Autologous peptides eluted from acute myeloid leukemia cells can be used to generate specific antileukemic CD4 helper and CD8 cytotoxic T lymphocyte responses *in vitro*

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Background and Objectives. The poor prognosis of acute myeloid leukemia (AML) treated with conventional chemotherapy justifies seeking additional immunotherapeutic approaches to eliminate minimal residual disease. Hence, we evaluated the feasibility of generating *in vitro* antileukemic immune responses, which would bypass the need for epitope identification and rely on antigen presentation by autologous dendritic cells.

Design and Methods. Naturally processed peptides were extracted by acid elution from circulating AML cells of six patients at diagnosis. Mature dendritic cells (mDC) were derived from autologous monocytes obtained when the patients were in complete remission, and were loaded with the pool of eluted peptides to stimulate autologous T lymphocytes *in vitro*.

Results. We were able to induce *in vitro* antileukemic Th1 responses characterized by $CD4^+$ T-cell proliferation, significant interferon-γ secretion by both $CD4^+$ and $CD8^+$ T lymphocytes by recognition of autologous AML cells and generation of cytotoxic $CD8^+$ T lymphocytes.

Interpretation and Conclusions. These results demonstrate that naturally processed peptides eluted from AML cells and presented by differentiated autologous mDC could be immunogenic *in vitro*. Although more *in vitro* data will be needed to check the safety of such an approach, notably to rule out possible autoimmune adverse effects, these results lay the basis for a potentially effective antileukemic immunotherapy for high-risk AML patients with minimal residual disease.

Key words: acute myeloid leukemia, antileukemic immune response, dendritic cells, eluted peptides.

Haematologica 2005; 90:1050-1062

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Acute myeloid leukemia (AML) is a malignant hematopoietic disorder characterized by a clonal proliferation of immature myeloid precursors. Although most adult patients achieve a first complete remission with current chemotherapeutic protocols, long-term survival is still uncertain for half of the patients. The main cause of mortality is the recurrence of leukemia due to persistence of minimal residual disease. To prevent relapse and to improve overall survival, many immunotherapeutic approaches have been explored, their success relying upon the ability to generate specific antitumor immune responses. Allogenic hematopoietic stem cell transplantation is a clear example of the capacity of the human immune system to eliminate cancer cells and especially hematologic malignancies. This activity, called the graft-*versus*-leukemia (GvL) effect, is the first evidence that leukemic cells are susceptible to cytotoxicity mediated by activated T lymphocytes. However, the GvL effect in this context is mediated by the recognition of alloantigens in recipients' cells and thus, this effect is not expected in the autologous setting. A therapeutic vaccine strategy using immunogenic peptides derived from tumor-associated antigens (TAA) is a classical immunotherapeutic approach but generally relies on the identification of antigens expressed by solid tumor cells. Recently, studies of AML-associated antigens have shown that some junctional peptides derived from chimeric proteins PML-RARα, DEK-CAN, and BCR-ABL encoded by frequent chromosomic translocations, t(15;17), t(6;9), and t(9;22), respectively, are immunogenic and thus can be considered tumor-specific

neoantigens.[1-3] However their expression is restricted to only a few subsets of AML patients. PRAME, an ubiquitous TAA, is also expressed in AML cells and is a potential target for the immune response.[4] Normal proteins over-expressed in leukemia may also provide targets for immunotherapy. Two proteins, proteinase 3 (PR3), and Wilms' tumor suppressor (WT1) have already been shown to elicit cytotoxic T lymphocyte (CTL) responses specific to AML cells.[5-7] Injection of *in vitro*-induced WT1-specific CTL can prevent the progression of leukemia in a murine model.[8] These studies, providing evidence for the possibility of inducing antileukemic responses, support the feasibility of immunotherapy, and may lead to the development of peptide vaccination protocols. However, these approaches are limited by the human leukocyte antigens (HLA) restriction of peptide presentation and by the variability of TAA expression among leukemic patients. Indeed, AML encompasses a wide range of heterogeneous diseases with differences in the stages of differentiation arrest, and in the oncogenic processes involved. Thus, methods using autologous tumor cells have been investigated in order to bypass the problems of TAA identification and HLA polymorphism.

Naturally processed peptides, presented by HLA molecules and eluted by acid treatment have been previously used in a melanoma murine model. It has been demonstrated that immunization of animals with dendritic cells (DC) pulsed with the pool of acid-eluted peptides extracted from tumor cells can induce rejection of an established melanoma.[9] In humans, vaccination of patients suffering from malignant glioma with eluted peptide-pulsed DC elicits systemic cytotoxic responses and intracranial T-cell infiltrations.[10] In AML patients, such an approach, using autologous DC loaded with the pool of naturally processed peptides eluted from leukemic cells, has never been tested.

We previously showed in one patient that, in a partially HLA-compatible allogenic situation, the pool of naturally processed peptides eluted from AML cells was able to induce an antileukemic HLA-restricted T-cell response.[11] In the present work, we evaluated in six patients with various AML subtypes: (i) the feasibility of elution of peptides from AML cells, (ii) the potential of eluted peptides from AML cells to induce antileukemic responses in the autologous setting, (iii) the Th1/Th2 profile of T lymphocytes stimulated by eluted peptides. We demonstrated that autologous mature DC (mDC) loaded with the pool of peptides eluted from autologous AML cells at diagnosis (mDC/EP) were able to elicit an antileukemic immune response *in vitro* mediated by both $CD4^+$ and $CD8^+$ T lymphocytes for the six patients studied, and characterized by a Th1 cytokine production. These results strongly support the concept of using mDC/EP to develop a therapeutic vaccine for patients with poor prognosis AML.

Design and Methods

Patients

After informed consent, and approval of the local ethics committee, peripheral blood mononuclear cells (PBMC) were obtained from blood of six patients at diagnosis by Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden). The leukocyte layer contained a majority of leukemic cells (checked by cytological, cytogenetic, or fluorescent analysis) (*data not shown*). Some of the AML cells (100×10^6) were frozen in liquid nitrogen, and the rest were stored as a cell pellet at –80°C until used for trifluoroacetic acid (TFA) elution treatment. After patients had achieved complete remission through standard chemotherapy protocols, blood leukocytes were collected and aliquots frozen. Thawed cells were used for preparing the monocyte $CD14^+$ enriched population from which mDC were generated *in vitro*, while lymphocytes were used for repeated stimulations *in vitro*.

Elution of peptides from AML cells with TFA

Peptides were isolated from AML frozen pellets. Elutions were performed using TFA (Sigma-Aldrich, St Quentin Fallavier, France) as previously described.[12] Briefly, frozen pellets of 500×10^6 to 2×10^9 leukemic cells were resuspended in 5 to 25 mL 0.1% TFA in distilled, deionized water (ddH_2O) at room temperature. The resulting cellular lysate was centrifuged for 20 minutes at 15000 g at 4°C. Each supernatant was recovered, supplemented with TFA at 10%, and was centrifuged as previously described. The clear supernatant was loaded on a C18 SepPak column (Walters, Milford, MA, USA). Bound peptides were eluted using 2.0 mL 60% acetonitrile (Sigma-Aldrich) in ddH_2O and lyophilized. The lyophilized powder was suspended in 1.0 mL ddH_2O, and stored at – 80°C until use.

