Clinical Cancer Research

A Live-Attenuated Listeria Vaccine (ANZ-100) and a Live-Attenuated Listeria Vaccine Expressing Mesothelin (CRS-207) for Advanced Cancers: Phase I Studies of Safety and Immune Induction

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Abstract

Purpose: *Listeria monocytogenes* (*Lm*)-based vaccines stimulate both innate and adaptive immunity. ANZ-100 is a live-attenuated *Lm* strain (*Lm* Δ*actA*/Δ*inlB*). Uptake by phagocytes in the liver results in local inflammatory responses and activation and recruitment of natural killer (NK) and T cells, in association with increased survival of mice bearing hepatic metastases. The *Lm* Δ*actA*/Δ*inlB* strain, engineered to express human mesothelin (CRS-207), a tumor-associated antigen expressed by a variety of tumors, induces mesothelin-specific T-cell responses against mesothelin-expressing murine tumors. These two phase I studies test ANZ-100 and CRS-207 in subjects with liver metastases and mesothelin-expressing cancers, respectively.

Experimental Design: A single intravenous injection of ANZ-100 was evaluated in a dose escalation study in subjects with liver metastases. Nine subjects received 1×10^6, 3×10^7, or 3×10^8 colony-forming units (cfu). CRS-207 was evaluated in a dose-escalation study in subjects with mesothelioma, lung, pancreatic, or ovarian cancers. Seventeen subjects received up to 4 doses of 1×10^8, 3×10^8, 1×10^9, or 1×10^{10} cfu.

Results: A single infusion of ANZ-100 was well tolerated to the maximum planned dose. Adverse events included transient laboratory abnormalities and symptoms associated with cytokine release. Multiple infusions of CRS-207 were well tolerated up to 1×10^9 cfu, the determined maximum tolerated dose. Immune activation was observed for both ANZ-100 and CRS-207 as measured by serum cytokine/chemokine levels and NK cell activation. In the CRS-207 study, listeriolysin O and mesothelin-specific T-cell responses were detected and 37% of subjects lived $\geq$15 months.

Conclusions: ANZ-100 and CRS-207 administration was safe and resulted in immune activation. *Clin Cancer Res; 18(3); 858–68. ©2011 AACR.*

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Introduction

Cancer vaccines aim to induce immunity specific to protein antigens that are differentially expressed by cancer cells relative to the normal cells from which they are derived. Through its network of specialized antigen-presenting and effector cells, the immune system has the ability to become activated to recognize and lyse cancer cells. Current vaccine strategies aim to provide a series of signals that activate and mature dendritic cells (DC) for efficient antigen processing and presentation which in turn activate effector cells of the adaptive immune response. *Listeria monocytogenes* (*Lm*)-based vaccine vectors directly target and activate DCs *in vivo*, but in addition, take advantage of the capability of immunogenic infectious vectors to stimulate both adaptive

Translational Relevance

Listeria monocytogenes (*Lm*)-based vaccine vectors can stimulate both innate and adaptive immune responses. In preclinical studies, administration of *Lm* vaccines results in enhanced tumor-specific immune responses, delayed tumor growth, and improved survival. Furthermore, *Lm* can be modified to encode heterologous tumor antigens resulting in recruitment and activation of tumor antigen–specific T cells. In these two first-in-human phase I clinical studies in patients with advanced cancer, ANZ-100 [a live-attenuated *Lm* strain (*Lm* Δ*actA*/Δ*inlB*)] and CRS-207 (the *Lm* Δ*actA*/Δ*inlB* strain engineered to express human mesothelin), were well tolerated with encouraging dose-dependent evidence of immune activation. These results provide valuable insight into the safety and dosing of this new vaccine approach that will advance the further development of *Lm* vaccines as anticancer agents for multiple tumor types.

and innate immune responses. *Lm* is an intracellular bacterium that has access to both class I and II antigen-processing pathways. *Lm* provides a potent stimulation of innate immunity and also stimulates an adaptive immune response through recruitment and activation of $CD4^+$ and $CD8^+$ T cells specific for encoded heterologous antigens (1–4). The ability of *Lm* to stimulate adaptive immunity is mainly based on its intracellular lifecycle and the ability to target DCs *in vivo* (4). ANZ-100 is a live-attenuated double-deleted *Lm* strain (LADD; *Lm* Δ*actA*/Δ*inlB*). This strain has deletions of 2 virulence genes, *actA* and *internalin B (InlB)*. These virulence determinants facilitate cell-to-cell spread and invasion of nonphagocytic cells, and their combined deletion results in 1,000-fold attenuation when compared with wild-type *Lm* (5). However, uptake of ANZ-100 by phagocytic cells in the liver and spleen is retained and results in a local proinflammatory cytokine response resulting in activation and recruitment of both innate and adaptive effector cells. This immune response results in delay in tumor growth and increased survival of mice bearing hepatic metastases (6, 7). Importantly, multiple doses of *Lm* further extend survival.

The LADD strain has also been engineered to express human mesothelin and the resulting strain has been termed CRS-207 (*Lm*-mesothelin). CRS-207 has been shown to efficiently deliver mesothelin antigen into both class I and II antigen-processing pathways. Mesothelin is a tumor-associated antigen present on normal mesothelial cells and highly expressed by many human tumor types, including mesotheliomas, pancreatic adenocarcinomas (PDA), non–small cell lung cancers (NSCLC), and ovarian cancers (8–16). This expression profile, combined with limited expression on the surface of normal tissues, makes mesothelin an attractive target for active tumor-specific immunotherapy. Support for mesothelin as a T-cell target comes from studies showing a correlation between positive clinical outcomes and the induction of mesothelin-specific cellular immunity in subjects with PDA following vaccination with an irradiated allogeneic whole-cell vaccine encoding granulocyte macrophage colony-stimulating factor (GM-CSF). In a phase I study, a dose-dependent systemic antitumor response was reported to be associated with anti-mesothelin $CD8^+$ T-cell responses (17, 18). In subsequent studies, the induction of mesothelin-specific T cells as well as the increased post-vaccination diversity and avidity of the T-cell repertoire were shown to be associated with improved disease-free (DFS) and overall survival (OS; refs. 19, 20). Furthermore, CRS-207 mediates the induction of mesothelin-specific T-cell responses that correlate with tumor regressions of mesothelin-expressing murine tumors (unpublished data).

