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ABSTRACT

The overall objective of this project is to study the safety, immunologic response and clinical effects of vaccinating breast cancer patients with dendritic cell (DC)/tumor fusions in conjunction with IL-12. Task 1 of the approved Statement of Work is to assess fusions of human breast carcinoma cells with autologous DC by evaluating cytokine production and potency of the fusions in generating tumor specific immunity in vitro. We showed that the DC/breast tumor fusions were effective in stimulating cytokine production and tumor-specific T cell responses. Work performed over the past year has extended these studies by assessing the effects of breast tumor/DC fusions on the generation of CD4+CD25+ T cells that regulate the development of anti-tumor immune responses. Importantly, our results show that, compared to unfused DC and tumor cells, the DC/breast tumor fusions are more effective in stimulating CD4+CD25+ T cells that express an activated CD69+ phenotype and contribute to the generation of potent anti-tumor effector T cells. Work performed over the past year has also included: i) responses to multiple reviews of clinical protocol 03-221 submitted in 2003; and ii) fulfilling CITI and clinfosource training requirements. Our plan is to now perform the Phase II trial of breast cancer cell/DC fusions with IL-12 as a vaccine for patients with metastatic breast cancer (Tasks 2-4).
# Table of Contents

Cover.................................................................................................................................

SF 298................................................................................................................................

Table of Contents.............................................................................................................

Introduction..................................................................................................................... 2

Body................................................................................................................................. 6

Key Research Accomplishments.................................................................................... 9

Reportable Outcomes...................................................................................................... 9

Conclusions.................................................................................................................... 9

References.......................................................................................................................10

Appendices.......................................................................................................................

INTRODUCTION

The overall objective of the project is to study the safety, immunologic response, and clinical effect of vaccination with the dendritic cell (DC)/tumor fusions in conjunction with IL-12 in patients with breast cancer. The hypothesis underlying the proposed study is that fusions generated with patient-derived breast carcinoma cells and autologous DC will stimulate a broad anti-tumor response that will be further augmented by the presence of IL-12. In the first phase of the project, we have conducted pre-clinical investigations to further define the biology and enhance the efficacy of the fusion cell vaccine. During this period, we have also been completing DOD mandated regulatory requirements in order to initiate the planned clinical trial.

In the first year of the project, we sought to define the optimal DC population to enhance vaccine efficacy. We examined the impact of DC maturation on the immunologic potency of the fusion vaccine. We demonstrated that polyethylene glycol (PEG)-mediated fusion results in maturation and activation of the DC fusion partner. Mature DC/tumor fusions exhibited prominent levels of expression of costimulatory molecules, IL-12, and chemokine receptors necessary for migration to draining lymph nodes. Based on these findings, we elected to use DCs matured with TNFα to generate fusion cells for the clinical trial.

During the second year of the grant, we continued efforts to define the biology of DC/breast carcinoma fusions and approaches to further enhance their potency as antigen presenting cells. In this regard, a significant limiting factor in developing an effective tumor vaccine is the immunocompromised milieu of the cancer patient. One important component is the increased presence of regulatory T cells that inhibit the development of effective anti-tumor immune responses.

Regulatory T cells (i) play a key role in the maintenance of immune tolerance to both self and foreign antigens; (ii) are a thymically derived population of T cells that co-express CD4 and the IL-2Rα chain (CD25) and (iii) are crucial for the control of autoreactive T cells in vivo (1, 2). Regulatory T cells also potently suppress the activation/proliferation of other CD4+ or CD8+ cells in vitro (3), which may be due, in part, to the inhibition of IL-2 transcription in the effector population. This suppression is abrogated by the addition of exogenous IL-2 or by enhancing endogenous IL-2 production with an anti-CD28 antibody (4). Conflicting evidence exists as to whether
immunosuppression is primarily mediated by cell contact or via secretion of inhibitory cytokines such as IL-10 and TGFβ (1, 5, 6).

Regulatory T cells constitutively express cell surface molecules, many of which are also associated with activated/memory cells. These include CD25, CD45RB^{low}, CD62L, CD103, cytotoxic T lymphocyte-4 (CTLA-4), and the glucocorticoid-induced TNFα receptor family-related gene (GITR) (4, 7, 8). Although previous studies had identified GITR as a constitutive expression marker for naturally occurring regulatory T cells, recent studies have shown that it is also upregulated on conventional activated CD4+ T cells and, in some settings, may act as a costimulatory signal (4, 9, 10). In some studies, binding of GITR by agonist antibodies results in the reversal of the immunosuppressive phenotype of regulatory populations (11). The natural ligand for GITR has now been shown to be also expressed on DC, B cells, and macrophages (12). FOXP3, a master regulator identified for regulatory T cell development, is also expressed in activated human CD25- cells (12). Other studies have similarly shown that, in some instances, CD25- human T cell activation by DCs results in FOXP3 upregulation (13, 14).