Biochemical profile of peptides eluted from AML cells

An aliquot of eluted peptides prepared as above was reconstituted in 200 µL of 0.1% TFA in ddH_2O, loaded on a column, and analyzed by reverse-phase high performance liquid chromatography (RP-HPLC) (HPLC LC200, Perkin Elmer Instruments LLC, Connecticut, USA) as previously described.[13] Briefly, an acetonitrile gradient in ddH_2O was applied containing 0.1% TFA (0% for 5 minutes, 0 to 10% for 5 minutes, 10 to 35% for 50 minutes, 35 to 60% for 10 minutes, 60 to 100% for 5 minutes, and 100 to 0% for 5 minutes). The elution profile was read at 214

nm with an absorbance detector in milli UV (Perkin Elmer Instruments LLC).

Generation of autologous mDC

mDC were generated from CD14+ monocytes, immunomagnetically enriched from PBMC obtained from patients in complete remission. CD14+ cells were incubated in DC-medium consisting of RPMI 1640 (Life Technologies Inc., Cergy Pontoise, France) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies), 500 U/mL recombinant human granulocyte macrophage colony-stimulating factor (rhGM-CSF; Leucomax, Novartis, Paris, France) and 500 U/mL recombinant human interleukin-4 (rhIL-4) (PeproTech Inc, Tebu, Le Perray en Yvelines, France) for six days at 37°C in a 5% CO_2 atmosphere. On day 3, 500 U/mL of rhGM-CSF and 500 U/mL of rhIL-4 were added. On day 6, immature DC (iDC) were matured by adding 1 μg/mL of lipopolysaccharide (LPS; from *Escherichia coli*, Calbiochem-Novabiochem Corp. La Jolla, CA, USA) for 24 hours. On day 7, mDC were incubated for 1 hour 30 minutes at 37°C in a 5% CO_2 atmosphere with peptides eluted from AML cells as indicated below. mDC were freshly generated every week.

Immunofluorescent analysis

AML cells and autologous mDC were labeled with specific anti-human monoclonal antibodies in phosphate-buffered saline (PBS; GIBCO BRL, Paisley, Scotland) supplemented with 2% FBS for 20 minutes at 4°C. Monoclonal antibodies were fluorescein isothiocyanate (FITC)-conjugated anti-HLA A,B,C, CD14, CD86, and phycoerythrin (PE)-conjugated anti-HLA-DR, CD1a, CD80 (Becton Dickinson-Pharmingen, San Diego, CA, USA), and CD83 (Immunotech, Beckman Coulter Inc., Marseille, France). Cells were also examined with corresponding isotype-matched control monoclonal antibodies (Immunotech). Samples were analyzed on a FACScalibur™ flow-cytometer with CELLQuest™ software (Becton Dickinson).

ELISA IL-12p70 assay

IL-12 secretion by iDC and mDC was assessed using the human IL-12p70 immunoassay kit according to the manufacturer's instructions (Biosource International Inc., Camarillo, CA, USA).[14] Briefly, 100 μL of standard hIL-12p70, control (standard diluent buffer alone), or samples (culture supernatant of iDC or mDC) were incubated with 100 μL of biotinylated anti-IL-12p70 solution in a 96-well microplate precoated with anti-IL-12 monoclonal antibody for 3 hours. After several washes, 100 μL of peroxidase-conjugated streptavidin were added for 30 minutes. After several washes, the presence of IL-12p70 was revealed by adding a chromogenic substrate and measured by absorbance at 450 nm with a Victor™ fluorometer (Wallac, Gaithersburg, MD, USA).

Table 1. ***In vitro*** **expanding capacity of stimulated lymphocytes after four weeks of culture (fold increase), and distribution of the CD4+ and CD8+ T cell populations (%).**

	P2	*P3*	*P4*	*P5*	*P6*
Lymphocyte increase (fold)	17.5	19.5	20	8	18
CD4+ T cells (%)	57	87	71	61	41
CD8+ T cells (%)	35	10	14	36	55

Stimulation of lymphocytes in vitro against autologous mDC/EP

PBMC (4×10^6) from patients in complete remission were seeded in culture with 4×10^5 autologous mDC pulsed with peptides eluted from 4×10^6 AML cells in 24-well culture plates (Costar, Cambridge, MA, USA) at 37°C in 5% $C0_2$, in RPMI 1640 medium supplemented with 10% Human AB serum (PAA Laboratories GmbH, Linaz, Austria). On day 4 of culture, 20 U/mL of rhIL-2 (Roche, Meylan, France) and 5 ng/mL of rhIL-7 (PeproTech Inc, Tebu) were added. Lymphocytes were maintained in culture for at least five weeks and submitted to four stimulations by freshly prepared autologous mDC/EP, and addition of cytokines every four days. On one occasion (patient P1), the fourth stimulation by autologous mDC/EP was delayed for one week. The *in vitro* expanding capacity of remission T lymphocytes after four stimulations with mDC/EP is shown in Table 1.

CD4+ and CD8+ T lymphocyte purification

CD4+ and/or CD8+ T lymphocytes were isolated from stimulated T cells after three *in vitro* mDC/EP stimulations using microbead-positive immunoselection (Miltenyi Biotec). Briefly, stimulated T cells recovered from 24 to 48-culture wells were pooled and washed three times with cold buffer (PBS supplemented with 0.5% bovine serum albumin (BSA; Sigma) and 2 mM ethylenediaminetetraacetic acid (EDTA; GIBCO BRL). Stimulated T cells were incubated with 80 μL of buffer and 20 μL of MACS CD4 or CD8 microbeads per 10^7 total cells for 15 minutes at 4°C. The purity of CD4+ and CD8+ T cells was analyzed by flow cytometry and was ≥90% for both populations after positive selection. The percentages of CD4+, and CD8+ T cells obtained after *in vitro* mDC/EP stimulations are shown in Table 1.

Interferon-γ (IFN-γ) ELISPOT assay

After four *in vitro* mDC/EP stimulations, unfractioned T cells or both CD4+ and CD8+ T-cell popula-

tions were tested for IFN-γ production in response to various targets by using an ELISPOT assay. Briefly, nitrocellulose plates (96-well) (Millipore, Bedford, MA, USA) were coated with capture mouse IgG1 anti-human IFN-γ monoclonal antibodies (1-D1K, Mabtech, Nacka, Sweden). After 20 hours of stimulated T lymphocytes and target cell co-cultures, biotinylated mouse IgG_1 anti-human IFN-γ monoclonal antibodies (7B6-1, Mabtech) were added, as previously described.[15] Spots were revealed with BCIP substrate (Sigma) in a gel overlay. Spots were counted with a computer-assisted video image analyzer (KS ELISPOT, ZEISS, Jena, Germany). A positive value was assigned to spot frequencies that were higher than the mean background of the individual assay plus two standard deviations.

CD4⁺ T-cell proliferation assay

$CD4^+$ T lymphocytes were sorted from 28-day cultured stimulated lymphocytes. The stimulated $CD4^+$ T cells (5×10^5) were co-cultured with increasing concentrations of autologous or allogenic AML cells at various effector:target (E:T) ratios for 72 h at 37°C in 5% CO_2. Cells were pulsed with 0.5 μCi [^{3}H]-thymidine for the last 18 hours of culture, and then harvested for liquid γ-scintillation counting with a 1450 Microbeta plus (Wallac).

