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*Report*

# Restoration of tumor-specific HLA class I restricted cytotoxicity in tumor infiltrating lymphocytes of advanced breast cancer patients by *in vitro* stimulation with tumor antigen-pulsed autologous dendritic cells

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*Key words:* breast cancer, CTLs, dendritic cells, TIL, tumor lysate

**Summary**

Breast tumor infiltrating lymphocytes (TIL) are enriched in tumor-specific cytotoxic T lymphocytes (CTL), and may represent a superior source of CTL compare to peripheral blood lymphocytes (PBL), for adoptive T cell immunotherapy of breast cancer. However, the immunocompetence of TIL and the possibility to consistently restore their tumor-specific lytic activity *in vitro* remains an open issue. In this study we evaluated the potential of tumor antigen-pulsed fully mature dendritic cell (DC) stimulation in restoring tumor-specific cytotoxicity in anergic TIL populations from advanced breast cancer patients. In addition we have compared tumor-specific T cell responses induced by tumor antigen-loaded DC stimulation of TIL to responses induced from PBL. Although TIL were consistently non-cytotoxic after isolation or culture in the presence of interleukin-2 (IL-2), in matched experiments from three consecutive patients, tumor-lysate-pulsed DC-stimulated CD8+ T cell derived from TIL were found to be significantly more cytotoxic than PBL ($p < 0.05$). In addition, cytotoxicity against autologous tumor cells was more significantly inhibited by an anti-HLA class I (W6/32) MAb in TIL compared to PBL ($p < 0.05$). CTL populations derived from TIL and PBL did not lyse autologous EBV-transformed lymphoblastoid cell lines, and showed negligible cytotoxicity against the NK-sensitive cell line K562. Furthermore, in both CD8+ T cell populations the majority of the tumor-specific CTL exhibited a Th1 cytokine bias (IFN-$\gamma^{high}$/IL-$4^{low}$). Taken together, these data show that tumor lysate-pulsed mature DC can consistently restore tumor-specific lytic activity in non-cytotoxic breast cancer TIL. These results may have important implications for the treatment of chemotherapy resistant breast cancer with active or adoptive immunotherapy.

*Abbreviations:* CTL: cytotoxic T lymphocyte; DC: dendritic cells; HLA: human leukocyte antigen; TIL: tumor infiltrating lymphocytes; LCL: lymphoblastoid B-cell line

## Introduction

Despite the use of cytoreductive surgery, and increasingly intense chemotherapeutic and radiotherapeutic regimens, early dissemination of treatment-resistant tumor cells remains a major cause of metastatic recurrence and death in breast cancer patients [1, 2]. Several reports have shown that most of the disseminated tumor cells commonly detected in the bone marrow of breast cancer patients rest in the G0 phase of the cell

cycle [1, 3, 4]. Because of the poor prognostic impact of detection of micrometastatic disease in breast cancer patients [2] and the high resistance to chemotherapeutic agents in non-proliferating cells [3–5], immunotherapy may represent an attractive treatment option to offer to these patients.

Dendritic cells (DC) constitute a complex system of professional antigen presenting cells (APC) uniquely able to induce primary T and B cell responses [6, 7]. Recently, the combination of GM-CSF and IL-4 has been shown to generate large numbers of myeloid monocyte-derived DC [8–10]. However, these DC are still immature. Although they may effectively capture antigens, immature DC 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, LPS, or a cocktail of inflammatory cytokines [7, 11, 12], DC 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 DC is critical for their optimal use in immunization strategies [13–15].

Tumor infiltrating lymphocytes (TIL) are present in most human breast carcinomas and large numbers of TIL have been found to express an activated phenotype implying that an immune response against cancer cells is occurring *in vivo* [16–18]. Consistent with this view, lymphocytes derived from tumor tissues of breast cancer patients have been shown to be enriched with tumor-specific cytotoxic T lymphocytes (CTL) that can be stimulated to specifically kill breast cancer cells *in vitro* [16, 18–20]. However, TIL have been shown to be defective in several immunologic functions when compared to lymphocyte populations derived from peripheral blood (PBL) [17, 21, 22]. Indeed, a poor ability to proliferate in the presence of mitogens or low doses of IL-2, lack of cytotoxic activity against autologous or allogenic tumor cells, and alterations in the expression and/or function of cytoplasmic CD3-ζ chain, which is essential for T cell receptor (TcR) signaling, have been previously reported in TIL recovered from breast and other cancers [16–22]. Importantly, however, in some cases, poor immune responses by explanted T lymphocytes could be normalized upon *in vitro* culture with high doses of recombinant IL-2 [23] or by T cell stimulation with anti-CD3 and anti-CD28 [24]. It is therefore possible that when stimulated by tumor-antigen-pulsed mature DC, which are endowed with high expression of HLA class I and II antigens and costimulatory molecules [8–11], TIL may reverse their anergic state and become a superior source of tumor specific CTL for adoptive T cell immunotherapy of advanced or metastatic breast cancer refractory to standard treatment modalities.

