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-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, including the G12D KRAS. The ADXS-HOT clinical program is comprised of several *Lm*-based immunotherapies designed to target multiple shared 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: 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 γδ 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 calipers.

Treatment Regimen for Efficacy Studies: On day 4 after tumor implantation, mice were treated with either *Lm*-KRAS_G12D (*Lm*-HOT) (1×10^8 CFU), an *Lm*-based vector targeting the KRAS G12D mutation found in the CT26 murine tumor cell line, LmddA 274 (1×10^8 CFU), an *Lm*-based vector expressing no tumor-specific antigen, PBS (Naïve), or ADXS-503 (a 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 (Steward) with Collagenase IV (Stem Cell Technologies). The resulting single-cell suspensions were subjected to immunophenotyping with the following antibodies using standard staining procedures: anti-CD45, anti-CD4, anti-CD8, anti-CD44, anti-CD25, anti-CD107a, anti-CTLA4, anti-PD-1, anti-CD3, OVA (SIINFEKL) peptide-MHC 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 SIINFEKL 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). TILs were defined as $CD45^+CD8^+CD4^-$ cells.

RESULTS

Lm-HOT treatment induces Ag-specific IFNγ responses in non-tumor bearing mice

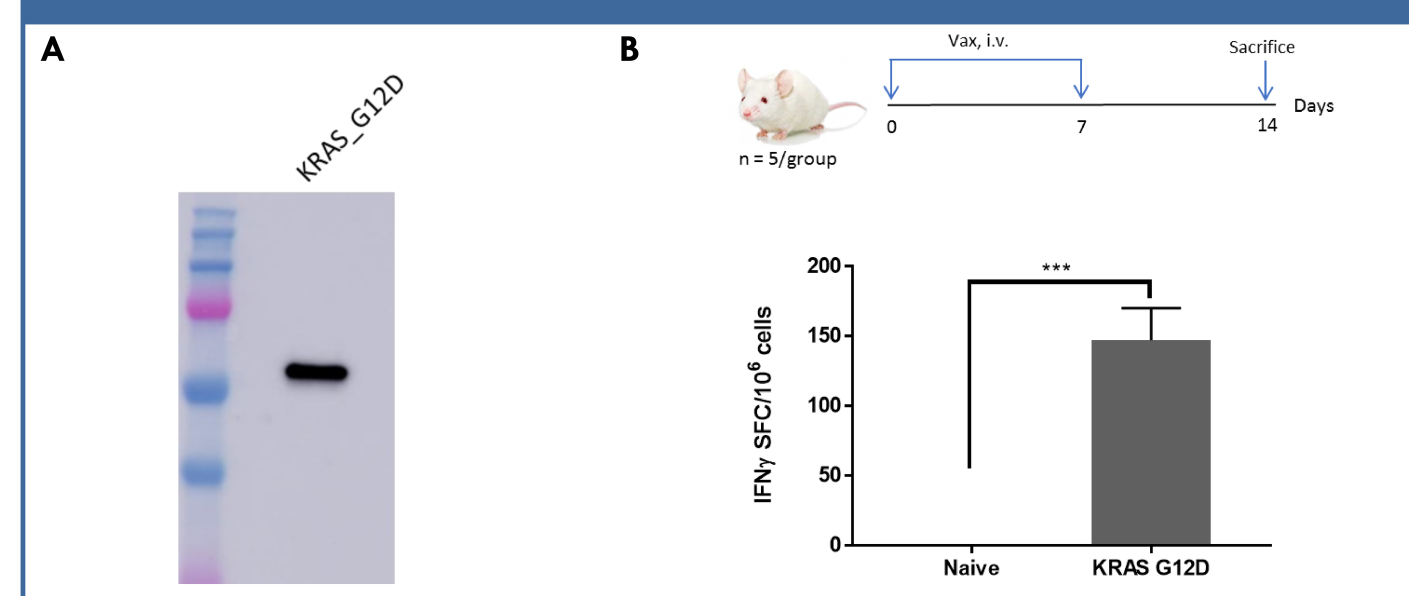

Figure 1. *Lm*-KRAS_G12D construct induces KRAS_G12D-specific IFNγ immune responses in non-tumor bearing mice. (A) Expression of *Lm*-KRAS_G12D constructs as examined by Western Blot analysis detected using an anti-FLAG mAb against a FLAG motif located at the C-terminal region of the target antigen. (B) Lm BALB/c mice (n = 5 per group) were immunized at days 0 and 7 and spleens were harvested one week after last immunization to assess the cellular immune responses. The frequency of KRAS_G12D-specific T cells producing IFNγ was measured via stimulation with a KRAS_G12D peptide pool and the predicted CTL epitope by IFNγ ELISpot. Error bars indicate SEM and experiments were performed independently at least 2 times with similar results.

