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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
The overall objective of this project is to study the safety, immunologic response and clinical effect of vaccinating breast cancer patients with dendritic cell (DC)/tumor fusions in conjunction with IL-12. The hypothesis underlying these studies is that tumor specific immunity can be generated by the presentation of breast tumor antigens in the context of the potent antigen presenting machinery of the DC. Task 1 of the approved Statement of Work is to assess fusions of human breast carcinoma cells with autologous DC by evaluating cytokine production (IL-12, IL-10) and potency of the fusions in generating tumor specific immunity in vitro (Months 1-12). Our results demonstrate that fusions of breast cancer cells with immature or mature DC are associated with induction of phenotypic characteristics consistent with DC maturation and activation. The fusion cells express IL-12, IL-10 and the chemokine receptor CCR7. The breast tumor/DC fusions were effective in stimulating i) autologous T cell proliferation, ii) cytokine production and iii) tumor-specific T cell responses. Based on these findings, our plan is to now perform the Phase I/II clinical trial of breast cancer cell/DC fusions with IL-12 as a vaccine for patients with metastatic breast cancer (Tasks 2-4; Protocol included in Appendix A).

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INTRODUCTION

The overall objective of the project is to study the safety, immunologic response, and clinical effect of vaccination with dendritic cell (DC)/tumor fusions in conjunction with IL-12 in patients with breast cancer. The hypothesis underlying the proposed study is that tumor specific immunity can be generated by the presentation of tumor antigen(s) in the context of the potent antigen presenting machinery of the DC. The generation of DC/tumor fusions allows for the simultaneous presentation of multiple tumor antigens, including those that have yet to be identified, in the context of co-stimulatory signals that are necessary for the activation of primary immune responses. In a clinical study, patients with breast cancer underwent vaccination with breast carcinoma cells that were fused with autologous partially mature DC generated with GM-CSF and IL-4. Vaccination was well tolerated and generated anti-tumor immunity and disease regression in a subset of patients.

We have subsequently focused on strategies to further enhance the efficacy of the fusion vaccine. Our studies have included exploring the role of cytokine adjuvants and optimizing the nature of the DC undergoing cell fusion. IL-12 plays a central role in the development of an TH1 responses and has been shown to augment responses to dendritic cell-based cancer vaccines. In animal models, administration of IL-12 in conjunction with the DC/tumor fusion vaccine significantly increased tumor specific CTL responses and resulted in durable regression of metastatic disease.

The optimal stage of DC maturation for generating tumor vaccines has not been defined and is likely dependent on the vaccination strategy. An effective DC-based vaccine requires the capacity to process and present tumor antigens, potency in stimulating T cell responses, stability of phenotype following in vivo administration, and the ability to migrate to sites of T cell activation. Vaccination with peptide pulsed mature DC results in antigen specific immunity while immature DC may induce inhibition of the immune response. In addition, tumor cells may express IL-10 that inhibits DC maturation and may blunt the capacity of fusion cells to induce tumor specific immunity. However, antigen-loading strategies that require internalization and cell processing are

optimized by the use of a partially mature DC that then undergo maturation. Animal studies have demonstrated that antigen loading with necrotic or apoptotic tumor cells may induce maturation and activation of the DC populations. The impact of cell fusion on DC maturation and the optimal stage of DC development for fusion cell-induced T cell responses has not been defined.

Task 1 in the approved Statement of Work is to assess fusions of human breast carcinoma cells with mature DC by evaluating cytokine production (IL-12, IL-10) and potency of the fusions in generating tumor specific immunity in vitro (Months 1-12). From work performed in Task 1, we defined the effects of DC maturation on the phenotypic and functional characteristics of the fusion cell vaccine. We examined the antigen presenting properties of the fusion cell preparations with regard to expression of costimulatory and maturation markers, IL-12, and the chemokine receptor, CCR7. We also studied their capacity to induce autologous T cell proliferation, to induce expression of TH1 and TH2 cytokines in responding T cell populations and to stimulate T cells to lyse autologous and semi-autologous tumor targets.

BODY

Preparation of breast cancer cells, immature DC and mature DC. Tumor specimens were obtained from breast cancer patients undergoing procedures for independent diagnostic or therapeutic indications as per a protocol approved by the hospital institutional review board for clinical investigation. Tumor cells were isolated from primary or metastatic lesions as well as malignant effusions. When necessary, tumor tissue was subjected to mechanical disruption, filtering, and then digestion with collagenase to generate a single cell suspension. Tumor cells were cultured in RPMI 1640 medium containing L-glutamine, gentamycin, regular human insulin (5 μg/ml) and 10% autologous plasma. In some cases, longer term cultures were established from patient-derived specimens. Tumor cells were found to express cytokeratin and/or MUC-1, but lacked expression of costimulatory molecules.

DC expressing an immature or mature phenotype were generated from peripheral blood progenitors obtained from patients with breast cancer (autologous) and from leukopak preparations acquired from volunteer donors (allogeneic). Adherent mononuclear cells were isolated and cultured in GM-CSF (1000U/ml) and IL-4 (1000U/ml) for 7 days. An aliquot of cells were subsequently cultured for 2 days with TNFα (25 ng/ml). Cells were harvested for phenotypic analysis. Following culture for 1 week with GM-CSF and IL-4, the resultant cell population demonstrated an immature DC phenotype. Maturation was effectively induced by exposure to TNFα and was associated with increased expression of CD80, CD86 and CD83 (Figure 1A). In 15 serial experiments, immature and mature DC prominently expressed CD86 (75% and 84%, respectively) (Figure 1B). Low levels of CD14 expression were observed for both DC populations. In contrast, mature DC demonstrated a statistically significant increase in mean expression of CD80 (20% vs. 9%, $p = 0.05$) and CD83 (31% vs. 7% $p=0.0003$). Maturation with TNFα also resulted in increased expression of CCR7. As a measure of their functional capacity as antigen presenting cells, DC preparations were examined for their ability to stimulate allogeneic T cell proliferation. In serial studies, mature as compared to immature DC demonstrated increased mean levels of allogeneic T cell proliferation. At a DC:T cell ration of 1:30, the mean T cell stimulation index was 12.4 and 22.9 for immature and mature DC, respectively.

