Understanding Tumor Infiltrating Lymphocytes: Sequestered Reactivity in T cells

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Abstract

Adoptive cellular therapy (ACT) uses the liberated force of greatly expanded T lymphocytes to mediate tumor regression. Regression is believed to be primarily driven by the recognition of neoantigens displayed by the major histocompatibility complex (MHC) molecules of tumor cells, making the identification of neoantigens an imperative goal to increase the efficacy in ACT. This thesis highlights a methodology to identify neoantigens, using whole genome sequencing and the construction of tandem minigenes. Two patients with metastatic uveal melanoma, with TIL readily recognizing autologous tumor, were screened for neoantigen reactivity. Screening of 232 and 87 nonsynonymous mutations in these two TIL respectively resulted in no identification of neoantigens, suggesting that alternative targets may also be critical for neoantigen identification.

Besides the lack of neoantigen reactivity one barrier for the success of immunotherapy is the down-regulation or loss of MHC molecules, a known mechanism of immune evasion. As such, we explore an unconventional type of T cell not MHC restricted, $\gamma\delta$ T cells, as an active component of the tumor microenvironment. Sparse $\gamma\delta$ presence across diverse cancer histologies (1-3% of $CD3^+$ cells) could be identified. The majority of these cells are Vδ1, with a similar Memory Effector to Terminally Differentiated Phenotype, as observed on $\alpha\beta$ T cells. Furthermore, they surprisingly expressed CD8 as well as CD56, variably CD69 and PD-1 but lack NKG2D expression, indicating a substantial difference from those obtained from the blood.

Keywords: TIL Therapy, Neoantigen Reactivity, $\gamma\delta$ T cells, Immunotherapy.

1. Introduction

Our cells and tissues have inherent mechanisms to promote genomic stability, recognize stressed cells, and limit uncontrolled proliferation. But when these mechanisms fail, uncontrolled proliferation leads to invasive growth and ultimately to the development of a malignant tumor. To counter the high mortality associated with cancer, in the last few decades, therapies evolved from the crude destruction of rapidly dividing cells to the refined targeting of molecular pathways.

Recently, a new wave of cancer therapies emerged called Immunotherapy. This type of anti-cancer treatment harnesses the bodies own immune system to effectively find, attack and destroy malignant cells. [1] This breakthrough can be traced back to early studies that showed that human tumor infiltrating lymphocytes (TIL) were capable of recognizing and killing autologous tumor cells, providing the rationale to infuse these cells, after *in vitro* expansion, back into the patient. [2, 3] The production of TIL for clinical treatment (**Figure 1**) starts with a biopsied or surgically resected tumor, which is cut into tiny fragments. These fragments are left in cell medium complemented with high doses of IL-2. Afterwards the T cells are rapidly expanded, with the use of irradiated feeders, and infused back into the patient, followed by doses of intravenous IL-2 to facilitate infused T cell survival. The use of TIL for the treatment metastatic melanoma has been shown to be surprisingly effective with a complete response (CR) rate of 24%, out of the 99 patients that received treatment, with a median follow-up of 40.9 months. [4]

However, the effective responses seen in TIL therapy for metastatic melanoma were not replicated in the vast majority of other solid cancers. [5, 6] Nevertheless, a few, rare patients do respond to TIL therapy with exceptional and durable regressions, have a commonality between them, the existence of tumor antigen reactive T cells. [7]

T cells are capable of recognizing tiny peptide molecules that are specific to cancer cells, called antigens, when they are displayed by an MHC molecule. There are many types of antigens, but the ones most interesting for cancer therapy are the

so called, neoantigens, which are the result of somatic mutations in normal gene products. [8] They can be due to single base substitutions, insertions and deletions of nucleotides (indels), and gene rearrangements and/or amplifications. In cancer, they may arise as a consequence of mutagen exposure, defects in the DNA replication or repair machinery.

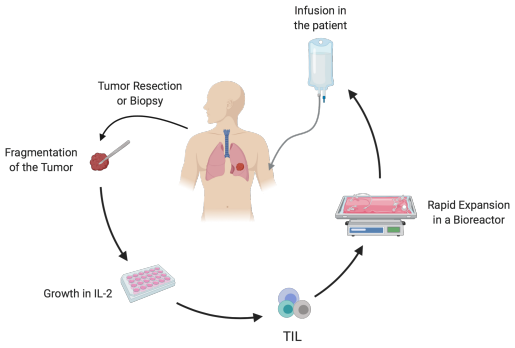

Figure 1: A summary of TIL production for clinical treatment.

This neoantigen reactivity provided rationale to use T cells transduced with isolated and well characterized tumor reactive TCRs as therapy, a strategy currently being evaluated in clinical trials. [9] With that in mind, driver mutations are often considered the ideal target for a less personalized, "off-the-shelf" type of therapy. However, it is important to point out that the vast majority of the neoantigens identified so far are unique for each patient.