In this study we have evaluated the potential of fully mature tumor lysate-pulsed autologous DC in restoring tumor-specific cytotoxicity in TIL from advanced breast cancer patients. Here we show for the first time that autologous DC have the potential to reverse the anergic state of TIL and that TIL constitute a superior source of tumor-specific CD8+ CTL compared to PBL.

## Patients and methods

Tumor tissue, TIL and PBL were obtained from three consecutive patients who had undergone total mastectomy and lymphadenectomy for invasive breast cancer. Specimens were obtained at the time of surgery through the Surgical Oncology Department and Pathology Department at the University of Arkansas for Medical Sciences (UAMS), Little Rock, AR, 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 age 35 and 54, respectively, while patient 3 was an African–American female with stage T2 N1 M0 ductal adenocarcinoma and was age 72. All three patients had received no treatment prior to surgery.

### *Tumor cell lines*

The natural killer (NK) sensitive target K562 (a human erythroleukemia cell line) was purchased from American Type Culture Collection (ATCC) and was maintained at 37°C, 5% $CO_2$ in complete media (CM) containing RPMI 1640 (Invitrogen, Grand Island, NY) and 10% fetal bovine serum (FBS, Gemini Bioproducts, Calabasas, CA). Fresh breast tumor cells were obtained from 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 I (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. Fresh tumor cell lines were maintained in keratinocyte serum-free medium (KSFM, Invitrogen), supplemented with 5 ng/ml epidermal growth factor and 35–50 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*

$(5–10) \times 10^6$ autologous tumor cells cultured in KSFM were washed twice with phosphate-buffered saline (PBS, pH 7.4) and harvested by scraping. Cells were lysed by 3–4 freeze cycles (in liquid nitrogen) and thaw cycles (room temperature). Lysis was monitored by light microscopy. Larger particles were removed by centrifugation (10 min, 600 rpm), supernatants were passed through a 0.2 μm filter, and stored at −80°C until use.

*Isolation of TIL from advanced breast cancer patients*

Single cell suspensions were obtained by processing solid tumor samples as described above. The resultant cell suspension containing tumor cells and TIL 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 harvest TIL and tumor cells as previously described by Whiteside et al. [27]. Enriched TIL preparations were then washed twice in RPMI 1640 plus 10% autologous plasma and either immediately stimulated with tumor lysate-loaded DC (see below) or cryopreserved.

*Isolation of PBL and generation of DC*

PBL were separated from heparinized venous blood by Ficoll-Hypaque (Sigma) density gradient centrifugation and either cryopreserved in RPMI 1640 plus 10% DMSO and 25% autologous plasma, or immediately used for DC generation. Briefly, PBMC obtained from 42 ml of peripheral blood were placed in six-well culture plates (Costar, Cambridge, MA) in AIM-V (Invitrogen) at $(0.5–1) \times 10^7$ per 3 ml per well. After 2 h at 37°C, non-adherent 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 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 and 500 U/ml, respectively. After 6 days of culture, final maturation of monocyte-derived DC was induced by exposure during the last 48 h of culture (i.e., day 6 to day 8) to TNF-α (1000 U/ml) IL-1β (500 U/ml) (R&D Systems) and PGE2a (0.5 μM/l) (Sigma). After final maturation, DC were harvested for pulsing with breast tumor lysate as described below.

*DC pulsing*

Following culture, fully mature DC 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 DC. The 500 μl of total cell extract derived from $5 \times 10^6$ to $10 \times 10^6$ tumor cells in AIM-V and DOTAP (125 μg in 500 μl of AIM-V) were mixed in 12 mm × 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 as described below.

