

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL

**IMMUNE PROFILING OF THE TUMOR
MICROENVIRONMENT IN A MURINE MELANOMA MODEL
TREATED WITH ANTI-TNF AND IMMUNE CHECKPOINT
INHIBITORS VIA scRNA-SEQ**

KAZIM KÜÇÜK

**A THESIS OF MASTER OF SCIENCE
DEPARTMENT OF BIOENGINEERING
BIOINFORMATICS AND SYSTEMS BIOLOGY PROGRAM**

ADVISOR: ASSOC. PROF. DR. PINAR PİR

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LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

ANTI-TNF VE CHECKPOINT İNHİBİTÖRLERİ İLE TEDAVİ
EDİLEN FARE MELANOMA MODELİNDE TÜMÖR
MİKROÇEVRESİNİN scRNA-SEQ İLE İMMÜN PROFİLİNİN
ARAŞTIRILMASI

KAZIM KÜÇÜK

YÜKSEK LİSANS TEZİ
BİYOMÜHENDİSLİK ANABİLİM DALI
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*To all outcasts and fragile
beings.*

This is for you.

ABSTRACT

The incidence of melanoma is rising worldwide, with advanced cutaneous melanoma driving significant mortality rates. While immune checkpoint inhibitor (ICI) therapies such as anti-programmed death receptor 1 (anti-PD-1) and anti-cytotoxic T cell-associated antigen 4 (anti-CTLA-4) have revolutionized cancer treatment, a substantial proportion of patients fail to benefit in the long term due to immune-related adverse events (irAEs), and resistance mechanisms.

Anti-Tumor Necrosis Factor (TNF) therapy effectively manages some irAEs, such as colitis, and, paradoxically, can improve the efficacy of ICI at least in some mouse cancer models, suggesting that anti-TNF combination with ICI may improve tumor responses, while reducing toxicities. It remains to clarify the pharmacodynamics impact of TNF inhibitors combined with ICI in mouse cancer models.

Recent research utilizing single-cell RNA sequencing to investigate immune cell populations in melanoma has revealed significant diversity in their composition. This diversity within the heterogeneous tumor microenvironment is likely to play a pivotal role in determining therapy outcomes.

In this study, analyses of single cell transcriptomics data from CD45⁺ Tumor-infiltrating leukocytes from B16-OVA melanoma murine models were conducted using computational tools to compare two therapy regimens: a bitherapy, combining anti-PD-1 and anti-CTLA-4, and a tritherapy, combining anti-PD-1, anti-CTLA-4 and anti-TNF. The workflow encompassed preprocessing, cell-type annotation, analyses of cell-type proportions, differentially expressed genes (DEGs), gene set enrichment, and over-representation, and finally, cell-cell interaction analysis.

Results showed that the Anti-TNF significantly reduced regulatory T cells, natural killer cells, and non-classical monocyte populations. However, the low number of DEGs between therapies within these populations suggests that anti-TNF primarily affects cell abundance rather than modulating gene expression profiles. Cell-cell interaction analysis revealed CCL3–CCR4/CCR5 interactions between CD8⁺ T cells and Tregs in the bitherapy group, indicating that the higher presence of Tregs may be driven by this chemokine-mediated recruitment. Intriguingly, CD8⁺ T cells in the bitherapy group exhibited a stronger effector profile, and a specific subpopulation of CD8⁺ T cells was identified as the most affected by treatment. Within this subset, TNFR1 signaling and apoptosis-related pathway enrichment were observed in the bitherapy group, whereas CD8⁺ cells in the tritherapy group showed increased proliferative activity. These findings suggest that the presence of TNF may contribute to the depletion of certain CD8⁺ subsets over a longer experimental timeframe. However, the fact that this study was conducted on samples obtained at a single time point makes it difficult to establish a generalizable conclusion, considering that immune cell responses may vary over time.

Keywords: Single Cell RNA Sequencing, Melanoma, Immunotherapy, Immune Checkpoint Inhibitors, Anti-TNF

ÖZET

Melanoma insidansı dünya genelinde artış göstermekte olup, ileri evre kutanöz melanoma önemli bir mortalite nedenidir. Programlanmış hücre ölümü reseptörü 1 (PD-1) ve sitotoksik T lenfosit ilişkili antijen 4 (CTLA-4) gibi immün kontrol noktası inhibitörleri (ICI) tedavileri kanser tedavisinde önemli bir ilerleme sağlamış olsa da, hastaların önemli bir kısmı bağışıklıkla ilişkili yan etkiler (irAE'ler) ve tedaviye karşı gelişen direnç mekanizmaları nedeniyle uzun vadede yeterli klinik yanıt alamamaktadır.

Anti-Tümör Nekroz Faktör (anti-TNF) tedavisi, kolit gibi bazı irAE'leri etkili biçimde kontrol altına alabilmekte ve bazı fare tümör modellerinde ICI tedavisinin etkinliğini artırabilmektedir. Bu durum, anti-TNF tedavisinin ICI ile kombinasyonunun hem tümör yanıtını artırabileceğini hem de toksisiteyi azaltabileceğini düşündürmektedir. Ancak, bu kombinasyonun farmakodinamik etkileri henüz yeterince aydınlatılamamıştır.

Bu çalışmada, B16-OVA melanoma fare modeli kullanılarak tümör mikroçevresindeki bağışıklık hücrelerinin tek hücreli RNA dizileme (scRNA-seq) yöntemiyle kapsamlı analizi gerçekleştirilmiştir. CD45⁺ tümör infiltrate eden lökositler incelenmiş ve iki farklı tedavi rejimi karşılaştırılmıştır: (1) anti-PD-1 ve anti-CTLA-4'ü içeren biterapi ve (2) bu rejime ek olarak anti-TNF içeren triterapi. Biyoinformatik analiz süreci; veri ön işleme, hücre tipi anotasyonu, hücre tipi miktarlarının karşılaştırılması, farklı şekilde eksprese edilen genlerin (DEG) belirlenmesi, gen seti zenginleştirme ve aşırı temsil analizi (ORA) ve hücreler arası etkileşim analizlerini kapsamaktadır.

Analizler sonucunda, anti-TNF tedavisinin düzenleyici T hücreleri (Treg) ve doğal öldürücü hücreler (NK) popülasyonlarını anlamlı ölçüde azalttığı saptanmıştır. Ancak bu hücre popülasyonlarında tedaviler arasında sınırlı sayıda farklı gen ifadesi gözlenmiş, bu da anti-TNF'nin hücre miktarları üzerinde etkili olduğunu, gen ekspresyon profillerini doğrudan değiştirmediğini düşündürmektedir. Hücreler arası etkileşim analizinde, biterapi grubunda CD8⁺ T hücreleri ile Treg'ler arasında CCL3–CCR4/CCR5 ekseninde bir etkileşim saptanmış, bu durum biterapi örneklerindeki Treg hücresi fazlalığının kemokin aracılı çekilme yoluyla gerçekleştiğini düşündürmektedir.

Ayrıca, biterapi grubundaki CD8⁺ T hücrelerinin daha belirgin bir efektör profile sahip olduğu ve bu hücreler içinde belirli bir alt grubun tedaviden en fazla etkilendiği belirlenmiştir. Bu alt popülasyonda, biterapi rejimi altında TNFR1 aracılı sinyal iletimi ve apoptozla ilişkili yolların zenginleştiği; buna karşın triterapi grubundaki CD8⁺ hücrelerde proliferatif aktivitenin arttığı gözlemlenmiştir. Bu bulgular, TNF varlığının uzun süreli deneysel süreçte bazı CD8⁺ alt popülasyonlarının tükenmesine neden olabileceğini ortaya koymaktadır. Ancak bu çalışmanın tek bir zaman diliminde elde edilen örnekler üzerinde yapılmış olması, immün hücre yanıtının zaman içinde değişebileceği bilgisi göz önüne alınınca genelleştirilebilir sonuçlar elde edilmesini zorlaştırmaktadır.

Anahtar Kelimeler: Tek Hücreli RNA Dizileme, Melanoma, İmmünoterapi, İmmün Checkpoint İnhibitörleri, Anti-TNF

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LIST OF SYMBOLS AND ABBREVIATIONS

AICD	: Activation-induced cell death
Ag	: Antigen
BCR	: B cell receptor
cDC1	: Conventional dendritic cell type 1
cDC2	: Conventional dendritic cell type 2
COLLECTRI	: Collection of Transcriptional Regulatory Interactions
CTL	: Cytotoxic T lymphocyte
CTLA-4	: Cytotoxic T-lymphocyte-associated protein 4
DEG	: Differentially expressed gene
DNT	: Double-negative T cell
EGFR	: Epidermal growth factor receptor
FDR	: False discovery rate
Foxp3	: Forkhead box P 3
GSEA	: Gene set enrichment analysis
GO	: Gene Ontology
ICI	: Immune checkpoint inhibitor
IFN	: Interferon
irAEs	: Immune-related adverse events
ILCs	: Innate lymphoid cells
ILTs	: Innate-like T cells
LMER	: Linear mixed-effects regression
MAPK	: Mitogen-activated protein kinase
MDSCs	: Myeloid-derived suppressor cells
MHC	: Major histocompatibility complex
mregDC	: Mature dendritic cells
moDC	: Monocyte-derived dendritic cell
msigDB	: Molecular Signatures Database
NK	: Natural killer cell
NF- κ B	: Nuclear factor kappa-light-chain-enhancer of activated B cells
ORA	: Over-representation analysis
PD-1	: Programmed cell death protein 1
PD-L1	: Programmed death-ligand 1
RNA	: Ribonucleic acid
scRNA	: Single-cell RNA
TAM	: Tumor-associated macrophage
TF	: Transcription factor
TCR	: T cell receptor
TGF- β	: Transforming growth factor β
TNF	: Tumor necrosis factor
TNFR1	: Tumor necrosis factor receptor 1
TNFR2	: Tumor necrosis factor receptor 2
TILs	: Tumor-infiltrating lymphocytes
Treg	: Regulatory T cell (T-regulatory cell)
TRAIL	: TNF-related apoptosis-inducing ligand
UMI	: Unique molecular identifier
VEGF	: Vascular endothelial growth factor

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1. INTRODUCTION

In this thesis, the aim was to investigate the effects of immune checkpoint inhibitors (ICIs) and anti-TNF agents on CD45+ immune cell populations in mouse melanoma model. The data used in this study is unpublished and has been analyzed for the first time in this work.

Unpublished results from Dr. Bruno Segui's laboratory have demonstrated that TNF blockade enhances the efficacy of ICIs in a mouse melanoma model (B16-OVA). However, the single-cell analysis conducted in this thesis allows for a more comprehensive examination of all genes and immune cell populations, aiming to provide more in-depth insights than those obtained from previous wet lab experiments.

The first section of this chapter provides an overview of melanoma, including its definition and the factors contributing to its development. This is followed by a discussion on ICI therapy, which has shown promising results in melanoma treatment. The mechanism of action of ICIs is explained, along with the combined use of multiple ICIs. The paradoxical effects of anti-TNF agents, which are used to mitigate the side effects of ICI therapies, are also addressed. The importance of single-cell studies in cancer research is emphasized, and findings from previous single-cell studies involving melanoma and ICI therapies are reported.

The second chapter of the thesis details the theoretical background of each method used, along with the rationale for their selection. Parameters used in the methodologies are provided, and the reasoning behind their selection is discussed. In the discussion section, the results are interpreted with reference to the existing literature.

To the best of our knowledge, this is the first study to investigate the effects of combining anti-TNF agents with ICIs on mouse immune cells collected from skin tumor samples at the single-cell level.

1.1. Cancer and Melanoma

Cancer usually starts when mutations occur in a single cell, disrupting the normal checks and balances that regulate cell growth, division, and death. These mutations

can activate genes that accelerate growth (oncogenes, such as RAS, RAF, and mTOR) or disable genes that normally act as brakes (tumor suppressor genes, like TP53). This imbalance leads to uncontrolled cell growth, resistance to natural cell death, and evasion of the body's mechanisms that typically prevent abnormal cell expansion **(Hanahan & Weinberg, 2011)**.

Melanoma is a type of cancer that develops from the pigment (melanin)-producing cells known as melanocytes; it usually occurs on the skin, but rarely in the mouth, intestines or eye (uveal melanoma). There are two primary types of melanoma, cutaneous and non-cutaneous. Cutaneous melanoma arises from skin melanocytes and is highly aggressive, being largely initiated by ultraviolet radiation exposure. Melanoma's development reflects a complex interplay between inherited susceptibility and environmental exposures, with UV radiation serving as the principal environmental risk factor due to its genotoxic impact on DNA **(Long et al., 2023; Rastrelli et al., 2014)**. Numerous epidemiological studies have shown that greater cumulative sun exposure is positively correlated with higher melanoma incidence.

Melanoma is labeled "advanced" once tumor cells move beyond the epidermis into nearby lymph nodes or surrounding tissues. When it spreads to distant sites like brain, survival rates fall sharply, and surgery by itself no longer offers a cure. That's why treating advanced melanoma depends on systemic therapies, most notably immune checkpoint inhibitors and targeted BRAF/MEK inhibitors **(Davis et al., 2019)**.

1.2. Immunotherapy

Traditional cancer treatments like chemotherapy and radiotherapy work by directly attacking tumor cells, but they can also harm healthy tissue and can have significant side effects. Immunotherapy takes a different track: it boosts the body's own defense system to seek out and destroy cancer. A cornerstone of this approach is the blockade of "immune checkpoints," which are molecular brakes the immune cells normally use to avoid over activation of T cells and turning on healthy tissues by mistake. Unfortunately, tumors can hijack and exploit these checkpoints to cloak themselves from immune attack. By using specially designed antibodies, immunotherapy can block these checkpoints, essentially taking the "brakes" off the immune system. This allows immune cells, particularly T cells, to recognize and eliminate cancer cells more

effectively, by reactivating the cytotoxic features of the immune response, especially, CD8⁺ T cells and natural killer (NK) cells and by rescuing them from exhaustion. Key targets in this strategy are molecules like cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1), both of which normally inhibit immune responses. Blocking them lead to promising results, especially in cancers like melanoma, by reactivating the immune attack against tumors (**Pardoll, 2012**).

Since anti-CTLA-4 therapy gained approval for metastatic melanoma in 2011, a wave of additional immune-modulating treatments has emerged. PD-1/PD-L1 blockade has shown consistent clinical success and paved the way for trials targeting new inhibitory checkpoints (e.g., LAG-3, TIM-3, VISTA, TIGIT) and agonistic antibodies for co-stimulatory receptors such as 4-1BB, GITR, CD40 and OX40 (**Salomon et al., 2018; Ziogas et al., 2023**).

1.2.1 Immune Checkpoint Inhibitors

Both checkpoint inhibitor immunotherapy and the MAPK pathway blockade via BRAF and MEK inhibitors have recently been the key factors for the advanced melanoma patients' increased survival rate. BRAF and MEK inhibitors are only recommended for people who have tumors with BRAF V600 mutations. On the other hand, the checkpoint inhibitors benefit is independent of the BRAF mutation status (**McArthur & Ribas, 2013**).

Immune checkpoint inhibitors (ICIs) are monoclonal antibodies designed to block CTLA-4 or PD-1/PD-L1 interactions. These pathways are, in normal immune responses, crucial for immune control to avoid autoimmunity. But tumors hijack these pathways through PD-L1 overexpression to evade the immune recognition, leading to suppression of T cell activation and immune tolerance. By disrupting these inhibitory signals, ICIs free the immune system to attack and kill melanoma cells, because T cells can continue on showing cytotoxicity, proliferation and anti-tumor cytokine production. PD-1 blockade restores the immune system's ability to recognize and destroy tumor cells by reinvigorating exhausted T-cells in peripheral tissues (**Carlino et al., 2021**).

Blocking PD-1 does not merely reinvigorate CD8⁺ T cells, it also shifts the balance of regulatory T cells in the tumor microenvironment. Several studies have found that

anti-PD-1 therapy actually expands intratumoral Tregs. For instance, scRNA and T cell receptor repertoire sequencing (TCR) analyses of melanoma tumor-infiltrating lymphocytes (TILs) before and after treatment reveal an expansion of Foxp3 positive Treg cell clusters in response to therapy **(Nagasaki et al., 2022)**. The rise in intratumoral Tregs is actually a byproduct of CD8 T cell reactivation: once PD-1 is blocked, those reinvigorated CD8 TILs unleash a burst of IL-2 secretion, and IL-2 cytokine then enhances Treg survival and proliferation. Another study also showed that reinvigorated CD8⁺ T cells after PD-1 blockade accumulate alongside with Tregs in the tumors and secrete interleukin-2 (IL-2), which induces the upregulation of inducible T-cell co-stimulator (ICOS), an anti-apoptotic receptor, on Tregs, thereby enhancing their survival and resistance to apoptosis **(Geels et al., 2024)**.

In summary, single-cell studies reveal that PD-1 blockade drives CD8⁺ T cell activation, exhaustion, and clonal expansion, while also promoting Treg accumulation, underscoring the need to counteract this compensatory immunosuppressive response.

Regulatory T cells (Tregs) maintain immune homeostasis and suppress anti-tumor responses. They are characterized by expression of CD4, CD25, and the transcription factor Foxp3, which is essential for their function and also drives CTLA-4 transcription. Tregs constitutively express high levels of CTLA-4, leading to the hypothesis that CTLA-4 blockade may impair their suppressive activity or deplete them through antibody-dependent cell-mediated cytotoxicity **(Takahashi et al., 2000; Wu et al., 2006; Zheng & Rudensky, 2007)**.

Blocking CTLA-4 with agents like ipilimumab, the first checkpoint inhibitor approved for melanoma, triggers a different immune response than PD-1 blockade. Single-cell profiling shows CTLA-4 inhibition drives an expansion of Icos positive CD4⁺ effector cells that secrete IFN- γ and TNF, whereas PD-1 inhibitors mainly reinvigorate CD8⁺ T cells. Although anti-CTLA-4 can also engage certain exhausted-like CD8 subsets, its dominant impact falls on the CD4 compartment. In mouse melanoma models, CTLA-4 blockades further boost the intratumoral CD8/Treg ratio **(Wei et al., 2017)**. A defining feature of anti-CTLA-4 therapy is its capacity to deplete regulatory T cells within the tumor itself **(Shklovskaya et al., 2022)**.

One of the most notable distinction between CTLA-4 and PD-1 blockade is how they shape long-term T cell memory. CTLA-4 inhibition tends to preserve a pool of TCF-

1⁺ progenitor T cells which are less exhausted, self-renewing cells that are key for lasting immunity. Whereas PD-1 blockade mainly drives the expansion of terminally differentiated TOX positive T cells, which are more exhausted. Anti-PD-1 therapy alone can draw upon and eventually deplete this progenitor pool as it drives their differentiation into terminal effectors. Functionally, this means CTLA-4 blockade supports the generation of long-lived, stem-like T cells capable of sustaining anti-tumor responses. On the other hand, PD-1 blockade predominantly boosts short-lived effector T cells (**Mok et al., 2025**).

Together, these findings highlight that CTLA-4 and PD-1 therapies mobilize distinct T cell populations within the tumor microenvironment.

Furthermore, preclinical studies show that anti-CTLA-4 therapy can induce Fcγ receptor (FcγR)-dependent cytotoxicity in vitro and deplete intratumoral Tregs in vivo. This depletion increases the ratio of intratumoral effector T cells to Tregs, thereby enhancing antitumor immunity (**Havel et al., 2019**).

Overall, blocking CTLA-4 on T cells promotes co-stimulation by allowing CD80/86 interactions with CD28, thereby enhancing T-cell activation at an early stage. Additionally, it impairs Tregs, which suppress antitumor immunity. However, CTLA-4 inhibition tends to cause more severe immune-related adverse events (irAEs) due to systemic over-activation of the immunity (**Wolchok et al., 2013**).

1.2.2 Mechanism of Immune Checkpoint Inhibitors

Naive T cells differentiate into activated T cells upon encountering peptide–Major Histocompatibility Complexes (MHC) expressed by antigen-presenting cells (APCs), through engagement of their T cell receptor (TCR) and the co-stimulatory receptor CD28. Once primed, these T cells have to navigate through the bloodstream, extravasate across the tumor’s permeable vasculature, and migrate through layers of stroma and other physical barriers until they establish direct immunological contact with cancer cells. Finally, upon encountering their targets, T cells must recognize and respond appropriately, such as cytotoxic T lymphocytes (CTLs) releasing lytic granules to kill the malignant cells. This final step depends not only on the tumor cells displaying enough peptide-MHC complexes but also on the T cells overcoming various immunosuppressive mechanisms within the tumor environment. Following

activation, T cells upregulate the expression of inhibitory receptors such as PD-1, CTLA-4, LAG3, and TIM3. During chronic antigen exposure, continuous stimulation leads to sustained expression of these inhibitory receptors, initiating immune checkpoint mechanisms through ligand-receptor interactions to regulate and restrain T cell activity (Pitt et al., 2016; Seidel et al., 2018). By blocking those inhibitory receptors, ICIs can stimulate the immune response towards cancer cells.

1.3. Dual Checkpoint Inhibition

Combining anti-CTLA-4 with anti-PD-1 (e.g., ipilimumab + nivolumab) improves melanoma responses by reshaping the intratumoral T cell landscape. A 2024 single-cell transcriptomics study found that anti-CTLA-4 alone induces a burst of Mki67⁺ TCF7⁺ progenitor exhausted CD8 T cells which are stem-like cells that sustain the tumor-infiltrating lymphocytes (TIL) pool. Adding anti-PD-1 drives these progenitors to differentiate into TIM3 (Havcr2) and granzyme-expressing terminally exhausted cells with potent cytotoxicity. In combination treated tumors, both progenitor and terminally exhausted T cell subsets are enriched, reflecting CTLA-4's role in broadening the T cell repertoire and PD-1's role in deep functional reinvigoration. Moreover, single-cell TCR study showed that dual blockade accelerates and amplifies clonal expansions compared to either therapy alone, which is essential because it amplifies the few tumor-specific T cells into a sufficiently large population (Wang et al., 2024). In another study, in the peripheral blood of patients undergoing therapy, combination treatment uniquely expanded a population of CD8 T cells with a terminally differentiated effector phenotype, cells that still express the cytotoxic granzyme and perforin genes, a finding not seen with either monotherapy alone (Wei et al., 2019).

1.4. Combination Therapy and Immune Adverse Events

The combined immunotherapy approach leverages synergistic mechanism, simultaneously enhancing T-cell activation and reducing immune suppression within the tumor microenvironment, to achieve superior antitumor effects.

In a clinical trial with patients who had not been treated for advanced melanoma before, the combination approach showed much better results, with a lot more patients

responding and staying progression-free longer compared to just using anti-CTLA-4 (ipilimumab) alone (61% vs. 11%). However, the trade-off was that more people in the combination group experienced serious side effects (grades 3–4), affecting 54% of them, compared to only 24% in the single therapy group (**Postow et al., 2015**).

While ICIs usually have fewer side effects compared to traditional chemotherapy, they can still cause immune-related issues that affect the liver (hepatitis), skin and endocrine organs (**Spain et al., 2016**). One of the more common and challenging side effects, especially with dual checkpoint inhibition, is colitis, which is inflammation in the colon. In fact, TNF levels tend to go up in the intestines of patients treated with both ipilimumab and nivolumab (**Perez-Ruiz et al., 2019**). Additionally, people with metastatic melanoma on anti-PD-1 therapy often show higher TNF gene activity in their tumor samples (**Chen et al., 2016**).

Immune related adverse events from ICI therapy are generally managed by stopping the agents and starting steroids; for severe or steroid-refractory cases, TNF blockade with infliximab, a monoclonal antibody (mAb) that blocks TNF is advised, except in patients with hepatitis. The impact anti-TNF antibodies have on anticancer immune responses in these settings are not known, and data on the safety of long-term TNF inhibitor usage for irAes are currently lacking (**Postow & Hellmann, 2018**).

1.5. Tumor Necrosis Factor

TNF is a critical cytokine in immune regulation, originally named for its ability to induce hemorrhagic necrosis in tumors. TNF exists in two distinct forms: (i) soluble TNF and (ii) membranebound or transmembrane TNF (**Horiuchi et al., 2010**). TNF plays a dual role, it can promote or inhibit the activities of the immune cells and cancer cells within tumors. The overall impact of TNF inhibition on tumor development can be either beneficial or harmful, depending on qualitative factors (such as the presence of particular cell types) and quantitative factors (such as the local levels of TNF). Earlier studies have also shown that TNF signaling impact depends on how long it is present and the concentration it reaches in TME (**Cruceriu et al., 2020**).

High levels of TNF can inhibit tumor growth. At high concentrations, TNF acts as an anti-tumor agent by inducing apoptosis in tumor endothelial cells, leading to vascular collapse and direct tumor necrosis by reducing the tumor cells' ability to absorb

nutrients and oxygen. Conversely, chronic low levels of TNF within the TME can actively drive cancer progression via several mechanisms including: (i) blood vessel formation by inducing the expression of vascular endothelial growth factor; (ii) immunosuppressive myeloid cells, Tregs, regulatory B lymphocytes (Bregs) and myeloid-derived suppressor cells (MDSCs), which help tumors evade immune detection (**Chen et al., 2021**). In the B16 mouse melanoma model, for instance, cancer cells secreting low amounts of TNF led to increased infiltration of myeloid cells that expressed endothelial markers that facilitate tumor vascularization and growth (**Li et al., 2009**).

TNF exerts its biological effects through two receptors: TNFR1, broadly expressed and involved in innate immunity, and TNFR2, selectively expressed on immune-suppressive cells (MDSCs, Tregs) and endothelial cells. Within tumors, stromal cells (including macrophages, fibroblasts, and endothelial cells) rather than malignant cells are the primary sources of TNF (**Bertrand et al., 2015**).

Multiple mechanisms have been suggested to underlie TNF's inhibitory effects. Evidence indicates that prolonged exposure to this cytokine can impair TCR signaling or trigger T cell exhaustion (**Salomon et al., 2018**).

Mechanistically, TNF produced during anti-PD-1 therapy promotes PD-L1 and TIM-3 (Havcr2) expression on CD4 and CD8 TILs and triggers TNFR1-dependent activation-induced cell death (AICD) of PD-1/TIM-3 co-expressing CD8 TILs. Blocking TNF/TNFR1 signaling increases melanoma-specific CD8⁺ T-cell populations. Additionally, TNF signaling through TNFR1 directly promotes AICD in cytotoxic T lymphocytes (CTLs), further depleting their antitumor activity (**Bertrand et al., 2015**). In mice injected with B16 melanoma cell line, anti-PD-1 treatment by itself only slowed tumor growth, and most tumors eventually relapsed. By contrast, either TNF-deficient mice or wild-type mice treated with TNF/TNFR1 blocking antibodies and combining this blockade with anti-PD-1 triggered robust tumor rejection (**Bertrand et al., 2017**).

TNF's immunosuppressive effects are generally attributed to its engagement of TNFR2, since signaling through this receptor often provides protection across a variety of immune-driven disorders (**Faustman & Davis, 2013**). In mouse cancer models, blocking TNF, whether with anti-TNF treatment or via TNFR2 knockout, slowed

tumor growth and coincided with fewer myeloid-derived suppressor cells(MDSCs), implying that TNF promotes the survival, recruitment, or suppressive activity of these cells against antitumor immunity **(Salomon et al., 2018)**.

When TNF engages TNFR2, it drives regulatory T cells to proliferate, survive longer, and upregulate Foxp3 by strengthening their suppressive affect on the immune response. TNFR2 positive Tregs express elevated levels of checkpoint receptors like CTLA-4 and CD73, as well as inhibitory cytokines IL-10 and TGF β **(Li et al., 2022)**. Yet, antibodies that target TNFR2 can selectively remove or disable these TNFR2 positive Tregs. In mouse pancreatic cancer models, pairing anti-TNFR2 with PD-1/PD-L1 blockade not only reduced Treg and MDSC infiltration but also achieved full tumor regressions with durable immunity **(Zhang et al., 2022)**.

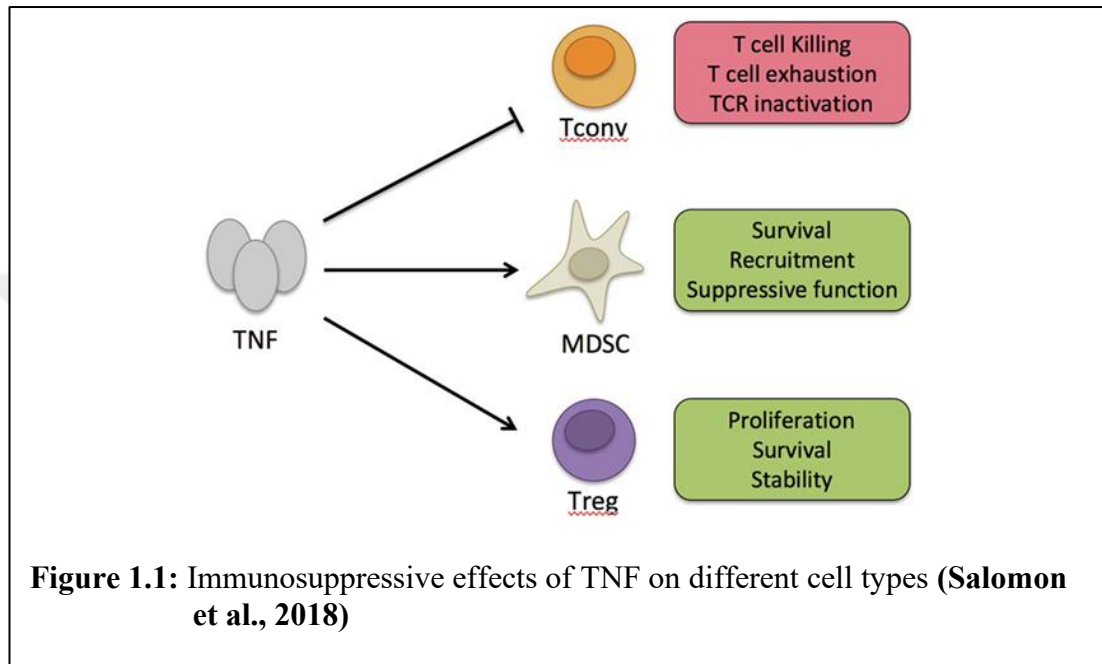
A recent analysis of patient data showed that elevated TNFR2 levels in the tumor microenvironment are linked to poorer responses to ICIs across multiple cancers, including melanoma **(Liao et al., 2023)**.

Additionally, TNF signaling in NK cells boosts the inhibitory receptor expression of TIM-3 while reducing levels of the activating receptor NKp46, which together undermine their anti-tumor functions **(Chen et al., 2021)**.

In melanoma cells, TNF promotes immune escape and tumor relapse through multiple, interconnected mechanisms. By signaling through TNFR2, TNF activates NF- κ B in melanoma cells, which both downregulates differentiation antigens required for T cell recognition and upregulates PD-L1 as an adaptive resistance strategy **(Li et al., 2024)**. The resulting increase in PD-L1 on tumor cells engages PD-1 on T cells to inhibit their activity, while concomitant TNF-driven dedifferentiation or induction of ectoenzymes such as CD73 further diminishes tumor immunogenicity **(Chen et al., 2019; Reinhardt et al., 2017)**.

To summarize, TNF can activate immune responses in acute contexts, but in chronic cancer settings TNF mainly acts as a “brake” on anti-tumor immunity by enhancing the expansion of Tregs and other suppressor cells while driving CD8 T and NK cells into a dysfunctional state in melanoma **(Montfort et al., 2019)**. But some studies paradoxically report that it can inhibit the suppressive activity of human Tregs **(Nagar et al., 2010; Nie et al., 2013; Valencia et al., 2006; Zanin-Zhorov et al., 2010)**. Moreover, while some *in vitro* studies suggest TNF can dampen Treg suppressive

function, *in vivo* this is overshadowed by TNF's dominant role in driving Treg population expansion (Bilate & Lafaille, 2010; Grinberg-Bleyer et al., 2010; Zanin-Zhorov et al., 2010; Zaragoza et al., 2016). However, it should be noted that assessing TNFR2's *in vivo* function in Treg biology is challenging due to the lack of a Treg-specific conditional TNFR2 knockout model (Salomon et al., 2018).



