Agonism of the CD137 co-stimulatory pathway synergizes with the *Listeria monocytogenes*-based immunotherapy, axalimogene filolisbac, to promote durable tumor regression in a murine HPV$^+$ tumor model

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INTRODUCTION

- Axalimogene filolisbac (AXAL), a live attenuated *Listeria monocytogenes (Lm)*-based immunotherapy targeting the human papillomavirus (HPV)16-E7 protein, is currently being evaluated as a monotherapy or in combination with co-inhibitory blockade in the treatment of HPV-associated cancers.
- Advaxis' *Lm*-based immunotherapies act by stimulating innate immunity through multiple innate immune sensors including STING, by reducing the frequencies and functions of immunosuppressive cells in the tumor microenvironment, and by inducing the generation of tumor antigen-specific T cells that infiltrate and destroy the tumor.[1]
- To identify targets other than co-inhibitory receptors to combine with Advaxis' *Lm*-based immunotherapies, we took a rational approach. Namely, we sought targets that have been shown to be involved in immunity to *Lm,* reasoning that agonism of such a target would enhance the antitumor activity of the *Lm*-based immunotherapy.
- Using this approach, **we identified CD137, an inducible T cell co-stimulatory receptor, whose interactions with its ligand CD137L are essential for immunity to *Lm*.**
  - CD137-deficient mice are more susceptible than wild-type mice to infection with LD_{50} and LD_{100} doses of *Lm*.[2]
  - Wild-type mice treated with an agonistic anti-CD137 monoclonal antibody (mAb) are more resistant than untreated-wildtype mice to infection with LD_{50} and LD_{100} doses of *Lm*.[2]
  - CD137L-deficient mice have impaired CD8$^+$ T cell responses during *Lm* infection.[3]
  - It is important to note that CD137-CD137L interactions **are not required** to generate CD8$^+$ T cells reactive to vaccinia,[4] another vector platform used in therapeutic cancer vaccines.

OBJECTIVE

To test our hypothesis that agonism of the CD137 co-stimulatory pathway enhances AXAL-mediated tumor control in a murine HPV$^+$ tumor model.

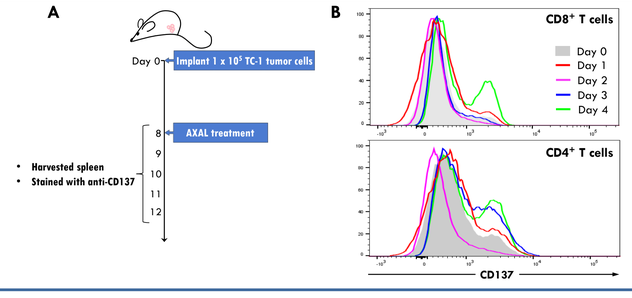
MATERIALS AND METHODS

- C57BL/6 female mice were implanted with 1 x 10^5 TC-1 tumor cells, which express the E6 and E7 proteins from HPV16. For tumor control experiments, we adopted a staggered treatment strategy, with AXAL administered first followed 4 days later with anti-CD137 mAb and/or another antibody -based immunotherapy (see **Figure 2A**). Briefly, TC-1 tumor-bearing mice were treated on day 8 post-tumor implantation with a single agent (including isotype mAb and empty vector (XFL7) controls) and at 7-day intervals thereafter for a total of 3 doses. For those experimental groups receiving an additional antibody-based immunotherapy (all mAbs were purchased from BioXcell and were used at subtherapeutic doses), anti-CD137 mAb (clone LOB12.3; 150 µg and 300 µg), anti-PD-1 mAb (clone RMP1-14; 100 µg) and/or anti-CTLA-4 mAb (clone 9H10; 50 µg) were administered on days 12, 19 and 26 post-tumor implantation. Tumor size was measured twice a week using digital calipers.
- Flow cytometric analysis of splenocytes or tumor-infiltrating leukocytes (TILs):
  - Tumors were dissociated into single cell suspensions using the Mouse Tumor Dissociation Kit (Miltenyi Biotec). Single cell suspensions of splenocytes were prepared by gently pressing the spleen through nylon mesh followed by red blood cell lysis. The resulting single cell suspensions were stained with fluorochrome-conjugated H-2D^b/HPV16-E7 peptide dextramer (Immudex) and fluorochrome-conjugated monoclonal antibodies against various surface and intracellular antigens. Ebioscience's Fixable Viability Dye eFluor™ 520 was used to discriminate live and dead cells.
  - To assess IFNγ production by tumor-infiltrating T cells, 2 x 10^6 TILs were cultured for 5 hours in the presence of Ebioscience's Cell Stimulation Cocktail (plus protein transport inhibitors) prior to flow cytometric analysis.
  - Cells were acquired on an Attune NxT flow cytometer (ThermoFisher). Data analysis was performed using FlowJo software (Tree Star, Inc.).
- Serum samples were collected at day 19 post-tumor implantation and tested for ALT and AST activity (Abcam) to assess the effect of various treatments on liver function.
- Statistical analyses were performed using GraphPad Prism software.

