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In Vitro Induction of Tumor-Specific HLA Class I-Restricted CD8+ Cytotoxic T Lymphocytes from Patients with Locally Advanced Breast Cancer by Tumor Antigen-Pulsed Autologous Dendritic Cells[1]

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Background. **Early dissemination of treatment-resistant tumor cells remains the major cause of metastatic recurrence and death in breast cancer patients. Dendritic cells (DCs) are the most powerful antigen-presenting cells, and recently DC-based vaccination has shown great promise for the treatment of human malignancies by immunological intervention.**

Materials and Methods. **CD8+ T lymphocytes derived from peripheral blood mononuclear cells stimulated *in vitro* with autologous breast tumor antigen-pulsed DCs were tested for their ability to induce a HLA class I restricted cytotoxic T lymphocyte (CTL) response against autologous tumor cells. To correlate cytotoxic activity by CTL with T cell phenotype, two-color flow cytometric analysis of surface markers and intracellular cytokine expression was performed.**

Results. **DC pulsed with breast tumor extracts consistently elicited a tumor-specific HLA class I restricted CTL response *in vitro* in three consecutive patients harboring locally advanced breast cancer. CTL expressed strong cytolytic activity against autologous tumor cells but did not lyse autologous Epstein Barr virus-transformed lymphoblastoid cell lines and showed variable cytotoxicity against the natural killer-sensitive cell line K-562. In all patients, two color flow cytometric analysis of surface markers and intracellular cytokine expression demonstrated that tumor-specific CTL exhibited an heterogeneous CD8+/CD56+ expression and a striking Th1 cytokine bias (IFNγ^{high}/IL-4 ^{low}).**

Conclusions. **Tumor lysate-pulsed DCs can consistently stimulate specific CD8+ CTLs able to kill autologous tumor cells in patients with locally advanced breast cancer *in vitro*. Tumor antigen-pulsed DC-based vaccinations may be appropriate for the treatment of residual and/or chemotherapy-resistant breast cancer refractory to standard salvage treatment modalities.**

Key Words: **breast cancer; CTL, dendritic cells; tumor lysate; cytokines; cytotoxicity assays.**

INTRODUCTION

Breast cancer remains the most common neoplasm and the second-leading cause of cancer death in women worldwide, with 211,300 new cases estimated in the United States alone in 2003 [1]. Although cytoreductive surgery, radiotherapy, and the use of aggressive regimens of chemotherapy has led to an increase in the survival rates of patients with breast cancer, early dissemination of treatment-resistant tumor cells is the major cause of metastatic recurrence and death [2]. Recent studies using immunocytochemical techniques have shown that micrometastatic breast cancer cells resistant to cytotoxic therapeutic agents may be detected in the bone marrow of early stage cancer patients [3, 4] and that most of these cells rest in the G0 phase of the cell cycle [4]. Because of the known reduced efficacy of chemotherapeutic agents against non-

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proliferating cells [4, 5], immunotherapy may represent an attractive treatment option for these patients.

Dendritic cells (DCs) are rare but highly potent antigen-presenting cells of bone marrow origin that can stimulate both primary and secondary T and B cell responses [6, 7]. Culture of monocytes with granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin (IL)-4 has been shown to generate large numbers of myeloid DCs [8–10]. However, these DCs are still immature. Although they may effectively capture antigens, immature DCs lack full T cell-stimulatory activity and are sensitive to the immunosuppressive effects of several immunoregulatory cytokines that can be produced by tumors [7, 11]. In contrast, when maturation is induced by appropriate stimuli, such as monocyte-conditioned medium, lipopolysaccharide, or a cocktail of inflammatory cytokines [7, 11, 12], DCs demonstrate a reduced ability to phagocytose antigens but show a significantly higher production of key cytokines (e.g., IL-12), increased resistance to the immunosuppressive effects of IL-10, increased expression of T cell adhesion and costimulatory molecules, and increased expression of chemokine receptors that guide DC migration into lymphoid organs for priming of antigen-specific T cells [7, 11]. It is thus apparent that the maturation stage of DCs is critical for their optimal use in immunization strategies [13–15].

Although the identity and distribution of antigenic peptides and CTL epitopes presented by freshly isolated epithelial breast tumors is still poorly understood, recent studies have unequivocally shown the existence of multiple tumor antigens that can be recognized by CTL and used as target molecules to induce autologous tumor cell killing *in vitro* [16, 17]. Therefore, an effective strategy for vaccinating patients against tumor may be the use of unfractionated tumor-derived antigens, such as whole tumor cells, proteins, or peptides isolated from tumor cells. Consistent with this view, DCs fused with breast cancer cells [18] or autologous DCs pulsed with HER-2/neu-derived peptides [16, 19] are able to activate CTL responses in patients with breast cancer. In addition, promising preliminary clinical data have been reported for several human malignancies when DCs pulsed with tumor-derived antigens have been used to induce antitumor immunity [20–24].

Recently, using autologous DCs pulsed with acid eluted peptides derived from tumor cells [25] or tumor lysate [26], we have reported the induction of tumor-specific HLA class I restricted CTL in advanced ovarian carcinoma patients, a malignancy known to share multiple antigens with breast cancer [16, 19, 27, 28]. In this study, using a similar approach we show the induction of autologous tumor-specific CTL responses against breast cancer cells in three consecutive patients harboring locally advanced breast cancer. Two color flow cytometric analysis of intracellular cytokine expression at the single cell level showed that a polarized type 1 pattern of cytokine expression (i.e., high IFN-γ/low IL-4) is inducible in the tumor lysate-pulsed DC-stimulated CD8+ T cell populations. This approach may therefore hold potential for the treatment with active or adoptive immunotherapy of breast cancer patients harboring residual or resistant disease after standard surgical and cytotoxic treatment.

