Neoantigens that fail to elicit measurable T cell responses following peptide immunization can control tumor growth when delivered using a Listeria-based immunotherapy platform

Brandon Coder[1], Hyewon Phee[2], Cristina Mottershead[1], Dipti Kelkar[1], Elena Filippova[1], Xiaoming Ju[1], Bryan Vander Lugt[2], Olga Pryshchep[2], Justin Lesch[2], Xian Liu[2], Jason DeVoss[2], Keegan Cooke[2], Claret Liu[2], Jinghui Zhan[2], Petia Mitchell[2], Kim Ramos[1], Daniel O Villarreal[1], Jim Johnston[3], Robert Petit[1], Michael F. Princiotta[1]

[1]Advaxis, Inc., Princeton, NJ | [2]Amgen, Inc., South San Francisco, CA | [3]Amgen, Inc., Thousand Oaks, CA

ABSTRACT

Introduction: Recent advances in the field of cancer immunotherapy have identified CD8$^+$ T cell responses against tumor-specific neoantigens as a key driver of tumor regression and prolonged survival. ADXS-NEO is a personalized *Listeria monocytogenes* (*Lm*)-based immunotherapy designed to generate immune responses against mutation-derived tumor-specific neoantigens.[1] Advaxis' *Lm*-based immunotherapies consist of live highly-attenuated bacterial vectors that are bioengineered to secrete a fusion protein consisting of a truncated non-hemolytic fragment of listeriolysin O, which has adjuvant properties[2], and tumor-specific neoantigens that harbor nonsynonymous point mutations (NSMs). The objective of this study is to demonstrate the feasibility of using the ADXS-NEO platform to target tumor-specific point mutations to generate neoantigen-specific T cells and control tumor growth.

Results: Whole-exome sequencing of the MC38 mouse tumor cell line identified 2870 unique NSMs. Among these, the IC_{50} of 138 NSMs were predicted to be less than 500 nM by the netMHCcons algorithm. We evaluated the immunogenicity of 37 NSMs, and found that 12 immunogenic NSMs elicited a CD8$^+$ T cell response following peptide immunization. Moreover, we identified 10 additional immunogenic NSMs in MC38-bearing mice treated with a check point inhibitor. Altogether, we identified 22 immunogenic and 23 non-immunogenic NSMs. Two ADXS-NEO vectors were constructed, Lm-19 & Lm-20, targeting 19 non-immunogenic and 20 immunogenic NSMs respectively. The ability of Lm-19 and Lm-20 to control MC38 tumor growth was evaluated in C57BL/6 mice. We found that both Lm-19 & Lm-20 led to an accumulation of neoantigen-specific CD8$^+$ TILs and significantly slowed tumor growth. Moreover, both Lm-19 and Lm-20 decreased the frequency and absolute number of intratumoral Tregs, TAMs, and MDSCs and increased the frequency and absolute number of effector CD8$^+$ T cells. Interestingly, expression of PD-L1 was decreased in TAMs and MDSCs and the frequency and total number of granzyme A$^+$ CD8$^+$ effector T cells was increased. Furthermore, the proportion of phenotypically exhausted PD-1^{hi}LAG-3$^+$ TILs was decreased. Together, these data suggest the tumor microenvironment in mice receiving Lm-19 and Lm-20 becomes more cytotoxic and less suppressive.

Conclusion: ADXS-NEO is a potent immunotherapy capable of driving immune responses against tumor-specific mutations and leading to tumor control. The effectiveness of the *Lm* platform is demonstrated by the generation of neoantigen-specific T cells to peptide sequences that were identified as "non-immunogenic" using a conventional peptide-adjuvant immunization. This study is a clear demonstration that T cell mediated anti-tumor responses can be generated by targeting tumor-derived NSMs with the ADXS-NEO *Listeria monocytogenes* vector.

OBJECTIVE

Evaluate the capacity of the "non-immunogenic" Lm19 and "immunogenic" Lm20 ADXS-NEO constructs to generate neoantigen-specific T cells and control tumor growth in the MC38 murine colorectal tumor model.

