

Antileukemic T-cell Responses Can Be Predicted by the Composition of Specific Regulatory T-cell Subpopulations

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Summary: Regulatory T cells (T_{reg}) are important regulators of immune responses. In acute myeloid leukemia (AML) patients before/after immunotherapy (stem cell transplantation or donor lymphocyte infusion), their suppressive role can contribute to suppress severe graft-versus-host reactions, but also to impair antileukemic reactions. As leukemia-derived dendritic cells (DC_{leu}) are known to improve the antileukemic functionality of T cells, we evaluated the composition and development of distinct T_{reg} subtypes in AML patients ($n = 12$) compared with healthy probands ($n = 5$) under unstimulated conditions and during stimulation with DC_{leu} -containing DC (DC) or blast-containing mononuclear cells (MNC) in 0- to 7-day mixed lymphocyte cultures by flow cytometry. T-cell subgroups in AML patients were correlated with antileukemic functionality before and after DC or MNC stimulation by functional fluorolysis assays. (1) AML patients' T cells presented with significantly higher frequencies of T_{reg} subgroups in unstimulated T cells compared with healthy probands. (2) After 7 days of DC or MNC stimulation, all T_{reg} subtypes generally increased; significantly higher frequencies of T_{reg} subtypes were still found in AML patients. (3) Antileukemic cytotoxicity was achieved in 36% of T cells after MNC compared with 64% after DC stimulation. Antileukemic activity after DC but not after MNC stimulation correlated with significantly lower frequencies of T_{reg} subtypes ($CD8^+T_{reg}/T_{eff/em\ reg}$). Furthermore, cut-off values for T_{reg} subpopulations could be defined, allowing a prediction of antileukemic response. We demonstrate a crucial role of special T_{reg} subtypes in the mediation of antileukemic functionality. High $CD8^+T_{reg}$, $T_{eff/em\ reg}$, and $CD39^+$ T cells correlated clearly with a reduced antileukemic activity of T cells. DC stimulation of T cells contributes to overcome impaired antileukemic T-cell reactivity. Refined analyses in the context of clinical responses to immunotherapies and graft versus host reactions are required.

Key Words: AML, regulatory T cells, dendritic cells, immune escape
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Acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS) are clonal disorders of hematopoietic stem cells characterized by an impaired cell

differentiation.¹ Although up to 70%–80% of patients younger than 60 years of age achieve a complete remission with induction therapy, the 2-year survival is only about 46%.² Despite intensive treatment options such as high-dose chemotherapy, stem cell transplantation (SCT), and transfusion of Donor T cells to maintain the antileukemic function, the rate of relapse or persistence of the disease is still unsatisfying in patients with AML.

Until now, several soluble or cellular factors are known that regulate immune mechanisms in healthy individuals. However, in tumor patients, unbalanced regulatory mechanisms can contribute to immune escape mechanisms through which leukemic blasts can escape the killing by the immune system³; downregulation of HLA class I expression on tumor cells⁴ or a defective antigen processing and expression on tumor cells protect them from elimination by cytotoxic T lymphocytes (CTL).⁵ Another escape mechanism uses the disruption of the Fas/FasL system, which induces apoptosis. Furthermore, soluble factors such as the immunosuppressive cytokines interleukin (IL)-10 or tumor growth factor- β , or FasL and 4-1BBL released by T cells, dendritic cells (DC), or tumor cells can limit the immune surveillance because of insufficient lymphocyte stimulation in an inhibitory microenvironment.^{6,7} Moreover, we and others have already shown that regulatory soluble factors produced by host T cells or DC contribute to the regulation and mediation of antileukemic T-cell function.³ Moreover, cellular factors such as regulatory T cells (T_{reg}) consisting of naturally occurring T_{reg} and several subgroups of extrathymically activation-induced T_{reg} are known to regulate immune responses. T_{reg} are classically divided into naturally occurring $CD4^+CD25^+FOXP3^+$ T_{reg} (nT_{reg}) and activation-induced T_{reg} (iT_{reg}) appearing after peripheral antigen stimulation.⁸ iT_{reg} can belong to $CD4^+$ and $CD8^+$ T cells, whereas nT_{reg} belong only to $CD4^+$ T cells, and form about 1%–2% of the peripheral blood cells.⁹ From a clinical point of view, T_{reg} is a 2-edged sword. On the one hand, T_{reg} physiologically maintain the peripheral immune tolerance and are able to prevent excessive immune responses in allergy or autoaggressive immune reactions in autoimmune diseases, transplantations, or graft-versus-host disease.^{10–13} In humans suffering from the IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome, a mutation in the *FOXP3* gene leads to a deficiency of T_{reg} function with severe clinical autoimmune inflammatory disease.^{14,15} On the other hand, increased amounts of T_{reg} can diminish effective antitumor T-cell responses in patients with cancer.^{16–18} In the past years, several attempts have been made to identify specific T_{reg} markers and to determine the mechanisms of T_{reg} function. Originally, regulatory T cells were defined as “ $CD8^+$

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suppressor T cells” and later on as CD25⁺CD4⁺ T cells.¹⁹ A more specific marker was found with the identification of the transcription factor FOXP3 and its key role in the development and function of T_{reg}.^{20,21} In addition, surface markers such as CTLA-4 (CD152⁺), LAG-3, CD39⁺ CD73⁺, and a low expression of the IL-7 receptor (CD127^{low}) have contributed to a refined definition of T_{reg}. A common disadvantage of all of these markers is their potentially “unspecific” upregulation during the activation of CD4⁺ T cells.²² Regarding the function of T_{reg}, 4 basic mechanisms are postulated for their suppressive function including their production of inhibitory cytokines such as tumor growth factor-β and IL-10,^{23,24} their potential of a granzyme B-dependent cytotoxicity,²⁵ their potential to downregulate IL-2-mediated metabolism and immunosuppressive adenosines generated through the hydrolysis of ATP by the ectoenzymes CD39⁺ and CD73⁺,^{26,27} and their suppression of DC-mediated T-cell stimulation through the upregulation of CD152.²⁸

Recent findings of our group showed that an inhibitory microenvironment as established by blasts can impair an antileukemic T-cell response, but can be overcome by the conversion of leukemic blasts to leukemia-derived DC (DC_{leu}).²⁹ Furthermore, we could work out relevant soluble factors (eg, released cytokines) or cells (eg, amounts of DC_{leu} and mature DC) that are important and predictive key players in the successful mediation of antileukemic, cytotoxic T cells or the clinical response to immunotherapy. However, there are still cases where no antileukemic function of T cells after DC stimulation could be achieved.^{30,31} Thus, the aims of this study were as follows: (1) to evaluate compositions of several T_{reg} subgroups (eg, CD4⁺ T_{reg}, CD8⁺ T_{reg}, T_{naive reg}) in uncultured peripheral blood-mononuclear cells from healthy probands and AML patients; (2) to investigate the development and recruitment of T_{reg} subgroups during the stimulation of T cells

with DC_{leu}-containing DC (“DC”) or blast-containing mononuclear cells (“MNC”) in AML patients compared with healthy probands; (3) to correlate T-cell expression profiles with the antileukemic function of “DC”-stimulated or “MNC”-stimulated T cells in AML patients; and (4) to evaluate potential antileukemically predictive values of T_{reg} subgroups.

MATERIALS AND METHODS

Sample Collection

After obtaining informed consent, heparinized blood samples were taken from patients. MNCs were isolated from whole blood samples by density gradient centrifugation (Ficoll-Hypaque; Biochrom, Berlin, Germany), and then washed and suspended in phosphate-buffered saline without Ca²⁺ and Mg²⁺ (Biochrom). Cell counts were quantified by Neubauer counting chambers, frozen with standardized procedures, and stored in liquid nitrogen until use.

Patients’ Characteristics and Diagnostics

Diagnosis of AML and MDS cases was based on the French-American-British (FAB) classification. Samples from 12 patients with AML in acute stages of the disease were used as sources for DC generation. Sources for T cells were healthy stem cell donors (“donors,” n = 1), patients before SCT, all of them in active stages of the disease (“autologous,” n = 6), or patients after SCT (“after SCT,” n = 5), with 3 of them in remission and 2 of them at relapse after SCT (Table 1). Patients’ age at diagnosis was on average 48 years (range, 5–70y), and the female to male ratio was 1:1. Three patients presented with undifferentiated leukemia (M0 n = 2, M1 n = 2) and 1 with immature granulocytic leukemia (M2 n = 1). Monocytic leukemia was diagnosed in 6 patients (M4 n = 4, M5 n = 1). The 1 MDS case was a chronic myelomonocytic

