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## DC-based vaccine loaded with acid-eluted peptides in acute myeloid leukemia: the importance of choosing the best elution method

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**Abstract** Tumor-associated peptides isolated by acid elution are frequently used for therapeutic immunization against various tumors both in mice and in humans. In acute myeloid leukemia (AML), the frequent accessibility of a large tumor burden allows for extraction of peptides from leukemia cells by using either citrate-phosphate (CP) or trifluoroacetic acid (TFA) buffer. To develop an optimal immunotherapeutic protocol for AML patients, we evaluated both in mice and in humans, the immunogenicity of peptides eluted from leukemia cells with the two acids (TFA or CP). Although *ex vivo* studies in mice showed that both prophylactic immunizations with mature dendritic cells (DC) loaded with TFA-peptides (DC/TFA), or CP-peptides (DC/CP), were able to stimulate specific antileukemia immune responses, only vaccination with DC/TFA was able to prevent leukemia outgrowth. Moreover, in humans, only DC/TFA generated significant antileukemia CD4<sup>+</sup> and cytotoxic CD8<sup>+</sup> T cell responses *in vitro*. In summary, these data demonstrate that the choice of the acid elution

procedure to isolate immunogenic peptides strongly influences the efficacy of the antileukemia immune responses. These findings raise essential considerations for the development of immunotherapeutic protocols for cancer patients. In our model, our results argue for the use of the TFA elution method to extract immunogenic AML-associated peptides.

**Abbreviations** Mature DC: Mature dendritic cells · TFA: Trifluoroacetic acid · CP: Citrate-phosphate · DC/TFA: Mature dendritic cells loaded with peptides extracted from leukemia cells using TFA buffer · DC/CP: Mature dendritic cells loaded with peptides extracted from leukemia cells using CP buffer · MST: Median survival time

### Introduction

The poor prognosis of patients with acute myeloid leukemia (AML) stresses the importance of exploring new therapeutic strategies to improve overall survival. The induction of antileukemia immune responses in patients during the period of minimal residual disease could prevent relapse. Among the immunotherapeutic protocols currently developed for generating antitumor immune responses, one is to use characterized leukemia-associated peptides as antigens [25]. However, only few shared tumor-associated antigens (TAA) have been identified so far (<http://www.cancerimmunity.org/peptidedatabase>), and most epitopes are restricted by specific HLA class I molecules (e.g., HLA-A2, and -A24) which limits their clinical use [15, 16, 19]. The use of material obtained from total tumor to promote the broadest range of anti-tumor T cell epitopic specificities is an alternative strategy potentially interesting for vaccination trials. Indeed, vaccination with a tumor antigen mixture may allow bypassing the previously described limitations. It would favor the generation of a polyclonal and polyepitopic immune response, which may limit the risk of tumor

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immunoediting [38]. Moreover, the immunodominant antigens on tumor cells being probably patient-specific, notably on account of the multitude of oncogenic events involved in malignant transformation [36], such autologous immunotherapeutic vaccination would provide a custom-made treatment.

As dendritic cells (DC) play a central role in inducing antitumor immune responses, DC-based vaccine strategies have been explored. They were successful in prophylactic use [29], and in some therapeutic murine models [42], and provided the basis for the conception of using DC in human vaccination. Several strategies have been investigated for DC loading: fusion of DC with tumor cells [1, 28], DC-derived exosomes [8, 46], transfection of DC with RNA encoding TAA [10, 12], or loading DC with apoptotic tumor cells, unfractionated tumor cell lysates [13, 22, 23], chaperone-enriched tumor extracts [44], or acid-eluted tumor peptides [9, 27, 45].

The efficiency of acid elution procedures to isolate immunogenic tumor-derived peptides has widely been described [33]. Recent data demonstrated that peptides extracted from ovarian tumor cells using trifluoroacetic acid (TFA) buffer, and subsequently loaded on DC, induced in vitro stimulation of CTL capable of eradicating established ovarian tumor when used for cellular adoptive immunotherapy in mice [17]. Nevertheless, the use of TFA-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. For this reason, TFA buffer [33], initially used to extract tumor peptides, has been replaced by citrate-phosphate (CP) buffer which was supposed to preserve the survival of tumor cells, and to allow several cycles of peptide extraction following regeneration of MHC/peptide complexes on the surface of the tumor cells in culture [37]. The use of CP-peptides loaded on DC in a murine model was able to induce rejection of an established melanoma tumor [45]. We have previously demonstrated that AML-associated peptides extracted using CP buffer, and loaded on peripheral blood mononuclear cells (PBMC) obtained from a partially HLA-compatible healthy donor, were able to induce a patient-specific antileukemia immune response in vitro [27]. Moreover, in a clinical trial, vaccination of patients suffering from malignant glioma using DC pulsed with CP-peptides eluted from tumor cells was shown to elicit systemic immune responses [43].

In AML patients, the frequent availability to a large number of circulating leukemia cells, which substantially express HLA class I and class II molecules [9, 26, 41], makes both TFA and CP antigen acid extraction techniques feasible. Our objective was to compare the efficacy of peptides extracted from leukemia cells by either the TFA- or the CP-buffers in inducing antileukemia immune responses. We first addressed this question using a relevant murine model in vitro and in vivo; we then extended the investigation in vitro to the human situation using cells from three AML patients.

## Patients, materials and methods

### Murine cell lines

The C1498 cell line arose spontaneously in a 10-month-old C57BL/6 female mouse in 1941, and was characterized as a myelomonocytic leukemia [5]. The C1498 cells expressed myelomonocytic markers (Gr-1, Mac-1/3), MHC class I, CD45, ICAM-1/2, FADD, and LFA-1 [35]. The expression of MHC class II molecules and the costimulatory molecules B7-1 (CD80) and B7-2 (CD86) was negative and not inducible by addition of IFN- $\alpha$ . The AML cell line C1498 was obtained from the American Type Culture Collection (ATCC, Rockville, MD), and was grown in AIM-V medium (Life Technologies; Inc. Cergy Pontoise, France) without FBS at 37°C in 5% CO<sub>2</sub>. The A20 BALB/c (H-2<sup>d</sup>) B cell lymphoma line originally derived from a spontaneous neoplasm type B [21] was used for allogeneic control. The NK cell-sensitive lymphoma cell line YAC-1 was used for nonspecific cytotoxicity. The A20 and YAC-1 cell lines were purchased from the ATCC. The two latest cell lines were maintained at 37°C in 5% CO<sub>2</sub>, in complete medium consisting of RPMI 1640 (Life Technologies) supplemented with 2 mM glutamine, 50  $\mu$ g/ml streptomycin, 50 U/ml penicillin, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50  $\mu$ M 2-mercaptoethanol (2-ME), and 10% FBS (Life Technologies).

### Animals

C57BL/6 (H-2<sup>b</sup>) female mice were purchased from the Janvier laboratory (Janvier, Le Genest-St-isle, France), and were used at 6–14 weeks. They were allowed to adapt to their environment for 1 week before initiating the experiments, and during the course of the experiments, animals were maintained under standard environmental conditions with free access to food and water.

