INTRODUCTION

- Advaxis’ Listeria monocytogenes (Lm)-based immunotherapies are live attenuated bacterial vectors that are bioengineered to secrete an antigen-adjunct fusion protein consisting of a truncated non-haemolytic fragment of listeriolysin O (LLO), which has adjuvant properties, and one or more tumor-associated or tumor-specific antigens.
- 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, such as regulatory T cells (Tregs), in the tumor microenvironment (TME), and by inducing the generation of tumor antigen-specific T cells that infiltrate and destroy the tumor.1
- The purpose of this study is to investigate further the mechanism by which Advaxis’ Lm-based immunotherapies alter intratumoral Treg homeostasis and function.

OBJECTIVE

To use flow cytometric analysis, immunofluorescence microscopy, and immune-related gene expression analysis to investigate the mechanism by which Advaxis’ Lm-based immunotherapies alter intratumoral Treg homeostasis and function.

MATERIALS AND METHODS

- C57BL/6 mice were implanted with TC-1 tumor cells, which express the E6 and E7 proteins from human papillomavirus (HPV)16. On day 8, when T cells were palpable, mice were treated with either one dose (1 x 10^6 CFU) of ADXS-701, an Lm-based vector expressing ILLO fused to the E6 and E7 proteins from HPV16 and HPV18, one dose (1 x 10^8 CFU) of LmddA 274, on Lm-based vector expressing ILLO but no tumor-specific antigen, or PBS. Five days post-treatment, tumor-infiltrating lymphocytes were phenotyped by flow cytometry and intact tumors were evaluated by immunofluorescence microscopy and immune-related gene expression analysis. For tumor control experiments, TC-1 tumor-bearing mice were treated with 3 doses of LmddA 274 or ADXS-701 at 7-day intervals. Tumor size was measured twice a week.
- Flow cytometric analysis of tumor-infiltrating lymphocytes
  - Tumors were dissociated into single cell suspensions using the Mouse Tumor Dissection Kit (Miltenyi Biotec). The resulting single cell suspensions were plated based on treatment group and then stained with fluorochrome-conjugated monoclonal antibodies against various surface and intracellular antigens. Ebciscis’s Flowscan Violed Eflow™ 320 was used to discriminate live and dead cells.
  - To assess IFNγ and IL-17A production by various infiltrating T cell subsets, 2 x 10^6 cells were cultured for 5 hours in the presence of Ebciscis’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.).
- Immunofluorescence microscopy of the TME
  - Intact tumors were embedded in Tissue Tek OCT and snap frozen in liquid nitrogen. Embedded tumor tissue was sectioned at 5 µm thickness, fixed in 4% formaldehyde, and then blocked with 10% normal rat serum prior to staining with fluorochrome-conjugated monoclonal antibodies at room temperature, overnight, in moist dark chambers.
- Stained sections were imaged using the Zeiss Axio Imager M2 microscope equipped with an ApoTome. The varying method was used to image the whole section, using a motorized stage.
- Immune-related gene expression analysis of the TME
  - Total RNA was extracted from intact tumors harvested on day 13 post-implantation, and the NanoString nCounter PanCancer Immune Profiling Panel was used to quantitate expression levels of 770 genes.
- Differential gene expression analysis was conducted on a normalized NanoString count data from tumors of ADXS-701- and PBT-treated mice. NanoString’s nSolver™ software was used to identify genes that were differentially expressed (p<0.05 log-fold change ≥ 2) between the 2 groups.
- Ingenuity Pathway Analysis (IPA) (Qiagen) was used to identify signaling pathways and networks that are activated in the tumors of ADXS-701-treated mice compared to PBS-treated mice.
- Statistical analyses were performed using GraphPad Prism software.

