Targeting frameshift mutations with a *Listeria monocytogenes* immunotherapy drives neoantigen-specific anti-tumor immunity in the MC38 and CT26 mouse tumor models

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ABSTRACT

Introduction: Neoantigens derived from tumor-specific mutations have been shown to drive tumor specific CD8$^+$ T cell responses leading to tumor regression and extending overall survival. Frameshift mutations are estimated to generate up to nine times more neoantigens per mutation compared to in-frame mutations[1]. However, it is not clear if vaccination against frameshift mutations induces neoantigen-specific CD8$^+$ T cell responses that result in control of tumor growth. ADXS-NEO is a personalized Listeria monocytogenes (Lm)-based immunotherapy designed to target mutation-derived tumor-specific neoantigens. Advaxis' Lm-based immunotherapies consist of live attenuated bacterial vectors that are bioengineered to secrete an antigen-adjuvant fusion protein consisting of a truncated non-hemolytic fragment of listeriolysin O, which has adjuvant properties, and tumor-specific antigens[2,3]. Here, we demonstrate the feasibility of using the ADXS-NEO platform to target tumor-specific frameshift mutations in order to generate neoantigen-specific T cells that control tumor growth.

Results: Whole-exome sequencing of the CT26 and MC38 mouse tumor cell lines identified 30 and 31 unique frameshift mutations respectively. Individual frameshift mutations ranged in size from 12 to as many as 150 amino acids (aa). Lm vectors targeting the two longest frameshift mutations were constructed for each tumor model. The therapeutic efficacy of Lm vectors expressing either a single 57 aa (Lm-57) or a single 150 aa (Lm-150) MC38 frameshift mutation were evaluated in C57BL/6 mice. Both Lm vectors generated multiple unique frameshift-specific TILs and slowed tumor growth. Furthermore, we evaluated the tumor microenvironment following Lm-57 or Lm-150 treatment and observed a decrease in the frequency and absolute number of Tregs, TAMs, and MDSCs and an increase in the frequency and absolute number of total cytotoxic granzyme A$^+$ effector CD8$^+$ T cells.

Similarly, Lm vectors expressing either a 64 aa (Lm-64) or a 93 aa (Lm-93) CT26 frameshift mutation were evaluated in the CT26 tumor model. Both Lm-64 and Lm-93 significantly controlled tumor growth. Additionally, an influx of neoantigen-specific TILs and a significant decrease in the frequency of intratumoral Tregs was observed.

Conclusion: ADXS-NEO induced potent immune responses against tumor-specific frameshift mutations and controlled tumor growth. Advaxis' Lm platform is able to target frameshift mutations ≥150 aa and generate multiple neoantigen-specific T cells per frameshift. ADXS-NEO controls tumor growth via multiple mechanisms, including the generation of tumor-specific cytotoxic TILs, by secreting tumor-derived neoantigens directly into dendritic cells and by attenuating the suppressive tumor microenvironment.

OBJECTIVE

Evaluate the feasibility of targeting full length frameshift mutations with the ADXS-NEO platform to generate neoantigen-specific T cells and control tumor growth in the MC38 & CT26 murine colorectal tumor models.

MATERIALS AND METHODS

Comparative Whole Exome Sequencing (WES): MC38 & CT26 murine colorectal cell lines and matched C57BL/6 or BALB/c normal tissue (tail snip) were sent out 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 frameshift amino acid changes present only in the tumor sample.

Frameshift identification : The somatic VCF files from MC38 was annotated against GRCm38 with Snpeff (version 4.3p) Predicted peptide sequences arising from frameshift mutations were generated by inserting or deleting the respective INDEL mutation into the annotated transcript. The resulting mutated cDNA sequence was translated and the peptide sequence representing an altered reading frame was obtained starting from the wild-type 10 amino acids upstream of the INDEL mutation site through to the first observed stop codon downstream.