1.6. Single Cell RNA Sequencing

Single-cell RNA sequencing (scRNA-seq) is a revolutionary genomics technology that profiles gene expression at the resolution of individual cells, unlike bulk RNA-seq which measures average gene expression across a mixture of many cells. Single-cell RNA sequencing workflows typically begin with the isolation of individual cell via microfluidic platforms, microwell arrays or fluorescence-activated cell sorting and followed by reverse transcription of polyadenylated transcripts and incorporation of a unique molecular barcode into each cell's cDNA. Barcoded libraries are then pooled for high-throughput sequencing, and computational demultiplexing reconstructs the transcriptome of each captured cell. By contrast, bulk RNA-seq merges RNA from heterogeneous cell populations into a composite "average" profile, obscuring cell-type-specific signals (Chang et al., 2024).

Recent improvements in cell-capture efficiency and library-preparation protocols now permit the profiling of thousands of single cells per tumor specimen, enabling detailed dissection of malignant, immune and stromal compartments that bulk approaches cannot resolve. However, scRNA-seq suffers from “dropout” effect in which some transcripts go missing and requires complex bioinformatics analysis, whereas bulk RNA-seq is simpler, more cost-effective (Sun et al., 2021). Despite these trade-offs, scRNA-seq’s ability to resolve cellular heterogeneity has made it indispensable in cancer research, with over 200 datasets generated across dozens of tumor types in the past decade (Aran, 2023; Y. Zhang et al., 2021).

1.6.1 Importance of scRNA-seq Studies in Cancer Research

Intratumoral heterogeneity is one of oncology’s toughest obstacles: even tumors of a single histological type harbor distinct malignant subpopulations with their own gene-expression signatures. That cellular complexity explain why one region of a tumor might shrink under treatment while another persists (Levitin et al., 2018). Thanks to scRNA-seq studies, researchers have uncovered coexisting cell states (mesenchymal, proliferative, stem-like) within individual tumors in glioblastoma and melanoma, demonstrating the technique’s power to dissect intratumoral cellular diversity (Tirosh & Suva, 2024).

Furthermore, by profiling individual cells across developmental stages, scRNA-seq can map out lineage trajectories and uncover the rare, stem-like subpopulations which are defined by quiescence and self-renewal programs that drive tumor growth, fuel treatment resistance (Sun et al., 2021).

In the Table 1.1, several immune cell types identifiable by scRNA-seq, together with their functions in the TME, are described.

Table 1.1: Functions of various cell types in tumor microenvironment (Chen et al., 2021).

Effect on Tumor	Cell type	Mechanism
Anti-tumor effects	CD8 CTLs	Kill cancer cells that display tumour-associated antigens through perforin/granzyme release and IFN- γ production
	NK	Eliminates cancer cells that over express ligands recognized by NK cell receptors
	CD4 Th1 helper	Enhance the development of tumour antigen-specific CTLs and produce IFN γ , which has antitumour effects
	Dendritic cells	Capture and process tumor antigens, then migrate to lymph nodes to present tumour antigens to naive T cells to induce their effector differentiation
Mixed effects	B cells	TLS- associated B cells present tumor antigens to and co-stimulate T cells. In contrast regulatory B cells (Bregs) secrete immunosuppressive cytokines (IL-10, TGF- β , IL-35) and promote Treg recruitment.
Pro-tumor effects	Treg	Act as immune brakes, damping effector T cell activity and inhibit differentiation of naive CD4 T cells
	MDSCs	Reduce the numbers of CTLs, CD4 T helper cells and NK cells; increase the numbers of Treg cells and inhibit the activity of CTLs by inactivating TCRs.
	TAMs	Produce CCL22 to recruit Treg cells and produce IL-10 and TGF β , which are known as immunosuppressive cytokines

1.6.2 scRNA-seq Melanoma Studies

Single-cell RNA sequencing has become an invaluable way to elucidate how diverse cell populations in melanoma tumors react to immunotherapy, by profiling each tumor

and immune cell, it uncovers the mix of cell types, their gene-expression programs, and new markers linked to treatment success or resistance **(He et al., 2024)**.

In a 2023 study, researchers used PD-1–knockout, melanoma-bearing mice and combined single-cell RNA sequencing with spatial transcriptomics to show that, within tumors, PD-1 deficient regulatory T cells become hyper-suppressive in the microenvironment. One of the major findings of this study was that, without PD-1, Tregs upregulate alternative coinhibitory receptors such as CD30 to boost their suppressive activity. This observation revealed that PD-1 normally acts as a brake on Treg function, and that PD-1 deficiency enhances STAT5 signaling in Tregs, thereby inducing CD30 expression. This compensatory induction of CD30 was also seen in human melanoma samples after PD-1/PD-L1 blockade. These findings reveal that while anti-PD-1 therapy reinvigorates exhausted CD8 T cells, it also induces the activation of hyper suppressive Tregs, suggesting that combining PD-1 inhibitors with a CD30 antagonist could overcome Treg-mediated resistance in refractory patients **(Lim et al., 2025)**.

Sade-Feldman and colleagues profiled immune cells from metastatic melanoma patients treated with anti–PD-1 or CTLA-4 antibodies and uncovered two distinct CD8⁺ T cell states. In patients who did not respond, T cells displayed an “exhausted” signature by showing high expression levels of TIM-3, PD-1, LAG-3 and ENTPD1. Whereas responders harbored a memory/progenitor population marked by TCF7 (also known as TCF1), IL7R and other memory-associated genes. Importantly, follow-up tissue analyses confirmed that TCF7 expression reliably predicts a positive outcome to anti–PD-1 therapy **(Sade-Feldman et al., 2018)**.

In another scRNA-seq study on melanoma, researchers examined tumor infiltrating B cells and they found evidence of clonal expansion of B cells in responders, indicating an active antigen-driven B cell response in the tumor **(Helmink et al., 2020)**.

Using scRNA-seq of the myeloid compartment in B16 melanoma models, another study uncovered a distinct subpopulation of tumor associated macrophages (TAMs) annotated as MerTK⁺ TAM. These cells characterized by high Arg1, CD206 and PD-L1 expression that correlates with tumor progression and resistance to checkpoint blockade **(Wu et al., 2024)**.

In their 2023 study, Phadke and colleagues used single-cell RNA sequencing in mouse melanoma models to pit PD-1 + CTLA-4 blockade against PD-1 + LAG-3 blockade. The PD-1 and CTLA-4 combination therapy showed more FOXP3 positive Tregs and fewer activated CD4 helper cells but enriched for highly cytotoxic interferon gamma secreting CD8 T cells and these CD8 cells remained effective even after CD4 T cell depletion, suggesting CTLA-4 blockade partly acts by increasing Treg mediated suppression. By contrast, PD-1 and LAG-3 combination blockade promoted CXCR4⁺IL21R⁺ helper CD4⁺ cells and CD8⁺ T cells with lower cytotoxic gene expression, and its anti-tumor activity depended entirely on CD4 helper population. These findings reveal that different checkpoint combinations elicit distinct TIL phenotypes like anti-CTLA-4 drives a CD8 centric response, whereas anti-LAG-3 relies on CD4 T cell support **(Phadke et al., 2023)**.

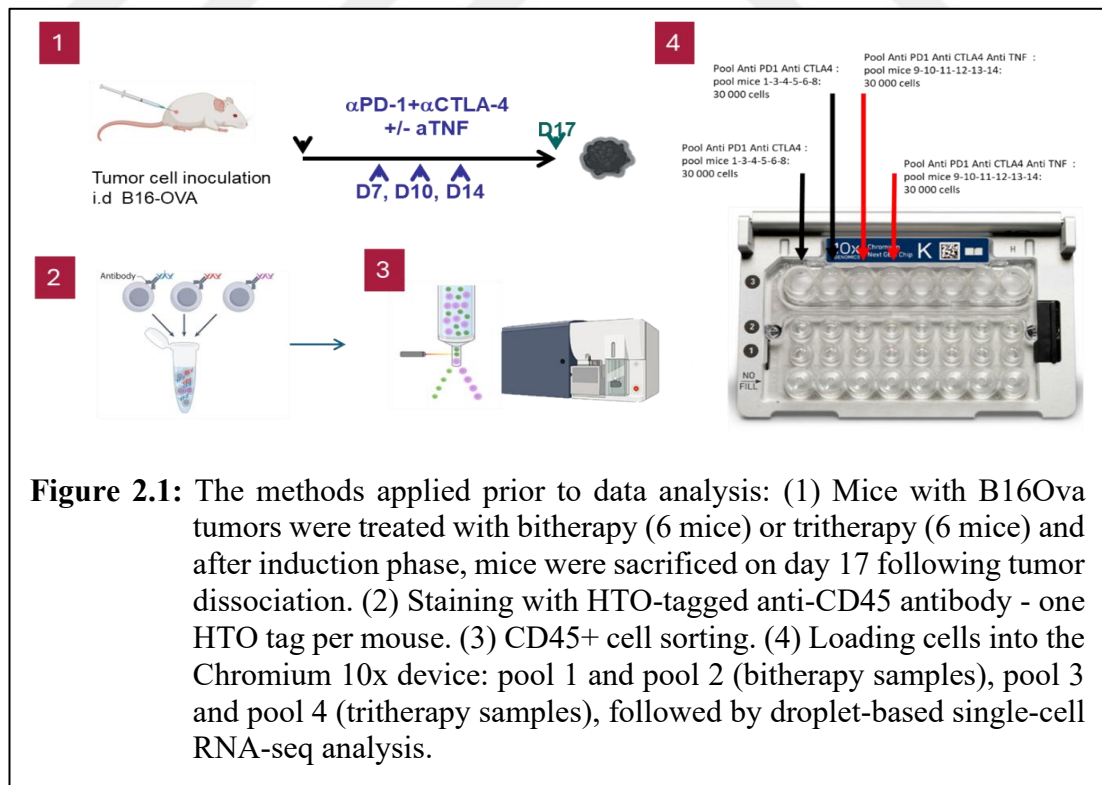
Another study was conducted on B16 mice melanoma models with the focus of age-related affects without applying any ICIs therapies and it was found that there is striking difference young versus aged mice. Intriguingly, old mice group harbored a more effector like microenvironment, with abundant cytotoxic CD8⁺ T cells, conventional dendritic cells and M1-like macrophages. In contrast, young mice's tumors were enriched for exhausted CD8⁺ T cells, M2 macrophages and Tregs. Trajectory analysis confirmed that CD8⁺ T cells in older mice preferentially differentiate into effector rather than exhausted states. Age-driven shifts in the tumor microenvironment mean older individuals may mount a stronger defense against melanoma, and these inherent differences could also shape their responsiveness to PD-1 and CTLA-4 checkpoint therapies **(C. Zhang et al., 2021)**.

In a recent study published in 2025, researchers found that melanoma patients whose CD8⁺ tumor-infiltrating lymphocytes were made up of more than 20% cells co-expressing PD-1 and CTLA-4 not only responded better to anti-PD-1 therapy but also showed longer survival. Intriguingly, single-cell analysis showed that the PD-1 and CTLA-4 positive cells are heterogeneous. They consist of a small proportion of TCF7⁺ progenitor-exhausted cells alongside proliferating cells, fully exhausted effectors, granzyme-producing cytotoxic cells, tissue-resident memory-like cells and a glycolysis-high subset. Despite comprising diverse exhausted subsets, the overall frequency of PD-1 and CTLA-4 positive TILs reliably predicts patient responsiveness to checkpoint blockade **(Mahuron et al., 2025)**.

2. METHODS

2.1. Experimental Workflow Prior to scRNA Analysis

Wild-type C57BL/6 mice were orthotopically injected in the skin with 3×10^5 B16-OVA melanoma cells and two groups of 6 mice each were constituted. At days 7, 10 and 14, the first group of mice received ICI combination therapy of anti-PD-1 and anti-CTLA-4 antibodies (bithérapie), while the second group was administered with anti-TNF in addition to ICI (trithérapie). At day 17, mice were euthanized and tumors were collected. Following tumor digestion, CD45⁺ cells were isolated and analyzed using the 10x Genomics single-cell RNA sequencing platform with the inclusion of intronic region (Figure 2.1). In particular, the individual barcoding of each mouse in the second stage and the details of how the samples were loaded onto the Chromium 10x device in the fourth stage are important for selecting the correct arguments when using certain bioinformatics packages. The experiments were conducted by Prof. Segui's team.



2.2. Single Cell RNA Analysis

2.2.1 Identification of Low Quality Droplets

The initial step of the analysis involved filtering low-quality droplets, characterized by low *nCount_RNA* (UMIs) and *nFeature_RNA* (detected genes), indicative of a lack of viable cells. To enhance the accuracy of this classification, the *DropletQC* package was employed.

In the *DropletQC* framework for single-cell RNA-seq analysis, the nuclear fraction is the key metric for classifying droplets as empty, containing damaged cells, or containing intact cells. It is defined as the ratio of intronic (unspliced, nuclear-derived) RNA fragments to the total RNA content (intronic + exonic). Empty droplets, which lack intact cells, typically contain ambient RNA released from lysed cells. This RNA is predominantly mature, cytoplasmic, resulting in both low UMI counts and low nuclear fraction scores. Damaged cells arise from partial cell lysis, often due to mechanical stress during sample preparation. These droplets retain nuclear RNA while losing much of their cytoplasmic content, yielding moderate UMI counts and elevated nuclear fraction scores. In contrast, intact cells preserve both nuclear and cytoplasmic RNA, displaying higher total RNA content and intermediate nuclear fraction values indicative of a balanced transcriptomic profile (**Muskovic & Powell, 2021**).

Each sample pool was analyzed independently. BAM files containing intronic and exonic regions were used to compute the nuclear fraction for each droplet. As a prerequisite to identify damaged cells, empty droplets were first detected and removed. The density plot (Figure 2.2 b) showed no clear bimodality, suggesting a minimal presence of empty droplets—as a result of prior CellRanger filtering, which excludes low-UMI droplets.

However, due to *DropletQC*'s tendency to overestimate empty droplets *via* automated thresholding, *barcodeRanks()* function from *DropletUtils* package was applied to the raw data for more accurate empty droplet classification (**Lun et al., 2019**). Droplets were considered empty if their nuclear fraction was below 0.05 and their UMI count fell below the inflection point of the barcode rank plot (Figure 2.2 a).

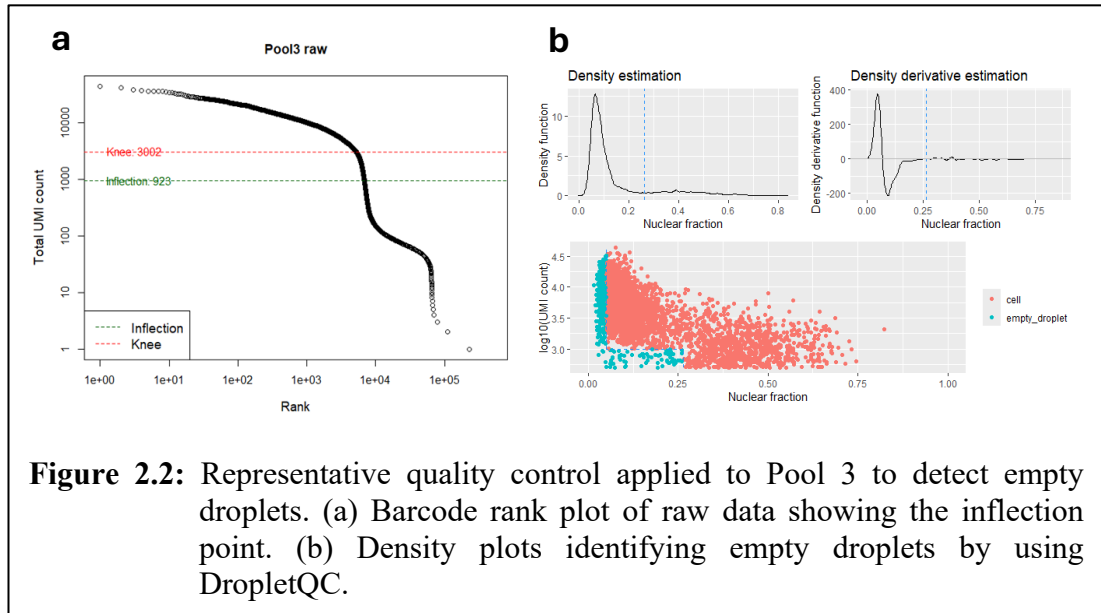
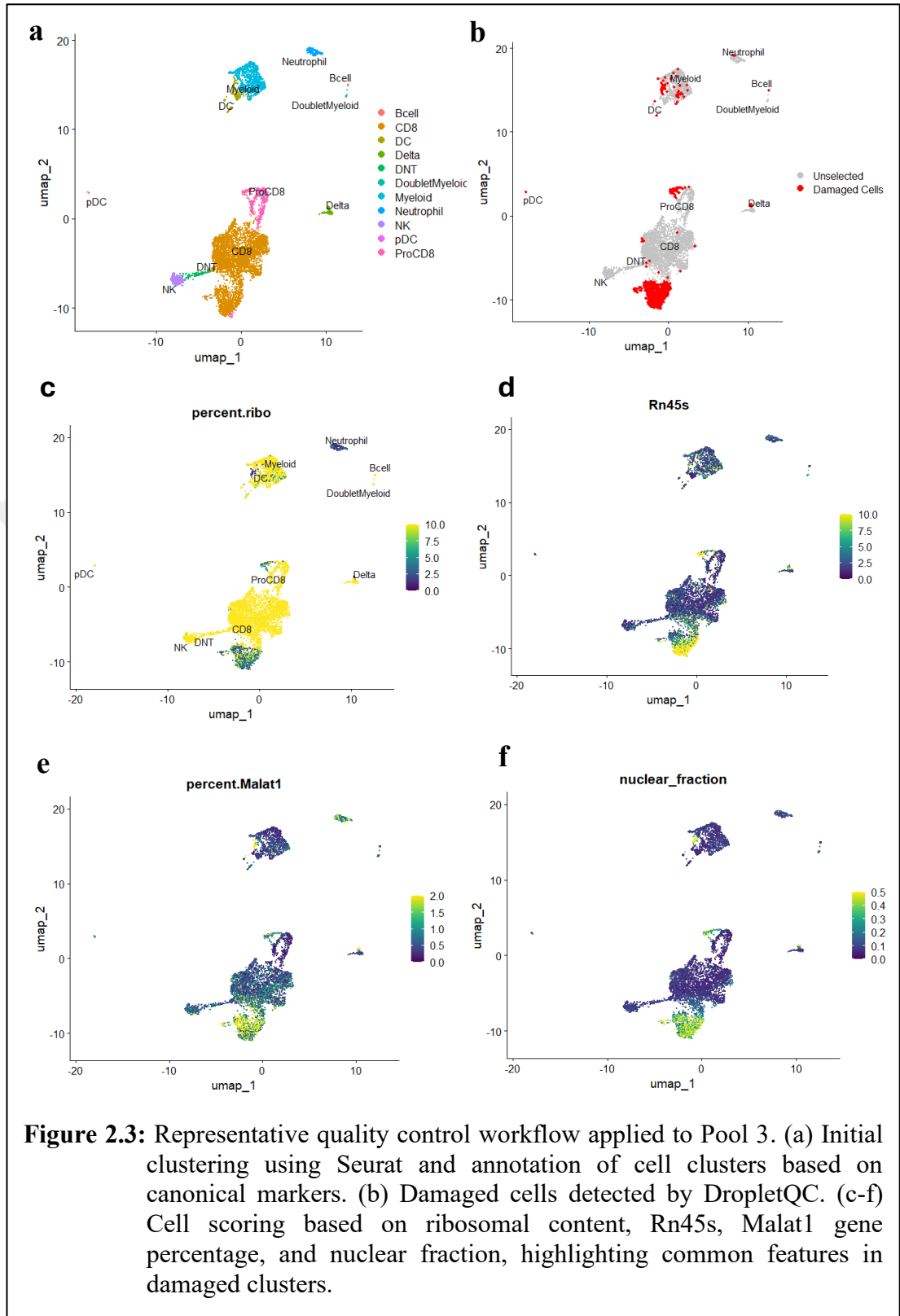


Figure 2.2: Representative quality control applied to Pool 3 to detect empty droplets. (a) Barcode rank plot of raw data showing the inflection point. (b) Density plots identifying empty droplets by using DropletQC.

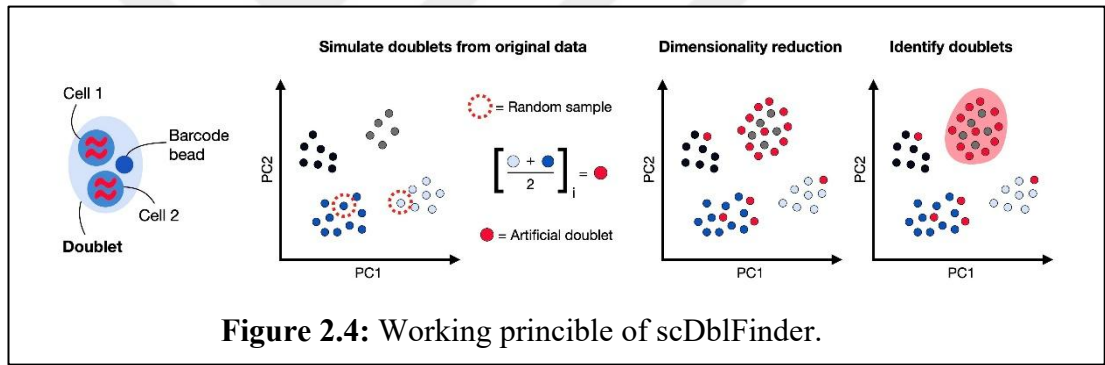
Since nuclear fractions vary by cell type, identifying damaged cells also required basic cell type annotation. Instead of using scGate, which assigns labels based on expression and gating, the standard Seurat workflow was used, and clusters from the resulting UMAP (Figure 2.3 a) were annotated using canonical markers.

Following the cell type annotation, *DropletQC* was used to identify damaged cells for each cell type (Figure 2.3 b). The identification suggested that tissue dissection or other technical factors may have introduced low-quality droplets into the dataset for each cell type. Damaged cells were characterized by elevated nuclear fraction scores, low ribosomal gene content (Figure 2.3 c), high *Rn45s* expression (Figure 2.3 d), and increased *Malat1* expression (Figure 2.3 e). Ribosomal scores were computed using Seurat's *PercentageFeatureSet* function with genes starting with "Rpl" and "Rps". For *Rn45s* content calculation, the genes *Gm42418* and *AY0361181*, known indicators of ribosomal RNA contamination and technical noise, were used. Marker identification via Seurat::FindAllMarkers on damaged clusters also revealed an abundance of long noncoding RNAs and genes poorly annotated in literature, complicating cell-type assignment for these unknown cell populations. Ultimately, damaged cells and empty droplets were excluded from the dataset, and the same quality control pipeline was applied across all sample pools.



2.2.2 Identification of Doublets

A doublet is found in the dataset when two or more cells are mistakenly encapsulated together, producing a hybrid transcriptomic profile. Doublets formed from different cell types or states are called heterotypic doublets. Their identification is crucial as these artifacts can distort downstream analyses by mimicking novel or intermediate cell types. On the other hand, homotypic doublets are formed by cells of the same type, these doublets are hard to detect but less problematic. Doublets can be identified either through their abnormally high number of detected features and read counts, or by computational approaches that generate synthetic doublets and compare them to the observed cell profiles. Among such methods, the *scDblFinder* package has demonstrated superior performance in benchmark evaluations (Germain et al., 2022; Xi & Li, 2021).



Working principle of *scDblFinder* is briefly explained in the Figure 2.4. It works by generating artificial doublets from real cell profiles by combining gene expression profiles of different droplets, mimicking what real doublets might look like. Then, it analyzes the structure of the data using a method called k-nearest neighbors (kNN), which helps find patterns in how cells are related based on their gene expression. Using these patterns, it trains a machine learning model to distinguish real cells from artificial doublets. Doublet detection algorithm assigns a doublet score to each droplet showing how likely it is to be a doublet based on its proximity to artificially created doublet cluster within a k-nearest neighbor (kNN). A final label (doublet or singlet) is assigned to each cell based on this (Germain et al., 2022).

scDblFinder was applied independently to each sample pool prior to downstream quality control procedures, such as the exclusion of cells with high mitochondrial gene

content. The `mice_no` variable was specified as the sample argument, reflecting the experimental design depicted in Figure 2.1, where individual barcodes were assigned to each mouse during the second step of library preparation. Consequently, inter-sample doublets—droplets containing cells from multiple mice—were effectively filtered out during the demultiplexing step performed by CellRanger, which removed droplets carrying multiple mouse-specific barcodes. As a result, `scDblFinder` was expected to detect only intra-sample (within-mouse) doublets.

The `multiSampleMode` parameter was set to `"singleModelSplitThres"`, indicating that the doublet classifier was trained using the full set of cells within each pool, while the threshold for doublet classification was computed separately for each individual mouse. Notably, varying this parameter had minimal impact on the overall doublet detection results. Additionally, the argument `removeUnidentifiable = TRUE` was enabled to prioritize the detection of heterotypic doublets—those composed of transcriptionally distinct cell types. The selected parameters were determined by the experimental setup illustrated in Figure 2.1. Ultimately, doublets detected by `scDblFinder` removed before downstream analysis for each pool separately.

Table 2.1: Possible doublet profiles in pool 1 given as example

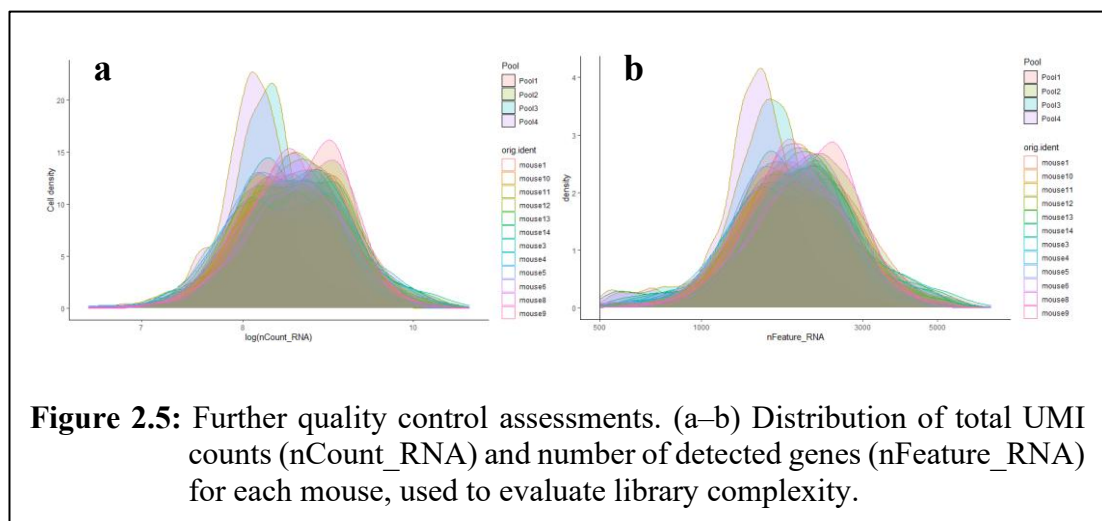
Potential doublet profile	Explanation
mouse1_Tcell + mouse2_Tcell	These cells must have been removed during previous demultiplexing step, as each mouse has its own hashtag.
mouse1_Tcell + mouse2_macrophage	The same applies to the first point.
mouse1_Tcell + mouse1_Tcell	These are difficult to detect. Doublet detection methods are generally ineffective for homotypic doublets, as they primarily target heterotypic doublets, which are considered more problematic.
mouse1_Tcell + mouse1_macrophage	Although these doublets are heterotypic, they could not have been filtered out in previous steps because the droplets involved share the same mouse barcode. However, they can be detected using doublet detection methods.

2.2.3 Further Quality Control Assessments

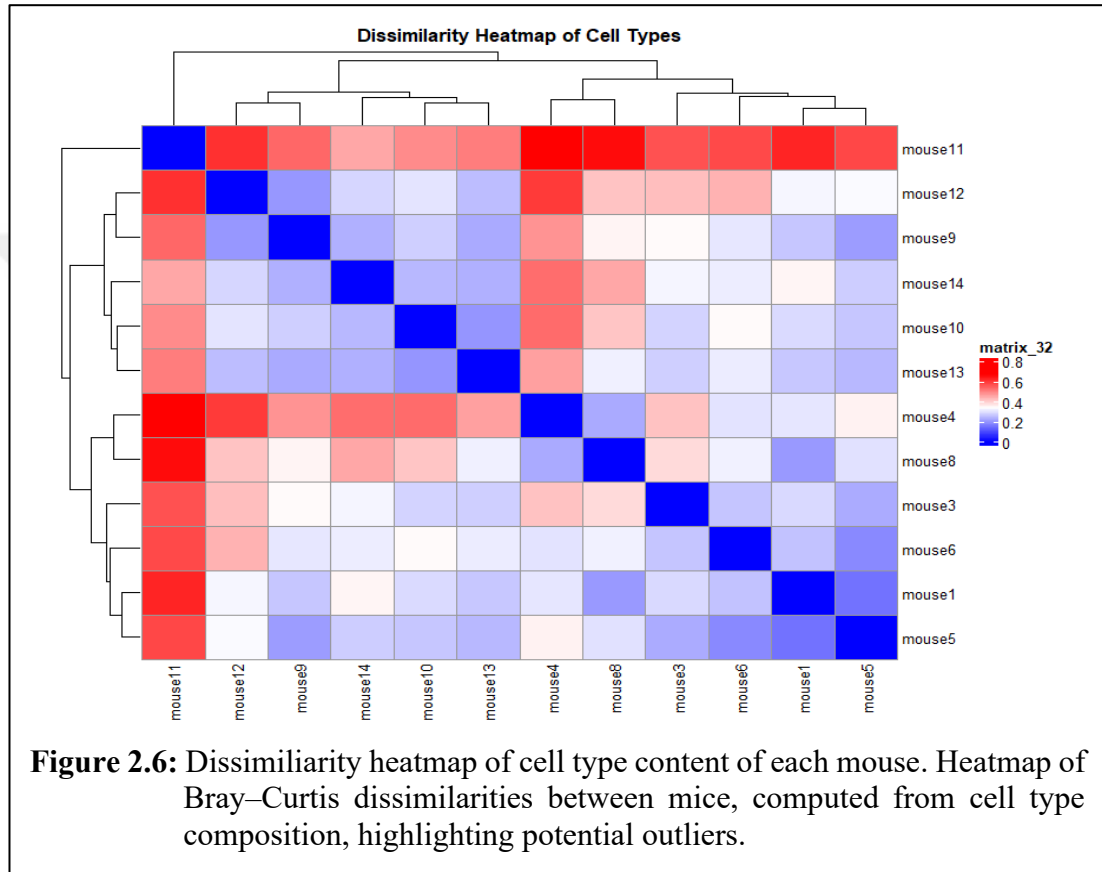
In single-cell RNA sequencing (scRNA-seq), it is common to filter out cells that show unusually high levels of mitochondrial gene expression, since this often signals that the cell is stressed or cell death is triggered. When a cell begins to break down, whether due to stress, programmed cell death, or damage, its outer membrane can lose integrity, allowing most of its RNA to leak out. However, mitochondrial RNA, enclosed by the mitochondria's double membrane, exhibits increased stability and persists for longer durations. As a result, these stressed or dying cells show an unusually high proportion of mitochondrial transcripts, making this a useful signal for identifying and removing low-quality cells from the analysis (Osorio & Cai, 2021). Droplets containing more than 10% mitochondrial gene expression (identified by the patterns "^mt-", "^Rmrp", or "^Mrp") were removed from the dataset.