Phenotype of immature and mature DC/tumor fusions. We subsequently examined the phenotypic characteristics of the fusion cell populations generated with immature and

mature DC. Expression of co-stimulatory and DC maturation markers was assessed. Immature and mature DC populations were fused with primary patient-derived breast cancer cells or the MCF-7 human breast carcinoma cell line by co-culture with PEG. Fusion cells were quanitified by determining the percentage of cells that co-expressed unique tumor (MUC-1 and/or cytokeratin) and DC (CD11c) antigens. In 12 serial studies, equivalent mean fusion efficiencies were observed following fusion of breast tumor cells with mature (11%) and immature (7%) DC. Fusion cells were isolated by FACS gating around cells that co-expressed DC and tumor derived antigens. Expression of CD86 was uniformly observed in both immature DC/breast carcinoma (mean 89%) and mature DC/breast carcinoma (mean 82%) fusion populations (Figure 2A, B). In 11 serial studies, the maturation marker, CD83, was detected in 46% and 51% of immature and mature DC/breast tumor fusion cell populations, respectively (p=0.5, NS) (Figure 2A, B). Immunocytochemical staining demonstrated prominent expression of DR, CD86 and CD83 by the immature and mature DC/tumor fusions (Figure 2C-H). These studies demonstrate that fusion of DC and breast carcinoma cells results in phenotypic characteristics consistent with maturation and activation.

Expression of IL-12 and IL-10 by immature and mature DC/tumor fusions. As a measure of their potency as antigen presenting cells and their capacity to stimulate TH1 and TH2 responses, we next examined intracellular expression of IL-12 and IL-10 by the fusion cell populations (Figure 3). Fusion cell preparations were labeled with CD11c and cytokeratin (CT) or MUC-1 and subsequently fixed, permeabilized and stained for IL-12 or IL-10. Fusion cells were isolated by FACS gating of cells that co-expressed DC and tumor derived antigens and analyzed for IL-10 and IL-12 expression. Fusion cells generated with immature and mature DC and breast cancer cells were compared in 12 separate experiments. The mean percentage of fusion cells that express IL-12 and IL-10 did not differ between the fusion cell populations. IL-12 was expressed by 40% and 49% of the immature and mature DC/breast carcinoma fusions, respectively (p= 0.35,

NS). IL-10 was expressed by 36% and 40% of the immature and mature DC/breast carcinoma fusion populations (p=0.7 NS).

Expression of CCR7 by immature and mature DC/tumor fusions. As a measure of migratory capacity, expression of CCR7 was determined for fusions generated with immature and mature DC (Figure 4). CCR7 was prominently expressed on both immature and mature fusion populations, suggesting that tumor-DC fusion resulted in the expression of an activated phenotype with the capacity to migrate to draining lymph nodes. In 10 serial experiments, mean CCR7 expression was observed in 33% and 38% of the immature and mature DC/breast cancer fusions, respectively.

Stimulation of autologous T cell proliferation and cytokine production by immature and mature DC fusions. The functional capabilities of mature and immature DC/tumor fusion populations were analyzed by assessing their abilities to stimulate T cell proliferation and cytokine production. Fusion cell populations were co-cultured with autologous T cells for 5 days and proliferation was determined by measuring uptake of tritiated thymidine after overnight pulsing. Proliferation was measured as the T cell stimulation index (SI)(Fusion stimulated T cells/T cells alone). In 6 serial experiments, both immature and mature DC/breast cancer fusions stimulated autologous T cell proliferation with mean SIs of 3.3 and 3.5, respectively (Figure 5). Stimulated T cell populations were analyzed by intracellular flow cytometry for expression of IFNγ, IL-2, IL-10, IL-4, and TNFα (Figure 6A,B). Expression of intracellular cytokines did not differ in T cell populations stimulated by immature and mature DC/breast cancer fusions. We also examined the percentage of regulatory T cells in the stimulated cultures. No differences were seen in the percent CD4+/CD25+ populations following stimulation with immature and mature DC/breast cancer fusions.

We also quantified cytokine production in supernatants of stimulated T cell populations using the BD cytometrix array bead system (BD Biosciences) (Figure 7). Mean levels of IFNg following stimulation with immature and mature DC/breast cancer fusions was 2188 and 2252 pg/ml, respectively. These levels were greater than that seen with T cells cultured with unfused autologous DC (685 pg/ml). Fusion cell preparations did not induce an increase in IL-12, IL-4, IL-10, IL-2 and TNFα production.

Stimulation of tumor specific CTL responses. Both immature and mature DC/tumor populations were capable of generating significant levels of target specific killing, as demonstrated by the lysis of autologous tumor or semi-autologous fusion targets. In 10 separate experiments, CTL activity did not differ between the fusion populations (Figure 8). Mean CTL lysis for effector:T cell ratio of 30:1 was 27% for T cell stimulated with mature and immature DC/breast cancer fusions.

KEY RESEARCH ACCOMPLISHMENTS

We have demonstrated that DC/breast tumor fusion results in the maturation and activation of the DC fusion partner. This effect of fusing DC with breast cancer cells is manifested by the increased expression of costimulatory and maturation markers, and IL-12. We have also found that 1) Fusion cells have the potential for migratory capacity as manifested by the prominent expression of CCR7; 2) Fusion cells stimulate autologous T cell proliferation and production of IFNα; and 3) Immature and mature DC/breast cancer fusions effectively stimulate CTL responses directed against tumor targets.

