Host intrinsic differences mediating cancer progression

Introduction

Head and Neck cancers of the squamous cells (HNSCC) are a common, heterogeneous group of cancers that usually begin in the squamous cells that line mucosal surfaces inside the head and neck. Mouse models are valuable tools for studying these cancers, which can be achieved by injecting mice with squamous cell carcinomas that mimic human HNSCC. A223 is one such cell line which mimics squamous cell head and neck cancer (White *et al.* 2013). This cell line can occasionally be rejected by the mouse, without therapeutic intervention. The tumors from mice that are able to kill the tumor without intervention are called "regressing" and the tumors from mice that are not able to kill the tumor are called "progressing".

When researching HNSCC the host immune response is widely studied, often focusing on T cells and T cell receptors (TCR). Cytotoxic T cells (CD8+ T cells) are important in the immune response to cancers. These CD8+ T cells express TCRs that can recognize specific antigens (Atta *et al.* 2015). When a TCR recognizes its specific antigen the T cell can become activated and undergo clonal expansion, in which the number of T cells with that specific TCR is increased. The TCR is made of a constant and variable domain. The variable domain contains V, D, and J region sequence elements. These genes undergo recombination to produce variable antigen recognition. Within the variable region are complementarity determining regions (CDRs) which determine which antigens can be bound by the TCR. Each chain (α and β) has 3 CDRs with CDR3 being the most important in determining differential antigen binding, as it spans the V and J junctions and is therefore hypervariable. The collection different TCRs present in a cell is referred to as the TCR repertoire.

Immunotherapies are widely studied to combat HNSCC and common immunotherapeutic targets are programmed cell death 1 (PD-1) and programmed death-ligand 1 (PD-L1). PD-L1 binds to the checkpoint molecule PD1, which transmits an inhibitory signal, reducing the proliferation of antigen specific T cells, and thus reducing the killing of tumor cells (American Cancer Society 2019). Anti-PD-L1 treatments aim to block this binding interaction and increase the immune response to cancers.

Unfortunately, the response to these treatments varies from patient to patient. It is hypothesized that Responder mice have different TCRs expanded, and their T cells are better able to bind to antigens, promoting tumor death. As a way to examine the host intrinsic differences between responders and non-responders to anti-PD-L1 treatment, single cell RNA sequencing (scRNA-seq) and TCR sequencing was performed on T-cells of a responder and non-responder mouse injected with A223 cells. These data were combined with previous sequencing data from progressors and regressors of the A223 cells. These data were then analyzed to determine the overall clonality of the samples, clonotype groups, and cell type clustering.

Methods

The VDJ sequencing data was analyzed in R. The clones in each sample were obtained by examining the nucleotide sequence and V/D/J gene usage. The clonotypes were obtained by

examining the nucleotide sequence of the CDR3. These clonotypes were compared using two different packages: Immunarch and UpSetR.

The clonotypes were then analyzed using GLIPH (Grouping of Lymphocyte Interactions by Paratope Hotspots) which is an algorithm that groups clonotypes into groups based on their predicted antigen binding. GLIPH was used to obtain groups of clonotypes predicted to bind the same MHC-restricted peptide antigen and significant motifs. The results from GLIPH were visualized in R.

Finally, scRNA-seq data was analyzed using Seurat in R. The Responder and Non-Responder mouse spleen and tumor infiltrating lymphocyte (TIL) samples were compared. The UMAP reduction and clustering was combined with the markers for each cluster to determine the cell state of each cluster.

Results

The spleens are sequenced as a baseline for naïve T cells. When the Responder and Non-responder samples were compared, there was very little overlap between the individual mice (Figure 1A-B). There was some overlap within tissues of the same individual, but almost zero

