TÉCNICO LISBOA

# Understanding Tumor Infiltrating Lymphocytes: Sequestered Reactivity in T cells

**Inês Ferreira e Silva**

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## Biological Engineering

Supervisors: Prof. Markus Maeurer, MD, PhD
Prof. Maria Margarida Fonseca Rodrigues Diogo, PhD

### Examination Committee

Chairperson: Prof. Jorge Humberto Gomes Leitão, PhD
Supervisor: Prof. Markus Maeurer, MD, PhD
Member of the Committee: Prof. Paula Alexandra Quintela Videira, PhD

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## Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

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# Resumo

A terapia de células usa a ”força” dos linfócitos T expandidos *in vitro* para provocar regressão tumoral. Esta regressão é conduzida pelo reconhecimento de neoantigénios, em exposição pelo complexo de histocompatibilidade. A identificação de neoantigénios é fundamental para melhorar a eficácia de tratamentos antitumorais e esta dissertação enaltece uma metodologia para identificar neoantigénios (com recurso a sequenciação de genoma e *tandem minigenes*). Dois pacientes com melanoma uveal metastático foram documentados como tendo reatividade tumoral. Para identificar os neoantigénios responsáveis pela reatividade 232 e 87 mutações, respetivamente, foram testadas não tendo sido identificada reatividade a nenhum dos candidatos.

Para além da falta de reactividade a neoantigénios, uma das barreiras para o sucesso da imunoterapia é a *downregulation* ou perda do complexo de histocompatibilidade. Neste cenário, foram exploradas outro género de células T, as gamma delta ($\gamma\delta$). Este trabalho demonstrou pouca infiltração de $\gamma\delta$ (menos de 3% de todos os linfócitos T), em diferentes tipos de tumor. A maior parte destas células são V$\delta$1, com perfis de memória semelhantes aos linfócitos convencionais. Para além disso, verifica-se expressão de CD8, em conjunto com CD56 e uma expressão variável de CD69 e PD-1. Verifica-se ainda baixa expressão de NKG2D que, em conjunto com os outros marcadores, demonstra que existem diferenças substanciais entre estas células no sangue e em ambiente tumoral.



**Palavras-chave:** TIL, Gamma-delta, Reatividade a Neoantigénios, Terapia Celular, Imunoterapia.

# 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 V$\delta$1, with a similar Memory Effector to Terminally Differentiated Phenotype, as observed on $\alpha\beta$ T cells. Furthermore, they surprisingly express CD8 as well as CD56, variably CD69 and PD-1 but lack NKG2D expression, indicating a substantial difference from $\gamma\delta$ obtained from the blood.



**Keywords:** Tumor Infiltrating Lymphocytes (TIL), Neoantigen Reactivity, Adoptive Cellular Therapy, $\gamma\delta$ T cells, Immunotherapy.

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# Nomenclature

## List of Abbreviations

| | |
|---|---|
| **ACT** | Adoptive Cellular Therapy |
| **APC** | Antigen Presenting Cell |
| **CAR** | Chimeric Antigen Receptor |
| **CR** | Complete Response |
| **DC** | Dendritic Cell |
| **ECM** | Extracellular Matrix |
| **ELISA** | Enzyme-Linked Immunosorbent Assay |
| **GFP** | Green Fluorescent Protein |
| **GI** | Gastrointestinal |
| **HLA** | Human Leukocyte Antigen |
| **i-TCR** | Induced T cell Receptor |
| **IFN-$\gamma$** | Interferon Gamma |
| **MHC** | Major Histocompatibility Complex |
| **mRNA** | Messenger RNA |
| **OR** | Objective Response |
| **PBL** | Peripheral Blood Lymphocyte |
| **PBMC** | Peripheral Blood Mononuclear Cell |
| **PMP** | Pseudomyxoma Peritonei |
| **RCC** | Renal Cell Carcinoma |
| **REP** | Rapid Expansion Protocol |

| | |
|---|---|
| **$T_{reg}$** | T regulatory Cell |
| **TAM** | Tumor Associated Macrophage |
| **TCM** | Central Memory T cell |
| **TCR** | T Cell Receptor |
| **TD** | Tumor Digest |
| **TEMRA** | Terminally Differentiated T cell |
| **TEM** | Effector Memory T cell |
| **TIL** | Tumor Infiltrating Lymphocytes |
| **TME** | Tumor Microenvironment |
| **TMG** | Tandem Mini Genes |
| **UM** | Uveal Melanoma |
| **WES** | Whole Exome Sequencing |
| **WGS** | Whole Genome Sequencing |

# Chapter 1

# Introduction

This chapter serves as an introduction on how cancer arises in spite of a functional immune system (the so-called Hellstrom paradox [1–3]), highlighting the duality that the immune system plays in cancer progression. It will also explore enhancing the adaptive immune system as a means to mediate tumor regressions, and showcase adoptive cell therapy as a form of anti-cancer therapy. Furthermore, it will cover some of the many aspects behind immune evasion, and focus on $\gamma\delta$ T cells as a novel way to overcome MHC loss or downregulation. At the end of the chapter there will be a brief overview of the methodologies employed in this dissertation.

## 1.1 Concepts of Cancer Immunology

> *Down to their innate molecular core, cancer cells are hyperactive, survival-endowed, scrappy, fecund, inventive copies of ourselves.*
>
> Siddhartha Mukherjee, *The Emperor of All Maladies*

Our cells and tissues have inherent mechanisms to promote genomic stability, recognize stressed cells, and limit uncontrolled proliferation. When these mechanisms fail, the uncontrolled proliferation leads to invasive growth and ultimately to the development of a malignant tumor. [4, 5] Many of the mechanisms that limit cancer establishment and progression have been extensively researched, reported and explored as advanced therapeutic targets with only modest degrees of success. Nevertheless, in the last few decades, cancer therapies evolved from surgical resection and the crude destruction of rapidly dividing cells to the refined targeting of molecular pathways, even if with limited success in metastatic forms of cancer.

Focusing on the patient's immune response had been a less effective strategy until recently, when immune checkpoint inhibition demonstrated that it was possible to target a specific molecular pathway to effectively make the immune system attack and destroy malignant cells. [6] But the notion that the immune system played a role in the bodies defense against cancer is not new. In fact, back in the sixties,

Burnet and Thomas speculated that lymphocytes could effectively recognize and eliminate continuously arising malignant tumors, an early description of *immune surveillance*. [7–9]

The increased incidence of cancer in immunocompromised individuals provided insights into the nature and importance of the host response. The Cincinnati Transplant Tumor Registry reported a twofold greater risk in transplant patients for developing melanoma, while 608 cardiac transplant patients followed by the University of Pittsburgh were found to have a 25-fold increase in the frequency of lung tumors. [10, 11]

Perhaps a more convincing piece of evidence was the recurrent presence of immune cells found infiltrating the cancerous mass. One could speculate that immune cells were recruited by the tumor to support its survival and expansion, as fibroblasts often are. However, early trials following patients with cancer reported significant correlation between the increased percentage of immune cells and improved survival.

A more recent trial classified cutaneous melanoma tumors into three distinct categories, brisk, non-brisk and absent of TIL. Patients with brisk infiltration survived 1.5-3 years longer than the patients with absent lymphocyte infiltration. [12] Another trial followed patients with advanced-stage ovarian carcinoma, initially treated with surgical resection followed by chemotherapy regimens, and found that 74% of patients survived past 5 years, when they had increased tumor lymphocyte infiltrates in their primary cancer. Comparatively, only 12% of patients with sparse lymphocyte infiltration were alive after 5 years. [13] This glaring disparity was later described across several cancer histologies, including colorectal, breast cancer and gliomas. [14–16] Studies like these culminated in the development of an ”Immunoscore”, a classification system based on the percentage of lymphocytes present in the tumor. This system is a better prognostic predictor in gastrointestinal (GI) malignancies, when compared with the classical staging system, and has now been implemented in oncology practice. [17, 18]

Together, these studies provide strong evidence that the immune system is capable of surveilling and controlling tumor cells. But if the human body is capable of immunosurveillance **why do immunocompetent individuals still develop malignant tumors?** This question is partly answered through the concept of immmunoediting, a dynamic process that encapsulates the paradox of the immune system both constraining and promoting tumor progression. [19, 20] It can be described through 3 phases:

- Elimination, where both adaptive and innate immune systems cooperate to recognize and kill transformed cells that have escaped intrinsic mechanisms of tumor suppression.
- Equilibrium, where the tumor subclones capable of surviving elimination are sparse, which in turn limits tumor growth. Ultimately pressure from the adoptive immune system together with the genomic instability of tumor cells leads to the survival of the fittest cells, a scenario resulting in tumor cells that evade detection and prepare the microenvironment for uncontrolled growth.
- Escape, where the immune system can no longer contain the tumor’s proliferation, likely causing the patient to start displaying symptoms of disease.

### 1.1.1 Immune cells in the TME: A delicate balance

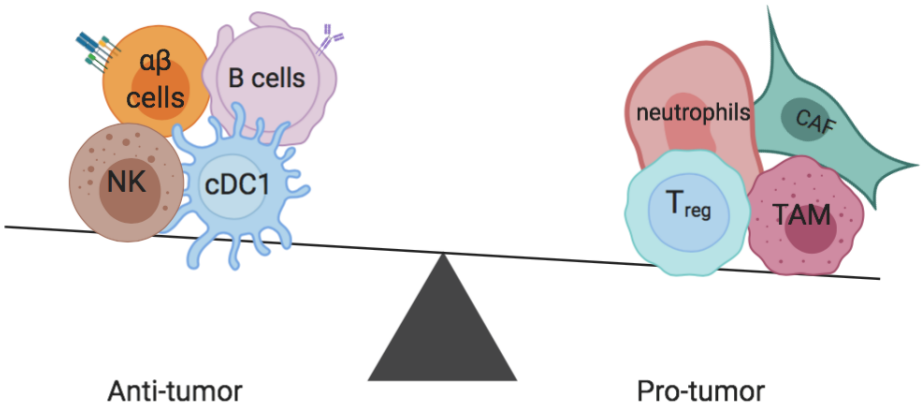



Figure 1.1: The delicate balance of the cellular components in the tumor microenvironment.
cDC1s are a subset of Dendritic Cells, NK stands for Natural Killer cells, CAF are Cancer Associated Fibroblasts, TAM means Tumor Associated Macrophage, Treg is a regulatory T cell and $\alpha\beta$ is the canonical TCR of a T cell.

The immune surroundings of an established tumor often have many parallels with chronic viral infections: the initial trigger (the first malignant cells) is not successfully eliminated leading to a build-up of inflammatory signals, with subsequent activation of immunoregulatory mechanisms. [21] To add fuel to the fire, cancer cells can manipulate both stromal and immune cells to enhance the already immunosuppressive environment. [22]

A cornerstone of any immune response is the recruitment of myeloid cells. Monocytes are recruited to the site of inflammation and locally differentiated into macrophages and DCs, capable of probing the surroundings and capturing antigens. One immune cell critical to mount both anti-viral and anti-tumor responses is the cDC1, a conventional DC subtype highly effective at transporting antigens to tumor-draining lymph nodes, thus mediating antigen cross priming and T cell priming. [23–25] Their exclusion from the tumor microenvironment (TME) correlates with decreased survival for the patient. Even when cDC1s are capable of infiltrating the tumor, they are highly dysfunctional. This dysfunction seems to be driven by IL-10 producing macrophages present in the TME, which in turn inhibit the DCs from producing IL-12. [26]

Macrophages, another type of myeloid cell, clear cellular debris resulting from tumor cell death through phagocytosis, while simultaneously recruiting other immune components to the clearing site. They help orchestrate immune responses and have a secondary role, serving as an antigen presenting cell (APC). Within the TME, many of the recruited inflammatory macrophages become tumor associated macrophages (TAM), a general term that covers several different phenotypic subcategories. [27] In mice and *in vitro* studies, TAMs aid tumor progression, by supporting angiogenesis and extra-cellular matrix (ECM) remodeling. [28] They also have a role in IL-10 production, leading to the suppression of *cross priming* by APCs within the TME. [26] Studies have also shown a role in the exclusion of T cells, as macrophages associate with CD8 cells within the stromal areas, thus trapping and impeding them from

moving further into the tumor. [29] Furthermore, macrophages can express both PD-L1 and PD-L2 which directly inhibit TCR signaling and cytotoxic T cell function. [30, 31]

TAMs have also been shown to recruit T regulatory cells (Tregs) and induce the expression of *foxp3*, a Treg transcription factor, in CD4+ T cells *in vitro*. [32] Tregs are a $CD4^+$ T cell subtype that have an immunosuppressive role. They are generally considered pro-tumoral, with recent studies pointing to their presence in TIL as the main driver for rapid tumor progression after checkpoint inhibitor treatment. [33] They are thought to consume most IL-2 available in the TME, leading to IL-2 depletion and promoting T cell apoptosis and anergy. [34] In addition, Tregs lead to the downregulation of CD80 and CD86 on antigen presenting cells (APCs), meaning they have a role that is not only prejudicial locally, but may also impact the cross priming in the lymph nodes. [35]

Perhaps the most adverse prognostic immune cell infiltrate is neutrophils, a phagocyte normally found in the bloodstream, as a meta-analysis of gene expression signatures by *Gentles et al.* shows its presence in the tumor microenvironment (TME) is associated with a worst prognosis. [36]

A helpful but perhaps 'over-simplistic' summary of the role each of these cells has in tumor progression can be seen in **Figure 1.1**, as each component is weighted on a scale as either pro or anti tumor, shifting the balance of the immune response.

#### Immune signatures in the TME

Distinct leukocytes are recruited to tumors, each with multiple subtypes that may play different and often conflicting roles in fostering or suppressing tumor growth. [37] This delicate balance, in which cell types that are normally considered anti-tumor may in some scenarios be associated with pro-tumor activities, is amplified by the fact that different tumor histologies will often have very distinct immune populations.

The 'immune landscape', a hallmark of the tumor microenvironment, suggests that different immune landscapes are associated with variable tumor progression, and likelihood of recurrence. [38] Through the analysis of the transcriptional profile of 681 genes (representative of most immune cell subpopulations) it was possible to distinguish 105 patients with colorectal cancer into two groups: Group 1 associated with prolonged disease-free survival and Group 2 associated with worst disease prognosis and unfavorable outcomes. Group 1 showed a clear signature of genes associated with cytotoxic T cell surface molecules, T helper cell surface molecules, and chemokine-related genes, whereas Group 2 was associated to genes that regulate the adaptive immune response associated with Tegs, Th2/Th17 and NK cells.

With the construction of The Cancer Genome Atlas (TCGA), both tumor genomic and transcriptomic data become readily available, permitting the examination of the immune infiltrate within the tumor microenvironment at an unprecedented scale. Exceptional work by *Thórsson et al.* characterized the immune TME across 33 cancers and identified six immune subtypes : [39]

- **Wound healing** signature with increased levels of angiogenesis, high proliferative capacity and a Th2 signature in the adaptive immune infiltrate. The cancers that were most strongly associated with this signature include colorectal cancers, lung squamous cell and breast invasive carcinomas.
- **IFN-$\gamma$** signature has pronounced CD8 signalling and broad TCR diversity, alongside a high M1

macrophage polarization[1]. It was comprised of highly mutated cancers: some BRCA, gastric, ovarian, and cervical tumors (CESC).

- **Inflammatory** signature shows reduced tumor cell proliferation and less somatic copy alterations. It has elevated levels of Th17 and Th1 and is typical in most kidney, prostate and pancreatic carcinomas.
- **Lymphocyte depleted** signature has a prominent macrophage signature, with high M2 levels. It characterizes histologies like some liver hepatocellular carcinomas and gliomas.
- **Immunologically quiet** signature is present in most lower grade gliomas, with a reduced lymphocyte count, but a high M2 response. Curiously, this signature was enriched in IDH mutations, suggesting an IDH role in decreased leukocyte chemotaxis.
- **TGF-$\beta$ dominant** signature can be found across different tumor types with high TGF-$\beta$ expression and increased lymphocyte infiltration.