REPORTABLE OUTCOMES

The results obtained from work performed in Task 1 are being integrated into a manuscript that will be submitted for publication.

CONCLUSIONS

In the subsequent aims of the project, we will conduct a clinical trial that examines the safety, immunologic response and clinical effects of vaccination with the DC/breast cancer fusion vaccine in conjunction with IL-12. We have completed preparation of a clinical protocol that has been filed under our IND with the FDA. In addition, a CMC section was prepared that outlines the methods for DC preparation, vaccine characterization, quality assurance, microbiological studies, and immunologic analyses.

We have submitted the study and informed consent to the Dana Farber/Harvard Cancer Institute scientific review committee and institutional review board. After responding to their comments, we received approval to initiate the study. We have also submitted the study to CTEP at the National Cancer Institute, who will be providing IL-12. After responding to their critique, we received approval to move forward with the protocol. Finally, we submitted the study to the Department of Defense Medical Research and Material Command Human Subjects Research Review Board. Upon receipt of their comments, we resubmitted a revised protocol and revised consent for review. As a part of this process the protocol was extensively revised and included updated information from the recently completed DC fusion and peptide vaccine trials. Toxicity, clinical and immunologic data from these trials underwent summary analysis for this purpose.

Figure 1

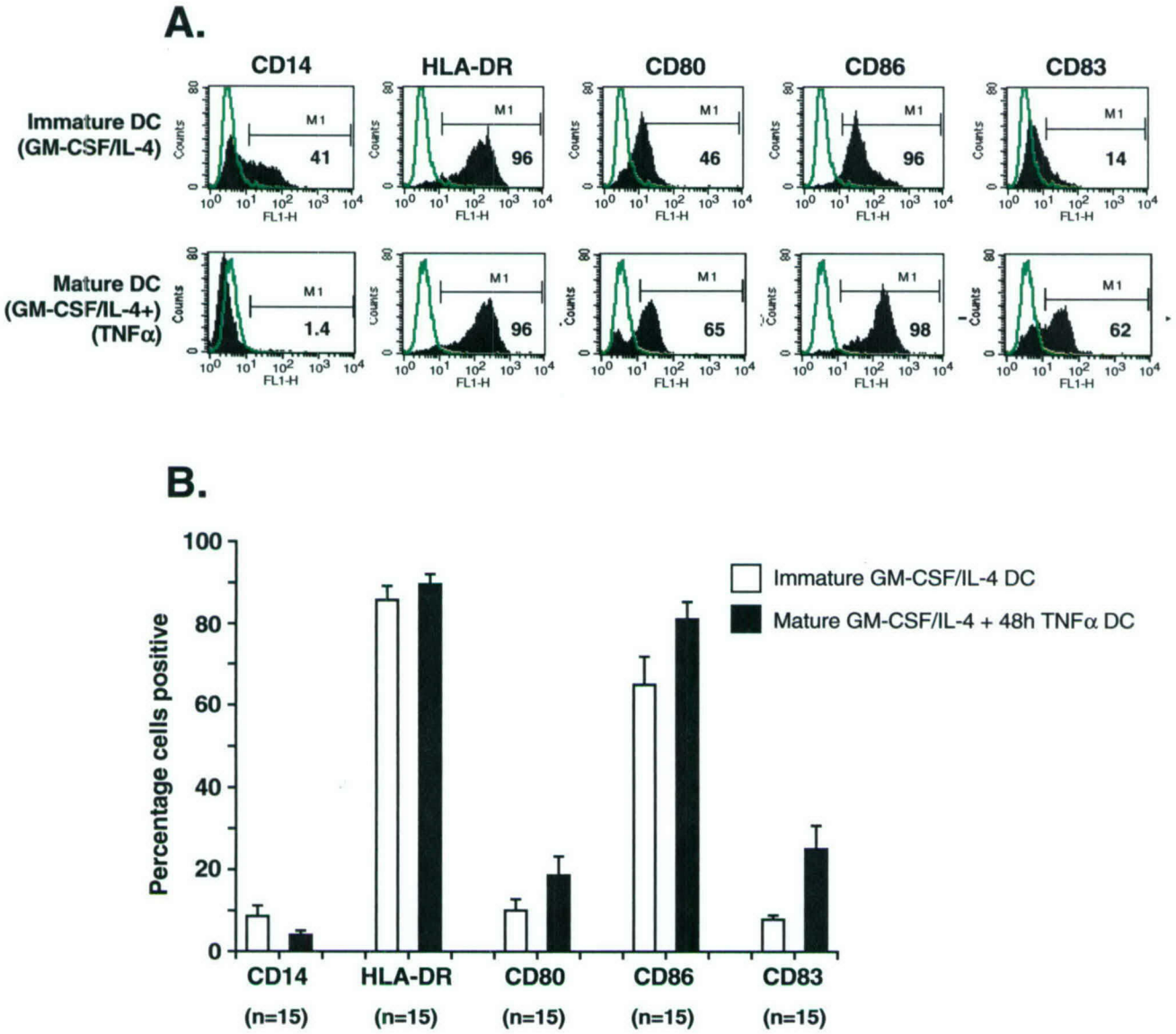
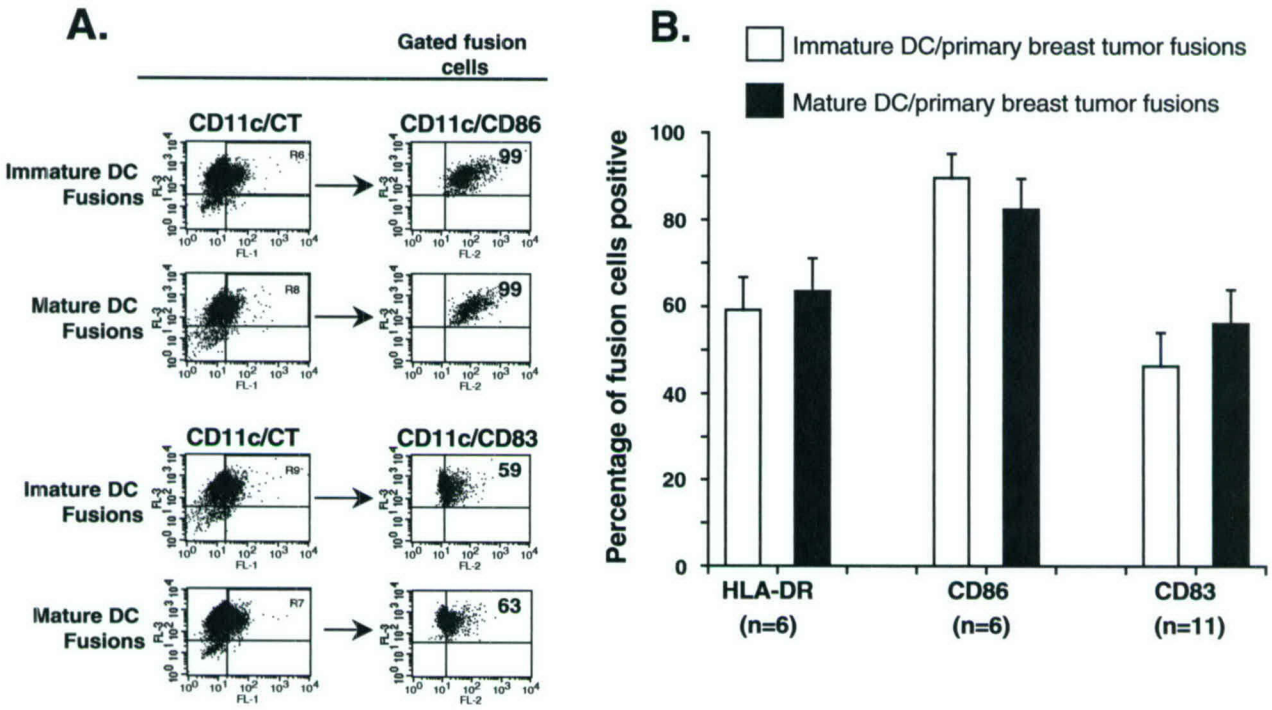


