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14. ABSTRACT
The purpose of the study was to determine the effectiveness, safety, immunologic response, and clinical activity of vaccination with the dendritic cell (DC)/breast cancer fusions administered in conjunction with IL-12 in patients with metastatic breast cancer. DC/breast carcinoma fusion cells present a broad array of tumor-associated antigens in the context of DC-mediated costimulation. Our pre-clinical studies showed that fusion of DCs with breast carcinoma cells up-regulates expression of costimulatory and maturation markers, results in high levels of expression of IL-12, and stimulates a mixed T cell response characterized by the expansion of both activated and regulatory T cell populations. Our results further showed that IL-12, IL-18, and TLR 9 agonist CpG oligodeoxynucleotides reduced the level of fusion-mediated regulatory T cell expansion and that sequential stimulation of fusion cells with anti-CD3/CD28 resulted in marked expansion of activated tumor-specific T cells. These findings indicated that DC/breast carcinoma fusions are effective in combination with IL-12 in activating antigen-specific T cells. In clinical studies, vaccination of patients with metastatic breast cancer with fusion cells was well tolerated and induced immunologic responses. Combination of the vaccine with IL-12 was delayed by a lengthy review process, issues relating to the availability of IL-12 from the NCI and the challenge of enrolling patients with accessible tumor tissue for generating the vaccine. As a result, the present trial was closed and the plan is to develop this approach for breast cancer patients as adjuvant therapy following primary surgery.

15. SUBJECT TERMS
None provided.

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Vaccination of Patients with Metastatic Breast Cancer

Department of Defense Grant # DAMD17-03-1-0487

FINAL Progress Report

October 2013

David Avigan, MD
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Table of Contents

A. INTRODUCTION

This final report for the Department of Defense Grant # DAMD17-03-1-0487 summarizes the main objectives and findings of the study. These pre-clinical and clinical studies were undertaken to determine the safety, immunologic response, and clinical effect of vaccination with dendritic cell (DC)/breast cancer fusions administered in conjunction with IL-12 in patients with metastatic breast cancer. DC/breast carcinoma fusion cells present a broad array of tumor-associated antigens in the context of DC-mediated co-stimulation. In clinical studies, vaccination with fusion cells was well tolerated, induced immunologic responses in a majority of patients, and results in disease regression in subset. We postulated that administration of the vaccine in conjunction with IL-12 would further enhance vaccine response by promoting T cell activation. In this project, we conducted preclinical studies and a phase I/II clinical trial in which patients with metastatic breast cancer underwent vaccination with DC/tumor fusions administered in conjunction with rhIL-12. An initial cohort of patients was treated with the DC/tumor fusions alone. In the subsequent cohort, fusion cells were administered with rhIL-12 given subcutaneously at the time of vaccine administration and on days 3 and 5. Measures of tumor specific cellular and humoral immunity were obtained at serial time points following vaccination as reported in the attached supplementary section of this report. Time to disease progression and RECIST measurable disease response were followed as a secondary endpoint.

In the clinical study, the targeted population included patients with histological proven stage IV breast cancer with RECIST measurable disease and accessible tumor (e.g. subcutaneous tissue, superficial lymph nodes, or peripheral lung nodule accessible by VATS) for vaccine generation. Patients undergoing more invasive procedures for independent diagnostic or therapeutic indications had tumor collected for the purposes of this study. Patients participating in the second cohort of the study received three fixed doses of $5x10^5$-$5x10^6$ subcutaneous fusion cells, dependent on cell yields in conjunction with escalating doses of rhIL-12. Participants enrolled onto successive Cohorts 1 and 2 received doses of rhIL-12: 0, and 30 ng/kg, respectively, in conjunction with the fusion vaccine.

B. BODY

Pre-Clinical Studies

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 (1, 2, 3). Tumor-reactive cytotoxic T lymphocytes are relatively depleted and lack evidence of functional potency, such as expression of IFNγ (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 assessed the effect of DC/breast carcinoma fusions on the relative expansion of regulatory and activated T cell populations and 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.

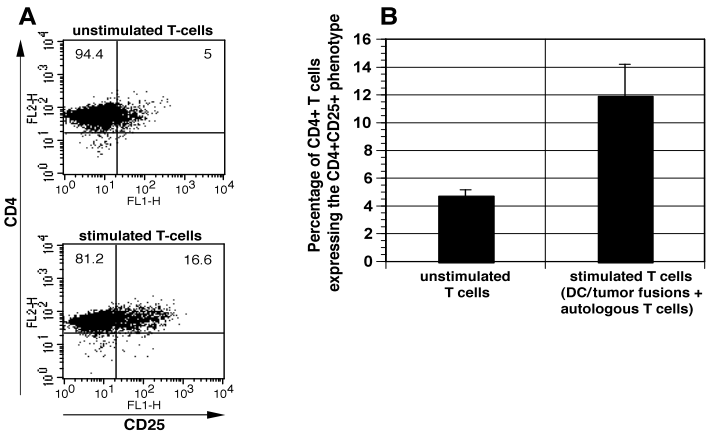


Figure 1. Induction of regulatory T cells in DC/breast carcinoma fusion cells cocultured with autologous T cells. *Fusions cells generated with GM-CSF/IL-4/TNFα treated DCs and ZR-75-1 breast cancer cells were cultured for 5 days with autologous nonadherent cells. CD4+ T cells were positively selected 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).

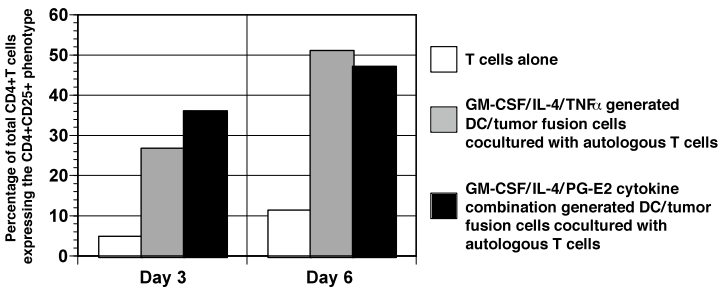


Figure 2. Induction and expansion of regulatory T cells during prolonged culture periods. *Expansion of regulatory T cells in response to fusion cells generated by DCs matured with TNFα or PG-E2 cytokine combination and breast cancer cells was assessed at day 3 and 6 following coculture with autologous 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.

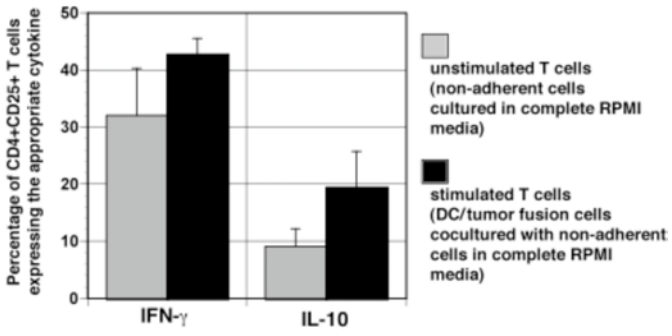
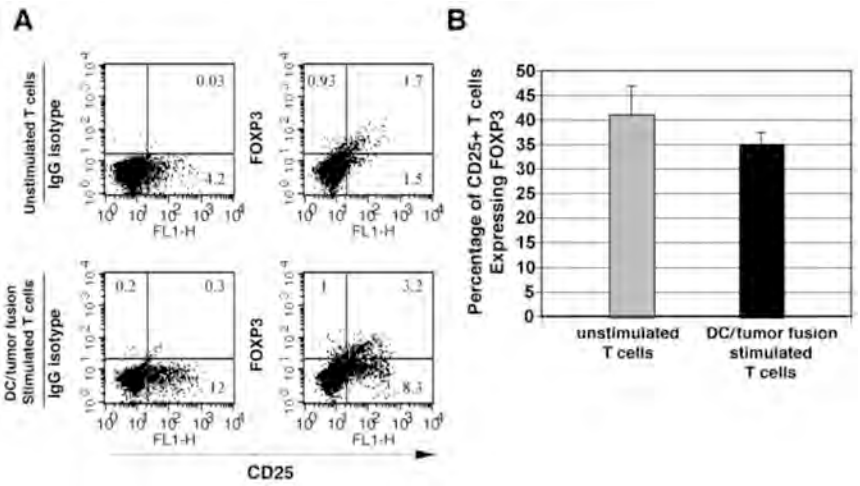


Figure 3. Intracellular expression of IFNγ and IL-10 in regulatory T cells*. Fusion cells generated from TNFα matured DCs and breast cancer cells were cocultured with autologous non-adherent cells for 5 days. CD4+ T cells were positively selected using the Miltenyi beads and stained with FITC conjugated 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 autlogous 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 48 h 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).

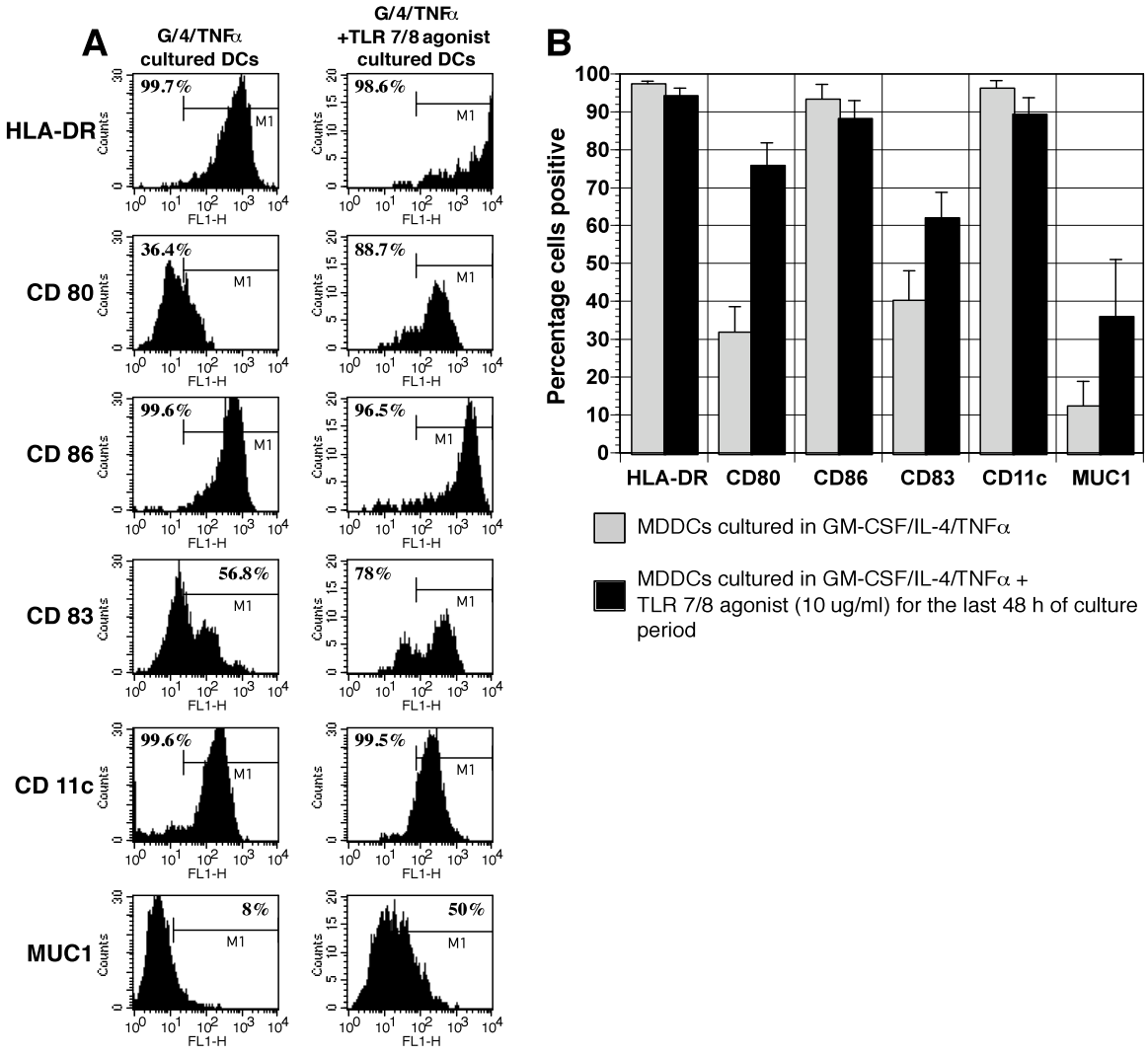


Figure 5. Phenotypic analysis of TNFα matured DCs treated with TLR 7/8 agonist (M003). *Day 5 DCs were treated with TNFα (25 ng/ml) or additionally with M003 (10 ug/ml) for the last 48 h of culture period. DCs were washed, incubated with primary antibody and stained with FITC conjugated secondary antibody. 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).

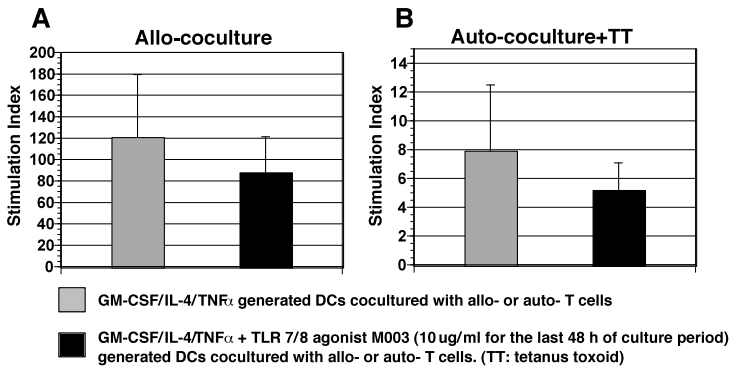


Figure 6. T cell proliferation in allo- and auto-coculture assays with TNFα and M003 treated DCs. TNFα matured DCs and those treated with M003 were cocultured with enriched allogeneic T cells (**A**) or with autologous T cells (**B**) in the presence of tetanus toxoid antigen (10 ug/ml) in a DC:T cell ratio of 1:10 in a 96 well tissue culture plate. T cell proliferation was determined by incorporation of [^{3}H]-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 [^{3}H]-Thymidine incorporation (mean of triplicates) over background [^{3}H]-Thymidine incorporation (mean of triplicates) of the unstimulated T cell population.

Comparision 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 IFNγ (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 IFNγ (1.95%; SEM± 0.5) without affecting the percentage of cells expressing IL-10 (1.2%; SEM± 0.6) as shown in Figure 7.

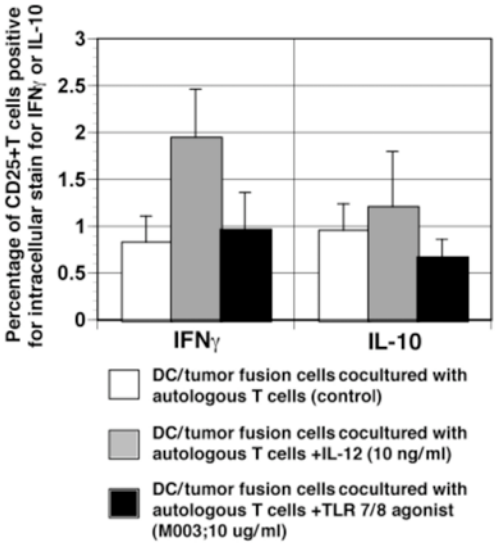


Figure 7. Effect of exogenous IL-12 and TLR 7/8 (M003) on DC/tumor fusion cell stimulation of activated and T regulatory cells. *DC/breast cancer fusion cells were cocultured with autologous non-adherent cells for 5 days in the presence of exogenous recombinant human IL-12 (10 ng/ml) or M003 (10 ug/ml) 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 IFNγ 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 IFNγ 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 comparision, we examined the effects of CpG ODN on DC maturation (Figure 8). 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).

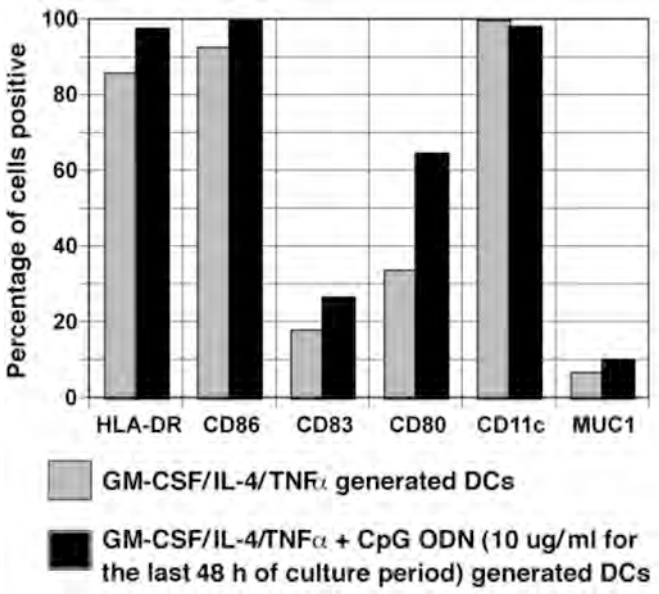


Figure 8. Phenotypic analysis of TNFα and CpG ODN treated monocyte derived DCs. *5 day GM-CSF/IL-4 cultured DCs were treated with TNFα (25 ng/ml) or additionally with CpG ODN (10 ug/ml) for the last 48 h of culture period. Cells were harvested and labeled with primary antibody for the surface markers 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

We also examined the effects of CpG ODN on promoting fusion-mediated expansion of activated as compared to regulating T cells (Figure 9). 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).

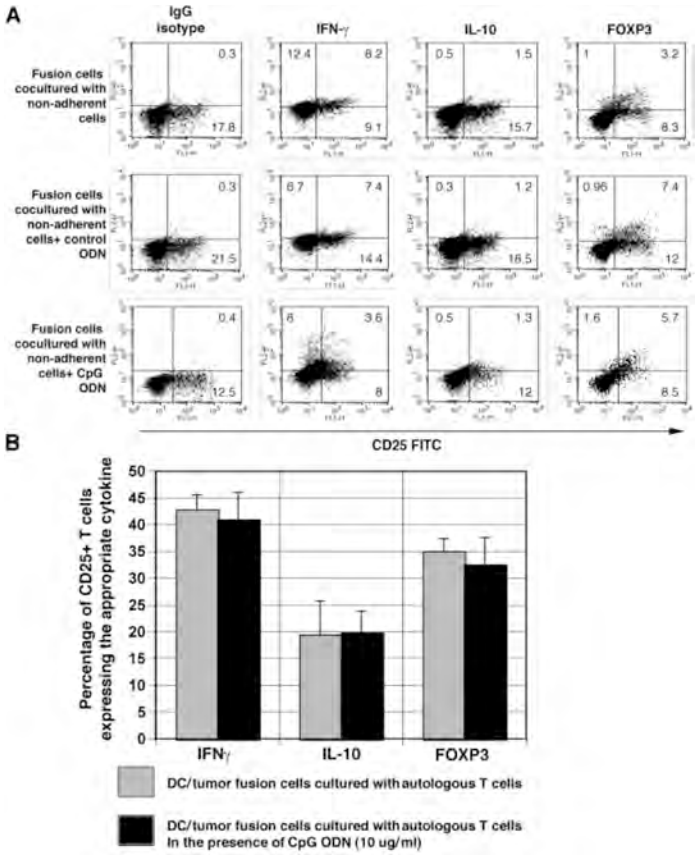


Figure 9. Expression of IFNγ, IL-10 and FOXP3 by regulatory T cells after stimulation with CpG ODN treated DC/tumor fusion cells*. CpG ODN treated DCs were fused with breast carcinoma cells and cocultured with autologous nonadherent T cells for 5 days. CD4+ T-cells were positively selected with magnetic 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.

Our pre-clinical studies thus far demonstrated 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/IFNγ 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 IFNγ 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 IFNγ production.

To further define the nature of the T cell response to DC/breast carcinoma fusions, we examined the functional characteristics of the expanded T cell population that co-express CD4 and CD25. Following stimulation with fusion cells, increased induction of $CD4^{+}CD25^{+high}$ with high mean fluorescence intensity are observed as shown in Figure 1A. To confirm the regulatory phenotype of this population of $CD4^{+}CD25^{+high}$ cells, their ability to suppress the activation of $CD4^{+}CD25^{-}$ cells was assessed using a co-culture invitro proliferation assay in a U-shaped 96 well plate.

Using strict gating parameters, regulatory T cells were identified as CD4 positive cells with a brighter CD25 staining than that of the CD4-negative or CD4 intermediate population (Figure 10A). The frequency of regulatory T cells was calculated as the percentage of $CD4^{+}CD25^{+high}$ cells in the CD4+ population, and was in the range of 0.09-3% of total CD4+ T cells analyzed. The $CD4^{+}CD25^{-}$ and $CD4^{+}CD25^{+high}$ population of cells were isolated from an average of between 40-50 $x10^{6}$ positively selected CD4+ T cells from the DC/tumor fusion cell coculture (>97 % purity) using a FACSVantage SE (BD Biosciences, San Jose, CA). These cells were incubated with 100 ul each of anti-CD4 TC (IgG2a, Invitrogen) and anti-CD25-FITC (IgG1, BD PharMingen). The analysis and sort gates were restricted to viable population of CD4+ T cells by means of their forward and side scatter properties. Large, activated CD4+ T cells were excluded. On reanalysis, the forward and side scatter properties of the $CD4^{+}CD25^{+high}$ cells were not appreciably different from those of the $CD4^{+}CD25^{-}$population suggesting that these cell population to be similar in size. Upon flow bidimensional flow cytometric analysis of CD4+ T cells positively selected from the DC/breast carcinoma fusion cell cocultures demonstrated the presence of highest level of CD25 ($CD4^{+}CD25^{+high}$ with high mean fluorescence intensity) appearing as a tail to the right from the major population containing both $CD4^{+}CD25^{+low}$ and $CD4^{+}CD25^{-}$ cells as compared to unstimulated T cells (Figure 10A). The $CD4^{+}CD25^{+high}$ represents between 1-3% of the total $CD4^{+}$ T cell population, whereas the $CD4^{+}CD25^{+low}$ cells can represent up to 16% of CD4+ T cells.

