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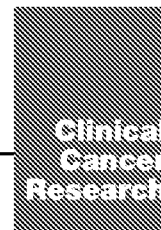
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## Phase I Trial of TGF- $\beta$ 2 Antisense GM-CSF Gene-Modified Autologous Tumor Cell (TAG) Vaccine

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### Abstract

**Purpose:** On the basis of the hypothesis that the combined expression of immunostimulatory granulocyte macrophage colony stimulating factor (GM-CSF) and antitumor suppressor TGF- $\beta$ 2 antisense (AS) transgenes can break tolerance and stimulate immune responses to cancer-associated antigens, we constructed an expression plasmid [the tumor-associated glycoprotein (TAG) plasmid] that coexpresses GM-CSF and TGF- $\beta$ 2 AS nucleotide sequences and which was incorporated into an autologous whole-cell vaccine.

**Experimental Design:** Patients undergoing resection were enrolled. Freshly harvested autologous tumor cells were mechanically and enzymatically disaggregated, then electroporated with the TAG vector. The resulting vaccine was irradiated, then aliquoted and cryopreserved until the time of injection. Patients received a minimum of 5 to a maximum of 12 monthly intradermal injections. Immune function was monitored at baseline and at months 3 and 6.

**Results:** Vaccine manufacturing efficiency was 84% (32/38). Twenty-three patients received at least 1 vaccination. There were no grade 3 or 4 toxicities, and grade 1 and 2 events were local in nature. Seventeen of 21 patients had stable disease (SD) at month 2 or later as their best response, and 1 patient with stage IVa malignant melanoma achieved a complete response (CR) following 11 vaccinations and remains without evidence of disease 2 years following initiation of therapy. Six of 13 patients displayed a positive enzyme-linked immunospot (ELISPOT) response to autologous TAG vaccine at week 12 including 3 patients with prolonged SD or CR. The 3 other patients survived through week 24, as compared with none of the 7 ELISPOT-negative patients.

**Conclusions:** On the basis of safety and clinical and immunologic results, further evaluation of bifunctional vaccines is warranted. *Clin Cancer Res*; 17(1): 183-92. ©2011 AACR.

### Introduction

Despite decades of immune-based therapies in solid tumor patients (1-7), only a minority have effectively translated into the clinic (8). Among the hypotheses accounting for this are the ineffective priming of tumor-specific T cells, a lack of high avidity of primed, tumor-specific T cells, and physical or functional suppression of primed, tumor-specific T-cell activity by host- and/or tumor-related mechanisms. Tumor-infiltrating lymphocytes (TIL) include immunosuppressive regulatory T cells

(Treg; CD4<sup>+</sup> CD25<sup>+</sup> Fox3p<sup>+</sup>; ref. 9) which, rather than cross-priming CD8<sup>+</sup> cytotoxic T cells, inhibit T-effector antitumor activities (9-11) in part through TGF- $\beta$ -dependent suppression of antigen-presenting dendritic cell (DC) processes (12). Further, both tumor-infiltrating, tolerogenic DCs and suppressor T lymphocytes express the TGF- $\beta$  receptor 1 (TGF- $\beta$ R1) and are therefore susceptible to immunosuppressive modulations by TGF- $\beta$ 1 and TGF- $\beta$ 2 produced by tumor cells (13-18). Circulating Th1-suppressive cytokines, including TGF- $\beta$  and IL-10, that are frequently elevated in patients with advanced cancer mediate immune suppression in tumor-bearing animal models by downregulating antigen recognition, Th1 activation, and antitumor immune effector functions (19-21).

We have previously demonstrated safety as well as response and survival benefits in 2 studies of gene-based vaccines in non-small cell lung cancer (NSCLC) patients (22-26). In the GVAX trial using GM-CSF-secreting allogeneic tumor cells, we documented complete responses (CR) in 3 of 33 patients. In a second randomized study, we observed a dose-related survival advantage in patients

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### Translational Relevance

Advances in the understanding of immune mechanisms have brought vaccine technology through the threshold of clinical application. The first immune approach approved by the Food and Drug Administration (FDA) has recently demonstrated significant survival advantage in advanced prostate cancer for patients receiving Provenge. Principles involving antigen education and immune activation were utilized. Tumor-associated glycoprotein (TAG) is the first clinical vaccine product reported which advances "clinically relevant" immune stimulatory support by providing antigen education (autologous tumor tissue) and immune activation (GM-CSF gene-induced stimulation) in combination with inhibition of cancer-induced immune suppression (TGF- $\beta$ 2 gene inhibition). Induction of prolonged disease stability and complete response in coordination with immune activation was demonstrated without toxic effect in patients with advanced cancer, thereby supporting a triple mechanism approach for immune management of cancer. Future personalized management of cancer will involve combination-targeted approaches that optimize immune control of cancer.

receiving an antisense (AS) TGF- $\beta$ 2 knockdown allogeneic vaccine (Belagenpumatucel-L). Enhanced tumor antigen recognition correlated with clinical benefit.

