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International Journal of Cancer Research and Treatment

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Ex Vivo Expansion of Antitumor Cytotoxic Lymphocytes with Tumor-associated Antigen Loaded Dendritic Cells

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Abstract. *Cell therapy with lymphocytes is an attractive approach for cancer immunotherapy. Methods to generate ex vivo effector cells directed against whole autologous tumor antigens are under investigation. Our procedure involved stimulation of autologous lymphocytes with antigen-pulsed dendritic cells (DC). Experimental conditions were established with DC, maturated with TNFα, LPS and CD40L, from healthy donors and the M74 melanoma cell line. DC were pulsed with either irradiated, apoptotic or necrotic tumor cells or fused with tumor cells. Increase of lymphocyte cytotoxicity and IFNγ production were repeatedly observed with tumor cells-loaded DC. Stimulation of tumor-associated antigen-specific lymphocytes was clearly shown. MelanA-MART1 (dominant melanoma-associated antigen) tetramer staining revealed a high frequency of specific T cells. Lymphocytes were able to efficiently lyse MelanA-MART1-pulsed T2 target and MelanA-expressing target cells (M74) after CD56+ cells depletion. We confirmed with other tumor cell lines that this DC-mediated procedure induced activation of cytolytic lymphocytes.*

T lymphocytes, NK and NKT cells are the final effector cells of a successful antitumor immune response. The concerted action of these classes of lymphocytes participates in the natural immune defense against tumor development. Each class exhibits distinct mechanisms capable of killing tumor cells. Different factors influence the precursor frequency of antigen-specific T cells and their differentiation into effector memory cells. The most effective activators of T cells are the professional antigen-presenting dendritic cells (DC)(1). DC elicit a polarized Th1 type T cell response (2, 3). Interestingly, *in vitro*, DC can acquire antigen from apoptotic cells and induce class I-restricted cytolytic lymphocytes (CTL) (4, 5). Induction of T CD8 CTLs is the goal of cancer vaccine strategies. Several findings have emerged from experimental studies with model antigens and DC (6). Experimental vaccination with tumor-antigen-presenting DC have induced tumor-specific T cell response (7). Occasional clinical regressions have been noted in initial feasibility studies, particularly in melanomas (8, 9), prostate cancers (10) and renal cell cancers (11). Most current vaccination protocols are focused on DC charged with synthetic tumor peptides in a specific HLA-binding context. DC maturation, an important step for protective activity against tumor development (9, 12), is induced by inflammatory and microbial stimuli (3, 13, 14). It has recently been shown that exposure to necrotic tumor cells induces maturation of immunostimulatory DC (15) but the involved mechanisms are still under investigation (16). Our objective was not to obtain DC to be re-injected but rather to focus on the production of ready-to-use effector cells for adoptive immunotherapy. The advantage of passive immunotherapy stategies stems from the fact that both phenotypic and functional activities of the cells can be controlled before injection (17, 18). *In vivo*, cancerous cells are heterogeneous in a tumor so, depending on their class I expression, NK, NKT or antigen-specific T cells would be of therapeutic interest. The methods currently used for *ex vivo* lymphocyte expansion have not been developed for their efficacy to expand tumor-specific CTL (19-23). Nevertheless, a recent trial in melanoma patients

Abbreviations: CTL, cytolytic T lymphocytes; FACS, fluorescence-activated cell sorter; Ab, antibody; BSA, bovine serum albumin ; PBS, phosphate buffered saline; NK: natural killer cells.

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Key Words: Dendritic cell, human, CTL, tumor immunity, antigen presentation.

demonstrated that the clinical efficacy of tumor infiltrating lymphocytes (TIL) was related to the percentage of tumor associated antigens (TAA)-specific T cells in the cell therapy product (18, 23).

Our approach was centered on the processing of whole tumor cell antigens. This method avoids selection of tumor cells subsequent to specific targeting of only one TAA. Our method can be applied no matter what HLA-type the patient expresses. DC were used to process and present whole TAA and provide co-stimulatory molecules. Our procedure was a coculture of DC and autologous lymphocytes in a controlled cytokinic environment. The objective of the work was to compare different treatments of tumor cells for an optimized TAA presentation by DC. Irradiation and treatment of tumor cells with apoptosis and necrosis inducing factors (sodium butyrate and hydrogen peroxide) before pulsing the DC and fusion of tumor cells with DC were compared for their ability to generate antitumor cytolytic effector cells. The experiments were conducted with HLA-A2 healthy volunteers and the M74 melanoma cell line as source of TAA. The experimental procedures were developed with clinical use in mind: effector cells need to be numerous, quite alive and are intended to continue to proliferate *in vivo* after injection.

