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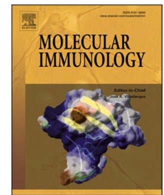

Dendritic/antigen presenting cell mediated provision of T-cell receptor gamma delta (TCRγδ) expressing cells contributes to improving antileukemic reactions ex vivo

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ABSTRACT

T-cell receptor gamma delta (TCRγδ) expressing T-cells are known to mediate an MHC-independent immune response and could therefore qualify for immune therapies. We examined the influence of dendritic cells(DC)/antigen presenting cell (APC) generated from blast-containing whole blood (WB) samples from AML and MDS patients on the provision of (leukemia-specific) TCRγδ expressing T-cells after mixed lymphocyte culture (MLC). Kit-M (granulocyte-macrophage colony-stimulating factor (GM-CSF) + prostaglandin E1 (PGE1)) or Kit-I (GM-CSF + Picibanil) were used to generate leukemia derived APC/DC (DC_{leu})from WB, which were subsequently used to stimulate T-cell enriched MLC. Immune cell composition and functionality were analysed using degranulation- (DEG), intracellular cytokine- (INTCYT) and cytotoxicity fluorolysis- (CTX) assays. Flow cytometry was used for cell quantification. We found increased frequencies of APCs/DCs and their subtypes after Kit-treatment of healthy and patients ́WB compared to control, as well as an increased stimulation and activation of several types of immune reactive cells after MLC. Higher frequencies of TCRγδ expressing leukemia-specific degranulation and intracellularly cytokine producing T-cells were found. The effect of Kit-M-treatment on frequencies of TCRγδ expressing cells and their degranulation could be correlated with the Kit-M-mediated blast lysis compared to control. We also found higher frequencies of TCRγδ expressing T-cells in AML patients ́samples with an achieved remission (compared to blast persistence) after induction chemotherapy. This might point to APC/DC-mediated effects resulting in the provision of leukemia-specific TCRγδ expressing T-cells: Moreover a quantification of TCRγδ expressing T-cells might contribute to predict prognosis of AML/MDS patients.

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1. Introduction

1.1. *Current therapy strategies for myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML)*

Risk adapted standard strategies for AML/ high-grade MDS result in impressive remission rates of 66–90% after 1–2 therapy cycles in AML. However, within two years, ensue in relapses in up to 80% of cases (Garcia-Manero et al., 2020, Estey, 2020). For patients with less tolerance for the induction therapy low-dose cytarabine or hypomethylating agents + venetoclax are potential therapy strategies (Dinardo et al., 2020). However, treatment options are limited and prognosis unfavorable for patients with relapsed or refractory disease (Thol et al., 2024). There is a high demand for development and adaption of immuno- and chemotherapies to stabilize remissions (or progredient disease) in AML/MDS cases, e.g. therapies utilizing specific chimeric antigen receptor (CAR)-T-cells, tyrosine kinase inhibitors, bispecific antibodies (BiTEs) and antigen-presenting cells (APCs) such as dendritic cells (DCs) (Estey, 2020, Garcia-Manero et al., 2020).

1.2. *The potential of DCs as APCs for immune therapies*

Hematopoietic stem cells and monocytes are able to differentiate into DCs as the result of complex maturation and activation processes. The differentiation processes involve upregulation of various receptors, such as cell adhesion receptors or chemokine receptors (e.g. CCR7), MHC-antigens and a variety of costimulatory factors (Behrends et al., 2016, Amberger et al., 2019, Amberger and Schmetzer, 2020).

DCs can be generated ex vivo: as monocyte-derived DC loaded with tumor antigens (Amberger and Schmetzer, 2020) or leukemia-derived DC (DC_{leu}) generated from myeloid blasts (presenting the patients׳ individual tumor antigens), which can be generated in cultures and adoptively transferred to patients. Combining designated response modifiers into Kits can generate APCs/DC/DC_{leu} from cells in whole blood (WB) (e.g. Kit-M: GM-CSF (granulocyte macrophage colony stimulating factor) + PGE1 (prostaglandin E1) or Kit-I: GM-CSF+Picibanil) (Amberger and Schmetzer, 2020, Ansprenger et al., 2020, Kremser et al., 2010). This APC/DC generation was shown to be achieved without an induction of blast-proliferation (blasts co-expressing CD71 ($Bla_{prol\text{-}CD71}$; transferrin receptor) or IPO38 ($Bla_{prol\text{-}IPO38}$; intracellular marker for proliferation); see Table 1) (Amberger et al., 2019, Grabrucker et al., 2010, Schmetzer et al., 2007, Plett et al., 2022). Moreover we could already show, that Kit-treated patients' WB lead to improved leukemia specific/antileukemic activity after MLC (Klauer et al., 2022; Rackl et al., 2022; Schutti et al., 2024).

Mode of action of these response modifier mediated APC/DC generation is given in Supplementary Figure 1: two combined response modifiers (GM-CSF+PGE1 (Kit M) or GM-CSF+Picibanil) are crucial to convert leukemic cells into DC_{leu}: The myeloid differentiation factor fosters the creation of DC progenitors (and in vivo hematological recovery) (Amberger et al., 2019, Schmetzer et al., 2022, Yu et al., 2022): The second factor (either PGE1 or Picibanil) mediate a danger signal as well as maturation of DCs/DC_{leu}s (Schwepcke et al., 2022). Both signals trigger the reprogramming of the cancer cell to an APC/DC_{leu} and boost healthy DCs (Amberger et al., 2019, Klauer et al., 2024). Supplementary Figure 1.**A**). Finally those APC/DC or DC_{leu} mediate an (antigen specific) activation of the innate and the adaptive immune system, are responsible for effector functions (kill of infected cells or of leukemic cells in

Table 1
Cell subpopulations.

group	subgroup	acronym	markers	abbrev. [referred to cell fraction]	reference
blasts	blasts	Bla	CD15, CD34, CD65, CD117,	Bla/cells	(Campana and Behm, 2000)
	proliferating blasts	$Bla_{prol\text{-}CD71}$	$CD71^+Bla^+DC^-$	$Bla_{prol\text{-}CD71}/Bla$	(Klauer et al., 2024)
	proliferating blasts	$Bla_{prol\text{-}IPO38}$	$IPO38^+Bla^+DC^-$	$Bla_{prol\text{-}IPO38}/Bla$	(Klauer et al., 2024)
dendritic cells	antigen presenting cells/ dendritic cells	APC/DC	CD80, CD206, CD209, CD83	DC/cells	(Schmetzer et al., 2007)
	mature DC	DC_{mat}	DC^+CCR7^+	DC_{mat}/cells DC_{mat}/DC	(Schutti et al., 2024)
	leukemia derived DC	DC_{leu}	Bla^+DC^+	DC_{leu}/cells DC_{leu}/DC DC_{leu}/Bla	(Schmetzer et al., 2007)
	mature leukemia derived DC	$D_{leu\text{-}mat}$	$Bla^+DC^+CCR7^+$	$DC_{leu\text{-}mat}$/cells $DC_{leu\text{-}mat}/DC_{leu}$ $DC_{leu\text{-}mat}/DC_{mat}$	(Klauer et al., 2024)
T-cells	T-cells	T	$CD3^+$	$CD3^{+/}$cells	(Schick et al., 2013)
	Transferrin-R positive late proliferating T-cells	$T_{prol\text{-}late}$	$CD3^+CD71^+$	$T_{prol\text{-}late}/CD3^+$	(Schick et al., 2013)
	Type II C- type lectin positive early proliferating T-cells	$T_{prol\text{-}early}$	$CD3^+CD69^+$	$T_{prol\text{-}early}/CD3^+$	(Schick et al., 2013)
	CD4 positive T-cells	T_{CD4+}	$CD4^+CD3^+$	$T_{CD4+}CD3^+$	(Schick et al., 2013)
	CD4 negative T-cells	T_{CD4-}	$CD4^-CD3^+$	$T_{CD4-}/CD3^+$	(Schick et al., 2013)
	non-naive T-cells	$T_{non\text{-}naive}$	$CD3^+CD45RO^+$	$T_{non\text{-}naive}/CD3^+$	(Vogt et al., 2014)
	naive T-cells	T_{naive}	$CD3^+CD45RO^-$	$T_{naive}/CD3^+$	(Vogt et al., 2014)
	central memory T-cells	T_{cm}	$CD3^+CD45RO^+CCR7^+$	$T_{cm}/CD3^+$	(Vogt et al., 2014)
	CD4 positive non-naive T-cells	$T_{non\text{-}naiveCD4+}$	$CD3^+CD4^+CD45RO^+$	$T_{non\text{-}naiveCD4+}/T_{CD4+}$	(Amberger et al., 2019)
	CD4 negative non-naive T-cells	$T_{non\text{-}naiveCD4-}$	$CD3^+CD4^-CD45RO^+$	$T_{non\text{-}naiveCD4-}/T_{CD4-}$	(Amberger et al., 2019)
TCRγδ	TCRγδ positive T-cells	$T_{TCR\gamma\delta+}$	$TCR\gamma\delta^+CD3^+$	$T_{TCR\gamma\delta+}/CD3^+$	(Bensussan et al., 1989)
others	cytokine induced killer cells	CIK	$CD3^+CD56^+$	CIK/cells	(Amberger et al., 2019)
	natural killer cells	NK	$CD3^-CD56^+$	NK/cells	(Amberger et al., 2019)
	monocytes	Mon	$CD14^+$	Mon/cells	(Amberger et al., 2019)
	proliferating monocytes	$Mon_{prol\text{-}CD71}$	$CD71^+CD14^+DC^-$	$Mon_{prol\text{-}CD71}/Mon$	
	B-cells	B	$CD19^+$	Bcell/cells	(Amberger et al., 2019)
DEG	CD107a positive TCRγδ positive T-cells	$T_{TCR\gamma\delta+107a+}$	$CD107a^+\ TCR\gamma\delta^+CD3^+$	$T_{TCR\gamma\delta+107a+}/T_{TCR\gamma\delta+}$	(Aktas et al., 2009, Schutti et al., 2024)
INTCYT	TNF alpha positive TCRγδ positive T-cells	$T_{TCR\gamma\delta+TNF\alpha+}$	$TNF\alpha^+\ TCR\gamma\delta^+CD3^+$	$T_{TCR\gamma\delta+TNF\alpha+}/T_{TCR\gamma\delta+}$	(Rodríguez-Caballero et al., 2004, Schutti et al., 2024)

abbrev. subpopulation; markers (measured by FACS) marker combinations;

case of leukemia (Supplementary Figure 1.**B)** (Doraneh-Gard et al., 2024, Unterfrauner et al., 2023, Klauer et al., 2024).

