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Biologic Activity of Autologous, Granulocyte-Macrophage Colony Stimulating Factor Secreting Alveolar Soft Parts Sarcoma and Clear Cell Sarcoma Vaccines

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Abstract

Purpose—Alveolar soft parts sarcoma (ASPS) and clear cell sarcoma (CCS) are rare mesenchymal malignancies driven by chromosomal translocations that activate members of the microphthalmia transcription factor (MITF) family. However, in contrast to malignant melanoma, little is known about their immunogenicity. To learn more about the host response to ASPS and CCS, we conducted a phase I clinical trial of vaccination with irradiated, autologous sarcoma cells engineered by adenoviral mediated gene transfer to secrete granulocyte-macrophage colony stimulating factor (GM-CSF).

Experimental Design—Metastatic tumors from ASPS and CCS patients were resected, processed to single cell suspensions, transduced with a replication defective adenoviral vector encoding GM-CSF, and irradiated. Immunizations were administered subcutaneously and intradermally weekly times three and then every other week.

Results—Vaccines were successfully manufactured for 11 of the 12 enrolled patients. Eleven subjects received from 3 to 13 immunizations. Toxicities were restricted to grade 1–2 skin reactions at inoculation sites. Vaccination elicited local dendritic cell infiltrates and stimulated T cell mediated delayed type-hypersensitivity reactions to irradiated, autologous tumor cells. Antibody responses to tissue-type plasminogen activator (tTPA) and angiopoietins-1/2 were detected. Tumor biopsies showed programmed death-1 (PD-1) positive $CD8^+$ T cells in association with PD ligand-1 (PD-L1) expressing sarcoma cells. No tumor regressions were observed.

Conclusions—Vaccination with irradiated, GM-CSF secreting autologous sarcoma cell vaccines is feasible, safe, and biologically active. Concurrent targeting of angiogenic cytokines and antagonism of the PD-1 negative regulatory pathway might intensify immune-mediated tumor destruction.

Keywords

sarcoma; cancer vaccine; microphthalmia-associated transcription factor; antibodies; angiopoietin

Introduction

ASPS and CCS are rare cancers of mesenchymal origin that primarily affect young adults (1–6). These neoplasms frequently arise in the head and neck region or extremities and frequently metastasize hematogenously to the lungs, central nervous system, and other sites. The cell of origin remains under active study, with some evidence suggesting neural crest derived lineages as a source (7, 8). Surgical excision may be curative for localized lesions, but disseminated disease, which may be relatively slow growing, is largely refractory to cytotoxic therapies and eventually proves fatal in most cases. The rarity of the neoplasms together with their indolent and variable natural histories present significant challenges for

understanding disease pathogenesis and identifying treatment regimens that might induce durable clinical benefits.

Genetic investigations have provided important insights into the biology of ASPS and CCS. Both tumors are driven by chromosomal translocations that activate members of the MITF family, a group of basic helix-loop-helix leucine zipper transcription factors that bind to a canonical CANNTG motif (9). ASPS is characterized by the non-reciprocal translocation t(X;17)(p11;q25) that fuses the gene encoding transcription factor E3 (TFE3), a member of the MITF family, on Xp11.2 to the gene encoding native alveolar soft part sarcoma chromosome region candidate 1 (ASPL) on 17q25, which appears to modulate the glucose transporter GLUT4 (10). The chimeric ASPL-TFE3 protein retains the nuclear import signal and DNA binding domain of TFE3, but replaces the N-terminal sequences with ASPL, thereby perturbing the normal regulation of TFE3 transcriptional activity. CCS is characterized by the balanced translocation t(12;22) (q13;12) that fuses the Ewing's sarcoma gene EWS on 22q12 with the cyclic AMP (cAMP) regulated transcription factor ATF1 on 12q13 (11). The chimeric EWS-ATF1 protein retains the DNA binding and heterodimerization domains of ATF, but replaces the N-terminal regulatory sequences with EWS, thereby constitutively upregulating the transcription of cAMP responsive genes. Among these is the melanocyte master transcription factor MITF, which also plays a critical role in the pathogenesis of malignant melanoma (12).

Because TFE3 and MITF bind to the same DNA motif, their dysregulation triggers alterations in oncogenic pathways that are shared between ASPS and CCS. For example, both transcription factors upregulate the receptor tyrosine kinase product of the c-Met proto-oncogene, which upon engagement by the cognate ligand hepatocyte growth factor signals to promote tumor cell proliferation, survival, and invasion (13, 14). Accordingly, tivantinib, a small molecule inhibitor of c-Met, has demonstrated some anti-tumor activity in ASPS and CCS patients, although the frequency and duration of clinical responses are modest (15, 16). The hypoxia inducible factor (HIF)-1α is a second target for both TFE3 and MITF, and activation of this transcription factor triggers the expression of an array of proteins critical to angiogenesis including vascular endothelial growth factor-A (VEGF-A), angiopoietin-1/2, platelet derived growth factor, and their cognate receptors (7, 17–21). Consistent with these findings, anti-angiogenic treatment with sunitinib shows clear anti-tumor activity in both ASPS and CCS patients (22–24). In addition, cediranib, a small molecule inhibitor of all three VEGF receptors induced an impressive 35% response and 84% disease control rate at 24 weeks of therapy in a cohort of 46 ASPS patients (25, 26). Cederanib and sunitinib are now being tested in a randomized trial targeting advanced ASPS (ClinicalTrials.gov identifier NCT01391962).

