Evaluation of total PD-1 expression using multi-color flow cytometry in Metastatic Non-Small Cell Lung Cancer patients treated with Multi-Neoantigen Vector (ADXS-503) in combination of Pembrolizumab to assess T cell subsets

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INTRODUCTION

Non-Small Cell Lung Cancer (NSCLC) – Current Challenges:

Patients who progress on PD-1/ -L1 blockade represent an unmet need with limited treatment options

- Checkpoint inhibitor (CPI) re-challenge after disease progression show a low ORR (3-13%, only PR) and disease control rate of ~ 45% in NSCLC (Katayama Y, 2020; Gobbini E, 2020)

Progression free survival and overall survival rates obtained with CPIs in 1st line therapy must improve

- The median PFS achieved with CPI alone in 1st line (i.e., 7-10 m) does not seem to be improved when CPI is combined with chemo ± bevacizumab (i.e., 5.2 -9 m) (Socinsky MA, 2018; Gadgeel S, 2020)

ADXS-503 Immunotherapy

- ADXS-503 is an off-the-shelf, live attenuated Listeria monocytogenes (Lm)- immunotherapy developed to 1) reverse the resistance to CPI in NSCLC patients progressing on PD-1/-1 blockade and 2) to increase the sensitivity to PD-1/-L1 blockade in 1st line therapy
- ADXS-503 is bioengineered to secrete an antigen-adjuvant fusion proteins (tLLO-503) consisting of a truncated fragment of listeriolysin O (tLLO) fused to 22 tumor antigens commonly found in NSCLC (Fig. 1)

Combination of ADXS-503 with PD-1/-L1 blockade

- Lm vectors are effective at inducing innate and adaptive immunity, generating T cells that target multiple neoantigens (Hecht JR et at, 2019)
- Published preclinical & clinical data have shown synergistic activity of the combination of ADXS Lm-based immunotherapies with a PD-1 blocking antibody (Bongiorno, 2017, Stein MN et.al., 2020)
- Lm vectors also neutralize Tregs and MDSCs in the tumor microenvironment and increase PD-1 expression
- ADXS-503-101 is an ongoing Phase 1 /2 clinical trial (NCT03847519), designed to evaluate the safety, tolerability and preliminary clinical and immunological activity of ADXS-503 alone and in combination with anti-PD-1 antibody therapy, in subjects with NSCLC (Figure 2)
- The administration of Pembrolizumab (Pembro) to patients in this study may interfere with the accurate quantification of PD-1 in peripheral blood mononuclear cells (PBMCs) obtained from Part B (progressing on Pembro) and Part C (1st line therapy) patients

BACKGROUND

Precision for Medicine (Precision) developed and qualified two multi-color flow immunophenotyping assays to quantify total PD-1 expression in cryopreserved peripheral blood mononuclear cells (PBMCs). The PD-1 expression, immune cell subsets composition and their activation status will be used as pharmacodynamic biomarkers for Advaxis clinical studies in patients with Metastatic Non-Small Cell Lung Cancer treated with ADXS-503 alone and in combination with Pembrolizumab. Pembrolizumab (Pembro) is a programmed death receptor-1 (PD-1)-blocking antibody approved for the treatment of advanced lung cancer. ADXS-503 and Pembro have complementary mechanisms of immune activation and reversal of immune tolerance.

The detection of free PD-1 and Pembro-bound PD-1 was achieved by co-staining a partially competing αPD-1 antibody (clone PD1.3.1.3) with a biotinylated αHu-IgG4 antibody. The assay conditions were optimized for sensitivity, optimal signal:noise ratio, detection of free and drug bound receptor by titrating and testing various commercial αPD-1 antibody clones and tertiary reagents to detect biotinylated αHu-IgG4.

These flow assays will facilitate the evaluation of both free and drug bound PD-1 expression as a pharmacodynamic biomarker in T-cells when PD-1 blockade is being used.

