirkstoffe sind für den klinischen Einsatz zugelassen und stellen daher attraktive Medikamente für die Immuntherapie bei myeloischer Leukämie dar.

Methoden/Ergebnisse: In vitro: Vollblutproben (WB) von Ratten oder Patienten mit AML wurden mit Kits kultiviert, um DC_{leu} zu erzeugen. Die Kit behandelten Proben wurden dann in gemischten Lymphozytenkulturen (MLC) mit T-Zellen der jeweiligen Blutdonoren inkubiert, um leukämiespezifische Immunzellen zu induzieren. Ihre Aktivität wurde mit verschiedenen Assays (Degranulation/intrazelluläre Zytokinproduktion) auf funktionelle Leukämiespezifität und Blastenzytotoxizität geprüft. Darüber hinaus wurde die zelluläre Zusammensetzung von Ratten-/Patientenproben im Verlauf der Kit-Behandlung durchflusszytometrisch untersucht.

1) AML-erkrankte Ratten in vivo: Je drei Ratten wurden mit den Kits I, K oder M behandelt oder unbehandelt gelassen (Kontrollen). In der Milz und im peripheren Blut (PB) der Kit behandelten Tiere wurde ein signifikanter Anstieg von DC_{leu} im Vergleich zu den unbehandelten Tieren festgestellt, ohne dass es zu einer Induktion der Blastenproliferation (Ki67-Positivität) kam. Eine (signifikante) Reduktion der Blasten wurde bei den mit Kit M ($p=0,03/0,0001$ in Milz/PB) und I behandelten Tieren festgestellt, nicht jedoch bei K. Die erfolgreiche Behandlung korrelierte mit einer Zunahme der $CD62L^+$ (memory like) T-Zellen ($p=0,07$) und einer Verringerung der CD4+ (regulatorischen) T-Zellen ($p=0,037$).

2) Ex vivo: In WB-Proben von therapierefraktären AML-Patienten (im Verlauf der Behandlung mit Decitabin/LD-AraC) wurde gezeigt, dass Kit M DC_{leu} erzeugt, immunreaktive Zellen aktiviert und leukämiespezifische/antileukämische Reaktionen vermittelt. Aktivierte oder leukämiespezifische Lymphozyten wurden in geringen Anteilen in Proben gesehen, die während aktiver Krankheitsstadien entnommen wurden, sowie in Proben von zwei Patienten während des weiteren Verlaufs der persistierenden Krankheit.

3) Therapie von refraktären AML-Patienten: Nach Genehmigung durch die Ethikkommission, Aufklärung und Zustimmung wurden zwei dieser Patienten eine individuelle systemische Salvage-Behandlung mit Kit M (als Dauerinfusion verabreicht) angeboten. Die Behandlung wurde von den Patienten gut vertragen und einer der behandelten Patienten (Patient 1482) verbesserte sich klinisch. Innerhalb von 24 Tagen stieg bei diesem unter Anderem der Anteil der Neutrophilen an den Leukozyten von 10 % auf 50 %, die Thrombozyten erreichten bis 100 G/l. Dieser Effekt war bei Patient 1602, der zum Zeitpunkt der Behandlung sehr hohe Blasten und niedrige Immunzellwerte präsentierte, nicht zu sehen. Ebenso erkennbar war bei Patient 1482 eine kontinuierliche,

leukämiespezifische Immunaktivierung im Immunmonitoring, was bei Patient 1602 nur in geringerem Maß zu sehen war. Im nicht-kitbehandelten Patienten fielen diese Werte kontinuierlich ab. Patient 1482 konnte nach 4 Wochen Behandlung in gutem klinischem Zustand entlassen werden, verstarb jedoch während der Behandlungspause an einer Sepsis bei wiederkehrenden Blasten. Im Gegensatz zur Situation vor Beginn der Behandlung (oder bei dem nicht mit Kit M behandelten Patienten) zeigte das Immunmonitoring einen kontinuierlichen Anstieg von potenziell leukämiespezifischen Immunzellen. Diese immunstimulierenden Effekte zeigten sich nach Beendigung der Therapie rückläufig.

Schlussfolgerung: Die Behandlung von leukämisch erkrankten Organismen mit blastenmodulierenden Kits (insbesondere GM-CSF und PGE1) wurde gut vertragen und führte zu einer klinischen Verbesserung. Ferner wurden vermehrt (leukämiespezifische) Zellen sowohl des adaptiven als auch des angeborenen Immunsystems nachgewiesen, während in den Proben unbehandelter Organismen nur geringe Zahlen von (leukämiespezifischen) aktivierten immunreaktiven Zellen gefunden wurden.

Abstract

Background: Kits (combinations of immune-modulatory compounds: GM-CSF + either Picibanil (Kit I), Prostaglandine E2 (PGE2, Kit K) or Prostaglandin E1 (PGE1, Kit M, patent DE102014014993) convert myeloid blasts into dendritic cells of leukemic origin (DC_{leu}). Upon stimulation with DC_{leu}, anti-leukemic (T) cells can be generated *ex vivo*. The compounds are approved for clinical use and are therefore attractive drugs for immunotherapy in myeloid leukemia.

Methods/Results: ***In vitro***: Whole blood (WB) samples from rats or patients with AML were co-cultured with Kits to generate DC_{leu}. The Kit-treated samples were then incubated in mixed lymphocyte cultures (MLC) with T cells from the respective hosts to induce leukemia-specific immune cells. Their activity was tested for functional leukemia specifity and blast cytotoxicity using various assays (degranulation/intracellular cytokine production). In addition, the cellular composition of rat/patient samples was tested during the course of Kit treatment by flow cytometry.

1) ***In vivo***: **AML-diseased rats**: Three rats each were treated with Kit I, K, or M, or left untreated (controls). A significant increase of DC_{leu} was detected in spleen/PB in Kit-treated compared to untreated animals without induction of blast proliferation (Ki67positivity). A (significant) reduction of blasts was seen in animals treated with Kit M (p=0.03/0.0001 in spleen/PB) and I, but not K. Successful treatment correlated with an increase of $CD62L^+$ (memory like) T cells, (p=0.07) and a reduction of $CD4^+$ (regulatory) T cells (p=0.037).

2) ***Ex vivo:*** **In WB samples from therapy-refractory AML patients** (during the course of decitabine/LD-AraC-treatment) Kit M was shown to generate DC_{leu}, activate immune-reactive cells and mediate leukemia-specific/antileukemic responses. Activated or leukemia-specific

lymphocytes were observed in low proportions in samples taken during active stages of the disease as well as in samples from two patients during the further course of persisting disease.

3) Therapy of refractory AML-Patients: After approval from the ethics committee and obtaining informed consent, two of these patients were offered an individual systemic salvage treatment with Kit M (applied as continuous infusions). The treatment was well tolerated by the patients and one of the treated patients (patient 1482) improved clinically. Within 24 days, among other things, the percentage of neutrophils in the leukocytes increased from 10% to 50%, and the platelets reached up to 100 G/L. This effect was not seen in patient 1602, who presented very high blasts and low immune cell counts at the time of treatment. Similarly, continuous leukemia-specific immune activation in immunomonitoring was evident in patient 1482, which was seen to a lesser extent in patient 1602. In the non-kit-treated patient, these values dropped continuously. Patient 1482 could be discharged in good clinical condition after 4 weeks of treatment, but died of sepsis in recurrent blasts during the treatment break. In contrast to the situation before the start of treatment (or in the patient not treated with Kit M), immune monitoring showed a continuous increase in potentially leukemia-specific immune cells. These immunostimulatory effects regressed after cessation of therapy.

Conclusion: Treatment of leukemic organisms with blast modulating kits (especially GM-CSF and PGE1) was well tolerated and resulted in clinical improvement. Furthermore, increased (leukemia-specific) cells of both the adaptive and innate immune systems were detected, whereas only low numbers of (leukemia-specific) activated immunoreactive cells were found in the samples of untreated organisms.

1. Introduction

1.1. Acute myeloid leukemia (AML)

Up to now prognosis of AML patients is unfavorable due to high relapse rates of about 70-80% after induction therapy. For therapy intolerable or refractory patients' prognosis is even worse **(Tamamyan et al., 2018, Yanada and Naoe, 2012)**. High-dose induction chemotherapy with cytarabine ± anthracycline followed by allogeneic hematopoietic stem cell transplantation (HSCT) is the only potential curative treatment and is the standard therapy, especially for young AML patients with fewer comorbidities **(Döhner et al., 2015, Döhner et al., 2017)** (**O'Donnell MR et al., 2017**). For patients with less tolerance for the induction therapy low-dose cytarabine or hypomethylating agents are potential therapy strategies **(Gil-Perez and Montalban-Bravo, 2019)** (**Burnett AK et al., 2018**). New immune therapeutic strategies have to be developed that address the dysfunctional reactivity of the immune system against leukemic blasts **(Ansprenger et al., 2020, Lichtenegger et al., 2017)**.

1.2. DC-based immunotherapy

DC cells play a central role in connecting and activating the innate and the adaptive immune system **(Wan and Dupasquier, 2005, Palucka and Banchereau, 1999, Klauer et al., 2019)**. DCs can be generated under Good Manufacturing Practice (GMP) from $CD14^+$ monocytes which can be pulsed with leukemia associated antigens (LAA), leukemic peptides or messenger RNA (mRNA, electroporation). Alternatively, DCs can be generated directly from leukemic blasts, rendering the complicated antigen loading process on DCs unnecessary. Those DC_{leu} are characterized by the expression of individual patients' whole leukemic antigen repertoire including known as well as unknown leukemic antigens. **(Amberger et al., 2019, Amberger and Schmetzer, 2020)** (**Kremser et al., 2014**). These DCs have to be re-administrated to patients as a 'vaccine' (**Van Acker et al., 2019**). Alternatively, DC_{leu} could be induced in patients in vivo after application of 'DC_{leu}-inducing Kits' (combinations of Granulocyte–Macrophage-Colony Stimulating Factor (GM-CSF) and a second response modifier (e.g., Picibanil, Prostaglandin E_1 or E_2), which triggers DC/DC_{leu} differentiation and maturation) **(Amberger et**

al., 2019, Amberger and Schmetzer, 2020, Schwepcke et al., 2022). Using AML patients' WB (containing individual patients' immune inhibitory or activating soluble and cellular factors, which might influence blasts' proliferation as well as anti-leukemic effects **(Hirn Lopez et al., 2019)**) resemble the in vivo situation most. Composition and modes of action of each individual Kits are given in **Table 1.**

Due to their immunomodulatory properties (in combination with GM-CSF) PGE_1 and PGE_2 have been shown to be highly efficient ex vivo DC-generating factors by providing a danger signaling, enhancing DCs' maturation and migratory capacity **(Okamoto and Sato, 2003, Conejo-Garcia et al., 2016, Amberger et al., 2019, Schwepcke et al., 2022)**.

1.3. Rat model

A suitable, 'slowly' growing model to study proliferation and differentiation of AML cells is the Brown Norway Rat Leukemia Model (BNML). The model was developed in 1977 in the radiobiological institute TNO in Rijswijk upon repeated injections of the carcinogenic 9,10-dimethyl-1,2-benzanthracene (DMBA) that led to the development of an aggressive form of acute promyelocytic-like leukemia. Since cellular, histological (suppression of healthy hematopoiesis) and clinical symptoms (anemia, bleeding, etc.) in leukemically diseased rats are comparable to human AML, this model has been used to study animals' response to chemotherapy or SCT, to investigate interactions of leukemic blasts with immune-reactive cells and to quantify minimal residual disease **(Martens et al., 1990)**. BNML cells have features of primary blasts, and are maintained via in vivo passages by transfer by i.v. to healthy BN rats. We selected the BNML model as an appropriate animal model to test our selected blast modulatory Kits in vivo. All experiments with rats were performed at the University of Oslo by A. Rabe, team M. Inngjerdingen and results provided for evaluation.

The aim of this study was:

1) to treat diseased (Brown Norway) Rats in parallel with different Kits infusion to convert blasts to DC_{leu} to evaluate their safety, efficacy (reduction of tumour cells) and immunity (immune activation, generation of an immunological memory).

2) to treat two patients with end-stage AML by intravenous application of Kit M in individual systemic salvage treatments to study safety as well as clinical and immunological efficacy of Kit M.

3) to potentially deduce Kit-based treatment options.

2. Material and Methods

2.1. Rats' experiments

Seven-week-old, inbred Brown Norway Myeloid Leukemia (BNML) (BN/OrlRj) rats were used as a model for promyelocytic myeloid leukemia that closely resembles the human promyelocytic subtype **(Martens et al., 1990)** and PVG.1N ($RT1^n$) rats were used for safety analyses (PVG.1N is a PVG rat strain with the MHC background as BN rats, in which AML cannot be induced). The use of animals was authorized by the Norwegian Animal Research Authority (NARA) under license numbers 12.4196 (in vitro) and 6060 (in vivo).

BNML cell suspensions were prepared from the spleen of rats suffering from acute leukemia, washed and frozen at -80˚C until further use. In male rats, BNML disease was induced by intravenous injection of approximately 8x106 BNML cells into the penile vein. Leukemic cells spread to all organs, and at day 23 infiltrating mainly the bone marrow (BM, 94% blasts), liver, spleen (88% blasts) and PB (65% blasts), replacing healthy hematopoiesis. BNML cells in WB were detectable after 15-17 days. Typical clinical symptoms of rats suffering from leukemia included enlarged spleen and liver, weight loss, fatigue, and a brittle coat.

Figure 1 shows leukemic cells positive for RM124 with partial positivity for CD86 and MHCII and negativity for DC marker CD103. **Figure 1B** shows the displacement of healthy hemopoietic cells between day 0-23 after injection of leukemic cells to rats. The average spleen size increased from 3 cm in healthy BN rats to 7.5 cm in terminally ill leukemically diseased rats (**data not shown**).

Figure 1: Injection of leukemic BNML cells into healthy rats induced development of leukemia in 14-17 days.

Figure 1A: Leukemic cells are positive for blast marker RM124 and in part for MHC II and negative for DC marker CD103.

