Dendritic cell viability is decreased after phagocytosis of apoptotic tumor cells induced by staurosporine or vaccinia virus infection

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Background and Objectives. Dendritic cells (DC) primed with tumor antigens can effectively mediate the regression of a variety of established solid malignancies in both murine and human models. Several experimental studies indicate that apoptotic bodies are an optimal source of tumor antigens for *ex vivo* priming of DC. However, the clinical use of killed tumor cells as a source of antigens will require an optimal methodology to induce effective tumor cell apoptosis.

Design and Methods. The goal of this study was to compare the efficiency of three agents for inducing neoplastic B lymphocyte apoptosis; staurosporine, infection by modified vaccinia (MVA) viral particles and ultraviolet C (UVC) radiation.

Results. The three methods were finely tuned to induce apoptosis in more than 90% of tumor cells after 24 hours of exposure. However, the viability of monocyte-derived DC, loaded with B-cell tumor apoptotic bodies induced by staurosporine or MVA viral particles, decreased dramatically within 48 hours after phagocytosis of the killed neoplastic cells. The persistence of the apoptosis-inducing agents in the apoptotic bodies and not in the tumor supernatant, was responsible for the observed damage to DC viability. In contrast, DC viability was not affected after uptake of tumor cells killed through UVC-irradiation. Furthermore, B-lymphoblastic cell line (LCL)-specific T cells were reactivated by DC loaded with apoptotic bodies induced by UVC-rays.

Interpretation and Conclusions. Since the method used to induce tumor cell apoptosis might be detrimental to DC viability, these findings should be considered when designing anticancer vaccination programs.

Key words: dendritic cells, anticancer vaccine, immunotherapy, non-Hodgkin's lymphoma, apoptosis.

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Dendritic cells (DC) are considered professional antigen-presenting cells (APC) and play a central role in regulating immune responses.[1,2] Recent evidence indicates that *ex vivo* generated DC primed with tumor antigens[3] can stimulate the regression of established renal cell carcinoma[4,5] and melanoma.[6,7] Several techniques are now available for creating tumor-specific DC vaccines, such as DC loading with peptides, or killed tumor cells, or by tumor-associated antigen gene transduction.[8-10]

Among these strategies, the use of whole tumor cells as the source of antigens has the advantage of transferring a wide spectrum of known and unknown antigens, characterizing the tumor cells in all stages of its development and differentiation, to the APC.[11] This strategy should enable vaccine preparations to be obtained even if the tumor cells are not immunologically well characterized. The additional presence in killed tumor cells of epitopes for T-helper lymphocytes may be beneficial since MHC II-restricted T-helper lymphocyte activation plays a pivotal part in the physiologic immune response to pathogens and might be of considerable importance in the tumor rejection process.[12,13] On the other hand, protocols based on immunization against a single tumor antigen carry a potential risk of inducing escape variants or being ineffective on heterogeneous tumor cell populations. Methods that yield a pool of tumor antigens include tumor lysate generation,[14] apoptotic bodies,[15] tumor-derived exosomes,[16] and tumor-DC fusion.[17] The use of apoptotic bodies of tumor cells seems to be an appropriate way to assure an adequate cross-presentation of tumor antigens by DC.[7,15] DC take up apoptotic and necrotic cells by an endocytic pathway allowing the processing of proteins eventually resulting in peptide loading of class I and II HLA molecules.[18] Several authors have recently reported that apoptotic tumor cells are better than cell lysate in promoting cross-priming of cytotoxic T lymphocytes (CTL) using different human tumor cell histotypes.[19-21]

However, to be useful in a clinical setting the method to induce apoptosis should kill all the neoplastic cells, thus preventing reinfusion of viable tumor cells into the patient. In the present study, we compared several methods for inducing apoptosis of primary leukemia and lymphoma cells for subsequent loading on monocyte-derived DC. In addition, we evaluated DC viability after these cells had been loaded with apoptotic bodies induced by vaccinia viral particle infection, staurosporine treatment or exposure to UVC-ray irradiation.

Design and Methods

Cell isolation and culture

Immature dendritic cells were generated from peripheral blood mononuclear cells (PBMC) of patients with chronic lymphatic leukemia (CLL) or follicular lymphoma (FL) as described by Sallusto and Lanzavecchia.[22] Briefly, $CD14^+$ cells obtained from PBMC using an immunomagnetic technique according to the manufacturer's instructions (CD14 microbeads kit, Miltenyi Biotec, Bergisch Gladbach, Germany) were incubated at 37°C in 5% CO_2 at a concentration of 10^6 cells/mL in 10% FCS (Hy Clone, Logan, UT, USA), RPMI (Bio Whittaker Europe, Walkersville, MD, USA), in the presence of recombinant human interleukin-4 (20 ng/mL; Schering Plough, Kenilworth, NY, USA) and recombinant human granulocyte-macrophage colony-stimulating factor (50 ng/mL; Molgramostim, Myelogen, Sandoz). On day +5, adherent and non-adherent cells were collected, labeled with anti-CD1a, anti-CD14, anti-CD83 monoclonal antibodies and analyzed by FACScalibur (Becton Dickinson, San José, CA, USA).

