Autologous dendritic cells loaded with apoptotic tumor cells induce T cell-mediated immune responses against breast cancer in vitro

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Abstract

Dendritic cell (DCs) based immunotherapy has received increased interest in the treatment of specific malignancies including breast cancer. In this in vitro study, T cell responses, which are induced by monocyte-derived DCs pulsed with apoptotic breast tumor cells (ApTC), were analyzed in terms of proliferation, specific cytotoxicity, and cytokine release. Nylon wool-enriched T lymphocytes from five patients with breast cancer stimulated with monocyte-derived DCs pulsed with apoptotic tumor cells in vitro and their proliferation response were analyzed by [3H] thymidine uptake and specific cytotoxic activity of tumor antigen-primed T cells after three rounds of weekly stimulation by flow cytometry. Interferon-γ (IFN-γ) and interleukin-4 (IL-4) cytokine release assay was carried out 24 h after the last stimulation. The supernatant from primed T cells was collected and analyzed using commercially available ELISA kits. cell proliferation assays revealed that DCs pulsed with apoptotic tumor cell could stimulate an autologous T cell proliferation response with stimulation indices of 5-21. The T cell-mediated cytotoxicity assay demonstrated that tumor antigen-primed T cells could kill significantly more autologous tumor cells than normal cells (P < 0.05). These cells had variable amounts of cytotoxic activity against K562 cells. Primed T cells released both IFN-γ and IL-4 in response to re-stimulation by antigen-pulsed DCs, but were dominated by IFN-γ production in two out of five patients and IL-4 production in three out of five patients. In conclusion, our results suggested that DCs pulsed with apoptotic breast tumor cells could elicit effective specific antitumor T cell responses in vitro. Therefore, vaccination with DCs pulsed with apoptotic tumor cells may be considered as a novel strategy for immunotherapy of patients with breast cancer refractory to standard modalities.

1. Introduction

Breast cancer is one of the most common cancers among females. The incidence of this type of cancer varies widely around the world with North America and European countries having the highest rates and Asian and African countries having the lowest. Current incidence rates predict that one in eight women in the United States will develop breast cancer during their lifetime. Breast cancer is the second leading cause of cancer death in American women following lung cancer [1].

The modern era of breast cancer treatment has developed with great rapidity due to the efforts of an extremely broad spectrum of basic and clinical scientists whose efforts have redefined our standards for appropriate therapeutic strategies. Immunotherapy is a therapeutic strategy that manipulates the host’s immune responses against tumor cells. This type of therapy marks a new area of cancer therapies that are directed passively or actively against tumor cells [2].

Dendritic cells (DCs) are the most potent antigen presenting cells for naive T cell activation [3]. DCs originate from the bone marrow and reside in a resting or immature state in non-lymphoid tissues in which they efficiently capture and process antigens. Upon stimulation with bacterial products, inflammatory cytokines, or CD40 ligation, DCs undergo a maturation process that results in enhanced antigen presenting capacity and expression of MHC, upregulation of co-stimulatory molecules, and migration into secondary lymphoid organs where they prime naive T cells [4,5]. The presence of DCs at the tumor site and regional lymph nodes suggested that these cells have a crucial role in the antitumor immune
response [6,7]. Because of their unique capacity to stimulate resting T cells, DCs are the most promising option for immunization protocols, especially since they can potentially induce antitumor immunity in patients with malignant disease [8,9].

The rationale for this approach is based on the observation that DCs can be pulsed with tumor antigen and subsequently administered as a cellular vaccine to induce a specific antitumor response [10]. Methods that allow for large scale in vitro generation of DCs from peripheral blood monocytes using granulocyte–macrophage colony stimulating factor (GM-CSF) and interleukin-4 (IL-4) have recently developed and facilitated induction of immune responses in vitro as well as in clinical vaccination trials [11].

