



Article

# Dendritic Cell-Triggered Immune Activation Goes along with Provision of (Leukemia-Specific) Integrin Beta 7-Expressing Immune Cells and Improved Antileukemic Processes

Elias Rackl <sup>1</sup>, Lin Li <sup>1</sup> , Lara Kristina Klauer <sup>1</sup> , Selda Ugur <sup>1</sup>, Elena Pepeldjiyska <sup>1</sup>, Corinna L. Seidel <sup>2</sup> , Carina Gunsilius <sup>1</sup>, Melanie Weinmann <sup>1</sup>, Fatemeh Doraneh-Gard <sup>1</sup>, Nina Reiter <sup>1</sup>, Caroline Plett <sup>1</sup>, Daniel Christoph Amberger <sup>1</sup> , Peter Bojko <sup>3</sup>, Doris Kraemer <sup>4</sup>, Jörg Schmohl <sup>5</sup>, Andreas Rank <sup>6</sup> , Christoph Schmid <sup>6</sup> and Helga Maria Schmetzer <sup>1,\*</sup>

<sup>1</sup> Department of Medicine III, University Hospital of Munich, 81377 Munich, Germany

<sup>2</sup> Department of Orthodontics and Dentofacial Orthopedics, University Hospital, LMU Munich, 80336 Munich, Germany

<sup>3</sup> Department of Hematology and Oncology, Rotkreuzklinikum Munich, 80634 Munich, Germany

<sup>4</sup> Department of Hematology and Oncology, St.-Josefs-Hospital, 58097 Hagen, Germany

<sup>5</sup> Department of Hematology and Oncology, Diakonieklinikum Stuttgart, 70176 Stuttgart, Germany

<sup>6</sup> Department of Hematology and Oncology, University Hospital of Augsburg, 86156 Augsburg, Germany

\* Correspondence: helga.schmetzer@med.uni-muenchen.de; Tel.: +49-0-89-4400-73137; Fax: +49-0-89-4400-76137



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**Abstract:** Integrin beta 7 ( $\beta 7$ ), a subunit of the integrin receptor, is expressed on the surface of immune cells and mediates cell–cell adhesions and interactions, e.g., antitumor or autoimmune reactions. Here, we analyzed, whether the stimulation of immune cells by dendritic cells (of leukemic derivation in AML patients or of monocyte derivation in healthy donors) leads to increased/leukemia-specific  $\beta 7$  expression in immune cells after T-cell-enriched mixed lymphocyte culture—finally leading to improved antileukemic cytotoxicity. Healthy, as well as AML and MDS patients' whole blood (WB) was treated with Kit-M (granulocyte–macrophage colony-stimulating factor (GM-CSF) + prostaglandin E1 (PGE1)) or Kit-I (GM-CSF + Picibanil) in order to generate DCs (DC<sub>leu</sub> or monocyte-derived DC), which were then used as stimulator cells in MLC. To quantify antigen/leukemia-specific/antileukemic functionality, a degranulation assay (DEG), an intracellular cytokine assay (INTCYT) and a cytotoxicity fluorolysis assay (CTX) were used. (Leukemia-specific) cell subtypes were quantified via flow cytometry. The Kit treatment of WB (compared to the control) resulted in the generation of DC/DC<sub>leu</sub>, which induced increased activation of innate and adaptive cells after MLC. Kit-pretreated WB (vs. the control) led to significantly increased frequencies of  $\beta 7$ -expressing T-cells, degranulating and intracellular cytokine-producing  $\beta 7$ -expressing immune cells and, in patients' samples, increased blast lysis. Positive correlations were found between the Kit-M-mediated improvement of blast lysis (vs. the control) and frequencies of  $\beta 7$ -expressing T-cells. Our findings indicate that DC-based immune therapies might be able to specifically activate the immune system against blasts going along with increased frequencies of (leukemia-specific)  $\beta 7$ -expressing immune cells. Furthermore,  $\beta 7$  might qualify as a predictor for the efficiency and the success of AML and/or MDS therapies.

**Keywords:** integrin beta 7; leukemia-derived dendritic cells; immune therapy; AML; MDS

## 1. Introduction

### 1.1. Acute Myeloid Leukemia (AML) and Myelodysplastic Syndrome (MDS)

AML and MDS are clonal stem cell disorders of hematopoiesis that lead to the uncontrolled proliferation of progenitor cells and the suppression of healthy, functional cells [1,2], going along with infections, bleeding, thrombosis and anemia [3]. Their prognosis depends on chromosomal aberrations, blast counts and patients' ages. Standard treatment for

AML/high-grade MDS is based on high-dose chemotherapy, hypomethylating agents and stem cell transplantation (SCT), leading to high rates of remission (66–90% of AML cases after 1–2 therapy cycles), however high rates of relapse have also been noted in up to 80% of cases in the following two years [4–6].

### 1.2. DC-Based Immunotherapeutic Approaches

DCs mediate immune responses via major-histocompatibility-complex (MHC)-associated antigen-presentation [7]. In vivo DCs derive from hematopoietic stem cells and monocytes and undergo an activation and maturation process in conjunction with the upregulation of cell adhesion receptors, chemokine receptors (e.g., CCR7), MHC antigens and other co-stimulatory factors [8–10].

DCs can be generated ex vivo from healthy monocytes or from myeloid blasts in patients with myeloid leukemia ( $DC_{leu}$ , leukemia-derived DC-expressing individual patients' leukemic antigens). DC/ $DC_{leu}$  and their subtypes, e.g.,  $DC_{leu}$ , mature DC ( $DC_{mat}$ ) and mature leukemia-derived DC ( $DC_{leu-mat}$ ) (Table 1), can be generated from whole blood (WB) using selected combinations of response modifiers (e.g., GM-CSF (granulocyte macrophage colony-stimulating factor) + PGE1 (prostaglandin E1) (Kit-M) or GM-CSF+Picibanil (Kit-I)) without the induction of blast proliferation (as detected by blasts' co-expression of transferin receptor CD71 ( $Bla_{prol-CD71}$ ) or intracellular proliferation marker IPO38 ( $Bla_{prol-IPO38}$ ); see Table 1) [10–13].

**Table 1.** Cell subpopulations.

Group	Subgroup	Acronym	Markers	Abbrev. (Referred to Cell Fraction)	Reference
<b>T-cells</b>	T-cells	T	CD3 <sup>+</sup>	CD3 <sup>+</sup> cells	[14]
	Transferrin-R-positive late-proliferating T-cells	$T_{prol-late}$	CD3 <sup>+</sup> CD71 <sup>+</sup>	$T_{prol-late}/CD3^+$	[14]
	Type II C- type lectin-positive early-proliferating T-cells	$T_{prol-early}$	CD3 <sup>+</sup> CD69 <sup>+</sup>	$T_{prol-early}/CD3^+$	[14]
	CD4-positive T-cells	$T_{CD4+}$	CD4 <sup>+</sup> CD3 <sup>+</sup>	$T_{CD4+}/CD3^+$	[14]
	CD4-negative T-cells	$T_{CD4-}$	CD4 <sup>-</sup> CD3 <sup>+</sup>	$T_{CD4-}/CD3^+$	[14]
	Non-naive T-cells	$T_{non-naive}$	CD3 <sup>+</sup> CD45RO <sup>+</sup>	$T_{non-naive}/CD3^+$	[15]
	Naive T-cells	$T_{naive}$	CD3 <sup>+</sup> CD45RO <sup>-</sup>	$T_{naive}/CD3^+$	[15]
	Central memory T-cells	$T_{cm}$	CD3 <sup>+</sup> CD45RO <sup>+</sup> CCR7 <sup>+</sup>	$T_{cm}/CD3^+$	[15]
	CD4-positive non-naive T-cells	$T_{non-naiveCD4+}$	CD3 <sup>+</sup> CD4 <sup>+</sup> CD45RO <sup>+</sup>	$T_{non-naiveCD4+}/T_{CD4+}$	[10]
	CD4-negative non-naive T-cells	$T_{non-naiveCD4-}$	CD3 <sup>+</sup> CD4 <sup>-</sup> CD45RO <sup>+</sup>	$T_{non-naiveCD4-}/T_{CD4-}$	[10]
<b>blasts</b>	Blasts	Bla	e.g. CD15, CD34, CD65, CD117,	Bla/cells	[16]
	Proliferating blasts	$Bla_{prol-CD71}$	CD71 <sup>+</sup> Bla <sup>+</sup> DC <sup>-</sup>	$Bla_{prol-CD71}/Bla$	[17]
	Proliferating blasts	$Bla_{prol-IPO38}$	IPO38 <sup>+</sup> Bla <sup>+</sup> DC <sup>-</sup>	$Bla_{prol-IPO38}/Bla$	[17]

Table 1. Cont.

