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Improved Effector Function of Leukemia-specific T-lymphocyte Clones Trained with AML-derived Dendritic Cells

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Abstract. *Recently it was shown that myeloid leukemic cells can be induced to differentiate into leukemia-derived dendritic cells (DC_{leu}), regaining the stimulatory capacity of professional DCs while presenting the leukemic antigen repertoire. But so far, the induced antileukemic T-cell responses have varied in specificity and efficacy, or have even mediated opposite effects. In an attempt to further characterize the DC/DC_{leu} induced T-cell response pattern, immunoscope spectratyping, a novel and powerful tool to detect T-cell receptor (TCR) rearrangements was used in combination with functional flow cytometry and non-radioactive fluorolysis assays. Human leucocyte antigen (HLA) matched donor T-cells were repeatedly stimulated, either with leukemic blasts (French-American-British, FAB M4eo) or the corresponding blast-derived DCs. Functional comparison revealed no significant difference in their T-cell stimulatory capacity, while the DC/DC_{leu} fraction favored T-cells with a higher lytic activity, comprising a higher proportion of T-memory $CD45R0^+$ cells. Stimulation with blasts and DC/DC_{leu} induced a similar TCR restriction pattern, while stimulation with DC/DC_{leu} favored the CD4 T-cell subset and seemed to cause a higher grade of restriction. In conclusion, a combined strategy using spectratyping with functional tests might not only provide useful information about the specificity and efficacy of the induced T-cell response, but also pave the way to gain effective T-cell clones for therapeutic use.*

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Acute myeloid leukemia (AML) as well as myelodysplastic syndromes (MDS) are clonal disorders of hematopoietic stem cells, characterized by impaired normal cell differentiation (1). About 70% of successfully chemotherapeutically treated AML patients soon relapse (2), indicating the need for additional therapeutic strategies in order to maintain stable remissions. To date, allogenic stem cell transplantation (SCT) is the only curative treatment option (3) with donor T-cells mediating the antileukemic reaction (4). But although transfusion of donor T-cells (DLT) can reinduce complete remissions in relapsed patients after SCT, there are still numerous patients who do not respond (5). Moreover the appearance of graft *versus* host (GvH) reactions can impair the efficacy of SCT or relapse-therapy (3, 5). The reasons for these varying T-cell effects have to be elucidated. An insufficient expression of costimulatory antigens, major histocompatibility complex (MHC) molecules and tumor-associated antigens (TAA) on the surface of cancer cells, as well as disturbed mechanisms of apoptosis might be the main reasons for an ineffective immune response in malignant diseases (6). As dendritic cells (DCs) are known to stimulate T-effector cells, especially tumor-cytotoxic T-cells (7, 8), they are of potential interest for antitumor or antileukemic vaccination strategies (9, 10). In contrast to solid tumor cells, myeloid leukemic cells can be induced to convert into leukemia-derived DCs (DC_{leu}) expressing DC-typical antigens (*e.g.* CD40, CD86, CD80, CD1a, CD83), thereby regaining the stimulatory capacity of mature professional DCs (11-13). Thus, the cumbersome identification and subsequent pulsing with tumor-specific antigens probably can be replaced because the leukemia-derived DCs might still present the complete leukemic antigen repertoire. Moreover we have already shown that it is technically possible to generate sufficient DCs from any myeloid leukemia under serum-free culture using one out of three previously tested DC-

generating methods (14-17). In most cases, stimulation with DC_{leu} induced a very effective cytotoxic T-cell response (18), but in some cases an opposite T-cell response pattern was observed with T-cells mediating anergy or even supporting blast proliferation *in vitro*. The different T-cell response pattern is not predictable. It might be expected that a different precursor frequency of leukemia specific T-cells may result in a different clonal composition and functional diversity after stimulation with DC/DC_{leu}. In an attempt to elucidate the mechanisms involved the clonal patterns of T-cells before and after antigenic stimulation were characterized.

The rearrangement of the T-cell receptor (TCR) permits 10^{16} possible combinations (19), due to the expressed chains (mostly α- and β-chains, rarely γ- and δ-chains), the gene-rearrangement of the different regions of the chains (52 variable (V), 2 diversity (D), 13 joining (J) and 2 constant (C) regions), and the insertion of a different number of nucleotide triplets. The most interesting highly variable, antigen recognizing complementarity-determining region 3 (CDR3) of the TCR can be analyzed by immunoscope spectratyping technology, a PCR-based method allowing the analysis of the TCR with respect to length heterogeneity of the CDR3 in combination with the V- to the J-region in the β-chain (20). *Via* immunoscope spectratyping (21), predominating T-cell clones can be identified, characterized and monitored *in vitro* and *in vivo*, even if they are clonally expanded on a polyclonal background and correlated with clinical or experimental observations (22) or possibly with their function in combination with functional *in vitro* cytotoxicity assays. Subsequent sequencing, would exclude overlaying T-cell clones and prove monoclonal expansion.

The objective of the present study was to characterize the T-cell response pattern of a patient with treatment refractory AML, induced by repeated stimulation with DC_{leu} or blasts *in vitro*. Thereby TCR rearrangement analysis and functional T-cell assays might contribute not only to the understanding of anti-leukemia directed immunoreactions in myeloid leukemia, but could also help to identify the DNA-regions of the TCR gene loci involved in these reactions, to further isolate leukemia specific T-cells for adoptive immunotherapy.

Materials and Methods

Patient characteristics, sample collection and diagnosis. Mononuclear cells (MNCs) from the heparinized peripheral blood (PB) (PB-MNCs) of a patient (# 502) with recurrent AML after SCT, were isolated from the interphase by density-gradient centrifugation (Ficoll-Hypaque, Biochrom, Berlin, Germany), washed and suspended in PBS without Ca^{2+} and Mg^{2+} (Biochrom). Diagnosis was based on the French-American-British (FAB) classification (23). Cytogenetic analyses were performed according to standard protocols and criteria defined by the International System for Human Cytogenetic Nomenclature (Mitelman 1995) (24). The samples were collected after obtaining informed consent. Patient #502 had presented with persisting AML-M4eo after chemotherapy, with 95% $CD34^+$, $CD117^+$, $CD65^+$, $CD15^+$ myeloid blasts and a complex aberrant karyotype (Table I).