For suppression assay, $CD4^+CD25^-$ T cells ($5x10^4$cells/well) were cocultured in triplicate in the presence or absence of $CD4^+D25^{+high}$ T cells ($5x10^4$cells/well, 1:1 ratio) with Tetanus Toxoid (10 ug/ml) or with I ug/ml of anti-CD3 antibody (clone UCHT1; BD PharMingen) in the presence of irradiated (3500 Rad) T-cell depleted autologous PBMNCs ($2.5x10^5$ cells/well) as antigen presenting cells (APCs). As a control, equal numbers of $CD4^+CD25^-$ cells were added. APCs were isolated by negative selection of autologous PBMNCs incubated with anti-CD3 coated magnetic beads (Miltenyi Biotec, CA) according to manufacturer's protocol. In addition, $CD4^+CD25^-$ T cells were also stimulated with PHA (4 ug/ml) in the presence or absence of $CD4^+D25^{+high}$ T cells. The ability of $CD4^+CD25^{+high}$ to suppress proliferation was assessed by [^{3}H]Thymidine (1 uCi [0.037 MBq] per well) incoporation pulsed on day 4 (day 3 for PHA stimulated cocultures) and quantified 18 hours later.

Presence of the $CD4^+D25^{+high}$ cells resulted in significant inhibition of proliferation as determined by thymidine uptake following overnight pulsing in cocultures stimulated with anti-CD3 and tetanus toxoid recall antigen (Figure 10 B). Similarly, mitogenic stimulation of $CD4^+CD25^-$ in the presence or absence of $CD4^+D25^{+high}$ cells at a 1:1 ratio also demonstrated significant inhibition in proliferation (Figure 10 B). The level of suppression seen correlated with the ratio of $CD4^+CD25^-$:$CD4^+D25^{+high}$ (1:1) cells in the culture, with more $CD4^+D25^{+high}$ cells resulting in more suppression of $CD4^+CD25^-$ cell proliferation (data not shown). These results are not due to exhaustion of resources within the culture system because the addition of the same amount of $CD4^+CD25^-$ cells instead of $CD4^+D25^{+high}$ cells does not cause suppression (Figure 10B).

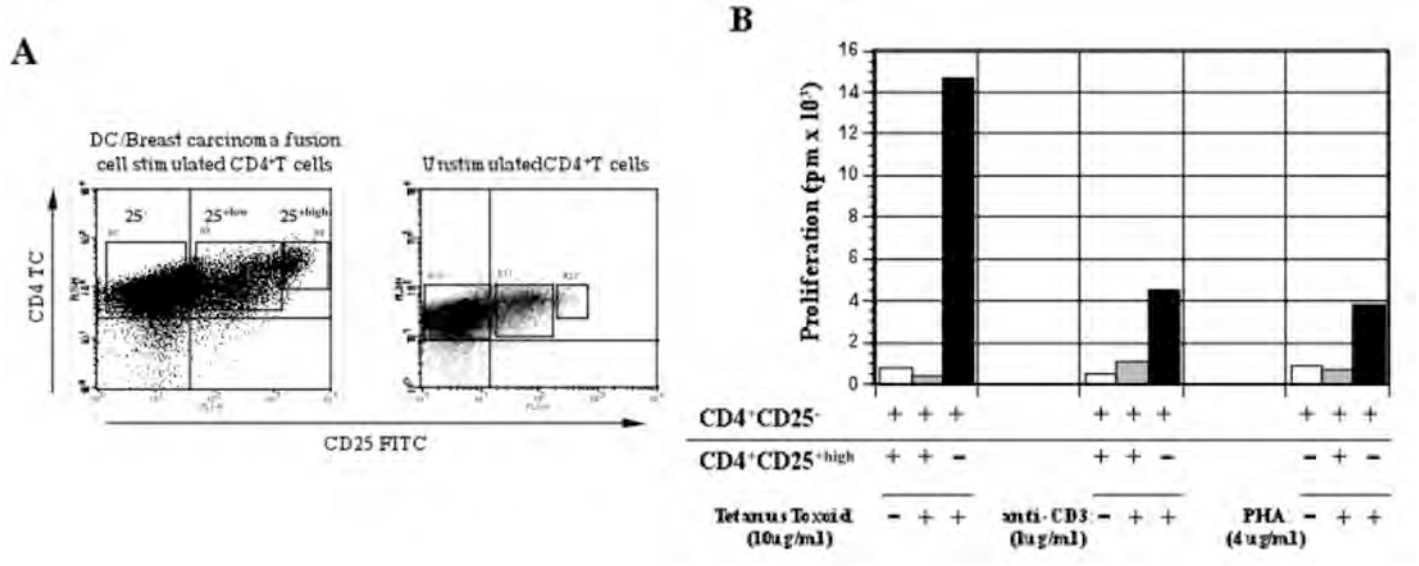


***Figure 10. Induction of $CD4^+CD25^{+high}$ regulatory T cells in the DC/breast carcinoma fusion cell cocultures**. **(A)** Representative bidimensional dot plot FACS analysis of unstimulated and DC/Breast carcinoma fusion cell stimulated T cells after positive selection of CD4 T cells followed by staining with anti-CD4 TC and anti-CD25 FITC antibodies. **(B)** Proliferation and suppression of $CD4^+CD25^-$ stimulated with anti-CD3 antibody, tetanus toxoid or PHA in the presence or absence of $CD4^+CD25^{+high}$ (1:1 ratio) cells. Control group of experiments constituted an addition of equal number of $CD4^+CD25^-$ cells. The bar graph represents the mean (±SEM) of four separate experiments.*

The $CD4^+D25^{+high}$ population was further analyzed by the expression of Foxp3. Greater than 75% of the FACS sorted $CD4^+D25^{+high}$ T cells expressed Foxp3, whereas the $CD4^+D25^-$ population showed virtually no expression (Figure 11), indicating that Foxp3 expression correlates well with the

regulatory activity of the $CD4^{+}D25^{+high}$ T cells. These data demonstrate that DC/breast carcinoma fusion cells induce the expansion of a T cell population with phenotypic and functional characteristics of regulatory T cells.

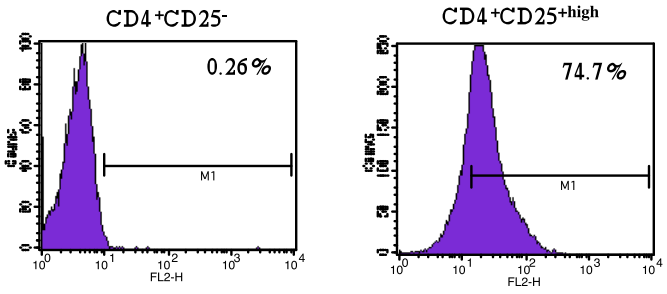


Figure 11. Expression of Foxp3 in $CD4^{+}D25^{+high}$ T cells. FACS sorted population of both $CD4^{+}D25^{+high}$ and $CD4^{+}D25^{-}$T cells were fixed and permeabilized followed by intracellular staining with PE-conjugated Foxp3 antibody. The cells were analyzed of a single FL2-H channel. No expression of Foxp3 was observed in the $CD4^{+}D25^{-}$ cells as compared to $CD4^{+}D25^{+high}$ T cells.

Selective expansion of activated T cells with DC/breast carcinoma fusions followed by anti-CD3/CD28

We also examined several strategies to enhance the capacity of DC/breast carcinoma fusions to stimulate anti-tumor immunity and limit the expansion of regulatory T cells. We hypothesized that combination of antigen specific stimulation with DC/tumor fusions and nonspecific ligation of the T cell costimulatory complex (CD3/CD28) would result in the activation of tumor specific lymphocytes. In preliminary studies, we demonstrated that combined stimulation with DC/breast carcinoma fusion and anti-CD3/CD28 resulted in the expansion of tumor reactive T cells with a predominantly activated phenotype.

We then expanded these studies to examine the phenotypic and functional characteristics of T cells undergoing sequential stimulation with DC/breast carcinoma fusions and anti-CD3/CD28 (Figure 12). Limited proliferation of T cells was observed following exposure to anti-CD3/CD28 alone (SI: 1.5 ±0.5 SEM; n=7) or DC/breast carcinoma fusions (SI 3.1 ±1.2 SEM; n=7). However, a marked increase in T cell expansion was noted when T cells were first stimulated with DC/breast carcinoma fusions and then expanded with anti-CD3/CD28 (SI: 23 ±8.73 SEM; n=7) (Figure 12). Of note, no increase in proliferation was observed when T cells were first exposed to anti- CD3/CD28 and then cultured with DC/breast carcinoma fusions (SI: 1.6 ±0.3 SEM; n=6).

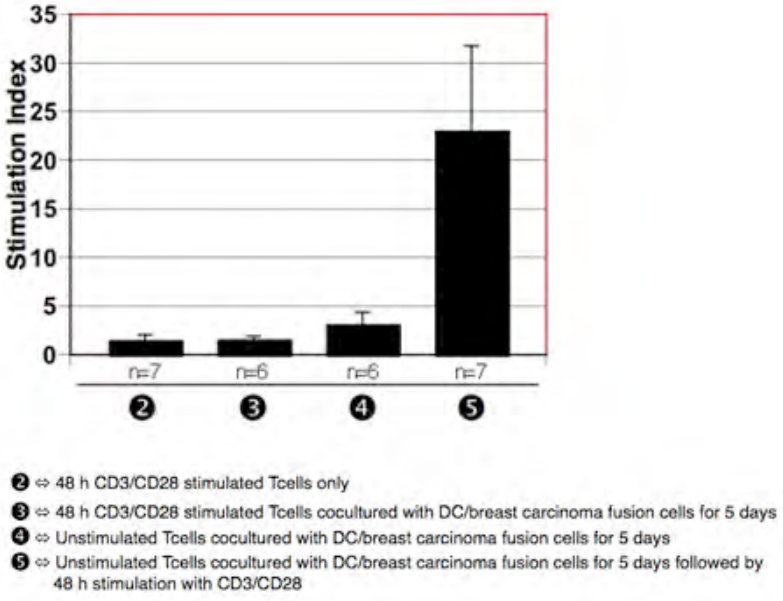


Figure 12. Combined stimulation with DC/breast carcinoma fusion cells and CD3/CD28 ligation*. Autologous T cells were stimulated by culture with: DC/breast carcinoma fusion cells for 5 days, anti-CD3/CD28 coated plates for 48 hours, anti-CD3/CD28 followed by DC/breast carcinoma fusions, or DC/breast carcinoma fusions followed by anti-CD3/CD28. Results were compared to unstimulated T cells. Mean T cell proliferation was determined for all culture conditions (n=6-7). T cells were aliquoted at 1x10^5/well in triplicate in a 96 well tissue culture plate and pulsed with 1 uCi/ml of ^{3}[H]-Thymidine for a period of 18-24 h. T cell proliferation was determined as described in materials and methods section. Results were normalized by calculation of stimulation index (SI) and is presented for each of the culture conditions listed.*

Sequential stimulation with DC/breast carcinoma fusion and anti-CD3/CD28 resulted in the specific expansion of tumor reactive T cells. Exposure to anti-CD3/CD28 following fusion cell stimulation induced a 13.7 mean fold increase in MUC1 tetramer binding cells (n=3) (Figure 13). The percentage of MUC1 tetramer positive cells remained at baseline levels following stimulation with anti-CD3/CD28 alone.

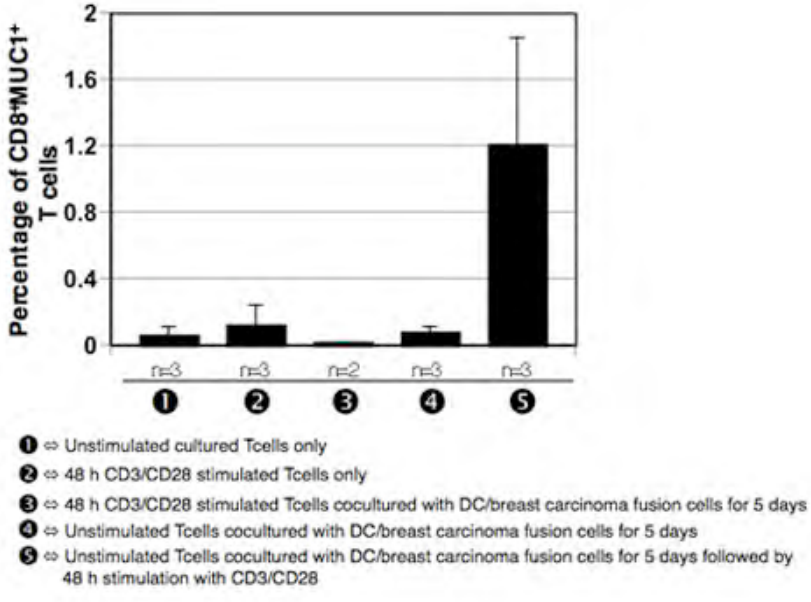


Figure 13. Combined stimulation with DC/breast carcinoma fusion cells and CD3/CD28 ligation*. Autologous T cells were stimulated by culture with: DC/breast carcinoma fusion cells for 5 days, anti-CD3/CD28 coated plates for 48 hours, anti-CD3/CD28 followed by DC/breast carcinoma fusions, or DC/breast carcinoma fusions followed by anti-CD3/CD28. Results were compared to unstimulated T cells. Mean expression of CD8+MUC1+ T cells using PE-conjugated MUC1 specific tetramers is presented is for each of the culture conditions listed.*

With regard to the phenotype of the expanded T cell population, the percentage of T cells expressing the CD4+CD25+ phenotype was markedly increased following sequential stimulation with DC/tumor

fusions and anti-CD3/CD28 (28%) as compared to T cell stimulated by anti-CD3/CD28 (11%) or fusions alone (10%) (n=6) (Figure 14A). As compared to fusion cells alone, sequential stimulation with DC/breast carcinoma fusions and anti-CD3/CD28 resulted in a 5 and 4 fold increase of CD4+CD25+ cells that coexpressed CD69 (Figure 14B) and IFNγ (Figure 14C). In contrast, an approximately 5 fold increase of regulatory T cells was also observed as manifested by an increase in CD4+CD25+ T cells that expressed Foxp3 (Figure 14D).

These results suggest that fusion mediated stimulation followed by anti-CD3/CD28 expansion induces increased levels of both activated and regulatory T cells.

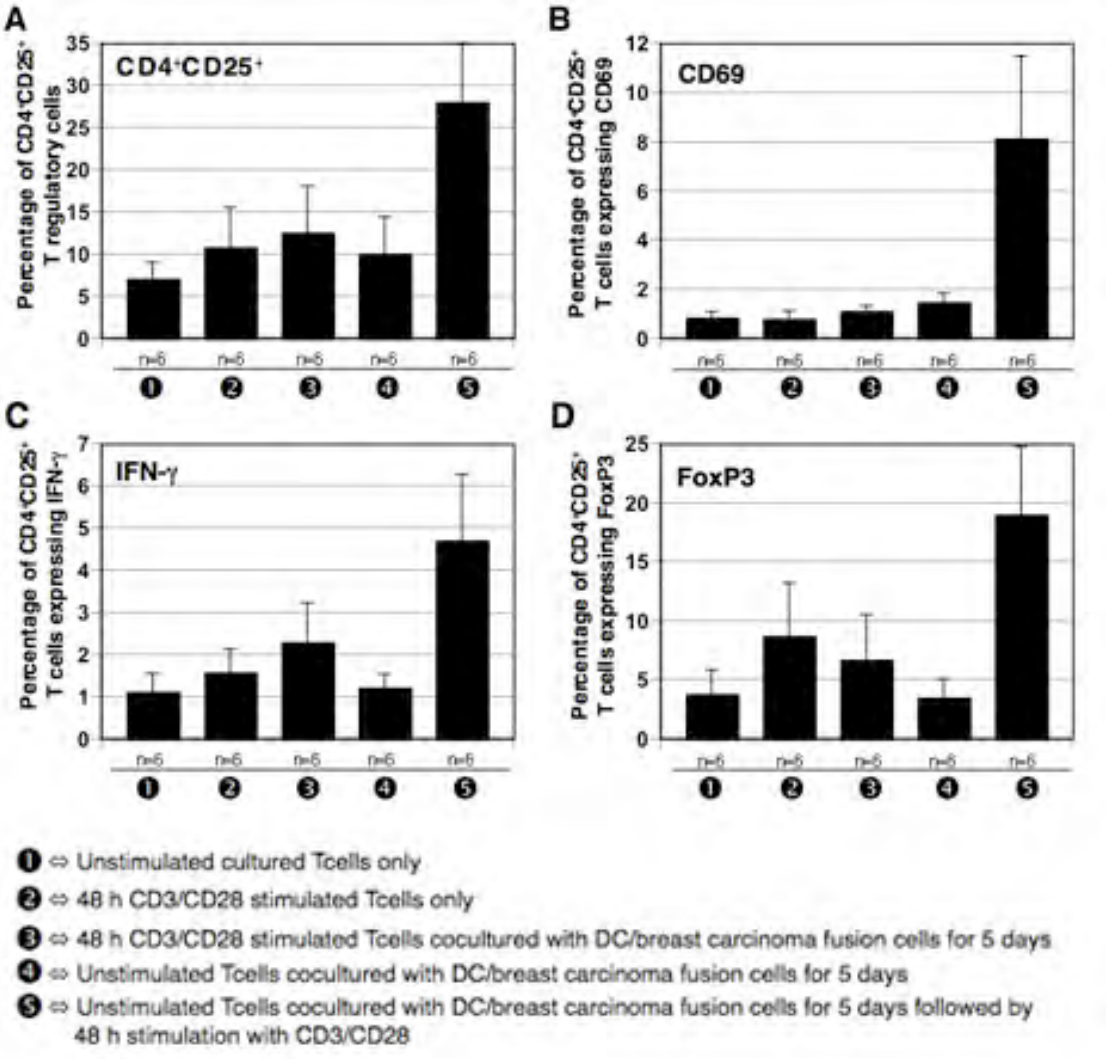


Figure 14. Combined stimulation with DC/breast carcinoma fusion cells and CD3/CD28 ligation*. Autologous T cells were stimulated by culture with: DC/breast carcinoma fusion cells for 5 days, anti-CD3/CD28 coated plates for 48 hours, anti-CD3/CD28 followed by DC/breast carcinoma fusions, or DC/breast carcinoma fusions followed by anti-CD3/CD28. Results were compared to unstimulated T cells. (**A**) Mean expression of CD4+CD25+ T cells (n=6), **(B)** CD4+CD25+CD69+ T cells (n=6), **(C)** IFNγ expressing CD4+CD25+ T cells (n=5), and **(D)** Foxp3 expressing CD4+CD25+ T cells is presented for each of the culture conditions listed.*

Clinical Trial

Eight patients were screened and seven were enrolled to the study. One patient consented to the study, but later elected to pursue only standard of care therapy and was not registered on the trial. Of the seven participants enrolled, three completed vaccinations; two were enrolled to study but later elected to pursue only standard of care therapy and were taken off study; two were taken off study for disease progression prior to the initiation of vaccination.

Summary of Subject Study Information

Table 1: Screen Failures to Date

SUBJECT	SCREENING#	DATE OF CONSENT	AGE	GENDER/RACE	REASON FOR WITHDRAWAL/ SCREEN FAILURE
PP	7	4/2/12	56	FEMALE/ CAUCASIAN	ELECTED TO PURSUE ONLY STANDARD OF CARE THERAPY

Table 2: Subjects Enrolled to Date

SUBJECT	SCREENING #	ENROLLMENT #	DATE OF CONSENT	DATE OF REGISTRATION	AGE	GENDER/ RACE	COHORT	OFF-STUDY DATE	REASON OFF-STUDY
BV01 (EG)	1	1	9/7/10	9/21/10	58	FEMALE/ CAUCASIAN	1	2/3/11	DISEASE PROGRESSION
BV02 (CB)	2	2	2/10/11	3/8/11	77	FEMALE/ CAUCASIAN	1	3/9/11	WITHDREW CONSENT
BV03 (DK)	3	3	4/26/11	4/29/11	48	FEMALE/ CAUCASIAN	1	6/9/11	DISEASE PROGRESSION
BV04 (JB)	4	4	6/30/11	7/14/11	56	FEMALE/ CAUCASIAN	1	10/31/11	DISEASE PROGRESSION
BV05 (ME)	5	5	8/22/11	9/8/11	81	FEMALE/ CAUCASIAN	1	10/21/11	DISEASE PROGRESSION
BV06 (LR)	6	6	2/6/12	2/16/12	47	FEMALE/ CAUCASIAN	1	8/17/12	DISEASE PROGRESSION
BV07 (CC)	7	7	6/26/12	6/29/12	39	FEMALE/ CAUCASIAN	2	N/A	N/A

Table 3: Participants Treated

SUBJECT	COHORT/DOSE	TREATMENT DATES	Clinical Outcome
BV01 (EG)	COHORT 1/ DOSE: $5x10^6$	#1. 11/23/10 #2. 12/14/10 #3. 01/04/11	Subject developed progressive disease at 1 month following final vaccine.
BV04 (JB)	COHORT 1/ DOSE: $5x10^6$	#1. 08/25/11 #2. 09/15/11 #3. 10/06/11	Subject developed progressive disease at 1 month following final vaccine.
BV06 (LR)	COHORT 1/ DOSE: $5x10^6$	#1. 05/10/12 #2. 05/31/12 #3. 06/21/12	Subject developed progressive disease at 2 months following final vaccine.

