

Investigating the role of IGF-1 on the anti-cancer immune response



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Declaration

I declare that this dissertation describes my own research work, except for the following experiments that were completed in collaboration and with others, detailed below:

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Established, monitored, administered treatments for MyC-CaP allograft experiments *in vivo* and extracted peripheral blood by cardiac puncture [2.2.9.1, 2.2.9.2, 2.2.9.3, 2.2.9.4].

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Whole blood collection, PBMC isolation and prostate-specific antigen testing [2.3.1.3].

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Co-ordinated patient data collection [2.3.1.1].

Abstract

Immune cells within the tumour niche can profoundly shape both disease progression and response to anti-neoplastic treatment. In prostate cancer (PCa), developmental pathways are exploited for tumour development, including the insulin-like growth factor-1 (IGF-1) axis. Conversely, fasting-mimicking dietary interventions can enhance anti-tumour immunity, mediated at least in part, by IGF-1 axis inactivation. In this study, the relationship between IGF-1 axis activity and immune cell function was explored in prostate cancer. Using *in vitro* cell-based assays, the effects of IGF-1 supply on Treg function and metabolism were investigated. Following which, the *in vivo* effects of combined therapeutic IGF blockade and immune checkpoint blockade (ICB) were evaluated. Then, peripheral blood immune cells were profiled by mass cytometry before and after treatment with an IGF neutralizing antibody, from PCa patients recruited to the WINGMEN trial (NCT05110495). The results from *in vitro* cell-based assays show that high IGF-1 supply facilitates the metabolic adaptation of regulatory T-cells (Tregs) to high-lactate and low-glucose conditions generated by cancer cells. Subsequent IGF neutralization in murine PCa reduced the frequency tumour-infiltrating Tregs, but did not reduce tumour growth, nor sensitize tumours to ICB. In patients with localized PCa, IGF neutralization boosted peripheral CD8⁺, MAIT and iNKT cell numbers. A low Treg, high memory CD4⁺ T-cell setpoint before treatment initiation correlated with anti-tumour effects from IGF neutralization. In conclusion, this work describes a hitherto unknown link between the IGF-1 axis and tumour-associated Treg metabolism, and defines how reduced IGF-1 levels contribute to anti-tumour immune responses induced by FMD interventions.

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List of abbreviations

2-NBDG	2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose
4E-BP1	Eukaryotic initiation factor 4E binding protein 1
ACADL	Acyl-Coenzyme A dehydrogenase, long-chain
ACADS	Acyl-Coenzyme A dehydrogenase, short chain
ACC1	Acetyl-CoA carboxylase 1
ADAM12	A disintegrin and metalloprotease-12
ADT	Androgen deprivation therapy
AKT	Protein kinase B
AMPK	AMP-activated protein kinase
APC	Antigen presenting cell
AR	Androgen receptor
ARSI	Androgen receptor signalling inhibitors
ASNS	Asparagine synthetase
AS-ODN	Antisense oligodeoxynucleotides
ATF	Activating transcription factor
ATP	Adenosine triphosphate
ATRA	All-trans-retinoic acid
BCAT1	Branched chain amino acid transaminase 1
BDCA1	Cluster of differentiation antigen 1c
BODIPY	4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene
Breg	Regulatory B-cells
C/EBPγ	CCAAT enhancer binding protein gamma
CAF	Cancer associated fibroblasts
Cbl	Cbl proto-oncogene
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
CCL17	C-C chemokine ligand 17
CCR4	Chemokine receptor type 4
CCR6	Chemokine receptor 6
CD	Cluster of differentiation
CD103	Cluster of differentiation 103
CD11b	Integrin alpha M
CD11c	Integrin alpha X
CD123	Interleukin-3 receptor alpha chain
CD14	Cluster of differentiation 14
CD141	Thrombomodulin
CD15	Fucosyltransferase 4
CD16	Low affinity immunoglobulin gamma Fc region receptor III-A
CD161	Killer cell lectin-like receptor subfamily B
CD19	Cluster of differentiation-19
CD20	B-lymphocyte antigen
CD206	Cluster of differentiation 206
CD25	interleukin-2 receptor alpha chain
CD27	Tumour Necrosis Factor Receptor Superfamily Member 7
CD28	Cluster of differentiation 28
CD33	Siglec-3

CD36	Cluster of differentiation 36
CD38	Cyclic ADP ribose hydrolase
CD39	Ectonucleoside triphosphate diphosphohydrolase-1
CD45RO	Protein tyrosine phosphatase receptor type C
CD56	Neural cell adhesion molecule 1
CD57	human natural killer-1
CD62L	L-selectin
CD66b	Carcinoembryonic antigen-related cell adhesion molecule 8
CD73	5'-ecto-nucleotidase
CD80	Cluster of differentiation 80
CD86	Cluster of differentiation 86
cDC1	Conventional type 1 dendritic cells
c-Ets	ETS Proto-Oncogene
CFSE	Carboxyfluorescein succinimidyl ester
CGAS	cyclic GMP-AMP synthase
CM	Conditioned medium
CPT1a	Carnitine palmitoyl transferase-1A
Cth	Cystathionine gamma-lyase
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
CXCL	C-X-C motif chemokine ligands
CXCL12	Chemokine stromal cell-derived factor 1
CyTOF	Mass cytometry by time-of-flight
DEG	Differential gene expression
DGAT1	Diacylglycerol acyltransferase-1
ECAR	Extracellular acidification rate
ECM	Extracellular matrix
EGF	Epidermal growth factor
Elk-1	ETS Like-1 protein
ERG	ETS-related gene
ERK	Extracellular-related kinase
ETC	Electron transport chain
FABP5	Fatty acid binding protein 5
FAK1	Focal adhesion kinase (FAK)
FAM21	WASH complex subunit 2A
FAO	Fatty acid oxidation
FAS	Fatty acid synthesis
FAS	Fatty acid synthesis
FCCP	Carbonyl-cyanide-p-trifluoromethoxyphenylhydrazine
FGF	Fibroblast growth factor
FnIII	Fibronectin type III domains
FOXA1	Forkhead box protein
FoxP3	Forkhead box P3
GADD45A	Growth arrest and DNA damage inducible alpha
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GGG	Gleason Grade Group
GH	Growth hormone
Glut1	Glucose transporter 1

Gpt2	Glutamic pyruvate transaminase
Grb2	Growth-factor-receptor-bound protein 2
GSEA	Gene-set enrichment analysis
GTP	Guanosine-5'-triphosphate
HADH	Hydroxyacyl-Coenzyme A dehydrogenase
HFD	High-fat diet
HGPIN	High-grade prostatic intraepithelial neoplasia
HIF1A	Hypoxia inducible factor 1 subunit alpha
HLA-DR	Human leukocyte antigen-DR
HSC	Haematopoietic stem cells
ICB	Immune checkpoint blockade
ICOS	Inducible T-cell co-stimulator
IFN-β	Interferon-beta
IFN-γ	Interferon-gamma
IgD	Immunoglobulin D
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor receptor 1
IGF-2	Insulin-like growth factor-2
IGFBP	IGF binding protein
IGFs	Insulin-like growth factors
IL-10	Interleukin-10
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-1β	Interleukin-1 beta
IL-2	Interleukin-2
IL-23	Interleukin-23
IL-4	Interleukin-4
IL-6	Interleukin-6
ILC	Innate lymphoid cells
InsR	Insulin receptor
IL-35	Interleukin-35
IRS	Insulin receptor substrate
ISR	Integrated stress response
iTreg	In vitro induced-regulatory T-cell
JUN	Transcription factor JUN
Ki67	Marker of proliferation Kiel 67
KLK	Kallikrein-related peptidase
LC-MS	Liquid chromatography-mass spectrometry
LD	Lipid droplets
LEF-1	Lymphoid enhancer-binding factor 1
LGPIN	Low-grade prostatic intraepithelial neoplasia
mAbs	Monoclonal antibodies
MAIT	Mucosal-associated invariant T cells
MAPK	Mitogen-activated protein kinases
MCT1	Monocarboxylate transporter-1
MDM2	MDM2 proto-oncogene
MDSC	Myeloid derived suppressor cell

MEK1	Mitogen-activated protein kinase 1
MEM	Marker enrichment modelling
MHC	Major histocompatibility complex
MMP	Matrix metalloprotease
MNK	MAP kinase-interacting serine/threonine-protein kinase
moDC	Monocytic dendritic cells
MSigDB	Molecular Signature Database
mTOR	Mechanistic target of rapamycin complex
mTORC1	Mechanistic target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
MYC	C-MYC oncogene
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NKG2D	Natural killer group 2D
NKG2DL	Natural killer group 2D ligand
OCR	Oxygen consumption rate
ORA	Over-representation analysis
OS	Overall survival
OX40	Tumour necrosis factor receptor superfamily, member 4
OXPHOS	Oxidative phosphorylation
PAPP-A	Pregnancy-associated plasma protein-A
PARP	poly-ADP-Ribose Polymerases
PCa	Prostate cancer
PD-1	Programmed death-1
PDAC	Pancreatic ductal adenocarcinoma
PDGF	Platelet-derived growth factor
PDK1	Phosphoinositide-dependent kinase-1
PD-L1	Programmed death-ligand-1
Phgdh	3-phosphoglycerate dehydrogenase
PHLPP	PH domain and leucine rich repeat protein phosphatases
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PPARγ	Peroxisome proliferator-activated receptor- γ
PPAT	Periprostatic adipose tissue
PSA	Prostate-specific antigen
Psph	Phospho-serine phosphatase
PTEN	Phosphatase and tensin homolog
PTEN	Phosphatase and tensin homolog
pTreg	Peripheral Treg
RA	Rheumatoid Arthritis
RACK1	Receptor for activated C kinase 1
RT	Radiotherapy
rpS6	Ribosomal protein S6
SHC	Src homology 2 (SH2)-containing-protein
sIGF-1	Serum insulin-like growth factor-1
SLC	Solute carrier family
SMC	Smooth muscle cells

SOAT2	Sterol o-acyltransferase 2
SOS	Son of sevenless
SPOP	Speckle type BTB/POZ Protein
SRC	Spare respiratory capacity
SREBP1	Sterol regulatory element-binding protein 1
STAT3	Signal transducer and activator of transcription 3
STING	Stimulator of interferon gene
SUMO-1	Small ubiquitin-like modifier protein-1
SV40T	Simian vacuolating virus 40 tag
TAA	Tumour associated antigens
TAM	Tumour-associated macrophages
TAN	Tumour associated neutrophil
TCA	Tricarboxylic acid
Tconv	Conventional CD4 T-cell
TCR	T-cell receptors
TCR	T-cell receptors
TCRgd	T-cell receptor gamma delta
TDLN	Tumour-draining lymph nodes
TFH	T-Follicular helper
TFR	Follicular regulatory T-cell
TGF-β	Transforming growth factor-beta
TGF-βR1	Transforming growth factor-beta receptor 1
TH1	T helper type-1
TH17	T-helper 17
TH22	T-helper 22
TI	Tumour-infiltrating
TIGIT	T Cell Immunoreceptor With Ig And ITIM Domains
TIL	Tumour-infiltrating lymphocyte
TIM-3	HAVCR2 hepatitis A virus cellular receptor 2
TI-Treg	Tumour-infiltrating Treg
TME	Tumour microenvironment
TMPRSS2	Transmembrane protease serine 2
TMRM	Tetramethylrhodamine methyl ester
TNFRSF	Tumour necrosis factor receptor superfamily
TNFSFRL	Tumour necrosis factor receptor superfamily ligand
TNF-α	Tumour necrosis factor alpha
TOFA	5-tetradecyloxy-2-furoic acid
TP53	Tumour protein P53
Treg	Regulatory T-cell
TREM2	Triggering Receptor Expressed on Myeloid Cells
t-SNE	t-distributed stochastic neighbour embedding
tTreg	Thymic Treg
UPR	Unfolded protein response
VAT	Visceral adipose tissue
VEGF	Vascular endothelial growth factor
VLIP	Viral-like insulin peptides

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Chapter 1 – Introduction

1.1 The anti-tumour immune response

1.1.1 Concepts of cancer immunity

A key aspect of the immune system's ability to defend against invading pathogens is the capacity to discriminate between self and nonself (1). Shortly after the discovery of cytotoxic T-cells in the late 1960s, Drs. Karl and Ingegerd Hellström published the first series of experiments demonstrating that lymphoid cells isolated from the peripheral blood of neuroblastoma patients were tumour reactive (2). The Hellström's experiments raised a paradox: if tumour reactive immune cells exist in the peripheral blood of neuroblastoma patients and inhibit cancer cell growth, at least *in vitro*, why then do their tumours continue to grow *in vivo*?

In the past two decades, significant progress has been made with respect to our understanding of the complex network of interactions that occur between cancer and the immune system as tumours develop (3–6). Tumour cells are continuously and spontaneously eradicated by the activated immune system, a concept referred to as cancer immunosurveillance (3). However, many clinically apparent tumours grow and develop in spite of immunosurveillance. This phenomenon is described in a process known as cancer immunoediting, where the immune response against tumours applies a selection pressure that results in the expansion of transformed cancer cells that edit, disable, or exploit host immunity either directly or through reshaping of their microenvironment (7,8). This dynamic process is often explained in phases that can

function independently, or in succession, called *elimination*, *equilibrium*, and *escape* (3).

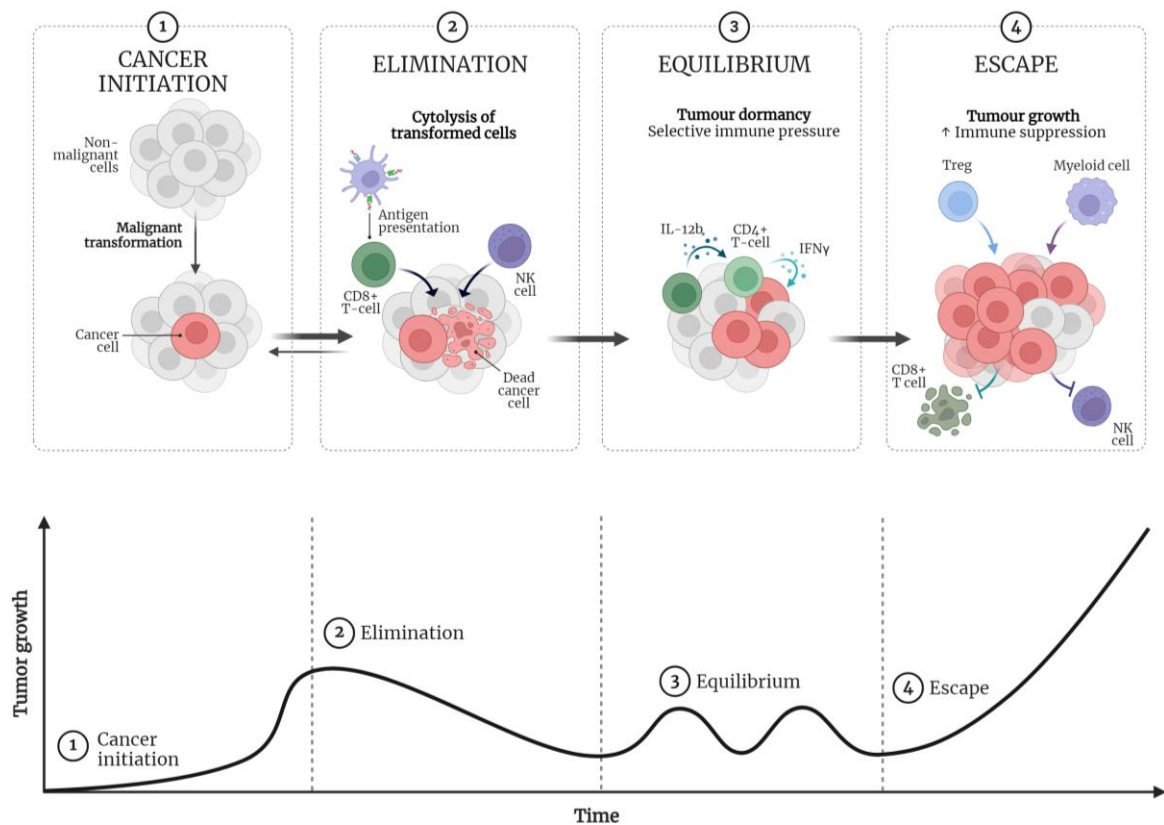


Figure 1.1.1.1 – Phases of anti-cancer immunity as tumours develop over time

The rate of tumour growth over time after cancer initiation is dynamically regulated by interactions with the host immune system, modelled by phases of tumour cell *elimination*, *equilibrium* and *escape*.

After malignant transformation and **1) cancer initiation**, tumour associated antigens (TAAs) acquired during oncogenesis are released upon cancer cell death (9). TAAs are captured and processed by antigen presenting cells (APCs) such as intratumoural conventional type 1 dendritic cells (cDC1s) and displayed on cell-surface major histocompatibility complex (MHC) molecules (10,11). In the presence of inflammatory cytokines, cDC1s mature and upregulate chemokine receptors for

migration to secondary lymphoid organs, where they interact with and activate TAA specific CD4⁺ T helper type-1 (Th1) and CD8⁺ T-cells (12,13). Activated Th1 and CD8⁺ T-cells enter the peripheral circulation and migrate to stromal/tumour tissue in response to chemokine gradients alongside other anti-tumorigenic cell types such as natural killer (NK) and NKT cells (14–18). Recognition of TAAs presented on MHC molecules by transformed cancer cells by cell-surface T-cell receptors (TCRs) of CD4⁺ Th1 and CD8⁺ T-cells induces cytokine production and/or lysis of tumour cells (19,20). Tumour infiltrating NK cells recognize both MHC deficient and stress-ligand expressing cancer cells (21,22), whilst NKT recognize glycolipid antigens expressed by tumour cells (17), inducing cytolysis and release of TAAs (23,24). Thus, subsequent iterations of antigen presentation by APCs and cycles of immunity by CD4⁺ Th1 and CD8⁺ T-cells form the cancer-immunity cycle (5), resulting in net tumour cell **2) elimination**.

The elimination of tumour cells vulnerable to immune-mediated cell killing applies an evolutionary bottleneck that selects for tumour cells that inhibit aspects of the cancer immunity cycle (25). Hence, the **3) equilibrium** phase describes a relative balance established between simultaneous eradication of tumour cells by anti-tumour immunity, and outgrowth and proliferation of tumour cells insensitive to immune-mediated killing, resulting in a latency period of no net tumour growth (3). The *equilibrium* phase potentially remains stable for years, even decades, before clinically detectable tumour growth becomes apparent (26,27).

The **4) escape** phase describes the progressive growth of tumours that have evolved mechanisms that evade anti-tumour immunity (3). Notable mechanisms of

immune *escape* include inhibition of antigen presentation (28,29), loss of tumour antigenicity (30), recruitment of immunosuppressive immune cells (31–33), and stroma-dependent extracellular matrix remodelling (34). At this stage, anti-tumour immunity is no longer sufficient to prevent tumour outgrowth, and clinically apparent tumours become detectable. However, the identification of mechanisms implicated in the tumour *escape* phase has led to the development of novel therapeutic strategies designed to restore or complement anti-tumour immunity and promote tumour elimination (35). Broadly referred to as cancer immunotherapy, therapeutic agents that promote anti-tumour immunity achieve long-lasting responses in a minority of patients, and are curative in a subset of malignancies (36,37).

1.1.2 Immunometabolism in the TME

Mitochondria are the site of three important energy generating pathways that require aerobic conditions: the tricarboxylic acid (TCA) cycle, oxidative phosphorylation (OXPHOS) and fatty acid β -oxidation (FAO), generating adenosine triphosphate [ATP] (38). Glycolysis describes the sequence of glucose catabolism in the cytoplasm that generates two ATPs, producing pyruvate. In oxygen-deprived tissue, pyruvate remains in the cytoplasm and is converted into lactate, to replenish the co-enzyme nicotinamide adenine dinucleotide (NAD⁺). Under oxygen-replete conditions, amino acid derivatives, fatty acids and pyruvate enter mitochondria and are used to generate acetyl-coenzyme-A (acetyl-CoA), which is oxidized through the TCA cycle and electron transport chain (ETC) to produce 36 ATP. Cytoplasmic long-chain fatty acids imported into mitochondria are used to fuel FAO (39). FAO describes a cyclical

series of reactions that shortens fatty acids, generates reducing equivalents for the ETC and produces an additional acetyl-CoA oxidized within the TCA cycle.

To meet the energy demands required for uncontrolled cell division, cancer cells are often heavily dependent on glycolytic metabolism and uptake high levels of glucose (40,41). Described as the “Warburg effect”, cancer cells actively generate high levels of lactate (200-fold higher than healthy tissue) even in oxygen-replete conditions (42). Dysregulation of multiple metabolic pathways is an important hallmark of cancer, which supports metabolic flexibility [reviewed in ref. (43)]. Ultimately, the nutrient deficient TME represents a profoundly challenging microenvironment for sustained Th1 and CD8⁺ TIL effector function (44–46), due to their bioenergetic reliance on glycolytic metabolism and amino acid availability for redox regulation and effector function within the TME (47–49). Consequently, metabolic dysfunction of Th1 and CD8⁺ cells within the TME is common (50–52). In contrast, glucose deprivation can prevent the development of pro-inflammatory transcriptional signatures and drive the acquisition of immunosuppressive phenotypes (53). Whilst beyond the scope of this introduction, it must be acknowledged that the co-ordinated regulation of metabolic pathways derived from both cell-extrinsic and intrinsic signals carefully controls immune cell differentiation, plasticity and function in a cell-type dependent manner [reviewed in refs. (54–60)]. Immune cell-type specific metabolic phenotypes in normal health and disease are often profoundly different within the TME, and cancer immunometabolism ultimately forms a central regulatory component of anti-tumour immune responses [reviewed in refs. (61–68)].

1.2 Prostate cancer

PCa is the most prevalent form of cancer among men in 112 countries, accounts for one in every 14 cancers diagnosed globally and caused 375,000 deaths worldwide in 2020 (69,70). Projected cases are estimated to rise from 1.4 million in 2020 to 2.9 million in 2040, attributed to changing age structures and improved longevity. A significant challenge in treating prostate cancer is the sporadic nature of the transition from low-grade localized tumours that do not require treatment for many years, to rapidly growing and highly lethal metastatic tumours (71,72). Risk factors of PCa development include age, ethnicity, height, chronic inflammation of the prostate, genetics, diet, obesity, serum testosterone and serum insulin-like growth factor-1 (sIGF-1) levels (73–79).

1.2.1 Pathogenesis of PCa

The prostate is a male reproductive gland that supports male fertility through the production of prostatic fluid high in Zn^{2+} , citrate and kallikreins that support the normal function of sperm in male semen (80). Localized at the base of the bladder and supported by androgens produced in the testes, the prostate gland is formed of four distinct histological zones known as the peripheral, transition, and central zones with an anterior fibromuscular stroma [figure 1.2.1.1] (81). Prostate tissue encompasses a branching duct system composed of glandular epithelium that consists of basal and luminal epithelial cells, embedded in a fibromuscular stroma and interspersed neuroendocrine cells (82). Up to 80% of prostate cancers are multifocal adenocarcinomas that develop in the peripheral zone, thought to arise from low-grade prostatic intraepithelial neoplasia (LG-PIN) that progress to high-grade intraepithelial

neoplasia [HG-PIN)] (83). Although poorly defined, characteristic features of HG-PIN include intraglandular proliferation of luminal or basal epithelial cells, with histological features of atypical epithelial cells that resemble invasive adenocarcinoma.

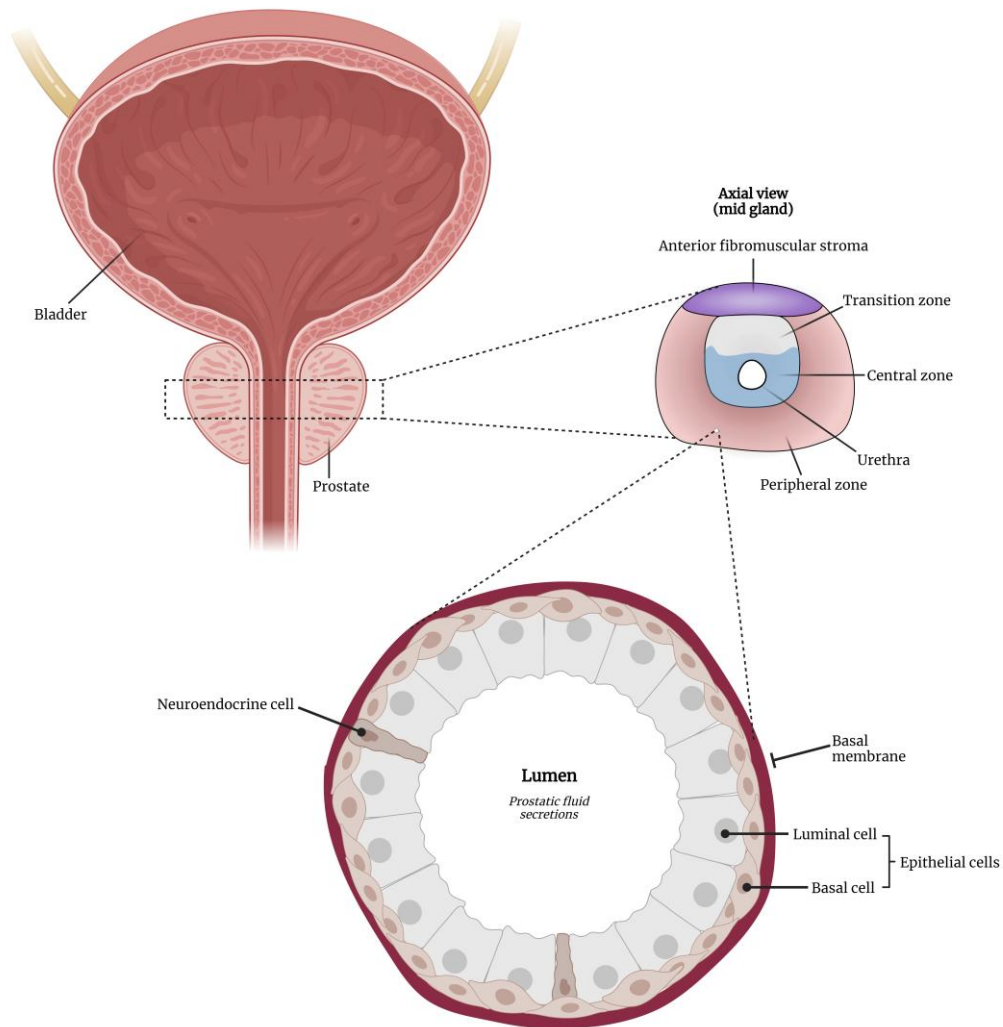


Figure 1.2.1.1 – Anatomy and histological structure of the normal human prostate

The prostate gland is positioned inferiorly to the neck of the bladder, and is comprised of four primary zones: the peripheral zone, transition zone, central zone and fibromuscular stroma. Within the peripheral zone, basal and luminal epithelial cells interspersed with neuroendocrine cells support prostatic fluid secretions, forming branching ducts surrounded by a basal lamina.

The acquisition of multiple genetic mutations in either basal or luminal prostate epithelial cells is thought to be a primary driver towards the development of invasive adenocarcinoma (84). The multifocal nature of invasive adenocarcinoma often leads to local extension into the periprostatic fat tissue and heterogeneous disease, whereby multiple tumour foci can harbour genetically distinct subclones (85–87). In localized adenocarcinoma, fusions of transmembrane protease serine 2 (TMPRSS2) with ETS-related gene (ERG) [*TMPRSS2-ERG*] is the most prominent molecular subtype [~21-50% of PCa] (88–94). The fusion of androgen responsive elements of TMPRSS2 to the ERG gene results in ERG overexpression, activation of C-MYC oncogene (MYC), tumour progression and transformation of the proximal stromal niche. Other common genomic alterations in localized PCa include loss of phosphatase and tensin homolog (PTEN) [~20%] (95), MYC amplification [~10-30%] (96–100), mutations in speckle type BTB/POZ Protein (SPOP) [~15%] (101), tumour protein P53 (TP53) [6-7%] (102,103) and forkhead box protein (FOXA1) [~4%] genes. The progression from localized to metastatic PCa is associated with amplification of the androgen receptor (AR) gene (seen in 30-50% of patients) and invasion of adenocarcinoma to the urinary bladder and pelvic soft tissue (104–107). The most common sites of PCa metastasis are the bone marrow [>80%], lymph nodes [~10%] and liver [~10%] (108).

1.2.2 Treatment of PCa

Upon diagnosis, Gleason Grade Group (GGG) scores, prostate-specific antigen (PSA) levels and clinical stage are used by clinicians to categorize low, intermediate and high risk PCa (109). The GGG system describes the degree of histological

abnormality from diagnostic biopsies (110). Serum PSA levels correlate with tumour size, caused by the production of PSA by malignant epithelial cells at a rate that is ten times higher the equivalent mass of benign epithelial cells (111). Hence, PSA levels are used both as a clinical biomarker for monitoring PCa growth, and as a screening tool for early detection of disease. Men diagnosed with localized disease (no identifiable lymph node or distant metastasis) and low-intermediate risk PCa are offered 3 primary treatment options: active surveillance, radical prostatectomy (partial or complete removal of the prostate), or external beam radiotherapy (84). Active surveillance describes the treatment of symptoms related to PCa with palliative intent and routine monitoring of the prostate gland through PSA testing, physical examinations and biopsies of the prostate. Of note, there is no significant difference in 10-year mortality from PCa after treatment with either active surveillance (1.5%), radical prostatectomy (0.9%) or radiotherapy [0.7%] (112). Men on active surveillance maintain better urinary control and sexual function, at the expense of increased rates of bowel dysfunction and nocturia. Conversely, radical prostatectomy and radiotherapy reduce the risk of clinical progression and metastasis.

For the ~15% of men that present with localized high risk PCa, first-line treatment is often radiotherapy (RT) combined with androgen deprivation therapy (ADT) or radical prostatectomy (113,114). Although no consensus has been reached on the optimal treatment strategy, treatment selection requires consideration of performance status and co-morbidities, as RT + ADT is associated with increased risk of cardiovascular disease (115). Chemotherapy is often ineffective in high-risk PCa, even when combined with ADT (116,117). For men with metastatic PCa, ADT is

offered as first-line standard of care treatment (118). The majority of patients eventually acquire metastatic castration-resistant PCa [mCPRC], resistant to ADT therapy (119). Treatment of mCPRC includes utilization of second-generation androgen receptor signalling inhibitors (ARSI), poly-ADP-Ribose Polymerases (PARP) inhibitors, taxanes or alpha/beta particle emitting radionuclide therapies (120–123). Whilst an improved arsenal of treatment options has improved the rate of overall survival for men with mCPRC, the aggressive nature of the disease means treatment at this stage is life-prolonging, not curative (124).

Immune checkpoint blockade (ICB) monotherapy or combination regimens targeting the programmed death-1 (PD-1)/ Programmed death-ligand-1 (PD-L1) axis and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) are widely used to treat heavily pre-treated and metastatic disease, including tumours that originate from the lung, kidney, bladder and skin (125–127). However, metastatic PCa remains a significant exception. To date, results from phase I-III trials of both monotherapy and combination ICB have failed to demonstrate an overall survival (OS) benefit in men with mCRPC [summarized in table 1.2.2.1] (128–133), although there is a small subset of patients who derive long-lasting clinical benefit after ipilimumab treatment (134). The clinical benefit of ICB is mediated by re-activation of anti-tumour immunity by tumour-infiltrating CD4⁺ T_H1 and CD8⁺ CTLs through blockade of PD-1/PD-L1 and CTLA-4 inhibitory pathways (135,136).

Table 1.2.2.1 - Summary of completed trials informing the use of ICB in biomarker-unselected mCPRC patients

Drug	Nature	Disease	Phase	Result	Refs.
Nivolumab	Anti-PD1	mCRPC	I	No objective response	(128)
Pembrolizumab	Anti-PDL1	mCPRC	II	No sig. objective response (3-5% ORR)	(129)
Ipilimumab	Anti-CTLA-4	mCRPC	III	No objective response	(131)
Ipilimumab	Anti-CTLA-4	mCRPC	III	No objective response	(130)
Ipilimumab	Anti-CTLA-4	mCRPC	III	No sig. objective response	(132)
+enzalutamide	+ARSI			(18 vs 8% ORR)*	
Nivolumab+	Anti-PD1 +	mCRPC	II	No sig. objective response	(133)
Ipilimumab	anti-CTLA-4			(10-25% ORR)	

ORR = Objective Response Rate, sig. = significant

*Combination treatment vs enzalutamide monotherapy

However, cautious optimism surrounds early prospective analysis of trial data. Complete and durable responses (~45% ORR) were observed in a subset of mCPRC patients with microsatellite instability-high/mismatch repair-deficiency tumours (3-5% of patients) treated with anti-PD-1/PD-L1 ICB (137). A high interferon-gamma (IFN- γ) gene signature and an intra-tumoural CD8⁺ T-cell density >400 cells/mm² in pre-treatment tumour samples also improves the rate of overall survival after anti-CTLA-4 ICB in mCPRC, implicating a pro-inflammatory and high CD8⁺ TIL setpoint with enhanced anti-PCa immunity (138). Furthermore, early clinical data from combination trials of durvalumab (anti-PD-1) and olaparib (PARP inhibitor) induce durable and promising anti-tumour effects in a significant minority of patients [~33% ORR] (139).

Notably, baseline levels of peripheral blood MDSCs and increased activation of CD4⁺ and CD8⁺ T-cells by cycle 3 of treatment are predictive of progression free survival (PFS). Taken together, durable and long-lasting responses are possible in mCPRC patients treated with ICB. However, failure of ICB trials in mCRPC appears to represent a composite of a low frequency of tumour-infiltrating CD8⁺ TILs, low tumour antigenicity and suppression of anti-tumour immunity by immunosuppressive cell types. Thus, one avenue of treatment optimisation of ICB in advanced PCa requires the identification and design of therapeutic combination strategies that target and/or transform aspects of the immunosuppressive TME.

1.3 The immune microenvironment of PCa

Epithelial glands within the healthy human prostate have a diverse lymphoid and myeloid immune infiltrate surrounded by non-immune stromal cells including fibroblasts, smooth muscle cells, telocytes and pericytes, supported by an extracellular matrix (ECM) that protects against pathogens originating from the urinary tract (140,141). As pre-malignant lesions develop, prostate epithelial cells continuously interact with their immune and stromal microenvironment (142). Early genomic and metabolic alterations in PCa cells further drives activation of the surrounding mesenchymal stroma that supports both PCa growth and transforms the immune infiltrate (143,144). For example, MYC-activated luminal neoplastic cells in the precursor state undergo a switch to downregulate pro-immunogenic transcriptional programs (145). This change is accompanied by an enrichment of immunosuppressive cells in the microenvironment, including MDSCs, Tregs, and TREM2 macrophages.

In this section, a brief outline of the diverse range of stromal and immune cell types in the PCa TME is provided, with key functional pathways and relative prognostic significance of each cell type highlighted.

1.3.1 Tumour associated macrophages

Macrophages are innate immune cells from the myeloid lineage that play a diverse role in tissue infection, wound repair, inflammation and clearance of dead cells (146). Cellular functions include the detection of pathogen and damage-associated molecular patterns, cytokine production, phagocytosis of extracellular matter and antigen presentation. Macrophages adopt appropriate phenotypes that are generalized into a dichotomous state of classical activation (M1) in response to the cytokine IFN- γ and/or bacterial lipopolysaccharide or alternative activation (M2) in response to interleukin-4 (IL-4) and interleukin-13 [IL-13] (147). M1 macrophages upregulate MHC-class II, produce pro-inflammatory cytokines including interleukin-1 β , interleukin-12 (IL-12) and tumour necrosis factor-alpha (TNF- α) display a high phagocytotic index (148,149). Conversely, M2 macrophages promote wound-healing, resolution of inflammation and fibrosis through production of transforming growth factor-beta (TGF- β), interleukin-10 (IL-10), epidermal growth factor (EGF) and IGF-1. Bone marrow derived blood monocytes are considered the precursor to tumour-associated macrophages (TAMs), that display remarkable plasticity in response to metabolic, cytokine and growth factor signals. Broadly, TAMs are often converted towards an M2-like phenotype that support tumour growth, angiogenesis and produce immunoregulatory cytokines that suppress anti-tumour immunity [reviewed in ref. (150)].

Single-cell RNA-seq has shown enrichment for two phenotypically distinct subsets of TAMs within the PCa TME (151,152). The first, named metallothionein (MT)-TAMs, delineates TAMs with high expression of zinc transporter genes from the metallothionein family, thought to represent a tissue-resident macrophage subset that regulate zinc homeostasis within the prostate. The second, named lipid loaded (LL)-TAMs, readily accumulate lipids and are transcriptionally enriched for pathways associated with lipid metabolism and OXPHOS. Curiously, MT-TAMs and LL-TAMs appear to play opposing roles within the TME. Increased MT-TAMs infiltration is associated with a lower Gleason score, driven by expression of chemokines C-X-C motif chemokine ligands (CXCL) 9 and 10 that mediate the proximal recruitment of CD8⁺ CTLs. Conversely, the abundance of LL-TAMs is associated with tumour progression. In murine models, LL-TAMs display an M2-like phenotype characterised by cluster of differentiation 206 (CD206), PD-L1, cluster of differentiation 36 (CD36) and high ectonucleoside triphosphate diphosphohydrolase-1 (CD39) expression (152). PD-L1 is an extracellular ligand that inhibits T-cell activation through engagement with PD-1 (136), whilst CD39 is an ectonucleotidase that converts extracellular ATP to adenosine, suppressing T-cell activation and expansion (153). CD36 is a scavenger receptor that facilitates the extracellular transport of free-fatty acids and oxidised low-density lipoprotein used to fuel fatty acid oxidation and mitochondrial metabolism in TAMs, required for their immunosuppressive function (154,155). In mouse models, LL-TAM abundance within the TME increases with a high-fat dietary intake (152). Thus, LL-TAMs could represent an important link between obesity and increased risk of death from PCa (156).

1.3.2 Myeloid derived suppressor cells

MDSCs are a heterogeneous population of pathologically activated monocytes and neutrophils that have immunosuppressive activity (157). Broadly, MDSCs suppress T-cell activation (158), promote the *de novo* conversion and expansion of peripheral Tregs [pTreg] (159), inhibit cytotoxicity of NK cells and the maturation of dendritic cells (160,161). Within the TME, MDSCs promote immunosuppression by secretion of TGF- β , interleukin-10 (IL-10) and depletion of extracellular cystine, cysteine, L-arginine required for T-cell activation (162–164).

In human PCa, the accumulation of myeloid-MDSCs (CD14⁺) and granulocytic-MDSCs (CD15⁺) that express Integrin alpha M (CD11b) and Siglec-3 (CD33) but not human leukocyte antigen-DR [HLA-DR⁻] in the peripheral blood and tumour correlates with both malignant transformation, tumour stage and more aggressive disease (165–169). Yet, the functional classification of MDSC subsets within PCa remains challenging in the absence of consensus functional markers that are unambiguously used by research groups to define MDSCs. Single-cell analysis has shown that suppressive myeloid cells with a high MDSC signature score are enriched in human prostate tumours [CD14^{hi} tumour-inflammatory monocytes (TIMo)] (151). TIMo cells within PCa recruit Tregs, T_H1 and T_H17 cells to the prostate TME via production of the chemokine ligand 20 (CCL20). Granulocytic MDSCs (CD11b⁺ CD15⁺) accumulate within the stromal niche of distant metastatic foci, hypothesized to facilitate metastatic dissemination through increased local vascularization (170). In mouse models, signal transducer and activator of transcription 3 (STAT3) activation in MDSCs (CD11b⁺ CD14⁺ HLA-DR^{-/lo}) in murine PCa induces IL-6, IL-1 β and IL-10

production, associated with immunosuppressive effects (171). MDSCs also have a direct effect on PCa progression through production of interleukin-23 (IL-23), which supports the development of castration resistant PCa through activation of AR signalling (172).

1.3.3 B-cells

B-cells (CD19⁺) are cells that mature in the bone marrow and protect against infection through production of antibodies after antigen specific activation by T-cells, primarily in secondary lymphoid organs, and are central to the adaptive immune response (173). Activated B-cells localize within germinal centres and undergo somatic hypermutation and affinity maturation, regulated by interactions with follicular helper CD4⁺ T-cells [T_{FH}] (174). Activated B-cells in the GC differentiate into long-lasting memory B-cells and short-lived plasmablasts that produce high affinity antibodies that protect against pathogens by neutralization, opsonization and complement activation. B-cells exert anti-tumour effects through production of tumour-inhibitory antibodies and formation of tumour-adjacent tertiary lymphoid structures that activate and recruit CD4⁺ T_H1 and CD8⁺ CTLs [reviewed in ref. (175)]. However, a subset of B-cells termed regulatory B-cells (Breg) suppress anti-tumour immunity through production of IL-10 and interleukin-35 (IL-35), inhibiting IFN γ production through STAT3 activation in CD8⁺ TILs [reviewed in ref. (176)].

B-cells remain largely understudied in PCa. In preclinical models of castration resistant PCa, the production of CXCL13 by PCa promotes B-cell recruitment and lymphotoxin production, which promotes the growth of androgen-deprived PCa (177). The transition of localized PCa to advanced disease is associated with reduced

peripheral blood CD19⁺ B-cells and a concomitant increase in peripheral lymph nodes, implicating a tumour-associated B-cell response with PCa progression (178). However, the overall prognostic significance of B-cell tumour-infiltrates in human PCa is less clear (178,179). Although by single-cell comparisons of malignant vs adjacent healthy tissue, total B-cell infiltrates decrease, others report total B-cell numbers increase within malignant lesions (151,180). Nevertheless, tumour-infiltrating B-cells display a more activated phenotype, with an abnormal polarization towards IgG4 production (181). The biological significance of IgG4 polarized B-cells in PCa is not known, but is suggested to contribute to immune-escape in other cancer types through local IL-10 production (182). In support of this, prognostic associations of suppressive Breg (CD19⁺ IL-10⁺) populations have been subsequently identified within malignant PCa lesions, and the number of Bregs within PCa correlates with tumour stage (183). Yet how, or why Bregs accumulate within human PCa is unclear.

1.3.4 CD4⁺ T-cells

CD4⁺ T-cells are important cells of the adaptive immune response that recognition of cognate antigens presented by MHC class II molecules on competent antigen presenting cells (184). Naïve CD4⁺ T-cells undergo clonal expansion and differentiation into distinct memory and effector subtypes that produce cytokines and chemokines that co-ordinate cytotoxic CD8⁺ T-cell activation, antibody mediated B-cell responses and directly cytolyze infected cells (185–187). The priming environment of naïve CD4⁺ T-cells and strength of T-cell receptor (TCR) stimulation has a profound effect on the development of CD4⁺ T-cells into subsets with specialized function [reviewed in ref. (188)]. Briefly, these include T_H1, T-helper 2, T-helper 9, T-helper 17

(T_H17) cells and T-helper 22 cells, that support distinct immunological responses against classes of pathogens (189–191), T_{FH} cells that support B-cell antibody production (192) and peripheral regulatory T-cells (pTreg)/Type-1 regulatory T (T_R1) cells that suppress immune responses to foreign/neoantigens (193,194). Glucose, lipid and amino acid metabolism has a profound effect on the fate of CD4⁺ T-cells during differentiation through direct and indirect interactions with signalling pathways, including mechanistic target of rapamycin complex (mTOR) and AMP-activated protein kinase [AMPK] (195–197). Although dependent on the organ of tumour development and stage of disease, broadly, T_H1 CD4⁺ T-cells promote anti-tumour immunity through priming of tumour-specific CD8⁺ CTLs, improving both the functional quality and magnitude of anti-cancer CD8⁺ CTLs activation and memory cell formation (19). CD4⁺ CTL and T_{FH} subsets are also generally associated with anti-cancer effects, whilst Tregs and T_H17 cells are associated with tumour promoting effects through suppression of anti-tumour CTLs [reviewed in ref. (198)].

Although the PCa TME is often referred to as an immunological desert for T-cells, CD4⁺ TILs are selectively enriched in prostate tumours mediated by production of CXCL9 by PCa cells (199). Increased infiltration of total CD4⁺ TILs correlates with poor prognosis (200), however, interpretations of these early associations were limited by the lack of delineation between pro/anti-tumorigenic CD4⁺ subsets, such as CD4⁺ CTLs from Tregs. Since then, analysis of conventional CD4⁺ T-cells (T_{conv}) [CD4⁺ FoxP3⁻] infiltration has shown a prognostically favourable association between T_{conv} infiltration within the tumour stroma, overall survival and a reduced risk of lymph node metastasis (201). However, compared with healthy glands, PCa-infiltrating naïve

and central memory CD4⁺ T-cells express significantly reduced levels of receptors that provide co-stimulatory activation signals upon engagement with APCs, including cluster of differentiation 28 (CD28), tumour necrosis factor receptor superfamily, member 4 (OX40) and inducible T-cell co-stimulator (ICOS), indicative of a reduced propensity for activation by APCs (202). PCa CD4⁺ TILs are skewed towards both T_H17 and Tregs (203), and downregulate perforin and IFN γ (204), indicative of a widespread attenuation of both CD4⁺ CTL and T_H1 cell function.

1.3.5 CD4⁺ regulatory T-cells

CD4⁺ Tregs are a heterogenous subset of T-cells that limit effector immune responses to foreign and autoantigens via cell-cell interactions and production of cytokines/metabolites (205). Most Treg subsets express the transcription factor Forkhead box P3 (FoxP3) that supports lineage specification, phenotypic stability, metabolic fitness and the expression of genes with regulatory function (206–209). The most commonly studied Treg subsets in cancer development are thymic Tregs (tTregs) that differentiate in the thymus from immature precursors and express interleukin-2 receptor alpha chain (CD25), and pTregs that differentiate from naïve or effector CD4⁺ T-cells extrathymically in peripheral tissues upon antigen stimulation in the presence of retinoic acid, TGF- β and IL-2 (210). Treg subsets can be further delineated by T_R1 (193), T-helper 3 [T_H3] (211), CD8⁺ (212), CD4⁺CD8⁺ alpha-beta ($\alpha\beta$) TCR⁺ (213), visceral adipose tissue [VAT] (214), gamma-delta [$\gamma\delta$] (215), and follicular [T_{FR}] (216) regulatory T-cells with specialized function. Within the TME, the current working model posits that tTregs recognise self-antigens, whilst pTregs recognise somatic mutation-derived antigens (neoantigens) with a different TCR repertoire (217–219).

Tumour infiltrating Tregs (TI-Tregs) have multiple origins, including circulating tTregs, tissue-resident Tregs and pTregs generated *in situ* and suppress antitumor CD8/CD4 CTLs, NK cells, NKT-cells and dendritic cells through multiple mechanisms (220). Treg expression of CTLA-4 depletes cluster of differentiation 80 (CD80) and cluster of differentiation 86 (CD86) expressed by APCs by trogocytosis, inhibiting T-cell co-stimulatory activity of APCs (221). Tregs also induce bystander suppression of proximal APCs and effector T-cells through i) competitive extracellular uptake of cysteine, inducing redox remodelling in a cell-contact and CTLA-4 dependent manner and ii) competitive consumption of interleukin-2 (IL-2) mediated by high expression of the cell-surface high-affinity interleukin-2 receptor complex (222,223). Other mechanisms include the secretion of immunosuppressive mediators' IL-10, IL-35, TGF- β , and extracellular adenosine generation by hydrolysis of ATP by cell-surface CD39 and 5'-ecto-nucleotidase (CD73) expression (224–226). Production of granzyme-B and perforin by Tregs also induces direct cytolysis of CD8⁺, NK and mature dendritic cells, inhibiting anti-tumour immunity *in vivo* (227).

There is a growing appreciation for the important role of metabolism in the phenotypic stability and suppressive function of Tregs (209). Relative to T_H1, T_H2 and T_H17 cells, Treg cells are less reliant on glycolysis for their bioenergetic needs, enhancing FAO fuelled OXPHOS (228,229), and display significant metabolic plasticity in response to mTOR and AMPK signalling (228,230–234). Whilst high lactate production and high glucose utilization by tumour cell metabolism inhibits tumour-infiltrating effector T-cell metabolism, Tregs are metabolically flexible and utilize lactate as a substrate to fuel mitochondrial OXPHOS and cytoplasmic fatty acid

synthesis [FAS] (235,236). Although glycolytic Treg metabolism appears both detrimental and beneficial in a tumour-dependent manner (237–240), lipid and cholesterol metabolism is indispensable for TI-Treg suppressive function (241,242).

Tregs are regarded as one of the most important cell types responsible for the development and maintenance of an immunosuppressive TME in PCa (243). Murine prostate allografts convert tumour-infiltrating T_{conv} cells into pTreg cells through production of TGF- β (244), and recruit circulating tTregs via the C-C chemokine ligand-17 (CCL17)-C-C chemokine receptor type 4 (CCR4) axis (245). In immunocompetent mouse models of PCa, depletion of TI-Tregs by anti-CCR4, anti-CD25 or pTreg migratory function by kruppel-like factor 2-deficiency enhances anti-tumour immunity, inhibits tumour growth and improves survival (217,245,246). In men, the presence of Tregs infiltrating the epithelium of normal prostate tissue is associated with a four-fold increased risk of developing PCa (247). In localized disease, Tregs infiltrate the malignant epithelium and the stromal niche to a greater extent than adjacent benign tissue. Tregs infiltrating localized PCa display a high suppressive activity score that exclusively and highly express genes from the tumour necrosis factor receptor superfamily (TNFRSF); OX40, TNFRSF9 (4-1BB) and TNFRSF18 (GITR). OX40, 4-1BB and GITR bind TNFSRF ligands (TNFSFRL) 4 [OX40L], 9 [4-1BBL] and 18 [GITRL] expressed by APCs respectively (151). High expression of OX40, 4-1BB and GITR is frequently reported in TI-Tregs with high suppressive potency (248–250). However, the consequence of OX40 and GITR receptor ligation with OX40L and GITRL is less clear (251–253). Treg infiltration in localized PCa correlates with increased risk of death (254), bone metastasis (255), whilst circulating Treg frequency correlates with

advanced tumour stage (243). Thus, Tregs are a central component to the immunosuppressive TME in PCa.

1.3.6 CD8⁺ T-cells

CD8⁺ T-cells are an important subset of lymphocytes and the adaptive immune response that protect against intracellular pathogens and tumours through recognition of peptide sequences presented by MHC class I molecules or MHC deficient cells by killer cell lectin-like receptor subfamily K, member 1 NKG2D (256,257). Naïve CD8⁺ T-cells are activated primarily by engagement of cell surface TCRs with antigens presented by APCs, facilitated by co-stimulation of CD80/86 surface receptors and secretion of cytokines IL-12 by APCs (258). The generation and programming of memory CD8⁺ T-cells requires cytokine production, co-stimulation and licensing of DCs by helper CD4⁺ T_{conv} cells (259). Upon activation, CD8⁺ T-cells rapidly differentiate and expand, and with IL-12 co-stimulation acquire high cytolytic capacity through secretion of perforin, granzymes, IFN- γ and TNF- α [referred to as T-cell (Tc) 1 cells] (260). CD8⁺ Tc1 cells are widely considered one of the most important cell types for cancer immunotherapy and most powerful effectors of the anti-cancer immune response through TAA guided cytotoxicity of tumour cells. However, persistent exposure to TAAs induces the expression of cell-surface immune checkpoint molecules which normally protect against uncontrollable CD8⁺ T-cell activation (136), inducing anergy, exhaustion or apoptosis.

In preclinical models, co-operation between CD4⁺ T_{conv} and CD8⁺ T-cells is required for effective inhibition of implanted human prostate xenografts (261), and effector CD8⁺ T-cell function inhibits tumour growth and mediates the anti-tumour

effects of ICB in syngeneic mouse models (262,263). However, rapid tolerization and exhaustion of TAA specific CD8⁺ TILs occurs in murine PCa through progressive DNA-methylation induced by chronic TCR stimulation (264), IL-8 mediated inhibition of glycolytic metabolism and inhibition of cytolytic function by direct and inhibitory TGF- β signalling (265,266). Although most cancer types describe a positive prognostic association between CD8⁺ TILs and clinical outcomes, this association is less clear in human prostate cancer. Independent cohort studies investigating both low and high-risk PCa have found inconsistent associations between CD8⁺ TIL infiltration with both advanced tumour stage and improved survival (267–269). The majority CD8⁺ T-cell infiltration is limited and proximal to the periphery of malignant epithelium (268). Similar to CD4⁺ TILs in PCa, concomitant analysis of the functional status and intratumoural heterogeneity of CD8⁺ TIL subsets is essential to understand why CD8⁺ TILs may not function as an independent prognostic factor in a straightforward manner. In LG-PIN, CD8⁺ T-cells display an activated and cytotoxic phenotype (270). However, progression to high-grade PCa is associated with the acquisition of an exhausted phenotype and co-expression of multiple immune-checkpoints, including TIM-3, PD-1, TIGIT and CTLA-4. Multiple CD8⁺ Treg subsets with suppressive function have also been described in men with PCa, thought to be derived from interactions between tolerogenic dendritic cells and/or extracellular adenosine generated by CD73 expression by PCa cells (271–274). High CTLA-4 expression in CD8⁺ T-cells marks a subset of CD8⁺ Treg cells that secrete high levels of IL-35 that suppress TAA specific CTL immunity, and TILs isolated as CD8⁺CD25⁺FoxP3⁺ from PCa tissue display *ex vivo* suppressive function. Retrospective analysis of 1080 patients

with localized PCa also identified CD8⁺ TILs that express cluster of differentiation 103 (CD103), produce high levels of IL-10, PD-1 and CTLA-4 and correlate with poor prognosis (275). Taken together, CD8⁺ TILs display a largely exhausted and immunoregulatory phenotype in PCa, emphasizing the tolerogenic and suppressive nature of the PCa TME.

1.3.7 Cancer associated fibroblasts

Fibroblasts are a heterogenous cell-type that function as progenitors for tissue specific mesenchymal cells such as osteoclasts and adipocytes, that maintain and remodel ECM rich connective tissue and secrete cytokines, growth factors and metabolites that support cell growth, wound healing and immune regulation (276). Fibroblasts at the site of a tumour are directly and indirectly reprogrammed towards an activated state by growth factors and chemokines produced by cancer cells, including TGF- β , EGF, fibroblast growth factor (FGF), IL-1 β and IL-6 (277). Broadly referred as cancer associated fibroblasts (CAFs), their activation and subsequent production of growth factors and cytokines are associated with suppression of anti-tumour immune responses across cancer types.

In pre-malignant PCa, production of kallikrein-related peptidase 4 (KLK4) by HG-PIN lesions drives stromal CAF transformation through degradation of IGFBPs and proteolytic maturation of TGF- β (141). KLK4 also activates proteinase-activated receptor 1 expressed by prostate CAFs, inducing the production FGFs and vascular endothelial growth factors (VEGFs) that promote the recruitment and proliferation of Tregs and TAMs (278). Paracrine TGF- β signalling is the most well-studied mechanism of epithelial and CAF crosstalk in malignant PCa, and the elevated levels of TGF- β in

both pre-malignant lesions and localized PCa is consistent with the development of a wound repair-like and fibrotic (ECM dense) stromal microenvironment (279). Uptake of lactate generated by glycolytic cancer cells by CAFs also promotes IL-6 production, a pro-inflammatory cytokine implicated in impaired CD4⁺ T_H1 anti-tumour immunity through skewing towards interleukin-17a producing CD4⁺ T_H17 cells and downregulation of MHC class II by tumour-infiltrating dendritic cells (280). The activation of CAFs also promotes stromal matrix stiffening through overproduction of collagens, fibronectin and hyaluronate in addition to the downregulation of the chemokine stromal cell-derived factor 1 (CXCL12), associated with the exclusion of CD8⁺ T-cells from the TME (281).

1.3.8 Other cell types

The prostate TME contains other immune and stromal cell types that influence anti-tumour immunity in PCa, albeit at a lower frequency relative to other cell types mentioned previously. Intra-tumoural dendritic cells are important antigen presenting cells that activate anti-tumour T-cells (13). However, tumour-infiltrating dendritic cells in PCa are rare, and those that migrate to and infiltrate PCa display an immature phenotype with low T-cell co-stimulatory capacity (282). Obesity, marked by the expansion of visceral white adipose tissue, is strongly linked to prostate cancer risk and progression (156). The prostate is surrounded by periprostatic adipose tissue (PPAT) formed of adipocytes that accumulate fatty acids (87). Progression and development of aggressive disease occurs in PCa that invades local PPAT, high in adipocytes that secrete IGF-1, IL-6, TNF- α and leptin in paracrine manner. PPAT also releases high levels of

extracellular free fatty acids, that drives CD8⁺ TIL dysfunction and the formation of LL-TAMs.

Innate lymphoid cells (ILCs) are a group of innate immune cells with lymphoid morphology but lack re-arranged antigen specific receptors and myeloid/lymphoid lineage markers that includes NK cells, ILC1, ILC2 and ILC3 cells (283). Analogous to T-cells, ILCs are subdivided into cells with cytotoxic function (NK cells), and helper subsets that resemble T_H1, T_H2 and T_H17 cells (ILC1, ILC2 and ILC3 respectively). Increased infiltration of NK cells in PCa is broadly associated with improved clinical outcomes and patients with *TMPRSS2-ERG* fusion (284), whilst reduced NK cell infiltration is observed in patients with mutations in *PTEN* and *p53*. Relative to normal prostate tissue, malignant PCa is enriched for exhausted and Neural Cell Adhesion Molecule 1 dim (CD56) NK cells, suggesting impaired effector function (202). The frequency of peripheral blood ILC2s also increases as benign prostate glands transition to localized PCa (285), and ILC2s infiltrate subcutaneous allografts of murine PCa (286). How ILC2s contribute to anti-tumour immunity in PCa is currently not known, but is hypothesized to contribute to IL-13 driven MDSC immune suppression (287).

The role of other stromal cells in malignant PCa has been largely understudied relative to immune cell types. Smooth muscle cells (SMCs) are abundant in the fibromuscular stroma, yet are less organized and sequentially replaced by CAFs in the stroma of PCa (288,289). Other cell types include telocytes, a stromal cell type with multiple extensive thin extrusions found interspersed between fibroblasts and SMCs in the prostate stroma (290). Although their function within the healthy prostate is still poorly understood, telocytes secrete TGF- β and vascular endothelial growth factors

(VEGFs), hypothesized to contribute to stromal and immune remodelling associated with PCa (291). For ease of understanding, a summary of the immune and stromal infiltrate of PCa outlined in this section is shown in fig. 1.3.8.1.

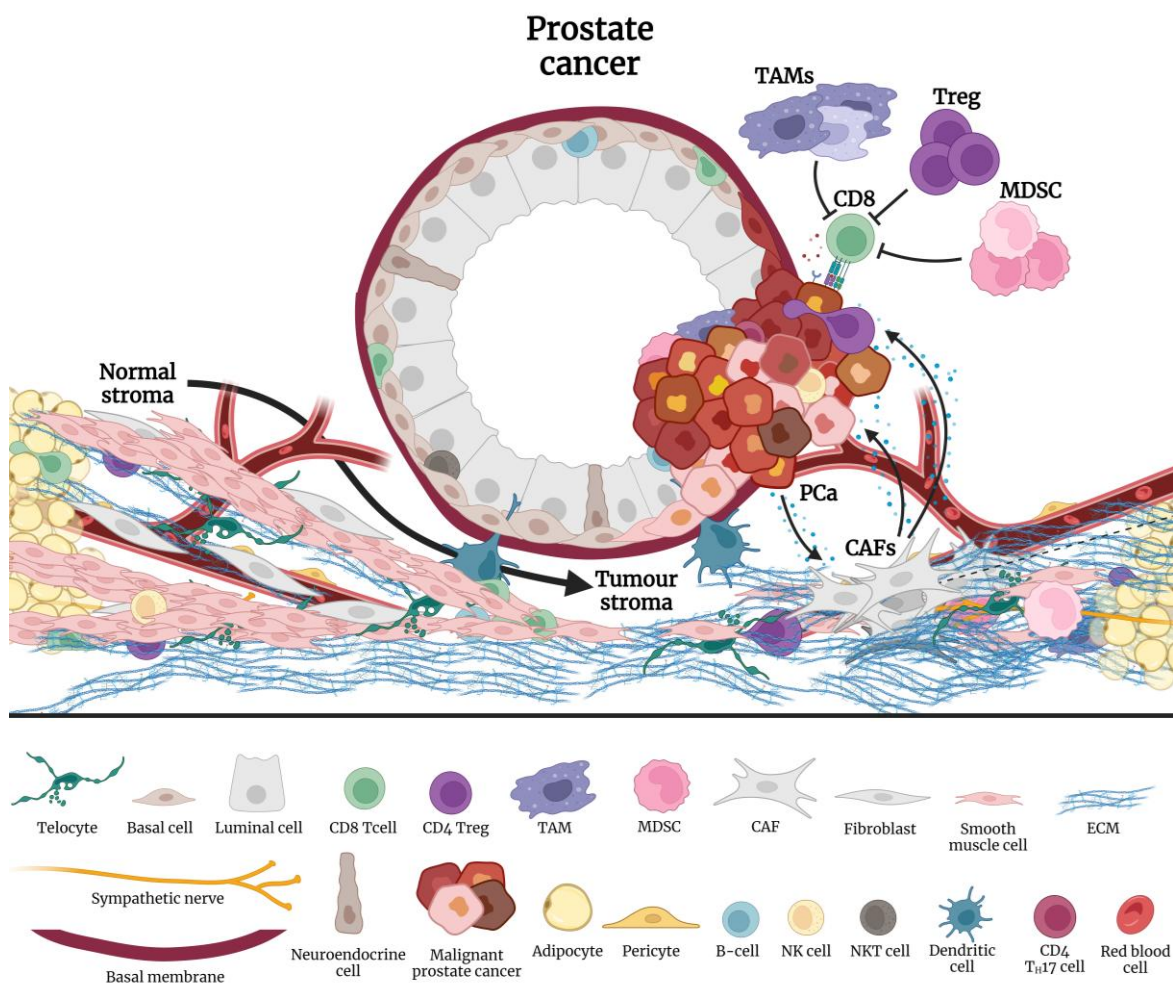


Figure 1.3.8.1 - The tumour microenvironment of malignant prostate cancer

The PCa tumour microenvironment is comprised of a non-cancerous immune infiltrate and proximal stromal niche that promotes immune evasion and cancer progression. Early pre-cancerous lesions promote the activation and transformation of the stromal niche, activating stromal cell production of growth factors. Malignant PCa development is further supported by ECM deposition and the production of immunoregulatory cytokines and chemokines that recruits and expands of immunosuppressive cell types, including MSDCs, Tregs and TAMs that inhibit CD8 T-cell immunity. Figure generated in BioRender.

1.4 The insulin-like growth factor-1 axis

1.4.1 Insulin-like growth factor-1

IGF-1 is a 7-kilodalton anabolic hormone that shares structural homology with both insulin-like growth factor-2 (IGF-2) and insulin [67% and ~45-52% respectively] (292). The functions of IGF-1 are mediated through association with the insulin-like growth factor receptor 1 (IGF-1R), a receptor that is expressed in almost all cell types with a notable exception for hepatocytes (293). IGF-1 plays an important role in normal growth and development by inhibiting apoptosis, promoting cell proliferation, stimulating protein synthesis and inducing cell differentiation (294). Synthesis and secretion of IGF-1 predominantly occurs by liver hepatocytes in response to binding of growth hormone (GH) to GH receptors (295). In addition to regulation by GH, circulating IGF-1 levels are also affected by dietary factors, such as dairy consumption, protein intake and fasting (296). Endogenous IGF-1 is also produced locally within tissues in both an autocrine and paracrine manner by macrophages, adipocytes, fibroblasts, bone marrow stromal cells and endothelial cells (297–299). In addition to supporting somatic growth and development, IGF-1 is implicated in a diverse range of biological processes related to central nervous system development, neuroprotection, adipogenesis, wound healing, inflammation, ageing and metabolism (297,300–302).

1.4.2 Insulin-like growth factor binding proteins

IGF-1 bioactivity is regulated by six IGF binding proteins (IGFBPs) that bind to insulin-like growth factors (IGFs) with high-affinity (303). Binding of IGFBPs to IGF-1 increases the half-life of IGF-1 in the circulation from 10 to 25 minutes (304), and

prevents IGF-1R activation by IGF-1. Around 70-80% of IGF-1 in the circulation forms a ternary complex with IGFBP3 and acid labile subunit (305), which prevents exit of bound IGF-1 through the capillaries, restricting it within the circulation and increasing the half-life to ~16 hours (306). IGFBP3 is produced by hepatocytes in the liver and its production is regulated by GH, which allows for a proportional absorption of IGF-1 to the circulation (307). Conversely, IGFBP1 is produced by hepatocytes in response to amino acid shortages, hypoxia, and starvation to dynamically inhibit the bioactivity of IGFs in response to stress or nutrient deprivation (308). IGFBPs also exert IGF independent regulation of cellular function through functional nuclear localization sequences and binding to cell surface receptors (309,310). Proteolysis of IGFBPs functions to increase the local concentration of bioactive IGFs to cell surface IGF receptors in a cell-type and tissue specific manner (311). IGFBP specific proteases pregnancy-associated plasma protein-A (PAPP-A) and PAPP-A2 cleave IGFBP-IGF complexes, liberating IGFs (312). Other proteases with reported IGFBP proteolytic function include complement components 1s, 1r, a disintegrin and metalloprotease-12, plasminogen, kallikrein-related peptidases (KLK) 2,3 and 4, cathepsin D-like protease and matrix metalloproteases (MMP)-2, 7, 9 and 12 (311).

1.4.3 Insulin-like growth factor receptor-1

IGF-1R is a ~440-kDa heterotetrameric transmembrane receptor with ~50% sequence homology with the insulin receptor (InsR) that is bound with high affinity by IGF-1 (1-5 nM), IGF-2 (17.8 nM) and with low affinity by insulin (> 100 nM) (313–315). Homodimeric IGF-1R is formed from the non-covalent association of two extracellular alpha subunits and two transmembrane beta subunits with tyrosine kinase domains (316). In the absence of IGF ligands, two membrane-proximal fibronectin type III domains (FnIII) of IGF-1R are spatially separated by ~67 angstrom (Å) that maintain an autoinhibited state (317). IGF binding triggers a conformational rearrangement and formation of an asymmetrical dimer that reduces the distance between the two beta subunits with tyrosine kinase domains, resulting in trans-autophosphorylation of tyrosine kinase residues and receptor activation.

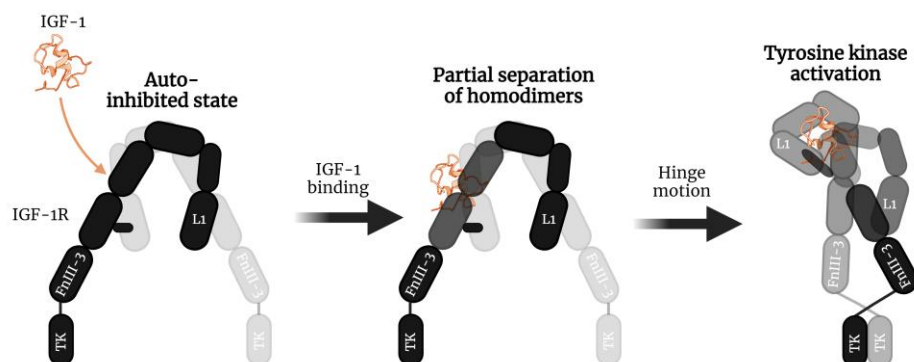


Figure 1.4.3.1 - Working model of IGF-1 mediated IGF-1R activation

IGF-1R protomers are coloured in black and grey, with IGF-1 in orange. L1 = leucine-rich repeat domain, FnIII-3 = fibronectin type III domains, TK = tyrosine kinase domain. Figure adapted from *Li et al*, made in BioRender.

Monomers of IGF-1R can also combine stochastically with InsR isoforms to form heterodimeric hybrid receptors, if both InsR and IGF-1R monomers are expressed in the same cell (318). Whilst the mechanism of formation or biological function of IGF-1R-InsR receptor is poorly understood, hybrid receptor formation is proportional to the relative mole fractions of IGF-1R/InsR in non-malignant cells (319). IGF-1 has a 50-times higher affinity for hybrid IGF-1R-InsR receptors than insulin (320), and is hypothesized to mediate the effects of IGF-1 on improved insulin sensitivity (321). Notable IGF-1R-InsR hybridization occurs in breast, prostate, and thyroid cancers and promotes cell growth through binding of IGF-1 (322–324).

1.4.4 Canonical IGF-1R signalling

Upon tyrosine kinase autophosphorylation, IGF-1R promotes the recruitment and phosphorylation of intracellular adapter proteins such as insulin receptor substrates (IRS-1/2) and src homology 2 (SH2)-containing-proteins [Shc] (325,326). First, phosphorylation of IRS1/2 proteins recruited to IGF-1R promotes interactions with the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K) class I, SH-PTP2 (a tyrosine phosphatase) and growth-factor-receptor-bound protein 2 (Grb2) (327). The recruitment of the p85 regulatory subunit of PI3K to phosphorylated IRS proteins leads to the activation of the catalytic subunit p110 and catalysis of phosphatidylinositol-3,4,5-triphosphate (PIP_3) from phosphatidylinositol-4,5-bisphosphate (PIP_2) at the inner cell membrane (328). Then, protein kinase B (Akt) is phosphorylated at threonine 308 by phosphoinositide-dependent kinase-1 (PDK1) and serine 473 by mammalian target of rapamycin complex 2 (mTORC2) (329). Phosphorylated Akt activates mTORC1, which in turn regulates the phosphorylation

of S6 kinases and the eukaryotic initiation factor 4E binding protein 1 (4E-BP1) – regulating cell growth, metabolism, protein synthesis and proliferation (330,331). Of note, IGF-1 cannot efficiently activate mTORC1 under amino acid deprivation conditions, and intracellular lysine is required for effective IGF induced mTORC1 activation (332,333). Although phosphorylation of both Thr308 and Ser473 is thought necessary for the complete activation of Akt, phosphorylation of Thr308, but not Ser473, leads to mTORC1 activation (334). Curiously, mTORC2 can also directly phosphorylate tyrosine residues 1131/1136 of IGF-1R and promote IGF-1R activation (335).

The second major pathway downstream of IGF-1R activation is mediated by Grb2 through recognition of SH2 domains from IRS and Shc, recruiting the guanine nucleotide exchange factor (GEF) son of sevenless (SOS) to the cell membrane (336). SOS interacts with Ras at the cell membrane, facilitating a conformational change that enables the exchange of guanosine diphosphate (GDP) for guanosine-5'-triphosphate [GTP] (336). GTP-bound Ras subsequently binds and activates the serine/threonine kinase Raf1 to the plasma membrane, which activates mitogen-activated protein kinase 1 (MEK1). MEK1, in turn, activates and phosphorylates the mitogen-activated protein kinases (MAPKs) extracellular-related kinase (ERK) 1 and 2 which phosphorylate and activate a wide range of nuclear transcription factors and kinases, including ETS Like-1 protein (Elk-1), ETS Proto-Oncogene (c-Ets) 1/2, S6 kinase (S6K) and MAP kinase-interacting serine/threonine-protein kinase (MNK) 1/2, promoting cellular proliferation and cell cycle progression. However, It must be acknowledged that considerable divergent regulation of PI3K-AKT and MAPK signalling occurs by

regulation of substrate activation and expression of catalytic and/or regulatory subunits, in cell-type dependent manner (337–339).