1.3. Innate and adaptive immune system

The immune system utilizes humoral and cellular defense mechanisms against pathogens and tumor cells (Parkin and Cohen, 2001, Dunn et al., 2002). Macrophages ($CD15^+$), monocytes ($CD14^+$), APCs/DCs ($CD80^+$, $CD206^+$, etc. (Azad et al., 2014, Paurević et al., 2024)) and cytokine induced killer cells (CIK, $CD56^+CD3^+$) and natural killer cells (NK, $CD56^+CD3^-$): the innate immune system responsible for a ́first-line ́ response against pathogens and tumors (Beutler, 2004, Grabrucker et al., 2010, Schmeel et al., 2015, Franks et al., 2020). The adaptive immunity is responsible for long-term immunity: B-cells ($CD19^+$) as well as T-cells ($CD3^+$) and their subpopulations (see Table 2): naive T-cells ($CD3^+CD45RO^-$: T_{naive}), non-naive T-cells ($CD3^+CD45RO^+$: $T_{non\text{-}naive}$; $CD3^+CD4^+CD45RO^+$: $T_{non\text{-}naiveCD4+}$ and $CD3^+CD4^-CD45RO^+$: $T_{non\text{-}naiveCD4\text{-}}$) after stimulation, $CD4^+CD3^+$ (T_{CD4+}) and $CD4^-CD3^+$ ($T_{CD4\text{-}}$) as mediators of immune responses and central memory cells ($CD3^+CD45RO^+CCR7^+$: T_{cm}) recognizing reoccurring antigens (Amberger et al., 2019, Boeck et al., 2017, Bonilla and Oettgen, 2010).

1.4. T-cell receptor gamma delta (TCRγδ)

T-cell receptors (TCR) are responsible for the antigen-induced proliferation and activation of T-cells (Alcover et al., 2018). While the alpha beta heterodimer of TCR (TCRαβ) is able to recognize antigens almost exclusively in association with major histocompatibility complex (MHC) molecules, TCRγδ has the ability of MHC-independently recognizing antigens (Alcover et al., 2018, hayday, 2000, Morath and Schamel, 2020). TCRγδ expressing T-cells can be activated by various mechanisms, including indirectly by APCs/DCs via cytokines (Hannani et al., 2012, Devilder et al., 2006) and by leukemic cells (Duval et al., 1995). TCRγδ expressing T-cells themselves are also able to modulate DCs and $CD8^+$ T-cell-activity via cytokines (Nakamizo et al., 2015). TCRγδ expressing T-cells have been accredited with a high potential for immune therapy (Saura-Esteller et al., 2022, Vantourout and Hayday, 2013, Mensurado et al., 2023) and antileukemic activity (Minculescu et al., 2019, Bensussan et al., 1989, Costa et al., 2023). Enhanced cytolytic activity of γ/δ TCR-T cells against tumor cells has also recently been observed (Davies et al., 2024). CD19-specific γ/δ TCR-T cells have been subject to studies within which it was hypothesized that they could induce remission in hemato-oncologic patients (Li et al., 2023). TCRγδ has also been the subject of studies where bispecific antibodies were used to recruit TCRγδ expressing T-cells, capable of targeting cells expressing tumor-antigens in AML, again demonstrating the promising possibilities of TCRγδ in the context of hemato-oncological immune therapies (Ganesan et al., 2021).

1.5. Assessing leukemia-specific and blastolytic functionality of immune cells

The potential for cell lysis (of, e.g., leukemic blasts) can be measured using cytotoxicity assays such as fluorolysis-, chromium-51-release- and LDH- assays (Kienzle et al., 2002, Kumar et al., 2018, Grabrucker et al., 2010). Lysosomal-associated membrane glycoproteins (LAMPs), such as CD107a, remain on the cell surface after the release of the lytic granules and can be quantified using the degranulation assay (DEG) (Betts et al., 2003, Pepeldjiyska et al., 2021, Schutti et al., 2024). Furthermore, the intracellular cytokine assay (INTCYT) can be used to detect and measure cytokines, such as tumor necrosis factor alpha (TNFα), which is known to induce cell apoptosis (Rath and Aggarwal, 1999, Letsch and Scheibenbogen, 2003, Pepeldjiyska et al., 2021, Schutti et al., 2024).

1.6. Aims of the study

TCRγδ expression on T-cells in peripheral WB from AML/MDS patients and healthy volunteers was analysed with a special focus on the role of ́Kit-pretreated ́ WB to influence the TCRγδ provision after mixed lymphocyte culture (MLC).

In detail, we investigated (the)

- APC/DC($_{leu}$), their subtypes and the generation of APC/DC in patients ́ and healthy WB after Kit-treatment
- innate and adaptive immune cells, with a focus on TCRγδ expressing T-cells, before and after MLC with Kit-pretreated vs. untreated (patients ́ and healthy) WB
- degranulation and intracellular cytokine production of TCRγδ expressing T-cells in uncultivated (patients ́ and healthy) WB and after MLC with Kit-pretreated vs. untreated (patients ́ and healthy) WB
- blastolytic functionality of immune cells after MLC with Kit-pretreated vs. untreated (patients ́) WB
- correlations between TCRγδ expressing T-cells, blastolytic activity and leukemia-specific activity in patients ́ samples
- correlations between TCRγδ expressing T-cells in uncultivated patients ́ samples and response to chemotherapy/cytogenetic risk of patients

2. Materials and methods

2.1. Acquisition of samples

Samples were acquired from the university hospitals in Munich (LMU), Tuebingen, Oldenburg, and Augsburg, the Diakonieklinikum in Stuttgart, and the Rotkreuzklinikum in Munich. Peripheral blood samples were collected from healthy volunteers and patients with diagnosed myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML). Patients gave consent in written and oral form in accordance with Helsinki guidelines and the vote (number: 33905) of the Ethic Committee of LMU in Munich.

2.2. Details of collected samples

Samples were collected from 42 AML or MDS patients and 21 healthy individuals. At sample collection, patients were on average 63.1(29−98) years old, healthy volunteers were 30.7(20−56) years old. The male: female ratio was 1:1 for patients and 1:1.33 for healthy volunteers. Patients’ peripheral blood (PB) contained on average %Bla/cells: 32 (10−94) (immunocytologically determined) blasts. The FAB classification system was applied to classify AML cases (Bennett et al., 1976), and the WHO classification system to classify MDS cases (Swerdlow et al., 2017). The ELN classification was used to assess the cytogenetic risk for AML patients (Döhner et al., 2017) and a multifactorial risk assessment according to the IPSS-R classification system (Greenberg et al., 1997, Garcia-Manero et al., 2020) was conducted for MDS patients. More detailed patient information can be found in Table 2.

2.3. Sample preparation

Density gradient centrifugation was used to isolate mononuclear cells (MNCs) from WB, pretreated according to standard procedures (Böyum, 1968, Amberger et al., 2019). An aliquot of cells was directly frozen, another aliquot of MNCs underwent a CD3+ T-cell-enrichment via MACS-microbead-technology (Miltenyi Biotec, Bergisch Gladbach, Germany) and were frozen for subsequent experimental use (Willasch et al., 2010, Pepeldjiyska et al., 2021, Amberger et al., 2019).

Table 2
Patients' and healthy individuals' characteristics.

