# Digital spatial profiling and antigen-dependent phenotypic analysis of IL15-engineered tumor-infiltrating lymphocytes (cytoTIL15™ therapy) in an allogeneic melanoma PDX model

Rachel Burga, Zheng Ao, Arman Aksoy, Scott Lajoie, Kyle Pedro, Jack Tremblay, Gauri Kulkarni, Alonso Villasmil Ocando, Benjamin Primack, Meghan Langley, Theresa Ross, Jeremy Tchaicha, Mithun Khattar, Michelle Ols, Jan ter Meulen

*Obsidian Therapeutics, Inc. 1030 Massachusetts Avenue, Cambridge, MA 02138*

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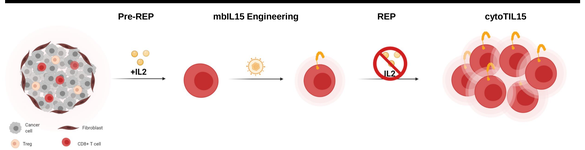
## Background

cytoTIL15™ therapy is an IL2-independent, engineered TIL product which allows pharmacological control of membrane-bound IL15 (mbIL15). We have previously shown that cytoTIL15 TILs demonstrate enhanced persistence and anti-tumor efficacy in a human allogeneic melanoma PDX model, utilizing the melanoma associated antigen, MART-1, as a model system based on conserved antigen reactivity. Here we use digital spatial profiling and single cell sequencing to characterize the RNA expression profile and phenotypic markers of tumor infiltrating immune cells as well as tumor cells in this model and compare the results to unengineered, IL2-dependent TIL.

## Rationale and Methods




cytoTIL15 therapy contains TILs engineered with mbIL15 under the control of a carbonic-anhydrase-2 drug responsive domain, regulated by the ligand acetazolamide (ACZ). cytoTIL15 cells were generated from human melanomas through a proprietary rapid expansion process. Expanded TILs were phenotyped and assayed for *in vitro* polyfunctionality, cytotoxicity, and frequency of tumor-associated antigen-specific TCR. *In vivo* phenotype and anti-tumor functionality was examined through adoptive transfer of TILs into NSG mice bearing subcutaneous, HLA-matched, patient-derived-xenograft (PDX) tumors expressing conserved melanoma-associated antigen (MAA) MART-1, in IACUC approved animal studies. Tumors, spleen, bone marrow, and blood were harvested 14-21 days following adoptive cell transfer and assessed by flow cytometry, GeoMx® (NanoString) digital spatial profiling, and single cell sequencing to characterize the TIL and the tumor microenvironment (TME).

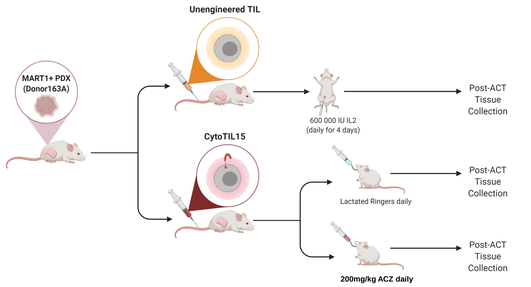



**Figure 1. Schema of PDX Model and cytoTIL15 treatment.** *Left to Right:* Melanoma patient-derived xenograft (PDX) tissue was serially passaged as 100 mg fragments implanted subcutaneously onto the flanks of female NSG mice. Animals were randomized 13-days following PDX-implant, and ACT with $10^6$ conventional TILs or cytoTIL15 was performed 14-days following PDX-implant. Unengineered TIL and cytoTIL15 were generated from two different HLA-matched, allogeneic donors. After adoptive cell transfer, animals received either IL2, ACZ, or vehicle dosing. At time points 14-21 days following adoptive cell transfer, tissues (tumor, cardiac blood, bone marrow) were harvested and assessed for downstream readouts. Assays included flow cytometry from individual fresh tissue cell suspensions, single cell RNA sequencing from pooled tumor suspensions, and GeoMx® digital spatial profiling from embedded and immunofluorescent stained tissue sections. Sequencing data was processed, normalized, and analyzed (for differential gene expression) using standard R packages: tidyverse, edgeR, limma, Seurat. All gene annotations were based on the GRCh38 reference genome and Ensembl 105 gene models.

