The Potential of ADXS-NEO: *Leveraging Listeria Monocytogenes as a Vector for Personalized Cancer Immunotherapy*

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Introduction

Over the last several years, the field of immuno-oncology has rapidly evolved to become one of the most exciting and promising areas of cancer research. The approval of the cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed cell death protein 1(PD-1)/PD-1 ligand 1 (PD-L1) checkpoint inhibitors has garnered extensive press because of the extraordinary effectiveness of these agents in select solid tumors. Recent data suggest that the majority of patients who benefit from immune checkpoint inhibitors have pre-existing T cells capable of targeting the unique mutations of their cancer, or neoepitopes (1). Unfortunately, not every patient has existing T-cell responses against his or her neoepitopes, and therefore, long-term clinical benefit from CTLA-4 and PD-1/L1 inhibitors may be restricted to individual patients with specific cancers that harbor a higher mutational burden. Mutations that occur in DNA within protein-coding regions cause cells to generate altered proteins that may not function correctly. The build up of these mutations and the resulting dysfunctional proteins can result in the cell becoming cancerous. These same mutations can also create a cancer-cell specific "handle," or neoepitope signature. The immune system can then recognize and target tumor-specific antigens without hindrance from central immune tolerance or harm to normal cells. The future of more-effective cancer immunotherapies may depend on the development of personalized treatments that generate and enhance a patient's immune response against these neoepitopes, which are unique to each individual patient's tumor.

The Problem of Tolerance

Previous attempts to vaccinate patients against tumor-associated targets have focused on naturally occurring tumor antigens that are inappropriately expressed or over-expressed, such as cancer/testis antigens or human epidermal growth factor receptor 2 (HER2), respectively. The challenge with these vaccinations is that they must overcome the central tolerance of the immune system, which eliminates any T-cell clones that might bind to naturally occurring peptides with high strength, thus preventing autoimmune attack on normal tissues. Actually, the best way to overcome central tolerance may be to avoid it altogether. The neoepitope mutations on cancers are completely foreign to the immune system, and highly reactive T-cell clones against them should not be deleted by central tolerance. Therefore, immunizing against these neoepitopes could be much more effective than immunizing against nonmutated targets.

Technological advances in genome sequencing have opened the door to identification of potential neoepitopes in an individual patient's cancer that may be used as targets for immune activation, effectively making personalized neoepitope-targeted cancer immunotherapies a realistic possibility in the near future. Several different methods are being pursued to achieve this ambitious goal, including whole peptide, dendritic cell, viral-based, and attenuated bacterial immunotherapies. The use of attenuated *Listeria monocytogenes* (*Lm*) transformed with specific plasmids encapsulating all, or a special subset, of a patient's immunogenic cancer neoepitopes (*Lm* Technology™) offers several advantages when compared with other methods under active investigation. This approach, called MINE™ (My Immunotherapy Neo-Epitopes), has resulted in development of ADXS-NEO, which has the potential to be a highly effective, practical, combinable, and affordable individualized neoepitope-based cancer immunotherapy.

Mutational Burden

Recently, the concept of mutational burden has become an important consideration in evaluating which patients might benefit the most from immune checkpoint inhibitor treatment. CTLA-4– and PD-1/L1–blocking antibodies seem to be the most effective in higher mutational burden tumors like melanoma and non-small cell lung cancer (NSCLC). In clinical trials with anti–CTLA-4 treatment of melanoma, nearly all patients who achieved durable tumor control had tumors with ≥200 nonsynonymous somatic mutations (1). A prominent publication in the journal *Science* discusses the somatic mutational burden associated with many cancers, and suggests that the recognition of neoantigens by the immune system is a major factor contributing to the success of immunotherapies (2). Further validation of the predictive role of high mutational burden in response to immune checkpoint blockade is the recent finding of profound and selective anticancer activity of PD-1 inhibition only in those patients with metastatic colorectal cancer (CRC) demonstrating deficient DNA mismatch repair mutations resulting in microsatellite instability (MSI) and a large mutational burden, whereas CRC without these mutations and with a lower mutational burden did not respond to PD-1 blockade treatment (3).

What is it about high mutational burden that enables immunotherapy success? Preclinical studies have demonstrated that only a subset of neoepitopes in tumors are capable of generating T-cell responses, and an even smaller group is capable of generating T-cell responses that can control tumor growth (4). It has also been shown that patients who have durable clinical responses after immune checkpoint inhibitor treatment had pre-existing T-cell responses to the neoantigens that were responsible for tumor control in low numbers and were expanded under immune checkpoint inhibition (1). Therefore, it seems that a high mutational burden may increase the likelihood that a patient will develop a low-level T-cell response to a neoepitope that is capable of tumor control when therapeutically expanded. Tumors are, however, known to be poor at presenting antigens, and low-level T-cell responses to neoepitopes do not seem to be capable of tumor control without the supplemental inhibition of immune checkpoints within the tumor microenvironment. It remains to be determined whether *prospective* immunization and boosting against neoepitopes with a system that is proficient at presenting antigens, like Advaxis' *Lm*-listeriolysin O (LLO) vectors, can significantly lower the mutational burden cutoff for targeted tumor control with checkpoint inhibitors, yielding an effective combination immunotherapy.