DC generation. DCs were generated in parallel using three different DC-differentiating methods, i) MCM-Mimic (14), ii) Picibanil and iii) Ca-Ionophore (15) (Table II). Subsequently, the method resulting in the best DC counts was chosen for the quantitative generation of DCs as described peviously (17, 25). MNCs obtained from the AML patient in an active, blast-rich phase of the disease were incubated in 12-well multiwell tissue culture plates in *ex vivo* 15 (BioWhittaker, Verviers, Belgium) FCS-free medium. i) The DCs were generated from 2.5×10^6 MNC/ ml in 'MCM-Mimic' medium containing 800 U/ml granulocyte macrophage colony-stimulating factor (GM-CSF), 500 U/ml interleukin (IL)-4 and 40 ng/ml FMS-related tyrosine kinase 3 ligand (FL). After 5 days, the same cytokines were added to the culture again. Half medium exchange was performed on day 8. Again GM-CSF, IL-4 and FL were added together with 150 ng/ml IL-6, 5 ng/ml IL-1β, 1 μg/ml prostaglandin (PG) E_2 and 5 ng/ml tumor necrosis factor (TNF) α. After 12 days in culture, the DCs were harvested for subsequent experiments (17). ii) The DCs were generated with 'Picibanil', a lysis product of *Streptococcus pyogenes*, which has non-specific immunmodulatory effects from $1\text{-}1.25\times10^6$ MNC/ml in the presence of 500 U/ml GM-CSF and 250 U/ml IL-4. After 7 days in culture, 5 μl/ml OK-432 (=*Picibanil*) and 1 μg/ml PGE_2 were added. The cells were harvested after 10 days in culture (17). iii) The DCs were generated from 7×10^5 MNC/ml in 'Ca-Ionophore' (15) and were cultured in the presence of 375 ng/ml A23187 and 250 U/ml IL-4 and harvested after 3 days (17).

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

Flow cytometry. Flow cytometric analyses with a panel of mouse monoclonal antibodies (moAbs) directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), tandem Cy7-PE-conjugation (PC7) or allophycocyanine (APC) were performed to evaluate and quantify the percentage and phenotypes of the leukemic cells, B-, T- and NK-cells and DCs in the PB/BM (bone marrow) samples analyzed. The antibodies were purchased from [a]Becton Dickinson (Heidelberg, Germany), [b]Immunotech/Beckmann Coulter (Krefeld, Germany) and [c]Caltag (Hamburg, Germany). FITC conjugated mAbs used were $CD1b^a$, $CD3^b$, $CD4^a$, $CD28^b$, $CD33^b$, $CD45RO^b$, $CD65^b$, $CD83^b$, $CD86^c$. $CD1a^b$, $CD8^a$, $CD56^b$, $CD80^b$, $CD154^b$ and $CD206^b$ were used as PE-conjugated mAbs. $CD3^b$, $CD14^a$, $CD19^b$, $CD25^a$, $CD34^b$ and $CD117^b$ were chosen as the PC7-conjugated mAbs. The APC-conjugated mAbs, used were $CD3^b$, $CD4^b$, $CD13^b$, $CD14^b$, $CD33^b$, $CD34^b$, $CD15^a$, $CD45RA^a$ and $CD71^a$. Regulatory T-cells (Tregs) were defined by CD4, CD25, CD122, CD62L positivity. CD4 cells were gated on CD25 expression. The ~10% highest expressing cells were further differentiated by CD122 and CD62L expression. $CD4^+$, $CD25^{++}$, $CD62L^+$, $CD122^+$ cells were rated as potentially being Tregs (26). The MNC or cultured cells were suspended in PBS with 20% FCS (Biochrom) and incubated with mAbs according to the manufacturer's instructions. Appropriate isotype controls were used. At least 5,000 events were evaluated on a FACS Calibur Flow Cytometer (Becton Dickinson) using Cell Quest data acquisition and analysis software (Becton Dickinson). For analysis and

Table I. *Patient characteristics*.

Patient	Diagnosis	Stage of disease	Cytogenetic marker	Blasts* (%)	Blast phenotype* (CD)	Monocytes* (%)	B-cells* (%)	T-cells* (%)	NK cells* (%)
#502	AML, M4eo	Persistance	t(2;20)qq, t(5;5)qp, del(5q), r7, t(12;16)pq	95	34, 117, 13, 33, 65, 15	2	nd	3	2

*Proportions of positive cells in the mononuclear cell (MNC) fraction.

Table II. *DC-differentiating methods*.

DC-generating method / medium	DC differentiation-stimulating substances	Mode of action	Culture time (days)	Reference
'MCM-mimic'	GM-CSF, IL-4, TNF, FL, IL-1β, IL-6, PGE2	Cytokine-based DC-differentiation, PGE2 increases CCR7-expression and improves migration	10-14	Lee *et al*. (14) Kufner *et al*. (28) Loibl *et al*. (17)
'Picibanil'	GM-CSF, IL-4, TNF, lysate from Streptococcus pyognes, OK-432, PGE2	Bacterial lysate and PGE2 stimulate DC differentiation	7-8	Sato *et al*. (16) Loibl *et al*. (17)
'Ca-Ionophore'	IL-4, A23187	Bypass of cytokine-driven DC differentiation	2-3	Houtenbos *et al*. (15) Loibl *et al*. (17)

MCM: monocyte-conditioned medium, GM-CSF: granulocyte macrophage colony-stimulating factor, IL: interleukin, TNFα: tumor necrosis factor α, PGE2: prostaglandin E2, A23187: Ca-ionophore, OK-432: Picibanil, FL: FMS-related tyrosine kinase 3 ligand.

quantification of the lymphocytes, monocytes and leukemic cells before culture the total MNC fractions were gated. An AML sample was considered as 'positive' for a surface marker if the percentage of positive events in a gate surrounding the blasts, lymphocytes and monocytes was more than 20%, as described previously (25, 27). The proportions of positive events in defined gates compared with the isotype controls were calculated using Cell Quest Software.