It is generally accepted that tumors growing in vivo naturally provide antigens to APCs either by shedding from the surface of viable cells or by fragmentation of dead tumor cells. Previous studies have shown that multiple tumor antigens do exist and can be used to induce autologous tumor-specific T cell responses in vitro. Thus, they present an alternative strategy for effective vaccination due to the use of unfractionated tumor-derived antigens such as tumor cell lysates [12], peptides eluted from tumor cell membrane [13], apoptotic tumor cells [14], and fusion of tumor and dendritic cells [15]. Indeed, feeding DCs with apoptotic tumor cells provides a full array of antigenic peptides that rapidly gain access to both MHC class I (cross-presentation) and class II pathways, leading to a diversified immune response involving cytotoxic T lymphocytes as well as CD4+ T cells [16–18]. This method does not require the identification of tumor-associated antigens. Despite protein alterations during apoptosis induction, cross-presentation of apoptotic bodies (apobodies) allows for the presentation of the MHC-peptide density as efficiently as peptide loading for priming, naive CTLs [19].

In the present study, we have evaluated whether DCs pulsed with apoptotic tumor cells derived from patients with breast cancer are able to elicit T cell responses in terms of proliferation, cytotoxicity, and cytokine release against autologous tumor cells. Our aim was to obtain initial preclinical evidence for the potential efficacy of DC therapy. Importantly, a major objective of our study was to establish an experimental model that would allow us to evaluate and subsequently optimize the immunostimulatory capacity of DCs under autologous conditions. We hope that such an approach may therefore hold potential for treatment with active or adoptive immunotherapy for patients with breast cancer who have residual or resistant disease after standard surgical and cytotoxic treatments.

2. Materials and methods

2.1. Patients

Tumor, normal tissues, and peripheral blood were obtained from five patients who had undergone radical mastectomy for invasive ductal carcinoma of the breast. Blood specimens were obtained at the time of surgery and two weeks later with weekly intervals (Surgery Department, Day Hospital, Tehran, Iran). Patients were 33–58-years-old (mean = 43 ± 8-years-old) with stage III disease (T3 N2 M0) and did not receive any treatment before surgery. All patients provided informed consent before obtaining blood and tumor specimens.

2.2. Media and reagents

Complete media (CM) including RPMI-1640 (Gibco, Germany) supplemented with 10% human AB serum (Blood Transfusion Organization, Tehran, Iran), 2.5 × 10^{-5} M 2ME, 2 mM L-glutamine (Sigma Chemical Co., Munich, Germany), 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma Chemical Co., Munich, Germany) were used to culture cells from peripheral blood mononuclear cells (PBMCs) as well as the K562 cell line. Organoid media (OM) including DMEM supplemented with 2.5 × 10^{-5} M 2ME, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM Hepes, 0.075% bovine serum albumin (BSA), 0.5 µg/ml hydrocortisone, 5 µg/ml insulin, and 5 µg/ml epidermal growth factor (EGF) (all from Sigma Chemical Co., Munich, Germany) were used to culture freshly isolated normal breast cells and tumor cells. Enzyme solution containing collagenase III (0.1 mg/ml), DNase (1 mg/ml), and hyaluronidase (1 mg/ml) (Sigma Chemical Co., Munich, Germany) were used for digestion of tumor and normal breast tissues. γ-Irradiation-induced apoptosis in tumor cells was detected using Hoechst 33342, Acridine orange, and Propidium iodide fluorescent dyes (Sigma Chemical Co., Munich, Germany). Recombinant human-GM-CSF (Novartis-Basel, Switzerland), IL-4, and tumor necrosis factor alpha (TNF-α) (Peprotech, London, UK) were used to derive DCs from peripheral blood monocytes. IL-2 (Peprotech, London, UK) was used to induce cytotoxic T lymphocytes (CTLs) from nylon wool-enriched T cells. T cell proliferation assay (TPA) was performed by [3H] thymidine (Amersham Pharma, London, UK) uptake test. PKH-26 and Annexin V/PI fluorescent dyes (Sigma Chemical Co., Munich, Germany) were used to label target cells and measure cytotoxic activity of T cells, respectively. Finally, interferon gamma (IFN-γ) and IL-4 cytokine release assay was performed using commercially available ELISA kits (R&D Co., Stockholm, Sweden).

