Immunotherapy for patients with acute myeloid leukemia using autologous dendritic cells generated from leukemic blasts

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Abstract. Vaccination with dendritic cells (DCs) as professional antigen presenting cells generated from autologous leukemic blasts might elicit anti-leukemic T cell responses in patients with acute myeloid leukemia (AML). To test this hypothesis, autologous AML-DC were generated under good manu-facturing practice (GMP) conditions and injected s.c. into five AML patients up to four times at a biweekly interval. No severe adverse side effects were observed. Three patients remained in a stable condition for 5.5-13 months and two patients died from rapidly progressive AML. Compared to the initial T cell frequency, enzyme linked immunosorbent spot (ELISPOT) assays revealed a significant increase of granzyme B releasing CD8^{+} T cells specifically recognizing the PRAME derived peptide (ALYVDSLFFL), a leukemia associated antigen expressed by AML blasts. The cytokine levels in the serum of vaccination AML patients as assessed by cytokine bead assay changed over the period of vaccination to an elevated type 1 T helper cell pattern. Interferon γ production by CD4^{+} T helper cells increased during vaccination. In summary, we demonstrated that autologous AML-DC vaccination is well tolerated and can result in an enhanced and specific response of cytotoxic T cells in AML patients.

Introduction

Acute myeloid leukemia (AML) is a hematological disease characterized by the clonal proliferation of undifferentiated

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myeloid progenitor cells. The prognosis for AML patients is rather poor with a overall survival of 20-25% depending on the individual risk profile (1). In a fervent need for novel treatment modalities, immunotherapies might constitute a therapeutic option in addition to chemotherapy.

A new hope for efficient immunotherapeutic approaches has emerged after the characterization of dendritic cells (DCs) as the most professional antigen presenting cells with a unique property to prime naive T cells (2). DCs are considered to be important elements also in the induction of specific anti-tumor immune responses, as they have a unique capacity to activate a broad range of immune effector cells including T cells, B cells, NKT cells and NK cells (2,3).

Recently, we have reported on the administration of tumor cell lysate or apoptotic body loaded DCs to B-cell chronic lymphocytic leukemia patients that led to immunological and even clinical responses (4). Several groups showed that DCs can be generated from the leukemic blasts of patients with AML or CML (5,6). Choudhury *et al* (7) and our group (8,9) demonstrated that such autologous AML-DCs can stimulate anti-leukemic T cell responses. At present, leukemia is the only known malignancy in which DCs can be generated directly from the cancer cells. AML-DCs constitutively express tumor/leukemia associated antigens (TAA/LAA) such as the antigen preferentially expressed in melanoma (PRAME) (10), the Wilms tumor gene 1 (WT-1) (11) or the receptor for hyaluronic acid mediated motility (RHAMM/CD168) (8). Vaccination with AML-DCs has been suggested as a novel treatment option for AML patients by others (12-16). We report here on the clinical and immunological results of the first clinical vaccination trial with AML-DCs.

Materials and methods

Cell samples. Three patients were treated at the University Hospital of Ulm (Germany) and two patients at the Medical University of Lublin (Poland) in the framework of our AML-DC vaccination protocol approved by the local ethics committees. Informed consent was obtained from all patients. All patients were treated with AML-DCs second or third line in a palliative setting. The clinical characteristics of the patients in this study are summarized in Table I.

Table I. Clinical characteristics of patients.

| Patient no. | Initials | Age (years) | Sex | FAB type | WBC (G/L) | | No. of DC vaccinations |
					Before	After	
1	S.S.	71	M	M2	1.9	4.9	4
2	M.R.	71	F	M0	29.5	NA[a]	1
3	T.L.	67	M	Sec AML	6	NA[a]	1
4	L.H.	70	F	M2	2.7	1.2	4
5	B.K.	54	F	M4	3.1	2.4	4

[a]NA, not available, as the patient died after the first vaccination. Sec AML, secondary AML from myelodysplastic syndrome (MDS). FAB type, type of AML according to the French-American-British classification. Before/after, before/after four vaccinations with AML-DC.