CD8⁺ T-cell cytotoxic responses

$CD8^+$ T lymphocytes were collected from 28-day cultured stimulated lymphocytes and studied for their potential cytotoxicity against autologous and allogenic AML cells using a standard four-hour ^{51}chromium (^{51}Cr)-release cytotoxicity assay at different E:T ratios. Briefly, 40 μL of supernatant were collected, and ^{51}Cr release was measured with a γ-scintillation counter (1450 Microbeta plus, Wallace). Spontaneous and maximal releases were defined by the incubation of target cells with complete medium in the absence or presence of 10% hydrochloric acid, respectively. In all our experiments, spontaneous release was <15%, and specific lysis (%) was calculated as [(cpm experimental ^{51}Cr release - cpm spontaneous ^{51}Cr release)/(cpm maximal ^{51}Cr release – cpm spontaneous ^{51}Cr release)]×100.

Cytokine quantification in supernatants of stimulated T lymphocytes

Cytokines were quantified in culture supernatants collected at the end (7th day) of each cycle of lymphocyte stimulation with mDC/EP for patient P1, or at the end of the 28 days of lymphocyte culture after $CD4^+$ and $CD8^+$ T-cell microbead separation for patients P4 to P6. IL-2, IL-4, IL-6, IL-10, tumor necrosis factor (TNF-α) and IFN-γ were quantified by using the Cytometric Bead Array kit according to the manufacturer's manual (Becton Dickinson). IL-2 was always detected in the lymphocyte culture supernatants. However, since we could not distinguish between endogenous and added IL-2, the quantification of IL-2 was not analyzed.

Table 2. Clinical characteristics of AML patients at diagnosis and outcome.

Patient	Age at diagnosis	FAB type	Leukocytosis 10^9/L	% of circulating AML cells	Complete remission obtained	Time since last chemotherapy (Mo)§
P1	23	AML 5	58.2	82	Yes	3.5
P2	60.5	AML 1	40	61	Yes	1
P3	54.5	AML 0	15.6	70	Yes	1.5
P4	60	AML 5	92	95	Yes	1
P5	60	sec AML*	43.5	46	No†	1.5
P6	48	AML 5	32	60	Yes	3

Secondary AML following refractory anemia with excess of blasts; †complete remission of short duration, relapsed to a refractory anemia with excess of blasts; §Mo: months.

Statistical analysis

Statistical analysis was performed using Student's t test. p values < 0.05 were considered statistically significant.

Results

Surface expression of HLA class I and II molecules by AML cells, and elution of peptides

AML cells from six patients at diagnosis (patients P1 to P6) with a variety of French-American-British (FAB) subtypes and clinical histories (Table 2) were examined for their expression of HLA molecules by flow cytometry. We observed a high surface expression of both HLA class I and II molecules in the majority of cells (>75%) (Figure 1A). The RP-HPLC elution profiles of the peptides extracted from AML cells by TFA treatment obtained from the six patients at diagnosis are shown in Figure 1B. The elution profiles were heterogeneous and varied for each patient.

Phenotype, and function of in vitro generated mDC

A $CD14^+$-enriched monocyte population was purified from PBMC of patients in complete remission, and cultured for 6 days with GM-CSF and IL-4 to generate iDC. Then, iDC were differentiated by 24-hour LPS treatment to mDC. The phenotype, and function of such mDC from one representative patient are shown in Figure 2. A high percentage of mDC from this patient expressed CD1a (61%), the co-stimulatory molecules CD80 (87%) and CD86 (97%), the mature DC-associated molecules CD83 (98%), and the HLA class I (99%) and class II molecules (99.5%) (Figure 2A). The production of IL-

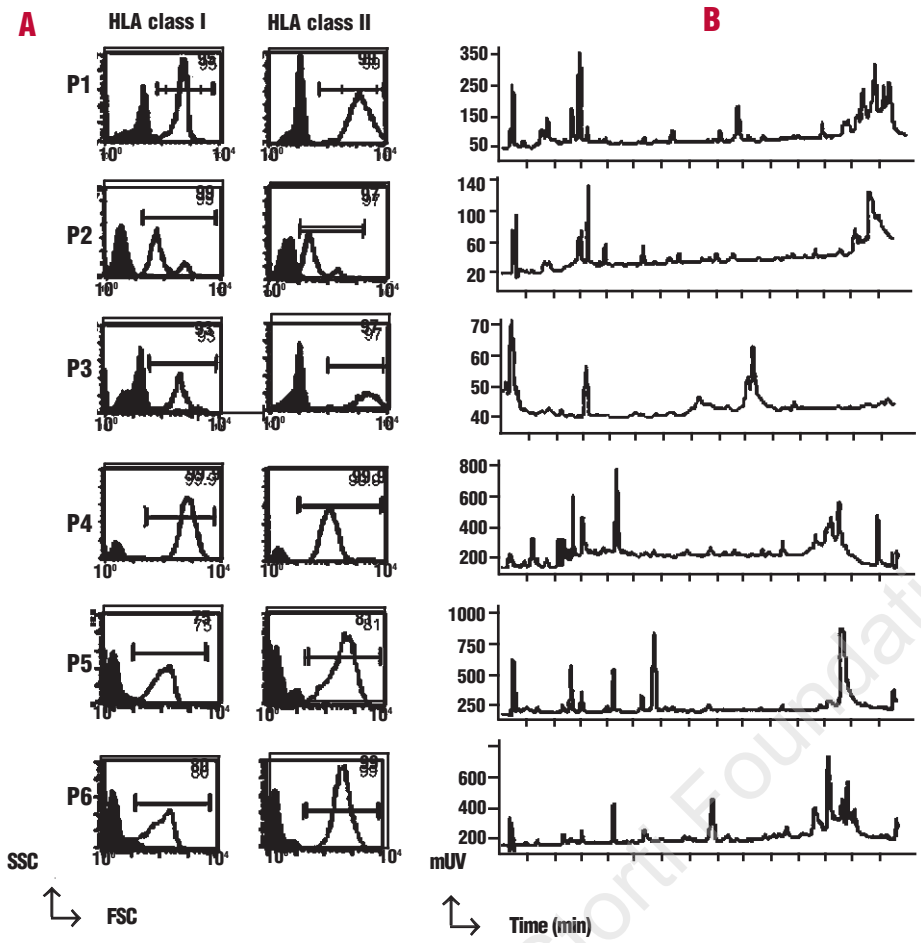


Figure 1. A. Expression of HLA class I and class II molecules on AML cells from patients P1 to P6 at diagnosis. AML cells were labeled with monoclonal antibodies as described in Design and Methods. AML cells were labelled with isotype-matched control antibodies (filled histogram) or with the HLA-specific antibodies (open histogram). The percentage of positive cells was obtained with statistical means and is indicated. B. RP-HPLC expression profile of naturally processed peptides from AML cells of patients P1 to P6 at diagnosis. Naturally processed peptides were obtained from AML cells by acid elution and were fractioned by RP-HPLC on an acetonitrile gradient for 80 minutes.

12p70 was also examined because of the crucial role of this cytokine in inducing Th1 immune responses (Figure 2B). The mDC from patient P2 secreted high levels of IL-12p70, while his iDC produced low levels of IL-12p70 ($p<0.001$). The levels of IL-12p70 secretion by mDC obtained from two additional patients were similar (*data not shown*).