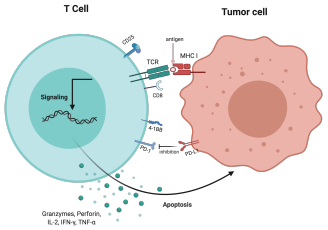

Figure 2: Cytotoxic T cell recognition and killing of tumor cells.

For T cell reactivity to occur, an antigen presenting cell (APC) has to migrate to the lymph nodes and stimulate the naive T cell. This naive T cell will then undergo differentiation and its progeny will make its way to the tumor site, via inflammation signalling. Inside the tumor microenvironment, as the T cell encounters its target, the TCR stimulation leads to transient 4-1BB up-regulation (also known as CD137), a key mediator in survival and proliferation of $CD8^+$ T cells (**Figure 2**). [10] In addition, IFN-γ is released by the neoantigen reactive cells 6-12 hours after stimulus, making it a reliable marker to access reactivity. [11, 12] Both 4-1BB up-regulation and IFN-γ release have been used to detect neoantigen reactivity, and are an important step in accessing how likely a patient is to respond to TIL therapy.

During this work, we aim to identify and characterize T cell reactivity in patients with metastatic uveal melanoma, which is crucial to give the scientific community key insights on how the immune system mediates tumor regression and, more importantly, opens the door on using neoantigen reactive T cells as a form of personalized therapy.

1.1. When T cell recognition fails: a job for $\gamma\delta$ T cells

An increasingly asked question in immunotherapy trials is what proportion of malignant T cells are actually expressing MHC molecules. Without the MHC molecules, cancer cells are thought to become invisible to T cells, a well document form of immune evasion. However, there is a small subset of T cells, called $\gamma\delta$ T cells, that are not MHC constricted. These cells also have a recombinant T cell receptor (TCR), but with a δ and a γ chain instead of the canonical α and β TCR chains. In the blood, $\gamma\delta$ T cells are mostly $\gamma 9\delta 2$, with important functions in recognising microbial agents and being a quick agent that mounts an inflammatory response. In the tissue, most $\gamma\delta$ T cells are thought to be $\delta 1$, but very few characteristics are known about them in the tumor microenvironment. They are hypothesized to directly recognize molecular indicators of stress in malignant cells, with this recognition being associated to potent cytolytic activity, even if there is no clear understanding of how $\gamma\delta$ mediate this recognition. [13]

These cells are an unexplored source of anticancer immunity which will be further explored and phenotypically characterized during this work.

2. Results and Discussion

The TIL used to screen for T cell reactivity were obtained from patients with metastatic uveal melanoma, through a liver metastasis resection. After initial outgrowth, several fragments were tested for autologous tumor recognition and the reactive cultures were pooled together and rapidly expanded, under GMP protocol (**Figure 1**).

2.1. Mutation Calling

In parallel the resected metastasis were sent for whole genome sequencing (WGS) to identify nonsynonymous mutations, the precursors of neoantigens. WGS from patient A resulted in 232 nonsynonymous mutations, whose mutational type diversity is represented in **Figure 3.A**, an abnormally high number, since Uveal Melanomas usually have between 20 to 81 nonsynonymous mutations. [6]

Patient's B WGS resulted in 87 nonsynonymous mutations, represented in **Figure 3.B**.

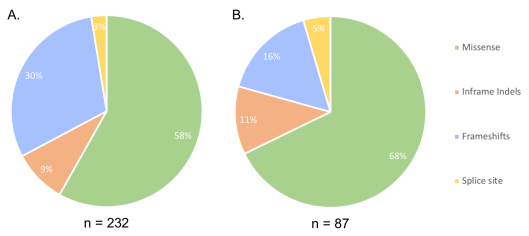

Figure 3: Type distribution of all nonsynonymous mutations for patient A and B identified by WGS. Both tumor samples are from a liver metastasis and autologous PBMCs were used for a normal genome control.

Both patients lacked mutations in either NRAS or BRAF, both common in cutaneous melanoma, but practically nonexistent in uveal melanoma (UM). [14] A missense mutation in GNAQ, altering codon 209, is present in patient A but not patient B. GNAQ mutations have been described in more than half of uveal melanomas, making it one of the most common known oncogenic mutations for this type of cancer. Furthermore, this gene is found to be mutated at different stages of malignant progression, suggesting that it is a driver mutation in UM. [15] GNAQ mutations also occur almost exclusively in codon 209, [16], making this point mutation an interesting target for an *off-the-shelf* TCR based adoptive cell therapy in uveal melanoma.

Patient B has a GNA11 mutation, in codon 209. This particular missense mutation is present in 57% of metastatic uveal melanomas, and is predicted to cause a leucine substitution in 97.3% of all mutations. Both GNAQ and GNA11 are the most common mutations in UM, present in 88% of all tested tumors. [17] The high frequency of this GNA11 mutation in patients with UM also makes it a highly desirable target for induced TCR based ACT in uveal melanoma.

