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# Fusions of Breast Carcinoma and Dendritic Cells as a Vaccine for the Treatment of Metastatic Breast Cancer

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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. Studies performed in Task 1 have shown that the DC/breast tumor fusions are effective in (1) stimulating cytokine production, and (2) inducing tumor-specific T cell responses. Work performed over the past year has extended these studies by further defining the effects of stimulation with IL-12, Toll-like receptor agonists, CpG oligodeoxynucleotide and CD3/CD28 ligation as approaches that could be used to enhance effectiveness of the DC/breast tumor fusion vaccine. The results support our original hypothesis that IL-12 should be used in conjunction with the DC/breast tumor fusion vaccine in clinical trials. Our clinical protocol has been approved by the FDA, the NCI Clinical Trials Evaluation Program and the Dana-Farber/Harvard Cancer Center IRB. Lengthy delays in the DOD human use approval process have necessitated NCI recertification of the IL-12 before we can begin the trial.

**Subject Terms:** Breast cancer cells, dendritic cells, fusions, vaccines, T cells

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Introduction.

The overall objective of this project is to study the safety, immunologic response, and clinical effect of vaccination with dendritic cell (DC)/breast cancer cell fusions administered in conjunction with IL-12 in patients with metastatic breast cancer. The hypothesis underlying the proposed study is that DC/breast cancer fusion cells will effectively present a broad array of tumor antigens in the context of DC-mediated costimulation resulting in a potent anti-tumor immune response. We postulate that administration of IL-12 as a vaccine adjuvant will further amplify the vaccine response by promoting T cell activation. In the first phases of the project, we have conducted pre-clinical studies to examine the phenotypic and functional characteristics of the DC/breast cancer fusion vaccine, define the nature of the T cell response, and assess the effect of immunostimulatory agents on vaccine-mediated immune responses. During this period, we completed preparation of the clinical protocol and the complex regulatory steps to initiate the clinical trial. These have included (1) obtaining approval for the protocol under an FDA supervised IND, (2) securing approval for the protocol and consent forms from the Dana-Farber/Harvard Cancer Center Institutional Review Board, (3) obtaining approval from the National Cancer Institute, Clinical Trials Evaluation Program (CTEP) of our protocol and their commitment to provide the recombinant human IL-12 for our trial, and (4) completing DOD mandated regulatory requirements in order to initiate the planned clinical trial.

Patients with malignancy exhibit functional deficiencies in cellular immunity that inhibit the ability of cancer vaccines to generate meaningful anti-tumor responses (1). The immunologic milieu of the cancer patient is characterized by the predominance of immature DCs in the tumor bed that present antigen in the absence of significant costimulation and thereby result in a tolerizing effect (2, 3). Tumor-reactive cytotoxic T lymphocytes are relatively depleted and lack evidence of functional potency, such as expression of IFN\(_{\gamma}\) (4, 5). In addition, the increased presence of immunosuppressive regulatory T cells has been demonstrated in the circulation and tumor bed of cancer patients (6, 7). A potential concern is that tumor cells in the fusion vaccine preparation will inhibit DC maturation and function, bias T cell response towards an inactivated state, and will lead to the relative expansion of regulatory T cells, all of which will blunt the immunologic response. In the first 2 years of the project, we examined the phenotypic and functional characteristics of the DC/breast cancer fusion vaccine, assessed the impact of DC maturation on the immunologic potency of the fusion vaccine, and defined the nature of fusion-mediated T cell responses in vitro. In the third year of the grant, we have assessed the effect of DC/breast carcinoma fusions on the relative expansion of regulatory and activated T cell populations and have examined strategies to enhance the capacity of the fusion vaccine to promote T cell activation and anti-tumor immunity. Depletion of regulatory T cells has been shown to augment responses to cancer vaccines in animal models and clinical studies(8, 9). In contrast, tumor vaccines may paradoxically expand regulatory T cells that ultimately inhibit response to vaccination. We therefore studied approaches to enhance vaccine potency by augmenting DC maturation and the expansion of activated, as compared to regulatory, T cells. In this
regard, we studied the effect of IL-12 and Toll like receptor (TLR) agonists (TLR 7/8 and TLR 9) that induce innate immune responses (10, 11). Activation of TLRs pathways has been shown to reverse the inhibitory effects of regulatory T cells, reverse the immunosuppression associated with expanding tumor lesions, and augment vaccine response in cancer bearing animals (11, 12). We also examined the effect of combined TLR 7/8 agonist and the CpG oligodeoxynucleotide (CpG ODN) which signals through TLR9 (12), on DC phenotype and vaccine-mediated T cell stimulation.
Effects of DC/breast cancer fusion cells on T cell populations