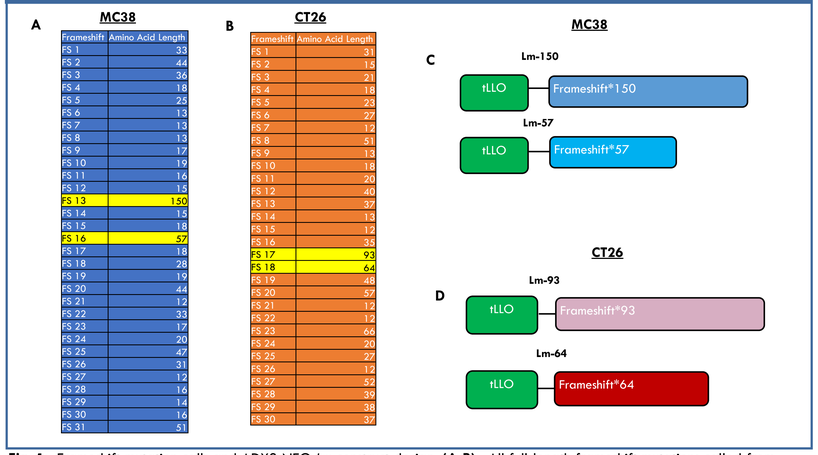
Tumor model and *Lm* treatment: CT26 (300,000) and MC38 (300,000) cells were implanted subcutaneously (s.c.) in the right flank of mice and given 3 weekly immunizations with *Lm* i.v. starting on day 8.

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 14-mer peptides corresponding to predicted MHC-I binding peptide identified using IEDB with a percent rank <1%. http://tools.iedb.org/mhci/

RESULTS

Figure 1: Frameshift Identification and Construct Design



MC38 Frameshift	Amino Acid Length
FS 1	33
FS 2	44
FS 3	36
FS 4	18
FS 5	25
FS 6	13
FS 7	13
FS 8	13
FS 9	17
FS 10	19
FS 11	16
FS 12	16
FS 13	150
FS 14	15
FS 15	18
FS 16	57
FS 17	18
FS 18	28
FS 19	19
FS 20	44
FS 21	12
FS 22	33
FS 23	17
FS 24	20
FS 25	47
FS 26	31
FS 27	12
FS 28	16
FS 29	14
FS 30	16
FS 31	51

CT26 Frameshift	Amino Acid Length
FS 1	31
FS 2	15
FS 3	21
FS 4	18
FS 5	23
FS 6	27
FS 7	12
FS 8	51
FS 9	13
FS 10	18
FS 11	20
FS 12	40
FS 13	37
FS 14	13
FS 15	12
FS 16	35
FS 17	93
FS 18	64
FS 19	48
FS 20	57
FS 21	12
FS 22	12
FS 23	66
FS 24	20
FS 25	27
FS 26	12
FS 27	52
FS 28	39
FS 29	38
FS 30	37

Fig. 1: Frameshift mutation calls and ADXS-NEO *Lm* construct design. **(A-B).** All full-length frameshift mutations called from WES of MC38 and CT26 cell lines, with the two longest frameshifts highlighted in yellow. **(C-D).** Cartoon describing the incorporation of full-length frameshift fusion protein into the ADXS *Listeria monocytogenes* platform.

Figure 2: Tumor Microenvironment Dosing Schedule and Early Tumor Control

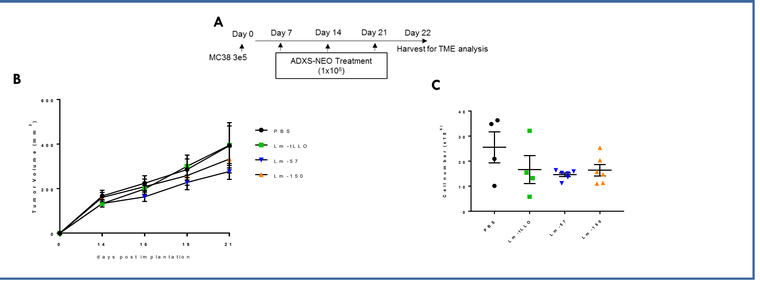


Fig. 2: **(A.)** Schematic detailing the tumor implantation, ADXS-NEO dosing, and tumor harvest schedule for evaluating the effects of therapeutic immunization with ADXS-NEO. **(B.)** Tumor growth curve prior to tumor harvest. **(C.)** Total cell number of harvested tumors 22 days post implantation. Groups: PBS vehicle control, Lm-tLLO empty vector control, Lm-57, and Lm-150.