Group	Subgroup	Acronym	Markers	Abbrev. (Referred to Cell Fraction)	Reference
dendritic cells	Dendritic cells	DC	CD80, CD206, CD209, CD83	DC/cells	[12]
	Mature DC	DC <sub>mat</sub>	DC <sup>+</sup> CCR7 <sup>+</sup>	DC <sub>mat</sub> /cells DC <sub>mat</sub> /DC	[13]
	Leukemia-derived DC	DC <sub>leu</sub>	Bla <sup>+</sup> DC <sup>+</sup>	DC <sub>leu</sub> /cells DC <sub>leu</sub> /DC DC <sub>leu</sub> /Bla	[12]
	Mature leukemia-derived DC	DC <sub>leu-mat</sub>	Bla <sup>+</sup> DC <sup>+</sup> CCR7 <sup>+</sup>	DC <sub>leu-mat</sub> /cells DC <sub>leu-mat</sub> /DC <sub>leu</sub> DC <sub>leu-mat</sub> /DC <sub>mat</sub>	[17]
others	Cytokine-induced killer cells	CIK	CD3 <sup>+</sup> CD56 <sup>+</sup>	CIK/cells	[10]
	Natural killer cells	NK	CD3 <sup>-</sup> CD56 <sup>+</sup>	NK/cells	[10]
	Monocytes	Mon	CD14 <sup>+</sup>	Mon/cells	[10]
	Proliferating monocytes	Mon <sub>prol-CD71</sub>	CD71 <sup>+</sup> CD14 <sup>+</sup> DC <sup>-</sup>	Mon <sub>prol-CD71</sub> /Mon	
	B-cells	B	CD19 <sup>+</sup>	CD19 <sup>+</sup> /cells	[10]
Integrin beta 7 (β7)	β7-positive T-cells	T <sub>β7+</sub>	β7 <sup>+</sup> CD3 <sup>+</sup>	T <sub>β7+</sub> /CD3 <sup>+</sup>	[15]
	β7-positive CIK-cells	CIK <sub>β7+</sub>	β7 <sup>+</sup> CD3 <sup>+</sup> CD56 <sup>+</sup>	CIK <sub>β7+</sub> /CIK	
	β7-positive NK-cells	NK <sub>β7+</sub>	β7 <sup>+</sup> CD3 <sup>-</sup> CD56 <sup>+</sup>	NK <sub>β7+</sub> /NK	[18]
	β7-positive non naive T-cells	T <sub>non-naiveβ7+</sub>	β7 <sup>+</sup> CD3 <sup>+</sup> CD45RO <sup>-</sup>	T <sub>non-naiveβ7+</sub> /T <sub>non-naive</sub>	[15]
	β7-positive naive T-cells	T <sub>naiveβ7+</sub>	β7 <sup>+</sup> CD3 <sup>+</sup> CD45RO <sup>+</sup>	T <sub>naiveβ7+</sub> /T <sub>naive</sub>	[15]
	β7-positive central memory T-cells	T <sub>cmβ7+</sub>	β7 <sup>+</sup> CCR7 <sup>+</sup> CD3 <sup>+</sup> CD45RO <sup>+</sup>	T <sub>cmβ7+</sub> /T <sub>cm</sub>	[15]
DEG	CD107a-positive β7-positive T-cells	T <sub>β7+107a+</sub>	CD107a <sup>+</sup> β7 <sup>+</sup> CD3 <sup>+</sup>	T <sub>β7+107a+</sub> /T <sub>β7+</sub>	[19]
	CD107a-positive β7-positive CIK-cells	CIK <sub>β7+107a+</sub>	CD107a <sup>+</sup> β7 <sup>+</sup> CD3 <sup>+</sup> CD56 <sup>+</sup>	CIK <sub>β7+107a+</sub> /CIK <sub>β7+</sub>	[19]
	CD 107a-positive β7-positive NK-cells	NK <sub>β7+107a+</sub>	CD107a <sup>+</sup> β7 <sup>+</sup> CD3 <sup>-</sup> CD56 <sup>+</sup>	NK <sub>β7+107a+</sub> /NK <sub>β7+</sub>	[19]
INTCYT	TNF alpha-positive β7-positive T-cells	T <sub>β7+TNFα+</sub>	TNFα <sup>+</sup> β7 <sup>+</sup> CD3 <sup>+</sup>	T <sub>β7+TNFα+</sub> /T <sub>β7+</sub>	[20]
	IFN gamma-positive β7-positive T-cells	T <sub>β7+IFNγ+</sub>	IFNγ <sup>+</sup> β7 <sup>+</sup> CD3 <sup>+</sup>	T <sub>β7+IFNγ+</sub> /T <sub>β7+</sub>	[21]

Abbrev. cell subpopulations; marker combinations (measured by FACS) defining the different cell subpopulations.

### 1.3. Key Players of Immune Defense and Their Detection

Immune defense is mediated by humoral and cellular mechanisms of the innate and adaptive immune system [22,23]. The key players of the (fast-acting) innate immune system are monocytes (CD14<sup>+</sup>), macrophages (CD15<sup>+</sup>), DCs (e.g., CD206<sup>+</sup>, CD80<sup>+</sup>, etc.) as well as NK-cells (CD56<sup>+</sup>CD3<sup>-</sup>) and CIK-cells (CD56<sup>+</sup>CD3<sup>+</sup>) [13,24–26]. The key players of the (antigen-specific/long-lasting) adaptive immune system are B-cells (CD19<sup>+</sup>), T-cells (CD3<sup>+</sup>) and their subsets: naive or non-naive T-cells (T<sub>naive</sub>, CD3<sup>+</sup>CD45RO<sup>-</sup>; T<sub>non-naive</sub>, CD3<sup>+</sup>CD45RO<sup>+</sup>; T<sub>non-naiveCD4+</sub>, CD4<sup>+</sup>CD3<sup>+</sup>CD45RO<sup>+</sup>; and T<sub>non-naiveCD4-</sub>, CD4<sup>-</sup>CD3<sup>+</sup>CD45RO<sup>+</sup>), which give rise to central memory cells (T<sub>cm</sub>, CD3<sup>+</sup>CD45RO<sup>+</sup>CCR7<sup>+</sup>), enabling faster reactivation of

the immune system against reoccurring targets (see Table 1) [10,27,28]. These cells can be detected via flow cytometry.

Leukemia-specific/antileukemic cells can be quantified using functional analyses. The degranulation assay (DEG) detects the CD107a molecule left on the cell surface after the release of granzymes and perforins [29,30]. The intracellular cytokine assay (INCYT) allows the intracellular quantification of cytokines (interferon gamma (IFN $\gamma$ ) and tumor necrosis factor alpha (TNF $\alpha$ )), which are considered specific triggers of the immune responses and mediators of cell apoptosis [21,30,31]. Antileukemic blast lytic effects can be detected, e.g., using a non-radioactive fluorolysis assay [13,32,33].

#### 1.4. Integrin Beta 7 ( $\beta$ 7)

The  $\beta$ 7 subunit, expressed on adaptive and innate immune cells, plays an important role in cell–cell-adhesion [15,34,35] (e.g.,  $\beta$ 7-expressing T-cells (T $_{\beta$ 7+), T $_{\text{non-naive}}$  (T $_{\text{non-naive}\beta$ 7+), T $_{\text{naive}}$  (T $_{\text{naive}\beta$ 7+), T $_{\text{cm}}$  (T $_{\text{cm}\beta$ 7+), NK-cells (NK $_{\beta$ 7+)- and CIK-cells (CIK $_{\beta$ 7+); see Table 1).  $\beta$ 7 expressed in hematopoietic stem cells (HSC) plays a role in HSC homing [36].  $\beta$ 7 is primarily known for its function in T-cell-trafficking to the gut via interaction with mucosal-addressin-cell-adhesion-molecule-1 (MAdCAM-1) [35,37]. Higher  $\beta$ 7 expression on T-cells seems to correlate with cytotoxic effects towards intestinal cells in inflammatory bowel diseases [38] and in NK-cells with higher cytotoxicity in immunodeficiency-virus-infected macaques [39].  $\beta$ 7 has been associated with the intraepithelial differentiation of cytotoxic as well as regulatory T-cells, and therefore, with pro- and anti-inflammatory functions [40]. In the past, higher  $\beta$ 7 expression in T-cells could be correlated with higher antileukemic, blastolytic potential in AML samples [15].

#### 1.5. Aim of This Study

The aim of this study was to further explore the role of  $\beta$ 7 expression in immune cell subpopulations in uncultured peripheral WB or after mixed lymphocyte culture (MLC) of patients' or healthy donors' T-cell-enriched cells with Kit-pretreated WB as stimulator cells.

In detail, we explored:

- DC subpopulations using (Kit-treated vs. untreated) WB from leukemia patients (or healthy individuals);
- ( $\beta$ 7-expressing) innate and adaptive immune cells before and after MLC with Kit-pretreated vs. untreated WB from leukemia patients (or healthy individuals);
- The antileukemic activity of cells using the cytotoxicity fluorolysis assay (CTX) after MLC of patients' T-cells with Kit-pretreated vs. untreated WB as stimulator cells;
- Leukemia-specific cells using DEG and INCYT assays in uncultivated WB from leukemia patients (vs. comparable cells for healthy individuals) and after MLC with Kit-pretreated vs. untreated WB;
- The correlations between antileukemic functionality, leukemia-specific activity and ( $\beta$ 7-expressing) immune cells;
- The correlations between patients' clinical outcomes/prognostic risk assessment and ( $\beta$ 7-expressing) immune cells.

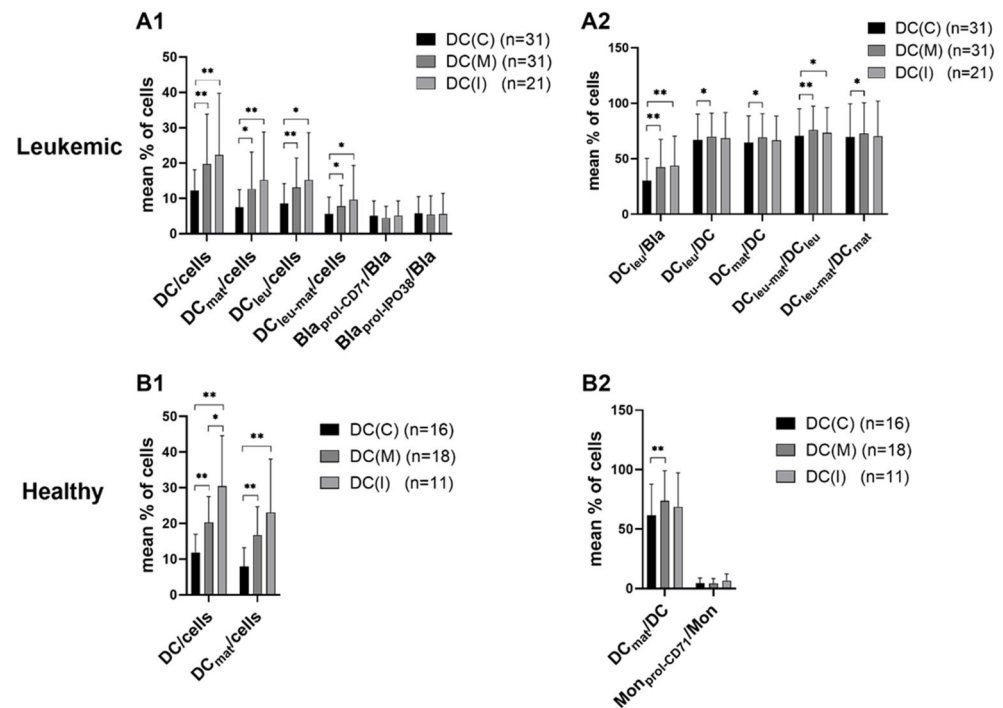
## 2. Results

We further explored the role of  $\beta$ 7-expressing (leukemia-specific) immune-reactive cell populations as prognostic markers to predict the clinical outcome, as well as to mediate antileukemic functionality after stimulation. Therefore, we studied the expression of  $\beta$ 7 on uncultured immune-reactive cells from AML patients and healthy blood donors (as a control), generated DC/DC $_{\text{leu}}$  from healthy and leukemic samples and studied their potential to activate ( $\beta$ 7-expressing) immune-reactive cells after T-cell-enriched MLC.

