

Systemic GM-CSF Recruits Effector T Cells into the Tumor Microenvironment in Localized Prostate Cancer

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Abstract

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is used as an adjuvant in cancer vaccine trials and has the potential to enhance antitumor efficacy with immunotherapy; however, its immunologic effects are not fully understood. Here, we report results from a phase I study of neoadjuvant GM-CSF in patients with localized prostate cancer undergoing radical prostatectomy. Patients received subcutaneous injections of GM-CSF (250 $\mu\text{g}/\text{m}^2/\text{day}$) daily for 2 weeks (cohort 1; $n = 6$), 3 weeks (cohort 2; $n = 6$), or 4 weeks (cohort 3; $n = 6$). Treatment was well tolerated with all grade 1 or 2 adverse events. Two patients had a

decline in prostate-specific antigen (PSA) of more than 50%. GM-CSF treatment increased the numbers of circulating mature myeloid dendritic cells, proliferating conventional CD4 T cells, proliferating CD8 T cells, and to a lesser magnitude FoxP3⁺ regulatory CD4 T cells. Although GM-CSF treatment did not augment antigen-presenting cell localization to the prostate, treatment was associated with recruitment of CD8⁺ T cells to the tumor. These results suggest that systemic GM-CSF can modulate T-cell infiltration in the tumor microenvironment. *Cancer Immunol Res*; 4(11); 948–58. ©2016 AACR.

Introduction

Prostate cancer remains the most prevalent noncutaneous malignancy and the second leading cause of death in men in the United States (1). Although localized prostate cancer is potentially curable with definitive surgery or radiation, up to one-third of patients treated with curative surgical intent ultimately relapse (2–4). Efforts have been made to study neoadjuvant approaches before radical prostatectomy (RP) to improve clinical outcomes (5). Neoadjuvant immunotherapy strategies are attractive approaches, because immunotherapy is generally well tolerated and may be more effective for patients with less advanced disease and lower disease burden (6). Neoadjuvant studies also provide

the opportunity to study treatment effects within the tumor and its microenvironment.

Many immunotherapeutic strategies focus on the generation of T cell-based antitumor immunity, and a subset of these utilize cytokines, whether alone or in combination with other therapeutic agents. Granulocyte-macrophage colony-stimulating factor (GM-CSF, sargramostim; Sanofi) is a pluripotent cytokine that regulates the differentiation and function of granulocytes and macrophages (7) and is an important growth factor and stimulator of dendritic cells (DCs; ref. 8). GM-CSF has been widely utilized as a cancer vaccine adjuvant as well as in combination with immune checkpoint blocking antibodies to facilitate antigen recognition and T-cell expansion (9–12); however, the benefit of GM-CSF remains unclear (13–15).

GM-CSF monotherapy has been studied as an immune modulator in several clinical studies of advanced refractory or biochemically relapsed prostate cancer (16–18). Significant declines in prostate-specific antigen (PSA) were observed in some patients, and sustained responses were seen in a small minority of patients. Although the exact mechanism by which GM-CSF contributes to control of disease remains unclear, clinical benefit of GM-CSF is thought to be mediated by enhanced antitumor immunity. In a follow-up analysis, GM-CSF was associated with a transient increase in the number of circulating monocytes and DCs (18). Although it has not been demonstrated conclusively whether the observed changes are clinically significant, these results raise the possibility that the positive clinical responses observed in some advanced prostate cancer patients reflect the ability of GM-CSF to enhance antitumor immunity via a T cell-mediated mechanism.

We have shown that neoadjuvant immunotherapy with sipuleucel-T can enhance the recruitment of T cells to the

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prostate (19). Utilizing a similar approach for analysis, here we characterize the systemic and tissue-specific immunologic effects in a cohort of prostate cancer patients treated with neoadjuvant GM-CSF.

Materials and Methods

Study design

This was a single-center phase I study of neoadjuvant GM-CSF prior to planned radical prostatectomy (RP) in patients with localized prostate cancer (NCT00305669). Eligibility criteria included appropriate candidacy for RP, adequate complete blood count, and adequate renal and hepatic functions. Patients were excluded if they had prior prostate cancer-directed therapy (hormonal, radiation, chemotherapy, immunotherapy, or other investigational therapy), were on systemic steroids, whose tumors had neuroendocrine or small cell features, or who had evidence of nodal or distant metastasis. During dose escalation, patients received GM-CSF (250 $\mu\text{g}/\text{m}^2/\text{day}$ subcutaneously) on days 1–14 (cohort 1), days 1–21 (cohort 2), or days 1–28 (cohort 3), with 6 patients in each cohort. A phase II dose expansion phase at the maximum tolerated dose (MTD) was originally planned; however, the study was terminated early due to slow accrual. All patients underwent RP within 5 days after the last dose of GM-CSF. Patients who failed to undergo RP within 5 days after the last dose of GM-CSF or withdrew from the study were replaced. All patients who received any dose of GM-CSF were included in the safety analysis. The study protocol was reviewed and approved by the Institutional Review Board at the University of California, San Francisco (UCSF) prior to initiation. Signed informed consents were obtained from all patients at the time of trial enrollment.

Study endpoints

The co-primary endpoints were safety and tolerability of daily GM-CSF prior to RP and differences in the number of infiltrating CD11c⁺ and CD68⁺ antigen-presenting cells (APC; number/ μm^2) between RP tissues of study patients and RP tissues from untreated control patients, quantified by immunohistochemistry (IHC) and image analysis. Secondary endpoints included differences in the number of infiltrating T-cell subsets (CD3⁺, cytotoxic CD3⁺CD8⁺, helper CD4⁺, and regulatory CD4⁺FOXP3⁺ T cells) between RP tissues of treated patients and untreated controls, quantified by IHC and image analysis; treatment-associated changes in circulating immune cell subsets measured by flow cytometry of peripheral blood mononuclear cells (PBMC); treatment-associated changes in PSA; changes in Gleason scores between pretreatment biopsy and posttreatment RP tissues; and surgical outcomes, including surgical complications and estimated blood loss.

Blood and tissue samples

Whole blood was collected via peripheral venipuncture at baseline (all cohorts), day 14 (all cohorts), and preoperatively (day 21 for cohort 2 and day 28 for cohort 3). PBMCs were isolated from whole blood by ficoll-hypaque density gradient centrifugation. Formalin-fixed and paraffin-embedded (FFPE) specimens from radical prostatectomy specimens were made available for the current study. Tissues from 12 patients who underwent RP at UCSF without preoperative therapy, matched to the study patients by the distribution of Cancer of the Prostate Risk Assessment (CAPRA) risk scores, were used as controls.