Although the percentage of ribosomal gene expression is another metric often used to filter out low-quality cells, it was not applied here, as ribosomal RNA levels can vary significantly between cell types—for example, neutrophils naturally have low ribosomal RNA content.

Additionally, as shown in Figure 2.5, the distribution of nCount_RNA and nFeature_RNA was examined for each mouse. Unlike the other samples, cells from mouse 11 did not display two distinct peaks, suggesting the absence of two different lineages—and the cellular diversity observed in other mice was not present.



The dissimilarity of cell type composition for each individual was calculated using the `vegdist(method = "bray")` function from the `vegan` package, and it was observed that the cell type distribution of mouse 11 was markedly different from that of the other mice (Figure 2.6). This analysis was done on merged samples of all mice and after clustering and annotation in another attempt of analysis of all data apart from the workflow mentioned in this thesis.



Although mouse 11 appeared to be an outlier, it was retained in the dataset considering the possibility that it might still hold biological relevance.

Finally, only droplets with fewer than 40,000 *nCount_RNA* (UMIs) were retained to eliminate potential homotypic doublets. The minimum number of unique genes (*nFeature_RNA*) was set to 500.

2.2.4 Clustering

Clustering is an essential method that groups individual cells according to their gene expression profiles. The underlying assumption is that cells with similar transcriptomic

signatures likely represent similar cell types. Prior to clustering, data from four individually filtered cell pools were merged, and clustering was performed using the Seurat R package. Genes expressed in fewer than three cells were excluded in the merged data to remove lowly expressed genes. For the initial clustering, standard preprocessing was performed with *NormalizeData()*, *FindVariableFeatures()*, *ScaleData()*, *RunPCA()*, *FindNeighbors()* and *FindClusters()* using default settings. Importance and explanations of these functions are explained in Table 2.2. UMAP visualization (Figure 2.7 a) showed 19 clusters with no treatment-specific grouping, indicating no batch effect, likely due to consistent lab conditions and equipment, hence no data integration method was applied. Cluster cell-type assignments in Figure 2.7.b were made according to the gene list provided in Table 2.3

Table 2.2 : Explanations of Seurat functions (Butler et al., 2018).

Function	Purpose	Descriptions
<i>NormalizeData()</i>	Normalize gene expression	Accounts for differences in sequencing depth and RNA content across cells.
<i>FindVariableFeatures()</i>	Identification of highly variable genes	Detects genes with high variance across cells and selects informative genes most likely to capture biological signal.
<i>ScaleData()</i>	Standardization of expression values	Ensures all genes are on a comparable scale, a prerequisite for Principal Component Analysis (PCA)
<i>RunPCA()</i>	Dimensionality reduction using PCA	Applies PCA on scaled, variable genes to capture the major sources of variation in fewer dimensions, simplifies the data structure, reduces noise.
<i>FindNeighbors()</i>	Construct similarity graph	Builds a k-nearest neighbor graph in PCA space to represent relationships between similar cells.
<i>FindClusters()</i>	Identify cell clusters	Applies a community detection algorithm (e.g., Louvain or Leiden) on the neighbor graph to group cells with similar expression profiles.

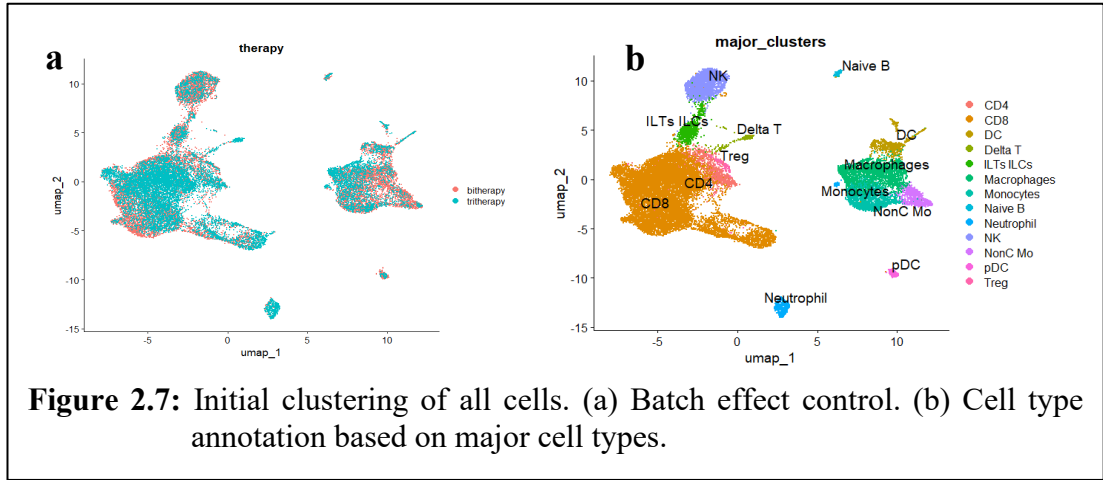


Figure 2.7: Initial clustering of all cells. (a) Batch effect control. (b) Cell type annotation based on major cell types.

Table 2.3: Markers used for cell type annotation collected from literature

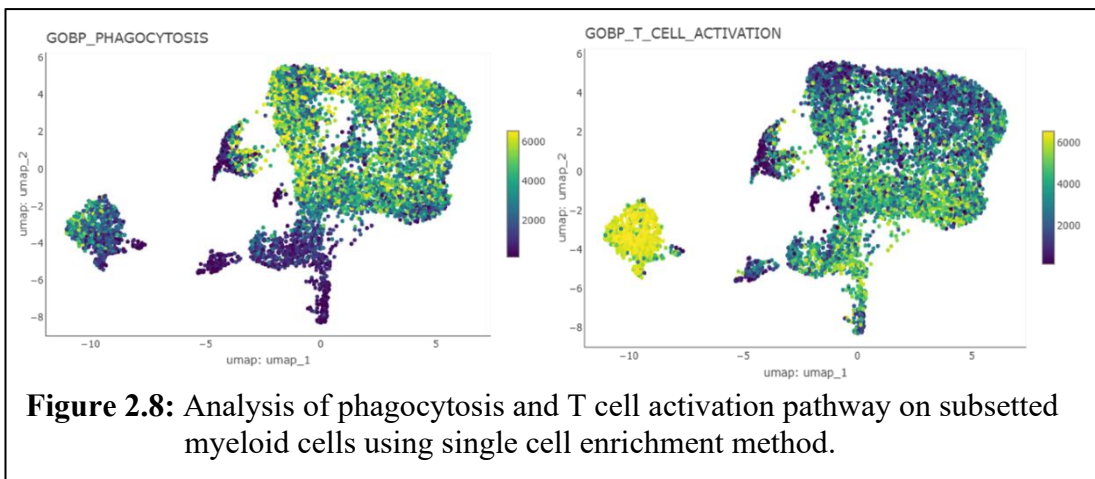
Cell Name	Positive Genes	Negative Genes
mregDC (mature dendritic cell)	Ccr7, Cxcl16, Fcsl1, Cd74, Itgax, Cacnb3, Il4i1, Tmem123, H2-Aa, H2-Eb1, H2-Ab1	Fcgr1, Itgam
cDC1	Clec9a, Cd209a, Cst3, Xcr1, Flt3, H2-Aa, H2-Eb1, H2-Ab1	Ccr7, Fcgr1, Itgam, Itgax
cDC2	Cd209a, Clec10a, Itgax(low), Sirpa(low), Mgl2, H2-Aa, H2-Eb1, H2-Ab1	Ccr7, Itgae
pDC	Siglech, Bst2, Ly6d, Kik1, Cox6a2, Flt3	
Non-classical monocyte	Trem14, Spn, Adgre4, Gngt2, Nr4a1, Itgal, Cx3cr1 (high compared to classical monocyte)	Ly6c2(low), Ccr2, Itgax(low)
Monocyte/Monocyte-derived macrophages	Lyz2, Ly6c2, Ccr2, F13a1, Fn1, Hp, Sell,	Spn
Macrophage	Adgre1, Mcl1, Cd163, Mmp genes, Arg1, Nos2, Mafk, Fcgr1, Msr1, Apoe, Lpl, Csf1r, Tgfbi, C1qc, C1qb, C1qa	
Neutrophil	G0s2, S100a8, S100a9, Clec4d	
Naive B cell	Cd79a, Ly6d, Cd19, Ebf1, Ms4a1	Cd27
Th17 Delta T	Cd3, Trdc, Terg-C1, Cd163l1, Il17a, Icos, Rora, Rorc, Il23r	Cd8a, Cd4, Ncr1
Double=Negative T cell / MAIT / Natural killer T cell / Innate like T cell	Cd3, Tcf7, Cd7, Xcl, Il17r, Mr1, Cd1d, Ikzf2, Ribosomal genes (very high)	Cd8a, Cd4, Klrk1c / Klrk1b / Klrk1 (low), Trbc1/Trbc2(low)
Proliferating T cells	Pclaf, Top2a, Mki67, Birc5	
Innate like cells	Ncr1, Klrk1c, Tbx21, Gata3	Cd3e, Cd3g, Cd3d, Cd5, Cd19, Ter119, Eomes(low), Itga2(low)
Natural Killer	Gzma, Klrk1c, Prf1, Ncr1, Klrk1, Eomes	
Keratinocytes	Krt15, Col17a1, Krt2, Krt5, Krt17	
Fibroblasts	Col1a2, Col1a1, Col3a1, Col6a1, Col6a2, Col6a3	
Endothelial	Selp, Sele, Cdh5, Cd34, Cd93	

When markers for keratinocytes, fibroblasts, endothelial cells, and melanoma cells were examined, no contamination was observed in the data.

2.2.5 Subclustering

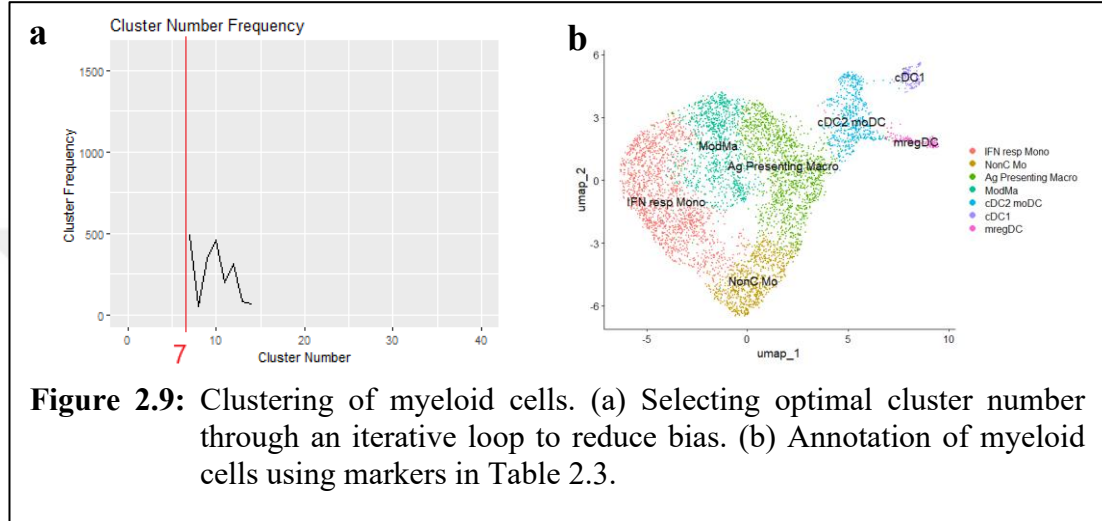
2.2.5.1 Subclustering of Myeloid Cells

Clusters were assigned based on marker genes to primary immune types: CD8, CD4, proliferating CD8, NK, innate-like T cells, pDCs, myeloid cells (macrophages, monocytes, dendritic cells), B cells, and neutrophils (Figure 2.7 b). Myeloid clusters were subsetted without using gating strategy by selecting myeloid clusters directly and subsetting them. Selected clusters re-clustered using default Seurat parameters starting from construction of new Seurat object from count matrix. This revealed a doublet cluster expressing both T cell and myeloid markers, undetected by *scDblFinder*. To interpret it, pathway enrichment was assessed using the *VISION* package, which scores individual cells based on gene expression (DeTomaso et al., 2019). Given that macrophage phagocytosis of T cells can produce such profiles, the phagocytosis pathway was evaluated using gene sets from the *Mus musculus* GO Biological Process section of MSigDB.



In one of the myeloid clusters, elevated T cell activation signatures were observed in the absence of phagocytosis pathway activity (Figure 2.8). This atypical expression profile suggested the presence of a technical doublet that evaded detection by standard doublet identification methods; therefore, the cluster was excluded from downstream

analyses. Therefore, clustering step was repeated. Optimal parameters were identified through an iterative loop testing combinations of PCA dimensions (10–30), k (34–41), resolution (0.4–1), and prune.SNN (0.06–0.1). PCA range was guided by Seurat's ElbowPlot() and JackStraw plots. After 2016 of iterations, 7 clusters were identified as optimal and most frequent, with 10 clusters as a secondary possibility (Figure 2.9.a). This method was intended to minimize analyst-driven bias (Schneider et al., 2021).



For the annotation of myeloid cells, each cluster was compared to all other myeloid cells and highly and significantly expressed genes are detected. For this purpose, two different approaches were utilized. In Figure 2.10, the pseudobulk EdgeR method is used, and the top 7 genes are shown in the plot. The genes were selected by ordering the p-values of each gene, calculated using the Quasi-likelihood F-test, in increasing order (Chen et al., 2020). In the second approach, Figure 2.11, FindAllmarkers() function from Seurat package using Wilcoxon test with default parameters and top 10 genes with $\text{avg_log2FC} > 1$ criteria selected for visualization in the heatmap. Final annotation of myeloid cells made according to the dot plot in Figure 2.12 and markers in Table 2.3.

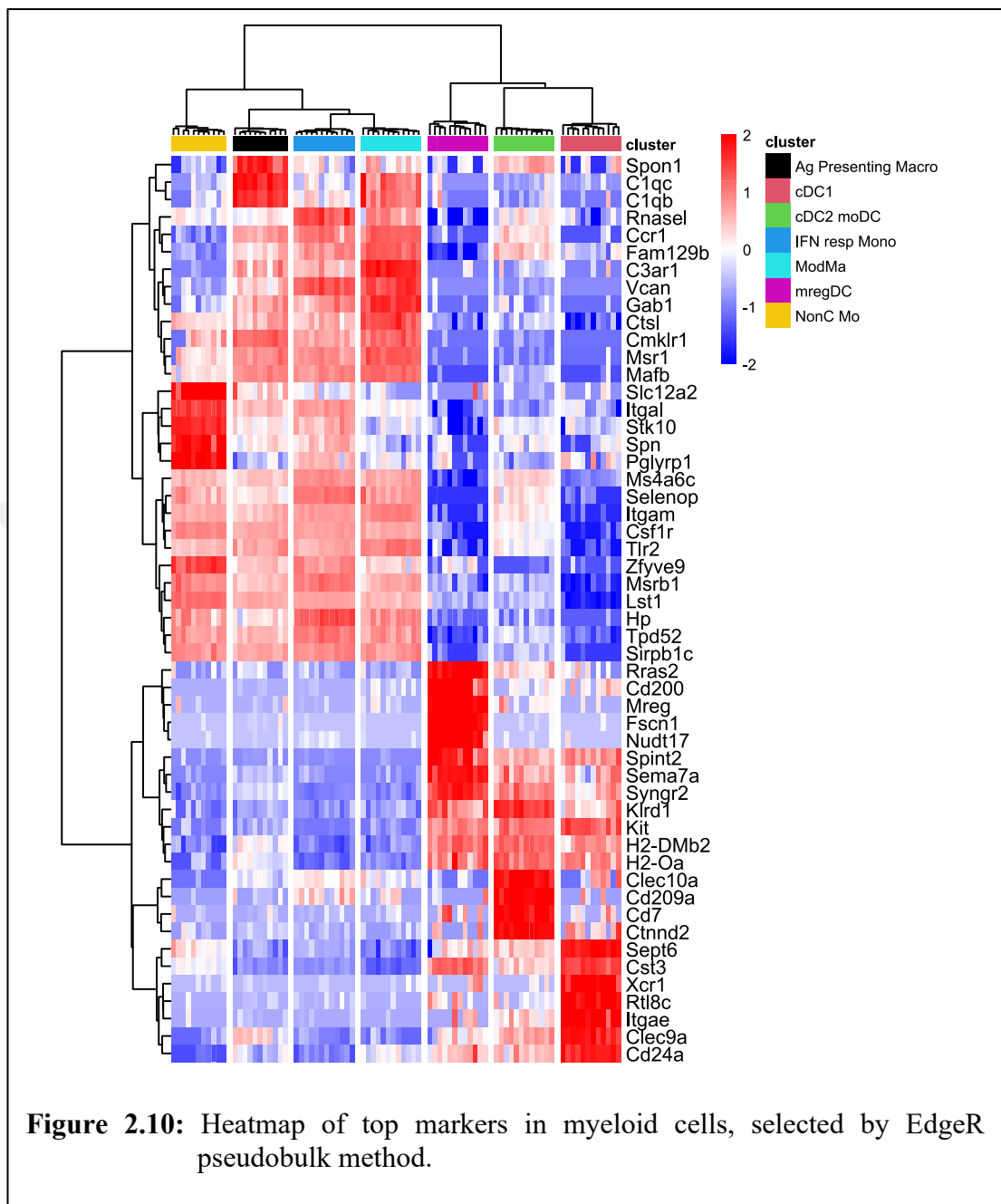
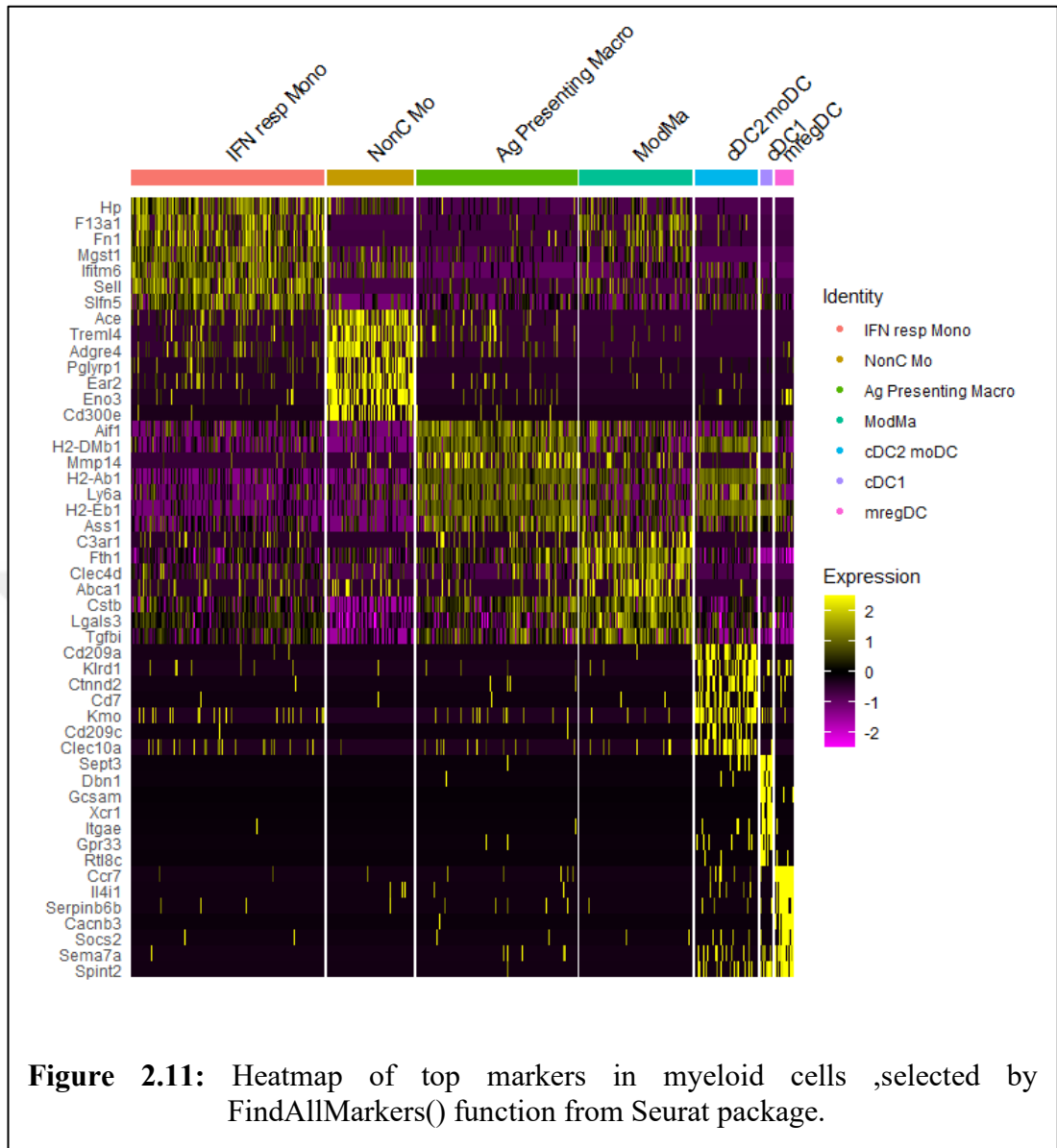


Figure 2.10: Heatmap of top markers in myeloid cells, selected by EdgeR pseudobulk method.



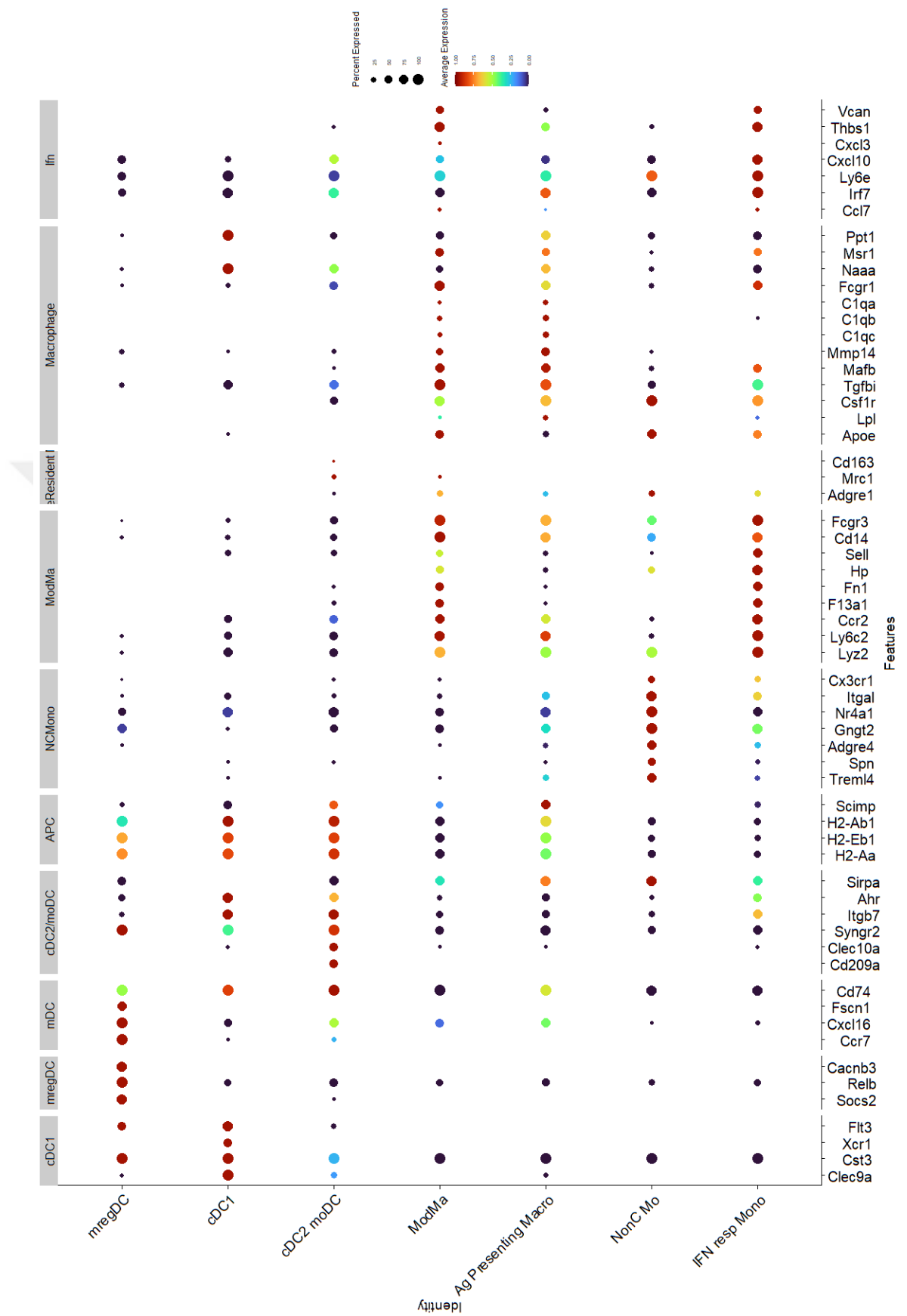


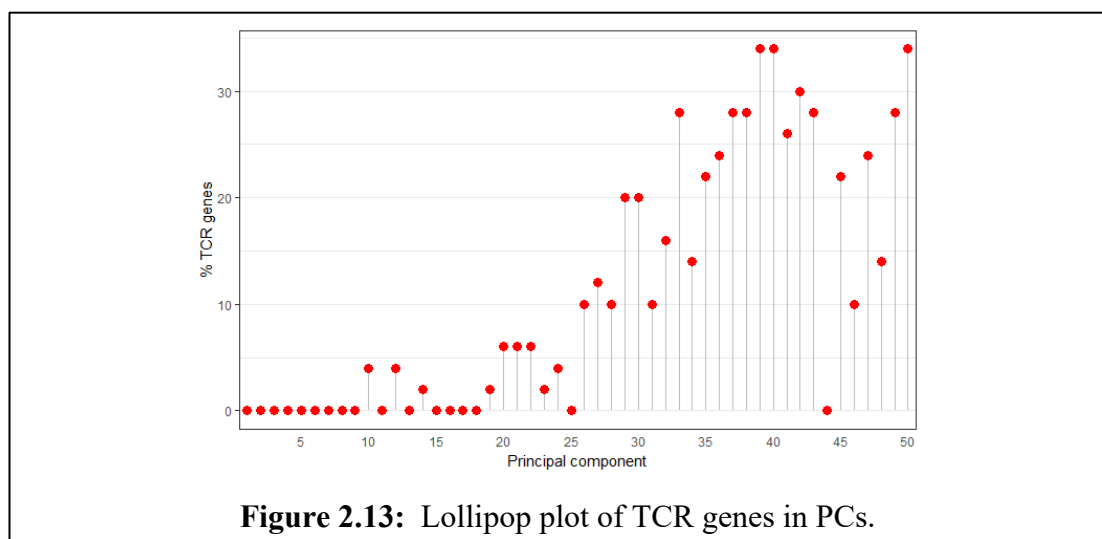
Figure 2.12: Dotplot of markers used for annotation of myeloid cells.

2.2.5.2 Subclustering of CD4 and CD8 Cells

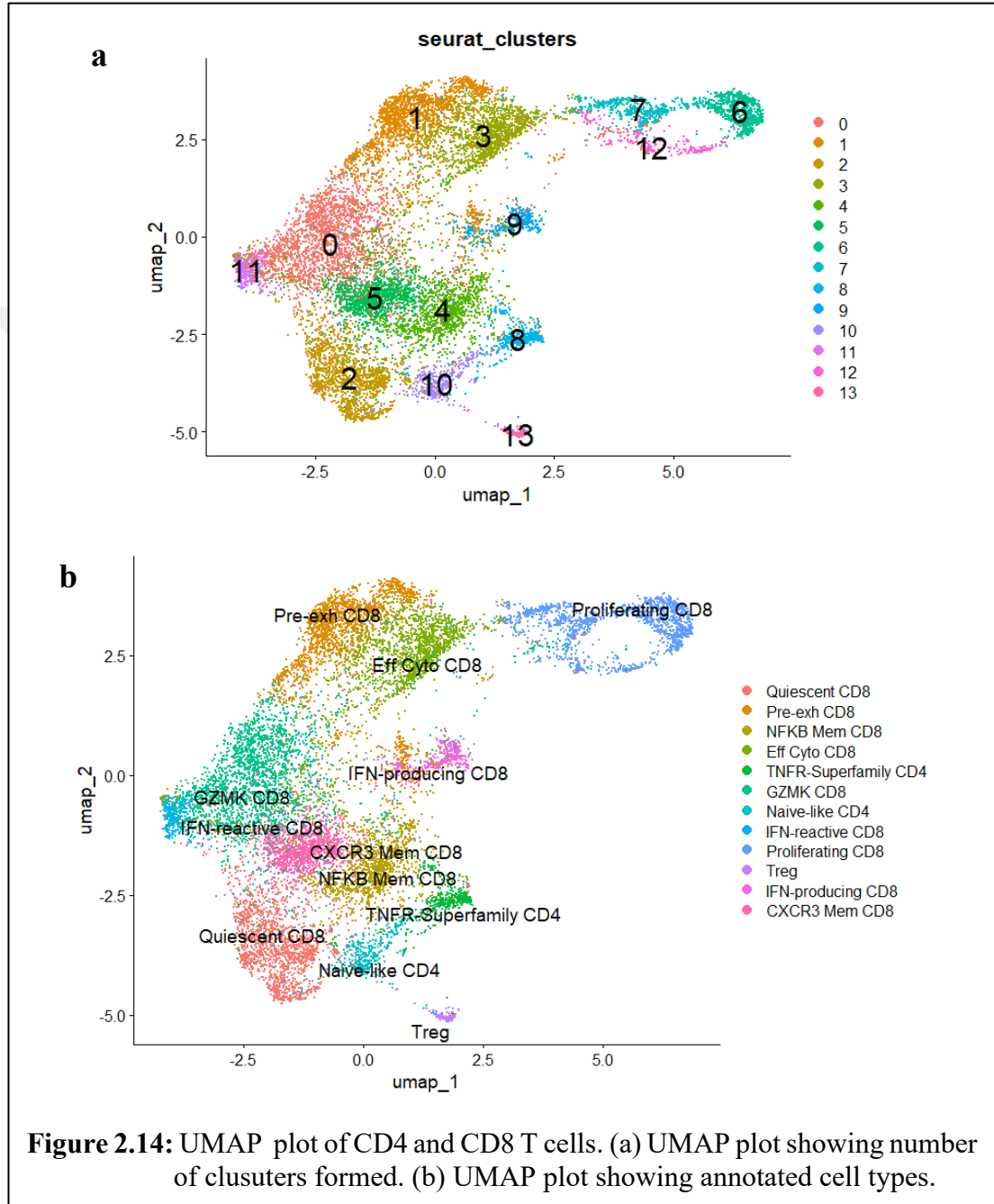
CD8 and CD4 T cell clusters were reclustered to find the subsets of cells. A new Seurat object was created from the count matrix using `min.cells = 3` and `min.features = 500`. Data was normalized with `NormalizeData()` and variable features identified using `FindVariableFeatures()`.

To avoid TCR gene-driven clustering, *Trav*, *Traj*, *Trbv*, and *Trbj* genes were assessed for their contribution to principal components (Figure 2.13) and excluded from variable features. These TCR genes, which reflect clonal identity rather than cell state, introduce technical noise and drive clustering by clonotype. Removing them ensures that clustering reflects the global transcriptional program of T cells, yielding biologically meaningful results (Sundell et al., 2023). Additionally, a heat shock gene module score was calculated using `AddModuleScore()` with genes starting with *Hspa*, *Hspb*, *Hsp90*, *Hspd*, and *Dnaj*. During data scaling with `ScaleData()`, both the heat shock module score and the nuclear fraction score (calculated as described in method section 2.1.1 using the *DropletQC* package) were regressed out.