RESULTS (cont.)

Lm-HOT treatment Impairs tumor growth and improves long-term survival

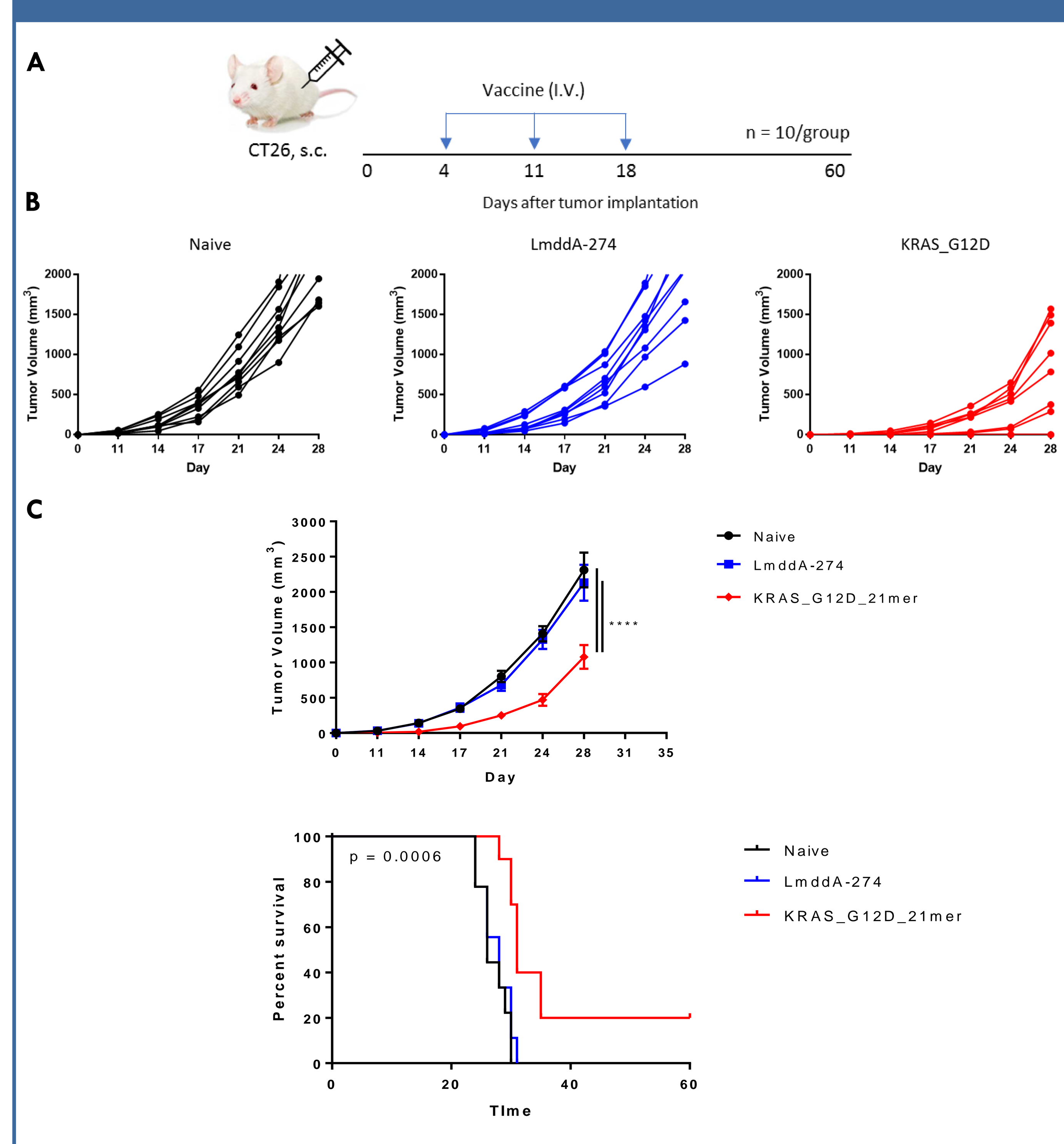

Figure 2. *Lm*-KRAS_G12D immunization promotes CT26 tumor control and improves long-term survival. (A) BALB/c mice implantation with CT26 tumor cells (3×10^5) received the indicated treatment regimen. (B) Individual tumor responses, group tumor measurements (mean +/- SEM) (C), and survival (D) were monitored over time. ****P<0.0001.