## REP and IL15 engineering expands MART-1-reactive TILs

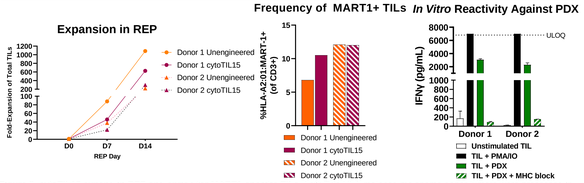
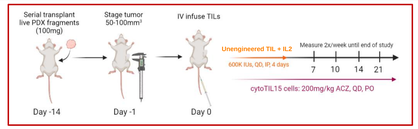



**Figure 2. cytoTIL15 expand in REP and enrich for MART1-specific TILs.** *Left:* Two HLA-matched melanoma donor tumors were processed in pre-REP, and cytoTIL15 and unengineered TILs (conventional TILs) were expanded in REP with feeder cells and supplements for 14-days. *Middle:* At the end of REP, unengineered and cytoTIL15 cells were assessed for the frequency of MART-1 specific TCR by tetramer staining. *Right:* Cryopreserved TILs were thawed and rested for 24 hours. A cryopreserved PDX tumor sample was digested into a single cell suspension and co-cultured at 1:1 target:effector ratio with rested TILs, with and without 80 ug/mL HLA ABC blocking reagent. After 24 h co-culture, supernatant was assessed for IFNγ content by MSD.

## cytoTIL15 therapy controls allogeneic melanoma PDX tumors in a MART-1-dependent manner



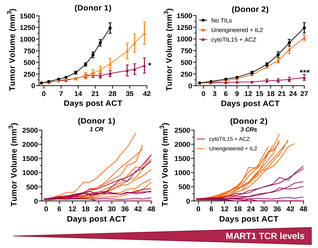




**Figure 3. cytoTIL15 therapy demonstrates superior tumor control *in vivo*, in an HLA-matched melanoma PDX model.** *Top:* MART1+ melanoma tumor tissue was excised from the donor and implanted subcutaneously into female NSG mice. Serial passaging was performed in vivo to establish a PDX model, and animals were randomized on day-13 following PDX implant. On Day 14 post-PDX implant, cytoTIL15 cells and unengineered TILs (expanded in REP with feeder cells +/- IL2 or ACZ for 14-days) from two HLA-matched and MART-1-reactive donors were adoptively transferred fresh intravenously. cytoTIL15 cells (animals dosed with 200 mg/kg ACZ PO daily), demonstrated improved anti-tumor efficacy as compared to unengineered TILs (supported with 600,000 IU IL2 QD IP for 4 days). *Bottom:* Individual spider plots for each animal on study reveals that anti-tumor efficacy was associated with increased frequency of MART-1-reactive TILs, as described in Figure 2. (n=8/arm; *p<0.05, ***p<0.001).

## cytoTIL15 cells infiltrate into tumors, circulate and accumulate in bone marrow, and are enriched for MART-1

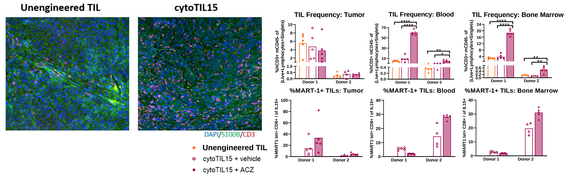
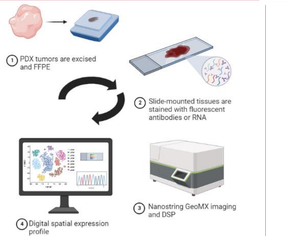



