Drug- and cell-mediated antitumor cytotoxicities modulate cross-presentation of tumor antigens by myeloid dendritic cells

Alessandra Galetto[a], Stefano Buttiglieri[a], Sarah Forno[b], Francesco Moro[c], Antonio Mussa[c] and Lina Matera[b]

The way a tumor cell dies is believed to influence both its engulfment by dendritic cells (DC) and access of the relevant antigen(s) to the cross-presentation pathway. Here we have studied the effect of lymphokine activated killer (LAK) cells, γ-radiation and the antimetabolite drug 5-fluorouracil (5-FU) on tumor uptake by HLA-matched DC, and DC presentation of tumor antigens to autologous T lymphocytes. LAK cells and radiation were the best inducers of apoptotic death (Annexin-V$^+$/propidium iodide$^-$) on the gastric cell line KATO III and a primary gastric carcinoma, respectively. The highest rate of tumor uptake by monocyte-derived, granulocyte macrophage colony stimulating factor/interleukin (IL)-4-driven DC was associated with 5-FU, followed by radiation. These treatments also induced high levels of heat shock protein (*hsp70*). In contrast, only DC that had been taken up 5-FU- or LAK-treated tumors up-modulated IL-12 and presented tumor-associated antigens with increased efficiency, as shown by class I MHC-restricted interferon-γ release and cytotoxic responses by autologous lymphocytes. Together, these data indicate that apoptotic death induced by anti-cancer therapies can induce distinct patterns of class I MHC cross-presentation of gastric carcinoma-associated antigens to cytotoxic T lymphocyte precursors. *Anti-Cancer Drugs* 14:833–843

Anti-Cancer Drugs 2003, **14**:833–843

Keywords: antigen presentation/processing, apoptosis, dendritic cell, tumor immunity, vaccination

[a]Department of Oncology, Centro OncoEmatologicoSubalpino (COES), [b]Laboratory of Tumor Immunology, Department of Internal Medicine and [c]Department of Surgical Oncology, University of Turin, Turin, Italy.

Sponsorship: Supported in part by MIUR, Regione Piemonte (Oncology project) and the CERMS/COES project funded by the 'Compagnia di San Paolo/FIRMS'.

Correspondence to L. Matera, Laboratory of Tumor Immunology, Department of Internal Medicine, University of Turin, corso A. M. Dogliotti, 14, 10126 Turin, Italy. Tel: +39 011 6961813; fax: +39 011 6634751; e-mail: lina.matera@unito.it

Received 10 June 2003 **Revised form accepted** 20 August 2003

Introduction

Cellular immunity mediated by antigen-specific cytotoxic T lymphocytes (CTL) is one of the major mechanisms of tumor clearance. Priming of both CD4 and CD8 T lymphocytes requires the presence of MHC–antigen complexes and co-stimulatory molecules on antigen-presenting cells, whereas recognition of the relevant antigen–MHC complex is sufficient for armed CTL to kill the tumor. A characteristic of dendritic cells (DC) [1] is their ability to present extracellular antigen in both class II and class I MHC pathways, thereby priming both CD4 and CD8 T cells (cross-priming) [2]. Animal studies and human cancer trials have shown that specific T cell responses against tumors as well as tumor regression can be achieved with vaccines based on DC. However, identification of an efficient antigen-loading strategy is still a critical challenge. Loading DC with defined peptides of known sequences has induced peptide-specific T cell responses and considerable anti-tumor effects [3–6]. However, this approach has potential drawbacks since: (i) it is limited to HLA-matched individuals, (ii) it recruits only the epitope peptide-specific T cell fraction and does not catch co-expressed tumor-associated antigens (TAA), and (iii) it fails to trigger CD4 T lymphocytes. In contrast, crude tumor material provides the complete array of TAA, thus targeting multiple specificities and reducing the risk of tumor escape variants. Native tumor antigens can be delivered to DC in the form of tumor cell lysates (i.e. necrotic death) or apoptotic tumor cells. Uptake by DC is restricted to their immature stage when they are well equipped to acquire antigen, but express low levels of the MHC and co-stimulatory molecules needed for T cell stimulation. Upon receipt of a maturation signal, DC downregulate relevant receptors, and upregulate MHC and co-stimulatory molecules [7]. The way a tumor cell dies is believed to influence both its engulfment by DC and access of the relevant antigen(s) to the cross-presentation pathway. It will also determine whether a permissive (pro-inflammatory) or non-permissive (suppressive) cytokine milieu is created [8]. Apoptotic cell death occurring during normal development is expected to induce inert clearance by scavenger cells, whereas non-physiological death should be perceived as threatening. Danger signal [9] has been associated with stress proteins (e.g. the family of heat shock protein *hsp*). Since *hsp* are

0959-4973 © 2003 Lippincott Williams & Wilkins DOI: 10.1097/01.cad.0000097711.69687.64

produced during necrotic death, this has been assumed to be highly immunogenic by definition [10,11]. However, the data indicating which form of death is more immunogenic are conflicting. Engulfed apoptotic cells suppress antigen presentation by DC [12] and reversion of suicide apoptotic death to non-apoptotic death by *bcl-2* transfection confers high immunogenicity on injected tumors [10]. However, direct evidence of an immunogenic potential of apoptotic tumors has been provided [13–24]. In some instances, a maturation signal [25–28] or previous exposure to heat stress [29] were necessary to induce maturation of DC, and hence appropriate processing and presentation of apoptotic tumors. Direct comparison displayed comparable effects on DC antigen processing and presentation by apoptotic (UVB light-treated) and necrotic (thaw/freezing lysates) tumors [24]. In a recent study, late apoptosis/secondary necrosis, but not early apoptosis, conferred immunogenic properties on DC-presented melanoma cells in the presence of a maturation signal [28].

Besides natural turnover and host immunogenic killing, tumor death is induced exogenously via radiotherapy, chemotherapy, gene suicide therapy and adoptively transferred native lymphokine activated killer (LAK)/specific (CTL) cytotoxic cells. By generating high amounts of apoptotic tumor cells, these treatments provide the immune system with a formidable source of native tumor antigens. Indeed, establishment of an immune memory against chemotherapy [30,31] and NK-killed [32] tumors has been described. Here we have addressed the relevance of these kinds of tumor cell death to DC uptake and antigen presentation *in vitro*. We show that apoptotic tumors, short-term treated with radiation, chemotherapy and LAK cells are taken up by myeloid DC, and induce distinct patterns of class I MHC cross-presentation of gastric carcinoma associated antigens to CTL precursors.

Materials and methods

Cytokines

Human recombinant (r) granulocyte-macrophage colony stimulating factor (GM-CSF) and r interleukin (IL)-4 were purchased from Sigma-Aldrich (MI, Italy), and rIL-2 from Chiron (MI, Italy).

Phenotype of DC

Cells were collected, washed in phosphate-buffered saline (PBS) and incubated (10^5 cells in 100 µl PBS) for 30 min at 4°C with 10 µl of the following mAb (all from Becton Dickinson, Mountain View, CA): FITC-conjugated anti-CD80; phycoerythrin (PE)-conjugated anti-CD86, anti-CD83, anti-HLA-DR and anti-CD36; and FITC/PE-conjugated anti-CD14 and irrelevant isotype-matched mAb (Becton Dickinson). After washing, the cells were fixed in PBS/1% paraformaldehyde and analyzed using a FACSCalibur (Becton Dickinson). A total of 10 000 events were analyzed for each sample.