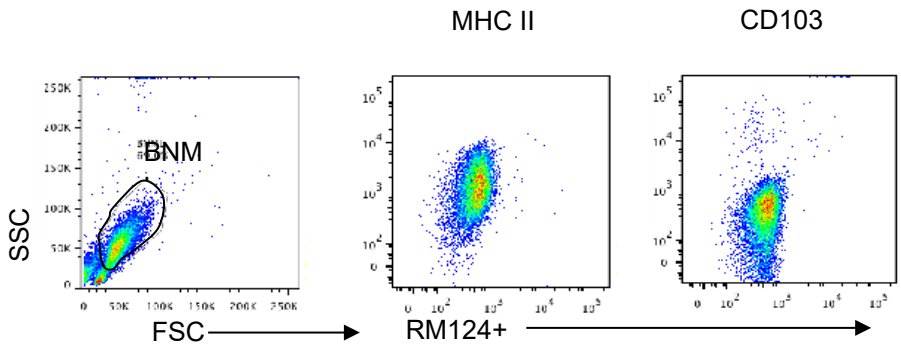
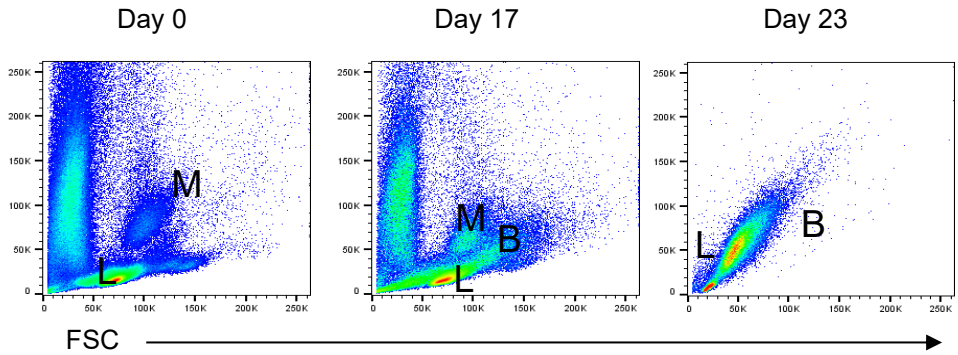


Figure 1B: Displacement of healthy lymphocytes (L), monocytes (M) and granulocytes by an increasing blast population.



SSC side scatter in flow cytometry; FSC forward scatter in flow cytometry; BNML brown Norway rat acute myelocytic leukemia; M monocytes; L lymphocytes; B blasts; FSC forward scatter in flow cytometry.

Figure 1: Figure 1A shows expression profiles of leukemic rat cells. Figure 1B shows displacement of healthy cells (monocytes, granulocytes, and lymphocytes) after injection of leukemic cells to rats over 23 days.

For BNML injections, the rats were anesthetized. To monitor the tumor load and immunoreactive cells, rats were sedated by inhalation of Isoflurane Baxter (Baxter International Inc., Deerfield, Illinois) and 0.2 ml of blood from the lateral tail vein was collected weekly. Rats were sacrificed after 23 days after BNML injections by asphyxiation with CO_2.

Blood samples of diseased and scarified rats were taken by puncture of the heart chambers. Monitoring and quantifying of BNML cells was performed using flow cytometry as described below.

Induction of leukemia, treatment with Kits, sacrifice and collections of cell samples were performed on the same days for all Kit treated or untreated rats.

Table 1: Different Kits used for cell biological experiments conducted with rats' and human whole blood.

	Kit	Composition	Concentration	Mode of Action	Culture Time	Reference
Rat	D	rat GM-CSF OK-432 PGE_2	800 U/ml 10 μg/ml 1 μg/ml	**(rat) GM-CSF:** induction of myeloid (dendritic cell) differentiation	7-8 days	(Amberger et al., 2019) (Schwepcke et al., 2022) (Ryoma et al., 2004) (Waller, 2007) (Simmet and Peskar, 1988)
	I_{low}	rat GM-CSF OK-432	800 U/ml 1 μg/ml	**OK-432:** lysis product from Streptococcus pyogenes; stimulation of dendritic cell differentiation	7-8 days	
	I_{high}	rat GM-CSF OK-432	800 U/ml 10 μg/ml		7-8 days	
	K	rat GM-CSF PGE_2	800 U/ml 1 μg/ml	**PGE_1:** Increase of CD197 expression and enhancement of dendritic cell migration	7-8 days	
	M	rat GM-CSF PGE_1	800 U/ml 1 μg/ml		7-8 days	
Human	I*	GM-CSF OK-432	800 U/ml 10 μg/ml	**PGE_2:** similar effects as with PGE_1	7-8 days	(Amberger et al., 2019) (Schwepcke et al., 2022)
	M*	GM-CSF PGE_1	800 U/ml 1 μg/ml		7-8 days	(Simmet and Peskar, 1988)

(rat/human) GM-CSF (rat/human) granulocyte macrophage colony stimulating factor (recombinant rat GM-CSF or recombinant human GM-CSF); OK-432 picibanil; PGE_1 prostaglandin E_1; CCR7 chemokine receptor type 7 (CD197); PGE_2 prostaglandin E_2; culture was restimulated during culture time (after 2/3 days and 5/6 days; * Modiblast GmbH (Oberhaching, Germany) holds the European patent 15 801 987.7-1118 and the US patent 15-517627 for the use of immunomodulatory effective compositions for the treatment of AML patients.

2.1.1. Ex vivo – animal experiments

DC/DC_{leu} generation

Three different Kits were used for the generation of DC/DC_{leu} from WB samples of BNML diseased rats ex vivo. A selected amount of WB (containing approx. 5 x 106 PBMNCs) was pipetted into the wells of a 12-multiwell-tissue culture plate and subsequently diluted 1:1 in X-Vivo medium (Lonza, Basel, Switzerland). Afterwards Kits were added as given in Table 1. Cell incubations were performed for 7-9 days at 37° C and 5% CO2 concentration.

DC/DC_{leu} were generated from WB samples using 800 U/ml rat GM-CSF and 10µg/ml Picibanil (*Kit I*), or 1µg/ml PGE_1 (*Kit M*), or 1µg/ml PGE_2 (*Kit K*). Three days later, the same amounts of drugs were added, and cells were harvested after a total of 7 days of incubation and used for subsequent experiments **(Schwepcke et al., 2022)**.

Mixed lymphocyte culture (MLC culture)

Healthy BN/OrlRj rats (n=3) served as T cell donors. T cells were prepared by Lymphoprep by incubation with nylon wool. The purity of $CD3^+$ T cells was 84 $\pm$ 4% (controlled by flow cytometry). T cells were co-cultured with WB cell suspensions from untreated or Kit-treated rats, which contained a mixture of blasts and blasts converted to DC/DC_{leu}. After a two-fold stimulation with rat IL-2 (50 U/mL), DC/DC_{leu} stimulated immune cells were harvested after 6-8 days, and a Cytotoxicity assay performed as described below.

2.1.2. In vivo – animal experiments

Application of single response modifiers to healthy rats (safety-analysis)

To observe possible side effects of the response modifiers Picibanil (OK-432) (c=0.175µg), PGE_1 (c=1.2µg) and PGE_2 (c=1.2µg) three rats were anaesthetized and each of the three drugs diluted in 500 µl PBS and injected in each rat. Injections of 500 µl PBS into control rats served as negative control. Monitoring of rats for possible side effects (weight changes, mobility, interaction with other rats, respiration, skin irritation and sleep) was performed 2h, 24h and 72h after injections. Rats were sacrificed using CO_2 3 days after the last injection. Cell samples were taken to study compositions of hematopoietic cells.

Kit treatment of rats diseased with leukemia

To study possible effects of Kits on the tumor load as well as on the composition of immunoreactive cells, leukemically diseased rats were split into 5 treatment subgroups (each 3 rats). 14 days after the injection of the BNML cells, the rats were anesthetized and treated as follows:

Group 1 received an injection of 1 µg rat GM-CSF and 0.175 µg Picibanil (Kit I_{low}), group 2 of 1 µg rat GM-CSF and 0.35 µg Picibanil (Kit I_{high}), group 3 of 1 µg rat GM-CSF and 1.2 µg PGE_1 (Kit M), group 4 of 1 µg rat GM-CSF and 1.2 µg PGE_2 (Kit K) and group 5 served as control without drug injections. Drug combinations were each dissolved in 500 µl PBS before injection.

A second dose of drugs was injected four days after the first injection. After 24 days, the rats were sacrificed. Blood samples were taken by heart punction, and their spleens were removed, weighed, and processed for cell preparation shortly after sacrifice. Frequencies of blasts and immune-reactive cells in blood and spleen were analyzed by flow cytometry. Compositions and mode of action of each individual Kit are given in **Table 1**.

2.2. Flow cytometry

WB samples were lysed using RBC lysis buffer, centrifuged, cells stained with rat specific mAbs and incubated for 15 minutes in the dark on ice. The measurements were performed on fluorescence activated cell sorting (FACS) machines Canto™ or LSR Fortessa™ using the FACS Diva software (BD): Verification of successful DC/DC_{leu} generation was obtained by staining the cells' surfaces with the blast marker Rm124 (FITC, BD Pharmingen), the DC markers CD86 (PE, BD Pharmingen), CD103 (Alexa 647, BD Pharmingen) and MHC class II (Strepatvidin-PerCP, BD Pharmingen). Moreover, T-, NK- and NKT-cell subtypes were quantified as given in ***Table 2.***

2.3. Cytotoxicity assay (Chromium release assay)

A standard 4h ^{51}Cr-release assay was performed to determine cytotoxicity (**Kim et al., 2007**).

Table 2: Subtypes of leukemic blasts, DC and DCleu, adaptive and innate immunity cell subsets in rats' blood.

		Name of Subgroups	Surface Marker	Referred to	Abbreviation of cell population	Reference
	Blast cells	Leukemic blasts	RM124+	WB	Bla/WB	(Martens et al., 1990)
		Proliferating blasts	Bla+Ki67+	WB	Bla_{prol}/WB	(Martens et al., 1990)
	Dendritic cells	Dendritic cells	DC e.g. CD86+, CD103+	WB	DC/WB	(Schmetzer et al., 2007)
		Leukaemia derived DC	DC+Bla+	WB or DC or Bla	DC_{leu}/WB DC_{leu}/DC DC_{leu}/Bla	(Schmetzer et al., 2007)
		MHC II	MHC II	WB	MHC+Bla/WB	(Martens et al., 1990, Schmetzer et al., 2007)
	T lymphocytes	CD3+ pan T cells	CD3+	WB	CD3+/WB	(Amberger et al., 2019)
Adaptive		CD4+ coexpressing T cells	CD3+CD4+	CD3+	CD4+CD3+/CD3+	(Amberger et al., 2019)
Immune system		CD8+ coexpressing T cells	CD3+CD8+	CD3+	CD8+CD4+/CD3+	(Amberger et al., 2019)
		Memory like T cells	CD3+CD4+CD62L++ CD3+CD4-CD62L++	CD3+ CD3+	$CD4+T_{mem}$/CD3+ $CD8+T_{mem}$/CD3+	(Sada-Ovalle, 2008)
		Regulatory T cells	CD3+CD4+Foxp3+CD25+	CD3+	$CD4+T_{reg}$/CD3+	(Shevach et al., 2006)
Innate	**Natural killer cells**	CD3-CD56+ NK cells	NKR-P1A+ CD3-	lymphocytes	NKcell/cells	(Ryan et al 1995)
Immune system	**Natural killer T cells**	6B11+ NKT cells	NKR-P1A+ CD3+	lymphocytes	NKTcell/cells	(Ryan et al 1995)

WB whole blood; MHC II major histocompatibility complex type II.

2.4. Individualized clinical treatments

Based on the results from animal experiments two patients with refractory AML were offered an individualized salvage treatment with Kit M. Treatments had been extensively discussed with the responsible ethical committees as well as the patients' health care providers. Before start of treatment, both patients (P1482, P1601) were elaborately informed by experienced hematologists on several occasions about the experimental nature as well as possible side effects of the treatment and had given written informed consent into the treatment as well as examinations on blood samples drawn in addition to routine monitoring. A third patient (P1511) in comparable clinical condition was treated only with palliative therapy but agreed in immune monitoring of multiple blood samples drawn during in addition to routine monitoring during his clinical course. Patients' data and treatments of P1482, P1601 and P1511 are given in the result section.

2.5. Experiments with human blood

2.5.1. Blood sampling

Heparinized peripheral blood samples were taken from the 3 patients in a therapy refractory, acute phase of AML. The university hospital Augsburg (P1482, P1511) and Stuttgart (P1601) provided patient blood samples. Anticoagulation was accomplished with Lithium-heparin-tubes (7.5ml, Sarstedt, Nuernberg, Germany) containing standardized concentrations of heparin (***Table 4***). Heparinized AML WB was either directly used for experiments or mononuclear cells (MNC) and T cells isolated and frozen for later use **(Schwepcke et al., 2022)**.

2.5.2. Cell analysis by flow cytometry

Flow cytometric analyses were used to determine and quantify different phenotypes of leukemic blasts, DC/DC_{leu}, T cell subsets, B cells and monocytes before, during, and after our therapy **(Klauer et al., 2021)**. Cellular subtypes are given in **Table 3**. Panels were labelled with Fluorescein isothiocyanat (FITC), phycoerythrin (PE), tandem Cy7-PE conjugation (Cy7-PE) and allophycocyanin (APC). Monoclonal antibodies (mAbs) for the different panels (FITC, PE, Cy7-PE, APC) were provided by Becton Dickinson, Heidelberg, Germany (a), Beckman Coulter, Krefeld, Germany (b), Miltenyi Biotech, Bergisch Gladbach, Germany (c), Santa Cruz Biotechnology, Heidelberg, Germany (d) and Thermo Fisher, Darmstadt, Germany (e). CD3b, CD15b, CD25b, CD33b, CD34b, CD45ROb, CD65b, CD71b, CD83b and IPO-38d were used for the FITC channel. As for PE-conjugated mAbs, we used CD3b, CD4b, CD19b, CD33b, CD34b, CD56b, CD80a, CD83b, CD117b, CD127b and CD206b. For Cy7-PE, mAbs against CD3b, CD4b, CD14a, CD15a, CD19b, CD25a, CD33b, CD34b, CD56b, CD65c, CD80a, CD117b and CD197a were used. For APC, mAbs against CD3b, CD4b, CD14a, CD15a, CD34b, CD45ROe, CD56b, CD65c, CD69a, CD83a, CD86a, CD117b, CD206a and CD209a were used. 7AADa was used to identify dead cells. For intracellular antigen staining, the FIX & PERM Cell Fixation and Cell Permeabilization kit (ThermoFisher Scientific, Darmstadt, Germany) was used.