RESULTS

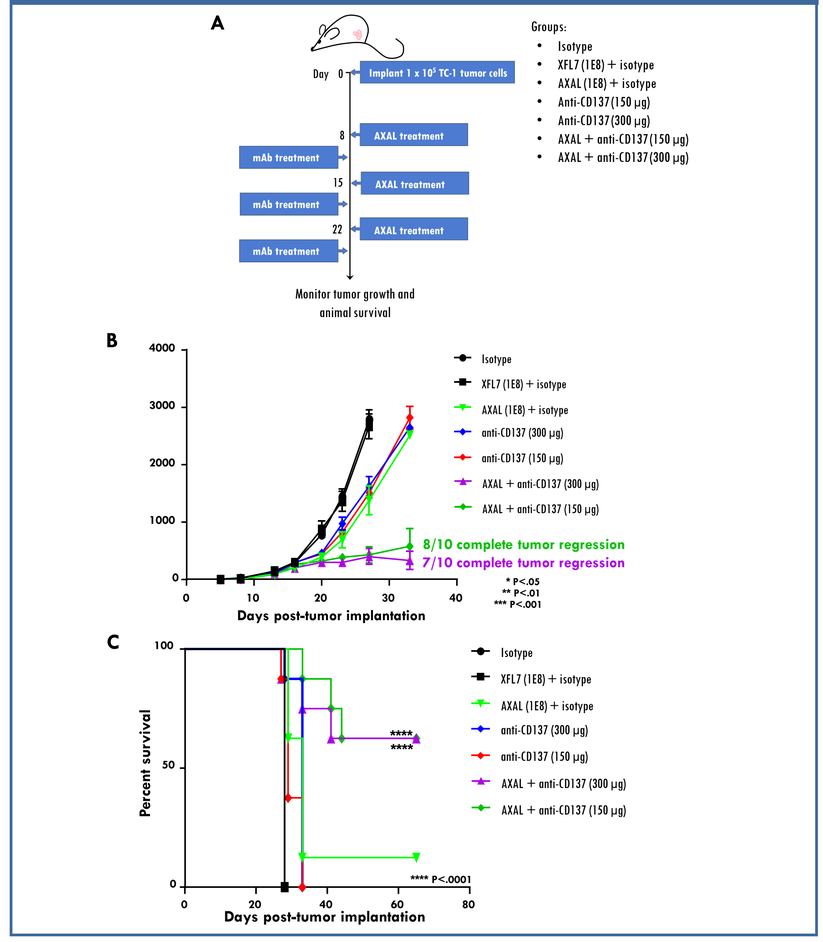
- To optimize the timing of anti-CD137 mAb treatment in combination with AXAL, we examined the kinetics of CD137 expression on T cell effectors in tumor-bearing mice after a single dose of AXAL (**Figure 1A**).
  - As shown in **Figure 1B**, AXAL induced the expression of CD137 on splenic CD8$^+$ T cells on Day 4 post-AXAL-treatment and on splenic conventional (Foxp3$^-$) CD4$^+$ T cells on Day 3 post-AXAL-treatment.
  - **Based on these findings, we adopted a staggered treatment regimen in which anti-CD137 mAb treatment is administered 4 days after AXAL treatment in order to coincide with the peak of AXAL-induced CD137 expression on antitumor effector cells (Figure 2A).**
- Next, we tested whether agonism of the CD137 co-stimulatory pathway, using two mAb concentrations (150 µg and 300 µg), synergized with AXAL to control tumor growth and to prolong animal survival (**Figure 2**)
  - Although treatment with AXAL or anti-CD137 mAb inhibited tumor growth, **only treatment with AXAL + anti-CD137 mAb resulted in tumor regression (Figure 2B).**

