



Research Article

Volatile Profiling using an eNose - Exploiting Breath Volatile Organic Compounds for Disease Monitoring in Refractory Acute Myeloid Leukemia Patients

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Abstract

Introduction: The demand for systemic tools to monitor disease-related biomarkers is ever more pressing. Volatile organic compound (VOC) analysis, capable of detecting individual molecules in human metabolism offers a non-invasive and easily collectible avenue for measurement via electronic nose (eNose) and might qualify as a systemic tool for disease monitoring. We analyzed VOC results from breath samples of AML patients (throughout the course of their disease, with/without treatment) with breath samples from healthy individuals. **Methods:** Clinical and immunological monitoring encompassed the observation of three refractory AML patients undergoing different therapies. Patient P1511 received chemotherapy, while patients P1482 and P1601 were treated with Kit M (GM-CSF and PGE₁).

Myeloid leukemic blasts can be transformed into dendritic cells of leukemic origin (DC_{leu}) ex vivo and in vivo, with the potential to stimulate and activate the immune system specifically against leukemia. These patients underwent repeated clinical and haematological/immunological assessments. Blood samples were periodically collected to monitor (leukemia-specific) immune cells using flow cytometry, cytokine secretion assay (CSA), and intracellular cytokine assay (InCyt). VOC monitoring involved the collection of VOCs in earloop masks containing exhaled air from AML patients and healthy volunteers measured using an

eNose. All samples were measured in triplicate, and the mean values were used for principal component analysis. Subsequently, linear discriminant analyses (LD) were performed. **Results:** The findings underscore Kit M as a safe clinical drug that fosters the generation of DC/DC_{leu} and augments the frequencies of leukemia-specific/antileukemic cells within the adaptive and innate immune systems, both ex vivo and in vivo, in AML patients. VOC profiles exhibited significant differences between healthy and AML breath donors, as well as between profiles during chemotherapy and Kit M therapy. Moreover, the eNose can detect differences in VOC profiles between breath samples taken with and without chemo-/immunotherapy. **Conclusion:** We posit that breath profiling with an eNose, capturing disease-related VOCs, could serve as a diagnostic or monitoring tool for leukemia patients. This approach entails the collection of VOCs using breath masks, eliminating the need for direct VOC measurement via an eNose during the course of the disease.

Keywords: Leukemia Derived DC; Acute Myeloid Leukemia; Anti-Leukemia Functionality; Leukemia-Specific Cells; Vocs of Exhaled Breath; Immune Monitoring in the Course of The Disease

Introduction

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 [1, 2]. 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 [3]. For patients with less tolerance for the induction therapy low-dose cytarabine or hypomethylating agents are potential therapy strategies [4, 5]. New immune therapeutic strategies address the dysfunctional reactivity of the immune system against leukemic blasts [6, 7].

DC-Based Immunotherapy

DC cells play a central role in connecting the innate and the adaptive immune system [8, 9]. DCs can be generated directly from leukemic blasts, rendering the complicated antigen loading process on (monocyte derived) DCs unnecessary [10]. Those DC_{leu} are characterized by the expression of individual patients' whole leukemic antigen repertoire including known as well as unknown

leukemic antigens [11, 12]. These DCs have to be re-administrated to patients as a 'vaccine'. 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 E1 or E2), which triggers DC/DC_{leu} differentiation and maturation) [11, 13, 14].

Due to their immunomodulatory properties (in combination with GM-CSF) PGE1 and PGE2 have been shown to be highly efficient ex vivo DC-generating factors by providing a danger signaling, enhancing DCs' maturation and migratory capacity [13-16].

The Immune System

The immune system comprises both the innate and adaptive branches. The innate immune system is composed of antigen-presenting cells (APCs) like monocytes and dendritic cells (DCs), as well as specialized cell types like cytokine-induced killer (CIK) cells, invariant natural killer T (iNKT) cells, and natural killer (NK) cells. These components play a crucial role in the initial defence against pathogens and tumour cells [14, 17-19]. The adaptive immune system involves B cells (CD19+) and T cells (CD3+) and their various subtypes. After activation they provide (cytotoxic) effector as well as cytotoxic cells of different lines. Additionally, they develop memory cells enabling a quicker reactivation of the immune system upon encountering recurring antigens [13, 18, 20]. Abbreviations are summarized in Table 1, supplement.

		Name of Subgroups	Abbreviation of Subgroups	Surface Marker	Referred to	Abbreviation	Reference
Adaptive immune system	Blast cells	Blasts	Bla	Bla e.g. CD34+, CD117+	WB	Bla/WB	[51]
		Proliferating blasts	Bla _{prol71}	Bla+DC-CD71+	Bla	Bla _{prol71} /Bla	[52]
		Proliferating blasts	Bla _{prolIPO38}	Bla+DC-IPO38+	Bla	Bla _{prolIPO38} /Bla	[6]
	Dendritic cells	Dendritic cells	DC	DC+ e.g. CD80+, CD206+	WB	DC/WB	[51]
		Leukaemia derived DC	DC _{leu}	DC+Bla+	WB	DC _{leu} /WB	[51]
		Mature migratory DC	DC _{mat}	DC+CD197+	WB	DC _{mat} /WB	[53]
		Mature migratory DC _{leu}	DC _{leu-mat}	DC+Bla+CD197+	WB	DC _{leu-mat} /WB	[53]
	B lymphocytes	CD19+ B cells _{memory}	Bcell _{memory}	CD19+CD27+IgD-	CD19+	Bcell _{memory} /CD19+	[54]
	T lymphocytes	CD3+ pan T cells	CD3+	CD3+	lymphocytes	CD3+/cells	[13]
		CD4+ T cells	T _{CD4+}	CD3+CD4+	CD3+	TCD4+/CD3+	[13]
		CD4- T cells	T _{CD4-}	CD3+CD4-	CD3+	TCD4-/CD3+	[13]
		T helper cells 1	TH ₁ +	CCR4-CXCR3+CCR5+CCR6-	CD4+	TH1+/CD4+	[55]
		Naive T cells	T _{naive}	CD3+CD45RO-	CD3+	Tnaive/CD3+	[17]
		Non-naive T cells	T _{non-naive}	CD3+CD45RO+	CD3+	T _{non-naive} /CD3+	[17]
			T _{non-naive CD4+}	CD3+CD45RO+CD4+	T _{CD4+}	T _{non-naive CD4+} /T _{CD4+}	
			T _{non-naive CD4-}	CD3+CD45RO+CD4-	T _{CD4-}	T _{non-naive CD4-} /T _{CD4-}	
		Central (memory) T cells	T _{cm}	CD3+CD45RO+CD197+	CD3+	T _{cm} /CD3+	[17]
			T _{cmCD4+}	CD3+CD45RO+CD197+CD4+	T _{CD4+}	T _{cm CD4+} /T _{CD4+}	
			T _{cmCD4-}	CD3+CD45RO+CD197+CD4-	T _{CD4-}	T _{cm CD4-} /T _{CD4-}	
		Effector (memory) T cells	T _{em}	CD3+CD45RO+CD197-	CD3+	T _{em} /CD3+	[17]
	T _{em CD4+}		CD3+CD45RO+CD197-CD4+	TCD4+	T _{em CD4+} /T _{CD4+}		
	T _{em CD4-}		CD3+CD45RO+CD197+CD4-	TCD4-	T _{em CD4-} /T _{CD4-}		
	Proliferating T cells - early	T _{prolCD69+}	CD3+CD69+	CD3+	T _{prolCD69+} /CD3+	[17]	
		T _{prolCD4-CD69+}	CD3+CD4-CD69+	TCD4-	T _{prolCD4-CD69+} /T _{CD4-}		
	Proliferating T cells - late	T _{prolCD71+}	CD3+CD71+	CD3+	T _{prolCD71+} /CD3+	[17]	

Innate immune system	Cytokine induced killer cells	CD3+CD56+ CIK cells	CIKcell	CD3+CD56+	lymphocytes	CIKcell/cells	[13]
	Natural killer cells	CD3-CD56+ NK cells	NKcell	CD3-CD56+	lymphocytes	NKcell/cells	[13]
	Invariant natural killer T cells	6B11+ iNKT cells	iNKTcell	6B11+	lymphocytes	iNKTcell/cells	[22]
Leukemia specific cells	T lymphocytes *	CD4+ coexpressing T cells _{leu}	T _{CD4+leu}	CD3+CD4+INFy+	T _{CD4+leu}	T _{CD4+leu} /T _{CD4+}	[17]
Adaptive immune system		CD8+ coexpressing T cells _{leu}	T _{CD4-leu}	CD3+CD4-INFy+	T _{CD4-leu}	T _{CD4-leu} /T _{CD4-}	[17]
Innate immune system	Cytokine induced killer cells **	CD3+CD56+ CIK cells _{leu}	CIKcell _{leu}	CD3+CD56+INFy+	CIKcell	CIKcell _{leu} /CIKcell	[17]
	Natural killer cells **	CD3-CD56+ NK cells _{leu}	NKcell _{leu}	CD3-CD56+INFy+	NKcell	NKcell _{leu} /NKcell	[17]

Table S1: Cell subsets and their abbreviations.

Leukemia-Specific Cells and Anti-Leukemic Processes

The cytokine secretion assay (CSA) and the intracellular cytokine assay (InCyt) enable the intracellular quantification of cytokines (e.g., interferon-gamma (IFN- γ)) on a single-cell level. These cytokines are key factors in immune responses and mediators of cell apoptosis [17, 18, 21] and provide a comprehensive analysis of cell functionality, particularly in terms of the activity and cytotoxicity of immune cells specific to leukemia [17].

Methodological Tools for Monitoring AML and Anti-Leukemic Processes

Understanding both leukemia-related and anti-leukemic processes requires the analysis of various cellular and humoral factors, whether activating or inhibitory, as well as soluble factors and even small molecules [13, 18, 21-25]. In recent years, the role of physical factors, such as physiological hypoxia [26] and circulating vesicles (e.g., extracellular vesicles (EVs)) [27, 28], has been investigated for their potential in refined monitoring immunological or tumor-associated processes.

VOC Analysis as a Novel Approach for Characterizing and Monitoring (Malignant) Diseases

Every living organism, whether human, plant, or animal, emits organic compounds into the environment. Exhaled molecules reflect metabolic changes in both healthy and pathological conditions, making exhaled air a potential tool for monitoring disease-related biomarkers [29]. Volatile organic compounds (VOCs) are organic chemicals containing hydrocarbon compounds. Exhaled VOCs

can be easily collected noninvasively, either by directly capturing exhaled breath using an electronic nose (eNose) or by analysing VOCs collected on carriers such as fleece or earloop masks. Subsequent analysis is conducted using an eNose. Proof of concept has demonstrated that VOC analysis can aid in the detection of disease-associated markers, such as AML (characterized by cell-molecular anomalies and metabolic changes resulting in different endogenous VOCs), Parkinson's disease, Alzheimer's disease, COVID-19 infection and lung cancer [30-35].

Objectives of this study

The aims of the studies presented here were to explore VOC profiles during in vivo treatment of refractory AML patients with DC_{leu}-inducing Kits compared to chemotherapy and compared to healthy breath donors' VOC profiles using an eNose. In addition, clinical parameters of refractory AML patients during the course of the disease under chemotherapy and/or Kit M treatment as well as (leukemia specific) immune cell compositions were collected.

Finally, a VOC-based breath profiling strategy was discussed for its further use to augment hematological and immunological profiling strategies.

Material, Methods and Patients

Sample Collection

The blood and breath sample collection for this study was carried out in collaboration with the University Hospitals of Augsburg (P1511, P1482) and the Diakonieklinikum in Stuttgart (P1601).

In accordance with ethical guidelines and the Declaration of Helsinki, patients provided written consent for the experimental use of their blood/breath donations. Whole blood (WB) samples were obtained from patients in refractory phases of Acute Myeloid Leukemia (AML). Ethical approval for this research was obtained from the local Ethics Committee of LMU in Munich (Pettenkofenstr. 8a, 80336 Munich, Ludwig-Maximilian-University Hospital in Munich; Protocol Number 339-05).

Characterization of Healthy Volunteers and Patients

Patient 1511, a 79-year-old Caucasian male diagnosed with AML in December 2015 at the age of 76, demonstrated refractoriness to several lines of therapy. Following his initial AML diagnosis, he underwent 22 cycles of decitabine treatment, subsequently followed by cytarabine and midostaurin therapies. In September 2018, a relapse of the disease was observed, characterized by more than 50% blasts in peripheral blood (PB). This patient was subjected to chemotherapy treatment (without the use of Kit M) and served as a control in this study. Over the entire course of the chemotherapy phase, the patient underwent comprehensive clinical, haematological, and immunological monitoring. Furthermore, periodic blood samples were collected to monitor the population of leukemia-specific immune cells using flow cytometry and cytokine secretion assays (CSA). Clinical and haematological data are given in Figure 1.

