WHITEPAPER

T CELL STATE PROFILING USING RNA MODELS

Introduction

Tumor Infiltrating Lymphocytes (TILs) detect tumors and, most importantly for human health, destroy them. As such, the presence of TILs, in particular CD8+ T cells, has been studied in cancer and found to be prognostic in a range of tumor types.[1-5] However, these cells sometimes fail to effectively eliminate cancer cells.[6] One cause of this immunity failure is T cell dysfunction, in particular T Cell Exhaustion.[7] In an exhausted state[8], T cells are unable to fully resolve infections or eliminate cancer cells due to excessive antigen stimulation.[9] Recently, however, researchers have had success in regaining cytotoxicity from exhausted T cells in mice and humans using checkpoint inhibitors.[10,11]

Cell therapies, in particular Chimeric Antigen Receptor T cell (CAR-T) therapies, seek to leverage the tumor clearance power of TILs. In the most prevalent paradigm of CAR-T, autologous T cells are isolated from a patient and engineered to respond to a specific epitope, such as CD19.[12] This type of therapy has been primarily used to treat B cell malignancies and has shown robust responses with high efficacy in some indications, but not in others.[13] To address current challenges, researchers continue to refine their understanding of positive and negative characteristics of CAR-T therapeutic products.[14] For example, dysfunction and exhaustion of CAR-Ts in the apheresis, manufactured, and administered products has been associated with non-response.[15,16] In addition, other T cell states such as activation status and memory status can also affect cytotoxicity[17], and subsequently the clearance of both blood malignancies and solid tumors.

It is clear that the state of endogenous and engineered T cells can affect therapy success in patients. To enable researchers to better characterize T cells, we have created five Health Expression Models that describe different states of T cells. The Unstimulated Model characterizes T cells that have yet to encounter antigen. The Activation Model characterizes T cells that have been subject to engagement of cognate antigen and costimulation. The Exhaustion Model characterizes T cells that have been over-stimulated and have reduced cytotoxic function. Additionally, the Central Memory and Effector Memory Models characterize two memory T cell subtypes critical to the adaptive immune response. These RNA models are used to measure the (dys)functional states of T cells and as such can serve as more convenient surrogates for complex, multi-faceted functional assays. Since these models are an RNAseq-based technology, T cells functional status can be characterized in FFPE, previously impossible. In this work, we show the performance of these five T cell state Health Expression Models.

Methods and Results

Exhaustion Model Performance

Un-stimulated, stimulated and exhausted CD8+ T cells were derived from human Naive CD8+ T cells by repeated activation with anti-CD3/anti-CD28/anti-CD2 tetramers over two weeks. Eight time points were investigated (unstimulated/Day 0, 2, 4, 6 ,8, 10, 12 and 14). In addition, cells from each time point were harvested and stained for cell surface marker expression of inhibitory receptors Programmed cell death protein 1 (PD-1), Lymphocyte-activation gene 3 (LAG3) and T-cell immunoglobulin and mucin-domain containing-3 (TIM3). The percentage of cells triple-positive for all three markers was measured by flow cytometry. Finally, the concentration of cytokines Interferon Gamma (IFNγ) and Interleukin-2 (IL2), were measured in the cell supernatant at each time point.

A hallmark of T cell exhaustion is the progressive increase in the expression of inhibitory receptors such as PD-1, TIM3 and LAG3[8], coupled with the progressive loss of proliferative and cytotoxic potential and a later stabilization or decrease in inhibitory receptor expression once exhaustion is established. Our results mirror this as shown in Figure 1, where the percentage of cells co-expressing these three inhibitory receptors increases from day 0 (unstimulated) through day 10 and then begins to decrease as the cells become almost completely exhausted. However, this metric alone is not a sufficient measure as both peak activation and peak exhaustion both show high levels of inhibitory receptor co-expression.

Upon activation, CD8+ T cells begin to secrete IFNγ and IL2, however with overstimulation the cells begin to become exhausted. Typically, IL2 production is lost early in the development of exhaustion, followed by decreased IFNγ production. This exact result is shown in Figure 1 where the production of IL2 is the greatest at day 4 (y-axis - percentage