Regulatory T cells are thought to play a significant role in suppressing anti-tumor immunity in patients with malignancy (15). Increased levels of regulatory T cells have been demonstrated in the tumor bed and in the circulation of cancer patients (16-18). In a recent report, blockade of IL-10 and TGFβ was partially effective in limiting tumor growth. Moreover, depletion of CD4+ regulatory cells from the tumor bed resulted in effective immunologic targeting and eradication of established metastatic disease (19). In a clinical study, administration of expanded tumor infiltrating lymphocytes following lymphodepletion therapy resulted in disease regression in 50% of patients with metastatic melanoma (20). Importantly, recent studies have shown that depletion of regulatory T cell populations enhances antitumor responses to tumor vaccination (21, 22).

During year 2 of the grant period, we sought to determine the effect of DC/breast carcinoma fusions on regulatory T cell populations and the impact of these regulatory T cells on induction of anti-tumor immunity. We examined the capacity of immature or mature dendritic cells to induce regulatory T cells upon stimulation of allogeneic T cells. We then quantified regulatory and activated T cells among autologous and allogeneic T cells that had been stimulated by DC/breast carcinoma.
fusions. CD4+/CD25+ cells were further characterized by examining expression of CD69, GITR, CTLA-4 and FoxP3. Finally, we studied the nature of cytokine expression in this population.
Induction of CD4+CD25+ T cells by dendritic cells in allo-coculture assay

The primary focus of our pre-clinical investigation was to identify the role of regulatory T cells in modulating the anti-tumor response to the DC/breast carcinoma fusion vaccine. As an initial step, we studied the ability of immature and mature DC to stimulate regulatory T cell responses. Immature DCs were generated by culturing adherent PBMNCs in GM-CSF and IL-4 for 1 week. Maturation was induced by exposure to TNFα (25 ng/ml) for 48 h on day 5. In a subset of DCs, cytokines were removed for 48 h prior to the T cell stimulation assay. DC and allogeneic T cells were co-cultured in a 96 well plate at a DC:T cell ratio of 1:10 for 5 days. The cocultures were harvested on day 5 and immediately prepared for staining with directly conjugated antibodies against CD4 and CD25 surface markers.

Immature and mature DCs prominently induced a distinct group of double positive CD4+CD25+ T cells as determined by bidimensional FACS analysis (Immature DC: Figure 1A. Mature DC: Figure 2A). The range of percent of CD4/CD25 T cells that were stimulated was 18-30% and 28-35% for immature and mature DC, respectively. DC maintained their capacity to stimulate CD4/CD25 T cells following withdrawal of cytokines (Figure 1B and Figure 2B).

CD25 expression is upregulated in both activated and suppressor CD4+ T cells (Shevach, 2002 #7274). To further characterize the CD4+CD25+ T cells that were generated, we performed three color FACS analysis to assess the expression of common regulatory and activation markers. Dual expressing CD4+CD25+ T cells were isolated by FACS gating in the upper right quadrant of the dotplots (as shown in Figures 1 and 2) and expression of CD69, GITR or CTLA-4 was quantified. Approximately 10% of T cells stimulated by immature DC expressed the activation marker, CD69. In contrast, only 8% and 4% of the CD4+CD25+ T cell population expressed GITR and CTLA-4, respectively, which are found commonly on regulatory cells (Figure 1A). No significant changes were seen in T cells stimulated by DC following cytokine withdrawal (Figure 1B). Following stimulation with mature DCs, 5 and 10% of cells expressed CD69 and GITR, respectively (Figure 2A). These findings indicate that isolated cultured DCs have the capacity to induce regulatory
(CD4+CD25+) T cells and its associated surface molecules upon stimulation of allogeneic T cells.