By inhibiting TGF- $\beta$ 2 expression, Belagenpumatucel-L reduces the cytokine-associated immune suppression that is well documented in cancer patients (13, 14, 27–30). TGF- $\beta$ 2 also antagonizes natural killer (NK) cells, lymphokine-activated killer (LAK) cells, and DC function (20, 21, 31–34). GVAX was shown to enhance tumor antigen expression and DC migration to the vaccination site (35–37). However, GM-CSF-induced maturation can be blocked by TGF- $\beta$  (38). On the basis of the hypothesis that the combined expression of GM-CSF and TGF- $\beta$ 2 AS transgenes can optimally stimulate immune responses to cancer-associated antigens, we have constructed an expression plasmid [the TAG (tumor-associated glycoprotein) plasmid] that coexpresses GM-CSF and TGF- $\beta$ 2 AS nucleotide sequences.

### Materials and Methods

The construction and cGMP manufacturing of TAG has been described (39). TAG vector utilizes the pUMVC3 vector backbone that contains an origin of replication, kanamycin resistance gene, a CMV (cytomegalovirus) promoter, and intron A driving the hGM-CSF cDNA and a 930-base pair AS fragment of the hTGF- $\beta$ 2 cDNA.

Freshly harvested autologous tumor cells were mechanically and enzymatically disaggregated, then electroporated with the TAG vector. The resulting vaccine was irradiated,

then aliquoted and cryopreserved until the time of injection (40).

### Study design

The primary objective of this trial was to evaluate the safety of TAG vaccine in advanced solid tumor patients without alternative standard therapy options. Following progression on preceding therapy, cancer patients were entered into 1 of 2 cohorts depending on tumor harvest and vaccine manufacturing cell yield. Cohort 1 patients received  $1 \times 10^7$  cells per injection and cohort 2 patients received  $2.5 \times 10^7$  cells per injection. A maximum of 12 intradermal injections were administered monthly alternating between the right and left upper arms. A safety assessment was made after 3 patients were entered into each cohort.

Study participation requirements included a minimum of 5 manufactured vaccine doses. Patients not meeting this requirement were deemed ineligible. Treatment of eligible patients was continued until progressive disease (PD) or up to a maximum of 12 injections.

### Study population

All eligible patients were treated in the outpatient facilities of Mary Crowley Cancer Research Centers (MCCRC). Inclusion criteria were as follows: a histologically confirmed, advanced or metastatic noncurable solid tumor following completion of 1 or more diseases appropriate standard of care therapies and recovery from all treatment-related toxicities to grade 1 or lower (except alopecia); availability of tumor in sufficient quantity (i.e.,  $\geq 2$  g) for vaccine processing; history of brain metastases allowed if treatment completed month 2 or later prior to enrollment with MRI confirmation of no active disease; presence of 1 or more measurable or evaluable lesion; patients of age 18 years and above; ECOG performance status of 0 to 1; a signed, IRB-approved, protocol-specific written informed consent document; a negative pregnancy test for women of child-bearing potential; and normal organ and marrow function defined as follows: absolute granulocyte count ( $\geq 1,500/\text{mm}^3$ ), platelets ( $\geq 100,000/\text{mm}^3$ ), total bilirubin ( $\leq 2$  mg/dL), AST (SGOT)/ALT (SGPT;  $\leq 2 \times$  institutional upper limit of normal), and creatinine ( $< 1.5$  mg/dL).

Exclusion criteria included the following: surgery involving general anesthesia, chemotherapy, radiotherapy, steroid therapy greater than 2-mg prednisone equivalents per day, or immunotherapy within 4 weeks of study entry; use of other investigational agents within 30 days prior to study entry; mucinous adenocarcinoma; prior splenectomy; prior malignancy (excluding nonmelanoma skin cancer) unless in remission for 2 years or more; Kaposi's sarcoma; uncontrolled intercurrent illness, for example, ongoing or active infection, symptomatic congestive heart failure, unstable angina pectoris, cardiac arrhythmia, or psychiatric illness/social situations that would limit compliance with study requirements; or confirmation that patient was pregnant or nursing, HIV positive, known to have chronic hepatitis B or C infection, or a history of autoimmune diseases.