2.5.3. Dendritic cell culture (DC culture)

DC/DC_{leu} were generated from 500 µl heparinized blast containing WB of AML patients. Response modifiers (Kit M, contains GM-CSF and PGE_1) were added at start and on day 2/3 of culture **(Klauer et al., 2021, Schwepcke et al., 2022)**. After culture cells were harvested, quantified, and used for subsequent experiments. Both the WB pretreated with Kit M ($DC^{WB(Kit}$

$^{M)}$) or without pretreatment with Kit M ($DC^{WB(Control)}$) were analyzed by flow cytometry **(Klauer et al., 2021)**.

2.5.4. Mixed lymphocyte culture (MLC culture) to activate immune reactive cells

DC cultures were harvested on day 7-8 and MLC cultures were set up. 1x 10^6 (previously frozen) T cells from healthy or AML patients' WB were co-cultured with approximately 2.5x 10^5 cells from DC/DC_{leu} generating culture ($DC^{WB(Control)}$, $DC^{WB(Kit\ M)}$) and RPMI in a total of 1 ml in the presence of IL-2 **(Amberger et al., 2019)**. Different immune cell subtypes after MLC were quantified by flow cytometry ***(Table 3)***.

2.5.5. Detection of antigen specific cells (INFy-cytokine Secretion Assay, CSA, intracellular INFy secretion assay, InCyt, ELISPOT)

To evaluate and quantify IFNγ secreting cells in PB from AML patients during Kit treatment, a CSA, InCyt and at some time points additionally INFy-ELISPOT analyses were performed. WB samples were in parallel tested for IFNγ secretion (with or without prior leukemic antigenic stimulation) (Klauer et al., 2021). Detected INFy producing cells are given in **Table 3**.

2.5.6. Cytotoxicity fluorolysis assay (CTX)

To investigate the ability of effector cells (T cell enriched cells, stimulated with or without Kit M- treated WB after MLC) to lyse target cells (thawed viable patients' MNCs stained with two different blast markers) the cytotoxicity fluorolysis assay was conducted. The lytic activity of effector cells was calculated and defined as the frequencies of viable target cells in the culture with co-cultured effector and target cells (for 3h and 24h) as compared to Control **(Amberger et al., 2019)**.

2.6. Statistical methods and evaluation of data

Graphics and statistical analyses were performed with the GraphPad Prism software. Data acquired by Flow cytometry were evaluated with FlowJo (version 7.6.5). Data were presented as mean ± standard deviation of the mean (SEM). Statistical comparisons between experimental groups were performed with the parametric One-way analysis of variance (ANOVA) in combination with a non-parametrical Mann-Whitney test using Microsoft Excel and IBM SPSS Statistics®. Differences were considered 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.

Table 3: Subtypes of leukemic blasts, DC and DC_{leu}, adaptive and innate immunity and leukemia specific cell subsets in human blood samples.

		Name of Subgroups	Surface Marker	Referred to	Abbreviation	Reference
	Blast cells	Blasts	Bla e.g. CD34+, CD117+	WB	Bla/WB	(Schmetzer et al., 2007)
		Proliferating blasts	Bla+DC-CD71+	Bla	Bla_{prol71}/Bla	(Plett et al., 2017)
		Proliferating blasts	Bla+DC-IPO38+	Bla	$Bla_{prolIPO38}$/Bla	(Ansprenger et al., 2020)
	Dendritic cells	Dendritic cells	DC+ e.g. CD80+, CD206+	WB	DC/WB	(Schmetzer et al., 2007)
		Leukaemia derived DC	DC+Bla+	WB	DC_{leu}/WB	(Schmetzer et al., 2007)
		Mature migratory DC	DC+CD197+	WB	DC_{mat}/WB	(Grabrucker et al., 2010)
		Mature migratory DC_{leu}	DC+Bla+CD197+	WB	$DC_{leu-mat}$/WB	(Grabrucker et al., 2010)
Adaptive immune system	**B lymphocytes**	CD19+ B $cells_{memory}$	CD19+CD27+IgD-	CD19+	$Bcell_{memory}$/CD19+	(Agematsu et al., 1998)
	T lymphocytes	CD3+ pan T cells	CD3+	lymphocytes	CD3+/cells	(Amberger et al., 2019)
		CD4+ T cells	CD3+CD4+	CD3+	T_{CD4+}/CD3+ or CD3+CD4+/CD3+	(Amberger et al., 2019)
		CD4- T cells	CD3+CD4-	CD3+	T_{CD4-}/CD3+ or CD3+CD4-/CD3+	(Amberger et al., 2019)
		T helper cells 1 T helper cells 17	CCR4-CXCR3+CCR5+CCR6- CCR4+CXCR3-CCR5-CCR6+	CD4+ CD4+	TH_1+/CD4+ TH_{17}+/CD4+	(Waidhauser et al., 2021) (Waidhauser et al., 2021)
		Non-naive T cells	CD3+CD45RO+ CD3+CD45RO+CD4+ CD3+CD45RO+CD4-	CD3+ T_{CD4+} T_{CD4-}	$T_{non-naive}$/CD3+ $T_{non-naïve\ CD4+}/T_{CD4+}$ $T_{non-naïve\ CD4-}/T_{CD4-}$	(Klauer et al., 2021)
		Central (memory) T cells	CD3+CD45RO+CD197+ CD3+CD45RO+CD197+CD4+	CD3+ T_{CD4+}	T_{cm}/CD3+ $T_{cm\ CD4+}/T_{CD4+}$	(Klauer et al., 2021)
		Effecor (memory) T cells	CD3+CD45RO+CD197- CD3+CD45RO+CD197-CD4+	CD3+ T_{CD4+}	T_{em}/CD3+ $T_{em\ CD4+}/T_{CD4+}$	(Klauer et al., 2021)
		Proliferating T cells - early	CD3+CD69+ CD3+CD4-CD69+	CD3+ T_{CD4-}	$T_{prolCD69+}$/CD3+ $T_{prolCD4-CD69+}/T_{CD4-}$	(Klauer et al., 2021)
		Proliferating T cells - late	CD3+CD71+	CD3+	$T_{prolCD71+}$/CD3+	(Klauer et al., 2021)
Innate immune system	**Cytokine induced killer cells**	CD3+CD56+ CIK cells	CD3+CD56+	lymphocytes	CIKcell/cells	(Amberger et al., 2019)
	Natural killer cells	CD3-CD56+ NK cells	CD3-CD56+	lymphocytes	NKcell/cells	(Amberger et al., 2019)
	Invariant natural killer T cells	6B11+ iNKT cells	6B11+	lymphocytes	iNKTcell/cells	(Boeck et al., 2017)
Leukemia specific cells						
	T lymphocyte cells *	CD4+ coexpressing T $cells_{leu}$	CD3+CD4+INFy+	$T_{CD4+leu}$	$T_{CD4+leu}/T_{CD4+}$	(Klauer et al., 2021)
Adaptive immune system		CD8+ coexpressing T $cells_{leu}$	CD3+CD4-INFy+	$T_{CD4-leu}$	$T_{CD4-leu}/T_{CD4-}$	(Klauer et al., 2021)
Innate immune system	**Cytokine induced killer cells ****	CD3+CD56+ CIK $cells_{leu}$	CD3+CD56+INFy+	CIKcell	$CIKcell_{leu}$/CIKcell	(Klauer et al., 2021)
	Invariant natural killer T cells **	6B11+ iNKT $cells_{leu}$	6B11+INFy+	iNKTcell	$iNKTcell_{leu}$/iNKTcell	(Klauer et al., 2021)

* Evaluated by cytokine secretion assay (P1482/P1511) and by intracellular cytokine assay (P1601) +LAA stimulation; ** Evaluated by cytokine secretions assay (P1482/P1511) and by intracellular cytokine assay (P1601) +LAA stimulation.

Table 4: Characteristics of acute myeloid leukemia (AML) patients are presented.

Status	Stage	Patient	Age at diagnosis	Sex	ELN-risk-stratification at first diagnosis	Clinical treatment at first diagnosis	Clinical treatment in the further course of relapse/persisting disease treatment	Blast phenotype (CD)	Blasts in PB/BM before chemotherapy or Kit M treatment (%)	Conducted cell biological experiments
AML	**Persisting Relapse**	P1511	76	m	intermediate	Chemotherapy (Decitabine (22 cycles)	Chemotherapy (Cytarabine+Midostaurin)	**34,117**,13, 33	54/40	DC, MLC, CTX, CSA
AML	**Persisting Relapse**	P1482	74	m	unfavourable	Chemotherapy (HMA and Daunorubicin/AraC (induction) + AraC (3 cycles))	Chemotherapy (Decitabine (2 cycles) + Hydroxyurea/Cytarabine) and Kit M treatment	**117,34**,15, 13,33,64	<5/68	DC, MLC, CTX, CSA
AML	**Persisting Relapse**	P1601	74	f	unfavourable	Chemotherapy (Azacytidine (4 cycles) and Venetoclax (1 cycle)	Chemotherapy (Hydroxycarbamid) and Kit M treatment	**34,117**,33, 13	90/68	DC, MLC, CTX, InCyt

ELN risk stratification risk evaluation based on assessments of EuropeanLeukemiaNet; PB peripheral blood; BM bone marrow; m male; f female; bold blast phenotype: blast markers used for DC_{leu} evaluation; DC dendritic cell culture measurements; MLC mixed lymphocyte culture measurements; CTX cytotoxicity assay; CSA cytokine secretion assay; InCyt intracellular cytokine assay; HMA Hypomethylating agents; Details of clinical treatments and hematological values are given in Figure 6,7 and 8.

3. Results

3.1. BNML rats as a model to study the efficacy of blast-modulatory Kits to induce anti-leukemic responses ex vivo and in vivo

DC/DC_{leu} can be generated ex vivo from myeloid blasts in WB from leukemically diseased BNML rats

We generated DC and DC_{leu} from WB samples of leukemically diseased BNML rats with Kits (Kit D, I, K, M) compared to control without added Kits. With all Kits on average comparable frequencies of DC in WB could be generated compared to control. Frequencies of e.g., DC_{leu}/WB were between 27-38% with Kits compared to 3% with control (***Figure 2A***). **Figure 2B** shows populations of DC_{leu}: cells coexpressing blast-marker RM124 and DC-markers (MHCII, CD103) after the influence of Kit M or I compared to control. Abbreviations for all cell subsets are given in **Table 2.**

Figure 2: DC/DC_{leu} could be generated ex vivo from (blast containing) rats' whole blood with Kits (Kit M, D, K and I). Kit treated WB led to improved antileukemic reactivity of rat T cells after MLC.

Figure 2A: DC subtypes could be generated from rats' WB and blasts with Kit M, D, K, and I (compared to Control).

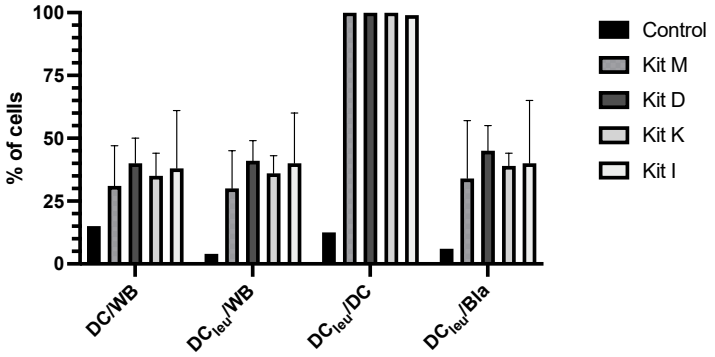


Figure 2B: Increased frequencies of DC (subtypes) after culture of rats´ blood with Kit M and Kit I compared to Control.

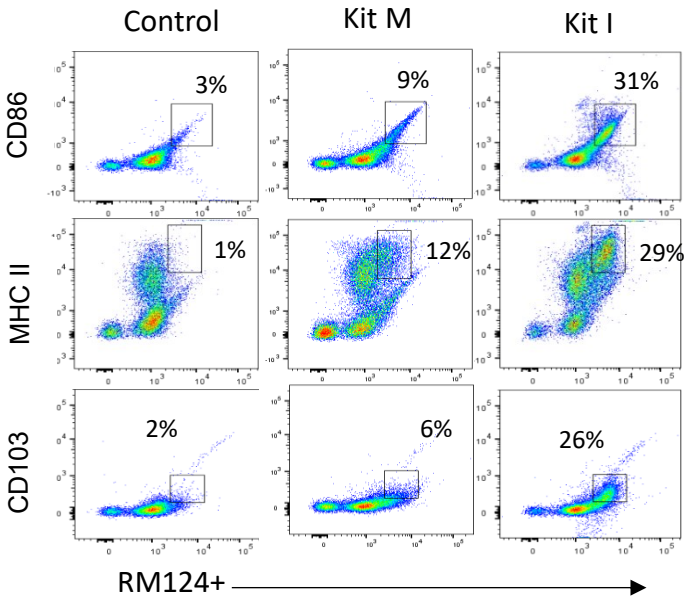


Figure 2C: Rat T cells primed with Kit pretreated (vs. untreated) whole blood achieved higher blast lysis after MLC.

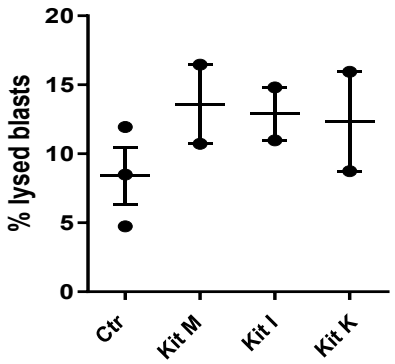


Figure 2: Figure 2A: Rats' blast containing WB was cultured with Kits (Kit-M, D, K and I) compared to Control without Kits. Mean results ± standard deviation of DC subtype frequencies obtained from each two rats are given. Figure 2B: Percentual increase of RM124+ blasts coexpressing CD103, MHC II and CD86 as evaluated by flow cytometry after culture with Kit I or M compared to Control (without added Kits) are given. Figure 2C: Improved antileukemic blast lytic activity was demonstrated (using a chrome release assay) for Kit pretreated rat WB samples after MLC. Average frequencies ± standard deviation of lysed blasts are given. Abbreviations for cell subtypes are given in Table 2.