### Tumor peptides acid elution

Aliquots of 10<sup>9</sup> murine C1498 cells or 10<sup>9</sup> human leukemia cells were submitted to the acid-elution procedure using the TFA or the CP buffer.

*TFA elution procedure* Peptides were extracted from leukemia cells with TFA buffer (Sigma-Aldrich) as previously described [18]. Briefly, leukemia cells were resuspended in 15 ml 0.1% TFA in distilled, deionized water (ddH<sub>2</sub>O), and dounce homogenized until cell disruption. The resulting lysate was centrifuged for 20 min at 15,000 rpm at 4°C. The supernatant was recovered, precipitated with TFA to obtain a 10% final solution and centrifuged again, under the same conditions. The supernatant was loaded on a C18 SepPak column (Walters, Milford, MA). Peptides were eluted using 2 ml 60% acetonitrile (ACN; Sigma-Aldrich) in ddH<sub>2</sub>O and lyophilized. The pool of peptides (identified as TFA-peptides)

was re-suspended in 1 ml RPMI 1640 medium (Life Technologies), fractionated in aliquots and stored at  $-80^{\circ}\text{C}$  until use.

**CP elution procedure** Leukemia cells were resuspended in 10 ml CP buffer (0.131 mol/l citric acid, 0.066 mol/l  $\text{Na}_2\text{HPO}_4$ , pH 3.3), and immediately centrifuged 10 min at 1,200 rpm at  $4^{\circ}\text{C}$ . Supernatant was centrifuged 30 min at 15,000 rpm at  $4^{\circ}\text{C}$  and loaded on a C18 SepPack column, as for the TFA procedure, and the pool of eluted peptides (identified as CP-peptides) was cryopreserved at  $-80^{\circ}\text{C}$ .

**RP-HPLC biochemical profile of peptides eluted from leukemia cells**

An aliquot of eluted peptides was fractionated by RP-HPLC (HPLC LC200, Perkin Elmer Instruments LLC, Connecticut, USA), as previously described [37]. Briefly, an ACN gradient (0% for 5 min, 0–10% for 5 min, 10–35% for 50 min, 35–60% for 10 min, 60–100% for 5 min, and 100–0% for 5 min) containing TFA in  $\text{ddH}_2\text{O}$  was used. The elution profile was read at 214 nm with an absorbance detector in milli UV (mUV) (Perkin Elmer Instruments LLC).

**Generation of DC**

**Murine bone marrow-derived DC** DC were generated from bone marrow cells with GM-CSF and IL-4, as initially described by Mayordomo et al. [7], with some modifications. Briefly, marrow cells from femurs and tibias of mice were flushed out with 1 ml RPMI 1640 medium (Life Technologies) with a syringe and a 25-gauge needle. Cells were then plated at  $10^6$  cells/ml for 2 h in complete medium consisting of RPMI 1640 supplemented with 10% FBS (Life Technologies), at  $37^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  atmosphere. After incubation, nonadherent cells were recovered and plated at  $7 \times 10^5$  cells/ml in complete medium supplemented with 10 ng/ml of GM-CSF (R&D system, Lille, France), and 500 U/ml of IL-4 (IL-4; R&D system). On days 2, and 4, two-thirds of the medium were replaced with additional GM-CSF and IL-4. On day 7, CD11c positive cells were isolated with immunomagnetic murine CD11c Microbeads by MACS<sup>®</sup> (Miltenyi Biotec, Paris, France) according to the manufacturer's instructions. CD11c-positive cells were then matured at  $10^6$  cells/ml for 6 h with 1  $\mu\text{g}/\text{ml}$  of lipopolysaccharide (LPS from *Escherichia coli*, Calbiochem-Novabiochem Corp., La Jolla, California). Mature DC were loaded with TFA- or CP-peptides during the last 90 min of maturation at a DC:AML cell-eluted peptide ratio of 1:100. Then, mature DC loaded with TFA- or CP-peptides (DC/TFA, and DC/CP, respectively) were washed three times with PBS (GIBCO BRL, Paisley, Scotland) and resuspended at  $5 \times 10^5$  mature DC/ml for immunization.

**Human monocyte-derived DC** DC were generated from CD14<sup>+</sup> monocytes, immunomagnetically enriched from

PBMC obtained from patients in complete remission as previously described [9]. Briefly, CD14<sup>+</sup> cells were incubated in DC-medium consisting of RPMI 1640 (Life Technologies) supplemented with 10% FBS (Life Technologies), 500 U/ml recombinant human GM-CSF (Leucomax, Novartis, Paris, France) and 500 U/ml rhIL-4 (PeproTech Inc, Tebu, Le Perray en Yvelines, France) for 6 days at  $37^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  atmosphere. On day 3, 500 U/ml of rhGM-CSF and 500 U/ml of rhIL-4 was added in the culture. On day 6, immature DC were matured by adding 1  $\mu\text{g}/\text{ml}$  of LPS for 6 h. During the last 90 min, mature DC were incubated at  $37^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  atmosphere with TFA- or CP-peptides. Then DC/TFA, and DC/CP were washed three times with PBS (GIBCO BRL) and used to stimulate PBMC of patients in complete remission.

**Murine model of AML and prophylactic vaccination**

All the in vivo experiments were conducted in mice randomized before prophylactic vaccination. DC dose and vaccination schedule were previously determined to provide a high degree of protection from a uniformly lethal dose of syngeneic C1498 injected intravenously. For the calibration of the murine model, C57Bl/6 syngeneic mice ( $n = 5$  per group) were injected intravenously with C1498 cells varying from  $2 \times 10^4$  to  $5 \times 10^5$ . The lethal dose of C1498 AML cells ( $6 \times 10^4$ ) was determined by using three times the dose that induced 60% of mortality ( $2 \times 10^4$  cells). The immunization protocol consisted of an intraperitoneal injection of  $10^5$  mature DC loaded with TFA- or CP-peptides extracted from  $10^7$  C1498 cells in 200  $\mu\text{L}$  of PBS on day  $-20$  and  $-10$  prior to the injection of the C1498 lethal dose. Control mice received PBS alone, unloaded mature DC, or eluted peptides alone extracted from  $10^7$  C1498 cells.

**Patients**

AML cells were collected from the blood of two AML 5, and one AML 1 (FAB subtype) patients at diagnosis, namely P1, P2, and P3, after they had given informed consent. Patients P1, P2, and P3 had 60, 95, and 91% of circulating blasts, respectively. The local ethics committee approved the protocol. Fifty milliliters of total blood from patients with hyperleucocytosis were separated by Ficoll-Hypaque gradient (Pharmacia Biotech, Uppsala, Sweden). The leukocyte layer contained a majority of leukemia cells (checked by cytological, cytogenetic, and fluorescent analysis) (Table 1). A total of  $100 \times 10^6$  AML cells were frozen and stored in liquid nitrogen. The rest of the cells were stored as a cell pellet at  $-80^{\circ}\text{C}$  until their use for acid elution treatment. PBMC were isolated from blood of patients in complete remission after standard chemotherapeutic protocols and blood leukocytes were collected. A monocyte-enriched population was used for in vitro generation of mature DC, while lymphocytes were cultured for generation of antileukemia T cell lines in vitro.

