Protection of Mice against Leukemia after Vaccination with Bone Marrow-derived Dendritic Cells Loaded with Apoptotic Leukemia Cells

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Abstract

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that need to be activated before they can function to initiate primary and secondary immune responses in vivo. DCs are also specialized to maintain peripheral tolerance to self after uptake of apoptotic material, likely corresponding to both apoptotic bodies and whole apoptotic cells. Here, we report that murine bone marrow-derived DCs can be activated in vivo by exogenous signals received from apoptotic leukemia cells expressing on the cell surface a model tumor-associated antigen. Injected in vivo, these exogenously activated DCs can function as adjuvants to protect mice against leukemia by stimulating an antigen-specific cellular-mediated cytotoxic immune response. To our knowledge, this is the first report indicating that DCs loaded with apoptotic leukemia cells protect mice against leukemia development.

Introduction

Tumor cells can evade the immune system by different mechanisms, including the expression of immunosuppressive cytokines (1–3), down-regulation, and constitutive expression of Fas and Fas ligand (4), and low expression of MHC molecules (5).

When the immune system encounters a new antigen in the periphery, the outcome is not necessarily activation. Indeed, the encounter of antigens by mature T cells often results in the induction of tolerance because of anergy (6). What determines the outcome of antigen encounter is the context in which the antigen is presented to the immune system. It appears that the immune response at the T-cell level is dependent on the costimulatory signals present at the time of antigen recognition.

Antigen-presenting cells express costimulatory molecules, such as B7, which promote T-cell activation (7). In the absence of the appropriate costimulatory signals, engagement of the T-cell receptor itself typically leads to anergy or apoptosis of the antigen-specific T cells.

On the basis of the emerging concept of the central role of antigen-presenting cells in the initiation of immune responses, DC-based vaccines are under active investigation (8, 9). Many factors appear to be responsible for the unique property of DCs in activating T cells (10). DCs express 50-fold higher levels of MHC molecules than macrophages, providing more peptide/MHC ligand for T-cell receptor engagement. Moreover, these cells express extremely high levels of adhesion and costimulatory molecules critical for T-cell activation (7).

DCs loaded with a TAA can induce a state of prophylactic, and even therapeutic, antitumor immunity in animal models. These experimental results have encouraged the first clinical attempts to exploit DCs for cellular immunotherapy against human cancers (11).

The form of antigen loaded onto DCs ranges from MHC class I-restricted TAAs, in the form of peptides (12), to proteins (13, 14). However, to explore the utility of this vaccination approach in the treatment of tumors expressing uncharacterized TAAs, antigens can be presented by fusion of DCs with whole tumor cells (15), by DC-derived exosomes (16), and by using RNA encoding TAAs (17) or unfractionated tumor cells extracts (14, 18). It has also been shown that DCs can phagocytose apoptotic cells and cross-present antigen from these sources to CD8+ T cells (19, 20).

Recent data demonstrated that DCs are also specialized to maintain peripheral tolerance to self antigens after uptake of apoptotic cells (21).

In this study, we demonstrate for the first time that murine BM-derived DCs can be activated in vivo by exogenous signals received from apoptotic leukemia cells and stimulate in vivo a cellular-mediated cytotoxic immune response protecting mice against leukemia development.

Materials and Methods

Animals. Six- to 8-week-old pathogen-free female DBA/2 (H-2d) mice were purchased from Ifla Credo (L’Arbresle, France). Mice were housed in a temperature-controlled, light-cycled room. All in vivo experiments were performed in accordance with local ethical guidelines.

