The Differential Influence of Allogeneic Tumor Cell Death via DNA Damage on Dendritic Cell Maturation and Antigen Presentation

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ABSTRACT

Dendritic cells (DCs) respond to danger signals from tissue injury by amplifying their immune-inducing capacity. In the cancer context, this may lead to in vivo antitumor synergy between DCs and DNA-damaging chemotherapeutic agents. Neither the interaction between DCs and dying tumor cells nor whether different ways of inducing cell injury can deliver danger signals of different strength to DCs nor the potential role of damaged DNA as a danger signal has been studied rigorously. Here we report that coculture of immature DCs with tumor cells treated with the alkylating agents melphalan and chlorambucil leads to enhanced autologous and allogeneic T-cell activation, up-regulation of surface expression of MHC and costimulatory molecules, and increased interleukin (IL)-12 secretion. Exposure of the same DCs to tumor cells killed by cytarabine or by freeze-thaw (primary necrosis) resulted in significantly less T-cell proliferation and IL-12 production, indicating that DCs are able to sense and respond differentially to the mode of cell death. Exposure of DCs to DNA purified from tumor cells treated with alkylating agents also increased their T-cell-stimulating capacity, expression of CD86, and IL-12 secretion, supporting the hypothesis that the activating effects of tumor cells are linked to the nature of the DNA damage. This is the first study that shows that DCs respond differentially to killed tumor cells, depending upon the mechanism of DNA damage and consequent cell death.

INTRODUCTION

It has become increasingly evident that alloreactivity of donor immune cells against host leukemic tumor cells plays a role in controlling the patient’s malignancy after allogeneic BMT (1, 2). Early GVL responses are probably mediated by direct donor T-cell allorecognition, whereas delayed responses leading to long-term tumor regression may be due to the indirect pathway, where donor-derivated antigen-presenting cells, DCs in particular, cross-present host leukemic cell alloantigens to syngeneic (donor-derived) T cells (3–6). The importance of this GVL effect (4) and response differentially to the mode of cell death. Exposure of DCs to DNA purified from tumor cells treated with alkylating agents also increased their T-cell-stimulating capacity, expression of CD86, and IL-12 secretion, supporting the hypothesis that the activating effects of tumor cells are linked to the nature of the DNA damage. This is the first study that shows that DCs respond differentially to killed tumor cells, depending upon the mechanism of DNA damage and consequent cell death.

MATERIALS AND METHODS

Antibodies. The following MoAbs were used: (a) CD1a [supernatant mouse MoAb, NA1/34, IgG2a; gift from Prof. A. McMichael (John Radcliffe Hospital, Oxford, United Kingdom)]; (b) CD2 (mouse MoAb MAS 593, IgG2b; Harlan Sera-Lab, Loughborough, United Kingdom); (c) CD3 [supernatant mouse MoAb UCH T1, IgG1; gift of Prof. P. C. L. Beverley (The Edward Jenner Institute for Vaccine Research, Berkshire, United Kingdom)]; (d) CD4 (supernatant mouse MoAb HB246, IgG2b; gift of Prof. P. C. L. Beverley); (e) CD19 (supernatant mouse MoAb BU12, IgG1; gift of D. Hardie (Birmingham University, Birmingham, United Kingdom)]; (f) CD40 (superna-
tiant mouse MoAb; Sigma Aldrich, Poole, Dorset, United Kingdom; (g) CD80 (supernatant mouse MoAb; Sigma Aldrich); (h) CD86 (supernatant mouse MoAb, BU63, IgG1; gift from D. Hardie (Birmingham Medical School, Birmingham, U.K.); and (i) HLA-DR (supernatant mouse MoAb L243, IgG2a; gift of Prof. P. C. L. Beverley). The secondary antibody for flow cytometry was a rabbit anti-mouse FITC-conjugated IgG (Dako A/S) at a 1:20 dilution of a 0.3 mg/ml stock to give a final concentration of 15 μg antibody/10^6 cells.