TABLE 1. Patients’ and Samples’ Characteristics

Patients’ Characteristics at the Time Point of DC Generation					Patients’ Characteristics at the Time Point of T-cell Preparation			
Patient No.	FAB-type	Stage	Blast Phenotype (CD)	IC Bla%	Source of T Cells and the Stage	Antileukemic Activity (%) of Unstimulated (u), MNC (“MNC”)–stimulated or DC–stimulated T Cells (“DC”)†		
						“u”	“DC”	“MNC”
m569	M0	pers.	15, 33, 13, 34, 19	11	CR, after SCT	–60	–76	–63
853	M0	rel. a. SCT	34, 33, 117	50	CR, after SCT	+15	–75	+10
m761	M1	pers.	34, 117, 15, 65, 2, 7, 53	95	CR, after SCT	–30	+150	+55
824	M1/M2	dgn.	7, 13, 34, 117, 123	68	First diagnosis, autologous	ND	–85	+100
748	M2	dgn.	34, 33, 117, 13, 38	50	First diagnosis, autologous	+120	+700	+800
610	M4	pers. rel.	34, 4, 33, 117, 13	80	Stem cell donor, allogeneous	+400	+220	+260
855	M4	dgn.	13, 33, 34, 65, 117	37	First diagnosis, autologous	ND	ND	ND
880	M4	rel. a. SCT	33, 64, 13, 15, 117, 34, 7	90	Relapse, after SCT*	–93	–51	–9
938	M4	rel. a. SCT	34, 117, 33, 4	74	Relapse, after SCT*	–60	–77	–80
948	M5	dgn.	14, 65, 15, 33	70	First diagnosis, autologous	+30	–55	+20
887	MDS/CMML	pers.	33, 34, 14, 64, 117	8	Persisting disease, autologous*	–58	–12	–12
914	sAML	rel.	34, 33, 15, 117	47	Relapse, autologous	+800	+250	+350

*T cells harvested at different time points at relapse (patients 880, 938) or during persisting disease (patient 887) and pooled for MLC.

†Negative values: percentual proportions of *lysed* blasts, positive values: percentual proportions of *increased* blasts.

AML indicates acute myeloid leukemia; CMML, chronic myelomonocytic leukemias; CR, complete remission; DC, dendritic cells; dgn., diagnosis; IC Bla, immune cytologically detected blasts; MDS, myelodysplastic syndromes; MLC, mixed lymphocyte cultures; MNC, mononuclear cells; ND, no data; pers. rel., persisting relapse; pers., persisting disease; rel. a. SCT, relapse after SCT; rel., relapse; sAML, secondary AML; SCT, stem cell transplantation.

leukemia (CMML, n = 1) and 1 patient suffered from a secondary AML (sAML n = 1) (Table 1). Five healthy probands served as controls.

Flow cytometric analysis of uncultured MNC fractions was performed to quantify the cellular composition of MNC in 12 AML patients and in healthy cell samples. The cellular composition of MNC in AML patients was 3% ± 2% B cells, 9% ± 6% T cells, 2% ± 2% natural killer cells, 5% ± 6% monocytes, and 56% ± 25% blasts. In cases with aberrant expression of CD19⁺, CD56⁺, or CD14⁺, B cells, natural killer cells, or monocyte counts, respectively, could not be quantified.

DC Generation

DC_{leu}-containing DC were generated from AML patients' MNC obtained in active stages of the disease (first diagnosis, persisting disease, relapse before or after SCT) and afterward used for mixed lymphocyte cultures (MLC) using stem cell donors' or patients' autologous T cells or T cells after SCT (Table 1).

Thawed MNCs were pipetted in 12-well tissue culture plates in 1 mL Xvivo 15 (Bio Whittaker Europe, Verviers, Belgium) FCS-free medium. DCs were generated from blood samples according to a refined strategy culturing cells in a minimized assay with 3 DC-generating methods (MCM-Mimic, Picibanil, Ca-Ionophore, Table 2) in parallel, as described by us.³¹⁻³³

All of the substances used for DC generation are approved for human treatment.

For the analysis of a successful generation of DC, cells were quantified by flow cytometry using patient-specific "blast"-staining antibodies (CD33, CD13, CD15, CD65, CD34, and CD117) according to diagnostic reports in combination with DC-staining antibodies (CD1a, CD1b, CD40, CD80, CD83, CD86, and CD206) as described.³⁴ We defined DC generation as successful if at least >10% DC and >5% leukemia-derived DC (DC_{leu}) could be generated in the MNC fraction.³² DC_{leu} were defined by the coexpression of blast-with DC-markers. The method resulting in the highest DC count was chosen for quantitative generation of DC.

We generated DC successfully in every given case of our 12 patient and 5 healthy proband samples according to

procedures already described by members of our group.^{30,32} AML samples presented with on average 20% ± 14% DC (range, 10%–51%) and 6% ± 2% DC_{leu} (range, 5%–10%) after cultivation in selected best DC medium for individual patients. We selected Picibanil, 3 times Ca-Ionophore, and 7 times MCM-mimic.

MLC

As shown in Table 1 CD3⁺ T cells (later on used as "effector" T cells) were positively selected (Milteney Biotech, Bergisch-Gladbach, Germany, 1 × 10⁶ cells/well) from MNC from patients (autologous n = 6), from matched stem cell donors (n = 1), or from patients in remission or at relapse after stem cell transplantation (n = 5, Table 1, right side) and cocultured with "stimulator-cells": irradiated (20 Gy) AML blast-containing MNC [2.5 × 10⁴ MNC (T'MNC')] and in parallel with irradiated DC_{leu}-containing AML DC [2.5 × 10⁴ DC (T'DC')] in 1 mL RPMI-1640 medium (Biochrom, Berlin, Germany) containing 15% human serum (PAA) and IL-2 50 U/mL (Proleukin R5, Chiron, Munich, Germany). T-cell stimulation was performed using a cell suspension containing a mixture of blasts-converted DC (DC_{leu}), blasts, DC, monocytes, and lymphocytes ('DC'). Total DC counts in the MLC were adjusted to 2.5 × 10⁴ DC and T-cell counts adjusted to 1 × 10⁶/well. After 2-fold restimulation with 2.5 × 10⁴ 'DC' or 'MNC', supplementation of IL-2 (50 U/mL), and half medium exchange on days 3 and 5, cells were harvested on days 7–8 and the cytotoxicity fluorolysis assay was carried out. DC (generated from 5 healthy monocytes containing MNC) or MNC served as healthy "stimulator cells" for autologous "effector" T cells prepared from these healthy donors, as described.^{30,32}

Cell Characterization by Flow Cytometry

Flow cytometric analysis was carried out using monoclonal mouse antihuman antibodies (moAbs) labeled with fluorescein isothiocyanate (FITC), phycoerythrin (PE), tandem Cy7-PE conjugation (PC7), or allophycocyanin (APC). Antibodies were provided by Immunotech/Beckman Coulter^a, Becton Dickinson^b, Caltag^c, Serotech^d, and Invitrogen^e. As FITC-conjugated moAbs, we used CD1b^b, CD80^{ab}, CD83^a, CD86^c, CD3^a, CD25^a, CD39^d, CD45RO^a,

TABLE 2. DC-differentiating Methods Selected for Individual AML Patients (After Pretesting)

DC-generating Method/Medium	DC Differentiation-stimulating Substances	Mode of Action	Culture Time (d)	References	DC Method for Individual Samples
MCM-Mimic	GM-CSF, IL-4, TNF α , IL-1 β , IL-6, PGE2	Cytokine-based DC-differentiation, PGE2 increases CCR7-expression and improves migration	10–14	Kremser et al ³² Dreyssig et al ³³	m569 (M0) 610 (M4) 748 (M2) m761 (M1) 853 (M0) 887 (MDS/CMML) 914 (sAML)
Picibanil	GM-CSF, TNF α , lysate from <i>Streptococcus pyogenes</i> , PGE2	Bacterial lysat and PGE2 stimulate DC differentiation	7–8	Kremser et al ³² Dreyssig et al ³³	824 (M1/M2) 855 (M4)
Ca-Ionophore	IL-4, A23187	Bypass of cytokine-driven DC differentiation	2–3	Kremser et al ³² Dreyssig et al ³³	880 (M4) 938 (M4) 948 (M5)

AML indicates acute myeloid leukemia; CMML, chronic myelomonocytic leukemias; DC, dendritic cells; MDS, myelodysplastic syndromes; sAML, secondary AML.

and CD122^d. As PE-conjugated moABs, we used CD80^a, CD83^a, CD86^{bc}, CD206^a, CD4^a, CD8^b, CD73^b, CD127^a, and CD152^a. As PC7-conjugated moABs, we used CD1a^a, CD40^c, CD3^a, CD4^a, CCR7^b, and 7AAD. CD34^{a,c}, CD117^a, CD33^a, CD1a^a, CD40^c, CD206^a, CD3^a, CD8^b, CD25^e, CD45RO^e, CD69^b, and CD71^b were used as APC-conjugated moABs.

For analysis and quantification of lymphocytes, monocytes, and leukemic blasts before culture, the total MNC fraction was gated. Proportions of positive events in defined gates compared with the isotype controls were calculated using the Cell Quest software. Quantification and characterization of DC and DC_{leu} were performed by flow cytometry according to our gating strategy already described by former groups.^{30–34} To quantify and characterize the several T-cell subpopulations, we defined a “lympho-gate” surrounding lymphocytes and monocytes. T-cell subgroups were defined by antigen coexpression before and after ‘MNC’ or ‘DC’ stimulation as shown in Table 3. Flow cytometric analyses were carried out on days 0, 1, 3, 5, and 7 followed by a functional cytotoxic fluorolysis assay on days 7 or 8 as described below.