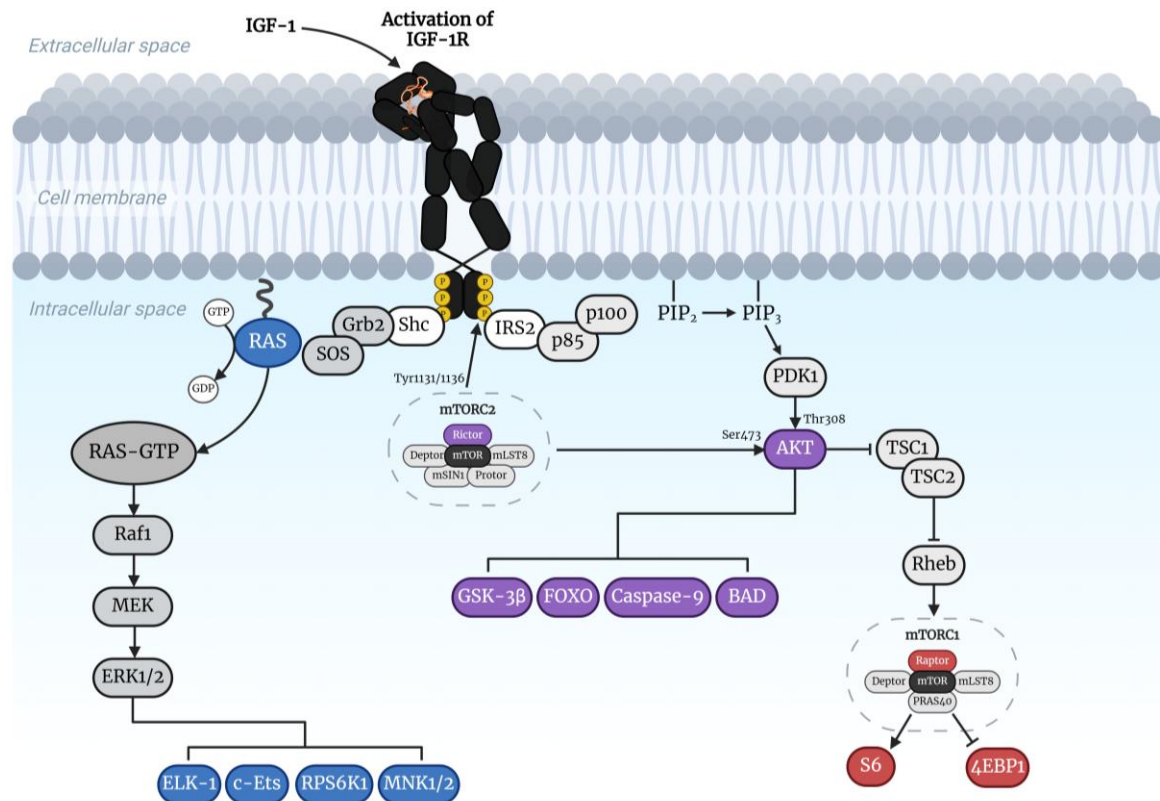


Figure 1.4.4.1 - Classical model of canonical IGF-1R signalling

Figure shows canonical pathways activated upon IGF-1 binding to IGF-1R, promoting tyrosine kinase autophosphorylation and subsequent PI3K-AKT-mTOR and MAPK signalling. Figure generated in BioRender.

1.4.5 Non-canonical IGF-1R signalling

In recent years it has become evident that IGF-1R functions as a hybrid receptor with both tyrosine kinase and G protein-coupled receptor activity. The latter is thought to be mediated through the additional recruitment of G-proteins and β -arrestins to IGF-1R (340). Upon IGF binding, GRK2 mediated phosphorylation of Ser1248 of IGF-1R can promote transient β -arrestin-2 binding and IGF receptor recycling. This, in turn, results in decreased ERK activation and promotes early phosphorylation of AKT. Conversely, GRK6 mediated phosphorylation of ser1291 on IGF-1R promotes a stable interaction of IGF-1R with β -arrestin-1, associated with increased ERK activation and late AKT phosphorylation. Thus, the differential expression of G-proteins/ β -arrestins that associate with IGF-1R could confer an indirect means of regulating both the magnitude and temporal nature of IGF-1R signalling, potentially facilitating cell-type specific effects. Further regulation of IGF-1R signalling can also occur after internalization of the receptor–ligand complex by modifications of the endocytic pathway (341). For example, low concentrations of IGF-1 (~1 nM IGF-1) promote clathrin mediated endocytosis and MDM2 proto-oncogene (MDM2)-mediated ubiquitination of IGF-1R, whilst high concentrations of ligand (~5–15 nM IGF-1) promote caveolin mediated endocytosis and Cbl proto-oncogene (Cbl)-mediated ubiquitination. This endocytic process can also be directly regulated by adapter proteins such as IRS-1, that sustain IGF-1R signalling by inhibiting clathrin-mediated endocytosis and receptor degradation (342).

Nuclear localization of internalized IGF-1R has also been described, a process that requires receptor SUMOylation by small ubiquitin-like modifier protein-1

[SUMO-1] (343). In cancer cells, nuclear IGF-1R functions as a transcriptional co-factor for lymphoid enhancer-binding factor 1 (LEF1) and interacts with RNA polymerase II at regions of genomic DNA, promoting expression of transcription factor Jun (JUN) and WASH complex subunit 2A [FAM21] (344). Tyrosine kinase activity of nuclear IGF-1R has also been reported, and the phosphorylation of histone H3 at tyrosine41 by nuclear IGF-1R induces chromatin remodelling through binding of brahma-related gene-1 and subsequent expression of snail family transcriptional repressor-2 (345). Curiously, IGF induced internalization and cytosolic mitochondrial translocation of IGF-1R has also been reported in central neurons, required for efficient regulation of mitochondrial-to-cytosolic calcium flux (346). Finally, IGF stimulated IGF-1R can also stabilize β 1 integrin interactions with the extracellular matrix, forming intracellular scaffolds with receptor for activated C kinase 1 (RACK1) that results in focal adhesion kinase (FAK) dephosphorylation in focal adhesions and STAT3 phosphorylation (347).

In conclusion, although our appreciation for the complexity of IGF-1R signalling has grown in recent years, significant gaps in our understanding remain. For example, non-canonical functions of IGF-1R are primarily derived from studies of transformed cancer cells stimulated with IGFs, with the aim of identifying targets for therapeutic exploitation (340,343,345). Yet, it remains unclear as to whether these processes are biologically meaningful to other cell types that express IGF receptors. Detailed and standardized comparisons of IGF-1R signalling across different cell types could provide valuable insight into how the IGF-1 axis shapes and regulates such diverse and cell-type specific biological effects in health and disease.

1.4.6 The IGF-1 axis and prostate cancer

There is a causative association in the general population between increased serum IGF-1 levels and prostate cancer risk (79). Due to the mitogenic effects of IGF-1 binding to IGF-1R, epidemiological studies have long acknowledged the contribution of serum IGFs to cancer growth, treatment resistance and propensity for metastasis (348). However, experimental studies have also shown the expression of several components of the IGF-1 axis change within the PCa TME during tumour development. First, HG-PIN driven stromal remodelling results in the secretion of proteases, including KLK2, KLK3, KLK4, MMP-2 and MMP-9 that liberate IGFs bound to IGFBPs proximal to the epithelium (141). Subsequent transformation of the stromal and immune infiltrate during the transition from benign to malignant PCa is associated with increased stromal: epithelial IGF-1 axis crosstalk by secretion of IGF ligands by CAFs, TAMs and adipocytes (151,349,350). Curiously, paracrine IGF-1 secretion by CAFs and autocrine IGF-1 production by PCa cells is transcriptionally upregulated by AR signalling, which increases IGF-1R expression and inhibits IGFBP3 production in transformed epithelial cells (351,352). Thus, stromal cells responsive to AR signalling are able to produce high levels of IGF ligands to support local tumour growth. Genetic abnormalities and/or chromosomal alterations including *TMPRSS2-ERG* fusion also result in upregulated expression of IGF-1R in PCa epithelial cells, further enhancing cell growth, angiogenesis and DNA damage repair (353).

First generation therapeutics targeting the IGF-1 axis for cancer treatment included the development of humanized monoclonal antibodies (mAbs) directed against IGF-1R that blocked receptor-ligand binding, or small molecule drugs that

inhibited IGF-1R tyrosine kinase activity and signalling (354). Despite promising preclinical efficacy, both classes of IGF-1R inhibitors demonstrated inconsistent overall survival benefits in clinical trials across multiple cancer types (355). Initially, the lack of therapeutic efficacy was thought hindered by dose-limiting hyperglycaemia caused by co-inhibition of InsR signalling (356). Thus, second generation humanized monoclonal antibodies MEDI-573 (dusigitumab) and BI-836845 (Xentuzumab) were subsequently developed that potently neutralize IGF-1 and IGF-2, but not insulin (357,358). Whilst dose-limiting toxicity and metabolic disorders were avoided, results from trials have thus far have failed to demonstrate clinical benefit in advanced disease, including in patients with mCPRC (359,360). Our current understanding is that for the majority of tumours, IGF-1 axis dysregulation occurs secondary to other molecular events that drive malignant progression (361). Thus, although high IGF-1 axis activity supports tumour growth, therapeutic inhibitors remain unlikely to be efficacious when administered as a monotherapy.

1.5 The IGF-1 axis and immunity

Conversely, it is becoming increasingly clear that IGF-1 plays a critical role in the function of the immune system. Administration of exogenous IGF-1 *in vivo* stimulates lymphopoiesis (362), granulopoiesis (363), and myelopoiesis (364). IGF-1R is expressed ubiquitously by immune cells (365–370), and the IGF-1 axis plays an important role in the regulation of immunity and inflammation (371–373). IGF-1 axis activation in immune cells is broadly associated with anti-inflammatory effects (374), but under certain conditions can provoke pro-inflammatory effects (375). In this section we outline key epidemiological and experimental studies that link the IGF-1

axis and immunity, and explore what is currently known about the IGF-1 axis and the regulation of anti-tumour immunity.

1.5.1 The IGF-1 axis and immune regulation

In 1986, Drs Hunt and Eardley performed the first series of experiments wherein IGF-1, normally associated with growth-promoting functions, inhibited IL-2 induced lymphocyte proliferation at concentrations ranging from 1-5 $\mu\text{g mL}^{-1}$ [130-600 nM] (376). Since then, epidemiological studies of sIGF-1 levels have long connected high sIGF-1 with protective effects against inflammatory driven pathology: including fatal COVID-19 infection (377), myocardial infarction (378), and autoimmune diseases such as Rheumatoid Arthritis [RA] (379) and Inflammatory Bowel Disease (380). More recently, five viral-like insulin peptides (VLIPs) with 30-50% homology to human IGF-1 have been discovered from the *Iridoviridae* family, hypothesized to regulate host-microbe immune interactions by selective agonism and antagonism of IGF-1R signalling (381).

Preclinical studies suggest that high IGF-1 axis activity supports the anti-inflammatory activity of Tregs, neutrophils, macrophages and myeloid cells. IGF-1 stimulates Treg proliferation (382,383), and in mouse models, ectopic expression of IGF-1 in skin cells in mice alleviates symptoms of allergic contact dermatitis and spontaneous type-1 diabetes (384). In both cases, IGF-1R activation in Tregs was required for protective immunosuppressive effects. In mouse models of sepsis, a pathogenic subset of CD200-receptor 1 expressing neutrophils were found to secrete IGF-1, enhancing systemic immunosuppression by increasing Treg numbers in secondary lymphoid organs (385). Furthermore, increased IGF receptor expression is

also associated with enhanced Treg suppressive activity. Tumour-infiltrating intrahepatic Tregs that express high levels of IGF-1R secrete more IL-10, TGF- β and display a transcriptional enrichment of genes required for OXPHOS and glycolysis relative to TI-Tregs that do not express IGF-1R (386). Hence, both IGF-1 stimulation and increased IGF-1R expression appear to enhance both Treg numbers and anti-inflammatory activity.

Broadly, the IGF-1 axis also plays an important role in the regulation of myeloid/granulocyte cell function. IGF-1, but not insulin, promotes a transcriptional response that phenocopies IL-4 stimulation in neutrophils that results in development of a pro-tumorigenic N2-like phenotype (387). Macrophages are also an important source of IGF-1 within local tissues, which is associated with improved anti-inflammatory properties (372). Macrophages increase IGF-1 production in response to IL-4 and IL-13 stimulation, which forms an autocrine feedback loop via IGF-1R that re-enforces M2-like polarization (388) and the resolution of inflammation generated by muscle-injury, neurodegenerative inflammation and wound healing (302). IGF-1 also enhances the suppressive function of intestinal monocytes by increased IL-10 production, reducing intestinal inflammation in mouse models of colitis (389). Conversely, compromised IGF-1R signalling in monocytes breaks immune tolerance, resulting in autoantibody production (390). Hence within the TME, high IGF-1 activity could promote the anti-inflammatory nature of cell types typically associated with suppression of anti-tumour immune responses.

However, IGF-1 axis activation can also enhance the activity of immune cells that promote inflammatory responses. For example, T-cells isolated from patients with

autoimmune RA and Graves' disease express increased levels of IGF-1R, associated with inflammation and increased serum levels of the pro-inflammatory cytokine IL-6 (391,392). These observations were supported experimentally by *DiToro et al.*, wherein low-dose IGF-1 (1 nM) co-stimulation promotes the *in vitro* differentiation and aerobic glycolytic capacity of pro-inflammatory T_H17 cells in response to TGF- β and IL-6 (366). These observations were mirrored in ILC3 cells, the innate equivalent of T_H17 cells, whereby IGF signalling was required for the production of pro-inflammatory cytokines IL-17a and IL-22 in response to IL-23 stimulation. Intriguingly, the ILC3-IGF-1 axis also appears essential for the development of pulmonary immunity in newborns (370). Mediated by the production of IGF-1 by alveolar fibroblasts, the pulmonary niche instructs the maturation and expansion of IGF-1R⁺ ILC precursors into ILC3s. Similarly, autocrine IGF-1 production by human uterine decidual NK cells enhances their cytotoxic function through increased expression of intracellular perforins and granzymes (369). However, others have shown that inhibition of IGF-1R has no effect on human NK cell function or cytotoxicity, and the subject remains under considerable debate (393). Nevertheless, these observations highlight the developmental influence of the IGF-1 axis on immune regulation, and that the inflammatory milieu at the point of activation or the relative magnitude of IGF-1R activation could be important determining factor shaping the immune response to IGF-1.

1.5.2 The IGF-1 axis and anti-tumour immunity

Considering the broad regulatory effects of IGF-1 axis activation on immune cell function, it is perhaps unsurprising that IGF-1 has emerged as an important factor involved in the regulation of anti-cancer immunity, and thus, response to cancer

immunotherapy. Multiple treatment modalities have been used to experimentally modulate the bioactivity of IGF-1 within the TME, albeit to a different degree. This includes the use of antisense oligodeoxynucleotides (AS-ODN) or pharmacological inhibitors directed against IGFs/IGF-1R and the use of intermittent hypocaloric dietary interventions (357,394,395).

Early preclinical observations also supported the hypothesis that high IGF-1 axis activity within the TME promotes an immunosuppressive microenvironment. For example, inhibition of autocrine/paracrine IGF-1 production in rat glioblastoma by AS-ODNs enhanced tumour cell MHC-I expression and promoted tumour-specific CD8⁺ T-cell immunity, implicating low IGF-1 bioactivity with improved anti-cancer immunity (394). These observations were supported experimentally in murine B16 melanoma, whereby the overexpression of IGF-1 within tumour cells reduced anti-tumour serum antibody titres and inhibited the formation of TAA-specific CD8⁺ T-cells (396). *In vitro*, stimulation of DCs with IGF-1 shapes their functional maturation, inhibiting tumour-antigen specific CD8⁺ CTL priming (397). Consequently, the regulation of dendritic cell function by IGF-1 in the TME provides a mechanistic basis as to how changes in IGF bioactivity could shape anti-tumour immune responses. However, IGF-1R expression by other immune cell types could remain an important regulatory component in the formation of their cellular identity within the TME (348). A notable example of this is illustrated in a model of Lewis-lung carcinoma, wherein induced IGF-1R-deficiency (in all cell types) led to the development of a less immunosuppressive TME; with reduced numbers of TI-Treg, TAM and TANs. Lastly, increased IGF-1R phosphorylation in the TME correlates with both poor prognosis and

a sparse CD8⁺ TIL, but high TI-Treg and TAM infiltrate in patients with breast cancer (398). Hence, high IGF-1 axis activity is broadly associated with poor disease control and the formation of a more suppressive TME.

In preclinical tumour models, treatment with IGF-1 axis inhibitors shows promise as a potential therapeutic approach to enhance cancer immunosurveillance. For example, administration of an IGF neutralizing drug in mice with orthotopic Pancreatic ductal adenocarcinoma (PDAC) enhanced CD8⁺ CTL infiltration in TME, although their cytotoxic phenotype did not improve (399). These observations were further supported in intrasplenic PDAC models of metastasis, whereby therapeutic inhibition IGFs enhanced CD8⁺ CTL infiltration within PDAC liver metastases, associated with increased PD-1 expression (400). The authors noted significant reductions of other suppressive cell types in the metastatic TME, including M2-like TAMs, G-MDSCs, N2-TANs and increased infiltration of anti-tumour MHC-II⁺ dendritic cells. Moreover, combination therapy with anti-PD-1 ICB stimulated IFN- γ production by CD8⁺ CTLs, and improved overall survival. Others have since corroborated observations that combined inhibition the IGF-1 axis and anti-PD-1 has a synergistic effect on enhanced anti-tumour immunosurveillance and tumour growth delay in epithelial ovarian tumours and murine MC-38 colon tumours (401,402).

One other possible mechanism as to how IGF-1 axis inhibition enhances anti-tumour immunity is through the induction of autophagy in tumour epithelial cells (401), which could link to small observation studies and randomized controlled trials that have long associated dietary intake with changes in IGF-1 bioactivity (296). For example, high protein and dairy intake is associated with increased sIGF-1, whilst

vegan diets (no dairy, reduced protein) and FMD (2-5 days of 300-600 Kcal/day followed by 16 to 23 days of refeeding) reduce sIGF-1 levels (403–405). In evolutionary terms, dietary linked fluctuations in IGF-1 levels likely represents an adaptation of humans/animals to survive in environments whereby food supply undergoes periods of relative scarcity. Notably, hepatocytes become insensitive to GH signalling upon hypocaloric intake and produce less IGF-1. Thus, IGF-1 production is linked to diet as a means of communicating nutrient abundance to cells, which biologically functions to facilitate metabolic adaptation, cellular stress resistance and cessation of proliferation (406).

Recent clinical studies have shown that FMD interventions, that reduce sIGF-1 levels, induce a striking protective effect on healthy cells to chemotherapy induced toxicity and signs of improved anti-tumour effects (395,403,407). This was accompanied by pre-clinical evidence that FMD-like interventions and caloric restriction mimetics (active substances that mimic some of the FMD-like effects) concomitantly decrease immunosuppression and boost anti-tumour immunity through TI-Treg depletion, TI-MDSC depletion, loss of M2-like macrophage polarization, increased TI-CD8 CTL infiltration and regeneration of haematopoietic stem cells [HSCs] (408,409). The relevance of this approach for improving cancer immunosurveillance can be seen in a recent trial inclusive of different cancer types. FMD, together with neoadjuvant chemotherapy, induced a contraction of peripheral blood/tumour MDSCs and Tregs, whilst augmenting tumour infiltration of CD8⁺ CTLs, DCs and NK cells (403). Crucially, the effects of FMD appear to be mediated by decreased levels of serum IGF-1, insulin, glucose and leptin. Importantly, the relative

contribution of individual hormones/metabolites to this immunological response is currently not known.

Nevertheless, preclinical observations suggest that changes in IGF-1 bioactivity are critical for the anticancer immunosurveillance effect of FMD-like interventions. Crucially, the anti-tumour and TI-Treg depleting effects of the hypocaloric mimetic hydroxycitrate were abolished upon administration of recombinant IGF-1 (409). Second, *Ajona et al.* show fasting cycles of 48 hours (referred herein as short-term starvation/STS) are sufficient to reduce sIGF-1 levels, and sensitize subcutaneous murine lung tumours to anti-PD-1 ICB (395). Although fasting alone did not affect the frequency of tumour-infiltrating CD8⁺ CTLs or Tregs, the combination of STS anti-PD-1 boosted the CD8⁺/Treg ratio and cancer inhibitory immunosurveillance that was CD8⁺ CTL dependent. Whilst both studies demonstrate the activation of autophagy in tumour cells by IGF-1R inhibition as the primary mechanism of improved immunosurveillance, both authors emphasise that further mechanistic studies are required to obtain a more complete understanding of cell-type specific roles in fasting-induced immunosurveillance.

1.6 Aims of this thesis

Tregs are essential to the establishment of immune tolerance within the PCa TME, and potently suppress anti-tumour CD8⁺ CTLs (246). Increased Treg infiltration correlates with poor clinical parameters, including PCa risk, advanced tumour stage and mortality (243,254). Hence, the identification of therapeutic strategies that selectively deplete immunosuppressive TI-Tregs, without affecting Tregs in healthy tissues, is a desirable but challenging goal in PCa. Observations from both preclinical cancer models and clinical FMD studies show that reducing sIGF-1 levels could be one method of effectively reducing TI-Treg numbers (403,409). Yet most, if not all studies to date have thus far only investigated and linked plausible biological mechanisms of TI-Treg depletion to changes in IGF-1R⁺ tumour epithelial cells. However, TI-Tregs also express IGF receptors (386). Thus, understanding how IGF-1 supply regulates the phenotype and suppressive function of IGF-1R⁺ Tregs proximal to cancer cells is important to characterize (see chapters 3 and 4).

The sparse CD8⁺ CTL infiltrate and immunosuppressive PCa TME is a barrier to clinical benefit from ICB in mCRPC (138). In other preclinical tumour models, reduced IGF bioactivity boosts CD8⁺ CTL infiltration and sensitizes tumours to anti-PD-1 ICB (395,409). Notably, the PCa TME is high in IGF-1 bioactivity relative to other cancer types. Therefore, there is potential for synergy whereby therapeutic neutralization of IGFs in PCa sensitizes tumours to ICB through enhanced CD8⁺ CTL infiltration and depletion of immunosuppressive TI-Tregs. Using a syngeneic mouse model of MYC-amplified PCa, the effects of IGF neutralization on T-cell infiltration will be explored, and combination strategies with ICB will be investigated (chapter 5).

The translation of research findings to clinically meaningful interventions is often limited by preclinical models that do not fully recapitulate aspects of human immunology or disease (410). Although a correlation exists between decreased serum IGF-1 levels and enhanced anti-tumour immune responses, it is currently not known if this relationship is causative. The WINGMEN trial is a phase 0 study of the medicinal product Xentuzumab, an IGF inhibitor, in men with localized PCa prior to radical prostatectomy. Utilizing single-cell mass cytometry, the relationship between IGF inhibition and peripheral immunity in men with PCa will be explored, and immune cell subsets and functional markers that correlate with anti-tumour effects will be investigated (chapter 6).

Hence, the main aims in this thesis:

- Investigate how IGF-1 influences Treg function.
- Explore therapeutic synergy between IGF blockade and ICB in murine PCa.
- Understand how IGF neutralization shapes peripheral immunity in men with PCa and explore immune correlates of anti-tumour efficacy.

Chapter 2 - Materials and methods

2.1 Materials

2.1.1 List of experimental consumables

Table 2.1.1.1 Reagents and consumables used in this thesis

Product	Manufacturer	Cat. # number
1.5ml conical bottomed microfuge tubes	Fisher Scientific	#10051232
15ml falcon tubes	Fisher Scientific	#10345521
Heat inactivated Fetal Bovine Serum	Sigma	#F9665- 100ML
50ml un-skirted falcon tubes	Fisher Scientific	#10420602
Dimethylsulfoxide	Sigma	# D8418
Phosphate buffered saline	Invitrogen	#10010056
Cryovials	Medline Scientific	#127-T311- 200
BD Vacutainer™ Hemogard™ Closure Plastic K2-Edta Tube	BD	#BD 367525
Ficoll-Paque PLUS	GE Healthcare	#17-1440-02
Plastic Pasteur pipette (2mL)	Fisher Scientific	#10646341
LS columns	Miltenyi Biotec	#130-095-130
Recombinant mouse IL-2	BioLegend	#575402
CD3 Monoclonal Antibody (OKT3), Functional Grade	eBioscience™	#16-0037-81
CD28 Monoclonal Antibody (37.51), Functional Grade	eBioscience™	#16-0281-82
2-Mercaptoethanol	eBioscience™	#21985023
UltraComp eBeads™ Compensation Beads	eBioscience™	#01-2222-41
ArC™ Amine Reactive Compensation Bead Kit (for use with LIVE/DEAD™ Fixable dead cell stain kits)	eBioscience™	#A10346

Recombinant Human TGF- β 1 (carrier-free)	BioLegend	#781802
IFN gamma Monoclonal Antibody (XMG1.2) functional grade	eBioscience™	#16-7311-81
IL-4 Monoclonal Antibody (11B11)	eBioscience™	#14-7041-81
Naive CD4+ T Cell Isolation Kit, mouse	Milteyni Biotech	#130-104-453
CD4+CD25+ Regulatory T Cell Isolation Kit, mouse	Milteyni Biotech	#130-091-041
Recombinant Human IL-2 (carrier-free)	BioLegend	#589102
T Cell TransAct™, human	Milteyni Biotech	#130-111-160
Sodium Butyrate	STEMCELL Technologies	#72242
SepMate™-50 (IVD)	STEMCELL Technologies	#85450
Lymphoprep™	STEMCELL Technologies	#07801
RPMI 1640 Medium	Gibco™	#11875093
RPMI 1640 Medium, no glucose	Gibco™	#11879020
Poly-L-lysine solution	Sigma-Aldrich	#25988-63-0
Seahorse XF T Cell Metabolic Profiling Kit	Agilent	#103772-100
Seahorse XF RPMI medium, pH 7.4	Agilent	#103576-100
Seahorse XF 1.0 M glucose solution	Agilent	#103577-100
Seahorse XF 100 mM pyruvate solution	Agilent	#103578-100
Seahorse XFe96/XF Pro FluxPak Mini	Agilent	#103793-100
Seahorse XF 200 mM glutamine solution	Agilent	#103579-100
Seahorse XF96 Cell Culture Microplate	Agilent	#101085-004
BODIPY™ 493/503 (4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene)	Invitrogen™	#D3922
MitoTracker™ Deep Red FM Dye, for flow cytometry	Invitrogen™	#M46753

MitoProbe™ TMRM Assay Kit for Flow Cytometry	Thermo Scientific™	#M20036
2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose)	Invitrogen™	#N13195
5-(Tetradecyloxy)-2-furoic acid, TOFA	Sigma-Aldrich	#613450-5MG
rac-Ethyl 2-[6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylate (Etomoxir)	Sigma-Aldrich	#236020
Rapamycin	Sigma-Aldrich	#553210
Lactate-Glo™ Assay	Promega	#J5021
Glucose-Glo™ Assay	Promega	#J6021
Corning™ Frosted Microscope Slides	Corning™	#11343835
ProLong™ Glass Antifade Mountant with NucBlue™ Stain	Invitrogen™	#P36983
Fisherbrand™ Glass Circle Coverslips	Fisherbrand™	#12313138
RNeasy Mini Kit (50)	Qiagen	#74104
Thermo Scientific™ DNase I, RNase-free (1 U/μL)	Fisher Scientific™	#10649890
gentleMACS™ C Tubes	Milteyni Biotech	#130-093-237
gentleMACS™ Tumor Dissociation Kit, mouse	Milteyni Biotech	#130-096-730
eBioscience™ Cell Stimulation Cocktail (plus protein transport inhibitors) (500X)	eBioscience™	# 00-4975-93
BD Microtainer® K ₂ EDTA 250-500μl	BD	#365975
Anti-Mouse CD16/CD32 (Mouse BD Fc Block™)	BD	#553141
Ficoll®-Paque PREMIUM 1.084	Merck	#GE17-5446-02
70μm cell strainer	Merck	#CLS431750
10x RBC lysis buffer	eBioscience™	#00-4300-54
Bovine serum albumin	Merck	#A9418-50G
Ethylenediaminetetraacetic acid	Merck	#E6758-100G

60x15mm dish non-treated	Fisher Scientific	#150340
5 mL Syringe PP/PE without needle	Merck	#Z683582-100EA

2.1.2 Software used

Table 2.1.2.1 – Description of source and provider of software used in experimental analysis

Product	Provider	Source
Seahorse Wave	Agilent	https://www.agilent.com/zh-cn/product/cell-analysis/real-time-cell-metabolic-analysis/xf-software/seahorse-wave-desktop-software-740897
	Cimini Lab/ Broad	
CellProfiler	Institute of MIT and Harvard	https://cellprofiler.org/
Galaxy v24.1.2.dev0	Galaxy team, Johns Hopkins University	https://usegalaxy.org/
Genome assembly and gene annotation	Ensembl	https://www.ensembl.org/Mus_musculus_FVB_NJ/Info/Index
StringTie	Kovaka et al. (411)	https://bio.tools/stringtie
HISAT2	Kim et al. (412)	https://bio.tools/hisat2
FeatureCounts	Liao et al. (413)	https://bio.tools/featurecounts

Gene Ontology	Young et al. (414)	https://bioconductor.org/packages/release/bioc/html/goseq.html
Gene set enrichment analysis	Broad Institute/UC San Diego (415)	https://www.gsea-msigdb.org/gsea/index.jsp
FlowJo_V10.10.0	BD	https://www.flowjo.com/solutions/flowjo/downloads
R (Version 4.3.3)	The R Project for Statistical Computing	https://www.r-project.org
R Studio	Posit	https://posit.co/download/rstudio-desktop/
FlowSOM (version 4.1.0)	Van Gassen et al. (416)	https://github.com/SofieVG/FlowSOM
XShift (version 1.4.1)	Samusik et al. (417)	https://github.com/nolanlab/vortex
Marker enrichment modelling (MEM)	Diggins et al. (418)	https://github.com/cytolab/mem
Graphpad Prism (v10)	Dotmatics	https://www.graphpad.com

2.2 Methods

2.2.1 Ethics

2.2.1.1 Animal studies

All mice used in this study were obtained from Charles River Laboratories. 5-6-week-old wild-type and immunocompetent FVB/NJ or C57BL/6 mice (Charles River Laboratories) were housed in groups of six or less, in individually ventilated cages, in

a pathogen-free facility [22°C, 50% humidity, 12 h light cycles]. Mice were monitored daily, weighed twice weekly and had access to food and water ad libitum. All experiments were performed in accordance with United Kingdom Animals (Scientific Procedures) Act 1986 at the Radiation and Radiobiology Institute, University of Oxford. Home Office-approved Project Licences: Professor Anderson Ryan (30-3395) and Dr. Eileen Parkes (PP7686743) at the University of Oxford.

2.2.1.2 Human primary immune cells

All primary immune cells used for *in vitro* experiments in this study were used in accordance with Human Tissue Act 2004 [under: Dr Kerry Fisher (Chief Investigator), REC – 19/LO/1848, Protocol number = ONC1NHSTblood1, Reference number – Oncology NHS blood].

2.2.2 Cell isolation and culture

All *in vitro* cell culture work was performed utilizing aseptic technique in Class II laminar flow hoods.

2.2.2.1 Cell lines

The Myc-CaP cell line was purchased from ATCC (CRL-3255™). KPC cells were a kind gift from Dr Jen Morton at the Cancer Research UK Glasgow: The Beatson Institute [KRas^{G12D}; Trp53^{R172H}; Pdx-1 Cre]. Cells used for experiments were low passage (5-8) and maintained in DMEM low glucose with 10% (v/v) fetal bovine serum, 2 mM glutamine, and 100 U/mL penicillin/streptomycin. Cells were sub-cultured every 3-4 days, or at >70% confluency. For sub-culturing, cell-culture medium was aspirated and cells were washed twice with PBS. Then, TrypLE™ dissociation reagent was added to

adherent cancer cell monolayers and incubated at 37°C, 5% CO₂ for 10 minutes. To quench the reaction, cell culture medium was added to cells and sub-cultured in a new flash at a 1:20 ratio. All cells were cultured in a humidified incubator, at 37°C, 5% CO₂ unless otherwise indicated.

2.2.2.2 Splenocytes

Mice were euthanized by schedule-1 cervical dislocation, and mice were positioned on their right side and sprayed with 70% ethanol. Aseptic forceps and scissors were used to make a small incision below the ribcage. The spleen was visualized under the translucent layer of muscle, excised away from surrounding connective tissue and placed in 15 mL falcon tubes containing 5 mL of ice-cold 0.5% BSA-PBS.

Isolated spleens were manually disaggregated in 2 mL ice-cold 0.5% BSA-PBS using the blunt-end insert of a 5 mL syringe in a 60x15mmx6 mL dish. Disaggregated splenocytes were passed through a 70µm cell strainer into a new 50 mL falcon tube. To collect residual cells, the 60x15mmx6 mL dish was gently rinsed x2 with 5 mL of ice-cold 0.5% BSA-PBS and passed through the 70µm cell strainer. Collected splenocytes were centrifuged at 350g for 5 minutes at RT and resuspended in 5 mL of 1x RBC lysis buffer for 3 minutes at RT. Cells were diluted in 15 mL of PBS, mixed by tube inversion, and passed through a 70 µm cell strainer before being placed on ice for cell isolations.

2.2.2.3 Bone marrow derived macrophages (murine)

After isolation of the spleen, lower limbs were sprayed with 70% ethanol. Aseptic forceps and scissors were used to first remove excess skin, and a cut was made

where the femur is at the base of the hip joint to remove the leg. The major muscles around the femur were excised using scissors, exposing the bone. Two further cuts were made at the knee joint and ankle, separating the femur, tibia and fibula. The epiphyses of each bone were cut, exposing the marrow. A 23G needle containing ice-cold PBS was inserted into the bone marrow cavity and was sequentially flushed until the bone marrow cavity turned clear into a 50 mL falcon tube. Bone marrow cells were pelleted at 300g for 4 minutes at 4°C.

2.2.2.4 Macrophage polarization

Cells were resuspended in 30 mL DMEM high glucose (10% FCS, 100 IU/mL penicillin, 100 mg/mL streptomycin, 50 μ M 2-mercaptoethanol, 20 ng mL⁻¹ M-CSF) and cultured in three separate 60x15mm $\times$ 6 mL dishes for 6 days (37°C, 5% CO₂). Fresh medium was added on D4 and D6. After 6 days of culture, BMDMs were polarized for 24 hours by addition of 20 ng mL⁻¹ M-CSF (M0), 20 ng mL⁻¹ LPS and 100 ng mL⁻¹ IFN- γ (M1) or 20 ng mL⁻¹ IL-4 [M2] (419). The next day, the supernatant was removed and replaced with fresh DMEM high glucose (10% FCS, 100 IU/mL penicillin, 100 mg/mL streptomycin, 50 μ M 2-mercaptoethanol) for a further 24 hours before use as M0, M1 or M2 conditioned medium.

2.2.2.5 Peripheral blood mononuclear cells (human)

NC24 leukocyte blood cones were collected from Oxford NHS Blood and Transplant Blood Donor Centre and transported to the laboratory at room temperature. 15 mL of LymphoprepTM was added through the central hole of the SepMateTM-50 (IVD) insert. Whole blood [7.5 mL] was diluted with an equal volume of PBS + 2% FBS and

was carefully layered on top of the density gradient medium. SepMate™-50 tubes were centrifuged at 1200 x g for 10 minutes at room temperature, with the brake on. The top layer was poured off, containing enriched mononuclear cells (MNCs). Enriched MNCs were washed x2 with PBS + 2% FBS and counted prior for use in cell culture.

2.2.2.6 Magnetic activated cell sorting (MACS)

All human and murine MACS isolations kits were purchased from Miltenyi Biotech. Freshly isolated splenocytes (murine) or PBMCs (human) were used to isolate T-cell subsets by negative selection by magnetic activated cell sorting (MACS) from single cell suspensions, according to the instructions provided by the respective Miltenyi Biotech kit. Briefly, non-target cells are magnetically labelled with a cocktail of biotinylated antibodies as a labelling agent, and then incubated with secondary anti-biotin microbeads. Target cells are left unlabelled and can pass through the magnetic column, with a relative purity/enrichment of 80-95% (naïve CD4⁺ T-cell isolation kit), 75-92% (CD8⁺ T-cell isolation kit) or 50-90% (CD4⁺ CD25⁺ regulatory T-cell isolation kit) target cells (data not shown).

2.2.2.7 iTreg differentiation

Flat-bottom TC treated flat-bottom 96-well plates were coated with anti-CD3 antibody (1 µg ml⁻¹) in 40 µL PBS overnight at 4°C prior to naïve CD4⁺ isolation. Naïve CD4⁺ T-cells were isolated from single cell suspensions of spleens of 5–6-week-old male FVB N/Ctrl or C56BL/6 mice by MACS separation. Excess anti-CD3 antibody from pre-coated 96-well plates was removed by gentle pipette aspiration with 100 µL of PBS. Then, 3 x 10⁵ naïve CD4⁺ T-cells were added to anti-CD3 coated plates in fully

supplemented RPMI 1640 (10% foetal calf serum, 100 IU/mL penicillin, 100 mg/mL streptomycin, 50 μ M 2-mercaptoethanol) with added soluble anti-CD28 (2 μ g ml⁻¹), rmIL-2 [$\sim$ 450 IU mL⁻¹] (20 ng ml⁻¹), rhTGF- β (2.5 ng ml⁻¹), anti-mIL-4 (10 μ g ml⁻¹), anti-IFN γ (10 ng ml⁻¹) for a total of 3 days under standard culture conditions. For treatments with IGF-1, LONG[®] R3 IGF-I (1, 10, 30, 50 nmol L⁻¹) or the equivalent dilution of solvent [hydrochloric acid] (0.03, 0.3, 10, 16 nmol L⁻¹) was added to culture medium in a total volume of 200 μ L per well.

For the generation of human iTregs, freshly isolated naïve CD4⁺ T-cells from human PBMCs were seeded at a density of 3 x 10⁵ cells per well (flat-bottom 96-well plate) and cultured in X-VIVO-15 (2% foetal calf serum, 100 IU/mL penicillin and 100 mg/mL streptomycin) supplemented with rhIL-2 (20 ng ml⁻¹), rhTGF- β (2.5 ng ml⁻¹), 4 μ L T-Cell TransAct[™] (colloidal polymeric nanomatrix conjugated to humanized CD3 and CD28 agonists) and $\pm$ 50 nmol L⁻¹ IGF-1 in a total volume of 200 μ L per well for 72 hours.

Where indicated, pathway inhibitors were added to culture medium at the start of iTreg differentiation; rapamycin (100 nM), etomoxir (1 μ M), 5-(Tetradecyloxy)-2-furoic acid [TOFA] (5 μ g mL⁻¹), SMS121 (150 μ M), TG100-155 (1 μ M), AZD5363 (300 nM), trametinib (50 nM), BMS-754807 (1 μ M) or BI-885578 (1 μ M). After 72 hours of culture under standard culture conditions, cells were collected and washed twice with 200 μ l PBS at 350g for 4 minutes at RT before analysis by flow cytometry.

2.2.2.8 tTreg stimulation

CD4⁺ CD25⁺ T-cells were isolated from single cell suspensions of spleens of 5–6-week-old male FVB N/Ctrl mice. To assess the impact of IGF-1 priming on suppressive potency, tTregs were seeded at a density of 3×10^5 cells per well (96-well plate) stimulated with anti-CD3/CD28 dynabeads at a 3.5:1 Treg: bead ratio and rmIL-2 (20 ng ml^{-1}) $\pm 50 \text{ nmol L}^{-1}$ IGF-1. After 72 hours, beads were magnetically removed prior to use in suppression assays.

2.2.3 Flow cytometry

2.2.3.1 Cytokine detection

For detection of IL-10, IL-17a, Granzyme-B, IFN- γ and TNF- α , cells were pre-treated with a protein transport inhibitor cocktail [eBioscience™, 00-4980-03, 1x] for 5 hours under standard culture conditions (37°C, 5% CO₂) prior to sample staining.

2.2.3.2 Surface and intracellular staining

Antibody concentrations, fluorophore-conjugates and clones used in this study for flow cytometry with murine or human reactivity are listed in table 2.2.3.1 or 2.2.3.2 respectively. Prior to surface staining, 1×10^6 cells were resuspended in 100 μL PBS and incubated with anti-CD16/32 (BD Pharmingen™ FC block) antibodies for 15 minutes at 4°C. Cells were washed once with 900 μL PBS (centrifugation at 350g, 4 minutes, 4°C), resuspended in 100 μL PBS, and stained with live-dead fixable viability dye and a cocktail of antibodies for surface antigens for 30 minutes at 4°C (protected from light). Then, cells were washed twice with 900 μL PBS and centrifuged at 350g for 4 minutes at 4°C. For fixation and permeabilization, cells were resuspended in 200 μL of

FoxP3 transcription factor buffer for 30 minutes at RT. Cells were washed twice with 900 μ L PBS, centrifuged at 350g for 4 minutes at RT. For intracellular antigen staining, cells were stained with a cocktail of antibodies for 30 minutes at room temperature in 100 μ L 1x permeabilization buffer. Then, cells were washed 2 times in 900 μ L PBS and centrifuged at 350g for 4 minutes at RT. For acquisition, cells were resuspended in 1 mL FACS buffer (1×10^6 cells/mL) [PBS - 2.5 mM EDTA, 0.5% FCS] and analysed using an Attune™ NxT flow cytometer. The same Attune™ NxT instrument and voltage settings were used for all experiments. Laser alignment, delays, detector sensitivities and cytometer quality control were checked prior to each experiment by using Attune™ Performance Tracking Beads. Compensation matrices were generated prior to every acquisition session using single stain compensation bead controls for the antibodies used in experimental analysis. Data visualisation and quantification was done using FlowJo v10.10.0. Fluorescence minus one (FMO) controls were used to quantify the percentage of cells staining positive for each marker.

2.2.3.3 Phospho-flow cytometry

Antibody concentrations, fluorophore-conjugates and clones used for phospho-flow cytometry are listed in table 2.2.3.3. For detection of phosphorylated markers, target cells were pelleted by centrifugation at 350g for 2 minutes at room temperature and immediately fixed in 200 μ L 4% PFA for 10 minutes at RT. Fixed cells were pelleted by centrifugation at 350g for 4 minutes at 4°C and permeabilized by addition of 500 μ L ice-cold 90% methanol for 10 minutes at 4°C, gently vortexing at both minute 2 and 5 of the incubation. Pelleted cells (350g for 4 minutes at RT) were washed twice with 200 μ L PBS and resuspended in 100 μ L 1x permeabilization reagent. Cells were incubated

with primary antibodies targeting phosphorylation sites for a further 45 minutes at room temperature. Cells were washed twice in 200 μ L PBS (centrifuged at 500g for 4 minutes at RT) and resuspended in 200 μ L FACS buffer [PBS - 2.5 mM EDTA, 0.5% FCS] before analysis by flow cytometry as previously described.

Table 2.2.3.1 Flow cytometry antibodies (Murine reactivity)

Target	Conc.	Fluorophore	Supplier	Clone	Cat. no.#
Dead cells	1 $\mu\text{L mL}^{-1}$	eFluor™ 506	eBioscience™	-	#65-0866-14
CD45	0.5 μg	Pacific Orange	eBioscience™	30-F11	#MCD4530
CD4	0.2 μg	APC-eFluor™ 780	eBioscience™	GK1.5	#47-0041-82
CD8a	0.2 μg	Alexa Fluor™ 700	eBioscience™	53-6.7	#56-0081-82
IGF-1R α	1 μg	Alexa Fluor™ 488	Santa Cruz	1H7	#sc-461 FITC
CD3	0.5 μg	eFluor™ 450	eBioscience™	145-2C11	#48-0031-82
GrzB	0.2 μg	eFluor™ 450	eBioscience™	NGZB	#48-8898-80
PD-1	0.2 μg	eFluor™ 450	eBioscience™	RMP1-30	# 48-9981-82
PD-1	0.125 μg	Super Bright 702	eBioscience™	J43	#46-9985-82
CD62L	0.015 μg	Super Bright 702	eBioscience™	MEL-14	#67-0621-82
IL-17a	0.25 μg	APC	eBioscience™	eBio64DEC17	#17-7179-42
ICOS	0.1 μg	APC	BioLegend	7E.17G9	#117419
CD39	0.25 μg	PE	eBioscience™	24DMS1	#12-0391-82
CTLA-4	0.2 μg	PE	eBioscience™	UC10-4B9	#12-1522-81
GrzB	0.2 μg	PE	eBioscience™	NGZB	#12-8898-80
CXCR5	0.125 μg	PE	eBioscience™	SPRCL5	#12-7185-80
FoxP3	0.4 μg	PE-Cyanine7	eBioscience™	FJK-16s	#25-5773-82
CD25	0.25 μg	PE-eFluor™ 610	eBioscience™	PC61.5	#61-0251-82
IFN- γ	0.3 μg	PerCP-Cyanine5.5	eBioscience™	XMG1.2	#45-7311-82
CD44	0.125 μg	PerCP-Cyanine5.5	eBioscience™	IM7	#45-0441-80
IL-10	0.4 μg	PerCP-Cyanine5.5	eBioscience™	JES5-16E3	#45-7101-82
CD73	0.125 μg	PerCP-Cyanine5.5	BioLegend	TY/11.8	#127213

Table 2.2.3.2 - Flow cytometry antibodies (Human reactivity)

Target	Conc.	Fluorophore	Supplier	Clone	Cat. no.#
CD4	0.2 µg	BV421	BioLegend	RPA-T4	#562425
CD8a	0.125 µg	APC-eFluor™ 780	eBioscience™	RPA-T8	#47-0088-42
CD25	0.125 µg	PE-eFluor™ 610	eBioscience™	CD25-4E3	#61-0257-42
FoxP3	0.2 µg	APC	eBioscience™	PCH10	#17-4776-42
HLA-DR	0.25 µg	PE-Cyanine7	eBioscience™	LN3	#25-9956-42
CD69	0.125 µg	PE	eBioscience™	FN50	#12-0699-42

Table 2.2.3.3 - Phospho-flow cytometry antibodies (Murine reactivity)

Target	Conc.	Fluorophore	Supplier	Clone	Cat. no.#
rpS6 (Ser235, Ser236)	0.125 µg	PE	eBioscience™	cupk43k	#12-9007-41
AKT1 (Ser473)	0.25 µg	PE	eBioscience™	SDRNR	#12-9715-41
ERK1/2 (Thr202/Tyr204)	0.2 µg	AF647	BioLegend	4B11B69	#675503
STAT3 (Tyr705)	0.2 µg	FITC	BioLegend	13A3-1	#651019
STAT5 (Tyr694)	0.25 µg	APC	eBioscience™	SRBCZX	#17-9010-41

2.2.4 Treg suppression assays

2.2.4.1 Responder CD8⁺ T-cells

First, differentiated iTregs or anti-CD3/CD28 stimulated tTregs (suppressor cells) were washed two times with 200 μ L PBS prior to co-culture suppression assays (centrifuged at 350g for 4 minutes at RT) to remove residual IGF-1 from culture medium. MACS sorted CD8⁺ T-cells isolated from the spleen of autologous donors were used as responder cells. To measure proliferation, CD8⁺ T-cells were resuspended in 1 mL of PBS (1×10^6) cells and stained with CFSE at a final concentration of 2 μ M for 10 minutes at RT. To quench the reaction, 9 mL of fully supplemented RPMI 1640 was added, and labelled cells were centrifuged at 350g for 4 minutes at RT and washed x2 with PBS to remove unlabelled dye.

To measure the suppression of responder CD8⁺ T-cells, 5×10^4 CFSE labelled CD8⁺ T-cells were plated in triplicate (96-well plate) in 200 μ L RPMI 1640 (10% FBS, penicillin-streptomycin, L-glut, β -ME) with rmIL-2 (20 ng ml⁻¹), anti-CD3/CD28 dynabeads (3.5:1 beads: responder cell ratio) and autologous Tregs (suppressor cells) at the indicated effector: target ratio (420). After 48 hours, CD8⁺ T-cell proliferation, activation and cytokine production was measured by flow cytometry. Responder CD8⁺ T-cells cultured in wells without a) dynabeads or b) Tregs were used as controls. The relative suppression of responder CD8⁺ T-cells after 48 hours induced by iTregs was calculated using the following formulae:

$$\% \text{ Suppression} = \frac{A - T}{A} \times 100$$

A = % positivity/% proliferation of responder CD8⁺ T-cells + dynabeads

T = % positivity/% proliferating of responder CD8⁺ T-cells + dynabeads + Tregs

Where:

- % positivity = the percentage of responder CD8⁺ T-cells staining positive for markers such as CD25⁺, PD-1⁺ or cytokines such as IFN- γ ⁺ or TNF- α ⁺.
- % proliferating = responder CD8⁺ T-cells that have a greater CFSE dilution than a negative control (unstimulated CFSE labelled responder CD8⁺ T-cells).

Area-under-curve analysis was performed in Graphpad prism as described by *Akimova et al.* (420).

2.2.4.2 Responder CD8⁺ T-cells with cancer cells

The Treg suppression assay from 2.2.4.1 was further adapted to use autologous cancer cells (Myc-CaP or KPC) as an activation stimulus for responder CD8⁺ T-cells (instead of anti-CD3/CD28 dynabeads). For suppression assays with cancer cells, 5 x 10³ cancer cells were pre-seeded per well of a 96-well plate the day prior to responder CD8⁺ isolation and co-culture with Treg suppressor cells. Responder CD8⁺ T-cells cultured in wells without a) cancer cells or b) Treg cells were used as controls.

After 48 hours of co-culture, non-adherent cells were collected and non-adherent cancer cells were dissociated as previously described (2.2.2.1). Residual non-adherent cells were washed twice by gentle aspiration with 200 μ L PBS and collected in the same 1.5 mL Eppendorf tube. Adherent cancer cells were then disaggregated and collected with non-adherent cells. Cell apoptosis was measured by co-incubation with 1 μ M ApoTracker Green dye during surface antibody staining as previously described (2.2.3.2). For all suppression assays, three technical replicates per condition were analysed and averaged for each donor.

For assays with cancer cells, A and T become:

***A** = % positivity/% proliferation of responder CD8⁺ T-cells + cancer cells*

***T** = % positivity/% proliferating of responder CD8⁺ T-cells + cancer cells + Tregs*

2.2.4.3 Responder CD8⁺ T-cells with conditioned medium

For Treg suppression assays utilizing conditioned medium, culture medium from 2.2.4.1 was replaced with the supernatant generated by MyC-CaP cells, detailed below:

- **PCa conditioned medium:** Filtered [0.7 μ m] supernatant from 2.5×10^5 MyC-CaP cells cultured for 48 hours in a 6-well plate in RPMI 1640 (10% FBS, penicillin-streptomycin, L-glut, β -ME).

2.2.5 Treg metabolism assays

2.2.5.1 Neutral lipid content and glucose uptake measurements

For neutral lipid measurements, cells were resuspended in serum free RPMI 1640 and incubated with 100 nM BODIPY™ 493/503 at 37°C 5% CO₂. For glucose uptake measurements, cells were resuspended in glucose free RPMI 1640 and incubated with 10 µM 2-NBDG (2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose) 37°C 5% CO₂. After 30 minutes of 2-NBDG or BODIPY™ 493/503 staining, cells were washed twice with 1 mL PBS [350g, 5 minutes, RT] to remove residual unbound dye before acquisition by flow cytometry.

2.2.5.2 Mitochondrial membrane potential measurements

For mitochondrial fitness tests, cells were incubated for 30 minutes at 37°C 5% CO₂ with both 20 nM Tetramethylrhodamine, Methyl Ester, Perchlorate (TMRM) and 10 nM MitoTracker DeepRed at 37°C 5% CO₂. After 30 minutes, cells were washed twice with 1 mL PBS [350g, 5 minutes, RT] to remove residual unbound dye before acquisition by flow cytometry.

2.2.5.3 Metabolic flux analysis

iTregs differentiated $\pm$ 50 nmol L⁻¹ IGF-1 (as in 2.2.2.7) were used for metabolic flux analysis. The day prior, XF extracellular flux sensory cartridges were pre-hydrated by adding 200 µl XF calibrant to wells of the utility plate and placed in a humidified non-CO₂ 37°C incubator overnight. Remaining wells were pre-coated with 100 µl poly-l-lysine 0.1% (w/v) overnight. Wells of the Seahorse XF96 cell culture microplates were washed twice with 200 µl PBS to remove residual poly-l-lysine and set to dry by

incubation for 3 hours at 37°C. 2.5×10^5 iT_{reg} cells were added per well [except for 4 background control wells A1, A12, H1, H12] and centrifuged at 350g for 4 minutes, RT, to adhere iTregs to the XF96 microplate. All wells were gently resuspended in 160 µl Seahorse XF RPMI medium and (pH 7.4, 2 mM glutamine, 1 mM pyruvate and 25 mM glucose). Cells were placed in a non-CO₂ humidified 37°C incubator for 45 minutes prior to placement of the XF96 microplate in a Seahorse XF Pro Analyzer.

Basal rates of oxygen consumption (OCR) and extracellular acidification (ECAR) were analysed for 20 minutes. OCR and ECAR of iTregs was calculated in response to sequential injection with Oligomycin (2 µM – Injection port A), FCCP (1 µM – Injection port B) and Rotenone/antimycin A (0.5 µM – Injection port C) using a 3/0/3 : mix/wait/measure protocol. Outliers were categorized as experimental wells that had a) low/no baseline oxygen consumption or b) no change in oxygen consumption in response to FCCP injection - and were removed from analysis. Results were exported using the Agilent Seahorse Wave Desktop software to GraphPad Prism.

Data normalization: After analysis - assay medium was removed, wells were gently washed with 160 µl PBS, and cells were stained with Hoechst 33342 at a concentration of 1 µg mL⁻¹ for 5 minutes in the dark at RT. Cells were gently washed twice with 160 µl PBS before imaging at x4 magnification by widefield microscopy. Cell numbers per well were quantified using CellProfiler and OCR and ECAR rates were normalized per 1000 cells (421).

The rate of basal respiration, max OCR and spare respiratory capacity were calculated using the following formula:

$$\text{Basal respiration} = B - RA$$

$$\text{Max OCR} = F - RA$$

$$\text{Spare respiratory capacity} = F - B$$

Where:

B = Oxygen rate measurement before oligomycin injection

RA = Oxygen rate measurement after rotenone/antimycin A injection

F = Oxygen rate measurement after injection with FCCP

2.2.5.4 Mitochondrial stress assays

To measure relative changes in Treg mitochondrial function, we first generated conditioned medium or made cell culture medium in the following manner:

- I. **CD8⁺ conditioned medium:** Filtered [0.7 μm] supernatant of $5 \times 10^4 \text{ mL}^{-1}$ CD8⁺ T-cells cultured in fully supplemented RPMI 1640, rmIL-2 (20 ng ml⁻¹) activated by anti-CD3/CD28 dynabeads (3.5:1 beads: responder cell ratio) in a 96-well plate for 48 hours. Used here to mimic metabolic conditions generated by CD8⁺ T-cells from suppression assays (2.2.4.1).
- II. **Restrictive medium:** Glucose free RPMI 1640 supplemented with 2% FBS. Used here as an experimental control to mimic nutrient stress.
- III. **PCa conditioned medium:** as in 2.2.4.3. Used here to mimic metabolic conditions generated by cancer cells from suppression assays (2.2.4.3).

To assess changes in mitochondrial function, iTregs differentiated $\pm 50 \text{ nmol L}^{-1}$ IGF-1 (as in 2.2.2.7) were plated in 200 μL of a) RPMI 1640 (10% FBS, penicillin-streptomycin, L-glut, β -ME) with rmIL-2 (20 ng ml^{-1}), b) CD8⁺ conditioned medium, c) restrictive medium, or d) PCa conditioned medium in a 96-well plate. Then, anti-CD3/CD28 dynabeads were added at a 3.5:1 beads: Treg cell ratio. After 48 hours, mitochondrial membrane potential was measured by flow cytometry as in 2.2.5.2.

2.2.5.5 Lactate and glucose measurements

Glucose and L-lactate levels from RPMI 1640 (10% FBS, penicillin-streptomycin, L-glut, β -ME) culture medium or conditioned medium (as generated in 2.2.5.4) was measured using Glucose-Glo[™] and Lactate-Glo[™] assay kits, as per instructions provided by the manufacturer, Promega.

2.2.5.6 Preparation of samples for confocal imaging

Coverslips were pre-coated with 300 μl poly-l-lysine 0.1% (w/v) overnight in 12 well plates, washed twice with 300 μl PBS, and incubated for 3 hours at 37°C 5% CO₂ to dry. 3×10^5 iTregs were added to wells containing poly-l-lysine coated coverslips, centrifuged at 350g for 3 minutes at RT, and rested for 1 hour at 37°C 5% CO₂. Cells were then incubated with 10 nM MitoTracker DeepRed and 100 nM BODIPY[™] 493/503 in PBS for 20 minutes at 37°C 5% CO₂, washed twice with PBS, and incubated with 4% (w/v) paraformaldehyde (PFA) for 10 minutes at 4°C. iTregs were gently washed twice with PBS and coverslips were mounted onto slides using SlowFade[™] Diamond Antifade Mountant with DAPI (Invitrogen[™]). Slides were stored in the dark at RT overnight before imaging.

2.2.5.7 Confocal microscopy

Slides were imaged on a Zeiss LSM 710 inverted confocal microscope (Department of Oncology, ORCRB, University of Oxford). Images were acquired by sequential 3 channel scanning (3 fluorescence channels). Z-stacks of 5-7 μm interslice distance were generated for all samples. Plan-Apochromat 63x/1.4 Oil DIC M27; 3.7940 pixels per micron, using filters for DAPI (358 – 463 nm), BODIPY FL (503 – 512 nm) and Alexa Fluor 647 (653 – 668 nm). Images used for analysis were exported as maximum intensity projections using Zeiss ZEN software.

2.2.5.8 CellProfiler imaging analysis

To quantify iTreg lipid foci, we used the *IdentifyPrimaryObjects* feature within CellProfiler (figure 2.2.5.1). A signal intensity threshold [0.114] for lipid foci detection was set on the basis of detection of background signal from negative controls (no BODIPY staining). Then, we measured individual foci intensity (average pixel intensity within foci) by adding measurements of *MeanIntensity* within the *IdentifyPrimaryObject* feature. Next, to quantify proximity of lipid foci with mitochondria, we utilized two methods. First, we used the *MeasureObjectNeighbors* tool, that first sets a central measurement point (centre of mitochondrial foci identified using the *IdentifySecondaryObject* feature). Then, *PercentTouching* expands this central point by a set number of pixels [$n = 20$) to define “proximal space” to individual mitochondria. It then measures the percentage of this proximal space that is now touching a neighbouring secondary object (lipid droplet). Alternatively, *FirstClosestDistance* measures and quantifies the shortest distance, in pixels, from individual mitochondria to the closest defined lipid droplet.

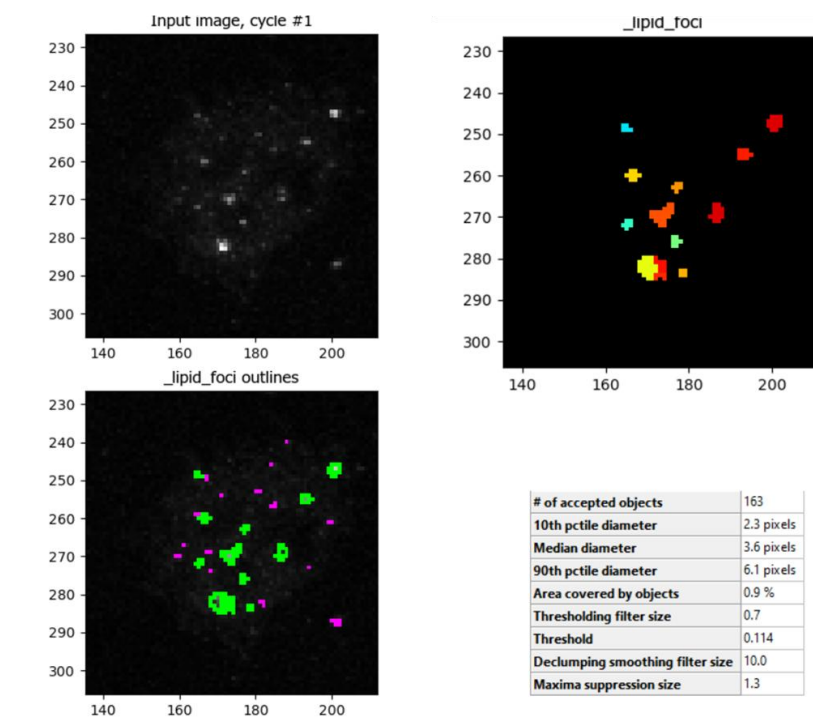


Figure 2.2.5.1 Lipid foci quantification using CellProfiler

Representative example of automated lipid foci identification using the *IdentifyPrimaryObjects* feature within CellProfiler, with accepted or rejected foci circled in green or purple respectively.

2.2.6 3' mRNA-sequencing

2.2.6.1 Fluorescence activated cell sorting

For cell sorting, iTregs were transferred to 15 mL falcon tubes, counted using a haematocytometer, and washed x2 in 5 mL of PBS (centrifuge: 350g, 4 minutes, RT) prior to resuspension in FACS buffer (PBS, 0.5% BSA, 2 mM EDTA) at a concentration of 1×10^6 cells mL^{-1} in 15 mL tubes. Collection tubes for each sample were prepared by addition of 1 mL of undiluted FBS to 15 mL falcon tubes. Cells were transported on ice to the flow cytometry facility at the Weatherall Institute of Molecular Medicine for cell

sorting using a BD FACSAria III. A total of 1×10^6 live, CD4⁺, CD25⁺ cells were sorted per sample [85 μ m nozzle size, normal purity sort, collection into 15 mL falcon tube with 1 mL FBS] and immediately placed on ice for transport and RNA isolation.

2.2.6.2 RNA isolation

RNA was isolated from 1×10^6 FACsorted Treg cells [Live, CD4⁺, CD25⁺] per sample using the RNeasy Mini Kit according to instructions provided by Qiagen, with a single modification at the final stage of RNA elution: eluting in a final volume of 25 μ L RNAase free water per sample. The purity of isolated RNA and concentration and A260/280 was quantified on a NanoDrop Microvolume Spectrophotometer. An A260/280 score of > 2.0 was used as a threshold for sufficient RNA purity to be used for RNA-sequencing. Where necessary, total RNA was subsequently further diluted in RNase free water at $> 30 \text{ ng } \mu\text{L}^{-1}$ and stored at -80°C until sequencing.

2.2.6.3 Library construction, Quality Control and Sequencing

RNA isolated from FACsorted live, CD4⁺ CD25⁺ cells was submitted for mRNA-sequencing with Novogene (UK), performed in-house. Briefly, messenger RNA was purified from total RNA using poly-T oligo-attached magnetic beads. After fragmentation, the first strand cDNA was synthesized using random hexamer primers followed by the second strand cDNA synthesis. The non-directional library was ready after end repair, A-tailing, adapter ligation, size selection, amplification, and purification. The library was checked using Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection. Quantified libraries were pooled and sequenced on an Illumina NovaSeq™ system by paired-end sequencing (PE150).

Quality control statistics for samples used in mRNA-sequencing analysis is shown in table 2.2.9.1.

Table 2.2.6.1 mRNA-sequencing data quality summary

Sample ID	Raw reads	Raw data (Gb)	Effective(%)	Error(%)	Q20(%)	Q30(%)	GC(%)
coTreg_1	60,475,770	9.1	95.66	0.02	95.52	88.28	50.02
coTreg_2	71,882,398	10.8	95.72	0.02	95.53	88.20	50.00
coTreg_3	62,574,488	9.4	96.12	0.02	95.69	88.55	50.16
IGF_coTreg_1	64,719,446	9.7	91.95	0.02	95.46	88.15	50.26
IGF_coTreg_2	58,760,390	11.6	95.91	0.02	95.72	88.69	49.97
IGF_coTreg_2	18,868,108		96.46	0.01	98.59	96.15	50.01
IGF_coTreg_3	106,760,598	16.0	94.22	0.01	98.33	95.73	48.02

Raw reads: total amount of reads of raw data.

Effective (%): (Clean reads/Raw reads)*100%

Error (%): base error rate

Q20 (%), Q30 (%): (Base count of Phred value > 20 or 30) / (Total base count)

GC (%): (G & C base count) / (Total base count)

2.2.6.4 Genome assembly and gene annotation

Genome assembly and differential gene expression analysis was performed using the cloud-based Galaxy platform, using default tool parameters unless otherwise specified. Adapter sequences were trimmed using *Trimmomatic* [Sliding window trimming, “number of bases to average across” = 4, “average quality required”=20] and *FASTQC* reports [“length of kmer to look for”=7] were generated to assess quality control (GC content, per base sequence quality, sequence duplication levels). Reference

genome and gene annotations were downloaded from Ensembl for the FVB_NJ_v1 mouse model (GCA_001624535.1). Paired end reads were aligned and mapped to the FVB_NJ_v1 genome using *HISAT2*. Then, *StringTie* and *StringTie-Merge* was used for de novo transcriptome assembly [“Use reference annotation”=Yes, GCA_001624535.1] and *GFFcompare* was used to annotate transcripts from the merged transcriptome [“Use reference annotation” = Yes, GCA_001624535.1]. Next, *FeatureCounts* was used to count reads aligning to exons (from *GFFcompare* analysis). Then, gene counts produced from *FeatureCounts* was used to perform differential gene expression testing using *DEseq2* [“Visualising the analysis results”=Yes, “Output normalized counts table”=Yes]. The variance between normalized counts used by *DESeq2* was used to plot principal component analysis (PCA) [fig. 2.2.6.4], indicative of the transcriptomic effect of IGF-1 priming.

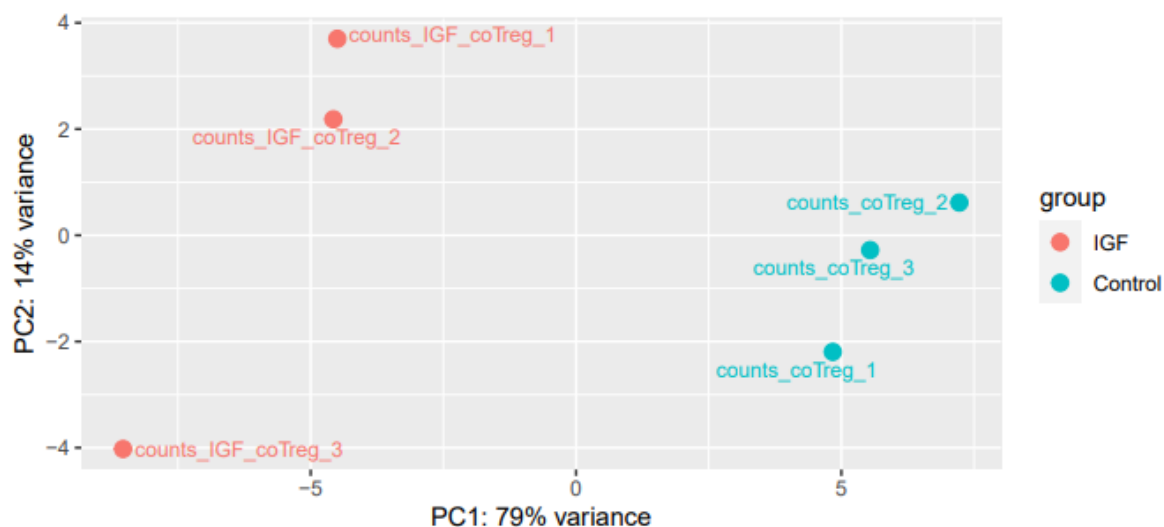


Figure 2.2.6.4 – Principal component analysis

Normalised gene counts from DESeq2 are plotted along the first two components retaining the highest variance, showing a distinct transcriptional separation of iTregs differentiated with ± 50 nmol L⁻¹ IGF-1, stimulated anti-CD3/CD28 dynabeads in CM from MyC-CaP cells]

2.2.6.5 Over-representation and gene set enrichment analysis

Over-representation analysis from differentially expressed genes (DEGs) was conducted using the cloud-based Galaxy platform. Gene Ontology (GO) analysis for cellular component, biological process and molecular function gene enrichment was performed using DEGs generated from *DESeq2* analysis, using the Wallenius method and Benjamini-Hochberg [FDR] (1995) correction for multiple hypotheses testing. An adjusted p-value of < 0.05 was considered statistically significant. Gene set enrichment analysis (GSEA) was performed within *GSEA 4.3.3* software, using the normalized read count output from *DESeq2* and using gene sets from *MSigDB*.

2.2.7 In vivo MyC-CaP allograft experiments

2.2.7.1 Tumour cell preparation and injection for in vivo experiments

Adherent Myc-CaP cells were dissociated using TrypLE™ Express Enzyme (1X), counted, washed, and 2×10^6 cells were mixed at a 1:1 ratio with cold Matrigel and DMEM (low glucose). Prior to tumour inoculation, mice were anaesthetised with 2% isoflurane in air/oxygen, ear punched for identification, and the injection site was sterilised using 70% ethanol. To establish prostate cancer allografts, an ice-cold insulin syringe was used to subcutaneously inject 100 μ L of the Matrigel-cell mix into the right flank of the mouse. Mice were closely monitored and placed on a warm mat until restoration of normal motor function. Tumour volume ($\text{width} \times \text{length} \times \text{height}$)/2 was measured twice weekly using a digital calliper.

2.2.7.2 Immunogenicity studies of IGF blockade

Mice were randomised into two treatment groups: PBS (control) or 100 mg/kg Xentuzumab (Boehringer Ingelheim, BI836845) [n=3 per group]. PBS or Xentuzumab was administered twice weekly by intraperitoneal injection (IP), initiated upon tumour volume reaching 80-100 mm³ in size. Upon reaching a tumour volume of 400 mm³, mice were euthanised by pentobarbital injection. Then, tumours were isolated, collected in 5 mL ice cold PBS 0.5% BSA, and processed for analysis by flow cytometry.

2.2.7.3 Tolerability and efficacy studies of IGF blockade with anti-PD-1

For tolerability analysis, tumour bearing mice (as in 2.2.9.1) were treated with either twice weekly or three-times weekly with 10 mg/kg anti-PD-1 (clone RMP1-14, BioXCell) and 100 mg/kg Xentuzumab [n=6 per group]. A subset of mice (n=3) were selected for TIL analysis after 7 days of treatment onset or a tumour volume > 400 mm³ and euthanized by cervical dislocation. Tumours were collected in 5 mL ice cold PBS 0.5% BSA, and processed for analysis by flow cytometry.

For efficacy studies, G*Power software was used to determine group size [n=8] based on anti-tumour immune effects induced by fasting-induced decreased sIGF-1 levels in a syngeneic murine model of lung adenocarcinoma (395). Mice were randomized to four treatment groups, summarized in table 2.2.9.1. Treatments were admixed within the same syringe to reduce stress for mice receiving two separate IP injections.