3.2. Ex vivo priming of rat T cells with Kit treated rat WB results in improved anti-leukemic reactivity

Stimulation of rats' T cells in T cell enriched MLC with Kit-pretreated rat WB was performed to assess the priming efficiency of generated DC/DC_{leu}-containing WB to specifically stimulate T cells' anti-leukemic activity. **Figure 2C** shows that every Kit-pretreatment (vs. untreated control) resulted in improved blast lysis (as demonstrated by Chrome release assay).

3.3. In vivo treatment of healthy rats with single Kit components and of BNML rats with Kits is safe

To identify possible adverse effects of Kits, the single drugs were injected into PVG.1N rats, the MHC identical (cheaper) rat strain (n=3). The dosages of the substances corresponded to those used for the treatment of BNML rats: 0.35 μg of Picibanil and each 1 μg PGE_1 or PGE_2 were applied per rat and injection. Rats were observed for 72 hours for various side effects such as interactions with other rats, skin irritations, altered breathing, sleeping behavior, weight, or mobility. No changes in physiological functions and behavior were found pointing to a good tolerability and safety of the drugs. No adverse events were found in BNML rats under Kit treatment compared to control rats (see below).

3.4. In vivo treatment of BNML rats with Kits leads to improved hematological and immunological effects

Rats suffering from leukemia were treated with Kit I (in two different concentrations: Kit I_{low}, Kit I_{high}), Kit M and Kit K on days 14 and 17 after induction of leukemia, while untreated rats served as control (each n=3). Compositions of immune reactive cells in PB and spleen of Kit

treated vs untreated rats were monitored after sacrifice of rats. Cell subsets were analyzed as described in **Table 3**.

Application of Kits to leukemically diseased BNML rats increased DC_{leu} counts in blood and spleen with all Kits. Blast proliferation was not induced after treatment with Kit I_{high} and M **(*Figure 3A*)**. Even more, in spleen and blood a significant reduction of blasts compared to control was seen after in vivo Kit M treatment (%blasts: PB: 10.7% ($p< 0.0001$); spleen: 15.8% ($p< 0.033$)) and tendencies after Kit I treatment (%blasts: PB: 4.4%; spleen: 6.3%). Frequencies of T_{reg} were stable in blood and (tendentially) significantly reduced after the influence of Kit I K or M. T_{mem} (memory like CD8+ CD62L++ T cells) were tendentially significantly increased in blood after Kit M treatment, but not under the influence of other Kits **(*Figure 3B*)**. Frequencies of NK and NKT cells did not change throughout Kit treatment (*data not shown*).

In summary, these results show that DC/DC_{leu} could be generated from blasts in rat WB using Kits, that improved anti-leukemic reactions after MLC ex vivo. Moreover, treatment of leukemically diseased rats with Kit M and I_{high}, but not with Kit I_{low} or Kit K, led to increased frequencies of DC/DC_{leu} as well as of T_{mem} and furthermore, to significant reduction of blasts in rat PB and/or spleen.

Figure 3: Treatment of leukemically diseased rats with Kits (especially Kit M and I_{high}) induced DC/DC_{leu}, activated immunoreactive and memory like T cells and reduced regulatory cells and blast cells compared to Control in PB or spleen.

Figure 3A: Increased frequencies of DC/DC_{leu} detectable under the influence of Kit M and Kit I_{high} (vs. Control) without induction of blasts proliferation led to (significant) reduction of blasts *in vivo*.

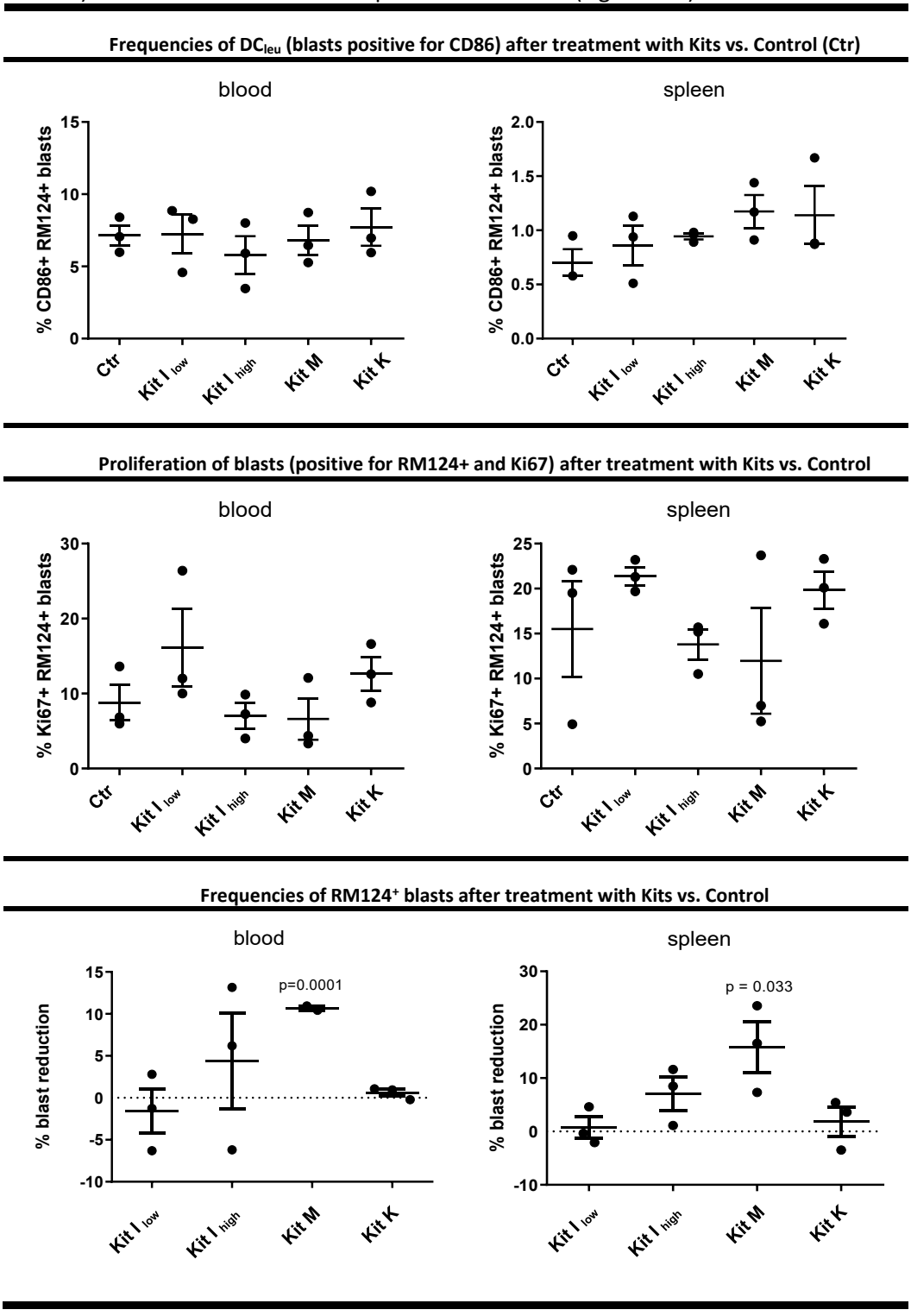


Figure 3B: Reduced frequencies of regulatory T cells and increased frequencies of memory like T cells detectable especially under the influence of Kit M and Kit I_{high} (preferentially in PB).

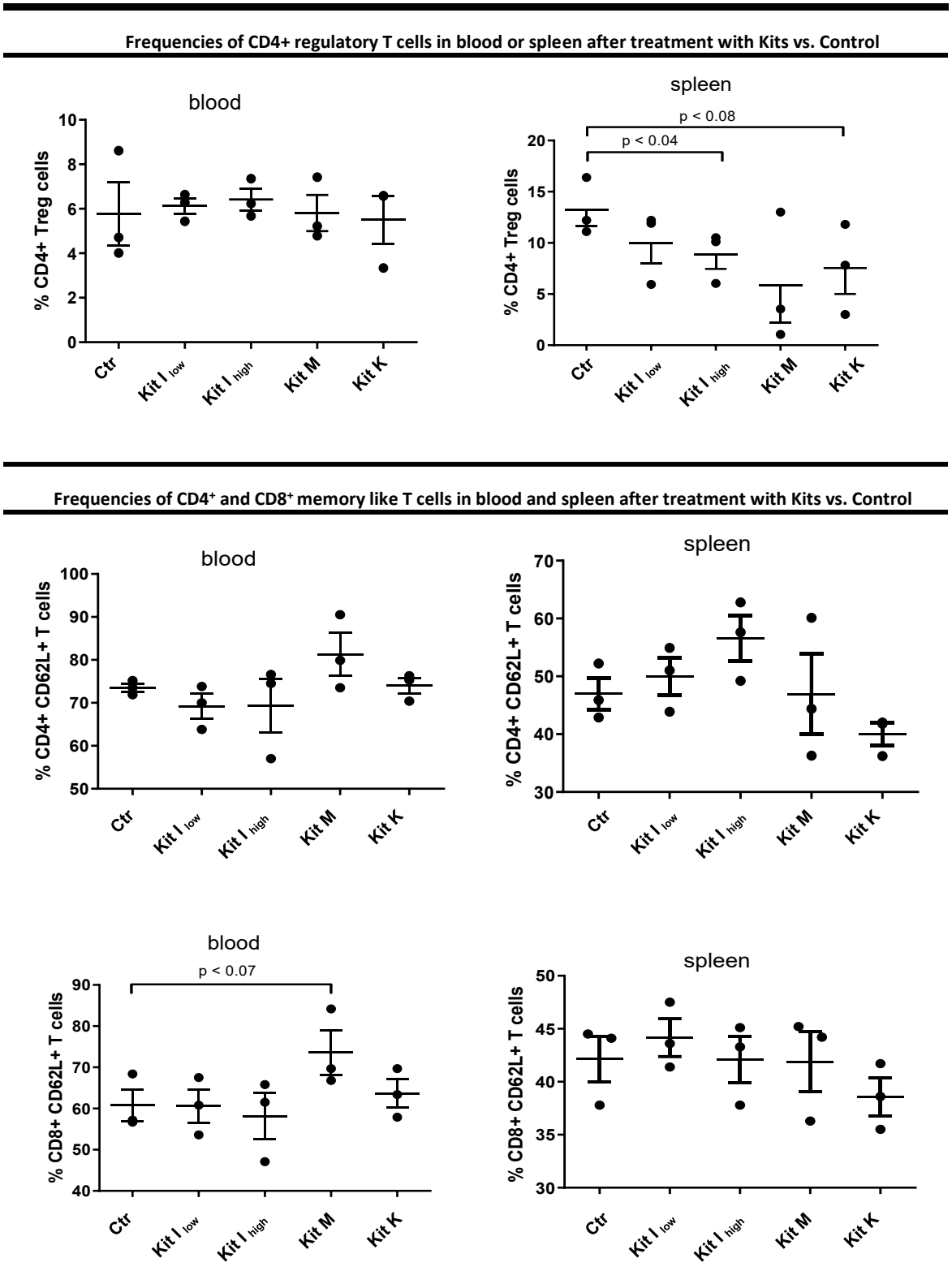


Figure 3: Each three leukemically diseased rats per group were treated with Kits for 9 Days (repeated injection of Kits after 5 Days) and sacrificed then. The composition of cells in PB and spleen was quantified by flow cytometry. Data was statistically analyzed using a paired t test. Given are mean values ± standard deviation and differences were defined as significant with p values <0.5. Abbreviations for cell subtypes are given in Table 2.

3.5. Human part

3.5.1. Two case reports about AML patients under the treatment with Kit M compared to one patient treated with chemotherapy

Two patients older than 70 years with refractory or relapsed AML without available standard treatment option were included and with a high load of myeloid blasts in peripheral blood and bone marrow were treated for four (P1482) or two weeks (P1601) with Kit M. Another patient (P1511) served as a control under the treatment with conventional chemotherapy. Patients' characteristics are given in ***Table 4***, individual patients' clinical and immunological courses under therapy are given below and in ***Figure 6-8***.

DC/DC_{leu} generation possible with Kit M and Kit I ex vivo

We could show that DC subtypes, especially mature leukemia derived DC ($DC_{leu\text{-}mat)}$, could be generated with Kit M and I in all three patients' samples without induction of blasts' proliferation (***Figure 4A***). Furthermore, we stimulated T cell enriched immunoreactive cells with those Kit pretreated (DC/DC_{leu} containing) WB and found an activation of T-cells, going along with induction of proliferating $T_{non\text{-}naive\ CD4+}/T_{CD4+}$ as well as of memory T cells ($T_{em/eff}$/CD3+ or T_{cm}/CD3+) compared to control (***data not shown***). Moreover, in all 3 cases an improved blast lysis (especially after 24h of target-effector cell incubation) was shown using those MLC as effector cells (***Figure 4B***).

In summary ex vivo Kits were shown to give rise to mature DC/DC_{leu} without induction of blasts' proliferation, followed by activation of (anti-leukemically) activated effector cells. We conclude that Kits could give rise to antileukemic processes in vivo.