The Rationale and Risks Associated with Use of Algorithms to Deselect Potential Neoantigens

The neoepitope repertoire in human cancer varies along a continuum based on the type of cancer. For each tumor type, there

is a distribution of somatic mutations that center on a mean. In general, higher mutational-load tumors like NSCLC, melanoma, or MSI-high CRC harbor hundreds of somatic mutations, with some ranging beyond 1000 (2). It has been reported that only about 8% of cluster of differentiation 8-positive ($CD8^+$) T-cell neoepitopes occur in oncogenes where they could be tumor drivers. The majority of $CD8^+$ T-cell neoepitopes, as well as all $CD4^+$ T-cell neoepitopes, appear to be passenger mutations (2). However, T-cell responses against passenger mutations have been shown to be capable of tumor control, and need to be considered as neoepitope targets (2). T-cell epitopes must be presented within the context of the patient's major histocompatibility complex (MHC) molecules, which impose limitations: MHC class I epitopes (for $CD8^+$ T cells) are 8–11 amino acids long, whereas MHC class II epitopes bind peptides that are 10–15 amino acids long. It has been shown that the vast majority of mutations within expressed genes do not lead to the formation of neoepitopes that are recognized by T cells, possibly because they are not efficiently cross-presented (5,6). Similarly, the technical challenge of presenting hundreds of patient-specific neoepitopes presents formidable manufacturing hurdles that would make it impractical or impossible with most vaccine platforms. Therefore, most groups attempting to develop personalized neoepitope immunotherapies or vaccines have been leveraging the creation of predictive algorithms intended to eliminate neoepitopes that would not ultimately be immunogenic and capable of tumor control. This technology is investigational, and thus far, most reported algorithms have been only partially successful, predicting 30% to 60% of neoepitopes that were ultimately immunogenic in patients. However, at this level of predictive power, it implies that at best, 40% of the effective neoepitopes could still be missed.

The Advaxis platform is capable of presenting hundreds of personalized neoepitopes. By employing the large capacity of ADXS-NEO in the absence of a predictive algorithm, the technology can minimize the risk of eliminating immunogenic neoantigens and optimize the chances for clinical efficacy.

Antigen Presentation

The efficacy of *Listeria* as a protein antigen delivery vehicle derives from the unique life cycle of the bacteria. Because *Listeria* enter the cytosol of the host antigen-presenting cell (APC) within minutes after initial infection, proteins secreted by the bacteria are readily accessible to the host cell's MHC class I and class II antigen-processing machinery. Any protein secreted by the bacteria will be rapidly degraded and peptides derived from these proteins will be directly presented on the surface of the infected cell by host cell MHC class I molecules. This process has been shown to be highly efficient for proteins expressed by *Listeria* when compared with other antigen-delivery systems, such as viral vectors. Furthermore, since *Listeria* primarily infect professional APCs such as dendritic cells, it obviates the need for cross-presentation of antigen, further enhancing the efficiency of the process (7).

Because MHC class I presentation of *Listeria*-derived peptides is highly efficient, it is an ideal vector for the delivery of larger antigenic polypeptides. It is not necessary to determine the minimal antigenic sequence for any given peptide using predictive algorithms, as virtually any peptide sequence capable of binding an MHC class I molecule contained within a longer polypeptide sequence will be efficiently processed and presented. As such, it is possible to include all amino acids surrounding a given nonsynonymous mutation, thus allowing for the generation of any possible MHC class I binding peptide, independent of human leukocyte antigen (HLA) haplotype, that contains the immunogenic mutation.

Turnaround Times

Advancement of personalized neoepitope-targeted immunotherapy has faced additional technical challenges, including the time required to develop a personalized neoepitope-based treatment for each patient. Many patients with advanced cancer may have only a few months to live without a successful intervention. Immunotherapies historically necessitate lead times that are considered too long to be practical to generate antitumor effects, and vaccination strategies may require priming and repeated boosters to obtain a maximal response. To deliver treatment to a patient, his or her DNA must be sequenced, compared, and run through bioinformatics filters and algorithms before manufacturing of a patient-specific neoepitope treatment can begin. The treatment then must be produced to Good Manufacturing Practice (GMP) specifications and pass stringent safety and manufacturing control testing before administration to the patient. For example, the duration from biopsy to administration of an immunotherapeutic vaccine via the peptide approach can be as long as 3 months.