MATERIALS AND METHODS

Patients

Tumor tissue and peripheral blood lymphocytes (PBLs) were obtained from three patients who had undergone total mastectomy and lymphadenectomy for invasive breast cancer. Specimens were obtained at the time of surgery through the Departments of Surgery and Pathology at the University of Arkansas for Medical Sciences, under approval of the Institutional Review Board. Patients 1 and 2 were Caucasian females with stage T4 N1 M0 and T2 N1 M0 ductal carcinoma and were ages 35 and 45, respectively, whereas patient 3 was an African-American female with stage T2 N1 M0 ductal adenocarcinoma and was age 65. All three patients had received no treatment before tumor biopsy.

Tumor Cell Lines

The natural killer (NK)-sensitive target K562 (a human erythroleukemia cell line) was purchased from American Type Culture Collection and was maintained at 37°C, 5% CO_2 in complete media containing RPMI 1640 (Invitrogen, Grand Island, NY) and 10% fetal bovine serum (Gemini Bioproducts, Calabasas, CA). Fresh breast tumor cells were obtained from tumor biopsy or surgical specimens. Single-cell suspensions were obtained by processing solid tumor samples under sterile conditions at room temperature as described [25, 26]. Briefly, viable tumor tissue was mechanically minced in RPMI 1640 to portions no larger than 1-3 mm^3 and washed twice with RPMI 1640. The portions of minced tumor were then placed into 250-ml trypsinizing flasks containing 30 ml of enzyme solution (0.14% collagenase Type 1 [Sigma, St. Louis, MO] and 0.01% DNase [Sigma, 2000 KU/mg]) in RPMI 1640 and incubated on a magnetic stirring apparatus overnight at 4°C. Enzymatically dissociated tumor was then filtered through 150-μm nylon mesh to generate a single cell suspension. The resultant cell suspension was then washed twice in RPMI 1640 plus 10% autologous plasma and placed on discontinuous Ficoll-Hypaque (75/100%) density gradients and centrifuged again to separate tumor infiltrating lymphocytes (TIL) and tumor cells. Fresh tumor cells were seeded and maintained in serum-free keratinocyte medium (Invitrogen), supplemented with 5 ng/ml epidermal growth factor and 35 to 50 μg/ml bovine pituitary extract (Invitrogen) at 37°C, 5% CO_2. All experiments were performed with fresh or cryopreserved tumor cultures which had at least 90% viability and contained >99% tumor cells.

Preparation of Tumor Lysate

Autologous tumor cells (5 to 10 $\times$ 10^6) cultured in serum-free keratinocyte medium were washed twice with PBS (pH 7.4) and harvested by scraping. Cells were lysed by three to four freeze cycles (in liquid nitrogen) and thaw cycles (room temperature). Lysis was monitored by light microscopy. Larger particles were removed by centrifugation (10 min, 78*g*), supernatants were passed through a 0.2-μm filter and stored at −80°C until use.

Isolation of PBLs and Generation of DCs

PBLs were separated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation and either cryopreserved in RPMI 1640 plus 10% DMSO and 25% autologous plasma or immediately used for DC generation. Briefly, PBLs obtained from 42 ml of peripheral blood were placed in 6-well culture plates (Costar, Cambridge, MA) in AIM-V medium (Invitrogen) at 0.5–1 $\times$ 10^7/3 ml per well. After 2 h at 37°C, nonadherent cells were removed, and the adherent cells were cultured at 37°C in a humidified 5% CO_2/95% air incubator, in AIM-V medium supplemented with recombinant human GM-CSF (800 U/ml, Immunex, Seattle, WA) and IL-4 (1000 U/ml, R & D Systems, Minneapolis, MN). Every 2 days, 1.5 ml of spent medium was replaced by 1.5 ml of fresh medium containing 1600 U/ml GM-CSF and 1000 U/ml IL-4 to yield final concentrations of 800 U/ml and 500 U/ml, respectively. After 6 days of culture, final maturation of DC was induced by exposure during the last 48 h of culture (i.e., day 6 to day 8) to tumor necrosis factor-α (1000 U/ml), IL-1β (500 U/ml; R & D Systems), and PGE2a (0.5 mM; Sigma). After final maturation, DCs were harvested for pulsing with breast tumor lysate as described below.

Antigen Loading of DCs

After culture, fully mature DCs were washed twice in AIM-V and added to 50-ml polypropylene tubes (Falcon, Oxnard, CA). The cationic lipid DOTAP (Boehringer Mannheim, Indianapolis, IN) was used to deliver the total cell extract to DCs. Total cell extract (500 μl) derived from 5 to 10 $\times$ 10^6 tumor cells in AIM-V and DOTAP (125 μg in 500 μl of AIM-V) were mixed in 12 $\times$ 75 mm polystyrene tubes at room temperature for 20 min. The complex was added to the DC in a total volume of 2-5 ml of AIM-V and incubated at 37°C in an incubator with occasional agitation for 3 h. The cells were washed twice with PBS and resuspended in AIM-V.