We first examined the capacity of the DC/breast cancer fusion vaccine to expand activated memory effector cells as compared to immunosuppressive regulatory T cell populations. These two populations are characterized by the co-expression of CD4 and CD25. Activated cells are further defined by the expression of CD69 and the expression of stimulatory cytokines, such as IFNγ. In contrast, regulatory T cells are characterized by expression of GITR, CD62L, CTLA-4 and FOXP3, inhibitory cytokines, such as IL-10 and TGFβ, minimal proliferation in response to mixed lymphocyte proliferation, and the suppression of autologous T cell responses.

We examined the capacity of DC/breast cancer fusions to stimulate expansion of activated as compared to regulatory T cells. Mature DCs were fused to human MCF-7 or ZR-75-1 breast carcinoma cells and cocultured with autologous T cells at a ratio of 1:10 (fusions: T cells) for 5 days. The coculture cells were harvested on day 5 and CD4+ T cells were positively selected from this population using CD4+ magnetic beads. FACS analysis of the resultant CD4+ T cells demonstrated a purity of greater than 97%. CD4+CD25+ T cells were quantified by flow cytometric analysis and further characterized with respect to expression of cell surface markers and cytokine profile.
In a series of 4 separate experiments, stimulation with DC/breast carcinoma fusions resulted in an increase in CD4+CD25+ T cells (11.9%; SEM ± 4.7) as compared to unstimulated T cells (4.7%; SEM ± 0.94) as shown in Figure 1.

**Figure 1: Induction of regulatory T cells in DC/breast carcinoma fusion cells cocultured with autologous T cells.** Fusions cells generated with GM-CSF/IL-4/TNFα treated DCs and ZR-75-1 breast cancer cells were cultured for 5 days with autologous nonadherent cells. CD4+ T cells were positively selected using Miltenyi magnetic beads and analyzed by bi-dimensional FACS analysis for the dual expressing CD4+CD25+ T cells. Nonadherent cells cultured alone served as a source for unstimulated CD4+ T cells. (A) Bidimensional FACS analysis profile of unstimulated and DC/breast cancer fusion cell stimulated T cells expressing the CD4+CD25+ phenotype. (B) Bar graph depicting the mean (SEM) of 4 separate experiments.
In additional studies, we assessed the expansion of CD4+CD25+ T cells in response to DC/breast carcinoma fusions generated with DCs matured with TNFα or a combination of TNFα, PGE2, IL-6, and IL-1β (PG-E2 cytokine combination). At day 3 of stimulation, CD4+CD25+ T cells comprised 27% and 36% of the total CD4+ population following exposure to fusions generated with TNFα and the PGE2 cytokine combination, respectively. Following another 3 days of culture, levels of CD4/CD25 expression rose to 51% and 47%, respectively (Figure 2).

**Figure 2. Induction and expansion of regulatory T cells during prolonged culture periods.** Expansion of regulatory T cells in response to fusion cells generated by DCs matured with TNFα or PG-E2 cytokine combination and breast cancer cells was assessed at day 3 and 6 following coculture with autologous non-adherent cells. CD4+ T cells were positively selected using the Miltenyi beads, stained for the dual expressing CD4+CD25+ phenotype and analyzed by bi-dimensional FACS analysis. The results are representative of two separate experiments.
**Intracellular expression of IFNγ and IL-10 in autologous CD4+CD25+ T cell populations following stimulation with DC/breast carcinoma fusion cells**

We assessed the profile of cytokine expression in the CD4+CD25+ T cell population following stimulation with DC/breast carcinoma fusions using intracellular flow cytometric analysis. In 4 serial studies, the mean percentage of CD4+CD25+ T cells expressing IFNγ rose from 32% (SEM± 16.4) to 43% (SEM± 5.5) following fusion cell stimulation. The percentage of CD4+CD25+ T cells expressing the inhibitory cytokine, IL-10, also rose from 9% (SEM± 6.2) to 19% (SEM± 12.6) as shown below in Figure 3.