Cytotoxicity Fluorolysis Assay

The lytic activity of effector T cells was measured by a fluorolysis assay by counting viable blast target cells, labeled with specific fluorochrome antibodies, before and after effector cell contact as described.³⁰ ‘DC’-stimulated or ‘MNC’-stimulated T cells from healthy matched donors (n = 1), from AML patients (autologous n = 5), or from AML patients after SCT (after SCT n = 5) and unstimulated T cells were cocultured with thawed blasts as target cells for 4 or 24 hours, as described. Before culture, blast-target cells were stained for 15 minutes with 2 FITC and/or PE-conjugated blast-specific antibodies and subsequently cocultured with effector cells. As a control, target and effector cells were cultured separately and mingled with T cells shortly before FACS analysis. To evaluate the amount of viable (7AAD⁻) target cells and to quantify the

cell loss after 24 hours of incubation, cells were harvested, washed in phosphate-buffered saline, and resuspended in a FACS flow solution containing 7AAD (BD, Biosciences Pharmingen) and a defined number of fluorospheres beads (Becton Dickinson, BD, Heidelberg, Germany). Viable cells were gated in an SSC/7AAD⁻ gate, and viable cells co-expressing the patient-specific blast phenotype were quantified later by taking into account defined counts of calibration beads as described. Cells were analyzed in a FACS Calibur Flow Cytometer using the CELL Quest software (Becton Dickinson). The percentage of lysis was defined as the difference between proportions of viable blasts before and after effector cell contact. Results are given in Table 1.

Statistical Methods

Means, SDs, and results with the 2-tailed *t* test were evaluated with a personal computer using Excel2007 (Microsoft). Differences were considered as significant if the *P*-value was <0.05.

RESULTS

We wanted to enlighten the development, activation, proliferation, and differentiation of T cells before and especially after ‘MNC’ or ‘DC’ stimulation in AML patients and healthy probands with special focus on the role of regulatory T cells and T_{reg} subtypes (as given in Table 3) in the mediation of antileukemic activities of ‘MNC’-stimulated, ‘DC’-stimulated, or unstimulated T cells.

‘DC’ Stimulation Leads to Alteration in T-cell Profiles

We and others have already demonstrated the variable blast-lytical outcome of T cells after ‘MNC’ or ‘DC’ stimulation.³⁰ We compared the expression of several T-cell markers (as listed in Table 2) on unstimulated T cells (day 0) and after stimulation (day 7) in MLC. To prevent any unwanted ‘MNC’ or ‘DC’ stimulation of T cells, measurements on day 0 were carried out “on ice.”

TABLE 3. Subtypes of T Cells and T_{reg} as Evaluated by Flow Cytometry

Names of T-cell Subgroups	Short Text Form	Surface Marker Combination
CD3 ⁺ T cells	CD3 ⁺ T cells	CD3 ⁺
CD4 ⁺ -expressing T cells	CD4 ⁺ T cells	CD4 ⁺ CD3 ⁺
CD8 ⁺ -expressing T cells	CD8 ⁺ T cells	CD8 ⁺ CD3 ⁺
Regulatory T cells	T _{reg}	
IL-2-R ⁺ IL-7-R ^{low} -expressing CD8 ⁺ T cells	CD8 ⁺ T _{reg}	CD8 ⁺ CD25 ⁺⁺ CD127 ^{low}
IL-2-R ⁺ IL-7-R ^{low} -expressing CD4 ⁺ T cells	CD4 ⁺ T _{reg}	CD4 ⁺ CD25 ⁺⁺ CD127 ^{low}
IL-2-R ⁺ IL-7-R ^{low} CCR7 ⁻ CD45RO ⁻ T cells	T _{naive reg}	CD25 ⁺⁺ CD127 ^{low} CD194 ⁻ CD45RO ⁻
IL-2-R ⁺ IL-7-R ^{low} CCR7 ⁻ CD45RO ⁺ T cells	T _{eff/em reg}	CD25 ⁺⁺ CD127 ^{low} CCR7 ⁻ CD45RO ⁺
IL-2-R ⁺ IL-7-R ^{low} CCR7 ⁺ CD45RO ⁺ T cells	T _{cm reg}	CD25 ⁺⁺ CD127 ^{low} CCR7 ⁺ CD45RO ⁺
IL-2-R ⁺ IL-7-R ^{low} Ecto-Apyrase, Ntpd-ase 1-expressing CD4 ⁺ T cells	CD39 ⁺ T _{reg}	CD39 ⁺ CD25 ⁺⁺ CD127 ^{low} CD4 ⁺
IL7-R ^{low} Ecto-5'-Nucleotidase-expressing T cells	CD73 ⁺ T _{reg}	CD73 ⁺
CTLA-4 ⁺ T cells	CD152 ⁺ T _{reg}	CD152 ⁺ CD3 ⁺
Activated T cells		
IL-2-R ⁺ T cells	CD122 ⁺ T cells	CD122 ⁺ CD3 ⁺
CD40L ⁺ T cells	CD154 ⁺ T cells	CD152 ⁺ CD3 ⁺
IL-2-R ⁺ CD4 ⁺ T cells	CD25 ⁺ CD4 ⁺ T cells	CD25 ⁺ CD4 ⁺
IL-2-R ⁺ CD8 ⁺ T cells	CD25 ⁺ CD8 ⁺ T cells	CD25 ⁺ CD8 ⁺
IL-7-R ⁺ CD4 ⁺ T cells	CD127 ⁺ CD4 ⁺ T cells	CD127 ⁺ CD4 ⁺
IL-7-R ⁺ CD8 ⁺ T cells	CD127 ⁺ CD8 ⁺ T cells	CD127 ⁺ CD8 ⁺
Proliferating T cells		
Type II C-type lectin ⁺ T cells	CD69 ⁺ T cells	CD69 ⁺ CD3 ⁺
Transferrin-R ⁺ T cells	CD71 ⁺ T cells	CD71 ⁺ CD3 ⁺

Profiles of Uncultured T Cells From AML Patients Compared With Healthy Proband

AML Patients Show Significantly Lower Levels of Both CD127⁺CD4⁺ and CD127⁺CD8⁺ T Cells Before DC or MNC Stimulation

We studied the expression of activation and proliferation markers on uncultured T cells from AML patients and from healthy probands. Low amounts of proliferating or activated T cells (CD3⁺ T cells expressing CD69, CD71, and CD122 or CD4⁺/CD8⁺ T cells expressing CD25) were found on unstimulated T cells (d0) in the MLC of healthy donors and AML patients (Fig. 1A). However, healthy probands showed significantly higher levels of CD127⁺CD4⁺ (93% ± 7% vs. 65% ± 21%) and CD127⁺CD8⁺ (86% ± 10% vs. 52% ± 17%) T cells compared with AML patients ($P < 0.005$, Fig. 1A).

In summary, this means that low amounts of proliferating T cells were found in unstimulated T cells from healthy donors or AML patients, but significantly higher proportions of CD4⁺/CD8⁺ T cells expressed CD127 in healthy compared with AML donors.

AML Patients Present With Significantly Higher Levels of Several T_{reg} Subgroups Before 'DC' or 'MNC' Stimulation

We compared the expression of common regulatory cell markers on uncultured T cells of AML patients and healthy probands.

Comparable amounts of unstimulated (d0) CD3⁺ T cells expressing CD152 or CD4⁺ T cells expressing CD39 (with or without CD25/CD127 expression) were found in healthy probands or AML patients (Fig. 1B, upper part). However, healthy probands showed significantly lower amounts of CD4⁺ T_{reg} in CD4⁺ T cells (11% ± 5% vs. 29% ± 11%, $P < 0.005$) or CD8⁺ T_{reg} in CD8⁺ T cells (4% ± 2% vs. 14% ± 9%, $P < 0.005$) or CD39⁺ cells in MLC fractions (10% ± 6% vs. 25% ± 6%, $P < 0.005$) compared with AML patients. In contrast, we found significantly higher amounts of CD73⁺ cells in MLC in healthy probands compared with AML patients (33% ± 5% vs. 19% ± 11%, $P < 0.005$, Fig. 1B, upper part).

Furthermore we found significantly lower amounts of all T_{reg} subsets in unstimulated cell fractions in healthy probands compared with AML patients: T_{naive reg} in MLC (1% ± 1% vs. 4% ± 4%, $P < 0.05$), T_{naive reg} in T_{naive} (12% ± 17% vs. 28% ± 17%, $P < 0.05$), T_{eff/em reg} in MLC (5% ± 3% vs. 10% ± 7%, $P < 0.05$), T_{eff/em reg} in T_{eff/em} (9% ± 7% vs. 19% ± 9%, $P < 0.005$), T_{cm reg} in MLC (1% ± 1% vs. 4% ± 2% $P < 0.005$), and T_{cm reg} in T_{cm} (17% ± 14% vs. 31% ± 13%, $P < 0.05$) (Fig. 1B, lower part).

In summary, we found significantly lower frequencies of several T_{reg} subgroups in unstimulated T cells in healthy probands compared with AML patients.

