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Clinical Development of *Listeria monocytogenes*–Based Immunotherapies

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Abstract

Active immunotherapy targeting dendritic cells (DCs) has shown great promise in preclinical models and in human clinical trials for the treatment of malignant disease. Sipuleucel-T (Provenge, Dendreon, Seattle, WA), which consists of antigen-loaded dendritic cells (DCs), recently became the first targeted therapeutic cancer vaccine to be approved by the US Food and Drug Administration (FDA). However, ex vivo therapies such as Provenge have practical limitations and elicit an immune response with limited scope. By contrast, live-attenuated *Listeria monocytogenes* (Lm) naturally targets DCs in vivo and stimulates both innate and adaptive cellular immunity. Lm-based vaccines engineered to express cancer antigens have demonstrated striking efficacy in several animal models and have resulted in encouraging anecdotal survival benefit in early human clinical trials. Two different Lm-based vaccine platforms have advanced into phase II clinical trials in cervical and pancreatic cancer. Future Lm-based clinical vaccine candidates are expected to feature polyvalent antigen expression and to be used in combination with other immunotherapies or conventional therapies such as radiotherapy and chemotherapy to augment efficacy.

The rationale for an immunologically based approach for the treatment of cancer rests on the supposition that immune effectors can be induced and directed toward antigens that are preferentially expressed or over-expressed by tumor cells, subsequently leading to their destruction. This premise was recently validated by sipuleucel-T (Provenge, Dendreon, Seattle, WA), which is composed of an enriched population of autologous dendritic cells (DCs) loaded ex vivo with a recombinant fusion protein of prostatic acid phosphatase (PAP) linked to granulocyte-macrophage colony-stimulating factor (GM-CSF). Provenge received approval by the US Food and Drug Administration (FDA) last year based on a controlled phase III clinical trial showing a 4.1-month survival benefit in patients with hormone-refractory prostate cancer. DC-targeting is a desirable mechanism of action of active immunotherapies because DCs activate tumor-specific cytotoxic T lymphocytes (CTLs), which in turn have been shown to be particularly effective in lysing tumor cells.[1,2] CTLs that recognize tumor cells have been isolated from patients with melanoma, breast, ovarian, renal, lung, and colorectal cancers.[3,4] In many instances, CTLs identified have exhibited specificity for oncogenic or normal proteins over-expressed in tumor cells; examples include the MAGE family, Her-*2neu,* MART-1, p53, *ras* proteins, mesothelin, and

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carcinoembryonic antigen, as well as PAP.[5–9] The presence of antigen-specific CTLs among these malignancies demonstrates unequivocally that an immune response to tumors can occur in individuals with cancer and indicates that enhancement of such responses would provide clinical benefit.

As the characterization of the origin of tumor antigens has increased, it has become apparent that many of the antigens defined with tumor-reactive T cells are products of non-mutated native genes with tissue-specific expression. The lesson that emerges is that the anti-tumor response is directed, for reasons that are as yet unclear, against normal tissue antigens to which the individual is normally tolerant. This has led to the concept that effective immunotherapy is not only directed against tumor-specific targets but also can be directed to gene products expressed by normal cells in the parent tissue. The induction of effective tumor-specific immunity relies on the optimal choice and combination of two independent elements: the target antigen(s) and the adjuvant or vaccine vector for delivery of the antigen(s) into the appropriate DC subset. This review will discuss specifically the development of a novel vaccine technology based on the intracellular bacterium *Listeria monocytogenes* (Lm). Lm-based vaccines have been shown by several groups to directly target DCs in vivo following immunization and to induce robust innate and adaptive immunity. The selection of optimal target antigens is beyond the scope of this review and has been extensively reviewed by others.[8,10]

BACTERIAL-BASED VACCINE VECTORS

The immunostimulatory properties of live bacteria or bacterial components have been recognized as potential cancer therapies since the early 20th century. Work pioneered by Dr William Coley demonstrated that live organisms or bacterial extracts from the bacteria *Streptococcus pyogenes* and *Serratia marscencens* had potent anti-tumor activities in cancer patients. To date, several FDA-approved products are based on live-attenuated or killed bacteria, including *Salmonella typhi* Ty21a for typhoid fever, *Vibrio cholerae* CVD 103-HgR for cholera, and *Mycobacterium bovis* BCG for tuberculosis and bladder cancer.[11] Although the therapeutic potential of bacterial-based vaccines was realized very early, clinical development of the majority these technologies lagged significantly behind other biologics. The full potential of bacterial-based therapeutics only started to be exploited with the advances made over the last 20 years in molecular biology that helped to overcome technical barriers related to manipulating bacterial genomes for safety and antigen expression. Furthermore, the increased understanding of host–pathogen interactions and the role of innate immune recognition of bacterial molecular patterns and how this relates to the induction of robust adaptive immunity has facilitated the development of more effective bacterial-based immunotherapies. Several bacterial-based vaccine vectors are in development, including bacillus Calmette-Guerin (BCG; an attenuated form of the bacterium *Mycobacterium bovis*), *Escherichia coli, Listeria monocytogenes* (Lm), *Salmonella, Shigella*, and *Yersinia.* These bacterial vectors have demonstrated activity in preclinical tumor models and some have been evaluated for safety and immunogenicity in early-stage clinical trials. The clinical development of Lm-based therapeutic vaccines is discussed below.

