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# Driving T cell dysfunction in vitro for rational immunotherapy design


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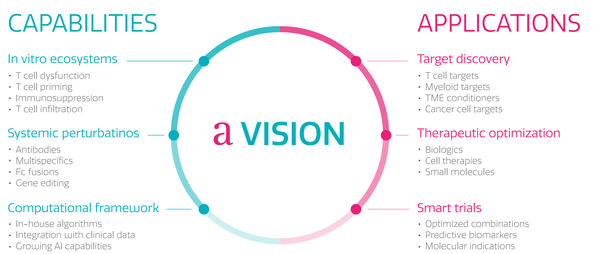
## Background

Immune checkpoint blockade (ICB) elicits durable responses in some cancer patients, but novel targets and combination approaches are needed to address resistance and broaden clinical benefit. Agenus is addressing this need with our Virtual Systems for Immuno-Oncology (VISION) platform. VISION is based on a collection of *in vitro* ecosystems which we deeply and systematically interrogate to discover novel targets, optimize our therapies and design smarter clinical trials. Here, we present an illustrative example of a VISION system modeling T cell dysfunction in a tumor microenvironment (TME)-like context for development of novel approaches to ICB resistance.

### Agenus VISION platform supports smart, streamlined drug discovery and development




## Methods

We developed a long-term human co-culture system comprised of primary T cells and cancer cells that enables controlled differentiation of naïve T cells to effector, memory and dysfunctional states. We longitudinally monitored T cell effector functions, protein and RNA expression across states and single cells. Finally, we challenged the system with anti-PD-1 antibody to uncover biomarkers and mechanisms of therapeutic resistance.

## Results

T cells in our system become activated and then gradually progress to a terminally dysfunctional state driven by multiple cancer antigen exposures. T cell cytotoxicity is maintained over several antigen exposures before sharply decreasing whereas cytokine secretion begins to decrease with only one prior antigen exposure. The expression of known T cell regulators and novel factors is altered over the time course, with known factors reflecting previous observations *in vivo*. Anti-PD-1 antibody prolongs cytotoxic capacity but T cells eventually fail to respond. Single cell mapping in the presence of anti-anti-PD-1 antibody reveals an expanded population of T cells that co-expresses PD-1, TIGIT and activation markers. Consistent with this, the combination of PD-1 and TIGIT blockade enhances cytotoxicity relative to monotherapies.

## Conclusions

These findings demonstrate the potential of VISION systems to deeply interrogate response and resistance to current and next-generation I-O therapies. In this case, we have applied a physiologically-relevant model of intratumoral T cell dysfunction to define a predictive biomarker signature for anti-PD-1 response and discover potential combination partners predicted to enhance durability of anti-PD-1 responses. With a growing repertoire of virtual systems and capabilities, VISION is poised to advance Agenus' multi-faceted approach to fighting cancer with immunotherapy.


**Disclosures**
Cailin E. Joyce, Matthew Hancock, Thomas Horn, Simarjot Pabla, Benjamin Duckless, Andrew Basinski, Dhan Chand, Jeremy D. Waight, Mariana Manrique, Tyler J. Curiel, Nicholas S. Wilson, Alex Duncan, Jennifer S. Buell, David A. Savitsky, Lukasz Swiech, John C. Castle – an Agenus Inc. current or former employment/consultancy and stock ownership



