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TAG Vaccine:
Autologous Tumor Vaccine Genetically Modified To Express GM-CSF and Block Production of TGFß2

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We have designed a novel autologous vaccine by combining two vaccine strategies that have each been previously tested in separate non-small cell lung cancer (NSCLC) clinical trials: 1) a GM-CSF gene transduced tumor cell vaccine[1]; and 2) a TGFβ2 antisense gene transduced cell vaccine.[2,3] Each has demonstrated similar beneficial effects without any evidence of significant toxicity in advanced cancer patients.

The GM-CSF transgene directly stimulates increased expression of tumor antigen(s) and enhances dendritic cell migration to the vaccination site. TGFβ2 blockade following intracellular TGFβ2 antisense gene expression reduces production of immune inhibiting activity at the vaccine site. These agents have never been used in combination but the rationale of integrating enhancement of an anticancer immune response concurrently with a reduction in cancer-induced immune suppression is conceptually sound. We harvest autologous cancer cells from patients with advanced refractory cancer. We have constructed a TGFβ2 antisense/GM-CSF (TAG) expression plasmid and have successfully demonstrated preclinical activity of the vector function following transfection by electroporation and irradiation of autologous cancer tissue from six patients. Phase I clinical trial is underway to treat advanced solid tumor cancer patients.

The autologous vaccine manufacturing process (Table 1) requires freshly procured tumor tissue (within 48 hours of surgery) and is completed within two days of initiation of the manufacturing process. The process entails the dissection and dissociation of the tumor into a single-cell suspension. Cells are washed, enumerated and then transfected with the TAG expression plasmid. Cells are incubated overnight to allow expression of the GM-CSF protein and the TGFβ2 antisense.

On the following day, the cells are harvested, enumerated and then irradiated. Following irradiation, the cells are washed, formulated in freeze media and then aliquoted into final containers for freezing and storage.

TABLE 1. The process for creating autologous vaccine.

Patient Identification and Consent
↓
Tumor Procurement and Transport
↓
Tumor Receipt
↓
Tumor Evaluation / Dissection
↓
Tumor Dissociation
↓
Cell Wash / Cell Count
↓
Cell Transfection
↓
Overnight Incubation
↓
Harvest / Cell Count
↓
Irradiation
↓
Cell Washes
↓
Final Fill
↓
Product Freezing
↓
QC Testing
↓
QA Release
↓
Shipment for Patient Treatment

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Description of the Manufacturing Process

Preparation of Single-Cell Suspension

Tissue processing is aseptically performed in an ISO Class 7 (Class 10,000) clean room with Level 2 gowning (*i.e.*, per MF-SOP-100, cleanroom access and gowning), under a certified biological safety cabinet (BSC) (Class 100, ISO 5). The tumor tissue is examined for nontumor tissue (*e.g.*, trim away fat, necrosis, and other nontumor tissue) and nonbiological elements (*e.g.*, staples, suture lines, etc.).

The tissue is weighed and then cut into fine pieces (about 1 mm cubes). This is performed as quickly as possible. The tumor is then mechanically and enzymatically dissociated into a single-cell suspension using GMP-grade collagenase and DNase I (Pulmozyme; Genentech, South San Francisco, CA). The tumor tissue dissociation is performed in a bag (Sartorius Stedim, Concord, CA), 1 or 3 L, depending on the volume of the mass and media used. The use of a strong closed bag allows for more secure manipulation of the dissociation and closed transfers to and from the BSC and incubator.

After dissociation is completed, the cells are washed, resuspended and enumerated (live and total cells) with trypan blue viability stain on a hemocytometer.

Plasmid Design and Construction

The TAG plasmid was used to transfect the autologous cells. This vector has been previously used in BB-IND 13401 (Gradalis, Inc.) for a xenograft-expanded autologous tumor cell vaccine. (*See companion article in this issue, pp 30-36.*)

Tumor Cell Transfection

Transfection is accomplished by electroporation of the tumor cells using an electroporator (Bio-Rad Laboratories, Hercules, CA). A mixture of 50 µg of plasmid (50 µl) is combined with 2 x 10^7 cells (500 µl) in a sterile 0.4 cm gap cuvette. An exponential decay pulse waveform is applied using the following conditions: electrical current of 300 V, capacitance of 1000 uF and resistance set to infinity (determined by testing various voltages on similar cells for optimum viability of tumor cells and transfection of vector). Time constants are recorded for each electroporated aliquot of tumor cells. Cuvettes are visually inspected following electroporation for telltale signs that the process has been successful. Following electroporation, tumor cells are incubated overnight at 37°C. The cells are incubated to allow transcription of the TGFβ2 antisense and the GM-CSF transgene.