Table 2.2.7.1 Summary of treatment groups for tumour-bearing mice

Group	Treatment administered	
Control	+ PBS [10 mL/kg]	+ rat IgG2a isotype control [+10 mg/kg]
Anti-PD-1	+ anti-PD-1 [10 mg/kg]	+ PBS [10 mL/kg]
Anti-IGF	+ PBS [10 mL/kg]	+ Xentuzumab [100 mg/kg]
Combo	+ anti-PD-1 [10 mg/kg]	+ Xentuzumab [100 mg/kg]

Prostate allografts were established as in 2.2.9.1, and treatment was initiated upon tumour volume reaching 80-100 mm³. At the experimental endpoint, the mice

were culled by pentobarbital injection followed by terminal cardiac puncture and peripheral blood was collected in 1 mL K2EDTA coated vacutainer tubes. Spleens and tumours were excised and placed in 1 mL ice-cold PBS 0.5% BSA for processing and analysis by flow cytometry (N = 8 tumours, N = 6 spleen/peripheral blood).

2.2.7.4 Peripheral blood isolation

300-450 μ L of whole blood was collected per mouse, transported at room temperature and processed within 2 hours of cardiac puncture. Whole blood was diluted with ice cold PBS at a 1:1 ratio, and mixed by tube inversion. Next, an equal volume of Ficoll-Paque was carefully layered on top of the whole-blood solution and subsequently centrifuged at 400g for 40 minutes at room temperature (acceleration speed set at 4, break off). The upper layer containing plasma and platelets was gently removed by pipette aspiration and the buffy coat at the interface was carefully transferred to a sterile 1.5 mL centrifuge tube. Cells were washed twice [350g, 4 minutes, RT] with 1 mL PBS and resuspended for cell counting and flow cytometry staining.

2.2.7.5 Tumour infiltrating lymphocyte analysis

Whole tumours were carefully cut into smaller 2 mm³ segments using a scalpel and transported in 5 mL PBS (2% FBS), on ice. Tumour segments were weighed using a balance, and 0.1-1g of tumour tissue per sample was added to a mixture of 2.35 mL RPMI 1640 (10% FBS, 100 IU/mL penicillin, 100 mg/mL streptomycin, 50 μ M 2-mercaptoethanol), 100 μ L Enzyme D, 50 μ L Enzyme R, and 12.5 μ L Enzyme A in a gentleMACS™ C-tube. Tumour tissues were dissociated using the gentleMACS™ Octo

Dissociator according to the gentleMACS program *37C_m_TDK_1*. Disaggregated cells were passed through a 70 µm cell strainer to collect non-cellular debris. Cell suspensions were washed x2 in PBS [500g, 5 minutes, RT] and residual red blood cells were lysed in x1 RBC lysis buffer for 3 minutes, at RT. Cells were washed twice [500g, 5 minutes, RT] with 1 mL PBS. For intracellular cytokine detection in TILs, cells were transferred to 96-well plates (1 x 10⁶ cells per well) and incubated with 1x eBioscience™ Cell Stimulation Cocktail (plus protein transport inhibitors) for 5 hours in fully supplemented RPMI-1640 and incubated under standard culture conditions (37°C, 5% CO₂) prior to antibody staining. For analysis by flow cytometry, tumour samples (with low CD45⁺ infiltrates) were transferred to 15 mL falcon tubes and subsequent staining volumes were scaled relative to 1 x 10⁶ cells / 100 µL PBS to allow for sampling of all available events and stained for flow cytometry analysis as previously described (2.2.4).

2.2.8 Cytometry by Time-Of-Flight (CyTOF)

2.2.8.1 The WINGMEN trial (NCT05110495)

Men ≥ 18 years old with localised T1-T3 prostate adenocarcinoma who opted for radical prostatectomy (surgery) as their treatment option were identified and approached to discuss participation in the WINGMEN trial (figure 2.3.1.1a). The trial offered surgical patients additional once weekly intravenous (i.v.) Xentuzumab infusions (1000mg), during the 4–6-week window they normally wait for prostatectomy. For research purposes, peripheral blood was sampled ~2-4 hours before the first i.v. infusion with Xentuzumab to evaluate pre-treatment levels of PSA, sIGF-1, serum metabolites and immune cells (PBMCs), referred herein as “*pre*” [figure 2.3.1.1b]. This was used to compare with peripheral blood samples collected after 4-6

cycles of Xentuzumab, ~2-4 hours prior to their surgery. As to investigate immune correlates and biomarkers of response to Xentuzumab treatment, mass cytometry (CyTOF) was used for deep interrogation of 37 markers and immunophenotyping of peripheral blood immune cells before, and after treatment with Xentuzumab. Although advances in spectral flow cytometry offer similar high-dimensional resolution and marker depth for immunoprofiling, we opted for mass cytometry as CD45 barcoding enabled the staining and acquisition of all 52 patient samples in a single batch, minimizing intra-patient variability.

2.2.8.2 Trial patient data

Table 2.2.8.1 - Overview of data from patients recruited to the WINGMEN trial

Patients (n=27)	Baseline (pre)	Before surgery (post)
Median age, years (range)	66 (50 - 74)	66 (50 - 74)
Eastern Cooperative Oncology Group performance score (%)		
0	92.6	92.6
1	7.4	7.4
Body mass index	27.43 (19.67 - 31.82)	27.09 (19.57 - 31.08)
Median presenting PSA, ng/ml (range)	7 (2 - 24)	6 (2 - 27)
Median baseline serum IGF-1, nmol L ⁻¹ (range)	18.05 (11 - 36)	4.05 (1.5 - 40)
Gleason score (n)	n=26	n=26
3+3	1	0
3+4	13	12
4+3	7	10
4+4	2	1
4+5	3	2
5+4	0	1
5+5	0	0
Grade group (n)	n=27	n=27
1	1	1
2	13	13
3	7	7
4	3	3
5	3	3
Tumour stage (n, AJC TNM)		n=26
pT1		0
pT2a		5
pT2b		2
pT2c		8
pT3a		9
pT3b		2

2.2.8.3 Antibodies

Table 2.3.1.2 includes a list of abbreviations for immune cell markers used herein. Table 2.3.1.3 includes a list of the full antibody panel and corresponding metal-isotope conjugates. Antibodies and Cell-ID™-cisplatin staining were used at recommended concentration provided by Fluidigm (acquired by Standard BioTools). For in-house conjugation [University of Oxford, Kennedy Institute of Rheumatology, Mass Cytometry facility], antibodies were labelled using Maxpar MCP9 Antibody Labelling Kits according to instructions provided by Fluidigm.

Table 2.2.8.2 Abbreviations for immune markers used in mass cytometry analysis

Marker	Full name	Lineage/purpose
CD45	PTPRC protein tyrosine phosphatase receptor type C	Pan-immune
CD19	Cluster of differentiation-19	B-cell
CD20	B-lymphocyte antigen	
IgD	Immunoglobulin D	
CD27	Tumour Necrosis Factor Receptor Superfamily Member 7	
CD3e	CD3 epsilon chain	
CD38	Cyclic ADP ribose hydrolase	
CD8a	T-cell surface glycoprotein CD8 alpha chain	T-cell
CD4	Cluster of differentiation 4	
CD45RA	PTPRC protein tyrosine phosphatase receptor type C (isoform RA)	
CD45RO	Protein tyrosine phosphatase receptor type C (isoform RO)	
CCR7	C-C Chemokine Receptor 7	
TCRgd	T-cell receptor gamma delta	
CCR4	C-C Chemokine Receptor 4	
CTLA4	Cytotoxic T-lymphocyte-associated protein 4	

FoxP3	Forkhead box P3	
CD66b	Carcinoembryonic antigen-related cell adhesion molecule- 8	Neutrophil
CD16	Low affinity immunoglobulin gamma Fc region receptor III-A	Monocyte
CD14	Cluster of differentiation-14	
CD11b	Integrin alpha M	
CD36	Cluster of differentiation-36	
BDCA1	Cluster of differentiation antigen 1c	Dendritic cell
CD11c	Integrin alpha X	
HLADR	Human Leukocyte Antigen-DR isotype	
CD141	Thrombomodulin	
CD123	Interleukin-3 receptor alpha chain	
CD161	Killer cell lectin-like receptor subfamily B	ILCs/ NK cells
CD56	Neural cell adhesion molecule 1	
CD57	Human natural killer-1	
CD127	Interleukin-7 receptor subunit alpha	
CD39	Ectonucleoside triphosphate diphosphohydrolase-1	Functional markers
CD69	Cluster of differentiation 69	
GrzB	Granzyme-B	
PD1	Programmed-death-1	
PDL1	Programmed-death-ligand-1	
CD25	Interleukin-2 receptor alpha chain	
Ki67	Marker of proliferation Kiel 67	
TIGIT	T-cell immunoreceptor with Ig and ITIM domains	

Table 2.2.8.3 - Mass cytometry antibodies (Human reactivity)

Mass tag	Marker	Labelling	Clone	Stain	Supplier	Cat. no #
89 Y	CD45	Pre-conjugated	HI30	Surface	Fluidigm	#3089003B
106 Cd	CD45	Pre-conjugated	HI30	Surface	Fluidigm	#3106001C
110 Cd	CD45	Pre-conjugated	HI30	Surface	Fluidigm	#3110001C
111 Cd	CD45	Pre-conjugated	HI30	Surface	Fluidigm	#3111001B
112 Cd	CD45	Pre-conjugated	HI30	Surface	Fluidigm	#3112001C
113 Cd	CD45	Pre-conjugated	HI30	Surface	Fluidigm	#3113001B
114 Cd	CD45	Pre-conjugated	HI30	Surface	Fluidigm	#3114001C
116 Cd	CD45	Pre-conjugated	HI30	Surface	Fluidigm	#3116001C
141 Pr	CD39	Pre-conjugated	A1	Surface	BioLegend	#328221
142 Nd	CD19	Pre-conjugated	HIB19	Surface	Fluidigm	#3142001B
143 Nd	CD127	Pre-conjugated	A019D5	Surface	Fluidigm	#3143012B
144 Nd	CD69	Pre-conjugated	FN50	Surface	Fluidigm	#3144018B
145 Nd	CD4	Pre-conjugated	RPA-T4	Surface	Fluidigm	#3145001B
146 Nd	IgD	Pre-conjugated	IA6-2	Surface	Fluidigm	#3146005B
147 Sm	CD11c	Pre-conjugated	Bu15	Surface	Fluidigm	#3147008B
148 Nd	CD16	Pre-conjugated	3G8	Surface	Fluidigm	#3148004B
149 Sm	CD38	In-house	HIT2	Surface	Fluidigm	#3141020D
150 Nd	BDCA1	In-house	L161	Surface	BioLegend	#331502
151 Eu	CD123	Pre-conjugated	6H6	Surface	Fluidigm	#3151001B
152 Sm	CD66b	Pre-conjugated	80H3	Surface	Fluidigm	#3152011B
153 Eu	TIGIT	Pre-conjugated	MBSA43	Surface	Fluidigm	#3153019B
154 Sm	CD3	Pre-conjugated	UCHT1	Surface	Fluidigm	#3154003B
155 Gd	CD36	Pre-conjugated	5-271	Surface	Fluidigm	#3155012B
156 Gd	TCRgd	Pre-conjugated	GL3	Surface	Fluidigm	#3159012B
158 Gd	CCR4	Pre-conjugated	205410	Surface	Fluidigm	#3158006A
159 Tb	CCR7	Pre-conjugated	G043H7	Surface	Fluidigm	#3159003A

160 Gd	CD14	Pre-conjugated	M5E2	Surface	Fluidigm	#3160006B
161 Dy	CTLA4	Pre-conjugated	14D3	Surf. + Cyto.	Fluidigm	#3161004B
162 Dy	FoxP3	Pre-conjugated	259D/C7	Nuclear	Fluidigm	#3162024A
163 Dy	CD56	Pre-conjugated	NCAM16.2	Surface	Fluidigm	#3163007B
164 Dy	CD161	Pre-conjugated	HP-3G10	Surface	Fluidigm	#3164009B
165 Ho	CD45RO	Pre-conjugated	UCHL1	Surface	Fluidigm	#3165011B
166 Er	CD141	Pre-conjugated	M80	Surface	Fluidigm	#3166017B
167 Er	CD27	Pre-conjugated	HIT2	Surface	Fluidigm	#3167006B
168 Er	CD8a	Pre-conjugated	SK1	Surface	Fluidigm	#3168002B
169 Tm	CD25	Pre-conjugated	2A3	Surface	Fluidigm	#3169003B
170 Er	CD45RA	Pre-conjugated	HI100	Surface	Fluidigm	#3169008B
171 Yb	CD20	Pre-conjugated	2H7	Surface	Fluidigm	#3171012B
172 Yb	ki67	Pre-conjugated	B56	Nuclear	Fluidigm	#3172024B
173 Yb	HLADR	Pre-conjugated	L243	Surface	Fluidigm	#3174001B
174 Yb	PD1	Pre-conjugated	EH12.2H7	Surface	Fluidigm	#3174020B
175 Lu	PDL1	Pre-conjugated	29E.2A3	Surface	Fluidigm	#3175017B
176 Yb	CD57	Pre-conjugated	HCD57	Surface	Fluidigm	#3176019B
194 Pt	Dead	NA	Cell-ID™ Cisplatin	-	Fluidigm	#201194
198 Pt	GrzB	Pre-conjugated	GB11	Cytoplasm	Fluidigm	#3198002C
209 BI	CD11b	Pre-conjugated	ICRF44	Surface	Fluidigm	#3209003B

2.2.8.4 Whole blood processing and PBMC isolation

Whole blood (6 mL) was sampled and collected in BD Vacutainer™ Hemogard™ Closure Plastic K2-Edta tubes and diluted with ice cold PBS at a 1:1 ratio. Then, 15 mL of Ficoll-Paque PLUS was added, and PBS-diluted whole blood was carefully layered on top before centrifugation at 800g for 20 minutes at room temperature (horizontal rotor, swing-out head, acceleration speed set at 2, break speed set at 2). The buffy coat (PBMC's) was carefully harvested from the Ficoll interface using a Pasteur pipette and diluted in 40 mL ice cold PBS, mixed by tube inversion and centrifuged at 500g for 10 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended in 5 mL ice-cold PBS for cell-counting by a haematocytometer. PBMC pellets were resuspended in 1.6 mL of freezing medium (90% Heat inactivated Fetal Bovine Serum, 10% DMSO) and dispensed into two cryovial aliquots of 800 µL each. Cryovials were transferred to liquid nitrogen storage within 1-5 days after being stored at $\leq -80^{\circ}\text{C}$.

2.2.8.5 Sample preparation and staining

Cryopreserved PBMCs were thawed in a water bath at 37°C, for 5 minutes. Cells were washed x2 with 10 mL PBS + 2% FBS by centrifugation at 300g for 5 minutes at RT. For dead cell exclusion, cells were incubated with a x2 solution of Cell-ID™ Cisplatin-194t, for 5 minutes at room temperature (2×10^7 cells per mL), quenched with 10 mL PBS + 2% FBS. Cells were washed a further x2 with 10 mL PBS + 2% FBS by centrifugation at 300g for 5 minutes at RT before CD45 barcoding. Individual samples were stained with a unique barcode of six metal-conjugate CD45 antibodies for 30 minutes at RT. Cells were washed x2 with 2 mL Maxpar Cell Staining Buffer and

centrifuged at 300g for 5 min, and collected into a single tube. Then, all barcoded samples were stained with a surface antigen antibody cocktail for 30 minutes at RT, in one tube. Cells were washed x2 with 2 mL Maxpar Cell Staining Buffer and centrifuged at 300g for 5 min. Cells were fixed for 10 minutes in 1x Maxpar-Fix I buffer and washed x2 with 2 mL of Maxpar Perm-S Buffer. Intracellular antigens (cytoplasmic, nuclear) were stained with an antibody cocktail for 30 minutes at RT. Cells were washed x2 in 2 mL Maxpar Cell Staining Buffer and resuspended at a concentration of 5×10^5 cells mL⁻¹. Samples were acquired on a CyTOF Helios™ mass cytometer at an event rate of <500 events/second, using dual count mode.

2.2.8.6 Data pre-processing

After acquisition, Helios™ normalization and de-barcoding tools were used to normalize signal intensity over time and generate individual FCS files based on CD45 barcodes. Data were exported as FCS files for downstream analysis, and gated to exclude residual normalization beads, debris, dead cells and doublets, leaving live, CD45⁺ events for subsequent analysis. A minimum event threshold of 5×10^4 live CD45⁺ cells per sample was set to allow for statistically meaningful comparisons of pre and post treatment samples. 17 of 26 patients had paired samples that were in abundance of this threshold and were used for analysis.

2.2.8.7 Boolean gating strategy for CyTOF analysis

CyTOF data from blood samples the WINGMEN trial were manually analysed using a gating strategy guided by the Human Immunology Project Consortium and

published guidelines for consensus identification and functional characterisation of immune cell subsets (summarized in table 2.2.1.4 and figures 2.2.8.1-8) [(422–428)].

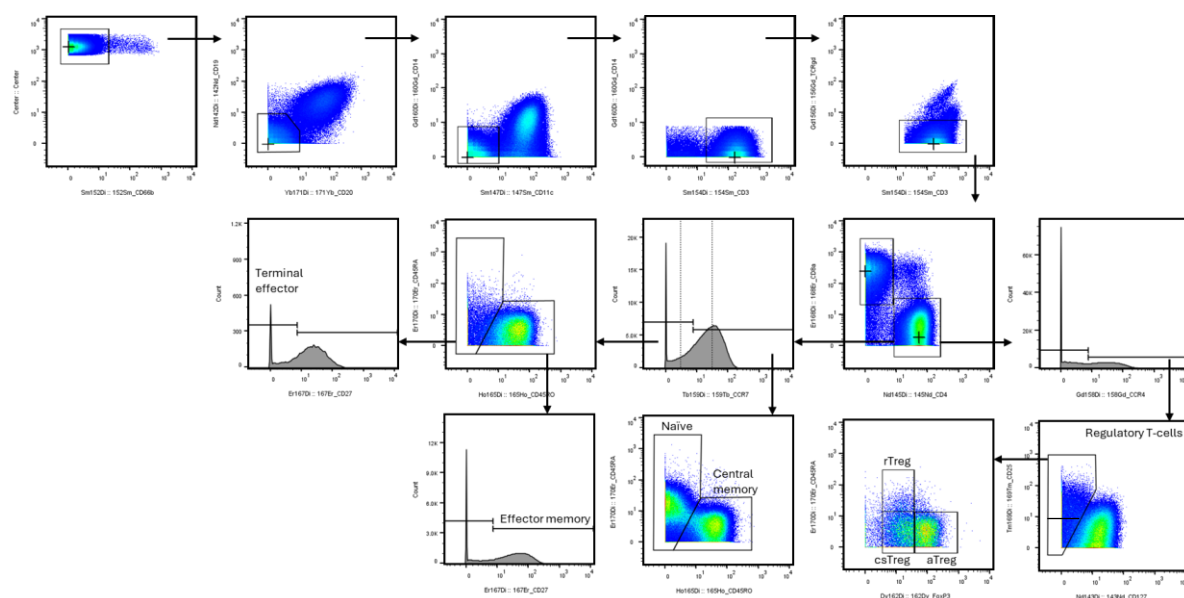


Figure 2.2.8.1 - Gating strategy used to identify subpopulations of CD4⁺ T-cells

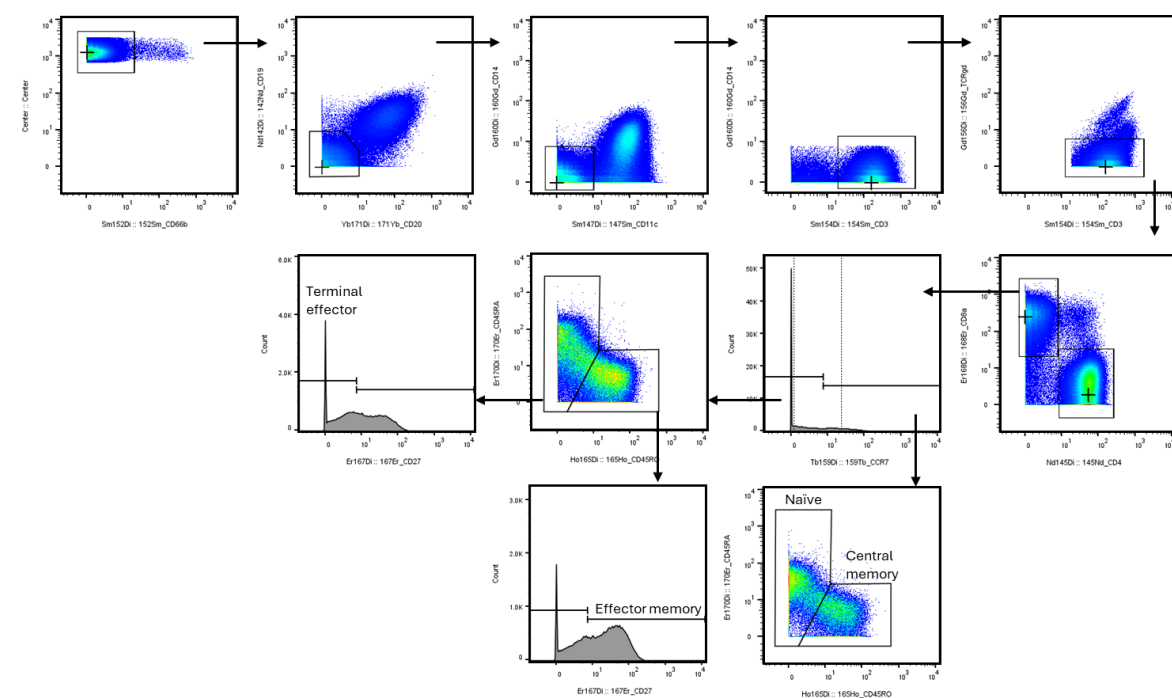
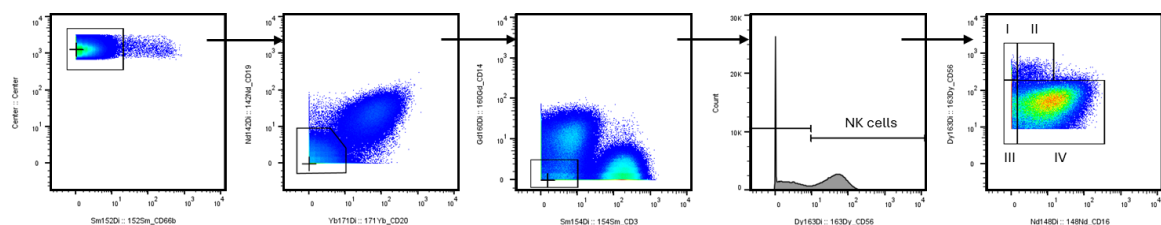
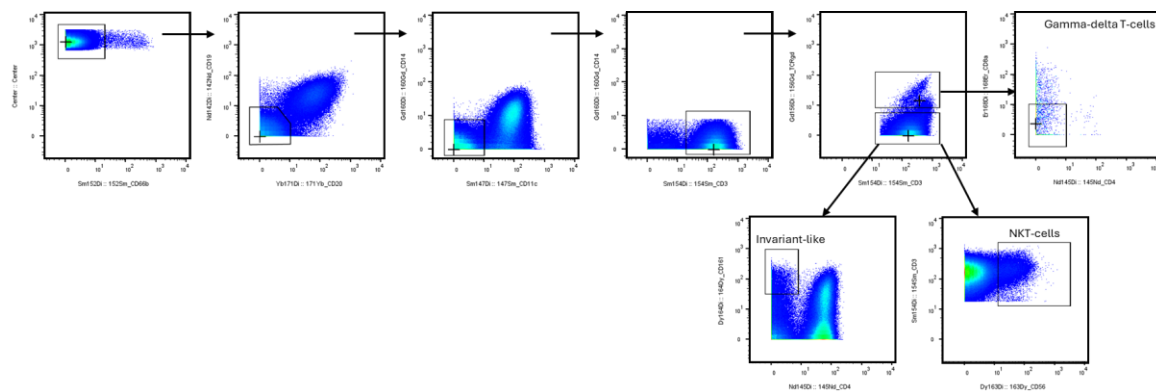
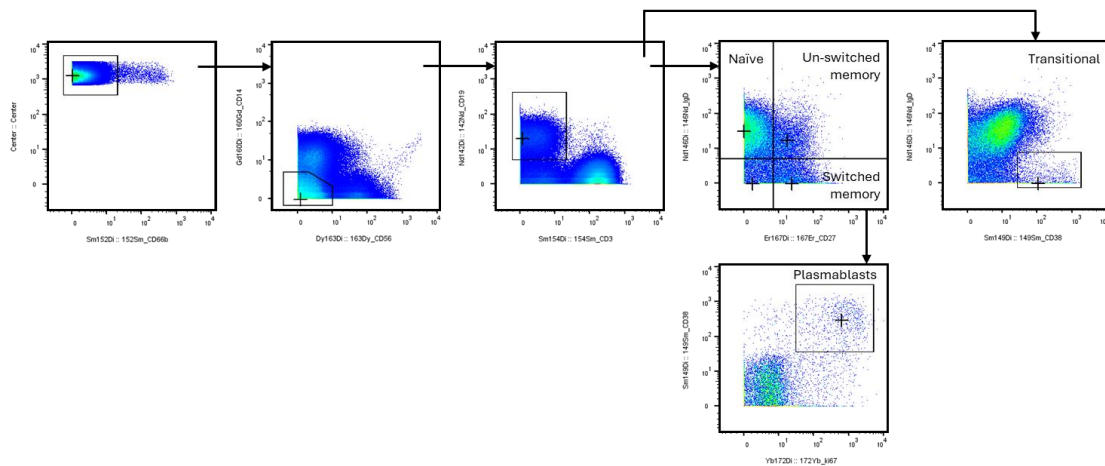


Figure 2.2.8.2 - Gating strategy used to identify subpopulations of CD8⁺ T-cells



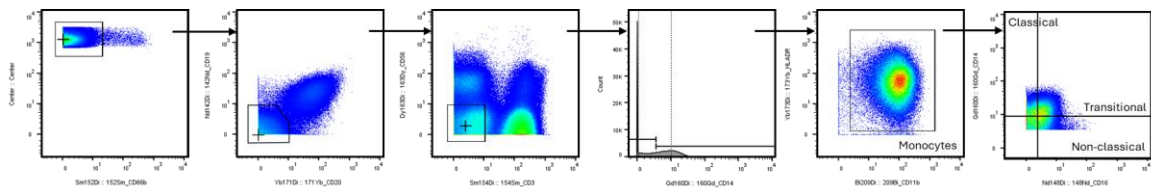


Figure 2.2.8.6 - Gating strategy used to identify subpopulations of monocytes

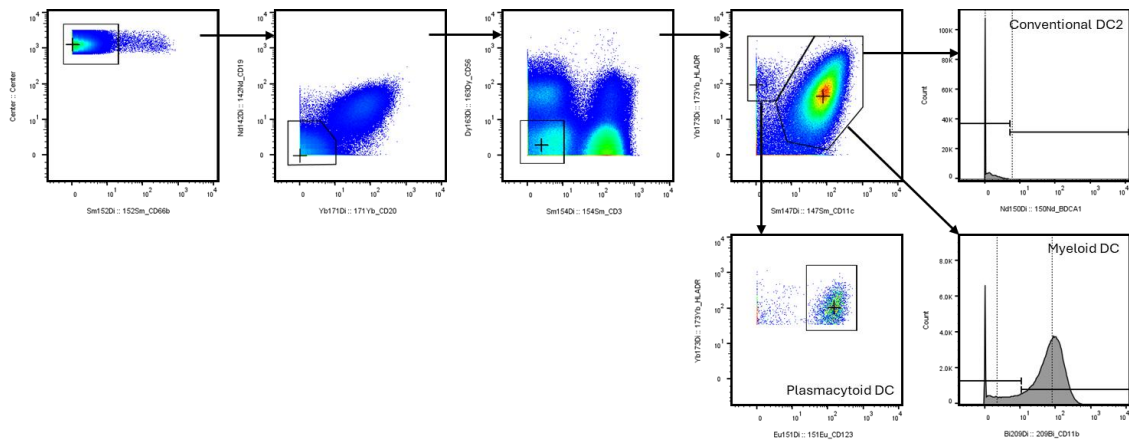


Figure 2.2.8.7 - Gating strategy used to identify subpopulations of dendritic cells

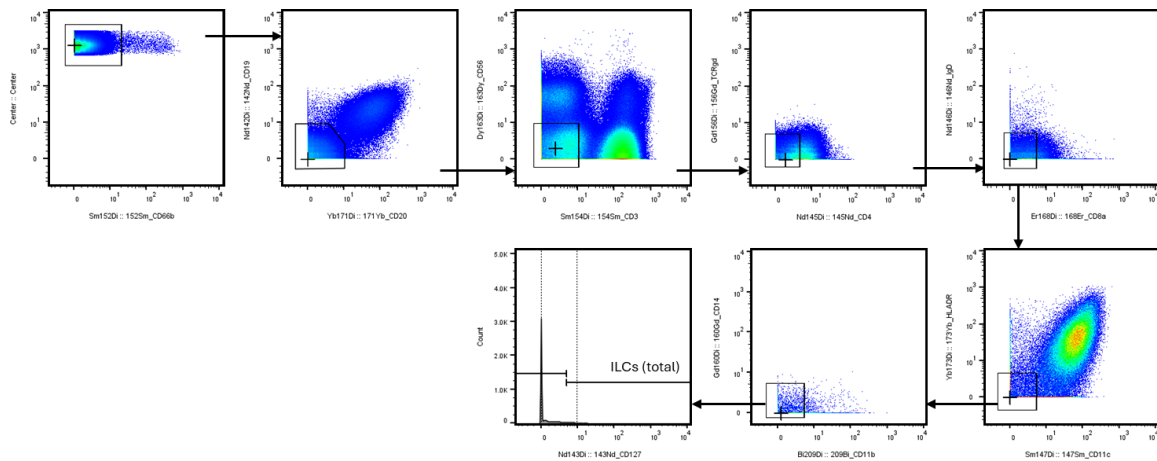


Figure 2.2.8.8 - Gating strategy used to identify innate lymphoid cells

Table 2.2.8.4 - Immune cell definitions used for mass cytometry analysis

Cell type	Sub-population	Definition
CD4 ⁺	CD4 ⁺ αβ T cells (total)	CD45 ⁺ CD66b ⁻
		CD19 ⁻ CD20 ⁻
		CD14 ⁻ CD11c ⁻
		CD3 ⁺
		CD3 ⁺ TCR-γδ ⁻
		CD4 ⁺ CD8 ⁻
	Naïve CD4 ⁺ αβ T cells	CD4 ⁺ CCR7 ^{hi}
		CD45RA ⁺ CD45RO ⁻
	Central-memory CD4 ⁺ αβ T cells	CD4 ⁺ CCR7 ^{hi}
		CD45RA ⁻ CD45RO ⁺
	Effector-memory CD4 ⁺ αβ T cells	CD4 ⁺ CCR7 ^{lo/-}
		CD45RA ⁻ CD45RO ⁺
		CD27 ⁺
CD8 ⁺	Terminal effector CD4 ⁺ αβ T cells	CD4 ⁺ CCR7 ^{lo/-}
		CD45RA ⁺ CD45RO ⁻
		CD27 ⁻
	T _{reg}	CD4 ⁺ CCR4 ⁺
		CD127 ⁻ CD25 ⁺
	Resting T _{reg} (rT _{reg})	CD45RA ⁺ FoxP3 ^{lo}
	Activated T _{reg} (aT _{reg})	CD45RA ⁻ FoxP3 ^{hi}
	Cytokine secreting T _{reg} (csT _{reg})	CD45RA ⁻ FoxP3 ^{lo}
CD8 ⁺	CD8 ⁺ αβ T cells (total)	CD45 ⁺ CD66b ⁻
		CD19 ⁻ CD20 ⁻
		CD14 ⁻ CD11c ⁻
		CD3 ⁺

		CD3 ⁺ TCR- $\gamma\delta$ ⁻
		CD4 ⁻ CD8 ⁺
	Naïve CD8 ⁺ $\alpha\beta$ T cells	CD8 ⁺ CCR7 ^{hi}
		CD45RA ⁺ CD45RO ⁻
	Central-memory CD8 ⁺ $\alpha\beta$ T cells	CD8 ⁺ CCR7 ^{hi}
		CD45RA ⁻ CD45RO ⁺
	Effector-memory CD8 ⁺ $\alpha\beta$ T cells	CD8 ⁺ CCR7 ^{lo/-}
		CD45RA ⁻ CD45RO ⁺
		CD27 ⁺
	Terminal effector CD8 ⁺ $\alpha\beta$ T cells	CD8 ⁺ CCR7 ^{lo/-}
		CD45RA ⁺ CD45RO ⁻
		CD27 ⁻
Invariant T-cells	$\gamma\delta$ T-cells	CD45 ⁺ CD66b ⁻
		CD19 ⁻ CD20 ⁻
		CD14 ⁻ CD11c ⁻
		CD3 ⁺ TCR- $\gamma\delta$ ⁺
		CD4 ⁻ CD8 ⁻
	Invariant-like T-cells	CD45 ⁺ CD66b ⁻
		CD19 ⁻ CD20 ⁻
		CD14 ⁻ CD11c ⁻
		CD3 ⁺ TCR- $\gamma\delta$ ⁻
		CD4 ⁻
	NKT-cells	CD161 ^{hi}
		CD45 ⁺ CD66b ⁻
		CD19 ⁻ CD20 ⁻
		CD14 ⁻ CD11c ⁻
		TCR- $\gamma\delta$ ⁻

		CD3 ⁺ CD56 ⁺
B-cells	B-cells (total)	CD45 ⁺ CD66b ⁻
		CD56 ⁻ CD14 ⁻
		CD19 ⁺ CD3 ⁻
	Naïve B-cells	IgD ⁺ CD27 ⁻
	Transitional B-cells	IgD ⁺ CD38 ^{hi}
	Un-switched memory B-cells	IgD ⁺ CD27 ⁺
	Switched memory B-cells	IgD ⁻ CD27 ⁺
	Plasmablasts	IgD ⁻ CD27 ⁺ Ki67 ⁺ CD38 ^{hi}
NK cells	NK cells (total)	CD45 ⁺ CD66b ⁻
		CD19 ⁻ CD20 ⁻
		CD3 ⁻ CD14 ⁻
		CD3 ⁻ CD14 ⁻
	NK cells I	CD56 ⁺
		CD56 ^{bright} CD16 ⁻
		CD56 ^{bright} CD16 ^{dim}
		CD56 ^{dim} CD16 ⁻
Monocytes	Monocytes (total)	CD45 ⁺ CD66b ⁻
		CD19 ⁻ CD20 ⁻
		CD3 ⁻ CD56 ⁻
		CD14 ⁺
	Classical monocytes	CD14 ^{hi} CD16 ⁻
	Transitional monocytes	CD14 ^{int/hi} CD16 ⁺
	Non-classical monocytes	CD14 ^{lo} CD16 ⁺
Dendritic cells	Dendritic cells (total)	CD45 ⁺ CD66b ⁻

		CD19 ⁻ CD20 ⁻
		CD3 ⁻ CD56 ⁻
		CD11c ⁺ HLA-DR ⁺
	Myeloid dendritic cells (mDC)	CD11b ⁺
	Conventional DC 2 (cDC2)	BDCA1 ⁺
		CD45 ⁺ CD66b ⁻
	Plasmacytoid dendritic cells (pDC)	CD19 ⁻ CD20 ⁻
Innate lymphoid cells		CD3 ⁻ CD56 ⁻
		CD11c ⁻ HLA-DR ⁺
		CD123 ⁺
		CD45 ⁺ CD66b ⁻
		CD19 ⁻ CD20 ⁻
		CD3 ⁻ CD56 ⁻
	Innate lymphoid cells (ILCs)	TCR- $\gamma\delta$ ⁻ CD4 ⁻
	[total]	CD8 ⁻ IgD ⁻
		CD11c ⁻ HLA-DR ⁻
		CD11b ⁻ CD14 ⁻
		CD127 ⁺

2.2.8.8 Unsupervised clustering by FlowSOM and X-Shift

Unsupervised clustering analysis by FlowSOM and XShift was performed in FlowJov10.10.0 and R (Version 4.3.3). Individual FCS files were assigned keywords and numbers to organise by treatment group and patient ID. For unsupervised clustering, equivalent nodes for immune cell populations of interest were concatenated into a single FCS file, summarized in table 2.2.8.5. Analysis was performed on ArcSinh transformed data.

Table 2.2.8.5 - Input for unsupervised clustering (FlowSOM)

Figures	Gate input for concatenation	Number of cells	(n) FlowSOM metacluster input
6.4.2	Live, CD45 ⁺	1,166,215	30
6.4.4	Live, CD45 ⁺ CD66b ⁻ CD19 ⁻ CD20 ⁻ CD14 ⁻ CD11c ⁻ CD3 ⁺ CD3 ⁺ TCR- $\gamma\delta$ ⁻ CD4 ⁺ CD8 ⁻	452,099	18
6.4.6	Live, CD45 ⁺ CD66b ⁻ CD19 ⁻ CD20 ⁻ CD14 ⁻ CD11c ⁻ CD3 ⁺ CD3 ⁺ TCR- $\gamma\delta$ ⁻ CD4 ⁺ CD8 ⁻ CD4 ⁺ CCR4 ⁺ CD127 ⁻ CD25 ⁺	24,080	5
6.4.9	Live, CD45 ⁺ CD66b ⁻ CD56 ⁻ CD14 ⁻ CD19 ⁺ CD3 ⁻	69,953	8

X-Shift analysis was run using default parameters within FlowJo v10.10.0 and all available markers, within FlowJo. FlowSOM analysis was performed using the following settings: number of FlowSOM Clusters (*n*), FlowSOM Dimension X: 10 Y: 10, Requested Plot Types MST, Requested Dendrograms Both, SOM Node Scale 100.0,

Radar Plot Colour Palette Rainbow, Seed Value 3, summary report (yes), using all available markers. Where (n) is the number of clusters generated by K-means X-Shift clustering on the same population. tSNE plots, dendrograms, relationship trees and heatmaps were automatically generated as an output within R (version 4.3.3) and saved in a pre-determined file directory upon completion of the FlowSOM algorithm. FlowSOM and X-Shift metaclusters generated as individual populations within FlowJo were applied to individual samples via use of the keyword function (Treatment # and Patient IDs) and exported for statistical comparison.

2.2.8.9 Marker enrichment modelling

Marker enrichment modelling (418) was used to calculate relative marker expression and feature enrichment within each cluster identified by FlowSOM within FlowJo_V10.10.0. The parent population (e.g. total CD19⁺ cells) was used as the MEM reference control for sub-populations, and values were mapped from a minimum of ± 1 to a maximum of ± 10 for relative marker enrichment to aid with population identification.

2.2.8.10 Hyperfinder

After identification of immune cell subsets by unsupervised clustering, Hyperfinder was used to generate gating strategies for immune cell clusters of interest [within FlowJo_V10.10.0, set to a maximum of 10 gates, target F-measure beta of 1]. This gating strategy was retroactively applied to the original parent population for comparison and exported for statistical analysis.

2.2.9 Statistical analysis

Except for data generated from mRNA-sequencing experiments, non-parametric paired T-tests, unpaired T-tests, one-way ANOVAs with multiple comparisons (Tukey test) or two-way ANOVAs were applied where indicated to determine statistical significance using Graphpad prism v10. Error bars are displayed as $\pm$ standard deviation (SD), representative of at least three independent experiments unless otherwise indicated. For mRNA-sequencing experiments, the Wald test was used for hypothesis testing when comparing two groups, corrected for multiple testing using the Benjamini and Hochberg method (in DEseq2). A p-value < 0.05 (or an adjusted p-value for mRNA-seq experiments) was considered statistically significant.

[Statistical annotations are denoted herein as; ns = $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$].

Chapter 3 - Investigating the role of IGF-1 on the generation and function of *in vitro* TGF- β -induced regulatory T-cells.

3.1 Background

Tregs are composite of tTregs induced by self-antigens in the thymus, and extrathymic induced pTregs, formed from FoxP3⁺ CD4⁺ T-cells in response to non-self-antigens in the presence of cytokines such as TGF- β , IL-2 and retinoic acid (210). Both tT_{regs} and pT_{regs} are found in human cancers and inhibit anti-tumour immunity by suppression of CTL activation, cytokine production and tumour cytolysis (217–219).

In PCa, Tregs are an important cell type that suppress Th1 and CD8⁺ CTLs and enable tumour outgrowth (246). The abundance of FoxP3⁺ Tregs within normal histological prostate lesions is associated with more than a four-fold increase in prostate cancer risk (247). Conversely, within malignant PCa, Tregs are enriched in the tumour stroma. A critical stage of pre-malignant prostate lesion transformation involves the production of KLK4 that drives local remodelling of the stromal niche (142). Autocrine KLK4 production in pre-malignant lesions liberates IGF-1 bound to IGFBPs and promotes the proteolytic maturation of TGF- β (429). Thus, a positive feedback loop is formed between CAFs and epithelial cells through paracrine TGF- β and IGF-1 signalling, that could represent an important mechanism that supports *de novo* pTreg generation in the PCa TME.

3.2 Chapter aims

Consequently, a key aim of this chapter is to explore how IGF-1 supply shapes the suppressive function of pTregs and tTregs proximal to PCa cells.

3.3 Investigating IGF-1 axis activity in murine T-cells

3.3.1 Expression of IGF-1R on murine T-cell subsets

With the aim of investigating effects of IGF-1 on Treg function, a 14-colour flow cytometry panel was designed for use on the Attune NXT flow cytometer to allow for multi-parameter immunophenotyping of T-cells (fig. 3.3.1.1a). For flow cytometry panel design and optimization, the systematic workflow by *Holmberg-Thyden et al.* was followed [data not shown] (430). Using this panel, the relative expression of the IGF-1R, the cognate receptor for IGF-1, on CD4⁺ and CD8⁺ T-cell subsets was evaluated by flow cytometry. Unstimulated wild-type splenic CD8⁺, Tconv and tTreg cells expressed detectable levels of cell surface IGF-1R relative to fluorescent minus one controls (fig. 3.3.1.1b). Relative to CD8⁺ and Tconv cells, tTregs express significantly higher levels of IGF-1R (fig. 3.3.1.1c). Thus, murine T-cells express IGF-1R, the receptor for IGF-1.

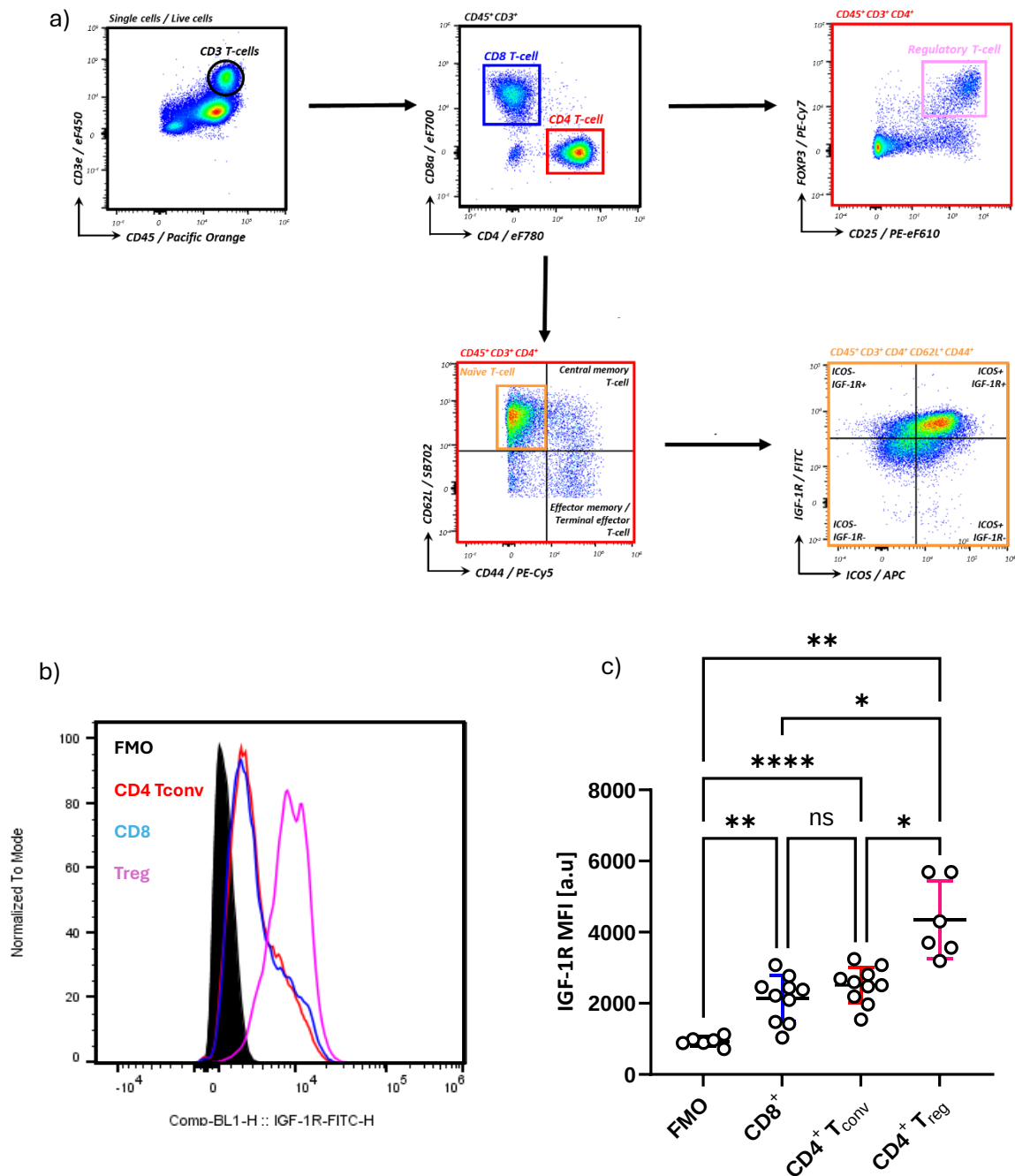


Figure 3.3.1.1 - Expression of the type-1 IGF receptor (IGF-1R) by murine T-cell subsets

Splenocytes isolated from 5–6-week-old male FVB mice were stained with T-cell lineage and phenotypic markers prior to analysis by flow cytometry. a) Representative flow cytometry dot plots display the unimodal/bimodal nature of FoxP3, CD25, CD44, CD62L, ICOS or IGF-1R staining. b-c) Representative histograms and quantification of IGF-1R MFI in CD8, CD4 Tconv or Treg subsets relative to fluorescent minus one controls. [$* = p < 0.05$, $**** = p < 0.0001$, by One-way ANOVA with multiple comparisons]

$n = 6$ independent donors for FMO controls and $CD4^+$ Tregs. $n = 10$ independent donors for $CD8^+$ and $CD4^+$ Tconv cells. Bars represent means $\pm$ SEM.

3.3.2 IGF-1 and CD4⁺ T-cell signalling

To test if detectable surface IGF-1R was functional and responsive to ligand-based activation, the phosphorylation of markers downstream of canonical IGF-1R signalling after IGF-1 stimulation were explored. Whilst traditional cell-signalling techniques such as Western immunoblots offer high sensitivity, we found they were not practical for our T-cell signalling experiments due to their requirements for high cell numbers. Alternatively, analysis by phospho-flow cytometry is a more high-throughput approach as it requires fewer cells, allowing for testing of more conditions per-assay.

To test the effects of IGF-1 on CD4⁺ T-cell signalling, naïve CD4⁺ T-cells were isolated and serum starved cells for 4 hours to reduce background phosphorylation levels (data not shown). CD4⁺ T-cells were then stimulated with physiological doses of IGF-1 (1, 10, 50, 100 nmol L⁻¹) for 30 minutes prior to fixation and permeabilization of cells and analysis by flow cytometry (fig. 3.3.2.1a). To test if downstream phosphorylation signals induced by IGF-1 were specific to IGF-1R activation, serum starved naïve CD4⁺ T-cells were pre-treated with the IGF-1R tyrosine kinase inhibitor BMS-754807 20 minutes prior to stimulation with IGF-1. IGF-1R signalling transmits signals through PI3K-mTOR, STAT3 and MAPK pathways. Hence, phosphorylation sites downstream of mTORC1 (phosphorylation of rpS6 at serine 235,236), mTORC2 (phosphorylation of AKT at serine 473), MAPK (phosphorylation of ERK1/2 at threonine 202/tyrosine 204) and STAT3 (phosphorylation of STAT3 at tyrosine 705) activation were selected to measure IGF-1R signalling.

IGF-1 stimulation induced a significant increase in the percentage of naïve CD4⁺ cells staining positive for phosphorylation of ribosomal protein S6 (rpS6) at concentrations ranging from 10 – 100 nmol L⁻¹ IGF-1 but this increase was not detectable at 1 nmol L⁻¹ (fig. 3.3.2.1b-c). This response was abrogated in the presence of 100 nM rapamycin, indicative that phosphorylation of rpS6 induced by IGF-1 occurs in an mTORC1 dependent manner (fig. 3.3.2.1d). IGF-1 stimulation did not lead to a significant increase in AKT phosphorylation at serine 473 in naïve CD4⁺ T-cells (fig. 3.3.2.1e-f). Hence, IGF-1/IGF-1R signalling promotes downstream phosphorylation targets of mTORC1, but not mTORC2 in naïve CD4⁺ T-cells.

IGF-1 stimulation did not significantly impact STAT3 phosphorylation at concentrations of IGF-1 up to 100 nmol L⁻¹ (fig. 3.3.2.2a-b) but did significantly increase the percentage of cells staining positive for phosphorylated ERK1/2 (fig. 3.3.2.2c-d) at concentrations ranging from 10 – 100 nmol L⁻¹. These results indicated that IGF-1/IGF-1R signalling promotes downstream MAPK activity, but does not lead to STAT3 phosphorylation in naïve CD4⁺ T-cells.

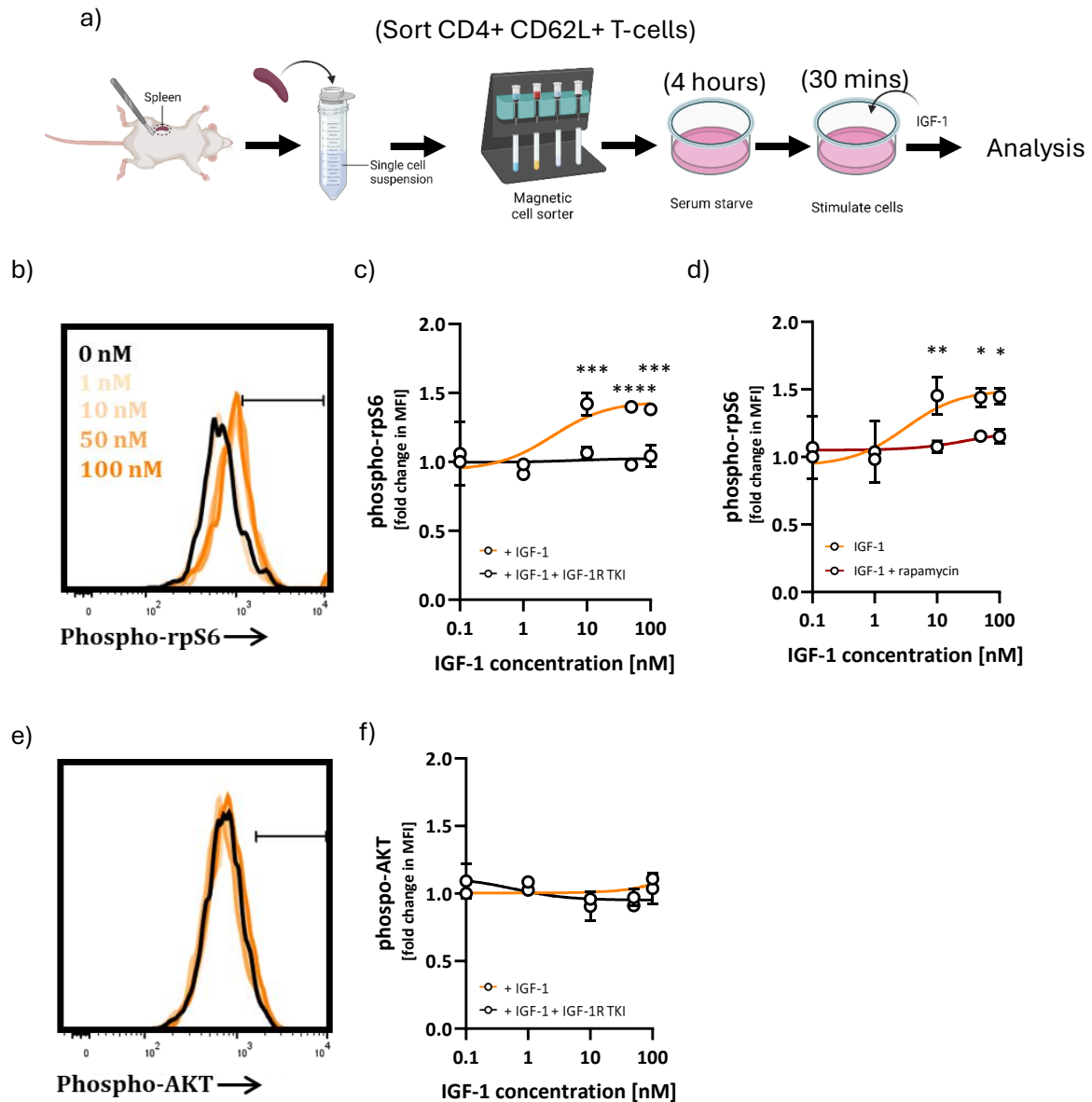


Figure 3.3.2.1 - IGF-1 stimulation activates mTORC1 but not mTORC2 in naïve murine CD4⁺ T-cells

a) Diagram illustrating experimental design. Naïve CD4⁺ T-cells were sorted magnetically from single-cell suspensions of 6–8-week-old (♂) FVB murine splenocytes. CD4⁺ T-cells were serum-starved for 4 hours and stimulated with IGF-I or solvent control for 30 minutes. Graphs show flow cytometric histograms and the relative MFI of phospho-rpS6 (b-d) or phospho-AKT (e-f).

Specificity of phospho-signal to bioactive IGF-1 was confirmed via addition of an IGF-1R tyrosine kinase inhibitor (BMS-754807) (c and f) or an mTORC1 inhibitor (rapamycin) [d] for 20 minutes prior to IGF-1 stimulation.

[**** = $p < 0.0001$, by Two-way ANOVA]

N = 3 independent experiments and donors. Bars represent means $\pm$ SD.

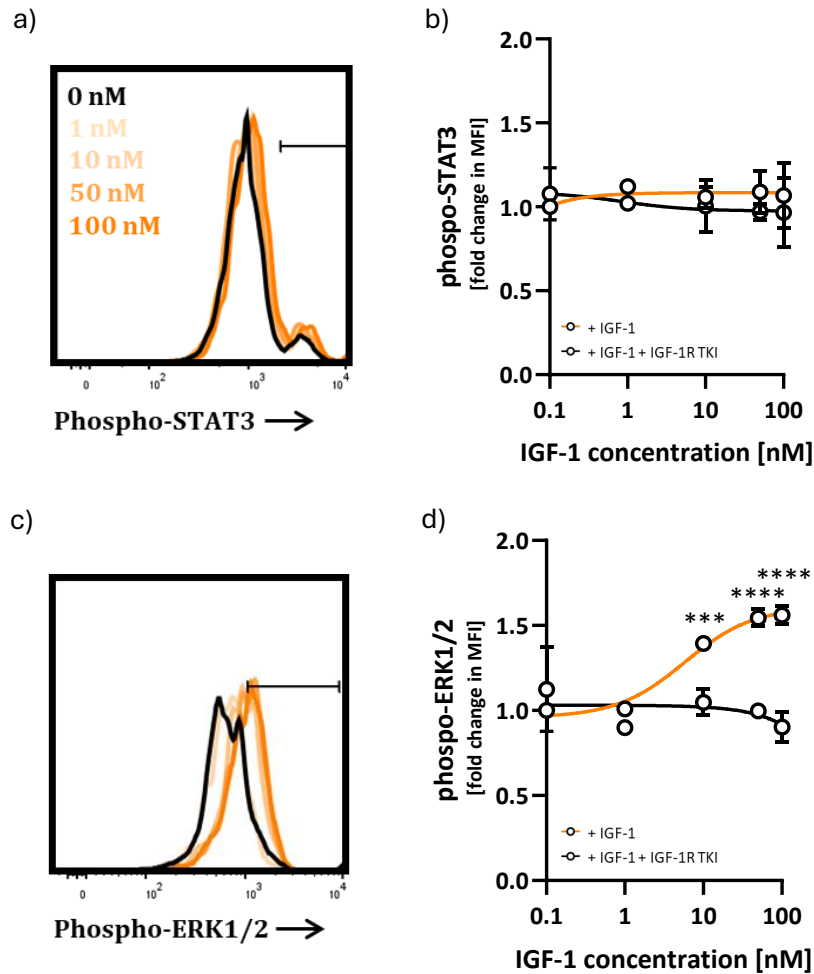


Figure 3.3.2.2 - IGF-1 stimulation activates MAPK but not STAT3 signalling in CD4⁺ T-cells

Naïve CD4⁺ T-cells were sorted magnetically from single-cell suspensions of 6–8-week-old (♂) FVB murine splenocytes. Cells were serum-starved for 4 hours, and stimulated with IGF-I (orange bars) for 30 minutes. Graphs show flow cytometric histograms and MFI of phospho-STAT3 (Tyr705) (a-b) or phospho-ERK1/2 (Thr202,Tyr204) (c-d).

Specificity of phospho-signal to bioactive IGF-1 was confirmed via addition of an IGF-1R tyrosine kinase inhibitor, BMS-754807, 20 minutes prior to IGF-1 stimulation (b, d).

[** = $p < 0.01$ *** = $p < 0.001$, **** = $p < 0.0001$, by Two-way ANOVA]

N = 3 independent experiments and donors. Bars represent means $\pm$ SD.

3.3.3 Macrophage: CD4⁺ T-cell crosstalk by the IGF-1 axis

Whilst the majority of circulating IGFs are produced by the liver, IGF-1 is also produced within local tissues by fibroblasts, macrophages and B-cells. In PCa, crosstalk exists between cancer-associated fibroblasts (IGF-1⁺) and tumour cells [IGF-1R⁺] (151). However, is not currently known if stromal and/or immune cells produce IGFs at physiological concentrations that activate IGF-1R signalling in CD4⁺ T-cells. Although CAF isolation would have represented a physiologically relevant source of IGFs to the PCa TME, their heterogeneity *in vivo* limits cell yields from FACsorting and their altered gene expression profile upon tissue culture presents challenges for reproducible *in vitro* experiments (431). In contrast, bone-marrow derived macrophages can be isolated in high numbers from autologous donors, are easy to culture, and can be polarized *in vitro* to produce IGF-1. Having established phosphorylation of rpS6 in murine CD4⁺ T-cells as a marker of IGF axis activation in 3.3.2, crosstalk between IGFs produced by stromal/immune cells, and CD4⁺ T-cells expressing IGF-1R was explored.

To test this, conditioned medium (CM) from macrophages (M ϕ) polarized under M0, M1 or M2 conditions was generated and added to serum starved naïve CD4⁺ T-cells. CM from M1 and M2, but not M0 macrophages significantly increased phosphorylation levels of rpS6 above baseline levels in CD4⁺ T-cells, indicative of the presence of cytokines and growth factors in M1 and M2 CM (data not shown). Next, to assess the relative levels of bioactive IGF-1 in different macrophage CMs, we utilized the potent IGFs neutralization properties of the antibody xentuzumab (358). We found that addition of Xentuzumab to M1 (fig. 3.3.3.1a-c) and M2 (fig. 3.3.3.1d-f), but not M0 (fig. 3.3.3.1g-i) macrophage CM significantly decreased rpS6 phosphorylation

percentage positivity in CD4⁺ T-cells. Notably, phosphorylation of rpS6 did not return to baseline levels upon Xentuzumab treatment, indicative of additional growth factors or cytokines produced by Mφ's that contribute to mTORC1 activation in these cells. Nevertheless, these data show that IGF ligands are produced at physiologically relevant doses by M1 and M2 macrophages that bind and activate IGF-1R expressed by CD4⁺ T-cells. Hence, paracrine IGF-1 axis crosstalk between IGF-1⁺ macrophages and IGF-1R⁺ CD4⁺ T-cells could theoretically function to fine-tune CD4⁺ T-cell fate through modulation of mTORC1 activity.

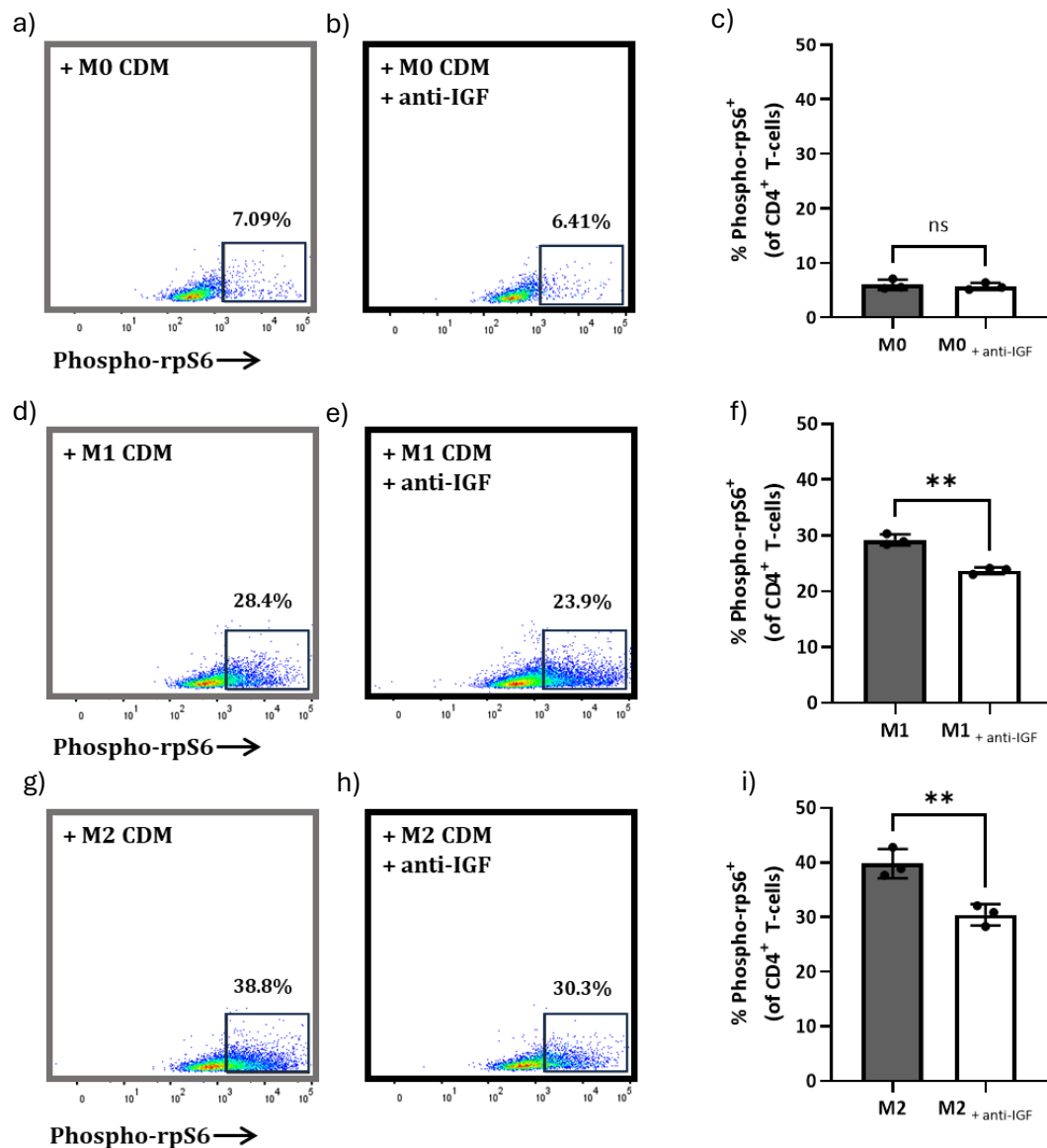


Figure 3.3.3.1 - IGFs produced by M1 and M2 macrophages contribute to rpS6 phosphorylation in CD4⁺ T-cells.

Macrophages flushed from the bone marrow of 6–8-week-old (♂) C57BL/6 mice were differentiated *in vitro* for 4 days with m-CSF prior to polarization under M0, M1 or M2 conditions. After 48 hours of polarization, fresh culture medium without polarizing cytokines was added for a further 24 hours to generate conditioned medium (CM). Naïve CD4⁺ T-cells were sorted magnetically from 6–8-week-old (♂) C57BL/6 murine splenocytes. CD4⁺ T-cells were serum-starved for 4 hours prior to addition of CM ± Xentuzumab for 30 minutes before analysis of phospho-markers by flow cytometry.

Graphs show flow cytometric histograms and percentage positivity of phospho-rpS6 upon addition of M2 CM ± Xentuzumab (a-c), M1 CM ± Xentuzumab (e-f) or M0 CM ± Xentuzumab (g-i).

[** = $p < 0.01$, by unpaired T-test] N = 3 independent experiments and donors. Bars represent means ± SD.

3.4 Investigating the effects of IGF-1 stimulation on iTreg differentiation and phenotype

3.4.1 IGF-1 and iTreg differentiation

In vitro activation of CD4⁺ T-cells with anti-CD3 and anti-CD28 antibodies in the presence of TGF- β 1 leads to the robust induction of FoxP3⁺ iTregs and is a useful tool recapitulate the development pTregs *in vivo* (210). First, the efficiency of *in vitro* induced Treg (iTreg) differentiation with TGF- β by evaluating FoxP3 and cell surface CD25 expression. We confirmed that FoxP3 upregulation in TCR stimulated CD4⁺ T-cells labelled with CellTrace Violet occurred in a TGF- β dependent manner (fig. 3.4.1.1a-c).

Next, a range of IGF-1 concentrations (0, 10, 30, and 50 nmol L⁻¹) was used based on measurements of IGF-1 bioactivity in tumours to recapitulate low (0, 10 nmol L⁻¹) and high (30, 50 nmol L⁻¹) IGF-1 microenvironments experienced *in vivo* (395,403,432,433). Although IGF-1 did not significantly impact the formation of FoxP3⁺ CD25⁺ double positive iTregs at concentrations at 10 or 30 nmol L⁻¹, at concentrations of 50 nmol L⁻¹ this was significantly increased (fig. 3.4.1.1c-d). Further exploration of individual markers showed IGF-1 did not significantly impact the expression of CD25 (fig. 3.4.1.1f) but had a significant effect on FoxP3 expression (fig. 3.4.1.1g).

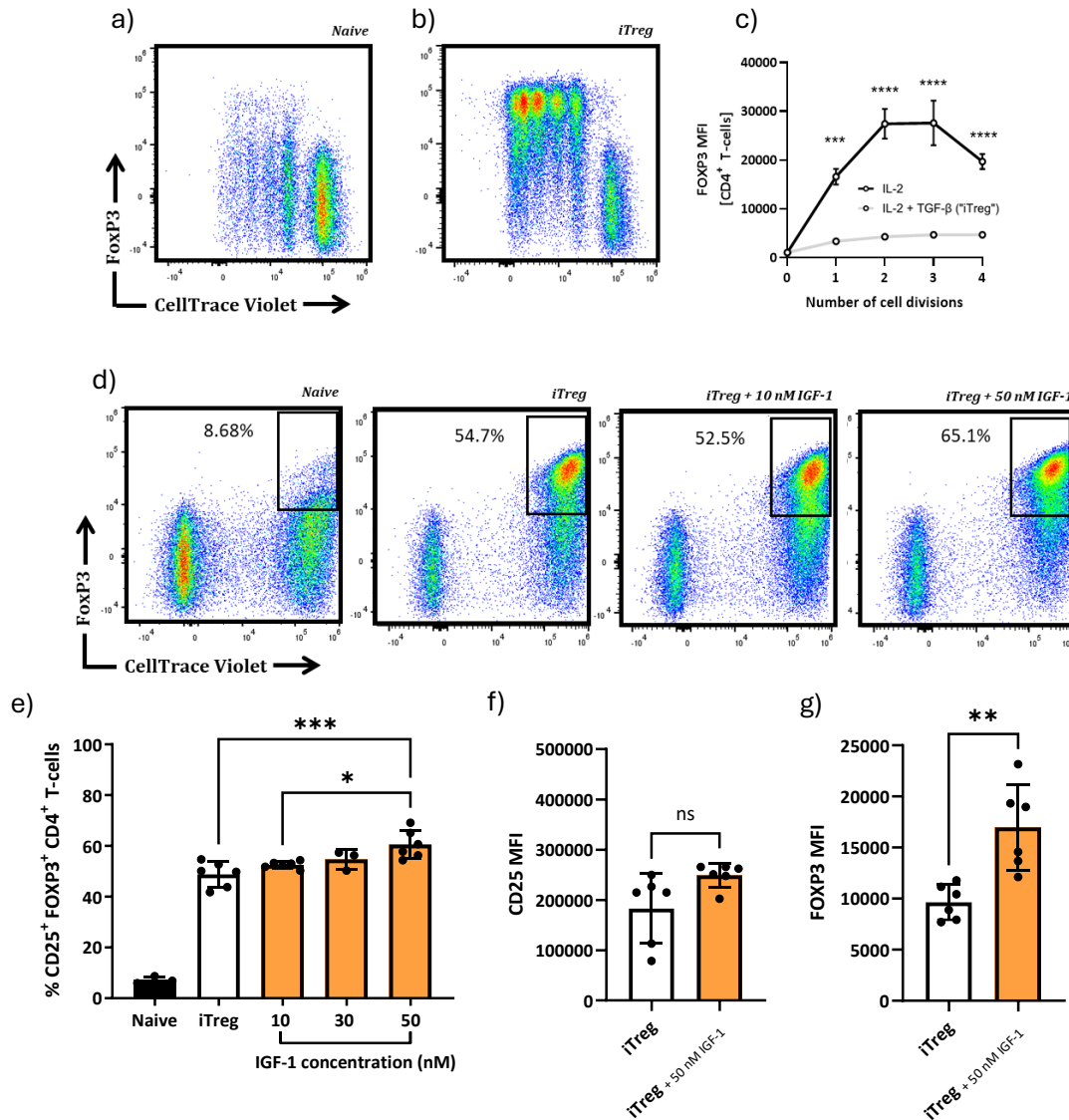


Figure 3.4.1.1 IGF-1 enhances FoxP3 expression during iTreg differentiation

Sorted naïve murine CD4⁺ T-cells from 6–8-week-old (σ) FVB mice were labelled with CellTrace Violet and differentiated under iTreg conditions or IL-2 alone for 72 hours. Expression of FoxP3, CD25 and CellTraceViolet was analysed by flow cytometry and representative dot for cells treated with IL-2 alone (a) or under iTreg conditions (b) are shown. CellTraceViolet peaks indicating cell divisions were individually gated and the MFI of FoxP3 expression was quantified for each division (c).