![Graph showing intracellular expression of IFNγ and IL-10](image)

**Figure 3. Intracellular expression of IFNγ and IL-10 in regulatory T cells.** Fusion cells generated from TNFα matured DCs and breast cancer cells were cocultured with autologous non-adherent cells for 5 days. CD4+ T cells were positively selected using the Miltenyi beads and stained with FITC conjugated CD25 antibody. Following fixation and permeabilization, the cells were stained with PE-conjugated anti-IFNγ or anti-IL-10 in parallel with matching isotype controls. The stained CD4 selected cells were analyzed by bidimensional FACS analysis and CD25+ T cells expressing IFNγ or IL-10 were determined. The bar graph shows mean (SEM) of 5 separate experiments.
Expression of FOXP3 transcription factor in CD4+CD25+ T cells after autologous coculture with DC/tumor fusion cells

We also assessed the effects of fusion cell stimulation on the intracellular expression of FOXP3, a marker thought to be most specific for regulatory T cells. FOXP3 expression was detected in a mean of 41% (SEM± 11.7) and 35% (SEM± 4.9) of the unstimulated and fusion cell-stimulated CD4+CD25+ T cell populations, respectively (Figure 4).

**Figure 4: Expression of FOXP3 in DC/breast cancer fusion cell stimulated regulatory T cells.** DC/breast cancer fusion cells were cocultured with non-adherent autologous cells for 5 days. CD4+ T-cells were positively selected with Miltenyi magnetic beads from the fusion coculture and from nonadherent cells (control). Cells were stained with FITC conjugated anti-CD25 and than fixed and permeabilized, followed by intracellular staining for PE-conjugated FOXP3 or its matching isotype antibody. (A). Dual expressing CD25+ and FOXP3+ cells were determined by bidimensional FACS anaysis. (B) Bar graph showing the mean (SEM) of 4 separate experiments.

**Phenotypic characteristics and the capacity to induce T-cell proliferation of monocyte derived DCs following a 48h exposure to TLR 7/8 (M003) agonist**

Based on the above results, we explored the impact of the TLR7/8 agonist (M003-3M) on the phenotypic characteristics of DCs undergoing maturation from peripheral blood precursor populations. Expression of costimulatory and maturation markers were compared for adherent peripheral blood mononuclear cells cultured with GM-CSF, IL-4 and TNFα in the presence or absence of M003. In 11 serial experiments, mean expression of the costimulatory molecule, CD80,
rose from 32% (SEM± 6.6; n=10) to 76% (SEM± 5.9; n=11) (Figures 5A and 5B). Similarly, the maturation marker, CD83, was detectable on 62% (SEM± 6.7; n=11) and 40% (SEM± 7.8; n=10) of DCs generated in the presence and absence of M003, respectively. In contrast, expression of CD11c (panmyeloid marker), HLA class II, and CD86 did not differ between the two populations. Of note, exposure to M003 also resulted in upregulation of the tumor associated antigen, MUC1, with mean expression of 36% (SEM± 15.9; n=4) and 12% (SEM± 6.5; n=4) observed in DCs cultured with and without M003, respectively (Figures 5A and 5B).

**Figure 5. Phenotypic analysis of TNFα matured DCs treated with TLR 7/8 agonist (M003).** Day 5 DCs were treated with TNFα (25 ng/ml) or additionally with M003 (10 ug/ml) for the last 48 h of culture period. DCs were washed, incubated with primary antibody and stained with FITC conjugated secondary antibody. The cells were analyzed for the indicated surface marker shown above. (A) A representative phenotypic histogram FACS profile (FL1-H channel) of TNFα treated DCs and those treated with M003. (B) Bar graph showing mean (SEM) of 10-11 separate experiments. Numbers in the histograms depict the percentage of cells positive for the indicated marker after gating.
In contrast, DCs generated in the presence of M003 did not demonstrate an enhanced capacity to stimulate allogeneic T cell proliferation or autologous T cells in the presence of tetanus antigen, as shown in Figure 6. There was no significant difference observed between the stimulation indexes in allo-coculture assays (n=4) using GM-CSF/IL-4/TNFα generated DCs as compared to those additionally activated with M003 (120.5, SEM ± 58.9 versus 87.5, SEM ± 33.7, respectively) (Figure 6). Similarly, no significant difference was observed in autologous coculture assays (n=4) pulsed with tetanus toxoid antigen (10 ug/ml) (7.8 SEM ± 4.6 versus 5.2 SEM ± 1.9, respectively). We are currently studying the phenotypic characteristics of fusion cells generated with M003 stimulated DCs.