## 2.1. Generation of DC (Subtypes) from WB

### 2.1.1. Significantly Higher Frequencies of DCs and Their Subtypes in Patients' and Healthy WB Samples after Kit Treatment Compared to Control (without Added Kits)

We found significantly higher frequencies of DC/DC<sub>leu</sub> subtypes under the influence of Kit-M (DC(M)) or Kit-I (DC(I)) compared to the control (DC(C) in healthy or AML patients' samples (e.g., %DC/cells, Figure 1A1,B1). Moreover, in AML samples, frequencies of (mature) DC<sub>leu</sub> were significantly increased compared to the control (Figure 1A1,A2).



**Figure 1.** Generation of DC/DC<sub>leu</sub> from (A1) leukemic and (B1) healthy WB with and without Kits. WB samples were cultured for 7 days with Kit-M or Kit-I or without added Kits as control. Results with Kit-M (DC(M)) or Kit-I (DC(I)) or without added Kits as control (DC(C)) are given. Mean frequencies  $\pm$  standard deviation of DC subtypes in (A2) leukemic (AML/MDS) and (B2) healthy samples are given; n—number of cases. Differences were considered as significant (\*) when  $p \leq 0.05$  and as highly significant (\*\*) when  $p \leq 0.005$ . Abbreviations of cell subpopulations are given in Table 1.

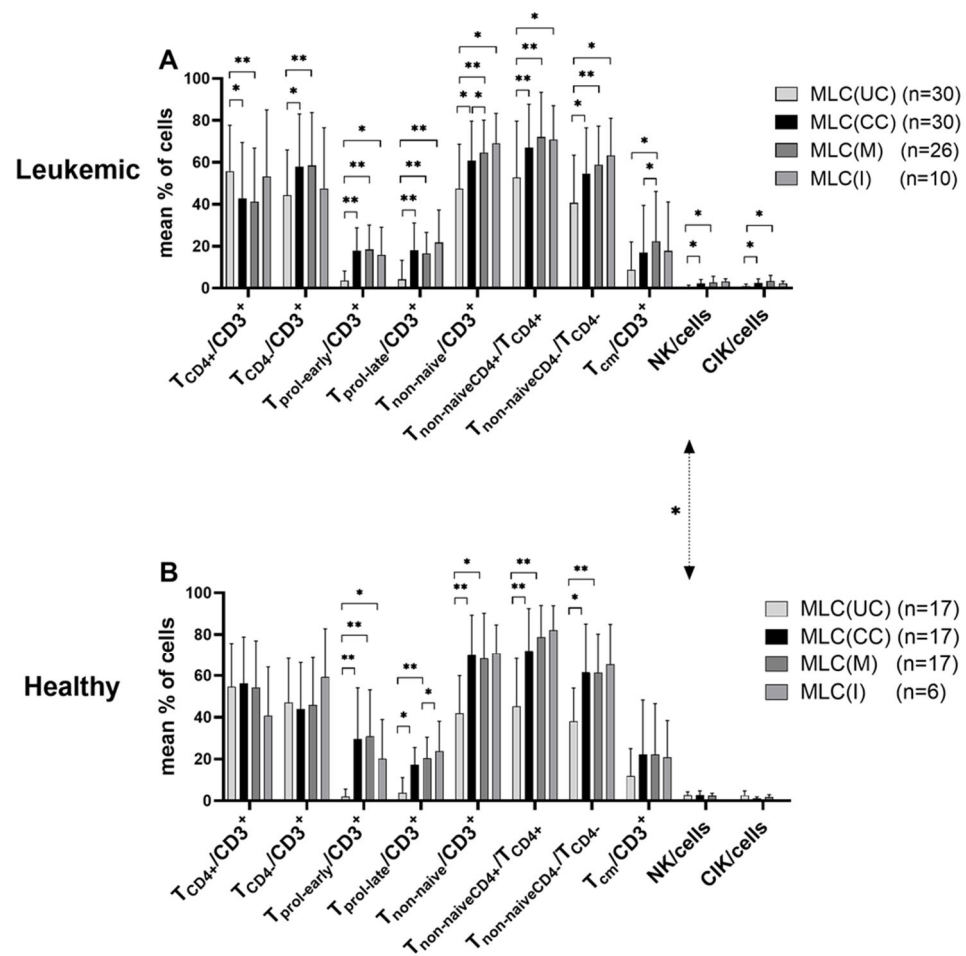
### 2.1.2. No Influence of Kit Treatment on Proliferation of Blasts or Monocytes

The frequencies of proliferating blasts (Bla<sub>prol-CD71</sub>, Bla<sub>prol-IPO38</sub>; Figure 1A1) were comparable in Kit-treated patients' WB and that of the control. Moreover, the frequencies of proliferating monocytes (Mon<sub>prol-CD71</sub>; Figure 1B2) were comparable in Kit-treated healthy donors' WB and that of the control.

In summary, we found higher frequencies of DCs and their subtypes (DC<sub>leu</sub>, DC<sub>mat</sub> and DC<sub>leu-mat</sub>) in Kit-treated WB when compared to the control. The (DC-independent) proliferation of blasts and monocytes was not induced.

### 2.1.3. Profiles of Immune-Reactive (and Especially $\beta 7$ -Expressing) Cells in Uncultured WB from AML vs. Healthy Blood Donors

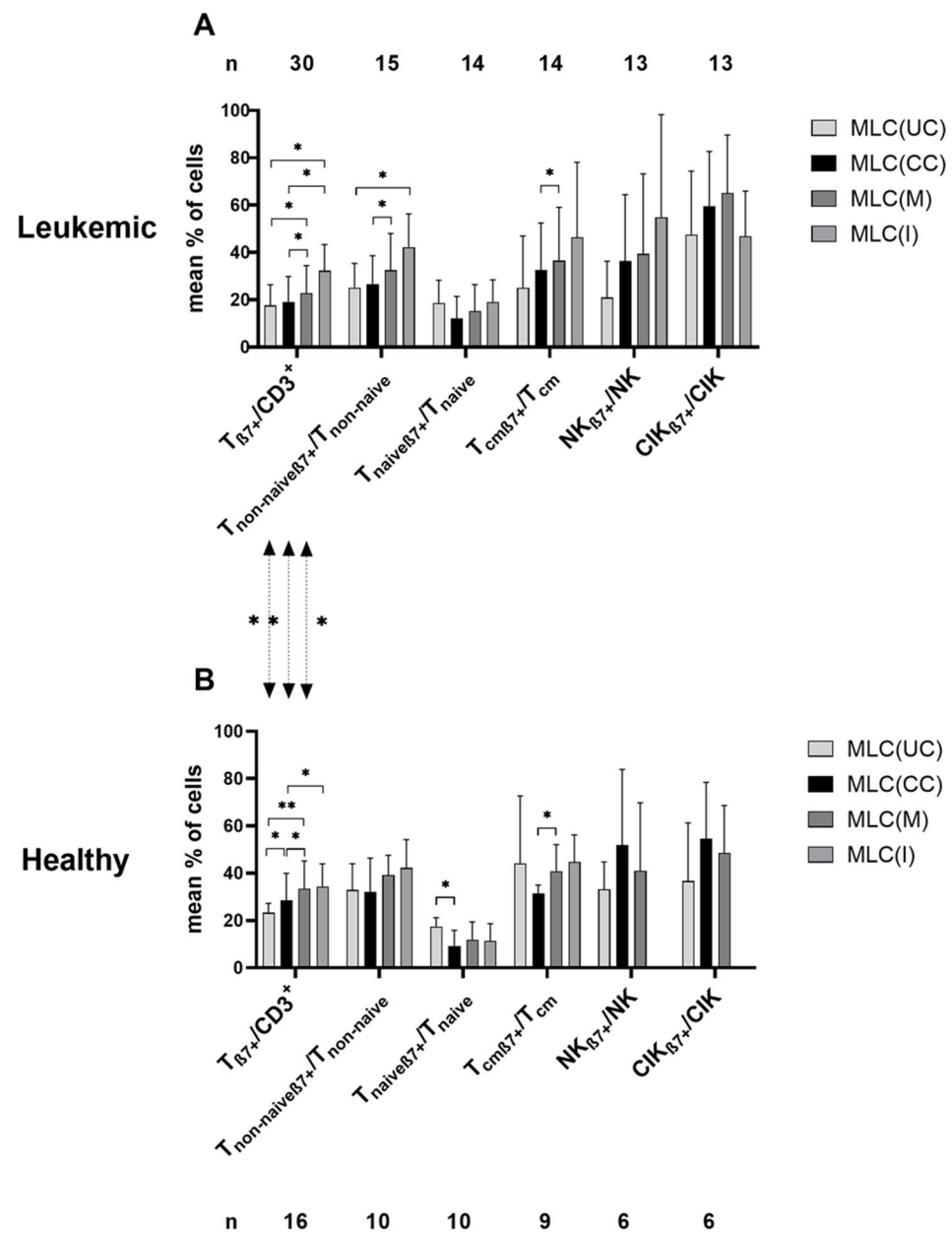
Low frequencies of proliferating T-cells (T<sub>prol-early</sub>/CD3<sup>+</sup>, T<sub>prol-late</sub>/CD3<sup>+</sup>), T<sub>cm</sub>/CD3<sup>+</sup> and innate immune cells were found in uncultured AML as well as in healthy samples. Significantly higher frequencies of NK-cells were found in (uncultured) healthy vs. AML WB samples (Figure 2, MLC(UC)).



**Figure 2.** Composition of immune-reactive cells before and after T-cell-enriched MLC using (A) leukemic and (B) healthy WB with or without Kit pretreatment. Cells were analyzed via flow cytometry before and after 7 days of (T-cell-enriched) MLC with Kit-pretreated or untreated WB and IL-2. Cells before MLC from WB without added Kits as control (MLC(UC)), and cells after MLC, from WB pretreated with Kit-M (MLC(M)), Kit-I (MLC(I)) or without added Kits as control (MLC(CC)), are given. Mean frequencies  $\pm$  standard deviation of immune-reactive cell subpopulations in (A) leukemic (AML/MDS) and (B) healthy samples are given; n—number of cases. Differences were considered as significant (\*) when  $p \leq 0.05$  and as highly significant (\*\*) when  $p \leq 0.005$ . Double-sided arrows give (significant) differences between defined healthy and leukemic immune-reactive cell subtypes. Abbreviations of cell subpopulations are given in Table 1.