Culture of cell lines. Human cell lines were cultured in a standard medium consisting of RPMI-1640 (Biochrom, Berlin, Germany) supplemented with 10% (v/v) AB serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 units/ml strepto-mycin. T2 cells and the K562 cell line were obtained from the 'Deutsche Sammlung von Zellen und Mikroorganismen' (DSZM, Braunschweig, Germany).

Cell isolation. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation. The viability of obtained PBMCs was always >95%, as determined by trypan blue staining. The viable cells were quantified in a Neubauer chamber (Zeiss, Oberkochen, Germany) and stored for RNA preparation and flow cytometric analysis in liquid nitrogen.

mRNA preparation, reverse transcription and reverse transcriptase polymerase chain reaction (RT-PCR). For the isolation of mRNA from the peripheral blood mononuclear cells (PBMC) isolated by Ficoll density gradient centrifugation, the μMACS mRNA isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) was used according to the manufacturer's instructions. Fifty ng of mRNA was reverse transcribed into 20 μl of cDNA using a First Strand cDNA synthesis kit for RT-PCR (AMV) (Roche Diagnostics, Mannheim, Germany). The expression of PRAME was determined as described previously (8).

Generation of AML-DCs. Patients underwent a single 3-h leukapheresis using a COBE Spectra cell separator (Gambro, Lakewood, USA), 5 to 70.8x10^9 peripheral blood mono-nuclear cells (PBMC) were harvested. DCs were generated from leukapheresis products and cryopreserved as previously described (8,9). Cells were seeded (1x10^6 cells/cm²) into culture flasks (Nunc, Roslild, Denmark) in RPMI-1640 medium (Biochrom AG, Berlin, Germany) supplemented with 2% human AB serum (German Red Cross Blood Center, Ulm, Germany), 2 mM L-glutamine, 100 units/ml penicillin and 100 units/ml streptomycin. After 2 h of incubation at 37˚C, non-adherent cells were removed and adherent blood monocytes (purity >80% of CD14+ cells by FACS analysis) were cultured in RPMI-1640 medium supplemented with 100 ng/ml human granulocyte-macrophage colony stimulating factor (GM-CSF, Leukomax, Novartis, Basel, Switzerland),

1,000 IU/ml interleukin-4 (Strathmann, Hannover, Germany) and 10% human AB serum. For maturation of the cells, a complete medium change was performed on day 6. The new medium contained GM-CSF and IL-4 in the concentrations mentioned above and additionally 50 ng/ml TNF-α (Strathmann). The AML-DC cultures were fed with fresh medium and cytokines every three days and cell differentiation was monitored using inverse light microscopy. The expression of cell surface molecules on the DCs was analyzed using flow cytometry after 8 days of culture.

Cell viability and morphologic studies. The viability of the DCs harvested after 8 days of culture was assessed by trypan blue staining using a Neubauer counting chamber, and the morphology of the cells was analyzed by light microscopy. For light microscopy, DC suspensions were spinned and stained with May-Gruenewald solution (Merck, Darmstadt, Germany).

Immunophenotyping of the cells. Harvested cells were washed in FACS medium [phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA)] and stained at 4˚C for 20 min by antibodies directly conjugated with Fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Thereafter, cells were washed three times with PBS and analyzed by FACScan (Becton-Dickinson, Heidelberg, Germany) using CellQuest™ software (Becton-Dickinson). Antibodies were the following: FITC-labeled anti-mouse IgG, anti-human HLA-DR, CD40 and CD83, as well as PE-labeled anti-mouse IgG, anti-human HLA-ABC, CD80 and CD86 (Becton-Dickinson).

Vaccination protocol. Eligible patients received up to 4 injections with 5x10^6 AML-DCs at a biweekly interval. The vaccine was administered s.c. in the vicinity of the inguinal lymph nodes. The treatment schedule is displayed in Fig. 1. Clinical examination, hematological blood testing and immunological screening were performed before each vaccination.

Assessment of intracellular interferon gamma (IFN-γ). The expression of intracellular IFN-γ by CD4 and CD8 positive cells was assessed by three color flow-cytometry. PBMC were stained with anti-CD4*PE and anti-CD8* peridinin chlorophyll protein (PerCP) moAbs (Becton-Dickinson). Following membrane staining, the cells were fixed and per-meabilized according to the manufacturer's protocol using

the IntraStain kit (DakoCytomation, Glostrup, Denmark) and simultaneously stained with anti-IFN-γ FITC-labeled moAbs (Becton-Dickinson). Thereafter, the cells were washed twice, acquired using a FACScan flow cytometer and analyzed using CellQuest software. Relevant mouse isotype controls were used for every experiment. At least 10,000 events gated on lymphocytes were collected.