In vitro *generation of tumor-specific CTL*

Fresh or cryopreserved responder PBL and TIL were washed and resuspended in AIM-V at $(10–20) \times 10^6$ cells/well in six-well culture plates (Costar) with tumor lysate-pulsed autologous DC (ratios from 20:1 to 30:1 responders 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. Control cultures consisted of

matched autologous populations of CD8+ T cells derived from PBL and TIL supplemented with cytokines only (i.e., in the absence of DC) and cultured *in vitro* for the same period of time. Human rIL-2 (10 U/ml) was added to the cultures every 3–4 days. After 8–10 days PBL 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 (Ortho Pharmaceutical Corp., Raritan, NJ) (0.2 μg/ml) in AIM-V plus 5% heat inactivated autologous plasma in 24-well plates (Costar) before being assayed for CTL activity. In some experiments, Dynabead-separated CD8+ T cells were restimulate once more with autologous lethally irradiated (10,000 cGy) tumor cells at a 20:1 lymphocyte/tumor ratios or tumor lysate pulsed DC before being tested in cytotoxicity assay or being further expanded with allogeneic irradiated PBL (5000 cGy) ($1 \times 10^6$ cells/well) and anti-CD3 monoclonal antibody (as described above). As negative control targets, autologous lymphoblasts were prepared by 3-day stimulation with Con-A (1 μg/ml, Invitrogen) in RPMI 1640 plus IL-2 (25 U/ml), while EBV-transformed autologous lymphoblastoid B-cell lines (LCL) were established by coculture of PBL with EBV-containing supernatant from the B95.8 cell line in the presence of 1 μg/ml cyclosporin A (Sandoz, Camberley, UK) and were maintained in AIM-V supplemented with 10% human AB serum (Gemini Bioproducts).

*Cytotoxic activity*

A standard 6 h chromium ($^{51}$Cr) release assay was performed to measure the cytotoxic reactivity of DC-tumor lysate stimulated CD8+ T lymphocytes derived from PBL and TIL. The K562 tumor cell line was used as a target for the detection of NK activity. Con-A activated peripheral blood lymphocytes and/or EBV-transformed LCL 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 effector cells and $^{51}$Cr-labeled targets were then incubated in a final volume of 200 μl/microwell at 37°C with 6% $CO_2$.

*Phenotypic analysis of T cells*

Flow cytometry was performed using fluorochrome-conjugated MAbs directed against the following human leukocyte antigens: Leu-4 (CD3, pan T cells); Leu-3 (CD4, T helper/inducer); Leu-2a (CD8, T cytotoxic/suppressor); Leu-19 (CD56, NK/K cells); Tac (CD25, the IL-2R); anti-HLA-DR (L-243); anti TcR-α/β or TcR-γ/δ (Becton Dickinson, San Jose, CA) and analyzed on a FACScan (Becton Dickinson).

*Flow cytometric analysis of intracellular cytokines*

Flow cytometric analysis of intracellular cytokine expression by stimulation with 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 ($7.5 \times 10^5$/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. The 10 μg/ml Brefeldin A was added for the final 3 h of incubation. Controls (non-activated 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+ cytotoxic 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 three assays for each patient. CD8+ T cell cytotoxicity against autologous tumor cell targets was demonstrated for all three patients, ranging from 22 to 38%, mean 30% (PBL) and from 39 to