Between 18 and 22% of T-cell (subtypes, e.g., T<sub>naive</sub>/T<sub>nonnaive</sub>/T<sub>cm</sub>) and even higher frequencies of innate cells co-expressed  $\beta 7$ . The differences in  $\beta 7$  expression were significantly lower in uncultured leukemic vs. healthy T-cells (Figure 2A vs. Figure 2B, MLC(UC)).

Comparing the expression profiles before vs. after T-cell-enriched MLC (using Kit pretreated (or untreated) patients' or healthy donors' WB as stimulator cells), we found higher frequencies of activated/proliferating/CD4<sup>-</sup> T-cells (e.g., T<sub>non-naive</sub>/CD3<sup>+</sup> (and T<sub>nonnaiveCD4+</sub>/T<sub>CD4+</sub> and T<sub>non-naiveCD4-</sub>/T<sub>CD4-</sub>) and T<sub>CD4-</sub>/CD3<sup>+</sup>) within patients' and healthy samples, as well as higher frequencies of CIK/cells and NK/cells (within patients' samples) after MLC compared to before (Figure 2). Higher frequencies of  $\beta 7$ -expressing immune-reactive cell subpopulations (except for T<sub>naive $\beta 7+$</sub> ) were found after MLC compared to before (Figure 3).



**Figure 3.** Composition of  $\beta 7$ -expressing immune-reactive cells before and after T-cell-enriched MLC using (A) leukemic and (B) healthy WB with and without Kit pretreatment as stimulator cells. Cells were analyzed via flow cytometry before and after 7 days of (T-cell-enriched) MLC with Kit-pretreated or untreated WB and IL-2. Cells before MLC from WB without added Kits as control (MLC(UC)), and cells after MLC, from WB pretreated with Kit-M (MLC(M)), Kit-I (MLC(I)) or without added Kits as control (MLC(CC)), are given. Mean frequencies  $\pm$  standard deviation of immune-reactive cell subpopulations in (A) leukemic (AML/MDS) and (B) healthy samples are given; n—number of cases. Differences were considered as significant (\*) when  $p \leq 0.05$  and as highly significant (\*\*) when  $p \leq 0.005$ . Double-sided arrows give (significant) differences between defined healthy and leukemic immune-reactive cell subtypes. Abbreviations of cell subpopulations are given in Table 1.

## 2.2. T-Cell-Enriched Mixed Lymphocyte Culture with Patients' or Healthy Donors' Kit-Pretreated (vs. Untreated) WB

### 2.2.1. Significant Activation and Provision of T-Cells after MLC of Patients' WB, but Not in Healthy Samples with Kit-Pretreated (vs. Untreated) WB

Comparing the influence of Kit treatment (vs. without) on the composition of immune-reactive cells in patients' samples, we found significantly higher frequencies of T $_{non-naive}$

and  $T_{cm}$  after MLC(M) compared to MLC(CC) (e.g., % $T_{non-naive}/CD3^+$ : MLC(M) vs. MLC(CC),  $p \leq 0.05$ ; and % $T_{cm}/CD3^+$ : MLC(M) vs. MLC(CC),  $p \leq 0.05$ ), whereas the frequencies of innate cells were not significantly different (Figure 2A).

No significantly different frequencies of T- or innate cells were found after MLC(M) or MLC(I) vs. the control (MLC(CC) in healthy samples) (Figure 2B).

### 2.2.2. Significantly Increased Provision of $\beta 7$ -Expressing Immune-Reactive Cells after MLC with Kit-Pretreated Patients' or Healthy WB Compared to Control (MLC(CC))

After MLC of Kit-treated (vs. untreated) patients' WB, we found higher frequencies of  $\beta 7$ -expressing T-cell subpopulations after MLC(M), e.g.,  $\beta 7$ -expressing  $T_{cm}$  in the  $T_{cm}$  fraction (%  $T_{cm\beta 7+}/T_{cm}$ : MLC(M):  $36.58 \pm 22.57$ ,  $p \leq 0.05$ ; MLC(CC):  $32.74 \pm 19.72$ ). The frequencies of  $\beta 7$ -expressing T-cell subpopulations were comparable after MLC(M) and MLC(I). No differences between MLC(M), MLC(I) and MLC(CC) were found in  $\beta 7$ -expressing innate immune-reactive cells (Figure 3A).

After MLC of Kit-treated (vs. untreated) healthy donors' WB, we found significantly higher frequencies of  $T_{\beta 7+}$  and  $T_{cm\beta 7+}$  after MLC(M) and MLC(I) and significantly increased frequencies of  $T_{\beta 7+}/CD3^+$  compared to MLC(CC) (e.g., % $T_{\beta 7+}/CD3^+$ : MLC(M):  $33.56 \pm 11.62$ ,  $p \leq 0.05$ ; MLC(CC):  $28.54 \pm 11.41$ ). The frequencies of  $\beta 7$ -expressing cells after MLC(M) and MLC(I) were comparable. No differences could be found in  $\beta 7$ -expressing innate immune cells (Figure 3B).

In healthy (compared to patients') samples, we found significantly higher frequencies of  $T_{\beta 7+}$  cells with MLC(UC), MLC(CC) and MLC(M) ( $p \leq 0.05$ , Figure 3).

In summary, Kit-treated (vs. untreated) patients' WB led to higher frequencies of  $\beta 7$ -expressing T-cell subtypes, with an induction of non-naive and memory  $\beta 7$ -expressing T-cells after MLC. Higher frequencies of  $\beta 7$ -expressing T-cell subtypes were found in healthy donors' (vs. patients') samples.

### 2.2.3. Detection of Antigen-Specific (Degranulating or Intracellularly $IFN\gamma$ -Producing) $\beta 7^+$ Immune-Reactive Cells in Uncultured WB from AML and Healthy WB Donors or in Immune-Reactive Cells after T-Cell-Enriched MLC

We found low frequencies of antigen-specific degranulating or intracellularly  $IFN\gamma$ -producing immune-reactive cells in uncultured WB from AML and healthy WB donors. Significantly higher frequencies of  $T_{\beta 7+IFN\gamma+}/T_{\beta 7+}$  were found in uncultured healthy WB samples compared to AML WB samples (Figure 4). (Non-significantly) higher frequencies of these antigen-specific cells were found after stimulation with LAA (in AML samples) and with SEB (in healthy samples). Here, we present data without antigen stimulation.

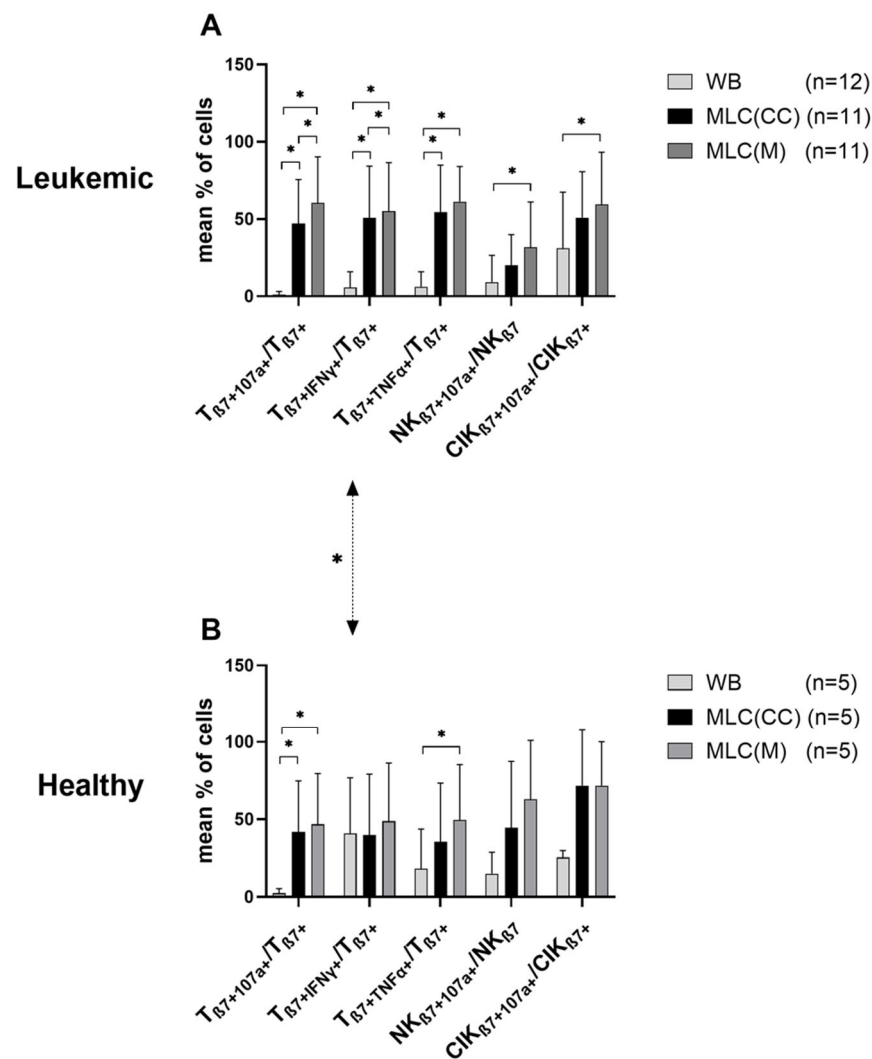
We found significantly increased frequencies in most of antigen-specific (TNF $\alpha$ - or  $IFN\gamma$ -producing or degranulating) T-cell or innate  $\beta 7$ -expressing cell types before vs. after MLC (using Kit pretreated (vs. untreated) patients' or healthy donors' WB as stimulator cells (e.g.,  $T_{\beta 7+IFN\gamma+}/T_{\beta 7+}$  in WB vs. MLC(CC) or WB vs. MLC(M)). This effect was especially evident within patients' samples, but was also found in healthy samples.

We found significantly higher frequencies of  $T_{\beta 7+107a+}$  and  $T_{\beta 7+IFN\gamma+}$  after MLC(M) compared to MLC(CC) in patients' samples without LAA stimulation (% $T_{\beta 7+107a+}/T_{\beta 7+}$ : MLC(M):  $60.62 \pm 29.62$ ,  $p \leq 0.05$ ; MLC(CC):  $47.15 \pm 28.39$ ;  $T_{\beta 7+IFN\gamma+}/T_{\beta 7+}$ : MLC(M):  $55.24 \pm 31.35$ ,  $p \leq 0.05$ ; MLC(CC):  $50.83 \pm 33.50$ ). The frequencies of  $T_{\beta 7+TNF\alpha+}$  and of  $NK_{\beta 7+107a+}$  and  $CIK_{\beta 7+107a+}$  were comparable after MLC(M) and MLC(CC) (Figure 4A). In patients' samples with additional LAA stimulation, the degranulation and intracellular cytokine production after MLC(M) and MLC(CC) were comparable (data not shown).

