Lm-LLO Immunotherapies Targeting Multiple Antigens and Their Impact on Different Mechanisms in the Tumor Microenvironment

Anu Wallecha and Poonam Molli

Research and Development, Advaxis Inc., 305 College Road East, Princeton, NJ-08540

Abstract

Overexpression of tumor associated antigens (TAA) such as carbonic anhydrase 9 (CA9), Her2/neu and high molecular weight melanoma associated antigen (HMW-MAA) is associated with aggressive high-grade tumors leading to disease progression and reduced survival. CA9 is a cell surface enzyme that catalyzes the reversible hydration of carbon dioxide to bicarbonate and is overexpressed in response to tumor hypoxia in many common tumor types. CA9 plays a critical role in hypoxia-associated tumor acidosis, which plays an important role in tumor progression and chemoresistance in various types of cancer. Current Her2/neu-directed therapies confer limited clinical benefits and most patients experience progressive disease indicating that additional therapeutic strategies targeting Her2/neu could have potential. HMW-MAA is reported to be a TAA as well as an angiogenesis associated protein, as it is expressed at high levels by activated pericytes and pericytes in tumor angiogenic vasculature that are associated with neovascularization *in vivo*. We hypothesized that an *Lm*-LLO immunotherapy, using attenuated *Listeria monocytogenes* (*Lm*)-LLO as the vector capable of delivering multiple antigens would likely have a synergistic effect on decreasing tumor growth by targeting independent mechanisms that support tumor growth. We created two bivalent *Lm*-LLO immunotherapies expressing two antigens such as cHer2/HMW-MAA or cHer2/CA9. These bivalent *Lm*-LLO immunotherapies efficiently secreted two antigens, grew intracellularly and escaped the phagolysosome, supporting that recombinant bacteria retained their ability to deliver antigen successfully in an antigen presenting cell. Preliminary antitumor therapeutic studies in the treatment of mice bearing established tumors expressing Her2 demonstrate that both of these bivalent *Lm*-LLO immunotherapies show an improvement in the reduction of tumor growth when compared to monovalent *Lm*-LLO immunotherapies. We will present data on the therapeutic efficacy of two bivalent *Lm*-LLO immunotherapies and provide evidence on the mechanisms likely responsible for the observed anti-tumor effects. Currently *Lm*-LLO immunotherapies are being evaluated in Phase 2 clinical trials for HPV-associated malignancies such as cervical, head and neck, and anal cancers.

Overview of the Immunotherapies Used in the Current Study

LmddA based Immunotherapy. *LmddA* is a non-pathogenic, attenuated and genetically modified *Lm* vector (*Lm* Δ *dal dat actA*), which does not have the ability to spread from cell to cell due to the *actA* deletion. The *dal dat* deletion in *LmddA* is complemented by a copy of the *dal* gene using a plasmid that also carries the TAA expression cassette. This complementation is essential for *in vitro* and *in vivo* growth of *LmddA*-based constructs and led to the development of an antibiotic-marker free plasmid. The TAA is fused to the first 441 residues of the LLO protein (tLLO).

Her2/neu. Her-2/neu overexpression or mutations have been associated with several types of human cancer, including breast, ovarian, pancreatic, gastric and colon cancers. The Her2/neu is a potential target for immunotherapy as it is overexpressed in tumors but has limited presence in other tissues, except for the heart.

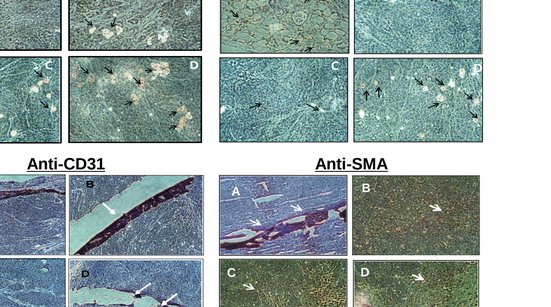
HMW-MAA or CSPG4. The human high molecular weight-melanoma associated antigen (HMW-MAA) is found to be overexpressed in many cancers. HMW-MAA is a useful antigen to target for the treatment of tumors as it is expressed at high levels in pericytes in tumor angiogenic vasculature.