Assessment of cytokine concentrations. The serum level of IL-2, IL-4, IL-5, IL-10, tumor necrosis factor-α (TNF-α) and IFN-γ in the PB was measured by the human Th1/Th2 cyto-kine cytometric bead array (CBA) (Becton-Dickinson) using a FACScan flow cytometer equipped with CellQuest software (Becton-Dickinson). The patients' plasma samples were frozen and stored at -192˚C in liquid nitrogen. Immediately before the experiments, samples were thawed and assessed for the concentration of cytokines according to the manufacturer's protocol.

Mixed lymphocyte peptide culture (MLPC) and IFN-γ and granzyme B ELISPOT assays. PBMCs from AML patients were separated by Ficoll and subsequently selected by CD8 magnetic beads through a MACS column (Miltenyi Biotec). Eight days of mixed lymphocyte peptide culture (MLPC) with PRAME- (pos. 300-309: ALYVDSLFFL) and IMP-derived peptides (pos. 58-66: GILGFVFTL) were followed by granzyme B ELISPOT assays as described previously (9,17).

Results

Quality control of the vaccine preparations. AML blasts were obtained by leukapheresis. After eight days of DC culture as described in Materials and methods, floating and semi-adherent
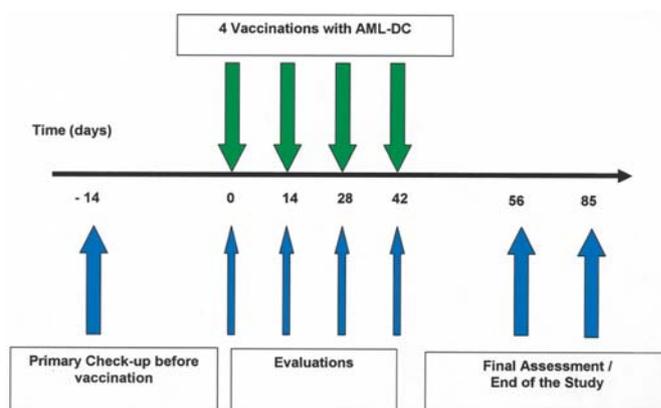
Figure 1. Schedule of clinical vaccinations with AML-DCs. Patients received up to 4 injections with $5x10^6$ AML-DCs at a biweekly interval. The AML-DCs were administered s.c. in the vicinity of inguinal lymph nodes. Blood samples were taken before every vaccination and on days +56 and +85.
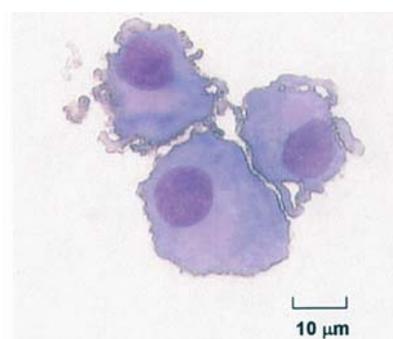
Figure 2. DCs generated from AML blasts visualized by light microscopy. DCs generated from AML blasts and visualized by light microscopy (x400) display typical dendritic veils and excentric nuclei.
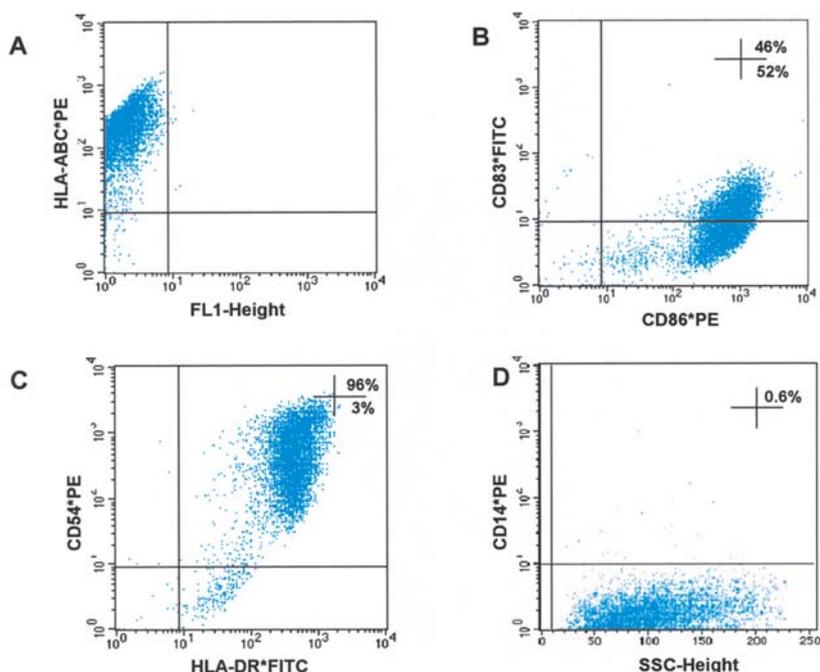
Figure 3. Phenotype of DCs generated from AML blasts. Representative results obtained by flow cytometric analysis of AML-DC vaccine preparations. Cells displayed a DC phenotype with high HLA-ABC expression (A), high expression of CD86+ with coexpression of CD83+ (B). Generated AML-DCs were clearly double-positive for HLA-DR+ and CD54+ (C), but did not express CD14 (D). For more details see Materials and methods, the percentage of positive cells is given in the respective panels, indicating the maturation of DCs from monocytic cells.