A total of 11,201 clustering results were generated by varying dimensions (15–30), `k.param` (20–44), `prune.SNN` (0.06–0.12), and resolution (0.4–1). For each result, the distribution of Mouse 11 cells, known to form a distinct cluster, was evaluated using the maximum cell proportion in any cluster and the Herfindahl-Hirschman Index used to assess distribution of Mouse 11. The formation of distinct CD4 Treg cluster with high percentage expression of *Fox3p* -known marker for Tregs- was also considered while selecting final parameters to get a distinct and pure Treg cluster.

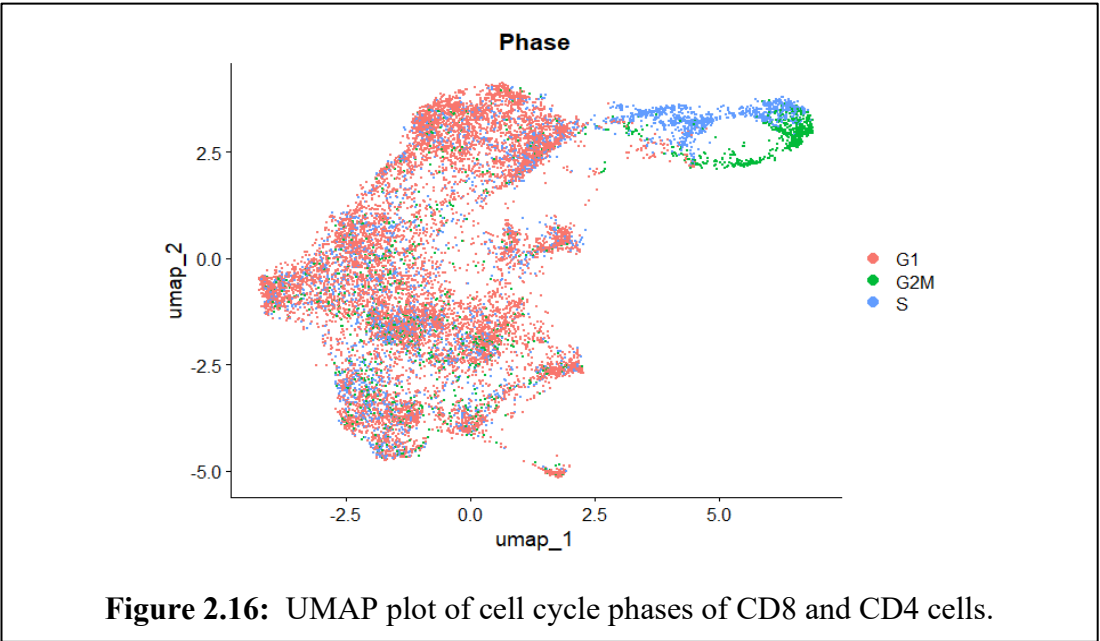
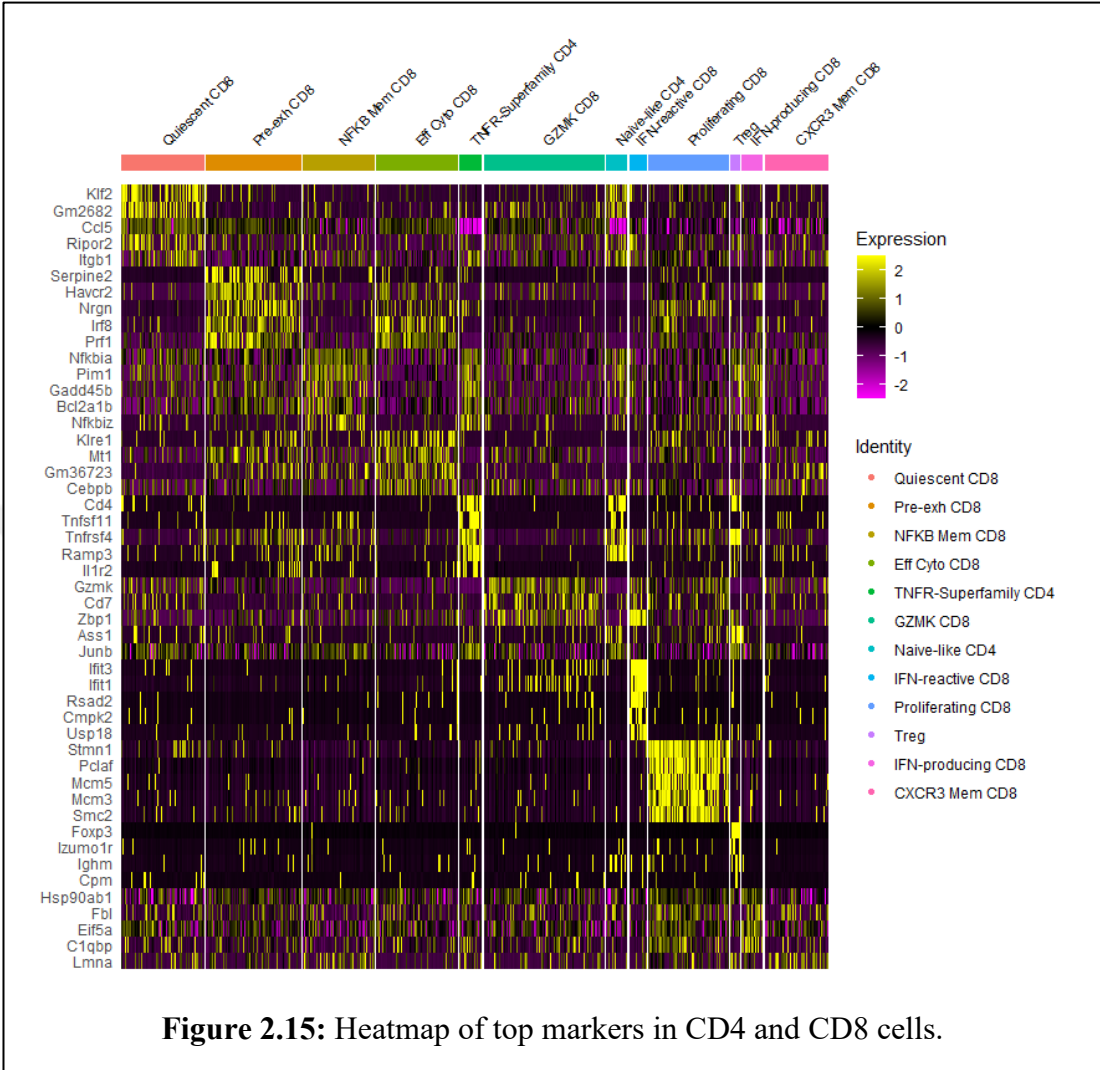


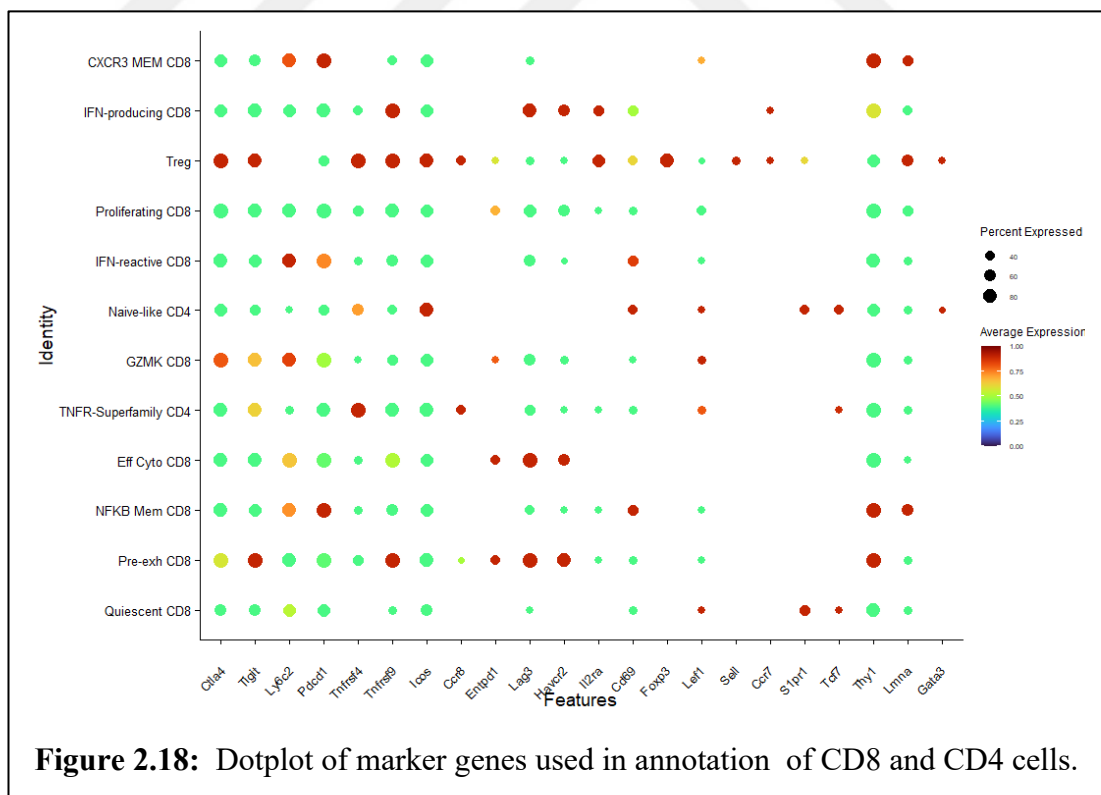
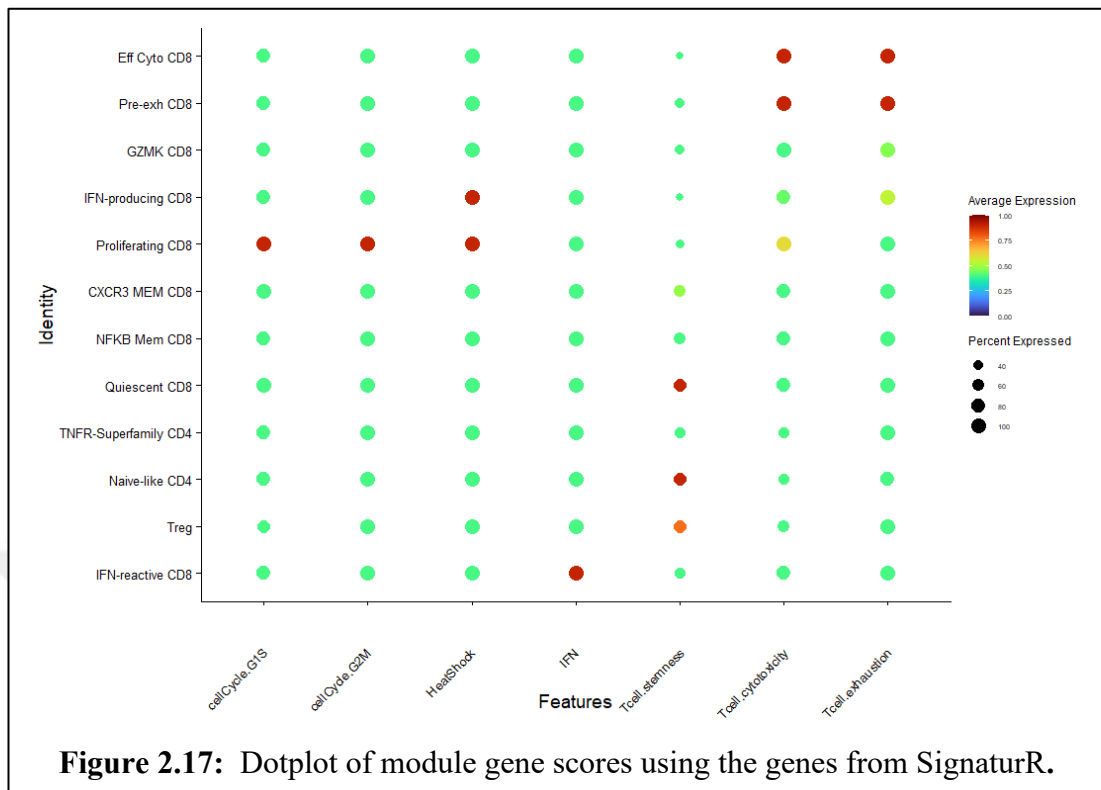
Finally, FindNeighbours() was run with `dims = 1:26`, `nn.method = "rann"`, `k.param = 21`, and `prune.SNN = 0.12`, followed by FindClusters() using `modularity.fxn = 1`, `resolution = 0.6`, and `algorithm = 2`. Optimal number of clusters is identified as 14 clusters as it shown in Figure 2.14.a.

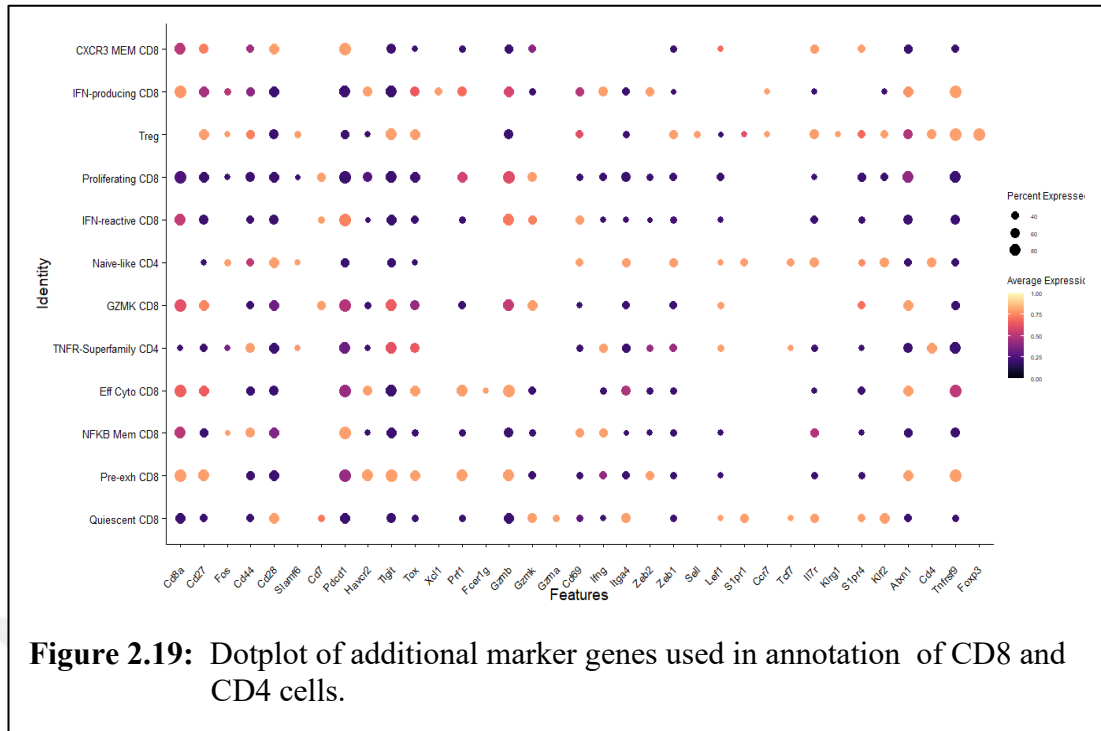


Cell type annotation in Figure 2.14.b was done using highly expressed genes in each clusters detected by FindAllMarkers function (Figure 2.15), cell cycle scoring (Figure 2.16), module gene sets from *SignatuR* package (Figure 2.17), known cell markers

(Figure 2.18 and Figure 2.19) and over-representation analysis by comparing each cluster to the others (Figure 2.20 and Figure 2.21).







Since identifying marker genes alone was not sufficient for annotating subclusters of CD8 cells due to their similar transcriptomic profiles, the functional characteristics of each cluster were also examined. GO pathway enrichment analysis was performed using the highly and significantly expressed genes obtained from the DEG results by comparing each CD8 cluster with the other CD8 clusters at the single-cell level. The identification of these genes was done using the *FindMarkers()* function, with the MAST test. Genes with a log fold change greater than 0.5, expressed in at least 25% of the cells within the cluster ($\text{min.pct} = 0.25$), and with an adjusted p-value less than 0.05 were selected. Prior to DEG analysis, non-protein-coding genes were removed from the dataset using the *AnnotationDbi* and *org.Mm.eg.db* package.

The *compareCluster* function from the *ClusterProfiler* package was used for the enrichment analysis. Reactome and Biological Pathways GMT files were downloaded from the Molecular Signatures Database (MSigDB) and used in the *compareCluster* function with the "fun" parameter set to "enricher". DEGs of each clusters provided as list to the *CompareCluster* function. Terms containing fewer than 15 genes ($\text{minGSSize} = 15$) were excluded from the gene sets. A p-value cutoff of 0.05 was applied, and the Benjamini-Hochberg method was chosen for p-value adjustment (pAdjustMethod). The background gene list included all genes in the DEG list.

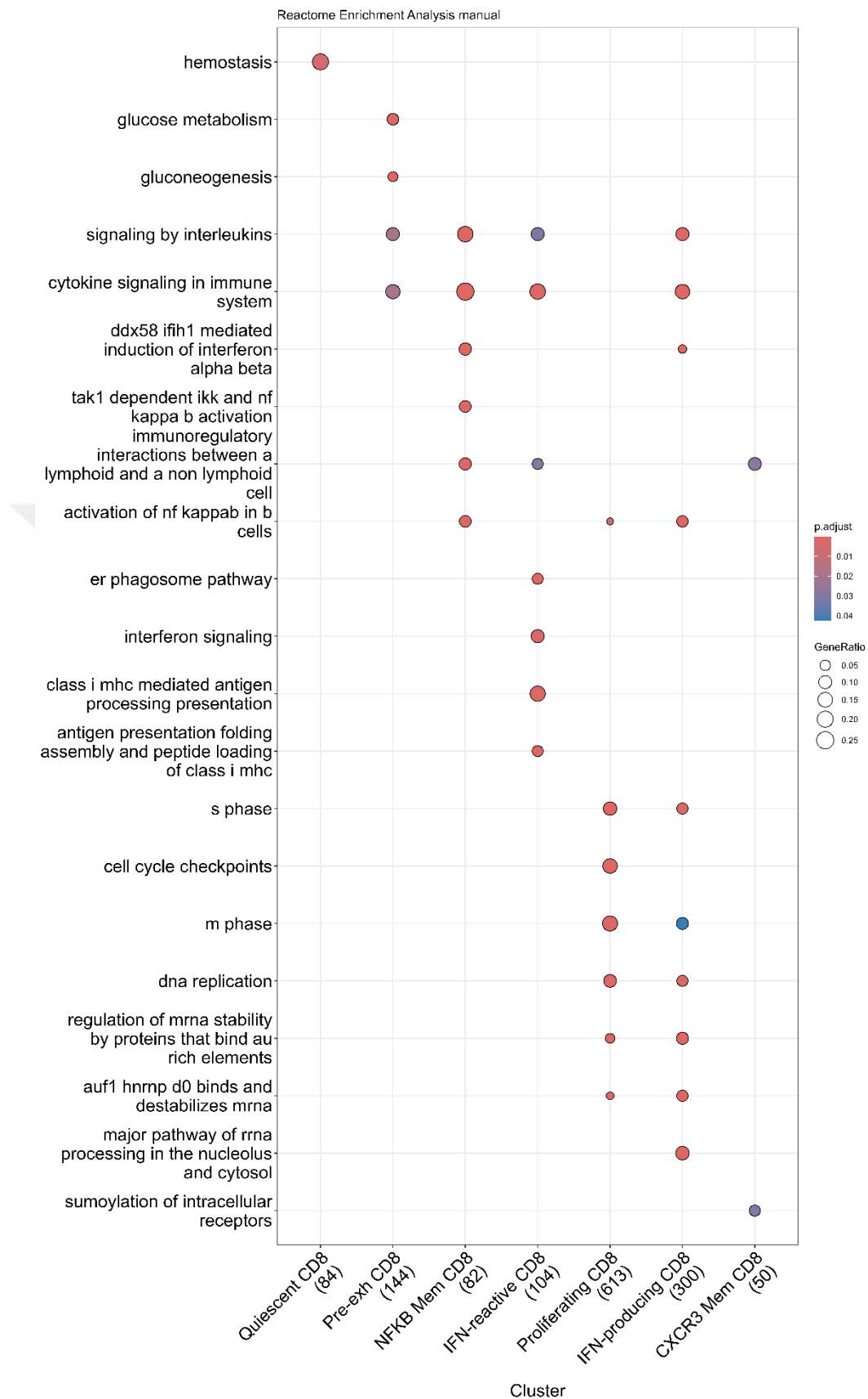


Figure 2.20: Bubble plot of GO Reactome pathways enriched terms for each CD8 clusters.

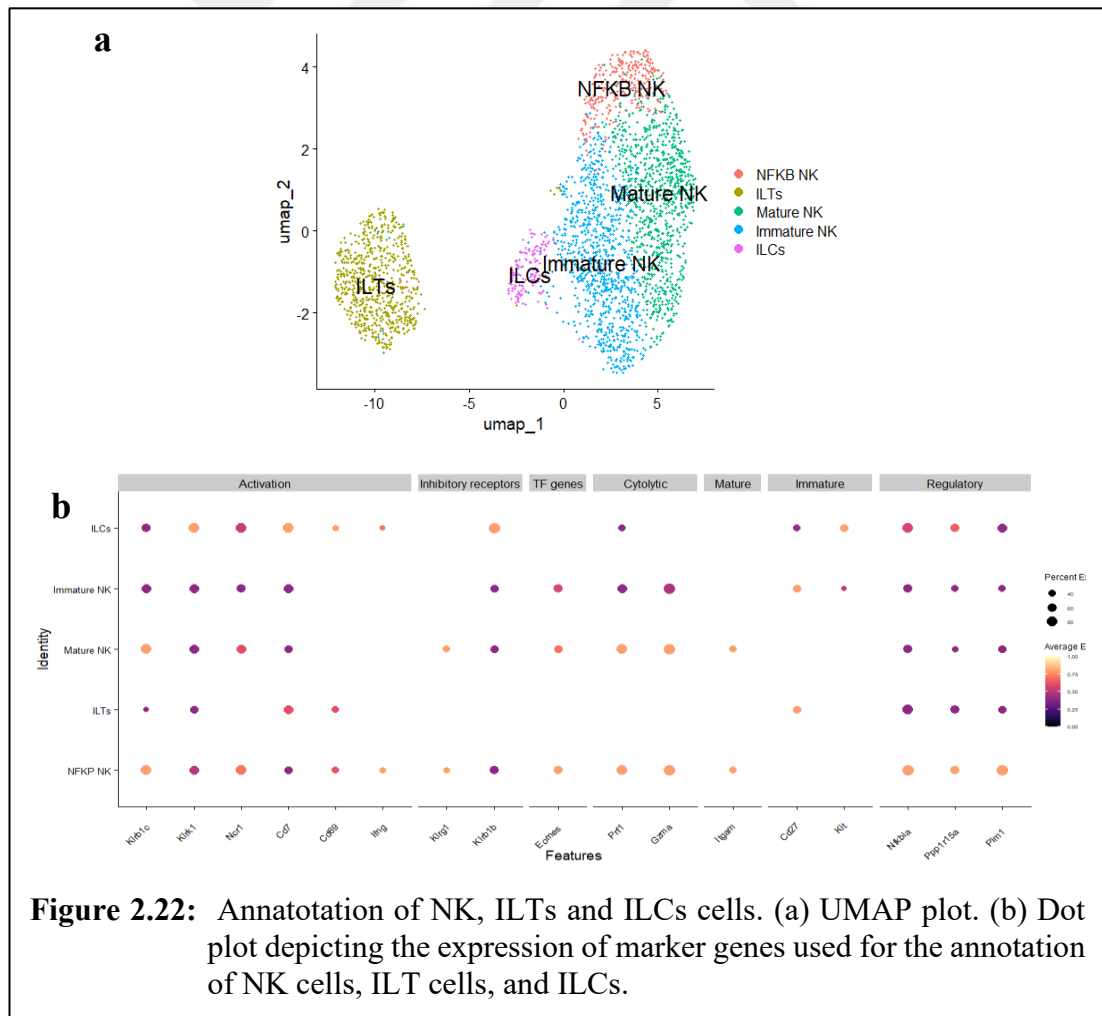


Figure 2.21: Bubble plot of GO Biological pathways enriched terms for each CD8 clusters.

2.2.5.3 Subclustering of NK, ILTs And ILCs Cells

The NK and ILTs/ILCs clusters in Figure 2.7.b were subsetted via subclustering and analyzed in a similar manner to the previous subclustering sections, but without regressing out any covariates. The `FindNeighbours()` function was run with `dims = 1:10`, `nn.method = "rann"`, `k.param = 33`, and `prune.SNN = 0.06`, followed by `FindClusters()` using `modularity.fxn = 1`, `resolution = 0.8`, and `algorithm = 2`. An annotated UMAP plot of these clusters is shown in Figure 2.22.a. Annotations were made based on the genes presented in Figure 2.22.b and Figure 2.23.

For the heatmap presented in Figure 2.23, the `FindAllMarkers()` function was employed using the Wilcoxon rank-sum test to identify the most significant marker genes for each cluster. Genes were selected based on an average log fold change (`avg_log2FC`) greater than 1 and expression in at least 80% of cells within the respective cluster (`pct.1  $\geq$  0.8`).



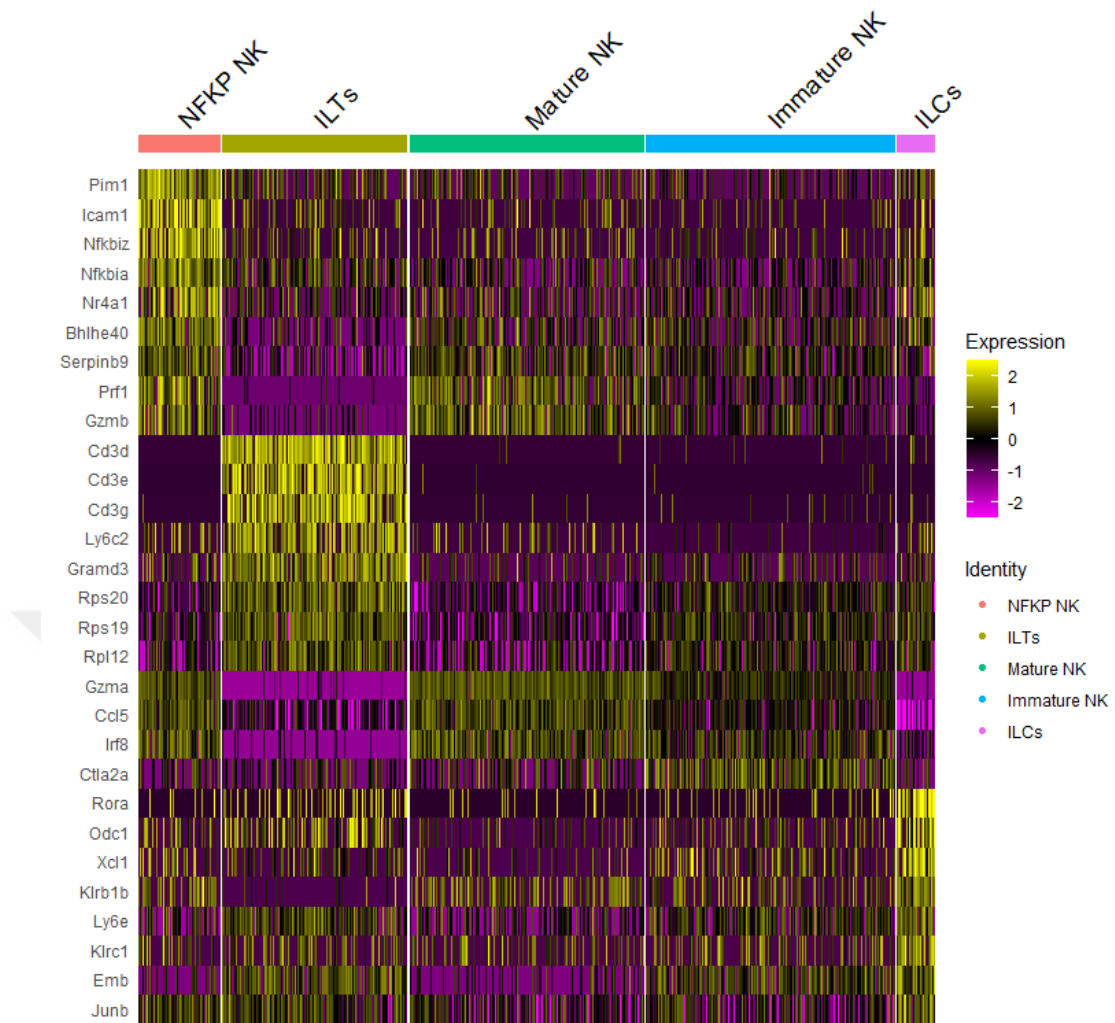


Figure 2.23: Heatmap of top markers in NK,ILTs and ILCs cells.

2.2.6 Methodology of Comparative Analysis Between Therapies

After the annotation of all cell types, several analytical methods were employed to compare differences in therapies.

2.2.6.1 Proportion Test

To determine which cell types exhibit statistically significant increases or decreases in abundance in response to therapy, the Propeller proportion test was applied. Propeller is a cluster-based differential-abundance testing approach from the *Speckle* package that calculates cell-type proportions per sample, applies data transformations (arcsine-square-root or logit) to normalize those proportions, and then performs statistical tests

(t-tests or ANOVAs) within an empirical Bayes framework to enhance power and accuracy—particularly when sample sizes are small or cell types are rare. The method outputs p-values and false discovery rates (FDRs) for each cell type while explicitly accounting for biological replication and the substantial sample-to-sample variability observed in biological data (**Phipson et al., 2022**).

For comparing cell-type abundances between therapeutic groups, *propeller()* function was used with the arcsine transformation on cell-type percentages to mitigate the influence of outlier mice. Here, an “outlier” is any sample in which a given cell-type proportion is unusually high or low relative to the rest of its group and might otherwise disproportionately influence the statistical test. The arcsine-square-root transform reduces the impact of extreme proportions in a gradual manner, ensuring that even if one sample exhibits a particularly aberrant cell-type fraction, it will not dominate the comparison.

2.2.6.2 Pseudobulking and DEG Analysis Within Each Cluster

Differential gene expression (DEG) analysis was conducted within each cluster using pseudobulking strategy, selected for its robust performance (**Squair et al., 2021**).

The “pseudobulk” approach in single-cell RNA-seq begins with an aggregation step in which raw counts for each gene are summed across all cells from a given biological replicate. For example, counts from all Treg cells in mouse 1 in bitherapy are combined into one profile, and counts from all Treg cells in mouse 8 in tritherapy form another. These aggregated profiles mimic bulk RNA-seq samples, enabling the use of differential-expression tools.

Pseudobulking at the replicate level is preferable to directly comparing all cells in one cluster under one condition to all cells in that cluster under another condition. Biological replicates naturally differ because of both technical variation and true biological heterogeneity. By aggregating counts by replicate, pseudobulk methods explicitly capture this between-replicate variance. In contrast, single-cell-specific differential expression methods treat each cell as an independent sample even if they belong to same replicate. When sample-to-sample variance ignored, highly expressed genes are particularly prone to be called differentially expressed even when there is no real difference. Pseudobulk workflows avoid this bias by focusing on differences

across replicates rather than across individual cells. As a result, they produce more reliable DEG lists and reduced false positives. This means that downstream analyses such as pathway enrichment will more accurately capture true treatment effects rather than technical noise (Squair et al., 2021).

Before aggregating gene counts, samples with fewer than 10 cells in a given cluster were excluded to avoid issues related to low library size. Next, the *AggregateExpression()* function from the Seurat package was used to sum counts for each cell type within each individual mouse. No gene-level filtering was applied before DEG analysis; instead, *DESeq2::results()* function was run with *independentFiltering = TRUE* to remove lowly expressed genes automatically during the analysis. Ultimately, differentially expressed genes were identified in each cluster when comparing tritherapy with bitherapy.