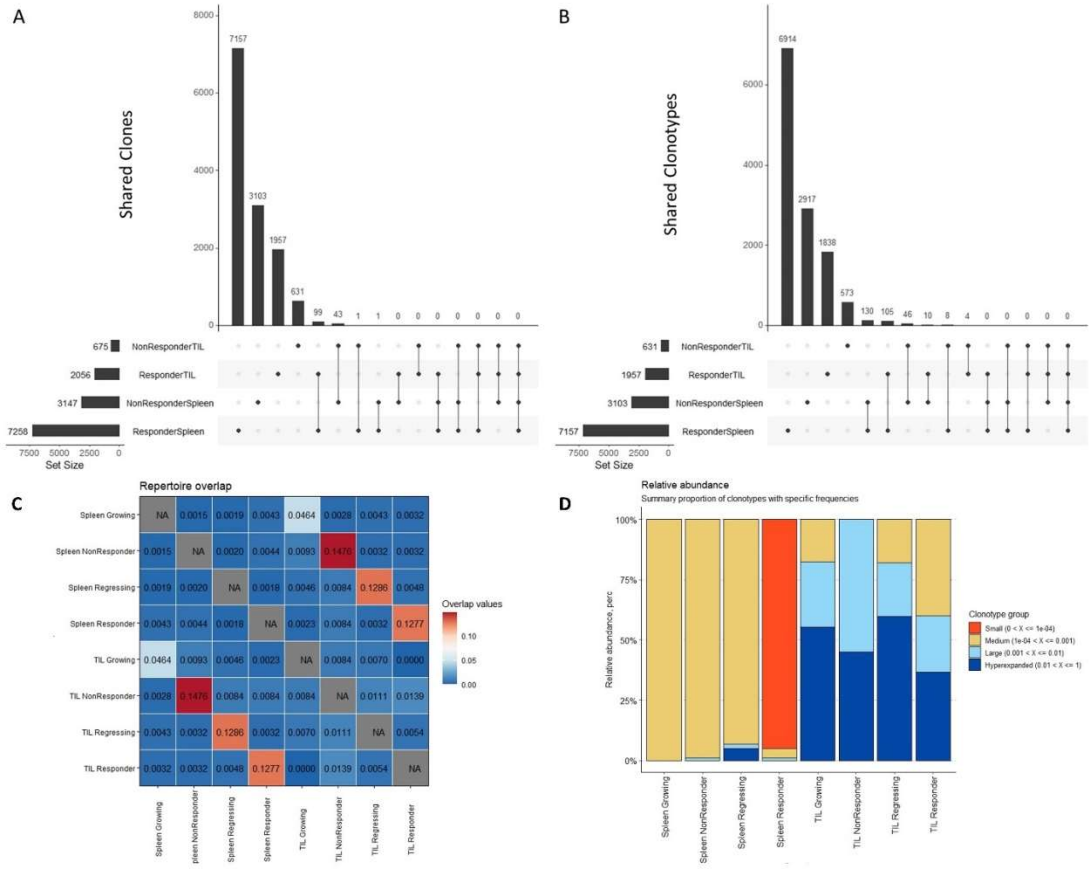

Figure 1) Differences in TCRs

overlap between individuals. As expected, there is more overlap between clonotypes than there is between clones (due to the redundancy in the code of life). The data was then combined with the sequencing samples from a mouse with a regressing tumor and a mouse with a progressing tumor (growing). Unsurprisingly, the samples from the two tissues from the same individual were the most similar to each other (Figure 1C). As expected, it was found that the spleens had a much larger and more diverse T cell repertoire. Small clonotypes (those that exist comprise a small fraction of the total repertoire) made up significantly more of the spleen samples than the TILs (Figure 1D). There were not many cells (<700) in the Non-Responder TIL sample, which suggested that there were problems with the VDJ library preparation or sequencing. The sequencing was repeated on the same library, yielding similar results.

GLIPH discovered many groups of immunologically similar CDR3 amino acid sequences in the Responder and Non-Responder sample (Figure 3). The top twenty groups were the same as in the previously sequenced progressing and regressing samples. Group 1, also as previously discovered, is prevalent in similar proportions in all samples. The other top groups are nearly mutually exclusive between Responder and Non-Responder mice.

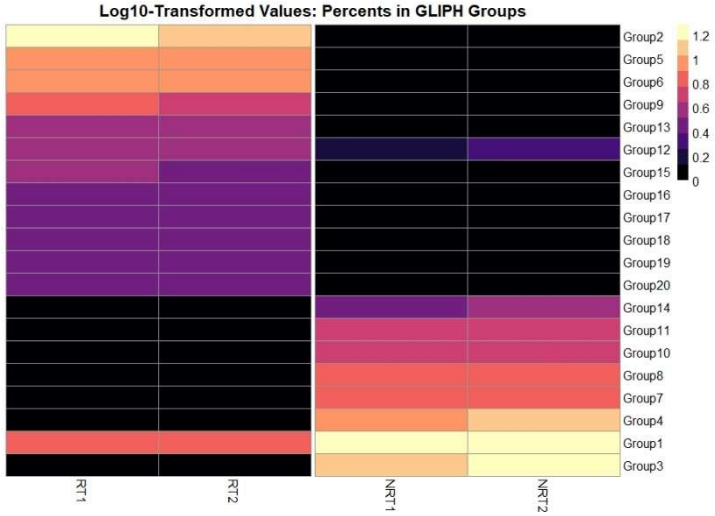

Figure 2) Percents of GLIPH groups in each sample

Examination of highly expressed genes in the scRNA-seq data discovered that there was significant contamination and poor sorting of the T cell samples (Figure 4). The selection failed to isolate T cells and many other macrophage cells were kept and subsequently sequenced. Additionally, some tumor cells were kept and sequenced. The TIL samples clustered together and the spleens also clustered together. The clustering revealed that the spleen samples had significantly less contamination of macrophages, neutrophils, and tumor cells. However, the TIL samples showed significant contamination, and the majority of the cells sequenced were macrophages (not T cells). The gene expression profiles of the myeloid cells were compared to