Irradiation of Transfected Tumor Cells

On the following morning, the cells are harvested, washed, enumerated by hemocytometer and then they are irradiated at 10,000 cGy in a standard blood bank gamma irradiator. Cell proliferation is arrested (post-10,000 cGy irradiation) and prevents the formation of new tumors when the vaccine is injected into the patient.

Preparation of Final Product

Following irradiation, cells are washed and resuspended in 1% human serum albumin ([HSA] Buminate; Baxter, Deerfield, IL) in Plasma-Lyte A, pH 7.4 (Baxter) at a cell concentration twice the final frozen concentration. Final cell concentration is at one of two dose levels: cohort 1 dose 1.0 x 10^7 or cohort 2 dose 2.5 x 10^7 cells/injection. The goal is to make a minimum of five doses of vaccine, and the optimal result is to generate 12 doses of vaccine at the higher dose level. The final vial for each dose of vaccine is a sterile 2.0 ml externally-threaded screw cap cryovial (Nalgene, Rochester, NY).

The freeze media consists of 20% dimethyl sulphoxide ([DMSO] Cryoserv; Edwards Lifesciences, Irvine, CA), 1% HSA (Buminate; Baxter) in Plasma-Lyte A, pH 7.4 (Baxter). The cold cell suspension and freeze media are mixed in equal proportions and placed in the cold freezing container (cryo 1°C freezing bath). The cells are gradually frozen to approximately −80°C. After freezing, the cells are stored in the vapor phase of liquid nitrogen until all release testing is completed, all necessary approvals are obtained, and the patient is ready for treatment.

Qualification of the Manufacturing Process

Five full-scale preclinical manufacturing processes and five clinical manufacturing processes have been performed. Table 2 depicts the types of tumors processed (tumors 6–10 are the clinical vaccines).

TABLE 2. Types of tumors processed.

Tumor Processed	Tumor ID	Diagnosis
1	ATCV-001	Melanoma
2	ATCV-002	Hepatocellular Carcinoma
3	ATCV-003	Non-Small Cell Lung Cancer Ascites Fluid
4	ATCV-004	Sarcoma
5	ATCV-005	Breast Cancer
6	ATCV-006	Non-Small Cell Lung Cancer
7	ATCV-007	Ovarian Cancer
8	ATCV-008	Neuroendocrine
9	ATCV-009	Adrenocortical
10	ATCV-010	Breast Cancer

The tumors processed range in size (and volume, in the case of the ascites), as well as type, and the resulting viable cell yield also varies greatly (Table 3). In two instances, there was contamination noted during manufacturing. In the first instance (tumor 1), contamination was consistent with an infection at the site of resection. The second contamination occurred during manufacturing due to Petri dish lid jarring as the dishes were transported back and forth between the BSC and incubator during tissue dissociation. As a result of that contamination event, the tissue dissociation process was switched from Petri dishes to a single closed Sartorius Stedim bag, eliminating the potential for contamination at that step. For the pre-clinical runs, vialing was not done based on clinical dose level (cohort 1 or 2).

The prefreeze viability of the transfected tumor cells (day 2 of manufacturing) ranged from 80–98% (data not shown). The clinical vaccines

(ATCV-006, 007, 008 and 010) were vialed at 2.5 x 10^7 cells (dose cohort 2). A minimum of five doses at the cohort 1 dose level is needed to consider the manufacturing process successful. Patients with multiple tumor harvests will be allowed to combine vials to qualify for minimum clinical dose requirement. A maximum of 12 doses at cohort 2 dose level will be made for patient treatment. Because tumor cell yield is highly variable due to tumor mass, cellularity, and processing compatibility, the minimum dose number and lower dose cohort (cohort 1) have been included. There are data to suggest that even a few doses at a lower cell number may have some clinical benefit.[4]

Cultures of pre- and post-transfection for autologous tumor cell vaccine were set up to test for the expression of GM-CSF and TGFβ2. In order to evaluate transfection, GM-CSF and TGFβ2 expression was determined by commercially-available ELISA kits (R&D Systems, Minneapolis, MN). The pretransfection sample (4 x 10^6 cells) is taken from day one of the manufacturing. This sample is stored on cold packs until the day one manufacturing is completed. Then the sample is removed from the manufacturing facility so that the cell cultures can be set up for generating the sample for ELISA.

