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Research Article

# Pulsing with blast cell lysate or blast-derived total RNA reverses the dendritic cell-mediated cytotoxic activity of cytokine-induced killer cells against allogeneic acute myelogenous leukemia cells

## Pulsen mit Blastenzelllysat oder Blasten-Gesamt-RNA richtet die durch dendritische Zellen vermittelte Aktivität von Zytokin-induzierten Killerzellen gegen allogene akute myeloische Zellen

## Abstract

Immunotherapeutic strategies may be a treatment option in patients with refractory acute myelogenous leukemia (AML) or, in cases of complete remission after conventional therapy regimens, may help to reduce disease recurrence or delay time to progression. Evidence suggests a key role of dendritic cells (DCs) in cancer immunotherapy due to their capacity to present tumour antigens to effector cells. We generated cytokine-induced killer (CIK) cells from healthy donors and examined their responses *in vitro* in an LDH release assay against three cell lines and allogeneic HLA non-matched blasts from three patients with *de novo* AML after coincubation with autologous peripheral blood monocyte-derived DCs. Although DCs were unable to enhance CIK cell effects against all three cell lines tested, the cytotoxic activity against the patients' AML cells increased after coculture with mature DCs, which was significant in two of three patients. However, neither prior pulsing of the DCs with blast cell lysates nor with leukemic cell-derived total RNA further enhanced the lytic capacity of the CIK cells. On the contrary, pulsing reduced or even reversed the cytotoxic activity of the effector cells. This decrease of allogeneic cytotoxicity led us to conclude that monocyte-derived DCs may be useful in autologous or allogeneic vaccine strategies for the treatment of AML or in priming donor lymphocytes in vitro, but unfractionated antigens as pulsing agents may have inhibitory effects on T cell efficiency and their employment in immunotherapeutic strategies for AML seems questionable.

**Keywords:** dendritic cells, cytokine-induced killer cells, AML, blast cell lysate, blast-derived RNA

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## Zusammenfassung

Immuntherapeutische Strategien können eine Behandlungsoption bei Patienten mit refraktärer akuter myeloischer Leukämie (AML) sein oder in den Fällen einer kompletten Remission nach konventionellen Therapieformen helfen, das Wiederauftreten der Krankheit zu verhindern oder die Zeit bis zur Progression zu verlängern. Es gibt Hinweise darauf, dass dendritische Zellen (DCs) eine zentrale Rolle in der Krebs-Immuntherapie spielen aufgrund ihrer Fähigkeit, tumorantigene Effektor-Zellen zu präsentieren. Wir stellten Zytokin-induzierte Killer (CIK)-Zellen von gesunden Spendern her und untersuchten deren Reaktionen *in vitro* in einem Laktatdehydrogenase (LDH)-Assay gegen Zelllinien und allogene HLA nicht übereinstimmende Blasten von drei Patienten mit *de novo* AML nach Koinkubation mit autologen aus dem peripheren Blut abgeleiteten DCs. Obwohl DCs die CIK Zellen Wirksamkeit gegen alle drei

getesteten Zelllinien nicht verbessern konnten, wurde die zytotoxische Aktivität gegen die Patienten-AML-Zellen nach Kokultur mit reifen DCs in zwei von drei Patienten signifikant erhöht. Doch weder ein Pulsen der DCs mit blastären Zelllysaten noch mit aus leukämischen Zellen gewonnener Gesamt-RNA konnten die lytische Kapazität der CIK-Zellen weiter verbessern. Im Gegenteil, gepulste DCs reduzierten sogar die zytotoxische Aktivität der Effektorzellen. Dieser Rückgang der allogenen Zytotoxizität führte uns zu dem Schluss, dass von Monozyten abgeleitete DCs nützlich sein könnten in autologen oder allogenen Impfstrategien zur Behandlung von AML. Unfraktionierte Antigene zum Pulsen von DC können dagegen hemmende Wirkung auf T-Zellen haben.

**Schlüsselwörter:** dendritische Zellen, Zytokin-induzierte Killerzellen, AML, Zelllysat, RNA

# Introduction

Chemotherapy and allogeneic bone marrow transplantation (BMT) are conventional options for the treatment of acute myelogenous leukemia (AML) [1], [2], [3], [4]. Although complete remissions can be achieved in the majority of patients, relapse of the disease remains a frequent cause of treatment failure and results in a poor prognosis [5]. Therapeutic options for patients with recurrent leukemia are limited. After BMT, second marrow transplants from the same donor may be considered, but the mortality, treatment-related morbidity and risk of further relapse are high [6], [7].

Alternative or additional strategies are provided by immunotherapeutic approaches. The importance of T cell reactions against leukemic cells is demonstrated by the successful employment of donor lymphocyte infusions (DLI) in patients with relapsed chronic myelogenous leukemia (CML) after allogeneic BMT. The application to acute leukemia patients also shows susceptibility of AML blasts to the donor lymphocytes, though the treatment turns out to be less effective [8], [9], [10], [11].

In solid tumours, antigen-specific immunotherapy has already emerged as a promising approach to control the disease. Evidence suggests the central role of dendritic cells (DCs) for mediating these immune reactions, as they are specialized to prime naive helper and cytotoxic T lymphocytes and directly trigger NK cell functions [12], [13], [14], [15]. DCs can stimulate T cells as they express high levels of major histocompatibility complex (MHC) class I and II molecules along with costimulatory molecules such as B7.1 (CD80) and B7.2 (CD86) on their surface. They are equipped to capture and process antigens – e.g., tumour-associated antigens – and present immunogenic MHC-peptide complexes to T lymphocytes and so enhance the otherwise low immunogenicity of these proteins [16], [17], [18], [19]. Mature DCs migrate to secondary lymphoid organs where they stimulate antigen-specific T cells [20].