Materials and Methods

Healthy donors. Peripheral blood samples were collected from ten different HLA-A2 healthy donors (EFS, Rennes, France). The cells were centrifuged on density gradient (UNISEP®, Novamed, Jerusalem, Israel). Mononuclear cells (MNC) were frozen in human serum albumin and 10% DMSO until use for DC and lymphocyte preparation.

Tumor cells. The HLA-A2 cell lines used in this study were: the MelanA-Mart1-expressing M74 and SK23 melanoma cell lines, the transfer associated with antigen processing (TAP) protein deficient HLA T2 cells, the human MCF7 breast adenocarcinoma cell line and the human R131 renal cell carcinoma cell line (established in our laboratory). These cell lines and the K562 NK-sensitive erythroleukemia cell line were maintained in RPMI 1640 medium (Eurobio, Les Ulis, France) containing 10% fetal calf serum (FCS)(Gibco, InVitrogen Corporation, Germany), 2% L-glutamine, 50 μg/mL streptomycin and 50 IU/mL penicillin (ICN Biomedicals, Aurora, USA).

M74 cells were used for tumor antigen DC pulsing (DC-Tu) either after 150 grays irradiation (M74irr) or by means of apoptosis and secondary necrosis-inducing treatment. Sodium butyrate treatment was adapted from Grégoire (24). Briefly, cells were treated with 5 mM sodium butyrate (Sigma-Aldrich) for three consecutive days. Supernatant cells were collected each day, pooled and kept at 4˚C. The collected cells (M74but) were used for DC pulsing. Hydrogen peroxide 10 μM (Sigma-Aldrich) treatment was done with a similar procedure (M74per) adapted from Lennon (25). Controls were performed with untreated M74 cells.

The SK23, MCF7 and R131 cells were treated with hydrogen peroxide before DC pulsing (DC-Tu per).

DC culture. DC were prepared from MNC according to the method described by Sallusto and Lanzavecchia (26). Briefly, 10.10^6 MNC were seeded in 5 ml serum-free X-Vivo 10 medium (Biowhittaker, Maryland, USA) in a 25-cm^2 culture flask (Cellstar®, Greiner Labortechnik, Frickenhausen, Germany). Non-adherent cells were collected after 2h for lymphocyte culture. Remaining adherent cells were cultured in DC medium: serum free X-Vivo 10 medium supplemented with 10% AB serum (EFS de Rennes), 50 μg/mL streptomycin and 50 IU/mL penicillin. 1000 IU/mL GM-CSF (Leucomax 400™ Novartis/Shering Plough, Switzerland) and 400 IU/mL IL-4 (Biosource International, CA, USA) were added in days 0, 2 and 5 of culture. DC were collected after seven days (immature DC: imm DC) and seeded in DC medium (density 10^6 cells/mL) in 24-well plates (Falcon®, Becton Dickinson, NJ, USA). Treated or untreated tumor cell lines (ratio 10:1), TNFα (25 ng/mL) (Pharmingen, CA, USA), LPS (10 μg/mL) (Sigma-Aldrich, Saint Quentin Fallavier, France) and CD40L (0.4 μg/mL) (Apotech Biochemicals, Alexis Corporation) were immediately added to DC for both antigen processing and maturation of DC. After an 18 h contact, supernatant cells (maturated DC: mat DC) were collected and added to lymphocytes in a 1:100 DC:lymphocyte ratio. DC were phenotypically characterized before lymphocyte stimulation (day 8).

Phagocytosis assays. Fluorescence was quantified in DC after phagocytosis of fluorescent latex microspheres according to the Atanassov method (27). Microspheres, fluoresbrite plain microspheres® 0.52 μm in diameter (Polysciences, PA, USA), were washed with water and centrifuged (4000 $trs.min^{-1}$ 5 min) before suspending the pellet in FCS (microspheres final dilution: 1/6) and submitting the final solution to 10 min ultrasound before use. 10^5 DC were incubated for 1 h with 10 μL microspheres. Cell fluorescence was analyzed by flow cytometry.