A Patient # 4

B Patient # 5
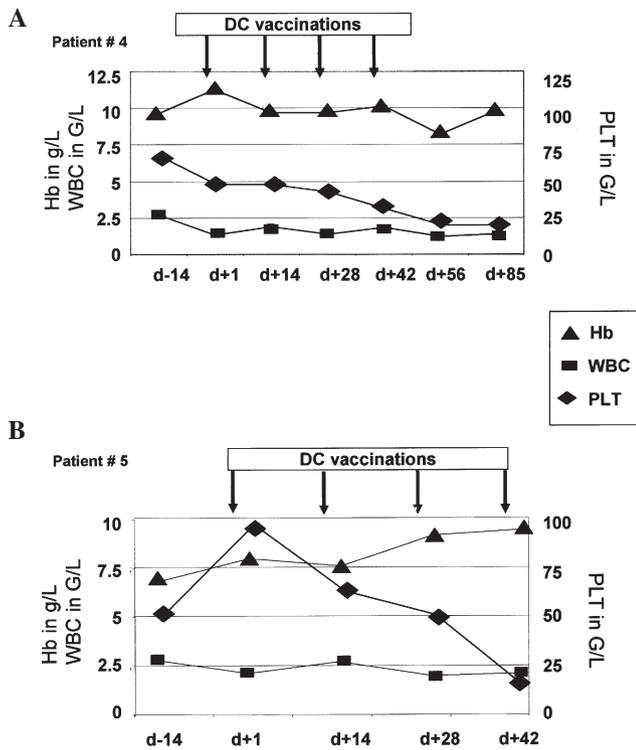
Figure 4. Clinical course of peripheral blood counts during AML-DC immunotherapy. Changes in the white blood cell (WBC) count in G/L (closed squares), hemoglobin (Hb) in g/L (closed triangles) and platelet (PLT) counts in G/L (closed diamonds) during AML-DC treatment. Stable blood parameters values were observed in all patients. A, results from patient no. 4; and B, from patient no. 5.

