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Exploiting Next-Generation Syngeneic Models to Enhance Responses to Cancer
Immunotherapy

A Dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Bryan Shing Hei Yung

Committee in charge:

Professor J. Silvio Gutkind, Chair
Professor Jack D. Bui
Professor Hannah Carter
Professor Matthew J. Hangauer

2024

The Dissertation of Bryan Shing Hei Yung is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2024

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List of Abbreviations

4NQO	4-nitroquinoline-1 oxide
ACC	adrenocortical carcinoma
ACT	adoptive cell therapy
APC	antigen presenter cell
ATP	adenosine triphosphate
BRCA	breast invasive carcinoma
BLCA	bladder urothelial carcinoma
cAMP	cyclic adenosine monophosphate
CAR-T	chimeric antigen receptor-T
cDC1	conventional DCs type 1
CESC	cervical squamous cell carcinoma and endocervical adenocarcinoma
CHOL	cholangiocarcinoma
CIMP	CpG island methylator phenotype
CIN	chromosomal instability
CNO	clozapine-N-oxide
COAD	colon adenocarcinoma
COX	cyclooxygenase
CREB	cAMP response element-binding protein
CTL	cytotoxic T lymphocytes
DC	dendritic cell
DCZ	deschloroclozapine
DLBC	lymphoid neoplasm diffuse large B-cell lymphoma
DMBA	7, 12-dimethylbenz(a) anthracene
DMH	1, 2-dimethylhydrazine dihydrochloride

DREADD	Designer Receptor Exclusively Activated by A Designer Drug
ESCA	esophageal carcinoma
FACS	fluorescent-activated cell sorting
FDA	Food and Drug Administration
fsk	forskolin
GBM	glioblastoma multiforme
GEF	guanine exchange factor
GEMM	genetically engineered mouse model
GPCR	G protein-coupled receptor
GTP	guanosine triphosphate
HNSC	head and neck squamous cell carcinoma
HPV	human papillomavirus
ICB	immune checkpoint blockade
IFN	interferon
IL	interleukin
IUPHAR	International Union of Basic and Clinical Pharmacology
KICH	kidney chromophobe
KIRC	kidney renal clear cell carcinoma
KIRP	kidney renal papillary cell carcinoma
LAML	acute myeloid leukemia
LCML	chronic myelogenous leukemia
LCMV	lymphocytic choriomeningitis virus
LGG	brain lower grade glioma
LIHC	liver hepatocellular carcinoma
LN	lymph node
LUAD	lung adenocarcinoma

LUSC	lung squamous cell carcinoma
MDSC	myeloid-derived suppressive cell
MESO	mesothelioma
MHC	major histocompatibility complex
MMRD	Mismatch-repair deficiency
MMTV	mouse mammary tumor virus
MSI	Microsatellite instability
NK	natural killer
NOD/SCID	nonobese diabetic/severe combined immunodeficiency
NSAID	nonsteroidal anti-inflammatory drug
NSCLC	non-small-cell lung cancer
OV	ovarian serous cystadenocarcinoma
OVA	ovalbumin
PAAD	pancreatic adenocarcinoma
PCA	principal component analysis
PCPG	pheochromocytoma and paraganglioma
PD-1	programmed death 1
PDAC	pancreatic ductal carcinoma
PD-L1	programmed death-ligand 1
PGE2	prostaglandin E2
PKA	protein kinase A
PRAD	prostate adenocarcinoma
READ	rectum adenocarcinoma
RNA-seq	RNA sequencing
SARC	sarcoma
SBS	single-base substitution

SCC	squamous cell carcinoma
scRNA-seq	single-cell RNA sequencing
SKCM	skin cutaneous melanoma
SNP	single-nucleotide polymorphism
STAD	stomach adenocarcinoma
TAM	tumor-associated macrophage
TCR	T cell receptor
TGCT	testicular germ cell tumors
TGF	tumor growth factor
THCA	thyroid carcinoma
THYM	thymoma
TIL	tumor-infiltrating lymphocyte
TMB	tumor mutational burden
TME	tumor microenvironment
TNF	tumor necrosis factor
Treg	regulatory T cell
UCS	uterine carcinosarcoma
UCEC	uterine corpus endometrial carcinoma
UV	ultraviolet
UVM	uveal melanoma

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Abstract of the Dissertation

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Immunotherapy

by

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Doctor of Philosophy in Biomedical Sciences

University of California San Diego, 2024

Professor J. Silvio Gutkind, Chair

Cancer immunotherapy has brought about a paradigm shift in the standard of care for cancer patients; however, the limited response rates in cancer patients underscores the persistent challenge in translating preclinical findings to effective clinical interventions. Here, we explore the imperative of 1) refining and developing preclinical animal models to better mirror human cancer and 2) identifying G protein-coupled receptors (GPCRs) as a promising avenue to improving responses to immunotherapy. Clinical success in the discovery and development of anti-cancer

drugs have relied on the use of preclinical animal models, namely mice, to recapitulate tumor growth kinetics and tumor-intrinsic elements of human disease. However, many of the most commonly used syngeneic models for cancer immunotherapy do not properly resemble the disease state for which they were designed, and this discrepancy has been associated with a high attrition rate of preclinical and early-stage drug candidates failing to gain Food and Drug Administration (FDA) approval. This underscores the need to validate pre-existing models for disease relevance as well as to develop new pre-clinical models that can translate to human responses. Using new and pre-existing preclinical models, our research has identified GPCRs as critical regulators of the immune response within the tumor microenvironment (TME). GPCRs are the most intensively studied drug targets since they play key roles in many physiological processes, and they have remained longstanding favorable pharmacological targets. Here, we target GPCRs that have been identified on dysfunctional T cells using RNA-seq to relieve the immunosuppressive tumor microenvironment. Preliminary data shows an upregulation of $G\alpha_s$ -coupled receptors, PTGER2, PTGER4, and ADRB2, on activated and exhausted T cells in HNSC patients, suggesting that these GPCRs that are coupled to the G protein $G\alpha_s$, and their downstream signaling cascades may be dampening anti-tumor cytotoxicity of CD8 T cells, leading to T cell dysfunction and exhaustion.

Introduction

Cancer Immunotherapy

Cancer immunotherapy has primarily focused on improving the capacity of T lymphocytes in their antigen-directed cytotoxicity of tumor cells. In the past two decades, advances in the field of cancer immunotherapy have solidified the use of immune-modulating agents in the treatment of cancer, alongside surgery, radiation, chemotherapy, and targeted therapy. Basic science discoveries have elucidated molecular and cellular biology of T cells that have led to new treatment strategies, including immune checkpoint blockade (ICB) and adoptive cellular therapy. These treatments represent a shift in the approach to cancer therapy as they enhance the immune system to respond to cancer cells, rather than targeting cancer cells directly. In 2011, ipilimumab (anti-CTLA-4 antibody) became the first successful immune checkpoint blockade (ICB) therapy approved by the Food and Drug Administration (FDA) for cancer, followed by two antibodies against programmed cell death 1 (PD-1) in 2014, pembrolizumab and nivolumab. By 2018, the Nobel Prize in Physiology or Medicine was awarded to Drs. James Allison and Tasuku Honjo for their contributions to these discoveries in which inhibiting negative regulators of T cell activation and function improved patient outcomes. ICB is now the standard of care for multiple indications, including advanced melanoma¹, non-small cell lung cancer (NSCLC)², and renal cell carcinoma³. For context, the objective response rate was 40% in the nivolumab (anti-PD-1) group compared to 13.9% in the dacarbazine group, showing a clear survival benefit (CheckMate-066, NCT01721772). In another avenue, two chimeric antigen receptor-T (CAR-T) therapies were approved by the FDA in 2017 for the treatment of relapsed or refractory diffuse large B-cell lymphoma (NCT02435849). These are a type of adoptive cell therapy (ACT) that involve genetically engineering patient T cells to express synthetic receptors on the cell surface to detect tumor-specific antigens and eradicate tumor cells, which are then adoptively transferred back into the patient have resulted in robust and durable responses. In some cases, complete response

rates of up to 74% were observed in patients⁴⁻⁶, compared to objective response rates of 26% (7% complete response) using rituximab-based regimens⁷.

Despite these rapid and life-saving advances in the field, the majority of patients still do not respond to these treatments. Therefore, the main goal for cancer immunotherapy is developing agents that are consistently effective in all patients and cancer types. To achieve this goal, we must overcome challenges in developing pre-clinical models that translate to human responses and uncovering molecular and cellular mechanisms of immune escape to immunotherapy.

Tumor Microenvironment

Cancer is a malady caused by genetic mutations resulting in uncontrolled division and survival of transformed cells. While we appreciate that these genetic alterations are necessary in initiation and disease progression, cancer is also a complex environment of extrinsic factors that extend to interactions with non-cancerous cells. The tumor microenvironment (TME) is a diverse ecosystem containing immune cells, cancer-associated fibroblasts, endothelial cells, pericytes, as well as tissue specific cells such as neurons and adipocytes. Cells composing the tumor express and secrete molecules that shape and influence the structure of the TME, and understanding the role of these molecules in pathogenesis or in tumor regression can reveal potential drug targets that may enhance clinical efficacy.

Due to the advances in cancer immunotherapy which demonstrated the efficacy of ICB in the modulation of immune cells within the TME leading to strong anti-tumor responses, retrospective analysis in patient populations treated with ICB revealed three broad classes of TME that are associated with differential responses to immunotherapy: Immune-inflamed (or hot), immune-excluded, immune-desert (**Figure 0.1**). Immune-infiltrated tumors are considered immunologically “hot” tumors and are characterized by the presence of cytotoxic lymphocytes (CTLs) in the central tumor, specifically CD8⁺ T cells, that express PD-1. Mismatch-repair

deficiency (MMRD)/Microsatellite instability (MSI) high cancers have higher rates of nonsynonymous single-nucleotide polymorphisms (SNPs), which lead to higher numbers of neoantigens and tumor-infiltrating lymphocytes (TILs). Neoantigens are foreign proteins that are not normally found in healthy tissue, but arise from tumors due to genetic mutations, transcriptomic variants, post-translational modifications, and viral-related genes which can be recognized by the host immune system. As a result, patients with MSI-high tumors generally respond better to ICB therapy compared to those with MSI-low⁷⁻⁹. Despite the presence of ongoing tumor immunity, suppression mechanisms are present to subdue the activity of anti-tumoral T cells, allowing these tumors to progress. The immune-desert phenotype is typically characterized by a lack of lymphocytes in the tumor or in the periphery, usually due to the lack of pre-existing anti-tumor immunity. The immune-excluded phenotype is characterized by an abundance of immune cells in the TME, but they are sequestered and confined in the stroma and are unable to penetrate the central tumor. The mechanisms by which tumors are able to exclude CTLs or escape anti-tumor immunity are still under active investigation, which include physical and functional barriers. Physical barriers such as stromal fibrosis in the periphery of the TME may lead to physical exclusion of immune cells. Functional barriers may include the recruitment of suppressive immune cell populations as well as altering the metabolic microenvironment to become more acidic and hypoxic can lead to functional impairment of CTLs. Furthermore, tumors can escape detection by altering the molecular gradient of chemokines such as CXCR3, which are used in the recruitment of CTLs into the TME. Both immune-cold and immune-excluded have been linked with patient prognosis to ICB, with the former corresponding to worst outcomes¹⁰⁻¹². While these tumor phenotypes are likely an oversimplification of the dynamic features of the TME, they are a useful classification system that provides insight into different mechanisms that influence tumor-immune interactions leading to differential responses to immunotherapy.

Suppressive mechanisms within the TME interfere with the cancer immunity cycle, facilitating tumor escape from immune surveillance (**Figure 0.2**). The cancer immunity cycle

begins with mutations accumulating within cancer cells, leading to the generation of neoantigens which are recognized by the immune system. Subsequently, professional antigen presenter cells (APCs) take up these antigens on major histocompatibility complex (MHC) I and II molecules and present them to T cells within lymph nodes. Antigen-specific T cells are then primed and activated against their cognate neoantigens, resulting in the upregulation of effector molecules such as cytolytic proteins, cytokines, co-signaling receptors, and chemokine receptors. These licensed T cells then traffic into blood vessels towards and into the tumor. Once infiltrated into the TME, these fully activated antigen-reactive T cells recognize and bind to cancer cells through interactions between their T cell receptor (TCR) and its cognate antigen bound to MHC I on the cancer cell surface, resulting in the release of cytokines and cytolytic molecules that kill their target cancer cell. The resulting cancer cell death results in the release of more neoantigens and tumor-associated antigens, leading to increased breadth and depth of the immune response in subsequent iterations of this cycle. This conceptual framework allows for a mechanistic understanding of how tumor-T cell interactions are disrupted in cancer patients, in which tumor cells can evade immune surveillance by affecting different phases of the cancer immunity cycle.

Cancer immunity as described by this cycle is self-propagating, where continual recognition and killing of tumor cells by T cells results in the release and generation of immune-stimulatory factors that should continually amplify and broaden T cell responses. However, tumor cells under constant survival pressure may evolve to escape immune surveillance, which in normal circumstances immune cells are able to identify and eliminate malignant cells, through a process named immunoediting. One of the main mechanisms of immunoediting involves downregulating the expression and presentation of tumor-specific antigens. This can occur through immune selection of cancer cells that lack or mutate immunogenic tumor antigens as well as through the acquisition of defects in antigen presentation, such as loss of MHC expression or dysregulation of antigen processing machinery^{13,14}. As such, downregulation of MHC-I has been found in approximately 20 to 60% of common solid tumors, and changes in MHC expression have

correlated with clinical response^{15,16}. Furthermore, while neoantigens themselves are inherently poorly immunogenic due to closely resembling self-proteins, loss of peptide:MHC as well as the presentation of poorly immunogenic neoantigens results in ineffective targeting of CTLs to cancer cells. Another method of immune escape that tumor cells leverage is upregulating co-inhibitor surface receptors that suppress the activity of CTLs. Homeostatic regulation of T cell activation through inhibitory feedback mechanisms are built into the immune response, to prevent over-active immunity leading to auto-immune disease (**Figure 0.3**). Under normal conditions, co-stimulatory molecules such as CD28 contribute to the second signal required after TCR recognition of peptide:MHC that is necessary to generate robust T cell response¹⁷. Co-inhibitory molecules are then subsequently expressed in activated T cells to moderate and constrain the intensity of activation for optimal immunity and tolerance. This includes one of the most well-studied immune checkpoint molecules PD-1, which can have inhibitory functions in the periphery after activation. Tumor cells have been demonstrated to upregulate the expression of PD-L1, the cognate ligands for PD-1, especially within immunologically hot tumors. Furthermore, tumoral PD-L1 expression has been used as a predictive biomarker of response to anti-PD-1 and anti-PD-L1 therapies in patients with a variety of tumor types, as demonstrated in early studies evaluating the safety and efficacy of nivolumab and atezolizumab^{12,18,19}. Other commonly expressed co-inhibitory receptors that are being investigated for clinical intervention include TIM-3, LAG-3, and TIGIT. Another contributor to immune escape of cancers is the creation immune tolerance through the recruitment of suppressive immune cell populations. While immunologically hot tumors are infiltrated with anti-tumor CTLs and are better prognostic predictors of responses to immunotherapy, the presence of tumor-specific T cells is not sufficient for tumor control, suggesting that other immune cell populations could contribute to the immunosuppressive TME. Indeed, numerous other immune cells have been reported to have suppressive functions in the TME, including regulatory T cells (Tregs), tumor-associated macrophages (TAMs), myeloid-derived suppressive cells (MDSCs), and neutrophils. Due to inflammation being a cornerstone of

cancer initiation and progression, these tumor-promoting immune cells are often seen in the context of wound-healing, to prevent the spread of inflammation and limit tissue damage. As part of their natural function, immune suppressive cells such as Tregs can inhibit anti-tumor immune responses through the production of cytokines, such as tumor growth factor(TGF)- β and interleukin (IL)-10. Furthermore, these cells can also contribute co-inhibitory signaling on responding anti-tumor T cells through the expression of co-inhibitory ligands and receptors. For example, CTLA-4, a CD28 homolog that binds to B7 family molecules CD80/CD86 and has been identified to be a key regulator of T cell activation by contributing to downstream signaling that interfere with CD28 signaling, is highly expressed on Tregs^{20,21}. While the development of immunotherapy has achieved great clinical results, the diverse repertoire of mechanisms cancer cells are able to employ to remodel the TME to escape immune surveillance remains an obstacle in achieving complete responses.

T cell Dysfunction

While cancer cells can immunoedit to become resistant to immune detection and elimination, the remodeling of the TME by cancer cells into a hostile environment for responding anti-tumor T cells also results in an impaired T cell differentiation state known as exhaustion. T cell exhaustion was initially identified in virus-specific CD8 T cells responding to chronic lymphocytic choriomeningitis virus (LCMV) that were persistent but functionally impaired²²⁻²⁴. Normally under acute infection or following vaccination, naïve CD8 T cells undergo robust proliferation and clonal expansion to differentiate into effector CD8 cells that directly kill target cells and control infections. Effector differentiation is accompanied by transcriptional, epigenetic, and metabolic reprogramming resulting in the acquisition of essential effector functions such as cytokine and cytotoxic molecule production. Following antigen clearance and resolution of inflammation, most of the effector CD8 population contracts while a small subset of memory precursors persists and differentiate into memory T cells. Memory T cells downregulate their

effector program and acquire more stem-like ability, allowing them to survive in the absence of antigen as long-lived cells that undergo homeostatic maintenance driven by IL-7 and IL-15 and to confer protective immunity^{25,26}. In contrast to acute infection and vaccination, chronic infection and cancer results in persistent antigen burden resulting in defects in the development of memory T cells, leading to T cell exhaustion^{27,28}.

Persistent exposure to antigen is a key feature that is shared between mouse models of chronic infections or cancer and human chronic infections and cancers (**Figure 0.4**). Studies have shown that CD8 T cells that are primed during a chronic infection can recover and develop into memory T cells if they are adoptively transferred into an antigen-free host during the first weeks of infection^{29,30}. This coincides with an inflection point in transcriptional and epigenetically reprogramming where CD8 T cells become locked into exhaustion differentiation, which can be observed in the timescale of hours in some models of cancer³¹⁻³³. As mentioned previously, cancer cells, immune cells, and non-immune cells that compose the TME could contribute to the suppressive environment by secreting soluble factors, including cytokines such as IL-10 and TGF- β which are associated with attenuating T cell activation and effector functions^{34,35}. In acutely resolved infections, a highly proinflammatory environment promotes the development of effector T cells, leading to the development of memory T cells as the inflammation resolves³⁶. However, sustained high levels of these cytokines in the context of cancer and chronic infection can induce the development of T cell exhaustion, but are not sufficient alone to drive T cell exhaustion³⁷. Currently, neutralizing therapies targeting both IL-10 and TGF- β have shown to enhance CD8 responses and reduced viral burden; both of which are currently being investigated for cancer immunotherapy^{38,39}. Other non-cytokine mediators have also been implicated in T cell exhaustion, such as prostaglandin E2 (PGE2) and adenosine. PGE2 is a lipid mediator that is reported to both a proinflammatory molecule and an immunosuppressor⁴⁰⁻⁴². Studies have shown that PGE2 suppress effector T cell survival and function, and that inhibition of its signaling through its receptors EP2 and EP4 on T cells synergizes with PD-1 blockade. Adenosine can also function

as an immunosuppressor in settings where extracellular adenosine concentration is high, such as in cancer^{43,44}. Adenosine can signal through adenosine A2A receptor (ADORA2A), where it has been shown to suppress T cell function and contribute to exhaustion⁴³. Furthermore, the expression of CD39 and CD73, two ectoenzymes that convert extracellular adenosine triphosphate (ATP) and adenosine diphosphate into adenosine, are highly expressed by exhausted CD8 T cells^{45,46}. While this is not an exhaustive list of all the possible contributors to the development of T cell exhaustion, sustained antigen stimulation leading to chronic TCR activation, exposure to soluble factors such as cytokines and proinflammatory mediators, and the presence of suppressive cells in the TME can all contribute to the development of T cell exhaustion.

Exhausted T cells are functionally, transcriptionally, and epigenetically distinct from effector and memory T cells, characterized by the impairment of effector functions, elevated and sustained expression of co-inhibitory receptors, altered expression of transcription factors, and poor response to homeostatic cytokines^{47,48}. Severely exhausted T cells have been shown to lose effector functions in a progressive pattern during chronic infection, where loss of IL-2 production occurs early followed by defects in tumor necrosis factor (TNF) production and in cytotoxic capacity by granzyme B production⁴⁹. Defects in interferon (IFN)- γ production occur at more severe and later stages of exhaustion and correlates with terminally exhausted T cells^{50,51}. Interestingly, exhausted T cell populations may retain the ability to degranulate (measured by CD107a) and more terminal subsets of exhausted T cells retain slightly better cytotoxic capacity⁵². The final stage of exhaustion can result in physical deletion in some settings where antigen burden is high²³.

As T cells become progressively more dysfunctional in the context of chronic antigen stimulation, loss of effector function into deeper stages of T cell exhaustion is often accompanied by elevated and sustained expression of co-inhibitory receptors, such as CTLA-4, PD-1, TIM-3, LAG-3 and TIGIT. Normally, the expression of these co-inhibitory receptors is transient in effector

T cells, where they normally function to constrain T cell activation and function at the end of infection to prevent autoimmunity. The expression of these receptors decreases as antigen is cleared, resulting in low expression in memory T cells that have experienced antigen. However, under chronic infection and cancer conditions, co-inhibitory receptor expression is high and sustained after antigen exposure, and exhausted T cells acquire a diverse and concurrent expression of multiple co-inhibitory receptors, significantly impacting their normal function. As described earlier, not only are these inhibitory receptors expressed on the T cell surface, but their ligands are also upregulated in APCs, tumor cells, and other cells during cancer as well as chronic infection, resulting in the inability of antigen-specific T cells to mount and sustain adequate responses. Targeting these inhibitors are the foundation of immune checkpoint blockade, in which PD-1 and CTLA-4 blocking antibodies show clinical efficacy in humans with chronic infections and cancer. These functional and phenotypic changes in exhausted T cell differentiation can be attributed to divergent transcriptional programs as compared to those from effector and memory T cell development.

Despite many of the key studies of T cell exhaustion where from models of infection, it is now clear that T cell exhaustion is a clinically relevant T cell differentiation state that occurs not only in chronic infection, but also in cancer, where tumor-specific CD8 T cells display hallmarks of dysfunction and exhaustion in mouse models of cancer as well as human cancers, including melanoma, leukemia, ovarian cancer, non-small cell carcinoma, and others⁵³⁻⁵⁸. Early work in tumor models showed the potential of blocking inhibitory receptor pathways, including CTLA-4 and PD-1 as mentioned earlier, to mediate tumor regression^{59,60}. Despite remarkable success pre-clinically leading to clinical application, the lack of complete responses in patients suggests that other immune suppressive pathways outside of PD-1 and CTLA-4, including LAG-3, TIM-3, and others, may be contributing to the challenge of reinvigorating exhausted T cell populations. Indeed, co-expression of multiple inhibitory receptors on exhausted T cells is common in the context of cancer and chronic infection, suggesting compounding negative regulation of

exhausted T cells by multiple inhibitory receptors and other pathways. There has been robust and synergistic effects of co-blockade of PD-1 and CTLA-4 in mouse models of cancer⁶¹ and has been clinically successful in melanoma patients⁶²⁻⁶⁴. Furthermore, similar co-blockade effects have been observed with targeting other combinations of co-inhibitory receptors simultaneously. While the precise pattern of exhausted T cell function may vary from disease to disease, key hallmarks of exhausted T cell differentiation can provide potential immunotherapeutic opportunities to target subtypes of exhausted T cells and insights in whether T cell exhaustion can be prevented or reversed. Identifying and targeting additional key contributors to T cell exhaustion, such as these soluble molecules and inhibitory receptors, could be a potential strategy to reverse the effects of the suppressive TME on T cell function and exhaustion.

Current Mouse Models in Cancer Immunotherapy Research

As mentioned previously, the clinical success of treating cancer with immunotherapies such as ICB and ACT have demonstrated the potential to transform treatment strategies and improve patient outcome. Despite these successes, response to immunotherapy has been restricted to a minority of patients. To broaden the populations that benefit from immunotherapy, a greater understanding is needed of the factors that accurately affect response. As discussed earlier, the level of T cell infiltration can vary between individual tumors, patients, and tumor subtypes (e.g. immune-excluded), and these tumor phenotypes can correlate with patient prognosis and outcome following immunotherapy. Our basic science knowledge of cancer immunotherapy and the advancements in the development of anticancer drugs has come from our ability to recapitulate characteristics of human disease, such as tumor growth, in mammalian organisms; in doing so, we are able to demonstrate measurable effects of new therapeutics targeting cancer and enhancing anti-tumor immunity. For cancer immunology, mice (*mus musculus*) have been the model organism to increase our understanding of the pathophysiology

of human cancers and to facilitate the pharmacological evaluation of approved and potential new drug candidates. The use of preclinical mouse models has led to many key discoveries in immunotherapy and cancer treatment, including the anti-tumor effects of monoclonal antibodies against CTLA-4 and PD-1^{59,65}. Though human cancer immunology may not always be faithfully mirrored in commonly employed models⁶⁶, various model systems have been employed over the years to simulate specific facets of human immunology.

One of the simplest and most cost-effective cancer models are the transplantable subcutaneous xenograft and syngeneic cell lines (**Figure 0.5**). These models originate from cultured cell lines for easy transplantation subcutaneously, allowing for easy observation as the disease advances beneath the skin surface. Cell-line xenografts are typically derived from human patient cancer cells, and as such can only be grown in immunodeficient mice (e.g., nonobese diabetic (NOD)/severe combined immunodeficiency (SCID)). While useful for determining the toxicity, dosing, and safety of cytotoxic and targeted therapies directed at cancer cells, these models are agnostic to the role of the immune system. Syngeneic implantable models surmount this obstacle, enabling the growth of tumors that allow for the intricate dynamics of immune infiltration and remodeling within the tumor microenvironment. The utilization of cell-line transplants ensures reliable reproducibility, as these clonal cell lines are generally implanted simultaneously, enhancing the consistency required for effective target validation. Furthermore, some models can be inoculated orthotopically, which may better recapitulate the human tumor microenvironment⁶⁷. Syngeneic mouse models can be generated from a variety of methods, including the use of genetic engineering, exposure to environmental agents (e.g. carcinogens, radiation), spontaneous growth, or a combination of these factors. Despite their feasibility, these models do not always reflect the complexity of the development of the original tumor and its microenvironment since many of these implantable models develop heterotopically rather than at their initial anatomical position. Another drawback of using implantable cell lines is the lack of clonal diversity found in the developing tumor, which does recapitulate the heterogeneity of cancer

cells in human patients. Furthermore, these models also do not represent the multistage process of carcinogenesis, which can be found in genetically engineered mouse models (GEMMs). Despite their ease of use, these commonly used mouse models may have poor predictive value, as observed by high clinical trial attrition rates. While taking into account these limitations, syngeneic mouse models offer opportunities to investigate whether a drug's mechanism of action aligns with its therapeutic effect in an expedient and cost-effective manner. This highlights an ongoing need for developing and profiling newer models that can be directly applicable for immunotherapy drug development.

GEMMs are models in which tumors may spontaneously arise by the action of genetic alterations in key oncogenic drivers (e.g., BRAF, KRAS, MYC) and tumor suppressor genes (TP53, PTEN, CDKN2A) (**Figure 0.5**). These models feature stochastic and early characteristics of tumorigenesis, resulting in tumors that may more closely recapitulate human diseases⁶⁸. Due to these key features, GEMMs have successfully predicted clinical efficacy in studies investigating the use of combination drug inhibitors to epidermal growth factor receptor and vascular endothelial growth factor in mutant KRAS-driven GEMMs of NSCLC and pancreatic ductal carcinoma (PDAC)⁶⁹. While GEMMs may have demonstrated better predictive value for clinical responses, their uptake in drug development has been limited due to the complexity of generating these models. Compared to syngeneic mouse models which may develop palpable tumors as early as days, tumors from GEMMs can require 2-10 months to reach an appropriate size for therapeutic intervention, therefore requiring a substantial financial and time investment in a breeding colony. Additionally, GEMMs also lack the tumor mutational burden (TMB) of human cancers, since they are primarily driven by a handful of genes; therefore, they do not fully capture the heterogeneity of human cancer and do not elicit proper immune surveillance. This may affect the predictive efficacy of these models since high tumor mutational burden has been associated with a better prognosis after treatment with ICB⁷⁰⁻⁷³.

Depending on the context for therapeutic intervention, each model system has benefits and drawbacks in their use. Syngeneic mouse models have shorter latency periods, reproducible, recapitulate TMB, and high throughput, whereas GEMMs recapitulate anatomical location and contain disease-specific mutations. In essence, there is an urgent need for advancing next-generation mouse models with enhanced predictive capabilities for clinical efficacy. Additionally, it is crucial to thoroughly characterize existing tumor models to identify their relevance to specific human cancers or disease processes.

Investigating G Protein-Coupled Receptors to Enhance Immunotherapy

While cancer immunotherapy has advanced the standard of care for the treatment of cancer, the majority of patients receiving these treatments do not fully respond. Indeed, responses across different indications have had mixed results in objective response rates to different immune checkpoint inhibitors targeting PD-1/PDL-1 and CTLA-4. This suggests that there are other additional inhibitory mechanisms that tumors can employ, other than activation of co-inhibitory signaling, to escape anti-tumor immunity. One potential area for therapeutic discovery is in the superfamily of G protein-coupled receptors (GPCRs).

GPCRs represent the largest protein family encoded by the human genome, with over 800 identified members. The common feature shared by all GPCRs is the presence of a 7-transmembrane domain structure, which includes an extracellular N terminus and an intracellular C terminus. As a result of advancements in alignment tools and gene ontology, 342 functional non-olfactory human GPCRs have been documented and classified into major receptor families based on sequence similarity and function⁷⁴⁻⁷⁶. GPCRs interact with a diverse array of agonists, spanning ions, amines, purines, lipids, peptides, and proteins. Upon agonist binding, conformational changes occur in the extracellular loops of the transmembrane region for ligand binding, and primarily in the second, third, and fourth intracellular loops, fostering receptor activation and coupling with $\alpha\beta\gamma$ G proteins^{77,78}. Upon activation, the receptor engages with

heterotrimeric G proteins, triggering the release of GDP from the $G\alpha$ subunit and its substitution with GTP (**Figure 0.6**)^{79,80}. This exchange leads to the functional detachment of the GTP-bound α subunit from the $\beta\gamma$ dimers. GPCRs can activate one or multiple $G\alpha$ proteins, which can be subdivided into four major families: $G\alpha_s$, $G\alpha_i$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$. $G\alpha_s$ activates adenylyl cyclases to catalyze the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP), which serves as a second messenger and initiates the activation of protein kinase A (PKA)^{81,82}. The $G\alpha_i$ family members mainly oppose the actions of $G\alpha_s$ by suppressing cAMP synthesis, as well as activating diverse phospholipases and phosphodiesterases, and facilitate the opening of multiple ion channels. Meanwhile, the $G\alpha_{q/11}$ family converts phosphatidylinositol 4,5-bisphosphate into DAG and inositol 1,4,5-trisphosphate, thereby triggering the activation of PKC and increasing intracellular Ca^{2+} concentrations. Lastly, $G\alpha_{12/13}$ signaling involves a family of Rho guanine exchange factors (GEFs) which associate with active $G\alpha_{12/13}$ to stimulate Rho GTPase⁸³. Once dissociated from the $G\alpha$ subunit, the $G\beta\gamma$ dimers also play a central signaling role, including ion channel regulation as well as GPCR signaling attenuation. Ultimately, activation of GPCR signaling initiates the activity of various $G\alpha$ and $G\beta\gamma$ subunits depending on each GPCR's G protein coupling specificity, which can also be distinct for each of their ligands (i.e. biased agonism), the intensity and duration of receptor activation, and the level of expression of each G protein subunit and the repertoire of signaling molecules expressed in each cell type.

The significance of GPCRs lies in their multitude of biological functions, encompassing key physiological processes such as vision, olfaction, neurotransmission, hormone and enzyme release, immune response, hemostasis, cardiac response and blood pressure regulation, epithelial cell renewal, stem cell fate decisions, tissue development, and homeostasis. Hence, GPCR dysfunction contributes to over 30 different human diseases, such as type 2 diabetes mellitus, obesity, depression, Alzheimer's disease, cancer, and many others⁸⁴. As a result, drugs targeting GPCRs represent approximately 34% of all FDA-approved drugs, with over 475 approved drugs targeting 108 unique GPCRs^{85,86}. Furthermore, the discovery of hotspot

mutations within G protein, possessing oncogenic properties, has accelerated research in this field. For example, GNAS (encoding the $G\alpha_s$ subunit) has been linked to pro-inflammatory functions, mimicking the chronic inflammatory state of during tumor development. $G\alpha_s$ is extensively studied for its involvement in the actions of inflammatory agents such as prostaglandins derived from cyclooxygenase (COX) 2. Its inflammatory impact in cancer is notably exemplified in colon neoplasia, where COX2-generated prostaglandin E2 (PGE2) accelerates colon cancer advancement by activating PI3K and AKT pathways and alleviating the inhibitory phosphorylation of β -catenin, thus contributing to $G\alpha_s$ -driven oncogenic signaling⁸⁷. Mutations in GNAQ and GNA11 (encoding $G\alpha_q$ and $G\alpha_{11}$, respectively) are particularly significant in the occurrence of uveal melanoma^{88,89}. Approximately 93% of patients exhibit mutations in these genes, leading to the production of constitutively active $G\alpha_q$ family members. Not only is aberrant G protein signaling driving oncogenesis, many GPCR ligands are produced in significantly higher amounts in the hypoxic and acidic tumor microenvironment, which may drive positive feedback mechanisms that enhance disease progression. Furthermore, the identify of these secreted ligands and their impact on the immune system has yet to be fully elucidated.

Despite being the largest family of targetable receptors, GPCRs have been understudied in the field of cancer immunology, having only recently being targeted for cancer. As described previously, discoveries surrounding the TME revealed the importance of the composition of its associated immune cells to patient responses to ICB. The best studied role of GPCRs have been focused on the role of chemokine receptors, which facilitate cell migration of lymphocytes towards chemokine gradients and localization into tissues and organs⁹⁰. For anti-tumor immunity, cytotoxic immune cells such as natural killer (NK) cells and CD8 T cells that are reactive to tumor antigens are guided by chemokine GPCRs to the tumor, where they can secrete cytotoxic molecules to induce tumor cell death. CXCR3 on CD8 T cells is a chemokine receptor that is upregulated following T cell priming and recall, facilitating the migration of cells to the periphery and interact with APCs within tissues. CXCR3 binds the ligands CXCL9, CXCL10, and CXCL11, which are

secreted by a variety of cells upon exposure to inflammatory cytokines (e.g. IFN α , β , and γ)⁹¹⁻⁹³. The expression of CXCL9, 10, and 11 are part of the interferon gene signature that is often used to predict favorable responses to anti-PD-1 treatment^{94,95}. These chemokines are used to facilitate interactions of T cells with dendritic cells (DCs), which capture cancer cell antigens on MHC-I and MHC-II molecules and present to antigen-reactive CD8 and CD4 T cells, respectively. A subset of DCs called conventional DCs type 1 (cDC1) exclusively express XCR1 (the chemokine receptor for XCL-1) and have been shown to drive anti-tumor responses by enhancing CD8 T cell-driven immune responses⁹⁶⁻⁹⁸. Conversely, tumors can deploy multiple mechanisms to avoid immune destruction, including the recruitment of MDSCs which can remodel the microenvironment to become highly immune-suppressive by secreting cytokines such as IL-6, IL-10, and transforming growth factor- β ⁹⁹. This can result in the accumulation of suppressive Tregs and the polarization of macrophages to a more suppressive phenotype, often referred to as M2-like or tumor-associated macrophage (TAM) phenotype¹⁰⁰. These suppressive immune cell populations also express ligands for inhibitory receptors on T cells, such as PD-L1, to restrain T cell activation and promote exhaustion. The chemokine receptor CCR8 is a receptor for CCL1 and is highly expressed in intratumoral Tregs. CCR8 signaling is thought to contribute to Treg cell suppressive function and has been found to promote Treg cell survival¹⁰¹. In essence, a range of chemokines orchestrates the infiltration of various anti-tumoral and immune suppressive cells into the tumor microenvironment. Through these mechanisms, tumors can adapt to evade detection and destruction by the innate and adaptive immune systems. This presents an opportunity to disrupt these immune evasion tactics by targeting chemokine receptors using a growing array of small molecule inhibitors, negative allosteric modulators¹⁰², and/or blocking antibodies. These therapeutic approaches are either in clinical use or undergoing evaluation for various chronic inflammatory conditions. Exploring their application in cancer immunotherapy promises to be a captivating frontier in the field of GPCR targeting.

As mentioned earlier, tumor cells also leverage soluble mediators within the TME to drive immune suppression to favor tumor growth. Adenosine is typically released from injured and necrotic cells by extracellular breakdown of ATP by CD39 and CD73. Normal physiological levels of adenosine are typically at the nanomolar level; however, levels of adenosine can rise to micromolar ranges within sites of injury and hypoxia such as the TME. Adenosine can signal through four GPCRs, A1, A2A, A2B, and A3 receptors (encoded by ADORA1, ADORA2A, ADORA2B, and ADORA3)¹⁰³. A1 and A3 receptors signal through G α_i , whereas the activation of A2A and A2B receptors, which are expressed on immune and endothelial cells, leads to signaling through G α_s proteins. Additionally, A2B can also signal through G α_q ¹⁰⁴. Among the four adenosine receptors, the A2A receptor (encoded by the ADORA2A gene) emerges as the predominant subtype expressed in most immune cells. Broadly speaking, activation of the A2A receptor delivers an immunosuppressive signal in T cells¹⁰³, NK cells¹⁰⁵, and DCs¹⁰⁶. A2A receptor activation disrupts the migration of T cells and NK cells by desensitizing chemokine receptors and diminishing levels of pro-inflammatory cytokines¹⁰⁷. Inhibiting the adenosine-producing pathway has exhibited regression of tumors in breast cancer, colorectal cancer, and melanoma, while both small molecule inhibitors targeting the A2A receptor and blocking antibodies against CD73 and CD39 are presently undergoing assessment for combination cancer immunotherapies^{108,109}. Despite the objective of these immunotherapies to enhance immune cell activity within the immunosuppressive tumor microenvironment, it remains crucial to acknowledge the impacts of tumor-induced inflammation, largely mediated by prostaglandins and prostaglandin receptors. Prostaglandins are a group of active lipid compounds found in almost every tissue in humans and animals, and they play a key role in the generation of an inflammatory response¹¹⁰. They are derived enzymatically from arachidonic acid by the COX isoforms, namely COX1 and COX2, and serve as potent vasodilators¹¹¹. Among them, PGE2 stands out as the most abundant prostaglandin produced in cancers. The prostanoid receptor family, comprising GPCRs, includes EP1 (PTGER1), EP2 (PTGER2), EP3 (PTGER3), and EP4 (PTGER4). EP1 is coupled to G α_q ;

EP3 is coupled to G α _i, while both EP2 and EP4 are coupled to G α _s. Binding of PGE2 to different EP receptors regulates the function of various immune cell types, including macrophages, DCs, T cells, and B cells. PGE2 synthesized by cancer cells correlates with increased expression of FOXP3 in Treg cells, thus promoting the immunosuppressive activity of Tregs¹¹². Additionally, PGE2 is associated with enhanced recruitment of MDSCs¹¹³, reduced CD8 T-cell activation¹¹⁴,¹¹⁵, and elevated expression of inhibitory markers like PD-1^{113,114}. PGE2 affects the differentiation, maturation, and cytokine secretion of DCs by upregulating CD25 (the alpha chain of the IL-2 receptor) and indoleamine-pyrrole 2,3-dioxygenase while decreasing expression of CD80, CD86, and MHCI maturation markers¹¹⁶. Recently, NSAIDs blocking COX2 and/or COX1 and COX2 have demonstrated beneficial effects in reducing the risk of esophageal, stomach, skin, and breast cancers, complementing their established role in preventing colorectal cancer^{117,118}. As we investigate the role of GPCRs on immune cells, we may uncover potential new roles outside of chemotaxis that can influence anti-tumor immune responses. Since revitalization of exhausted T cells remains incomplete, understanding the role of GPCRs on immune cell function and the potential for them to drive T cell differentiation states may provide attractive therapeutic targets to enhance current immunotherapies for cancer treatment.

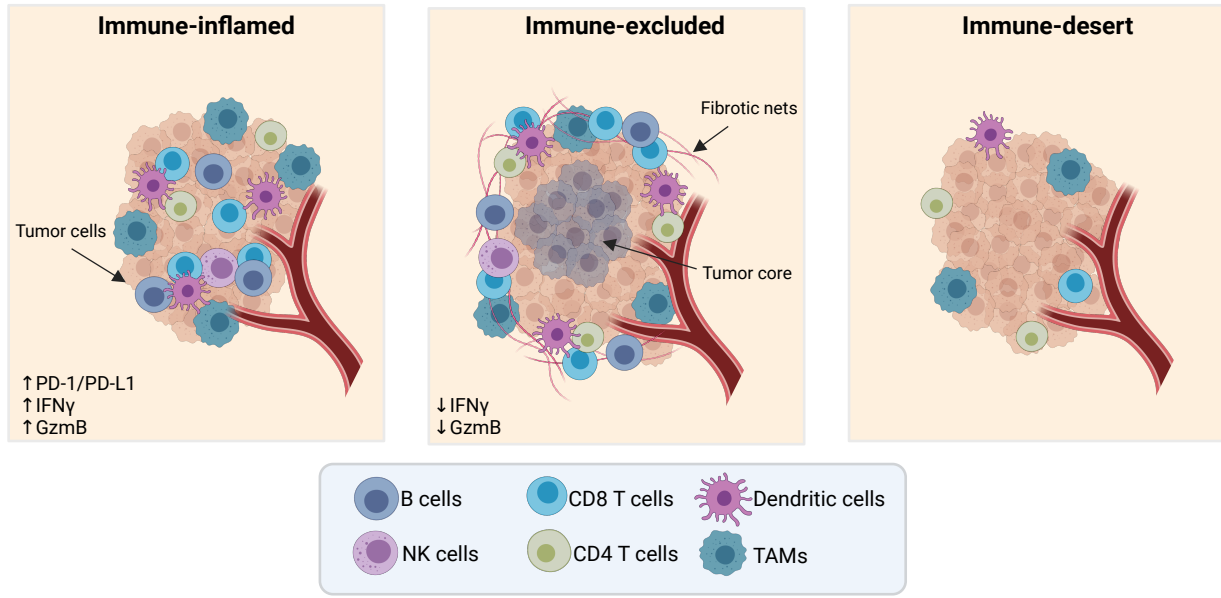


Figure 0.1: Tumor immune phenotypes have differential responses to immune checkpoint blockade.

Three types of tumor immune microenvironments. Left, immune-inflamed TME exhibits elevated levels of PD-L1 expression on both tumor and myeloid cells, accompanied by robustly activated CTLs expressing markers like granzyme B, IFN- γ , and PD-1. Certain I-I TIME subsets may also display defects in DNA mismatch repair (MSI-H), leading to an increased presence of neoepitopes. Middle, immune-excluded show a characteristic pattern of keeping CTLs away from the tumor core. Instead, CTLs are situated around the edges of the tumor, where they interact with tumor-associated macrophages (TAMs) or are confined within fibrotic structures. Right, immune-deserts are characterized by an overall lack of CTLs and pre-existing immunity.

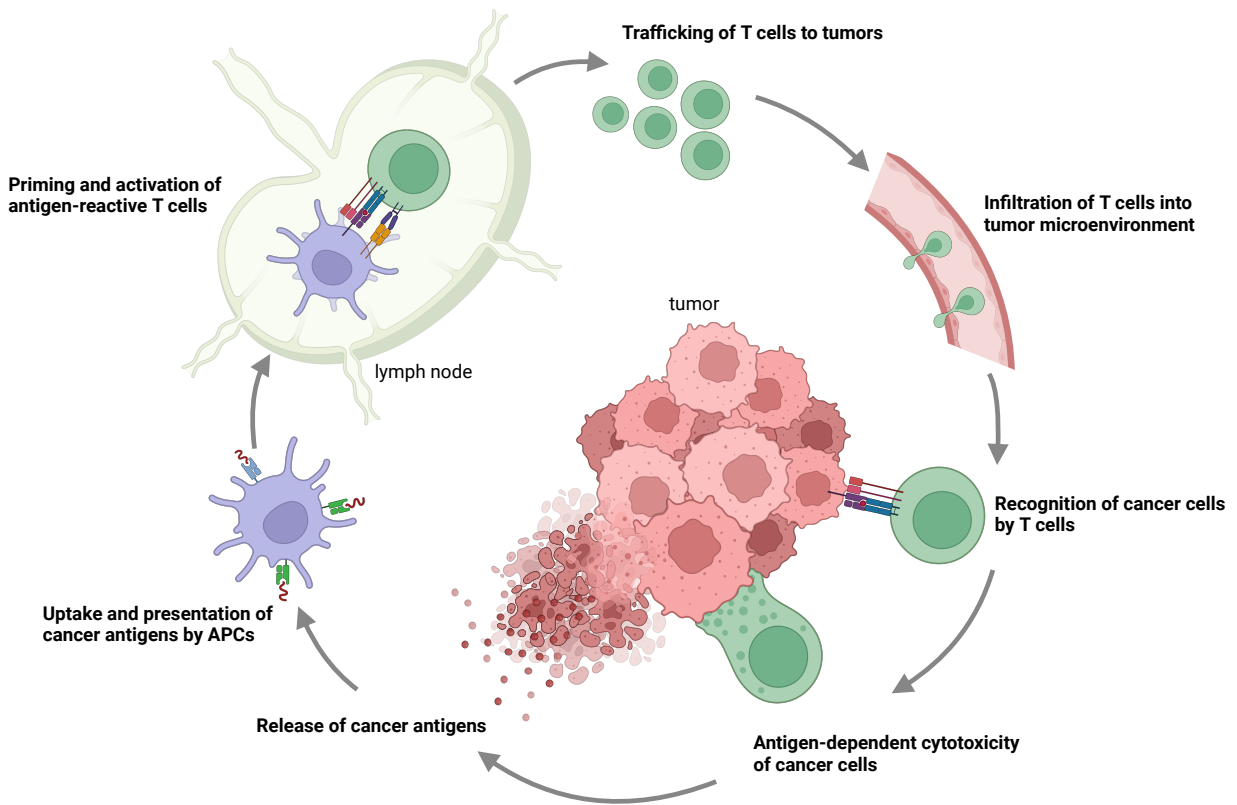


Figure 0.2: The anti-cancer immune response.

The immune system detects and eliminates cancer cells, beginning with the release of cancer cell antigens, which are presented to T cells by antigen-presenting cells. Activated T cells then infiltrate the tumor, recognize cancer cells, and eliminate them. This process leads to the formation of memory T cells, providing long-term protection against tumor recurrence. The cycle underscores the dynamic interaction between cancer cells and the immune system and guides the development of immunotherapies aimed at enhancing antitumor immune responses.

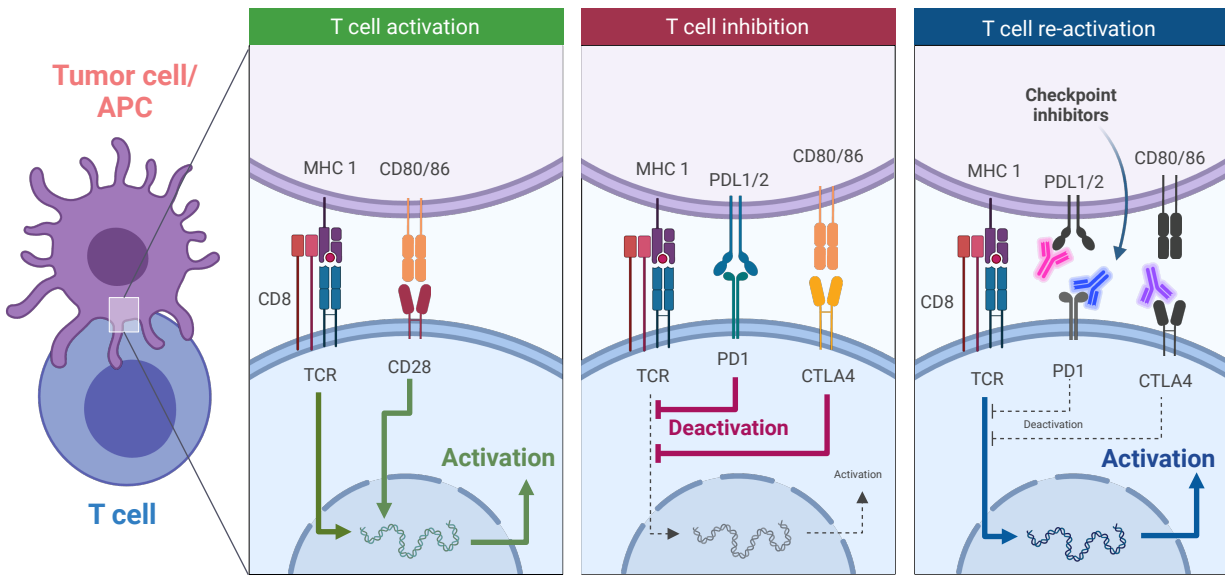


Figure 0.3: Immune checkpoint inhibitors restore T cell effector functions.