A single intravenous dose of ANZ-100 underwent evaluation in a phase I dose escalation study of safety and tolerability in adults with carcinoma and liver metastases (NCT00327652). A total of 9 subjects received single-dose infusions at 3 dose levels [1×10^6, 3×10^7, 3×10^8 colony-forming units (cfu)]. Subsequently, CRS-207 underwent evaluation in a phase I, open-label, multiple dose, dose-escalation study in subjects with mesothelioma, NSCLC, PDA, or ovarian cancer (NCT00585845). Seventeen subjects were enrolled into 4 cohorts (1×10^8, 3×10^8, 1×10^9, and 1×10^{10} cfu). Here, we report the safety, shedding and clearance data, clinical activity, and the induction of immunologic responses to both ANZ-100 and CRS-207.

Subjects and Methods

Construction of ANZ-100 and CRS-207

ANZ-100 (LADD; *Lm* Δ*actA*/Δ*inlB*), *Lm* strain CERS 382.20, was constructed by deletion of the *actA* and *inlB* genes from the Streptomycin-resistant wild-type strain DP-L4056. Using standard techniques, deletions of *actA* and *inlB* were made by homologous recombination of the mutant alleles into the wild-type chromosome (21). Deletion mutations were confirmed by PCR.

CRS-207, *Lm* strain hMeso38, was constructed by the addition of a mesothelin expression cassette into the CERS 382.20 strain. The same homologous recombination approach used to delete *inlB* was used to insert the mesothelin antigen. Mesothelin is expressed as an ActA fusion protein under the transcriptional control of the *actA* promoter. The *actA* promoter is strongly induced in host cells, resulting in efficient production of the heterologous antigen. All genomic modifications were confirmed by PCR and DNA sequencing, and the attenuated phenotype of both strains were showed *in vivo* by LD_{50} (tested in CD-1, C57BL/6, and BALB/c mice: LD_{50} of 8.0×10^7, 1.2×10^8, and 8.4×10^7 cfu, respectively, compared with 3.0×10^4 cfu of the wild-type *Lm* strain) and clearance in liver and spleen of mice and *in vitro* by infectivity and intracellular growth kinetics. Clinical grade material of ANZ-100 and CRS-207 was manufactured at the Waisman Clinical BioManufacturing Facility.

Study design

Nine subjects were enrolled into the ANZ-100 study at Johns Hopkins University (JHU), Baltimore, MD, and Mary Crowley Cancer Center, Dallas, TX, between October 9, 2006, and January 7, 2008. The primary objective of the ANZ-100 study was to determine the maximum tolerated dose (MTD) of a single dose of ANZ-100 in subjects with carcinoma and liver metastases. Using a standard 3 + 3 design, eligible subjects received a single 2-hour intravenous infusion of ANZ-100 (22).

Seventeen subjects were enrolled into the CRS-207 study at JHU; the National Cancer Institute (NCI), Bethesda, MD; the University of Pennsylvania, Philadelphia, PA; Hadassah-Hebrew University Medical Center, Jerusalem, Israel; and at Mary Crowley Cancer Center between December 13, 2007, and January 5, 2009. The primary objective of the CRS-207 study was to determine the MTD of multiple doses of CRS-207 in subjects with malignancies known to express mesothelin. Secondary objectives included assessing safety, biodistribution and clearance of *Lm*, immunologic endpoints, and antitumor activity. Using a 3 + 3 design, sequential cohorts of 3 to 6 subjects received up to 4 doses of CRS-207 administered 3 weeks apart.

These multi-institutional, first-in-human, phase I, dose-escalation studies were reviewed and approved by local Institutional Review Boards, Institutional Biosafety Committees, the U.S. Food and Drug Administration, and the NIH Recombinant DNA Advisory Committee. All participating subjects signed informed consent.

Subject selection

In the ANZ-100 study, eligible subjects had treatment-refractory carcinoma and hepatic metastases. In the CRS-207 study, eligible subjects had treatment-refractory mesothelioma, PDA, NSCLC or ovarian cancer. For both studies, main eligibility criteria included: no cancer therapy for 4 weeks, age $\geq$18 years old, a life expectancy of $\geq$12 weeks, an Eastern Cooperative Oncology Group performance status of 0 to 1 or Karnofsky performance status of 80% to 100%, adequate organ function, no ongoing infections, history of brain metastases, or history of autoimmunity. Concurrent antineoplastic therapies, history of listeriosis or vaccination with a *Lm*-based vaccine, known allergy to both penicillin and sulfa, and artificial implants (except biliary stents) were not permitted. Subjects who were HIV, HTLV-1, HCV, or HBV positive were excluded.

Procedures and treatment

Tests were conducted for baseline toxicity (complete blood counts and chemistry profile) and tumor assessment (computerized tomography scan). The intervention and data collection schedules are shown in Supplementary Figs. S1 (ANZ-100) and S2 (CRS-207).

Nine subjects [6 with colorectal cancer (CRC), 2 with PDA, 1 with melanoma] with treatment-refractory carcinoma and liver metastases received a single-dose 2-hour infusion at 1 of 3 dose levels (1×10^6, 3×10^7, 3×10^8 cfu) of ANZ-100 (Table 1). Subjects were observed in an in-patient facility for 5 days and evaluated for toxicity on days 6, 9, 16, and 28.

Seven subjects with PDA, 5 with mesothelioma, 3 with NSCLC, and 2 with ovarian cancer received up to 4 intravenous infusions of CRS-207 in 21-day intervals and were observed for toxicities in an in-patient facility for 24 to 48 hours (Table 1). Subjects were evaluated for toxicity on days 4 and 7 in the clinic and by phone on day 14. Following the final administration, subjects were evaluated by phone 21 days after dosing and returned for a final clinic visit on the 28th day after the final dose (day 91).

A 10-day course of oral amoxicillin or trimethoprim/sulfamethoxazole in penicillin-allergic subjects was initiated 6 to 7 days following the subject's last dose. Subjects were restaged radiographically [Response Evaluation Criteria in Solid Tumors (RECIST) 1.0] at days 28 and 91 (CRS-207 study only). Subjects with progressive disease at day 28 on the CRS-207 study were allowed to continue on study if clinically stable.

Assessments

Toxicities. Adverse events were graded using the NCI Common Terminology Criteria for Adverse Events (CTCAE) v3.0. Initially, a dose-limiting toxicity (DLT) was defined as the occurrence of any NCI CTCAE (Version 3.0) $\geq$grade III that were determined to be possibly or probably related to the agent, during the 28 days after the first dose. For individuals who had alanine aminotransferase (ALT), aspartate aminotransferase (AST), or alkaline phosphatase elevations $\leq$ grade I severity at study entry, a DLT was defined as enzyme elevations >5 $\times$ upper limit of normal (ULN) that were determined to be related and persisted for more than 7 days. For individuals who had ALT, AST, or alkaline phosphatase levels that were above 2.5 $\times$ ULN to 3.5 $\times$ ULN at study entry, a DLT was defined as enzyme elevations above 10 $\times$ ULN that were determined to be related and persisted for more than 7 days. Early initiation of antibiotics coincident with isolation of *Lm* from a sterile body site, other than blood (e.g., CSF, joint fluid) was considered a DLT. DLT criteria were modified several times during the CRS-207 study. In addition to the liver enzyme criteria mentioned, a DLT was defined as a treatment-related $\geq$ grade III laboratory abnormality lasting for more than 48 hours, fever above 40°C lasting greater than 24 hours, hypotension unresponsive to intravenous fluids, and grade IV lymphocyte decreases that persisted for more than 4 days. The MTD was the highest dose at which no more than 1 of 6 subjects experienced a DLT.