Hence, the use of DCs in vaccine strategies for the treatment of malignancies promises to be helpful to overcome the resistance of tumours to the immune system. For this purpose, active DCs can be generated *ex vivo* in large quantities. In fact, several studies have demonstrated tumour responses after vaccination with antigen-pulsed DCs in a variety of mouse tumour models and even in human melanoma, non-Hodgkin's lymphoma (NHL) and prostate cancer [21], [22], [23], [24], [25]. Vaccine strategies may use DCs prepared with either defined tumour-associated antigens such as HPV-16 E6/E7 [26], [27] or proteins of the MAGE family in melanoma patients [28], or with undefined tumour antigens such as whole tumour lysates [24], [25] or tumour-derived total RNA [29]. The use of whole tumour lysate or complete RNA in cancer immunotherapy provides some advantages as compared with the use of defined tumour antigens: The identification of the effective antigen(s) is not required and treatment strategies are feasible even for malignancies (such as AML) in which only few more or less specific tumour-associated antigens have been characterized [30], [31]. Furthermore, the probable presence of multiple antigens reduces the risk of a tumour cell escape [32]. Finally, in patients with acute leukemia tumour material required for the generation of lysate or RNA can easily be obtained in sufficient quantities from peripheral blood or bone marrow aspirates. One major drawback in the use of unfractionated antigens is the possible occurrence of autoimmune reactions directed against 'self-antigen' included in the lysate or total RNA [33], [34].

In this study, we used peripheral blood monocyte-derived DCs from healthy donors pulsed with either whole blast cell lysate or blast cell-derived total RNA to stimulate autologous cytokine-induced killer (CIK) cell responses *in vitro* against three AML cell lines and against allogeneic blasts from three HLA-unmatched patients with *de novo* AML.

# Materials and methods

## AML cells

AML cell lines HL-60, KG-1 and the CML cell line K-562 (chronic myelogenous leukemia in blast crisis) were purchased from the Deutsche Sammlung für Mikroorganismen und Zellkultur (DSMZ, Braunschweig, Germany). Peripheral blood from patients (Table 1) at *de novo*

leukemia stage with >65% myeloblastic cells was drawn after informed consent in accordance with our local ethic committee. Cells were isolated by Ficoll density gradient centrifugation and maintained in RPMI 1640 (Gibco BRL, Berlin, Germany) supplemented with 10% FCS (PAA, Linz, Austria), 100 U/mL penicillin and 100 µg/mL streptomycin (Biochrom, Berlin, Germany). Patients' AML cells could be maintained for 6 to 35 d.

**Table 1: Characteristics of AML patients**

| Patient | 1 | 2 | 3 |
|---|---|---|---|
| Sex | F | F | M |
| Age | 52 | 42 | 24 |
| Sample | PB | PB | PB |
| % Blasts | 63 | 96 | 72 |
| FAB | M1 | M4 | M4 |
| Blast cell phenotype | | | |
| CD80 | <2.0 | 3.0±1.4 | <2.0 |
| CD86 | 7.4±1.2 | 15.5±2.3 | <2.0 |
| HLA-ABC | 96.2±2.1 | 94.5±4.8 | 86.8±5.7 |
| HLA-DR | 92.8±3.1 | 78.8±6.4 | 16.0±1.4 |

Percentage of blasts was determined by examination of a peripheral blood smear. Blast cell phenotype was determined by flow cytometric analysis and is shown as percent expression. Flow cytometric data represent the mean of 3 separate experiments ± SEM. Abbreviations: M, male; F, female; PB, peripheral blood

## Dendritic cells

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors' buffy coats (day 0) by Ficoll density gradient centrifugation (Lymphoprep, Nycomed, Oslo, Norway). Cells were allowed to adhere in six-well-plates (Becton Dickinson, Heidelberg, Germany) at a density of 5 x $10^6$ cells/mL for 1 hr at 37 °C in a humidified atmosphere of 5% $CO_2$ in RPMI 1640 medium with 10% autologous serum. Non-adherent cells were collected for generating cytotoxic lymphocytes (see below). Adherent monocytes were cultured in 2 mL RPMI 1640 with 10% autologous serum, 25 mM Hepes (hydroxyethylpiperazine ethane sulfonic acid), 100 U/mL penicillin and 100 µg/mL streptomycin supplemented with 750 U/mL human granulocyte-macrophage colony-stimulating factor (GM-CSF) and 500 U/mL human interleukin-4 (IL-4, Essex Pharma, Nürnberg, Germany) for 7 d. Medium was changed on day 4 and 500 U/mL tumour necrosis factor-α (TNF-α) was added on day 5.

To generate DCs from leukemic blasts, patients' AML cells were isolated and cultured as described above. Only samples containing more than 65% leukemic blasts were used. GM-CSF, IL-4, and TNF-α were repetitively added at the above mentioned concentrations every 3 to 4 d. Medium was changed when necessary.

## Effector cells

Effector cells, termed cytokine-induced killer cells (CIK cells), were generated as described previously [35]. Briefly, non-adherent Ficoll separated human PBMC were grown in RPMI 1640 medium consisting of 10% fetal calf serum, 25 mM Hepes, 100 U/mL penicillin and 100 µg/mL streptomycin (hereafter referred to as complete medium) in tissue culture flasks (Becton Dickinson) and incubated at 37 °C in a humidified atmosphere of 5% $CO_2$ at 3x$10^6$ cells/mL. 1,000 U/mL human recombinant interferon-γ (IFN-γ, Roche, Mannheim, Germany) was added immediately (day 0). After 24 hrs of incubation, 50 ng/mL of an antibody against CD3 (Orthoclone OKT-3, Cilag GmbH, Sulzbach, Germany), 100 U/mL IL-1β and 300 U/mL IL-2 (Roche) were added. IL-2 was used for repetitive stimulation every 3 to 4 d at a concentration of 300 U/mL. Fresh medium was added when required. Additionally, for comparing different effector cell types, peripheral blood lymphocytes (PBLs) and lymphokine-activated killer cells (LAK cells) were generated from cryopreserved non-adherent Ficoll separated human PBMC. PBLs were thawed 1 d before use and grown within complete medium without cytokines, LAK cells were generated by thawing 6 d before use and culturing within complete medium supplemented with IL-2 (1,000 U/mL) [36].

## Phenotypic analysis of cells by flow cytometry

Cells were washed with PBS and single cell suspensions were stained with various combinations of fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated mouse antihuman monoclonal antibodies in a total volume of 50 µl for 15 min on ice. Stained cells were washed with PBS and flow cytometric analysis was subsequently performed on a Coulter Epics XL Cytometer (Coulter-Immunotech, Krefeld, Germany). Background staining using FITC- and PE-conjugated mouse IgG was <2%. Data from 20,000 cells were collected and analyzed. DCs were phenotyped with the following markers: CD14, CD80, CD83 (Coulter-Immunotech), CD86, HLA-ABC, and HLA-DR (Pharmingen, Hamburg, Germany). Effector cells were analyzed for the expression of the phenotypic markers CD3, CD4, CD8, CD28, and CD56 (Coulter-Immunotech). Phenotypic characterization of the patients' AML cells was performed with monoclonal antibodies against CD14, CD80 (Coulter-Immunotech), CD13, CD64, CD86, HLA-ABC, HLA-DR (Pharmingen), and CD34 (Becton Dickinson).