T cells are activated by two signals: the first comes from the recognition and binding of the T cell receptor to peptide:MHC molecules present on the cell surface. A second signal is provided by co-stimulatory receptors such as CD28 to generate robust immune responses. Following activation, T cells will upregulate co-inhibitory receptors such as PD-1 and CTLA-4 to regulate the intensity and duration of the immune response to prevent excessive inflammation and autoimmunity. Tumors cells can express the ligands for these receptors to suppress T cell function and escape immunological cell death. Checkpoint inhibitors are monoclonal antibody therapies designed to block the activation of inhibitory receptors on T cells, thereby restoring T cell function and anti-tumor responses.

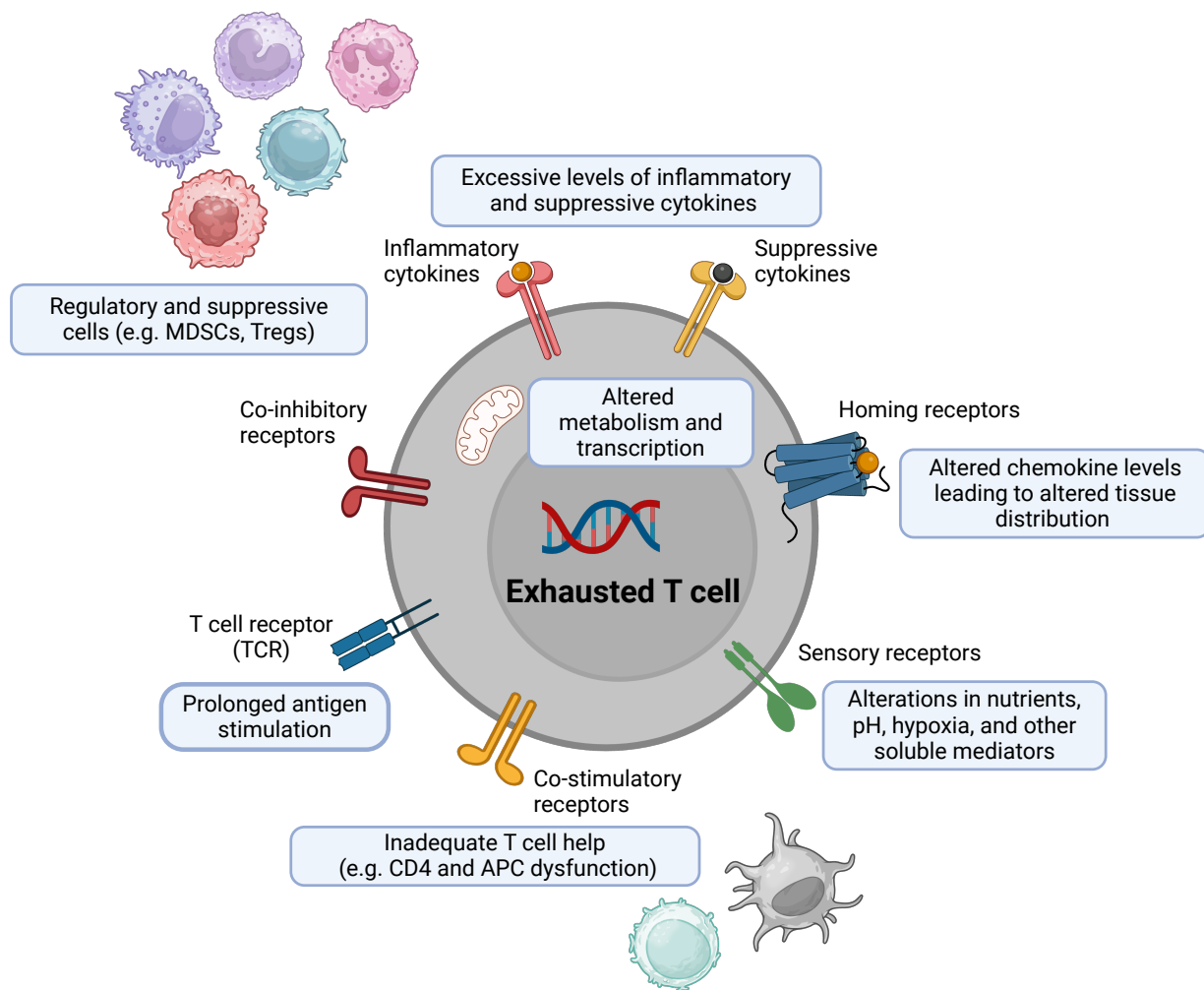


Figure 0.4: Contributors to T cell exhaustion.

In both chronic infection and cancer models, a critical factor driving T cell exhaustion is the persistent exposure to antigen. Additionally, factors such as the absence of CD4 and antigen-presenting cell (APC) support, along with signals from inhibitory receptors, further contribute to this state of exhaustion. Notably, suppressive immune cell populations not only provide ligands for these inhibitory receptors but also release suppressive cytokines and other soluble mediators that directly impede T cell function. Moreover, dysregulation of chemokines, nutrients, and metabolites can exacerbate T cell dysfunction, amplifying the overall impact on immune response.

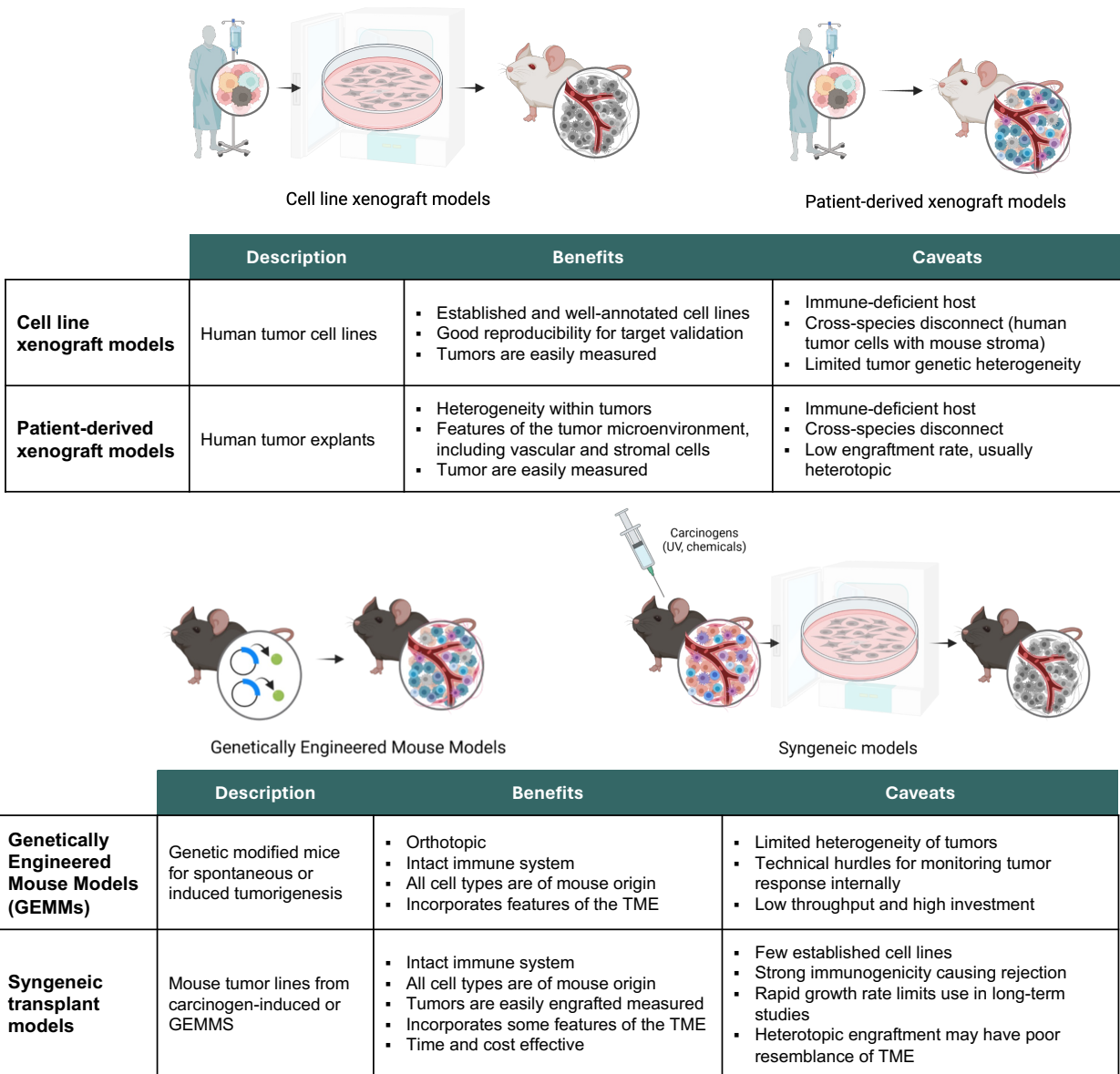


Figure 0.5: Commonly used mouse models for preclinical cancer research.

(Top), Human-derived tumor cell lines and patient-derived xenografts were used in immune-deficient mice to examine the effect of drug candidates directly on tumor cells (e.g. cytotoxic and targeted therapy). These models typically investigated the efficacy of compounds during later-stage disease, and therefore cannot be used to study early and stochastic development of tumors. (Bottom), Due to advancements in the field of cancer immunology, models with intact immune systems were needed to investigate tumor-immune cell interactions. Genetically engineered mouse models allowed for the synchronous and controlled initiation of tumorigenesis, in which developing tumors represent early-stage disease. However, long latency periods and large breeding programs have limited their use. Syngeneic implantable models were developed from carcinogen-induced tumors or GEMMs. These can be rapidly deployed and cost-effective; however, there are limited numbers of well-established cell lines. Furthermore, their rapid growth kinetics limit their ability for long-term use.

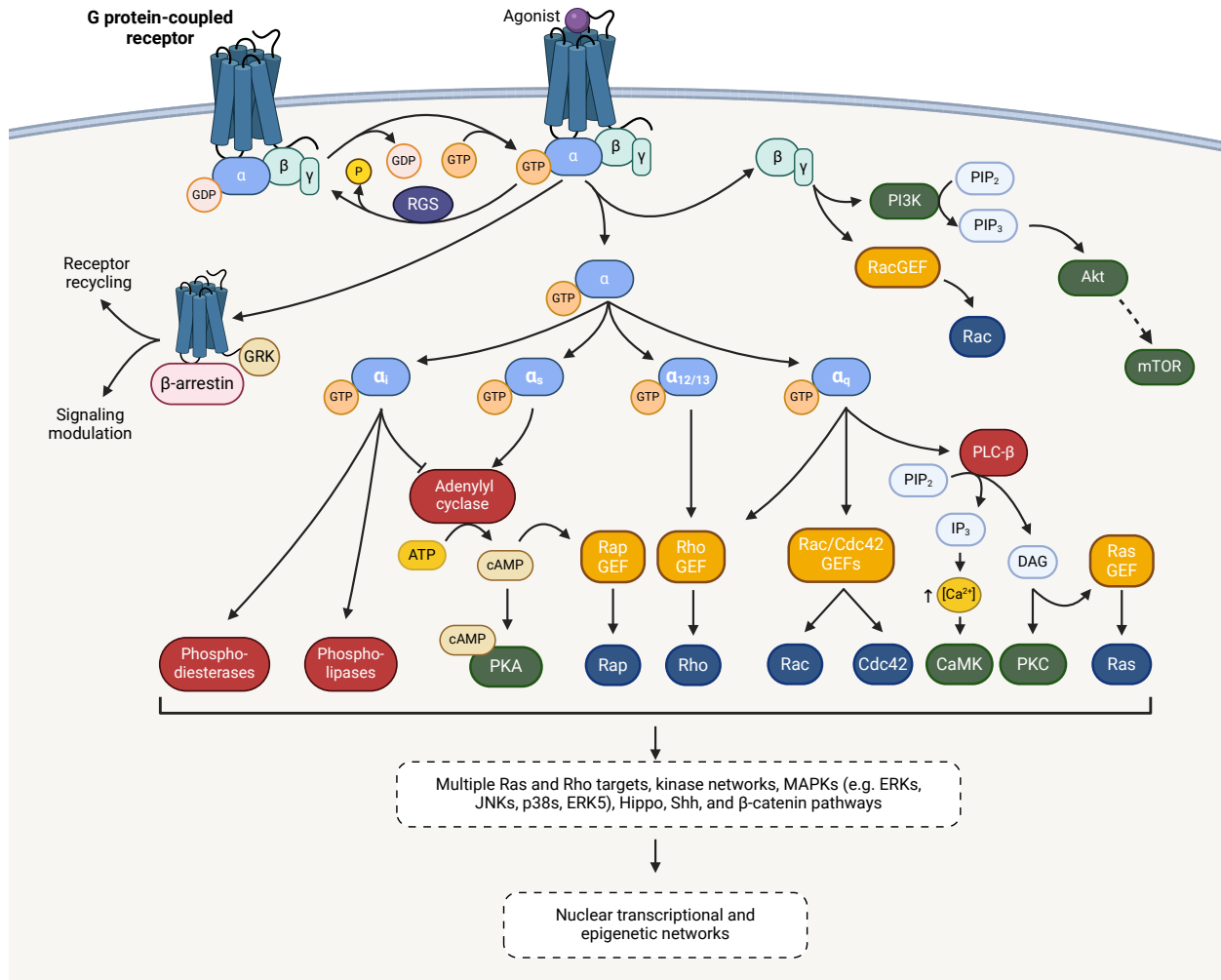


Figure 0.6: GPCR coupling and signaling pathways.

After agonist binding, GPCRs promote the exchange of GTP for GDP from the Gα subunit. The GTP-bound Gα subunit dissociates from the Gβγ dimer, and each separately mediates downstream signaling activities. The Gα subunit can be divided into four main subfamilies, and a single GPCR can couple to one or more families of Gα subunits. Downstream effectors regulated by their targets include a variety of second messenger systems (red), GEFs (yellow), and Rho and Ras GTPases (blue), which will result in the stimulation of multiple kinase cascades (green) regulating key cellular functions. These include members of the MAPK, AKT, and mTOR, second messenger regulated kinases and phosphatases, and multiple kinases regulated by Rho and Ras GTPases. Ultimately, these large numbers of signaling mediators can have multiple effects in the cytosol and nucleus to regulate gene expression, cell metabolism, migration, proliferation, and survival.

Chapter 1

Mouse syngeneic cancer atlas for immune oncology discovery

Abstract

Mouse syngeneic models have been indispensable for the advancement of immunotherapy, yet their molecular and immunologic characteristics remain poorly defined. This lack of in-depth characterization and understanding of key features of tumor-immune cell interactions may hamper the selection of appropriate models for preclinical testing, resulting in the impediment of drug development programs. Our study examines the mutational and transcriptional landscape of commonly used syngeneic tumor models in order to determine the translational relevance to their corresponding human disease. Using integrative genomic and transcriptional analysis of cell line and tumor tissue, we identify mutation signatures and gene expression patterns of diverse mouse tumor models and assess their relationship to human disease. This characterization highlights the importance of extensive profiling and will enable investigators to select appropriate experimental models and apply their experimental findings to better predict the success of potential therapies in clinical settings.

Introduction

Over the past decade, therapies targeted at engaging the immune system have advanced the standard of care for the treatment of cancer patients. In particular, monoclonal antibodies targeting PD-1 (e.g. nivolumab, pembrolizumab) and CTLA-4 (ipilimumab) have demonstrated remarkable clinical efficacy and durable responses in patients with melanoma, bladder cancer, kidney cancer, lung cancer, head and neck cancer, and Hodgkin lymphoma^{12,119-123}. Despite this success, most patients do not have complete and durable responses to treatment, and the underlying mechanisms for heterogeneous responses to immune checkpoint blockade remain

unclear. To expand clinical responses to these therapies, a greater understanding is needed of the diverse factors that contribute to differential responses.

Cancer drug discoveries have relied on preclinical animal models to study mechanism of action, delivery approaches, dosing, and toxicity. Mice (*mus musculus*) have been the model system to study cancer because of their relatively low cost, short reproductive cycle, and are easily genetically modified. Preclinical efficacy in experimental animal models of cancer has been essential to the development of treatment strategies, but the translational relevance of these models is not fully understood. Furthermore, the success of our tumor model systems hinges on how accurately the positive efficacy data obtained in preclinical studies predict efficacy in the clinic. In a study, carried out of oncology trials, it was observed that a lack of validated preclinical models or an unclear disease linkage was the most common reason that a drug failed to demonstrate clinical efficacy¹²⁴. As such, the attrition rate of cancer therapeutic drug development is substantial; 95% of candidates that enter phase 1 of clinical development fail to gain market authorization^{125,126}. This observation can be partly attributed to the poor predictive value of our preclinical models, remaining one of the top challenges facing the field of cancer immunotherapy¹²⁷. Implantable syngeneic tumor models have been the gold standard of cancer immunology, due to their short latency periods, reproducibility, and high throughput^{59,128}. As such, they have been invaluable in our evaluation of preclinical proof-of-concept studies for immunotherapy drug candidates, which have led to our understanding of mechanism of action and identifying potential biomarkers of clinical response^{59,94,129,130}. While these models have been the workhorse of preclinical drug discovery, the genotypes and phenotypes of these syngeneic murine tumor models as well as their ability to recapitulate human disease is still understudied¹³¹. Ultimately, a better understanding and characterization of these commonly used syngeneic mouse models is required to enable appropriate model selection in order to extrapolate preclinical data to clinical responses.

Here, we profiled the genomic and transcriptomic landscape of several murine syngeneic tumor models. Using whole genome sequencing of the cell lines, we characterized the driver mutations that corresponded to their corresponding human disease as well as identified mutations that were found in other human cancers. We have also leveraged mutational signature analysis to identify single-base substitution events. Lastly, we performed bulk RNA sequencing (RNA-seq) on mouse tumor tissue to identify mouse-human similarities from associated transcriptional perturbations. This work aims to uncover the disease relevance of commonly used murine syngeneic models of human cancer in order to refine future mouse research endeavors.

Results

Mutational and transcriptomic landscape of syngeneic mouse models.

We collected 22 syngeneic tumor models representing nine different cancer subtypes: 4MOSC1, 4MOSC2, MEER, MOC1, and MOC2 for head and neck cancer; B16F10, YUMM1.7, YUMMER1.7, and YUMMER.G for melanoma; 4T1, DDHER2, and Met-1 for breast cancer; MC38 and CT26 for colorectal cancer; ID8 and ID8-Vegf-Defb29 for ovarian cancer; TRAMPC1 and TRAMPC2 for prostate cancer; GL261 for glioma; LLC for lung cancer; and KPC4580 and PAN02 for pancreatic cancer. We then performed whole genome sequencing on the cell lines with a coverage depth of 30x as well as bulk RNA-seq (30 million reads) on tumors that developed after orthotopic or heterotopic implantation (**Figure 1.1**).

To compare the properties of somatic alterations in mouse models with those found in human patients, we identified and characterized mutated driver genes across all The Cancer Genome Atlas (TCGA) cohorts (**Table 1.1**) to establish commonly mutated driver genes within each cancer subtype as well as in all cancers (**Figure 1.2a**). From this list of genes, we performed genome-wide analysis on the *in vitro* murine tumor cell lines to gain an understanding of broad genetic perturbations (e.g. missense, nonsense, frame-shift deletions and insertions) (**Figure 2b**). We identified *Gm21190* as the gene with the highest mutation frequency from all cell lines,

identified as a missense mutation in 17 models (**Figure 1.3a**). Other highly mutated genes include *Ttn*, *Cilp*, *Kif12*, *Crb1*, *Trp53*, *Fat4*, and *Fras1*. Interestingly, some of the genes have genetic aberrations that are consistent among all cell lines, such as *Gm21190* commonly existing as a missense mutation and *Kif12* as a nonsense mutation, whereas others such as *Crbf* occur as different types of mutations. Many of these commonly mutated driver genes were identified in matched human-mouse data (**Table 1.2**). However, some models did not have or had few driver mutations that were identified from their respective human disease (e.g. ID8, DDHER2, TRAMPC1, TRAMPC2).

Next, we leveraged the Pan-Cancer Analysis of Whole Genomes (PCAWG) database, comprising of 2,780 human whole genomes from 20 different cancer types, as a reference for the mutational analysis of the mouse tumor cell lines¹³². From these whole genomes, we determined the single-base substitutions (SBS), doublet-base substitutions (DBS), and small indels for mutational signature analysis for both human and mouse samples. We identified the tumor mutational burden (TMB) of each PCAWG cohort in which there is a corresponding syngeneic model (**Figure 1.3b**). Here, we observed that the TMB, measured as mutations per megabase, of mouse tumor cell lines was typically much higher compared to their corresponding human patient data. The average TMB in the human cohorts analyzed ranged from 0.7 to 9 mutations per megabase, whereas the mouse tumor cell lines ranged from 11 to 108 mutations per megabase.

We then generated SBS signatures for each of the mouse tumor cell lines for comparative analysis against their equivalent human cohort. For SBSs, the primary classification comprised 96 classes constituted by the 6 base substitutions C>A, C>G, C>T, T>A, T>C and T>G (in which the mutated base is represented by the pyrimidine of the base pair), plus the flanking 5' and 3' bases. Signatures were extracted from each type of cancer individually, from all cancer types together, as separate SBS signatures. We determined a matched signature using a cosine similarity score with a threshold of 0.85. From this analysis, we determined that some mouse

tumor models had signatures that reflected a percentage of human patient data, while other models have SBS mutation signatures that do not reflect any human signature at all within PCAWG (**Figure 1.3c** and **Table 1.2**). Of note, the YUMMER models had signatures that matched 13.1% of the melanoma cohort and PAN02 matched to 9.5% of patients with pancreatic cancer. Other models, such as CT26, GL261, LL2, MC38, MOC1, and MOC2, had SBS signatures that did not match to any patient within their respective cohort.

Transcriptomic alterations mirror the combined impact of genomic and epigenomic changes that accumulate within tumor cells. Therefore, we compared the RNA-seq-derived transcriptomic similarity of human cancers from TCGA and the mouse tumor tissue samples. To do this, we implanted the cell lines using their most common method of transplantation from existing literature, either orthotopically or heterotopically, and allowed the tumors to establish for at least 14 days. This was accomplished by determining optimal inoculation cell doses for each model to prevent premature ulceration and end-point volume criteria. To assess the similarity of tumor transcriptional patterns between human tumors and mouse models, we computed pair wise correlations across all gene expression profiles of mouse tumors and their corresponding human TCGA project. Samples cluster together based on their similarity in terms of gene expression across all 1:1 mouse to human genes. Interestingly, most mouse tumor models clustered predominantly with lung squamous cell carcinoma (LUSC) and skin cutaneous melanoma (SKCM) (**Figure 1.3c**). Mouse models that clustered according to their respective human disease include 4MOSC1, 4MOSC2, MOC1, and MOC2 for head and neck squamous cell carcinoma (HNSC); GL261 for glioblastoma multiforme (GBM); B16F10, YUMM1.7, YUMMER1.7, and YUMMER.G for SKCM; and ID8 and ID8-Vegf-Defb29 for ovarian serous cystadenocarcinoma (OV). It is unclear why many of these tumor models have transcriptional characteristics that represent LUSC and SKCM, which may be dependent on differences in etiology and the generation of these models. Next, we applied CIBERSORT, a deconvolution method that resolves fractions of diverse immune cell subsets using gene expression profiles from tumor tissue¹³³, to

determine immunological characteristics of each tumor model (**Figure 1.4**). Here, we were able to identify populations of immune cells that could modulate responses to immunotherapies that have been previously reported by other studies. For example, the 4MOSC2, B16F10, 4T1, LL2, and MC38 have been previously reported to have immunosuppressive microenvironments resulting in immune checkpoint insensitivity^{67,134-137}. CIBERSORT analysis reveals amplification of transcripts from immunosuppressive myeloid populations that have infiltrated these tumors, suggesting that these immune cells may be responsible for limiting responses to ICB (**Figure 1.4**). Based on the genetic and transcriptional landscape of these tumor models, we may begin to identify mouse tumor models that may be appropriately selected to infer preclinical data to their respective human cancers.

Head and neck cancer

HNSC is derived from the mucosal epithelium in the oral cavity, pharynx, and larynx, and is primarily associated with risk factors such as tobacco and alcohol use, along with human papillomavirus (HPV) infection¹³⁸. Recently, our team described the optimization of a carcinogen-induced oral cancer mouse model (4MOSC) based on 4-nitroquinoline-1 oxide (4NQO), a DNA adduct-forming agent that acts as a tobacco-mimetic¹³⁵. These models can be transplanted orthotopically into the tongue of immunocompetent C57BL/6 mice, which led to the formation of well-differentiated HNSC tumors. The MEER tumor cell line was generated by transforming murine pharyngeal epithelial cells with HPV16 E6 and E7 oncogenes and H-ras¹³⁹, and typically implanted heterotopically in the flank of C57BL/6 mice. The MOC cell lines were derived from exposing C57BL/6 mice to 7,12-dimethylbenz(a) anthracene (DMBA), another carcinogen that is found in tobacco smoke. MOC1 was generated from a mucosal lip lesion and MOC2 from a floor-of-mouth mass¹⁴⁰. While the MOC tumor models can be implanted orthotopically in the mouth, they are typically inoculated heterotopically in the flank. MEER, MOC1, and MOC2 develop tumors that driven by *Ras* family oncogenes (*Kras* in MEER and

MOC2, and *Hras* in MOC1) (**Figure 1.4a**), which are infrequently mutated in human HNSC¹⁴¹. These Ras mutations are not found in 4MOSC1, which contains mutations impacting *Trp53*, *Fat1/3/4*, and *Notch2*.

WGS of these HNSC tumor cell lines revealed distinct mutational patterns across each model, depending on the methods used to generate each model. The SBS signature of lesions induced by 4NQO have been shown to bear remarkable similarity (93.9%) to human cancer signature SBS4 (COSMICv99, available at <https://cancer.sanger.ac.uk/signatures/sbs/>) , which is strictly associated with tobacco smoking typically seen in HNSC, esophageal carcinoma (ESCA), and lung cancer¹⁴². The SBS signature of the 4MOSC1 and 4MOSC2 reflects previous work done by our lab¹³⁵, and these models matched to 5.3% and 3.5%, respectively, of patients identified in PCAWG based on cosine similarity (**Figure 1.5b** and **Table 1.2**). Within the HNSC PCAWG group, 4MOSC1 and 4MOSC2 matched to the same patient with a cosine similarity score of 0.89 and 0.88, respectively. While the mutational signature of their most matched patient revealed 63,185 SBS, 4MOSC1/2 both had SBSs well over 400,000. In contrast to the 4MOSC models, the mutational signature of squamous cell carcinomas (SCC) caused by DMBA has been previously shown to reflect 39.7% similarity with that of cancer signature 4. Our analysis of the SBS signatures of the MOC1 and MOC2 cell lines reflect the DMBA signature that was previously described¹³⁵ and, due to its limited ability to imitate tobacco smoking, these models did not achieve a similarity threshold against any HNSC patient in PCAWG. Despite being induced by the same carcinogen, MOC1 has a substantial mutational burden (196,293 SBSs) compared to MOC2 (55,804 SBSs) (**Figure 1.5b** and **Table 1.2**). Lastly, the MEER model has an SBS signature that matched with 14% of the PCAWG HNSC project, scoring a maximum cosine similarity of 0.90. While a human cancer signature with a proposed etiology of HPV infection has yet to be defined, the MEER mouse model signature resembles human cancer signature SBS40a and may reflect an HPV mutational signature due to the expression of the E6/7 oncogenes.

When we overlayed the transcriptional patterns of the HNSC mouse tumor tissue with human tumors, we observed that the 4MOSC1/2 and MOC1/2 clustered to group 1 – mesenchymal subtype, while MEER clustered to group 3 – basal subtype (**Figure 1.6a**). Interestingly, normal mouse tongue tissue clustered more with group 2 - classical and group 4 – atypical, rather than human “normal”. As this clustering was the result of similarities between the human and mouse transcriptomes, our aim was to investigate whether these clusters held any biological or clinical features that could characterize them. To do this, correlations were calculated according to genes that are significantly differentially expressed by samples of each mouse tumor model to those of each TCGA sample (grouped by subtypes determined by annotations (**Figure1.6b**)). We observed gene expression patterns in 4MOSC1 and MOC2 that were enriched in patients with smoking status. Not surprisingly, the transcriptional pattern of MEER tumors was enriched in patients with HPV. Taken all together, the 4MOSC1 tumor model may better represent tobacco-associated HNSC, whereas the MEER model may establish an HPV signature and be appropriately selected for HPV+ HNSC tumor studies.

Skin Cutaneous Melanoma

Skin cancers encompass carcinomas of all layers of the skin with the most common being basal cell and SCC. Melanoma skin cancers are much less common but are the most serious form of skin cancer that begins in melanocytes and is associated with exposure to UV light. Furthermore, malignant melanoma is highly aggressive and rapidly metastasizes to other parts of the body. The B16F10 mouse model is the most used metastatic melanoma model for preclinical studies. The B16 parent cell line was established from a spontaneous lesion in C57BL/6 mice, which was then serially passaged 10 times to generate the F10 variant¹⁴³. While the B16 models have been extraordinarily useful since its development in 1930, its genetic drivers have not been well documented and its response to therapies targeted at treating skin cutaneous melanoma have had mixed results^{67,144,145}. To address these limitations, the YUMM1.7 mouse model was

generated from male melanoma GEMMs carrying three relevant human melanoma driver mutations: an activating *Braf*^{V600E} mutation, and *Pten*^{-/-} and *Cdkn2*^{-/-} deletions. Furthermore, YUMM1.7 has been demonstrated to be sensitive to BRAF and MEK inhibitors, which are commonly used to treat BRAF-mutant human cancers. The YUMMER1.7 cell line was generated based on the observation that YUMM1.7 had a low number of somatic genetic changes, typically not seen in skin cutaneous melanoma where it ranks highest in TMB among all human cancers^{132,146}. To that end, YUMMER1.7 was generated from the YUMM1.7 cell line after being exposed to three rounds of ultraviolet (UV)-B irradiation to induce passenger mutations that would enhance the immunogenicity of the model¹⁴⁶. Characterization of the DNA contents of YUMM1.7 and YUMMER1.7 models revealed that YUMM1.7 contains both diploid and tetraploid clones, whereas YUMMER1.7 is a tetraploid clone. Lastly, the YUMMER.G model is related to the YUMMER1.7 model, but instead is of female origin, diploid, and contains *Mc1r*^{e/e} (pheomelanin).

Mutational analysis from WGS of the melanoma cell lines revealed *Braf* and *Pten* mutations in the B16F10 cell line; however, this *Braf* mutation was not analogous to the V600E mutation that is typically responsible for BRAF inhibitor sensitivity (**Figure 1.7a**)⁶⁷. Interestingly, B16F10 was also observed to have an *Apc* mutation that is typically seen in colorectal adenocarcinomas (COAD). Surprisingly, the *Braf*, *Pten*, and *Cdkn2* mutations that were used to generate the YUMM models were not identified by our analysis. This may be due to the limitation of our mutation calling pipelines which call short insertions and deletions that are less than 50bp, making large gene knockouts undetectable. From the SBS signature analysis, T>G transitions were enriched in both B16F10 and YUMM1.7 (**Figure 1.7b**), closely reflecting human SBS17b. Interestingly, this mutational pattern is often observed in patients with acral melanoma, a rare type of melanoma that occurs under nails, palms, and soles of the feet¹⁴⁷, which could be reflected in the single patient out of 328 in the skin PCAWG group matched by both models (**Table 1.2**). Interestingly, the mutational burden of B16F10 (196,081 SBSs) was much higher compared to the YUMM1.7 (35,928 SBSs), despite matching the same patient (cosine similarity 0.92 and 0.86,

respectively). In contrast, the mutational signature of both YUMMER1.7 and YUMMER.G is dramatically different compared to the non-UV-irradiated melanoma models. Unsurprisingly, these models reflect the human UV light exposure signatures (SBS7a/b/c/d) (**Figure 1.7b**), which are typically found in cancers of the skin from sun-exposed areas^{132,148}. The mutational signature of YUMMER1.7 and YUMMER.G was well represented in the PCAWG skin cohort, both matching with a cosine similarity > 0.85 to 13.1% of the 328 patients. The patients with the highest matched score to YUMMER1.7 bore a similar mutational burden (SP82532 212,889 SBSs vs 231,120 SBS), whereas YUMMER.G resembled a patient with a lower SBS amount (MELA_0021 7,915 SBSs vs 74,855 SBSs).

We next performed bulk RNA-seq on these tumor models after orthotopic intradermal implantation of these models in wild-type female (B16F10 and YUMMER.G) and male (YUMM1.7 and YUMMER1.7) mice. Examination of the transcriptional patterns of these melanoma mouse models revealed that the YUMM and YUMMER models overlapped more with group 2, whereas B16F10 clustered with group 1 (**Figure 1.8a**); however, the identify of these subtypes are still unknown and under further investigation. While the YUMM and YUMMER models overlapped with the same group in the PCA analysis, their differentially expressed genes determined by patient annotation revealed major differences based on the Broad Firehose pipeline clustering (<https://gdac.broadinstitute.org/>). Furthermore, YUMM1.7 and YUMMER1.7 exhibit specific gene patterns that correlated with BRAF mutation status (**Figure 1.8b**). While the transcriptional data reveals clear distinctions in significantly differentially expressed genes among the different melanoma tumor tissues based on Firehose clustering, further investigation is needed to link mutational and transcriptional data to determine the appropriate subtype of melanoma that these models can recapitulate.

Breast invasive carcinoma

Breast cancers that have spread into surrounding breast tissue are known as invasive breast cancers, with invasive ductal carcinoma as the most common subtypes (80% of all invasive cancers). Major risk factors for breast cancer include age, genetic predisposition, and reproductive history. Invasive breast cancer can be further subdivided into molecular subtypes independent from histological phenotype, with the four main subtypes being luminal, HER2-enriched, basal-like/triple-negative, and normal breast-like. The 4T1 cell line is one of the most commonly used models to study advance stage invasive breast cancer, due to its ability to metastasize from the mammary pad to other organs, such as the lymph nodes and lungs^{149,150}. 4T1 was derived from the 410.4 cell lines that were formed from a spontaneously arising mammary tumor in BALB/c background mice. The DDHER2 breast tumor cell lines are typically used to study HER-2 positive breast carcinomas and were derived from a spontaneous breast tumor in transgenic BALB/c mice which had mouse mammary tumor virus (MMTV) promoter-driven breast epithelium-specific expression of the activated oncogenic form of Her-2/neu¹⁵¹. Lastly, the Met-1 mouse model is generated from MMTV-polyoma middle tumor-antigen GEMMs, which develop spontaneous tumors which lose the expression of estrogen receptor (ER) and progesterone receptor (PR), that is typically used to study ER-negative luminal human breast cancer^{152,153}.

Driver gene analysis of the models revealed that only 4T1 harbored the *Tp53* mutation (**Figure 1.9a**), which is mutated in 30% of all invasive breast cancers^{154,155}. Interestingly, the Met-1 model was found to contain the *Brca2* mutation. Germline mutations in the BRCA family of genes has been show to lead to hereditary breast and ovarian cancer syndrome, which accounts for approximately 6% of all breast cancer cases¹⁵⁶. Unsurprisingly, *ErbB2* (encoding HER-2) is found as a mutation in the DDHER2 cell line, which contains the mutant oncogenic rat HER2 gene. SBS analysis of the breast cancer cell lines revealed that 4T1 had mutational signature that represented 3% of the 698 patients within the breast cancer cohort in PCAWG (**Table 1.2**).

DDHER2 had the highest maximum cosine similarity score (0.94) and matched 8 patients. Despite containing human breast cancer driver genes, the mutational landscape of Met-1 failed to reach the 0.85 threshold against any breast cancer patient. SBS signature analysis shows an enrichment in T>C and T>G transitions in both 4T1 and DDHER2 mouse models, albeit having highest cosine similarity scores against different patients (**Figure 1.9b**). Interestingly, T>G transitions in both models reflect SBS17b, which was present in the B16F10 and YUMM1.7 models. This signature may suggest an etiology that may be consistent with spontaneously generated mouse tumors. The mutational signature of Met-1 is dominated by only C>G substitutions and moderately reflects that of SBS13, which may be caused by the APOBEC family of cytidine deaminases found in human cancer. Compared to the mutational burdens of previously described cell lines, these breast cancer models have SBSs ranging from 12,672 to 87,875 SBSs, likely due to the lack of carcinogen treatment.

These breast cancer cell lines were then implanted orthotopically into the mammary fat pad of female mice to generate tumor tissue for transcriptional profiling. Regrettably, only the tumors generated by the 4T1 and DDHER2 cell lines were successfully extracted; implantation of Met-1 cells led to tumor rejection and thus was excluded from the analysis. The PCA of these two models revealed that 4T1 clustered with Her2 molecular subtype (as defined by TCGAblinks), whereas DDHER2 clustered more with Luminal type A breast cancers (**Figure 1.10a**). Furthermore, 4T1 expresses genes that correlates strongly with ER, PR, and HER2 status in human breast cancer patient data from TCGA (**Figure 1.10b**). Interestingly, the transcriptional profile of DDHER2 reflects the inverse of 4T1.

Colorectal adenocarcinoma

More than 90% of all colorectal carcinomas are adenocarcinomas originating from epithelial cells of the colorectal mucosa, and can be characterized based on glandular formation¹⁵⁷. These adenocarcinomas also contain variants, such as mucinous, signet ring,

medullary, micropapillary, serrated, cribriform comedo-type, adenosquamous, spindle cell, and undifferentiated. These cancers are affected by many life-style related factors, including obesity, physical activity, diet, smoking, and alcohol use. Here, we profile the two of the most used tumor models: MC38 and CT26. The MC38 adenocarcinoma model was isolated from a colon tumor from female C57BL/6 mice following long-term exposure to 1,2-dimethylhydrazine dihydrochloride (DMH)¹⁵⁸. CT26 is an N-nitroso-N-methylurethane (NNMU) induced undifferentiated colon carcinoma cell line that was established from BALB/c mice¹⁵⁹.

Examination of the driver mutations from WGS of these cell lines revealed that both MC38 and CT26 contain certain driver mutations that are typically seen in colorectal cancer, such as *Kras*, *Trp53*, and *Braf* mutations (**Figure 1.11a**)¹⁶⁰. Other mutations that were identified in these models that were also represented in other human and mouse models include *Gnas*, *Smarca4*, *Brca2* *Pten*, and *Fat3/4*. Furthermore, MC38 and CT26 share multiple driver mutations despite being induced by different carcinogens, including *Fat3/4*, *Mtor*, *Card11*, *Dicer1*, *Epha3*, *Lats1*, and *Smarca4*. When we profiled the SBS signatures in both models, we observed that neither model reached the 0.85 cosine similarity threshold within the colorectal cancer group in PCAWG, with MC38 achieving a maximum score of 0.75 and CT26 reaching 0.69, despite bearing driver mutations common in human colorectal cancer (**Table 1.2**). SBS analysis also revealed an enrichment of C>A and T>G substitutions in MC38 (**Figure 1.11b**). The mutational pattern of MC38 bears a resemblance to the combination of two human SBS signatures: SBS29 which is found in cancer samples from individuals with a tobacco-chewing habit and SBS17b. CT26 also seems to bear SBS17b signature, along with the C>T alkylating agent exposure signature SBS11. SBS11 is also associated with larger numbers of somatic mutations, which is reflected in the 424,226 SBSs seen in CT26.

While these colorectal models can be implanted orthotopically, these are typically inoculated subcutaneously in the flanks of mice, due to surgical implantation into the colorectal mucosa as a limited factor for this approach. Furthermore, it remains a challenge to monitor tumor

growth without expressing transgenes which may further increase immunogenicity. As such, we implanted the MC38 and CT26 tumor cell lines subcutaneously in C57BL/6 and BALB/c mice, respectively. Principal component analysis showed MC38 clustering to COAD patient samples from TCGA with chromosomal instability (CIN) and invasive status (**Figure 1.11c**), whereas CT26 clustered more closely with MSI/CpG island methylator phenotype (CIMP), which is found in 20% of malignant colorectal tumors¹⁶¹. Patterns in the differentially expressed genes between MC38 and CT26 also revealed genes linked with anatomical location of human tumors, which may be informative in understanding characteristics of tumorigenesis as well as guiding treatments aimed at different tumor microenvironments (**Figure 1.11d**).

Pancreatic adenocarcinoma

Pancreatic adenocarcinomas carry the lowest survival rate of all major organ cancers and accounts for 90% of all pancreatic carcinomas¹⁶². These carcinomas can be further subdivided into main variants, which include adenosquamous, colloid/mucinous, undifferentiated/anaplastic, signet ring, medullary, and hepatoid carcinomas. Risk factors associated with pancreatic cancer include tobacco use, obesity, diabetes, and exposure to certain chemicals. For our analysis, we leveraged the PAN02 and KPC4580 pancreatic cancer models. The PAN02 model is a well-established adenocarcinoma model that was developed by chemical induction with 3-methylcolanthrene (3-MCA) in male C57BL/6 mice¹⁶³. In contrast to the PAN02 carcinogen model, the KPC4580 model belongs to the family of KPC cell lines that was established from a spontaneous tumor that developed from male GEMMs with pancreas-targeting p48-Cre recombinase with human-relevant *Kras*^{G12D}, and *Trp53*^{R172H} mutations¹⁶⁴.

Mutational profiling from WGS of these pancreatic cell lines revealed that PAN02 and KPC4580 harbor mutations commonly found in pancreatic cancers, including *Kras* and *Trp53* (**Figure 1.12a**). PAN02 bears more driver mutations compared to the three identified in KPC4580, likely due to carcinogenic induction compared to genetic transgene expression of *Kras* and *Trp53*

in the KPC models. Identified driver mutations in PAN02 that are also present in other carcinogen-induced models include: *Fat1/3/4*, *Lats1*, *Brca2*, *Pcbp1*, *Smarca4*, and *Gnas*. Furthermore, both models matched well to the pancreatic cancer cohort within PCAWG, with PAN02 scoring at least 0.85 against 9.5% of the 431 patients and KPC4580 matching to 3.4% (**Table 1.2**). The differences between the carcinogen-induced PAN02 model and the GEMM-derived KPC4580 model is further reflected in SBS analysis, where 178,400 SBSs were observed in PAN02 compared to the 24,922 SBSs in KPC4580 (**Figure 12.b**). When compared to human SBS signatures, it is unclear which previously-defined SBS signatures could reflect the enrichment of C>T and T>C substitutions of PAN02's signature, which may define a mutational pattern caused by 3-MCA exposure. Candidate human SBS signatures include SBS26, which is caused by defective DNA-mismatch repair mechanisms. The SBS signature of KPC4580 shows amplification of C>T, T>V, and T>G substitutions. Potential human SBS signatures that could contribute to the signature of KPC4580 include SBS9 (mutations induced by polymerase η) and SBS17b.

Both PAN02 and KPC4580 were implanted into male C57BL/6 mice heterotopically in the flank; however, only KPC4580 developed tumors that were able to be extracted for downstream analysis. Regression of PAN02 tumors despite high inoculation numbers could be explained by the higher mutational burden, leading to neoantigen-mediated graft rejection. Bulk RNA sequencing of KPC4580 tumor samples resulted in no discernable clustering within the PDAC TCGA study (**Figure 1.12c** and **1.12d**).

Ovarian carcinoma

Ovarian cancer is the leading cause of death in women with gynecological cancers¹⁶⁵. There are four main histological types of epithelial ovarian cancers: serous, endometrioid, clear-cell, and mucinous carcinoma. These are further subdivided into Type I and Type II tumors. Type I tumors include low-grade serous, endometrioid, clear-cell, and mucinous carcinomas, which

mainly arise from atypical proliferative tumors. Type II encompasses the more fatal variants, which include high-grade serous, carcinosarcoma, and undifferentiated carcinoma, which originate from serous tubal intraepithelial carcinoma. The main risk factors associated with ovarian cancer are positive family history of breast or ovarian cancer, as well as increased risk from smoking. The main syngeneic model for ovarian cancer research has been the ID8 model. This cell line was derived from spontaneous *in vitro* transformation of C57BL/6 mouse ovarian surface epithelial cells^{165,166}. The ID8-Vegf-Defb29 variant introduces *Vegf* and *Defb29* genes to enhance angiogenesis and tumorigenesis, allowing for a more aggressive model of high-grade serous carcinoma. Both models are typically implanted intraperitoneally, resulting in solid tumor and ascites development.

From our WGS data of these cell lines, only ID8-Vegf-Defb29 contained a driver mutation that was found in the TCGA database, *Atrx*. Interestingly, *Atrx* is commonly found in lower-grade gliomas¹⁶⁷, and is only found in about 2% of all ovarian cancers¹⁶⁸. We observed that ID8 and ID8-Vegf-Defb29 had cosine similarity scores >0.85 to 3 and 2 of 135 patients within the ovarian cancer cohort of PCAWG, approximately 2 and 1.5% respectively (**Table 1.2**). The mutational signature of ID8 and ID8-Vegf-Defb29 showed broad SBS of every type (**Figure 1.13a**). Again, the SBS17b signature is reflected in the T>G substitution signature in both models.

To obtain tumor tissue for bulk RNA-seq analysis, we implanted these cell lines intraperitoneally in female C57BL/6 mice. After 14 days, there was noticeable ascites development as well as the development of solid masses within the peritoneal space. Profiling of the gene expression patterns of these two models along with the OV TCGA group demonstrated that ID8 clustered more with group 1 – mesenchymal subtype whereas ID8-Vegf-Defb29 clustered more with group 3 – differentiated and immunoreactive subtype (**Figure 1.13b**). This is also reflected when we clustered the differentially expressed genes from these two models based on patient annotation (**Figure 1.13c**), demonstrating that expression of *Vegf* and *Defb29* resulted in a transcriptional landscape that is distinct from that of parental ID8.

Prostate adenocarcinoma

Prostate adenocarcinoma arises from the glandular cells of the prostate gland and typically affect middle-aged men around the ages of 45-60¹⁶⁹. These cancers are either classified as androgen-sensitive or androgen-insensitive, which is an indicator of testosterone stimulation. The main risk factors associated with these cancers include genetic predisposition, ethnicity, age, tobacco and alcohol use, obesity, and other environmental factors. The TRAMP cell lines were developed as a syngeneic transplantable model to study prostate adenocarcinoma. These models harbor a rat probasin promoter driving prostate-specific epithelial expression of the SV40 large T antigen. TRAMP males develop prostatic intraepithelial neoplasms by 8-12 weeks of age, from which TRAMPC1 and TRAMPC2 were developed¹⁷⁰.

Previous studies have identified *SPOP*, *TP53*, *FOXA1*, and *PTEN* as driver mutations found in most cases of primary prostate cancer¹⁷¹. However, WGS of the TRAMPC1 and TRAMPC2 cell lines revealed *Trp53* as the only driver gene mutated in these cell lines. The SBS signature of TRAMPC1 and TRAMPC2 are nearly identical, both scoring a maximum of 0.85 against the same patient within PCAWG (**Figure 1.14a** and **Table 1.2**). Overall, both TRAMPC1 and TRAMPC2 have SBS signatures that matched with 0.85 cosine similarity to 3 out of 404 prostate cancer patients. When examining these signatures, both TRAMPC1 and TRAMPC2 have T>G substitutions consistent with SBS17b, which has been demonstrated over other mouse models arising from spontaneous tumors, as well as an enrichment in C>A, C>T, and T>C substitutions. Furthermore, these models had similar SBS events compared to their human counterpart.

The TRAMP models have been typically deployed as heterotopic subcutaneous tumors in male C57BL/6 mice. After implantation, outgrowth, and bulk RNA-seq of TRAMPC1 and TRAMPC2 tumor tissue, we observed nonspecific clustering when compared to the PRAD TCGA group (**Figure 1.14b**). The transcriptional landscape of these models further bears no consistent

resemblance to any gene expression profile using annotations from the TCGA dataset (**Figure 1.14c**).

Glioma

Gliomas are primary brain tumors that can be classified by their presumed cell-of-origin, such as astrocytic tumors, oligodendrogliomas, ependymomas, and mixed gliomas¹⁷². These make up the most commonly occurring tumors of the central nervous system. Of these, glioblastoma multiforme (GBM) is the most malignant and frequently occurring type of astrocytomas. The etiology of brain neoplasm remains to be elucidated, with no underlying carcinogenetic causes, and remains largely incurable. Clinical molecular characteristics of GBM include amplification of *EGFR*, *MDM2*, *PDGFR*, and *RB*, as well as loss of *PTEN*, *IDH*, *TP53*, and *ATRX*. The GL261 is the most frequently used model for the study of GBM. GL261 was generated via chemical induction with 3-MCA, similar to PAN02, pellets into the brains of C57BL/6 mice. These tumors histologically resemble ependymoblastomas. Otherwise, this model recapitulates GBM phenotypes, including sensitivity to radiotherapy and resistant to temozolomide treatment^{173,174}.

Mutational analysis of the GL261 cell line revealed missense mutations in *Trp53*, *Stag2*, *Ptpn11*, *Bcor*, *Fat3*, and *Nf* as mutations previously identified in human GBM patient data (**Figure 1.15a**). When we examined the SBS patterns, GL261 did not match to any patient data within the brain cancer cohort of PCAWG, despite bearing driver mutations found in GBM cancers (**Table 1.2**). GL261 also had about 300x the amount of SBSs compared to its most matched human sample (333,349 SBSs vs 1,370 in SJHGG002), showing an enrichment in C>A and T>A substitutions (**Figure 1.15b**). The composite mutational signature of GL261 resembles a combination of both SBS4 (tobacco smoking) and SBS34.