**Cell Lines and Culture Conditions.** U937 monoblastoid leukemia cells (American Type Culture Collection, Manassas, VA) were maintained at 37°C/5% CO2 in RPMI 1640 (Invitrogen, Paisley, United Kingdom) supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine (all from Clare Hall Laboratories, Imperial Cancer Research Fund, London, United Kingdom) to comprise RPMI-CM. U937 cultures were tested regularly and found to be negative for Mycoplasma infection (Mycoplasma Detection Kit; American Type Culture Collection). U937 was selected for this study because (a) it is a histogenic tumor cell line that does not up-regulate CD95 after chemotheraphy and is thus insensitive to Fas ligation-mediated apoptotic death, and (b) it expresses low surface MHC class I and no class II molecules (30), thus limiting the possibility of direct tumor cell-derived allopeptide presentation.

**Induction of Tumor Cell Death.** U937 cells were suspended in RPMI-CM at 10^6 cells/ml and treated for 18 h with the alkylating agents mechlorethamine, L-phenylalanine mustard (Mel), Chlor, or the S-phase-specific agent ARA-C (Sigma Aldrich). Of note, CTX is a more commonly used alkylating agent in clinical practice than Mel; however, because CTX requires hepatic metabolism to its active form and Mel does not, the latter agent was chosen for these in vitro studies. U937 cells were also treated with MM-C. In selected experiments, apoptosis was induced by treatment of 5 x 10^5 cells for 120 s, at a distance of 6 cm, with a short wavelength UV-B light source delivering 20 mW/cm².

**Quantification of Cell Death.** To identify apoptosis, cells were washed once and resuspended in HBSS at a final cell concentration of 10^6 cells/ml. Aliquots were fixed and permeabilized by dropwise addition of an equal volume of cold 70% ethanol (stored at −20°C) to give a final concentration of 35% ethanol. The cells were left on ice for 30 min, washed twice, and treated with DNase-free RNase A (Sigma Aldrich; final concentration, 500 μg/ml; 37°C, 10 min). PI (Sigma Aldrich) was added (final concentration, 50 μg/ml), and the cells were analyzed immediately by flow cytometry.

**Preparation of DNA.** For DNA extraction from untreated U937 cells, 5–7 x 10^7 cells were washed twice with HBSS and resuspended in 0.5 ml of lysis buffer [0.2% SDS, 0.1 M Tris (pH 8.5), 5 mM EDTA, and 200 mM NaCl]. The cell suspension was incubated at 55°C for 3 h and then tipped into 2 ml of isopropanol. The solution was mixed by inversion; the DNA was removed, sterilized in 70% ethanol, and then allowed to dry prior to dissolving in AIM-V-CM (overnight, 37°C) before use in assays. For DNA extraction from treated cells (i.e., fragmented and drug-modified DNA), three rounds of standard phenol chloroform extraction were used. The purified DNA was precipitated in 70% ethanol and then dissolved in 500 μl of AIM-V medium as described above. Purity and concentration of DNA were measured by spectrophotometry. DNA was diluted to a stock concentration of 500 μg/ml in AIM-V, which was added to DC cultures to achieve a final concentration of 100 μg/ml. For gel electrophoresis, purified DNA was resuspended in TE buffer [10 mM Tris-HCl (pH 7.6) and 1 mM EDTA (pH 8.0)] and loaded on a 0.7% agarose gel containing 0.5 μg/ml ethidium bromide. The gel was run at 90 V with a 1-kb DNA ladder control (Invitrogen) for 20 min at 20°C and photographed under UV transilluminaton.

**DC Preparation.** Monocyte-derived DCs were prepared from 120 ml of fresh whole blood from healthy volunteers. Mononuclear cells were separated on Ficoll lymphoprep (Nycomed Pharma, Oslo, Norway; 400 x g, 30 min, brake off) and incubated in RPMI-CM for 2 h at 37°C/5% CO2. Nonadherent cells were removed by gentle washing, and adherent cells were cultured for up to 7 days in AIM-V medium supplemented with 100 μg/ml NaHCO3, (Clare Hall Laboratories, Imperial Cancer Research Fund, London, United Kingdom), 100 units/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, 100 ng/ml human recombinant granulocyte macrophage colony-stimulating factor, and 50 ng/ml IL-4 (gifts from Schering-Plough Research Institute, Kenilworth, NJ) to comprise AIM-V-CM. Treated tumor cells were added to T-cell- and B-cell-depleted or undepleted DC cultures on day 1 at a DC: tumor cell ratio of 1:4.