The involvement of MITF family members in the pathogenesis of ASPS and CCS highlights a potential relationship with malignant melanoma. Indeed, genomic profiling revealed shared mRNA expression patterns between CCS and melanoma that included upregulation of melanocyte differentiation antigens, the transcription factor SOX10, and the growth factor receptors ERBB3 and FGFR1 (27). Correspondingly, transcriptional analysis of ASPS demonstrated increased expression of melanoma inhibitor of apoptosis protein (ML-IAP), a MITF target gene that promotes melanoma cell survival (17, 28). While these findings

underscore common aspects of ASPS, CCS, and melanoma biology, they also raise the possibility that elements of the host response to these neoplasms might also be similar. However, while malignant melanoma is perhaps the most intensively studied cancer from an immunologic perspective, little is known regarding the anti-tumor response to ASPS or CCS. A recent genomic analysis of ASPS uncovered high-level expression of the innate activating receptors TLR2 and TLR9, suggesting that host factors might be involved in disease pathogenesis (7).

We previously reported that vaccination with irradiated autologous tumor cells engineered to secrete GM-CSF enhances cellular and humoral anti-melanoma responses in some patients with advanced disease (29, 30). Metastatic lesions resected after, but not before therapy manifested dense intra-tumoral cellular infiltrates composed of $CD4^+$ and $CD8^+$ T lymphocytes and $CD20^+$ B cells that effectuated tumor destruction. Based upon the ability of this vaccination strategy to augment immunity in advanced melanoma patients and the shared biology of MITF related tumors, we undertook a phase I trial of autologous, GM-CSF secreting tumor cell vaccines in patients harboring advanced ASPS or CCS.

Materials and Methods

Clinical protocol

The clinical protocol received approval from the Dana-Farber/Harvard Cancer Center Institutional Review Board, the Food and Drug Administration, and the Recombinant DNA Advisory Committee. The study was registered at clinicaltrials.gov (NCT00258687) and conducted at the Dana-Farber Cancer Institute, Brigham and Women's Hospital, and Boston Children's Hospital according to institutional and federal guidelines. Written informed consent was obtained from all patients before study participation. For patients under the age of 18 years for whom it was developmentally appropriate, assent was also obtained. Patients of any age with histologically confirmed CCS or ASPS were eligible if they were considered to have unresectable and thereby incurable disease. The trial was also open to rare patients with translocation-associated renal cell carcinoma that involves activating mutations in TFE3 (9) and patients with melanoma less than 18 years of age, but no patients with these diseases were enrolled. Subjects were allowed to have any number of prior therapies, provided they were more than 4 weeks from the last treatment. Additional key inclusion criteria were: Eastern Cooperative Oncology Group (ECOG) performance status 0 to 1; estimated life expectancy of at least 6 months; and adequate hematological, hepatic, and renal function. Major exclusion criteria included pregnant or nursing mothers and infections with HIV, Hepatitis B or Hepatitis C. Patients with brain metastases were excluded unless these were stable at least 3 months off of treatment, and the patient had no neurological symptoms.

Vaccine preparation

Solid tumors were placed into sterile media and transported on ice to the Cell Manipulation Core Facility at the Dana-Farber Cancer Institute, where they were dissected into small fragments and processed to single cell suspension with collagenase and mechanical digestion. When sufficient cells were obtained (see below), 2×10^6 tumor cells were

irradiated (10,000 rads) and cryopreserved (90% fetal calf serum, 10% DMSO) in 1×10^6 cell aliquots for use in delayed-type hypersensitivity testing. The remaining tumor cells were placed in media (α-MEM, 10% fetal calf serum, gentamicin) and infected overnight at 37°C with a replication defective adenoviral vector encoding human GM-CSF (Ad-GM) at a multiplicity of infection of 10. Ad-GM contains a GM-CSF expression cassette in the E1 region of adenovirus type 5 and a second deletion in the E3 region. The GM-CSF expression cassette contains the CMV immediate early promoter/enhancer, a shortened human beta-globin second intron, the human GM-CSF gene, and the beta-globin polyadenylation signal and 3′ untranslated region (29). After overnight infection, the tumor cells were extensively washed and irradiated (10,000 rads). 1×10^6 cells were placed into culture for 48 hours, the supernatants collected, and GM-CSF levels determined with an ELISA (Endogen EH-GMCSF) according to the manufacturer's instructions. Individual vaccine aliquots were cryopreserved based upon overall tumor cell yield as follows: $\geq 6\times10^5$ to $< 6\times10^6$ total, 1×10^5 aliquots (dose level 1); $\geq 6\times10^6$ to $\leq 3\times10^7$ total, 1×10^6 aliquots (dose level 2); 3×10^7 to 1×10^8 total, 4×10^6 aliquots (dose level 3); $\geq 1\times10^8$ total, 1×10^7 aliquots (dose level 4). Samples of non-transfected and infected tumor cells were tested for sterility, endotoxin, and mycoplasma. Prior to clinical administration, cryopreserved cells were thawed, washed extensively, and resuspended in 1 ml of sterile saline for the vaccines and 0.5 ml for the non-transduced cells used for delayed-type hypersensitivity analysis.

Treatment and evaluation

Irradiated, autologous, engineered cellular vaccines were administered intradermally (0.5 ml) and subcutaneously (0.5 ml) into normal skin on the limbs and abdomen on a rotating basis. Injections were given weekly times three and then every other week until the vaccine supply was exhausted or the patient was removed from study. A minimum of six immunizations was required to consider a patient evaluable for biologic activity. Disease evaluation was conducted at baseline, week 10 and then at 4 month intervals or whenever clinically indicated. Blood was drawn for immune monitoring before, during, and after administration of the vaccine. Irradiated, dissociated, non-transduced tumor cells were injected intradermally (0.5 ml) into normal skin at the time of beginning vaccination and with the fifth vaccination to evaluate delayed-type hypersensitivity. Punch biopsies were obtained 2–3 days after injections. When possible, distant metastases were biopsied after vaccination to assess immune infiltrates.