diagn.	no.	age	sex	subtype	stage	ic bla	blast phenotype [CD]	risk class.	response	exp.
AML	1444	35	f	p/M1	dgn.	41	33, 65, **15**, **34**, 117	favourable	yes	DC; MLC; CTX;
AML	1540	83	f	p/M1	dgn.	32	13, 34, 33, 15, **117**, **56**	favourable	no	DC; MLC; CTX;
AML	1618	63	m	p/M1	dgn.	13	**117, 33,** 13, 11b, 15	favourable	no	CTX; D/I;
AML	1427	52	m	p/M2	dgn.	94	13, 33, 34, **117**	favourable	no	DC; MLC; CTX;
AML	1541	82	f	p/M2	dgn.	15	15, **34, 117**	interm.	no	DC; MLC; CTX;
AML	1442	73	f	p/M4	dgn.	15	33, 13, 34, **117**, **15**	interm.	yes	DC; MLC; CTX;
AML	1459	54	m	p/M4	dgn.	51	33, 64, **15**, 4, 56, 14	favourable	yes	DC; MLC; CTX;
AML	1614	53	f	p/M4	dgn.	35	34, **117,** 14, 33, 13	interm.	yes	CTX; D/I;
AML	1430	79	m	p/M5	dgn.	62	13, 33, **34, 117**	favourable	nd	DC; MLC; CTX;
AML	1432	34	m	p/M5	dgn.	57	**34**, 13, 33, 64, 4	interm.	yes	DC; MLC; CTX;
AML	1466	47	f	p/M5	dgn.	11	33, 15, 13, **117**, **34**	adverse	yes	DC; MLC; CTX;
AML	1575	62	f	p/M5a	dgn.	75	14, **56,** 64, **65,** 4	interm.	yes	DC; MLC; CTX;
AML	1452	44	m	p/nd	dgn.	11	**34**, **117**, 33, 13	interm.	no	DC; MLC; CTX;
AML	1568	29	m	p/nd	dgn.	69	**34,** 117, 33, 13, 19, 20, **65**	interm.	no	DC; MLC; CTX;
AML	1570	36	f	p/nd	dgn.	11	**34, 117, 65,** 13, 33	favourable	no	DC; MLC; CTX;
AML	1608	61	f	p/nd	dgn.	23	34, **117**, 13, 33, 56, **65**	favourable	yes	CTX; D/I;
AML	1612	76	m	p/nd	dgn.	20	**34**, **65**, 117, 133, 13	adverse	no	CTX; D/I;
AML	1617	81	f	p/nd	dgn.	51	13, 33, 64, **34, 117,** 56,	interm.	yes	CTX; D/I;
AML	1492	52	f	s/M2	dgn.	38	117, **34**, 13, 33, 7, **15**	nd	no	DC; MLC; CTX;
AML	1542	58	f	s/M4	dgn.	52	13, 33, **34**, 117, 15, 65, 64, 2, **56,** 14	adverse	no	DC; MLC; CTX;
AML	1426	61	f	s/M5	dgn.	34	13, 33, **34**, 64, **117**, 14	adverse	yes	DC; MLC; CTX;
AML	1464	72	m	s/nd	dgn.	38	**34,** 117, 13	nd	nd	DC; MLC; CTX;
AML	1555	46	f	s/nd	dgn.	20	33, 14, **15**, 11, **117,** 13	favourable	nd	DC; MLC;
AML	1574	56	m	s/nd	dgn.	41	**34, 117,** 15, 19	nd	no	DC; MLC; CTX;
AML	1604	59	f	s/nd	dgn.	12	**117, 34,** 13, 33, **65**, 7	interm.	nd	CTX; D/I;
AML	1609	71	m	s/nd	dgn.	46	**117, 56,** 65, 33, 13, 7	favourable	yes	CTX; D/I;
AML	1610	82	f	s/nd	dgn.	14	**117**, 33, 14, **56**	nd	nd	CTX; D/I;
AML	1615	79	f	s/nd	dgn.	70	**34, 117**, 13	interm.	no	CTX; D/I;
AML	1616	69	f	s/nd	dgn.	14	**34, 117,** 33, 20, 23	interm.	no	CTX; D/I;
AML	1621	71	m	s/nd	dgn.	21	**34**, **117**, 5, 13,	adverse	no	CTX; D/I;
AML	1571	61	m	p/M2	rel.	18	**117,** 33, 13, 7	nd	yes	DC; MLC; CTX;
AML	1424	37	f	p/M4	rel.	13	13, 14, 33, **117**	nd	nd	DC; MLC; CTX;
AML	1548	87	m	p/M5a	rel.	12	33, **15,** 117, 34, **56**	nd	no	DC; MLC; CTX;
AML	1449	78	m	s/nd	rel.	32	65, 14, **15,** 33, 56, **34**	nd	nd	DC; MLC; CTX;
AML	1482	75	m	s/nd	rel.	12	117, 13, 64, **15**, **117,** 33	nd	nd	DC; MLC; CTX;
AML	1546	80	m	p/nd	pers.	22	33, **34,** 13, 117, 14, **65**	nd	no	DC; MLC;
AML	1470	67	m	p/nd	PR a. SCT	38	33, 117, **34,** 56, **65**	nd	no	DC; MLC;

(*continued on next page*)

Table 2 (*continued*)

diagn.	no.	age	sex	subtype	stage	ic bla	blast phenotype [CD]	risk class.	response	exp.
AML	1457	63	m	s/nd	rel. a. SCT	37	34, 117, 13, **65, 15**	nd	no	DC; MLC; CTX;
AML	1543	61	m	p/nd	rel. a. SCT.	38	13, 33, **117**, 56, **34**	nd	no	DC; MLC; CTX;
MDS	1567	98	f	MDS	dgn.	14	34, 117, **15**, **65**, 56, 14	very high	nd	DC; MLC; CTX;
MDS	1573	61	m	MDS	dgn.	12	**34, 117,** 65, 13	high	no	DC; MLC; CTX;
MDS	1572	63	f	MDS-EB2	dgn.	10	**34, 117,** 65, 33, 13	very high	no	DC; MLC; CTX;
healthy	1417	34	f							DC; MLC;
healthy	1418	22	m							DC; MLC;
healthy	1420	26	f							DC; MLC;
healthy	1421	27	f							DC; MLC;
healthy	1422	20	f							DC; MLC;
healthy	1425	27	m							DC; MLC;
healthy	1428	56	f							DC; MLC;
healthy	1429	22	f							DC; MLC;
healthy	1431	22	m							DC; MLC;
healthy	1436	25	m							DC; MLC;
healthy	1448	27	f							DC; MLC;
healthy	1458	21	f							DC; MLC;
healthy	1544	22	m							DC; MLC;
healthy	1545	32	m							DC; MLC;
healthy	1547	46	f							DC; MLC;
healthy	1566	54	f							DC; MLC;
healthy	1576	55	m							DC; MLC;
healthy	1578	32	m							DC; MLC;
healthy	1611	26	f							D/I;
healthy	1613	24	f							D/I;
healthy	1619	25	m							D/I;

diagn. diagnosis; no. sample number; m male f female; subtypes: p primary AML; s secondary AML; AML (FAB class.); MDS (WHO class.); rel. a. SCT relapse after stem cell therapy; rel. relapse; dgn. first diagnosis; pers. persisting disease; PR partial remission; nd no data; ic bla immunocytologically determined blasts; bold numbers: blast markers used for DC characterization; risk class. risk classification cytogenetic (ELN AML) and multifactorial (IPSS-R MDS); response, response to chemotherapy; exp. experiments conducted; DC dendritic cell generation; MLC Mixed lymphocyte culture; D/I degranulation and intracellular cytokine assay; CTX cytotoxic fluorolysis assay;

2.4. Generation of APC/DC($_{leu}$)

WB samples were mixed with a combination of response modifiers (Kit-M (GM-CSF (concentration (conc.): 800 U/ml, granulocyte macrophage colony stimulating factor, Sanofi-Aventis, Frankfurt, Germany) and PGE1 (conc.: 1 µg/ml, prostaglandin E1, Santa Cruz Biotechnology, Dallas, Texas, US)) and Kit-I (GM- CSF (conc.: 800 U/ml) and Picibanil (conc.: 10 µg/ml, Chugai Pharmaceutical Co., Kajiwara, Japan))), or without any Kits as control (the same amount of cells were added to the cultures compared), in accordance with our APC/DC-generating protocols (Amberger et al., 2019, Schmetzer et al., 2007, Boeck et al., 2017). Kits were added on the first day and again after 2–3 days of incubation. APC/DC-cultures were harvested after 6–8 days, quantified and utilized in subsequent experiments (Amberger et al., 2019, Boeck et al., 2017). In order to simplify abbreviations, Kit-M treated WB is referred to as DC(M) (instead of APC/DC(M)), Kit-I-treated WB as DC(I) (instead of APC/DC(I)), and untreated WB (control) as DC(C) (instead of APC/DC(C)),

2.5. MLC- T-cell enriched mixed lymphocyte cultures

T-cells were thawed (as described previously) and cultured with DC-containing Kit-pretreated WB (containing approximately 250.000 cells), and 50 U/ml IL-2 (PeproTech, Berlin, Germany) to stimulate T-cell activation and maturation. After six to eight days of incubation time, the cultures were harvested, and the composition of immune reactive cells quantified by flowcytometry. In addition, intracellular cytokine production (INTCYT), and degranulation assays (DEG) were evaluated and

the cytotoxicity fluorolysis assay (CTX) was performed (Amberger et al., 2019, Boeck et al., 2017, Schutti et al., 2024)). In this study, MLC(UC) refers to cells before MLC, and after MLC with untreated WB they are referred to as MLC(CC), with Kit-M-pretreated WB as MLC(M), and with Kit-I-pretreated WB as MLC(I).

2.6. INTCYT- intracellular cytokine- and DEG- degranulation assay

For the DEG and INTCYT, uncultured WB or cells after MLC with and without Kit-pretreatment were used (Schutti et al., 2024). The intracellular cytokine production, in this study specifically TNFα, can be used to characterize immune cell functionality (Rodríguez-Caballero et al., 2004). According to manufactors ́ instruction, the release of cytokines (TNFα) was inhibited by adding Brefeldin A solution (conc.: 5 μg/ml, BioLegend, San Diego, California, US). Cells were stained using PE/Cy7-conjugated TNFα antibodies (BioLegend, San Diego, California, US) (Pepeldjiyska et al., 2021). CD107a on the cell surface can be used as a measure of degranulation and therefore of immune cell functionality (Aktas et al., 2009). Monensin solution (conc: 2 μmol/l, BioLegend, San Diego, California, US) was added according to manufactors` instruction to inhibit endocytosis processes possibly weakening the fluorescence of CD107a antibodies on the cell surface over time. Cells were stained using FITC-conjugated CD107a antibodies (BioLegend, San Diego, California, US) (Pepeldjiyska et al., 2021).

2.7. CTX- cytotoxicity fluorolysis assay

The blastolytic potential of effector cells (immunoreactive cells after T-cell enriched MLC with or without Kit-pretreated WB) was quantified using the CTX. Therefore, effector cells and target cells (patients ́ blast containing MNC, stained with blast markers) were incubated together (́group 1 ́) and separately for comparison (́group 2 ́). Effector and target cells were quantified after 0, 3, and 24 hours of co-culture. Cell viability was quantified via flow cytometry using 7-AAD Staining Solution (7AAD, BD Biosciences, San Jose, California, US) as a viability marker. The ́blast lysis ́ was determined by comparing viable blast cells in ́group 1 ́ to ́group 2 ́, and ́lysis improvement ́ defined by blast lysis after MLC using Kit-pretreated to untreated WB (Kienzle et al., 2002, Amberger et al., 2019, Schutti et al., 2024, Klauer et al., 2022, Unterfrauner et al., 2023).

2.8. Cell quantification via flow cytometry

Cell quantification was performed via FACSCalibur four channel flow cytometer. Cell staining was carried out using monoclonal antibodies labelled with FITC (fluorescein- isothiocyanate), PE (phycoerythrin), PE/Cy 7 (phycoerythrin/ cyanine 7) and APC (allophycocyanin).