Found in patient A is a missense mutation present in SF3B1, a gene encoding a component of the spliceosome, in codon 625. Through RNA sequencing of uveal melanoma specimens it was revealed that SF3B1 mutations are associated with differential alternative splicing of protein coding genes. [18]

2.2. Neoantigen Reactivity Assay

The nonsynonymous mutations were used to build tandem minigenes (TMG). After confirming that the TMGs are fully translated, the screening process for mutation specific T cell reactivity can begin.

Autologous monocytes were differentiated into immature DCs and electroporated with the corresponding TMGs. After, a co-culture with electroporated DCs and TIL was set-up and left overnight. With the mRNA inside the cells, the ribosomal machinery translates the TMG in aberrant proteins that are quickly cleaved by the cell's proteasome, leading to the formation of small peptides. The excess of peptides in the cytosol (8-16 a.a) leads to their incorporation in the MHC molecules, which will then be transported to the cell surface, where they are presented to the T lymphocytes. [19]

When a T cell recognizes a specific neoantigen through the TCR, coupled with CD28 co-stimulation (by CD80 & CD86 in the APC), it becomes activated, up-regulating 4-1BB and triggering IFN-γ release. Thus, 4-1BB levels were measured for both patients through flow cytometry, as seen in **Figure 4**. IFN-γ ELISAs were also run on the co-culture supernatants for both patients, represented on the bottom of **Figure 4**.

The IFN-γ results appear consistent with the flow cytometry data, with a moderate IFN-γ release associated with a slight up-regulation of 4-1BB for the co-culture with tumor digest. Likewise both parameters (4-1BB and IFN-γ) increase when the TIL was stimulated with anti-CD3 anti-CD8 coupled beads. The increased IFN-γ associated to TMG6, in **Figure 4.B** is likely the result of a technical error during the ELISA, and another ELISA will be run to confirm that. This screening concluded that there was no TIL reactivity to any of the TMG, in both patients, as measured by the lack of IFN-γ production and 4-1BB up-regulation.

2.3. Error 404: Antigen not found

This lack of TIL reactivity was somewhat unexpected, due to high percentage of tumor reactivity (around 20% for each of the tested TIL). Even so, there are a few plausible explanations to the lack of neoantigen reactivity for all of the mutations tested:

- The TIL may be reactive to self antigen;
- The neoantigen(s) behind T cell reactivity was(were) not identified by WGS;
- TMG processing did not lead to the formation of the right epitope;

The coverage used in WGS was sufficient to identify the vast majority of nonsynonymous mutations. Likewise, the bioinformatic analysis has been validated internally and not a cause for concern. It also seems unlikely that the mutation would be dimly expressed, as both TIL specimens are reproducibility reactive to the single cell tumor digests.

One hypothesis would be that the epitope(s) inducing reactivity is (are) a result of alternative transcript splicing, a regulated process that excludes particular exons of a gene in premature mRNA.