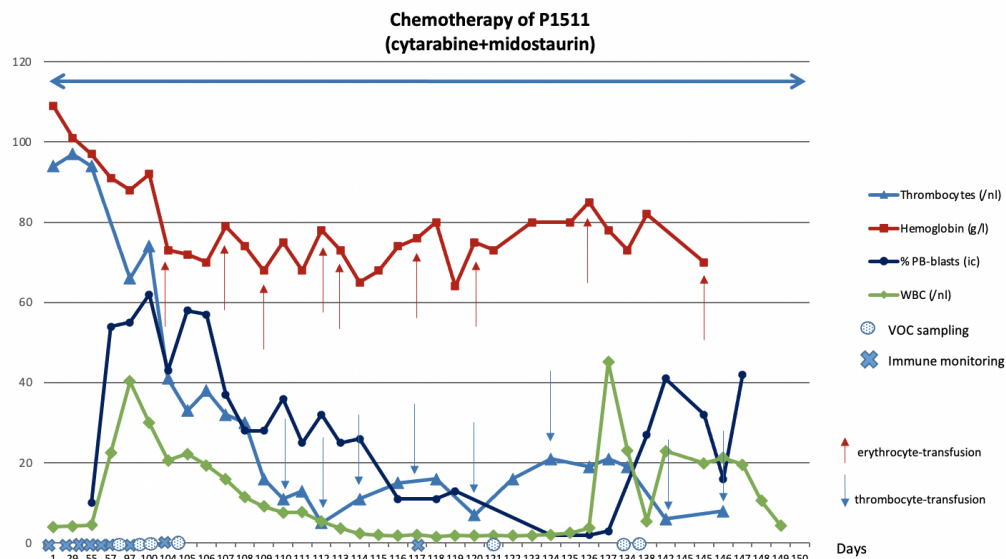


Figure 1: Clinical course of P1511 during chemotherapy treatment (without Kit M). Chemotherapy (cytarabine, midostaurin) was given from the start until the end of observation. Blood cells (thrombocytes, hemoglobin, blasts) in peripheral blood (PB) and leukocytes/white blood cells (WBC) are given. ↑ Timepoints of erythrocyte-transfusion, ↓ timepoints of thrombocyte-transfusion.

Kit M treated patients: P1482 and P1601 presented with pancytopenia and were deemed clinically unfit for further intensive therapy. In the absence of established treatment options, we offered them an individualized salvage treatment involving the systemic administration of blast modulatory drugs. These patients received daily intravenous doses of GM-CSF (Granulocyte-Macrophage Colony-Stimulating Factor) and PGE1 (Prostaglandin E1), referred to as Kit M. The decision to pursue this treatment was made based on recommendations from medical specialists. Before start of treatment both patients had been elaborately informed by experienced haematologists 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. Treatment plans were adapted to individual conditions (Table 2, supplement).

Day	Drug	Dosage (iv)	Schedule
11	GM-CSF	50 mg/m ² /4 hours	8-12 am
	PGE ₁	20 mg (in total)	1-3 pm
12	PGE ₁	20 mg (in total)	8-10 am
	GM-CSF	50 mg/m ² /4 hours	11 am-3 pm
	PGE ₁	20 mg (in total)	8-10 pm
13	PGE ₁	20 mg (in total)	8-10 am
	GM-CSF	50 mg/m ² /4 hours	11 am-3 pm
	PGE ₁	20 mg (in total)	8-10 pm
14	PGE ₁	20 mg (in total)	8-10 am
	GM-CSF	75 mg/m ² /4 hours	11 am-3 pm
	PGE ₁	40 mg (in total)	8-10 pm
15	PGE ₁	40 mg (in total)	8-10 am
	GM-CSF	75 mg/m ² /4 hours	11 am-3 pm
	PGE ₁	40 mg (in total)	8-10 pm
16-38		Percede as day 15	

a) P1482

Day	Drugs	Dosage (iv)	Schedule
9	GM-CSF	50 mg/m ² /4 hours	8-12 am
		(77.5 mg in total)	
	PGE ₁	20 mg (in total)	1-3 pm
10	PGE ₁	20 mg (in total)	8-10 am
	GM-CSF	50 mg/m ² /4 hours	11 am- 3 pm
		(77.5 ug in total)	
	PGE ₁	20 mg (in total)	6-8 pm
11	PGE ₁	20 mg (in total)	8-10 am
	GM-CSF	50 ug/m ² /4 hours	11 am- 3 pm
		(77.5 mg in total)	
	PGE ₁	20 mg (in total)	6-8 pm
12	PGE ₁	20 mg (in total)	8-10 am
	GM-CSF	75 mg/m ² /4 hours	11 am- 3 pm
		(116 mg in total)	
	PGE ₁	40 mg (in total)	6-8 pm

13	PGE ₁	40mg (in total)	8-10 am
	GM-CSF	75 mg/m ² /4 hours	11 am- 3pm
	PGE ₁	40 mg (in total)	6-8 pm
14	PGE ₁	40 mg (in total)	8-10 am
	GM-CSF	75 mg/m ² /4 hours	10 am- 12 pm
	PGE ₁	40 mg (in total)	12- 1 pm
15-26		Percede as day 14	

b) P1601

Table S2: Treatment regime for P1482 and P1601 using Kit M (leukine (GM-CSF) and Prostavasin (PGE1). Courses of the disease are given in Figures 2 and 3.

Patient 1482 a 72-year-old Caucasian male, was initially diagnosed with AML in April 2015. After achieving complete remission through induction chemotherapy, a relapse occurred in May 2017. Clinical assessments, including morphological and cytological evaluations, revealed leucocytosis with 90% peripheral blood (PB) blasts and 70% bone marrow (BM) blasts. To address this relapse, the patient underwent treatment with two cycles of decitabine at a dosage of 20 mg/m² for 11 days, followed by hydroxyurea and cytarabine (100 mg/m²). Although this treatment led to a reduction in blast counts, complete remission was not achieved. Subsequently, the patient received GM-CSF at an initial dose of 50 µg/m², administered intravenously over 4 hours for three days, starting on day 11 of treatment. The GM-CSF dosage was gradually increased to 75 µg/m² from day 14 to day 38. Additionally, the patient received intravenous doses of PGE1 (Alprostadi, Prostavasin®) ranging from 20 to 80 µg daily. PGE₁ was administered over a duration of 2 hours, once or twice daily (Table 2, supplement).

The patient underwent comprehensive clinical, haematological, and immunological monitoring throughout the entire treatment phase. Additionally, blood samples were collected at various intervals during the observation period to assess the population of immune cells specific to leukemia, employing techniques such as flow cytometry and cytokine secretion assays (CSA).

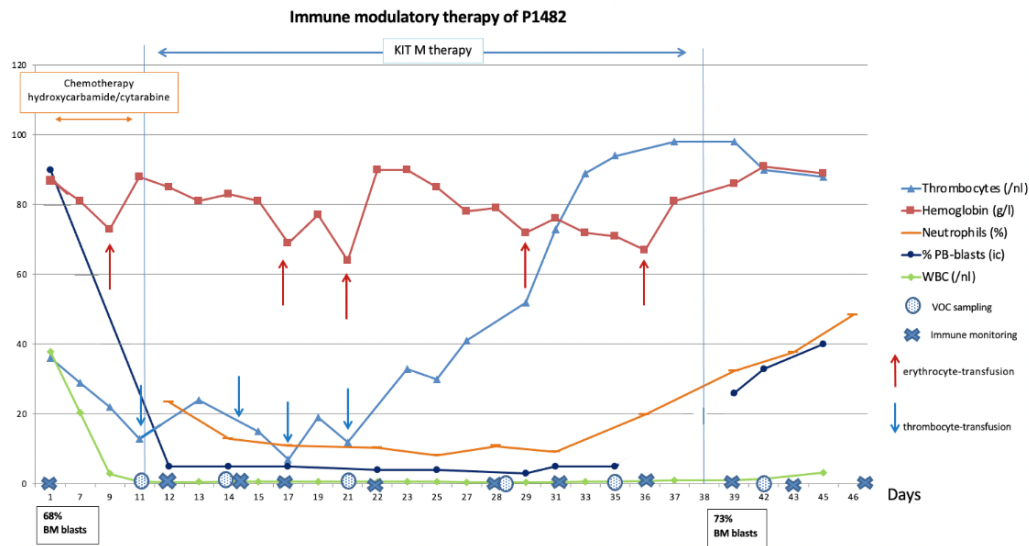


Figure 2: Clinical course of P1482 during chemotherapy or Kit M treatment. Chemotherapy (hydroxycarbamide, cytarabine) was given from the start of observation till day 11. Kit M treatment between day 11 and 38, no treatment from day 38 till the end of observation. Blood cells (thrombocytes, hemoglobin, 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.

Patient 1601: Patient 1601, a 74-year-old Caucasian female, received an AML diagnosis in January 2020. She underwent treatment with azacitidine (75 mg/m² day 1-7) for four cycles and, additionally, Venetoclax (initial dose 400 mg/day) for one cycle. Due to developing pancytopenia, treatment with both medications was discontinued after five cycles. The patient demonstrated refractoriness to this therapy, evidenced by 90% blasts in peripheral blood (PB) and a concurrent bout of pneumonia, aggravated by a history of heavy smoking.

P1601 received GM-CSF at an initial dose of 50 µg/m², administered intravenously over 4 hours for three days, commencing on day 9 and concluding on day 11. This GM-CSF dosage was escalated to

75 µg/m² from day 12 to day 26. Additionally, the patient received intravenous infusions of PGE1 (Alprostadi, Prostavasin®) ranging from 20 to 80 µg daily, administered over 2 hours, either once or twice daily (refer to Table 2 for details). Between day 19 and day 26, the patient underwent additional chemotherapy, receiving 500 mg of hydroxycarbamide twice a day. Following the cessation of Kit M treatment, hydroxycarbamide was continued until day 29.

Throughout the entire treatment phase, the patient underwent comprehensive clinical, haematological, and immunological monitoring. Periodic blood samples were collected during the observation period to assess leukemia-specific immune cells, employing techniques such as flow cytometry and intracellular cytokine assays (InCyt).

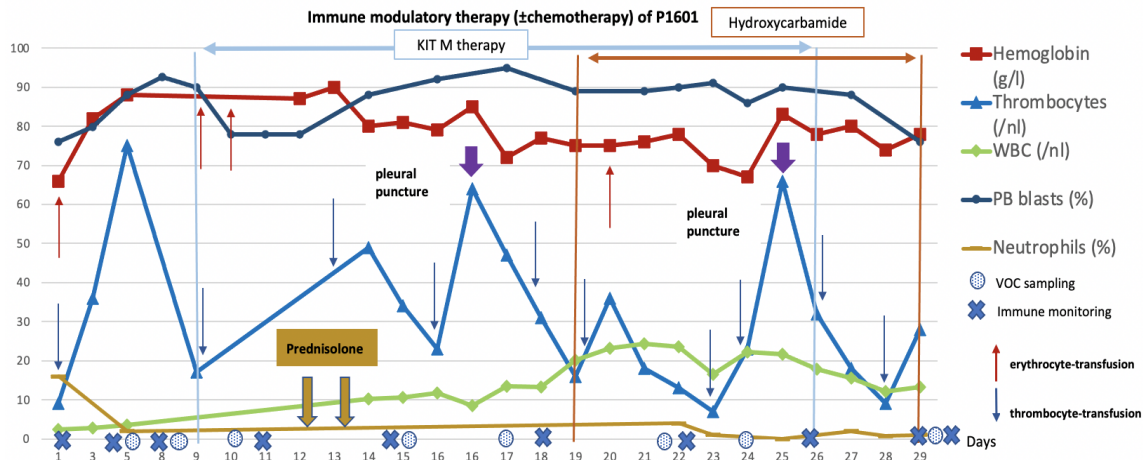


Figure 3: Clinical course of P1601 during Kit M treatment ± chemotherapy. No treatment was given between day 1 and 9, Kit M treatment from day 9-26, chemotherapeutical treatment (hydroxycarbamide) from day 19-29. Patients’ pneumonia was additionally daily treated by prednisolone on day 12-14. Blood cells (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.