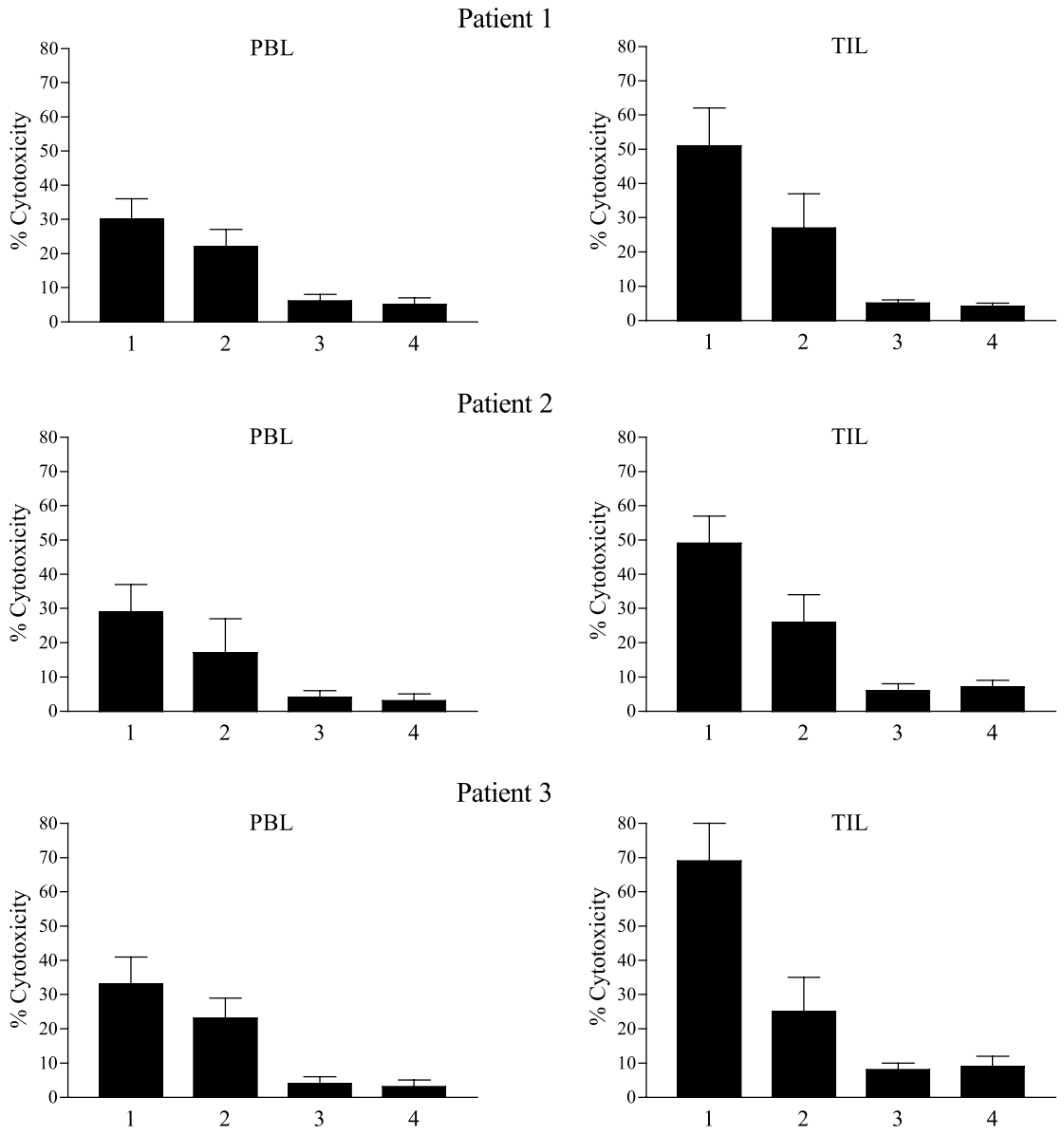


*Figure 1.* Tumor-specific CD8+ CTL responses induced in PBL and TIL by tumor lysate-pulsed DC in three patients with advanced breast cancer, measured in a 6 h $^{51}$Cr-release assay. Percentage lysis (±standard deviation) at a 20:1 effector/target cell ratio is shown. Anti-HLA class I blocking antibody (W6/32) and isotype control MAb (data not shown) were used at 50 µg/ml. Patients 1–3; 1, autologous tumor; 2, autologous tumor + W6/32 anti-class I MAb; 3, LCL control; 4, K562.

64%, mean 51% (TIL) (PT1), from 20 to 35%, mean 29% (PBL) and from 41 to 57%, mean 49% (TIL) (PT2), and from 23 to 42%, mean 51% (PBL) and from 58 to 80%, mean 69% (TIL) (PT3) at 20 effectors per target. In contrast, pure populations of CD8+ T cells isolated from TIL cultured in low amounts of IL-2 (i.e., not stimulated with DC) showed no cytotoxicity against autologous tumor targets (data not shown). DC-stimulated CD8+ T cell cytotoxicity against the NK-sensitive cell line K562 was detectable at a low level in all patients. The absence of significant cytotoxicity against autologous EBV-transformed LCL (Figure 1) 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 tumor-specific lytic activity against autologous tumor targets was significantly inhibited by pre-treatment

of tumor targets with MAb specific for HLA class I (W6/32) (range of inhibition: from 16 to 52% (PBL) and from 41 to 85% (TIL)) (Figure 1) ($p < 0.05$ by Student's *t*-test for all three patients) but not with pre-treatment by isotype controls (data not shown).