Fig 1. Generation and phenotypic analysis of monocyte derived dendritic cells (DC). DC were prepared from peripheral blood mononuclear cells by plastic adherence of monocytes that were cultured in GM-CSF (1000U/ml) and IL-4 (1000U/ml) for 7 days and subjected to FACS analysis (**A**; upper panel). DC maturation was induced by adding TNFα (25 ηg/ml) to the DC cultures on day 5 and subjected to FACS analysis 48 h later (**A**; lower panel). In 15 separate experiments the mean (± SEM) percentage of cells expressing the appropriate surface markers is shown (**B**).

Figure 2



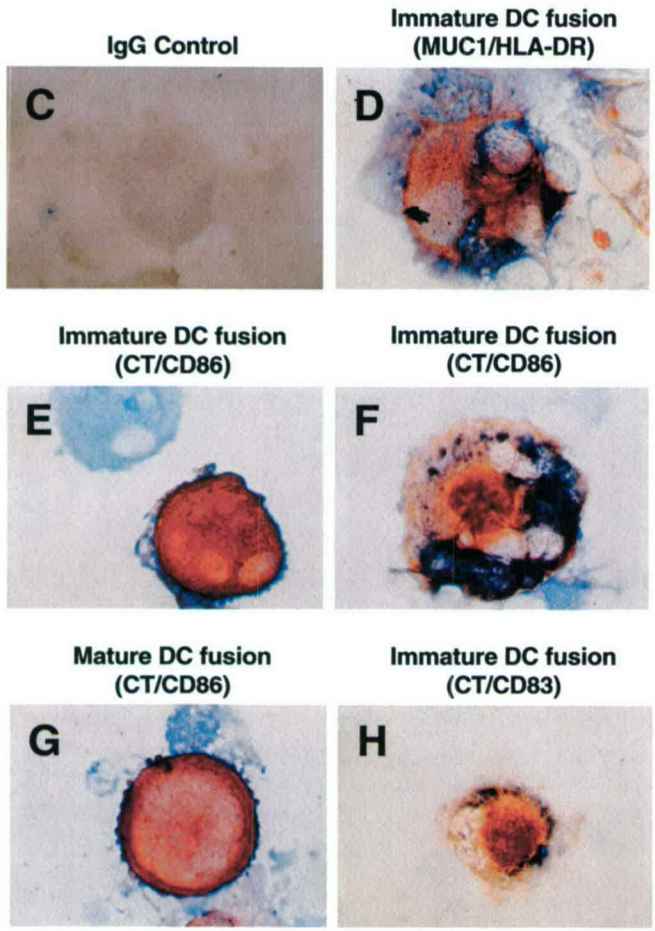


Fig 2. DC-Breast Tumor fusions. Tumor cells were fused with immature or mature DC preparations and analyzed by bidimensional FACS analysis for Cytokeratin (CT) and CD11c (**A**, left). The dual positive fusion cells were gated and analyzed for expression of HLA-DR, CD86 and CD83 (A, right) and the mean percentage (±SEM) of 6-11 separate experiments is shown (**B**). **C-H**. Immunohistochemical analysis of DC-tumor fusion cells was performed following cytospin preparation. The fusion cells were stained for Isotype matched IgG control (**C**). Immature DC fusions cells were stained for MUC1/HLA-DR (**D**), CT/CD86 (**E** and **F**) and for CT/CD83 (**H**). An example of mature DC fusion stained for CT/CD86 Is shown (**G**).