Tumor Cells. The murine L1210/hCD4 B lymphocytic leukemia cell line (14) was genetically modified by retroviral-mediated gene transfer to express the HSV1/TK gene. After 72 h of cocultivation with the packaging cell line PSIIRIP/TK-RES-Neo (Génopoïétique, Paris, France), in the presence of 8 µg/ml of polybrene, leukemia cells were serially diluted in the presence of 0.75 mg/ml G418. After 14–21 days of selection, some L1210/hCD4/TK clones were isolated, propagated in vitro in culture medium (RPMI 1640 supplemented with 10% decomplemented FCS, 1% l-glutamine, and 1% antibiotics) and subsequently tested for sensitivity to GCV. For further studies, only one of the isolated L1210/hCD4/TK clones was used and apoptosis death was induced as described below.

Induction of Apoptotic Death. L1210/hCD4/TK cells were cultured for 7 days in the presence of 10 µM GCV. Cell death was confirmed using the Annexin V-FITC Apoptosis Detection Kit (PharMingen., San Diego, CA). Cells were stained with Annex V and PI. Early apoptosis is defined by Annex V+/PI− staining as determined by flow cytometric analysis (FACScan; Becton Dickinson, Mountain View, CA). Basal apoptosis and necrosis were identified using Annexin V−/PI−. For further studies, only one of the isolated L1210/hCD4/TK clones was used and apoptosis death was induced as described below.

Generation and Loading of BM-derived DCs. BM cells were obtained from DBA/2 mice as described elsewhere (14). Briefly, they were cultured at 7 × 106 cells/ml in RPMI 1640 supplemented with 5% FCS, 1% l-glutamine, 20 µg/ml gentamicin, 50 µM 2-mercaptoethanol, and 2000 units/ml recombi-
naut murine GM-CSF (Genzyme, Cambridge, MA) in 24-well plates at 37°C in 5% CO₂. On days 2, 4, and 6, 90% of the medium was replaced by GM-CSF-containing medium.

For coculture experiments, loosely adherent aggregates of growing DCs were harvested on day 4, counted, washed in PBS, and characterized by flow cytometry. These cells were used for phagocytosis of apoptotic leukemia cells, as described below, before being injected as vaccine into animals.

Loading of BM-derived DCs with shCD4 protein was performed as previously described (14). Briefly, on day 5, cultured cells were loaded by addition of 10 μg/ml of the purified protein, and on day 7 cells were characterized by flow cytometry and used for animal vaccination. Control-unloaded BM-derived DCs were harvested on day 7, characterized by flow cytometry, and used for animal vaccination.

Flow Cytometric Analysis. Cells (5 × 10⁶) were suspended in PBS/3% FCS/0.02% sodium azide, centrifuged at 1350 rpm for 5 min, resuspended in 100 μl of buffer, and then single or double labeled with saturating concentrations of mAb. Depending on the analysis performed, either unconjugated M5/114 mAb (rat IgG, ATCC TIB120), revealed by a FITC-conjugated M5/114 mAb (rat IgG, ATCC TIB120), or unconjugated hamster IgG (ATCC HB224) revealed by a phycoerythrin-conjugated F(ab')₂ goat anti-hamster IgG (Caltag Laboratories). Unconjugated rat mAbs against DEC-205 (NLDC 145; Serotec, Oxford, United Kingdom), B7-1 (IG10; Pharmingen), and B7-2 (GL1; Pharmingen) were also used and revealed with an FITC-conjugated F(ab')₂ goat anti-rat IgG (Caltag Laboratories). To minimize nonspecific binding, cells were preincubated with 2.4G2 (Fc-yII/III, ATCC HB 197) mAb. The following immunoglobulin isotype controls were used: unconjugated hamster IgG and biotin-conjugated rat IgG2a from Caltag Laboratories. Cells were fixed in 1% paraformaldehyde and analyzed on a FACScan (Becton Dickinson).

Phagocytosis of Apoptotic Cells. Apoptotic leukemia cells were dyed red using PKH26-GL (Sigma, Saint Quentin Fallavier, France) and cocultured with phagocytic DCs that were dyed green on day 4 of culture using PKH67-GL (Sigma) at a ratio of 1:1. After 24 h, flow cytometric analysis was performed and double positive cells were enumerated.