Figure 1. AXAL treatment induces CD137 expression on both CD4$^+$ and CD8$^+$ T cells within 4 days



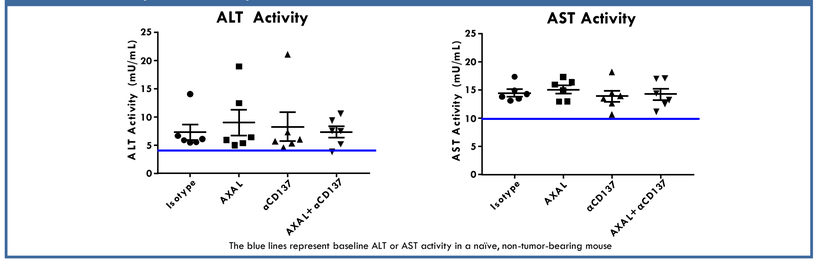
- Notably, both doses of anti-CD137 mAb synergized with AXAL to inhibit tumor growth and to prolong animal survival (**Figure 2B and 2C**). Consequently, all subsequent studies were performed using the lower dose (150 µg) of anti-CD137 mAb.

Figure 2. Synergistic effects of combining AXAL and anti-CD137 mAb on tumor growth and animal survival



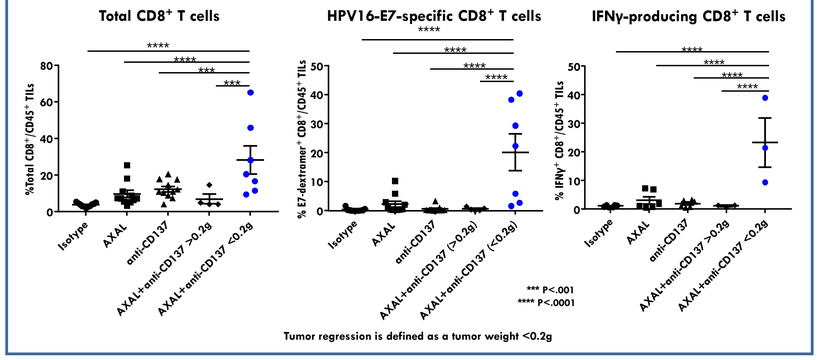
- Given that treatment with anti-CD137 mAbs can lead to liver toxicity in both mice and humans[5] and that *Lm* have a tropism for the liver, we assessed liver function in tumor-bearing mice treated with isotype control, AXAL, anti-CD137 mAb (150 µg), or AXAL + anti-CD137 mAb (150 µg) on day 19 post-tumor implantation by measuring serum liver enzyme (ALT and AST) activity (**Figure 3**).
  - **No differences in serum ALT or AST activity were observed among the treatment groups, indicating that combining AXAL with the lower dose of anti-CD137 mAb does not result in liver damage.**

Figure 3. Combining AXAL and anti-CD137 immunotherapies does not elevate serum liver enzyme activity



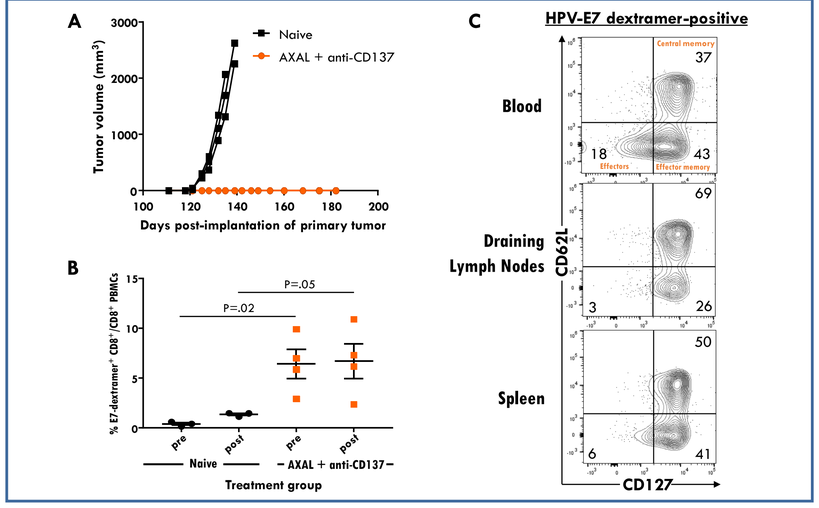
- To elucidate the mechanism(s) by which AXAL + anti-CD137 mAb mediate tumor regression, phenotypic analysis of the tumor-infiltrating immune cells was performed on day 19, at the start of apparent tumor growth inhibition.
  - **Significant increases in the frequencies of tumor-infiltrating CD8$^+$ T cells, HPV-E7-specific CD8$^+$ T cells, and IFNγ-producing CD8$^+$ T cells were observed in AXAL + anti-CD137 mAb-treated mice with regressed tumors compared to mice treated with either agent alone or with isotype control (Figure 4).**