Listeria monocytogenes—Potent Stimulator of Innate and Adaptive Immunity

Effective priming of cell-mediated immunity depends on integration of three signals that must be provided by the appropriate antigen-presenting cell: (1) antigen, (2) costimulation, and (3) transient inflammation.[12] The quality and quantity of these three signals orchestrate priming of $CD4^+$ and $CD8^+$ T cells, differentiation into effector and memory cells, and an ability to protect against pathogen challenge or recognize and kill malignant cells within the host.

The ability of Lm to effectively stimulate robust, multi-functional, cell-mediated (adaptive) immunity is based primarily on its intracellular lifecycle[13,14] and ability to target DCs in vivo (Figure 1).[15,16] The critical importance of DCs to prime naïve T cells is well established. However, several subsets of DCs that have been defined in the mouse and the individual contribution of each subset to the induction of antigen-specific immunity have not been fully elucidated. There are two major subsets of DCs in mice that can be distinguished by the expression of CD11c: conventional DCs that express high levels of CD11c and plasmacytoid DCs (pDCs) that lack CD11c cell surface expression. The presence of CD8α and CD4 further defines subsets of the conventional DCs. CD8α^+ DCs are necessary and sufficient to prime an adaptive immune response induced by Lm[17] and highly susceptible to Lm infection, whereas pDCs are not infected in vivo.[18,19] This finding was confirmed by intravital microscopy studies demonstrating that blood-borne Lm rapidly associated with DCs in the subcapsular red pulp.[20] Within the first few minutes following intravenous administration, Lm was found inside of spleen DCs or associated with their cell surfaces, resulting in a significant decrease in bacterial mobility. Very few live bacteria were associated with neutrophils or monocytes.

Upon encounter with host DCs, Lm triggers several pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) on the bacterium. Three main families of PRRs have been described to date: toll-like receptors (TLRs), the NOD-like receptors (NLRs), and Stimulator of Interferon Gene (STING). Lm-derived components such as peptidoglycan, bacterial nucleic acids, and flagellin signal through host cell TLRs, culminating in the activation of transcription factors such as nuclear factor-κB (NF-κB), mitogen-activated protein (MAP) kinase, and Jun kinase, and hence, the transcription of a plethora of inflammation-associated genes.[21,22] After phagocytosis, Lm is able to escape the phagolysosome and make its way into the cytosol, primarily by secretion of the pore-forming hemolysin listeriolysin O (LLO).[23] In response to bacterial vacuolar escape, host intracellular PAMP sensors recognize bacterial products in the cytosol, leading to activation of the NK-κB and IRF3 pathways.[24] NK-κB signaling results in secretion of proinflammatory cytokines and IRF3 (together with IRF7) leads to production of interferon-beta (IFN-β), initiating a powerful immuno-stimulatory cascade.[25] The intracellular localization and triggering of cytosolic NLRs are critical to the induction of a robust cell-mediated adaptive immune response, supported by the observation that phagolysosome-confined Lm mutants fail to induce a functional T-cell response.[14,26] Prolonged presence of the bacteria within the phagolysosomal compartment induced the expression of interleukin (IL)-10 through a MyD88-dependent signaling pathway. The inability of phagolysosome-confined Lm to provide functional and protective immune responses can be reversed by IL-10 in vivo blockade.

Wild-type as well as designated attenuated strains of Lm induce robust CD4$^+$ and CD8$^+$ T-cell immunity. Upon infection of host antigen-presenting cells, the majority of the bacteria are subsequently degraded, and processed Lm antigens are presented on the surface of the antigen presenting cell via the class II endosomal pathway.[27,28] This cascade results in subsequent presentation of Lm-related proteins on class I major histo-compatibility complex (MHC) via direct presentation following direct infection of DCs or cross-presentation as shown by Reinicke et al,[29] resulting in CD8$^+$ T cell activation. Efficient targeting of both class II and class I MHC processing pathways augments the induction of a robust and multi-functional T-cell memory response. This simultaneous targeting of both class I and class II MHC processing pathways is not unique to native Lm proteins but extends to recombinant strains engineered to express tumor- or infectious disease-related antigens.[30–36] The induced T-cell response specific for the encoded antigen also can result in a broader tumor-specific immune response through epitope spreading.[37,38]

Lm-BASED IMMUNOTHERAPIES

The striking potency of Lm-based immunotherapies has been demonstrated in multiple animal models of cancer and infectious disease.[30,31,34,37,39–43] In addition to its potency, Lm offers a number of features favorable for tumor immunotherapy: (1) the ease of strain engineering that allows for the expression of multiple tumor antigens[44]; (2) Lm is not neutralized by an antibody response and is immunogenic upon repeat administration[45–47]; (3) antibiotic sensitivity, an important consideration in terms of patient safety; and (4) cost-effective and straightforward manufacturing. Despite these remarkable features, Lm remains a food-borne pathogen. While the incidence of human infection is only about five cases per million in the United States, acute infection (known as listeriosis) has a high mortality rate of 20% to 30%,[48–51] and cancer patients are particularly susceptible.[51] For successful clinical application in a therapeutic setting, it is essential to develop a Lm platform vaccine strain that is safe, yet retains the potency of vaccines based on wild-type bacteria.[52] The strategies currently under development to attenuate the pathogenicity of the wild-type organism can be organized into four categories: (1) deletion of virulence genes affecting bacterial growth and cell-to-cell spread in vivo; (2) aberrant expression of PrfA, the master transcriptional activator of Lm virulence genes; (3) engineering of auxotrophic mutant strains with limited capacity for intracellular growth; and (4) inactivation. The goal of these strategies is to segregate the immunogenicity from the toxicity of wild-type Lm.