$CD19^+$ tumor cells harvested from ten peripheral blood (PB) and ten bone marrow (BM) samples from patients with CLL (n=5) and FL (n=5) were selected using a high-gradient immunomagnetic technique according to the manufacturer's instructions (CD19 microbeads kit, Miltenyi Biotec, Bergisch Gladbach, Germany) and then frozen. Tumor cell purity in the final preparation was evaluated by κ/λ light chain analysis (κ/λ simultest, Becton Dickinson, San José, CA, USA).

A human lymphoblastic cell line (LCL) was generated and cultured in RPMI with 10% human serum, as described elsewhere.[23] All patients were informed about the study and gave oral consent to the procedures.

Tumor antigen preparation

Three different methods to induce apoptosis were tested: (i) $CD19^+$ tumor cells were infected by modified *Ankara vaccinia virus* (MVA, Bavarian Nordic, Germany) as reported elsewhere.[9] Briefly, pelleted tumor cells were incubated for 2 hrs at 37°C in 5% CO_2 with sonicated MVA viral particles at different multiplicities of infection (MOI) (from 5 to 500 MOIs). After washing three times, the cells were incubated at 37°C in 5% CO_2 for 24 hrs; (ii) $CD19^+$ tumor cells were incubated at 37°C in 5% CO_2 for 16 hrs with a microbial alkaloid, staurosporine (Sigma Chemicals Co., Steinheim, Germany), a strong inhibitor of protein kinases,[24] at different concentrations (1, 2.5 and 5 mM). After washing three times, the cells were incubated at 37°C in 5% CO_2 for 24 hrs; (iii) $CD19^+$ tumor cells or LCL were exposed to a dose of 10 J and 15 J of UVC irradiation from a 9W UVC lamp (T-UV9 UV-C Germicidal Lamp Philips, Holland) and then incubated at 37°C and 5% CO_2 for 24 hrs. After 24 hrs of incubation, apoptosis was evaluated by FACScan analysis using an annexin V-propidium iodide (PI) assay (MedSystems Diagnostics GmbH, Vienna, Austria). We calculated the percentage of apoptotic cells as the sum of early (annexin V^+/PI^-) and late apoptotic cells (annexin V^+/PI^+).

Tumor antigen uptake by DC

Immature DC were incubated for 48 hrs with apoptotic tumor cells (1:2 ratio) at a concentration of 1.5×10^6 cells/mL. The percentage of DC containing apoptotic bodies was evaluated in two ways: fluorescent microscopy (FM) and FACScan analysis. When FM was used, DC and tumor cells were labeled with two different stains: green CMFDA (Molecular Probe, Eugene, Oregon, 1 ng/mL) and red CMFTR (Molecular Probe, Eugene, Oregon, 5 ng/mL) fluorescent stains, respectively. DC containing apoptotic tumor bodies appeared as yellow spots, due to the mixture of red and green fluorescence signals.

To calculate the exact number of DC which could take up apoptotic bodies, we used FACscan analysis after labeling DC and tumor cells with red CMFTR (10 ng/mL) or green CMFDA (1 ng/mL). Trypan blue (Sigma Chemicals Co., Steinheim, Germany) was added to quench the green fluorescence in order to evaluate the apoptotic bodies attached to the DC surface.[25] The trypan blue only entered the stained cells and stopped the fluorescent emission if the apoptotic bodies were on live DC. As a negative control, the same experiments were performed at 4°C to prevent phagocytosis.

DC viability after phagocytosis of apoptotic tumor cells

In order to evaluate whether apoptotic bodies might cause toxic damage to DC, the viability of the DC, after they have been loaded with apoptotic bodies, was tested using propidium iodide staining (MedSystems Diagnostics GmbH, Vienna, Austria, 10 μg/mL). Briefly, the DC were labeled with CMFDA green stain and then incubated for 48 hrs with apoptotic tumor cells. To rule out that toxic damage to DC could result from the presence of staurosporine or MVA viral particles in the supernatant, DC were also incubated for 48 hours with the supernatant of neoplastic cells previously exposed to staurosporine or MVA viral particles. After both incubations, the DC were extensively washed and, after staining with propidium iodide and CD19 PE (Becton Dickinson, San José, CA, USA), were analyzed by flow-cytometry.