MATERIALS AND METHODS

Comparative Whole Exome Sequencing (WES), neoantigen prediction, and construct design: WES: MC38 murine colorectal cell line and matched C57BL/6 normal tissue (tail snip) were sent for nucleic acid extraction, whole exome sequencing sample preparation, and comparative whole exome sequencing. Following WES, the whole exome sequence of the normal sample was used as a reference dataset to identify non-synonymous amino acid changes present only in the MC38 tumor sample.

Neoantigen selection: Non-synonymous mutations were run through netMHCcons (http://tools.iedb.org/mhci/) and NSMs with an IC_{50} <500nm were selected for further experimentation. Peptides corresponding to the minimal 9-mer epitope from 37 NSMs identified from netMHCcons were used to immunize C57BL/6 mice with CpG adjuvant. Splenocytes were harvested and CD8$^+$ T cell responses were evaluated using IFNγ ELISpot with 9-mer epitope re-stimulation. Additional neoantigens were identified in MC38 tumors following treatment with checkpoint inhibitors and ex-vivo IFNγ ELISpot with minimal epitope re-stimulation.

Tumor model and *Lm* treatment: MC38 (300,000) cells were implanted subcutaneously (s.c.) in the right flank of mice and given 3 weekly immunizations with *Lm* i.v. starting on day 8.

Flow analysis: Tumors were enzymatically dissociated into single cell suspensions using a gentleMACS and MACS tissue dissociation kit (Miltenyi Biotec). The resulting single-cell suspensions were immunophenotyped with the conjugated antibodies using standard staining procedures. Intratumoral immune cells were defined as CD45$^+$ cells.

***ELISpot*:** Tumors were enzymatically dissociated into single cell suspensions using a gentleMACS and MACS tissue dissociation kit (Miltenyi Biotec). T cells were isolated using pan-T isolation kit. ELISpot plates (Mabtech) were prepared using standard methods, and 2e5 sample T cells were incubated with minimal 9-mer epitopes corresponding to the predicted MHC-I K^b, or D^b binding peptide identified using netMHCcons.

RESULTS

Figure 1: Neoantigen Identification and Construct Design

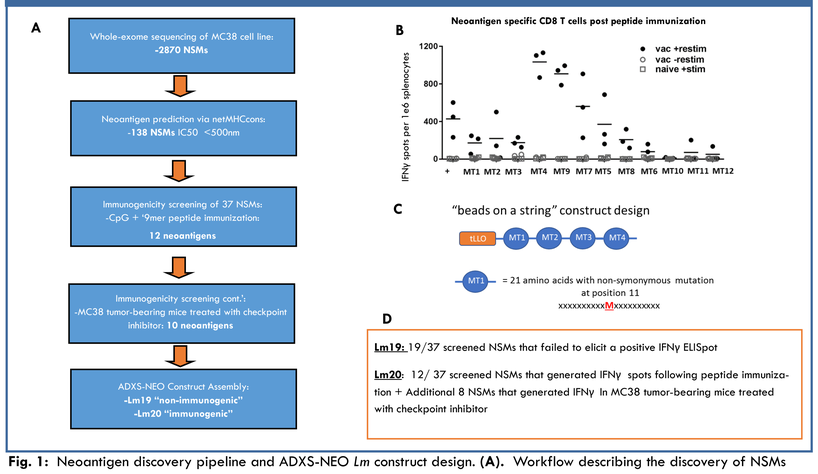

Fig. 1: Neoantigen discovery pipeline and ADXS-NEO *Lm* construct design. **(A).** Workflow describing the discovery of NSMs and selection and validation of potential neoantigens to be incorporated into ADXS-NEO constructs Lm19 and Lm20. **(B).** IFNγ ELISpot screening showing the 12 positive neoantigens following peptide + CpG immunization of C57BL/6 mice. **(C).** Cartoon describing the incorporation of neoantigen fusion protein into the ADXS *Listeria monocytogenes* platform. **(D).** Description of the NSMs included in the experimental vectors Lm19 and Lm20.