Profiles of 'MNC'-stimulated Versus 'DC'-stimulated T Cells After 7 Days of Culture in MLC in Cases With AML or in Healthy Proband

AML Patients Show Significantly Lower Levels of CD127⁺CD4⁺ and CD127⁺CD8⁺ T Cells After 'DC' or 'MNC' Stimulation Compared With Healthy Proband

We compared the expression of common regulatory cell markers on T cells from AML patients and healthy probands after stimulation with 'MNC' or 'DC', respectively (Fig. 2A).

In the AML patients' cohort, after 7 days with 'DC' stimulation, comparable, although lower, amounts of CD3⁺ T cells coexpressed CD69⁺, CD71⁺, and CD122⁺ compared with healthy donors. Moreover, comparable amounts of both CD4⁺ and CD8⁺ T cells coexpressing CD25 were found (Fig. 2A). However, compared with AML patients, healthy probands showed significantly higher levels of CD127⁺CD4⁺ (78% ± 20% vs. 26% ± 19%, $P < 0.05$) and CD127⁺CD8⁺ T cells on day 7 (62% ± 27% vs. 17% ± 14%, $P < 0.05$, Fig. 2A). After 7 days of 'MNC' stimulation, similar expression patterns could be observed (data not shown).

AML Patients Present With Significantly Higher Levels of T_{cm reg} and T_{eff/em reg} After 'DC' or 'MNC' Stimulation Compared With Healthy Proband

We compared the expression of various regulatory cell markers on T cells from AML patients and healthy probands after stimulation with 'MNC' or 'DC', respectively (Fig. 2B).

Concerning the expression of CD73⁺, CD152⁺, CD39⁺CD4⁺, and CD39⁺CD25⁺CD127^{low} T cells, no significant differences between AML patients and healthy probands could be observed after 7 days with 'DC' stimulation (Fig. 2B). Tendentially, healthy probands showed lower amounts of CD4⁺ T_{reg} (referred to CD4⁺ cells) and CD8⁺ T_{reg} (referred to CD8⁺ cells) compared with AML patients ($P < 0.1$) (Fig. 2B, upper part). However, compared with AML samples, healthy probands presented with significantly lower amounts of T_{cm reg} (26% ± 8% vs. 58% ± 21%, $P < 0.005$) and T_{eff/em reg} (33% ± 14% vs. 59% ± 16%, $P < 0.05$) and tendentially lower amounts of T_{naive reg} (53% ± 31% vs. 78% ± 22%, $P \leq 0.1$, Fig. 2B, lower part) after 7 days of 'DC' stimulation. Values after 7 days of 'MNC' stimulation in AML patients and healthy probands were comparable to values after 'DC' stimulation (data not shown).

In summary, we still found significantly higher levels of T_{reg} subtypes in AML patients compared with healthy probands on day 7.

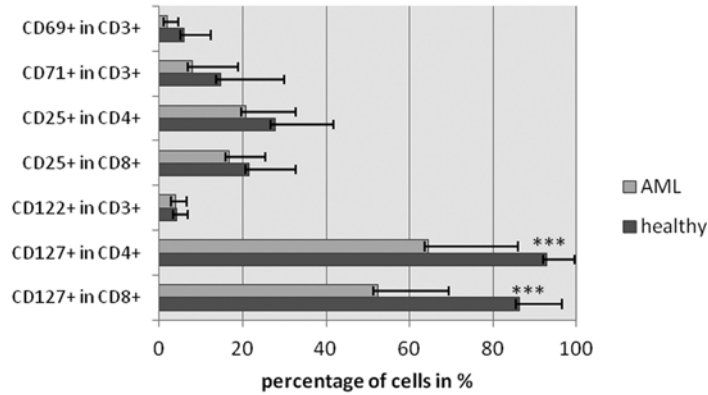
Kinetic Profiles of Surface Markers on T Cells in the Time Course of 'DC' or 'MNC' Stimulation (Day 0–7)

We examined the kinetic development of surface marker profiles, performed measurements on days 0, 1, 3, 5, and 7, and compared statistical profiles of day 0 and 7 (Fig. 3).

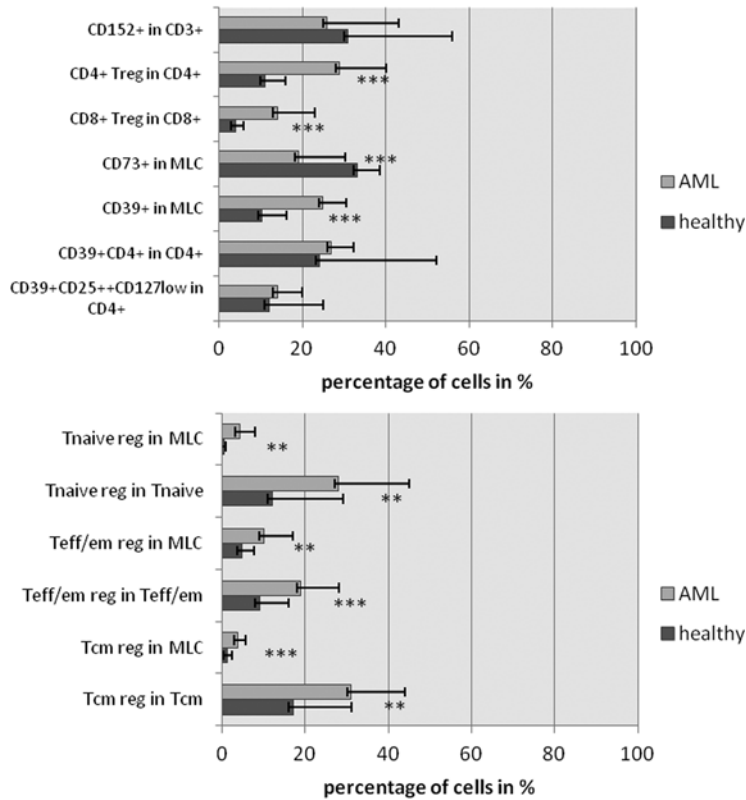
Expression Profiles of Activation and Proliferation Are Comparable in AML Patients and Healthy Proband

Proportions of CD25⁺ T cells stayed rather constant during 'DC' stimulation between day 0 and day 7 in both groups (Fig. 3A). In general (with the exception of CD127), T cells from healthy probands showed a similar kinetic development during 'DC' stimulation compared with AML patients. As shown in Fig. 3A, the kinetics of healthy probands showed no significant decrease in CD127⁺ T cells, whereas the expression on CD4⁺ T cells decreased significantly from 63% ± 23% to 26% ± 19% in AML patients ($P < 0.005$) between day 0 and 7. We could demonstrate a significant increase in CD69⁺ T cells in AML patients (2% ± 2% to 18% ± 20%, $P < 0.05$) and healthy probands (6% ± 7% to 21% ± 9%, $P < 0.05$) between day 0 and 7 of 'DC' stimulation ($P < 0.05$, Fig. 3A). CD71⁺ T cells of AML patients (8% ± 9% to 28% ± 19%, $P < 0.05$) and healthy probands (15% ± 15% to 39% ± 22%, $P < 0.1$) and CD122⁺ T cells of AML patients (4% ± 2% to 12% ± 9%, $P < 0.05$) and healthy probands (4% ± 2% to 12% ± 8%, $P < 0.1$) showed a significant continuous increase, reaching high values on days 3–5.

A activation and proliferation



B regulation



*p=0.05-0.1, **=p<0.05, ***=p<0.005

FIGURE 1. Profiles of uncultured T cells from AML patients versus healthy probands on day 0. Unstimulated T cells before MLC with ‘DC’ or ‘MNC’ of AML or healthy samples were stained with different fluorochrome-labeled antibodies, and subpopulations characterizing activation and proliferation (A) or regulation (B) were specifically gated and quantified. AML indicates acute myeloid leukemia; DC, dendritic cells; MLC, mixed lymphocyte cultures; MNC, mononuclear cells.

During stimulation with ‘MNC’, we could demonstrate an analogous kinetic development as with ‘DC’ stimulations in healthy and AML samples (data not shown).

In summary, we could show that after 7 days of stimulation with ‘DC’ or ‘MNC’, all 17 MLC presented with an increase in immunologic activation markers. Compared with day 0, AML patients and healthy probands showed a significant upregulation of CD69⁺, CD71⁺, and CD122⁺ T cells and a significant downregulation of

CD127⁺ T cells (only in AML) after ‘DC’ stimulation and after ‘MNC’ stimulation (data not shown).