In most experiments, DCs were not depleted of T and B cells until after tumor cell exposure, to reflect the in vivo situation. However, to eliminate the possible effects of contaminating lymphocytes, e.g., via CD40 ligation, as a potential source of DC activation, DC cultures were depleted of T or B cells on day 0 in selected experiments (i.e., before exposure to treated tumor cells). Thus, DC cocultures were purified on day 0, 3, or 5 by incubation with CD19, CD2, and CD23 MoAb for 45 min on ice. Cells were washed in HBSS and mixed with anti-IgG-coated magnetic microbeads (Dynal, Merseyside, United Kingdom; 4 x 10^6/10^6 cells) before separation on magnetic columns. DCs purified on days 3 and 5 are referred to as day 1–(3) DCs and day 1–(5) DCs (the first number refers to the day of DC culture when tumor cells were added; the number in parentheses refers to the day of DC culture that DCs were harvested, respectively; subtraction of these numbers yields the number of days of DC/tumor cell coculture). In selected experiments, DCs not exposed to tumor cells were matured in 100 ng/ml LPS (Sigma Aldrich) for 18 h.

DCs were cultured in serum free AIM-V CM as it is used in DC vaccine protocols. DCs cultured in AIM-V mature more rapidly compared with DCs grown in serum-supplemented media, as evidenced by lower CD1a expression (31). In the in vivo postchemotherapy/allogenetic BMT state, undifferentiated myeloid DC precursors are immersed in an environment of massive alloegenetic tumor cell death. To model this in vivo state, HLA-mismatched (MHC class I loci) DCs were cocultured with tumor cells at a 1:4 DC:tumor cell ratio.

In some experiments, whole injured tumor cells were pretreated with 100 μg/ml DNase and/or 100 μg/ml proteinase K for 30 min at 37°C and then washed thoroughly with HBSS before DC coculture. In DNA experiments, the DNA purified from treated tumor cells was added to day 1 DCs at a final concentration of 100 μg/ml for a total of 4 days. In certain groups, DNA was pretreated with DNase at a concentration of 100 μg/ml for 30 min at 37°C before DC coculture.

**T-cell Proliferation Assays.** Autologous T cells were obtained from the nonadherent population of peripheral blood mononuclear cell fraction and cryopreserved in FCS containing 10% DMSO (Sigma Aldrich) at −70°C. Cells were thawed rapidly (37°C), and B cells, monocytes, and macrophages were depleted by incubation with CD19, HLA-DR, and CD14 MoAb for 45 min on ice. Cells were washed and then mixed with magnetic microbeads and separated on magnetic columns. T cells were used immediately after purification. Allogenetic T cells from HLA-mismatched donors were prepared in an identical fashion.

Titrations of purified DCs, either untreated or exposed to tumor cells, were incubated at 37°C/5% CO2 with autologous T cells (10^3 cells/well) in the presence of 1.25 μg/ml con-A (Sigma Aldrich) or with allogenetic T cells in flat-bottomed 96-well microtiter plates. The DC autologous and allogenetic T-cell cocultures were incubated for 3 and 6 days, respectively. Both assays were then pulsed with 1 μCi of [3H]thymidine (ICN Biomedical, High Wycombe, United Kingdom) for the final 18 h of culture. Cells were harvested, and T-cell proliferation was measured by liquid scintillation counting (Microbeta Systems). All assays were performed in triplicate. Results were expressed as cpm x 10^-3. Error bars represent the SE.