Treatment Related Adverse Events to Date

Patient ID	AE	Onset	CTC Grade	Relationship	Action Taken Regarding TX	Outcome
BV01 - EG	Rash, R leg	1/13/11	1	Possibly	Seen by dermatologist, biopsy was negative; treated with Triamcinolone ointment	Ongoing- Off study 2/3/11
BV04 – JB	ESR, elevated	9/15/11	1	Possibly	None	Ongoing- off study 10/31/11
BV04 – JB	Myalgias, generalized	9/17/11 10/7/11	1 1	Possibly	None	Resolved
BV04 – JB	Ecchymosis, injection site	9/17/11 10/7/11	1 1	Definitely	None	Resolved
BV04 – JB	Induration, L thigh injection site	9/16/11	1	Definitely	None	Resolved
BV04 – JB	Erythema, L thigh injection site	9/16/11	1	Definitely	None	Resolved
BV04 – JB	Pain, sternum	9/26/11	1	Possibly	None	Resolved
BV06 – LR	Nausea	5/10/12	1	Definitely	None	Resolved
BV06 – LR	Ecchymosis, injection site	5/14/12	1	Possibly	None	Resolved
BV06 – LR	Nausea	5/31/12	1	Definitely	None	Resolved

Treatment Related Serious Adverse Events

There have been no serious adverse events related to study treatment.

Detailed Immune monitoring of patients BV01, BV04 and BV06

Immune monitoring of Patient BV01:

Induction of tumor-reactive lymphocytes by DC/breast cancer cell fusion vaccine

To assess the effects of fusion cell vaccination on the capacity of patients to mount a tumor-specific CTL response, we measured the immunologic response to vaccination by determining the percentage of circulating $CD4^+$ and $CD8^+$ T cells that recognize autologous breast carcinoma cells as manifested by the percentage of cells that express IFN-γ after ex-vivo exposure to autologous tumor lysate. Immunologic assessments were performed before each vaccination (three time points) and at one month after the last vaccine (Figure 1). The peak response after vaccination was compared with pre-vaccination levels of tumor-reactive T cells to assess the fold increase in tumor-reactive T cells after vaccination. At each time point, mononuclear cells were isolated from peripheral blood by Ficoll density centrifugation and cryopreserved. After completion of the study, PBMCs were thawed, and 1×10^6 cells were cultured with lysate generated by repeated freeze/thaw cycles of 1×10^5 autologous breast carcinoma cells. As a control, PBMCs were cultured with tetanus toxoid (10 µg/mL) or media alone. After 5 days of coculture, expression of IFN-γ by $CD4^+$ and $CD8^+$ populations was determined by intracellular FACS analysis. Cells were restimulated with tumor lysate for 4 hours in the presence of 1 µg/mL GolgiStop (BD Biosciences). The cells were stained with FITC-conjugated CD4 or CD8 antibodies and permeabilized with Cytofix/Cytoperm Plus (BD Biosciences). Cells were thereafter stained with PE-conjugated anti–human IFN-γ, fixed in 2% paraformaldehyde, and analyzed by flow cytometry. A slight increase in the percentage of CD4 and CD8 T cells expressing IFNγ was observed at the pre-vaccination #2 time point, but this response tapered off after the patient received the third vaccine (Figure 1).

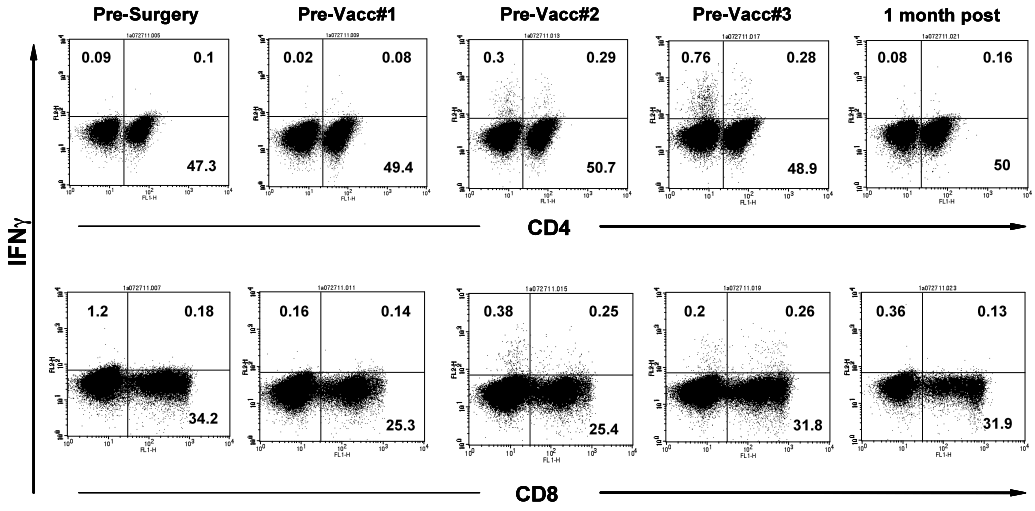


Figure 1. ***Expression of IFNγ by CD4 and CD8 populations before and after vaccination.*** *PBMCs isolated before each vaccination and at serial time points after vaccination were cocultured with autologous tumor lysate, pulsed with GolgiStop, labeled with FITC-conjugated CD4 or CD8 antibodies, and then permeabilized by incubation in Cytofix/Cytoperm Plus. Cells were then incubated with PE conjugated anti–IFNγ or a matched isotype control antibody, fixed in 2% paraformaldehyde and thereafter analyzed by flow cytometry. Percentage of $CD4^+$ (upper panel) and $CD8^+$Tcells expressing IFNγ after ex vivo exposure to autologous tumor tumor lysate is shown. Numbers in the quadrants depict the percentage of cells positive.*

Vaccine potency and Phytohemagglutinin and tetanus-induced induced T-cell proliferation

As a measure of potency of the generated vaccine as antigen-presenting cells, the capacity of the DC, breast carcinoma, and fusion cells to stimulate proliferation of allogeneic T cells was measured as shown below. In addition, freshly isolated patient PBMCs (1×10^5) isolated at serial time points (as outlined above) were cocultured in 200 µl of medium in 96-well U-bottomed plates for 4 days with 4 µg/ml PHA and tetanus toxoid (10 µg/mL (Figure 2). Proliferation was determined by measuring incorporation of [^{3}H]-Thymidine after overnight pulsing of triplicate samples. Data is presented as Stimulation Index. DC/Tumor fusion cells showed higher stimulation index as compared to tumor or dendritic cells alone. PHA stimulation was more enhanced at one month post-vaccination #3, whereas Tetanus Toxoid response to patient PMNCs did not change after the second vaccine as shown below.

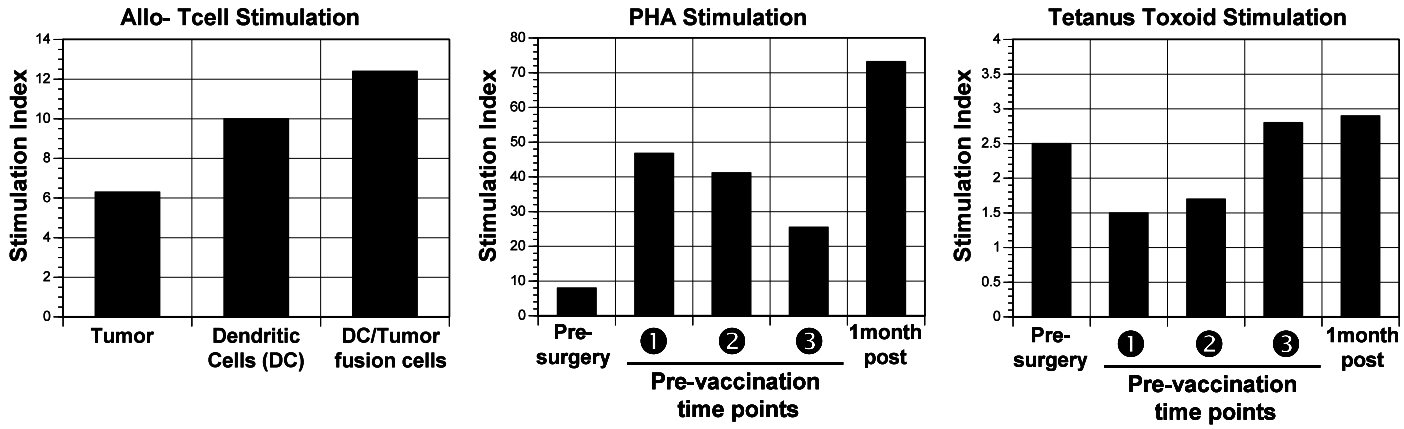
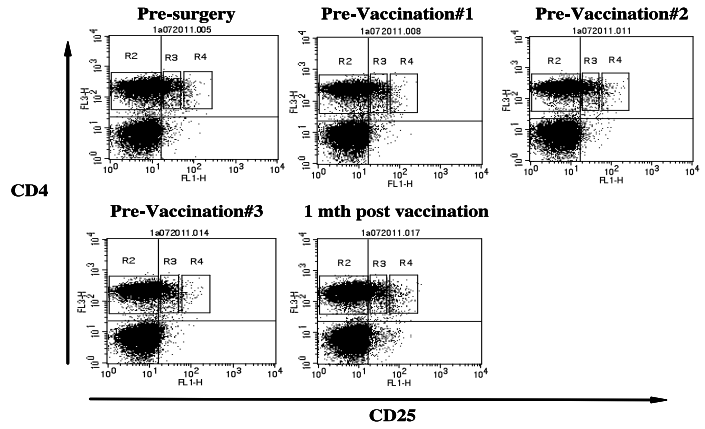


Figure 2. Potency of fusion cells in the stimulation of allogeneic T-cell proliferation and proliferation capacity of patient PBMCs pre- and post-vaccination*. Patient-derived DCs, breast carcinoma cells, and fusion cells were cocultured with T cells from a healthy donor at a T-cell:target ratio of 1:10. Patient derived PBMCs at serial time points pre- and post-vaccination were thawed and subjected to PHA and Tetanus Toxoid stimulation. Cocultures were incubated for 5 days, and T-cell proliferation was determined by incorporation of [^{3}H]-thymidine (1 µCi/well) after overnight pulsing. Stimulation index (SI) represents counts per minute (CPM) of sample per CPM of unstimulated T cells.*

Quantification of regulatory T cells

As outlined above, PBMCs were isolated at serial time points pre- and post-vaccination, and regulatory T cells were quantified by determining the percentage of CD4/CD25^{high} T cells by bidimensional FACS analysis (Figure 3). Expression of FOXP3 by CD4/CD25 cells was measured in by intracellular FACS analysis (Figure 4). No significant changes were observed in the expression of CD4/CD25^{high} T cells at pre-surgery and post-vaccination time points. Similarly, the expression of CD4/CD25 expressing FOXP3 did not change dramatically as depicted in Figure 3.



BV01-Tregs	Percentage of Cells positive		
	Gate R2	Gate R3	Gate R4
	CD4+Cd25-	CD4+CD25+low	CD4+CD25+high
Pre-Surgery	40.2	4.8	0.63
Pre-Vaccination # 1	39.4	4.9	0.63
Pre-Vaccination # 2	37.4	4.6	0.66
Pre-Vaccination # 3	35.8	3.8	0.38
I mth post vaccination	39.1	3.5	0.86

Figure 3*. Phenotypic characterization of $CD4^{+}CD25^{-}$, $CD4^{+}CD25^{+low}$, and $CD4^{+}CD25^{+high}$ T cells pre- and post-vaccination. Thawed PBMCs were cultured for 2 hours and then incubated with anti-CD4 TC and anti-CD25 FITC. Tcells were then separated into $CD4^{+}CD25$, $CD4^{+}CD25^{+low}$,and $CD4^{+}CD25^{+high}$ fractions by bidimensional FACS sorting as shown in a representative dot plot. Quadrant gates were set up to distinguish the three observed populations of Tregs and the percentage of each cell type determined. The Table depicts the percentage of cells positive in each of the three quadrants at serial time points.*

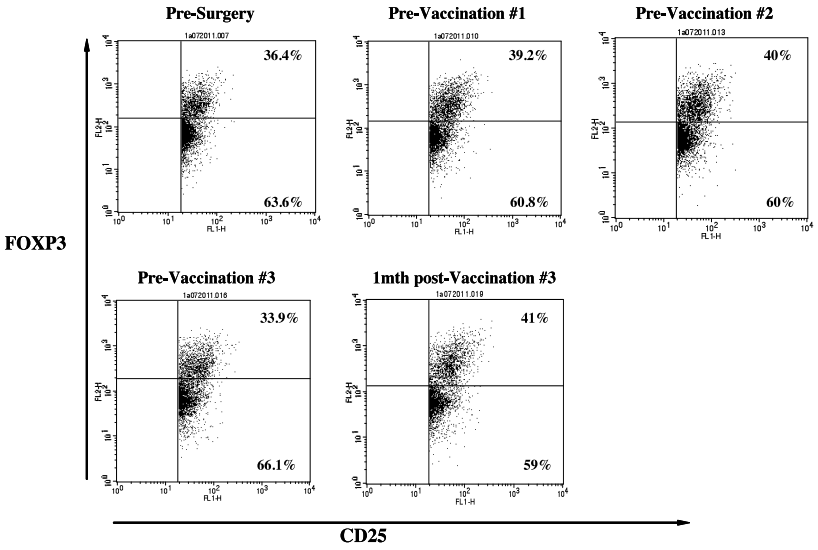


Figure 4. *Thawed PBMCs at serial time points were cultured for 2 hours and stained with anti-CD4 TC and anti-CD25 FITC followed by treatment with Cytofix Cytoperm Plus. Expression of FOXP3 by CD4/CD25 cells was measured by intracellular staining for FOXP3.*

Patient BV01 with metastatic breast cancer was vaccinated with fusion cells generated from patient-derived tumor cells and autologous DCs. The results show that both CD4 and CD8 T cells expressed IFNγ post-vaccination suggesting an establishment of anti-tumor activity that tapered off at 1 month post-vaccination # 3. Furthermore, weak responses to PHA and Tetanus Toxoid were observed post vaccination, whereas, no dramatic change was observed in the levels of regulatory T cells or those that expressed FOXP3 pre- and post-vaccination.

Immune monitoring of Patients BV04 and BV06:

Induction of tumor-reactive lymphocytes by DC/breast cancer cell fusion vaccine

To assess the effects of fusion cell vaccination on the capacity of patients to mount a tumor-specific CTL response, we measured the immunologic response to vaccination by determining the percentage of circulating $CD4^+$ and $CD8^+$ T cells that recognize autologous breast carcinoma cells as manifested by the percentage of cells that express IFN-γ after ex-vivo exposure to autologous tumor lysate. Immunologic assessments were performed before each vaccination (three time points) and at one month after the last vaccine. The peak response after vaccination was compared with pre-vaccination levels of tumor-reactive T cells to assess the fold increase in tumor-reactive T cells after vaccination. At each time point, mononuclear cells were isolated from peripheral blood by Ficoll density centrifugation and cryopreserved. After completion of the study, PBMCs were thawed, and 1×10^6 cells were cultured with lysate generated by repeated freeze/thaw cycles of 1×10^5 autologous breast carcinoma cells. As a control, PBMCs were cultured with tetanus toxoid (10 μg/mL) or media alone. After 5 days of coculture, expression of IFN-γ by $CD4^+$ and $CD8^+$ populations was determined by intracellular FACS analysis. Cells were restimulated with tumor lysate for 4 hours in the presence of 1 μg/mL GolgiStop (BD Biosciences). The cells were stained with FITC-conjugated CD4 or CD8 antibodies and permeabilized with Cytofix/Cytoperm Plus (BD Biosciences). Cells were thereafter stained with PE-conjugated anti–human IFNγ, fixed in 2% paraformaldehyde, and analyzed by flow cytometry. An increase in the percentage of CD4 and CD8 T cells expressing IFNγ was observed at the pre-vaccination #2 and #3 time point, but this response tapered off after the patient received the third vaccine (Figure 5). As an additional measure of anti-tumor immunity in HLA-A 2.1 positive patients, the number of CD8+ T cells binding the MUC1 tetramer was determined by bidimensional FACS analysis using CD8-FITC and MUC1 tetramer-PE antibody (Figure 6).

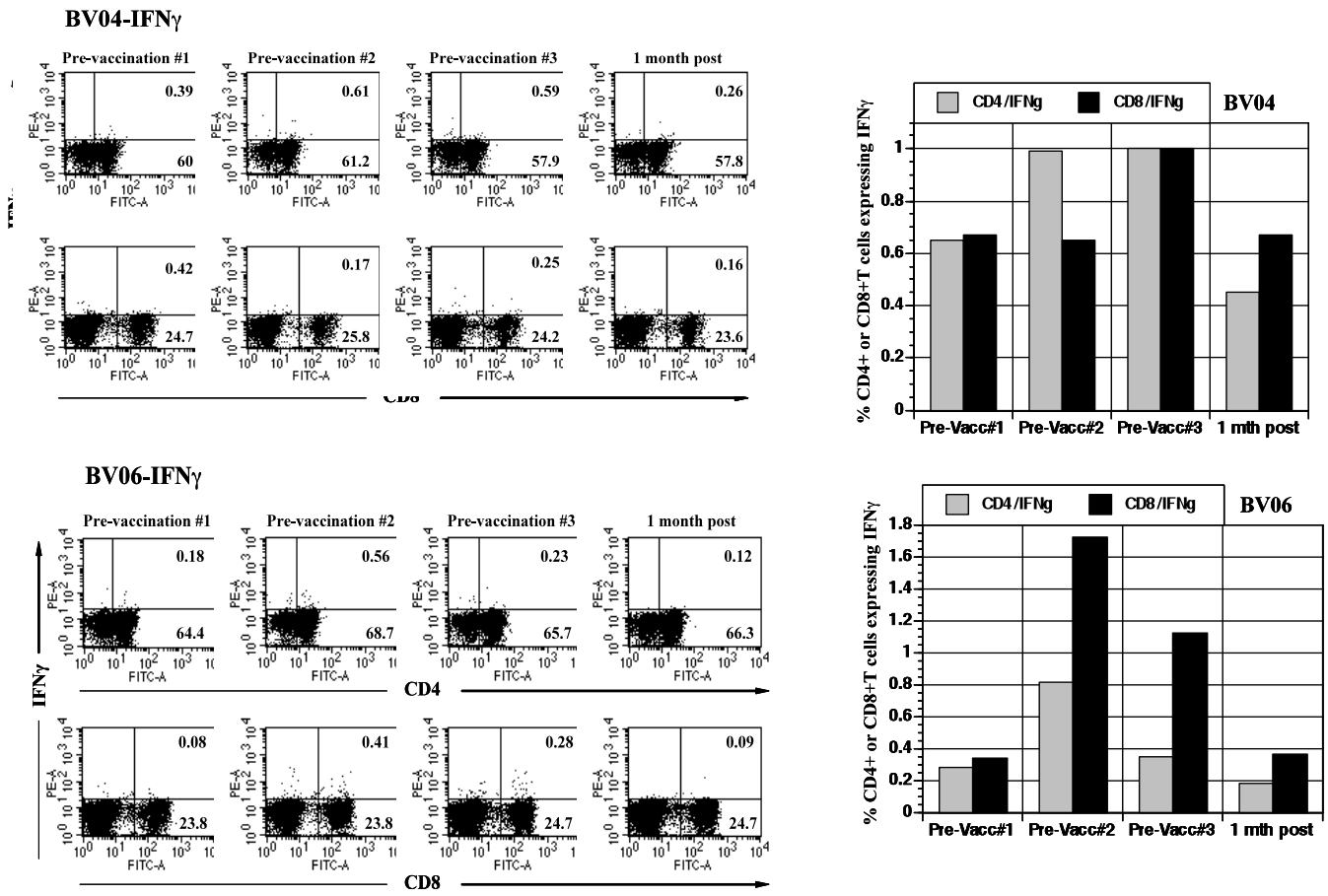


Figure 5. ***Expression of IFNγ by CD4 and CD8 populations before and after vaccination in patient number BV04 and BV06.*** *PBMCs isolated before each vaccination and at serial time points after vaccination were cocultured with autologous tumor lysate. After a 5 day culture period, the cells were re-stimulated with autologous tumor lysate in the presence of GolgiStop for four hours. Thereafter, the cells were harvested and labeled with FITC-conjugated CD4 or CD8 antibodies, and then permeabilized by incubation in Cytofix/Cytoperm Plus. Cells were then incubated with PE conjugated anti–IFNγ or a matched isotype control antibody, fixed in 2% paraformaldehyde and analyzed by flow cytometry. Dot plot analysis shows $CD4^+$ and $CD8^+$Tcells expressing IFNγ after ex vivo exposure to autologous tumor tumor lysate for patients BV04 and BV06. Corresponding bar graphs of the right depicts the total number of CD4+ and CD8+ T cells expressing IFNγ at serial time points. Numbers in the quadrants depict the percentage of cells positive.*

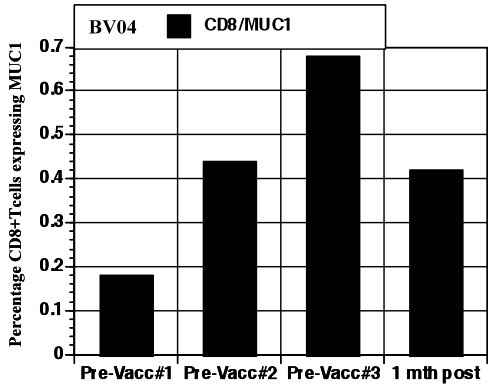


Figure 6. ***Expansion of MUC1 tetramer positive cells following vaccination in patient BV04.*** *CD8+ T cells binding the MUC1 tetramer were quantified at serial time points (prior to each vaccination and at 1month post vaccination) in patient BV04 who was determined to be HLA-A2.1. Binding to a control tetramer was quantified in parallel and the control value was subtracted from that obtained for the MUC1 tetramer.*

Vaccine potency and Phytohemagglutinin and tetanus-induced induced T-cell proliferation

As a measure of potency of the generated vaccine as antigen-presenting cells, the capacity of the DC, breast carcinoma, and fusion cells to stimulate proliferation of allogeneic T cells was measured as shown below. In addition, freshly isolated patient PBMCs (1×10^5) isolated at serial time points (as outlined above) were cocultured in 200 µl of medium in 96-well U-bottomed plates for 4 days with 4 µg/ml PHA and tetanus toxoid (10 µg/mL) (Figure 7). Proliferation was determined by measuring incorporation of [^{3}H]-Thymidine after overnight pulsing of triplicate samples. Data is presented as Stimulation Index. DC/Tumor fusion cells showed higher stimulation index as compared to tumor or dendritic cells alone. PHA stimulation was more enhanced at one month post-vaccination #3, whereas Tetanus Toxoid response to patient PMNCs did not change after the second vaccine as shown below.