Naïve CD4⁺ T-cells were differentiated under the following conditions: IL-2 alone (Naïve), iTreg conditions (iTreg) or iTreg conditions $\pm$ 10, 30 or 50 nmol L-1 IGF-1 for 72 hours before analysis by flow cytometry. Representative dot plots (d) show sample gating for FoxP3⁺ CD25⁺ co-expressing CD4⁺ T-cells after Treg differentiation. Expression of FoxP3 and CD25 was quantified and displayed as percentage positivity of CD4⁺ T-cells (e) or MFI (f-g) respectively.

[* = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001, by Two-way ANOVA (c), one-way ANOVA with multiple comparisons (e) or unpaired T-test (f,g).

N = 6 independent donors over 3 independent experiments. Bars represent means $\pm$ SD.

3.4.2 IGF-1 and iTreg proliferation

Given that IGF-1 regulates Treg proliferation both *in vitro* and *in vivo* (382,383), and increased IGF-1R expression in Tregs (fig. 3.3.1) we first considered whether the upregulation of FoxP3 was mediated by the preferential expansion and proliferation of FoxP3⁺ cells. To test this, naïve CD4⁺ T-cells were labelled with a proliferation dye CellTrace Violet (CTV). With each cell division, the CTV dye intensity halves, forming sequential peaks of dye intensity representative of the number of T-cell divisions. Thus, we measured the number of cell divisions during iTreg differentiation with IGF-1 stimulation by quantifying the relative change in dye dilution by flow cytometry. We found that addition of 10 and 30 nmol L⁻¹ IGF-1 significantly increased the rate of proliferation during CD4⁺ iTreg differentiation (fig 3.4.2.1a-b). However, this difference was not statistically significant at 50 nmol L⁻¹ IGF-1, although there was a trend towards increased proliferation (fig 3.4.2.1c). These results show that low-mid range concentrations of IGF-1 stimulate proliferation during iTreg differentiation.

To decouple the relationship between FoxP3 expression and iTreg proliferation, FoxP3 expression was compared within populations of CD4⁺ T-cells with the same number of cell divisions. We found that 10-30 nmol L⁻¹ IGF-1 did not significantly impact FoxP3 expression at any number of cell divisions relative to controls (fig 3.4.2.1d-e). However, 50 nmol L⁻¹ induced enhanced FoxP3 expression in iTregs with at least 2 cell divisions (fig 3.4.2.1f). Thus, the effects of IGF-1 on FoxP3 upregulation at high doses (50 nmol L⁻¹ IGF-1) is not necessarily dependent on an increased rate of Treg proliferation.

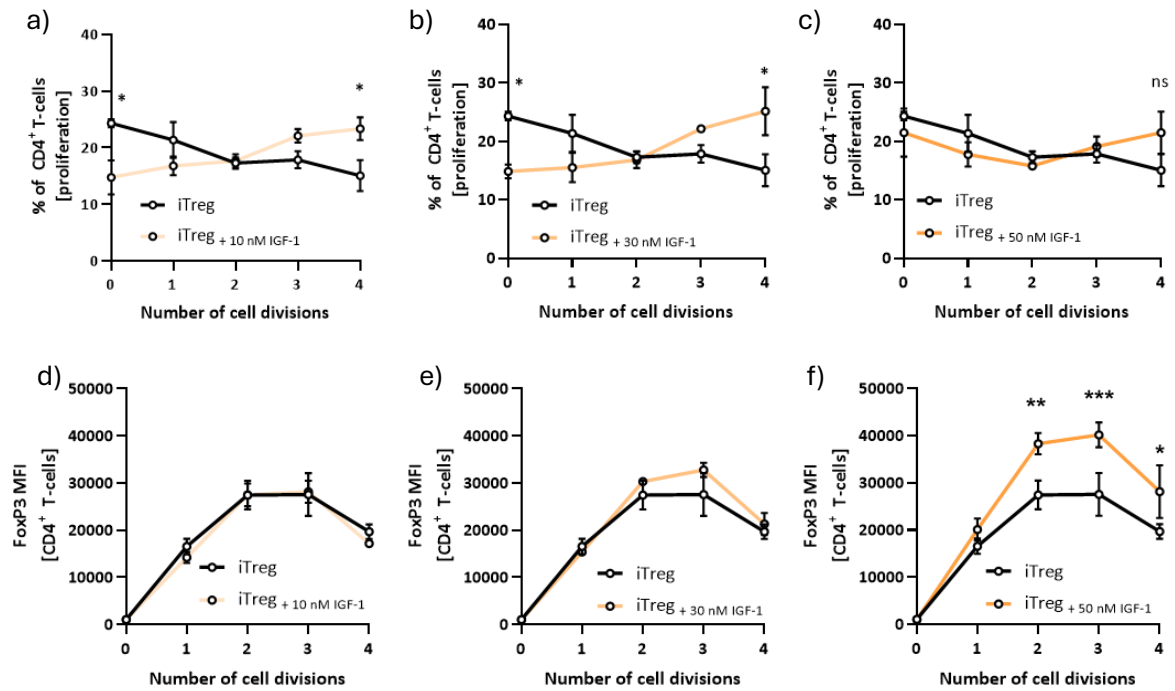


Figure 3.4.2.1 - IGF-1 has a dose-dependent effect on iTreg proliferation and FoxP3 expression

Peaks of CellTrace Violet dilution from proliferating cells were individually gated and FoxP3 expression was quantified and compared within each proliferation peak.

The number of cell divisions in CD4⁺ iTreg cells treated with (a) 10 nmol L⁻¹ IGF-1, (b) 30 nmol L⁻¹ IGF-1, or (c) 50 nmol L⁻¹ IGF-1 relative to control iTreg cells.

The median fluorescence intensity of FoxP3 expression of CD4⁺ iTreg cells treated with (d) 10 nmol L⁻¹ IGF-1, (e) 30 nmol L⁻¹ IGF-1, or (f) 50 nmol L⁻¹ IGF-1 relative to control iTreg cells.

Comparisons were made between cells with the same number of cell divisions identified by CTV peaks (0-4 divisions, data not shown).

[* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, by Two-way ANOVA]

N = 3 independent experiments and donors. Bars represent means $\pm$ SD.

3.4.3 IGF-1 and phospho-signalling during iTreg differentiation

Whilst mTORC1 activity alone supports the formation of effector FoxP3⁺ iTregs (434), concomitant mTORC1 and mTORC2 activity (435), or MAPK activation negatively impacts the stability of FoxP3 expression in response to TGF- β stimulation (436). In human tTreg cells, IGF-1 enhances IL-2 mediated STAT5 phosphorylation (382), an important pathway for Treg stability. Hence, we also considered whether increased FoxP3 expression in response to high doses of IGF-1 could be mediated by enhanced phosphorylation of STAT5. Thus, we quantified phosphorylation of rpS6 (figure 3.4.3.1a), FoxP3 (figure 3.4.3.1b), phospho-STAT5 [downstream of IL-2 signalling] (figure 3.4.3.1c), phospho-STAT3 (figure 3.4.3.1d), phospho-ERK1/2 (figure 3.4.3.1e) and phospho-AKT (figure 3.4.3.1f) by flow cytometry at 0, 4, 24, 48 and 72-hours post-differentiation upon co-stimulation with 0 or 50 nmol L⁻¹ IGF-1.

After 4 hours, co-stimulation with 50 nmol L⁻¹ IGF-1 induced significant increase in phospho-rpS6, and a significant decrease in phospho-AKT and FoxP3 expression. By 24 hours, phospho-rpS6 and phospho-AKT levels returned to controls, whilst FoxP3 expression significantly increased in IGF-1 stimulated iTregs. FoxP3 expression peaked by 24 hours, and was sustained at slightly higher levels from 48-72 hours with iTregs stimulated with IGF-1. ERK and AKT signalling remained largely inactive throughout iTreg differentiation were not significantly activated with IGF-1 co-stimulation.

The lack of MAPK activity observed in IGF-1/TCR stimulated iTregs could be explained the digital nature of the phospho-ERK response, forming an “all or nothing” response (437). However, IGF-1 stimulation induced a notable, but transient increase

in phospho-rpS6 levels. The temporal nature of this activation likely relates to observations in L6 myoblast cells, whereby IGF-1-induced mTORC1 activation initiates a negative feedback loop via degradation of the adapter protein IRS1 after 3-6 hours, required for the internalization of activated IGF-1R (438). Of note, low-dose IGF-1 (10 nmol L⁻¹) had no effect on phospho-rpS6 levels throughout iTreg differentiation (appendix fig. 8.1.1a), and the effect of 50 nmol L⁻¹ IGF-1 on increased phospho-rpS6 at 4 hours was abrogated in the presence of rapamycin (appendix fig. 8.1.1b). Thus, a threshold of ligand availability is required for IGF-1 mediated phosphorylation of rpS6, and is mTORC1 dependent.

Inhibition of mTORC1 with rapamycin did not impact the IGF-1 mediated FoxP3 upregulation, nor did the addition of other small molecule inhibitors of canonical pathways downstream of IGF-1R activation [AKT isoforms 1-3, PI3K γ/δ or MEK1/2] (appendix fig. 8.1.1b). This response was only abolished in the presence of inhibitors of IGF-1R tyrosine kinase activity. These results could be indicative of a requirement for non-canonical IGF-1R signalling, such as nuclear translocation after receptor internalization. Although speculation, nuclear IGF-1R has binding sites in regions of DNA in the promoter region of JUN, which interacts with an enhancer region within intron 1 of the *FoxP3* locus (344).

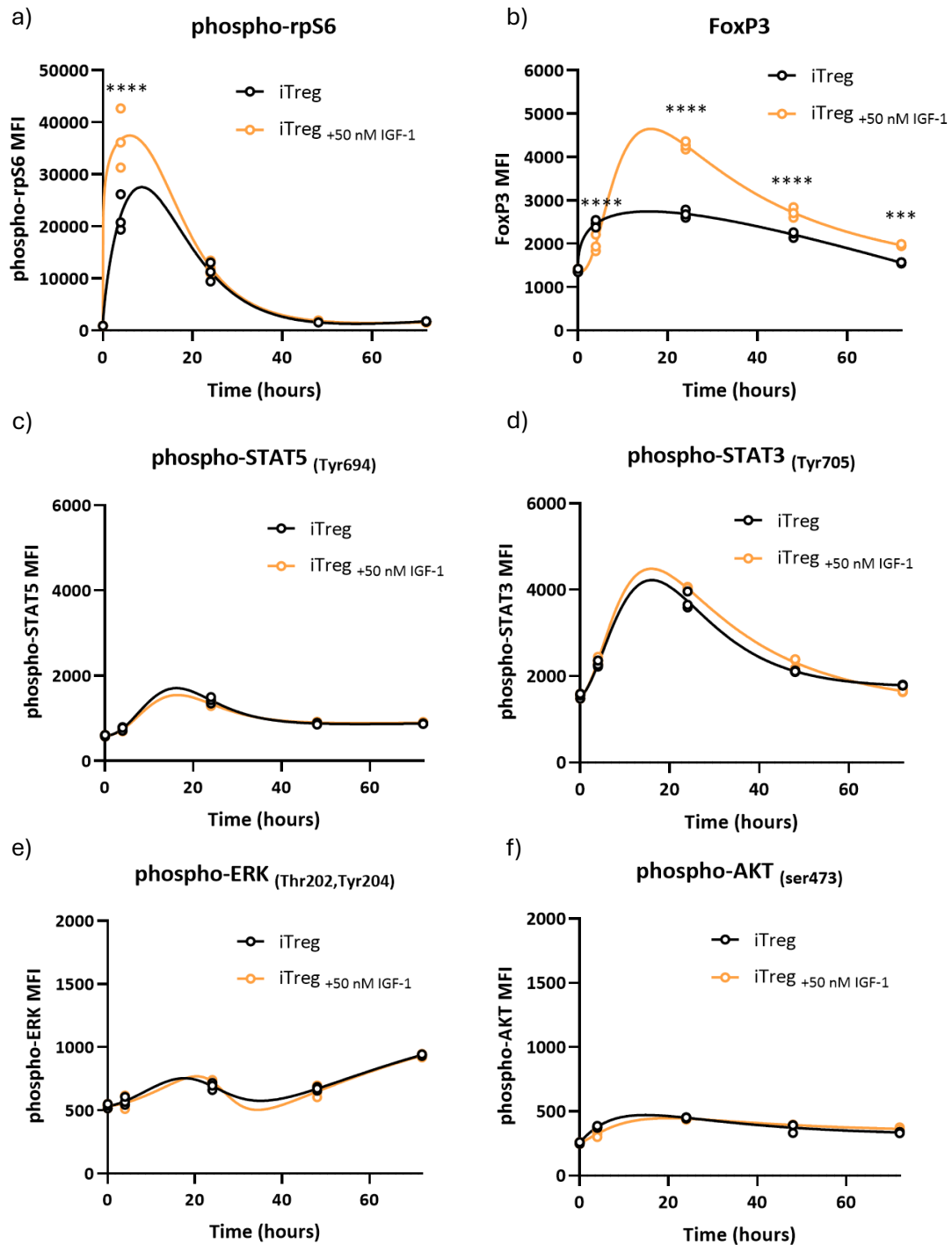


Figure 3.4.3.1 Co-stimulation with 50 nM IGF-1 promotes mTORC1 activation during iTreg differentiation

Sorted naïve murine CD4⁺ T-cells from 6–8-week-old (♂) FVB mice were differentiated under iTreg conditions $\pm$ 50 nM IGF-1 and analysed by flow cytometry. The MFI of phospho-rpS6 (a), FoxP3 (b), phospho-STAT5 (c), phospho-STAT3 (d), phospho-ERK1/2 (e) and phospho-AKT (f) was quantified at 0, 4, 24, 48 and 72 hours post differentiation. A non-linear bell-shaped dose response curve was fitted to visually model phosphorylation and expression dynamics, not for statistical comparisons.

[*** = $p < 0.001$, **** = $p < 0.0001$, by Two-way ANOVA]

3.4.4 IGF-1 and iTreg phenotype

Of functional importance, the suppression of anti-tumour immunity mediated by effector TI-Tregs is dependent on mTORC1 activity in syngeneic mouse models of melanoma (434). Hence, the activity of mTORC1 in eTregs directly correlates with the capacity to suppress anti-tumour immunity *in vivo*. Having observed increased early rpS6 phosphorylation in iTregs differentiated with high IGF-1 supply, the expression of proteins and transcription factors upregulated by effector Treg cells was investigated, in addition to markers of Treg suppressive function.

We found that 50 nmol L⁻¹ IGF-1 did not significantly impact the expression of cell-surface BTLA, PD-1, ICOS, CTLA-4, CD73, IL-17a or intracellular IL-10 after 72 hours (appendix fig. 8.1.1.2a-f). However, high IGF-1 supply enhanced the expression of intracellular Granzyme-B (fig. 3.4.4.1a-c), nuclear Ki67 (fig. 3.4.4.2d-f) and cell-surface PD-L1 (fig. 3.4.4.2a-c) and TIM-3 expression (fig. 3.4.4.2d-f). Furthermore, stimulation with 50 nmol L⁻¹ IGF-1 significantly increased the percentage of iTregs that were effector cells (appendix fig 8.1.2a). Taken together, high IGF-1 supply promotes the development of effector-iTregs, consistent with observations with increased mTORC1 activation.

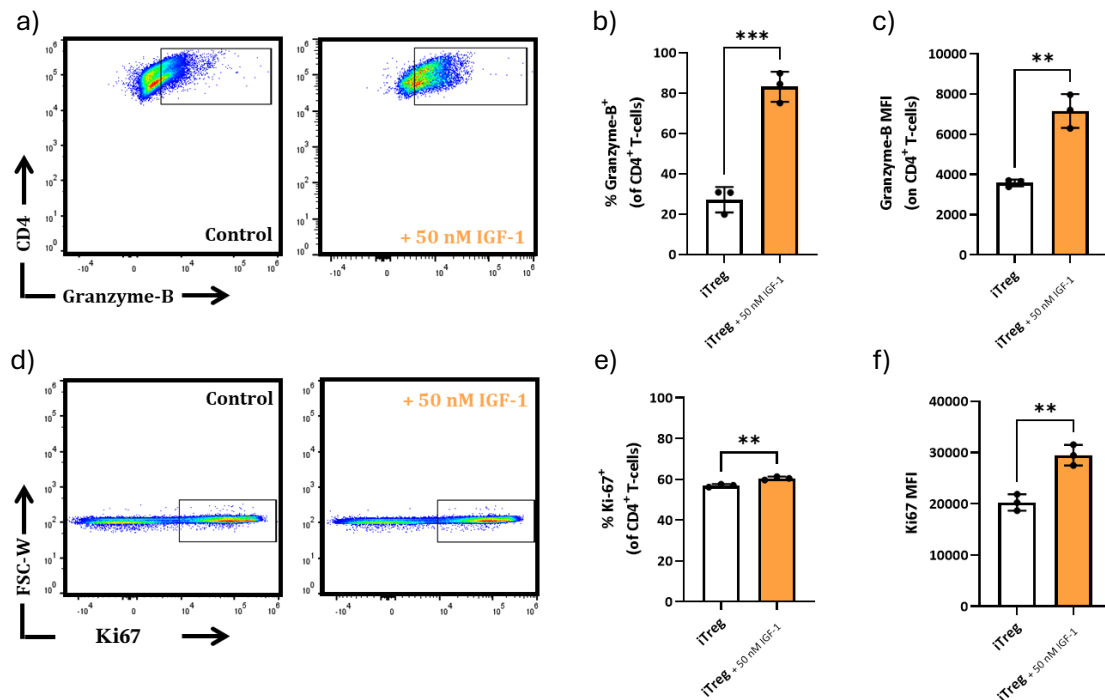


Figure 3.4.4.1 IGF-1 upregulates Granzyme-B and Ki67 expression in iTregs

Sorted naïve murine CD4⁺ T-cells from 6–8-week-old (♂) FVB mice were differentiated under iTreg conditions $\pm$ 50 nM IGF-1 for 72 hours and analysed by flow cytometry.

Representative dot plots show sample gating strategy for identification of Granzyme-B⁺ (a) or Ki67⁺ (c) Tregs, alongside quantification of percentage positivity and MFI for Granzyme-B (b-c) and Ki67 (e-f).

[** = $p < 0.01$, *** = $p < 0.001$, by unpaired T-test]

N = 3 independent experiments and donors. Bars represent means $\pm$ SD.

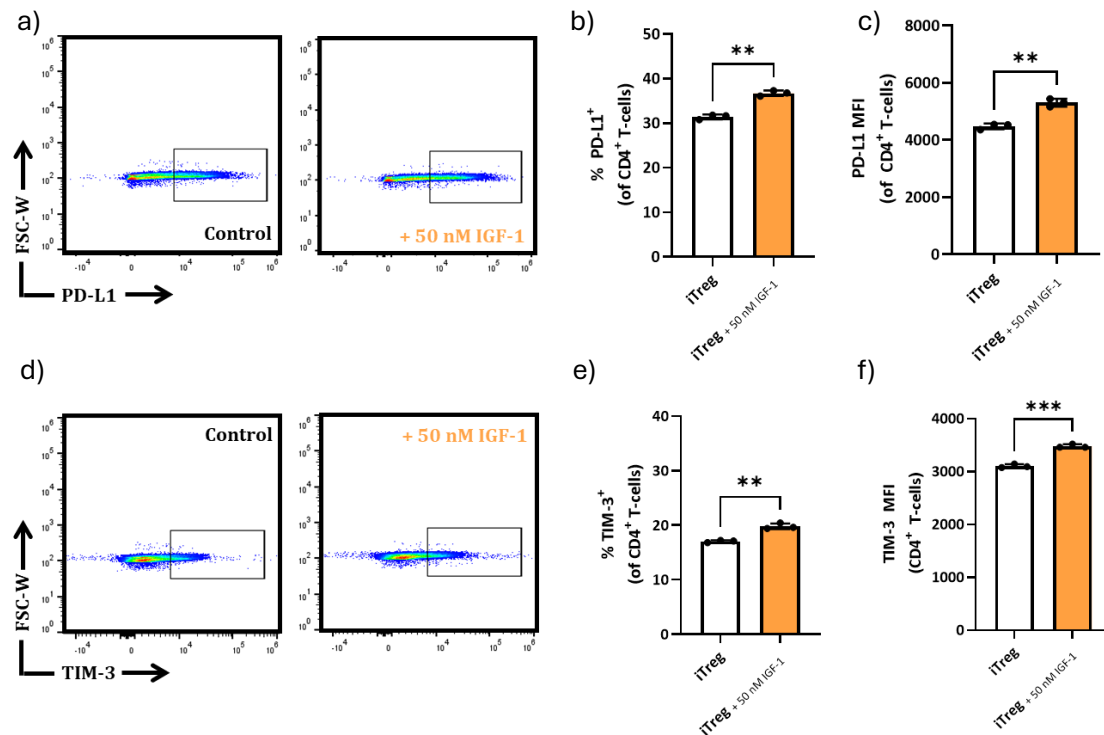


Figure 3.4.4.2 - IGF-1 upregulates PD-L1 and TIM-3 expression in iTregs

Sorted naïve murine CD4⁺ T-cells from 6–8-week-old (♂) FVB mice were differentiated under iTreg conditions $\pm$ 50 nM IGF-1 for 72 hours and analysed by flow cytometry.

Representative dot plots show sample gating strategy for identification of PD-L1⁺ (a) or TIM-3⁺ (c) Tregs, alongside quantification of percentage positivity and MFI for PD-L1 (b-c) and TIM-3 (e-f).

[** = $p < 0.01$, *** = $p < 0.001$, by unpaired T-test]

N = 3 independent experiments and donors. Bars represent means $\pm$ SD.

3.5 Investigating how IGF-1 priming shapes the *in vitro* suppressive potency of regulatory T-cells.

3.5.1 IGF-1 primed iTregs retain suppressive function against autologous CD8⁺ T-cells.

Tregs possess a wide range of mechanisms utilized to suppress immune responses. Whilst Treg derived Granzyme-B inhibits CD8⁺ T-cell mediated tumour clearance via cytolysis of CD8⁺ cells (227), PD-L1 ligation with its receptor PD-1 functions delivers inhibitors signals to CD8⁺ T-cells, inhibiting activation (136). Consequently, how IGF-1 associated changes in iTreg phenotype influenced their suppressive capacity were investigated.

First, we first confirmed that iTregs differentiated from naïve CD4⁺ T-cells (differentiated without IGF-1) were functionally immunosuppressive (appendix fig. 8.1.1.4b). For all subsequent suppression assays, generated iTregs were collected, centrifuged, and fresh media was added to remove residual IGF-1 prior to co-culture to omit potential effects of IGF-1 on CD8⁺ T-cells. To test the effects of IGF-1 priming on the suppressive potency of iTregs, CD8⁺ T-cell activation, cytokine production and proliferation was measured by flow cytometry after 48-hours of co-culture. We observed that iTregs primed with 50 nmol L⁻¹ IGF-1 did not significantly impact the ability of iTregs to suppress responder CD8⁺ T-cell proliferation (fig. 3.5.1.1a-b), cell surface CD25 expression (fig. 3.5.1.1c-d), or production of IFN- γ (appendix fig. 8.1.1.4c) and TNF- α (appendix fig. 8.1.1.4c). Of note, CD8⁺ cell viability did not significantly

after co-culture (data not shown). Hence, IGF-1 priming does not appear to affect the suppressive potency of iTregs in conventional TCR-based suppression assays.

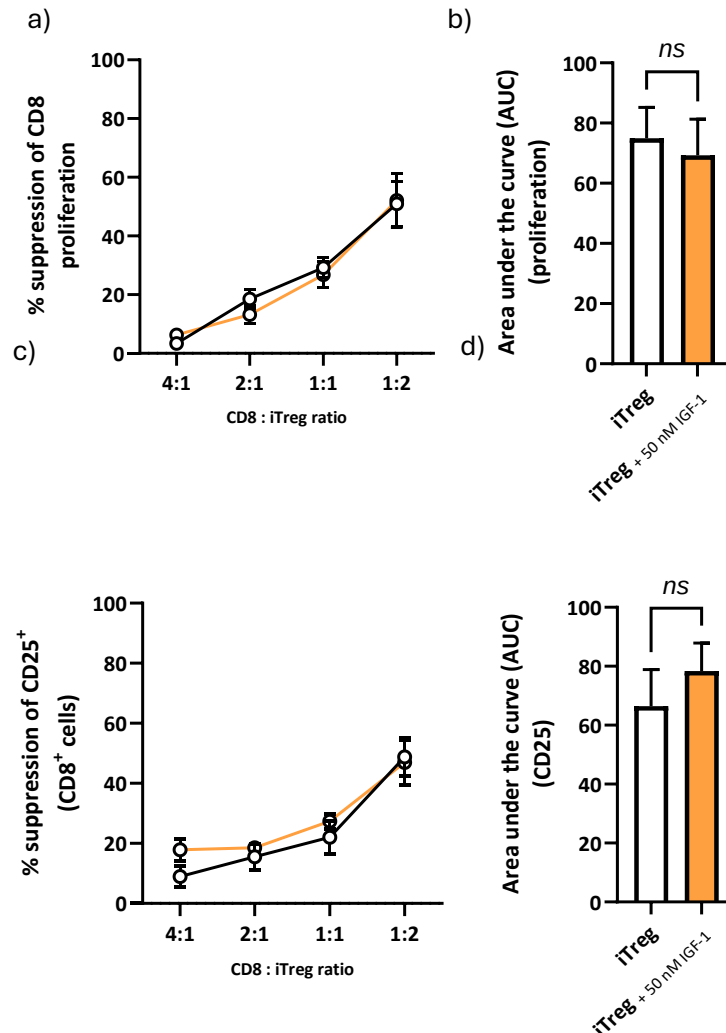


Figure 3.5.1.1 - IGF-1 primed iTregs retain suppressive function against TCR stimulated CD8⁺ T-cells.

Sorted naïve murine CD4⁺ T-cells from 6–8-week-old (♂) FVB mice were differentiated under iTreg conditions $\pm$ 50 nmol L⁻¹ IGF-1 for 72 hours and co-cultured with responder CD8⁺ T-cells + dynabeads (1:1 ratio) at the indicated ratio before analysis by flow cytometry after 48 hours. Graphs show both the % suppression and area-under the curve comparisons of CD8⁺ T-cell proliferation (a-b) and CD25⁺ (c-d) CD8⁺ T-cells from a 4:1 to a 1:2 CD8 : iTreg ratio.

[N = 4 independent experiments, ns = $p > 0.05$, by Two-way ANOVA (c,d) or paired-T test (d-f)]

N = 4 independent experiments and donors. Bars represent means $\pm$ SEM.

3.5.2 IGF-1 primed iTregs more effectively suppress CD8⁺ T-cells activated by cancer cells.

Whilst TCR-based co-culture suppression assays are an effective method to compare suppressive function, they do not entirely reflect conditions experienced by Tregs in the prostate TME *in vivo*. Hence, a more-physiologically relevant stimulus experienced by CD8⁺ T-cells in prostate cancer was investigated.

Primed CD8⁺ T-cells are activated by both neoantigen specific MHC-1 (MHC-I) recognition (14), and antigen/MHC-I independent interactions by NKG2D (256), killing cancer cells devoid of MHC-1 expression. To test this, CD8⁺ T-cells were labelled with CFSE to evaluate proliferation and markers of T-cell activation were evaluated (CD25, TIM-3, TIGIT). CD8⁺ T-cells co-cultured with MyC-CaP cells expressed significantly higher levels of activation markers TIGIT and TIM-3, but not CD25, and had an increased rate of proliferation (appendix fig. 8.1.1.5a-e), relative to controls.

Next, the effects of high IGF-1 priming on iTreg suppression of CD8⁺ T-cell activation by prostate cancer cells was explored. Upon co-culture, iTregs differentiated with 50 nmol L⁻¹ IGF-1 more effectively suppressed activation induced expression of both TIM-3 (fig. 3.5.2.1a-c) and TIGIT (fig. 3.5.2.1d-f) in responder CD8⁺ T-cells. Thus, iTregs primed with high IGF-1 supply more effectively suppress CD8⁺ T-cells activated by autologous prostate cancer cells.

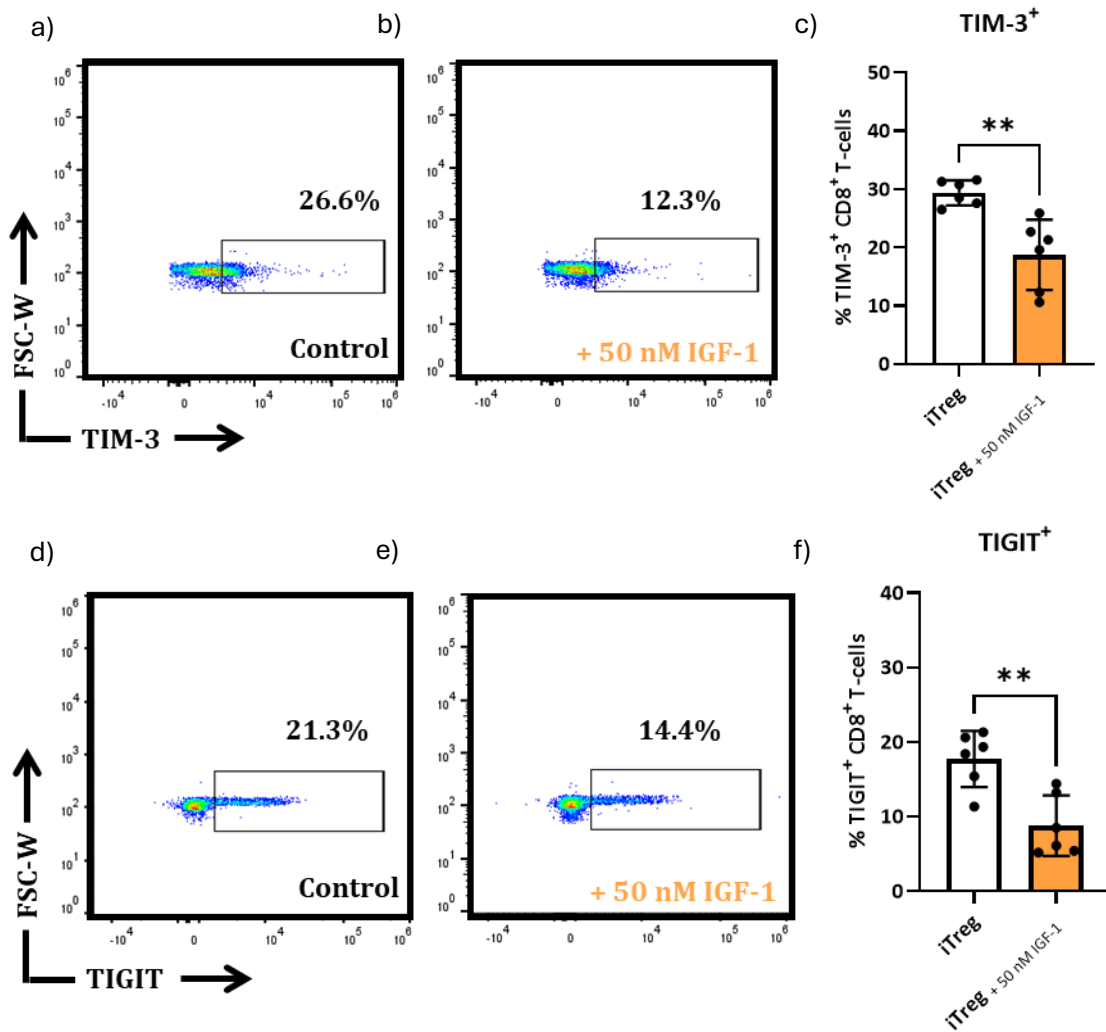


Figure 3.5.2.1 – IGF-1 priming enhances iTreg function in suppression assays with prostate cancer cells.

CD8⁺ T-cells were sorted magnetically from single-cell suspensions of 6–8-week-old (♂) FVB murine splenocytes and co-cultured with MyC-CaP cells and iTregs differentiated $\pm$ 50 nmol L⁻¹ IGF-1 at a 1:1:0.1 iTreg: CD8⁺: cancer cell ratio for 48 hours prior to analysis by flow cytometry.

Representative dot plots show sample gating strategy for identification of TIGIT⁺ (a-b) or TIM-3⁺ (d-e) CD8⁺ T-cells, quantified in (c) and (f) respectively.

[** = $p < 0.01$, by unpaired T-test]

N = 6 independent experiments and donors. Bars represent means $\pm$ SD.

3.5.3 IGF-1 primed iTregs more effectively suppress CD8⁺ T-cells in the presence of proximal cancer cells.

Cancer cells produce cytokines, chemokines and cell-surface proteins that can influence the activity of both Treg and CD8⁺ T-cells (35). Thus, the presence of prostate cancer cells could have a direct effect on the suppressive activity of iTregs primed with high IGF-1 supply in TCR-based suppression assays. To test this, we reproduced experimental conditions from 3.5.1, but plated MyC-CaP cell monolayers overnight prior to onset of suppression assays. Intriguingly, iTregs differentiated with 50 nmol L⁻¹ IGF-1 were significantly more effective at suppressing the induction of cancer cell apoptosis by CD8⁺ T-cells (fig. 3.5.3.1a-b) and CD8⁺ T-cell activation measured by CD25⁺ and IFN-γ⁺ expression in responder cells (fig. 3.5.3.1c-d). However, the relative proliferation of responder CD8⁺ T-cells was not significantly different (data not shown). iTregs primed with 50 nmol L⁻¹ IGF-1 also expressed significantly greater levels of PD-1 after suppression assays with MyC-CaP cells (appendix fig. 8.1.1.6a-b).

To test if this effect was specific to prostate cancer, or perhaps more generalizable to other cancer types, co-culture suppression assays with iTregs and autologous CD8⁺ T-cells and pancreatic cancer cells were performed (from C57BL/6 mice). Similarly, iTregs primed with 50 nmol L⁻¹ IGF-1 more potently inhibited the induction of cancer cell apoptosis by CD8⁺ T-cells, reduced activation induced CD25⁺ expression in responder CD8⁺ T-cells and more effectively suppressed CD8⁺ proliferation in a KPC dependent manner (appendix fig. 8.1.1.6c-e). Differential suppression of responder CD8⁺ T-cell activation required KPC cell monolayers (data

not shown). Thus, high IGF-1 priming enhances the suppressive properties of iTregs proximal to cancer cells.

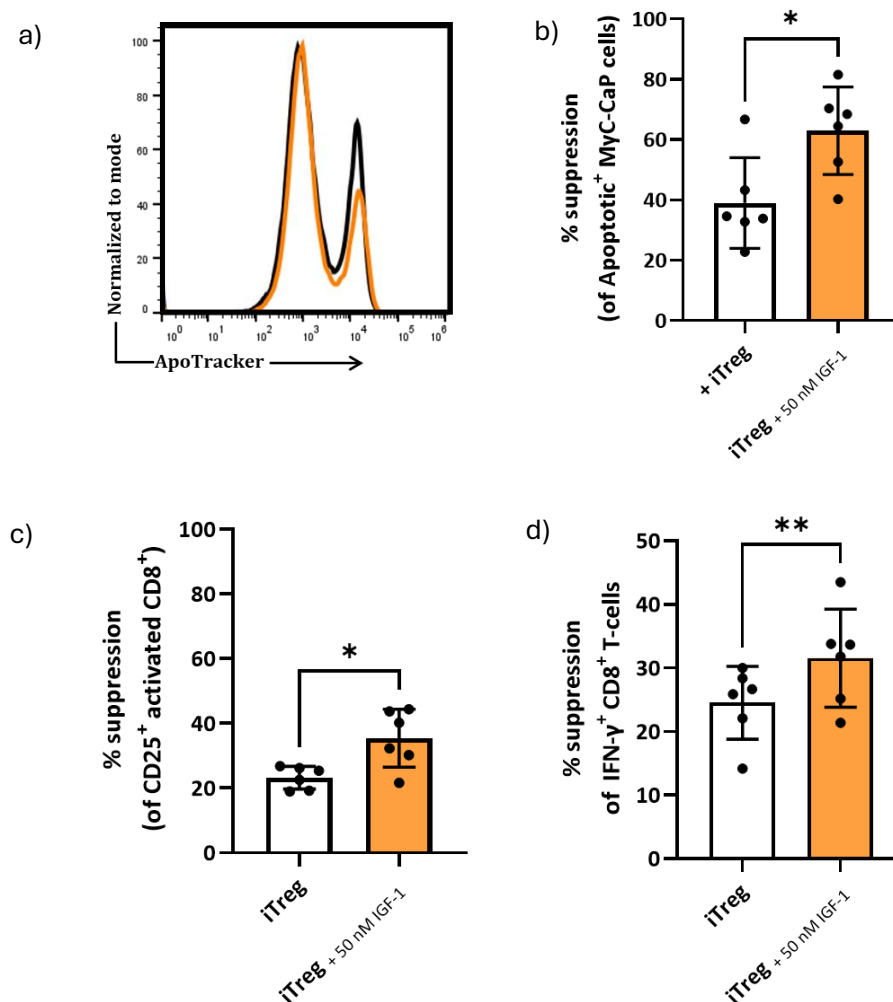


Figure 3.5.3.1 – High IGF-1 priming enhances iTreg suppression of CD8⁺ T-cell activation when co-cultured with prostate cancer cells

iTregs $\pm$ 50 nmol L⁻¹ IGF-1 generated from FVB mice were co-cultured with responder CD8⁺ T-cells (+CD3/CD28 dynabeads) at a 1:1 ratio, in the presence of MyC-CaP monolayers for 48 hours prior to analysis of cancer cell apoptosis and T-cell function by flow cytometry. Representative histograms of ApoGreen staining for cancer cells [CD45⁺, CD4⁺, CD8⁻] are shown (a), and relative suppression of cancer cell apoptosis is quantified and shown in (b).

(c) Relative suppression of activation induced CD25 or (d) IFN γ expression in responder CD8⁺ T-cells after co-culture with iTregs or iTregs differentiated with 50 nmol L⁻¹ IGF-1.

[* = $p < 0.05$, ** = $p < 0.01$, unpaired T-test, N = 6 independent experiments and donors, bars represent means $\pm$ SD.]

3.5.4 PCa conditioned medium shapes the suppressive potency of Tregs primed with IGF-1.

Tumour cells could modulate the suppressive function of iTregs through direct ligation with TIM-3 or PD-1, or indirectly through the secretion of cytokines, growth factors or metabolites into the cell culture medium (35). Consequently, suppression assays were next performed in conditioned medium (CM) generated from MyC-CaP cells, to assess whether changes in suppressive function required direct iTreg cell-contact with cancer cells. Concentration dependent effects were determined by sequential dilution of PCa CM with fully supplemented RPMI-1640.

Consistent with previous observations, priming of iTregs with 50 nmol L⁻¹ IGF-1 significantly enhanced the relative suppression of CD25 expression in responder CD8⁺ T-cells cultured in PCa CM (fig. 3.5.4.1a). However, changes in iTreg suppressive potency with high IGF-1 priming were abrogated in suppression assays performed with partial (25-75%) or complete (100%) dilution with fresh RPMI 1640. Next, we explored if the effects of 50 nmol L⁻¹ IGF-1 on iTreg suppressive function were conserved in tTreg cells. Consistent with observations from iTregs, tTregs primed with 50 nmol L⁻¹ IGF-1 more effectively suppressed responder CD8⁺ T-cell activation in undiluted PCa CM (fig. 3.5.4.1b). Furthermore, there was no significant difference in IGF-1 primed tTreg suppression upon partial (25-75%), or complete (100%) dilution of PCa CM with fresh RPMI-1640.

In conclusion, high IGF-1 supply enhances Treg *in vitro* suppressive capacity under conditions generated by cancer cells. However, this does not require direct cell

contact between Tregs and cancer cells. Curiously, the suppressive potency of Tregs primed without IGF-1 decreased as concentrations of PCa CM increased. Thus, the mechanism by which IGF-1 mediates this response appears to be through the preservation of Treg function, rather than enhancement of suppressive. Of note, IGF-1 priming did not significantly impact the survival of iTreg or tTregs, measured by cellular viability, after suppression assays in PCa CM (data not shown).

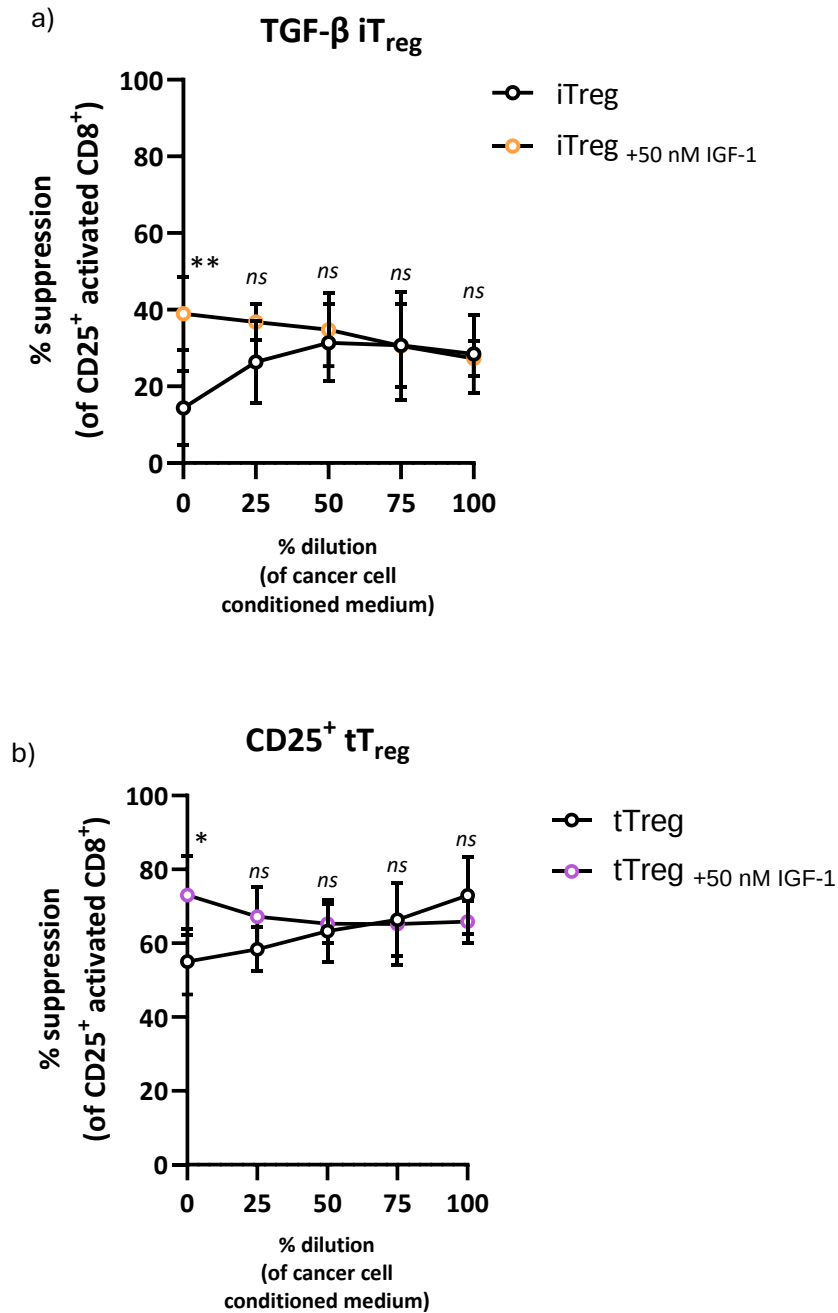


Figure 3.5.4.1 PCa conditioned medium shapes the suppressive phenotype of iTregs differentiated with low or high IGF-1 supply.

MyC-CaP cancer cell conditioned medium was diluted by 0, 25, 50, 75 or 100% by fully supplemented RPMI 1640.

The relative suppression of CD25⁺ expression in CD8⁺ T-cells induced by CD3/CD28 dynabeads was quantified after co-culture with iTregs (a) or tTregs (b) primed $\pm$ 50 nmol L⁻¹ IGF-1 (1:1 Treg:CD8 ratio) [ns = $p > 0.05$, * = $p < 0.05$, by two-way ANOVA] [N = 3 independent experiments and donors]

3.6 Discussion

In this chapter, we explore how changes in IGF-1 bioactivity shapes the differentiation and function of TGF- β 1 induced-Treg cells, and explore their suppressive function under *in vitro* conditions generated by MYC-amplified prostate cancer cells. This study addresses a gap in our understanding as to how microenvironmental cytokines and growth factors generated in prostate tumorigenesis could contribute to the development and suppressive phenotype of Treg cells that inhibit anti-tumour immune responses.

A significant challenge we faced was the selection of experimental culture conditions for iTreg generation. Whilst low-dose anti-CD3 TCR stimulation and TGF- β 1 are indispensable for efficient iTreg generation (439), published protocols vary significantly due to either the desire to recapitulate disease-relevant Treg inducing conditions *in vivo* (440), or to optimise their *ex vivo* expansion (441). For the protocol used in this study, we explored the available literature on the abundance of *in vitro* iTreg polarizing co-factors relevant to the PCa TME to define conditions most likely experienced by pTregs in prostate cancer. We noted reduced levels of all-trans-retinoic acid [ATRA] (442), hydrogen sulphide (443) and a lack of available literature on vitamin C/D3, butyrate and progesterone levels. Whilst we cannot rule out the presence of these co-factors in the TME, nor secondary lymphoid organs, at the time of writing they do not appear to play a primary role in the transformation or development of the early PCa immune microenvironment. Hence, whilst in this chapter we opted for a TGF- β driven *in vitro* iTreg protocol, findings from this study led us to explore the

effects of IGF-1 on Treg infiltration and function in mouse models of MYC-amplified prostate cancer (shown in chapter 5) that more accurately represents the PCa TME.

A limitation of this study that we are unable to uncouple the biological effects from cross-reactivity and activation of InsR or InsR-IGF-1R heterodimer receptors by IGF-1. BMS 754807, the IGF-1R tyrosine kinase inhibitor used in this study, also has inhibitory effects on InsR, albeit with reduced potency (444). Whilst we considered using targeted knockdown approaches for IGF-1R and InsR prior to IGF-1 stimulation, unoccupied IGF-1R and InsR receptors also have kinase independent effects on the susceptibility to apoptosis, DNA methylation patterns and the phosphorylation of over 1400 intracellular phosphosites (445). Hence, deletion of surface IGF-1R or InsR introduces a confounding variable that could influence the transcriptional response to IGF-1, that occurs independently of the desired targeted knockdown. Hence, we conclude that whilst our *in vitro* suppression assays are representative of microenvironments with low/high IGF-1 bioactivity, we cannot conclusively determine if this effect on regulatory T-cell function is dependent on IGF-1R.

Nevertheless, an unexpected finding was the phosphorylation of rpS6, but not AKT at serine 473 upon IGF-1 stimulation in both CD4⁺ T-cells and iTregs. These observations are consistent with visceral pre-adipocytes (446), yet contrast studies of IGF-1R signalling in epithelial cells (447), transformed cancer cells and macrophages (355,388). These data could be explained by cell-type dependent differences in IRS adapter proteins, or expression of AKT de-phosphorylating proteins such PH domain and leucine rich repeat protein phosphatases (PHLPPs) which actively dephosphorylate AKT at serine 473 and are expressed at higher levels by CD4⁺ and

FoxP3⁺ T-cells (448,449). However, both the route of mTORC1 activation by IGF-1 stimulation is not yet known, and possible activation of mTORC2/MAPK signalling at earlier timepoints of iTreg differentiation onset cannot be conclusively ruled out.

An important finding from this study was that both iTregs and tTregs activated with high levels of IGF-1 more effectively suppressed responder CD8⁺ activation in the presence of prostate cancer cell conditioned medium. Changes in Treg suppression did not occur in conventional CD8⁺ suppression assays with fully supplemented medium, suggesting that the suppressive effect was also not caused by direct ligation with CD8⁺ T-cells (e.g. PD-1, PD-L1) nor by changes in immunosuppressive cytokines or metabolites produced directly by iTregs. Considering that MYC-amplified cancer cells are highly glycolytic and generate high levels of lactate (145), changes in suppressive function could relate to the relative mitochondrial fitness of IGF-1 pre-treated Treg cells driven by mTORC1 activation (231). Hence, in the next chapter we will explore the role of IGF-1 on TGF- β -iT_{reg} metabolism, and how this relates to changes in the suppressive potency in MYC-amplified cancer cell conditioned medium.

Chapter 4 – Investigating IGF-1 as a metabolic growth factor for regulatory T-cells

4.1 Background

In PCa, abnormal lipid metabolism is also considered a hallmark of disease (450). High rates of glycolysis, lipid uptake and increased endogenous neutral lipid synthesis are mediated by a complex interaction between AR signalling and MYC amplification (451). Nevertheless, TI-Tregs remain highly immunosuppressive, partly attributed to a FoxP3 dependent shift towards lipid-fuelled FAO, enhanced OXPHOS, and the capacity to utilize lactate as a nutrient for mitochondrial metabolism (209,241).

In parallel to its mitogenic effects, IGF-1/IGF-1R signalling also has functions as a metabolic signal of nutrient abundance in adipose and muscle tissue (452,453). In these cells, IGF-1R activation promotes a transcriptional response that facilitates nutrient uptake, metabolite synthesis and metabolic programmes of increased energy expenditure, such as mitochondrial biogenesis and OXPHOS by activation of mTOR (454). The regulation of cellular metabolism has implicated the IGF axis in both the incidence of metabolic disease such as obesity, type-2 diabetes, cachexia and metabolic syndrome and dyslipidaemia (156,455–457).

There is growing evidence that the IGF-1/IGF-1R axis also directly regulates the metabolism of immune cells. Early work showed that ablation of IGF-1R in myeloid cells significantly reduced the energy expenditure of cells and inhibited macrophage activation in response to immunometabolic challenge (458). Furthermore, in in

murine T_H17 cells IGF-1R activation promotes aerobic glycolysis, increasing basal oxygen consumption and extracellular acidification rates (366). Dynamic regulation of IGF-1R activity also occurs by intracellular concentrations of the metabolite mevalonate in monocytes, the downstream product of acetyl-CoA and precursor of cholesterol, regulating the epigenetic modification of pro-inflammatory genes (459).

Previously, we showed that 50 nmol L⁻¹ IGF-1 promotes mTORC1 activation during iTreg differentiation, enhancing *in vitro* suppressive potency in PCa CM. Crucially, mTORC1 is a critical regulator of Treg lipid and cholesterol synthesis, OXPHOS and glycolysis (231). Thus, we hypothesized that the IGF-1-mTORC1 axis in Tregs could function as a metabolic link between Tregs and the endocrine system, which could function to support the metabolic transition required for TI-Treg effector function.

4.2 Chapter aims

As a result, the principal aim of this chapter was to investigate how high IGF-1 supply and PCa CM shapes the metabolism of Tregs.

4.3 IGF-1 and iTreg bioenergetics

As highlighted in chapter 3, iTreg differentiation with 50 nmol L⁻¹ IGF-1 promotes early rpS6 phosphorylation, a downstream target of mTORC1, but not AKT at serine 473 (indicative of mTORC2 activity). Whilst mTORC1 activity in tTreg cells regulates the rate of OXPHOS and lipid/cholesterol synthesis (231), mTORC2 is associated primarily with changes in aerobic glycolysis (460). Consequently, the mitochondrial fitness of Treg cells primed with IGF-1 (used at 50 nmol L⁻¹ herein) was investigated.

4.3.1 Mitochondrial fitness and metabolic flux

To test this, we first utilized a high-throughput method of measuring the relative mitochondrial energy generating capacity. Redox transformations of metabolites within the mitochondria result in the generation of an electrical charge difference across the inner membrane, required for ATP generation (38). This charge difference is referred to as the mitochondrial membrane potential ($\Delta\Psi_m$), and can be measured using the cell-permeant dye Tetramethylrhodamine methyl ester (TMRM). Differentiated iTreg cells were treated with or without carbonyl cyanide m-chlorophenylhydrazone (CCCP) during TMRM incubation (an oxidative phosphorylation uncoupler).

CCCP significantly reduced the mitochondrial membrane potential of murine iTreg cells (fig. 4.3.1.1a), indicative that TMRM dye accumulation in Tregs correlates with the rate of mitochondrial OXPHOS. Priming with IGF-1 significantly increased

the relative median fluorescence intensity (MFI) of TMRM staining in both murine iTreg (fig. 4.3.1.1b) and tTreg (fig. 4.3.1.1c) subsets.

Whilst murine iTreg cells are largely dependent on FAO (229), glycolysis is indispensable for the generation and function of human iTreg cells (461). Consequently, whether the effects of IGF-1 on enhanced mitochondrial membrane potential were conserved in human iTregs were investigated. Conditions for effective and stable FoxP3 induction in human iTregs by TGF- β , IL-2 and anti-CD3/CD28 stimulation were first optimized (fig. 4.3.1.1d-e, appendix fig. 8.1.2.1a-d). Of note, we could not generate functionally suppressive human iTreg cells *in vitro*, including protocols of TGF- β 1, rapamycin and/or sodium butyrate (data not shown). Consistent with murine iTregs, IGF-1 significantly increased the mitochondrial membrane potential of human iTregs (fig. 4.3.1.1f). Thus, the effects of IGF-1 on mitochondrial function could be generalizable to human Treg subsets.

Although TMRM staining is a useful tool to guide investigations into mitochondrial fitness, differences in the rate of TMRM dye extrusion through efflux pumps in mitochondria can lead to results that are not directly caused by changes in the relative rate of mitochondrial OXPHOS (462). Thus, real-time rates of oxygen consumption (OCR) and extracellular acidification (ECAR) were assessed to investigate the relative rates of OXPHOS and glycolysis in iTregs differentiated $\pm$ IGF-1 (463). Basal oxygen consumption, indicative of the resting ATP demand, was slightly higher in IGF-1 primed iTregs, but this difference was not statistically significant (fig. 4.3.1.1h). Subsequent injections of oligomycin, an inhibitor of mitochondrial ATP synthase, allows for the calculation of ATP-linked rate of OXPHOS after subtracting

the rate of basal oxygen consumption. IGF-1 did not impact the ATP-linked rate of OXPHOS in iTregs differentiated with IGF-1.

Carbonyl-cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) is a protonophore that uncouples the mitochondrial respiratory chain activity from cellular ATP requirements, effectively allowing all hydrogen ions to pass through the mitochondrial membrane. As a result, FCCP is injected after oligomycin to calculate the maximal possible activity of the respiratory transport chain. Furthermore, the difference in OCR that follows FCCP injection allows for the calculation of mitochondrial spare respiratory capacity (SRC), indicative of the capacity of Tregs to metabolically adapt to stress conditions (464). Intriguingly, IGF-1 priming significantly increased the maximal OCR (fig. 4.3.1.1i) and spare respiratory capacity (fig. 4.3.1.1j) of iTreg cells. Finally, injection of rotenone and antimycin A, inhibitors of complex I and III of the respiratory transport chain respectively, completely shuts down mitochondrial respiration, allowing for the calculation of non-mitochondrial oxygen consumption. Following rotenone and antimycin A injections, the rate of OCR significantly decreased, confirming that most OCR measurements made were of mitochondrial OXPHOS. Of note, IGF-1 priming did not significantly impact the basal rate of ECAR, indicative of the rate of glycolysis at steady state in TGF- β -iTregs (appendix fig. 8.1.2.2a). In conclusion, although high IGF-1 priming does not change rate of basal rate of OXPHOS or glycolysis, it does enhance iTreg mitochondrial spare respiratory capacity, indicative of increased cellular substrate availability for oxidative metabolism, improved capacity to respond to oxidative stress, or improved mitochondrial homeostasis (464).

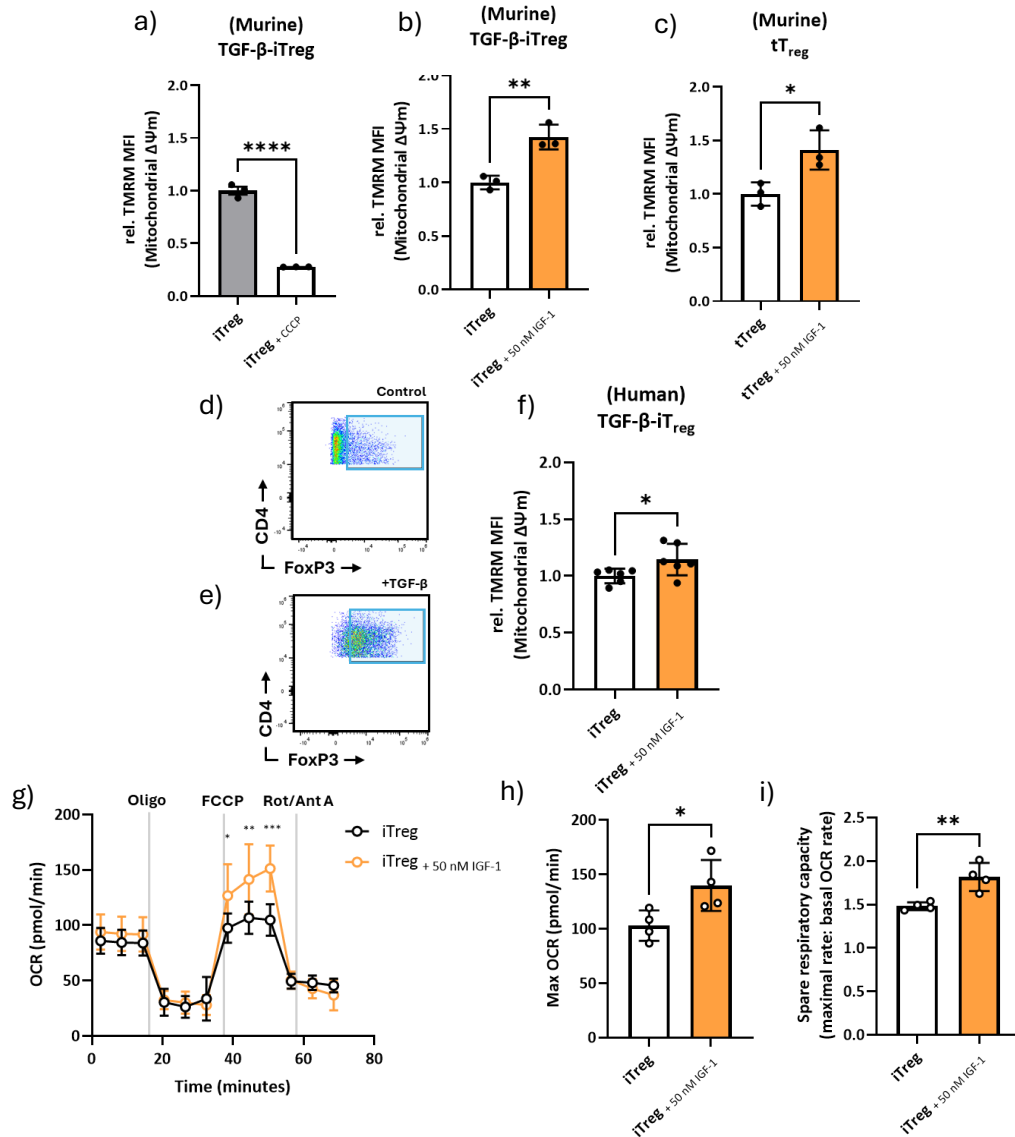


Figure 4.3.1.1 - High IGF-1 supply increases the spare respiratory capacity of TGF-β-iTreg cells

a) Relative mitochondrial TMRM MFI of murine TGF-β iTreg cells in the presence or absence of the oxidative phosphorylation uncoupler CCCP (a), or (b) 50 nmol L⁻¹ IGF-1. (c) Relative mitochondrial TMRM MFI 72 hours post-activation of CD4⁺ CD25⁺ tTregs by anti-CD3/CD28 dynabeads ± 50 nmol L⁻¹ IGF-1. (d) FoxP3 expression in human CD4⁺ T-cells after 5 days of stimulation with T-Cell TransAct™ + IL-2 and (e) + 2.5 ng mL⁻¹ TGF-β. (f) Human iTregs were stained with TMRM, and the relative MFI was quantified. (g) Oxygen consumption rate of TGF-β iTreg cells differentiated ± 50 nmol L⁻¹ IGF-1 under basal conditions and in response to oligomycin (Oligo), fluorocarbonyl-cyanide phenylhydrazine (FCCP), rotenone (Rot) and antimycin-A (Ant A) injections. The maximal oxygen consumption rate and spare respiratory capacity was calculated and shown in (h) and (i) respectively.

[* = $p < 0.05$, ** = $P < 0.01$, **** = $p < 0.0001$, by unpaired T-test (a,b,c,g,h) or two-way ANOVA (f)]. N = 3 independent experiments and donors (a-e), N = 6 independent experiments (f) N = 2 independent experiments with 4 independent donors (g-i). Bars represent means ± SD.

4.3.2 Glucose uptake and neutral lipid accumulation

Active mTORC1 signalling results in the transcriptional activation of multiple regulatory factors that regulate both glucose and lipid metabolism, such as sterol regulatory element-binding protein 1 (SREBP1), peroxisome proliferator-activated receptor- γ (PPAR γ) and hypoxia inducible factor 1 subunit alpha [HIF1A] (465,466). Whilst SREBP1 and PPAR γ regulate lipogenic metabolism, HIF1a transcription in T-cells stimulates programmes of glucose metabolism and transport. Thus, changes in mitochondrial SRC upon IGF-1 priming could be mediated through increased availability of glucose or fatty acids that are oxidized within mitochondria.

Flow cytometry compatible fluorescent probes can be used to measure metabolic parameters, such as glucose uptake by 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) and lipid levels by 4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene [BODIPYTM 493/503] (467). First, the reporter sensitivity of 2-NBDG and BODIPYTM 493/503 fluorescent probes were tested. As a positive experimental control for 2-NBDG, iTregs were differentiated with 50 nmol L⁻¹ insulin, a potent stimulant of glucose uptake (468). The relative MFI of 2-NBDG was significantly higher in iTreg cells stimulated with insulin (appendix fig. 8.1.2.2b). Tregs contain significantly higher levels of intracellular neutral lipids relative to Tconv cells. Thus, as a positive control for BODIPYTM 493/503, we compared BODIPY staining in differentiated iTreg cells with T_H0 Tconv cells. Consistent with these observations, iTreg the relative MFI of BODIPYTM 493/503 was significantly higher in iTreg cells than T_H0 Tconv cells (fig. 4.3.2.1a).

Next, the effects of high IGF-1 supply on the rate of iTreg glucose uptake and total neutral lipid content were tested. IGF-1 stimulation significantly increased the relative MFI of both BODIPY[™] 493/503 (fig. 4.3.2.1b) and 2-NBDG (fig. 4.3.2.1b). Thus, IGF-1 increases the total neutral lipid content of iTregs, indicative of a lipogenic metabolic programme. Furthermore, given that we did not observe changes in the rate of ECAR (appendix fig. 8.1.2.2a). Hence, these results suggest that the increase in glucose uptake induced by IGF-1 are not caused by an increased rate of glucose demand from glycolysis, but could be indicative of changes in the expression of glucose transport proteins.

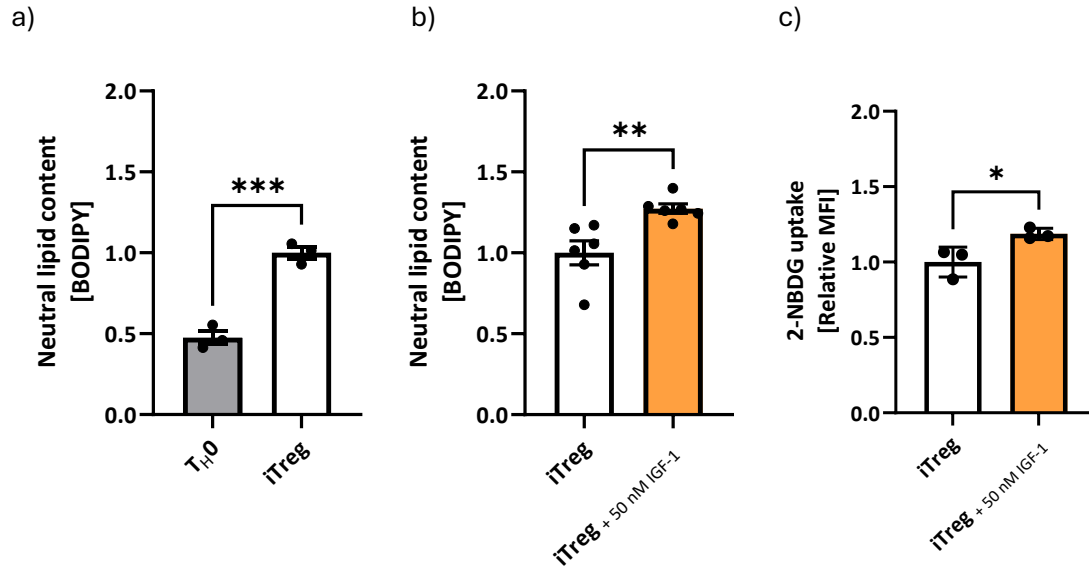


Figure 4.3.2.1 – High IGF-1 supply increases the neutral lipid content and glucose uptake in differentiated iTregs

Sorted naïve murine CD4⁺ T-cells from 6–8-week-old (♂) FVB mice were differentiated under T_H0 or iTreg conditions ± 50 nmol L⁻¹ IGF-1 for 72 hours.

a) Relative MFI of BODIPY[™] 493/503 in CD4⁺ T-cells differentiated under T_H0 or (b) iTreg conditions, ± 50 nmol L⁻¹ IGF-1 [bars = mean ± SEM].

c) Relative MFI of 2-NBDG after iTreg differentiation ± 50 nmol L⁻¹ IGF-1.

[* = p < 0.05, ** = P < 0.01, *** = p < 0.001, by unpaired T-test]

(a,c) N = 3 independent experiments and donors.

(b) N = 6 independent experiments and donors.

Bars represent means ± SEM.

4.4 IGF-1 and lipid metabolism

Although murine induced regulatory T-cells preferentially utilize free fatty acids for energy production by FAO, high intracellular levels of free fatty acids cause oxidative stress, inflammation and stress of the endoplasmic reticulum (469). This is circumvented by storage of free fatty acids within subcellular lipid droplets (LDs), which serve as a subcellular organelle that protect Tregs from free fatty acid lipotoxicity and enhance FoxP3 stability (470).

4.4.1 Lipid droplet biogenesis

To test the subcellular localization of intracellular neutral lipids, iTregs were stained with BODIPY™ 493/503 and imaged by confocal microscopy. Representative images show the maximal intensity projections of Z-stack confocal images of cellular nuclei (DAPI) and LDs (BODIPY) after 72 hours from control and IGF-1 treated iTregs (fig. 4.4.1.1a). Next, we quantified the number of lipid droplets per cell and the relative staining intensity of BODIPY™ 493/503 per lipid droplet using CellProfiler, an open-source software that can be used to quantitatively measure phenotypes from hundreds of images automatically.

Consistent with observations of increased neutral lipid staining by flow cytometry, iTregs differentiated with 50 nmol L⁻¹ IGF-1 formed significantly more lipid droplets per cell after 48 hours (fig. 4.4.1.1b) and 72 hours (fig. 4.4.1.1c) of differentiation with TGF- β . Furthermore, lipid droplets in IGF-1 stimulated iTregs had a significantly higher average BODIPY™ 493/503 staining intensity per droplet after 48 hours (fig. 4.1.1.2a) and 72 hours (fig. 4.1.1.2b). These results indicate that a function

of IGF-1 could be to promote an adaptive response that increases the packaging of excessive lipids into LDs that can be used for energy reserve, whilst protecting cells against lipotoxic effects of free fatty acids.

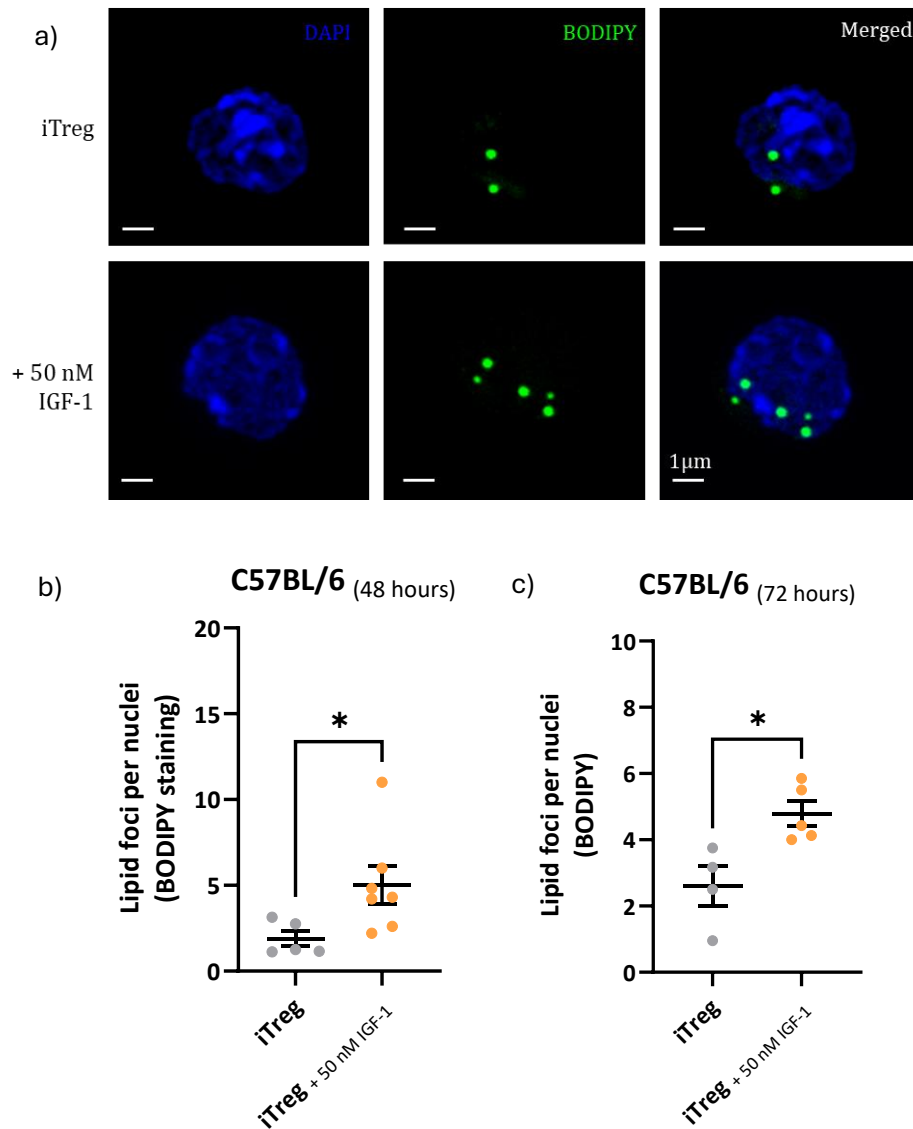


Figure 4.4.1.1 - IGF-1 increases the number of lipid droplets per cell in differentiating Tregs

Sorted naïve murine CD4⁺ T-cells from 6–8-week-old (♂) C57BL/6 were differentiated under iTreg conditions $\pm$ 50 nmol L⁻¹ IGF-1.

a) Representative confocal images [Z-Stack, maximal intensity projections] of differentiated iTregs $\pm$ 50 nM IGF-1 showing DAPI (blue) and BODIPYTM 493/503 (green) and merged channels (DAPI + BODIPYTM 493/503) used to quantify subcellular LDs.