RESULTS

- We reasoned that Lm-based immunotherapies, to be effective at tumor control, must reprogram the immunosuppressive TME early during the course of treatment in order to induce and sustain infiltration of tumor-specific T cells. Accordingly, to investigate the mechanism by which Advaxis’ Lm-based immunotherapies alter intratumoral Treg homeostasis and function, we analyzed the TME 5 days after a single dose of LmddA 274 (no tumor-specific antigen control) or ADXS-701.
- On day 13 post-implantation, we observed significant reductions in the frequencies of intratumoral Tregs (defined as CD4^+ Foxp3^+ CD3^+) relative to total CD4^+ T cells and to total CD45^+ cells in LmddA 274- and ADXS-701-treated mice compared to PBS-treated mice (Figure 1A).
- The extent of reduction in Treg frequency observed in LmddA 274- and ADXS-701-treated mice on day 13 post-implantation was comparable to those previously observed in LmddA 274- and ADXS-701-HVLP7-treated mice on day 24 post-implantation (Figure 1B).2
- To determine whether the reduction in intratumoral Treg frequency is the result of impaired Treg homeostasis, we compared the proliferative status and viability of Tregs in tumors of mice treated with PBS, LmddA 274 or ADXS-701 by flow cytometry.
- The percentage of proliferating (defined as Ki-67^+) Tregs was comparable between the 3 treatment groups (Figure 2A), but the percentage of dead Treg (defined as Flodex Viability Dye eFlour™ 520-positive), assayed ex vivo and after a 5-hour culture period in the presence of PMA and ionomycin, was significantly higher in ADXS-701-treated mice than in PBS-treated mice (Figure 2B).
- Further phenotypic analysis revealed that the percentage of Tregs expressing CCR8, a chemokine receptor whose expression is essential for Treg survival and function,4 was significantly reduced in LmddA- and ADXS-701-treated mice compared to PBS-treated mice (Figure 3A-C).
- Expression levels of other Treg phenotypic markers, namely Foxp3 and CD39, were also reduced in total Tregs and in CCR8-negative Tregs from LmddA-274- and ADXS-701-treated mice compared to PBS-treated mice (Figure 3D-E).
- The phenotypic changes in intratumoral Tregs from LmddA 274- and ADXS-701-treated mice were associated with a dramatic increase in the infiltration of activated cytokine-producing T cell effectors into the tumor.
- Increases in the frequencies of total T cells, CD8^+ T cells and γδ T cells relative to total CD45^+ cells were observed in LmddA 274- and ADXS-701-treated mice compared to PBS-treated mice (Figure 4; P<0.05).
- Increases in the frequencies of proliferating T cells, CD4^+ T cells and CD8^+ T cells were observed in LmddA 274- and ADXS-701-treated mice compared to PBS-treated mice (Figure 4A; P<0.05).
- Increases in the percentages of IFNγ-producing CD4^+ and CD8^+ T cells, and CD8^+ T cells with a high level of IL-17A-producing γδ T cells were observed in LmddA 274- and ADXS-701-treated mice compared to PBS-treated mice (Figure 5B; P<0.05).
- The increased infiltration of activated cytokine-producing T cell effectors into the tumors of LmddA 274- and ADXS-701-treated mice may result in a bias in Treg suppressive activity, T cell effector reactivity to Treg suppression, or both.
- Immunofluorescence microscopy revealed the presence of T cell clusters in the tumors of all three treatment groups (Figure 6). While the clusters in PBS-treated mice were composed predominantly of Tregs with very few T cell effectors, those in LmddA 274- and ADXS-701-treated mice were composed predominantly of T cell effectors with very few Tregs.
- Many of the top 32 genes that were found to be differentially expressed in tumors from ADXS-701- and PBS-treated mice were consistent with the changes in T cell quantity and function that were demonstrated by flow cytometric analysis (Figure 7).

SUMMARY AND CONCLUSIONS

- Within 5 days of administration, a single dose of an Advaxis’ Lm-based vector alters the tumor microenvironment by impairing Treg survival and function and by promoting effector T cell recruitment, activation and differentiation.
- Changes in the suppressive Treg phenotype, specifically the downregulation of CCR8 expression, may be a key mechanism by which Advaxis’ Lm-based immunotherapies impair intratumoral Treg homeostasis and function.

REFERENCES