Shedding and clearance. Specimens were obtained for culture to assess the *Lm* distribution and clearance. With each administration of ANZ-100, blood, urine, stool, and sputum specimens were cultured at baseline, 6 hours, days 1–5, days 8, 16, and 28. An additional blood culture was taken at 2 hours. With each administration of CRS-207, urine and stool cultures were obtained at baseline, 4 hours, days 1, 4, and 7. Blood cultures were obtained at baseline, 4 hours, days 1 and 4.

Table 1. Patient characteristics

ID	Age (sex)	Cancer type	Dose level, cfu	ECOG PS	Prior therapies	Mesothelin IHC[a]	No. of doses
ANZ-100							
01-002	57 (F)	Pancreatic	10^6	0	2	NA	1
02-003	53 (M)	Colorectal	10^6	0	2	NA	1
02-006	69 (M)	Colorectal	10^6	1	5	NA	1
01-005	62 (M)	Pancreatic	3×10^7	0	2	NA	1
02-054	66 (M)	Melanoma	3×10^7	0	4	NA	1
02-058	71 (M)	Colorectal	3×10^7	0	4	NA	1
01-008	49 (F)	Colorectal	3×10^8	0	4	NA	1
02-062	57 (M)	Colorectal	3×10^8	0	5	NA	1
02-064	60 (M)	Colorectal	3×10^8	0	5	NA	1
CRS-207							
001-001	65 (M)	Pancreatic	10^8	0	5	50%, 2–3+	4
001-002	61 (F)	Pancreatic	10^8	0	4	NE	4
003-001	65 (F)	Mesothelioma	10^8	0	2	25%, 1–2+	4
002-001	64 (F)	Ovarian	10^8	1	8	NE	2
002-002	60 (M)	Mesothelioma	10^8	1	1	NE	2
004-001	55 (M)	Mesothelioma	10^8	0	4	NE	3
005-002	56 (F)	NSCLC	3×10^8	0	4	70%, 2–3+	1
001-004	68 (F)	Pancreatic	3×10^8	0	1	NE	3
001-005	60 (M)	Pancreatic	3×10^8	1	1	75%, 2–3+	2
005-003	79 (F)	Pancreatic	3×10^8	0	0	80%, 2–3+	2
004-002	59 (M)	Pancreatic	10^9	1	4	NE	4
005-001	56 (F)	NSCLC	10^9	0	5	50%, 2+	4
001-003	61 (M)	Pancreatic	10^9	0	2	NE	4
002-003	72 (F)	NSCLC	10^9	1	5	100%, 3+	4
002-004	75 (M)	Mesothelioma	10^9	1	4	NE	2
003-002	52 (F)	Ovarian	10^9	1	10	NE	4
003-003	40 (M)	Mesothelioma	10^{10}	1	2	NE	1

Abbreviations: ECOG, Eastern Cooperative Oncology Group; NA, not applicable; NE, not evaluable; PS, performance status.
[a]Mesothelin IHC: the percentage of tumor cells showing membranous staining were evaluated for intensity (0, no staining; 1+, weak staining; 2+, moderate staining; 3+, strong staining).

Immunohistochemistry. Mesothelin expression on the tumor was not required for CRS-207 study entry, but immunohistochemistry (IHC) was conducted on available archived tissue. IHC was conducted using a monoclonal antibody (2C6) on a Leica BondMax autostainer. The staining intensity and extent were scored. The percentage of tumor cells showing membranous staining (predominantly luminal) were evaluated for intensity (0, none; 1+, weak thin; 2+, moderate; and 3+, strong and thick membranous staining).

Immunologic assessments

Multiplexed serum chemokine assay. Chemokines were detected using a custom Cytokine Bead Array (BD) using frozen serum samples collected before, 4 hours, and 24 hours postinfusion with ANZ-100 and at baseline and at 24 hours after CRS-207. The array was specific for interleukin (IL)-6, IL-8, IL-9, IL-10, IFN-γ–induced protein 10 (IP-10), lymphotoxin-α (LT-α), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), and MIP-1β. Upon collection of all samples within a patient cohort, samples were tested by a blinded operator. Data are presented as change in fold concentration because predose chemokine concentration in sera varied considerably between subjects.

Multiplexed serum cytokine assay. Cytokines were detected using the Meso Scale Discovery (MSD) platform using frozen serum samples collected before, 4 hours, and 24 hours postinfusion with ANZ-100 or CRS-207. ANZ-100 samples were tested using a 9-plex proinflammatory kit (GM-CSF, IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12p70, TNF-α) and CRS-207 samples were tested using a 7-plex kit (IFN-γ, IL-1β, IL-6, IL-8, IL-10, IL-12p70, TNF-α). Upon collection of all samples within a patient cohort, samples were tested by a blinded operator. Results are expressed as mean ± SD.

Lymphocyte counts. For the ANZ-100 study, a BD FACSCalibur was used to determine absolute numbers of lymphocyte subpopulations after applying BD Multitest CD3/CD16, CD56/CD45/CD19 with Trucount,

and BD Multitest CD3/CD8/CD45/CD4 with Trucount reagent cocktails.

Determination of cellular activation. For the ANZ-100 study, a BD FACSCalibur was used to determine the expression level of CD38 on natural killer (NK) cells in blood using CD3-FITC, CD38-PE, CD4-PerCp, CD16-APC, CD56 APC, and IgG Isotype-PE. CD38 expression levels were analyzed by a blinded operator and reported as a ratio of peak CD38 MESF value between 48 and 96 hours postdosing and predose value. A histogram is also presented for subjects 001–005.

Detection of antigen-specific T cells. Peripheral blood mononuclear cells (PBMC) were prepared within 4 to 6 hours after collection and cryopreserved at each clinical site.

