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Interferon Gamma Secretion of Adaptive and Innate Immune Cells as a Parameter to Describe Leukaemia-Derived Dendritic-Cell-Mediated Immune Responses in Acute Myeloid Leukaemia in vitro

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Keywords

Leukaemia-derived dendritic cells · Acute myeloid leukaemia · Anti-leukaemic functionality · Leukaemia-specific cells · Cytokine secretion assay

Abstract

Introduction: Myeloid leukaemic blasts can be converted into leukaemia-derived dendritic cells (DC_{leu}), characterised by the simultaneous expression of dendritic- and leukaemia-associated antigens, which have the competence to prime and enhance (leukaemia-specific) immune responses with the whole leukaemic antigen repertoire. To display and further specify dendritic cell (DC)- and DC_{leu}-mediated immune responses, we analysed the interferon gamma (IFNy) secretion of innate and adaptive immune cells. ***Methods:*** DC/DC_{leu} were generated from leukaemic whole blood (WB) with (blast)modulatory Kit-I (granulocyte-macrophage colony-stimulating factor [GM-CSF] + Picibanil [OK-432]) and Kit-M (GM-CSF + prostaglandin E1) and were used to stimulate T cell-enriched immunoreactive cells. Initiated anti-leukaemic cytotoxicity was investigated with a cytotoxicity fluorolysis assay. Initiated IFNy secretion of T, NK, CIK, and iNKT cells was investigated with a cytokine secretion assay (CSA). IFNy positivity was additionally evaluated with an intracellular cytokine assay (ICA). Recent activation of leukaemia-specific cells was verified through addition of leukaemia-associated antigens (LAA; WT-1 and Prame) ***Results:*** We found Kit-I and Kit-M competent to generate mature DC and DC_{leu} from leukaemic WB without induction of blast proliferation. Stimulation of immunoreactive cells with DC/DC_{leu} regularly resulted in an increased anti-leukaemic cytotoxicity and increased IFNy secretion of T, NK, and CIK cells, pointing to the significant role of DC/DC_{leu} in leukaemia-specific alongside anti-leukaemic reactions. Interestingly, an addition of LAA did not further increase IFNy secretion, suggesting an efficient activation of leukaemia-specific cells. Here, both the CSA and ICA yielded comparable frequencies of IFNy-positive cells. Remarkably, the anti-leukaemic cytotoxicity positively correlated with the IFNy secretion in T^{CD3+}, T^{CD4+}, T^{CD8+}, and NK^{CD56+} cells. ***Conclusion:*** Ultimately, the IFNy secretion of innate and adaptive immune cells appeared to be a suitable parameter to assess and monitor the efficacy of in vitro and potentially in vivo acute myeloid leukaemia immunotherapy. The CSA in this regard proved to be a convenient and reproducible technique to detect and phenotypically characterise IFNy-secreting cells. In respect to our studies on DC-based immunomodulation, we were able to display the potential of DC/DC_{leu} to induce or improve leukaemia-specific and anti-leukaemic activity.

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Introduction

Acute myeloid leukaemia (AML) is a malignant disorder of haematopoietic stem cells characterised by the uncontrolled clonal expansion of abnormally differentiated myeloid blasts. Displacing the physiological haematopoiesis, the accumulation of leukaemic blasts causes typical complications such as erythrocytopenia, thrombocytopenia, and leukocytopenia [1, 2]. Standard therapy for AML consists of chemotherapy with or without allogeneic haematopoietic stem cell transplantation [3, 4]. With overall 5-year survival rates of 28.7%, mainly due to infections and relapses initiated by leukaemic stem cells and residual blasts, the outcome though remains unsatisfactory [5–7].

In recent years, immunotherapeutic approaches in the treatment of AML have gained attention. Different strategies have been developed with the attempt to redirect the immune system in order to overcome the leukaemic immune escape and enforce a tumour-specific immune response. One of the most auspicious approaches in this regard involves the use of dendritic cell (DC)-based vaccines [8–11]. DCs are some of the most potent antigen-presenting cells of the immune system. As they link the innate and the adaptive immune system, they are pivotal in initiating and regulating an antigen-specific immune response [12, 13]. As such, DCs can be exploited to present tumour antigens in order to induce a potent anti-tumour immunity.

DCs can be generated ex vivo from leukaemic myeloid blasts resulting in mature leukaemia-derived DC (DC_{leu}) [14–16], characterised by the simultaneous expression of dendritic cell antigens, leukaemia-specific antigens, and CCR7 as maturation and migration marker [17, 18]. Thus, presenting the whole leukaemic antigen repertoire, mature DC_{leu} have the competence to activate T cells and likely natural killer cells (NK cells), cytokine-induced killer cells (CIK cells), and invariant natural killer cells (iNKT) to (re)gain leukaemia-specific activity [15, 16, 18–20].

Leukaemia-specific activity or rather cytotoxicity implies a complex synergy of the innate and adaptive cellular immune system as well as the humoral immune system. Cytotoxicity is exerted by various innate and adaptive immune cells via different mechanisms: through the (rather early and fast) release of the cytolytic molecules perforin and granzyme by degranulation, through the (rather late and slow) interaction with the Fas ligand (FasL) and TNF-related apoptosis-including ligand (TRAIL), and/or through the secretion of tumour necrosis factor alpha (TNFa) and interferon gamma (IFNy) [21–23]. Especially the latter, IFNy, has a wide-ranging role: it is not only essential to regular immunity, promoting innate and adaptive immune responses and thereby licensing immune cells to exert cytotoxicity, but also strongly associated with anti-tumour immunity, promoting tumour surveillance, recognition, and elimination [24]. However, it has been noted before that under certain circumstances IFNy can also stimulate immune-suppressive mechanisms in tumour cells, including upregulation of indoleamine 2,3-dioxygenase and of checkpoint inhibitors such as programmed cell-death ligand 1, leading to immune escape [25, 26].

In the early phase of an immune response, IFNy is mainly secreted by innate immune cells such as NK, iNKT, DC, and macrophages upon activation [27, 28]. It not only stimulates innate immune cells by enhancing their effector mechanisms and IFNy secretion, but also facilitates tumour recognition and elimination. By up-regulation of the major histocompatibility complex (MHC) class I and II antigen- and cross-presentation pathways, as well as the MHC-II expression on classically non-MHC-II-expressing cells, it increases the perceptibility of tumour cells to adaptive immune cells. Moreover, by up-regulation of the expression of FasR, FasL, TRAIL, caspase-1, -3, -7, -8, and BIM and down-regulating the expression of survivin, it increases the susceptibility of tumour cells to extrinsic and intrinsic pathways of apoptosis [25, 26, 29–33]. Importantly, by promoting the differentiation, proliferation, and activation of helper T cells type 1 (Th1) and cytotoxic T cells (Tc), IFNy links the innate and adaptive immunity, thereby commencing the advanced phase of an immune response. Moreover, it inhibits the differentiation of helper T cells type 2 (Th2) and regulatory T cells [26–28, 34]. In the advanced phase of an immune response, IFNy is mainly secreted by adaptive immune cells such as Th1 and Tc cells upon primary activation by DC or secondary activation by its specific antigen. IFNy thereupon not only stimulates adaptive immune cells by enhancing their effector mechanisms and IFNy secretion, but also innate immune cells [27, 28, 35]. That way, IFNy creates a positive feedback loop and, together with further cytokines, is able to stimulate and sustain an effective immune response, incorporating both innate and adaptive immune cells.

IFNy is considered as sine qua non for the immune system. It is an important mediator of the innate and adaptive immunity and plays a pivotal role in anti-tumour immunity. A blockade of this critical cytokine restrains an effective immune defence [36, 37]. This inseparable connection between IFNy and cell-based immunity has lead us to hypothesise that IFNy could be a suitable parameter to display leukaemia-specific activity and cytotoxicity. Particularly in respect to future applications, IFNy readouts might have the potential to be a convenient parameter to assess and monitor the efficacy of AML immunotherapy.

Table 1. Patients' characteristics

Patient No.	Age	Sex	FAB type	Stage	Blast phenotype (CD)	IC blasts, %	Cyto-, molecular genetics	Source	Conducted experiments
AML									
1489	55	f	p–M0	dgn	13, 33, **34**, 65, **117**	61	46, XX; FLT3-ITD mut, NMP1 mut	MNC	DCC, MLC, CTX, CSA
1490	65	m	s–M?	dgn	**15**, 33, 56, **65**	46	46, XY; del(20) (q12q13)[12]; NPM1 wt	MNC	DCC, MLC, CTX, CSA
1509	60	m	p–M2	dgn	13, 33, **34**, 65, **117**	48	46, XY; FLT3-TKD wt, NPM1 mut	WB	DCC, MLC, CTX, CSA, ICA
1511	78	m	p–M4	rel	13, 15, 33, **34**, 65, **117**	54	FLT3-ITD mut, RUNX1 mut	WB	DCC, MLC, CTX, CSA
1514	68	m	s–M?	dgn	33, 56, **117**	36	46, XY; FLT3-TKD wt, NPM1 mut	WB	DCC, MLC, CSA
1515	67	f	p–M2	dgn	33, **34,** 65, **117**	80	46, XX; FLT3-TKD wt, NPM1 mut	WB	DCC, MLC, CSA
1518	83	f	p–M5	dgn	14, 15, **34**, **65**	72	46, XX; FLT3-TKD wt, NPM1 mut	WB	DCC, MLC, CSA, ICA
1521	56	m	p–M4	dgn	13, **15**, 33, **34**, 65, **117**	72	46, XY; FLT3-TKD mut, NPM1 mut	WB	DCC, MLC, CTX, CSA
1525	77	m	p–M1	dgn	13, 15, 33, **34**, **117**	78	46, XY; FLT3-ITD wt, FLT3-TKD mut, NPM1 mut	WB	DCC, MLC, CSA
1526	74	f	p–M?	dgn	15, 33, **34**, 56, **65**, **117**	59	46, XX; FLT3-TKD wt, NPM1 mut	WB	DCC, MLC, CSA
1527	42	m	p–M2	dgn	7, 13, 15, 33, **34**, **65**, **117**	51	46, XY; FLT3-ITD mut, NPM1 mut	WB	DCC, MLC, CTX, CSA, ICA
1531	71	m	p–M4/5	dgn	13, 33, **34**, **117**	24	n.d.	WB	DCC, MLC, CSA, ICA
1536	61	m	p–M5	dgn	14, **34**, 56	73	46, XY; NPM1 mut	WB	DCC, MLC, CTX, CSA
1562	37	m	p–M1	dgn	2, 7, 13, **34**, 117	82	46, XY; del(2) (q21), der(14) t(2; 14) (q21; q32)[7]/46,XY[3]; FLT3-TKD mut, RUNX1 mut	WB	DCC, MLC, CTX, CSA
1565	62	f	p–M4	dgn	13, 15, **34**, 65, **117**	31	46, XX	WB	DCC, MLC, CTX, CSA
1567	98	f	p–M?	dgn	14, 15, **34**, 56	57	46,XX del(5q31), del(5q32-33), MECOM rearrangement inv(3) (q21q26.2)/t(3; 3) (q21;q26.2); FLT3-TKD wt, NPM1 wt	WB	DCC, MLC, CTX, CSA
1568	29	m	p–M?	dgn	10, 13, 33, **34**	79	46, XY	WB	DCC, MLC, CTX, CSA
1570	36	f	p–M?	dgn	7, 13, 14, 33, **34**, **117**	33	46, XX; NPM1 mut	WB	DCC, MLC, CTX, CSA
1572	64	f	p–M?	dgn	13, 33, **34**, 65, **117**	50	46, XX; RUNX1 mut, EZH2 mut, BCOR mut, U2AF1 mut, ASXL1 mut	WB	DCC, MLC, CTX, CSA, ICA
Healthy									
1486	57	f						MNC	CSA
1499	21	f						MNC, WB	DCC, MLC, CSA
1505	22	m						MNC, WB	DCC, MLC, CSA
1523	17	m						WB	DCC, MLC, CSA

The aim of the study was to generate DC/DC$_{leu}$ with immunomodulatory Kit-I and Kit-M (DC/DC$_{leu}$-Kit-I and DC/DC$_{leu}$-Kit-M) from leukaemic whole blood (WB) and therewith stimulate autologous T cell-enriched immunoreactive cells. We investigated the resulting anti-leukaemic cytotoxicity with a cytotoxicity fluorolysis assay (CTX) and the resulting IFNγ secretion of innate and adaptive immune cells with a cytokine secretion assay (CSA). IFNγ production was additionally evaluated with an intracellular cytokine assay (ICA). Ultimately, we correlated the IFNγ secretion with the anti-leukaemic cytotoxicity of DC/DC$_{leu}$-stimulated immunoreactive cells.