Phenotypic characteristics of positively selected CD4+ T cells from auto and allo-cocultures with mature DC/tumor fusion cells

We next examined the T cell response to stimulation with DC/breast cancer fusions with regard to the presence of regulatory T cell populations. Mature DCs were fused to human MCF-7 breast carcinoma cells by coculture with PEG. The DC/tumor fusion cells were cocultured with autologous or allogeneic T cells at a ratio of 1:10 (fusions: T cells) for 5 days. T cells stimulated by unfused DC and MCF-7 cells and unstimulated T cells were used as controls. Initial attempts to isolate CD25+ cells using the Miltenyi magnetic bead system from the fusion/T cell cocultures were unsuccessful due to very low yields. Attention was thereafter directed at isolating CD4+ cells for the experiments. The coculture cells were harvested on day 5 and CD4+ T cells were positively selected from this population using the CD4+ magnetic beads (Miltenyi Corporation) according to manufacturer’s protocol. FACS analysis of the resultant CD4+ T cells demonstrated a purity of greater than 97%.

Following isolation, CD4+ T cells were aliquoted equally and stained with directly conjugated antibodies to determine the population of CD4+CD25+ T cells. The CD4+ T cells were then subjected to two-color analysis to determine the expression of CD69, GITR and CTLA-4 on CD25+ T cells as depicted in a representative experiment in Figure 3.

In a series of 5 separate experiments, the mean percentage of autologous CD4+CD25+ T cells did not significantly differ between unstimulated T cells, T cells stimulated by fusion cells, and T cells stimulated by unfused DC and MCF-7 cells (Figure 4A). Mean values were 6.48% (SEM± 1.09), 7.26% (SEM± 1.14) and 8.96% (SEM± 2.6) following stimulation by fusion cells, unfused DC and MCF-7 cells and an unstimulated T cell culture, respectively (Figure 4A). However, a greater percentage of the fusion stimulated CD4+ cells coexpressed CD25 and CD69, as compared to GITR or CTLA-4, supporting the predominance of an activated phenotype (Figures 3 and 4B). In addition, coculture of fusion cells and autologous T cells resulted in a significantly (p=0.01) greater percentage of CD4+/CD25+/CD69+ cells (4.78%; SEM± 0.43) as compared to T cells cocultured with unfused DC and MCF-7 cells (1.35%;
SEM± 0.2) or in the unstimulated culture (1.12%; SEM± 0.54). The percent of CD4+ cells expressing CD25+ and GITR was also highest following stimulation with fusion cells (Figure 4B). Similarly, the percent of CD4+CD25+ regulatory T cells expressing CD69 (mean 29.5%; SEM± 9.1) was highest following stimulation with fusion cells (Figure 4C). Expression of CTLA-4 was minimal in all of the stimulated autologous T cell population (Figures 4B and 4C).

Fusion cells more potently stimulated allogeneic CD4+CD25+ T cells (mean 10%; SEM± 3.7; n=3) than unfused DC and MCF-7 cells (mean 3.67%; SEM± 1.05; n=3) (p=0.05) (Figure 4D). In contrast to the autologous T cell cultures, a greater percentage of the fusion stimulated CD4+ cells co-expressed CD25+ and GITR (10.6%) as compared to CD69 (4.2%) (Figure 4E). Coexpression of CD25 and GITR by allogeneic T cells was more pronounced following stimulation by fusion cells as compared to unfused DC and MCF-7 cells (Figure 4F). These findings indicate that DC/tumor fusions cells have the potent capacity to induce the expression of an activated phenotype population of dual CD25+/CD69+ expressing T cells.

Expression of FOXP3 in CD25+ T cells in DC/tumor fusion cells cultured with autologous and allogeneic T cells

FOXP3 has been shown to be the master control gene for the development and function of natural CD4+CD25+ regulatory T cells (12). We therefore extended our studies to determine the nature of induction of FOXP3 expression in CD25+ T cells following coculture of DC/tumor fusion cells with autologous and allogeneic T cells. As described above, CD4+ cells were positively selected by magnetic beads from the coculture assays and analyzed for intracellular FOXP3 staining with surface staining for CD25. A minority of resting T cells expressed FOXP3 (Figure 5). Following stimulation of autologous or allogeneic T cells with DC/MCF-7 fusions, FOXP3 expression was seen in nearly all of the CD4+ population, including the CD25+ and CD25- subsets. (Figures 5 and 6).