### Administration of TAG vaccine

Eligible patients received monthly intradermal injections of TAG vaccine (either  $1 \times 10^7$  or  $2.5 \times 10^7$  cells per injection for  $\geq 5$  doses). Sites of injection were rotated between the right and left upper arms. If the ipsilateral axillary lymph nodes were radiated during prior therapy, alternative sites (e.g., anterior thigh) were used. Patients were observed for at least 30 minutes following vaccination with vital signs monitored every 10 minutes. If patients were deemed clinically stable, vaccine administration was continued for up to 12 monthly doses as long as sufficient material was available.

### Assessments

The following evaluations were performed within 2 weeks prior to therapy: a complete medical history, physical examination, ECOG assessment, and chest X-ray, chest/abdominal/pelvic computed tomography (CT) or magnetic resonance imaging (MRI), brain MRI or CT, and radionuclide bone scan, if indicated. A complete blood count (CBC) with differential and platelet count was also performed, as well as serum chemistries [creatinine, glucose, total protein, blood urea nitrogen (BUN), total carbon dioxide ( $\text{CO}_2$ ), albumin, total and direct bilirubin, alkaline phosphatase, and AST and/or ALT] and electrolytes (total calcium, chloride, potassium, sodium). Urinalysis, pregnancy test for females of child-bearing potential, EKG (electrocardiogram), and immune function analysis, including enzyme-linked immunospot (ELISPOT) analysis of cytotoxic T-cell function to autologous tumor antigens, TGF- $\beta 2$  levels and GM-CSF levels were also obtained.

Evaluations performed every  $28 \pm 3$  days during therapy included the following: physical examination; ECOG performance status assessment; CBC with differential and platelet count; serum chemistry and electrolytes; toxicity assessment; and clinical assessment of tumor response. Radiological assessments of tumors were done quarterly. Immune function, specifically serum cytokine analysis (IFN $\gamma$ , IL-4, IL-6), DC-mediated response (ELISPOT assay of CD8 $^+$  cells to autologous tumor antigens) and TGF- $\beta 2$  and GM-CSF levels, was monitored at baseline and at months 3 and 6.

### Tumor response

Tumor response in patients with measurable disease was reported using RECIST (response evaluation criteria in solid tumors) 1.0 criteria. Any objective response to treatment required confirmatory scans at least 4 weeks apart.

Complete response was defined as disappearance of all target lesions; partial response (PR), a 30% decrease in the sum of the longest diameters (SLD) of target lesions, taking as reference the baseline SLD; and PD, a 20% increase in the SLD of target lesions, taking as reference the nadir SLD recorded since the treatment started; or the appearance of  $\geq 1$  new lesion. SD met neither PD nor PR criteria.

### Immune assessment

ELISPOT assay was performed using ELISPOT Assay for Interferon Gamma (BD Biosciences). Ninety-six-well plates from the kit were coated with primary anti-IFN $\gamma$  monoclonal antibody and incubated overnight at 4°C. Cryopreserved pretreatment and posttreatment peripheral blood mononuclear cells (PBMC) collected at weeks 12 and 24 after vaccination were thawed and revitalized in culture medium overnight at 37°C stimulation, then dispensed at  $1 \times 10^4$  cells per well into microwells that were previously treated with human AB serum (2 hours). Coincubation was carried out with the mitogen phorbol myristate acetate and the Ca $^{2+}$  ionophore ionomycin (PMA-I; 5 and 500 ng/mL, respectively) or target cells (TAG autologous vaccine;  $3 \times 10^4$  cells per well) for 48 hours at 37°C to attain target to effector cell ratios of 3:1. The wells were washed and incubated with a biotinylated detection antibody (BD Biosciences ELISPOT Set), enzyme reagent, and the chromogenic substrate according to manufacturer's protocol. Positive reactions were analyzed using ELISPOT reader system (Carl Zeiss) with KS ELISPOT Software 4.9, service provided by ZellNet Consulting, Inc.

### Results

TAG vaccine manufacturing was successful in 32 of 38 patients. Five vaccines were rejected due to contaminants ( $n = 3$ ; all from resections involving colon wall) or inadequate harvest of tumor cells ( $n = 2$ ); 1 vaccine was intentionally manufactured for research purposes. Twenty-three patients received at least a single vaccine since June 2, 2008, in the outpatient facilities of MCCRC. Table 1 provides demographics of treated patients as well as tissue site, dose level, number of vials manufactured, cell viability, GM-CSF expression, and percent TGF- $\beta 2$  and TGF- $\beta 1$  knockdown of transfected product.

### Safety

There was no obvious difference in the rate of serious adverse events across the 2 dose cohorts. No grade 3/4 treatment-related events were observed. Grade 1/2-related events and serious adverse events are shown in Tables 2 and 3, respectively.