Phenotypes of mDC obtained from patients P2 to P6 in complete remission were generally comparable (Table 3), except for patient P5 whose generated mDC showed a decreased expression of CD83 and CD86 molecules and persistence of a few $CD14^+$ cells. Of note, this patient relapsed soon after to refractory anemia with excess of blasts.

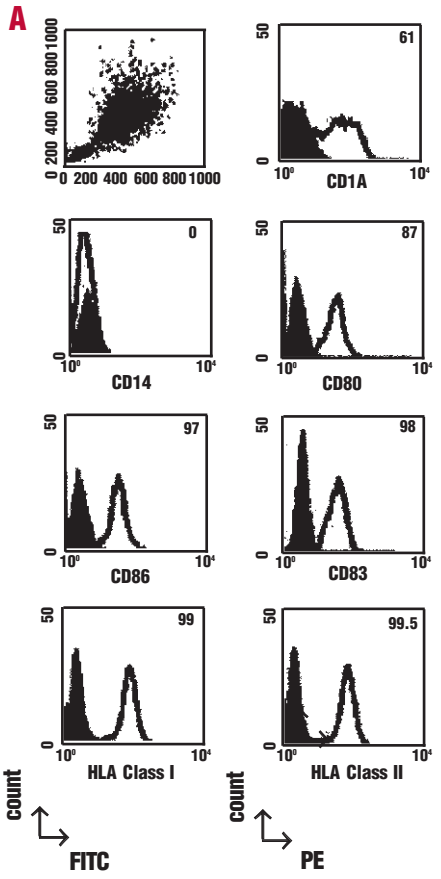
These data suggest that mDC generated *in vitro* from monocytes of AML patients in complete remission have the principal phenotypic and functional characteristics of mDC derived from healthy donors, including the capacity to produce IL-12p70.

In vitro stimulation of lymphocytes by autologous mDC/EP induced a Th1 antileukemic response

In order to evaluate the immunogenicity of peptides eluted from AML cells, lymphocytes from patient P1 in complete remission were stimulated *in vitro* four times weekly with autologous mDC/EP, and were examined for their ability to produce IFN-γ in response to various stimuli, using an IFN-γ ELISPOT assay. As shown in Figure 3, the production of IFN-γ by stimulated lymphocytes derived from patient P1 in response to autologous mDC/EP was significantly higher than that observed in response to mDC loaded with an irrelevant peptide (mDC/IrP) ($p<0.001$), or in response to eluted peptides alone (EP) ($p<0.05$). Importantly, those stimulated lymphocytes also produced IFN-γ in response to autologous AML cells (Figure 3). These results suggest that mDC/EP were able to stimulate autologous lymphocytes *in vitro*, leading to the generation (or expansion) of specific autologous antileukemic T cells producing IFN-γ. The production of such cytokines indicated that the autologous mDC/EP-induced immune response was, at least in part, of the Th1 type.

In vitro stimulation of lymphocytes by autologous mDC/EP-generated antileukemic specific $CD4^+$ T-cell responses

We further examined which T-cell subsets were involved in the antileukemic response induced by autologous mDC/EP stimulations. $CD4^+$ T cells were



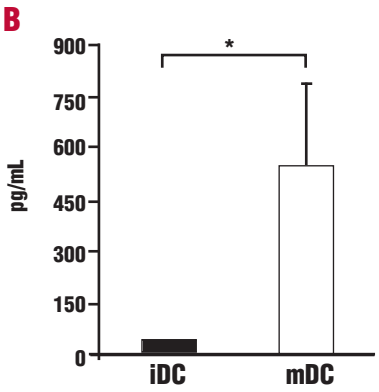


Figure 2. Phenotypic and functional characteristics of mDC derived from monocytes of AML patients in complete remission. A. The mDC phenotype is shown for one representative patient (patient P2). Results for the five tested patients are summarized in Table 3. mDC were labeled with isotype-matched control monoclonal antibody (filled histogram) or with the indicated monoclonal antibody (open histogram). Values indicated represent the mean percentage of positive cells obtained for each marker. This experiment was performed twice, giving the same results. B. Quantification of iDC and mDC IL-12 secretion by a p70-IL-12 ELISA. Results for a representative patient (P2) of three analyzed are shown. Three independent experiments were performed and showed a mean IL-12 secretion of 500 pg/mL (from 120 pg to 960 pg/mL) by mDC (open bar) and 39.3pg/mL (from 37 to 40 pg/mL) by iDC (black bar). *, $p< 0.001$.

Table 3. Phenotype of mDC generated from PBMC of patients in complete remission.

*Patient**	*CD14†*	*CD1a†*	*CD86†*	*CD83†*	*CD80†*	*HLA-I†*	*HLA-II†*
P2	0	61	97	98	87	99	99.5
P3	0.3	65	96	90	92	98.9	99
P4	0	58	83	96.5	91.3	98.6	99.4
P5	17.8	53	77	57	94.7	98	97
P6	0.1	63	97.8	95.5	73.3	98.8	99.9

**The phenotype of patient P1 could not be determined; †% of total positive cells for the marker.*

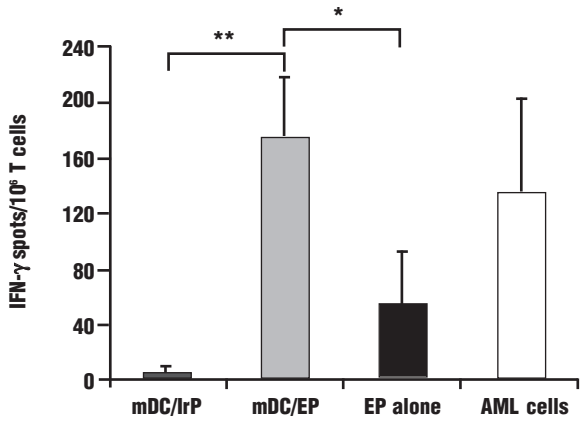


Figure 3. Specificity and IFN-γ production of T lymphocytes from patient P1 stimulated with mDC/EP. Each bar represents the mean number of IFN-γ-secreting cells per 10^6 T cells stimulated either with mDC/IrP (irrelevant peptides), mDC/EP, eluted peptides (EP) alone, or autologous AML cells, and analyzed with an IFN-γ ELISPOT assay. The number of spots obtained when T cells were cultured alone, or when T cells were stimulated with unloaded mDC, represents the non-specific background for the calculation of positive value, depending on the targets. Results from patient P1 were confirmed in two independent experiments. *, $p< 0.05$ and **, $p<0.001$.

isolated from stimulated lymphocytes after four weeks of culture, and analyzed for their proliferation in response to autologous or allogenic AML cells (Table 4). As shown in Figure 4A, both autologous and allogenic AML cells triggered the proliferation of stimulated $CD4^+$ T cells *in vitro* in a dose-dependent manner. Strikingly, for the five patients analyzed, the proliferation of their $CD4^+$ T cells was always significantly higher in response to autologous AML cells than in response to allogenic AML cells, at all E:T ratios examined ($p<0.05$ to $p<0.001$). We also analyzed the production of IFN-γ by stimulated $CD4^+$ T cells from four patients using an IFN-γ ELISPOT assay. These $CD4^+$ T cells were challenged *in vitro* by either autologous or allogenic AML cells, or normal autologous unloaded mDC or PBMC. As shown in Figure 4B, *in vitro* stimulation by autologous AML cells always induced a significantly higher number of