## Isolation of blast cell lysates and RNA

AML cell lines and patients' samples containing more than 65% leukemic blasts were used. Patients' AML cells

were isolated by Ficoll density gradient centrifugation at *de novo* leukemia stage and cultured in RPMI 1640 supplemented with 10% FCS. Cells were harvested, washed with phosphate-buffered saline (PBS, PAA, Cölbe, Germany) and alternately frozen in liquid nitrogen and thawed at 37 °C five times. The blast cell extract solution was filtered (0.2 µm) and protein concentration was measured using a Bradford protein assay (Bio-Rad, Munich, Germany). The protein solution was then stored at –20 °C until used in DC-pulsing experiments.
Total RNA from leukemic blasts was extracted using the RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. After isolation, the RNA was reconstituted in sterile, endotoxin- and RNase-free water and quantitated by measuring OD at 260 and 280 nm. OD 260/280 ratios were typically 1.65-2.0. RNA was stored at –70 °C.

## Pulsing of DCs

Blast cell-derived lysate at various concentrations was added to the DCs on day 1. Antigen exposure was stopped after 4 hrs by medium replacement or on day 4 when the medium was changed routinely.
Pulsing with tumour-derived RNA was performed on day 2 in serum-free RPMI 1640 medium. The cationic liposome DOTAP (N-[1-(2,3-Dioleoyloxy)]-N,N,N-trimethylammonium propan methylsulfate, Roth) was employed to transfer the RNA into the DCs. Various amounts of RNA and DOTAP were mixed in a constant ratio of 1 µg RNA to 2 µl transfectant at room temperature for 15 min, but not more than 8 µl DOTAP were used even for transfection with the highest RNA concentrations due to toxic effects. The complexes were added to the DCs in 500 µl of serum-free medium, and the cells were slightly shaken at 37 °C for 1 hr. Serum-free medium was added to a final volume of 2 mL, and the DCs were incubated at 37 °C in a humidified atmosphere of 5% $CO_2$. Pulsing was stopped after 24 hrs by adding 10% autologous serum to the medium. Replacement of medium and addition of TNF-α were performed as described above.

## Coculturing of effector cells with DCs

Lymphocytes were harvested on day 7 and cocultured with autologous DCs for 7 d using stimulator to responder ratios of 1:5 to 1:10 which had been found to be optimal. IL-2 was added immediately or up to 48 hrs after coincubation and once again 3 d later.

## Cytotoxicity assay

A CytoTox 96 non-radioactive assay (Roche) was used to measure cytotoxic activity. This enzymatic assay quantitatively measures lactate dehydrogenase (LDH) which is released upon cell lysis and has been shown to be of identical efficiency as compared to standard $^{51}$[Cr] chromium release assay [37], [38]. Target cells (cell lines or AML patients' allogeneic blasts) were plated in triplicates in a 96-well U-bottom tissue culture plate ($10^4$ cells/well) and coincubated with lymphocytes at final effector to target cell ratios of 2.5:1, 5:1, 10:1 and 20:1. After 4.5 hrs of incubation, cells were centrifuged and 50 µl supernatant from each well was transferred to a fresh 96-well plate, 50 µl of the substrate mix was added and incubated at room temperature in the dark for 15 to 30 min. Before measuring, 50 µl of a stop solution was added to each well. Maximal release of LDH was performed by incubating the target cells with 0.1% anionic detergent (IGEPAL, Sigma, Deisenhofen, Germany). Target cells without effector cells were used as negative control (spontaneous release). Absorbency data were collected at 490 nm using a 96-well plate reader. Cytotoxicity was calculated according to the following formula:

$$\text{Cytotoxicity [\%]} = \frac{(ET - E_{spont}) - T_{spont}}{T_{max} - T_{spont}} \times 100$$

where ET is the experimental release, $E_{spont}$ is the spontaneous release by effector cells, $T_{spont}$ is the spontaneous release by target cells, and $T_{max}$ is the maximal release by target cells.

## Statistical analysis

Wilcoxon's signed rank test was used to analyze for statistical significance. A P value of $<0.05$ was considered significant.

# Results

## Flow cytometric analysis of DCs

DCs were generated from monocytes using GM-CSF, IL-4 and TNF-α as described in "Materials and methods". After 7 d of culture the yield of cells was 0.4-1 x $10^6$/well and flow cytometric analysis revealed 94.7±2.4% of the cells expressing CD80, 39.6±4.4% CD83, 85.3±2.7% CD86, 98.5±2.1% HLA-ABC and 96.4±3.1% HLA-DR while 68.4±7.0% of the cells were negative for CD14 (Figure 1). These data represent the mean ± standard error of the mean (SEM) of five separate experiments. Pulsing of the DCs with blast cell lysate (10 µg/mL) or total RNA (10 µg/mL) did not alter the percentage of MHC molecule, CD80, CD86 or CD83 expression (data not shown).
On DCs generated from the patients' leukemic blasts with GM-CSF, IL-4 and TNF-α, flow cytometric analysis was performed when >60% of the cells showed typical DC morphology (between days 11 and 21). The flow cytometric phenotype of the cells was compared to autologous leukemic blasts cultured for equivalent periods without cytokines (see below). Cells developed or upregulated expression of costimulatory and MHC molecules, but the percentage varied strongly from patient to patient, and CD83 expression did not exceed 20% in any of the three patients (data not shown).