Material and Methods

Sample Collection

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 Hospital Munich (vote No. 33905). Samples in form of heparinised peripheral WB were provided by the University Hospitals of Augsburg, Oldenburg, and Munich.

Patients' Characteristics

Blood samples were obtained from AML patients (n = 19) with a mean age of 62.2 years (range 29–98 years) and a female-to-male ratio of 1:1.4, and from healthy volunteers (n = 4) with a mean age of 29.3 (range 16–57 years) and a female-to-male ratio of 1:1. AML patients were characterised by the French-American-British (FAB) classification (M1-M7), the aetiology (primary AML, secondary AML), the stage of disease (first diagnosis, relapse), the blast phenotype, the blast frequency in peripheral blood, and the cyto- and molecular genetics. An overview is given in Table 1.

AML patients presented in WB/mononuclear cells (MNC) with an average of 57.6/53.6% leukemic blasts (range 23.7–80.4/45.9–61.2%), 11.4/1.4% T^{CD3+} cells (range 0.9–19.4/0.5–2.2%), 3.3/7.6% B^{CD19+} cells (range 0.1–3.9/0.7–14.6%), 2.8/0.7% NK^{CD56+} cells (range 0.3–8.1/0.5–0.9%), and 5.3/2.7% monocytes^{CD14+} (range 0–16.3/1.6–3.7%). In cases with aberrant expression of T, B, NK, or monocytoid antigens, proportions were not included in the analyses.

Cell Characterisation by Flow Cytometry

Flow cytometric analyses were implemented to evaluate and quantify frequencies, phenotypes, and subsets of leukaemic blasts,

Table 2. Cells and cell subsets as evaluated by flow cytometry

	Abbreviation of subgroups	Surface marker	Referred to	Abbreviation	Ref.
Blast cells					
Blasts	BLA	BLA^+ ($CD15^+$, $CD34^+$, $CD65^+$, $CD117^+$)	WB or MNC	BLA/WB or/MNC	[17]
Proliferating blasts	BLA_{prol}	$BLA^+DC^-CD71^+$	BLA	BLA_{prol}/BLA	[39]
Dendritic cells					
Dendritic cells	DC	DC^+ ($CD80^+$, $CD206^+$)	WB or MNC	DC/WB	[17]
Leukaemia-derived DC	DC_{leu}	DC^+BLA^+	WB or MNC DC BLA	DC_{leu}/WB or /MNC DC_{leu}/DC DC_{leu}/BLA	[17]
Mature DC	DC_{mat}	DC^+CCR7^+	WB or MNC DC	DC_{mat}/WB or /MNC DC_{mat}/DC	[18]
Mature DC_{leu}	$DC_{mat\text{-}leu}$	$DC^+BLA^+CCR7^+$	WB or MNC DC_{leu} DC_{mat}	$DC_{leu\text{-}mat}$/WB or /MNC $DC_{leu\text{-}mat}/DC_{leu}$ $DC_{leu\text{-}mat}/DC_{mat}$	
Monocytoid cells					
$CD14^+$ monocytes	$monocytes^{CD14+}$	$CD14^+$	WB	$monocytes^{CD14+}$/WB	[18]
T cells					
$CD3^+$ pan T cells	T^{CD3+}	$CD3^+$	WB or MNC	T^{CD3+}/WB or MNC	[75]
$CD4^+$-coexpressing T cells	T^{CD4+}	$CD3^+CD4^+$	$CD3^+$	$T^{CD4+}/CD3^+$	[75]
$CD8^+$-coexpressing T cells	T^{CD8+}	$CD3^+CD8^+$	$CD3^+$	$T^{CD8+}/CD3^+$	[75]
Naive T cells	T_{naive}	$CD3^+CD45RO^-$	$CD3^+$	$T_{naive}/CD3^+$	[41]
Non-naive T cells	$T_{non\text{-}naive}$	$CD3^+CD45RO^+$	$CD3^+$	$T_{non\text{-}naive}/CD3^+$	[41]
Central (memory) T cells	T_{cm}	$CD3^+CD45RO^+CCR7^+$	$CD3^+$	$T_{cm}/CD3^+$	[41]
Effector (memory) T cells	T_{em}	$CD3^+CD45RO^+CCR7^-$	$CD3^+$	$T_{em}/CD3^+$	[41]
Proliferating T cells – early	$T_{prol\text{-}early}$	$CD3^+CD69^+$	$CD3^+$	$T_{prol\text{-}early}/CD3^+$	[75]
Proliferating T cells – late	$T_{prol\text{-}late}$	$CD3^+CD71^+$	$CD3^+$	$T_{prol\text{-}late}/CD3^+$	[75]
B cells					
$CD19^+$ B cells	B^{CD19+}	$CD19^+$	WB or MNC	B^{CD19+}/WB or /MNC	[20]
CIK cells					
$CD3^+CD56^+$ CIK cells	CIK^{CD56+}	$CD3^+CD56^+$	WB or MNC	CIK^{CD56+}/WB or /MNC	[20]
$CD3^+CD161^+$ CIK cells	CIK^{CD161+}	$CD3^+CD161^+$	WB or MNC	CIK^{CD161+}/WB or /MNC	
NK cells					
$CD3^-CD56^+$ NK cells	NK^{CD56+}	$CD3^-CD56^+$	WB or MNC	NK^{CD56+}/WB or /MNC	[20]
$CD3^-CD161^+$ NK cells	NK^{CD161+}	$CD3^-CD161^+$	WB or MNC	NK^{CD161+}/WB or /MNC	
iNKT cells					
$6B11^+$ iNKT cells	iNKT	6B11+	WB or MNC	iNKT/WB or /MNC	[20]
IFNy-secreting cells					
IFNy-secreting $CD3^+$ pan T cells		$CD3^+IFNy^+$	$CD3^+$	$T^{CD3+}IFNy^+/T^{CD3+}$	
IFNy-secreting $CD4^+$-coexpressing T cells		$CD3^+CD4^+IFNy^+$	$CD3^+CD4^+$	$T^{CD4+}IFNy^+/T^{CD4+}$	
IFNy-secreting $CD8^+$-coexpressing T cells		$CD3^+CD8^+IFNy^+$	$CD3^+CD8^+$	$T^{CD8+}IFNy^+/T^{CD8+}$	
IFNy-secreting $CD3^+CD56^+$ CIK cells		$CD3^+CD56^+IFNy^+$	$CD3^+CD56^+$	$CIK^{CD56+}IFNy^+/CIK^{CD56+}$	
IFNy-secreting $CD3^+CD161^+$ CIK cells		$CD3^+CD161^+IFNy^+$	$CD3^+CD161^+$	$CIK^{CD161+}IFNy^+/CIK^{CD161+}$	
IFNy-secreting $CD3^-CD56^+$ NK cells		$CD3^-CD56^+IFNy^+$	$CD3^-CD56^+$	$NK^{CD56+}IFNy^+/NK^{CD56+}$	
IFNy-secreting $CD3^-CD161^+$ NK cells		$CD3^-CD161^+IFNy^+$	$CD3^-CD161^+$	$NK^{CD161+}IFNy^+/NK^{CD161+}$	
IFNy-secreting $6B11^+$ iNKT cells		$6B11^+IFNy^+$	$6B11^+$	$iNKT^+IFNy^+/iNKT^+$	

Surface marker combinations for flow cytometric staining and analysis.

DCs, monocytes, B cells, T cells, NK cells, CIK cells, and iNKT cells. Abbreviations of all cell types are given in Table 2.

Cells were stained with various monoclonal antibodies (moAbs) labelled with fluorescein isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin-cyanine7 tandem conjugate (PE-Cy7), or allophycocyanin (APC). Antibodies were provided by Beckman Coulter[a] (Krefeld, Germany), Becton Dickinson[b] (Heidelberg, Germany), Miltenyi Biotec[c] (Bergisch Gladbach, Germany), BioLegend[d] (Koblenz, Germany), and Santa Cruz Biotechnology[e] (Heidelberg, Germany). For analyses FITC-conjugated moAbs CD3[b], CD14[a], CD15[a], CD45RO[a], CD65[a], CD71[a], CD161[b], IPO38[e]; PE-conjugated moAbs CD3[a], CD4[b], CD34[a], CD56[a], CD65[c], CD80[a], 6B11[b], IFNy[d]; PE-Cy7-conjugated moAbs CD3[a], CD4[a], CD14[b], CD33[a], CD34[a], CD65[c], CD117[a], CD197[b]; APC-conjugated moAbs

Table 3. DC/DCleu-generating protocols

DC/DC_{leu} protocol	DC/DC_{leu} source	Composition (total)	Time of addition	Time of culture	Reference
Pici-$_{PGE1}$	MNC	GM-CSF 500 U/mL IL-4 250 U/mL OK-432 10 µg/mL PGE_1 1 µg/mL	d0 d0 d7 d7	9 days	[38]
Kit-I	WB	GM-CSF 800 U/mL OK-432 10 µg/mL	d0, d2–3 d0, d2–3	7–8 days	[Unpublished data] European Patent No. 15 801 987.7-1118
Kit-M	WB	GM-CSF 800 U/mL PGE_1 1 µg/mL	d0, d2–3 d0, d2–3	7–8 days	[Unpublished data] European Patent No. 15 801 987.7-1118
Mode of action	GM-CSF IL-4 OK-432 PGE_1	Induction of myeloid and DC differentiation Induction of DC differentiation Danger signalling, stimulation of DC differentiation Danger signalling, stimulation of DC maturation and migration (via CCR7 expression)			[16, 38]

GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-4, interleukin 4; OK-432, Picibanil; PGE_1, prostaglandin E_1; d day.

CD3[a], CD14[a], CD19[a], CD34[a], CD56[a], CD69[b], CD117[a], CD206[b], 6B11[d] were used. Non-viable cells were detected with 7AAD[b].

In preparation of staining, erythrocytes in WB samples were lysed using lysing buffer (Becton Dickinson) according to the manufacturer's instructions. Staining was performed by a 15-min incubation of cells with the corresponding moAbs in the dark at room temperature using a staining medium containing 95% PBS (Biochrom, Berlin, Germany) and 5% FCS (Biochrom). Intracellular staining (e.g., IPO38, IFNy) was performed with the FIX&PERM Cell Fixation and Cell Permeabilisation Kit (Thermo Fisher Scientific, Darmstadt, Germany).

Stained cells were analysed with the fluorescence-activated cell sorting flow cytometer FACS Calibur (Becton Dickinson) and the acquisition and analysis software CellQuestPro (Becton Dickinson). Isotype controls were conducted according to the manufacturer's instructions.