### *Phenotypic analysis of lymphocytes from peripheral blood and tumor tissue*

Flow cytometric analysis was used to characterize the phenotype of the populations of lymphocytes obtained from PBL and TIL before and after the *in vitro* stimulation with autologous tumor antigen-loaded DC. The proportions of CD3+, CD8+ and CD4+ T cells in PBL and TIL from the three breast cancer patients evaluated in this study before DC activation are described in Table 1. CD3+ T cells were the major lymphocyte population in each tissue, with the percentage of CD4+ T cells greater than the percentage of CD8+ T cells in PBL ($p < 0.01$) (Table 1). In contrast, in all three patients, TIL contained a similar number of CD4+ and CD8+ T cells (Table 1). The PBL had a CD4+ to CD8+ ratio of 2:1, while TIL showed a ratio of 1:1 (Table 1). Starting lymphocyte populations were also different in the percentage of CD56 and CD19 positive lymphocytes, with PBL containing more CD56+ and CD19+ cells compared to TIL ($p < 0.05$ and $p < 0.02$ for CD56 and CD19, respectively). The expression of CD56 on T lymphocytes was further analyzed by two-color immunofluorescence. By this technique, the CD8+ T cells were compared for co-expression of CD56. PBL contained more CD8+/CD56+ T cells compared to TIL (Table 1, $p < 0.05$).

After 21–28 days' culture of autologous tumor antigen-pulsed DC-stimulated PBL and TIL, the striking majority of cells were found to be TcR-αβ+/CD3+ T cells (90–97%). PBL contained more CD4+ T cells (range from 58 to 89%) than CD8+ T cells. In contrast, in the TIL populations derived from all three patients, CD8+ T cells (range from 51 to 71%) were predominant over CD4+ T cells. Enriched

*Table 1.* Subpopulations of mononuclear cells in PBL and TIL from breast cancer patients[a]

| Specific MAb | Percentage of Phenotype | | | | | |
|---|---|---|---|---|---|---|
| | Patient 1 | | Patient 2 | | Patient 3 | |
| | PBL | TIL | PBL | TIL | PBL | TIL |
| CD3 | 75 | 52 | 68 | 49 | 67 | 50 |
| CD4 | 50 | 25 | 46 | 23 | 44 | 26 |
| CD8 | 25 | 27 | 22 | 25 | 23 | 24 |
| CD19 | 11 | 2 | 14 | 3 | 10 | 2 |
| CD56 | 9 | 3 | 16 | 2 | 8 | 3 |
| CD8/CD56 | 6 | 2 | 8 | 2 | 5 | 1 |
| CD4/CD8 ratio | 2.0 | 0.9 | 2.1 | 0.9 | 1.9 | 1.1 |

[a] Values are percentage of PBL and TIL that are positive for specific markers used in the cell staining before stimulation with tumor lysate-pulsed autologous dendritic cells.

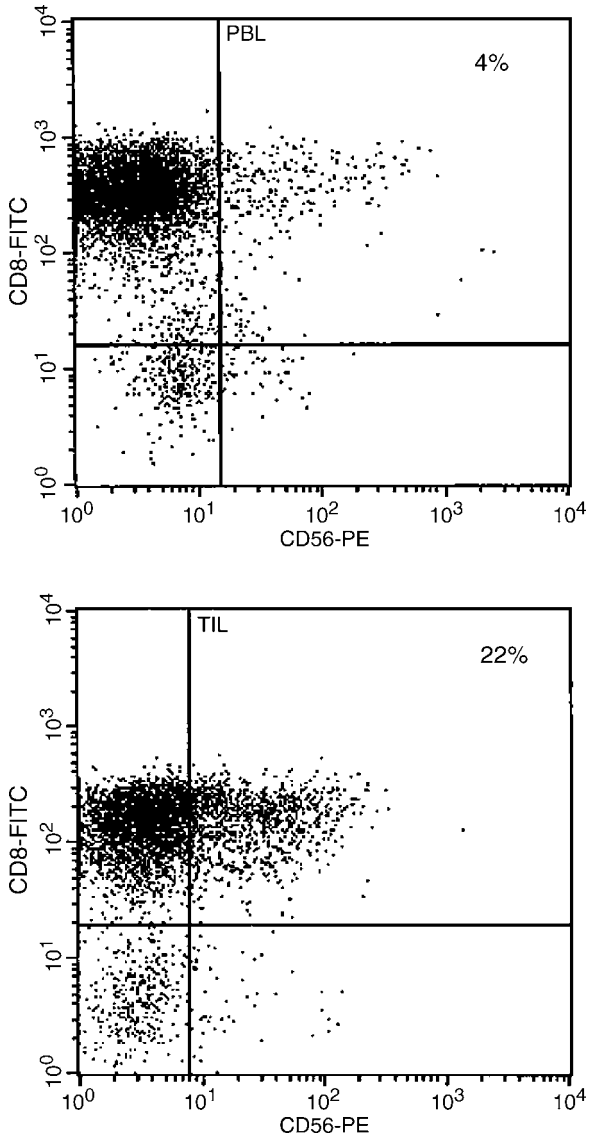


*Figure 2.* Two-color flow cytometric analysis of CD56 expression by breast tumor-specific CD8+ T cells in PBL and TIL from a representative patient after *in vitro* stimulation with tumor antigen-loaded DC.