Figure 2: Tumor Microenvironment Dosing Schedule and Early Tumor Control

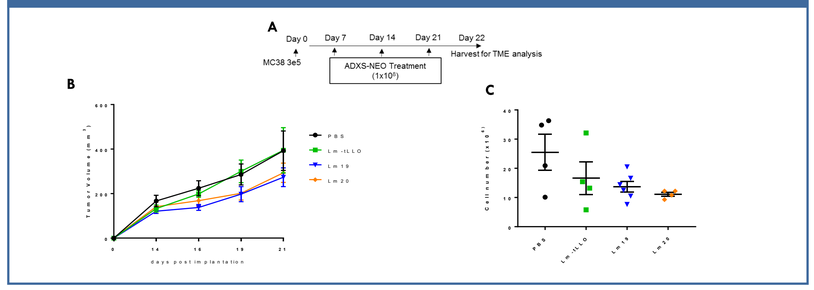

Fig. 2: **(A.)** Schematic detailing the tumor implantation, ADXS-NEO dosing, and tumor harvest schedule for evaluating the effects of therapeutic immunization with ADXS-NEO. **(B.)** Tumor growth curve prior to tumor harvest. **(C.)** Total cell number of harvested tumors 22 days post implantation. Groups: PBS vehicle control, Lm-tLLO empty vector control, Lm19, and Lm20.

Figure 3: Intratumoral Regulatory T cells are Attenuated Following Treatment with ADXS-NEO

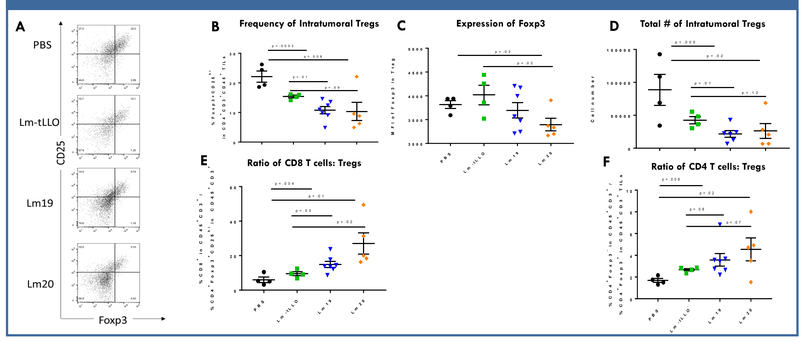

Fig. 3: Immunophenotyping of regulatory T cells found within the TME harvested on day 22 post implantation. **(A.)** Representative dot plot of Foxp3 and CD25 Treg gating within CD45$^+$CD3$^+$CD4$^+$ TILs. **(B.)** Percentage of CD4$^+$ TILs that are Foxp3$^+$CD25^{hi}. **(C.)** Mean fluorescence intensity of Foxp3 within the Treg population. **(D.)** Total # of Tregs within the tumor. **(E-F.)** Ratio of effector CD8 or CD4 (Foxp3$^-$) : Tregs (Foxp3$^+$).

Figure 4: ADXS-NEO Therapy Attenuates the Suppressive Tumor Microenvironment

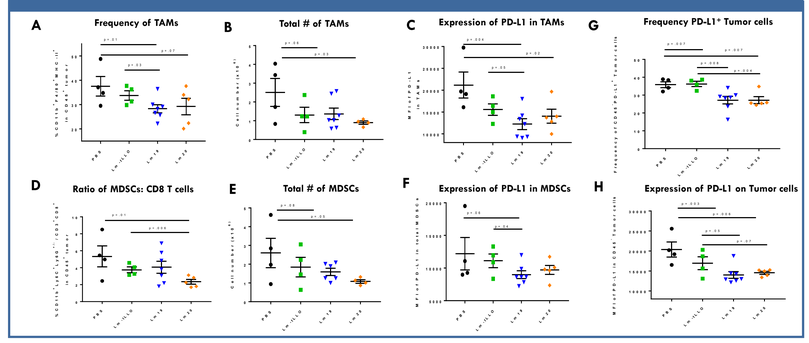

Fig. 4: Immunophenotyping of the myeloid compartment of the TME on day 22 post implantation. **(A-C.)** The frequency and total number of Tissue Associated Macrophages and expression of PD-L1 on a per cell basis. **(D-F.)** The CD8:MDSC ratio and total number of granulocytic and monocytic myeloid derived suppressor cells found in the tumor and expression of PD-L1 on a per cell basis. **(G-H).** The frequency of PD-L1$^+$ CD45$^-$ tumor cells and the expression of PD-L1 within CD45$^-$ tumor cells on a per cell basis.