**Flow Cytometry.** For phenotype studies, DCs were purified of necrotic debris by separation on Ficoll Lymphoprep, resuspended in ice-cold staining buffer (HBSS, 10% sodium azide), and incubated first with the relevant MoAbs for 30 min at 4°C. Cells were washed, and secondary immunolabeling was performed using FITC-conjugated rabbit antimmunoglobulin (30 min, 4°C). Cells were washed twice and fixed in 3.8% paraformdehyde and collected within 5 days on a FACScan flow cytometer (Becton Dickinson). Data were analyzed using CellQuest software.

**IL-12 Production.** For measurement of IL-12 production, DCs were exposed to injured tumor cells or the DNA thereof for up to 7 days. At days 2, 3, and 5 of culture, supernatants were collected and purified by ultracentrifugation at 1600 x g for 5 min, and IL-12 concentrations were measured using a commercial ELISA kit (R&D Systems Europe, Abingdon, United Kingdom) that detects the p40 subunit of IL-12. Detection limits were 20 pg IL-12/ml.

**Statistical Analysis.** In T-cell proliferation assays, the SE of triplicates is reported. The statistical significance in T-cell proliferation assays and release of IL-12 by DCs in response to treated tumor cells or the DNA thereof was determined using a Student's t test. P values are shown for one-tailed t tests.
RESULTS

Effects of DNA-damaging Chemotherapy Agents on Tumor Cells. Initial experiments were performed to characterize the effects of DNA-damaging agents on U937 cells. U937 were seeded at 10⁶ cells/ml and treated for up to 18 h with an escalating dose of Mel, Chlor, NM, MM-C, or ARA-C, all of which have known apoptosis-inducing mechanisms of action (32). To quantify the kinetics of drug-induced U937 apoptosis and secondary necrosis, mock- or Mel-treated tumor cells were stained with PI at discrete time points during drug exposure. In apoptosis, DNA fragmentation occurs and elutes out of permeabilized cells, resulting in lower PI fluorescence (M₁ gate). Fig. 1 shows that Mel-treated cells demonstrate an apoptosis pattern of DNA fluorescence (by PI staining) with complete elution from ethanol-permeabilized cells at 18 h of treatment. Furthermore, there is no cell cycle specificity (cells in G₁, S, and G₂ are equally affected, a characteristic of alkylating agents) because all cells progressively lose FL-2 fluorescence due to DNA elution from permeabilized cells (indicated by the M₁ gate). This is in contrast to the preferential effect of ARA-C on cells in the S phase with relative sparing of G₁-phase cells (data not shown). Secondary necrosis does not occur up to 3 h of drug exposure because unpermeabilized tumor cells exclude PI (Fig. 1, second row, second column); secondary necrosis does not occur until 6 h of treatment (data not shown). At 18 h, approximately 95% of the cells demonstrate secondary necrosis, as evidenced by positive PI staining in the unpermeabilized group (Fig. 1, fourth row, second column). Most cells fluoresce brightly (arrow), indicating a full complement of intact DNA that may be drug-modified (cross-linked, oxidized, and so forth). The remaining cell population with low fluorescence in the unpermeabilized sample represents cells with moderately fragmented DNA. At this highest concentration of drug, treated cells include trypan blue at 18 h, confirming their secondary necrotic state (data not shown).

Because both apoptosis and secondary necrosis were quantifiable at 18 h of drug exposure, the dose-response profiles of the different agents were measured at this time point (Fig. 1b). As expected, dose-response curves for alkylating agents with a similar mechanism of action, i.e., Mel and Chlor, are nearly identical. In contrast, the increased potency of ARA-C and NM is seen at lower concentrations relative to alkylating agents. The number of cells falling within the low-fluorescence (M₁) gate declined at the highest concentrations of NM. This may be due to the direct cytotoxicity by NM and is indicative of primary necrosis. Treatment of U937 cells with the closely related alkylators NM and Chlor and the atypical alkylator, MM-C, at concentrations of 420, 266, and 243 μM, respectively, induces the equivalent percentage of cell death as ARA-C and Mel at 289 and 237 μM, respectively. Therefore, these concentrations were selected, allowing for direct comparisons of DCs exposed to the same proportion of dead tumor cells treated by the different agents.