Co-culture of DC with autologous PBMC and interferon-γ ELISPOT assay

In order to evaluate the cross-priming of tumor

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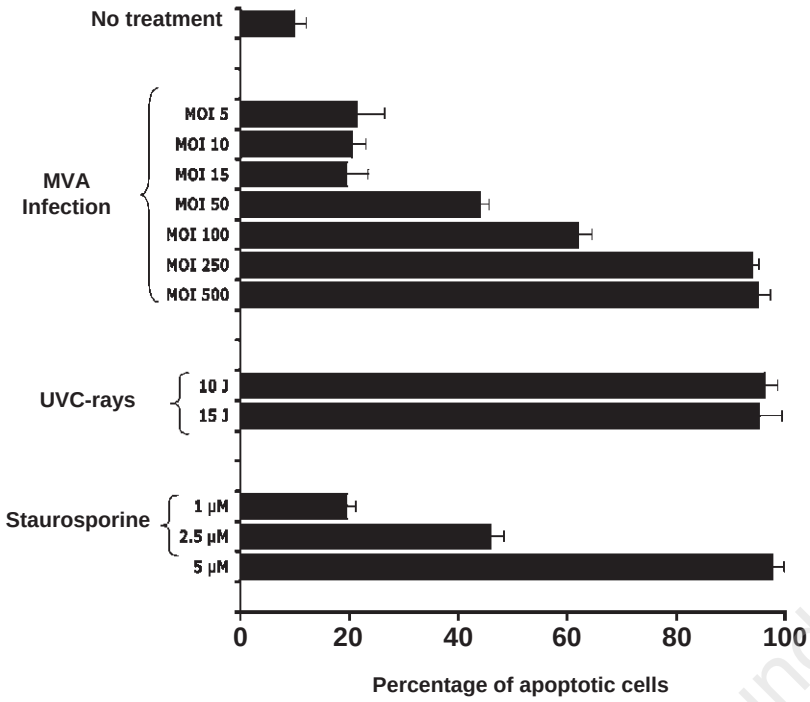

Figure 1. Percentage of apoptotic neoplastic B lymphocytes. Neoplastic B lymphocytes were: a) incubated with sonicated MVA viral particles at different MOI (5, 10, 15, 50,100, 250, 500) for 2 hrs at 37°C; b) incubated with staurosporine at different doses (1, 2.5 and 5 µM) for 16 hrs; or c) exposed to UVC irradiation (10 J -15 J). After three washes, the cells were incubated for 24 hrs before evaluation of the percentage of early (annexin V^+/propidium $iodide^-$) and late (annexin V^+/propidium $iodide^+$) apoptosis by FACScan analysis using the annexin V-propidium iodide assay. The results show the total fraction of apoptosis (early + late) observed in the different conditions and the data are expressed as mean ±SD of triplicates of ten separate experiments.

antigens by DC after phagocytosis of apoptotic bodies, PBMC were activated against an autologous Epstein-Barr virus (EBV)-induced lymphoblastoid cell line.[26] Immature DC were cultured for 24 hrs with LCL induced to undergo apoptosis by exposure to 10 J UVC-irradiation. After extensive washing, the DC were matured by 12-hr incubation with tumor necrosis factor (TNF)-α (10 ng/mL) and then co-cultured with autologous PBMC (ratio 1:4) in 10% heat-inactivated human serum RPMI. During 4 weeks of culture, fresh DC loaded with killed LCL were added to the culture every 7 days to restimulate autologous PBMC. On days +1, +3, +5 of every week, the cultures were fed with rh-IL2 (Proleukin; Chiron, Amsterdam, Netherlands, 10 U/mL). The lymphocytes activated by DC loaded with UVC-ray-induced apoptotic autologous LCL were 41.5% $CD8^+$ cells and 58.5% $CD4^+$ cells. Enzyme-linked immunospot (ELISPOT) assays were performed to assess the interferon γ (IFN-γ) production by lymphocytes activated by autologous DC loaded with apoptotic autologous LCL induced by UVC-ray exposure, as described elsewhere.[27] Briefly, wells in 96-well plates with nitrocellulose membrane (Millipore, Bedford, MA, USA) were coated with primary anti-INF-γ antibody (clone MAB1-D1K; Mabtech, Nacka, Sweden) diluted in $NaHCO_3$ buffer at pH 9.2 and incubated at 4°C overnight. To remove the primary antibody in excess, the plates were washed six times with PBS containing 0.5% of FBS before seeding the effector and target cells. The assay was performed in triplicate and responder fourth-week activated T cells were seeded at 10^4, 2.5×10^4 or 5×10^4 cells/well.

DC loaded with UVC-induced apoptotic LCL were seeded in a fixed number of 1.67×10^4 cells/well. As controls, unloaded DCs, apoptotic or live LCL alone were cultured with the responders.

After 24 hrs of incubation at 37°C in 5% CO_2, the cells were removed from the plate by washing six times with PBS supplemented with 0.5% FBS. A biotinylated secondary anti-INFγ-antibody (clone Mab7-B6-1; Mabtech) was added for 2 h at room temperature to each well. After extensive washing, the plates were incubated for 1 hr at room temperature with the avidin-alkaline phosphatase (AP) complex reagent (Mabtech, Sweden). The AP substrate was added and incubated for 20 minutes to develop spots. The reaction was stopped under running tap water. When the plate was completely dry, the spots were counted by computer-assisted image analysis (AID, Strassberg, Germany).