On day two, the post-transfection, post-irradiation, pre-freeze sample (6 x 10^6 cells) is used for expression analysis. This sample is also stored on cold packs until the day two manufacturing is completed. The sample is then removed from the manufacturing facility so that the cell cultures can be set up for generating the sample for ELISA.

Five vaccines (ATCV-006, -007, -008, -009, and -010) have been manufactured as part of the pre-clinical qualification process. These vaccines have been evaluated for GM-CSF and TGFβ2 antisense expression using post-transfection, pre-irradiation samples. During FDA review, it was determined that all vaccines need to be evaluated for GM-CSF and TGFβ2 expression using post-transfection, post-irradiation samples. Because these patients are alive and can be eligible for treatment under this IND, these patient vaccines are considered a distinct cohort by virtue of the unique expression qualification assay samples. As such, any of these five patients who receive vaccine will be tracked as a separate cohort.

GM-CSF expression is detected throughout the 14 day post-transfection assay. GM-CSF concentration is plotted as ng/ml. A summary of GM-CSF expression for all manufacturing processes (Figure 1) indicates that the median level of expression is about 1 ng/ml. In all manufactured prod-

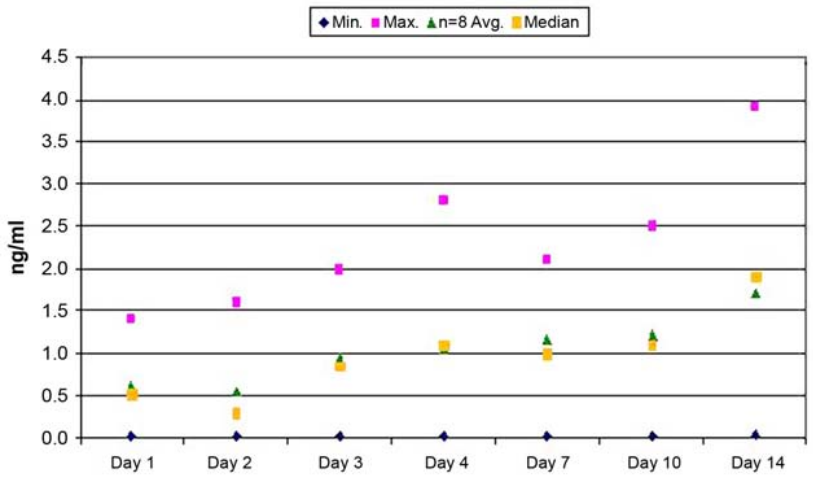
TABLE 3. Tumor mass versus cell yield.

Tumor ID	Weight/Volume	Number of Viable Tumor Cells	Number of Vials[c]
ATCV-001	5.60 g	Aborted on day 2 due to contamination[a]	0
ATCV-002	8.00 g	5.70 x 10^7	5
ATCV-003	1030 ml	6.60 x 10^7	6
ATCV-004	36.00 g	Aborted on day 2 due to contamination[b]	0
ATCV-005	4.95 g	6.00 x 10^7	6
ATCV-006	45.00 g	1.75 x 10^8	7
ATCV-007	14.30 g	1.75 x 10^8	7
ATCV-008	23.70 g	3.25 x 10^8	13
ATCV-009	21.20 g	8.00 x 10^7	8
ATCV-010	18.70 g	2.80 x 10^8	11

(a) Contamination due to skin infection (yeast) at site of resection.

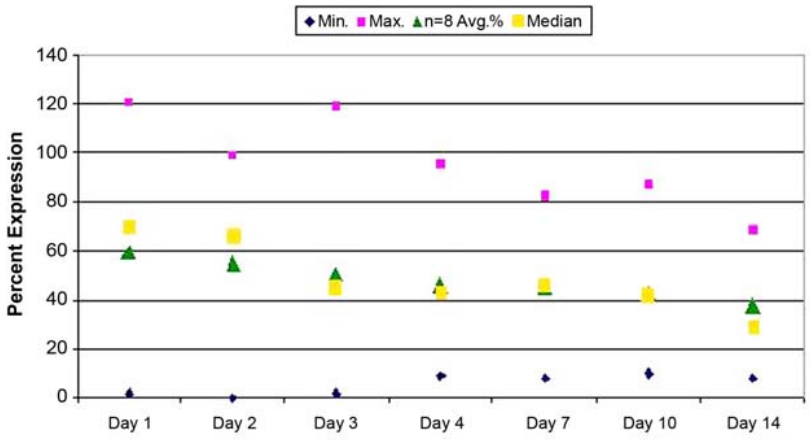
(b) Contamination due to Petri dish lid jarring between BSC and incubator. Switched to Sartorius Stedim bags for dissociation to eliminate issue.