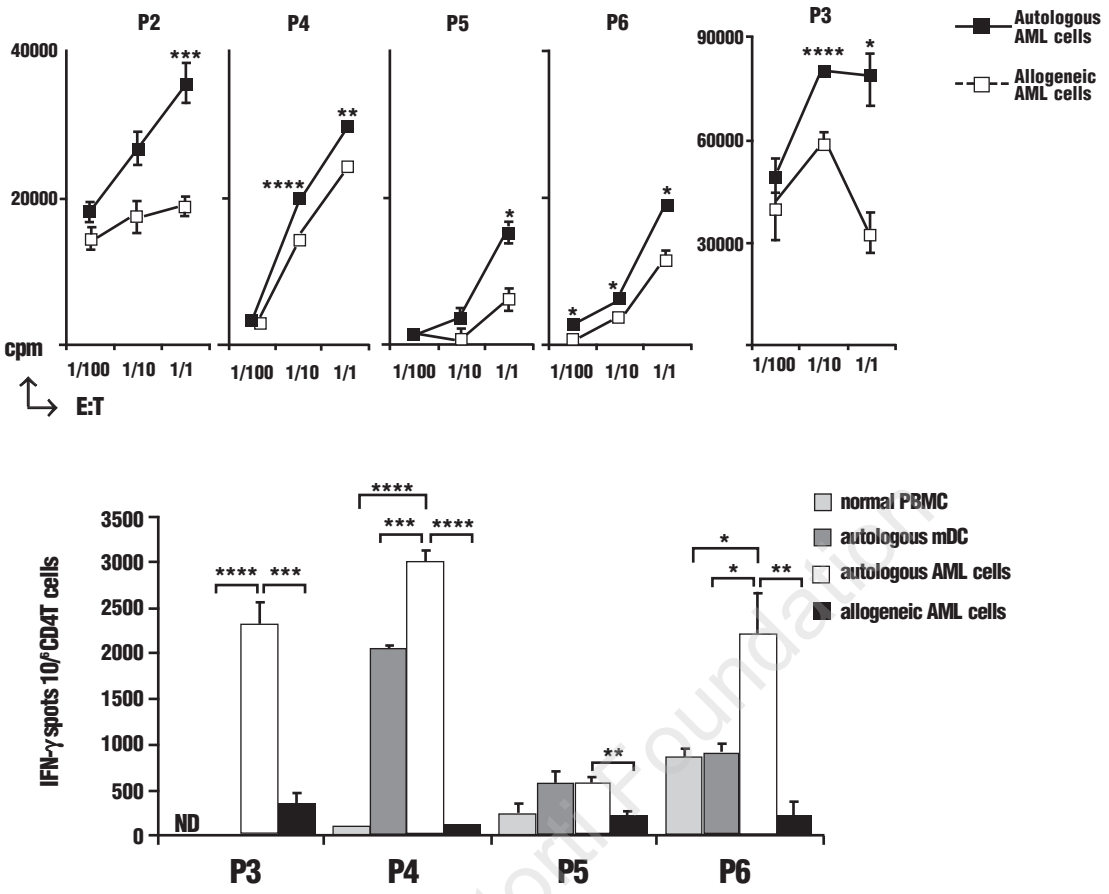


Figure 4. Involvement of CD4+ T lymphocytes in the antileukemic response induced by stimulation with mDC/EP. A. Proliferation of CD4+ T cells isolated from *in vitro* stimulated T lymphocytes of patients P2 to P6 in response to autologous and allogenic AML cells at the indicated effector:target (E:T) ratios. Results represent mean cpm of triplicate measurements. [3H]-thymidine incorporation corresponding to proliferation of T cells alone and AML cells alone were subtracted. B. IFN-γ ELISPOT assay of isolated CD4+ T cells from patients P3 to P6 stimulated with either autologous or allogenic AML cells, or with either normal autologous PBMC, or unloaded mDC. Each value represents the mean of triplicate measurements for 10^6 CD4+ T cells. *, $p<0.05$; **, $p<0.02$; ***, $p<0.01$; ****, $p<0.001$.

IFN-γ secreting T cells than stimulation with either allogenic AML cells ($p<0.01$ for P5 and P6, and $p<0.001$ for P3 and P4), or normal autologous unloaded mDC ($p<0.05$ for P6, $p<0.01$ for P4, and $p<0.001$ for P3), or normal PBMC ($p<0.05$ for P6, and $p<0.001$ for P4). Moreover, stimulation of CD4+ T cells from patients P4 and P6 with autologous PHA-blasts induced a low level of IFN-γ production, comparable to the production observed following stimulation with normal PBMC (*data not shown*). Interestingly, stimulated CD4+ T cells from patient P5 who soon had a relapse of his refractory anemia with excess of blasts, displayed a reduced proliferative response and a smaller production of IFN-γ compared to stimulated CD4+ T cells from the other patients (Figure 4A). These results suggest that for AML patients in complete remission, repeated *in vitro* stimulations of lymphocytes with autologous mDC/EP were able to induce an autologous antileukemic CD4+ immune response.

Table 4. HLA phenotype of autologous and allogenic AML cells used in functional assays.

Patient	*Autologous AML cells*	*Allogenic AML cells used as control*
P2	Not Determined	A3/31, B35/40, DR01/04
P3	A2/24, B39/62 DR08/15	A3/31, B35/40, DR01/04
P4	A29/30, B35/39 DR04/16	A3/24, B39/53, DR01/08
P5	A2/28, B39/53,DR NDa	A3/A24, B45/63, DR7/4
P6	A2/30, B51/35, C4/16, DR04/13	A2/28, B39/53, DR01

In vitro stimulation of lymphocytes by autologous mDC/EP generated CD8+ T cells specific for autologous AML cells

We further investigated whether repeated *in vitro* stimulations of lymphocytes by autologous mDC/EP also induced antileukemic cytotoxic CD8+ T cells. Hence, CD8+ T cells were isolated from stimulated