In vitro Generation of Tumor-Specific CTLs

Fresh or cryopreserved responder PBLs were washed and resuspended in AIM-V at 10-20 $\times$ 10^6 cells/well in 6-well culture plates (Costar) with tumor lysate-pulsed autologous DC (ratios from 20:1 to 30:1 responder PBL:DC). The cultures were supplemented with recombinant human GM-CSF (250 U/ml) and recombinant human IL-2 (10 U/ml-Aldesleukin, Chiron Therapeutics, Emeryville, CA) and incubated at 37°C. Human rIL-2 (10 U/ml) was added to the cultures thereafter every 3-4 days. After 8-10 days, PBLs were restimulated once with tumor lysate loaded DC at a 20:1 ratio. At day 21, CD8+ cells were separated from the bulk cultures by positive selection with CD8-Dynabeads (Dynal Inc., Lake Success, NY) and further expanded in number for 10-14 days using autologous or allogeneic irradiated PBL (5000 cGy; 1 $\times$ 10^6 cells/well) and anti-CD3 monoclonal antibody (0.2 μg/ml, Ortho Pharmaceutical Corp, Raritan, NJ) in AIM-V plus 5% heat inactivated autologous plasma in 24-well plates (Costar) before being assayed for CTL activity. In some experiments, CD8+ T cells were restimulated once more with autologous lethally irradiated (i.e., 10,000 cGy) tumor cells at a 20:1 lymphocyte/tumor ratios or tumor lysate pulsed DCs before being tested in cytotoxicity assays or being further expanded with allogeneic irradiated PBLs (5000 cGy; 1 $\times$ 10^6 cells/well) and anti-CD3 monoclonal antibody. As negative control targets, Epstein Barr virus (EBV)-transformed autologous lymphoblastoid B-cell lines (LCLs) were established by coculture of PBLs with EBV-containing supernatant from the B95.8 cell line in the presence of 1 μg/ml cyclosporin A (Sandoz, Camberley, United Kingdom) and were maintained in AIM-V supplemented with 10% human AB serum.

Cytotoxicity Assays

A standard 6-h chromium (^{51}Cr) release assay was performed to measure the cytotoxic activity of DC-tumor lysate stimulated CD8+ T lymphocytes against autologous tumor cells [29]. Briefly, ^{51}chromium-labeled breast tumor target cells were plated at 1 $\times$ 10^4 cells/well in triplicate on 96-well U-bottomed plates (Costar) with 2 $\times$ 10^5 effector cells/well in a final volume of 200 μl. The maximally allowed spontaneous release of ^{51}chromium by breast target tumor cells was 35%. Assays characterized by a higher release of chromium by tumor cells were either repeated or excluded from the final evaluation. The K562 tumor cell line was used as a target for the detection of NK activity. EBV-transformed LCLs were used as autologous control targets. For determination of HLA class I restriction of lysis, monoclonal antibodies (MAbs) were used to block cytotoxicity. ^{51}Cr-labeled tumor targets were preincubated with MAbs specific for monomorphic HLA class I (W6/32, 50 μg/ml) or isotype control (50 μg/ml). The percentage of specific ^{51}Cr release was calculated as [^{51}Cr (experimental) $-$ ^{51}Cr (background)] $\times$ 100/[^{51}Cr (total) $-$ ^{51}Cr (background)]. The results represent the mean of not less than four assays for each patient.

Phenotypic Analysis of T Cells

Flow cytometry was performed using fluorochrome-conjugated MAbs directed against the following human leukocyte antigens: CD3 (pan T cells); CD4 (T helper/inducer); CD8 (T cytotoxic/suppressor); CD56 (NK/K cells); CD25 (IL-2 receptor); HLA-DR; TcR-α/β or TcR-γ/δ (Becton Dickinson, San Jose, CA) and analyzed on a FACScan (Becton Dickinson).

Flow Cytometric Analysis of Intracellular Cytokine Expression

Frequencies of breast tumor antigen-responsive cytokine-expressing CD8+ T cells were determined by a modification of the method of Maino & Picker [30]. Briefly, 2 $\times$ 10^5 DC-tumor lysate stimulated CD8+ T lymphocytes were incubated overnight (16 h) with 0.5 $\times$ 10^5 autologous tumor cells in a 24-well plate in AIM-V medium, 5% human AB serum, 50 U/ml of IL-2, and 1 μg/ml of costimulatory MAbs to CD28 and CD49d (BD Biosciences, San Jose, CA). Controls included unpulsed DC-stimulated CD8+ T lymphocytes, DC-tumor lysate stimulated CD8+ T cells incubated in the absence of tumor cells, and/or costimulatory MAbs, or stimulation with solid phase OKT3 (1 μg/ml). Brefeldin A (Sigma) was added 2 h after the beginning of the assay at a concentration of 10 μg/ml. After 16 h, lymphocytes were harvested, washed, and stained for superficial markers with the CD8 phycoerythrin MAb and then fixed with 2% paraformaldehyde in PBS for 20 min at room temperature. Intracellular staining was performed after permeabilization by incubation in PBS plus 1% bovine serum albumin (BSA) and 0.5% saponin (S-7900, Sigma) for 10 min at room temperature with MAbs against IFN-γ FITC and/or IL-4 FITC. After a final wash in PBS, samples were resuspended in 200 ml of 2% paraformaldehyde in PBS and analyzed by flow cytometry within 24 h. Files were acquired using a logical gate on lymphocytes (i.e., CD8-positive cells). Results were reported as cytokine-producing CD8+ cells as a percentage of gated CD8+ cells. Two-color (CD8-PE/IFN-γ-FITC) flow cytometric acquisition was performed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences). Between 30,000 and 60,000 CD8+ T cells were typically collected. Cells were gated on CD8 versus side scatter to collect only lymphocytes. A response region was defined based on the staining profile of cells responding to a