### Response

To date, 23 patients have received vaccine (Table 4). Seventeen of 21 patients had SD at month 2 or later as their best response. Two withdrew early for personal reasons with SD after 1 cycle and were not considered evaluable; 2 had PD at month 2 of assessment; and 1 with PD at month 3 withdrew consent.

One 78-year-old male patient with metastatic melanoma (013) achieved a CR of all target (abdominal, lymph node) and nontarget (bone metastases) lesions (Fig. 1). He had previously failed standard therapy. The CR was ascertained after completion of 11 low-dose vaccine injections and confirmed 3 months later by repeat PET-CT (Fig. 1). Subsequent PET-CT scans continue to show disease-free status.

Table 1. Demographics of TAG-treated patients (n = 23)

Patient ID	Cancer	Age, y	Sex	Prior XRT	Prior surgery	Number of prior investigational and/or chemotherapy regimens (single or multiple agent)	Tissue origin	Dose (low/high)	Number of vaccine vials manufactured	Cell viability, %	GM-CSF expression, pg/1 × 10 <sup>6</sup> cells	TGF-β2 knock-down, %	TGF-β1 knock-down, %
007	Ovarian	52	F	No	Yes	5	Omentum and peritoneum	High	7	87	30	32	6
008	Neuro-endocrine	28	M	No	Yes	0	Pancreas	High	13	92	1,600	41	0
009	Neuro-endocrine	33	F	Yes	Yes	2	Adrenal gland	Low	8	79	2,800	19	0
010	Breast	46	F	No	Yes	10	Metastasis in liver	High	11	99	230	9	0
012	Melanoma	51	M	No	Yes	1	Lung tissue and lymph node	Low	10	95	7,300	47	23
013	Melanoma	77	M	No	Yes	0	Metastasis in peritoneum	Low	11	92	6,500	41	33
014	Lung	71	F	Yes	Yes	3	Lung tissue and lymph node	High	11	87	300	25	8
017	Lung	79	F	Yes	Yes	2	Lung tissue	High	8	89	571	41	0
018	Colorectal	61	M	No	Yes	6	Metastasis in liver	High	26	89	384	17	22
020	Breast	48	F	Yes	Yes	7	Metastasis to lymph nodes	High	21	90	459	61	14
021	Colon	69	F	No	Yes	4	Tissue from abdominal wall	High	24	94	117	44	6
023	Neuro-endocrine	39	F	No	Yes	1	Tumor tissue from liver	High	15	92	91	88	7
024	Colon	57	F	Yes	Yes	3	Pelvic lymph node resection	High	12	97	7	33	0
026	Colon	75	F	No	Yes	2	Lymph node deep chest wall	Low	9	96	13	84	0
029	Neuro-endocrine	30	F	Yes	Yes	2	Tumor tissue from liver	High	12	97	876	80	33
031	Breast	64	F	Yes	Yes	6	Mets from lung	High	7	94	1,243	98	0
032	Gastric	59	M	Yes	Yes	2	Mets from omentum	Low	10	97	403	89	0
033	Leiomyo-sarcoma	58	F	No	Yes	4	Peritoneal mets	Low	8	97	134	98	65
034	Melanoma	56	M	No	Yes	2	Lymph node left thigh	High	10	98	532	98	15
035	Bladder	80	F	No	Yes	2	Lung tumors	High	10	96	116.5	99	0
037	Bladder	56	F	No	Yes	5	Vaginal tumor	Low	11	88	85	100	0
041	Hemangiopericytoma	65	M	Yes	Yes	0	Brain	High	12	90	2,563	96	16
043	Cervical	59	F	No	Yes	5	Uterine/cervical	High	7	84	1,288	100	0

Abbreviation: XRT, X-ray therapy.

**Table 2.** Grade 1, 2 adverse events, definitely, probably, and or possibly related

Adverse event	Grade	Relationship to TAG	Number of patients
Edema	1	Definitely related	1
Fatigue	1	Possibly related	1
	2	Possibly related	1
General pain (NOS)	1	Definitely related	1
Injection site reaction	1	Definitely related	4
	1	Probably related	1
	2	Probably related	1
Rash	1	Possibly related	1
	2	Possibly related	1
Left arm soreness	1	Probably related	1
Left arm weakness	1	Probably related	1

Two vaccine recipients (041 and 043) are currently receiving treatment on trial. Seven of 20 evaluable patients have survived for more than 1 year following initiation of treatment. Four of these patients (008, 013, 023, and 037) have successfully received all manufactured vaccines.