Pathology

Tissues were fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin. Immunohistochemistry was performed using standard techniques with monoclonal antibodies to CD1a, CD11c, CD4, CD8, CD20 (all from Ventana), FoxP3 (Abcam), PD-1, and PD-L1 (31). Vaccination and delayed-type hypersensitivity responses were graded on a semi-quantitative scale (0–4+) based on the presence of: mononuclear cells admixed with eosinophils and basophils accumulated around blood vessels; endothelial cells that were swollen or necrotic, or showing vessel luminal occlusion; and dermal edema and fibrin exudation. The scoring was graded according to the density of the mononuclear cells. Null referred to no cells per high power field (HPF); trace to the presence of a rare cell per HPF;

1+ to 3–5 cells around a vessel or scattered between vessels; 2+ to approximately 10 cells per HPF; 3+ to >10 to 19 cells per HPF; 4+ to greater that 20 cells per HPF.

cDNA expression library screening

The construction of the K008 melanoma-derived cDNA expression library was reported (32). Post-vaccination sera from a long-term surviving patient was pre-cleared against *Escherichia coli* and lambda phage lysates and used at a 1:1,000 dilution in TBST (50 mM Tris/138 mM NaCl/2.7 mM KCl/0.05% Tween 20, pH 8.0). Positive plaques were detected with an alkaline phosphatase-conjugated polyclonal goat anti-human pan-IgG antibody (Jackson ImmunoResearch) and 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) (Promega). Reactive clones were plaque-purified and the inserts matched to the NCBI Entrez Nucleotide database.

ELISAs

Previously described procedures were used for the ELISAs with some modifications (32, 33). EIA/RIA plates (Corning Incorporated, Corning, NY) were coated with 100 μL of purified recombinant protein at a concentration of 5 μg/mL in coating buffer (0.05% sodium azide containing PBS) overnight at 4°C. Angiopoietin-1 and angiopoietin-2 were from R&D, tissue-type plasminogen activator was from Abnova, and recombinant ML-IAP and NY-ESO-1 were prepared in house. The plates were washed with PBST (0.05% Tween-20 containing PBS) and blocked for two hours at room temperature with 200 μL/well blocking solution (PBST, 2% nonfat milk, 0.05% sodium azide). After the plates were again washed, longitudinal sera samples were added at a final dilution of 1:500 in blocking solution (100 μL/well) and incubated at 4°C overnight. After several further washes, the plates were incubated with 100 μL/well of a 1:2000 diluted alkaline phosphatase–conjugated goat anti-human IgG antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for one hour at room temperature. Finally, the plates were washed again, incubated with 100 μL/well of the PNPP substrate (Sigma, St. Louis, MO) for 25 minutes at room temperature, and then the OD (405 nm) was determined (Spectramax 190 Microplate Reader; Molecular Devices, Sunnyvale, CA).

Statistics

The main considerations for this single stage design study were the feasibility of vaccine manufacture and the safety of administration of the agent. Given limited information about the patient population, which was comprised of patients with rare cancers, we assumed that the feasibility and safety of administering GVAX would not differ between CCS and ASPS patients. The trial was originally designed to include 20 patients, but was stopped early at 12 patients due to slow accrual of patients with these rare tumors. Feasibility goals were set at 90% for vaccine manufacture, 85% for vaccine initiation, and 75% for delivering at least 6 vaccines.

Results

Patients, vaccine production and administration

Twelve patients were enrolled onto this phase I vaccine study (Table 1). Three subjects had CCS and nine had ASPS. There were 4 females and 8 males with a mean age of 25.1 years (range 10–50). All patients had metastatic disease and failed at least one prior therapy. Involved sites included soft tissues, lung, central nervous system, bone, viscera, retroperitoneum, adrenal, breast, and spleen.

Metastatic lesions were harvested from all subjects for vaccine manufacture. Tumors were most commonly obtained from the lungs or soft tissues. Resected metastases were processed to single cell suspensions with mechanical and enzymatic digestion, transduced overnight with a replication defective adenoviral vector encoding GM-CSF at a multiplicity of infection of 10, irradiated at 10 cGy, and cryopreserved in individual aliquots for subsequent administration. At least 6 vaccines were successfully produced for 11 patients (92%). The tumor preparation for ASPS-12 was contaminated with *Proprionibacterium acnes*, likely from a skin source, and was not administered; the subject was withdrawn from study. Vaccines were manufactured at dose level I (1×10^5) in one case, dose level III (4×10^6) in 2 cases, and dose level IV (1×10^7) in 9 cases (including ASPS-12). The average GM-CSF secretion rate was 347 ng/10^6 cells/24 hours. Other than the samples from ASPS-12, all vaccine preparations were negative for endotoxin, mycoplasma and sterility testing. The average pre-freeze viability was 84%.

Vaccines were administered intradermally and subcutaneously into the limbs or trunk on a rotating basis every week times three and then every other week until the supply was exhausted or the patient removed from study. Six vaccinations were required to determine biologic activity. Eleven subjects received at least one vaccine and were evaluable for toxicity (92%). Rapid disease progression resulted in the withdrawal of one subject (CCS-2) after the third vaccination. Ten patients received at least 6 vaccines and were evaluable for biologic activity (83%; 90% exact binomial confidence interval 56% to 97%). The largest number of vaccines received by any one subject was 13.

Toxicities

Vaccination consistently induced grade 1–2 erythema and induration at injection sites. Mild local pruritus was easily controlled with emollients. Occasional grade 1–2 fatigue and flu-like symptoms were reported. One subject was withdrawn after 6 vaccines for grade 2 urticaria deemed possibly related to vaccination, but this resolved with local therapy over several weeks. There were no significant hepatic, renal, pulmonary, cardiac, hematologic, gastrointestinal, or neurologic toxicities attributable to immunization. One patient (CCS-8) was diagnosed with type 1 insulin-dependent diabetes one year after completing a course of 12 vaccinations. A cryopreserved serum sample obtained prior to treatment revealed the presence of anti-beta cell antibodies, demonstrating that the autoimmunity predated vaccination. Whether immunization impacted the kinetics of disease development remains unclear, but the diabetes was effectively managed with an insulin pump. No other autoimmune toxicities were observed.