2.2.6.3 Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) is a statistical approach aimed at identifying biologically meaningful changes in gene expression by examining predefined groups of genes such as pathways. Unlike traditional analyses that focus on individually significant genes, GSEA considers the entire ranked list of genes without imposing an arbitrary significance cutoff. This allows the method to detect subtle but coordinated shifts in gene expression across entire pathways. Essentially, GSEA asks whether groups of functionally related genes collectively display meaningful changes between biological condition, revealing pathway level insights that might remain hidden if each gene is considered independently (Subramanian et al., 2005).

GSEA was then performed using the Hallmark gene sets from MSigDB. For each cluster, genes were ranked by the score $\text{sign}(\log_2\text{FoldChange}) * -\log_{10}(p\text{value})$. Each ranked list was passed, in decreasing order, to the *fgseaMultilevel()* function from the *fgsea* package. Pathways containing fewer than 10 genes or more than 500 genes were excluded from the analysis.

2.2.6.4 Over Representation Analysis

The *enricher* function in the clusterProfiler package performs a two step Over Representation Analysis (ORA) on a given filtered gene list: First, it compares the

given set of differentially and significantly expressed genes against predefined gene sets such as pathways and counts how many genes overlap. It then applies a hypergeometric test (Fisher's exact test) to determine whether the number of overlapping genes in each category is higher than would be expected by chance (Wu et al., 2021; Yu et al., 2012).

Second, because we are testing many categories at once, it adjusts the resulting p values using a false discovery rate method with Benjamini–Hochberg correction. This yields “adjusted p-values” (or q-values) that control for multiple comparisons, allowing us to identify which biological categories are truly enriched in our gene list rather than false positives.

Using the same DEG results described in Methods Section 2.2.6.2, we performed ORA by selecting only those genes that remained significantly differentially expressed after Benjamini–Hochberg adjustment of the p-values and that met a fold-change threshold of 0.5. Because the DEG analysis was performed using DESeq2 with the independentFiltering function, the resulting DEG list includes genes with missing adjusted p-values. These genes were excluded from the p-value correction step due to their low expression. Therefore, since a background gene list must be provided for the ORA analysis, genes with missing adjusted p-values were removed from the background list. Since this is a comparative analysis between tritherapy and bitherapy, the genes upregulated in bitherapy and those upregulated in tritherapy were each used separately in the analysis.

2.2.6.5 PROGENy Pathway Analysis

PROGENy is an acronym for “Pathway Responsive GENes” and serves as a database of pathway–gene relationships derived from perturbation studies. Rather than providing direct information about genes that activate a pathway, PROGENy has the information of downstream “responsive” genes whose expression changes when a pathway is activated or inhibited. Each target gene within a pathway has an associated weight and sign, indicating the strength and direction of its relationship with the pathway. Consequently, some genes contribute more to the calculation of pathway activity, while others have minimal affect. Additionally, each pathway–target gene pairing is accompanied by a p-value reflecting the reliability of the association, so

interactions with the lowest p-values can be retained for analysis. PROGENy contains information for only 12 pathways. These pathways are: androgen, estrogen, epidermal growth factor receptor (EGFR), hypoxia, JAK–STAT, mitogen-activated protein kinase (MAPK), nuclear factor kappa B (NF- κ B), p53, PI3K, transforming growth factor beta (TGF- β), tumor necrosis factor alpha (TNF- α), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), vascular endothelial growth factor (VEGF) and WNT pathways (Schubert et al., 2018).

The analysis began by retrieving, for each pathway, the top 500 target–pathway interactions with the lowest p-values via the *decoupleR::get_progeny()* function. Pathway scores were then computed from these interactions by fitting a univariate linear model with the *decoupleR* package’s *run_ulm()* function (Badia-i-Mompel et al., 2022). Because the analysis was conducted at the single-cell level, each cell yielded a set of 12 pathway scores. The result yielded a nested structure. For example, Mouse 1 in the bitherapy group has 26 Treg cells, and therefore 26 single-cell scores for each pathway were calculated. Similarly, Mouse 10 in the tritherapy group has 21 Treg cells, resulting in 21 single-cell scores per pathway.

To perform a statistical comparative analysis between therapies for each cell type and pathway, a linear mixed-effects model (LMER) from the *lme4* package is used. In this model, treatments are considered as fixed effects, and individual mice are included as random effects. The choice of this model is based on the following criteria: cell scores from the same mouse are not independent, and the number of cells per mouse is unequal due to the nature of single-cell data. The ‘estimate’ value provided by the model was used to show the difference in pathway scores between tritherapy and bitherapy.

2.2.6.6 Transcription Factor Activity Analysis

Transcription factors (TFs) are proteins that bind specific DNA sequence, usually in a gene’s promoter or enhancer region and TFs act as molecular “switches” that turn genes on or off to regulate the expression of genes. To analyze TF activity in each cell, CollecTRI network is used. CollecTRI (Collection of Transcription Regulation Interactions) is a gene-regulatory-network resource, curated from experiments and the literature. It contains transcription factor–target gene interactions and enables the

accurate estimation of TF activities from transcriptomic data. Each interaction is assigned a sign, +1 for activation and -1 for repression, reflecting its regulatory effect (Müller-Dott et al., 2023).

The CollecTRI network was used with the `run_viper()` function from the decoupleR which utilizes VIPER (Virtual Inference of Protein-activity by Enriched Regulon) algorithm. VIPER is an enrichment algorithm that takes a pre-defined regulation network as its input (Alvarez et al., 2014).

The scores calculated for each cell using the `run_viper()` function yield a nested structure similar to that of PROGENy pathway analysis. For comparative analysis between therapies, LMER is applied, as described in the previous section.

2.2.6.7 Cell-Cell Interaction Analysis

MultiNicheNet is a package that detects and ranks changes in cell–cell signaling across different samples and conditions using single-cell transcriptomic data. MultiNicheNet identifies cell–cell interactions not only based on gene expression profiles but also by modeling ligand activity, which incorporates both molecular interactions and the downstream transcriptional responses they induce in target cells. Unlike conventional tools such as CellChat, which aggregate all cells from a given condition into a single pseudo-sample, MultiNicheNet retains sample-level resolution. Specifically, it constructs pseudobulk profiles for each cell type within each sample and performs differential expression analysis using edgeR. This enables the identification of condition-specific ligand–target interactions by capturing biologically meaningful variation across individual samples (Browaeys et al., 2023).

For the cell–cell interaction analysis, subpopulations of similar cell types were first merged into a single group, as suggested in the MultiNicheNet tutorial. Samples that have less than 10 cells were excluded from the analysis for that specific cell type. For the `multi_nichenet_analysis()` function, the `p_adj_val` argument was set to FALSE due to the low number of samples in each condition. For cell interaction scoring, a specific scenario was selected to prioritize ligand activity. Therefore, the `scenario` argument was set to "*lower_DE*", which reduces the weight of DEGs by half and doubles the weight assigned to ligand activity

2.2.6.8 Immune Cell Recruitment Capacity Analysis of NK Cells

To score the immune cell recruitment capacity of natural killer (NK) cells, NK cells were first aggregated into metacells to enable correlation analysis (Morabito et al., 2023). The gene Ccl5, known for its role in immune cell recruitment, was selected as the anchor gene (Böttcher et al., 2018). Weighted gene co-expression analysis (WGCNA) was applied to identify a co-expression module containing Ccl5. Within this module, genes showing a Pearson correlation greater than 0.7 with Ccl5 were selected, and the top 25 were used to calculate the recruitment scores. The average UMI percentage of these selected genes is calculated for each mouse and Student's t-test was applied to compare therapies.

2.2.6.9 UCell Scoring Using Defined Gene Sets

UCell is a scoring method for single-cell data based on a given list of genes. During the calculation step, for each cell, UCell first ranks all genes by their expression levels. It then asks, “Are my signature genes highly expressed in this cell?” using the Mann–Whitney U test. The result is a single signature score per cell: a higher score means the cell more strongly matches the provided gene list (Andreatta & Carmona, 2021).

Scores were calculated for each cell using the genes in Table 2.4, and the mean score was then computed for each mouse. Wilcoxon rank sum test was subsequently used for statistical comparison.

Table 2.4 : Genes used for UCell cell scoring (Bagaev et al., 2021).

Process	Genes
T cell stemness	Tcf7, Lef1, Sell, Il7r
Co-activation molecules	Cd28,Cd40,Tnfrsf4,Icos, Tnfrsf9,Cd27,Cd80,Cd86,Cd40lg,Cd83,Tnfsf4,Icosl,Tnfsf9, Cd70
T cell exhaustion	Havcr2, Lag3, Pdcd1, Tigit, Ctla4
Effector cells	Ifng, Gzma, Gzmb,Prfl, Gzmk, Zap70, Fasl, Tbx21, Eomes
Effector cell traffic	Cxcl9, Cxcl10, Cx3cl1, Ccl3, Ccl4, Cx3cr1, Ccl5, Cxcr3
Pro-tumor cytokines	Il10, Tgfb1, Tgfb2, Tgfb3, Il22, Mif
Checkpoint proteins	Pdcd1, Cd274, Ctla4, Lag3, Pdcd1lg2, Btla, Havcr2, Tigit, Vsr

Descriptions of the processes in Table 2.4 is given below (**Bagaev et al., 2021**):

- Co-activation molecules: Co-stimulatory molecules deliver essential secondary activation signals when T-cell and B-cell receptors (TCRs and BCRs) engage peptide-MHC complexes on antigen-presenting cells. These signals are critical for driving the proliferation and differentiation of T and B lymphocytes following their initial activation.
- Effector cells: CD8⁺ cytotoxic T lymphocytes and NK cells can identify and directly eliminate tumor cell, a function essential for a robust antitumor immune response. This activity is driven by genes characteristic of effector T and NK cells, which encode cytotoxic effector proteins (such as granzymes, perforin, granulysin, and FAS ligand), key cytokines (notably interferon gamma).
- Effector cell traffic: This process involves genes that produce chemokines and their receptors, which direct effector cells to migrate into the tumor microenvironment.
- Pro-tumor cytokines: The tumor microenvironment is a dynamic community of diverse cell types that “talk” to one another by releasing and sensing cytokine. This process involves genes that produce chemokines and their receptors, which direct effector cells to migrate into the tumor. A pro-tumor immune response occurs when immune signals actually help the tumor grow and spread instead of fighting it.
- Checkpoint proteins: This list involves genes encoding these checkpoint proteins, which can inhibit the antitumor ability of T cells to fight cancer.

2.2.6.10 Immune Response Enrichment Analysis

In diseases or conditions affecting the immune system, identifying the specific cytokines involved is crucial. The Immune Dictionary developed by Cui and colleagues offers a comprehensive resource that maps, for a wide range of cell type–cytokine combinations, the gene expression responses triggered by stimulation with each cytokine (**Cui et al., 2024**).

The database created by Cui and colleagues was downloaded from Supplementary Table 3 of the online version of their paper. This file contains lists of genes responsible

for cytokine responses in each specific cell type. Using this file, enrichment analyses can be performed to assess the overlap between cytokine response genes and differentially expressed genes detected in our analysis, typically using statistical methods such as the hypergeometric test.

Immune response enrichment analysis is specifically applied to a selected cell cluster to narrow the analytical focus, prioritizing clusters most affected by treatment. Initially, TNF α scores calculated via PROGENy analysis are averaged per cell type for each mouse. This analysis results in a dataframe with cell types in rows, individual mouse identifiers in columns, and their respective mean TNF α scores as matrix entries. Using this dataframe, to identify the cell types whose TNF α activity best distinguishes between bitherapy and tritherapy groups, feature selection was performed using a Random Forest algorithm

The T-cell cluster identified as the most important feature by Random Forest analysis for distinguishing between treatment groups was selected for downstream cytokine enrichment analysis. DEGs identified through the pseudobulk approach described in methods section 2.2.6.2 are utilized for cytokine enrichment analysis. The enricher function from the clusterProfiler package is used to perform hypergeometric testing.

2.2.6.11 Projection of Proliferating CD8 Cells

In the Proliferating CD8 cluster, cell cycle genes mask the underlying transcriptional identity; however, this cluster actually consists of multiple distinct CD8 cell types (Giles et al., 2022). To resolve this issue, the Proliferating CD8 cells were processed prior to being projected onto previously annotated non-proliferating CD8 cells.

The proliferating CD8 cells were subsetted, normalized, and scaled. During scaling, cell-cycle-related genes were regressed out after scoring with the CellCycleScoring() function. Cell-cycle genes (S-phase and G2/M-phase) were obtained from the specified reference and combined with the SignaturR package's cell-cycle gene list.

Next, the proliferating CD8⁺ cells were projected onto the UMAP embedding of non-proliferating CD8⁺ cells using Seurat's FindTransferAnchors() and MapQuery() functions. MapQuery() internally calls TransferData(), IntegrateEmbeddings(), and ProjectUMAP(). Based on transcriptional similarity, proliferating cells were mapped to the previously annotated CD8 subpopulations. For FindTransferAnchors(), the

parameters `dims = 1:26`, `k.anchor = 5`, `k.filter = 150`, and `k.score = 30` were selected. Twenty-six dimensions were used to match the clustering of the reference dataset (annotated CD8 cells), and the other parameters were tuned to minimize overcorrection.

2.2.6.12 Tumor Size Comparison

The tumor sizes for each mouse are shown in Table 2.5. Normality was assessed using the Shapiro–Wilk test ($p > 0.05$), indicating a normal distribution. Equality of variances was evaluated with Levene’s test ($p > 0.05$), suggesting equal variances. Therefore, an independent two-sample t-test assuming equal variances (`var.equal = TRUE` in R’s `t.test()` function) was used to compare tumor sizes between the groups.

Table 2.5: Tumor sizes of each mice

Mice hashtag	Therapy	Tumour volume (mm ³)
1	Bitherapy	45.86
3	Bitherapy	9.14
4	Bitherapy	54.8
5	Bitherapy	55.04
2	Bitherapy	22.36
6	Bitherapy	45.86
7	Tritherapy	30.33
8	Tritherapy	26.75
9	Tritherapy	25.48
10	Tritherapy	53.92
11	Tritherapy	33.79
12	Tritherapy	20.55

3. RESULTS

In this section, the results of cell-type proportion analysis, DEGs between the two therapies, and enrichment terms associated with these DEGs identified through ORA are presented.. This is complemented by GSEA, PROGENy-based pathway activity inference, and transcription factor activity analysis, all performed in a comparative manner between tritherapy and bitherapy. Throughout the comparisons, positive values indicate enrichment or upregulation in the tritherapy condition.

Subsequently, immune-relevant gene signatures previously defined in the literature are used to score individual cells, allowing comparison of immune functional states between treatments. Given the critical role of NK cells in immunotherapy, the analysis includes an assessment of which treatment condition possess greater immune recruitment capacity in NK cells. Additionally, the T-cell subset most affected by therapy is identified, along with the cytokines predominantly produced within this population. The section concludes with an evaluation of cell–cell interactions specific to each treatment, highlighting differences in intercellular communication dynamics between bitherapy and tritherapy. Finally, projection analysis was performed to determine the predominant T-cell subtypes contributing to proliferating CD8 T cells.

3.1. Proportion Test

This analysis was performed as described in Section 2.2.6.1 of the Methods. The propeller proportion test analysis, using arcsine transformation,, cDC2-moDC, NK, nonclassical monocytes, naive-like CD4 cells, and IFN-producing CD8 cells in bitherapy samples.

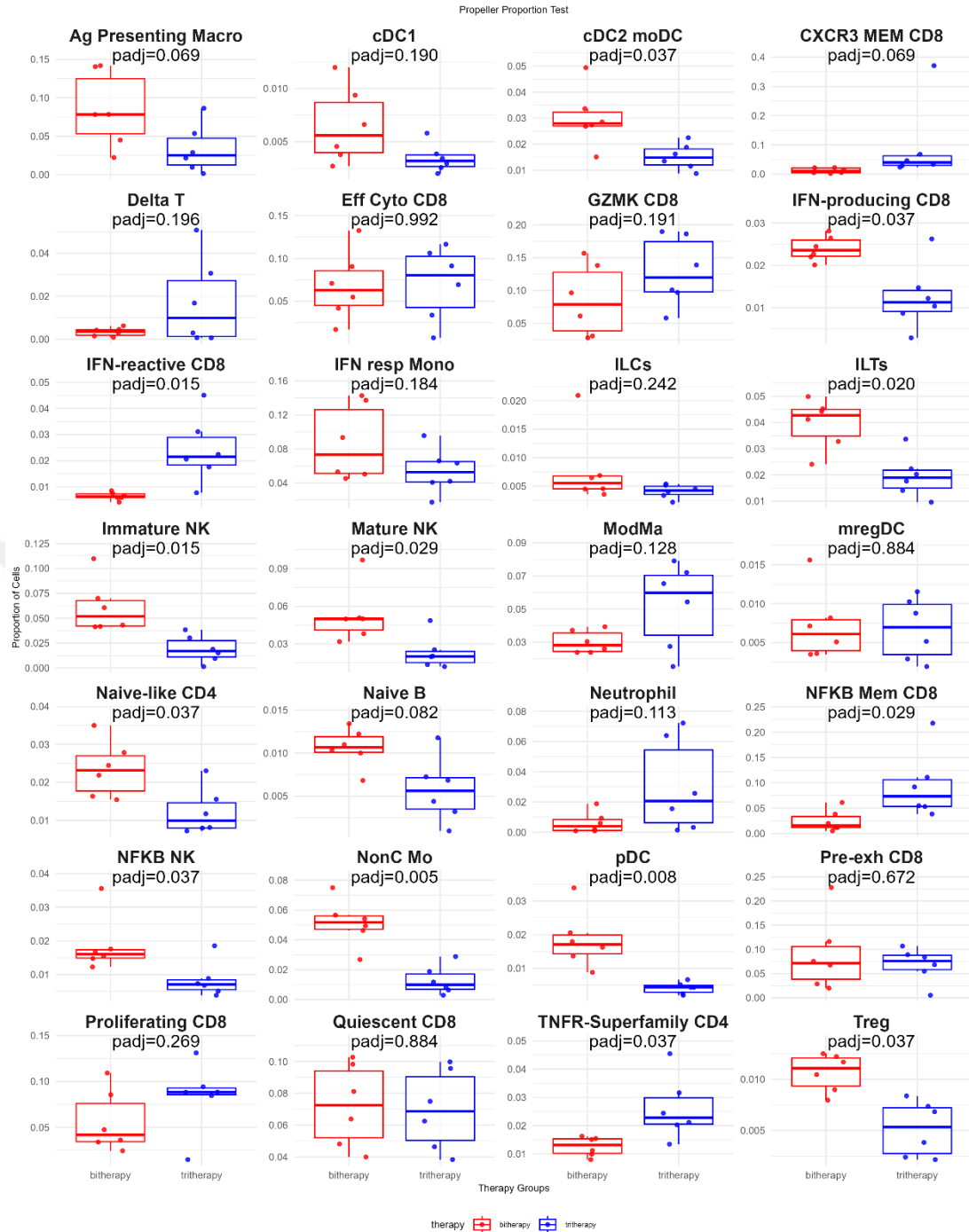


Figure 3.1: Proportion test.

In tritherapy samples, a significant increase was observed in IFN-reactive CD8 cells expressing interferon-stimulated genes, NF- κ B memory CD8 cells, and TNFR-superfamily CD4 cells with a TH1 profile which express Tbx21 and Cd40lg genes.

No significant differences were detected in pre-exhausted CD8 cells expressing and effector genes or in highly cytotoxic Eff-Cyto CD8 cells.

3.2. DEG and ORA Results

DEG analysis using Deseq2, as described in Section 2.2.6.2 of the Methods, was performed at pseudobulk level on the clusters exhibiting statistically significant changes in terms of proportions revealed a relatively low number of DEGs, even in the most significant clusters. Conversely, clusters without statistically significant changes displayed a higher number of DEGs.

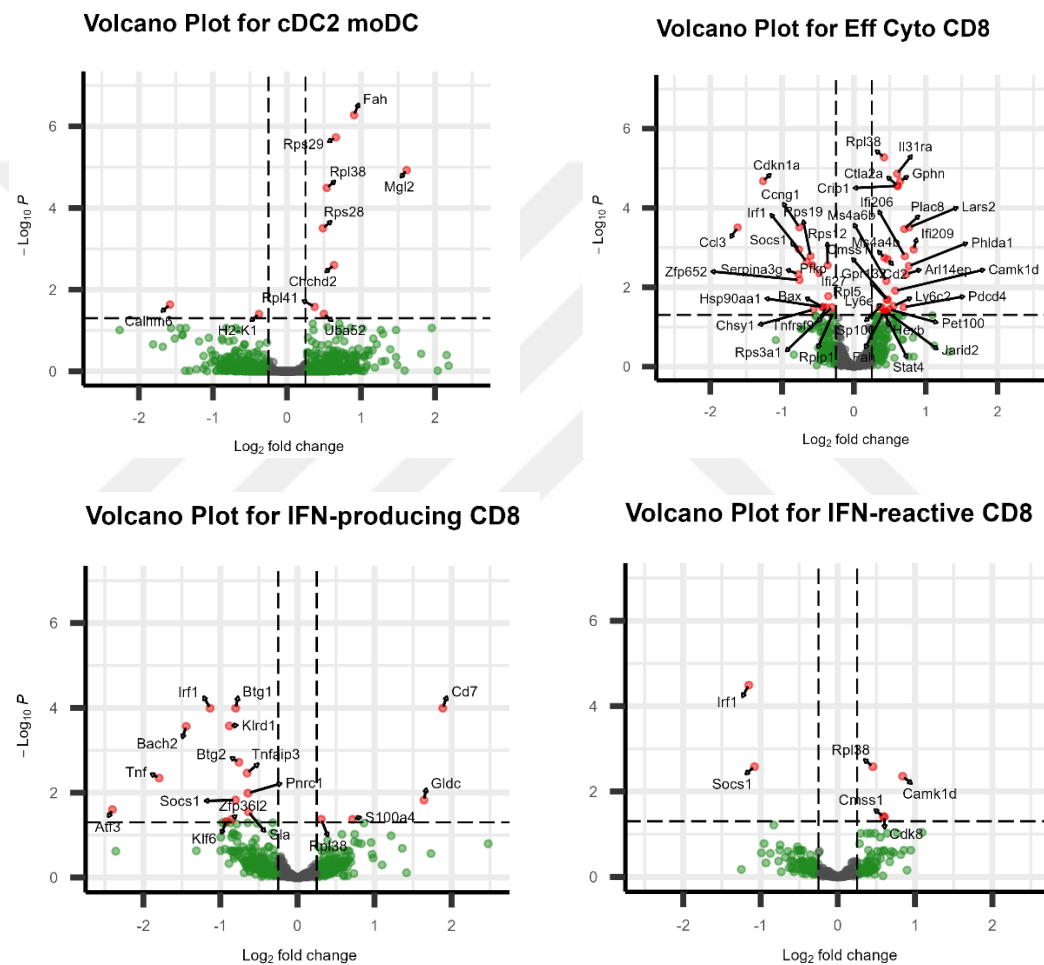


Figure 3.2: Volcano plots of DEGs for proportionally changed clusters.

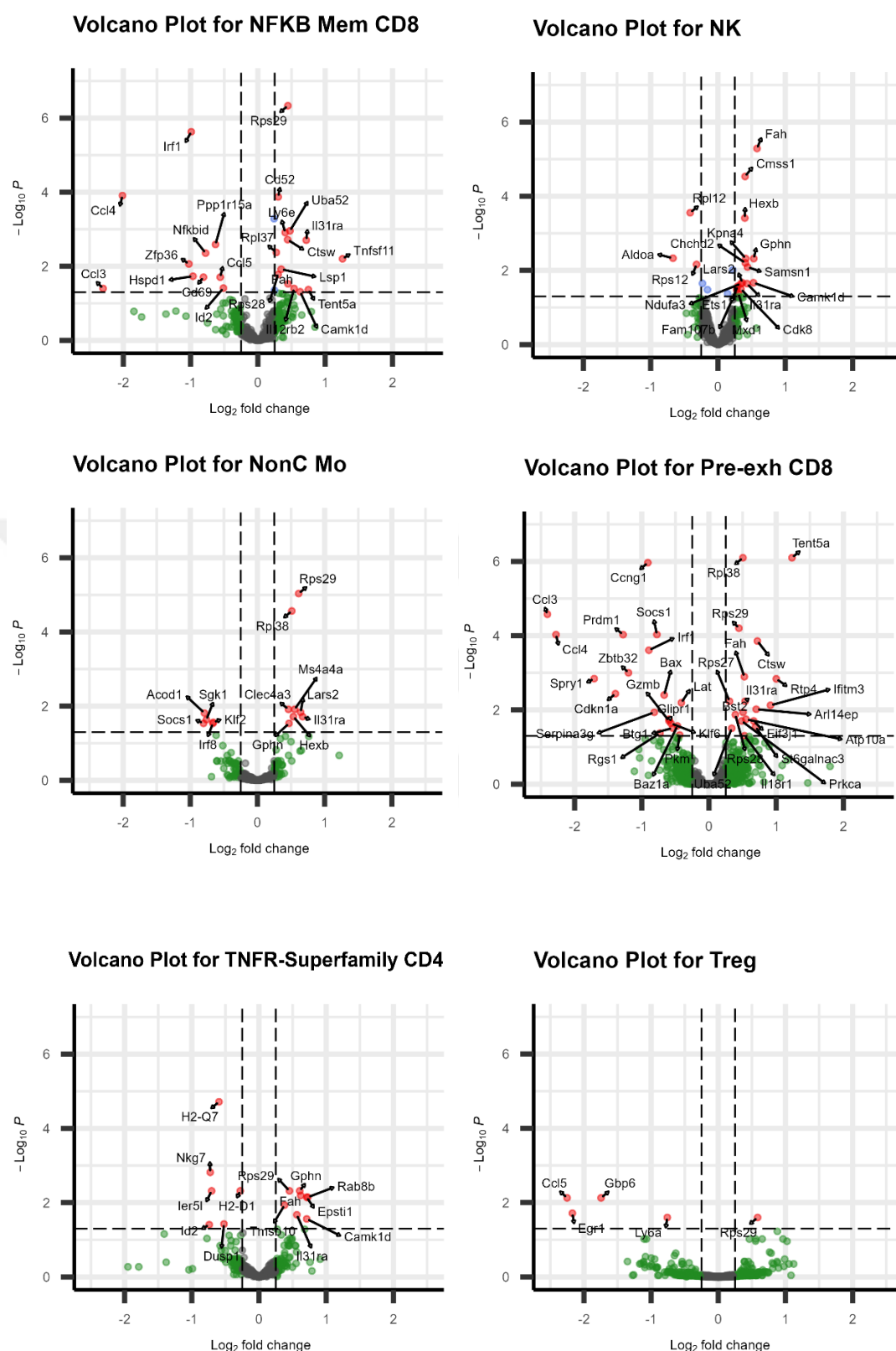


Figure 3.2: Volcano plots of DEGs for proportionally changed clusters.

Over-representation analysis (ORA) was performed on proportionally changed clusters to identify Reactome pathways associated with significantly increased genes

in the Figure 3.2. In the IFN-producing CD8 cluster from bitherapy samples, enrichment was observed in TNF signaling, regulation of TNFR1 signaling, and TNFR1-induced NF- κ B signaling pathways. In Treg cells from bitherapy samples, enriched pathways included signaling by GPCR (Figure 3.3.a).

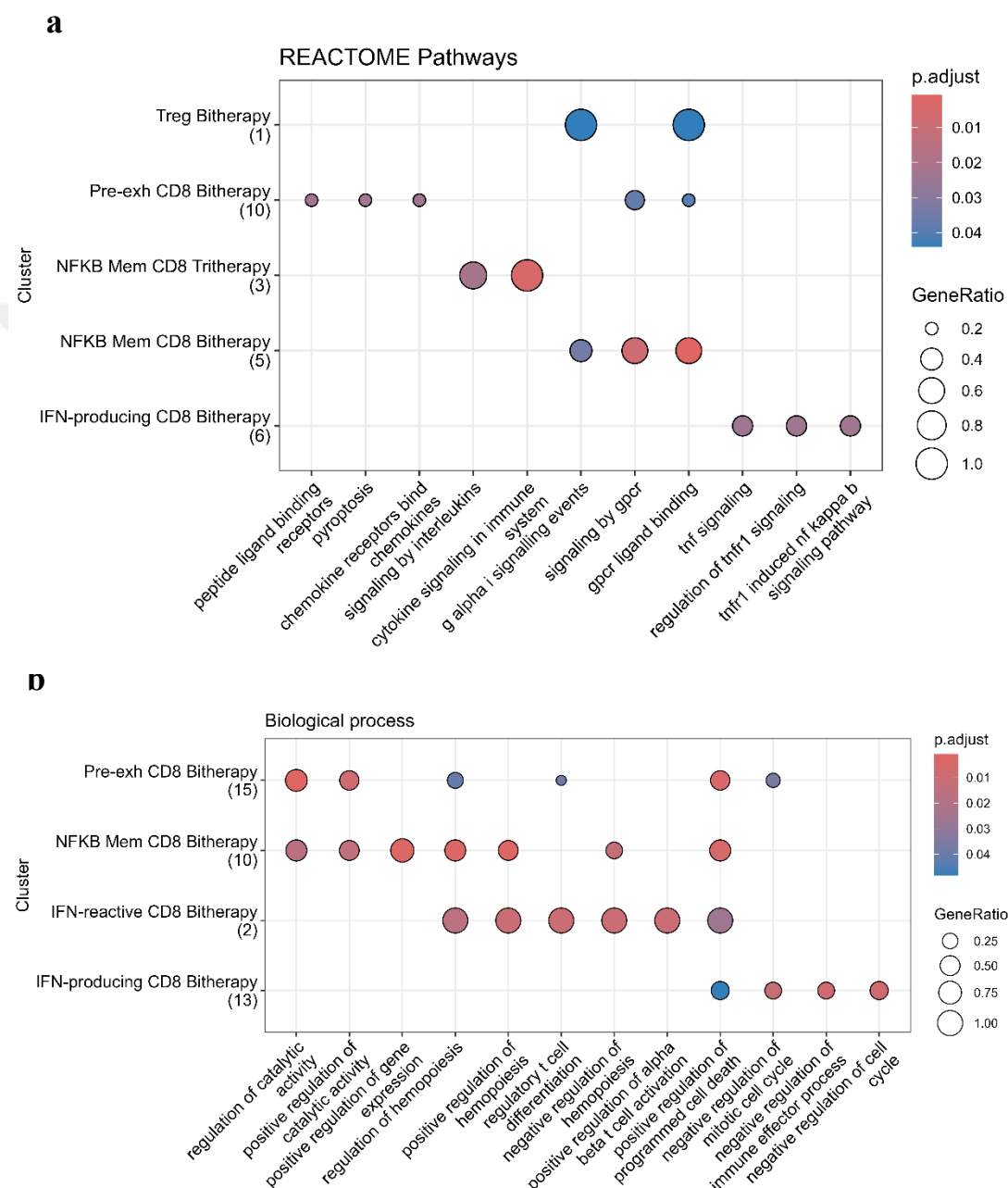


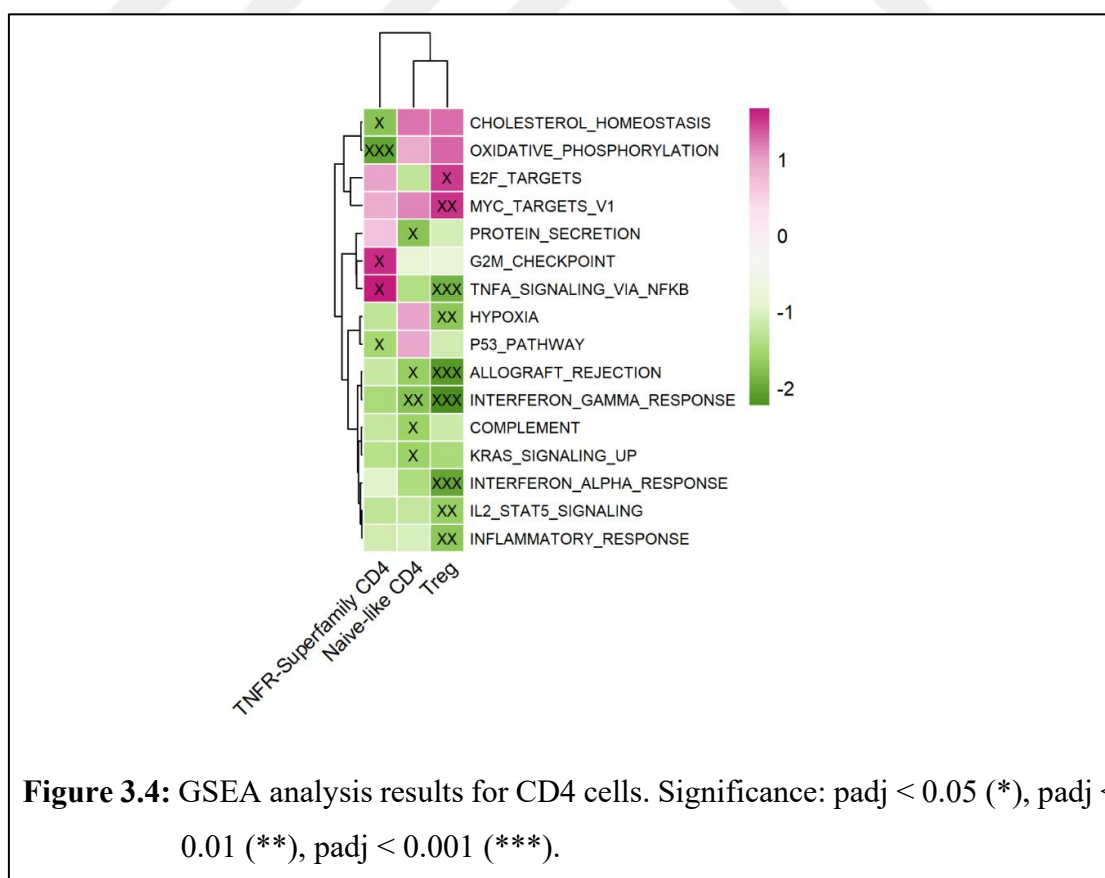
Figure 3.3: ORA analysis results. (a) ORA analysis using Reactome pathways database. (b) ORA analysis using Biological Pathways database.