1. Deletion of Virulence Genes

Because Lm has been studied and characterized for decades, it is not surprising that multiple virulence genes have been identified. ActA mediates the polymerization of host cell actin at the pole of cytosolic bacterium, resulting in motility and invagination of neighboring cells through formation of cell protrusions known as listeriopods.[23,53] More than a decade ago, *actA* deletion mutants (Lm Δ*actA*) were shown to be attenuated 1,000-fold in mice—determined by the median lethal dose—but retained the ability to elicit protective immunity.[54] The safety of Lm Δ*actA* was enhanced by deletion of *plcB* (Lm Δ*actA*/Δ*plcB*), a gene encoding one of the two bacterial phospholipases C, and required for efficient release from the secondary vacuole.[55] Lm Δ*actA*/Δ*plcB* bacteria are absolutely deficient in cell-to-cell spread, and have a toxicity-immunogenicity profile that is similar to Lm Δ*actA*-based vaccine strains. Notably, Lm Δ*actA*/Δ*plcB* has been evaluated in human clinical studies given via the oral route (Table 1). While both Lm Δ*actA* and Lm Δ*actA*/Δ*plcB* are significantly attenuated in mouse virulence compared to wild-type Lm, the overall tissue tropism of both strains is likely not affected (particularly after intravenous or intraperitoneal administration), which may lead to toxicities that could impede clinical development.

The multi-gene family of proteins known as internalins has been shown to contribute to the broad tropism of the bacterium by mediating invasion of particular cell types. Internalin B (InlB), for example, mediates entry into host cells that express the hepatocyte growth factor receptor (c-Met).[23,56] The combined de letions of *actA* and *inlB* (Lm Δ*actA*/Δ*inlB*) largely blocked growth in hepatocytes, either by direct infection or through cell-to-cell spread from adjacently infected Kupffer cells.[31] Aduro BioTech, Inc (Berkeley, CA) developed the Lm Δ*actA*/Δ*inlB* vaccine platform strain that has been the basis for three FDA-approved phase I clinical studies and more recently one FDA-approved phase II clinical study for patients with metastatic pancreas cancer (Table 1; see section below).

2. Aberrant PrfA Expression

Another approach to attenuate wild-type Lm pathogenicity was developed by Paterson and colleagues and utilizes aberrant expression of PrfA.[41] PrfA is a transcription factor activated intracellularly that acts as a central virulence regulator and PrfA knock-out strains are

completely avirulent.[57] The *prfA* gene has been deleted from the bacterial chromosome and has been complemented by its placement on a pAM401-based multi-copy plasmid, also encoding a designated heterologous antigen.[41] The potency of this vaccine platform has been evaluated extensively in preclinical studies and reduces virulence in mice by 3 to 4 logs.[58] However, the mechanism of the attenuation, is not completely understood, but it is likely due to aberrant PrfA expression—either its level or temporal pattern of expression compared to wild-type Lm—due to placement of the *prfA* gene on a plasmid. Moreover, because the full genetic complement of wild-type Lm is retained, the strain has the conceptual possibility of reversion to wild-type in the event of recombination of the plasmid with the host bacterial chromosome. A vaccine strain expressing HPV 16 E7, known as Lm-LLO-E7 (Lovaxin C, Advaxis, Princeton, NJ) and developed by Advaxis, has completed a phase I clinical study and is currently under investigation in two phase II clinical studies in women with cervical intraepithelial neoplasia (CIN) grade 2/3 or advanced cervical cancer due to HPV 16 (Table 1).

3. Auxotroph Mutant Strains

Auxotrophic strains lack the capability to synthesize a required metabolic enzyme and must be exogenously supplied with the missing metabolite in order to survive. A unique approach for deriving a highly attenuated Lm vaccine strain was developed by Frankel and colleagues, and is based on deletion of two genes, *dal* and *dat*, which are required for biosynthesis of D-alanine, a component of peptioglycan and lipoteichoic acid.[59] The vaccine strain, known as Lm Δ*dal*/Δ*dat*, was further engineered using either isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible or recombinase-sensitive expression of the *B. subtilis dal* race-mase to transiently and only partially complement the conditionally lethal phenotype.[60] The modified vector is attenuated in mice by 4 logs compared to wild-type Lm, and was shown to elicit strong functional mucosal cellular immunity in the vagina of mice when used as a component of a heterologous prime-boost vaccination regimen with an adenovirus vector.[61] Although not described in this work, the potential toxicity of the modified Lm Δ*dal*/Δ*dat* vector could be reduced further by targeted deletion of particular bacterial virulence genes that are not required for vaccine immunogenicity.