Vaccination and delayed-type hypersensitivity reactions

Biopsies of vaccination sites 2–3 days after the first and fifth inoculations were obtained in 8 patients. The reactions to the fifth immunization were more intense. Histopathology revealed brisk infiltrates of dendritic cells, macrophages, eosinophils, neutrophils, and lymphocytes that extended throughout the dermis and sometimes into the subcutaneous fat (Figure 1). Endothelial cell activation and damage were observed in the superficial venules of the upper dermis, and there was evidence of dermal edema and fibrin deposition. A semi-quantitative scoring system that integrated these morphologic features (range 0–4+) indicated that reactions in 6 of 8 subjects evaluated were of 3+ or 4+ intensities (Table 1). Dendritic cells were identified in H&E sections based on an ovoid or dendritic shape with prominent pale-gray cytoplasm; an oval, sometimes indented nucleus with clear nucleoplasm; and a single, small blue nucleolus often apposed to a delicate nuclear membrane. Immunohistochemistry for CD1a and CD11c confirmed the strong dendritic cell response. Significant numbers of $CD4^+$ and $CD8^+$ T cells and, to a lesser extent, $FoxP3^+$ Tregs were present, but $CD20^+$ B cells were rare (not shown).

Irradiated, autologous non-transfected sarcoma cells were available for delayed-type hypersensitivity testing in 7 patients (insufficient cells precluded these studies in 4 patients). Injections of non-transfected sarcoma cells failed to elicit significant cellular infiltrates (or clinical reactions) in all patients tested at the time of beginning treatment. However, biopsies of the injection sites at the time of the fifth vaccination demonstrated responses that were graded at least 1+ intensity (range 0–4+) in all 7 patients tested (Table 1). The more modest reactivity compared to vaccine sites might reflect the injection of smaller numbers of tumor cells and/or the absence of enforced GM-CSF expression. Histopathologically, the responses were characterized by brisk infiltrates of T lymphocytes, eosinophils, and macrophages throughout the dermis (Figure 2). Immunohistochemistry revealed the presence of $CD11c^+$ dendritic cells, $CD4^+$ and $CD8^+$ T cells, and scattered $FoxP3^+$ T cells.

Vaccine-induced humoral responses

Prior studies of autologous GM-CSF secreting tumor cell vaccines in advanced melanoma patients revealed the development of B cell infiltrates in distant metastases (29, 30). Consistent with these results, analysis of a pulmonary metastasis resected after completion of 12 vaccines in patient CCS-8 disclosed a prominent plasma cell component, with the characteristic eccentric nuclei and cartwheel-like chromatin pattern (Figure 3A). We thus investigated whether the vaccinated ASPS and CCS patients generated antibodies to ML-IAP or NY-ES0-1, two targets of humoral immunity in melanoma and sarcoma (34–36). However, analysis of sera samples obtained longitudinally revealed only low levels of antibodies to ML-IAP in one patient and no responses to NY-ES0-1 (not shown).

To characterize the antibody responses in more detail, we sought to identify targets using an unbiased approach. Towards this end, we screened a tumor-derived cDNA expression library with post-vaccination sera obtained from a long-term surviving patient (ASPS-6). Because no ASPS or CCS cDNA expression library was available, we used a previously constructed melanoma-derived cDNA expression library (K008) that has proved informative for antigen discovery efforts in several other tumor types (32, 33, 37, 38). While the use of

the K008 library limits the ability to detect sarcoma-specific antigens, it favors the identification of shared tumor antigens that may include proteins commonly involved in transformation by the MITF-related transcription factor family.

The library screening yielded 13 distinct gene products, 12 of which encode known proteins (Table 2). As in prior studies of advanced melanoma patients, the antibody targets were primarily intracellular proteins that functioned in fundamental aspects of cancer cell biology, such as transcription/translation, signaling, cell division, metabolism, and intracellular trafficking. Of particular interest was the identification of tissue-type plasminogen activator (tTPA) as an antibody target. tTPA is a secreted protein that plays a critical role in fibrinolysis, which is involved in angiogenesis and tumor cell invasion (39, 40). The development of an ELISA with recombinant tTPA protein confirmed the presence of specific antibodies in ASPS-6, but the titers were not altered with vaccination (Figure 3B). Evaluation of the entire cohort demonstrated that several patients harbored higher anti-tTPA antibody titers than ASPS-6, but no impact of immunization could be discerned.

Because tTPA is involved in angiogenesis, a prominent aspect of ASPS and CCS biology, and these tumors are sensitive to angiogenesis inhibition, we wondered whether the immune response might be directed towards other vascular targets. Indeed, prior work in advanced melanoma patients showed that GM-CSF secreting tumor cell vaccines elicit antibodies to angiopoietin-1 and -2, and expression profiling analysis showed that these cytokines were elevated in the sarcomas (7, 17–21, 33). In accordance with these findings, 9 of the 10 ASPS and CCS patients evaluable for biologic activity generated antibodies to angiopoietin-1 and -2 as a function of treatment. Together with the histopathology, these results reveal the ability of GM-CSF secreting tumor cell vaccines to elicit humoral immunity in sarcoma patients.

Tumor-induced immunosuppression

Notwithstanding the plasma cell infiltrates observed in the post-vaccination resection sample from patient CCS-8, minimal tumor necrosis was detected. A breast metastasis obtained after vaccination on patient ASPS-6 similarly showed only modest tumor destruction. We thus considered whether immunosuppressive mechanisms operative in the tumor microenvironment might limit the activity of effector T cells (41). Immunohistochemical analysis of the metastasis from ASPS-6 showed scattered $CD8^+$ T cells that were juxtaposed to sarcoma cells, but without clear evidence of cytotoxic effect (Figure 4). However, the infiltrating $CD8^+$ T cells were positive for the negative T cell co-stimulatory receptor PD-1 (42, 43). Furthermore, the adjacent sarcoma cells expressed the cognate ligand PD-L1, with a pattern of staining that appeared to include both cytoplasmic and membrane compartments. Insufficient material was available for immunohistochemical evaluation of the post-vaccination sample from patient CCS-8, but a pre-vaccination sample from patient ASPS-9 showed comparable PD-1 expressing $CD8^+$ T cells and PD-L1 expressing sarcoma cells as the ASPS-6 specimen. Together, these results raise the possibility that the PD-1 pathway might contribute to immunosuppression in ASPS and CCS patients.