METHODS

Step 1. Separation and Storage of PBMCs
- For clinical application, clinical sites collected patient whole blood
- Centralized PBMC isolation using SepMate™ tubes- Ficoll density gradient separation
- PBMCs cryopreserved and stored in vapor phase of LN2 to maintain viability
- Longitudinal samples from each subject intended to be batch tested together

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Step 2. Thaw and Stain PBMCs
- PBMCs are thawed in complete medium, cells counted for viability
- Assay Controls included: single color controls for compensation controls
- Inter-assay healthy PBMC controls: full panel stain and fluorescence minus 1 or more markers, e.g. FMO stains, for objective setting of gates

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Step 3. Detection of PD-1
- For patient PBMCs assumptions are they have Pembro-bound PD-1
- Healthy PBMC control –pretreated with and without 10ug/mL of Pembro for assay control
- Pembro bound receptor are detected using a biotinylated anti-Hu-IgG4 antibody, followed with staining of fluorochrome conjugated to anti-biotin antibody
- Free PD-1 receptors are quantified using a commercial anti-PD-1 antibody (Miltenyi clone PD1.3.1.3)
- Total PD-1 reported when evaluating PD-1 marker

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Step 4. Full Panel Stain
- For PBMCs full flow panel staining for each of the two flow panels described below:
  - ❑ Panel 1 T cell/T cell memory/T-reg (16-marker)- Viability dye, CD3, CD4, CD8, CD45RO, CCR7, CD127, CD25, FoxP3, TIGIT, CD28, CD56, Ki67, Granzyme B, CD95, PD-1, αIgG4
  - ❑ Panel 2 T cell/NK cell/T cell-activation (15-color)- Viability dye, CD3, CD4, CD8, CD45RA, CCR7, CD127 CD25, CD28, CD56, CD16, CD154, CD38, PD-1, HLA-DR, αIgG4
- PBMCs are stained, washed and prepared for acquisition on flow cytometer

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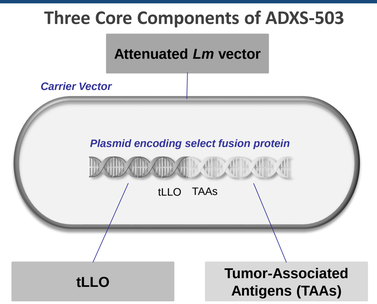
Step 5. Cell Acquisition and Detection with BD LSRFortessa™ 5-laser 20-parameter system

Figure 1. ADXS-503 Vector Design based on the *Lm* platform to Trigger Strong Immune Responses Against Tumor Associated Antigens (TAAs)

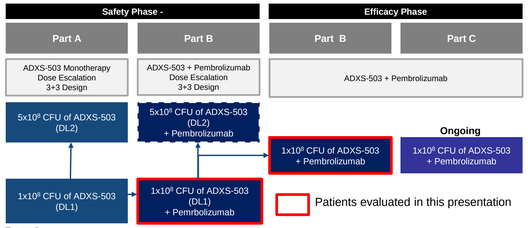


***Listeria monocytogenes* (*Lm*) bacteria induce an innate immune response**
- Carrier vector; irreversibly attenuated (≥ 4-log)
- Tropism to spleen & lymph nodes
- Increases PD-L1 expression in the tumor microenvironment (TME)

Truncated Listerolysin (tLLO-503)
- Promoter and adjuvant properties
- Neutralizes Tregs & MDSCs in the TME (Wallecha, 2013)

ADXS-503 harbors highly prevalent TAAs in NSCLC to induce adaptive immunity
- 22 TAAs total with 11 hot spot mutations and 11 oncofetal (OFAs)/cancer testis antigens (CTAs) *Lm* based-neoantigen vectors induce CD8+ T cell responses and antigen spreading (Goldman JW, 2021)

Figure 2. A Phase 1/2, Open-Label Study of ADXS-503 Alone and in Combination with Pembrolizumab in Subjects with Metastatic Squamous or Non-Squamous NSCLC



Part A
- Patients with relapsed, refractory metastatic non-small cell lung cancer (NSCLC) who received up to 3 prior lines of therapy, were eligible to receive ADXS-503 alone (n = 7 subjects).