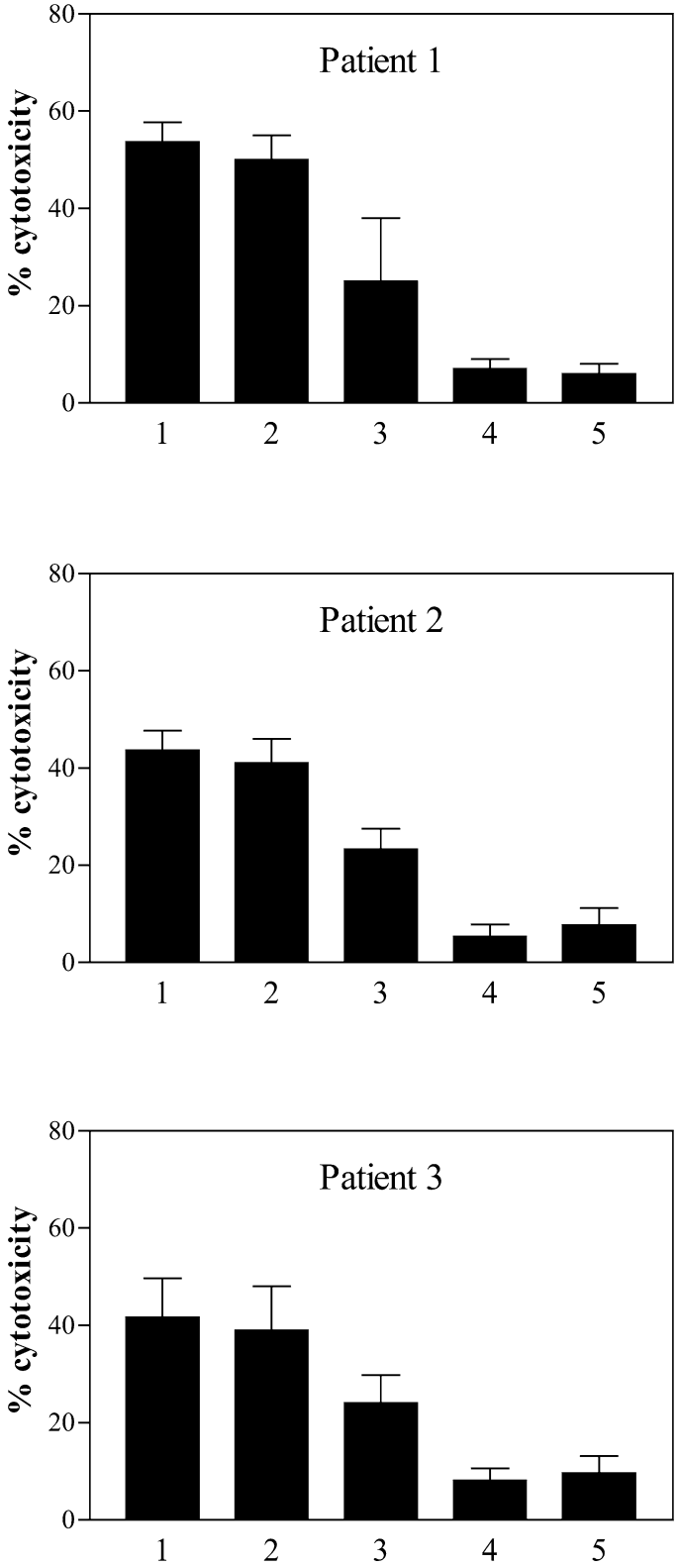

FIG. 1. Tumor-specific CD8+ CTL responses induced by tumor lysate-pulsed DCs from PBLs derived from patients with advanced breast cancer, measured in a 6-h ^{51}Cr-release assay. Percentage lysis (mean ± standard deviation) at a 20:1 effector/target cell ratio is shown for four different experiments for each patient. Anti-HLA class I blocking antibody (W6/32) and isotype control antibody were used at 50 μg/ml. Patients 1-3; 1, autologous tumor; 2, autologous tumor + isotype control antibody; 3, autologous tumor+W6/32 anti-Class I MAb; 4, LCL control; 5, K562.

positive control, such as anti-CD3 MAb.

Flow cytometric analysis of intracellular cytokine expression by stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin was conducted essentially as previously described [25, 26]. CD8+ T cells were tested after resting for 14 days after the last antigen stimulation prior to activation by PMA and ionomycin. Briefly, T cells (7.5×10^5/ml) were incubated at 37°C for 6 h in AIM-V plus 5% autologous plasma and 50 ng/ml PMA and 500 ng/ml ionomycin. Brefeldin A (10 μg/ml) was added for the final 3 h of incubation. Controls (nonactivated cultures) were incubated in the presence of Brefeldin A only. The cells were harvested, washed, and fixed with 2% paraformaldehyde in PBS for 20 min at room temperature, after which they were washed and stored overnight in PBS at 4°C. For intracellular staining, the cells were washed and permeabilized by incubation in PBS plus 1% BSA and 0.5% saponin (S-7900, Sigma) for 10 min at room temperature. Activated and control cells were stained with FITC-anti-IFN-γ, and PE-anti-IL-4, and isotype-matched controls (FITC-anti-Igγ2a and PE-anti-Igγ1) from Becton-Dickinson. After staining, cells were washed twice with PBS plus 1% BSA and 0.5% saponin, once with PBS plus 0.5% BSA, and fixed a second time with 2% paraformaldehyde in PBS.