Status	Stage	Patient	Age at first diagn	Sex	ELN-risk-stratification at first diagnosis	Clinical treatment	Blast phenotype (CD)	Blasts in PB/BM (%)*	Conducted cell biological experiments	Sources for VOC-measurements in the course of disease
AML	Persisting Relapse	P1511	76	m	Intermediate	chemotherapy (cytarabine+ midostaurin)	34,117, 13,33	54/40	DC, MLC, CTX, CSA	7x masks
AML	Persisting Relapse	P1482	72	m	Unfavourable	chemotherapy (hydroxycarbamid+ cytarabine) and immune therapy (Kit M)	117,34,15, 13, 33,64	90/68	DC, MLC, CTX, CSA	6x masks
AML	Persisting Relapse	P1601	74	f	Unfavourable	chemotherapy (hydroxycarbamide) and immune therapy (Kit M)	34,117, 33,13	76/43	DC, MLC, CTX, InCyt	8x masks
Healthy Stuttgart		HS1	35	f						1x mask
		HS2	31	f						1x mask
		HS3	53	f						1x mask
		HS4	39	m						1x mask
		HS5	35	m						1x mask

Augsburg	HA1	46	m						1x mask
	HA2	33	f						1x mask
	HA3	39	f						1x mask
	HA4	20	m						1x mask
	HA5	32	m						1x mask
	HA6	28	f						1x mask
	HA7	40	f						1x mask
	HA8	23	m						1x mask
	HA9	37	f						1x mask
	HA10	21	f						1x mask
ELN risk stratification risk evaluation based on assessments of EuropeanLeukemiaNet; PB peripheral blood; BM bone marrow; m male; f female; bold: blast markers used for DCleu evaluation; DC dendritic cell culture; MLC mixed lymphocyte culture; CTX cytotoxicity assay; CSA cytokine secretion assay; InCyt intracellular cytokine assay; Details of clinical treatments and hematological values are given in Figure 1,2 and 3; ▯last value before start of chemotherapy (P1511) or Kit M treatment (P1482, P1601).									

Table 3: Characteristics of acute myeloid leukemia (AML) patients and healthy controls.

Cell Characterization by Flow Cytometry

To assess and quantify the phenotypes of DC/DC_{leu}, leukemic blasts, monocytes, and various subsets of immune-reactive cells within the adaptive and innate immune systems (as given in table 1, supplement), flow cytometry analyses were conducted using the FACSCalibur™ fluorescence-activated cell sorting flow cytometer. Employing a refined gating technique and CellQuestPro analysis software (Becton Dickinson, Heidelberg, Germany), the functionalities of cells, including proliferation, cytokine production, and cytotoxicity, were investigated [17, 18].

Preparation of Cells

AML whole blood (WB) samples were either used directly for experiments or subjected to mononuclear cell (MNC) isolation and frozen for future use (all blood sample processing was routinely performed under a hood). MNCs were isolated from WB following standard procedures [17] and used for the isolation of T cells via MACS-microbeads as shown before [36].

Dendritic Cell Culture (DC Culture)

DC/DC_{leu} were cultured following established protocols using 'Kit M' (800 U/ml granulocyte macrophage colony-stimulating factor (GM-CSF, Sanofi-Aventis, Frankfurt, Germany) and 1 µg/ml prostaglandin E1 (PGE1, Santa Cruz Biotechnology, Dallas, Texas, USA)) vs control without added Kit M, as shown before and analyzed by flow cytometry [7,11,22 ,17].

Mixed Lymphocyte Culture (MLC)

After 7-8 days DC cultures containing approximately 2.5x10⁵ DCs and MLC were started adding patients' previously frozen Tcells (1x10⁶) and 5 µl of 50 U/ml interleukin 2 in RPMI medium as

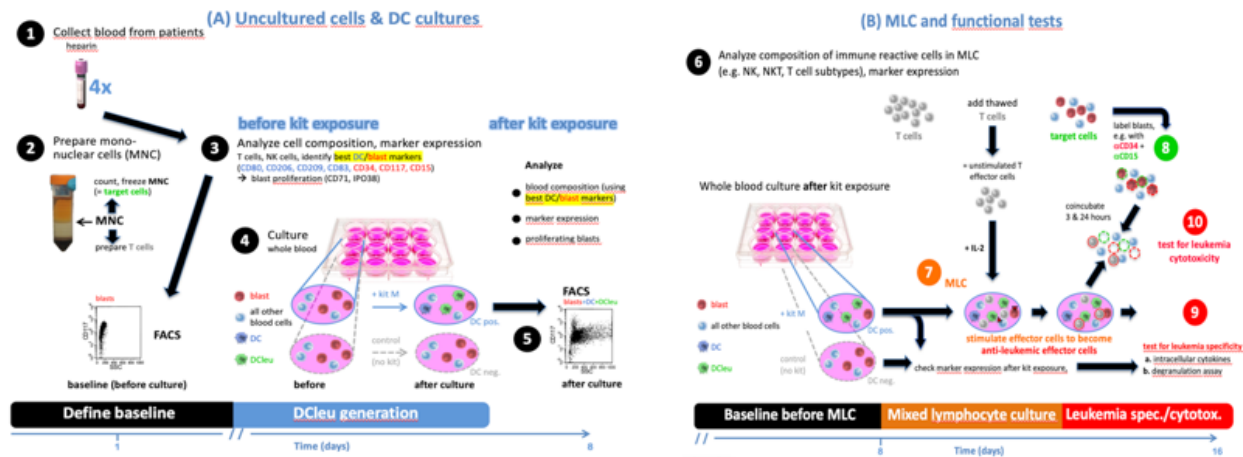
shown before [17]. After culture, measurements were conducted with Kit M (MLC^{WB-DC(Kit-M)}) and Control (MLC^{WB-DC(Control)}), and cells were utilized for the CSA, the InCyt, and the CTX assays [13]. Flow cytometry was employed to quantify different immune cell subtypes after MLC. For a reference to abbreviations, please refer to Table 1, supplement.

Cytokine Secretion Assay (CSA) and Intracellular Assay (InCyt) to detect antigen specific

CSA and InCyt assays were used to quantify intracellularly producing or secreting IFN γ , with or without simultaneously stimulated with two leukemia-associated antigens (LAA): 2 µg/ml Wilms tumour 1 protein (WT-1) and an additional 2 µg/ml PRAME ("preferentially expressed antigen in melanoma," as previously described [17]. CSA and InCyt were carried out to quantify potentially leukemia specific cells after MLC ex vivo or in patients' PB the course of the disease. Subtypes are given in table 1, supplement [17].

Cytotoxicity Fluorolysis Assay (CTX)

To assess the ability of effector cells (T cell-enriched cells, stimulated with or without Kit M-treated whole blood after MLC) to lyse target cells (thawed viable patients' mononuclear cells [MNCs] stained with two different blast markers), the cytotoxicity fluorolysis assay was conducted, as previously described [17]. For each test, equal amounts of effector cells and target cells were combined within a medium. The lytic activity of effector cells was calculated as the percentage of viable target cells in the culture with co-cultured effector and target cells (for 3 hours and 24 hours) compared to the control [13]. A flowchart summarizing the cell biological experiments is provided in Baudrexler et al. [30] and is given in the supplement (Flow chart 1).



Flow Chart S1: ear cells) and T cells were gained from WB and later on used for MLC (‘T cell enriched’ mixed lymphocyte culture) and CTX; 3./4./5. DC cultures were set up with leukemic WB and measured by flowcytometry before and after culture with (vs. without) Kit M; 6./7./8. MLC culture were set up with harvested DC culture + T cells, stimulated with IL-2 and measured by flowcytometry before and after culture; 9./10. Several immune assays were performed after MLC: CSA + InCyt as well as CTX after mixture of MNCs and MLC.

Collection of AML Patients’ and Healthy Donors’ Breath Masks

To obtain samples for volatile organic compound (VOC) analysis, we collected earloop masks containing exhaled air from both AML patients and healthy individuals (Table 3). This breath sampling took place in rooms within the hospitals of Stuttgart and Augsburg to ensure consistent background air quality. We used fleece earloop masks (Henry Schein Medical GmbH, Hamburg, Germany) as a medium for transmission, which were stored in plastic cups. Participants were instructed not to brush their teeth or use any cosmetics, such as perfume or lipstick, within the two hours preceding sampling. During the sampling process, individuals wore unpowdered latex gloves (Meditrade) while breathing through the masks for a duration of five minutes. The collected masks were then placed in plastic cups with lids, stored in zipper bags, and kept in a dark environment at 10°C until they were ready for measurement. All breath samples collected were sent to the University Hospital Marburg, where VOC analyses were conducted [32].

Healthy Donors

We collected one earloop mask from each of the 15 healthy Caucasian volunteers (labelled as ‘Healthy’: n=15). Specifically, we collected 5 masks from healthy donors in Stuttgart (‘Healthy Stuttgart’: n=5) and 10 masks from healthy donors in Augsburg (‘Healthy Augsburg’: n=10). On average, the healthy donors from Stuttgart were 39 years old (with a range of 31-53), and those

from Augsburg were 32 years old (with a range of 20-46). The female-to-male ratio was 1:0.6 in both hospitals. The masks from healthy individuals served as a control group. Characteristics of the healthy donors are summarized in Table 3.

AML-patients’ samples

A total of 7 earloop masks were collected from P1511 during the course of treatment under chemotherapy (n=7). Specific sampling dates are provided in Figure 1.

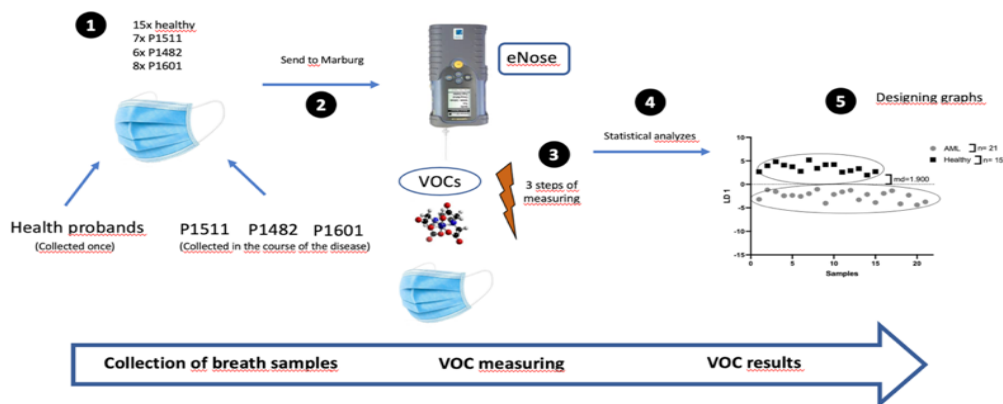
We collected 6 earloop masks during the course of treatment from P1482 under both chemotherapy and Kit M therapy. This included 1 mask before Kit M therapy and 1 mask after Kit M therapy. Additionally, we collected 4 masks during Kit M treatment. Sampling dates are detailed in Figure 2.

In total, we collected 8 earloop masks during the course of disease management from P1601, including Kit M therapy, Kit M therapy with simultaneous chemotherapy, and phases without any treatment. Among these, 3 masks were sampled before and after Kit M/chemotherapy treatment (referred to as ‘without therapy’). Furthermore, 3 masks were collected during Kit M therapy, and 2 masks during Kit M therapy with simultaneous chemotherapy. Sampling dates are provided in Figure 3.

Experimental Set-up of the eNose

Earloop masks containing breath samples were transported to Marburg for measurement and analysed in a sealed room using the Cyranose 320® electronic nose (eNose) [37, 38]. The Cyranose

320[®] is categorized as a polymer sensor-based eNose, featuring 32 thin-film carbon polymer chemiresistors (NoseChip). These sensors operate by detecting volatile organic components (VOCs) based on their structure, size, polarity, and proton affinity. Upon exposure to gases, the polymer layer swells as it absorbs analytes. Each sensor responds differently to various analytes, and no single sensor typically responds to only one analyte. The sensors generate a pattern of signals, referred to as smell-prints, composed of 32 individual signals for single substances or mixtures. The measurements are based on changes in resistance experienced by each sensor when exposed to VOCs. The eNose technology used in this study followed a standardized approach, ensuring that medical air did not affect the measured VOCs. This approach maintained a standard to prevent contamination by ambient air. Before initiating the study, the sensors were tested with defined olfactory substances to ensure their proper functioning. Only when all 32 sensors indicated correct reference ranges could the study proceed. The sensors were also calibrated once a month using a test battery of odours, including liquorice, menthol, and aromatics, among others. Each measurement consisted of three steps: establishing a reference value using medicinal air (Aer medicinalis), measuring the VOCs in the volatile surface of cell supernatants, and cleaning the eNose sensors with ambient air. All samples were measured in triplicate, and the mean values were used for principal component analysis. Subsequently, linear discriminant analyses (LD) were performed, and the LD results were employed for further analyses, especially contingency table analyses. The Mahalanobis distance between groups was determined as well [37, 38]. An overview of the VOC sampling, measurement, and analysis process is provided in Flow Chart 2.



Flow chart 2: Overview about the VOC experiments

1. Collection of breath masks in the course of the disease of AML patients and healthy probands; 2. Transportation to Marburg; 3. Measurement of VOCs above breath masks by eNose; 4./5. Statistical analyzes of VOC data and presentation of VOC differentiations in graphs.