AML Patients and Healthy Probands Show a Continuous Increase in T_{reg} Subgroups

Similarly, no significant changes in the proportion of CD152⁺ T cells between d0 and d7 were seen in AML patients and healthy probands (Fig. 3B). A significant increase in the proportion of T-cell subtypes between d0 and d7 was found in

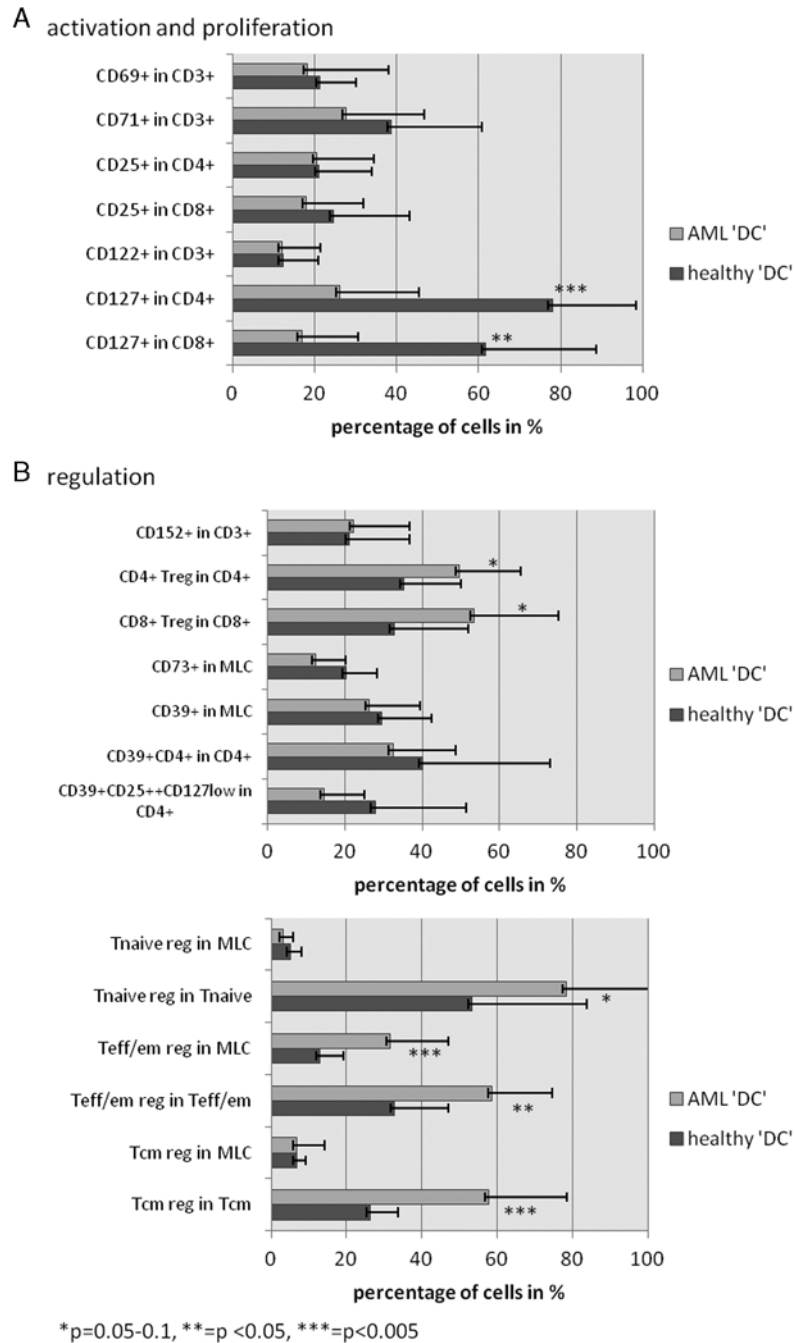


FIGURE 2. Profiles of 'DC'-stimulated T cells from AML patients versus healthy probands on day 7. T cells after MLC with 'DC' of AML or healthy samples were stained with different fluorochrome-labeled antibodies, and subpopulations characterizing activation and proliferation (A) or regulation (B) were specifically gated and quantified. AML indicates acute myeloid leukemia; DC, dendritic cells; MLC, mixed lymphocyte cultures.

AML patients and in healthy probands for T_{naive reg} (AML: 32% ± 20% to 78% ± 22%, *P* < 0.005; healthy: 13% ± 22% to 53% ± 31%, *P* < 0.05), T_{cm} (AML: 35% ± 14% to 58% ± 21%, *P* < 0.05; healthy: 18% ± 17% to 26% ± 8%, *P* < 0.1), T_{eff/em reg} (AML: 20% ± 10% to 59% ± 16%, *P* < 0.005; healthy: 11% ± 8% to 33% ± 14%, *P* < 0.05), CD8⁺ T_{reg} (AML: 15% ± 9% to 53% ± 22%, *P* < 0.005; healthy: 4% ± 2% to 33% ± 19%, *P* < 0.05), and CD4⁺ T_{reg} (AML: 31% ± 11% to 50% ± 16%, *P* < 0.005; healthy:

12% ± 6% to 35% ± 15%, *P* < 0.05). Regarding the ectoenzymes CD39⁺ and CD73⁺, we could detect a continuous and tendentially significant decrease in CD73⁺ cells in AML patients and a significant decrease in healthy probands (AML: 19% ± 11% to 13% ± 08%, *P* < 0.1; healthy: 33% ± 5% to 20% ± 8%, *P* < 0.05). CD39⁺ cells, CD39⁺CD4⁺, or CD39⁺CD127^{low}CD25⁺⁺CD4⁺ in CD4⁺ T cells showed no significant increase in AML patients. However, as shown in Fig. 3B, in healthy probands, we found

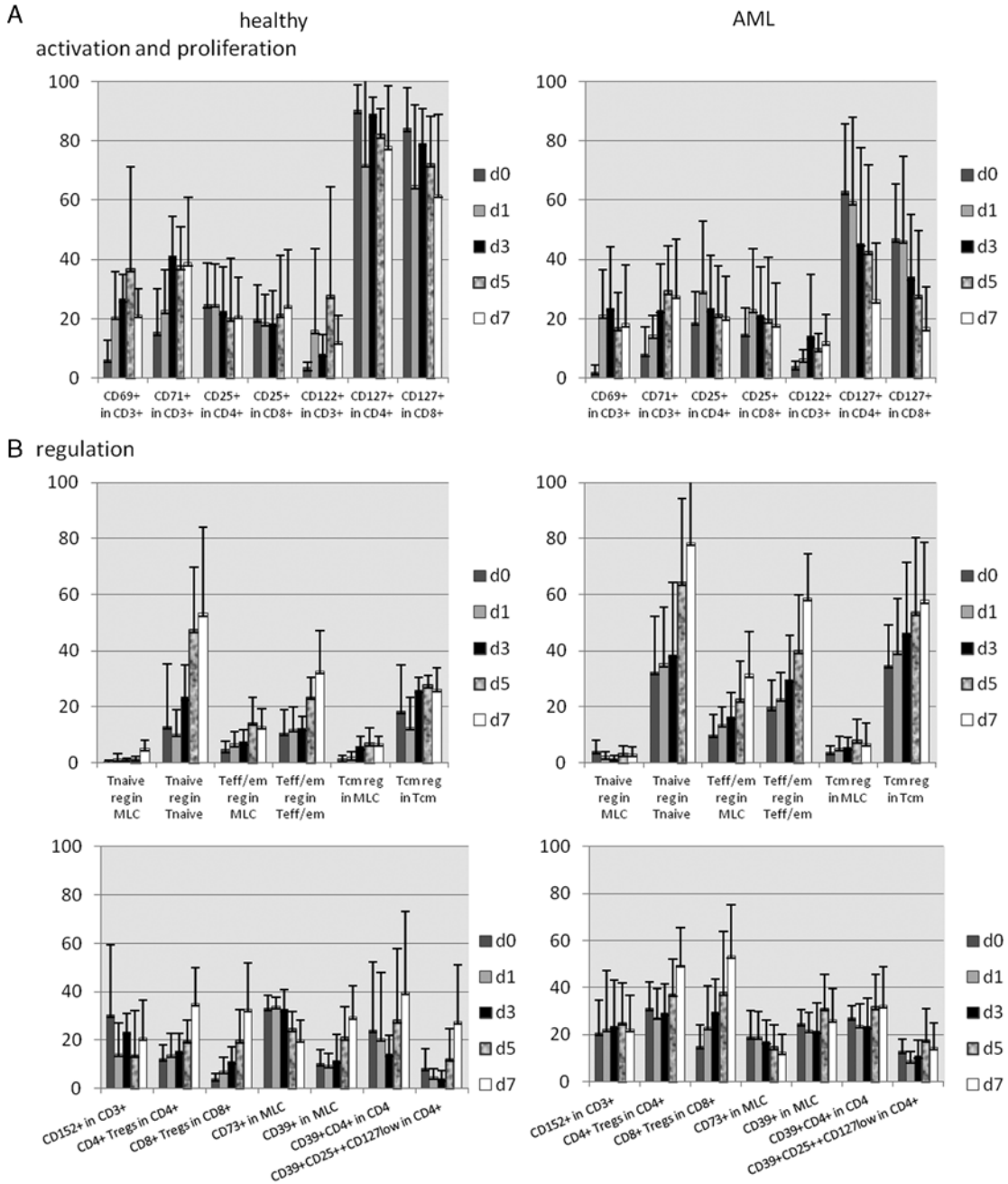


FIGURE 3. Kinetic profiles of surface markers on T cells from AML patients versus healthy probands (in the time course of ‘DC’ stimulation). Expression profiles of activation and proliferation markers (A) and regulation markers (B) in the course of ‘DC’ stimulation in healthy (left side) or AML samples (right side) after specific gating of T-cell subtypes are given. AML indicates acute myeloid leukemia; DC, dendritic cells.

a significant and continuous increase in CD39⁺ cells (10% ± 6% to 29% ± 13%, *P* < 0.05) and CD39⁺CD25⁺⁺CD127^{low}CD4⁺ cells (8% ± 8% to 28% ± 23%, *P* < 0.1).