For transcriptomic analysis of GL261 tumors, we implanted GL261 into the right cerebral cortex of C57BL/6 mice. From the tumor tissue, we performed bulk RNA-seq and observed that

two tissue samples clustered with group 2 – non-codel, mutant IDH status, whereas one sample clustered with group 3 – codel, mutant IDH status (**Figure 1.15c** and **1.15d**). Differences in gene expression pattern could be due to slight variability in the anatomical location of which these tumors were engrafted.

Lung carcinoma

Lung cancer refers to tumors originating in the lung parenchyma or within the bronchi, and is the most commonly diagnosed cancer worldwide¹⁷⁵. It is primarily associated with smoking; other risk factors include exposure to other carcinogens, such as asbestos. The most common type of lung cancer are non-small cell lung cancers, which can be further subdivided into lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC). LUAD comprises about 40% of all lung cancer cases and usually affects non-smokers. LUSC is the second most common malignancy among tobacco smokers. Mutations in *KRAS* and *EGFR* are common in LUAD, but rare or absent in *LUSC*. For lung cancer, LL2 is a cell line that has been derived from an implantation of primary Lewis lung carcinoma that is widely used as a model for metastasis. Parental Lewis lung carcinoma was derived from a spontaneously developed epidermoid carcinoma in the lung of C57BL/6 mice¹⁷⁶

Our WGS of the LL2 cell line revealed 8 driver mutations that are also commonly mutated in human lung cancer: *Trp53*, *Fat1/3*, *Maml2*, *Nras*, *Nrk*, *Polq*, and *Phpn2* (**Figure 1.16a**). SBS comparisons to lung cancer PCAWG data revealed that LL2 did not match to any of the 48 patients in the cohort (**Table 1.2**). LL2 is also the most mutated cell line that has been derived from a spontaneous tumor, with 259,650 SBS substitutions. Furthermore, LL2 has an enrichment of T>C and T>G SBS, which closely resembles the human SBS17b signature that has been previously represented in other spontaneous arising mouse models (**Figure 1.16b**).

LL2 *in vivo* tumor models have been utilized in many different settings. LL2 can be orthotopically implanted, albeit more technically challenging. The most commonly used method is

subcutaneous injection into the flank, which has been shown to retain its metastatic characteristic. Furthermore, tail vein injections of this model facilitated metastatic lesions in the lungs¹⁷⁷. For this model, we used heterotopic flank tumors to profile the transcriptional landscape of the tumor tissue. We compared LL2 tumor samples to both LUAD and LUSC cohorts within TCGA. We saw observed clustering LL2 samples to terminal respiratory unit (TRU) – bronchoid and proximal-inflammatory – squamoid groups within LUAD (**Figure 1.16c**). When compared to LUSC patient data, LL2 clustered broadly with secretory, classical, and basal subtypes (**Figure 1.16d**). Examination of differentially expressed genes using paper_RNA_clusters of both LUAD and LUSC revealed gene expression patterns that varied between the three LL2 samples.

Discussion

Syngeneic mouse models of cancer are indispensable for the advancement of innovative immunotherapies and treatment approaches for cancer patients. They have been widely used for evaluating efficacy, mechanism of action, and identifying predictive biomarkers of response to inform clinical drug development. However, there is still a need to fully elucidate the molecular and immunologic characteristics of these models to increase their predictive value in clinical settings. In this study, our goal is to analyze the genetic and transcriptional features of frequently used syngeneic models, with the aim to establish a reference for selecting mouse models suitable for testing hypotheses relevant to specific disease indications. We provide molecular annotation of these models to describe the specific driver mutations that are commonly found in their respective human disease, the SBS signatures that can reflect the etiology of human disease, and the gene expression profiles that can correlate to specific human disease subtypes. Taken all together, we can leverage this information to determine which models are better suited for certain disease subsets and whether or not they can be used accurately to translate preclinical findings into clinical response.

Our analysis of the somatic mutation profile of the mouse cell lines reveals genetic disparities when compared with the prevalent mutations in the corresponding clinical disease. Examples of these include *Apc* in B16F10, and *Kras* and *Atrx* in PAN02. However, several genes frequently mutated in clinical cases are also present in the preclinical models we examined, providing a chance to explore the effects of these mutations in a preclinical syngeneic environment. These findings will allow researchers to explore other models exhibiting the same signaling pathway addictions to specific molecular targets (e.g. BRAF, MEK) as well as identify new markers for pharmaceutical intervention, such as the *Fat* family of proteins. We have also identified the single-base substitution signatures of our mouse models and determined their ability to reflect their human equivalents.

Historically, B16F10, GL261, LL2, ID8, and MC38 have been the most widely used models for their respective disease indications. Our analysis indicated that despite originating from similar tissues as those in humans, the mutational patterns of these models diverge notably from their human counterparts. This discrepancy can be attributed to their varied methods of derivation, including carcinogen exposure, serial passaging on plastic, or spontaneous oncogene modulation, which may not accurately reflect the etiology of human diseases. Interestingly, the human SBS17b signature was enriched in spontaneously developed mouse tumor models, such as B16F10, YUMM1.7, 4T1, KPC4580, TRAMPC1/2, ID8, and LL2. It is currently unclear how the SBS17b mutational signature is induced in human disease. We also observed that the relevance of tumor initiation techniques used to develop these cell lines can recapitulate certain disease etiologies. The data shown in our study, along with previous studies from our lab, demonstrated the use of DMBA-induced tumor models poorly reflected the human SBS7 tobacco-smoking signatures compared to 4NQO¹³⁵. Furthermore, use of UVB irradiation on GEMMs bearing human-relevant driver mutations recapitulated mutation patterns consistent with UV-exposure signatures found in human skin cutaneous melanoma.

Our study also highlights the gene expression profile of these tumor models *in vivo* and their ability to reflect the transcriptional landscape of human disease. While murine tumor models have been broadly applied to cancers defined by anatomical region, our goal is to delineate specific subtypes of human cancer in which preclinical evaluation and data can be applied. Upon further examination of our head and neck cancer models, 4MOSC1 not only reflects the somatic mutations induced by tobacco-smoking, but it also expresses gene patterns that correlated to smoking status in HNSCC patients. While an HPV-induced mutational signature has yet to be defined, the MEER tumors have differentially expressed genes associated with patient HPV status.

There are still limitations for the application of these syngeneic models for cancer research. Despite most of these models bearing driver mutations commonly found within their respective disease, they rarely contain the same variant observed in human tumors and harbor mutations from other cancer subtypes. The mutational landscape of these mouse models also does not accurately reflect the mutational pattern seen in their human disease equivalents. MC38 and CT26 harbor many commonly mutated genes in colorectal cancer, including *Trp53* and *Kras*; however, the mutational patterns from these models did not match any patient data within the PCAWG database. Furthermore, many of these models have TMBs that are much higher than what is observed in humans, which may result in higher immunogenicity leading to tumor regression and graft rejection observed in our experiments. This could be in part attributed to extended culturing time, freeze-thaw cycles, and the extent of carcinogenesis. From the bulk RNA sequencing data, we also see variability between biological replicates from the same cell line. This could be due in part to differences between immune cell recruitment and stromal remodeling of the TME at the anatomical location of implantation. These limitations present many challenges for modeling immunotherapy responses for human disease. Thus, we conclude that careful considerations must be taken to ensure that new and next-generation syngeneic mouse models

reflect human-relevant driver genes and passenger mutations that reflect clinical presentation of disease.

In conclusion, our study illuminates the potential for specific subsets of human diseases to benefit from preclinical data derived from appropriate model systems. Through our analysis, we are beginning to identify these subsets, paving the way for targeted preclinical investigations. Moreover, in silico data analysis combined with further characterization of tumor tissue holds promise in identifying specific TME and spatial immune neighborhoods within these model systems. This deeper understanding of the immune microenvironment will enable researchers to interrogate responses to immune-modulating treatments more effectively, ultimately advancing our ability to translate preclinical findings into clinical applications.

Methods

Cell lines

The 4MOSC1 and 4MOSC2 cell lines were previously generated by our lab¹³⁵. The B16F10, ID8, ID8-Vegf/Defb29, DDHER2, CT-26, 4T1, and GL261 cell lines were gifted by N. Steinmetz (University of California San Diego, La Jolla, California). The MEER cell line was gifted by J. Califano (University of California San Diego, La Jolla, California). The 4T1 cell line was gifted by J. Yang (University of California San Diego, La Jolla, California). The KPC4580 and Pan02 cell lines were gifted by R. White (University of California San Diego, La Jolla, California). The MC38 cell line was gifted by A. Sharabi (University of California San Diego, La Jolla, California). The MOC1 and MOC2 line was gifted by R. Uppaluri (Washington University School of Medicine, St. Louis, Missouri). The YUMM1.7, YUMMER1.7, and YUMMER.G cell lines were gifted by Z. Ronai (Sanford Burnham Prebys Medical Discovery Institute, La Jolla, California). LL/2 (LLC1), TRAMPC1, and TRAMPC2 were purchased from American Type Culture Collection (ATCC) and were cultured according to their specified guidelines.

Mouse tumor models

All animal studies involving tumor implantation were approved by the Institutional Animal Care and Use Committee (IACUC) of University of California San Diego with protocol ASP#S15195. Mice at Moores Cancer Center, UCSD are housed in micro-isolator and individually ventilated cages supplied with acidified water and fed 5053 Irradiated Picolab Rodent Diet 20 from lab diet. Temperature for laboratory mice in our facility is mandated to be between 65–75 °F (~18–23 °C) with 40–60% humidity. All animal manipulation activities are conducted in laminar flow hoods. All personnel are required to wear scrubs and/or lab coat, mask, hair net, dedicated shoes, and disposable gloves upon entering the animal rooms.

Depending on tumor model, male and female mice (C57BL/6 or BALB/c) between six and eight weeks of age were obtained from Charles River Laboratories. Mice were shaved three days prior to implantation when necessary. All subcutaneous, intradermal, and intraperitoneal injections were performed with a final volume of 100 μ L; intralingual injections were performed at 40 μ L; intracranial injections were performed at 10 μ L. For the 4MOSC cell lines, 1×10^6 cells were implanted intralingually in female C57BL/6 mice. LLC1 (5×10^5), MC38 (1×10^5), MOC1 (5×10^6), MOC2 (1×10^5), MEER (1×10^6), and PAN02 (1×10^6) were implanted subcutaneously in the flank of female C57BL/6 mice. B16F10 (1×10^5) and \

YUMMER.G (1×10^6) were implanted intradermally in female C57BL/6 mice. YUMM1.7 (1×10^5) and YUMMER1.7 (1×10^6) were implanted intradermally in male C57BL/6 mice. 4T1 (1×10^5) was implanted orthotopically in the mammary fat pad of female BALB/c mice. CT26 (1×10^5) and DDHER2 (1×10^6) was implanted heterotopically in the flank of female BALB/c mice. For TRAMPC1 and TRAMPC2, 5×10^6 cells were resuspended in a 1:1 mixture of PBs and Matrigel (Corning #356231) and implanted subcutaneously in the flank of male C57BL/6 mice. KPC4580 (5×10^5) was also implanted subcutaneously in the flank of male C57BL/6 mice. For GL261, 5×10^4 cells were implanted in the right cerebral cortex of female C57BL/6 mice at a

depth of 3.5mm. For ID8 and ID8-Vegf-Defb29, 5×10^6 were implanted intraperitoneally in female C57BL/6 mice.

Each tumor model contained three biological replicates and tumors were harvested after 14 days to allow for proper engraftment. Tumor tissue was divided into two parts for subsequent downstream analysis: tissues were harvested directly into Trizol and physically homogenized immediately post-euthanasia for bulk RNA sequencing and formalin-fixed and paraffin-embedded.

DNA and mRNA sequencing analysis

DNA was extracted using PureLink Genomic DNA Mini kits (Invitrogen) and RNA was extracted using RNeasy Mini kits (Qiagen). WGS was performed by Novogene using their 350bp plant and animal whole genome library preparation with NovaSeq 6000 PE150. Bulk RNA sequencing was performed by the Institute of Genomic Medicine at University of California San Diego using mRNA stranded library with 30 million reads using NovaSeq 600 PE150.

Whole genome raw sequencing data were aligned to mm39 (C57BL/6) and BALB_cJ_v1 (BALB/c) using BWA¹⁷⁸. Somatic mutations were identified by comparing the sequencing data from each cancer sample to the sequencing data from a normal tissue derived from the tail of one mouse per each strain (all mice were genetically identical). To ensure robustness of the results, a consensus variant calling strategy was leveraged in which somatic mutations were identified using three independent bioinformatics tools: Strelka2¹⁷⁹, Varscan2¹⁸⁰, and GATK4 Mutect2¹⁸¹.

For bulk RNA sequencing, FASTQ files were mapped to the mm39 reference genome using STAR¹⁸². The resulting aligned BAM files were quantified using HTSeq¹⁸³ to generate the gene expression counts. Transcriptomic data for cancers in humans was sourced from the Cancer Genome Atlas. For comparison, 12 TCGA projects were selected which corresponded with the 9 cancers represented among the mouse models. For each project, all samples with gene expression data were exported from the Genomic Data Commons (GDC)¹⁸⁴ along with clinical

data. Tissue-matched normal samples were also included if available. For each TCGA project, expression-based subtypes were compiled from both the TCGAbiolinks^{184,185} and consensus hierarchical clustering analysis performed by the Broad Firehose Pipeline¹⁸⁶. Subtypes with fewer than five data points within any project were removed along with their corresponding data.

Mutational signature analysis

Any mutation found in two out of the three variant callers was considered a bona fide somatic mutation. Additional filtering to remove any residual germline contamination was applied and any mutation found in Mouse Genome Project or shared among all four cancers was discarded. Somatic mutational profiles were derived using the immediate sequencing context by evaluating the base 5' and the base 3' to each single point mutation. In addition, transcriptional strand bias was evaluated by considering all protein coding genes. Mutational signatures were extracted using our previously developed computational framework SigProfiler^{132,187}.

Principal component analysis and visualization

Gene expression counts were filtered by 1:1 mouse to human genes, transformed using DESeq2¹⁸⁸, batch-corrected using limma¹⁸⁹ to correct for species-specific differences in expression, and scaled. Principal component analysis was performed with DESeq2 to generate a PCA plot containing all human and mouse samples. This process was repeated individually for each cancer type in which TCGA samples were grouped by subtype. Comparisons within the PCA plot were limited to significantly differentially expressed genes between different subtypes of cancer among TCGA samples.

Differential expression analysis and visualization

Gene expression counts were filtered by 1:1 mouse to human genes, normalized by effective library sizes using EdgeR¹⁹⁰, batch-corrected using limma to correct for species-specific

differences in expression, and scaled. For individual analysis of cancer, each human sample was compared to each mouse sample from models of that same cancer. A Pearson correlation coefficient was calculated based on significantly differentially expressed genes between the subtype of cancer associated with that human sample compared to all other subtypes (including tissue-matched normal samples if applicable) using EdgeR. For global analysis, all human samples were compared to all mouse samples. Again, a Pearson correlation coefficient was calculated based on significantly differentially expressed genes between the cancer associated with that human sample compared to all other cancers using EdgeR. Correlation values were visualized using ComplexHeatmap¹⁹¹ in R. Immune infiltration levels were quantified from expression using CIBERSORT¹³³.

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Prebys Medical Discovery Institute, La Jolla, California) for the YUMM1.7, YUMMER1.7, and YUMMER.G cell lines.

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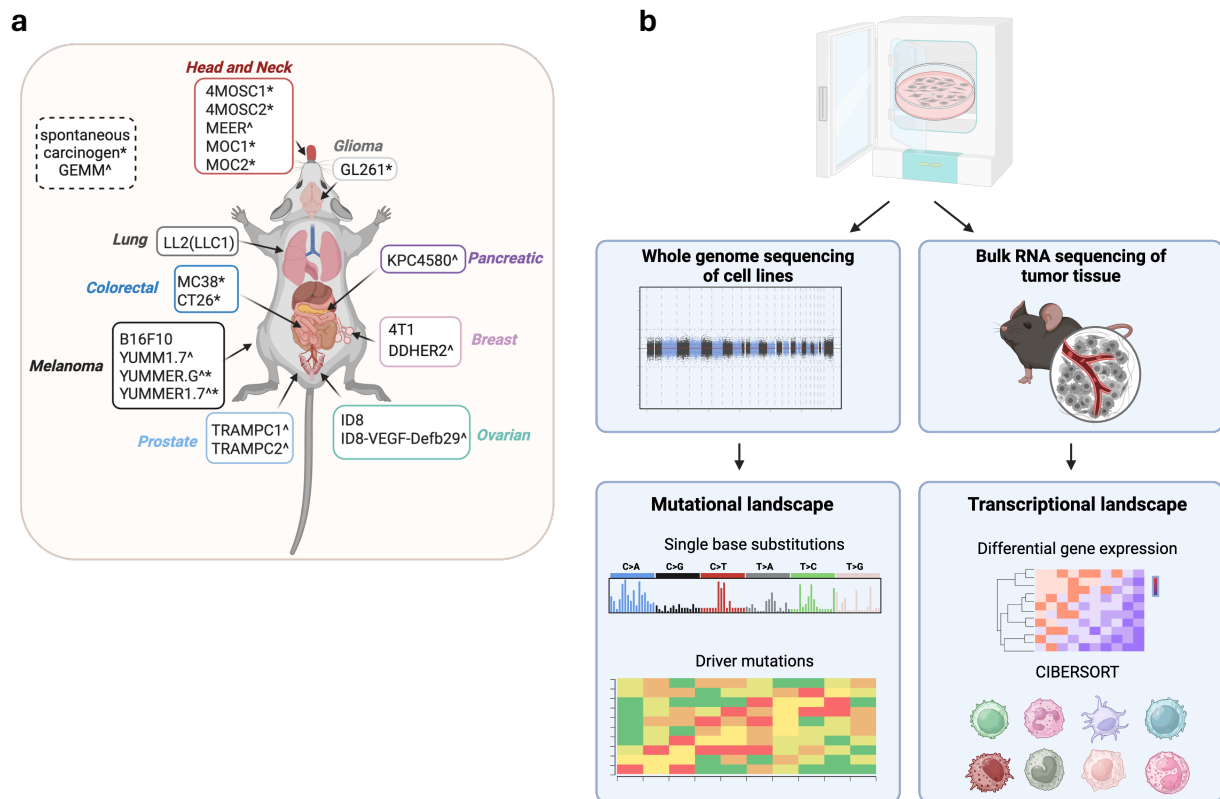


Figure 1.1: Commonly used syngeneic models and bioinformatic pipeline.

a, Overview of syngeneic models included in the study, organized by organ system. Models are derived from spontaneous, carcinogen-induced, and genetically manipulated tumor systems from C57BL/6 and BALB/c backgrounds. **b**, Bioinformatic pipeline for somatic mutation and gene expression analysis. Driver mutations and single-base substitutions were identified from whole genome sequencing was performed on tumor cell lines. Differential gene expression was performed on bulk RNA sequencing of tumor tissue generated from syngeneic mouse cell lines. CIBERSORT was also performed to infer immune cell populations within tumor tissue.

Figure 1.2: Driver mutations found across all TCGA project cohorts.

a, Identified driver mutations found in TCGA cohorts: adrenocortical carcinoma (ACC), bladder urothelial carcinoma (BLCA), breast invasive carcinoma, cervical squamous cell carcinoma (CESC), cholangiocarcinoma (CHOL), colon adenocarcinoma and rectum adenocarcinoma (COADREAD), lymphoid neoplasm diffuse large b-cell lymphoma (DLBC), esophageal carcinoma (ESCA), glioblastoma multiforme (GBM), head and neck squamous cell carcinoma (HNSC), kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), acute myeloid leukemia (LAML), brain lower grade glioma (LGG), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), mesothelioma (MESO), ovarian serous cystadenocarcinoma (OV), pancreatic adenocarcinoma (PAAD), pan-cancer (PANCAN), pheochromocytoma and paraganglioma (PCPG), prostate adenocarcinoma (PRAD), sarcoma (SARC), skin cutaneous melanoma (SKCM), stomach adenocarcinoma (STAD), thymoma (THYM), thyroid carcinoma (THCA), uterine carcinosarcoma (UCS), uterine corpus endometrial carcinoma (UCEC), uveal melanoma (UVM). **b**, Identified somatic mutations in driver genes and their frequencies in all 22 syngeneic models.

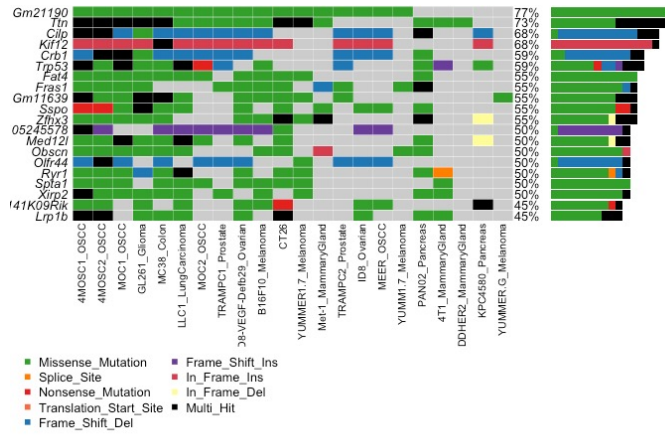
Input Sample	B cells naive	B cells memory	Plasma cells	T cells CD8	T cells CD4	T cells CD4 memory resting	T cells CD4 memory activated	T cells CD4 follicular helper	T cells regulatory (Tregs)	T cells gamma delta	NK cells resting	NK cells activated	Monocytes	Macrophages M0	Macrophages M1	Macrophages M2	Dendritic cells resting	Dendritic cells activated	Mast cells resting	Mast cells activated	Eosinophils	Neutrophils	P-value	Correlation
4MOSR1_1	0.043		0.021	0.034		0.022	0.01	0.031	0.016		0.01	0.018	0.059	0.076	0.042	0.09	0.016					0.000	0.000	0.125
4MOSR1_2	0.046		0.009	0.013		0.038	0.023	0.045	0.037		0.028	0.015	0.087	0.09	0.037	0.097	0.01	0.02				0.000	0.000	0.095
4MOSR1_3	0.024		0.009	0.013		0.058	0.033	0.085	0.075		0.075		0.057	0.131	0.058	0.035	0.01			0.029	0.005	0.000	0.000	0.078
4MOSR2_1	0.031		0	0.14			0.09	0.049		0.036	0.02			0.006	0.153		0	0.029			0.062	0.000	0.138	
4MOSR2_2	0.082					0.05	0.007	0.032		0.073	0.019			0.019	0.046	0.133			0.016		0.025	0.000	0.318	
4MOSR2_3	0.064					0.193	0.009	0.036		0.135				0.174	0.024	0.054			0.085		0.094	0.000	0.122	
4T1_1	0.098					0.178	0.037	0.007		0.111	0.003	0.123	0.259			0.136	0.037	0.01		0.099		0.000	0.000	0.185
4T1_2	0.243		0.077	0.002		0.006	0.074			0.016			0.12			0.181	0.059	0.03	0.019		0.001	0.000	0.267	
4T1_3	0.101					0.109	0.016			0.011	0.033	0.222				0.128	0.014	0.036		0.018		0.000	0.000	0.167
B16F10_1	0.102			0.059		0.126	0.064		0.064	0.046		0.1	0.008	0.113	0.016				0.01	0.022	0.000	-0.093		
B16F10_2	0.093			0.075		0.123	0.085	0.009		0.022	0.032	0.084	0.023	0.058	0.09	0.273			0.013	0.02	0.000	0.000	-0.081	
B16F10_3	0.103		0.009	0.087		0.077	0.023	0.009		0.055		0.293	0.011	0.037	0.066	0.231			0.03	0.008	0.000	0.000	-0.071	
CT26_1	0.042		0.009	0.008		0.065	0.011		0.008	0.052		0.014		0.012	0.143	0.069			0.082		0.000	0.000	0.200	
CT26_2	0.064		0.011			0.058	0.033			0.043	0.008	0.001	0.024	0.015	0.191	0.069			0.103		0.000	0.000	0.205	
CT26_3	0.11		0.082		0.033		0.039			0.053	0.027	0.271			0.045	0.138	0.114		0.088		0.000	0.000	0.274	
DOHER1_1			0.017			0.198				0.064			0.161			0.124	0.138		0.038		0.000	0.000	0.129	
DOHER1_2	0.042		0.024			0.147				0.096	0.001	0.001	0.001	0.036	0.025	0.073	0.167		0.028		0.000	0.000	0.164	
DOHER1_3	0.028		0.017	0.005		0.103				0.026	0.001	0.001		0.011	0.208		0.008	0.049		0.000	0.000	0.194		
GL261_1		0.128	0.031	0.081			0.042	0.116		0.029	0.057	0.001	0.001	0.066	0.026		0.102		0.031		0.000	0.000	-0.084	
GL261_3		0.169	0.022	0.029		0.009	0.092	0.045		0.021	0.001	0.225			0.084	0.027	0.235		0.039		0.000	0.000	0.034	
GL261_6	0.223		0.077	0.012		0.036	0.124			0.014	0.064	0.202	0.122	0.036	0.055			0.034		0.000	0.000	-0.015		
ID8_1	0.127		0.001	0.006		0.047	0.064			0.019	0.016	0.122	0.012	0.01	0.024	0.03		0.035		0.000	0.000	0.808		
ID8_2	0.11					0.055				0.058	0.184	0.025				0.023		0.047		0.000	0.000	0.142		
ID8_3	0.071			0.009	0.009	0.029				0.047	0.199	0.034				0.057		0.028		0.000	0.000	0.225		
ID8_VEGF_DEF829_2	0.062					0.043	0.029			0.055	0.117	0.037				0.031		0.029		0.016	0.000	0.414		
ID8_VEGF_DEF829_4	0.127		0.001	0.001		0.064	0.042			0.042	0.086	0.03				0.025		0.043		0.019	0.000	0.416		
ID8_VEGF_DEF829_5	0.168					0.056	0.032				0.057	0.08	0.037			0.017		0.037		0.01	0.000	0.402		
KPC4580_1	0.088		0.136		0.012	0.087				0.049			0.223			0.078	0.054		0.074		0.030	0.000	0.398	
KPC4580_2	0.157		0.14	0.063		0.092		0.005	0.071		0.01		0.22	0.01	0.1	0.018		0.125		0.000	0.000	0.320		
KPC4580_3	0.084		0.239	0.032		0.04	0.059			0.053		0.068	0.155	0.007	0.168	0.001	0.018	0.023		0.032	0.000	0.321		
LLC2_1	0.06		0.035	0.004			0.086	0.009		0.044		0.001		0.02	0.278	0.144	0.001	0.035		0.000	0.000	0.132		
LLC2_2	0.022			0.007	0.008	0.046	0.009			0.02	0.009	0.001			0.205	0.106		0.069		0.008	0.000	0.079		
LLC2_3	0.004				0.093		0.024			0.012	0.013		0.001	0.086	0.272		0.026			0.000	0.000	0.095		
MC38_1	0.054		0.012	0.107		0.147	0.014		0.047	0.014	0.15	0.176	0.034	0.207			0.038		0.000	0.000	0.112			
MC38_2	0.049		0.004	0.04		0.121	0.021		0.038	0.013	0.206	0.127	0.017	0.201			0.063		0.000	0.000	0.057			
MC38_3	0.068		0.004	0.037		0.009	0.155		0.03	0.023	0.152	0.174	0.039	0.268			0.05		0.000	0.000	0.111			
MEER_1	0.096		0.082	0.223			0.063	0.058		0.047	0.086	0.027	0.052	0.025	0.115	0.088	0.029		0.008	0.000	0.063			
MEER_2	0.098		0.104	0.175			0.047	0.042		0.07	0.023		0.202	0.032	0.085	0.111		0.011		0.000	0.000	0.129		
MEER_3	0.121		0.229	0.099	0.008	0.008	0.037	0.061		0.046	0.038		0.158		0.1	0.001	0.057		0.025	0.015	0.000	0.222		
MOCL1_1	0.02		0.03	0.099		0.02	0.047	0.026		0.07	0.004		0.055	0.005		0.09		0.032		0.000	0.000	0.074		
MOCL1_2	0.075		0.01			0.035	0.071			0.02	0.019		0.011	0.038		0.032		0.054		0.000	0.000	0.083		
MOCL1_3	0.109			0.117		0.149		0.009		0.026		0.092	0.248	0.009	0.2			0.029		0.009	0.000	0.111		
MOCL2_1	0.038					0.018	0.008	0.026		0.034			0.001		0.117			0.146		0.027	0.000	0.378		
MOCL2_2	0.034					0.063		0.034		0.077			0.001		0.136			0.12		0.049	0.000	0.262		
MOCL2_3	0.108			0.006		0.049		0.028	0.026	0.074	0.017		0.001	0.162				0.078		0.007	0.000	0.435		
TRAMP1_1	0.107		0.038	0.028		0.05	0.047	0.019		0.073			0.231		0.213	0.062	0.001	0.045		0.000	0.000	0.118		
TRAMP1_2	0.048		0.029			0.147	0.035		0.028		0.216	0.045		0.241	0.174		0.039		0.000	0.000	0.098			
TRAMP1_3	0.118		0.204	0.027		0.076	0.012	0.009		0.005	0.041	0.216			0.221	0.057		0.017		0.000	0.000	0.222		
TRAMP2_1	0.188		0.252	0.016		0.012	0.044			0.005	0.007	0.076	0.021		0.167	0.16	0.037	0.015		0.000	0.000	0.410		
TRAMP2_2	0.039		0.011	0.015	0.009	0.018	0.008			0.015	0.153		0.001	0.252	0.145	0.001	0.037		0.000	0.000	0.345			
TRAMP2_3	0.146		0.072			0.042	0.033			0.014	0.01	0.075		0.01	0.29	0.201		0.016		0.000	0.000	0.121		
YUMR11_1	0.158		0.114	0.009		0.008	0.016	0.046	0.055	0.05	0.001		0.24	0.034	0.241			0.025		0.002	0.000	0.459		
YUMR11_2	0.125			0.025		0.002	0.026	0.021	0.021	0.016	0.104	0.192	0.042	0.194	0.075		0.086			0.000	0.000	0.306		
YUMR11_3	0.175		0.069			0.022	0.046	0.04	0.038	0.055		0.054	0.153	0.031	0.294			0.049		0.000	0.000	0.322		
YUMR17_1	0.086		0.024	0.041		0.028	0.092	0.009		0.052		0.235	0.045	0.01		0.017	0.058			0.000	0.000	0.195		
YUMR17_2	0.022			0.147			0.031		0.021	0.043	0.069	0.001	0.042	0.086	0.099	0.006		0.129		0.000	0.000	0.198		
YUMR17_3	0.162			0.015			0.094		0.008	0.085		0.159	0.121	0.045	0.252			0.058		0.000	0.000	0.256		
YUMR18_1	0.085		0.079	0.092		0.115	0.044	0.006		0.059	0.019	0.143	0.041	0.021	0.225		0.009	0.062		0.000	0.000	0.230		
YUMR18_2	0.136		0.059	0.078			0.066	0.072		0.06	0.013	0.005	0.223	0.005	0.221		0.037	0.023		0.002	0.000	0.317		
YUMR18_3	0.091		0.028	0.127		0.114	0.015			0.049	0.006	0.257	0.001	0.035	0.202			0.068		0.000	0.000	0.187		

Figure 1.3: CIBERSORT reveals immune landscape of murine syngeneic tumors.
Leukocyte signature matrix from immune cell profiling of mouse tumor tissue by CIBERSORT enumerating the composition of 22 immune cell subsets.

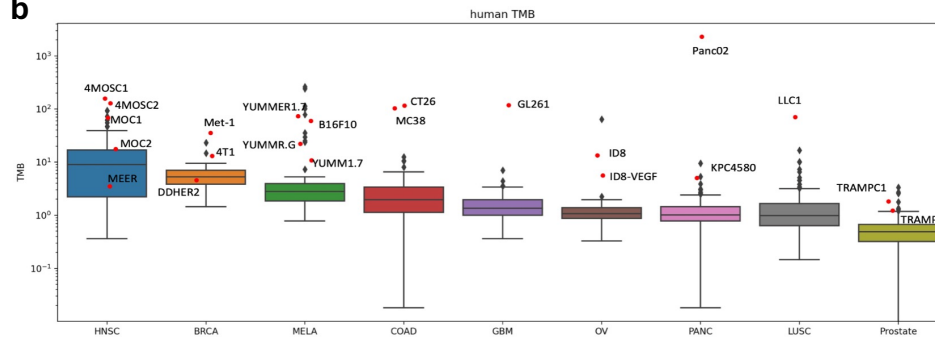
Figure 1.4: Mutational landscape of syngeneic tumor models.

a, Driver gene plot displaying the most frequently mutated pan-cancer driver genes, colored by mutation type, within each cell line. **b**, Whisker-box plot of tumor mutation burden (number of mutations per megabase) of each patient sample within each of PCAWG cohort. Plotted in red is the mutational burden of matched syngeneic cell lines. **c**, Cosine similarity scores of each mouse syngeneic model compared to each patient within the corresponding PCAWG cohort. A threshold of 0.85 cosine similarity is used to determine a similar match. **d**, PCA plot of gene expression from human samples within the TCGA database overlayed with those of each mouse model sample. Samples cluster together based on their similarity of gene expression across significantly differentially expressed genes between subtypes of cancer among TCGA samples.

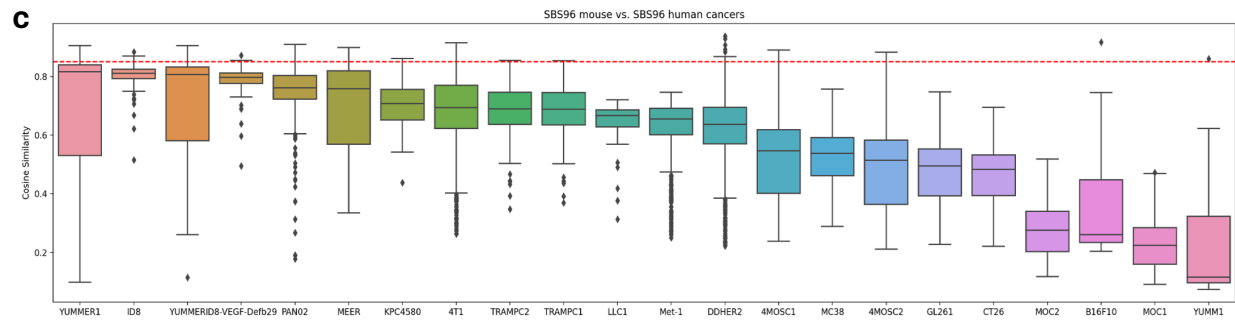
a



b



c



d

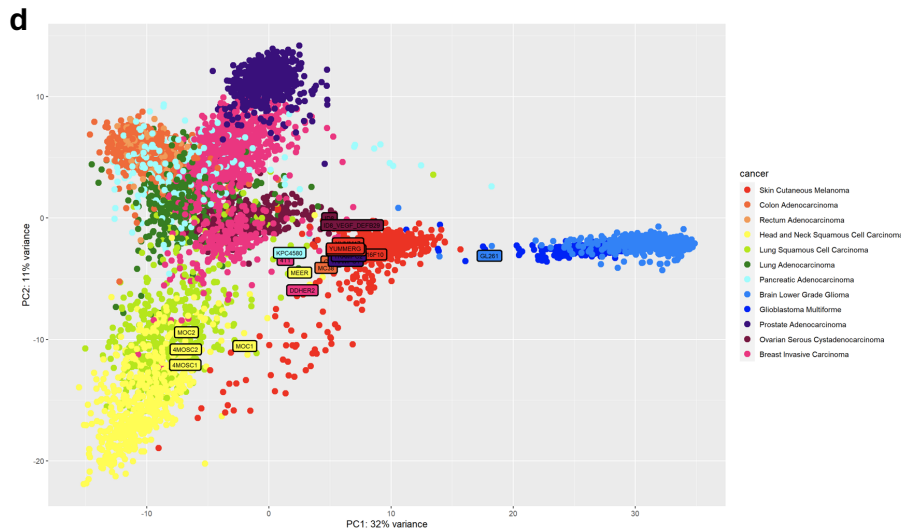
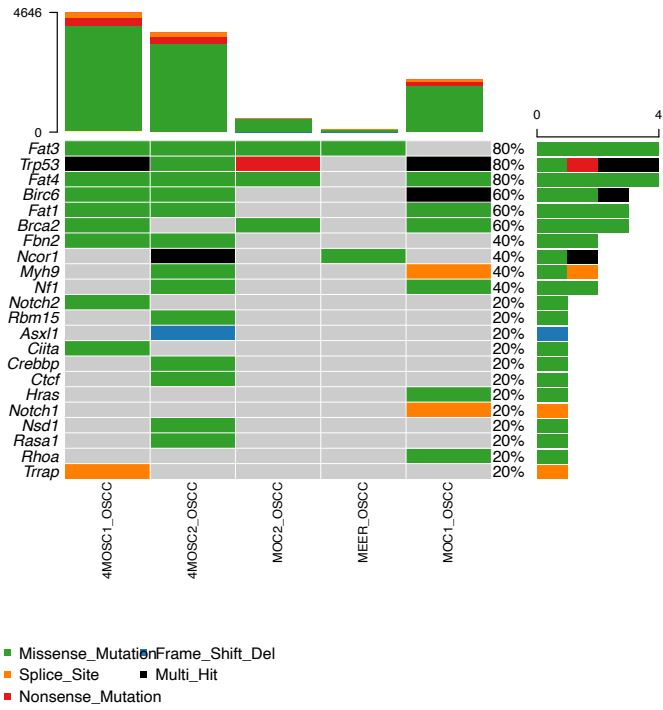


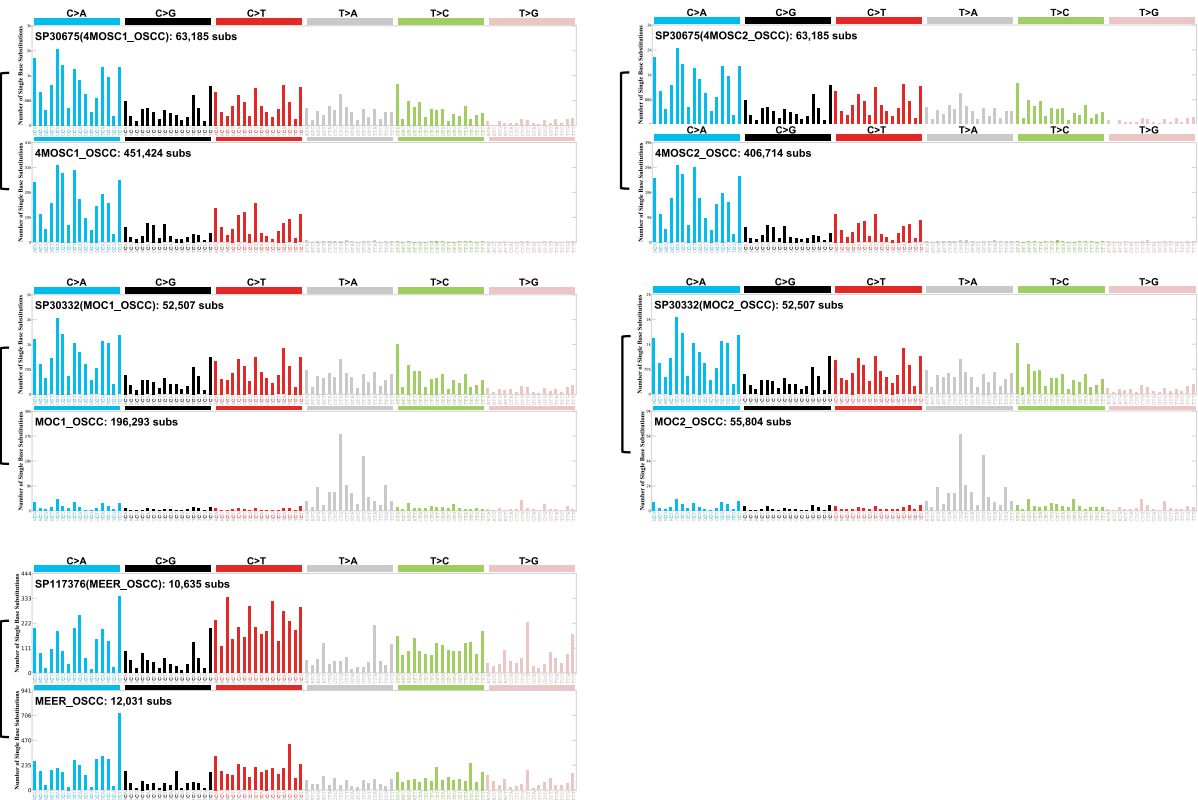
Figure 1.5: Mutational landscape of 4MOSC, MEER, and MOC head and neck cancer models.

a, Driver plot of mutated head and neck cancer driver genes found in each mouse head and neck cell line, colored by mutation type. **b**, Single base substitution signatures of each mouse model against their highest cosine similarity scored human patient within the head and neck cancer PCAWG cohort.

a



b



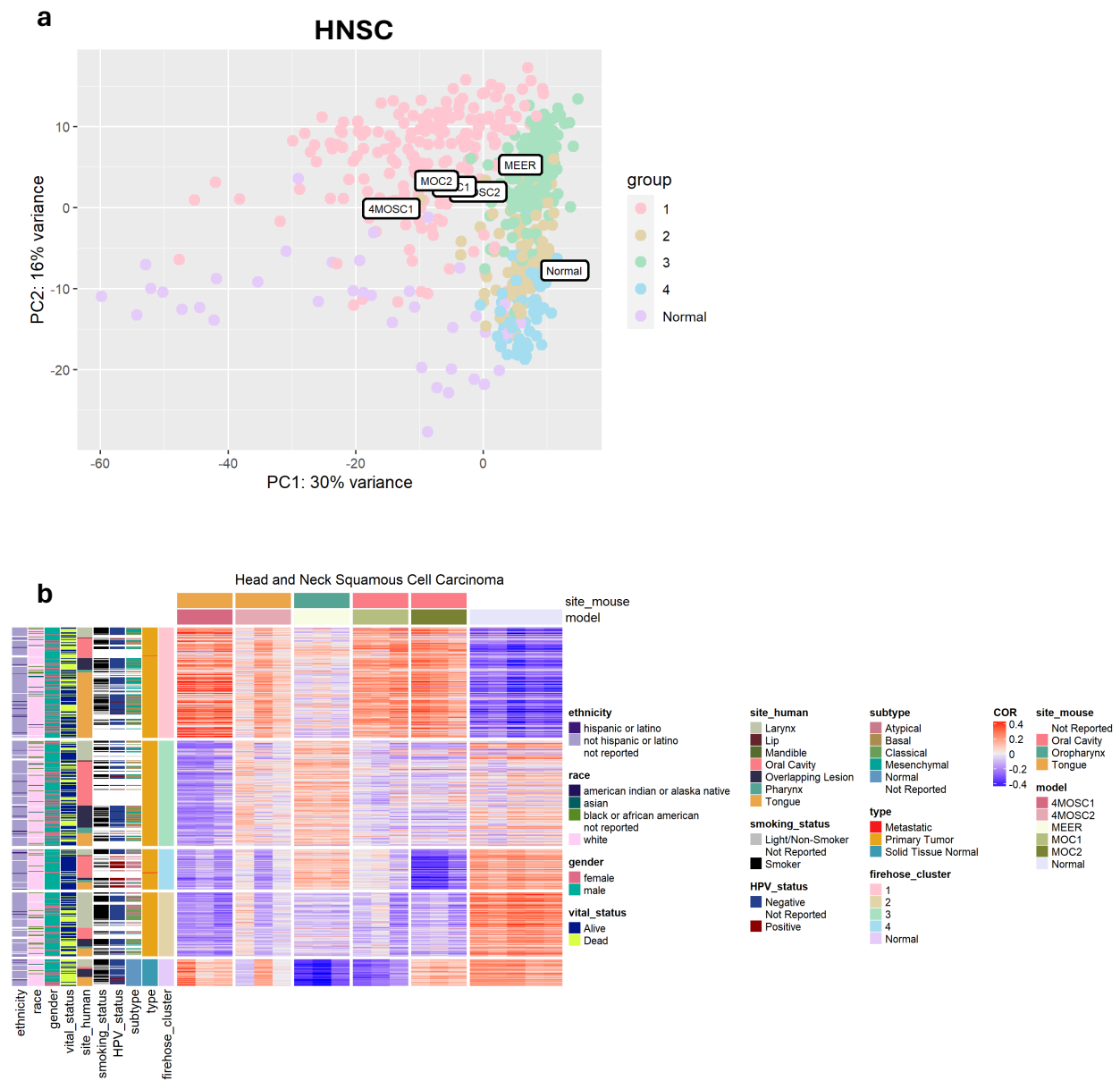


Figure 1.6: Gene expression profile of 4MOSC, MEER, and MOC head and neck cancer models.

a, PCA plot of gene expression profiles of 4MOSC1, 4MOSC2, MEER, MOC1, and MOC2 against those of the HNSC TCGA group. **b**, Heatmap visualizing correlations in gene expression between each TCGA sample (grouped by subtypes indicated in annotations and each mouse sample. Strength of correlation is indicated by color. For each row, correlations are calculated according to genes that are significantly differentially expressed by mouse samples compared to TCGA expression data.

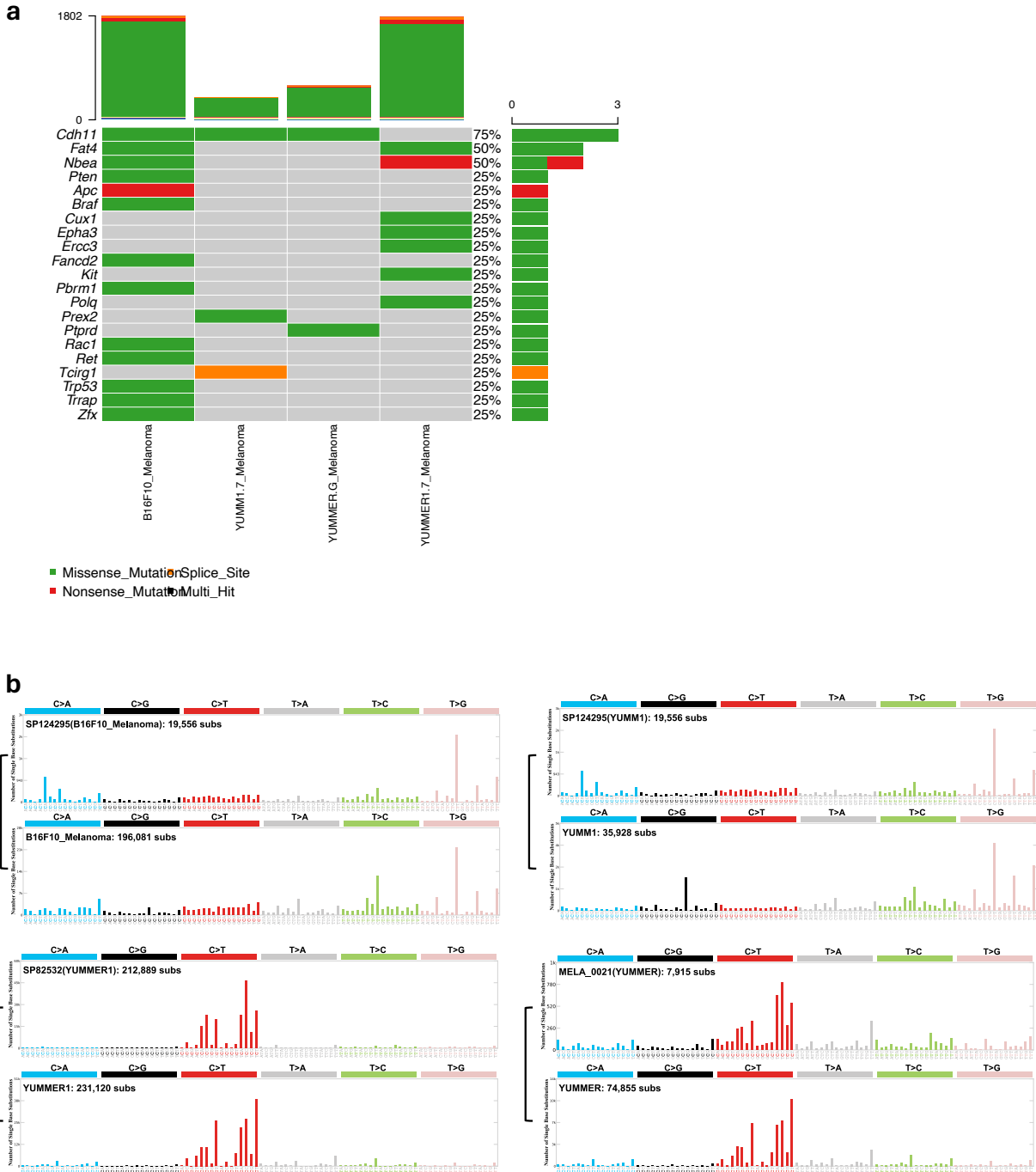


Figure 1.7: Mutational landscape of B16F10 and YUMM family skin cutaneous melanoma models.

a, Driver plot of mutated skin melanoma driver genes found in B16F10, YUMM1.7, YUMMER1.7, and YUMMER.G, colored by mutation type. **b**, Single base substitution signatures of each mouse model against their highest cosine similarity scored human patient within skin cancer PCAWG cohort.