Measurement of LD frequency in iTreg cells treated $\pm$ 50 nM IGF-1 after (b) 48 or (c) 72 hours using the #IdentifyPrimaryObjects feature in CellProfiler.

[* = $p < 0.05$, ** = $P < 0.01$, *** = $p < 0.001$, by unpaired T-test]

N = 3 independent experiments, and > 50 cells were analysed per donor, per experiment.

Bars represent means $\pm$ SEM.

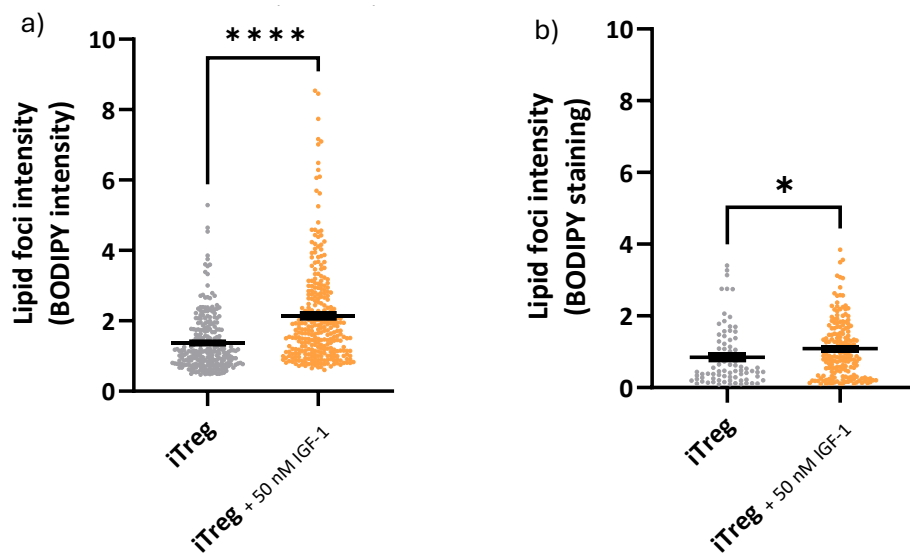


Figure 4.4.1.2 - IGF-1 increases the amount of neutral lipids stored per droplet during iTreg differentiation

Measurements of LD foci intensity in iTreg cells treated $\pm$ 50 nM IGF-1 after (a) 48 or (b) 72 hours, quantified using CellProfiler.

N = 3 independent experiments, > 50 cells were used for quantification per donor, per experiment.

[* = $p < 0.05$, ** = $P < 0.01$, **** = $p < 0.0001$, by unpaired T-test]

Bars represent means $\pm$ SEM.

4.4.2 Lipid droplet proximity with active mitochondria

Whilst LDs have traditionally recognised roles as both a source of lipids that can be utilized under nutrient deprivation and function to protect against free fatty acid lipotoxicity (471), they are now also recognized as highly dynamic organelles that can form both stable and dynamic and interactions with mitochondria (472). Mitochondria anchored to LDs (referred herein as peridroplet mitochondria, PDM) are metabolically distinct from mitochondria alone in the cytosol (referred herein as cytoplasmic mitochondria, CPM), and display an increased basal rate of OXPHOS and SRC relative to CPM. Consequently, we were interested in exploring whether changes in SRC after IGF-1 stimulation were also associated with changes in the proximity of subcellular LDs and mitochondria.

To test this, iTregs differentiated $\pm$ IGF-1 were stained with MitoTracker™ DeepRed and BODIPY™ 493/503, and imaged by confocal microscopy (fig. 4.4.2.1a). To investigate changes in the proximity of LDs and mitochondria, we used two measurement features within CellProfiler: *PercentTouching* and *FirstClosestDistance*. Analysis using *PercentTouching* showed that in control iTregs (no IGF-1), < 10% of individual mitochondria had proximal LDs (fig. 4.4.2.1b). However, in iTregs stimulated with IGF-1, the percentage of individual mitochondria with proximal LDs significantly increased to > 35%. These findings were corroborated by *firstclosestdistance* analysis, where 50 nmol L⁻¹ IGF-1 significantly reduced the closest distance measured from mitochondria to lipid droplets in iTregs (figure 4.4.2.1c). Thus, IGF-1 promotes closer associations between intracellular LDs and active mitochondria in iTreg cells.

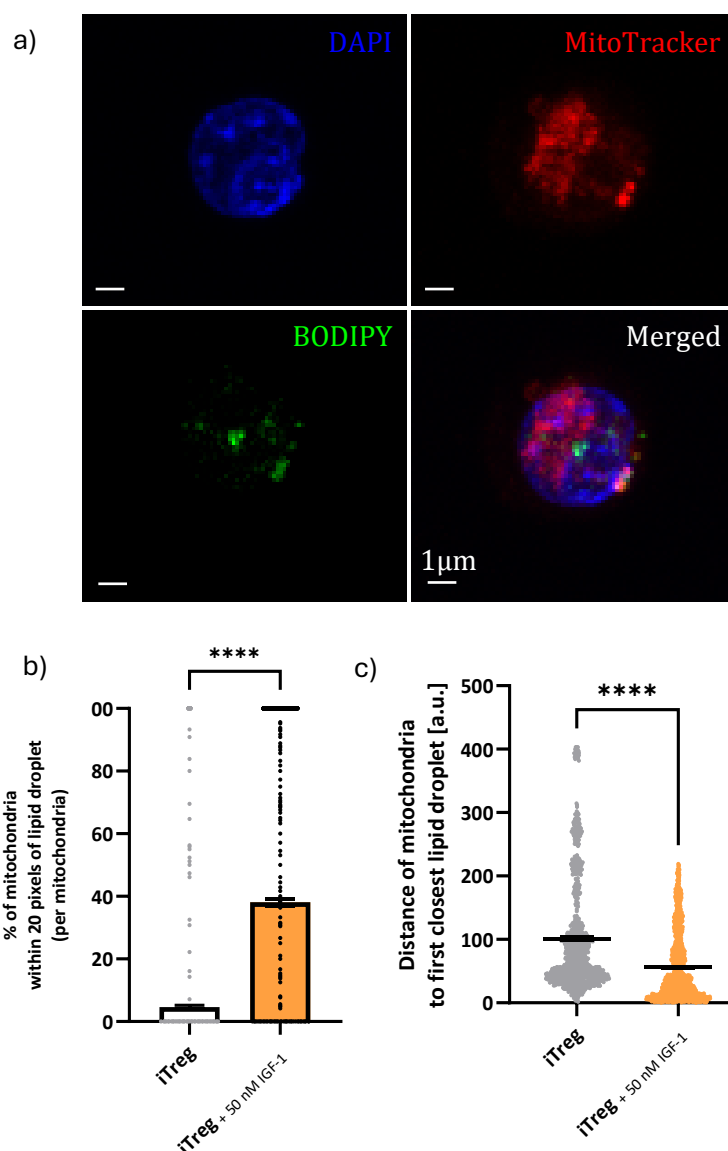


Figure 4.4.2.1 - IGF-1 increases the proximity of lipid droplets to active mitochondria during TGF- β iTreg differentiation

Sorted naïve murine CD4⁺ T-cells from 6–8-week-old (σ) FVB mice were differentiated under iTreg conditions $\pm$ 50 nmol L⁻¹ IGF-1.

(a) Representative confocal images [Z-Stack, maximal intensity projections] of differentiated iTregs [DAPI (blue) and BODIPYTM 493/503 (green), MitoTrackerTM Deep Red (red) and merged channels (MitoTrackerTM Deep Red, DAPI, BODIPYTM 493/503).]

(b) *PercentTouching* and (c) *FirstClosestDistance* analysis were performed under the *MeasureObjectNeighbors* module in CellProfiler, with quantification of mitochondrial and lipid droplet proximity and closest distance measurements shown.

[**** = $p < 0.0001$, by unpaired T-Test]

N = 3 independent experiments and donors. Bars represent means $\pm$ SEM.

4.4.3 Utilizing small molecule inhibitors to explore how IGF-1 shapes iTreg lipid metabolism

Different metabolic pathways contribute to the formation and relative abundance of intracellular neutral lipids, including endogenous synthesis of fatty acids from acetyl-CoA, exogenous fatty acid uptake by transport proteins and the rate of fatty acid oxidation within mitochondria (473). Lipid biosynthetic and oxidative pathways are regulated by the relative activity of the nutrient sensor mTORC1 and AMPK, which function as metabolic checkpoints that integrate environmental cues from their surrounding microenvironment and facilitate the metabolic plasticity of Tregs (231,234). mTORC1 activity is required for lipid and cholesterol biosynthesis in tTregs, and CD36 mediated fatty acid uptake in activated CD4⁺ T-cells, whilst AMPK activation promotes FAO (231,234,466). Consequently, we next investigated the impact of small molecule inhibitors of fatty acid synthesis (FAS), fatty acid uptake (FAU), FAO and/or mTORC1 on changes in iTreg neutral lipid levels induced by IGF-1.

Briefly, rapamycin exhibits selectivity for mTORC1 inhibition at 0.5 – 100 nmol L⁻¹ whilst TOFA allosterically inhibits acetyl-CoA carboxylase 1 (ACC1) activity (474–476), required for the cytosolic conversion of acetyl-CoA to malonyl-CoA, a rate limiting step of long-chain fatty acid synthesis (FAS). SMS121 is small molecule inhibitor of the long-chain fatty acid transporter CD36, required for extracellular FA uptake, and etomoxir is a widely used inhibitor of the mitochondrial long-chain fatty acid importer carnitine palmitoyl transferase-1A (CPT1a), a rate limiting step of long-chain FAO (477). Of note, we confirmed that the addition of rapamycin, SMS121, TOFA

and etomoxir at concentrations used in this study did not significantly impact the viability of murine iTregs after 72 hours (data not shown).

Analysis of iTregs differentiated in the presence of small molecule inhibitors for 72 hours revealed inhibition of mTORC1, ACC1 and CPT1a, but not CD36, abrogated the effect of IGF-1 on increased neutral lipid levels (figure 4.4.3.1a). Thus, the IGF-1 driven increase in neutral lipid levels in iTregs is mTORC1 dependent, and mediated primarily through endogenous *de novo* lipid synthesis rather than exogenous fatty acid uptake. Whilst unexpected, the effects of CPT1a inhibition could be indicative of cellular depletion ATP or nicotinamide adenine dinucleotide phosphate (NADPH) levels from limited FAO, reducing the rate of IGF-1 induced *de novo* lipid synthesis. Conversely, a spectrum of off-target effects of etomoxir have been described including inhibition of diacylglycerol acyltransferase-1 [DGAT1] (478), required for the synthesis of medium chain fatty acids/triacylglycerols. Whilst we used a significantly lower concentration of etomoxir (2.5 μ M) than studies identifying inhibitory effects of etomoxir on DGAT1 (50 μ M), from these data alone we cannot conclusively rule out that in addition to CPT1a inhibition, the inhibitory effects on neutral lipid levels in the presence of IGF-1 may also be caused by co-inhibition of off-target fatty acid biosynthetic enzymes.

Given that the breakdown of neutral lipid stores can be used as a source of endogenous LC-FAs for mitochondrial oxidation, the effects of IGF-1 on increased iTreg mitochondrial membrane potential could be regulated by changes in the rate of endogenous lipid synthesis, LC-FA mitochondrial import, or mTORC1 activity. Hence, the relative accumulation of mitochondrial TMRM dye was measured after co-

incubation with small molecule inhibitors for 72 hours. Similarly, iTregs differentiated in the presence of small molecule inhibitors of ACC1 and CPT1a, but not CD36, abrogated the effect of IGF-1 on increased TMRM staining (figure 4.4.3.1b). Unexpectedly, co-stimulation with IGF-1 in the presence of rapamycin induced a significant decrease in TMRM staining relative to iTregs differentiated with rapamycin alone. Thus, the metabolic effects of IGF-1 requires the activity of mTORC1, endogenous lipid biosynthesis and LC-FA mitochondrial import, but not extracellular FA uptake. These observations could be indicative that IGF-1 promotes metabolic phenotype in iTregs that resembles memory CD8⁺ T-cells, whereby extracellular glucose is used for lipid biosynthesis to provide LC-FA substrates for mitochondrial FAO, rather than through import of extracellular FAs (479).

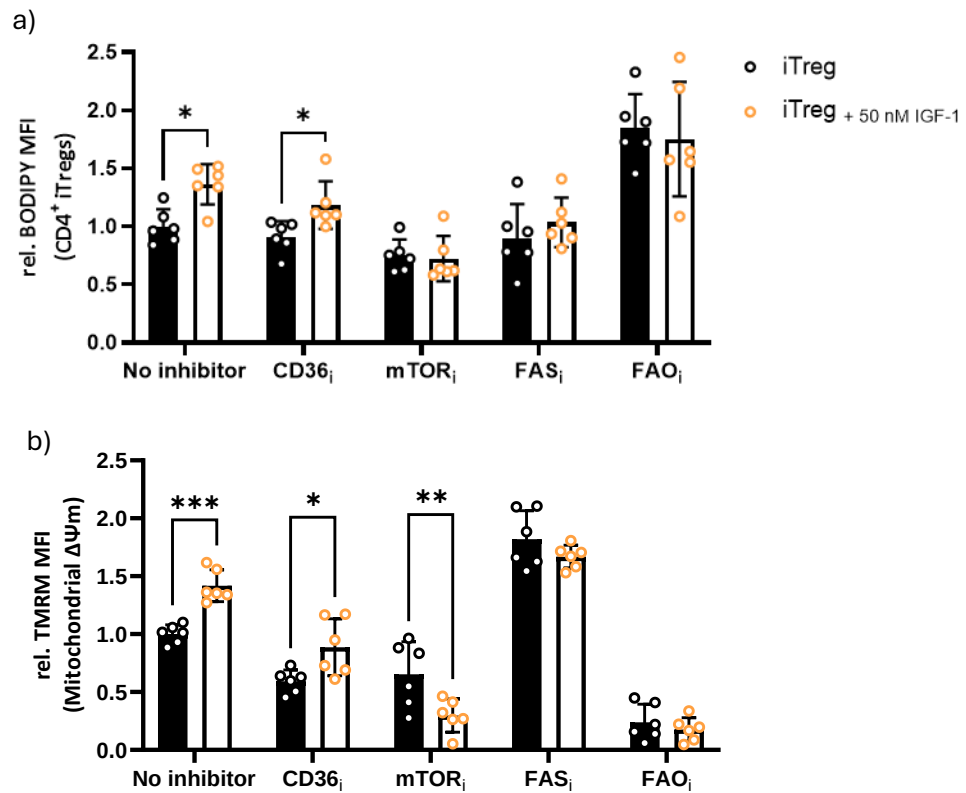


Figure 4.4.3.1 - The metabolic effects of IGF-1 on iTregs require mTORC1, ACC1 and CPT1a activity.

Sorted naïve murine CD4⁺ T-cells from 6–8-week-old (♂) FVB mice were differentiated under iTreg conditions ± 50 nmol L⁻¹ IGF-1 in the presence of either no inhibitor (DMSO), 150 μM SMS121, 100 nM rapamycin, 5 μM TOFA or 2.5 μM etomoxir.

a) Relative BODIPY™ 493/503 (green) MFI in differentiated iTregs after 72 hours, quantified by flow cytometry.

b) Relative TMRM MFI in MitoTracker⁺ iTregs after 72 hours, quantified by flow cytometry.

[* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, by multiple unpaired T-Tests]

N = 6 independent experiments from 3 independent donors.

Bars represent means ± SD.

4.5 IGF-1 and mitochondrial fitness under stress conditions

In chapter 3, we characterized a context dependent effect of PCa conditioned medium on enhanced suppressive potency of iTregs primed with high IGF-1. Consequently, an important next step was to investigate whether these observations related to the IGF-1 driven increase in cellular spare respiratory capacity (see 4.3.1), indicative of an enhanced capacity to respond to mitochondrial stress induced by conditions generated by PCa cells.

4.5.1 Testing the mitochondrial fitness of iTregs exposed to PCa conditioned medium

To test this, iTregs were stimulated with anti-CD3/CD28 dynabeads in different cell culture mediums, representative of different experimental conditions from suppression assays, and mitochondrial TMRM staining was measured by flow cytometry. Stimulation of iTregs cultured in CM from CD8⁺ T-cells had no effect on the relative mitochondrial TMRM MFI of iTregs, relative to stimulation in normal culture medium (fig. 4.5.1.1a). However, stimulation of iTregs in both restrictive medium (no glucose, 2% FBS) and MyC-CaP CM caused a significant decrease in relative mitochondrial TMRM MFI. Thus, MYC-amplified PCa cells generate conditions that inhibit the mitochondrial fitness of iTregs; indicative of impaired redox regulation, mitochondrial stress or a reduced rate of OXPHOS.

MYC-amplification in prostate cancer promotes glycolytic metabolism, enhancing extracellular glucose uptake and lactate production (451). Others have shown that high lactate levels induce redox stress in Tconv and iTreg cells through

depletion of intracellular NAD⁺ levels (242). Hence, it was important to characterize whether PCa CM generated and used in our experiments were in fact representative of glycolytic MYC-amplified cancer cells. Measurements of glucose and lactate levels using Glucose/Lactate-Glo™ assays confirmed MyC-CaP CM had significantly decreased glucose and increased lactate concentrations relative to both fully supplemented RPMI 1640 and CD8⁺ T-cell CM (fig. 4.5.1.1b, appendix fig. 8.1.2.2c).

Next, the relative mitochondrial fitness of iTregs differentiated $\pm$ 50 nmol L⁻¹ IGF-1 was assessed after anti-CD3/CD28 stimulation in MyC-CaP CM. Intriguingly, we observed that priming with IGF-1 rescued the effect of MyC-CaP CM on decreased mitochondrial TMRM staining (fig. 4.5.1.1c). Taken together, priming with IGF-1 preserves the mitochondrial fitness of iTregs exposed to low-glucose and high-lactate conditions from MYC-amplified cancer cells, consistent with an increased mitochondrial SRC.

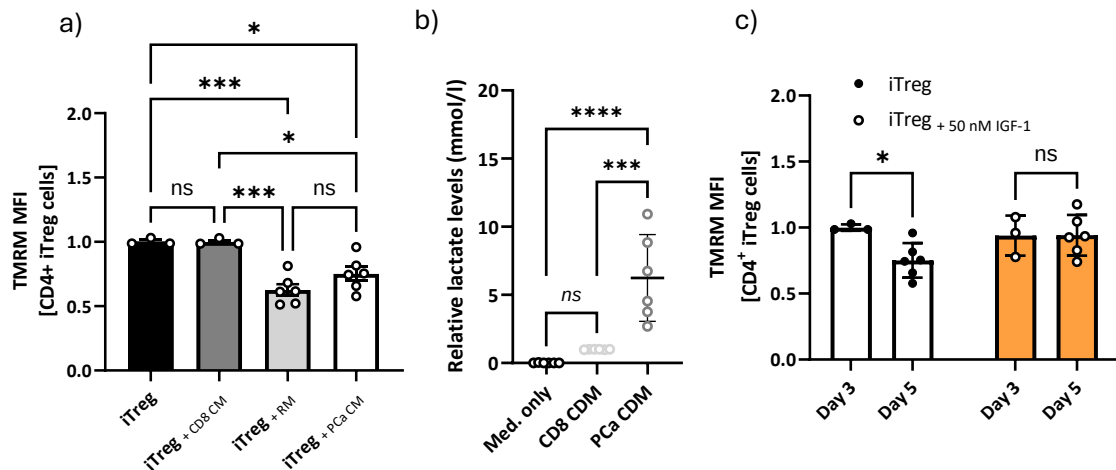


Figure 4.5.1.1 High IGF-1 supply preserves mitochondrial function in iTregs exposed to low-glucose, high lactate PCa CM

Sorted naïve murine CD4⁺ T-cells from 6–8-week-old (♂) FVB mice were differentiated under iTreg conditions $\pm$ 50 nM IGF-1 for 72 hours.

a) Relative TMRM MFI in active mitochondria of iTreg cells after culture with fully supplemented RPMI 1640 [iTreg], CM from CD8⁺ T-cells (fully supplemented RPMI 1640), restrictive medium (Glucose free RPMI 1640 supplemented with 2% FBS), or prostate cancer cell conditioned medium (fully supplemented RPMI 1640) for 48 hours (n=6).

b) Quantification of lactate concentrations in fully supplemented RPMI 1640 (10% FBS), CD8⁺ conditioned medium, and PCa conditioned medium by Lactate-GloTM (n=6).

c) Relative TMRM MFI of iTreg cells differentiated with $\pm$ 50 nM IGF-1 before and after culture with prostate cancer cell conditioned medium for 48 hours. (n=3 control, n=6 PCa CM).

[* = $p < 0.05$, ** = $P < 0.01$, **** = $p < 0.0001$]

[(a-b) one-way ANOVA with multiple comparisons, (c) two-way ANOVA] N = 3 (D3) or 6 (D5) independent experiments and donors. Bars represent means $\pm$ SD.

4.5.2 Transcriptional profiling of iTregs activated in low-glucose, high-lactate PCa conditioned medium.

Under nutrient deprivation, neutral lipids from LDs can be broken down by lipolysis for β -Oxidation and energy generation (471). However, mitochondrial stress from high levels of lactate and glucose depletion also impact a broad range of metabolic, redox and cellular stress responses that could impact iTreg function (480). As to take a more discovery based experimental approach to explore possible mechanisms of IGF-1 priming on Treg mitochondrial fitness, transcriptomic analysis was performed on iTregs primed $\pm 50 \text{ nmol L}^{-1}$ IGF-1, stimulated with anti-CD3/CD28 dynabeads in PCa CM. mRNA was isolated from FACSsorted live, CD4⁺, CD25⁺ cells after 48 hours.

Differential gene expression (DEG) analysis by *DESeq2* revealed 1349 genes were significantly differentially expressed after culture with PCa CM [p-adj < 0.05] (appendix table 8.1.5.1), with log2fold changes of all DEGs represented visually on a heatmap (fig. 4.5.2.1a) and volcano plot (fig. 4.5.2.1b). To explore pathways enrichment in the list of DEGs, over-representation analysis (ORA) was performed. ORA revealed significant enrichment of genes required for biological processes involved in the regulation of inflammatory responses, T-cell differentiation and amino acid biosynthesis (appendix fig. 8.1.2.3) and molecular functions related to cytokine binding, DNA-binding transcription repressor activity (RNA-pol-II specific), actin binding and growth factor binding (appendix fig. 8.1.2.4). These findings were consistent with an IGF-1-mTORC1 driven transcriptional phenotype (T-cell

differentiation, amino acid biosynthesis, RNA-pol-II) and IGF-1R activation (growth factor binding, RNA-pol-II).

Gene-set enrichment analysis (GSEA) uses pre-defined gene sets representative of biological processes or molecular functions and differs from ORA in that it incorporates both directionality of gene expression changes and uses normalized gene counts of all genes, not just DEGs (415). Exploration of the iTreg transcriptome by GSEA using gene sets from *Hallmark* gene sets revealed OXPHOS, the unfolded protein response (UPR), mTORC1 signalling, hypoxia and adipogenesis as the top upregulated pathways in IGF-1 primed iTregs, and IL-2/STAT-5, genes downregulated by KRAS, mitotic spindle assembly, TGF- β signalling and early response to oestrogen as the top downregulated pathways (fig. 4.5.2.1c). These results were indicative that one mechanism by which IGF-1 could facilitate metabolic adaptation is through enhanced mitochondrial OXPHOS that replenishes intracellular NAD⁺ levels. Conversely, decreased IL-2/STAT5 and TGF- β signalling could be indicative of decreased lineage stability.

To investigate key response elements driving the transcriptional response to IGF-1, GSEA was performed using gene sets from the MSigDB master regulators/transcription factor database. Priming with IGF-1 drove a transcriptional enrichment for CCAAT enhancer binding protein gamma (C/EBP γ , CHOP), DNA damage inducible factor 3 (DDIT3) and growth arrest and DNA damage inducible alpha (GADD45A) activity (fig. 4.5.2.1d) [p-adj < 0.05, FDR < 0.25]. Conversely, cyclic GMP-AMP synthase (CGAS) was the only significantly enriched regulatory element in iTregs primed in the absence of IGF-1. The collective enrichment of CHOP, DDIT3 and

GADD45A as predicted regulatory elements were indicative of an activated integrated stress response (ISR), consistent with significant enrichment for UPR related genes by GSEA and the differential upregulation of activating transcription factors 3, 4 and 5 (ATF3, ATF4, ATF5) in IGF-1 primed iTregs (481). Of note, although a primary function of GADD45A is stress-related DNA damage repair, it also promotes adipogenesis and lipid metabolism through interaction with PPAR γ , a transcription factor also differentially upregulated in IGF-1 primed iTregs (482). In contrast, the transcriptional response in iTregs differentiated without IGF-1 were indicative of increased cytosolic DNA sensing through cGAS-STING (stimulator of interferon gene, STING) signalling and speculatively, a failure to respond to cellular stress (483).

As to cross-reference findings from GSEA of the *Hallmark* gene sets, we also performed GSEA using *Gene Ontology*, *WikiPathways* and *Reactome* gene sets. Over 200 pathways were significantly different [p.adj < 0.05] (data not shown), which made further interpretation challenging in the absence of a more hypothesis driven approach. A subset of the top differentially enriched pathways are shown in appendix table 8.1.5.2 [p.adj < 0.05, FDR < 0.25]. The ER is an important site for lipid and cholesterol synthesis, in response to ER stress the UPR can regulate both adaptive lipogenesis and lipolysis, in a manner dependent on both the type of stimulus and cellular context (473). Consequently, the enrichment of genes indicative of UPR activation could drive transcriptional changes in lipid and cholesterol metabolic pathways in iTreg primed with IGF-1. Conversely, the relative enrichment for CGAS responsive genes in iTregs primed without IGF-1 could be indicative that a reduced

capacity to respond to mitochondrial stress promotes cGAS-STING activation by release of mitochondrial DNA (483).

Manual exploration of significantly enriched *GO: Biological Process* pathways revealed IGF-1 priming drove a transcriptional enrichment towards cholesterol storage, very low-density lipoprotein particle assembly, fatty acid β -oxidation, lipoprotein localisation and lipid oxidation (fig. 4.5.2.1e) [p-adj < 0.05, FDR < 0.25]. In contrast, there was a significant downregulation of pathways related to response to interferon beta (IFN- β), cellular response to prostaglandin stimulus, neutral lipid catabolic process, response to cholesterol, cholesterol efflux and the lipoxygenase pathway [p-adj < 0.05, FDR < 0.25]. These findings were indicative that a transcriptional response to IGF-1 involves the sequestering and packaging of free FAs and cholesterol through lipid droplet formation and an upregulation of enzymes required for long-chain FAO. However, in the absence of IGF-1 priming, pathway enrichment suggests iTregs break down neutral lipid stores and regulate the subsequent release of lipotoxic intracellular cholesterol through upregulated efflux transporters, associated with increased IFN- β signalling.

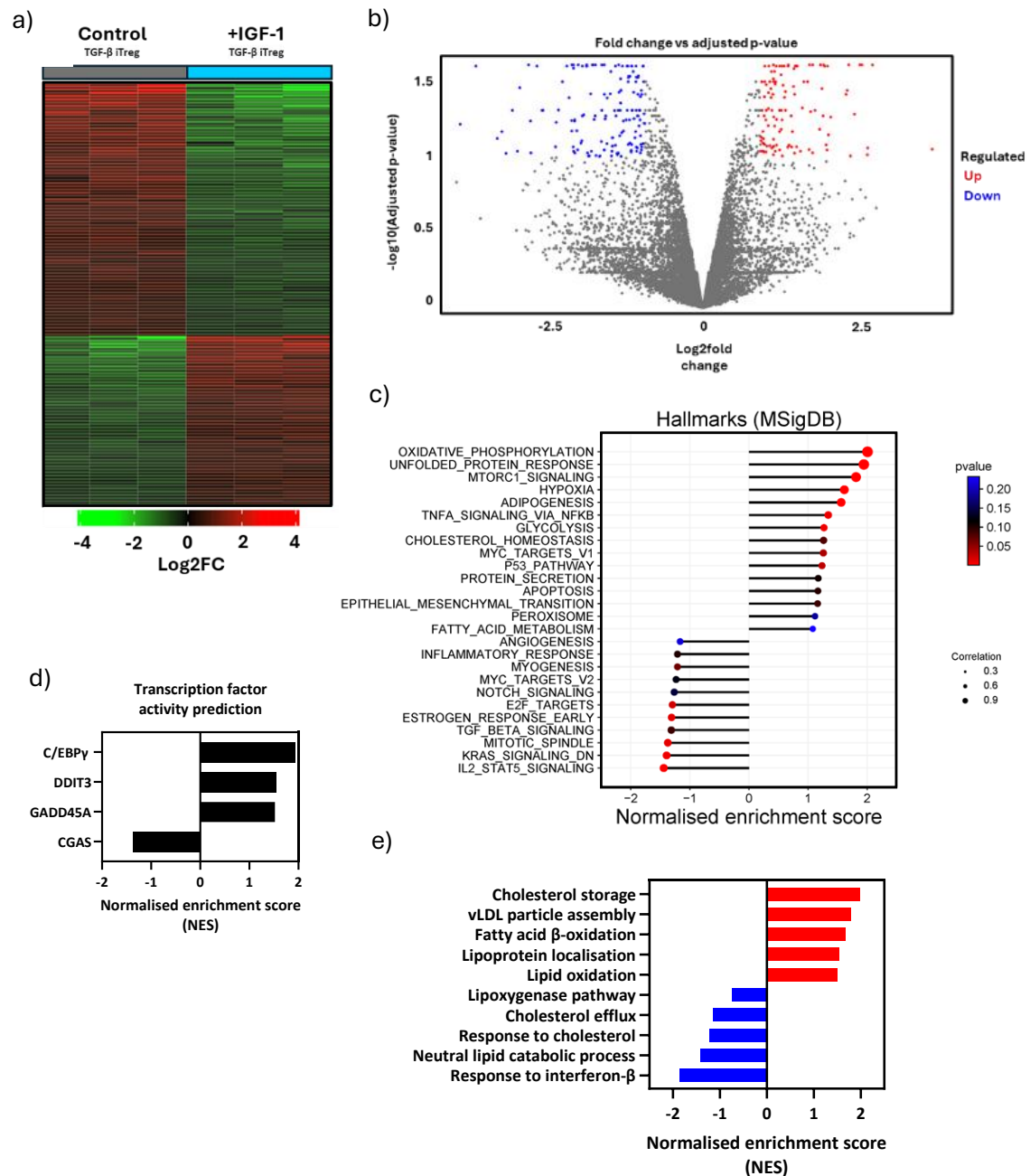


Figure 4.5.2.1 - High IGF-1 supply shapes the transcriptional response of iTregs to PCa conditioned medium

Sorted naïve murine CD4⁺ T-cells from 6–8-week-old (♂) FVB mice were differentiated under iTreg conditions $\pm$ 50 nM IGF-1 for 72 hours and cultured with PCa conditioned medium for 48 hours before FACSsorting of CD4⁺, CD25⁺ cells, RNA isolation and mRNA-sequencing. a) Heatmap of visually representative of the log2fold change in gene expression of 1349 DEGs identified by DESeq2 analysis. B) Volcano plot of DEGs [log2-fold change and -log10(adjusted p-value)]. Top 60 up/downregulated genes are highlighted in red and blue respectively. c) Significantly enriched pathways from GSEA of MSigDB *Hallmark* gene sets. d) Significantly enriched regulatory elements from GSEA of MSigDB *transcriptional response elements* gene sets. e) Selected enriched pathways from GSEA of GO: *Biological Process* gene sets.

Notable upregulated genes induced by IGF-1 priming were solute carrier family (SLC) 2 member 3 (solute SLC2A3), an amino acid transport protein, followed by asparagine synthetase (ASNS), an enzyme that converts aspartate and glutamine to asparagine and glutamate (484). Intracellular asparagine levels are an important regulatory element of electron transport chain and mTORC1 activity, through an ATF4-mediated relief of mitochondrial stress (485). Considering the transcriptional enrichment for amino acid biosynthesis by ORA, and OXPHOS and mTORC1 pathways by GSEA, the transcriptional regulation of amino acid metabolism was considered as an important component of preserved mitochondrial fitness in response to IGF-1. *GO: Biological Process* GSEA revealed a significant transcriptional enrichment for amino acid activation, biosynthesis, cell redox homeostasis and amino acid transmembrane transport pathways in IGF-1 primed iTregs (figure 4.5.2.2a). Leading DEGs of note include ASNS, cystathionine gamma-lyase (Cth), 3-phosphoglycerate dehydrogenase (Phgdh), phospho-serine phosphatase (Psph), glutamic pyruvate transaminase (Gpt2) and branched chain amino acid transaminase 1 (Bcat1). The transcriptional upregulation of genes required the serine-cysteine-glutathione redox buffer system (Cth, Psph, Gpt2) may function to preserve mitochondrial fitness in iTregs through an enhanced capacity for redox regulation mediated by glutathione (486).

Finally, upon exploration of leading DEGs from GSEA of amino acid transmembrane transport pathway, we noted the differential upregulation of several SLC-transport proteins related to cysteine (SLC7A1, SLC38A2, SLC1A4) and arginine (SLC7a1, SLC7a3) transport. Tregs can utilize non-specific competitive cysteine

deprivation to suppress responder CD8⁺ T-cell activation in a CTLA-4 dependent manner (222), and the depletion of extracellular arginine levels also inhibit CD8⁺ T-cell proliferation (487). Thus, a possible mechanism of enhanced suppressive potency in IGF-1 primed iTregs cultured in PCa CM (identified in chapter 3) could be mediated by non-specific competitive nutrient deprivation or redox imbalance in proximal responder CD8⁺ T-cells.

To test this, responder CD8⁺ T-cells were co-cultured in PCa CM with iTregs primed $\pm$ IGF-1. The mitochondrial fitness of responder CD8⁺ T-cells was measured after 48 hours by TMRM staining, and partial (50%) and complete (100%) dilution of PCa CM with fully supplemented RPMI 1640 were used as experimental controls. In PCa CM, responder CD8⁺ T-cells co-cultured with IGF-1 primed iTregs had significantly reduced levels of mitochondrial TMRM staining, indicative of reduced mitochondrial activity (figure 4.5.2.2b). This effect was abrogated upon both partial (50%) and complete dilution of PCa CM (100%) with fully supplemented RPMI 1640. Therefore, a possible mechanism by which the suppressive potency of iTregs primed with IGF-1 changes in the presence of low-glucose, high-lactate PCa CM could be through a metabolic shift that drives metabolic dysfunction in proximal responder CD8⁺ T-cells.

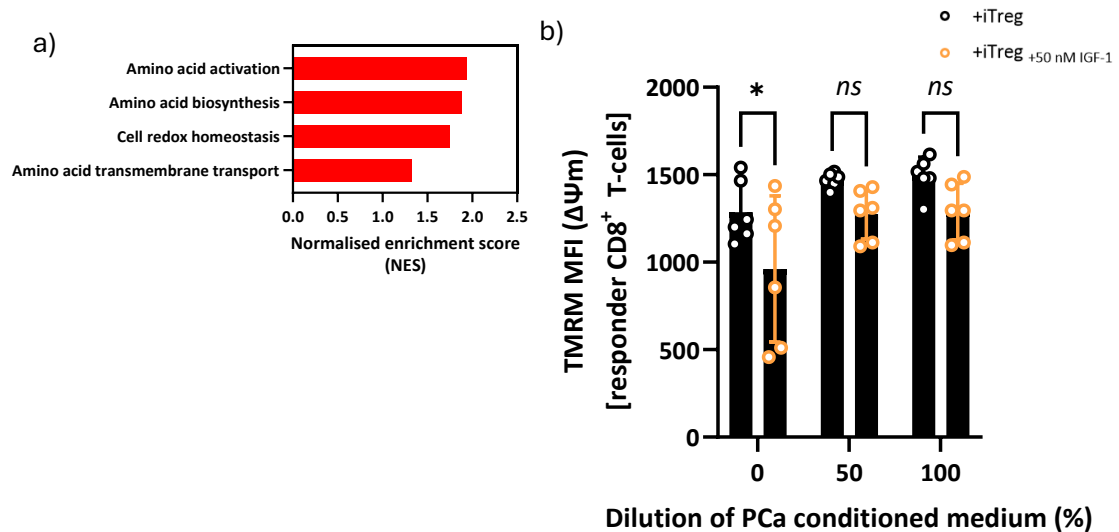


Figure 4.5.2.2 - High IGF-1 supply upregulates the transcription of genes required for nutrient uptake and amino acid metabolism in iTregs activated in PCa CM

a) Selected enriched pathways from GSEA of GO: Biological Process gene sets ($p_{adj} < 0.05$) related to amino acid metabolism and redox homeostasis.

iTregs differentiated $\pm 50 \text{ nmol L}^{-1}$ IGF-1 were co-cultured with autologous CD8⁺ T-cells +CD3/CD28 dynabeads at a 1:1 ratio in 0, 50, or 100% diluted MyC-CaP CM (dilution by fully supplemented RPMI 1640). Mitochondrial fitness of responder CD8 T-cells was measured by flow cytometry after 48 hours of co-culture by TMRM staining.

b) Relative mitochondrial TMRM MFI of responder CD8⁺ T-cells after co-culture with iTregs differentiated $\pm 50 \text{ nmol L}^{-1}$ IGF-1.

[ns = $p > 0.05$, * = $p < 0.05$, by two-way ANOVA] [N = 6 independent donors over 3 independent experiments]

4.6 Discussion

Whilst TI-Tregs display a unique dependence on fatty acid synthesis, cholesterol metabolism and free fatty acid uptake for effective suppression of anti-tumour immune responses (241,242), very little is known about how co-factors produced within the TME influence this metabolic transition. In this chapter, the implications of high IGF-1 bioactivity on Treg metabolism was explored at steady state and upon exposure to low-glucose and high-lactate conditions generated by MYC-amplified prostate cancer cells. Together, these data highlight a hitherto unknown role of high IGF-1 supply in the metabolic adaptation of iTregs to low-glucose, high-lactate conditions generated by PCa cells.

We speculate that the biological response to IGF-1 in our study likely reflects an IGF-1-mTORC1-mediated priming of ATF4 and C/EBP γ -mediated upregulation of integrated stress response genes (488), which increases the capacity of iTregs to respond to oxidative stress. In preclinical tumour models, ATF4 facilitates the *de novo* conversion of activated CD4⁺ TILs into FoxP3⁺ cells through enhanced redox balance of oxidative metabolism (489), and elevated ATF4 expression is associated with increased Treg infiltration in patients with breast cancer (490). Furthermore, TI-Tregs are dependent on glutathione-mediated ROS scavenging for the suppression of anti-tumour immunity in B16-melanoma (491). Given that TI-Tregs are dependent on endogenous lipid synthesis for their immunosuppressive function in the TME (241), the activation and regulation of the UPR and ER stress is likely an important regulatory component of lipogenic metabolism in TI-Tregs. Yet to our knowledge, this

relationship remains completely uncharacterized in TI-Tregs, and remains an important direction for future work.

In contrast, low IGF-1 bioactivity is associated with a failure to maintain mitochondrial membrane potential in TGF- β -iTregs upon exposure to low-glucose and high-lactate conditions. FABP5 expression has been identified as a critical metabolic checkpoint for Treg mitochondrial integrity, that enhances OXPHOS in iTregs (492). Intriguingly, the loss of mitochondrial integrity through downregulation of FABP5 is also associated with increased cGAS-STING signalling in Tregs, promoting suppressive activity through type I IFN signalling. Hence, in iTregs primed without IGF-1, pathway enrichment for cGAS and response to IFN- β could be further indicative of a failure to regulate mitochondrial homeostasis, speculatively through the reduced relative expression of FABP5. With respect to anti-tumour immunity, the sensing of mitochondrial DNA by cGAS-STING in response to Treg mitochondrial dysfunction drives enhanced immunosuppression *in vivo*. These findings were corroborated by observations that failure to regulate oxidative stress causes Treg apoptosis and extracellular adenosine generation in the TME, which suppresses anti-tumour immunity *in vivo* (493). Hence, it is important to consider that low levels of IGF-1, and the failure to regulate mitochondrial integrity in response to oxidative stress, could alternatively improve the suppressive potency of pTregs *in vivo* through IFN- β signalling, apoptosis and extracellular adenosine generation.

There are several limitation of our work in this chapter. First, we rely heavily on the use of small molecule inhibitors to inhibit metabolic or signalling pathways to draw conclusions related to mitochondrial fitness and lipid metabolism. Whilst off

target and non-specific effects were considered where possible, our findings should be used as a guide for future investigation, rather than definitive conclusions. Validation experiments, including profiling of the lipidome/metabolome by liquid chromatography–mass spectrometry and carbon tracing of exogenous glucose/amino acids are essential next steps (467). Second, *in vitro* metabolic assays using flow cytometry compatible fluorescent probes were performed in the absence of exogenous sodium palmitate and with 10% FBS, which supplements 2-3% of the free fatty acid, cholesterol and 1% of the poly-unsaturated fatty acids available to most cells in the body (494). Due to the lack of exogenous free fatty acid supply, from our data alone we cannot conclusively rule out the contribution of CD36-mediated lipid uptake as an additional contributing factor to intracellular neutral lipid levels in response to IGF-1, and should be tested in future experiments. Third, measurements of 2-NBDG uptake alone are not sufficient to confirm an IGF-1 induced upregulation of glucose uptake (495). Finally, although IGF-1 priming reduces the proximity of LDs with active mitochondria, we do not control for the increased frequency of LDs in Tregs differentiated with IGF-1, nor do we co-stain for FoxP3. Hence, increased proximity of mitochondria with LDs cannot be interpreted as direct interactions, important for interpretations related to CPM and PDM metabolism, nor a Treg specific effect. Nevertheless, our work highlights an area of novel investigation of IGF-1 priming on Treg LD biogenesis, mitochondrial metabolism, and highlights the UPR as an important component of the transcriptional response to PCa CM.

In conclusion, our findings suggest that high local concentrations of IGF-1 within the prostate TME could support the mitochondrial adaptation of TI-Treg cells

in vivo. A summary of theorized interactions between enriched RNA-seq pathways and DEGs in the presence, or absence of IGF-1 priming can be found below (fig. 4.6.1.1 and 4.6.1.2 respectively).

4.6.1 Supplementary figures

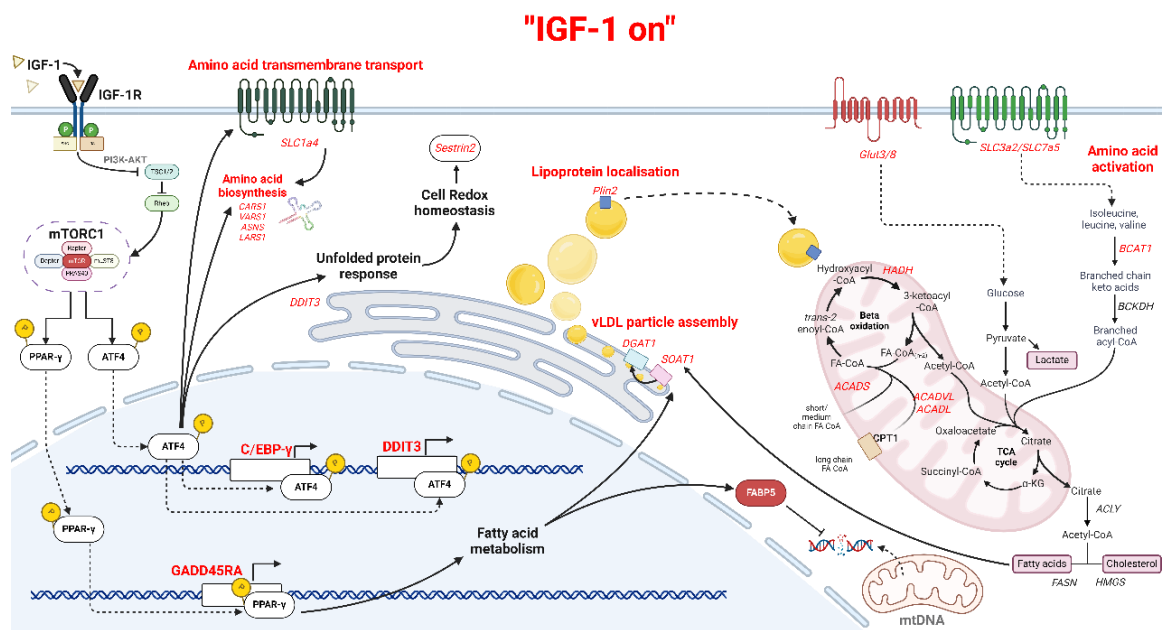


Figure 4.5.2.1 Proposed model for the effects of high IGF-1 priming on exposure of iTregs to PCa CM

Activation of IGF-1R by high IGF-1 supply promotes an mTORC1-mediated priming of ATF4 and PPAR- γ , which interact with C/EBP γ /DDIT3 or GADD45RA respectively, upregulating their transcriptional activity. Then, upon exposure to PCa CM, primed ATF4 mediates the activation of the ISR and transcription of genes related to the UPR, amino acid metabolism, redox regulation and nutrient transport. Simultaneously, PPAR- γ -GADD45RA promotes the transcription of genes related to adipogenesis and lipoprotein particle assembly at the ER. The enhanced transcription of FABP5, and enzymes required for mitochondrial FAO and branched chain amino acid catabolism likely further support mitochondrial integrity and capacity for mitochondrial OXPHOS.

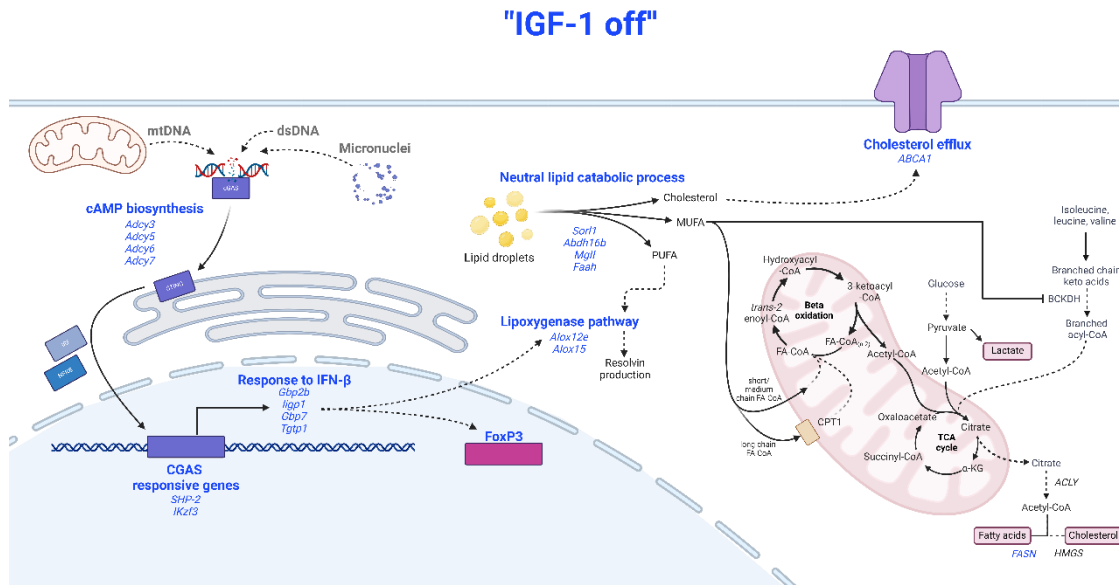


Figure 4.6.1.2 - Proposed model for the effects of low IGF-1 priming on exposure of iTregs to PCa CM

Low IGF-1 bioactivity results in the catabolism of intracellular neutral lipid stores, with the intracellular rise of lipotoxic free-FAs and cholesterol within the cytoplasm mitigated, in part, by increased cholesterol efflux and FAO. However, the failure to maintain redox regulation within mitochondria results in the release of mitochondrial DNA and sensing by cytoplasmic CGAS. Activated CGAS produces of the secondary messenger 2',3'-cGAMP, which in turn activates STING, which phosphorylates IRF3 via TBK1 and promotes type-1 IFN- β signalling. Autocrine or paracrine IFN- β signalling could then support FoxP3 stability, and/or drive the production of anti-inflammatory resolvins from polyunsaturated fatty acids by the 15-lipoxygenase pathway.

Chapter 5 - Investigating the effects of IGF blockade and anti-PD-1 immunotherapy on the anti-tumour immune response in murine prostate cancer

5.1 Background

Immune checkpoint blockade (ICB) with PD-1/PD-L1 and/or CTLA-4 inhibitors induces durable tumour regression in some cancer patients with advanced disease (30,125,137). Unfortunately, results from trials of ICB in prostate cancer have been largely disappointing, with little to no survival benefit observed in clinical trials (131,133,134). Hence, a current unmet need for patients with advanced PCa is the identification of treatment modalities that improve sensitivity to ICB by inhibiting the immunosuppressive PCa immune infiltrate, and/or boosting the CD8⁺ CTL infiltrate (138). There is evidence of IGF inhibition being immunogenic and therapeutically synergistic with ICB in preclinical tumour models (400–402). Moreover, non-small cell lung carcinoma patients with durable clinical benefit post-immune checkpoint blockade (ICB) had significantly lower circulating IGF-1 and lower tumour IGF-1R than those with no durable benefit (395). Thus, therapeutic IGF blockade could promote anti-tumour effects mediated by ICB in prostate tumours, currently under evaluation in advanced human PCa.

Preclinical prostate cancer (PCa) research has been restricted by a deficit of clinically relevant syngeneic models for the evaluation of immunotherapy (496). This is caused by both the rarity of spontaneous prostate cancer in mice, and developmental differences in murine prostate biology. However, some genetically engineered and immunocompetent mouse models of prostate cancer do reflect aspects of the development and immunology of human PCa, including the TRAMP-C1 and MyC-CaP models (497,498). The Tramp-C1 mouse model is driven by a transgenic viral oncogene simian vacuolating virus 40 tag (SV40T), which forms spontaneous tumours in the mouse prostate by 16-28 weeks, modelling the development neuroendocrine driven human prostate cancers, representative of 1-5% of human PCa (499). Limitations include the expression of the SV40T antigen, which is immunogenic, and the resistance of primary subcutaneously implanted tumours to androgen deprivation, which is not representative of human disease (500).

Alternatively, the syngeneic MyC-CaP model is driven by overexpression of the c-MYC oncogene, representative of chromosomal MYC amplification in PCa patients [~10-30%] (96–100). The MyC-CaP model phenocopies the most common type of deadly PCa; tumours that initially respond to androgen deprivation, become castration resistant, but remains sensitive to transcriptional regulation by androgen receptor signalling (498). Furthermore, MyC-CaP allografts are widely resistant to ICB monotherapy (501–504), and retain a sparse CD3⁺ T-cell infiltrate that remains at the periphery of tumours (498). For these reasons, the syngeneic MyC-CaP allograft model was used evaluate the effects of IGF inhibition.

5.2 Chapter aims

The aims of this chapter were two-fold. First, to assess if IGF inhibition promotes CD8⁺ CTL infiltration and depletes TI-Tregs in murine PCa, and second, to assess the anti-tumour efficacy of combined IGF inhibition with ICB.

5.3 Investigating the immunogenicity of IGF blockade in PCa allografts

5.3.1 Immunophenotyping of TILs

Given that high IGF-1 supply enhances the *in vitro* suppressive function and metabolism of Tregs activated in conditioned medium generated from MyC-CaP cells, we hypothesized that blockade of IGFs could inhibit TI-Treg function within MyC-CaP allografts, facilitating the activation and expansion of anti-tumour CD8⁺ CTLs. Thus, to inform synergistic strategies of combinatorial therapy with Xentuzumab (an IGF neutralizing antibody), the relative infiltration and function of TILs was first investigated in a small cohort of mice inoculated with subcutaneous MyC-CaP allografts (n = 3 per group). Treatment of tumour bearing wild-type FVB/NJ mice with Xentuzumab (referred to as IGF blockade herein) did not have a significant impact on the rate of tumour growth (figure 5.3.1.1a). Exploration of the immune infiltrate of prostate allografts by flow cytometry at a tumour volume of 400 mm³ revealed that IGF blockade did not change the total number of CD45⁺ immune cells (appendix fig. 8.1.3.2a), but enhanced CD3⁺ TIL infiltration (figure 5.3.1.1b). Although not statistically significant, this was likely caused by an increased number of CD8⁺ TILs. There was a trend towards reduced FoxP3⁺ expression in CD4⁺ TILs, but this was also not statistically significant (data not shown). For all subsequent experiments described in this chapter, TILs were stimulated with a cocktail of phorbol 12-myristate 13-acetate (PMA), ionomycin, brefeldin A and monensin (as described in 2.2.7.5) to measure their overall capacity to produce intracellular cytokines and granzymes.

Further analysis of CD4⁺ T_{conv} [Foxp3⁻] TILs revealed treatment with IGF blockade increased the percentage of central memory CD4⁺ TILs (CD62L⁺ CD44⁺), at the expense of effector CD4⁺ TILs (CD62L⁻ CD44⁺) [figure 5.3.1.1c]. However, CD4⁺ TILs expressed reduced PD-1 but higher CTLA-4 expression, whilst the expression of pro-inflammatory cytokines IFN- γ and TNF- α did not change (figure 5.3.1.1d). In contrast, IGF blockade had no effect on the relative proportion of effector or memory CD8⁺ TILs (figure 5.3.1.1e). Furthermore, IGF blockade increased both PD-1 and CTLA-4 expression, but not IFN- γ or TNF- α in CD8⁺ TILs (figure 5.3.1.1f). Taken together, whilst two-times (2x) weekly treatment with Xentuzumab does not appear to have anti-tumour effects in murine PCa, it does promote TIL infiltration.

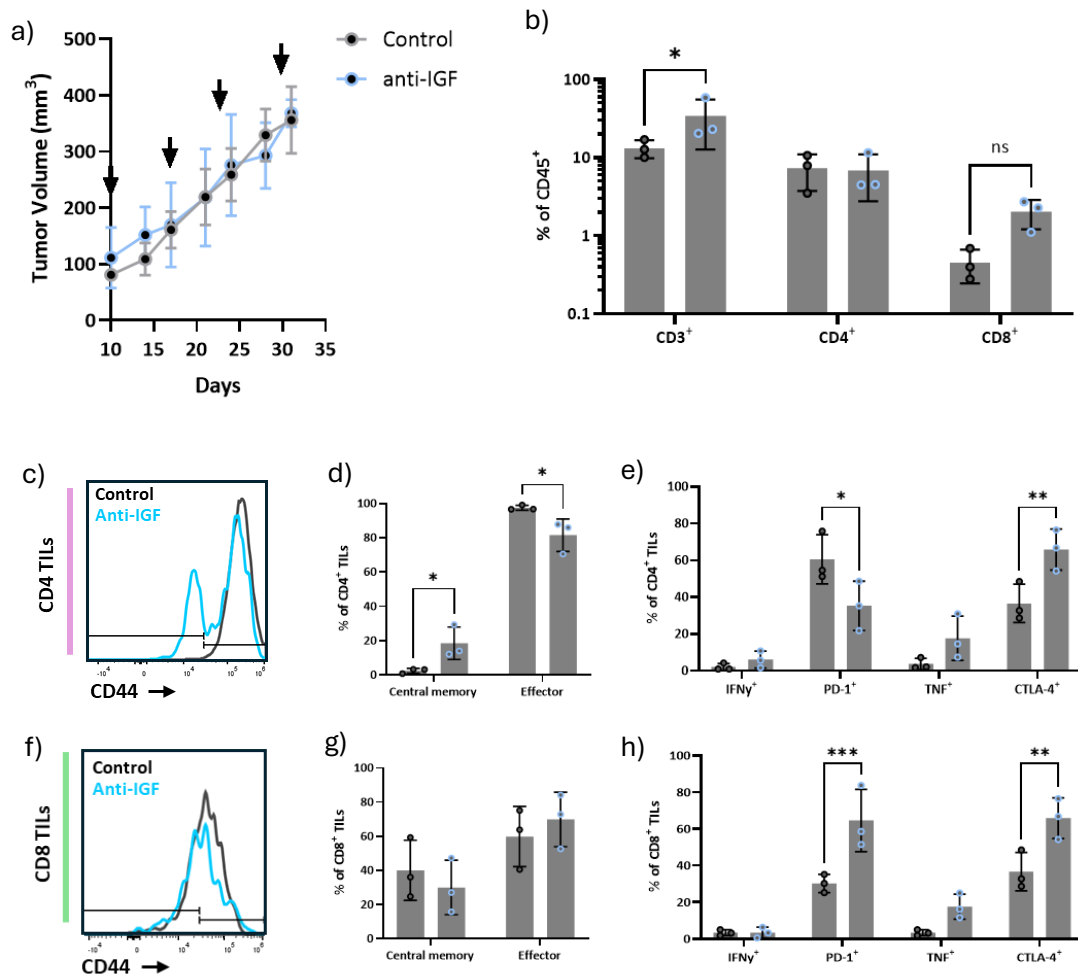


Figure 5.3.1.1 - Effects of IGF blockade on the infiltration and phenotype of tumour-infiltrating T-cells in MyC-CaP allografts

1 × 10⁶ MyC-CaP cells were implanted subcutaneously to the right flank of 5–6-week-old (male ♂) FVB mice. Tumour bearing mice received either x2 weekly intra-peritoneal injections of PBS (control) or 100 mg/kg Xentuzumab (anti-IGF) once palpable tumours reached 100 mm³. At a tumour volume 400 mm³ mice were euthanized, and TILs were analysed by flow cytometry.

a) MyC-CaP allograft growth in mice treated with either PBS (control) or Xentuzumab (anti-IGF). Days = Number of days after tumour implantation, arrows indicate PBS/Xentuzumab administration. b) Percentage of total CD3⁺, CD3⁺ CD4⁺ or CD3⁺ CD8⁺ T-cells of tumour infiltrating CD45⁺ cells after treatment with PBS or Xentuzumab. c) Representative histograms of CD44 expression in CD4⁺ TILs after treatment with PBS or Xentuzumab, with quantification of the percentage of CD4⁺ TILs that were (d) central memory or effector/effector memory cells or that expressed (e) IFN-γ⁺, PD-1⁺, TNF-α⁺ or CTLA-4⁺. f) Representative histograms of CD44 expression in CD8⁺ TILs after treatment with PBS or Xentuzumab, with quantification of the percentage of CD8⁺ TILs that were central memory or effector/effector memory cells (g) or IFN-γ⁺, PD-1⁺, TNF-α⁺ or CTLA-4⁺ (h).

[* = p < 0.05, ** p < 0.01, [(a) mixed-effects analysis with Šidák's multiple comparisons test, (b,d,e,g, h) two-way ANOVA, N = 3 mice per group. Bars represent means ± SD.]

5.4 Therapeutic evaluation of IGF blockade with anti-PD-1 in a syngeneic mouse model of MYC-amplified PCa

Anti-PD-1 ICB was selected for therapeutic evaluation with Xentuzumab in murine PCa on the basis of three lines of supporting evidence. First, work from our group has shown acute IGF-1 stimulation upregulates the *in vitro* expression of the PD-1 ligand, PD-L1, in MyC-CaP cells, but not ligands of CTLA-4 [*B7-H1*], TIGIT or TIM-3 (*Caecam-1*, *Galectin-9*) [unpublished, data not shown]. Second, IGF-blockade induced a selective increase in PD-1 expression in CD8⁺ TILs, but not CD4⁺ TILs. In non-small cell lung cancer, an increased ratio of PD-1 expression on CD8⁺ relative to CD4⁺ TILs is associated with clinical benefit from PD-1/PD-L1 blockade (505). Third, anti-PD-1 ICB has therapeutic synergy in combination with IGF-1 axis inhibitors in other preclinical tumour models (395,401,402,409). Taken together, these data suggested that combined administration of anti-PD-1 and Xentuzumab could have a biologically synergistic effect in murine PCa.

5.4.1 Tolerability of IGF blockade with anti-PD-1

Prior to therapeutic evaluation of combination treatment, the safety and tolerability of anti-PD-1 and Xentuzumab combination protocols in MyC-CaP tumour bearing mice was assessed in a pilot tolerability study. TIL phenotype was also investigated at a tumour volume of 400 mm³. Administration of combination treatment (anti-PD-1 and Xentuzumab) to mice with MyC-CaP allografts three-times weekly was more immunogenic relative to a two-times weekly protocol, with an increased CD4⁺ Tconv and decreased Treg tumour infiltrate (appendix fig. 8.1.3.2b-d). Whilst

combination treatment was well tolerated in mice receiving twice-weekly treatments, unfortunately mice receiving three-times weekly treatments experienced significant adverse effects after 10-17 days ($> 10\%$ bodyweight loss, hunched posture, piloerection, splenomegaly). In light of these safety data, we opted for a twice weekly combination treatment protocol for efficacy studies.

5.4.2 Efficacy of IGF blockade with anti-PD-1 ICB

Next, the anti-tumour efficacy of anti-PD-1 monotherapy (RMP1-14 + IgG), Xentuzumab monotherapy (Xentuzumab + IgG) or combination treatment of anti-PD-1 and Xentuzumab (RMP1-14 + Xentuzumab) was assessed in wild-type FVB/NJ mice with subcutaneous MyC-CaP allografts, summarized in figure 5.4.2.1a. Treatments were administered at a tumour volume of $80\text{--}100\text{ mm}^3$, and the experimental endpoint was reached upon tumour growth to a volume of 800 mm^3 . Neither anti-PD-1 nor Xentuzumab (monotherapy, or combination therapy) had a significant effect on MyC-CaP tumour growth delay relative to treatment with PBS (control) [fig. 5.4.2.1b-c]. Analysis of tumour sections ($N > 100$ sections) by immunohistochemistry (IHC) revealed a significant increase in tumour necrosis with combination therapy not with neither anti-PD-1 nor Xentuzumab monotherapy (data not shown). Hence, whilst combination treatment did not significantly delay MyC-CaP tumour growth, there was evidence of some anti-tumour effects.

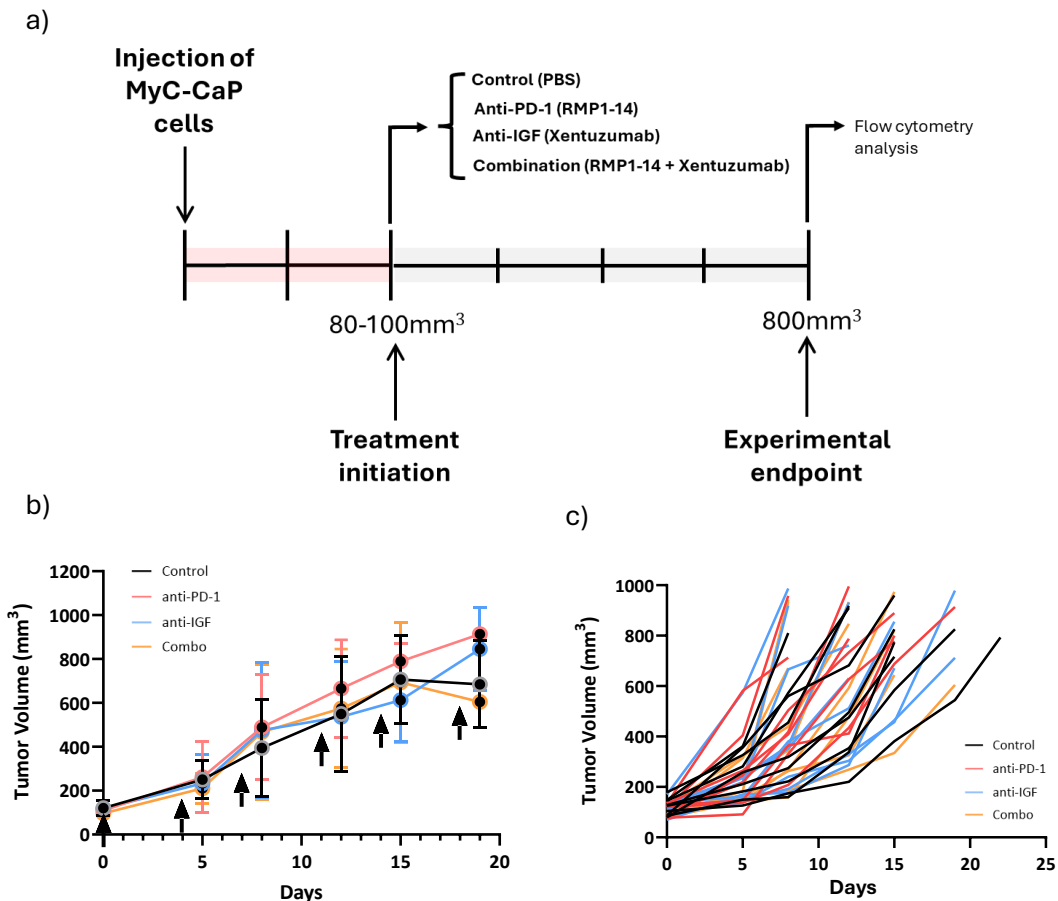


Figure 5.4.2.1 - Effects of anti-PD-1 and Xentuzumab monotherapy and combination treatment on MyC-CaP allograft tumour growth

1×10^6 MyC-CaP cells were implanted subcutaneously to the right flank of 5–6-week-old (male ♂) FVB mice. Tumour bearing mice received either x2 weekly intra-peritoneal injections of PBS (control) or 100 mg/kg Xentuzumab (anti-IGF) once palpable tumours reached 80-100 mm³.

a) Diagram of experimental design and MyC-CaP allograft treatment protocol.

b) MyC-CaP allograft growth in mice treated with either PBS (control), anti-PD-1 (RMP1-14), Xentuzumab (anti-IGF) or anti-PD-1 and Xentuzumab (combo). Days = number of days after treatment initiation, arrows indicate therapeutic treatment administration.

c) Growth curves for individual MyC-CaP allografts treated with either PBS (control), Xentuzumab (anti-IGF), anti-PD-1 (anti-PD-1) or anti-PD-1 and Xentuzumab (Combo).

[(b) mixed-effects analysis with Šidák's multiple comparisons test, N = 10, bars represent means $\pm$ SD.]

5.4.3 Immunophenotyping of TI-Tregs

A key aim of this chapter was to explore if neutralization of IGF bioactivity *in vivo* had an inhibitory effect on TI-Treg infiltration and function in MyC-CaP allografts. Hence, the expression of FoxP3 was first evaluated in CD4⁺ TILs. Representative histograms display the gating strategy for identification of FoxP3 positivity in CD4⁺ TILs (figure 5.4.3.1a). IGF blockade significantly reduced the percentage positivity and MFI of FoxP3 expression in tumour-infiltrating CD4⁺ T-cells compared with treatment with PBS or anti-PD-1 monotherapy (figure 5.4.3.1b-c). Although the percent positivity of FoxP3⁺ CD4⁺ TILs after combination treatment was not significantly different to Xentuzumab alone, there was a significant decrease in the relative MFI of FoxP3. Of note, the relative infiltration of other CD45⁺, CD4⁺ or CD8⁺ T-cells did not significantly differ in monotherapy or combination therapy groups (appendix fig. 8.1.3.3a-c).

To test whether this correlated with a total reduction in the TI-Treg infiltrate proportional to the whole tumour mass, the total number of Tregs per gram of tumour mass was calculated. Treatment with Xentuzumab led to a significant decrease (~2-fold-reduction) in the total number of TI-Tregs per gram of prostate tumour tissue relative to treatment with PBS or anti-PD-1 monotherapy (fig. 5.4.3.2a-b), but this did not correlate with a change in the CD8:Treg ratio. There was also a trend towards a decrease in the H-score of FoxP3 expression by IHC upon treatment with Xentuzumab, however this was not statistically significant (data not shown).

Hence, IGF blockade *in vivo* inhibits TI-Treg infiltration in MyC-CaP allografts. Furthermore, the addition of anti-PD-1 to Xentuzumab promotes a further decrease in

FoxP3 expression by MFI, but not percentage positivity, which could be indicative of a possible synergistic effect between anti-PD-1 with Xentuzumab.

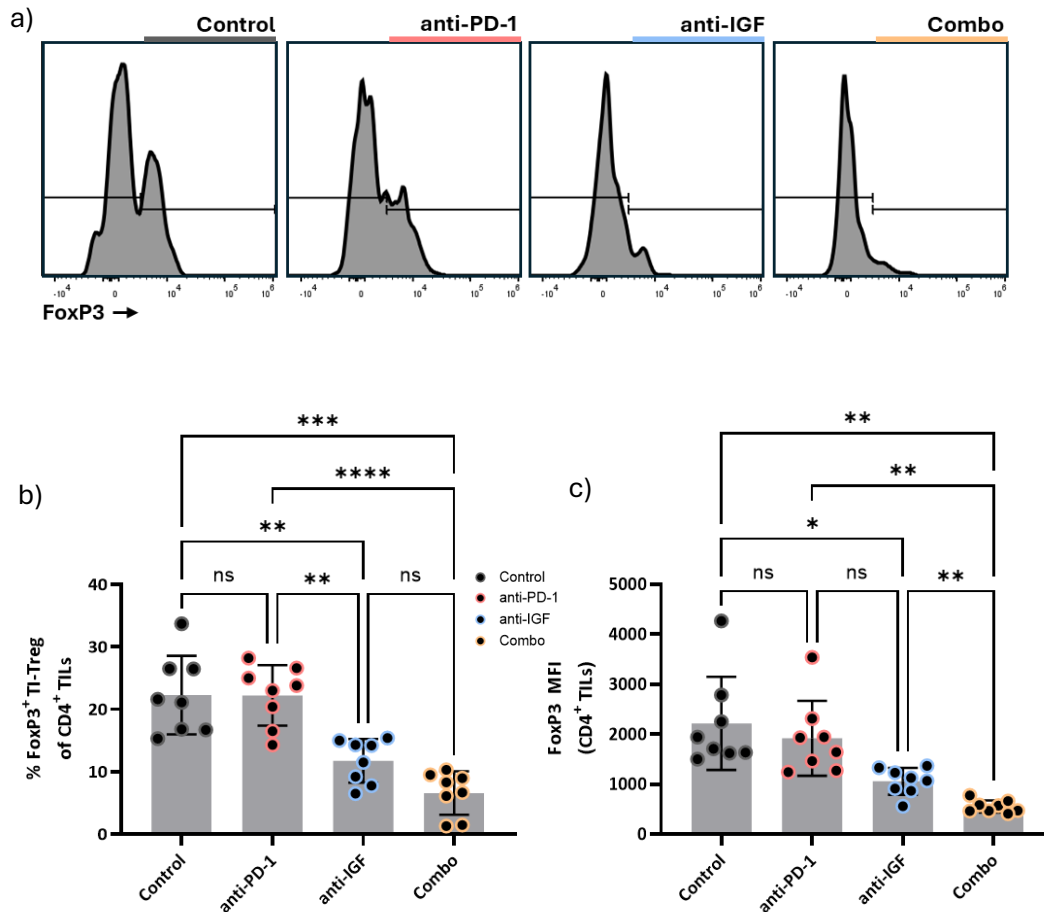


Figure 5.4.3.1 - Investigating the effects of anti-PD-1 and Xentuzumab on TI-Treg infiltration in MyC-CaP allografts

a) Representative histograms of FoxP3 staining in CD4⁺ TILs.

b) Quantification of the percentage of CD4⁺ TILs staining positive for FoxP3.

c) Quantification of the median fluorescence intensity of FoxP3 expression in CD4⁺ TILs

[(b-c) One way ANOVA with multiple comparisons, N = 8 tumour, bars represent means $\pm$ SD.]