Kit M improves clinical course of AML patient compared to chemotherapy alone

Patient 1482: A 74-year-old Caucasian male was diagnosed with AML (unfavorable subtype) in April 2015. After failure to respond to hypomethylating agents (HMA), he achieved CR upon classical induction chemotherapy (Daunorubicine/AraC) as well as 3 courses of consolidation with high-dose AraC. In May 2017 a relapse was diagnosed. The patient was in need for multiple transfusions of erythrocytes and thrombocytes. Pronounced leukocytosis with 90% PB-blasts and 70% BM-blasts, as evaluated by morphological and cytological examination was shown. The patient was treated with 2 cycles of Decitabine (20mg/m^2), but AML proved to be HMA refractory. 11 days before start of Kit M Hydroxyurea was applied for 7 days, followed

by Cytarabine (100 mg/m^2, as continuous infusion) for 3 days, which led to control of leukemic proliferation, but not to remission**.** During pancytopenia (with <1% blasts) and in absence of any established therapy, he was offered an individual systemic salvage treatment. The entire treatment was carried out on an inpatient basis to immediately counteract possible adverse events. P1482 received 50 µg/m² GM-CSF (leukine), transfused iv. over 4 hours from day 11-13 for 3 days, which was escalated to 75 µg/m² GM-CSF from day 14-38. In addition, the patient received 20-80 µg PGE_1 (Alprostadil (Prostavasin®) per day iv. over each 2 hours one or two times daily, as given in ***Table 5***. For safety reasons strict stopping rules for the experimental treatment had been defined: 1) Discontinuation of GM-CSF therapy in case of >10000 blasts/µl or >50% of leukocytes, in case of lung-toxicities (severe dyspnea, severe affections of blood gas parameters). 2) Discontinuation of PGE_1 therapy was determined in case of drop in blood pressure (<100mmHg systolic or 50mmHg diastolic) or clinical signs of cardiac insufficiencies (Dyspnea, pulmonary edema). 3) Discontinuation of infusion of both drugs in case of any other toxicities (>CTC2) with restart of infusions only in case of complete normalization of parameters.

Routine clinical and laboratory parameters showed that the treatment was clinically well tolerated, and the patient improved clinically. Asymptomatic decrease of systolic blood pressure was the only CTC II° toxicity, seen with PGE_1 given over 2 hours, but resolved after prolongation of the infusion. Neutrophils in WBC increased from 10% to 50%, thrombocytes reached 100 G/l after 24 days (no need for platelet transfusions), whereas WBC and Hemoglobin counts remained low. Treatment was stopped after 4 weeks, and the patient was discharged in good clinical conditions. 8 days later, progression of AML was seen with high blast counts in PB (40%) and BM (73%). The patient developed severe sepsis and died few days later (***Figure 7***).

Figure 4: DC/DC_{leu} could be generated ex vivo from (blast containing) AML patients' whole blood with Kits (Kit-M and I) compared to Control. Kit treated WB led to improved antileukemic reactivity of human T cells after MLC.

Figure 4A: Increased frequencies of DC/DC_{leu} subtypes could be generated from (blast containing) AML patients' WB with Kit M and I compared to Control without induction of blasts' proliferation.

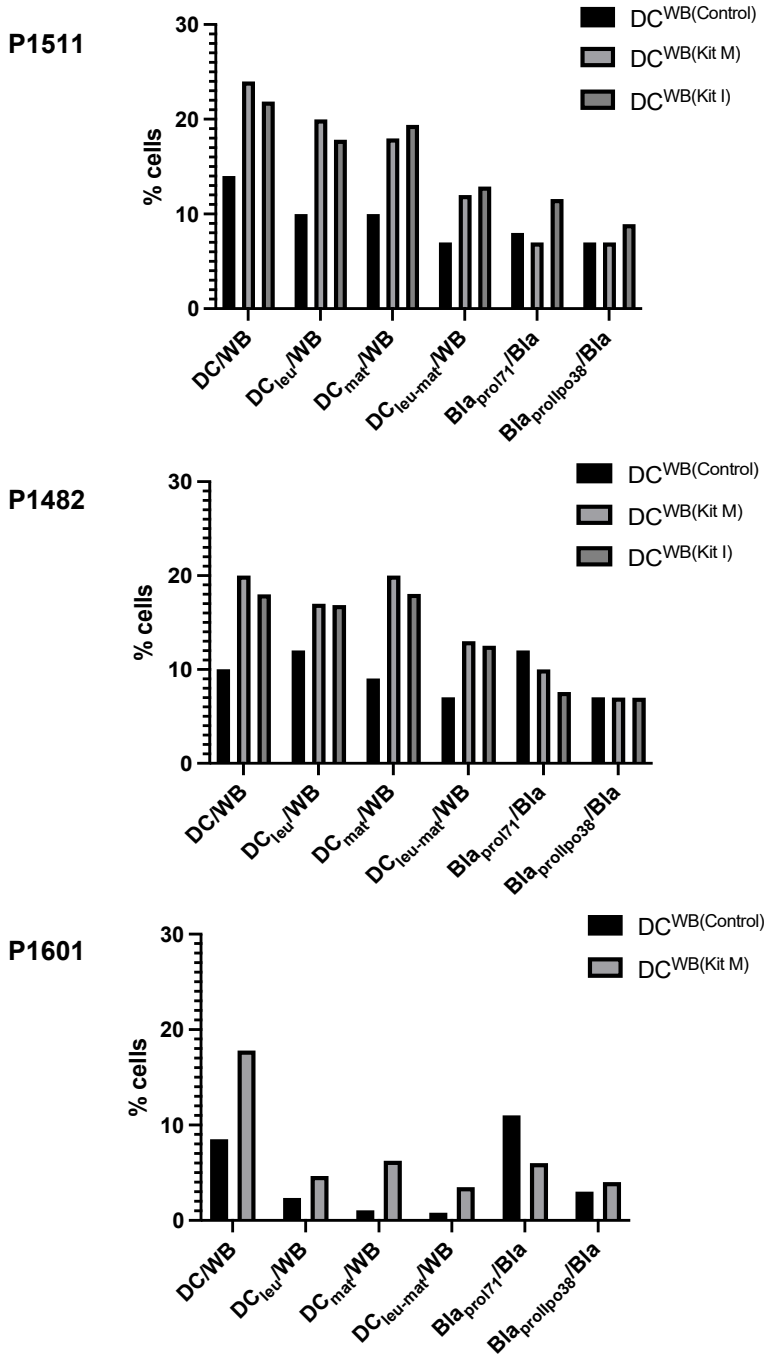


Figure 4B: Stimulatory effect of Kit M treated (vs. untreated WB) on antileukemic reactivity.

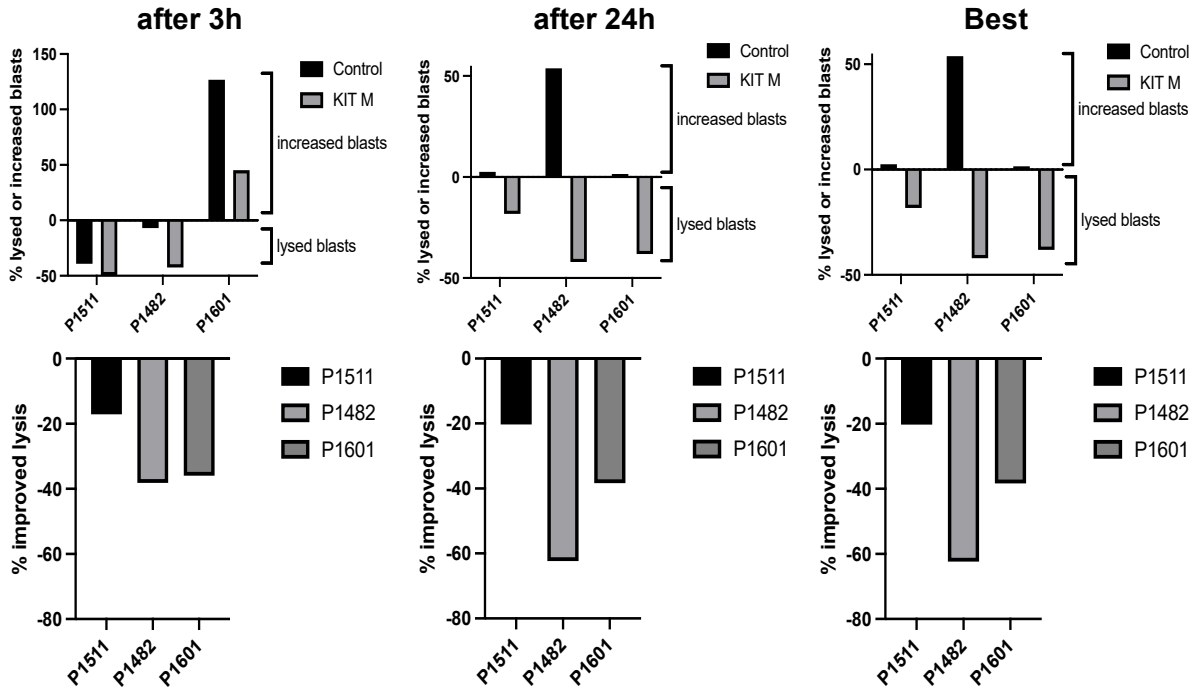
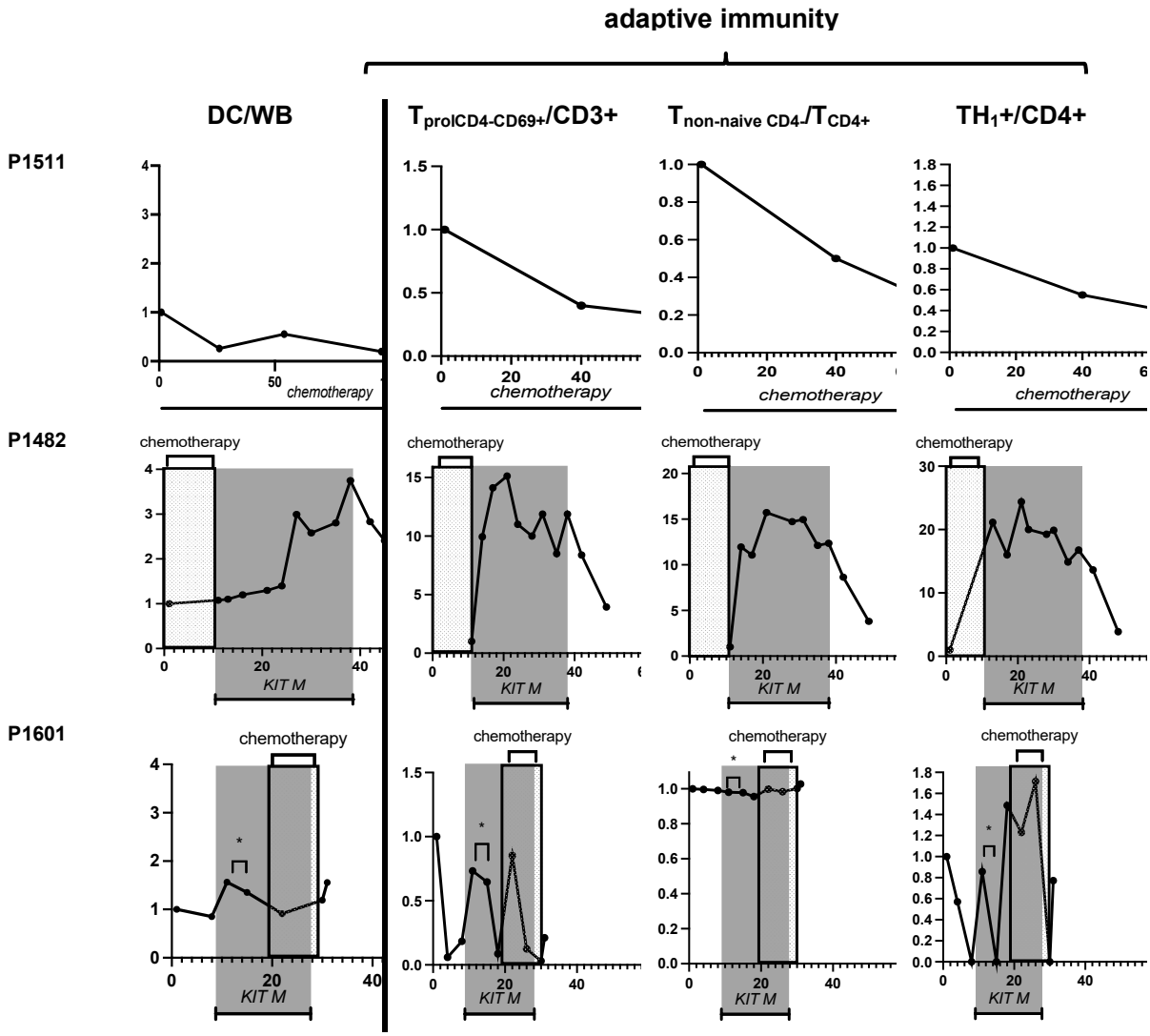


Figure 4: Figure 4A shows the frequencies of generated DC/DC_{leu} (subtypes) after *ex vivo* treatment with Kit I ($DC^{WB(Kit\text{-}I)}$) and Kit M ($DC^{WB(Kit\text{-}M)}$) compared to Control (without Kit treatment, $DC^{WB(Control)}$) for P1511, P1482 and P1601. Figure 4B demonstrates the improved antileukemic blast lytic activity (using a non-radioactive fluorolysis assay) for Kit M pretreated (vs. untreated) patients' WB samples after MLC. Frequencies of lysed or increased blasts as detected after 3 or 24 hours of effector-target cells incubation (and the best achieved antileukemic reactions in addition) are given in the upper part. Proportion of improved lysis is given in the lower part. Abbreviations are given in Table 3.