Enrichment analysis in IFN-producing CD8 cells bitherapy samples revealed enrichment in pathways related to *the positive regulation of programmed cell death* and *the negative regulation of the cell cycle* (Figure 3.3.b)

In contrast, biological pathway or Reactome analyses did not identify any enriched pathways for natural killer and non-classical monocyte clusters. This suggests that proportional changes in cell populations are not always accompanied by transcriptional changes in the cells when considering only significantly altered genes.

3.3. GSEA Analysis

GSEA analysis revealed that among CD4 subsets, Treg cells appeared to be the most affected by 'TNF α signaling via NF-K β ' pathway (Figure 3.4). Several activity related pathways like IFN alpha and gamma response, inflammatory response and IL2-STAT5 pathway is enriched in bitherapy Tregs cells, showing that Tregs in bitherapy samples might be more immunosuppressive (Burchill et al., 2007). Intriguingly, TNF pathway appeared to be enriched in TNFR-superfamily CD4 cells in anti-TNF treated (tritherapy) group.



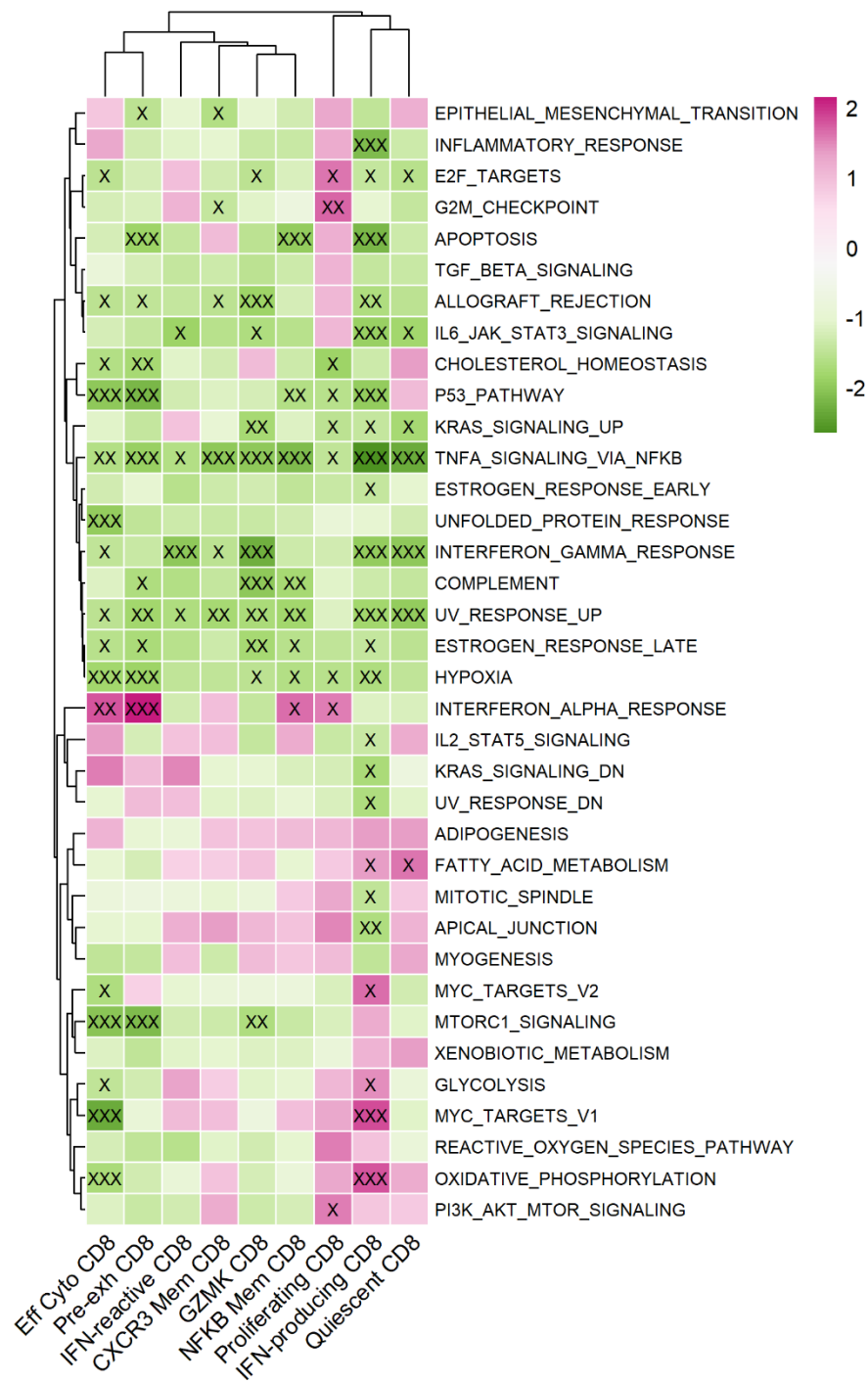
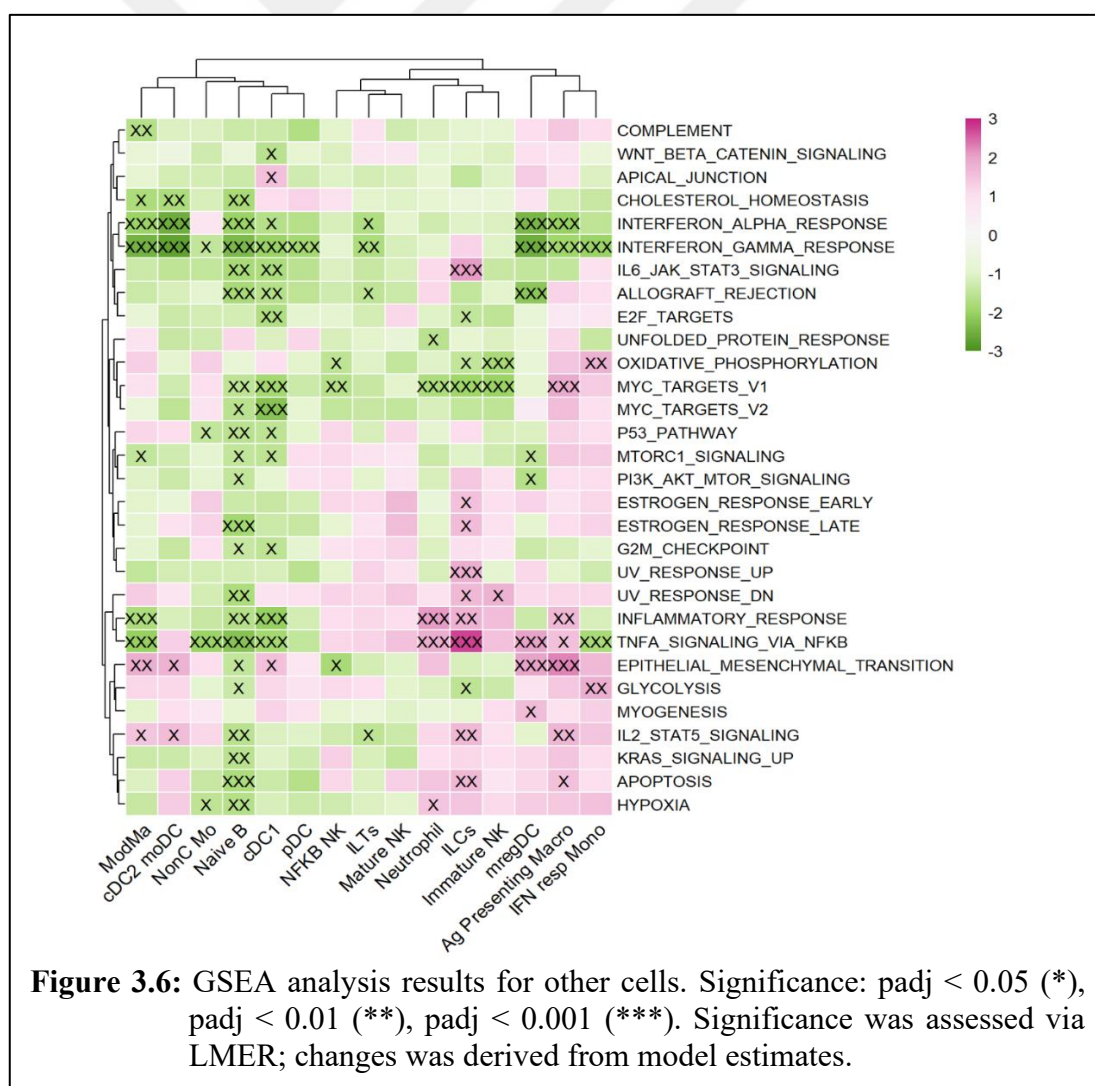


Figure 3.5: GSEA analysis results for CD8 cells. Significance: $\text{padj} < 0.05$ (*), $\text{padj} < 0.01$ (**), $\text{padj} < 0.001$ (***)

GSEA analysis showed pathway 'TNF α signaling via NF-Kb', 'Hypoxia', 'TGF Beta Signalling', 'UV response UP' and 'Interferon Gamma Response' enrichment in all CD8 subsets under bithrapy conditions. 'Apoptosis' and 'P53' pathway showed

enrichment in bitherapy Pre-exhausted CD8, IFN-producing CD8 and NFKB Memory CD8 cells. “Interferon Alpha Response” is significantly enriched in tritherapy Pre-exhausted and Eff-Cyto CD8 cells and “MTORC1 signalling” significantly enriched in bitherapy within these clusters. “Oxidative Phosphorylation” and “MYC Targets” are enriched in tritherapy IFN-producing CD8 (Figure 3.5)

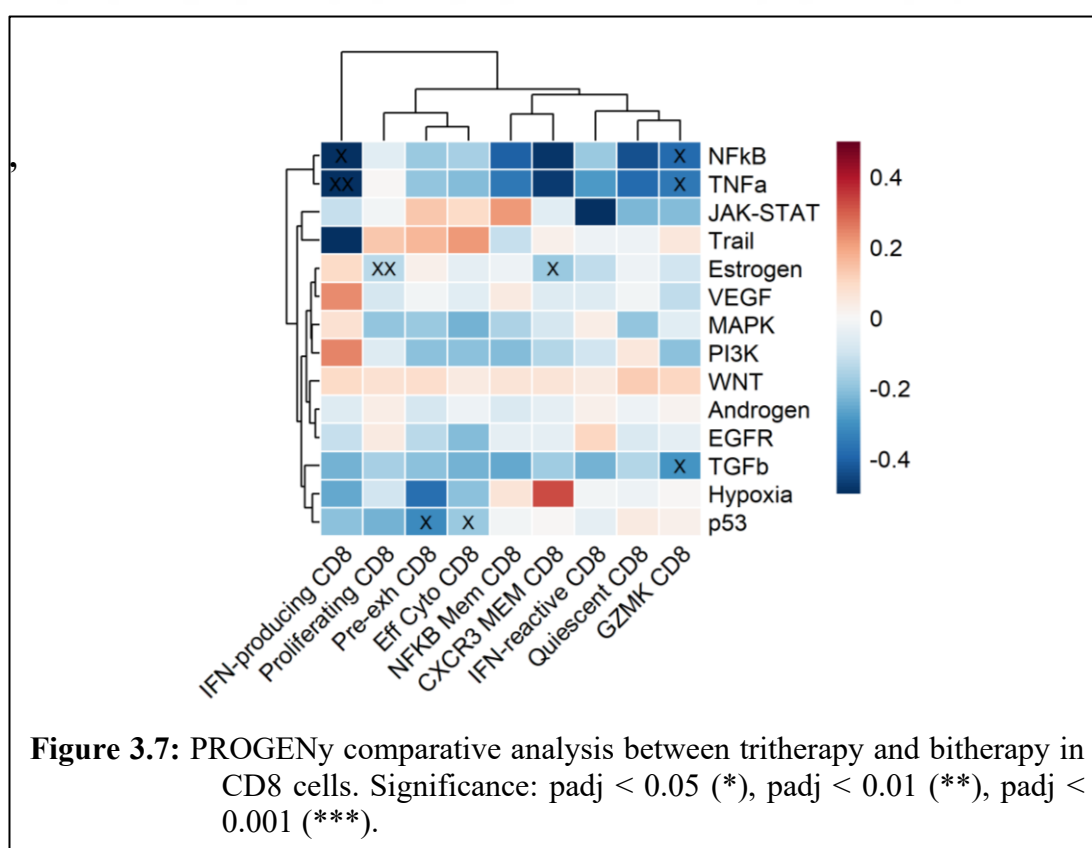
The same analysis on myeloid compartments via GSEA showed that, intriguingly, in all NK cell subsets, the 'TNF α signaling via NF- κ B' pathway was enriched in the anti-TNF treated group but not significantly (Figure 3.6). Additionally, although the TNF pathway in the ILCs cluster appeared significantly enriched in the tritherapy group, differential expression analysis between tritherapy and bitherapy within this cluster revealed only a significant upregulation of Egr1 in the tritherapy group, suggesting that the TNF pathway enrichment observed in the Hallmark GSEA may not always reflect broad transcriptional changes between therapies (Appendix 1).

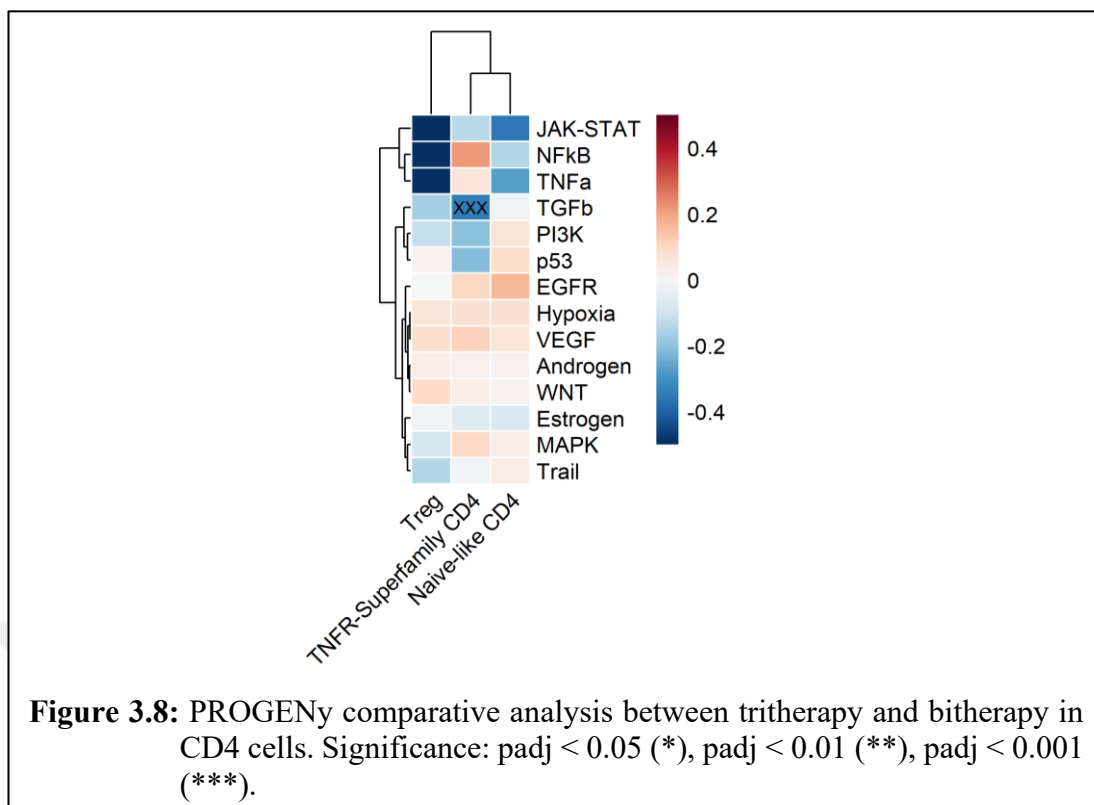


Although GSEA analysis using Hallmark gene sets allows us to identify which pathways differ between therapies, it is incorrect to make direct inferences about the directionality of these changes between therapies without specifically examining the leading edge gene lists that constitute these pathways. This is because Hallmark gene sets are known to include both activating and suppressing genes related to a given pathway. Therefore, the most accurate interpretation of such results is that one therapy induces higher expression of genes associated with a particular pathway compared to another therapy. However, these upregulated genes may influence the pathway's activity in either a positive or negative direction.

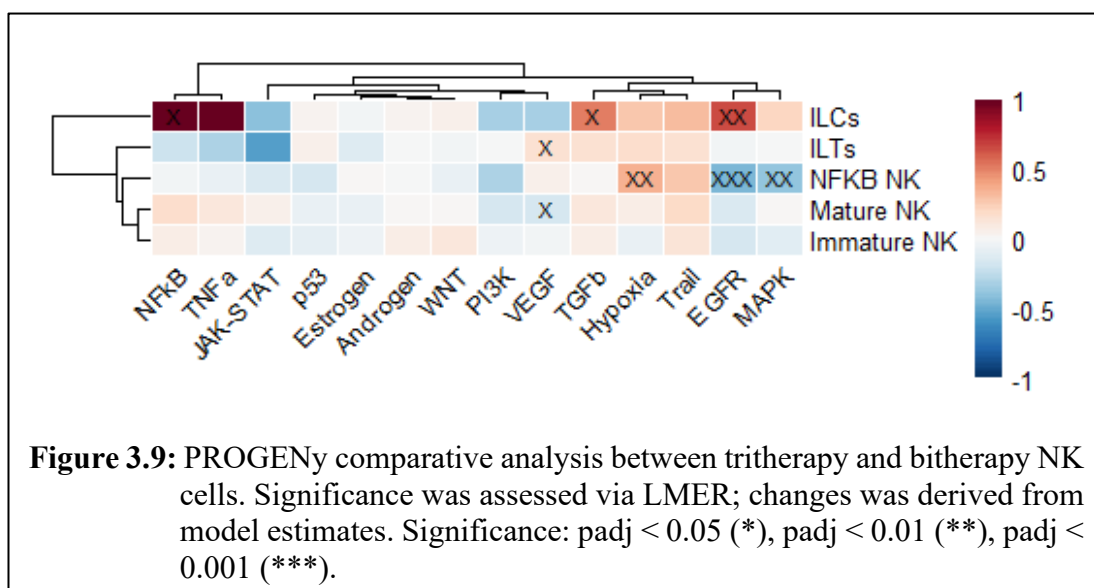
3.4. PROGENy and Transcription Factor Analysis

PROGENy analysis indicated increased TNF α , NF- κ B, and TGF- β signaling across all CD8 subsets with bitherapy. WNT signaling was elevated in CD8 cells under tritherapy, though not significantly. The p53 pathway was significantly upregulated in Pre-exh and Eff-Cyto CD8 cells (Figure 3.7), consistent with GSEA results (Figure 3.5). IFN-producing CD8 cells from bitherapy showed significant increases in TNF α and NF- κ B activity.

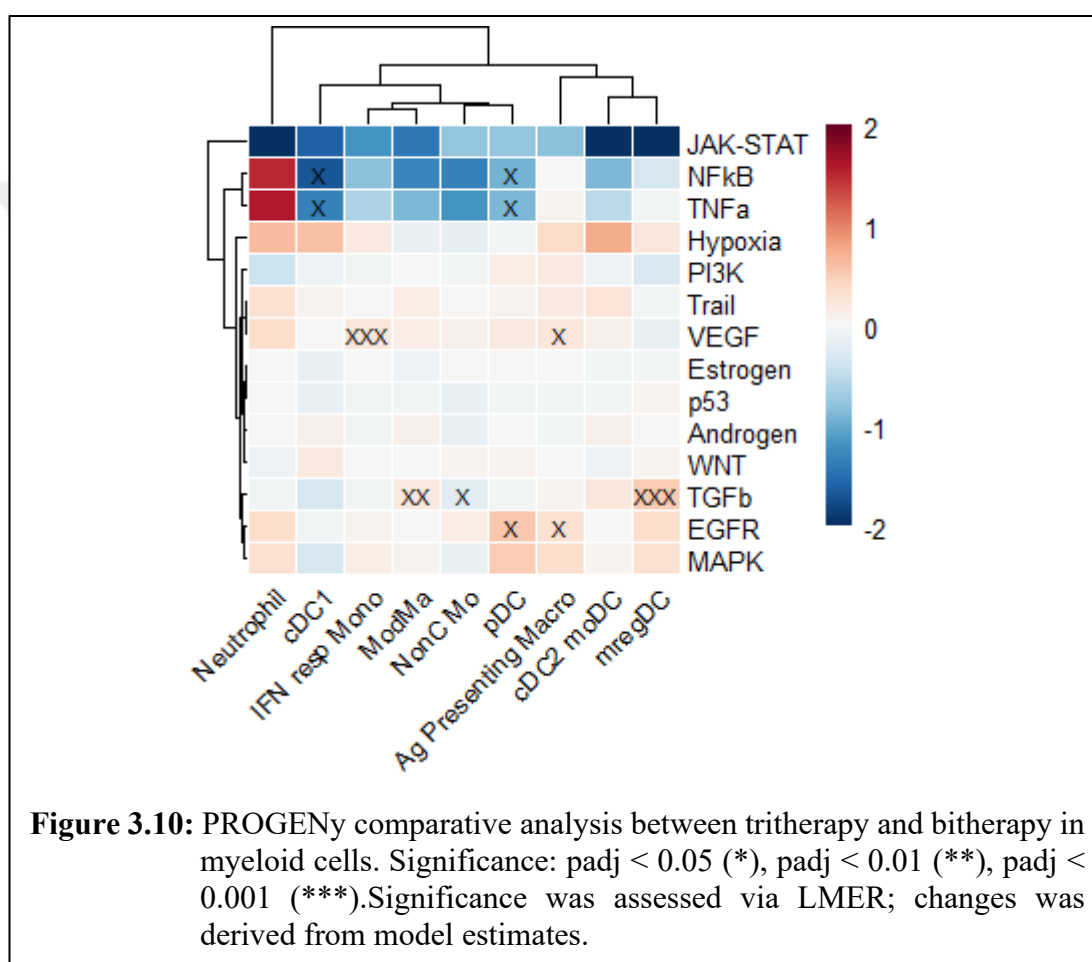




In Tregs, JAK-STAT, TNF α , and NF- κ B signaling were elevated under bitherapy (Figure 3.8). Although these changes were not significant in the LMER test due to low statistical power from limited cell numbers, TNF α activity was significantly increased in bitherapy based on a t-test with 0.01 pvalue. NK cells exhibited no alterations in TNF α or NF- κ B pathway activity (Figure 3.9).

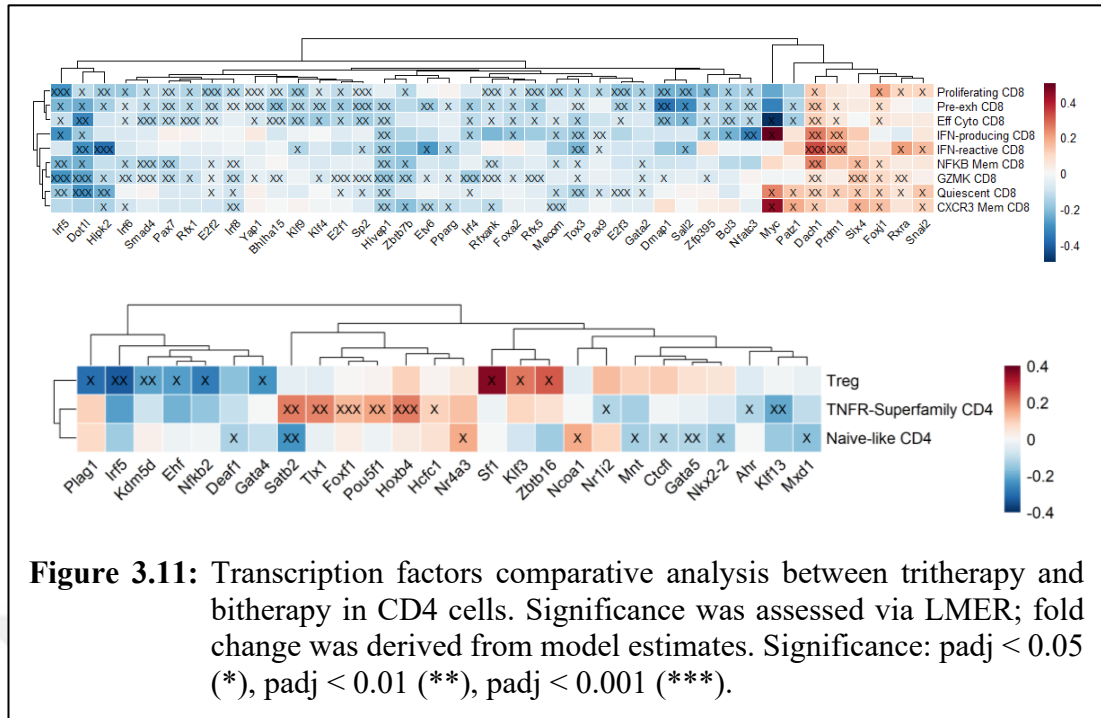


In bitheraPy, the JAK-STAT pathway was found to be active in all myeloid cells. In the NF- κ B and TNF- α pathways, a general increase was observed in the bitheraPy group without anti-TNF treatment. However, in tritheraPy, although these pathways were highly active in neutrophil cells, the p-value was not significant possibly due to the low number of cells in the comparison. In the ModMa (monocyte-derived macrophages) cluster, the TGF- β pathway was significantly increased in the tritheraPy group (Figure 3.10).



The transcription factors showing the most significant changes are presented in Figure 3.11. Dach1 was relatively increased in all CD8⁺ T cells under tritheraPy, while Irf5, Dot1l, Tox3, and Hive1 were elevated in the bitheraPy group. Eomes did not show significant changes, whereas Tbx21 was significantly upregulated in pre-exhausted CD8⁺ T cells under bitheraPy (data not shown).

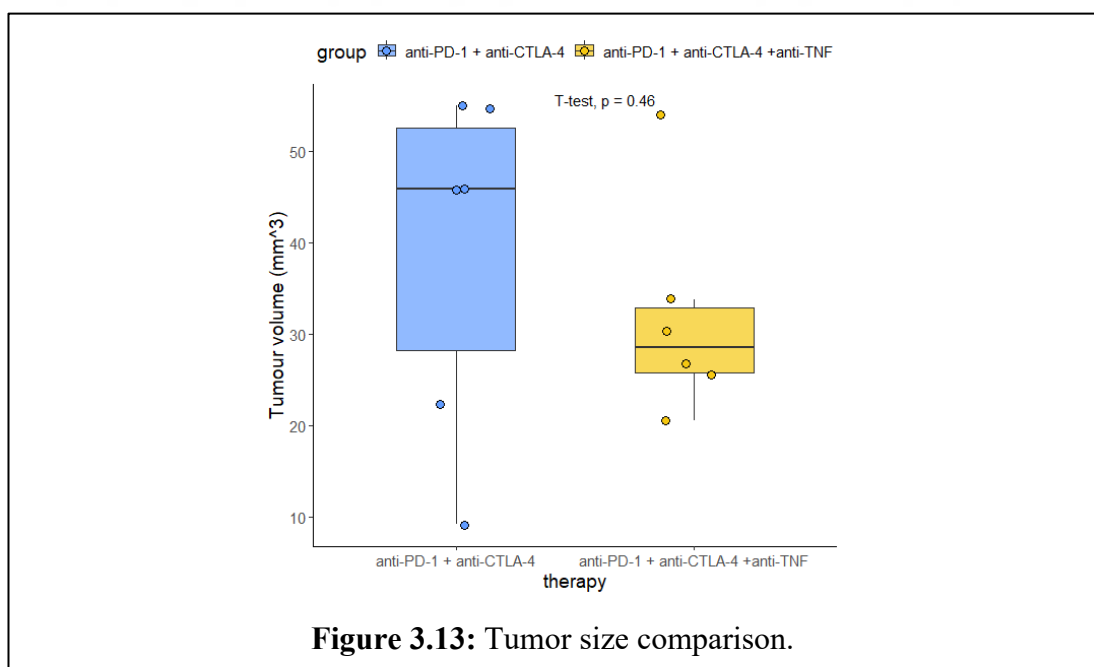
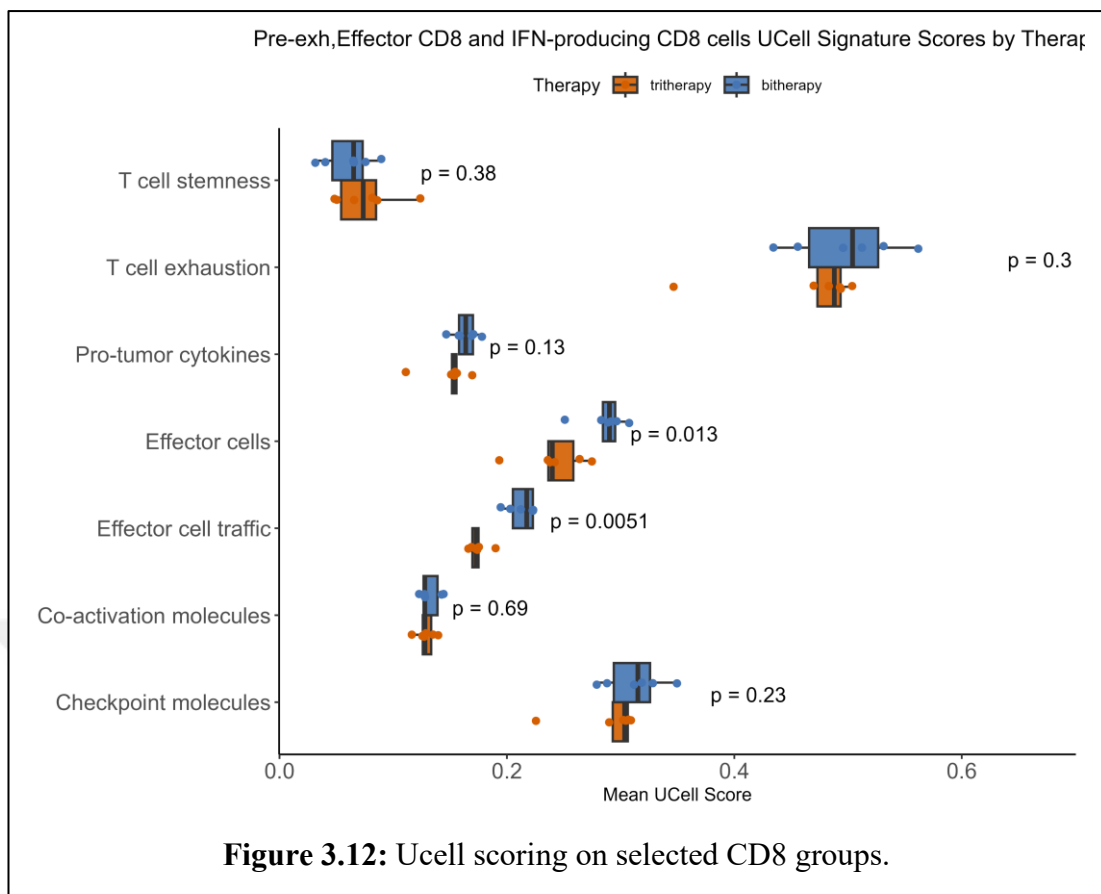
In Treg cells, IRF5, Nfkb2 is significantly increased in bitheraPy group.