Detection of listeriolysin O–specific T cells by IFN-γ ELISPOT. PBMCs were analyzed using a 2-step IFN-γ ELISPOT assay in which first autologous DCs were obtained through *in vitro* culture (23). Monocytes were isolated from PBMCs using a 1-hour adherence step. Nonadherent cells were collected and cryopreserved. Adherent cells were cultured for 2 days in DC induction medium (RPMI with 1% autologous plasma and GM-CSF and IL-4) and then a cocktail containing IL-1β, IL-6, and prostaglandin E2-α (24). After 2 days of culture, mature DCs were harvested, counted, and added at a 1:10 ratio to 2×10^5 nonadherent thawed PBMCs. Antigen was added as pools of 15-mer peptides whose sequences overlap by 11 amino acids and cover the entire sequence of the listeriolysin O (LLO), a 130 amino acid protein (25, 26). The CEF pool was used as a positive control. The CEF is a pool of 32 peptides of defined $CD8^+$ epitopes against cytomegalovirus (CMV), Epstein-Barr virus (EBV), and influenza (27). Cells were cultured for 24 hours in RPMI containing 10% human AB serum before they were washed and spots visualized using BD Biosciences' Human IFN-γ ELISPOT and AEC substrate kits. Spots were enumerated in an ELISPOT reader (Cellular Technology, Ltd.) and analyzed using a software package (Immunospot software v. 3.6). T-cell responses to LLO were considered positive when specific T-cell frequencies were $\geq$1 in 10^5 PBMCs and increased by at least 2-fold compared with baseline.

Detection of mesothelin-specific T cells by IFN-γ ELISPOT. The methodology for the synthesis of peptides, ELISA assay for identifying reactive mesothelin peptides, and ELISPOT assays have previously been described (18–20). Samples were tested from subjects with HLA-A1, A2, A3, and A24 alleles if pre- and posttreatment samples were available. T-cell responses to mesothelin were considered positive when the frequency of specific responses were $\geq$1 in 10^5 $CD8^+$ peripheral blood lymphocytes (PBL) above the control sample and increased by at least 2-fold compared with baseline. The maximal response to a single best peptide is reported.

Statistical considerations

The main objectives of these studies were to determine the MTD of ANZ-100 or CRS-207 in subjects with cancer. A standard 3 + 3 design was used for dose-escalation (22). The incidence of toxicities is summarized by cohort. Exploratory analyses included evaluation of RECIST response, OS, cytokine/chemokine responses, immune cell phenotyping, and T-cell responses. The NK cell and lymphocyte values before and after treatment are plotted for each individual. Log-linear models are used to compare fold-upregulation and induction between dose levels. For analysis incorporating multiple time points, linear mixed-effects models are used to account for the within-individual repeated measurements. The numbers of individuals with LLO-specific and mesothelin-specific T-cell responses were tabulated. The survival is documented for each individual and individuals with survival $\geq$15 months are considered "long-term" survivors. The relationship between disease and immunologic characteristics are explored by tabulating the number of long-term survivors in different subcategories.

Results

Subject characteristics

Subject characteristics for both studies are shown in Table 1. For ANZ-100, 6 subjects with CRC, 2 with PDA, and 1 with melanoma received a single dose of either 10^6, 3×10^7, or 3×10^8 cfu of *Lm*. Their median age was 60 (range, 49–71). The median number of prior therapies was 4. For CRS-207, 5 subjects with mesothelioma, 7 with PDA, 3 with NSCLC, and 2 with ovarian cancer received at least one infusion of CRS-207 (6 subjects at 1×10^8, 4 subjects at 3×10^8, 6 subjects at 1×10^9, and 1 subject at 1×10^{10} cfu). Their median age was 61 (range, 40–79). The median number of prior therapies was 4.

Treatment-related toxicities and DLT events

A detailed description of treatment related grade $\geq$II toxicities for both studies is provided in Supplementary Table S1. ANZ-100 was well tolerated at all dose levels. The most frequent adverse events of any grade were transient grade $\leq$III lymphopenia (9 patients, 100%), grade $\leq$III hyperglycemia (8 patients, 89%), hypophosphatemia (5 patients, 56%), and fever (7 patients, 78%). No DLTs were observed and ANZ-100 was well tolerated up to the maximum planned dose.

CRS-207 was also well tolerated. The most frequent adverse events of any grade were transient lymphopenia (17 patients, 100%), hypophosphatemia (6 patients, 35%), transaminitis (7 patients, 41%), fever (9 patients, 53%), chills/rigors (9 patients, 53%), nausea (9 patients, 53%), fatigue (6 patients, 35%), and hypotension (6 patients, 35%). All of these adverse events were grade $\leq$II except for transient $\geq$grade III lymphopenia and hypophosphatemia, one grade III transaminitis, and one grade III fever. The first dose cohort received 1×10^8 cfu of CRS-207 at 3-week intervals for 4 doses. Two adverse events occurred in subjects dosed in cohort 1 (1×10^8 cfu) which met the initial protocol-defined criteria of DLTs. One subject experienced transient grade III hypophosphatemia 4 hours following the second infusion of CRS-207. A second subject experienced a grade III temperature approximately 22 hours after

receiving the first infusion. The subject was treated with acetaminophen and temperature returned to baseline within 24 hours. DLT criteria were amended to allow for transient grade III hypophosphatemia and fever. After transient grade IV lymphopenia was identified in the 1×10^9 cohort, grade IV lymphopenia was considered as a DLT only if it persisted for more than 4 days. Following dosing of 3 subjects in cohort 2 (1×10^9 cfu) without reaching a DLT, one subject was dosed in cohort 3 at 1×10^{10} cfu dose. This subject experienced a grade II cytokine release syndrome requiring aggressive fluid resuscitation. Because of the nature of the event, this was considered a DLT and the dose level was considered too toxic for further recruitment. The MTD was determined to be 1×10^9 cfu. To better characterize toxicities, additional subjects were enrolled into cohort 2 (1×10^9 cfu, $n = 3 + 3 = 6$). Manageable hypotension and modest elevations in liver function tests (LFT) were observed. An intermediate dose of 3×10^8 cfu was also added. Four subjects were enrolled into this cohort before the study was terminated. Manageable hypotension and transient LFT elevations (3 of 4 subjects) was also observed at the new dose level. One subject had elevations up to 11 × ULN after a second dose in the absence of any changes in bilirubin. The LFTs improved without intervention. The subject was not redosed.

In general, subjects treated at all dose levels experienced symptoms that might be expected from a cytokine release–like syndrome from a bacteremia. While not all subjects experienced the same events, a constellation of symptoms was common. *Lm* was administered intravenously over a 2-hour period. Subjects typically had a temperature peak at 2 to 4 hours, sometimes associated with rigors, nausea, headaches, dehydration, and dry mouth. Mild hypotension was self-correcting or corrected with intravenous fluids. The most consistent laboratory abnormalities were transient, self-correcting electrolyte abnormalities, and lymphopenias with nadirs at 4 hours postinfusion. The degree of lymphopenia was dose-dependent and the most significant hypotension occurred at the highest dose level. The transient, self-correcting nature suggests that these abnormalities are the result of electrolytes and lymphocytes transiently shifting out of the blood compartment. Overall review of the safety data from the trial did not identify any significant toxicity with *Lm or Lm*-mesothelin that was not reversible or unexpected from either previous studies in cynomologus monkeys or based on mechanism of action.