In Vitro Cytotoxicity Assay. Groups of 12 DBA/2 mice were injected i.v. into the retro-orbital sinus with phagocytic DCs loaded with apoptotic leukemia cells, with shCD4–loaded or unloaded BM-derived DCs, and with apoptotic leukemia cells alone, respectively (1–2 × 10⁵ cells/100 μl PBS/mouse). Fourteen days postvaccinations, two mice of each group were sacrificed to test CTL reactivities as previously described (14) with some modifications. Briefly, splenocytes were incubated in vitro for 3 days with 10 μg/ml of the purified shCD4 protein in the presence of 25 units/ml recombinant murine interleukin 2 (Genzyme). After restimulation, L1210/hCD4 or unmodified L1210 cells were labeled with 100 μCi/ml Na⁹⁰⁰⁰CrO₄ (Amersham, Arlington Heights, IL) for 45–60 min and incubated with the stimulated splenocytes for 4 h at 37°C. CTL activity was finally evaluated at an E:T ratio ranging from 1:1 to 50:1. Supernatant samples were harvested and counted in triplicate in a Wallac 1450 MicroBeta liquid scintillation counter (Turku, Finland). Percent specific ⁹⁰⁰⁰Cr release was determined by subtracting spontaneous release from experimental group release and dividing by maximum release minus spontaneous release. Maximum release was determined by treatment of target cells with 1% Triton X-100 containing medium.

In Vivo Antileukemia Protection Effect. Fourteen days postvaccinations, 10 mice of each group were challenged with 3 × 10⁷ L1210/hCD4/TK cells in 100 μl of PBS, injected i.v. into the retro-orbital sinus, and survival was recorded until > day 84.

Statistical Analyses. Survival data were compared using the Fisher exact test to calculate the statistical significance, determined at the <0.05 level.

Results and Discussion

The ability of DCs to generate antitumor immune responses in vivo has been documented in many experimental tumor models (8, 9). DCs loaded with tumor lysates, TAA-derived peptides, synthetic MHC class I-restricted peptides or RNA, have all been demonstrated to generate tumor-specific immune responses and antitumor activity. These studies have established the rationale for evaluating tumor antigen-bearing DCs as therapeutic vaccines in humans (11).

We recently demonstrated that murine BM-derived DCs loaded with either purified antigen in the form of protein or unfractionated tumor extracts induced an efficient in vivo antitumoral immune response (14). We used the murine L1210 B lymphocytic leukemia
genetically modified to express on the cell surface the hCD4 molecule as a model TAA.

The hCD4 protein is a prototypic member of the immunoglobulin superfamly gene and a molecule central to the immune system. This molecule was previously used in an antitumor vaccination approach based on the direct immunization of mice with an hCD4-expressing plasmid DNA (22). DNA immunization was able to induce protection from tumor cell challenge through the generation of specific cellular and humoral immune responses directed against the hCD4 displayed on the tumor cells. These data indicate that the hCD4 protein is specifically recognized by T and B cell immunity generated in vaccinated animals, demonstrating the ability of hCD4 to induce relevant immune responses against this model antigen.

In the present study, we have evaluated murine BM-derived DCs for their ability: (a) to take up apoptotic material from apoptotic hCD4-expressing L1210 cells and (b) to present the hCD4, non-self TAA, to T cells to initiate a protective antileukemia effect in vivo.

As a source of apoptotic cells, we used the leukemia L1210/hCD4 cell line (14) genetically modified to express the HSV1/TK gene (L1210/hCD4/TK cells). GCV treatment (Fig. 1) induced >90% apoptotic death in these cells within 7 days (Fig. 1A), as compared to untreated L1210/hCD4/TK cells that showed a basal apoptotic death ranging from 10 to 15% (Fig. 1B).