The Advaxis team has been working diligently to minimize the time between DNA sequencing and the ability to provide the first neoepitope treatment. Turnaround time has been reduced to 8 weeks in pre-Investigational New Drug (IND) process development testing, and additional methods are continually being evaluated to shorten the time frame.

Manufacturing Complexity and Cost of Goods Sold (COGS)

Innovative methods have been applied to enhance ADXS-NEO manufacturing capabilities and enable processes that are compatible with personalized neoepitope immunotherapy. Once the genetic engineering is completed, the culture of the live attenuated bacterial vectors is straightforward and highly scalable. There is no need for manufacturing specific proteins in quantity through biochemical means, as the bacteria themselves produce and secrete the peptides directly within APCs. There is no concern about characterization of viral insertions or expression of mRNA inside critical cells employing host cell mechanisms. Similarly, there is no time required or complexity involved to collect and cultivate autologous cells and modify them ex vivo for re-administration to the patient. This allows Advaxis to keep costs low and manufacturing controls simple, and permits efficient, cost-effective, and timely production of patient-specific ADXS-NEO treatments, allowing for multiple retreatments from the initial production. ADXS-NEO personalized immunotherapies can then be priced on par with other cancer treatments while still allowing an adequate margin.

Treatment Rounds

T cells resulting from immunizations are amplified upon repeat immunizations, or "boosting." In order to generate as many neoepitope-targeting T cells as possible to fight an advanced cancer, patients should receive multiple immunizations to boost the initial response. Some vector-based approaches can generate neutralizing antibodies, which may limit a specific vector to 1 or 2 administrations. Advaxis' vectors do not generate neutralizing antibodies and can therefore be given repeatedly and mixed with or replaced by other *Lm*-LLO vectors that present different targets. Additionally, part of the Advaxis *Lm*-LLO mechanism of action includes a transient reduction of regulatory T cell (Treg) and myeloid-derived suppressor cell (MDSC) activity within tumors, which is yet another way in which repeated administration facilitates tumor control by T cells (8).

Potential for Combination Treatments

The most potent combination regimens in cancer treatment are those where 2 active agents can be combined to provide complementary mechanisms of action. ADXS-NEO has the potential to contribute

to multiple combination regimens. Animal models and early data from clinical trials have shown that *Lm*-LLO immunotherapies have the potential for significant synergy with active immunotherapy agents, in particular PD-1– and/or PD-L1–blocking antibodies.

Data published in the *Journal for ImmunoTherapy of Cancer* in 2013 (9) demonstrated that the combination of an *Lm*-LLO–based vaccine with anti–PD-1 antibody leads to increased antigen-specific immune responses and tumor-infiltrating $CD8^+$ T cells, along with a decrease in immune suppressor cells (Tregs and MDSCs). The combination regimen led to synergistic activity, with significant inhibition of tumor growth and prolonged survival/complete regression of tumors in treated animals. The combination of an *Lm*-LLO–based vaccine with blocking of PD-1/L1 can lead to overall enhancement of the efficacy of antitumor immunotherapy over either agent alone. It was also shown that in vitro infection with *Lm* results in significant upregulation of surface PD-L1 expression on human monocyte-derived dendritic cells, which suggests the translational capacity of this finding.

Data presented at the American Association for Cancer Research annual meeting in 2016 (10) provided evidence supporting the upregulation of PD-1 and activation of T cells by an Advaxis *Lm*-LLO agent in human head and neck tumors. Data from the study showed increased immune activation within the tumor microenvironment, including upregulation of PD-1 and PD-L1 expression, reduction of Tregs and MDSCs, and infiltration of $CD8^+$ and $CD4^+$ T cells. These observations suggest potentially strong synergy with an anti–PD-1 antibody (9,10). Preclinical data also suggest synergy with immune costimulatory agonists like OX40 and GITR (11).

Synergy of *Lm*-LLO vectors with radiation therapy has been demonstrated in preclinical models (12) and has also been observed in ongoing veterinary trials in nonresected canine osteosarcoma (Nicola Mason, personal communication). Advaxis treatments can also be given sequentially with chemotherapies, provided there has been sufficient hematopoietic recovery.

ADXS-NEO: The Future of Immunotherapy

The Advaxis MINE™ technology leverages ADXS-NEO, a novel investigational immunotherapy treatment, to target specific neoepitopes found in individual tumors utilizing the *Lm*-LLO vector.