Sample Preparation

MNC were isolated from WB by density gradient centrifugation using the Ficoll-Hypague technique and a separating solution with a density of 1.077 g/mL (Biocoll, Biochrom). T cells were isolated from MNC using the MACS microbead and column-based immunomagnetic cell separation technology (Miltenyi Biotec) via positive selection of $CD3^+$ cells according to the manufacturer's instructions. Purity of isolated T cells was on average 80.5% (range 57.7–95.3%). MNC and T cells, unless directly used, were frozen with 70% RPMI-1640 medium (Biochrom), 20% human serum (HealthCare Europa GmbH, Vienna, Austria), 10% dimethyl sulfoxide (Sigma Aldrich Chemie GmbH, Steinheim, Germany), stored at −80°C and thawed when required.

Dendritic Cell Culture

The generation of DC/DC_{leu} was performed by the stimulation of MNC or WB with specific response modifiers, including granulocyte-macrophage colony-stimulating factor (GM-CSF) (Sanofi-Aventis, Frankfurt, Germany), interleukin 4 (IL-4) (PeproTech, Berlin, Germany), Picibanil (OK-432) (Chugai Pharmaceutical Co., Kajiwara, Japan), and prostaglandin E1 (PGE_1) (PeproTech). Compositions of DC/DC_{leu}-generating protocols are given in Table 3.

DC/DC_{leu} from healthy and leukaemic MNC were generated with the DC/DC_{leu}-generating protocol Pici-$_{PGE1}$ [38]. For this, 3–4 × 10^6 MNC were cultured in 12-multiwell culture plates (ThermoFisher Scientific) and diluted with 2 mL x-vivo 15 medium (Lonza, Basel, Switzerland). Response modifiers were added to the cultures (further referred to as $MNC^{DC(P)}$) according to the protocol. A culture without added response modifiers served as negative control ($MNC^{DC(Control)}$). A half-medium exchange was carried out every 2–3 days. Cells were harvested after 9 days.

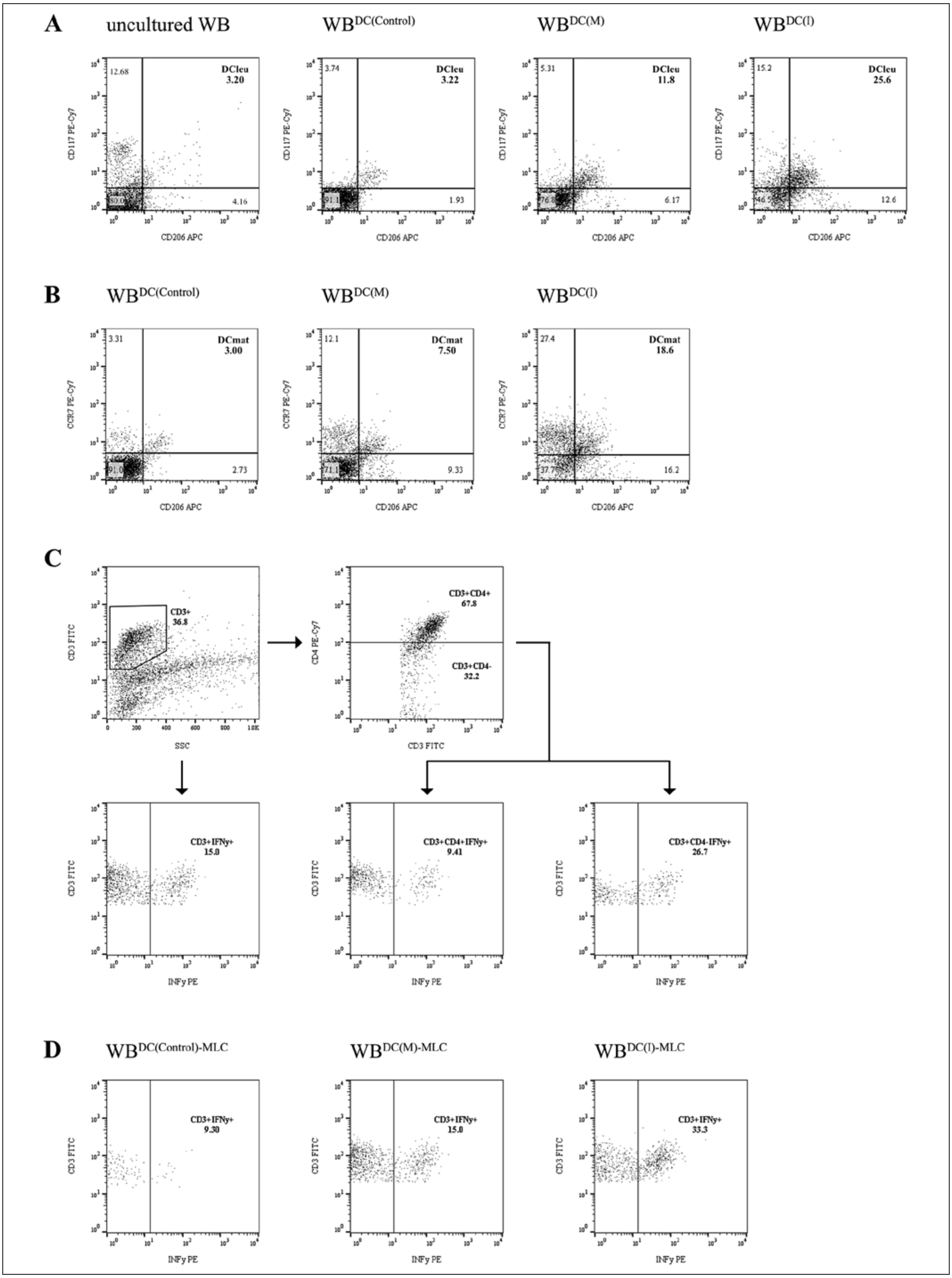
DC/DC_{leu} from healthy and leukaemic WB were generated with the DC/DC_{leu}-generating protocols Kit-I and Kit-M [unpublished data] [38]. For this, 500 µL WB (corresponding to 5.0–30.3 × 10^6 MNC) were cultured in 24-multiwell culture plates (ThermoFisher Scientific) and diluted with 500 µL x-vivo 15 medium. Response modifiers were added to the cultures (further referred to as $WB^{DC(I)}$, $WB^{DC(M)}$) according to the protocol. Likewise, a culture without added response modifiers served as negative control ($WB^{DC(Control)}$). Cells were harvested after 7–8 days.

DC cultures as well as subsequent mixed lymphocyte cultures (MLC) were incubated at 37°C, 21% O_2, and 5% CO_2. In some cases, additional cultures were incubated simultaneously in hypoxia-like conditions (37°C, 10% O_2, 10% CO_2).

Flow cytometric analyses of leukaemic blasts, DC, DC_{leu}, and DC_{mat} were performed before and after dendritic cell culture

Fig. 1. Flow cytometric analyses. **A** Conversion of myeloid blasts into DC_{leu} through blastmodulatory Kit-I and Kit-M. Exemplary plots show DC_{leu}, characterised by the co-expression of the blast marker CD117 and the DC marker CD206, in uncultured WB, $WB^{DC(Control)}$, $WB^{DC(M)}$, and $WB^{DC(I)}$. **B** Gain of maturation through Kit-I and -M. Exemplary plots show DC_{mat}, characterised by the co-expression of the DC marker CD206 and the maturation marker CCR7, in $WB^{DC(Control)}$, $WB^{DC(M)}$, and $WB^{DC(I)}$. **C** Gating of IFNy-secreting T^{CD3+} ($CD3^+IFNy^+$), T^{CD4+} ($CD3^+CD4^+IFNy^+$), and T^{CD8+} ($CD3^+CD4^-IFNy^+$) cells as detected by CSA. **D** Comparison of IFNy-secreting T^{CD3+} ($CD3^+IFNy^+$) cells in $WB^{DC(Control)\text{-}MLC}$, $WB^{DC(M)\text{-}MLC}$, and $WB^{DC(I)\text{-}MLC}$. Abbreviations of all cell types are given in Table 2.

(For figure see next page.)


A
uncultured WB
WBDC(Control)
WBDC(M)
WBDC(I)
12.68
DCleu
3.20
80.0
4.16
3.74
DCleu
3.22
91.1
1.93
5.31
DCleu
11.8
76.8
6.17
15.2
DCleu
25.6
46.5
12.6
CD117 PE-Cy7
CD206 APC
B
WBDC(Control)
WBDC(M)
WBDC(I)
3.31
DCmat
3.00
91.0
2.73
12.1
DCmat
7.50
71.1
9.33
27.4
DCmat
18.6
37.7
16.2
CCR7 PE-Cy7
CD206 APC
C
CD3+
36.8
CD3 FITC
SSC
CD3+CD4+
67.8
CD3+CD4-
32.2
CD4 PE-Cy7
CD3 FITC
CD3+IFNy+
15.0
CD3+CD4+IFNy+
9.41
CD3+CD4-IFNy+
26.7
INFy PE
D
WBDC(Control)-MLC
WBDC(M)-MLC
WBDC(I)-MLC
CD3+IFNy+
9.30
CD3+IFNy+
15.0
CD3+IFNy+
33.3
CD3 FITC
INFy PE
1

(DCC) using a refined gating strategy [unpublished data] [16, 17, 39]. DC_{leu} were analysed by the co-expression of at least one blast marker (CD15, CD34, CD65, CD117) including lineage-aberrant markers (CD 56) and one DC marker that had not been expressed on naive blasts (CD80, CD206; of which CD80 qualified in 82.4%, CD206 in 94.1%, and both CD80 and CD206 in 76.5% of cases). Maturation of DC/DC_{leu} was analysed by the further co-expression of CCR7 (Fig. 1A, B). Premise for DC subgroup analyses was the presence of ≥5% DCs in the total cell fraction.

We conducted preliminary experiments to assess the feasibility and comparability of DC/DC_{leu} generation in different settings. As these experiments and previous studies [unpublished data] [38, 40] affirmed the feasibility and comparability with $Pici\text{-}_{PGE1}$ in healthy and leukaemic MNC, with Kit-I and Kit-M in healthy and leukaemic WB, as well as under hypoxia-like and normoxia-like conditions (data not shown), further experiments were conducted on leukaemic WB to adapt to more physiological conditions, under normoxia-like conditions.

Mixed Lymphocyte Culture

Consecutive generation of T cell-enriched immunoreactive cells was performed by the stimulation of autologous T cells with DC/DC_{leu}-containing MNC^{DC} or WB^{DC}.

Based on a MNC model, 1×10^6 T cells and a fraction of MNC^{DC} containing 0.25×10^6 DC/DC_{leu} were co-cultured in a 24-multiwell culture plate (total cell count 1.7–4.8 × 10^6 MNC) and diluted in 1 mL RMPI-1640 medium containing 100 U/mL penicillin (Biochrom) and 15% human serum. 50 U/mL IL-2 were added on day 0 and day 2–3 to all cultures (further referred to as $MNC^{DC(P)\text{-}MLC}$, $MNC^{DC(Control)\text{-}MLC}$). A half-medium exchange was carried out every 2–3 days. Cells were harvested after 7–9 days.

Based on a WB model, 1×10^6 T cells and a fraction of WB^{DC} containing 0.25×10^6 DC/DC_{leu} were co-cultured in a 24-multiwell culture plate (total cell count 1.8–9.4 × 10^6 MNC) and diluted in 1 mL RMPI-1640 medium containing 100 U/mL penicillin. 50 U/mL IL-2 were added on day 0 and day 2–3 to all cultures (further referred to as $WB^{DC(I)\text{-}MLC}$, $WB^{DC(M)\text{-}MLC}$, $WB^{DC(Control)\text{-}MLC}$). Cells were harvested after 6–7 days.

Flow cytometric analyses of T-cell subsets were performed before and after MLC using a refined gating strategy [18, 19, 41].