Lm-HOT treatment alters the tumor immune microenvironment

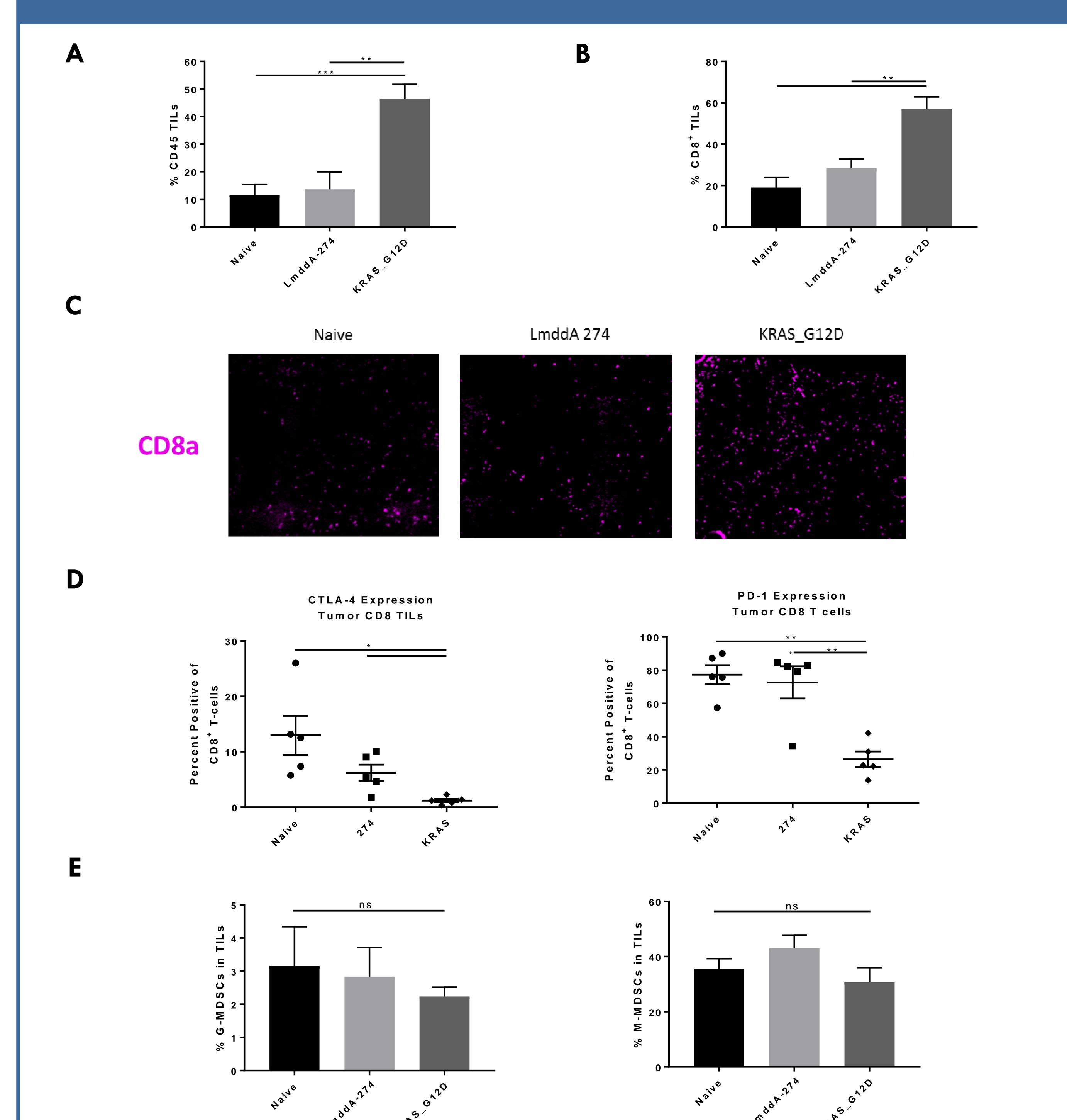

Figure 3. Vaccination altered the cellular composition of the tumor immune microenvironment in the CT26 colorectal model. TILs from tumors of CT26 mice were harvested 15 days after tumor implantation. (A) Bar graphs show the frequency of $CD45^+$ leukocyte infiltrate into the tumor. (B) $CD8^+$ TILs as percentage of total $CD45^+$ cells are shown in treated versus untreated groups. (C) Representative immunofluorescent image of tumor tissues from control groups or KRAS_G12D immunized mice for CD8 expression. (D) Frequency of CTLA-4 and PD-1 expression on tumor infiltrating $CD8^+$ T cells (E) TIL populations of G-MDSC ($CD11b^+Ly6C^-Ly6G^+$) and M-MDSC ($CD11b^+Ly6C^+Ly6G^-$) were identified by flow cytometry

RESULTS (cont.)