populations of CD8+ T cells were isolated at this time, and analyzed for expression of CD56 after a further two or more weeks of culture. A variable proportion of CD8/CD56-antigen positive cells was detected in all CD8+ T cells derived from PBL and TIL. However, as representatively shown for patient 1 (Figure 2), autologous tumor antigen-DC-stimulated TIL consistently expressed a higher percentage of CD8+/CD56+ T cells compared to PBL ($p < 0.01$). Further analysis revealed the populations of CD8+ T cells from PBL and TIL to be CD25+, HLA-DR+ and CD16− (data not shown).

### *Two-color flow cytometric analysis of intracellular IFN-γ and IL-4 expression by tumor-specific T cells*

To determine the pattern of Type 1 and Type 2 cytokine expression in the populations of CD8+ T lymphocytes collected from PBL and TIL, two-color flow cytometric analysis of intracellular IFN-γ or IL-4 expression by CD8+ T cells was performed before and after *in vitro* culture with DC-tumor lysate-loaded autologous DC. As described in Table 2, at the beginning of culture, significantly more CD8+ T cells from TIL expressed IFN-γ than PBL-derived CD8+ T cells following PMA and ionomycin activation ($p < 0.05$). In contrast, IL-4 expression was found at low, but significantly higher levels in CD8+ T cells from PBL ($p < 0.05$) when compared to TIL (Table 2). When pure populations of CD8+ T cells were tested after stimulation with tumor lysate-loaded DC and further *in vitro* expansion, significantly higher numbers of CD8+ T cells from TIL were found to contain intracellular IFN-γ compared to CD8+ T cells derived from PBL (Table 2 and Figure 3) ($p < 0.05$). IL-4 expression was again significantly higher in CD8+ T cells derived from PBL when compared to IL-4 expression by CD8+ T cells obtained from TIL with higher numbers of double positive (i.e., IFN-γ+/IL-4+) CD8+ T cells from PBL (Figure 3) ($p < 0.05$). Similar results were consistently obtained in several repetitive analyses for all patients. Unactivated (i.e., resting) CD8+ T cells failed to stain for IFN-γ or IL-4, and similarly, FITC-anti-IgG2a and PE-anti-IgG1 isotype

*Table 2.* Percentage of IFN-γ and IL-4 positive CD8+ T cells from PBL and TIL derived from breast cancer patients[a]

| | Patient 1 | | Patient 2 | | Patient 3 | |
|---|---|---|---|---|---|---|
| | IFN-γ | IL-4 | IFN-γ | IL-4 | IFN-γ | IL-4 |
| PBL | 11 | 4 | 19 | 3 | 14 | 3 |
| TIL | 57 | 0.5 | 52 | 1 | 76 | 0.5 |

[a] Percentages of IFN-γ and IL-4 positive CD8+ T cells from PBL and TIL before *in vitro* stimulation with tumor lysate-pulsed autologous dendritic cells. The difference in IFN-γ expression in PBL versus TIL before DC-stimulation was significant at $p > 0.05$. The difference in IL-4 expression in PBL versus TIL before DC-stimulation was significant at $p > 0.05$.