Flow cytometry

PBMCs were thawed into FACS wash (PBS, 2% BSA) and washed twice with FACS wash. Samples were stained with designated panels for 20 minutes at 4°C and washed twice with FACS wash. Cells requiring intracellular staining were fixed and permeabilized with BD Cytofix/Cytoperm buffer (Cat #554722) according to the manufacturer's protocol. Intracellular staining with antibodies was carried out for 30 minutes at 4°C and washed twice with FACS wash. Cells were acquired on a LSRII flow cytometer (BD Biosciences) and data were analyzed with FlowJo analysis software version 9.7.5 (FlowJo, LLC). DCs were gated from both the lymphocyte and myeloid populations in the forward scatter (FSC)-A by side-scatter (SSC)-A plots. Monocytes and granulocytes were gated from the myeloid populations in the FSC-A by SSC-A plots. T cells were gated from the lymphocyte population in the FSC-A by SSC-A plots. The absolute counts for each cell type were calculated by multiplying (i) the percentage of cells gated with (ii) the percentage of the preceding subsets, and (iii) absolute monocyte count plus absolute lymphocyte count for DCs; absolute monocyte count for monocytes and myeloid-derived suppressor cells (MDSC); and absolute lymphocyte count for T-cell subsets. The absolute monocyte and lymphocyte counts were obtained from the complete blood count with differential of respective patients on the day that blood was drawn. The sources for fluorochrome-conjugated antibodies to human CD3, CD8, CD14, CD11c, CD25, CD45, CD66b, CD69, CD86, CD123, FoxP3, HLA-DR, INOS, and Ki-67 are summarized in Supplementary Table S1.

Immunohistochemistry

Immunohistochemistry was performed on 5- μm FFPE sections from RP specimens. For CD68 single staining, slides were incubated with anti-CD68 mouse monoclonal antibody (Dako #M0876, Clone #PG-M1) for 30 to 60 minutes at room temperature, and antigen-antibody complexes were visualized with the EnVision⁺ Dual Link System-HRP (Dako #K4063). For double stains, slides were first incubated with primary antibody to CD8, FoxP3, or CD83 (Supplementary Table S2) for 30 to 60 minutes at room temperature, and visualized with horseradish peroxidase (HRP) using DAB⁺ as chromogen (EnVision G|2 Doublestain System; Dako #K5361). This was followed by incubation with secondary antibody to CD3, CD4, or CD11c (Supplementary Table S2) for 60 minutes and visualized with alkaline phosphatase (AP) using Permanent Red as chromogen (Envision G|2 Doublestain System; Dako #K5361). Specimens were counterstained with hematoxylin prior to analysis. Immune infiltration in RP tissue was evaluated by immunohistochemistry, where tissue was designated into three distinct compartments: benign glands, tumor interface, and tumor center. Five randomly selected fields from each compartment were captured with ImageScope software (Aperio), and automatic cell counts for single- and double-stained cells were determined with AxioVision software (Zeiss).

Statistical analysis

Statistical analysis was performed with Prism software Version 6.0. Baseline patient characteristics were compared among cohorts using the Fisher exact test for categorical variables and the Kruskal–Wallis test for continuous variables. Due to the study design, peripheral blood immune subset data were available at baseline and day 14 for all three treated cohorts, day 21 for cohort

2 only, and day 28 for cohort 3 only. Pairwise comparisons between each time point were performed by the Wilcoxon matched-pairs signed rank test. To analyze differences in tissue immune cell infiltration by tissue compartment (benign glands, tumor interface, and tumor center), the Wilcoxon matched-pairs signed rank test was also used. The Mann-Whitney test was used to perform comparisons of tissue immune cell infiltration between the untreated controls and each of treated cohorts. The Kruskal-Wallis test was implemented to determine whether there was a significant difference among the three treated cohorts, and the Wilcoxon matched-pairs signed rank test was then used to perform pairwise comparisons between treated cohorts if significant. A *P* value of < 0.05 was considered statistically significant. No multiple testing adjustment was performed.

Results

Patients

Between November 16, 2004, and February 15, 2011, 18 patients were enrolled at UCSF. Overall, 7 (38.9%) patients had low-risk disease, 8 (44.4%) patients had intermediate-risk disease, and 3 (16.7%) patients had high-risk disease (Table 1). Patients were enrolled into cohort 1 (GM-CSF 250 $\mu\text{g}/\text{m}^2/\text{day}$ s.c. for 14 days), cohort 2 (GM-CSF 250 $\mu\text{g}/\text{m}^2/\text{day}$ s.c. for 21 days), and cohort 3 (GM-CSF 250 $\mu\text{g}/\text{m}^2/\text{day}$ s.c. for 28 days), sequentially. Due to the small sample sizes and nonrandomized nature of the study, baseline characteristics were not completely balanced

among the cohorts. Gleason score and NCCN risk group were statistically different among the cohorts, largely driven by 2 patients with Gleason 8 disease in cohort 3 (Table 1). All 18 patients underwent radical prostatectomy (RP) within 5 days of the last GM-CSF injection. Four additional patients who were enrolled were replaced due to delayed RP or withdrawal from the study. One patient had acute grade 2 hypoxemia during anesthesia for planned RP related to body habitus; surgery was aborted and the patient was taken off of the study. Two patients were removed from the study due to asymptomatic leukocytosis between $30 \times 10^9/\text{L}$ and $40 \times 10^9/\text{L}$. One patient withdrew consent in the middle of treatment. All enrolled patients who received any dose of GM-CSF were included in the safety analysis.

Safety

Adverse events (AE) determined to be related or possibly related to GM-CSF treatment that occurred prior to RP were summarized in all 22 patients. All AEs were grade 1 or grade 2 and transient (Table 2). The most common AEs (>10% of patients) were injection site reaction (81.8%), fatigue (63.6%), bone pain (27.3%), and headache (13.6%). Neoadjuvant GM-CSF treatment did not appear to affect operative complications, duration of surgery, or estimated blood loss during surgery. Leukocytosis was not considered an AE because GM-CSF treatment was anticipated to cause a rise in total white blood cell count.

Table 1. Baseline demographics and disease characteristics

	Cohort 1 (N = 6)	Cohort 2 (N = 6)	Cohort 3 (N = 6)	Total	P
Age, year					0.150
Median	60	57	63	60	
Range	57-66	51-63	53-68	51-68	
Weight, lbs					0.103
Median	196.2	228.4	188.4	202.3	
Range	159.7-224.3	178.5-269.9	151.2-227.3	151.2-269.9	
Race, N (%)					1.000
Caucasian	6 (100.0)	6 (100.0)	6 (100.0)	18 (100.0)	
ECOG					1.000
PS = 0	6 (100.0)	6 (100.0)	6 (100.0)	18 (100.0)	
Gleason sum, N (%)					0.023
≤ 6	2 (33.3)	1 (16.7)	4 (66.7)	7 (38.9)	
7	4 (66.7)	5 (83.3)	0 (0.0)	9 (50.0)	
≥ 8	0 (0.0)	0 (0.0)	2 (33.3)	2 (11.1)	
Clinical stage, N (%)					0.100
T1c	2 (33.3)	5 (83.3)	2 (33.3)	9 (50.0)	
T2a	4 (66.7)	0 (0.0)	3 (50.0)	7 (38.9)	
T3a	0 (0.0)	1 (16.7)	1 (16.7)	2 (11.1)	
PSA, ng/mL					0.466
Median	5.7	5.9	4.2	5.0	
Range	3.0-9.1	3.6-13.2	3.2-8.9	3.0-13.2	
LDH, U/L					0.927
Median	151	139	151	150	
Range	128-170	129-237	95-199	95-237	
Hemoglobin, g/dL					0.654
Median	15.1	15.4	15.1	15.2	
Range	14.3-15.5	14.7-16.9	12.6-15.9	12.6-16.9	
Alkaline phosphatase, U/L					0.548
Median	68	65	75	72	
Range	46-112	47-88	50-97	46-112	
NCCN risk group, N (%)					0.007
Low	2 (33.3)	1 (16.7)	4 (66.7)	7 (38.9)	
Intermediate	4 (66.7)	4 (66.7)	0 (0.0)	8 (44.4)	
High	0 (0.0)	1 (16.7)	2 (33.3)	3 (16.7)	

Abbreviations: ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase.