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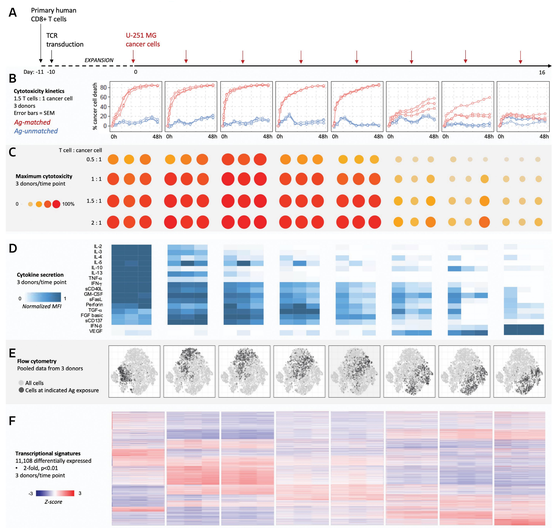
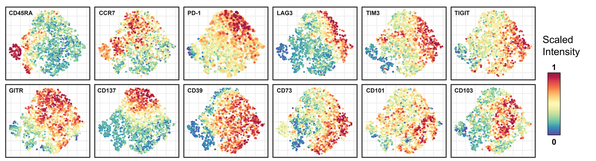
## Figure 1. Chronic cancer antigen exposure drives CD8+ T cell dysfunction in vitro



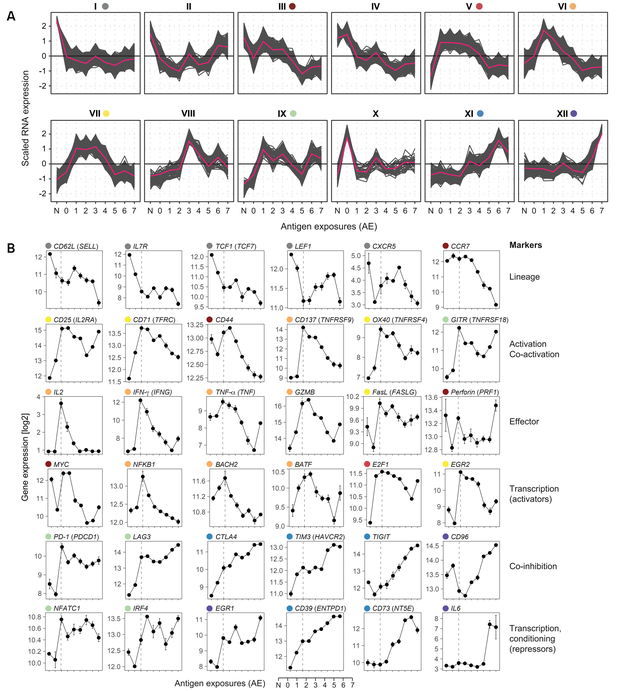

**A) Method for driving T cell dysfunction in vitro**. *T cell preparation:* PBMCs were isolated from three healthy donors on a Ficoll gradient and rested overnight. CD8+ T cells were isolated on magnetic beads, stimulated with CD3/CD28, transduced with NY-ESO-1 lentivirus, and expanded. TCR expression was confirmed by flow cytometry. *Cancer cell preparation:* U251 MG cell lines were transduced with lentivirus encoding a fusion protein of b2-microglobulin, HLA2-A2 and NY-ESO-1 or MART-1 peptide and selected on blasticidin. Antigen expression was confirmed with an NFAT reporter system in Jurkat T cells. For co-culture, a fixed number of irradiated U251 MG cells were cultured for 24h. CD8+ T cells were added to achieve the desired cancer:T cell ratio. The co-culture was monitored every 24h. If cancer cells were depleted, CD8+ T cells were transferred to a fresh well of irradiated U251 MG cells and the remainder were collected for downstream analyses. If cancer cells were not depleted, media was changed and reassessed the following day. **B) Cytotoxicity kinetics** *as monitored by live cell fluorescence microscopy. Cancer cells were stained with CellTrace Far Red and incubated overnight. CD8+ T cells were added to achieve the desired cancer:T cell ratio. Whole-well imaging in the Cy5 channel was assessed in confocal mode using an ImageXpress Micro Confocal High-Content Imaging System. Image analysis was performed in MetaXpress software. Briefly, intensity and object size thresholds were applied and the total cell area above these thresholds was calculated and normalized against the 0h time point.* **C) Maximum cytotoxicity** *across four T cell : cancer cell ratios, as calculated by area under the curve in 'B'.* **D) Secreted effector molecules.** *T cells were exposed to fresh antigen for 24h and supernatants were analyzed using the Human XL Cytokine Discovery Kit, Human Magnetic Luminex Assay or Human CD8+ T-Cell Magnetic Bead Panel. Analysis was performed using FlexMap 3D software. Briefly, mean fluorescence intensity (MFI) for each analyte was normalized to the maximum MFI observed over the course of the assay, and the resulting data was scaled between 0 and 1.* **E) tSNE visualization of flow cytometry measurements** *for twelve proteins. Assay details in Figure 2 legend.* **F) RNA expression** *for 11,108 differentially expressed genes. Assay details in Figure 3 legend.*