Quantification and characterization of DC. For the analysis and quantification of the DC and especially the DC_{leu} a refined gating strategy was applied and the DCs were quantified by flow cytometry as already described (25). For quantification those DC markers were selected that were not expressed on the naïve blasts (Figure 1A, left side). A 'blast gate' was set surrounding the blast population and residual lymphocytes or monocytes (Figure 1Bi, left side). The remaining cells were gated in a 'DC gate'. DCs were generated as described, harvested and counted. The cells which displayed the typical scatter of DCs and expressed typical DC antigens (*e.g.* CD86, CD80, CD40, CD1a, CD83) were counted. Moreover the 'blasts' could be quantified using blast-specific markers. The blasts converted to DC_{leu} could be quantified estimating those blast cells that had gained a DC antigen (Figure 1A and Bii, right side). In addition, the blasts that had not been converted to DC_{leu} or DCs of non-leukemic origin could be quantified with this gating strategy. DC_{opt}, the DC marker with the highest expression rates on the DCs gained after culture, which was not expressed on the naïve blasts was also quantified. DCs coexpressing CD83 were defined as 'mature DCs'. Other maturation criteria were the loss of CD14 or CD206 positivity. The viable DCs (7-amino-actinomycin D (7AAD)-negative/DC marker expressing (DC^+)) and migratory DCs (chemokine (C-C motif) receptor (CCR) 7-positive/DC^+) were also quantified. In the samples with an immunophenotypically detectable blast population in the MNC-fractions, the percentage of blasts being converted to DCs co-expressing specific blast antigens (*e.g.* CD56, CD117) were quantified. Microscopic controls were regularly performed and revealed the DC-typical morphology of large cells with irregular shapes and cytoplasmic projections.

Mixed lymphocyte cultures (MLC): generation of leukemia-cytotoxic T-cells. Positively selected $CD3^+$ T-cells (1×10^6 cells/well) (Milteney Biotech, Bergisch-Gladbach, Germany) from the healthy stem cell donor of patient #502 were cocultured with irradiated (20 Gy) cell suspensions containing 25,000 DCs generated from the patient's MNCs or the same number of irradiated MNCs as a control in 1 ml RPMI-1640 medium (Biochrom) containing 15% human serum (PAA Laboratories, Pasching, Austria) and 50 U/ml IL-2 (Proleukin R5, Chiron, Munich, Germany) as described. The cells were harvested after a 10 day training period of the T-cells coculture and 2-fold restimulated with 5×10^4 DC/DC_{leu} ($T^{*DC/DCleu}$) or 5×10^4 blasts ($T^{*blasts}$) under supplementation with IL-2, as described previously (28). Half medium exchange was carried out every 3-4 days. Six days after the last restimulation, the cells were harvested and the cytotoxicity assay was carried out.

The antigen expressions on the (allogeneic) $CD3^+$ T-cells were evaluated by FACS analyses comparing their coexpression of CD4, CD8, CD45RA, CD45RO, CCR7, CD71, CD25, CD122 and CD62L before and after blast or DC contact. This contributed to the evaluation of the proportions of proliferating ($CD71^+$), naïve, memory, central memory, regulatory, CD4/8 or migratory T-cells before or after MNC or DC coculture (28-30).