Gel electrophoresis of purified DNA from injured cells demonstrates DNA fragmentation (Fig. 1c). The peak of “intact” DNA seen by PI staining was confirmed by the low-mobility DNA band seen at the same size as genomic DNA from untreated cells (Fig. 1c, white arrow). Indeed, the majority of DNA from injured cells is at this “genomic size,” further supporting the existence of intact, drug-modified DNA rather than complete fragmentation.

The Level of Autologous T-cell Proliferation Induced by DCs Exposed to Injured Tumor Cells Depends on the DNA-damaging Agent Used and the Period of Coculture. Tumor cells were treated with ARA-C or Mel and cocultured with day 1 DCs. At day 3 [day 1-(3) DCs] or day 5 [day 1-(5) DCs], DCs were purified and assayed for their ability to stimulate autologous T-cell stimulation in the presence of con-A. Fig. 2a shows that day 1-(3) DCs exposed to...
Mel-treated cells induced 2–4-fold greater autologous T-cell proliferation compared with control day 3 DCs (not exposed to injured tumor cells). There was 2–3-fold greater proliferation in the ARA-C group over control DCs. As expected, there is an increase in immunostimulatory power from days 3 to day 5 in the control DC group. However, this is increased further when DCs are cocultured for an additional 2 days [day 1–(5)] with Mel-treated tumor cells, which induced up to 4-fold greater proliferation (Fig. 2b). In the ARA-C treatment group, the increased T-cell proliferation induced by day 1–(3) DCs was unsustained because equivalent T-cell proliferation levels were induced by control and day 1–(5) DCs. We thus focused on day 1–(5) DCs cocultured for 4 days with Mel-injured tumor cells.

The next experiments focused on other DNA-damaging agents that have similar mechanisms of action to Mel, Chlor, the closest structural and mechanistic analogue to Mel, and the atypical alkylators NM and MM-C were used. Fig. 2b shows autologous naïve T-cell proliferation induced by day 1–(5) DCs exposed to tumor cells injured with the various agents. At all DC:T cell ratios, both Mel- and Chlor-treated tumor cells endowed DCs with increased and equivalent T-cell-stimulatory capacity. Exposure to NM-treated tumor cells induced less T-cell proliferation than the Mel group, although proliferation was still 2-fold greater than that of controls. At the highest DC:T cell ratio, day 1–(5) DCs exposed to MM-C-injured tumor cells stimulated T-cell proliferation comparable with proliferation induced by control DCs, providing further evidence that the observed effects are due to agent-specific tissue injury signals, and not to potential immunomodulatory effects of the drugs themselves. At the highest DC:T cell ratio, the differences between treatment groups were less striking, probably due to saturation of T-cell activation.

During DC-tumor cell coculture, contaminating T and B cells remain in the culture (although relatively low in number compared with DCs) until their depletion at day 5. To exclude the possibility that DC activation was secondary to CD40 ligation by contaminating T cells, DCs were depleted of T and B cells on day 0 and then exposed on day 1 to tumor cells injured with Mel, Chlor, UV-B irradiation, or two cycles of freezing/thawing to induce primary necrosis. The T-cell immunostimulatory capacity was assayed at a 40:1 autologous T-cell:DC ratio in the presence of con-A (statistics are for each group relative to control DC level; *, P < 0.01; **, P < 0.001) or in an allogeneic mixed lymphocyte reaction [control, Mel, UV-B, Chlor, crumbs, freezing/thawing, X]. To test the role of damaged DNA, cells injured with Mel were pretreated with DNase before exposure to DCs. Data are representative of at least four independent experiments.