RESULTS

Tumor-Specific CD8+ CTL Responses

Cytotoxicity assays were conducted after a minimum of 5 weeks after initiation of T lymphocyte cultures. The results presented in Figure 1 represent the mean of not less than four assays for each patient. Only results from CTL cultures tested in cytotoxicity without *in vitro* re-stimulation by irradiated autologous tumor cells are shown. CD8+ T cell cytotoxicity against autologous tumor cell targets was demonstrated for all three patients, ranging from 41 to 58% (PT1), from 28 to 45% (PT2), and from 24 to 47% (PT3) at 20 effectors per target. T cells that were not stimulated with antigen-pulsed DC were non-cytotoxic against autologous breast cancer cells (data not shown). Cytotoxicity against the NK-sensitive cell line K562 was detectable at a low level in all patients. The absence of cytotoxicity against autologous EBV-transformed LCL demonstrated that, although these cells were highly cytotoxic against autologous tumor cells, they failed to kill autologous cells infected with EBV. Blocking studies demonstrated in all cases that the lytic activity against autologous tumor targets was significantly inhibited by pretreatment of tumor targets with MAb specific for HLA class I range of inhibition: from 28 to 75% [PT1], from 41 to 65% [PT2], from 21 to 55% [PT3]; Fig. 1; ($P < 0.05$ by Student's t test for all three patients) but not by pretreatment with isotype controls (P = not significant; Fig. 1).

Phenotypic Analysis

Flow cytometric analysis was used to determine the phenotype of the populations of tumor lysate-

pulsed-DC stimulated T cells. Almost all of the lymphocytes were CD3-positive T cells (85% to 95%). Tumor lysate-pulsed DC-activated lymphocytes at 3 weeks of culture consisted of subpopulations with a predominance of CD4+ T cells (range from 52 to 72%) over CD8+ T cells, (range from 25 to 45%). *In vitro* cytotoxicity assays were conducted using pure CD8+ T cell populations (i.e., more than 95% CD8+). All of the cells were CD3+/CD8+ and CD4−, with a variable proportion of CD56+ cells. Further analysis revealed the populations of CD8+ T cells also to be CD25+, HLA-DR+, and CD16− (data not shown). CD8+/CD56+ cells ranged from 12 to 45% in several different experiments from all three patients. Figure 2 depicts a representative experiments of CD8+/CD56+ expression in CTL populations derived from all three patients.

Two-Color Flow Cytometric Analysis of Intracellular IFNγ and IL-4 Expression by Tumor-Specific T Cell Cultures

To evaluate whether cytokine expression by DC-tumor lysate stimulated CD8+ T cells derived from patients with advanced stage breast cancer segregated in discrete IFN-γ+/IL-4− and IFN-γ-/IL-4+ subsets, detection of intracellular cytokine expression at the single cell level was evaluated by flow cytometric techniques after stimulation with PMA and ionomycin as described in the Methods section. Two-color flow cytometric analysis of intracellular IFN-γ and IL-4 expression by CD8+ CTLs was performed after at least 6 weeks of culture. As representatively shown in Figure 3, the majority of CD8+ T cells from all patients contained intracellular IFN-γ, whereas a second subset contained only IL-4 and a third minor subset contained both. In this regard, the percentages of IFN-γ/CD8+ T cells in multiple experiments were 71 ± 9 (mean ± SD) for patient 1, 73 ± 11 for patient 2, and 62 ± 9 for patient 3. Similar results were consistently obtained in several repetitive analyses from different primings for all patients. FITC-anti-IgG2a and PE-anti-IgG1 isotype controls did not stain either activated or unactivated CD4+ or CD8+ T cells (data not shown).

Frequencies of Intracellular Cytokine-Positive Tumor Antigen-Responsive CD8+ T Lymphocytes from Breast Cancer Patients

To evaluate the frequency of DC-tumor lysate stimulated CD8+ T cells that expressed intracellular IFN-γ or IL-4 upon stimulation with autologous breast tumor cells, T cells were stimulated for 16 h in the presence of viable tumor cells and costimulatory MAbs. As representatively shown in Figure 4 for patients 1 and 2, the percentage of CD8+/IFN-γ-positive CTL after two restimulations with tumor antigen-pulsed DC was in the range of 0.8% to 3.5%. Unpulsed DC-stimulated CD8+ T cell controls showed IFN-γ expression in the range of unstimulated controls (0.1% to 0.3%; Fig. 4). No detectable IL-4 expression was found in either antigen-pulsed or unpulsed DC-stimulated CD8+ T cells (data not shown).