Fusion of DC and tumor cells. ImmDC were incubated for 5 min with M74 in a 3:1 ratio in 50% diluted polyethylene glycol (Sigma-Aldrich) as adapted from Gong (28). Hybrids (DC-Fu) were washed twice with RPMI and submitted to the 18 h DC maturation procedure as described above. DC-Fu were used for lymphocyte stimulation. Hybrids were analyzed by flow cytometry: DC were stained with PE conjugated anti-HLA-DR mAb and M74 with FITC anti-HLA-ABC mAb separately before fusion. Additive microscopic observations were performed after labelling of DC and tumor cells with, respectively, red or green fluorescent probes (CellTracker™, Molecular Probes, OR, USA). Cytospin was done and DAPI-antifading dye (Oncor, MD, USA) was applied before observation with an epifluorescence microscope. Tumor cells fused with DC appeared as yellow.

Lymphocyte culture. Lymphocytes were cultured from non-adherent MNC in lymphocyte medium: RPMI 1640 containing 10% AB serum, 2% L-glutamine (Biowhittaker, Maryland, USA), 100 μg/mL streptomycin, 100 IU/mL penicillin and 150 IU/mL IL-2 (Proleukin®, Chiron, Suresnes, France). After 8 days in culture (density 10^6 cells/mL), lymphocytes were stimulated with antigen-pulsed DC or simply with irradiated tumor cells. The number,

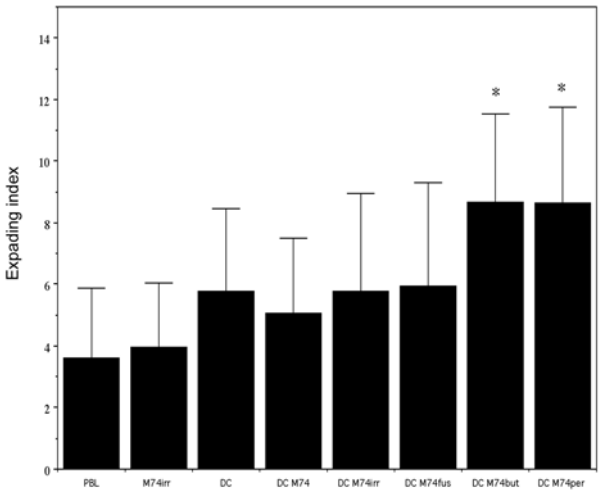

Figure 1. *Expanding index (EI) of lymphocytes seven days after stimulation with TAA-pulsed DC. EI was day 7/day 0 cell count. PBL were stimulated with: DC pulsed with non-irradiated (DC M74), irradiated tumor cells (DC M74 irr) or pulsed with butyrate (DC M74but) or peroxide (DC M74per) treated tumor cells; DC fused with tumor cells (DC M74fus). Controls were non-stimulated PBL (PBL) and PBL stimulated with irradiated tumor cells (M74irr) or with non-pulsed DC (DC). Data are the mean EI of assays conducted with PBL from 7 different donors. *significantly different from PBL with p<0.01.*

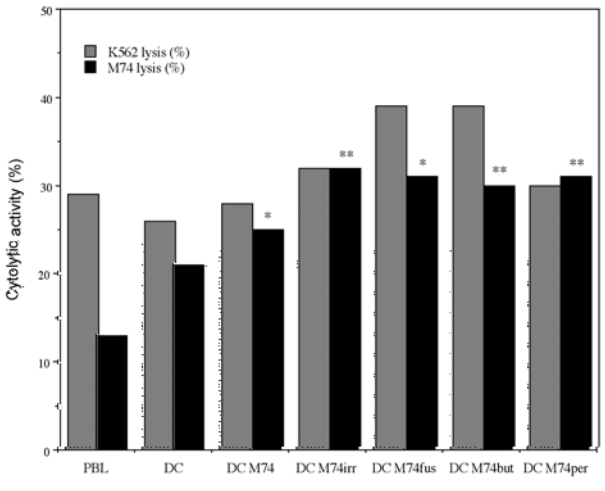

Figure 2. *Cytolytic activity against M74 and K562 (NK sensible) tumor cell line. PBL were stimulated with DC pulsed with irradiated tumor cells (DC M74 irr) or pulsed with butyrate (DC M74but) or peroxide (DC M74per) treated tumor cells; DC fused with tumor cells (DC M74fus). Controls were non-stimulated PBL (PBL) and PBL stimulated with non-pulsed DC (DC). Data are mean values from 9 (M74 lysis) or 7 different donors (K562 lysis). E/T ratio: 50/1 *significantly different from non-stimulated PBL (p<0.05), ** (p<0.01).*

phenotypic and functional characteristics were evaluated 7 days after DC stimulation. Viability was evaluated by the trypan blue exclusion test. Controls were performed with non-stimulated lymphocytes (PBL) and non-pulsed DC stimulated lymphocytes (DC).