Mel-treated cells induced 2–4-fold greater autologous T-cell proliferation compared with control day 3 DCs (not exposed to injured tumor cells). There was 2–3-fold greater proliferation in the ARA-C group over control DCs. As expected, there is an increase in immunostimulatory power from days 3 to day 5 in the control DC group. However, this is increased further when DCs are cocultured for an additional 2 days [day 1–(5)] with Mel-treated tumor cells, which induced up to 4-fold greater proliferation (Fig. 2b). In the ARA-C treatment group, the increased T-cell proliferation induced by day 1–(3) DCs was unsustained because equivalent T-cell proliferation levels were induced by control and day 1–(5) DCs. We thus focused on day 1–(5) DCs cocultured for 4 days with Mel-injured tumor cells.

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capacity to Mel. This suggests oxidative mechanisms of tumor cell injury as a possible mediator of DC activation. Furthermore, because DNA damage is the common basis for tumor cell death in this system, the variable functional up-regulation by DCs exposed to injured tumor cells may be due to a response to non-protein signals, such as damaged DNA. To test this, Mel-injured tumor cells were treated with 100 μg/ml DNase for 30 min and then washed thoroughly before exposure to DCs. Interestingly, there is complete abrogation of the enhanced allostimulatory capacity of DCs exposed to injured tumor cells after their treatment with DNase (see Fig. 4).

Day 1-(5) DCs Respond to Tumor Cells by Up-Regulating Surface MHC and Costimulatory Molecules. In parallel with the functional assays, the phenotype of day 1-(3) and day 1-(5) DCs cocultured with Mel-injured tumor cells was examined. Fig. 3 shows that surface CD1a expression of day 1-(5) DCs declines over time and in parallel to control DCs. The decline in CD1a expression of control DCs is most likely due to accelerated maturation and culture in serum-free AIM-V medium (33) and is not enhanced by exposure to tumor cells. There is markedly enhanced expression of surface CD86 and HLA-DR and slightly enhanced CD80 expression in DCs exposed to Mel-treated tumor cells, with levels peaking at day 5. Expression of these surface markers on day 1-(5) DCs reaches levels comparable with those seen on LPS-matured DCs. MHC class I expression (HLA-ABC) is typically not enhanced (data not shown). However, we have noticed significant interdonor variability where enhancement of class I expression relative to control DCs is not uncommon. CD86 expression is enhanced consistently in DCs across virtually all healthy donors assayed. Importantly, CD14 expression declines over time and parallels the trend in control DCs, indicative of maturation along the DC, and not macrophage, lineage.

Enhanced Phenotypic Expression of CD86 on DCs May Be Abrogated by Treating Tumor Cells Injured by Alkylating Agents with DNase, but not Proteinase K. To further investigate the potential role of damaged DNA as well as protein mediators as immunostimulatory signals to DCs, tumor cells were injured with Mel, washed thoroughly, and immediately treated with DNase, proteinase K, or both. After DNase or proteinase digestion, tumor cells were washed thoroughly before coculture with DCs for 4 days [day 1-(5) DCs], after which DC surface CD86 expression was quantified. As shown in Fig. 4a, there is CD86 up-regulation upon LPS stimulation and with exposure to Mel-injured (non-DNase-/non-proteinase K-treated) whole tumor cells. Pretreatment of injured tumor cells with proteinase K did not affect their immunomodulatory effect on CD86 expression by DCs, whereas pretreatment with DNase abrogated CD86 up-regulation (Fig. 4b). Simultaneous treatment with DNase and proteinase K only partially reversed the DNase effect, likely due to the enzymatic digestion of DNase by proteinase K. Treatment of DCs with DNase alone has no effect on surface CD86 expression (data not shown); furthermore, cells were thoroughly washed to rid them of residual DNase before the addition of treated cells to DC cultures. These results provide further support to the role of modified DNA as the mediator of enhanced DC function.