Apart from defining six subtypes that encompass the diversity of the tumor immune landscape, this work also intimately characterized the TME. It reaffirmed that tumors with a greater leukocyte fraction (LF) were more responsive to immune checkpoint inhibitors and found a reduced LF in tumors with a higher proportion of stromal fraction, as is the case with pancreatic adenocarcinomas and low grade gliomas, further highlighting the role TIL have in prognosis. [39]

[1] M1 to M2 macrophages is a binary way of looking at macrophages as either pro-inflammatory (effective at phagocytosis and secretion of pro-inflammatory cytokines) or anti-inflammatory (immune suppressive and angiogenesis inducing), respectively.

## 1.2 TIL, an (often) effective ”living drug” therapy

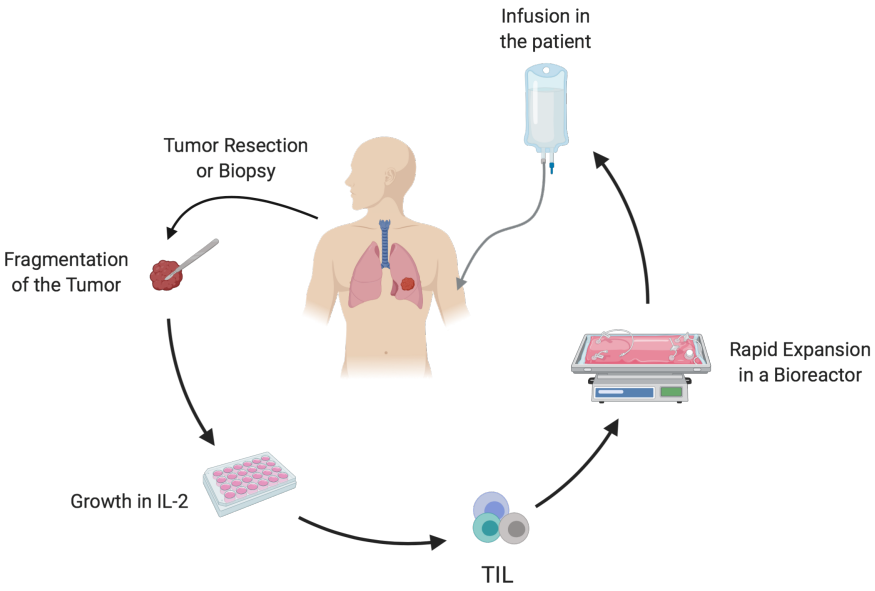



Figure 1.2: A summary of TIL production for clinical infusion and treatment.

The first evidence of an ability to grow tumor infiltrating lymphocytes, in mice, were those of Ilana Yron in the Surgery Branch of the NCI, in 1980. [40] Early studies showed that human tumor infiltrating lymphocytes (TIL) obtained from resected melanomas, and cultured in IL-2, were capable of recognizing and killing autologous tumor cells. [41] This finding was both exciting and puzzling. On one hand it showed that our own immune system could effectively recognize and kill tumor cells.

But if lymphocytes had that capacity all along why was the tumor still progressing? While the answer remained elusive at the time, those early studies provided the rationale for the first TIL clinical trials in patients with advanced melanoma, refractory to all other therapies including IL-2 dosage, spearheaded by Professors Rosenberg, Topalian, and Lotze in the Surgery Branch at the NCI. The very first TIL trial, published in 1988, showed that the stimulated TIL infusion could lead to measurable regressions in patients with metastatic melanoma, although of relatively short duration. Out of the 20 patients enrolled, 9 had an objective regression that lasted 2 to 13 months. [42]

Overall TIL production for therapy, represented in **Figure 1.2**, 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, leading to the migration and survival of the lymphocytes out of the fragment. Afterwards the cells are rapidly expanded, with the use of irradiated allogeneic feeders, and infused back into the patient, followed by several doses of intravenous high dose IL-2 to facilitate infused T cell survival.

However, durable responses remained elusive. Several iterations and combinations were trialed to improve the therapy’s efficacy, including various methods to obtain TIL and distinct expansion protocols. TIL obtained from fragments and tumor digests was compared in clinical trials, with no significant differences although in general fragments have appeared to be superior. [43] The introduction of specialized culture flasks allowed for more effective gas transfer thus permitting faster expansion rates.

And by starting the rapid expansion with ”younger” TIL, meaning cells that had spent less time in culture, the expansion had increased numbers, with a less exhausted phenotype. [44]

In the end the biggest alteration that lead to significant improvement in durable responses was completely depleting the patient's own lymphoid compartment right before *in vivo* transplantation, a practice termed nonmyeloablative (NMA) lymphodepletion. [45] Lymphodepletion, achieved by chemotherapy regimens or a combination, eradicates the bodies lymphoid cells and is thought to eliminate lymphoid cells that aid the tumor and provide space for the new transplanted cells to re-populate and lead the reconstitution of the immune system. In fact, the first reported patients treated with preemptive lymphodepletion were described to have persistent re-population with the infused T-cells. Furthermore, these clonal cells proliferated, had a capacity to traffic to tumor sites and mediated cytotoxic activity. [45]

Ultimately, 51% out of the 35 lymphodepleted patients experienced objective clinical responses, with a medium duration of 11.5 months. Most of those with complete responses did not have recurrences for up to 10 years. [46] More recently, clinical trials using TIL to treat metastatic melanoma report a complete response (CR) rate of 24%, out of the 99 patients that received treatment, with a median follow-up of 40.9 months. [47] This is substantially greater than with IL-2 infusion alone, which led to durable complete regressions in less than 10% of patients. [48]

**But how does TIL therapy compare to the use of immune checkpoint inhibitors?**

Immune checkpoint inhibitors, weaponize the immune system against cancer, and have revolutionized cancer treatment in the last decade. They do so by targeting the ”brakes” of T lymphocytes, through the use of humanized monoclonal antibodies against cytotoxic T-cell lymphocyte associated protein-4 (CTL-4), programmed cell death protein 1 (PD-1 ) or its corresponding ligand (PD-L1). [6]

The largest reported trial of ipilimumab, an antibody targeting CTL-4, led to objective responses (ORs) in 7% of patients, and CRs in 3 out of the 540 patients treated. [49] More importantly, a posterior analysis of the patients that responded to ipilimumab therapy showed consistent durable responses, with a 3-year survival of 22% that remained stable for up to 10 years. [50] Yet, treatment with anti-CTL4 had severe adverse effects for many patients (66%), with 10-15% having grade 3 or 4 immune-related adverse events. As for antibodies targeting the PD-1 and PD-L1, both Nivolumab and Pembrolizumab have mediated ORs in 32%-26% of ipilimumab-refractory patients and 40%-33% of treatment-naive patients respectively, with lesser side effects. [51]

In comparison, the 2016 Melanoma TIL trial had 51% of patients alive after 3 years, including patients that had progressed through prior immune checkpoint treatment (targeting CTL-4, PD-1 or both). [47] This validates TIL therapy as a valid option for patients, particularly for those that have exhausted all other courses of treatment.

Outside of Melanoma, the efficacy of TIL has been tested in other advanced solid cancers with varied but generally low overall response rates. [52, 53] Patients diagnosed with refractory metastatic cervical cancer were treated with a single TIL infusion. 33% of the nine enrolled patients experienced ORs, and 22% had a CR ongoing 22 and 15 months after treatment. Interestingly, HPV reactivity in the infusion product correlated positively with clinical response. [52] Another TIL trial enrolled 21 patients, this time for the treatment of patients with metastatic uveal melanoma. Despite being considered by many an immunotherapy resistant tumor, 35% of patients achieved an OR. One patient had a complete response, as numerous hepatic metastases cleared after infusion, on-going at 21 months post-therapy at the time of publication. [53]

There are also singular reports of individual patients whose response to therapy is so significant that it warrants further research. One such case was a patient with metastatic cholangiocarcinoma that received a predominant $CD4^+$ TIL infusion, achieving a decrease in target lesions with prolonged stabilization of disease. [54] A single patient with colorectal cancer also enjoyed a substantial response to TIL therapy, shown to be driven by CD8 T cells. [55] More recently, a patient with metastatic breast cancer refractory to multiple lines of chemotherapy was treated with TIL resulting in complete durable cancer regression, on-going at 22 moths post infusion. [56]

Thus, **Adoptive Cell Transfer (ACT)**, utilizing *in vitro* expanded lymphocytes infused in a patient to mediate an immune response can lead to dramatic and durable tumor regressions. ACT involves either the *ex vivo* activation of tumor infiltrating lymphocytes (TIL) or the genetic engineering of peripheral blood lymphocytes, either by the addition of a chimeric antigen receptor (CAR) or the induction of an endogenous T cell receptor (i-TCR). [57]

### 1.2.1 T cell Reactivity

The dramatic responses reported in some of these pioneer trials provided clear evidence that the adaptive immune system has the intrinsic capacity of identifying and killing tumor cells.

This direct killing of tumor cells is largely mediated by $\alpha\beta$ T cells, as their TCRs recognize antigens displayed by the Major Histocompatibility Complexes Class I and Class II (MHCs I&II) present on tumor cells. This recognition of epitopes had been readily demonstrated in virus infected cells as a hallmark of the adaptive capacity of the immune system. But if malignant cells are a version of our own cells **what are T cells recognizing?**

It is established by now that each cancer cell has an altered genome, resulting in an unstable transcriptome, fertile ground to give rise to ”cancer identifiers” (Figure 1.3). These identifiers can arise from non-mutated proteins, known as ”self-antigen”, or peptides not coded by the ”healthy” human genome, so-called neoantigens. [58]

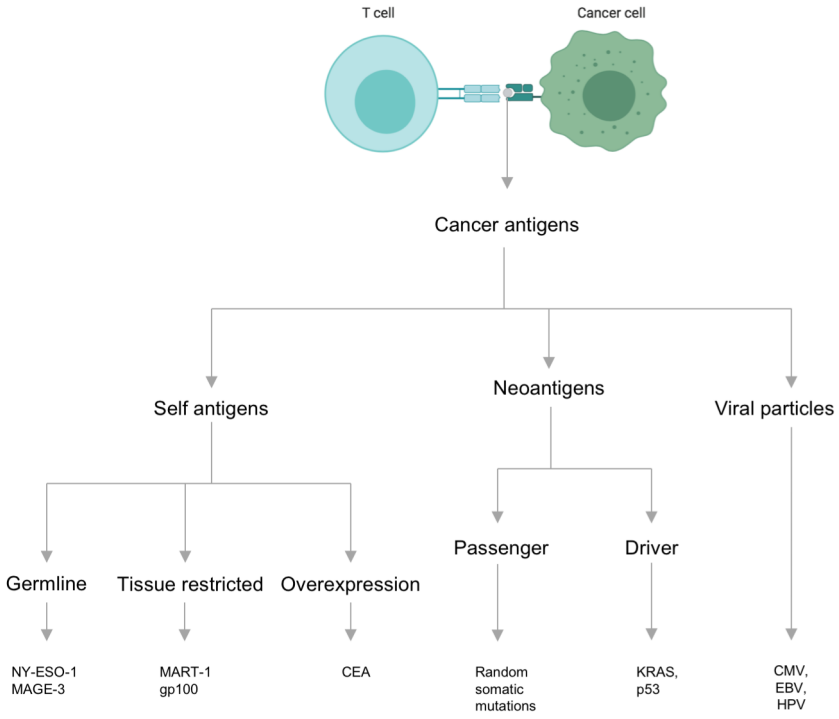



Figure 1.3: The diversity of cancer identifiers completed with examples of already identified antigens

**Self antigens** can arise from genes involved in cell potency (genes expressed in stem cells but typically silenced in differentiated cells), genes whose expression pattern is tissue constricted or genes expressed at very low levels in somatic tissues. In all scenarios, the transcriptionally dysfunctional cancer cell is thought to express some of these genes, thus permitting potential epitope formation and triggering an adaptive immune response.

In view of their shared presence across multiple cancer histologies, self antigens have been a prominent target of cancer vaccine trials, with little to no evidence of their clinical effectiveness in

established tumors. The working hypothesis to explain this disappointing result was that self antigens were subject to negative selection, meaning that self reactive T cells were likely eliminated during thymic development. However, some vaccine trials were able to show that the vaccines were capable of eliciting both CD8+ and CD4+ T cells responses, meaning that there are, at least in part, T cells with appropriate TCRs available to specifically recognize cancer cells. [59, 60] This corroborated early evidence that self antigen responses may form due to incomplete tolerance, leading to low avidity T cell receptors. [61, 62]

In contrast, **neoantigens** are the result of somatic mutations in normal gene products. 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. Independently of their origin, somatic mutations have the potential to generate new, 'non-self', epitopes, which can be divided into two main categories:

- **Passenger mutations**, which result from somatic mutations that are specific to the tumors of each patient. These mutations reflect the genetic and transcriptomic instability of tumor cells.
- **Driver mutations**, arise in genes that when altered give the cancer cell a growth advantage and are positively selected. As such, they are typically known oncogenes and can be shared across different cancer histologies, like TP53 and KRAS or be specific drivers like GNA11 or GNAQ in uveal melanoma. [63]

Somatic mutations seem to induce a preferential immune response. [64] Frameshifts mutations in particular generate a large number of non-self epitopes, and appear to be more immunogenic than self single point mutation derived antigens. [65, 66]

Driver mutations are considered vital for the malignant capabilities of the cancerous cell. They are also more likely to be expressed by most cancer cells, as driver genes have identical mutations across metastases, and across histologies. [67, 68] The most frequent peptides predicted to bind with MHC proteins were those from driver mutations, such as BRAF, KRAS, PIKC3A and TP53. [39] Consequently, driver mutations are often considered the ideal target for a less personalized, "off-the-shelf" type of therapy. Having said that, the frequency of the 20 neoantigens identified in a tumor reactivity screening was less than 0.005% across a cohort of 20 thousand human tumor samples. [58] This finding is also seen in various screenings of tumor reactivity at the NCI. Out of the 75 neoantigens identified in both TIL and PBMCs from patients with melanoma none were shared, nor corresponded to driver mutations. [69] A similar result was obtained when screening for neoantigen reactivity with TIL obtained from GI malignancies; out of the 73 neoantigens found in the screening only one epitope was shared between two patients out of 35 patients, a driver mutation in $KRAS^{12}$. [70] The resounding conclusion up to this point is that the vast majority of the identified neoantigens are unique for each patient.

Another category of tumor specific antigens are those derived from viral particles, likely due to the incorporation of viral genetic material into the cells' genome. Virus like HPV, CMV and EBV are thought to have a prominent role in the oncogenic development of some malignancies, and **viral antigens** have been shown to be expressed in tumor cells and by the cell's HLA molecules. [71] Moreover, there has been viral reactivity found in TIL, suggesting that viral reactive T cells have an active role in the TME. On

that note, vaccination against HPV has proven to be an effective way of preventing tumor progression in patients with premalignant lesions arising in the cervix. [72] Moreover, the two patients (out of nine) with durable regressions in the cervical cancer TIL trial had received HPV reactive T cells. [52] These studies culminated in the design and testing of several strategies to target viral epitopes as a means to combat tumor development, with several clinical trials underway to test the efficacy of induced TCRs targeting known viral epitopes.