## Figure 2. Chronic cancer antigen exposure drives expression of activation and dysfunction markers on CD8+ T cells



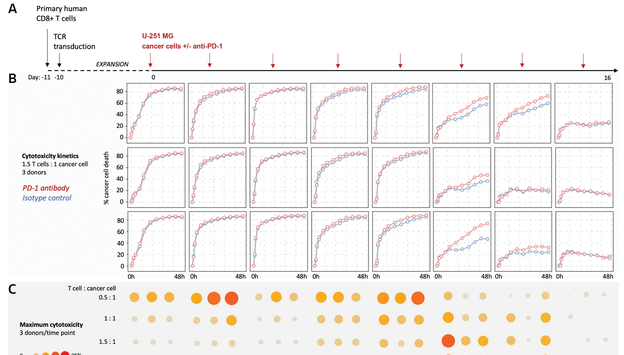

*Cells were washed, stained with 1) Near IR Live/Dead dye and 2) a mix of antibodies against CD3, CD8 and the markers listed below or isotype controls, and finally fixed in 1.6% paraformaldehyde. To accommodate integrated analysis of different time points all samples were processed and run in parallel. Data were analyzed in R using a custom workflow and functions from flowCore and flowDensity packages. Briefly, we gated on viable CD3+CD8+ cells, calculated a scaled mean fluorescence intensity and transformed using the hyperbolic arcsine to accommodate for negative values. Transformed data was used for k-means clustering (k=8) and t-Distributed Stochastic Neighbor Embedding (t-SNE) visualization (perplexity=20). Transformed expression data was further scaled between 0 and 1 for each marker.*

## Figure 3. Chronic cancer antigen exposure drives transcriptional signatures of activation and dysfunction in CD8+ T cells



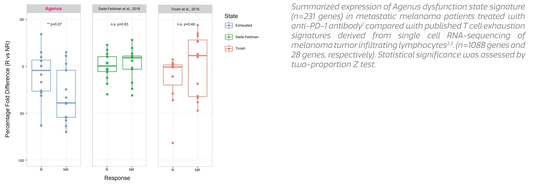

*cDNA libraries were prepared using the TruSeq RNA Library Prep Kit v2 and sequenced on a NextSeq 550 (76 bp paired end run) generating an average of 20 million paired reads per sample. Reads were mapped against NCBI Human Reference Genome Build GRCh37 (hg19) using the STAR aligner in BaseSpace (Illumina). Data normalization and differential gene expression analysis was performed using DESeq2 functions in R. p-values were adjusted using the Benjamini–Hochberg method. K-means clustering was performed in R on scaled count data (k=12 based on correlation analysis). A)* **K-means cluster profiles**, *with mean cluster expression shown in red and individual genes in grey. B)* **Normalized log2 RNA expression** *for selected genes. Error bars = SEM across three donors.*