These were: FITC-conjugated antibodies against IgG*, CD34*, CD65*, CD33*, CD117***, CD15*, CD56***, CD3**, CD71*, ipo38****, CD19*, CD107a***, CD4**, CD45RO*, CD14*; PE-conjugated antibodies against IgG*, TCRγδ**, CD 117*, CD80*,CD83*, CD56*, CD206*, CD3*, CD4**; PE/Cy7-conjugated antibodies against IgG*, CD15**, CD117*, CD19*, CD34*, CD197**, CD56*, CD4*, TNFα***, CD3*, CD14**; APC-conjugated antibodies against IgG*, CD206**, CD80***, CD209**, CD83**, CD34*, CD117*, CD14*, CD56*, CD69**, CD45RO***, CD4**, CD3*, CD19*. Antibodies were supplied by Beckman Coulter (*, Brea, California, US), BD Biosciences (**, San Jose, California, US), BioLegend (***, San Diego, California, US), Santa Cruz Biotechnology (****, Dallas, Texas, US). 7AAD** was used to detect viable cells.

For APC/DC($_{leu}$) quantification, blast markers with high expression (e.g. CD34, CD117, CD65, CD56 or CD15) and APC/DC markers with low/no expression on blasts (e.g. CD80, CD83, CD206 or CD209) were individually chosen for each patient (Campana and Behm, 2000, Schmetzer et al., 2007).

FIX & PERM Cell Fixation and Cell Permeabilization Kit (ThermoFisher Scientific, Darmstadt, Germany) was used to detect intracellular markers (IPO38, INTCYT). Isotype samples were added as controls (Kufner et al., 2005, Amberger et al., 2019, Schutti et al., 2024).

2.9. Data analysis

BD CellQuest Pro (Becton Dickinson, Heidelberg, Germany) was used to analyse the flowcytometric data. Excel 2010 (Microsoft, Redmond, Washington, USA) and SPSS Statistics 26 (IBM, Armonk, New York, USA) were utilized for statistical analysis. Data was presented including means, standard deviations, and statistical significances. Analyses of differences between groups were conducted with the paired-t-test and the Wilcoxon-Mann-Whitney-U-test. The Pearson-correlation and the Spearman-correlation were used for correlation analyses. Significances were defined by $p \leq 0.05$ as significant and $p \leq 0.005$ as highly significant.

3. Results

3.1. Prologue

We analysed immune cell profiles with a focus on TCRγδ expression on T-cells before and after MLC, and further correlated immune cell profiles with blastolytic functionality, as well as clinical risk groups and outcomes of patients. Abbreviations for all subsequently mentioned cell subpopulations are given in Table 1.

3.2. Generation of APC/DC($_{leu}$) and their subtypes using WB

3.2.1. Significantly higher frequencies of APC/DC(subtypes) generated with Kit vs. control

Frequencies of APC/DC_{leu} and their subtypes in patients ́ and healthy samples were (highly) significantly higher after DC(M) and DC(I) compared to DC(C) (e.g. %DC_{leu}/cells; %DC_{mat}/cells) (Fig. 1). Moreover, we found significantly higher frequencies of mature DC subsets compared to control (Fig. 1)-thereby confirming data as shown before (Klauer et al., 2022, Schutti et al., 2024).

3.2.2. Blast/monocytes ́ proliferation was not increased after Kit-treatment compared to control

In Kit-treated as well as untreated WB, we found no significant differences in frequencies of proliferating blasts ($Bla_{prol\text{-}CD71}$, $Bla_{prol\text{-}IPO38}$; Fig. 1.A1) in patients ́ WB and in frequencies of proliferating monocytes ($Mon_{prol\text{-}CD71}$; Fig. 1.B2), as already shown before (Plett et al., 2022).

In conclusion, Kit-treated WB (compared to the untreated control) led to higher frequencies of APC/DCs and DC-subtypes (DC_{leu}, DC_{mat}, $DC_{leu\text{-}mat}$) without induction of (APC/DC-independent) proliferation of blasts (patients) or monocytes (healthy).

3.2.3. Cells of the innate and adaptive immunity in uncultured WB

While we could find low frequencies of different T-cell subtypes and innate immune cells in healthy and AML WB samples, frequencies of NK/cells were significantly higher in healthy samples compared to patients ́ samples (%NK/cells) (Fig. 2). Furthermore, we found significantly higher frequencies of TCRγδ expressing T-cells in healthy samples (%$T_{TCR\gamma\delta+}$/$CD3^+$: 7–8%) compared to patients ́ samples (%$T_{TCR\gamma\delta+}$/$CD3^+$: 5–6%).

After MLC, frequencies of TCRγδ expressing T-cells in healthy and patients ́ samples were comparable (Fig. 3.A), as were the frequencies of innate immune cells (Fig. 2). We generally found more innate as well as proliferating, non-naive and/or memory T-cells (e.g., $T_{non\text{-}naive}$/$CD3^+$ (and $T_{nonnaiveCD4+}$/T_{CD4+} and $T_{non\text{-}naiveCD4\text{-}}$/$T_{CD4\text{-}}$) and $T_{CD4\text{-}}$/$CD3^+$) after MLC compared to before (Fig. 2), which is also true for TCRγδ expressing T-cells (Fig. 3.A).

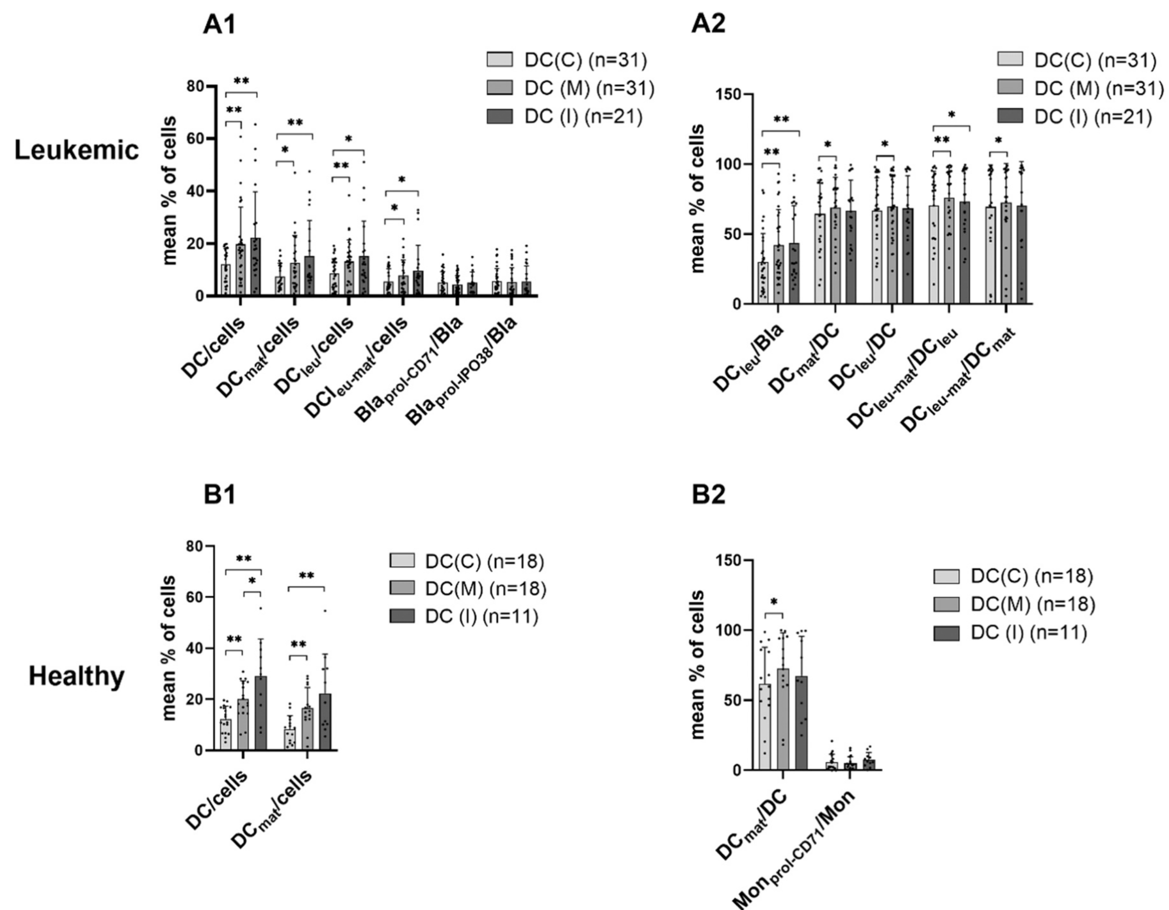

Fig. 1. DC$_{(leu)}$-generation using leukemic (A) and healthy (B) WB with and without Kit-treatment; WB-samples were cultivated with and without (control) Kits for 7 days. DC(M), DC(I), DC(C) refer to Kit M, Kit I and control WB-cultures; Average frequencies ± standard deviation and dot plots of DC-subtype values in (A) leukemic (AML/MDS) and (B) healthy samples within (1) all cells and (2) other DC$_{(leu)}$ subpopulations; (n) number of cases; Significance is defined as $p \leq 0.05$ (significant/ *) and $p \leq 0.005$ (highly significant/ **), all other comparisons were not statistically significant; Cell subpopulations are given in Table 2.

3.3. Immune cell stimulation after MLC with Kit-treated (compared to untreated) WB

3.3.1. Significantly higher frequencies of T- and CIK-cells found after MLC with Kit-pretreated (vs. untreated) WB

We analysed the composition of immune cells after MLC with Kit-pretreated WB and compared the results with the control after MLC with untreated WB. In patients' samples, $T_{non\text{-}naive}$ and T_{cm} could be found in significantly higher frequencies after MLC(M) compared to control (MLC(CC)) (%$T_{non\text{-}naive}$/$CD3^+$: MLC(M) vs. MLC(CC): $p \leq 0.05$; and %T_{cm}/$CD3^+$: MLC(M) vs. MLC(CC): $p \leq 0.05$). Frequencies of innate immune cells were comparable. (Fig. 2.A)

No differences were found in healthy samples comparing MLC(M) or MLC(I) vs. the control (Fig. 2.B)

3.3.2. Significantly higher frequencies of TCRγδ expressing immune reactive cells found after MLC with Kit-M-pretreated (vs. untreated) WB

In patients' samples, we were able to find highly significantly higher frequencies of TCRγδ expressing cells within T-cells after MLC(M) compared to MLC(CC) (%$T_{TCR\gamma\delta+}$/$CD3^+$: MLC(M): 24.58±15.80, $p \leq 0.005$; MLC(CC): 19.67±14.82). No significant differences were found between MLC(I) and MLC(CC). Similar results could be found in healthy donors' samples. (Fig. 3.A)

We summarize that Kit-M-treatment of WB resulted in higher frequencies of TCRγδ expressing T-cells compared to control. Furthermore, frequencies of TCRγδ expressing T-cells were higher within healthy (compared to patients') before MLC.