2.3. Tumor and normal breast cell isolation

Fresh breast tumor and normal cells were isolated from surgical specimens. Single cell suspensions were obtained by processing solid tissues under sterile conditions at room temperature as described previously [20]. Briefly, tumor and normal tissues were mechanically minced to portions no larger than 1–3 m³ in OM and washed twice. The minced tissue was then placed into a T25 culture flask containing 5 ml enzyme solution and incubated overnight at 37 °C and 5% CO₂. Enzymatically dissociated tissues were then subjected to serial centrifugation to obtain a cell suspension, and the resultant cells were washed twice in OM, counted, and subjected to gamma irradiation.

2.4. Antibodies and flow cytometry

Immunophenotyping of monocyte-derived DCs and primed T cells was performed by direct immunofluorescence staining of cell surface antigens using FITC or RPE conjugated mouse antibodies against CD1a, CD11c, CD14, CD83, HLA-DR, CD3, CD4, CD8, and the appropriate isotype matched controls (Serootech, London, UK). Samples were analyzed on FACSScan Calibur (Becton Dickinson, USA) using CellQuest software.

2.5. Preparation of apoptotic tumor cell

We took 6 × 10⁶ freshly isolated tumor cells and cultured them in three T25 flasks (2 × 10⁶ cells in each flask) with OM media supplemented with 10% human AB serum (Iranian Blood Transfusion Organization, Tehran, Iran). The optimum dose and post-irradiation incubation period to induce the maximum number of apoptotic tumor cells was determined and then the cells were irradiated up to 8 Gy (optimum dose) by gamma emitting ⁶⁰Co radioisotope source (Gamma Irradiation Center of Iranian Atomic Energy Organization, Tehran, Iran). The irradiated cells were incubated for 48 h (optimum incubation period) at 37 °C and 5% CO₂ and the resulting apoptotic tumor cells were frozen in liquid nitrogen until use.
Apoptosis induction was detected by epifluorescence microscopy using Hoechst 33342 (5 μg/ml) and Propidium Iodide (1.5 μM) fluorescent dyes [21]. Quantification of live, apoptotic and dead cells was determined by flow cytometric analysis (FACS Calibur (Becton Dickinson, USA)) using Acridine Orange (0.1 μM) and Propidium Iodide (1.5 μM)) [22].

2.6. Generation of DCs pulsed with apoptotic tumor

Monocyte-derived DCs were generated as described [23]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated using lymphoprep (1.077 g/ml) (Nycomed Pharma, Oslo, Norway) resuspended in CM supplemented with 10% AB serum and cultured in T25 flask for 2 h at 37 °C and 5% CO2. The non-adherent cells were then removed and used for T cell enrichment by nylon wool, and the adherent cells were cultured in CM containing GM-CSF and IL-4 at final concentrations of 1000 U/ml and 800 U/ml, respectively. Cultures were fed by removing 2 ml of medium and adding back 3 ml fresh medium with cytokines every other day. On day 4, apoptotic tumor cells were added to immature DC at a ratio of 1:1 and incubated overnight. Maturation factors including 25% V/V MCM (Monocyte Conditioned Medium; supernatant from overnight culture of autologous, adherent PBMCs) and TNF-α (10 ng/ml) were added on day 5 and tumor antigen-pulsed mature DCs were harvested on day 7.

2.7. T cell proliferation assay

T cell proliferation assay was performed using autologous DCs pulsed with apoptotic tumor cells which were irradiated with 30 Gy as stimulator and nylon wool-enriched autologous T cells as responder cells at a ratio of 1:5, 1:10, and 1:20. The purity of T cells was about 80%, which was determined by monoclonal anti-CD3 antibody staining. Phytohemagglutinin (PHA) stimulated T cells (2.5%) (Sigma Chemical Co., Munich, Germany) and DC or T cells alone served as positive and negative controls, respectively. Cultures were established in V bottom 96-well plates at a final volume of 200 μl of CM supplemented with 10% AB serum for 5 days and [3H] thymidine was added at a concentration of 1 μCi/well for at the last 18 h of culture. Proliferative responses were measured by a liquid scintillation counter (Wallac Inc., Turku, Finland) and expressed as mean counts per minute and the stimulation index (SI) obtained for triplicate wells.