We were able to find (non-significantly) higher frequencies of  $T_{\beta 7+107a+}$  and  $NK_{\beta 7+107a+}$  after MLC(M) compared to MLC(CC) in healthy samples without SEB stimulation (Figure 4B). In healthy samples with additional SEB stimulation, the degranulation and intracellular cytokine production of cells after MLC were comparable (data not shown).

We found comparable frequencies of degranulating or cytokine-producing  $\beta 7^+$  immune cells in healthy compared to patients' samples (Figure 4A vs. Figure 4B)





**Figure 4.** Composition of  $\beta 7$ -expressing degranulating or intracellular cytokine-producing immune-reactive cells in uncultivated WB and after T-cell-enriched MLC using (A) leukemic and (B) healthy WB with or without Kit pretreatment as stimulator cells. Degranulation and intracellular cytokine production were quantified via flow cytometry in untreated and uncultivated WB as well as after 7 days of (T-cell-enriched) MLC with Kit-pretreated or untreated WB and IL-2. Results without LAA or SEB stimulation are given. Uncultivated cells in WB and cultivated cells after MLC from WB pretreated with Kit-M (MLC(M)) or without added Kits as control (MLC(CC)) are given. Mean frequencies  $\pm$  standard deviation of immune-reactive cell subpopulations in (A) leukemic (AML/MDS) and (B) healthy samples; n—number of cases. Differences were considered as significant (\*) when  $p \leq 0.05$ . Double-sided arrows give (significant) differences between defined healthy and leukemic immune-reactive cell subtypes. Abbreviations of cell subpopulations are given in Table 1.

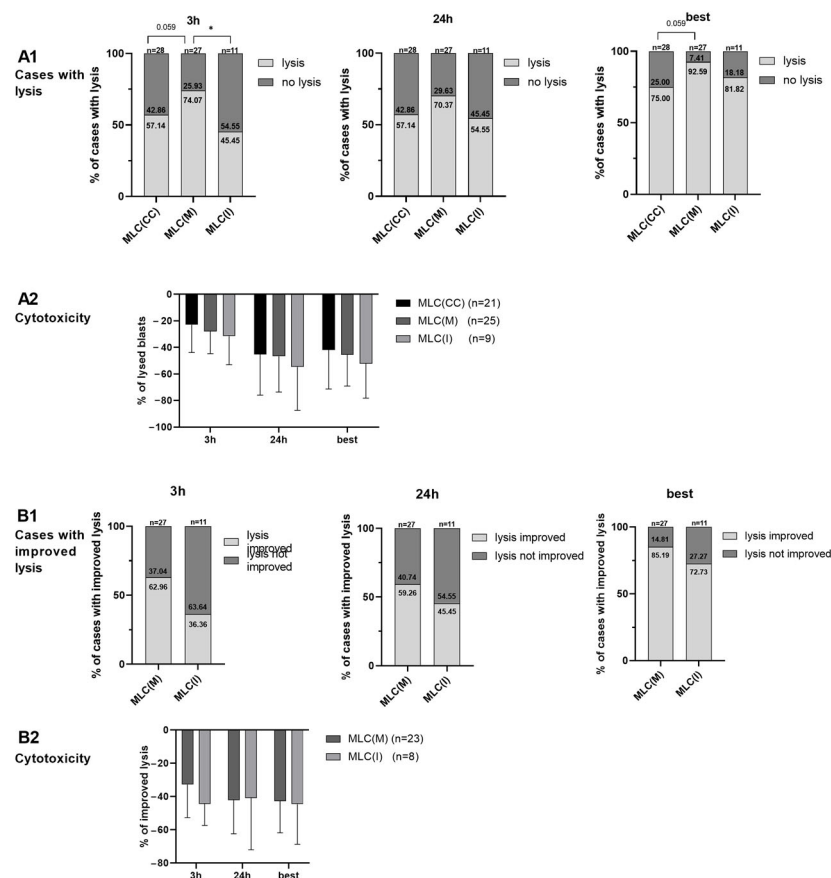
In summary, we found an induction of degranulating and intracellular cytokine-producing  $\beta 7^+$  T-, NK and CIK-cells after MLC using Kit-pretreated WB compared to the control. We also found more degranulation and intracellular cytokine production in healthy donors' (vs. patients') samples.

#### 2.2.4. Improved Antileukemic and Blastolytic Functionality of Immune Cells after T-Cell-Enriched MLC with Kit-M-Pretreated (vs. Untreated) WB

We compared the achieved blast lysis ('lysis') of MLC(CC), MLC(I) and MLC(M) using the cytotoxicity fluorolysis assay after the co-cultivation of effector (T-cell-enriched MLC stimulated with Kit-pretreated or untreated WB) and target (blast-containing MNC) cells.

We quantified the frequencies of viable blasts after 3 and 24 h of incubation time and selected the superior antileukemic effectivity after either 3 or 24 h incubation time as a ‘best of’ value.

After 3 (24) h of incubation of effector with target cells, blast lysis was found in 74.07 (70.37)% of cases after MLC(M) compared to 57.14 (57.14)% after MLC(CC) and 45.45 (54.55)% after MLC(I) (Figure 5A1), with comparable frequencies of blast lysis after MLC(M), MLC(I) and MLC(CC) in cases with achieved lysis (Figure 5A2). After 3 h of incubation of effector with target cells, we found nearly significantly more cases with lysis after MLC(M) compared to MLC(CC) ( $p = 0.059$ ). Figure 5A1). Improved lysis (compared to MLC(CC)) was found in 62.96 (59.26) % of cases after MLC(M) vs. in 36.36 (45.45) % after MLC(I) (Figure 5B1), going along with comparable frequencies after MLC(M) and MLC(I) (compared to MLC(CC)) in cases with improved lysis (Figure 5B2).



**Figure 5.** Blastolytic potential of immune-reactive cells after T-cell-enriched MLC using leukemic WB with and without Kit pretreatment as stimulator cells. For the cytotoxicity assay, target and effector cells were coincubated for a total of 24 h. Results after 3 h and 24 h and the ‘best of’ values after coincubation are given. Results after MLC from WB pretreated with Kit-M (MLC(M)) or Kit-I (MLC(I)) or without added Kits as control (MLC(CC)) are given. Percentages of cases (A1) with achieved (vs. non-achieved) blast lysis and (B1) with improved (vs. non-improved) blast lysis are given. Mean frequencies  $\pm$  standard deviation of (A2) lysed blasts (in cases with lysis) and (B2) lysis improvement (in cases with improved lysis) are given; n—number of cases. Differences were considered as significant (\*) when  $p \leq 0.05$ . Abbreviations of cell subpopulations are given in Table 1.

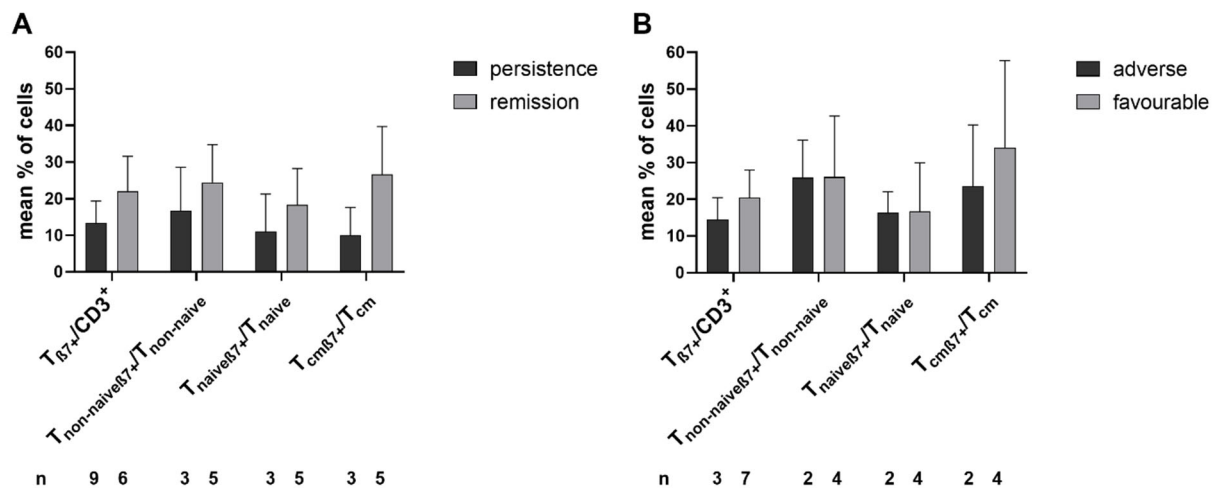
Selecting the best achieved lysis after 3 and 24 h of incubation of effector with target cells (best), we found nearly significantly more cases with lysis after MLC(M) compared to MLC(CC) ( $p = 0.059$ ). Figure 5A1). Frequencies of lysed blasts after MLC(M), MLC(I) and MLC(CC) in cases with achieved lysis were not significantly different (Figure 5A2). After MLC(M) vs. MLC(I) (compared to MLC(CC)), blast lysis could be improved in 85.19% of

cases after MLC(M) and in 72.73% of cases after MLC(I) (Figure 5B1), going along with the comparable frequencies of lysis improvement after MLC(M) and MLC(I) (compared to MLC(CC)) in cases with improved lysis (Figure 5B2).

In summary, Kit-M treatment indicates clearly (although not significantly) improved blast lysis after MLC when compared to the control in our patient cohort.