**Table 1** Clinical characteristics of AML patients at diagnosis and outcome

| Patient | Age at diagnosis | FAB type | Leucocytosis (10 <sup>9</sup> /l) | Percentage of circulating blasts | Obtention of complete remission |
|---------|------------------|----------|-----------------------------------|----------------------------------|---------------------------------|
| P1      | 48               | AML5     | 32                                | 60                               | Yes                             |
| P2      | 60               | AML5     | 92                                | 95                               | Yes                             |
| P3      | 54               | AML1     | 25                                | 91                               | Yes                             |

#### In vitro stimulation of human lymphocytes with DC/TFA or DC/CP

PBMC ( $4 \times 10^6$ ) from patients in complete remission were seeded in culture with  $4 \times 10^5$  autologous mature DC pulsed with peptides extracted from  $4 \times 10^6$  AML cells in 2 ml of RPMI 1640 medium. This co-culture was then supplemented with 10% human AB serum (PAA Laboratories GmbH, Linaz, Austria) in a 24-well culture plate (Costar, Cambridge, MA) at 37°C in a 5% CO<sub>2</sub> atmosphere. On day 4, 20 U/ml of rhIL-2 (Roche, Meylan, France) and 5 ng/ml of rhIL-7 (PeproTech Inc, Tebu) were added. Lymphocytes were submitted to four stimulations once a week with freshly prepared autologous DC/TFA or DC/CP and maintained in culture for 4 weeks. rhIL-2 and rhIL-7 were added every 4 days. T lymphocytes stimulated with DC/TFA or DC/CP are referred to as DC/TFA-T cell lines, and DC/CP-T cell lines, respectively. After four in vitro stimulations with autologous DC/TFA or DC/CP, cultured lymphocytes were positively sorted into CD4<sup>+</sup> or CD8<sup>+</sup> T cells (> 90% purity) using mAb-conjugated magnetic beads according to the manufacturer's instructions (Myltenyi Biotec), and each lymphocyte subpopulation was studied in functional assays (proliferation, IFN- $\gamma$  production, and cytotoxicity).

#### Interferon- $\gamma$ ELISPOT assay

After 28 days of culture, CD4 and CD8 T cell subpopulations were tested for interferon- $\gamma$  (IFN- $\gamma$ ) production in response to various targets: normal autologous PBMC, autologous unloaded mature DC, autologous AML cells, and allogeneic AML cells. T cell lines alone were used to measure the background signal. Nitrocellulose plates (96-well) (Millipore, Bedford, MA) were coated with 1  $\mu$ g/ml capture mouse IgG<sub>1</sub> anti-human IFN- $\gamma$  mAb (1-D1K, Mabtech, Sweden). 50,000 T cells were co-cultured with 5,000 target cells for 20 h at 37°C in 5% CO<sub>2</sub> atmosphere. Released cytokine molecules were trapped by adding 1  $\mu$ g/ml biotinylated mouse IgG<sub>1</sub> anti-human IFN- $\gamma$  mAb (7B6-1, Mabtech) for 2 h at room temperature, followed by incubation with alkaline phosphatase-conjugated streptavidin (Extravidin<sup>®</sup>, 1/3,000 diluted, Sigma). Spots were revealed with 5-bromo-4-chloro-3-indolyl-phosphate-4-toluidine substrate (Sigma). Spots were counted with a computer-assisted video image analyzer (KS ELISPOT, ZEISS, Jena, Germany). A positive value was assigned to spot frequencies greater than the mean background of the assay plus two standard deviations.

#### In vitro cytotoxic responses

**In mice** Cytotoxic responses were evaluated with a 4 h chromium (<sup>51</sup>Cr)-release cytotoxicity assay. Seven days after tumor challenge, spleen cells from mice that received DC or nonvaccinated controls were harvested, and prepared at  $1.5 \times 10^6$  per ml in complete medium. Cells were cultured in 100-mm petri dishes with  $1.5 \times 10^5$  irradiated C1498 and 1 ng of rhIL-2 (Roche, Meylan, France) in 20 ml final, at 37°C for 6 days. At days 2 and 5, rhIL-2 was added. At the end of the culture, cells were harvested, purified through a Ficoll-Hypaque (Pharmacia Biotech), washed in PBS, and were thereafter referred to as effector cells.  $5 \times 10^3$ /100  $\mu$ l of <sup>51</sup>Cr-labeled target cells (100  $\mu$ Ci for  $2 \times 10^6$  cells), either C1498, allogeneic control A20, or NK-sensitive YAC-1 cells were incubated with the effector cells in 96-well plates (Costar, No. 3799, Cambridge, MA). After 4 h incubation, 50  $\mu$ l of supernatant was collected and <sup>51</sup>Cr release was measured with a  $\gamma$ -scintillation counter (1450 Microbeta plus, Wallac).

**In humans** Stimulated CD8<sup>+</sup> T lymphocytes were studied for their potential cytotoxicity against autologous AML cells in a 4 h <sup>51</sup>Cr-release cytotoxicity assay. A total of  $5 \times 10^3$  <sup>51</sup>Cr-labeled AML cells were co-cultured with effector cells. After 4 h of co-culture, 50  $\mu$ l of supernatant was collected and <sup>51</sup>Cr release measured with a  $\gamma$ -scintillation counter (1450 Microbeta plus).

For all experiments, both in mice and humans, spontaneous and maximal releases were defined by the incubation of target cells in complete medium in the absence or presence of 10% HCl. The spontaneous/maximal release ratio was <20% in all experiments. Specific lysis (%) was calculated as (experimental <sup>51</sup>Cr release – spontaneous <sup>51</sup>Cr release)/(maximal <sup>51</sup>Cr release – spontaneous <sup>51</sup>Cr release)  $\times$  100.

#### Statistical analysis

For the study in mice, the Kaplan–Meier product-limit method was used to calculate survival rates. Differences between groups were determined using the generalized Log rank test. Survival data are also presented as median survival time (MST), the time point when half of the mice remain alive. For the study in humans, statistical analysis was performed using the Student's *t* test. For all statistical analysis, *P* values < 0.05 were considered statistically significant.