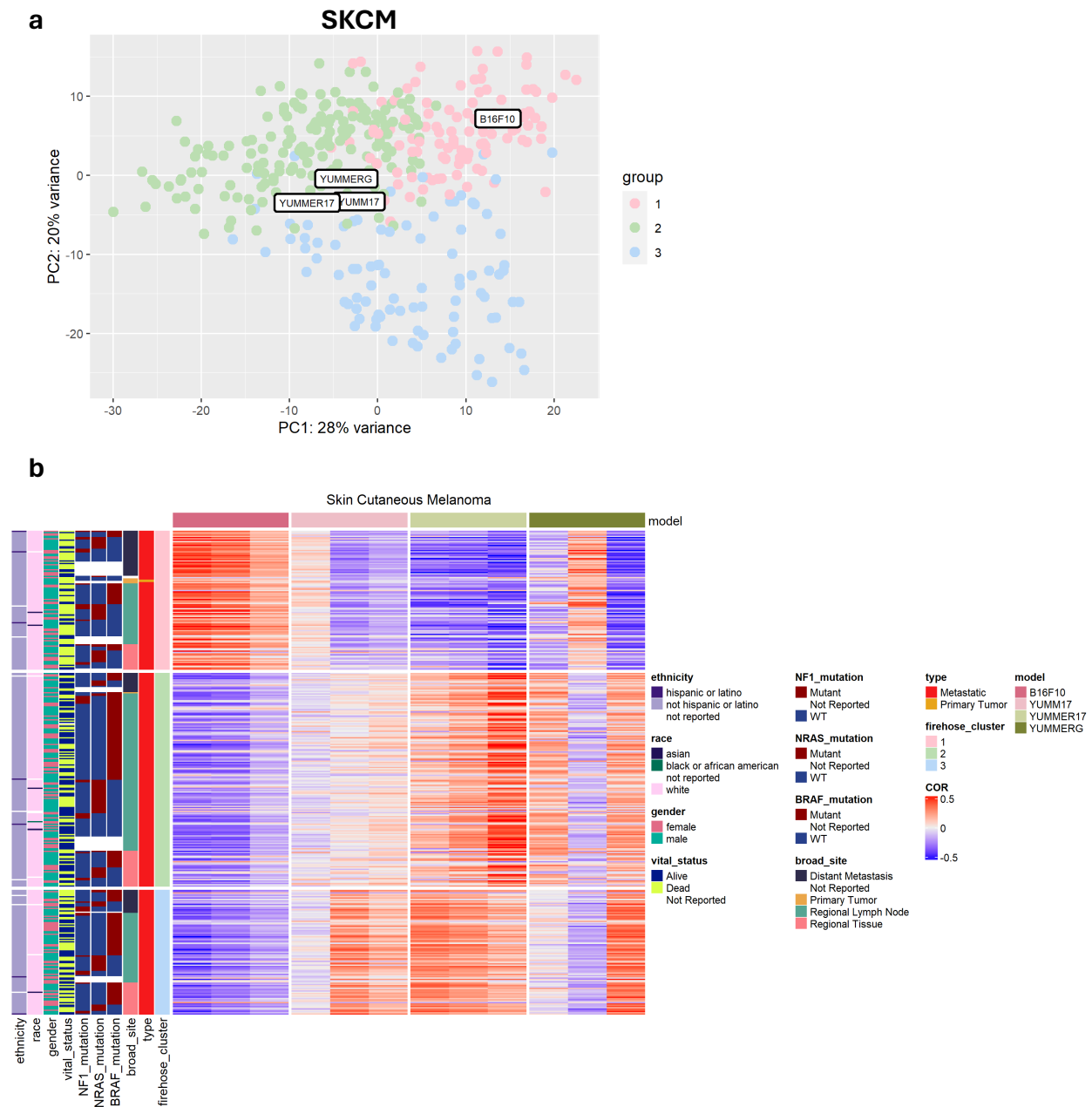


Figure 1.8: Gene expression profile of B16F10 and YUMM family skin cutaneous melanoma models.

a, PCA plot of gene expression profiles of B16F10 and YUMM models against those of the SKCM TCGA group. **b**, Heatmap visualizing correlations in gene expression between each TCGA sample (grouped by subtypes indicated in annotations) and each mouse sample. Strength of correlation is indicated by color. For each row, correlations are calculated according to genes that are significantly differentially expressed by mouse samples compared to TCGA expression data.

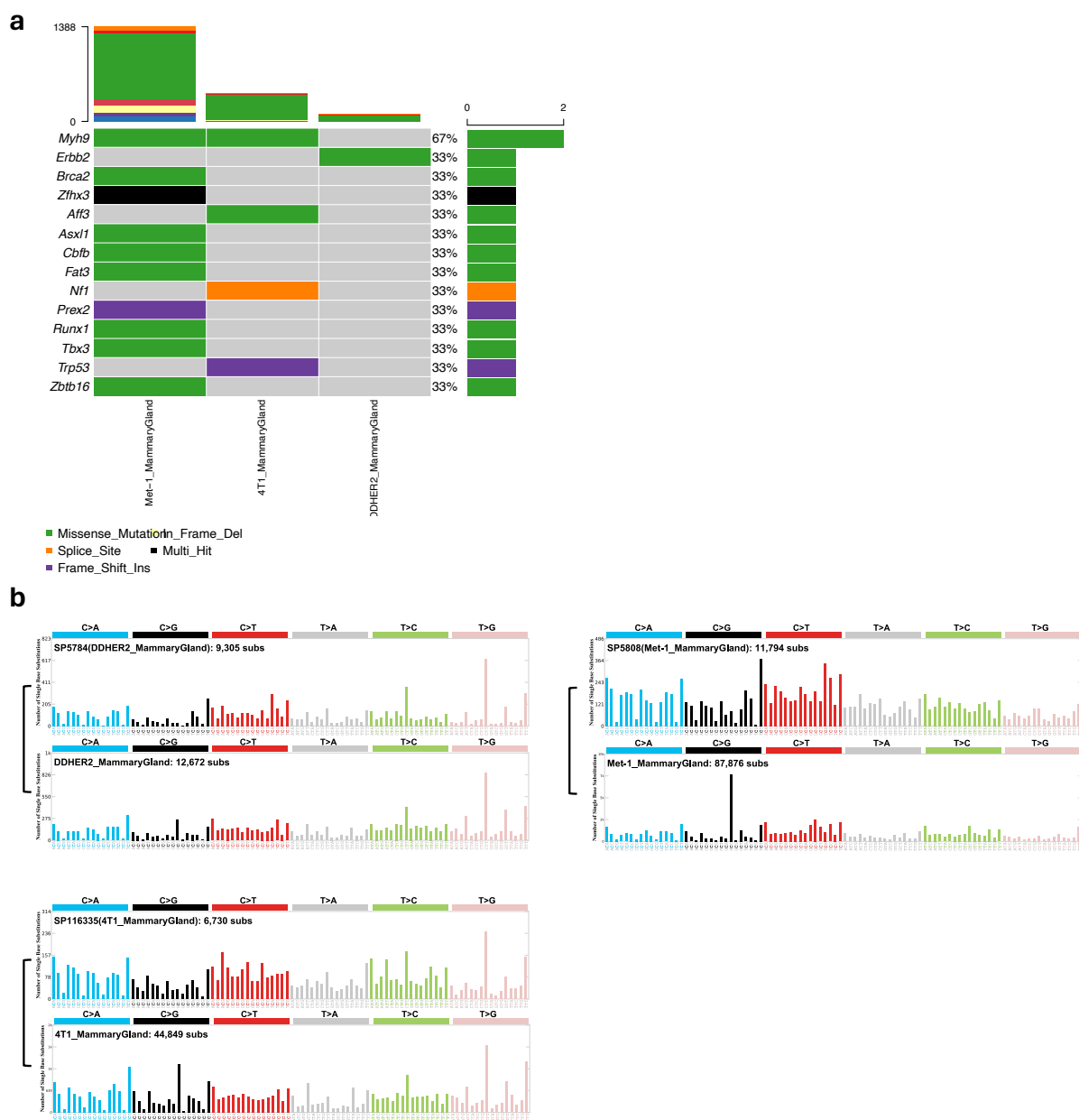


Figure 1.9: Mutational landscape of 4T1, DDHER2, and Met-1 invasive breast cancer models.

a, Driver plot of mutated breast cancer driver genes found in each mouse breast cancer cell line, colored by mutation type. **b**, Single base substitution signatures of each mouse model against their highest cosine similarity scored human patient within the breast cancer PCAWG cohort.

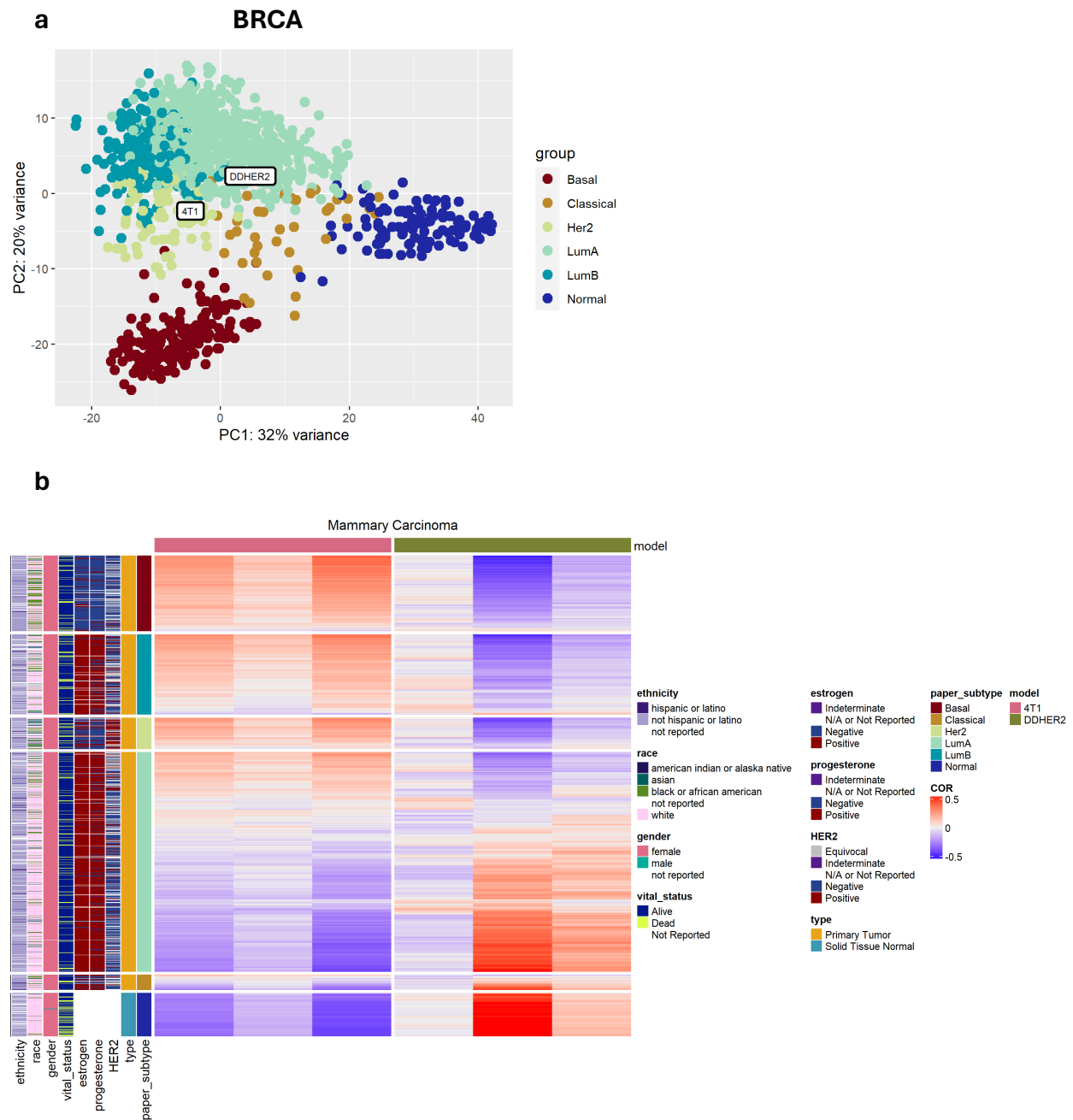
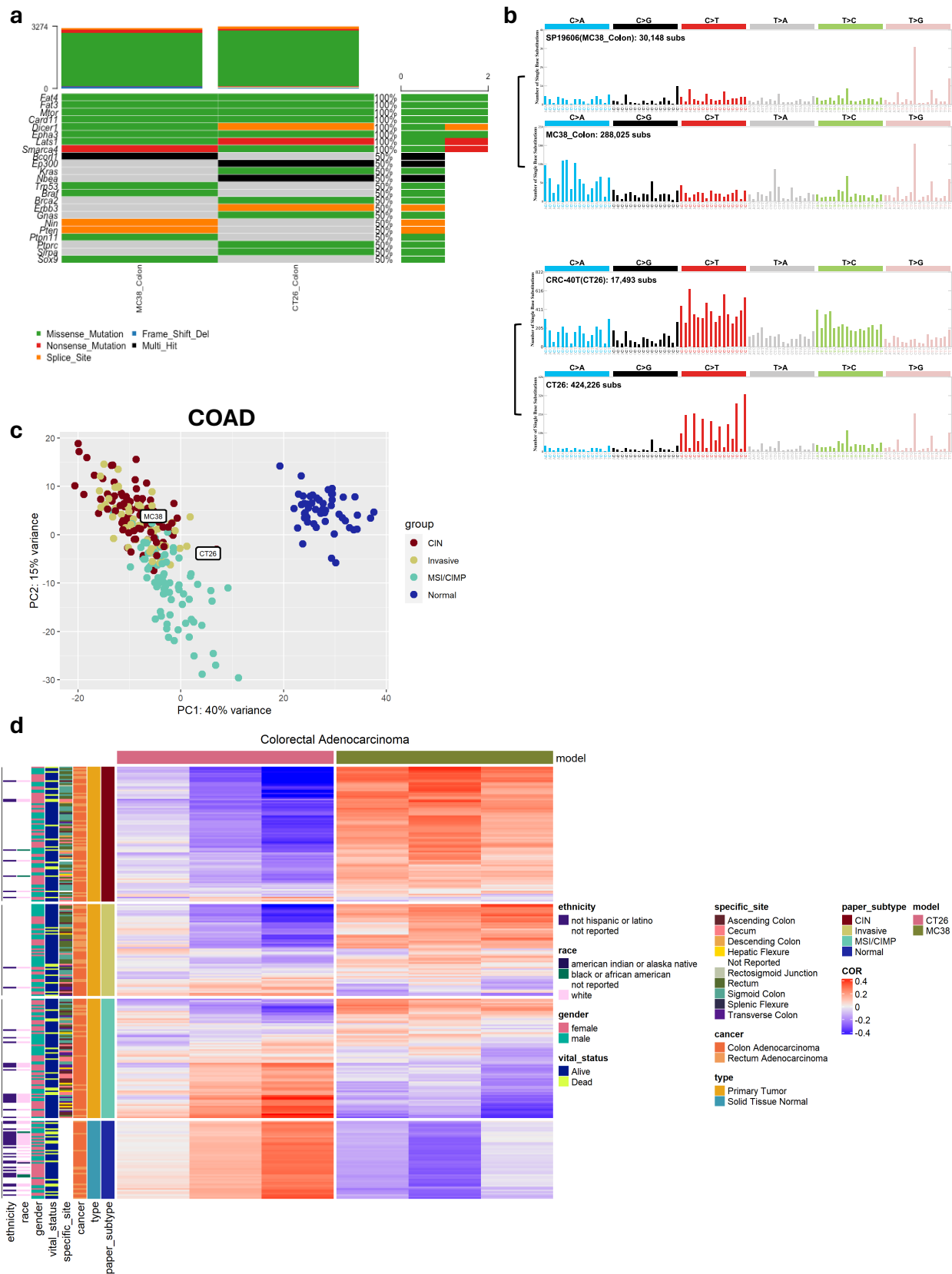


Figure 1.10: Gene expression profile of invasive 4T1 and DDHER2 breast cancer models
a, PCA plot of gene expression profiles of 4T1 and DDHER2 models against those of the BRCA TCGA group. **b**, Heatmap visualizing correlations in gene expression between each BRCA TCGA sample (grouped by subtypes indicated in annotations and each mouse sample. Strength of correlation is indicated by color. For each row, correlations are calculated according to genes that are significantly differentially expressed by mouse samples compared to TCGA expression data.

Figure 1.11: Mutational landscape and gene expression profile of MC38 and CT26 colorectal adenocarcinoma models

a, Driver plot of mutated colorectal cancer driver genes found in MC38 and CT26 cell lines, colored by mutation type. **b**, Single base substitution signatures of each mouse model against their highest cosine similarity scored human patient within the corresponding human cancer cohort. **c**, PCA analysis of gene expression profiles of MC38 and CT26 against those of the COAD TCGA group. **d**, Heatmap visualizing correlations in gene expression between each COAD TCGA sample (grouped by subtypes indicated in annotations and each mouse sample. Strength of correlation is indicated by color. For each row, correlations are calculated according to genes that are significantly differentially expressed by mouse samples compared to TCGA expression data.



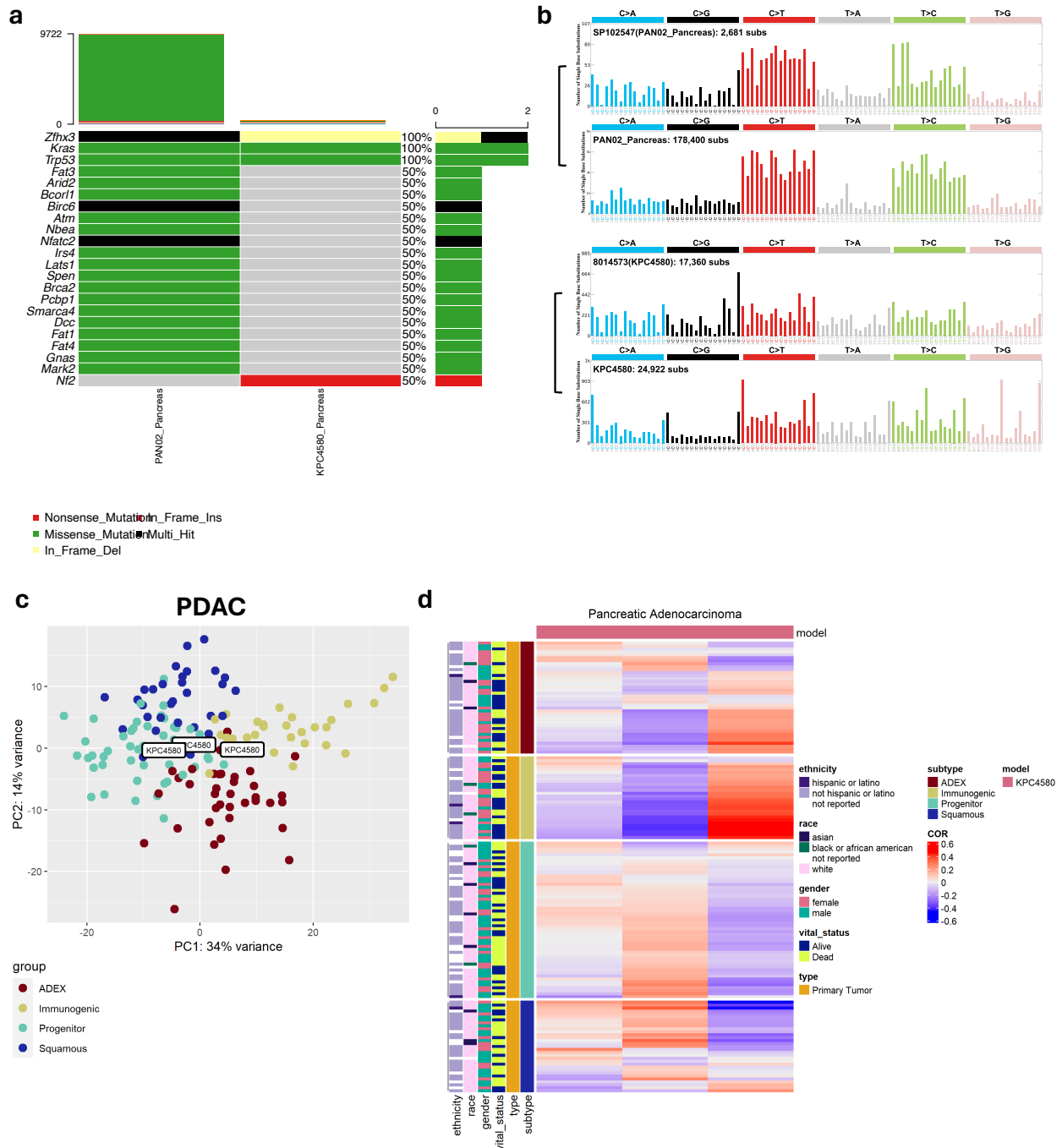


Figure 1.12: Mutational landscape and gene expression profile of PAN02 and KPC4580 pancreatic adenocarcinoma models.

a, Driver plot of mutated pancreatic cancer driver genes found in PAN02 and KPC4580 cell lines, colored by mutation type. **b**, Single base substitution signatures of each mouse model against their highest cosine similarity scored human patient within pancreatic cancer PCAWG cohort. **c**, PCA analysis of gene expression profiles of KPC4580 against those of the PDAC TCGA group. **d**, Heatmap visualizing correlations in gene expression between each PDAC TCGA sample (grouped by subtypes indicated in annotations) and each mouse sample. Strength of correlation is indicated by color. For each row, correlations are calculated according to genes that are significantly differentially expressed by mouse samples compared to TCGA expression data.

Figure 1.13: Mutational landscape and gene expression profile of TRAMPC prostate adenocarcinoma models

a, Single base substitution signatures of the TRAMPC models against their highest cosine similarity scored human patient within the prostate cancer PCAWG cohort. **b**, PCA analysis of gene expression profiles of TRAMPC1/2 against those of the PRAD TCGA group. **c**, Heatmap visualizing correlations in gene expression between each PRAD TCGA sample (grouped by subtypes indicated in annotations and each mouse sample. Strength of correlation is indicated by color. For each row, correlations are calculated according to genes that are significantly differentially expressed by mouse samples compared to TCGA expression data.

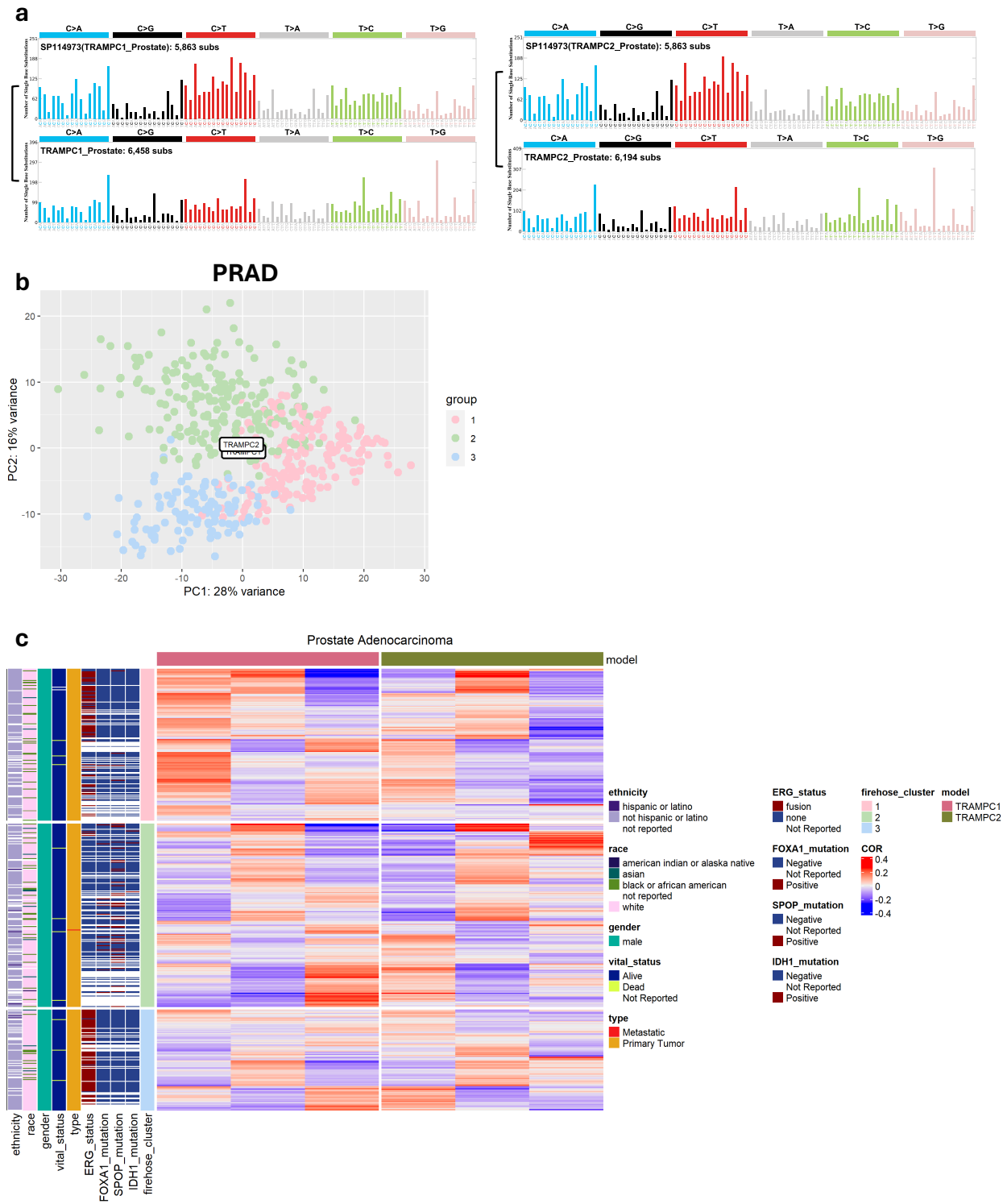


Figure 1.14: Mutational landscape and gene expression profile of ID8 ovarian carcinoma models

a, Single base substitution signatures of each mouse ovarian cancer model against their highest cosine similarity scored human patient within the ovarian cancer PCAWG cohort. **b**, PCA analysis of gene expression profiles of ID8 and ID8-Vegf-Defb29 against those of the OV TCGA group. **c**, Heatmap visualizing correlations in gene expression between each OV TCGA sample (grouped by subtypes indicated in annotations and each mouse sample. Strength of correlation is indicated by color. For each row, correlations are calculated according to genes that are significantly differentially expressed by mouse samples compared to TCGA expression data.

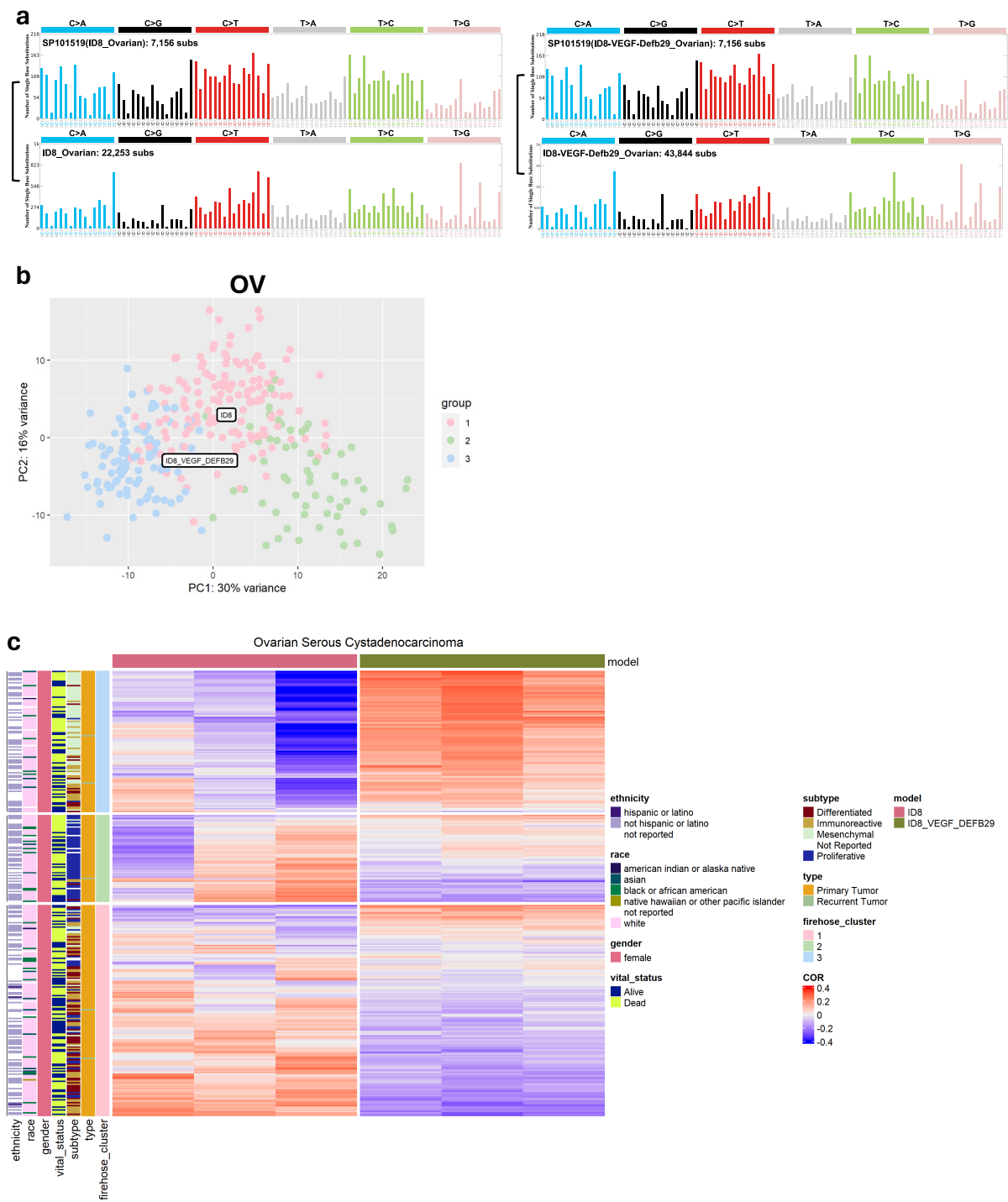


Figure 1.15: Mutational landscape and gene expression profile of the GL261 glioma/glioblastoma multiforme model.

a, Driver plot of mutated glioma cancer driver genes found in GL261, colored by mutation type. **b**, Single base substitution signature of GL261 against its highest cosine similarity scored human patient within the brain cancer PCAWG cohort. **c**, PCA analysis of gene expression profile of GL261 against those of the GBM TCGA group. **d**, Heatmap visualizing correlations in gene expression between each GBM TCGA sample (grouped by subtypes indicated in annotations and GL261 biological replicates. Strength of correlation is indicated by color. For each row, correlations are calculated according to genes that are significantly differentially expressed by mouse samples compared to TCGA expression data.



Figure 1.16: Mutational landscape and gene expression profile of the Lewis lung carcinoma model.

a, Driver plot of mutated lung cancer driver genes found in LL2/LLC1, colored by mutation type. **b**, Single base substitution signature of LL2 against its highest cosine similarity scored human patient within the lung cancer PCAWG cohort. **c**, PCA analysis of gene expression profile of LL2 against those of the LUAD TCGA group (left). Heatmap visualizing correlations in gene expression between each LUAD TCGA sample (grouped by subtypes indicated in annotations and LL2 biological replicates. Strength of correlation is indicated by color. For each row, correlations are calculated according to genes that are significantly differentially expressed by mouse samples compared to TCGA expression data (right). **d**, PCA analysis of gene expression profile of LL2 against those of the LUSC TCGA group (left). Heatmap visualizing correlations in gene expression between each LUSC TCGA sample (grouped by subtypes indicated in annotations and LL2 biological replicates. Strength of correlation is indicated by color. For each row, correlations are calculated according to genes that are significantly differentially expressed by mouse samples compared to TCGA expression data (right).

Table 1.1: List of TCGA studies and their abbreviations

STUDY ABBREVIATION	STUDY NAME
ACC	Adrenocortical carcinoma
BLCA	Bladder Urothelial Carcinoma
BRCA	Breast invasive carcinoma
CESC	Cervical squamous cell carcinoma and endocervical adenocarcinoma
CHOL	Cholangiocarcinoma
DLBC	Lymphoid Neoplasm Diffuse Large B-cell Lymphoma
COAD	Colon adenocarcinoma
CNTL	Controls
ESCA	Esophageal carcinoma
GBM	Glioblastoma multiforme
HNSC	Head and Neck squamous cell carcinoma
KICH	Kidney Chromophobe
KIRC	Kidney renal clear cell carcinoma
KIRP	Kidney renal papillary cell carcinoma
LAML	Acute Myeloid Leukemia
LCML	Chronic Myelogenous Leukemia
LGG	Brain Lower Grade Glioma
LIHC	Liver hepatocellular carcinoma
LUAD	Lung adenocarcinoma
LUSC	Lung squamous cell carcinoma
MESO	Mesothelioma
MISC	Miscellaneous
OV	Ovarian serous cystadenocarcinoma
PAAD	Pancreatic adenocarcinoma
PCPG	Pheochromocytoma and Paraganglioma
PRAD	Prostate adenocarcinoma
READ	Rectum adenocarcinoma
SARC	Sarcoma
SKCM	Skin Cutaneous Melanoma
STAD	Stomach adenocarcinoma
TGCT	Testicular Germ Cell Tumors
THYM	Thymoma
THCA	Thyroid carcinoma
UCS	Uterine Carcinosarcoma
UCEC	Uterine Corpus Endometrial Carcinoma
UVM	Uveal Melanoma

Table 1.2: Characterization of driver mutations, mutational signatures, and tumor mutational burdens of 22 syngeneic mouse models.

Cell line	Organ System	# driver genes found in matched human cancer	# of PCAWG samples of matched cancer type (>0.85 cosine similarity)	# of PCAWG samples of matched cancer type with +/- 10% TMB
4MOSC1	Mouth	10	3/57	0/56
4MOSC2	Mouth	15	2/57	0/56
4T1	Breast	4	21/698	0/211
B16F10	Skin	13	1/328	3/107
CT26	Colorectal	16	0/73	1/52
DDHER2	Breast	1	8/698	2/211
GL261	Brain	6	0/71	0/39
ID8	Ovary	0	3/135	0/110
ID8-Vegf-Defb29	Ovary	1	2/135	0/110
KPC4580	Pancreas	5	3/87	1/232
LL2/LLC1	Lung	8	0/48	0/47
MC38	Colorectal	15	0/73	2/52
MEER	Mouth	2	8/57	4/56
Met-1	Breast	10	0/698	0/211
MOC1	Mouth	10	0/57	0/56
MOC2	Mouth	4	0/57	0/56
PAN02	Pancreas	21	41/431	0/232
TRAMPC1	Prostate	1	3/404	1/199
TRAMPC2	Prostate	1	3/404	5/199
YUMM1.7	Skin	3	1/328	11/107
YUMMER.G	Skin	2	43/328	4/107
YUMMER1.7	Skin	7	43/328	1/107

Chapter 2

The GPCR- G_{α_s} -PKA signaling axis promotes T cell dysfunction and cancer immunotherapy failure

Abstract

Immune checkpoint blockade (ICB) targeting PD-1 and CTLA-4 has revolutionized cancer treatment. However, multiple cancers fail to respond to ICB, prompting the search for additional strategies to achieve durable responses. G protein-coupled receptors (GPCRs) are the most intensively studied drug targets but are largely underexplored in immune-oncology. Here, we cross-integrated large single-cell RNA sequencing (scRNA-seq) datasets from CD8 T cells covering 19 distinct cancer types, and identified a significant enrichment of G_{α_s} -coupled GPCRs on exhausted CD8 T cells. These include EP₂, EP₄, A_{2A}R, β_1 AR, and β_2 AR, all of which promote T cell dysfunction. We developed transgenic mice expressing a chemogenetic CD8-restricted G_{α_s} -DREADD (Designer Receptor Exclusively Activated by A Designer Drug) to activate CD8-restricted G_{α_s} signaling, and discovered that the G_{α_s} -PKA signaling axis promotes CD8 T cell dysfunction and immunotherapy failure. Our studies reveal that G_{α_s} -GPCRs represent druggable immune checkpoints that can be targeted to enhance the response to ICB immunotherapies.

Introduction

Breakthrough discoveries over the past few decades have begun to unravel the complexity of the anti-tumor immune response, leading to the implementation of cancer immunotherapy in patients^{59,192}. Specifically, immune checkpoint blockade (ICB) by antibodies targeting PD-1 (e.g., nivolumab, pembrolizumab, cemiplimab), its ligand programmed death-ligand 1 (PD-L1) (e.g., atezolizumab, avelumab, durvalumab), and CTLA-4 (ipilimumab & tremelimumab) have been

approved for multiple cancer types ^{64,193}. As monotherapies, these checkpoint inhibitors have shown remarkable efficacy in a subset of patients, but responsiveness is restricted to certain tumor types, and many patients who initially respond do not have subsequent durable tumor control ¹⁹⁴. This raises the possibility that additional immune checkpoints may exist, which prevent ICB from achieving its full potential and leads to primary or acquired resistance. In this regard, tumors may deploy multiple immune evasion strategies to bypass anti-tumor immune responses that may need to be blocked concomitantly with ICB in order to achieve durable tumor remission.

G protein-coupled receptors (GPCRs) represent the largest family of cell surface receptors involved in signal transduction, and are the target of >30% of all FDA-approved drugs ⁸⁵. GPCRs have remained the most pharmacologically favorable drug targets primarily due to their druggability and relevance to a broad spectrum of physiological processes and highly prevalent disease conditions ⁸⁵. However, the role of GPCRs in immune-oncology is understudied; while aberrant GPCR signaling has been implicated in cancer, their roles and expression patterns on tumor-infiltrating immune cells and potential as a target for immunotherapies are less understood ^{81,195}. There are 800 known human GPCRs, and their signaling cascades are primarily determined by the activation of one or multiple heterotrimeric Gα proteins (e.g., Gα_s, Gα_{i/o}, Gα_{q/11}, and Gα_{12/13}) ⁷⁹. The nature of the immune cell infiltrating the TME is largely dictated by chemokines and their associated GPCRs that guide and recruit different pro- or anti-tumoral immune cells to the tumor, thereby orchestrating the balance between cytotoxicity and immunosuppression ¹⁹⁵. Most chemokine receptors are coupled to Gα_i, inhibiting cyclic AMP (cAMP) production. These include CXCR3, a Gα_i-coupled GPCR on T cells that binds three chemokines, CXCL9/10/11, to promote the migration of T cells into the tumor ¹⁹⁶. These chemokines are induced by IFNα, β, and γ, and are part of the interferon gene signature that has predictive value for a favorable response to pembrolizumab ⁹⁴.

In contrast to these anti-tumor chemokine receptors, other GPCRs expressed on T cells may override chemokine-coordinated intratumoral CTL migration, and instead, display immune

suppressive functions. In this regard, metabolites and inflammatory mediators that accumulate in the TME, such as adenosine and prostaglandins, act on $G\alpha_s$ -coupled receptors and display immunosuppressive activity^{197,198}, albeit their direct impact on CD8 T cell has not been fully elucidated as their respective receptors are expressed in multiple innate and adaptive immune cell types. Here, we developed a computational pipeline to integrate large datasets of intratumoral T cell single-cell RNA sequencing (scRNA-seq), and combined it with a synthetic biology approach to show that activation of $G\alpha_s$ -coupled GPCRs and the $G\alpha_s$ signaling axis is sufficient to drive a hyporesponsive T cell state. In turn, our findings revealed that concomitant inhibition of $G\alpha_s$ -coupled GPCRs with ICB may be necessary to reactivate the anti-tumor immune response, thereby providing a multi-modal immunotherapy approach for cancer treatment.

Results

$G\alpha_s$ -coupled GPCR expression and T cell dysfunction

Building on our previous analysis shedding light on the onco-GPCRome and aberrant GPCR signaling and activity on tumor cells¹⁹⁵, we investigated the landscape of GPCR expression on each tumor-infiltrating CD8 T cell subtype. We collected data and performed an integrated analysis of scRNA-seq datasets from 19 cancer types (**Figure 2.1a and Table 2.1a**). The cancer types covered in this analysis encompass those in which patients show higher (e.g., ~25-50% in NSCLC and CM) and lower (e.g., <20% in OC and PDAC) response rates to immunotherapy¹⁹⁹, and thus provides a comprehensive overview of expression landscape of tumor-infiltrating CD8 T cells. Using the Seurat SCTransform scRNA-seq integration method, we jointly analyzed 217,953 total CD8 T cells, which were stratified into naïve (N), proliferating (P), cytotoxic (C), effector memory (EM), and exhausted (EX) based on landmark genes and nearest neighbor analysis, ProjecTIL²⁰⁰ (**Figure 2.1b, 2.1c, Figure 2.2a, 2.2b, and Table 2.1b**). This resulted in delineation of functional (cytotoxic, effector memory) and dysfunctional (exhausted) CD8 subtypes, nonspecific naïve T cells that stochastically migrate through the tumor, and proliferating T cells

that express features of both effector memory and exhausted T cells^{201,202}. When we analyzed the relative expression of 386 non-olfactory GPCR genes, many GPCRs showed a distinct expression pattern in each CD8 T cell subtype (**Figure 2.1d and Tables 2.1c and 2.1d**). Terminally exhausted CD8 T cells in both cancer and chronic viral infection most commonly have been shown to express high levels of PD-1, TIM-3, CXCR6, LAG3, CTLA4, and CXCL13 proteins, all of which are reflected at the transcriptomic level in our combined analysis, as previously documented and validated in the individual datasets (**Tables 2.1a, 2.1b, and 2.1c**). Within EX CD8 T cells, we found that *CXCR6*, *PTGER4*, *GPR65*, *ADGRE5*, and *F2R* were among the most highly expressed GPCRs, with expression patterns similar to those of signature exhaustion genes (**Figure 2.1e**).

A transcriptional gene module predicting T cell dysfunction programs linked to *LAG3* expression (e.g., *TIGIT*, *PDCD1*, *LAG3*, and *CXCL13*) was established in human melanoma patients²⁰³. To identify GPCRs most relevant to this dysfunction program, we generated dysfunction scores for all CD8 T cells from our integrated analyses (**Figure 2.1f, Table 2.1e**). As expected, EX CD8 T cells across 19 cancer types displayed the highest dysfunction scores, significantly higher as compared with proliferating, cytotoxic, and effector memory CD8 T cells (**Figure. 2.1g, Figure. 2.2c**). We next investigated the GPCR genes most strongly associated with T cell dysfunction scores. *CXCR6*, which is expressed on PD-1^{hi} effector and exhausted CD8 T chronic viral infection, was the GPCR most significantly correlated with T cell dysfunction²⁰⁴ (**Figure 2.1h**). In total, 35 GPCRs were shown to be significantly correlated with the dysfunction score, with *GPR56*, *CCRL2*, *GIPR*, and *F2R* among the top candidates to contribute to T cell exhaustion (**Figure 2.1h and Table 2.2a**). We next aimed to distinguish GPCR expression patterns based on heterotrimeric G protein coupling information to begin defining functional gene sets. Based on the International Union of Basic and Clinical Pharmacology (IUPHAR) classification, we grouped all GPCRs into G protein programs based on their primary G protein coupling to $G\alpha_{12/13}$, $G\alpha_i$, $G\alpha_{q/11}$, or $G\alpha_s$ (**Table 2.2b**). Intriguingly, when we calculated the mean

correlation of each pathway, we found that the $G\alpha_s$ program was the most enriched with T cell dysfunction while the $G\alpha_i$ program was the least correlated (**Figure 2.1i**). Multiple $G\alpha_s$ -coupled GPCRs were positively correlated with T cell dysfunction with significant Spearman correlations, including *GIPR*, *ADORA2A*, *PTGER4*, *GPR65*, and *TSHR* (**Figure 2.1j**). These data raise the possibility of a GPCR- $G\alpha_s$ -signaling program that correlates with T cell dysfunction in tumor-infiltrating CD8 T cells.

Endogenous $G\alpha_s$ -GPCRs diminish effector T cell function

Next, we investigated the relevance of $G\alpha_s$ -GPCRs to T cell exhaustion phenotypes. We analyzed bulk transcriptomic data from the murine lymphocytic choriomeningitis chronic viral infection model (LCMV), which represents the experimental model system most central to recent, landmark discoveries defining T cell exhaustion ⁴⁷ (**Figure 2.3a and Table 2.3a**). In this model, an acute strain of LCMV infection (Armstrong) generates robust, proliferative, and activated effector CD8 T cells that are able to resolve infection ²⁰⁵. Contrastingly, the chronic strain of LCMV infection (clone 13) leads to persistent antigen exposure, generating a distinct exhausted T cell state, with sustained expression of inhibitory receptors and hierarchical loss of effector functions ²⁰⁵. We combined 3 RNA-sequencing datasets of fluorescent-activated cell sorting (FACS)-purified effector CD8 T cells from acute LCMV infection and exhausted T cells from chronic LCMV infection and used DESeq2 to directly compare differential expression of GPCRs between the two CD8 subtypes. This revealed that multiple $G\alpha_s$ -GPCRs are upregulated in exhausted T cells compared to effector T cells, including *Glp1r*, *Ptger2*, *Ptger4*, and *Gpr65*, which were also significantly correlated with T cell dysfunction in human tumor infiltrating CD8 T cells (**Figure 2.1g**, above, and **Figure 2.3b and Table 2.3b**). This suggests that the $G\alpha_s$ -GPCRs that are expressed on both exhausted tumor-infiltrating CD8 T cells and exhausted CD8 T cells from chronic viral infection are intrinsically similar.

Recently, we have determined coupling across 148 human GPCRs for 11 specific human G proteins and used a machine learning approach to augment a GPCR coupling predictions for Class A GPCRs²⁰⁶. Here, we curated gene sets stratifying for their predictive G protein coupling as designated by IUPHAR and our reported assay. We found that the $G\alpha_s$ -coupling gene set by IUPHAR had the most significant enrichment with upregulated GPCRs on exhausted T cells in LCMV infection (**Figure 2.3c**). Additional gene sets were also significantly enriched for upregulated GPCRs on exhausted T cells, including GNAQ predicted coupling, $G_{q/11}$ primary IUPHAR coupling, and GNA14 predicted coupling (**Figure 2.3c and Table 2.3c**). Altogether, this suggests that in addition to T cell dysfunction, the expression of these $G\alpha_s$ -coupled GPCRs may also play a role in driving T cell exhaustion.

To gain a better understanding of how the expression of these $G\alpha_s$ -GPCRs modulate CD8 T cells, we first sought to model impairment of T cell function resulting from persistent TCR signaling by modulating T cell activation *in vitro* (**Figure 2.3e**). CD8⁺ T cells from splenocytes of C57Bl/6 mice were activated with anti-CD3 and anti-CD28 for 48 hours. Subsequently, cells were subjected to additional restimulation (chronic stimulation) to model reactivation of T cells upon TCR engagement at the tumor. As a control, CD8 T cells were also expanded in culture with interleukin 2 (IL-2) (activated) without additional restimulation. Characteristic of terminally exhausted cells, the expression of PD-1, CTLA-4, TIM3, and LAG3 on CD8 T cells were all significantly elevated in chronically versus acutely stimulated CD8s, concomitant with a decrease in IFN γ and TNF, confirming in this assay that chronically activated CD8 T cells begin acquiring an exhaustion-like phenotype (**Figure 2.4a**).

To simulate exposure of $G\alpha_s$ ligands at the TME, we added ligands stimulating $G\alpha_s$ -GPCRs in our chronic exhaustion assay (Prostaglandin E2 (P) for EP2 and EP4, dobutamine (D) for β_1 AR and β_2 AR, and CGS-21860 (C) for A_{2A} R). After 48 hours of the initial activation, CD8 T cells were replated for an additional 48 hours of activation with or without $G\alpha_s$ ligands, and we

measured the functional capacity by flow cytometric analysis of IFN γ , TNF, and granzyme B. Continuous stimulation and the addition of PGE2, dobutamine, or CGS-21680 all significantly reduced IFN γ , TNF, and granzyme B single positivity as well as highly cytotoxic, polyfunctional CD8 T cells, as measured by cells doubly expressing IFN γ and TNF (**Figure 2.3f, 2.3g, Figure 2.4b**). A similar reduction was seen in the viability and proliferative capacity of CD8 T cells, as indicated by the reduction in Ki-67 positivity (**Figure 2.4c**). Moreover, stimulation with PGE2 and dobutamine significantly elevated PD-1 and Tim-3 expression (**Figure 2.3g, Figure 2.4d**). The simultaneous reduction in T cell function and proliferation and increase of inhibitory receptor expression suggests that G α_s ligands augment the dysfunctional phenotypes in CD8 T cells polarizing towards exhaustion.

In order to evaluate the functional suppression by G α_s ligands on cytotoxic T cell killing, we activated purified T cells from OT-1 transgenic mice, whose TCRs are specific for the ovalbumin (OVA) peptide, SIINFEKL (OVA₂₅₇₋₂₆₄), and co-cultured them with MC38 tumor cells expressing OVA (MC38-OVA) at a 1:5 effector to target ratio (**Figure 2.3h**). G α_s ligands significantly diminished the cytotoxicity of OT-1 CD8 T cells toward MC38-OVA cells as measured by viability of tumor cells after 2 days (**Figure 2.3i**). Together, our in vitro experiments identify an inhibitory effect of ligands for G α_s -GPCRs on T cell function and cytotoxicity.