Intracellular cytokine profiles of CD4+CD25+ T cells following coculture of DC/tumor fusion cells with autologous or allogeneic T cells

To further characterize the CD4+/CD25+ cells that were stimulated by coculture with DC/breast carcinoma fusions, we examined their profile of intracellular
cytokines. Following stimulation with fusion cells, T cells underwent CD4 magnetic bead separation. CD4+ cells were stained with CD25, permeabilized and fixed using Cytofix/CytoPerm solution (BD Biosciences), and then subjected to intracellular staining for IFN, IL-4, and IL-10. Ten percent of the total CD4 population and 90% of the CD4/CD25+ cells expressed IFNγ (Figure 7A). In addition, 8% of the total CD4 population and approximately 60% of the CD4/CD25+ cells expressed the inhibitory cytokine IL-10. In contrast, 5% of the CD4 cells and 40% of the CD4/CD25+ expressed IL-4. Therefore, while nearly all of the fusion stimulated CD4/CD25+ cells express IFN, consistent with an activated phenotype, coexpression of inhibitory cytokines was also observed (Figures 7A and 7B). These results raise the question as to how this cell population might modulate the overall response to the fusion vaccine. Functional studies are presently underway to assess this issue.
KEY RESEARCH ACCOMPLISHMENTS

We have demonstrated that stimulation of autologous T cells with DC/breast carcinoma fusions results in the presence of a subset of CD4+ cells that coexpress CD25. As compared to unfused DC and tumor cells, the fusion vaccine stimulates a higher percentage of CD4+CD25+ T cells that express an activated CD69+ phenotype. Of note, however, a subset of CD4+CD25+ cells is also induced that expresses GITR and is associated with both an immunosuppressive and activated phenotype. Ongoing studies are further characterizing these populations with respect to their capacity to regulate anti-tumor immunity. In this regard, we are examining the impact of depleting CD4+CD25+ T cells prior to secondary stimulation with the fusion vaccine. Based on these results, we will monitor the levels of circulating CD4+CD25+ T cells prior to and following fusion cell vaccination of breast cancer patients and assess the impact of adjuvant IL-12 in this setting. Notably, approaches to limit the impact of suppressive T cells in vivo are available, including the use of immunostimulatory agents such as anti-CTLA-4.

REPORTABLE OUTCOMES

The results obtained from preclinical work performed in Task 1 during months 1-24 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. During year 2 of the grant, we have been completing the regulatory and logistical requirements to initiate the clinical study. These efforts have included submission of the protocol, informed consent and manufacturing component for FDA approval, application to the NCI and Wyeth corporation to secure access to rhIL-12, obtaining approval for the clinical protocol and informed consent from the Harvard Cancer Center Office of Protection of Human Subjects, and a lengthy review process with the DOD consisting of multiple protocol and consent revisions. As a part of the DOD review process and upon request by the medical reviewer and review committee, we have submitted multiple substantial protocol revisions over the course of the year. The clinical research staff
underwent CITI recertification. At the conclusion of this process, we learned that the NCI had suspended their arrangement with Wyeth to distribute rHL-12 for investigator initiated trials. As a result, we have approached Wyeth independently and secured their approval for providing rHL-12 for the clinical study. Once this arrangement has been concluded, we will resubmit the study in its final form for re-review to the Harvard Cancer Center Office of Protection of Human Subjects and the FDA.

REFERENCES


FIGURE LEGENDS

Figure 1. Induction of regulatory T cells by immature dendritic cells in allo-coculture. (A) Immature dendritic cells were generated with GM-CSF/IL-4, cocultured for 5 days with allogeneic T cells and analyzed for the presence of CD4+CD25+ T cells. Dual expressing cells (CD4+CD25+) were gated in the upper right quadrant and analyzed for the expression of common activation and regulatory T cell markers, CD69, GITR or CTLA-4 on CD25+ T cells in a three color FACS analysis. (B) A subset of GM-CSF/IL-4 cultured DCs were subjected to 48 h culture without cytokines prior to allogeneic T cell stimulation. Regulatory T cells were analyzed by three color FACS analysis as above.

Figure 2. Induction of regulatory T cells by TNFα matured dendritic cells in allo-coculture. (A) Mature DCs were generated by subjecting 5d GM-CSF/IL-4 cultured DCs to 48 h culture with TNFα. Dual expressing CD4+CD25+ T cells were quantified and isolated by FACS gating for a three color analysis to determine the percentage of CD25+ T cells expressing CD69, GITR and CTLA-4. (B) A subset of matured DCs were subjected to 48 h culture without cytokines prior to allogeneic T cell stimulation. Regulatory T cells were analyzed as before in a three color FACS analysis.