2.8. Induction of tumor-specific CTLs

We pulsed 1 × 10⁵ γ-irradiated (30 Gy) DCs with apoptotic tumor cells and cultured them with 1 × 10⁶ nylon wool-enriched autologous T cells (ratio = 1:10) in CM supplemented with 10% AB serum in a 24 well microtiter plate for 19 days at 37 °C and 5% CO2. Responder T cells were re-stimulated twice with DCs loaded with apoptotic tumor cells at a ratio of 1:20 every other week. The cultures were fed every 3 days with fresh media and recombinant human IL-2 (20 IU/ml), the latter was added after a second round of re-stimulation. On day 19, tumor antigen-primed T cells were harvested and subjected to phenotypic analysis using anti-CD4 and anti-CD8 antibodies.

2.9. Cytotoxicity assay

Isolated breast tumor and normal cells were thawed and enriched for viable cells using percoll gradient centrifugation. The viability of enriched cells was about 80–90%. Fluorometric assessment of T lymphocyte antigen-specific cytotoxicity was performed using PKH-26. Annexin V and propidium iodide (PI) fluorescent dyes with some modifications in the procedure described by Fischer [24]. Briefly, target cells including tumor and normal breast cells as well as the K562 cell line were washed twice with serum-free media before staining with PKH-26. The cells were then resuspended in loading buffer (Diluent C) and incubated for 60 min with 1 mM freshly prepared PKH-26 dye at 37 °C, with shaking every 15 min. The staining reaction was stopped by incubation with 500 μl human AB serum for 30 s at room temperature. The cell pellet was transferred into a fresh 50 ml tube and washed twice with 40 ml CM containing 10% human AB serum twice. PKH-26 labeled target cells were incubated with primed T cells in 96-well V bottom plates at an effector:target ratio of 20:1 in a final volume of 200 μl at 37 °C and 5% CO2 for 3 h. After co-incubation, cells were harvested and washed with phosphate buffered saline (PBS) and resuspended in 100 μl high calcium content Annexin V binding buffer. Staining with anti-FITC was performed for 20 min at room temperature in the dark and PI was added immediately before flow cytometric analysis.

Three-color FACS analysis was carried out on a Coulter cytometer (Coulter Co., London, UK). Unstained, PKH-26 stained, and PKH-26 and Annexin V/PI stained target cells were used as controls. Triplicate wells were averaged and the percentage of specific lysis was calculated as follows:

\[
\text{Specific Lysis} = \frac{\text{Total} - \text{Control}}{\text{Control}} \times 100
\]

Control, target cell lysis in the absence of effectors; Total, target cells stained with PKH-26 alone.

2.10. Cytokine assay

Twenty-four hours after the last stimulation of autologous T cells with DCs pulsed with apoptotic tumor cells (described above), the supernatants were collected to measure IFN-γ and IL-4 release using commercially available sandwich ELISA kits according to the manufacturer’s instructions (R&D Co., Stockholm, Sweden). Cytokine release was reported as mean picograms ± SEM of IFN-γ and IL-4 for triplicate wells.

2.11. Statistical analysis

The data depicted in each figure corresponds to one representative experiment of at least five independently performed experiments. Student’s t test was used to determine the significance of data comparison.

3. Results

3.1. Apoptosis induction

To investigate the effects of dose-rate and post-irradiation incubation time on radiation-induced apoptosis, breast cancer tumor cells were exposed to 4, 8, 12, and 16 Gy γ-radiation and incubated for 24, 48, and 72 h at 37 °C and 5% CO2. Our results indicated that irradiation with 8 Gy and an incubation time of 48 h post-irradiation was the optimum dose and incubation time (Figs. 1 and 2). Freshly isolated breast tumor cells were irradiated with 8 Gy and incubated for 48 h at 37 °C and 5% CO2 prior to pulsing DCs.