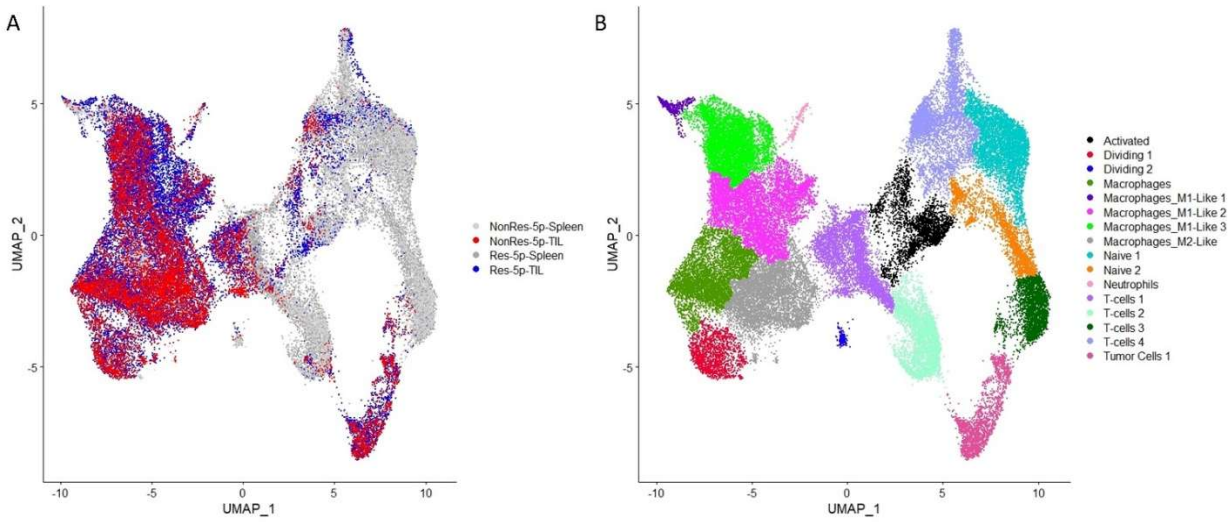

Figure 3) UMAP clustering of scRNA-seq data by sample and cell type

the T cells and there were significantly different profiles. However, when the Responder myeloid cells were compared to the Non-Responder myeloid cells, there was not a clear difference in gene expression profiles. The expression profiles were also compared for the Responder T cells and Non-Responder T cells (Figure 4). These had distinct expression profiles, and some genes that are immunologically relevant were upregulated in the Responders. Additionally, the gene expression profiles were compared for the top clonotypes discovered in the Responder and Non-