We conducted preliminary experiments to assess the feasibility and comparability of DC/DC_{leu} stimulation on immunoreactive cells and its resulting anti-leukaemic cytotoxicity in different settings. As these experiments affirmed the feasibility and comparability in (healthy and) leukaemic MNC and WB (data not shown), further experiments were conducted on leukaemic WB to adapt to more physiological conditions.

Cytotoxity Fluorolysis Assay

A fluorolysis assay was performed to analyse the lytic activity of T cell-enriched immunoreactive cells against leukaemic blasts (further referred to as anti-leukaemic cytotoxicity) of $MNC^{DC\text{-}MLC}$ and $WB^{DC\text{-}MLC}$ [18]. Therefore, a fraction of $MNC^{DC\text{-}MLC}$ and $WB^{DC\text{-}MLC}$ containing 1×10^6 T cells (effector cells) and 1×10^6 thawed autologous leukaemic blasts (target cells) were co-cultured (total cell count 2.3–6.0 × 10^6 MNC) diluted in 1 mL RMPI-1640 medium containing 100 U/mL penicillin and 15% human serum for 3 and 24 h at 37°C, 21% O_2, 5% CO_2. Target cells were stained with FITC-, PE-, or APC-conjugated blast-specific moAbs before culture, and with 7AAD and a defined number of fluorosphere beads (Beckman Coulter) after culture when harvested. All assays were performed in combination with a control, for which effector and target cells were cultured analogously but separated and only merged prior to flow cytometric analyses.

Flow cytometric analyses were performed using a refined gating strategy [18]. Achieved anti-leukaemic cytotoxicity is described as "blast lysis" defined as the percentual difference of viable target cells between the effector-target cell culture and the control, "cases with blast lysis" defined as the proportion of cases with blast lysis >0%, "improved blast lysis" defined as the percentual difference of the blast lysis of $WB^{DC(I)\text{-}MLC}$ or $WB^{DC(M)\text{-}MLC}$ and $WB^{DC(Control)MLC}$, and "cases with improved blast lysis" defined as the proportion of cases with improved blast lysis >0%.

Cytokine Secretion Assay

For the detection of IFNy-secreting cells in MNC, $MNC^{DC\text{-}MLC}$, WB and $WB^{DC\text{-}MLC}$, an IFNy secretion assay (Miltenyi Biotec) was performed. According to the manufacturer's instructions, cells were firstly labelled with an IFNy Catch Reagent (Miltenyi Biotec), a bi-specific moAB directed against the panleukocytic marker CD45 and IFNy. By connecting to leukocytes during a non-IFNy-secretion period (10 min, on ice), followed by connecting to IFNy during an IFNy-secretion period (45 min, 37°C), IFNy could be bound to the positive secreting cells. For detection by flow cytometry, cells were secondly labelled with an IFNy-specific PE-conjugated IFNy Detection Antibody (Miltenyi Biotec).

In some cases, an additional stimulation of MNC, $MNC^{DC\text{-}MLC}$, WB, and $WB^{DC\text{-}MLC}$ was performed prior to the CSA, for which cells were incubated for 4 h with a leukaemia-associated antigen (LAA) suspension containing 50 µg/mL WT-1 (Miltenyi Biotec) and 50 µg/mL PRAME (Miltenyi Biotec) or with 1 µg/mL staphylococcal enterotoxin B (SEB, Sigma Aldrich Chemie GmbH).

For flow cytometric analyses of IFNy-secreting cells, cells were co-stained with FITC-, PE-Cy7-, and APC-conjugated moAbs. Analyses of IFNy-secreting T, NK, CIK, and iNKT cells were performed with a gating strategy described in Figure 1C, D.

We conducted preliminary experiments to assess the feasibility of the CSA in different settings. As these experiments affirmed the feasibility in uncultured and cultured, healthy and leukaemic, MNC and WB, as well as under hypoxia-like and normoxia like conditions, with comparability in MNC and WB and under hypoxia-like and normoxia-like conditions (data not shown), further experiments were conducted on leukaemic WB to adapt to more physiological conditions, under normoxia-like conditions.

Intracellular Cytokine Assay

For the detection of intracellular IFNy in WB and $WB^{DC\text{-}MLC}$, an intracellular cytokine assay was performed. To avoid cytokine secretion during the assay, cells were firstly incubated with brefeldin A (1000X, BioLegend) concentrated at 1:1,000 for 15 h. Intracellular staining of IFNy subsequently was procured using the FIX&PERM Cell Fixation and Cell Permeabilisation Kit according to the manufacturer's instructions. For flow cytometric analysis of IFNy-producing cells, cells were co-stained with FITC-, PE-Cy7-, and APC-conjugated moAbs. Analyses of IFNy-secreting T, NK, CIK, and iNKT cells were performed with the same gating strategy as used for the CSA.

Statistical Methods

Data is presented as mean ± standard deviation (SD). Statistical comparisons for two groups were performed using the two-tailed *t* test and the Pearson correlation coefficient. Significance was defined as "not significant" (n.s.) with *p* values >0.10, as "borderline significant" with *p* values 0.10 to 0.05, and as "significant" with *p* values <0.05. Correlation was defined as "negligible" with *r* values 0.00 to 0.30 (−0.00 to −0.30), as "low" with *r* values 0.30 to 0.50 (−0.30 to −0.50), as "moderate" with *r* values 0.50 to 0.70 (−0.50 to

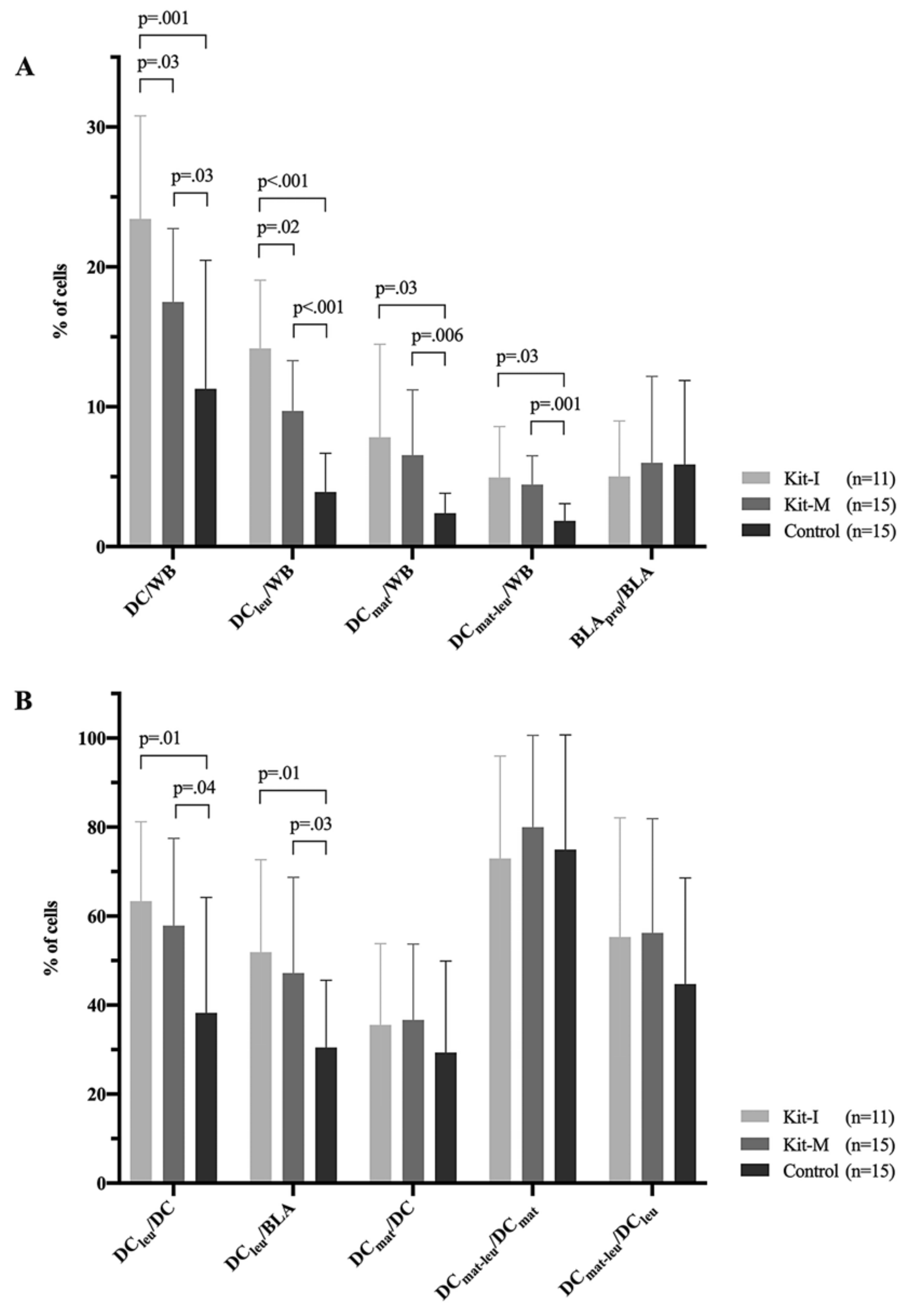


Fig. 2. Generation of (mature) DC and DC_{leu} from leukaemic WB with blastmodulatory Kit-I and Kit-M without induction of blast proliferation. Given are the mean ± SD of DC, DC_{leu}, DC_{mat}, and $DC_{mat\text{-}leu}$ in the WB fraction and BLA_{prol} in the blast fraction in $WB^{DC(I)}$, $WB^{DC(M)}$, and $WB^{DC(Control)}$ (**A**), and the mean ± SD of DC_{leu} in the DC and blast fraction, DC_{mat} in the DC fraction, and $DC_{mat\text{-}leu}$ in the DC_{mat} and DC_{leu} fraction in $WB^{DC(I)}$, $WB^{DC(M)}$, and $WB^{DC(Control)}$ (**B**). Statistically significant (*p* values <0.05) and borderline significant (*p* values 0.10 to 0.05) differences are given. Abbreviations of all cell types are given in Table 2.

−0.70), and as “high” with *r* values 0.70 to 1.00 (−0.70 to −1.00). Statistical analyses and figures were implemented with GraphPad Prism 8 (GraphPad Software, California, USA) and Pages 8.2 (Apple, California, USA).

Results

Generation of Mature DC/DC_{leu} from Leukaemic WB without Induction of Blast Proliferation

We were able to generate significantly higher frequencies of DC/WB and DC_{leu}/WB with Kit-I and Kit-M compared to control, with significantly higher frequencies in $WB^{DC(I)}$ than $WB^{DC(M)}$. Differentiating DC_{leu} in its subgroups showed significantly higher frequencies of DC_{leu}/BLA as well as of DC_{leu}/DC in $WB^{DC(I)}$ and $WB^{DC(M)}$ compared to $WB^{DC(Control)}$. Frequencies of DC_{leu}/BLA and DC_{leu}/DC did not differ significantly in $WB^{DC(I)}$ compared to $WB^{DC(M)}$ (Fig. 1A, 2A, B).

We furthermore evaluated the maturation of DC and found significantly higher frequencies of DC_{mat}/WB and $DC_{mat\text{-}leu}$/WB and no significantly different frequencies of DC_{mat}/DC in $WB^{DC(I)}$ and $WB^{DC(M)}$ compared to $WB^{DC(Control)}$. Differentiating $DC_{mat\text{-}leu}$ in its subgroups showed no significantly different frequencies of $DC_{mat\text{-}leu}$/DC_{mat} and no significantly different frequencies of $DC_{mat\text{-}leu}$/DC_{leu} in $WB^{DC(I)}$ and $WB^{DC(M)}$ compared to $WB^{DC(Control)}$. Frequencies of DC_{mat} and $DC_{mat\text{-}leu}$ in depicted cell groups did not differ significantly in $WB^{DC(I)}$ compared to $WB^{DC(M)}$ (Fig. 1B, 2A, B).