Figure 3. Phenotypic analysis of positively selected CD4+T cells from auto and allo-cocultures following stimulation with DC/tumor fusion cells. Bi-dimensional FACS analysis was performed on positively selected CD4+ T cells to determine the induction of CD4+CD25+ regulatory T cells in auto or allo-coculture conditions. CD4+ T cells were further analyzed for the expression of regulatory T cell activation and common markers of CD69, GITR and CTLA-4 on CD25+ T cells. This was compared in parallel with positively selected CD4+ T cells from a culture of unstimulated T cells (control). Representative dotplot analyses from a series of 5 separate experiments is shown.

Figure 4. Bi-dimensional FACS analysis of positively selected CD4+ T cells from auto and allo-cocultures following stimulation with DC/tumor fusion cells. (A and D) Mean percentage (± SEM) of expression of CD4+CD25+ regulatory T cells induced in auto (A) or allo- (D) cocultures after stimulation with DC/tumor fusion cells for 5 d. (B and E) Mean percentage (+ SEM) of the total CD4+ T cells expressing CD69,
GITR or CTLA-4 on CD25+ T cells in auto (B) or allo- (E) cocultures. (C and F) Mean percentage (±SEM) of total CD25+ T cells expressing CD69, GITR or CTLA-4 surface molecules in auto (C) or allo- (F) cocultures. Data is representative (mean±SEM) of 5 separate experiments.

**Figure 5. Expression of FOXP3 in CD25+ T cells.** Intracellular expression of FOXP3 was examined in positively selected CD4+ T cells from auto and allo-cocultures stimulated with DC/tumor fusion cells for 5 d. FOXP3 expression was determined in CD25+ cells. IgG Isotype matched control intracellular analysis was performed in parallel. Upper panel shows an example of FOXP3 expression in CD25+ T cells from CD4+ selected from autologous T cell coculture stimulated for 5 d with DC/tumor fusion cells. Lower panel shows an example of FOXP3 expression in CD25+ T cells from unstimulated T cells following CD4+ T cell selection (control).

**Figure 6. FOXP3 expression in positively selected CD4+ T cells from auto and allo- cocultures after stimulation with DC/tumor fusion cells.** (A) CD4+ T cells were positively selected from autologous cocultures stimulated with fusion cells and from cocultures with unfused DC and MCF-7 cells and stained for CD25 followed by intracellular staining for FOXP3. These results were compared with CD4+ T cells selected from unstimulated T cells (control). (B) FOXP3 staining in CD25+ T cells from allo-cocultures stimulated with DC/tumor fusion cells or unfused DC and MCF-7 cells following positive selection of CD4+ T cells. Data is representative (mean±SEM) of 5 separate experiments.

**Figure 7. Expression of IFN, IL-4 and IL-10 in CD4+CD25+ regulatory T cells.** (A) Positively selected CD4+ T cells from auto cocultures with DC/tumor fusion cells were stained with CD25 antibody. After fixing and permeabilizing the cells with Cytofix/Cytoperm, the cells were stained for IFN, IL-4 or IL-10 and subjected to bi-dimensional FACS analysis. The results were compared in parallel with CD4+ T cells selected from unstimulated T cells (control). (B) Bar graph depicting the percentages of CD4+CD25+ T cells expressing IFN, IL-4 or IL-10.
Figure 1

GM-CSF/IL-4 cultured DCs followed by 48 hr of culture in complete RPMI media (without cytokines) co-cultured with allo-T cells for 5 days.

A) Allo-T cells

B) CD69

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-15-
Figure 2

GM-CSF/IL-4/TNFα cultured DCs followed by 48 hr of culture in complete RPMI media (without cytokines) cocultured with allo-T cells (1:10) for 5 days.
Figure 4

A  Autologous cocultures

B  Autologous cocultures

C  Autologous cocultures

D  Allogeneic cocultures

E  Allogeneic cocultures

F  Allogeneic cocultures

- DC/MCF-7 fusion cells cultured with autologous or allo- T cells
- DC+MCF-7 cells cultured with autologous or allo- T cells
- Control T cells only
Figure 5

DC/MCF-7 fusions + auto T cells

Control T cells (CD4+) only

CD25/IgG  CD25/FOXP3

CD25 FITC
Figure 6

A. Autologous coculture

CD4+CD25+FOXP3+ cells

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B. Allo- coculture

CD4+CD25+FOXP3+ cells

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Cocultured with autologous T cells
Figure 7

A

DC/MCF-7 fusion cells + autologous T cell coculture (CD4+ selected)

Control T cells (CD4+ selected)

B

CD25 FITC

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