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# *In Vitro* Antitumor Lymphocyte Generation Using Dendritic Cells and Innate Immunity Mechanisms as Tumor Cell Treatments

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**Abstract.** *Dendritic cells play a central role in the initiation and regulation of acquired and innate immunity, playing an important role in immunosurveillance and antitumor reaction. This reaction is mediated by effector cells and soluble factors. We chose to investigate four dendritic cell loading methods by mimicking innate immunity mechanisms and using whole tumor cell treatments in order to stimulate lymphocytes: sodium hypochlorite, TNFα and IFNγ and IgG opsonization. These methods were compared in an HLA.A2 model of healthy donors and with the M74 melanoma cell line. Treated tumor cell-loaded DC were able to increase proliferation of lymphocytes. Moreover, a CTL population was stimulated, as shown by their specific cyotoxicity against tumor cells (with w6/32 antibody assays), against MelanA/MART-1 loaded T2 cells and using MelanA/MART-1 tetramer. IgG osponization seemed to be less efficient than other tumor cell treatments. These loaded DC, or the obtained effector cells, could be interesting for therapeutic applications in antitumor cell therapy.*

Immunotherapy of cancer is a recent concept and dendritic cells (DCs), as a result of their central role in immunity (1, 2), are being fully exploited to develop new therapeutic tools (3). Indeed, when they are immature, DC have characteristics common to other phagocytes, such as TLR, CR and FcR molecule expression, allowing recognition of pathogens, opsonized or not. DCs are also capable of phagocytosis but their cytotoxic capacities are limited. They are not effector but stimulator cells. After loading cell-target associated antigens, DCs become the most powerful T-lymphocyte activators and can induce a specific cellular immune response (4). But DCs can also stimulate B-lymphocytes or NK and NKT cells, leading to humoral and innate immune responses.

There are many DC loading methods, *i.e.* the means of facilitating incorporation, process and presentation of tumor associated antigens (TAA). These methods can be classified into two categories: determined antigens and whole treated tumor cells. The most common techniques for determined antigens are the use of synthetic peptides and gene transfection. For whole cell treatments, fusion, induction of apoptosis or necrosis (5) and production of exosomes (6) or lysates are the most used. These exogenous antigens are then phagocytosed and presented to CD8+ T-lymphocytes on major histocompatibility complex (class I) molecules through the cross-presentation process (7, 8). The cellular mechanisms of cross-presentation are not completely understood but ospsonization and IFNγ treatment are known to improve this process (9).

The innate immune system is regarded as the first line of defense against infection by pathogens. Moreover, it plays an important part in the process of immunosurveillance against tumor development (10).

This immunity includes effector cells and soluble molecules. Soluble factors are cytokines, often inflammatory like TNFα or IFNγ, and opsonins, like immunoglobulins (Ig) and complement molecules. Cells involved in non-specific immunity are phagocytes, of which the most important are neutrophiles and monocytes/macrophages, and NK and NKT cells. These cells are able to recognize many pathogens expressing conserved molecules, such as pathogen-associated molecular patterns (PAMP), with a panel of receptors like Toll-like receptors (TLR) (11).

*Abbreviations:* Ab: Antibody; BSA: Bovine Serum Albumin; CR: Complement Receptor; CTL: Cytolytic T-Lymphocyte; DC: Dendritic Cell; FcR: Fc Fragment Receptor; LU: Lytic Unit; NK: Natural Killer; PAMP: Pathogen Associated Molecules Pattern; PBL: Peripheral Blood Lymphocyte; TAA: Tumor Associated Antigens; TLR: Toll-Like Receptor.

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*Key Words:* Cancer, dendritic cell, innate immunity, lymphocyte, tumor-associated antigen, immunotherapy.

Phagocytes kill targets, after phagocytosis or not, by degranulation while releasing bactericidal molecules. These molecules can be associated with oxygen atoms such as the hydrogen peroxide ($H_2O_2$) and hypochloric acid (HClO), which are powerful bactericides. NK and NKT cells kill infected or abnormal cells by induction of apoptosis through mechanisms like perforine-granzyme or Fas-Fas L. All these cells are also able to lyse cells by antibody-dependant cellular cytotoxicity (ADCC), by recognizing targets opsonized by Ig (thanks to FcR) or by complement molecules (CR).