To elucidate the underlying mechanisms of G α_s -mediated CD8 T cell dysfunction, we next characterized the pathways downstream of G α_s /cAMP that drive immune suppression, focusing on PGE2 as this inflammatory mediator led to the most pronounced inhibition of function from all G α_s -GPCR ligands tested. Addition of PGE2 and forskolin (fsk), a direct adenylyl cyclase-cAMP activator, during chronic stimulation led to a significant increase of the cAMP response element-binding protein (pCREB), which becomes phosphorylated following increased intracellular cAMP and PKA activation (**Figure 2.3j**). EP₂ or EP₄ inhibitors (EP₂i and EP₄i, respectively) significantly decreased PGE2- but not fsk-induced activation of pCREB, supporting the receptor mediated

effects of PGE2 on PKA (**Figure 2.3k**). Similarly, addition of EP₂i or EP₄i antagonists restored production of IFN γ and TNF secretion following inhibition by PGE2 but not in response to fsk (**Figure 2.3l**). Interestingly, the addition of EP₂ and EP₄ antagonists together more significantly reduced PGE2-mediated IFN γ and TNF inhibition as compared to each antagonist alone, suggesting that concomitant blockade of G α_s activation by multiple G α_s receptors may afford better rescue from immune suppressive effects on CD8 T cells. Next, we sought to determine whether inhibitory effects by PGE2/cAMP-induced inhibition could be rescued by activation of an endogenously expressed G α_i -GPCR, CXCR3, with its ligand, CXCL10. Although CXCL10 alone did not significantly affect IFN γ and TNF secretion, addition of CXCL10 with PGE2 alleviated inhibition by PGE2 (**Figure 2.4e**). Together, these data strongly suggest that G α_s activation leads to dampening of CD8 T cell function, which can be rescued either by blocking G α_s -GPCRs or by stimulating the G α_i axis.

To begin exploring the role of the G α_s -PKA axis in PGE2-mediated inhibition of T cell function, we generated conditional CD8-specific *Gnas* knockout mice (**Figure 2.3l**). We used mice with CD8 T cell-restricted Cre recombinase temporally controlled with tamoxifen²⁰⁷ (*E8i-CreERT2*) crossed with mice with loxP sites flanking *Gnas* exon one (referred to as CD8-*Gnas* KO, **Figure 2.3l** and **Table 2.4**). *E8i-CreERT2* mice express the reporter gene in mature CD8 T cells but not in CD4 T cells, B cells, myeloid cells, dendritic cells, or natural killer cell populations²⁰⁸. CD8 T cells were isolated and chronically stimulated following administration of tamoxifen. As expected, CD8 T cells with functional G α_s express less IFN γ and TNF after treatment with PGE2 (**Figure 2.3m**), but this inhibition was rescued in CD8-*Gnas* KO mice (**Figure 2.3m**). In search of the potential mechanisms, we examined the involvement of PKA, a key downstream signaling effector of G α_s . For these studies, we leveraged our protein kinase inhibitor peptide (PKI) mouse model²⁰⁹. *Tet-GFP-PKI* mice were crossed with *E8i-CreERT2-ROSA26-LSL-rtTA* mice to generate conditional, tetracycline-inducible PKI system to specifically inhibit PKA in CD8 T cells (referred

to as CD8-*PKI*) (**Figure 2.5a, 2.5b, 2.5c** and **Table 2.5**). CD8 T cells were isolated from the CD8-*PKI* mice following tamoxifen and doxycycline administration and chronically stimulated as above. When GFP-*PKI* was expressed in chronically activated CD8 T cells, it prevented the decrease in IFN γ ⁺ TNF⁺ double-positive CD8 T cells caused by PGE2 (**Figure 2.5d**), which mirrors *Gnas* deletion (see above, **Figure 2.3m**). This strongly suggests that *Gnas* and its downstream signaling target, PKA, are necessary for PGE2-mediated inhibitory effects on CD8 T cells.

A chemogenetic model reveals immune suppressive G α_s signaling

The use of DREADDs (designer receptors exclusively activated by designer drugs), which are GPCRs engineered to be non-responsive to endogenous ligands but responsive to a designer drug, clozapine-N-oxide (CNO), can be exploited to achieve G protein activation in a tissue specific fashion²¹⁰. To bypass potential off-targeted effects elicited by CNO, we utilized the recently developed deschloroclozapine (DCZ), which affords higher affinity and more selective agonist activity for DREADDs²¹¹. To specifically interrogate the function of the G α_s signaling axis in CD8 T cells, we expressed G α_s -DREADD by crossing *E8i-CreERT2* with *ROSA26-LSL-Gs-DREADD* mice (**Figure 2.6a, Figure 2.7a, Table 2.6**). The resulting mice, referred to as CD8-GsD, were dosed with tamoxifen every day for 3 days and G α_s -DREADD expression and activation was subsequently verified (**Figure 2.6b**). By qPCR, only in mice treated with tamoxifen, was there a demonstrable expression of G α_s -DREADD in CD8 T cells and not in CD4 T cells (**Figure 2.6c**). This confirmed tamoxifen-inducible recombination by the CRE recombinase for CD8-restricted G α_s -DREADD expression.

To confirm activation of the G α_s -DREADD, mice were first dosed with tamoxifen, and then 0.01 mg/kg of DCZ was administered intraperitoneally (**Figure 2.6b**). After administration of tamoxifen and DCZ, mice were subsequently bled to assess pCREB induction by flow cytometry. Whereas the frequency of pCREB⁺ in NK1.1⁺ NK cells, CD11b⁺ myeloid cells, and CD4⁺ T cells were low and did not differ between DCZ-treated and non-treated mice, the frequency of CD8⁺

pCREB⁺ cells significantly increased in DCZ-treated mice (**Figure 2.6d**). Mice treated with DCZ did not show significant changes in frequency of CD8, CD4, NK cells, or CD11b myeloid cells in the peripheral blood (**Figure 2.7b**). When we isolated peripheral blood from mice dosed with tamoxifen, addition of DCZ in vitro also increased pCREB exclusively in CD8 T cells (**Figure 2.6e**). These results confirm tamoxifen-inducible, CD8-specific expression and activation of Gα_s-DREADD by DCZ in CD8-GsD mice. Aligned with our data for Gα_s-GPCRs ligands, only in mice with tamoxifen-induced expression of Gα_s-DREADD, CD8-Gα_s activation led to a significant decrease of IFN γ , TNF, Ki-67, and granzyme B concomitant with increase in PD-1 and Tim-3 expression (**Figure 2.6f-h and Figure 2.7c**). In order to gain an understanding of downstream transcriptional modulations stimulated by Gα_s signaling in CD8 T cells, we assessed gene expression of dual-specificity phosphatase 1 (*Dusp1*), a CREB target shown to negatively regulate T cell activation and function through inactivation of JNK and reduced NFATc1^{212,213} (**Figure 2.6i**). Stimulation of the Gα_s-DREADD significantly increased expression of *Dusp1*, in addition to *Tigit* and *Tox*, both of which are highly expressed on terminally exhausted CD8 T cells^{214,215} (**Figure 2.6j**). Altogether, activation of Gα_s-DREADD on CD8 T cells was sufficient to exacerbate exhaustion-related phenotypes, as indicated by decreased cytotoxic function in tandem with increased expression of exhaustion-related genes.

To assess biological relevance of the inhibitory Gα_s signaling in CD8 T cells in the tumor setting, we utilized the ovalbumin (OVA) tumor model system to investigate the effect of Gα_s signaling on recruitment and function of antigen-specific CD8 T cells. To study native T cell trafficking and function, we took advantage of an orthotopic head and neck cancer syngeneic mouse model, 4MOSC1, which recapitulates human head and neck cancer mutational signatures with ~93% similarity¹³⁵. CD8-GsD or littermate control mice were given 3 doses of tamoxifen before tumor implantation with 4MOSC1-OVA, and DCZ was given every day starting 1 day after tumor implantation (**Figure 2.6k**). CD8-GsD mice dosed with tamoxifen and with or without DCZ were sacrificed at an early time point to quantify tumor-infiltrating SIINFEKL-tetramer⁺ CD8 T

cells. There was a significant decrease of antigen-specific CD8 T cells infiltrating the tumor, and IFN γ and TNF from the bulk CD8 population were both significantly decreased in DCZ-treated mice compared to untreated (**Figure 2.6l and 2.6m**). Our *in vitro* and *in vivo* data collectively indicate sufficiency of G α_s -activation to drive exhaustion-related phenotypes and prevent trafficking of tumor-specific CD8 T cells.

G α_s -signaling promotes CD8 T dysfunction and immunotherapy failure

We next investigated the effect of CD8-specific G α_s -signaling on immunotherapy response and tumor killing *in vivo*. CD8-GsD mice were dosed with tamoxifen and on day 0, and 4MOSC1 cells or MC38-OVA cells were implanted into the tongue and flank of mice, respectively (**Figure 2.8a**). DCZ (0.01 mg/kg) was given daily to mice starting 1 day after tumor implantation. In the absence of agonist stimulation CD8-GsD mice with 4MOSC1 partially responded to anti-PD-1, similar to WT mice as reported ¹³⁵, but activation of the G α_s -DREADD by DCZ administration abolished any anti-tumoral responses (**Figure 2.8b**). Additionally, though anti-PD-1 afforded a survival advantage in tumor-bearing mice, activation of the G α_s signaling axis in CD8 T cells led to survival rates not significantly different from untreated mice (**Figure 2.8c**). As controls, neither DCZ nor tamoxifen affected growth of tumors in littermate control mice (**Figure 2.9**).

These findings prompted us to determine whether activation of G α_s signaling in CD8 T cells was sufficient to limit the long-term anti-tumor immunity from combined PD-1 and CTLA-4 ICB¹³⁵. In the absence of DREADD ligand stimulation, 90% of CD8-G α_s -DREADD mice responded to dual ICB with durable tumor regression (**Figure 2.8b, c**). This was abrogated by the administration of DCZ (**Fig 2.8b, c, Figure 2.10**), indicating that activation of the G α_s signaling axis on CD8 T cells is sufficient to limit T cell responses to ICB. Intriguingly, this was paralleled by a significant decrease of tumor growth in CD8-*Gnas* KO mice compared to littermate control mice (**Figure 2.11a**). In these mice, there was a significant decrease in CD8 T cell exhaustion

markers, such as PD-1+ TIGIT+ double positive tumor-infiltrating and experienced CD8 T cells lacking *Gnas* (**Figure 2.11b, c**).

Next, we determined whether diminished response to immunotherapies following activation of $G\alpha_s$ signaling was associated with diminished T cell functionality. Similar to our observations in the 4MOSC1 model, anti-PD-1 provided significant anti-tumor activity in CD8-GsD mice with MC38-OVA tumor cells in absence of stimulation, but DCZ-treated mice led to failed responsiveness and worse overall survival (**Figure 2.8c, e**). Treatment with anti-PD-1 in CD8-GsD mice led to a significant increase in tetramer-positive CD8 T cells, IFN γ and granzyme B secretion (**Figure 2.8f**), which were abolished by DCZ treatment concurrent with anti-PD-1 (**Figure 2.8f**). These data suggest that activation of $G\alpha_s$ signaling exclusively on CD8 T cells leads to functional impairment as well as decreased recruitment of antigen-specific CD8s at the tumor, which ultimately leads to failure to respond to immunotherapies.

$G\alpha_s$ -signaling and reduced response to ICB therapy in patients

To begin exploring the clinical relevance of $G\alpha_s$ -GPCR expression, we first investigated the correlation of expression between PD-1 and various $G\alpha_s$ -GPCRs. In the TCGA skin cutaneous melanoma cohort, we found that *GPR65*, *PTGER2*, *PTGER4*, *ADRB2*, and *ADORA2A* were all significantly positively correlated with PD-1 expression in bulk tumors (**Figure 2.12a**). This suggests that PD-1 expression is likely concurrent with the expression of these GPCRs. We next asked whether the expression of these GPCRs could predict immunotherapy response in patients. We analyzed a cohort of 32 patients with metastatic melanoma (total biopsies = 48) treated either anti-PD-1 or anti-CTLA-4, or combination therapy, where single-cell RNA-seq was performed on the tumors pre- and post- therapy²¹⁶. We observed that the expression levels of *GPR65*, *PTGER2*, *PTGER4*, *ADRB2*, and *ADORA2A* align with non-responders to immunotherapy, with *PTGER2* and *ADORA2A* expressed significantly higher in CD8 T cells from non-responders compared to responders, and *GPR65* nearing significance (**Figure 2.12b, c**). We

found that 4 of 5 of these G α s-GPCRs (*PTGER4*, *GPR65*, *ADORA2A*, and *PTGER2*) have a significant predictive power to identify melanoma patients who would not respond to immunotherapy. In this analysis, *PTGER2* ranked at the top with the highest area under the curve (AUC) of 0.78 (**Figure 2.12c**). To test the extent of the role of immune suppression in immunotherapy response by G α s-GPCRs pathway across pan-cancer, we next computed the correlation between mean G α s-GPCRs pathway levels in a cancer type and immunotherapy objective response rate (ORR) observed across 16 cancer types^{70,199,217}. Remarkably, aligned with our hypothesis, we found that the mean levels of G α s-GPCRs pathway levels in a cancer type is most negatively correlated with the immunotherapy ORR out of all the G protein signaling programs (**Figure 2.12d**).

In summary, we propose a G α s-PCR signaling axis that when activated in CD8 T cells, is sufficient to decrease cytotoxic function, exacerbate exhaustion-related phenotypes, and abolish responses to immunotherapy (**Figure 2.13**). Thus, the concomitant blockade of G α s-PCRs with other inhibitory receptors, including PD-1 and CTLA-4, may be needed to garner more effective and durable response to immunotherapy.

Discussion

Numerous recent studies have investigated the expression profile of intratumoral T cells at the single-cell resolution, revealing gene expression programs characterizing each individual T cell population and their functional consequences²¹⁸. While we have not validated these findings individually, we have developed computational pipelines enabling the integration of these scRNA-seq datasets from hundreds of thousands of intratumoral immune cells and transcriptomic information delineating response to ICB. By combining this wealth of information with the development of a chemogenetic approach to stimulate CD8-restricted G α s-PCRs, we have now uncovered a CD8-T cell GPCR-G α s-signaling axis promoting T cell dysfunction and immunotherapy failure.

GPCR signaling networks in the TME orchestrate anti-cancer immune defense mechanisms, as demonstrated by chemokines and chemokine receptors displayed on both anti-tumoral and pro-tumoral immune cells ¹⁹⁵. On the other hand, metabolites and inflammatory mediators accumulating in the TME can bind GPCRs that exert immunosuppressive effects. These include the nucleoside, adenosine, a product from the breakdown of ATP by ectonucleotidases CD39 and CD73, and the inflammatory mediator, PGE2, that is enzymatically derived from the cyclooxygenase (COX) isoforms, COX1 and COX2, which are upregulated in most solid cancers ²¹⁹. Both adenosine and PGE2 stimulate GPCRs expressed in many immune cell types, and exert their immunosuppressive activity by acting primarily on G_{α_s} -coupled receptors, $A_{2A}R$ (*ADORA2A*) and EP_2 (*PTGER2*) and EP_4 (*PTGER4*), respectively ^{197,198}. Stimulation of A_{2A} receptor provides a broad immunosuppressive signal through multiple cell types including T cells, NK cells, DCs, and neutrophils ¹⁹⁸, and blocking antibodies anti-CD73 and anti-CD39 are under current evaluation for combination with ICB¹⁹⁸. PGE2 has been linked to recruitment of MDSCs, and decreased CD8 T cell activation and NK cell recruitment, among others ^{195,220}. Hence, nonsteroidal anti-inflammatory drugs (NSAIDs) that block COX2 and/or COX1 and COX2, and EP receptors represent promising targets for combination with ICB²²¹. However, the precise role of these GPCRs in CD8 T cell function is much less understood, as most studies have relied on global gene knock out strategies and systemic inhibition of receptors or biosynthetic pathways involved in ligand production. This may limit the ability to define key regulated events in CD8 T cells, and overlook the existence of redundant or compensatory mechanisms that may render therapeutic interventions blocking these GPCRs ineffective.

Given the complexity of receptor expression in multiple immune and tumor cells in the TME and difficulty of unraveling the function of these G_{α_s} -linked GPCRs in CD8 T cells, we took a synthetic biology approach to build chemogenetic DREADDs and gain spatial and temporal control of G_{α_s} signaling specifically in CD8 T cells. The uniqueness of the CD8-GsD mouse model afforded us the opportunity to interrogate functions of G_{α_s} /cAMP/PKA irrespective of the GPCR

that provides the activation. We found that activation of $G\alpha_s$ -linked GPCRs in CD8 T cells is sufficient to limit antigen-specific CD8 T cell recruitment, abolish cytotoxic function, and abrogate the anti-tumor responses to both anti-PD-1 and anti-CTLA-4. $G\alpha_s$ stimulation also augmented expression of terminal exhaustion-related receptors, such as PD-1 and Tim-3. Indeed, our studies utilizing the chemogenetic $G\alpha_s$ -DREADD, $G\alpha_s$ -knockout, and PKA inhibition models all link the CD8- $G\alpha_s$ /PKA axis to T cell dysfunction in effector CD8s and tumor-infiltrating CD8s.

Although the underlying mechanisms by which $G\alpha_s$ -PKA may exert these inhibitory functions are yet to be fully elucidated, it is likely that in the TME, $G\alpha_s$ -PKA can directly inhibit T cell migration and contribute to the expression of CD8 T cell exhaustion programs. In turn, the implication of $G\alpha_s$ -linked GPCRs as negative regulators and heightening the dysfunction of CD8 T cells in the TME provides a strong foundation for the future exploration of the GPCR- $G\alpha_s$ -PKA axis for the development of precision cancer immunotherapy in combination with ICB. In this regard, although the abundance of ligands in the TME and GPCR expression on tumor-infiltrating CD8 T cells is likely tumor-type specific, our pan-cancer computational analysis provides a shared landscape of GPCRs broadly associated with intratumoral exhausted CD8 T cells, which includes *ADORA2A*, *PTGER2*, *PTGER4*, *ADRB1*, *ADRB2*, and *GPR65*, all of which converge to the initiation of CD8 T cell exhaustion programs by the activation of the $G\alpha_s$ -PKA pathway (**Figure 2.13**). This may have a direct clinical impact, as it may provide a rationale for the use of readily available β -adrenergic blockers, such as propranolol, in cancer patients with elevated circulating adrenaline/noradrenaline, which are typical of physical and emotional stress conditions, in lesions exhibiting *ADRB1* or *ADRB2* expression in intratumoral CD8 T cells ²²². Intriguingly, GPR65, a proton-sensing GPCR, also emerged as a $G\alpha_s$ -GPCR significantly correlated with T cell dysfunction. Hypoxia and acidosis resulting from the enhanced anaerobic metabolism, referred to as the “Warburg effect”, is a hallmark of most solid tumors ²²³, and has been associated with immune exclusion. Therefore, expression of GPR65 on CD8 T cells may provide a mechanism of intrinsic resistance to cytotoxic CD8 T cells, which can be disrupted, for example, by the future

development of GPR65 inhibitors. Another unexpected finding from our study is that all of the $G\alpha_s$ -GPCRs that we identified as part of our pan-cancer analysis were significantly correlated with *PDCD1* (PD-1) expression, and that 5 out of the 6 $G\alpha_s$ -GPCRs that we analyzed had significant predictive power to predict responses to immunotherapy in melanoma patients. Thus, our studies may provide a valuable resource for the future development and exploration of targeting CD8 T cell specific GPCRs as part of combination immunotherapies. Our data also suggest that many $G\alpha_s$ -GPCRs expressed on CD8 T cells may be functionally redundant, which raises the question of whether to target individual receptors based on the patient-specific expression profile, or simultaneously. As an alternative approach, perhaps targeting the shared downstream mechanisms, specifically cAMP/PKA, may provide a more feasible approach to targeting the $G\alpha_s$ -immune checkpoint thus circumventing the emerging receptor-ligand redundancy. As the prospect of cancer immunotherapies will likely rely on combinatorial strategies blocking additional targets, studies interrogating downstream signaling mechanisms of T cell dysfunction and exhaustion will become important for uncovering these targets. Our work here investigating the pan-cancer GPCR repertoire of tumor infiltrating CD8 T cells combined with the use of synthetic biology approaches to reveal the specific immune suppressive functions of $G\alpha_s$ -signaling in CD8 T cells can now provide opportunities to achieve multitargeted $G\alpha_s$ -GPCRs inhibition or $G\alpha_s$ /cAMP/PKA pathway modulation with the overall goal of enhancing responses to immunotherapies.

Methods

Study Design

The sample size for each experiment was selected based on historical data and previous publications using the same tumor models^{135,224}. Mice from in vivo experiments were randomized based on tumor volume prior to initiation of treatment or data collection. Data collection and analysis were not performed blind to the conditions of the experiments. Data reported for all

experiments were not subjective but rather based on quantitative analyses. All data collected was included and represented in the main figures or supplementary materials.

Cell lines, antibodies and other reagents

The 4MOSC1 cell lines were previously generated in-house by our lab. The MC38 cell line was generously gifted by Dr. Andrew Sharabi. The MC38-OVA cell line was generated by retroviral transduction with pMSCV-OVA (gift from Andrew Sharabi). 4MOSC1-SIINFEKL was generated by lentiviral transduction with the pLenti-CMV GFP DEST vector. 4MOSC1 cell lines were grown in keratinocyte media with growth supplement, cholera toxin, EGF, and antibiotics. MC38-OVA were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 1% antibiotics/antimycotics and 1 µg/mL blasticidin. All cell lines were grown at 37°C and 5% CO₂. PD-1 antibody (clone J43, catalog #BE0033-2; clone RMP1-14, catalog #BE0146), CTLA-4 antibody (clone 9H10, catalog #BP0131), isotype antibody (Armenian hamster IgG isotype control, catalog #BE0091; Rat IgG isotype control, catalog #BE0251; Syrian hamster IgG isotype control, catalog #BE0087), and CD8 depletion antibody (Clone YTS 169.4, catalog #BE0117) were obtained from Bio X Cell (West Lebanon, NH, USA). Deschloroclozapine (DCZ) was purchased from Tocris (catalog #7193).

Mice

All the animal experiments used in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, San Diego, with protocol ASP #S15195. Mice at Moores Cancer Center, UCSD are housed in micro-isolators and individually ventilated cages supplied with acidified water and fed 5053 Irradiated Picolab Rodent Diet 20 from LabDiet. Temperature for laboratory mice in our facility is mandated to be between 65–75°F (~18–23°C) with 40–60% humidity, and a 12 light/12 dark cycle was maintained for the facility. All animal manipulation activities are conducted in laminar flow hoods. All personnel are required to

wear scrubs and/or lab coat, mask, hair net, dedicated shoes, and disposable gloves upon entering the animal rooms. All animal studies conducted in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, San Diego, with protocol ASP #S15195.

The *E8i-CreERT2* mice were obtained from Dr. Dario Vignali, University of Pittsburgh²⁰⁷. The *Gnas-exon 1^{fl/fl}* mice were obtained from Dr. Ramiro Iglesias-Bartolome, National Institutes of Health²⁰⁹. CD8-Gnas-KO mice were generated by crossing *E8i-CreERT2* with *Gnas-exon 1^{fl/fl}*. Information regarding genotyping of CD8-Gnas-KO mice is listed in **Table 2.4**. The Tet-GFP-PKI mice were obtained from Dr. Ramiro Iglesias-Bartolome, National Institutes of Health. Transgenic mice were generated as previously described using a codon-optimized sequence for the 1-24 amino acids from PKIA fused to GFP²⁰⁹. The *ROSA26-rtTA-IRES-EGFP* mice were obtained from Dr. Andras Nagy, Samuel Lunenfeld Research Institute²²⁵. Information regarding genotyping of CD8-PKI mice is listed in **Table 2.5**. OT-1 mice (C57BL/6-Tg(TcraTcrb)1100Mjb/J, stock no: 003831) were purchased from the Jackson Laboratory and bred in house. The *ROSA26-Gs-DREADD-LSL* mice were obtained from Dr. Rebecca Berdeaux, The University of Texas, Houston²²⁶. *ROSA26-Gs-DREADD* mice were generated by crossing *E8i-CreERT2* mice with *ROSA26-Gs-DREADD-LSL*. Information regarding genotyping of CD8-GsD mice is listed in **Table 2.6**.

Tamoxifen, doxycycline, and DCZ treatment

Tamoxifen was purchased from Sigma-Aldrich. Where indicated, CD8-GsD mice were dosed with tamoxifen at 75 mg/kg body weight. A stock solution of 15 mg/mL was prepared by dissolving 75 mg of tamoxifen in 5 mL of miglyol and dissolved at 37°C. After dissolving, the solution was stored at -20°C protected from light. Mice were given 1.5 mg in 100 µl by intraperitoneal injection (i.p.) for 3 consecutive days before tumor implantation or cell isolation. Mice were delivered 25 mg/kg doxycycline in the diet (Bio-Serv) beginning the same day as initial

tamoxifen administration. Mice consume doxycycline for 5 consecutive days before cell isolation. Deschloroclozapine (DCZ) was purchased from Tocris. Mice were dosed with DCZ at 0.01 mg/kg. A stock solution of 10 mg/mL was prepared by dissolving 10 mg of DCZ into 1 mL of DMSO. Subsequently, a working concentration of 0.002 mg/mL of DCZ in PBS was prepared, and mice were given 0.0002 mg of DCZ in 100 μ L i.p. daily.

In vivo mouse tumor models.

For 4MOSC1 and 4MSOC1-SIINFEKL tumor xenograft, WT female C57Bl/6 mice (4-6 weeks old) were purchased from Charles River Laboratories (Worcester, MA, USA). WT mice or age-matched CD8-GsD mice were first given tamoxifen i.p. every day for 3 days. On the fourth day, 5×10^5 tumor cells were injected into the tongue of mice and when tumors reached $\sim 30 \text{ mm}^3$ (4-5 days post-implantation), mice were treated by i.p. injection of either isotype control antibody, anti-PD-1 (clone J43), or anti-CTLA-4 (clone 9H10) (i.p. 10 mg/kg three times a week). Where indicated, DCZ (i.p. 0.1 mg/kg) was started on the fifth day and given daily.

For MC38-OVA tumor xenograft studies, WT female C57Bl/6 mice (4-6 weeks old) were purchased from Charles River Laboratories (Worcester, MA, USA). WT mice or age-matched CD8-GsD mice were first given tamoxifen i.p. every day for 3 days. On the fourth day, 1×10^5 cells were injected subcutaneously into the flanks of mice. When tumors reached $\sim 100 \text{ mm}^3$ (8-10 days post-implantation), mice were treated by i.p. injection of either isotype control antibody or anti-PD-1 (clone RMP1-14) (i.p. 10 mg/kg three times a week). Where indicated, DCZ treatment was started on the fifth day and given daily.

All mice were euthanized after the completion of the treatment, or when control-treated mice succumbed to tumor burdens, or when tumors have ulcerated. Mice were also sacrificed when tongue tumors reached 8mm in greatest diameter, or when flank tumors reached 1500 mm^3 , as determined by the ASP guidelines).

Tumors were dissected as previously described²²⁴. In brief, tumors were isolated, minced, and resuspended into the Tumor Dissociation Kit (Miltenyi Biotec, San Diego, CA) and were processed with the gentleMACS Octo Dissociator, according to the manufacturer's protocol for tumor dissociation. Digested tumors were then passed through 70 μ m strainers to generate single-cell suspensions and processed further for flow cytometric analysis.

***In vitro* T cell cultures and isolations**

Splenocytes were isolated from 4-6-week-old mice and mechanically disrupted. Red blood cells were lysed by RBC lysis buffer (BioLegend) according to manufacturer's instructions. CD8 T cells were isolated by EasySep CD8 Isolation Kit by negative selection. For determining expression of transgenes in CD4 or CD8 T cells, CD8 T cells were first isolated through positive selection with CD8a MicroBeads (Miltenyi Biotec), followed by positive selection with CD4 MicroBeads of the prior eluted fraction. For activation, they were then cultured at 1×10^6 cells per well in 1 mL in 24-well plates with Dynabeads Mouse T-Activator CD3/CD28 beads at a 1:1 cells to beads ratio with 25 U/mL hIL-2 (PeproTech) for 48 hours. Naïve CD8 T cells were cultured with 25 U/mL hIL-2 alone. After 48 hours, activated cells were collected and counted and either split into the "acute" or "chronic" activation groups. For acute activation, CD8s were cultured at 5×10^4 cells per well in 200 μ l in 96-well round bottom plates with 25 U/mL hIL-2 without beads. For chronic activation, CD8s were cultured at the same cell density with 25 U/mL hIL-2 and with CD3/CD28 beads at a 1:2.5 cells to beads ratio. Where indicated, GPCR agonists were added at the following concentrations: 1 μ M 16, 16-di-methyl prostaglandin E2 (PGE2) (Tocris), 5 μ M dobutamine hydrochloride (dobutamine) (Tocris), 5 μ M CGS 21680 hydrochloride (CGS 21680) (Tocris). For PGE2 experiments, where indicated, inhibitors or CXCL10 were added at the following concentrations: 1 μ M forskolin (Tocris), 1 μ M PF 04418948 (EP₂ inhibitor; EP₂i) (Tocris), 1 μ M ONO AE3 208 (EP₄ inhibitor; EP₄i) (Tocris), 10 nM CXCL10 (gifted by Tracy Handel); PGE2 and inhibitors were added in the last 24 hours of culture. Inhibitors were first added for 30 min

and then 1 μ M of PGE2 was added in the last 24 hours of culture. For CD8-GsD *in vitro* experiments, 2 μ M of 4-hydroxytamoxifen (Sigma-Aldrich) was included in culture. Additionally, DCZ was added at a concentration of 0.002 mg/mL.

***In vitro* co-culture tumor killing assay**

Splenocytes were isolated from OT-1 mice (4-6 weeks old) and activated with 100 nM of OVA Peptide (257-264) (GenScript) and 50 U/mL of hIL-2 (PeproTech) for 48 hours. After 48 hours, fresh media with 50 U/mL IL-2 was added to culture and incubated for an additional 24 hours. Cells were then collected and replated and expanded at 1×10^6 cells/mL.

For the specific cytotoxicity assay, tumor cells (target) were plated at 50,000 cells per well in a 24-well plate. OT-1 T cells (effector) were then added at a 1:5 target:effector ratio. Where indicated, GPCR agonists were added at the following concentrations: 1 μ M 16, 16-di-methyl prostaglandin E2 (PGE2) (Tocris), 5 μ M dobutamine hydrochloride (dobutamine) (Tocris), 5 μ M CGS 21680 hydrochloride (CGS 21680) (Tocris). The co-culture was left for 36 hours, and cell viability was assessed by flow cytometric staining with Zombie Aqua Viability Dye (BioLegend).

Flow cytometry

The following flow cytometry antibodies (Mouse) were purchased from BioLegend, San Diego, CA: CD45 (30-FII) (1:100), CD3 (145-2CII) (1:200), CD8a (53-6.7) (1:200), PD-1 (29F.1A12) (1:100), Tim-3 (RMT3-23) (1:100), IFN-gamma (XMGI.2) (1:100), Granzyme B (GBII) (1:100), TNF-alpha (MP6-XT22) (1:100), CTLA-4 (UC10-4B9) (1:100), LAG-3 (C9B7W) (1:100), Ki-67 (16A8) (1:100). Anti-CREB (pS133) / ATF-1 (pS63) (1:20) was purchased from BD Biosciences, Franklin Lakes, NJ. For viability stain of CD8 T cells *in vitro*, cells were washed once with PBS and stained with Zombie Aqua Viability Dye (BioLegend) according to manufacturer's instructions. Cell surface staining was done for 30 min at 4°C. For intracellular and transcription factor staining, cells were stimulated with 1x Cell Activation Cocktail with Brefeldin A (BioLegend)

in media for 4-6 hours at 37°C prior to viability staining. Unstimulated cells were used as a control. After cell surface staining, cells were fixed with FOXP3/Transcription factor buffer set and stained with intracellular antibodies for 45 min at room temperature.

For flow cytometry acquisition using the Agilent NovoCyte Advanteon, the voltage and photodetector gains were automatically determined by calibration and quality control (QC) of the instrument. Automatic compensation was then performed using single-stained samples of OneComp eBeads™ Compensation Beads (ThermoFisher Scientific), and voltage parameters were automatically determined based on pre-determined photodetector gains from the calibration. Optimal dilutions for antibodies used in this study were determined based on titrating the antibody amount and adjusting the cell number used. The gating strategy for flow cytometry experiments is shown in **Figure 2.14a and 2.14b**, and gates were drawn based on non-activated and unstimulated control cells.

Detection of pCREB

For CD8-GsD pCREB activation experiments *in vivo*, CD8-GsD mice were first given tamoxifen i.p. every day for 3 days. DCZ (i.p. 0.01 mg/kg) was started on the fifth day and given daily for 5 days. Blood was collected from mice with retro-orbital bleeding and lyse and fixed with Lyse/Fix Buffer (BD Biosciences). Cells were then permeabilized with Perm Buffer II (BD Biosciences) and stained with the anti-CREB (pS133)/ATF-1 (pS63) antibody (BD Biosciences) according to manufacturer's instructions.

For CD8-GsD pCREB activation experiments *in vitro*, CD8-GsD mice were first given tamoxifen i.p. every day for 3 days. On the fourth day, blood was collected from mice with retro-orbital bleeding. Red blood cells were lysed by RBC lysis buffer (BioLegend) according to manufacturer's instructions. 0.002 mg/mL of DCZ was added to culture for 15 min, and cells were fixed by CytoFix buffer (BD Biosciences) according to manufacturer's instructions. Cells were then permeabilized with Perm Buffer II (BD Biosciences) and stained with extracellular antibodies and

with the anti-CREB (pS133)/ATF-1 (pS63) antibody (BD Biosciences) according to manufacturer's instructions.

For PGE2 pCREB activation experiments *in vitro*, CD8 T cells from chronic activation scheme were serum-starved for 1 hour. 1 μ M of PGE2 was then added for 15 min in the presence or absence of inhibitors and cells were fixed by CytoFix buffer (BD Biosciences) according to manufacturer's instructions. Cells were then permeabilized with Perm Buffer II (BD Biosciences) and stained with extracellular antibodies and with the anti-CREB (pS133)/ATF-1 (pS63) antibody (BD Biosciences) according to manufacturer's instructions.

Quantitative PCR

RNA was extracted from naïve, activated, chronically stimulated, and chronically stimulated CD8 T cells treated with DCZ by the RNeasy Mini kit following manufacturer's instructions (Qiagen). 100ng of RNA was converted to cDNA using SuperScript™ VILO™ cDNA Synthesis Kit (ThermoFisher Scientific). qPCR was performed using the SYBR™ Select Master Mix (ThermoFisher Scientific). *Actb* was used for normalization. The following primers were used for qPCR: *Dusp1* fwd 5'- GTTGTGGATTGTCGCTCCTT, *Dusp1* rev 5'- TTGGGCACGATATGCTCCAG, *Tox* fwd 5'- GCTCCCGTTCCATCCACAAA, *Tox* rev 5'- TCCCAATCTCTTGCATCACAGA, *Tigit* fwd 5'- GAATGGAACCTGAGGAGTCTCT, *Tigit* rev 5'- AGCAATGAAGCTCTCTAGGCT, *Actb* fwd 5'- GGCTGTATTCCCCTCCATCG, *Actb* rev 5'- CCAGTTGGTAACAATGCCATGT.

Bioinformatics analysis

For scRNA-seq integration, we used the Seurat scRNA-seq SCTransform integration method²²⁷. We collected 30 datasets with publicly available raw counts across 19 cancer types. Each dataset was transformed individually with the SCTransform function to regress out mitochondrial percentage, S score, G2M score and DIG score. The S, G2M and DIG scores were

obtained from a recent publication. The data was then prepared for integration with the `SelectIntegrationFeatures`, `PrepSCTIntegration` and `FindIntegrationAnchors` functions. The data was then integrated with the `IntegrateData` function. Principal Component Analysis (PCA) and dimensionality reduction were performed with the `RunPCA` and `RunUMAP` functions, respectively. CD8 T cell states were identified using the `ProjectTILs` package, the multimodal reference mapping workflow in `Seurat` and key marker genes, resulting in 5 major functional states: naive, cytotoxic, effector memory, proliferative, and exhausted. To examine the relative expression of GPCRs between CD8 T cell subtypes, the average expression of each GPCR was calculated for each of the 5 groups, row-normalized for each GPCR and visualized on a circular heatmap using *circlize v0.4.12* and *ComplexHeatmap v2.6.2*. Package versions: *circlize v0.4.12*, *ComplexHeatmap 2.6.2*, *ggplot2 3.3.3*, *Seurat 4.0.1*.

For calculation of T cell dysfunction score, the average expression of all genes in the T cell dysfunction gene set was calculated and plotted for each CD8 T cell from the integrated CD8 dataset. Spearman correlations for each GPCR and the dysfunction score were calculated by correlating expression of the GPCR with mean expression of the dysfunction gene set for each CD8 T cell from the melanoma dataset (GSE120575).

For differential expression analysis, 3 datasets of LCMV RNA-seq data were batch corrected with `ComBat (v3)`. Subsequently, `DESeq2 (version 1.32)` was used to analyze all effector CD8 T cells versus all exhausted CD8 T cells. p-values from this analysis have been adjusted for multiple hypothesis correction and false discovery rates with Benjamini-Hochberg testing^{228,229}

For gene set enrichment analysis, we created GPCRs gene-sets on the basis of their G-protein coupling mechanisms. We considered either G-protein family level transduction mechanisms from IUPHAR or individual G-protein couplings from recent experimental TGF α shedding assay that we augmented through machine learning-based predictions^{206,230}. For each G-protein family coupling from IUPHAR, we created gene-sets based on primary and secondary

mechanisms either in isolation or combined. For experimental TGF α shedding assay, we considered as couplings the binding with LogRAi values greater than -1.0. We defined the predicted couplings by considering either a looser (0.5) or a more stringent (0.9) cutoff of the coupling probabilities outputted from PRECOG²³⁰. For each individual G-protein couplings, we created gene-sets by considering the experimental and predicted couplings either in isolation or in combination. We created gene sets in the .gmt format, by considering corresponding Entrez IDs, and we performed gene set enrichment analysis through *ClusterProfiler* (v.4.6.2)²³¹ giving as an input the list of genes ranked according to Log2Fold change values from differential expression analysis.

For analysis of objective response rates (ORR), expression profiles of tumors from 2277 patients across 14 cancer types treated with immune checkpoint inhibitors were collected^{199,217,232}. Mean expression of each GPCR was calculated and were classified based on coupling information from IUPHAR.

Statistical analysis

Graphs were plotted using GraphPad Prism v9.2.0 (GraphPad Software Inc.) and R (v4.2.2). Where indicated, data is expressed as mean $\pm$ s.e.m. The following tests were performed (see Figure legends for details): correlation, unpaired two-tailed student's t-test, one- or two-way ANOVA, Log-rank (Mantel-Cox) for Kaplan-Meier survival curves. Statistical significance was determined by: *, $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data distribution was assumed to be normal, but this was not formally tested.

Data Availability

GEO accessions regarding the human datasets used in this study are listed here: GSE116256, GSE114727, GSE110686, GSE14018, GSE123813, GSE22898, GSE123139, GSE115978, GSE146771, GSE134520, GSE140228, GSE125449, GSE117570, GSE127465,

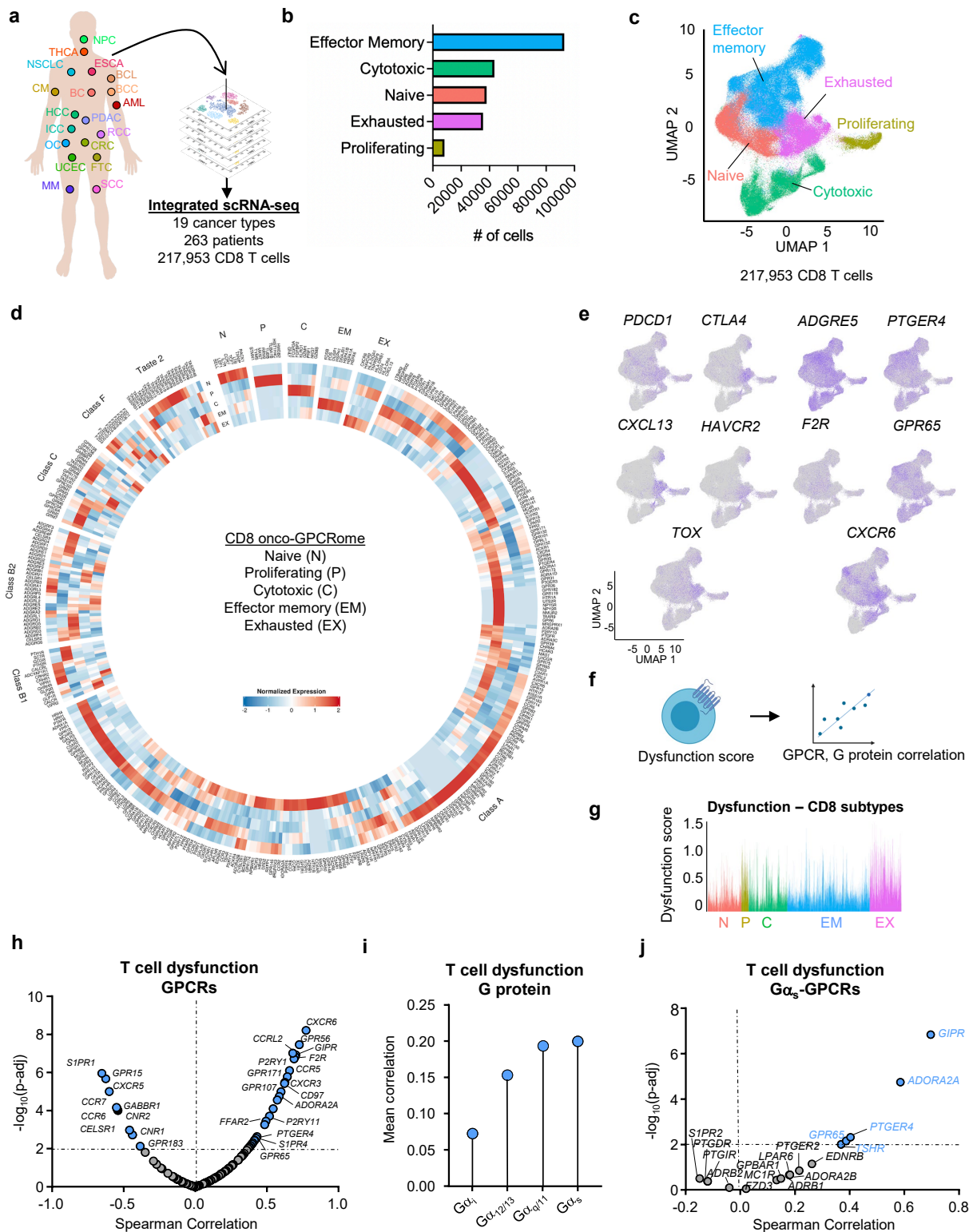
GSE99254, GSE123813, GSE156728, GSE120575, GSE84820, GSE123235, GSE122969, GSE88987, GSE141299. EGA archive identifiers regarding the human datasets used in this study are listed here: EGAS00001002171, EGAS00001002486, EGAS00001002325, EGAS00001002553. Additional information regarding these datasets can be found in Supplementary Table 1. The remaining data is available within the article and supplementary information. Source data are provided with this paper.

Acknowledgements

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Bartolome R, Carter H, Ruppin E, Mesirov JP, Gutkind JS. The GPCR-G α s-PKA signaling axis promotes T cell dysfunction and cancer immunotherapy failure. *Nat Immunol.* 2023 Aug;24(8):1318-1330. doi: 10.1038/s41590-023-01529-7. Epub 2023 Jun 12. PMID: 37308665; PMCID: PMC10735169. The dissertation author was the primary investigator and author of this paper.

Figure 2.1: $G\alpha_s$ -GPCRs are correlated with T cell dysfunction and terminally exhausted T cells. **a**, Schematic of integrated analysis of 19 single-cell RNA-seq datasets (n=263 patients, n=217,953 CD8 T cells). Full name of cancer types and their abbreviations are listed in the text and in **Table 2.1a**. This figure was generated, in part, with BioRender.com. **b**, Integration of all CD8 expressing cells and the stratification into 5 different CD8 subtypes (n=217,953 CD8 T cells analyzed). **c**, Visualization of integrated CD8 T cells using dimensionality reduction. **d**, The CD8 onco-GPCRome. Normalized average expression of 367 GPCR genes, with blue representing lower and red representing higher expression, organized by receptor family and aligned with annotated landmark genes from different CD8 subtypes. **e**, Visualization of landmark genes for terminally exhausted CD8 T cells with the top 5 most highly expressed GPCRs in the terminally exhausted CD8 population. **f**, Schematic explaining analysis of correlation of GPCRs and G protein genes with the T cell dysfunction score²⁰³. **g**, Quantification of T cell dysfunction score across all subtypes of CD8 T cells. **h**, Spearman correlation of 119 GPCR genes with the T cell dysfunction score and the statistical *p*-values were calculated and plotted from tumor-infiltrating CD8 T cells from human melanoma (GSE120575)²¹⁶. Blue dots indicate GPCRs with Spearman correlations with *p*<0.01 and grey dots indicate GPCRs with Spearman correlations with *p*>0.01. A full list of *p*-values and Spearman correlation values is listed in **Table 2.1d**. **i**, The mean correlation values of GPCRs were calculated based on their G protein coupling designation from IUPHAR. These values were then ranked and plotted, and included $G\alpha_i$, $G\alpha_{12/13}$, $G\alpha_{q/11}$, and $G\alpha_s$ G protein couplings. **j**, Spearman correlation of 367 GPCR genes with the T cell dysfunction score was the statistical *p*-values were calculated, and the $G\alpha_s$ -coupled GPCRs (primary coupling as designated by IUPHAR) were plotted. Blue dots indicate GPCRs with Spearman correlations with *p*<0.01 and grey dots indicate GPCRs with Spearman correlations with *p*>0.01. Figures 1a and 1f were generated, in part, with BioRender.com.



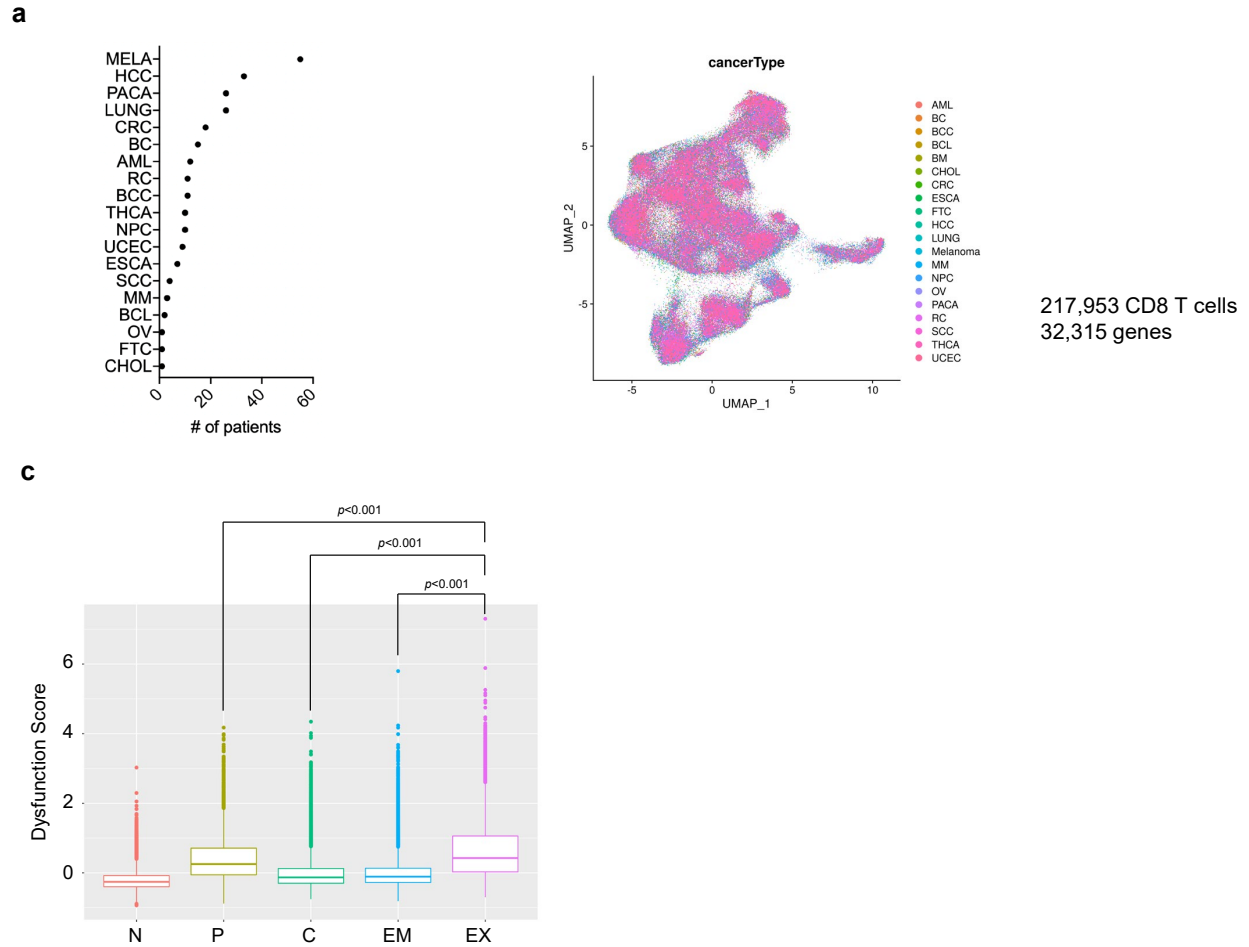


Figure 2.2: Patient and cancer information for integration analysis of CD8 T cells.**a**, Information about patients, cancer type, and dataset used in the single-cell RNA-seq integration. **b**, Visualization of 217,953 CD8 T cells after integration from 30 single-cell RNA-seq datasets. **c**, Statistical comparison of calculated dysfunction score from tumor-infiltrating populations of CD8s characterized from **Figure 2.1b**. Each dot represents one cell from groups listed in **Table 2.1a**. Naïve: lower bound=0.147, middle bound=0.229, upper bound=0.337, 25th percentile=0.228, 75th percentile=0.231. Proliferating: lower bound=0.372, middle bound=0.564, upper bound=0.790, 25th percentile=0.556, 75th percentile=0.571. Cytotoxic: lower bound=0.184, middle bound=0.290, upper bound=0.447, 25th percentile=0.288, 75th percentile=0.292. Effector Memory: lower bound=0.209, middle bound=0.304, upper bound=0.431, 25th percentile=0.303, 75th percentile=0.305. Exhausted: lower bound=0.361, middle bound=0.555, upper bound=0.800, 25th percentile=0.551, 75th percentile=0.559.