4. Inactivation

The above strategies to attenuate wild-type Lm are based on live bacteria. For prophylactic administration or for otherwise healthy patients with chronic disease, the safety profile of a killed vaccine may be preferable, but the well-known dichotomy in the mouse listeriosis model is that only live strains of Lm prime potent T-cell memory that protects mice against bacterial challenge.[62–64] One strategy to inactivate Lm-based vaccines with the intent to retain immunogenicity is γ-irradiation of the vaccine vector.[65] Mice vaccinated with γ-irradiated Lm elicited a Lm-specific immune response with partial protection against wild-type challenge (1–2 log reduction in bacterial load). Because the secondary expansion of $CD8^+$ T cells was minimal following multiple vaccinations, it appears that γ-irradiated Lm induce memory T cells of only partial functionality, similar to the phagolysosome-confined Lm Δ*hly* mutants. Aduro BioTech developed another approach to provide the safety of a killed vaccine with the potency of a live vaccine, known as “killed but metabolically active” (KBMA) vaccines. KBMA vaccines are based on Lm Δ*actA*/Δ*inlB* strains with defective nucleotide excision repair.[66,67] KBMA vaccines are exquisitely sensitive to photochemical inactivation by the combined treatment with psoralen, a DNA crosslinking reagent, and long-wave UV light. KBMA vaccines are unable to replicate, providing another level of attenuation but still elicit functional cellular immunity comparable to Lm Δ*actA*/Δ*inlB*.[67]

Lm-BASED IMMUNOTHERAPIES UNDERGOING CLINICAL EVALUATION

Once Lm strains that had been modified to decrease virulence while maintaining immunogenicity became available, the introduction into human hosts both healthy and cancer bearing became possible. The Lm platforms based on deletion of virulence genes and aberrant expression of PrfA have been evaluated in human clinical trials. The early clinical trials were intended to demonstrate safety and assess for signals of immune activation, but two trials also provided anecdotal evidence of efficacy. Current phase II clinical trials are intended to confirm safety and to formally evaluate efficacy. Completed and ongoing clinical trials with Lm in both cancer and infectious disease indications are described below and in Table 1.

BMB72 (Lm Δ*actA*/Δ*plcB* expressing influenza A nucleoprotein [NP]) and BMB54 (Lm Δ*actA*/Δ*inlB* expressing influenza A NP)

In 2002, Hohmann and colleagues reported on their experiences with the Lm Δ*actA*/Δ*plcB* parent vector (LH1169) in 20 healthy adult subjects who received single escalating oral doses of LH1169.[55] The oral doses tested ranged from 1×10^6 to 1×10^9 colony-forming units (cfu). Subjects were monitored for 14 days in the hospital and then as outpatients for an additional 6 weeks. There were no fevers or positive blood cultures and all but one subject cleared their stool cultures in 4 days or less. This subject, who was in the 1×10^6 cfu cohort, had an asymptomatic positive stool culture at 8 weeks that cleared after 3 days of antibiotics. Three subjects developed transaminitis <2.5× the upper limit of normal (ULN). The elevations occurred around study day 10 and resolved without intervention. One of these subjects was in the 1×10^7 cfu cohort and the other two subjects were in the 1×10^9 cfu cohort. In the 1×10^9 cfu cohort, two of the four subjects had a humoral response to Lm and one of these subjects also had cellular immune responses. The investigators concluded that attenuated Lm can be studied in adult subjects without serious long-term health sequelae.

More recently, in 2011, these investigators reported their experiences with two attenuated Lm vaccine vectors expressing influenza A NP.[68] These strains were evaluated in vitro, in mice, and in 22 healthy subjects. The subjects received single oral doses of either BMB72 (Lm Δ*actA*/Δ*plcB* NP) or BMB54 (Lm Δ*actA*/Δ*inlB* NP) and were then observed in the hospital for 7 days and as outpatients for an additional 6 weeks. The doses tested ranged from 1×10^8 to 1×10^{10} cfu. The vaccines were found to be safe without any serious or unexpected adverse events. Similar to the prior experience, there were no fevers, positive blood cultures, or prolonged shedding. Four of 12 subjects who received BMB72 had asymptomatic serum transaminase elevations <1.4× ULN. The transaminitis did not appear to be related to the dose level as the abnormalities occurred in the 1×10^8, 1×10^9, and 4×10^9 cfu cohorts but not the 1×10^{10} cfu cohort. Similar to the study of the parental strain, the peak occurred around day 7–10 in three of the subjects. Interestingly, there were no cases of transaminitis using orally administered BMB54, which was specifically altered by deleting *inlB* to decrease hepatocyte injury. Six of six subjects who received 4×10^9 cfu or more of either strain had detectable mucosal immune responses to Lm antigens but not to the influenza antigen. Half of the subjects had IFN-γ ELISpot responses to a complex listerial sonicate antigen but not to influenza NP. The authors concluded that similar to their prior study, mucosal responses to Lm were present at low levels at the highest doses administered. Systemic humoral responses to vector and NP were not detected. These investigators are now assessing whether alternate forms of delivery may improve immune responses. There is an ongoing study, testing BMB72 using transcutaneous immunization with native purified cholera toxin as a cutaneous adjuvant.

Lovaxin C (*ADXS11–001*, Lm-*LLO-E7*)