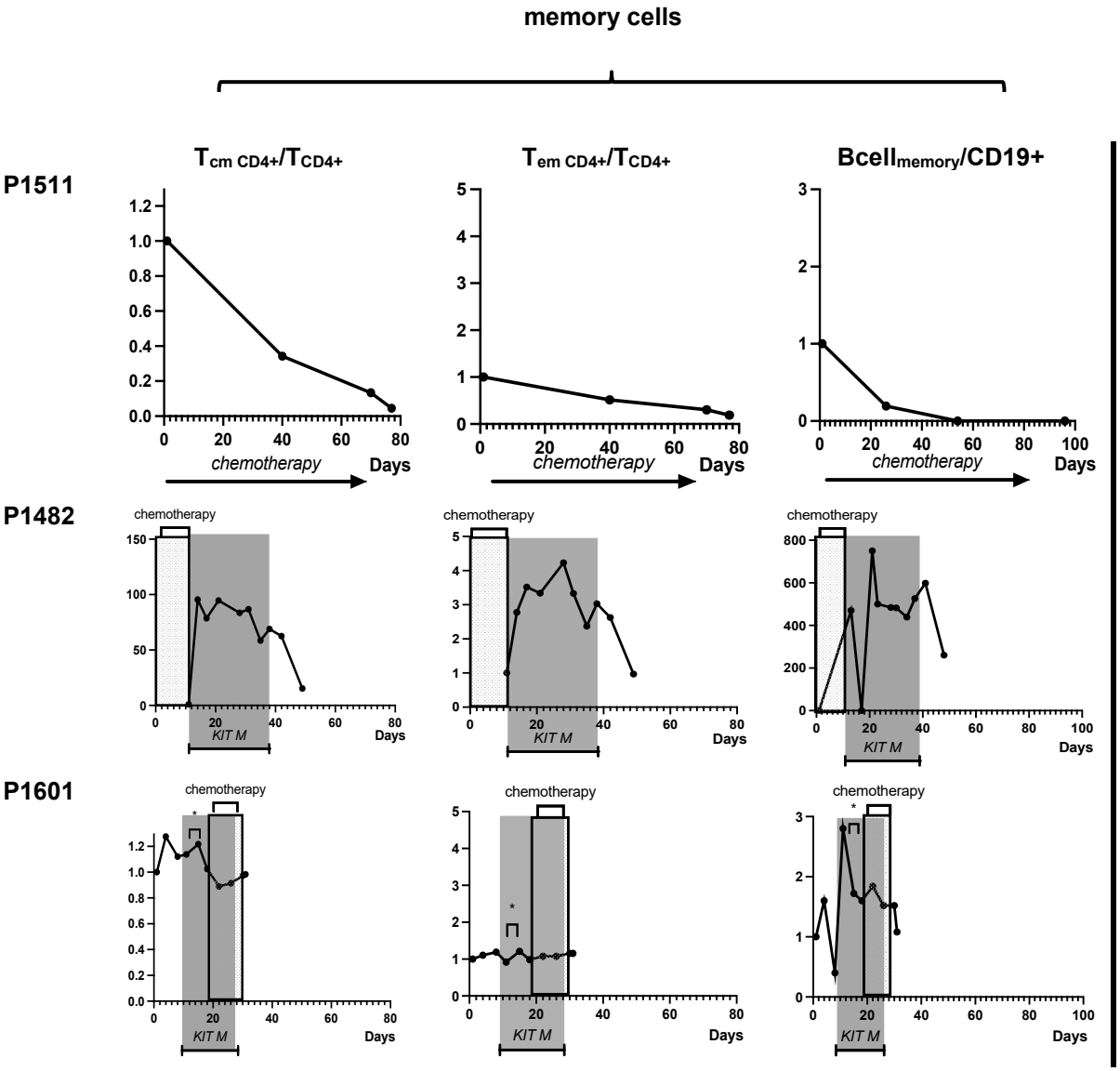
Figure 5: Treatment of therapy refractory patients with Kit M (P1482, P1601) activated cells of the adaptive and innate immune system compared to not Kit M treated patient (P1511).

Figure 5A: Effects of Kit M treatment (P1482, P1601) (vs. no Kit M treatment (P1511)) on patients' DC cells or cells of the innate and adaptive immunity in the course of disease.

a) Effects on frequencies of DCs and cells of the adaptive immunity



b) Effects on memory cells and cells of the innate immunity.



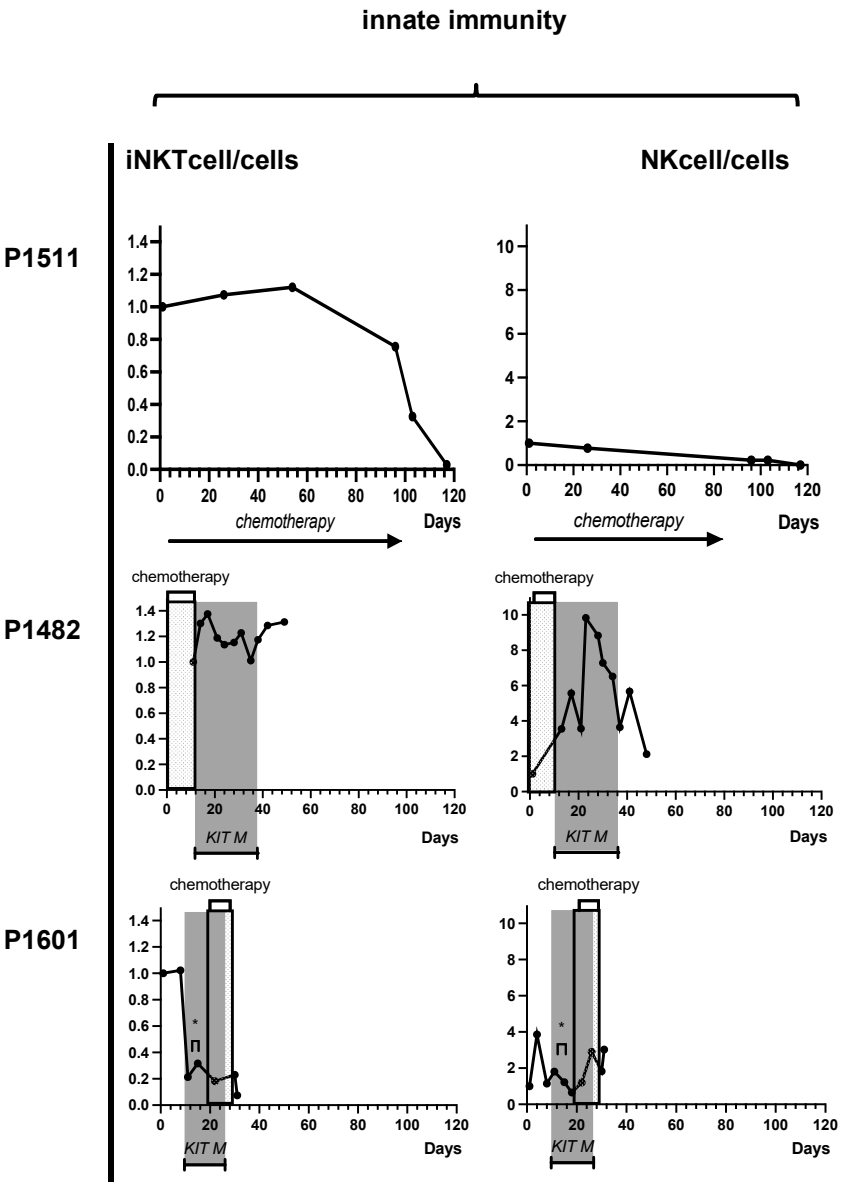

innate immunity
iNKTcell/cells
NKcell/cells
P1511
P1482
P1601
chemotherapy
Days
KIT M
*
Π

Figure 5B: Effects of Kit M treatment (P1482, P1601) (vs. no Kit M treatment (P1511)) on the provision of leukemia specific cells.

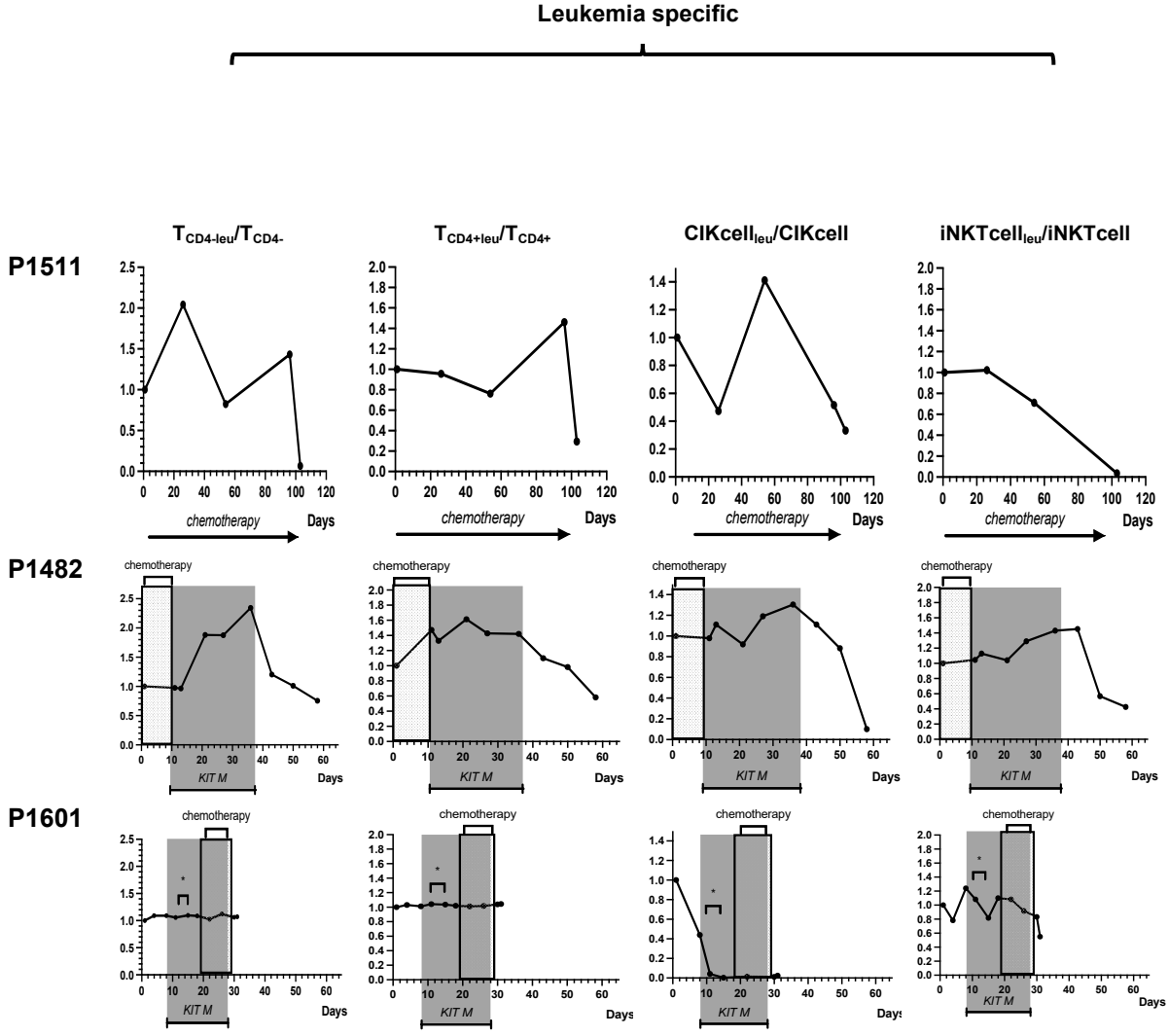


Figure 5: Figure 5A shows the effects of Kit M (vs. no Kit M) treatment on DC cells and cells of the innate and adaptive immunity in the course of disease of P1511, P1482 and P1601.

All values in the course of disease are given as 'fold change' values referred to the value at the beginning of observation.

P1511: chemotherapy during the whole observation time.

P1482: chemotherapy from day 1-11, Kit M treatment between day 11 and 38, no treatment from day 38 till the end of observation.

P1601: no treatment between day 1 and 9, Kit M treatment from day 9-26, chemotherapy from day 19-29, * application of prednisolone from day 12-14.

Details of the clinical courses of all patients are given in Figure 6,7 and 8. Abbreviations of cell subtypes are given in Table 3.

Immune monitoring (including standard immune status, leukemia specific cell monitoring (by ELISPOT and CSA + LAA stimulation) at defined time points showed (other than before treatment and in the patient without Kit-M-treatment) a continuous increase of DCs and proliferating $CD8^+$ T cells, of $T_{non\text{-}naive}$ (and a decrease of T_{naive}) of the CD8 as well as the CD4-lines could be seen during Kit M treatment. In addition, Th_1+ and Th_{17}+ (***data not shown***) $CD4^+$ T cells as well as $Bcell_{memory}$ increased over the 4-week-treatment. The same was true for T_{cm} and T_{em} of the CD8 as well as the CD4-lines. Regarding cells of the innate immune system, increased frequencies of NK cells (either $CD161^+$ or $CD56^+$), of CIK cells (either $CD161^+$ or $CD56^+$) and iNKT cells (of the NK- as well as the CD3-type) were seen during Kit M treatment (***Figure 5A***).

Antigen specific cells were monitored after LAA stimulation by CSA. Overall (slightly) increasing frequencies of IFN-γ-producing CD4, CD8 and CIK and iNKT cells of the innate immune system were seen suggesting an in vivo production/activation of (potentially leukemia specific) cells. Immune stimulatory effects decreased after discontinuation of therapy, although not to the base line before start of treatment (***Figure 5B***).

Patient 1601, a 74-year-old Caucasian female was diagnosed with AML (unfavorable subtype) in January 2020. She achieved one cycle of induction therapy with Daunorubicin (45 mg/m²) and Cytarabine (100 mg/m²). Unfortunately, persistence of disease had to be diagnosed at the end of treatment. A consecutive palliative treatment was induced with 4 cycles with Azacytidine (75 mg/m^2, day 1-7) and additionally one cycle of Venetoclax (initial dose 400 mg/day). This patient was refractory (90% blast in PB and 68% blasts in BM) and therapy was stopped due to pancytopenia. The patient suffered from pneumonia (heavy smoker) and was in need of multiple transfusions of erythrocyte and platelet concentrates. She received prednisolone between day 12-14 (50 mg/day) and had to undergo two pleural punctures (going along with thrombocyte infusions) (day 16 and 25). As a rescue therapy P1601 received 50 µg/m² GM-CSF (Leukine), transfused iv. over 4 hours for 3 days from day 9-11, which was escalated to 75 µg/m² GM-CSF from day 12-26. In addition, the patient received 20-80 µg PGE_1 (Alprostadil (Prostavasin®) per day iv. over each 2 hours one or two times daily, as given in ***Table 5***. Other than P1482, P1601 received drugs from day 14-26 between 8 am and 2 pm

(***Table 5***). Stopping rules for the experimental treatment in case of adverse events were defined as given for patient P1482.

Routine clinical and laboratory parameters were obtained and showed that the treatment was clinically well tolerated. No decrease of blood pressure or other clinical parameters were seen. Neutrophils, Hemoglobin and thrombocyte-values stayed low, and the patient had to receive erythrocyte and platelet transfusions. PB blasts counts were constantly high (>75%) but decreased soon after application of Kit M treatment between day 9-10 (from 90% to 78%) and between day 17-19 (from 95% to 89%). Interestingly, the blast counts increased by application of prednisolone (day 12-14; from 78% to 88%) and were not further reduced under hydroxycarbamide therapy (1g/day) between day 19-29 (***Figure 8***). On day 29 the patient decided to stop all treatments and died with refractory disease on day 31.