## Results

Differences in C1498-eluted peptide composition according to the elution procedure

To examine the peptide composition extracted from C1498 cells with the two main acid buffers currently

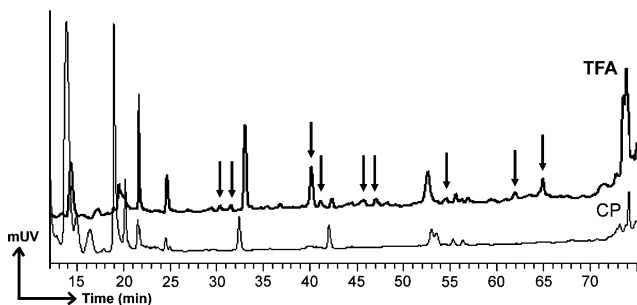


used, we compared the RP-HPLC elution profiles of TFA- and CP-eluted peptides. We observed that the RP-HPLC elution profiles of peptides eluted from C1498 cells differed according to the acid buffer used (Fig. 1). More peaks were observed in the elution profile of TFA-eluted peptides than in the CP-eluted peptides. However, each acid-elution technique led to superposable profiles over time demonstrating that this technique is highly reproducible (data not shown).

These results show that the composition of acid-eluted peptides was different depending on the buffer used, and that elution using TFA buffer generated a higher diversity of peptides from the C1498 cells than elution using CP buffer.

#### Vaccination of mice with DC/TFA or DC/CP induced cytotoxic antileukemia immune response ex vivo

Differences in the composition of peptides extracted from C1498 cells by TFA- and CP-treatment may result in differences in the capacity of these pools of peptides to induce antileukemia immune responses. To address this question, we considered whether CTL elicited by various immunization regimens in mice were equally able or not to lyse specifically syngeneic C1498 cells. Mice were immunized intraperitoneally with CP-eluted peptides, TFA-eluted peptides, unloaded mature DC, DC/CP, and DC/TFA at days -20 and -10 before challenge with C1498 leukemia cells. We observed that CTL derived from mice immunized by mature DC, DC/CP or DC/TFA were more effective for lysing the C1498 cells (69, 72, and 59% lysis, respectively) than CTL derived from mice immunized by CP- or TFA-peptides alone (50 and 45% lysis, respectively) or with PBS (28% lysis) (Fig. 2). A marked NK cytotoxicity against YAC-1 cells and a nonspecific lysis of allogeneic A20 cells were also detected in all groups. However, this nonspecific cytotoxicity was higher in splenocytes isolated from mice immunized with DC/CP (58% lysis of YAC-1) compared to those isolated from mice immunized with DC/TFA



**Fig. 1** RP-HPLC profiles of eluted peptides extracted from murine C1498 cells with TFA or CP buffer. Peptides were eluted from the same sample of murine C1498 myelomonocytic cell lines using TFA (*bold line*) or CP (*fine line*) buffer. The pools of leukemia-associated peptides were fractionated by RP-HPLC on an ACN gradient for 80 min. The TFA-elution procedure generated greater diversity of peptides

(36% lysis). The specific lysis of C1498 was significantly different from the nonspecific lysis of YAC-1 in the DC/TFA, the DC/CP and the TFA-peptides groups. However, DC/TFA immunization induced CTL with a higher specificity against C1498 cells than the DC/CP immunization ( $P < 0.01$  for DC/TFA group, and  $P < 0.05$  for the DC/CP group).

These results demonstrate that both TFA- and CP-peptides loaded on mature DC are able to induce antileukemia immune responses in mice.

#### Vaccination of mice with DC/TFA but not with DC/CP prevented leukemia progression

Since the strength of ex vivo T cell responses was comparable in mice immunized with either DC/TFA or with DC/CP, we examined whether these two vaccine strategies would induce a similar immune protection in vivo. To answer this question, we evaluated the efficacy of a prophylactic vaccination using TFA or CP-eluted peptides loaded (or not) on mature DC to prevent leukemia outgrowth. At days -20 and -10, groups of mice received two injections of either TFA- or CP-peptides alone, or mature DC loaded with either TFA- or CP-peptides, or PBS, or unloaded mature DC. Mice were then challenged with the syngeneic C1498 cells at day 0. Vaccination with TFA-peptides alone (MST 28 days) or unloaded mature DC (MST 35 days) induced a significant survival advantage compared to the PBS control group ( $P = 0.047$  and  $0.0005$ ) (Fig. 3). Interestingly, immunization with CP-peptides alone (MST 29,5 days), or mature DC/CP vaccines (MST 30 days) were not effective to induce a protective immune response, since the survival of these two groups were not significantly different from the survival of the PBS control group (MST 27 days). On the other hand, we observed a prolonged survival of mice vaccinated with DC/TFA (MST not reached) as compared to the TFA-peptides vaccinated group ( $P < 0.001$ ), to the mature DC vaccinated group ( $P = 0.02$ ), and to the DC/CP vaccinated group ( $P = 0.0007$ ).

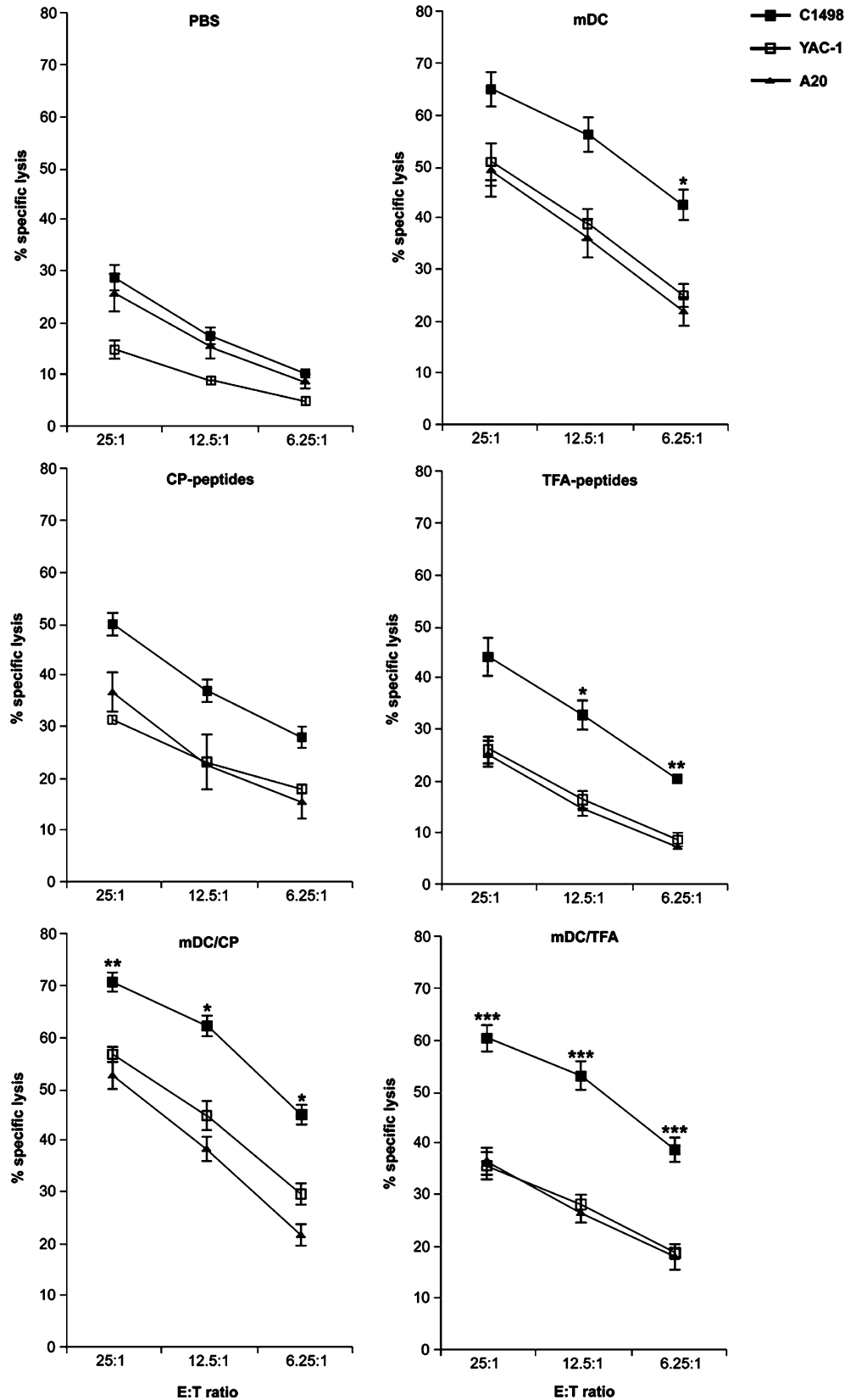
Furthermore, the weight of mice with prolonged survival after immunization remained remarkably stable over time suggesting the absence of auto-immune disease (data not shown).