Statistical Methods

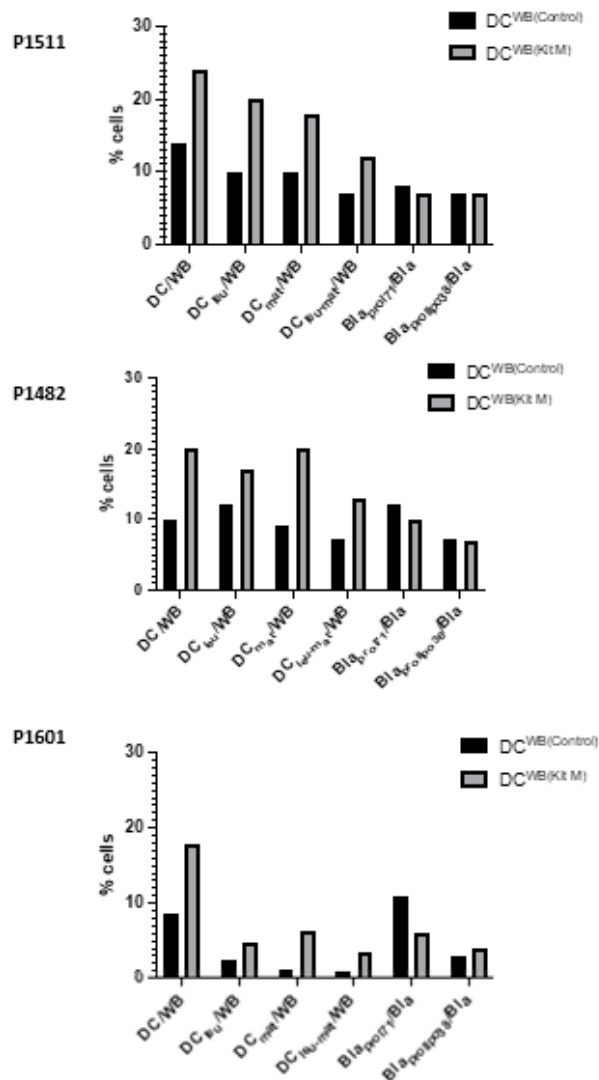
All cell biological measurements were performed using flow cytometry (FACSCalibur[™], Becton Dickinson, Heidelberg, Germany) with BD CellQuestPro software. Statistical analyses were conducted using Microsoft Excel (version 16.52, Redmond, USA) and Prism 9 (GraphPad Software, version 9.1.1, San Diego, USA). Data are presented as mean \pm standard deviation [11]. Statistical investigations of VOC data, calculated by linear discriminant analyses, were conducted using Prism 9 (GraphPad Software, version 9.1.1, San Diego, USA). Principal component analysis (PCA) and linear differential analysis (LDA) were conducted for data modeling and statistical analysis for the VOC results. PCA is an unsupervised multi-variable statistical analysis method for linear data compression, decreasing the data dimension, and feature extraction. This method is used for presenting the response of an olfactory machine to offer qualitative information about emitted organic compounds for pattern recognition [39]. LDA is a supervised classification approach, to find a linear combination of features that characterizes or separates two or more classes of objects [39]. Data from VOC analyses are presented as sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). Fisher's exact test was used for statistical comparisons in contingency table analyses. The Mahalanobis distance (md) between groups was used as a distance measure for multidimensional data. An MD value greater than 1.96 was considered significant, as it corresponds to a p-value of < 0.05 . A MD value greater than 2.58 indicated a p-value of < 0.01 [40]. Significance was considered at p values ≤ 0.05 , and borderline significance was

considered at p values between 0.05 and 0.10.

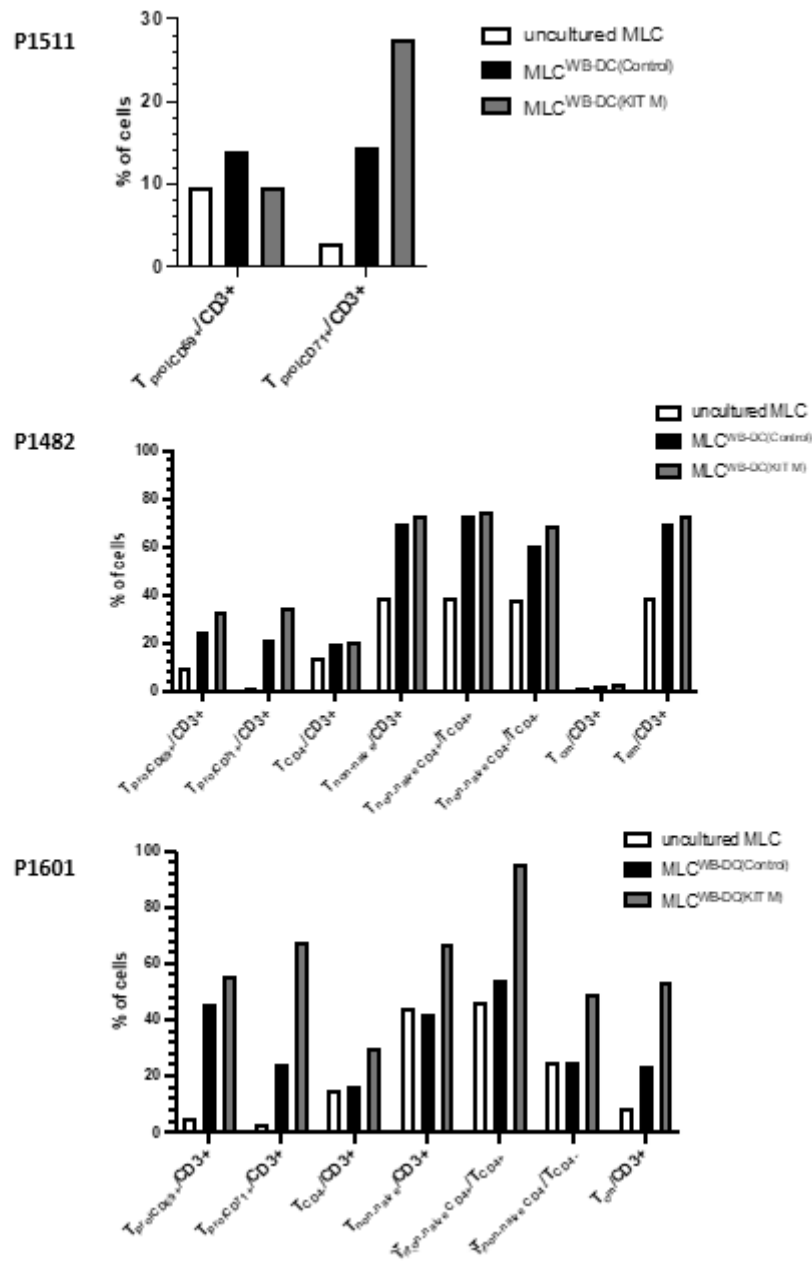
Results

Ex vivo generation of DC- and T cell subtypes with (vs. without) Kit M pretreated WB of AML patients' blood samples and increased antileukemic activity

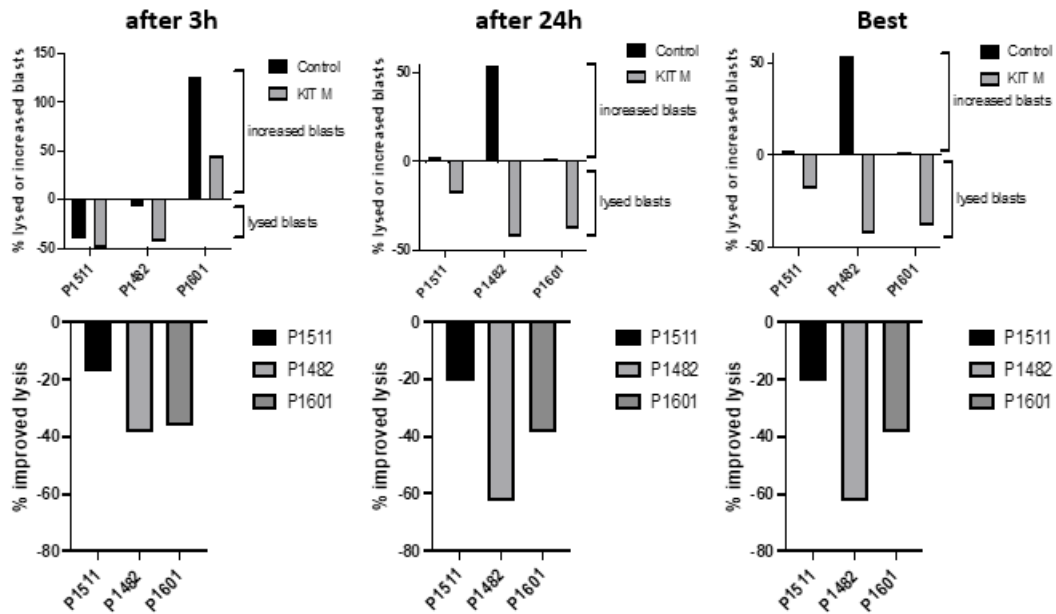
Treating blasts containing leukemic WB with (vs. without) blast modulating Kit M we found higher frequencies of mature leukemia derived DC ($DC_{leu-mat}$) in $DC^{WB(Kit M)}$ compared to $DC^{WB(Control)}$ in all three patients without induction of blasts' proliferation (Figure S4A, supplement). Moreover, we stimulated T cell enriched immunoreactive cells with Kit M pretreated (DC/DC_{leu} containing) WB. Immune reactive cells before (uncultured MLC) and after MLC ($MLC^{WB-DC(Control)}$ or $MLC^{WB-DC(Kit M)}$) showed that T cells were activated and particularly induced to proliferate and to create memory cells in Kit M pretreated settings (Figure S4B, supplement). Abbreviations for cell populations are given in Table 1.



S4A: Increased frequencies of DC/ DC_{leu} subtypes could be generated from (blast containing) AML patients' WB pretreated with Kit M ($DCWB(Kit M)$) compared to Control ($DCWB(Control)$) without induction of blasts' proliferation.



S4B: Stimulation of immunoreactive T cell subtypes could be achieved from patients' WB pretreated with Kit M (MLC^{WB-DC}(Kit M)) compared to Control (MLC^{WB-DC}(Control)) after MLC.



S4C: Stimulatory effect of Kit M treated (vs. untreated) WB on antileukemic reactivity after MLC.

Figure 4: Figure 4A shows the frequencies of generated DC/DC_{leu} (subtypes) after ex vivo treatment of WB with Kit M (DCWB(Kit M)) compared to Control (without Kit treatment, DCWB(Control)) for P1511, P1482 and P1601. Figure 4B shows the frequencies of T cell subsets after stimulation of T cell enriched immunoreactive cells with Kit M pretreated WB (MLCWB-DC(Kit M)) compared to not pretreated WB (MLCWB-DC(Control)) and uncultured cells (uncultured MLC). Figure 4C demonstrates the improved antileukemic blast lytic activity (using a non-radioactive fluorolysis assay) for Kit M pretreated (vs. untreated) patients' WB samples after T cell enriched 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) are given in the upper part. Proportion of improved lysis is given in the lower part. Abbreviations are given in Table 1.

The cytotoxic impact of T cell enriched MLC (with and without pretreatment with Kit M) showed in all 3 cases an improved blast lysis (especially after 24 hours of target-effector cell incubation) after MLC^{WB-DC(Kit M)} compared to MLC^{WB-DC(Control)}, as evaluated via CTX and (Figure S4C, supplement).

We show that Kit M gives rise to antileukemic immune cells ex vivo without induction of blasts' proliferation. We conclude that Kit M application to patients probably is safe and could give rise to antileukemic processes in vivo.