Figure 3

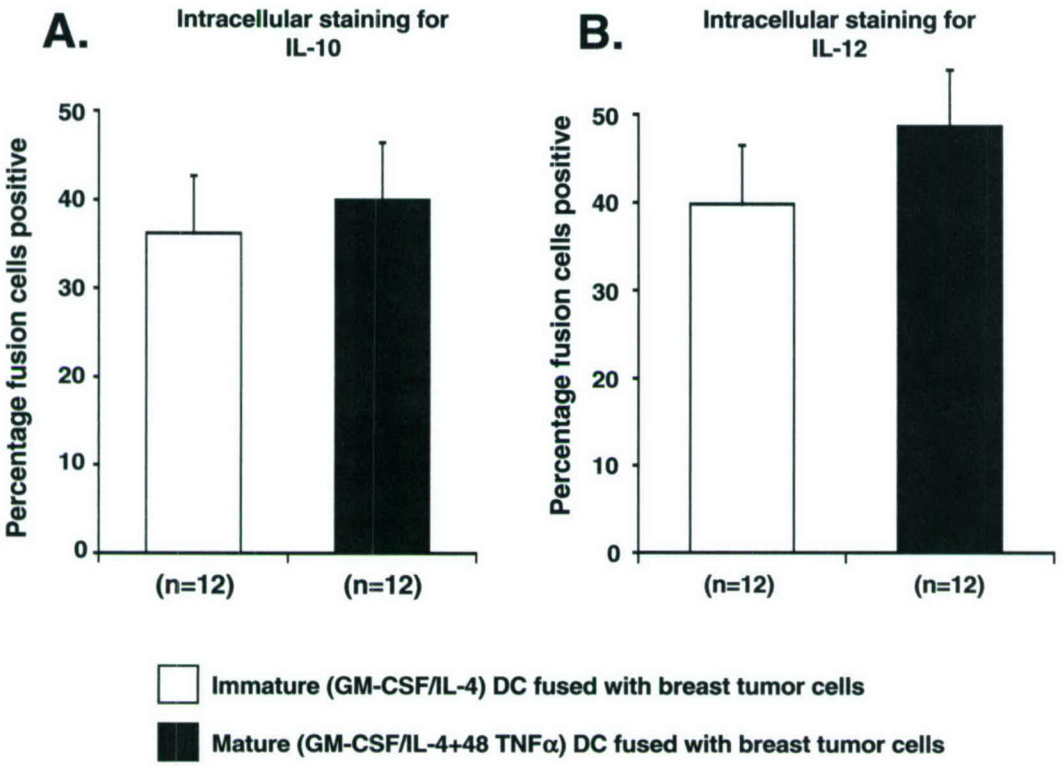


Fig 3. Expression of IL-10 and IL-12 in DC-Tumor fusion cells prepared using immature or mature DC. Fusion cells were stained with CT and CD11c and subsequently fixed, permeabilized, and stained for intracellular IL-10 or IL-12. Fusion cells co-expressing CT or MUC1 and CD11c were gated and analyzed for the expression of IL-10 and IL-12. In 12 separate experiments, the mean percentage (±SEM) of immature and mature DC fusion cells expressing IL-10 (**A**) and IL-12 (**B**) is shown.

Figure 4

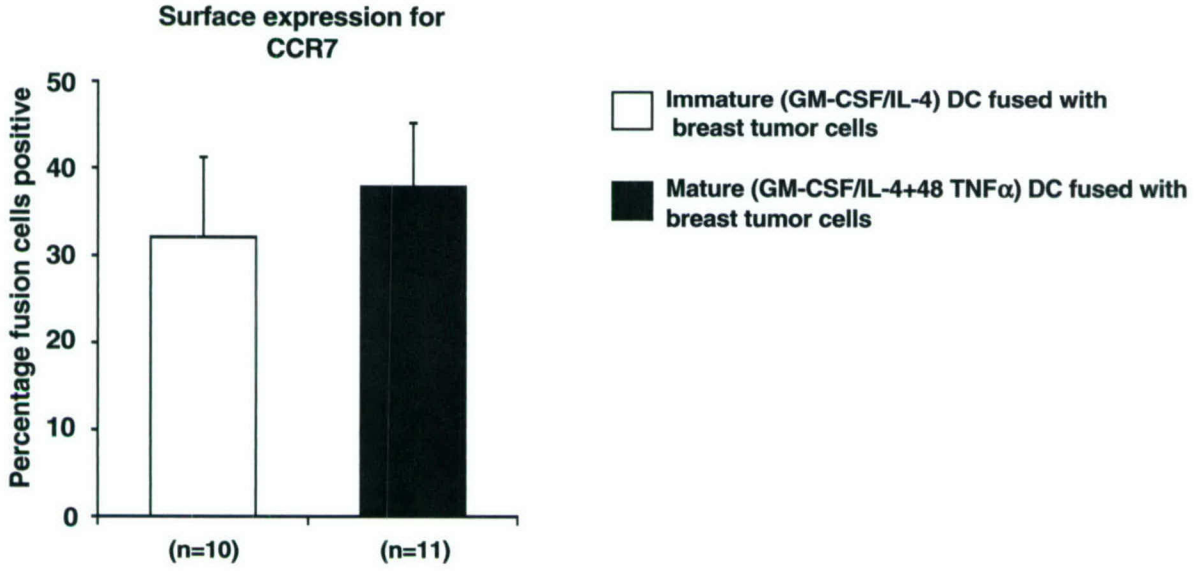


Fig 4. Expression of CCR7 on fusion cells prepared with either immature or mature DC.
Immature and mature DC fusion cells were labeled with CT or MUC1, CD11c, and CCR7. Fusion cells co-expressing CD11c and CT or MUC-1 were gated and analyzed for the expression of CCR7.