#### Immune responses

IFN $\gamma$  ELISPOT assessments were retrospectively performed to compare quantified CD8<sup>+</sup> T-cell activity in weeks 12 and 24 postvaccination blood to baseline samples from 16 patients (008, 009, 010, 012, 013, 014, 017, 023, 024, 026, 029, 031, 032, 033, 034, and 035) in a blinded fashion. *In vitro* IFN $\gamma$  production was determined following phorbol myristate acetate (PMA)/ionomycin-induced polyclonal T-cell differentiation (41, 42) or cocubation with the patient's irradiated, autologous TAG vaccine (Fig. 2A and B, respectively).

Most patients (11/16) responded to PMA plus ionomycin stimulation ( $\geq 50\%$  increase at week 12), including the 2 patients with prolonged SD (008 and 023) and 1 patient (013) with CR (Fig. 2A). Therefore, a majority of the advanced cancer patients retained immunoresponsiveness by these *in vitro* criteria.

Six of 13 patients displayed a positive ELISPOT response to autologous TAG vaccine ( $>10$ -fold increase over baseline) at week 12 postvaccination (Fig. 2B). These included the 3 patients with prolonged SD or CR, who also demonstrated further elevated ELISPOT activities at week 24 (4-, 5-, and 14-fold increase over the week 12 values). Of the 3 other ELISPOT-responsive patients, 2 (032 and 033) survived through week 24, as compared with none of the 7 ELISPOT-negative patients.

#### Discussion

Safety and tolerability of the TAG vaccine have been demonstrated in the 23 reported patients. Despite the poor prognosis of advanced cancer patients enrolled into trial, an unexpectedly high proportion of patients survived with SD of 3 months or longer and survival of more than 1 year was observed in 35% of the evaluable patients. Moreover, the CR observed in a patient with extensive metastatic melanoma confirms the clinical activity of the TAG vaccination.

Rejection of antigen-expressing tumor cells is effected primarily by specific host CTL (cytotoxic T lymphocyte;

**Table 3.** Serious adverse events

Patient ID	Serious adverse event	Start date	Stop date	Grade	Relation to therapy
007	Small bowel obstruction	June 5, 2008	June 8, 2008	3	Not related
	Dehydration	June 28, 2008	June 30, 2008	3	Not related
010	Seizure	November 13, 2008	November 13, 2008	3	Not related
	Brain metastasis	November 13, 2008	Ongoing	3	Not related
	GI bleed	November 18, 2008	November 29, 2008	3	Not related
017	Pneumonia resulting in death	November 26, 2008	November 28, 2008	5	Not related
031	Pericardial effusion	March 26, 2009	March 29, 2009	4	Not related
032	Weight loss	June 17, 2009	June 30, 2009	2	Not related
	Vomiting	June 17, 2009	June 30, 2009	3	Not related
	Aspiration pneumonia	June 21, 2009	June 30, 2009	3	Not related

Table 4. Response of treated patients (n = 23)

Patient ID	Time to construct vaccine, d <sup>a</sup>	No. of vaccines received	Best response	Survival since consent of tissue procurement, d <sup>b</sup>	Survival since treatment start, d <sup>b</sup>	Reason off Study treatment	Survival status
007	79	1	NE	240	51	Voluntary withdrawal from study	Expired
008	47	12	SD	930	764	Completed all vaccines	Alive
009	71	5	SD	882	765	Disease progression	Alive
010	79	3	SD	344	136	Disease progression	Expired
012	42	5	SD	805	751	Disease progression	Alive
013	50	11	CR	763	681	Completed all vaccines	Alive
014	33	3	SD	465	320	Disease progression	Expired
017	32	3	SD	141	87	Disease progression	Expired
018	37	2	PD	265	169	Disease progression	Expired
020	28	1	NE	693	247	Voluntary withdrawal from study	Alive
021	40	2	PD	533	169	Disease progression	Expired
023	30	12	SD	694	583	Completed all vaccines	Alive
024	66	4	SD	268	159	Disease progression	Expired
026	59	3	SD	528	431	Disease progression	Expired
029	43	4	SD	210	135	Disease progression	Expired
031	30	3	SD	183	120	Disease progression	Expired
032	47	6	SD	246	190	Disease progression	Expired
033	42	6	SD	594	499	Disease progression	Alive
034	29	5	SD	212	162	Disease progression	Expired
035	29	3	PD	133	91	Voluntary withdrawal from study	Expired
037	44	11	SD	356	282	Completed all vaccines	Alive
041	25	4	SD	233	135	On study	Alive
043	28	3	SD	202	65	On study	Alive

<sup>a</sup>Time lapse = time from date of harvest to passing all product release criteria for clinical acceptability per FDA release criteria.

<sup>b</sup>Data current as of 7/7/10.

Abbreviation: NE, not evaluable.