Part B
- Patients with metastatic NSCLC who have progressive disease (PD) on initial scan while on Pembro as last therapy, are eligible to receive ADXS-503 + Pembro (n= 9 across efficacy & safety phases)
- 6 evaluable patients, one with partial response (PR) and three with stable disease (SD) for an overall response rate of 17% and disease control rate of 67% (Goldman JW, 2021)
- These patients have been evaluated with the novel flow cytometry assay in this presentation

Part C
- Patients with metastatic NSCLC in the 1st line setting who have PD-L1 expression ≥1% and no EGFR mutations or ALK translocations are eligible to receive ADXS-503 + Pembro (n=~2 patients)

Table 1. ADXS-503 Lm construct was designed to elicit T cell responses against highly prevalent tumor antigens in squamous and non-squamous NSCLC tumors

Proprietary TAA Peptides	
TAA	HLA Allele
CEACAM5	A*02:01
CEACAM5	A*24:02
CEACAM5	A*03:01
CEACAM5	B*07:02
STEAP1	A*02:01
STEAP1	A*24:02
RNF43	B*07:02
MAGE-A6	A*03:01
NY-ESO1	A*02:01
MAGE-A4	B*07:02
GAGE1	B*07:02

Hotspot peptides	
Gene	Hotspot
KRAS	G12C
KRAS	G12V
KRAS	G12A
EGFR	L858R
KRAS	G12D
U2AF1	S34F
BRAF	V600E
PIK3CA	E545K
TP53	R158L
EGFR	L861Q
TP53	R273L

- 11 antigens derived from somatic Hot Spot Mutations (HSM)
  - HSM selection process for ADXS-503 used an agnostic approach and has relied solely on ranking based on prevalence
- 11 Oncofetal and Cancer Testis antigens that are overexpressed/ differentially expressed in NSCLC
  - Proprietary, sequence-optimized peptides of these TAAs, (also referred to as heteroclitic peptides), were generated by modifying their anchor-residue positions in order to increase their binding affinity to MHC class I molecules
- ADXS-503 will potentially elicit T cell responses in practically all NSCLC patients as 42% of patients express ≥1 hotspot antigen and >90% express ≥1 TAA targeted by ADXS-503

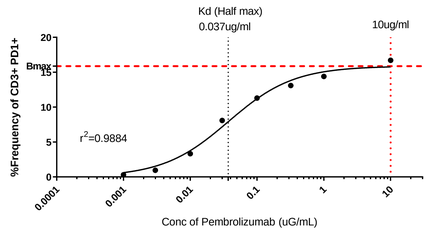
RESULTS: DETECTION OF PD-1 WITH PEMBROLIZUMAB IN FLOW ASSAYS

Figure 3. Develop a multi-color flow cytometry method for the accurate quantification of total PD-1 expression in cryopreserved peripheral blood mononuclear cells (PBMCs)

Evaluation of T cell memory, proliferation and activation, and NK cells and NK activation. Two flow panels both containing PD-1 marker were used in sample testing. The markers for each panel are described in the methods section.
- Panel 1 T cell/T cell memory/T-reg (16-marker)
- Panel 2 T cell/NK cell/T cell-activation (15-marker)

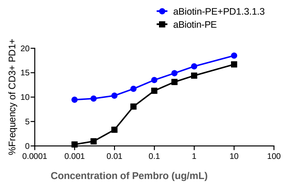
Legend

Figure 4. Saturation curve of Pembrolizumab: Healthy PBMCs treated with Pembro (0.001-10ug/mL) detected with Biotin α-IgG4 and α-Biotin-PE



- The maximum specific binding (Bmax) and half-maximum binding (Kd) of Pembrolizumab were calculated using specific binding with hill slope.
- Bmax = 10ug/mL
- Kd (Half-max) = 0.037ug/mL