Tumor cells

The human gastric carcinoma cell lines KATO III [33] (HLA-A2$^+$), hereafter referred to as KATO, and GTL-16 (HLA-A2$^-$) were grown as suspension-adherent culture in RPMI 1640 medium (Life Technologies, Paisley, UK) containing 20% heat inactivated FCS (Life Technologies), L-glutamine, penicillin and streptomycin (Sigma-Aldrich), and subcultured every 6–8 days. The adherent cells were dislodged by gently scraping. The T leukemia cell line Jurkat was grown as suspension culture in RPMI 1640 medium containing 10% FCS and split every 4 days. Studies were also performed on cells isolated from surgically resected tissues (here referred to as K) of gastric cancer patients, who had not received chemotherapy, radiation therapy, immunotherapy or immunosuppressive medications within the prior 4 weeks. The patients provided signed informed consent. Tumor tissues were excised as an adjuvant surgical procedure and thereafter judged of transformed nature by the local anatomopathologist unit. The surgical samples were incubated for 2 h at 37°C in PBS with 0.1% collagenase type IV, 0.002% DNase type I and 0.01% hyaluronidase type V (Sigma-Aldrich). The recovered cells were separated on a Ficoll-Hypaque gradient (Pharmacia, Uppsala, Sweden), and after flow cytometric screening for the HLA-A2$^+$ aplotype with the B77.2 mAb (kindly provided by Imperial Cancer Research Fund, Oxford, UK), frozen until use. One of these tumors was found to be HLA-A2$^+$ and used in this study.

Cell isolation and cultures

Generation of DC from adherent peripheral blood mononuclear cells (PBMC) was performed as described previously [23]. Briefly, PBMC were isolated from heparinized blood of HLA-A$^+$ blood bank donors by standard Ficoll-Hypaque density gradient centrifugation, washed with RPMI 1640 supplemented with 5 mM EDTA (Sigma-Aldrich) and 2% heat-inactivated FCS, and suspended at 5×10^6/ml in RPMI 10% FCS. After a 2 h incubation at 37°C in a humidified, 5% CO_2 incubator, non-adherent cells were removed by two washings with pre-warmed (37°C) 10% FCS RPMI medium and the adherent cells were cultured in the above complete medium supplemented with rGM-CSF (30 ng/ml) and rIL-4 (10 ng/ml). Cytokines were replaced on day 3 and immature (i) DC was collected on day 6.

Killing of tumor cells

KATO cells or the thawed K cells received the following treatments: γ-radiation (9000 rad), 5-fluorouracil (5-FU) at a concentration of 78 µg/ml, which has been described to correspond to the blood level of the drug during continuous infusion [34], and LAK at an effector:target

ratio of 10 : 1. This ratio was shown in pilot experiments to produce 30–45% ^{51}Cr-specific release by KATO targets. LAK cells were prepared from PBMC of healthy blood donors, cultured for 5 days with 200 U/ml of rIL-2.

Analysis of tumor death

After 4 h from exposure to radiation, 5-FU or LAK, tumor cells were stained with Annexin-V–FITC (Ann-V) and propidium iodide (PI) using the Annexin-V-FLUOS staining kit (Roche, MI, Italy), followed by flow cytometric analysis.

Uptake of dying tumors by iDC

The tumor cells were stained with PKH26 (Sigma-Aldrich) according to the manufacture's instructions, before receiving the above killing treatments. After 4 h tumor cells were washed (two centrifugations of 10 min at 300 *g*) and incubated for 24 h at 37°C with 1×10^5 iDC at a 2 : 1 ratio, and the mixed culture was stained with the FITC mAb CD80 (20 min at 4°C). Engulfment of tumor cells by DC was assessed by flow cytometric analysis as the percentage of double-stained versus green cells on a total of 10 000 events.

Analysis of *hsp70* expression

For immunoblot analysis, 2×10^6 cells/sample were washed twice with PBS and lysed with RIPA buffer. Equal amounts of protein for each sample were separated by SDS–PAGE (12% resolving gel) and blotted onto nitrocellulose. The filters were incubated with a mouse mAb against *hsp70* (W27; Santa Cruz Biotechnology, Santa Cruz, CA) and the primary mAb was recognized by horseradish peroxidase-conjugated sheep anti-mouse (Amersham Pharmacia Biotech, Rainham, UK). Proteins were detected by ECL (Amersham) and analyzed by Kodak 1D Image Analysis System (Kodak, Cinisello Balsamo, MI, Italy). Results were expressed as net intensity (NI), according to the manufacturer's instructions.

Analysis of IL-12, tumor necrosis factor (TNF)-α and IL-10 release

The 24 h supernatant of treated or untreated tumor cells and of iDC, unpulsed or cultured at the ratio of 1 : 2 with KATO/K cells, as indicated, was collected and analyzed by standard ELISA for IL-10 (CLB, Amsterdam, The Netherlands), TNF-α (Bender MedSystem, Vienna, Austria) or IL-12 p70 (PharMingen, Immucor, MI, Italy) release.

Induction of antigen-specific CTL response using dying KATO or an autologous tumor

Four hours after the indicated treatments (see above), KATO and K cells, either untreated or treated with radiation, 5-FU or LAK, were washed (two centrifugations of 10 min at 300 *g*) and mixed to DC from MHC-matched (HLA-A2$^+$) donors at a ratio of 2 : 1 or left alone in culture. Incubation proceeded for 24 h, after which 40% monocyte conditioned medium (MCM) [35] was added to induce a mature DC phenotype and the cultures were incubated for a further 24 h. Unpulsed/tumor-pulsed DC and DC-free tumors were irradiated (6000 and 9000 rad, respectively) and added to T lymphocytes (DC : lymph ratio 1 : 10; tumor : lymph ratio 1 : 5). rIL-2 (20 U/ml) was added to the culture after 24 h and the sensitization procedure was repeated weekly, for up to three total stimulations.

Analysis of interferon (IFN)-γ release by ELISPOT

The frequency of IFN-γ-secreting T lymphocytes in cultures was assessed in an ELISPOT assay, as previously described [23,36]. Briefly, nitrocellulose membrane 96-wells microtiter plates (Multiscreen; Millipore, Vimodrome, MI, Italy) were coated with anti-IFN-γ mAb (PharMingen, San Diego, CA) and incubated overnight at 4°C. The remaining binding sites were blocked by incubation with 10% FCS. After extensive washings, 10^5 T cells (initial number in culture) were seeded in medium alone, medium containing the target tumors (KATO, GTL-16 or Jurkat, as indicated) at a 2 : 1 ratio or medium containing phorbol myristate acetate (PMA; 50 ng/ml) and ionomycin (1 μg/ml). Triplicate wells of these combinations were set up in the presence of the anti-human MHC class I blocking mAb W6.32 (DBA Italia, MI, Italy) at the 1 : 80 dilution or of the equally diluted isotype-matched antibody. After 24 h, cells were washed and a biotinylated anti-IFN-γ mAb (PharMingen) was added. After 1 h incubation, plates were washed with PBS/Tween and avidin–horseradish peroxidase conjugate (PharMingen) was added for 30 min following another PBS/Tween washing, the substrate solution (3-amino-9-ethylcarbazole) (Sigma-Aldrich) was added for 5–10 min. Rinsing with tap water stopped the reaction. After the plates had dried overnight, red spots (indicative of reactive lymphocytes) were enumerated by computer-assisted video image analysis [37] with the AID Elispot-Reader (Bioline Amplimedical, MI, Italy).