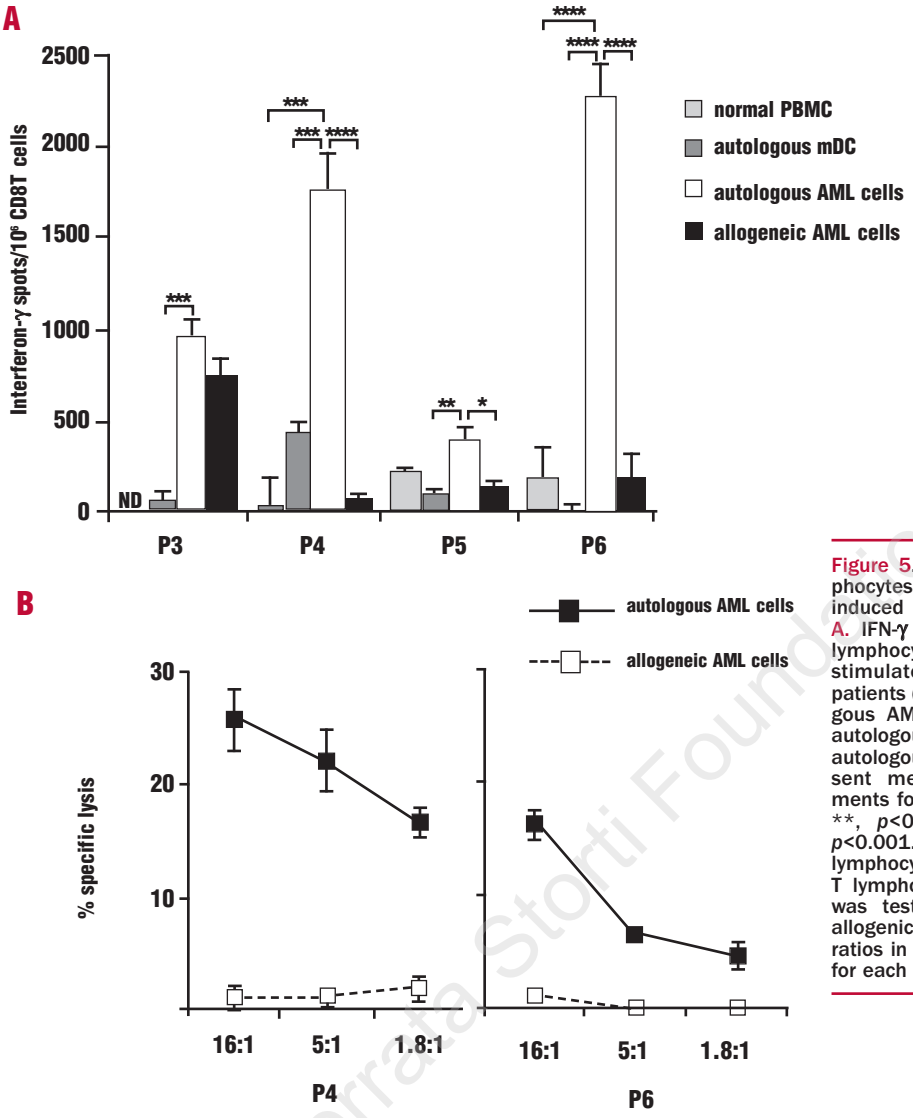


Figure 5. Involvement of CD8⁺ T lymphocytes in the antileukemic response induced by stimulation with mDC/EP. **A.** IFN-γ ELISPOT analysis of CD8⁺ T lymphocytes isolated from *in vitro* stimulated T lymphocytes of four patients (P3 to P6) triggered by autologous AML cells, allogenic AML cells, autologous unloaded mDC, or normal autologous PBMC. The values represent means of triplicate measurements for 10^6 CD8⁺ T cells. *, $p<0.05$; **, $p<0.02$; ***, $p<0.01$; ****, $p<0.001$. **B.** Cytotoxic activity of CD8⁺ T lymphocytes, isolated from stimulated T lymphocytes of patients P4 to P6, was tested against autologous and allogenic AML cells at the indicated E:T ratios in a ^{51}Cr release assay. $p<0.001$ for each E:T ratio. ND, not determined.

lymphocytes after four weeks of culture, and were analyzed for their IFN-γ production in response to autologous or allogenic AML cells, or in response to normal autologous unloaded mDC or PBMC. As shown in Figure 5A, the number of IFN-γ-secreting CD8⁺ T cells that responded to stimulation by autologous leukemic targets was significantly higher than that induced by either allogenic AML cells ($p<0.05$ for P5, and $p<0.001$ for P4 and P6), or autologous mDC ($p<0.02$ for P5, $p<0.01$ for P3 and P4, and $p<0.001$ for P6), or normal autologous PBMC ($p<0.01$ for P4, and $p<0.001$ for P6). One exception was observed with stimulated lymphocytes from patient P5 that produced IFN-γ in response to challenge with autologous PBMC. For this patient however, we cannot rule out that there were some circulating cells derived from the malignant clone since he relapsed to refractory anemia with excess of blasts soon after chemotherapy. We also observed that the autologous PHA-blasts of patients P4 and P6 did not induce IFN-γ production by their autologous CD8⁺ T stimulated cells (*data not shown*).

Finally, a ^{51}Cr release cytotoxicity assay was performed for three patients who had provided a sufficient number of CD8⁺ T cells. The CD8⁺-stimulated T cells obtained previously from patients P4 and P6 were able to lyse 25% and 17% of autologous AML cells respectively, at a T cell:AML cell ratio of 16:1 (Figure 5B). The CD8⁺ stimulated T cells from patient P5 were not cytotoxic *in vitro* (*data not shown*). NK-cell-mediated cytotoxicity was probably not involved since we did not detect the expression of either CD16⁺ or CD56⁺ NK cell markers on any CD8⁺ stimulated T cells, as assessed by flow cytometry (*data not shown*), and since we observed no cytotoxicity directed against allogenic AML cells at any E:T

ratio studied (Figure 5B). These results suggest that repeated stimulation with autologous mDC/EP of lymphocytes from AML patients in complete remission was able to induce *in vitro* antileukemic CD8+ immune responses.

In vitro expansion of stimulated lymphocytes after four weeks of culture, and distribution of the CD4+ and CD8+ T cells

We evaluated the *in vitro* expansion of lymphocytes after four mDC/EP stimulations (Table 1). This increase varied from 8 to 20 fold, depending on the patients, after five weeks of *in vitro* culture. The median percentage of CD4+ T cells among stimulated lymphocytes was 61% (range 41-87%). We never observed expansion of CD16+ NK cells in the culture (< 1%) (*data not shown*).

Production of Th1 and Th2 cytokines by in vitro lymphocytes stimulated with autologous mDC/EP

Production of Th1 and Th2 cytokines such as IFN-γ, TNF-α, IL-10, IL-6, and IL-4 by stimulated lymphocytes of patient P1 was measured during each stimulation cycle. Each week, a sample of culture supernatant was collected three days after the last addition of fresh autologous mDC/EP. We observed that IFN-γ and IL-6 were the main cytokines produced, while TNF-α, IL-10 and IL-4 were barely detectable (Figure 6A). IFN-γ production testified that the autologous mDC/EP-induced immune response was mainly of Th1 type. IL-6 was probably produced by the mDC, as suggested by its very low concentration in the supernatant of lymphocytes collected ten days after the third stimulation by mDC/EP. Production of Th1/Th2 cytokines was evaluated in culture supernatants of stimulated CD4+ and CD8+ T cells isolated from stimulated T cells of patients P4 to P6. The supernatants used for the titration of cytokines were collected three days after the last stimulation (Figure 6B). Again, IFN-γ and IL-6 were the main cytokines detected while IL-4 was never detected in the culture supernatants of CD4+ T cells. However, the IFN-γ secretion by CD8+ T cells from patient P4 was weak, contrasting with the production by his CD4+ T cells. Nevertheless, a high level of IFN-γ was detected in supernatants of CD8+-stimulated T cells from patients P5 and P6. IL-6 was detected in supernatant of CD8+-stimulated T cells from patients P4 and P6, but not from patient P5. As observed for CD4+-stimulated T cells, IL-4 was never detected in CD8+ T-cell supernatants. This cytokine secretion profile was characteristic of cytotoxic T-cell phenotypes (producing IFN-γ but not IL-4).

