Targeting shared hotspot cancer mutations with a *Listeria monocytogenes* immunotherapy induce potent anti-tumor immunity

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**INTRODUCTION**

Introduction: Virtually all tumors contain somatic mutations that can result in novel antigenic sequences that may be targeted by the host cellular immune response. Some of these mutations occur in preferential regions of specific genes commonly referred to as hotspot mutations. Hotspot mutations are commonly shared by cancer patients both within and across multiple tumor types. These hotspot mutations often confer loss or gain of function contributing to oncogenesis, which makes them promising therapeutic targets. One such mutation commonly found in several human tumor types is an aspartic acid substitution for glycine at position 12 (G12D) in KRAS. This same mutation occurs in the CT26 murine colorectal tumor model. To determine if expression of the KRAS G12D sequence in a bacterial immunotherapy vector can control tumor growth in the CT26 murine model, the Advaxis *Listeria monocytogenes* (Lm)-based platform was engineered to express a 21-mer amino acid KRAS sequence peptide containing the G12D mutation (Lm-Hot KRAS, G12D). In addition, we evaluated control of tumor growth using an ADXS-HOT construct (ADXS-503) that expresses multiple shared human hotspot and tumor-associated antigens commonly found in specific cancer types. In this study, we demonstrate control of tumor growth in a mouse model by targeting a commonly shared hotspot mutation using an Lm-based immunotherapy.

**RESULTS**

Results: We show that the Lm-Hot KRAS, G12D therapy significantly delayed tumor growth and improved long-term survival in the murine CT26 colon carcinoma model. This response was associated with an increase in the frequency of tumor infiltrating antigen-specific CD8+ T cells and y6 T cells within the tumor microenvironment and a decrease in the frequency of intratumoral regulatory T cells (Tregs). Furthermore, tumor-specific CD8+ T cells displayed lower expression of exhaustion markers as well as increased functionality upon restimulation. Interestingly, our proprietary ADXS-503 (a clinical ADXS-HOT construct) which includes KRAS G12D as one of its multiple targets, was also capable of significantly suppressing tumor growth in the CT26 tumor model.

Conclusion: These results suggest that our ADXS-HOT platform is a promising approach to target shared hotspot mutations. ADXS Lm constructs targeting a single hotspot mutation can significantly control tumor growth whether it is in a single or multi-target construct. These data describe an exciting translatable discovery with the potential for broad utility across multiple tumor types and patients who share common hotspot mutations.

**MATERIALS AND METHODS**

Tumor Model: C57Bl/6 (B6) female mice were used for Figure 1 immunogenicity study. CT26 murine colon carcinoma cells (ATCC) were implanted subcutaneously (s.c.) in the right flank of female BALB/c mice and tumor growth was monitored twice a week with electronic callipers.

Treatment Regimen for Efficacy Studies: On day 4 after tumor implantation, mice were treated with either Lm-KRAS_G12D (Lm-HOT) (1 x 10^6 CFU), an Lm-based vector targeting the KRAS G12D mutation found in the CT26 murine colorectal tumor cell line, LmddA-274 (1 x 10^6 CFU), an Lm-based vector expressing no tumor-specific antigen, PBS (Naïve), or ADXS-503 (clinical construct) with KRAS_G12D sequence along with other HOT spot and TAA targets.

Flow Analysis: Tumors were enzymatically dissociated into single cell suspensions using a Stomacher machine (Seward) with Collagenase IV (Stem Cell Technologies). The resulting single-cell suspensions were subjected to immunophenotyping with the following antibodies used in standard staining procedures: anti-CD45, anti-CD4, anti-CD8, anti-CD44, anti-CD125, anti-CD90, anti-CTLA4, anti-PD1, anti-CD3, DVA (SINFEKL) peptide-NH2 class I tetramer, anti-CD11b, anti-Ly6G, anti-Ly6C, anti-IFNg, anti-TNFa, and Invitrogen LIVE/Dead fixable Violet Fluorescent Reactive Dye. For IFNg staining, cells were stimulated with either SINFEKL peptide, and/or cell stimulation cocktail plus protein transport inhibitors (Invitrogen). Events were acquired using the Attune flow cytometer (Thermo Fisher Scientific) and analyzed using FlowJo software (Tree Star). TIIIs were defined as CD45+CD8+CD44+ cells.

**RESULTS (cont.)**

Figure 2. Lm-KRAS_G12D immunization promotes CT26 tumor control and improves long-term survival. (A) BALB/c mice implantation with CT26 tumor cells (3 x 10^6) received the indicated treatment regimen. (B) Individual tumor responses, group tumor measurements (mean +/- SEM) (C), and survival (D) were monitored over time. ****p<0.0001.

**RESULTS (cont.)**

Figure 4. Lm-KRAS_G12D therapy reduces frequency of Tregs in CT26 tumors. (A-B) Cohorts of CT26 tumor-bearing mice were treated as in Figure 1A and TILs from tumors of CT26 mice were harvested 15 days after tumor implantation. (A-B) Representative flow dot plots and summary data show the percentage of Tregs (Foxp3+CD25+Foxp3+CD25+CD44+) of CD45+ cells. *p<0.05, ***p<0.001, ****p<0.0001 Error bars indicate SEM and experiments were performed n = 5/group.

**SOMMARIO**

- Lm-Hot therapy enhanced antitumor efficacy and improved long-term survival.
- Lm-Hot therapy increased tumor-specific T cells and significantly decreased tumor-resident Tregs.
- ADXS-503 therapy delays tumor growth similar to that of the KRAS_G12D_21mer Construct.
- ADXS-503 elicits effective anti-tumor immunity whether it is in a single or multi-target construct.
- ADXS-HOT platform is a promising approach to target shared hotspot mutations.

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1) LmddA-274 (Control), 2) Clinical HOT-503, or 3) HOT-Lm KRAS_G12D, followed with a boost one week after initial immunization. The data show tumor measurements for the individual experimental groups.