3.5. Cell Scorings and Tumor Size Comparisons

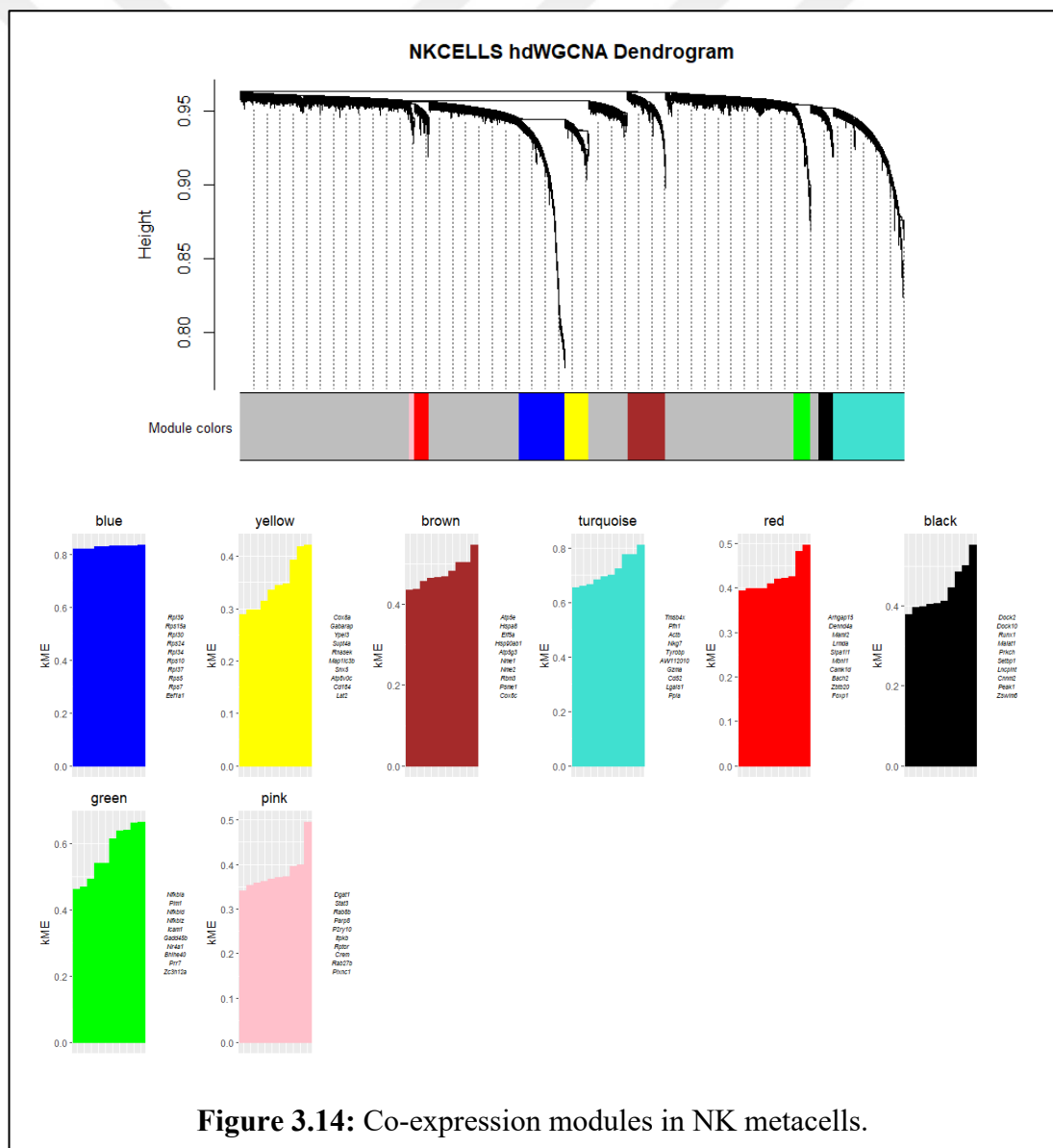
To further assess cytotoxicity and exhaustion levels in CD8⁺ T cells, UCell scoring was performed using the gene lists in Table 2.3, and statistical differences were evaluated. In highly exhausted and effector-like clusters, bitherapy significantly increased effector and trafficking scores; however, exhaustion profiles did not show a significant difference (Figure 3.12). Similar patterns were observed in total CD8⁺ T cells (data not shown).

Although the bitherapy group exhibited higher effector activity, tumor size comparison analysis showed no significant differences between the therapies (Figure 3.13).



3.6. Immune Cell Recruitment Capacity Analysis of NK Cells

This analysis was performed as described in Section 2.2.6.8 of the Methods. Co-expression modules are identified using WGCNA after turning NK cells into metacells. Each colour in Figure 3.14 represents different set of genes with possible similar functions. *Ccl5* gene, known for its role in immune cell recruitment, is identified within the turquoise module and pearson correlation between *Ccl5* and other genes within the module calculated (Figure 3.15.a). Immune recruitment capacity calculation based on *Ccl5*-correlated genes in NK metacells showed no significant change (Figure 3.15.b)



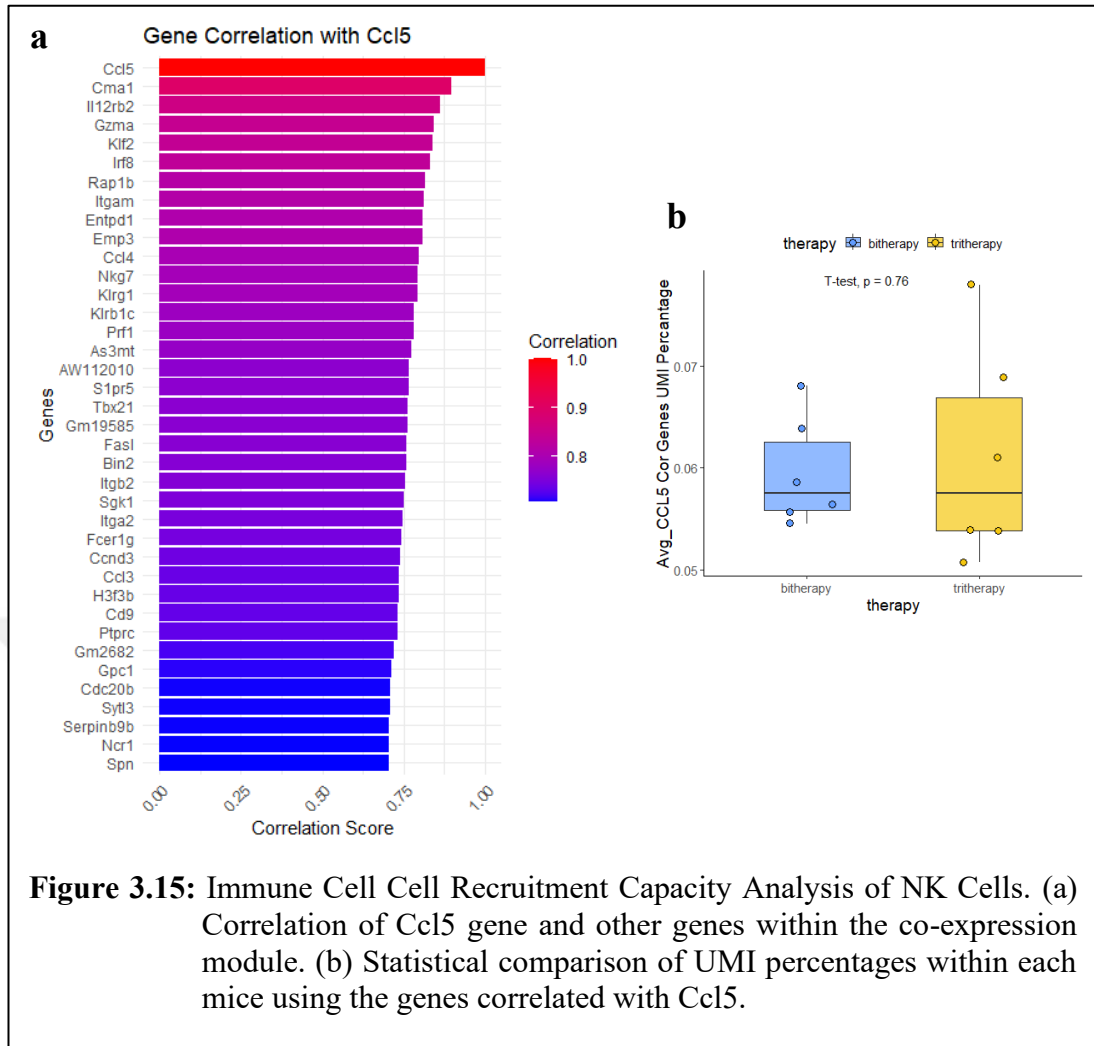
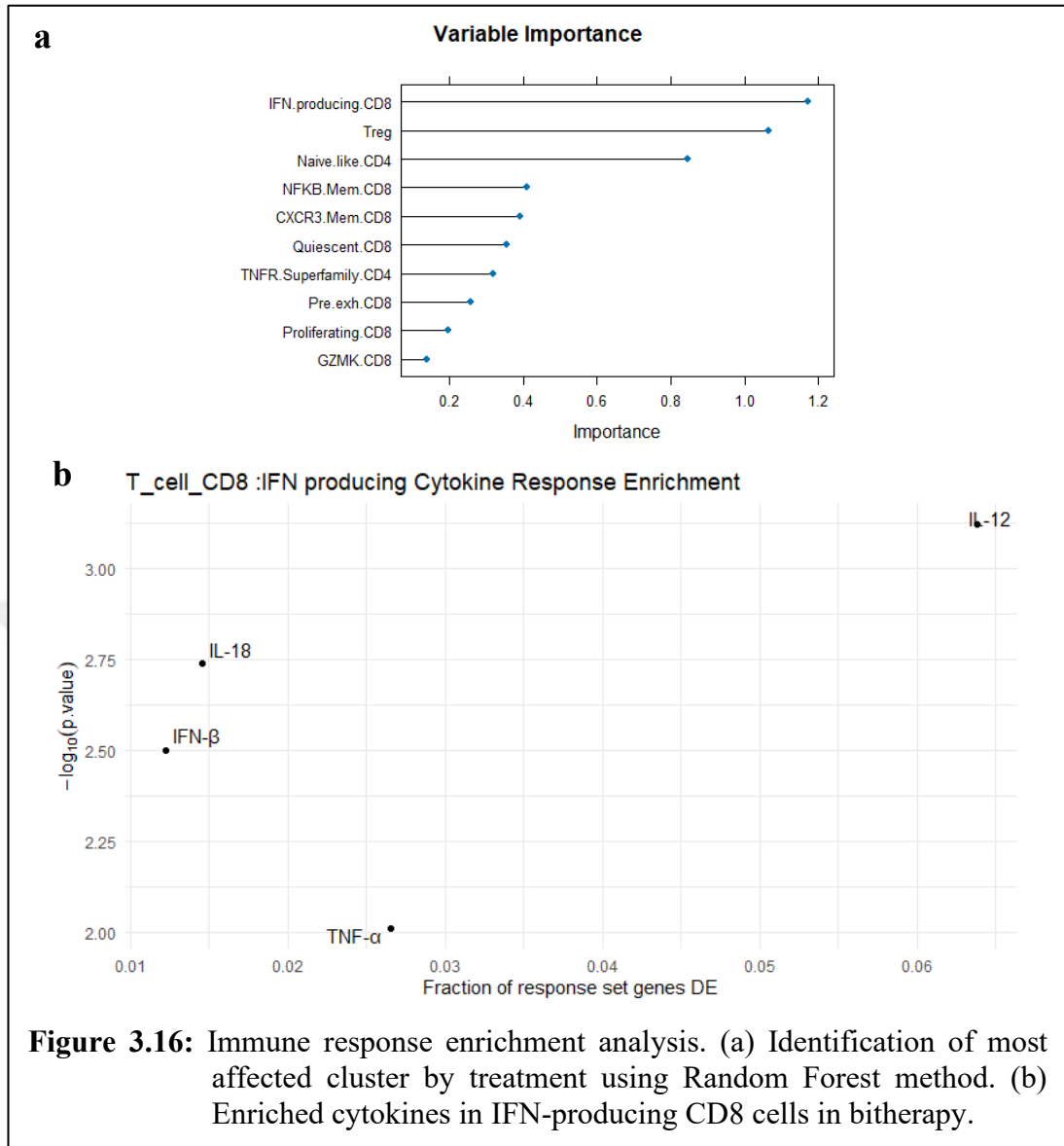


Figure 3.15: Immune Cell Cell Recruitment Capacity Analysis of NK Cells. (a) Correlation of Ccl5 gene and other genes within the co-expression module. (b) Statistical comparison of UMI percentages within each mice using the genes correlated with Ccl5.

3.7. Immune Response Enrichment Analysis

This analysis was performed as described in Section 2.2.6.10 of the Methods. Feature selection, here applied to cell types, using Random Forest method revealed that the "IFN-producing CD8" cell cluster was the most altered by the therapy and therefore immune response cytokine enrichment analysis was applied only to this cluster (Figure 3.16.a). Analysis indicated enrichment of IL-12, IL-18, IFN- β , and TNF- α in the bitherapy group, suggesting that the DEGs increased in bitherapy IFN-producing CD8 cells may be driven by the production of these cytokines (Figure 3.16.b).



3.8. Cell-Cell Interaction Analysis

Figure 3.17 shows the top 25 interactions with the highest prioritization scores for each therapy, calculated using MultiNicheNet. MultiNicheNet was utilized to identify and rank ligand–receptor interactions potentially mediating cell–cell communication within each therapy. The plots display therapy-specific interactions. In Figures 3.18 and 3.19, the pseudobulk expression profiles of these and additional interactions in each mouse, as well as the ligand activities of the interactions, are shown. The effects of these interactions on the therapy are discussed in the next chapter.

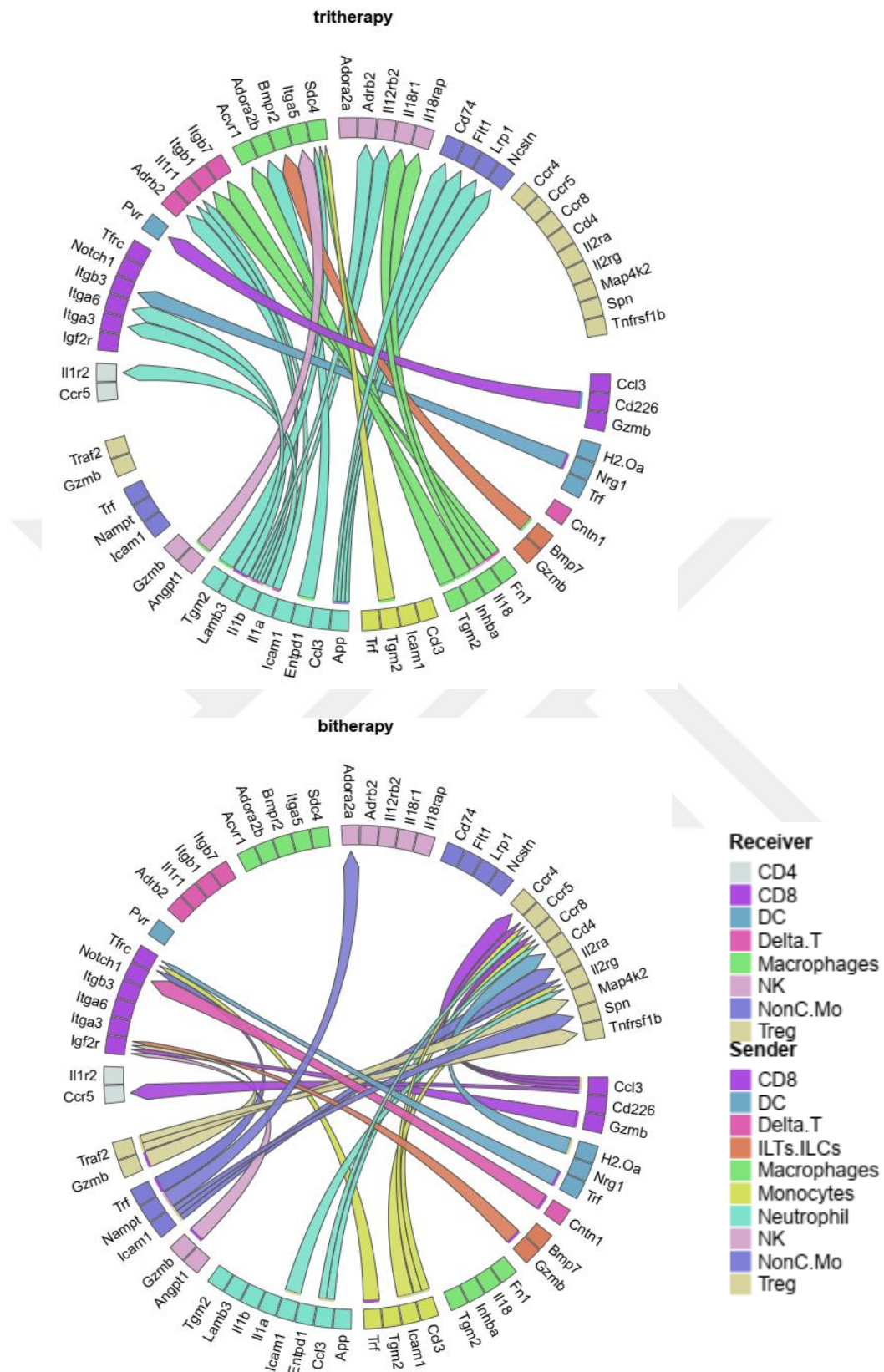


Figure 3.17: Cell-cell interaction analysis in tritherapy and bitherapy.

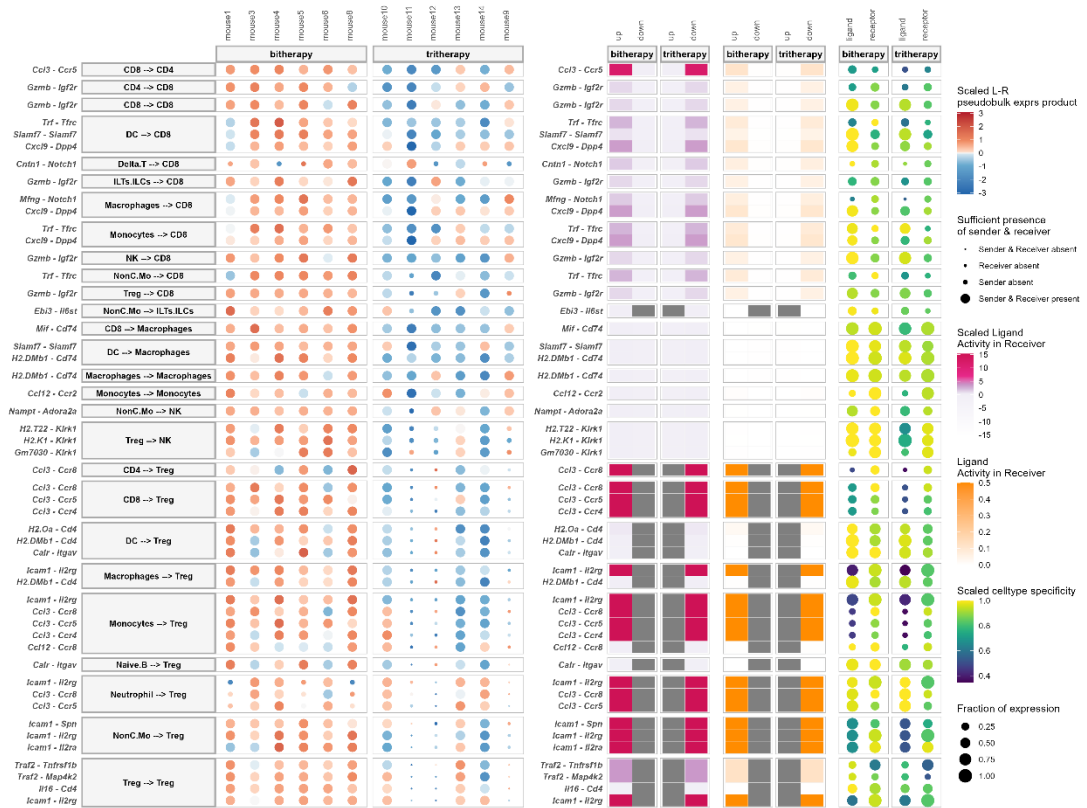


Figure 3.18: MultiNichenet plot of top bitherapy interactions and ligand activities

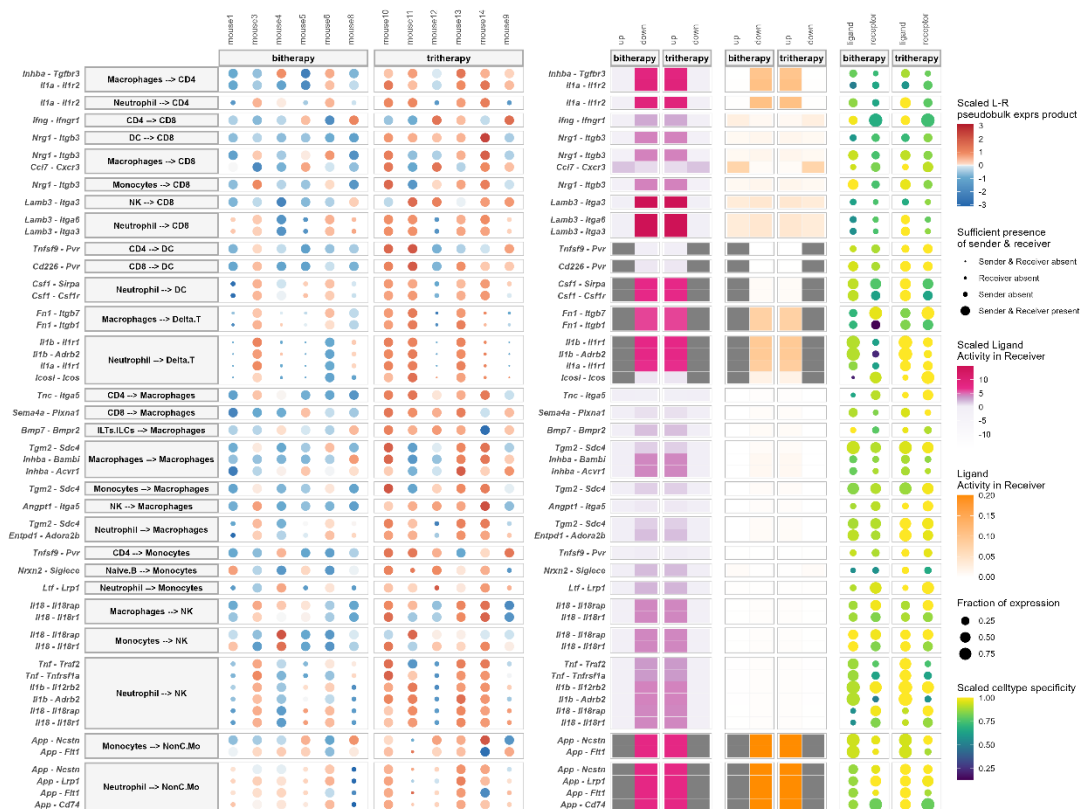


Figure 3.19: Multinichenet plot of top tritherapy interactions and ligand activities

3.9. Projection Analysis

As described in Methods section 2.2.6.11, proliferating CD8 cells were projected onto non-proliferating CD8 cells for cell subtype assignment. As shown in Figure 3.20, this cluster of 1,580 cells overlapped predominantly with GZMK-expressing CD8 cells and, to a lesser extent, with IFN-producing, pre-exhausted, and effector cytotoxic CD8 cells, indicating that proliferating CD8 cells share similar transcriptomic profiles with these subsets.

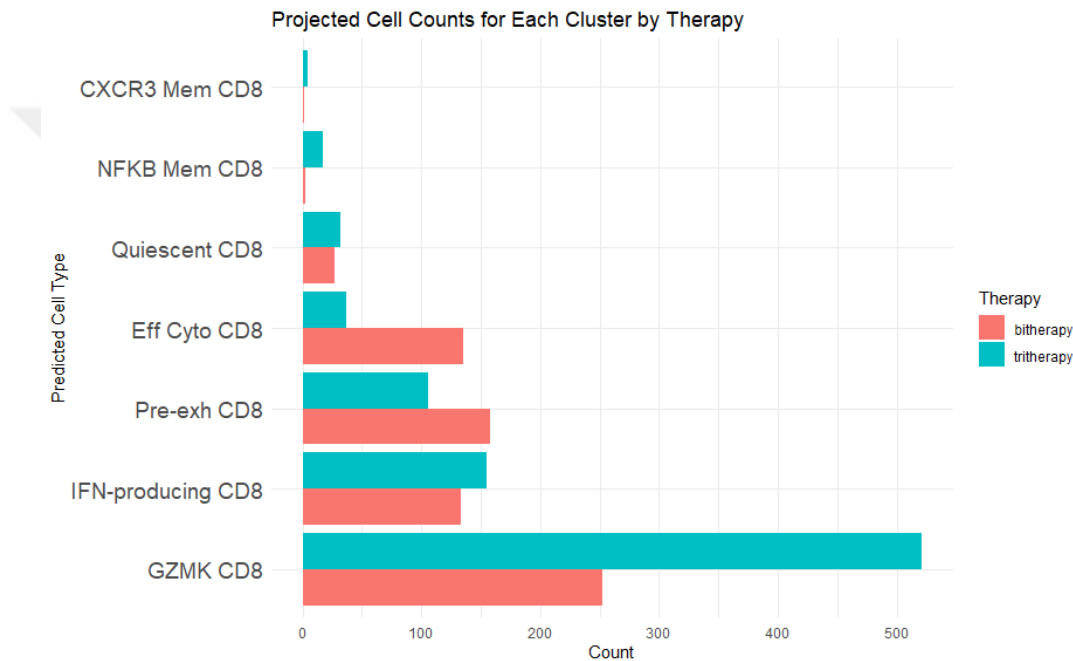


Figure 3.20: Predicted cluster identity of proliferating cells shown as the number of cells per cluster.

4. DISCUSSION

4.1. Biological Interpretation of Results

A proportional decrease in Treg cells, key mediators of immunosuppression, was observed in the anti-TNF-treated group. However, this significant reduction did not lead to a greater number of differentially expressed genes (DEGs) between treatment groups. This may be attributed to the general effect of immune checkpoint inhibitors (ICIs) in suppressing Treg cell activity, resulting in a low number of Treg cells overall, which may have limited the statistical power of differential expression analyses. In the dataset, a total of 189 Treg cells were detected—130 from the bi-therapy group and 59 from the tri-therapy group. Some mouse samples contained fewer than 10 Treg cells, leading to low library sizes when pseudobulk analysis was performed. Therefore, the experimental design should have accounted for the known Treg-reducing effects of ICI therapies by including a larger number of samples. This is particularly important because Treg cells, due to their immunosuppressive functions, are among the most studied cell types in immunotherapy research.

DEG analysis between Treg cells from the tritherapy and bitherapy groups revealed that only a few genes (Ly6a, Ccl5, Egr1, Gbp6) were significantly upregulated in the bitherapy group. Upregulation of Ly6a (also known as Sca1) has been associated with heightened suppressive function in Treg cells (**Grinberg-Bleyer et al., 2010**). Moreover, recent studies have demonstrated that Egr1 activation directly contributes to Treg differentiation and function by binding to regulatory elements near Foxp3, thereby promoting its expression and enhancing the suppressive capacity of Tregs (**Yang et al., 2025**).

The results of GSEA and PROGENy analyses are consistent with the genes associated with increased Treg activation observed in the bitherapy condition. In Treg cells from the bitherapy group, enrichment of activity-related pathways such as “IL2-STAT5 signaling,” “TNF α signaling via NF- κ B,” and “Interferon gamma response” was observed. Similarly, PROGENy pathway analysis revealed upregulation of the TNF α , NF- κ B, and JAK-STAT pathways. These pathways have been linked to enhanced

immune suppression in Tregs in the literature (**Burchill et al., 2007; Nagar et al., 2010; Oshi et al., 2022**).

Furthermore, transcription factor analysis revealed an increase in *Irf5* and *Nfkb2* in the bitherapy condition. In murine CD4⁺ T cells, IRF5 has been shown to be expressed at low levels in the resting state, but its expression increases following TCR activation (**Yan et al., 2020**). In another study, deletion of *Nfkb2* in a mouse model was shown to increase autoimmunity. Therefore, in the context of immunotherapy, *Nfkb2* expression enhances the suppressive functions of Treg cells by activating the alternative NF- κ B pathway (**Grinberg-Bleyer et al., 2018**).

Through ORA analysis, “G alpha (I) signaling” and “GPCR binding” pathways were found to be enriched in Treg cells under bitherapy. These pathways have also been reported in the literature to be associated with T cell migration and crosstalk with other immune cells (**Gurusamy et al., 2021**). This observation is also consistent with the results obtained from the cell–cell interaction analysis as discussed below.

Cell-cell interaction analysis revealed that interactions from CD8 to Treg cells were observed only in the bitherapy group, specifically through the Ccl3-Ccr4/Ccr5/Ccr8 interaction (Figure 3.17). Upon antigen stimulation, activated CD8⁺ T cells secrete CCL4/CCL3 in particular recruiting CCR5 positive leukocytes, including macrophages, dendritic cells, and T cells (**Trifilo et al., 2003**). In melanoma, however, CCR5 is also expressed on suppressive Tregs and MDSCs (**Adams et al., 2021**). In melanoma, tumor-infiltrating Tregs commonly express CCR4 and CCR8 which directs their migration into the tumor microenvironment (**Patterson et al., 2016**). Spranger and colleagues found in a B16 melanoma mouse model that activated CD8 T cells prompt tumor and stromal cells to release CCR4-binding chemokines. Those chemokines not only attract Foxp3 regulatory T cells but also drive their expansion right inside the tumor, boosting intratumoral Treg density. Since high numbers of Tregs in melanoma are linked with poorer patient outcomes, this sets up an inflammatory feedback loop: effector T-cell activation and the chemokines it produces paradoxically pulls in even more Tregs (**Spranger et al., 2013**). Conversely, CCL3 can also recruit beneficial cells. CCL3 is a potent attractant for dendritic cells, promoting antigen presentation (**Kang et al., 2021**).