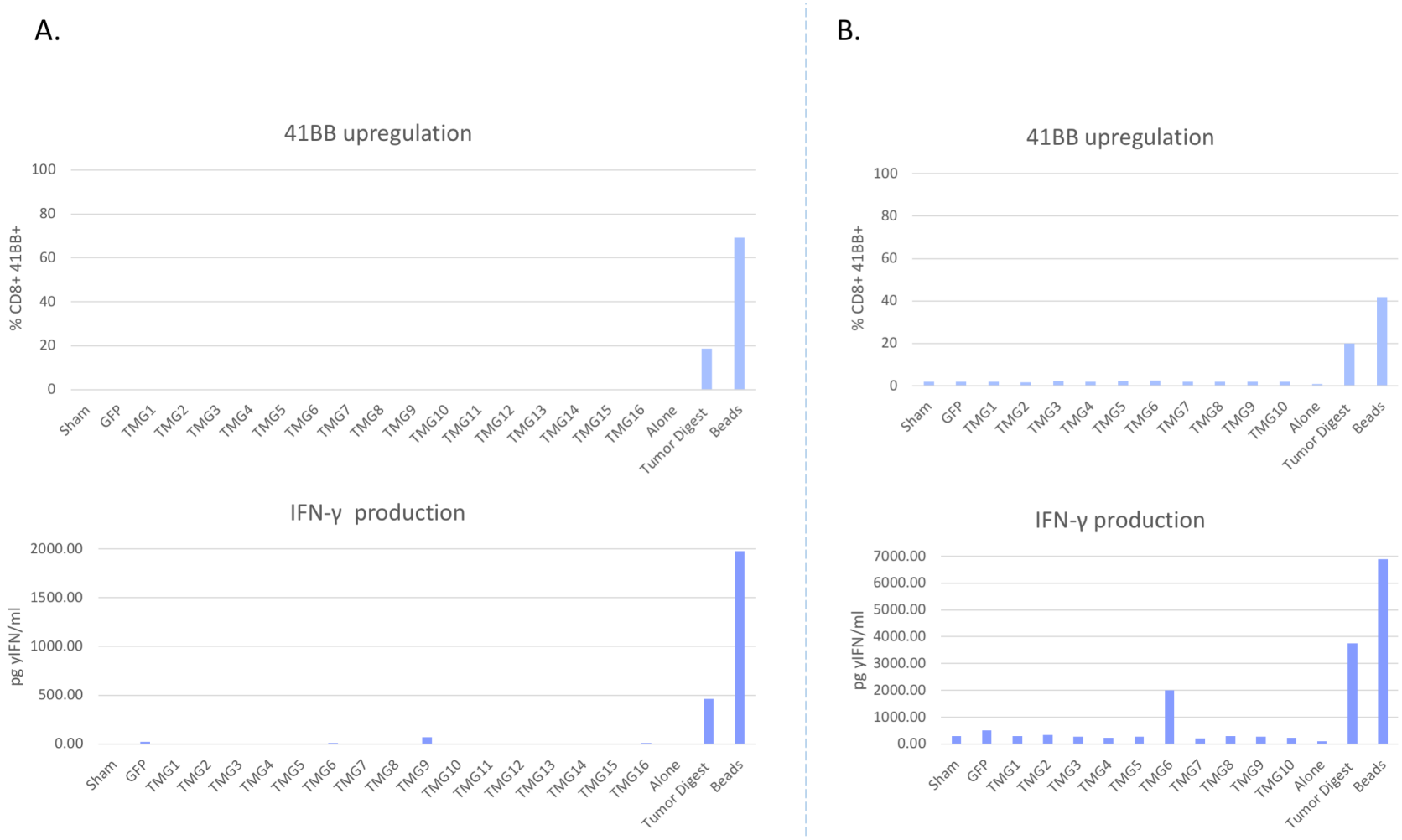

Figure 4: 4-1BB and IFN-γ screening for the TMG screening assay, with A and B corresponding to patient A and B, respectively.

This process has been described as an important source of neoantigens. [20] Work by *Smart et al.* experimentally validates this concept by showcasing that this type of neoepitope can be presented by MHC molecules and elicite T cell responses. [21] This source of neoantigen formation might be particularly important in cancers with somatic variants in splicing factors, such as SF3B1 and U2AF1, as they significantly alter the landscape of splicing in tumor cells. [18] With a high frequency of SF3B1 somatic mutations this might be a particularly overlooked driver of neoantigen formation in uveal melanoma.

To overcome this, one could RNA-seq the tumor sample and use bioinformatic tools to identify altered transcriptome derived neoantigen candidates, thus identifying potential T cell targets that the typical WGS or WES dependent methodologies would miss.

A more ambitious and experimental alternative would be isolate neoantigen reactive T cells TCRs directly. A possible methodology would be to co-culture the tumor digest with TIL and isolate the 4-1BB positive cells through sorting. The resulting cells would undergo single cell sequencing to identify and isolate the corresponding TCRs. Likely, if the cells have been 4-1BB sorted it's likely there would be skewed clonality, meaning you would expect many cells to have the same TCR as they are reacting to the same neoantigen. The sequencing would permit the assembly of full length TCR chains, while also giving key insight into the state and functionality of neoantigen reactive T cells. With the isolated TCRs one could reverse engineer potential epitopes and test them against the TCR to prove recognition. If successful, this approach would permit isolation of TCRs in an unbiased way.

To address self antigen reactivity, TIL could be tested against autologous APCs electroporated with mRNA encoding genes known to cause self reactivity, as is the case with gp100 and MART-1.

2.4. Characterizing Tumor Infiltrating Lymphocytes

Specimens from six patients with Pseudomyxoma Peritonei (PMP) were fragmented and successfully grown with 3000 IU/ml of IL-2 in order to analyze viable T cell populations. Initially, the inner and outer parts of the tumor were grown separately, but the practice was discontinued since the resulting TIL products were similar. Three additional gastrointestinal TIL specimens from cryopreserved tumor digests were also grown in IL-2.

Specimens from four patients with Renal Clear Cell Carcinoma (RCC) were also collected, fragmented and placed into culture. Three of the patients had undergone a radical nephrectomy, thus allowing the separation of fragments from central tumor, periphery of the tumor, adjacent and normal tissue. Growth of lymphocytes out of all fragments was possible, but variable. The fragments originating from the central part of the tumor mass disintegrated and gave rise to very limited cell numbers, which may be a by-product of the intense necrosis found within this site.

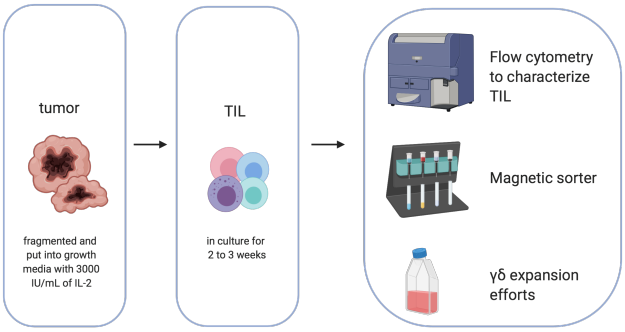

Figure 5: Workflow of TIL samples.