CTL assay

The cytolytic activity of induced CTL was analyzed in a standard ^{51}Cr-release assay, performed as previously described [23,38]. KATO cells were incubated for 1 h with 100 μCi $Na_2^{51}CrO_4$ (Perkin Elmer, Life Science, Boston, MA) and washed. Different amounts of effector cells were added to triplicate wells of a V-bottomed 96-well microtiter plate to give 20 : 1, 10 : 1, 5 : 1 and 2.5 : 1 effector : target ratios. ^{51}Cr-labeled KATO cells (5×10^3) were added to each well and the mixture was incubated for 4 h. At the end of the assay the supernatants (100 μl) were harvested and counted in a γ-counter. Percent specific cytotoxicity was calculated by the formula: $(E - S)/(M - S) \times 100$, in which E is the ^{51}Cr release

in the presence of effectors, S is the ^{51}Cr release with medium alone and M is the ^{51}Cr release with Triton X-100. Experiments with spontaneous release higher than 10% were not considered. For statistical analysis, CTL activity was converted to lytic units (LU)/10^8 effectors which reduce the ^{51}Cr response to linearity [39], a LU being the number of effector cells required to produce 30% specific cytotoxicity of 4×10^3 target cells. The LUs of control and treated cultures were logarithmically transformed to reach near to normal distribution [39], and compared using Student's t-test for paired data.

Statistical analysis

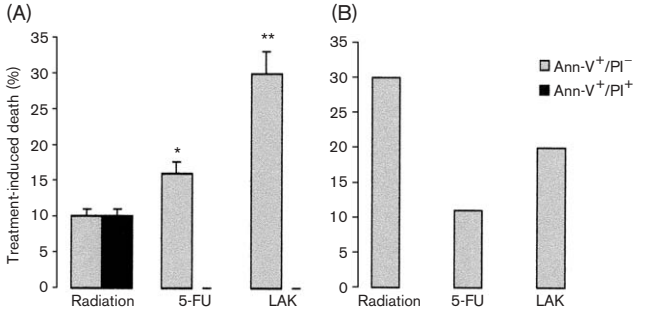
Paired Student's t-test was employed through the study to assess the statistical significance of changes induced by a given treatment.

Results

Both the treatment and the cell target can determine the kind of tumor death

KATO and K cells were treated as indicated, and after 4 h apoptotic and necrotic cells were defined by flow cytometric analysis as Ann-V^+/PI^- and Ann-V^+/PI^+, respectively. The short incubation time chosen allowed estimation of primary necrosis, since secondary necrosis occurs later [29]. Figure 1(A) shows death of KATO induced by each treatment (mean ± SD of four experiments) net of the basal death of untreated cultures (8 ± 1 Ann-V^+/PI^- and 4 ± 0.5 Ann-V^+/PI^+). The highest level of apoptotic death (31 ± 4, $p < 0.001$) was induced by LAK, followed by 5-FU (16 ± 2, $p < 0.005$). After treatment with radiation, small fractions of KATO cells were in early apoptosis (11 ± 4) or had progressed to late apoptosis/secondary necrosis (12 ± 2). The effect of treatments on death of the primary tumor (K) was tested on samples thawed on two occasions (basal values: 20 and 22% Ann-V^+/PI^- and 9 and 7% Ann-V^+/PI^+). The data from one of these experiments are depicted in Figure 1(B). Radiation induced the highest levels of apoptotic figures (treatment-induced values 30 and 34%), followed by LAK (21 and 22%), whereas 5-FU induced negligible changes (9.5 and 8%). Necrotic figures were not induced by any of the treatments. These results indicate a stability of the death mechanism employed by LAK cells, regardless of the target cell, as opposed to the target dependence of the mechanism of the cytostatic agents.

Fig. 1



Necrotic and apoptotic death of tumor cells treated with radiation, 5-FU or LAK cells. The gastric carcinoma cell line KATO (A) or fresh K cells (B) were exposed to the indicated antitumor treatments for 4 h and analyzed by flow cytometry. The frequency of early apoptotic (Ann-V^+/PI^-) and late apoptotic/secondary necrotic (Ann-V^+/PI^+) cells induced by each treatment is calculated by subtracting the values of parallel untreated tumor cultures (KATO: 8 ± 1% Ann-V^+/PI^- and 4 ± 0.5% Ann-V^+/PI^+; K: 20% Ann-V^+/PI^- and 9% Ann-V^+/PI^+). For LAK tumor co-cultures, the values of control cultures with LAK alone have also been subtracted to evaluate the death parameters of the tumor components. (A) Mean ± SD (*$p < 0.005$, **$p < 0.002$) of four experiments. (B) Results from an individual experiment, repeated once with similar results.

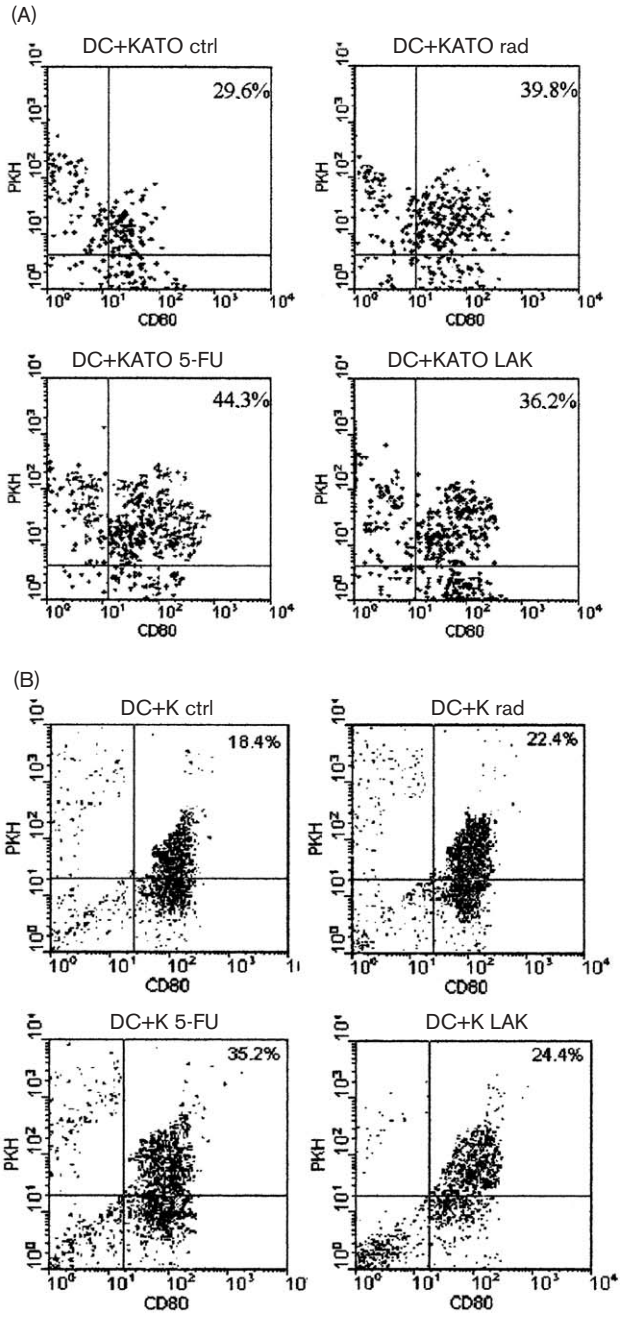
The uptake of tumors is differentially affected by the treatments