Lovaxin C is based on a platform that is attenuated by aberrant expression of PrfA and the antigen incorporated in this vector is the human papilloma virus (HPV) serotype 16 E7 oncoprotein. The intravenous route of administration has been tested in a completed phase I/II study and is being tested in ongoing phase II studies. The phase I/II target population for the first study of Lovaxin C was subjects with advanced cervical carcinoma. HPV 16 and 18 are the most prevalent sero-types in malignant lesions. Preclinical studies suggested that genetically fusing an antigen to a fragment of LLO enhances immunogenicity and results in improved efficacy against established tumors than expression of E7 alone.[33] The construct was studied in 15 subjects with previously treated, metastatic, refractory, or recurrent invasive carcinoma of the cervix.[69] Two intravenous doses given over 30 minutes were given 3 weeks apart. The dose levels tested were 1×10^9, 3.3×10^9, and 1×10^{10} cfu. Five subjects were treated at each dose level. Subjects were admitted inpatient for 5 days and received a course of ampicillin 5 days after each administration. Subjects were then followed for 3 months after the second dose. All patients experienced a flu-like syndrome including fevers, nausea/vomiting, chills, headache, tachycardia, and musculoskeletal pain. In most patients, these were of short duration, resolved within 12 hours of infusion and responded to symptomatic management. Two subjects were given antibiotics on day 3 instead of day 5 per protocol due to fevers present at 48 hours. Abnormal liver function tests of <4× ULN were observed on day 8 or later in two subjects in the 3.3×10^9 cfu cohort and one subject in the 1×10^{10} cfu cohort with the subsequent transaminase peaks being lower after the second dose. There were no dose-limiting toxicities (DLT) in the first two dose cohorts but three DLTs in the 1×10^{10} cfu cohort. All of these events were episodes of grade 2 diastolic hypotension that required therapeutic intervention. No shedding was detected in urine or feces and only one subject had a positive blood culture on the second day, which cleared on the third day after infusion. E7-specific T-cell responses were detected by ELISpot in one of three subjects tested. The median overall survival for the subjects was 347 days. Fifty-three percent of subjects had stable disease and 30.8% had reduction in tumor size, with no dose response effect. The investigators concluded that Lovaxin C is a safe therapeutic cancer vaccine in end-stage cervical cancer patients at doses of up to 3.3×10^9 cfu. Based on the safety conclusions of this trial and the interesting signals of activity, Advaxis, Inc has initiated a single-blind, placebo-controlled phase II study testing the safety of Lovaxin C for the treatment of CIN 2/3. CIN 2/3 lesions are high-risk but pre-invasive. Theoretically, better immune responses and clinical effect could be elicited in a healthier patient population with a minimal disease burden and fewer established immune tolerance mechanisms. The primary endpoint will be histologic determination of CIN 2/3 regression from baseline. Secondary endpoints include determination of changes in HPV DNA levels. Three intravenous infusions 28 days apart will be administered at one of three dose levels (5×10^7, 3.3×10^8, and 1×10^9 cfu). There is also a placebo arm. A second phase II study with Lovaxin C has been initiated for the treatment of persistent or recurrent squamous or non-squamous cell carcinoma of the cervix. The primary outcome measures are safety and the proportion of patients who survive for at least 12 months. These subjects will receive the same 28-day schedule for three courses.

ANZ-100 (A live-attenuated Lm strain [Lm Δ*actA*/Δ*inlB*])

ANZ-100 is an attenuated Lm that does not express a tumor antigen. 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.[37,40] NK cell depletion in vivo abro-grated the anti-tumor efficacy of ANZ-100 in a CT26 liver metastases model. Therefore, the innate immune response induced by ANZ-100 is thought to play a major role in the increase in survival of these mice. Furthermore, mice cured of their liver metastases resisted tumor rechallenge that was T-cell–

dependent, suggesting that adaptive immunity contributed to the anti-tumor immune response. A single intravenous dose of ANZ-100 was evaluated in a dose escalation study in adults with liver metastases refractory to standard therapy. Nine subjects received 1×10^6, 3×10^7, or 3×10^8 cfu. Subjects were observed in the hospital for 5 days and a 10-day course of oral amoxicillin was initiated 6 days following the subject's dose. A single infusion of ANZ-100 was well tolerated to the maximum planned dose. Adverse events included fevers and transient laboratory abnormalities such as lymphopenia. No DLTs were observed. Development of this construct is currently on hold in favor of allocating resources to the antigen expressing vaccine strains. However, this study has demonstrated that the most consistent induction of biologic activity, such as increased levels of proinflammatory cytokines and NK cell activation, occurs at the highest dose level tested in this protocol.

CRS-207 (Lm Δ*actA*/Δ*inlB*-*mesothelin*)

CRS-207 is an Lm Δ*actA*/Δ*inlB* strain engineered to express human mesothelin, a tumor-associated antigen present on normal mesothelial cells and highly expressed by many human tumor types, including pancreatic and ovarian cancer, mesothelioma, and non-small cell lung cancer. This expression profile, combined with limited expression on the surface of normal tissues, makes mesothelin an attractive target for active tumor-specific immunotherapy.[70] Support for mesothelin as a T-cell target comes from studies demonstrating a correlation between positive clinical outcomes and the induction of mesothelin-specific cellular immunity in subjects with pancreatic cancer following vaccination with an irradiated allogeneic whole cell vaccine encoding GM-CSF.[9] CRS-207 was evaluated in a dose escalation study in subjects with treatment-refractory tumors known to express mesothelin, including mesotheliomas, pancreatic adenocarcinomas, non-small cell lung cancers (NSCLC), and ovarian cancers.[71] Subjects were observed in an inpatient facility for 24 hours and received antibiotics 7 days after the last infusion. Seventeen subjects were enrolled into four dose cohorts ranging from 1×10^8 cfu to 1×10^{10} cfu. Up to four intravenous infusions of CRS-207 were well tolerated up to 1×10^9 cfu, the determined maximum tolerated dose (MTD). While this is an entirely different Lm-based vaccine strain, the flu-like or cytokine release symptoms and the dose range of tolerability appears similar to that found in the Lovaxin C study. Transient elevations of transaminase levels were also observed in this study but were not felt to be clinically relevant. A total of six subjects lived >15 months, and multiple biomarkers were evaluated for potential correlation (also see Next Steps for a discussion of the role of combination therapies). Mesothelin-specific $CD8^+$ T-cell responses were induced, although there was no correlation with survival. Interestingly, five of the six patients who lived >15 months mounted a LLO-specific T-cell response, indicating a potential biomarker of immune competency. Aduro BioTech has initiated a randomized, controlled phase II study of CRS-207 in patients with metastatic pancreatic cancer that includes extensive immune and disease monitoring. The study is a 90-patient, randomized, open-label study comparing the administration of CRS 207 following cyclophosphamide (CY) and a GM-CSF–secreting allogeneic pancreatic cancer cell vaccine against administration of CY/GM-CSF whole tumor cell vaccine alone in a 2:1 randomization (see section FUTURE COMBINATIONS).