3.2. Monocyte-derived DC generation

Human DCs was generated from peripheral blood monocytes of five patients with stage III breast cancer (Table 1). Three days after culturing plastic adherent monocytes in the presence of GM-CSF and IL-4, clusters of non-adherent cells appeared and increased in size and number thereafter. Maturation factors (TNF-α alone
or TNF-α and MCM) were added to immature DCs pulsed with apoptotic tumor cells on day 5 and 60–70% of cells appeared to loosely adhere to each other in clumps or were isolated floating cells with typical dendritic morphology by day 7. These cells exhibited typical cytologic features of DC, i.e., large irregular cells with numerous cell membrane processes as viewed by light microscopy (Fig. 3). The yield ranged from 5% to 6.6% (mean ± SEM = 5.8 ± 0.6%) of plated PBMCs and the viability of harvested DCs was more than 98% as determined by trypan blue staining.

Flow cytometric analysis of DCs revealed significant differences in the expression of surface molecules crucially involved in DC functions. Compared to immature DCs, mature DCs consistently showed a substantially enhanced expression of HLA-DR and CD83 and decreased expression of CD1a, CD14, and CD11c. The results showed that TNF-α and MCM induced higher levels of expression of maturation markers such as HLA-DR and CD83 compared to TNF-α alone (Fig. 4), so the former conditions were used as maturation conditions thereafter.

3.3. T cell proliferation

To address whether breast tumor antigen would be able to induce a proliferative response in autologous T cells, nylon wool-enriched autologous T cells were stimulated with DCs pulsed with apoptotic tumor cells at a ratio of 1:5, 1:10, and 1:20. Our results showed that T cell proliferative responses were elicited in three respective ratios for all five examined patients. However, proliferation rate and stimulation index varied from patient to patient (Fig. 5). The lowest and highest stimulation indices were 5 and 21, respectively (median ± SEM = 8.1 ± 3).

3.4. Cytotoxic activity

To examine the capacity of DCs pulsed with tumor apoptotic cells to elicit cytotoxic T cell response in vitro, we stimulated autologous T cells with antigen-pulsed DCs three times in weekly intervals.

Table 1

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (year)</th>
<th>Diagnosis</th>
<th>Grade</th>
<th>Stage</th>
<th>Tumor size (cm)</th>
<th>Surgical operation</th>
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<tbody>
<tr>
<td>1</td>
<td>33</td>
<td>IDC(R2)</td>
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<td>III</td>
<td>2.5</td>
<td>RM4</td>
</tr>
<tr>
<td>2</td>
<td>58</td>
<td>IDC(L3)</td>
<td>III</td>
<td>III</td>
<td>3</td>
<td>RM</td>
</tr>
<tr>
<td>3</td>
<td>37</td>
<td>IDC(L1)</td>
<td>III</td>
<td>III</td>
<td>5</td>
<td>RM</td>
</tr>
<tr>
<td>4</td>
<td>47</td>
<td>IDC(L1)</td>
<td>III</td>
<td>III</td>
<td>4</td>
<td>RM</td>
</tr>
<tr>
<td>5</td>
<td>41</td>
<td>IDC(L1)</td>
<td>III</td>
<td>III</td>
<td>1.5, 2</td>
<td>RM</td>
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<tr>
<td>Mean ± SD</td>
<td>43 ± 8</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>3.6 ± 0.8</td>
<td>—</td>
</tr>
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1, invasive ductal carcinoma; 2, right; 3, left; 4, radical mastectomy.
Cytotoxicity assays were conducted with unpurified, primed T cells at a minimum of 19 days after stimulation of T cell cultures with DCs pulsed with apoptotic tumor cells. As shown in Fig. 6, cytotoxicity against autologous tumor cells was demonstrated for all five patients at an effector:target ratio of 20:1, ranging from 21% to 65% (median ± SEM = 28 ± 9.5%). Lysis of NK-sensitive K562 cells was also observed (5.2–18%, median ± SEM = 7.1 ± 2.6%); however, lysis of autologous tumor cells was significantly higher than lysis of K562 cells (P < 0.05). In contrast to the cytotoxic responses generated against autologous tumor cells, tumor antigen-primed T cells failed to kill autologous normal cells of the breast (P < 0.05), with specific lysis against normal cells ranging from 1.2% to 4.3% (median ± SEM = 1.3 ± 0.7%). Our findings also showed that the patients who expressed lower levels of the CD4+ T:CD8+ T ratio after priming exhibited higher levels of cytotoxic activity (patients 2 and 3) and vice versa (patients 1, 4, and 5).