Table 2. Summary of on-study AEs

Adverse events, N (%)	Grade 1	Grade 2	Total
Injection site reaction	17 (77.3)	1 (4.5)	18 (81.8)
Fatigue	13 (59.1)	1 (4.5)	14 (63.6)
Bone pain	5 (22.7)	1 (4.5)	6 (27.3)
Headache	3 (13.6)	0 (0.0)	3 (13.6)
Back pain	2 (9.1)	0 (0.0)	2 (9.1)
Extremity pain	2 (9.1)	0 (0.0)	2 (9.1)
Muscle pain	1 (4.5)	1 (4.5)	2 (9.1)
Diarrhea	1 (4.5)	0 (0.0)	1 (4.5)
Abdominal pain	1 (4.5)	0 (0.0)	1 (4.5)
Fever (non-neutropenic)	1 (4.5)	0 (0.0)	1 (4.5)
Chills	1 (4.5)	0 (0.0)	1 (4.5)
Diaphoresis	1 (4.5)	0 (0.0)	1 (4.5)
Rash	1 (4.5)	0 (0.0)	1 (4.5)
Chest pain	1 (4.5)	0 (0.0)	1 (4.5)
Joint pain	1 (4.5)	0 (0.0)	1 (4.5)
Pruritus	1 (4.5)	0 (0.0)	1 (4.5)
Urinary frequency	1 (4.5)	0 (0.0)	1 (4.5)
Urinary incontinence	1 (4.5)	0 (0.0)	1 (4.5)
Dehydration	1 (4.5)	0 (0.0)	1 (4.5)
Hoarseness	1 (4.5)	0 (0.0)	1 (4.5)

NOTE: Patients could have had more than one AE. No grade ≥ 3 AEs were observed.

Clinical correlates

Two patients (11.1%) had a >50% decline in PSA during neoadjuvant GM-CSF treatment (Fig. 1). One patient in cohort 1 had baseline PSA of 6.4 ng/mL, which decreased to 3.09 ng/mL after 2 weeks of neoadjuvant therapy. A cohort 3 patient had baseline PSA of 5.1 ng/mL, which decreased to 3.8 ng/mL after 2 weeks, then further decreased to 2.4 ng/mL after 4 weeks of therapy. One patient (5.6%) in cohort 1 had a >50% increase in PSA during treatment (Fig. 1), from 4.2 ng/mL to 7.97 ng/mL. Because the baseline PSA values for all patients were relatively low (range, 3.03–13.2 ng/mL), the clinical significance of these PSA changes are unclear. Overall, no significant downstaging was observed at the time of RP. Five patients (27.8%) had downgrading of Gleason scores, and 5 patients (27.8%) had upgrading of Gleason scores at the time of RP compared with pretreatment biopsies (Supplementary Fig. S1). All patients had pN0 ($n = 11$) or pNX ($n = 7$) disease at the time of RP.

Changes in the peripheral APC compartment

The numbers of circulating DCs and monocytes have been reported to increase after GM-CSF administration in patients (18). There is also preclinical evidence that high doses of GM-CSF may

recruit MDSCs and impair antitumor immunity (20). In this study, PBMCs from study subjects at baseline (before treatment), day 14, and at completion of treatment (day 21 for cohort 2, and day 28 for cohort 3) were stained for APC markers and analyzed by flow cytometry to assess changes in immune subsets during treatment.

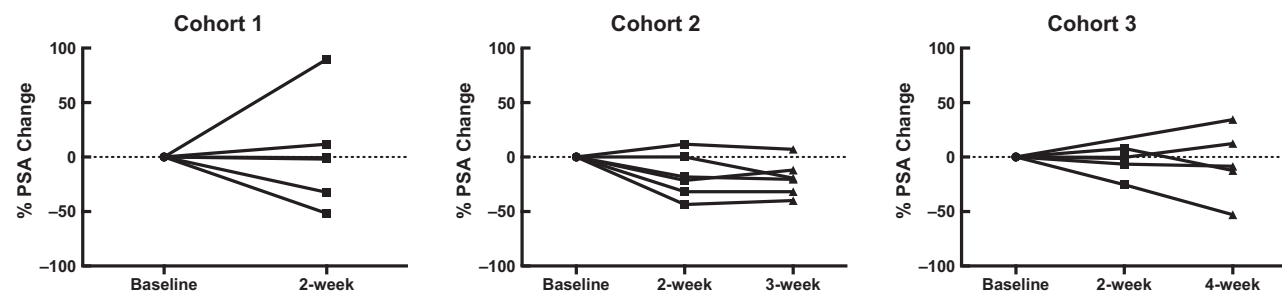
The HLA-DR⁺ DCs were separated into myeloid (CD11c⁺CD123⁻) and plasmacytoid (CD11c⁻CD123⁺) lineages. CD86 positivity was utilized to identify activated and mature DCs. GM-CSF treatment led to a significant decrease in the frequency of circulating myeloid and plasmacytoid DCs at day 14 compared with baseline ($P = 0.0002$ and $P = 0.0151$, respectively; Fig. 2A and B, top left). However, the absolute number of myeloid and plasmacytoid DCs (Fig. 2A and B, top right) and the frequencies and numbers of their respective CD86⁺ subsets (Fig. 2A and B, bottom) did not significantly decrease. The absolute number of CD86⁺ myeloid DCs overall increased from baseline to day 14 ($P = 0.0302$), indicating that the decrease in frequency of DCs did not adversely affect the number of activated DCs.

The frequency of CD14^{hi}HLADR⁺CD66b⁻ monocytes overall decreased from baseline to day 14 ($P = 0.0052$); however, the absolute number of these monocytes was not significantly changed (Fig. 2C). The frequency and absolute number of monocytic MDSCs (CD14^{hi}HLADR⁻CD66b⁻INOS⁺) did not change significantly with GM-CSF treatment (Fig. 2D).

Changes in the peripheral T-cell compartment

The study hypothesized that GM-CSF induces the uptake and processing of relevant prostate cancer antigens by DCs, which in turn crossprime anti-prostate cancer T cells. The effects of neoadjuvant treatment with GM-CSF on peripheral T-cell subsets were assessed by flow cytometry. PBMCs from trial patients at baseline, day 14, and at completion of treatment were stained for T-cell markers, Ki67 as a proliferative marker, and CD69 as an activation marker.

The administration of GM-CSF did not lead to an overall increase in the frequencies or numbers of total cytotoxic T cells (CD3⁺CD8⁺; Fig. 3A, left) or activated cytotoxic T cells (CD3⁺CD8⁺CD69⁺; Fig. 3A, middle). However, the frequency and number of proliferative cytotoxic T cells (CD3⁺CD8⁺Ki67⁺) increased after GM-CSF treatment between baseline and day 14 ($P = 0.0052$ and $P = 0.0125$, respectively; Fig. 3A, right). In contrast, the frequencies and numbers of CD3⁺CD8⁻ helper T cells ($P = 0.0002$ and $P = 0.0353$, respectively; Fig. 3B, left),

**Figure 1.**

Percent PSA change from baseline by cohort. Cohort 1: GM-CSF, 250 $\mu\text{g}/\text{m}^2/\text{day}$ s.c. for 14 days. Cohort 2: GM-CSF, 250 $\mu\text{g}/\text{m}^2/\text{day}$ s.c. for 21 days. Cohort 3: GM-CSF, 250 $\mu\text{g}/\text{m}^2/\text{day}$ s.c. for 28 days.