(c) One dosage is 2.5^7 viable cells per vial (high dose) and 1^7 per vial (low dose).



	Min.	Max.	n = 8 Avg.	Median
Day 1	0.01	1.40	0.60	0.50
Day 2	0.02	1.60	0.55	0.30
Day 3	0.02	2.00	0.33	0.85
Day 4	0.02	2.80	1.04	1.10
Day 7	0.02	2.10	1.16	1.00
Day 10	0.01	2.50	1.20	1.10
Day 14	0.03	3.90	1.72	1.90

FIGURE 1. GM-CSF expression in TAG-transfected autologous tumor cell vaccines.



	Min.	Max.	n = 8 Avg.	Median
Day 1	2.0	120.5	59.4	69.5
Day 2	0.0	98.0	54.9	66.5
Day 3	2.0	118.8	50.4	45.2
Day 4	9.0	94.7	46.3	42.3
Day 7	8.0	82.0	45.0	46.0
Day 10	10.0	86.8	42.5	41.6
Day 14	8.0	68.7	38.2	29.6

FIGURE 2. TGFß2 suppression in TAG plasmid transfected, irradiated cells in culture compared to non-transfected cells.

ucts, GM-CSF expression is observed although the level of expression is highly variable between manufacturing processes (and tumor types). In addition to documented variability in levels of GM-CSF expression between manufacturing processes, the levels of expression achieved with the TAG vaccine are deemed clinically relevant as: 1) use of a plasmid rather than a viral vector obviates the obfuscating effects of elicited anti-viral neutralizing antibodies; 2) use of a plasmid likewise prevents the development of elicited antibodies interfering with long-term gene expression; and 3) concurrent suppression of TGFβ2 will minimize tumor-associated inhibition of GM-CSF-induced dendritic cell maturation.[5]

To determine the suppression of endogenous TGFβ2 expression by the TGFβ2 antisense, a TGFβ2 ELISA was performed. There is significant variability in the level of endogenous TGFβ2 expression between the different tumor types, but all tumors have detectable TGFβ2 expression and all transfected products demonstrate knockdown of TGFβ2. The median percent TGFβ2 in transfected products is about 60% of the non-transfected and the suppression of TGFβ2 expression is maintained over the 14 day assay (Figure 2).

Table 4 lists the p-values for the difference in pre- and post-transfection levels for GM-CSF and TGFβ2, respectively, for each sample. Six patient samples of various cancer types had pre-transfection levels and post-transfection levels of GM-CSF and TGFβ2 measured. After transfection, both viable and total cell proliferation levels were measured pre-irradiation and post-irradiation at days 0, 1, 2, 3, 4, 7, 10, and 14. A paired t-test was used to compare differences in pre- and post-transfection levels per patient, and average pre- and post-irradiation levels for viable cell and

TABLE 4. Differences in expression levels pre- and post-transfection for GM-CSF and TGFß2.

Sample ID	Cancer Type	P-Value for Differences in GM-CSF Pre-/Post-Transfection	P-Value for Differences in TGFß2 Pre-/Post-Transfection
002	Hepatocellular	0.2190	0.094
003	NSCLC	0.0030	0.002
005	Breast	< 0.0001	0.001
006	Lung	< 0.0001	0.011
007	Ovarian	0.5520	0.451
008	Neuroendocrine	0.0020	0.004