Figure 5: Influx of Cytotoxic TILs Following Treatment with ADXS-NEO

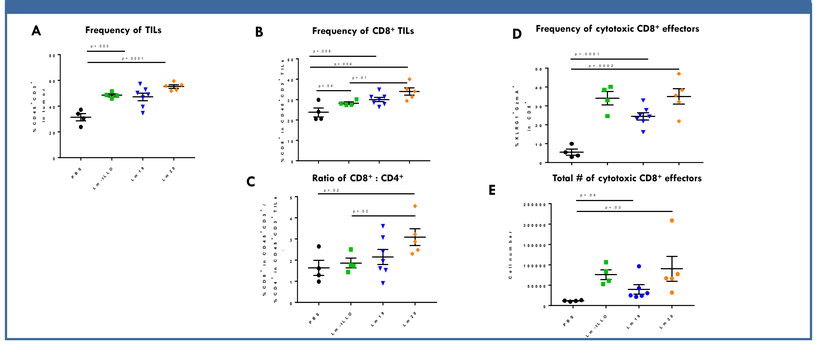

Fig. 5: Immunophenotyping of cytotoxic tumor infiltrating lymphocytes. **(A-C.)** The frequency of CD45$^+$CD3$^+$ TILs, frequency of CD45$^+$CD3$^+$CD8$^+$ TILs, and the ratio of CD8$^+$:CD4$^+$ TILs **(D-E)** The frequency and total number of cytotoxic effector CD8 T cells (CD45$^+$CD3$^+$CD8$^+$KLRG1$^+$Granzyme A$^+$).

Figure 6: Frequency of Exhausted CD8$^+$ TILs Decreases Following ADXS-NEO Treatment

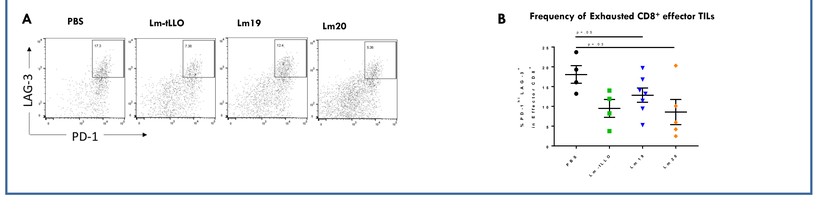

Fig. 6: Immunophenotyping of exhausted CD8$^+$ effector TILs. **(A.)** Representative dot plot of LAG-3 and PD-1 exhausted TIL gating within CD45$^+$CD3$^+$CD8$^+$KLRG1$^+$ TILs. **(B.)** Frequency of exhausted CD8 T cells (PD-1^{hi}LAG-3$^+$CD45$^+$CD3$^+$CD8$^+$KLRG1$^+$ found in the tumor microenvironment.