These results demonstrated that DC/TFA was the more effective vaccine able to induce a prolonged survival in vivo.

In AML patients, in vitro repeated stimulations of remission lymphocytes with DC/TFA, but not DC/CP, induced specific antileukemia immune responses mediated by IFN- $\gamma$  secreting T cells

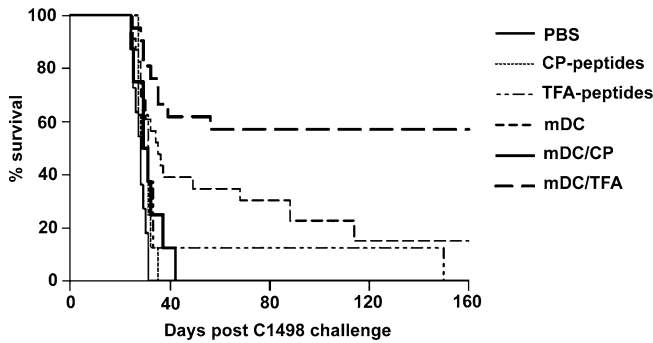
We have previously demonstrated that in vitro stimulations of PBMC obtained from patients in complete remission with mature DC loaded with peptides eluted by TFA from autologous leukemia cells induced a patient-specific CD4<sup>+</sup> and cytotoxic CD8<sup>+</sup> antileukemia

**Fig. 2** Specific CTL activity induced by various types of immunization. Mice were immunized at days - 20 and - 10 with PBS ( $n = 6$ ), CP-peptides ( $n = 4$ ), TFA-peptides ( $n = 4$ ), unloaded mature DC ( $n = 10$ ), DC/CP ( $n = 6$ ), or DC/TFA ( $n = 10$ ) before the injection of a lethal dose of C1498 cells. Seven days post-tumor cells challenge, spleen cells from recipient mice were harvested and stimulated in vitro with irradiated C1498 cells and rhIL-2. After 6 days of culture, cytotoxic activity was determined by 4-h  $^{51}\text{Cr}$  release cytotoxicity assay using C1498, A20, and YAC-1 as target cells. The results show the mean of three independent experiments performed on the pool of splenocytes isolated from a variable number of vaccinated mice (4-10 mice). Vaccination with DC/TFA induced a more specific recognition of autologous C1498 cells than DC/CP. \* $P < 0.05$ , \*\* $P < 0.02$ , and \*\*\* $P < 0.01$



immune response [9]. However, the results obtained in mice in the present study led us to ask whether the leukemic peptide-acid elution procedure could influence the

quality of human antileukemic immune responses. We therefore compared the ability of DC/TFA versus DC/CP in inducing autologous AML-specific  $\text{CD4}^+$  and



**Fig. 3** Survival of mice immunized with the different vaccines in a prophylactic setting. DC were derived from bone marrow cells after 6 days of culture with GM-CSF and IL-4. On day 7, CD11c positive cells were matured 6 h with LPS and loaded with CP-peptides or TFA-peptides for the last 90 min of maturation. PBS ( $n = 11$ ), unloaded mature DC ( $n = 23$ ), CP-peptides alone ( $n = 8$ ), TFA-peptides ( $n = 8$ ), DC/CP ( $n = 8$ ), or DC/TFA ( $n = 21$ ) were administered intraperitoneally 20 and 10 days prior to tumor challenge. Peptides eluted from  $10^7$  C1498 cells and  $10^5$  DC were used for each mouse. Neither DC/CP vaccination (MST of 30 days) nor CP-peptide vaccination (MST of 28 days) was able to prolong the survival of mice compared to the nonvaccinated group ( $n = 11$ , MST 28 days). In contrast, mice receiving DC/TFA had a significantly improved survival compared with the control group of nonvaccinated mice ( $P < 0.0001$ ). Results shown here are the cumulative results of two separate experiments

CD8<sup>+</sup> T cells in vitro. PBMC collected from the blood of two patients in complete remission were stimulated once a week, four times with DC/TFA or DC/CP to obtain primed lymphocytes referred to as DC/TFA-T cell lines and DC/CP-T cell lines respectively. After 28 days of culture, the resulting T cell lines were fractionated into CD4<sup>+</sup> and CD8<sup>+</sup> T cells by positive selection for the in vitro assays.

ELISPOT analysis demonstrated that PBMC repeatedly stimulated by DC/TFA contained a higher number of AML-specific IFN- $\gamma$ -secreting CD4<sup>+</sup> T cells ( $P < 0.02$  for patient P1) (Fig. 4a), and AML-specific IFN- $\gamma$ -secreting CD8<sup>+</sup> T cells ( $P < 0.05$  for patient P2) (Fig. 4b) than PBMC repeatedly stimulated by DC/CP. Surprisingly, we observed for the two patients analyzed that DC/CP-T cell lines contained CD4<sup>+</sup> T cells which also recognized autologous unloaded mature DC (Fig. 4a). This was also observed with CD8<sup>+</sup> T cells from the DC/CP T cell line from patient P2 (Fig. 4b). In contrast, INF- $\gamma$  production by both CD4<sup>+</sup> and CD8<sup>+</sup> T cells isolated from lymphocytes repeatedly stimulated with DC/TFA were highly specific for autologous AML cells, and responded weakly to the other autologous targets (Fig. 4a, b).

These results suggest that TFA-peptides loaded on mature DC were able to generate in vitro a more specific CD4<sup>+</sup> and CD8<sup>+</sup> antileukemia immune response. Indeed, the antiself recognition was more obvious when using CP-peptides for loading DC than using TFA-peptides.