To determine whether the death mechanism influenced uptake of tumor cells by DC, KATO and K cells were red dyed with PKH26 prior to the death stimuli. After 4 h, tumor cells were incubated with monocyte-derived HLA-related iDC and after 24 h the mixed cultures were stained with the DC-specific anti-CD80 mAb. Engulfment was judged as the frequency of double-stained cells evaluated by flow cytometry (Fig. 2). Almost 70% of cells were $CD80^+$ in cultures set up with DC alone (not shown). An apparent decrease of this percentage was observed in DC–KATO mixed cultures, due to dilution by tumor cells, which do not bear the CD80 marker. The fraction of KATO-taking up DC (Fig. 2A illustrates one experiment representative of three) was significantly increased after treatment of KATO cells with 5-FU (30.2 ± 3 versus 43.2 ± 5, $p < 0.01$, mean ± SD of three experiments), radiation (30.2 ± 3 versus 40.2 ± 5, $p < 0.02$) and LAK (30.2 ± 3 versus 36.9 ± 3, $p < 0.05$). A superimposable pattern was observed with the primary K cells: 18.4% uptake of the untreated tumor, and 22.4, 35.2 and 24.4% engulfment after radiation, 5-FU and LAK, respectively (Fig. 2B). A second experiment using DC from a different HLA-A^+ donor gave 19% uptake of the untreated K, and 28, 24 and 29% engulfment after radiation, 5-FU and LAK respectively (not shown). Comparison of Figures 1 and 2 shows that tumor uptake by DC is a characteristic of a given treatment and not dependent on the killing efficiency of the agents. A non-significant trend toward a negative correlation between apoptotic death and tumor uptake was observed. Expression of the DC maturation markers MHC class II, CD80 CD86 and CD83 and the receptor CD36 involved in DC phagocytosis was not affected (not shown).

Increase of IL-12 by DC loaded with tumor cells receiving different treatments

Antigen uptake is a maturation signal for DC, which, in turn, produces the T_h1 maturating cytokine IL-12.

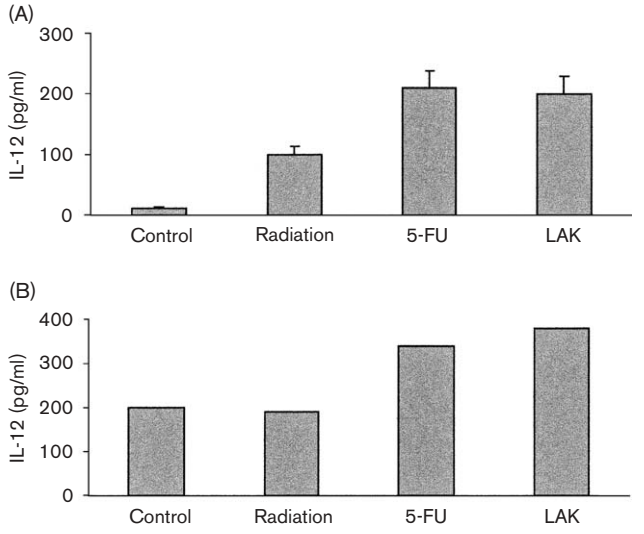
Fig. 2



Flow cytometric analysis of tumor-phagocytosing DC. KATO cells (A) and K cells (B) were red stained with PKH26 before receiving the described treatments, washed and incubated with DC. After 24 h, cultures were stained with the DC-specific FITC anti-CD80 mAb and double-stained cells were scored as percent of tumor phagocytosing (green) DC. (A) Results of an experiment representative of three using DC from different HLA-A2$^+$ donors. Increased uptake of KATO-loaded DC was observed after 5-FU ($p<0.01$), radiation ($p<0.02$) and LAK ($p<0.05$) treatments. (B) Results from a representative experiment of two using DC from different HLA-A$^+$ donors.

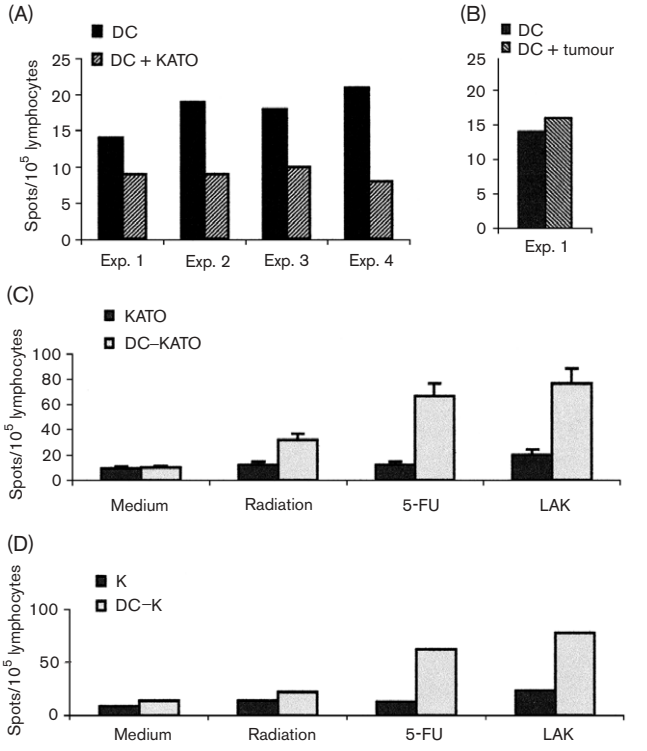
Figure 3 shows that production of IL-12 by tumor-loaded DC is differentially influenced by the treatments given to the tumor, the most effective treatments being 5-FU ($p < 0.002$) and LAK ($p < 0.005$) for both the KATO (A, means ± SD of four independent experiments using the HLA-A2$^+$ donors of Fig. 2) and K cells (B, results from one experiment representative of two, see Fig. 2).

Fig. 3



Increased IL-12 release by DC loaded with tumor cells receiving different treatments. DC were incubated with KATO cell line (A) or K cells (B) treated as indicated, and IL-12 contained in the 24 h supernatant was evaluated in a standard ELISA assay. (A) Mean ± SD of four experiments. (B) Data from one experiment, repeated once with similar results.

Fig. 4



Effect of the treatment given to tumor cells on T lymphocyte activation by tumor-loaded DC. Lymphocytes from healthy HLA-A$^+$ blood bank donors received a three-round stimulation with autologous DC alone (A and B) or with autologous DC loaded with the KATO (A and C) or K cells (B and D) untreated (A and B) or treated with the indicated chemotherapeutics or LAK cells (C and D). Parallel cultures stimulated by treated/untreated DC-free tumors are shown in (C; KATO) and (D; K). Five days after the third stimulation, IFN-γ release was evaluated in a 24-h ELISPOT assay using the sensitizing cells (KATO or K) as targets. The mean size of spots was much higher in cultures stimulated by antigen-presenting DC compared to cultures stimulated by the polyclonal activators PMA/ionomycin (not shown). Spots in the cultures with medium (negative control) and PMA/ionomycin were 10 ± 3 and 180 ± 15, respectively. (C) Mean ± SD of four experiments. (D) Results from an individual experiment, repeated once with similar results.

5-FU Does not influence the release of TNF-α by tumor cells or tumor-loaded DC

TNF-α is a strong maturation factor for DC. It therefore seemed pertinent to address its influence on the increased triggering of DC induced by 5-FU. A 24-h incubation of DC with untreated KATO cells (two experiments) or with K (one experiment) strongly increased TNF-α production after 24 h (from 70 to 150 and from 30 to 480 pg/ml for KATO, and from 30 to 470 for K). However, no consistent increase was observed when DC were loaded with 5-FU-treated KATO or with K cells (not shown). KATO cells did not produce TNF-α in the control or in the 5-FU-stimulated conditions. Trace amounts of TNF-α found in the K cells were not modified by 5-FU.