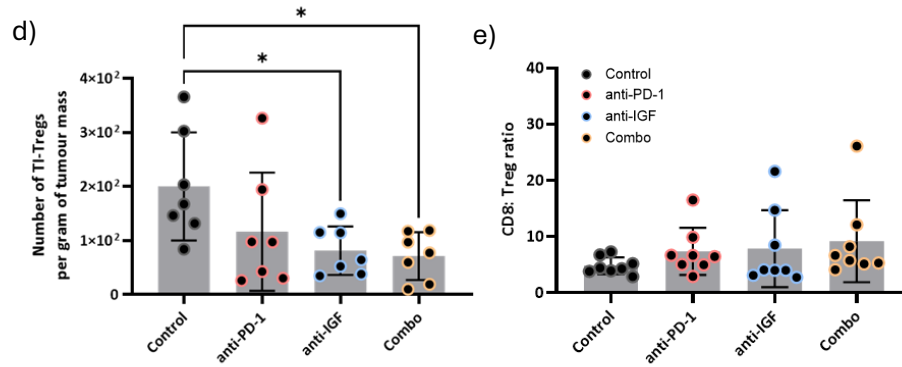


Figure 5.4.3.2 - Investigating the effects of anti-PD-1 and Xentuzumab on TI-Treg infiltration in MyC-CaP allografts

a) Quantification of the number of FoxP3⁺ CD4⁺ TILs identified by flow cytometry normalized per gram of tumour mass analysed.

b) Quantification of the ratio of CD8⁺ TILs to FoxP3⁺ CD4⁺ TILs identified by flow cytometry.

[(a-b) One way ANOVA with multiple comparisons, N = 8 (control, anti-PD-1) N = 9 (anti-IGF, Combo), bars represent means $\pm$ SD.]

Activation of mTORC1 upregulates granzyme-B expression in Tregs (506), a serine protease that promotes the suppression of anti-tumour CD8⁺ TILs by direct cytotoxicity (227). In chapter 3, we identified *in vitro* that IGF-1 upregulates granzyme-B expression in murine induced-Tregs. Thus, expression of granzyme-B was assessed in TI-Tregs. Figure 5.4.3.1a shows representative histograms of granzyme-B expression in TI-Tregs across treatment groups. IGF blockade induced a significant reduction in both the percentage positivity and MFI of granzyme-B in TI-Tregs compared with mice treated with either PBS or anti-PD-1 monotherapy (figure 5.4.3.1b-c). Of note, the expression of PD-1, CTLA-4, IGF-1R or CD25 did not change after treatment with anti-PD-1 or Xentuzumab in TI-Tregs (appendix fig. 8.1.3.3d-g).

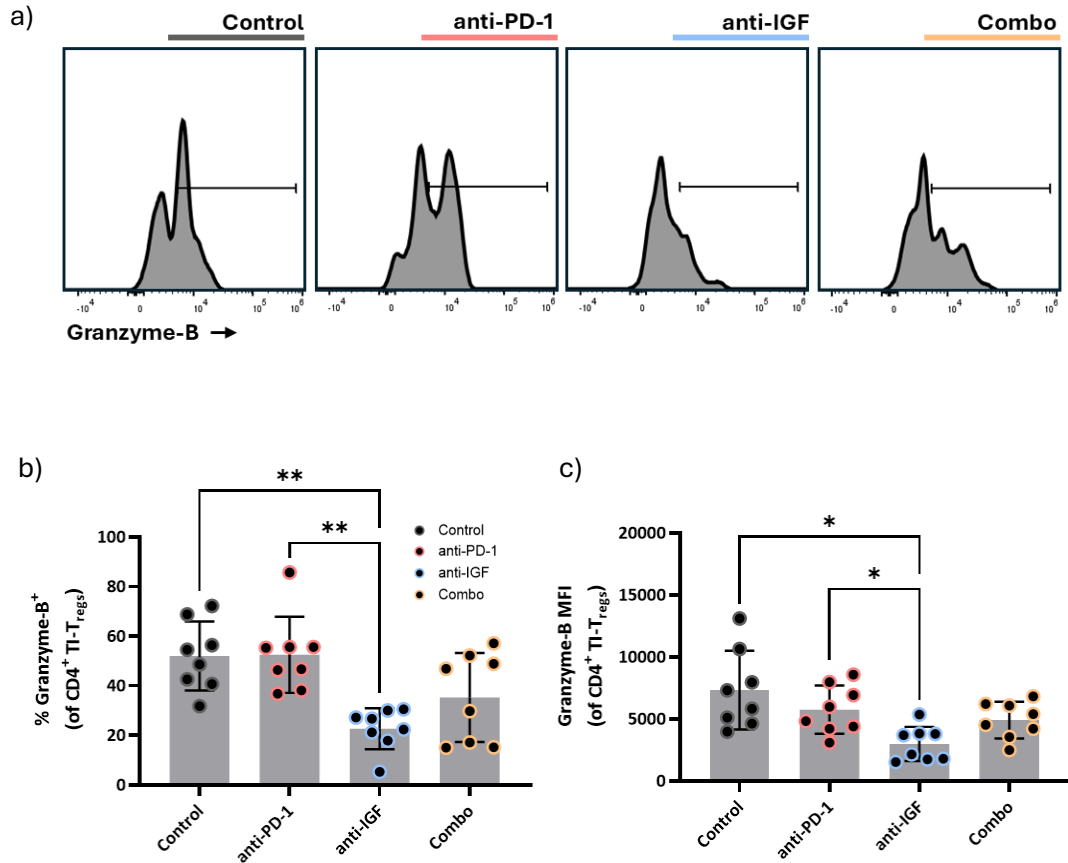


Figure 5.4.3.3 - IGF blockade inhibits Granzyme-B expression in TI-Tregs

a) Representative histograms of Granzyme-B staining in TI-Tregs.

b) Quantification of the percentage of TI-Tregs staining positive for Granzyme-B.

c) Quantification of the median fluorescence intensity of Granzyme-B expression in TI-Tregs

[(b-c) One way ANOVA with multiple comparisons, N = 8, bars represent means $\pm$ SD.]

A challenging paradigm of IGF immunology is the apparent divergent regulation of both T_H17 and Treg function in murine models of autoimmunity (366,383,384). Yet whether IGFs also contribute to tumour-infiltrating T_H17 differentiation and function *in vivo* is not yet known. Consequently, the effect of IGF blockade on IL-17a production, an effector pro-inflammatory cytokine produced by T_H17 cells, was investigated. Figure 5.4.3.4a shows representative dot plots showing relevant gating strategies used to define CD4⁺, T_H17 cells (IFN γ IL-17a⁺) or T_H1 (IFN γ ⁺ IL-17a⁻) cells. Treatment with anti-PD-1 or Xentuzumab did not significantly impact the percentage of T_H1 cells (figure 5.4.3.4b). However, Xentuzumab monotherapy significantly reduced the frequency of tumour-infiltrating T_H17 cells, with a ~5-fold decrease in T_H17 cells relative to treatment with PBS, anti-PD-1 monotherapy or combination treatment. Treatment with Xentuzumab or anti-PD-1 did not have a significant impact on the percentage of FoxP3⁺ Tregs that were T_R17 cells (IFN γ IL-17a⁺) or T_R1 (IFN γ ⁺ IL-17a⁻)-like (figure 5.4.3.4c), although a similar trend was observed towards a decrease in T_R17 cells (IFN γ IL-17a⁺) with Xentuzumab treatment.

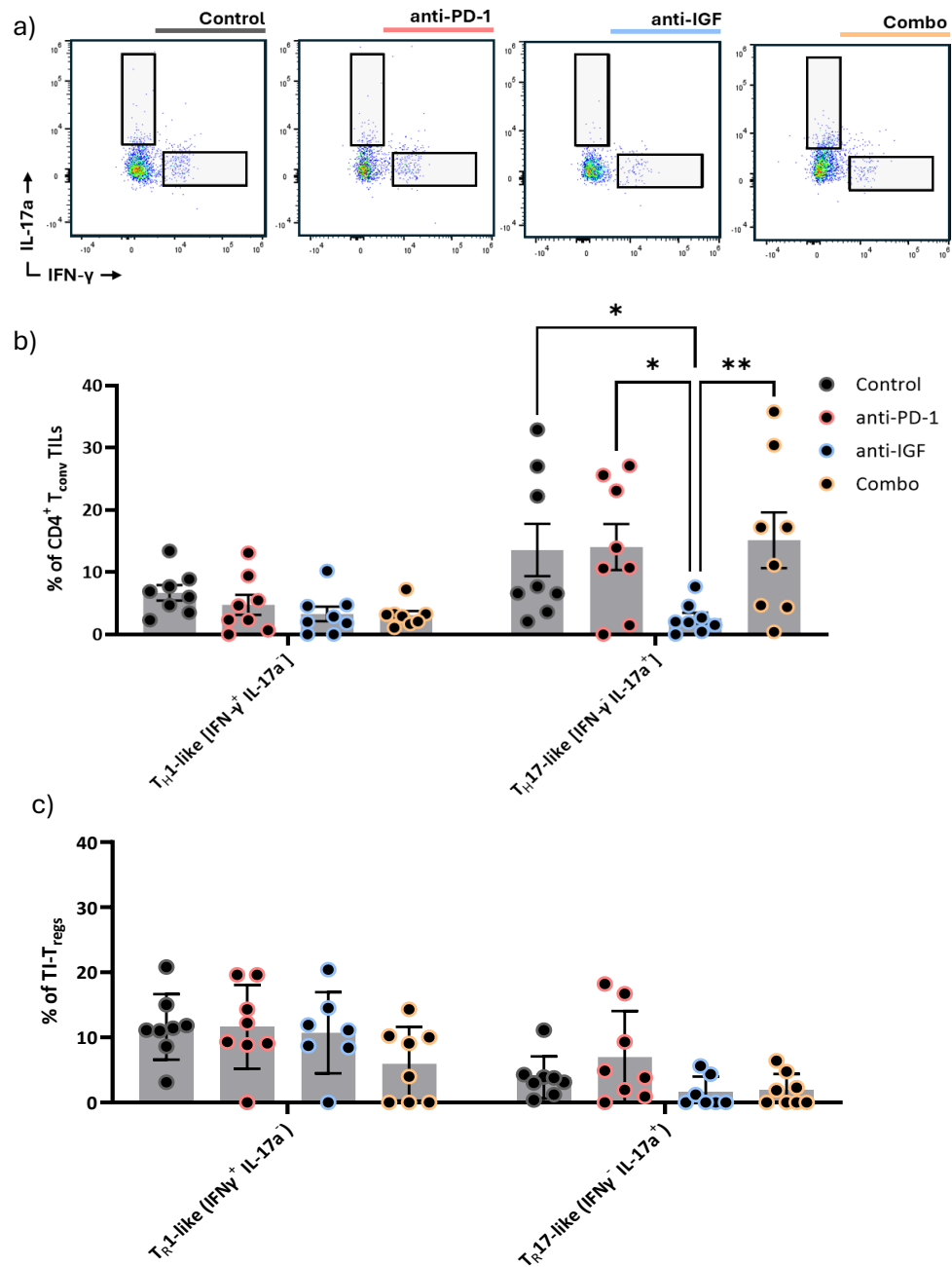


Figure 5.4.3.4 - IGF blockade inhibits TI-TH17 infiltration

a) Representative flow cytometry dot plots and gating strategy for of IL-17a⁺ IFN-γ⁻ and IL-17a⁻ IFN-γ⁺ cells in FoxP3⁻ CD4⁺ TILs.

b) Quantification of the percentage of FoxP3⁻ CD4⁺ TILs or (c) TI-Tregs co-staining for IL-17a⁺ IFN-γ⁻ or IL-17a⁻ IFN-γ⁺.

[(b-c) Two-way ANOVA, N = 8 , bars represent means ± SD.]

5.4.4 Immunophenotyping of TI-CD8⁺ CTLs

The cytotoxic CD8⁺ T-cell response is an important determinant of response to anti-PD-1 ICB (136). Whilst there were not a significant change in total CD8⁺ T-cell infiltrate, the functional status of CD8⁺ TILs was explored by staining for PD-1, CTLA-4, IFN- γ and Granzyme-B. Treatment with Xentuzumab or anti-PD-1 did not impact the percentage of CD8⁺ TILs expressing PD-1 or IFN- γ (appendix fig. 8.1.3.3h-i). However, expression of CTLA-4 in TI-CD8⁺ TILs significantly increased in mice treated with a combination of anti-PD-1 and Xentuzumab (fig. 5.4.4.1a-b). Conversely, CD8⁺ TILs from combination treated mice displayed a more cytotoxic phenotype, with enhanced Granzyme-B expression (figure 5.4.4.1c-d). In human PCa, CD8⁺ CTLA4⁺ TILs produce high levels of IL-35, and are functionally immunosuppressive *ex vivo* (274). Thus, the expansion of CD8⁺ CTLA-4⁺ TILs in MyC-CaP allografts could be representative of a mechanism of acquired resistance mediated by the *de novo* conversion of CD8⁺ TILs into cells with regulatory function.

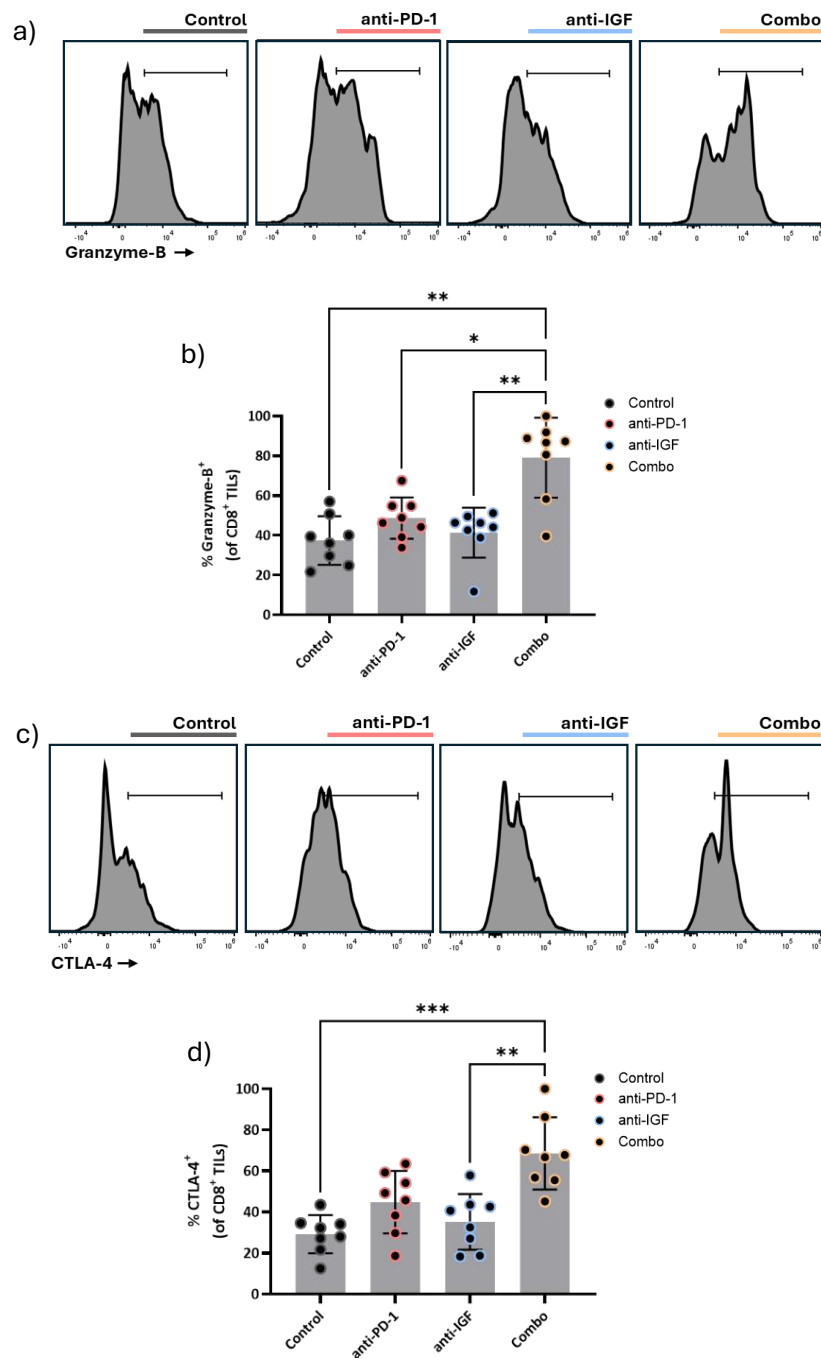


Figure 5.4.4.1 – IGF blockade and anti-PD-1 combination treatment upregulates Granzyme-B and CTLA-4 expression in CD8⁺ TILs.

a) Representative histograms of Granzyme-B staining in CD8⁺ TILs

b) Quantification of the percentage of CD8⁺ TILs staining positive for Granzyme-B.

c) Representative histograms of CTLA-4 staining in CD8⁺ TILs

d) Quantification of the percentage of CD8⁺ TILs staining positive for CTLA-4.

[(b, d) One-way ANOVA with multiple comparisons, N = 8, bars represent means ± SD.]

5.4.5 Peripheral T-cell immunity

Secondary lymphoid organs (SLOs) such as the spleen and tumour-draining lymph nodes (TDLN) have been recognised as important immunological sites for the expansion of tumour-reactive T-cells (259). TAA specific T-cells primed by APCs within SLOs transition through the peripheral blood, prior to trafficking to and infiltrating tumours (13,198). Whilst practical experimental constraints did not allow for the reliable isolation of sufficient numbers of TDLNs, we did investigate the peripheral immune response of T-cell subsets ($CD8^+$, $CD4^+$ T_{conv} , $CD4^+$ Treg, $CD4^+$ T_{FH} and T_{FR} cells). At the experimental endpoint, peripheral blood was collected by cardiac puncture and spleens were extracted for immunophenotyping by flow cytometry. Follicular $CD4^+$ subset markers were selected for flow cytometry analysis for two reasons; first, peripheral $CD8^+$ / $CD4^+$ T_{FH} crosstalk can restore $CD8^+$ dependent anti-tumour immunity to anti-PD-1/PD-L1 blockade (507), and second, mTOR signalling regulates their differentiation and function *in vivo* (508).

First, the abundance of peripheral blood and splenic $CD4^+$ T_{conv} cells was investigated (figure 5.4.5.1a). IGF blockade induced a significant expansion of both peripheral blood and splenic central memory $CD4^+$ T_{conv} cells (figure 5.4.5.1b), with a concomitant decrease in naïve $CD4^+$ T_{conv} cells ($CD62L^+ CD44^-$) (appendix fig. 8.1.3.4a). Similarly, IGF blockade promoted the expansion of peripheral blood and splenic effector/effector memory $CD8^+$ T-cells ($CD62L^- CD44^+$) [figure 5.4.5.1c]. In contrast, the differentiation status of T_{FH} , T_{reg} or T_{FR} subsets did not change after treatment with anti-PD-1, nor Xentuzumab (appendix fig. 8.1.3.4b).

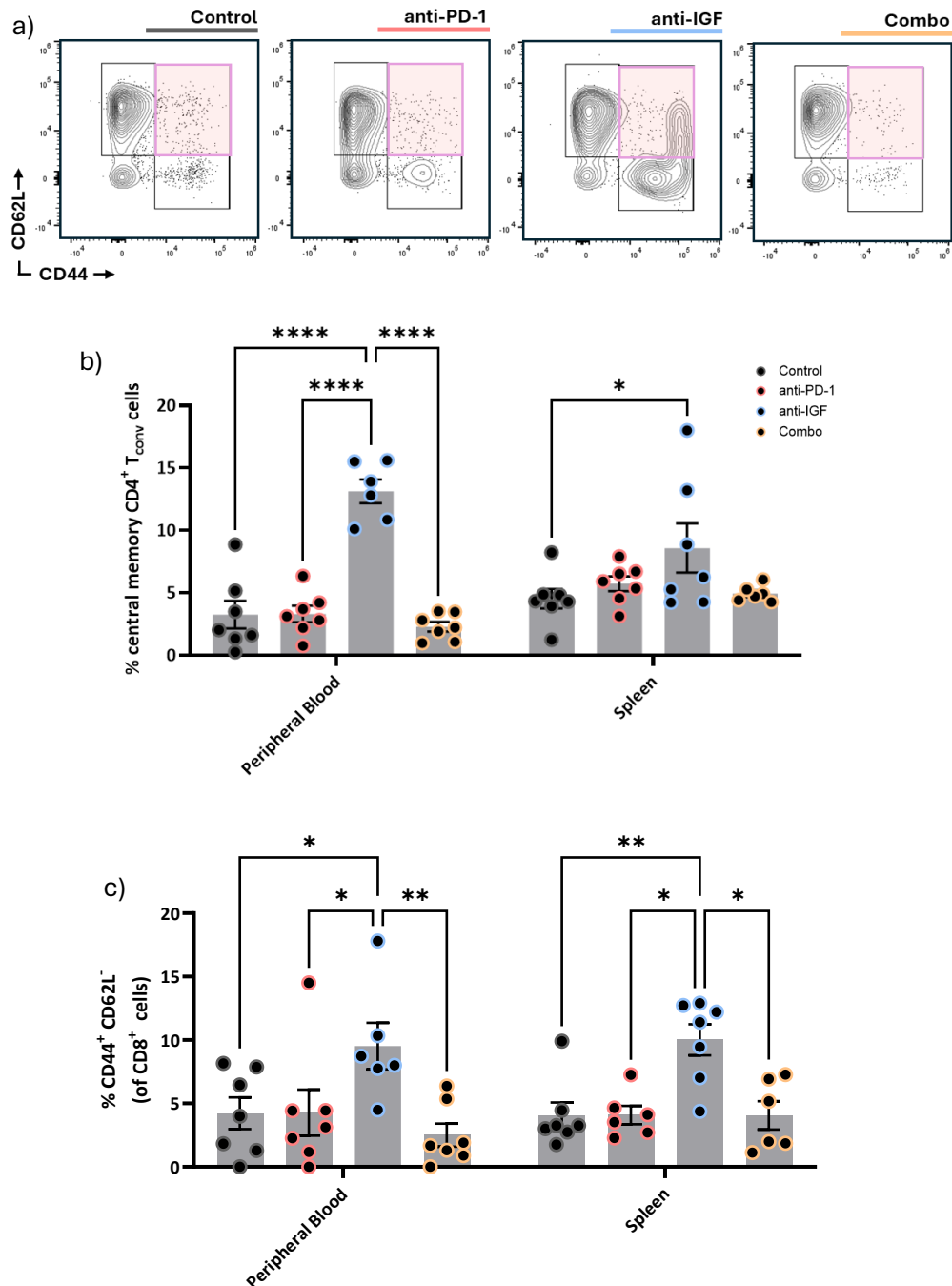


Figure 5.4.5.1 - IGF blockade promotes the expansion of peripheral central memory CD4⁺ T-cells and effector/effector memory CD8⁺ T-cells

a) Representative flow cytometry contour plots of CD44 and CD62L staining in FoxP3⁻ CD4⁺ Tconv cells in the peripheral blood or spleen of tumour bearing mice after treatment with PBS, anti-PD-1, Xentuzumab or combination treatment.

b) Quantification of the percentage of FoxP3⁻ CD4⁺ Tconv cells isolated from the peripheral blood and spleen that are central memory cells (CD44⁺ CD62L⁺).

c) Quantification of the percentage of CD8⁺ T-cells isolated from the peripheral blood that are effector/effector memory cells (CD44⁺ CD62L⁻).

[(b-c) One-way ANOVA with multiple comparisons, N = 6, bars represent means $\pm$ SD.]

We next considered the possibility that the expansion and differentiation of memory CD8⁺ and CD4⁺ Tconv cells in response to Xentuzumab could be indicative of reduced Treg suppressive function, generating an environment more conducive to T-cell activation and effector cell differentiation. However, upon evaluation of the frequency of Tregs, we found Xentuzumab enhanced the frequency of splenic Tregs, but not Tregs in the peripheral blood (figures 5.4.5.2a-b). Furthermore, splenic Tregs maintained expression of PD-1, inducible T-cell co-stimulator (ICOS), CD44 and CD25, indicative of retention of their functional status (appendix fig. 8.1.3.5a-c).

Neither Xentuzumab nor anti-PD-1 (monotherapy or combination treatment) had a significant impact on the frequency of total splenic or peripheral blood T_{FH} cells, nor T_{FH} cells responsive to IL-2 signalling (appendix fig. 8.1.3.5d). Intriguingly, IGF blockade induced the expansion of ICOS⁺ T_{FH} cells in both the peripheral blood and spleen relative to treatment with PBS (figure 5.4.5.3a). Expression of ICOS, a co-stimulatory receptor, promotes functional differentiation of T_{FH} cells through high affinity binding to ICOS-ligand [ICOSL] expressed by B-cells (509). Thus, a consequence of IGF blockade could be the potentiation of peripheral T_{FH} and B-cell interactions through the ICOS-ICOSL axis.

Humoral immunity mediated by T_{FH} cells is negatively regulated by T_{FR} cells, which function to regulate foreign/autoantigen antibody responses within germinal centres (216). Hence, we next wanted to investigate whether the increase in ICOS expression by T_{FH} cells correlated with a reduction of T_{FR} cells as a percentage of CD4⁺ T-cells in the peripheral blood or spleen. Figure 5.4.5.3b displays the bi-axial gating strategy used to identify T_{FR} cells in the spleens and peripheral blood of tumour bearing

mice. In contrast with our hypothesis, there was an expansion of splenic T_{FR} cells in mice treated with Xentuzumab, but not in the peripheral blood (figure 5.4.5.3c). Of note, due to the rarity of circulating T_{FR} (cT_{FR}) cells in the peripheral blood and limited blood sampling, statistical comparisons made here may not accurately reflect the underlying immunology due to a low frequency of detectable T_{FR} events.

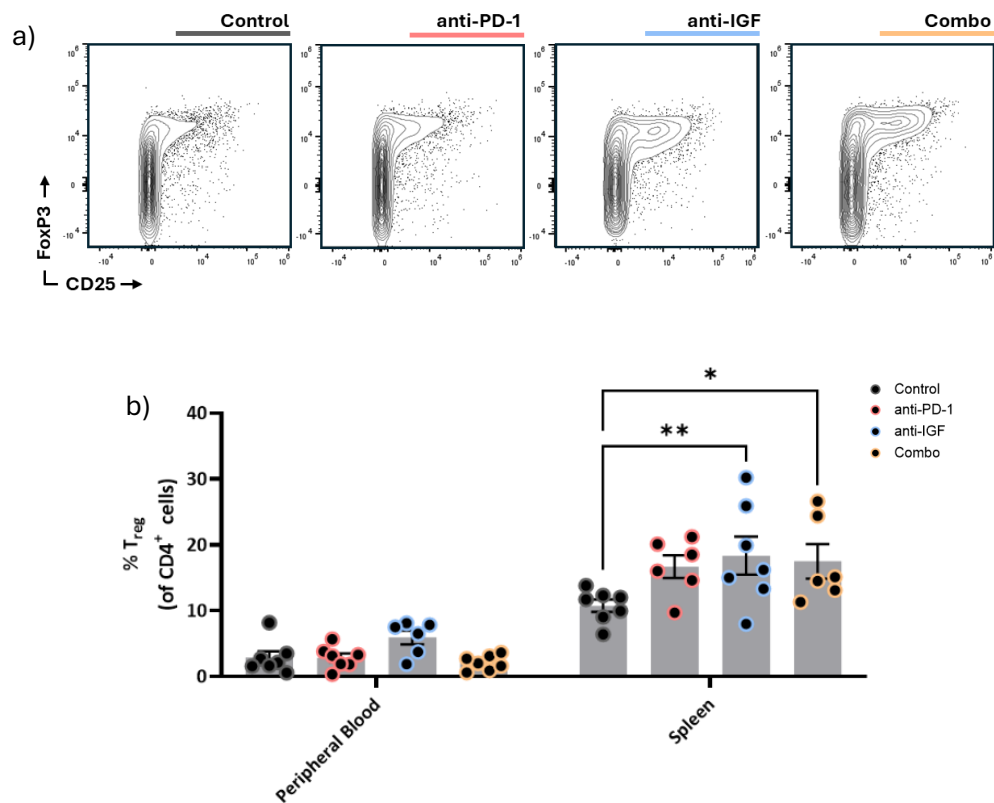


Figure 5.4.5.2 - IGF blockade increases the frequency of splenic Tregs

a) Representative flow cytometry contour plots of FoxP3 and CD25 staining in CD4⁺ T-cells in the peripheral blood or spleen of tumour bearing mice after treatment with PBS, anti-PD-1, Xentuzumab or combination treatment.

b) Quantification of the percentage of CD4⁺ cells that are FoxP3⁺ in cells isolated from the peripheral blood, or the spleen.

[(b-c) Two-way ANOVA, N = 6, bars represent means $\pm$ SD.]

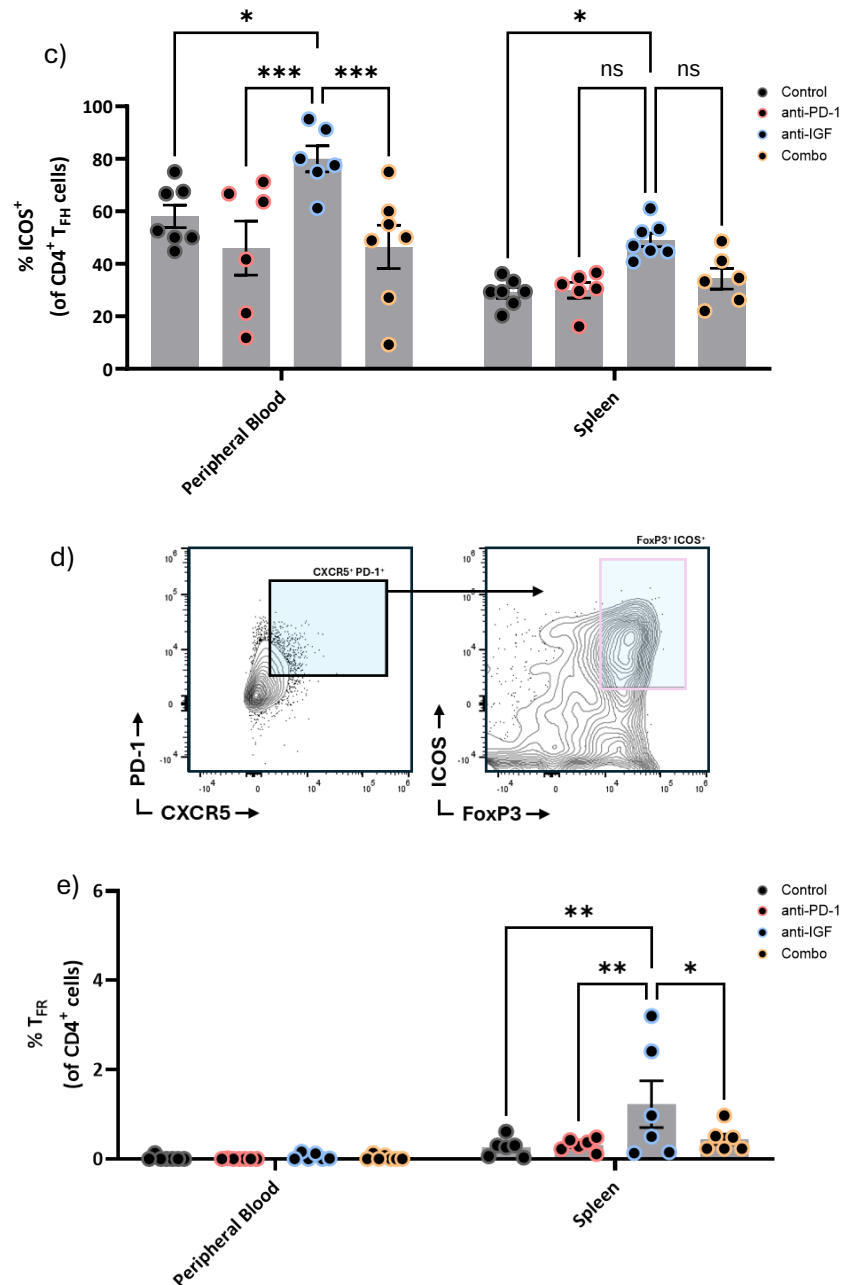


Figure 5.4.5.3 - IGF blockade promotes ICOS expression in peripheral follicular helper cells and expands splenic follicular Treg cells

a) Quantification of the percentage of follicular helper cells that are ICOS⁺ in cells isolated from the peripheral blood, or the spleen.

b) Contour plots showing the gating strategy for identification of follicular regulatory T-cells (defined as PD-1⁺, CXCR5⁺, ICOS⁺, FoxP3⁺)

c) Quantification of the percentage of CD4⁺ T-cells isolated from the peripheral blood, or the spleen, that are follicular regulatory T-cells.

[(a,c) Two-way ANOVA, N = 6, bars represent means $\pm$ SD.]

5.4.6 Investigating the relationship between IGF-1R expression and T-cell function *in vivo*.

Finally, to explore whether the differential sensitivity of CD4⁺ T-cell subsets isolated from the tumour, peripheral blood and spleen related to the relative expression of IGF receptors, we compared IGF-1R expression in T-cells isolated from different sites in PBS treated tumour bearing mice (figure 5.5.1.1a). There was no difference in IGF-1R expression in Tconv cells isolated from the spleen, peripheral blood or tumour, IGF-1R. Conversely, IGF-1R expression was differentially upregulated in TI-Tregs relative to splenic Tregs (figure 5.5.1.1b), which could be indicative of a greater dependency or sensitivity to IGF signalling of Treg cells within the TME.

Finally, the phenotype of IGF-1R⁺ TI-Tregs from MyC-CaP allografts was explored, by comparison with IGF-1R⁻ TI-Tregs from PBS treated mice. IGF receptor expressing Tregs displayed a highly activated phenotype, with increased CTLA-4, PD-1, CD25 but not granzyme-B expression (figure 5.5.1.1c). Hence, the expression of IGF receptors by Tregs within tumours correlates with markers of suppressive function, consistent with observations from murine models of hepatocellular carcinoma (386).

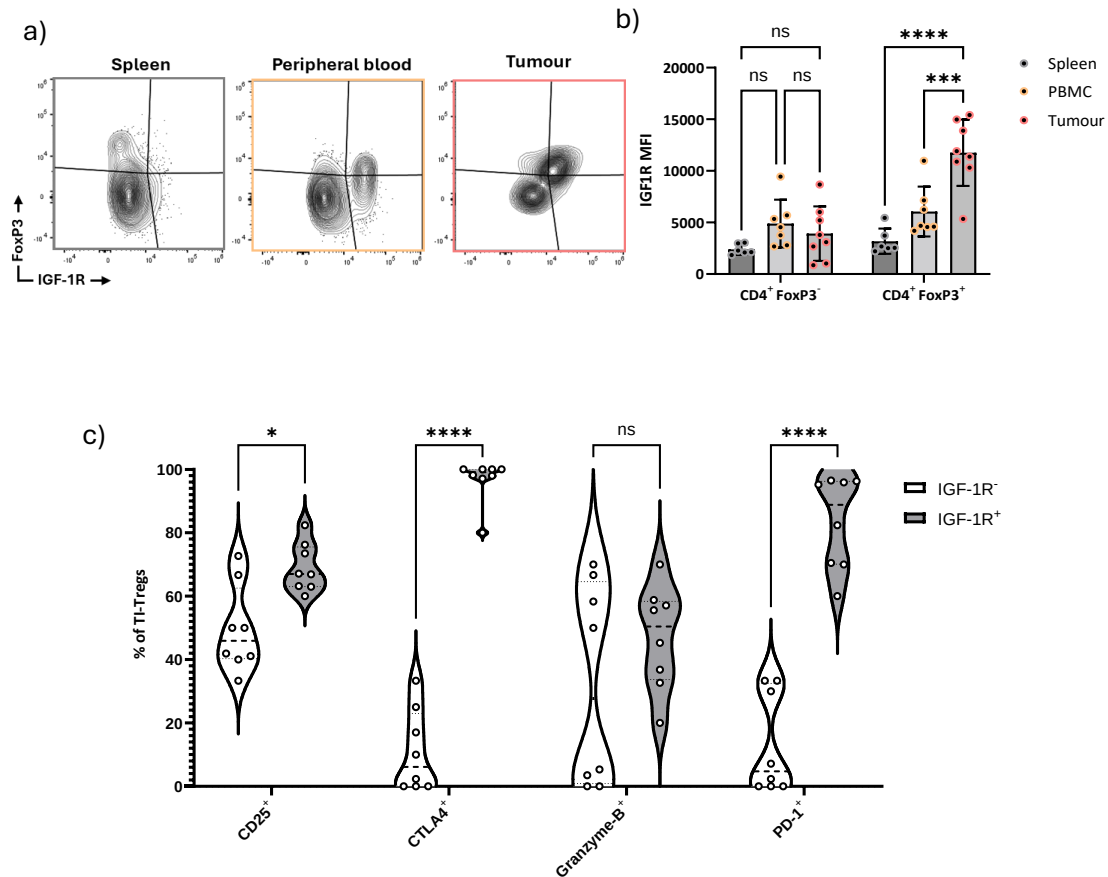


Figure 5.4.6.1 - IGF-1R is upregulated by TI-Tregs , and IGF-1R⁺ TI-Tregs display a highly activated phenotype.

a) Representative flow cytometry contour plots of IGF-1R and FoxP3 expression in CD4⁺ T-cells isolated from the peripheral blood, spleen or tumours of MyC-CaP tumour bearing mice treated with PBS.

b) Quantification of IGF-1R expression in FoxP3⁺ or FoxP3⁻ CD4⁺ T-cells isolated from the peripheral blood, spleen and MyC-CaP tumours treated with PBS.

c) Quantification of the percentage of IGF-1R⁻ or IGF-1R⁺ TI-Tregs from PBS treated mice staining positive for CD25, CTLA-4, Granzyme-B or PD-1.

[(b,c) Two-way ANOVA, (peripheral blood, spleen - N = 6, tumour - N = 8) bars represent means ± SD.]

5.5 Discussion

Although immune checkpoint blockade has shown significant clinical promise in other cancer types, evidence for durable antitumour effects is limited in patients with advanced PCa (132–134). Using a syngeneic and c-MYC driven model of murine prostate cancer (MyC-CaP), the effects of IGF blockade with anti-PD-1 immunotherapy was investigated. Consistent with results from recent human trial of both breast and prostate cancers (359,360), xentuzumab treatment did not induce significant anti-tumour effects as a monotherapy, nor when combined with anti-PD-1. This lack of therapeutic efficacy is also largely consistent with a failure of combinatorial treatments to overcome resistance to anti-PD-1 in MyC-CaP allografts (501–504).

The timing, not the dosing frequency, appears essential for demonstrable anti-tumour effects of anti-PD-1 in the MyC-CaP model of PCa. For example; *Serganova et al.* do show evidence of anti-tumour effects of anti-PD-1 when administered as a monotherapy to MyC-CaP allografts (510). These findings were seemingly inconsistent with observations from other groups (501–503), and those from this study. However, *Serganova et al.* noted that anti-PD-1 only remains efficacious in the MyC-CaP model if treatment is initiated at a tumour volume less than 50 mm³, unlike other groups that initiate treatment at a tumour volume of 100 mm³. Although speculation, this may relate to the relative phase of the immune infiltrate; whilst early murine MYC-driven PCa lesions are associated with pro-inflammatory programs, this is closely followed by a sustained immunosuppressive state driven by the recruitment of TREM2 macrophages, myeloid-derived suppressor cells and Tregs (145). Hence, the efficacy of

anti-PD-1 administered to MyC-CaP tumours at 50 mm³ could be representative of an early-stage MYC activated PCa TME, with an immune infiltrate more permissible to treatment with anti-PD-1. Nevertheless, these data reflect a challenging paradigm of preclinical prostate cancer research. Whilst demonstrable anti-tumour immunity in response to ICB is desirable in preclinical research models, this does not accurately represent the clinical context of ICB, under evaluation in advanced and metastatic PCa (511).

The principle aim of this study was not necessarily to evaluate whether blockade of the IGF axis was synergistic with anti-PD-1, as this has been shown in other tumour models (395,401,402,409). Rather, a main aim of this chapter was to evaluate if IGF blockade could evoke treatment sensitivity to immunotherapy in prostate cancer, an immunologically challenging model with a TME with a sparse CTL infiltrate that remains at the periphery of allografts (498). Whilst IGF blockade did initially increase CD8⁺ CTL infiltration early in tumour development, at later stages of tumour growth this was lost. Hence, IGF inhibition appears to be an insufficient immunological stimulus for the effective generation anti-tumour immune responses against c-MYC driven prostate cancer and is unlikely to improve the effectiveness of anti-PD-1 immunotherapy at doses that are safe or well tolerated. Nonetheless, future work could utilize other allograft or orthotopic mouse models of murine PCa to explore whether the absence of therapeutic effect is specific to immune microenvironments formed in MYC-amplified murine PCa, or more generalisable to the immunosuppressive microenvironment and low-neoantigen burden of PCa (410).

Conceptually, our findings do support an immunoregulatory role of IGFs in the formation of T-cell identity within the PCa tumour-immune microenvironment and peripheral immunological sites. The effects of IGF blockade on T-cell subsets also appears tissue-specific and dependent on their localization. For example, Xentuzumab treatment depletes TI-Tregs, but promotes splenic Treg expansion. Whilst the underlying mechanism of this tissue specific effect remains unclear, the concomitant expansion of peripheral memory CD4⁺, effector/effector memory CD8⁺ T-cells and ICOS⁺ T_{FH} cells could be indicative of an adaptive Treg response in the spleen to protect mice from therapy-induced immune activation and speculatively, protection against the loss of self-tolerance (390). Strikingly, IGF blockade reduced the abundance of both tumour-infiltrating Treg and T_H17 cell subsets *in vivo*. Although speculation, it seems plausible that levels of IGF-1 bioactivity within tumours may indicate a physiological state to CD4⁺ T-cells that are integrated alongside co-factors that allow for the fine-tuning of both tumour-infiltrating Treg (FoxP3⁺) and T_H17 (RAR-related orphan receptor gamma, RORγ⁺) responses through lipogenic metabolism and mTORC1 (231,241,242,512). Whilst beyond the scope of this study, the existence of stable and suppressive FoxP3⁺ RORγ⁺ pTreg cells *in vivo* raises an interesting question as to whether IGF-1 bioactivity could promote the development of FoxP3⁺ RORγ⁺ suppressive Tregs described in colorectal cancer (513). Due to time constraints, we could not assess mTORC1 activity, lipogenic metabolism nor the relative activation of cellular stress (related to *in vitro* observations from chapters 3-4). Further exploration of these pathways in relation to both tumour-infiltrating Treg and T_H17 cells in response to IGF blockade remains an important direction for future work.

Nevertheless, observations that Xentuzumab promotes the expansion of memory CD4⁺ T-cells in the peripheral blood *in vivo* and ICOS⁺ follicular helper cells, which play a supportive role in the initiation of anti-tumour immunity, guided investigations into how IGF inhibition could impact the peripheral anti-cancer immune response in men with prostate cancer.

Chapter 6 – Investigating how IGF blockade shapes the anti-cancer immune response in men with prostate cancer

6.1 Background

There is significant clinical interest in the identification of treatment protocols that enhance anti-tumour immune responses (511). One possibility includes fasting-mimicking dietary interventions that promote both systemic and intratumoural anti-tumour immunity from anti-neoplastic treatments through a concomitant depletion of peripheral blood MDSCs and Tregs, whilst boosting peripheral blood and tumour-infiltrating CD8⁺ CTLs and Tregs (403). Key changes in metabolites and blood hormones induced by FMD include reduced serum levels of leptin, insulin, IGF-1 and blood glucose. However, it is currently not known if reductions in IGF-1 represent a passive reflection of a fasted-state, or are essential to the anti-tumour immune response induced by FMD. Hence, a primary aim of this chapter is to explore how decreased IGF levels contribute to changes in peripheral immunity in men with localized PCa, guiding further optimisation of FMD protocols.

To test this, we analysed patient samples from the Windows trial of INsulin-like Growth factor neutralising antibody Xentuzumab in MEN scheduled for radical prostatectomy (WINGMEN trial), a trial for men with localized PCa scheduled for radical prostatectomy [NCT05110495]. The investigational medical product Xentuzumab (Boehringer Ingelheim), is an IGF neutralising antibody. The primary

endpoint of the trial is to measure the amount of IGF axis pathway inhibition in tumours induced by Xentuzumab, by comparing pre-treatment tumour biopsies with post-treatment samples after prostatectomy. A tertiary trial endpoint will also be investigated; to assess whether the phenotype of peripheral blood mononuclear cells (PBMC) can be used as a liquid biomarker of decreased IGF axis activity.

6.2 Chapter aims

Accordingly, there are two principal aims of this chapter. First, to investigate if IGF inhibition influences the frequency or phenotype of peripheral blood immune cells in patients with localized PCa. Second, explore if immunophenotyping can be used to predict patients more likely to experience a decrease in PSA score, indicative of anti-tumour effects.

6.3 Development of an immune-profiling pipeline for analysis of PBMC's using mass cytometry

6.3.1 Considerations for analysis of CyTOF data

A challenge of analysing mass cytometry data is that valuable immunological complexity can be lost upon bi-axial analysis with Boolean gating strategies alone (514). As a result, an analysis pipeline was generated that utilized both conventional manual gating strategies and unsupervised clustering analysis (figure 6.3.1.1a). A wide variety of both semi-supervised and unsupervised clustering methods exist, including Accense, DEPECHE, PhenoGraph, flowmeans, FlowSOM, X-Shift and Kmeans (515). Based on comprehensive performance comparisons of CyTOF analysis methodologies by *Liu et al.*, we opted for one of the top performing tools, FlowSOM, for our analysis. However, one limitation of FlowSOM is that it requires the number of clusters to be pre-determined by the user, which is important because an over/underestimation of clusters can lead to a loss of biologically relevant immune populations. For these reasons, we also utilized X-Shift, the tool with the best accuracy for unsupervised cluster definition, to first identify and generate an optimal number of clusters, used as an input for FlowSOM clustering. Marker enrichment modelling (MEM) was used throughout to quantify the magnitude of marker expression (on a -10 to +10 scale) to inform the labelling of cell-type/subset identified by FlowSOM (418). t-distributed stochastic neighbour embedding (t-SNE) is used for dimensionality reduction, which converts high dimensional cytometry data into a two-dimensional map (516). Within t-SNE plots, the distance of cells in two-dimensional space represents their relative immunological similarity. Thus, tSNE plots are utilized in this study to aid with the

visualization of immunological similarity of clusters identified by FlowSOM, shown in figure 6.3.1.1b (517).

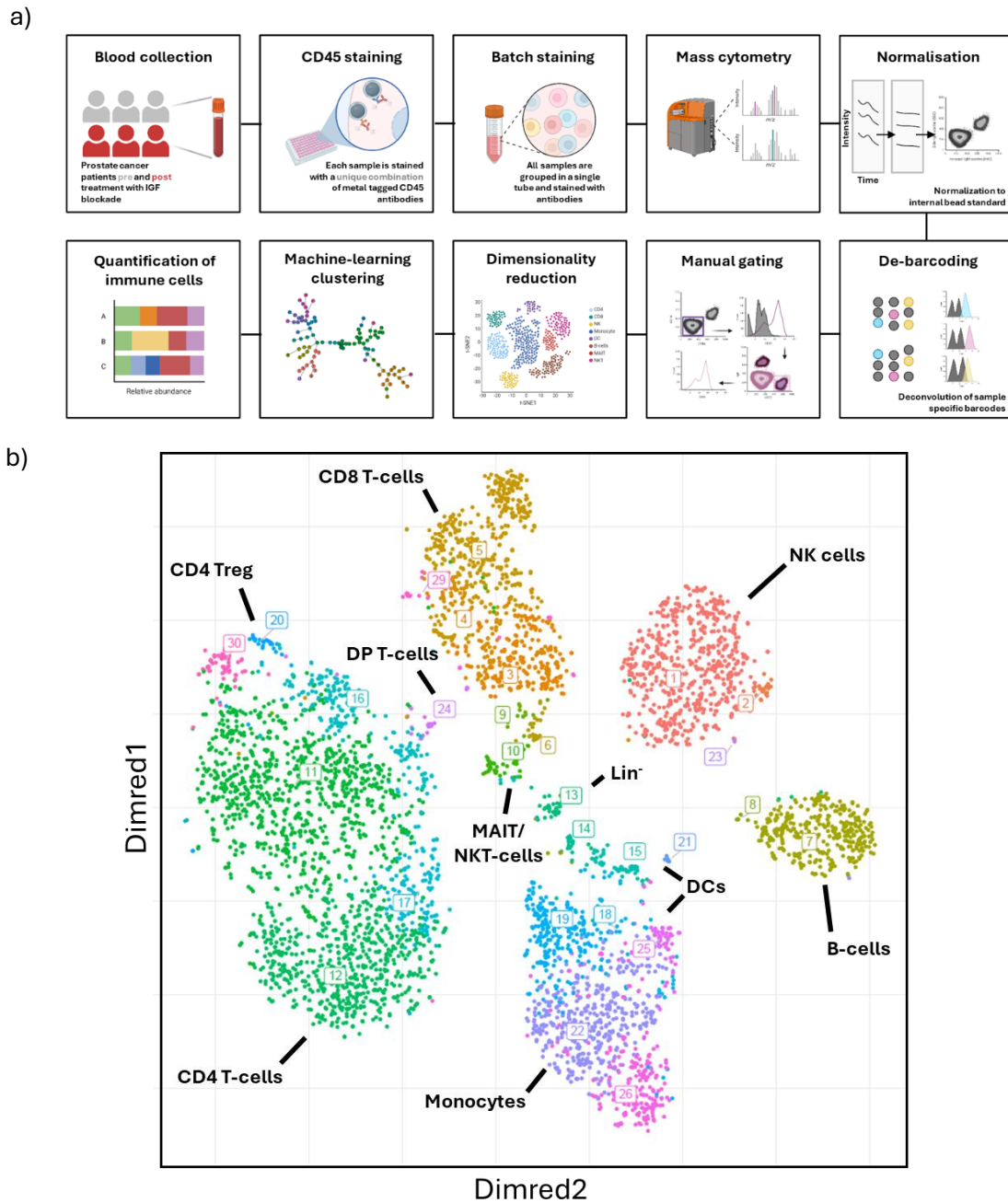


Figure 6.3.1.1 - Analysis pipeline for PCa patient PBMcs by mass cytometry

(a) Workflow of sample handling and analysis: PBMcs were isolated from whole blood collection samples from prostate cancer patients recruited to the WINGMEN trial. Individual samples were thawed and stained with a CD45 barcode, and all 52 samples were stained together with a total of 36 metal tagged antibodies. PBMcs were atomized and analysed by mass cytometry in a single batch. Expression intensity was normalized over time by using an internal bead control, and individual FCS files were generated after de-barcoding. Manually labelled cytometry data was used to feed high-dimensional analysis techniques and automatically identify immune cell populations for quantification.

b) Representative tSNE plot of pre-treatment CD45⁺ immune cell subsets defined by FlowSOM clustering (perplexity = 30, cells = 5000).

6.4 Immunophenotyping of PCa patient PBMCs before and after treatment with Xentuzumab

6.4.1 CD45⁺ cells

To test if IGF blockade shaped peripheral immune lineage development, patient samples were first interrogated by manual gating. Intriguingly, neutralization of IGFs significantly increased the levels of circulating CD8⁺ T-cells, but had no effect on other major immune lineages (figure 6.4.1.1a). A before and after plot is shown in figure 6.4.1.1b to illustrate the consistency of the increase in CD8⁺ cells numbers relative to pre-treatment levels.

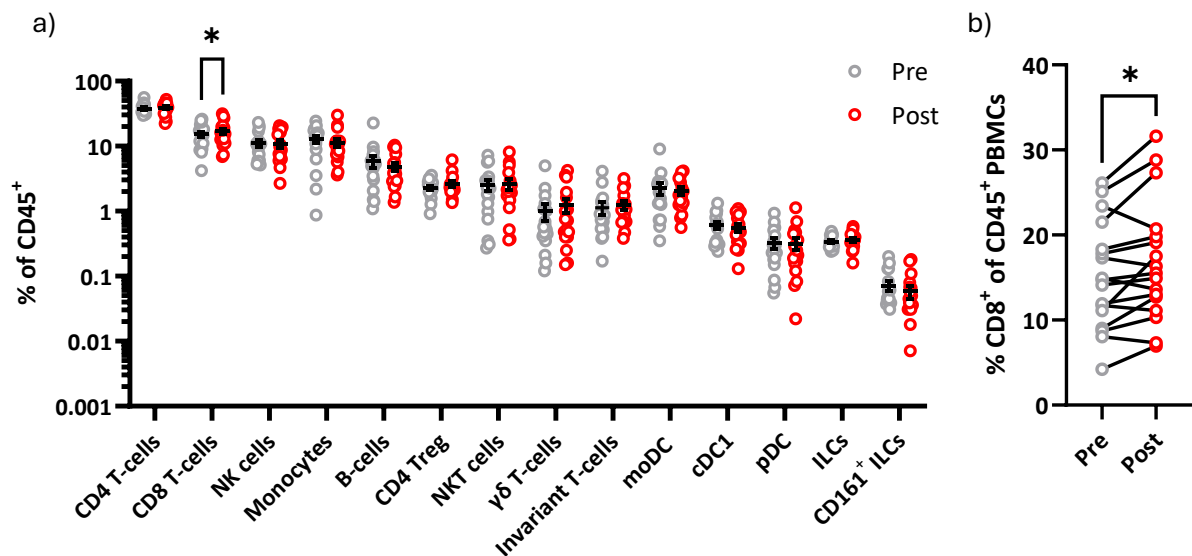


Figure 6.4.1.1 - Xentuzumab treatment increases the frequency of circulating CD8⁺ T-cells in patients with prostate cancer

- Frequency of CD4⁺ T-cells [CD3⁺, CD4⁺], CD8⁺ T-cells [CD3⁺, CD8⁺], NK cells [CD56⁺], monocytes [CD14⁺], B-cells [CD19⁺], NKT cells [CD3⁺, CD56⁺], $\gamma\delta$ T-cells [CD3⁺, $\gamma\delta$ TCR⁺], invariant-like T-cells [CD3⁺, CD161⁺], moDCs [HLA-DR⁺, CD11c⁺, CD1c⁺], cDC1s [HLA-DR⁺, CD11c⁺, CD141⁺], pDCs [HLA-DR⁺, CD11c⁺, CD123⁺], ILCs [Lineage⁻, CD127⁺] and CD161⁺ ILCs [Lineage⁻, CD127⁺, CD161⁺] as a percentage of live CD45⁺ cells in the peripheral blood of patients with prostate cancer before and after treatment with Xentuzumab.
- CD8⁺ T-cells as a percentage of CD45⁺ immune cells after treatment with Xentuzumab.

[* = $p < 0.05$, a) Wilcoxon signed-rank test b) paired T-test, N = 17, bars represent mean $\pm$ SEM.]

6.4.2 Unsupervised clustering of CD45⁺ cells

To supplement manual Boolean gating analysis, CD45⁺ cells were subsequently clustered by FlowSOM. Of the 30 distinct cell metaclusters (p1-p30) identified (figure 6.4.2.1), three populations significantly increased after treatment with Xentuzumab (figures 6.4.2.1a-b). Individual cluster cellular identity was then further investigated and explored using both marker enrichment modelling (MEM) and manual investigation by FlowJo. The first, population4 (p4), resembled an effector memory CD8⁺ T-cell subset (CD45RO⁺, CD27⁺) that co-expressed high levels of co-inhibitory receptors TIGIT and PD-1. The second, population5 (p5), identified a cluster of CD8⁺ T-cells that were a combination of naïve, stem cell memory and central memory CD8⁺ T-cells. Finally, population 10 (p10), expressed markers that resembled mucosal-associated invariant T-cells [MAIT] (CD3⁺, CD161⁺⁺) (518). Further resolution of this population revealed three distinct sub-populations of 1) CD161⁺⁺ CD8⁺ CD56⁻ MAIT-cells, 2) CD161⁺⁺ CD8⁺ CD56⁺ invariant-NKT cells and 3) CD161⁺⁺ CD56⁺ CD8⁻ NKT-cells. Thus, p10 is representative of an invariant T-cell population comprised of both NKT cells and MAIT-like cells. MAIT cells express semi-invariant $\alpha\beta$ T cell receptors and recognize major histocompatibility complex, class I-related (MR1) molecules, whilst invariant NKT (iNKT) cells recognize glycolipid antigens by cluster of differentiation 1 (CD1d). Taken together, these results confirmed observations from bi-axial analysis with respect to the expansion of CD8⁺ T-cells, but also revealed the expansion of iNKT and MAIT cells, immune subsets with high cytolytic potential and anti-tumour potency (519–521).

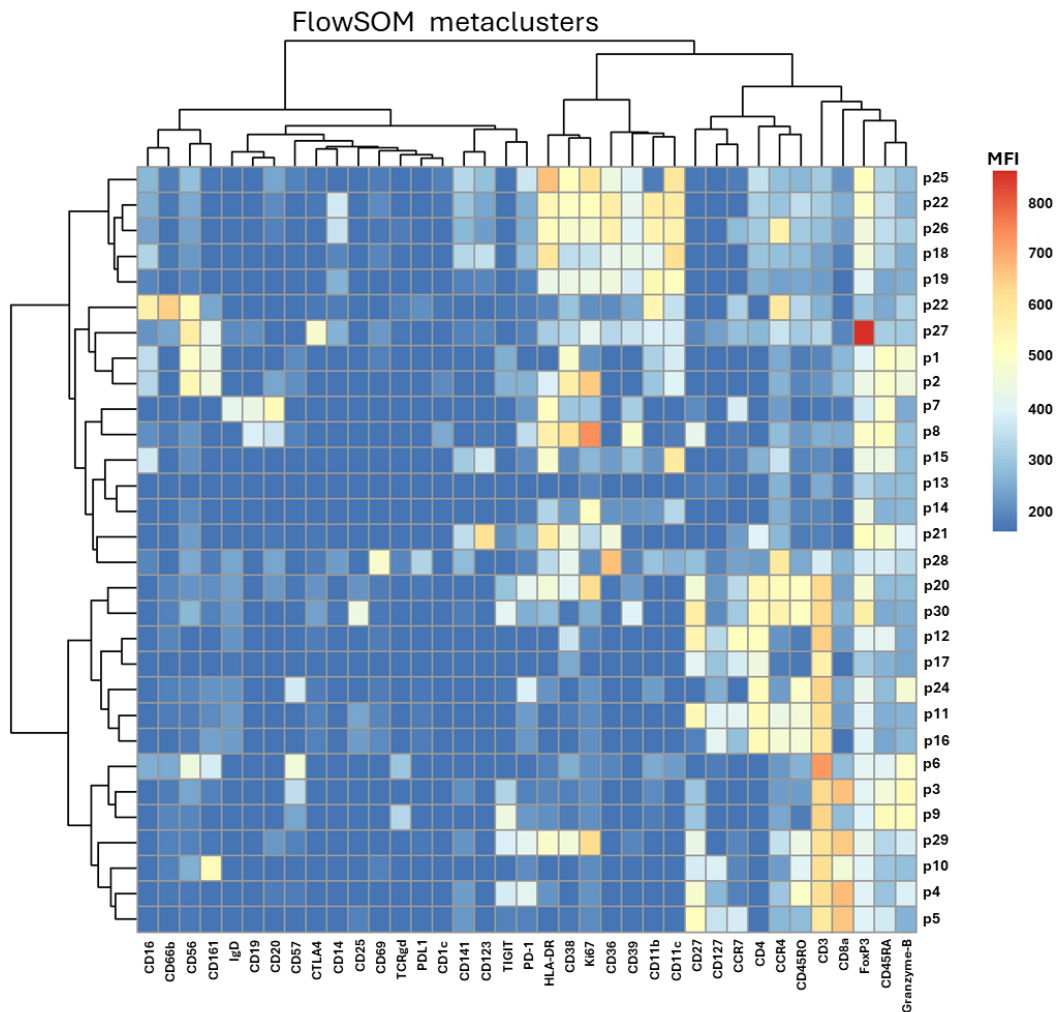
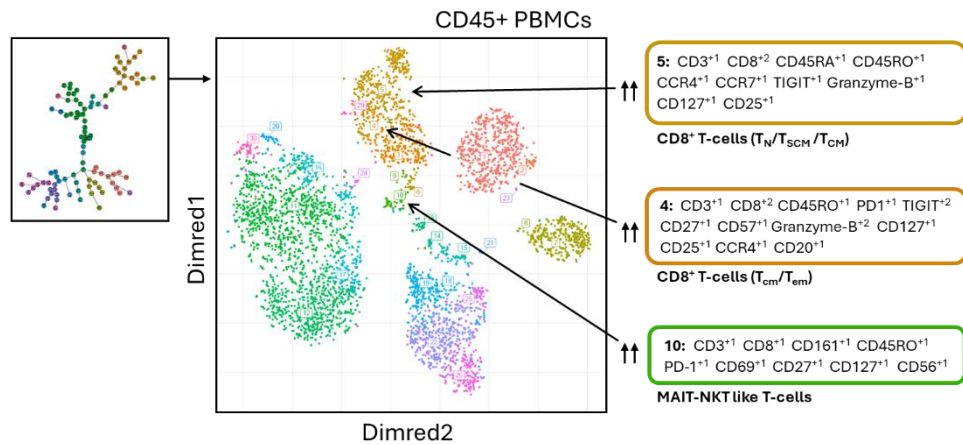


Figure 6.4.2.1 - Unsupervised clustering of CD45⁺ PBMCs from prostate cancer patients

A dead⁺, CD45⁺ gate was applied to each sample and positive cells were concatenated into a single data file. This was used for meta-clustering analysis by FlowSOM, which defined 30 distinct metaclusters (p1-30).

(a) FlowSOM metaclustering of CD45⁺ cells illustrated by a heatmap. Dendrograms present clustering of immune cells (rows) and markers (columns) which is based on hierarchical clustering. The median expression intensity for each marker within metaclusters is shown and colour coded.

a)



b)

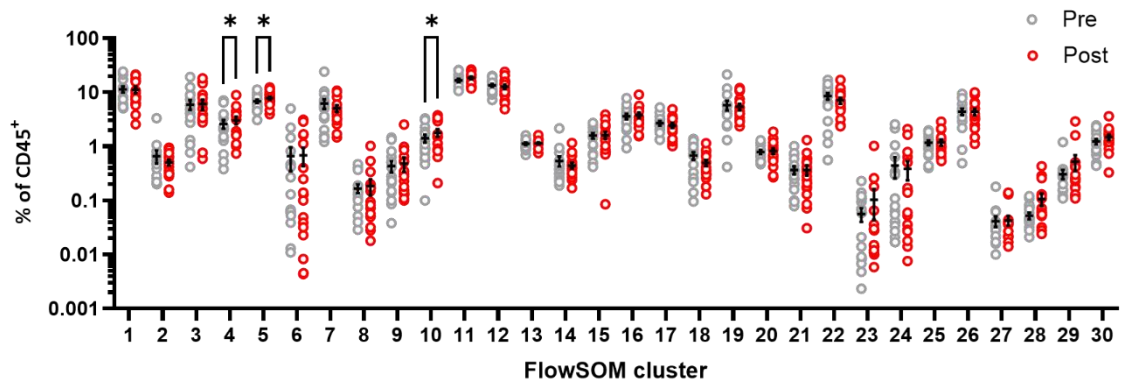


Figure 6.4.2.2 - Unsupervised clustering of CD45⁺ PBMCs from prostate cancer patients

a) Representative FlowSOM tree and tSNE plot after metaclustering of CD45⁺ cells. Defined metaclusters that significantly expand in response to Xentuzumab treatment are labelled with annotations from marker enrichment modelling, with arrows pointing to their corresponding location within the tSNE plot.

b) Quantification of the relative abundance of each FlowSOM metacluster as a percentage of CD45⁺ immune cells before and after treatment with Xentuzumab.

[* = $p < 0.05$, Wilcoxon signed-rank test, $N = 17$, bars represent mean $\pm$ SEM.]

6.4.3 Manual gating of total CD4⁺ T-cells

We next explored the effects of Xentuzumab treatment on circulating CD4⁺ T-cell populations, with representative sample gating used to identify CD4⁺ T-cell subsets shown in figure 6.4.3.1a. Whilst Xentuzumab treatment did not impact the frequency of total CD4⁺ T-cells as a percentage of CD45⁺ immune cells (figure 6.4.3.1b), it significantly reduced the number of naïve CD4⁺ T-cells. This corresponded with an expansion of effector memory CD4⁺ T-cells (fig. 6.4.3.1c-d). Consistent with changes in peripheral T-lymphocytes in preclinical studies of tumour bearing mice with PCa (chapter 5), we concluded that IGF blockade promotes peripheral memory CD4⁺ T-cell formation in human PCa.

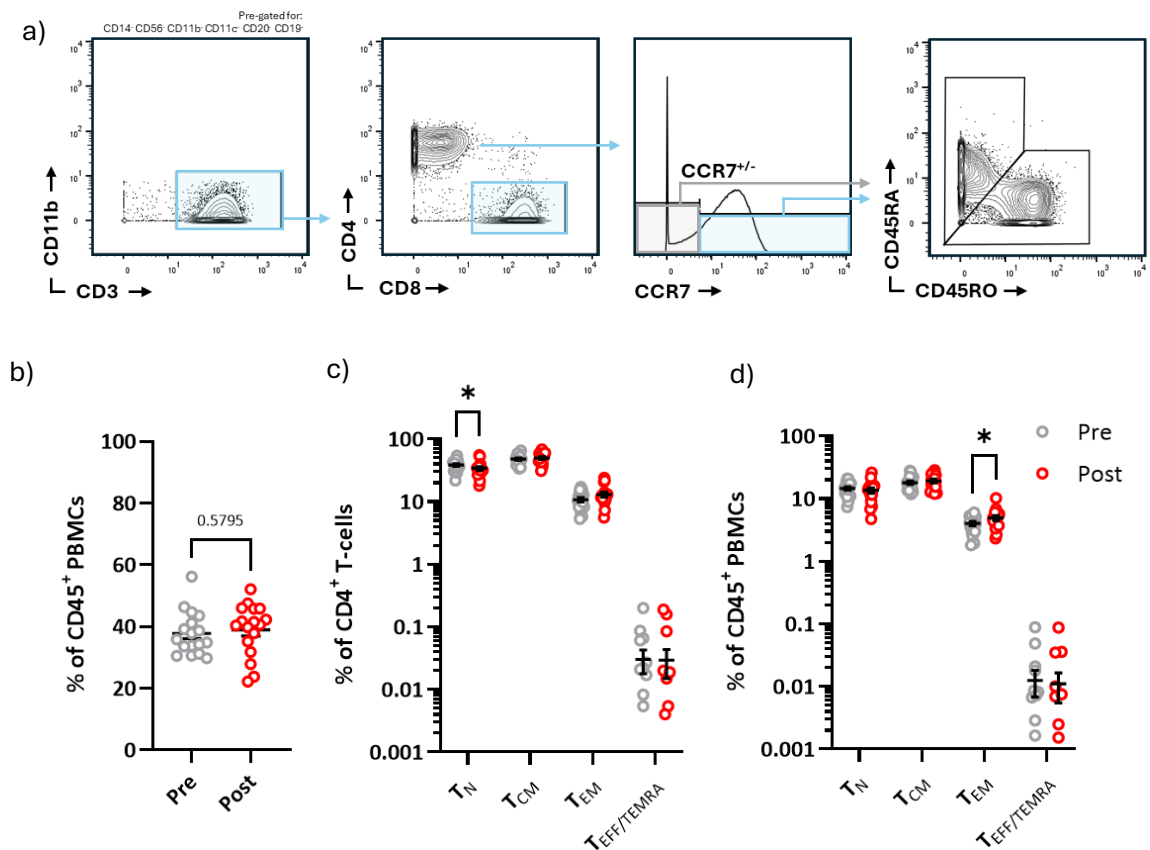


Figure 6.4.3.1 - Xentuzumab promotes effector memory CD4⁺ T-cell expansion

- Sample dot plots showing the gating strategy used to define memory CD4⁺ T-cells [CD3⁺, CD4⁺, CCR7⁺, CD45RA⁺, CD45RO⁺].
- Frequency of CD4⁺ T-cells as a percentage of CD45⁺ cells before and after treatment with Xentuzumab.
- Frequency of memory T-cell subsets as a percentage of all CD4⁺ T-cells.
- Frequency of memory CD4⁺ T-cell subsets as a percentage of all CD45⁺ PBMCs.

[* = $p < 0.05$, Wilcoxon signed-rank test, $N = 17$, bars represent mean $\pm$ SEM.]

6.4.4 Unsupervised clustering of total CD4⁺ T-cells

Supplementary unsupervised clustering analysis by FlowSOM revealed 18 distinct metaclusters within the CD4⁺ T-cell compartment (CD4_1-18) [figure 6.4.4.1]. Two CD4⁺ metaclusters were found to be differentially abundant after treatment with Xentuzumab (figures 6.4.4.2a-b). Consistent with manual gating, the first population, CD4_1, were naïve-like CD4⁺ T-cells. The second, CD4_16 was a subset of highly activated subset of effector memory CD4⁺ T-cells that co-expressed co-inhibitory receptors PD-1 and TIGIT. Taken together with earlier observations, IGF blockade promotes the expansion of effector memory PD-1⁺ TIGIT⁺ co-expressing cells in both CD4⁺ and CD8⁺ T-cell compartments.