Figure 2.3: G α_s -coupling augments an exhaustion-like dysfunctional state in CD8 T cells. **a**, Schematic of curated RNA-seq datasets from different subtypes of T cells sorted out from the murine chronic infection model, LCMV. **b**, Differential expression analysis of GPCRs not significantly ($p > 0.05$; grey) or significantly ($p < 0.05$) upregulated in effector (orange) versus exhausted (purple) CD8 T cells from LCMV datasets. Statistical significance was determined by the Wald test as part of the DESeq analysis package. **c**, Gene set enrichment analysis (GSEA) showing normalized enrichment scores (NES) of GPCRs significantly upregulated in either effector or exhausted T cells. Statistical significance was calculated as part of the GSEA analysis, see **Table 2.3c** for p -values; *, $p < 0.05$. ** $p < 0.01$, **d**, GSEA mountain plots illustrating significant enrichment of gene sets from **Figure 2.3c**. **e**, Experimental scheme illustrating *in vitro* chronic stimulation assay of CD8 T cells. **f**, Representative plots showing expression of IFN γ and TNF in chronically stimulated CD8 T cells after treatment with G α_s agonists. Statistical significance was determined by two-tailed unpaired Student's t -test comparing to control samples; ** $p < 0.01$, **** $p < 0.0001$; see **Figure 2.3g**. **g**, Quantification of IFN γ and TNF, granzyme B, Ki-67, PD-1 and Tim-3 in CD8 T cells treated with G α_s agonists. The average frequency and s.e.m. are shown ($n = 6$ biologically independent samples). **h**, Schematic illustrating *in vitro* co-culture tumor killing assay. **i**, Percent killing by OT-1 T cells in the presence or absence of G α_s agonists. The average frequency and s.e.m. are shown ($n = 6$ biologically independent samples). **j**, Representative bar plot showing the specificity of phospho-CREB (pCREB) induction (**j**) and IFN γ and TNF (**k**) inhibition by PGE2 in the presence or absence of EP2 (EP $_2$ i) or EP4 inhibitor (EP $_4$ i). The average frequency and s.e.m. are shown ($n = 3$ biologically independent samples). Statistical significance was determined by one-way ANOVA. **l**, Schematic depicting generation of CD8-*Gnas* KO mice. **m**, Quantification of IFN γ and TNF in CD8 T cells from CD8-*Gnas*^{+/+} or CD8-*Gnas* KO mice. The average frequency and s.e.m. are shown ($n = 6$ biologically independent samples). Unless indicated otherwise, statistical significance was determined by two-way ANOVA. Figures 2a and 2h were generated, in part, with BioRender.com.

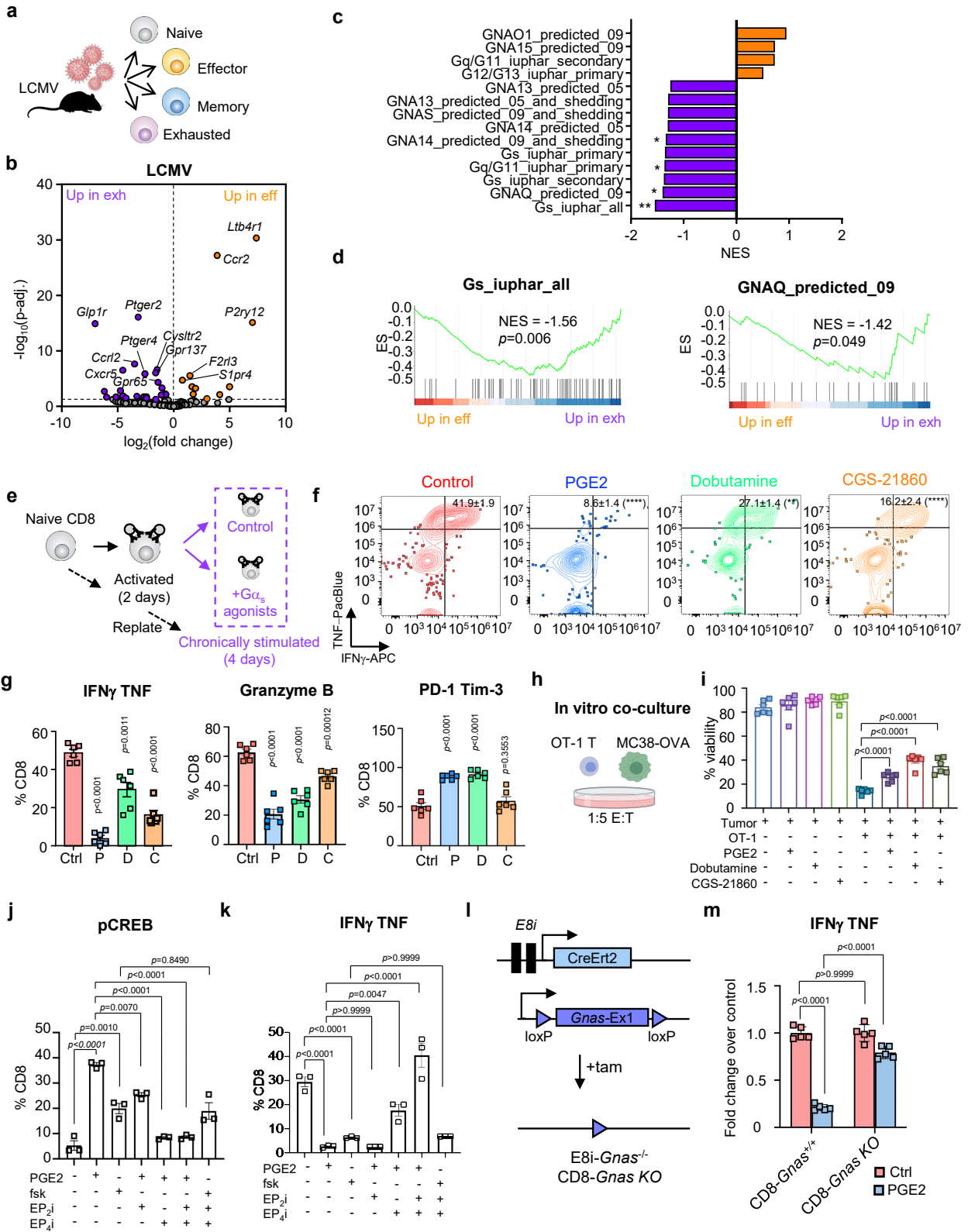


Figure 2.4: Effect of targeting the $G\alpha_s$ /PKA signaling pathway in CD8 T cells.**a**, Upregulation of inhibitory receptors and decrease of IFN γ and TNF in chronically versus acutely stimulated CD8 T cells. The average relative expression and s.e.m. are shown (n=6 biologically independent samples). **b**, Significant decrease of IFN γ or TNF with $G\alpha_s$ agonists in chronically stimulated CD8 T cells. The average relative expression and s.e.m. are shown (n=6 biologically independent samples). **c**, Significant decrease of Ki-67 and viability with $G\alpha_s$ agonists in chronically stimulated CD8 T cells. The average relative expression and s.e.m. are shown (n=6 biologically independent samples). **d**, Representative flow cytometry plots showing expression of Tim-3 and PD-1 in chronically stimulated CD8 T cells after treatment with 1 μ M PGE2 (P), 5 μ M Dobutamine (D), or 5 μ M CGS-21860 (C). **e**, Effect of CXCL10 on PGE2-mediated decrease in IFN γ and TNF. The average frequency and s.e.m. are shown (n=3 per group). Statistical significance was determined by two-way ANOVA. Unless indicated otherwise, statistical significance was determined by two-tailed unpaired Student's *t*-test.

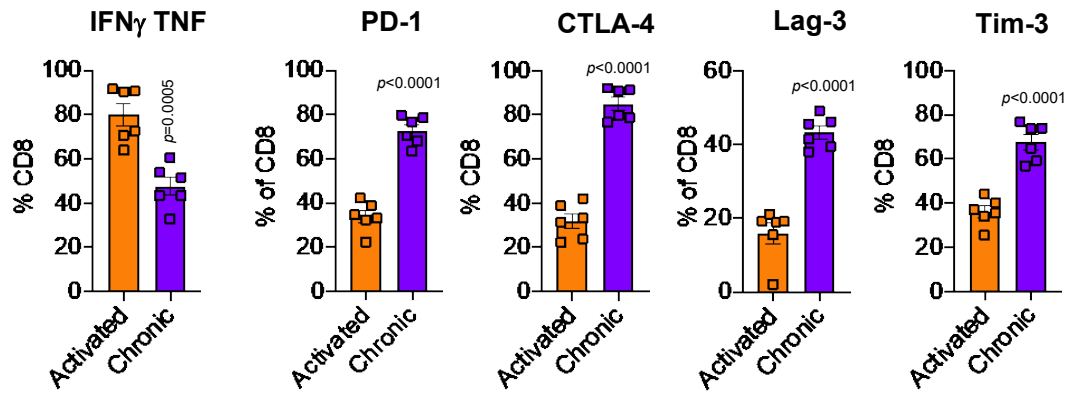
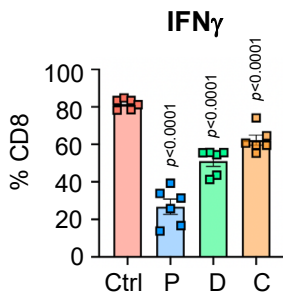
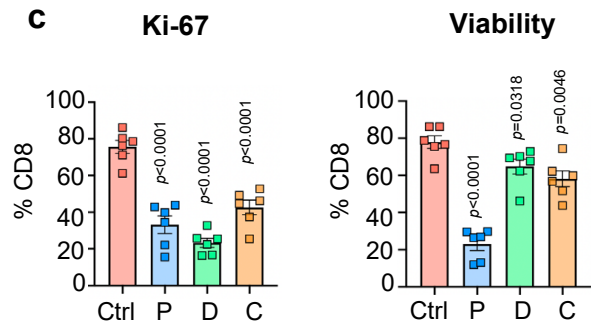
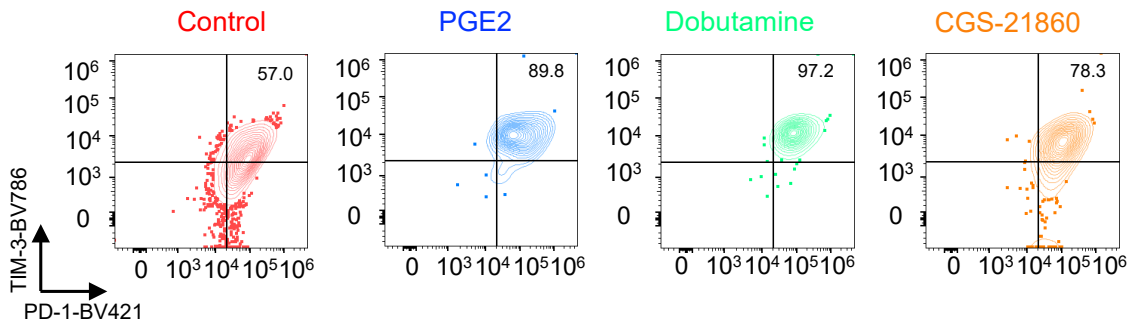
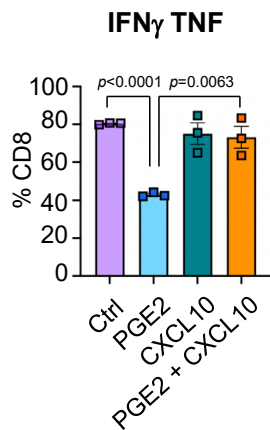
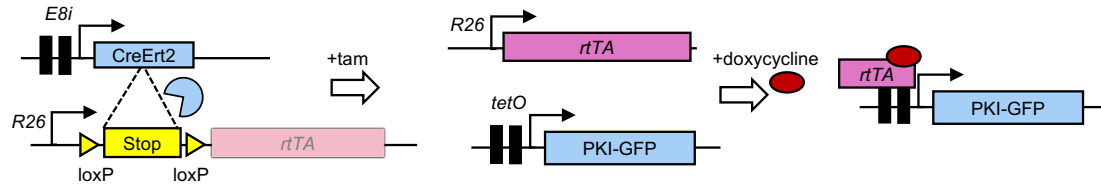
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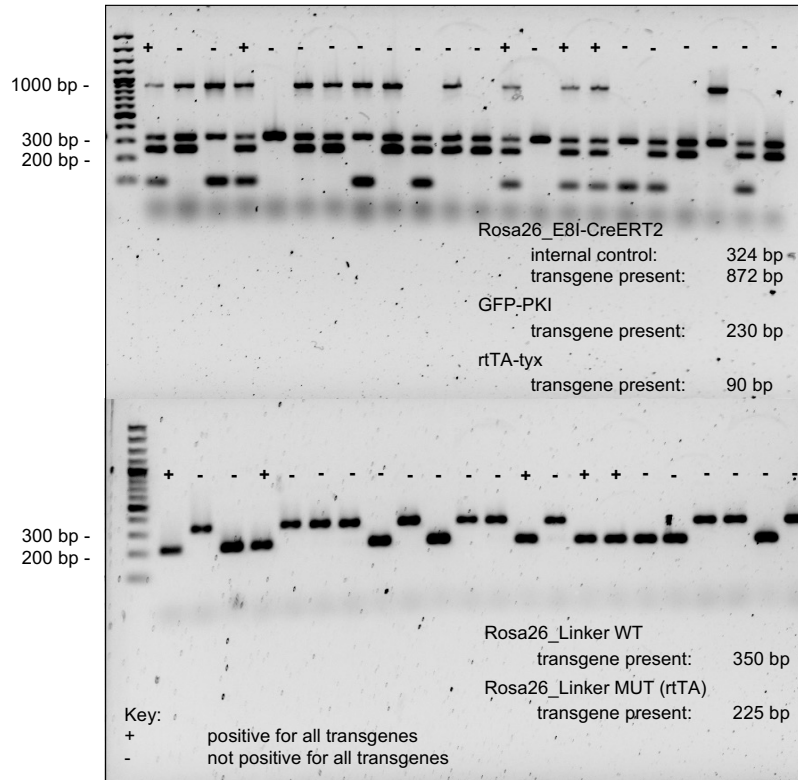
Figure 2.5: Development of a CD8-restricted PKI transgenic mouse model. **a**, Scheme illustrating the generation of CD8-PKI mice. **b**, Genotyping information for CD8-PKI mice. **c**, Confirmation of PKI expression in CD4 or CD8 T cells isolated from splenocytes of CD8-PKI mice and littermate controls after induction by doxycycline and tamoxifen. The average relative expression and s.e.m. are shown (n=6 mice per group). **d**, Quantification of IFN γ and TNF inhibition by PGE2 in chronically stimulated CD8 T cells from CD8-PKI mice. The average relative expression and s.e.m. are shown (n=3 biologically independent samples). Statistical significance was determined by two-way ANOVA.

a

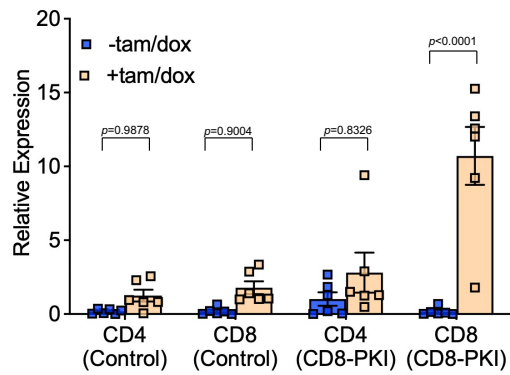
E8i^{CreErt2}Rosa26^{LSL}Tet-GFP-PKI (CD8-PKI) mice



b



c



d

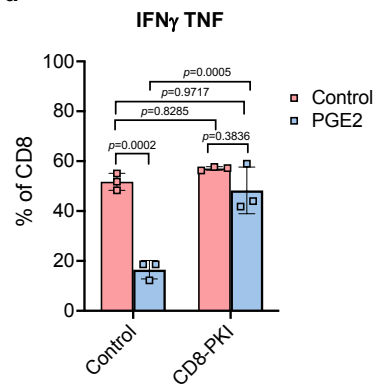


Figure 2.6: Mechanisms of immune suppression in CD8 T cells uncovered by Gα_s-DREADD. **a**, Schematic illustrating generation of *E8i^{CreErt2}Rosa26^{LSL GsDREADD}* (CD8-GsD) mice. **b**, Experimental scheme showing confirmation of expression and activation of CD8-specific Gα_s-DREADD by tamoxifen and deschloroclozapine (DCZ), respectively. **c**, Confirmation of Gα_s-DREADD expression in CD4 or CD8 T cells purified from peripheral blood of CD8-GsD mice dosed with or without tamoxifen. The average relative expression and s.e.m. are shown (n=8 mice per group). Statistical significance was determined by two-way ANOVA. **d**, Confirmation of CD8-restricted Gα_s-DREADD activation following tamoxifen and DCZ treatment in CD11b⁺, NK1.1⁺, CD4⁺, or CD8⁺ cells from peripheral blood. The average frequency and s.e.m. are shown (n=4 mice per group). Statistical significance was determined by two-way ANOVA. **e**, Representative histograms showing P-CREB induction from 0.002 mg/mL DCZ *in vitro*. **f**, Experimental scheme showing *in vitro* chronic stimulation assay with CD8 T cells purified from CD8-GsD mice. **g**, Representative flow cytometry plots of IFN γ and TNF (left panel) and quantification (right panel) in chronically stimulated CD8 T cells with or without 0.002 mg/mL DCZ. The average relative expression and s.e.m. are shown (n=3 biologically independent samples). **h**, Quantification of granzyme B, Ki-67, and PD-1 and Tim-3 in chronically stimulated CD8 T cells treated with or without 0.002 mg/mL DCZ. The average frequency and s.e.m. are shown (n=3 mice per group). **i**, Scheme illustrating CREB activity downstream of cAMP/PKA. **j**, Quantitative PCR data showing relative expression of *Dusp1* and exhaustion-associated genes. The average frequency and s.e.m. are shown (n=3 biologically independent samples). **k**, Experimental scheme of CD8-GsD mice implanted with 4MSOC1-SIINFEKL. **l**, **m**, Representative flow cytometry plots (left panel) and quantification (right panel) of OVA-tetramer positive (**l**) or IFN γ and TNF double positive (**m**) CD8 T cells in CD8-GsD implanted with 4MSOC1-OVA treated with or without 0.01 mg/kg DCZ (n=4 mice per group). The average frequency and s.e.m. are shown. Unless indicated otherwise, statistical significance was determined by two-tailed unpaired Student's *t*-test. Figures 3b, 3f, 3i, and 3k were generated, in part, with BioRender.com.

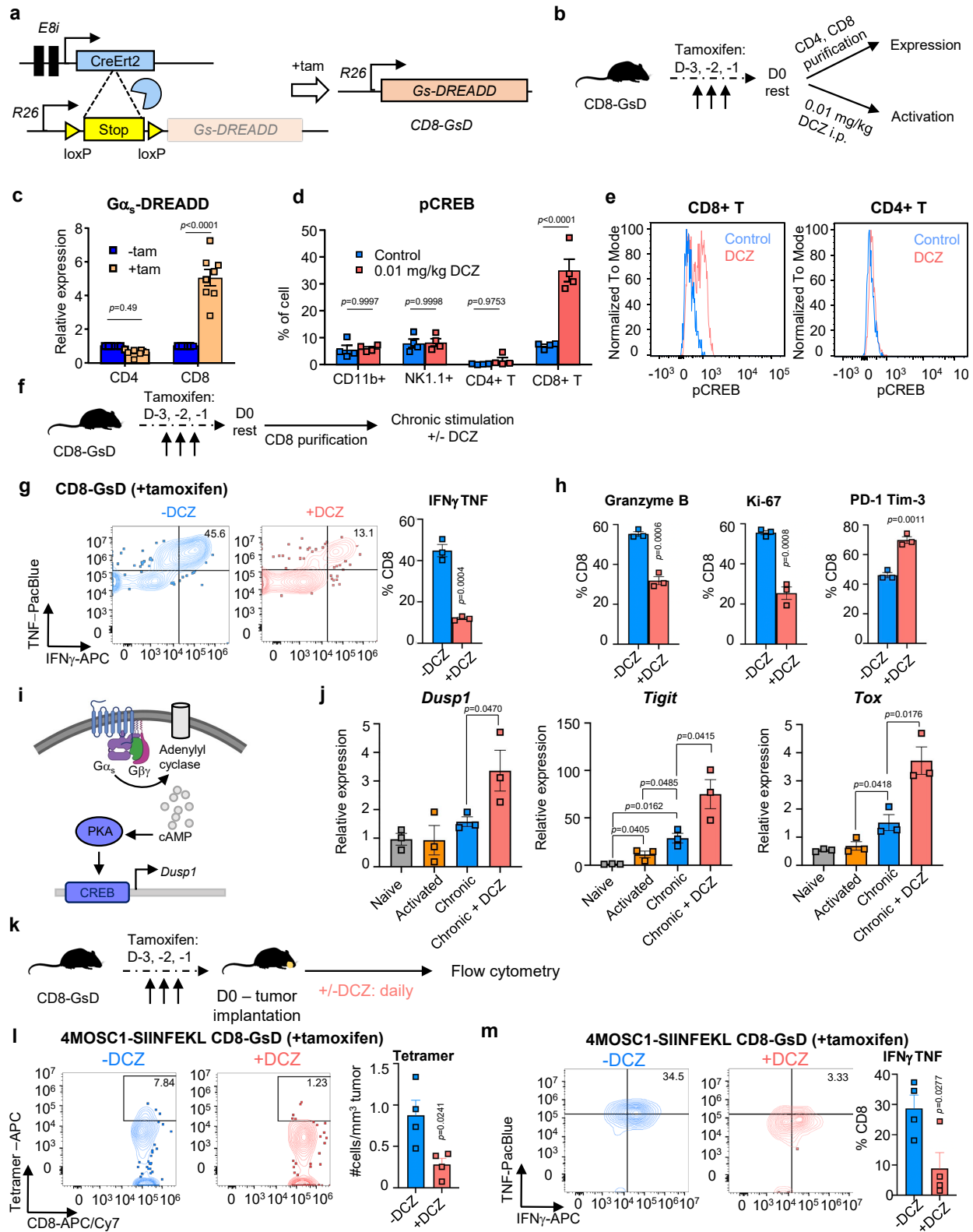
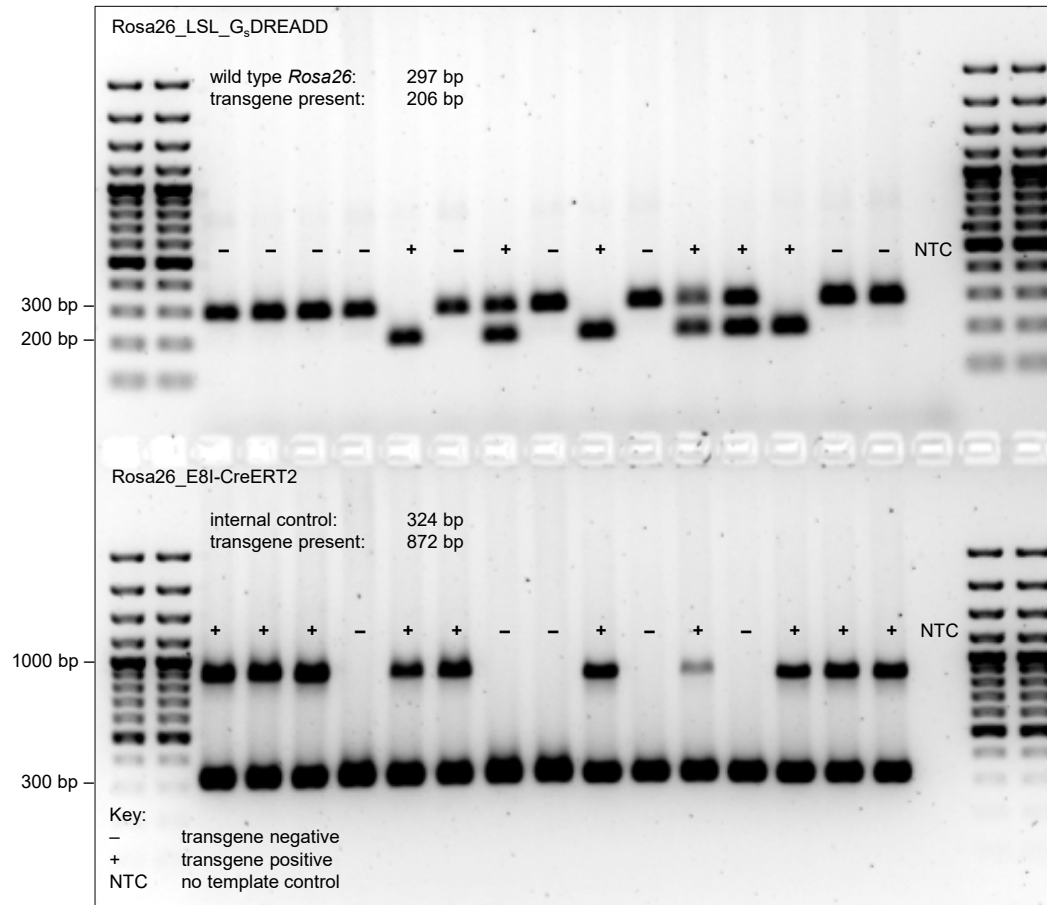


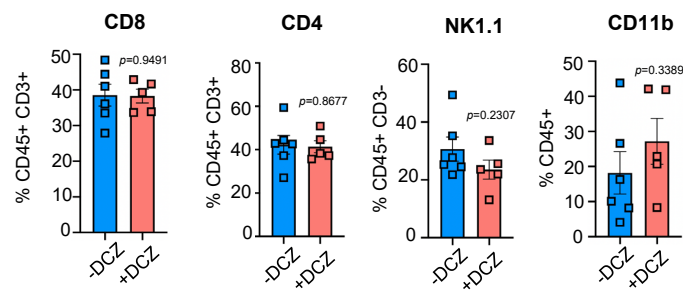
Figure 2.7: Development of a CD8-restricted $G\alpha_s$ -DREADD transgenic mouse model. **a**, Genotyping confirmation for CD8-GsD mice. Primers detecting the $G\alpha_s$ -DREADD, ROSA26, and *E8i-Cre* were used to confirm recombination by the Cre-recombinase. Information about primers and genotyping is listed in **Table 2.4**. **b**, Effect of DCZ on circulating CD8, CD4, NK cells, and CD11b myeloid cells in the peripheral blood of CD8-GsD mice treated with tamoxifen and 5 doses of DCZ (n=5 mice for -DCZ; n=6 mice for +DCZ). **c**, Effect of DCZ on non-tamoxifen-treated CD8-GsD mice. Quantification of IFN γ and TNF and PD-1 and Tim-3 in non-tamoxifen-treated CD8 T cells treated with or without DCZ. The average frequency and s.e.m. are shown (n=3 biologically independent samples). Statistical significance was determined by two-tailed unpaired Student's *t*-test.

a

***E8i^{CreErt2}Rosa26^{LSL}GsDREADD* (CD8-GsD) mice**



b



c

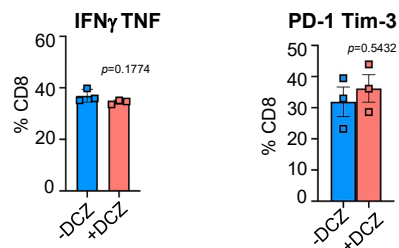
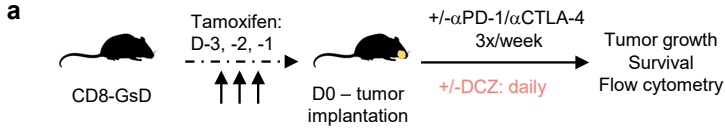
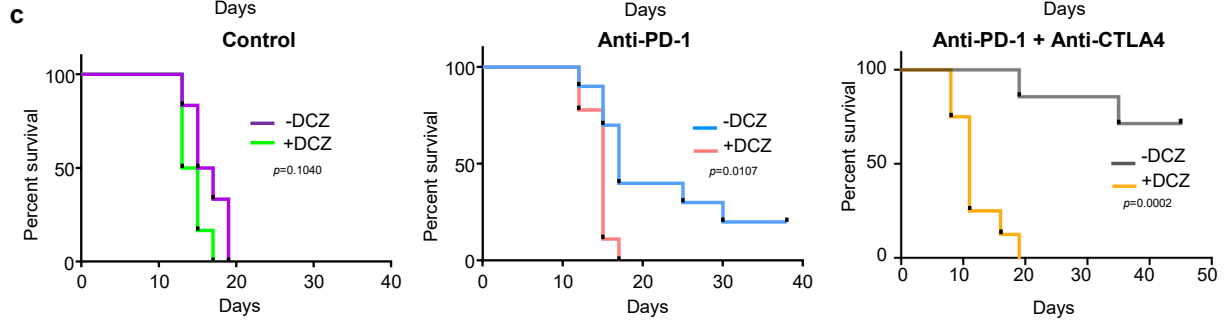
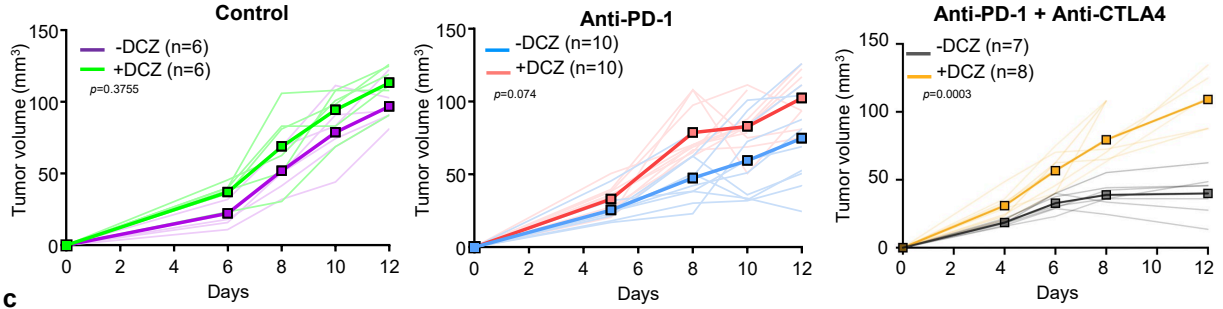


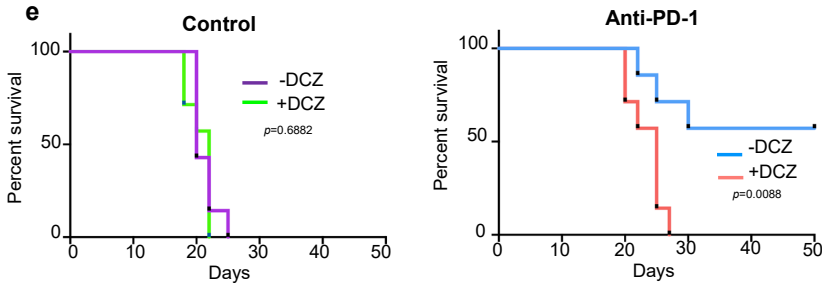
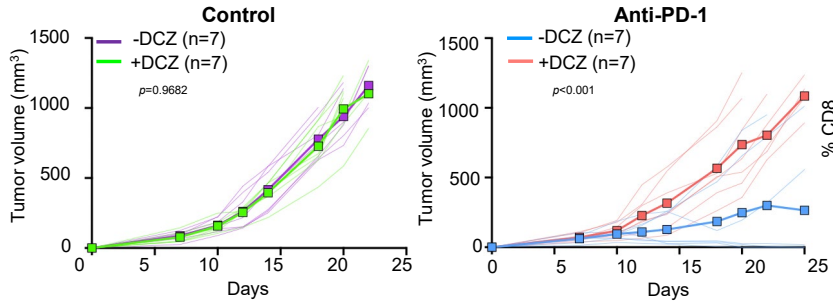
Figure 2.8: CD8-restricted $G\alpha_s$ stimulation leads to immunotherapy failure. **a**, Experimental scheme of CD8-GsD mice implanted with tumors and treated with DCZ, anti-PD-1, or anti-CTLA-4. This figure was generated, in part, with BioRender.com. **b, c**, Tumor growth curve (**b**) and survival curves (**c**) of CD8-GsD mice implanted with 4MOSC1 tumors treated with or without immunotherapy. Mice were treated with either hamster IgG (left panel) (n=5 mice per group), 10 mg/kg anti-PD-1 (middle panel) (n = 10 mice per group), or both anti-PD-1 and 10mg/kg anti-CTLA-4 (right panel) (n=7 mice group for -DCZ, 8 mice per group for +DCZ). **d, e**, Tumor growth curve (**d**) and survival curves of CD8-GsD mice implanted with MC38-OVA tumors treated with or without immunotherapy (n=7 mice per group). Mice were given 3 doses of tamoxifen, and 1×10^5 MC38-OVA cells were implanted into the flanks of mice. Mice were treated with either hamster IgG (left panel) or 10 mg/kg anti-PD-1 (middle panel). **f**, Mice from (**d**) were taken down and tumors were dissected for flow cytometric analysis. Shown is the quantification of OVA (SIINFEKL)-positive (left panel), IFN γ -positive, and granzyme B-positive tumor-infiltrating CD8 T cells following DCZ and/or anti-PD-1 treatment. The average frequency and s.e.m. are shown (n=4 mice per group). Statistical significance was determined by two-way ANOVA. Statistical significance of survival data was calculated by the log-rank test.



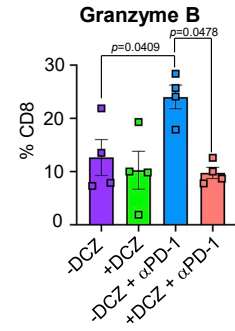
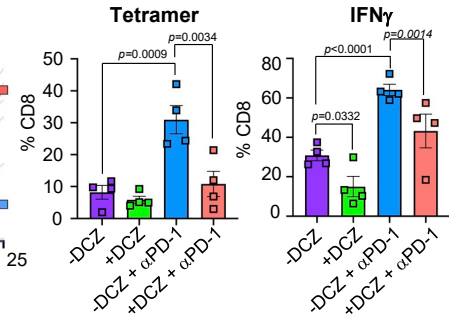
b 4MOSC1 – CD8-GsD (+tamoxifen)



d MC38-OVA – CD8-GsD (+tamoxifen)



f MC38-OVA – CD8-GsD (+tamoxifen)



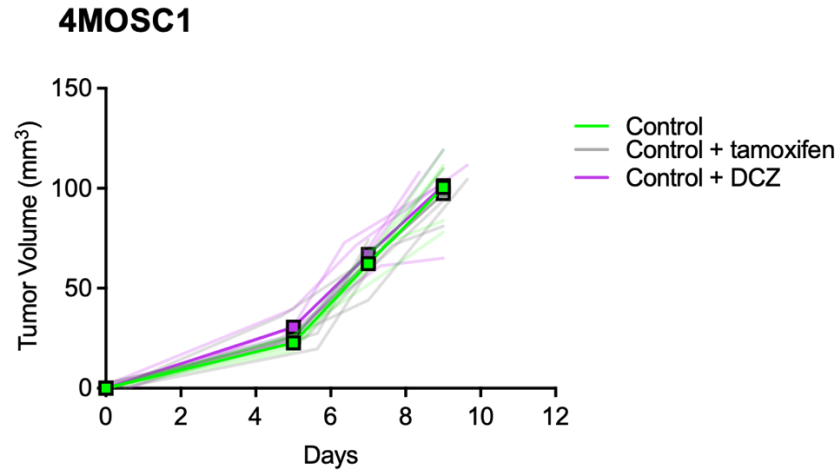


Figure 2.9: Effect of tamoxifen and DCZ on tumor growth. Tumor growth curve of CD8-GsD littermate control mice implanted with 4MOSC1 tumors treated with or without tamoxifen or DCZ. Mice were given 3 doses of tamoxifen, and 5×10^5 4MOSC1 cells were implanted into the tongue. Where indicated, 0.01 mg/kg DCZ was administered daily starting one day after tumor implantation.

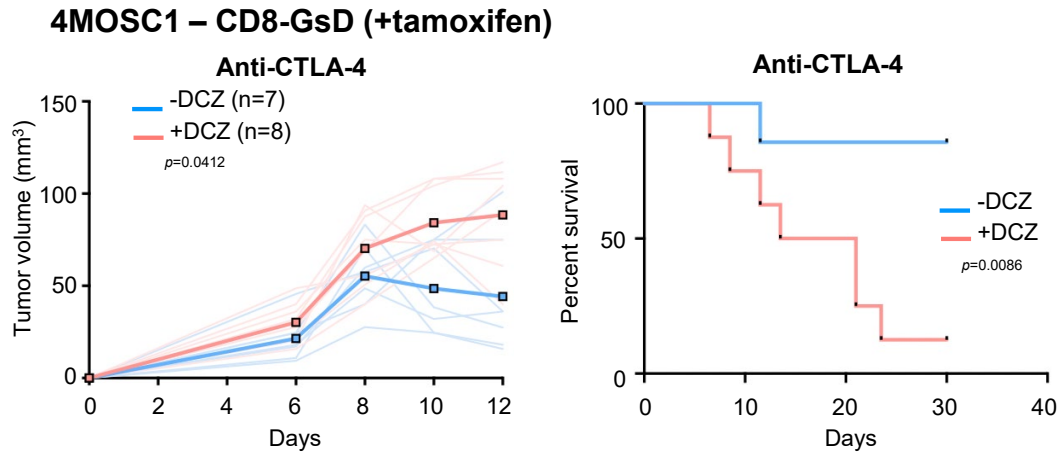
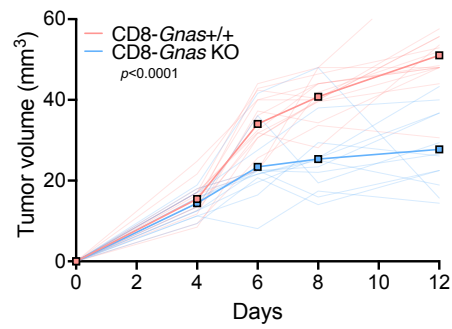


Figure 2.10: Anti-CTLA-4 response in CD8-GsD mice bearing 4MOSC1 tumors. Tumor growth curve (left panel) and survival plot (right panel) of CD8-GsD mice implanted with 4MOSC1 tumors treated with anti-CTLA-4 with or without DCZ (n=7 mice per group). Mice were given three doses of tamoxifen before orthotopic tumor implantation and treated with checkpoint inhibitors and DCZ as previously described. Statistical significance was determined by two-way ANOVA. Statistical significance of survival data was calculated by the log-rank test.

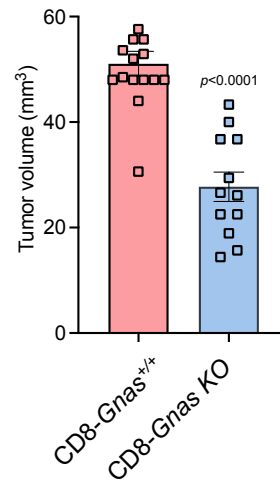
Figure 2.11: Effect of *Gnas* deletion CD8 T cells in mice bearing 4MOSC1 tumors. **a**, Tumor growth curve (left panel) and quantification of endpoint tumor volume (right panel) of CD8-*Gnas*^{+/+} (n=14 mice) and CD8-*Gnas*^{-/-} mice (n=12 mice) implanted with 4MOSC1 tumors. Mice were given 3 doses of tamoxifen prior to orthotopic tumor implantation. The average tumor volume and s.e.m. are shown. Statistical significance was determined by two-way ANOVA. **b**, Quantification of PD-1⁺TIGIT⁺ CD8 T cells in 4MOSC1 tumors and draining lymph nodes at endpoint. The average frequency and s.e.m. are shown (n=5 mice per group). Statistical significance was determined by two-tailed unpaired Student's *t*-test. **c**, Frequency of CD8⁺ T cells in 4MOSC1 tumors in CD8-*Gnas* KO mice versus littermate controls. The average frequency and s.e.m. are shown (n=5 mice per group). Statistical significance was determined by two-tailed unpaired Student's *t*-test.

a

4MOSC1

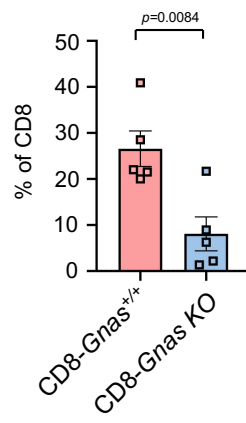


Day 12

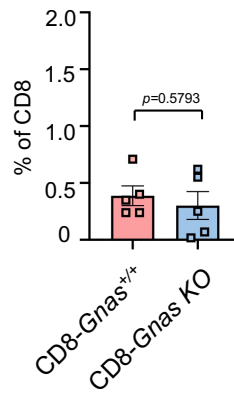


b

Tumor
PD-1+ TIGIT+



LN
PD-1+ TIGIT+



c

Tumor

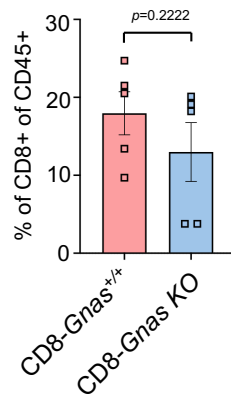
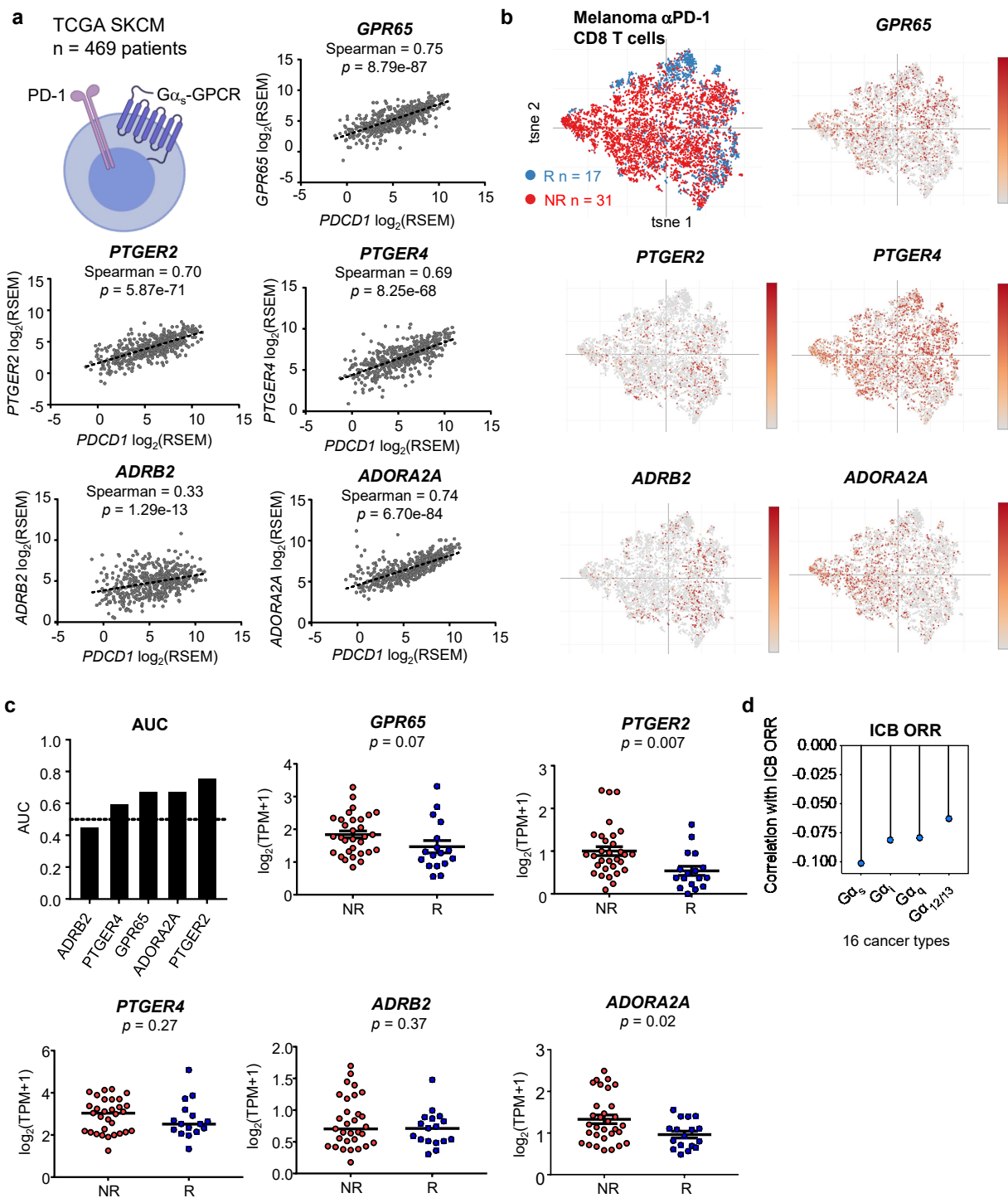
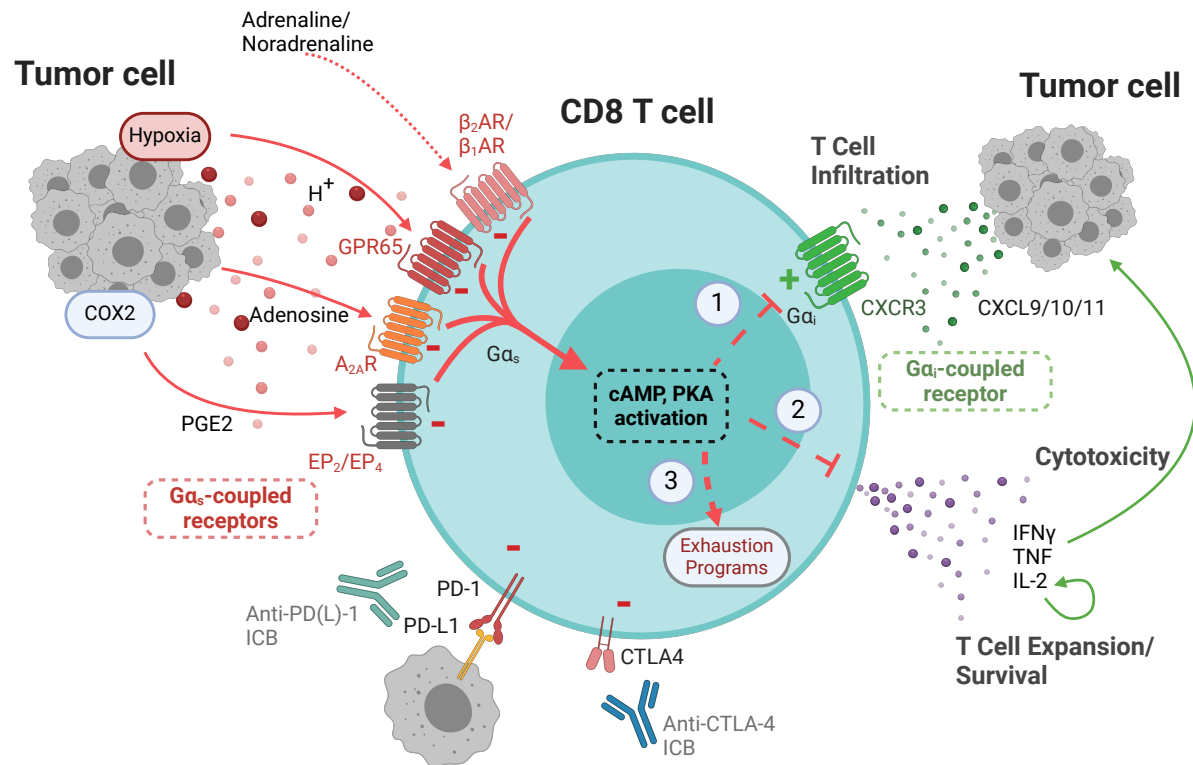


Figure 2.12: $G\alpha_s$ -GPCRs in cancer patients correlates with decreased survival and ICB response. **a**, Correlation of various $G\alpha_s$ -GPCRs to *PDCD1* in melanoma tumors from the TCGA Skin Cutaneous Melanoma cohort (n=469 patients). Spearman correlations and p-values are listed. This figure was generated, in part, with BioRender.com. **b**, t-distributed stochastic neighbor embedding (tsne) visualization of responders (R) and (NR) to immunotherapy in melanoma patients from GSE120575 (top left panel). Expression patterns of various $G\alpha_s$ -GPCRs are shown accordingly. **c**, Area under the curve (AUC) analysis of predictive power of $G\alpha_s$ -GPCRs in predictive response to immunotherapy (top left panel; n=469 patients). Expression of the GPCRs were calculated as $\log_2(\text{TPM}+1)$ and expression levels between R and NR was compared. Statistical significance was determined by two-tailed unpaired Student's *t*-test. The average frequency and *p*-values are shown. **d**, Predicted correlation of objective response rate (ORR) to ICB for each G protein coupling pathway across 16 cancer types.