Shedding and clearance

ANZ-100 was not detected in the blood, stool, urine, or sputum specimens collected at any time point. *Lm* suspected to be CRS-207 was detected in blood cultures of 4 subjects. In 2 of the subjects, the cultures were negative by 24 hours. An ovarian cancer subject who received a dose of 1×10^8 cfu had positive cultures at 4 and 24 hours after the first intravenous infusion. Subsequent blood cultures 4 days after dosing were negative. Blood cultures taken after her second infusion were negative. *Lm* suspected to be CRS-207 was detected in blood cultures of a mesothelioma subject receiving 1×10^9 cfu at day 4 after the second intravenous infusion. Subsequent cultures were negative. All remaining blood, stool, or urine specimens collected throughout the study for all subjects were negative.

Immunohistochemistry

Mesothelin membranous staining was detected in all 7 available archived samples for subjects enrolled into the CRS-207 study. The extent of staining is listed in Table 1.

PBMC phenotype analysis after ANZ-100 administration

Peripheral blood was analyzed prior to treatment with ANZ-100, daily for 5 days following treatment, and then weekly for 1 month. Immune activation was determined by phenotypic analysis of NK cells ($CD3^-CD16/56^+$). Interestingly, there was a transient reduction in peripheral lymphocyte and NK cell numbers (Fig. 1A and B) that reached a nadir at day 2 following treatment, suggesting the possibility that ANZ-100–induced activation results in lymphocyte and NK cell margination from the peripheral blood to other compartments. A significant upregulation of the activation marker CD38 was noted on NK cells for all dose levels ($P = 0.0008$; Fig. 1C). There appears to be a dose-dependent trend but it was not statistically significant ($P = 0.1238$). This level increased at the 96-hour time point as shown for one patient (Fig. 1D).

Cytokine/chemokine induction after ANZ-100 and CRS-207 administration

Serum samples were collected prior to and at 2 hours, 6 hours, and daily for 5 days following ANZ-100 administration and analyzed for the presence of MCP-1, MIP-1α, MIP-1β, LT-α, IP-10, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IFN-γ, and TNF-α. At the highest dose level of 3×10^8 cfu, a significant induction of cytokines and chemokines such as MCP-1 and MIP-1β was observed ($P = 0.0006$ and 0.0002, respectively; Fig. 2A and B). The response peaked at 2 hours after the completion of the 2-hour intravenous ANZ-100 infusion and returned to baseline within 48 to 72 hours. Subjects at the highest dose level also had a consistent induction of the TH1 cytokines IFN-γ and IL-12p70 (Fig. 2C and D). The upregulation of the CD38 activation marker suggests biologic activity of ANZ-100 at doses as low as 1×10^6 cfu. However, a more consistent induction of proinflammatory cytokines and chemokines was observed in subjects receiving the higher dose level of 3×10^8 cfu.

In the CRS-207 study, chemokine induction was noted for all dose levels including the starting dose level of 1×10^8 cfu. For MCP-1, MIP-1β, and IP-10, the pattern was consistently elevated for all dose levels ($P = 0.0050$, $P < 0.0001$, and $P < 0.0001$, respectively) and did not vary significantly across time points (Fig. 3A–C). The degree of upregulation is less than that observed in the ANZ-100 study, which may be due to the fact that the levels were taken at 24 hours rather than at the expected 2-hour postinfusion peak (Fig. 3A–C). The induction of IL-10 ($P = 0.049$), IL-12p70 ($P = 0.100$),