## Figure 4. Chronic cancer antigen exposure ultimately drives CD8+ T cells to a anti-PD-1 refractory state



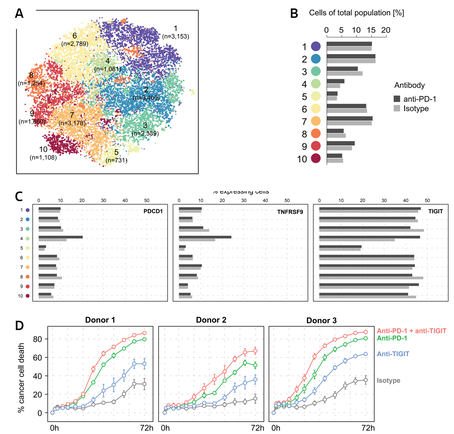

**A) Method for evaluating the effect of anti-PD1 antibody on T cell dysfunction in vitro**. *Co-cultures were supplemented with 5ug/mL Nivolumab or an in-house IgG4 isotype control antibody. Additional assay details in Figure 1A legend. B)* **Cytotoxicity kinetics** *and C)* **maximum cytotoxicity** *across four T cell : cancer cell ratios, in the presence or absence of PD-1 antibody. Assay details in Figure 1B,C legend.*

## Figure 5. A gene expression signature associated with the anti-PD-1 refractory state predicts anti-PD-1 response in melanoma patients



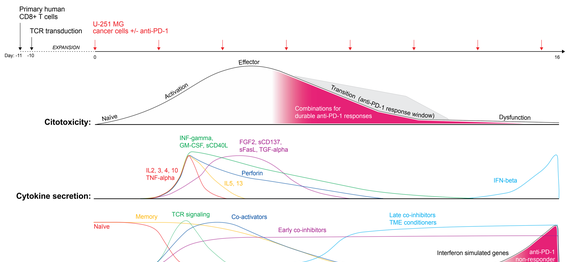

*Summarized expression of Agenus dysfunction state signature (n=231 genes) in metastatic melanoma patients treated with anti-PD-1 antibody<sup>1</sup> compared with published T cell exhaustion signatures derived from single cell RNA-sequencing of melanoma tumor infiltrating lymphocytes<sup>2,3</sup>. (n=1088 genes and 28 genes, respectively). Statistical significance was assessed by two-proportion Z test.*

## Figure 6. Deep profiling of anti-PD-1 responsive T cell subsets identifies combination approaches to enhance durability of anti-PD-1 responses




*CD8+ T cells from three donors were co-cultured through five antigen exposures and harvested. Single cell cDNA libraries were constructed using the 10X Genomics single cell 3' reagent kit v2 and sequenced on a NextSeq 550 (26 bp/98 bp paired end run) generating >50,000 paired reads per cell. Reads were aligned to the NCBI Human Reference Genome Build GRCh38 (hg38) and processed with counts of unique molecular identifiers (UMIs) using Cell Ranger software and downstream analysis was performed using the R package Seurat.* **A) tSNE visualization of ~20k cells** *from three donors, based on variably expressed genes. Cells are colored based on k-means cluster assignment.* **B) Frequency of cells** *in each cluster in the presence or absence of 5ug/ml Nivolumab.* **C) Frequency of PDCD1, TNFRSF9 & TIGIT expression** *across K-means clusters. Assay details in Figure 2 legend.* **D) Cytotoxicity kinetics** *as monitored by live cell fluorescence microscopy in the presence of 5ug/ml Nivolumab and/or anti-TIGIT antibodies. Assay details in Figure 1B legend.*

## Figure 7. I-O target discovery and translational insight from deep, integrative profiling of the tumor:immune interface in vitro





**References**
1. Hugo W, Zaretsky JM, Sun L, Song C, Moreno BH, Hu-Lieskovan S, et al. Genomic and transcriptomic features of response to anti-PD-1 therapy in metastatic melanoma. Cell. 2016;165(1):35-44.
2. Sade-Feldman M, Yizhak K, Bjorgaard SL, Ray JP, de Boer CG, Jenkins RW, et al. Defining T cell states associated with response to checkpoint immunotherapy in melanoma. Cell. 2018;175(4):998-1013 e20.
3. Tirosh I, Izar B, Prakadan SM, Wadsworth MH, 2nd, Treacy D, Trombetta JJ, et al. Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. Science. 2016;352(6282):189-96.



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