On the basis of previous observations that immature DCs are the cells responsible for uptake of apoptotic cells (19–21), we analyzed the immunophenotype of murine BM-derived DC cultures to predict the requisite stage of DC development for the acquisition of apoptotic material. Unloaded BM-derived DCs were analyzed on days 4, 5, and 7 of the culture (Fig. 2). As early as 4 days of culture (Fig. 2, A–E), two populations of the easily dislodged cells were distinguished according to their level of expression of MHC-II molecules, B7-1, B7-2, and DEC-205 markers. On the other hand, only a weak expression of the CD11c marker was observed. From day 5 (Fig. 2, F–J) to day 7 (Fig. 2, K–O) of culture, marked changes involved the expression of CD11c and MHC-II markers that increased and of DEC-205 that decreased. This latter marker corresponds to an integral membrane protein homologous to the macrophage mannose receptor which is able to mediate endocytosis (23). For this reason, we considered DEC-205 as a marker of DC development, which is down-regulated during DC maturation. Minor changes involved B7-1 and B7-2 markers.

BM-derived DC cultures were also tested in a phagocytosis assay that allowed us to visually detect the uptake of latex beads and to compare the phagocytic capacity of DCs at days 4, 5, and 7 of the culture. Cultures at day 4 engulfed latex beads more efficiently than cultures at the other time points (data not shown).

On the basis of these results, BM-derived DCs at day 4 were cocultured with apoptotic L1210/hCD4/TK leukemia cells for 24 h. Phagocytosis of apoptotic cells by DCs was assessed by flow cytometric analysis (Fig. 3) based on the ability of green PKH67GL-labeled DCs (Fig. 3A) to take up apoptotic material from red PKH26GL-labeled apoptotic tumor cells (Fig. 3B). After 24 h in coculture (Fig. 3C), cells were analyzed and phagocytic uptake was defined by the percentage of double positive cells. Results show that 82% of DCs engulfed apoptotic material from apoptotic leukemia cells in coculture. This is one representative of four experiments.

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to both apoptotic bodies and whole apoptotic cells, as previously described (20, 24).

After antigen uptake, the maturation process of DCs in vivo involves cell migration, up-regulation of MHC-II and costimulatory molecules, and down-regulation of endocytosis. As a result, antigen acquired in peripheral sites is retained, processed, and presented to T cells in secondary lymphoid organs, where activated DCs attract naive and memory T cells for priming.

Here, we evaluated in mice the effects of vaccination with BM-derived DCs loaded with apoptotic leukemia cells expressing the hCD4 molecule as a non-self antigen model in terms of in vivo and memory T cells for priming. The efficacy of this vaccination was compared to that of vaccination with shCD4-loaded and unloaded DCs, as well as with apoptotic leukemia cells directly injected as vaccine into mice (Fig. 4). Only mice vaccinated with loaded DCs generated an antigen-specific cell-mediated cytotoxic immune response against hCD4-expressing L1210 target cells (Fig. 4A). Controls, tested against the unmodified L1210 target cells, showed absence of lysis (Fig. 4B). After challenge with L1210/hCD4/TK cells, the mean survival of mice vaccinated with DCs loaded with apoptotic leukemia cells or shCD4-loaded DCs was significantly prolonged to >84 days (P = 0.01 and P = 0.005, respectively), as compared to control mice vaccinated with unloaded DCs or with apoptotic cells alone that succumbed to the tumor with a mean survival of 35 and 28 days, respectively (Fig. 4C).

Altogether, our data demonstrate that murine BM-derived DCs loaded with apoptotic tumor cells can induce an antileukemia protective effect in vivo against a non-self antigen (hCD4), as efficient as with DCs loaded with the purified antigen in the form of protein.

In conclusion, we believe that loading of DCs with apoptotic leukemia cells provides an unlimited satisfactory supply of antigens for vaccination; it also increases the probability of inducing immunity to more than one TAA, either molecularly characterized or uncharacterized.

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