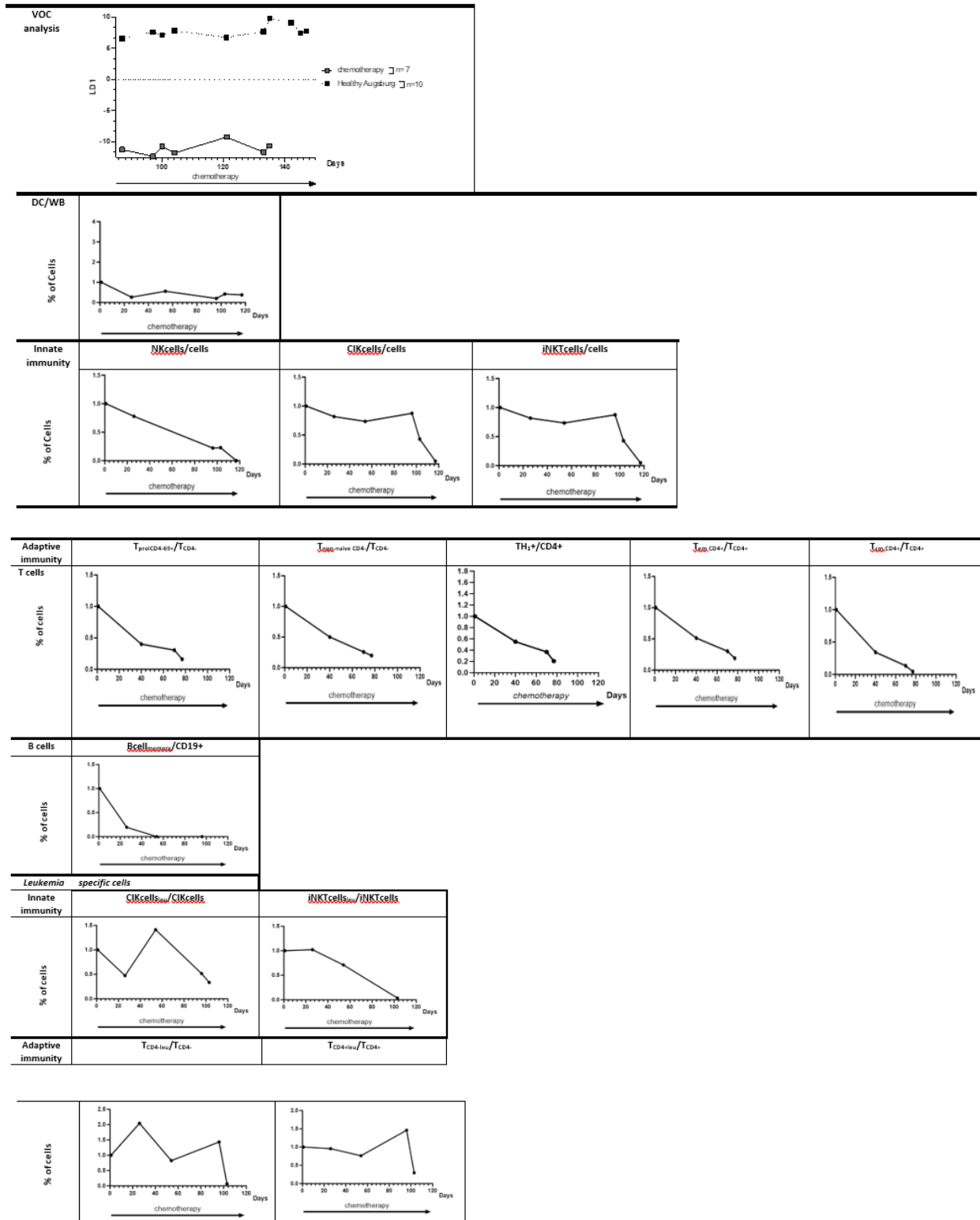
In Vivo Kit M Improved Clinical Courses Of AML Patients Compared To Control-Patient

P1511 (Patient without immunomodulatory treatment):

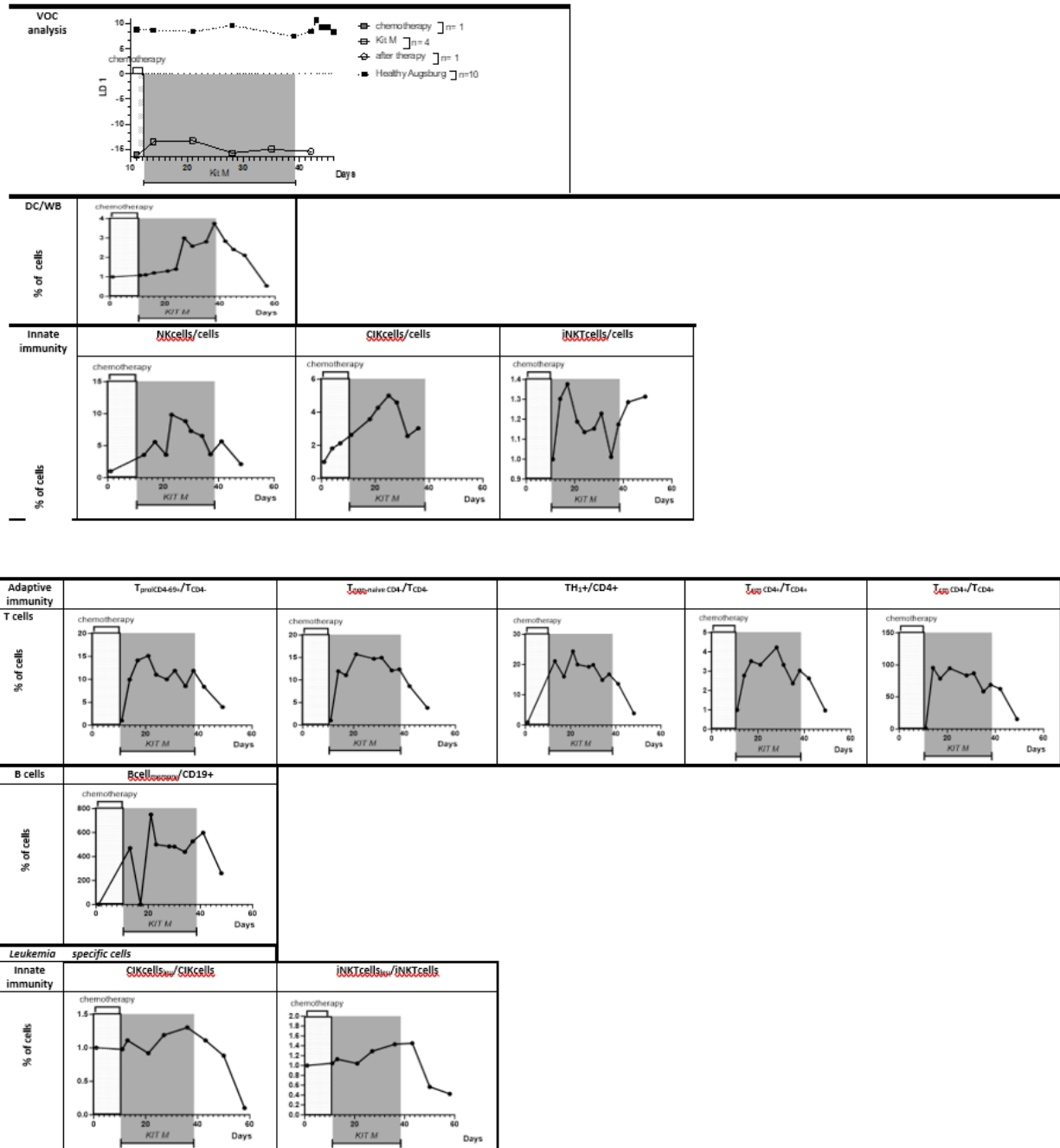
The clinical course of P1511 was observed over a period of 4 months, while he was being treated with chemotherapy (without Kit M). During this time, the evaluated laboratory parameters

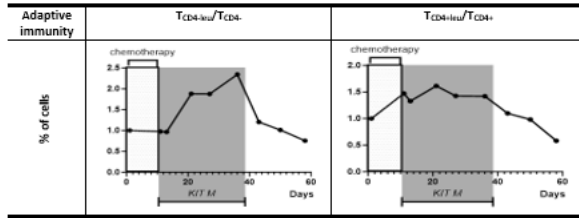
(thrombocytes, hemoglobin, white blood cells) were continuously reduced, and 9 erythrocyte and 8 platelet transfusions were given. In addition, the patient was consistently suffering from high frequencies of PB blasts (in general: >3%; mostly >20%) (Figure 1).

Immune monitoring: During chemotherapy we found a decrease of DCs, of proliferating T cells and T_{non-naive CD4⁺/T_{CD4⁻}. Other adaptive immune cell subtypes (like TH₁⁺/CD4⁺ as well as T_{em CD4⁺/T_{CD4⁺} and Bcell_{memory}/CD19⁺) decreased under chemotherapy. Regarding cells of the innate immune system, frequencies of NK cells decreased, whereas frequencies of CIK cells and iNKT cells, stable over 100 or 60 days, decreased in the further course of observation. Antigen specific cells were monitored after LAA stimulation by CSA: In general, decreasing frequencies of leukemia specific cells of the adaptive and innate lines were found (Figure 5A and 5B lower part, for details see supplement).}}

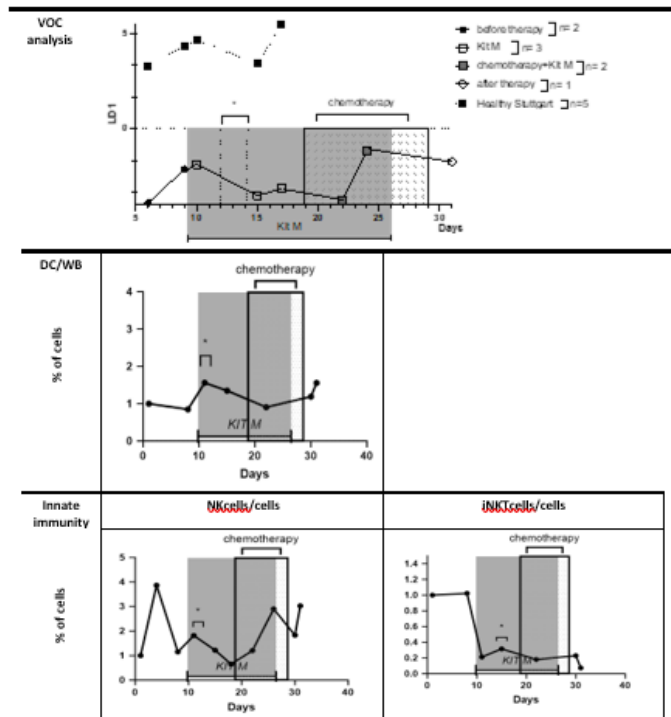


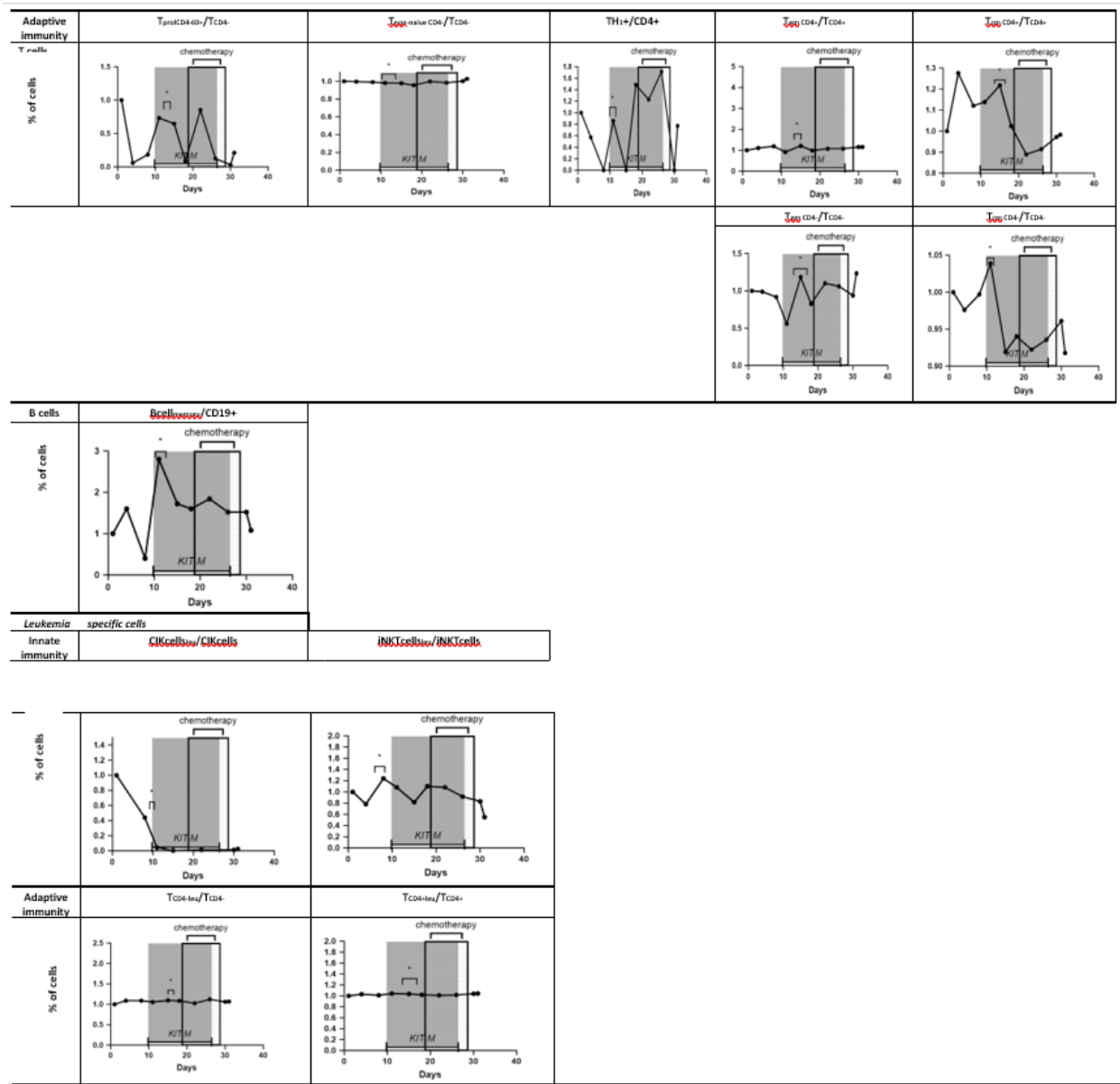
S5A: Profiles obtained from samples from P1511 under chemotherapy vs. healthy controls: healthy and P1511 VOC samples could be clearly differentiated. Decreased frequencies of (leukemia specific) immunoreactive cell subtypes could be detected in the course of disease.