### Tumor Mutational Burden and Response to Checkpoints Inhibition

If T cells are, for the most part, reacting to mutations in tumors, immunotherapy would be most effective in tumors with a higher amount of mutations. This concept is encapsulated by the term **Tumor Mutational Burden**, TMB, a measurement of the number of mutations carried by tumor cells. [73] And indeed, when looking at the effectiveness of immunotherapies there is a correlation between higher TMB and increased response to immunotherapy, **Figure 1.4**. [74, 75]

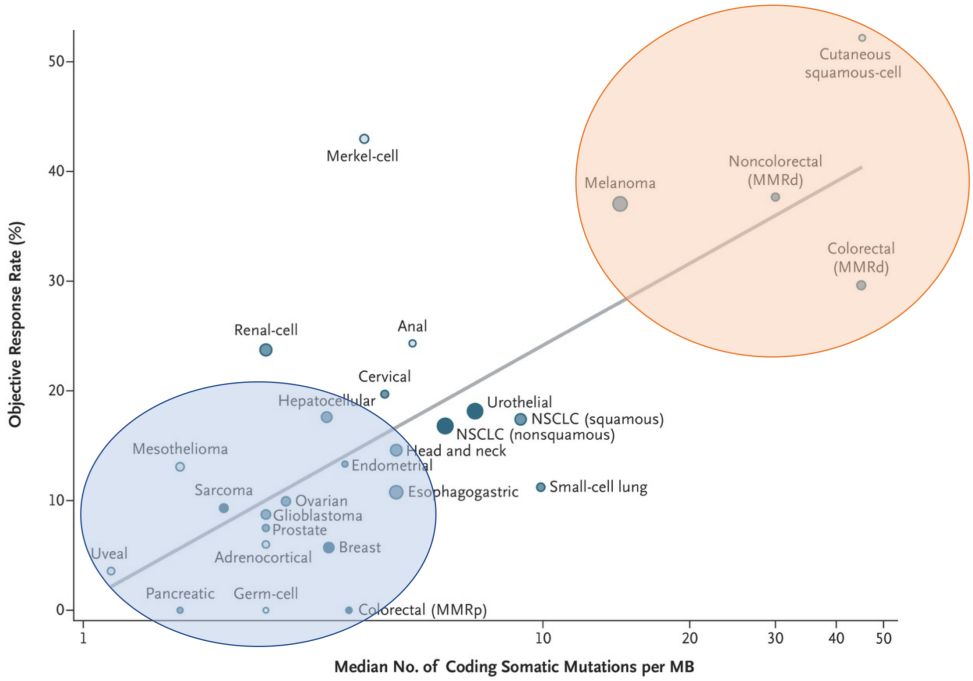



Figure 1.4: Correlation between Tumor Mutational Burden and Objective Response Rate with Anti–PD-1 or Anti–PD-L1 Therapy across 27 Tumor Types. Orange marks tumor considered very responsive to immmunotherapy, while blue marks tumors that are considered non responsive. Adapted from [74].

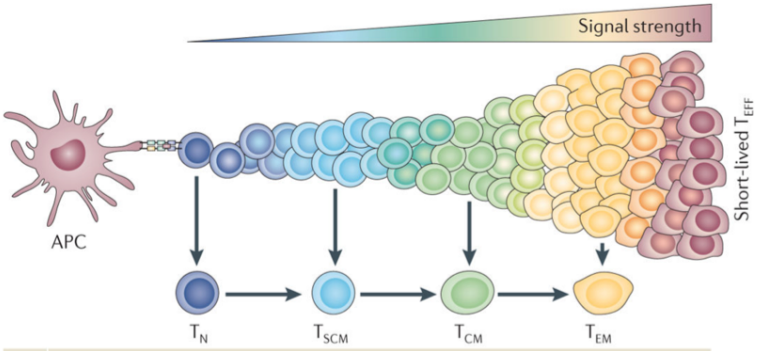
Even within the same cancer type, patients whose tumors had a higher TMB, had improved survival. [76] And while the previous study was restricted to melanoma patients, a recent meta-analysis by *Samstein et al.* demonstrated that a higher somatic TMB is associated with overall better survival after treatment with checkpoint inhibitors across different histologies. [77]

In the case of ACT, both higher mutational and neoantigen burden seem to forecast clinical benefit in patients with checkpoint resistant stage IV melanoma. [78] Whereas the TMB can predict responses in

ACT is still unknown, but there is a clear trend of increased OR for immunotherapies in histologies with a higher mutational load.

### Characterizing CD8$^+$ T cells in the TME

Tumor specific CD8$^+$ T cells are primed in tumor draining lymph nodes, after which they migrate to the tumor where they exert their cytotoxic, effector function. This process is known to drive their differentiation. While the lineage relationship of cytotoxic lymphocytes is debatable, the general consensus is that naive T cells begin to proliferate and differentiate into memory and effector progeny after being engaged by APCs, as schematized in **Figure 1.5**.




| | | $T_N$ | $T_{SCM}$ | $T_{CM}$ | $T_{EM}$ |
|---|---|---|---|---|---|
| Phenotype | CCR7 | +++ | +++ | ++ | – |
| | CD62L | +++ | +++ | ++ | – |
| | CD27 | +++ | +++ | ++ | +/– |
| | CD28 | ++ | +++ | +++ | +/– |
| | CD45RA | + | + | – | +/– |
| | CD45RO | – | – | + | + |
| | CD122 | – | ++ | +++ | +++ |
| | CD95 | – | ++ | +++ | +++ |
| | KLRG1 | – | – | +/– | ++ |
| Function | IFNγ | – | + | ++ | +++ |
| | IL-2 | – | ++ | +++ | +/– |
| | Cytoxicity | – | +/– | + | +++ |
| | Telomere | +++ | +++ | ++ | + |
| | Self-renewal | + | +++ | ++ | + |

Figure 1.5: T cell memory lineage. Adapted from [79].

A central memory T cell (TCM) phenotype is linked to a more proliferative state, with less cytotoxic capacity. These cells are generally CD62L positive, a molecule involved in the homing to the lymph nodes. They are also associated with the co-stimmulatory molecules, CD27 and CD28, which are progressively lost throughout the differentiation process. [79] As central memory cells differentiate to effector memory and effector T cells (TEM and Teff) they decrease the capacity to produce IL-2 and increase their capacity to produce IFN-$\gamma$ and perforins/granzymes.

Recent work suggests the existence of antigen experienced memory T cells, with stem cell like features such as multipotency, asymmetric division and proliferative capacity. These cells are thought to be powerful allies to contain tumors and disease progression, and further studies showed that high extracellular potassium levels associated with the necrosis of the tumor microenvironment might be

essential for the surge/maintenance of these cells. [80]

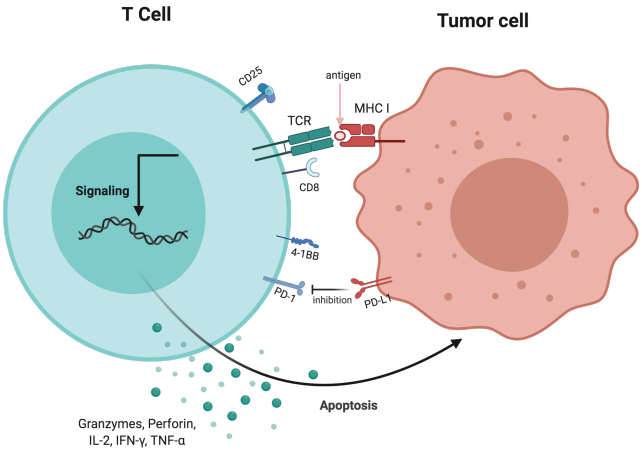



Figure 1.6: Cytotoxic T cell recognition and killing of tumor cells.

After migration, 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. [81] Its expression is so consistently associated with TCR stimulation that it has been used for the detection and isolation of antigen reactive T cells for almost 2 decades. [82, 83] The alpha chain of the IL-2 receptor, or CD25, may also be up-regulated after TCR stimulation, likely as a way for the cell to receive more IL-2 stimulation, and initiate local clonal expansion. [84]

Both TCR stimulation and T cell differentiation have been associated with the expression of inhibitory receptors such as PD-1, CTLA-4, LAG-3 and TIM-3. [85, 86] In a normal clearing operation these inhibitory receptors receive an environmental signal that tells cytotoxic cells that they destroyed their enemy and can stop the attack, thus avoiding unnecessary tissue damage. The issue starts when the immune response is not able to clear the ”troublemakers”, leading to repeated stimulation of T cells. This repeated stimulation is a characteristic of the TME and is thought to lead to T cell exhaustion, a concept used to characterize T cells with impaired proliferation, that are no longer capable of cytotoxic function (lack of IFN-$\gamma$, IL-2 and impaired granzyme/perforin production). [87, 88] Chronic infection studies have shown that CD8 T cells with multiple inhibitor receptors have not only impaired effector functions but may even start to actively produce IL-10, a powerful anti-inflammatory cytokine. [89]

## 1.3 Limitations of Immunotherapy

By now it is clear that lymphocytes are capable of both recognizing and killing tumor cells in the context of immunotherapy, ultimately decimating the tumor mass and mediating durable regressions in some patients. However, even in patients with a high mutational load, immunotherapy often does not work. Some of this lack of responses is due to a highly immunosuppressive tumor microenvironment, previously covered in Section 1.1.1.

But on top of a frankly suppressive TME, there seems to be a functional impairment of tumor specific lymphocytes, likely experiencing chronic activation and exhaustion. [88] This notion is supported by reports from *Goff and Tran et. al*, which reveal that tumor reactivity does not necessarily translate into a response, thus suggesting reactive T cells are impaired. [47, 55] On this line of thought, work by *Miller & Sen et al.* identified a subpopulation of "progenitor exhausted" T cells that retain polyfunctionality and persist long term. These progenitors are capable of responding to anti-PD-1 therapy while their progeny, the 'terminally exhausted' TIL cannot. [90] This is consistent with a report by *Philip et al.* that found that PD-1 positive cells were epigenetically reprogrammed into chronic dysfunction. [91] At an initial stage, tumor specific T cells (TST) showed signs of dysfunction, measured by the lack of IFN$-\gamma$ production and downregulation of CD62L. However that dysfunction was reversible, as the chromatin remained plastic and reprogrammable. But as the TST cells remained in the tumor, the chromatin remodeled again, as seen by the gain and loss of ATAC-seq peaks in gene loci that included *Pdcd1*, *CTLA4* and *Tcf7*, thus showing that prolonged stimulation in a suppressive environment resulted in fixed epigenetic changes, that could no longer be reversed by changing culture conditions (like *in vitro* TIL expansion) or checkpoint inhibitors. Furthermore, this work suggests that only PD-1$^{high}$ cells expressing CD38 and CD101, could be responsive to therapeutic reprogramming, and thus remain effective in leading tumor regression. [91]

Another concern with immunotherapy is that the majority of T cells present in the tumor are not the tumor reactive, but the so called 'bystanders'. Work by *Simoni et al.* shows that TIL not only recognize neoantigens, but also a wide range of epitopes unrelated to cancer. [92] These non-tumor specific T cells, are characterized by the lack of CD39 expression, in association with the lack of exhaustion markers (PD-1, TEMRA associated phenotype). It is currently unknown what role do these cells play in tumor clearance by the immune system.

#### HLA Downregulation and Loss

An increasingly asked question in immunotherapy trials is what proportion of malignant T cells are actually expressing HLA molecules. Neoplastic cells are known to downregulate or completely lose MHC molecules, particularly in the context of advanced disease. [93]

This is in part due to the effective role of T cells, as they pressure the tumor into shedding the MHC molecule to avoid being targeted and destroyed. This 'evolutionary pressure' was demonstrated in a patient with metastatic colorectal cancer, after TIL therapy. The therapy caused most lesions to shrink and the patient went into remission, but 9 months after treatment one lesion started growing again. That lesion was resected, and further analysis showed that the tumor cells had lost chromosome 6 haplotype,

which encoded the HLA-C*08:02 class I molecule. [55] This provided direct evidence of tumor immune evasion by MHC loss.

Besides HLA loss, there are reports of abnormalities in the expression of MHC I molecules in cancer cells. Dissociation of MHC class I heterodimers in acidic pH, an established hallmark of the TME. [94] In melanoma, tumor cells resistant to checkpoint inhibitors were found to be insensitive to IFN-$\gamma$, due to JAK1 or JAK2 genetic alterations, keeping MHC expression low. [95] In Merkel cell carcinoma, transcriptional suppression of HLA genes has been documented as a consequence of CD8 adoptive cellular therapy combined with checkpoint inhibitors, although it was reversible by the use of IFN-$\gamma$ or 5-azacitadine. [96] Additionally, in the context of pancreatic adenocarcinomas, there has been evidence that MHC-I molecules are degraded through lysossomes by autophagy-dependent mechanisms. [97]

If (and when) tumor cells stop expressing an HLA molecule, direct tumor targeting by the canonical $\alpha\beta$ T lymphocytes ceases. This has became a major roadblock in the battle against cancer recurrence, with no other available medical treatments. In this scenario, $\gamma\delta$ T cells, believed to thread the line between adaptive and innate immunity, might be a possible line of attack.

## 1.4 $\gamma\delta$ T cells

$\gamma\delta$ T cells are unconventional T lymphocytes, as they are not restricted by MHC antigen presentation even though they have a somatically re-arranged TCR. Their TCR is a heterodimer composed of a $\gamma$ and a $\delta$ chain. In the blood, most $\gamma\delta$ T cells are V$\gamma$9V$\delta$2 while in the tissue the majority has a V$\delta$1 chain.

$\delta$1 T cells, unlike $\delta$2s, have limited high-frequency clonotypes, suggesting clonal expansion overtime. These clonotypes are associated with an effector phenotype, proliferative capacity in response to IL-15 stimulation coupled with CX3CR1 expression and granzyme secretion, suggesting heightened capacity for tissue homing and increased cytotoxicity. This is suggestive of long-lived, highly specific and functional $\gamma\delta$ T-cell memory that permits efficient responses to recurrent stress challenges. [98]

The majority of $\gamma\delta$ T cells in the TME have been described as V$\delta$1, although their role is somewhat controversial. Gene expression signatures associated with $\gamma\delta$ T cells were found to be the best indicator for improved survival across a cohort of 18 thousand human tumors from 39 different cancer histologies. [36] Furthermore, $\gamma\delta$ T cells are capable of displaying antigen and providing co-stimulatory signals, thus driving their $\alpha\beta$ counterparts to differentiate and proliferate. [99, 100] But some authors report $\gamma\delta$ T cells as agents of tumor progression by production of IL-10 and IL-17 or the inhibition by cell-to-cell contact with their $\alpha\beta$ T cell counterparts. [101]. $\gamma\delta$ T cells isolated from colorectal carcinoma samples have an effector phenotype but reduced capacity to produce IFN-$\gamma$. [102] This is in stark contrast with the high cytotoxicity demonstrated by $\gamma\delta$ *in vitro*, a dichotomy expertly reviewed by *Zhao and colleagues*. [103]

$\gamma\delta$ T cells are thought to directly recognize molecular indicators of stress in malignant cells, with this recognition being associated to potent cytolytic activity. However, there is no clear understanding of how $\gamma\delta$ mediate this recognition. One hypothesis is that they use a wide range of NK receptors, which are briefly summarized in **Table 1.1**.

Table 1.1: NK receptors and their presence/role in $\gamma\delta$ T cells.

| Ligand | Description |
|---|---|
| DNAM-1 | Recognizes both PVR and Nectin-2 on the surface of cells. [104] It has been shown to be widely expressed on $\gamma\delta$ T cells, particularly in V$\delta$2, and is thought to play a role in their capacity to lyse tumor cells. [105, 106] |
| NKG2D | Recognizes MIC-A, MIC-B and UL-16 binding proteins, molecules that are up-regulated by stressed cells (malignant or infected). [107] It is expressed by $\gamma\delta$, and even some $\alpha\beta$, T cells. |
| NKp30 | Thought to trigger NK activation and anti-tumor cytotoxicity, the receptor recognizes B7--H6, expressed in the surface of tumor cells. [108] The induction of NKp30 in $\gamma\delta$ T cells *in vitro* is thought to increase their cytotoxic capacity. |
| NKp44 | Involved in NK anti-tumor cytotoxicity by recognition of a growth factor, PDGF-DD, over-expressed in tumor cells. [109] Its induction *in vitro* is also thought to increase $\gamma\delta$ cytotoxicity. |
| NKp46 | Thought to recognize hemagglutinins on the surface of virus infected cells, the activation of this receptor drives potent cytotoxicity in NK cells. [110] Its presence in V$\delta$1 cells is thought to mark a subset of cytotoxic intraepithelial $\gamma\delta$ cells. [111] |

Whereas NKp30 and NKp44 expression has been shown to be induced in V$\delta$1 cells, [112] through the use of IL-15 after *in vitro* expansion, Mikulak at al. was able to demonstrate that intraepithelial $\gamma\delta$ T cells, naturally possess NKp46. [111] Coupled together these findings increase the likelihood that NK receptors are at least partially responsible for the powerful anti-tumor cytotoxicity V$\delta$1s are capable of.