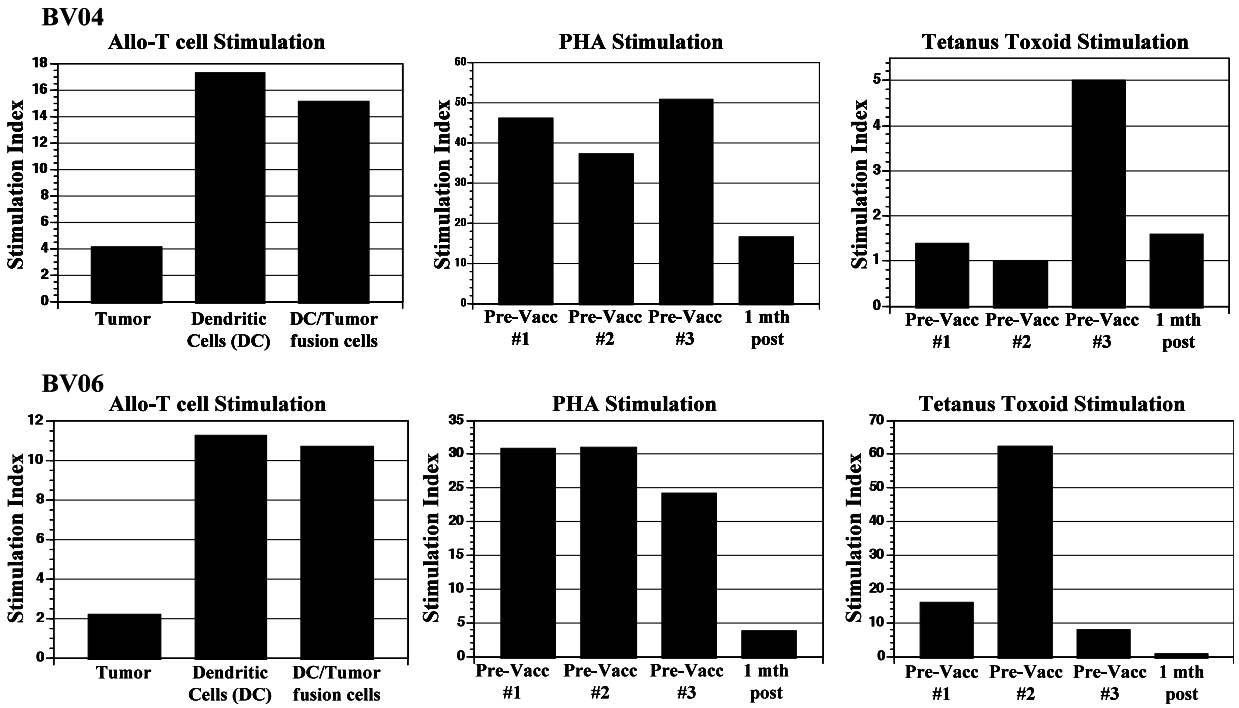
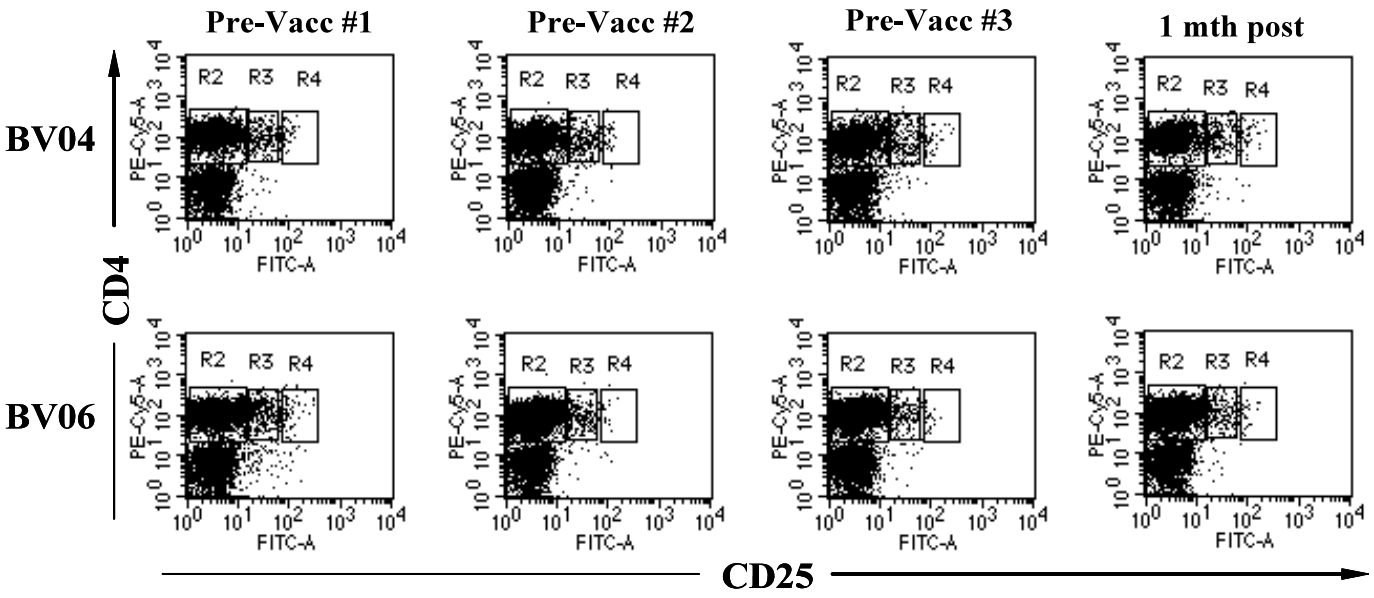


Figure 7*. Potency of fusion cells in the stimulation of allogeneic T-cell proliferation and proliferation capacity of patient PBMCs pre- and post-vaccinationfor patient number BV04 and BV06**. *Patient-derived DCs, breast carcinoma cells, and fusion cells were cocultured with T cells from a healthy donor at a T-cell:target ratio of 1:10. Patient derived PBMCs at serial time points pre- and post-vaccination were thawed and subjected to PHA and Tetanus Toxoid stimulation. Cocultures were incubated for 5 days, and T-cell proliferation was determined by incorporation of [^{3}H]-thymidine (1 µCi/well) after overnight pulsing. Stimulation index (SI) represents counts per minute (CPM) of sample per CPM of unstimulated T cells.*

Quantification of regulatory T cells

As outlined above, PBMCs were isolated at serial time points pre- and post-vaccination, and regulatory T cells were quantified by determining the percentage of CD4/CD25^{high} T cells by bidimensional FACS analysis (Figure 8). Expression of FOXP3 by CD4/CD25 cells was measured in

by intracellular FACS analysis (Figure 9). No significant changes were observed in the expression of CD4/CD25^{high} T cells at pre-surgery and post-vaccination time points, although and increase in CD4/CD25^{high} T cells was observed at 1 month (see table below). Similarly, the expression of CD4/CD25 expressing FOXP3 did not change dramatically as depicted in Figure 9.



	Percentage of Cells positive		
	Gate R2	**Gate R3**	**Gate R4**
	CD4+CD25-	CD4+CD25+low	CD4+CD25+high
BV04			
Pre-Vaccination #1	41.3	2.84	0.5
Pre-Vaccination #2	42.2	2.68	0.38
Pre-Vaccination #3	37.2	2.73	0.52
1 month post	38.6	2.89	0.77
BV06			
Pre-Vaccination #1	47.8	3.52	0.75
Pre-Vaccination #2	57.4	2.11	0.18
Pre-Vaccination #3	48.7	2.13	0.22
1 month post	49.86	2.48	0.46

Figure 8. ***Phenotypic characterization of CD4^{+}CD25^{-}, CD4^{+}CD25^{+low}, and CD4^{+}CD25^{+high} T cells pre- and post-vaccination.*** *Thawed PBMCs were cultured for 2 hours and then incubated with anti-CD4 TC and anti-CD25 FITC. T cells were then separated into CD4^{+}CD25, CD4^{+}CD25^{+low}, and CD4^{+}CD25^{+high} fractions by bi-dimensional FACS sorting as shown in a representative dot plot. Quadrant gates were set up to distinguish the three observed populations of Tregs and the percentage of each cell type determined. The Table depicts the percentage of cells positive in each of the three quadrants at serial time points.*

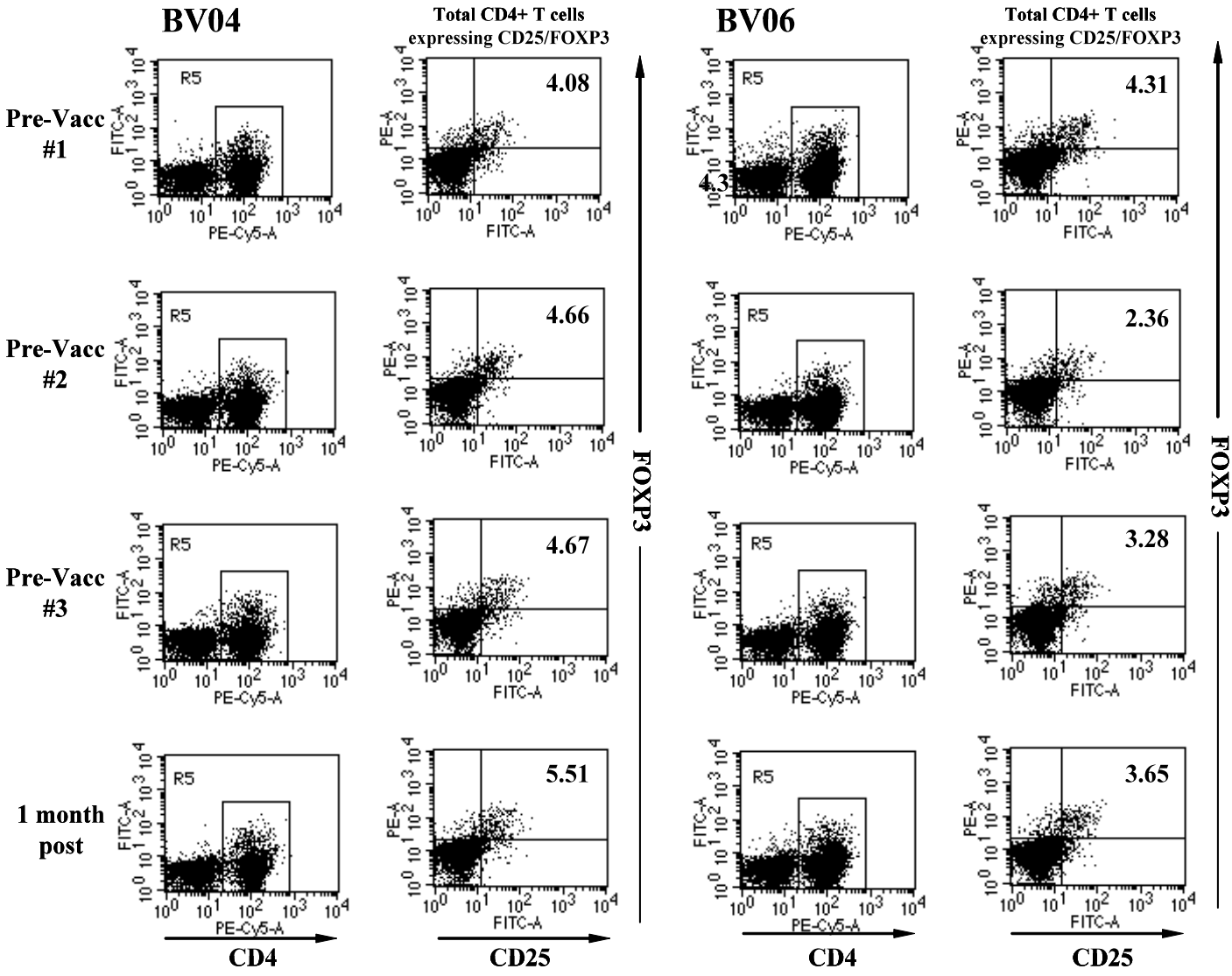


Figure 9. ***Total CD4+ T cells expressing CD25/FOXP3 in patients BV04 and BV06***. *Thawed PBMCs at serial time points were cultured for 2 hours and stained with anti-CD4 TC and anti-CD25 FITC followed by treatment with Cytofix/Cytoperm Plus. Expression of FOXP3 by CD4/CD25 cells was measured by intracellular staining for FOXP3. Total CD4+ t cells were gated as shown in the left panels for both patients BV04 and BV06 and analyzed for dual expressing CD25 and FOXP3 expressing cells in the upper right quadrant of dot plots as shown in the right panels.*

C. KEY RESEARCH ACCOMPLISMENTS

- Our pre-clinical studies demonstrated 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/IFNγ and IL-10/FOXP3, respectively.

- We found that an agonist of TLR7/8 increased expression of costimulatory and maturation markers on DCs. However addition of the TLR7/8 agonist to the fusion/T cell coculture did not significantly increase the relative percentage of activated T cells.

- Addition of IL-12 was associated with an increase in IFNγ producing cells.

- 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 IFNγ production.

D. REPORTABLE OUTCOMES

The results obtained from preclinical studies performed during the first 2 Tasks together with data obtained in the first 2-4 year of the award, have been integrated into a manuscript that was published in the Journal of Immunology (*J Immunol* 2008; 181:808-821). A copy of the manuscript is attached in the appendices section of this report. Moreover, our results were presented at the BCRP Era of Hope meetings in 2008 and 2011. Abstracts pertaining to these meeting are attached with this report.

E. CONCLUSION

During the course of this award leading up to the clinical trials, we were subjected to a lengthy review process and received approval from the FDA, IRB, and DOD to conduct a clinical trial in which patients with metastatic breast cancer would undergo vaccination with DC/breast carcinoma fusions in conjunction with IL-12. However, opening of the protocol was significantly delayed because issues relating to the availability of IL-12 from the NCI arose during the review process. We worked closely with Drs. Zweibel and Streicher at CTEP who assumed control of the IL-12 stocks and completed necessary potency testing for their release. In anticipation of trial initiation, the protocol and consent were updated and the revised editions were re-submitted to the IRB and FDA. Enrollment in the clinical trial was a challenge because of the requirement for patients with metastatic disease to have accessible tissue for which removal was clinically indicated and who met criteria for clinical stability. A significant number of patients were screened who could not go forward. As a result, the study was closed and we are considering the development of a future trial in which patients undergo vaccination as adjuvant therapy following primary surgery.

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G. APPENDICES

Abstracts of *Era of Hope* meetings attended

DOD Breast Cancer Research Program *Era of Hope* 2008

Dendritic cell (DC)/Breast carcinoma fusion cells in conjunction with IL-12, CPG ODN, or anti-CD3/CD28 results in the selective expansion of activated tumor reactive T cells.

Baldev Vasir, Zekui Wu, Adam Bissonnette, Jacalyn Rosenblatt, Donald Kufe, David Avigan

We have previously shown that vaccination of patients with DC/breast carcinoma fusions stimulates anti-tumor immune responses in a majority of patients with metastatic disease; however, only a subset demonstrate evidence of tumor regression. To define the factors that limit vaccine efficacy, we examined the biological characteristics of DC/breast carcinoma fusions as antigen presenting cells and the nature of the vaccine-mediated T cell response. Fusion of DCs with breast carcinoma cells results in increased expression of costimulatory and maturation markers, IL-12 and the chemokine receptor, CCR7. Of note, fusion cells also coexpressed IL-10, supporting their ability to deliver stimulatory and inhibitory signals to reactive T cell populations. DC/breast cancer fusions stimulate a mixed T cell response characterized by the expansion of both activated and regulatory T cell populations. Coculture of fusion cells and autologous T cells resulted in a statistically significant increase in CD4+CD25+ T cells that expressed CD69 and IFNγ as well as FOXP3 and CTLA-4, consistent with an activated and regulatory T cell phenotype. To further define the T cell response to DC/breast carcinoma fusions, regulatory and activated T cells were separated by flow cytometric sorting of $CD4^{+}CD25^{+high}$ and $CD4^{+}CD25^{+low}$ populations. $CD4^{+}CD25^{+high}$ but not $CD4^{+}CD25^{+low}$, cells uniformly expressed FOXP3. Consistent with a regulatory T cell phenotype, $CD4^{+}CD25^{+high}$ cells inhibited the proliferative responses of $CD4+CD25^{-}$ T cells to tetanus toxoid, anti-CD3, and PHA. These observations suggested that the increased presence of regulatory cells may potentially inhibit the in vivo efficacy of the fusion cell vaccine. As such, we examined several strategies to bias the fusion-mediated T cell response towards activated cells. We found that addition of IL-12, TLR7/8 agonists, CPG ODN, or IL-18 increased the relative presence of fusion-activated T cells and reduced the expansion of fusion-mediated regulatory T cells. We also found that the sequential stimulation with DC/breast carcinoma fusions and anti-CD3/CD28 results in the relative expansion of tumor specific T cells with an activated phenotype. These strategies thus provide the basis to enhance vaccine efficacy and represent a platform for the successful use of adoptive immunotherapy for patients with breast cancer.

DC-Breast Fusion cell Vaccination in conjunction with IL-12: Preclinical Studies and a Clinical Trial

David Avigan, MD; Baldev Vasir, PhD; Jacalyn Rosenblatt, MD, Heidi Mills, Poorvi Somaya, Lynne Uhl, Carol Delaney, Katerina Luptakova, Donald Kufe, MD

Background: We have developed a potent tumor vaccine in which patient derived tumor cells are fused with autologous DCs, and demonstrated that vaccination with DC/tumor fusions results in the eradication of established metastatic disease in animal models. The immunologic potency of fusion cells may be enhanced by the use of cytokine adjuvants, such as IL-12. **Methods:** In this project, we examined the nature of immune response to DC/breast fusion cells with respect to the stimulation of activated versus regulatory T cells, and examined strategies to limit the expansion of regulatory T cells both in vitro and in an ongoing clinical trial. **Results:** Stimulation with DC/breast carcinoma fusions in vitro elicits a complex response characterized by the expansion of CD4/CD25^{low} effector cells and CD4/CD25^{high} T cells that exhibit functional characteristics of regulatory T cells. CD4/CD25^{high} cells uniformly expressed FOXP3 and demonstrated phenotypic characteristics consistent with regulatory T cells as manifested by inhibition of T cell responses to PHA, allogeneic DCs, and tetanus toxoid. In contrast, CD4/CD25^{low} cells did not inhibit responses by CD4/CD25- cells. Stimulation of T cells with DC/tumor fusions in the presence of IL-12 resulted in the expansion of fewer regulatory T cells, suggesting that the use of IL-12 may enhance the response to vaccination. Based on preclinical studies, we have initiated a phase 1 clinical trial in which patients with metastatic breast cancer are treated with DC/tumor fusion cells and escalating doses on IL-12. An initial cohort of 3 patients are treated with the DC/tumor fusions alone. In the subsequent cohorts, fusion cells will be administered with rhIL-12 given subcutaneously at the time of vaccine administration and on days 3 and 5. The study was recently activated and one patient has been enrolled to date. The patient is 58 year old woman with metastatic breast cancer. Breast carcinoma cells were isolated from a malignant pleural effusion and were identified by expression of MUC1. Mature DC and tumor cells were co-cultured with PEG and fusion cells were quantified by determining the percentage of cells that co-express unique DC and tumor antigens. Fusion cells potently stimulated allogeneic T cell proliferation in vitro. To date, she has received 2 of 3 scheduled vaccinations. Measures of tumor specific cellular and humoral immunity will be obtained following vaccination. Time to disease progression and RECIST measurable disease response will be followed. **Conclusion:** DC/breast fusion vaccination holds promise as a potent as a means of inducing immunologic and clinical responses in patients with breast cancer.