Collectively our results suggest that specific cytotoxicity against autologous tumor cells is a major component of tumor antigen-primed T cells. Although an NK-like cytotoxic response was detected, autoreactive cytotoxic response against autologous, normal cells was mostly negligible.

Fig. 3. Monocyte-derived dendritic cells pulsed with tumor antigen. Peripheral blood mononuclear cells (PBMCs) from five patients with breast cancer were incubated for 2 h at 37 °C and the adherent cells were cultured in the presence of GM-CSF and IL-4. Immature DCs were pulsed with apoptotic tumor cells on day 4 and maturation factors (TNF-α plus monocyte condition medium (MCM)) were added on day 5. Tumor antigen-pulsed mature DCs were harvested on day 7 and reviewed by light (a) and phase contrast (b) microscopy.

Fig. 4. Representative histograms of flow cytometric analysis of monocyte-derived DCs. Peripheral blood mononuclear cells (PBMCs) from five patients with breast cancer were incubated for 2 h at 37 °C and the adherent cells were cultured in the presence of GM-CSF and IL-4. Immature DCs were pulsed with apoptotic tumor cells on day 4 and maturation factors were added on day 5. Immature DCs (a) and mature DCs pulsed with tumor antigen (b) using either TNF-α alone (fine line) or TNF-α plus monocyte condition medium (MCM) (coarse line) as maturation factors, were harvested on day 7 and analyzed by FACS using anti-CD1a, CD11c, CD14, CD83, and HLA-DR antibodies. After we obtained the results shown in this figure, TNF-α and MCM were used as maturation factors for all five patients.
3.5. Cytokine release

It has been reported that tumor antigen-primed T cells could secrete cytokines upon specific apoptotic tumor cell pulsed DC stimulation in vitro which appeared to correlate with their antitumor response efficacy. To address this issue, we examined cytokine profiles. The production of cytokines was determined 24 h after the third round of stimulation of autologous T cells in the supernatant of primed cells using a commercially available sandwich ELISA. As shown in Fig. 7, all five patients released variable amounts of either IFN-γ or IL-4 in response to re-stimulation. Two of these patients secreted higher levels of IFN-γ than IL-4 (335 ± 38 pg/ml vs. 29 ± 8 pg/ml) and three others secreted higher levels of IL-4 than IFN-γ (1393 ± 1218 pg/ml vs. 90 ± 81 pg/ml). Interestingly, IFN-γ production by primed T cells correlated well with the high level of cytotoxic activity (r = 0.95). Meanwhile IFN-γ or IL-4 production was proportional to the CD4+ T:CD8+ T cell ratio (Fig. 8). Altogether, our results indicated that stimulation of T cells with DCs pulsed with apoptotic tumor cells could elicit proliferation of predominantly CD8+ T cells, specific cytotoxicity against autologous tumor cells, and Th1 biased cytokine in some, but not all patients with breast cancer.

4. Discussion

It is now well established that DCs play a unique role in antitumor immunity [2, 25]. They are potent inducers of CD4+ and CD8+ T cell-mediated responses against tumor cells. Additional evidence suggests that breast tumor antigen-loaded DCs may yield enhanced antitumor immunity in vitro as well as in vivo [26]. The number of DCs in peripheral blood is not high enough to use in experimental or clinical settings, instead large numbers of DCs are generated from either bone marrow-derived CD34+ precursors.
or peripheral blood monocytes [11,27]. Given the unique properties and potent ability of DCs generated in vitro to stimulate naive T cells, it is not surprising that different DC-based protocols have already been applied to in vitro analysis or clinical trials for a number of cancers [2,28]. In the present study, peripheral blood monocyte-derived dendritic cells were used to load tumor antigens and stimulate T cell-mediated responses in vitro in patients with breast cancer.