Figure 5

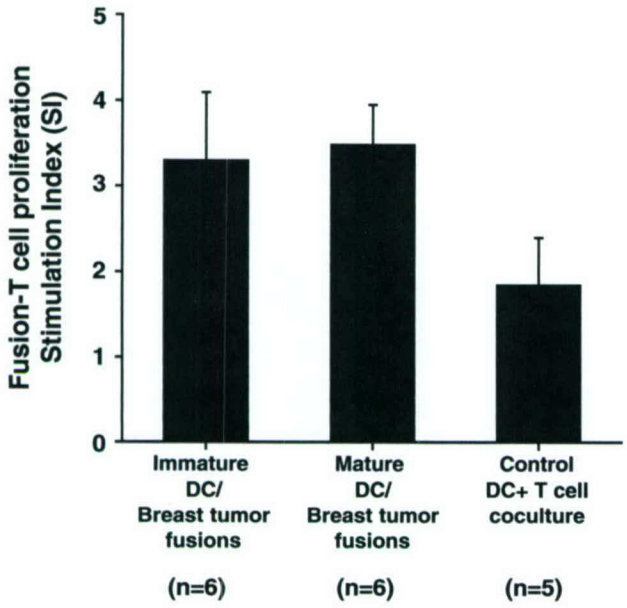


Fig 5. Induction of T cell proliferation by fusion cells prepared with immature or mature DC. Fusion cells were co-cultured with T cell and proliferation was measured by 3[H]-Thymidine uptake. Results were normalized by calculation of the stimulation index (SI).

Legends for Figure 6 and Figure 7

Fig 6. Intracellular cytokine staining of cocultured stimulated T cells. An aliquot of T cells were retrieved from cocultures with DC, immature (IM) and mature (M) DC breast tumor fusion cells and analyzed for intracellular TH1 and TH2 cytokines and for the expression of CD4+CD25+ as shown in an example from one experiment (**A**). In a series of 7-12 experiments, the mean percentage (±SEM) cells positive for IL-2, IL-4 IFNγ, IL-10 and TNFα (**B**) is shown. Percentage of CD4+CD25+ T cell population was determined as shown (**A** and **B**).

Fig 7. Detection of TH1 and TH2 cytokines in supernatants of DC and immature (IM) and mature (M) DC-fusion cells cocultured with T cells. The TH1 and TH2 cytokine profiles in the supernatants of the cocultures was quantitated using the TH1/TH2 and the Inflammation Cytometric Bead Array (CBA) analysis Kit, as shown in an example from one CellQuest analyzed experiment (**A**). The data was acquired with BD CellQuest software and formatting and subsequent analysis of data was performed using the BD CBA software. In a series of 4 (DC+T cell cocultures) and 11 (immature and mature DC fusion cell cocultures) separate experiments, the mean (±SEM) concentration of cytokine (pg/ml) is shown (**B**).