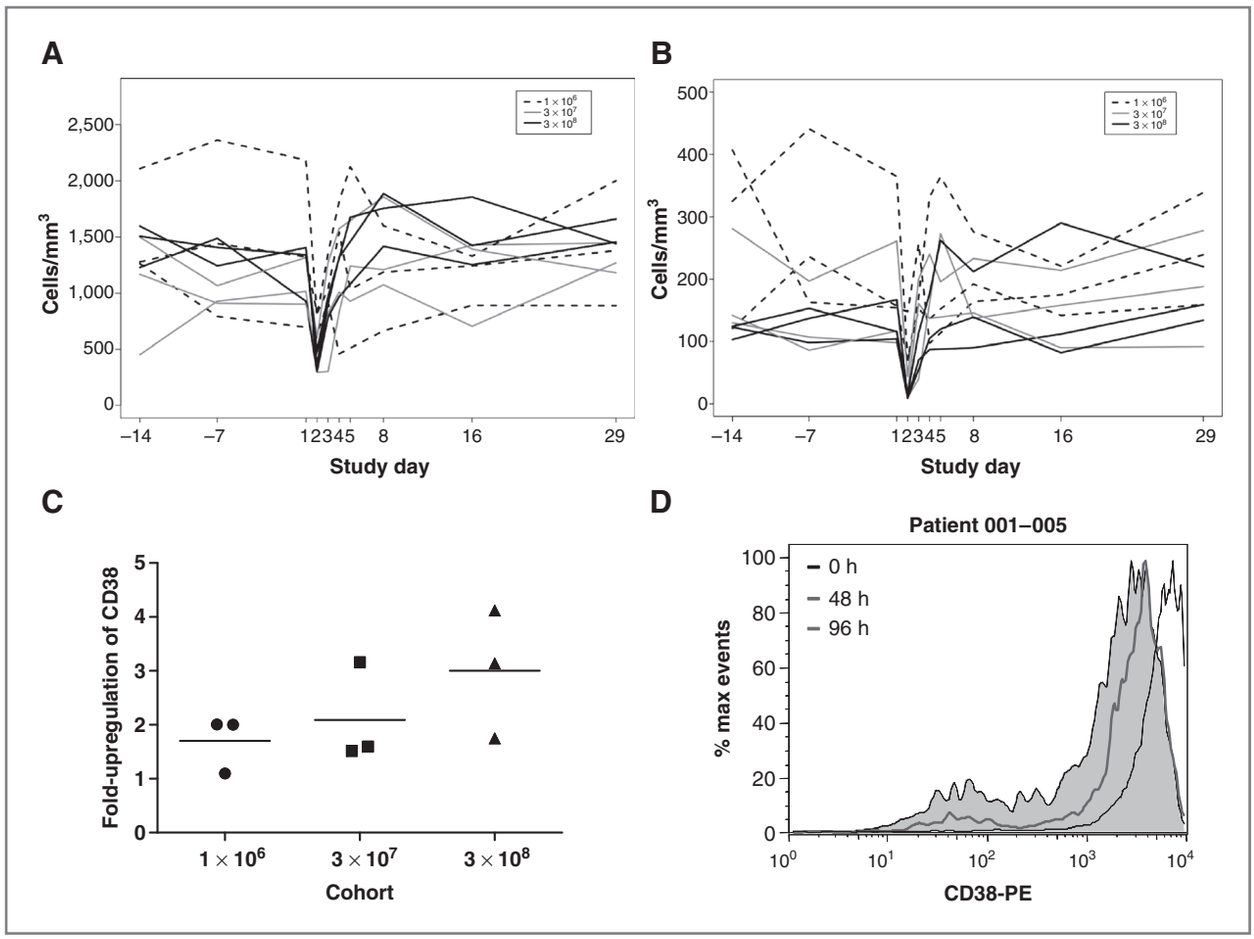

Figure 1. ANZ-100 administration results in lymphocyte and NK cell declines in the peripheral blood suggesting margination into tissue and induces NK cell activation as evidenced by CD38 expression on $CD3^-CD16/56^+$ cells. A, measurement of lymphocytes in the peripheral blood shows transient lymphocyte declines following ANZ-100 administration. B, measurement of NK cells in the peripheral blood shows transient NK cell declines following ANZ-100 administration. Legend refers to cohort dose level. C, CD38 expression on NK cells by cohort dose level. Ratio of peak CD38 MESF value between 48 and 96 hours postdosing and predose value. A significant upregulation of CD38 was noted for all dose levels ($P = 0.0008$). There appears to be a slight dose-dependent trend but it was not statistically significant ($P = 0.1238$). D, histogram showing CD38 expression on NK cells after ANZ-100 administration in subjects 001–005. PE, phycoerythrin.

IL-6 ($P = 0.059$), and TNF-α ($P = 0.092$) are higher in the 1×10^9 cfu dose level than in the 3×10^8 dose level (Fig. 3D). There was no significant difference in the induction levels for IFN-γ ($P = 0.375$) or IL-8 ($P = 0.171$). The data for the single subject with a dose of 1×10^{10} cfu are included in the plot for reference.

Detection of LLO-specific and mesothelin-specific T-cell responses after CRS-207 administration

LLO-specific T-cell responses were analyzed in 8 subjects with viable samples pretreatment and after the second and fourth infusion of CRS-207 (Fig. 4A). Final T-cell responses are reported. Six of the subjects were positive for vaccine-induced *Lm*-specific responses. The CEF-specific responses are provided in Supplementary Fig. S3. In the CRS-207 study, mesothelin-specific $CD8^+$ T-cell responses were induced in 6 of the 10 evaluable subjects (Fig. 4B).

Efficacy and survival

While the CRS-207 study enrolled subjects with multiple disease types and was not powered to assess survival, 37% of this phase I patient population survived for ≥15 months, with 3 subjects alive as of October 14, 2010 (Table 2). Of the 6 long-term survivors, 3 had PDA, 2 had NSCLC, and 1 had mesothelioma. These 6 subjects had prior immunotherapy or subsequent local radiation. Five of 6 subjects received all 4 doses of CRS-207 and all 5 evaluable subjects showed vaccine-induced *Lm*-specific responses. One subject had been discontinued from study after 1 dose because of a protocol violation and samples were not collected for immunologic evaluation. In addition, 4 of the 5 evaluable subjects among the long-term survivors had stable disease by RECIST at day 91 (end of study). Eight of 8 evaluable subjects in the group that survived <15 months had progressive disease by day 91. All 5 evaluable subjects who lived