Clinical outcomes

Restaging evaluation of the 10 patients who completed at least 6 vaccinations revealed 7 with stable and 3 with progressive disease at week 10. No tumor regressions were observed. The variable and sometimes indolent natural history of ASPS and CCS limits the ability to draw inferences regarding the impact of vaccination on disease activity or survival. Eight patients succumbed to progressive disease from 4 to 101 months after study entry. Patient ASPS-7 received ipilimumab and sunitinib after this vaccination trial. Two patients are alive at 103 and 104 months; ASPS-6 is currently being treated with cediranib. ASPS-11 was lost-to follow-up 11 months after study entry, but was known to have progressive disease at that time.

Discussion

Our studies were undertaken in an effort to learn more about the host response to ASPS and CCS. These soft tissue sarcomas often affect young adults and are usually fatal, but their rarity presents challenges to unraveling disease pathogenesis and testing the potential activity of novel treatments. Investigations of the characteristic chromosomal translocations that activate TFE3 and MITF have helped delineate key oncogenic mechanisms and guide the selection of targeted therapies such as small molecule inhibitors of c-Met and angiogenesis (12, 22). Nonetheless, little is known regarding the immunogenicity or potential sensitivity to immunotherapy of ASPS and CCS. Because MITF is a major driver of malignant melanoma, we hypothesized that strategies that provided insights into anti-melanoma immunity might similarly illuminate the host reaction to these genetically related sarcomas.

Our phase I clinical trial established the feasibility and safety of irradiated, autologous GM-CSF secreting ASPS and CCS vaccines. Metastatic lesions were processed to single cell suspensions and efficiently transduced with a replication defective adenoviral vector encoding GM-CSF, resulting in sufficient numbers of cytokine producing tumor cells to constitute at least six immunizations for all 12 subjects. One preparation was contaminated with *Proprionibacterium acnes*, likely reflecting colonization of the tumor, and was not administered. Patients received from 3 to 13 vaccinations, which were well tolerated. Toxicities were limited to mild or moderate local skin reactions and constitutional symptoms. One subject was diagnosed with type 1 diabetes a year after completing therapy, but autoantibodies to islet cell antigens were present before treatment, indicating that vaccination did not provoke the loss of tolerance. No other serious inflammatory pathologies were noted.

Tumor regressions were not observed on the trial, but several indices of anti-tumor immunity revealed the biologic activity of vaccination. Injections of irradiated, GM-CSF secreting tumor cells strongly elicited local myeloid and lymphoid cell infiltrates. The prominent dendritic cell component together with $CD4^+$ and $CD8^+$ T cells suggest that immunization may have enhanced tumor antigen presentation and activated anti-tumor T cells (44). Consistent with this idea, vaccination triggered delayed-type hypersensitivity reactions to irradiated, autologous, non-transduced sarcoma cells. These responses were composed of $CD4^+$ and $CD8^+$ T cells, dendritic cells, macrophages and eosinophils, a

cellular profile that likely reflects the mixed Th1 and Th2 cytokine profile characteristic of GM-CSF secreting tumor cell vaccines (30, 45).

Examination of vaccination and delayed-type hypersensitivity reactions also disclosed the presence of FoxP3$^+$ Tregs, a distinct cell population that restrains T effectors (46). GM-CSF elicits Tregs through a mechanism that involves myeloid cell production of milk fat globule epidermal growth factor-8 (MFG-E8), a secreted protein that binds phosphatidylserine on the surface of apoptotic cells (47). MFG-E8 acts as a bridge that promotes the ingestion of apoptotic cells by mononuclear phagocytes, which in turn release TGF-β and CCL22 to support Treg homeostasis. In preclinical models, blockade of this GM-CSF driven suppressive pathway using a dominant negative MFG-E8 mutant intensifies tumor destruction through inhibition of Treg activity, and efforts to translate this combinatorial vaccine strategy to testing in patients are underway. Additional strategies that are being explored to enhance the potency of these cellular vaccines include the co-delivery of other dendritic cell activating agents such as toll-like receptor ligands, type I interferon, or STING agonists (48).

Histopathologic analysis of a metastasis resected after therapy disclosed a prominent plasma cell infiltrate, raising the possibility that vaccination evoked a humoral response. Because minimal reactivity was detected against ML-IAP and NY-ESO-1, two immunogenic antigens in melanomas and sarcomas (34–36), we pursued an unbiased approach to target discovery and screened a melanoma-derived cDNA expression library with post-vaccination sera from a long-term surviving patient. This work uncovered high titer antibodies against an array of gene products that participate in diverse aspects of tumor cell biology. Of particular interest was the identification of tTPA, a central regulator of fibrinolysis, given the prominent angiogenesis characteristic of ASPS and CCS (39, 40). While antibody titers to tTPA were not impacted with therapy, extension of the analysis to other tumor vasculature-associated factors revealed angiopoietin-1 and -2 as common targets of vaccine responses. Future studies will examine whether the antibodies block functional activities of the angiogenic cytokines, as we previously demonstrated for immunized melanoma patients (33).

The induction of humoral reactions to angiopoietin-1 and -2 might have therapeutic relevance in view of the sensitivity of ASPS and CCS to angiogenesis inhibition, particularly with the VEGFR antagonists sunitinib and cediranib (25). VEGF and angiopoietins cooperate during tumor angiogenesis and promote immunosuppression through skewing dendritic cells towards Treg stimulation (49, 50). Combination therapy with VEGFR blockade and vaccination might thus exert a potent effect on the tumor vasculature while intensifying anti-tumor immunity. In accordance with this idea, a recent phase I clinical trial that evaluated concurrent administration of blocking antibodies to VEGF-A and cytotoxic T lymphocyte associated antigen-4 (CTLA-4) in advanced melanoma patients revealed marked tumor endothelial cell activation and high levels of anti-melanoma cellular and humoral responses (51).