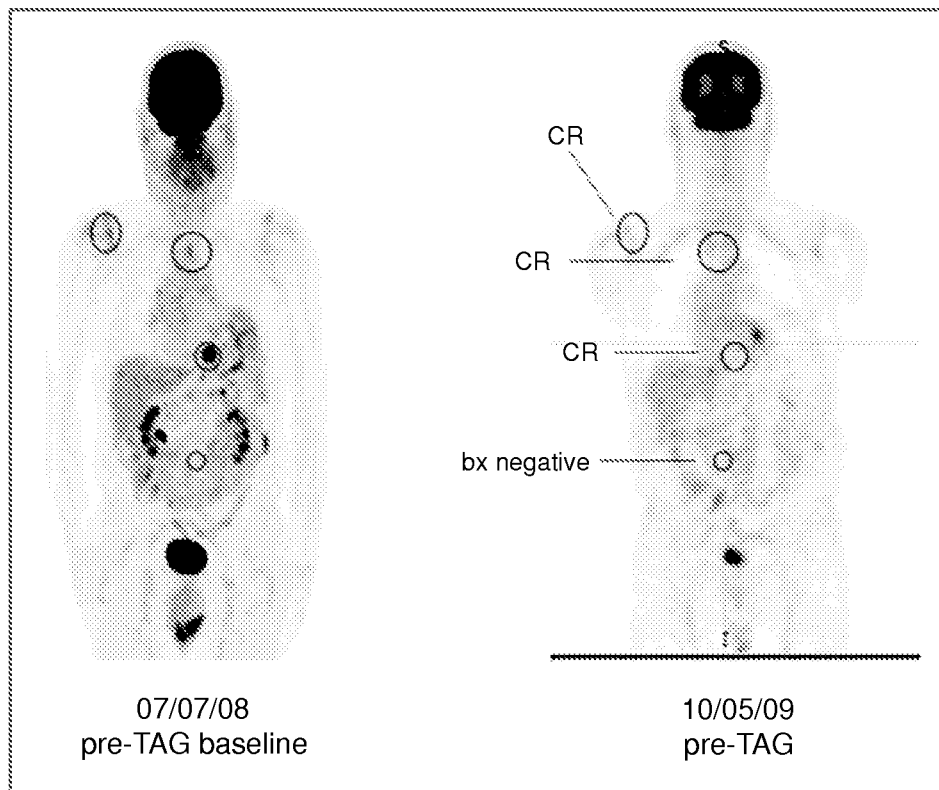
refs. 43, 44). TILs have been shown to mediate durable regression of established tumors in mice with advanced tumor burdens (45, 46). In patients with metastatic tumors, various investigators have documented the existence of antitumor CTL effectors in PBMC and TIL that are able to lyse autologous tumor cells, but not NK targets, allogeneic tumor cells, or autologous fibroblasts (47–51). These findings support the premise that tumor-associated antigens expressed by metastatic human tumors can stimulate a specific T-cell response by immune effectors that can be expanded *ex vivo* to achieve clinical objective responses. This is particularly evident within the subset of patients with prolonged SD or CR postvaccination, who displayed progressively elevated recall responses at weeks 12 and 24 as compared with prevaccination levels (Fig. 2B).

Overexpression of 2 or more of the TGF- $\beta$  isoforms has been demonstrated in melanoma, gliomas, prostate, gastric, colorectal, ovarian, and gastric cancers (15–17, 52).

TGF- $\beta$ 1 and TGF- $\beta$ 2 bind to TGF- $\beta$  receptor 2 (TGF- $\beta$ R2) which phosphorylates TGF- $\beta$ R1. This heterotetrameric receptor complex is then able to suppress DC and helper T-cell function through regulation of the Smad complex and non-Smad MAP (mitogen-activated protein) kinases (12, 18). Polak et al. recently showed that tumor-infiltrating, tolerogenic DCs and suppressor T-cell lymphocytes in malignant melanoma correlate with immunosuppressive TGF- $\beta$ 1, TGF- $\beta$ 2, and IL-10 expression (15). This mechanism of tumor-associated immunosuppression is likely to contribute to tumor escape.