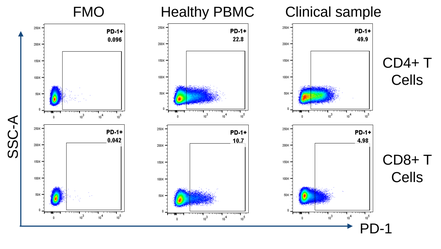
Figure 5. % Frequency of PD1+ CD3+ T cells on healthy PBMCs treated with a dose titration Pembro (.001-10ug/mL), Biotin α-IgG4, α-Biotin-PE, and Miltenyi clone PD1.3.1.3



%PD-1+ Frequency

Pembro ug/mL	αBiotin-PE +PD1.3.1.3	αBiotin-PE
0.000	9.15	0.23
0.001	9.47	0.31
0.003	9.71	0.96
0.010	10.30	3.33
0.030	11.70	8.08
0.100	13.50	11.30
0.320	14.90	13.10
1.000	16.30	14.40
10.000	18.50	16.70

Figure 6. PD-1 Detection in CD4+ and CD8+ T cells: Healthy PBMCs treated with 10ug/mL of Pembro before staining with Biotin α-IgG4 and α-Biotin-PE antibody, and staining with Miltenyi clone PD1.3.1.3



RESULTS: CLINICAL SAMPLE FLOW CYTOMETRY RESULTS

Figure 7. Expansion of NK cells supported by modulation of PD-1 and CD38 observed in NSCLC patients achieving stable disease with ADXS-503 + Pembro

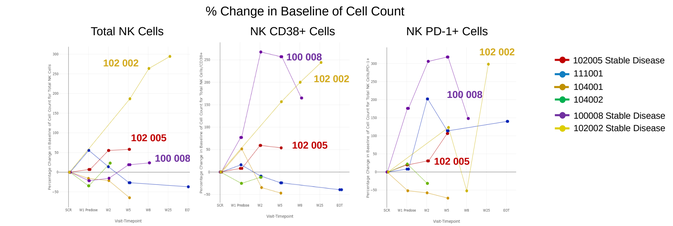


Figure 8. Expansion of CD8+ & CD4+ T-cells by modulation of PD-1 was observed in NSCLC patients treated with ADXS-503 + Pembro and achieving stable disease

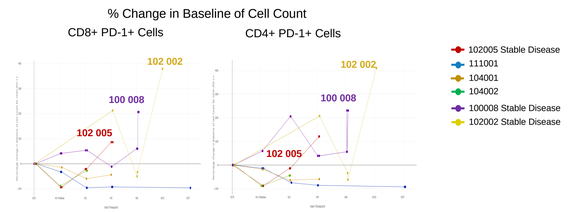


Figure 9. CD8+ T cell proliferation, cytotoxicity and memory cells correlates with stable disease in patients treated ADXS-503 with Pembro

CONCLUSION

- ❑ A novel flow cytometry analysis was developed to accurately identify PD-1 on PBMCs of NSCLC patients receiving PD1/PD-L1 blockade therapy with pembrolizumab + ADXS-503.
  - These assays performed as expected and no interference in PD-1 detection due to Pembrolizumab PD-1 blockage was observed.
  - The advantage of this PD-1 detection format is that PD-1 expression can be determined independently of PD-1 receptor status: both free and drug-bound PD-1 are accounted for in this detection method.
- ❑ The preliminary flow cytometry data confirmed the on-mechanism activation of innate and adaptive immune responses to *Lm* vectors like ADXS-503. The flow analysis of the three patients with clinical benefit (stable disease) after the addition of ADXS-503 to ongoing Pembrolizumab therapy, showed :
  - Proliferation and activation of NK cells
  - Increased PD-1 expression on circulating CD4+, CD8+ and NK cells
  - Increased counts of CD8+ T cells, including proliferative, cytotoxic and memory CD8+ T cell
  - Further analysis of patients in Part B and Part C is ongoing

ACKNOWLEDGEMENTS

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