Activation of Gα_s- coupled GPCRs on CD8 T cells:

1. inhibits infiltration into the TME
2. reduces effector functions
3. initiation of T cell exhaustion programs

Figure 2.13: Scheme illustrating the Gα_s-signaling axis as an immune checkpoint in cancer. Gα_s-GPCRs, like EP₂, EP₄, A₂AR, β₁AR, and β₂AR, expressed on CD8 T cells that ligands in the TME activates cAMP and PKA and augments T cell exhaustion-related programs and diminishes T cell proliferation, cytotoxicity, and infiltration into the tumor. These receptors may need to be blocked in combination with PD-1 and CTLA-4 to overcome T cell dysfunction and exhaustion. This figure was generated, in part, with BioRender.com.

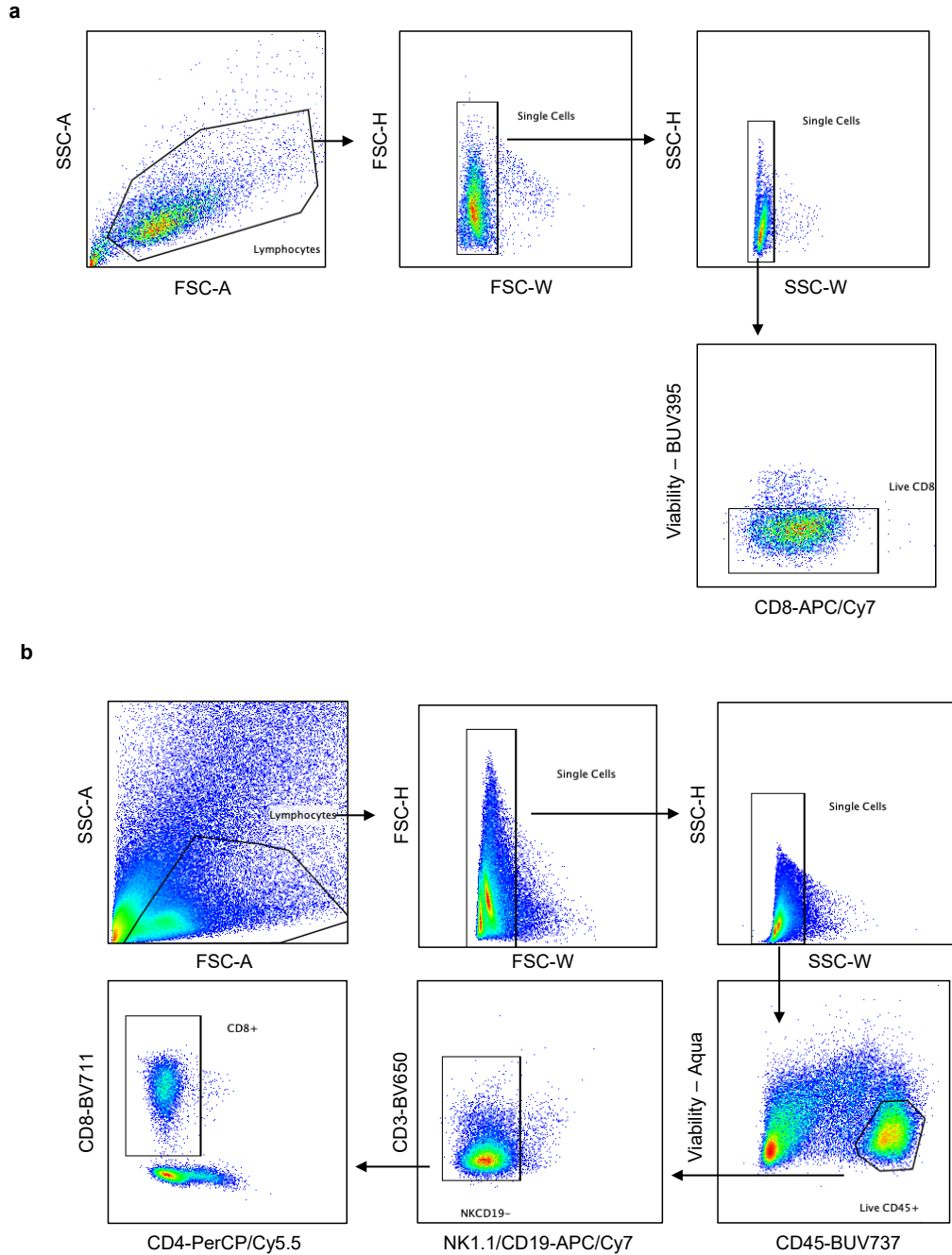


Figure 2.14: Gating strategy for chronic stimulation and in vivo experiments. **a**, For chronic stimulation experiments, lymphocytes were gated from forward scatter area (FSC-A) and side scatter area (SSC-A). Single cells were distinguished from doublet cells in forward scatter height (FSC-H) and forward scatter width (FSC-W), and then side scatter height (SSC-H) and side scatter width (SSC-W). Live CD8 cells were then gated. **b**, For in vivo experiments, lymphocytes were gated from forward scatter area (FSC-A) and side scatter area (SSC-A). Single cells were distinguished from doublet cells in forward scatter height (FSC-H) and forward scatter width (FSC-W), and then side scatter height (SSC-H) and side scatter width (SSC-W). Live CD45 cells were then gated. T cells were distinguished by NK1.1 negative, CD19 negative, and CD3 positive. CD8 T cells were then gated as CD4 negative.

Table 2.1a: Integrated dataset information and number of CD8 cells.

Cancer Type	Abbr.	PMID/GEO	# of Patients	Naïve	Proliferating	Effector Memory	Cytotoxic	Exhausted
<i>Acute Myeloid Leukemia</i>	AML	30827681	16	463	30	261	80	76
<i>Breast Cancer</i>	BC	29961579	3	2267	167	4634	1814	1584
<i>Breast Cancer</i>	BC	29961579	8	863	27	905	220	306
<i>Breast Cancer</i>	BC	29942092	2	307	94	377	393	279
<i>Breast Cancer</i>	BC	34653365	2	622	24	2359	726	560
<i>Basal Cell Carcinoma</i>	BCC	31359002	11	1740	215	4897	2029	1441
<i>B-cell Lymphoma</i>	BCL	23292937	2	960	76	1226	635	585
<i>Cutaneous Melanoma</i>	CM	30595452	24	1688	797	2114	1460	2147
<i>Cutaneous Melanoma</i>	CM	30388455	31	230	113	575	143	337
<i>Colorectal Cancer</i>	CRC	30479382	12	683	124	1316	792	623
<i>Colorectal Cancer</i>	CRC	30479382	6	1336	103	2630	1501	811
<i>Esophageal Cancer</i>	ESCA	34489433	7	1725	609	6391	2063	1738
<i>Fallopian Tube Carcinoma</i>	FTC	30383866	1	74	34	264	283	112
<i>Hepatocellular Carcinoma</i>	HCC	31675496	5	2308	212	4529	2862	1392
<i>Hepatocellular Carcinoma</i>	HCC	31675496	5	142	15	318	140	136
<i>Hepatocellular Carcinoma</i>	HCC	31675496	5	355	60	386	301	337
<i>Hepatocellular Carcinoma</i>	HCC	34216724	18	89	93	233	28	58
<i>Intrahepatic Cholangiocarcinoma</i>	ICC	31588021	1	34	7	77	123	59
<i>Multiple Myeloma</i>	MM	GSE141299	3	1332	360	3451	2209	1277
<i>Nasopharyngeal Carcinoma</i>	NPC	33531485	10	4131	1672	12077	5864	4929
<i>Lung Cancer</i>	NSCLC	31033233	4	124	6	505	170	44
<i>Lung Cancer</i>	NSCLC	30979687	7	222	81	569	117	214
<i>Lung Cancer</i>	NSCLC	29942094	15	817	101	1049	1351	743
<i>Ovarian Cancer</i>	OC	30383866	1	393	229	1306	1041	548
<i>Pancreatic Cancer</i>	PDAC	31273297	20	223	116	131	112	146
<i>Pancreatic Cancer</i>	PDAC	31932730	6	664	177	3230	915	971
<i>Renal Carcinoma</i>	RCC	34162837	5	595	94	1227	621	602
<i>Renal Carcinoma</i>	RCC	32103181	6	2268	720	6444	4562	2550
<i>Squamous Cell Carcinoma</i>	SCC	31359002	4	2141	337	4639	2175	1633
<i>Thyroid Carcinoma</i>	THCA	34663816	10	6360	531	15074	5810	5675
<i>Uterine Corpus Endometrial Carcinoma</i>	UCEC	32103181	9	2788	993	9610	2950	3585

Table 2.1b: Top 15 landmark genes for each CD8 subtype.

<i>Gene</i>	<i>p_val</i>	<i>avg_log2FC</i>	<i>cluster</i>
<i>HSPA1A</i>	0	2.533561467	Effector Memory
<i>HSPA1B</i>	0	2.230933969	Effector Memory
<i>HSPA6</i>	0	2.050751606	Effector Memory
<i>DNAJB1</i>	0	1.854314176	Effector Memory
<i>FOS</i>	0	1.740725227	Effector Memory
<i>FOSB</i>	0	1.33507599	Effector Memory
<i>DUSP1</i>	0	1.252101305	Effector Memory
<i>HSPH1</i>	0	1.222636427	Effector Memory
<i>HSP90AA1</i>	0	1.134874918	Effector Memory
<i>NR4A1</i>	0	1.123253261	Effector Memory
<i>LMNA</i>	0	1.111369416	Effector Memory
<i>PPP1R15A</i>	0	1.027595327	Effector Memory
<i>JUN</i>	0	0.991751032	Effector Memory
<i>HSPE1</i>	0	0.973700548	Effector Memory
<i>CD69</i>	0	0.963455447	Effector Memory
<i>SELL.1</i>	0	1.23897444	Naïve
<i>CCR7</i>	0	1.16347472	Naïve
<i>IL7R.1</i>	0	0.878039401	Naïve
<i>TXNIP.1</i>	0	0.823084477	Naïve
<i>LEF1</i>	0	0.795159638	Naïve
<i>TCF7</i>	0	0.789892309	Naïve
<i>LTB</i>	0	0.748669108	Naïve
<i>GIMAP7.1</i>	0	0.632345606	Naïve
<i>KLF2</i>	0	0.613851459	Naïve
<i>RPL34</i>	0	0.578260052	Naïve
<i>RPS6</i>	0	0.563198008	Naïve
<i>RPS8</i>	0	0.561461705	Naïve
<i>RPL31</i>	0	0.547433073	Naïve
<i>RPL32</i>	0	0.545994654	Naïve
<i>EEF1B2</i>	0	0.542728155	Naïve
<i>CXCL13.2</i>	0	1.620950967	Exhausted
<i>HLA-DRA.2</i>	0	1.218872548	Exhausted
<i>TNFRSF9.2</i>	0	1.144817679	Exhausted
<i>HLA-DRB1.2</i>	0	1.092589039	Exhausted
<i>CD74.2</i>	0	1.029335106	Exhausted
<i>HLA-DQA1.2</i>	0	1.019835142	Exhausted
<i>HAVCR2.2</i>	0	0.971055326	Exhausted
<i>CXCR6.1</i>	0	0.958608015	Exhausted
<i>KRT86</i>	0	0.954068486	Exhausted
<i>CTLA4.2</i>	0	0.920948786	Exhausted

Table 2.1b: Top 15 landmark genes for each CD8 subtype (continued).

<i>Gene</i>	<i>p_val</i>	<i>avg_log2FC</i>	<i>cluster</i>
<i>DUSP4.1</i>	0	0.91765913	Exhausted
<i>HLA-DRB5.2</i>	0	0.902553762	Exhausted
<i>TIGIT.2</i>	0	0.89589929	Exhausted
<i>VCAM1.2</i>	0	0.893825734	Exhausted
<i>LAG3.2</i>	0	0.858391983	Exhausted
<i>GNLY.3</i>	0	3.479790846	Cytotoxic
<i>FGFBP2.3</i>	0	2.268422808	Cytotoxic
<i>FCGR3A.3</i>	0	1.545373811	Cytotoxic
<i>GZMB.3</i>	0	1.222870063	Cytotoxic
<i>PRF1.2</i>	0	1.199893169	Cytotoxic
<i>KLRD1.3</i>	0	1.174208677	Cytotoxic
<i>GZMH.2</i>	0	1.159035966	Cytotoxic
<i>NKG7.3</i>	0	1.072501582	Cytotoxic
<i>IFI6.2</i>	0	0.977612202	Cytotoxic
<i>ISG15.2</i>	0	0.944073206	Cytotoxic
<i>TYROBP</i>	0	0.940553826	Cytotoxic
<i>CX3CR1</i>	0	0.92828352	Cytotoxic
<i>SPON2.2</i>	0	0.885798811	Cytotoxic
<i>ADGRG1.2</i>	0	0.839670186	Cytotoxic
<i>MX1.2</i>	0	0.792540599	Cytotoxic
<i>STMN1.3</i>	0	3.61866144	Proliferating
<i>TUBB.3</i>	0	3.128831025	Proliferating
<i>TUBA1B.3</i>	0	3.032249421	Proliferating
<i>TYMS</i>	0	2.790142539	Proliferating
<i>HIST1H4C.3</i>	0	2.691705046	Proliferating
<i>RRM2</i>	0	2.642782709	Proliferating
<i>MKI67</i>	0	2.500434033	Proliferating
<i>HMGB2.3</i>	0	2.425946088	Proliferating
<i>HMGN2.3</i>	0	2.408238764	Proliferating
<i>UBE2C</i>	0	2.333854386	Proliferating
<i>TOP2A</i>	0	2.255498383	Proliferating
<i>NUSAP1</i>	0	2.079144186	Proliferating
<i>CENPF</i>	0	2.036909931	Proliferating
<i>KIAA0101</i>	0	1.908186478	Proliferating
<i>DUT</i>	0	1.807422206	Proliferating

Table 2.1c: Top 100 GPCRs expressed in exhausted CD8s.

Gene	Naive	Proliferating	Cytotoxic	Effector Memory	Exhausted
<i>CXCR4</i>	16.1446	11.8699	17.5503	29.7444	21.8718
<i>CXCR6</i>	1.5211	1.7238	1.8720	2.2450	4.7955
<i>ADGRE5</i>	2.1609	2.4135	3.5619	3.6641	3.5959
<i>CXCR3</i>	1.6901	1.8067	1.4298	1.7051	1.8591
<i>GPR65</i>	1.2092	1.0019	2.1737	1.9492	1.7352
<i>PTGER4</i>	1.6045	1.0588	1.7689	2.6443	1.7348
<i>GPR171</i>	1.1646	0.7674	1.0760	1.2644	1.3221
<i>GPR183</i>	1.8273	0.6435	1.1811	2.6911	1.3089
<i>P2RY10</i>	0.8309	0.6755	0.7181	1.0264	1.0893
<i>F2R</i>	0.7230	0.7583	0.8821	0.8120	1.0015
<i>CCR5</i>	0.4733	0.5911	0.4482	0.4924	0.7824
<i>ADGRG1</i>	0.2637	0.3461	1.4488	0.2897	0.6909
<i>S1PR4</i>	0.8187	0.5967	1.0366	0.6208	0.5999
<i>CCR7</i>	3.0071	0.2925	0.3866	1.1454	0.4647
<i>GPR132</i>	0.5207	0.2742	0.5625	0.5988	0.4643
<i>P2RY8</i>	0.6456	0.2833	0.8114	0.6347	0.4424
<i>GPR18</i>	0.3582	0.2643	0.3320	0.4761	0.4231
<i>LPAR2</i>	0.3806	0.2231	0.3026	0.4248	0.3826
<i>GPR25</i>	0.1763	0.4677	0.2715	0.2365	0.3608
<i>ADORA2A</i>	0.2317	0.2447	0.1993	0.2388	0.3530
<i>ADRB2</i>	0.3290	0.1769	0.8779	0.4989	0.2777
<i>PTGER2</i>	0.6628	0.1653	0.8173	0.5388	0.2428
<i>CCR1</i>	0.1208	0.2668	0.2196	0.1211	0.2350
<i>CCRL2</i>	0.0972	0.1458	0.1231	0.1552	0.2218
<i>GPR68</i>	0.1480	0.1188	0.1723	0.1258	0.1987
<i>GPR35</i>	0.0983	0.0890	0.1504	0.2253	0.1649
<i>LPAR6</i>	0.2711	0.1169	0.2884	0.1722	0.1596
<i>LPAR5</i>	0.1239	0.1193	0.1341	0.1049	0.1512
<i>CCR6</i>	0.2569	0.0954	0.0896	0.4086	0.1508
<i>ADGRG5</i>	0.0743	0.0940	0.2382	0.0816	0.1473
<i>CCR4</i>	0.1748	0.0591	0.0414	0.1566	0.1329
<i>S1PR1</i>	0.7637	0.1268	0.7805	0.3715	0.1246
<i>PTGDR</i>	0.3299	0.0575	0.4215	0.2788	0.1192
<i>CCR2</i>	0.1163	0.0574	0.0822	0.1001	0.1190
<i>C3AR1</i>	0.0989	0.0474	0.1660	0.1387	0.1146
<i>P2RY11</i>	0.0849	0.1011	0.0744	0.0756	0.1145
<i>GPR174</i>	0.0802	0.0629	0.0705	0.0666	0.1071
<i>GPR34</i>	0.0370	0.0543	0.0338	0.0624	0.0781

Table 2.1c: Top 100 GPCRs expressed in exhausted CD8s (continued).

Gene	Naive	Proliferating	Cytotoxic	Effector Memory	Exhausted
<i>S1PR5</i>	0.1600	0.0536	0.9678	0.1690	0.0698
<i>OPN3</i>	0.0768	0.1255	0.0705	0.0715	0.0692
<i>CRHR1</i>	0.0711	0.0402	0.0522	0.0562	0.0590
<i>CXCR5</i>	0.0856	0.0177	0.0205	0.1057	0.0576
<i>GPR82</i>	0.0740	0.0492	0.0476	0.0706	0.0565
<i>FZD6</i>	0.0206	0.0605	0.0126	0.0179	0.0559
<i>CYSLTR1</i>	0.0642	0.0526	0.0345	0.0420	0.0557
<i>S1PR2</i>	0.0374	0.0279	0.0434	0.0504	0.0476
<i>NMUR1</i>	0.0553	0.0382	0.1430	0.0634	0.0469
<i>CX3CR1</i>	0.1291	0.0381	1.0191	0.0409	0.0462
<i>GPR15</i>	0.0578	0.0282	0.0333	0.0675	0.0404
<i>LTB4R</i>	0.0607	0.0540	0.0735	0.0510	0.0403
<i>LGR6</i>	0.0582	0.0087	0.1336	0.0820	0.0377
<i>LTB4R2</i>	0.0446	0.0377	0.0584	0.0505	0.0368
<i>FZD3</i>	0.0507	0.0268	0.0414	0.0385	0.0353
<i>TSHR</i>	0.0127	0.0316	0.0054	0.0120	0.0353
<i>ADRB1</i>	0.0123	0.0213	0.0221	0.0236	0.0344
<i>CCR8</i>	0.0129	0.0151	0.0050	0.0057	0.0297
<i>VIPR2</i>	0.0340	0.0132	0.0395	0.0615	0.0292
<i>ADGRE2</i>	0.0141	0.0203	0.0250	0.0296	0.0288
<i>F2RL2</i>	0.0243	0.0148	0.0328	0.0288	0.0283
<i>GPR141</i>	0.0095	0.0153	0.0857	0.0124	0.0271
<i>GIPR</i>	0.0217	0.0101	0.0220	0.0201	0.0271
<i>CELSR3</i>	0.0244	0.0334	0.0252	0.0161	0.0264
<i>GPR19</i>	0.0130	0.0622	0.0133	0.0122	0.0253
<i>GPR22</i>	0.0217	0.0090	0.0191	0.0133	0.0191
<i>P2RY1</i>	0.0035	0.0260	0.0046	0.0066	0.0171
<i>GPR146</i>	0.0165	0.0073	0.0118	0.0089	0.0147
<i>C5AR1</i>	0.0111	0.0105	0.0115	0.0120	0.0144
<i>GPR55</i>	0.0485	0.0069	0.0373	0.0278	0.0131
<i>ADGRE1</i>	0.0110	0.0088	0.0042	0.0044	0.0129
<i>HCRTR1</i>	0.0105	0.0102	0.0140	0.0067	0.0125
<i>OPRM1</i>	0.0166	0.0056	0.0113	0.0100	0.0119
<i>P2RY14</i>	0.0174	0.0090	0.0090	0.0272	0.0115
<i>CYSLTR2</i>	0.0142	0.0134	0.0130	0.0183	0.0094
<i>GALR2</i>	0.0038	0.0144	0.0025	0.0045	0.0092
<i>LGR4</i>	0.0019	0.0132	0.0071	0.0042	0.0092
<i>ADGRF3</i>	0.0082	0.0159	0.0082	0.0116	0.0089

Table 2.1c: Top 100 GPCRs expressed in exhausted CD8s (continued)

Gene	Naive	Proliferating	Cytotoxic	Effector Memory	Exhausted
<i>ADGRL1</i>	0.0219	0.0099	0.0248	0.0142	0.0088
<i>GABBR1</i>	0.0233	0.0064	0.0166	0.0150	0.0086
<i>MC1R</i>	0.0110	0.0060	0.0128	0.0085	0.0084
<i>GPR160</i>	0.0291	0.0101	0.0156	0.0151	0.0083
<i>PTAFR</i>	0.0069	0.0111	0.0053	0.0064	0.0081
<i>FPR1</i>	0.0062	0.0121	0.0061	0.0061	0.0071
<i>KISS1R</i>	0.0095	0.0050	0.0069	0.0128	0.0070
<i>CCR9</i>	0.0089	0.0024	0.0028	0.0088	0.0068
<i>FFAR3</i>	0.0017	0.0034	0.0010	0.0043	0.0067
<i>S1PR3</i>	0.0056	0.0164	0.0043	0.0058	0.0066
<i>CELSR2</i>	0.0093	0.0113	0.0135	0.0075	0.0064
<i>GNRHR2</i>	0.0062	0.0063	0.0033	0.0058	0.0062
<i>GPR63</i>	0.0066	0.0066	0.0067	0.0074	0.0062
<i>TAS2R14</i>	0.0057	0.0094	0.0061	0.0037	0.0060
<i>CNR2</i>	0.0183	0.0063	0.0116	0.0089	0.0058
<i>CCR10</i>	0.0109	0.0020	0.0046	0.0048	0.0058
<i>NTSR1</i>	0.0011	0.0029	0.0035	0.0026	0.0058
<i>ACKR3</i>	0.0067	0.0039	0.0083	0.0049	0.0056
<i>FZD1</i>	0.0013	0.0030	0.0008	0.0015	0.0054
<i>SSTR2</i>	0.0097	0.0133	0.0041	0.0050	0.0054

Table 2.1d: GPCR genes.

Symbol								
BAI1 (ADGRB1)	AGTR2	DRD4	GPR171	HRH1	MTNR1A	PTGFR	TAS2R50	FZD1
BAI2 (ADGRB2)	APLNR	DRD5	GPR173	HRH2	MTNR1B	PTGIR	TAS2R60	FZD10
BAI3 (ADGRB3)	AVPR1 A	EDNRA	GPR174	HRH3	NMBR	QRFPR	TAS2R7	FZD2
CD97 (ADGRE5)	AVPR1 B	EDNRB	GPR176	HRH4	NMUR1	RXFP1	TAS2R8	FZD3
CELSR1	AVPR2	F2R	GPR18	HTR1A	NMUR2	RXFP2	TAS2R9	FZD4
CELSR2 (EGFL2)	BDKRB 1	F2RL1	GPR182	HTR1B	NPBWR1	RXFP3	TBXA2R	FZD5
CELSR3 (EGFL3)	BDKRB 2	F2RL2	GPR183	HTR1D	NPBWR2	RXFP4	TRHR	FZD6
ELTD1 (ADGRL4)	BRS3	F2RL3	GPR19	HTR1E	NPFFR1	S1PR1	TSHR	FZD7
EMR1 (ADGRE1)	C3AR1	FFAR1	GPR20	HTR1F	NPFFR2	S1PR2	UTS2R	FZD8
EMR2 (ADGRE2)	C5AR1	FFAR2	GPR21	HTR2A	NPSR1	S1PR3	XCR1	FZD9
EMR3 (ADGRE3)	CCBP2	FFAR3	GPR22	HTR2B	NPY1R	S1PR4	ADCYAP1R 1	SMO
EMR4P (ADGRE4P)	CCKAR	FPR1	GPR25	HTR2C	NPY2R	S1PR5	CALCR	GPR10 7
GPR110 (ADGRF1)	CCKBR	FPR2	GPR26	HTR4	NPY5R	SSTR1	CALCRL	GPR13 7
GPR111 (ADGRF2)	CCR1	FPR3	GPR27	HTR5A	NPY6R	SSTR2	CRHR1	GPR14 3
GPR112 (ADGRG4)	CCR10	FSHR	GPR3	HTR6	NTSR1	SSTR3	CRHR2	GPR15 7
GPR113 (ADGRF3)	CCR2	GALR1	GPR31	HTR7	NTSR2	SSTR4	GCGR	OR51E 1
GPR114 (ADGRG5)	CCR3	GALR2	GPR32	KISS1R	OPN3	SSTR5	GHRHR	TPRA1
GPR115 (ADGRF4)	CCR4	GALR3	GPR34	LGR4	OPN4	SUCNR 1	GIPR	
GPR116 (ADGRF5)	CCR5	GHSR	GPR35	LGR5	OPN5	TAAR1	GLP1R	
GPR123 (ADGRA1)	CCR6	GNRHR	GPR37	LGR6	OPRD1	TAAR2	GLP2R	
GPR124 (ADGRA2)	CCR7	GNRHR2	GPR37L 1	LHCGR	OPRK1	TAAR5	PTH1R	
GPR125 (ADGRA3)	CCR8	GPBAR1	GPR39	LPAR1	OPRL1	TAAR6	PTH2R	
GPR126 (ADGRG6)	CCR9	GPBR	GPR4	LPAR2	OPRM1	TAAR8	SCTR	
GPR128 (ADGRG7)	CCRL1	GPR1	GPR42	LPAR3	OXER1	TAAR9	VIPR1	

Table 2.1d: GPCR genes (continued)

Symbol							
GPR133 (ADGRD1)	CCRL2	GPR101	GPR45	LPAR4	OXGR1	TACR1	VIPR2
GPR144 (ADGRD2)	CHRM1	GPR109A	GPR50	LPAR5	OXTR	TACR2	CASR
GPR56 (ADGRG1)	CHRM2	GPR109B	GPR52	LPAR6	P2RY1	TACR3	GABBR1
GPR64 (ADGRG2)	CHRM3	GPR119	GPR55	LTB4R	P2RY10	TAS2R1	GABBR2
GPR97 (ADGRG3)	CHRM4	GPR12	GPR6	LTB4R2	P2RY11	TAS2R10	GPR156
GPR98 (ADGRV1)	CHRM5	GPR120 (FFAR4)	GPR61	MAS1	P2RY12	TAS2R13	GPR158
LPHN1 (ADGRL1)	CMKLR1	GPR132	GPR62	MAS1L	P2RY13	TAS2R14	GPR179
LPHN2 (ADGRL2)	CNR1	GPR135	GPR63	MC1R	P2RY14	TAS2R16	GPRC5A
LPHN3 (ADGRL3)	CNR2	GPR139	GPR65	MC2R	P2RY2	TAS2R19	GPRC5B
ADORA1	CX3CR1	GPR141	GPR68	MC3R	P2RY4	TAS2R20	GPRC5C
ADORA2A	CXCR1	GPR142	GPR75	MC4R	P2RY6	TAS2R3	GPRC5D
ADORA2B	CXCR2	GPR146	GPR77	MC5R	P2RY8	TAS2R30	GPRC6A
ADORA3	CXCR3	GPR148	GPR78	MCHR1	PPYR1	TAS2R31	GRM1
ADRA1A	CXCR4	GPR149	GPR81	MCHR2	PRLHR	TAS2R38	GRM2
ADRA1B	CXCR5	GPR15	GPR82	MLNR	PROKR1	TAS2R39	GRM3
ADRA1D	CXCR6	GPR150	GPR83	MRGPRD	PROKR2	TAS2R4	GRM4
ADRA2A	CXCR7	GPR151	GPR84	MRGPRE	PTAFR	TAS2R40	GRM5
ADRA2B	CYSLTR1	GPR152	GPR85	MRGPRF	PTGDR2 (GPR44)	TAS2R41	GRM6
ADRA2C	CYSLTR2	GPR153	GPR87	MRGPRG	PTGDR	TAS2R42	GRM7
ADRB1	DARC	GPR160	GPR88	MRGPRX1	PTGER1	TAS2R43	GRM8
ADRB2	DRD1	GPR161	GRPR	MRGPRX2	PTGER2	TAS2R45	TAS1R1
ADRB3	DRD2	GPR162	HCRT1	MRGPRX3	PTGER3	TAS2R46	TAS1R2
AGTR1	DRD3	GPR17	HCRT2	MRGPRX4	PTGER4	TAS2R5	TAS1R3

Table 2.1e: Dysfunctional score gene module.

Gene set
HAVCR2
PDCD1
PTMS
FAM3C
IFNG
AKAP5
CD7
PHLDA1
ENTPD1
SNAP47
TNS3
CXCL13
RDH10
DGKH
KIR2DL4
LYST
MIR155HG
RAB27A
CSF1
CTLA4
TNFRSF9
CD27
CCL3
ITGAE
PAG1
TNFRSF1B
GALNT1
GBP2
MYO7A

Table 2.2a: Correlation of GPCRs to T cell dysfunction score (from GSE120575 Sade-Feldman, et al.)

GPCR	P-VALUE	SPEARMAN CORRELATION
CXCR6	0	0.773664785
GPR56	3.42E-08	0.726443769
CCRL2	1.15E-07	0.701910551
GIPR	1.46E-07	0.696699957
F2R	1.91E-07	0.690729483
P2RY1	9.61E-08	0.681676597
CCR5	7.82E-07	0.659248806
GPR171	1.65E-06	0.642531481
CXCR3	3.26E-06	0.62700825
CD97	3.71E-06	0.623968736
GPR107	1.01E-05	0.599978289
ADORA2A	1.79E-05	0.585757707
FZD6	2.80E-05	0.574142423
CCR1	7.96E-05	0.545484151
P2RY11	0.000195722	0.518779852
FFAR2	0.000361523	0.49379957
GPR114	0.000541283	0.485887972
GPR174	0.002293735	0.433239253
GPR137	0.002853574	0.424554928
TPRA1	0.00361604	0.414893617
GPR19	0.003731542	0.413590968
PTGER4	0.004779182	0.403169779
S1PR4	0.005670171	0.395788102
GPR65	0.006902243	0.387103778
CCR8	0.00803123	0.380264872
LPAR2	0.00868441	0.376682588
TSHR	0.009968122	0.368472481
GPR68	0.012296936	0.360290925
LPAR5	0.017546207	0.342705167
C3AR1	0.021038581	0.333369518
EMR2	0.022995284	0.328701693
CELSR3	0.024649447	0.325010855
CCR2	0.026562519	0.320994355
GPR113	0.036961438	0.302648719
P2RY10	0.039121083	0.299392097
LTB4R2	0.067463282	0.266391663

Table 2.2a: Correlation of GPCRs to T cell dysfunction score (from GSE120575 Sade-Feldman, et al.) (continued).

GPCR	P-VALUE	SPEARMAN CORRELATION
ADRA2B	0.071734968	0.262264999
EDNRB	0.072185825	0.261864242
GPR84	0.07496202	0.259439258
HRH1	0.079457824	0.255658457
CYSLTR2	0.10466158	0.237107807
P2RY2	0.113825439	0.231220877
GPR133	0.118749944	0.228205408
PTGER2	0.141255888	0.215371255
LTB4R	0.152822775	0.209400782
ADORA3	0.161504484	0.205328795
FPR2	0.201661947	0.187598417
P2RY6	0.20459093	0.186407557
FPR3	0.211741932	0.183549603
GPR25	0.216118356	0.181833691
ADORA2B	0.216829068	0.181557326
FPR1	0.218883082	0.180762133
ADRB1	0.219192514	0.180642789
LPAR6	0.223023049	0.178897091
GPR146	0.225028615	0.178413439
P2RY13	0.266427904	0.163635383
GPBAR1	0.313941506	0.148451706
C5AR1	0.340754916	0.140527044
GPR75	0.349369358	0.138063961
F2RL2	0.36406104	0.133630048
MC1R	0.367209084	0.132761615
SUCNR1	0.386860188	0.127753616
GPR126	0.458634665	0.109532916
CMKLR1	0.465597135	0.107852737
GPR132	0.502010667	0.099001303
CCR4	0.529212765	0.092813721
PTAFR	0.598469905	0.077724707
GPRC5B	0.606073196	0.076336421
CXCR4	0.623804675	0.072405558
GPR141	0.641799235	0.06887942
LPAR1	0.664914559	0.064144454
NMUR1	0.704771406	0.056123972
P2RY14	0.848103606	0.02833261

Table 2.2a: Correlation of GPCRs to T cell dysfunction score (from GSE120575 Sade-Feldman, et al.) (continued).

GPCR	P-VALUE	SPEARMAN CORRELATION
FZD3	0.885708511	0.021276596
CX3CR1	0.921814625	0.014546244
GPR97	0.942703168	0.010654502
CXCR2	0.943703585	0.01046817
FZD1	0.952559434	0.008819392
GPR82	0.98035165	0.003690838
EMR1	0.984809758	0.002822482
OPN3	0.988899136	0.002062583
GPR157	0.992403928	0.001411356
OPRL1	0.947866756	-0.009692929
GPR31	0.930495836	-0.012929572
GPR34	0.90024378	-0.018562744
CALCRL	0.896547908	-0.019273577
GPR35	0.847527475	-0.028441164
ADRB2	0.78639984	-0.040056448
P2RY12	0.691145329	-0.058846942
HRH2	0.655958207	-0.06578376
CCR9	0.57616804	-0.082725479
GPR42	0.535933898	-0.091566784
GPR18	0.521503925	-0.094550586
PTGIR	0.417967083	-0.119639562
PTGDR	0.415967224	-0.119843682
CCR10	0.39844968	-0.124687756
GPR22	0.364453643	-0.133521494
S1PR2	0.312638145	-0.148847615
FFAR3	0.302460051	-0.151976204
S1PR5	0.283918903	-0.15785474
CCKBR	0.193963394	-0.190787822
PTGDR2	0.112644508	-0.231958718
LGR6	0.103306894	-0.238011236
CYSLTR1	0.064861625	-0.268888406
GPR160	0.044980204	-0.291250543
P2RY8	0.015548723	-0.348784195
GPR183	0.007492982	-0.38341294
CNR1	0.00185255	-0.437923015
CELSR1	0.001051338	-0.458381958
CNR2	9.69E-05	-0.53983934

Table 2.2a: Correlation of GPCRs to T cell dysfunction score (from GSE120575 Sade-Feldman, et al.) (continued).

GPCR	P-VALUE	SPEARMAN CORRELATION
CCR6	9.06E-05	-0.541793313
GABBR1	8.06E-05	-0.545158489
CCR7	6.84E-05	-0.549826314
CXCR5	9.88E-06	-0.600629614
GPR15	2.10E-06	-0.624475548
S1PR1	1.09E-06	-0.651758576

Table 2.2b: Predicted GPCR coupling specificity.

GPCR	Coupling_type	GNAI3	GNA12	GNAQ	GNAS
<i>ADORA3</i>	IUPHAR	PC	-	-	-
<i>ADRA1A</i>	IUPHAR	-	SC	PC	-
<i>ADRA1B</i>	IUPHAR	-	-	PC	-
<i>ADRA1D</i>	IUPHAR	-	-	PC	-
<i>AGTR1</i>	IUPHAR	PC	-	PC	-
<i>AGTR2</i>	IUPHAR	PC	-	-	-
<i>APLNR</i>	IUPHAR	PC	-	-	-
<i>AVPR1A</i>	IUPHAR	-	-	PC	-
<i>AVPR1B</i>	IUPHAR	-	-	PC	-
<i>BAI1</i>	IUPHAR	-	-	-	-
<i>BDKRB1</i>	IUPHAR	PC	-	PC	-
<i>BRS3</i>	IUPHAR	-	-	PC	-
<i>C3AR1</i>	IUPHAR	-	-	-	-
<i>C5AR1</i>	IUPHAR	PC	-	SC	-
<i>CALCRL</i>	IUPHAR	-	-	-	-
<i>CASR</i>	IUPHAR	PC	PC	PC	-
<i>CCKBR</i>	IUPHAR	-	-	PC	-
<i>CCR1</i>	IUPHAR	PC	-	-	-
<i>CCR10</i>	IUPHAR	PC	-	-	-
<i>CCR2</i>	IUPHAR	PC	-	-	-
<i>CCR3</i>	IUPHAR	PC	-	-	-
<i>CCR4</i>	IUPHAR	PC	-	-	-
<i>CCR5</i>	IUPHAR	PC	-	-	-
<i>CCR6</i>	IUPHAR	PC	-	-	-
<i>CCR7</i>	IUPHAR	PC	-	-	-
<i>CCR8</i>	IUPHAR	PC	-	-	-
<i>CCR9</i>	IUPHAR	PC	-	-	-
<i>CHRM1</i>	IUPHAR	-	-	PC	-
<i>CHRM3</i>	IUPHAR	-	-	PC	-
<i>CHRM4</i>	IUPHAR	PC	-	-	-
<i>CHRM5</i>	IUPHAR	-	-	PC	-
<i>CMKLR1</i>	IUPHAR	PC	-	-	-
<i>CX3CR1</i>	IUPHAR	PC	-	-	-
<i>CXCR1</i>	IUPHAR	PC	-	SC	-
<i>CXCR2</i>	IUPHAR	PC	-	-	-
<i>CXCR3</i>	IUPHAR	PC	-	-	-
<i>CXCR4</i>	IUPHAR	PC	-	-	-
<i>CXCR5</i>	IUPHAR	PC	-	-	-

Table 2.2b: Predicted GPCR coupling specificity (continued)

GPCR	<i>Coupling_type</i>	<i>GNAI3</i>	<i>GNA12</i>	<i>GNAQ</i>	<i>GNAS</i>
<i>CXCR6</i>	IUPHAR	PC	-	-	-
<i>CYSLTR1</i>	IUPHAR	SC	-	PC	-
<i>CYSLTR2</i>	IUPHAR	SC	-	PC	-
<i>DRD2</i>	IUPHAR	PC	-	-	-
<i>DRD3</i>	IUPHAR	PC	-	-	-
<i>DRD4</i>	IUPHAR	PC	-	-	-
<i>EDNRA</i>	IUPHAR	-	-	PC	-
<i>F2RL1</i>	IUPHAR	-	-	-	-
<i>F2RL2</i>	IUPHAR	-	-	-	-
<i>F2RL3</i>	IUPHAR	-	-	-	-
<i>FFAR2</i>	IUPHAR	SC	-	PC	-
<i>FFAR3</i>	IUPHAR	PC	-	-	-
<i>FPR1</i>	IUPHAR	PC	-	-	-
<i>FPR2</i>	IUPHAR	PC	-	SC	-
<i>FPR3</i>	IUPHAR	PC	-	-	-
<i>FZD1</i>	IUPHAR	SC	-	SC	-
<i>FZD10</i>	IUPHAR	-	SC	-	-
<i>FZD2</i>	IUPHAR	PC	-	-	-
<i>FZD4</i>	IUPHAR	-	PC	-	-
<i>FZD5</i>	IUPHAR	-	-	-	-
<i>FZD6</i>	IUPHAR	PC	-	PC	-
<i>FZD8</i>	IUPHAR	-	-	-	-
<i>FZD9</i>	IUPHAR	SC	-	-	-
<i>GABBR1</i>	IUPHAR	-	-	-	-
<i>GABBR2</i>	IUPHAR	-	-	-	-
<i>GALR1</i>	IUPHAR	PC	-	-	-
<i>GALR2</i>	IUPHAR	SC	SC	PC	-
<i>GALR3</i>	IUPHAR	PC	-	-	-
<i>GHSR</i>	IUPHAR	SC	SC	PC	-
<i>GLP2R</i>	IUPHAR	-	-	-	-
<i>GNRHR</i>	IUPHAR	SC	-	PC	-
<i>GPR107</i>	IUPHAR	-	-	-	-
<i>GPR132</i>	IUPHAR	-	-	-	-
<i>GPR135</i>	IUPHAR	-	-	-	-
<i>GPR137</i>	IUPHAR	-	-	-	-
<i>GPR139</i>	IUPHAR	-	-	PC	-
<i>GPR143</i>	IUPHAR	-	-	-	-
<i>GPR156</i>	IUPHAR	-	-	-	-

Table 2.2b: Predicted GPCR coupling specificity (continued)

GPCR	Coupling_type	GNAI3	GNA12	GNAQ	GNAS
<i>GPR157</i>	IUPHAR	-	-	-	-
<i>GPR158</i>	IUPHAR	-	-	-	-
<i>GPR17</i>	IUPHAR	PC	-	SC	-
<i>GPR171</i>	IUPHAR	-	-	-	-
<i>GPR173</i>	IUPHAR	-	-	-	-
<i>GPR179</i>	IUPHAR	-	-	-	-
<i>GPR18</i>	IUPHAR	PC	-	PC	-
<i>GPR183</i>	IUPHAR	PC	-	-	-
<i>GPR20</i>	IUPHAR	PC	-	-	-
<i>GPR22</i>	IUPHAR	PC	-	-	-
<i>GPR27</i>	IUPHAR	-	-	PC	-
<i>GPR31</i>	IUPHAR	PC	-	-	-
<i>GPR34</i>	IUPHAR	PC	-	-	-
<i>GPR37</i>	IUPHAR	PC	-	-	-
<i>GPR37L1</i>	IUPHAR	PC	-	-	-
<i>GPR42</i>	IUPHAR	-	-	-	-
<i>GPR50</i>	IUPHAR	-	-	-	-
<i>GPR52</i>	IUPHAR	-	-	-	-
<i>GPR55</i>	IUPHAR	-	PC	PC	-
<i>GPR68</i>	IUPHAR	PC	-	PC	-
<i>GPR75</i>	IUPHAR	-	-	PC	-
<i>GPR84</i>	IUPHAR	PC	-	-	-
<i>GPRC5A</i>	IUPHAR	-	-	-	-
<i>GPRC5B</i>	IUPHAR	-	-	-	-
<i>GPRC5C</i>	IUPHAR	-	-	-	-
<i>GPRC5D</i>	IUPHAR	-	-	-	-
<i>GPRC6A</i>	IUPHAR	-	-	PC	-
<i>GRM2</i>	IUPHAR	PC	-	-	-
<i>GRM3</i>	IUPHAR	PC	-	-	-
<i>GRM4</i>	IUPHAR	PC	-	-	-
<i>GRM6</i>	IUPHAR	PC	-	-	-
<i>GRM7</i>	IUPHAR	PC	-	-	-
<i>GRM8</i>	IUPHAR	PC	-	-	-
<i>GRPR</i>	IUPHAR	-	-	PC	-
<i>HRH1</i>	IUPHAR	-	-	PC	-
<i>HRH3</i>	IUPHAR	PC	-	-	-
<i>HRH4</i>	IUPHAR	PC	-	-	-
<i>HTR1A</i>	IUPHAR	PC	-	-	-

Table 2.2b: Predicted GPCR coupling specificity (continued)

GPCR	Coupling_type	GNAI3	GNA12	GNAQ	GNAS
<i>HTR1B</i>	IUPHAR	PC	-	-	-
<i>HTR1D</i>	IUPHAR	PC	-	-	-
<i>HTR1E</i>	IUPHAR	PC	-	-	-
<i>HTR1F</i>	IUPHAR	PC	-	-	-
<i>HTR2A</i>	IUPHAR	SC	-	PC	-
<i>HTR2B</i>	IUPHAR	-	-	PC	-
<i>HTR2C</i>	IUPHAR	SC	-	PC	-
<i>HTR5A</i>	IUPHAR	PC	-	SC	-
<i>KISS1R</i>	IUPHAR	-	-	PC	-
<i>LGR4</i>	IUPHAR	-	-	-	-
<i>LPAR1</i>	IUPHAR	PC	PC	PC	-
<i>LPAR2</i>	IUPHAR	PC	PC	PC	-
<i>LPAR3</i>	IUPHAR	PC	-	PC	-
<i>LPAR5</i>	IUPHAR	-	PC	PC	-
<i>LTB4R</i>	IUPHAR	PC	-	PC	-
<i>LTB4R2</i>	IUPHAR	PC	-	SC	-
<i>MAS1</i>	IUPHAR	PC	-	PC	-
<i>MCHR2</i>	IUPHAR	-	-	PC	-
<i>MLNR</i>	IUPHAR	-	PC	PC	-
<i>MRGPRD</i>	IUPHAR	PC	-	PC	-
<i>MRGPRG</i>	IUPHAR	-	-	-	-
<i>MRGPRX1</i>	IUPHAR	-	-	PC	-
<i>MRGPRX2</i>	IUPHAR	PC	-	PC	-
<i>MRGPRX3</i>	IUPHAR	-	-	PC	-
<i>MRGPRX4</i>	IUPHAR	-	-	PC	-
<i>MTNR1A</i>	IUPHAR	PC	-	SC	-
<i>MTNR1B</i>	IUPHAR	PC	-	-	-
<i>NMBR</i>	IUPHAR	-	-	PC	-
<i>NMUR1</i>	IUPHAR	-	-	PC	-
<i>NMUR2</i>	IUPHAR	-	-	PC	-
<i>NPBWR1</i>	IUPHAR	PC	-	-	-
<i>NPBWR2</i>	IUPHAR	PC	-	-	-
<i>NPFFR1</i>	IUPHAR	PC	-	-	-
<i>NPFFR2</i>	IUPHAR	PC	-	-	-
<i>NPY1R</i>	IUPHAR	PC	-	-	-
<i>NPY2R</i>	IUPHAR	PC	-	SC	-
<i>NPY5R</i>	IUPHAR	PC	-	-	-
<i>NPY6R</i>	IUPHAR	-	-	-	-

Table 2.2b: Predicted GPCR coupling specificity (continued).

GPCR	Coupling_type	GNAI3	GNA12	GNAQ	GNAS
<i>NTSR2</i>	IUPHAR	-	-	PC	-
<i>OPN4</i>	IUPHAR	PC	-	PC	-
<i>OPN5</i>	IUPHAR	PC	-	-	-
<i>OPRD1</i>	IUPHAR	PC	-	-	-
<i>OPRK1</i>	IUPHAR	PC	SC	-	-
<i>OPRL1</i>	IUPHAR	PC	-	-	-
<i>OPRM1</i>	IUPHAR	PC	-	SC	-
<i>OR51E1</i>	IUPHAR	-	-	-	-
<i>OXER1</i>	IUPHAR	PC	-	-	-
<i>OXGR1</i>	IUPHAR	-	-	PC	-
<i>OXTR</i>	IUPHAR	SC	-	PC	-
<i>P2RY1</i>	IUPHAR	SC	-	PC	-
<i>P2RY13</i>	IUPHAR	PC	-	-	-
<i>P2RY14</i>	IUPHAR	PC	-	-	-
<i>P2RY2</i>	IUPHAR	SC	SC	PC	-
<i>P2RY4</i>	IUPHAR	-	-	PC	-
<i>P2RY6</i>	IUPHAR	-	SC	PC	-
<i>P2RY8</i>	IUPHAR	-	-	-	-
<i>PRLHR</i>	IUPHAR	-	-	-	-
<i>PTAFR</i>	IUPHAR	PC	-	PC	-
<i>PTGDR2</i>	IUPHAR	PC	-	-	-
<i>PTGER1</i>	IUPHAR	SC	-	PC	-
<i>PTGER3</i>	IUPHAR	PC	-	SC	-
<i>PTH2R</i>	IUPHAR	-	-	-	-
<i>QRFP3</i>	IUPHAR	PC	-	PC	-
<i>RXFP3</i>	IUPHAR	PC	-	-	-
<i>RXFP4</i>	IUPHAR	PC	-	-	-
<i>S1PR1</i>	IUPHAR	PC	-	-	-
<i>S1PR3</i>	IUPHAR	PC	PC	PC	-
<i>S1PR4</i>	IUPHAR	PC	PC	-	-
<i>S1PR5</i>	IUPHAR	PC	PC	-	-
<i>SCTR</i>	IUPHAR	-	-	-	-
<i>SMO</i>	IUPHAR	PC	PC	-	-
<i>SSTR1</i>	IUPHAR	PC	-	-	-
<i>SSTR2</i>	IUPHAR	PC	-	SC	-
<i>SSTR3</i>	IUPHAR	PC	-	SC	-
<i>SSTR4</i>	IUPHAR	PC	-	-	-
<i>SSTR5</i>	IUPHAR	PC	-	SC	-
<i>SUCNR1</i>	IUPHAR	PC	-	PC	-

Table 2.2b: Predicted GPCR coupling specificity (continued).