Results

Apoptosis of leukemia and lymphoma cells is induced efficiently by several agents

We observed a powerful induction of apoptosis of CLL and FL cells after treatment with all three methods investigated (MVA viral particles infection, staurosporine treatment, and UVC-ray exposure). No significant differences were observed between the different cell types (CLL or FL cells and BM or PB cells). As shown in Figure 1, staurosporine treatment and MVA infection induced apoptosis of tumor cells in a dose-dependent manner. Using staurosporine, we observed the highest percentage

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of apoptotic tumor cells (97.8±5.1%) after 24-hr culture with a dose of 5 μM. Using MVA viral particles with MOI ranging between 5 and 500, the apoptotic tumor cell fraction increased to between 21.3±3.9% and 95.8±2.3%, respectively. Moreover, 94.6±1.5% of the tumor cells were rendered apoptotic using an MOI of only 250. Finally, the percentage of apoptotic tumor cells was 96.3±2.3% after 24-hrs exposure to 10 J UVC-rays. These results did not change significantly when the UVC dose was increased to 15 J. Comparable percentages of apoptotic cells were obtained when LCL were used. Thus, on the basis of these results, we used staurosporine at 5 μM, MVA infection with an MOI of 250 and 10 J UVC-ray exposure for the subsequent experiments.

Immature monocyte-derived DC efficiently take up apoptotic tumor bodies

The uptake of fluorescent latex beads, detected by FM or FACScan analysis, was used to identify the maturation state of the DC with the highest phagocytic capacity. In all subsequent experiments were used 5-day cultured monocyte-derived DC since they were found to have the highest uptake capacity (*data not shown*).

Flow cytometry and FM analysis were also use to assess tumor cell uptake by DC. After labeling the DC with red CMFTR and the apoptotic tumor cells with green CMFDA stains, the uptake of apoptotic bodies by DC was documented by flow-cytometry (Figure 2, Panel A). Furthermore, the addition of trypan blue to quench the green fluorescence, before FACScan analysis, enabled us to exclude that double positive events reflected apoptotic cells adhering to the DC membrane rather than phagocytosed cells (Figure 2, Panel A). As a negative control, we performed the same experiments at +4°C to inhibit DC phagocytic activity (Figure 2, Panel B). Table 1 summarizes the percentage of DC (ranging from 84% to 96%) that contained killed tumor cells: the efficiency of DC loading did not differ significantly according to the method used to induce tumor cell apoptosis.

DC viability may be reduced after uptake of apoptotic tumor cells

After intensive washing, apoptotic tumor cells were co-cultured for 48 hrs with monocyte-derived DC. As shown in Figure 3, DC viability was dramatically reduced when the DC were co-cultured with apoptotic cells induced by MVA viral infection or staurosporine treatment. In fact, the dead CMFDA$^+$/PI$^+$ labeled DC were 90±5% and 92.7±6%, respectively. The very low amounts of viable DC prevented evaluation of their phagocytic or APC activity. DC cultured for 48 hours with UVC-induced apoptotic cells were minimally damaged (only 10±5% of CMFDA$^+$/PI$^+$ DC died). In addition, the supernatant harvested after exposure of neoplastic B cells to staurosporine or MVA viral particles did not induce DC damage (Table 2).

Table 1. Percentage of DC containing apoptotic bodies.

	% of DC containing tumor apoptotic bodies			
	37°C		*4°C*	
Apoptotic body inducer	*without trypan blue*	*with trypan blue*	*without trypan blue*	*with trypan blue*
UVC rays (10 J)	99±3	96±2	12±2	9±3
MVA infection (MOI 250)	90±3	84±3	10±2	6±3
Staurosporine (5 μM)	95±6	93±3	14±3	11±2

Data are expressed as mean ±SD of triplicates of five separate experiments.

Table 2. Supernatant from MVA-infected or staurosporine-treated neoplastic B cells does not affect DC viability.

	% of dead DC	
DC cultured	*With*	*Without*
Supernatant from MVA-infected tumor cells (MOI 250)	10±3	9±3
Supernatant from staurosporine-treated tumor cells (5 μM)	15±3	12±3

[a]*Percentage of dead DC after culture for 48 hours with supernatant from MVA-infected or staurosporine-treated neoplastic B cells. Data are expressed as mean ±SD of triplicates of five separate experiments.*

Reactivation of B-LCL-specific T cells by DC loaded with UVC-killed B-LCL

In an autologous system, ELISPOT analysis was used to evaluate the frequency of IFN-γ producing lymphocytes induced by DC loaded with UVC-induced apoptotic LCL. The numbers of IFN-γ-producing lymphocytes increased after three and four weeks of stimulation with antigen-pulsed DC, but not in cultures with unpulsed DC, or in cultures containing lymphocytes with apoptotic bodies