Lm-HOT treatment reduces frequency of Tregs in CT26 tumors

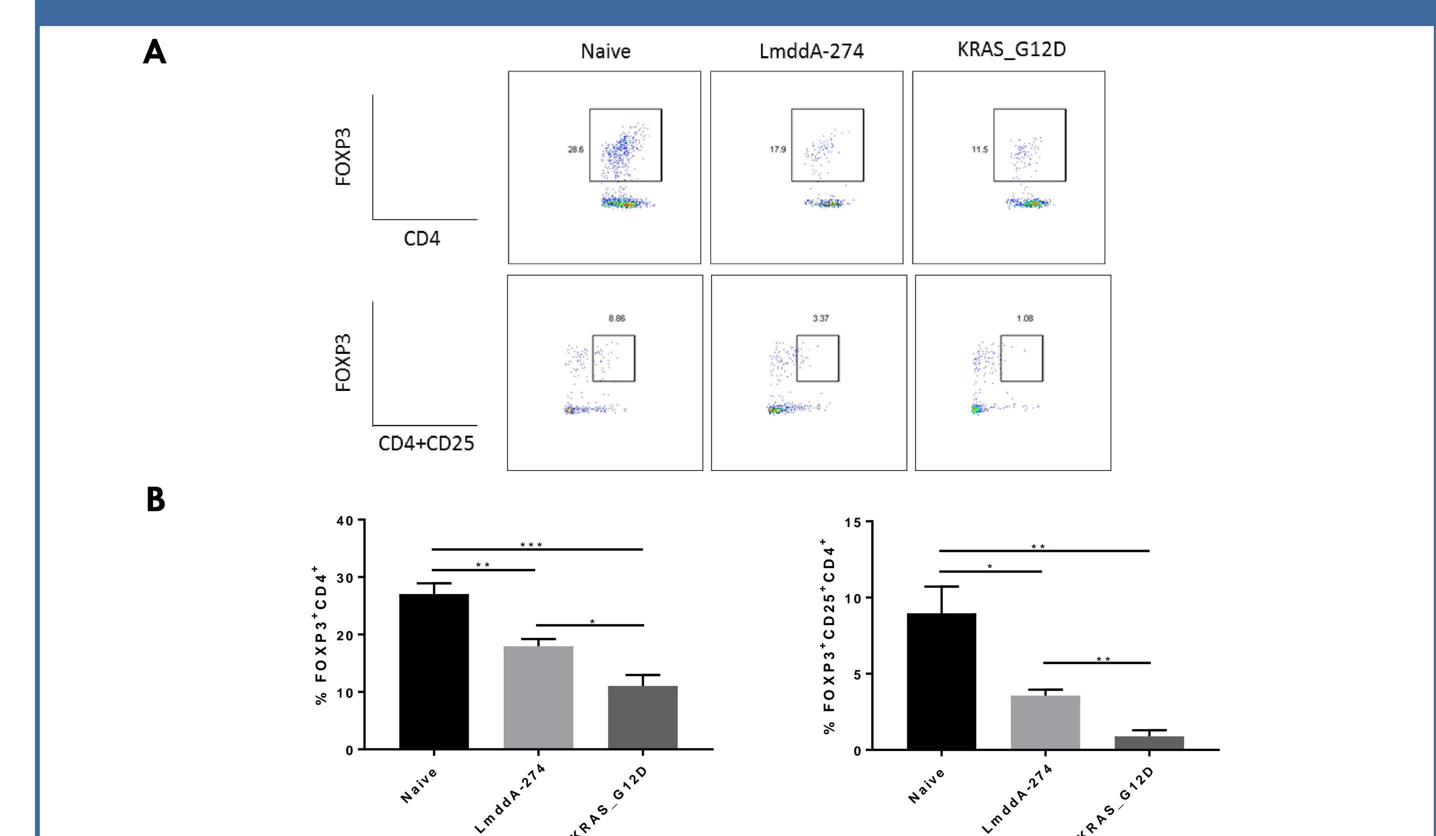

Figure 4. *Lm*-KRAS_G12D therapy reduces frequency of Tregs in CT26 tumors. (A-B) Cohorts of CT26 tumor-bearing mice were treated as in Figure 1A and TILs from tumors of CT26 mice were harvested 15 days after tumor implantation. (A-B) Representative flow dot plots and summary data show the percentage of Tregs ($Foxp3^+CD25^+$ and $Foxp3^+CD25^+CD44^+$) of $CD45^+$ cells. . *P<0.05; **P<0.01; ***P<0.001; Error bars indicate SEM of n = 5/group.

Lm-HOT treatment increased the frequency and functionality of tumor-infiltrating T cells