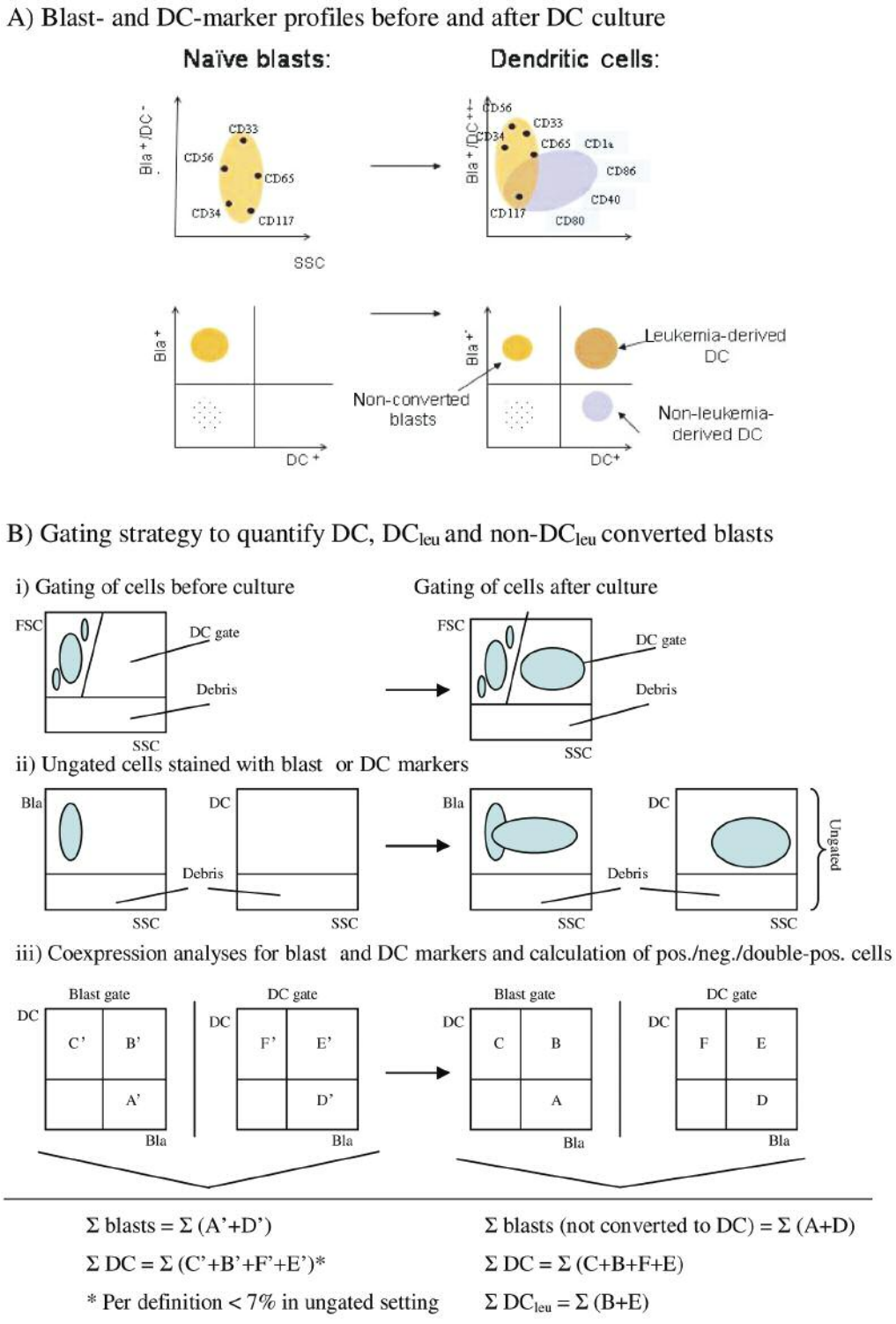

Figure 1. *A schematic presentation of a blast population before (left side) and after (right side) conversion to dendritic cells (DC). A blast population can be characterized by the expression of patient-typical blast antigens (e.g. CD34, CD117, CD65), including also lineage-aberrant markers (e.g. CD56, CD7, CD19) (A left). The expression of DC markers (e.g. CD80, CD86, CD40, CD1a, CD83, CD206, CD1b, CD137L) (A, right) has to be studied before culture and those markers selected that are not expressed on the uncultured blasts of the patient (A, left; Bii, left). After culture in DC medium, cells gain an increased side scatter (SSC) (Bi, ii right) and in addition express several co-stimulatory DC antigens not expressed on uncultured cells (A, right; Bi, ii right). A coexpression analysis of selected blast with DC markers allows quantification of 'leukemia-derived DC', expressing both blast and DC markers (Biii, E), of blasts not converted to DC (Biii, Σ(C+F)) and of DC without leukemic derivation (Biii, Σ (D+A)).*

Table III. *DC-characteristics after blast conversion.*

Patient	Blast and DC markers selected (CD)	DC preculture (% DC generated)			Best DC method	DC/MNC (%)	Blasts conv./ MNC (%)	DC_{leu}/ MNC (%)	DC_{leu}/ DC (%)	Via. DC/ DC (%)	Mat. DC/ DC (%)	Mig. DC/ DC (%)
		MCM	Picibanil	Ca-Ionophore								
#502	CD34, CD206	13	24	5	Picibanil	20	25	10	70	88	71	48

DC/MNC: proportions of dendritic cells in the total mononuclear cell suspension after culture; Blasts conv./MNC: proportion of blasts converted to DCs; DC_{leu}/MNC: proportion of leukemia-derived DCs in the total MNC suspension after culture; DC_{leu}/DC: proportion of leukemia-derived DCs in the DC fraction; Via. DC/DC: proportion of viable DCs in the DC fraction; Mat. DC/DC: proportion of mature DCs in the DC fraction; Mig. DC/DC: proportion of migratory DCs in the DC fraction; Blast and DC markers were selected for the quantification of DCs, DC_{leu} and the conversion of blasts to DC_{leu}.

Cytotoxicity assay (Fluorolysis). The lytic activity of effector T-cells was measured by a Fluorolysis assay by counting the viable target cells labeled with specific fluorochrome-labeled antibodies, before and after effector (E) cell contact. The DC- or MNC-trained donor T-cells or T-cells obtained from the AML -patient or untrained T-cells as a control were cocultured in 1.5 ml Eppendorf tubes with blasts as target cells. The E:T ratio was adjusted to 1:1 and the cells were incubated for 3 h or overnight at 37˚C and 5% CO_2. Before culture the blast-target cells were stained for 15 min with two FITC- and/ or PE-conjugated 'blast' specific antibodies and cocultured for 3 h with E-cells (T-cells or DCs as target cells were stained with T-/ DC-specific antibodies). As a control, target and E-cells were cultured separately and afterwards mixed with T-cells. To evaluate the percentage of viable ($7AAD^-$) target cells and to quantify the cell loss after the 3 h incubation time, the cells were harvested, washed in PBS and resuspended in a FACS flow solution containing 7AAD (BD, Heidelberg, Germany) and a defined number of Fluorosphere beads (Becton Dickinson). Viable, 7AAD-negative cells coexpressing specific blast marker (combinations) were quantified taking into account defined counts of calibration beads as described (28, 31). The cells were analyzed in a FACS Calibur Flow Cytometer using Cell Quest software (Becton Dickinson). The percentage of lysis was the difference between the proportions of viable blasts before and after the E-cell contact (28, 31).

CDR3 immunoscope spectratyping of TCR-Vβ transcripts. After isolation of the unstimulated and restimulated T-cells on day 10 by CD4- and CD8-positive selection (Dynabeads; Dynal Biotech ASA, Oslo, Norway), the total RNA was prepared from 1×10^5 cells by using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesized by random priming, using a cDNA synthesis kit (QuantiTect; Qiagen, Hilden, Germany) and subjected to TCR-Vβ gene family-specific PCR in 26 separate reactions, each containing one of the 26 Vβ families in combination with a universal Cβ-specific primer (32). In a second step, run-off products were generated from every Vβ-specific PCR product by each of the 13 fluorescence-labeled, Jβ-specific oligonucleotides (33). The fragments were separated on an automated 48-capillary DNA-sequencer (3730 DNA-Analyzer; ABI, Foster City, CA, USA). The length and fluorescence intensities of the CDR3 of the different Vβ-Jβ combinations were determined using Genescan 500LIZ (Applied Biosystems, Warrington, UK) as standard and analyzed by using *GeneMapper* software (Applied Biosystems). In a normal population of T-cells, CDR3 length analysis produces approximately 5 to 10 identifiable peaks spaced by 3 nucleotides, with fluorescence intensity following a quasi-Gaussian distribution. Although this method does not allow exact quantitative evaluations of clonal DNA in distinct peak positions, fluorescence intensities of single peaks can at least be used for semiquantitative estimations of the values of the distinct (clonally restricted) TCR DNA-peaks.

The grade of restriction (normal and restricted) and the size of the restricted peaks was described by visual evaluation. The complexity of restriction within the Vβ-families was determined by counting the number of discrete peaks (34). The percentages of restricted DNA in the screened Vβ-region were given for better comparability of the results in different settings.