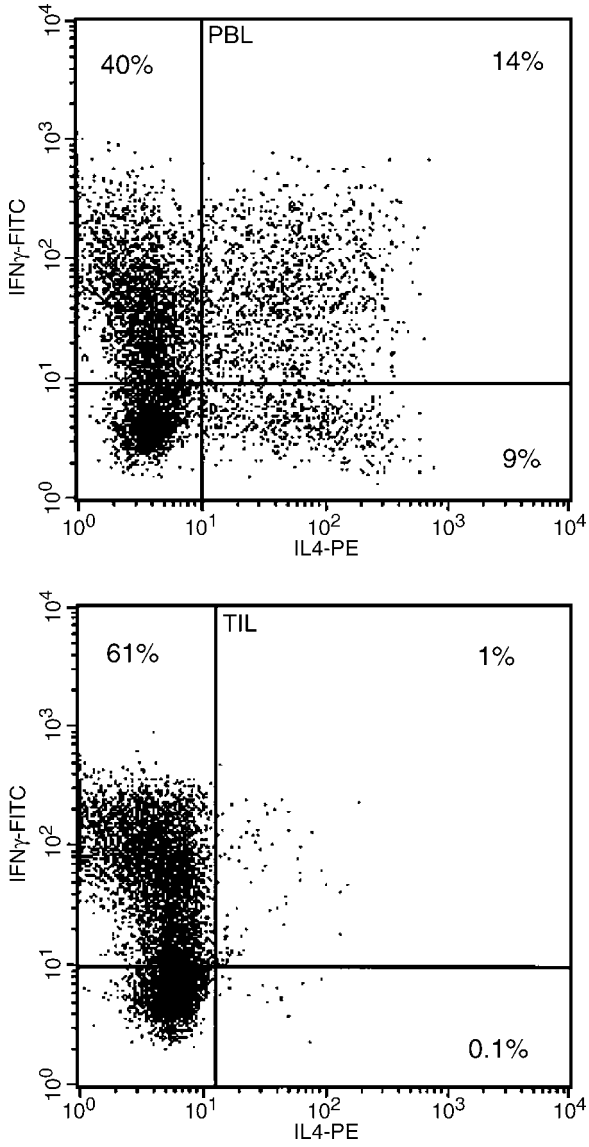


*Figure 3.* Representative dot plot analysis of intracellular IFN-γ and IL-4 expression by CD8+ T cells from PBL and TIL for an individual patient after *in vitro* culture with tumor antigen-loaded DC. Lymphocytes were activated by PMA and ionomycin as described in the Methods Section. Numbers in the quadrants represent the percentage of CD8+ cytokine positive T lymphocytes.

controls did not stain either activated or unactivated CD8+ T cells (data not shown).

## Discussion

DC play a crucial role during the priming and reactivation of antigen-specific immune responses [6–10]. This unique function combined with the recent standardization of *in vitro* DC culture conditions may offer the opportunity to test in large groups of cancer patients refractory to standard treatment modalities the potential of DC-based immunotherapy. In this regard, the use of mature versus immature DC may be critical for their optimal use in immunization strategies [11–15]. Indeed, recent clinical studies have shown that monocyte-derived mature DC may rapidly generate broad T cell immunity in healthy subjects vaccinated with less than $3 \times 10^6$ antigen-pulsed autologous DC [13, 14]. In contrast, the administration of immature DC has been shown to result in inhibition of preexisting effector T cell function [15] with experiments also providing direct evidence that antigen loaded immature DC may silence T cells, either by peripheral deletion, or expansion of regulatory T cells [28]. Several human phase I/II trials have been initiated using tumor antigen pulsed DC, and the induction of antitumor immune responses and therapeutic benefit in the absence of toxicity has been consistently observed in at least a proportion of patients [29–34]. However, it has become apparent that many late stage cancer patients may not be eligible for active immunization protocols because of either profound treatment-induced immunosuppression or tumor-induced subversion of the immune system [35–37]. Adoptive transfusions of tumor-specific *in vitro* activated T cells, which avoid the potential problems associated with inducing a CTL response *in vivo*, might therefore be a more effective approach for control of tumor growth in patients harboring advanced stage breast cancer.

Although the identity and distribution of antigenic peptides and CTL epitopes presented by freshly isolated breast tumors is still poorly known, recent studies have unequivocally shown that multiple tumor antigens do exist that can be recognized by CTLs and used as target molecules to induce autologous tumor cell killing *in vitro* [38–41]. In addition, several studies in breast and other cancers support the notion that the accumulation of lymphocytes in tumor tissue represents a specific immune response against tumor cells [16–24]. In agreement with this view, T lymphocytes infiltrating human solid tumors represent an enriched population of antigen-experienced CD8+ T cells which have been previously demonstrated to be endowed with 50–100 times stronger antitumor activity compare to *in vitro* activated PBL [42]. TIL could therefore represent a superior source of tumor-specific CTL to be used in adoptive immunotherapy for breast cancer patients. Unfortunately, several reports have shown that T cells derived from tumor tissue are commonly defective in several immunologic functions including a persistent lack of cytotoxicity when challenged against autologous or allogeneic tumor cells [16–24]. The reason why TIL become anergic *in vivo* is not well understood. However, tolerogenic immature DC endowed with low or absent costimulatory activity and the ability to secrete high levels of immunosuppressive cytokines (i.e., IL-10 and TGF-β) as well as the presence of regulatory T cells able to mitigate the immune response against breast cancer cells have been recently identified in the peripheral blood and tumor microenvironment of breast cancer patients [36, 37, 43, 44].