Our interest was focused on four methods of DC loading using whole tumor cells and by mimicking innate immunity mechanisms to stimulate lymphocytes. These methods were investigated and compared in an HLA.A2 healthy donor model with the HLA.A2 melanoma cell line M74. The first method used sodium hypochlorite treatment in order to recreate a cytotoxic effect of the neutrophiles and other phagocytes (12). Second and third treatments were the use of TNFα and IFNγ, known for their cytostatic and cytotoxic effects and induction of immunogenicity, as shown by increasing expression of class II and I MHC molecules after IFNγ treatment (13, 14). The last, target cell IgG opsonization allows their recognition and endocytosis by DCs.

Contrary to IgM, IgG does not need complement molecules to be recognized by DCs. Furthermore, opsonization of tumor cell apoptotic bodies for DC loading was recently shown to be efficient at generating an *in vivo* antitumor response (15). Loaded DCs were then used for autologous lymphocyte stimulation.

## Materials and Methods

*Healthy donors.* Peripheral blood samples were collected from 11 different HLA-A2 healthy donors (EFS, Rennes, France). Cells were centrifuged on a density gradient (UNISEP®, Novamed, Israel). Mononuclear cells (MNC) were frozen in human serum albumin and 10% dimethylsulfoxide (DMSO) until use for DC and lymphocyte preparation.

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Immunotech (France); anti-CD83 (HB15e) and anti-Lin1 (anti-CD3, CD14, CD16, CD19, CD20 and CD56) from Becton Dickinson/Pharmingen (USA). Cells were washed and resuspended in 250 μL PBS added with 0.3% formol.

CD4+CD25+CTLA4+ was considered as the regulatory T-cell phenotype according to Jonuleit *et al.* (18). Data analysis was performed on a FACScan flow cytometer (Becton Dickinson).

*Cytotoxicity assays.* T-cell-mediated cytotoxicity was assayed by measuring the cytolytic activity of lymphocytes in a 4 h $^{51}Cr$ release assay. The assays were carried out in U-bottomed microtiter plates. Target cells (tumor cell lines and K562) were incubated at 37˚C with 1 mCi of [$^{51}Cr$] sodium chromate /$10^6$ cells (Amersham Life Sciences, England), washed 3 times and counted. In some experiments, MHC class I-dependant cytotoxicity was evaluated using 50 μg/mL w6/32 antibody (Serotec, England) for 45 min, at 37˚C, before the third wash. Five thousand target cells per well were mixed with effector cells (E/T: 50/1) and incubated for 4 h. Chromium release was assessed in culture supernatants using a gamma counter (Topcount, Packard Instrument, France). All assays were run in triplicates. Specific release was calculated as follows: (mean experimental cpm – mean spontaneous cpm)/(mean maximum cpm – mean spontaneous cpm) x100. Spontaneous release and maximum release were respectively assessed by incubating target cells in culture medium alone or with 20% HCl supplemented Triton solution. One lytic unit (LU) was defined as the number of effector cells required to lyse 40% of 5000 targets; the number of LU per culture (LU(40)/culture) was calculated.

To assess the role of NK cells in cytolytic activity, assays were performed after magnetic depletion of CD56+ cells (MACS CD56 Microbeads, Miltenyi Biotec, France). Depletion was controlled by flow cytometry.

After CD56+ cell depletion, less than 1% of the cells were CD3–/CD56+ (more than 65% in the sorted bulk) with less than 10% of cytotoxicity against the K562 cell line.

The TAP-deficient T2 cell line was used to determine HLA-A2-mediated specific lysis of effectors. Relevant MelanA/MART-$1_{27-35}$ (AAGIGILTV) and control GP2 HER2/$Neu_{654-662}$ (IISAVVGIL) (from Neosystem, France) peptides were used at 50 μg/mL during 1 h at 37˚C to load T2 cells. Controls were non-loaded T2 cells.