Activation of T cell response by tumor-loaded DC correlates with IL-12 release

Phagocytosis is a property of both macrophages and DC, whereas only DC can efficiently present the processed antigen to T lymphocytes. To confirm that tumor engulfment had occurred in DC, we studied the effect of tumor treatment on DC presentation of putative TAA to autologous T lymphocytes. iDC that had been incubated for 24 h with untreated/treated tumor cells were further matured in the presence of a monocyte supernatant to optimize their antigen-presenting activity [35] and used as stimulators for autologous lymphocytes. Stimulation was repeated twice and specific antitumor response was assessed 5 days after the third stimulation in a 24-h ELISPOT assay, in the presence of the untreated KATO cells as targets.

Figure 4(A–D) shows the results of these experiments. The stimulatory activity of unloaded DC was decreased when they were loaded with the untreated KATO cells (Fig. 4A, results of four individual experiments), but not with the untreated fresh gastric carcinoma cells (K) (Fig. 4B, one experiment of two with similar results). Loading of DC with KATO (Fig. 4C, mean ± SD of three experiments) or K (Fig. 4D, data from one of two experiments with similar results) that had been treated with 5-FU or LAK significantly increased the IFN-γ release of co-cultured T lymphocytes (untreated KATO versus 5-FU, $p < 0.002$ and versus LAK, $p < 0.005$). Tumor cells not internalized by DC and still present in

the co-cultures of lymphocytes with tumor cell-pulsed DC were not contributing to lymphocyte activation, since a negligible level of IFN-γ release was found in control cultures of lymphocytes with DC-free tumor cells (Fig. 4C and D). Radiation treatment was much less immunogenic for both KATO (Fig. 4C) and K (Fig. 4D), and this pattern paralleled IL-12 release by tumor-loaded DC (Fig. 3).

MHC restriction and tumor specificity of the T lymphocyte response

To demonstrate that increased stimulation by 5-FU-treated tumors was MHC restricted, the IFN-γ ELISPOT assay was set up in the presence or absence of the anti-MHC class I mAb W6.32. In three independent experiments using different HLA-A2$^+$ donors (Table 1), DC loaded with 5-FU-treated KATO was better stimulators than DC loaded with untreated KATO ($p < 0.001$). The mean ± SD spot number was significantly decreased ($p < 0.002$) in cultures containing the W6.32 mAb. IFN-γ release was also significantly increased by DC loaded with LAK-treated KATO as opposed to untreated KATO ($p < 0.002$) and this increase was also W6.32 sensitive (untreated versus W6.32-treated cultures, $p < 0.005$). To confirm that recognition of DC-presented antigen is restricted to class I MHC and to show the tumor specificity of the response, IFN-γ release was also tested by using as targets the MHC-unrelated (HLA-A2$^-$), histologically matched GTL16 gastric carcinoma and the MHC-matched (HLA-A2$^+$), histologically unrelated Jurkat T leukemia cell lines (Fig. 5, data from one experiment representative of two).

Generation of CTL by DC is influenced by the kind of treatment given to the tumor cells

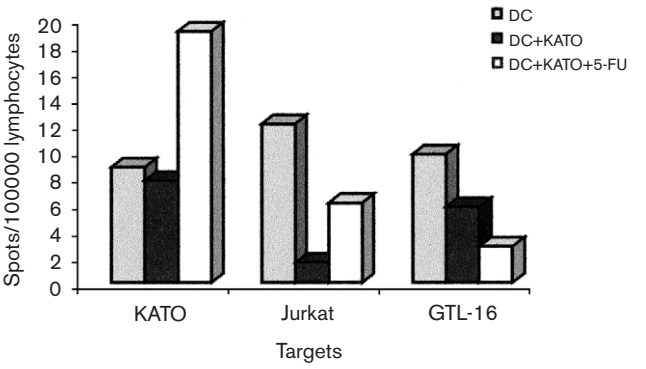
Cytotoxicity against untreated KATO cells was studied in cultures stimulated with DC alone, DC exposed to untreated KATO, DC exposed to 5-FU-treated KATO cells, untreated KATO and 5-FU-treated KATO. Figure 6 shows the results of one representative experiment of three, using four E : T ratios. The lytic activity correlated with the amount of IFN-γ found in the culture medium. Loading of DC with the untreated KATO cells decreased their stimulatory activity (320 ± 90 LU, mean ± SD for unloaded versus 80 ± 29 LU for KATO-loaded DC, $p < 0.01$). Inhibition shifted to stimulation when DC was loaded with 5-FU-treated KATO cells (80 ± 29 versus 740 ± 68 LU, $p < 0.001$) (Fig. 6A). Overall, the cytotoxic potential of cultures stimulated by DC + KATO–5-FU was significantly higher than that of cultures stimulated by DC + KATO ($p < 0.001$). Cytotoxicity induced by these DC preparations, but not that induced by DC-free tumors, was partially class I MHC restricted, as shown by the 50% inhibition induced by the W6.32 mAb (Fig. 6B). A similar inhibition pattern was induced by the anti-HLA-A mAb B77,2 (not shown).

Table 1 **MHC restriction of the IFN-γ response**[a]

Stimulators	Experiment 1	Experiment 2	Experiment 3
DC	9 ± 2[b]	11 ± 4	14 ± 5
DC + W6.32	8 ± 1 (9 ± 2)	10 ± 1 (9 ± 1)	16 ± 2 (15 ± 2)
DC + KATO	5 ± 0.6	6 ± 1	9 ± 2
DC + KATO + W6.32	5 ± 0.5 (6 ± 1)	5 ± 0.6 (6 ± 1)	9 ± 1 (10 ± 2)
DC + KATO–5-FU	22 ± 3	31 ± 3	36 ± 4
DC + KATO–5-FU + W6.32	10 ± 1 (23 ± 3)	18 ± 2 (30 ± 4)	21 ± 2 (35 ± 4)
KATO	3 ± 0.2	18 ± 2 (30 ± 4)	6 ± 0.5
K–5-FU	3 ± 0.3	5 ± 0.3	5 ± 0.4
DC + KATO + LAK	24 ± 3	29 ± 3	29 ± 4
DC + KATO + LAK W6.32	15 ± 3 (25 ± 3)	18 ± 2 (27 ± 4)	17 ± 3 (28 ± 3)
DC + LAK	21 ± 2	24 ± 2	26 ± 4
DC + LAK + W6.32	20 ± 2 (22 ± 3)	25 ± 3 (30 ± 1)	23 ± 6 (22 ± 3)

[a]Lymphocytes were cultured as detailed in legend to Figure 3. Five days after the third stimulation they were used as responders in a 24-h IFN-γ ELISPOT assay with untreated KATO cells as targets, in the presence of 1 : 80 diluted anti-MHC I mAb W6.32 or 1 : 80 diluted isotype control mAb (in brackets).
[b]Mean ± SD spots/10^5 of triplicate wells.