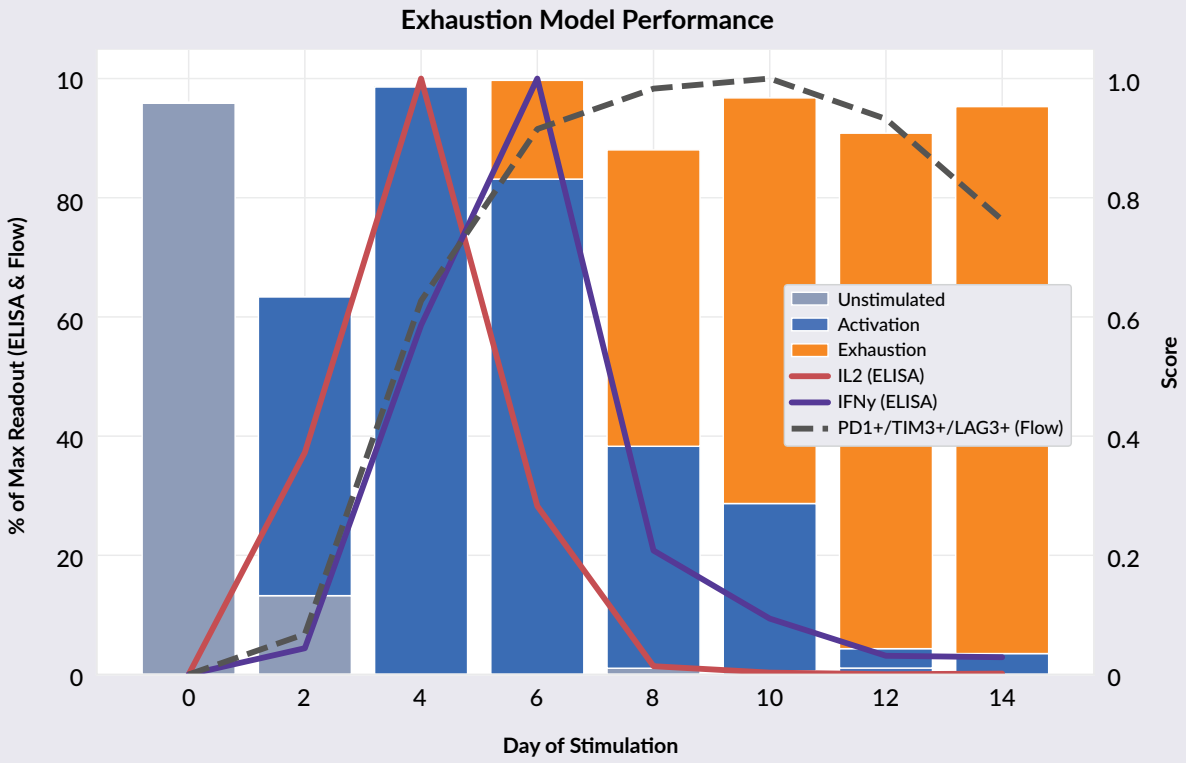

Figure 1. Levels of T Cell States, Cytokines, and Inhibitory Receptors in CD8+ T Cells during chronic stimulation measured using traditional functional assays (left axis) and Cofactor's technology (right axis).

of max readout), when nearly all cells are activated, and decreases greatly at day 6 as cells develop an exhausted cell state. The production of IL2 is nearly absent at day 8, the first day where the largest proportion of cells are identified as exhausted as shown in Figure 1 - day 8. In parallel, INFγ production follows IL2, however peak production is seen at day 6 and decreases greatly by day 8 and falls precipitously until it is nearly absent at day 12. As mentioned above, inhibitory receptor expression alone is not sufficient to determine the exhaustion state of a cell (or proportion of exhaustion in a group of cells). However, when taken together with the measurement of secreted cytokine levels, inhibitory receptor expression enables one to approximate the exhaustion state of a group of cells. This sort of multi-faceted approach is laborious. In addition, these types of measurements are not feasible in FFPE tumor samples. To address these challenges, we propose our Exhaustion Model as a new method to provide a direct, accurate, and precise measurement of exhaustion.

As CD8+ T cells are activated, one would expect to see state changes from Unstimulated to Activated, and as stimulation continues, from Activated to Exhausted. Figure 1 shows the results of the above experiments including data for T cell states (Unstimulated, Activation, and Exhaustion), percent max readout of cytokine concentration and expression of inhibitory receptors. We attempted to determine the cell states of the CD8+ T cells at each time point using our Health Expression Models. The bar chart shows that at day 0 (unstimulated) all of the cells are identified as "Unstimulated", however as CD8+ T cells are successively stimulated, starting at day 2, there is a concomitant increase in the percentage of cells identified as "Activation" through day 4. Even at day 6, the majority of cells are identified as "Activation". In contrast, day 6 begins to show an increase in the "Exhaustion" state model and the proportion of this cell state model increases all the way through to the final time point at day 14. The x-axis provides the T Cell State Profiling Score (out of 1) for each of the cell states at each time point. The results of this experiment show that our T cell state models are more robust than cytokine level or inhibitory receptor expression alone, and can be used to assess the contribution of each cell model in a pure mixture of *in vitro* CD8+ T cells.