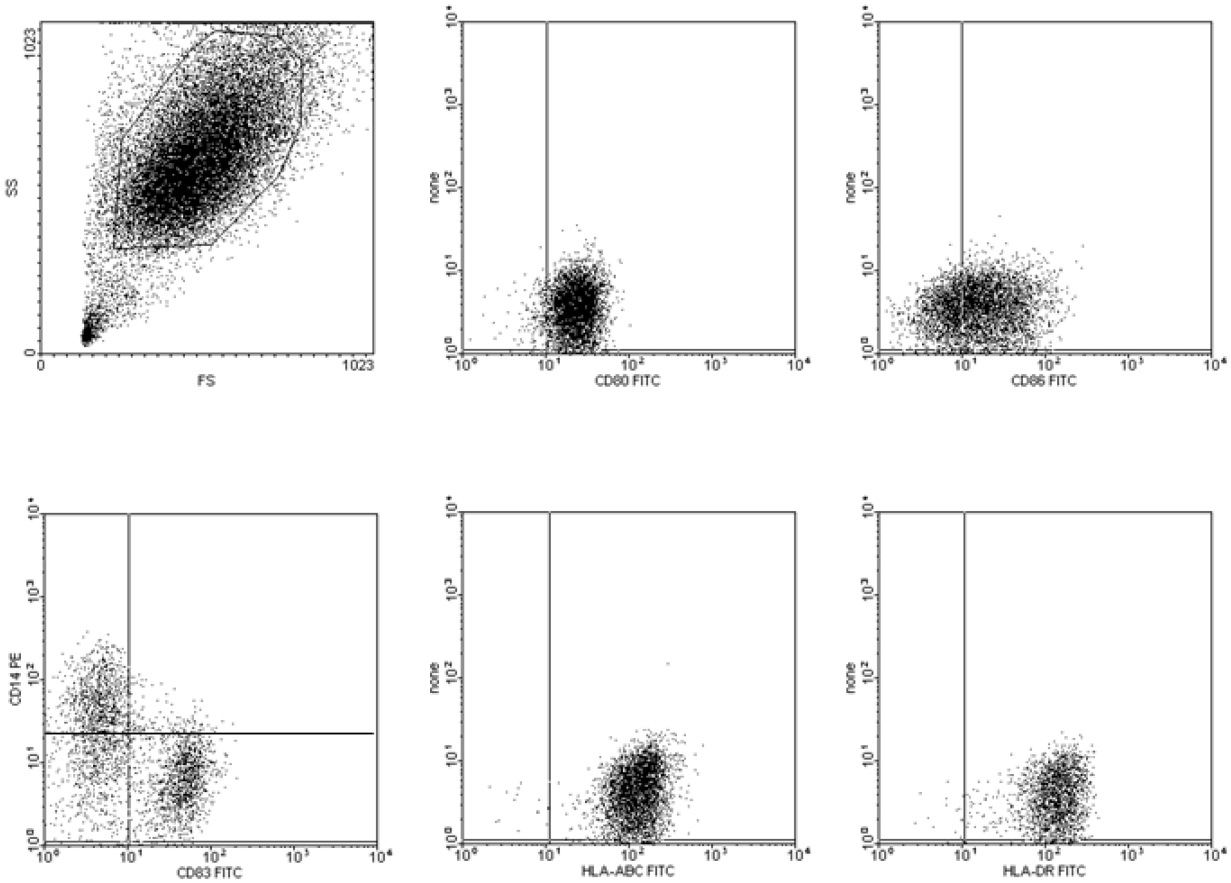



**Figure 1: Flow cytometric analysis of monocyte-derived DCs from healthy donors**
DCs were generated and cultured as described in "Materials and methods". On day 7, cells were stained with monoclonal antibodies against CD80, CD83, CD86, HLA-A,B,C, HLA-DR, and CD14 and analyzed. One representative of five experiments is illustrated.

## Flow cytometric analysis of CIK cells

The effector cell population was generated as described in "Materials and methods" using IFN-γ, antibody against CD3, IL-1ß, and IL-2. Results of the flow cytometric analysis are shown in Figure 2. From day 1 to day 14 of culture, CD3 expression increased from 73.5±2.5% to 94.5±3.4%. Co-expression of CD3 and the costimulatory signal CD28 remained unchanged (day 1: 68.0±5.5%; day 14: 71.4±6.3%). While 42.5±11.2% of the cells were positive for CD8 on day 1 and 66.5±2.7% on day 14, CD4 expression decreased from 36.3±9.4% to 28.9±4.4%, resulting in a mean $CD8^+$ to $CD4^+$ cell ratio of 2.3 on day 14 as compared to 1.2 on day 1. Coculture of the effector cell population with DCs on day 7 led to a further but not significant increase in this ratio on day 14 (data not shown). Percentage of $CD3^+CD56^+$ cells was <3.2% (mean of 1.9%) on day 1 and ranged from 5.1% to 14.3% (mean of 7.2%) on day 14. Data represent the mean ± SEM of five separate experiments.

## MHC and costimulatory molecule expression on AML cells

Phenotypic analysis of AML cells derived from patients is shown in Table 1. Blast cells were $HLA\text{-}ABC^+HLA\text{-}DR^+CD80^-CD86^-$ in patients 1 and 2, and $HLA\text{-}ABC^+HLA\text{-}DR^-CD80^-CD86^-$ in patient 3. HL-60, KG-1, and K-562 cells were $HLA\text{-}ABC^+HLA\text{-}DR^-$, $HLA\text{-}ABC^+HLA\text{-}DR^+$, and $HLA\text{-}ABC^-HLA\text{-}DR^-$, respectively, while all cell lines were $CD80^-CD86^-$ (data not shown).

## CIK cell activity in comparison to PBLs and LAK cells

First, we determined the basic allogeneic cytotoxicity of the CIK cell population in comparison to PBLs and LAK cells against myeloblastic cells from one AML patient chosen representatively. For this purpose, we performed an LDH-release assay with the different effector cell types generated from three healthy donors, using AML cells from patient 2 as target cells. At an effector to target cell ratio (E:T ratio) of 20:1 mean blast cell lysis in three separate experiments was 3.4%, 17.0% and 25.3% using PBLs, LAK cells and CIK cells, respectively (Figure 3),