In AML patients, repeated stimulation of remission lymphocytes with DC/TFA was more efficient at generating antileukemia cytotoxic CD8<sup>+</sup> T cell immune responses than DC/CP

To analyze the cytolytic potency of CD8<sup>+</sup> T cells isolated from lymphocytes repeatedly stimulated with either DC/TFA or DC/CP, we compared their ability to lyse autologous AML cells. CD8<sup>+</sup> T cells were positively isolated after four DC/TFA or DC/CP in vitro stimulations of T lymphocytes from patients in complete remission. Cr<sup>51</sup>-release assay showed that CD8<sup>+</sup> T cells isolated from DC/TFA-T cell lines were able to lyse significantly more efficiently autologous AML cells compared to CD8<sup>+</sup> T cells isolated from DC/CP-T cell lines ( $P < 0.05$ – $0.001$  at all  $E:T$  ratios) (Fig. 5a).

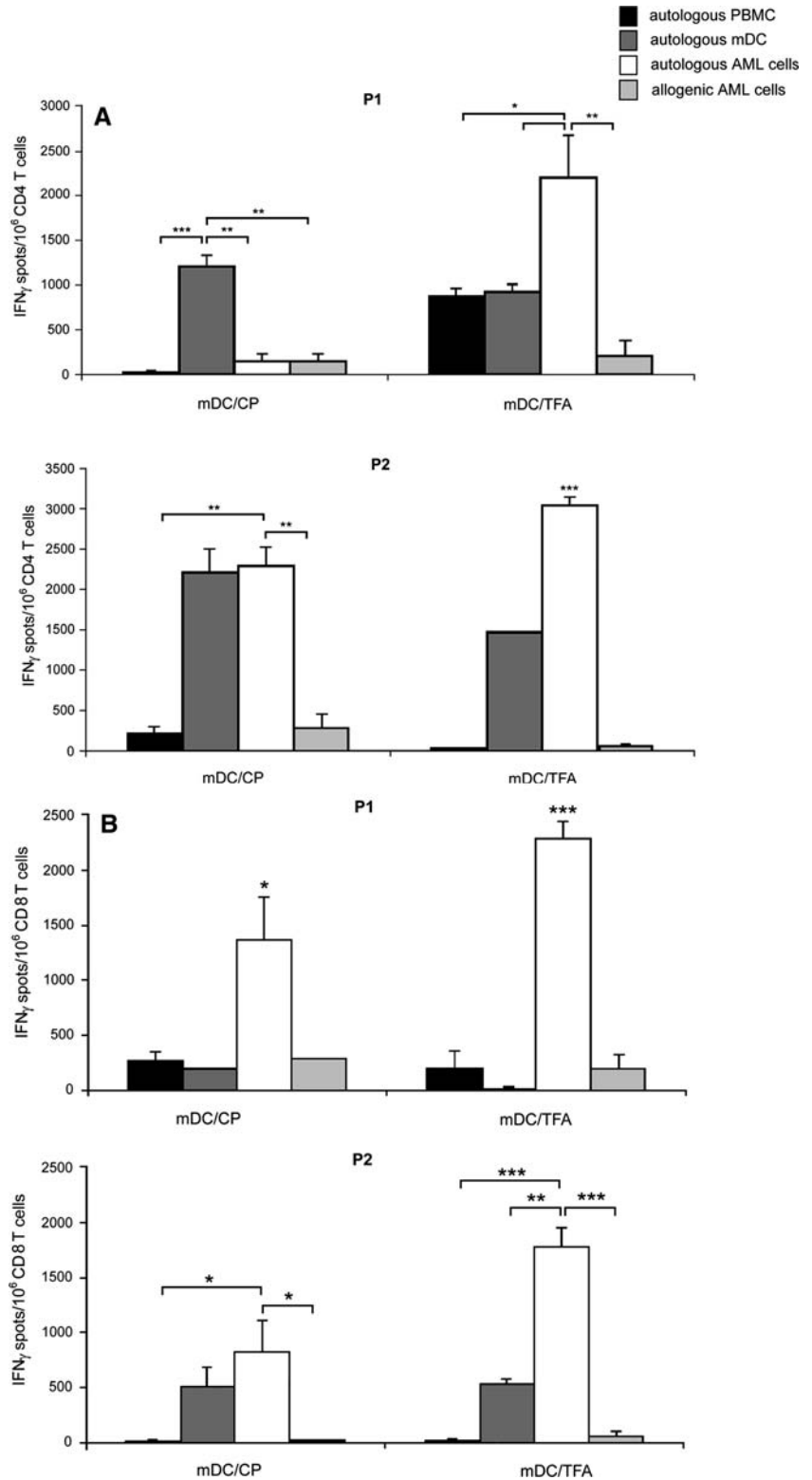
In order to confirm the specificity of the response induced by in vitro DC/TFA stimulation, we evaluated by ELISPOT and cytotoxic assays the recognition of the CD8<sup>+</sup> T cells isolated from a DC/TFA cell line of patient P3 against various targets. The ELISPOT IFN- $\gamma$  assay showed that the CD8<sup>+</sup> T cells produced IFN- $\gamma$  in response to DC/TFA or autologous AML cells but not in response to normal autologous targets as unloaded mature DC, PBMC, and PHA blasts or to allogeneic AML cells (Fig. 5b). Furthermore, the CD8<sup>+</sup> cells of patient P3 were cytotoxic against autologous AML cells but not against allogeneic AML cells or NK sensitive K562 cell line as controls (Fig. 5c).

These results suggested that stimulation with DC/TFA was more efficient than DC/CP at inducing specific CD8<sup>+</sup> T cells able to recognize autologous AML cells, and gave a specific antileukemia immune response in vitro.

## Discussion

Induction of a specific antileukemia cell immune response in AML patients during the period of minimal residual disease might improve the life-threatening evolution of this disease, which often relapses within weeks or months. We have previously established in vitro that autologous mature DC loaded with peptides eluted by acid elution from leukemia cells could efficiently generate an antileukemia immune response from patients' remission lymphocytes [9]. Two acid elution techniques with either TFA or CP acidic buffers are well documented in the literature [11, 37]. The goal of the present study was to compare their relative efficiency in generating specific antileukemia cell immunization. Furthermore, since immunization with peptides eluted from autologous cells has the potential drawbacks of generating an antiself detrimental response, we also examined the autoimmune responses generated using these two peptide-elution techniques. This comparative study was performed in two different experimental settings: first, in vitro and in vivo, using the murine AML model based upon injection

**Fig. 4** Human antileukemia IFN- $\gamma$ -secreting CD4<sup>+</sup> and CD8<sup>+</sup> T cell immune responses induced by in vitro stimulation with DC/TFA or DC/CP. IFN- $\gamma$  ELISPOT analysis was performed on CD4<sup>+</sup> (a) and CD8<sup>+</sup> T cells (b) isolated from DC/TFA and DC/CP T cell lines. Each value represents the mean of triplicate measurements for 10<sup>6</sup> T cells. In vitro stimulation of human lymphocytes with DC/TFA induced more IFN- $\gamma$ -producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells in co-culture with AML cells than stimulation with DC/CP. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$