NEXT STEPS

With completion of multiple phase I trials showing acceptable safety profiles and a variable degree of immune activation and clinical activity, several phase II studies are underway to confirm safety and assess for signs of clinical activity. An additional phase I study is also examining the transcutaneous route of administration in healthy subjects. Many questions remain unanswered with regard to antigen selection, dose selection, dose interval, number of doses, requirement for inpatient versus outpatient administration, appropriate patient

populations, need for antibiotic administration, immune monitoring methods, and optimal combination strategies.

In regard to dose selection as well as interval and number for intravenous administration, studies by both Advaxis and Aduro BioTech are eliminating the 1×10^{10} cfu dose levels. While the studies using the Lm Δ*actA*/Δ*inlB* constructs support a dose-dependent induction of chemokines and cytokines, the optimal dosing for induction of T-cell responses in human subjects is not yet apparent. Aduro BioTech will be testing the MTD of 1×10^{9} cfu at 3-week intervals for four doses in its phase II study. Advaxis is testing 5×10^{7}, 3.3×10^{8}, and 1×10^{9} cfu at 4-week intervals for three doses in its CIN study.

The collective experience with the use of Lm as an investigational agent in humans has established the administration of the attenuated bacteria to both healthy subjects and advanced cancer patients as a reasonably safe therapeutic and feasible option. The first studies with the oral administration of just a single dose to healthy subjects incorporated a 14-day hospitalization. This evolved now to the intravenous administration of multiple doses of Lm to advanced cancer patients with only a 24-hour stay. Current plans are to administer Lm entirely on an outpatient basis. Shedding was not detected in any of the studies of intravenous administration alleviating environmental concerns. Furthermore, in the studies of Lm Δ*actA*/Δ*inlB* constructs, antibiotics were only administered after the fourth dose of Lm or when the subjects came off study, which supports the preclinical data that hosts do not necessarily need antibiotics to clear the attenuated organisms. This preliminary safety data is making it possible for Advaxis to test Lovaxin C in relatively healthy women with CIN 2/3 lesions, a patient population that is more likely to benefit due to lower disease burden and more time to develop and effective immune response.

As with other immunotherapy trials, immune monitoring remains a challenge. Lm is expected to work through its capacity to elicit both innate and adaptive immunity. In addition, its activity is dependent on both $CD4^+$ and $CD8^+$ T cells. The translation of correlative immune responses from preclinical models with restricted HLA types and known immunodominant epitopes to heterogeneous patient populations is complex. Investigators testing the NP vaccine could not detect NP-specific responses. It is unknown if this is related to the route of administration, number of doses, pre-existing influenza immunity, or purely ineffective vaccine. Methods for detecting $CD4^+$ T-cell responses to these agents are not well established. In the CRS-207 study, methods using the isolation of subjects' peripheral blood mononuclear cells and overlapping mesothelin peptide libraries were not effective in measuring T-cell responses. Mesothelin-specific T-cell responses were not detected until methods using HLA-restricted T2 cells as antigen-presenting cells and $CD8^+$ T cells (isolated by negative selection) were used. Furthermore, the epitopes used may not have included epitopes relevant to a Lm-based vaccine as the epitopes were selected based on prediction methods and screening methods used from lymphocytes from subjects vaccinated with a GM-CSF–secreting pancreatic cancer cell vaccine. Performing comprehensive epitope mapping to mesothelin in the preclinical mouse model, we have observed differences in epitope recognition in mice vaccinated with Lm and an adenovirus encoding mesothelin (unpublished data). The preclinical data further support the notion that immunodominance might vary based on the vaccine platform used to elicit the response. In the Lovaxin C study, E7-specific responses were detected in only one subject but only a total of three were tested. Exploratory correlative work will continue to be an integral part of these early trials.

In addition, methods to optimize antigen expression and secretion from engineered Lm vaccine strains is an area of active development. Initial challenges in efficient expression have largely been overcome by using multiple strategies, including: (1) bacterial promoters

that are highly induced in Lm upon escaping into the cytosol of the infected APCs; (2) codon optimization; (3) fusion of the antigen to bacterial signal sequences; (4) removal of hydrophobic domains (although this strategy may eliminate some immunogenic epitopes); and (5) placement of antigen expression cassettes into distinct subgenomic regions within the Lm chromo-some. These advances in expressing single antigens can be applied to multiple antigens. Aduro BioTech has developed polyvalent strains that are currently undergoing preclinical testing in prostate cancer and melanoma models. Theoretically, targeting multiple antigens simultaneously may result in better results by decreasing the potential for immune escape and increasing the likelihood of targeting a relevant antigen.