Morphological, phenotypic, and functional characterization of monocyte-derived DCs were performed and revealed dendritic morphology with numerous cell membrane processes, CD14lo, CD83hi, and HLA-DRhi expression on the cell surface, and a high mixed leukocyte reaction (MLR) stimulation index. DC yield was 5–6% of the initial culture of PBMCs, which was comparable to results obtained by others [29].

Tumor-specific immune responses to a variety of epithelial tumors have recently been generated in vitro from the peripheral blood of cancer patients [10,30]. In an attempt to perform a preliminary investigation on DC-based breast tumor vaccines, we examined T cell-mediated immune responses induced by DCs pulsed with breast tumor antigens in vitro. In the last few years, evidence that many murine and human tumors may express immunogenic tumor antigens and that they can be used as targets for tumor-specific T lymphocytes have led to the development of different vaccination strategies for antitumor therapy. Because tumor-associated antigens are frequently not available, most immunization approaches have charged DCs in vitro with whole tumor antigen [12,14,30,31]. It should be noted that we performed our experiments using autologous cells to do in vitro studies in which DCs, T cells, breast tumor cells, and normal cells were derived from the same individual.

**Fig. 7.** IFN-γ and IL-4 cytokine production by breast tumor antigen-primed T cells. IFN-γ and IL-4 released by autologous T cells stimulated by DCs pulsed with apoptotic tumor cells were measured in the supernatant of T cells 24 h after the third stimulation using commercially available ELISA kits. The results from five patients are expressed as a mean of triplicates.

**Fig. 8.** CD4+ T:CD8+ T ratios in patients who predominantly produced IL-4 or IFN-γ. Autologous T cells from five patients with breast cancer stimulated with DCs pulsed with apoptotic breast tumor cells, three times with weekly intervals, followed by flow cytometric analysis using anti-CD4 and anti-CD8 monoclonal antibodies along with cytokine assays in the culture supernatant. BP, before priming, ApTC, apoptotic tumor cell.
Previous studies have shown that apoptotic tumor cells could elicit tumor-specific T cell responses in vitro [19,32]. The use of apoptotic tumor cells as an antigen delivery approach has already been successfully addressed in various cancer models including melanoma [33], leukemia [34], as well as colorectal and prostate cancers [35]. Various types of chemicals and irradiation sources have been used to induce apoptosis in tumor cells. In the present study, we used gamma irradiation as an apoptosis-inducing agent; however, we first had to determine the optimum dose and post-irradiation incubation time to produce the maximum number of apoptotic cells. As shown in Fig. 2, we found that the optimum dose and post-irradiation incubation time were 8 Gy and 48 h, respectively. Therefore, we pulsed immature DCs with apoptotic breast tumor cells which were exposed to 8 Gy γ-irradiation and incubated at 37 °C for 48 h.

DCs loaded with apoptotic cells trigger melanoma-specific helper- and cytotoxic T cell responses and provide long-term protection against poorly immunogenic tumors in mice [33]. Despite epitope competition and protein alteration during apoptosis induction, phagocytosis of apoptotic cells results in an MHC-peptide density that is as efficient in priming T cells as peptide loading. Several reports have shown that phagocytosis of cells undergoing apoptosis could not induce maturation in antigen presenting DCs; however, these cells are still responsive to DC maturation factors [19]. Therefore, we used TNF-α and MCM as maturation factors for antigen-pulsed immature DCs in the present study.

DCs are capable of inducing a lymphocyte proliferation response, especially when they are pulsed with the appropriate antigen. Our previous experiment showed that monocyte-derived dendritic cells from patients with breast cancer were capable of eliciting an allogenic mixed leukocyte reaction (MLR) in vitro (data not shown). In the present study, we demonstrated that DCs pulsed with apoptotic tumor cells could generate a T cell proliferative response in vitro (Fig. 5). Proliferation induction by antigen-loaded DCs revealed that an apoptotic tumor cell preparation would contain immunogenic epitopes that were properly processed and presented by DCs. In accordance to previous studies [26,36,37], we showed that proliferative responses varied from patient to patient which expressed different stimulation indices. Furthermore, the responses were increased by increasing the ratio of DC:T cells [38]. Collectively, our findings as well as the results from other studies, suggests that breast tumor antigens in the form of apoptotic tumor cell or tumor-dendritic cell hybrids could elicit autologous T cell proliferative responses in vitro [37].