A Patient # 4
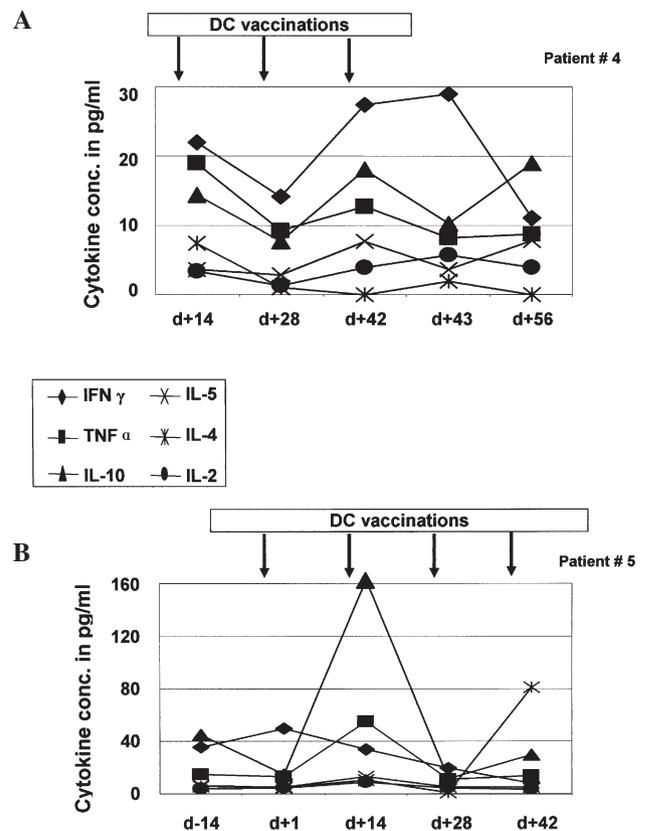
B Patient # 5

Figure 5. Changes in serum levels during AML-DC treatment as assessed by cytokine bead assay. Concentrations (pg/ml) of IFN-γ, TNF-α, IL-10, IL-5, IL-4 and IL-2 in the serum of patient no. 4 (A) and patient no. 5 (B) during AML-DC vaccination.

A Patient # 4
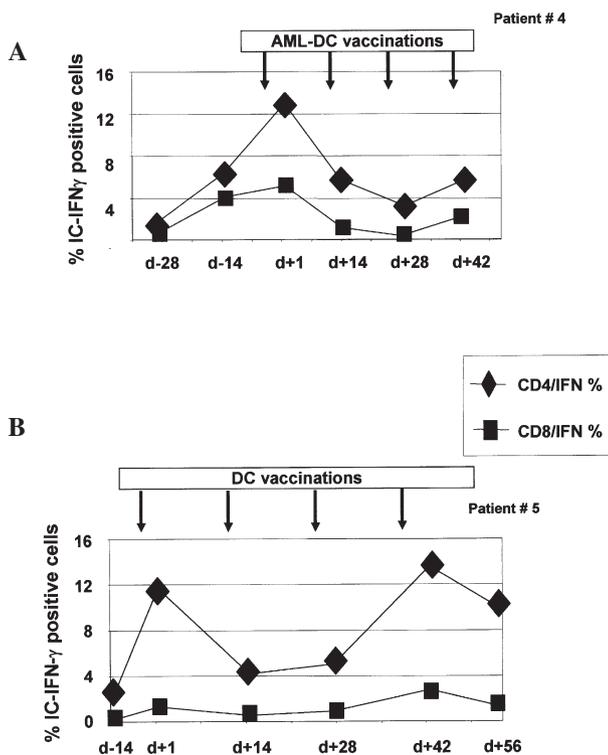
B Patient # 5

Figure 6. Changes in the intracellular IFN-γ level of CD4+ and CD8+ cells during AML-DC vaccination. Percentages of intracellular IFN-γ positive CD4+ (diamonds) and CD8+ (squares) at the indicated time-points in patient no. 4 (A) and patient no. 5 (B) as assessed by FACS analysis. While IFN-γ levels in CD8+ T cells remained rather stable, an elevation of the IFN-γ levels in CD4+ T cells was observed.

cells generated from AML blasts (AML-DC) showed typical DC morphology with characteristic veils, an excentric nucleus and a diameter of 15-20 μm. A typical microphotograph of AML-DCs is shown in Fig. 2. AML-DC vaccine preparations were highly enriched with mature DCs. The mean viability of generated DCs was 93% (SD ± 6%), the mean yield of viable DCs was 32×10^6 (SD ± 18×10^6; minimum, 9.3×10^6; maximum, 87.2×10^6) cells. Fig. 3 shows a typical immunophenotype of DC generated from the AML patients by FACS analysis. The DC highly expressed CD54, CD83, CD86, HLA-DR and HLA-ABC; expression of CD14 was absent. AML-DC vaccines were checked for the existence of TAA detected before preparation on the AML blast to confirm the blast origin of AML-DC (data not shown).