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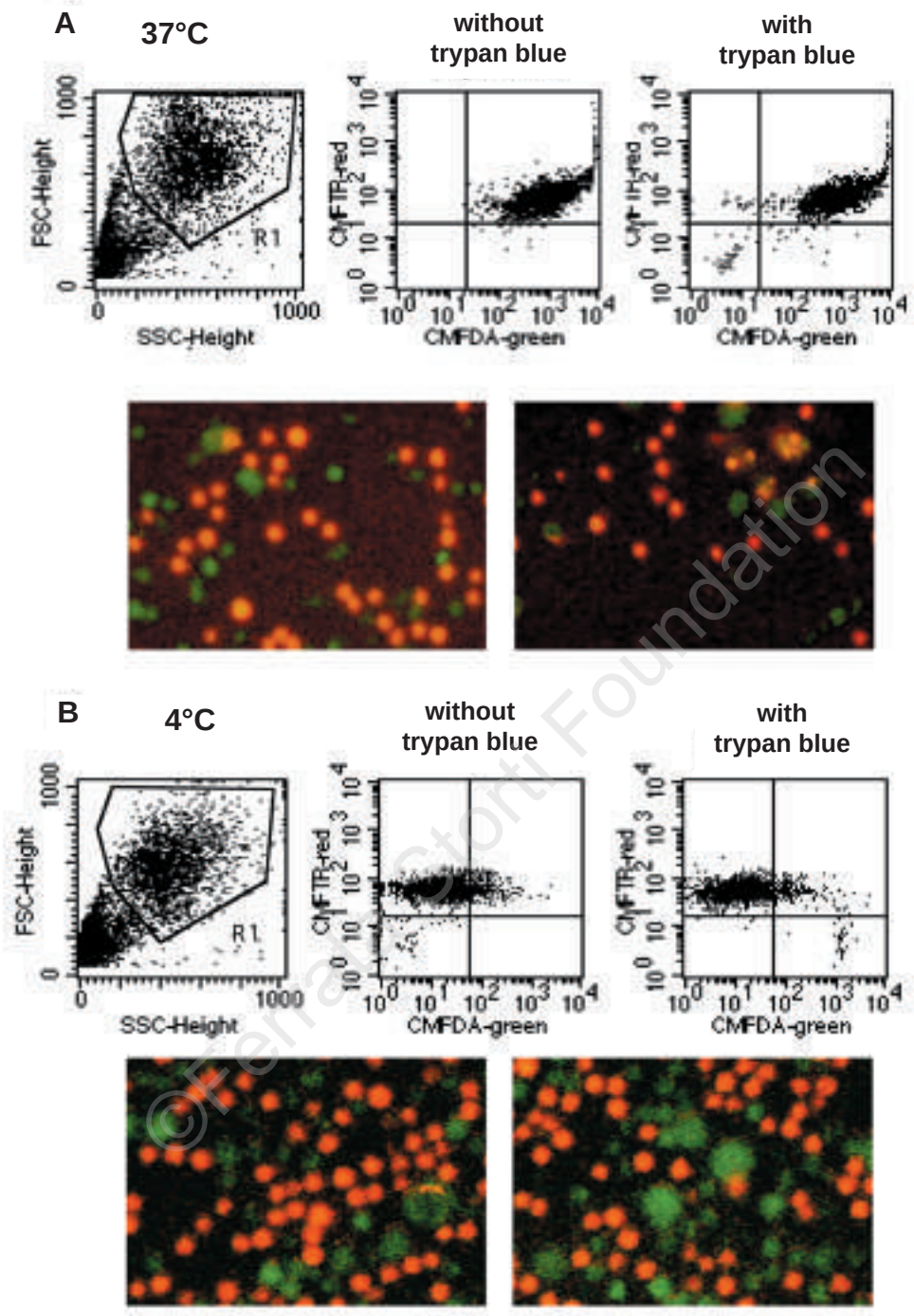

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Figure 2. Detection of DC containing UVC-induced apoptotic bodies by flow cytometry and fluorescent microscopy analysis. DC were stained by CMFTR red while UVC-induced apoptotic cells were labeled with CMFDA green. After 48 h of co-culture with apoptotic bodies, DC were analyzed by flow-cytometry. In the upper part of Panel A, DC containing apoptotic bodies resulted CMFTR$^+$/CMDA$^+$. The trypan blue dye exclusion test was used to quench the green florescence and discriminate between ingested apoptotic bodies and those merely adherent to the membrane of DC. The analysis was performed on gated cells derived from typical side and forward scatters of DC. Fluorescent microscopy (bottom part of Panel A) was used to confirm the presence of DC containing apoptotic bodies (yellow spots). In Panel B, the same experiment was performed at +4°C to inhibit DC phagocytosis. In this condition, the apoptotic bodies remained outside the DC and no CMFTR$^+$/CMDA$^+$ DC (upper part of Panel B) or yellow spots (bottom part of Panel B) were detectable. Data are from one representative experiment out of five.