Figure 7: Neoantigen-specific CD8$^+$ TILs Following Immunization with ADXS-NEO

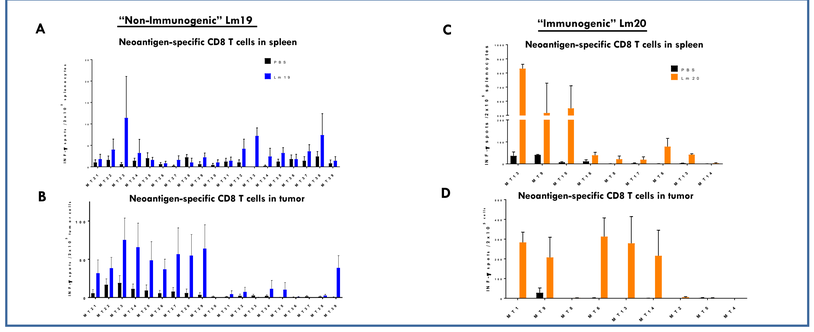

Fig. 7: Neoantigen-specific TILs in MC38 tumor bearing mice following ADXS-NEO treatment. Tumors from MC38 tumor bearing mice were harvested and re-stimulated with minimal predicted epitopes previously used to identify neoantigens (Fig. 1). IFNγ ELISpot assay was used to identify CD8$^+$ TILs responding to predicted neoantigens **(A.)** ELISpot results from the spleens of MC38 tumor bearing mice immunized with Lm19 and re-stimulated with the minimal 19 "non-immunogenic" epitopes. **(B).** ELISpot results from the tumors of MC38 tumor bearing mice immunized with Lm19 and re-stimulated with the minimal 19 "non-immunogenic" epitopes. **(C.)** ELISpot results from the spleens of MC38 tumor bearing mice immunized with Lm20 and re-stimulated with the minimal 20 "immunogenic" epitopes. **(D.)** ELISpot results from the tumors of MC38 tumor bearing mice immunized with Lm20 and re-stimulated with the minimal 20 "immunogenic" epitopes.

Figure 8: ADXS-NEO Controls Tumor Growth Targeting Either "Immunogenic" or "Non-immunogenic" NSMS

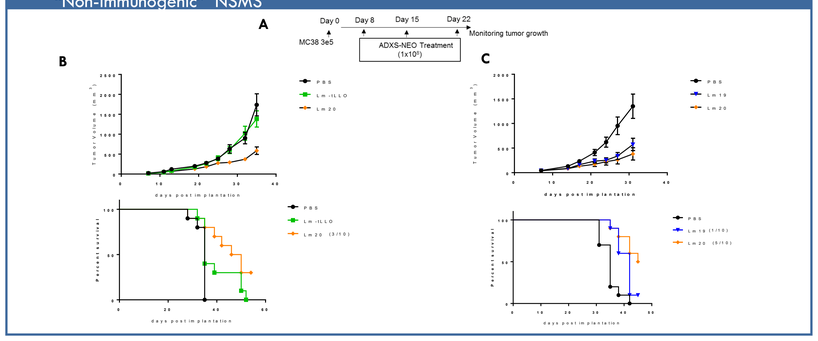

Fig. 8: Therapeutic efficacy of ADXS-NEO in MC38 tumor bearing mice. **(A.)** Schematic detailing the tumor implantation and ADXS-NEO dosing schedule. **(B.)** Tumor growth and survival curves for animals treated with Lm-tLLO empty vector and "immunogenic" Lm20. **(C.)** Tumor growth and survival curves for animals treated with Lm-tLLO empty vector, "non-immunogenic" Lm19, and "immunogenic" Lm20.

SUMMARY AND CONCLUSIONS

- ADXS-NEO controls tumor growth by targeting NSMs expressed as 21-mers in *Listeria monocytogenes* bacterial vector
- ADXS-NEO can control tumor growth and generate neoantigen-specific CD8$^+$ T cells by targeting neoantigens that fail to elicit immunogenicity when immunized as peptide + CpG adjuvant
- ADXS-NEO immunotherapy platform is a potent inhibitor of the suppressive TME, by reducing numbers and suppressive phenotype of Tregs, MDSCs, and TAMs
- ADXS-NEO generates a robust accumulation of cytotoxic and neoantigen-specific CD8$^+$ T cells that control tumor growth

REFERENCES

1. Wood LM, Paterson Y. Attenuated *Listeria monocytogenes*: a powerful and versatile vector for the future of tumor immunotherapy. *Front Cell In-fect Microbiol*. 2014: https://doi.org/10.3389/fcimb.2014.00051.

2. Chen Z, et al. Episomal expression of truncated listeriolysin O in LmddA-LLO-E7 vaccine enhance antitumor efficacy by preferentially inducing expansions of CD4+ Foxp3- and CD8+ T cells. *Cancer Immunol Res*. 2014:2:911-922.