Immune monitoring (including standard immune status, leukemia specific cell monitoring (by ELISPOT and InCyt + LAA Stimulation) at defined time points showed (other than before treatment and in the patient without Kit-M-treatment) a slight increase of DCs, proliferating T cells, stable $T_{non\text{-}naive}$ of the CD8 as well as the CD4-lines during Kit M treatment. In addition, Th_1+ and Th_{17}+ (***data not shown***) $CD4^+$ T cells as well as $Bcell_{memory}$ increased during treatment. Whereas T_{cm} decreased and T_{em} stayed on a stable line. Regarding cells of the innate immune system, stable frequencies of NK cells (either $CD161^+$ or $CD56^+$), of CIK cells (either $CD161^+$ or $CD56^+$) and decreased frequencies of iNKT cells (of the NK- as well as the CD3-type) were seen during Kit M treatment (***Figure 5A***).

Antigen specific cells were monitored after LAA stimulation by InCyt. Overall stable frequencies of IFN-γ-producing CD4 and CD8 cells and decreasing frequencies of CIK-cells were seen, whereas frequencies of iNKT cells slightly increased during Kit M treatment but decreased under chemotherapy suggesting a slight in vivo production/activation of (potentially leukemia-specific) or at least stable frequencies of cells (***Figure 5B***).

Patient 1511, a 79-year-old Caucasian male with AML (diagnosed in December 2015, at the age of 76) was refractory to several lines of therapy. Since his AML diagnosis, he received 22 cycles of Decitabine, followed by Cytarabine and Midostaurin. In September 2018 he relapsed with more than 50% blasts in PB. This patient was treated with standard chemotherapy (without Kit M) and severed as a control. The clinical course of P1511 was reviewed over a

period of 4 months and showed hematopoietic insufficiently decreased thrombocyte and erythrocyte values resulting in needs of multiple erythrocyte and platelet transfusions. At the same time, high frequencies of blasts could consistently be detected in PB (in general: >3%; mostly >20%) **(*Figure 6*)**.

Immune monitoring including standard immune status, leukemia specific cell monitoring (by ELISPOT and CSA + LAA stimulation) at defined time points showed (other than before treatment and in the patients with Kit-M-treatment) a decrease of DC, of proliferating and $T_{non\text{-}naive}$ of the CD8 as well as the CD4-lines. Th_1 and Th_{17} $CD4^+$ T cells as well as T_{cm}, T_{em} and $Bcell_{memory}$ decreased in the course of the disease. Regarding cells of the innate immune system, frequencies of NK cells decreased, whereas frequencies of iNKT cells kept stable over 60 days, however decreased in the further course of observation (***Figure 5A***).
Antigen specific cells were monitored after LAA stimulation by CSA. Varying, however, in general decreasing frequencies of leukemia-specific cells of the T- and innate lines were found (***Figure 5B***).

Table 5: Treatment regime for P1482 and P1601 using Kit M (leukine (GM-CSF) and Prostavasin (PGE1). Courses of the disease are given in Figure 7 and 8

a) P1482

Day	Drug	Dosage (iv)	Schedule
11*	GM-CSF	50 μg/m²/4 hours	8-12 am
	PGE_1	20 μg (in total)	1-3 pm
12	PGE_1	20 μg (in total)	8-10 am
	GM-CSF	50 μg/m²/4 hours	11 am-3 pm
	PGE_1	20 μg (in total)	8-10 pm
13	PGE_1	20 μg (in total)	8-10 am
	GM-CSF	50 μg/m²/4 hours	11 am-3 pm
	PGE_1	20 μg (in total)	8-10 pm
14	PGE_1	20 μg (in total)	8-10 am
	GM-CSF	75 μg/m²/4 hours	11 am-3 pm
	PGE_1	40 μg (in total)	8-10 pm
15	PGE_1	40 μg (in total)	8-10 am
	GM-CSF	75 μg/m²/4 hours	11 am-3 pm
	PGE_1	40 μg (in total)	8-10 pm
16-38		Percede as day 15	

b) P1601

Day	Drugs	Dosage (iv)	Schedule
9*	GM-CSF	50 μg/m²/4 hours (77.5 μg in total)	8-12 am
	PGE_1	20 μg (in total)	1-3 pm
10	PGE_1	20 μg (in total)	8-10 am
	GM-CSF	50 μg/m²/4 hours (77.5 ug in total)	11 am- 3 pm
	PGE_1	20 μg (in total)	6-8 pm
11	PGE_1	20 μg (in total)	8-10 am
	GM-CSF	50 ug/m²/4 hours (77.5 μg in total)	11 am- 3 pm
	PGE_1	20 μg (in total)	6-8 pm
12	PGE_1	20 μg (in total)	8-10 am
	GM-CSF	75 μg/m²/4 hours (116 μg in total)	11 am- 3 pm
	PGE_1	40 μg (in total)	6-8 pm
13	PGE_1	40μg (in total)	8-10 am
	GM-CSF	75 μg/m²/4 hours	11 am- 3pm
	PGE_1	40 μg (in total)	6-8 pm
14	PGE_1	40 μg (in total)	8-10 am
	GM-CSF	75 μg/m²/4 hours	10 am- 12 pm
	PGE_1	40 μg (in total)	12- 1 pm
15-26		Percede as day 14	

***Observation time and monitoring of blasts starts at Day 1; Kit M treatment starts at Day 11 and 9.**

Figure 6: Clinical course of disease of P1511 during chemotherapy.

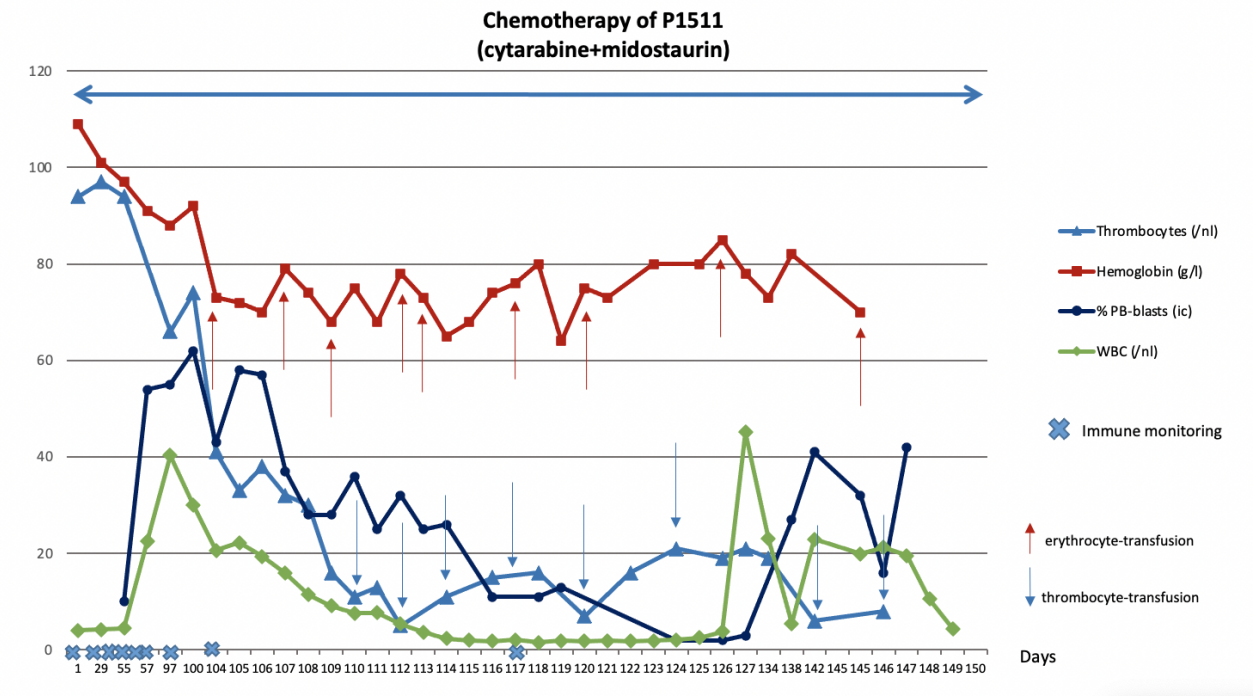


Figure 6: Clinical course of P1511 during chemotherapy (without Kit M). Chemotherapy (cytarabine, midostaurin) was given from the start of observation to the end of observation. Blood cells (thrombocytes, hemoglobin, blasts) in peripheral blood (PB) and leukocytes/white blood cells (WBC) are given. ↑ Timepoints of erythrocyte-transfusion, ↓ timepoints of thrombocyte-transfusion.

Figure 7: Clinical course of disease of P1482 during chemotherapy and Kit M treatment.

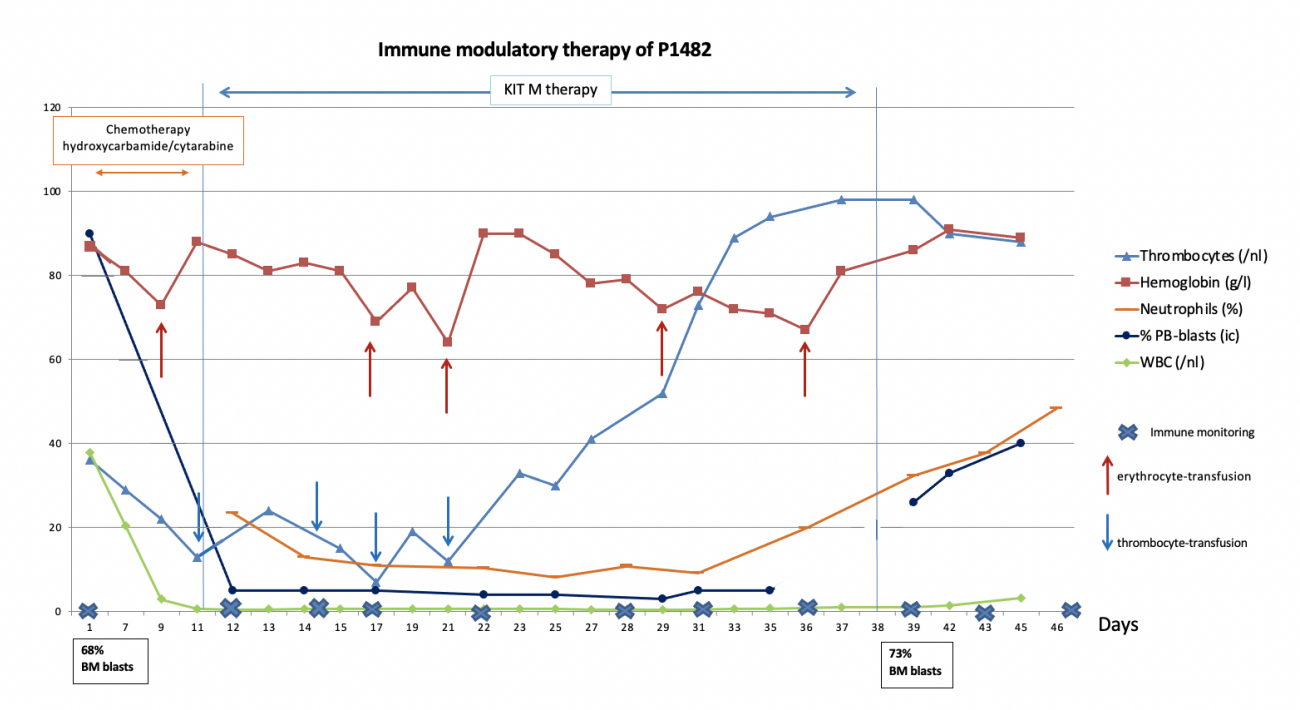


Figure 7: Clinical course of P1482 during chemotherapy and Kit M treatment. Chemotherapy (hydroxycarbamide, cytarabine) was given from the start of observation till day 11. Kit M treatment between day 11 and 38, no treatment from day 38 till the end of observation.

Blood cells (thrombocytes, hemoglobin, neutrophils, blasts) in peripheral blood (PB), frequencies of BM blasts and leukocytes/white blood cells (WBC) are given. ↑ Timepoints of erythrocyte-transfusion, ↓ timepoints of thrombocyte-transfusion.

Figure 8: Clinical course of disease of P1601 during Kit M treatment ± chemotherapy.

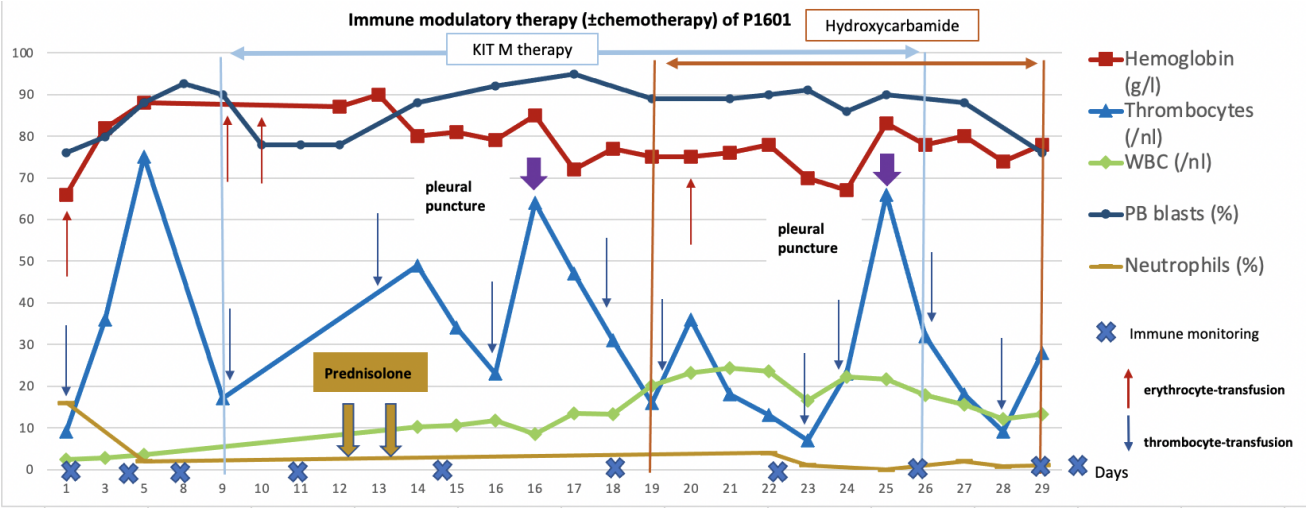


Figure 8: Clinical course of P1601 during Kit M treatment ± chemotherapy. No treatment was given between day 1 and 9, Kit M treatment from day 9-26, chemotherapeutical treatment (hydroxycarbamide) from day 19-29. Patients' pneumonia was additionally daily treated by prednisolone on day 12-14. Blood cells (thrombocytes, hemoglobin, neutrophils, blasts) in peripheral blood (PB) and leukocytes/white blood cells (WBC) are given. ↑ Timepoints of erythrocyte-transfusion, ↓ timepoints of thrombocyte-transfusion, ⇓ pleural punctions.