FUTURE COMBINATIONS

For most cancer types, such as NSCLC and pancreatic cancer, any single agent vaccine, especially in the advanced disease setting, is unlikely to be of major therapeutic benefit. This is borne out in preclinical models more representative of tolerant tumors. Optimal combinations will likely have to induce a diverse repertoire of high avidity T cells. In addition to combinations with standard therapies such as radiation and chemotherapy, investigators have evaluated both heterologous prime-boost strategies and combining vaccines with antibodies targeting negative T-cell regulators such as CTLA-4 and PD-1/B7–H1. The theoretical reasons that heterologous prime-boost is preferential to homologous prime-boost include the reduction of anti-vector immune responses and the potential for inducing complementary arms of the immune system (B cell, T cell, NK cell, etc). Bot et al reported their findings that using a DNA vaccine as a prime resulted in low PD-1 expressing central memory T cells and boosting with a peptide vaccine resulted in expansion of tumor-specific T cells and their conversion to effector T cells equipped with broad migratory and functional capabilities.[72] In this proposed model, priming regimens should include limited antigen exposure and robust optimal costimulation resulting in high avidity T cells with memory recall features, restricted migration and refractory to negative regulatory mechanisms. The boosting regimen should include substantial exposure to antigen with facultative costimulation resulting in the expansion of those high avidity T cells with broad functionality and widespread migration patterns, yet more susceptible to negative regulatory mechanisms. Interestingly, while the precise mechanism has not been determined, Lm vaccines work very well in pre-clinical models as a boost to a number of vaccine platforms.[73–76] When Lm is used as a boost to DNA, protein, adenovirus, vaccinia, dendritic cell, or GMCSF–secreting whole cell vaccines, the vaccines work synergistically to enhance T-cell responses and anti-tumor responses in tumor challenge models including mesothelin-based models (unpublished data). As seen with other prime-boost models, the order of the vacci-nation administration is crucial. In fact, the CRS-207 randomized, controlled phase II study currently being conducted in patients with in pancreatic cancer is in combination with low-dose cyclophosphamide (CY) and GM-CSF–secreting allogeneic pancreatic cancer cell vaccine as a prime followed by CRS-207 as a boost. CY is thought to primarily inhibit regulatory T cells and the combination of CY with the whole cell vaccine approach has been shown to induce higher avidity T cells in a HER2/neu mouse model and in patients with pancreatic cancer. Interestingly, of the seven pancreatic cancer subjects enrolled on the CRS-207 study, three were previously treated with the GM-CSF whole cell vaccine and all three of these subjects were in the group that lived 15 months or longer.

An additional finding from that study that supports a combination strategy with Lm vaccines is that in the two subjects with NSCLC who lived 15 months or longer, received subsequent radiation. One of these patients had focal radiation to a symptomatic rib metastasis. This resulted in regression of all of her nonirradiated bony lesions and substantial declines in her CEA levels. Given these interesting survival findings and the unexpected generalized response, there is speculation that these findings are immune-mediated. Radiation may have

effects on antigen release, cross-presentation of tumor antigens or sensitization of tumor cells to T-cell–mediated killing by upregulating tumor-associated antigens, Fas, adhesion molecules, and apoptotic genes.[77,78]

Finally, as with many other vaccine platforms, combinations with immune regulation modifiers such as CTLA-4 antibody and antibodies targeted at the PD-1 pathway are of interest given their synergistic potential in preclinical models. The CTLA-4 antagonist antibody Yervoy (ipilimumab, BMS) is now commercially available for patients with melanoma and antibodies targeting the PD-1 pathway are showing signs of efficacy in early clinical testing. In vivo blockade of CTLA-4 during $CD8^+$ T-cell priming using Lm in mice led to an increased expansion and maintenance of the antigen-specific T-cell response without a negative impact on the overall T-cell repertoire.[79] Interestingly, CTLA-4–mediated increase in long-term $CD8^+$ T-cell memory required cell-extrinsic CTLA-4 blockade, but not direct blockade of CTLA-4 on the $CD8^+$ T memory cell itself. While the regulatory and study design issues combining agents in various stages of clinical development remain a challenge, the simultaneous development of vaccine combinations aimed at priming and expanding high avidity tumor-specific T cells and antibodies targeting immune checkpoints may ultimately provide optimal efficacy. Other vaccine platforms such as peptide and whole cell vaccines are already being studied with immune regulation modifiers. As more experience is gained with Lm, an additional avenue of clinical development would be with this class of agents.

The road to development of tumor immunotherapy has multiple barriers. However, in the light of the most recent approvals for the prostate cancer vaccine, Provenge, and the melanoma immunotherapeutic, Yervoy, there is renewed enthusiasm for the therapeutic potential of the immune system. Now that several firstin-human studies of Lm constructs have been completed, the initial concerns about patient safety and feasibility of agent administration have been tempered. The potential for Lm to be used in a clinical setting will need to be tested through a series of rationally designed, scientifically driven clinical trials.