After 2 to 3 weeks of lymphocyte growth in 3000 IU/mL of IL-2, the resulting TIL product was immunophenotyped using spectral flow cytometry. This technique, unlike conventional flow cytometry, is capable of differentiating fluorochromes with significant spectral overlapping without the use of compensation allowing the use of multiple antibodies in complex flow panels (often used with 22-30 fluorochromes at a time).

To briefly summarize the gating strategy (after selecting alive, single cells) T cells were distinguished from NK cells based on CD56 versus CD3 expression. T lymphocytes were considered to be $CD3^+$, while NK cells were $C3^-CD56^+$. $CD3^+$ cells were then segregated based on expression of $\alpha\beta$ or $\gamma\delta$ TCR. $\alpha\beta$ cells were distinguished based on $CD4^+$ and $CD8^+$ expression, while $\gamma\delta$ lymphocytes were separated based on which antibodies attached to the delta chain of the TCR ($\delta 1$, $\delta 2$ or $\delta 1^-\delta 2^-$).

Standard TIL protocols may also lead to the expansion of a modest population of NK cells, as these cells also consume IL-2 and are a component of the TME. This population is typically under 15% of all lymphocytes grown and it often disappears during rapid expansion, a reflection of NK cells being a much smaller component of the TME with less affinity for IL-2 when compared with T lymphocytes and the utilization of OKT3, a CD3 antagonist in the REP. Their sparse infiltration is a characteristic of advanced human tumours, particularly when in comparison with the non malignant adjacent regions, suggesting these cells do not home efficiently to malignant tissues. [22, 23]

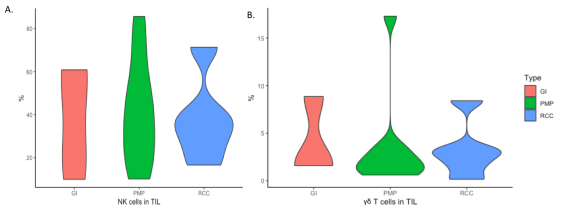

Figure 6: Violin Plot of: A. the percentage of NK cells; B. the percentage of $\gamma\delta$ in TIL across different cancer histologies after 2-3 weeks culture in IL-2

Unexpectedly, there was a high number of NK cell outgrow in this patient cohort, **Figure 6.A**. When contextualizing the TIL populations according to cancer histology it seems that the PMP TIL cohort had a particularly pronounced growth of NK cells (median of 40.9%). A similar finding can be seen in the TIL specimens grown from GI malignancies (median of 40.9%), albeit with a smaller sample size.

Interestingly the GI tumor digests used to produce TIL in this work resulted from the resection of peritoneal metastasis, the same anatomical location of the PMP resections, perhaps suggesting that NK cells may be a large component of the tumor immune infiltrate in the context of the peritoneum. As for $CD3^+$ cells, the TIL composition in PMP and GI seems to be patient specific with some key differences between the two histologies. The majority of $CD3^+$ $\alpha\beta$ TIL are $CD4^+$ (median of 59.0%), with sparse $\gamma\delta$ infiltration/outgrowth (median of 2.8%) in PMP. For the GI malignancies, $CD3^+CD4^+$ represents a median of 20% $\alpha\beta$ T cells in the TIL, with a $\gamma\delta$ infiltration/outgrowth of 1.76%. To the best of my knowledge, in the case of PMP there are no reports of tumor infiltrating lymphocytes growth, making these findings novel.

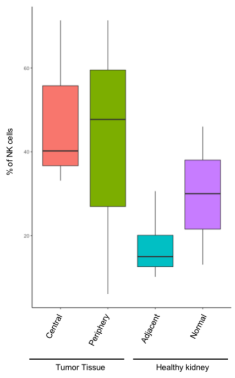

Figure 7: Box Plot of the percentage of NK cells in TIL cultures grown from different kidney regions.

In RCC, *Esendagli et al.* reports that NK cells infiltrates are more prominent in the adjacent and healthy regions of a kidney, compared to the tumor margins and interior. [22] TIL specimens grown in this cohort seem to contradict this report, as both central and peripheral regions of the tumor led to increased NK outgrowth compared with the regions adjacent or distant to the tumor, **Figure 7**. It is important to note however that only three samples were used in making this observation, thus requiring more samples to draw definitive conclusions.

If confirmed in a larger cohort, this NK outgrowth could be explained by different factors, from different tumor biology to TIL growth conditions. RCC tumors are highly heterogeneous, with different oncogenic drivers and different cellular components in the TME amongst them. [24] It could be possible that some RCC may have an increased infiltration of NK cells when compared with the surrounding healthy tissue. Another possibility could be the nature of the specimen itself and how its condition affects immune cellular growth. The center and periphery of these RCC specimens were far more necrotic than the associated adjacent and normal tissue, conditions normally associated with a reduced amount of T cells. This may lead to a hostile environment for T cell recruitment and growth, thus allowing for the preferential infiltration and subsequent expansion of NK cells.