CA9 or G250. Carbonic anhydrase 9 (CA9) is a transmembrane protein overexpressed in a variety of tumor types and is induced by hypoxia which has been a major cause for the failure of radiotherapy.

Anti-Tumor Efficacy of Different Monovalent and Bivalent *Lm* Based Immunotherapy

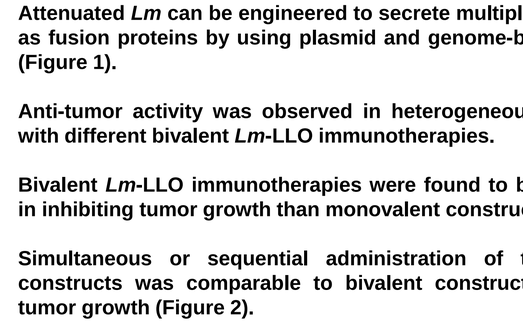
A **4T1 cells**

Groups	4T1 Tumor Implantation in mammary fat pad ($7x10^3$)	Immunotherapy		Measurement Dates
		Dose 1 ($1x10^8$ CFU)	Dose 2 ($1x10^8$ CFU)	
Naïve - PBS	Day 0	Day 3	Day 10	1X/ Week
cHer2	Day 0	Day 3	Day 10	1X/ Week
CA9	Day 0	Day 3	Day 10	1X/ Week
cHer2/ CA9	Day 0	Day 3	Day 10	1X/ Week



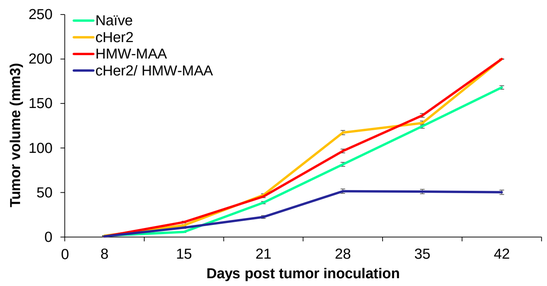
B **NT2 cells**

Groups	NT2 Tumor Implantation ($1x10^6$)	Immunotherapy			Measurement Dates
		Dose 1 ($1x10^8$ CFU)	Dose 2 ($1x10^8$ CFU)	Dose 3 ($1x10^8$ CFU)	
Naïve - PBS	Day 0	Day 7	Day 14	Day 21	2X/ Week
cHer2	Day 0	Day 7	Day 14	Day 21	2X/ Week
HMW-MAA	Day 0	Day 7	Day 14	Day 21	2X/ Week
cHer2/ HMW-MAA	Day 0	Day 7	Day 14	Day 21	2X/ Week



C **4T1-HMW-MAA cells**

Groups	4T1-HMW-MAA Tumor Implantation ($1x10^4$)	Immunotherapy			Measurement Dates
		Dose 1 ($1x10^8$ CFU)	Dose 2 ($1x10^8$ CFU)	Dose 3 ($1x10^8$ CFU)	
Naïve - PBS	Day 0	Day 1	Day 8	Day 15	1X/ Week
cHer2	Day 0	Day 1	Day 8	Day 15	1X/ Week
HMW-MAA	Day 0	Day 1	Day 8	Day 15	1X/ Week
cHer2/HMW-MAA	Day 0	Day 1	Day 8	Day 15	1X/ Week