Fig. 5

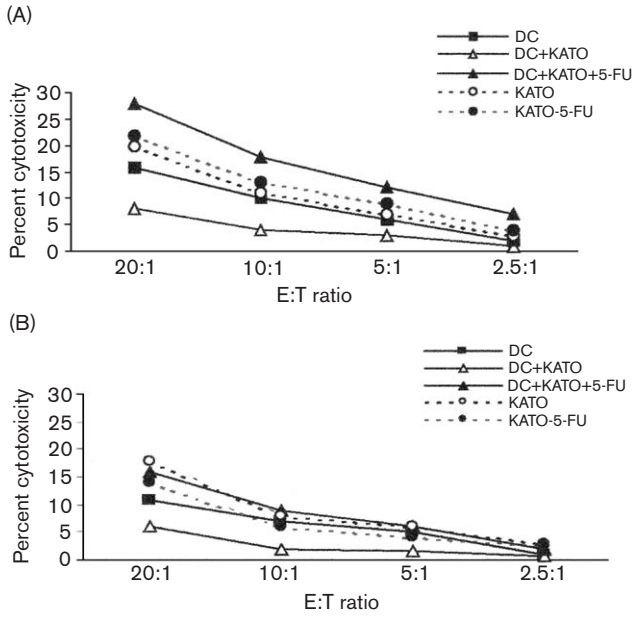


Antigen specificity of the response elicited by DC loaded with 5-FU-treated tumor cells. IFN-γ release by lymphocytes stimulated with DC, DC + KATO, DC + 5-FU–KATO was evaluated in a 24-h ELISPOT assay using as targets the KATO, the MHC-unrelated (HLA-A$^-$), histologically matched GTL16 gastric carcinoma and the MHC-matched (HLA-A$^+$), histologically unrelated Jurkat T leukemia cell lines. Results (from one of two superimposable experiments) show that recognition of DC-presented antigens is restricted to class I MHC and is specific for antigens associated to gastric carcinoma.

IL-10 production by DC is induced after KATO loading, but not diminished by 5-FU

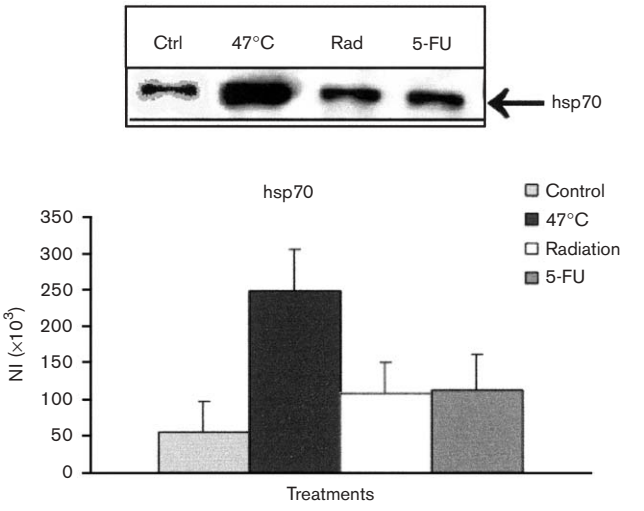
To gain more insight into the immunosuppression observed with KATO-pulsed DC, production of IL-10 was evaluated before and after pulsing. KATO cells alone did not produce IL-10 when unstimulated. Loading with untreated KATO induced IL-10 release at levels higher than those induced by lipopolysaccharide. However, treatment of KATO with 5-FU also increased IL-10 production by DC, thus ruling out a role of this cytokine in the inhibition observed with untreated KATO (data not shown).

Fig. 6



Generation of CTL by DC cultured with untreated or 5-FU-treated KATO cells. Lymphocytes were stimulated 3 times with DC loaded with the KATO tumor, which had received the indicated treatments. Five days after the last re-stimulation, lymphocytes were tested for cytotoxicity against the KATO cell line in a 4-h ^{51}Cr-release assay, with the NK target K562 cell line acting as cold target blocker. In addition, to establish the MHC dependence of the killing, the hot targets were incubated for 20 min at 4°C with medium containing a 1 : 80 dilution of the anti-MHC mAb W6.32 (B) or with isotype-matched mAb (A) before being added to the effector/cold target mixture. Results from one representative experiment of three show that cytotoxicity of cultures stimulated by DC + KATO–5-FU was significantly higher than that of cultures stimulated by DC + KATO ($p < 0.001$) and was at least in part class I MHC restricted.

Fig. 7



Modulation of *hsp70* induced by cytostatic treatment of tumor cells. KATO cells received the indicated treatments, and after 4 h the lysate of treated and untreated cells was analyzed in Western blotting for expression of *hsp70* using the mAb W27. (A) Representative results from one of three experiments, given in (B) as mean ± SD NI (Kodak 1D Image Analysis System). Both radiation and 5-FU treatment significantly ($p < 0.05$) increased the expression of *hsp70* over the control (untreated). Positive control was the 47°C (1 h) heated KATO.

Increased immunogenicity of 5-FU- and radiation-treated KATO cells is associated with up-modulation of *hsp70*

Hsps facilitate the response against tumor antigens, while not acting as antigens themselves [10,11]. We then studied the effect of treatments on *hsp70* expression by KATO cells. Western blotting showed significantly increased expression after 5-FU (56 000 ± 40 894 for untreated versus 112 355 ± 47 971 for FU-treated KATO, $p < 0.05$) and radiation (56 000 ± 40 894 untreated versus 108 576 ± 1361 for radiation-treated KATO, $p < 0.05$) (mean ± SE NI, from four experiments) (Fig. 7).

Discussion

It is well accepted that both the source of tumor antigen and the microenvironment determine the efficiency of DC antigen presentation. However, the events involved in tumor uptake and DC maturation after *in vivo* tumor death have not been precisely defined. The questions behind this study were whether apoptotic anticancer agents differentially activate *in vitro* a DC-boosted lymphocyte response after a DC maturation step induced by exogenous cytokines. The treatments influenced the DC cross-presentation of tumor antigens to CD8$^+$ cells to a different extent. Tumor phagocytosis by allogeneic MHC-matched DC, DC maturation and antigen-specific, MHC-restricted T lymphocyte response were all associated with a given treatment, but no significant correlation was found between the magnitude of tumor death and tumor immunogenicity.

The treatments induced distinct prevalences of tumor death. The antimetabolite 5-FU was chosen because it is the mainstay of adjuvant therapy for gastrointestinal tumors. In our model 5-FU was a good inducer of death with the KATO cell line, but was ineffective on the fresh cancer, which was instead sensitive to radiation. Activation of proteases, the effector molecules of apoptosis, occurs via intrinsic and extrinsic pathways, which originate from mitochondrial (cytochrome *c*) or cell surface signals (Fas receptor), respectively. Our results are in line with the contention that the action of cancer drugs (and radiation) may involve several levels of interference with these pathways [40], depending on the target cell. For example, disruption of the p53-related (intrinsic) pathway renders human cancer cells strikingly resistant to 5-FU [41].

In our experiments, tumor uptake occurred in 29.6–44.3% (KATO) and in 18.4–35.2% (K) of DC, in agreement with

data from other groups [25]. Engulfment by DC was negatively correlated with the extent of apoptotic death, though this trend was not statistically significant. Mild apoptosis induced by 5-FU led to the best uptake of both the KATO cell line and the fresh K cells. As expected [42,43], LAK cells were the best inducers of apoptosis on both the KATO cell line and the K. However, engulfment of LAK-treated tumor cells by DC tended to be low, 5-FU being by far the most efficient factor.

Cells dying by apoptosis are recognized by DC through the $\alpha_V\beta_3$, CD36 and phosphatidylserine receptors, as opposed to cells dying by necrosis, which are recognized through the *hsp* receptor CD95 [44]. In our study, no changes in CD36 receptor expression were observed after 5-FU treatment of KATO cells (not shown).