Reviewing the effect of Kits on the proliferation of non-converted blasts during DCC, we found no significant shift of BLA_{prol}/BLA in $WB^{DC(I)}$ and $WB^{DC(M)}$ compared to $WB^{DC(Control)}$ (Fig. 2A).

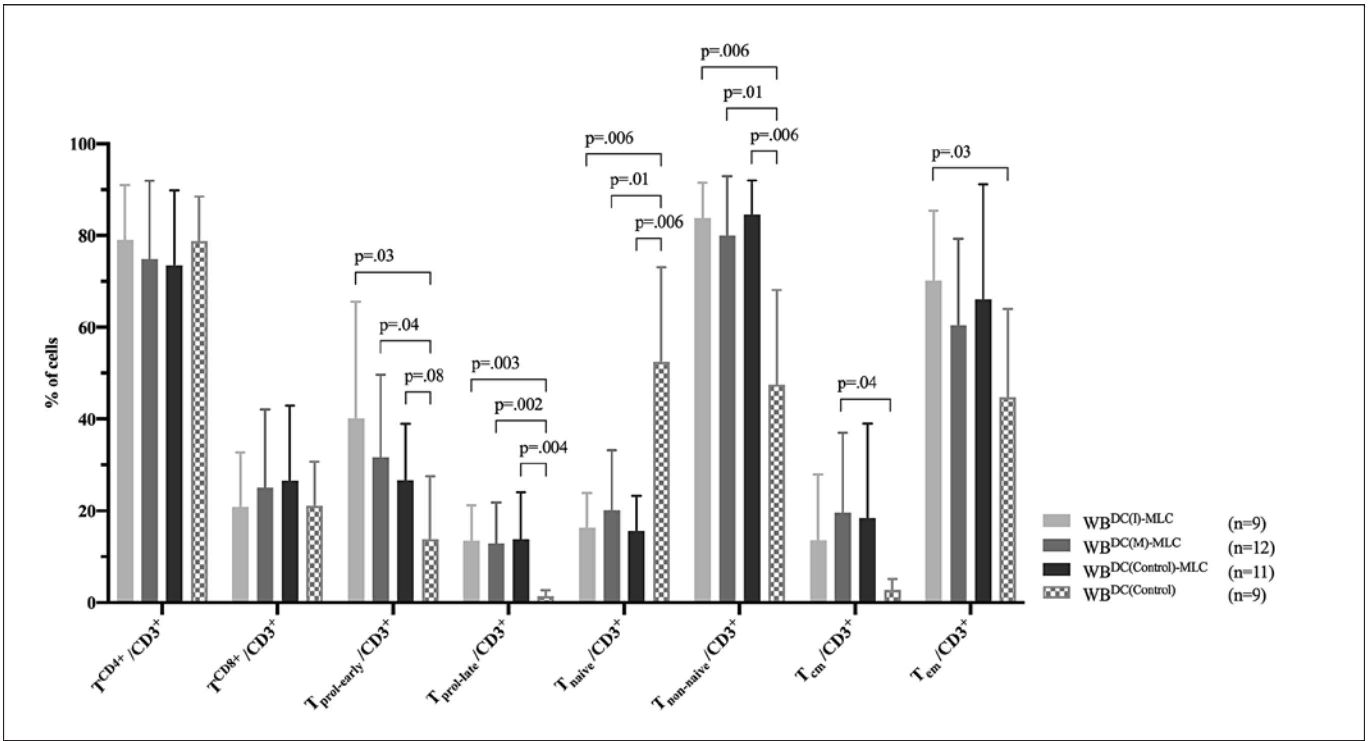
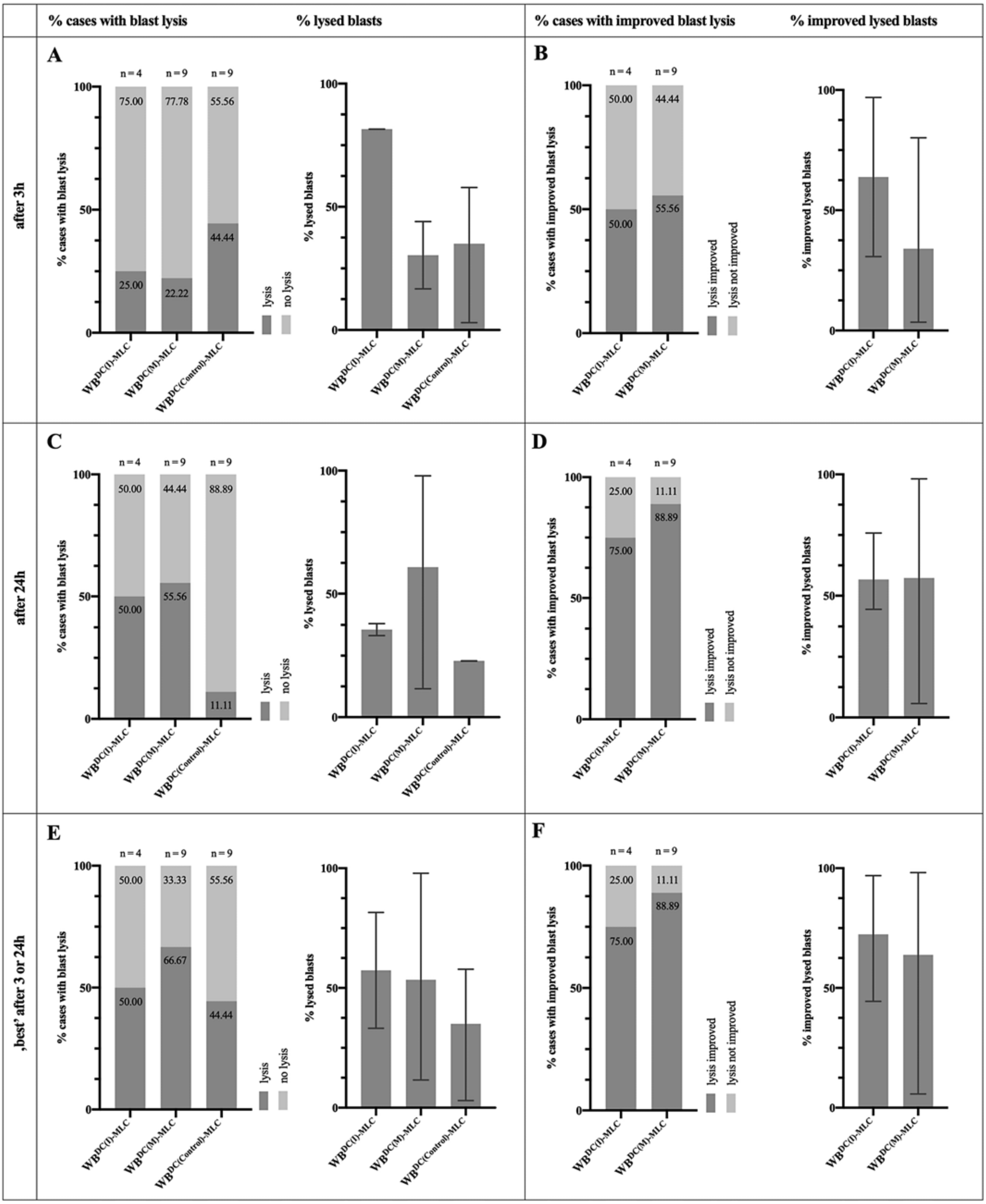


Fig. 3. Stimulatory effect of DC/DC_{leu} on the composition of immunoreactive cells. Given are the mean ± SD of T-cell subsets in the $CD3^+$ cell fraction before ($WB^{DC(Control)}$) and after ($WB^{DC(I)\text{-}MLC}$, $WB^{DC(M)\text{-}MLC}$, $WB^{DC(Control)\text{-}MLC}$) DC/$DC_{leu}$ stimulation. Statistically significant (*p* values <0.05) and borderline significant (*p* values 0.10 to 0.05) differences are given. Abbreviations of all cell types are given in Table 2.

Fig. 4. Stimulatory effect of DC/DC_{leu} on the cytotoxic activity of immunoreactive cells as measured by CTX. Given are the proportions of cases with blast lysis (“% cases with blast lysis”) and the mean ± range of lysed blasts (“% lysed blasts”) in $WB^{DC(I)\text{-}MLC}$, $WB^{DC(M)\text{-}MLC}$, and $WB^{DC(Control)\text{-}MLC}$ after 3 h (**A**) and 24 h (**C**), and the “best” achieved blast lysis after 3 h or 24 h (**E**); the proportions of cases with an improvement in blast lysis (“% cases with improved blast lysis”) and the mean ± range of improved lysed blasts (“% improved lysed blasts”) in $WB^{DC(I)\text{-}MLC}$ and $WB^{DC(M)\text{-}MLC}$ in relation to $WB^{DC(Control)\text{-}MLC}$ after 3 h (**B**) and 24 h (**D**), and the “best” achieved improvement in blast lysis after 3 h or 24 h (**F**).

(For figure see next page.)


% cases with blast lysis
% lysed blasts
% cases with improved blast lysis
% improved lysed blasts
after 3h
after 24h
‚best' after 3 or 24h
A
B
C
D
E
F
n = 4
n = 9
% cases with blast lysis
% lysed blasts
% cases with improved blast lysis
% improved lysed blasts
75.00
77.78
55.56
25.00
22.22
44.44
50.00
44.44
55.56
88.89
11.11
33.33
66.67
25.00
75.00
lysis
no lysis
lysis improved
lysis not improved
WB^DC(I)-MLC
WB^DC(M)-MLC
WB^DC(Control)-MLC
0
50
100

4

Stimulatory Impact of DC/DC_{leu} on T Cell-Enriched Immunoreactive Cells

DC/DC_{leu} and IL-2 Stimulation Increases T-Cell Activation

To assess the potential stimulating effect of generated DC/DC_{leu} on immunoreactive cells in the presence of IL-2, T-cell compositions were compared before ($WB^{DC(Control)}$) and after ($WB^{DC(I)-MLC}$, $WB^{DC(M)-MLC}$, $WB^{DC(Control)-MLC}$) MLC. Frequencies of T^{CD4+}, T^{CD8+}, $T_{prol-early}$, $T_{prol-late}$, T_{naive}, $T_{non-naive}$, T_{cm}, and T_{eff} cells were analysed in reference to T^{CD3+} cells.

We noticed a generally higher activation status of cells in $WB^{DC(I)-MLC}$, $WB^{DC(M)-MLC}$ as well as in $WB^{DC(Control)-MLC}$ compared to $WB^{DC(Control)}$, characterised by a significant increase of early and late proliferating T cells, a significant shift from naive to non-naive T cells, and a (significant) increase of central and effector memory T cells. T^{CD4+} and T^{CD8+} cells did not show any significant transformations. When comparing $WB^{DC(I)-MLC}$, $WB^{DC(M)-MLC}$, and $WB^{DC(Control)-MLC}$, no significant differences in T cell compositions could be found (Fig. 3).

DC/DC_{leu} Stimulation Increases Anti-Leukaemic Cytotoxicity

We analysed the lytic activity of $WB^{DC(I)-MLC}$, $WB^{DC(M)-MLC}$, and $WB^{DC(Control)-MLC}$ through CTX after 3 h and 24 h of incubation of effector and leukaemic target cells, to assess the anti-leukaemic cytotoxicity of DC/DC_{leu}-stimulated immunoreactive cells.