Clinical responses. Three of five patients completed the treatment with four AML-DC vaccinations. One patient lived for 13 months after 4 vaccinations with a stable WBC count and without peripheral blasts but eventually died from pneumonia. Two patients received a single AML-DC vaccination and died thereafter due to intracranial hemorrhage. These serious adverse effects were most likely due to the progression of the underlying disease. Thrombocytopenia in these patients was preexisting. No anti-platelet antibodies could be detected or occurred after vaccination.

The fourth patient showed a drop of blasts from 8% to 0% in the peripheral blood over the period of AML-DC therapy. One month after four vaccinations, blasts reappeared (6%). The
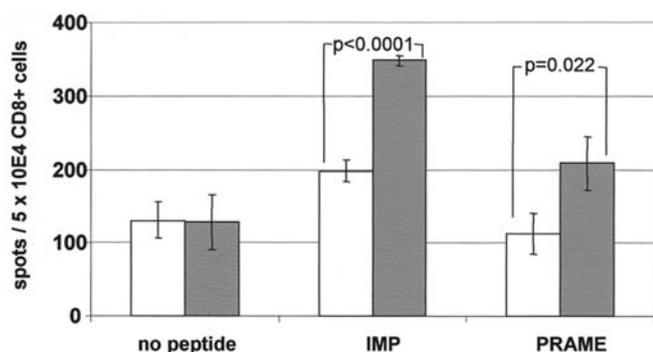
Figure 7. Activation of specific T lymphocytes after vaccination with AML-DCs. Enhanced specific CD8+ T cell responses against PRAME after vaccination with AML-DCs were assessed using an ELISPOT assay for granzyme B. Numbers stand for the mean value of spots per 50,000 CD8+ cells in triplicate, error bars indicate the standard deviation. Before (white bars) and after four vaccinations (black bars), CD8+ T cells of the peripheral blood were evaluated for their specific secretion of granzyme B in ELISPOT assays against T2 cells pulsed with a PRAME-derived peptide, an influenza matrix protein (IMP)-derived peptide as a positive control or without peptide as a negative control. After vaccination with AML-DC, a significant increase of CD8+ T cells recognizing the PRAME-peptide was observed, indicating an enhancement of the anti-leukemic T cell response. P-values were calculated using the unpaired t-test.

disease progressed and the patient died from sepsis 6 months after his first AML-DC vaccination. The fifth patient developed a stable WBC count during immunotherapy. After the last vaccination, the peripheral leukemic blast count initially dropped from 20% to 0%. However, one month after the last AML-DC vaccination, blasts reappeared in the PB of this patient. Low dose Ara-C (2x20 mg s.c.) was started as palliative treatment but, 5.5 months after his first AML-DC vaccination, the patient died in the phase of aplasia due to gastrointestinal hemorrhage. During immunotherapy, a stable WBC count was observed (Fig. 4).

Toxicity. All vaccinations were well tolerated and no severe side effects were associated with the s.c. injections of AML-DCs. Patients developed a mild local reaction (erythema, induration, pruritus) at the vaccination site which, in all cases, resolved within a few days. Renal function and liver function were not impaired. No signs of autoimmunity were detected as measured by rheumatoid factor, C-reactive protein and anti-nuclear antibodies.

Immunological response and Th1/Th2 response. The levels of IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ were measured by FACS analysis as described in Materials and methods. For patients 4 and 5, we observed a wide fluctuation of Th1/Th2 cytokine secretion during the AML-DC based immunotherapy (Fig. 5). To specify the IFN-γ secreting cells, intracellular FACS staining was performed. After vaccination, intracellular levels of IFN-γ in CD4 were 2- to 4-fold higher than before treatment (Fig. 6). Interestingly, after the 4th vaccination, an increase of IFN-γ in patient no. 4 was noticed and, in patient no. 5, there was a peak of IL-4 which correlated with a drop of blasts in the PB. In patient no. 5, an 8-fold increase of IL-10 and a 4-fold elevation of TNF-α was observed in the serum.