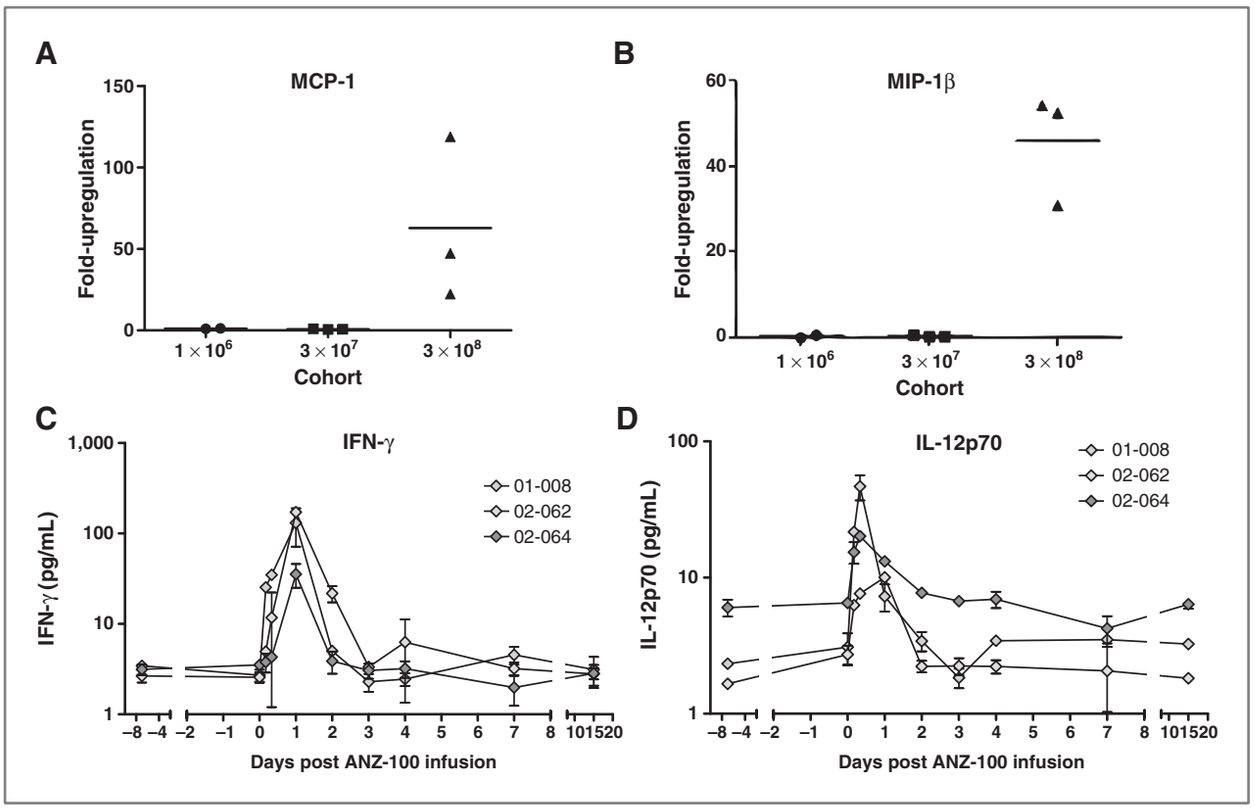

Figure 2. ANZ-100 induction of chemokines/cytokines is dose dependent and favors the induction of Th1 cytokines. A, MCP-1 chemokine expression presented by cohort dose level. Ratio calculated from peak chemokine value 2 hours postdose compared with mean chemokine value of 2 predose measurements. At the highest dose level of 3×10^8 cfu, a significant induction of MCP-1 was observed ($P = 0.0006$). B, MIP-1β chemokine expression presented by cohort dose level. Ratio calculated from peak chemokine value 2 hours postdose compared with mean chemokine value of 2 predose measurements. At the highest dose level of 3×10^8 cfu, a significant induction of MIP-1β was observed ($P = 0.0002$). C, IFN-γ cytokine expression after ANZ-100 administration in subjects at the 3×10^8 dose level. D, IL-12p70 cytokine expression after ANZ-100 administration in subjects at the 3×10^8 dose level.

≥15 months developed LLO responses. One additional subject lived ≥15 months but was not tested because a posttreatment sample was not collected. Thus, the induction of LLO-specific T-cell responses may serve as a biomarker of immunocompetency in future studies. In this small subset of 10 subjects with multiple histologic types of cancer, the induction of mesothelin-specific responses did not correlate with survival. The induction of mesothelin-specific T-cell responses as a marker of response to CRS-207 requires further investigation in a larger study of more homogenous subjects. These data provide the rationale for further evaluation of this *Lm*-mesothelin vaccine in a phase II study.

Discussion

These data from the phase I studies of ANZ-100 and CRS-207 *Lm* vaccines support the following conclusions. First, both vaccines are safe and tolerable in subjects with advanced, treatment-refractory cancers at immune activating doses. Second, there is a dose-dependent augmentation of systemic cytokine and chemokine responses that may serve as biomarkers of *Lm* vaccine bioactivity. Finally, a tumor antigen–modified *Lm* can induce tumor antigen–specific T-cell responses in subjects with advanced cancer. As such, *Lm* vaccine responses require further evaluation as a candidate biomarker of improved clinical outcomes.

These studies support that an attenuated bacteria can be given safely to subjects with advanced cancer with transient side effects. This is in marked contrast to many conventional options in which the toxicities can be cumulative and impairment in quality of life have to be weighed against potential benefit. Defining the tolerability of these constructs as single agents lays important groundwork for future studies in which these vaccines will be used in combinations. There are a number of unpublished preclinical studies testing *Lm* vaccines in combination with either other vaccine constructs or immune-modifying agents which show enhanced efficacy of the combination. A clinical trial has recently opened to enrollment testing CRS-207 in combination with an allogeneic GM-CSF–secreting PDA vaccine in subjects with advanced PDA. The study concept is based on mouse models which show that the combination of the GM-CSF and *Lm*-based vaccines in a heterologous prime/boost regimen results in the induction of antigen-specific T-cell responses of greater magnitude than either

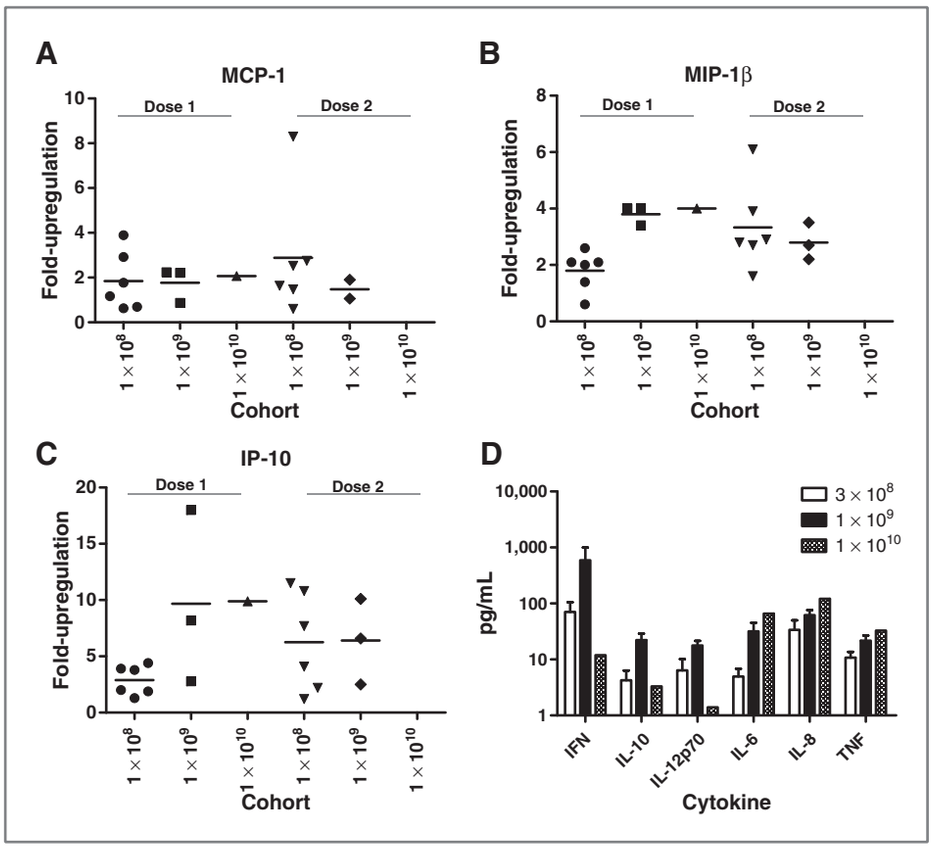

Figure 3. CRS-207 induction of chemokines/cytokines is present at all dose levels tested. A, MCP-1 chemokine expression presented by cohort dose level after dose 1 and 2. B, MIP-1>β chemokine expression presented by cohort dose level after dose 1 and 2. C, IPhemokine expression presented by cohort dose level after dose 1 and 2. D, cytokine expression 24 hours after first dose of CRS- resented by cohort dose level. The data for the single subject with a dose of 1×10^{10} cfu are included in the plot for reference.<

agent alone and correlates with superior antitumor responses. Interestingly, all 3 PDA subjects on the CRS-207 study who lived ≥15 months had received prior GM-CSF vaccine therapy.