As early as 3 h, we could observe a lysis of target cells in $WB^{DC(I)-MLC}$ as well as $WB^{DC(M)-MLC}$ in about a quarter of the cases, but in $WB^{DC(Control)-MLC}$ in about a half of the cases. Average frequencies of lysed blasts were (n.s.) higher in $WB^{DC(I)-MLC}$ than in $WB^{DC(M)-MLC}$ and $WB^{DC(Control)-MLC}$ (Fig. 4A). After 24 h, more cases of $WB^{DC(I)-MLC}$ and $WB^{DC(M)-MLC}$, but less cases of $WB^{DC(Control)-MLC}$ attained lysis. Average frequencies of lysed blasts decreased (n.s.) in $WB^{DC(I)-MLC}$ and $WB^{DC(Control)-MLC}$, but increased (n.s.) in $WB^{DC(M)-MLC}$, whereby frequencies in $WB^{DC(I)-MLC}$ and $WB^{DC(M)-MLC}$ were (n.s.) higher than in $WB^{DC(Control)-MLC}$ (Fig. 4C). Notably, in cases without lysis, frequencies of blasts showed no significant difference between $WB^{DC(I)-MLC}$, $WB^{DC(M)-MLC}$, and $WB^{DC(Control)-MLC}$.

Concerning the lysis of target cells in $WB^{DC(I)-MLC}$ and $WB^{DC(M)-MLC}$ in relation to $WB^{DC(Control)-MLC}$, after 3 h, we could observe an improvement in lysis in $WB^{DC(I)-MLC}$ as well as $WB^{DC(M)-MLC}$ in about half of the cases. Average improved lysed blasts were (n.s.) higher in $WB^{DC(I)-MLC}$ than in $WB^{DC(M)-MLC}$ (Fig. 4B). After 24 h, more cases of $WB^{DC(I)-MLC}$ and $WB^{DC(M)-MLC}$ attained an improvement in lysis. Average improved lysed blasts in $WB^{DC(M)-MLC}$ thereby increased (n.s.) to levels of $WB^{DC(I)-MLC}$ (Fig. 4D). Notably, in cases without an improvement in lysis, frequencies of blasts showed no significant difference between $WB^{DC(I)-MLC}$ and $WB^{DC(M)-MLC}$.

Overall, choosing the best anti-leukaemic cytotoxicity after 3 or 24 h, we found higher numbers of cases with lysis in $WB^{DC(M)-MLC}$ than in $WB^{DC(I)-MLC}$ and $WB^{DC(Control)-MLC}$. Average frequencies of lysed blasts were (n.s.) higher in $WB^{DC(I)-MLC}$ and $WB^{DC(M)-MLC}$ than in $WB^{DC(Control)-MLC}$, with equal averages in $WB^{DC(I)-MLC}$ and $WB^{DC(M)-MLC}$ (Fig. 4E). $WB^{DC(I)-MLC}$ and $WB^{DC(M)-MLC}$ attained equal proportions of cases with an improvement in lysis and equal averages of improved lysed blasts (Fig. 4F). There was no significant difference in the frequencies of blasts in cases without lysis between $WB^{DC(I)-MLC}$, $WB^{DC(M)-MLC}$, and $WB^{DC(Control)-MLC}$ and in cases without an improvement in lysis between $WB^{DC(I)-MLC}$ and $WB^{DC(M)-MLC}$.

Stimulatory Impact of DC/DC_{leu} on the IFNy Secretion of T Cell-Enriched Immunoreactive Cells Detected via CSA

Analysis of IFNy Secretion

The IFNy secretion of innate and adaptive immune cells was determined through CSA before (uncultured WB) and after ($WB^{DC(I)-MLC}$, $WB^{DC(M)-MLC}$, $WB^{DC(Control)-MLC}$) DC/DC_{leu} stimulation. Frequencies of IFNy-secreting T^{CD3+}, T^{CD4+}, T^{CD8+}, CIK^{CD56+}, NK^{CD56+}, CIK^{CD161+}, NK^{CD161+}, and iNKT cells were analysed.

No Spontaneous Activation of Immunoreactive Cells during DCC and MLC

We compared frequencies of IFNy-secreting immunoreactive cells in uncultured WB to $WB^{DC(Control)-MLC}$, to assess the effect of cultivation (DCC and MLC). We found low frequencies of IFNy-secreting cells in uncultured WB. Comparing uncultured WB to $WB^{DC(Control)-MLC}$ showed no significant differences, besides significantly lower frequencies of $NK^{CD161+}IFNy^{+}/NK^{CD161+}$ in $WB^{DC(Control)-MLC}$ (Fig. 5).

DC/DC_{leu} Stimulation Increases IFNy Secretion of Adaptive and Innate Immunoreactive Cells

To assess the effect of DC/DC_{leu} stimulation on the secretion of IFNy, we compared frequencies of IFNy-secreting immunoreactive cells in $WB^{DC(I)-MLC}$, $WB^{DC(M)-MLC}$ to uncultured WB as well as to $WB^{DC(Control)-MLC}$.

Regarding cells of the adaptive immune system, we found significantly higher frequencies of $T^{CD3+}IFNy^{+}/T^{CD3+}$ in $WB^{DC(I)-MLC}$ and $WB^{DC(M)-MLC}$ compared to uncultured WB and $WB^{DC(Control)-MLC}$, with $WB^{DC(I)-MLC}$ holding significantly higher frequencies than $WB^{DC(M)-MLC}$. Moreover, we could detect significantly higher frequencies of $T^{CD4+}IFNy^{+}/T^{CD4+}$ and

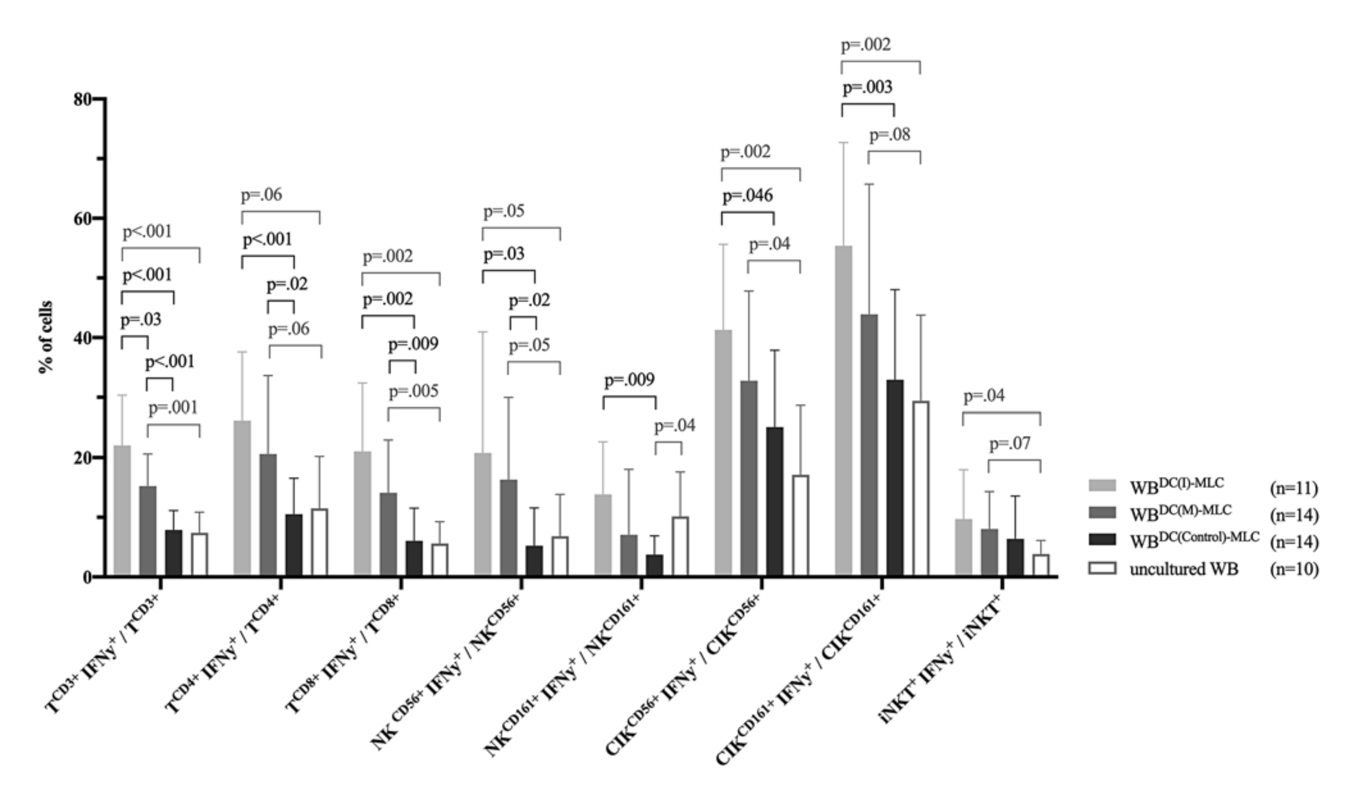


Fig. 5. IFNy secretion of immunoreactive cells before (uncultured WB) and after ($WB^{DC(I)\text{-}MLC}$, $WB^{DC(M)\text{-}MLC}$, $WB^{DC(Control)\text{-}MLC}$) DC/DC_{leu} stimulation as measured by CSA. Given are the mean ± SD of frequencies of IFNy-secreting T, NK, CIK, and iNKT cells. Statistically significant (*p* values <0.05) and borderline significant (*p* values 0.10 to 0.05) differences are given. Abbreviations of all cell types are given in Table 2.

$T^{CD8+}IFNy^{+}/T^{CD8+}$ in $WB^{DC(I)\text{-}MLC}$ and $WB^{DC(M)\text{-}MLC}$ compared to uncultured WB and $WB^{DC(Control)\text{-}MLC}$. Frequencies of both cell groups were (n.s.) higher in $WB^{DC(I)\text{-}MLC}$ compared to $WB^{DC(M)\text{-}MLC}$ (Fig. 1C, 5).

Regarding cells of the innate immune system, we found (significantly) higher frequencies of $NK^{CD56+}IFNy^{+}/NK^{CD56+}$ and $NK^{CD161+}IFNy^{+}/NK^{CD161+}$ in $WB^{DC(I)\text{-}MLC}$ and $WB^{DC(M)\text{-}MLC}$ compared to uncultured WB and $WB^{DC(Control)\text{-}MLC}$, beside $NK^{CD161+}IFNy^{+}/NK^{CD161+}$ showing no significant difference compared to uncultured WB and $WB^{DC(Control)\text{-}MLC}$, with (n.s.) higher frequencies in $WB^{DC(I)\text{-}MLC}$ than $WB^{DC(M)\text{-}MLC}$. Moreover, we could detect (significantly) higher frequencies of $CIK^{CD56+}IFNy^{+}/CIK^{CD56+}$ and $CIK^{CD161+}IFNy^{+}/CIK^{CD161+}$ in $WB^{DC(I)\text{-}MLC}$ and $WB^{DC(M)\text{-}MLC}$ compared to uncultured WB and $WB^{DC(Control)\text{-}MLC}$, with (n.s.) higher frequencies in $WB^{DC(I)\text{-}MLC}$ than $WB^{DC(M)\text{-}MLC}$. No significant differences could be found in the frequencies of $iNKT^{+}IFNy^{+}/iNKT^{+}$ in $WB^{DC(I)\text{-}MLC}$ and $WB^{DC(M)\text{-}MLC}$ compared to $WB^{DC(Control)\text{-}MLC}$, though compared to uncultured WB (Fig. 5).

No Impact of Age, Sex, and Blast Frequency on Stimulation of IFNy Secretion

Overall, the stimulation of IFNy secretion was possible with both DC/DC_{leu}-Kit-I and -Kit-M and independent from patients' age, sex, or blast frequency (data not shown).