D **Combination vs Sequential Therapy- NT2 cells**

Groups	NT2 Tumor Implantation ($1x10^6$)	Immunotherapy	Measurement Dates
		Doses ($1x10^8$ CFU) starting on Day 7	
Naïve- PBS	Day 0	PBS; 5 doses; one week apart	2X/ Week
cHer2	Day 0	5 doses; one week apart	2X/ Week
HMW-MAA	Day 0	5 doses; one week apart	2X/ Week
cHer2 + HMW-MAA	Day 0	5 doses; one week apart	2X/ Week
cHer2 followed by HMW-MAA	Day 0	Doses one week apart; 3 doses of cHer2 followed by 3 doses of HMW-MAA	2X/ Week

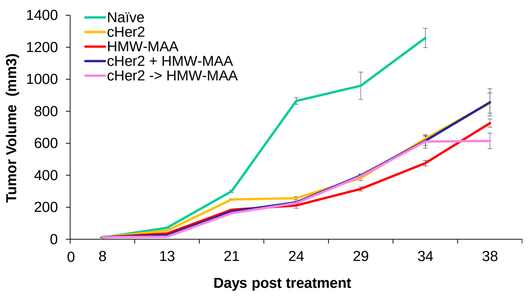
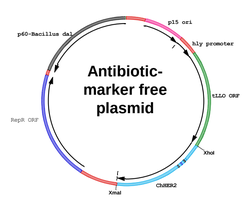


Figure 2*. Line plots showing effect of monotherapy versus bivalent therapy on anti-tumor efficacy using different cell line models. A) monovalent constructs- cHer2 & CA9 and bivalent construct– cHer2/CA9 was tested in 4T1 tumor model. Similarly, monovalent constructs- cHer2 & HMW-MAA and bivalent construct- cHer2/ HMW-MAA was tested in NT2 (B) and in 4T1-HMW-MAA tumor model (C). Effect of administering cHer2 or HMW-MAA monovalent therapy separately, simultaneously or sequentially was evaluated using NT2 tumor models (D).*

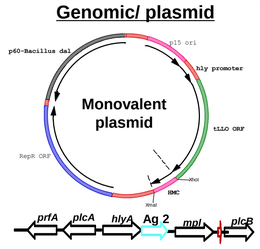
Construction of Bivalent Plasmid that Concomitantly Delivers Two Antigens

10403S Δ *dal dat actA /LmddA*



Protein secreted
tLLO-cHer2

Genomic/ plasmid



Proteins secreted
Plasmid tLLO-Antigen1 (HMW-MAA or CA9)
Genomic LLO-Antigen 2 (cHer2)

Figure 1. *Schematic representation of monovalent and bivalent plasmids. Restriction sites that were used for cloning of antigen 1 (XhoI and SpeI) and antigen 2 (XbaI and SacI or BglII) are indicated. The black arrow represents the direction of transcription. p15 ori and RepR refers to E.coli and Listeria origin of replication. tLLO is truncated Listeriolysin O protein (1-441aa). Bacillus-dal gene codes for D-alanine racemase which complements for the synthesis of D-alanine in Lm Δ dal dat strain.*

IHC Staining

Anti- CD8 **Anti-CD4**

Anti-CD31 **Anti-SMA**

Figure 3*. Immunohistochemical staining showing infiltration of CD8+ and CD4+ T cells, blood vessels (CD31) and pericytes (Smooth muscle actin) in different treatment groups. The different groups are represented as A: Naïve; B: cHer2; C: HMW-MAA and D: cHer2/ HMW-MAA*

Conclusions

- **Attenuated *Lm* can be engineered to secrete multiple tumor antigens as fusion proteins by using plasmid and genome-based expression (Figure 1).**
- **Anti-tumor activity was observed in heterogeneous tumor models with different bivalent *Lm*-LLO immunotherapies.**
- **Bivalent *Lm*-LLO immunotherapies were found to be more effective in inhibiting tumor growth than monovalent constructs (Figure 2).**
- **Simultaneous or sequential administration of two monovalent constructs was comparable to bivalent constructs in controlling tumor growth (Figure 2).**
- **Tumor-bearing mice treated with bivalent immunotherapy showed an increased infiltration of CD4+, CD8+ T cells and reduced blood vessels and pericytes (Figure 3).**