2.5. Gamma delta in TIL

TIL analysis from renal cell carcinoma, GI malignancies and pseudomyxoma samples shows that most TIL have some $\gamma\delta$ T cells present, typically in much smaller amounts (generally <3% of $CD3^+$ cells) than the characteristic predominant $\alpha\beta$ lymphocyte infiltrate, **Figure 6.B**.

The distribution of Vδ families in $\gamma\delta$ T cells is represented in **Figure 8.A**. The vast majority of $\gamma\delta$ T cells in the characterized TIL are Vδ1, with an average Vδ1 expression of 80.63% (n=13, ST.DEV=19.3%) with no meaningful variation across tumor types. This is consistent with a recent report from *Wu et al.* in breast cancer. [25] The median in our cohort is 83.6% compared with the reported $\approx$ 55% (n=44) by *Wu et al.*, suggesting an enrichment in Vγ1 populations. Whether this enrichment is due to the culture conditions, mainly IL-2, or an accurate representation of the lymphocyte infiltrate remains to be seen.

The phenotypic data reported in **Figures 8.B** was constricted to Vδ1 cells, as they represent the majority of $\gamma\delta$ T cells across the sample cohort. Just like in the $\alpha\beta$ TIL, the majority of $\gamma\delta$ cells are effector memory, TEM ($CD45RO^+CD62L^-$). This is consistent with data from *Daley et al.*, showing that $\gamma\delta$ TIL have very little $CD62L^+$ expression, and seem to be mostly TEM. [26] Two $\gamma\delta$ TIL samples have a majority of cells with a TEMRA ($CD45RO^-CD62L^-$) memory phenotype, typically

associated with terminally exhausted cells in $\alpha\beta$ TIL. [27]

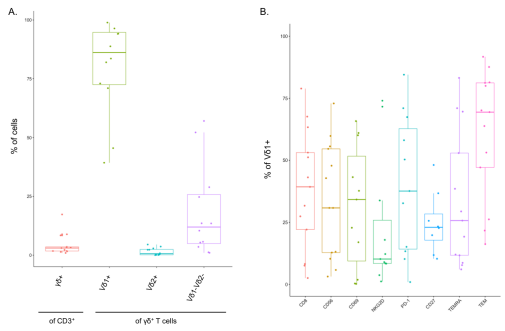

Figure 8: Box plot of: A. the distribution of $\gamma\delta$ T cells; B. Phenotypic characterization of Vδ1 in TIL across different cancer histologies.

Consistent with a TEM and TEMRA memory phenotype is the low expression of CD27, with an average of 25% (**Figure 8.B**). CD27, a member of the tumor necrosis factor receptor superfamily and a co-stimulatory immune molecule, has been described in $\alpha\beta$ T cells as a marker of "younger" memory T cells, cells with a greater potential for proliferation. *Dayley et al.* describes $\gamma\delta$ TIL in pancreatic cancer to be mostly CD27$^-$, [26] which is consistent with the flow cytometry data presented in this work. This is in stark contrast with the $\gamma 9\delta 2$ T cells found in the blood, as they have high expression of CD27, associated with a generally younger memory profile. [28]

CD69 expression, a marker of tissue resident T cells, was moderate, with some TIL samples having very low CD69 expression (<25% for n=4). As Vδ1 are considered to be tissue resident cells, CD69 was expected to be a prevalent marker of these cells. It is difficult to interpret this finding, but it differs significantly from the data presented in *Wu et al.*, where all $\gamma\delta$ samples had at least 69% of CD69 expression. [25] Also inconsistent with the findings reported in *Wu et al.* is the NKG2D expression which is consistently low across all samples. An initial hypothesis was that the prolonged IL-2 exposure necessary for the *ex vivo* expansion of TIL cultures could lead to downregulation of NKG2D. However, experimental evidence seems to indicate that IL-2 exposure leads to NKG2D upregulation. [29, 30] Another explanation might be the immunological imprinting caused by the tumor microenvironment. As for PD-1, it is the surface molecule with the biggest variation between TIL samples. PD-1 has been described as a reflection of tumor reactivity, increased T cell dysfunction, and shown to be upregulated in TIL across many cancer histologies. [31] For $\gamma\delta$ it is unclear whether PD-1 has the same phenotypic significance and it is something to address in the future.

An interesting observation from this dataset is the consistent presence of CD8$^+$ $\gamma\delta$ T cells (**Figure 8.B**). This is practically unreported in $\gamma\delta$ T cells, as they are canonically considered to be CD8$^-$CD4$^-$. Furthermore, use of nonlinear dimensionality reduction algorithms shows that the CD8 $\gamma\delta$ T cells are typically associated with CD56 expression, a NK marker that when expressed in T cell generally marks activation and cytotoxicity capacities. One report of $\gamma\delta$ T cells in the intestinal epithelium describes a subpopulation with high CD8$^+$ expression, coupled with CD56, CD25 and CD69 expression and high cytotoxicity capacity. This suggest that CD8$^+$ $\gamma\delta$ T cells are activated and capable of cytotoxic effector functions. [32]