3.3.3. Degranulation and intracellular cytokine production significantly increased within TCRγδ expressing immune reactive cells after MLC with Kit-M-pretreated (vs. untreated) WB in patients' samples

We found higher frequencies of degranulating and intracellular cytokine producing TCRγδ expressing T-cells after MLC compared to uncultivated WB in patients' samples.

Furthermore, we found highly significantly higher frequencies of degranulating (%$T_{TCR\gamma\delta+CD107a+}$/$T_{TCR\gamma\delta+}$: MLC(M): 59.43±31.39, $p \leq 0.005$; MLC(CC): 53.24±29.09) and intracellular cytokine producing (%$T_{TCR\gamma\delta+TNF\alpha+}$/$T_{TCR\gamma\delta+}$: MLC(M): 50.07±27.90, $p \leq 0.005$; MLC(CC): 38.87±27.67) TCRγδ expressing T-cells after MLC(M) compared to MLC (CC) in patients' samples. Differences in Kit-pretreated and untreated healthy samples and differences between healthy and patients' samples were not significant. (Fig. 3.B)

We summarize that Kit-M-pretreatment (compared to control) led to higher frequencies of degranulating and TNFα producing TCRγδ expressing T-cells in patients' samples.

3.3.4. Immune cells showed a superior blastolytic functionality after MLC with Kit-M-pretreated (vs. untreated) WB

The cytotoxicity fluorolysis assay was used to analyse the blastolytic potential ('lysis') of effector cells after MLC(M), MLC(I) and MLC(CC). We co-cultivated target (blast containing MNC) and effector cells (T-cell enriched MLC after culture) for 24 hours and analysed viable blast frequencies after 3 hours and 24 hours of incubation time and selected the best achieved blastolytic effect after either 3 hours or 24 hours of incubation time.

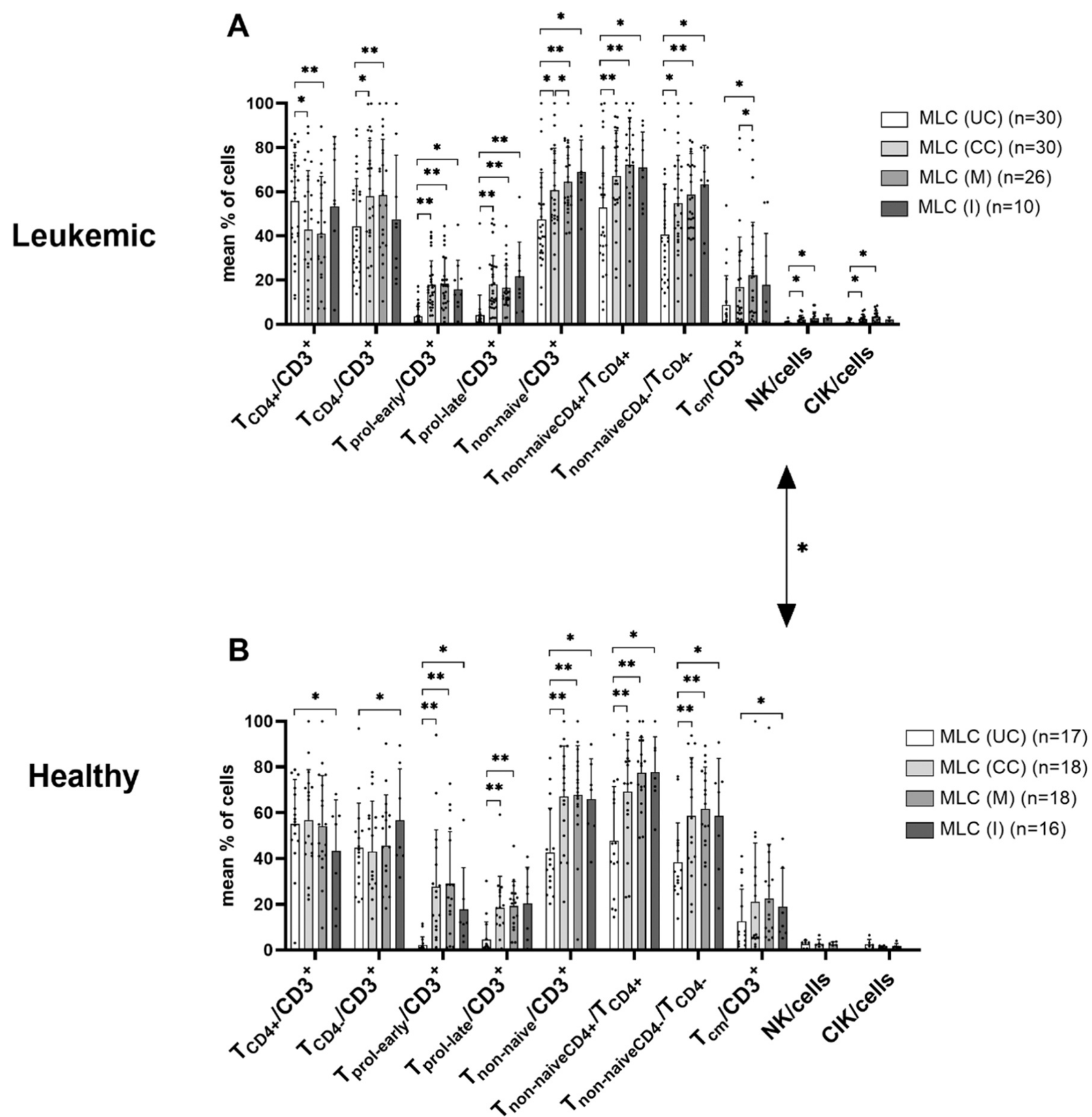

Fig. 2. Immune cell composition before and after T-cell enriched MLC with (A) leukemic and (B) healthy WB with and without Kit-pretreatment as stimulator cells. Immune cells were cultivated with Kit-pretreated and control WB for 7 days and quantified before and after MLC. MLC(M), MLC(I) and MLC(CC) refer to Kit M, Kit I and control MLC cultures after incubation, MLC(UC) to control culture before incubation; Average frequencies ± standard deviation and dot plots of immune cell subpopulations in (A) leukemic (AML/MDS) and (B) healthy samples; (n) number of cases; Significance is defined as $p \leq 0.05$ (significant/ *) and $p \leq 0.005$ (highly significant/ **), all other comparisons were not significant;; Cell subpopulations are given in Table 2.

After 3 (24) hours of incubation time, blast lysis could be achieved in 71.05 (76.32)% of cases after MLC(M) compared to 56.41 (64.10)% of cases after MLC(CC) and 45.45 (54.55)% of cases after MLC(I). Selecting the best blastolytic effect, blast lysis could be achieved in 94.74% of cases after MLC(M) compared to 79.49% of cases after MLC(CC) and 81.82% of cases after MLC(I). Significantly more cases with best achieved blast lysis were found after MLC(M) compared to MLC(CC) ($p \leq 0.05$). (Fig. 4.A). Frequencies of lysed blasts were comparable after Kit-pretreated and control MLC in cases with achieved lysis.

After 3 (24) hours of incubation time, improved lysis (MLC(M)/MLC(I) compared to MLC(CC)) could be achieved in 60.53 (63.16)% of cases after MLC(M) compared to 36.36 (45.45)% of cases after MLC(I). Selecting the best blastolytic effect, improved lysis (MLC(M)/MLC(I) compared to MLC(CC)) could be achieved in 86.84% of cases after MLC (M) compared to 72.73% of cases after MLC(I). Improved lysis (MLC (M)/MLC(I) compared to MLC(CC)) could be found in significantly more cases after MLC(M) compared to MLC(I) after 3 hours ($p \leq 0.05$) (Fig. 4. B1). Lysis improvement was comparable after Kit-M-pretreated and Kit-I-pretreated MLC in cases with improved lysis (Fig. 4.B2).

In conclusion, we observed a superior blastolytic functionality of effector cells after Kit-M-pretreated MLC compared to the control and Kit-I-pretreated MLC.