After ‘MNC’ stimulation, we could demonstrate an analogous kinetic development (data not shown).

In summary, all subtypes of T_{reg} increased significantly after ‘DC’ or ‘MNC’ stimulation among AML patients and healthy probands. In AML patients, CD39⁺ T_{reg} showed no increase after 7 days of ‘DC’ or ‘MNC’ stimulation, whereas a tendentially significant increase in CD39⁺ T_{reg} and a significant

increase in CD39⁺ cells was observed after ‘DC’ stimulation in healthy probands. We could not detect any significant differences in the pattern of regulatory T cells after ‘DC’ or ‘MNC’ stimulation in AML patients and in healthy probands.

Correlation of T-cell Expression Profiles With the Antileukemic Function of ‘MNC’-stimulated, ‘DC’-stimulated, or Unstimulated T Cells in AML Cases

We have already demonstrated that stimulation with DC_{leu} improves the antileukemic function of T cells. Thus,

we wanted to examine the potential differences of surface marker expression concerning the activation and regulation of T cells in the context of the antileukemic function of 'DC'-stimulated/'MNC'-stimulated T cells. We subdivided our cases into those that gained lytic activity ("lysis") and those that were not able to lyse blasts ("nonlysis") after 'DC' or 'MNC' stimulation and compared the surface marker profiles regarding the expression of activation, proliferation, and regulation markers on "MNC-stimulated," "DC-stimulated," or unstimulated T cells.

Functional assays showed that 'MNC' stimulation induced antileukemic cytotoxicity in 4 of 11 cases (36%) compared with 7 of 11 cases (64%) after 'DC' stimulation. Unstimulated T cells showed lytic activity in 5 of 10 cases (50%). Unstimulated T cells showed an average lytic efficiency of 60% of blasts (range, 30%–93%), 'MNC'-stimulated T cells of 41% (range, 9%–80%), and 'DC'-stimulated T cells of 62% (range, 12%–85%) of blasts. Cases with lysis after 'DC' stimulation consisted of T cells after SCT (n = 4) and autologous T cells (n = 3). Cases with lysis after 'MNC' stimulation were T cells from patients after SCT (n = 3) and in autologous T cells in 1 case. T-cell sources of cases with lysis of unstimulated T cells were T cells after SCT (n = 4) and in 1 case autologous T cells. Results of antileukemic activity in individual cases are given in Table 1 (right side).

T-cell Profiles of T Cells Before Stimulation With 'DC' or 'MNC' in AML Cases

Expression Profiles of Activation and Proliferation Markers on T Cells Before 'DC' or 'MNC' Stimulation in Lysis and Nonlysis Groups of AML Cases Do Not Differ

On day 0 before 'DC' stimulation, we found no significant differences between the lytically active and nonactive groups regarding the expression profiles of activation and proliferation markers (Fig. 4A, left side).

Tendentially Lower Proportions of CD8⁺ T_{reg} in the Lysis Group Before 'DC' But Not Before 'MNC' Stimulation

On day 0 before 'DC' stimulation, we found no significant differences between the lytically active and nonactive groups regarding the expression profiles of CD152⁺, CD4⁺ T_{reg}, CD39⁺, CD73⁺, CD39⁺CD4⁺, CD39⁺CD25⁺⁺CD127^{low}, T_{naive reg}, T_{eff/em reg}, and T_{cm reg} (Fig. 4B, left side). However, we found tendentially lower proportions of CD8⁺ T_{reg} (referred to CD8⁺ cells) before 'DC' stimulation in the later-on lysis group compared with the nonlysis group (10% ± 3% vs. 23% ± 12% *P* ≤ 0.1; Fig. 4B, left side).

Before 'MNC' stimulation, we could not detect any significant differences in the levels of any regulatory subgroups between the later on lysis and nonlysis groups (data not shown).

T-cell Profiles After 'MNC' or 'DC' Stimulation in AML Cases

Expression Profiles of Activation and Proliferation Markers Do Not Show Significant Differences After 'DC' or 'MNC' Stimulation in AML Cases

No significant differences in proportions of activated T cells (positive for CD69⁺, C71⁺, CD25⁺, CD122⁺, and CD127⁺) were found between the lytically active and nonactive groups after 'DC' stimulation (Fig. 4A, right side).

The Lysis Group Presents With Significantly Lower Amounts of CD8⁺ T_{reg} and T_{eff/em reg} After 'DC' But Not After 'MNC' Stimulation in AML Cases

No significant differences in proportions of regulatory T-cell subtypes were found in proportions of the following markers in the lytically active compared with the nonactive group after DC stimulation (Fig. 4A, right side): CD152⁺ T cells, CD4⁺ T_{reg}, CD73⁺, CD39⁺, or CD39⁺CD25⁺⁺CD127^{low}, T cells, and T_{naive reg} and T_{cm reg} T cells. Tendentially, significantly lower proportions of CD39⁺CD4⁺ T cells (referred to as CD4⁺ cells) were found in cases with lysis than in cases without lysis after DC stimulation (29% ± 14% compared with 44% ± 13%, *P* ≤ 0.1). Significantly lower proportions of CD8⁺ T_{reg} (referred to as CD8⁺ cells, 45% ± 15% compared with 76% ± 13%, *P* < 0.05) and T_{eff/em reg} (referred to as T_{eff/em}; 49% ± 12% compared with 75% ± 7%, *P* < 0.005) were found in the lysis compared with the nonlysis group after 'DC' stimulation (Fig. 4B, right side). It is interesting to note that these correlations between expression profiles concerning regulatory T cells and antileukemic functionality were not detectable after 'MNC' stimulation.

In summary, we found that low proportions of T_{reg} subpopulations (CD8⁺ T_{reg}, T_{eff/em reg}, and CD39⁺ CD4⁺) in unstimulated and even more in 'DC'-stimulated cell fractions went along with good antileukemic ex vivo activity (Fig. 4B).

Cut-off Values of T_{reg} Subpopulations Contribute to Predict the Antileukemic Reactivity of Unstimulated and Even More of 'DC'-stimulated T Cells in AML Cases

To gain a better prediction of the antileukemic behavior of 'DC'-stimulated or unstimulated T cells, we subgrouped our cases according to their antileukemic function and evaluated predictive cut-off values. These values could be defined for the following regulatory subgroups (before or after 'DC' stimulation): CD8⁺ T_{reg} (referred to as CD8⁺ cells), T_{eff/em reg} (referred to as T_{eff/em}), T_{cm reg} (referred to as T_{cm}), and T_{naive reg} (referred to as T_{naive}; Fig. 5). In detail, we could show that cases with <60% of CD8⁺ T_{reg} in all CD8⁺ cells, those with <60% of T_{eff/em reg} in all T_{eff/em} cells, and those with <70% of T_{cm reg} in all T_{cm} cells showed an 80%–100% antileukemic T-cell activity. Cases with higher proportions of these regulatory T cells had a high probability to belong to the nonlytic group (range, 67%–100%). These cut-off values became more distinctive after 'DC' stimulation compared with unstimulated samples and could not be defined after 'MNC' stimulation. Moreover, we could not define any cut-off values regarding the proportions of the activation or the proliferation markers expressed.

Furthermore, we tried to compare these cellular findings with the patients' clinical outcomes. Five of our 12 patients received immunotherapy, of whom 3 patients did not respond, 1 responded, and 1 died of SCT-related complications. The patient responding to immunotherapy had lower proportions of CD8⁺ T_{reg} and T_{eff/em reg} before stimulation and higher proportions of T_{naive reg} before and after DC stimulation compared with the nonresponders (data not shown).

In summary, our findings show that cases with an antileukemic function presented in general with lower amounts of T_{reg} subgroups (CD8⁺ T_{reg} and T_{eff/em reg}) in unstimulated and even more in 'DC'-stimulated cases.