*Tetramer.* The frequency of MelanA-Mart1 specific TCD8 lymphocytes was evaluated in the polyclonal population by the use of MHC/peptide tetramers (19) The PE-labeled A2 / MelanA/Mart1 tetramer was a kind gift from F. Lang (INSERM U463, Nantes, France). Effector cells ($10^5$) were incubated at room temperature with 40 μg/mL tetramer in 0.1% BSA supplemented PBS. 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.* The unpaired Student's *t*-test was used to test for statistical significance of the differences in the mean values (one or two-tailed). Values for $p<0.05$ were considered statistically significant.

## Results

*DC phenotype.* After 7 days in culture with GM-CSF and IL-4, cells were CD11c$^{high}$, HLA-DR$^{high}$ and Lin1$^{negative}$ as myeloid DCs. Maturation with pro-inflammatory cocktail (IL-1β, IL-6, TNFα and PGE2) induced a significant increase (n=5) in the CD80$^{high}$ (4%±4 to 65%±27) and CD83$^{high}$ (3%±4 to 67%±16) DC population, leading to a phenotype of mature myeloid DCs. A high percentage of CD86$^{high}$ and CD40$^{high}$ expressing cells was similarly observed in mature and immature DCs (88%±11 to 97%±6 and 88%±12 to 96%±5, respectively).

*Tumor cell death via apoptosis and/or necrosis.* At the time of DC pulsing, tumor cells were undergoing apoptosis or secondary necrosis, depending on the treatment. When tumor cells were collected after three consecutive sodium hypochlorite treatments, most were in secondary necrosis (more than 90% were A+IP+). With IFNγ and TNFα treatments, about 30-35% were necrotic (A+IP+) and 10-15% were apoptotic (A+PI–). Opsonization did not induce significative apoptosis or necrosis.

*Tumor associated antigen-loaded DCs stimulated T-lymphocytes*

*DCs stimulated T-cell growth.* TAA-loaded DCs significantly increased the expanding index of autologous lymphocytes after 7 days' *in vitro* culture (Figure 1). For TNFα-treated tumor cell-loaded DCs, the difference was not statistically significant but expansion was increased for 4/5 donors. T-lymphocytes alone had a high expanding index (x 4.3±2.9).

Loaded DCs do not alter T-cell phenotype. T-lymphocyte and NK cell percentages remained similar after DC stimulation, whatever the antigen pulsing assay. Moreover, use of DCs did not modify the respective percentages of CD4+ and CD8+ T-cell subpopulations. The percentage of lymphocytes with the CD4+CD25+CTLA4+ phenotype was very low (<1%) and was not increased by DC-Tu stimulation (data not shown).

*Loaded DCs stimulated CTL response against tumor cells.* When lymphocytes were stimulated with TAA-loaded DCs, their cytolytic activity against the M74 cell line was significantly ($p<0.01$) enhanced 3- to 5-fold (Figure 2).

This cytotoxicity was in part MHC class I-mediated as shown by the consistently observed decrease of lymphocyte cytotoxic activity in the presence of w6/32 antibody for sodium hypochlorite, IFNγ and TNFα (Figure 2). No significant decrease was observed for opsonization. Remaining lysis indicates that a significant part of cytotoxicity was due to NK activity. But TAA-loaded DC-stimulated lymphocyte cytotoxicity against the K562 cell line was not significantly enhanced (data not shown). Depletion of the NK cell population significantly decreased cytolytic activity against M74 only for controls but not for lymphocytes prepared with TAA-loaded DCs (Figure 3).