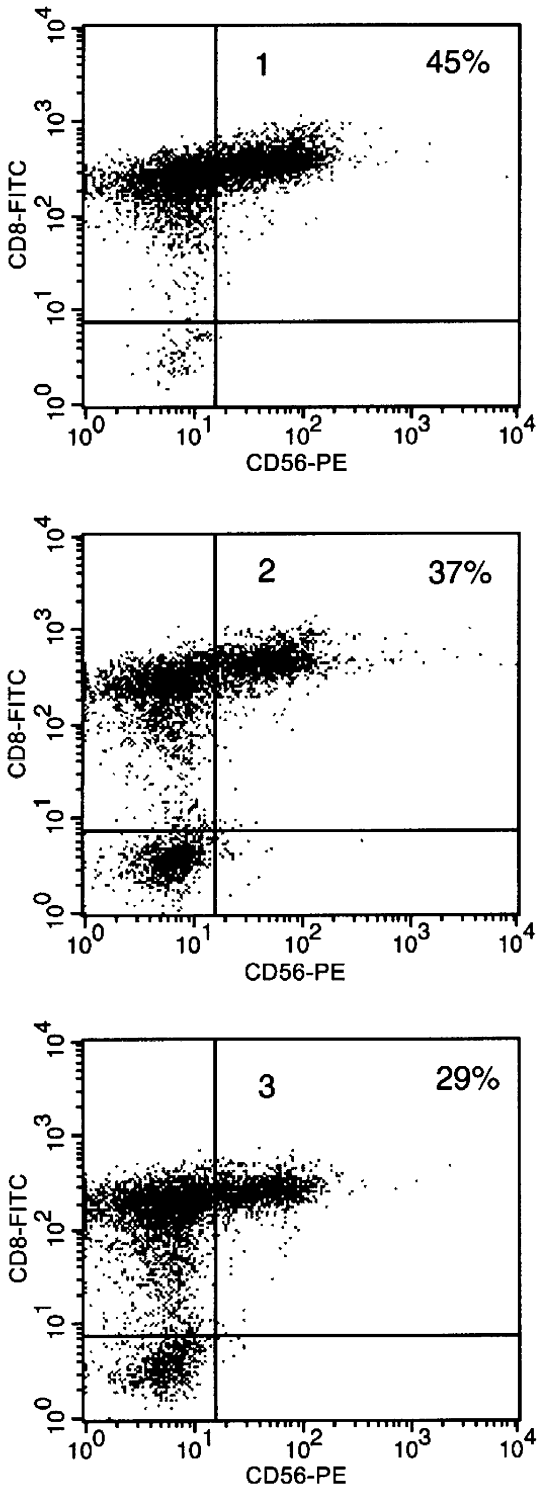

FIG. 2. Pure populations of breast tumor-specific CD8+ T cells derived from patients 1, 2, and 3 were tested 5 weeks after initiation of T lymphocyte cultures for CD56 expression by two-color flow cytometric analysis. A representative experiment for each patient is shown.

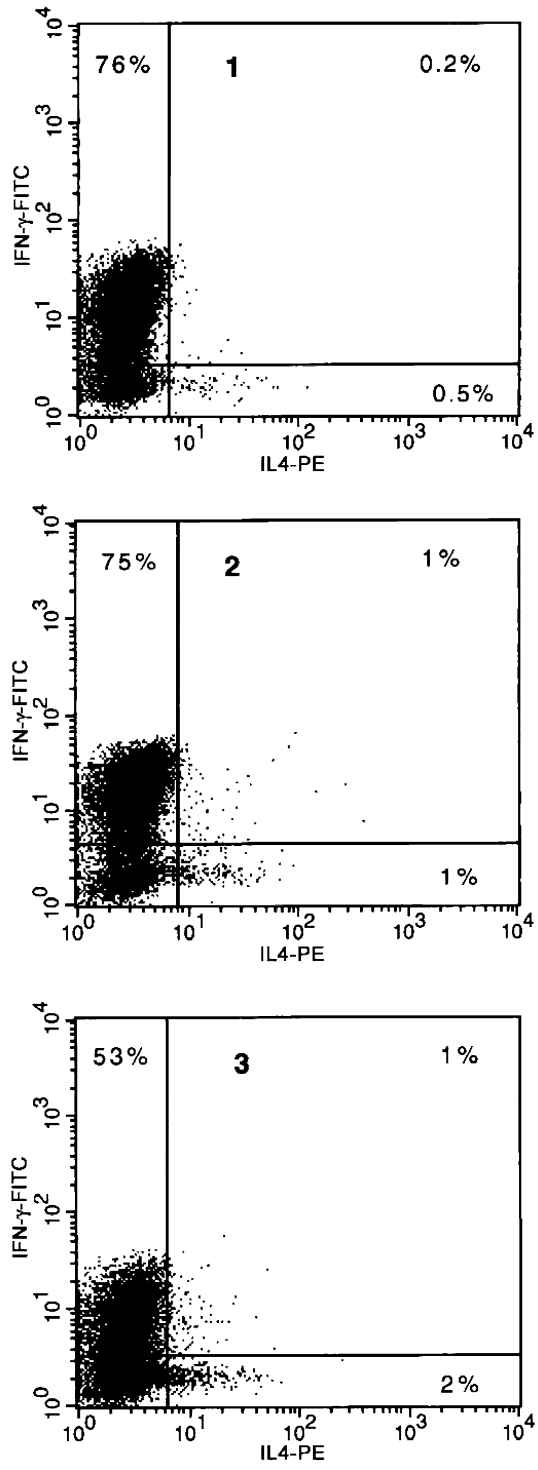

FIG. 3. Two-color flow cytometric analysis of intracellular IFN-γ and IL-4 expression by tumor specific CD8+ T cells derived from PBL from patients 1, 2, and 3 after stimulation by PMA and ionomycin. A representative experiment for each patient is shown.