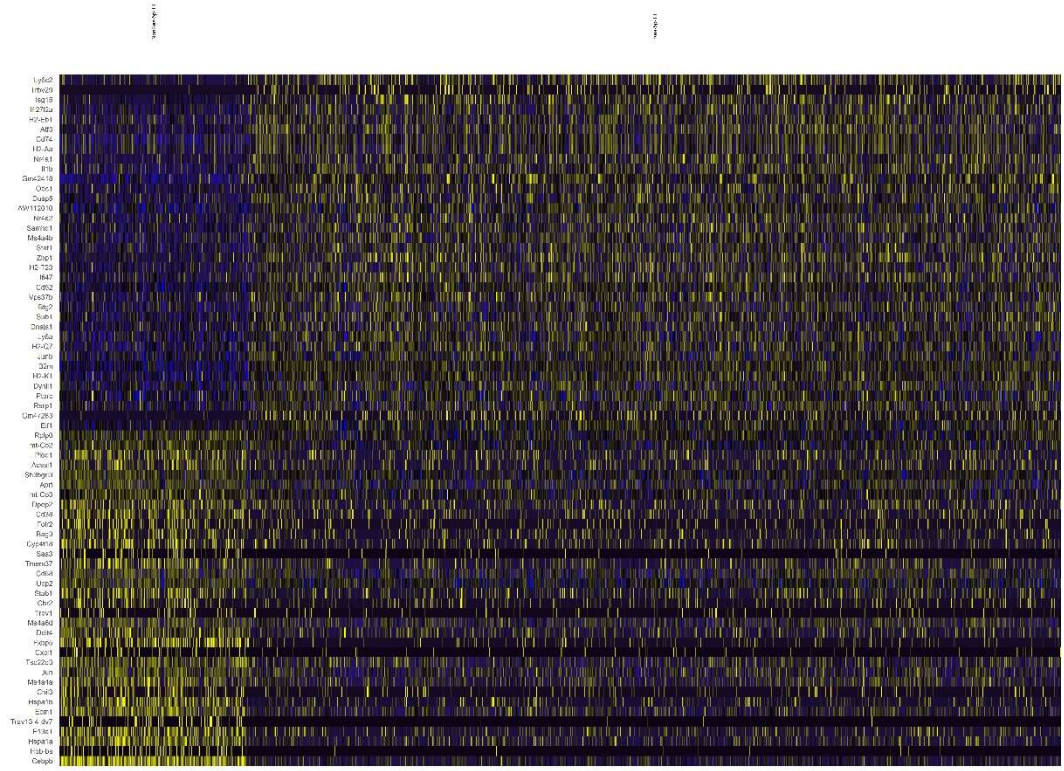

Figure 4) Top differential gene expression

Responder samples. These expression profiles also grouped by response to anti-PD-L1 treatment response as well as distinct clonotype profiles.

Discussion and Future Directions

These data serve as an exploration into the host intrinsic differences that may mediate response to anti-PD-L1 treatment. This experiment showed that there are significant differences in the immune response of Responder and Non-Responder mice. As shown by the repertoire overlaps, there is little overlap between the Responder and Non-Responder TCR repertoires. This suggests that individual mice respond differently to the anti-PD-L1 treatment in which TCRs are expanded. Looking further into the immunogenicity of the TCRs, GLIPH was used to group the TCRs into groups that respond similarly to antigens. This confirmed a previous finding that group 1 which was most prevalent and exists in all samples at similar proportions. This suggests that this TCR group is relevant to tumor recognition in general, but not to the actual response, or killing, of the tumor. The other top GLIPH groups were nearly mutually exclusive, once again providing evidence that Responder mice expand significantly different T cells than Non-Responders.

The scRNA-seq revealed that the cell sorting had not worked well and that many cells that were not T cells were kept and subsequently sequenced. The spleen samples contained a higher percentage of T cells than the TIL samples. The T cells and myeloid cells were separated, and the gene expression profiles were analyzed. The T cells and myeloid cells had significantly different gene expression profiles. When the Responder myeloid cells were compared to Non-Responder myeloid cells there were not significant differences in the overall expression profiles, but there were some significantly differently expressed genes relevant to antigen presentation. In contrast, the Responder T cells had significantly different gene expression profiles to the Non-Responder T cells. This is different from the previous experiment with progressing and regressing tumors. Additionally, when the top clonotypes from the Responder and Non-Responders were compared, they too had significantly different expression profiles. This showed that there are different genes being up- and down-regulated in mice that respond to anti-PD-L1 treatment compared to those that do not respond.

This study showed that there are significant differences in the TCR repertoires of Responder and Non-Responder mice. In all, this shows that there are host intrinsic differences in the response to cancer immunotherapies. These result in different T cells being expanded, and those T cells have distinct gene expression profiles which may elucidate why some mice respond to the therapy and some do not.

The single cell experiments are being repeated using a selection kit, to improve purity for T cells compared to the cell sorting. These analyses will be repeated to determine if the differences are still observed. Future directions will include repeating these analyses on more individual Responder and Non-Responder mice to ensure reproducibility. Additionally, studies may be performed on identified genes of interest to determine their function in the anti-tumor response.

References

American Cancer Society. (2019). Immune Checkpoint Inhibitors and Their Side Effects. https://www.cancer.org/treatment/treatments-and-side-effects/treatment-types/immunotherapy/immune-checkpoint-inhibitors.html

White, R. A., Neiman, J. M., Reddi, A., Han, G., Birlea, S., Mitra, D., Dionne, L., Fernandez, P., Murao, K., Bian, L., Keysar, S. B., Goldstein, N. B., Song, N., Bornstein, S., Han, Z., Lu, X., Wisell, J., Li, F., Song, J., Lu, S. L., … Wang, X. J. (2013). Epithelial stem cell mutations that promote squamous cell carcinoma metastasis. The Journal of clinical investigation, 123(10), 4390–4404. https://doi.org/10.1172/JCI65856

Attaf, M., Huseby, E., and Sewell, A. (2015). αβ T cell receptors as predictors of health and disease. Cell Mol Immunol 12, 391-399. https://doi.org/10.1038/cmi.2014.134