DCs Exposed to the DNA Purified from Tumor Cells Injured by Alkylating Agents, but not from Normal Uninjured Cells, Have Enhanced CD86 Expression and Induce Enhanced Autologous T-cell Proliferation. Day 1 DCs were exposed for 4 days to the DNA from normal tumor cells or to DNA from Mel-injured tumor cells (Fig. 4b). There was enhanced CD86 expression on DCs exposed to the DNA from Mel-treated tumor cells (Fig. 4a). This was confirmed by treating day 1 DCs with the DNA derived from Chlor and NM. The DNA from Chlor-treated cells endowed day 1-(5) DCs with the same degree of immunostimulatory power as Mel. DNA from NM-treated cells endowed day 1-(5) DCs with the greatest ability to induce T-cell proliferation. Parallel to the lack of phenotypic up-regulation upon exposure to the DNA from normal tumor cells, there was no enhancement of T-cell proliferation induced by DCs exposed to the DNA from ARA-C-injured cells.
DCs Release IL-12 When Exposed to Whole Treated Tumor Cells or the DNA Thereof. To further investigate the DC-modulating effects of chemotherapy-treated whole tumor cells and the DNA thereof, the in vitro release of the cytokine IL-12 was assayed. Fig. 5 shows that there is maximal release of IL-12 at day 5 of coculture, and day 1–5 DCs exposed to Mel-killed tumor cells release nearly 10-fold more IL-12 than control DCs. In parallel with their enhanced T-cell-stimulatory capacity, DCs exposed to UV-B-treated whole tumor cells induced 8-fold greater IL-12 release by these DCs relative to control DCs. DCs exposed to tumor cells treated with ARA-C released a low (but still significant) amount of IL-12. Although treatment with purified damaged DNA yields far less IL-12 than that with whole cells, there is 2–3-fold greater IL-12 release by DCs exposed to DNA from Mel-injured cells compared with day 5 control DCs.

**DISCUSSION**

Tumor cells dying via both apoptotic and necrotic mechanisms have been shown to be a plentiful source of antigens for processing and presentation by DCs to mediate T-cell immunity or tolerance. Nevertheless, controversy remains with respect to the immunomodulatory effects that dying tumor cells have on DCs after tumor antigen uptake. These conflicting findings may point to a delicate balance of DC activation that is biased in response to variations in the microenvironmental milieu. Biasing the response toward immunity may be reflected by the in vivo observations that direct anticancer therapy in combination with allogeneic BMT are necessary to drive powerful, and potentially curative, GVL responses. Although direct allogrecognition of host tumor tissue by donor leukocytes is thought to play a prominent role in acute tumor regression (Ref. 34; after tumor debulking), the cross-presentation of allogeneic tumor peptides by donor DCs to mediate autologous GVL T-cell responses probably plays a key role in long-term rejection (35). This is supported by the observation that tumor regression induced by combining DC vaccination with chemotherapy or radiation therapy is synergistic, not merely additive, compared with single treatment alone (27). It has been suggested that DC vaccine strategies using allogeneic tumor-loaded DCs as an adjuvant to boost and maintain antitumor activity may be a powerful immunotherapeutic option (36–38). In this study, the familiar notion that DCs respond to injured tumor
cells has been examined, although from a different angle. It is clear from our data that different mechanisms of cell death may provide danger stimuli of different strengths to DCs developing from myeloid precursors. The effect of exposure of day 1 DCs to Mel-treated tumor cells was to accelerate their acquisition of T-cell-stimulatory capacity with phenotypic up-regulation, and cytokine secretion. This reached a peak at day 5 of culture (4 days of exposure) and was associated with enhanced expression of HLA-DR, CD80, and CD86. The functional changes were duplicated using Chlor, a drug with a similar mechanism of action to Mel, which supports a differential effect of alkylating agents compared with purine analogues, atypical alkylators, or primary necrosis by simple freeze-thaw. Pretreatment of whole cells with proteinase K did not affect CD86 up-regulation, thus excluding the role of protein mediators, whereas treatment of Mel-killed tumor cells with DNase abolished their immunostimulatory activity. Both CD14 and CD14a were down-regulated, and DCs showed striking dendritic morphology, suggesting that monocytes respond to injured tumor cells with accelerated activation and maturation without deviation from DC lineage commitment. Taken together, these data suggest that DCs sense and respond to various types of tumor cell death via DNA damage in a differential manner.