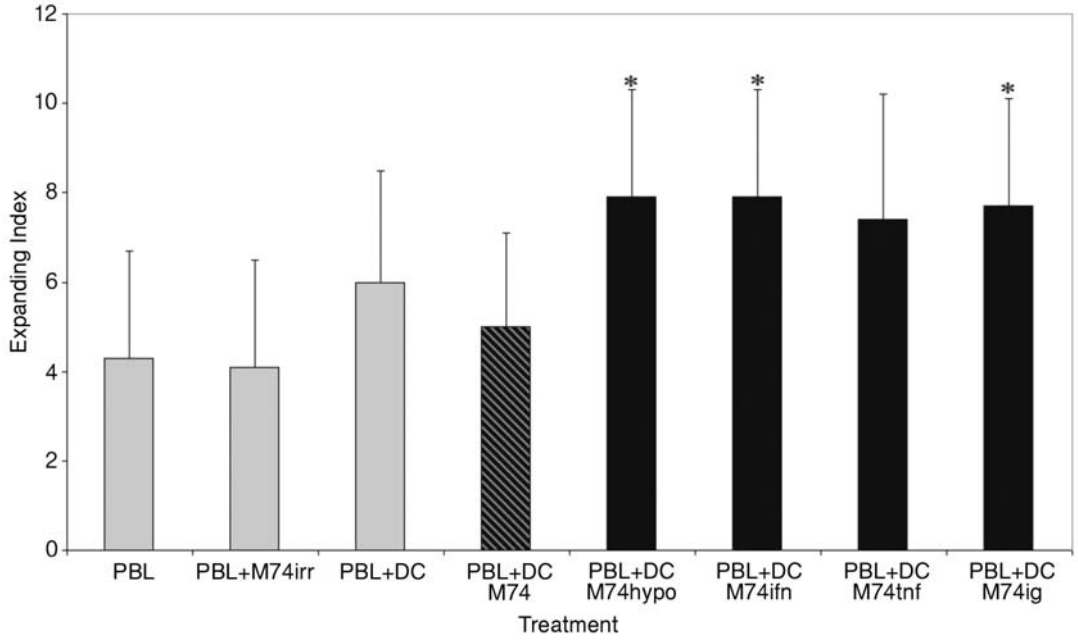


Figure 1. *Expanding index (EI) of lymphocytes seven days after stimulation with or without DC. Lymphocytes (PBL) were stimulated with: DCs pulsed with sodium hypochlorite (DC M74 hypo), IFNγ (DC M74ifn) or TNFα (DC M74tnf) -treated tumor cells or IgG-coated tumor cells (DC M74ig). Expanding index (EI) was day 7/day 0 cell count. Controls were non-stimulated lymphocytes (PBL), lymphocytes stimulated with irradiated tumor cells (M74irr), non pulsed-DCs (DC) or DCs pulsed with untreated tumor cells (DC M74). Data are the mean from 5 different donors. *Significantly ($p<0.05$) enhanced compared to PBL+DC M74.*

To determine if cytotoxicity against the M74 cell line was improved in part by stimulation of TAA-specific CTL and not only by presentation of allo-antigens, we assessed the cytolytic activity of lymphocytes against MelanA/MART-1 (a major melanoma-associated antigen)-pulsed T2 cells. Only CTL stimulated with TAA-loaded DCs killed these targets. Cytotoxicity was significantly ($p<0.01$) increased for lymphocytes stimulated with hypocholorite-, IFNγ- and TNFα- treated tumor cell-loaded DCs (Figure 4). No significative cytotoxicity was detected against the irrelevant peptide (HER2-Neu)-pulsed T2 cells or non-pulsed T2 cells.

In addition, the percentage of MelanA/MART-1-specific T-lymphocytes was enhanced in the lymphocyte population stimulated with TAA-loaded DCs, regardless of the tumor cell treatment. The MelanA/MART-1-specific CD8+ population was significantly increased (1.3%±0.1, $p<0.01$) with hypocholorite-treated tumor cell-loaded DCs than untreated tumor cell-loaded DC-stimulated lymphocyte (0.3%±0.1). The same results were observed with IFNγ- and TNFα-treated tumor cell- and opsonized-tumor cell-loaded DCs (1.4%±0.3, $p<0.01$; 1.3%±0.4, $p<0.05$; 1.2%±0.5, $p<0.05$ respectively).

## Discussion

When cultured with such pretreated tumor cells, DCs were able to stimulate autologous lymphocytes. The expanding index between stimulated and non-stimulated lymphocytes was significantly increased in 1 week, only by a mean of 1.7-fold, but there were great differences between donors. DCs are famous for their capacity to stimulate lymphocyte growth, in particular by secreting cytokines such as IL-12 and IL-2 (20). But under our conditions, with 150 U/mL IL-2, non-stimulated lymphocytes showed significant proliferation.