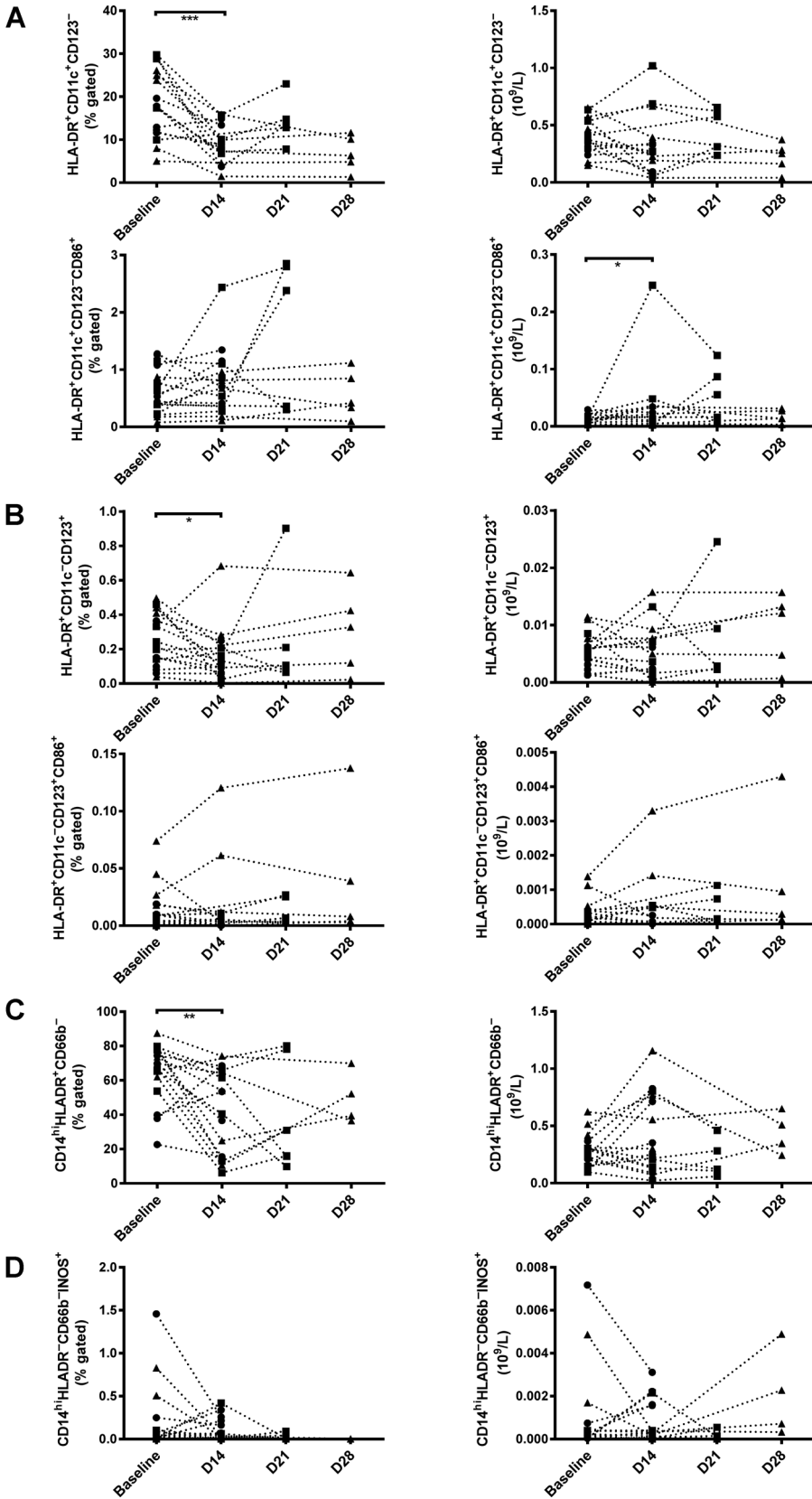


Figure 2.

Changes in peripheral DC, monocyte, and MDSC subsets. HLA-DR⁺CD11c⁺CD123⁻ myeloid DCs (A), HLA-DR⁺CD11c⁻CD123⁺ plasmacytoid DCs (B), and their respective mature subsets (bottom) are expressed in terms of frequency among gated PBMCs (left), and as absolute numbers (right). CD14^{hi}HLADR⁺CD66b⁻ monocytes (C) and CD14^{hi}HLADR⁺CD66b⁻INOS⁺ monocytic MDSC (D) are shown as frequencies among gated PBMCs (left), and as absolute counts (right). Circle, cohort 1; square, cohort 2; triangle, cohort 3. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