LAA Stimulation Does Not Further Increase IFNy Secretion of DC/DC_{leu}-Stimulated Immunoreactive Cells

To assess whether DC/DC_{leu}-stimulated adaptive immunoreactive cells have been subject to LAA-dependent activation, we added the two LAA WT-1 and PRAME to uncultured WB, $WB^{DC(I)\text{-}MLC}$, $WB^{DC(M)\text{-}MLC}$, and $WB^{DC(Control)\text{-}MLC}$. The addition of LAA did (n.s.) increase frequencies of IFNy-secreting cells in uncultured WB and $WB^{DC(Control)\text{-}MLC}$, whereas it did not further increase frequencies of IFNy-secreting cells in $WB^{DC(I)\text{-}MLC}$ and $WB^{DC(M)\text{-}MLC}$ (data not shown).

Positive Correlation of IFNy-Positive Immunoreactive Cells Obtained by CSA and ICA

In order to validate frequencies of IFNy-producing immunoreactive cells obtained by CSA, we performed

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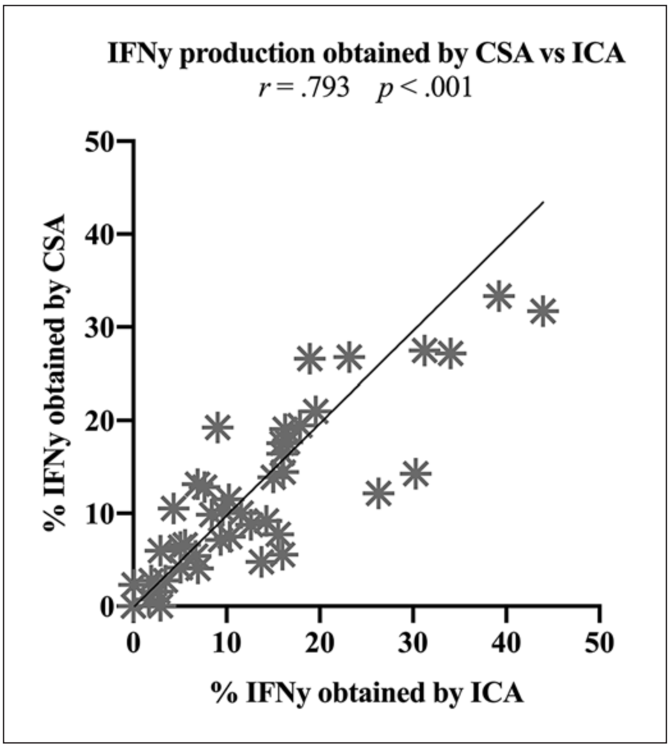

Fig. 6. Correlation of the IFNy production of immunoreactive cells (T^{CD3+}, T^{CD4+}, T^{CD8+}, CIK^{CD56+}, NK^{CD56+}, iNKT cells) obtained by CSA and ICA. Statistically significant (*p* values <0.05) correlations are given. Abbreviations of all cell types are given in Table 2.

parallel ICAs and correlated the results. There was a significantly high positive correlation (r = 0.793) between frequencies obtained by CSA and frequencies obtained by ICA (Fig. 6). Both the CSA and ICA hereby yielded comparable frequencies of IFNy-positive T^{CD3+}, T^{CD4+}, T^{CD8+}, CIK^{CD56+}, NK^{CD56+}, and iNKT cells.

Positive Correlation of IFNy Secretion and Anti-Leukaemic Cytotoxicity of DC/DC_{leu}-Stimulated Immunoreactive Cells

To assess the relationship between the IFNy secretion and anti-leukaemic cytotoxicity of DC/DC_{leu}-stimulated immunoreactive cells, we correlated the absolute improvement of IFNy secretion with the relative improvement of blast lysis (= improved blast lysis) in $WB^{DC(M)-MLC}$ in proportion to $WB^{DC(Control)-MLC}$. Unfortunately, both groups could not be correlated in $WB^{DC(I)-MLC}$ due to low case numbers.

The IFNy secretion did not correlate with anti-leukaemic cytotoxicity after 3 h, but after 24 h: within the adaptive immune cells, we found a significantly moderate positive correlation between $T^{CD3+}IFNy^{+}/T^{CD3+}$ and blast lysis (r = 0.600) and between $T^{CD8+}IFNy^{+}/T^{CD8+}$ and blast lysis (r = 0.596), and a significantly high positive correlation between $T^{CD4+}IFNy^{+}/T^{CD4+}$ and blast lysis (r = 0.716) (Fig. 7A–C). Moreover, within the innate immune cells, we found a significantly high positive correlation between $NK^{CD56+}IFNy^{+}/NK^{CD56+}$ and blast lysis (r = 0.976). Other innate immune cells showed no further significant correlations (Fig. 7D).

Discussion

DC-Based Immunotherapy for AML

Based on the realisation that the immune system can be exploited to take control over AML, different immunological strategies have been developed to prompt a potent anti-leukaemic immunity. Here, targeted immunotherapeutic strategies relying on antibodies, engineered T-cell receptors and T cells engineered to express chimeric antigen receptors have shown promising results [42–44]. Targeted immunotherapy, however, depends on a competent target antigen to assure on-tumour effectivity but prevent off-tumour toxicity. Yet, selecting a proper leukaemic target antigen proves to be difficult due to a pervasive expression pattern overlapping with healthy tissues and haematopoiesis [10, 43, 45, 46]. However, there are strategies which are able to overcome this obstacle, most notably DC-based strategies. DCs generated from myeloid leukaemic blasts (DC_{leu}) are able to simultaneously express dendritic- and leukaemia-specific antigens and thereby prime and enhance leukaemia-specific immune responses with the whole leukaemic antigen repertoire ex and in vivo [11, 47–52], breaking the burden of finding an appropriate target antigen.

DC/DC_{leu} Generation and Their Stimulatory Impact on the Anti-Leukaemic Activity of T Cell-Enriched Immunoreactive Cells

We generated DC and DC_{leu} from leukaemic WB ex vivo with immunomodulatory Kit-I and Kit-M. Frequencies of DC and DC_{leu} were significantly higher in $WB^{DC(M)}$ and $WB^{DC(I)}$ compared to $WB^{DC(Control)}$, with significantly higher frequencies in $WB^{DC(I)}$ than $WB^{DC(M)}$. Both DC and DC_{leu} showed a significant proportion of mature DC. This stimulation of maturation and CCR7-dependent migration to lymph nodes, as achieved by Kit-I and Kit-M, is essential for DC and DC_{leu} to activate T cells and other immunoreactive cells [53–55]. Though both Kits are able to generate significant frequencies of DC/DC_{leu}, it appears that the combination of GM-CSF + Picibanil (OK-432) in Kit-I has a stronger danger signalling and stimulatory impact on DC differentiation than the combination of GM-CSF + PGE_1 in Kit-M, but a similar stimulatory impact on DC maturation. Importantly, the proliferation of non-converted leukaemic blasts was not induced by Kit-I and Kit-M during DCC. All these findings have already been demonstrated in larger studies [unpublished data] [38, 39]. Comparable results were found under hypoxia-like conditions [40].

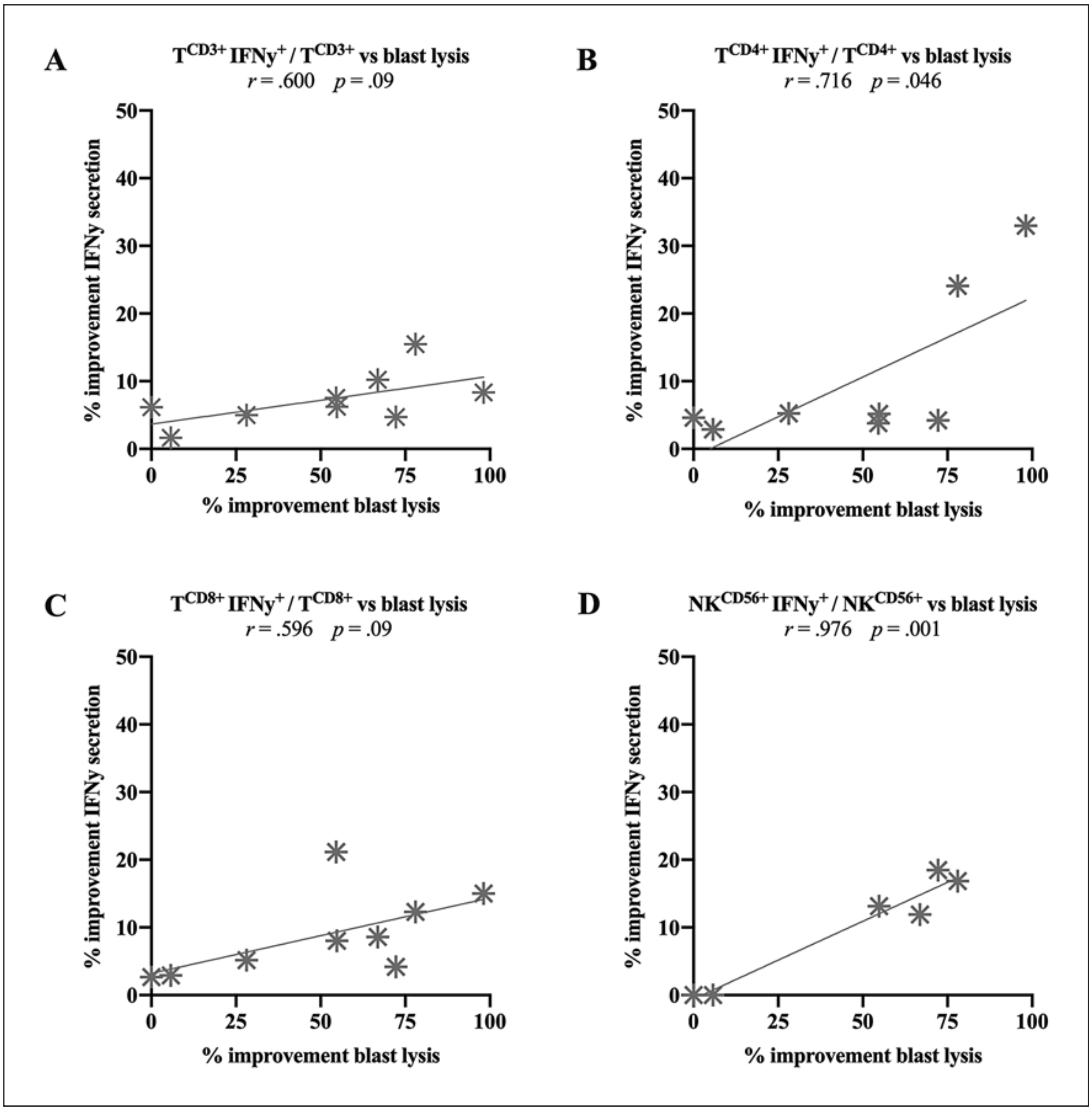

Fig. 7. Correlation of the absolute improvement of IFNy secretion with the relative improvement of blast lysis (= improved blast lysis) in $WB^{DC(M)\text{-}MLC}$ compared to $WB^{DC(Control)\text{-}MLC}$. Given are the correlation of $T^{CD3+}IFNy^+/T^{CD3+}$ (**A**), $T^{CD4+}IFNy^+/T^{CD4+}$ (**B**), $T^{CD8+}IFNy^+/T^{CD8+}$ (**C**), and $NK^{CD56+}IFNy^+/NK^{CD56+}$ (**D**) with blast lysis. Statistically significant (*p* values <0.05) and borderline significant (*p* values 0.10 to 0.05) correlations are given. Abbreviations of all cell types are given in Table 2.