S5B: Profiles obtained from samples from P1482 under treatment with Kit M vs. healthy controls: healthy and P1482 VOC samples could be clearly differentiated and increased frequencies of (leukemia specific) immunoreactive cell subtypes could be detected in the course of disease under Kit M treatment.

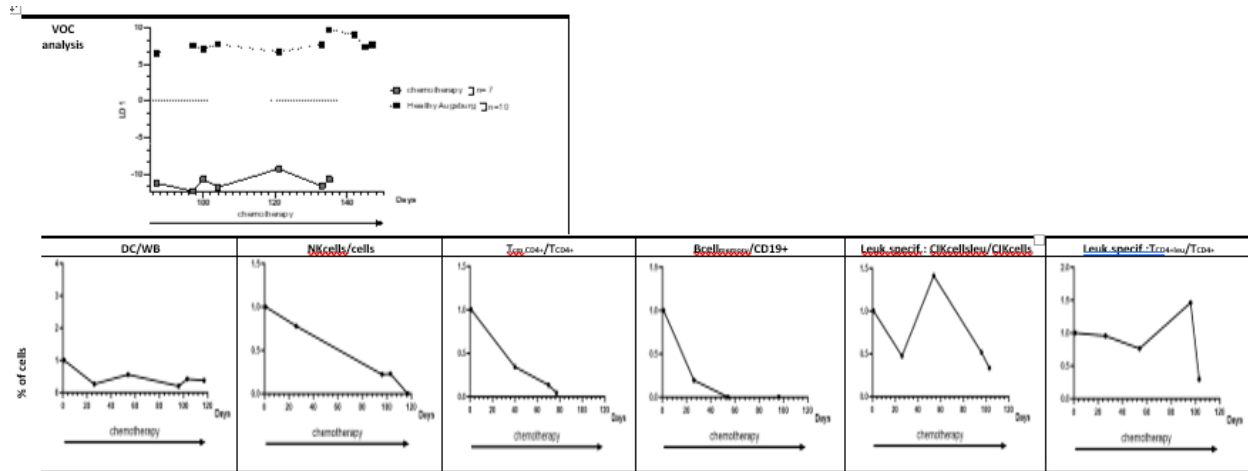




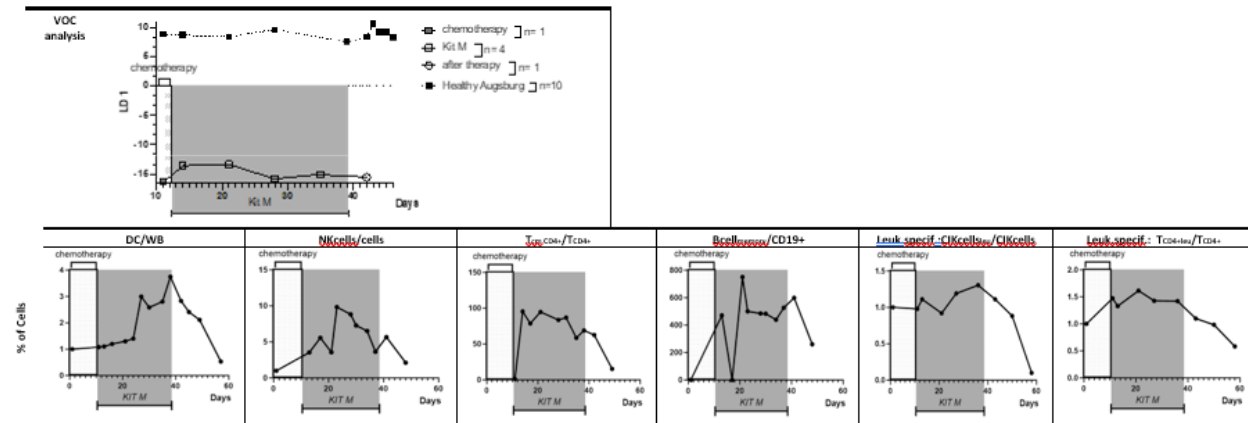
S5C: Profiles obtained from samples from P1601 under treatment with Kit M vs. healthy controls: healthy and P1601 VOC samples could be clearly differentiated and slightly increased frequencies of (leukemia specific) immunoreactive cell subtypes could be detected in the course of disease under Kit M treatment (\pm prednisolone(*))/chemotherapy).

Figure S5: Influences of patients' treatment with chemotherapy and/or Kit M on the provision of (leukemia specific) immunoreactive cells in the course of disease of therapy refractory patients (P1511, P1482, P1601) are given. All values in the course of disease are given as 'fold change' values referred to the value at the beginning of observation. VOC breath samples were collected with earloop masks. Phases under chemotherapy are given in hatched grey, under Kit M therapy in grey and single prednisolone applications as '*'. Details of the clinical courses including treatment phases of all patients are given in Figure 1,2 and 3. Abbreviations of cell subtypes are given in Table 1.

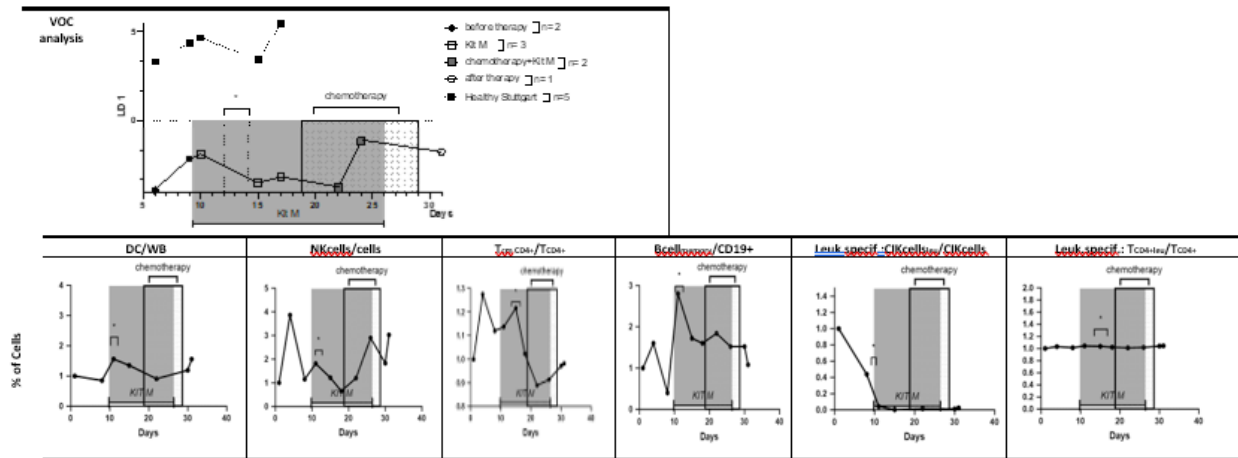
VOC Monitoring: During observation, we compared breath samples collected from healthy probands from Augsburg and from P1511 in the course of the disease. We found clearly differentiated healthy and leukemic VOC results during the whole observation time with stable distances between healthy and leukemic LD values. Nevertheless, we found an approximation of leukemic to healthy VOCs around day 10, 100 and 120 (Figure 5A, upper part).



5A: Profiles obtained from samples from P1511 under chemotherapy vs. healthy controls: healthy and P1511 VOC samples could be clearly differentiated. Decreased frequencies of (leukemia specific) immunoreactive cell subtypes could be detected in the course of disease.



5B: Profiles obtained from samples from P1482 under treatment with Kit M vs. healthy controls: healthy and P1482 VOC samples could be clearly differentiated and increased frequencies of (leukemia specific) immunoreactive cell subtypes could be detected in the course of disease under Kit M treatment.



5C: Profiles obtained from samples from P1601 under treatment with Kit M vs. healthy controls: healthy and P1601 VOC samples could be clearly differentiated and slightly increased frequencies of (leukemia specific) immunoreactive cell subtypes could be detected in the course of disease under Kit M treatment (\pm prednisolone(*))/chemotherapy).

Figure 5: Influences of patients' treatment with chemotherapy and/or Kit M on the provision of (leukemia specific) immunoreactive cells in the course of disease of therapy refractory patients (P1511, P1482, P1601) are given. All values in the course of disease are given as 'fold change' values referred to the value at the beginning of observation. VOC breath samples were collected with earloop masks. Phases under chemotherapy are given in hatched grey, under Kit M therapy in grey and single prednisolone applications as '*'. Details of the clinical courses including treatment phases of all patients are given in Figure 1,2 and 3. Abbreviations of cell subtypes are given in Table 1.

P1482: 11 days before start of Kit M therapy the patient was treated, as a rescue medication, with 2 cycles of decitabine (20 mg/m²), followed by hydroxyurea and cytarabine (100 mg/m²). During pancytopenia he was offered an individual therapy option with Kit M. During observation time the patient was in need for 5 erythrocyte and 4 platelet transfusions. Routine clinical and laboratory parameters showed that Kit M treatment was well tolerated, and the patient improved clinically: Neutrophils in white blood cells (WBC) increased from 10% to 50%, thrombocytes reached 100/nl after 24 days going along with no need for new platelet transfusions, whereas WBC and hemoglobin values (need for new erythrocyte transfusions) stayed low. After 4 weeks of Kit M treatment, 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 suffered with severe sepsis and died few days later (Figure 2).

Immune monitoring: In the course of Kit M treatment a continuous increase of DC, Tpro_{CD4-69+}/T_{CD4+?}, Tnon-naive CD4-/T_{CD4+} as well as T_{em}CD4+/T_{CD4+} and T_{cm}CD4+/T_{CD4+} could be shown. TH_{1+?}CD4+ and Bcell_{memory}/CD19+ increased during the 4-week treatment. Moreover, we found increased frequencies of NK-, CIK- and iNKT cells. Antigen specific cells analyzed after LAA stimulation by CSA showed increasing frequencies of innate and adaptive immune cells during Kit M therapy. We found rising frequencies of T_{CD4-leu}/T_{CD4-}, T_{CD4+leu}/

T_{CD4+?}, CIKcells_{leu}/CIKcells and iNKTcells_{leu}/iNKTcells suggesting an in vivo production of (potentially leukemia-specific) cells.

Immune stimulatory effects decreased after discontinuation of Kit M treatment, although not to the base line before start of therapy (Figure 5B lower part, for details see supplement).

VOC monitoring: During observation, we compared breath samples collected from healthy probands from Augsburg and from P1482 in the course of the disease. We found clearly differentiated healthy and leukemic VOC results during the whole observation time. Nevertheless, we found an approximation of leukemic to healthy VOCs in the beginning of Kit M treatment (day 11-28) (Figure 5B, upper part).

P1601: 8 weeks after treatment with azacytidine and venetoclax a therapy refractory status of the disease (90% blasts in PB) was confirmed, and the patient was offered a treatment with Kit M. During the observation time 4 erythrocytes and 10 platelet-transfusions were necessary. On day 12-14 the patient was treated with (50 g/day) prednisolone and had to undergo two times a pleural puncture (going along with platelet transfusion before puncture at day 16 and 25). Kit M was first applied as continuous infusion on day 9. On day 19 the patient received a simultaneous chemotherapy with 1 g/day hydroxycarbamide to reduce the tumor-load.

Clinically the Kit M treatment was well tolerated without adverse events: Neutrophils in WBC (<4%), hemoglobin and thrombocytes remained low during Kit M and chemotherapy. PB blast counts were constantly high (>75%) but decreased in the course 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 between day 19-29. On day 29 the patient decided to stop all treatments and died with refractory disease on day 31 (Figure 3).