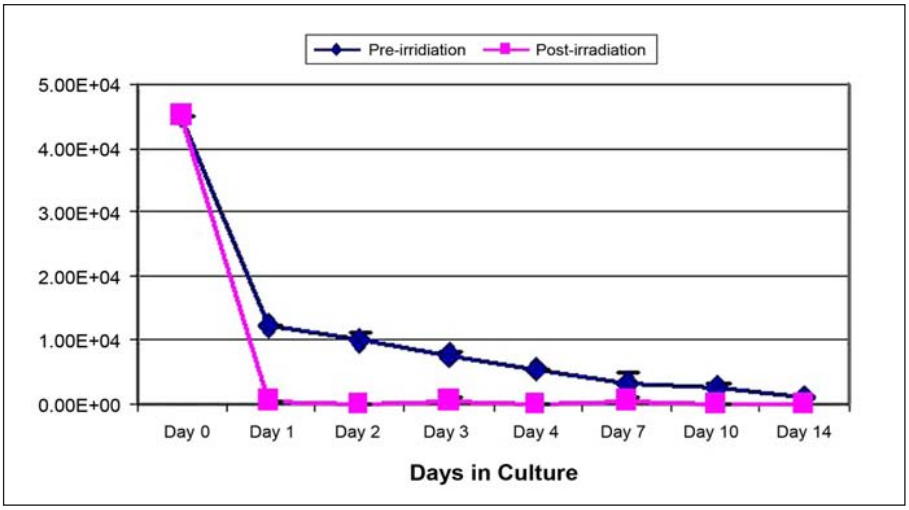


FIGURE 3. ATCV-003 pre- and post-irradiation cell proliferation.

total cell counts were determined.

Inhibition of tumor cell proliferation was accomplished by gamma irradiation at 10,000 cGy. One of two hospital blood bank irradiators was used to deliver the gamma irradiation dose. In order to assess the inhibition of proliferation, a cell culture-based proliferation assay was used. For the assay, an aliquot of 1 x 10^6 cells was removed on day 2 before irradiation, and another aliquot of 1 x 10^6 cells was removed post-irradiation. Both cell aliquots were kept on cold packs until the manufacturing process was finished. After that, the aliquots were used to set up the post-irradiation cell proliferation assay. Figure 3 depicts the results of one proliferation assay. The data clearly demonstrates that primary tumor cells, whether pre- or post-irradiation, rapidly diminish in viable cell number during the 14 day assay.

Average Viable Cell Proliferation

Linear regression analysis was performed on the pre- and post-irradiation viable cell time course (Figure 4 depicts post-irradiation time course). The correlation coefficients of these two linear regressions are strong, 0.9277 and 0.9506, which indicate that total and viable cell numbers decline in a predictable, linear manner *in vitro*. The slope of the post-irradiation linear regression is steeper than the pre-irradiation regression, indicating the effect of irradiation on tumor cell viability.

Stability of the Final Product

The pre-clinical manufacturing runs (ATCV-002 and -003) have been stored in the vapor phase of liquid nitrogen for at least three months. An aliquot of each has been thawed and assessed for cell number, viability and GM-CSF and TGFβ2 by ELISA and reverse transcription polymerase chain reaction (RT-PCR). In addition, one aliquot of vaccine from our xenograft-expanded autologous vaccine program (IND #13401) has been used for this study and represents a seven-month stability time point (transfected by the same expression plasmid and frozen with the same methodology as the current pre-clinical vaccine samples).

Total and viable cell numbers post-thaw are shown in Table 5. Total cell recovery ranged from 65–73%. Viable cell recovery ranged from 38–64%.

Thawed samples were placed in culture for 24 and 48 hour incubations to generate media samples for GM-CSF and TGFβ2 ELISA assays. Cell numbers continued to decrease during these incubation periods (data not shown). The protein from media samples were at the lower limit of detection for the GM-CSF ELISA and undetectable for