Detection of apoptotic and necrotic cells. Treated tumor cells were examined for the degree of apoptosis and secondary necrosis using a standard FACS assay (Annexin V-FITC detection kit, Immunotech, Marseille, France) which detects the binding of Annexin V (A) and inclusion/exclusion of propidium iodide (PI). A+PI- labeling was indicative of early apoptosis, A+PI+ of secondary necrosis and A-PI- of viable cells. It is known that apoptotic and necrotic bodies are phagocytosed by DC and acquire antigen for induction of class I-restricted CTL (4).

Flow cytometry analysis. Cells (10^5) were suspended in PBS supplemented with 0.5% BSA and labelled for characterization of the lymphocyte or DC phenotype by incubation at 4°C for 30 min with the following PE-, FITC-, or PC5-conjugated Abs (clone) and corresponding isotypes: anti-CD3 (clone UCTH1), anti-CD4 (13B8.2), anti-CD8 (B9 11), anti-CD25 (B1.49.9), anti-CD40 (mAb 89), anti-CD56 (NKH-1), anti-CD80 (MAB 104), anti-CD83 (HB15A) and anti-CTLA-4 (BNI3 used after saponin permeabilization) from Immunotech; anti-CD11c (S-HCL-3), anti-HLA-DR (L243) and Lin1 (anti-CD3, CD14, CD16, CD19, CD20 and CD56) from Becton Dickinson/Pharmingen (CA, USA) and CD86 (BU63) from Serotec (Oxford, UK). Cells were washed and suspended in 250 µL PBS added with 0.3% formol. CD4+CD25+CTLA4+ was considered as the T regulatory cell phenotype according to Jonuleit (29). Data analysis was performed on a FACScan flow cytometer (Becton Dickinson).

Cytotoxicity assays. T cell mediated cytotoxicity was tested in triplicate in standard ^{51}Cr release assay. The assays were carried out in U-bottomed microtiter plates. Depending on the assays, target cells were the M74, SK23, MCF7, R131 tumor cell line, K562 cells, or TAP-deficient T2 cells pulsed with ^{51}Cr for 1h (^{51}Cr]sodium chromate, specific activity 200 mCi/mg, Amersham Life Sciences, Buckinghamshire, England). T2 cells were previously pulsed with peptide (50 µg/mL, 1h, 37°C): HLA A0201-restricted Melan A-Mart 1_{27-35} melanoma peptide (AAGIGILTV, Neosystem, Strasbourg, France) or HLA A0201-restricted Her2neu irrelevant peptide (GP2). Her2neu is not overexpressed by M74 cells. Controls were non-pulsed T2.

Five thousand target cells per well were mixed with effector cells (E/T: 50/1 or 25/1) and incubated for 4h. Chromium release was assessed in culture supernatants using a gamma counter (Topcount, Packard Instrument, Rungis, France). Specific release was calculated as follows: (mean experimental cpm – mean spontaneous cpm)/(mean maximum cpm – mean spontaneous cpm) x 100. Spontaneous release was less than 30% of maximal release in all the assays.

To assess the role of NK cells in cytolytic activity, assays were done after magnetic sorting of CD56+ cells (MACS CD56 Microbeads, Miltenyi Biotec). Separation was controlled by cytometry: NK-depleted lymphocytes contained less than 1% of CD3-/CD56+ cells and sorted bulk more than 65% CD56+. NK-depleted lymphocytes provided less than 10% cytotoxicity against the K562 cell line.

IFNγ production. Responder cells were evaluated for their IFNγ production in response to contact with antigenic cells. Analyses were performed eight days after stimulation with TAA pulsed

Table I. *Effect of NK cell depletion on cytolytic activity against M74 tumor cell line*[a].

		M74 lysis (%)						
Donor		PBL	DC	DC M74	DC M74irr	DC M74fus	DC M74but	DC M74per
7	total lymph.	0	24	37	12	17	14	13
	NK depleted	0	11	15	19	0	15	28
8	total lymph.	14	24	21	23	18	18	23
	NK depleted	2	4	0	17	8	31	35
9	total lymph.	13	24	20	21	14	27	20
	NK depleted	5	0	1	20	9	34	33
10	total lymph.	11	24	23	19	15	26	24
	NK depleted	8	3	4	16	13	36	31

[a]M74 lysis was evaluated before (total lymphocytes) or after NK cell depletion by magnetic sorting (CD56). PBL were stimulated with DC pulsed with irradiated tumor cells (DC M74 irr) or pulsed with butyrate (DC M74but) or peroxide (M74per) treated tumor cells; DC fused with tumor cells (DC M74fus). Controls were non-stimulated PBL (PBL) and PBL stimulated with non-pulsed DC (DC). E/T ratio: 50/1. Data are from 4 different donors.