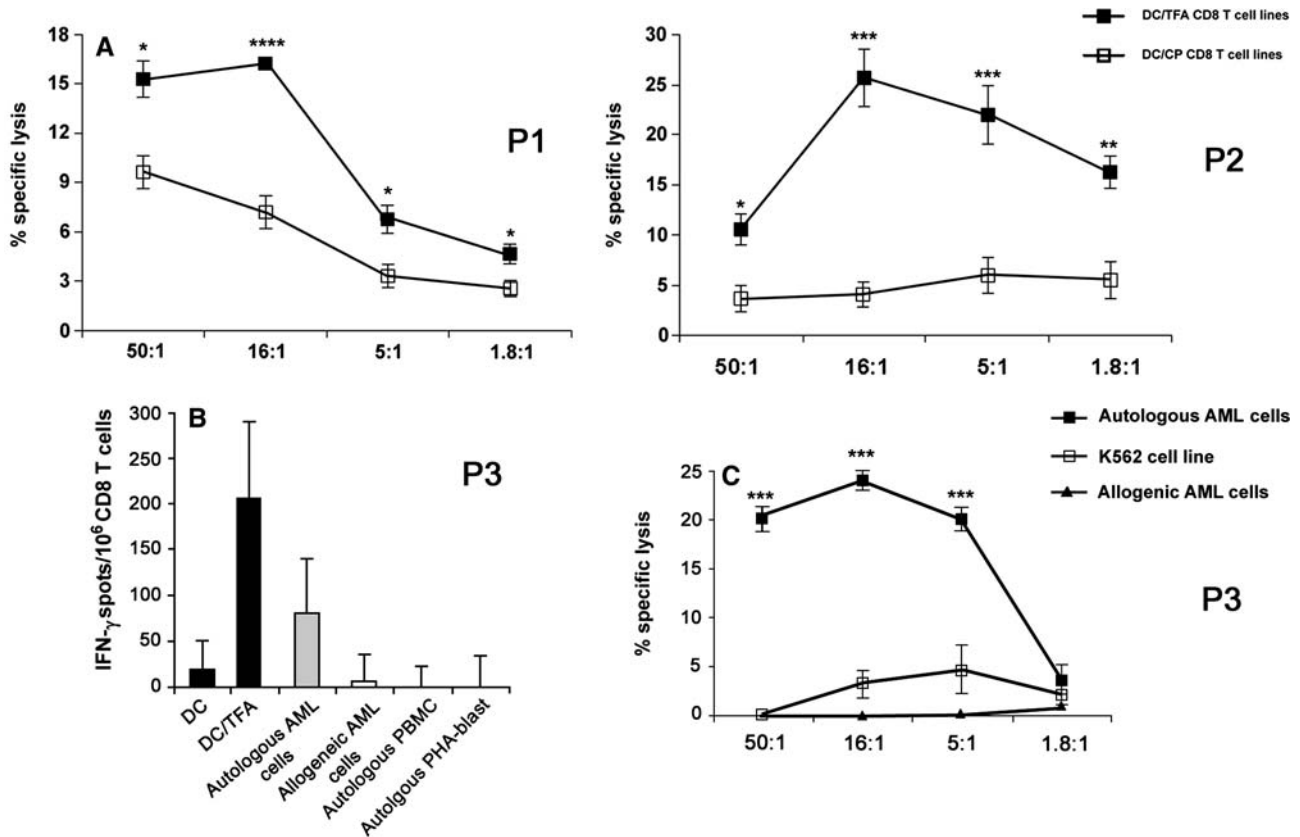


of C1498 cells, and second, in vitro only, using human cells obtained from AML patients in complete remission.

In this study, we have shown that the composition of peptides eluted from AML cells using TFA or CP is different, and influences the quality of the antileukemia immune response. The superiority of DC/TFA in inducing

antileukemia immune responses was demonstrated both in mice and in humans. Indeed, only DC/TFA could efficiently prevent leukemia outgrowth in mice. Furthermore, in vitro preliminary data in humans suggested that DC/TFA induced more specific antileukemia CD4<sup>+</sup> and cytotoxic CD8<sup>+</sup> T cells than DC/CP.





**Fig. 5** Human antileukemia CD8<sup>+</sup> T cell immune responses induced by stimulation with DC/TFA or DC/CP. **a** Cytotoxic activity of CD8<sup>+</sup> T cell lines, isolated from stimulated T lymphocytes of patients P1 and P2, was tested against autologous AML cells at the indicated *E:T* ratios in a <sup>51</sup>Cr release assay. **b** ELISPOT IFN- $\gamma$  of CD8 T cells isolated from a DC/TFA T cell line of patient P3 in response to various targets: autologous mature DC, DC/TFA,

autologous AML cells, allogeneic AML cells, autologous normal PBMC, or autologous PHA blasts (T cells stimulated by phytohemagglutinin A). **c** Cytotoxicity of CD8 T cells isolated from a DC/TFA T cell line of patient P3 against autologous AML cells, NK sensitive K562 cell line, and allogeneic AML cells at the indicated *E:T* ratios in a <sup>51</sup>Cr release assay. \**P* < 0.05, \*\**P* < 0.02, \*\*\**P* < 0.01, and \*\*\*\**P* < 0.001

Acid elution was initially used to identify new TAA [11, 37]. The first demonstration that acid-eluted peptides could be useful for treating cancer came from Bellone et al. [3] who reported that TFA-peptides eluted from poorly immunogenic B16 melanoma cells could be loaded on RMA cells as antigen-presenting cells to obtain antitumor CTL in vitro. Thereafter, several studies extended these results showing that acid-eluted peptides could induce efficient antitumor immune responses both in murine models and in clinical trials. For instance, TFA-eluted peptides loaded on autologous mature DC were shown to stimulate in vitro specific antitumor CTL that were able to induce the rejection of an established ovarian tumor after cellular adoptive immunotherapy in mice [17]. In one representative in vitro experiment in human, DC pulsed with CP-eluted peptides from ovarian tumor cells were able to induce CTL responses against autologous tumor cells [34]. Two clinical trials have validated the safety of using DC loaded with CP-eluted peptides in vaccination of glioblastoma patients, and confirmed that this extraction method had therapeutic applications [24, 43].