Ultimately there is not much known about $\gamma\delta$ T cells in the context of the human tumor microenvironment, a lack of knowledge this dissertation aims to partially address by analyzing phenotypic surface markers of $\gamma\delta$ in TIL.

## 1.5 Neoantigen Reactivity: The Methodology Behind

The overall process of identifying conventional antigen reactivity is based on identifying nonsynonymous mutations that result in the formation of novel epitopes. This process is highly dependent on genome sequencing technologies, and subsequent alignment algorithms, in order to reliably determine somatic mutations. The resulting set of candidate neoantigens is screened by co-culture of T cells with HLA-matched cells (either tumor or myeloid derived), in order to determine if lymphocytes are capable of recognizing the epitope.

This reactivity screening was typically done with individual, long synthetic peptides pulsed in HLA-matched, preferentially autologous, cells. However, tumors often have hundreds to thousands of mutations, making this process laborious, expensive and overall ineffective. Fortunately, several technological improvements, introduced and discussed in this section, have made this process more efficient and cost-effective.

### 1.5.1 Identifying Cancer Mutations

The first step in finding and characterizing neoantigen reactivity is to uncover non-synonymous mutations through tumor sequencing, usually by whole genome sequencing (WGS). This step can be challenging due to the inherent genomic heterogeneity of the tumor cells within the cancerous mass. In addition, the biopsied or resected tumor often comes with adjacent healthy tissue, further diluting less frequent tumor mutations. Hence, it is fundamental to ensure that there is a high number of unique reads for each region of a sequence, a concept known as deep sequencing.

Several reports suggest that whole exome sequencing, WES, a technique that assesses mutations in expressed genes is adequate to uncover most neoantigens. In fact, the coverage used in standard exome sequencing has been shown to effectively detect the majority of mutations that give rise to neoantigens, as most are thought to originate within exonic sequences. [113, 114]

Nonetheless, newer reports have questioned whether this is accurate. Work by *Laumont and Vincent et al.* described 36 antigens derived from noncoding regions, mutations that would have been entirely missed by standard WES. [115] This is in agreement with a newer notion, one that sees cancer cells as a consequence of severely altered transcription, likely causing a large number of epitopes not ”seen” by genomic sequencing, a notion expertly reviewed by *Zhou et al.* [116]

After WGS or WES, several possible neoantigen candidates may be ranked by overall expression (through RNA-seq) and by predicting peptide MHC binding affinity. This is typically done to reduce the amount of candidates tested, as a substantial amount of cancer mutations are individual [117] and the odds of a mutation generating a potent epitope is estimated to be less than 1/300 per haplotype. [61, 118] As the TIL screened for reactivity in this dissertation arose from uveal melanoma, the amount of expected mutations is quite low. Thus, measures to examine gene expression were not employed during this work.

#### From cancer mutations to epitope prediction

For epitopes to be formed, the mutated protein first needs to be processed and presented by the MHC molecules. In fact, the life of an antigen often starts with translation. Many proteins are imperfectly made, compromising their biological function and thus leading to their degradation into 8 to 16 aminoacids (a.a) by the proteosome. After this 'priming', the small peptides are pumped, via TAP1/TAP2 into the endoplasmic reticulum (ER), to bind to a MHC class I molecule. Incidentally, proteins with low levels of expression are unlikely to survive the proteases activity and may ultimately not be recognizable by the immune system.

An improving understanding of these mechanisms allowed for the construction of algorithms that can predict epitope formation to a given HLA allele. [119] Furthermore, machine learning promises to revolutionize the field, as the build-up of large databases of immunogenic neoantigens permits the continued improvement of the prediction models. However, the accuracy of these methods is currently up for debate. [58] As such, and since there was a relatively low amount of candidate neoantigens to screen, no peptide MHC binding algorithms were used.

### 1.5.2 Neoantigen Screening

With the candidate antigens established it is now possible to screen for T cell reactivity, with several, not mutually exclusive, experimental approaches. This "screening" of reactivity was typically done with individual, long synthetic peptides pulsed in HLA-matched, preferentially autologous, cells. However, tumors have hundreds to thousands of mutations, making this process laborious, expensive, costly in patient derived material and overall ineffective (very few antigens found per hundreds of mutations screened).

#### Tandem minigenes

An alternative to the use of peptides is the use of tandem minigenes (TMG), first described in *Tran et. al*, a construct that fuses together several minigenes capturing each of the putative neoantigens, as schematized in **Figure 1.7**. [54]

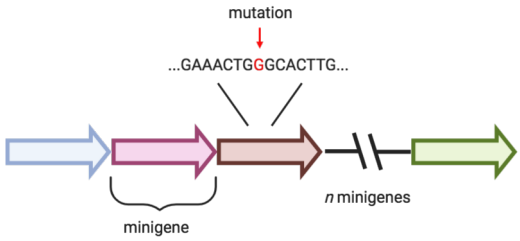



Figure 1.7: Schematics of a Tandem Minigene. Each colored arrow represents an individual minigene, composed of a mutation flanked by 12 a.a on each side. Adapted from [69].

A minigene, in the case of a single point mutation, is a mutated amino acid flanked by 12 amino acids of the wild-type protein. After a single open reading frame, 12 to 24 minigenes are then fused

together, resulting in a TMG. This construct is then transcribed as RNA and transfected into presenting cells, typically *in vitro*-derived immature dendritic cells, where neoantigen recognition by T cells can be accessed. Unlike the use of short peptides, the use of TMG is better resemble the natural antigen processing and presentation of neoepitopes on HLA molecules. Nevertheless, the chimeric proteins that result from the fused minigenes could create 3D structures that severely alter the processing by the proteasome. This would generate novel neoantigens that do not resemble the ones in the malignant cells. This drawback, although worth considering, does not seem to be significantly impeditive, as multiple screenings using TMGs led to the identification of cancer neoepitopes.

With this methodology, after T cells are found to be reactive to a particular TMG, this TMG is then deconstructed and all mutations are tested individually, either with the use of long peptides or the corresponding minigene RNA.

#### Selecting antigen reactive CD8 T cells

In Section 1.2.1 we explored how CD8 T cells exposed to antigen express various surface markers.

4-1BB (also known as CD137) is a transient marker expressed predominantly on $CD8^+$ T cells and the most commonly used in neoantigen reactive T cell screenings. [82] Expression of PD-1 on $CD8^+$ TIL is also a hallmark of tumor-reactive lymphocytes in melanoma. [120] This however, is only valid in uncultured T cells, as cells that undergo *in vitro* expansion frequently upregulate their exhaustion markers. Consequently, 4-1BB expression remains the gold standard to identify tumor-reactive cells in co-culture assays, as T cells typically undergo some *in vitro* growth or expansion before being used for neoantigen screening. [121]

As single cell techniques evolved, several other markers have emerged as important for the function and sub-categorization of CD8 T cells in the TME. Tumor specific $CD8^+$ TIL are also enriched in $CD103^+CD39^+$. These cells have a tissue resident memory phenotype ($CD69^+CD103^+$), express high levels of both PD-1 and have the capacity to kill autologous tumor cells (measured by 4-1BB up-regulation and IFN-$\gamma$ secretion), making them interesting phenotypic markers to examine tumor reactivity. [122]

## 1.6 Research Aims

TIL therapy has been shown to be an effective form of therapy for patients with melanoma but leaves much to be desired for other solid cancer histologies.

Patients who respond to TIL therapy, as well as other forms of immunotherapy, seem to have tumor reactive T cells. This tumor reactivity is thought to be driven by the presentation of neoantigens, small peptides that are the result of non-synonymous mutations, by the MHC molecules of cancer cells. As such, it is key to understand more about neoantigens, from how they are processed and presented to their expression level in tumor cells and how likely they are to be shared between patients. This is particularly important as it would likely allow for the isolation of tumor reactive TCRs to be isolated and used as *off-the-shelf* personalized cancer therapy.

However, not all patients with tumor reactive T cells respond to therapy, likely due to the downregulation or loss of the HLA alleles in cancer cells, a known and established mechanism of cancer evasion.

To learn more about TIL efficacy and how T cells drive tumor response, two patients with tumor reactive T cells were studied to find the neoantigen(s) driving tumor reactivity. If achieved, these neoantigen reactive T cells will be isolated and characterized, and their respective TCR sequenced.

Furthermore, several cancer histologies will be studied to evaluate and characterize their immune populations, with special attention to an understudied subpopulation of immune cells in the TME, the $\gamma\delta$ T cells. This work aims to highlight their frequency, and most commonly expressed markers, drawing a striking comparison from what is known in the blood and healthy tissue.

# Chapter 2

# Materials & Methods

## 2.1 Sample Processing

### 2.1.1 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). After initial TIL growth (between 2-4 weeks), select cultures to be grown in GMP conditions were rapidly expanded (a process known as REP) in gas-permeable G-Rex100 flasks using irradiated PBMC (40 Gy) 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). 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). All cells were cultured at 37°C in 5% $CO_2$.

### 2.1.2 Tumor Digestion

Tumor Digestion was executed as described in the Tumor Dissociation Kit from Miltenyi (product #130-095-929). Briefly: ≈ 1-2 mm fragments were put into a gentleMACS C Tube containing an enzyme mix (200 $\mu$l Enzyme H + 100 $\mu$l Enzyme R + 25 $\mu$l Enzyme A in 4.7 ml of RPMI). The tube was inserted into the gentleMACS$^{TM}$ Dissociator, which kept the sample in mild rotation at 37°C for 2 hours. Afterwards, the resulting suspension is passed through a 70 $\mu$m strainer (SmartStrainer, Miltenyi) to remove larger, undigested pieces and extracellular matrix remains. After washing the strainer with medium, the liquid is spun down (400g for 5 minutes). Cells are 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 as described in Section 2.1.4.

### 2.1.3 PBMC Processing

#### Monocyte isolation

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. Briefly, cells were resuspended in buffer and stained with anti-CD14 Antibody (40 $\mu$l for every $10^7$ cells) plus 10 $\mu$l of FcR Blocking (Miltenyi). 10 $\mu$l of Biotin-Antibody Cocktail was added and incubated for 5 minutes in the refrigerator (2-8 °C). After adding 20 $\mu$l of Anti-Biotin MicroBeads and 30 $\mu$l of buffer, cells were refrigerated for 10 min. Magnetic cell separation was done in LS columns using a MidiMACS$^{TM}$ magnet. Cells were used fresh or quickly frozen.

#### $\gamma\delta$ T cell isolation

T cells grown in CM with IL-2 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). Briefly, cells were resuspended in buffer and stained with either anti-$\gamma\delta$ or -$\alpha\beta$ TCR Biotin tagged antibody (20 $\mu$l for every $10^7$ cells) for 10 minutes, in the refrigerator (2-8 °C). After, 10$\mu$l of Anti-Biotin-Antibody (tagged with MicroBeads) was added and incubated for 5 minutes in the refrigerator (2-8 °C). After adding 50 $\mu$l of buffer, magnetic cell separation was done in LS columns using a MidiMACS$^{TM}$ magnet. Afterwards cells were either stained for flow acquisition or grown using cytokine cocktails.

#### DC generation

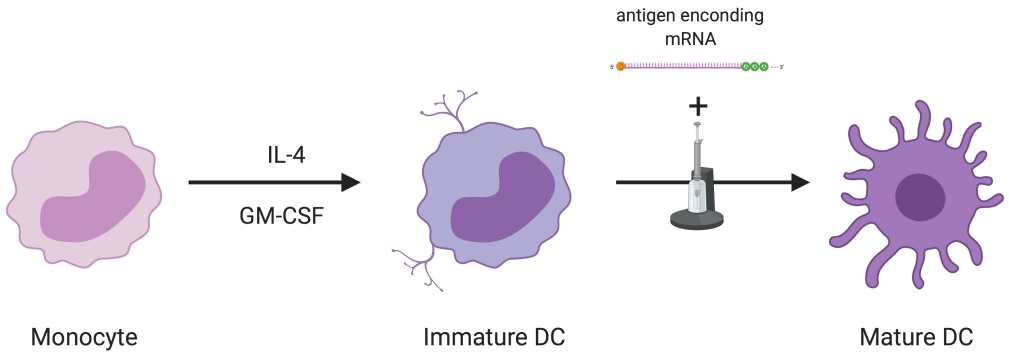



Figure 2.1: **Schematic of the Dendritic Cell Maturation Process.**

Cells, resulting from either apheresis or PBMC isolation after a blood draw, 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 flask washed with RPMI. 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, generating immature DCs. Fresh or freeze/thawed DCs were used in experiments. To complete the maturation process, cells were electroporated using the Neon™ Transfection System. Briefly, cells were were counted ($10^5$ cells per well), washed with PBS and re-suspended with Neon Buffer at the desired concentration ($10^5$cells per 10 $\mu$l). 10 $\mu$l of cells were mixed with 2 $\mu$l of mRNA and the resulting mix was pulsed twice at 1050 mV for 20 ms and platted in a 96 well plate (Thermo Fisher) in CM.

### 2.1.4 Cell Culture

All primary cells, samples and HEK293gp were kept in adherent plates or flasks at 37°C with 5% $CO_2$, when in culture, unless stated otherwise. Trypsin (ThermoFisher) was used when adherent cells reached confluency. IL-4 (400 IU/mL), IL-15 (125 IU/mL), IFN-$\gamma$ (1400 IU/mL) and IL-21 (25 IU/mL), all from Peprotech, were occasionally used to supplement T-cell cultures.

Generally, cells were frozen using CoolCell (Corning) in freezing medium (90% Human Serum + 10% DMSO) at -80°C for 24h and kept in liquid nitrogen at -180°C afterwards. To thaw cells, refrigerated media with 10% Recombinant DNASE I (Merck) was used. After spinning, the cells were platted at the desired confluency with the appropriate media for each cell type. Cells were counted

All co-cultures were set up in 96 well plates with $10^5$ adherent cells (HEK cells, autologous DCs, tumor digest) plus $10^5$ T cells (TIL) in complete media without cytokines. Subsequent analysis was done 16-18h after co-culture set up.

## 2.2 Whole Genome Sequencing

The samples were processed following the standard whole genome sequencing pipeline at the UPMC Genome Center. Genomic DNA was isolated from tumor tissue and PBMCs on the automated Chemagic 360 (Perkin Elmer) instrument according to the manufacturer's instructions. Extracted DNA was quantified using Qubitn™ dsDNA BR Assay KIt (ThermoFisher Scientific). DNA libraries were prepared using the KAPA Hyper Plus Kit (KAPA Biosystems). 500 ng of genomic DNA was processed through fragmentation, enzymatic end-repair and A-tailing, ligation, followed by quality check using Fragment Analyzer (AATI). Libraries with an average size of 450 bp (range: 300-600 bp) were quantified by qPCR on the LightCycler 480 (Roche) using the KAPA qPCR quantification kit (KAPA Biosystems). Libraries were normalized and pooled as per manufacturer protocol (Illumina). Sequencing was performed using NovaSeq 6000 platform (Illumina) with 151 paired-end reads to an average target depth of 70x coverage.

#### Mutation Calling

Samples were mapped with Edico Dragen (v2.1). Somatic variants were called by Mutect2 (GATK 4.0.5) at tumor-normal according to best practices. Variants were annotated with Funcotator (GATK 4.0.5) and a certain range of aa and nt sequence were retrieved around the detected variants using a UPMC Genome

Center internal script. All computation was carried out in a linux based AWS ec2 instance on DNAnexus platform.