Genetic modification with a TGF- $\beta$ 2 AS-encoding plasmid represents 1 of many approaches currently being evaluated to inhibit local TGF- $\beta$  activity. Others include the use of neutralizing antibodies, soluble receptors, receptor kinase antagonist drugs, AS reagents, and a number of less-specific drugs such as angiotensin II antagonist and tranilast (53). Systemic TGF- $\beta$  blockade could potentially interfere with healing, exacerbate the inflammatory disease

Figure 1. PET CT scans comparing baseline scan and second follow-up scan involving patient 013. Results demonstrate CR of all PET and CT identifiable metastatic melanoma lesions.



response, or neutralize the TGF- $\beta$  tumor suppressive effect in normal cells (54). Our results suggest the effectiveness of incorporating a *de novo* gene-modifying moiety into the vaccinating cancer cells *ex vivo* to produce localized TGF- $\beta$  blockade so as to enhance activation of the afferent arm of the immune response while bypassing the pharmacokinetic and toxicity concerns potentially associated with systemic anti-TGF- $\beta$  neutralization. We observed an objective response in only 1 of 23 patients entered into trial, despite encouraging SD achievement. Although these results would suggest an induced state of immunoequilibrium, another factor affecting this balance is the immunosuppressive activity of other TGF- $\beta$  isoforms (TGF- $\beta$ 1 and TGF- $\beta$ 3) that may override TGF- $\beta$ 2 knockdown. Thus, an alternate gene-modifying moiety that can globally attenuate TGF- $\beta$ 1, TGF- $\beta$ 2, and TGF- $\beta$ 3 may further potentiate the knockdown effects of all TGF- $\beta$ -related immunosuppressive activities of the malignant cell. We are currently investigating this strategy for future vaccine development.

We observed consistent GM-CSF expression by manufactured autologous TAG vaccines per  $1 \times 10^6$  cells at 24 hours, albeit at lower levels than observed in our adenoviral transgene GVAX studies. Although in 1 study, there was a suggestive correlation between survival of patients who received GVAX with GM-CSF expression (22), this relationship was not subsequently confirmed (35). Variations in GM-CSF expression are likely attributable to differences in constructs (plasmid-based vs.

viral-based expression). Nonetheless, local GM-CSF expression levels by the TAG vaccine are deemed clinically relevant as 1) use of a plasmid rather than a viral vector obviates the neutralizing effects of elicited antiviral antibodies, 2) use of a plasmid-based vaccine can minimize the development of humoral responses that interfere with long-term gene expression, and 3) concurrent suppression of TGF- $\beta$ 2 can abrogate tumor-induced inhibition of GM-CSF-dependent DC maturation (38). Conversely, we observed no objective tumor responses with the bystander GVAX vaccine that produced a 25-fold higher level of GM-CSF than the autologous vaccine, indicating that high levels of vaccine-based GM-CSF protein expression is not a necessary criterion for achieving clinical activity (55).

Mechanistically, tumor cells are able to promote the proliferation of immunosuppressive Treg cells directly via TGF- $\beta$  production or through the conversion of DCs into regulatory cells that secrete TGF- $\beta$  (56). As noted, GM-CSF is a key immunostimulatory agent in both antigen  $\rightarrow$  DC interaction and DC  $\leftrightarrow$  T-cell activation (57, 58). It is also potentially the most active of the immunostimulatory cytokines tested (35, 57, 58). However, GM-CSF-induced DC maturation is effectively blocked by TGF- $\beta$  as measured by decreased expression of ICAM-1, B7-2, and MHC class II expression (38). Furthermore, mixed lymphocyte reaction-stimulating activity of cells was blocked when TGF- $\beta$  was added during the first 4 to 6 days to cells