Table II. *Lymphocyte IFNγ production in response to tumor cells*[a].

	IFNγ (pg/mL)							
Donor	PBL	M74irr	DC	DC M74	DC M74irr	DC M74fus	DC M74but	DC M74per
2	21	0	30	130	55	23	227	133
3	0	nd	nd	nd	50	40	65	195
4	52	107	0	0	129	157	358	257
5	642	191	1159	1422	1500	108	1418	1500

[a]IFNγ production (pg/mL) was compared in PBL stimulated with DC pulsed with irradiated tumor cells (DC M74irr) or pulsed with butyrate (DC M74but) or peroxide (DC M74per) treated tumor cells; DC fused with tumor cells (DC M74fus). Controls were non-stimulated PBL (PBL) and PBL stimulated with irradiated tumor cells (M74irr) or with non-pulsed DC (DC). Data are from 4 different donors (nd=not determined).

DC. Briefly 2.10^5 M74 cells were seeded in 24-well plates for 12 h. The supernatant was discarded before adding 10^5 lymphocytes in a final volume of 500 μL of lymphocyte medium without IL-2. The plates were then incubated at 37°C for 72h and IFNγ was measured in the supernatant by ELISA methods according to the manufacturer's instruction (Ready-set-Go®, eBioscience, San Diego, CA, USA). The assays were performed with duplicate wells for each assay.

Tetramer. The number of MelanA-Mart1-specific TCD8 lymphocytes was evaluated in the polyclonal population by the use of MHC/peptide tetramers (30). The PE labelled A2 / MelanA-Mart1 tetramer was a gift from F. Lang (INSERM U463, Nantes, France). 10^5 effector cells were incubated at room temperature with 40 μg/mL tetramer in 0.1% BSA supplemented PBS. The cells were counterstained with anti-CD8 and anti-CD3, then washed twice and suspended in 250 μL PBS added with 0.3% formol. Data analysis was performed on a FACScan flow cytometer (Becton Dickinson).

Statistical analysis. Each cell treatment category and analysis was assayed with at least 3 different donors. The non-parametric Mann and Whitney ranking test was used for statistical analysis. We also noticed when a similar change (when compared to PBL control) was observed with each of the donors.

Results

Characteristics of DC. After 7 days in culture with GM-CSF + IL-4, about 10% of the blood MNC differentiated into imm DC. These cells were CD11c+, HLA-DR+ and Lin1- as myeloid DC. In preliminary experiments, we evaluated different doses of maturating agents (data not shown) and synergistic treatment with TNFα (25 ng/mL), CD40L (0.4 μg/mL) and LPS (10 μg/mL) for 18 h was finally chosen. Maturation induced a significant increase in expression of CD80 (6±7% to 45±32%, n=7)) and CD83 (2±2% to 34±24%) DC marker. A high percentage of CD86 (87±13% to 89±15%) and CD40 (91±9% to 92±16%)-expressing cells was similarly observed in mat and imm DC. The phagocytosis capacity of DC was not changed after maturation. The numbers of cells incorporating latex microspheres were 63±9% and 78±23% (n=3) for imm

Table III. *Yield of Melan A-Mart 1 tetramer-positive lymphocytes after a single DC-Tu or DC-Fu stimulation*[a].

Donor	Melan A-Mart 1 tetramer-positive CTL (%)				
	PBL	DC M74irr	DC M74fus	DC M74but	DC M74per
5	0.5	0.9	0.8	1.2	4.2
6	0.1	0.1	0.6	0.4	1.7
7	0.3	nd	0.6	0.2	1.9
9	0.1	0.3	0.2	0.3	1

[a]PBL were stimulated with DC pulsed with irradiated tumor cells (DC M74 irr) or pulsed with butyrate (DC M74but) or peroxide (DC M74per) treated tumor cells; DC fused with tumor cells (DC M74fus). Controls were non-stimulated PBL. Data are from 4 different donors.

and mat DC, respectively. The mean fluorescence intensity was 217±20 and 216±27 for imm and mat DC, respectively. Imm DC were round non-adherent cells. After maturation, a few of them exhibited a typical mature veiled morphology.