Immune monitoring: In the course of Kit M treatment, we found a slight increase of DCs, proliferating T cells (T_{CD4+} and stable $T_{non-naive\ CD4-}$, T_{CD4+} . Additionally, TH_{1+} , $CD4+$ and $B_{cell\ memory}/CD19+$ increased during Kit M therapy. Whereas T_{cm} subtypes decreased, and T_{cm} subtypes stayed on a stable line. Regarding cells of the innate immunity, stable frequencies of NK cells and decreased frequencies of iNKT cells were seen during Kit M therapy. Leukemia specific cells, detected by intracellular production of INF γ after LAA stimulation using the InCyt showed stable frequencies of $T_{CD4-leu}/T_{CD4-}$ and $T_{CD4+leu}/TCD4+$ and decreased frequencies of CIK cells. Frequencies of iNKT cells, proliferating T cells and B-memory-cells slightly increased during Kit M therapy but decreased under chemotherapy. Interestingly, prednisolone had an immunosuppressive effect on frequencies of DCs, NK cells, proliferating T, TH_{1+} , central memory T cell subtypes, B memory cells as well as INF γ producing iNKT and CIK cells (day 12-14) (Figure 5C, for details see supplement).

VOC Monitoring: During observation, we compared breath samples collected from healthy probands from Stuttgart and from P1601 in the course of the disease. We found clearly differentiated healthy and leukemic VOC results during the whole observation time. Nevertheless, we found an approximation of leukemic to healthy VOCs in the beginning of Kit M treatment (around day 10) that flattened out by application of prednisolone (day 12-14).

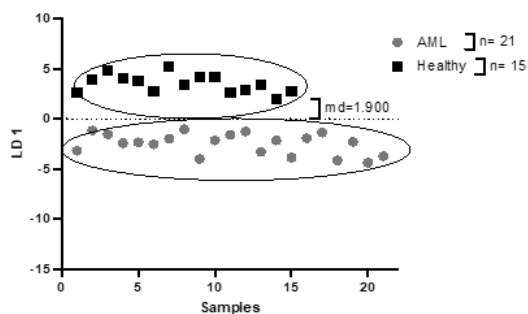
Leukemic VOCs showed an approximation to healthy VOCs in the beginning of supportive chemotherapy (day 22-24) (Figure 5C, upper part).

VOC comparisons

As a source for VOC analyzes conducted by eNose, we used healthy and leukemic breath samples collected by earloop masks. An overview about the collected breath samples of every proband is given in Figure 6. Various comparisons were analyzed by linear discriminant analyses (LD) and the smell-prints were graphically shown in two-dimensional principal component analysis (PCA) plots. Sensitivity, specificity, Mahalanobis distance (md), negative and positive predictive values as well as the p-value calculated by Fishers' exact test are given.

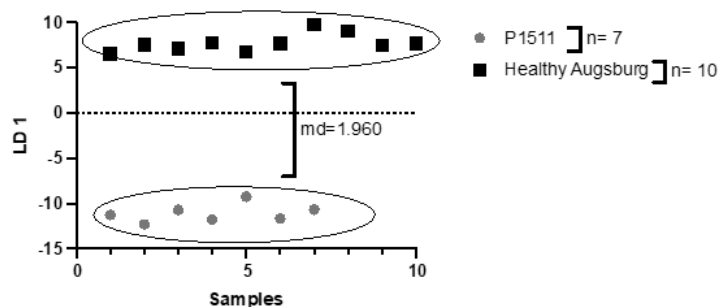
Significant differences between healthy and AML patients' breath samples

The eNoses showed a significant differentiation between 21 leukemic-collected from 3 AML patients in the course of the disease- and 15 healthy breath samples (%sensitivity: 100; %specificity: 100; $p=.0001$). To compare AML patients' breath samples with healthy probands' under comparable hospital conditions (e.g., comparable background smell) we analyzed these samples in the course of the disease of P1511 (patient from Augsburg) with 10 healthy probands' breath samples from Augsburg. We found significantly different VOC results (%sensitivity: 100; %specificity: 100; $p=.0001$). Comparing 10 healthy breath samples from Augsburg with breath samples from P1482 (patient from Augsburg) the eNose showed significant differences (%sensitivity: 100; %specificity: 100; $p=.0001$). Comparable results could be found in healthy ($n=5$) and leukemic (P1601 in the course of the disease) breath samples from Stuttgart (%sensitivity: 100; %specificity: 100; $p=.0008$). Detailed information about VOC results and a graphical overview are given in Figure 6.



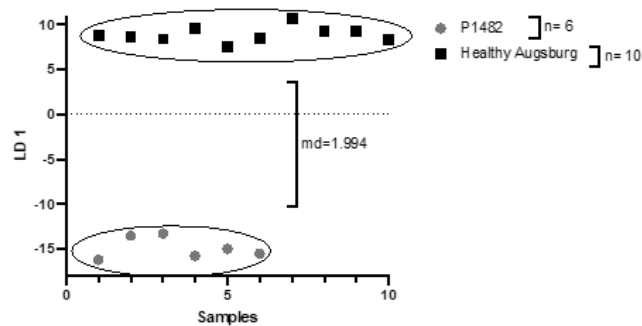
	AML P1511, P1482, P1601	Healthy Augsburg, Stuttgart	PPV ³ (%)	NPV ⁴ (%)	p ⁵
	Correct/false (%) ¹	Correct/false (%) ²			
eNose results	21/0 (100)	15/0 (100)	100	100	.0001

6.1 VOC profiles of healthy and AML patients' masks were significantly different



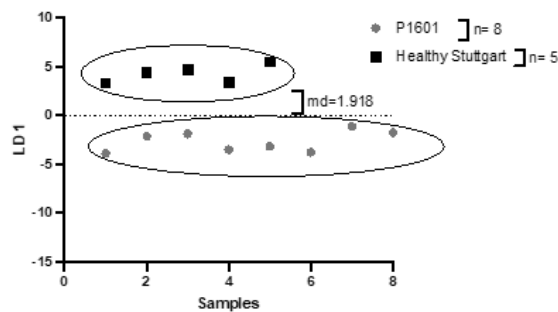
	AML P1511	Healthy Augsburg	PPV ³ (%)	NPV ⁴ (%)	p ⁵
	Correct/false (%) ¹	Correct/false (%) ²			
eNose results	7/0 (100)	10/0 (100)	100	100	.0001

6.1 VOC profiles of healthy and AML patients' masks were significantly different



	AML P1482	Healthy Augsburg			
	Correct/false (% ¹)	Correct/false (% ²)	PPV ³ (%)	NPV ⁴ (%)	P ⁵
eNose results	6/0 (100)	10/0 (100)	100	100	.0001

6.3 VOC profiles of P1482 and healthy probands in Augsburg were significantly different



	AML P1601	Healthy Stuttgart			
	Correct/false (% ¹)	Correct/false (% ²)	PPV ³ (%)	NPV ⁴ (%)	P ⁵
eNose results	8/0 (100)	5/0 (100)	100	100	.0008

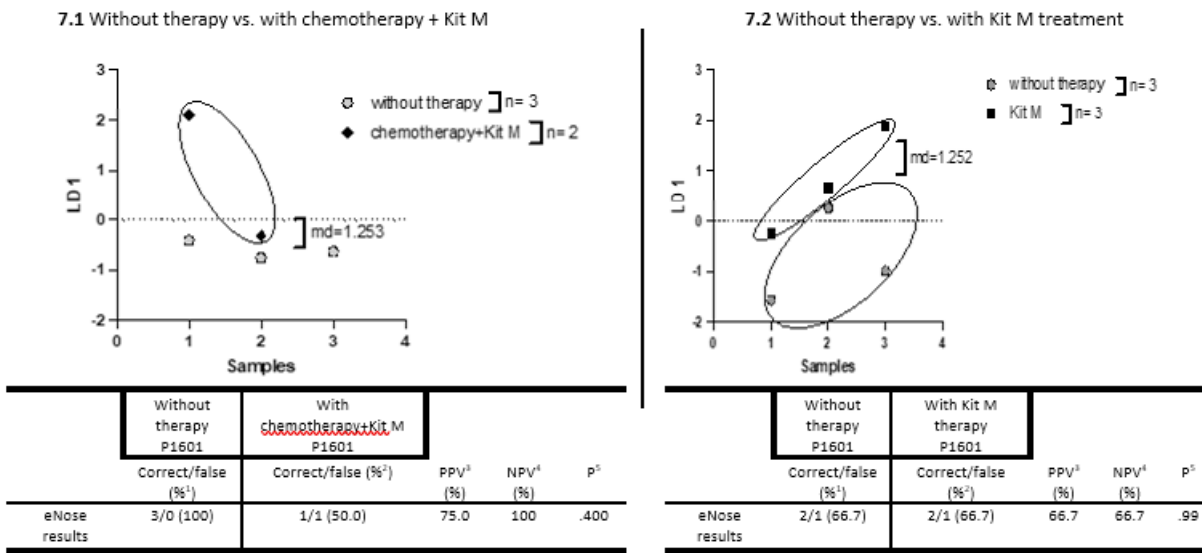
6.4 VOC profiles of P1601 and healthy probands in Stuttgart were significantly different

- 1 Sensitivity.
- 2 Specificity.
- 3 positive predictive value.
- 4 negative predictive value.
- 5 p values calculated with Fisher's exact test.
- 6 md, Mahalanobis distance

Figure 6: Breath samples were collected from healthy donors (n=10 from Augsburg, n=5 from Stuttgart) and from 3 AML patients (P1511 and P1482 from Augsburg, P1601 from Stuttgart) in the course of disease (irrespective of applied therapies) using earloop masks collecting exhaled breath for 5 minutes. VOC analyses were conducted using an eNose. Differences in VOC profiles were calculated by linear discriminant (LD-) analyses (dimensionless LD 1 values). Differences in VOC profiles between AML patients and their respective healthy donors' sample are given. Tables underneath giving an overview about the sensitivity, specificity, negative and positive predictive value. Statistical tests were performed using Fisher's exact test. Differences are considered as borderline significant with p values $\leq .1$ and significant with p values $\leq .05$.

Influence of Kit M ± chemotherapy on VOC profiles in P1601

To understand the influence of Kit M with or without simultaneous chemotherapy we compared VOCs of P1601s' breath samples. The eNose could (not significantly) differentiate breath samples before and after therapy (=no therapy; n=3) from breath samples taken during chemotherapy and simultaneous Kit M treatment (n=2) (%sensitivity: 100; %specificity: 50; p=.4). Moreover, the eNose could (not significantly) differentiate breath samples under no therapy from samples collected during Kit M treatment (%sensitivity: 66.7; %specificity: 66.7; p=.99). An overview about VOC results and treatment periods are given in Figure 7 and 3.



- 1 Sensitivity.
- 2 Specificity.
- 3 positive predictive value.
- 4 negative predictive value.
- 5 p values calculated with Fisher's exact test.
- 6 md, Mahalanobis distance.

Figure 7: Breath samples were collected from P1601 in course of disease using earloop masks collecting exhaled breath for 5 minutes. VOC analyses were conducted using an eNose. Differences in VOC profiles were calculated by linear discriminant (LD-) analyses (dimensionless LD 1 values). Differences in VOC profiles collected under chemotherapy ± Kit M therapy or without therapy were evaluated. Tables underneath giving an overview about the sensitivity, specificity, negative and positive predictive value. Statistical tests were performed using Fisher's exact test. Differences are considered as borderline significant with p values ≤.1 and significant with p values ≤.05.

Significantly different VOC results during chemotherapy compared to Kit M treatment

Using breath samples from P1511 under chemotherapy and from P1482 and P1601 under Kit M treatment, we could significantly distinguish these two therapy options by an eNose using VOC profiles (%sensitivity: 100; %specificity: 100; p=.0006). Detailed information is given in Figure 8A.

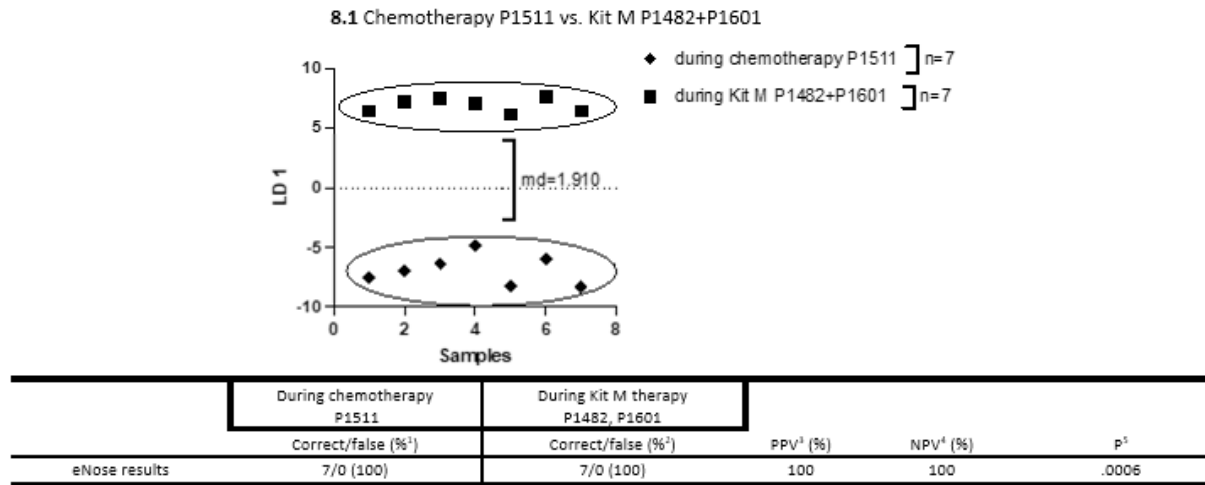


Figure 8A: Breath samples from 3 AML patients collected under chemotherapy and under Kit M therapy were significantly different.