**Figure 4. cytoTIL15 demonstrate enhanced infiltration and accumulation.** *Left:* Subcutaneous PDX tumors were harvested 20-days following ACT with unengineered TILs (+IL2) or cytoTIL15 therapy. Tumors were formalin-fixed and paraffin-embedded, and immunofluorescence was performed to identify the TILs (CD3+, red color) infiltrating the tissue. *Right Top:* 14-days following ACT, PDX tumors, cardiac blood, and bone marrow were harvested, processed into single cell suspension, and stained and assessed via flow cytometry. Staining for the fraction of cells positive for anti-human CD3 and negative for anti-mouse CD45 revealed significant tumor infiltration by all TILs, and improved trafficking and accumulation into blood and bone marrow by cytoTIL15 therapy + ACZ. *Right Bottom:* Flow cytometry was also used to evaluate the fraction of transduced TILs (IL15+) staining positive with MART-1 TCR tetramer; MAA-reactive TILs enriched within all compartments, supporting the correlation of superior MAA-reactive TIL infiltration and improved anti-tumor efficacy. (n=5/arm; *p<0.05, **p<0.01, ***p<0.0001).

## cytoTIL15 cells exhibit a distinct differential gene expression profile from unengineered TIL

### Digital spatial profiling workflow




### Single cell RNA sequencing (scRNAseq) workflow

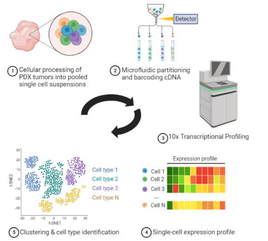



**Figure 5. Workflow for gene expression readouts.** *Left:* Digital spatial profiling was performed on FFPE PDX tumors, excised from mice 14-20 days following ACT with unengineered or cytoTIL15 TIL therapy, using the NanoString GeoMx® digital spatial profiling platform. Comparative analyses were restricted to the immune compartment identified by CD45+ cells. *Right:* Fresh tumors were excised, processed, and pooled per condition, and single cell suspension was obtained, barcoded, and assessed by 10X Genomics single-cell RNA sequencing (scRNAseq). Only cells that are CD3+CD56- were included in the comparative analyses. For the below figures, GeoMx results will be presented on the left panels, and scRNAseq results will be presented on the right panels.

### cytoTIL15 cells show enrichment in genes associated with enhanced infiltration and anti-tumor effector function

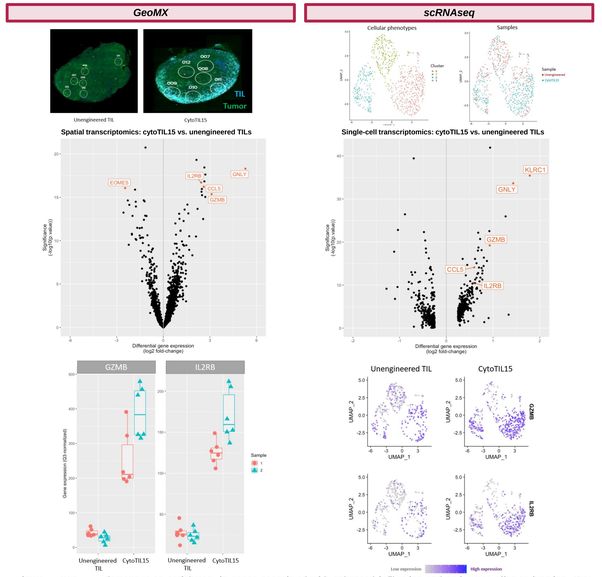



**Figure 7. cytoTIL15 demonstrates enrichment in genes associated with enhanced infiltration and anti-tumor effector function.** *Top:* Representative microscopy of digital spatial profiling revealing region of interest (ROI) selection for TIL (blue) and tumor (green) regions for PDX tumors treated with unengineered TILs or cytoTIL15 therapy. Similarly, representative unsupervised clustering of unengineered TIL- and cytoTIL15 therapy-treated tumors. *Middle:* Spatial and single-cell transcriptomics reveal a differential gene expression profile in cytoTIL15 cells vs. unengineered TILs, underscored by an enrichment in genes associated with pro-effector function and a favorable TIL phenotype such as IL2RB, GNLY, CCL5, GZMB, and KLRC1, and a decrease in exhaustion-associated genes such as EOMES. *Bottom:* GeoMX and scRNAseq reveal a significant ($p < 0.05$) enrichment in GZMB and IL2RB occurring in infiltrating cytoTIL15 cells but not unengineered cells, which is associated with the distinct cluster profiles of each cell treatment.