DC pulsing or fusion with M74 tumor cells. At the time of DC pulsing, the tumor cells were undergoing apoptosis or secondary necrosis, depending on their treatment. When collected after three days with 5 mM butyrate, 83% of the cells were in apoptosis (A+IP-) and 4% in secondary necrosis (A+IP+). With 10 μM peroxide, 16% were apoptotic and 50% necrotic (A+IP+). Four hours after 150 grays irradiation, 78% of the cells were surviving, only 14% were apoptotic and 8% necrotic. Internalisation of tumor cell bodies could be microscopically observed by the use of fluorescent cell probes.

Incubation of DC and M74 cells in the presence of PEG generated 22±9% (n=4) fused cells providing both DC and M74 markers as evaluated by cytometric analysis. The use of fluorescent cell probes showed that other hybrids could result from more than two cells. In addition, polycarions from identical cells (tumor-tumor and DC-DC) were observed.

DC-mediated T cell growth. Stimulation with TAA-pulsed autologous DC improved the expanding index (EI) of lymphocytes (Figure 1). EI was maximal (mean 8.6 after 7 days) with TAA prepared from butyrate- or peroxide-treated tumor cells. Whatever the conditions of TAA preparation for DC stimulation, the T (CD3+CD56-) cell percentages remained similar (mean from 58 to 67%). DC-Tu stimulation did not modify the respective percentages of TCD4 and TCD8 subpopulations. However, reduction in NK cells was consistently observed after stimulation with DC pulsed with TAA prepared from butyrate- or peroxide- treated tumor cells (mean 8% in both conditions compared to 13% in non-stimulated PBL). The percentage of lymphocytes with CD4+CD25+CTLA4+ phenotype was below 1% in all the assays and not changed by the DC-mediated stimulation procedure (data not shown).

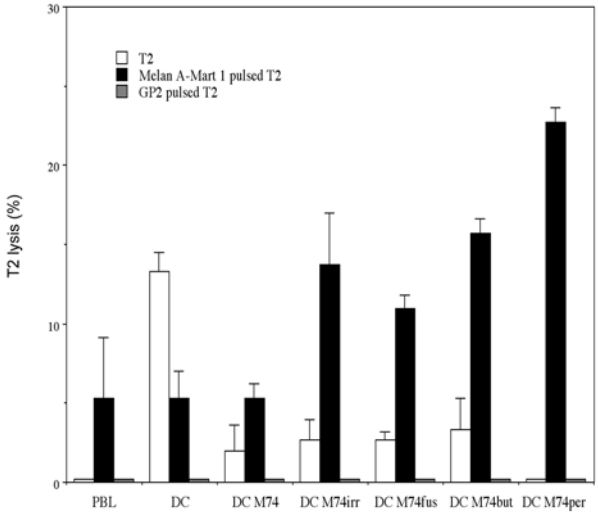

Figure 3. *Cytolytic activity against T2 cells. T2 were pulsed with Melan A-Mart 1 melanoma-associated antigen or with control peptide (Her 2-neu GP2). Control target were non-pulsed T2. E/T ratio 25/1. PBL were stimulated with DC pulsed with non-irradiated tumor cells (DC M74), irradiated tumor cells (DC M74irr) or pulsed with butyrate (DC M74but) or peroxide (DC M74per) treated tumor cells; DC fused with tumor cells (DC M74fus). Controls were non-stimulated PBL (PBL) and PBL stimulated with non-pulsed DC (DC). Data are mean values obtained from donors n°8, 9 and 10 (n=3). Similar difference for each of the 3 donors was observed between controls and DCM74irr, DCM74fus and DCM74per.*

Generation of CTL response against tumor cells. DC-Tu and DC-Fu stimulation enhanced effector cells able to lyse M74 tumor cells ($p<0.01$ and $p<0.05$, respectively, Figure 2). The cytolytic activity was a mean of two-fold increased. K562 lysis was not changed after stimulation with DC-Tu and DC-Fu (Figure 2). After NK cell depletion, M74 lysis was reduced in controls and DC-Fu but not in DC-Tu (Table I). This indicates that, when lymphocytes were stimulated with non-pulsed DC, M74-pulsed DC or DC-Fu, NK cells were responsible for the majority of the M74 lysis. On the contrary, when TAA were prepared from