To further explore the role of these cells, it would be necessary to isolate and expand this subset, work that this work began but left much to be continued. Briefly, efforts to isolate $\gamma\delta$ for posterior expansion were successful by both $\gamma\delta$ positive sorting or $\alpha\beta$ depletion. In addition, with the isolated $\gamma\delta$ T cells, two expansion protocols were tested. The fist one was similar to the standard $\alpha\beta$ T cell expansion protocol (CD3 antagonist stimulation coupled with irradiated feeders, and IL-2 supplementation). With it, no $\gamma\delta$ expansion was achieved suggesting the standard TIL expansion protocol is inadequate for $\gamma\delta$ survival and proliferation. The second one used IL-4, IL-15 and IL-21, together with CD3 antagonist stimulation every 3 to 5 days to propagate $\delta 1$ T cells isolated from the blood. [30] Two attempts of expansion were made using this latter protocol, with neither resulting in expansion of $\gamma\delta$ T cells.

Ultimately, this preliminary work suggests that $\gamma\delta$ cells rising from the TME might require further research into new culture and expansion methods.

3. Conclusion

This dissertation work showcased a methodology currently used in clinical trials to determine neoantigen reactivity in TIL. Surprisingly, out of all candidate neoantigens identified by WGS and tested through the use of tandem minigenes, none were shown to drive neoantigen recognition, even though these TIL effectively reacted against the autologous tumor digest (20% reactivity).

This demonstrates that there are still improvements necessary to better identify neoantigen reactivity. An additional screen for self antigen could be advantageous to pre-screen TIL reactivity, particularly if it was in TMG format as it would allow for testing of several known self antigens that drive cancer reactivity. In addition, with the use of RNA-seq it would be possible to look at transcriptional changes that may be driving tumor reactivity,

particularly in the context of uveal melanoma with SF3B1 mutations.

Perhaps a more ambitious screen would be the isolation of TCRs by single cell technologies. Coupled with phenotypic markers, or skewed clonality, the isolation of tumor reactive TCRs could reverse engineer the neoantigens responsible for driving tumor reactivity, thus providing an unbiased approached to characterise T cell tumor reactivity.

In addition, this work characterized several $\gamma\delta$ TIL samples across different histologies. All TIL samples show sparse but recurrent $\gamma\delta$ presence. In doing so, it showed that the overwhelming majority of $\gamma\delta$ T cells in TIL are $\delta 1$, thus suggesting these cells are the major subset of $\gamma\delta$ that persists in the tumor microenvironment. A surprising aspect of this work as the consistent existence of $CD8^+$ $\gamma\delta$ T cells in TIL. Moreover, these cells showed varied expression of CD56, CD69 and PD-1.

Other T cells markers are hypothesised to have a role in $\gamma\delta$ T cells and should be studied in the context of $\gamma\delta$ TIL, namely the three NK receptor molecules, NKp30, NKp44, Nkp46.

Unfortunately due to the difficulties in expanding $\gamma\delta$ T cells from TIL, the analysis of their cytokine expression profile in response to various stimuli was not performed during this work, but it is of the utmost priority as the expansion efforts come to fruition, particularly against autologous tumor tissue. Accordingly, it is crucial to get a better understanding of what targets they recognise in tumor, and how permissive they are to reprogramming by the tumor microenvironment.

4. Methodology

4.1. TIL Generation

TIL were generated from surgically resected metastasis or primary tumors. Tumors were cut into ≈1-2 mm fragments, placed individually into wells of a 24-well plate containing 2 ml of complete media (CM) containing 3000 IU/ml of IL-2 (Miltenyi, GMP Grade). CM consisted of RPMI (Gibco) supplemented with 5% AB human serum (Valley Biomedical) , 2 mM L-glutamine (Sigma-Aldrich) and 1% Pen-Strep (Sigma-Aldrich). For laboratory scale of *in vitro* expansion, select cultures were expanded in T25 Flasks (ThermoFisher) at a ratio of 1 to 300 in 400 ml of 50% RPMI and 50% AIM-V medium (ThermoFisher), supplemented with 5% human AB serum, 3000 IU/ml of IL-2, and 30 ng/ml of OKT3 antibody (Miltenyi, GMP grade).

4.2. Sample Processing

Tumor Digestion was executed as described in the Tumor Dissociation Kit from Miltenyi (product #130-095-929). Cells, resulting from either apheresis or PBMC isolation after a blood draw, were washed and sorted based on CD14 expression following the pan Monocyte Isolation kit (Miltenyi) instructions. Afterwards, cells (from either tumor or blood) were resuspended in 5-10 mL of Red Cell Lysis buffer (Ack lysis buffer, Gibco) and spun down again. The resulting pellet is resuspended in freezing medium and frozen.

4.3. $\gamma\delta$ T cell isolation

T cells grown from TIL were sorted to isolate $\gamma\delta$ T cells. Positive Sort, where $\gamma\delta$ are retained in the column, and Negative Sort, where $\gamma\delta$ go through the column, kits were tested and used (Miltenyi, ref 130-050-701 and 130-092-892, respectively). Afterwards cells were either stained for flow acquisition or grown using cytokine cocktails.