T Cell State Estimations of CD8 and CD4

To test our models on in vivo samples, blood was drawn from multiple donors and cell types were isolated using flow cytometry. CD8+ Naive (Naive; n=5), CD8+ Effector Memory (EM; n=5) and CD8+ Central Memory (CM; n=3) populations were isolated and sequenced to test the effectiveness of our models in identifying these CD8+ T cell subtypes. Figure 2, below, shows that our cell differentiation models are able to identify pure CD8+ subtypes with high accuracy (first three bars; Naive = ~90%, EM = ~70% and CM = ~80%) in representative samples. We also applied our models to purified CD8+ cells derived from healthy donor PBMCs (n=3) in order to determine the percentages of CD8+ cell subtypes.

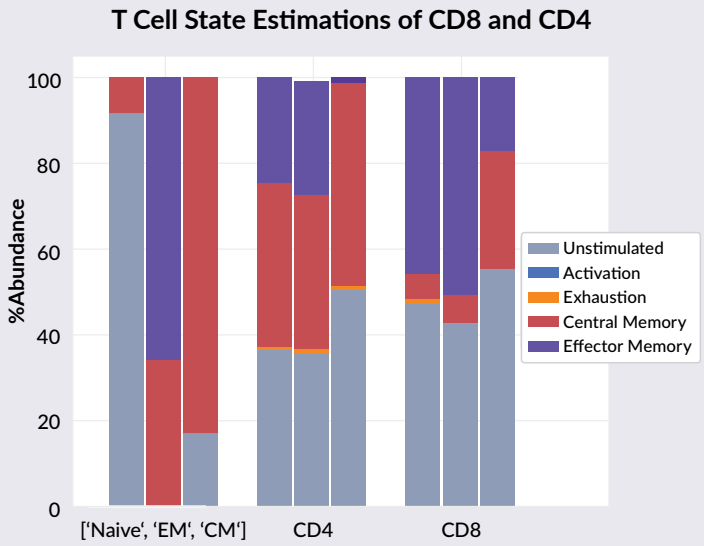

Figure 2. Levels of T Cell States in Purified PBMC Cell Types.

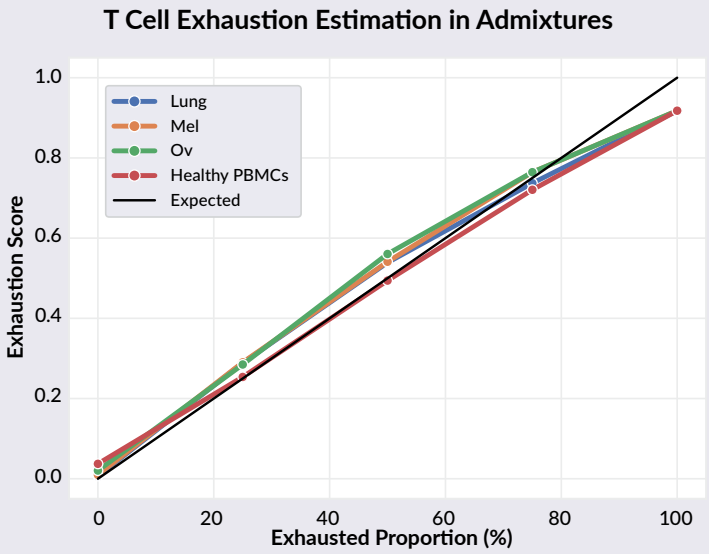

Figure 3. T Cell Exhaustion Estimation in Admixtures of Control Exhaustion Samples and Non-exhausted Negative Control Samples.

Figure 2 (bars 7-9) shows the percentage of these cell subtypes. The approximate percentages of CD8+ cell subtypes in healthy donors have been reported as Naive = 60% + 15%, EM = 30 + 15%, CM = 10% + 5%, Activated = 0% and Exhausted = 0%.[18] The percentages estimated using our state models corroborate these findings with Naive approximately 50%, EM between 20-45%, and CM between 10-25%. A similar experiment was performed as above, but using CD4+ cells from PBMCs of healthy donors (n=3). The approximate percentages of CD8+ cell subtypes in healthy donors have been reported as Naive = 50% + 12%, EM = 20% + 10%, CM = 30% + 5%, Activated = 0% and Exhausted = 0%. Figure 2 (bars 4-6) show that our models can distinguish cell states in both CD4+ and CD8+ T cells and corroborate the approximate percentages seen in CD4+ T cells from healthy donor PBMCs with Naive approximately 35-50%, EM between 0-15%, and CM between 25-30%. These results exhibit the validity of using cell state models to estimate T cell subtype abundance.