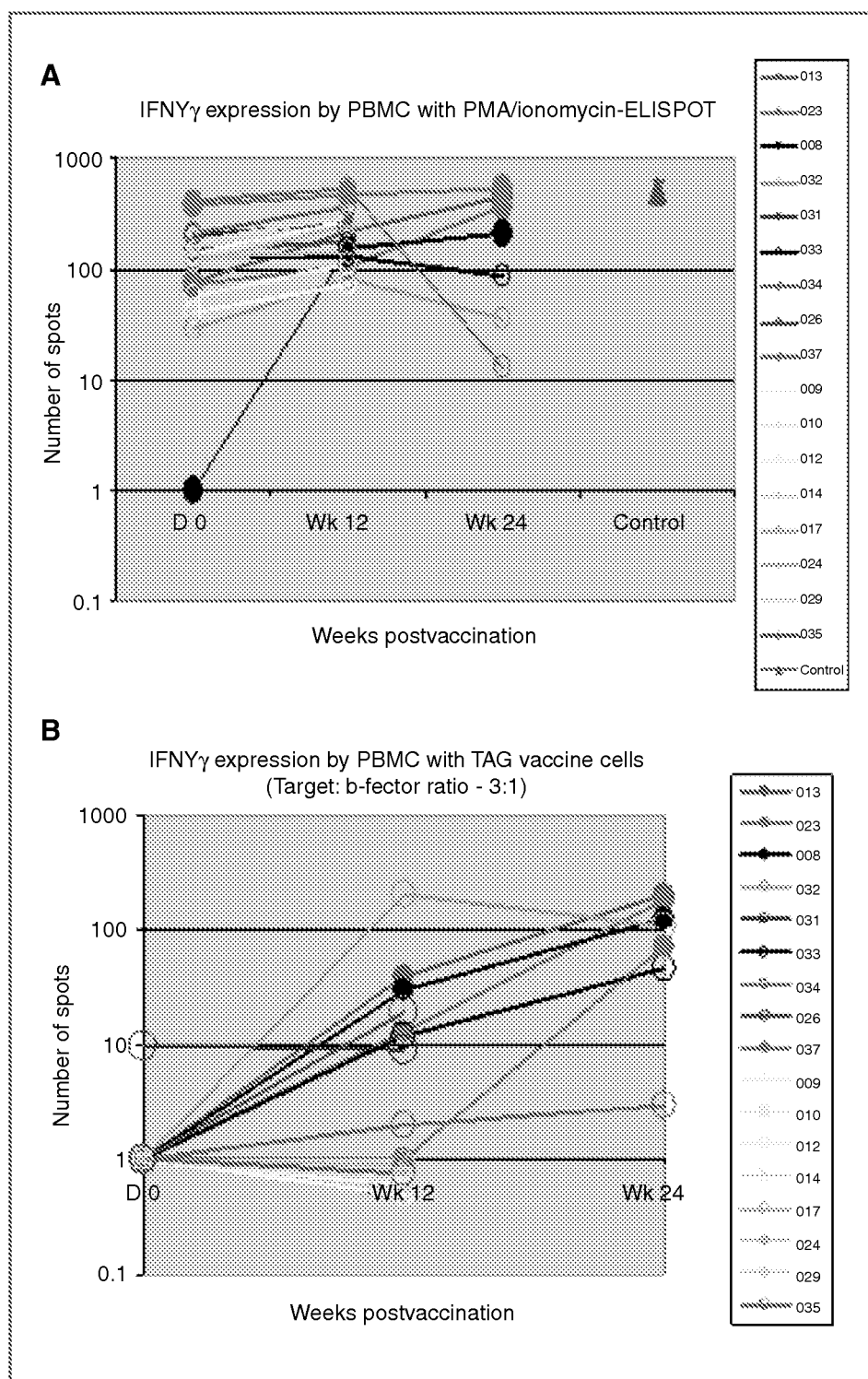


Figure 2. IFN $\gamma$  expression by ELISPOT assay demonstrating the activity of patients PBMC to PMA/ionomycin (A) or autologous TAG vaccine cells (at 3:1 ratio; B) incubation. Patient peripheral blood was collected before vaccination and at weeks 12 and 24 postvaccination. IFN $\gamma$  production was determined after 48 hours of incubation and quantified by spot enumeration.

cultured in GM-CSF, where a dose-response effect was evident (38).

Others have also demonstrated that gene-based immunotherapy has the potential for local and systemic management of disease (59). Our initial phase I/II trial results with

TGF- $\beta$ 2 AS gene vaccine suggested a dose-related survival response correlating with enhancement of tumor antigen recognition in advanced stage NSCLC patients. Although the dose of  $\geq 2.5 \times 10^7$  cells per injection, compared with  $1 \times 10^7$  cells per injection, given on a monthly basis in the

Belagenpumatucel-L NSCLC study, was associated with a survival advantage, an earlier study in patients with recurrent glioma using the same vector, documented responses at doses at and below  $1 \times 10^7$  cells per injection (60). There was no correlation of survival and cell dose in GVAX trials at MCCRC at or above  $1 \times 10^7$  cells per injection (25, 26). Thus, 2 dose levels,  $1 \times 10^7$  cells per injection and  $2.5 \times 10^7$  cells per injection, were defined as acceptable for purposes of this protocol, especially given the presumptive GM-CSF function-modulating effect of TGF- $\beta$ 2 silencing. Our results, thus, support the hypothesis that a combined plasmid with GM-CSF gene and TGF- $\beta$ 2 AS is safe and, furthermore, provide preliminary evidence of durable clinical benefit justifying further clinical investigation. Insofar as TGF- $\beta$ 1 is the dominant immune inhibiting cytokine in most solid tumors, TGF- $\beta$  blockade limited to the TGF- $\beta$ 2 isoform with TAG vaccine may limit immunomodulatory potency (15, 61). Further elucidation of post-vaccination-activated immune subsets (DCs, T effectors, Tregs) will serve to address the mechanistic role of GM-CSF plus TGF- $\beta$ -AS modifications in promoting a clinically relevant antitumor immune response. Methods to block both

TGF- $\beta$ 1 and TGF- $\beta$ 2 are under current clinical investigation by our team.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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