4.4. DC generation and electroporation

Monocytes were thawed washed and set to 5-10 M cells/ml in RPMI. Cells were platted in a T-175 Flask (Thermo Fisher) and incubated at 37°C, 5% CO_2 for 90 min. Afterwards, non-adherent cells were retrieved and the remainaing, adherent cells were incubated with DC media. DC media comprised of RPMI containing 5% human serum, 1% Pen-Strep, 2 mM L-glutamine, 800 IU/ml GM-CSF (Leukine) and 200 U/ml IL-4 (Peprotech). Fresh DC media was added to the cultures every 2-3 days for a total of 10 days. To complete the maturation process, cells were electroporated using the Neon™ Transfection System. Briefly, cells were washed with PBS and re-suspended with Neon Buffer at the desired concentration (10^5cells per 10 μl). 10 μl of cells were mixed with 2 μl of mRNA and the resulting mix was pulsed twice at 1050 mV for 20 ms and platted in a 96 well plate (Thermo Fisher).

4.5. Whole Genome Sequencing and Mutation Calling

Samples were processed following the standard whole genome sequencing pipeline at the UPMC Genome Center. Sequencing was performed using NovaSeq 6000 platform (Illumina) with 151 paired-end reads and an average target depth of 70x coverage. Samples were mapped with Edico Dragen (v2.1). Somatic variants were called by Mutect2 (GATK 4.0.5). Variants were annotated with Funcotator (GATK 4.0.5) and a certain range of a.a and nt sequence were retrieved around the detected variants using an internal script. All computation was carried out in a linux based AWS ec2 instance on DNAnexus platform.

4.6. Tandem minigene construction and synthesis

The encoding TMG DNA sequences were synthesized and cloned into eGFP tagged pcDNA3.1+ using EcoRI and XhoI restriction sites. TMG have 10 to 20 minigenes fused together, and each encodes the 25 amino acid long peptide with the mu-

tation. TMG plasmid constructs were transcribed *in vitro* and resuspended at 2-3 μg/l concentration in DNAse and RNAse free water.

4.7. Neoantigen Recognition Screening

Upregulation of 4-1BB as well as release of IFN-γ were used as measures to assess recognition of TMGs. Co-culture was performed 5-6 hours after DC electroporation. Expanded T cells (10^5) were cultured with (10^5) DCs, one well for each TMG. To confirm tumor specific reactivity, TIL was cultured alone and with autologous tumor digest in parallel. Anti-CD3-CD28 antibody was added to T cells alone for a positive control. After 16 h, supernatants were harvested to be analyzed by IFN-γ enzyme-linked immunosorbent assay (ELISA) and the cells were collected, stained and analyzed by flow cytometry.

4.8. ELISA

ELISA plates were prepared by coating 96 well plates (NUNC Band Products) with 100 μl/well primary IFN-y antibody from Endogen (clone 2G1) and left to incubate overnight at 4°C. 300 μl/well of blocking solution were added for an hour. After washing, standards and samples were added to the plate and left for an hour and a half. After washing, 50 μl/well of 1X Biotenylated antibody (Endogen, clone B133.5) solution were added to the precoated plate, followed by an 1 hour incubation. 100 μl/well of Streptadivin-HRP (Invitrogen) solution incubated for 30 min. 100 μl/well of TMB solution (Dako Product) was added, and after 10 min, 100 μl/well of 0.18M of H_2SO_4 was added in order to stop the reaction. Plates were read at 450 nm using a Tecan Infinite F50 spectral reader.

4.9. Staining & Flow Cytometry Acquisition

Expression of immune cell markers was assessed by flow cytometry. Briefly, cells were pelleted, washed with FACS buffer (1X PBS supplemented with 2% FBS and 2 mM EDTA), and then stained with the appropriate antibodies for $\approx$ 30 min, at 4°C in the dark. Cells were washed once with FACS buffer prior to acquisition on BD LSRFortessa^{TM} flow cytometer or Cytek^{TM} Aurora. All data was gated on PI/Zombie negative to excluded dead cells. For co-cultures 2-8 x 10^4 events were collected, while for tumor digest and TIL analysis 0.2 to 2 x 10^6 events were collected. Acquired flow data was analyzed using FlowJo 10.6.1 (BD).

4.10. Data Plots & Statistical Analysis

Data was plotted using Excel and/or R Studio, using the ggplot2 and reshape packages. Figures were created with Biorender.com.

4.11. Sample Information

All patients were part of on-site clinical trials, and provided written informed consent to the use of tissue samples and the generation of sequencing data. Two patients had metastatic Uveal Melanoma, and underwent surgical resection of liver metastasis. Four Patients had Metastatic Renal Clear Cell (RCC) carcinomas. Six patients had Pseudomyxoma Peritonei (PMP), a condition caused by mucinous low grade adenocarcinomas, and underwent cytoreductive surgery. Three patients had metastatic Gastrointestinal (GI) malignancies including, 2 Appendiceal and 1 Colorectal carcinoma.

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