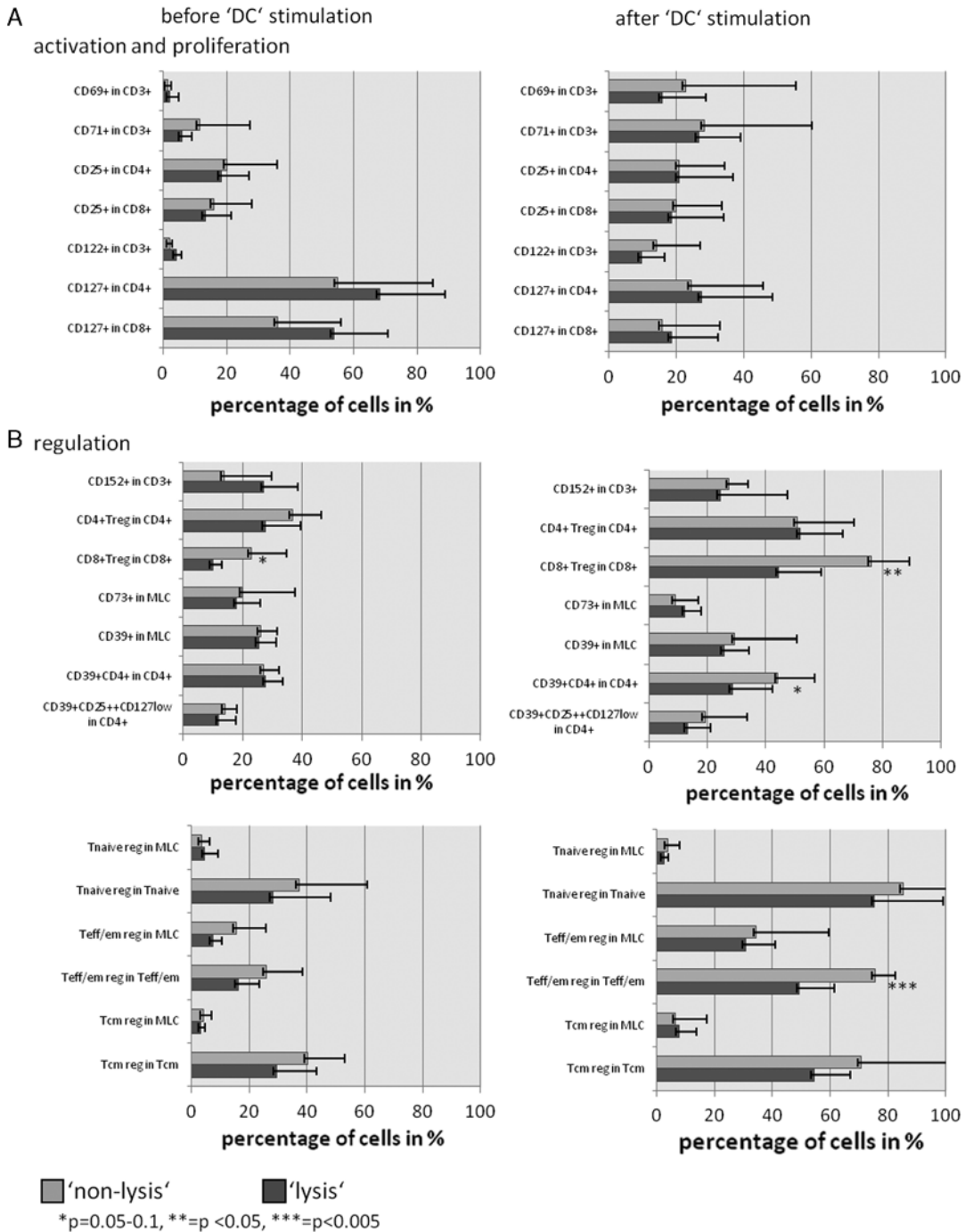


FIGURE 4. Correlation of T-cell subgroups with the antileukemic function of 'DC'-stimulated T cells in AML cases. Expression profiles of activation and proliferation markers (A) or regulation markers (B) on T cells from AML patients before (left side) or after 'DC' stimulation (right side) are given with cases subdivided into those with or without antileukemic functionality. AML indicates acute myeloid leukemia; DC, dendritic cells.

DISCUSSION

Regulatory T cells (iT_{reg}) and their subgroups play a critical role in the mediation of immune responses. With respect to AML patients, the role of T_{reg} is of special interest: on the one hand, the suppressive role of T_{reg} and their ability to suppress lethal graft-versus-host disease could be demonstrated,³⁵⁻³⁷ but on the other, high

T_{reg} counts after SCT, respectively, after adoptive T_{reg}-cell transfer could be correlated with reduced graft-versus-leukemia effects.³⁸⁻⁴⁰ Recently, our research group demonstrated that the conversion of leukemia blasts into leukemia-derived DC (DC_{leu}), being effective professional antigen-presenting cells by inducing activation, proliferation, and differentiation of naïve T cells into different

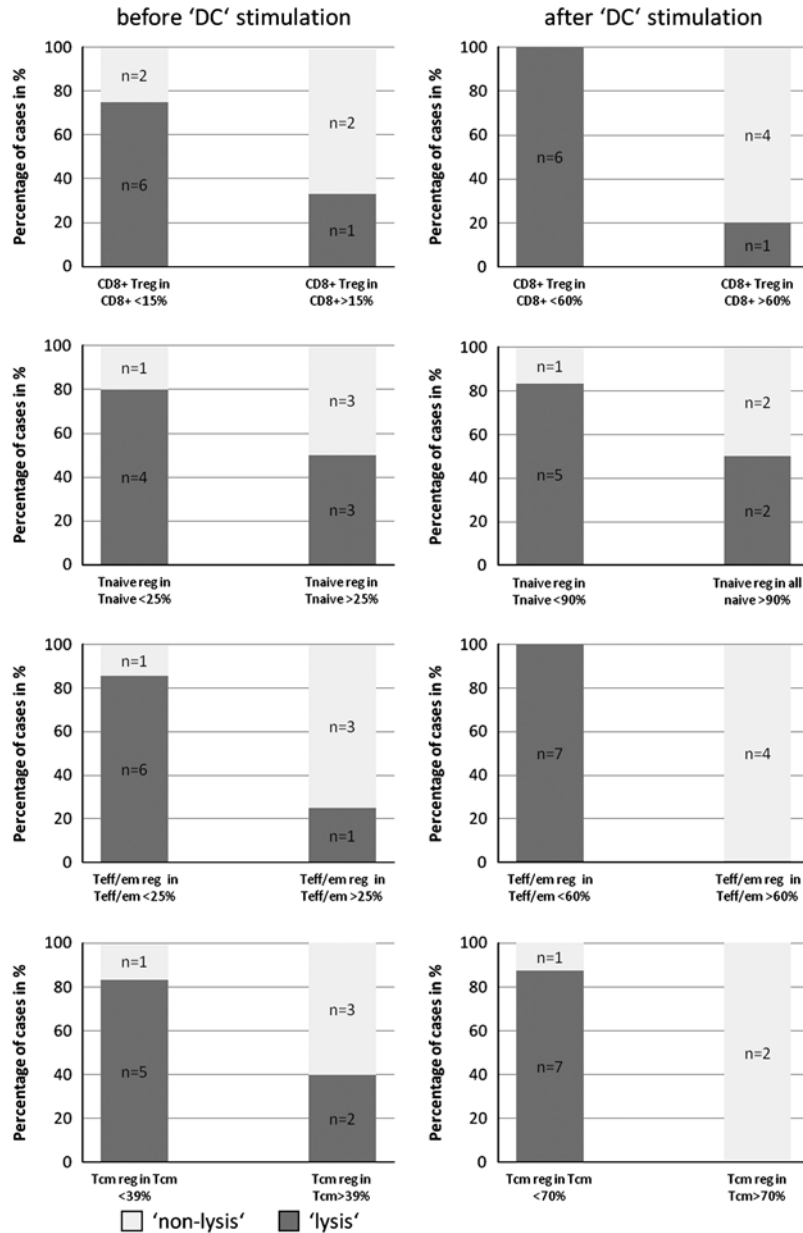


FIGURE 5. Cut-off values of T_{reg} subpopulations contribute to the prediction of the antileukemic reactivity of unstimulated and even more of 'DC'-stimulated T cells in AML cases. Proportions of selected T_{reg} subtypes before (left side) or after 'DC' stimulation (right side) that correlate with the lytic or the nonlytic function of T cells are given. AML indicates acute myeloid leukemia; DC, dendritic cells.

effector T cells, can improve the mediation of an antileukemic T-cell response and provides a good ex vivo model to study stimulatory influences on T cells.³⁰ To gain new insights into the role of T_{reg} and the mediation of antileukemic T-cell responses, we investigated the profile of unstimulated T cells, with ex vivo DC_{leu} containing DC ('DC') or leukemic blasts containing 'MNC'-stimulated T cells in AML patients compared with healthy probands. Finally, we correlated the T_{reg} distribution with the mediation of an effective antileukemic T-cell cytotoxicity (lysis vs. nonlysis).

First of all, the present study confirms our former findings, showing that 'DC'-stimulated T cells improve the mediation of an antileukemic T-cell response compared with

'MNC'-stimulated T cells. The present study verified that unstimulated T cells showed a reduced induction of antileukemic cytotoxicity compared with 'DC'-stimulated T cells, but a higher percentage of antileukemic function compared with 'MNC'-stimulated T cells.^{30,31} These findings contribute to the assumption that the inhibitory microenvironment induced by blasts could be abolished after blast conversion to DC_{leu}.²⁹

General Activation and Proliferation After 'DC' or 'MNC' Stimulation

T-cell stimulation with 'DC' or 'MNC' leads to a general T-cell activation and proliferation.³¹ During 'DC' or 'MNC' stimulation, we could observe an upregulation of

activation and proliferation surface markers. According to former studies, we also found the typical pattern of an early upregulation of CD69⁺ already on day 1 and a delayed upregulation of the proliferation marker CD71⁺, reaching the maximum expression on T cells on day 5.^{41,42} The surface expression of CD25⁺ already had a relatively high base level on unstimulated T cells compared with other surface markers and showed no significant upregulation on activated T cells during the whole time course. Nguyen and colleagues postulated the coexpression of CD71⁺ and CD25⁺ as reliable markers for T-cell proliferation. Our data suggest the single expression of CD71⁺ as the most reliable marker to evaluate T-cell proliferation. As already described, we could observe a downregulation of CD127⁺, the IL-7-receptor, during T-cell proliferation.^{43,44} Our observation of a significantly higher surface expression of CD127⁺ on unstimulated T cells in healthy probands compared with AML patients might express the higher proliferation potential of healthy T cells compared with T cells in AML patients.