3.4. Correlation analyses

3.4.1. Patients with remission (vs. blast persistence) after chemotherapy showed higher frequencies of TCRγδ expressing T-cells in uncultivated cells

We analysed AML patients´samples at first diagnosis before MLC and compared frequencies of TCRγδ expressing T-cells in patients with an achieved vs. a non-achieved remission after induction chemotherapy. Even though differences were not significant (due to low number of cases), we could find nearly significantly higher frequencies of TCRγδ

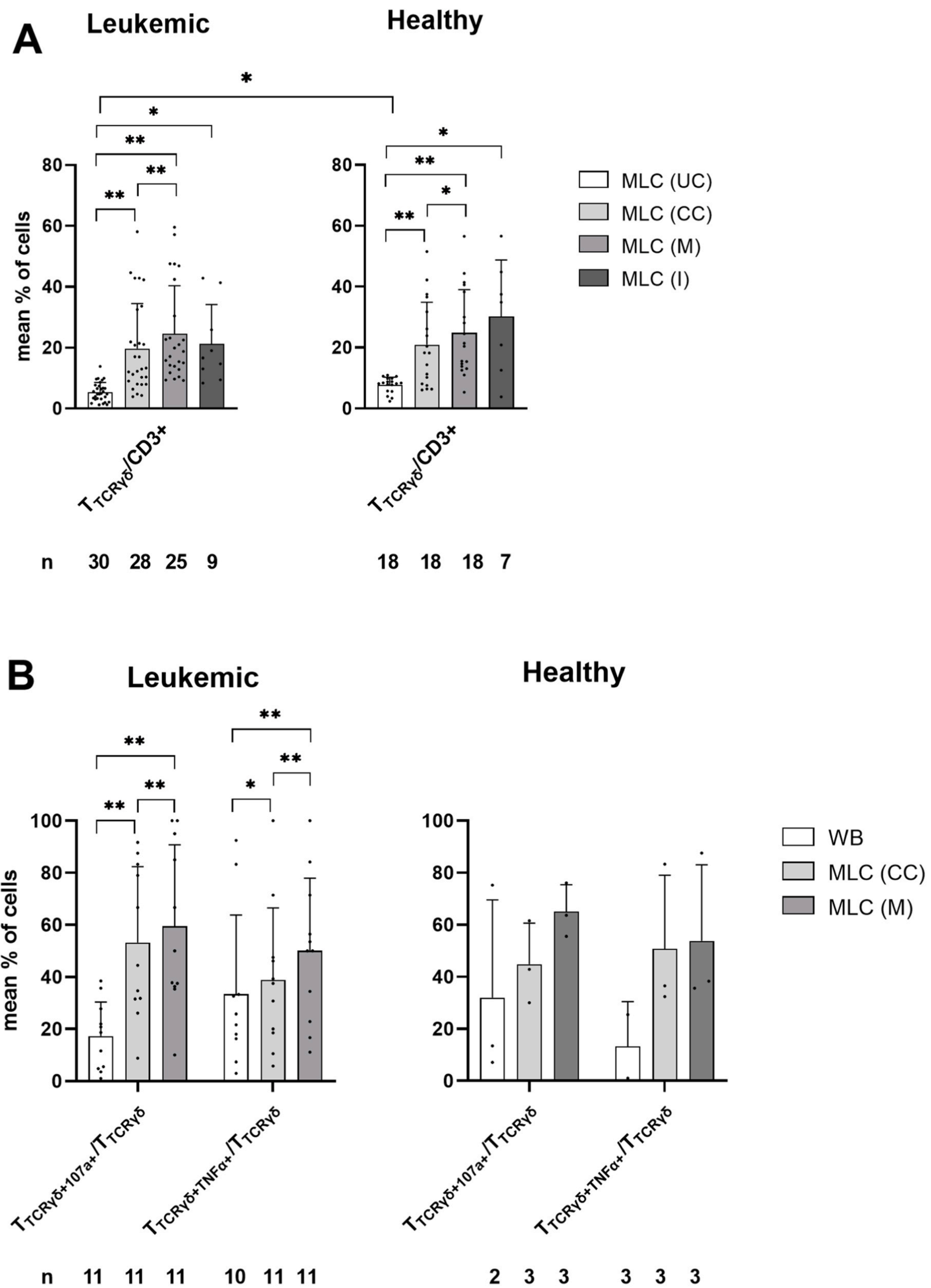

Fig. 3. Composition of (A) TCRγδ expressing T-cells and (B) degranulating and intracellular cytokine producing of TCRγδ expressing T-cells before and after T-cell enriched MLC with leukemic and healthy WB with and without Kit-pretreatment as stimulator cells Immune cells were cultivated with Kit-pretreated and control WB for 7 days and quantified before and after MLC. DEG and INTCYT were quantified in uncultivated WB and after MLC. MLC(M), MLC(I) and MLC(CC) refer to Kit M, Kit I and control MLC of immune cells co- cultivated with DC culture, MLC(UC) to the control culture before cultivation, WB to whole blood; Average frequencies ± standard deviation and dot plots of (A) TCRγδ co-expressing T-cells and (B) degranulating and intracellular cytokine producing TCRγδ co-expressing T-cells; (n) number of cases; Significance is defined as $p \leq 0.05$ (significant/ *) and $p \leq 0.005$ (highly significant/ **), all other comparisons were not statistically significant; Cell subpopulations are given in Table 2;.

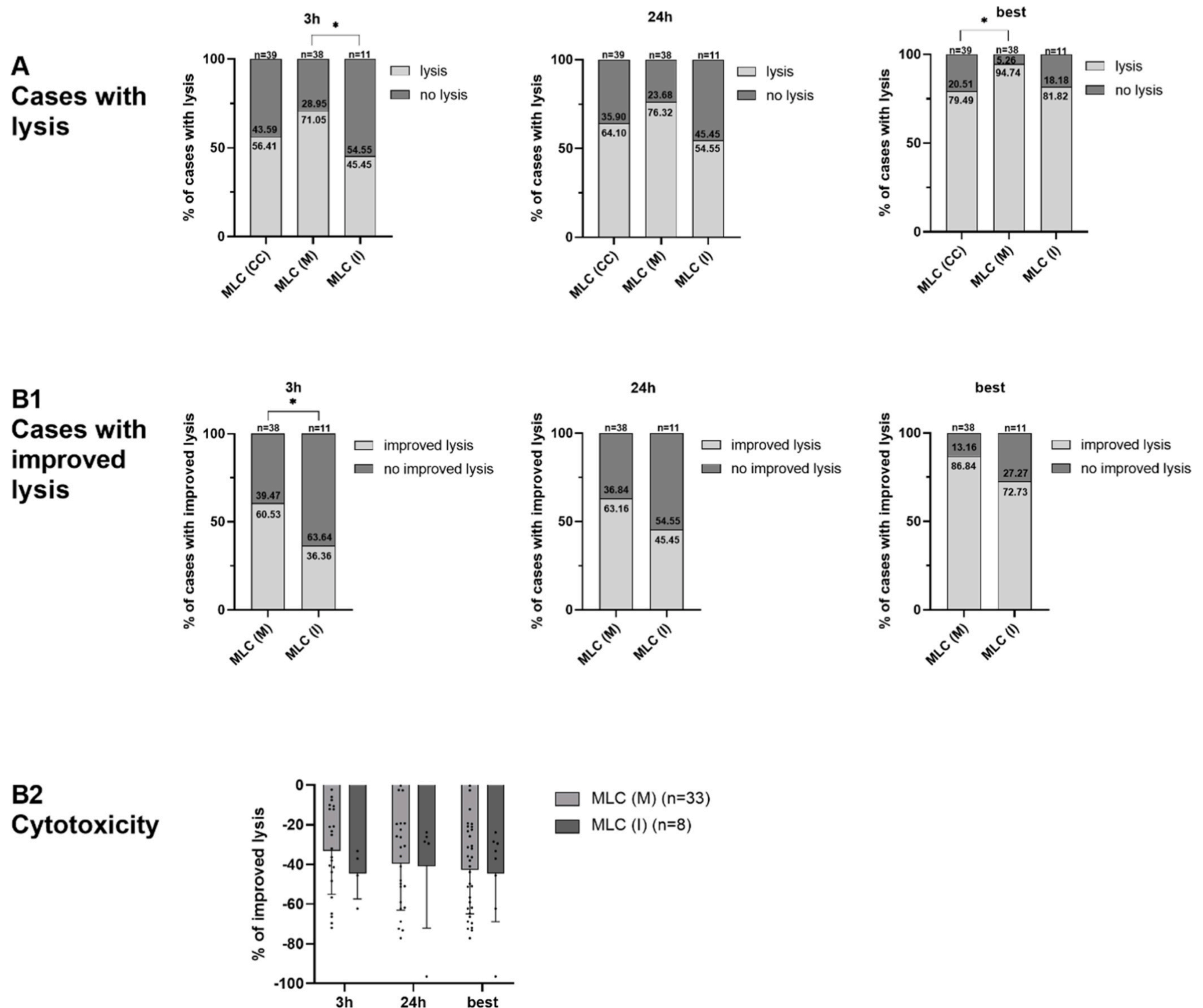

Fig. 4. Blastolytic potential of immune cells after T-cell enriched MLC with leukemic WB with and without Kit-pretreatment as stimulator cells. Target and effector cells were co-cultivated for 24 hours. Figures show results after 3 hours, 24 hours and the best of achieved/improved lysis values after either 3 hours or 24 hours of incubation time. MLC(M), MLC(I) and MLC(CC) refer to Kit M, Kit I and control cultures; Percentage of cases (A) with (lysis) and without (no lysis) lysis and (B1) with (improved lysis) and without (no improved lysis) improvement of blast lysis compared to MLC(CC); Averages ± standard deviation and dot plots of (B2) improvement of blast lysis compared to MLC(CC) within cases with improved blast lysis; (n) number of cases; Significance is defined as p≤ 0.05 (significant/ *), all other comparisons were not statistically significant; Cell subpopulations are given in Table 2;.

expressing T-cells in patients with an achieved remission ($p<0.1$) (Fig. 5.A).

3.4.2. Positive correlation of increased TCRγδ expression on T-cells and blastolytic functionality

We correlated TCRγδ expression on T-cells with blastolytic functionality as evaluated by a cytotoxicity assay. Increased frequencies of TCRγδ expressing T-cells nearly significantly correlated with increased blast lysis after MLC(M) compared to MLC(CC) ($r= 0.411$; $p= 0.058$) (Fig. 5.B).

3.4.3. Positive correlation of TCRγδ expression, blastolytic functionality and degranulation

We correlated frequencies of TCRγδ expressing T-cells, of degranulating TCRγδ expressing T-cells and blastolytic cytotoxicity. We found a non-significant positive correlation between frequencies of TCRγδ expressing T-cells and frequencies of degranulating TCRγδ expressing T-cells after Kit-M-pretreated MLC ($r= 0.542$; $p= 0.085$) (Fig. 5.C1). However, we found a significant positive correlation of frequencies of degranulating TCRγδ expressing T-cells with frequencies of lysed blasts after Kit-M-pretreated MLC ($r= 0.670$; $p= 0.024$) (Fig. 5.C2). Degranulating TCRγδ expressing T-cells after Kit-M-pretreated MLC correlated non-significantly positively with lysis improvement after Kit-M-pretreated MLC compared to control ($r= 0.565$; $p= 0.070$) (Fig. 5.C4). Moreover, increased frequencies of degranulating TCRγδ expressing T-cells correlated highly significantly positively with increased blast lysis after Kit-M-pretreated MLC compared to control ($r= 0.882$; $p< 0.001$) (Fig. 5.C3).