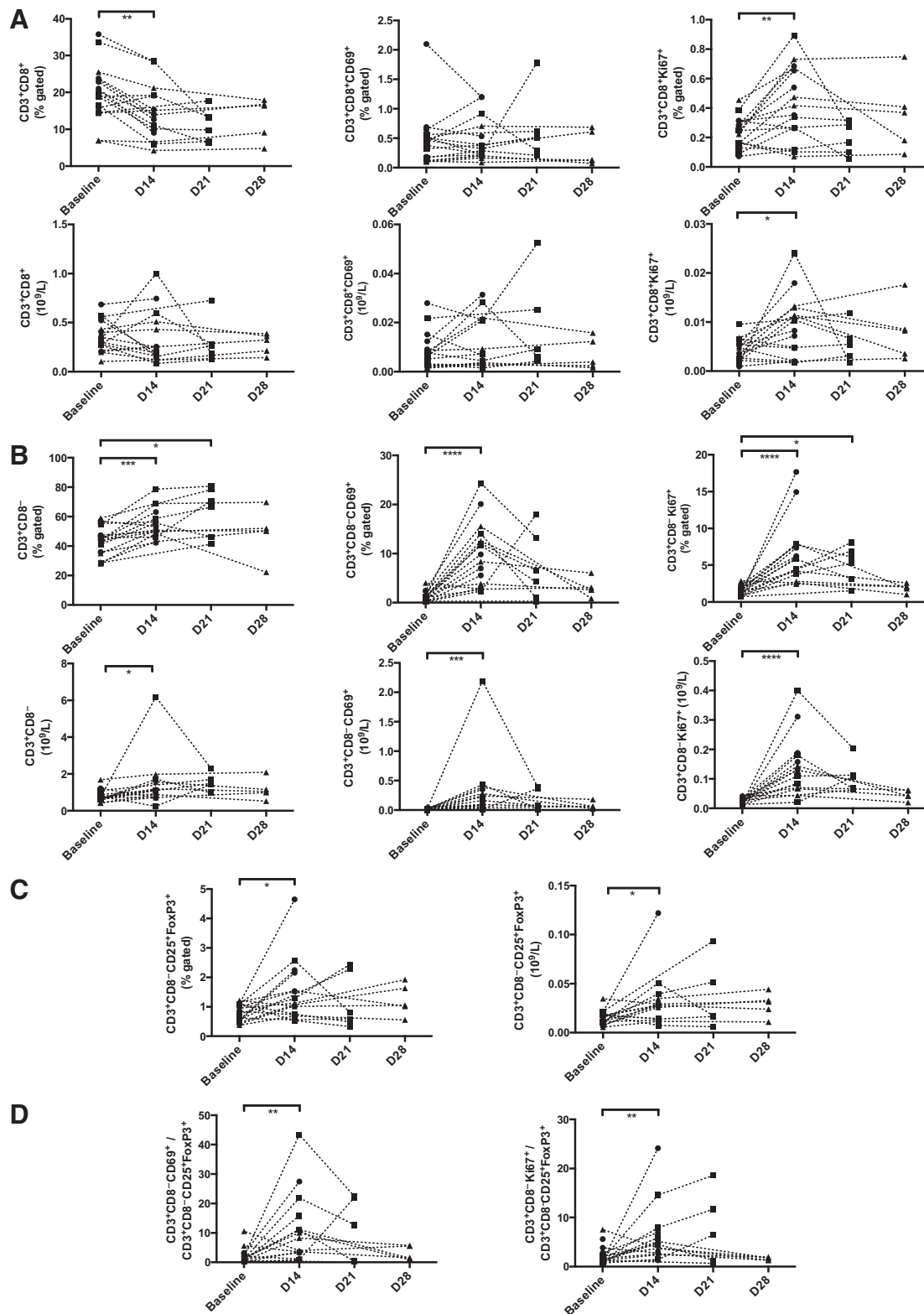


Figure 3. Changes in peripheral T-cell subsets. CD3⁺CD8⁺ cytotoxic T cells (A), CD3⁺CD8⁻ helper T cells (B), and CD3⁺CD8⁻CD25⁺FoxP3⁺ regulatory T cells (C) are expressed in terms of frequency among gated PBMCs, and as absolute numbers. The ratio of activated and proliferative CD3⁺CD8⁻ helper T cells to regulatory T cells are shown in D. Circle, cohort 1; square, cohort 2; triangle, cohort 3. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001.

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activated helper T cells (CD3⁺CD8⁻CD69⁺; $P < 0.0001$ and $P = 0.0002$, respectively; Fig. 3B, middle), and proliferative helper T cells (CD3⁺CD8⁻Ki67⁺; $P < 0.0001$ and $P < 0.0001$, respectively; Fig. 3B, right) all increased after GM-CSF treatment. Of note, there also appeared to be an increase in the frequency and number of regulatory T cells (CD3⁺CD8⁻CD25⁺FoxP3⁺; $P = 0.0182$ and $P = 0.0103$, respectively; Fig. 3C). A higher ratio of activated CD3⁺CD8⁻ helper T cells to regulatory T cells, as well as proliferative CD3⁺CD8⁻ helper T cells to regulatory T cells, was observed from baseline to day 14 ($P = 0.0067$ and $P = 0.0054$, respectively; Fig. 3D). No significant differences were observed in the ratio of activated and proliferative cytotoxic T cells to regulatory T cells (data not shown). Most of the observed circulating T-cell changes appeared to be transient; differences compared with pretreatment baseline were most pronounced at day 14.

APC localization in the tumor microenvironment

Untreated control RP and post-GM-CSF RP specimens were stained for the presence of CD11c⁺CD83⁺ DCs and CD68⁺ macrophages/monocytes. Representative images are shown (Fig. 4A and B, top). Overall, there was very little APC infiltration within the tumor or its microenvironment, with particularly minimal CD83 staining in both groups. No significant quantitative differences were seen in CD11c and CD68 staining between untreated control RP and post-GM-CSF RP specimens within the tumor interface or tumor (Fig. 4A and B, bottom). CD11c staining was observed to be lower in the post-GM-CSF RP specimens compared with untreated control RP tissues within the benign peritumoral tissue. These findings indicate that systemic GM-CSF did not significantly enhance APC localization to the tumor or its microenvironment.

T-cell localization in the tumor microenvironment

RP tissues from untreated controls and study patients treated with GM-CSF were stained for CD3⁺, CD3⁺CD8⁺, CD4⁺, and CD4⁺FoxP3⁺ T-cell subsets (Fig. 4C). Immunohistochemical specimens were quantified for positive cells in the benign peritumoral tissue, at the tumor interface, and within the tumor center. Overall, the administration of neoadjuvant GM-CSF significantly increased the number of CD3⁺ cells at the tumor interface, and to a lesser degree in the tumor center (Fig. 4D, top left). At the tumor interface, the increase in CD3⁺ cells were comprised of CD3⁺CD8⁺ cytotoxic T cells (Fig. 4D, top right; $P < 0.0001$); there was also a trend toward increase in conventional CD4⁺ T cells (Fig. 4D, bottom left; $P = 0.0646$). In the tumor center, the increase in CD3⁺ T cells was comprised of both CD3⁺CD8⁺ cytotoxic T cells (Fig. 4D, top right; $P = 0.008$) and conventional CD4⁺ T cells (Fig. 4D, bottom left; $P = 0.008$). Treatment was also associated with an increase in CD4⁺FoxP3⁺ regulatory T cells in the tumor center, although to a much lower magnitude than cytotoxic or conventional T cells (Fig. 4D, bottom right; $P = 0.0234$).

T-cell localization was analyzed by cohort to assess for any differential effects of GM-CSF by duration of treatment (Fig. 5). An increase in CD3⁺CD8⁺ T-cell infiltration within the tumor interface compared with untreated controls was observed in all cohorts ($P = 0.0268$ for cohort 1; $P = 0.0008$ for cohort 2; and $P = 0.0008$ for cohort 3; Fig. 5B, middle). An increase in CD3⁺, CD3⁺CD8⁺, and CD4⁺ T-cell infiltration within the tumor center compared with untreated controls was significant only in cohort 3 ($P =$

0.0320, 0.0182, and 0.0001, respectively; Fig. 5A–C, right). Although small in magnitude, the increase in CD4⁺FoxP3⁺ T-cell infiltration into the tumor interface and center compared with untreated controls was statistically significant only in cohort 3 ($P = 0.0415$ and 0.0020, respectively; Fig. 5D, middle and right). When comparing the three treated cohorts, differences were observed only in CD4⁺ T-cell infiltration in the benign peritumoral tissue and tumor interface. Specifically, CD4⁺ T-cell infiltration in the benign peritumoral tissue and tumor interface was significantly higher in cohort 3 compared with shorter-duration treatment cohorts (Fig. 5C, left and middle). Taken together, these data suggest that whereas peripheral effects of GM-CSF appeared to be transient, longer durations of GM-CSF have differential effects within the tumor microenvironment. It should be noted that in addition to treatment duration, differences in baseline characteristics among study cohorts may have contributed to these observations (Table 1).

Discussion

GM-CSF is a pleotropic cytokine that is FDA approved for the mobilization of granulocytes (7). The role of GM-CSF has expanded to cancer immunotherapeutics in recent years. Tvec, an attenuated herpes simplex 1 virus (HSV-1) that expresses GM-CSF, is FDA approved for the treatment of metastatic melanoma (21). Sipuleucel-T is an autologous cellular vaccine that is FDA approved for the treatment of asymptomatic or minimally symptomatic metastatic castration-resistant prostate cancer, which consists of autologous PBMCs cultured and activated with a recombinant protein PAP-GM-CSF (22, 23). More recently, GM-CSF combined with local radiotherapy was reported to lead to abscopal responses in 26.8% (95% CI, 14.2–42.9) patients with metastatic solid tumors (24). Despite these advances, the benefit of GM-CSF remains unclear, in part because of the heterogeneity of different clinical studies, and a lack of direct investigation of the role of GM-CSF.