Figure 6

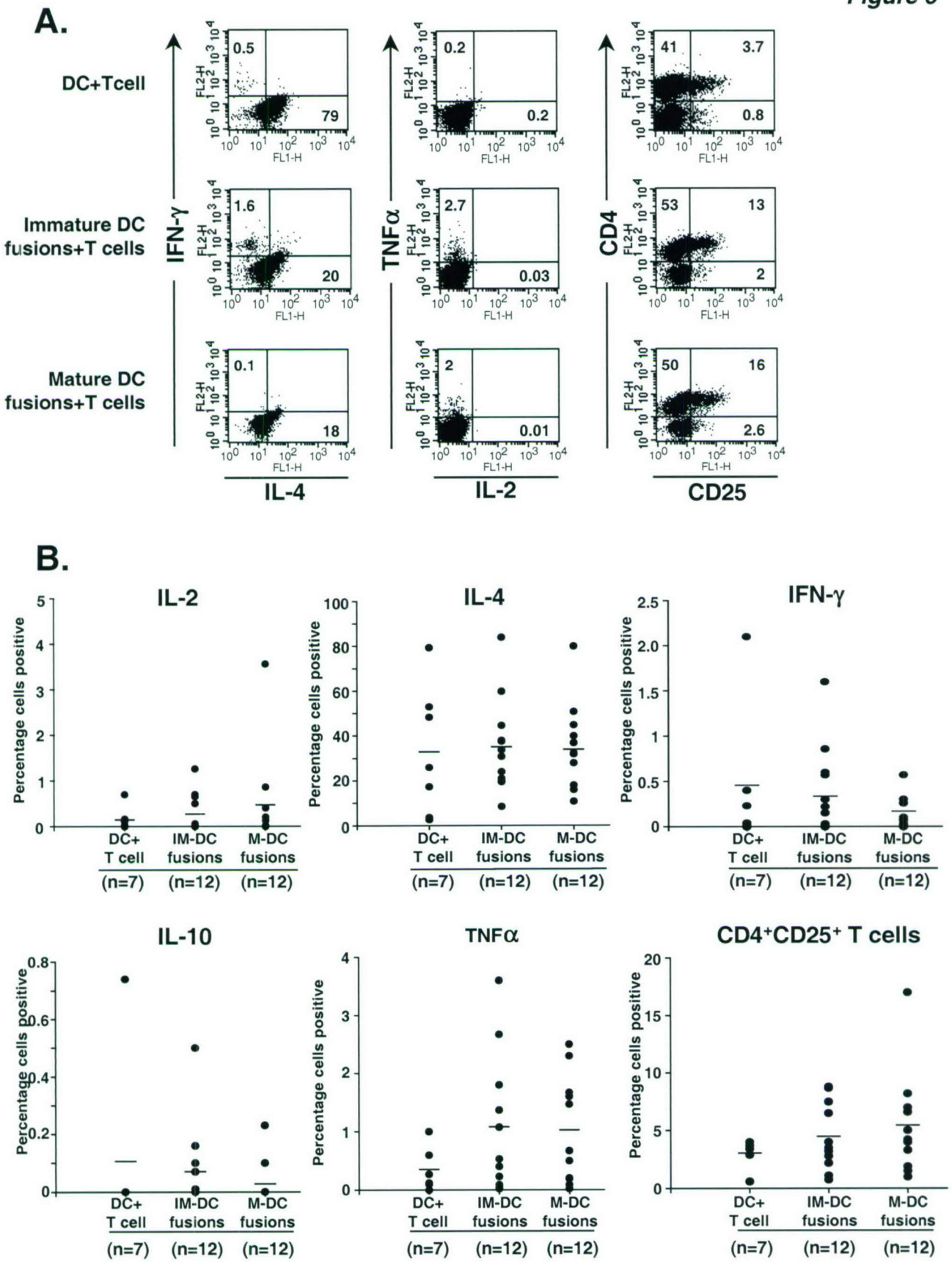
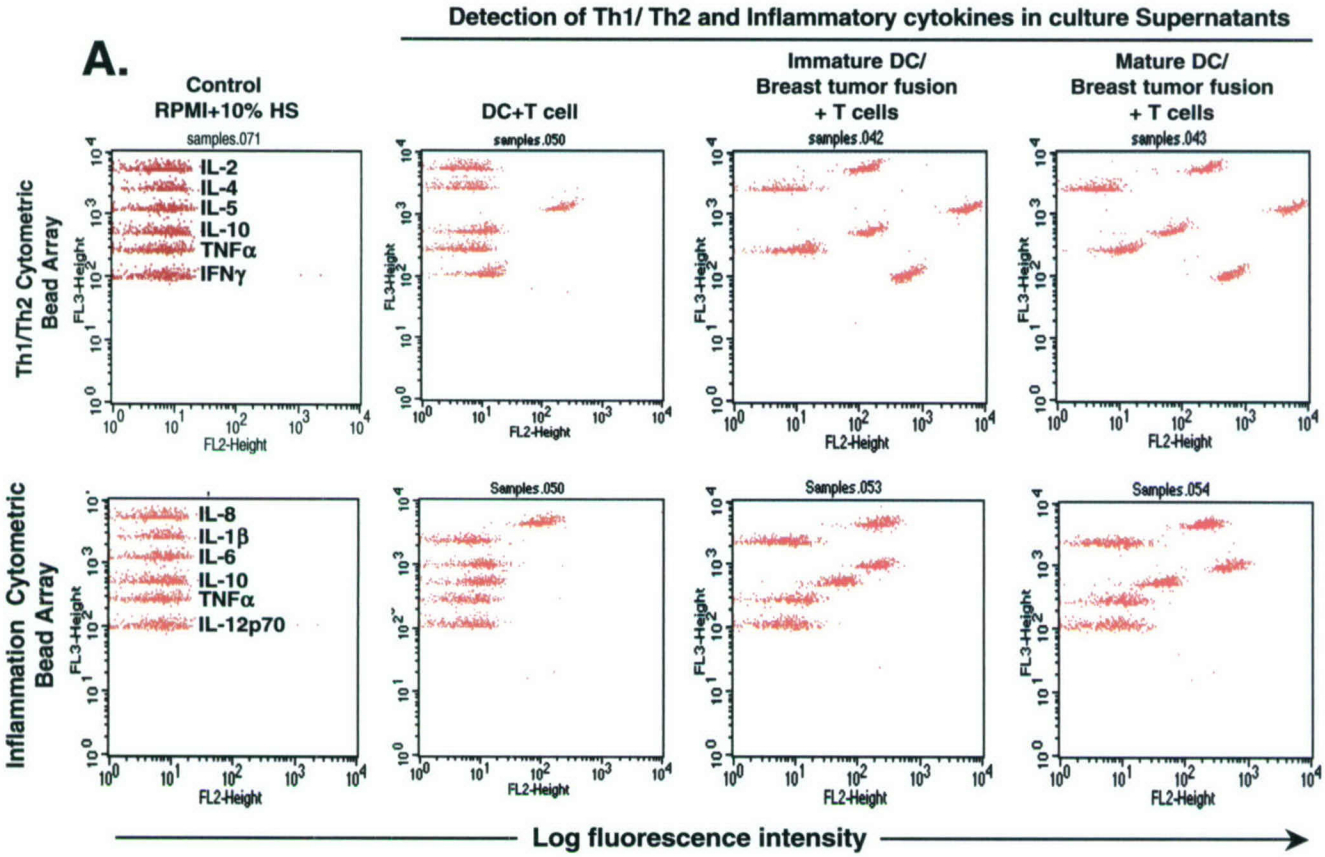

A.
DC+Tcell
Immature DC fusions+T cells
Mature DC fusions+T cells
IFN-γ
TNFα
CD4
IL-4
IL-2
CD25
FL1-H
FL2-H
0.5
79
0.2
0.2
41
3.7
0.8
1.6
20
2.7
0.03
53
13
2
0.1
18
2
0.01
50
16
2.6
B.
IL-2
IL-4
IFN-γ
IL-10
TNFα
CD4+CD25+ T cells
Percentage cells positive
DC+ T cell
IM-DC fusions
M-DC fusions
(n=7)
(n=12)
(n=12)