In conclusion, compared to control, higher frequencies of $DC_{(leu)}$ and their subtypes were generated with Kit-treatment of WB, leading to improved stimulation and activation of immune reactive cells including TCRγδ expressing T-cells. These TCRγδ expressing T-cells showed higher levels of degranulation and intracellular cytokine production after Kit-pretreated MLC (vs. control). Kit-M-pretreatment also led to a superior

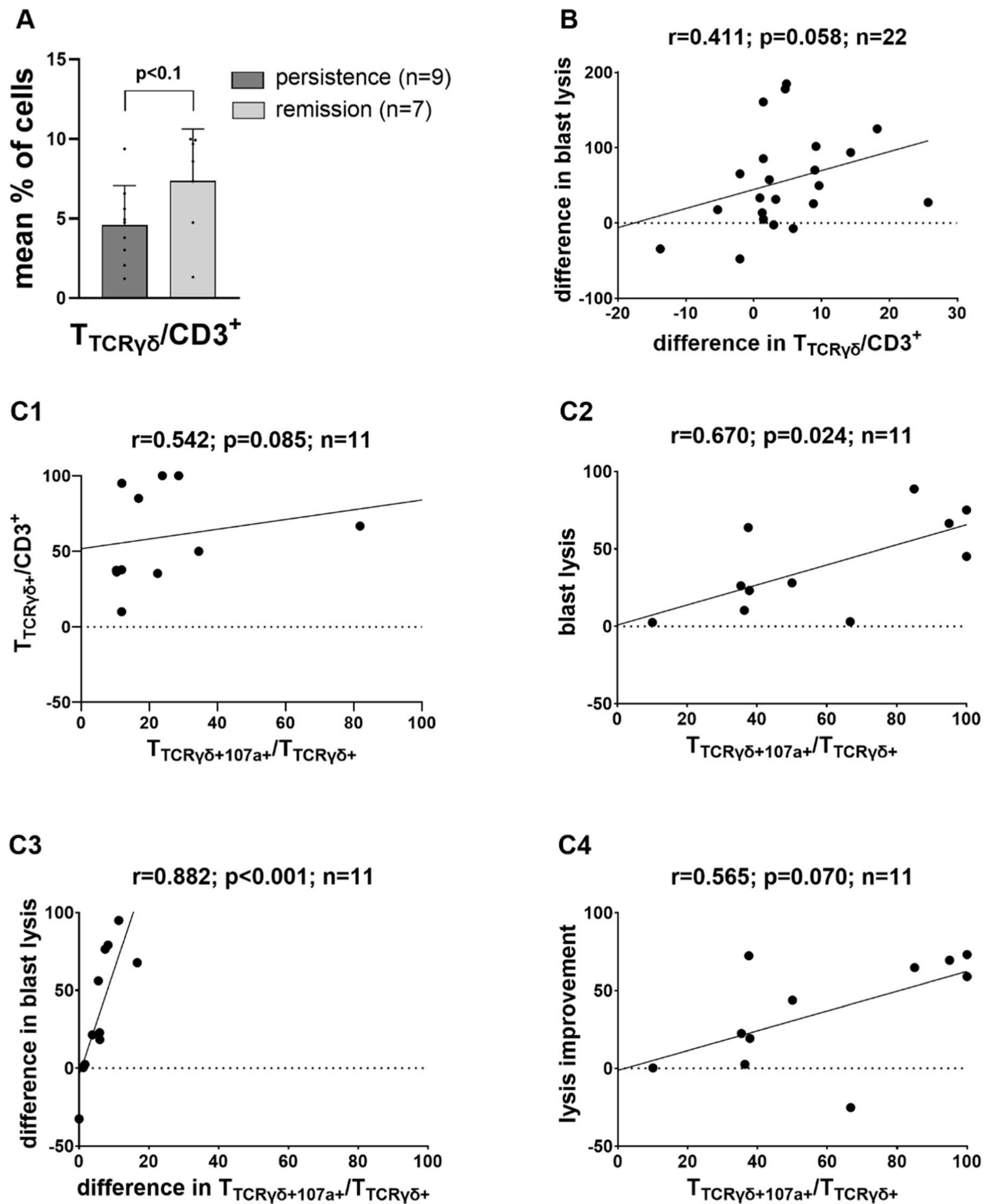

Fig. 5. Correlations of frequencies of TCRγδ expressing T-cells within clinical/ functional subgroups or with blastolytic functionality. Immune cells were cultivated with Kit-pretreated and control WB for 7 days and quantified before and after MLC. MLC(M) and MLC(CC) refer to Kit M and control MLC of immune cells co-cultivated with DC culture, MLC(UC) to control culture before cultivation. Lysis (improvement/difference) refers to the best of value of MLC(M) (vs. MLC(CC)). Lysis improvement refers to a relative increase, difference refers to an absolute increase in blast lysis in MLC(M) compared to MLC(CC). Average frequencies ± standard deviation and dot plots of TCRγδ co-expressing T-cells in AML patients′ WB (MLC(UC)) at initial diagnosis with (A) differences in response to chemotherapy; (B) correlations between differences in blast lysis and increase in frequencies of TCRγδ expressing T-cells after MLC(M) (compared to MLC(CC)); (C) correlations between (frequencies after MLC(M)/increased frequencies after MLC(M) vs. control) of degranulating TCRγδ expressing T-cells and frequencies of TCRγδ expressing T-cells (C1), blast lysis (C2), the difference in blast lysis (C3), or lysis improvement (C4) after MLC(M) (vs. control (C3, C4)); (n) number of cases; Significance is defined as $p \leq 0.05$ (significant/ *) and $p \leq 0.005$ (highly significant/ **); Cell subpopulations are given in Table 2;.

blastolytic cytotoxicity that correlated with higher frequencies of degranulating TCRγδ expressing T-cells which also correlated positively with higher frequencies of TCRγδ expressing T-cells. Additionally, the Kit-M-triggered promotion of frequencies of (degranulating) TCRγδ expressing T-cells after MLC correlated with the increased blastolytic functionality. Finally, higher frequencies of TCRγδ expressing T-cells in uncultivated AML patients′ samples at first diagnosis tendentially pointed to a higher chance to achieve remission for patients after

induction chemotherapy.

4. Discussion

4.1. Immune therapies as a focus of hematological research

DC/DC_{leu} have shown high potential for immune therapies due to their special natural role facilitating immune response (Behrends et al., 2016, Amberger et al., 2019, Estey, 2020, Garcia-Manero et al., 2020). DC-generating methods can be used to derive DCs from myeloid blasts or monocytes (Schmetzer et al., 2007, Kremser et al., 2010), and have been shown to have various effects on the (leukemia specific) activation/composition of immune cells as well as their marker/antigen profiles (Klauer et al., 2022; Rackl et al., 2022; Vogt et al., 2014; Pepeldjiyska et al., 2021). Further developments in immune therapeutic approaches, e.g. to induce DC/DC_{leu} in vivo, might help to improve therapy options and outcomes for patients with AML/MDS (Amberger and Schmetzer, 2020; Estey, 2020; Garcia-Manero et al., 2020; Klauer et al., 2022; Rackl et al., 2022; Röllig et al., 2019).

4.2. Immune cell stimulation mediated by Kit-treated WB

4.2.1. Generation of APCs/DCs and stimulation of immune cells

We could confirm that Kit-treatment (vs. control) of (healthy and patients') WB led to an increased generation of APCs/DCs and their subtypes (Amberger et al., 2019, Boeck et al., 2017, Hirn Lopez et al., 2019) (Fig. 1). We attribute this to the influence of GM-CSF and PGE1 (Kit-M) or GM-CSF and Picibanil (Kit I) on the intricate interplay of response modifiers (such as interleukins and chemokines) inherent in the microenvironment already contained in WB (Schwepcke et al., 2022, Amberger et al., 2019). Whereas frequencies of mature, leukemia-derived DC using Kit-treatment of WB (vs. control), were increased, no induction of monocytes' or blasts' proliferation was found, supporting preliminary data that Kit-treatment of WB does not induce the proliferation of blasts/monocytes (Plett et al., 2022, Amberger et al., 2019) (Fig. 1). These results are independent of patients' sex, age, mutation status cytogenetic risk or blast counts, and might therefore prove and qualify to stabilize patients' remissions, which remains to be proven clinically (Klauer et al., 2022, Klauer et al., 2024).

We could furthermore observe a (significant) stimulation of the adaptive and innate immunity after MLC with Kit-pretreated WB (vs. control): This includes significantly increased frequencies of non-naive and central memory T-cells in patients' samples (Fig. 2). This might indicate that Kit-pretreated WB might activate immune cells and give rise to the immunological memory, as shown before (Amberger et al., 2019, Amberger and Schmetzer, 2020, Boeck et al., 2017, Klauer et al., 2022, Plett et al., 2022, Klauer et al., 2024). Furthermore, similar to Boeck et al., we found higher frequencies of innate immune cells in healthy (vs. patients') samples (Boeck et al., 2017) (Fig. 2), which might be due to various bacterial, mycotic or viral antigens presented by healthy DC, leading to the activation of healthy immunoreactive cells (Logan et al., 2020, Teague and Kline, 2013, Rabinovich et al., 2007). The observed broad immune cell activation after MLC (vs. before) might be caused by the effect of IL-2 added to cultures (Schorle et al., 1991) (Fig. 2, Fig. 3.A).

4.2.2. MLC with Kit-M-pretreated WB (vs. control) led to increased frequencies of TCRγδ expressing T-cells, which were more prevalent in healthy (vs. patients) samples before cultivation

TCRγδ has shown high potential in the context of potentially new and already known immune therapies (Hannani et al., 2012, Vantourout and Hayday, 2013, Ganesan et al., 2021). Significantly higher frequencies of TCRγδ expressing T-cells were found in healthy (vs. patients') samples before cultivation (Fig. 3.A), which might be explained by the general immunosuppressive conditions in neoplastic diseases like AML as already shown (Logan et al., 2020, Rabinovich et al., 2007), which might point towards healthy, TCRγδ expressing T-cells having a functional, antileukemic role in healthy individuals.