GM-CSF monotherapy may possess single-agent activity in advanced prostate cancer (16–18) and is being used as an adjuvant in multiple investigational trials for advanced prostate cancer, including with DNA plasmid vaccines (25, 26) and PROSTVAC, a PSA-targeted poxviral vaccine for prostate cancer (27). The phase II randomized controlled trial of PROSTVAC showed promising results, including improvement in overall survival (27). The phase III randomized, double-blind study of PROSTVAC with or without GM-CSF is ongoing (NCT01322490) and should provide insights into the contribution of GM-CSF to PROSTVAC vaccination strategy.

In this study, we showed that neoadjuvant GM-CSF administration is well tolerated and enhances CD4⁺ and CD8⁺ T-cell infiltration but not APC recruitment into the tumor microenvironment in prostate cancer patients. In the circulation, systemic GM-CSF administration led to transient increases in mature myeloid DCs, proliferating CD8⁺ T cells, and activated and proliferating CD4⁺ T cells. These results suggest that APCs activate T cells outside of the tumor microenvironment; this may be occurring in the lymphoid tissues, but would need to be formally examined. It is also possible that GM-CSF exerts qualitative effects on APCs by promoting maturation and antigen presentation efficiency, leading to the generation of more effective T-cell responses. To our knowledge, this is the first study to demonstrate that systemic administration of GM-CSF leads to an increase

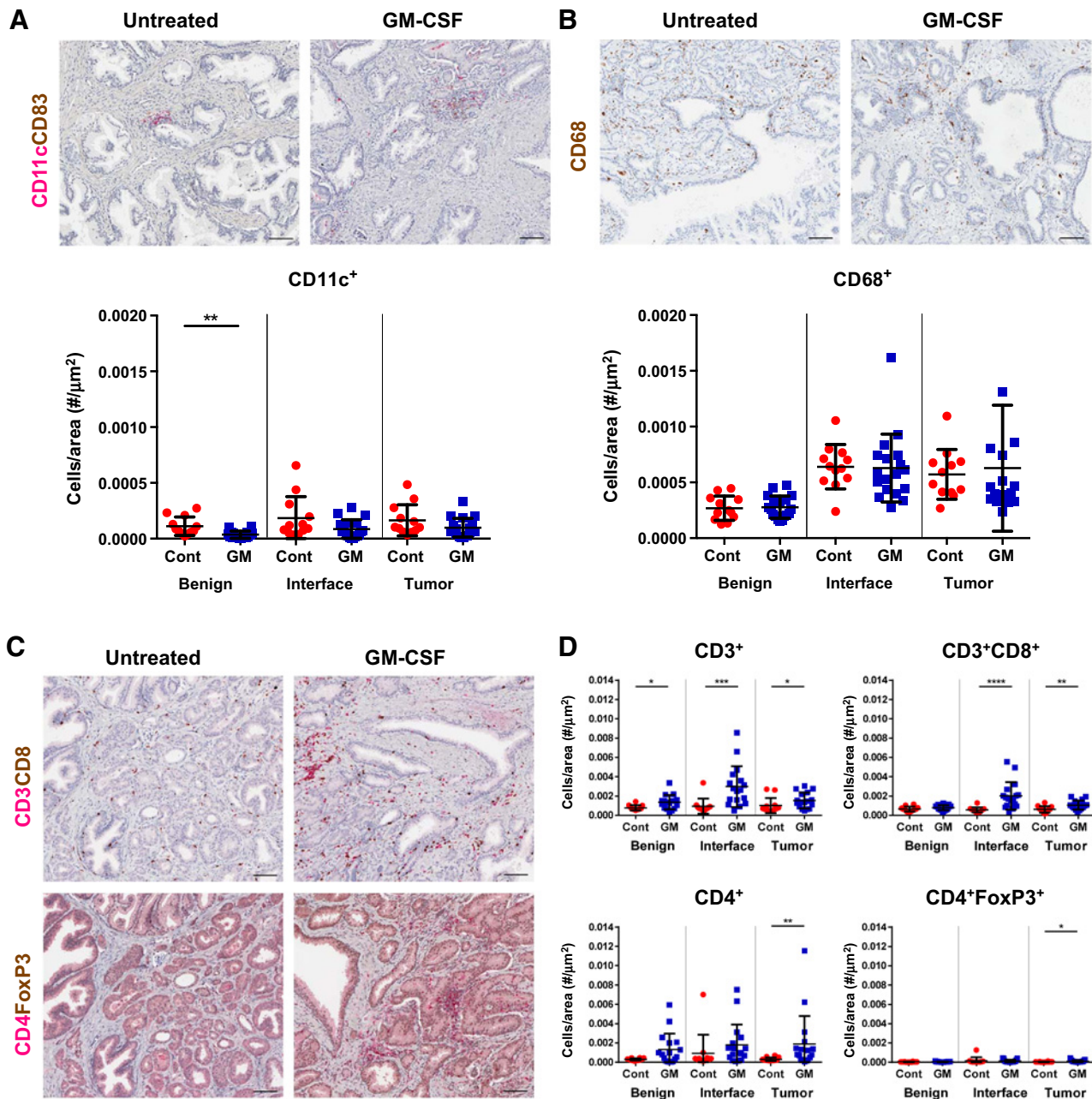


Figure 4. APC infiltration and T-cell infiltration in tissue compartments. CD11c and CD83 immunohistochemical double staining is shown in RP tumor specimens obtained from untreated and GM-CSF-treated patients (A, top), with quantitation of CD11c⁺ events (A, bottom). CD68 immunohistochemical single staining is shown in tumor specimens from untreated and GM-CSF-treated patients (B, top), with quantitation of CD68⁺ events (B, bottom). Immunohistochemical double stains of CD3 and CD8 (C, top) and CD4 and FoxP3 (C, bottom) in RP tumor specimens obtained from untreated and GM-CSF-treated patients. Quantitation of CD3⁺ (D, top left), CD3⁺CD8⁺ (D, top right), CD4⁺ (D, bottom left), and CD4⁺FoxP3⁺ (D, bottom right) subsets is shown. Scale bar, 100 μ m. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

in T-cell infiltration at the tumor site. This study provides a potential immunologic mechanism to explain the ability of GM-CSF to control prostate cancer in at least a subset of patients in prior clinical trials (16–18).