Fusions of Dendritic Cells with Breast Carcinoma Stimulate the Expansion of Regulatory T Cells while Concomitant Exposure to IL-12, CpG Oligodeoxynucleotides, and Anti-CD3/CD28 Promotes the Expansion of Activated Tumor Reactive Cells

Baldev Vasir, Zekui Wu, Keith Crawford, Jacalyn Rosenblatt, Corrine Zarwan, Adam Bissonnette, Donald Kufe and David Avigan

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Fusions of Dendritic Cells with Breast Carcinoma Stimulate the Expansion of Regulatory T Cells while Concomitant Exposure to IL-12, CpG Oligodeoxynucleotides, and Anti-CD3/CD28 Promotes the Expansion of Activated Tumor Reactive Cells[1]

Baldev Vasir,* Zekui Wu,* Keith Crawford,† Jacalyn Rosenblatt,‡ Corrine Zarwan,‡ Adam Bissonnette,* Donald Kufe,* and David Avigan[2‡]

Vaccination of patients with dendritic cell (DC)/breast carcinoma fusions stimulated antitumor immune responses in a majority of patients with metastatic disease but only a subset demonstrate evidence of tumor regression. To define the factors that limit vaccine efficacy, we examined the biological characteristics of DC/breast carcinoma fusions as APCs and the nature of the vaccine-mediated T cell response. We demonstrate that fusion of DCs with breast carcinoma cells up-regulates expression of costimulatory and maturation markers and results in high levels of expression of IL-12 consistent with their role as activated APCs. Fusion cells also express the chemokine receptor CCR7, consistent with their ability to migrate to the draining lymph node. However, DC/breast cancer fusions stimulate a mixed T cell response characterized by the expansion of both activated and regulatory T cell populations, the latter of which is characterized by expression of CTLA-4, FOXP3, IL-10, and the suppression of T cell responses. Our results demonstrate that IL-12, IL-18, and TLR 9 agonist CpG oligodeoxynucleotides reduce the level of fusion-mediated regulatory T cell expansion. Our results also demonstrate that sequential stimulation with DC/breast carcinoma fusions and anti-CD3/CD28 results in the marked expansion of activated tumor-specific T cells. These findings suggest that DC/breast carcinoma fusions are effective APCs, but stimulate inhibitory T cells that limit vaccine efficacy. In contrast, exposure to TLR agonists, stimulatory cytokines, and anti-CD3/CD28 enhances vaccine efficacy by limiting the regulatory T cell response and promoting expansion of activated effector cells. ***The Journal of Immunology,*** **2008, 181: 808–821.**

Breast cancer cells express unique Ags that are recognized by the host immune repertoire and serve as potential targets for tumor immunotherapy. However, tumor cells evade host immunity, in part, by presenting Ag in the absence of costimulation and the suppression of native APCs (1). Dendritic cells (DCs)[3] represent a complex network of APCs that are primarily responsible for initiation of primary immunity and modulation of the immune response (2, 3). Partially mature DCs are located at sites of Ag capture, excel at the internalization and processing of exogenous Ags, but are poor stimulators of T cell responses. Upon activation, DCs undergo maturation characterized by the increased expression of costimulatory molecules and CCR7, the chemokine receptor which promotes migration to sites of T cell traffic in the draining lymph nodes (4). Presentation of Ag by immature DCs may induce T cell tolerance (5). Breast carcinomas inhibit DC development through the secretion of IL-10, TGF-β, and vascular endothelial growth factor, resulting in the accumulation of immature DCs in the tumor bed that potentially suppress antitumor responses (6–9). Conversely, activated DCs can be generated by cytokine-mediated differentiation of DC progenitors ex vivo. DC maturation and function can be further enhanced by exposure to CpG oligodeoxynucleotides (ODN) (10, 11).

We have developed a cancer vaccine in which patient-derived tumor cells are fused with autologous DCs generated ex vivo. DC/tumor fusions express a broad array of tumor Ags presented in the context of DC-mediated costimulation. In animal models, vaccination with DC/tumor fusions protects against an otherwise lethal challenge of tumor cells and effectively eradicates established disease (12–15). Fusions of patient-derived breast carcinoma cells and DC stimulate T cell-mediated lysis of autologous tumor cells in vitro (16). In a clinical trial for patients with metastatic breast carcinoma, vaccination with autologous DC/tumor fusions induced antitumor immunity in a majority of patients while clinical responses were observed in a only subset of patients (17).

An effective cancer vaccine must have the capacity to present tumor Ags in the context of stimulatory signaling, migrate to sites of T cell traffic, and induce the expansion of activated effector cells with the ability to lyse tumor targets. One concern regarding the DC/breast carcinoma fusions is that tumor cells in the vaccine preparation may inhibit its function as an APC. Another potential

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[3] Abbreviations used in this paper: DC, dendritic cell; ODN, oligodeoxynucleotide; PEG, polyethylene glycol; SI, stimulation index; CBA, cytometric bead array; GITR, glucocorticoid-induced TNF receptor.

issue limiting response to vaccination is the presence of regulatory T cells that suppress T cell activation (18–20). Regulatory T cells deliver inhibitory signals via direct cell contact and the release of cytokines that play a role in mediating tumor-associated anergy. Regulatory T cells are increased in the circulation, tumor bed, and lymph nodes of patients with malignancy and their presence has been associated with worse outcomes (21–23). Paradoxically, studies have demonstrated that vaccination may lead to the expansion of regulatory T cells that ultimately blunt response. In animal models, depletion of regulatory T cells resulted in enhanced response to tumor vaccines (24, 25). Several strategies have been examined to enhance vaccine efficacy and promote T cell polarization toward an activated phenotype. Activation of innate immunity through ligation of TLR 9 potently stimulates T cell responses (26–31). Similarly, exposure to stimulatory cytokines, such as IL-12 and IL-18, results in T cell polarization toward a Th1 phenotype (14, 32–42). Ligation of the T cell/costimulatory complex (CD3/CD28) has also been shown to promote the activation of T cells when administered in the context of other stimulatory signals (43–46).

In the present study, we examined the phenotypic characteristics of DC/breast carcinoma fusions with respect to their function as APCs. We demonstrate that DC/breast carcinoma fusions exhibit features consistent with activated DCs as manifested by high levels of expression of costimulatory molecules, IL-12, and the chemokine receptor CCR7. In fact, immature DCs undergo maturation following polyethylene glycol (PEG)-mediated fusion with breast carcinoma cells. However, we also demonstrate that DC/tumor fusions stimulate a mixed response of activated and regulatory T cells, the latter of which potentially interfere with the development of antitumor immunity. We show that DC/breast carcinoma fusion cells induce the expansion of $CD4^+CD25^{+high}$ T cells, which uniformly express FOXP3 and are potent inhibitors of $CD4^+CD25^-$ T cells in a coculture system in vitro. Additionally, we also investigated whether the administration of a second stimulatory signal would favor the development of an activated antitumor immune response. We show that the addition of IL-12, IL-18, and the TLR 9 agonist CpG ODN decreases fusion-mediated expansion of regulatory T cells. Most notably, combined stimulation with DC/breast carcinoma fusions and anti-CD3/CD28 results in the dramatic expansion of tumor-specific T cells with an activated phenotype.

Materials and Methods

Generation of monocyte-derived DCs

PBMCs were isolated from leukopaks from normal donors and from peripheral venous blood collected from patients with breast cancer as per an institutionally approved protocol. PBMCs underwent Histopaque −1077 (Sigma-Aldrich) density gradient centrifugation and were plated in tissue culture flasks (BD Biosciences) in RPMI 1640 culture media containing 2 mM L-glutamine (Mediatech) and supplemented with heat-inactivated 10% human AB male serum (Sigma-Aldrich), 100 U/ml penicillin, and 100 μg/ml streptomycin (Mediatech) (complete medium) for 2 h at 37°C in a humidified 5% CO_2 incubator. The monocyte-enriched adherent fraction was cultured in complete medium containing GM-CSF (1000 U/ml) (Berlex) and IL-4 (1000 U/ml) (R&D Systems) for 5 days to generate immature DCs. A fraction of the DC preparation underwent further maturation by culturing the cells for an additional 48 h in the presence of TNF-α (25 ηg/ml) (R&D Systems) or the combination of TNF-α (25 ηg/ml), IL-1β (10 ηg/ml), IL-6 (1000 U/ml) (R&D Systems), and PGE_2 (1 μg/ml) (Calbiochem).

Isolation and culture of T cells

T cells were isolated from the nonadherent PBMC fraction using a T cell enrichment column (R&D Systems) or nylon wool column (Polysciences). Purity of T cells by both methods was >90% as determined by FACS analysis of CD3 surface expression. T cells were classified as allogeneic when derived from a third party donor and autologous when derived from the same donor from whom the DC fusion partner was derived.

Isolation and culture of tumor cells

Primary breast carcinoma cells were obtained from malignant effusions or resected tumor lesions as per an institutionally approved protocol. Human breast carcinoma cell lines MCF-7 and ZR-751 were purchased from ATCC. All tumor cell lines were maintained in DMEM (high glucose) or RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FBS (HyClone).

Preparation of DC/breast carcinoma fusion cells

DC/breast carcinoma fusions were prepared as previously described (16). Tumor cells were mixed with immature or mature DC preparations at ratios of 1:3-1:10 (dependent on cell yields) and washed in serum-free prewarmed RPMI 1640 culture media. The cell pellet was resuspended in 50% PEG solution (molecular mass: 1450)/DMSO solution (Sigma-Aldrich). After 3 min at room temperature, the PEG solution was progressively diluted with prewarmed serum-free RPMI medium and washed twice with serum free media. The fusion preparation was cultured for 5–7 days in 5% CO_2 at 37°C in complete medium with GM-CSF (500 IU/ml). Fusion cells were isolated for subsequent analyses by FACS gating around the population that coexpressed unique DC and tumor Ags as outlined below.

Characterization of DC, breast carcinoma, and DC/breast carcinoma fusion preparations by flow cytometry

DCs and breast carcinoma cells were incubated with primary mouse anti-human mAbs directed against HLA-DR, CD11c, CD14, CD80, CD86, CD83, CD40, CD54, MUC1, cytokeratin (CT), and matching isotype controls (BD Pharmingen), washed, and cultured with FITC-conjugated goat anti-mouse IgG1 (Chemicon International). Cells were fixed in 2% paraformaldehyde (Sigma-Aldrich) and underwent flow cytometric analysis using FACScan (BD Biosciences) and CellQuest Pro software (BD Biosciences). DC/breast carcinoma fusions preparations were subjected to dual staining to quantify the percentage of cells that coexpressed unique DC (CD11c-Cychrome) and tumor Ags (MUC1 or CT-FITC). Fusion cells were isolated by FACS gating and stained with PE-conjugated mouse anti-human Abs directed against CCR7, CD80, CD86, or CD83. The percentage of fusion cells expressing these markers was determined by multi-channel flow cytometric analysis. Alternatively, an aliquot of fusion cells was pulsed with GolgiStop (1 μg/ml; BD Pharmingen), permeabilized by incubation in Cytofix/Cytoperm plus (containing formaldehyde and saponin; BD Pharmingen), and washed in Perm/Wash solution (BD Pharmingen). The cells were then incubated with PE-conjugated anti-human IL-10 or IL-12 (Caltag Laboratories) or a matched isotype control Ab for 30 min, washed twice in Perm/Wash solution, and fixed in 2% paraformaldehyde (Sigma-Aldrich). The IL-12 Ab recognizes the IL-12 p40 monomers, homodimers, and the p70 heterodimers, but not the p35 subunit. A minimum of 1×10^4 events were acquired for analysis. To determine whether DC/breast carcinoma fusion cells coexpressed IL-10 and IL-12, fusion cells were labeled with PE Cy7-conjugated HLA-DR (eBioscience) and Cy5-conjugated DF3 Ab (DF3-conjugated using the Cy5 reactive Dye Kit; Amersham Biosciences) followed by intracellular staining with IL-10 (PE-conjugated) and IL-12 (FITC-conjugated) or matched isotype controls. The fusion cells were analyzed by 4-color flow cytometry using the BD LSR II analyzer (BD Biosciences) and data was analyzed using the BD FACSDiva software (BD Biosciences).

Immunohistochemical analysis of immature and mature DC, breast carcinoma, and DC/breast carcinoma fusion cells

Approximately 1.2×10^4 cells were spun onto slides (Cytospin; Thermo Shandon), allowed to dry, and fixed with acetone. The slides were incubated with primary mouse anti-human mAbs MUC1 and CT and an isotype-matched negative control at room temperature for 1 h, washed, incubated with 1:100 biotinylated $F(ab')_2$ of horse anti-mouse IgG (Vector Laboratories), washed, and incubated for 30 min with avidin-biotin complex reagent solutions (Vector Laboratories) followed by 3 amino-9-ethyl carbazole solution (Vector Laboratories). Cells were then stained for HLA-DR, CD86, or CD83 with the avidin-biotin complex-alkaline phosphatase kit (Vector Laboratories). Slides were washed, fixed in 2% paraformaldehyde (Sigma-Aldrich), and analyzed using an Olympus AX70 microscope.

Stimulation of allogeneic T cell proliferation by DC, tumor, and DC/breast carcinoma fusions

To assess their capacity to stimulate allogeneic T cell proliferation, immature and mature DCs and DC/breast carcinoma fusion cell preparations

were cocultured with allogeneic normal donor-derived T cells at a ratio of 1:10, 1:30, 1:100, 1:300, and 1:1000 in 96-well U-bottom culture plates (Costar) for 5 days at 37°C and 5% CO_2. T cell proliferation was determined by incorporation of [^{3}H]thymidine (1μCi/well; 37kBq; NEN-DuPont) added to each well 18 h before the end of the culture period. Thereafter, the cells were harvested onto glass fiber filter paper (Wallac) using an automated TOMTEC harvester (Mach II), dried, placed, and sealed in BetaPlate sample bag (Wallac) with 10 ml of ScintiVerse (Fisher Scientific). Cell bound radioactivity was counted in a liquid scintillation counter (Wallac; 1205 Betaplate). Data are expressed as stimulation index (SI). The SI was determined by calculating the ratio of [^{3}H]thymidine incorporation (mean of triplicates) over background [^{3}H]thymidine incorporation (mean of triplicates) of the unstimulated T cell population.

Cytokine expression by T cells stimulated by immature and mature DC/breast carcinoma fusions

The profile of secreted cytokines by T cells cultured with immature and mature DC/breast carcinoma fusions was determined using the cytometric bead array (CBA) kits (BD Biosciences). Supernatants from unstimulated T cells or cells exposed to unfused DC and breast carcinoma served as controls. Supernatants were collected before cell harvest and frozen at −80°C. Concentrations of IL-2, IL-4, IL-5, IL-10, IFN-γ, TNF-α, IL-12, IL-6, IL-1β, and IL-8 were quantified using an inflammatory CBA kit as per standard protocol. Briefly, the kits provided a mixture of six microbead populations with distinct fluorescent intensities (FL-3) that are precoated with capture Abs specific for each cytokine. Culture supernatant or the provided standardized cytokine preparations were added to the premixed microbeads and then cultured with secondary PE-conjugated Abs. Individual cytokine concentrations were indicated by their fluorescent intensities (FL-2) and then computed using the standard reference curve of Cellquest and CBA software (BD Pharmingen). Interassay reproducibility was assessed using two replicate samples of three different levels of the human standards in three separate experiments.

CTL response following stimulation with immature and mature DC/breast carcinoma fusions

DC/breast carcinoma fusion cell preparations generated with immature and mature DCs were cocultured with autologous T cells at a ratio of 1:10 for 7–10 days. DC/breast carcinoma fusions generated with DC autologous to T cell effectors were used as target cells in a standard 5-h ^{51}Cr-release assay. Target cells (2×10^4 cells/well) were incubated with ^{51}Chromium (NEN-DuPont) for 1 h at 37°C followed by repeated washes. ^{51}Cr release was quantified following 5-h coculture of effector and target cell populations at a ratio of 30:1 or 10:1. Percentage cytotoxicity was calculated using mean of triplicates by a standard assay as follows: % specific cytotoxicity = [(sample counts − spontaneous counts)/(maximum counts − spontaneous counts)] × 100. Spontaneous release was <25% of the maximum ^{51}Cr uptake. As a control, lysis of targets by unstimulated T cells and T cells stimulated by unfused DCs were assessed.

Tetramer staining

Ag-specific MUC1$^+$CD8$^+$ T cells were identified using PE-labeled HLA-A*0201$^+$ iTAg MHC class I human tetramer complexes (Beckman Coulter) composed of four HLA MHC class 1 molecules each bound to MUC1-specific epitopes M1.2 ($MUC1_{12-20}$) LLLLTVLTV (47). An A*0201 irrelevant peptide MHC class I tetramer with no known specificity was provided by Beckman Coulter as a negative control. Nonadherent cells were cocultured with DC/breast carcinoma fusion cells for 5 days, harvested, incubated with the MUC1 or control tetramer, and then stained with FITC-conjugated CD8 Ab. Cells were washed and analyzed by bidimensional FACS analysis. A total of 3×10^5 events were collected for final analysis. Similarly, nonadherent unstimulated cells were analyzed in parallel.

Analysis of regulatory and activated T cell responses to stimulation with DC/breast carcinoma fusions

Autologous and allogeneic T cell preparations were cocultured with mature DC/breast carcinoma fusions for 5 days at a 10:1 ratio. The cell preparations were incubated with FITC-conjugated anti-CD4, Cychrome-conjugated anti-CD25, and PE-conjugated anti-CD69, anti-glucocorticoid-induced TNF receptor (GITR), or anti-CTLA-4. Alternatively, cells were permeabilized and cultured with PE-conjugated Ab directed against IFN-γ, IL-10, IL-4, or FOXP3. Cells were subsequently analyzed by multi-channel flow cytometry. In some studies, CD4$^+$ T cells were isolated by magnetic microbead isolation (Miltenyi Biotec), and the resultant population were subjected to a two staining procedure with anti-CD25 Ab and the indicated marker.

Phenotypic and functional characterization of CD4$^+$CD25$^-$, CD4$^+$CD25^{+low}, and CD4$^+$CD25^{+high} T cells

In an effort to segregate activated and regulatory T cell populations based on the expression of CD4$^+$CD25$^-$, CD4$^+$CD25^{+low} and CD4$^+$CD25^{+high}, $40–50 \times 10^6$ of the CD4$^+$ T cells were positively selected (>97% purity) from resting and fusion-stimulated T cell populations and sorted using a BD FACSAria cell sorting instrument (BD Biosciences). The cells were incubated with anti-CD4 TC (IgG2a; Invitrogen) and anti-CD25-FITC (IgG1; BD Pharmingen) and separated into CD4$^+$CD25$^-$, CD4$^+$CD25^{+low}, and CD4$^+$CD25^{+high} fractions by FACS sorting as previously described (18). Intracellular staining for FOXP3 was performed on cells directly after sorting using the PCH101 anti-FOXP3 Ab (eBioscience) per the manufacturer's instructions. CD4$^+$CD25$^-$ T cells (5×10^4 cells/well) were cocultured in triplicate with equal numbers of CD4$^+$D25^{+low}, CD4$^+$D25^{+high}, or CD4$^+$CD25$^-$ T cells (as controls) in a 1:1 ratio, in the presence of irradiated (3500 Rads) autologous T cell depleted PBMCs as a source of APCs. The cultures were pulsed with tetanus toxoid (10 μg/ml), anti-CD3 Ab (1 μg/ml) (clone UCHT1; BD Pharmingen), or PHA (4 μg/ml) (without autologous PBMCs). T cell proliferation was quantified after 4 days of culture period by uptake of [^{3}H]thymidine (1 μCi [0.037 MBq] per well) following overnight pulsing.

Effects of exogenous IL-12, IL-18, and CpG ODN (TLR 9 agonist) on the fusion-mediated stimulation of autologous T cells

DC/breast carcinoma fusions were cocultured for 5–7 days with autologous T cells in the presence or absence of IL-12 (10 ng/ml; R&D Systems), IL-18 (10 ng/ml; R&D Systems), or CpG ODN (10 μg/ml; Coley Pharmaceutical Group). The recombinant human IL-12 added to the cultures was the p70 heterodimeric cytokine (R&D Systems). The CpG ODN (C-2395) consisted of a hexameric CpG motif, 5′-TCGTCGTTTT-3′, linked by a T spacer to the GC-rich palindrome sequence 5′-CGGCGCGCGCCG-3′ (48). A control CpG ODN (class B-2137) without stimulatory sequences was simultaneously tested in parallel in each experiment. Regulatory and activated T cell populations were quantified as outlined above.