4. Discussion

Immunotherapies for acute myeloid leukemia are a promising treatment option to achieve remission and stabilize the disease. These approaches can also be used in elderly or debilitated patients for whom conventional chemotherapy and radiotherapy are unsuccessful or have too severe side effects. Further research and development in this area will help improve the efficacy and safety of immunotherapies in AML and expand treatment options for patients.

4.1. DC/DC_{leu} generating and blast modulating protocols (Kits) lead to antileukemic reactions

Treating AML patients with ex vivo produced DC (loaded with leukemia associated antigens) or with DC_{leu} has been shown to stabilize the decrease/remission. These DC/DC_{leu} must be produced under comparable conditions, followed by an adoptive transfer to patients. An elegant solution to improve these DC-based strategies is to directly convert (residual) blasts in the patients in vivo to DC_{leu} resulting in activation of immune cells (as proposed here).

We and others could prove that DC/DC_{leu} can be generated ex vivo from blast containing MNC or WB without inducing blast proliferation on a regular basis using different DC/DC_{leu} generating protocols and Kits **(Schwepcke et al., 2022, Plett et al., 2022)**. The generation process is independent of patients' characteristics like age, sex, disease/prognostic classifications. Moreover, these Kits have already shown promising results as they are able to induce leukemia-specific/anti-leukemic cells after T cell enriched MLC **(Klauer et al., 2021, Plett et al., 2022)**.

We could show that DC/DC_{leu} can also be successfully generated from leukemic rat WB without induction of blasts' proliferation using different Kits ex vivo. An astonishing finding was, that nearly all the created DC/DC_{leu} were leukemia-derived, pointing to a highly efficient leukemic antigen-presentation and leading to leukemia-cytotoxicity of stimulated effector cells. In addition, blast lysis could be improved with all Kit-pretreated WB-samples used as stimulator cells in MLC, as already shown for human AML-samples **(Plett et al., 2022,**

Schwepcke et al., 2022, Klauer et al., 2021). We could confirm these preliminary findings as well as findings with rats by testing leukemic WB samples from P1601, P1482 and P1511 we could evaluate Kit M as 'best kit' inducing DC/DC_{leu} without induction of blasts' proliferation, inducing T cells' proliferation and improving anti-leukemic reactions compared to control **(Figure 4A+B)**.

These findings prompted us to deduce a Kit M-based treatment protocol for these patients.

4.2. Treatment of refractory AML patients with blast modulatory Kit M is safe, induces platelet regeneration and improves the composition of (anti-leukemic) cells directed immunoreactive cells

Two patients (P1482 and P1601) were finally able to undergo blast modulatory rescue therapy with Kit M (GM-CSF + PGE_1), being the best selected Kit in terms of DC/DC_{leu} generation and achieved blast lysis without induction of blast proliferation in WB-cultures.

P1482: Shortly before start of blast-modulatory treatment the patient was treated with Decitabine (20mg/m^2), followed by Hydroxyurea and Cytarabine (100mg/m^2) to minimize PB- (nearly 90%) and BM-blasts (nearly 70%). The in vivo treatment over 4 weeks started with application of single drugs in low concentration with dose-escalation during treatment (***Table 5***). An asymptomatic and transient decrease of blood pressure, known as a possible effect of PGE_1, was seen on the first day of application, but not during the remaining phase of therapy. No other adverse events were seen during treatment. (The patient even could leave the hospital after the 4 weeks' treatment phase in a good general condition).

Positive effects documented during the phase of treatment were normalization of (chemotherapy- and disease related) leukocytopenia, thereby minimizing risks for infections: Neutrophil-counts increased (without induction of blasts' proliferation) – probably an effect of GM-CSF. An astonishing finding was the soon recovery of platelets, which made the patient independent of transfusion requirements. This could be a result of the combined application of GM-CSF and PGE_1, since neither GM-CSF nor PGE_1 are known to contribute to induce thrombocytopoiesis. Meanwhile, requirements for erythrocyte-concentrates remained. Moreover, leukemic blasts were below the (immunological) detection limit over the whole phase of treatment. However, the patient presented with a relapse one week later, going along with reoccurrence of blasts in PB. In the BM around 70% blasts were detectable before start of Kit M treatment. Since no BM-aspiration had been performed during the 4-week

treatment neither monitoring of the tumor load (e.g., transient blast-decrease), nor the immune constellation was possible.

Monitoring of the patient's PB revealed an increase of DCs and DC_{leu} - although with variations (probably due to the DCs' migration to tissue, as already discussed in the rat system). No blast-proliferation, but instead decreased blast counts was seen underlining the safety of these drugs. Moreover, our data showed a continuous increase of several lines of cells of the adaptive (B- and T-linear) (leukemia-specific) immune system during the phase of treatment resulting in increased frequencies of B- and T-memory cells as well as of cells of the (leukemia-specific) innate immunity, although these cells were reduced after discontinuation of therapy, however remained on a higher level compared to start of treatment. These results suggest an induced or improved anti-leukemic response of the immune system by Kit treatment (– during the complete phase of Kit-treatment the patient did not receive any additional chemotherapy), which ultimately may have led to improved clinical parameters of the patient. The induction of 'leukemic specific' immune reactions as described here was already described as an important step towards stabilization of the disease or the remission. It remains to be discussed, whether the patient would have profited from a longer duration of treatment with Kit M.

P1601: Weeks before start of Kit M treatment, P1601 was treated by Azacytidine (75 mg/m^2, day 1-7) and additionally with Venetoclax (400 mg/day). However, the patient was therapy-refractory. Shortly before Kit M application blasts in PB increased to 93%. As in P1482 the in vivo Kit M therapy started with application of (single) lower dosage of GM-CSF and PGE_1 with final concentrations being applicated from day 13-26. Moreover, from day 14-26 all infusions were applicated before 1pm (***Table 5***). No adverse events were seen during treatment-pointing to good safety of the drugs (even if applicated in 5 hours). An interesting finding was the blast reduction between day 9-12 under the only treatment with Kit M (in low concentration): This could be interpreted by an (immunological) antileukemic mechanism induced with low dosage of Kit M drugs. Between day 12-14 prednisolone was given to treat pneumonia. The immune suppressive effect however, lead to a 'knock out' of immunological antileukemic effect- leading to an increase of blasts until day 17. Between day 17-19 a blast reduction probably due to the influence of Kit M was seen. Other than in P1482 a potential positive influence on thrombocyte-recovery was not seen in P1601: Thrombocyte counts in the course of treatment were low (probably due to patients' invasive, bleeding inducing

pleural punctures that needed platelets transfusions). Immunoreactive cells only slightly improved (or at least stable cell counts were seen) in the treatment phase with Kit M (without prednisolone). These results might suggest a (potentially) induced antileukemic response after Kit M application. Whether lower or higher dosages of GM-CSF and/or PGE_1 could be sufficient to activate antileukemic reactions in vivo, must be worked out by a clinical trial.

P1511 (control-patient): A potential study patient (P1511), who finally received further chemotherapy treatment due to a worsening of his condition, was ultimately used as a control for P1482 and P1601: This patient showed low platelets, hemoglobin, and neutrophil counts, while blast counts persisted in high frequencies throughout the phase of chemotherapy **(Figure 6)**. In contrast to P1482 and P1601 monitoring of P1511s' PB revealed low levels of DC and DC_{leu}, continuously decreasing frequencies of immune reactive cells of all lines: decreasing proliferating T cells, memory T cells, NK-, NKT- and CIK-cells as well as of leukemia-specific immune cells. All these findings suggest lower anti-leukemic competence in this not-kit-treated patient leading to the immune systems' inability to cope with the disease.

5. Conclusion

In summary, we could show that Kits (containing clinically approved drugs) produce DC/DC_{leu} ex vivo and in vivo in both leukemically diseased rats and humans. Compared to controls, we have demonstrated improved anti-leukemic responses ex vivo and in vivo. Kit M as rescue therapy was shown to be safe and to lead to neutrophils' and platelets' recovery without increasing blast counts. At the same time, immune cells of the adaptive and innate lines were activated and induced to give rise to memory- as well as to leukemia-specific/antileukemic cells.

Although further experiments and treatments (phase I trial is in preparation) are required, this in vivo strategy aiming at the conversion of (residual) blasts to leukemia-derived DC (without need of GMP procedures) appears to be promising, since DC/DC_{leu} can migrate to tissues and prevent (extramedullary) relapses. DC/DC_{leu}-generation is independent of patients' age, MHC, mutation, cytogenetic risk, transplant-, or FAB status and could contribute to stabilization of the disease or of remissions by induction of antileukemic cells and immunological memory. We show promising results in the field of immunotherapy for AML, that could contribute to stabilizing the disease or remission with a well-tolerated therapy.

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Abbreviations

ALL	acute lymphatic leukemia
AML	acute myeloid leukemia
AP	accelerated phase CML
APC	Allophycocyanin
APC	antigen presenting cell
BC	blast crisis CML
CARs	chimeric antigen receptors
CD	differentiation antigen (cluster of differentiation)
CML	chronic myeloid leukemia
CMML	chronic myelomonocytic leukemia
CP	chronic phase CML
CR	complete remission
DC	dendritic cell
DC_{leu}	leukemia derived dendritic cell
DC_{leu}/bla	blast converted to dendritic cell in blast-fraction
DC_{leu}/DC	leukemia derived dendritic cell in dendritic cell-fraction
DC_{migr}/DC	migratory mature dendritic cell in dendritic cell-fraction
DC_{viable}/DC	viable dendritic cell in dendritic cell-fraction

dgn.	diagnosis
DLI	donor lymphocyte infusion
FAB	French-American-British classification
FACS	fluorescence activated cell shorting
FCS	fetal calf serum-free medium
FITC	fluorescein isothiocyanate
FL	FLT3 ligand
GM-CSF	granulocyte magrophage - colony stimulating factor
GVHD	graft-versus-host disease
HBV	hepatitis-B
HCV	hepatitis-C
IC	immunocytological
IFN	interferon
IFN-I	DC-culture media (interferon alfa)
IFN-GI	DC-culture media (granulocyte magrophage - colony stimulating factor + interferon alfa)
IFN-GIT	DC-culture media (granulocyte magrophage - colony stimulating factor + interferon alfa + tumor necrosis factor alfa)
IFN-GITZ	DC-culture media (granulocyte magrophage - colony stimulating factor + interferon alfa + tumor necrosis factor alfa + Zylexis)
IL	interleukin
LAA	leukemia associated antigen
M0	acute myeloblastic leukemia
M1	acute myeloblastic leukemia with minimal maturation

M2	immature granulocytic leukemia
M3	acute promyelocytic leukemia
M4	acute myelomonocytic leukemia
M5	acute monocytic leukemia
M6	erythroid leukemia
MAF	magrophage-activating factor
MCM	DC-culture media (MCM-Mimic)
MDS	myelodysplastic syndrome
MHC I	major histocompability complex I
MHC II	major histocompability complex II
MLC	mixed lymphocyte culture
MNC	mononuclear cell
mAbs	monoclonal antibodies
moAbs	mouse monoclonal antibodies
NAP-1	neutrophil-activating protein-1
NK-cell	natural killer cell
NR	non-responder
OS	overall survival
PBS	phosphate buffered saline
PC7	tandem Cy7-PE conjugation
PE	Phycoerythrin
Peg-IFNα2b	pegylated interferon alfa
PGE_2	Prostaglandin E2

Pici	DC-generation media (Picibanil)
SCT	stem cell transplantation
TCGF	T-cell growth factor
T_{eff}	effector T-cell
TGF	transforming growth factor
TNF	tumor necrosis factor
T_{reg}	regulatory T-cell
WB	whole blood

List of publications

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Atzler M, Westhofen T, Tamalunas A, Schott M, Keller P, Ebner B, Stief C, Magistro G. Die Rolle des Mikrobioms bei urologischen Erkrankungen [The role of the microbiome in urological diseases]. Aktuelle Urol. 2021 Aug;52(4):338-344. German. doi: 10.1055/a-1478-2960. Epub 2021 Jun 8. PMID: 34102684.

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Atzler M, Rank A, Inngjerdingen M, Rabe A, Deen D, Wang R, Eiz-Vesper B, Schmid C, Schmetzer H. Increased detection of (leukemia-specific) adaptive and innate immune-reactive cells under treatment of AML-diseased rats and one therapy-refractory AML-Patient with clinically approved, blast modulating response modifiers. 2023 in preparation.

T. Baudrexler, T. Boeselt, M. Atzler, A. Hartz, U. Boas, C. Schmid, A. Rank, J. Schmohl, R. Koczulla, H. M. Schmetzer: Volatile profiling using an eNose allows differentiation of healthy and leukemic breath samples Submitted for publication.

S. Ugur, L. K. Klauer, C. Blasi, F. Doraneh-Gard, C. Plett, C. Gunsilius, D. C. Amberger, M. Weinmann, O. Schutti, Z. Fischer, E. Özkaya, M. Atzler, E. Pepeldjiyska, A. Völker, J. Schmohl, A. Rank, C. Schmid, H. M. Schmetzer: 'Kit'-mediated blastmodulation to leukemia-derived DC significantly improves antileukemic activities in whole blood independent of AML-patients' subtypes.
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Eidesstaatliche Versicherung

Ich, Michael Atzler, erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema

Increased detection of (leukemia-specific) adaptive and innate immune-reactive cells under treatment of AML-diseased rats and therapy-refractory AML-Patients with clinically approved, blast modulating response modifiers

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