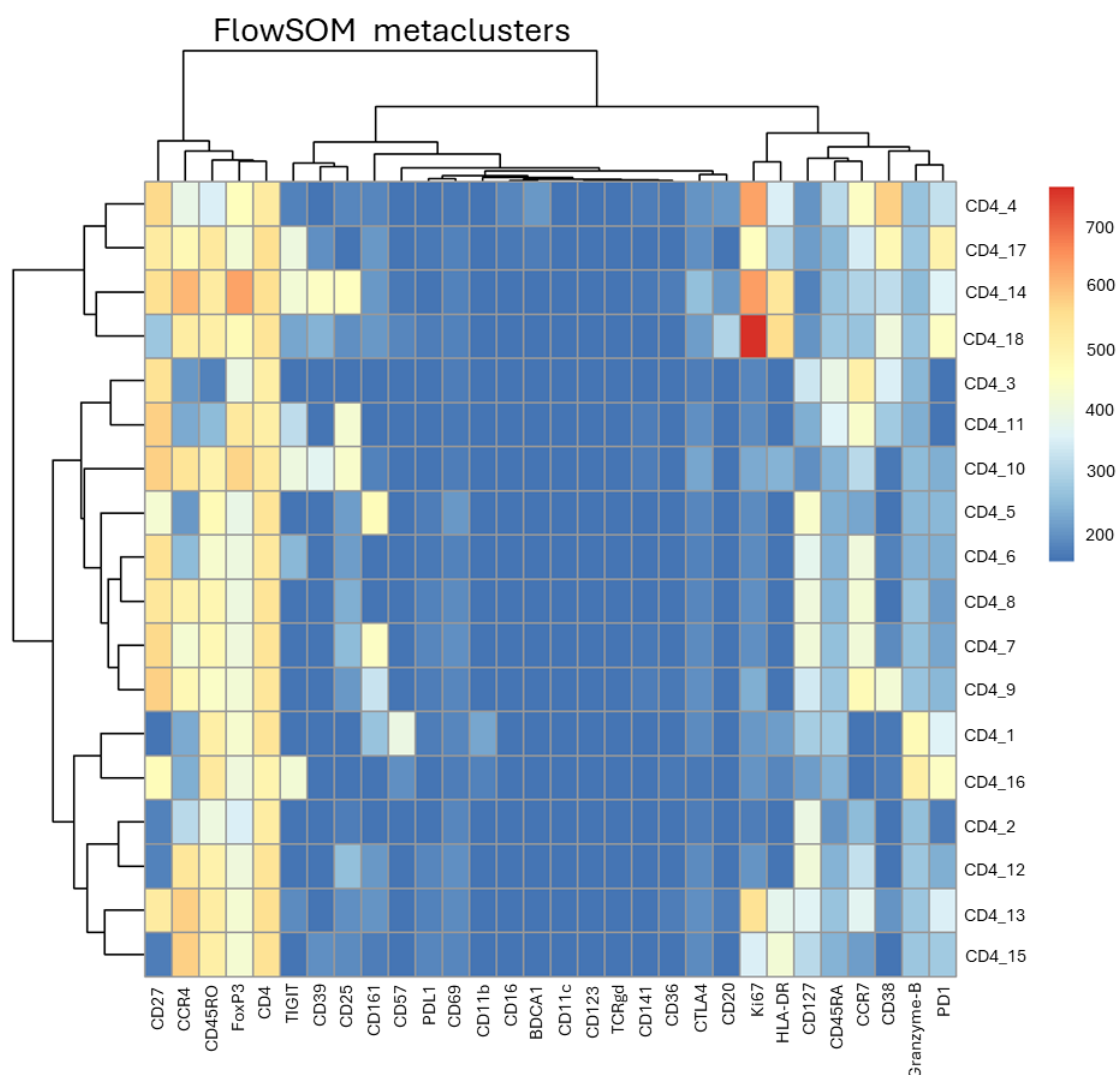


Figure 6.4.4.1 - Unsupervised clustering of CD4⁺ T-cells by FlowSOM

A Lineage⁻, CD3⁺, CD4⁺ gate was applied to each sample and positive cells were concatenated into a single data file. This was used for meta-clustering analysis by FlowSOM, which defined 18 distinct metaclusters (CD4_1-18).

FlowSOM metaclustering of Treg cells illustrated on a heatmap. Dendrograms present clustering of immune cells (rows) and markers (columns) which is based on hierarchical clustering. The median expression intensity for each marker within metaclusters is shown and colour coded.

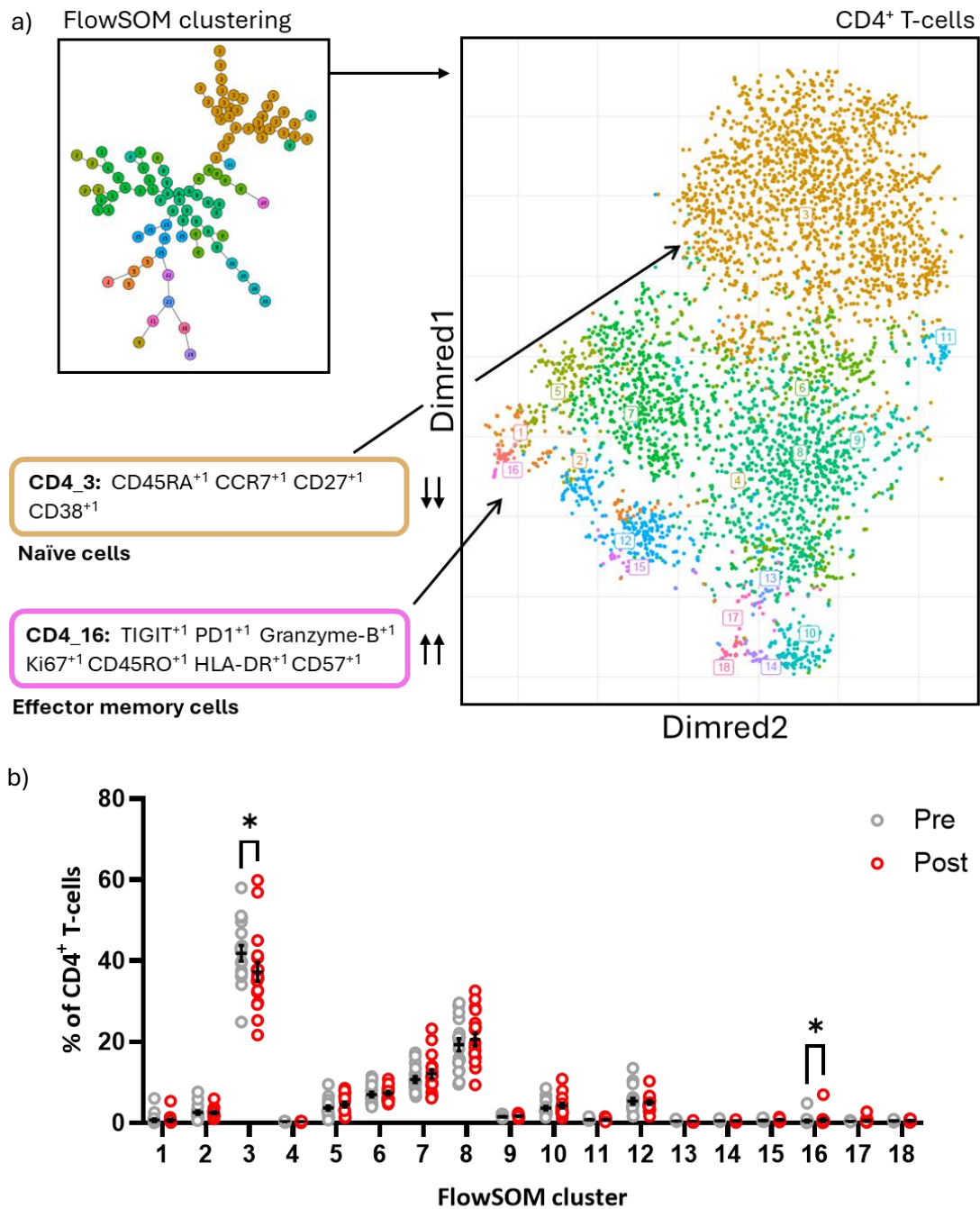


Figure 6.4.4.2 - Unsupervised clustering reveals a shift towards TIGIT⁺ PD-1⁺ effector memory CD4⁺ T-cell differentiation after treatment with Xentuzumab

a) Representative FlowSOM tree and tSNE plot after metaclustering of CD4⁺ cells. Defined metaclusters that significantly expand in response to Xentuzumab treatment are labelled with annotations from marker enrichment modelling, with arrows pointing to their corresponding location within the tSNE plot.

b) Quantification of the relative abundance of each FlowSOM metacluster as a percentage of CD4⁺ T-cells before and after treatment with Xentuzumab.

[* = $p < 0.05$, Wilcoxon signed-rank test, $N = 17$, bars represent mean $\pm$ SEM.]

6.4.5 Manual gating of regulatory T-cells

In light of *in vitro* and *in vivo* observations from previous results chapters (3-5), an important aim of this chapter was to explore if IGF blockade shapes peripheral Treg frequency or phenotype in men with PCa, and were identified by manual gating (figure 6.4.5.1a). Whilst the circulating Treg numbers did not change (fig. 6.4.5.1b), nor did the abundance of memory Tregs (figures 6.4.5.1c-d), Tregs expressed significantly reduced levels of the ectonucleotidase CD39 after treatment with Xentuzumab (figure 6.4.5.1e). Hence, whilst IGF inhibition does impact the abundance of total circulating Tregs in men with PCa, reduced levels of CD39 suggested that IGF blockade may reduce the abundance of a subset of ecto-enzyme expressing Tregs.

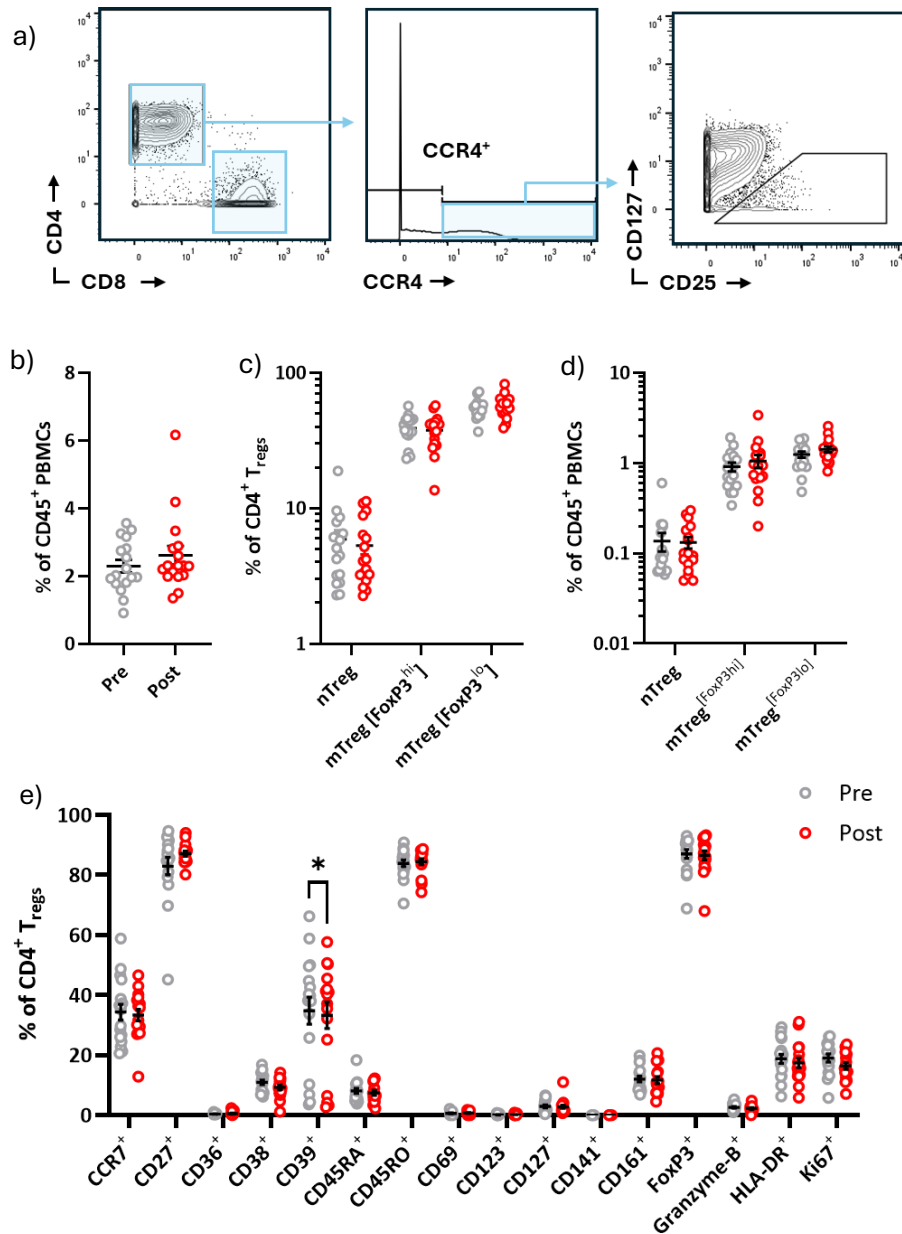


Figure 6.4.5.1 - Xentuzumab treatment reduces the frequency of CD39⁺ Tregs

a) Sample dot plots showing the gating strategy used to define regulatory T-cells [CD3⁺, CD4⁺, CCR4⁺, CD25⁺ CD127⁺]. b) Frequency of Tregs as a percentage of CD45⁺ cells before and after treatment with Xentuzumab.

Tregs were further subdivided into CD45RA⁺FoxP3^{lo} naïve Treg (nTreg), CD45RA⁻ CD45RO⁺ FoxP3^{hi} memory Tregs (mTreg FoxP3^{hi}) or CD45RA⁻ CD45RO⁺ FoxP3^{lo} memory Tregs (mTreg FoxP3^{lo}). The frequency of these memory Treg subsets was calculated as a percentage of Tregs (c) or total CD45⁺ cells (d) before and after treatment with Xentuzumab. e) The percentage of Tregs staining positive for expression of CCR7, CD27, CD36, CD38, CD39, CD45RA, CD45RO, CD69, CD123, CD127, CD141, CD161, FoxP3, Granzyme-B, HLA-DR and Ki67 before and after treatment with Xentuzumab.

[* = $p < 0.05$, Wilcoxon signed-rank test, $N = 17$, bars represent mean $\pm$ SEM.]

6.4.6 Unsupervised clustering of CD4⁺ Tregs

Tregs were then clustered by FlowSOM, which revealed 5 distinct peripheral blood Treg metaclusters (Treg_1-5) (figure 6.4.6.1). Two of the five FlowSOM Treg metaclusters significantly decreased after treatment with Xentuzumab (figures 6.4.6.2a-b). The first population, Treg_1, was a population of highly proliferative effector-memory Tregs that co-expressed high levels of TIGIT, HLA-DR and ecto-enzymes CD39 and CD38. The second, Treg_5, was also a highly proliferative Treg population, but lacked expression of TIGIT and CD27. These observations indicate that IGF blockade reduces the abundance of specific peripheral Treg sub-populations, rather than total Treg numbers.

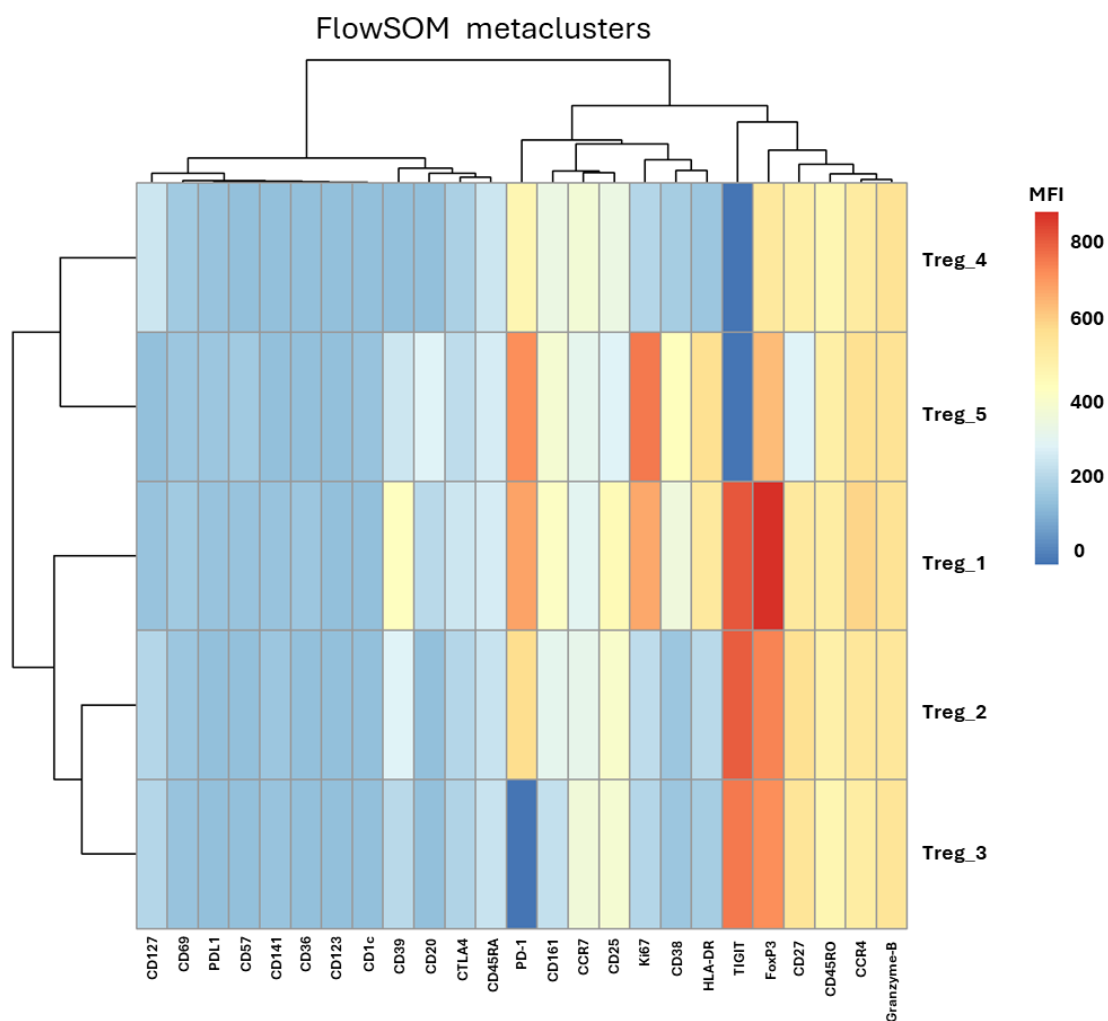


Figure 6.4.6.1 - Unsupervised clustering of circulating regulatory T-cells identifies 5 phenotypically distinct metaclusters

Lineage⁻, CD3⁺, CD4⁺, CCR4⁺, CD25⁺ CD127⁻ cells were used for meta-clustering analysis by FlowSOM, which defined 5 distinct metaclusters (Treg_1-5).

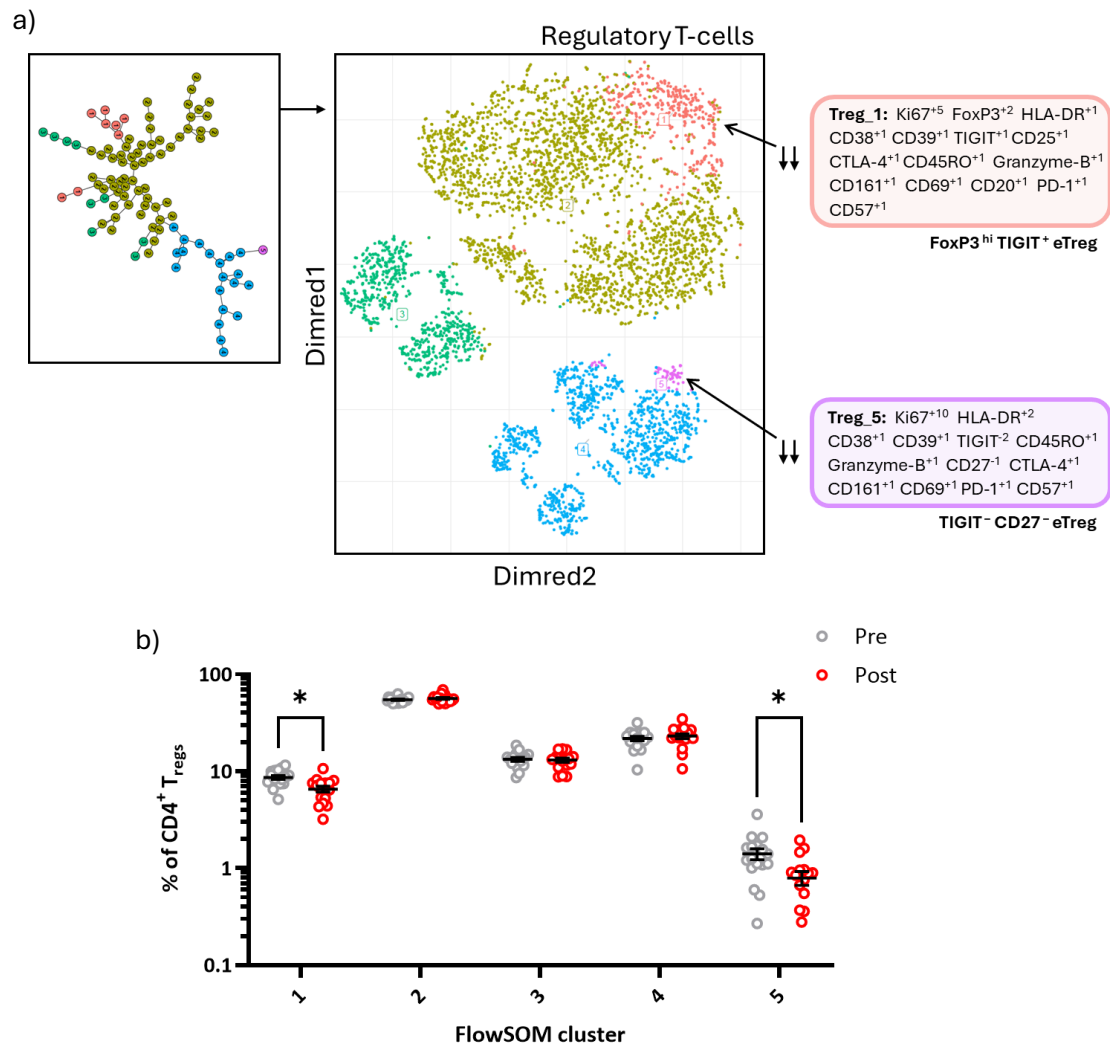


Figure 6.4.6.2 - The frequency of Treg_1 and Treg_5 metaclusters significantly decreases after treatment with Xentuzumab

a) Representative FlowSOM tree and tSNE plot after metaclustering of Treg cells. Defined metaclusters that significantly expand in response to Xentuzumab treatment are labelled with annotations from marker enrichment modelling, with arrows pointing to their corresponding location within the tSNE plot.

b) Quantification of the relative abundance of each FlowSOM metacluster as a percentage of CD4⁺ Tregs before and after treatment with Xentuzumab.

[* = $p < 0.05$, Wilcoxon signed-rank test, $N = 17$, bars represent mean $\pm$ SEM.]

6.4.7 Generating Boolean gating strategies for Treg subsets responsive to IGF blockade

To aid future investigation of Treg sub-populations sensitive to IGF blockade by other research groups, bi-axial gating strategies for Treg_1 and Treg_5 metaclusters were explored with markers frequently used in Treg flow cytometry panels. First, Hyperfinder was used, but this did not reproduce the phenotype of FlowSOM Treg_1 or Treg_5 populations after treatment with Xentuzumab (data not shown). Next, differentially expressed markers from the metacluster heatmap were considered. Both Treg_1 and Treg_5 expressed high levels of Ki67. Treg_1 expressed higher levels of TIGIT, PD-1 and CD45RO, whilst Treg_5 lacked expression of both TIGIT and CD27. Consequently, we arrived at a gating strategy for Treg_1 as: Ki67⁺ TIGIT⁺, PD-1⁺, CD45RA^{lo} CD45RO⁺ (referred to as cluster 1 [Boolean]) and Treg_5 as Ki67⁺ TIGIT⁻, CD27⁻ (referred to as cluster 5 [Boolean]) (fig. 6.4.7.1a). Treg sub populations defined by manual bi-axial gating significantly decreased after Xentuzumab treatment (fig. 6.4.7.1b-c), and phenotypically resembled their equivalent FlowSOM population (fig. 6.4.7.1d).

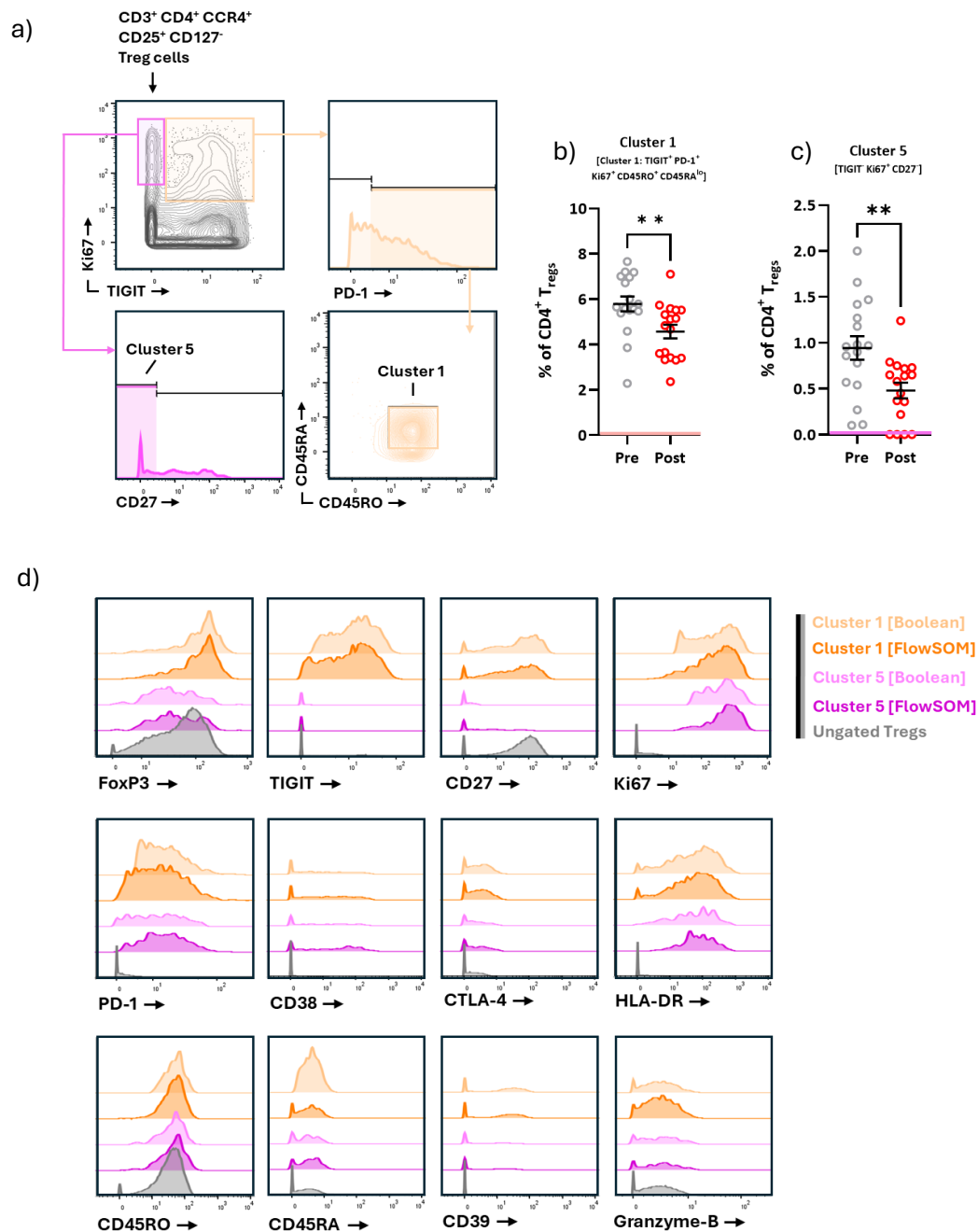


Figure 6.4.7.1 - IGF responsive Treg metaclusters 1 and 5 can also be identified using markers commonly used in flow cytometry panels

a) Sample dot plots showing the gating strategy used to define metacluster 1 [Treg_1] (Ki67⁺ TIGIT⁺, PD-1⁺, CD45RA^{lo} CD45RO⁺) and metacluster 5 [Treg_5] (Ki67⁺ TIGIT⁺, CD27⁻). b) Frequency of Ki67⁺ TIGIT⁺, PD-1⁺, CD45RA^{lo} CD45RO⁺ or Ki67⁺ TIGIT⁺, CD27⁻ (c) Tregs as a percentage of total Treg cells before and after treatment with Xentuzumab. d) Representative histograms comparing the relative expression levels of FoxP3, TIGIT, CD27, Ki67, CD38, CTLA-4, HLA-DR, CD45RO, CD45RA, CD39 and Granzyme-B in total Tregs (CCR4⁺ CD25⁺ CD127⁻), Treg_1 (FlowSOM), Treg_5 (FlowSOM), cluster 1 (Boolean) and cluster 5 (Boolean)

[** = $p < 0.01$, paired T-test, N = 17, bars represent mean $\pm$ SEM.]

6.4.8 Manual gating of B-cells

The impact of IGF blockade on peripheral blood B-cell immunity was then explored by manual gating. Xentuzumab did not significantly impact the frequency of total B-cells, CD19⁺ (figure 6.4.8.1a), nor the differentiation of memory B-cell subsets (fig. 6.4.8.1b).

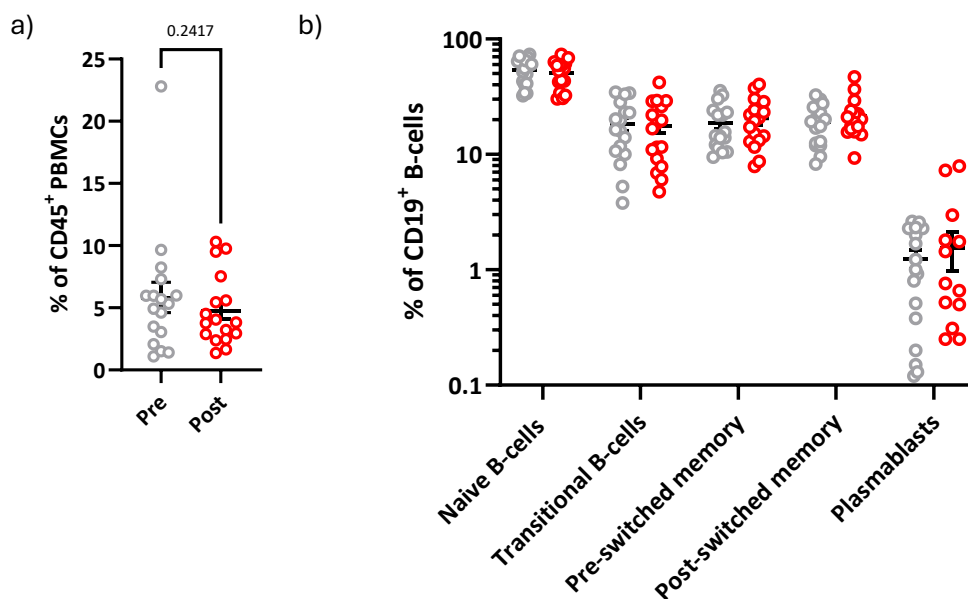


Figure 6.4.8.1 – **Manual gating of circulating B-cells before and after Xentuzumab treatment**

a) Frequency of CD19⁺ T-cells as a percentage of CD45⁺ cells before and after treatment with Xentuzumab.

b) The percentage of CD19⁺ cells that were naïve (IgD⁺ CD27⁻) transitional (IgD^{lo} CD38^{hi}), un-switched memory (IgD⁺ CD27⁺), switched memory (IgD⁻ CD27⁺) or plasmablasts (IgD⁻ CD27⁺ Ki67^{hi} CD38^{hi}) before and after Xentuzumab treatment.

[(a) Paired T-Test, (b) Wilcoxon signed-rank test, N = 17, bars represent mean ± SEM.]

6.4.9 Unsupervised clustering of B-cells

Subsequent clustering by FlowSOM revealed 8 distinct B-cell metaclusters (B-cell_1-8) (figure 6.4.9.1). Unexpectedly, 5 of the 8 B-cell metaclusters were found to be differentially abundant after treatment with Xentuzumab (figures 6.4.9.2a-b). B-cell_1 (naïve/T3-like) and B-cell_6 (atypical B-cells) significantly decreased after Xentuzumab, whilst B-cell_4 (unswitched memory B-cells), 7 (early-plasmablast) and 8 (plasmablasts) significantly increased as percentage of total CD19⁺ B-cells after Xentuzumab treatment (figure 6.4.9.2b). The expansion of unswitched memory B-cells and plasmablasts and concomitant decrease in naïve B-cells implicated the expansion antigen experienced cells and speculatively, activation of an anti-tumoural B-cell response in response to Xentuzumab. These data also highlight that manual gating strategies alone may not fully capture the true heterogeneity of peripheral blood B-cell subsets in human PCa.

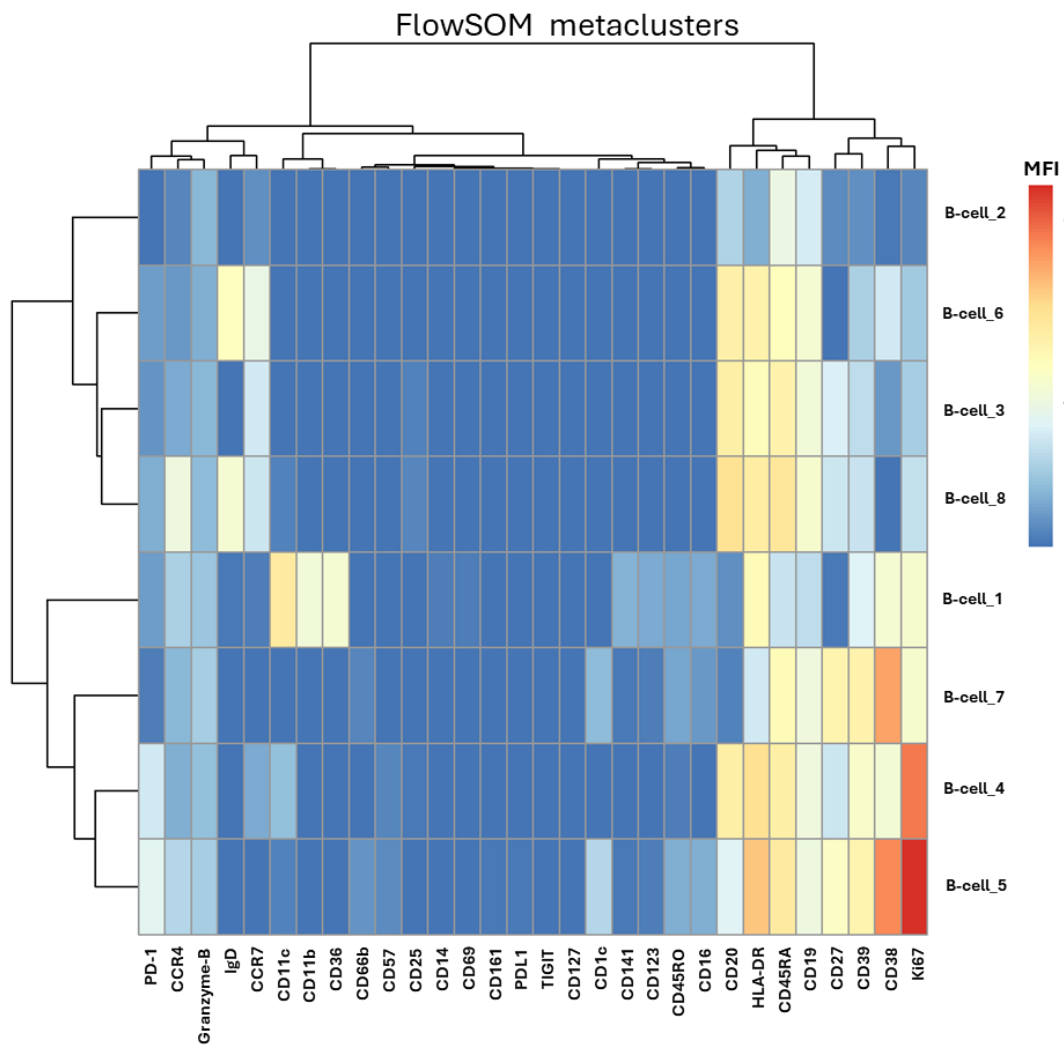


Figure 6.4.9.1 - Unsupervised clustering of CD19⁺ B-cells by FlowSOM

A Lineage[®], CD19⁺ gate was applied to each sample and positive cells were concatenated into a single data file. This was used for meta-clustering analysis by FlowSOM, which defined 18 distinct metaclusters (CD4_1-18).

(a) FlowSOM metaclustering of B-cells illustrated as a heatmap. Dendrograms present clustering of immune cells (rows) and markers (columns) which is based on hierarchical clustering. The median expression intensity for each marker within metaclusters is shown and colour coded.

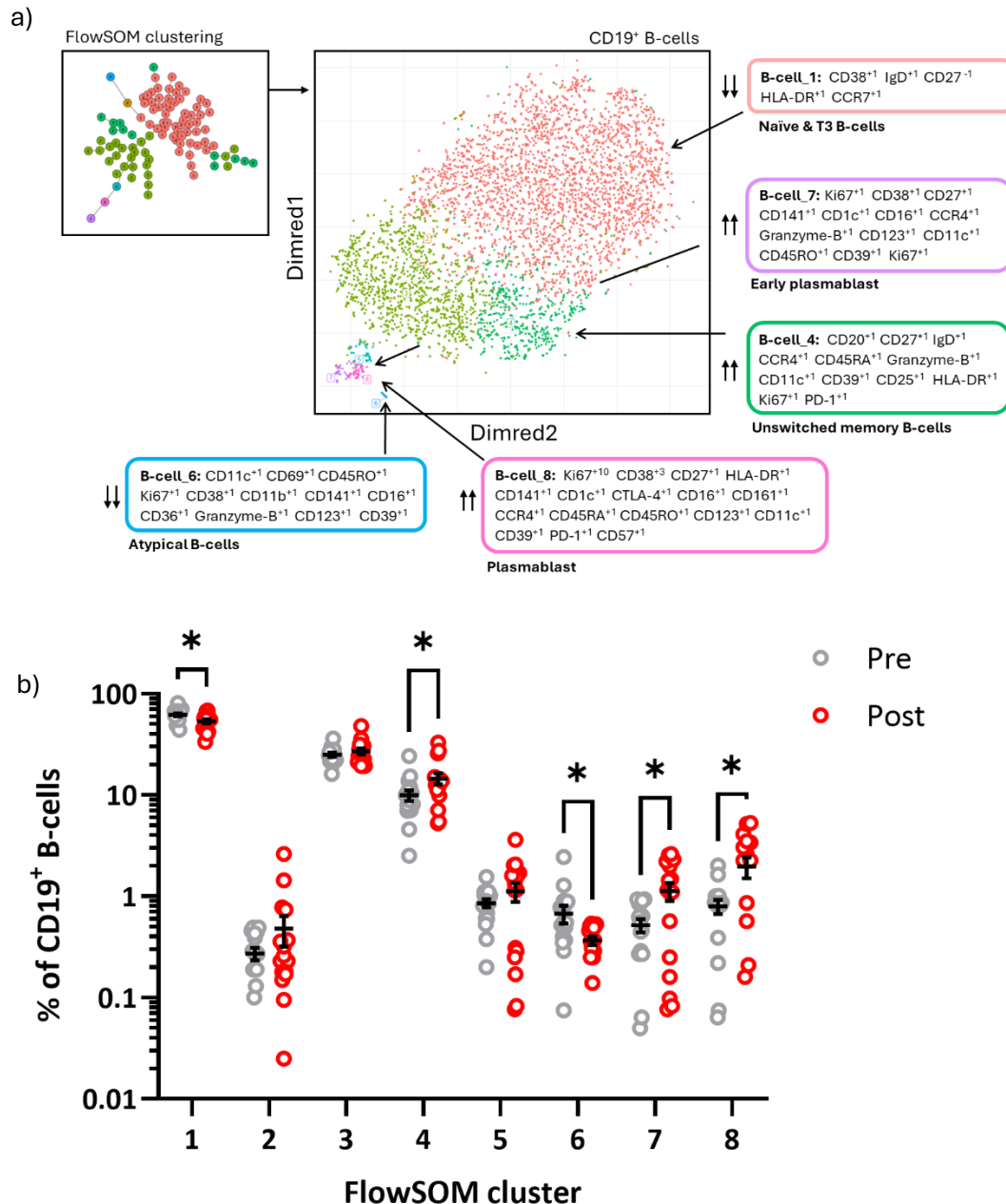


Figure 6.4.9.2 - Unsupervised clustering shows IGF blockade promotes the expansion of unswitched memory B-cells and plasmablasts

a) Representative FlowSOM tree and tSNE plot after metaclustering of CD19⁺ B-cells. Defined metaclusters that significantly expand in response to Xentuzumab treatment are labelled with annotations from marker enrichment modelling notations, with arrows pointing to their corresponding location within the tSNE plot.

b) Quantification of the relative abundance of each FlowSOM metacluster as a percentage of CD19⁺ B-cells before and after treatment with Xentuzumab.

[* = $p < 0.05$, Wilcoxon signed-rank test, $N = 17$, bars represent mean $\pm$ SEM.]

6.4.10 Manual gating of ILCs

Finally, the abundance and phenotype of peripheral blood ILCs (lin⁻ CD127⁺) were explored (figure 6.4.10.1a). Treatment with Xentuzumab did not significantly impact the frequency of ILCs as a percentage of CD45⁺ cells (figure 6.4.10.1b), significantly reduced CD161 and CD45RA expression (figure 6.4.10.1c), which could be representative of a decrease in the number of non-proliferative and quiescent ILCs (522). Of note, FlowSOM analysis of blood monocytes, NK cells, dendritic cells or $\gamma\delta$ -T-cell sub-populations revealed no statistically significant changes occurred after treatment with Xentuzumab (data not shown).

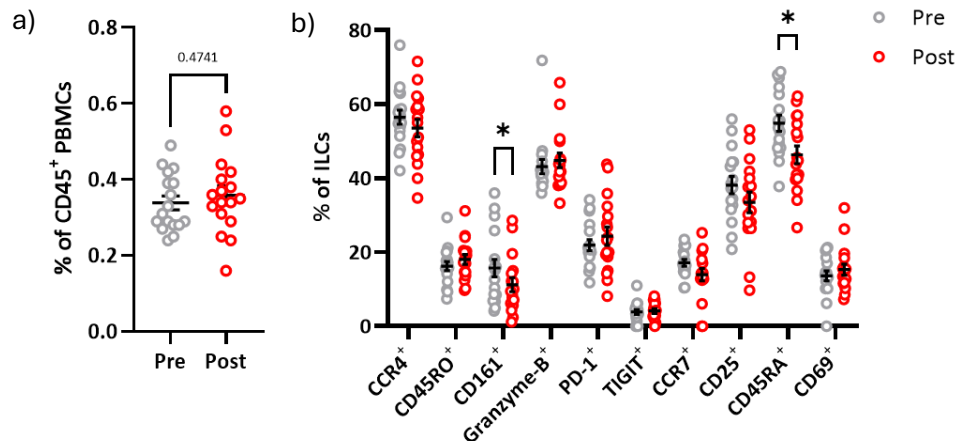


Figure 6.4.10.1 - Xentuzumab significantly reduces CD45RA and CD161 expression on ILCs

a) Frequency of total ILCs [lineage⁻ CD127⁺] as a percentage of CD45⁺ cells before and after treatment with Xentuzumab.

b) The percentage of ILCs staining positive for expression of CCR4, CD45RO, CD161, Granzyme-B, PD-1, TIGIT, CCR7, CD25, CD45RA, CD69.

[* = $p < 0.05$, (b) Paired T-Test, (c) Wilcoxon signed-rank test, N = 17, bars represent mean $\pm$ SEM.]

6.5 Immune correlates of anti-tumour responses

6.5.1 Defining “responders” and “non-responders” to Xentuzumab treatment

The identification of circulating immune biomarkers that correlate with PSA response after Xentuzumab treatment could provide valuable insight into possible mechanisms or important phenotypes associated with clinical benefit or immunosurveillance in men with localized PCa. To explore this, patients were first categorized by the relative change in the PSA score, indicative of PCa tumour size (111), before and after treatment with Xentuzumab (figure 6.5.1.1a). Of the 26 trial patients; 2 patients had incomplete data (missing either one or more PSA sampling). After Xentuzumab treatment, 7 patients experienced an increase in PSA score, whilst 11 patients had a decrease in PSA score. The PSA score did not change in 7 patients after Xentuzumab treatment.

Non-responders were categorized as patients with a PSA score increase ≥ 1 (n=7), and responders were categorized as patients with a PSA score decrease ≤ -1 (n=11) after Xentuzumab treatment. After grouping, the relative change in PSA score in patients classified as responder's (R) vs non-responders (NR) was significantly different (figure 6.5.1.1b). Of these patients, 5 out of 7 “non-responders”, and 7 out of 11 “responders” had paired CyTOF data that passed quality controls, and were used in subsequent analysis.

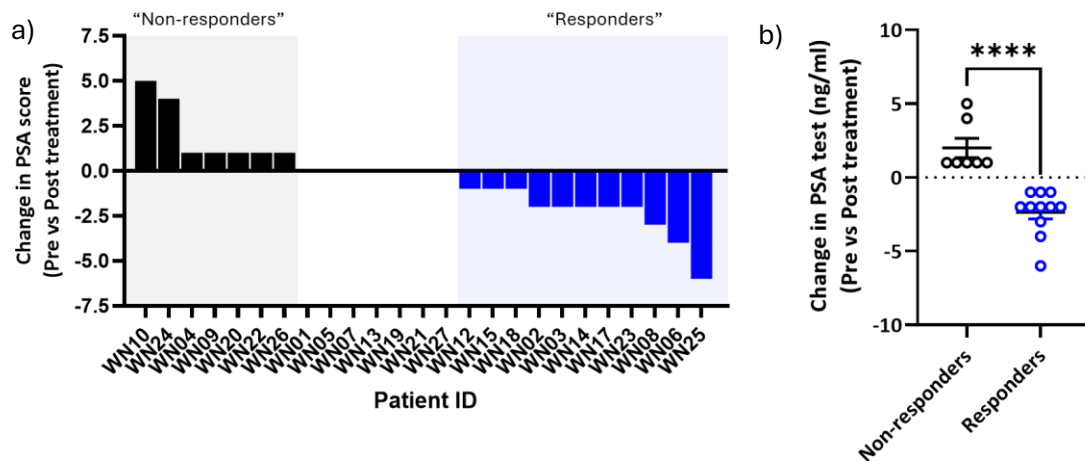


Figure 6.5.1.1 - Changes in PSA levels after treatment with Xentuzumab

a) Individual changes in PSA score (ng/mL) after treatment with 4-6 cycles of Xentuzumab. Non-responders were categorized as patients with a PSA increase ≥ 1 , and responders were categorized as patients with a PSA decrease ≤ -1 .

b) Comparison of the relative change in PSA score (ng/mL) in patients categorized as responder's vs non-responders.

[Bars represent mean $\pm$ SEM. Statistical comparisons made between responders (N = 11) and non-responders (N = 7) were done by unpaired T-tests, * = $p < 0.05$, ** = $p < 0.01$]

6.5.2 Investigating pre-treatment immune correlates of PSA response to Xentuzumab

The phenotype and abundance of immune cell subsets before treatment with anti-neoplastic cancer treatments and ICB has emerged as a useful tool to identify and categorize patients more likely to respond to treatment (523–525). Thus, the relative abundance of immune cell populations identified by manual gating and FlowSOM were compared within pre-treatment samples of responders and non-responders. After analysis, the frequency of four immune cell populations were significantly different in pre-treatment samples of responders compared with non-responders.

Non-responders had significantly higher levels of circulating Tregs, naive CD4⁺ T-cells and terminally differentiated CD8⁺ T-cells that expressed high levels of the ectoenzyme CD38 (fig. 6.5.2.1). In contrast, high levels of circulating CD161⁺⁺ effector memory CD4⁺ T-cells correlated with a decrease in PSA score after Xentuzumab treatment. Thus, the abundance of pre-existing memory CD161⁺⁺ CD4⁺ T-cells and a low Treg setpoint may function as important parameters of anti-tumour immunity in response to Xentuzumab treatment.

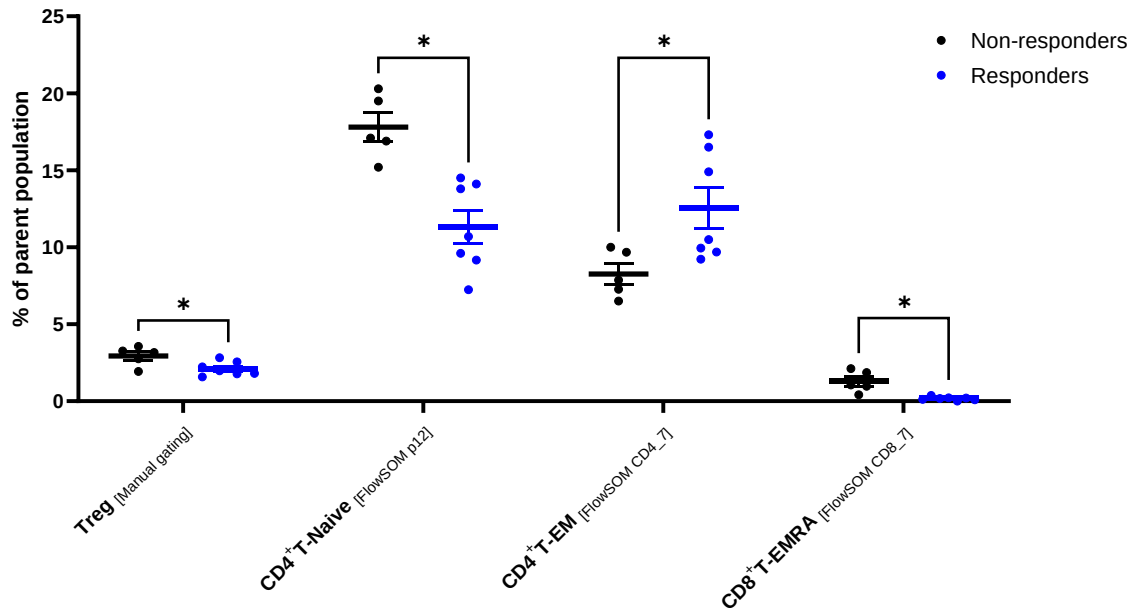


Figure 6.5.2.1 - Investigating pre-treatment immune correlates with response to Xentuzumab

The composition and abundance of immune cell subsets was compared between responders vs non-responders before they received Xentuzumab treatment.

Subsets defined:

- 1) The percentage of total CD45⁺ immune cells that are CD4⁺ CD25⁺ CD127⁻ CCR4⁺ T_{regs} in responders vs non-responders.
- 2) The percentage of total CD45⁺ immune cells defined by FlowSOM as metacluster p12 (CD4⁺ CD45RA⁺ CCR7⁺ CD38⁺ CD127⁺) in responders vs non-responders.
- 3) The percentage of total CD4⁺ T-cells defined by FlowSOM as metacluster CD4_7 (CD4⁺ CD45RO⁺ CD161⁺ CD27⁺ Ki67⁺ CD25⁺)
- 4) The percentage of total CD8⁺ T-cells defined by FlowSOM as metacluster CD8_7 (CD8⁺ CD45RA⁺ Granzyme-B⁺ TIGIT⁺ CD38⁺ PD-1⁺ HLA-DR⁺ CD57⁺ CD69⁺) in responders vs non-responders.

[Bars represent mean $\pm$ SEM. Statistical comparisons made between responders (N = 7) and non-responders (N = 5) were done by unpaired T-tests, * = $p < 0.05$, ** = $p < 0.01$]

6.5.3 Investigating post-treatment immune correlates of PSA response to Xentuzumab

Finally, we explored immune signatures associated with anti-tumour effects in post-treatment PBMCs. The frequency of three immune subpopulations were different in responders and non-responders after treatment with Xentuzumab. Responders to Xentuzumab had significantly higher levels of circulating NKT-cells and CCR4^{hi} monocytes, whilst non-responders had significantly higher levels terminally differentiated CD8⁺ T-cells that expressed high levels of the ectoenzyme CD38. Thus, high circulating levels of the population defined by FlowSOM as CD8_7 is seemingly an important correlate of a failure to respond to Xentuzumab treatment in men with PCa.

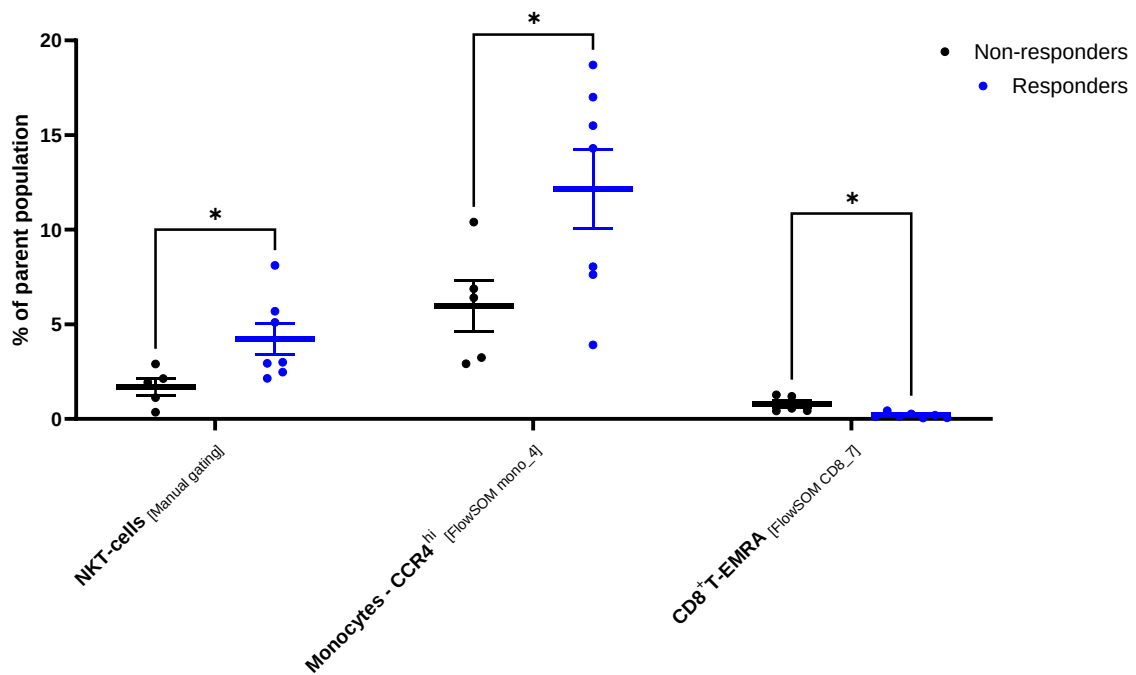


Figure 6.5.3.1 - Investigating post-treatment immune correlates with response to Xentuzumab

The composition and abundance of immune cell subsets was compared between responder's vs non-responders after they received Xentuzumab treatment.

- 1) The percentage of total CD45⁺ immune cells that are CD3⁺ CD56⁺ NKT-cells in responders vs non-responders.
- 2) The percentage of total CD14⁺ monocytes defined by FlowSOM as metacluster mono_4 (CD14⁺ CD11b⁺ CCR4⁺ CD36⁺ CD141⁺ CD1c⁺ CCR7⁺ PD-1⁺).
- 3) The percentage of total CD8⁺ T-cells cells defined by FlowSOM as metacluster CD8_7 in responders vs non-responders.

[Bars represent mean ± SEM. Statistical comparisons made between responders (N = 7) and non-responders (N = 5) were done by unpaired T-tests, * = p < 0.05, ** = p < 0.01]

6.6 Discussion

In this chapter, the peripheral immune response of 17 men with localized PCa was explored before and after treatment with Xentuzumab, as a part of the Phase 0: WINGMEN trial (NCT05110495). Using a combination of manual and unsupervised clustering methods, we show that peripheral blood immunophenotyping can be used as a surrogate biomarker of IGF bioactivity, meeting a tertiary trial endpoint. Conceptually, we show that systemic neutralization of IGFs has a primary effect on the abundance and phenotype of circulating populations of T-cells, B-cells, NKT-cells and ILCs, but not circulating NK cells, monocytes, or dendritic cells. Importantly, both pre-treatment and post-treatment immunophenotyping can be used to identify patients that are more likely to experience anti-tumour effects from treatment with Xentuzumab. Finally, manual gating strategies were generated that define peripheral immune cell subsets responsive to IGF neutralization to help guide future research efforts aimed at optimising FMD protocols for cancer patients.

We hypothesize that the effects of Xentuzumab treatment observed in this study are representative of a physiological response in peripheral immune cells that mimics pseudo-starvation, that recapitulates at least in-part, the response to caloric restriction and nutrient starvation (403,409). Changes observed in this study are consistent with both preclinical and clinical observations in short-term fasting and FMD studies, whereby reduced IGF-1 and blood glucose levels were associated with a similar expansion of peripheral CD8⁺ T-cells (403,526). Given the exquisite selectivity of Xentuzumab for IGF-1 and IGF-2 ligands (358), decreased serum IGF-1 levels are likely crucial to the FMD response with respect to changes in circulating CD8⁺ T-cells. In

mouse models, serum IGF-1 levels regulate the development and function of human hematopoietic progenitor cells (408). Whilst chronic IGF-1R activation by sIGF-1 promotes haematopoietic stem cell (HSC) exhaustion, prolonged fasting cycles can restore long-term HSC self-renewal. This, in turn, promotes the restoration of normal lymphocyte counts and reversal of immunosenescence in an IGF-1 dependent manner. Thus, the selective increase in CD8⁺ T-cells after IGF blockade in men with PCa could be representative of a restoration of normal lymphocyte counts by reversal of immunosenescence, rather than the lineage selective expansion of CD8⁺ T-cells. However, this does not sufficiently explain how IGF blockade also promotes the differentiation of both CD4⁺ and CD8⁺ T-cells towards an effector memory phenotype that co-express TIGIT and PD-1. Polarization towards effector memory subsets could be reflective of a loss of mTORC1 activity by reduced IGF-1R activation, diverting cells away from effector cell differentiation, inducing metabolic reprogramming and modifying tissue homing properties (527–529). These changes may also have important function consequences, as the frequency of circulating CD8⁺ T-cells co-expressing PD-1 and TIGIT are predictive of response to anti-PD-1 ICB in cancer patients (530). Thus, the expansion of TIGIT⁺ PD-1⁺ effector memory CD8⁺ T-cells after Xentuzumab treatment could represent an important mechanism by which reduced IGF-1 levels enhance the response to anti-PD-1 ICB in patients with non-small cell lung carcinoma (395).

Whilst Xentuzumab did not significantly impact the number of B-cells, Tregs or ILCs, there was a significant change in their subset composition. Within the B-cells compartment, Xentuzumab treatment induced the expansion of circulating

unswitched memory B-cells and plasmablasts. The enrichment of circulating unswitched memory-B-cells are associated with response to anti-PD-1 ICB in patient with colorectal cancer (531), whilst the expansion of plasmablasts could be representative of an expansion of neoantigen-reactive plasma cells (532). Conversely, in mouse models IGF-1R inhibition promotes autoantibody production from plasmablasts through enhanced follicular B-cell transition in SLOs (390). Thus, the expansion of plasmablasts highlight an important safety concern with respect to breaking of tolerance and potential for autoantibody production, and autoimmunity should be carefully monitored in future FMD trials.

In circulating Tregs, Xentuzumab significantly reduced the frequency of a subset of circulating Tregs characterised by high co-expression of TIGIT, PD-1, CD39 and CD38. In sepsis patients, circulating Tregs co-expressing CD39, CD38 and PD-1 are associated with increased mortality and immunosuppression (533). A similar population of circulating CD4⁺ co-expressing CD38 and CD39 T-cells (referred to as ectoenzyme-expressing T cells [Tee]) have been isolated and characterized in patients with metastatic melanoma (534). CD4⁺ Tee cells are highly suppressive *ex vivo* and are associated with both resistance to anti-PD-1 ICB and poor overall survival. Therefore, the reduced abundance of circulating CD39⁺ CD38⁺ Treg subset identified by FlowSOM after Xentuzumab treatment could represent targeted reduction of a subset with high suppressive potency. Further investigations into the relative abundance of intra-tumoural ecto-enzyme expressing (eeTregs) in pre-diagnostic vs post-treatment tumour biopsies will be an important next step in defining how IGFs contribute to Treg function in human PCa.

A main aim of this study was to test if peripheral immunity could be used to identify patients most likely to benefit from Xentuzumab treatment. Strikingly, of the broad spectrum of immune cell subsets that displayed sensitivity to Xentuzumab treatment, none correlated with anti-tumour effects. Hence, whilst inhibiting IGFs promotes a peripheral immune response that would appear favourable with respect to anti-cancer immunity, these changes appear insufficient to drive anti-tumour effects alone. Our findings are indicative that a pre-existing setpoint of high memory CD4⁺ T-cells, low Treg and low exhausted CD8⁺ T-cells correlates with a favourable anti-tumour response to Xentuzumab treatment. We hypothesize the abundance of circulating memory CD161⁺⁺ CD4⁺ T-cells in PCa patients may reflect an increased number of pre-existing tumour-reactive CD4⁺ T-cells that expand in response to Xentuzumab treatment, contributing to anti-tumour effects. These data support a model whereby IGF blockade does not intrinsically generate TAA-specific responses, rather, boosts pre-existing TAA-specific function. Alternatively, several studies have linked the abundance of NKT cells with protective immune effects against cancer (519), and the transition of benign prostatic hyperplasia (BPH) to localized prostate cancer is associated with a decrease in circulating NKT-cells and a reciprocal increase in circulating Tregs (535). Consequently, the correlation between responders to Xentuzumab and increased levels of NKT-cells and decreased Tregs could be representative of selecting patients with tumour microenvironments permissive to efficient anti-tumour immunosurveillance against PCa.

There are some limitations of our work. First, correlates of immune subsets with anti-tumour effects of Xentuzumab are dependent on changes in PSA levels, and

categorization of responders and non-responders should be further refined by investigating changes in malignant tumour cell viability by cleaved-caspase-3 staining. Second, we cannot conclusively resolve mucosa-associated invariant (MAIT) T-cells from iNKT cells identified by FlowSOM (p10). Future work should look to include invariant V α 7.2-J α 33 chain TCR staining to further investigate/resolve these populations (536). Furthermore, an important direction for future work is the integration of this CyTOF analysis with concomitant multiplexed immunofluorescent imaging of tumour-infiltrating immune cells, mRNA-sequencing of patient tumour biopsies, and metabolomics analysis of patient serum. Nevertheless, these findings have significant clinical implications for the optimisation of FMD interventions for cancer patients (403), and show for the first time how inhibiting IGFs contribute to the peripheral immune response in PCa. A visual summary of the key immunological changes is shown in fig.

6.6.1 Summary of chapter findings

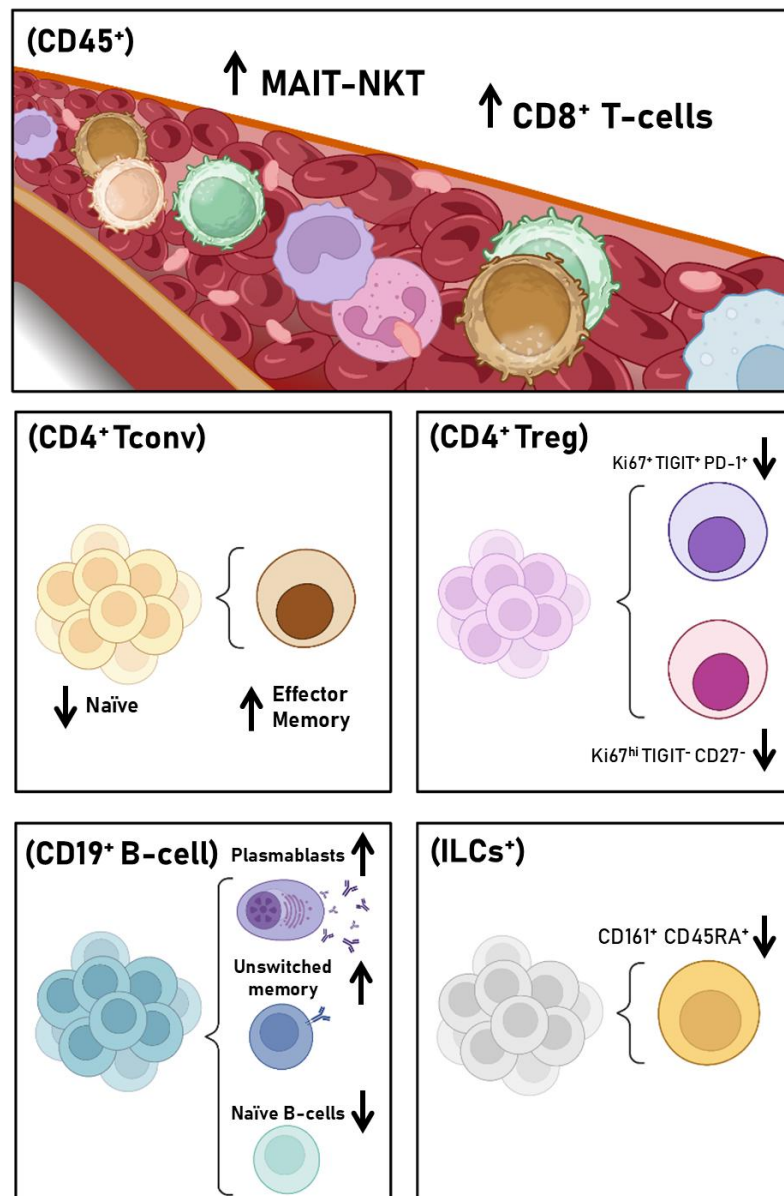


Figure 6.5.3.1 - Visual summary of changes within patient PBMCs induced by Xentuzumab treatment in men recruited to the WINGMEN trial.

Chapter 7 – General discussion

7.1 Conclusion

In this thesis, we set out to answer four main questions. How does IGF-1 influence Treg function? Is there therapeutic synergy between IGF blockade and ICB in murine PCa? How does IGF blockade shape peripheral immune responses in men with PCa? Do circulating immune subsets correlate with anti-tumour effects from IGF blockade? Results from this thesis help answer these questions. High IGF-1 supply promotes Treg suppressive function and preserves mitochondrial fitness in low-glucose, high-lactate conditions generated by cancer cells (chapters 3-4). IGF blockade does not promote anti-tumour immunity when combined with anti-PD-1, but reduced the number of TI-Treg and T_H17 cells infiltrating murine PCa allografts (chapter 5). In human PCa, IGF blockade induced the expansion of peripheral blood CD8⁺, MAIT and iNKT cells and changed the subset composition of circulating B-cells, Tregs and ILCs. Finally, low levels of circulating exhausted CD8⁺ T_{EMRA} cells emerged as an important immune correlate of response to treatment with Xentuzumab (chapter 6). Our findings have three principal implications. First, the IGF-1/IGF-1R axis functions as an important metabolic link between the endocrine system and Tregs through activation of mTORC1. Second, inhibiting IGFs *in vivo* has a targeted inhibitory effect on the frequency of tumour-infiltrating, but not peripheral or splenic Tregs. Third, inhibition of IGFs promotes a favourable immune response in the peripheral blood of men with localized prostate cancer and represents an important component of the immune response to FMD.

7.2 A biological role

We speculate that the relatively small effect sizes observed both *in vitro* and in patients with PCa is important to the fundamental physiology and regulation of immune cell function by the IGF-1-endocrine system *in vivo*. First, it would be detrimental for human health and disease if immune cells were dependent on IGFs for their homeostatic function, as fluctuations in IGF-1 levels caused by diet or exercise could have life-threatening effects on peripheral tolerance established by Tregs (194,537), or response to infection mediated by CD8⁺, MAIT and iNKT cells (538,538,539). Rather, co-stimulatory factors and regulation of ligand availability by IGFBPs likely form an essential layer of regulation of IGF bioactivity that allows for sensitive fine-tuning of immune cell function within local tissues.

In many respects, the wound healing response bears many similarities to the TME (540), and we speculate that the response of Tregs to high levels of IGFs observed in this study may reflect a conserved physiological response of wound-healing (146). Inflamed and ischemic tissue is hypoxic, and the subsequent influx of glycolytic inflammatory macrophages that occurs generates a microenvironment low in glucose and high in lactate. In the late-phase wound, polarization of M2-like macrophages results in secretion of TGF- β and IGFs by M2-like macrophages that normally promotes wound granulation and re-epithelialization (541). Hence, a local concentration gradient of IGF-1 is likely formed, proximal to the site of wound healing. As Tregs/CD4⁺ T-cells migrate in response to chemokines, high local bioactivity of IGF-1 ($> 50 \text{ nmol L}^{-1}$) could fine-tune mTORC1 activation, which could function to prime the integrated stress response and the differentiation of effector-Tregs that retain

suppressive capacity in the presence of the hypoxic, high-lactate wound microenvironment. We speculate that this response is likely exploited by tumours, often considered a wound that does not heal. Although crosstalk between IGF-1R⁺ epithelial cells and IGF-1⁺ TAMs and CAFs has been established (151), an important discovery in chapter 3 was that polarized macrophages produce IGF ligands at concentrations that stimulate IGF-1R-mTORC1 activity in CD4⁺ T-cells. Given the abundance of CAFs and TAMs within the PCa TME (202), stromal-immune derived IGFs could contribute to a substantial amount of mTORC1 activity in tumour infiltrating CD4⁺ T-cells over time.

On the other hand, the effects of high IGF-1 supply on endogenous lipid synthesis could be indicative of a conserved adipogenic programme that phenotypically resembles visceral adipose tissue (VAT) Treg differentiation (214). Our RNA-seq data revealed that IGF-1 primed iTregs expressed significantly more PPAR γ transcripts (1.75 log₂FC) the transcriptional regulator of adipocyte and VAT-Treg differentiation (542). Known regulatory factors of VAT-Treg differentiation include leptin, insulin and interleukin-33 (543,544). Whilst IGF-1 regulates pre-adipocyte differentiation, insulin is required for adipocyte maturation (545). Intriguingly, VAT-Treg differentiation requires a priming step in the spleen, before diversification in VAT in response to microenvironment cues (546). Thus, the regulation of PPAR γ activity and fatty acid metabolic reprogramming by mTORC1 during early CD4⁺ T-cell activation could function as a key priming step for pre-VAT-Treg differentiation (466), similar to the regulatory role of mTORC1 signalling in follicular regulatory T-cell (Tfr) differentiation (547). Furthermore, insulin receptors (InsR) and IGF1R also combine

to form active heterodimeric hybrid receptors, which can result in similarities between their biological effects, and overlap between the effects of IGF-1 and insulin has been described in other systems (321,323,453,455). Given that adipocytes also secrete IGF-1 in a paracrine manner (297), investigations of PPAR γ binding to response elements in CD4⁺ T-cells stimulated with high levels of IGF-1 supply remains an important direction for future work.

What functional relevance would a lipid driven transcriptional programme have for tumour infiltrating Tregs? Although the identification of the VAT-Treg subset within tumours remains elusive, TI-Tregs display a unique dependence on fatty acid synthesis, cholesterol metabolism and free fatty acid uptake for effective suppression of anti-tumour immune responses (241,242). Contextually, this could have implications for obesity-related endocrine tumours such as prostate cancer, which is surrounded by adipose tissue, a metabolically active endocrine organ that has a complex role in supporting PCa tumour growth (87). PPAT not only secretes free FAs that support PCa lipid metabolism, but also promotes tumour growth by IGF-1 production. Although speculation, the phenotype of iTregs primed in high IGF-1 observed in our study phenotypically resemble those of LL-TAMs from murine and human models of PCa (151,152), and represent an important link with diet, and obesity. Analysis of single cell RNA-sequencing data from colorectal and gastric cancers revealed that the transcription factor *MAF* was predicted to be an important regulatory component in induction and formation of LA-TAMs (548). Interestingly, we found that *MAFG*, a paralog of *MAF*, was significantly upregulated in IGF-1 primed iTregs cultured in PCa CM. Thus, future investigation into response elements required

for lipid-driven metabolic phenotypes in tumour-infiltrating suppressive immune cells could provide important insight for targeted therapies aimed at depleting these cell types.