Our analysis of ASPS biopsies also identified the PD-1 pathway as a potential contributor to immunosuppression (42, 43, 52). Scattered tumor-infiltrating CD8$^+$ T cells expressed PD-1,

whereas adjacent tumor cells showed cytoplasmic and surface PD-L1 staining. PD-1 is upregulated on T cells upon stimulation and is both a marker for a nascent anti-tumor response as well as a mediator of T cell exhaustion. Whether PD-L1 expression on sarcomas reflects cell autonomous oncogenic signaling or induction through interferon producing infiltrating $CD8^+$ T cells will require further study. In either case, PD-1 engagement restricts effector T cell proliferation, cytokine production, and cytotoxicity. Blocking antibodies to PD-1 have accomplished durable regressions in multiple cancer types, and our results suggest that this treatment strategy should also be investigated in ASPS and CCS patients.

The variable natural history of the sarcomas complicates interpretation of survival data in this phase I trial. Nonetheless, the survival of five subjects with advanced disease for at least two years after study enrollment together with the safety and immunologic activity of this vaccination scheme should motivate more detailed evaluation of immunotherapy for these rare tumors. Our clinical and laboratory investigations suggest that a combination of autologous cancer vaccination, VEGFR inhibition, and PD-1 blockade might effectively antagonize major host factors that impede immune-mediated tumor destruction.

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Statement of translational relevance

Alveolar soft parts sarcoma (ASPS) and clear cell sarcoma (CCS) are rare but frequently fatal mesenchymal malignancies that typically affect young adults. Little is known regarding the host response to these tumors. We conducted a phase I clinical trial investigating the biologic activity of irradiated, autologous sarcoma cells engineered to secrete granulocyte-macrophage colony stimulating factor (GM-CSF) in 12 patients with advanced ASPS or CCS. Vaccine manufacture was feasible and immunization was well tolerated. Vaccination enhanced immune responses as assessed through biopsies of immunization sites, delayed-type hypersensitivity reactions, and metastases. Analysis of humoral responses revealed angiopoietin-1 and -2 as targets of vaccine-induced antibodies. Engagement of the programmed death-1 (PD-1) immunoinhibitory pathway in the tumor microenvironment may impede effector T cell responses. These results provide new insights into the immune response to ASPS and CCS and suggest potential combination therapies to increase anti-tumor activity.

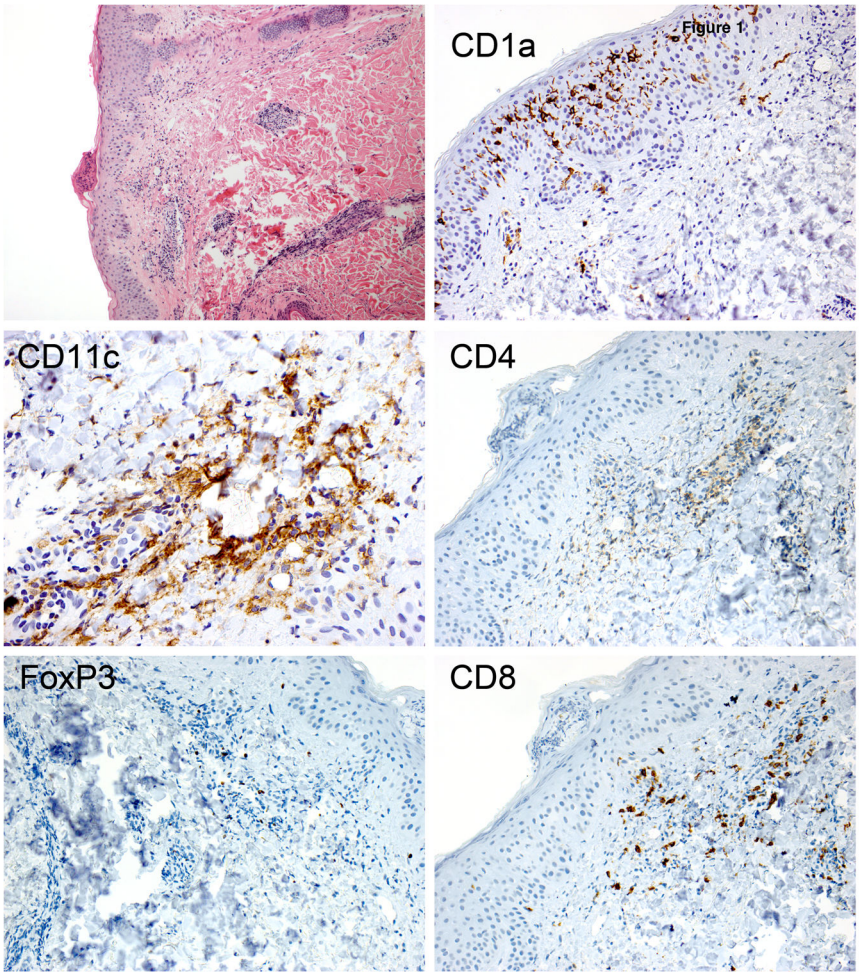

Figure 1. Autologous, GM-CSF secreting sarcoma cell vaccines stimulate local cellular infiltrates

A representative analysis of a skin biopsy obtained 2–3 days after the fifth vaccination. Shown are the H&E staining and immunohistochemistry for CD1a, CD11c, CD4, FoxP3, and CD8 expressing dendritic cells and T cells (400×). A strong dendritic cell reaction is evident.