Specific cellular immune responses. To demonstrate a leukemia-specific T cell immune response, ELISPOT assays for CD8+ T lymphocytes specifically reactive against the antigen, PRAME, were performed, a TAA which is expressed on AML-blasts, but not on cells of the nomal hematopoiesis. T cell reactivity to an influenza matrix protein peptide served as a positive control, an assay against unloaded CD8 negative antigen presenting cells as a negative control. The assays clearly demonstrate a general increase of T cell activation as measured by IMP specific T cells, but also a significant (p=0.022) increase of anti-leukemic T cell response as measured by PRAME specific T cell frequencies.

ELISPOT measuring the release of granzyme B was used to detect the reactivity of PRAME derived peptide pre-sensitized CD8+ T cells before and after vaccination. After vaccination, an increased granzyme B secretion by T cell reacting against T2 target cells pulsed with a PRAME epitope peptide was observed (Fig. 7).

Discussion

The donor lymphocyte infusion (DLI) and graft versus leukemia (GVL) effects suggest the existence of immunogenic tumor associated antigens in AML patients (18). Several clinical TAA peptide vaccination trials for AML patients have entered phase I/II studies, including the RHAMM/CD168 peptide R3 vaccination trial initiated at our institution. After vaccination with a Wilms tumor gene 1 (WT-1) peptide, encouraging results and even complete remissions were observed in AML patients (19,20).

We demonstrated previously that DCs generated from leukemic blasts express costimulatory molecules (CD40, CD80, CD86), HLA class I and II molecules, and TAA, thus indicating the potential of AML blasts to act as immune targets (9,21). Several other groups have detected the potency of DC derived from blasts to induce specific immune responses against TAA *in vitro* (7-9,12-15). Moreover, vaccination with irradiated AML cells transfected with B7.1 or GM-CSF was efficient *in vivo* to reject established leukemia in a murine AML model (22).

The general safety of DC vaccination has been proven in clinical trials for patients with several tumor entities, such as B-cell lymphoma, renal cell carcinoma, melanoma and prostate cancer (23-28). In AML patients, DCs pulsed with tumor lysates were shown to be safe and effective in eliciting an immunological response but no clinical benefit was demonstrated (29).

In the present study, we demonstrate that clinical vaccination is feasible and safe in patients with AML. Three of our five patients who completed a course of vaccination were subjected to functional T cell assays. In one patient, results obtained by ELISPOT assay indicate an increased granzyme B production to target T2 cells pulsed with a PRAME derived peptide. The lysis of tumor cells by activated IFN-γ producing T lymphocytes through granzyme B plays a main role in tumor rejection (30). In two other patients, the intracellular IFN-γ production increased during and after the DC vaccination and could be assigned to CD4+ cells rather than CD8+ T cells with higher levels of IFN-γ production in CD4+ cells. Activation of CD4+ cells plays an important role in anti-tumor immunity,

as CD4+ T helper cells are able to provide regulatory signals required for the priming of MHC I restricted CD8+ T cells, which are crucial effector cells in tumor rejection (31). Our data underline the importance of CD4+ T cells in the anti-leukemic action of the immune system.

The cytokine response shows fluctuations during therapy with some interesting findings; both types of cytokines Th1 and Th2 were noted in elevated levels and, in patient no. 5, Th1 response was revealed to be more pronounced. In patient no. 4, the increased IFN-γ production was maintained for one day after vaccination (Fig. 5). Interestingly, the boost of IL-4 correlated with a drop of blasts in the peripheral blood of patient no. 5 (Fig. 5B).

In this study, we demonstrated immunological responses in AML patients treated with AML-DC vaccine. An increased specific CTL response against TAA PRAME was observed after four vaccinations.

Our clinical approach of vaccination with autologous AML-DCs is neither restricted to any HLA type nor limited to any particular TAA expressed on leukemic blasts since most of the evaluable TAA/LAA are maintained or even upregulated during DC generation, as we have reported previously (8,9). AML-DC developed the potential to activate both CD4+ and CD8+ cells and to induce both Th1 and Th2 responses. We also recently described the expression of toll-like receptors on AML blasts as prerequisite for CpG action (Li L, *et al*, Blood, ASH abs. no. 1812, 104, 2004). Therefore, as a future perspective, AML patients might benefit from AML-DC vaccinations enhanced by powerful adjuvants such as keyhole limpet hemocyanin (KLH) or CG rich oligo-nucleotides (CpG-ODNs) (32).

Acknowledgements

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