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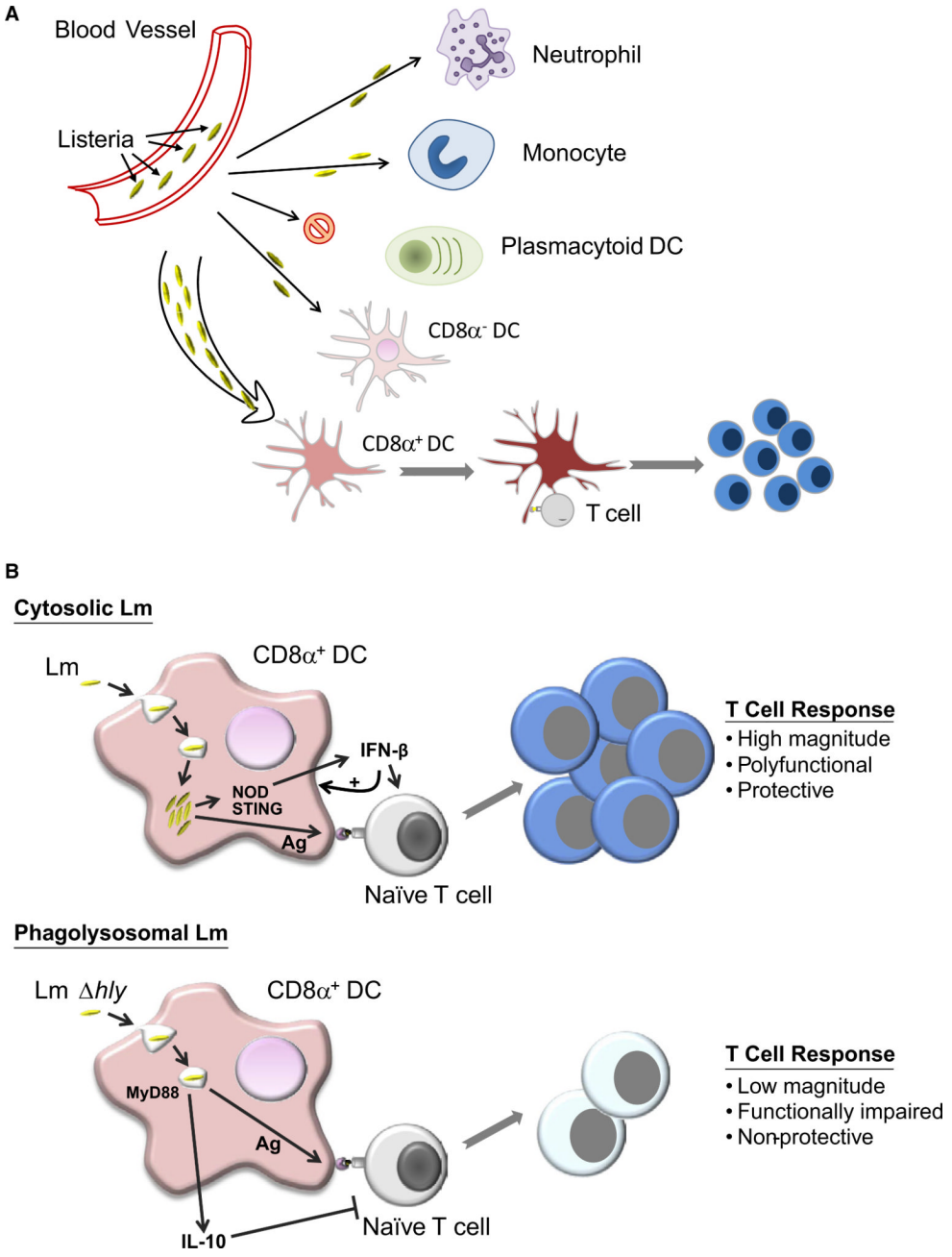

Figure 1.

Listeria monocytogenes (Lm)-based vaccines induce robust innate and adaptive immunity. Two key attributes contribute to Lm's potency: the ability to target CD8α^+ DCs in vivo, and the intracellular localization and triggering of cytosolic NLRs and STING host cell PAMP sensors. (A) Upon intravenous administration the majority of live Lm are found within CD8α^+ DCs that have been shown to be critical for the induction of antigen-specific T-cell responses in vivo. (B) The quality and magnitude of the induced T-cell response depends on the subcellular localization of Lm upon infection of the host antigen-presenting cell (APC). Lm strains that are able to escape from the phagolysosome into the cytosol of the APC (cytosolic Lm) induce robust T-cell immunity of high quality. Lm strains that remain in the

phagolysosome fail to induce protective T-cell immunity, partly due to the MyD88-dependent induction of IL-10.

Table 1

Clinical Evaluation of Lm-Based Immunotherapies

Strategy	Description	Indication	Clinical Status	References
Deletion of virulence genes	Δ*actA*/Δ*plcB* NP (BMB72) attenuated Lm administered orally	Healthy volunteers	Phase I	55,68
	Δ*actA*/Δ*inlB* NP (BMB54) attenuated Lm administered orally	Healthy volunteers	Phase I	68
	Δ*actA*/Δ*inlB* (ANZ-100); administered intravenously	Subjects with metastatic disease to the liver	Phase I	
	Δ*actA*/Δ*inlB* mesothelin (CRS-207); administered intravenously	Subjects with cancer to the ovary and pancreas, non-small cell lung cancer and mesothelioma	Phase I	
		Subjects with metastatic pancreatic cancer	Phase II (ongoing)	
Aberrant expression of PrfA	Lm-LLO-E7 (ADXS11-001; Lovaxin C); administered intravenously	Subjects with advanced cervical carcinoma	Phase I	80,81
		Subjects with cervical intraepithelial neoplasia grade 2/3	Phase II (ongoing)	
		Subjects with recurrent squamous or non-squamous cell carcinoma of the cervix	Phase II (ongoing)	