Despite their different impact on uptake, LAK and 5-FU were highly active inducers of the tumor-mediated maturation of DC, as judged by the release of IL-12. It is somehow surprising that IL-12 release, a marker of DC maturation, was not accompanied by increased expression of antigen-presenting (MHC class II), co-stimulatory (CD80, CD86) and maturation (CD83) membrane markers. However, other authors have also failed to find significant changes of maturation markers by DC pulsed with apoptotic tumors, despite the increased secretion of IL-12 [24].

Upregulation of the maturation marker IL-12 was accompanied by a specific antitumor response by MHC-restricted T lymphocytes as shown by IFN-γ release and CTL activity. Both the cytokine and the cytolytic response were affected by CD8 lymphocytes, since they were inhibited by anti-class I MHC mAb. Thus, DC uptake of 5-FU/LAK-treated tumors must have increased exposure of appropriate MHC–antigen complexes to the CTL precursors. This could have been possible without apparent upmodulation of MHC molecules if increased tumor antigen epitopes were made available during the MHC–antigen-processing pathway. *Hsp*s chaperon tumor antigen to DC for presentation in the MHC–antigen complex [10,11]. However, hsp70 was consistently upmodulated on KATO cells after 5-FU, but also after radiation, a treatment that failed to induce a strong antitumor response. This lack of correlation can be explained by the fact that in our study *hsp70* was evaluated in the lysate of cells dying by apoptosis. *Hsp*s confined within apoptotic cell bodies [10] are released and become active only when cells are disrupted and undergo secondary necrosis [28].

Loading of DC with the untreated KATO cell line, but not with the fresh tumor, consistently decreased the lymphocyte response compared to DC alone and this effect was accompanied by increased release of IL-10. However, even higher levels of IL-10 were achieved when DC were loaded with 5-FU-treated KATO (data not shown), thus making involvement of this cytokine unlikely in the tumor suppression effect observed here. In our previous study [23], DC from five colon and gastric cancer patients were exposed to the irradiated autologous tumor or to normal mucosa, matured with a MCM and used as stimulators of autologous lymphocytes. Tumor-specific stimulation, suppression or no response was observed, depending on the patients. Tumor-induced suppression was overcome by addition of IL-12. Our present observations corroborate those data by showing that reversal of the suppressing effect of KATO cells after 5-FU and LAK is accompanied by increased IL-12 secretion.

Our observation that anticancer molecules/cells modulate the immunogenicity of DC-presented TAA is in line with a recent demonstration of the enhanced antitumor effect of intratumoral injection of DC combined with chemotherapy in a murine model [31]. The fact that the stimulatory effect of treatments on DC antigen presentation, consistently observed with the KATO cell line, was confirmed on a surgically ablated gastric carcinoma strengthens the relevance of this phenomenon to a clinical setting. Our data corroborate reports of establishment of an immune memory against 5-FU-treated tumor in a murine system [30]. Further studies are needed to establish whether this property extends to drugs sharing the same mechanisms of action and whether the same changes are induced on normal cells.

The simplest explanation for the effect of LAK cells is their production of maturating factors for DC. Although the activatory effect of DC on NK cells has received more attention [45,46], the DC maturation properties of factors actively produced by LAK cells (e.g. IFN-γ) cannot be underestimated. The fact that MHC dependence of the T cell response was observed with tumor-loaded, but not with tumor-free DC–LAK mixed cultures indicates that LAK cells may indeed act by modifying the rate of tumor uptake by DC. Tumor killing by LAK cells proceeds through well-defined steps involving perforation of the cell membrane mediated by perforating esterase enzymes, followed by the entry of apoptotic molecules [42,43]. This process clearly affects the membrane structure and may lead to prompt capturing of the damaged cell by DC. CTL-mediated (14) and NK-mediated [32] destruction of tumor cells leads to enhanced access of antigen to cross-presentation pathway *in vivo*.

In conclusion, our data show that current anticancer regimens confer immunogenic properties on a tumor and overcome DC suppression. In addition, the observation that a gastric tumor cell line primes a class I MHC-

restricted response in MHC-matched healthy subjects supports the possibility of expanding CTL *in vivo* by using allogeneic tumors and strengthens the feasibility of this vaccination procedure in gastric cancer patients.