## Tumor-infiltrating and peripheral cytoTIL15 cells demonstrate enhanced stem-like anti-tumor phenotype

### Infiltrating and circulating cytoTIL15 cells produce potent effector cytokines

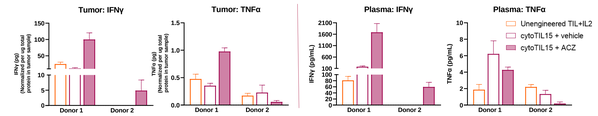



**Figure 8. cytoTIL15 cells demonstrate improved intratumoral and circulating cytokine production.** Cytokines (IFNγ and TNFα) were measured from excised PDX tumor suspension lysate (left) or from plasma obtained from cardiac blood (right) following ACT with unengineered or cytoTIL15 cells.

### Infiltrating and circulating MART1-specific cytoTIL15 cells have an activated, stem-like, memory phenotype

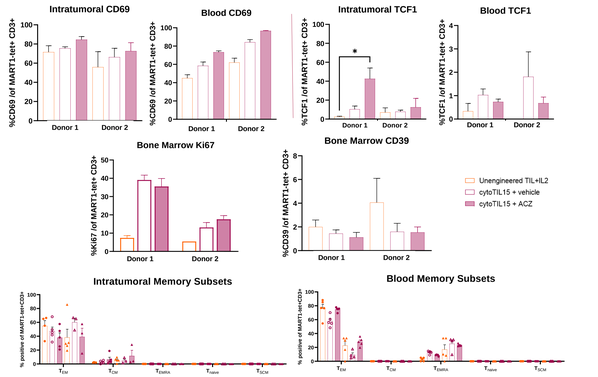



**Figure 9. Infiltrating and circulating MAA-specific cytoTIL15 cells have an activated, stem-like phenotype and exhibit memory formation.** *Top:* Infiltrating and circulating TILs are highly activated, expressing robust levels of CD69 (left), and cytoTIL15 cells demonstrate increased expression of transcription factor TCF1, which is associated with an acquisition of stem-like potential and in melanoma patients correlates with favorable therapeutic outcomes and responses to immunotherapy (right). *Middle:* Further supporting this distinct phenotype, cytoTIL15 cells accumulating in bone marrow niches have higher levels of Ki67, a nuclear protein associated with increased proliferative capacity, and have decreased levels of CD39 expression, which is associated with T cell terminal differentiation. *Bottom:* Intratumoral and circulating TILs have a distinct profile of T cell memory subsets, with an increase in central memory cells identified in infiltrating TILs, in contrast to the largely effector memory and TEMRA populations that were present in the TILs at the end of REP, and are reflected in the blood and bone marrow distributions. *p<0.05

## Conclusions

- cytoTIL15™ therapy demonstrates potent **anti-tumor cytotoxicity** against allogeneic tumor targets *in vitro* and *in vivo*.
- Anti-tumor efficacy is associated with reactivity to the conserved melanoma associated antigen (MAA), MART-1, in this antigen-specific model
- cytoTIL15 cells expand and infiltrate into PDX tumors *in vivo* and produce **cytokines** (IFNγ and TNFα) at the tumor site
- Adoptive cell therapy (ACT) **controls tumor growth** in this allogeneic setting, and enriched MAA-reactive TILs demonstrate a favorable anti-tumor phenotype.
- Specifically, the subpopulation of cytoTIL15 cells reactive to tumor-associated antigen displayed **increased expression of TCF-1**, which in melanoma patients has been associated with responses to immune checkpoint blockade, as well as progression free survival (PFS) and overall survival (OS)[1]
- cytoTIL15 cells showed a distinct profile of **RNA expression** consistent with their increased persistence and anti-tumor efficacy (*e.g.* IL2RB, GNLY, CCL5, GZMB, and KLRC1)



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*For questions, please reach out to Rachel Burga (rburga@obsidiantx.com).* *Schematics generated from biorender.io.* [1]*Zhou et al. Immunity 2010.*

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