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

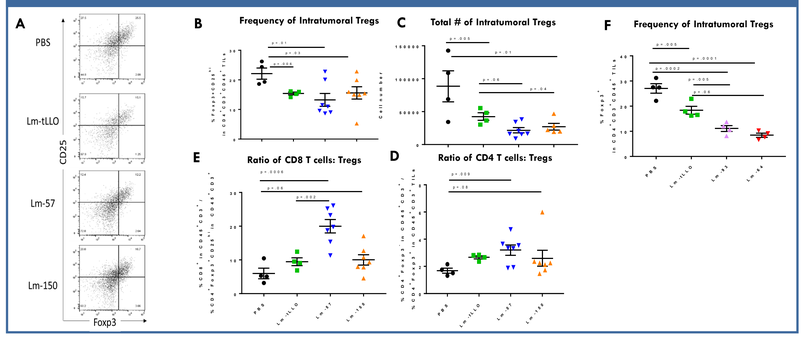


Fig. 3: Immunophenotyping of regulatory T cells found within the TME harvested on day 22 post implantation. **(A-D.)** Immunophenotyping from MC38 tumors. **(A.)** Representative dot plot of Foxp3 and CD25 Treg gating within CD45$^+$CD3$^+$CD4$^+$ TILs. **(B.)** Percentage of CD4$^+$ TILs that are Foxp3$^+$CD25^{hi}. **(C.)** Total # of Tregs within the tumor. **(E-D.)** Ratio of effector CD8 or CD4 (Foxp3$^-$) : Tregs (Foxp3$^+$). **(F.)** Percentage of CD4$^+$ TILs that are Foxp3$^+$CD25^{hi} from tumors of CT26 tumor-bearing mice.

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

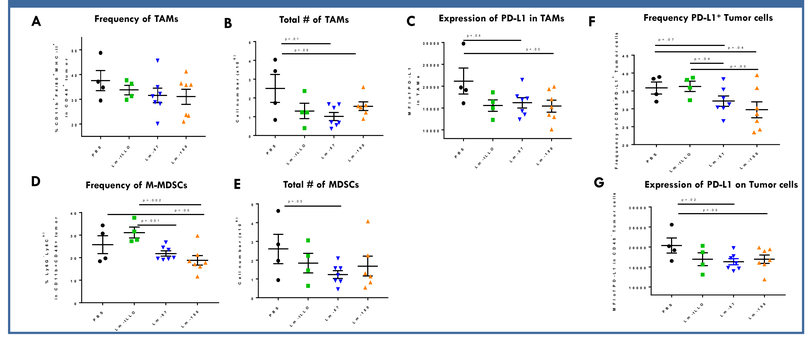


Fig. 4: Immunophenotyping of the myeloid compartment of the TME on day 22 post implantation. **(A-C.)** The frequency and total number of Tissue Associated Macrophages and expression of PD-L1 on a per cell basis. **(D-E.)** The frequency and total number of monocytic myeloid derived suppressor cells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

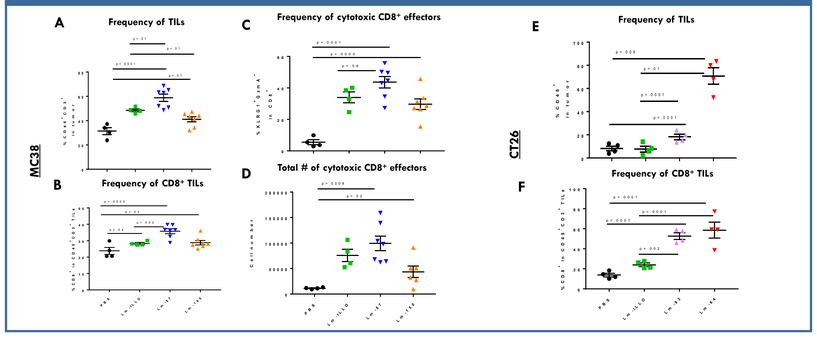


Fig. 5: Immunophenotyping of cytotoxic tumor infiltrating lymphocytes. **(A-B.)** The frequency of CD45$^+$CD3$^+$ TILs, frequency of CD45$^+$CD3$^+$CD8$^+$ TILs in MC38 tumors. **(C-D)** The frequency and total number of cytotoxic effector CD8 T cells CD45$^+$CD3$^+$CD8$^+$KLRG1$^+$Granzyme A$^+$) in MC38 tumors. **(E-F).** The frequency of CD45$^+$CD3$^+$ TILs, frequency of CD45$^+$CD3$^+$CD8$^+$ TILs from CT26 tumors.

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

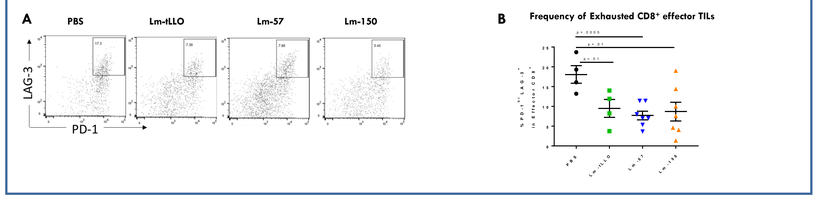


Fig. 6: Immunophenotyping of exhausted CD8 effector TILs. **(A.)** Representative dot plot of LAG-3 and PD-1 exhausted TIL gating within CD45$^+$CD3$^+$CD8$^+$KLRG1$^+$ TILs. **(B.)** Frequency of exhausted CD8 T cells (PD-1^{hi}LAG-3$^+$CD45$^+$CD3$^+$CD8$^+$KLRG1$^+$ found in the tumor microenvironment.