Results

Effect of repeated stimulation of T-cells with leukemic blasts or blast derived dendritic cells. As summarized in Table III, first the patient specific blast marker profile of patient #502 was determined to further identify and characterize the leukemia-derived DC/DC_{leu} population. Using the three standard procedures, 5-24% DCs were generated and the Picibanil method provided the best results. Quantitative DC-preparation resulted in 20% DCs, 70% of which displayed a DC_{leu}, 71% a mature and 48% a $CCR7^+$ migratory DC phenotype as summarized in Table III.

To further analyse the impact of these different stimulator cells (blasts, DC/DC_{leu}) on the T-cell response pattern, MLC were set up with the corresponding human leukocyte antigen (HLA)-matched donor T-cells. The T-cells were phenotypically characterized as shown in Figure 2. More than 95% viable, 7AAD-negative T-cells were found in the blast- and DC/DC_{leu}-trained T-cell fractions. T-cell proliferation, as measured by coexpression of CD71, increased during the blast- and DC-training period. Memory $CD45RO^+$ T-cells increased after blast- and to an even higher extent after DC-training. Whereas in the blast-trained cultures the proportion of $CD8^+$ T-cells increased compared to the untrained fractions, the proportion of $CD4^+$ T-cells increased after DC training. The percentage of T-cells expressing chemokines varied, but stayed rather low in the

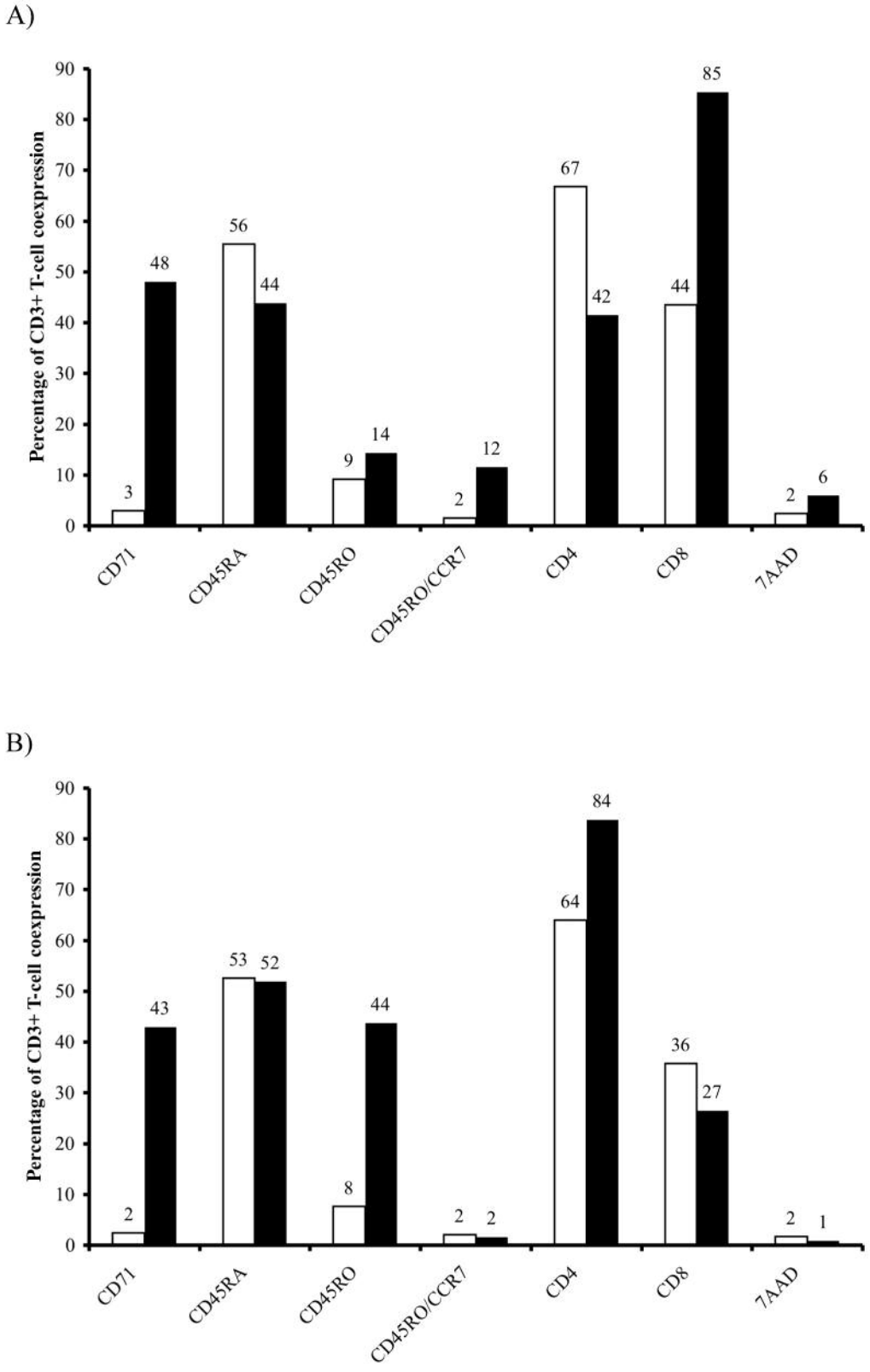

Figure 2. *Composition of T-cell subsets before (open columns) and after (closed columns) blast (A) or DC/DC_{leu} (B) training.*