Phenotypic analysis of primed T cells at the end of 19 days culture and weekly stimulation with DCs pulsed with tumor antigen revealed that such a process results in proliferation of either CD4+ T or CD8+ T cells. Consistent with other studies [39], comparison of the CD4+/CD8+ T ratio before and after priming showed that it was increased in some patients [16] and decreased in others [3] (Fig. 8).

Our next approach was to determine the cytotoxic activity of tumor antigen–primed T cells. There are numerous procedures to assay such activity, in which 51Cr-release assay is known as the gold standard test; however, in this study, we used a simple and non-radioactive flow cytometric analysis of cytotoxic activity (see Section 2) which is believed to be as sensitive and reproducible as 51Cr-release assay [24]. In order to mimic what may happen in patients immunized with DC-based vaccines, we used unfractonated primed T cells as effector cells in the cytotoxic activity assay. Target cells were autologous breast tumor cells, and normal cells, as well as the NK-sensitive K562 cell line that were labeled with PKH-26.

Flow cytometric analysis of specific lysis using Annexin V and PI showed that DCs pulsed with apoptotic tumor cells could elicit specific cytotoxic T lymphocyte response against autologous tumor cells (specific lysis = 21–65%). In all five patients with breast cancer, the primed T cells failed to kill autologous normal cells (specific lysis = 1.2–4.3%) (p < 0.05) and exhibited a variable amount of cytotoxic activity against the NK-sensitive K562 cell line (specific lysis = 5.2–18%) (Fig. 6). Previous studies reported that T cells primed by DCs pulsed with apoptotic tumor cells exhibit variable amounts of cytotoxicity in different cancers. For example, DCs loaded with apoptotic melanoma cell elicited CTLs that killed melanoma target cells more efficiently than CTLs generated with melanoma peptide loaded DCs [40]. The same results have been obtained in the case of leukemia as well [41].

The benefit of using whole tumor cells as an antigen source for loading DC has been demonstrated in many cases without development of autoreactivity toward self-antigens [34]. Our potential concern was the possible induction of autoimmune reactivity to self or normal tissues, as a consequence of processing of apoptotic tumor cell by potent antigen presenting DC; however, studies by us and others have shown negligible cytolytic reactivity against normal controls (Fig. 6) [30].

The strategy of antigen loading clearly influences the ability of DCs to polarize T cell for a Th1/Th2 response and determines the outcome of an elicited immune response [41]. T cell-mediated control of tumor is thought to be promoted by type 1 and impaired by type 2 cytokine responses [26]. Recent studies, however, have shown significant dysfunction of type 1 T cell response in tumor bearing hosts [42], suggesting that tumor progression may be associated with preferential type 2 T cell responses. In this study, we evaluated IFN-γ and IL-4 cytokine production by primed T cells as representatives of type 1 and type 2 cytokine patterns, respectively. Our result showed that two out of five patients predominantly produced IFN-γ and three others predominantly produced IL-4 (Figs. 7 and 8). These two patterns of cytokine production have been previously reported using either ELISPOT or cytoplasmic flow cytometry (CFC) analysis [35,36,43].

As reported previously, we found that autologous DCs pulsed with breast tumor antigens could elicit two patterns of immune responses in vitro, which are generally known as Th1 and Th2 type responses. These patterns are characterized by high level production of IFN-γ, predominant proliferation of CD8+ T cells, and strong and specific cytotoxic activity against autologous tumor cells in one group (two patients) and high level production of IL-4, predominant proliferation of CD4+ T cells, and modest to weak cytotoxicity against tumor cells in other group (three patients).

In conclusion, our findings demonstrated that autologous DCs pulsed with apoptotic tumor cells could elicit T cell-mediated immune responses in patients with breast cancer in vitro. This novel approach may have important implication for the treatment of residual or resistant disease with active or adoptive immunotherapy after standard surgical and cytotoxic treatments. In particular, this modality is recommended for patients who induce a type 1 immune response, however, the future design and implementation for clinical trials will ultimately determine the validity of this approach.

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