Whatever tumor cell treatment was used prior to loading, stimulation by DC-Tu did not involve a change in lymphocyte populations, such as T-lymphocytes (CD4+ and CD8+) or NK cells. DCs are known to stimulate CD4+ and CD8+ T lymphocytes (21), as well as NK cells (22), by secreting cytokines and by cell-cell contacts. Some loading methods were described as preferentially stimulating some subpopulations such as CD8+ T lymphocytes, due to cross-priming for example (23). After tumor cell treatments, we also observed an induction of CTL. Indeed, there was a significant increase of lymphocyte cytotoxicity against tumor cells. This lysis was partially mediated by a MHC class I recognition pathway since M74 cytotoxicity was reduced by w6/32 MHC class I blocking. Similar results were obtained from authors using tumor cell lysate-loaded DCs (24, 25). Cytotoxicity was also mediated by NK cells, as shown by the phenotype of effectors (approximately 20% of cells were CD3–/CD56+) and the important lysis of the K562 cell line (30-40%). Tumor cells are known to be able to decrease their expression of MHC class I molecules or to express

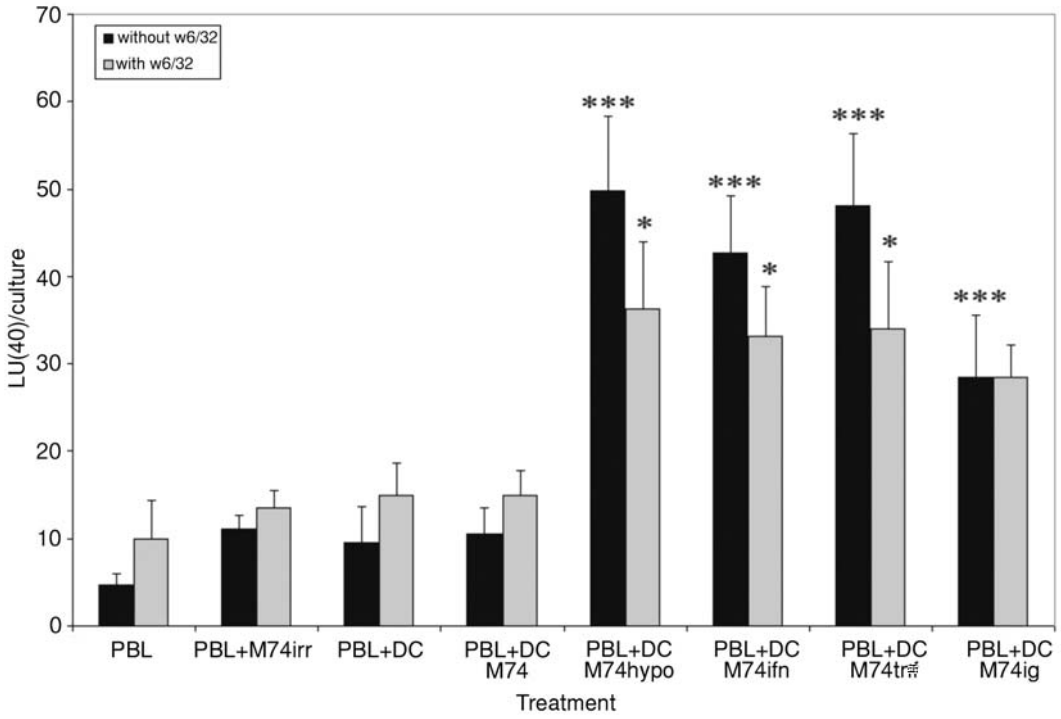


Figure 2. *Cytolytic activity against the M74 tumor cell line, with or without w6/32 MHC class I blocking. Lymphocytes were stimulated with: DCs pulsed with sodium hypochlorite (DC M74 hypo), IFNγ (DC M74ifn) or TNFα (DC M74tnf) -treated tumor cells or IgG -coated tumor cells (DC M74ig). Controls were non-stimulated lymphocytes (PBL), lymphocytes stimulated with irradiated tumor cells (M74irr), non pulsed-DCs (DC) or DCs pulsed with untreated tumor cells (DC M74). Data are the mean from 5 different donors. ***Significantly enhanced compared to PBL+DC M74 at $p<0.001$, *Significantly reduced compared to cytotoxicity without w6/32 ($p<0.05$).*

ligands for NK receptors, such as NKG2D, which could explain this NK-dependent lysis (26).