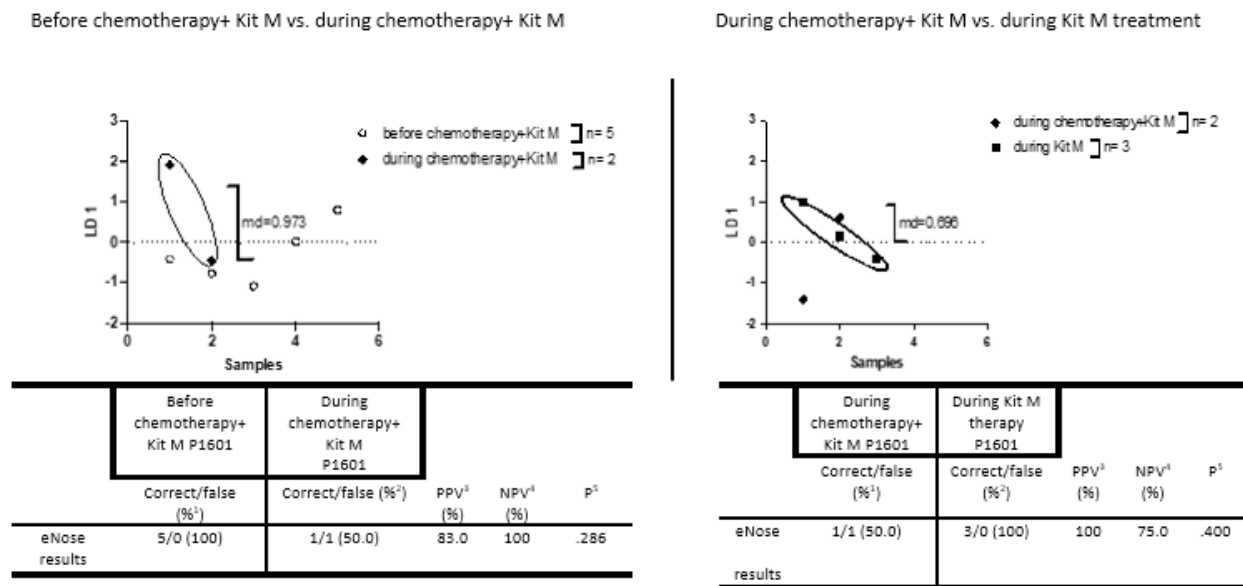


Figure 8B: Breath samples from P1601 collected before simultaneous therapy, under chemotherapy+ Kit M therapy and under Kit M therapy were different.

- 1 Sensitivity.
- 2 Specificity.
- 3 positive predictive value.
- 4 negative predictive value.
- 5 p values calculated with Fisher's exact test.
- 6 md, Mahalanobis distance

Figure 8: Breath samples were collected from 3 AML patients (P1511 and P1482 from Augsburg, P1601 from Stuttgart) in the course

of disease (respective of applied therapies) using earloop masks collecting exhaled breath for 5 minutes. VOC analyses were conducted using an eNose. Differences in VOC profiles were calculated by linear discriminant (LD-) analyses (dimensionless LD 1 values). Figure 8A shows significant differences in VOC profiles collected under chemotherapy from P1511 compared to under Kit M therapy from P1482 and P1601. Figure 8B shows differences in VOC profiles collected from P1601 before simultaneous therapy compared to under chemotherapy+ Kit M therapy and shows differences in VOC profiles collected from P1601 under chemotherapy+ Kit M therapy compared to under Kit M therapy. Tables underneath giving an overview about the sensitivity, specificity, negative and positive predictive value. Statistical tests were performed using Fisher's exact test. Differences are considered as borderline significant with p values ≤ 1 and significant with p values ≤ 0.05 .

Different breath samples from P1601 collected before therapy, under combined (chemo- and Kit M) therapy and under Kit M therapy

We found different VOC profiles in breath samples from P1601 before simultaneous therapy (Kit M and chemotherapy) compared to during chemotherapy and simultaneous Kit M therapy (%sensitivity: 100; %specificity: 50; $p=.286$). Furthermore, we found different VOC profiles in breath samples during chemotherapy and simultaneous Kit M treatment compared to under Kit M therapy (%sensitivity: 50; %specificity: 100; $p=.4$). Graphs and more details are shown in Figure 8B and 3.

In summary, these findings suggest that the eNose can effectively differentiate between healthy individuals and AML patients based on their breath samples. Additionally, it indicates that the eNose can detect differences in VOC profiles between breath samples taken with and without therapy (Kit M/chemotherapy). Furthermore, it demonstrates the eNoses' ability to distinguish between breath samples collected during chemotherapy and those collected under Kit M therapy.

Discussion

After induction therapy the relapse rate of AML patients is about 70-80% [6, 41, 42]. Therefore, there is a great need for new (non) immunotherapeutic options [43].

DC/DC_{leu} Based Immunotherapy

DC/DC_{leu} can be generated ex vivo from monocytes (loaded with LAA) or leukemic blasts and have the advantage of already containing the patients' specific leukemic antigens needed for an appropriate immunological answer and therefore bypassing the need of antigen-loading [13]. We and others could prove that DC/DC_{leu} can be generated ex vivo from patients' blast containing WB independent of patients' sex, age, MHC, leukemic subtype, or risk profile [12, 13, 17, 30, 44]. DC vaccinations were shown to induce leukemia specific immune responses and to improve the outcome of patients [7, 11, 45].

Ex vivo generation of DC/DC_{leu} with Kit M pretreated patients' WB resulting in increased antileukemic activity

We can confirm preliminary data [13, 14, 17, 18, 30, 44], that DC/

DC_{leu} could be generated ex vivo in Kit M pretreated (compared to not pretreated) AML patients' WB (P1511, P1482, P1601) without inducing blast proliferation. In addition, blast-lytic activity was shown to be regularly improved using Kit M pretreated WB samples as 'stimulator cells' in MLC [13, 14, 17, 18, 22, 30, 46] (Figure 4A-C). These findings prompted us to deduce a Kit M-based treatment protocol for P1482 and P1601: Our data show, that treatment of (therapy refractory) patients (P1482, P1601) with Kit M stabilized or even reduced blast counts in PB, increased thrombocyte counts (P1482), compared to a patient without Kit M treatment (P1511) (Figure 1-3). Even more we could show a (leukemia-specific) activation of cells of the innate and adaptive immune system in P1482 and in part in P1601, but not in P1511 (Figure 5A-C). These data could point to antileukemic effects after Kit M treatment (induced by blast-conversion to DC_{leu}, leading to leukemia specific immune effects as shown before ex vivo [6, 13, 14, 17]. These effects were abrogated by infusion of (immune suppressive) prednisolone (to treat pneumonia) in P1601. Under that influence of prednisolone these effects were diminished. This could point to an effect of cortisone on the exhalation of VOCs (Figure 5A-C). In vivo immunostimulant effects might be more efficient in cases with low blast counts/in remission and might contribute to stabilize disease or maintain remissions, as discussed by us before [13, 17].

VOC Profiling to Differentiate Healthy and AML Patients' Breath

It is well known that age, gender as well as smoking behavior, infections (e.g: pneumonia) or in the presence of impaired/damaged tissue (e.g: COPD) does not disturb the differentiation of malignant vs. non-malignant breath profiles/breath donors [32, 34, 47]. Our data show, that earloop masks qualify as carriers to be used for VOC profiling, as already shown before in patients with lung cancer, COPD vs. healthy donors [32]. There is no need to breath directly in an eNose. We have already demonstrated that a VOC profiling can differentiate between malignant (lung cancer) and nonmalignant breath donors [32, 34]. It has already been investigated that volatile samples above urine samples (to detect bladder tumor [48]) or above serum supernatants from diseased patients (to detect AML [30]) can be clearly separated from healthy samples. Moreover, we can add, that breath samples from

healthy breath donors could not only be clearly differentiated from breath samples from lung cancer, COPD [34] or Alzheimer's and Parkinson's disease [29, 40], but also from breath samples from AML patients (independent of disease, stage and treatment), as we could show here (Figure 6).

A clear differentiation of AML patients (treated by chemotherapy or Kit M or chemotherapy combined with Kit M or without any treatment) from healthy breath samples was possible- thereby pointing to volatile profiles (differentiating AML and healthy breath) being in general independent from applied therapies. This differentiation between healthy and AML samples was significantly different and independent of different institutions, where the healthy and AML breath samples were collected (Figure 6.2 and 6.3 in Hospital in Augsburg, Figure 6.4 in Hospital in Stuttgart). An interesting finding was, that differences between healthy and leukemic breath were lower soon after application of Kit M. This was true for P1482 (Figure 5B) and P1601 (Figure 5C). This might point to a positive influence of immune modulation on disease activity-shifting AML patients' breath closer to healthy breath patterns.

We moreover found clear (although due to low sample numbers not significant) differences between breath samples from AML patients without any therapy vs. with chemotherapy combined with Kit M or vs. Kit M alone (Figure 7). The same was true for samples taken before vs. during applied chemotherapy combined with Kit M (Figure 8B). The most impressive and highly significant differences were seen comparing breath samples during chemotherapy vs. during Kit M treatment (Figure 8A).

This means, that breath profiling in AML patients not only allows a clear differentiation between healthy and leukemic breath samples but can also differentiate between breath obtained under chemotherapy vs. during immune modulating therapy.

For the future use of eNose-based breath profiling, it should be examined whether breath samples from AML patients in different phases of remission (e.g. early remission, long-term remission, remission shortly before relapse) may allow conclusions to be drawn about a change of VOC profiles to monitor the quality or the stability of a remission. These results could contribute to detect imminent relapses early.

Colombo et al. examined cytokines in condensed exhaled breath and could correlate immune reactions with exhaled air profiles in these patients [49]. Moreover, our group could show, that an eNose can differentiate VOCs in healthy vs. leukemic DC- and Mixed lymphocyte culture supernatants [30].

Here we confirm, that immune- and VOC-profiling in the course of patients' diseases is possible and allows the detection of quantitative changes of immune cells as well as of volatile markers-independent of smoking behavior and AML subtype. For

the future we therefore recommend correlating immune with VOC profiles to contribute to a refined monitoring to estimate the quality of remissions.

We conclude, that systemic effects in healthy vs. AML PB might be not only monitored on the cellular side (by monitoring cell subsets [50]), on the soluble side (by monitoring chemokines/cytokines/extracellular vesicles (EVs) [24]30,52), but also on the volatile side (ex vivo) [30] Table or directly using breath samples collected with earloop masks from patients. Dysregulated release of pro- and anti-inflammatory cytokines/EVs in AML might influence anti- or pro-leukemic mechanism of the immune system [51]. In addition, EVs released by immune or tumor cells- detectable in serum from patients with leukemia- might contribute to a refined monitoring [28, 52]. These EVs might lead to increased release of cytokines and going along with different VOCs in exhaled air [53, 54].

Conclusion

VOCs can be collected on earloop masks and allow a differentiation of healthy and leukemic breath donors. Moreover, breath samples in different phases of the disease or under (chemo- or immune modulatory) therapy can be differentiated. These findings could contribute to deduce a refined VOC based monitoring strategy (optional in conjunction with immune monitoring), that allows a monitoring of the efficacy of applied therapies, of the quality of achieved responses/remissions to (immune)therapies or might contribute to detect imminent relapses on a volatile basis.

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Ethics: Sample collection was conducted after obtaining written informed consent of the blood donor and in accordance with the World Medical Association Declaration of Helsinki and the ethic committee of the Ludwig-Maximilian-University Munich (vote no. 339-05).

Conflict of Interests: Modiblast Pharma GmbH (Oberhaching, Germany) holds the European Patent 15 801 987.7-1118 and US Patent 15-517627 'Use of immunomodulatory effective compositions for the immunotherapeutic treatment of patients suffering from myeloid leukemias', with whom Schmetzer H. is involved with.

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