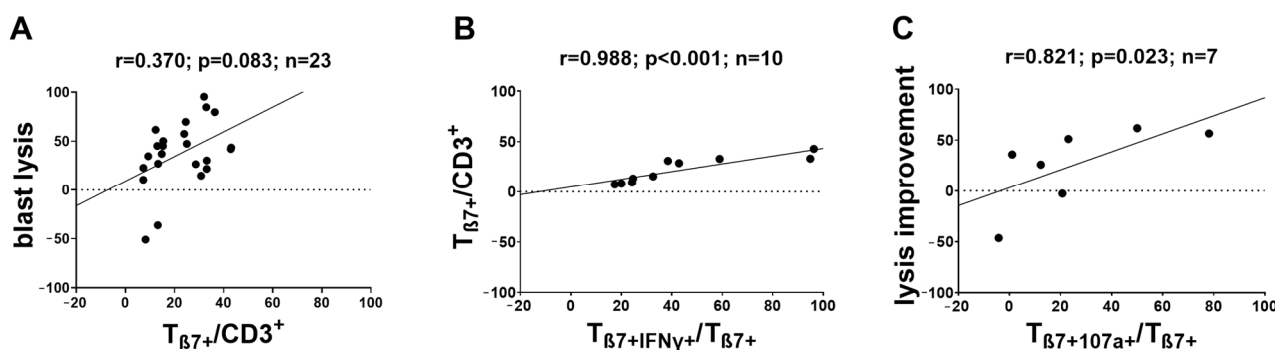
### 2.3. Correlation of (Antigen-Specific) $\beta 7$ Expression with Patients' Allocation to Risk Groups, Response to Induction Chemotherapy and Achieved Antileukemic (Ex Vivo) Functionality

We compared the  $\beta 7$  expression in T-cells and their subtypes in uncultured immune cells in the samples from AML patients at first diagnosis with allocation to the ELN risk groups and response to induction chemotherapy. Patients who achieved ( $n = 6$ , vs. those who did not achieve ( $n = 9$ )) remission were characterized by clear, although non-significantly higher frequencies of  $T_{\beta 7+}$  cells. In patients with favorable ( $n = 7$ ) vs. adverse ELN risk stratification ( $n = 3$ ), clear, although non-significantly higher frequencies of  $T_{cm\beta 7+}/T_{cm}$  were found (Figure 6A,B).



**Figure 6.** Composition of uncultivated  $\beta 7$ -expressing immune-reactive cells in AML patients' samples with patients subdivided into different groups at first diagnosis. Uncultured cells (MLC(UC)) were analyzed via flow cytometry. Mean frequencies  $\pm$  standard deviation of  $\beta 7$ -expressing immune-reactive cell subpopulations in patients with AML at first diagnosis with respect to patients' (A) responses to chemotherapy and (B) allocation to cytogenetic ELN risk groups are given; n—number of cases. Abbreviations of cell subpopulations are given in Table 1.

We correlated the degranulation and intracellular cytokine production (frequencies of (leukemia-specific)  $\beta 7$ -expressing immune-reactive cells) with (improved) antileukemic functionality after MLC. We found a clear (although not significant) positive correlation between  $T_{\beta 7+}/CD3^+$  after Kit-M pretreatment and improved blast lysis in MLC(M) (but not in MLC(I)) (compared to MLC(CC)) ( $r = 0.370$ ;  $p = 0.083$ ) (Figure 7A). We found significant, positive correlations between the frequencies of  $\beta 7$ -expressing cells and the frequencies of intracellular cytokine-producing  $\beta 7$ -expressing cells ( $T_{\beta 7+IFN\gamma+}$ :  $r = 0.988$ ,  $p < 0.001$ ;  $T_{\beta 7+TNF\alpha+}$ :  $r = 0.952$ ,  $p < 0.001$ ) in MLC(M) (Figure 7B). We also found a positive correlation between increased frequencies of  $T_{\beta 7+107a+}$  and lysis improvement ( $r = 0.821$ ;  $p = 0.023$ ) (Figure 7C) with the blastolytic functionality and frequencies of  $\beta 7$ -expressing cells.



**Figure 7.** Correlations of antileukemic functionality with frequencies of (leukemia-specific)  $\beta 7$ -expressing immune-reactive cells before and after T-cell-enriched MLC using Kit-M-pretreated (vs. untreated as control) leukemic WB as stimulator cells. Cells were analyzed via flow cytometry before and after 7 days of (T-cell-enriched) MLC with Kit-pretreated or untreated WB and IL-2. Results of uncultured cells before MLC (MLC(UC)), and cells after MLC, from WB pretreated with Kit-M (MLC(M)) or without added Kits as control (MLC(CC)), are given. Lysis (improvement) is given as the best of value after MLC(M) (compared to MLC(CC)). **(A)** Clear positive correlation of frequencies of  $T_{\beta 7+}/CD3^{+}$  with best blast lysis after MLC (with Kit-M-pretreated leukemic WB). **(B)** Significant positive correlation of frequencies of  $T_{\beta 7+}/CD3^{+}$  with  $T_{\beta 7+IFN\gamma+}/T_{\beta 7+}$  after MLC (with Kit-M-pretreated leukemic WB). **(C)** Significant positive correlation of frequencies of  $T_{\beta 7+107a+}/T_{\beta 7+}$  with improved blast lysis after MLC (with Kit-M-pretreated leukemic WB).  $r$ —correlation coefficient,  $p$ —significance,  $n$ —number of cases. Differences were considered as significant when  $p \leq 0.05$  and as highly significant when  $p \leq 0.005$ . Abbreviations of cell subpopulations are given in Table 1.

In summary, Kit treatment of WB led to the increased generation of DC subtypes (DC,  $DC_{leu}$ ,  $DC_{mat}$  and  $DC_{leu-mat}$ ), to activated immune cells and to increased frequencies of  $\beta 7$ -expressing cells compared to the control. Additionally, Kit treatment led to increased frequencies of degranulating and intracellular cytokine-producing  $\beta 7$ -expressing immune cell (subtypes). Finally, (partly significant) we saw positive correlations of  $\beta 7$ -expressing immune cell (subtypes) with the provision of leukemia-specific/antileukemic cells after T-cell-enriched MLC, with patients' responses to chemotherapy or with their allocations to risk types in our small patient cohort.

### 3. Discussion

Expressions of several leukemia-associated antigens (e.g., CD318 and CD11b) have been studied via flow cytometry to determine their value in classifying the disease and to monitor (residual) leukemic cells [41,42]. The evaluation of (leukemia-specific) immunoreactive cells has been shown to further contribute to evaluating therapy efficiency, to quantifying antileukemic reactions and to improving therapy options and prognoses [43,44].

#### 3.1. DC-Based Therapies as Promising Therapy Options

Due to their ability to mediate innate and the adaptive immune response, DCs' and  $DC_{leu}$ s' therapeutic potential has been widely recognized [5–7,9]; DC vaccinations with monocyte-derived DCs, as well as  $DC_{leu}$  converted from patients' myeloid blasts, have shown immunological effects in vivo [9,10,45,46].

#### 3.2. Improved Activation of the Adaptive and Innate Immune System with Kit-Treated WB

##### 3.2.1. Ex Vivo DC Generation and (Antileukemic) Immune Cell Activation

DC (subtypes) from healthy as well as patients' WB samples can be generated in the presence of Kits (compared to controls) [10,27,47,48] (Figure 1). Combinations of GM-CSF and PGE1 (Kit-M) or GM-CSF and Picibanil (Kit I), added to healthy/leukemic whole blood, use the soluble microenvironment in WB (containing, e.g., cytokines and chemokines) as an additional source for response modifiers to generate DC/ $DC_{leu}$ . Both Kits provide danger

signaling combined with maturation signaling, which guide cells' differentiation towards DC or DC<sub>leu</sub>, respectively, as published before [10,48]. The detailed functional pathways of (PGE1-/Picibanil-containing) DC-generating methods are not known. The induction of monocyte or blast proliferation was not seen, thereby indicating that Kits do not induce blast or monocyte proliferation; however, they give rise to significantly increased frequencies of leukemia- or monocyte-derived, mature DC, as already shown [10,11] (Figure 1). According to our findings (also confirmed here), we can deduce that Kit-M might trigger, with higher efficacy, improved/mediated antileukemic reactions compared to Kit I. Moreover, we show that the achieved antileukemic activities (achieved using patients' samples at first diagnosis) are independent of patients' sex, cytogenetic risk and blast counts (patients with leukemia in remission might profit from these Kit-mediated effects; (residual) blasts are converted to DC<sub>leu</sub> and trigger the immune system specifically against blasts). This has to be proven in a clinical trial [17,21].

Furthermore, we confirm that adaptive and innate immune cells from healthy and patients' samples pretreated (vs. not pretreated) with Kits were regularly (significantly) activated after MLC, giving rise to (significantly) increased frequencies of activated cells of the innate and adaptive immune system in AML samples, pointing to Kit-mediated activation of immune cells and the generation of memory cells (Figure 2), as shown before [9–11,21,27,30,48]. We also found higher frequencies of activated immune-reactive cells in healthy (compared to patients') samples, as already shown [27], possibly pointing to immunological activation against various bacterial, viral or mycotic targets [49,50] (Figure 2). Compared to uncultured cells, activation of immune cell subtypes after MLC was seen, due to the influence of IL-2, as expected [51] (Figures 2 and 3).

### 3.2.2. Higher $\beta 7$ Expression in Immune Cells after MLC with Kit-I and Kit-M Treatment of Healthy and Patients' WB Samples

$\beta 7$  expression in uncultured T-cells has already been shown to correlate with cell cytotoxicity against leukemic (blasts) and other (intestinal/intraepithelial cells) targets [15,38–40]. We found that healthy donors' samples showed significantly higher frequencies of  $\beta 7$  in T-cells compared to patients' samples before cultivation, as well as in cells after culture (control, Kit-M) (Figure 3); this confirms the findings of Vogt et al. obtained before/after MLC with blast-containing MNC pretreated using DC-generating methods (MCM-Mimic, Picibanil and Ca-Ionophore) as stimulator cells [15]. These increased frequencies of  $\beta 7$  expression in healthy cells compared to those of patients might indicate a detrimental effect of leukemic immunosuppression on  $\beta 7$  expression in immune cells [49]. Moreover, our data show (significantly) increased frequencies of  $\beta 7$ -expressing CD3<sup>+</sup>, T<sub>non-naive</sub> and T<sub>cm</sub> in healthy and patients' samples after MLC with Kit-M- and Kit-I-pretreated (vs. control) WB (Figure 3). These data might indicate the involvement of  $\beta 7$ -expressing immune cells in immune functionality in healthy as well as patients' samples (Figure 3).

Comparing the DC-generating potential of Kit-I and Kit-M, we found higher frequencies of DC after Kit-I treatment in healthy samples and lower frequencies of DC<sub>leu</sub> in patients' samples. This might point to lower efficiency of Kit-I in generating functional DC (Figure 1). These results confirm the unpublished data of Ugur et al. [17], who also showed lower functionality compared to Kit-M.