Although we have not further characterized the observed T-cell populations in the current study, it is likely that at least

some of the infiltrating lymphocytes preferentially home to the tumor site after GM-CSF administration, a portion of which are likely to be of an activated effector T-cell phenotype. Additional studies are needed to further characterize these T-cell populations to conclusively demonstrate if they are tumor specific, and to determine if they are effective for tumor

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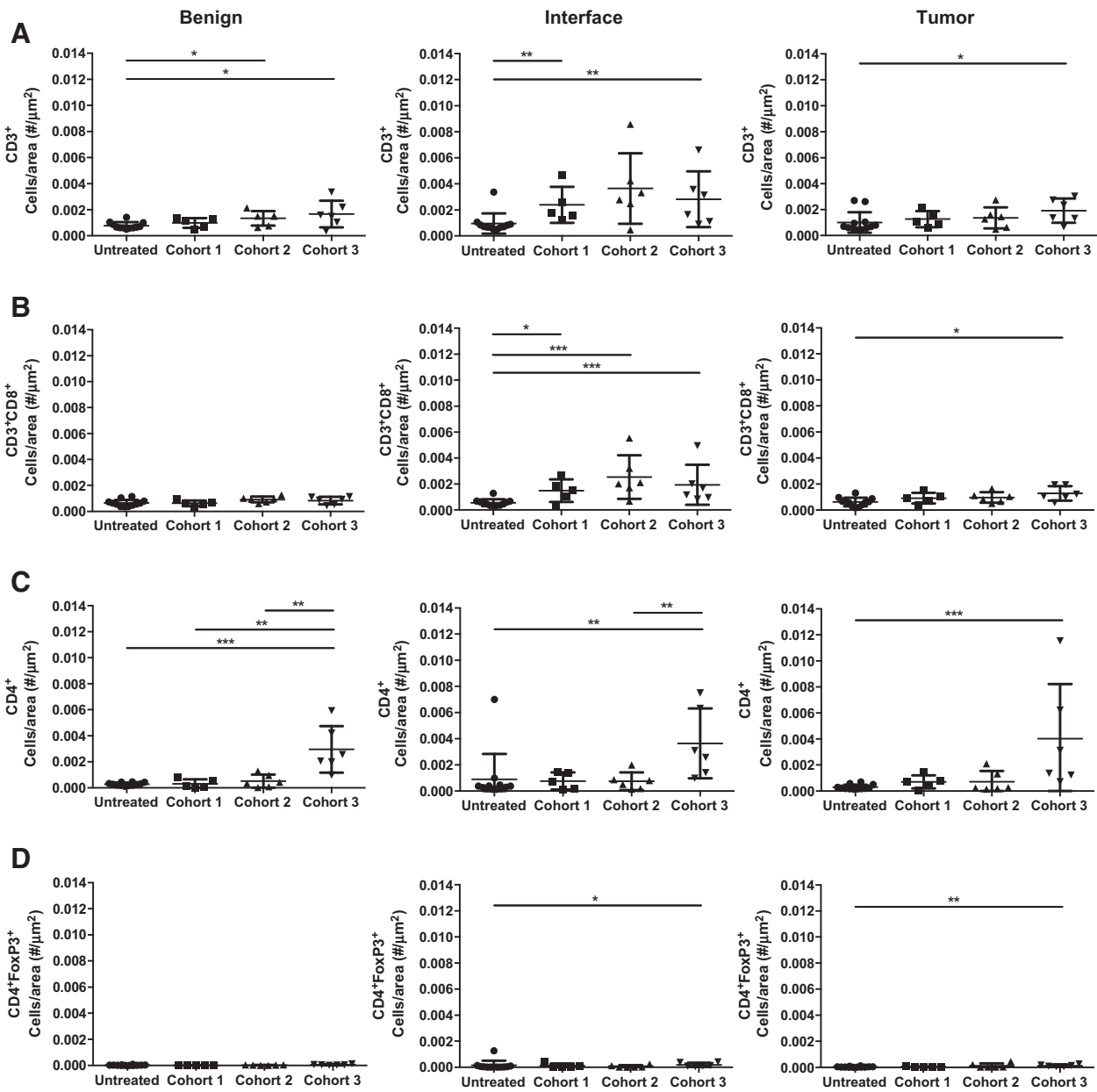


Figure 5. Comparison of T-cell infiltration in tissue compartments across treatment cohorts. Frequencies of CD3⁺ (A), CD3⁺CD8⁺ (B), CD4⁺ (C), and CD4⁺FoxP3⁺ (D) T-cell subsets are quantitated in benign peritumoral tissues (left), at the tumor interface (middle), and within the tumor center (right) in untreated patients and patients treated with GM-CSF by study cohort. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

control. Most likely, clinically significant antitumor immunity will require both sufficient numbers of infiltrating T cells and the "right" activation phenotype.

Systemic GM-CSF administration may also affect other immune cell subsets systemically, including DCs (18) and immunosuppressive cells such as regulatory T cells (28) and MDSC (20). In this article, we have shown that the number of circulating proliferative CD8⁺ T cells and regulatory T cells increases following GM-CSF, and the overall frequency of myeloid and plasmacytoid DCs decreased following GM-CSF. Parallel findings were reported in a phase I study of prostate

GVAX, consisting of two allogeneic GM-CSF-transduced prostate cancer cell lines (LN-CaP and PC3), combined with six infusions of escalating doses of ipilimumab, a monoclonal antibody to CTLA-4, in patients with metastatic castration-resistant prostate cancer (mCRPC; refs. 29, 30). However, given the multiple components in their immunotherapy strategy, it is difficult to attribute the observed changes to GM-CSF alone, and the influence of ipilimumab and the allogeneic cells cannot be discerned. Regardless, these investigators also made the clinically important observation that increases in activated T cells, low frequencies of MDSCs, as well as high DC

activation, were predictive of improved overall survival (29, 30). We have not yet correlated changes in immune cell subsets with clinical outcomes in our patient population. Thus, the clinical impact of our observations remains unknown and will be a focus of future studies.

As in our study with neoadjuvant sipuleucel-T (19), all of the patients on this trial underwent RP shortly after immunologic intervention, as specified by the trial protocol. Thus, the kinetics of the T-cell responses after GM-CSF have not been well defined. For example, it is not known how rapidly the activated T cells home to and infiltrate the tumor, and how durable the observed immunologic effects may be. In addition, it is not clear if a memory T-cell response was generated by systemic GM-CSF as given on this trial, given the relatively short follow-up of these patients and the absence of long-term immunomonitoring.

Despite the study limitations, our data show the potential for enhancing T cell-mediated immune effects by systemic GM-CSF administration. These results provide the rationale for immunotherapy trials utilizing GM-CSF in combination with other immunologic agents. In particular, systemic GM-CSF may help to convert a "non-inflamed" tumor microenvironment to an "inflamed" tumor microenvironment comprised of infiltrating T cell, leading to more effective tumor control. Future studies should further investigate the role of GM-CSF, including varying effects of different dosing schedules. Clinical development of combination strategies involving GM-CSF should include a control arm lacking GM-CSF, when possible. Careful design of immunomonitoring protocols, in both the peripheral and tumor compartments, may help to identify features of the immune response that serve as useful biomarkers to predict tumor control and long-term clinical outcomes.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA Cancer J Clin* 2015;65:5–29.
2. Ohori M, Goad JR, Wheeler TM, Eastham JA, Thompson TC, Scardino PT. Can radical prostatectomy alter the progression of poorly differentiated prostate cancer? *J Urol* 1994;152:1843–9.
3. Catalona WJ, Smith DS. 5-year tumor recurrence rates after anatomical radical retropubic prostatectomy for prostate cancer. *J Uro* 1994;152:1837–42.
4. Zincke H, Oesterling JE, Blute ML, Bergstralh EJ, Myers RP, Barrett DM. Long-term (15 years) results after radical prostatectomy for clinically localized (stage T2c or lower) prostate cancer. *J Urol* 1994;152:1850–7.
5. Pietzk EJ, Eastham JA. Neoadjuvant treatment of high-risk, clinically localized prostate cancer prior to radical prostatectomy. *Curr Urol Rep* 2016;17:37.
6. Schellhammer PF, Chodak G, Whitmore JB, Sims R, Frohlich MW, Kantoff PW. Lower baseline prostate-specific antigen is associated with a greater overall survival benefit from sipuleucel-T in the Immunotherapy for Prostate Adenocarcinoma Treatment (IMPACT) trial. *Urology* 2013;81:1297–302.
7. Markowicz S, Engleman EG. Granulocyte-macrophage colony-stimulating factor promotes differentiation and survival of human peripheral blood dendritic cells in vitro. *J Clin Invest* 1990;85:955–61.
8. Waller EK. The role of sargramostim (rhGM-CSF) as immunotherapy. *Oncologist* 2007;12:22–6.
9. Van den Eertwegh AJ, Versluis J, van den Berg HP, Santegoets SJ, van Moorselaar RJ, van der Sluis TM, et al. Combined immunotherapy with granulocyte-macrophage colony-stimulating factor-transduced allogeneic prostate cancer cells and ipilimumab in patients with metastatic castration-