Discussion

In order to study the feasibility of an immunotherapeutic intervention in AML patients, we investigated whether AML cells could trigger antitumor response *in vitro* from autologous lymphocytes collected from patients in complete remission. A second aim was to develop a convenient and broadly applicable method for tumor antigen extraction and presentation, which would be clinically usable. Peptides were extracted from AML cells by TFA elution. Such peptides likely represent a pool of naturally processed peptides, including potentially immunogenic leukemia antigens. Those eluted peptides were loaded *in vitro* onto mDC differentiated from monocytes obtained from patients in complete remission. Using this antigen mixture, and antigen presenting cells, lymphocytes displayed a specific autologous antileukemic immune response after four rounds of stimulation *in vitro*. Both antileukemic CD4+ and CD8+ T lymphocytes were generated (or expanded), as testified by the proliferation of CD4+ T cells, the IFN-γ secretion by CD4+ and CD8+ T cells, and the cytotoxicity mediated by CD8+ T cells. Importantly, the CD4+ and CD8+ T lymphocytes did not recognize non-leukemic autologous cells, such as normal PBMC or unloaded mDC.

Extracting naturally processed peptides from tumor cells and loading them on DC is a relatively simple process. It has already been shown in mice that immunization with EP loaded on DC generated both CD4+ helper and CD8+ cytotoxic leukemia-specific T cells *in vivo*, resulting in a 100% long-term survival of treated animals.[9] In humans, several studies with EBV-induced cell lines, CMV-infected cells or melanoma tumors have shown that the acid-elution method is able to extract immunodominant epitopes.[12,13,16–18] A similar immunotherapeutic protocol has been used recently in a phase I/II clinical trial for patients with glioma.[10] Recently, this technique was also able to identify a new TAA in chronic myeloid leukemia.[19] Taken together, these data suggest that it is clinically feasible to use various class I- and class II-restricted immunogenic tumor-associated peptides extracted by acid-elution to induce a polyclonal and polyepitopic immune response. However, the use of eluted peptides for immunotherapeutic approaches in humans has been limited by the need for a large number of purified tumor cells to perform the elution procedure. In AML patients, the frequent accessibility to a large number of circulating leukemic cells that substantially express HLA class I and class II molecules,[20,21] makes this antigen acid extraction technique

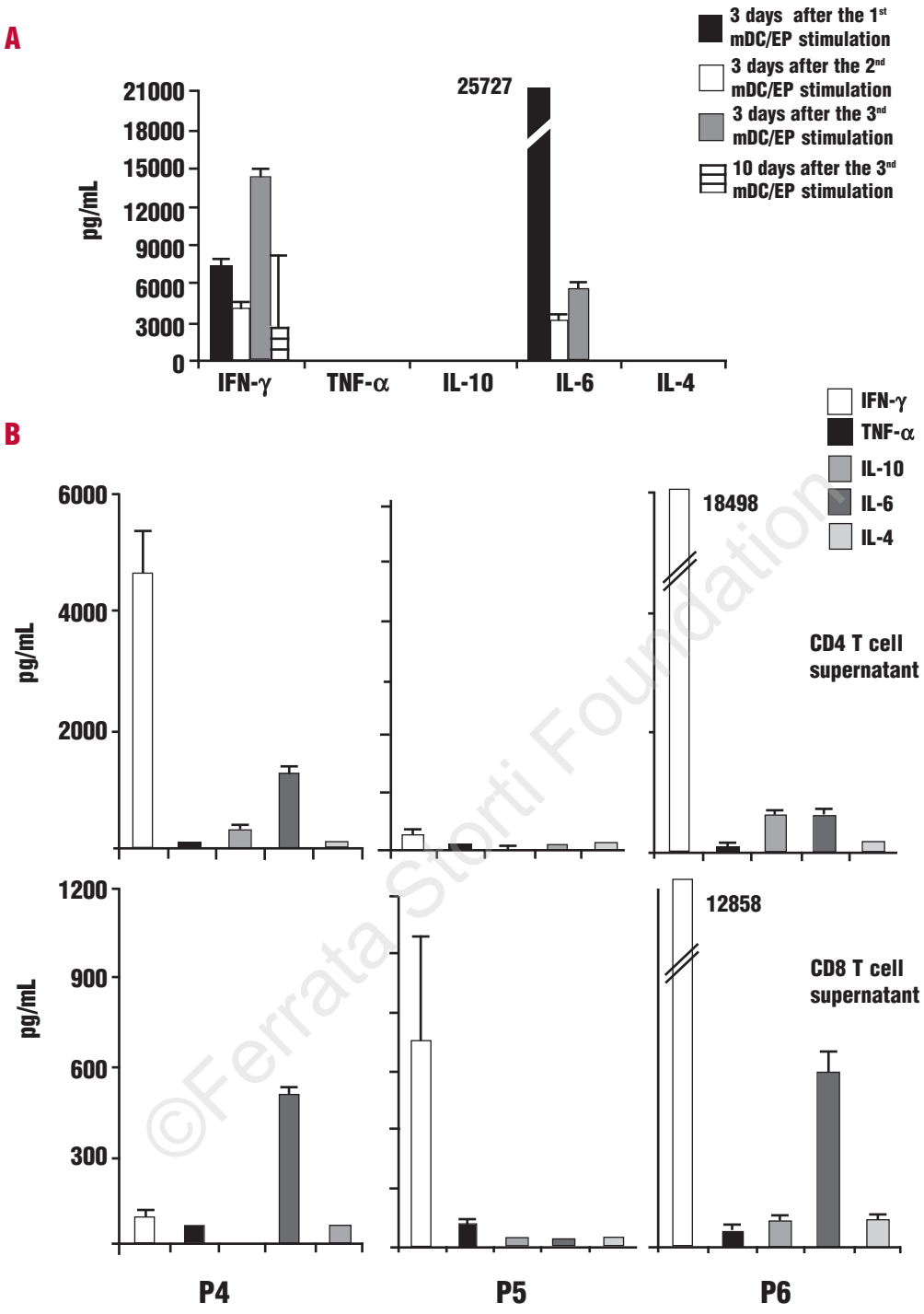


Figure 6. Th1/Th2 cytokine quantification in supernatant of stimulated T lymphocytes from patients P1, and P4 to P6. A. Cytokine production by stimulated T lymphocytes of patient P1 at several culture times: after one mDC/EP stimulation, after the second mDC/EP stimulation, after the third mDC/EP stimulation, or two weeks later. B. Th1/Th2 cytokine quantification in supernatant of $CD4^+$ and $CD8^+$ T cells isolated from stimulated T lymphocytes of patients P4 to P6. Each value represents the mean cytokine concentration of duplicate measurements.

more applicable than for solid tumors.