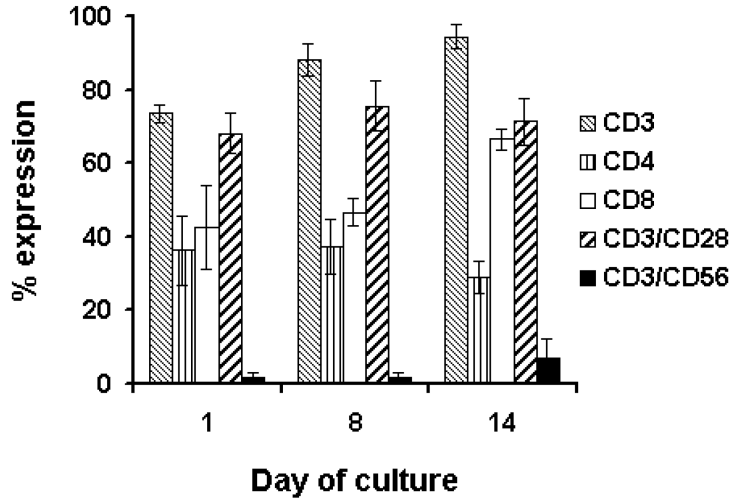



**Figure 2: Flow cytometric characterization of CIK cells**
Effector cells were cultured as described in "Materials and methods". On days 1, 8 and 14 cells were stained with monoclonal antibodies against CD3, CD4, CD8, CD28 and CD56 and analyzed. Results are presented as mean values ± SEM from five independent experiments.

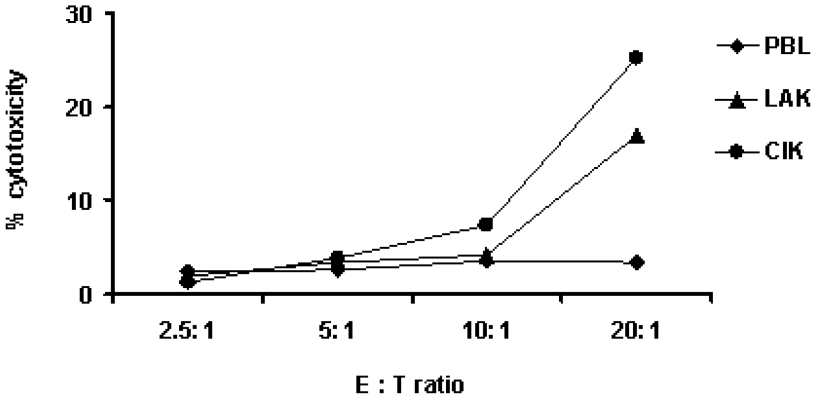



**Figure 3: Cytotoxic activity of PBLs, LAK cells and CIK cells against leukemic blasts from one patient with AML (patient 2)**
The distinct effector cells were generated from healthy donors according to the protocols described in "Materials and methods". Lysis was measured by an LDH-release assay. PBLs were used on day 1, LAK cells on day 6 of culture, CIK cells had been grown for 14 d. Results represent the mean of three independent experiments (SEM <20% of the mean).

which led us to perform the following studies with the latter effector cell population.

## Effector cell cytotoxicity against AML cell lines

CIK cells from healthy donors were cocultured with autologous either non-pulsed DCs or blast cell lysate- or total RNA-pulsed DCs and tested for cytotoxicity against cell lines HL-60, KG-1 and K-562. Successful delivery of RNA into DCs was demonstrated by flow cytometric analysis 24 hrs after transfection of the cells with enhanced green fluorescent protein RNA (eGFP-RNA) using DOTAP. Mean transfection efficiency was 15% as determined by evaluating the percentage of positive cells in a flow cytometric analysis (data not shown). Toxic effects of DOTAP on the DCs were excluded by adding 8 µl of the transfectant to non-pulsed DCs before coincubation with CIK cells which did not result in a decrease of effector cell number or cytotoxicity.

Coculture with DCs led to a 1.5–3.5fold increase in lymphocyte count, and no difference was detectable between cultures with pulsed and non-pulsed DCs. Lymphocytes proliferated preferably in the vicinity of DC clusters. In Figure 4, the cytotoxicity of the effector cell population against the three different cell lines is demonstrated. At an E:T ratio of 20:1, a mean of 24.4% of HL-60 cells, 9.3% of KG-1 cells and 18.6% of K-562 cells were lysed by effector cells that had not been coincubated with DCs. Neither coculture with non-pulsed DCs nor with DCs that had been pulsed with blast cell lysate or RNA could increase the cytotoxicity significantly.