Disclosure of Potential Conflicts of Interest

T.W. Friedlander has received honoraria from speakers bureau of Sanofi Aventis. E.J. Small is a consultant/advisory board member for Dendreon, Inc. No potential conflicts of interest were disclosed by the other authors.

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- resistant prostate cancer: a phase 1 dose-escalation trial. *Lancet Oncol* 2012;13:509–17.
10. Hodi FS, Lee S, McDermott DF, Rao UN, Butterfield LH, Tarhini AA, et al. Ipilimumab plus sargramostim vs ipilimumab alone for treatment of metastatic melanoma: a randomized clinical trial. *JAMA* 2014;312:1744–53.
 11. Hodi FS, Butler M, Oble DA, Seiden MV, Haluska FG, Kruse A, et al. Immunologic and clinical effects of antibody blockade of cytotoxic T lymphocyte-associated antigen 4 in previously vaccinated cancer patients. *Proc Natl Acad Sci U. S. A.* 2008;105:3005–10.
 12. Kwek SS, Lewis J, Zhang L, Weinberg V, Greaney SK, Harzstark AL, et al. Preexisting levels of CD4 T cells expressing PD-1 are related to overall survival in prostate cancer patients treated with ipilimumab. *Cancer Immunol Res* 2015;3:1008–16.
 13. Parmiani G, Castelli C, Pilla C, Santinami M, Colombo MP, Rivoltini L. Opposite immune functions of GM-CSF administered as vaccine adjuvant in cancer patients. *Ann Oncol* 2007;18:226–32.
 14. Faires MB, Hsueh EC, Ye X, Hoban M, Morton DL. Effect of granulocyte/macrophage colony-stimulating factor on vaccination with an allogeneic whole-cell melanoma vaccine. *Clin Cancer Res* 2009;15:7029–35.
 15. Slingluff CL, Petroni GR, Olson WC, Smolkin ME, Ross MI, Haas NB, et al. Effect of granulocyte/macrophage colony-stimulating factor on circulating CD8⁺ and CD4⁺ T-cell responses to a multipeptide melanoma vaccine: outcomes of a multicenter randomized trial. *Clin Cancer Res* 2009;15:7036–44.
 16. Small EJ, Reese DM, Um B, Whisenant S, Dixon SC, Figg WD. Therapy of advanced prostate cancer with granulocyte macrophage colony-stimulating factor. *Clin Cancer Res* 1999;5:1738–44.

17. Rini BI, Weinberg V, Bok R, Small EJ. Prostate-specific antigen kinetics as a measure of the biologic effect of granulocyte-macrophage colony-stimulating factor in patients with serologic progression of prostate cancer. *J Clin Oncol* 2003;21:99–105.
18. Rini BI, Fong L, Weinberg V, Kavanaugh B, Small EJ. Clinical and immunological characteristics of patients with serologic progression of prostate cancer achieving long-term disease control with granulocyte-macrophage colony-stimulating factor. *J Urol* 2006;175:2087–91.
19. Fong L, Carroll P, Weinberg V, Chan S, Lewis J, Corman J, et al. Activated lymphocyte recruitment into the tumor microenvironment following preoperative sipuleucel-T for localized prostate cancer. *J Natl Cancer Inst* 2014;106:dju268.
20. Serafini P, Carbley R, Noonan K, Tan G, Bronte V, Borrello I. High-dose granulocyte-macrophage colony-stimulating factor-producing vaccines impair the immune response through the recruitment of myeloid suppressor cells. *Cancer Res* 2004;64:6337–43.
21. Andtbacka RH, Kaufman HL, Collichio F, Amatruda T, Senzer N, Chesney J, et al. Talimogene laherparepvec improves durable response rate in patients with advanced melanoma. *J Clin Oncol* 2015;33:2780–8.
22. Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med* 2010;363:411–22.
23. Wei XX, Fong L, Small EJ. Prostate Cancer Immunotherapy with Sipuleucel-T: current standards and future directions. *Expert Rev Vaccines* 2015;14:1529–41.
24. Golden EB, Chhabra A, Chachoua Adams S, Donach M, et al. Local radiotherapy and granulocyte-macrophage colony-stimulating factor to generate abscopal responses in patients with metastatic solid tumours: a proof-of-principle trial. *Lancet Oncol* 2015;16:795–803.
25. Pavlenko M, Roos A-K, Lundqvist A, Palmborg A, Miller AM, Ozenci V, et al. A phase I trial of DNA vaccination with a plasmid expressing prostate-specific antigen in patients with hormone-refractory prostate cancer. *Br J Cancer* 2004;91:688–94.
26. Miller AM, Ozenci A, Kiessling R, Pisa P. Immune monitoring in a phase I trial of a PSA DNA vaccine in patients with hormone-refractory prostate cancer. *J Immunother* 2005;28:389–95.
27. Kantoff PW, Schuetz T, Blumenstein BA, Glode LM, Bilhartz DL, Wyand M, et al. Overall survival analysis of a phase II randomized controlled trial of a Poxviral-based PSA-targeted immunotherapy in metastatic castration-resistant prostate cancer. *J Clin Oncol* 2010;28:1099–105.
28. Vasu C, Dogan RN, Holterman MJ, Prabhakar BS. Selective induction of dendritic cells using granulocyte macrophage-colony stimulating factor, but not fms-like tyrosine kinase receptor 3-ligand, activates thyroglobulin-specific CD4⁺/CD25⁺ T cells and suppresses experimental autoimmune thyroiditis. *J Immunol* 2003;170:5511–22.
29. Santegoets SJ, Stam AG, Loughheed SM, Gall H, Scholten PE, Reijm M, et al. T cell profiling reveals high CD4⁺CTLA-4⁺ T cell frequency as dominant predictor for survival after prostate GVAX/ipilimumab treatment. *Cancer Immunol Immunother* 2013;62:245–56.
30. Santegoets SJ, Stam AG, Loughheed SM, Gall H, Jooss K, Sacks N, et al. Myeloid derived suppressor and dendritic cell subsets are related to clinical outcome in prostate cancer patients treated with CVAX and ipilimumab. *J Immunother Cancer* 2014;2:31.