Figure 4. Tumor regression in the AXAL + anti-CD137 mAb treatment group is associated with increased levels of tumor-infiltrating HPV-E7 specific CD8$^+$ T cells



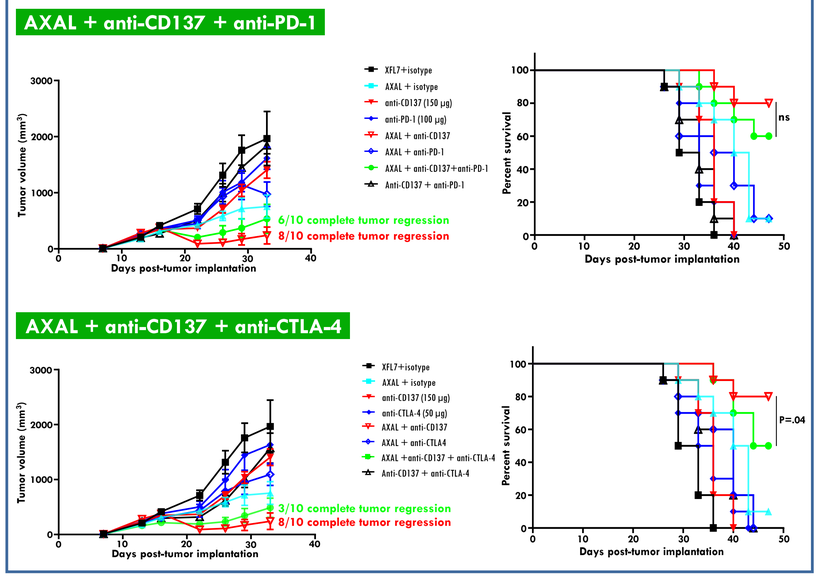
- To evaluate the durability of the antitumor T cell responses, mice with complete tumor regression were re-challenged in the opposite flank with TC-1 tumor cells on day 111.
  - **All 4 re-challenged mice remained tumor-free in both flanks for an additional 10 weeks, the time of study termination (Figure 5A).**
  - **High frequencies of HPV-E7-specific CD8$^+$ T cells were detected in the blood of the 4 mice, both 1 day before, and 7 days after, tumor re-challenge (Figure 5B).**
  - **On day 182 post-implantation of primary tumor, HPV-E7-specific CD8$^+$ T cells in the lymphoid organs of the re-challenged mice were phenotyped and found to be primarily central memory T cells (Figure 5C), a memory T cell population that has more efficient antitumor activity than effector memory T cells.[6]**

Figure 5. Durable tumor regression induced by AXAL + anti-CD137 mAb is associated with a high frequency of circulating memory HPV-E7-specific CD8$^+$ T cells



- Last, we investigated whether adding a checkpoint inhibitor, either anti-PD-1 mAb or anti-CTLA-4 mAb, to the AXAL + anti-CD137 mAb combination further enhances the ability of this combination to control tumor growth or to prolong animal survival.
  - **Neither of the triple combinations were more effective than AXAL + anti-CD137 mAb at controlling tumor growth or at prolonging animal survival (Figure 6).**

Figure 6. Triple combinations are not more effective at tumor growth inhibition and at prolonging animal survival than AXAL + anti-CD137 mAb



SUMMARY AND CONCLUSIONS

- AXAL treatment induces CD137 expression on both CD4$^+$ and CD8$^+$ T cells within 4 days.
- Combining AXAL and an agonistic CD137 mAb in a staggered fashion leads to the expansion of tumor-antigen specific CD8$^+$ T cells and to durable antitumor immunity in the mouse TC-1 tumor model, in the absence of liver toxicity. **These data support this combination as a potential new therapeutic strategy.**
- Adding a checkpoint inhibitor to the AXAL + anti-CD137 mAb combination **does not enhance** the ability of AXAL + anti-CD137 mAb to inhibit tumor growth and prolong animal survival.

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