Typical immunotherapeutic strategies load DCs toward the end of their maturation process. Our results suggested that DCs respond vigorously to injured tumor cells in an early stage of maturation (day 1). This is logical because immature DCs, and myeloid DC precursors for that matter, have the ability to efficiently take up antigen and cellular debris and thereby respond in a highly plastic manner by acquiring immunostimulatory or immunotolerizing qualities. The observations may help to explain the dichotomy reported in the literature about how DCs respond to dying tissue cells. Indeed, DC interaction with injured tumor cells is more complex than has been appreciated: alterations in the extracellular milieu of DCs may dictate the balance between potent antitumor immune responses and undesirable tolerogenic vaccinations. Furthermore, in the context of DC vaccine design whereby immature DCs are loaded with killed tumor cells (39) before being matured with exogenous inflammatory stimuli (tumor necrosis factor α, CD40 ligand, and so forth), the judicious choice of how tumor cells are injured may obviate the need for the ex vivo maturation step. Clearly, both the nature of tumor cell death and the DC maturation states are important as variables in responses to dying cells.

In this study, we have also demonstrated that DCs respond to the DNA isolated from treated tumor cells by CD86 up-regulation, induction of enhanced T-cell proliferation, and secretion of IL-12. These DC responses to exogenously added damaged DNA were similar to responses to whole cells. However, DC responses to DNA were markedly lower than their responses to whole treated tumor cells, particularly because the amount of exogenous DNA was purified from 20-fold more tumor cells than the number used in the whole cell treatment studies. This may reflect the relatively higher efficiency with which DCs take up whole cells or a possible synergism between damaged DNA and other cell-derived proinflammatory mediators.

The role of vertebrate DNA in danger signaling is controversial. In the murine system, Ishii et al. (6) reported that intact, unmodified double-stranded DNA released from necrotic tissue cells had immunostimulatory properties on DCs, and fragmentation or strand separation abrogated these properties. Genomic DNA from normal cells had little effect on DCs in this human study, but the requirement for intact DNA is underscored by the finding that DNase treatment of both whole injured cells and their DNA abrogated the effects on DCs, and fragmented DNA had no immunostimulatory properties. Whereas the precise DNA modification caused by many antineoplastic cytotoxic agents has long been known, at present one can only speculate about the in vivo effects that DNA adducts or oxidized DNA have on DCs and the immune system as a whole. However, in this context, it is noteworthy that there are isolated clinical reports of autoimmune side effects concomitant to antineoplastic therapy (40, 41), which could represent in vivo DNA stimulation of immune cells. It is feasible that, in a manner similar to the response to CpG oligodeoxynucleotide or viral double-stranded RNA via pattern recognition, DNA modification by induction of reactive oxygen intermediate (32) oxidation, alkylation, or cross-linking may enhance its immunogenicity by exposing otherwise cryptic immunostimulatory sites in DNA sequences to which the corresponding DC receptors may bind. This resembles Toll-like receptor ligation by autologous DNA sequences that may be critical in the pathogenesis of systemic lupus erythematosus (42).

The implications of the finding that modified DNA may act as a danger signal extend beyond tumor immune therapy strategies. In the cancer context, the findings are particularly striking, showing clearly that the type of cell death invoked may be a significant determinant of danger detected at the tumor site (43). Tumor-free survival may be mediated by T-cell responses that are primed by activated DCs during the initial antitumor treatment. This may theoretically account, at least in part, for the long-term effectiveness of one chemotherapy regimen over another. Therefore a better understanding of the molecular details concerning various forms of stress-induced death and their effects on dynamic, i.e., time-dependent DC immunobiology is needed. An improved understanding of the nature of tumor-elicted danger signals may lead to the discovery of “natural” immunological adjuvants that mimic those signals or else generate the appropriate type of cancer cell damage that is necessary to elicit them (43, 44).

REFERENCES


9. Gravina, E., and Kanfer, E. J. Selection and use of chemotherapy with hematopoietic cell damage that is necessary to elicit them (43, 44).


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