Effect of sequential stimulations with DC/breast carcinoma fusions and anti-CD3/CD28 on T cell responses

T cells were activated for 48 h by exposure to the immobilized mAbs, anti-CD3 (clone-UCHT1; BD Pharmingen), and anti-CD28 (clone-CD28.2; BD Pharmingen; CD3i/CD28i). Twenty-four-well non-tissue culture-treated plates (Falcon) were coated with each of the Abs (1 μg/ml in PBS) at 0.5 ml/well and left overnight at 4°C. The plates were blocked with 1% BSA and T cell preparations were added at a density of 2×10^6 cells/well. T cells were stimulated with anti-CD3/CD28 (48 h) or DC/breast carcinoma fusions alone (5–7 days), fusions followed by exposure to anti-CD3/CD28, or anti-CD3/CD28 followed by fusion cells. T cells were harvested and proliferation was determined by uptake of tritiated thymidine. T cells binding the MUC1 tetramer were quantified by FACS analysis as outlined above. The percentage of T cells expressing markers consistent with a regulatory (FOXP3) and activated (CD69, IFN-γ) phenotype were quantified.

Statistical analysis

Results are expressed as mean ± SEM. For comparisons, Student's *t* test was used and values of $p < 0.05$ were considered as significant.

Results

Phenotype of DC/tumor fusions generated with immature and mature DCs

Tumor cells suppress host immunity, in part, by disrupting the development and function of APCs. A potential issue concerning the effectiveness of the DC/tumor fusion vaccine is whether the tumor cell fusion partner will inhibit DC differentiation and interfere with Ag presentation by the fusion vaccine. To assess this question, we examined the phenotypic and functional characteristics of fusions generated with immature and mature DCs. Immature and mature DCs were generated from patients with breast

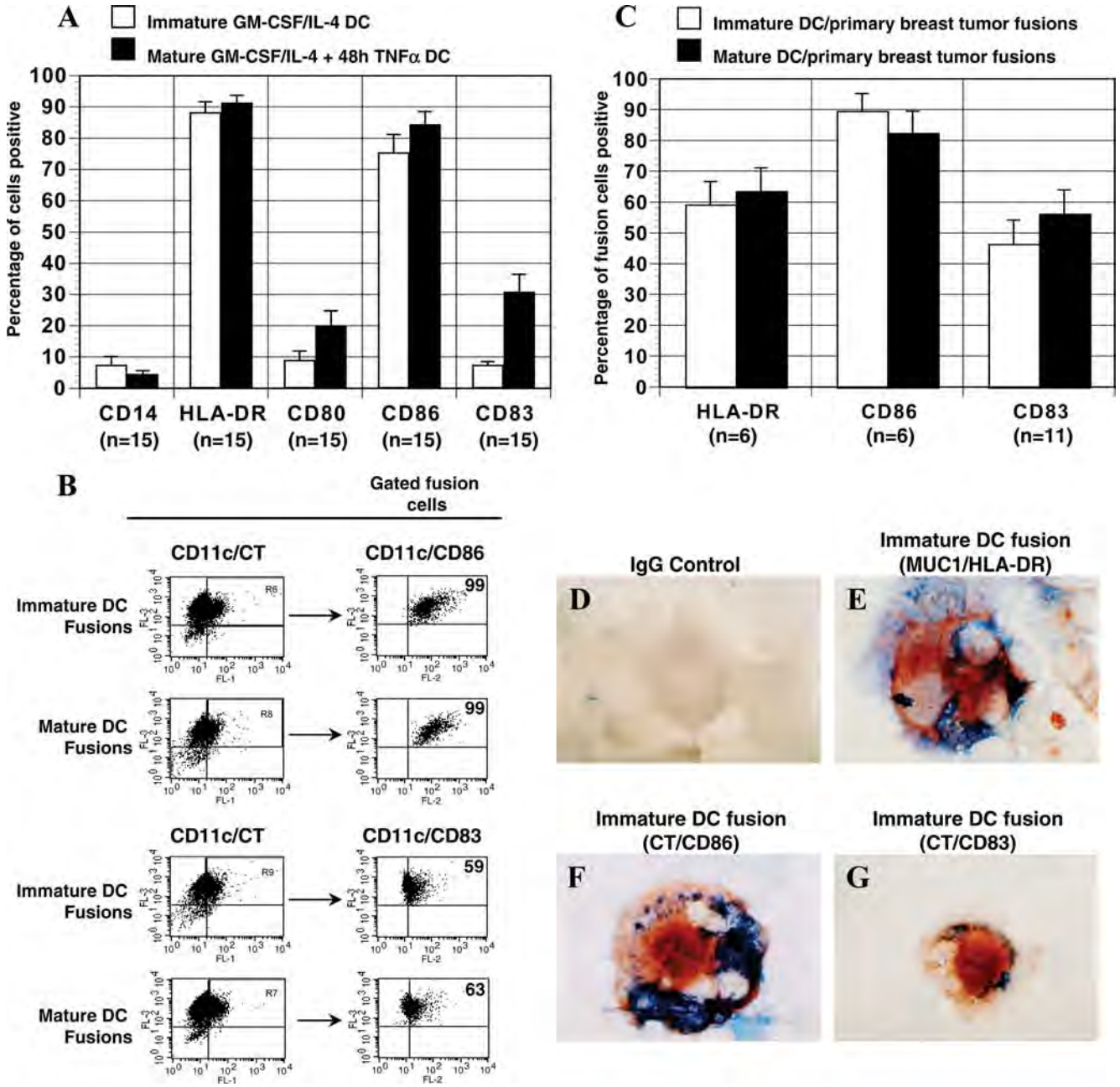

FIGURE 1. Phenotypic analysis of monocyte-derived DCs. DCs were generated from adherent mononuclear cells isolated from peripheral blood of breast cancer patients and leukopaks obtained from normal donors. Cells were cultured with GM-CSF (1000 IU/ml) and IL-4 (1000 IU/ml) for 5–7 days (immature DCs) and a subset underwent maturation with TNF-α (25 ng/ml) for 48–72 h. Immature and mature DCs underwent FACS analysis to assess expression of costimulatory and maturation markers. *A*, Mean percentage ($\pm$SEM) of cells expressing the indicated surface markers for 15 experiments is shown. Maturation results in increased expression of costimulatory (CD80, CD86) and maturation (CD83) markers. Patient-derived primary tumor cells or MCF-7 cells were fused with immature or mature DCs by coculture in the presence of PEG. *B*, In a representative experiment using patient-derived tumor cells, fusion cells were isolated by gating around cells that coexpressed CT and CD11c *(left panel)*. Expression of CD86 and CD83 by the fusion cells was determined (*right panel*). *C*, The mean percentage ($\pm$SEM) of immature and mature DC/breast carcinoma fusions expressing DR, CD86, and CD83 was determined. *D–G*, Immunohistochemical analysis of DC-tumor fusion preparations was performed following cytospin preparation. Immature DC/breast carcinoma fusions were stained for: (*D*) Isotype matched IgG control; (*E*) MUC1/HLA-DR; (*F*) CT/CD86; and (*G*) CT/CD83. Numbers in the *upper right quadrant* of the dot plots represent cells positively staining for the indicated surface markers.

cancer and from leukopak preparations acquired from volunteer donors. Adherent PBMC were cultured for 1 wk with GM-CSF and IL-4 to generate partially mature DCs. Maturation was induced by exposure to TNF-α for 48–96 h. Both immature and mature DC preparations strongly expressed the costimulatory molecule CD86, [75% (range: 25–98%) and 84% (45–99%), respectively] and exhibited low levels of CD14 expression ($n = 15$) (Fig. 1*A*). However, mature DC demonstrated a statistically significant increase in mean expression of CD80 [20% (3–65%) vs 9% (2–46%); $p = 0.05$] and CD83 [31% (3–77%) vs 7% (3–15%); $p = 0.0003$]. Similar phenotypic changes were observed following DC maturation with CD40L (data not shown). As a measure of their functional capacity as APCs, DC preparations were examined for their ability to stimulate allogeneic T cell proliferation. Mature as compared with immature DCs stimulated higher levels of allogeneic T cell proliferation (data not shown).

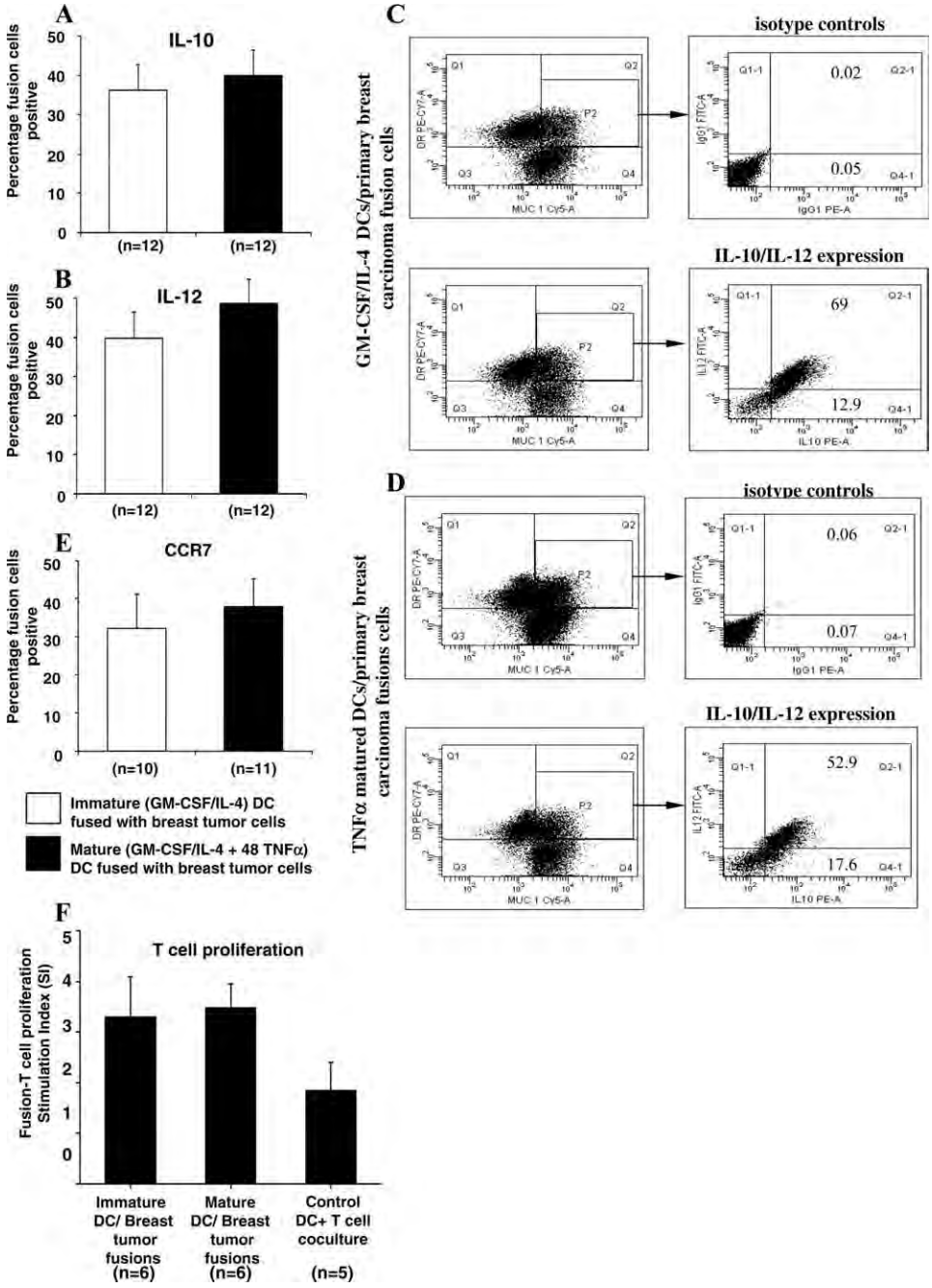

FIGURE 2. Expression of IL-10, IL-12, and CCR7 in DC/breast carcinoma fusion cells generated with either immature or mature DCs. Fusion cell preparations generated with primary breast carcinoma cells and immature or mature DCs were stained with CT and CD11c and subsequently fixed, permeabilized, and stained for intracellular IL-10 and IL-12. Unfixed fusion cells were analyzed for the surface expression of CCR7. Fusion cells were isolated by FACS gating and analyzed for expression of IL-10, IL-12, and CCR7. The mean percentage (±SEM) of immature and mature DC/breast carcinoma cells expressing IL-10 (*A*) and IL-12 (*B*) is shown for 12 experiments. *C*, Fusion cells were isolated by FACS gating around cells that coexpressed MUC1 and HLA-DR. Intracellular expression of IL-10 and IL-12 by the immature (*C*) and mature (*D*) DC/breast carcinoma fusions was determined by multi-channel FACS analysis. Numbers in the *upper right quadrant* of the dot plots represent the percentage of gated cells positively staining for intracellular markers for both IL-10 and IL-12. The mean percentage (±SEM) of immature and mature DC/breast carcinoma cells expressing CCR7 (*E*) is presented. Induction of autologous T cell proliferation by DCs, immature or mature DC/breast carcinoma cells as measured by [^{3}H]thymidine uptake (*F*). Results were normalized by calculation of SI as explained in *Materials and Methods* section.

We subsequently examined the phenotypic characteristics of the fusion cell populations generated with immature and mature DC. Immature and mature DC populations were fused with primary patient-derived breast cancer cells or MCF-7 human breast carcinoma cells by coculture with PEG. Fusion cells were quantified by determining the percentage of cells that coexpressed unique tumor (MUC1 and/or CT) and DC (CD11c) Ags (Fig. 1*B*). Equivalent mean fusion efficiencies were observed following fusion of tumor cells with mature (11% ± 1.6 SEM) and immature (7% ± 1.2 SEM) DC ($n = 12$). Using primary tumor cells obtained from patient derived samples, fusion cells were isolated by FACS gating of cells that coexpressed DC and tumor-derived Ags (Fig. 1*B*). Expression of CD86 was uniformly observed in both immature DC/breast carcinoma (89%) and mature DC/breast carcinoma (82%) fusion populations ($n = 6$) (Fig. 1*C*). The maturation marker, CD83, was detected in 46 and 51% of immature and mature fusion cell populations, respectively ($p = 0.5$, NS) (Fig. 1, *B* and *C*). A significant increase in CD83 and CD86 expression was observed in immature DC/breast carcinoma fusions as compared with immature DCs. Immunocytochemical staining demonstrated expression of DR, CD86, and CD83 by the immature DC/tumor fusions (Fig. 1, *D–G*). These studies demonstrate that fusion of DC with breast carcinoma cells results in phenotypic characteristics consistent with maturation and activation, and not inhibition of DC differentiation.

Expression of IL-12 and IL-10 by immature and mature DC/tumor fusions

As a measure of their potency as APCs and their capacity to stimulate Th1 responses, we next examined expression of IL-12 and IL-10 by the fusion cell populations generated with primary tumor cells obtained from patient samples (Fig. 2, *A* and *B*). Fusion cells

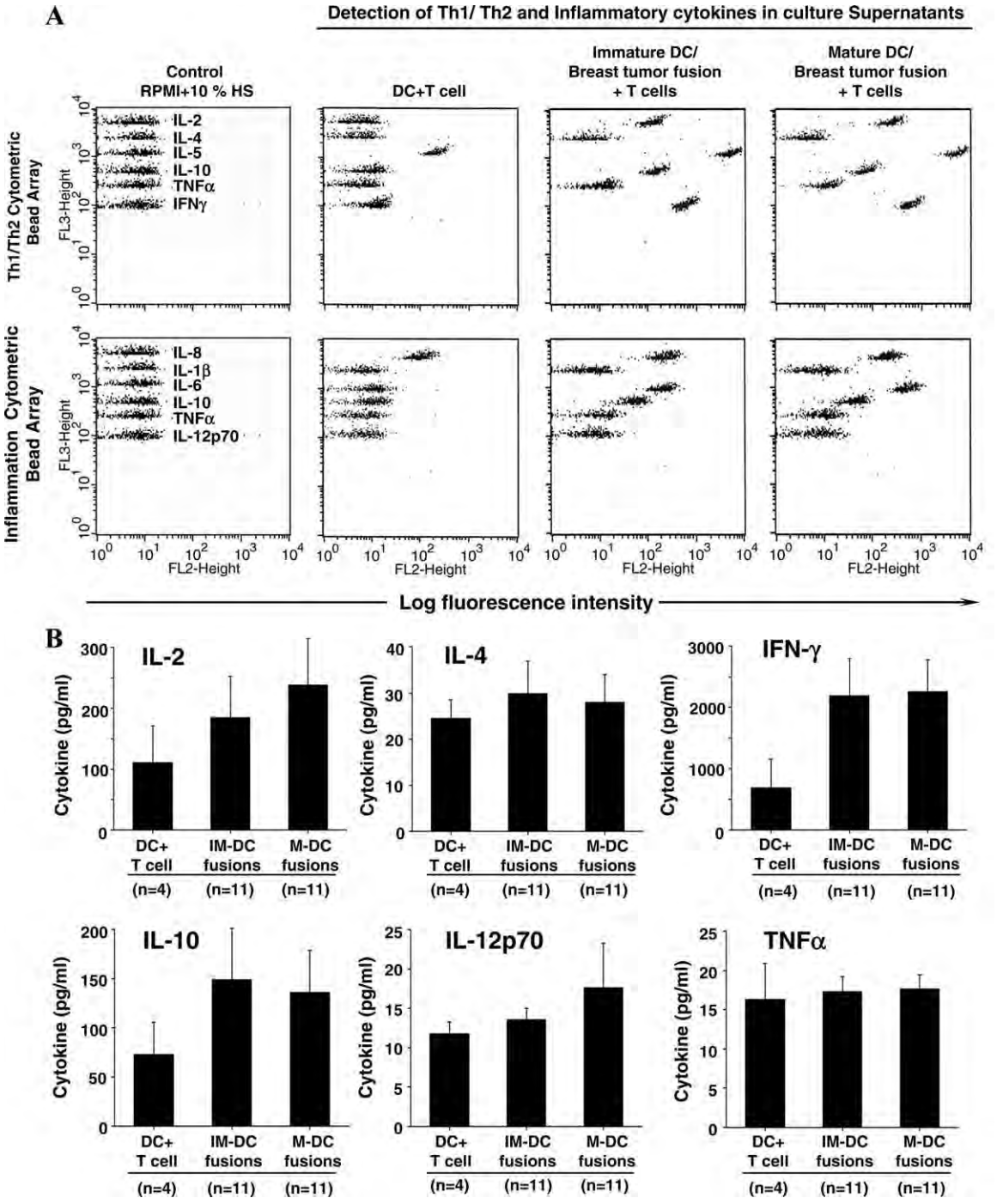

FIGURE 3. Culture supernatant expression of cytokines following autologous T cell stimulation with DC/breast carcinoma fusions The Th1 and Th2 and the inflammatory cytokine profiles of culture supernatants of immature and mature DC/breast carcinoma fusion cells (generated with primary breast carcinoma cells) cocultured with autologous nonadherent cells were quantitated using the CBA analysis kit. *A*, Representative example from a single experiment depicting the fluorescence bead array dot-plot assay display for Th1/Th2 (*upper panels*) and inflammatory cytokines (*lower panels*) after data acquisition with BD CellQuest software followed by data formatting and subsequent analysis using the BD CBA software. *B*, Mean (±SEM) concentration of IL-2, IL-4, IL-10, IL-12, TNF-α, and IFN-γ cytokine (pg/ml) in culture supernatants is presented from a series of 4 (DCs plus autologous nonadherent cell cocultures as controls) and 11 (immature and mature DC/breast carcinoma fusion cells cocultured with autologous nonadherent cells) separate experiments. IM-DC fusions: immature dendritic cell fusions; M-DC fusions: mature dendritic cell fusions.

were isolated by FACS gating of cells that coexpressed DC and tumor-derived Ags. 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 (±6.7 SEM) and 49% (±6.3 SEM) ($p = 0.35$, NS) and IL-10 by ~36.3 (±6.4 SEM) and 40% (±6.4 SEM; $n = 11$) (p = NS) of the immature and mature DC/breast carcinoma fusions, respectively ($n = 12$). We subsequently analyzed whether IL-12 and IL-10 was expressed by distinct populations of DC/breast carcinoma fusions. Immature and mature DC/breast carcinoma fusions were isolated by FACS gating of cells that coexpressed HLA-DR and MUC1. The gated cells underwent intracellular FACS analysis for IL-12 and IL-10 expression. IL-12 and IL-10 were expressed by a single DC/breast carcinoma fusion population (Fig. 2, *C* and *D*).