Synthetic tumor-associated peptides bound or loaded onto DC have been used in humans and mice leading to effective antitumor vaccines.[22-24] However, such strategies required prior identification of the immunogenic sequence within the TAA. In acute leukemia, only a few shared TAA have been identified so far (WT1, proteinase 3, PRAME, survivin, hTER, BAGE, RHAMM), and most epitopes described to date are restricted by some HLA class I

molecules (eg HLA-A2, and -A24) which limits their clinical use.[25-27] The acid-elution technique that we investigated here has the advantage of bypassing the need to characterize TAA and immunogenic epitopes and of being applicable without taking account of the patient's HLA phenotype.[2,3,7]

Acid elution of leukemic cell peptides might be more effective for inducing specific antitumor immune responses compared to simple tumor cell lysate. An *in vitro* study of six AML patients suggested that AML cell lysates loaded onto autologous DC could mediate inhibitory effects.[28] In another model, tumor lysate-pulsed DC were poorly efficient at inducing a T-cell immune response and antitumor protection, and were weak inducers of a Th1 response that was outweighed by an IL-5 Th2 response.[29] In another murine model using DC pulsed with acid-eluted tumor peptides it was shown that this technique provided a superior source of immunogenicity than did DC pulsed with tumor cell lysates, probably because the cell lysates contained an unsuitably complex mixture of peptides, and possibly intracellular proteases or inhibitory cytokines.[9] In human, immunotherapeutic strategies using DC pulsed with tumor cell lysates also led to heterogeneous results. Immunization induced transient IL-12 secretion that did not seem sufficient to maintain T-cell activation.[30]

The use of DC to present tumor antigen to patient's lymphocytes has been explored in different protocols. Several groups have investigated the feasibility of differentiating leukemic cells into DC called leukemic DC (LDC).[31] However, this approach might be difficult because of LDC heterogeneity,[32] variable induction of CTL,[33] and possible paradoxical induction of immune escape.[34] Our strategy was to use autologous functional mDC which might behave as a natural adjuvant[35,36] for the generation of an antileukemic response. Our data indicate, as reported by other groups, that mDC differentiated *in vitro* from monocytes in complete remission were functional and expressed all markers associated with appropriate peptide presentation.[28,32,37] Such mDC are well-known inducers of T-cell activation and immunity.[38-40] They secreted high levels of IL-12p70, which has been shown to play a central role in antitumor immune responses in murine models,[41-43] inducing the differentiation of $CD4^+$ T cells into high-level IFN-γ-producing Th1 cells, stimulating the generation of cytotoxic T lymphocytes,[44] and enhancing the avidity and concomitant tumor recognition by $CD8^+$ T cells.[45] Of note, although a final 24-hour LPS stimulus was used here to induce the maturation of monocyte-derived DC, obviously another clinical grade DC maturation procedure will be used in future clinical trials. The existence of a natural immune surveillance against AML remains difficult to demonstrate in patients. The presence of antileukemic T-cell precursors has been shown in active AML, but these bone-marrow-derived T-cell clones released little IFN-γ, and expressed inhibitory cytokines.[46] Thus, until now, circulating specific antileukemic T cells equivalent to tumor-infiltrating lymphocytes (TIL) have not been identified clearly *in vivo* in humans, which classifies acute leukemias as poorly immunogenic tumors. Many parameters may hinder the generation of a specific natural antileukemic response in patients. Indeed, there is likely a deficit in the quality or quantity of DC in AML patients at diagnosis.[47] Nevertheless, a recent study demonstrated that T lymphocytes from AML patients in complete remission after intensive chemotherapy were able to proliferate *in vitro* following anti-CD3 and anti-CD28 stimulation as effectively as lymphocytes from healthy controls, thus offering good prospects for vaccination.[48] In our limited series of patients, we found that repeated *in vitro* stimulation of remission lymphocytes with mDC/EP was efficient at inducing $CD4^+$ and $CD8^+$ T cells specifically reacting to the stimulation by autologous AML cells. The intensity of the specific antileukemic activities was in the same range of magnitude for five out of the six patients. The weakest response came from patient P5. His DC displayed a rather immature phenotype, his $CD8^+$ T cells secreted high levels of IFN-γ although displaying only a weak cytotoxic response, and his $CD4^+$ T cells stimulated by autologous AML cells were only weakly responsive. However, this patient was probably not in complete remission when his blood was collected since he went into relapse with a refractory anemia with excess of blasts soon after the end of chemotherapy. This might also explain our inability to properly differentiate his $CD14^+$ circulating cells into mDC, which then did not sufficiently immunostimulate his lymphocytes *in vitro*. Since the AML cell peptides obtained by TFA extraction may also contain myeloid cell-associated self-peptides, we are well aware that an autoimmune response against nonleukemic cells from the hematopoietic lineage is a potential adverse effect which must be carefully considered. We demonstrated in a previous study the absence of response of specific antileukemic lymphocytes against patient's EBV transformed cells and PHA-blasts.[11] In the present experiments, AML cells but neither normal autologous PBMC nor PHA-blasts induced a significant increase in IFN-γ production by both $CD4^+$ and $CD8^+$ T cells. The only exception was patient P5 whose $CD8^+$ T cells able to secrete IFN-γ when stimulated with PBMC obtained in remission. However, in this particular patient, it was possible that some PBMC belonging to the malignant clone were circulating since he soon relapsed with an

excess of blasts. Interestingly, although patients P1, P4 and P6 had AML FAB type 5 (monocytic), as shown in Table 2, their autologous PBMC containing monocytes were not recognized in the ELISPOT assays. Thus, the correlation between the elution of potential auto-antigens, and autoimmune recognition of the normal hematopoietic cells from the same lineage is not so obvious. We suspect that the risk of autoimmune recognition will be extremely heterogeneous between patients, depending on the FAB type of AML, and the proportion of regular auto-antigens in the pool of eluted peptides. Adverse autoimmune effects were not observed in a recent clinical study of glioblastoma using eluted peptides.[10] Of course this issue will require further investigation before clinical application.

Based on these results, we believe that mDC pulsed with acid-eluted peptides derived from autologous tumors represents a feasible approach for the treatment of established, weakly immunogenic tumors. We demonstrate for the first time in humans that this procedure is able to induce specific Th1-mediated antileukemic T-cell responses *in vitro*. These results provide good evidence for using mDC/EP to vaccinate AML patients and will be soon evaluated in a phase I/II clinical trial for high-risk patients with minimal residual disease.

SD, LT, and AB contributed to the conception and design of the study and the interpretation of the data, and wrote the first version of the paper. SD performed most of the experiments regarding induction of the antileukemic immune response, and created all the tables and figures in the manuscript. BV and AB managed patients and analyzed clinical data. CB performed technical experiments and collected patients' samples. JGG managed the Department of Immunology at the Institute Cochin. DF gave advice on the writing of the final version of this manuscript.

The authors declare that they have no potential conflicts of interest. The authors are indebted to Francine Connan for her excellent technical assistance with RP-HPLC, and to Nicolas Cagnard for statistical analysis. They are also grateful to Dr Francoise Audat and Dr Vassili Soumelis who ensured the inclusion of patients in the study, and to Emilie Floch' for her technical support. They thank Jean-Pierre Abastado, scientific director of Immuno-Designed Molecules (IDM) for constructive scientific input, and Marie-Thérèse Rubio for helpful advice on the manuscript. The authors also thank all the patients who were included in this study.

This work was supported by the "Institut National de la Santé et de la Recherche Médicale" (INSERM). The laboratory is associated with the "Ligue contre le cancer", Comité Ile de France. S. Delluc and L. Tourneur are supported by the "Fondation de France" (FDF), Comité Leucémie. S. Delluc received financial support from France Intergroupe de la Leucemie Myeloide Chronique (FI LMC). The "Delegation Regionale à la Recherche Clinique (DRRC)" and the "Société Francaise de Greffe de Moelle et de Thérapie Cellulaire" (SFGM-TC) promoted of this study.

Manuscript received January 19, 2005. Accepted June 28, 2005.

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