![Figure 6. T cell proliferation in allo- and auto-coculture assays with TNFα and M003 treated DCs.](image)

**Figure 6. T cell proliferation in allo- and auto-coculture assays with TNFα and M003 treated DCs.** TNFα matured DCs and those treated with M003 were cocultured with enriched allogeneic T cells (A) or with autologous T cells (B) in the presence of tetanus toxoid antigen (10 ug/ml) in a DC:T cell ratio of 1:10 in a 96 well tissue culture plate. T cell proliferation was determined by incorporation of [³H]-Thymidine added 18 h before the end culture period. Data are expressed as mean Stimulation Index (SI) with ± SEM. The SI was determined by calculating the ratio of [³H]-Thymidine incorporation (mean of triplicates) over background [³H]-Thymidine incorporation (mean of triplicates) of the unstimulated T cell population.

**Comparison of TLR 7/8 (M003) and IL-12 for induction of activated and inhibitory T cell populations by DC/breast cancer fusion cells**

The capacity of M003 and IL-12 to modulate fusion mediated stimulation of activated and inhibitory T cell populations was examined by assessing IFNγ and IL-10 expression, respectively. In 5 serial studies, autologous T cells were cocultured with DC/breast carcinoma fusions in the presence or absence of M003 or IL-12. The addition of M003 did not increase the mean percentage of
CD4+CD25+ T cells expressing IFN\(_{\gamma}\) (0.97%; SEM ± 0.4) or IL-10 (0.67%; SEM ± 0.2) (Figure 7). Importantly, however, addition of IL-12 to the coculture of DC/breast carcinoma fusions and autologous T cells resulted in a greater than two-fold increase in the percentage of CD4+CD25+ expressing IFN\(_{\gamma}\) (1.95%; SEM ± 0.5) without affecting the percentage of cells expressing IL-10 (1.2%; SEM ± 0.6) as shown in Figure 7.

**Figure 7. Effect of exogenous IL-12 and TLR 7/8 (M003) on DC/tumor fusion cell stimulation of activated and T regulatory cells.** DC/breast cancer fusion cells were cocultured with autologous non-adherent cells for 5 days in the presence of exogenous recombinant human IL-12 (10 ng/ml) or M003 (10 ug/ml) for 5 days. Unpulsed cocultures served as controls. CD4+ T cells were positively selected using Miltenyi magnetic beads and stained for FITC conjugated CD25. Following fixation and permeabilization, the cells were labeled for intracellular expression of IFN\(_{\gamma}\) or IL-10. Labeling with matching isotype controls was performed in parallel. Cells were analyzed by bidimensional FACS analysis and the percentage of CD25+ T cells positive for IFN\(_{\gamma}\) or IL-10 were determined. Data is presented as mean (SEM) of 5 separate experiments.
Phenotypic characteristics of monocyte derived DCs following exposure to CpG ODN

For further comparison, we examined the effects of CpG ODN on DC maturation (Figure 8) and promoting fusion-mediated expansion of activated as compared to regulatory T cells (Figure 9). Like the effects of TLR 7/8 on DC maturation, treatment of DCs with CpG ODN similarly demonstrated upregulated expression of costimulatory (CD80) and maturation (CD83) markers (Figure 8).

![Graph showing the percentage of cells positive for various markers.](image)

**Figure 8.** Phenotypic analysis of TNFα and CpG ODN treated monocyte derived DCs. 5 day GM-CSF/IL-4 cultured DCs were treated with TNFα (25 ng/ml) or additionally with CpG ODN (10 ug/ml) for the last 48 h of culture period. Cells were harvested and labeled with primary antibody for the surface markers shown in the bar graphs, followed by labeling with FITC conjugated secondary antibody. Labeled DCs were analyzed by FACS analysis after gating and the percentage of cells positive for the marker were determined. Data is representative of two separate experiments utilizing independent samples.

The effect of CpG ODN on the expression of IFNγ, IL-10 and FOXP3 in CD4+CD25+ T cells following coculture of autologous T cells with DC/breast cancer fusion cells

In 4 serial experiments, autologous T cells were cultured with DC/breast cancer cell fusions cells in the presence or absence of CpG ODN (10 ug/ml). CpG ODN had no detectable effect on the percentage of CD4+CD25+ T cells expressing IFNγ, IL-10 or FOXP3 (Figure 9).
Figure 9. Expression of IFN\(\gamma\), IL-10 and FOXP3 by regulatory T cells after stimulation with CpG ODN treated DC/tumor fusion cells. CpG ODN treated DCs were fused with breast carcinoma cells and cocultured with autologous nonadherent T cells for 5 days. CD4+ T-cells were positively selected with magnetic beads and labeled with CD25. After fixation and permeabilization, the cells were labeled with antibodies.
against IFNγ, IL-10 and FOXP3 or matching isotype controls and analyzed by bidimensional FACS analysis. (A) Bidimensional dot plot analysis of CD4 selected T cells labeled with CD25 and the indicated intracellular cytokine and FOXP3. (B) Bar graph depicting the mean (SEM) of 4 separate experiments.