Through the stimulation of immunoreactive cells with DC/DC_{leu} we could observe a generally higher activation status in $WB^{DC(I)\text{-}MLC}$, $WB^{DC(M)\text{-}MLC}$ as well as $WB^{DC(Control)\text{-}MLC}$ compared to $WB^{DC(Control)}$, characterised by a significant increase of proliferating T cells, a significant shift from naive to non-naive T-cell subsets, and a (significant) increase of central and effector memory T cells. This general transformation is most likely caused by the addition of IL-2 to all MLCs, as IL-2 triggers the proliferation and differentiation of T cells as well as the activation and differentiation of other immunoreactive cells [56]. In addition to that, previous larger studies, however, have found an increase of T^{CD8+} cells and a corresponding decrease of T^{CD4+} cells in $WB^{DC(I)\text{-}MLC}$ and $WB^{DC(M)\text{-}MLC}$ compared to $WB^{DC(Control)\text{-}MLC}$ and $WB^{DC(Control)}$ [38].

Even though the stimulation of immunoreactive cells with DC/DC_{leu} had no impact on the composition of T-cell subsets, it had an impact on the anti-leukaemic activity. We could demonstrate that the anti-leukemic cytotoxicity of immunoreactive cells could be notably improved through the stimulation with DC/DC_{leu}-Kit-I and -Kit-M in most of the cases. Interestingly, some cases achieved lysis or improved lysis after 3 h and some cases only after 24 h, whereas average lysis was the highest in $WB^{DC(I)\text{-}MLC}$ after 3 h and in $WB^{DC(M)\text{-}MLC}$ after 24 h. This occurrence might be due to different killing mechanisms of the immunoreactive cells: the early and fast-acting perforin-granzyme pathway and the late and slow-acting Fas/FasL pathway, which can run separately or synergistically [21, 22]. $WB^{DC(I)\text{-}MLC}$ hereby appears to perform via the former pathway, while $WB^{DC(M)\text{-}MLC}$ appears to operate via the latter pathway. Overall, pooling the best anti-leukaemic cytotoxicity after 3 or 24 h, DC/DC_{leu}-Kit-I and -Kit-M, however, appear to be equally efficient. Taken together, the CTX allows to quantify the acquired anti-leukaemic cytotoxicity, but it does not display participating cells of the immune response, like the CSA does.

Stimulatory Impact of DC/DC_{leu} on the IFNy Secretion of T Cell-Enriched Immunoreactive Cells

DC/DC_{leu} activate adaptive immune cells (T^{CD4+}, T^{CD8+}) by presenting leukaemia-specific antigens over MHC class I and II [35] and presumably innate immune cells (NK^{CD56+}, NK^{CD161+}, CIK^{CD56+}, CIK^{CD161+}, iNKT) by yet unknown MHC-unrestricted mechanisms [57, 58], thereby enhancing their effector mechanisms and IFN secretion, the latter also resulting in cross-stimulation. An increase of IFNy secretion upon DC/DC_{leu} stimulation can hence display functionally active leukaemia-specific cells.

With the CSA we were able to detect and phenotypically characterise IFNy-secreting adaptive and innate immunoreactive cells, and thereby evaluate the effect of DC/DC_{leu} stimulation. Uncultured leukaemic WB, as a starting point, already showed low frequencies of IFNy-secreting cells representing a physiological (and conceivably partially leukaemia-specific) immunological base activity. Through stimulation of immunoreactive cells by DC/DC_{leu}-Kit-I and -Kit-M, we were able not only to increase the anti-leukaemic cytotoxicity, but also to significantly increase the IFNy secretion of adaptive immune cells (T^{CD3+}, T^{CD4+}, T^{CD8+} cells) in $WB^{DC(I)\text{-}MLC}$ and $WB^{DC(M)\text{-}MLC}$ compared to uncultured WB as well as $WB^{DC(Control)\text{-}MLC}$. Further, the IFNy secretion of innate immune cells (CIK^{CD56+}, NK^{CD56+}, CIK^{CD161+}, NK^{CD161+} cells) was (significantly) increased in $WB^{DC(I)\text{-}MLC}$ and $WB^{DC(M)\text{-}MLC}$ compared to uncultured WB as well as $WB^{DC(Control)\text{-}MLC}$, beside NK^{CD161+} cells showing no significant difference compared to uncultured WB and iNKT cells showing no significant difference compared to $WB^{DC(Control)\text{-}MLC}$. The IFNy secretion induced with DC/DC_{leu}-Kit-I hereby emerged to be greater than with DC/DC_{leu}-Kit-M. Noteworthy, the stimulation of IFNy secretion was independent from patients' age, sex, or blast frequency. Overall, we found an increased immunological activity of innate and adaptive immunoreactive cells after DC/DC_{leu} stimulation, pointing to an induction of leukaemia-specific cells.

Moreover, we compared frequencies of IFNy-secreting cells in uncultured WB and cultured $WB^{DC(Control)\text{-}MLC}$ to assess the effect of cultivation on the secretion of IFNy. We could not find significant differences in any cell types, besides lower levels of IFNy-secreting NK^{CD161+} cells after cultivation. This might be due to an IL-2-induced expression of lectin-like transcript 1 (LLT1) on various cells, as cross-linking of LLT1 and CD161 on NK^{CD161+} cells results in an inhibition of IFNy production and cytotoxicity [59, 60]. However, it seems that cells stimulated with DC/DC_{leu}-Kit-I and -Kit-M can slightly compensate this effect. In regard to future clinical application, we suppose the effect of IL-2-induced NK^{CD161+} inhibition by LLT1 in vitro to be negligible in vivo, as IL-2 was supplemented. Overall, these findings show that no spontaneous activation of immunoreactive cells during DCC and MLC occurs.

We furthermore investigated the effect of LAA (WT-1 and PRAME) stimulation on DC/DC_{leu}-stimulated adaptive immunoreactive cells. We hypothesised, that the addition of LAA can only increase the IFNy secretion of cells that have not been subject to LAA-dependent activation. Conformably, the addition of WT-1 and PRAME did (n.s.) increase frequencies of IFNy-secreting cells in uncultured WB and $WB^{DC(Control)\text{-}MLC}$. In contrast, the addition of LAA did not further increase frequencies of IFNy-secreting cells in $WB^{DC(I)\text{-}MLC}$ and $WB^{DC(M)\text{-}MLC}$, affirming that these immunoreactive cells have been subject to activation through leukaemia-specific antigens presented by DC_{leu}-Kit-I and -Kit-M, in this case the known LAAs WT-1 and PRAME, whereby no further effect was possible. Remarkably, WT-1 and PRAME are only two of hundreds of leukaemic blast antigens presented by DC_{leu}, giving DC_{leu} the exceptional potential to initiate a comprehensive leukaemia-specific immune response.

Correlation of IFNy-Positive Cells Obtained by CSA and ICA

In order to validate frequencies of IFNy-producing immunoreactive cells obtained by CSA, we performed parallel ICAs and correlated the results. We found a significantly high correlation of obtained frequencies between both methods. Both the CSA and ICA yielded comparable frequencies of IFNy-positive T^{CD3+}, T^{CD4+}, T^{CD8+}, CIK^{CD56+}, NK^{CD56+}, and iNKT cells.

Correlation of IFNy Secretion and Anti-Leukaemic Cytotoxicity of DC/DC_{leu}-Stimulated Immunoreactive Cells

We conclusively correlated the IFNy secretion with the anti-leukaemic cytotoxicity in $WB^{DC(M)\text{-}MLC}$ and found a significantly positive correlation between the IFNy secretion of T^{CD3+}, T^{CD4+}, T^{CD8+} as well as NK^{CD56+} cells and the 24-h anti-leukaemic cytotoxicity. Though the other immune cells (NK^{CD161+}, CIK^{CD56+}, CIK^{CD161+} cells) did not show a direct correlation to cytotoxicity, they showed a (n.s.) increased secretion of IFNy pointing towards an increased activity, which may contribute indirectly to the overall leukaemia-specific activity. Ultimately, cytotoxicity remains dependent on the great interaction of various cells, cytokines, and other factors [12, 35]. Nevertheless, IFNy can reflect the cytotoxic activity of T^{CD3+}, T^{CD4+}, T^{CD8+}, and NK^{CD56+} cells. With this knowledge, we suppose that IFNy readouts of these specific cell groups are sufficient to assess and monitor the efficacy of AML immunotherapy.

Evaluation of the CSA

There are various techniques to investigate the cytokine production of immune cells on a single cell level, including the CSA, ICA, and Elispot technology. The central technique used in this study was the CSA, which allowed us to detect and phenotypically analyse IFNy-secreting immunoreactive cells on a single cell level and moreover display DC/DC_{leu}-induced leukaemia-specific activity and cytotoxicity. It hereby presented itself as a convenient, valid, and reliable method [61].

The ICA has, like the CSA, the potential to detect and phenotypical characterise IFNy-producing cells, however, potentially with a lower sensitivity than the CSA, especially when dealing with low frequencies of IFNy-positive cells [61–64]. Overall though, we and others found the IFNy production obtained by the CSA and ICA highly correlating [65]. Notably, the ICA holds the capacity to simultaneously analyse further cellular markers like TNFa, but, contrary to the CSA, is more time consuming and inevitably kills analysed cells [66]. The Elispot technology, an enzyme-linked cytokine capture assay, enables the detection of IFNy-producing cells with a high sensitivity, but lacks their phenotypical characterisation if cells are not previously sorted into cellular subsets of interest [67]. Noteworthily, the CSA, contrary to the ICA and the Elispot, holds the potential to isolate viable IFNy-expressing cells, if the interest lies in expansion, further functional analyses (e.g., MHC/peptide-tetramer staining), or cell therapy (e.g., adoptive T-cell transfer) [68, 69].

With IFNy assays being able to display leukaemia-specific cells as well as anti-leukaemic cytotoxicity, they are in advantage of regular cytotoxicity assays. Assays like fluorochrome-labelled assays, 51CR-labelled assays, degranulation assays, and LDH assays only allow the evaluation of achieved cytotoxicity but lack the characterisation of overall participating (leukaemia-specific) cells [70–72]. However, they best depict functionally active Tc, as they directly measure the lysis of target cells [73]. Nonetheless, these assays are often very labour-intensive and unsuited for upscaling as needed in clinical applications. A more specific identification of Tc can only be accomplished by MHC/peptide-tetramer staining, yet this method requires a specific target antigen, in contrary to IFNy and cytotoxicity assays, and does not assure the functionality of the specific T-cell receptor [73, 74].

All in all, the CSA technology holds multiple advantages compared to other cytokine and cytotoxicity assays, especially by combining characterising and functional data.

Conclusion

We were able to describe the potential of DC/DC_{leu} to induce or improve leukaemia-specific and anti-leukaemic activity through the detection of IFNy-secreting innate and adaptive immune cells ex vivo. The CSA in this regard proved to be a convenient and reproducible technique to detect and phenotypically characterise IFNy-secreting cells. As such, we believe that IFNy could become a very valuable parameter to assess and monitor the efficacy of AML immunotherapy in future clinical applications.

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Statement of 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 Hospital Munich (vote No. 33905).

Conflict of Interest Statement

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", in which H.M.S. is involved.

Author Contributions

L.K.K conducted DCC, MLC, CTX, and CSA experiments and all flow cytometric and statistical analyses. O.S., S.U., F.D.-G., and N.R. performed additional DCC, MLC, CTX, and CSA experiments, which were analysed by L.K.K. O.S. conducted ICA experiments, which were analysed by L.K.K. D.K., A.R., and C.S. provided leukaemic whole blood samples and corresponding diagnostic reports. D.C.A. and B.E.-V. supported functionality assays. H.M.S. designed the study. L.K.K and H.M.S. drafted the manuscript.

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