## 2.3 Tandem minigene construction and synthesis

The TMGs encoding DNA sequences were synthesized and cloned into eGFP tagged pcDNA3.1+ using EcoRI and XhoI restriction site, by Dr. Yadav and Dr. Bhaskarla. Each TMG was constructed by putting either 20 or 10 minigenes together. A Minigene (DNA sequence) encodes the 25 amino acid long peptide with the mutation. For point mutations the mutant amino acid is flanked by 12 wild amino acids on both sides. For frameshift mutations all the altered aminoacids are included until a STOP codon.

#### mRNA synthesis

500-1000 ng of linearized DNA (TMG constructs) was used during *in vitro* transcription reaction using the Message Machine T7 Ultra kit (Life Technologies) as instructed by the manufacturer protocol. After reaction completion, RNA was precipitated using $LiCl_2$ and resuspended at 2-3 $\mu$g/l concentration in DNAse and RNAse free water.

## 2.4 Cell Imaging

Both DCs and HEK293gp cells were imaged using a Zeiss Axiovert200 microscope. Acquisition of GFP expression was done with 20 s of exposure, while images acquired in Bright-field mode have 30-55 ms of exposure. FIJI was used to process the images as needed. [123]

## 2.5 Neoantigen Recognition Screening

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

## 2.6 ELISA

ELISA plates were prepared by coating 96 well plates (NUNC Band Products) with 100 $\mu$l/well primary IFN-y antibody from Endogen (clone 2G1) and left to incubate overnight at 4$^\circ$C. After removing all liquid, 300 $\mu$l/well of blocking solution (5% FBS in PBS) were added, followed by an hour of incubation. Afterwards the plates were washed (3 times per plate) with wash buffer (). Standards of 1000, 500, 250,

125, 62.5, 31.25, 15.63, 0 pg/$\mu$l were prepared from an IFN-$\gamma$ standard (Invitrogen). Standards and samples are added to the plate and left to incubate for an hour and a half. After washing, 50 $\mu$l/well of 1X Biotenylated antibody (Endogen, clone B133.5) solution were added to the pre-coated plate, followed by an 1 hour incubation. Next, the plate was washed. 100 $\mu$l/well of Streptadivin-HRP (Invitrogen) solution was added followed by an incubation of 30 min. The plate was re-washed and 100 $\mu$l/well of TMB solution (Dako Product) was added. After a brief incubation (10 min), 100 $\mu$l/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. All incubations were done at room temperature, except when stated otherwise.

## 2.7 Flow Cytometry

### 2.7.1 Antibodies

The following titrated anti-human antibodies were used for cell surface staining:

Table 2.1: Antibody list

| Marker | Clone | Fluorochrome | Brand |
|---|---|---|---|
| $\alpha\beta$ TCR | REA318 | VioBlue | Miltenyi |
| $\gamma\delta$ TCR | B1, 11F2 | APC, APC | Biolegend, Miltenyi |
| V$\delta$1 | REA173 | PE | Miltenyi |
| V$\delta$2 | B6 | APC-Fire50 | Biolegend |
| 4-1BB | 4B4-1 | APC | BD |
| CD3 | SK7, UCHT1 | APC-Cy7, PerCP-Cy5.5 | BD, BD |
| CD4 | SK3, OKT4 | PE, AF488 | BD, BD |
| CD8 | SK1, RPA-T8 | PE-Cy7, BV480 | BD, BD |
| CD25 | 2A3 | BV711 | BD |
| CD27 | M-T271 | BV711 | BD |
| CD45RO | UCHL1 | BV650 | BD |
| CD56 | 5.1H11, HCD56 | BV785, BV510 | BD, BD |
| CD62L | DREG-56 | BV750 | BD |
| CD69 | FN50 | BV421 | Biolegend |
| LAG-3 | 11C3C65 | BV421 | Biolegend |
| NKG2D | NCAM16.2 | BV510 | BD |
| OX40 | ACT35 | FITC | BD |
| PD-1 | EH12.1 | BV605 | BD |

Propidium iodide, PI (Thermofisher), or Zombie NIR (Biolegend) were used to mark dead cells.

### 2.7.2 Staining & 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™ flow cytometer or Cytek™ 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 200k to 2M events were collected. Acquired flow data was analyzed using FlowJo 10.6.1 (BD).

## 2.8 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.

## 2.9 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 (A and B) had metastatic Uveal Melanoma, and underwent surgical resection of liver metastasis. Four Patients (19-201, 19-202, 19-203 and 19-104) had Metastatic Renal Clear Cell (RCC) carcinomas and underwent either partial or complete kidney resections. Six patients (19-102 to 19-107) 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 (15-008, 16-019 and 14-011, respectively).

# Chapter 3

# Results & Discussion

This project had two distinct objectives. The first was to find and characterize tumor reactivity by identifying the specific antigen(s) that T cells recognize in the tumors of two patients with metastatic uveal melanoma. If successful, it intended to isolate and clone the reactive TCR(s), for additional testing and potential clinical use. The second was to grow and characterize TIL from various malignancies including GI malignancies (colon and appendiceal cancer), PMP and RCC, with a spotlight on $\gamma\delta$ as an 'underappreciated' immune population in the TME.

## 3.1 Finding Tumor Reactivity in TIL

This section is aimed at demonstrating that different TIL products obtained and expanded from a liver metastasis are capable of recognizing the autologous tumor, and thus ideal to identify neoantigen reactive T cells. It will also lay the necessary groundwork for the completion of the neoantigen screening. This includes the identification of nonsynonymous mutations from the resected metastasis from both patients and the test of expression of the mRNA encoding all tandem minigenes.

### 3.1.1 TIL Production

TIL from patient A was obtained from a resected liver metastasis, as mentioned in Section 2.1.1. After initial outgrowth, several fragments were tested for autologous tumor recognition by 4-1BB up-regulation. The cells that up-regulated 4-1BB were sorted and rapidly expanded, originating three different TIL products thought to be highly reactive to the autologous tumor.

To confirm tumor reactivity after expansion, the three TIL products were co-cultured with tumor digest (TD) and autologous monocytes (as a control). The experiment shows clear and specific tumor reactivity, as shown by the up-regulation of 4-1BB in **Figure 3.1**. TIL culture B was chosen to be tested for neoantigen reactivity, as it had more 4-1BB up-regulation.

TIL from patient B was obtained from a resected liver metastasis. After initial outgrowth, several fragments were tested for autologous tumor recognition (data not shown) and the reactive TIL were pooled together and rapidly expanded, under GMP protocol, described in Section 2.1.1.

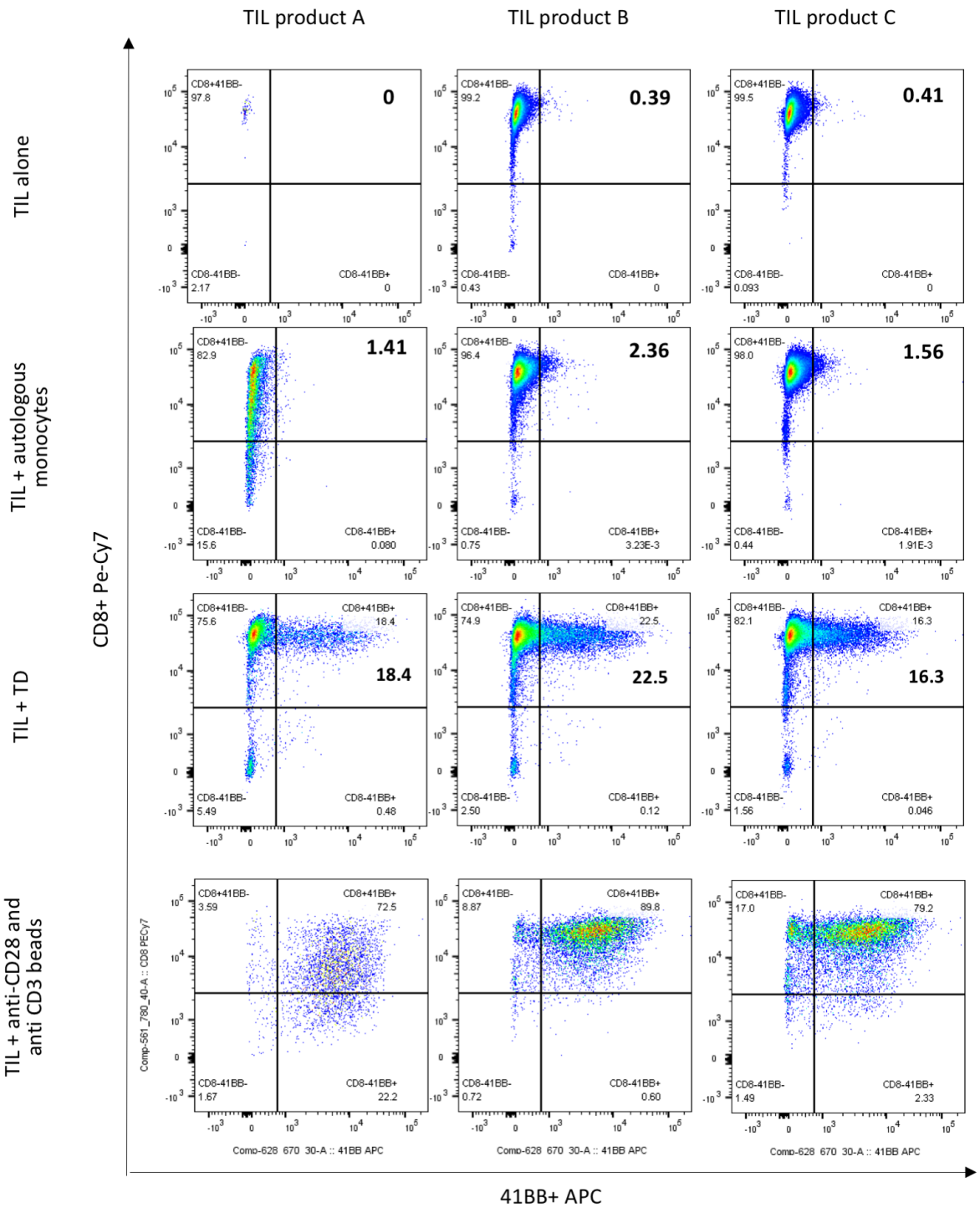



Figure 3.1: Up-regulation of 4-1BB after TIL co-culture against cells from the autologous tumor digest (TD).

### 3.1.2 Mutation Calling

Next-generation sequencing and TIL generation were derived from the same metastatic nodule, in both cases. WGS of the resected metastasis from patient A resulted in 232 nonsynonymous mutations, whose mutational type diversity is represented in **Figure 3.2.A**. This number of mutations is considered abnormal for an uveal melanoma patient, as the tumors reported in the Uveal Melanoma TIL trial had between 20-81 nonsynonymous mutations. [53] This particular patient's tumor also had a high percentage of frameshifts. Patient's B WGS resulted in 87 nonsynonymous mutations, represented in **Figure 3.2.B**.

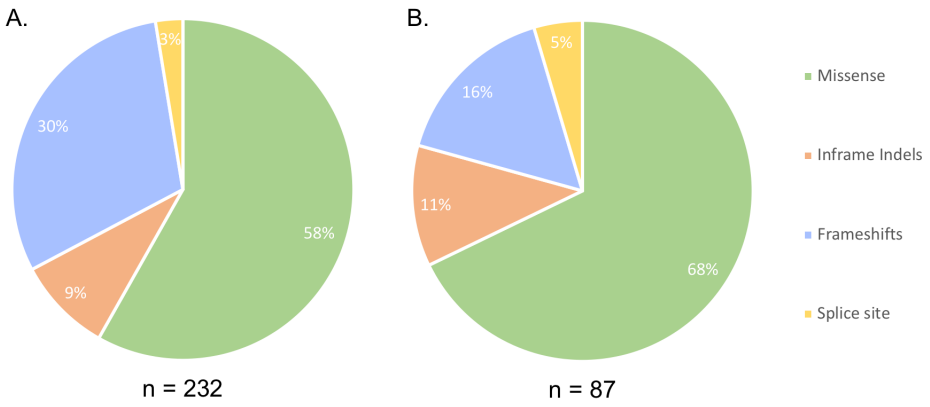



Figure 3.2: Type distribution of all nonsynonymous mutations for patient A and B identified by WGS. Both tumor samples are from a liver metastasis and autologous PBMCs were used for a normal genome control.

Both patients lacked mutations in either NRAS or BRAF. While these two genes are commonly mutated in cutaneous melanoma, mutations are practically nonexistent in uveal melanoma (UM), highlighting the different oncogenic pathways associated with both diseases. [124]

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. [125] GNAQ mutations also occur almost exclusively in codon 209, [63], making this point mutation an interesting target for an *off-the-shelf* TCR based adoptive cell therapy in uveal melanoma.

Also 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. [126] In addition, mutations occurring in this gene have been associated with a better prognosis. Indeed, mutations at codon 625, which encodes splicing factor 3B subunit 1, are associated with a good prognosis in low-grade UM. [127]

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.

[128] The high frequency of this mutation in patients with UM also makes it a highly desirable target for induced TCR based ACT in uveal melanoma.

### 3.1.3 Testing the Tandem Minigenes

Each individual mutation was screened in a high-throughput manner by the design of tandem minigenes, as detailed in Section 1.5.2.

To efficiently determine the neoantigens recognized by the reactive TIL, the mRNA containing the minigenes has to be fully translated into peptides that would then be processed and presented on a MHC molecule by a professional antigen presenting cell. As such, the tandem minigene mRNA has to successfully permeate inside the cell and be completely translated into peptides that will be cleaved and ultimately presented in an HLA molecule.

While the cleaving of the novel peptides is not an experimental parameter that can be controlled, the mRNA translation can be confirmed by adding a GFP protein to the end of the TMG construct and checking for GFP expression after electroporation. HEK cells were initially used as surrogates, since monocytes and/or monocyte-derived DCs are patient specific and have limited availability.

The result of the test, seen in **Figures 3.3** and 3.4, shows clear but variable expression of GFP per tested TMG. To ensure that the variation was not caused by the electroporation, some TMGs were tested twice (data not shown). All TMGs were deemed of acceptable quality, thus proceeding to the next step of the neoantigen screening process.

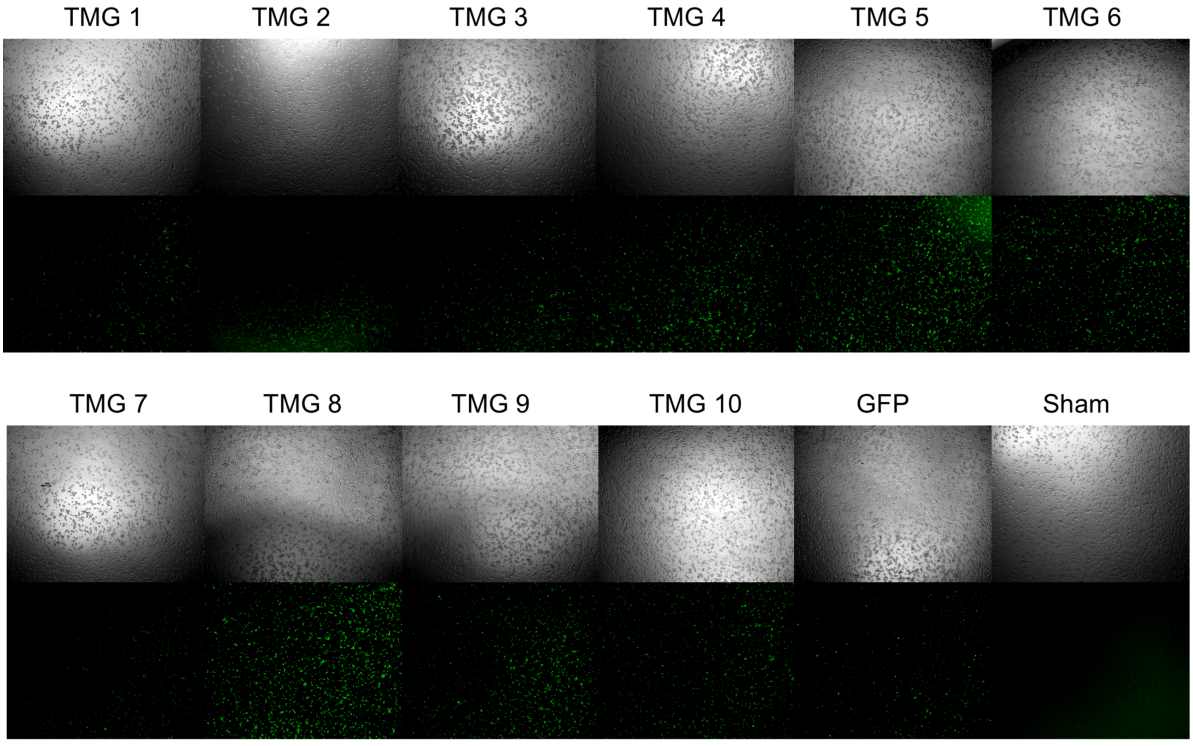



Figure 3.3: Expression of each individual TMG from patient B in HEK cells, 4 hours after electroporation.