**Effect of CD3/CD28 activation on the induction of regulatory T cells following autologous coculture of T cells with DC/breast carcinoma fusion cells**

As another approach, we examined the effect of activating of T cells by antibody-mediated ligation of CD3 and CD28 on response to the DC/breast carcinoma fusion vaccine. This pathway provides a strong antigen independent stimulus to T cells. Persistent exposure in the absence of antigen specific stimulation has resulted in the generation of regulatory T cells. We examined whether the combined stimulation with DC/breast carcinoma fusions and CD3/CD28 ligation results in a more pronounced expansion of tumor reactive T cells with an activated as compared to regulatory cell phenotype. We also studied the effect of the sequence of fusion cells and CD3/CD28 stimulation on the nature of the T cell response. In serial studies, only limited proliferation of T cells was observed following exposure to CD3/CD28 or fusion cells alone. In contrast, stimulation of T cells with both signals resulted in significantly greater proliferative responses.

Following stimulation of autologous T cells with DC/breast carcinoma cell fusions or CD3/CD28 ligation, the percent of CD4+CD25+ T cells was 7.7% and 9.7%, respectively. In contrast, stimulation with the combination of CD3/CD28 ligation and fusion cells resulted in 23.6% CD4+CD25+ T cells. Expression of CD69 was detected on 4.4%, 2.2%, and 9.5% of CD4+CD25+ T cells stimulated by fusion cells, CD3/CD28 ligation, and the combination, respectively. In a similar study in which additional fusions were generated with primary breast cancer cells, 39% of CD4+ cells coexpressed CD4+CD25+ and CD69 in contrast to 3.8% of cells following stimulation with fusion cells alone.

In the assessment of sequencing, expression of CD69 on CD4+CD25+ cells was 1%, 0.5%, and 19% following stimulation by fusion cells alone, CD3/CD28 ligation followed by fusion cells, and fusion cell stimulation followed by CD3/CD28 ligation, respectively. Similarly, IFNγ expression was detected in 1.4%, 1.2%, and 9.4% of CD4+CD25+ T cells stimulated by fusion cells alone, CD3/CD28 ligation followed by fusion cells, or fusion cells followed by CD3/CD28 ligation, respectively. These data suggest that antigen specific stimulation followed by antigen independent expansion by antibody mediated CD3/CD28 ligation is the most effective strategy to induce activated T cell responses.

**Key research accomplishments**

Our pre-clinical studies thus demonstrate that fusion cells induce expansion of CD4+CD25+ T cells. Similar levels of expansion were found using DCs generated with TNFα and PG-E2 cytokine combination and that expansion increases with longer periods of stimulation. Analysis of the CD4+CD25+ T cell
population demonstrated that both activated and regulatory T cells are increased as detected by CD69/IFN\(\gamma\) and IL-10/FOXP3, respectively. We found that an agonist of TLR7/8 increases expression of costimulatory and maturation markers on DCs. However, the addition of the TLR7/8 agonist to the fusion/T cell coculture did not significantly increase the relative percentage of activated T cells. Similarly, CpG ODN did not increase the number of activated as compared to regulatory cells. In contrast and importantly, the addition of IL-12 was associated with an increase in IFN\(\gamma\) producing cells. We also found that stimulation of T cells with DC/breast carcinoma fusions and antibody mediated CD3/CD28 activation results in a significant increase in T cell proliferation and a relative increase in activated memory effector cells as manifested by CD69 and IFN\(\gamma\) production.

Reportable Outcomes

The results obtained from preclinical studies performed during the first 2 Tasks together with data obtained in the past year, have been integrated into a manuscript and will be submitted for publication.

Conclusions

Our clinical protocol has received approval by the FDA, NCI/CTEP (distributor of IL-12) and Dana-Farber/Harvard Cancer Center. We have also met the requirements as outlined in the DOD review process. However, during the protracted period of DOD review, the availability of IL-12 was suspended for recertification, which has significantly delayed the initiation of the clinical trial. CTEP is recertifying lots of IL-12 that will be made available to us for the clinical trial. We are awaiting the completion of their testing so that IL-12 can be released for clinical use. Once available, we will begin the clinical trial which is projected for 2-3 years from study initiation to completion.

References