CD4⁺CD25⁺CD127^{low} T_{reg} Instead of FOXP3⁺ T_{reg}

Originally, T_{reg} were defined through the expression of the intracellular transcriptional factor FOXP3⁺, which is an important regulator of the development and function of T_{reg} and is classified as one of the most reliable T_{reg} markers.^{21,45} However, several studies described the existence of suppressive T cells not expressing FOXP3.⁴⁶ Several studies have demonstrated a high correlation between the expression of FOXP3⁺ and the low surface expression of the IL-7 receptor α chain (CD127^{low}) on T_{reg}.⁴⁷⁻⁴⁹ It is interesting to note that Hartigan-O'Connor et al⁴⁹ found robustly suppressive CD25⁺ CD127^{low} T cells despite the low expression of FOXP3. Therefore, we decided to use CD127^{low} as the T_{reg} marker in our model to address FOXP3⁺ and FOXP3-T_{reg}. Moreover, by focusing on extracellular surface markers, our methodological procedures were not impaired through permeabilization techniques.

High Counts of Distinct T_{reg} Subgroups Correlate With Cancer

It has been demonstrated that increased frequencies of T_{reg} or a loss of T_{reg}-mediated immune tolerance correlates with several solid cancers and hematologic diseases.⁴⁰ In the present study, we demonstrated that AML patients presented with significantly higher frequencies of several T_{reg} subgroups in unstimulated T cells compared with healthy probands, correlating with the findings of former studies.^{17,50} Shenghui et al⁵¹ have already shown that an elevated frequency of T_{reg} in the peripheral blood and bone marrow is associated with a poor prognosis in AML patients. However, although most of these studies were restricted to the well-investigated CD4⁺CD25^{high}FOXP3⁺T_{reg}, there have been some recent reports of other subpopulations of clinically interesting regulatory T cells. Beside CD4⁺ T_{reg}, recently, the existence of cytotoxic CD4⁺T cells was demonstrated, contradicting the paradigm of CD4⁺ T-helper cells and CD8⁺ cytotoxic T cells (Steger B, unpublished data, 2012). Recently, more investigations have been conducted to determine the suppressive role of the less well-described CD8⁺ T_{reg} population in cancer.^{52,53} There is already increasing evidence indicating that CD8⁺ T_{reg} might accumulate in the *tumor microenvironment* in solid tumor diseases such as prostate or ovarian cancer.⁵³⁻⁵⁵ Moreover, Chaput et al⁵⁶ demonstrated significantly higher

proportions of circulating CD8⁺ T_{reg} in the *peripheral blood* of colon cancer patients compared with healthy probands. Actually, less is known about circulating CD8⁺ T_{reg} in AML patients' peripheral blood.

Recently, several working groups have described T_{reg} subgroups, consisting of phenotypically and functionally distinct subtypes such as naive T_{reg} and effector T_{reg}, because of their ability to differentiate into effector/memory cells after antigen contact.^{46,57-59} In the present study, we could demonstrate higher frequencies of CD4⁺ T_{reg} and CD8⁺ T_{reg} and also of T_{naive reg}, T_{cm reg}, and T_{eff/em reg} in AML patients compared with healthy probands. These findings implicate that regulatory and effector T-cell subsets have a greater plasticity than originally thought.

Another T_{reg} subgroup is defined to mediate the suppressive mechanism of adenosine produced through the ectoenzymes CD39⁺ (ectonucleoside triphosphate diphosphohydrolase) and CD73⁺ (ecto-5'-nucleotidase 1), which catalyze the sequential hydrolysis of ATP/ADP.⁶⁰ In humans, the expression of CD39⁺ is found on B cells, leukemic blasts, and T cells and T_{reg} with restriction to T_{eff/em reg}.²⁶ Szczepanski et al¹⁷ have shown that T_{reg} in AML patients express CD39⁺ and CD73⁺ and consequently hydrolyze ATP more efficiently than T_{reg} in healthy probands. In unstimulated T cells, we found a higher expression frequency of CD39⁺ and a lower expression frequency of CD73⁺ in AML patients compared with healthy probands as most of our patients were studied during chemotherapeutic treatment. These data indicate that because of tumor lysis effects, free nucleosides influence the surface expression frequencies of CD39⁺ and CD73⁺ in AML patients compared with healthy probands. Because of the different expression frequencies, we recommend the monitoring of CD39⁺ T_{reg} only in AML patients without high levels of free nucleotides (due to chemotherapeutic intervention).

CTLA4 (CD152⁺) mediates suppression by binding to CD80⁺ and CD86⁺ on DC, through which these costimulatory surface molecules for T-cell activation are reduced on DC.⁶¹ We could detect neither a significant difference in the surface expression profile of CD152⁺ on healthy T_{reg} compared with the T_{reg} of AML patients nor an increase of this surface marker during DC or MNC stimulation. These findings suggest that in this setting, CTLA-4 does not play a crucial role. Similar findings have been reported formerly.⁶²

Kinetic Development of T_{reg} Subgroups Under Stimulation

The common problem of all T_{reg} markers is their general upregulation on activated T cells, which reduces their specific correlation to T_{reg}. Recent publications have also shown a general in vitro expansion of CD4⁺ T_{reg} by 'DC' stimulation during MLC.⁶³ Mahic et al⁶⁴ demonstrated an increase in CD8⁺ T_{reg} by continuous antigen stimulation. During 'DC' or 'MNC' stimulation, we found a continuous increase in most of the T_{reg} subgroups in AML patients and in healthy probands. After stimulation by MLC, we still found significantly higher levels of T_{cm reg} and T_{eff/em reg} and tendentially higher levels of CD4⁺ and CD8⁺ T_{reg} in AML patients compared with healthy probands.

Previous data of our group showed an upregulation of CD39⁺ during ex vivo of healthy T cells with nucleosides, whereas the expression of CD73⁺ was downregulated (Weiler M, unpublished data). During 'DC' and 'MNC'

stimulation, in the present study, we also observed an upregulation of CD39⁺ and a downregulation of CD73⁺ in healthy probands. It is interesting to note that AML patients did not show significant changes in the expression frequencies of CD39⁺ and CD73⁺ during stimulation, probably due to a high expression of CD39⁺ and a low expression of CD73⁺ antigens before culture.

The Antileukemic Functionality Correlates With Frequencies of T_{reg}

Our data showed that an antileukemic T-cell response was achieved in 60% of AML cases after 'DC' stimulation compared with 40% after 'MNC' stimulation. Consequently, 'DC' stimulation seems to be an option to reverse T-cell anergy and to reestablish an antileukemic T-cell response, as already shown by our group.^{29,30} In the context of the present study, we could correlate differences in T_{reg} profiles with the antileukemic functionality after 'DC', but not after 'MNC' stimulation. Moreover, ex vivo cut-off values predicting the antileukemic functionality were definable. The present study could demonstrate significantly higher proportions of CD8⁺T_{reg} and T_{eff/em reg} in the nonlysis group compared with the lysis group after 'DC' stimulation. The expression frequencies of these T_{reg} subgroups in the lysis group were comparable to the expression frequencies in our healthy probands. Surprisingly, we could not detect any correlations in the proportions of CD4⁺T_{reg} with the lysis or the nonlysis group.

CONCLUSIONS

In summary, our results provide new insights into the cellular profiles and the antileukemic T-cell functionality of T_{reg}. Our findings suggest a crucial role of CD8⁺T_{reg} and T_{eff/em reg} in the mediation of the immune escape in AML patients. Moreover, we showed that frequencies of distinctly decreased levels of T_{reg} subtypes correlate with the reestablishment of the antileukemic functionality in a significant proportion of patients. We could even define cut-off values for T_{reg} subtypes, which allow an estimation of the lytic potential of individual patients' T cells. CD39⁺ and CD73⁺ expression on T_{reg} subgroups have to be discussed critically in patients with a high cellular turnover because of chemotherapy.

From a clinical point of view, our data recommend the monitoring of the distinct T_{reg} subgroups (CD8⁺T_{reg}, and T_{eff/em reg}) and defining cut-off values in vivo. In the context of immunotherapeutic strategies, further experiments are necessary to learn about mechanisms involved in the suppression of antileukemic graft-versus-leukemia reactions and to create strategies to manipulate T_{reg} subtypes (eg, by abolishing an inhibitory blast microenvironment in vivo) or to learn about mechanisms responsible for diminished graft versus host reactions and to manipulate them (eg, by elimination of autoreactive T cells).

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CONFLICTS OF INTEREST/FINANCIAL DISCLOSURE

All authors have declared there are no financial conflicts of interest in regards to this work.

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