Figure 7: Neoantigen-specific CD8+ TILs Following Immunization with full-length frameshifts using ADXS-NEO

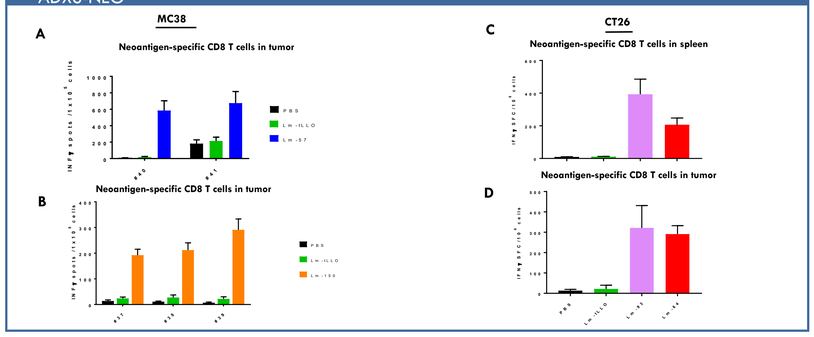


Fig. 7: Neoantigen-specific TILs in MC-38 tumor bearing mice following ADXS-NEO treatment targeting full-length frameshift mutations. Tumors from MC38 tumor bearing mice were harvested and re-stimulated with predicted 14-mer epitopes based on MHC-I IC_{50} rankings. IFNγ ELISpot assay was used to identify CD8$^+$ TILs responding to predicted neoantigens **(A.)** ELISpot results from the tumors of MC38 tumor bearing mice immunized with Lm-57 and re-stimulated with two individual 14-mer epitopes. **(B).** ELISpot results from the tumors of MC38-tumor bearing mice immunized with Lm-150 and re-stimulated with three individual 14-mer epitopes. **(C-D.)** ELISpot results from the spleens and tumors of CT26 tumor bearing mice immunized with Lm-64 & Lm-93 and re-stimulated with an overlapping peptide library of 14-mers.

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

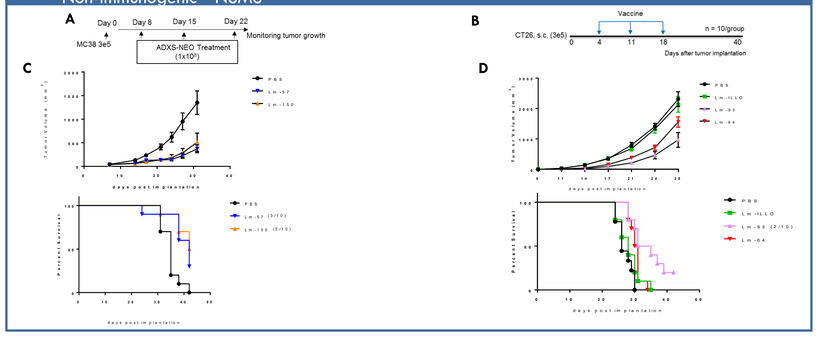


Fig. 8: Therapeutic efficacy of ADXS-NEO in MC38 & CT26 tumor bearing mice treated with ADXS-NEO targeting full-length frameshift mutations present in the tumor. **(A-B.)** Schematic detailing the tumor implantation and ADXS-NEO dosing schedule. **(B.)** MC38 Tumor growth and survival curves for animals treated with Lm-57 and Lm-150. **(C.)** CT26 Tumor growth and survival curves for animals treated with Lm-64 and Lm-93.

SUMMARY AND CONCLUSIONS

- ADXS-NEO *Listeria monocytogenes* immunotherapy platform has the capacity to express and target full-length frameshift mutations present in the tumor
- ADXS-NEO can control tumor growth and generate multiple neoantigen-specific CD8$^+$ T cells against frameshift mutations detected using whole exome sequencing
- ADXS-NEO immunotherapy platform is a potent inhibitor of the suppressive TME, by reducing numbers and suppressive phenotype of Tregs, MDSCs, and TAMs

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