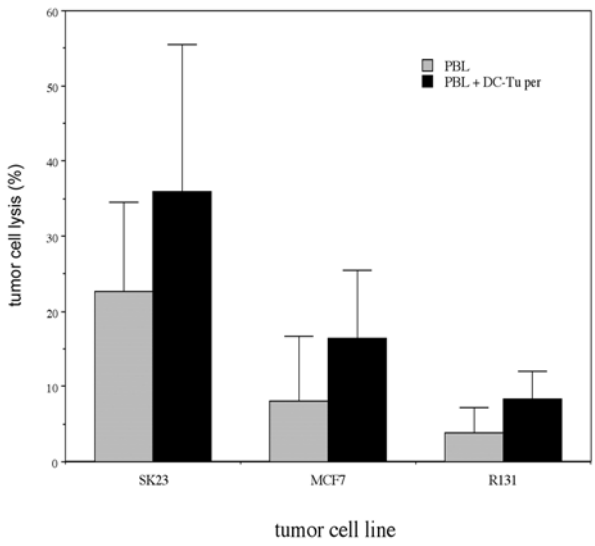

Figure 4. *Cytolytic activity of lymphocytes stimulated with DC pulsed with different necrotic tumor cells. PBL were stimulated with DC pulsed with SK23, MCF7 or R131 cells treated with 10 μM peroxide (PBL + DC-Tu per) or not (PBL). Data are cytolytic activity of the lymphocytes against the corresponding tumor cell line. Mean of assays (±SD) conducted with PBL from 4 different donors. E/T ratio: 50/1. Similar change for each of the donors was observed between PBL controls and DC-Tu-stimulated PBL for SK23 and R131.*

butyrate- or peroxide-treated tumor cells, the specific cytolytic activity was enhanced by 30% after CD56+ cell depletion (Table I). This is a sign of enhancement of CTL CD56- in these populations: a higher absolute number of CTL in a fixed effector:target ratio can explain that the final activity was found increased. In addition, DC-Tu-or DC-Fu-stimulated lymphocytes had 0% cytolytic activity against autologous monocytes (data not shown).

Specificity of CTL. IFNγ production in the presence of the M74 target was higher in DC-Tu-and DC-Fu-stimulated lymphocytes compared to non-stimulated PBL (Table II). When TAA were prepared from butyrate- or peroxide-treated tumor cells, CTL showed unfailingly strong IFNγ release, consistent with a Tc1 phenotype. This was not observed with PBL stimulated with irradiated tumor cells, with non-pulsed DC or DC pulsed with non-irradiated tumor cells.

Furthermore, the percentages of one dominant melanoma associated antigen (MelanA-Mart1) specific CTL were enhanced in DC-Tu (3/4 donors in DC M74but, 4/4 in DC M74per) and DC-Fu (4/4) (Table III). MHC-MelanA-Mart1 tetramer staining revealed a frequency up to 4.2% lymphocytes when DC were pulsed with peroxide-treated M74.

To control wheter enhancement of the cytolytic activity against M74 cells was not due to cross presentation of allo-antigens by DC at the time of stimulation, we compared the cytolytic activity of lymphocytes against T2 target pulsed with MelanA-Mart1 or with irrelevant peptide GP2. We could observe that CTL were able to efficiently lyse MelanA-Mart1-pulsed T2 target but not GP2-pulsed T2 (Figure 3). The highest cytolytic activity was observed when DC were pulsed with peroxide-treated M74.

Application to other tumor cell lines. The particular interest of a peroxide treatment was revealed with the above-described data, collected with the M74 cell line. Similar assays conducted with PBL from 4 different donors were applied to other cell lines. For melanoma (SK23), breast cancer (MCF7) and renal cell carcinoma (R131) cell lines, a 10 μM peroxide treatment of tumor cells before DC pulsing resulted in a repeated increase in cytolytic activity of lymphocytes against the corresponding tumor (Figure 4).

Discussion

Adoptive immunotherapy with immune effector cells is an attractive approach in cancer. Pioneering clinical trials have been conducted (17, 21, 31, 32), but methods to expand *ex vivo* autologous effector cells specifically directed against whole autologous tumor antigens remained to be developed (17, 23, 33). Our choice was to use DC pulsed with whole tumor cell antigens to expand lymphocytes with an optimal final antitumor activity. DC maturation was achieved with a cytokine cocktail of TNFα, LPS and CD40L. The expression of CD80 and CD83 cell surface markers were significantly enhanced compared to immature DC and co-stimulatory signals were expressed as revealed by the T cell priming functional activity of the DC.

Using DC pulsed with either irradiated, apoptotic or necrotic tumor cells or DC-Fu, we repeatedly observed the induction of cytolytic response. Cytolytic activity against M74 increased more than two-fold in both DC-Tu- and DC-Fu-stimulated lymphocytes. Effector cells have no cytolytic activity on normal cells. IFNγ production in response to contact with M74 cells was also significantly higher in DC-Tu- and DC-Fu-stimulated lymphocytes compared to non-stimulated PBL. Neither of these effects were observed with lymphocytes simply stimulated with irradiated tumor cells or with non-pulsed DC. In all the assays, lymphocytes provided an additive NK-like activity.