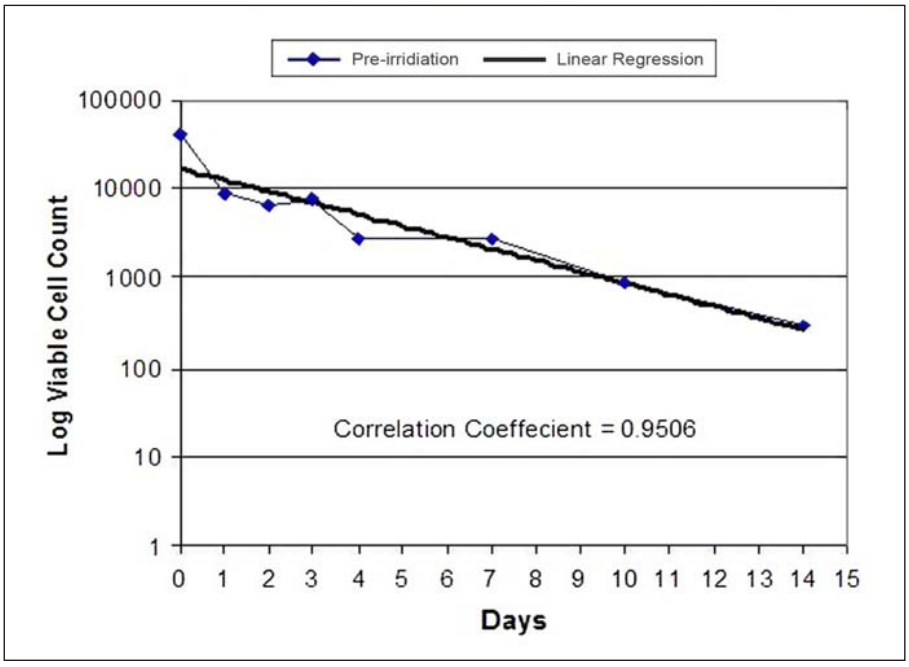


FIGURE 4. Post-irradiation viable cells with linear regression.

TABLE 5. Thawed cell recovery after frozen storage.

Vaccine ID	Months Post-Freeze	Live Cells/ Frozen Vial	Pre-Freeze % Viability	Total Cells Post-Thaw	Total Cell % Recovery	Live Cells Post-Thaw	Live Cell % Recovery
Xeno	7	2.50 x 10^7	98	1.87 x 10^7	73	9.50 x 10^6	38
ATCV-002	3	5.50 x 10^6	100	2.50 x 10^6	71	2.25 x 10^6	64
ATCV-003	3	1.30 x 10^7	87	9.70 x 10^6	65	7.90 x 10^6	61

the TGFβ2 ELISA (data not shown).

RT-PCR for GM-CSF mRNA and TGFβ2 antisense was performed on selected samples. GM-CSF mRNA was detected (Figure 5A) for the xeno-vaccine at: T0, ATCV-003 at T0, T24 and T48; and ATCV-001, T0 and ATCV-005, T0. Quantitation of the GM-CSF mRNA is shown in Figure 5B. It is interesting to note that for the ATCV-003 samples, the GM-CSF mRNA signal increases with incubation time.

RT-PCR for TGFβ2 antisense is depicted in Figure 6 (A and B). The results were analogous to the GM-CSF RT-PCR with positive detection of the antisense transcript in all samples. Also, the same signal increase was observed in ATCV-003 samples (with increasing incubation time) as was noted above for GM-CSF mRNA.

These initial stability results indicate that appreciable cell recovery is possible after three to seven months frozen storage and that GM-CSF and TGFβ2 antisense mRNA are readily detectable by RT-PCR.

In-Process Controls, Testing, and Specifications

During manufacturing, the visual inspection of the tumor tissue and cells (macro and microscopic) provides the first level of assurance that the manufacturing process is successful. Final product integrity is based on our Quality