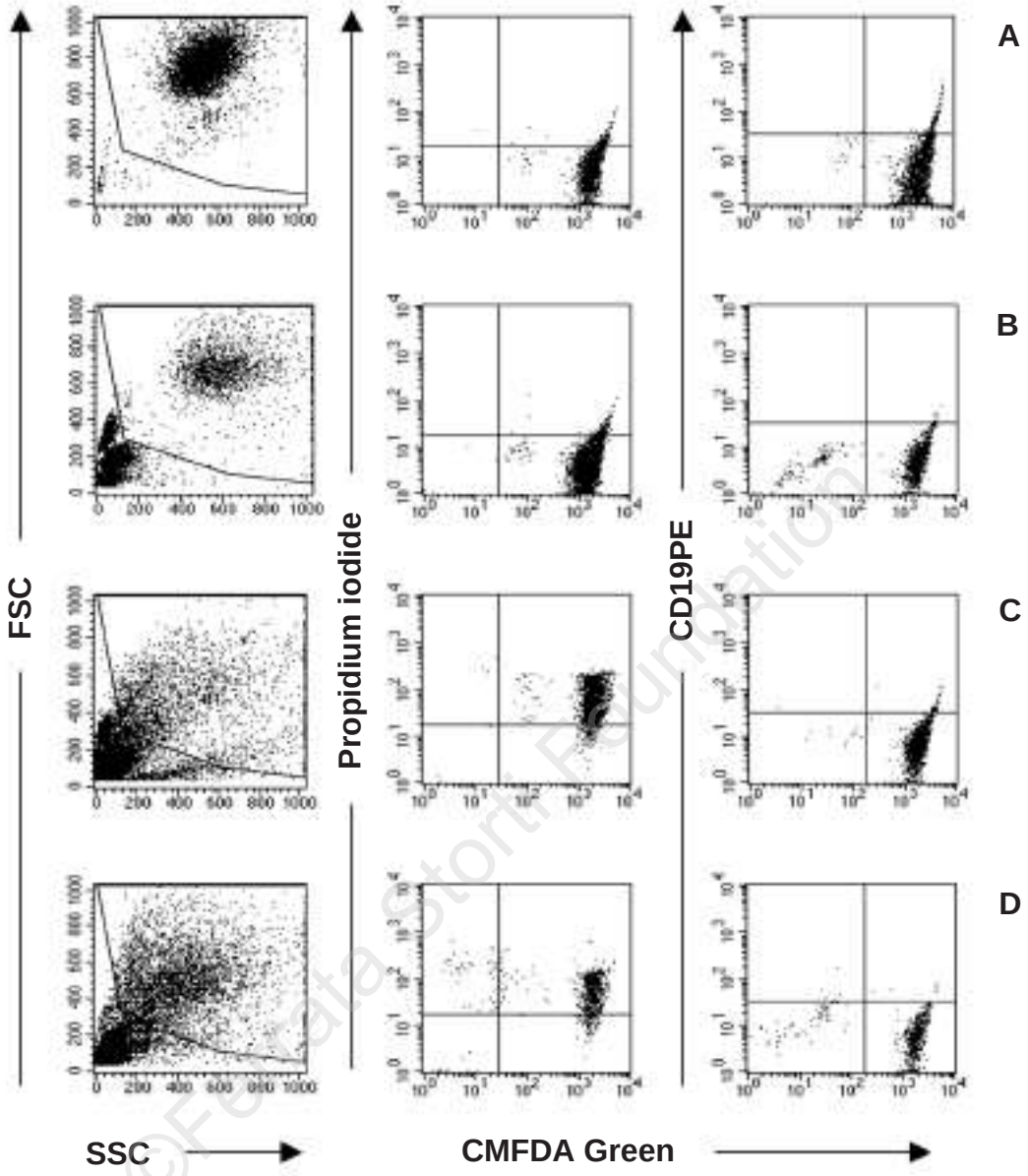

Figure 3. DC viability after loading with apoptotic bodies. DC were stained with green CMFDA and, after 48 hrs of incubation without (Panel A) or with (Panel B, C, D) apoptotic bodies, were labeled with proidium iodide and analyzed by flow-cytometry. The percentage of $CMFDA^+/PI^+$ DC loaded with UVC-induced apoptotic bodies was negligible (Panel B) whereas the percentage of $CMFDA^+/PI^+$ DC loaded with apoptotic bodies induced by 5 µM staurosporine treatment (Panel c) or by 250 MOI MVA viral infection (Panel D) resulted to be ≥ 90%. The absence of $CD19^+/CMFDA^+$ cells indirectly excluded $CD19^+$ necrotic tumor cells adherent to the DC membrane from the count. Data are from one representative experiment out of five.

without DC, or lymphocytes alone. As shown in Figure 4, at the fourth week of stimulation, $133\pm5/10^4$ lymphocytes activated by pulsed DC produced INF-γ spots. The number of INF-γ-producing lymphocytes after activation by DC loaded with apoptotic bodies was 7 and 3 times greater than when apoptotic bodies alone (19.5 ± 2 spots/10^4 cells; $p = 0.005$) or unpulsed DC (46 ± 5 spots/10^4 cells; $p = 0.005$) were used as stimulators. As expected, live LCL were optimal antigen presenting cells, inducing ten times more spots than did the apoptotic LCL (Figure 4).