We could also find higher frequencies of TCRγδ expressing T-cells after MLC with Kit-M-pretreated (vs. control) healthy and patients' WB (Fig. 3.A; $\%T_{TCR\gamma\delta+}/CD3^{+}$: MLC(M): 24.58 ± 15.80, $p\leq0.005$; MLC(CC): 19.67 ± 14.82 in patients), pointing to a possible functional role of TCRγδ expressing T-cells enabling Kit-M-mediated effector mechanisms.

Klauer et al. showed that Kit-I-treatment (vs. Kit-M-treatment) might lead to increased frequencies of DC, but possibly going along with a weaker induced functionality (Klauer et al., 2024). Similarly, we found higher frequencies of DC (in healthy samples) after DC(I) (vs. DC(M)) (Fig. 1).

4.2.3. MLC with Kit-M-pretreated patients' WB (vs. control) led to increased frequencies of degranulating/ intracellular cytokine producing TCRγδ-expressing T-cells

Immune cell degranulation and the production of intracellular cytokines (e.g. TNFα) can be quantified via DEG and INTCYT assays and are valuable tools to describe (specific) immune cell activation (Klauer et al., 2022, Pepeldjiyska et al., 2021), and can therefore link the activating effect of DCs after MLC with improved antileukemic effects, increased immune cell activation and ultimately, reduced blast counts (Klauer et al., 2022; Rackl et al., 2022; Pepeldjiyska et al., 2021).

We found significantly higher frequencies of degranulating and intracellular cytokine producing TCRγδ expressing T-cells after MLC with patients' Kit-M-pretreated (vs. control) WB (Fig. 3.B)⊡ Kit-M-pretreatment of WB was already shown to increase the degranulation and the cytokine production of immune cells compared to control, which might indicate an increased specifically induced antileukemic functionality of these Kit-M-induced TCRγδ expressing T-cells (Pepeldjiyska et al., 2021, Klauer et al., 2022). No differences of frequencies of degranulating and intracellular cytokine producing T-cells could be found within healthy samples and comparing healthy and patients' samples (Fig. 3.B), which might point to comparable activation levels of degranulating and intracellular cytokine producing TCRγδ expressing T-cells after MLC with Kit-pretreated vs. control WB in healthy samples (but could also be explained by the low number of healthy samples).

4.2.4. MLC with Kit-M-pretreated WB (vs. control) led to increased blastolytic functionality of immune reactive cells

Comparing the blast cytotoxicity of immune cells after MLC, we found significantly more cases with an achieved blast lysis after MLC(M) compared to control, but not after MLC(I) (Fig. 4). We conclude that Kit-M-pretreatment (vs. control) improves the antileukemic activation and the blastolytic functionality of immune cells after MLC, confirming results presented before (Pepeldjiyska et al., 2021, Klauer et al., 2022, Klauer et al., 2024). The achieved and improved blast lysis was superior after MLC(M) compared to MLC(I) with comparable frequencies of lysed blasts and lysis improvement after MLC with Kit-M- and Kit-I-pretreatment. We conclude that Kit-M-pretreatment might be superior compared to Kit-I-treatment to achieve and improve blast lysis (Fig. 4).

Some cases showed a superior blast lysis after 3 hours, while other cases showed a superior blast lysis after 24 hours (of target and effector cell coincubation). This might be explained by different pathways of cell apoptosis (lysis) induction, including fast (granzyme/perforin) pathways and slow (Fas/FasL) pathways (Klauer et al., 2022, Lowin et al., 1994).

4.3. Connecting TCRγδ expressing T-cells with the blastolytic functionality

4.3.1. Higher frequencies of TCRγδ expressing T-cells might indicate effective (chemo)therapy

In uncultured samples from healthy donors (vs. patients) with a functional immune system not affected by tumor immunosuppression (Rabinovich et al., 2007, Teague and Kline, 2013), we found higher

frequencies of TCRγδ expressing T-cells. Tendentially significantly higher frequencies of TCRγδ expressing T-cells were seen in uncultured WB-samples of patients (AML, first diagnosis) that achieved (vs not achieved) remission after induction chemotherapy (Fig. 5.A). These findings might point to a functional (antileukemic) role of TCRγδ expressing T-cells or at least a predictive marker to monitor the response to induction chemotherapy, adding further leads to the scientific discourse on the role of TCR in combating hematological malignancies (Goyal and Nardi, 2022, Minculescu et al., 2019, Saura-Esteller et al., 2022).

4.3.2. Kit-M-mediated increase in TCRγδ expression linked to a higher blastolytic functionality

We found positive correlations between the Kit-M-mediated increased frequencies of degranulating TCRγδ expressing T-cells and the Kit-M-mediated increased blast lysis (Fig. 5.B and 5.C3). This indicates that the increase in frequencies of TCRγδ expressing degranulating T-cells, detected after Kit-M-pretreatment of WB directly correlated with improved blastolytic functionality (Klauer et al., 2022) and might therefore be one of the leukemia specific immunological effects and/or mechanisms after Kit-treatment of WB (Fig. 5. C1, C2, C4). Additionally to the cytotoxic effects mediated by activated cells of the adaptive and innate immune (after stimulation with Kit M pretreated leukemic blood as demonstrated before (e.g: Klauer et al., 2022), this could indicate that TCRγδ T cells also play a role in the mediation of antileukemic reactivities in Kit M pretreated settings -in addition to other antitumor reactivities as shown before (Bensussan et al., 1989, Li et al., 2023) or could be relevant via modulatory effects of TCRγδ expressing T-cells towards other, e.g. cytotoxic effects of innate or adaptive (e.g. $CD8^+$) T-cells, an effect that has been described in the past (Nakamizo et al., 2015). No (tendentially) significant correlations were found after MLC(I), possibly due to an inferior effect of Kit-I-pretreatment on the blastolytic functionality of immune cells after MLC compared to Kit-M-pretreatment, which has been described in the past (Klauer et al., 2022, Klauer et al., 2024).

The potential of TCRγδ for immune therapies has been discussed in the past (Duval et al., 1995, Hannani et al., 2012, Vantourout and Hayday, 2013, Goyal and Nardi, 2022, Saura-Esteller et al., 2022). Even though the induced/increased cytotoxicity could not be assigned to a single mediation by TCRγδ T cells, we can add that leukemia specific TCRγδ expressing T-cells are induced by Kit-M-pretreatment of patients' WB- correlating with increased blastolytic functionality (Fig. 5.B, 5.C3), which might be mediated by a DC/DC_{leu} triggered activation of TCRγδ expressing T-cells (Fig. 3.A) and/ or consecutive immunomodulatory effects as shown in previous works (Hannani et al., 2012, Devilder et al., 2006), which might mean a synergistic effect between TCRγδ T cells and activated cells of the adaptive (e.g.: CD4, CD8 or B cells) or innate immune system (e.g.; NK, CIK, iNKT cells). The role of these cells in (Kit M related) improved blast lysis was shown before (e.g: Klauer et al., 2022, Schutti, 2024, Böck 2017). These findings could indicate that these TCRγδ expressing T-cells possess a cytotoxic potential towards myeloid blasts, which might contribute to develop and refine strategies addressing antitumor immunity (Bensussan et al., 1989, Saura-Esteller et al., 2022).

5. Conclusion

In conclusion, TCRγδ-expressing T-cells could be found in (uncultured) healthy and patients' samples. DCs and their subtypes could be generated using Kit-M-treatment of WB, leading to the activation of immune cells after MLC – thereby increasing frequencies of degranulating and intracellular cytokine producing TCRγδ expressing T-cells and resulting in an increased blastolytic functionality. Furthermore, we found a correlation of the Kit-M-mediated effects on the provision of TCRγδ expressing T-cells and blast lysis, making TCRγδ related strategies interesting for future immunotherapies. These data point to (DC-induced) leukemia-specific TCRγδ T-cells contributing to the improving antileukemic functionality.

Author contributions

E.R. provided great parts of experimental data and all flow cytometric and statistical data analyses. A.H., H.A.R., L.L., L.K.K., S.U., E.P., C.A., M.W., F.D.-G., C.P. and D.C.A. contributed DC, MLC, CTX, and DEG/INTCYT experiments, which were analysed by E.R., as well as whole blood samples and patients' reports. P.B., D.K., J.S., A.R., and C.S. contributed leukemic whole blood samples and patients' reports. The study was designed by H.M.S. The manuscript was drafted by E.R. and H.M.S., and edited by E.R., H.M.S, N.R. and J.S.

Statement of ethics

Collection of samples was conducted with patients' informed consents according to Helsinki guidelines and the vote of the Ethic Committee of LMU in Munich (vote number: 33905).

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CRediT authorship contribution statement

Christoph Schmid: Data curation. **Corinna L Seidel:** Data curation. **Anne Hartz:** Data curation. **Nina Reiter:** Data curation. **Elias Rackl:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation. **Julian Stein:** Writing – review & editing. **Helga Maria Schmetzer:** Writing – review & editing, Validation, Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Fatemeh Doraneh-Gard:** Data curation. **Doris Kraemer:** Data curation. **Selda Ugur:** Data curation. **Peter Bojko:** Data curation. **Lara Kristina Klauer:** Data curation. **Daniel Christoph Amberger:** Data curation. **Lin Li:** Data curation. **Caroline Plett:** Conceptualization. **Hazal Aslan Rejesky:** Data curation. **Melanie Weinmann:** Data curation. **Andreas Rank:** Data curation. **Carina Amend:** Data curation. **Jörg Schmohl:** Data curation. **Elena Pepeldjiyska:** Data curation.

Declaration of Competing Interest

H.M.S. is involved with Modiblast Pharma GmbH (Oberhaching, Germany), holder of the European Patent 15 801 987.7–1118 and the US Patent 15–517627 "Use of immunomodulatory effective compositions for the immunotherapeutic treatment of patients suffering from myeloid leukemias".

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.molimm.2024.09.007.

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