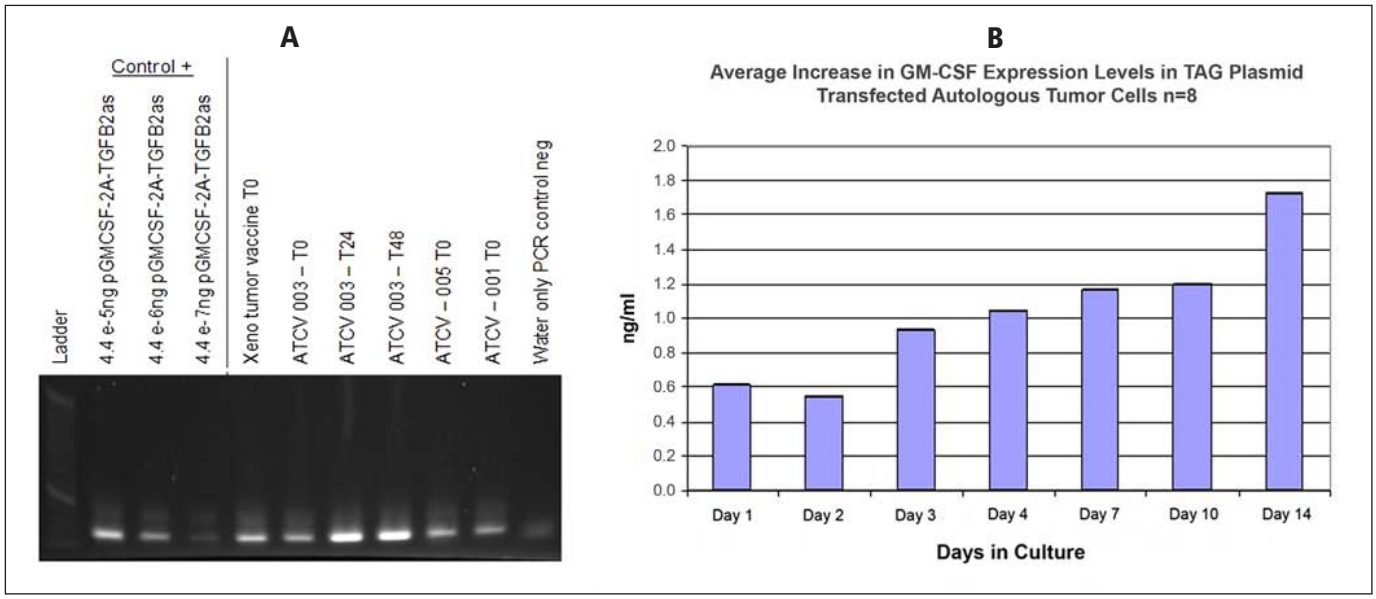


FIGURE 5. GM-CSF RT-PCR results from stability test samples.

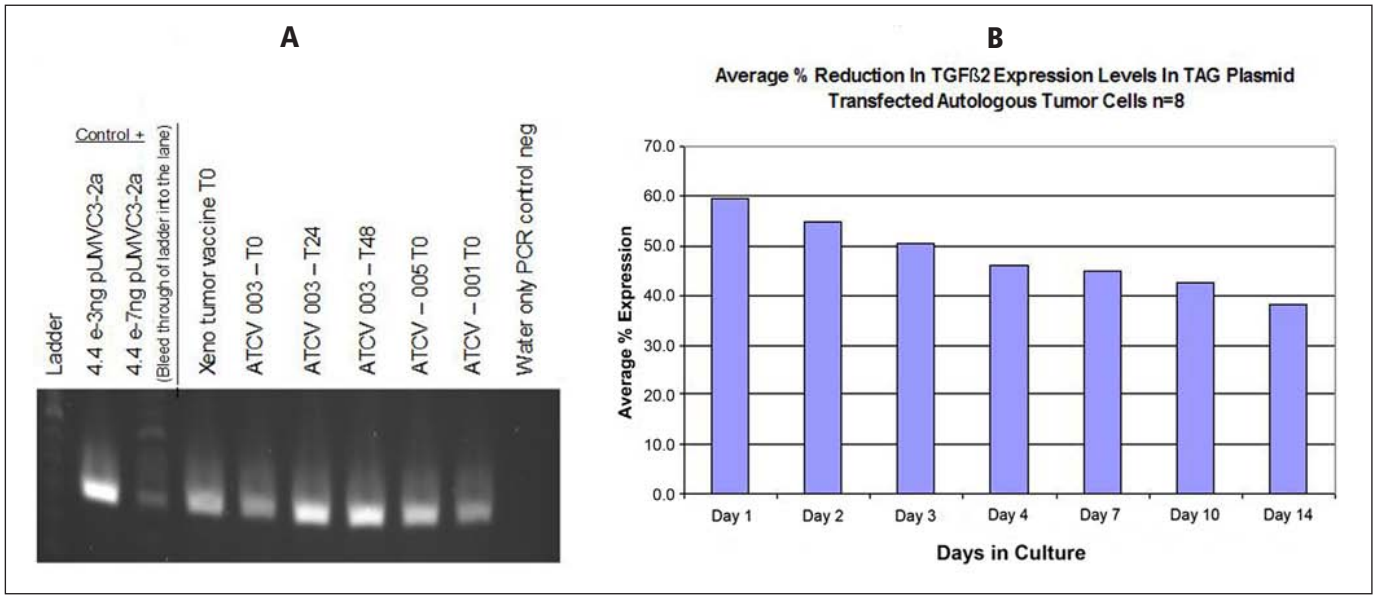


FIGURE 6. TGFß2-antisense PCR results from stability test samples.

System design and implementation (summarized in the next section).