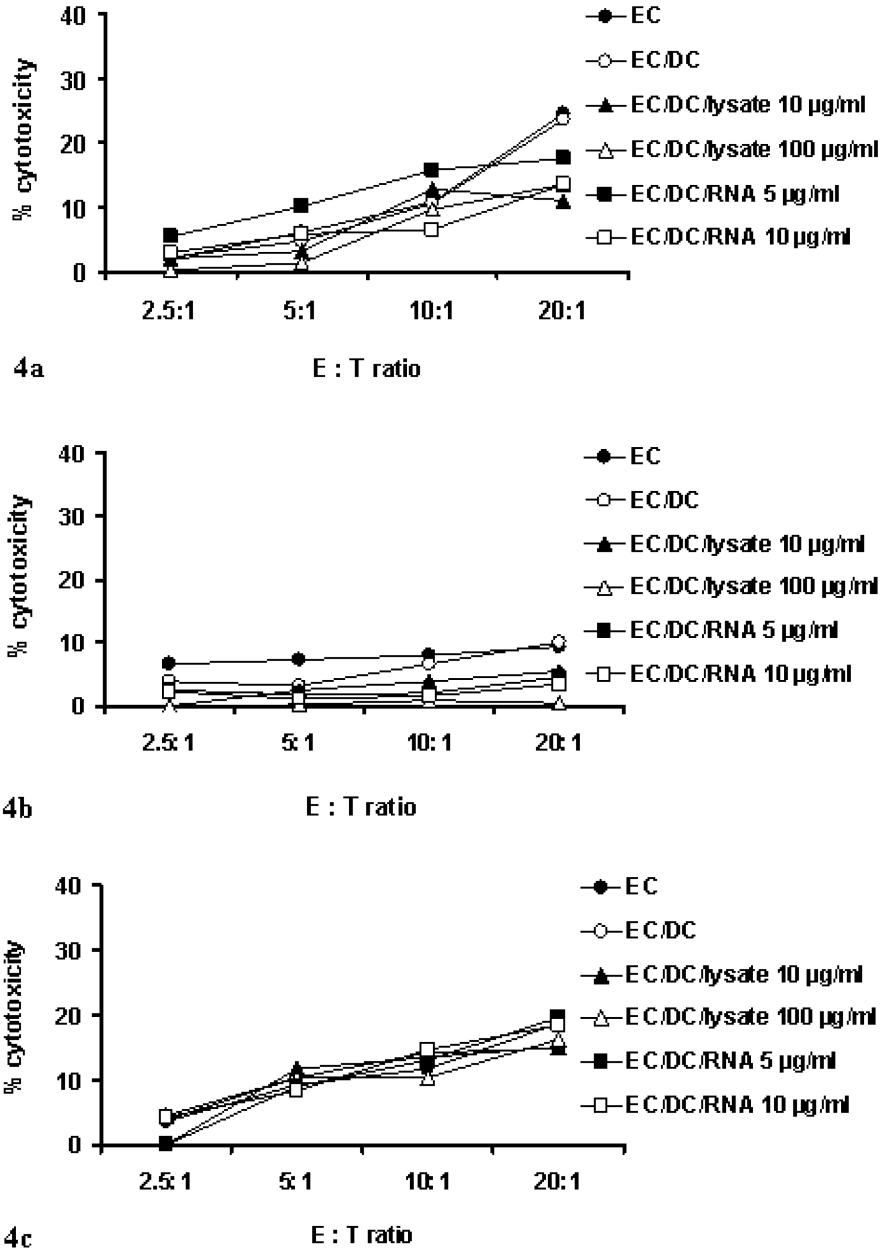
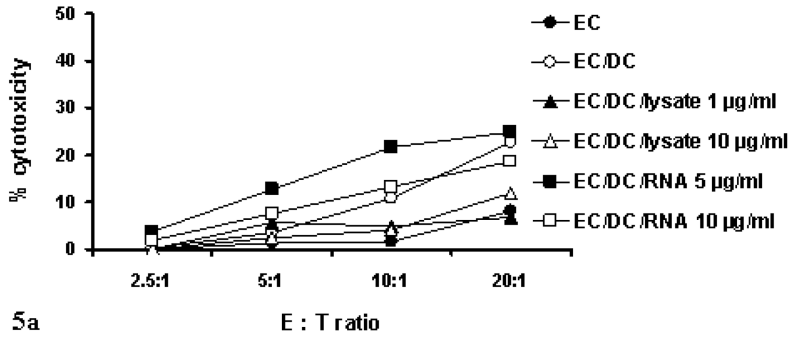



**Figure 4a–c: Cytotoxic activity of T cells against cell lines HL-60 (a), KG-1 (b) and K-562 (c)**

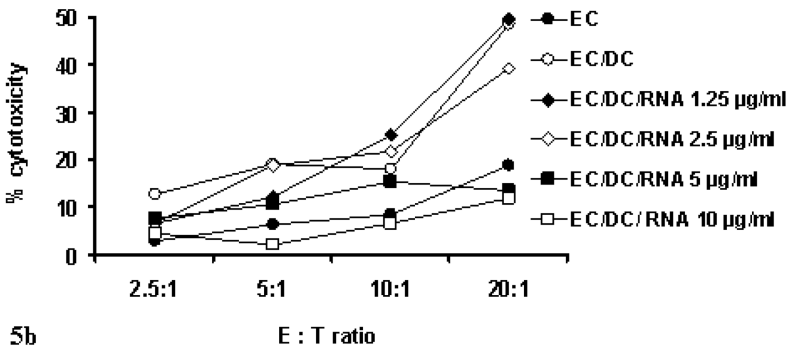
CIK cells and DCs were generated from various healthy donors as described in "Materials and methods". Effector cells were cocultured on day 7 with either non-pulsed DCs or with DCs that had been exposed to blast cell lysates or AML cell-derived RNA, respectively. Lysis of AML blasts was measured in an LDH-release assay on day 14 of cell culture. Effector cells not coincubated with DCs were used as control. (EC, effector cells (control); EC/DC, effector cells cocultured with non-pulsed DCs; EC/DC/lysate, effector cells cocultured with DCs pulsed with blast cell lysate; EC/DC/RNA, effector cells cocultured with DCs pulsed with blast cell-derived total RNA). Results represent the mean of at least three separate experiments (SEM <20% of the mean).

## Effector cell cytotoxicity against blast cells derived from AML patients

To investigate the allogeneic activity of CIK cells derived from healthy donors against myelogenous leukemic blasts from patients with AML, we performed an LDH-release assay on day 14 of CIK cell culture and used myeloblastic cells from three separate patients as targets. CIK cells were cocultured with autologous either non-pulsed DCs or with DCs that had been stimulated with the respective blast cell lysates or leukemic cell-derived total RNA. Effector cells not cocultured with DCs were used as control. The increase in CIK cell number ranged from 1.5-4.0fold after coculture with DCs, independent of prior pulsing. For patients 1, 2 and 3, the basic CIK cell activity at an E:T ratio of 20:1increased from 8.1% to 22.6% ($p$=0.043), from 18.7% to 48.3% ($p$=0.028) and from 29.4% to 32.8% ($p$>0.05), respectively, after coincubation with non-pulsed DCs (mean of at least four experiments; SEM <20% of the mean), as shown in Figure 5.




5a

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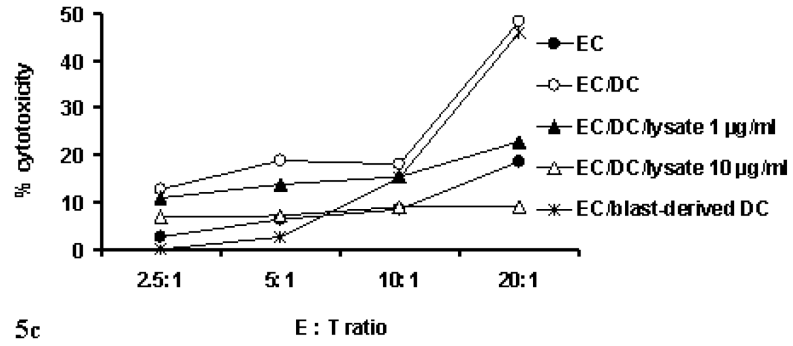