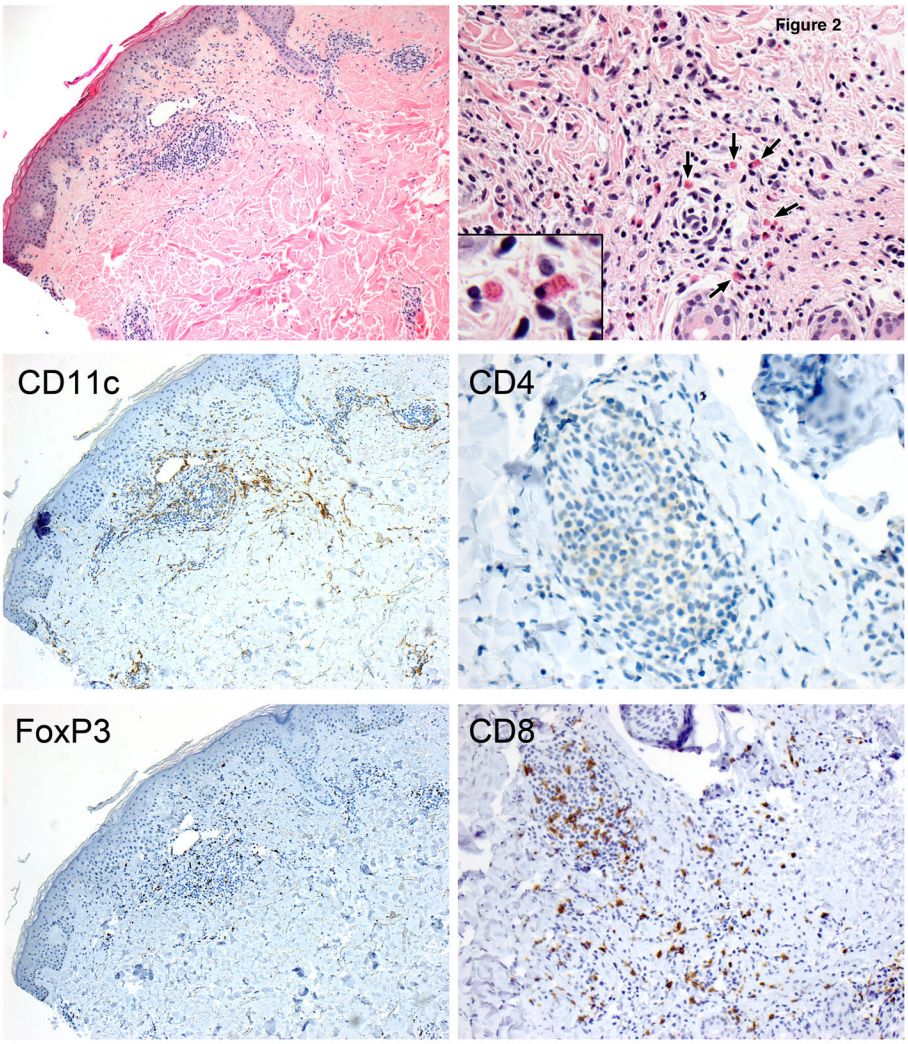

Figure 2. Vaccine-induced delayed-type hypersensitivity reactions to irradiated autologous sarcoma cells

A representative analysis of a skin biopsy obtained 2–3 days after the second injection of irradiated autologous sarcoma cells. Shown are the H&E staining and immunohistochemistry for CD1a, CD11c, CD4, FoxP3, and CD8 expressing dendritic cells and T cells (400×). A prominent interface perivascular infiltrate is seen with eosinophils.

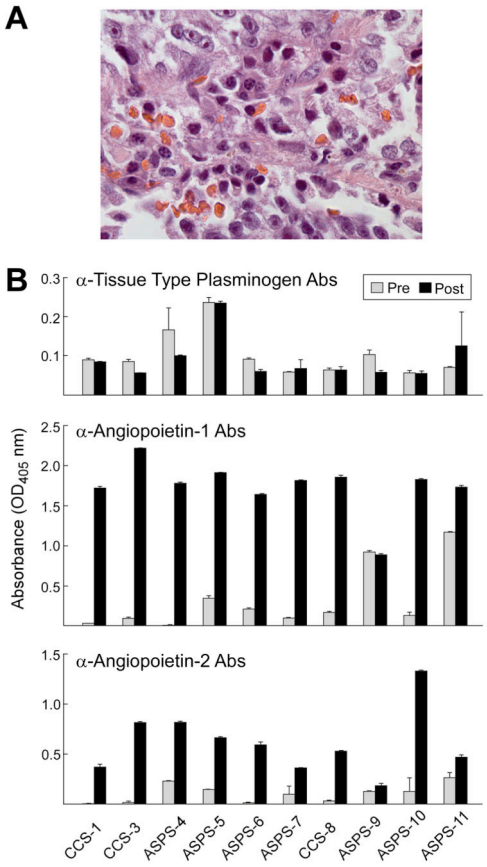

Figure 3. Vaccination elicits humoral immunity

A). Pulmonary metastasis obtained after completion of vaccination (patient CCS-8). Numerous plasma cells (with characteristic eccentric nuclei and cartwheel-like chromatin pattern) are admixed with the tumor cells. B). Pre- and post-vaccination sera samples were diluted 1:100 and evaluated for reactivity against recombinant tTPA, angiopoietin-1, and angiopoietin-2 in ELISAs.