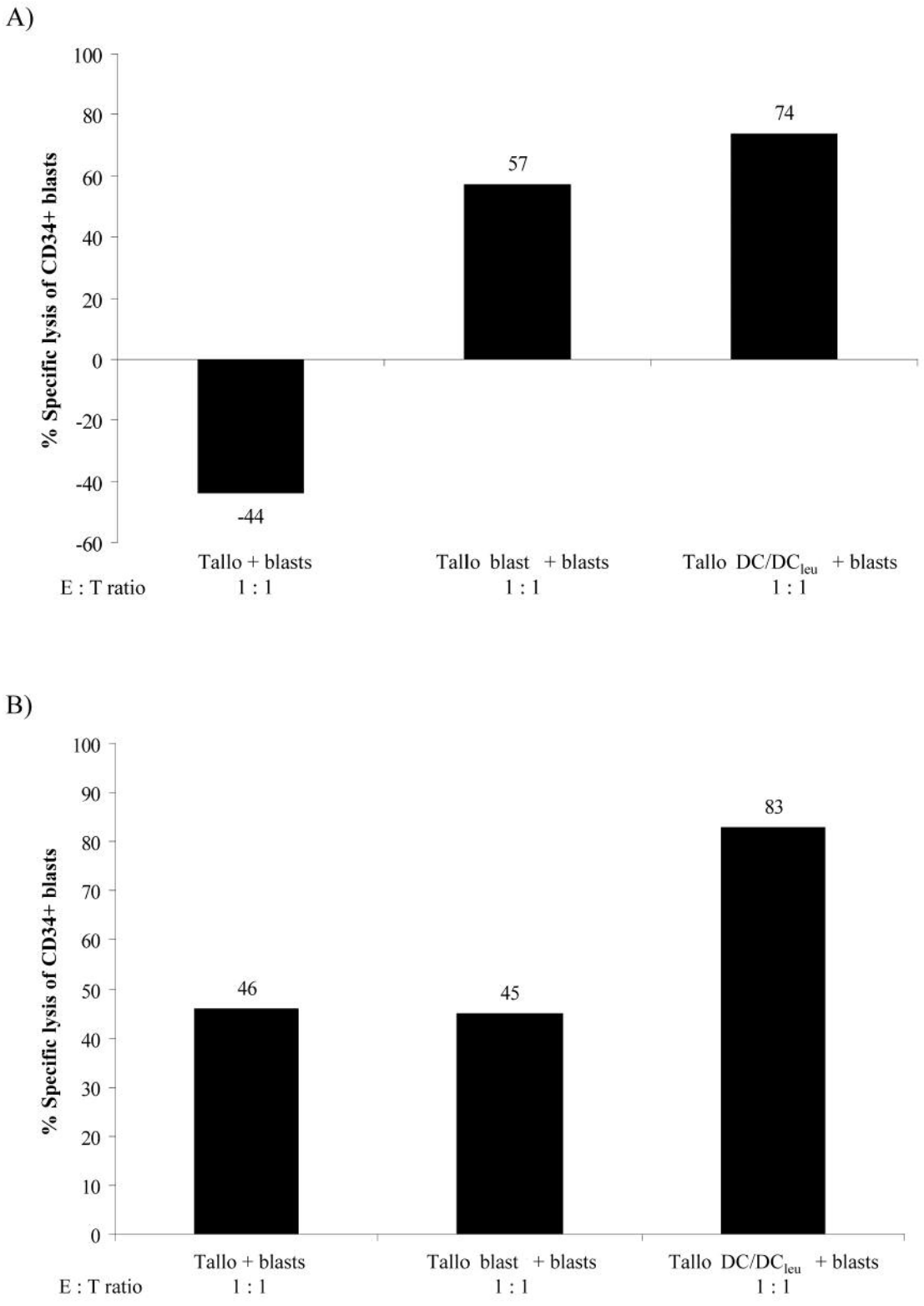

Figure 3. *Lytic activity of blast- or DC-trained allogeneic donor T-cells after 3 (A) and 24 (B) hours. Antileukemic activity of DC (Tallo 'DC/DC$_{leu}$') or MNC (Tallo 'blast') trained T cells was evaluated by a non-radioactive fluorolysis assay. Positive values indicate lysis of blasts and negative values show increase of blasts under the influence of T-cells.*

Table IV. *Peaks detected by immunoscope spectratyping within the Vβ families of CD4+ or CD8+ lymphocytes with or without stimulation with blasts or DC/DC$_{leu}$ contribute to clonal restrictions of T-cell receptor repertoire. Peak counts within Vβ families of positively selected CD4+ and CD8+ T-cells are presented before ('day 0') and after stimulation for 10 days ('day 10') with blasts or DC/DC$_{leu}$. The overall count of the peaks are summarized as a score. Peaks are presented in absolute numbers for each Vβ family, in brackets as percentage referring to the total number of peaks.*

Vß Family	CD4 day 0	CD4 (blast) day 10	CD4 (DC/DC$_{leu}$) day 10	CD8 day 0	CD8 (blast) day 10	CD8 (DC/DC$_{leu}$) day 10
Vβ1	3	2	0	4	3	1
	(2.1)	(2.3)		(3.4)	(5.4)	(3.3)
Vβ18	5	4	3	3	0	4
	(3.6)	(4.6)	(8.3)	(2.5)		(13.3)
Vβ23	5	4	1	5	3	3
	(3.6)	(4.6)	(2.8)	(4.2)	(5.4)	(10.0)
Vβ2	7	5	3	7	4	1
	(5.0)	(5.7)	(8.3)	(5.9)	(7.1)	(3.3)
Vβ4	5	3	2	4	2	1
	(3.6)	(3.4)	(5.6)	(3.4)	(3.6)	(3.3)
Vβ8	6	3	3	6	2	0
	(4.3)	(3.4)	(8.3)	(5.1)	(3.6)	
Vβ3	5	3	2	5	2	1
	(3.6)	(3.4)	(5.6)	(4.2)	(3.6)	(3.3)
Vβ13.1	7	6	1	6	4	1
	(5.0)	(6.8)	(2.8)	(5.1)	(7.1)	(3.3)
Vβ5.2	7	5	5	5	3	3
	(5.0)	(5.7)	(13.9)	(4.2)	(5.4)	(10.0)
Vβ5.1	4	4	3	1	1	1
	(2.9)	(4.6)	(8.3)	(0.8)	(1.8)	(3.3)
Vβ6	6	5	2	5	4	1
	(4.3)	(5.7)	(5.6)	(4.2)	(7.1)	(3.3)
Vβ20	4	0	0	1	0	0
	(2.9)			(0.8)		
Vβ7	6	4	0	5	2	0
	(4.3)	(4.6)		(4.2)	(3.6)	
Vβ22	7	5	2	6	3	1
	(5.0)	(5.7)	(5.6)	(5.1)	(5.4)	(3.3)
Vβ9	7	3	1	6	3	1
	(5.0)	(3.4)	(2.8)	(5.1)	(5.4)	(3.3)
Vβ16	6	3	1	6	1	0
	(4.3)	(3.4)	(2.8)	(5.1)	(1.8)	
Vβ11	7	3	1	5	2	0
	(5.0)	(3.4)	(2.8)	(4.1)	(3.6)	
Vβ12	4	2	1	4	1	0
	(2.9)	(2.3)	(2.8)	(3.4)	(1.8)	
Vβ15	5	3	0	3	0	2
	(3.6)	(3.4)		(2.5)		(6.7)
Vβ13.2	6	5	0	5	2	1
	(4.3)	(5.7)		(4.1)	(3.6)	(3.3)
Vβ14	6	5	1	7	5	0
	(4.3)	(5.7)	(2.8)	(5.9)	(8.9)	
Vβ17	6	5	1	6	5	1
	(4.3)	(5.7)	(2.8)	(5.1)	(8.9)	(3.3)
Vβ19	1	1	0	5	0	0
	(0.7)	(1.1)		(4.1)		
Vβ24	6	1	1	5	0	2
	(4.3)	(1.1)	(2.8)	(4.1)		(6.7)
Vβ10	3	2	1	1	2	3
	(2.1)	(2.3)	(2.8)	(0.8)	(3.6)	(10.0)
Vβ21	6	2	1	3	2	2
	(4.3)	(2.3)	(2.8)	(2.5)	(3.6)	(6.7)
Score	140	88	36	119	56	30