With both biologic and targeted agents, dose selection can be complex as the usual drug development philosophy of using the MTD may not be relevant. The maximum dose may not be the most biologically effective dose. There does

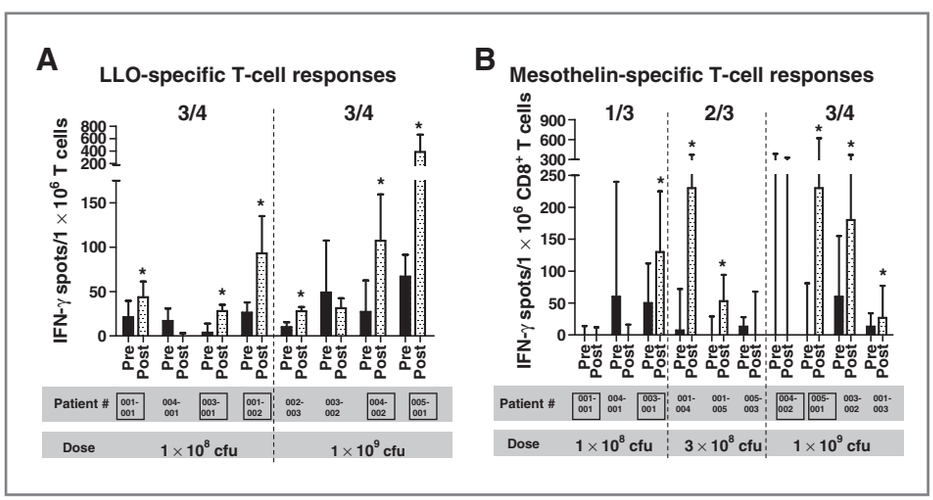

Figure 4. CRS-207 induces both LLO-specific and mesothelin-specific T-cell responses. A, LLO-specific T-cell responses were analyzed using IFN-γ ELISPOT. B, mesothelin-specific T-cell responses were analyzed using IFN-γ ELISPOT. *, T-cell responses were considered positive when the frequency of specific responses were ≥1 in 10^5 PBMCs (LLO ELISPOT) or $CD8^+$ PBL (mesothelin ELISPOT) and increased by at least 2-fold compared with baseline. Final T-cell responses are reported. The maximal response to a single best peptide is reported. The boxed patient identification numbers represent subjects who survived ≥15 months.

Table 2. CRS-207 clinical and immune response summary

ID	Cancer	Dose level	LLO response	Mesothelin response	RECIST	Survival, mo	Prior immunotherapy
≥15 mo OS							
001-001	PDA	10^8	Yes	No	PD	15	GVAX
001-002	PDA	10^8	Yes	NE	SD	30+	GVAX
003-001	Mesothelioma	10^8	Yes	Yes	SD	29+	IFN-β gene transfer[a]
005-002	NSCLC	3×10^8	NE	NE	NE	23	No
004-002	PDA	10^9	Yes	No	SD	17	GVAX
005-001	NSCLC	10^9	Yes	Yes	SD	26+	No
<15 mo OS							
002-001	Ovarian	10^8	NE	NE	PD	NE[b]	No
002-002	Mesothelioma	10^8	NE	NE	PD	4	No
004-001	Mesothelioma	10^8	No	No	PD	5	No
001-004	PDA	3×10^8	NE	Yes	PD	3	No
001-005	PDA	3×10^8	NE	Yes	NE	3	No
005-003	PDA	3×10^8	NE	No	NE	7	No
001-003	PDA	10^9	NE	Yes	PD	7	No
002-003	NSCLC	10^9	Yes	NE	PD	5	No
002-004	Mesothelioma	10^9	NE	NE	PD	1	No
003-002	Ovarian	10^9	No	Yes	PD	4	IFN-β gene transfer[a]
003-003	Mesothelioma	10^{10}	NE	NE	NE	5	No

Abbreviations: GVAX (GM-CSF–based whole-cell vaccine); NE, not evaluable; +, subjects alive as of October 14, 2010. Survival reported as of October 14, 2010.

[a]Adenovirus-mediated IFN-β gene transfer.

[b]Censored because of subject withdrawal of consent.

appear to be a dose-dependent augmentation of cytokine and chemokine responses. However, it remains unknown whether there is a dose-dependent induction of T-cell responses. Importantly, these studies show not only safety but also immune activity in the range of doses selected for testing. Of note, a phase I study of a different *Lm*-based vaccine has been reported (28). This study evaluated *Lm*-LLO-E7, a live-attenuated *Lm* that secretes the HPV-E16 E7 antigen fused to LLO, in subjects with previously treated cervical carcinoma and reported a similar adverse event profile and similar dose range for the MTD.

The transient transaminitis cases were expected on the basis of mechanism of action and preclinical studies. Another example of transaminitis in the context of immunotherapy is the flares in chronic hepatitis B patients induced by Peg-IFN-α-2b (29). Interestingly, host-induced flares which were followed by HBV DNA decreases were highly associated with response. These flares are thought to be due to the stimulatory effect of IFN, which is capable of increasing T-cell cytolytic activity and NK cell function. Likewise, with *Lm*-based therapies, the transaminitis is likely to be inflammatory in nature and not necessarily a negative finding. This will be monitored in future studies.

With the recent approval of Provenge for the treatment of metastatic castrate-resistant prostate cancer, there is mixed enthusiasm and continued skepticism about vaccination as a treatment for cancer. Provenge has been shown to prolong survival without evidence of appreciable RECIST response or prolongation of time to progression (30). In addition, another recently approved immunotherapy, ipilimumab, an antagonist antibody to cytotoxic T-lymphocyte–associated-4 (CTLA-4), has also shown a survival benefit in melanoma despite 5.7% to 10.9% response rates (31). In these studies, some subjects show increases in tumor volume before a delayed response and therefore response rate is likely to underestimate the activity of these agents. Therefore, OS is currently the best endpoint to evaluate immunotherapeutic agents in advanced cancer. While the survival data presented here are only hypothesis generating, it is provocative.

Conclusions

In summary, *Lm*-based vaccines, ANZ-100 and CRS-207, are well tolerated in subjects with advanced cancers. There is encouraging evidence of immune activation and potential clinical benefit, thus warranting further clinical studies.

Disclosure of Potential Conflicts of Interest

Under licensing agreements between Aduro BioTech, Inc. (Aduro) and the JHU, E.M. Jaffee and JHU have the potential to receive royalties received on sales of products/technology described in this article. The terms of this arrangement are being managed by JHU in accordance with its conflict of interest policies. D.G. Brockstedt, A.L. Murphy, and T.W. Dubensky have ownership interests in Aduro. T.W. Dubensky is a member of the scientific advisory board of Aduro. J.E. Eiden has ownership interests in Cerus

Corporation. P. Illei is a consultant for Leica Microsytem. No potential conflicts of interests were disclosed by other authors.

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