The percentages of T cells, TCD4 and TCD8 subpopulations were not modified in the assays after DC-Tu or DC-Fu stimulation. Large individual differences were noticed in the percentages of NK cells and basic

cytolytic activity of PBL from healthy donors against the M74 cell line after 15 days in culture with 150 UI/ml IL-2. As shown with assays of NK depletion, a great part of this activity was aspecific. Nevertheless MelanA-Mart1 antigen-specific lymphocytes could be basically detected as >0.1% in some donors. Similar observations were previously reported by other authors (33, 34). Regulatory T cells accounted for less than 1% of the cells in all our assays, before or after DC stimulation.

When TAA were prepared from butyrate- or peroxide-treated tumor cells, the percentage of CD56+ was significantly reduced in DC-Tu-stimulated lymphocytes. Treatment with butyrate induced apoptosis in more than 80% of the tumor cells, while treatment with peroxide induced secondary necrosis in 50% of the cells.

In both these conditions, the expanding index of lymphocytes was significantly enhanced (8 in seven days). Moreover, the lymphocytes were cytolytic against MelanA-expressing target cells (M74) up to 50% in one donor. This was not observed with DC loaded with untreated tumor cells. M74 lysis remained after CD56 depletion by cell sorting, indicating induction of CTL by DC-but or DC-per stimulation. This was confirmed by the increase in number of at least one dominant melanoma-associated antigen-specific lymphocytes: the MelanA-Mart1-specific TCD8 were up to 4.2% when TAA were prepared with necrotic cells (peroxide-treated tumor cells). These TAA specific lymphocytes were both specific and cytolytic: they were able to efficiently lyse the MelanA-Mart1-pulsed T2 target but not T2 pulsed with irrelevant peptide. One may suppose that similar activation was also accounted for other, but not identified, specific TAA. Taken together, these data confirm that enhancement of M74 lysis was not simply supported by potential differences in minor antigens between HLA-A2 donors and the M74 cell line, but real induction of TAA-specific CTL.

The data show that, in our procedure conditions, maturated DC processed and cross-presented tumor cell antigens and were functional to activate specific CTL. The procedure for TAA preparation was important for optimal activation and differentiation of specific effector cells by DC (3, 28, 35). The final amount of loaded antigen could not be strictly compared between DC-Fu and DC-Tu, but this could be responsible for differences in the final number of specific CTL. Characterization (HSP70, stress and danger signals expression) of the necrotic cells need also to be investigated to draw conclusions about the DC activation mechanism (36). Differences were observed in the mechanism of cytolytic activity of lymphocytes when pulsed TAA were prepared from irradiated and fused tumor cells (NK-like activity) or from necrotic tumor cells (CTL). Since induction of secondary necrosis by peroxide gave the best results when considering not only cytotoxic activity, IFNγ production but also MHC/MelanA-Mart1 tetramer binding and MelanA-Mart1 pulsed T2 lysis, a similar procedure was applied to other tumor cell lines. For SK 23 melanoma, MCF7 breast cancer and R131 renal cell carcinoma, induction of cytolytic effectors was achieved by the same procedure. However, differences in constitutive expression of immune suppressive factors by tumor cells of different origin (37) could participate in the observed variability in final functional activity of antigen-presenting and effector cells. Furthermore, the peroxide dose, standardized with the M74 cell line could be not sufficient to induce apoptosis in all cell lines. For example, in colon carcinoma cell lines a 1mM dose of peroxide was needed to induce post-apoptotic necrosis (personal data). As a conclusion, it is clear that improvement of specific activity of effector cells can be achieved when tumor antigens are prepared from necrotic tumor cells. The method is under evaluation with cells of patients in order to conclude about improvement when compared to simple irradiation of autologous tumor cells (38), a procedure we are using in our present clinical trial in renal cell carcinoma patients (trial n° AFFPAPS TC11, France).

Acknowledgements

We thank F. Lang (INSERM U 463, Nantes, France) for the gift of A2 / MelanA-Mart1 tetramer. This work was supported by grants from Le Comité 22 and Grand Ouest de la Ligue Contre le Cancer and from la Faculté de Médecine de Rennes.

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Received August 19, 2004
Revised March 29, 2005
Accepted April 4, 2005

ANTICANCER RESEARCH

International Journal of Cancer Research and Treatment

ISSN: 0250-7005

Editorial Office: International Institute of Anticancer Research,
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