### 3.2.3. Increased Production of (Antigen-Specific) Degranulating or Intracellular Cytokine-Producing Immune Cells after MLC of Kit-M-Pretreated Healthy and Patients' WB Samples

According to their biological function, DCs can help to overcome the anergy of immune-reactive cells and prime effector cells against their targets. Efficacy can (and has to) be demonstrated by induced/increased immune-reactive cells and decreased blast counts. (Functional) specific effects, mediated by DC/DC<sub>leu</sub> have to be quantified (compared to controls). In the case of DC (loaded with tumor antigens) or DC<sub>leu</sub>, their capability to activate the immune system specifically against leukemic cells has to be evaluated after (T-cell-enriched) mixed lymphocyte culture (MLC) using DC/DC<sub>leu</sub> as stimulator cells. The

assays to detect leukemia-specific activations are cytokine secretion assays, degranulation assays, intracellular cytokine assays, ELISPOT, TETRAMER, etc. [52]. Adding leukemia-associated antigens (e.g., WT1 and PRAME) to cultures with/without the cultivation of cells can help to detect/enrich low frequencies of specific cells [21,30,53].

DEG und INTCYT assays are useful for demonstrating the antigen-specific activation of immunoreactive cells by measuring cell degranulation and intracellular cytokine production (with and without stimulation with LAA) [21,30]. We already showed significant activation of antigen-specific degranulating or intracellularly cytokine-producing innate and adaptive immune cells after MLC with Kit-M-pretreated (vs. untreated) WB (and low frequencies in uncultured cells) [21,30]. Studying  $\beta 7$ -expressing immune cells, we observed higher frequencies of degranulating  $\beta 7$ -expressing T- and NK-cells, CIK-cells and intracellularly cytokine-producing T-cells (in healthy and/or patients' samples) after MLC(M) (compared to the control) (Figure 4A,B), with higher frequencies in healthy compared to patients' samples (Figure 4A,B). Together with the findings after the Kit-M-mediated MLC of increased  $\beta 7$  expression, this might indicate (specific) involvement of these cells in immune reactivation, in healthy as well as in AML patients.

While the addition of LAA (to patients' samples) or SEB (to healthy samples) antigen stimulation [21,29] led to higher frequencies of antigen-specific cells, especially in uncultured cell samples, it did not significantly enhance the Kit-M-mediated effects after MLC with respect to induced antigen-specific cells compared to the control (data not shown). This confirms the previous data, which show that Kit-M pretreatment of WB (going along with the generation of DC) stimulates and activates immune-reactive cells and compensates for LAA- or SEB-triggered activating effects [21].

In principle, the induction of leukemia-specific cells can be also detected *in vivo* by applying the methods given above [54]. Several groups have shown that AML patients treated with DC/DC<sub>leu</sub> showed significant induction of (leukemia-specific) cellular and humoral immunity, going along with reduced blasts and prolonged overall survival (e.g., [54–59], all cited in [52]). Depending on the methods used, significant induction of the leukemia-specific immune response was defined as a 25 (50)% increase in the frequency of leukemia-specific cells compared to the initial count, or significantly higher frequencies of induced specific cells compared to untreated controls (e.g., [55,60,61]).

#### 3.2.4. Increased Blastolytic Functionality of Immune-Reactive Cells in Kit-Pretreated Samples after MLC

The hardest proof of induced or improved antileukemic activity (compared to controls) is the detection of improved blast lysis compared to the controls (e.g., after MLC). This can be achieved via chrome release, fluorolysis or other assays [62]. The Kit pretreatment of blasts in WB has already been shown to improve the antileukemic activation of immunoreactive cells after MLC, going along with significantly increased blast lysis compared to the controls [11,17,21,30,48].

Here, we found (nearly significantly) more cases with a superior blastolytic effect of immune-reactive cells after MLC(M) compared to the control ( $p = 0.056$ ). Kit-I pretreatment was less effective compared to Kit-M pretreatment in giving rise to improved blastolytic functionality of the immune-reactive cells (Figure 5).

Blast lysis was, for some cases, superior after 3 h of incubation of target with effector cells, and for other cases, after 24 h. These effects could be attributed to different, independent blastolytic mechanisms, namely the faster perforin/granzyme pathways (leading to blast lysis predominantly after 3 h of coincubation of target with effector cells) and the slower Fas/FasL pathways (leading to blast lysis predominantly after 24 h of coincubation of target with effector cells) [17,21,63].

Summarizing the blastolytic effects achieved (after 3 h or 24 h of coincubation of effector with target cells), our data support previous data, which show that the Kit-M pretreatment of WB improves the blastolytic activity of immunoreactive cells in cell cultures [17,21].

### 3.3. Potential of $\beta 7$ Monitoring

#### 3.3.1. $\beta 7$ Expression in Immune-Reactive Cells as a Clue to Higher Susceptibility to Chemotherapy and Kit Treatment

Compared to patients with AML/MDS, the frequencies of  $\beta 7$ -expressing cells were higher in healthy donors with a healthy immune system, unaffected by the immunosuppressive effect of neoplastic cells [50,64]. Generally, although the differences were not significant due to low case numbers, AML patients at first diagnosis who achieved (vs. those who did not achieve) leukemia remission after induction chemotherapy presented with higher frequencies of  $\beta 7$ -expressing immune cell subtypes in uncultured WB samples; moreover, patients' assignment to the favorable vs. adverse cytogenetic risk group went along with higher frequencies of  $\beta 7^+$   $T_{cm}$  in uncultured patients' WB (Figure 6). This could point to an influence of  $\beta 7$ -expressing cells in contributing to a healthy functional immune system [15,38,40].

#### 3.3.2. $\beta 7$ Expression as a Marker for Improved Blast Cytotoxicity

We were able to find positive correlations of achieved blast lysis with frequencies of  $\beta 7$ -expressing cells after MLC(M). These results confirm previous findings that showed higher blastolytic cytotoxicity in samples with higher frequencies of  $\beta 7$  expression using different (Kit-independent) DC-generating methods, and could also point to involvement of  $\beta 7$  expression in the mediation of superior blastolytic functionality in immune cells [15]. In addition, the improved blast lysis correlated positively with the increased degranulation activity (Figure 7C), which further demonstrates the antileukemic potential of these Kit-induced  $\beta 7$ -expressing immune cells and the enhancing effect of Kit-M pretreatment on antileukemic functionality. Similar correlations could also be found for cells after MLC (Ugur [17] and personal communication; publication in preparation).

Possible correlations of  $\beta 7$  expression with cytotoxicity have been discussed in the context of autoimmune processes [38,40]. In the case of malignant diseases such as AML, it was hypothesized that a correlation between  $\beta 7$  expression and cell toxicity might have potential as a marker indicating the antileukemic functionality of immune cells [15]. This hypothesis could be further supported by our finding that  $\beta 7$  expression also correlated with more pronounced antileukemic intracellular cytokine production of  $\beta 7$ -expressing cells after MLC(M) (Figure 7B) (which also correlated with higher antileukemic functionality, as shown before [21]).

We can state that the Kit-M-induced activation of immune cells after T-cell-enriched MLC goes along with increased  $\beta 7$  expression in (leukemia-specific) immune cells and correlates with improved antileukemic activity.

## 4. Materials and Methods

### 4.1. Patients and Healthy Sample Acquisition

Heparinized peripheral WB samples (provided by the university hospitals of LMU in Munich, Augsburg, Oldenburg and Tuebingen, as well as the Rotkreuzklinikum in Munich) were taken from patients diagnosed with acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS) and healthy volunteers. Patients' consent was given according to Helsinki guidelines and the vote of the Ethics Committee of LMU in Munich (vote number: 33905).

We included 31 patients with AML or MDS in acute stages of disease and 18 healthy volunteers (as given in Table 2). Patient age at sample acquisition was, on average, 60.8 (29–98) years, and the age of healthy probands was 31.3 (20–56) years. The male:female ratio of patients was 1:0.8, and 1:1.25 in healthy individuals. The average peripheral blood (PB) blast count of patients was 33 (10–94) %Bla/cells. AML cases were classified according to the FAB classification system [65], and MDS cases according to the WHO classification system [66]. Assessments for AML patients were further risk-classified according to the ELN classification [67] in favorable, intermediate and adverse subgroups, and for MDS patients according to the IPSS-R classification system [5,68] (Table 2).

**Table 2.** Characteristics of patients and healthy individuals.

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	favorable	yes	DC; MLC; CTX
AML	1540	83	f	p/M1	dgn.	32	13, 34, 33, 15, 117, 56	favorable	no	DC; MLC; CTX; D/I
AML	1427	52	m	p/M2	dgn.	94	13, 33, 34, 117	favorable	no	DC; MLC; CTX
AML	1541	82	f	p/M2	dgn.	15	15, 34, 117	interm.	no	DC; MLC; CTX; D/I
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	favorable	yes	DC; MLC; CTX
AML	1430	79	m	p/M5	dgn.	62	13, 33, 34, 117	favorable	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; D/I
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; D/I
AML	1570	36	f	p/nd	dgn.	11	34, 117, 65, 13, 33	favorable	no	DC; MLC; 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; D/I
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, 117, 13	favorable	nd	DC; MLC; D/I
AML	1574	56	m	s/nd	dgn.	41	34, 117, 15, 19	nd	no	DC; MLC; CTX; D/I
AML	1571	61	m	p/M2	rel.	18	117, 33, 13, 7	nd	yes	DC; MLC; CTX; D/I
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; D/I
AML	1449	78	m	s/nd	rel.	32	65, 14, 15, 33, 56, 34	nd	nd	DC; MLC; CTX



Table 2. Cont.

Diagn.	No.	Age	Sex	Subtype	Stage	Ic bla	Blast Phenotype [cd]	Risk Class.	Response	Exp.
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
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; D/I
MDS	1573	61	m	MDS	dgn.	12	34, 117, 65, 13, 61	high	no	DC; MLC; CTX; D/I
MDS	1572	63	f	MDS-EB2	dgn.	10	34, 117, 65, 33, 13	very high	no	DC; MLC; CTX; D/I
healthy	1417	34	f							DC; MLC
healthy	1418	22	m							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	1440	20	f							DC; MLC
healthy	1448	27	f							DC; MLC
healthy	1458	21	f							DC; MLC
healthy	1544	22	m							DC; MLC; D/I
healthy	1545	32	m							DC; MLC; D/I
healthy	1547	46	f							DC; MLC; D/I
healthy	1566	54	f							DC; MLC; D/I
healthy	1576	55	m							DC; MLC; D/I
healthy	1578	32	m							DC; MLC

Diagn.—diagnosis; No.—sample number; m—male; f—female. Subtypes: p—primary AML; s—secondary AML. AML (FAB classification); MDS (WHO classification); rel. a. SCT—relapse after stem cell transplantation; rel.—relapse; dgn.—first diagnosis; pers.—persisting disease; PR—partial remission; nd—no data; Ic Bla—immunocytologically determined blasts; Risk Class: cytogenetic (AML ELN) and multifactorial (MDS IPSS-R) risk classification; Response—response to (induction) chemotherapy; Exp.—conducted experiments; MLC—mixed lymphocyte culture; DC—dendritic cell generation; CTX—cytotoxic fluorolysis assay; D/I—degranulation and intracellular cytokine assay.