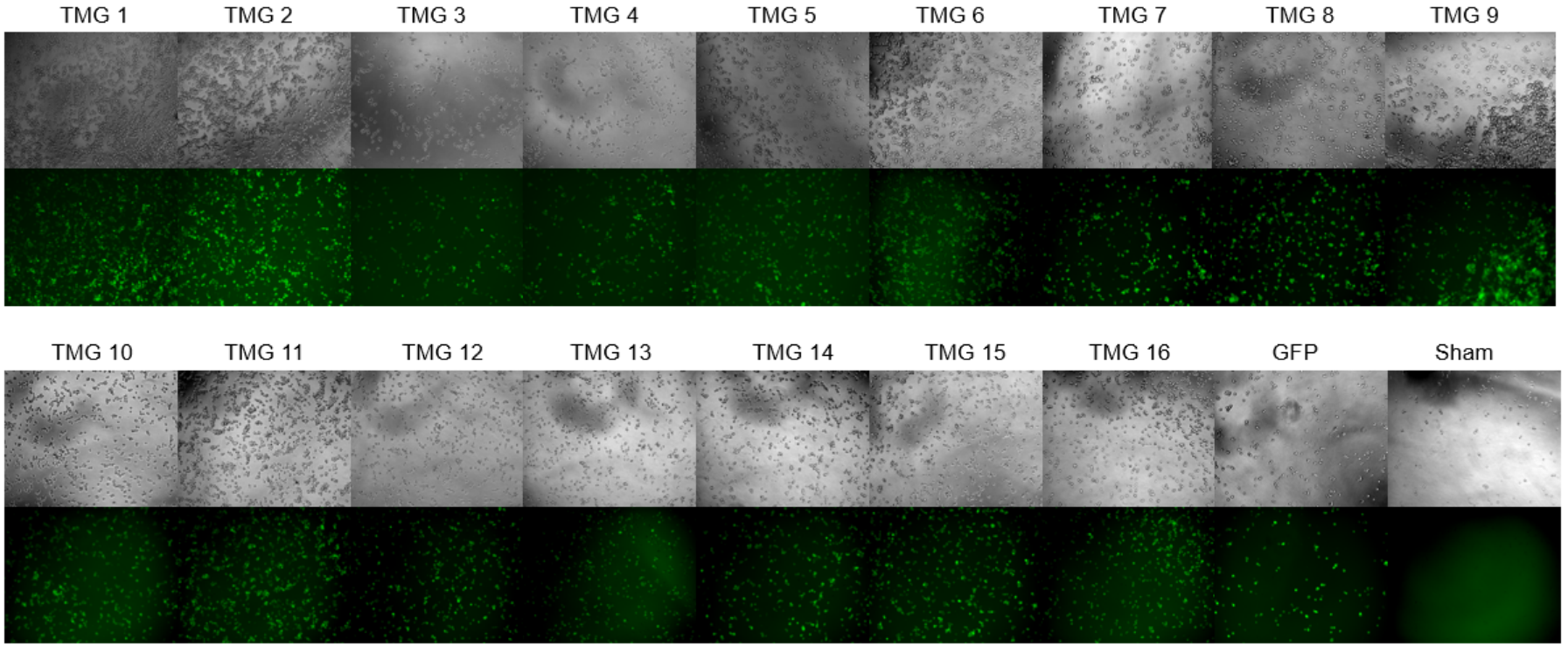



Figure 3.4: Expression of each individual TMG from patient A in HEK cells, 4 hours after electroporation.

## 3.2 TMG Screening

After confirming that the TMGs are fully translated, the screening process for mutation specific T cell reactivity begins. Autologous monocytes were differentiated into immature DCs and electroporated with the corresponding TMGs, in a 96 well plate, as described in Section 2.1.3. After confirming GFP expression for all electroporated DCs (data not shown) a co-culture with T cells was set-up and left overnight, as detailed in Section 2.5.

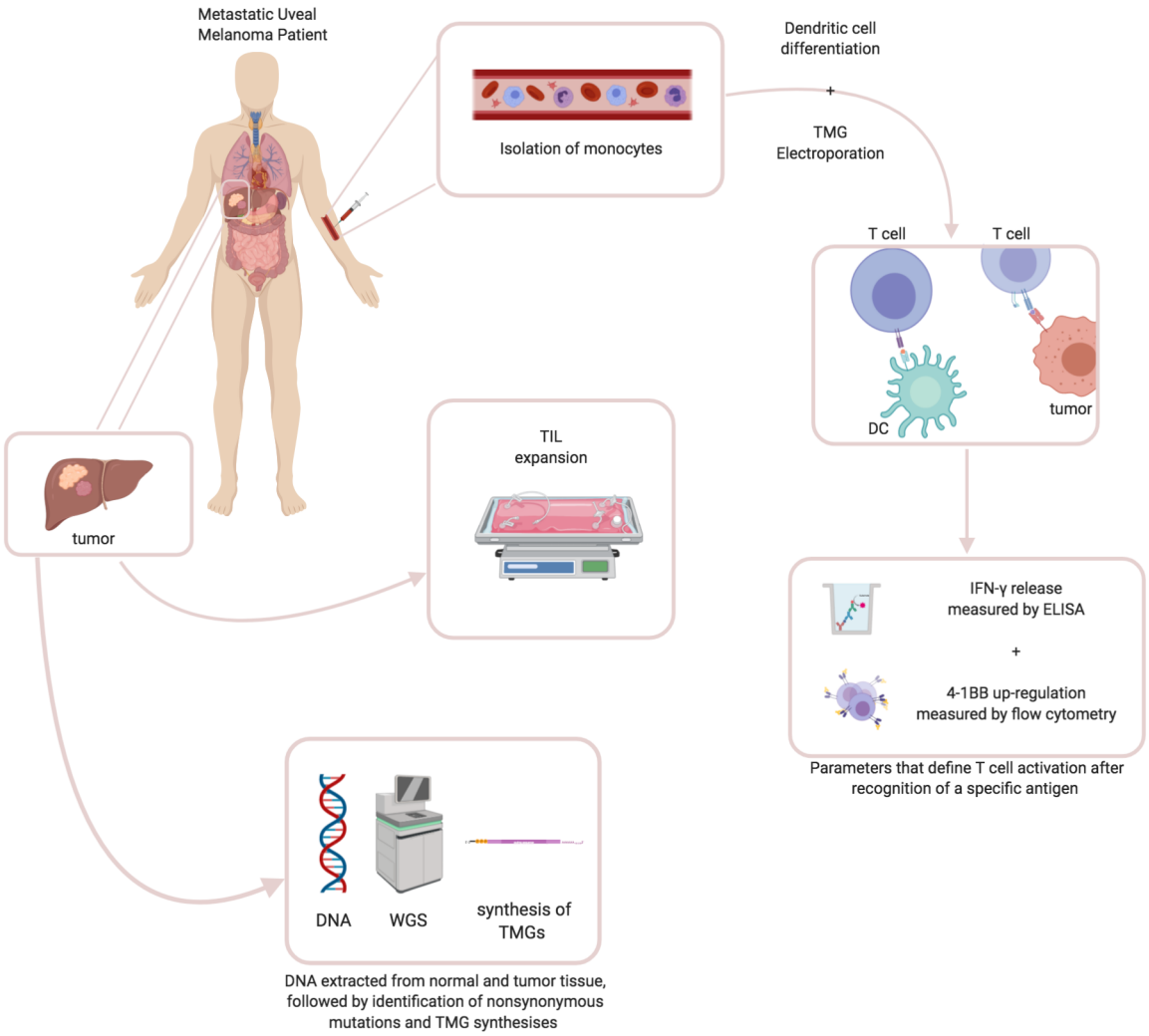



Figure 3.5: Workflow to identify TMG reactivity.

With the mRNA inside the cells, the ribosomal machinery starts translating it into proteins. These aberrant proteins 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. [129]

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-$\gamma$ release, a process described in Section 1.2.1. Thus, 4-1BB levels were measured for both patients through flow cytometry, as seen in **Figure 3.6**. IFN-$\gamma$ ELISAs were also run on the co-culture supernatants for both patients, represented on the bottom of **Figure 3.6**.

The IFN-$\gamma$ results appear consistent with the flow data, with a moderate IFN-$\gamma$ release associated with a slight up-regulation of 4-1BB for the co-culture with tumor digest. Likewise both parameters (4-1BB and IFN-$\gamma$) increase when the TIL was stimulated with anti-CD3 anti-CD8 coupled beads. The increased IFN-$\gamma$ associated to TMG6, in **Figure 3.6.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-$\gamma$ production and 4-1BB up-regulation.

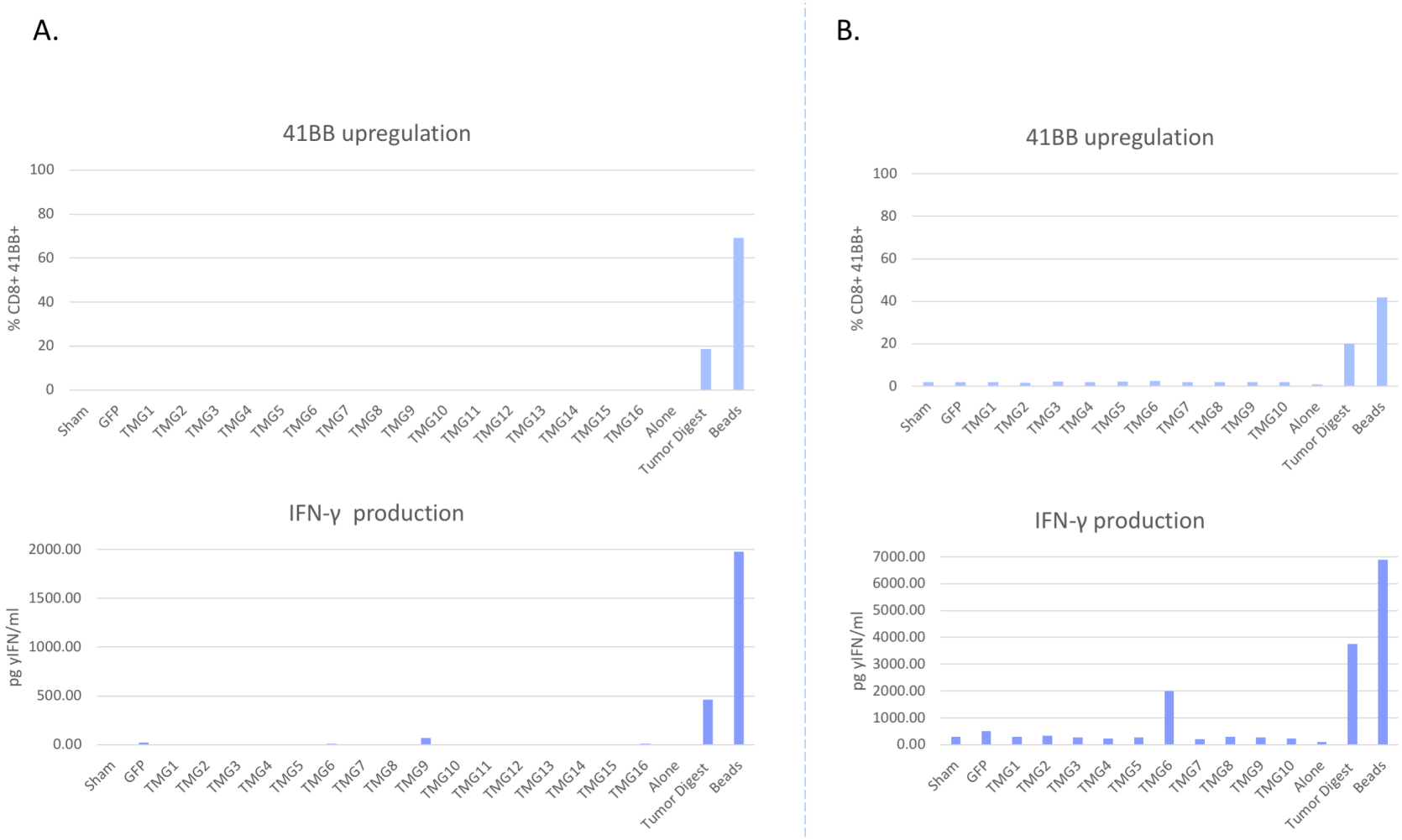



Figure 3.6: 4-1BB and IFN-$\gamma$ screening for the TMG screening assay, with A and B corresponding to patient A and B, respectively.

**Error 404: Antigen not found**

The lack of TIL reactivity was somewhat unexpected, due to high percentage of tumor reactivity. 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, and the bioinformatic analysis has been validated internally. It also seems unlikely that the mutation would be dimly expressed, as both TIL specimens are reproducibly reactive to the respective single cell tumor digests.

Perhaps 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. This process has been described as an important source of neoantigens by *Khales et al*. [130] Work by *Smart et al.* experimentally validates this concept by showcasing that this type of neoepitope can be presented by MHC molecules and elicit T cell responses. [131]

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. [126] 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 few other experiments could be planned to overcome the lack of neoantigen reactivity in the future. 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.

A more ambitious and experimental project would be to 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 providing key insights into the state and functionality of neoantigen reactive T cells.

With the assembled TCRs one could reverse engineer potential epitopes and test them, either with the use of mRNA as shown in this work or long peptides in autologous DCs. This concept has partially been demonstrated by *Scheper et al*., as they isolated TCRs from 4 patients and demonstrated tumor digest reactivity from 2 of those patients, without defining the epitope driving reactivity. [132] In parallel, work by *Dash et al*. has demonstrated that it is possible to reverse engineer the epitope to which the TCR responds to, by having the paired TCR sequencing. [133]

Consequently, this approach is likely to be technologically possible in the next few years. If successful it would permit unbiased identification of neoantigen reactivity while simultaneously allowing for TCR isolation.

## 3.3 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 CM culture with 3000 IU/mL of IL-2. 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. The other one underwent a nephrectomy of the diseased tissue, securing central and peripheral tumor 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.

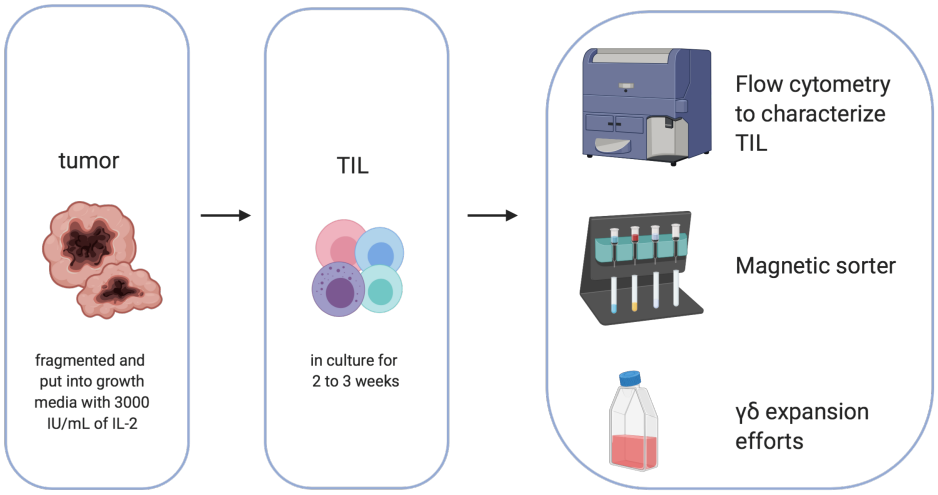



Figure 3.7: Workflow of TIL samples.