DISCUSSION

HLA-A2-restricted peptide epitopes derived from tumor antigens potentially overexpressed in breast carcinomas, e.g., carcinoembryonic antigen or HER2/neu proto-oncogene, have been previously shown to induce specific CTL responses against carcinoembryonic antigen-expressing target cells [31] or HER2/neu-positive autologous breast cancer cell lines [16, 19]. However, the use of synthetic peptides as antigens has a number of limitations. Because peptide vaccination protocols rely on knowledge of the HLA type of each patient as well as on the HLA restriction of the few breast cancer-specific peptides available today, this approach may not be practical for broadly inclusive vaccination protocols. Furthermore, tumor antigen heterogeneity in disseminated breast cancer cells has been reported [3, 4], and constitutes a major barrier to synthetic peptide-based immunotherapy of minimal residual disease. In contrast, autologous DC pulsed with whole tumor extracts derived from autologous breast tumor cells or fusions of autologous breast tumor cells with DC [18] may offer the significant advantage of allowing DC to present multiple immunogenic CTL epitopes without the need for knowledge of the individual HLA type. Furthermore, because the selective loss of one HLA-restriction element on tumor cells is a much more frequent finding than the total loss of HLA expression [32], it is likely that the use of DC pulsed with whole tumor extracts, incorporating peptides able to bind multiple HLA class I restriction elements, will afford a superior form of tumor-specific vaccination.

In this study, we demonstrated that autologous DC pulsed with breast cancer tumor lysate can stimulate a specific CD8+ CTL response from PBLs that is capable of killing autologous tumor cells in patients with advanced breast cancer. Importantly, a large proportion of the autologous tumor specific cytotoxicity was inhibited by anti-HLA class I MAb. These results, therefore, indicate that most of the cytotoxicity against autologous tumor cells was mediated by antigen specific HLA class I restricted CTLs. The lack of a complete block in cytotoxicity by anti-HLA class I MAb can be at least partially explained by the presence of a heterogenous population of CTL at the time of the cytotoxicity assays. The possibility of HLA-unrestricted lysis of target cells by at least some tumor-specific CD8+ T cells cannot be excluded [33, 34]. Indeed, high-avidity/high affinity tumor specific CD8+ CTL with the ability to kill tumor target cells using either HLA-class I restricted or a HLA class I unrestricted mechanisms have been previously reported [33, 34]. Autologous LCLs were not killed by tumor-specific CTL, demonstrating that although these CTLs were highly cytolytic for autologous tumor cells, they failed to kill autologous normal cells. NK activity was low for all patients when tested 5 weeks from DC priming, showing that at this time point CD8+ T cell populations do not retain significant NK-like cytotoxicity.