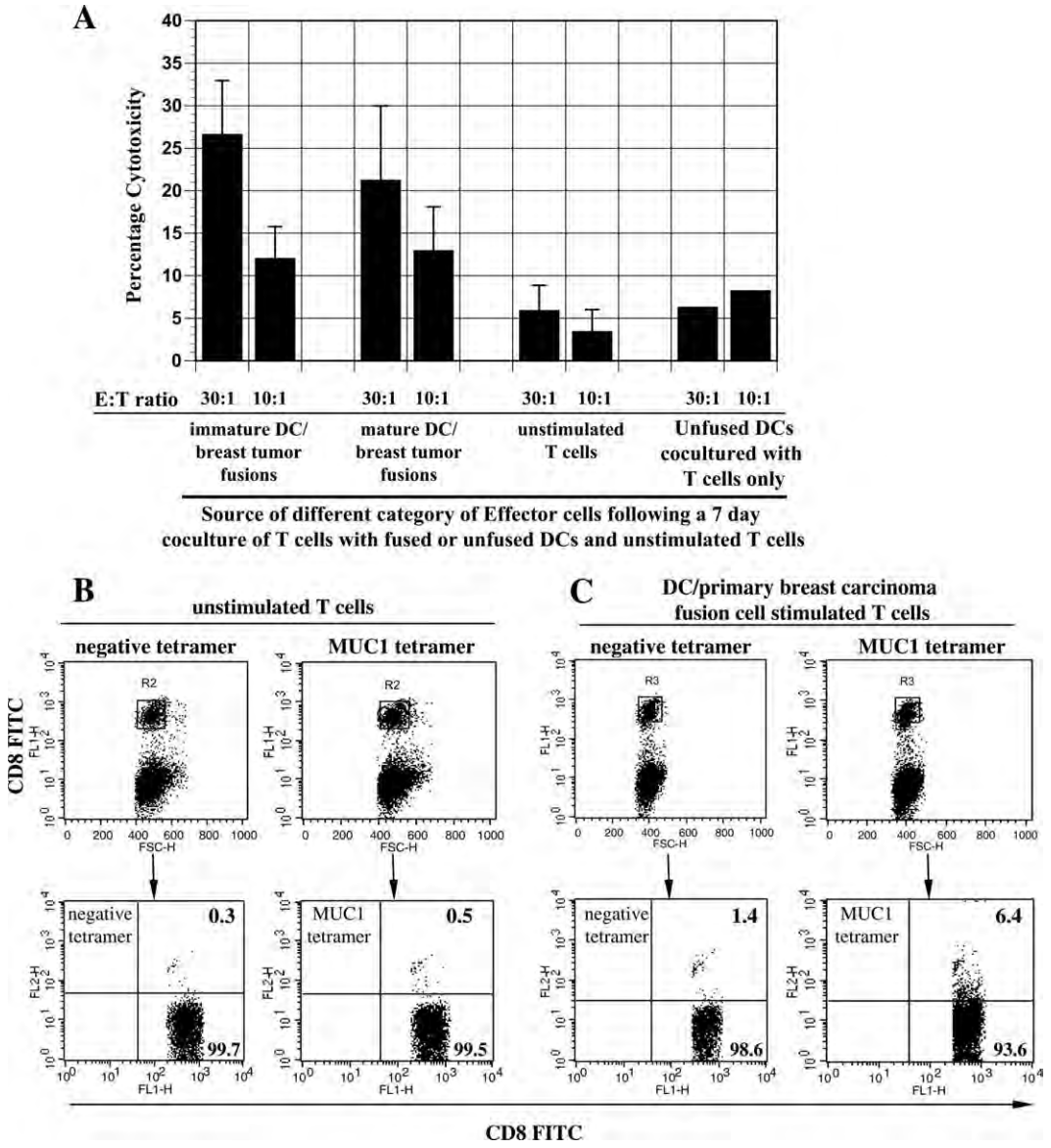

FIGURE 4. Immature and mature DC/breast carcinoma fusions stimulate lysis of tumor targets and expansion of MUC1-specific T cells. *A*, Immature and mature DC/breast carcinoma fusion cells were cocultured with autologous T cells at a ratio of 30:1 for 7–10 days. T cells were incubated with ^{51}Cr-labeled autologous breast tumor cells or semiautologous DC/breast carcinoma fusion cells. Lysis of the labeled cells was determined by chromium release assay. The mean percentage cytotoxicity (±SEM) following stimulation with immature or mature DC/breast carcinoma fusion cells is presented. *B*, Stimulation with mature DC/breast carcinoma fusions results in the expansion of T cells binding the MUC1 tetramer. DCs generated from HLA*0201 donors were fused with primary breast carcinoma cells and cultured with autologous T cells for 5 days. The percent of $CD8^+$ T cells binding the MUC1 tetramer before and following fusion cell stimulation was determined by bidimensional FACS analysis and compared with that seen with a control tetramer. The results are representative of three independent experiments using different samples. Numbers in the *upper right quadrant* of the dot plots represent cells positively staining for the indicated surface markers.

Expression of CCR7 by immature and mature DC/tumor fusions

The chemokine receptor, CCR7, directs cell migration to sites of T cell traffic in the draining lymph nodes and is characteristically expressed by DCs that are undergoing maturation and activation. As a measure of their migratory capacity, expression of CCR7 was determined for fusions generated with primary breast tumors and immature or mature DC (Fig. 2*E*). CCR7 was prominently expressed on both immature and mature fusion populations, suggesting that tumor-DC fusion resulted in the expression of a mature and activated phenotype. Mean CCR7 expression was observed in 33 (±9 SEM) and 38% (±7.3 SEM; $n = 11$) of the immature and mature DC/breast cancer fusions, respectively ($n = 12$). In contrast, mean expression of CCR7 by immature DCs was 3.8%.

Stimulation of autologous T cell proliferation and cytokine production by immature and mature DC fusions

The functional capabilities of mature as compared with immature DC/tumor fusion preparations were analyzed by comparing their capacity to stimulate T cell proliferation and cytokine production. Fusion cell populations were cocultured with autologous T cells for 5 days and proliferation was determined by measuring uptake of tritiated thymidine after overnight pulsing (Fig. 2*F*). Proliferation was measured as the T cell SI (Stimulated T cells/Unstimulated T cells). Both immature and mature DC/breast cancer fusions stimulated autologous T cell proliferation with SI of 3.3 (±1.4 SEM; $n = 6$) and 3.5 (±1.4 SEM; $n = 6$), respectively. We also quantified cytokine secretion generated by coculture of DCs,

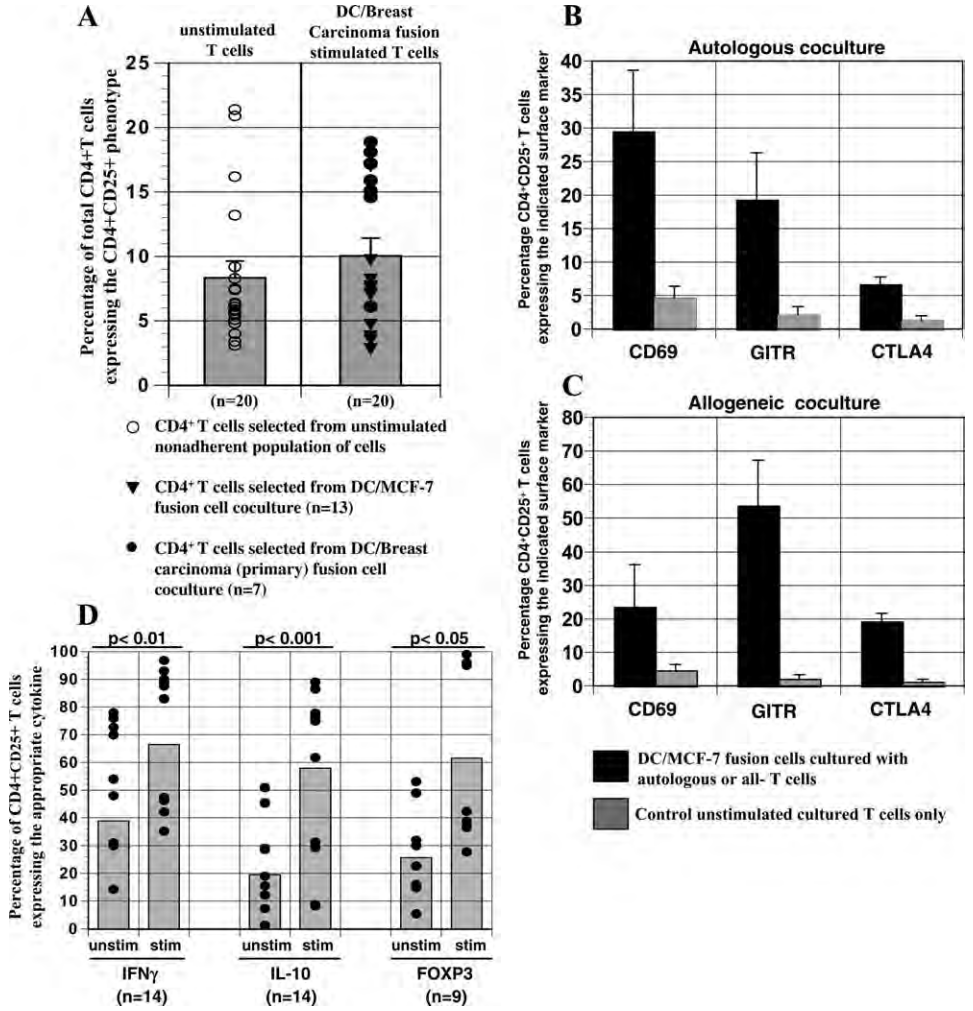

FIGURE 5. Stimulation with DC/breast carcinoma fusions results in the expansion of activated and regulatory T cells. *A*, T cells were stimulated with DC/breast carcinoma fusion cells for 5 days (13 generated with MCF-7 and 7 with primary breast carcinoma cells). $CD4^+$ T cells were selected using magnetic microbeads (Miltenyi Biotec) and labeled with PE-conjugated CD4 and FITC-conjugated CD25 Abs. $CD4^+CD25^+$ cells were quantified by bidimensional FACS analysis for unstimulated and fusion stimulated T cells. Data is presented for 20 experiments with associated mean values (overlaying shaded histogram). Autologous (*B*) or allogeneic (*C*) T cells were cultured with DC/breast carcinoma fusion cells for 5 days and $CD4^+$ T cells were isolated by magnetic bead separation. The mean percentage (±SEM) of cells that coexpressed CD25/CD69, CD25/GITR, and CD25/CTLA-4 was determined by bidimensional flow cytometry. Data is representative (mean ± SEM) of five separate experiments. *D*, Autologous T cells were cocultured with DC/breast carcinoma fusions for 5–7 days. Following selection of $CD4^+$ T cells using magnetic microbeads, cells were stained with FITC-conjugated CD25, permeabilized with Cytofix/Cytoperm solution, and stained with PE-conjugated IFN-γ, IL-10, or FOXP3 Abs. Data is presented as a stacking dot plot graph with mean values (shaded histogram) for a series of 9–14 experiments.

immature or mature DC/primary breast carcinoma fusions with autologous T cell populations using the BD CBA system (BD Biosciences) (Fig. 3, *A* and *B*). Mean levels of IFN-γ following stimulation with immature and mature DC/breast cancer fusions were 2188 and 2252 pg/ml, respectively. These levels were significantly greater than those seen with T cells cultured with unfused autologous DC (685 pg/ml). In contrast, secretion of IL-12, IL-4, IL-10, IL-2, and TNF-α was not increased following stimulation with fusion cells.

Stimulation of tumor-specific CTL responses and MUC1-specific 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 semiautologous fusion targets. CTL activity did not differ between the fusion populations ($n = 5$; stimulated by DC/breast carcinoma fusions). Mean CTL lysis for effector:T cell ratio of 30:1 was 27 and 21% for T cells stimulated with immature and mature DC/breast cancer fusions, respectively (p = NS) (Fig. 4*A*). In contrast, only background levels of killing were observed following coculture of targets with unstimulated T cells or those stimulated with unfused DCs. To assess the capacity of the fusion vaccine to stimulate T cell responses directed against a specific tumor Ag, we assessed whether HLA-A2.1$^+$ T cells stimulated by DC/breast carcinoma fusions recognized MUC1. Selective expansion of $CD8^+$ T cells binding the MUC1 tetramer was observed following stimulation with fusions generated with DCs and primary breast carcinoma cells (Fig. 4*B*). In a single experiment, 23.6% of the $CD8^+$MUC1 tetramer$^+$ T cells were observed to express IFN-γ. In a series of experiments, the mean percentage of $CD8^+$ T cells binding the MUC1 tetramer were 5.49% (±0.46 SEM; $n = 3$) as compared with 1.3% (±0.06 SEM; $n = 3$) of $CD8^+$ T cells binding the negative tetramer ($p = 0.005$). These results indicate that DC/breast carcinoma fusions exhibit an activated phenotype with strong expression of costimulatory molecules, stimulatory cytokines, and chemokine receptors

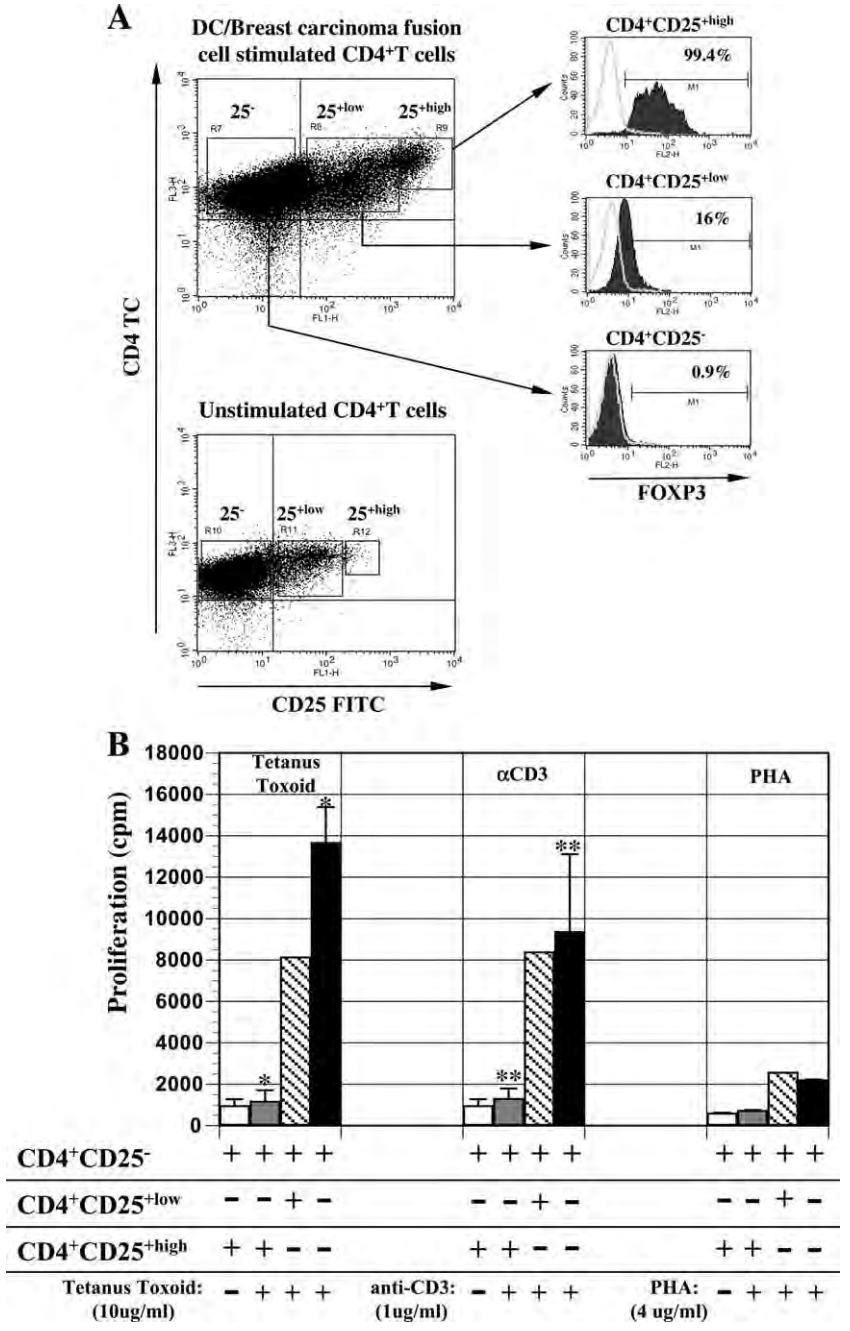

FIGURE 6. Phenotypic and functional characterization of $CD4^+CD25^-$, $CD4^+CD25^{+low}$, and $CD4^+CD25^{+high}$ T cells. $CD4^+$ T cells were isolated from resting and fusion-stimulated T cell populations and then incubated with anti-CD4 TC and anti-CD25 FITC. *A*, T cells were then separated into $CD4^+CD25^-$, $CD4^+CD25^{+low}$, and $CD4^+CD25^{+high}$ fractions by bidimensional FACS sorting as shown in a representative dot plot. Cells then underwent intracellular staining for FOXP3. *B*, The capacity of the T cell fractions to suppress T cell proliferation was then determined. $CD4^+CD25^-$ T cells (5×10^4 cells/well) were cocultured with $CD4^+D25^{+low}$, $CD4^+D25^{+high}$, or $CD4^+CD25^-$ cells (5×10^4 cells/well, 1:1 ratio) in the presence of irradiated (3500 Rad) autologous PBMCs which underwent T cell depletion using anti-CD3 coated magnetic beads. The cultures were pulsed with tetanus toxoid (10 μg/ml), anti-CD3 Ab (1 μg/ml) (clone UCHT1; BD Pharmingen), or PHA (4 μg/ml) (without autologous PBMCs). T cell proliferation was quantified after 4 days culture by uptake of [^{3}H]thymidine (1 μCi [0.037 MBq] per well) following overnight pulsing. The bar graph represents the mean ($\pm$SEM) of between 2 and 5 separate experiments. (*: $p < 0.001$; **: $p < 0.05$).

enabling them to migrate to sites of T activation. In addition, DC/breast carcinoma fusions stimulate antitumor CTL responses including the expansion of T cells targeting defined tumor Ags.

Fusion cells stimulate the expansion of activated and regulatory T cells

Having characterized DC/breast carcinoma fusions as potent APCs with the capacity to elicit T cell responses, we examined whether DC/tumor fusions induce the expansion of regulatory as compared with activated T cells. Stimulation with DC/breast carcinoma fusions using MCF-7 ($n = 13$) or primary breast carcimona cells ($n = 7$) did not result in an increase in the percentage of total $CD4^+CD25^+$ T cells (10% $\pm$ 1.3 SEM; $n = 20$) as compared with unstimulated T cells (8.3% $\pm$ 1.1 SEM; $n = 20$) (Fig. 5*A*). Noteworthy, higher levels of $CD4^+CD25^+$ cells were observed following stimulation with fusions generated with primary breast carcinoma cells as compared with the MCF-7 cell line. Although both activated memory effector cells and regulatory T cells coexpress CD4 and CD25, regulatory T cells may be differentiated by their relatively high level of CD25 expression and the presence of other markers such as GITR, CTLA-4, and FOXP3. In contrast, CD69 is characteristically expressed by activated T cells. Mature DCs were fused to a human breast carcinoma cell line (MCF-7) and cocultured with autologous or allogeneic T cells for 5 days. $CD4^+CD25^+$ cells were quantified by flow cytometric analysis and further characterized with respect to expression of cell surface markers and cytokine profile. $CD4^+$ 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%. However, coculture of fusion cells and autologous T cells resulted in a 6.3-fold increase in $CD4^+CD25^+$ T cells that expressed CD69, (4.7%-unstimulated T cells; 29.5-fusion

stimulated cells, $n = 5$; $p = 0.01$) consistent with an activated phenotype (Fig. 5*B*). Stimulation with mature DC/breast carcinoma fusions also resulted in 9- and 5.2-fold increase in $CD4^+CD25^+$ T cells that expressed GITR and CTLA-4, respectively (Fig. 5*B*). These findings suggest that both activated and inhibitory T cell populations are expanded by DC/breast carcinoma fusions (Fig. 5*B*). Noteworthy, fusion stimulation of allogeneic T cells resulted in a similar increase in $CD4^+CD25^+CD69^+$ T cells (5-fold), but a significantly greater expansion of GITR-(25-fold) and CTLA-4-(15-fold) positive populations (Fig. 5*C*).

We also assessed the profile of cytokine expression in the $CD4^+CD25^+$ T cell population following stimulation with DC/breast carcinoma (MCF-7 or ZR75) fusions using intracellular flow cytometric analysis. The mean percentage of $CD4^+CD25^+$ T cells expressing IFN-γ was 40 (±6.9 SEM) and 68% (±6.1 SEM) before and following fusion cell stimulation ($p = 0.005$), respectively ($n = 14$) (Fig. 5*D*). Similarly, the percentage of $CD4^+CD25^+$ T cells expressing the inhibitory cytokine, IL-10 (Fig. 5*D*) rose from 20 (±4.9 SEM) to 59% (±8.4 SEM) ($p = 0.0002$). Finally, we assessed the impact of fusion cell stimulation on the intracellular expression of FOXP3, a marker considered to be specific for regulatory T cells. FOXP3 expression increased from 26.5 (±5.4 SEM; $n = 9$) to 63% (±10.6 SEM; $n = 9$) ($p = 0.01$) of the unstimulated and fusion stimulated $CD4^+CD25^+$ T cell populations, respectively (Fig. 5*D*). As such, fusion cells induce the expansion of both immunostimulatory and immunosuppressive elements resulting in a complex response in which regulatory T cells may prevent the development of sustained effective antitumor immunity.