In this study we have carefully analyzed the ability of fully mature DC to restore tumor-specific lytic activity in TIL populations from advanced breast cancer patients. In addition, as a basis for the development of more effective immunotherapy protocols for the treatment of chemotherapy resistant disease, we have performed a comparative phenotypic and functional analysis of lymphocytes derived from PBL and TIL before and after *in vitro* stimulation with tumor antigen-pulsed autologous mature DC. We found TIL to be a consistently superior source of tumor-specific CTL compared to PBL. Indeed, although not cytotoxic immediately after collection or when maintained in low levels of IL-2 (data not shown), suggesting that a state of anergy exists *in vivo*, induction of higher cytotoxic activity against autologous breast tumor cells was consistently noted in TIL populations from all three patients studied when compared to CD8+ T cells derived from PBL. The lack of significant cytotoxicity against HLA-identical autologous LCL confirmed that, although TIL-derived CTL were highly cytolytic against autologous tumor cells, they failed to kill autologous control target cells expressing different antigens. Furthermore, the specificity of the TIL-derived populations of CTL was also confirmed by the significantly higher block in cytotoxic activity detected using anti HLA class I MAb when compared to PBL-derived CTL, and by their low cytotoxic activity against K562 NK sensitive target cells.

It is worth noting that while CD3+ T cells were the major lymphocyte population detected in both tissues, lymphocytes used at the time of *in vitro* stimulation with tumor antigen-pulsed DC were predominantly CD4+ T cells in PBL but not in TIL (CD4:CD8 ratio 2:1 v.s. 1:1, respectively). After *in vitro* restimulation with antigen loaded fully mature DC, CD4+ T cells remained predominant in PBL cultures, while in TIL cultures a predominant population of CD8+ T cells was consistently noted. CD56+/CD16+/CD3− NK cells and CD8+/CD56+ T cells were predominant in PBL compared to TIL at the beginning of culture. However, after *in vitro* stimulation and expansion of CD8+ T cells, TIL contained a significantly higher percentage of CD8+/CD56+ T cells compared to PBL. These results were found to correlate with a significantly higher cytotoxic activity in the CD8+ CTL populations derived from TIL compared to those derived from PBL. Taken all together these data support the view that, as previously described by us [25, 26] as well as others for CD8+ T cells derived from peripheral blood [45], CD56 expression on CD8+ T cells derived from TIL is an activation antigen associated with higher 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 [46]. 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 [47]. Recent studies, however, have shown significant dysfunction of Type 1 T cell responses in tumor bearing hosts [48, 49], 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-$\gamma$ 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 expressors in both the PBL and TIL derived CD8+ CTL populations. Indeed, IL-4+ T cells were detected in low numbers and mainly in PBL. These data, therefore, suggest that this approach is able, at least *in vitro*, to stimulate IFN-$\gamma$ production in CD8+ T cell populations derived from TIL as well as to induce the differentiation from PBL of Type 1 CD8+ T cells secreting IFN-$\gamma$ and endowed with cytotoxic activity against autologous tumor cells. More importantly, these results suggest that the anergy commonly seen in TIL population derived from breast cancer patients [17, 21] may be reversible when CD8+ T cells are removed from the tolerizing environment and repetitively stimulated *in vitro* with fully mature tumor antigen-pulsed-DC in the presence of low amount of IL-2. Finally, these data suggest that the lack of *in vitro* cytotoxic activity commonly detected in fresh TIL populations is not secondary to a Th2 cytokine switch.

In conclusion, although poor immune responses by explanted T lymphocytes might be expected, it seems possible to normalize *in vivo* defective immunologic responses against breast tumors by *in vitro* T cell stimulation with professional APC such as fully mature DC. These results, combined with the evidence that large numbers of late stage and heavily pretreated cancer patients are severely immunocompromised, and that lymphocytes derived from TIL represent a superior source of tumor-specific HLA class I-restricted CTL compared to PBL, further support the use of TIL in adoptive cellular immunotherapy of breast cancer patients harboring disease refractory to standard treatment modalities. The future design and implementation of clinical trials will ultimately determine the validity of this approach.

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