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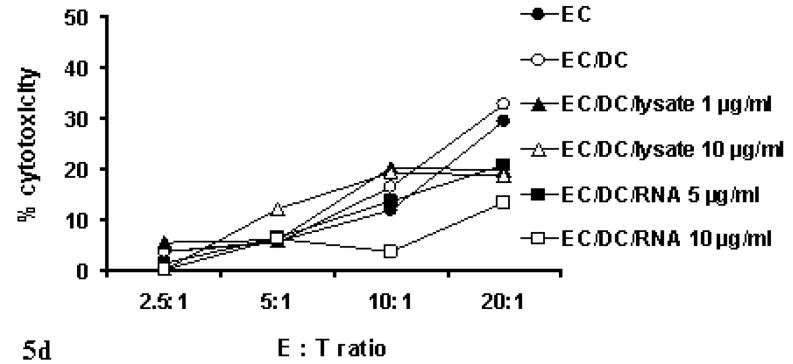


5d

**Figure 5a–d: Allogeneic antileukemic CIK cell activity against AML blasts derived from three different AML patients**
CIK cells and DCs were generated from different healthy donors as described in "Materials and methods". Effector cells were cocultured on day 7 with either non-pulsed DCs or with DCs that had been exposed to the respective blast cell lysates or AML cell-derived total RNA. Lysis of AML blasts was measured using an LDH-release assay on day 14 of cell culture. Effector cells not coincubated with DCs were used as control. (EC, effector cells (control); EC/DC, effector cells cocultured with non-pulsed DCs; EC/DC/lysate, effector cells cocultured with DCs pulsed with blast cell lysate; EC/DC/RNA, effector cells cocultured with DCs pulsed with blast cell-derived total RNA). Results represent the mean of at least four independent experiments (SEM <20% of the mean). The difference between control and effector cells cocultured with non-pulsed DCs at an E:T ratio of 20:1 was significant for patient 1 and 2 ($p$=0.043 and $p$=0.028, respectively).

However, in none of the three patients could the DC-mediated increase in the antileukemic CIK cell effect be further enhanced by prior pulsing of the DCs with the respective antigens. On the contrary, in patient 1 the DC-mediated effect remained unchanged or was even reduced – if not reversed – by using DCs pulsed with various concentrations of lysate or RNA (Figure 5a). In patient 2, the decrease of blast cell lysis after coculture with pulsed DCs was antigen concentration-dependent as demonstrated in Figure 5b+c. Coincubation of effector cells with lysate-pulsed monocyte-derived DCs resulted in a decrease of target cell lysis to 22.9% using 1 µg/mL lysate and to 9,0% using 10 µg/mL lysate at an E:T ratio of 20:1 (Figure 5b). Lysis of the same patient's blast cells by CIK cells coincubated with RNA-pulsed DCs was 49.5%, 39.3%, 13.5% and 12.0% using 1.25 µg/mL, 2.5 µg/mL, 5 µg/mL or 10 µg/mL RNA, respectively (Figure 5c). Lower antigen concentrations than those indicated did not influence the DC-mediated effect on the effector cells. Results represent the mean of at least four independent experiments (SEM <20% of the mean). For this patient, sufficient amounts of DCs derived from autologous leukemic blasts could be generated and were used comparatively. Mean target cell lysis at an E:T ratio of 20:1 was 48.3% using CIK cells cocultured with autologous, non-pulsed monocyte-derived DCs (see above) and 46.1% using CIK cells cocultured with autologous blast-derived DCs, indicating the equivalent effect of DCs of both origins. Finally, the antileukemic effect of allogeneic CIK cells against blast cells derived from patient 3 was diminished to two thirds or less of baseline levels with the strongest reduction appearing when 10 µg/ml RNA were used (Figure 5d). In all experiments, neither the period of time DCs were exposed to antigen nor the time point of IL-2 addition to the effector cells during coculture had influence on the effects described.

# Discussion

In this study, we generated CIK cells from various HLA non-matched healthy donors that exhibited considerable cytotoxicity against two of three cell lines tested (HL-60 and K-562) and allogeneic cytotoxic activity against blast cells derived from three AML patients. We could enhance the capacity of these CIK cells to lyse the patients' leukemia cells using autologous, peripheral blood monocyte-derived DCs for coincubation with the effector cells, whereas no increase in activity could be observed against all three cell lines. Neither employment of blast cell lysate nor leukemic cell-derived total RNA for pulsing the DCs led to a further increment of target cell lysis. On the contrary, either antigens mostly reduced or even reversed the cytotoxic activity of the effector cell population.

## Antileukemic activity of CIK cells

The effector cell population generated for our experiments, termed cytokine-induced killer cells (CIK), consists of up to 15% $CD3^+CD56^+$ cells which are rarely found in the peripheral blood. CIK cells have been shown to exhibit enhanced cytotoxic activity and to proliferate more rapidly than LAK cells [35], [39], [40]. As compared with other lymphocyte populations used for cytotoxicity studies with patients' AML cells, CIK cells showed basic lytic activity that was considerably high (8.1%, 18.7% and 29.4%) at comparably low E:T ratios (20:1). For example, LAK cells from healthy donors and patients with AML showed <15% mean target cell lysis against autologous and allogeneic myeloblastic leukemia cells at an E:T ratio of 60:1, and unactivated control lymphocytes lysed <5% of the same targets [36]. This discrepancy between the distinct lymphocyte populations was confirmed by our own studies. It apparently depends, on the one hand, on the amount of $CD56^+$ – i.e., non-MHC-restricted – cells within the respective population. On the other hand, although CIK cells appeared to be more effective than LAK cells lysing one representative patient's AML blasts in our experiments, natural killer (NK) cell-sensitive K-562 cells were lysed to a lower extent (18.6%) as compared to approximately 50% of K-562 cells lysed by LAK cells at an E:T ratio of 20:1 [41]. Hence, the high percentage of MHC-restricted $CD8^+$ cells and their activation by cytokines must also have decisive impact on the lytic capacity of the whole effector cell population. This presumption is supported by the fact that the cytotoxic activity of CIK cells can be partially abrogated by blockage of MHC class I molecules on DCs with monoclonal antibodies (Märten, unpublished data). Recent investigations and the results presented here demonstrate that CIK cells can be stimulated by autologous, non-pulsed monocyte-derived DCs to exhibit significantly increased lytic activity against various malignant cells such as pancreatic and colon carcinoma cell lines, colon carcinoma cells derived from patients [42] and patients' AML cells. This activation depends on the release of IL-12 by DCs upon coculture and on direct cell-cell interactions during the coincubation period, as coculture of DCs and effector cells performed with cell culture inserts does not enhance CIK cell cytotoxic activity [43]. The reasons why AML cell lines used in our experiments did not undergo enhanced lysis by effector cells cocultured with DCs remain unclear.