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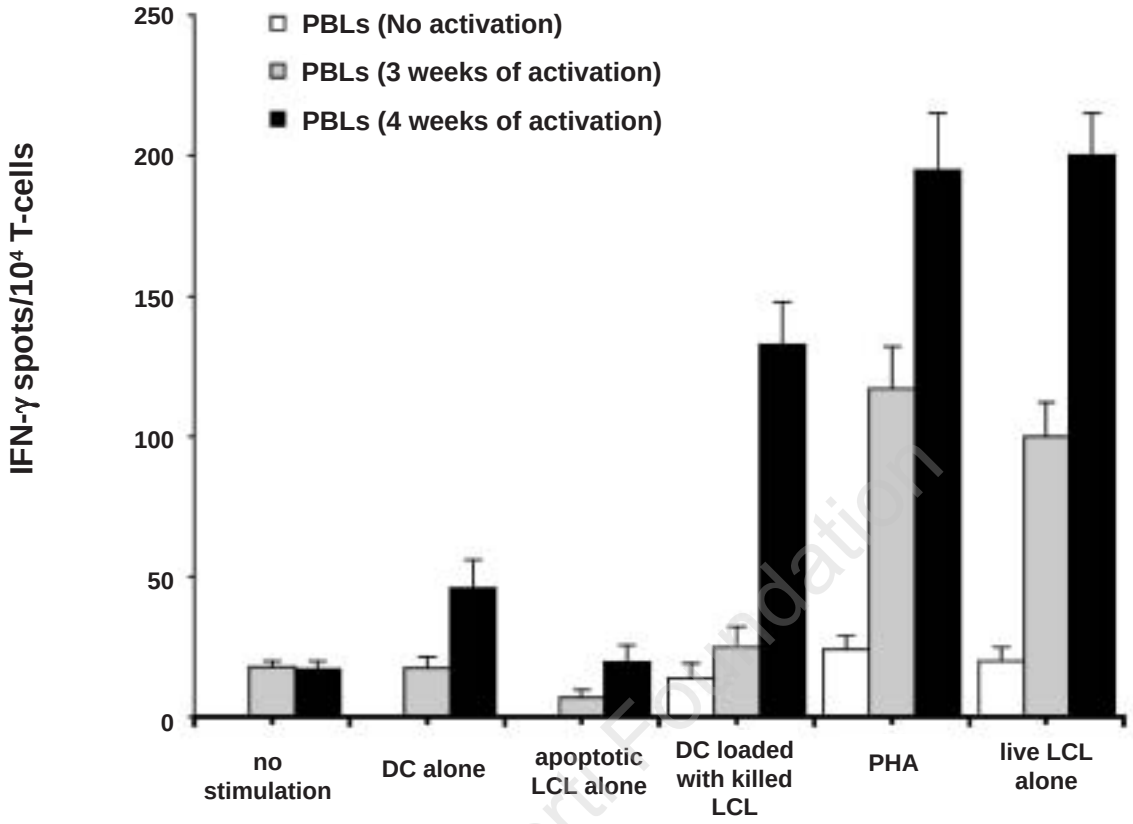

Figure 4. Activation of INF-γ-producing lymphocytes after culture with DC loaded with LCL apoptotic bodies. DC loaded with LCL apoptotic bodies induced by UVC-ray exposure were used as stimulators of autologous PBMC. After 3 and 4 weeks of culture, ELISPOT assays were performed to evaluate the number of INF-γ producing autologous lymphocytes. At the fourth week, 133±5/10⁴ lymphocytes produced INF-γ spots after activation by DC pulsed with apoptotic bodies, i.e. 7 times more than was obtained using apoptotic bodies alone (19.5±2 spots/10⁴ cells; p = 0.005) and 3 times more than obtained using unpulsed DCs (46±5 spots/10⁴ cells; p = 0.005). Data are from one representative experiment out of three.