#### 4.2. Initial Sample Preparation

Mononuclear cells (MNCs) [10,69] were isolated from WB according to standard preparations. T-cells were prepared using MACS microbead technology (Miltenyi Biotec, Bergisch Gladbach, Germany) [70]. MNC and T-cells were frozen for later experiments, as described [10,69,70].

#### 4.3. Cultivation of Dendritic Cells (DC) and Leukemia-Derived Dendritic Cells (DC<sub>leu</sub>)

DC and DC<sub>leu</sub> were cultured with (vs. without) combinations of response modifiers (Kit-M and Kit-I), as described [10].

Kit-M consisted of GM-CSF (final concentration (fc): 800 U/mL, granulocyte macrophage colony stimulating factor, Sanofi-Aventis, Frankfurt, Germany) and PGE1 (fc: 1 µg/mL, prostaglandin E1, Santa Cruz Biotechnology, Dallas, Texas, USA), and Kit-I consisted of GM-CSF (fc: 800 U/mL) and Picibanil (fc: 10 µg/mL, Chugai Pharmaceutical Co., Kajiwarra, Japan). Response modifiers were added on Day 0 and on Day 2–3 [27]. After 6–8 days, the cells were harvested, evaluated via flow cytometry and used for further experiments [10]. Cells will be referred to as DC(M) for Kit-M-treated WB, DC(I) for Kit-I-treated WB and DC(C) for the control with untreated WB.

#### 4.4. Cultivation of Cells in Mixed Lymphocyte Cultures (MLC)

After seven days of DC generation (Kit-treated or untreated WB) the cultivation of MLC was started with previously frozen/thawed T-cells in the presence of 50 U/mL IL-2, as described [21,30].

After 6–8 days, cells were harvested, immune cell subtypes were quantified, and degranulation (DEG), intracellular cytokine (INTCYT) and cytotoxicity fluorolysis assays (CTX) were performed, as described [10,27]. Cells before MLC will be referred to as MLC(UC) and those after MLC as MLC(CC) for the control with untreated WB. MLC(M) will refer to MLC with Kit-M-pretreated WB, and MLC(I) to MLC with Kit-I-pretreated WB.

#### 4.5. Degranulation Assay (DEG) and Intracellular Cytokine Assay (INTCYT)

Cells (uncultured WB or T-cell-enriched MLCs, stimulated with or without Kit-treated WB after MLC) were mixed and incubated in parallel with those without leukemia-associated antigens (LAA) (PepTivator WT1 (fc: 0.6 nmol/mL, Miltenyi Biotec, Bergisch Gladbach, Germany) and PepTivator PRAME (fc: 0.6 nmol/mL, Miltenyi Biotec, Bergisch Gladbach, Germany)) for patients' samples and with staphylococcal enterotoxin B (SEB, fc: 10 µg/mL, Sigma-Aldrich, St. Louis, Missouri, USA), as described [21,30,71].

An FITC-conjugated antibody against CD107a (BioLegend, San Diego, CA, USA) was used to detect cell degranulation as a marker for cell cytotoxicity, as described [19,72]. Finally, cells were harvested and analyzed via flow cytometry [30].

To quantify intracellular cytokine production, antibodies against tumor necrosis factor alpha (TNFα) [20] and interferon gamma (IFNγ) [21] were used: PE/Cy 7-TNFα antibodies (BioLegend, San Diego, CA, USA) and PE-IFNγ antibodies (BioLegend, San Diego, CA, USA). To stop the cellular cytokine secretion, after one hour of incubation time, Brefeldin A solution (fc: 5 µg/mL, BioLegend, San Diego, CA, USA) was added according to the manufacturer's instructions. Finally, cells were harvested and analyzed via flow cytometry [21].

#### 4.6. Cytotoxicity Fluorolysis Assay (CTX)

CTX was performed to test the ability of effector cells (T-cell-enriched cells, stimulated with Kit-M-treated or untreated WB) to lyse blast target cells.

Kit-M-treated (vs. untreated as a control) patients' WB (as 'stimulator cells') and patients' T-cells (frozen at the start of the experiments) were used as the main 'effector cells' to be activated by MLC. AFTER MLC functionality was measured: cells in MLC (after stimulation with Kit-M-treated (vs. untreated) WB cells) were 'effector cells'. Blast-containing MNC (frozen at the start of the experiments) were added as 'target cells'. Blasts were stained using patient-related blast markers and were quantified via flow cytometry.

Finally, the frequencies of viable (7AAD-negative) blast cells after the incubation of ‘target cells’ (blast-containing MNC) with ‘effector cells’ (T-cell-enriched MLC stimulated (vs. not stimulated) with Kit-M before were quantified, and blast lysis calculated according to previous publications, e.g., [21].

Finally, the difference in viable blast-cells in the main and the control samples was defined as ‘blast lysis’. The ‘lysis improvement’ was determined by comparing the achieved ‘blast lysis’ after MLC with, compared to without, Kit-pretreated WB [10,17,32].

#### 4.7. Quantification of Cells Using Flow Cytometry

Before or after culture, cells were stained using fluorochrome-labeled monoclonal antibodies and quantified using a FACSCalibur four-channel flow cytometer as described [12, 16]. The antibodies were conjugated with FITC (fluorescein-isothiocyanate), PE (phycoerythrin), PE/Cy 7 (phycoerythrin/cyanine 7) and APC (allophycocyanin). The FITC-labeled antibodies were: IgG\*, CD34\*, CD65\*, CD33\*, CD117\*\*\*, CD15\*, CD56\*\*\*, CD3\*\*, CD71\*, ipo38\*\*\*\*, CD19\*, CD107a\*\*\*, CD4\*\*, CD45RO\* and CD14\*. The PE-labeled antibodies were: IgG\*, CD 117\*, CD80\*, CD83\*, CD56\*, CD206\*, CD3\*, IFN $\gamma$ \*\*\* and CD4\*\*. The PE/Cy 7-labeled antibodies were: IgG\*, CD15\*\*, CD117\*, CD19\*, CD34\*, CD197\*\*, CD56\*, CD4\*, TNF $\alpha$ \*\*\*, CD3\* and CD14\*\*. The APC-labeled antibodies were: IgG\*, CD206\*\*, CD80\*\*\*, CD209\*\*, CD83\*\*, CD34\*, CD117\*, CD14\*, CD56\*, CD69\*\*,  $\beta$ 7\*\*, CD45RO\*\*\*, CD4\*\*, CD3\* and CD19\*. The antibodies were supplied by Beckman Coulter (\*, Brea, CA, US), BD Biosciences (\*\*, San Jose, CA, US), BioLegend (\*\*\*, San Diego, CA, US) and Santa Cruz Biotechnology (\*\*\*\*, Dallas, TX, US). For the detection of viable cells, 7AAD\*\* was used.

For every patient, highly expressed blast markers (e.g., CD34, CD117, CD65, CD56 or CD15) and DC markers with low/no expression of blasts (e.g., CD80, CD83, CD206 or CD209) were selected to quantify DC or DC<sub>leu</sub> subpopulations after culture [12,16].

For the detection of intracellular markers (ipo38 and INTCYT), the FIX & PERM Cell Fixation and Cell Permeabilization Kit (Thermo Fisher Scientific, Darmstadt, Germany) was used. Isotype samples served as controls [10,73].

#### 4.8. Statistical Analysis

The analysis of flow cytometric data was conducted using BD CellQuest Pro software (Becton Dickinson, Heidelberg, Germany). Statistical analyses, including the calculation of means, standard deviations and significance, were conducted using Excel 2010 (Microsoft, Redmond, Washington, USA) and SPSS Statistics 26 (IBM, Armonk, New York, NY, USA). Differences and correlations between groups were analyzed using the paired-*t*-test and the Wilcoxon–Mann–Whitney U test. Correlation analyses were conducted using Pearson correlation and Spearman correlation. Highly significant differences were defined in cases with *p*-values  $\leq 0.005$  and significantly different cases with *p*-values between 0.05 and 0.0051.

## 5. Conclusions

$\beta$ 7, as a subunit of the integrin receptor, is expressed in several subtypes of healthy and patients’ cells in the adaptive and innate immune system. Pretreatment of AML/MDS patients’ WB samples with blast-modulating Kits (vs. no Kits) increased the frequencies of DC/DC<sub>leu</sub>, which ultimately increased the frequencies of (leukemia-specific degranulating or cytokine-producing)  $\beta$ 7-expressing T- or NK/CIK-cell subtypes after T-cell-enriched MLC. The frequencies of the generated/activated  $\beta$ 7-expressing cells correlated *ex vivo* with the provision of leukemia-specific/antileukemic cells after (T-cell-enriched) MLC with Kit-pretreated (vs. untreated) WB, and *in vivo* with achieved (vs. not achieved) remission after induction chemotherapy, and with patients’ allocations to favorable (vs. unfavorable) risk types. (Due to the low case numbers available, not all results showed significant results; however, there were always clear differences between groups.)

**Author Contributions:** E.R. performed a great portion of the experiments and analyzed all flow cytometric and statistical data. L.L., L.K.K., S.U., E.P., C.G., M.W., F.D.-G., C.P. and D.C.A. contributed data to the DC, MLC, CTX and DEG/INTCYT experiments, which were evaluated by E.R., C.S. and N.R. helped with manuscript preparation. P.B., D.K., J.S., A.R. and C.S. provided the patients' samples and reports. H.M.S. was responsible for the study design. E.R. and H.M.S. contributed to drafting. C.L.S. contributed to critical revision and discussion and helped with submission. N.R., C.L.S., C.S., E.R. and H.M.S. contributed to editing the manuscript. All authors have read and agreed to the published version of the manuscript.

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