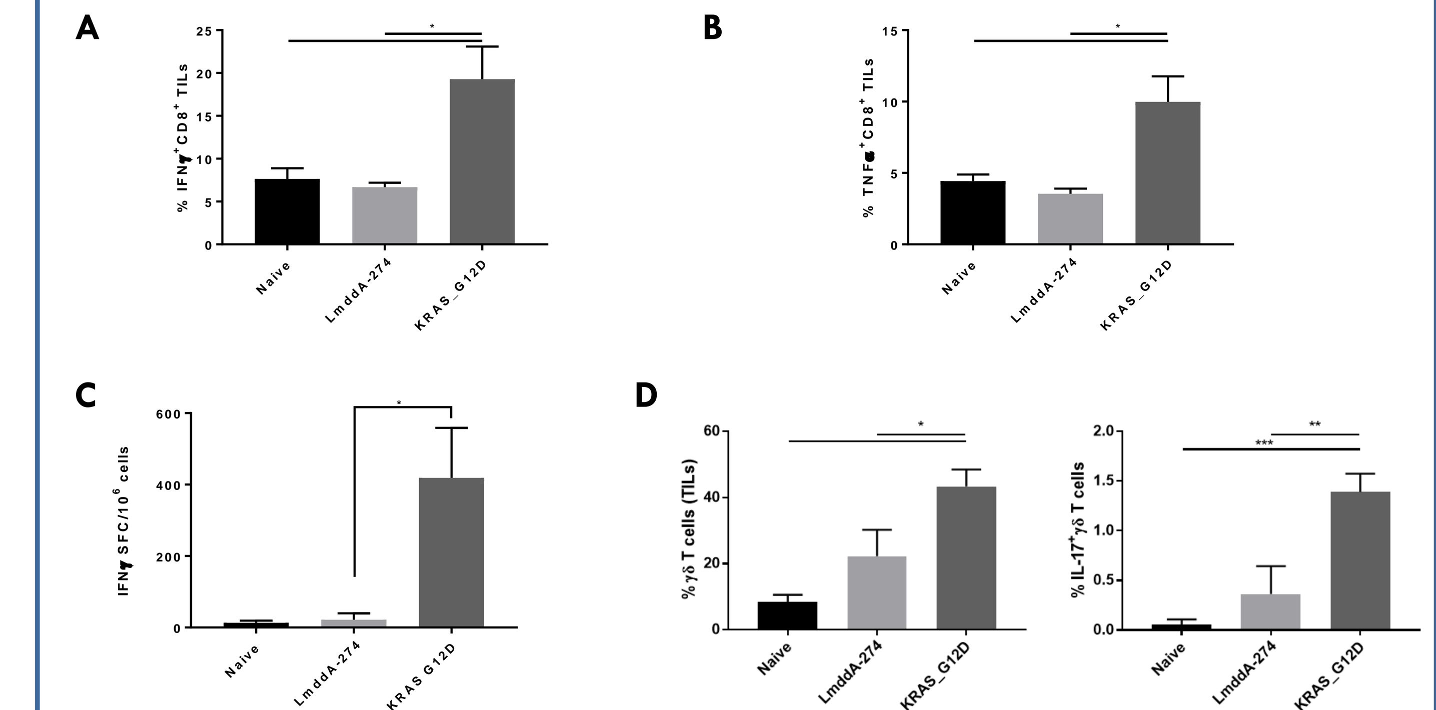

Figure 5. Immunization with *Lm*-KRAS_G12D increased the frequency and function of specific inflammatory cytokine production of tumor infiltrating T cells. (A-B) Summary data showing percentage of $CD8^+$ T cells releasing IFNγ and TNFα following incubation with PMA/ION stimulation. (C) ELISpot analysis of KRAS_G12D-specific IFNγ responses from tumors stimulated with KRAS_G12D peptide pool and its predicted CTL epitope. (D) Graphs showing tumor infiltrating γδ T cells and γδ T cells releasing IL-17 following PMA/ION stimulation.