Furthermore, Traf2-Tnfrsf1b interaction between Tregs is observed in bitherapy. Activated Treg cells produce membrane-bound TNF, which signals to neighboring Treg cells via TNFR2. This interaction loop enables them to continue proliferating; *in vitro* TNF blockade studies have shown that, in the absence of this signal, Tregs stop proliferating (Nelson et al., 2018). Thus, TNFR2–TRAF2 interaction activates the canonical NF-κB pathway, thereby enhancing the expression of Foxp3 and other suppressive genes, polarizing Treg cells toward a pro-tumor state, increasing immune suppression, and inhibiting the activity of effector CD8⁺ T cells (Albini et al., 2025; Medler et al., 2022).

Under bitherapy, classical and non-classical monocytes engage Tregs via Icam1–Il2rg ligand–receptor interactions, with IL-2 acting as a critical driver of Treg proliferation and suppressive capacity and sustaining Foxp3 expression. Moreover, strong IL-2R–STAT5 signalling is indispensable for Treg suppressor function (Chinen et al., 2016). Through ICAM1-mediated adhesion, monocytes can further amplify IL-2R signals in Tregs (Vyth-Dreese et al., 1993). Zhang et al. also showed that Icam1 expressing macrophages establish physical contacts with Tregs via this interaction, coinciding with upregulation of activation genes such as CTLA-4 (Ni et al., 2023). Consistent with these findings, gene set enrichment analysis in the Figure 3.4 reveals significant enrichment of IL-2–STAT5 signalling in bitherapy treated Tregs.

Unexpectedly, the only evidence of altered Treg activity in the tritherapy was that the GSEA plot showed enrichment of “MYC Targets V1” in tritherapy Tregs, yet transcription factor analysis did not reveal any significant upregulation of the MYC transcription factor.

Nevertheless, the increased suppression observed with bitherapy did not translate into a reduced tumor volume compared to tritherapy (Figure 3.13). Additionally, increased effector activity was observed in CD8 T cell clusters in bitherapy, further highlighting the complexity of cellular behavior within the immune microenvironment (Figure 3.12).

For instance, NK cells are typically associated with favorable outcomes in immunotherapy due to their cytotoxic activity (Chen et al., 2021). Intriguingly, their relative abundance significantly increased in the bitherapy group. However, DEG analysis did not reveal a substantial number of DEGs between treatment groups, nor

did it identify enrichment of pathways directly associated with cytotoxicity. Although GSEA suggested alterations in the TNF- α signaling pathway within this cell population, the functional implications remain unclear because PROGENy analysis did not reveal any significant changes in the TNF pathway. Correlation-based analysis of CCL5 which used to assess immune cell recruitment capacity, showed no significant differences between treatment groups. These findings suggest that an increase in NK cell numbers may not necessarily reflect a shift in their functional phenotype however different interactions between NK and other cells are observed which are open to interpretations. Such as:

Angpt1-Itga5 interaction between NK cells and macrophages is observed in tritherapy samples. Although NK cells are best known for cytotoxic functions, subsets of tumor-infiltrating NK cells can adopt pro-angiogenic phenotypes by producing ANGPT1 (Zecca et al., 2021). Macrophages in injured or tumor tissues upregulate integrins to sense the extracellular matrix. Thus integrin Itga5 expression marks a pro-angiogenic macrophage subset. Analysis of human tumors shows high ITGA5 expression correlates with M2-like TAM infiltration and immunosuppressive TME (Li et al., 2023; Liu et al., 2024). In Figure 3.10, the significant increase of the TGF- β pathway in the ModMa (monocyte-derived macrophage) cluster supports this finding and may indicate that this cluster exhibits an M2-like phenotype.

Another interaction suggesting the presence of an M2 phenotype in macrophages under tritherapy is the Entpd1–Adora2b ligand–receptor interaction, which was observed from neutrophils to macrophages. Neutrophil derived Entpd1 hydrolyzes extracellular ATP to AMP, promoting adenosine accumulation in the TME, which can activate Adora2b on macrophages and induces a tumor promoting M2 like phenotype with increased VEGF expression (Antonioli et al., 2013; Morello et al., 2016). As shown in Figure 3.10, macrophages display heightened VEGF pathway activity which matches with this cell-cell interaction observation (Zhang et al., 2023).

As mentioned, the presence of an M2 phenotype in macrophages is associated with poor therapeutic outcomes (Zhang et al., 2023). Unfortunately, MDSC populations, which could have provided further insights into myeloid cell-related dynamics and suppression, were not detected in the dataset.

Regarding CD8 T cells, the most pronounced cellular response to therapy was observed in a distinct subset of CD8 T cells characterized by interferon production. Low level expression of the XCL1 gene was detected in IFN-producing CD8 T cells, suggesting that this population may represent progenitor-exhausted CD8 T cells (Tpex). This inference was further supported by a projection analysis using the ProjectTILS package, which showed that this cell population overlaps with the Tpex cluster (**Andreatta et al., 2021**). However, progenitor CD8 T cells (Tpex) are expected to express TCF1, and because TCF1 was not detected in this cluster, we concluded that these cells are not Tpex. (**L. Sun et al., 2023**). In the projection analysis, this cluster was labeled as Tpex because projection methods attempt to map every cell onto reference populations even when the confidence scores are low (see Appendices 2 and 3). Consequently, no true Tpex cluster was identified, which limits the conclusions that can be drawn from the analysis.

This IFN-producing CD8 population demonstrated elevated expression of metabolic markers (e.g., Fbl, C1qbp, Gadd45g) and effector molecules (e.g., Ccl3, Ccl4, Ifng), indicating high functional activity. Immune-response enrichment analysis revealed that the transcriptional shifts within this cluster are driven by production of IL-12, IL-18, IFN- β , and TNF- α . Notably, the synergistic action of IL-12 and IL-18 has been linked to enhanced interferon- γ secretion, indicating that these cells in bitherapy adopt a predominantly effector-like phenotype (**Nakahira et al., 2002**). However, sustained activation of the TNF pathway may predispose them to activation-induced cell death over time (**Otano et al., 2020**). Likewise, in the bitherapy group, this cluster was associated with activation of TNFR1 signaling and pathways related to apoptosis, as shown in ORA analysis (Figure 3.3). Additionally, GSEA analysis of IFN-producing CD8⁺ cells in the bitherapy group showed enrichment of “Apoptosis” and “TNF α signaling via NF- κ B,” and PROGENy analysis revealed upregulation of the TNF, NF- κ B, and TRAIL pathways (Figure 3.5 and Figure 3.7). These results are consistent and mutually supportive.

Conversely, in the tritherapy group, enrichment of MYC target gene sets and upregulation of the MYC transcription factor were observed. These findings suggest that prolonged TNF signaling in the bitherapy group may contribute to depletion of CD8 T cells over time, whereas MYC-driven transcriptional programs in the tritherapy group may confer a survival advantage to this population (**Wang et al., 2011**).

However, enrichment of the TNFR1-mediated apoptosis pathway was not observed in the other CD8 bitherapy clusters. Performing TCR repertoire analysis could provide more detailed insights into the IFN-producing CD8 T cells and help us understand why this cluster specifically affected by treatment differences.

In the cell–cell interaction analysis of bitherapy samples, the only interaction that could support the induction of apoptosis was the GZMB–IGF2R interaction between NK cells/ILCs/ILTs/Treg/CD8 and CD8 cells. Granzyme B (GZMB) can engage IGF2R on CD8 T cells, triggering apoptotic contraction of the cytotoxic T cell pool through a perforin-independent mechanism of GZMB uptake. Blocking the GZMB–IGF2R interaction inhibits GZMB binding, internalization, and subsequent apoptosis induction. As demonstrated by Veugelers et al., IGF2R is essential for efficient GZMB-mediated cytotoxicity, and its blockade significantly reduces apoptosis in target cells **(Motyka et al., 2000)**.

Figure 3.12 demonstrates that, under bitherapy, CD8 cells exhibit a more effector-like profile. Another piece of evidence supporting that CD8 cells exhibit a more effector-like profile in bitherapy is the observation of TRF–TRFC interactions from monocytes and non-classical monocytes to CD8 cells (Figure 3.17). Transferrin–transferrin receptor (TRF–TRFC) signaling between monocytes and CD8 T cells in the melanoma tumor microenvironment exerts dual, context-dependent effects on antitumor immunity. TRFC-mediated iron uptake is essential for T cell proliferation, activation and effector function: upon activation, human and mouse CD8⁺ T cells markedly upregulate TRFC to import iron, supporting their proliferation and cytotoxic activity and correlating with granzyme B expression in tumor-infiltrating lymphocytes. Conversely, blockade or genetic loss of TRFC impairs T cell function and leads to severe immunodeficiency **(Berg et al., 2020; Heintzman et al., 2022; Sacco et al., 2021)**.

However, excessive iron uptake can be detrimental. When too much iron builds up inside cells, it fuels Fenton reactions that produce reactive oxygen species and lipid peroxides, which undermine T cell survival and function. In mouse melanoma models, high iron concentrations intravenously boosted tumor iron stores, sped up tumor growth, and weakened anti-tumor CD8 and Th1 responses **(Tymoszuk et al., 2020)**.

These findings suggest that CD8 cells in bitherapy exhibit a complex dynamic, displaying interactions associated both with cell death and with activation. On the other hand, the only interaction supporting CD8 cell activation in tritherapy was the CD226–PVR interaction between CD8 and DC cells, which was observed exclusively in tritherapy. It was shown that engaging CD226 amplifies TCR signaling in CD8T cells, driving their proliferation, boosting interferon gamma release and directing lytic granule polarization. By lowering the activation threshold, CD226 costimulation allows these cells recognize even weak tumor antigens. In mouse models, CD226 not only enhances CD8⁺ T-cell responses but also broadens the range of tumor targets that both CD8⁺ T cells and NK cells can detect (Gilfillan et al., 2008; Jo et al., 2024). However, CD226 has to compete with the inhibitory receptor TIGIT for binding to PVR and because TIGIT has higher affinity for PVR, upregulated TIGIT on T cells essentially sequesters PVR and blocks CD226 engagement. In melanoma-infiltrating lymphocytes, TIGIT is often coexpressed with PD-1 while CD226 is downregulated, creating a TIGIT–CD226 imbalance that tips the balance toward inhibition and exhaustion (Braun et al., 2020; Chiang & Mellman, 2022; Tang et al., 2023).

Another point of discussion regarding the overall experiment concerns the heterogeneous nature of CD8 T cells in their immune response and the timing of sample collection. In this study, immune cells were harvested at a single time point, on day 17 after tumor inoculation. However, the number and phenotype of TILs may vary depending on the timing of sample collection, complicating direct comparisons with other studies. Extending the experimental duration could potentially allow for the expansion or emergence of certain immune populations within the TME that are underrepresented at earlier time points. In particular, CD8 T cells are known to undergo continuous phenotypic transitions, from naïve to effector and exhausted states, throughout the course of an immune response. Therefore, as conclusion, a single time point analysis, as performed in this study, may not fully capture the dynamic nature of T cell differentiation and plasticity. This limitation makes it challenging to generalize findings across broader immunological contexts or treatment timelines.

4.2. Technical and Methodological Considerations

Technical and biological limitations may have influenced the interpretation of these findings.

Immune cell infiltration varies across tumor regions, and single-region sampling may not fully represent TME heterogeneity; multi-region sampling would enhance robustness. Other studies have demonstrated the importance of multi-region sampling for robust and comprehensive results (Jia et al., 2018; Meiller et al., 2021).

Rare or therapy-depleted cell populations reduce statistical power, emphasizing the need for more biological replicates. For example, Treg cells decreased in both treatment groups as a general effect of ICI therapy, and analyses based on a small number of cells have reduced statistical power due to the low library size.

Prior to filtering, the dataset contained 29,825 cells, and after filtration this number dropped to 23,323. This is a relatively low cell count for single-cell data.

Transcriptional similarities among closely related cell types, such as CD8 subtypes, complicate accurate annotation. This issue, along with inconsistent nomenclature and the limitations of automated annotation tools in novel conditions, underscores the importance of manual, context-specific analysis.

Although efforts were made in this study to minimize bias during clustering and to identify the most appropriate clustering parameters, it is well understood that the overall analysis outcome can vary depending on the clustering approach. A different analyst using an alternative method may derive a different number of clusters, which would consequently alter cell type annotations. Furthermore, in this study, after clustering and annotation of major cell types based on canonical markers, subclustering was performed by collectively subsetting cell populations. For example, CD4 and CD8 T cell clusters were jointly subsetted without applying any expression threshold. However, with this approach, droplets that do not purely represent CD8⁺ or CD4⁺ cells may also be included due to the dropout effect inherent in single-cell RNA sequencing. An alternative strategy would have been to subset CD4⁺ and CD8 T cell clusters separately and also by putting expression threshold to CD4 and CD8 markers. As shown in the violin plot in Appendix 4, some droplets expressing CD4 transcripts are transcriptionally similar to clusters annotated as CD8⁺ T cells, leading to their misclassification. Unfortunately, there is currently no definitive method to fully prevent this issue.

Another technical issue concerns the outlier sample, Mouse 11. In this analysis, Mouse 11 formed its own cluster (Cxcr3⁺ memory CD8⁺ T cells) due to its distinct cell-type

composition; it also exhibited a lower myeloid cell count, as well as divergent numbers of unique genes and total UMI counts compared to the other samples. Consequently, Mouse 11 disproportionately affects the results of comparative statistical tests. Therefore, an alternative version of the analysis should be conducted with this sample excluded.

Another major technical issue concerns the “damaged cell” identification described in Methods Section 2.2.1. In this analysis, cells were classified as damaged based on their nuclear fraction values using the DropletQC package and subsequently removed from the dataset. However, this quality-control metric is novel and not yet widely adopted in the literature. In an alternative and previous version of this analysis, these cells were retained and the clusters initially labeled as “damaged cells” were instead annotated as “Unknown,” since no established marker could be assigned to them. To investigate their identity, a reference dataset from the literature was integrated with our data in an attempt to re-annotate these ambiguous cells (**Giles et al., 2022**). This “Unknown CD8” (or “damaged cells”) cluster showed no resemblance to any cell type in the reference dataset (Appendix 5). Moreover, in the projection analysis using ProjectTILS, this cluster exhibited very low confidence scores and could not be biologically interpreted (data not shown).

In other studies, clusters containing long non-coding RNAs have also been identified and associated with contamination. Various strategies have been tested to handle these clusters, which highly express genes such as Gm42418, AY036118, and Gm26917. These approaches include:

- Filtering based on the percentages of the expression of these genes (**Kimmel et al., 2019**)
- Removing these genes from data before normalization (**Kimmel et al., 2020**)
- Regressing out these genes to reduce the effect on clustering (**Ye et al., 2022**)
- Removing clusters highly expressing those genes (**J. Sun et al., 2023**)

None of the methods described above (regressing out, filtering by expression threshold, or completely removing these genes from the dataset) were able to prevent these clusters from re-emerging in our analysis. Furthermore, these clusters show significantly higher Malat1 expression than other clusters, and the literature has linked

elevated Malat1 levels to low-quality droplets (**Montserrat-Ayuso & Esteve-Codina, 2024**).

In another analytical approach in which unknown cells were retained in the dataset, chromosomal abnormalities in the “Unknown CD8” population were assessed using inferCNV (Appendix 6). For inferCNV, a reference cell group, where anomalies are least expected, must be defined first. Here, innate-like cells (ILCs) and double-negative T cells (DNTs) were selected as the reference because their expression profiles most closely resembled those of naïve T cells. In the upper panel of Appendix 6, no gains or losses are observed in the chromosomes of the reference cells; in contrast, the lower panel shows both gains and losses in multiple chromosomes of the Unknown-CD8 cells. The heatmap intensity indicates that more than half of these cells harbor alterations in specific chromosomal regions (**Tickle et al., 2019**). However, it is unclear whether these represent genuine biological events or artifacts arising from the low number of genes expressed by this cluster. Furthermore, because inferCNV is most commonly used to compare tumor cells with naïve T cells, the applicability of these results to the Unknown CD8 population remains uncertain.

Another technical concern is the proliferating CD8⁺ T cell population. This group forms a distinct cluster in the analysis due to high expression of cell cycle–related genes; however, in reality, these cells represent proliferating versions of other CD8⁺ T cells in the dataset. Regressing out the cell cycle effect to allow these cells to redistribute into their respective functional clusters would yield more biologically meaningful results. Although this approach was attempted, a complete gene list covering all cell cycle–associated genes was not available. As a result, a small residual cluster of proliferation-associated CD8⁺ T cells consistently emerged. A similar observation was reported in another study, where cell cycle regression was performed, yet a small proliferative CD8⁺ cluster still appeared. In that study, additional confounding factors such as hypoxia, interferon content, and cell stress were also regressed out (**Nederlof et al., 2024**). Performing regression with a sufficiently comprehensive gene list is essential to enable biologically accurate cell type annotations that are consistent with cytometry analyses. This approach is particularly important for ensuring meaningful comparisons with cell classifications reported in the existing literature.

5. CONCLUSIONS

In this study, a comparative single-cell analysis was performed between a mouse melanoma model treated with anti-CTLA-4 and anti-PD-1 (bithérapie) and treated with anti-CTLA-4, anti-PD-1, and anti-TNF (trithérapie). The aim was to identify and characterize the differences induced by these therapeutic regimens at the single-cell level. The results obtained from these analyses can be summarized as follows:

- The proportion of immunosuppressive, Foxp3⁺ Treg cells was significantly increased in the bithérapie samples. This expansion appears to be driven by the Ccl3–Ccr4/Ccr5/Ccr8 chemokine–receptor interactions observed between CD8 T cells and Tregs under bi-thérapie conditions.
- Moreover, pathway and enrichment analyses have shown that Tregs in the bithérapie group display a more immunosuppressive phenotype. This enhanced activation may be driven either by autocrine NF- κ B signaling via a TNFR2–TRAF2 interaction in Tregs, or by paracrine IL-2–STAT5 pathway activation mediated through ICAM1–IL2RG interactions between monocytes and Tregs. Moreover, transcription factor analysis showed that Nfkb2 expression was significantly upregulated, indicating that the alternative NF- κ B pathway is active in the bithérapie group and may further enhance immunosuppressive function.
- CD8⁺ T cells in the bithérapie group exhibited an effector phenotype, and no differences in exhaustion levels were observed between the two treatment regimens. Although Treg cells were more suppressive under bithérapie, these changes did not translate into a significant difference in tumor size when comparing the two regimens.
- In the bithérapie group, CD8 T cells exhibited interactions associated with both apoptosis and activation. A perforin-independent mechanism of granzyme B (GZMB) uptake, specifically the pro-apoptotic GZMB–IGF2R interaction between NK/ILC/ ILTs/Tregs and CD8 T cells, was observed. Additionally, the TFR–TFRC interaction between monocytes and CD8 T cells, which is related to iron uptake, was detected in the bithérapie group and was associated with CD8 T cell activation in bithérapie.

- Apoptosis via TNFR1 and TNF signaling was observed exclusively in a metabolically active and effector-like CD8 subpopulation annotated as IFN-producing CD8 T cells. Within this cluster, cells from the bitherapy group displayed a stronger effector phenotype, but this heightened activation may lead to activation-induced cell death (AICD). In contrast, cells from the tritherapy group within the same cluster exhibited a more proliferative profile, associated with MYC upregulation. These findings suggest that CD8 T cells in the bitherapy group may become depleted over time due to AICD. Immune response analysis showed that changes in this cluster were also due to TNF cytokine expression in the bitherapy group.
- NK cell subpopulations which are typically associated with favorable therapy responses, were found to be increased under the bitherapy regimen. However, when DEGs identified from these NK cells were used in ORA, no enrichment of cytotoxicity-related pathways was detected. Furthermore, immune cell recruitment analysis revealed no significant differences between the two therapy groups. However, cell–cell interaction analysis revealed that NK cells in the tritherapy group may promote an M2-like macrophage phenotype via Angpt1–Itga5 interaction. A similar observation was made with the Entpd1–Adora2b interaction, where neutrophils appeared to drive macrophages toward an M2 profile. Supporting these findings, pathway analyses showed increased activity of the TGF- β and VEGF pathways in the tritherapy group.

Although numerous findings were obtained from this data analysis, it is difficult to conclude that one therapy is definitively superior to the other. One of the major limitations of this study is that it was conducted based on a single time point. Therefore, all results reflect only the immune cell profiles present in the TME at that specific day of sample collection. Considering the dynamic nature of immune cells, conducting a time-course study would provide broader and more generalizable insights into the role of anti-TNF therapy in melanoma treatment.

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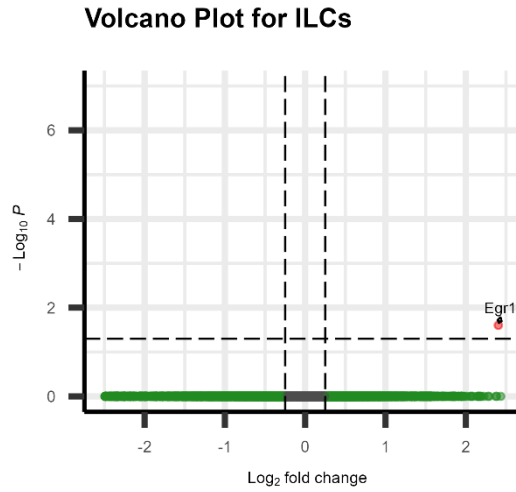
BIOGRAPHY

Kazım KÜÇÜK got his B.Sc. degree in Bioengineering from Yıldız Technical University in 2020. He started his M.Sc. in Bioinformatics and Systems Biology program at the Bioengineering Department of Gebze Technical University in 2022. He was a scholarship holder in a TUBITAK-1002-A Project during the dissertation year of his M.Sc.



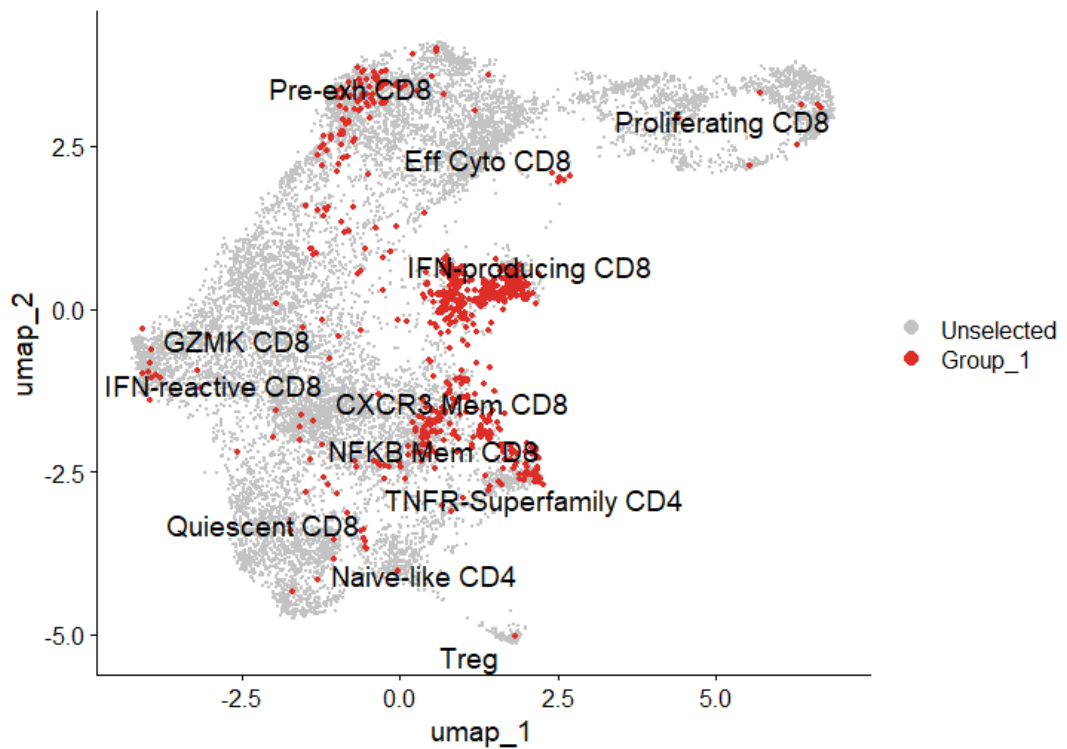
APPENDICES

APPENDIX-1: DEGs between ILCs cells (Tritherapy vs bitherapy)

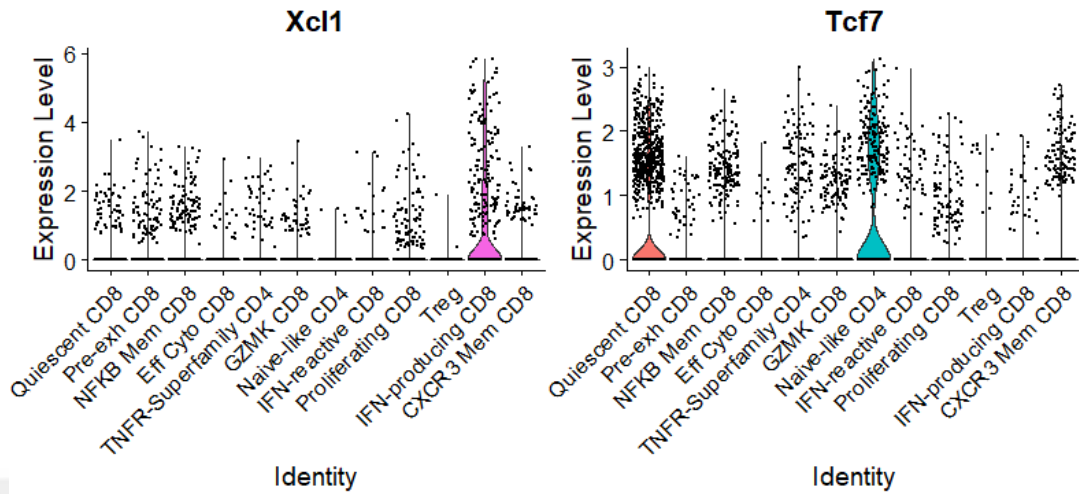


APPENDIX-2: ProjectTILs mouse data Tpex overlapped with IFN-producing cells.

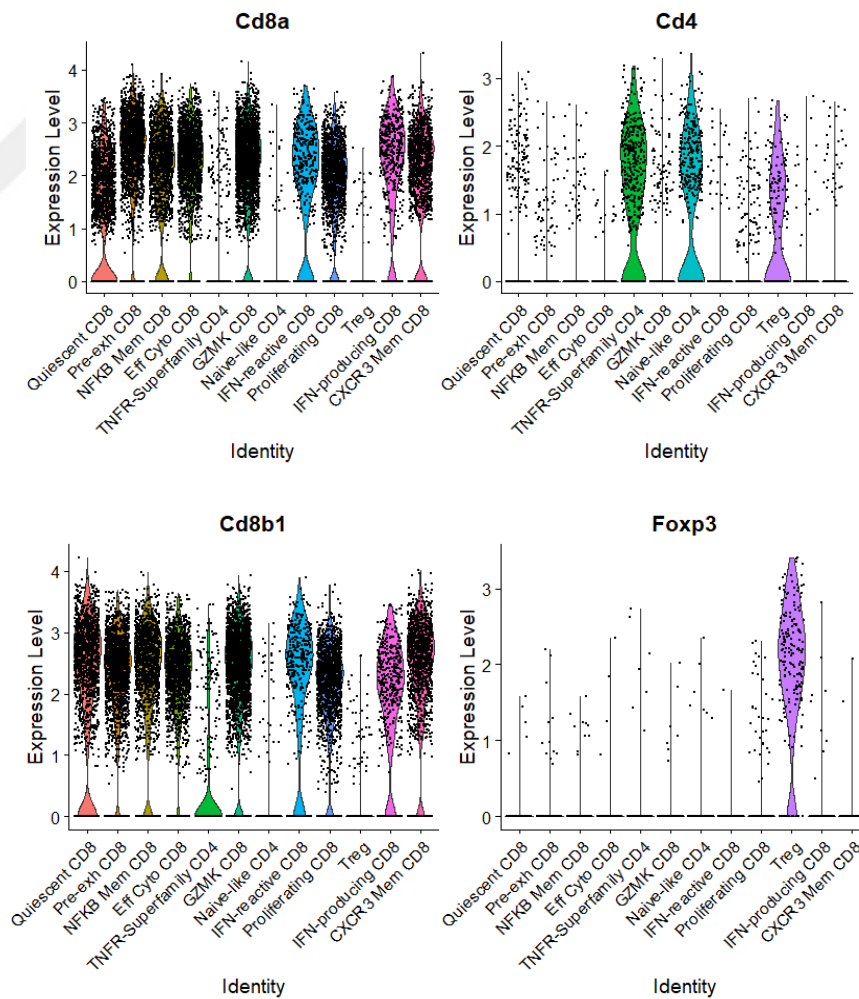
ProjectTILs mouse data Tpex cells overlapped with our data



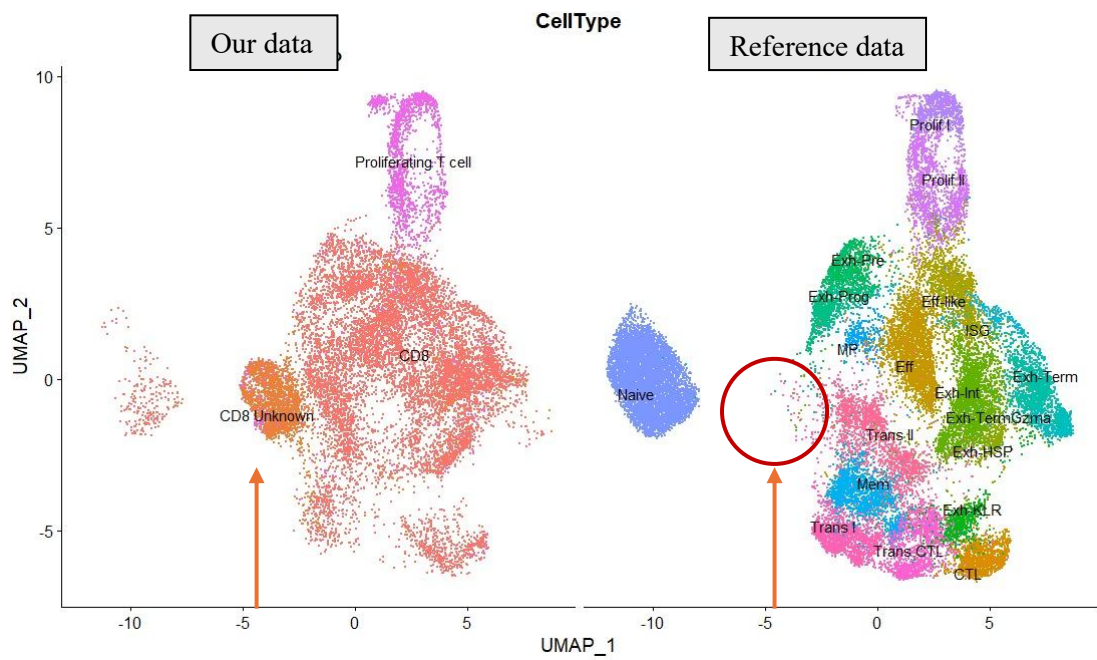
APPENDIX-3: Violin plot of normalized gene expressions of Xcl1 and Tcf7 in CD8 subsets



APPENDIX-4: Violin plot of normalized gene expression of CD4 and CD8 markers in T cell subsets



APPENDIX-5: Integration of our data and reference data



APPENDIX-6: InferCNV results comparing Unknown CD8 and ILCs/DNT cells