References

1 Banchereau J, Steinman R. Dendritic cells and the control of immunity. *Nature* 1998; **392**:245–252.
2 Ridge JP, Di Rosa F, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a $CD4^+$ T-helper and a T-killer cell. *Nature* 1998; **393**:474–478.
3 Mukherji B, Chakraborty NG, Yamasaki S, Okino T, Yamase H, Sporn JR, *et al.* Induction of antigen-specific cytolytic T cells *in situ* in human melanoma by immunization with synthetic peptide-pulsed autologous antigen presenting cells. *Proc Natl Acad Sci USA* 1995; **92**:8078–8082.
4 Nestle F, Alijagic S, Gilliet M, Sun Y, Grabbe S, Dummer R, *et al.* Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat Med* 1998; **4**:328–332.
5 Lodge PA, Lori AJ, Bader RA, Murphy GB, Salgaller ML. Dendritic cell-based immunotherapy of prostate cancer: immune monitoring of a phase II clinical trial. *Cancer Res* 2000; **60**:829–833.
6 Brossart P, Heinrich KS, Stuhler G, Behnke L, Reichardt VL, Stevanovic S, *et al.* Identification of HLA-A2-restricted T cell epitopes derived from MUC-1 tumor antigen for broadly applicable vaccine therapies. *Blood* 1999; **93**:4309–4318.
7 Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigens from apoptotic cells and induce class I-restricted CTLs. *Nature* 1998; **392**: 86–89.
8 Larsson M, Fonteneau JF, Bhardwaj N. Dendritic cells resurrect antigens from dead cells. *Trends Immunol* 2001; **22**:141–148.
9 Melcher A, Todryk S, Hardwick N, Ford M, Jacobson M, Vile RG. Tumor immunogenicity is determined by the mechanism of cell death via induction of heat shock protein expression. *Nat Med* 1998; **4**:581–587.
10 Basu S, Binder R, Suto R, Anderson K, Srivastava P. Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NK-κB pathway. *Int Immunol* 2000; **12**:1539–1546.
11 Gallucci S, Lolkema M, Matzinger P. Natural adjuvants: endogeneous activators of dendritic cells. *Nat Med* 1999; **5**:1249–1255.
12 Voll RE, Hermann M, Roth EA, Stach C, Kalden JR, Girkontaite I. Immunosuppressive effects of apoptotic cells. *Nature* 1997; **390**: 350–351.
13 Albert ML, Pearce SF, Francisco LM, Sautern B, Royb P, Silverstein RL, *et al.* Immature dendritic cells phagocytose apoptotic cells via $\alpha_V\beta_5$ and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J Exp Med* 1998; **188**:1359–1368.
14 Kurts C, Miller JF, Subramaniam RM, Carbone FR, Heath WR. Major histocompatibility complex class I-restricted cross-presentation is biased towards high dose antigens and those released during cellular destruction. *J Exp Med* 1998; **188**:409–414.
15 Restifo N. Building better vaccines: how apoptotic cell death can induce inflammation and activate innate and adaptive immunity. *Curr Opin Immunol* 2000; **12**:597–603.
16 Nouri-Shirazi M, Banchereau J, Bell D, Burkeholder S, Kraus ET, Davoust J, Palucka KA. Dendritic cells capture killed tumor cells and present their antigens to elicit tumor-specific immune response. *J Immunol* 2000; **165**:3797–3803.
17 Rubartelli A, Poggi A, Zocchi MR. The selective engulfment of apoptotic bodies by dendritic cells is mediated by $\alpha_V\beta_5$ integrin and requires intracellular and extracellular calcium. *Eur J Immunol* 1997; **27**:1893–1900.
18 Rovere P, Vallinoto C, Bondanza A, Crosti MC, Rescigno M, Ricciardi-Castagnoli P, *et al.* Bystander apoptosis triggers dendritic cell maturation and antigen-presenting function. *J Immunol* 1998; **161**:4467–4471.
19 Ronchetti A, Rovere P, Iezzi G, Galati G, Heltai S, Protti MP, *et al.* Immunogenicity of apoptotic cells *in vivo*: role of antigen load, antigen-presenting cells, and cytokines. *J Immunol* 1999; **163**:130–136.
20 Russo V, Tanzarella S, Dalerba P. Dendritic cells acquire the MAGE-3 human tumor antigen from apoptotic cells and induce a class I-restricted T cell response. *Proc Natl Acad Sci USA* 2000; **97**:2185–2190.
21 Henry F, Boisteau O, Bretaudeau L, Lieubeau B, Meflah K, Gregoire M. Antigen-presenting cells that phagocytose apoptotic tumor-derived cells are potent tumor vaccines. *Cancer Res* 1999; **59**:3329–3332.
22 Chang JW, Peng M, Vaquerano JE, Zhou YM, Clinton RA, Hyun WC, *et al.* Induction of T_h1 response by dendritic cells pulsed with autologous melanoma apoptotic bodies. *Anticancer Res* 2000; **20**:1329–1336.
23 Galetto A, Contarini M, Sapino A, Cassoni P, Consalvo E, Forno S, *et al. Ex-vivo* host response to gastrointestinal cancer cells presented by autologous dendritic cells. *J Surg Res* 2001; **100**:32–38.
24 Kotera Y, Shimizu K, Mulé JJ. Comparative analysis of necrotic and apoptotic tumor cells as a source of antigens in dendritic cell-based immunization. *Cancer Res* 2001; **61**:8105–8109.
25 Sauter B, Albert LM, Francisco L, Larsson M, Somersan S, Bhardwaj L. Consequences of cell death. Exposure to necrotic tumor cells, but not primary tissue cells, or apoptotic cells induces the maturation of immunostimulatory dendritic cells. *J Exp Med* 2000; **191**:423–434.
26 Hoffmann TK, Meidenbauer N, Dworacki G, Kanaya H, Whiteside TL. Generation of tumor-specific T lymphocytes by cross-priming with human dendritic cells ingesting apoptotic tumor cells. *Cancer Res* 2000; **60**: 3542–3549.
27 Schnurr M, Scholz C, Rothenfusser S, Galambos P, Dauer M, Röbe L, *et al.* Apoptotic pancreatic tumor cells are superior to cell lysates in promoting cross-priming of cytotoxic T cells and activate NK and γδ T cells. *Cancer Res* 2002; **62**:2347–2352.
28 Pietra G, Mortarini R, Parmiani G, Anichini A. Phases of apoptosis of melanoma cells, but not of normal melanocytes, differently affect maturation of myeloid dendritic cells. *Cancer Res* 2001; **61**:8218–8226.
29 Feng H, Zeng Y, Whitesell L, Katsanisn E. Stressed apoptotic tumor cells express heat shock proteins and elicit tumor-specific immunity. *Blood* 2001; **97**:3505–3512.
30 Consalvo M, Mullen CA, Modesti A, Musiani P, Allione A, Cavallo F, *et al.* 5-Fluorocytosine-induced eradication of murine adenocarcinomas engineered to express the cytosine deaminase suicide gene requires host immune competence and leaves an efficient memory. *J Immunol* 1995; **154**:5302–5312.
31 Tong Y, Song W, Crystal RG. Combined intratumoral injection of bone marrow-derived dendritic cells and systemic chemotherapy to treat pre-existing murine tumors. *Cancer Res* 2001; **61**:7530–7535.
32 Tanaka F, Hashimoto W, Okamura H, Robbins PD, Lotze MT, Tahara H. Rapid generation of potent and tumor-specific T lymphocytes by Interleukin 18 using dendritic cells and natural killer cells. *Cancer Res* 2000; **60**: 4838–4844.
33 Sekiguchi M, Sakakibara K, Fujii G. Establishment of cultured cell lines derived from a human gastric carcinoma. *J Exp Med* 1978; **48**:61–68.
34 O'Shaughnessy JA, Tolcher A, Riseberg D, Venzon D, Zujewski J, Noone M, *et al.* Prospective, randomized trial of 5-fluorouracil, leucovorin, doxorubicin and cyclophosphamide chemotherapy in combination with the interleukin-3/granulocyte-macrophage colony-stimulating factor (GM-CSF) fusion protein (PIXY321) versus GM-CSF in patients with advanced breast cancer. *Blood* 1996; **87**:2205–2211.
35 Romani N, Reider D, Heuer M, Ener S, Eibl B, Schuler G. Generation of mature dendritic cells from human blood: an improved method with special regard to clinical applicability. *J Immunol Methods* 1996; **196**: 137–151.
36 Romero PJ, Cerottini C, Aaanders G. A novel method to monitor antigen-specific cytotoxic T-cell responses in cancer immunotherapy. *Mol Med Today* 1998; **4**:305–312.
37 Herr WB, Linn N, Leister E, Wandel KH, Zum Buschenfelde M, Wolfel T. The use of computer-assisted video image analysis for the quantification of $CD8^+$ T lymphocytes producing tumor necrosis factor alpha spots in response to peptide antigens. *J Immunol Methods* 1997; **203**:141–152.
38 Matera L, Bellone G, Lebrun JJ, Kelly PA, Hooghe Peters EL, Francia Di Celle P, *et al.* Role of prolactin in the *in vitro* development of interleukin-2-driven anti-tumoural lymphokine-activated killer cells. *Immunology* 1996; **89**:619–625.
39 Trinchieri G. Biology of natural killer cells. *Adv Immunol* 1989; **47**:187–376.
40 Bunz F. Cell death and cancer therapy. *Curr Opin Pharmacol* 2001; **1**: 337–341.
41 Bunz FP, Hwang M, Torrance C, Waldman T, Zhang Y, Dillehay L, *et al.* Distruption of p53 in human cancer cells alters the responses to therapeutic agents. *J Clin Invest* 1999; **104**:263–269.
42 Roger R, Issaad C, Pallardy M, Leglise MC, Turhan AG, Bertoglio J, *et al.* BCR-ABL does not prevent apoptotic death induced by human natural killer or lymphokine-activated killer cells. *Blood* 1996; **87**:1113–1122.
43 Whiteside TL, Sung MW, Nagashima S, Chiamatsu K, Okada K, Vujanovic NL. Human tumor antigen-specific T lymphocytes and interleukin-2-activated natural killer cells: comparisons of antitumor effects *in vitro* and *in vivo*. *Clin Cancer Res* 1998; **4**:1135–1145.

44 Thery C, Amigorena S. The cell biology of antigen presentation in dendritic cells. *Curr Opin Immunol* 2001; **13**:45–51.
45 Amakata Y, Fujiyama Y, Andoh A, Hodohara K, Bamba T. Mechanism of NK cell activation induced by coculture with dendritic cells derived from peripheral blood monocytes. *Clin Exp Immunol* 2001; **124**:214–222.
46 Ziske C, Marten A, Schottker B, Buttgereit P, Schakowski F, Gorschluter M, *et al.* Resistance of pancreatic carcinoma cells is reversed by coculturing NK-like T cells with dendritic cells pulsed with tumor-derived RNA and CA 19-9. *Mol Ther* 2001; **3**:54–60.