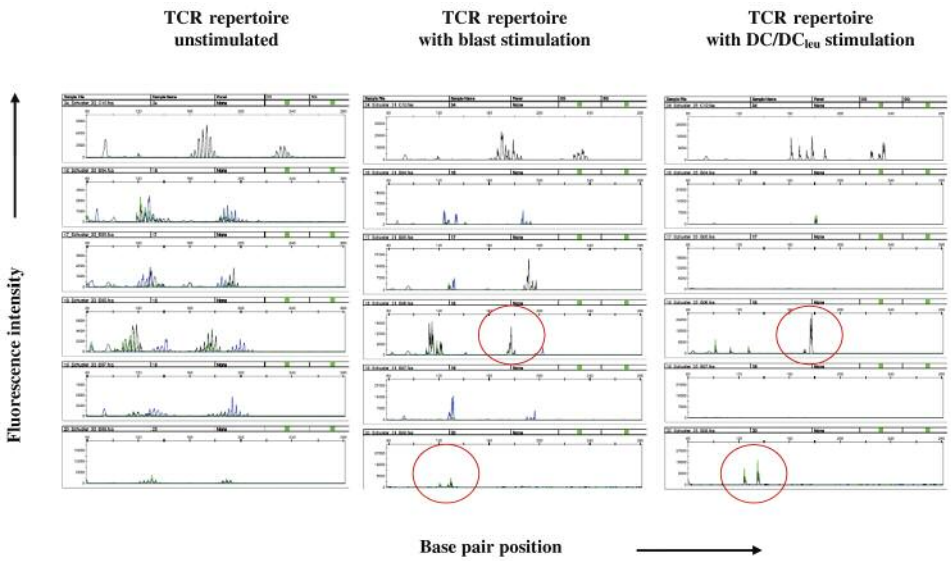

Figure 4. *Analysis of CD4+ lymphocytes using the immunsocope technique. The TCR repertoire for the unstimulated sample was normal. The blast- and DC/DC$_{leu}$-stimulated fractions both showed similar TCR restrictions, while restriction and fluorescence intensity was higher in the DC/DC$_{leu}$-stimulated fractions (marked by red circles).*

settings compared (Figure 2). The proportion of Tregs was 0.4% in the uncultured cell fractions and increased about 12-fold during DC stimulation (data not shown).

Both the blast- and DC/DC$_{leu}$-trained T-cells mediated blast lysis. But interestingly, stimulation with DC/DC$_{leu}$ resulted not only in a more effective, but also in a more prolonged lytic activity, as measured after 3 h and 24 h incubation periods (Figure 3).

TCR-Vβ profiles during training phase of T-cells with blasts or DC/DC$_{leu}$. The TCR-Vβ gene profiles analysed *via* immunoscope spectratyping are summarized in Table IV and Figure 4. The unstimulated CD4+ and CD8+ T-lymphocytes derived from the HLA-matched donor of patient #502 displayed a normal Gaussian distribution of the CDR3 region of all Vβ-Jβ gene combinations of the TCR. After stimulation with either blasts or DC/DC$_{leus}$, a significant TCR restriction was observed, whereas the CD8+ T-cell subset revealed a stronger TCR restriction as compared to the CD4+ subset (56/30 peaks in the CD8^{*blast}/CD8$^{*DC/DC_{leu}}$ fraction *vs*. 88/36 peaks in the CD4^{*blast}/CD4$^{*DC/DC_{leu}}$ fraction, Table IV). However, training with DC/DC$_{leu}$ induced an even more skewed TCR pattern, affecting both the CD4+ and CD8+ T-cell subsets (88 peaks in the CD4^{*blast} fraction *vs*. 36 peaks in the CD4$^{*DC/DCleu}$ fraction, and 56 peaks in the CD8^{*blast} fraction *vs*. 30 peaks in the CD8$^{*DC/DC_{leu}}$ fraction). Moreover, the percentage distribution of clonal DNA was highest for Vβ5.2 DNA (13.9%) and Vβ18, Vβ2, Vβ8 and Vβ5.1 DNA (8.3% each) in the CD4$^{*DC/DC_{leu}}$ fractions, whereas the percentages of clonal DNA stayed at ≤6.8% in the CD4^{*blast} or CD4+ untrained T-cell subsets (Table IV). The highest percentage distributions of clonal DNA in the CD8$^{*DC/DCleu}$ fractions were found in the Vβ18 DNA (13.3%) and in the Vβ23, Vβ5.2 and Vβ10 DNA (10% each), whereas the values stayed under 9.0% in the CD8^{*blast} fraction (Table IV). In the untrained CD4+ and CD8+ cell fractions, the Vβ subunits remained under 6.0%. The strongest restriction was therefore seen in the CD8+ cells after training with DC/DC$_{leu}$ (CD8$^{*DC/DC_{leu}}$).