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Discussion

Whole tumor cells, as a source of antigens, may be an attractive priming strategy for vaccine approaches since they provide a wide spectrum of tumor-specific and tumor-associated antigens.[15,28] In addition, this approach may in principle counteract the ability of neoplastic cells to escape immune recognition by down modulation of one or more antigenic determinants. On the other hand, emphasis on the clinical applications of this approach is reduced by uncertainty regarding the optimal means for treating tumor cell preparations, by the possibility of inducing autoimmunity against self antigens and by the debate regarding the optimal tumor: DC ratios for effective priming of T cells.[29] In spite of these uncertainties several whole tumor cell preparation methods have been shown to be effective for loading DC and then eliciting antitumor immune responses. Cross-priming of CTL with antitumor activity has been demonstrated with DCs pulsed with tumor cell lysate[30] and apoptotic tumor cells.[21,32-34] Specifically, Schnurr *et al*.[19] demonstrated that antigens from apoptotic pancreatic carcinoma cell lines, either in the form of whole cells or as released particles, were more potent than tumor lysates in inducing T-cell priming and activation by DC. In addition, Hoffman *et al*.[21] reported stronger CTL responses with apoptotic tumor cells than with cell lysates in a squamous cell carcinoma model.

The enhanced CTL activation by antigens from apoptotic cells may be attributed to several mechanisms. After ingestion, most particulated antigens requiring phagocytosis are digested into peptides associating with MHC class-II molecules in the endocytic compartments and are presented to T-

helper cells.[35] This is believed to be the predominant cell lysate processing pathway. Conversely, scavenger receptor-mediated phagocytosis of apoptotic tumor cells allows antigens to gain access to MHC class-I compartments, resulting in cross-presentation of the antigens to CTL.[31] In addition, enhanced CTL responses to tumors might be mediated by heat shock proteins expressed by stress-induced apoptotic tumor cells.[36] On the basis of this theoretical background and reported observations,[19,21] there is a scientific consensus that antigen preparations from apoptotic tumor cells represent a promising alternative to tumor lysate in DC-based tumor vaccines.

Taken together the existing data on preclinical models strongly suggest that DC loading with killed tumor cells may be an effective strategy for priming T-cell-mediated anti-tumor responses.

However, the best method for inducing apoptosis of neoplastic cells, with the efficiency required by a clinical protocol, remains to be assessed. To this end, we tested three strategies for inducing apoptosis in B-cell tumors. First, neoplastic B lymphocytes were transduced with MVA viral particles. We chose this agent since viral infection could even induce $CD4^+$ T helper response against viral determinants, a response that in principle could behave as an adjuvant in the antitumor response. Second, we cultured neoplastic B lymphocytes in the presence of staurosporine,[37] since this drug has already been successfully used for the treatment of lymphoproliferative diseases. Finally, neoplastic B cells were exposed to UVC-rays, a well-known method for promoting apoptosis.[19] By fine tuning the conditions to induce apoptosis by the three methods, highly effective tumor killing was achieved in all instances: less than 1% of the tumor cells were still alive after 48 hours of exposure to the different agents. However, we observed toxic damage of DC after the phagocytosis of apoptotic bodies whose apoptosis had been induced by staurosporine or MVA viral particles. A significant loss of DC viability was observed only when we utilized concentrations of the latter agents which caused $\geq$ 95% of apoptosis of the tumor cells. Under these experimental conditions, the percentage of DC still alive after 48 hrs of co-culture with apoptotic bodies was very low. According to our experiments, it can be suggested that apoptotic bodies internalized by DC release viral particles as well as staurosporine which in turn induce toxic damage of DC. In contrast, the viability and APC function of the DC were preserved when these cells were loaded with UVC-induced apoptotic bodies. In fact, DC loaded with killed autologous B-LCL were able to activate B-LCL-specific T cells.

In conclusion, we demonstrated that even though several agents could be used to induce apoptosis of neoplastic human B lymphocytes, the persistence of apoptosis inducers inside the apoptotic bodies affects DC viability. The percentage of viable DC after loading with apoptotic bodies induced by MVA infection or staurosporine, at concentrations required for clinical purposes, thus drops to extremely low levels. UVC radiation is consequently the best means for inducing tumor cell apoptosis for use in a clinical setting, since it had no observable harmful effect on the viability or APC function of DC. For these reasons, we are starting a clinical protocol of vaccination using autologous monocyte-derived DC loaded with UVC-killed autologous tumor cells in indolent non-Hodgkin's lymphoma patients.

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MDN wrote and revised the manuscript. MD, CC, AA, and AMG planned the experiments reported. They critically revised the manuscript and approved the submitted manuscript. SN performed experiments forming the basis of several data presented in the publication. She critically revised the manuscript and approved the submitted manuscript. LR, RM and MM performed apoptosis experiments. They critically revised the manuscript and approved the submitted manuscript. PB, IB and PM performed ELISPOT experiments. They critically revised the manuscript and approved the submitted manuscript. Primary responsibility for the paper: MDN; primary responsibility for Tables 1 and 2: MDN, AA; primary responsibility for Figure 1: MDN; primary responsibility for Figures 2-3: MDN, SN; primary responsibility for Figure 4: MDN, AA.

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