This NK activity, even if it took part in the total cytotoxicity against the tumor cells, was not necessary for M74 lysis, as shown after NK cell depletion. Moreover, HLA.A2-mediated specific cytotoxicity was generated against at least one tumor antigen. This was clearly shown by cytotoxicity against melanoma antigen MelanA/MART-1-loaded T2 cells, demonstrating that cytotoxicity against M74 cells was not simply due to presentation of unspecified allo-antigens by DCs.

To further investigate this, we also assessed the percentage of specific lymphocytes of MelanA/MART-1 using tetramer. The percentage was significantly increased in all our lymphocyte populations stimulated with DC-Tu. We use matured DCs in our experiments and they are known to be more efficient at stimulating MelanA-specific lymphocytes (27). Although lymphocytes were stimulated once with pulsed DCs, we observed high rates of MelanA/MART-1 tetramer-positive cells. Moreover, it is surprising that 0.1-0.5% tetramer-positive cells from healthy donors were observed, but it has been reported that, in the case of MelanA/MART-1, specific lymphocytes are usually found in the blood of healthy donors (28, 29).

With hypochlorite and IFNγ treatments, DC-Tu-stimulated lymphocytes provided a significant increase in their IFNγ production, in the presence of M74 cells (data not shown), but not with TNFα and opsonization treatments. DCs, stimulated by necrotic or apoptotic bodies, are able to stimulate lymphocyte IFNγ production by secreting IL-12 (27). However the mechanisms of DC activation by apoptotic or necrotic cells are not fully understood (30). Lymphocyte IFNγ production can depend on antigen concentration and the DC maturation state (expression of co-stimulatory molecules) (31).

But in our case, DCs were matured in the same way so the concentration of TAA may have been different depending on the treatment. IFNγ production was very high with lymphocytes stimulated with DC M74ifn. It's possible that IFNγ, by stimulating the expression of MHC class II molecules on tumor cells, enhanced CD4+ T-cell stimulation and the 'helper' effect and increased the cytokine production of lymphocytes (32). Although antigen immune complexes have been described to be efficient for DC loading and lymphocyte cytokine secretion (15), IFNγ production was very low with lymphocytes stimulated with DC M74ig. In our case, most of the IgG opsonized tumor cells were still alive and were not apoptotic or necrotic. A complementary experiment