At the final fill step of the manufacturing process, in-process environmental monitoring is performed. Tryptic soy agar (TSA) and Sabouraud dextrose agar (SDA) plates are placed in the BSC (where filling occurs) and at points around the manufacturing suite. In addition, an in-house 14-day sterility check of the post-irradiation cell centrifugation supernatant is performed using the BBL Septi-Check TSB Media (BD Biosciences, San Jose, CA). The SDA plates are incubated for 14 days at 25°C. The TSA plates and the Septi-Chek slide media and bottle are incubated at 37°C. The plates and Septi-Chek are monitored every work day for growth for the 14-day incubation period.

Vaccine Quality Control Tests

(See Table 6.)

Final Product Release Specifications and Characterization

(See Table 7.)

Potency

Cell number is used as the primary indicator of potency. Based on previous vaccine trials, cell dosage was a significant factor in determining response to vaccine treatment.

Identity and Specificity

Cell viability is used as the primary indicator of identity and specificity. The cells liberated from the tumor tissue are distinct in size and morphology and readily distinguishable throughout the manufacturing process. Because many tumor types are being processed for vaccines, no common marker is readily available at this time to denote identity or specificity more conclusively.

Endotoxin

The level of endotoxin present in the final product is determined by the Limulus amebocyte lysate (LAL) kinetic chromogenic method according to USP <85> Bacterial Endotoxins test.

Sterility

Sterility is confirmed by USP Sterility 14-day test as detailed in 21 CFR 610.12.

Mycoplasma

Mycoplasma detection is performed by Touchdown PCR (TD-PCR) GLP rapid assay (WuXi AppTec, Philadelphia, PA) for release. The limit of detection is ten copies. The US FDA "Points To Consider" *Mycoplasma* testing has been performed on all products manufactured to date, as a bridge to the *Mycoplasma* PCR, but will not be performed in the future.

For Information Only (FIO) Testing

Gradalis, Inc. will collect additional samples for ELISA and RT-PCR assays as well as additional assays (*e.g.,* proteogenomic analysis, cell morphology/marker studies). These test results will allow Gradalis, Inc. to better define potency, identity, and specificity aspects of the vaccines.

Quality Control Test Results To Date

(See Table 8.)

TABLE 6. Vaccine quality control tests.

Description of Test	Testing Facility	Test Results
Bacterial Endotoxins	WuXi AppTec	No inhibition or enhancement of test system shown
Environmental Testing	Gradalis, Inc.	No growth
Sterility Test	Gradalis, Inc.	No growth
Sterility Test Validation[a] (B/F)	WuXi AppTec	No bacteriostatic/fungistatic activity demonstrated
Detection of *Mycoplasma* DNA By PCR: GLP (Rapid)	WuXi AppTec	No *Mycoplasma* DNA sequences detected

(a) Performed only on first vaccine manufacturing run submitted for sterility testing (ATCV-002).

TABLE 7. Final product release specifications and characterization.

Release Test	Test Method	Specifications
Cell Number	Hemocytometer	Dose Cohort 1 or 2
Cell Viability	Trypan Blue Dye Exclusion	≥ 70% Viable
Endotoxin	GLP Kinetic Chromogenic LAL	≤ 5 EU/ml (Dose)
Sterility	21 CFR 610.12	No Growth
Mycoplasma	GLP Rapid PCR	No *Mycoplasma* DNA Detected

TABLE 8. Quality control test results to date.

Test Method	ATCV-002	ATCV-003	ATCV-005	ATCV-006	ATCV-007	ATCV-008
EM Monitoring	P	P	P	P	P	P
In-House Sterility	P	P	P	P	P	P
USP Sterility	P	P	P	P	P	P
Endotoxin	P	P	P	P	P	P
Mycoplasma PCR	P	P	P	P	P	P
Mycoplasma PTC	P	P	P	P	P	P

P = Passing Result

Clinical Use of Vaccine

Patients with viable cells in sufficient numbers of 1 x 10^7 cells/injection (low dose cohort) for five doses will receive monthly intradermal injections of the tumor cell vaccine as long as sufficient material is available. Patients who do not have a minimum of five doses manufactured will not undergo treatment. Patients may combine separate manufactured lots of vaccine from two or more harvests to achieve a qualifying number of doses.

Conclusion

Pre-clinical data has been presented in support of the autologous solid tumor TAG vaccine. These data have been submitted to FDA and the Phase I protocol has been approved and initiated (BB-IND 13650).

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