## Reversal of DC-mediated effector cell activity

To explain the reduction in the DC-mediated cytotoxic effector cell activity against AML cells after pulsing of the DCs with unfractionated antigens, inhibitory or toxic effects on the DCs of (parts of) the antigens have to be assumed. Although we shortened the time of DC-antigen contact to 4 hrs in some experiments, the impairment of cytotoxic activity persisted. Obviously, even this period commonly chosen for antigen exposure seems to be sufficient for critical components to exhibit inhibitory or toxic effects. However, we could not detect morphological, significant numerical, or phenotypic alterations of the DCs after antigen exposure (such as down-regulation of

MHC class I and II or CD80, CD83 and CD86 expression). In any case, a loss of DC function may not explain the reversal of target cell lysis to a value below the control levels (effector cells not coincubated with DCs). Interestingly, Schui et al. [44] also observed the mediation of inhibitory effects on cytotoxic T lymphocytes by blast cell lysates from some of their AML patients used for pulsing autologous DCs. They presumed undetermined inhibitory cytokines or TGF-β to be possibly responsible for this effect. This may also explain our observations in the allogeneic system when blast cell lysates were used. The reversal of target cell lysis after coculture with RNA-pulsed DCs, however, needs further reflection. For toxic effects of the transfectant could be excluded, we have to postulate, on the one hand, the MHC I-restricted presentation of inhibitory peptides by the DCs after blast cell RNA inoculation and transcription. Our results implicate that AML cell lysates and RNA effects may have to be critically distinguished from unfractionated antigens generated from solid tumours.

Furthermore, the exhibition of immune escape mechanisms by a tumour (such as downregulation of MHC molecules or the lack of costimulatory molecules) helps it to evade the host's immune response and failure of tumour cell recognition by T cells may occur when prior exposition to tumour-associated antigens happened in the absence of costimulatory factors (such as DCs expressing CD80 and CD86 on their surface) [45]. The use of DCs in our experimental setting helped us to overcome this mechanism. But, a lack of helper effects may also cause the ignorance of antigens [46]. Grohmann and colleagues demonstrated that even presentation by DCs of a tumour-associated antigen and 'self-protein' (P815AB from murine mastocytoma cells) led to a transient and sustained state of functional T cell non-responsiveness, partially contributed to by a considerable decrease of IFN-γ production by $CD8^+$ cells [47]. Hence, another reason for our observations may be the induction of T cell unresponsiveness. The underlying mechanisms of T cell tolerance range from ignorance of the antigen to anergy and clonal T cell deletion [48], [49], [50], [51]. We can speculate that in our experiments the use of whole blast cell protein or RNA may have led to effector cell tolerance and loss of target cell detection, for unfractionated AML cell extracts may consist of proteins and sequences recognized as 'self-antigens' besides some leukemia-associated antigens.

Concerning T cell unresponsiveness, IL-2 addition to the cocultured T cells has been shown to be critical. On the one hand, the stimulation of $T_H1$ cells with high doses of IL-2 has been demonstrated to induce a refractory period during which the cells could not be stimulated by neither antigen nor antigen-presenting cells in a murine model [51]. Taking this into account, we varied the time point of IL-2 addition to the T cells after coculture in order to avoid early IL-2 receptor occupancy during antigen presentation, but no influence on the effector cell activity could be detected. On the other hand, high concentrations (300–1,000 U/mL) of the cytokine have been reported to prevent and reverse antigen-induced unresponsiveness in human T lymphocytes [52].

We and others have looked at the percentage of NKT cells and Tregs in CIK cell populations. Tregs can be found in 0–10%, NKT cells in up to 50% of CIK cell cultures [40], [53], [54]. Interestingly, dendritic cells reduce number and function of CD4+CD25+ cells in cytokine-induced killer cells [55].

It may be that some AML samples may be even able to anergize DC activation by soluble factors. In future it may be fruitful to identify such soluble factors.

## Impact on clinical projects

We conclude that monocyte-derived DCs may be useful in – allogeneic or autologous – vaccine strategies for the treatment of AML as they can easily be generated from PBMC in sufficient numbers and as we were able to demonstrate their capacity to stimulate the cytotoxic activity of lymphocytes against myelogenous leukemic blasts. We achieved a target cell lysis comparable to that obtained with leukemic blast-derived DCs and CTLs [56], [57]. In contrast, according to our experiments the employment of unfractionated leukemia-antigens for arming the DCs reduced cytotoxic effector cell activity and may not be feasible for vaccine strategies in the treatment of AML. Lysate concentrations and RNA quantities usually used for DC-based cancer vaccines in solid tumours [24], [29] showed an inhibitory effect on the cytotoxic lymphocytes. Our data emphasize the necessity to search for leukemia-associated and leukemia-specific antigens and their employment in future therapeutical strategies. For instance, minor histocompatibility antigens HA-1 and HA-2 have been shown to be leukemia-associated (rather than leukemia-specific) antigens to which HLA-identical T cell responses could be induced by Mutis and colleagues [58]. Maximal AML cell lysis could be achieved by using peripheral blood-derived DCs pulsed with synthetic HA-1 and HA-2 to prime autologous HLA-A*0201-restricted CTLs against leukemic cells from AML (and acute lymphoblastic leukemia (ALL)) patients [31].

Our data are in accordance with observations from other groups. PBSCs obtained for autologous stem cell transplantation can constitute a novel source of MDCs to design feasible vaccination trials [59], [60]. DC-based vaccines against AML could be improved by CD25 T cell depletion allowing the induction of a long-lasting T cell response [61]. Dendritic cells pulsed or fused with AML cellular antigen provided comparable in vivo antitumour protective responses [62], [63].

Furthermore, adoptive immunotherapy strategies using infusions of either autologous or allogeneic, *ex vivo* generated cytotoxic effector cells according to our protocol could be a therapeutical option. Prior coincubation and/or simultaneous vaccination with non-pulsed DCs or with DCs stimulated with defined leukemia-associated antigens may help to optimize the antileukemic effect.

# Notes

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## Competing interests

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