However, to our knowledge, no comparative study has reported the superiority of one acid-elution procedure compared to another, for clinical applications. Only one study demonstrated that TFA-eluted peptides from an Epstein-Barr virus-transformed B-lymphoblastoid cell line were more potent at inducing in vitro IFN- $\gamma$  secreting T cells than CP-eluted peptides, although tumor lysate was more immunogenic than eluted peptides in this model [18]. In AML, tumor cell lysates might not be ideal since they could contain immunosuppressive cytokines, such as TGF- $\beta$ , that could impair induction of Th1 immune responses [14] or favor a transient IL-12 secretion insufficient to maintain T cell activation [40]. These above reports led us to investigate the use of more purified tumor-derived material such as acid-eluted peptides from leukemia cells, but this required close examination of the optimal conditions for extracting immunogenic peptides. The need to compare the TFA versus CP elution procedure was prompted by the observation that RP-HPLC profiles of the eluted peptides differed according to the acid buffer used, suggesting variability in the peptide composition generated by these two elution procedures. We observed in our model that

TFA-elution generated a higher diversity of peptides than CP-elution. This result can certainly be explained by the fact that TFA acidity (pH 2.0 to 2.1) can solubilize most peptides contained both in the cytoplasm and on MHC molecules, whereas CP acidity (pH 3.0–3.3) can only release peptides bound to MHC molecules at the cell surface, which possibly preserves cell viability [2]. Furthermore, TFA elution could extract intracellular peptides retained by Hsp molecules, which may favor a more efficient anti-tumor immune response [44].

In our murine model of AML, we demonstrate that a preventive protocol consisting of vaccination with DC/TFA protected mice from leukemia outgrowth, whereas no survival benefit was obtained in mice vaccinated with DC/CP. These results were surprising considering our *in vitro* data which demonstrated that both DC/TFA and DC/CP vaccines can induce cytotoxic antileukemia immune responses. It is possible that stimulated T cells generated following DC/CP were submitted to fast apoptosis *in vivo*. Indeed, it has been reported that multiple injections of DC transduced with the *MART-1* gene in a melanoma model are less efficient in protecting mice than a protocol with one simple injection, due to *in vivo* Fas-mediated apoptosis of specific anti-MART-1 IFN- $\gamma$ -secreting T cells [32]. Another explanation could be that the CP elution procedure is less potent for extracting immunogenic peptides than TFA elution in our murine leukemia model. Moreover, it has been reported that immunotherapy, using DC loaded with CP-peptides eluted from various tumor cells which express peptides derived from lymphocytic choriomeningitis virus glycoprotein, was only efficient when the immunogenic peptides were highly represented on the surface of tumor cells. This suggests a correlation between induction of efficient antitumor immune responses and the amount of tumor-specific peptides expressed at the cell surface [31]. In our study, this hypothesis is substantiated by the fact that TFA treatment generated more peptide diversity than CP treatment, and by the fact that *in vitro* CTL derived from DC/TFA vaccinated mice were more specific for syngeneic C1498 cells than CTL derived from DC/CP vaccinated mice. Nonspecific protection from leukaemia in this model was previously reported by Blazar et al. [4] relying on NK cell activation. We observed that vaccination with unloaded mature DC was able to delay leukemia outgrowth of mice maybe because of an enhanced nonspecific immune response. This relative protection was not observed when mice were immunized by DC/CP, since they died more quickly than with unloaded mature DC vaccination. One hypothesis could be that the loading with CP-peptides changes the functional quality of mature DC. The other hypothesis could be that CP-peptides contain a majority of self-antigens in this model which could have induced auto-immune adverse effects worsening the clinical outcome of these mice.

These results in mice were in good agreement with our *in vitro* results obtained in humans with AML patients' cells. *In vitro*, we observed that DC/TFA was more

efficient than DC/CP stimulation for inducing specific antileukemia immune responses. Indeed, testing the intensity of the antileukemia immune response of patients' primed lymphocytes in terms of IFN- $\gamma$  production (ELISPOT), we observed that the number of AML-specific IFN- $\gamma$ -CD4<sup>+</sup> and CD8<sup>+</sup> T cells was significantly higher when T cell lines were stimulated with DC/TFA compared to DC/CP (Fig. 4a, b). In the present study, TFA-eluted peptides from autologous AML cells were the most efficient in stimulating human antileukemia immune responses, especially since we observed that only stimulation of T cell lines by DC/TFA was able to induce cytotoxic CD8<sup>+</sup> T cells against autologous AML cells (Fig. 5a). Such results may appear at variance with our previous results, which demonstrated in humans that the use of CP-eluted peptides could induce antileukemia immune responses *in vitro* [27]. However, this observation was in an allogeneic setting where the antitumor immune response was elicited mostly upon allogeneic recognition of minor histocompatibility antigens [27].

An important goal of the present study was to evaluate the fine specificity of the antitumor immune responses induced by stimulation of T lymphocytes with human mature DC/peptides, since one major concern with the use of such AML cell-eluted peptides is the possible triggering of anti-self reactive lymphocytes, which might induce autoimmune pathologies. We have previously shown that *in vitro* stimulation of T lymphocytes with DC/TFA was able to induce antileukemia CD4<sup>+</sup> and CD8<sup>+</sup> immune responses specific of autologous AML cells with minimal anti-self mature DC and PBMC responses [9]. In the present study, we extended these data by comparing the fine specificity of antileukemia immune responses induced by either human DC/CP or DC/TFA. Note worthily, evaluation by IFN- $\gamma$  ELISPOT assay of repeated *in vitro* stimulation by DC/CP of lymphocytes from patient in complete remission generated CD4<sup>+</sup> and CD8<sup>+</sup> T cells which showed that they recognized autologous unloaded mature DC, but not autologous PBMC. In sharp contrast, autoimmune recognition by T cells repeatedly stimulated with DC/TFA was less marked, since recognition of autologous normal PBMC or unloaded mature DC was not statistically comparable to recognition of autologous leukemic cells. These results suggested that the immune response induced by DC/TFA was more specific for autologous AML cells, whereas DC/CP stimulation induced recognition of the monocyte lineage, undetectable in the resting PBMC. The problem of anti-self reactivity is inherent in all immunotherapeutic strategies using autologous whole tumor cells containing potential autoantigens. Indeed, the efficacy of antitumor immuno-intervention relies upon the balance between autoimmune recognition and the specific antitumor immune response, which may vary depending on the target cancer cells, and the type of vaccinating agents (peptides, cell lysates, genetically modified cells, etc.) [39]. This is well illustrated by the treatment of leukemia patients with bone marrow grafting when the graft-versus-leukemia effects are associated to the graft-versus-host disease

[6]. This is a critical issue for antitumor immunity, and it remains a major concern for most vaccination protocols in cancers. The benefit of vaccination compared to the risk of inducing an autoimmune disease has been evaluated in many clinical trials, and has always been found to be moderate or absent. Moreover, treatments using passive immunization with monoclonal antibodies against self-antigens have already been successful in objectively shrinking established tumors in patients with cancer, without serious toxicities [20, 30].

Altogether, our results suggest that acid-eluted peptides could be used in immunotherapeutic protocols in the AML setting, and show the importance of carrying out preclinical studies to define the best strategies of mature DC loading tailored to the tumor to be treated. Loading DC with TFA peptides might be preferred in future vaccination trials for high-risk AML patients.

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