GPCR	Coupling_type	GNAI3	GNA12	GNAQ	GNAS
TAAR2	IUPHAR	-	-	-	-
TAAR5	IUPHAR	-	-	-	-
TAAR6	IUPHAR	-	-	-	-
TAAR8	IUPHAR	-	-	-	-
TAAR9	IUPHAR	-	-	-	-
TACR3	IUPHAR	-	-	PC	-
TAS1R1	IUPHAR	-	-	-	-
TAS1R2	IUPHAR	-	-	-	-
TAS1R3	IUPHAR	-	-	-	-
TAS2R1	IUPHAR	-	-	-	-
TAS2R10	IUPHAR	-	-	-	-
TAS2R13	IUPHAR	-	-	-	-
TAS2R14	IUPHAR	-	-	-	-
TAS2R16	IUPHAR	-	-	-	-
TAS2R19	IUPHAR	-	-	-	-
TAS2R20	IUPHAR	-	-	-	-
TAS2R3	IUPHAR	-	-	-	-
TAS2R30	IUPHAR	-	-	-	-
TAS2R31	IUPHAR	-	-	-	-
TAS2R38	IUPHAR	-	-	-	-
TAS2R39	IUPHAR	-	-	-	-
TAS2R4	IUPHAR	-	-	-	-
TAS2R40	IUPHAR	-	-	-	-
TAS2R41	IUPHAR	-	-	-	-
TAS2R42	IUPHAR	-	-	-	-
TAS2R43	IUPHAR	-	-	-	-
TAS2R45	IUPHAR	-	-	-	-
TAS2R46	IUPHAR	-	-	-	-
TAS2R5	IUPHAR	-	-	-	-
TAS2R50	IUPHAR	-	-	-	-
TAS2R60	IUPHAR	-	-	-	-
TAS2R7	IUPHAR	-	-	-	-
TAS2R8	IUPHAR	-	-	-	-
TAS2R9	IUPHAR	-	-	-	-
TBXA2R	IUPHAR	-	-	PC	-
TPRA1	IUPHAR	-	-	-	-
TRHR	IUPHAR	-	-	PC	-
UTS2R	IUPHAR	-	-	PC	-
XCR1	IUPHAR	PC	-	-	-

Table 2.2b: Predicted GPCR coupling specificity (continued).

GPCR	Coupling_type	GNAI3	GNA12	GNAQ	GNAS
<i>ADCYAP1R1</i>	IUPHAR	-	-	PC	PC
<i>ADORA2A</i>	IUPHAR	-	-	SC	PC
<i>ADORA2B</i>	IUPHAR	-	-	SC	PC
<i>ADRB1</i>	IUPHAR	SC	-	-	PC
<i>ADRB2</i>	IUPHAR	SC	-	-	PC
<i>ADRB3</i>	IUPHAR	SC	-	-	PC
<i>AVPR2</i>	IUPHAR	-	-	-	PC
<i>BDKRB2</i>	IUPHAR	PC	-	PC	PC
<i>CALCR</i>	IUPHAR	-	-	SC	PC
<i>CRHR1</i>	IUPHAR	-	-	SC	PC
<i>CRHR2</i>	IUPHAR	-	-	SC	PC
<i>DRD1</i>	IUPHAR	-	-	-	PC
<i>DRD5</i>	IUPHAR	-	-	-	PC
<i>EDNRB</i>	IUPHAR	PC	-	PC	PC
<i>FSHR</i>	IUPHAR	PC	-	PC	PC
<i>FZD3</i>	IUPHAR	-	-	-	PC
<i>FZD7</i>	IUPHAR	PC	-	-	PC
<i>GCGR</i>	IUPHAR	-	-	-	PC
<i>GHRHR</i>	IUPHAR	-	-	-	PC
<i>GIPR</i>	IUPHAR	-	-	-	PC
<i>GLP1R</i>	IUPHAR	-	-	-	PC
<i>GPBAR1</i>	IUPHAR	-	-	-	PC
<i>GPR101</i>	IUPHAR	-	-	-	PC
<i>GPR119</i>	IUPHAR	-	-	-	PC
<i>GPR12</i>	IUPHAR	PC	-	-	PC
<i>GPR26</i>	IUPHAR	-	-	-	PC
<i>GPR3</i>	IUPHAR	-	-	-	PC
<i>GPR4</i>	IUPHAR	PC	PC	PC	PC
<i>GPR6</i>	IUPHAR	PC	-	-	PC
<i>GPR65</i>	IUPHAR	-	-	-	PC
<i>GPR78</i>	IUPHAR	-	-	-	PC
<i>HCRTR1</i>	IUPHAR	PC	-	PC	PC
<i>HCRTR2</i>	IUPHAR	PC	-	PC	PC
<i>HTR4</i>	IUPHAR	-	SC	-	PC
<i>HTR6</i>	IUPHAR	-	-	SC	PC
<i>HTR7</i>	IUPHAR	-	-	-	PC
<i>LHCGR</i>	IUPHAR	-	-	SC	PC
<i>LPAR4</i>	IUPHAR	PC	PC	PC	PC
<i>LPAR6</i>	IUPHAR	PC	PC	-	PC

Table 2.2b: Predicted GPCR coupling specificity (continued).

GPCR	Coupling_type	GNAI3	GNA12	GNAQ	GNAS
<i>MC1R</i>	IUPHAR	-	-	-	PC
<i>MC2R</i>	IUPHAR	-	-	-	PC
<i>MC3R</i>	IUPHAR	-	-	-	PC
<i>MC4R</i>	IUPHAR	-	-	-	PC
<i>MC5R</i>	IUPHAR	-	-	-	PC
<i>MCHR1</i>	IUPHAR	PC	-	PC	PC
<i>PTGDR</i>	IUPHAR	-	-	-	PC
<i>PTGER2</i>	IUPHAR	-	-	-	PC
<i>PTGER4</i>	IUPHAR	SC	-	-	PC
<i>PTGIR</i>	IUPHAR	SC	-	SC	PC
<i>PTH1R</i>	IUPHAR	-	-	SC	PC
<i>RXFP1</i>	IUPHAR	PC	-	-	PC
<i>RXFP2</i>	IUPHAR	PC	-	-	PC
<i>S1PR2</i>	IUPHAR	-	PC	PC	PC
<i>TAAR1</i>	IUPHAR	-	-	SC	PC
<i>TACR1</i>	IUPHAR	-	-	PC	PC
<i>TACR2</i>	IUPHAR	-	-	PC	PC
<i>TSHR</i>	IUPHAR	-	-	SC	PC
<i>VIPR1</i>	IUPHAR	SC	-	SC	PC
<i>VIPR2</i>	IUPHAR	-	-	-	PC
<i>ADORA1</i>	IUPHAR	PC	-	SC	SC
<i>ADRA2A</i>	IUPHAR	PC	-	-	SC
<i>ADRA2B</i>	IUPHAR	PC	-	-	SC
<i>ADRA2C</i>	IUPHAR	PC	-	-	SC
<i>CCKAR</i>	IUPHAR	-	-	PC	SC
<i>CHRM2</i>	IUPHAR	PC	-	SC	SC
<i>CNR1</i>	IUPHAR	PC	-	-	SC
<i>CNR2</i>	IUPHAR	PC	-	-	SC
<i>FFAR1</i>	IUPHAR	SC	-	PC	SC
<i>GPR39</i>	IUPHAR	-	SC	PC	SC
<i>GRM1</i>	IUPHAR	SC	-	PC	SC
<i>GRM5</i>	IUPHAR	SC	-	PC	SC
<i>HRH2</i>	IUPHAR	-	-	PC	SC
<i>NPSR1</i>	IUPHAR	-	-	PC	SC
<i>NTSR1</i>	IUPHAR	SC	-	PC	SC
<i>P2RY11</i>	IUPHAR	-	-	PC	SC
<i>PROKR1</i>	IUPHAR	-	-	PC	SC
<i>PROKR2</i>	IUPHAR	SC	-	PC	SC
<i>PTGFR</i>	IUPHAR	-	-	PC	SC

Table 2.3a: LCMV exhausted versus effector CD8 T cells dataset information.

<i>Study</i>	# of effector samples	# of exhausted samples
GSE84820	4	2
GSE123235	0	5
GSE122969	3	3
GSE88987	2	2

Table 2.3b. Differential expression results for GPCRs in effector versus exhausted CD8 T cells from LCMV infection for Figure 2B.

GPCR	baseMean	log2FoldChange	lfcSE	stat	pvalue
<i>Ltb4r1</i>	23.5372	7.3664	0.6036	-12.2048	0.0000
<i>Ccr2</i>	1538.4428	3.8650	0.3339	-11.5741	0.0000
<i>Ptger2</i>	265.3204	-3.1795	0.3522	9.0278	0.0000
<i>P2ry12</i>	81.6635	7.0262	0.8029	-8.7515	0.0000
<i>Glp1r</i>	535.2234	-7.0283	0.8081	8.6970	0.0000
<i>Ccrl2</i>	363.5038	-3.4958	0.5534	6.3166	0.0000
<i>Cysltr2</i>	359.2163	-1.4898	0.2525	5.8997	0.0000
<i>Cxcr5</i>	553.5609	-4.5633	0.7799	5.8510	0.0000
<i>Gpr137</i>	77.2490	-1.6171	0.2869	5.6368	0.0000
<i>Ptger4</i>	1517.6854	-2.5698	0.4623	5.5588	0.0000
<i>F2rl3</i>	169.5287	1.4432	0.2667	-5.4118	0.0000
<i>S1pr4</i>	3005.5624	0.7673	0.1536	-4.9946	0.0000
<i>Gpr65</i>	545.4911	-1.4060	0.2937	4.7872	0.0000
<i>S1pr1</i>	2249.7681	1.6369	0.3782	-4.3286	0.0000
<i>Gpr25</i>	3.5845	4.9763	1.1518	-4.3203	0.0000
<i>Tpra1</i>	539.1148	-1.0653	0.2542	4.1905	0.0000
<i>Cx3cr1</i>	3770.9628	1.9416	0.4672	-4.1554	0.0000
<i>Gpr22</i>	6.6955	-4.7645	1.2387	3.8464	0.0001
<i>Gpr3</i>	6.5660	-6.1987	1.6432	3.7723	0.0002
<i>Lpar6</i>	304.4629	-0.7806	0.2261	3.4518	0.0006
<i>Gpr146</i>	91.4458	1.7370	0.5092	-3.4111	0.0006
<i>Lpar5</i>	319.1773	-0.7062	0.2120	3.3318	0.0009
<i>P2ry13</i>	32.5358	4.1042	1.2363	-3.3196	0.0009
<i>Gpr19</i>	48.4330	-1.1138	0.3410	3.2668	0.0011
<i>Ccr6</i>	179.4275	-4.5344	1.4418	3.1450	0.0017
<i>Celsr3</i>	10.5039	-3.2928	1.0735	3.0672	0.0022
<i>Oprm1</i>	7.3517	-2.6919	0.9002	2.9903	0.0028
<i>Tas1r3</i>	21.6005	-2.4505	0.8231	2.9770	0.0029
<i>Gpr161</i>	3.9443	-5.1444	1.7382	2.9596	0.0031
<i>Gpr63</i>	2.4020	-5.1606	1.7554	2.9398	0.0033
<i>Hrh1</i>	4.3318	-6.0005	2.0561	2.9183	0.0035
<i>Gprc5c</i>	5.2186	-4.2643	1.5568	2.7391	0.0062
<i>Mrgpre</i>	101.1292	-2.4437	0.8964	2.7260	0.0064
<i>Gpr87</i>	2.8890	2.9640	1.1347	-2.6122	0.0090
<i>Gpr62</i>	6.9019	-3.0475	1.1760	2.5915	0.0096
<i>F2rl1</i>	93.6205	-1.6027	0.6375	2.5139	0.0119

Table 2.3b: Differential expression results for GPCRs in effector versus exhausted CD8 T cells from LCMV infection for Figure 2B (continued).

GPCR	baseMean	log2FoldChange	lfcSE	stat	pvalue
<i>Nmur1</i>	2.9802	-5.4572	2.1840	2.4987	0.0125
<i>Cxcr2</i>	3.4922	4.9231	2.0103	-2.4489	0.0143
<i>Tbxa2r</i>	494.7445	-0.7858	0.3232	2.4313	0.0150
<i>Cxcr3</i>	4133.0170	-0.5021	0.2140	2.3466	0.0189
<i>Mc1r</i>	3.3056	-4.8883	2.1578	2.2654	0.0235
<i>Grm2</i>	1.5423	-4.4750	2.0219	2.2133	0.0269
<i>S1pr5</i>	815.4985	1.8455	0.8354	-2.2090	0.0272
<i>Htr2a</i>	1.5245	-4.4774	2.0449	2.1896	0.0286
<i>Vipr1</i>	16.3466	1.7029	0.7932	-2.1470	0.0318
<i>P2ry14</i>	39.4478	1.5288	0.7238	-2.1123	0.0347
<i>Gpr162</i>	2.8438	-3.0955	1.4766	2.0963	0.0361
<i>Grm5</i>	1.7289	-4.6526	2.2534	2.0647	0.0389
<i>Gpr171</i>	1027.2957	0.6628	0.3272	-2.0257	0.0428
<i>Ntsr1</i>	2.5098	-5.1937	2.5806	2.0126	0.0442
<i>Adrb2</i>	257.3619	-0.8085	0.4038	2.0023	0.0453
<i>Npy1r</i>	1.5967	-4.5400	2.2963	1.9771	0.0480
<i>Fzd4</i>	3.3494	-2.9702	1.5026	1.9767	0.0481
<i>Pth2r</i>	2.1605	-4.9811	2.5299	1.9689	0.0490
<i>Gpbar1</i>	5.4324	-4.1692	2.1219	1.9648	0.0494
<i>Gpr156</i>	1.4413	-4.3871	2.2716	1.9313	0.0534
<i>Gpr132</i>	568.6693	0.5704	0.2964	-1.9247	0.0543
<i>Tshr</i>	1.4928	-4.4347	2.3067	1.9225	0.0545
<i>Fzd9</i>	2.1682	-4.9764	2.6211	1.8986	0.0576
<i>Oprl1</i>	2.1348	-3.9571	2.1244	1.8627	0.0625
<i>Chrm4</i>	5.6080	2.0192	1.0862	-1.8589	0.0630
<i>P2ry2</i>	2.3000	-4.3624	2.3560	1.8516	0.0641
<i>Gpr26</i>	1.9022	-4.7985	2.6280	1.8259	0.0679
<i>Oprd1</i>	1.7614	-4.2592	2.3625	1.8028	0.0714
<i>Chrm1</i>	2.0775	-4.2176	2.3432	1.7999	0.0719
<i>Gpr141</i>	5.0984	2.7836	1.5603	-1.7840	0.0744
<i>Ptafr</i>	1.8526	2.6894	1.5280	-1.7601	0.0784
<i>Calcr1</i>	135.0970	0.9078	0.5160	-1.7593	0.0785
<i>Lgr6</i>	3.9094	-2.0151	1.1489	1.7540	0.0794
<i>Gpr151</i>	1.7144	-2.9625	1.7244	1.7180	0.0858
<i>Lgr5</i>	1.5524	-4.4999	2.6328	1.7091	0.0874
<i>Gipr</i>	1.5006	-4.4536	2.6112	1.7056	0.0881
<i>Adrb1</i>	69.7447	-1.4505	0.8668	1.6734	0.0943
<i>Mc5R</i>	1.3312	-4.2900	2.5642	1.6731	0.0943

Table 2.3b: Differential expression results for GPCRs in effector versus exhausted CD8 T cells from LCMV infection for Figure 2B (continued).

GPCR	baseMean	log2FoldChange	lfcSE	stat	pvalue
<i>Ccr4</i>	2.0391	-3.4966	2.1249	1.6456	0.0999
<i>C5Ar1</i>	1.3229	3.9169	2.4299	-1.6120	0.1070
<i>Bdkrb1</i>	2.9588	-3.4815	2.1609	1.6111	0.1072
<i>C3Ar1</i>	2.7589	-2.5007	1.5618	1.6011	0.1093
<i>Ccr5</i>	1461.5874	0.6788	0.4277	-1.5872	0.1125
<i>Gpr135</i>	1.9407	-2.1387	1.3849	1.5444	0.1225
<i>Sctr</i>	1.8743	-2.3620	1.5625	1.5116	0.1306
<i>Ffar1</i>	3.2965	-2.6923	1.8362	1.4662	0.1426
<i>Ccr8</i>	9.2811	-1.7463	1.1979	1.4578	0.1449
<i>Gpr21</i>	3.6513	-1.5155	1.0577	1.4329	0.1519
<i>Prokr2</i>	1.3537	-4.2945	3.0869	1.3912	0.1642
<i>Ccr7</i>	236.8923	-1.7194	1.2514	1.3741	0.1694
<i>Gpr45</i>	3.4807	-1.4786	1.0825	1.3659	0.1720
<i>Xcr1</i>	52.2076	0.9396	0.6973	-1.3476	0.1778
<i>Htr2B</i>	1.6903	-2.1383	1.7361	1.2316	0.2181
<i>Gpr107</i>	475.2301	0.2547	0.2121	-1.2010	0.2297
<i>Kiss1R</i>	4.0120	0.9626	0.8140	-1.1826	0.2370
<i>Galr2</i>	2.6296	-1.7196	1.5596	1.1026	0.2702
<i>Gpr182</i>	3.2474	-1.4577	1.3222	1.1025	0.2702
<i>Gpr83</i>	1.5488	-2.1386	1.9808	1.0797	0.2803
<i>Vipr2</i>	3.6617	-1.7742	1.6543	1.0725	0.2835
<i>Hrh2</i>	277.8349	-0.2906	0.2712	1.0715	0.2839
<i>Gpr157</i>	2.3711	-1.1549	1.0857	1.0637	0.2875
<i>Adora3</i>	2.3972	-1.7225	1.6201	1.0632	0.2877
<i>Cysltr1</i>	2.0606	-1.7129	1.6218	1.0562	0.2909
<i>Pth1R</i>	12.2183	-0.7270	0.7263	1.0009	0.3169
<i>Avpr2</i>	7.5608	1.1015	1.1350	-0.9704	0.3318
<i>Ccr1</i>	4.4961	1.4222	1.4848	-0.9578	0.3382
<i>F2R</i>	1780.8225	0.4096	0.4348	-0.9421	0.3461
<i>Opn3</i>	5.5939	1.1281	1.2041	-0.9369	0.3488
<i>Gpr35</i>	2.2193	1.0377	1.1722	-0.8852	0.3760
<i>Gpr68</i>	1155.4571	0.2132	0.2426	-0.8788	0.3795
<i>Lgr4</i>	3.2298	-0.9334	1.0886	0.8574	0.3912
<i>Gpr18</i>	926.8691	-0.2757	0.3500	0.7877	0.4309
<i>Cmklr1</i>	229.5789	0.7316	0.9358	-0.7818	0.4344
<i>Adora2A</i>	873.8334	-0.3342	0.4300	0.7771	0.4371
<i>Gpr4</i>	2.2899	-1.2625	1.6308	0.7742	0.4388

Table 2.3b: Differential expression results for GPCRs in effector versus exhausted CD8 T cells from LCMV infection for Figure 2B (continued).

GPCR	baseMean	log2FoldChange	lfcSE	stat	pvalue
<i>Cxcr6</i>	6442.5266	0.4800	0.6392	-0.7510	0.4527
<i>Gpr15</i>	3.1095	-1.1827	1.6281	0.7264	0.4676
<i>Chrm3</i>	1.6506	-0.8956	1.3524	0.6622	0.5078
<i>F2RI2</i>	281.5584	0.3398	0.5470	-0.6211	0.5345
<i>Ccr9</i>	41.7449	0.4313	0.7103	-0.6072	0.5437
<i>P2Ry10</i>	611.8933	-0.1243	0.2269	0.5477	0.5839
<i>Ptger1</i>	9.4884	-0.4614	0.8641	0.5340	0.5933
<i>Gpr179</i>	2.2228	-0.5242	1.0675	0.4910	0.6234
<i>Lpar2</i>	42.5827	-0.4780	0.9890	0.4833	0.6289
<i>S1Pr2</i>	293.8964	-0.2280	0.4961	0.4596	0.6458
<i>Gpr34</i>	12.4221	0.5779	1.2721	-0.4543	0.6496
<i>Ccr3</i>	7.9873	0.4425	1.0543	-0.4197	0.6747
<i>Gabbr1</i>	128.9964	0.2911	0.7669	-0.3796	0.7043
<i>Fzd5</i>	60.0803	0.3431	0.9257	-0.3707	0.7109
<i>Tas1R1</i>	3.5204	0.3255	1.1332	-0.2872	0.7739
<i>Cnr2</i>	5.8164	-0.3167	1.2660	0.2502	0.8025
<i>Gpr183</i>	499.5805	-0.1683	0.6789	0.2480	0.8042
<i>Cxcr4</i>	104.6638	-0.1703	0.6888	0.2472	0.8048
<i>Gpr174</i>	568.4135	0.0763	0.3195	-0.2389	0.8112
<i>Galr3</i>	8.3051	0.2344	1.1255	-0.2083	0.8350
<i>Gpr52</i>	3.2643	-0.2404	1.1962	0.2009	0.8407
<i>Gprc5A</i>	12.8005	0.2150	1.1014	-0.1952	0.8452
<i>Gpr153</i>	1.5852	-0.2925	1.6455	0.1778	0.8589
<i>Gpr55</i>	315.0789	0.0893	0.5090	-0.1755	0.8607
<i>Celsr2</i>	12.5753	0.1399	0.9770	-0.1432	0.8861
<i>Fzd6</i>	2.4511	0.1692	1.2121	-0.1396	0.8890
<i>Celsr1</i>	330.5655	-0.0424	0.6474	0.0654	0.9478
<i>Gpr160</i>	211.1207	0.0031	0.2934	-0.0105	0.9917
<i>Oprk1</i>	0.8288	-2.8701	1.8386	1.5610	0.1185
<i>Npbwr1</i>	1.0576	-3.9369	3.2696	1.2041	0.2285
<i>Fzd7</i>	0.7610	-2.7095	2.7369	0.9900	0.3222
<i>Gpr1</i>	0.4832	-2.8896	3.2850	0.8796	0.3791
<i>Cxcr1</i>	1.0150	-3.8621	3.1608	1.2219	0.2218
<i>Htr5B</i>	0.5540	-2.1885	3.2744	0.6684	0.5039
<i>Adora1</i>	0.4111	-2.6063	3.2862	0.7931	0.4277
<i>Chrm5</i>	0.6646	-2.8896	3.2850	0.8796	0.3791
<i>Gpr176</i>	0.4699	-1.9320	3.2760	0.5898	0.5553
<i>Adra2B</i>	0.6687	-2.4879	3.2723	0.7603	0.4471

Table 2.3b: Differential expression results for GPCRs in effector versus exhausted CD8 T cells from LCMV infection for Figure 2B (continued).

GPCR	baseMean	log2FoldChange	lfcSE	stat	pvalue
<i>Sstr4</i>	0.7888	-3.5098	3.2772	1.0710	0.2842
<i>Mc3R</i>	1.0720	-3.9625	3.1112	1.2736	0.2028
<i>Ghsr</i>	0.9846	-2.8947	3.2588	0.8883	0.3744
<i>Qrfpr</i>	0.8390	-3.5886	2.7238	1.3175	0.1877
<i>P2Ry1</i>	0.9399	-0.4056	1.8467	0.2196	0.8262
<i>Gpr149</i>	0.6646	-2.8896	3.2850	0.8796	0.3791
<i>Rxfp1</i>	0.4470	-2.6028	2.8551	0.9116	0.3620
<i>Rxfp4</i>	1.1593	-4.0697	3.2676	1.2454	0.2130
<i>Gpr61</i>	0.8038	-2.5197	3.2718	0.7701	0.4412
<i>Tacr3</i>	0.8660	-3.6469	3.2745	1.1137	0.2654
<i>Ptgfr</i>	0.9734	-3.8231	3.2714	1.1686	0.2426
<i>Cnr1</i>	0.6227	-3.2017	3.2827	0.9753	0.3294
<i>Gabbr2</i>	0.8737	-2.9565	2.7402	1.0789	0.2806
<i>Htr1D</i>	0.7898	-0.6269	2.3119	0.2712	0.7863
<i>Htr6</i>	0.4050	-2.0801	2.8998	0.7173	0.4732
<i>Cckar</i>	0.6454	-3.1903	3.2829	0.9718	0.3312
<i>Fzd10</i>	1.1739	-1.6063	2.0670	0.7771	0.4371
<i>Rxfp2</i>	0.8439	-3.6121	3.2049	1.1270	0.2597
<i>Calcr</i>	0.6317	-2.9986	2.7419	1.0936	0.2741
<i>Gpr85</i>	0.5450	0.2386	2.3940	-0.0996	0.9206
<i>Grm8</i>	1.0264	-3.8959	3.1456	1.2385	0.2155
<i>Opn1Sw</i>	1.0109	-1.5671	1.9561	0.8011	0.4231
<i>Smo</i>	0.5602	-0.0509	1.5462	0.0329	0.9738
<i>Chrm2</i>	0.5467	-2.8896	3.2850	0.8796	0.3791
<i>Crhr2</i>	0.7679	-3.4627	3.2781	1.0563	0.2908
<i>Ghrhr</i>	0.6227	-3.2017	3.2827	0.9753	0.3294
<i>Adcyap1R1</i>	0.9860	-3.8333	3.1611	1.2126	0.2253
<i>Tacr1</i>	0.2801	-1.4654	3.2723	0.4478	0.6543
<i>Prokr1</i>	1.0568	0.2816	2.1556	-0.1307	0.8961
<i>Oxtr</i>	0.6289	-3.1966	3.2828	0.9737	0.3302
<i>Rho</i>	0.9510	-3.7838	3.2721	1.1564	0.2475
<i>Gprc5D</i>	0.7035	-3.3553	3.2800	1.0229	0.3063
<i>C5Ar2</i>	1.2575	-3.1548	2.1386	1.4752	0.1402
<i>Ptgir</i>	1.1908	2.3605	1.6111	-1.4652	0.1429
<i>Ffar2</i>	0.6868	0.3509	1.8377	-0.1909	0.8486
<i>P2Ry6</i>	0.9028	0.7133	1.8361	-0.3885	0.6976
<i>Cckbr</i>	0.9060	-3.7170	3.2732	1.1356	0.2561
<i>Gprc5B</i>	0.8483	-2.9228	2.2842	1.2796	0.2007

Table 2.3b: Differential expression results for GPCRs in effector versus exhausted CD8 T cells from LCMV infection for Figure 2B (continued).

GPCR	baseMean	log2FoldChange	lfcSE	stat	pvalue
<i>Mrgprg</i>	0.6096	-2.8896	3.2850	0.8796	0.3791
<i>Mrgprf</i>	0.6123	-3.1847	3.2830	0.9701	0.3320
<i>Ednra</i>	0.9791	-3.8236	3.1677	1.2071	0.2274
<i>Hcctr2</i>	0.4841	-2.8896	3.2850	0.8796	0.3791
<i>Ackr4</i>	1.2482	-2.2479	1.8546	1.2121	0.2255
<i>Grm1</i>	0.5907	-3.1206	3.2837	0.9503	0.3419
<i>Nmbr</i>	1.2168	-1.8179	1.6001	1.1361	0.2559
<i>Gpr6</i>	0.8059	-3.5389	3.2766	1.0801	0.2801
<i>Gprc6A</i>	0.0000	0.0000	0.0000	0.0000	1.0000
<i>Grm6</i>	0.4056	-2.6063	3.2862	0.7931	0.4277
<i>Adora2B</i>	0.5889	-0.5455	2.9067	0.1877	0.8511
<i>Glp2R</i>	0.9382	-3.7663	2.6906	1.3998	0.1616
<i>Ccr10</i>	0.9991	-0.3084	2.3098	0.1335	0.8938
<i>Crhr1</i>	0.3978	-1.6230	3.2771	0.4952	0.6204
<i>Gcgr</i>	0.4382	-2.6063	3.2862	0.7931	0.4277
<i>Uts2R</i>	0.4404	-2.6063	3.2862	0.7931	0.4277
<i>Gpr33</i>	0.8375	-1.1928	2.8562	0.4176	0.6762
<i>Agtr1A</i>	0.4873	-2.6063	3.2862	0.7931	0.4277
<i>S1Pr3</i>	0.7856	-2.4298	2.0881	1.1636	0.2446
<i>Ltb4R2</i>	0.3714	-0.4596	2.5031	0.1836	0.8543
<i>Fzd3</i>	0.4356	-0.9321	2.0963	0.4446	0.6566
<i>Adra1A</i>	0.8445	-3.6137	2.7198	1.3287	0.1840
<i>Ednrb</i>	0.7833	-3.5006	3.2773	1.0681	0.2855
<i>Trhr</i>	0.7971	0.1885	3.2290	-0.0584	0.9534
<i>Gpr20</i>	0.8387	-3.5930	3.2755	1.0969	0.2727
<i>Sstr3</i>	0.2519	-0.6588	3.1756	0.2075	0.8356
<i>Gpr84</i>	0.4933	-2.8896	3.2850	0.8796	0.3791
<i>Casr</i>	1.0077	-3.8727	2.6862	1.4417	0.1494
<i>Drd3</i>	0.5390	-2.8896	3.2850	0.8796	0.3791
<i>Mas1</i>	1.1350	-2.7634	2.7842	0.9925	0.3209
<i>Fpr1</i>	0.6089	2.6028	2.3040	-1.1297	0.2586
<i>Fpr2</i>	1.0943	3.3586	2.5620	-1.3109	0.1899
<i>Fpr3</i>	0.4636	-2.6063	3.2862	0.7931	0.4277
<i>Sstr5</i>	0.2604	0.1700	3.2688	-0.0520	0.9585
<i>Lhcgr</i>	0.9101	-3.7022	3.1927	1.1596	0.2462
<i>Fshr</i>	0.7011	-3.3514	3.2801	1.0217	0.3069
<i>Fzd8</i>	0.5582	-2.1885	3.2744	0.6684	0.5039
<i>Hrh4</i>	0.2192	0.6321	3.2594	-0.1939	0.8462

Table 2.3b: Differential expression results for GPCRs in effector versus exhausted CD8 T cells from LCMV infection for Figure 2B (continued).

GPCR	baseMean	log2FoldChange	lfcSE	stat	pvalue
<i>Htr4</i>	0.9007	-3.2883	2.3638	1.3911	0.1642
<i>Gpr152</i>	0.5400	-2.9803	2.7991	1.0647	0.2870
<i>Htr7</i>	0.2997	1.6030	3.2348	-0.4955	0.6202
<i>Ffar4</i>	1.2016	-1.5793	2.8447	0.5552	0.5788
<i>Adra2A</i>	0.4881	-1.9084	2.8765	0.6635	0.5070
<i>Brs3</i>	0.8074	-3.5415	3.2765	1.0809	0.2797
<i>Opn1Mw</i>	0.7439	-2.7043	3.2687	0.8273	0.4081

Table 2.3c: GSEA results for enrichment of upregulated GPCRs in exhausted CD8 T cells for Figure 2C, D.

<i>Description</i>	<i>Enrichment Score</i>	<i>NES</i>	<i>pvalue</i>	<i>qvalues</i>	<i>rank</i>
<i>Gs_iuphar_all</i>	-0.4666	-1.5612	0.0056	0.3596	102
<i>GNA14_predicted_09_and_shedding</i>	-0.3897	-1.3524	0.0383	0.5384	65
<i>Gq/G11_iuphar_primary</i>	-0.4128	-1.3770	0.0452	0.5384	93
<i>GNAQ_predicted_09</i>	-0.4771	-1.4155	0.0491	0.5384	53
<i>Gs_iuphar_primary</i>	-0.4259	-1.3672	0.0521	0.5384	72
<i>GNA14_predicted_05</i>	-0.3786	-1.3139	0.0573	0.5384	87
<i>GNA13_predicted_05_and_shedding</i>	-0.3774	-1.3091	0.0645	0.5384	63
<i>GNAS_predicted_09_and_shedding</i>	-0.3854	-1.3125	0.0671	0.5384	71
<i>Gs_iuphar_secondary</i>	-0.5150	-1.3901	0.0774	0.5519	102
<i>GNA13_predicted_05</i>	-0.3665	-1.2683	0.0947	0.6078	63
<i>GNA14_predicted_05_and_shedding</i>	-0.3612	-1.2524	0.1092	0.6107	65
<i>GNA14_predicted_09</i>	-0.3770	-1.2630	0.1163	0.6107	85
<i>GNAL_predicted_09_and_shedding</i>	-0.3620	-1.2322	0.1331	0.6107	83
<i>GNAZ_predicted_05</i>	-0.3516	-1.2063	0.1596	0.6107	118
<i>Gq/G11_iuphar_all</i>	-0.3544	-1.2063	0.1636	0.6107	93
<i>GNAL_predicted_05_and_shedding</i>	-0.3422	-1.1878	0.1675	0.6107	80
<i>GNAS_predicted_05_and_shedding</i>	-0.3414	-1.1846	0.1771	0.6107	80
<i>GNAS_predicted_09</i>	-0.3728	-1.2138	0.1801	0.6107	71
<i>GNAL_predicted_09</i>	-0.3656	-1.2153	0.1825	0.6107	83
<i>Gio_iuphar_secondary</i>	-0.4236	-1.2243	0.1902	0.6107	25
<i>GNAQ_predicted_09_and_shedding</i>	-0.3487	-1.1707	0.2108	0.6447	66
<i>GNAS_predicted_05</i>	-0.3309	-1.1485	0.2239	0.6535	80
<i>GNA14_shedding_assay_coupled</i>	-0.3463	-1.1601	0.2431	0.6695	65
<i>GNAQ_predicted_05</i>	-0.3274	-1.1279	0.2520	0.6695	56
<i>GNAL_predicted_05</i>	-0.3227	-1.1200	0.2611	0.6695	80
<i>GNAQ_predicted_05_and_shedding</i>	-0.3218	-1.1167	0.2711	0.6695	66
<i>GNAL_shedding_assay_coupled</i>	-0.3574	-1.1315	0.2899	0.6894	67
<i>GNAS_shedding_assay_coupled</i>	-0.3430	-1.1109	0.3100	0.7110	67
<i>GNAQ_shedding_assay_coupled</i>	-0.3322	-1.0964	0.3316	0.7300	65
<i>GNAZ_predicted_05_and_shedding</i>	-0.3113	-1.0760	0.3411	0.7300	118
<i>GNAI1_predicted_09</i>	-0.3183	-1.0737	0.3747	0.7762	88
<i>GNAZ_predicted_09</i>	-0.3446	-1.0564	0.3977	0.7980	118
<i>GNA15_shedding_assay_coupled</i>	-0.3436	-1.0359	0.4236	0.8242	81
<i>GNAI3_predicted_05_and_shedding</i>	-0.2820	-0.9720	0.5240	0.9879	66
<i>GNAO1_predicted_09</i>	0.2202	0.9617	0.5741	0.9879	5
<i>GNAI3_predicted_05</i>	-0.2738	-0.9443	0.5800	0.9879	66

Table 2.3c: GSEA results for enrichment of upregulated GPCRs in exhausted CD8 T cells for Figure 2C, D (continued).

<i>Description</i>	<i>Enrichment Score</i>	<i>NES</i>	<i>pvalue</i>	<i>qvalues</i>	<i>rank</i>
<i>GNAZ_predicted_09_and_shedding</i>	-0.2751	-0.9302	0.6190	0.9879	118
<i>GNAI1_predicted_09_and_shedding</i>	-0.2664	-0.9241	0.6529	0.9879	99
<i>Gio_iuphar_all</i>	-0.2598	-0.9012	0.6942	0.9879	99
<i>GNAI1_predicted_05</i>	-0.2484	-0.8629	0.7530	0.9879	121
<i>G12/G13_iuphar_all</i>	-0.2787	-0.7975	0.7738	0.9879	87
<i>GNA12_shedding_assay_coupled</i>	-0.2551	-0.7939	0.7954	0.9879	65
<i>GNA12_predicted_09_and_shedding</i>	-0.2551	-0.7939	0.7954	0.9879	65
<i>GNAZ_shedding_assay_coupled</i>	-0.2417	-0.7870	0.8219	0.9879	117
<i>GNAO1_predicted_05</i>	-0.2295	-0.7856	0.8413	0.9879	105
<i>GNA13_shedding_assay_coupled</i>	-0.2415	-0.7586	0.8592	0.9879	63
<i>Gio_iuphar_primary</i>	-0.2192	-0.7503	0.8847	0.9879	98
<i>Gq/G11_iuphar_secondary</i>	0.1880	0.7408	0.8966	0.9879	18
<i>GNAI3_predicted_09</i>	-0.2229	-0.7001	0.9056	0.9879	92
<i>GNAI1_predicted_05_and_shedding</i>	-0.2124	-0.7350	0.9157	0.9879	121
<i>GNA13_predicted_09_and_shedding</i>	-0.2105	-0.6854	0.9234	0.9879	121
<i>GNAO1_shedding_assay_coupled</i>	-0.2040	-0.6715	0.9298	0.9879	105
<i>GNAO1_predicted_05_and_shedding</i>	-0.2027	-0.6997	0.9354	0.9879	105
<i>GNA12_predicted_05_and_shedding</i>	-0.1958	-0.6784	0.9497	0.9879	66
<i>GNA15_predicted_09</i>	0.1461	0.7433	0.9545	0.9879	23
<i>GNAI3_predicted_09_and_shedding</i>	-0.1881	-0.6459	0.9646	0.9879	93
<i>GNA13_predicted_09</i>	-0.1946	-0.5624	0.9663	0.9879	50
<i>GNA15_predicted_05_and_shedding</i>	-0.1869	-0.6401	0.9676	0.9879	88
<i>GNA12_predicted_05</i>	-0.1787	-0.6195	0.9729	0.9879	66
<i>GNAI1_shedding_assay_coupled</i>	-0.1837	-0.6236	0.9745	0.9879	105
<i>GNA15_predicted_09_and_shedding</i>	-0.1602	-0.5375	0.9888	0.9879	56
<i>GNA15_predicted_05</i>	-0.1646	-0.5628	0.9889	0.9879	88
<i>GNAI3_shedding_assay_coupled</i>	-0.1694	-0.5751	0.9898	0.9879	93
<i>GNAO1_predicted_09_and_shedding</i>	-0.1491	-0.5008	0.9929	0.9879	117
<i>G12/G13_iuphar_primary</i>	0.1553	0.5236	1.0000	0.9879	197

Table 2.4: Genotyping, primer, and PCR information for CD8-Gnas KO mice.

Transgene	Tm	Sequence	Band sizes
Rosa26_LSL_HA-GsDREADD-GFP			
Olg-Rosa-9F	56.9	CTCGAAGTACTCGGCGTAGG	Knock in=206bp
Olg-Rosa-9R	55.3	CTTGGCAATCCGGTACTGTT	
gt-tomato-wt-F	58.2	AAGGGAGCTGCAGTGGAGTA	WT= 297bp
gt-tomato-wt-R	53.5	CCGAAAATCTGTGGGAAGTC	
Rosa26_E8i-Cre-ERT2-IRES-GFP			
E8i-Cre-IC-1	62	CTAGGCCACAGAATTGAAAGATCT	IC = 324bp
E8i-Cre-IC-2	64	GTAGGTGGAAATTCTAGCATCATC C	
E8i-Cre-ER7-For	69	CCACCGAGTCCTGGACAAGATCA C	
E8i-Cre-IRES-Rev	65	CCTCGACTAAACACATGTAAAGCA TG	E8i-Cre = 872 bp
Gnas_flox			
Gs Flox P1 fwd	57.2	GAGAGCGAGAGGAAGACAGC	GsF= 400 bp
Gs Flox P2 rev	66.4	TCGGGCCTCTGGCGGAGCTT	wt= 320 bp

Cycling conditions

Transgene	Step #	Temp °C	Time	Note
HA-GsDREADD-GFP	1	95	4 min	30 cycles
	2	95	1 min	
	3	55	1 min	
	4	72	1 min	
	5	72	10 min	
	6	4	hold	
2% Agarose				

Transgene	Step #	Temp °C	Time	Note
E8I-CreERT-GFP	1	95	3 min	35 cycles
	2	95	30 sec	
	3	60	1 min	
	4	72	1 min	
	5	72	7 min	
	6	4	hold	
2% Agarose				

Table 2.4: Genotyping, primer, and PCR information for CD8-Gnas KO mice (continued).

Transgene	Step #	Temp °C	Time	Note
Gs fl/fl	1	95	3 min	
	2	95	30 sec	repeat
	3	60	1 min	steps 2-4
	step + 0.2/cycle			for 35
	4	72	30 sec	cycles
	5	72	5 min	
		6	4	hold
2% Agarose				

Table 2.5: Genotyping, primer, and PCR information for CD8-PKI mice.

Transgene	Tm	Sequence	Band sizes
Rosa26 E8i-Cre-ERT2-IRES-GFP			
E8i-Cre-IC-1	62	CTAGGCCACAGAATTGAAAGATCT	IC = 324bp
E8i-Cre-IC-2	64	GTAGGTGGAAATTCTAGCATCATCC	
E8i-Cre-ER7-For	69	CCACCGAGTCCTGGACAAGATCAC	
E8i-Cre-IRES-Rev	65	CCTCGACTAAACACATGTAAAGCATG	E8i-Cre = 872 bp
Tet-GFP-PKI			
PKI_J_FWB	62	GGGGAAGGGGATGCTACCTA	PKI-WT = 230bp
PKI_J_RVB	62	TCGAATTTCACTTCTGCCCGA	
ROSA26-rtTA-IRES-EGFP (Linker)			
Linker WT fwd (ROSA5)	57.6	GAGTTCTCTGCTGCCTCCTG	WT = 350bp
Linker WT REV (ROSA3)	54.8	CGAGGCGGATACAAGCAATA	Mut = 225bp
Linker MUT REV (RTTA3)	55.3	AAGACCGCGAAGAGTTTGTC	

Cycling conditions

Cycling conditions				
Transgene	Step #	Temp °C	Time	Note
E8I-CreERT-GFP	1	95	3 min	35 cycles
	2	95	30 sec	
	3	60	1 min	
	4	72	1 min	
	5	72	7 min	
	6	4	hold	
Cycler program				
GENO2		2% Agarose		

Transgene	Step #	Temp °C	Time	Note
ROSA26-rtTA-IRES-EGFP (Linker)	1	94	1 min	35 cycles
	2	94	1 min	
	3	63	45 sec	
	4	72	1 min	
	5	72	5 min	
	6	4	hold	
Cycler program				
Linker	2% Agarose			

Table 2.5: Genotyping, primer, and PCR information for CD8-PKI mice (continued).

Transgene	Step #	Temp °C	Time	Note
Tet-GFP-PKI	1	95	2 min	
	2	95	30 sec	29 cycles
	3	62	45 sec	
	4	72	45 sec	
	5	72	2 min	
	6	12	hold	
Cycler program				
<i>GFP-PKI</i>		2% Agarose		

Table 2.6: Genotyping, primer, and PCR information for CD8-GsD mice.

Transgene	Tm	Sequence	Band sizes
Rosa26_LSL_HA-GsDREADD-GFP			
Olg-Rosa-9F	56.9	CTCGAAGTACTCGGCGTAGG	Knock in=206bp
Olg-Rosa-9R	55.3	CTTGGCAATCCGGTACTGTT	
gt-tomato-wt-F	58.2	AAGGGAGCTGCAGTGGAGTA	WT= 297bp
gt-tomato-wt-R	53.5	CCGAAAATCTGTGGGAAGTC	
Rosa26_E8i-Cre-ERT2-IRES-GFP			
E8i-Cre-IC-1	62	CTAGGCCACAGAATTGAAAGATCT GTAGGTGGAAATTCTAGCATCATC	IC = 324bp
E8i-Cre-IC-2	64	C	
E8i-Cre-ER7-For	69	CCACCGAGTCCTGGACAAGATCAC CCTCGACTAAACACATGTAAAGC	E8i-Cre = 872 bp
E8i-Cre-IRES-Rev	65	ATG	

Cycling conditions

Transgene	Step #	Temp °C	Time	Note
HA-GsDREADD-GFP	1	95	4 min	
	2	95	1 min	30 cycles
	3	55	1 min	
	4	72	1 min	
	5	72	10 min	
	6	4	hold	
Cycler program				
GENO2		2% Agarose		

Transgene	Step #	Temp °C	Time	Note
E8i-CreERT-GFP	1	95	3 min	
	2	95	30 sec	35 cycles
	3	60	1 min	
	4	72	1 min	
	5	72	7 min	
	6	4	hold	
Cycler program				
GENO2		2% Agarose		

Conclusion

Cancer immunotherapy has emerged as a distinct pillar in the treatment of cancer alongside surgery, radiation, chemotherapy, and targeted therapy. While immunotherapies such as immune checkpoint inhibitors and CAR-T adoptive therapy have had breakthrough and lasting clinical responses in a variety of advanced and refractory cancers, the majority of patients receiving these therapies do not respond or inevitably develop primary or acquired resistance. These poor outcomes underscore the complexity of tumor-immune cell reactions that have yet to be fully explored in our preclinical models of disease. To broaden the application of successful immune-modulatory treatments to achieve complete and durable responses (cure) in patients, we must prioritize the development and thorough characterization of preclinical models that are the foundation of our ability to predict clinical response. Furthermore, the identification of other inhibitory mechanisms that are exploited by tumor cells to escape immune surveillance and destruction is necessary to achieve robust anti-tumor immunity. Here, we explore both avenues to enhance patient responses to immunotherapy.

In Chapter 1, we profiled the genomic and transcriptomic landscape of 22 existing and next-generation syngeneic models for cancer to examine their relevance to human disease as well as possible cancer subtypes. Whole genome sequencing analysis revealed matched cancer driver genes in many of the tumor models from the TCGA database. Depending on the methods used to generate these syngeneic cell lines, tumor models with human-relevant driver mutations as well as defined etiologies of human cancer (tobacco smoking, UV exposure) better reflected the mutational signature of human diseases, as seen with the 4MOSC and YUMMER models. This observation was also evident in the transcriptional profiles of tumors derived from the implantation of these cell lines. For example, the 4MOSC1 cell line generated from the exposure to 4NQO, a tobacco-mimetic, and the HPV E6/E7-expressing MEER cell line had gene expression profiles that correlated to the smoking and HPV status of head and neck cancer patients,

respectively. With this analysis, we hope to uncover features of these syngeneic models that can better be applied to translating preclinical findings into the clinic. Further characterization of the tumor microenvironment to understand the spatial neighborhoods as well as the organization of immune infiltrates and stroma will further aid in understanding mechanisms of immune suppression by the TME.

In Chapter 2, we identified a GPCR-G protein signaling pathway in CD8 T cells that is responsible for limiting immune responses to ICB. GPCRs have been highly valuable as pharmacological targets due to their druggability, involvement in numerous physiological processes, and their ability to modulate various cell signaling pathways. Furthermore, their structural diversity allows for the design of selective ligands that can specifically target individual receptor subtypes. Despite representing the largest protein family targeted by FDA-approved drugs, they have been undervalued in the field of cancer immunology and their role on immune cells remains to be fully elucidated. Therefore, our studies profiled GPCR expression patterns on CD8 T cells from patients across 19 distinct cancer types, revealing an association between $G\alpha_s$ -coupled GPCRs and T cell dysfunction. Using various orthogonal approaches, we demonstrated that signaling through $G\alpha_s$ in CD8 T cells polarized them to a more dysfunctional state, resulting in decreased cytokine secretion, cytotoxic protein expression, co-expression of multiple inhibitory receptors, and immunotherapy failure. Conversely, inhibition of this signaling pathway by activation of $G_{\alpha i}$ -coupled receptors, or by inhibiting downstream $G\alpha_s$ signaling through *Gnas* gene deletion or PKA inhibition led to restoration of T cell function. By showing that tumor cells avoid immune elimination by stimulating GPCRs on infiltrating CD8+ T cells, we identified vulnerabilities that can be exploited for therapeutic intervention. If $G\alpha_s$ signaling in CD8+T cells within the TME can be interrupted, we might be able to convert 'cold' immunophenotypes into 'hot' immune-infiltrated environments that correlate with improved clinical response to ICB, and perhaps overcome the immune-suppressive TME of solid tumors that is a barrier to adoptive cell therapies, including CAR-T cells. Our findings can inform new treatment strategies to improve existing ICB

therapies, including the concomitant use of existing FDA-approved GPCR-targeting drugs or the development of new GPCR-modulating agents as part of combination immunotherapies. However, targeting immune-suppressive GPCRs expressed primarily on CD8⁺ T cells remains a challenge. We aim to further profile the expression patterns of GPCRs on each T cell subset, with the hope of identifying new druggable targets. Elucidating the downstream mediators of Gα_s-induced T cell dysfunction is essential to identify T cell-specific signaling mechanisms amenable to pharmacological intervention. Finally, characterization of the complete immune-cell GPCRome could aid in the identification of new immune-modulating strategies to increase the response to ICB for cancer treatment.

In conclusion, this dissertation highlights important avenues for the improvement of patient responses to cancer immunotherapy. Identifying and developing preclinical models that translate to human responses as well as identifying mechanisms of immune suppression in the TME remain key challenges for the field. As we elucidate the complexities of tumor-immune cell interactions and are better able to model them preclinically, we are paving the way for the development of novel treatment strategies and methodologies that may, one day, cure cancer.

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