Functional characterization of T cells stimulated by DC/breast carcinoma fusions

To further define the functional characteristics of T cells stimulated by DC/breast carcinoma fusions, $CD4^+CD25^-$, $CD4^+CD25^{+low}$, and $CD4^+CD25^{+high}$ T cells were separated by flow cytometric sorting (Fig. 6*A*). Fusion stimulation resulted in an increase in the $CD4^+CD25^{+low}$ and $CD4^+CD25^{+high}$ fractions. Consistent with a regulatory T cell phenotype, $CD4^+CD25^{+high}$ T cells uniformly expressed FOXP3. In contrast, FOXP3 expression was seen in only a minority of $CD4^+CD25^{+low}$ cells and was absent from $CD4^+CD25^-$ cells. In a series of experiments, the mean percentage of FACS sorted $CD4^+CD25^{+high}$ T cells that expressed FOXP3 were 86.9% (±7.1 SEM; $n = 3$) as compared with 11.73% (±2.6 SEM; $n = 3$) of $CD4^+CD25^{+low}$ and 0.52% (±0.2 SEM; $n = 3$) of $CD4^+CD25^-$, respectively. To assess the functional properties of these populations, we examined their ability to suppress T cell proliferation in response to TCR ligation with anti-CD3 or exposure to the tetanus toxoid recall Ag. Consistent with regulatory T cell phenotype, addition of $CD4^+CD25^{+high}$ cells to $CD4^+CD25^-$ cells at a ratio of 1:1 significantly inhibited proliferative responses to anti-CD3 (7-fold decrease; $p = 0.03$) and tetanus toxoid (11.5-fold decrease; $p = 0.0002$) (Fig. 6*B*). Modest inhibition was also observed of PHA-mediated stimulation of $CD4^+CD25^-$ cells. The degree of suppression correlated with the levels of $CD4^+CD25^{+high}$ cells added to the culture (data not shown). In contrast, significant inhibition of T cell responses to anti-CD3, tetanus toxoid, and PHA was not observed following the addition of $CD4^+CD25^{+low}$ or $CD4^+CD25^-$ cells. These data demonstrate that DC/breast carcinoma fusion cells induce the expansion of distinct T cell populations with phenotypic and functional characteristics of regulatory and activated T cells, respectively.

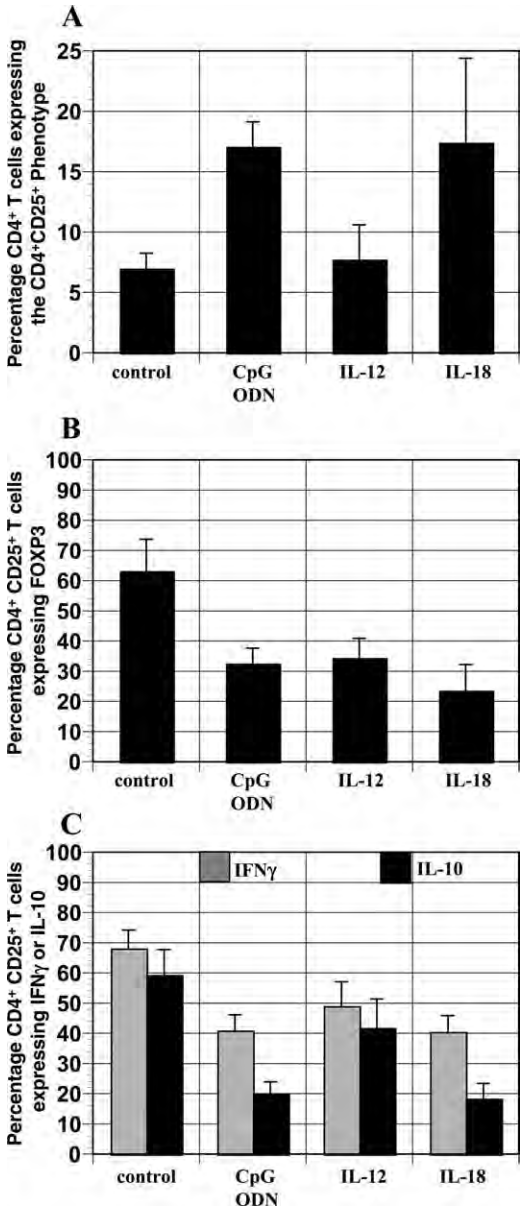

FIGURE 7. Addition of CpG ODN, IL-12, and IL-18 results in decreased expansion of regulatory T cells by DC/breast carcinoma fusions. DC/breast carcinoma fusion cells were cocultured with autologous T cells in the presence or absence of CpG ODN, IL-12, or IL-18 for a period of 5 days. *A*, Following selection of $CD4^+$ T cells, the percentage of $CD4^+/CD25^+$ was determined by bidmensional FACS analysis for each of the conditions. *B*, Mean percentage (±SEM) of $CD4^+CD25^+$ T cells expressing FOXP3 for each of the conditions was determined by intracellular FACS analysis. *C*, Mean percentage (±SEM) of $CD4^+CD25^+$ T cells expressing IFN-γ and IL-10 for each of the conditions was determined by intracellular FACS analysis.

Effect of CpG ODN, IL-12, and IL-18 on DC maturation and fusion-mediated stimulation of T cell populations

In an effort to bias the T cell response toward an activated phenotype and limit the influence of regulatory T cells, we studied the effect of the TLR 9 agonist, CpG ODN on vaccine response. TLR agonists activate elements of the innate immune response and have been shown to augment vaccine efficacy. We examined the capacity of CpG ODN to modulate fusion-mediated stimulation of activated and inhibitory T cell populations by quantifying expression of IFN-γ as compared with IL-10 and FOXP3 in $CD4^+CD25^+$

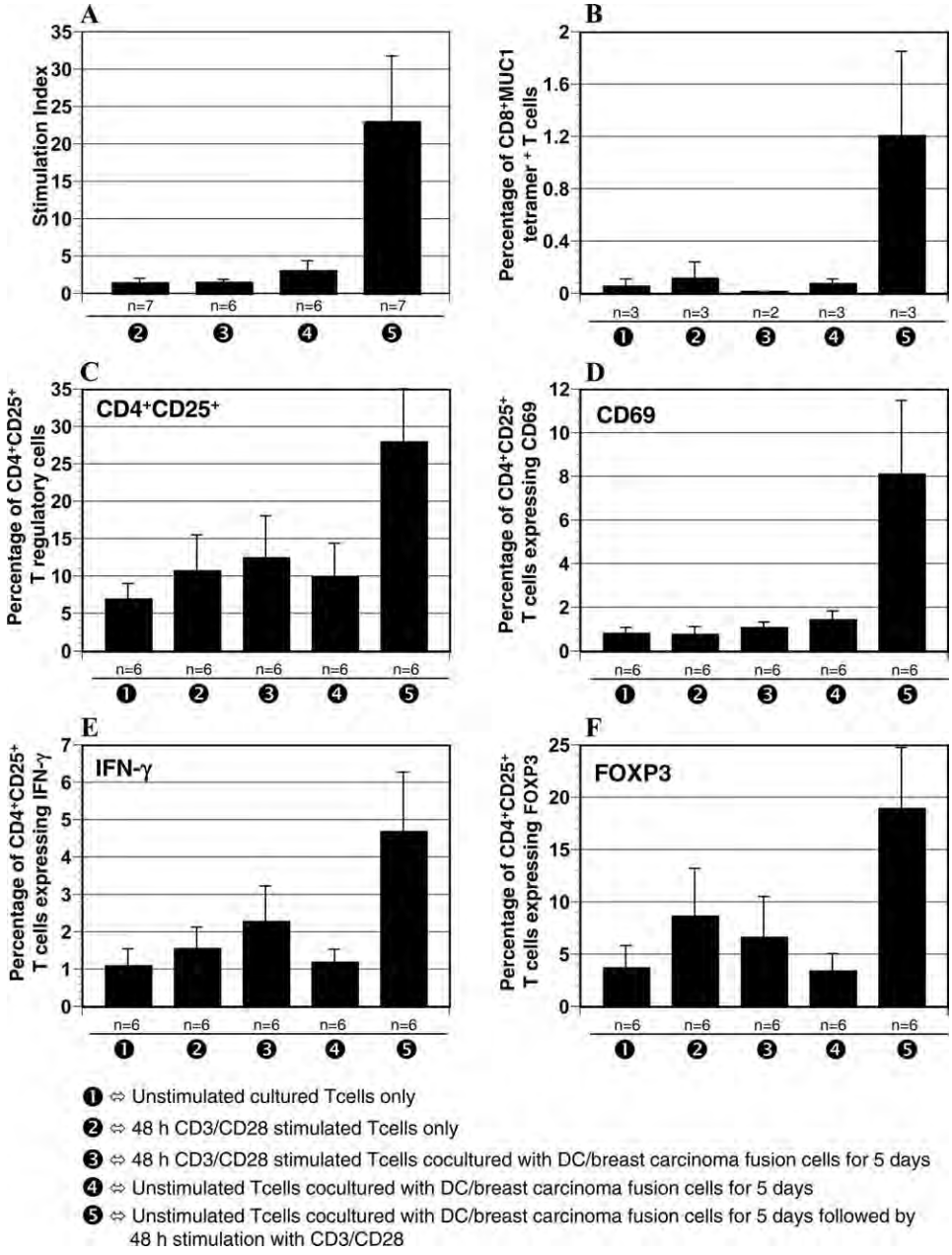

FIGURE 8. Combined stimulation with DC/breast carcinoma fusion cells and CD3/CD28 ligation. Autologous T cells were stimulated by culture with: DC/breast carcinoma fusion cells for 5 days, anti-CD3/CD28 coated plates for 48 h, anti-CD3/CD28 followed by DC/breast carcinoma fusions, or DC/breast carcinoma fusions followed by anti-CD3/CD28. Results were compared with unstimulated T cells. *A*, Mean T cell proliferation was determined for all culture conditions ($n = 6–7$). T cells were aliquoted at 1×10^5/well in triplicate in a 96-well tissue culture plate and pulsed with 1 uCi/ml [^{3}H]thymidine for a period of 18–24 h. T cell proliferation was determined as described in *Materials and Methods* section. Results were normalized by calculation of SI. Mean expression of (*B*) $CD8^+MUC1^+$ T cells using PE-conjugated MUC1-specific tetramers, (*C*) $CD4^+CD25+$ T cells ($n = 6$), (*D*) $CD4^+CD25^+CD69^+$ T cells ($n = 6$), (*E*) IFN-γ-expressing $CD4^+CD25^+$ T cells ($n = 5$), and (*F*) FOXP3-expressing $CD4^+CD25^+$ T cells is presented for each of the culture conditions listed.

cells. We also examined the effect of adding the stimulatory cytokines IL-12 and IL-18 on the phenotypic profile of T cells cocultured with DC/breast carcinoma fusions. A 2.5-fold increase was seen in the fusion stimulated $CD4^+CD25^+$ T cells in the presence of CpG ODN and IL-18, respectively ($p = 0.0004$ and $p = 0.006$). In contrast, no significant increase in $CD4^+CD25^+$ cells was observed when IL-12 was added to the cocultures of T cells and DC/breast cancer fusions (Fig. 7*A*).

The addition of CpG, IL-12, or IL-18 decreased the percentage of $CD4^+CD25+$ with phenotypic characteristics of regulatory T cells as determined by FOXP3 expression ($p = 0.024$, $p = 0.042$, and $p = 0.016$, respectively). In concert with these findings, expression of IL-10 in the $CD4^+CD25^+$ T cells was significantly lowered in cocultures pulsed with CpG ODN (19.8% ±4.1 SEM, $n = 7$; $p = 0.002$) and IL-18 (18.3% ±5.1 SEM, $n = 4$; $p = 0.0004$) as compared with T cells stimulated by DC/breast carcinoma fusions alone (59.3% ±8.4 SEM, $n = 14$) (Fig. 7*B*). Notably, a decrease in the mean percentage of $CD4^+CD25^+$ T cells expressing IFN-γ was also seen following the addition of CpG and IL-18 to cocultures of fusions and autologous T cells (Fig. 7*C*). These results indicate that the addition of IL-12 or TLR agonists enhances vaccine efficacy by limiting the presence of immunosuppressive regulatory cells.

Effect of CD3/CD28 ligation on fusion-mediated stimulation of T cells

As another strategy to bias the vaccine response toward immune activation, we examined the effect of ligation of the TCR/costimulatory complex using Abs directed against CD3 and CD28. Exposure to anti-CD3/CD28 provides an Ag-independent stimulus resulting in the expansion of activated or inhibitory T cells, dependent on the nature of the surrounding immunologic milieu. We hypothesized that sequential stimulation with DC/breast carcinoma fusions followed by anti-CD3/CD28 would amplify the response of T cells that had been primarily activated by the fusion vaccine.

Limited proliferation of T cells was observed following exposure to anti-CD3/CD28 alone (SI: 1.5 ± 0.5 SEM; $n = 7$) or DC/breast carcinoma fusions (SI: 3.1 ± 1.2 SEM; $n = 7$) (Fig. 8*A*) However, a marked increase in T cell expansion was noted when T cells were first stimulated with DC/breast carcinoma fusions and then expanded with anti-CD3/CD28 (SI: 23 ± 8.73 SEM; $n = 7$). Noteworthy, no increase in proliferation was observed when T cells were first exposed to anti-CD3/CD28 and then cultured with DC/breast carcinoma fusions (SI: 1.6 ± 0.3 SEM; $n = 6$). Sequential stimulation with DC/breast carcinoma fusions generated with primary tumor cells and anti-CD3/CD28 resulted in the specific expansion of tumor reactive T cells. Exposure to anti-CD3/CD28 following fusion cell stimulation induced a 13.7 mean-fold increase in MUC1 tetramer binding cells ($n = 3$) (Fig. 8*B*). The percentage of MUC1 tetramer$^+$ cells remained at baseline levels following stimulation with anti-CD3/CD28 alone. With regard to the phenotype of the expanded T cell population, the percentage of T cells expressing the $CD4^+CD25^+$ phenotype was markedly increased following sequential stimulation with DC/tumor fusions and anti-CD3/CD28 (28%) as compared with T cell stimulated by anti-CD3/CD28 (11%) or fusions alone (10%) ($n = 6$) (Fig. 8*C*). As compared with fusion cells alone, sequential stimulation with DC/breast carcinoma fusions and anti-CD3/CD28 resulted in a 5- and 4-fold increase of $CD4^+CD25^+$ T cells that coexpressed CD69 (Fig. 8*D*) and IFN-γ (Fig. 8*E*). In contrast, an ~5-fold increase of regulatory T cells was also observed as manifested by an increase in $CD4^+CD25^+$ T cells that expressed FOXP3 (Fig. 8*F*). These results suggest that fusion-mediated stimulation followed by anti-CD3/CD28 expansion induces increased levels of both activated and regulatory T cells.

Discussion

DC based vaccines represent a promising approach to stimulating antitumor immunity and disease response (2, 49–52). Strategies in which individual tumor Ags are loaded onto DCs are limited by the relative lack of defined immunogenic targets and the risk of immunologic escape through down-regulation of the target Ag. In contrast, DC/breast cancer fusions effectively present a broad array of tumor Ags (12, 16). Endogenously and internalized Ags are presented in the context of the MHC class I and II pathways resulting in a balanced helper and CTL response (53). In a phase I/II trial, 23 patients with metastatic breast and renal carcinoma underwent vaccination with partially mature DCs fused with autologous tumor cells harvested from sites of accessible tissue (17). Fusion cells demonstrated coexpression of tumor-specific Ags, such as MUC1 and DC-derived costimulatory molecules. Vaccination resulted in antitumor immune responses in 10/18 evaluable patients as manifested by an increase in IFN-γ following ex vivo exposure to tumor lysate. Two patients demonstrated disease regression and six patients had stabilization of metastatic disease. Therefore, although the vaccination with DC/breast cancer fusions stimulated antitumor immune responses in a majority of patients, only a subset demonstrated a clinically meaningful disease response.

A major challenge to developing an effective cancer vaccine strategy is overcoming the intrinsic immune deficiencies that limit immunologic response in tumor-bearing patients. Two central elements of tumor-mediated immune suppression include inhibition of DC maturation and the increased presence of regulatory T cells (7–9, 21–23). A potential concern is that tumor cells in the DC/breast carcinoma fusion preparation may inhibit DC development and Ag presentation and induce the expansion of regulatory T cells that would subsequently blunt vaccine response. In previous studies, vaccination with Ag-pulsed immature DCs induced tolerance in Ag-specific T cells (5). In contrast, fusion of immature DCs with multiple myeloma cells resulted in further maturation of the DC fusion partner (54).

In the present study, fusion of DC with breast carcinoma cells resulted in enhanced expression of the costimulatory markers CD80 and CD86, and the maturation marker CD83. Fusion cells generated with immature and mature DCs demonstrated similar levels of maturation, suggesting that the fusion process itself promotes DC activation. Significant expression of IL-12 was observed in both populations consistent with their role as potent APCs with the capacity to stimulate primary immune responses. Expression of CCR7 by the fusion cell populations supports their capacity to migrate to sites of T cell traffic in the draining lymph node. DC/breast carcinoma fusions potently stimulated autologous T cell proliferation with associated secretion of high levels of IFN-γ. Noteworthy, coexpression of IL-12 and IL-10 was observed in the immature and mature DC/breast carcinoma fusion cells. This suggests that the DC/tumor fusions exhibit a mixed phenotype with the capacity to deliver both stimulatory and suppressive signals.

We subsequently examined the effect of DC/breast carcinoma fusions on the relative expansion of activated as compared with regulatory T cells. Stimulation with fusion cells resulted in an increase of $CD4^+CD25^+$ cells. Immunophenotyping of this population revealed the presence of activated ($CD69^+$) as well as inhibitory (CTLA-4^+, FOXP3) T cells. Stimulation with DC/breast carcinoma fusions resulted in the expansion of $CD4^+CD25^{+low}$ and $CD4^+CD25^{+high}$ cells, the latter of which demonstrated characteristic findings of regulatory T cells including uniform expression of FOXP3 and suppression of T cell responsiveness. In concert with these findings, a relative increase in both IFN-γ and IL-10 producing cells was observed.

Regulatory T cells play a significant role in mediating tolerance to self Ags in the normal host (55). In patients with malignancy, their increased presence is thought to mediate tumor-associated suppression of host immune responses (18–20). Precise definition of regulatory cells is complex as many markers such as GITR and CD25 are shared between regulatory and activated T cell populations. Regulatory cells are identified by a panel of markers, including $CD25^{high}$, GITR, CTLA-4, and FOXP3, lack of response to mixed lymphocyte reactions, and the ability to suppress autologous T cell responses in vitro (56–58). DC vaccines may paradoxically increase the presence of regulatory cells that subsequently blunt vaccine response (59). In contrast, depletion of regulatory cells is associated with enhanced vaccine response in diverse tumor models (24, 25). Noteworthy, in a clinical trial, vaccination with DCs pulsed with tumor-specific RNA generated antitumor immunity only when administered in conjunction with an agent (ontak) that eliminates circulating $CD25^+$ cells (25).

Animals models have demonstrated that coadministration of IL-12 and IL-18 promotes T cell activation and augments the efficacy of the DC-based cancer vaccines (14, 32–42). Another strategy to minimize the effect of regulatory T cells is through the activation of innate immunity by ligation of the TLRs (26–31). Administration of CpG ODN to activate TLR9 was shown to overcome the immunosuppression resulting from an expanding tumor burden, decrease the presence of regulatory cells and promote vaccine response. In the present study, addition of the TLR 9 agonists (CpG), IL-12 and IL-18 reduced the levels of regulatory T cells following fusion-mediated stimulation.

In an effort to further enhance vaccine-mediated expansion of tumor reactive lymphocytes, we subsequently examined the effect of sequential stimulation with DC/breast carcinoma fusions and anti-CD3/CD28. CD3/CD28 ligation results in activation of the diverse signaling pathways including those mediated by NFκB

(60, 61). Exposure to anti-CD3/CD28 results in T cell expansion and restoration of the complexity of the T cell repertoire in patients with malignancy and HIV (46, 62–64). Adoptive immunotherapy with anti-CD3/CD28 expanded T cells have been examined in patients with renal carcinoma and those undergoing donor lymphocyte infusions after allogeneic hematopoietic stem cell transplantation (65, 66). Therapy was well tolerated and associated with disease response in a subset of patients. However, CD3/CD28 ligation may also deliver an inhibitory signal that promotes the expansion of T cells with an immunosuppressive phenotype. The nature of the T cell response is dependent on the other signals present at the time of stimulation (43). Stimulation with anti-CD3/CD28 alone or in the presence of inhibitory cytokines may induce the expansion of regulatory T cells (67).

We hypothesized that sequential stimulation with DC/breast carcinoma fusions followed by anti-CD3/CD28 would induce an Ag-specific primary response with associated inflammatory cytokines that would facilitate CD3/CD28 expansion of the activated T cell compartment. In the present study, this pattern of stimulation resulted in the dramatic expansion of tumor reactive T cell populations far in excess to that seen with either modality alone or when T cells were exposed to anti-CD3/CD28 before the DC/breast carcinoma fusions. The resultant T cell population primarily manifested an activated phenotype.

In summary, DC/tumor fusions exhibit characteristics of potent APCs and stimulate tumor-reactive T cells in vitro. However, fusion cells also induce the expansion of regulatory T cells that potentially inhibit vaccine response. Addition of IL-12, IL-18, and CpG ODN biases the T cell response toward an activated phenotype, limiting the influence of regulatory cells. Ligation of the CD3/CD28 markedly stimulates expansion of tumor-specific memory effector cells. As such, these strategies provide a promising strategy to enhance vaccine efficacy and provide an important platform for adoptive immunotherapy with activated T cells.

Disclosures

The authors have no financial conflict of interest.

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