After 2 to 3 weeks of lymphocyte growth in mid-dose IL-2 (3000 IU/mL), 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).

A representative initial gating strategy is shown in **Figure 3.8**. CD56 versus CD3 expression is used to distinguish T lymphocytes (all $CD3^+$) from NK cells ($C3^-CD56^+$). $CD3^+$ cells are then separated based on expression of $\alpha\beta$ or $\gamma\delta$ TCR. $\alpha\beta$ cells are distinguished based on $CD4^+$ and $CD8^+$ expression, while $\gamma\delta$ lymphocytes are segregated based on the antibodies attaching to the delta chain of the TCR (either $\delta 1$ or $\delta 2$).

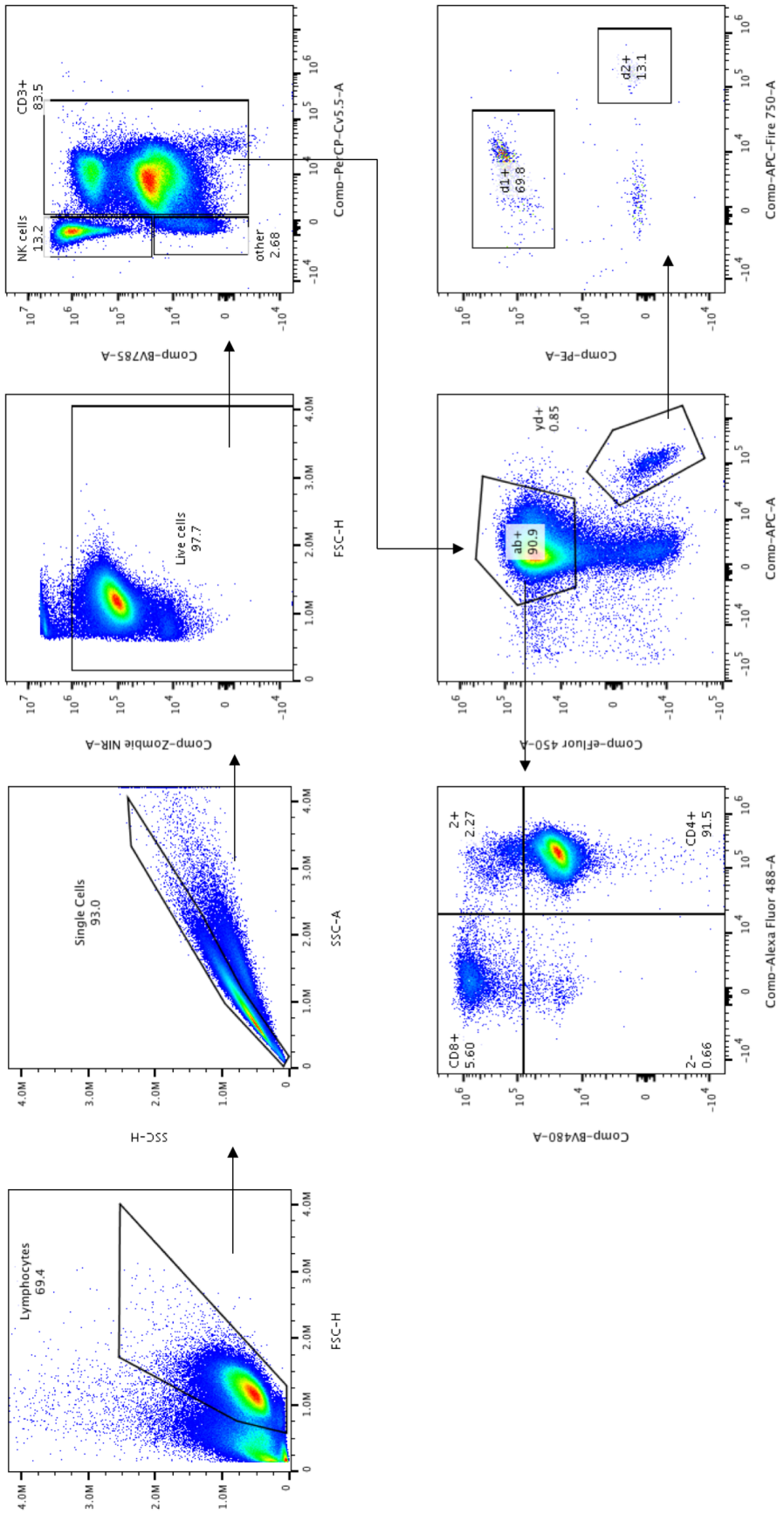



Figure 3.8: Initial gating strategy to differentiate subtypes of lymphocytes present in TIL.

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 tumors, particularly when in comparison with the non malignant adjacent regions, suggesting these cells do not home efficiently to malignant tissues. [134, 135]

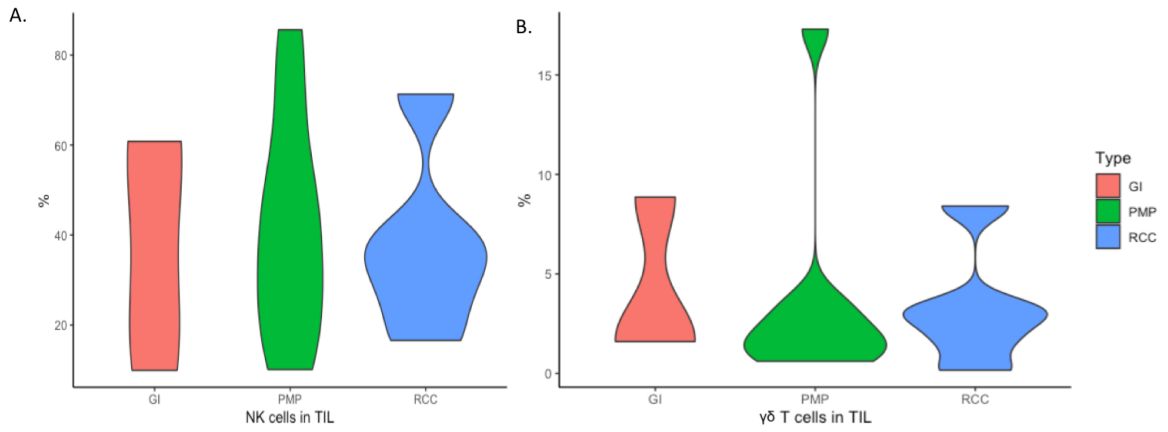



Figure 3.9: Violin Plot of: A. the percentage of NK cells; B. the percentage of $\gamma\delta$ in TIL across different cancer histologies after 2-3 weeks culture in IL-2, Section 2.1.1

Unexpectedly, there was a high number of NK cell outgrow in this patient cohort, **Figure 3.9.A**. When contextualizing the TIL populations according to cancer histology it seems that the PMP TIL cohort has 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 completely novel.

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. [134] 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 3.10**. It is important to note however that only three samples were used in making this observation, thus requiring more samples to draw definitive conclusions.

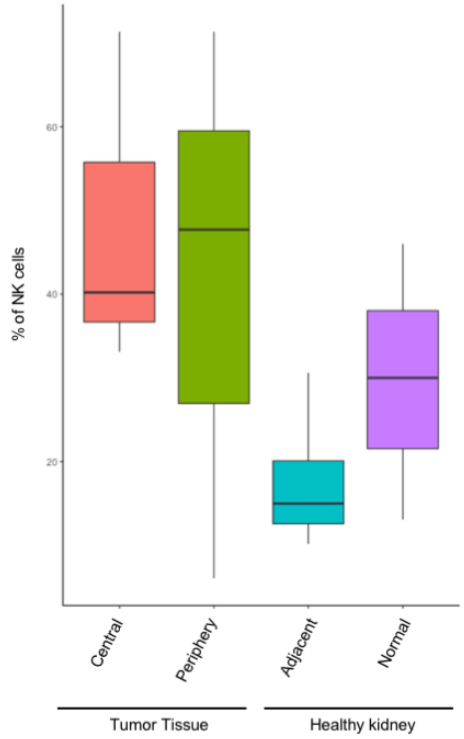



Figure 3.10: Box Plot of the percentage of NK cells in TIL cultures grown from different kidney regions.

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. [136] 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.

### 3.3.1 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 3.9.B**.

The distribution of V$\delta$ families in $\gamma\delta$ T cells is represented in **Figure 3.11A**. The vast majority of $\gamma\delta$ T cells in the characterized TIL are V$\delta$1, with an average V$\delta$1 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. [137] The median in our cohort is 83.6% compared with the reported $\approx$ 55% (n=44) by *Wu et al.*, suggesting an enrichment in V$\gamma$1 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.

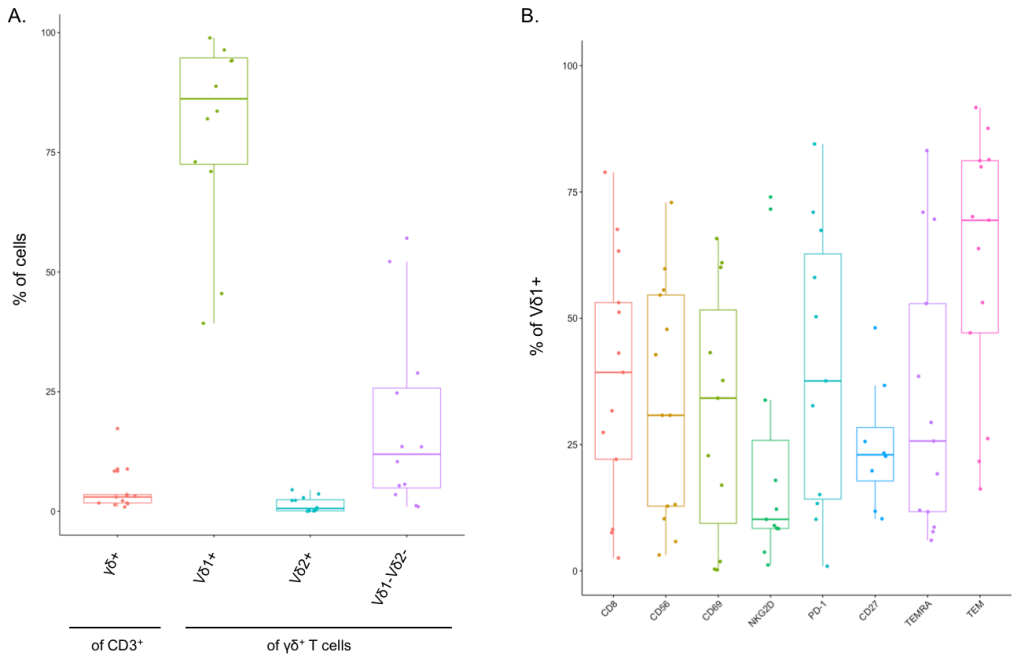



Figure 3.11: Box plot of: A. the distribution of $\gamma\delta$ T cells; B. Phenotypic characterization of V$\delta$1 in TIL across different cancer histologies.

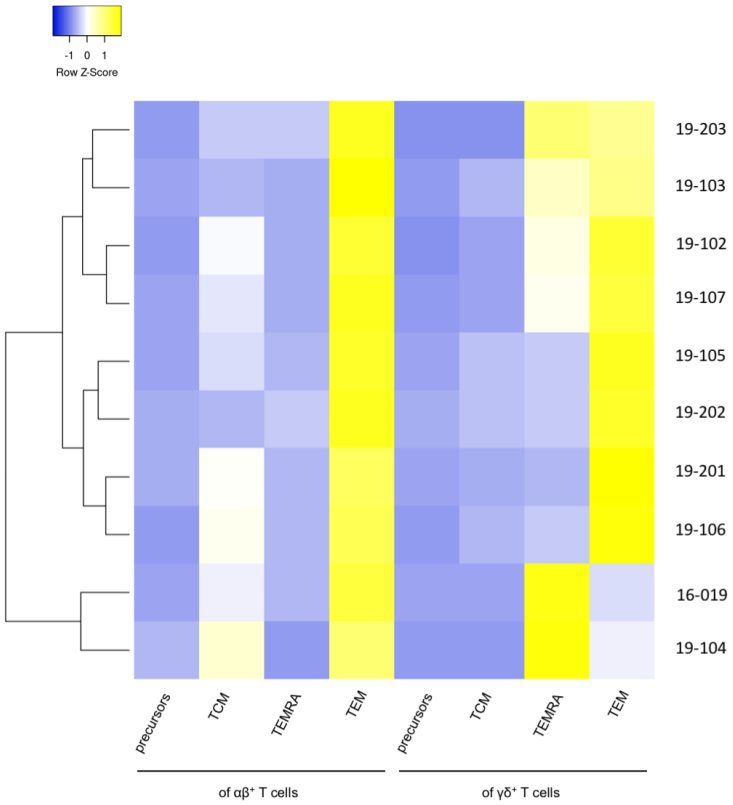



Figure 3.12: Heatmap with the distribution of the memory phenotype across $\alpha\beta$ and $\gamma\delta$ T cells, clustered by average linkage using an euclidean distance.

Very little of the examined $\gamma\delta$ TIL contain V$\delta$2, a population that is far more common in the peripheral blood. Of notice is a frequency of V$\delta1^{-}$V$\delta2^{-}$ cells in some samples, accounting for 50% of all $\gamma\delta$ T cells in two PMP samples. These cells are likely V$\delta$3, a highly specialized subset of $\gamma\delta$ T cells present in the liver and gut epithelium. If seen in a bigger cohort of patients, their role in the tumor microenvironment might warrant further investigation as these cells have been associated with the promotion of tissue homeostasis and wound healing. [138]

The phenotypic data reported in **Figures 3.11.B and 3.12** was constricted to V$\delta$1 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$^{-}$), **Figure 3.12**. 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. [101] 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. [139]

Consistent with a TEM and TEMRA memory phenotype is the low expression of CD27, with an average of 25% (**Figure 3.11B**). 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$^{-}$, [101] which is consistent with the flow 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. [140] Curiously, in murine models, CD27$^{+}$ $\gamma\delta$ T cells have been shown to be producers of IFN-$\gamma$, whereas CD27$^{-}$ populations produce IL-17 when stimulated. [141] However, this distinction has not been confirmed in humans.

CD69 expression, a marker of tissue resident T cells, was moderate, with some TIL samples having very low CD69 expression ($<$25% for n=4). As V$\delta$1 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. [137]

Also contradicting findings 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. [111, 112] 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 antigen exposure, tumor reactivity and increased T cell dysfunction, and shown to be upregulated in TIL across many cancer histologies. [142] But in $\gamma\delta$ T cells the role of PD-1 remains unclear.

No other trends or conclusions can be made from the V$\delta$1 surface markers as expression varies across patients and cancer histologies with no discernible trends.

### $\gamma\delta$ Subsets in TIL

To discern patterns in highly heterogeneous samples across a diverse cohort, one can look at samples from individual patient's in order to identify trends and establish possible TIL subpopulations. To do so, reduction methods were employed in the flow data of $\gamma\delta$ T cells containing at least 2000 events. In particular, t-distributed stochastic neighbor embedding (t-SNE) maps were created, with a few $\gamma\delta$ subpopulations of interest emerging.