Advaxis vectors are superior antigen presenters that focus on the generation of a predominantly $CD8^+$ T-cell immune response. *Listeria* directly deliver the immunogenic peptides within the cytosol of multiple APCs simultaneously. Each vector triggers multiple Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NODs), pathogen-associated molecular pattern (PAMP) receptors, etc, which alter the immunologic macroenvironment. Furthermore, the multicopy DNA plasmids, coupled with the presence of bacterial DNA in the cytosol of the APC, are a potent agonist of stimulator of interferon genes (STING), which enhances immune recognition of tumors.

Listeria are known to induce T-cell responses against weak antigens and, in transgenic animal models, have been shown to prevent the development of autochthonous tumors despite central tolerance. It is highly likely that neoepitopes that are not immunogenic when given by different vaccine platforms will be immunogenic when presented using Advaxis' *Lm* Technology™. The inhibition of Tregs and MDSCs within the tumor microenvironment by Advaxis' truncated LLO overrides a mechanism of tumor-mediated tolerance that is distinct from immune checkpoint inhibition, leading to the potential for excellent synergy in combination-based therapies.

Because there is no development of neutralizing antibodies, repeated treatments with a single *Listeria* vector or simultaneous or sequential treatment with multiple vectors is possible. The Advaxis plasmid has the capacity to accommodate thousands of amino acids, and by making multiple vectors, virtually every neoepitope in a patient can be delivered. These treatments are synergistic with PD-1/L1 blockade, as well as other active forms of cancer treatment, lending themselves well to combination regimens. Prospectively immunizing a patient against *all* of his or her potential neoepitopes, followed by PD-1/L1 treatment, may significantly lower the mutational burden threshold above which immune checkpoint inhibitor treatment can contribute to potent and durable tumor control.

Next-generation Advaxis personalized immunotherapies have the potential to revolutionize the treatment of cancer by providing highly efficacious, targeted attacks on tumor cells with little to no impact on healthy cells. Tumor immunotherapies take advantage of the most effective cancer-fighting agents that nature has devised: the host's own immune cells. With the knowledge of cancer immunology gained over the last 10 years, science has already taken the first steps in altering the immunosuppressive capabilities of cancer; the next step is initiating a direct counterattack to eradicate this "emperor of all maladies" (13).

1. Snyder A, et al. Genetic basis for clinical response to CTLA-4 blockade in melanoma. *N Engl J Med*. 2014;371(23):2189-2199.

2. Schumacher TN, Schreiber RD. Neoantigens in cancer immunotherapy. *Science*. 2015;348(6230):69-74.

3. Le DT, et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N Engl J Med*. 2015;372(26):2509-2520.

4. Castle JC, et al. Exploiting the mutanome for tumor vaccination. *Cancer Res*. 2012;72(5):1081-1091.

5. Linnemann C, et al. High-throughput epitope discovery reveals frequent recognition of neo-antigens by CD4+ T cells in human melanoma. *Nat Med*. 2015;21(1):81-85.

6. Lu YC, et al. Efficient identification of mutated cancer antigens recognized by T cells associated with durable tumor regressions. *Clin Cancer Res*. 2014;20(13):3401-3410.

7. Wolf BJ, Princiotta MF. Processing of recombinant Listeria monocytogenes proteins for MHC class I presentation follows a dedicated, high-efficiency pathway. *J Immunol*. 2013;190(6):2501-2509.

8. Wallecha A, Singh R, Malinina I. Listeria monocytogenes (Lm)-LLO immunotherapies reduce the immunosuppressive activity of myeloid-derived suppressor cells and regulatory T cells in the tumor microenvironment. *J Immunother.* 2013;36(9):468-476.

9. Mkrtichyan M, et al. Anti-PD-1 antibody significantly increases therapeutic effect of Listeria monocytogenes (Lm)-LLO immunotherapy. *J Immunother Cancer*. 2013;1:15.

10. Krupar R, et al. HPV E7 antigen-expressing Listeria-based immunotherapy (ADXS11-001) prior to robotic surgery for HPV-positive oropharyngeal cancer enhances HPV-specific T cell immunity. American Association for Cancer Research (AACR) annual meeting; April 16–20, 2016; New Orleans, Louisiana. Abstract 7632.

11. Mkrtichyan M, et al. Agnostic antibodies to co-stimulatory molecules, OX40 and GITR, significantly enhance the anti-tumor efficacy of Listeria monocytogenes (Lm-LLO)-based immunotherapy. American Association for Cancer Research (AACR) annual meeting; April 18–22, 2015; Philadelphia, Pennsylvania. Abstract LB-229.

12. Hannan R, et al. Combined immunotherapy with Listeria monocytogenes-based PSA vaccine and radiation therapy leads to a therapeutic response in a murine model of prostate cancer. *Cancer Immunol Immunother*. 2012;61(12):2227-2238.

13. Mukherjee, S. *The Emperor of All Maladies: A Biography of Cancer*. New York, NY: Scribner; 2010.