A detailed analysis of the individual peaks revealed increasing immunofluorescence levels after the training periods of both CD4^{*blast} and CD4$^{*DC/DC_{leu}}$ fractions, however with the highest levels found in the CD4$^{*DC/DC_{leu}}$ fraction (Figure 4). The TCR-repertoires of the CD4+ lymphocytes detected by the immunoscope technique are illustrated as fluorescence intensity at distinct base pair positions. In the upper line of Figure 4, the results of the Vβ3 (Gaussian curve between base pair 160-180) and Vβ13.1 (Gausian curve between base pair 220-240) are shown. In the lower part, rows 2-6, the corresponding Jβ families (row 2: Jβ1.1, 1.2, 1.3; row 3: Jβ1.4, 1.5, 1.6; row 4: Jβ2.1, 2.2; 2.3; row 5: Jβ2.4, 2.5, 2.6 and row 6: Jβ2.7) are presented. In rows 4 and 6, the restricted repertoires with similar patterns are highlighted with red circles. In the DC/DC$_{leu}$-stimulated fraction, an increased restriction fluorescence intensity compared to the blast-stimulated fraction can be seen (Figure 4). Similar results were found with CD8^{*blast} and CD8*$^{DC/DC_{leu}}$ fractions, although peak intensity decreased in

the $CD8^{*DC/DC_{leu}}$ fractions (data not shown). Thus, in the $CD8^{*blast}$ and $CD4^{*DC/DC_{leu}}$-trained cell fractions, clonal DNA increases as shown in increasing immunofluorescence levels. Moreover, restricted DNA peaks were found at the same position after blast or DC-training (middle compared to right side). In summary, the data showed that polyclonal proportions of Vβ subunits were found in the untrained $CD4^+$ and $CD8^+$ cells and that in general DC/DC_{leu} training gave rise to different, restricted clones compared to blast-trained T-cells. However, a few identical peaks could be detected in the blast- and DC-trained T-cell fractions, with more of these peaks detectable after DC/DC_{leu} training.

Discussion

Allogenic SCT provides a potential curative treatment option in myeloid leukemia, taking advantage of a graft *versus* leukemia (GvL) effect mediated by donor lymphocytes (3). Even in recurrent myeloid leukemia after allogeneic SCT, subsequent transfusion of donor lymphocyte can induce sustained remissions in some patients (5). But so far the requirements to successfully induce an antileukemic immune response *in vivo* are not well understood. Several reasons for resistance have been put forward, some related to tumor cells, some to host factors. Down-regulation of HLA-antigens and co-stimulatory molecules as well as secretion of inhibitory cytokines such as TGF-β might be involved and disturb the generation of alloreactive T-cell immunity.

In the present study, conversion of the leukemic blasts isolated from the patient with recurrent AML resulted in 20% DCs, 70% of which were characterized as DCs of leukemic origin (DC_{leu}) (18, 25). Interestingly, the capacity of the DC/DC_{leu} fraction to induce T-cell proliferation was not superior to that of the blast fraction, whereas the T-cell subsets appeared to be differentially affected. Stimulation with the DC/DC_{leu} cell fraction favored the expansion of $CD4^+$ T-cells, whereas stimulation with the leukemic blasts predominantly gave rise to expansion of $CD8^+$ T-cells. In addition, the cytotoxicity assays reflected a different response pattern. The DC/DC_{leu}-stimulated T-cells mediated not only a more efficient leukemic blast lysis than the blast-trained T-cells (74% *vs.* 57%), but also exhibited sustained killing activity (83% *vs.* 45%), after 3 or 24 hours of co-incubation with the leukemic target cells. The importance of cytotoxic $CD4^+$ T-cells to suppress leukemia colony formation *in vitro* has been discussed previously (35, 36) and might be in line with the observation that $CD8^+$ T-cells can be depleted from the DLT without jeopardizing the GvL effect, while concurrently diminishing graft *versus* host disease (GvHD) (37). Whether or not *in vivo* $CD4^+$ T-cells additionally have to recruit leukemia-reactive, minor H-antigen-specific-$CD8^+$ T-cells to exert effective cytolytic activity (38) is currently under investigation. In the present case, analysis of the induced T-cell repertoire using immunoscope spectratyping revealed a clonal restriction in both, the $CD4^+$ and $CD8^+$ T-cell subsets after stimulation. Interestingly, stimulation with blasts favoured a TCR restriction in the $CD8^+$ T-cell subset, whereas stimulation with DC/DC_{leu} predominantly resulted in a TCR restriction in the $CD4^+$ T-cell subset. Although these results are intriguing, the data have to be interpretated carefully. Firstly, mRNA levels in activated T-cells are generally higher compared to resting T-cells and could bias the results (39). Secondly, oligoclonality can also occur in healthy people during transient phases of restricted immunity or shortly after chemotherapy (40). In contrast to the stimulation with blasts, stimulation with DC/DC_{leu} resulted in a significant increase of $CD45RO^+$ T memory cells as well as Tregs. The reasons for this different T-cell development are unclear but might be caused by a different cytokine release pattern induced after blast, or DC/DC_{leu} stimulation. The mere phenotypic characterisation of T-cell subsets (*e.g.* CD4, CD8, memory cells, Tregs) did not allow any prediction of their acquired functional capacities, although after DC/DCleu stimulation a marked increase of $CD4^+$ T-cells was observed, with a significant proportion of $CD4^+$ T-cells immunophenotypically characterized to represent Tregs, the lytic capacity of the induced $CD4^+$ T-cells dominated the immune response. Similarly, spectratyping, an emerging powerful tool to analyze and track changes of the T-cell repertoire during a clinical course, as a stand alone technique seemed not to be sufficient to predict functional capabilities. In conclusion, this means that the detection of any clonal restriction alone is neither sufficient nor predictive for delineating a leukemia-specific or unspecific T-cell response pattern. In combination with FACS, cytokine- or additional functional assays, spectratyping might provide useful information about the clonal behaviour of cells involved in productive immunity. Thus, advanced T-cell selection strategies using Vβ/Jβ-specific antibodies or streptamers might pave the way for targeted adoptive immunotherapy reducing the risk of GvHD. Moreover monitoring of these antileukemic T-cells during a clinical course could provide the opportunity to anticipate the risk of relapse at an early stage.

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