T Cell Exhaustion Estimation in Admixtures

Our models have shown to be effective in estimating the cell states in CD4+ and CD8+ T cells in purified cell mixes in vitro and in vivo. However, we wanted to further investigate the use of our exhaustion model in increasingly complex samples. Accordingly, we mixed sequencing data from positive control exhaustion samples into non-exhausted negative control samples at increasing proportions (0%, 25%, 50% and 100%). Negative samples included the CD45- (non-immune) component of dissociated tumor cell samples (DTCs) from Lung (Lung), Melanoma (Mel) and Ovarian (OV) and an immune cell mix from healthy PBMC donors (Healthy PBMCs). The positive exhaustion sample was the fully exhausted timepoint (day 14) of the stimulated CD8+ T cell experiment in Figure 1. The CD45- component from cancer samples and immune cells from PBMCs is expected to have no exhaustion component and thus serves as a negative exhaustion data set in the context of complex samples and several immune cell types. Figure 3 shows the comparison between the fraction of exhausted cells estimated by our models (y-axis), at each dilution level, versus the known percent of exhausted cells from the data mix reported above (x-axis). As shown in Figure 3, our model estimations match the expected readouts (Expected - black line). The Exhaustion Model is unaffected by noise from non-exhausted data (CD45- and PBMC immune cell mixes) and is able to estimate, with high accuracy and dynamic range, the fraction of exhaustion that exists in complex cancer samples from multiple indications. Next, we aimed to validate our Exhaustion Model in cancer samples that are expected to have differing levels of exhaustion. Head and Neck Squamous Cell Carcinoma (HNSC) biopsies have a higher level of exhaustion in Human papillomavirus-positive (HPV+) patients than HPV-negative (HPV-) patients.[19, 20] Using data from The Cancer Genome Atlas (TCGA), samples were grouped by HNSC HPV+ (n=36) and HNSC HPV- (n=241) status.

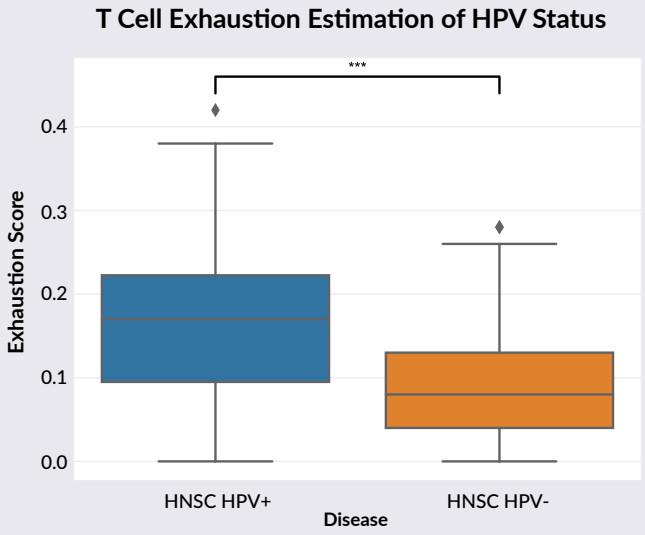

Figure 4. Exhaustion Estimation of Head and Neck Squamous Cell Carcinoma TCGA Samples

T Cell Exhaustion Estimation of HPV Status

By comparing expression data to the five Health Expression Models we estimated the level of exhaustion for each sample (y-axis). Figure 4 shows that indeed, our models estimate statistically more exhaustion (Student's t-test; p-value < 0.001) in HNSC samples with HPV+ status (median = 17; blue box) versus those with HPV-status (median = 8; orange box). These results show that our exhaustion models corroborate primary research findings in "real world" data. Gene Set Enrichment Analysis (GSEA) was used to validate the higher exhaustion state for HNSC HPV+ relative to HNSC HPV- samples in the TCGA dataset (data not shown).

Conclusion

In this work, we showed how novel Health Expression Models can be used to estimate five different T cell states: Unstimulated, Activation, Exhaustion, Central Memory, and Effector Memory. These states were validated in an *in vitro* model of T Cell activation and chronic stimulation, as well as in purified PBMC samples. We further validated these results in complex admixtures derived from fully exhausted T cells and tumor derived CD45- cells or PBMC cells. Finally, we showed the utility of the Exhaustion Model in measuring a relevant difference in exhaustion in "real world" HNSC tumor samples.

The validated Health Expression Models presented here offer a unique way to measure T cell functional states. With regards to measuring T cell exhaustion, this technique can provide a more direct measurement of T cell state than traditional functional assays and obviate several different tools and workflows. In addition, since this technique is based on a Health Expression Model derived from RNA, it can be applied to FFPE samples which comprise ~95% of the world's clinical samples. These T cell State Models offer a new capability to aid researchers in the development of new immuno-oncology and cell therapies.

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