Figure 7


Detection of Th1/ Th2 and Inflammatory cytokines in culture Supernatants
A.
Control
RPMI+10% HS
DC+T cell
Immature DC/
Breast tumor fusion
+ T cells
Mature DC/
Breast tumor fusion
+ T cells
Th1/Th2 Cytometric
Bead Array
samples.071
samples.050
samples.042
samples.043
IL-2
IL-4
IL-5
IL-10
TNFα
IFNγ
FL3-Height
FL2-Height
Inflammation Cytometric
Bead Array
Samples.050
Samples.053
Samples.054
IL-8
IL-1β
IL-6
IL-10
TNFα
IL-12p70
Log fluorescence intensity

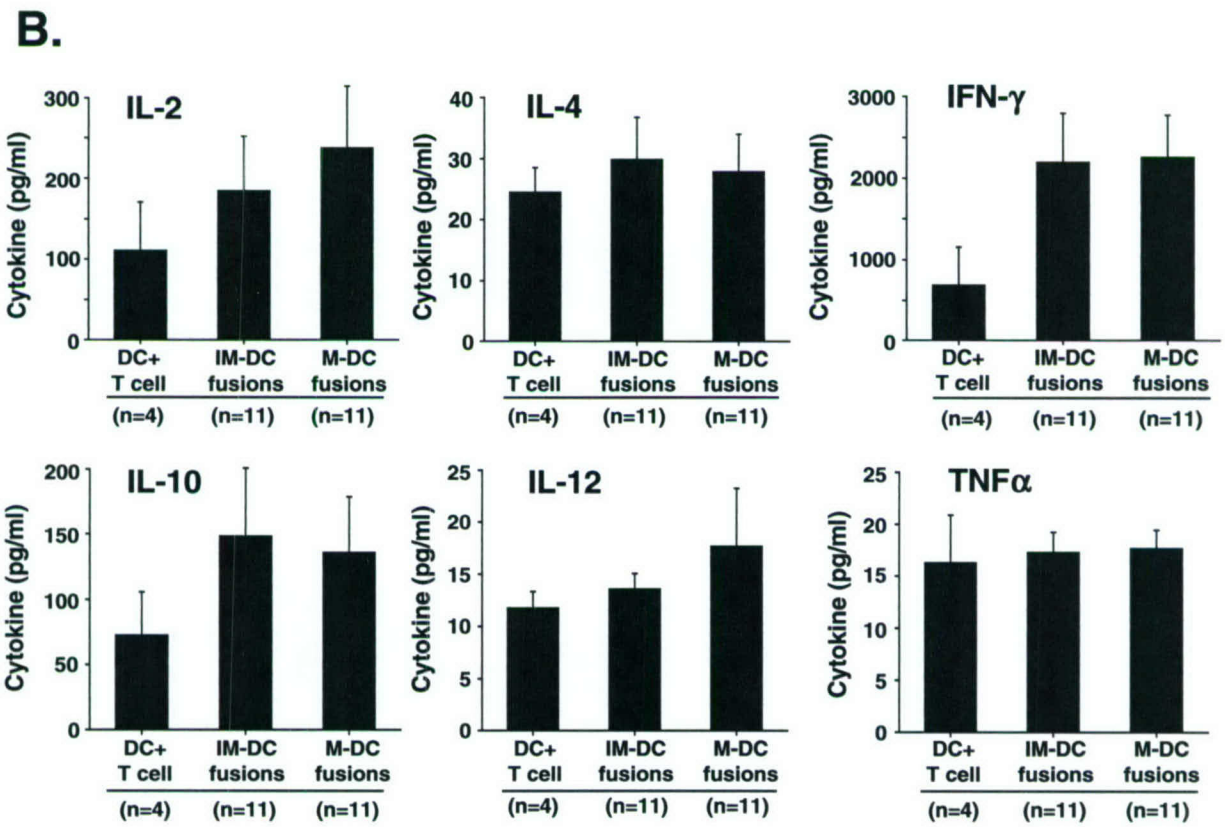

B.
IL-2
IL-4
IFN-γ
IL-10
IL-12
TNFα
Cytokine (pg/ml)
DC+
T cell
IM-DC
fusions
M-DC
fusions
(n=4)
(n=11)
(n=11)

Figure 8

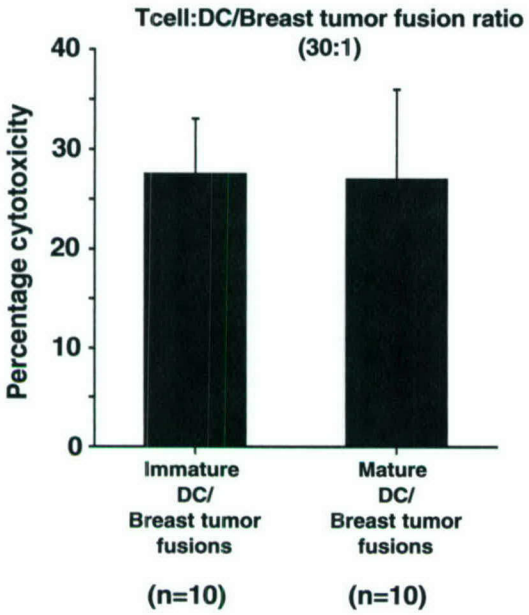


Fig 8. Cytotoxic T lymphocyte assay. Immature and mature DC-tumor cell fusions were co-cultured with autologous T cells at a ratio of 30:1 for 7-10 days. Lysis of autologous breast tumor cells or semiautologous DC/breast carcinoma fusions was determined by chromium release assay. In 10 separate experiments each with immature and mature DC tumor fusion cells, the mean percentage cytotoxicity (±SEM) observed is shown.