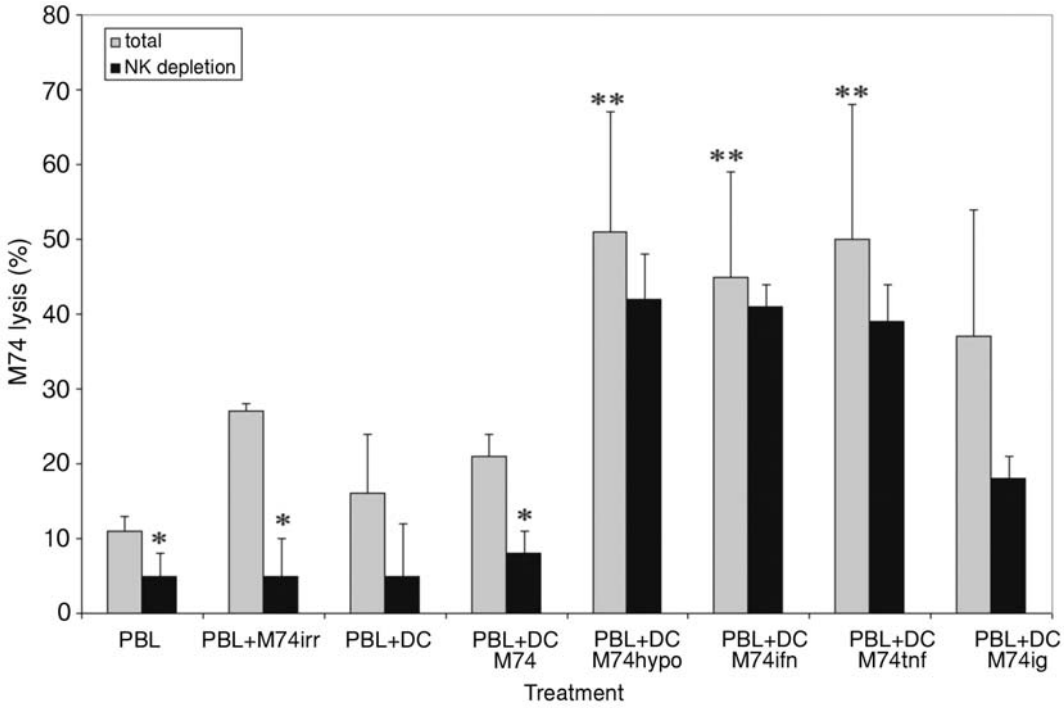


Figure 3. *Cytolytic activity against the M74 tumor cell line, before and after depletion of NK cells by magnetic cell sorting (CD56+). Lymphocytes were stimulated with DCs pulsed with sodium hypochlorite (DC M74 hypo), IFNγ (DC M74ifn) or TNFα (DC M74tnf) -treated tumor cells or IgG-coated tumor cells (DC M74ig). Controls were non-stimulated lymphocytes (PBL), lymphocytes stimulated with non pulsed-DCs (DC) or DCs pulsed with untreated tumor cells (DC M74). Data are the mean from 3 different donors. **Significantly increased compared to PBL+DC M74 with p<0.01, *Significantly reduced compared to cytotoxicity without NK depletion (p<0.05).*

Figure 4. *Cytolytic activity against peptide-loaded T2 cells. T2 cells were loaded with melanoma associated antigen MelanA/MART-1. Lymphocytes were stimulated with DCs pulsed with sodium hypochlorite (DC M74 hypo), with IFNγ (DC M74ifn) or TNFα (DC M74tnf)-treated tumor cells or IgG-coated tumor cells (DC M74ig). Controls were non-stimulated lymphocytes (PBL), lymphocytes stimulated with non pulsed-DCs (DC) or DCs pulsed with untreated tumor cells (DC M74). Data are from 4 different donors. **Significantly different from PBL at p<0.01.*

(ELISA) showed that in the presence of IgG opsonized tumor cells, DCs secreted more IL-10 than in other cases. The opsonization of live 'compatible' (HLA.A2) cells may lead to a partial immunoregulation phenomenon by DCs. Complementary experiments were carried out with other HLA.A2 tumor cell lines (renal cell carcinoma, breast cancer and colorectal cancer) and the same results were observed for expanding index, phenotype and cytotoxicity against tumor cells (data not shown). Furthermore, no significant cytotoxicity was detected with these methods against autologous monocytes (data not shown). More investigations must be carried out to complete our understanding of this response.

## Conclusion

Our results show that the use of non-specific immune mechanisms to treat tumor cells can be achieved *in vitro* and allow incorporation, processing and presentation of TAA by DCs to lymphocytes. Specific and cytotoxic effectors can be collected with such procedures.

These techniques are not restricted to a precise HLA type and could be used for any type of cancer. These loaded DCs, or the obtained effector cells, could be interesting for therapeutic applications in antitumor cell therapy.

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**April 4, 2007**

**Dr. Catros-Quemener**

Re: Your manuscript No. **7770-G** entitled «***In Vitro* Antitumor Lymphocyte...»**

**Dear Dr**

Referring to your above manuscript for publication in AR, please allow us to use this form letter in reply:

1. *Referee's recommendations:*
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   - □ Accepted with minor changes.
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We would appreciate your prompt reply.
With many thanks,
Yours sincerely,

*J.G. Delinassios*
*Managing Editor*

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