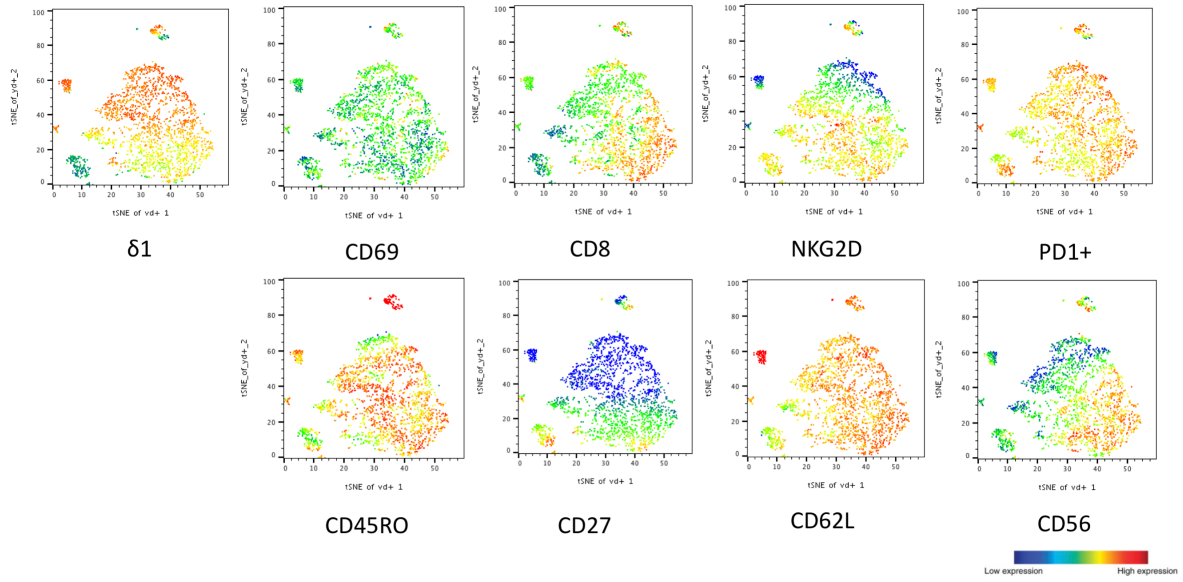



Figure 3.13: Relative expression of different markers after t-distributed stochastic neighbor embedding (t-SNE) map of $\gamma\delta$ TIL (gated on live, CD3$^+$ $\gamma\delta$-TCR$^+$).

In particular, a subset of V$\delta$1 seems to reliably express CD8 molecules, a canonical $\alpha\beta$ TCR co-receptor that is characteristic of cytotoxic T cells. Data from human $\gamma\delta$ populations in TIL is scare, but as seen in **Figure 3.11.B** the majority of TIL in this cohort have at least some CD8$^+$ expression.

This CD8 expression was to some extent unexpected, since $\gamma\delta$ T cells are generally considered to be both CD8$^-$ and CD4$^-$. One exception to this rule is a subset of intestinal intraepithelial $\gamma\delta$ T lymphocytes ($\gamma\delta$ iIELs) in mice, that has been shown to express CD8$\alpha\alpha$ homodimer. As for humans, there is one report by *Kadivar et al.* describing a population of $\gamma\delta$ expressing CD8$\alpha\beta$ heterodimer. This population is further characterized as mostly $\delta$1 with increased CD69 and CD56 expression plus heightened production of granzyme B and perforin. [143]

As the antibody clones used to detect CD8$^+$ cells in this work attach to the $\alpha$ chain of the molecule, it is impossible to know whether $\gamma\delta$ in TIL express the CD8$\alpha\beta$ heterodimer, and further work should be done to clarify this. Even so, this finding has direct consequences in the reports for TIL products used in the context of adoptive cellular therapy, as some of these products likely have $\gamma\delta$CD8+ TIL, that are being assumed to be canonical cytotoxic CD8 T cells (with an $\alpha\beta$TCR).

In **Figure 3.13**, this $\gamma\delta$ CD8$^+$ subset is shown to be associated with CD56 expression and PD-1

up-regulation. A similar pattern is found in other 3 of the TIL samples. Although a conventional marker of NK cells, CD56 expression has also been reported in $\alpha\beta$ and $\gamma\delta$ T lymphocytes, as a marker proportional to the cells' level of activation. [144, 145]. This suggests that at least a subset of the V$\delta$1 in TIL are activated. Another possibility is that the up-regulation of CD56 could be a transient consequence of the culture conditions, as described by *Acker et al.*, in the context of IL-15 stimulation. [146] This however seems unlikely, as different patients have very different V$\delta$1CD56 levels **(Figure 3.11.B)** even though all TIL was grown under the same conditions, and for a similar duration of time (Complete Media with 3000 IU/mL of IL-2 for 2-3 weeks). All things considered, V$\delta$1 CD8$^+$ co-expression with CD56$^+$ might indicate that the V$\delta$1CD8$^+$ population, present in some of the TIL, could correspond to an activated $\gamma\delta$ T cell subset.

**Isolation and expansion of $\gamma\delta$ in TIL**

To better understand the functionality of $\gamma\delta$ cells, it is necessary to isolate and expand them from the growing TIL. Only then it would be possible to test for functional target cell recognition. As such, efforts ensued to develop protocols for isolation and expansion.

Different commercially available isolation kits from Miltenyi were tested. Briefly, after 2-3 weeks of TIL growth in IL-2, the cells were sorted through columns, using the MidiMACS$^{TM}$ system. These efforts were hindered by the sparse number of naturally growing $\gamma\delta$ TIL which made the number of isolated cells insufficient for both flow analysis of the sample and further expansion, with many samples being used for one or the other.

Positive sorting, where anti $\gamma\delta$ TCR antibodies are retained in the column through magnetic forces, resulted in purities of 96% $\gamma\delta$ T cells, but were associated with increased cell death, as noted by substantial decreases in cell number in the days following the sort.

A negative sorted kit also commercially available by Miltenyi was tested. After TIL sorting, flow cytometry was used to determine what cells were isolated, resulting in 92% of CD3$^+$ cells, $\approx$ 98% of those $\gamma\delta$ positive. Of note is that NK cells (CD3$^-$CD56$^+$) also passed through the column. This sorting method improved survival of the cells in IL-2 culture.

Due to the clear reduction in $\gamma\delta$ TIL number, post magnetic sort, cultured in CM supplemented with 3000 IU/mL IL-2, across multiple samples, a sample with a large yield post sort was examined a week after sorting, with curious results. While the percentage of V$\delta$1 remained somewhat similar there was a clear shift in memory phenotype showing a clear shift from TEMRA to TEM, **Figure 3.14**[1]. This coupled with the reduction of cells suggests that the majority of TEMRA cells died when cultured under these conditions.

[1] Acquisition was on different days so to guarantee that the results are comparable each memory subpopulation was determined with a healthy donor PBMCs (as positive control), thus permitting a clear distinction of naive, TEM and TCM populations.

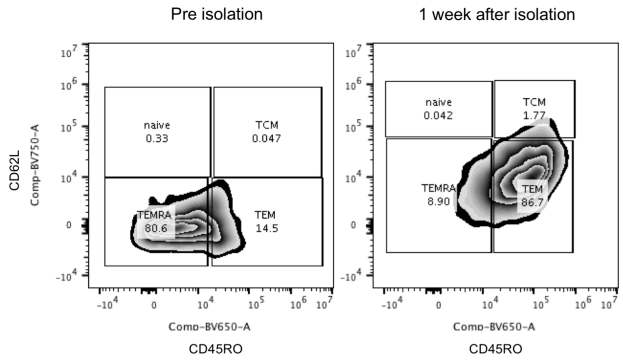



Figure 3.14: Zebra plot of the memory profile of $\gamma\delta$ T cells, pre and one week post sort.

Different expansion protocols, with varying cytokine cocktails, were employed after $\gamma\delta$ isolation, with little additional success. IL-2 and the standard REP with irradiated feeders was initiated, a protocol that has shown moderate success in expanding V$\delta$2 from the blood. Of the 5 $\gamma\delta$ TIL samples expanded with this protocol all resulted in a typical $\alpha\beta$TCR TIL product, likely due to the few remaining $\alpha\beta$ T cells after isolation overgrowing the isolated $\gamma\delta$ T cells.

Another tested strategy was a method described by *Almeida et al.* where sorted $\gamma\delta$ cells were cultured with no feeders in IL-4, IL-15 and IL-21 with OKT3 stimulation every 3 to 5 days. [112] This method resulted in an unhealthy culture, without expansion. Although it cannot be discarded that this might have happened due to the very limited number of $\gamma\delta$ as a starting population, it is worth nothing that the V$\delta$1 expansion protocol by *Almeida et al.* cells originating from the blood, and that these cell have a younger memory profile and are less likely to have been exposed to the suppressive factors present in the TME (see Section 1.1.1).

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

# Chapter 4

# Conclusions

## 4.1 Neoantigen Screening

This dissertation work showcased a methodology currently used in the clinical trials to determine neoantigen reactivity. Doing so required the mastery of several techniques from DC differentiation and electroporation to the use of ELISA and flow cytometry.

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. This demonstrates that there are still improvements necessary to better identify neoantigen reactivity, as will be discussed further ahead (Section 4.3).

The analysis of nonsynonymous mutations in two patients with metastatic Uveal Melanoma reiterated the frequency of both GNAQ and GNA11 hotspot mutations in this cancer histology. Also relevant is the SF3B1 mutation, which could be an unexplored source of neoantigens driving T cell targetting in Uveal Melanoma, as discussed in Section 3.2.

## 4.2 $\gamma\delta$ TIL Characterization

This work is one of the first to look at immune infiltrates in pseudomyxoma peritonei and demonstrate that growth of TIL is possible in this histology. In doing so, it characterizes its lymphocyte infiltrate, showing a large NK population. From the technical point of view, it required the mastery of spectral flow cytometry, a relatively new way of obtaining complex multi-parameter flow data.

Together with the RCC and GI malignancies samples this is one of the largest datasets looking at $\gamma\delta$ T cells in TIL. All 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 V$\delta$1, suggesting these cells are the major subset of $\gamma\delta$ that persists in the tumor microenvironment. Further analysis showed V$\delta$1 T cells routinely expressed CD8, in stark opposition to what is currently described in immunology books ($\gamma\delta$ T cells are canonically considered to be CD4$^-$CD8$^-$). This has important consequences for clinical trials using TIL therapy. TIL infusions are generally characterized as a mix of CD8 and CD4, with very little double negative cells, with

the assumption that the majority of CD3 positive cells in TIL are $\alpha\beta$ T cells. However, this could be untrue in some patients.

As for the phenotypic characteristics of $\gamma\delta$ T cells it showed that CD27 expression seems to be consistent with a predominately TEM to TEMRA phenotype. In addition, it demonstrated varied expression of CD56, CD69 and PD-1, similar to what has been described in $\alpha\beta$ TIL, but not in $\gamma 9\delta 2$ T cells ($\gamma\delta$ T cells mostly in the blood). Curiously it shows a generally low expression of NKG2D, which is inconsistent with data reported in the literature. Together, this data suggests that $\gamma\delta$ T cells in TIL may in fact be very different from V$\delta$1 derived from blood, perhaps highlighting the effect that the TME has in reprogramming infiltrating immune cells.

Furthermore, this work was able to demonstrate efficient isolation of $\gamma\delta$ T cells, Section 3.3.1, but struggled to expand $\gamma\delta$ TIL through the $\alpha\beta$ TCR T cell REP protocols (use of high concentration of IL-2 coupled with the use of irradiated feeders and CD3 antagonist stimulation), suggesting that new protocols are necessary to effectively expand these cells.

It is clear that $\gamma\delta$ T cells are a diverse and complex subset of the TIL population, and this work showcases the very first steps taken to get a better understanding of this T cell subset in TIL.

## 4.3 Future Work

### 4.3.1 Neoantigen Reactivity

If any T cells were identified as reactive to a tandem minigene that tandem minigene would have been deconvoluted into the various minigenes that composed it. This would allow the individual test of each of those minigenes, and thus identifying the specific neoantigen(s) driving T cell recognition. Subsequent work would have isolated and sequenced the TCR in order to fully characterize it and possibly use it in the context of *off-the-shelf* adoptive cell transfer therapies.

The identification of tumor neoantigens is fundamental to move forward the immunotherapy field, but it can be particularly important in moving forward cancer vaccine trials. This is not only because neoantigens can be used in these trials but also to improve the current epitope prediction algorithms, as they are currently based on the processing of viral antigens by APCs and not on the processing of nonsynonymous mutations and altered transcription.

This methodology will likely continue to be used to screen TIL samples of patients enrolled in clinical trials, as the presence of neoantigen reactive T cells is an indicator of immunotherapy response and prolonged survival. Furthermore, the isolation of reactive TCRs is key to understanding more about chronic T cell dysfunction and the mechanisms by which tumor cells evade our immune system.

To improve the neoantigen reactivity screening of tumor reactive TIL this work proposes a few additional steps, detailed in Section 3.2, including the screen of self-antigen. This could also be done in TMG format in order to maximize the amount of tested self antigen per autologous monocytes used (decreasing both cost and use of patient derived material). To both identify neoantigen reactive TCRs and gain knowledge of the state of these cells in TIL it could be interesting to reverse engineer the neoantigen

driving recognition. By using single cell RNA sequencing technologies, one could isolate the TCR and reverse engineer the corresponding neoantigen through the TCR. This methodology would also allow the identification of unknown markers for reactivity, while allowing key insight into the transcription networks of tumor reactive T cells.

### 4.3.2 $\gamma\delta$ TIL Studies

This work characterized several TIL samples across different histologies, but much remains to be done.

The functional role of CD56 and CD8 expression in $\gamma\delta$ T cells is unclear and it warrants further investigation. CD56 has been linked to an activation marker but its role remains unknown. Likewise the role of CD8 is unclear in $\gamma\delta$ T cells. Although it likely marks a subtype of $\gamma\delta$ that has been activated, it is unclear whether the CD8 molecule is a homodimer or a heterodimer and if the CD8 acts as a co-receptor to the TCR.

The low expression of CD27, a marker reported to differentiate IL-17 from IFN-$\gamma$ producing $\gamma\delta$ T cells in mice, is consistently low and seems to be associated to memory. It is worth noting that memory in $\gamma\delta$ T cells is assumed to work similarly to $\alpha\beta$, but future studies are needed to assert that. A puzzling marker is the variable CD69 expression seen in the $\gamma\delta$ TIL. Recent reports suggest CD69 associated with CD103 expression mark tissue resident T cells. One could speculate that V$\delta$1s, as tissue surveillant cells, would have high expression of CD69 but that does not seem to be the case.

Apart from the markers here presented, many other phenotypic markers remain interesting candidates and may warrant further study. CD122, the $\beta$ chain of the IL-2 receptor has been linked to enhanced IFN-$\gamma$ in $\gamma\delta$ T cells, in mice. Its human homolog has been associated with co-expression of V$\delta$1CD8$^{+}$ by *Kadivar et al.*, and marks cells that possess potent cytotoxic capacities. [143] There is also much unknown in regards to the existence and role of other NK receptors in $\gamma\delta$ TIL. As such, a more complex flow panel should be designed to include the 3 NK receptor molecules, NKp30, NKp44, Nkp46 and clarify how these markers are expressed in TIL.

Regarding the phenotypic characterization of $\gamma\delta$ TIL it might be worth considering analysing these cells as fresh as possible, meaning right after tumor digestion. For this work this option was disregarded due to the observation of highly autofluorescent tumor digests obtained from samples in the peritoneum. This might be worth revising, with different protocols for digestion and T cell subset enrichment available.

Another crucial focal point is the development of an expansion protocol that consistently allows for the survival and growth of $\gamma\delta$ TIL. The biggest limiting factor in using $\delta$1 in adoptive cellular therapy is the difficulty in expanding these cells using clinical grade protocols. Work by *Almeida et al.* looks promising for cells from the blood, but it might require modifications to be adapted to $\gamma\delta$ from the TIL. It might also be worth considering strategies to enhance the $\gamma\delta$ growth in the early stages of TIL development to start with a higher population.

Unfortunately due to the difficulties in expanding $\gamma\delta$ T cells, 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 TME reprogramming.

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