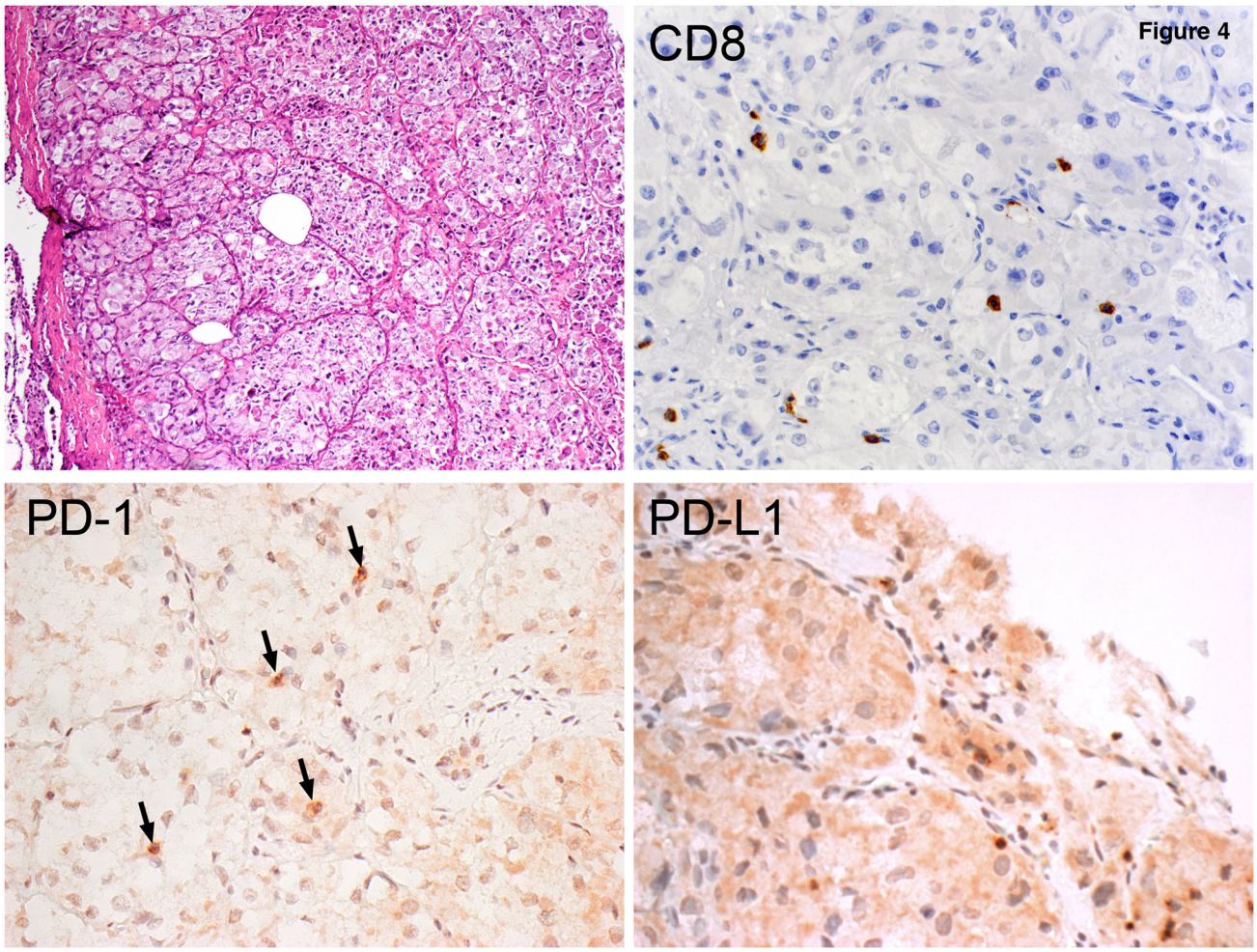

Figure 4. The PD-1 pathway is involved in ASPS

Breast metastasis obtained after completion of vaccination (patient ASPS-6). Shown are the H&E staining and immunohistochemistry for CD8, PD-1, and PD-L1 expressing cells (400×).

Table 1

Patient characteristics and vaccine manufacturing and administration

GM-CSF secretion rates are ng/10^6 cells/24 hours. VAX and DTH reactions were scored on a semi-quantitative scale from 0–4+ as described in Methods.

Patient	Age	Tumor source	Other disease sites	VAX dose	VAX #s	GM-CSF	Clinical outcome	VAX site	DTH
CCS-1	10	Lung	soft tissue	1×107	13	79	DOD 24 mos	4+	1+
CCS-2	31	Breast	viscera	1×105	3	23	DOD 4 mos		
ASPS-3	23	retroperitoneum	bone, lung	1×107	13	182	DOD 30 mos	3+	3+
ASPS-4	41	Adrenal	Lung, soft tissue	1×107	13	130	DOD 17 mos	4+	2+
ASPS-5	28	Lung	brain, soft tissue	1×107	7	787	DOD 21 mos	3+	
ASPS-6	24	Lung	brain, soft tissue	1×107	12	1401	Alive 104 mos	2+	1+
ASPS-7	15	Lung	soft tissue	1×107	6	322	DOD 101 mos	2+	2+
CCS-3	10	Leg	lung	1×107	12	27	Alive 103 mos		
ASPS-9	23	Lung	soft tissue	4×106	9	120	DOD 41 mos		
ASPS-10	34	Lung	viceral, soft tissue	1×107	7	478	DOD 13 mos	3–4+	1+
ASPS-11	50	Spleen	lung, soft tissue	1×107	6	394	LTF 11 mos	3+	3+
ASPS-12	12	Arm	lung	4×106	0	227	N/A		

Table 2

Antibody targets identified through cDNA library screening with patient ASPS-6 post-vaccination sera.

Gene Product	Function
tTPA (tissue type plasminogen activator)	Fibrinolysis
CCDC46 (coiled-coil domain containing 46)	Centrosomal protein
TGS1 (trimethylguanosine synthase homolog)	mRNA splicing
PHF20 (plant homeodomain finger protein 20)	Lysine acetyltransferase complex
KTN1 (kinesin-binding protein)	ER membrane protein
KIF16B (kinesin family member 16B)	Subcellular trafficking
BECN1 (beclin)	Autophagy
PUF60 (poly-u binding splicing factor)	mRNA splicing
PRKAG1 (protein kinase, AMP-activated, gamma 1 non-catalytic subunit)	Signaling
GARNL1 (GTPase-activating Rap/Ran-GAP domain-like 1)	Signaling
FUCA1 (alpha-fucosidase)	Metabolism
PKC (protein kinase C)	Signaling
CM37413	Unknown