A clinical ADXS-503 HOT construct inhibits tumor growth

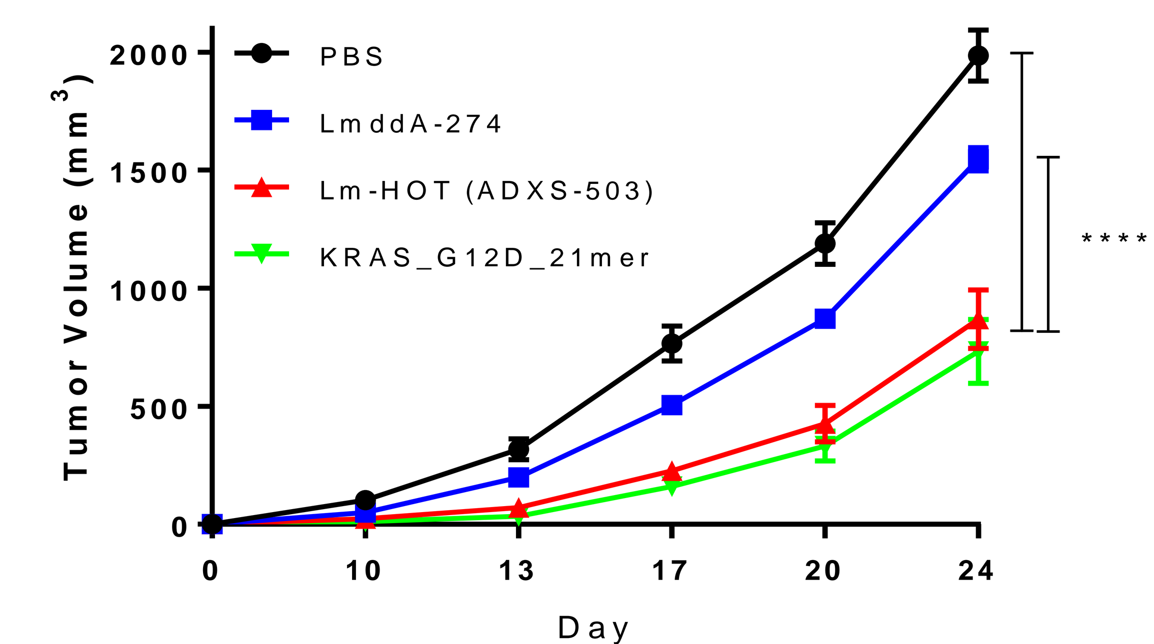

Figure 6. Therapy using a clinical *Lm*-HOT (ADXS-503) construct significantly impairs the growth of CT26 tumors. Naïve BALB/c mice were implanted with 300,000 CT26 colorectal tumor cells in the flank and 4 days after tumor implantation, mice were immunized with one of the following *Lm*-constructs 1) 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.

SUMMARY

- *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.