Phenotypic analysis of tumor-lysate DC-activated

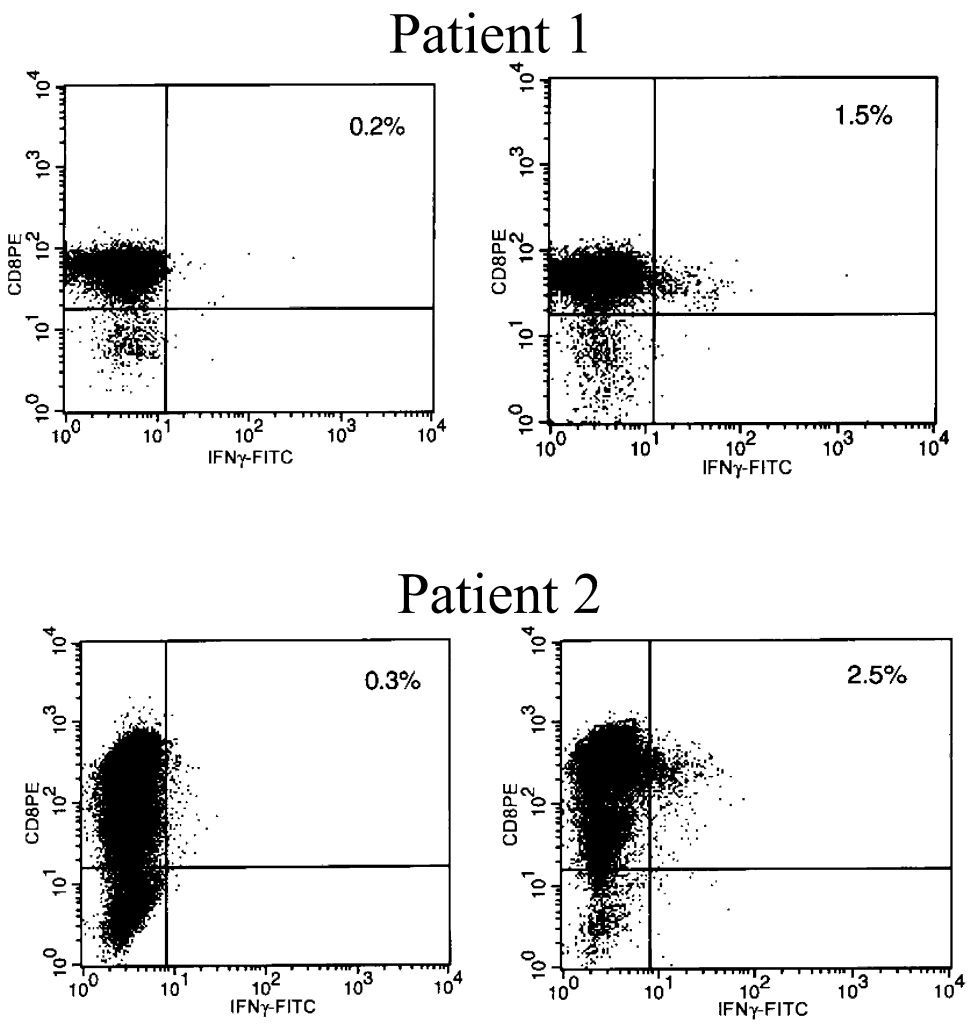

FIG. 4. Two-color flow cytometric analysis of intracellular IFN-γ expression by tumor antigen-specific CD8+ T cells. The percentage of tumor lysate antigen-specific CD8+ T cells producing IFN-γ in response to autologous tumor stimulation versus DC-stimulated controls (right and left, respectively) is indicated. Patient 1 (upper panels); patient 2 (lower panels). CD8+ T cells 3 weeks after priming were stimulated with autologous tumor cells plus costimulatory MAbs to CD28 and CD49d for 16 h. Brefeldin A was added for the final 14 h.

lymphocytes after 3 weeks of culture revealed a predominance of CD4+ T cells over CD8+ T cells. Interestingly, pure populations of CD8+ T cells from all patients revealed a variable CD8+/CD56+ subpopulation after expansion. The origin of these cells expressing both the T cell markers CD3/CD8 and the NK cell marker CD56 is still uncertain, but previous reports have suggested that these T cells could be extrathymically differentiated [35–37]. In this regard, homodimeric CD8 α/α expression has been previously shown to be inducible in multiple cell types by activation events in the periphery, whereas T cells of thymic origin express the CD8 α/β heterodimer [35–37]. We found that CD56+ CTL express heterodimeric CD8 α/β molecules (data not shown), confirming the thymic origin of these highly cytotoxic HLA-restricted T cells. These results are in agreement with our previous findings in patients with cervical cancer [29] and the findings of Pittet *et al.,* [38], who also observed that CD56 expression by human CD8+ T cells closely correlated with cytotoxic effector function. We would suggest, therefore, that CD56 expression on tumor specific CD8+ CTL generated by tumor-antigen-pulsed DC against different human tumors, may be regarded as an activation antigen associated with cytotoxic function, rather than a lineage-specific marker.

T cell-mediated protection from viral infection as well as control of tumors is thought to be promoted by Type 1 cytokine responses and impaired by Type 2 cytokine responses [39]. Consistent with this view, recent data have demonstrated that CD8+ T-cell inhibition of tumor growth *in vivo* correlates more with type 1 cytokine production than with cytotoxic function [40]. Recent studies, however, have shown significant dysfunction of Type 1 T cell responses in tumor bearing

hosts [41, 42], suggesting that tumor progression may be associated with a preferential Type 2 T cell response. In this report, two color flow cytometric analysis of intracellular IFN-γ and IL-4 expression by tumor lysate-pulsed DC stimulated CD8+ T cells demonstrated that tumor-specific T cells from advanced breast cancer patients showed even at this advanced stage of the disease a large proportion of Type 1 cytokine expressor in the CD8+ CTL populations of all patients. These data, therefore, show that this vaccination approach is able, at least *in vitro*, to induce the differentiation from PBL of Type 1 CD8+ T cells secreting IFN-γ and endowed with cytotoxic activity against autologous tumor cells.

The use of intracytoplasmic staining for evaluating the percentages of antigen specific cytokine expressing T cells has previously been demonstrated for mitogens, superantigens and viral peptides [30], but only recently for tumor immunotherapy [43, 44]. In this study we evaluated the percentage of tumor lysate antigen-specific CD8+ T cells producing IFN-γ and IL-4 in response to autologous tumor stimulation. We found that a low but consistent percentage of CD8+ T cells were able to express IFN-γ when challenged with tumor cells. In contrast, no detectable IL-4 was found in the breast tumor specific populations, in agreement with the results obtained when CTL were activated by PMA plus ionomycin. Collectively, these results showed that intracellular cytokine expression by flow cytometry may be a useful method to evaluate the frequency of breast tumor specific cytokine-expressing CTL following *in vitro* activation and expansion by tumor antigen-pulsed autologous DC.

DCs pulsed with specific peptides, tumor lysate, full-length proteins, or fused with autologous tumor cells have already shown great promise in the presence of minimal toxicity for the effective treatment of human malignancies by immunological intervention [20–24]. In breast cancer fusion of autologous DCs with tumor cells [18], or transduction of DCs with retrovirus encoding for the HER2/neu gene [45], have recently shown great promise for the generation of tumor specific cytotoxic responses against breast cancer cells. Our findings illustrate the feasibility of either tumor lysate-pulsed DC vaccines or adoptive immunotherapy with tumor antigen pulsed DC-stimulated T cells as novel strategies for the treatment of chemotherapy resistant breast cancer after standard surgical–cytotoxic treatment. The future design and implementation of clinical trials will ultimately determine the validity of this approach.

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