Although we have assessed the acute response to low and high physiological IGF-1 levels, it is also important to consider the implications of temporal fluctuations in IGF bioactivity caused by daily exercise or the frequency of food intake in patients with PCa (537). Fluctuating levels of perfusing serum IGF-1 could contribute to oscillatory mTORC1 activation in TI-Tregs, contributing to decreased anergy to proliferative stimuli and subsequent expansion (549). Conversely, it is also important to consider that the effects of high IGF bioactivity on Tregs in PCa tissue may conversely be beneficial in patients with pre-malignant benign prostatic hyperplasia, characterised by chronic inflammatory infiltrates, associated with an increased risk of developing PCa (550). Accordingly, it remains possible that whilst the high IGF supply could have detrimental effects for patients with malignant PCa, it could simultaneously be beneficial for immune responses in patients with pre-malignant inflammatory lesions (551).

Whilst there was significant early clinical interest in evaluating the efficacy of various iterations of IGF-1 axis inhibitors in phase I-III trials of cancer patients, to our knowledge, there have been no retrospective investigations into potential changes in immune infiltrates. Thus, most of our current understanding of how IGFs shape intra-tumoural immunity relies heavily on data from preclinical mouse models. Nevertheless, results from this study are consistent with others that implicate CD8⁺ T-cells and CD4⁺ Tregs as major respondents to pharmacological inhibition of the IGF-1

axis within the TME (395,398–400). However, it is worth noting that this response is not entirely consistent. For example, both *Freeman et al.* and *Hashimoto et al.* use pharmacological inhibitors of IGF ligands, using an IGF-1R fusion protein (IGF-trap) and a neutralizing antibody (MEDI-573) respectively. Both groups independently identified an increase in CD8⁺ TIL infiltration in mouse models of PDAC in response to IGF inhibition, thought to be driven by increased chemokine production by intra-tumoural CAFs and macrophages. Conversely, *Ajona et al.* and *Wu et al.* utilize pharmacological inhibitors of IGF-1R tyrosine kinase activity; PQ401 (a diaryl urea) and picropodophyllin (PPP) [a cyclolignan alkaloid] respectively. Intriguingly, IGF receptor tyrosine kinase inhibition in mouse models of lung cancer did not change CD8⁺ TIL or TI-Treg infiltrates when administered as a monotherapy. Rather, both groups identified that IGF receptor inhibition sensitizes CD8⁺ TILs and TI-Tregs to combinatorial treatments with anti-PD-1, through modulation of cancer cell autophagy (395,398). Hence, inhibitors of IGF ligands at first glance would appear to exert different immunological effects to IGF receptor tyrosine kinase inhibitors.

This thesis utilizes xentuzumab, a potent inhibitor of IGF ligands biologically similar to MEDI-573 and the IGF-trap. Consistent with other inhibitors of IGF ligands, in this study we also observed an increase in CD3⁺ TIL infiltration in early tumours (fig. 5.3.1), yet this effect was later lost once tumours reached 800 mm³ (appendix fig. 8.3.1.1). Subsequent investigation into immune infiltrates of human PCa (from the WINGMEN trial) by multiplex immunofluorescence confirmed xentuzumab treatment significantly enhances intra-tumoural CD8⁺ TIL infiltration (data not shown). Given the concomitant expansion of CD8⁺ T-cells also detected in this study by CyTOF (fig.

6.4.1), it seems highly plausible that increased numbers of tumour-infiltrating CD8⁺ T-cells are derived from the peripheral blood. Taken together, these data cohesively highlight CD8⁺ T-cells as one of the most consistent cell types responsive to IGF inhibitors in cancer.

Still, divergence within preclinical studies could be caused by plausible differences in the selectivity of pharmacological inhibition. Whilst xentuzumab, MEDI-573 and the IGF-trap are highly selective inhibitors of IGF ligands (357,358,552), off-target effects of IGF-1R tyrosine kinase inhibitors [PQ401 and PPP] cannot be discounted (553,554). Alternatively, these conflicting observations could represent tumour/organ specific effects of IGF inhibition. For example, if CD8⁺ TIL chemotaxis is induced by chemokines produced by intra-tumoral CAFs and macrophages in response to IGF inhibition, then the relative immune/stromal abundance within different tumours may influence the magnitude of the chemokine gradient formed, and thus the relative influx of CD8⁺ TILs. These data highlight important aspects of IGF biology that still require detailed investigations; i) a uniform characterisation of IGF bioactivity across different tissues/cancer types and ii) the proportion of tissue/tumour IGF bioactivity derived from either perfusing serum IGFs or endogenous/locally produced IGFs from stromal/epithelial/immune cells. These data could provide valuable insight into why we still observe variability in the response of tumour-infiltrating immune cells to IGF inhibitors.

Intriguingly, results from the WINGMEN trial suggest IGF blockade shifts intra-tumoural CD8⁺ T-cells towards a T-resident memory (T_{RM}) programme (data not shown). In human PDAC, mass cytometry revealed a significant population of tumour

infiltrating CD8⁺ T_{RM} cells that co-expressed high levels of TIGIT and PD-1 (555). It is tempting to speculate that a similar population of T_{RM} cells could be representative within FlowSOM p4 (TIGIT⁺ PD-1⁺ memory CD8⁺ T-cells), that expand in the peripheral blood after xentuzumab treatment (fig. 6.4.2), and potentially within PCa tumours. Given the convergence of PD-1 and TIGIT inhibitory pathways in inhibiting TIL effector function (556), future work could look to further optimise the activation and function of infiltrating CD8⁺ TILs within the PCa TME through the combination of xentuzumab with anti-PD-1 and anti-TIGIT. This combination regimen is of particular clinical interest, as a co-formulation of anti-PD-1 and anti-TIGIT is currently under evaluation in MK-3475-365/KEYNOTE-365, a phase Ib/II for patients with PCa (557). Whilst limitations on resources did not permit the exploration or pursuit of additional combinatorial regimens, other immunomodulatory treatments of interest include co-inhibition of IGFs and TGF- β s. Combined inhibition of these pathways could therapeutically exploit aspects of pTreg suppressive function (chapters 3 and 4) and co-inhibit crosstalk between IGF and TGF- β pathways described in fibroblasts and PMN-MDSCs [CD11b⁺ Ly6G⁺] (400,558).

Finally, although we provide *in vitro* evidence that the effect of high IGF-1 on Tregs may be exploited in physiological conditions relevant to PCa tumours, we do not show that this is dependent on IGF-1R expression by Tregs *in vivo*. Thus, immunophenotyping of TI-Tregs or CD4⁺ TILs isolated from MyC-CaP allografts inoculated in transgenic mice with a targeted deletion of IGF-1R by cre-recombinases is an important next step [as in ref. (366)]. Alternatively, VLIPs have been isolated with a fascinating range of both agonistic and antagonistic effects on IGF-1R activation

(381). For example, VLIPs isolated from Mandarin fish ranavirus stimulate IGF-1R autophosphorylation, stimulating MAPK activation, but not AKT and mTORC1. Thus, VLIPs could represent a powerful research tool that could enable selective inhibition/agonism of pathways downstream of IGF-1R.

7.3 Concluding remarks

In this thesis, we used cell-based assays, mouse models and patient samples to investigate the effects of low and high IGF supply on the anti-tumour immune response to prostate cancer. We have identified a novel link between IGF-1 supply, metabolism, and Treg function in the TME, and show in prostate cancer patients that systemic IGF blockade has an immune-stimulatory effect on peripheral blood CD8⁺ MAIT, and iNKT-cell numbers, whilst inhibiting the abundance of peripheral ectoenzyme-expressing Tregs (summarized in figure 7.3.1). Altogether, we foresee that targeted inhibition of IGFs by pharmacological agents, or decreasing IGF-1 bioactivity through dietary interventions, will be a useful therapeutic tool to boost anti-tumour immunity in cancer patients.

7.3.1 Visual summary of thesis findings

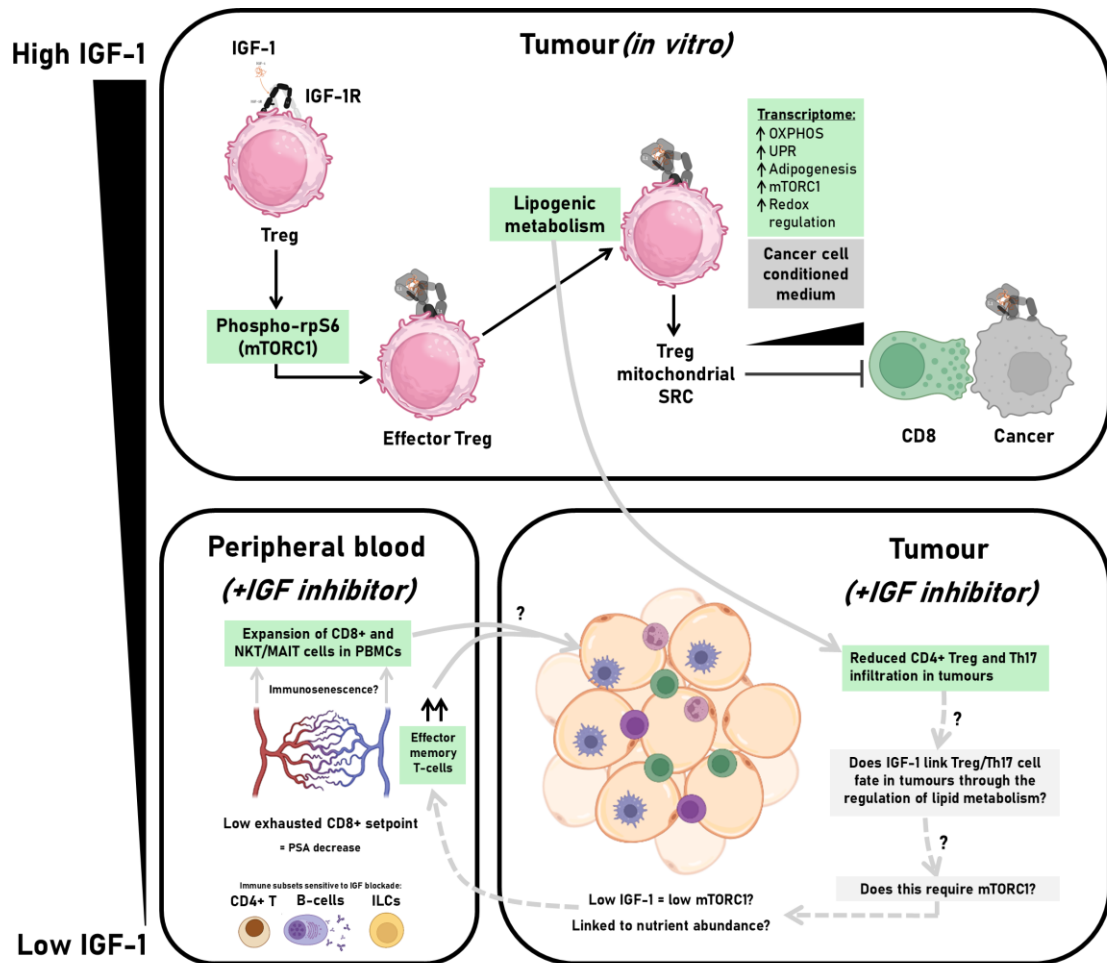


Figure 6.6.1.1 – Summary of thesis findings: IGF-1 as an immunometabolic growth factor?

In vitro, high levels of IGF-1 (50 nM) promote the activation of mTORC1 and phosphorylation of rpS6 during Treg differentiation. This results in the differentiation of Tregs with an effector-like phenotype, and a bioenergetic shift towards lipogenic metabolism with increased mitochondrial spare respiratory capacity (SRC). Tregs primed with IGF-1 more effectively retained their suppressive function upon exposure to low-glucose, high lactate conditioned medium from cancer cells, associated with a transcriptional upregulation of genes related to OXPHOS, the UPR, adipogenesis, mTORC1 and redox regulation. Depletion of IGFs in murine prostate tumours by treatment with xentuzumab caused a significant reduction in the frequency of both tumour-infiltrating CD4⁺ Th17 cells and Tregs, but did not significantly reduce the rate of tumour growth. These findings could relate to a convergent dependency on lipogenic metabolism by Th17 and TI-Tregs, regulated by IGF-1R/mTORC1. Results from PCa patient PBMCs suggest that the identity of peripheral blood T-cells, NKT/MAIT-cells, B-cells and ILCs are sensitive to IGF inhibition, and that a reduction in IGF levels forms a central component of the immune response induced by dietary fasting.

Chapter 8 – Appendix

8.1 Supplementary figures

8.1.1 Chapter 3 supplementary data

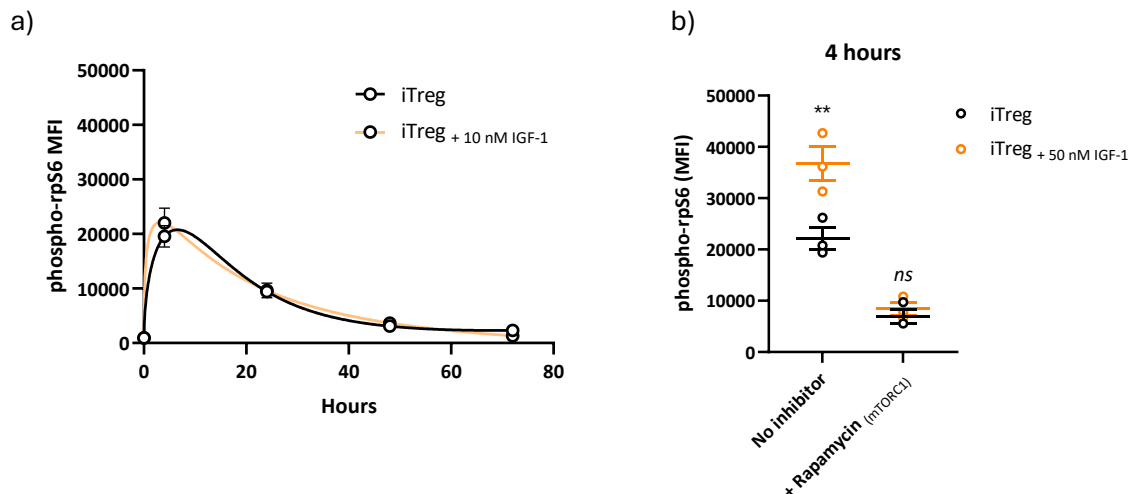


Figure 8.1.1.1 - 10 nM IGF-1 does not significantly increase rpS6 phosphorylation during iTreg differentiation

Sorted naïve murine CD4⁺ T-cells from 6–8-week-old (♂) FVB mice were differentiated under iTreg conditions $\pm$ IGF-1 and analysed by flow cytometry.

- The MFI of phosphorylated-rpS6 was quantified at 0, 4, 24, 48 and 72 hours $\pm$ 10 nM IGF-1 during iTreg differentiation. A non-linear bell-shaped dose response curve was fitted to visually model phosphorylation and expression dynamics, not for statistical comparisons.
- The MFI of phosphorylated-rpS6 was quantified during iTreg differentiation after 4 hours $\pm$ 50 nM IGF-1 in the presence or absence of 100 nM rapamycin (mTORC1 inhibitor).

[ns = $p > 0.05$, ** = $p < 0.01$, statistical significance determined by Two-way ANOVA]

N = 3 independent experiments and donors.

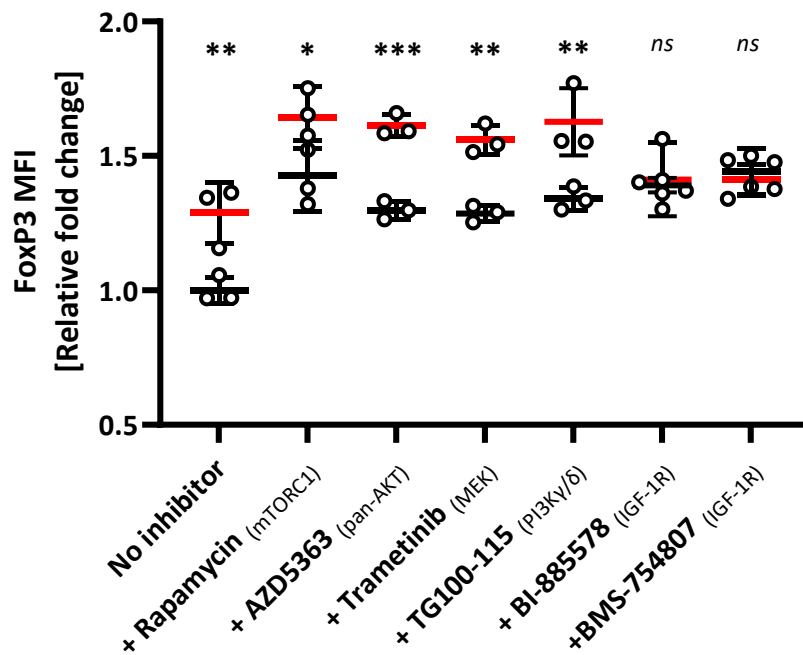


Figure 8.1.1.2 - FoxP3 upregulation during iTreg differentiation with 50 nM IGF-1 is abrogated in the presence of IGF-1R tyrosine kinase inhibitors

Sorted naïve murine CD4⁺ T-cells from 6–8-week-old (♂) FVB mice were differentiated under iTreg conditions $\pm$ 10 nM IGF-1 and analysed by flow cytometry. Where indicated, pathway inhibitors were added to culture medium at the start of iTreg differentiation; rapamycin (100 nM), etomoxir (1 μ M), 5-(Tetradecyloxy)-2-furoic acid [TOFA] (5 μ g mL⁻¹), SMS121 (150 μ M), TG100-155 (1 μ M), AZD5363 (300 nM), trametinib (50 nM), BMS-754807 (1 μ M) or BI-885578 (1 μ M).

[ns = $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $P < 0.001$, by Two-way ANOVA]

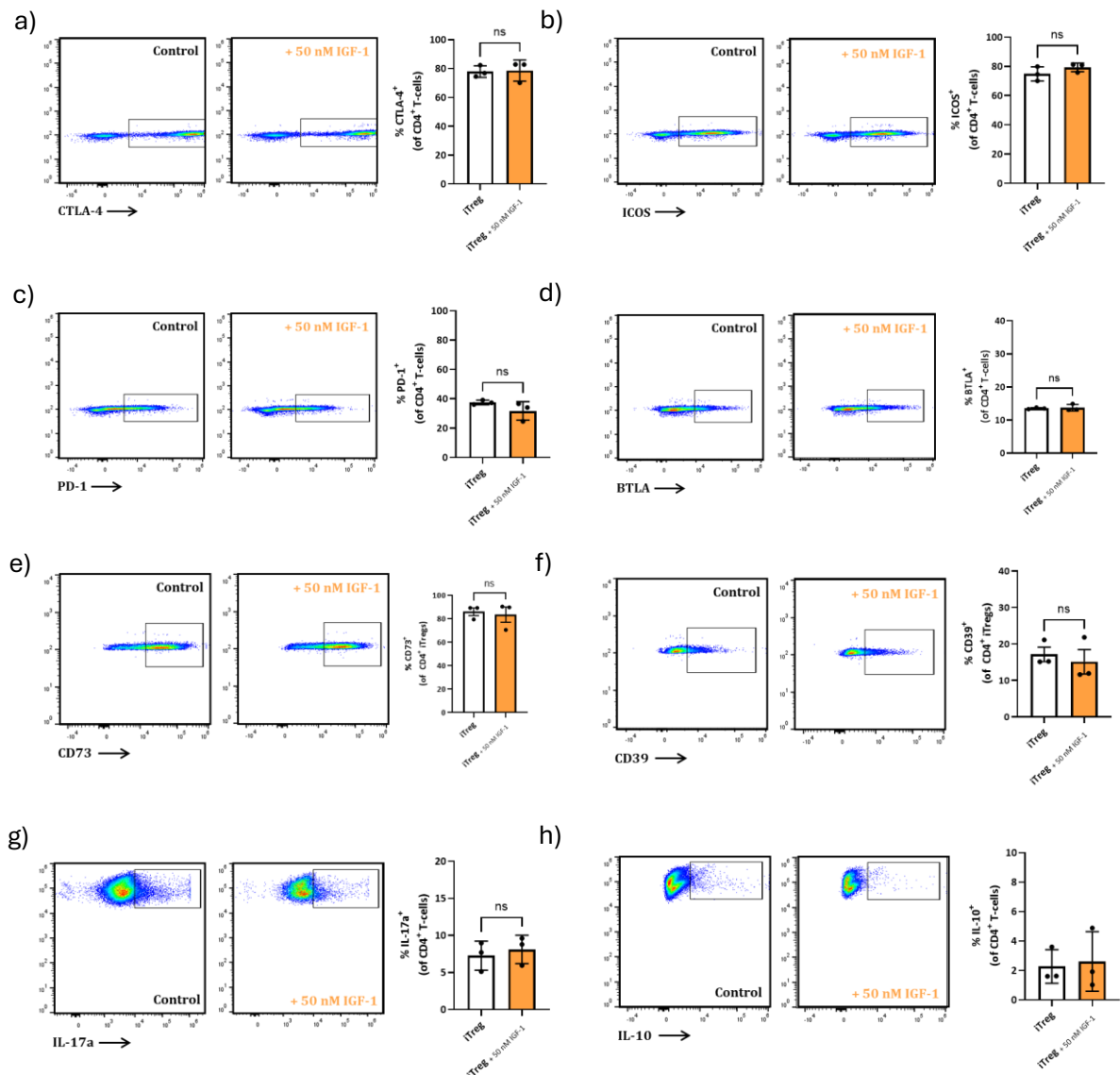


Figure 8.1.1.3 – 50 nM IGF-1 has no effect on other immunoregulatory cytokines and cell-surface markers expressed by iTreg cells

Sorted naïve murine CD4⁺ T-cells from 6–8-week-old (♂) FVB mice were differentiated under iTreg conditions $\pm$ 50 nM IGF-1 for 72 hours and analysed by flow cytometry.

Representative dot plots show sample gating strategy and quantification of percentage positivity for a) CTLA-4, b) ICOS, c) PD-1, d) BTLA, e) CD73, f) CD39, g) IL-17a or h) IL-10 relative to FMO controls.

[ns = $p > 0.05$, by unpaired T-test]

N = 3 independent experiments and donors. Bars represent means $\pm$ SD.

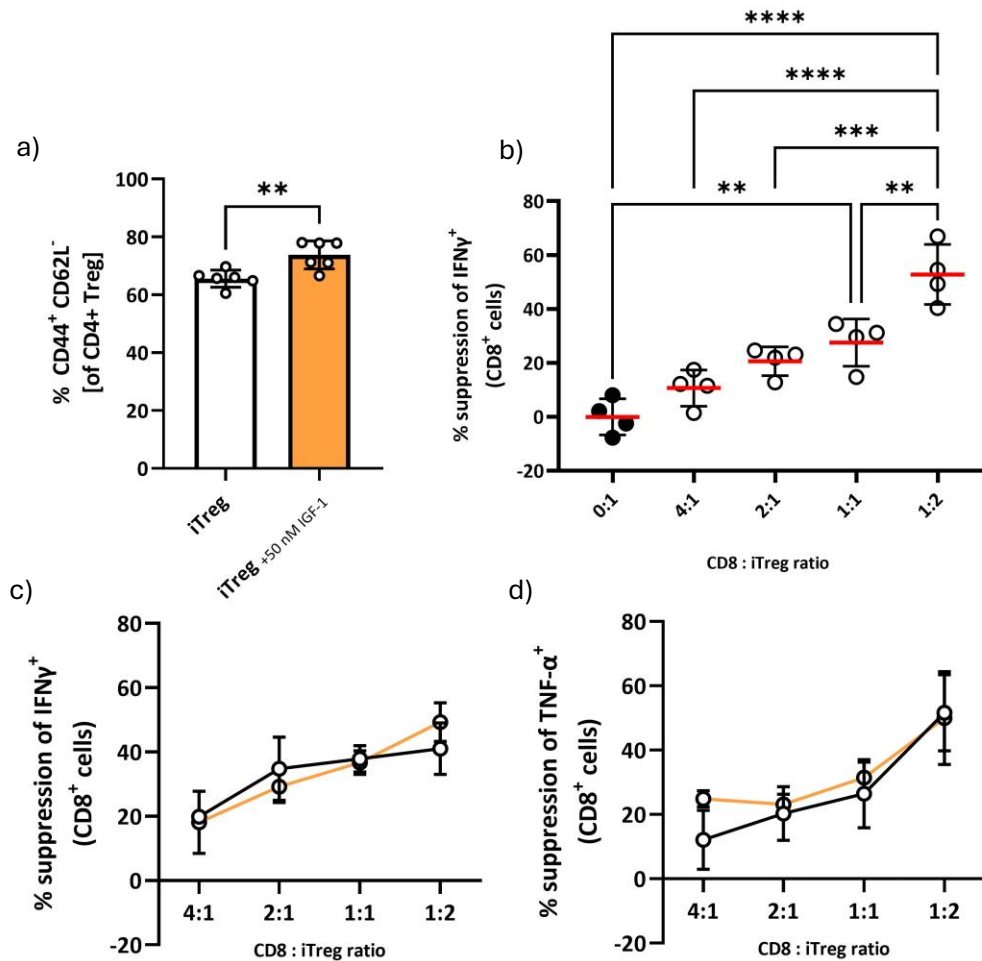


Figure 8.1.1.4 Effects of 50 nM IGF-1 on effector iTreg cell differentiation and suppressive potency

Sorted naïve murine CD4⁺ T-cells from 6–8-week-old (♂) FVB mice were differentiated under iTreg conditions $\pm$ 50 nM IGF-1 for 72 hours.

- The percentage of iTreg cells staining positive for CD44 and negative for CD62L after 72 hours $\pm$ 50 nM IGF-1 (n = 6 independent experiments).
- The percentage suppression of IFN- γ ⁺ in responder CD8⁺ T-cells co-cultured with autologous iTreg cells (differentiated without IGF-1) at the indicated CD8: iTreg ratio after 48 hours (N = 4 independent experiments).
- The percentage suppression of IFN- γ ⁺ in responder CD8⁺ T-cells co-cultured with autologous iTreg cells differentiated $\pm$ 50 nM IGF-1, at the indicated CD8: iTreg ratio after 48 hours (N = 4 independent experiments)
- The percentage suppression of TNF- α ⁺ in responder CD8⁺ T-cells co-cultured with autologous iTreg cells differentiated $\pm$ 50 nM IGF-1, at the indicated CD8: iTreg ratio after 48 hours (N = 4 independent experiments).

[ns = p > 0.05, * = p < 0.05, ** = p < 0.01, *** = P < 0.001, **** = p < 0.0001 by unpaired T-Test (a), one-way ANOVA with multiple comparisons (b) or by Two-way ANOVA (c)]

Bars represent means $\pm$ SD.

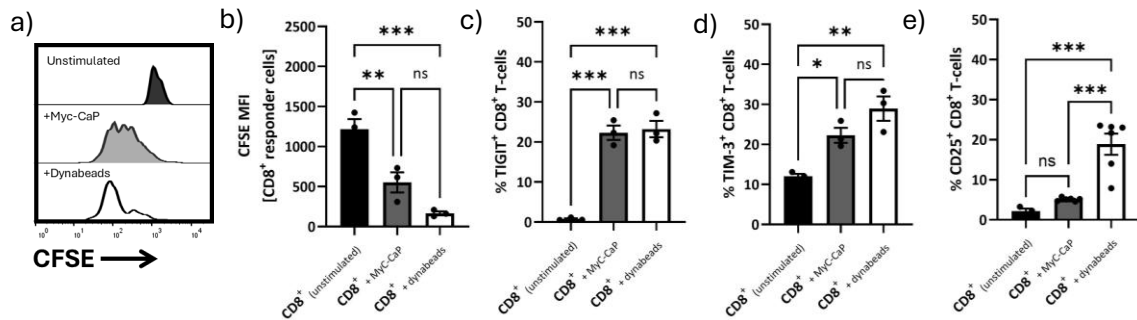


Figure 8.1.1.6 - MyC-CaP cells promote responder CD8⁺ T-cell proliferation and expression of TIGIT, and TIM-3.

CD8⁺ T-cells were sorted magnetically from single-cell suspensions of 6–8-week-old (♂) FVB murine splenocytes, labelled with CFSE, and co-cultured alone (unstimulated), with MyC-CaP cells or anti-CD3/CD28 dynabeads prior to analysis by flow cytometry.

a) Representative histograms of CFSE expression after 48 hours.

b) Quantification of CFSE MFI, c) TIGIT⁺, d) TIM-3⁺ or CD25⁺ (e) responder CD8⁺ T-cells after 48 hours.

[ns = $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $P < 0.001$, by one-way ANOVA with multiple comparisons, N = 3-6 independent experiments and donors. Bars represent means $\pm$ SD.]

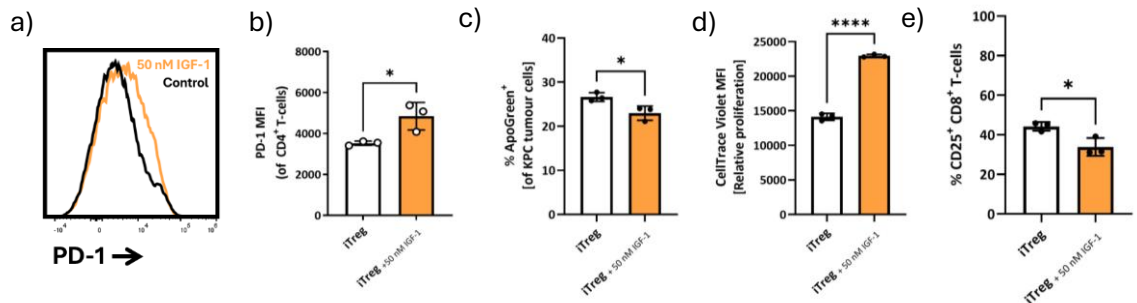


Figure 8.1.1.5 – iTreg cells primed with 50 nM IGF-1 upregulate PD-1 and suppress CD8⁺ CTLs in syngeneic cancer cell co-culture models

iTregs $\pm$ 50 nmol L⁻¹ IGF-1 generated from FVB (a-b) or C57BL/6 (c-e) mice were co-cultured with responder CD8⁺ T-cells (+CD3/CD28 dynabeads), in the presence of MyC-CaP monolayers prior to analysis of cancer cell apoptosis and T-cells by flow cytometry.

a) Representative histograms of PD-1 expression in iTreg cells after 48 hours, quantified in (b).

c) Percentage positivity of ApoGreen KPC cells [CD45⁺, CD4⁺, CD8⁺] after 48 hours.

d) Quantification of responder CD8⁺ T-cell CellTrace Violet proliferation dye MFI, or CD25 percentage positivity (e)

[* = $p < 0.05$, ** = $p < 0.01$, **** = $p < 0.0001$ by unpaired T-test, N = 3 independent experiments and donors, bars represent means $\pm$ SD.]

8.1.2 Chapter 4 supplementary data

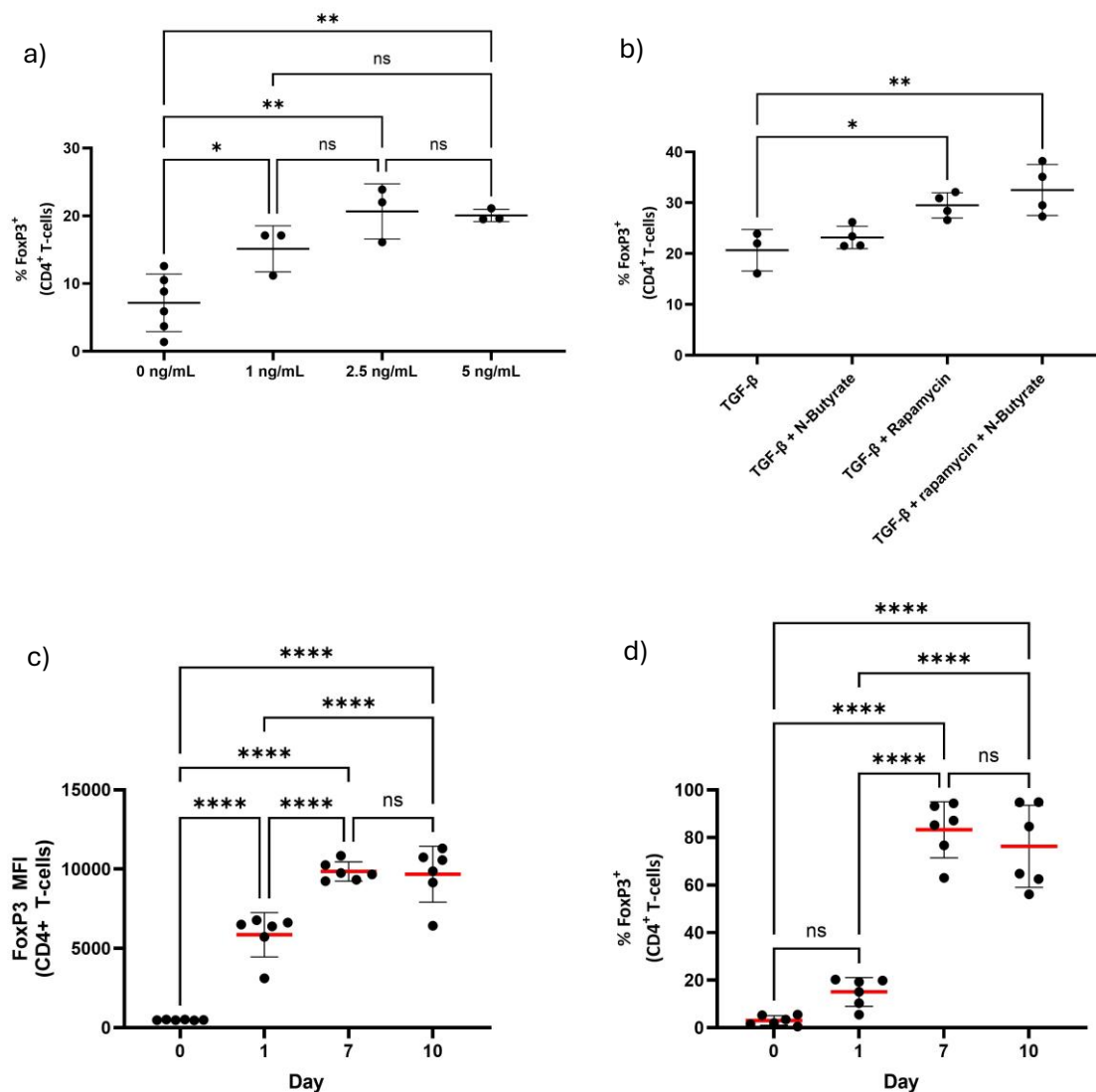


Figure 8.1.1.1 Optimisation of human iTreg differentiation

To assess the optimal concentration of TGF-β, naïve CD4⁺ T-cells isolated from healthy donor human PBMCs were stimulated as described in 2.2.2.7, in the presence of 0, 1, 2.5 or 5 ng / mL TGF-β. The percentage of CD4⁺ T-cells expressing FoxP3 after 72 hours was quantified by flow cytometry (a). Then, we tested the effects of adding sodium butyrate (100 μM) and/or rapamycin (100nM) with 2.5 ng/mL TGF-β. The percentage of CD4⁺ T-cells expressing FoxP3 after 72 hours was quantified by flow cytometry (b).

Finally, we assessed the stability of FoxP3 expression in iTregs differentiated as in 2.2.2.7. To test this, we replaced iTreg culture medium with fresh medium without anti-CD3/CD28 or TGF-β stimulation at day 7, and quantified the MFI (c) and percentage positivity (d) of FoxP3 in CD4⁺ T-cells by flow cytometry at PBMC isolation (day 0), day 1, day 7 and day 10.

[ns = $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$ by one-way ANOVA with multiple comparisons, N = 3-6 independent experiments and donors, bars represent means ± SD.]

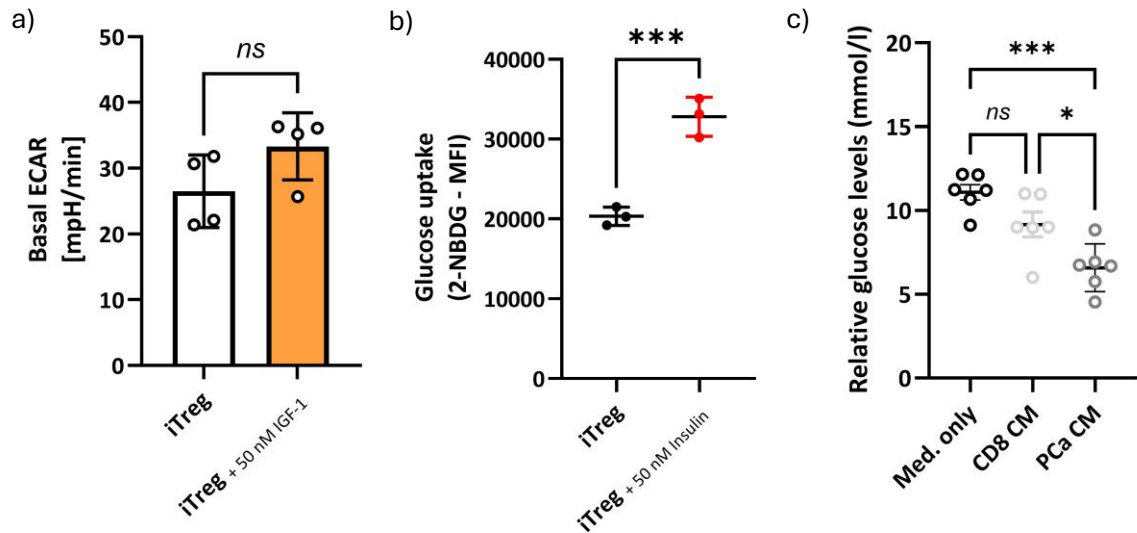


Figure 8.1.2.2 – Glycolysis and glucose uptake in murine iTreg cells, glucose measurements of conditioned medium

iTregs were differentiated as in 2.2.2.7 from FVB mice $\pm$ 50 nM IGF-1 (a) or insulin (b).

a) Quantification of the basal extracellular acidification rate prior to FCCP injection by metabolic flux analysis (2.2.5.3) where mpH/min = extracellular acidification rate. (N = 2 independent experiments with 4 different donors).

b) Quantification of glucose uptake (2.2.5.1) by analysis of 2-NBDG staining by flow cytometry, 72 hours post differentiation. (N = 3 independent experiments and donors).

c) Quantification of extracellular glucose concentrations in fully supplemented RPMI 1640, CD8⁺ CM or MyC-CaP CM (see 2.2.5.4) by Glucose-Glo assay. (N = 6 independent experiments and donors) .

[ns = $p > 0.05$, * = $p < 0.05$, *** = $p < 0.001$, by unpaired T-test (a-b) or one-way ANOVA with multiple comparisons (c). Bars represent means $\pm$ SD.]

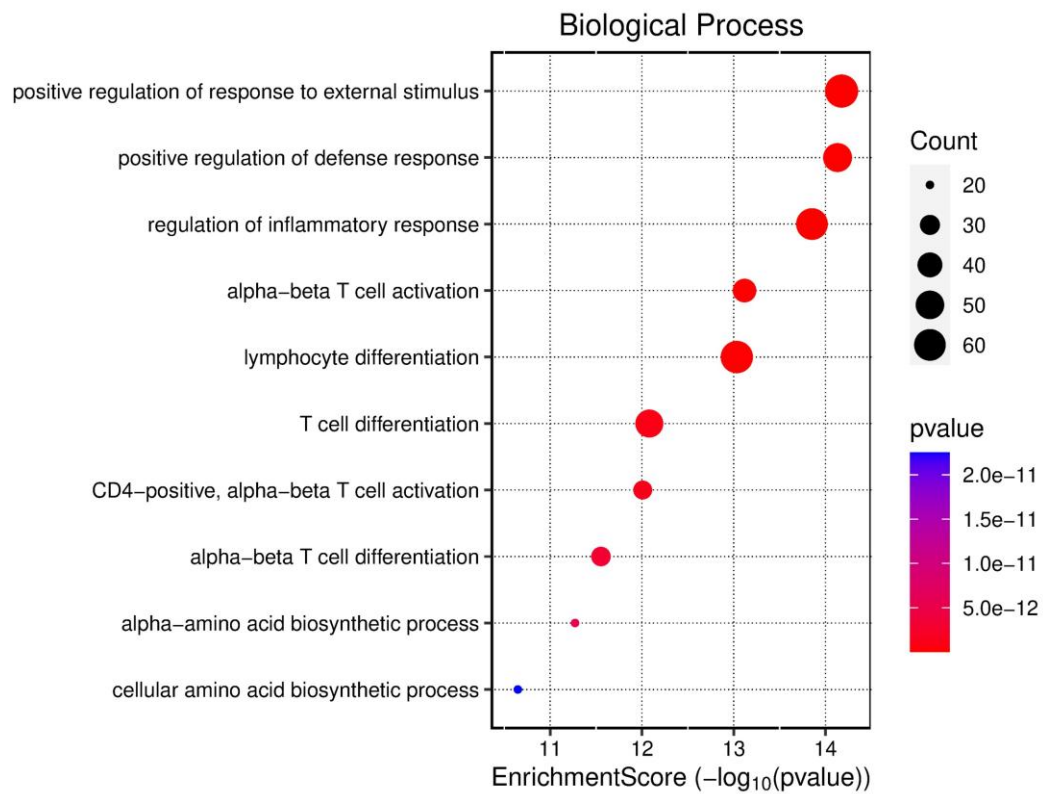


Figure 8.1.2.3 – Over representation analysis of GO: Biological Processes pathways

GO enrichment plot displaying the top 10 biological processes pathways from mRNA-Seq DEGs in iTreg cells stimulated with anti-CD3/CD28 dynabeads in PCa CM $\pm$ 50 nM IGF-1.

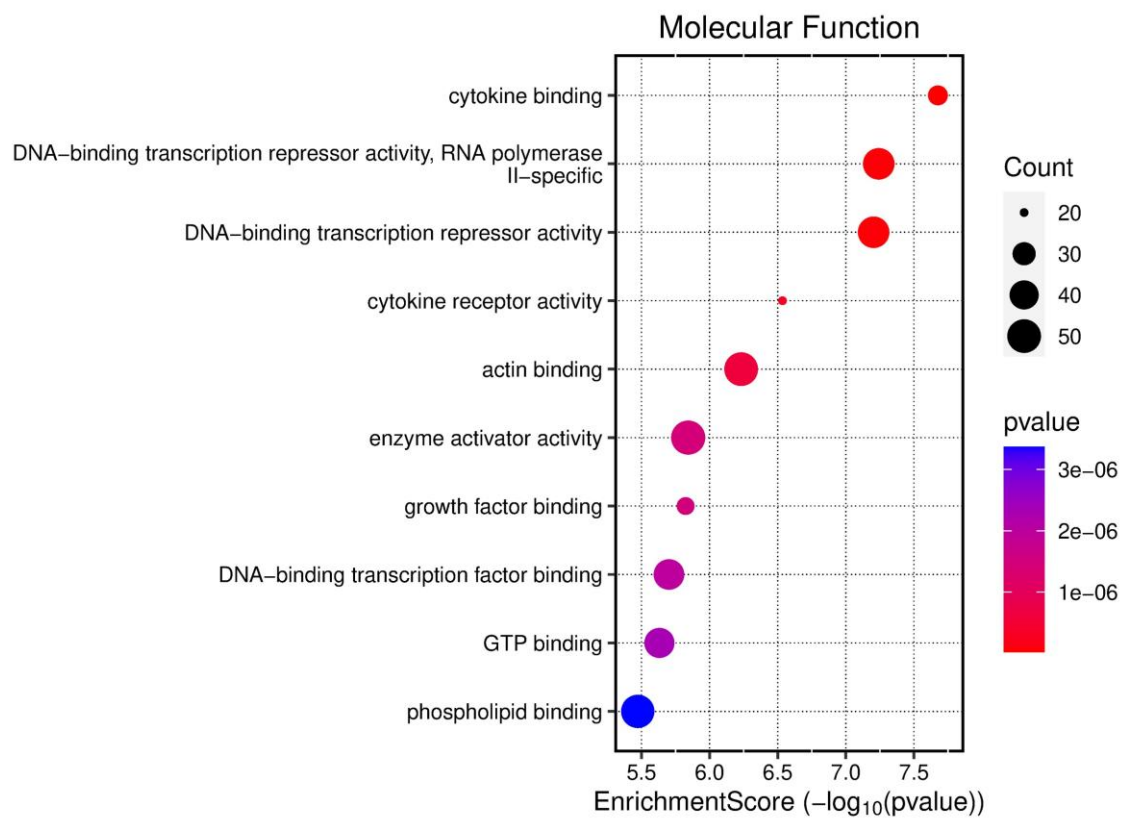


Figure 8.1.2.4 - Over representation analysis of GO: Molecular function pathways

GO enrichment plot displaying the top 10 molecular function pathways from mRNA-Seq DEGs in iTreg cells stimulated with anti-CD3/CD28 dynabeads in PCa CM $\pm$ 50 nM IGF-1.

8.1.3 Chapter 5 supplementary data

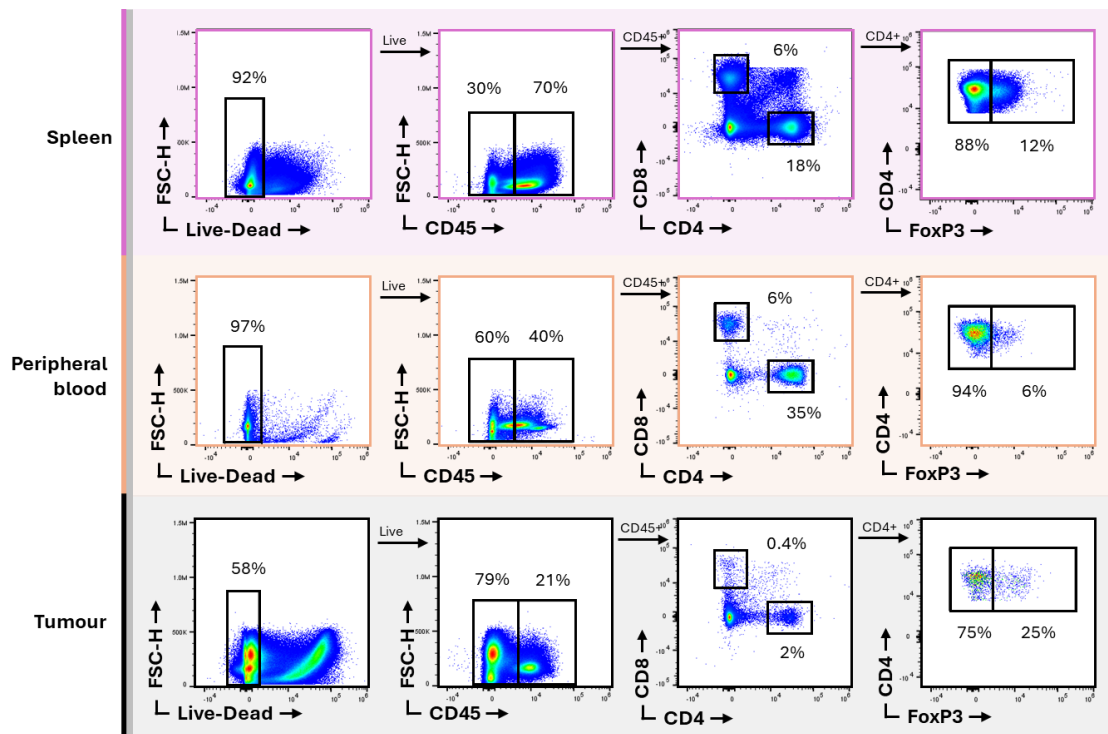


Figure 8.1.3.1 – Sample bi-axial gating strategy used for immunophenotyping of T-cell subsets from the spleen, peripheral blood or tumour from FVB mice.

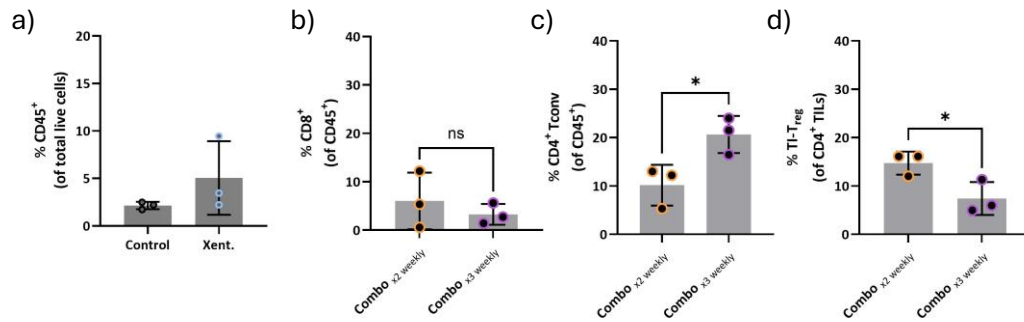


Figure 8.1.3.2 – Quantification of immune cell tumour infiltrates in MyC-CaP allografts.

a) Quantification of CD45⁺ cells as a percentage of total live cells from MyC-CaP tumours treated with PBS (control) or Xentuzumab (Xent.) at a tumour volume of 400 mm³.

b) Quantification of the percentage of CD8⁺ or CD4⁺ Tconv (c) TILs as a percentage of total CD45⁺ immune cells, or c) TI-Tregs as a percentage of CD4⁺ TILs in tumour-bearing mice (5.4.1) treated with twice-weekly or three-times weekly anti-PD-1 and Xentuzumab treatments.

[N=3, by unpaired T-test, bars represent means $\pm$ SD.]

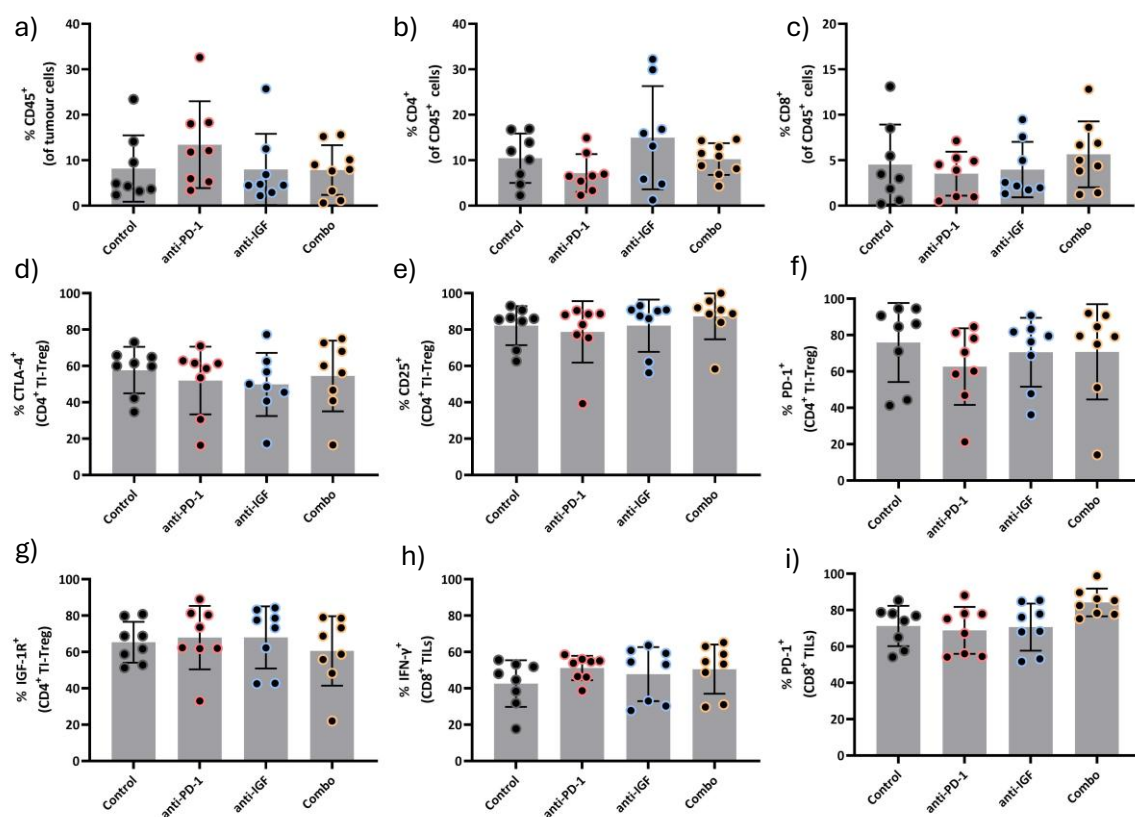


Figure 8.1.3.3 – Supplementary TIL analysis from efficacy studies of Xentuzumab with anti-PD-1

Tumour bearing mice received either x2 weekly intra-peritoneal injections of PBS (control), anti-PD-1, Xentuzumab (anti-IGF) or anti-PD-1 and Xentuzumab (combo). At a tumour volume of 800 mm³, tumours were disaggregated and TILs analysed by flow cytometry.

- a) The percentage of total tumour cells that are CD45⁺ immune cells.
- b) The percentage of TI-CD45⁺ cells that are CD4⁺ T-cells.
- c) The percentage of TI-CD45⁺ cells that are CD8⁺ T-cells.
- d) The percentage of TI-Tregs cells that are CTLA-4⁺, CD25⁺(e), PD-1⁺ (f) or g) IGF-1R⁺.
- h) The percentage of CD8⁺ TILs staining positive for IFN-γ or PD-1 (i).

[Statistical significance determined by one-way ANOVA with multiple comparisons, N = 8].

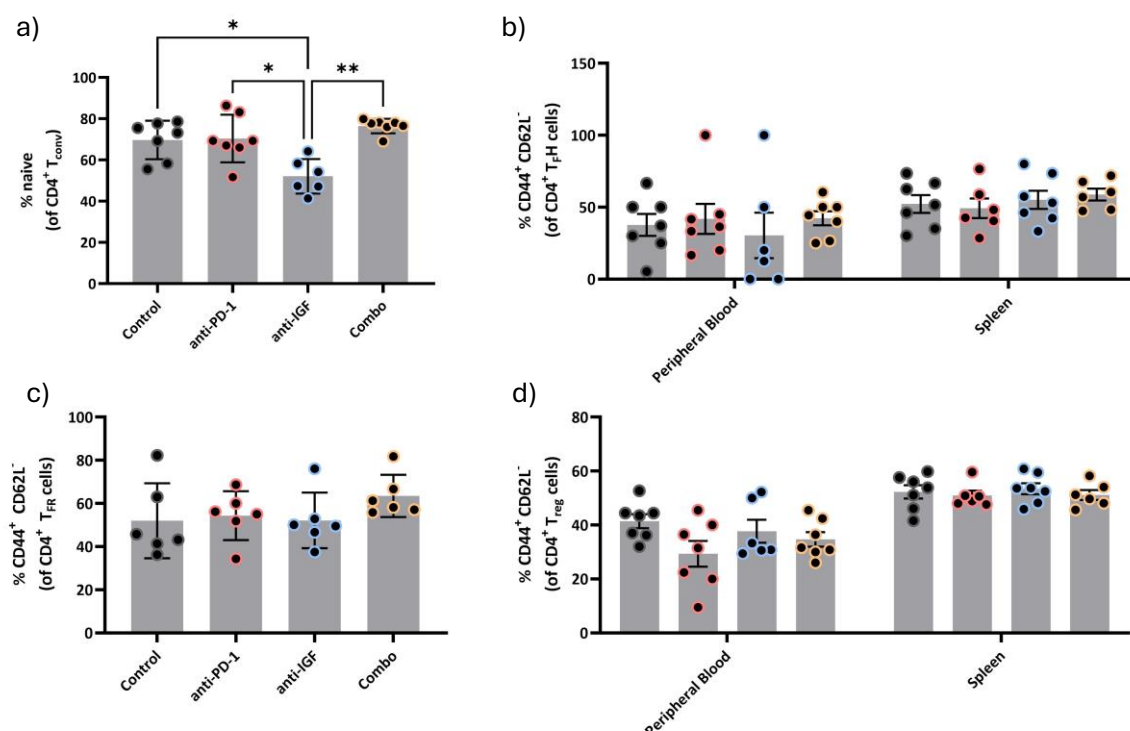


Figure 8.1.3.4 – Peripheral T-cell subset immunophenotyping

Tumour bearing mice received either x2 weekly intra-peritoneal injections of PBS (control), anti-PD-1, Xentuzumab (anti-IGF) or anti-PD-1 and Xentuzumab (combo). Peripheral blood and splenocytes were analysed by flow cytometry at a tumour volume of 800 mm³.

- The percentage of naïve cells (CD44⁻ CD62L⁺) of total CD4⁺ T_{conv} cells in the peripheral blood.
- The percentage of effector T_{FH} cells (CD44⁺ CD62L⁻) in the peripheral blood and spleen.
- The percentage of effector T_{FR} cells (CD44⁺ CD62L⁻) in the spleen.
- The percentage of effector Treg cells (CD44⁺ CD62L⁻) in the peripheral blood and spleen.

[* = $p < 0.05$, ** = $p < 0.01$ - with statistical significance determined by one-way ANOVA with multiple comparisons (a,c) or by two-way ANOVA (b-d), N = 6-8].

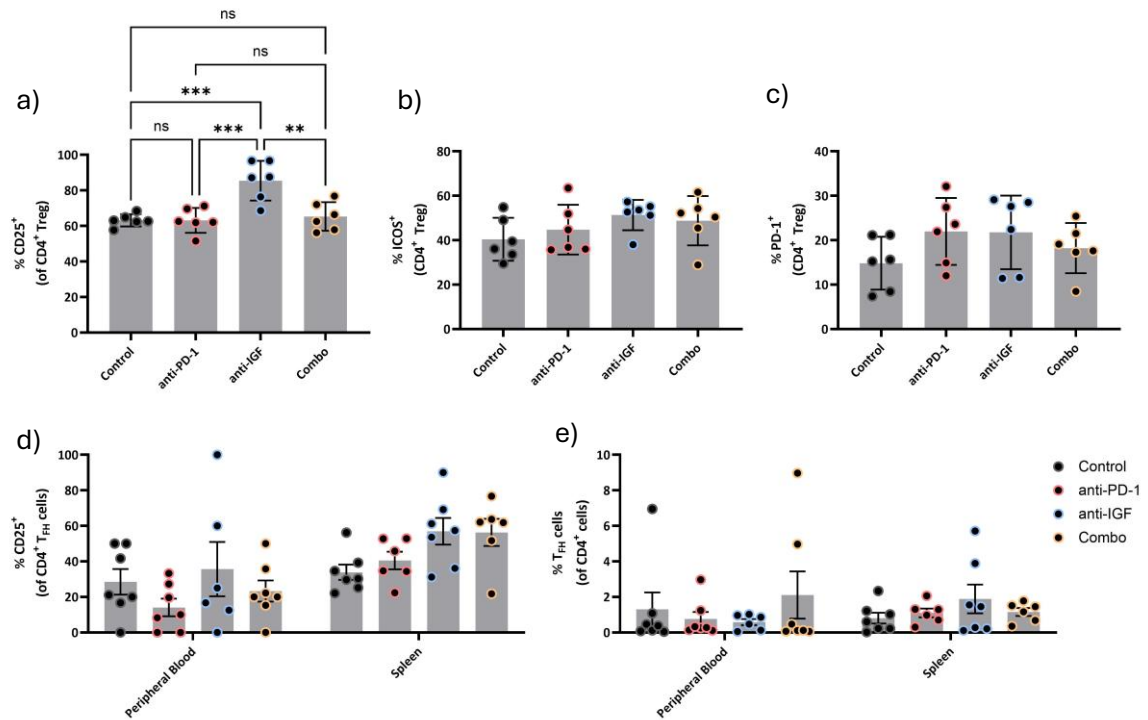


Figure 8.1.3.5 – Treg and T_{FH} peripheral immunophenotyping

Tumour bearing mice received either x2 weekly intra-peritoneal injections of PBS (control), anti-PD-1, Xentuzumab (anti-IGF) or anti-PD-1 and Xentuzumab (combo). Peripheral blood and splenocytes were analysed by flow cytometry at a tumour volume of 800 mm³.

a) The percentage positivity of CD25, ICOS (b) or PD-1 (c) in splenic Tregs.

d) The percentage positivity of CD25 in T_{FH} cells in the peripheral blood and spleen.

e) The percentage of total CD4⁺ T-cells that are T_{FH} cells in the peripheral blood and spleen.

[ns = $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ - with statistical significance determined by one-way ANOVA with multiple comparisons (a-c) or by two-way ANOVA (d-e), N = 6-8].

8.1.4 Chapter 6 supplementary data

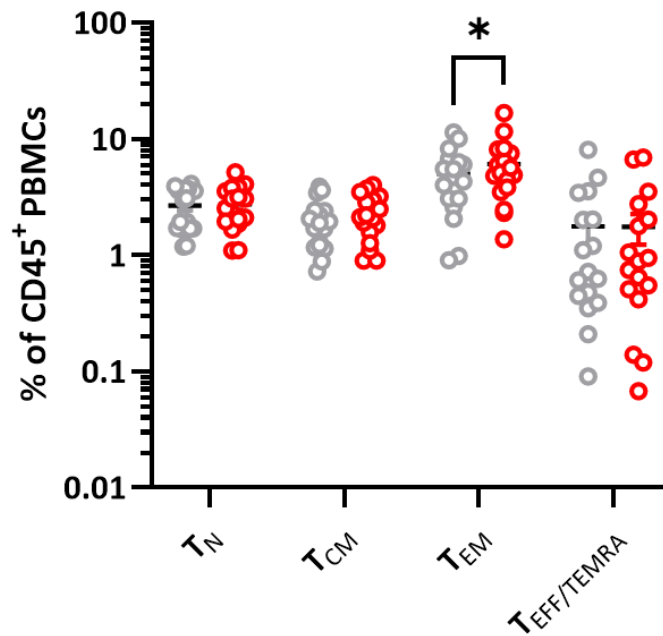


Figure 8.1.3.1 Xentuzumab treatment promotes the expansion of peripheral blood effector memory CD8⁺ T-cells as a percentage of total CD45⁺ cells

T_N = CCR7^{hi} CD45RA⁺ CD45RO⁻

T_{CM} = CCR7^{hi} CD45RA⁻ CD45RO⁺

T_{EM} = CCR7^{lo/-} CD45RA⁻ CD45RO⁺ CD27⁺

T_{EFFECT/EMRA} = CCR7^{lo/-} CD45RA⁺ CD45RO⁻ CD27⁻

[* = p < 0.05, Wilcoxon signed-rank test, N = 17, bars represent mean ± SEM.]

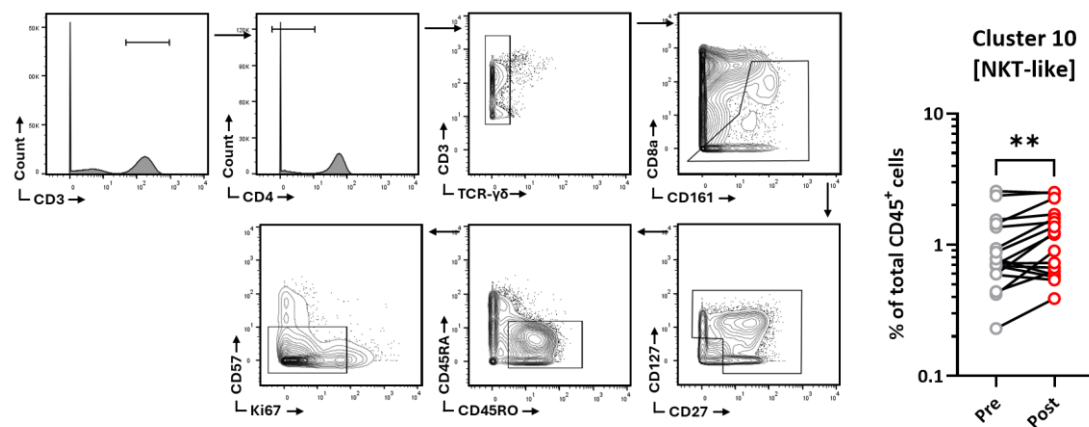


Figure 8.1.4.2 – Hyperfinder gating strategy to identify an NKT-like population that expands as a percentage of total CD45⁺ cells after treatment with Xentuzumab

[** = $p < 0.01$, paired T-test, $N = 17$]

8.1.5 Supplementary tables

Table 8.1.5.1 List of differentially expressed genes from DESeq2 analysis (see 2.6.6)

Gene symbol	Base mean	Log2FC	StdErr	Wald-Stats	P-Value	P-Adj
Slc3a2	4392.008762	1.72361	0.10596	16.2666	1.70E-59	2.19E-55
Asns	2242.040142	2.70597	0.172519	15.68504	1.91E-55	1.23E-51
Sell	7043.508047	-1.85236	0.125193	-14.7961	1.55E-49	6.65E-46
Apold1	546.7254954	2.7549	0.186761	14.75096	3.03E-49	9.74E-46
Slc7a5	6944.493916	2.13784	0.150508	14.20414	8.64E-46	2.22E-42
Map3k20	1718.542892	1.845652	0.130187	14.17695	1.27E-45	2.72E-42
Ddit3	1097.397843	1.907563	0.140364	13.59011	4.58E-42	8.41E-39
Pdlim4	1275.021561	-2.00313	0.147509	-13.5797	5.28E-42	8.48E-39
Sh3bp5	2078.264581	-1.44974	0.107232	-13.5196	1.20E-41	1.71E-38
Slc6a9	257.0383954	2.956771	0.220645	13.40056	6.00E-41	7.70E-38
Shmt2	5290.19652	1.479094	0.113923	12.98329	1.52E-38	1.78E-35
Atf4	3930.084352	1.225713	0.094811	12.92798	3.13E-38	3.35E-35
Nod1	205.6834764	-3.04403	0.240012	-12.6829	7.36E-37	7.27E-34
Drc1	165.9965313	-3.95926	0.319004	-12.4113	2.27E-35	2.08E-32
Slc1a4	316.1312662	2.528336	0.207062	12.21052	2.73E-34	2.34E-31
Cars1	2357.919668	1.41404	0.11587	12.20367	2.97E-34	2.38E-31
Dgat1	6496.172201	1.274535	0.105936	12.03123	2.44E-33	1.80E-30
Tg	8742.816221	1.14531	0.095217	12.02846	2.52E-33	1.80E-30
H1f0	10748.79139	1.074221	0.091905	11.68842	1.46E-31	9.87E-29
Nfkbia	4422.996675	-1.15416	0.098976	-11.661	2.02E-31	1.29E-28
Paxbp1	12751.25886	1.365498	0.117394	11.63174	2.84E-31	1.74E-28
Gbp7	656.2190656	-1.5799	0.137687	-11.4746	1.77E-30	1.03E-27
Bcat1	1024.508295	1.483936	0.130027	11.4125	3.62E-30	2.02E-27
Rasa3	9410.314273	-1.27552	0.112697	-11.3181	1.07E-29	5.71E-27
Nars1	5981.418338	1.244014	0.110067	11.30236	1.28E-29	6.56E-27
Psat1	8026.232425	1.237084	0.114377	10.81582	2.90E-27	1.43E-24
Slpr1	5676.361954	-1.14449	0.105906	-10.8067	3.20E-27	1.52E-24
Gipr	835.7134744	2.057955	0.194084	10.60343	2.87E-26	1.32E-23
Ipcefl	1108.278028	-1.39457	0.131876	-10.5749	3.90E-26	1.73E-23
Mars1	2938.451703	1.21741	0.116688	10.43306	1.75E-25	7.50E-23
Stc1	423.2741741	-1.7444	0.167279	-10.4281	1.85E-25	7.64E-23
Soat2	409.7406074	2.131543	0.20637	10.32874	5.22E-25	2.10E-22
Vipr1	580.9979889	-2.27672	0.225228	-10.1085	5.07E-24	1.97E-21
Abcg1	1662.674083	-1.25578	0.124328	-10.1005	5.50E-24	2.08E-21
Ms4a6c	1134.284061	-1.22582	0.124245	-9.86612	5.84E-23	2.08E-20
Pck2	4021.435332	1.024535	0.103845	9.866029	5.84E-23	2.08E-20
Ms4a4b	285.4078561	-2.0635	0.209355	-9.85644	6.43E-23	2.23E-20
Gpt2	535.2285885	1.607215	0.165331	9.721169	2.45E-22	8.28E-20
Xpot	2225.235443	1.157072	0.119085	9.716341	2.57E-22	8.46E-20
Iars1	2960.071259	1.102888	0.113696	9.700311	3.01E-22	9.65E-20
Tnfrsf9	12451.51652	0.97296	0.100434	9.687582	3.40E-22	1.07E-19
Tgfb3	1429.555118	1.584589	0.164805	9.614905	6.92E-22	2.11E-19
Foxp3	1213.534462	-1.20444	0.125295	-9.61279	7.06E-22	2.11E-19

Tgm2	17741.2041	1.456617	0.152113	9.57591	1.01E-21	2.95E-19
Il18r1	1411.528817	-1.42625	0.149112	-9.56494	1.12E-21	3.20E-19
Cd93	272.461008	2.505609	0.262695	9.538099	1.45E-21	4.06E-19
Pde2a	794.2869429	-1.41652	0.150591	-9.40637	5.14E-21	1.40E-18
Emp1	1113.524133	-1.43818	0.154252	-9.32357	1.12E-20	3.01E-18
Nfil3	2877.095506	0.954751	0.102528	9.312118	1.25E-20	3.28E-18
Ripor2	4229.429664	-1.06295	0.114912	-9.25016	2.24E-20	5.76E-18
Trib3	1713.059425	1.187263	0.128985	9.204675	3.43E-20	8.63E-18
Osm	1310.475209	-1.15444	0.126213	-9.1468	5.86E-20	1.44E-17
Sars1	8528.430531	0.843637	0.092251	9.14502	5.96E-20	1.44E-17
Mthfd2	4085.629061	1.076689	0.118161	9.112024	8.09E-20	1.92E-17
Spry1	595.5558625	-1.68363	0.186018	-9.05094	1.42E-19	3.31E-17
Gars	6131.355091	0.850935	0.094154	9.037655	1.60E-19	3.67E-17
Pycr1	193.9345439	2.039471	0.22735	8.970609	2.95E-19	6.64E-17
Ramp3	962.5897611	1.26482	0.143269	8.828315	1.06E-18	2.35E-16
Dmwd	334.6879141	1.61339	0.182852	8.823464	1.11E-18	2.41E-16
Galc	468.1425759	1.383594	0.158175	8.747265	2.19E-18	4.68E-16
Yars1	2637.647991	0.99285	0.115553	8.592159	8.54E-18	1.80E-15
Arhgef18	3252.498702	-0.98234	0.114708	-8.56383	1.09E-17	2.26E-15
Slc7a3	212.4583089	2.519362	0.29447	8.555572	1.17E-17	2.39E-15
Myc	5462.141803	-0.97292	0.114323	-8.51026	1.74E-17	3.48E-15
Tlr1	1316.079467	-1.06438	0.12519	-8.50214	1.86E-17	3.68E-15
Samd9l	346.7245133	-1.5072	0.177442	-8.49403	2.00E-17	3.88E-15
Nedd4l	504.971849	-1.31308	0.154956	-8.47384	2.37E-17	4.55E-15
Aqp3	1097.104641	-1.41996	0.168364	-8.43386	3.34E-17	6.31E-15
Atf5	595.2769278	1.434819	0.170568	8.412032	4.03E-17	7.50E-15
Rasgrp2	1469.673935	-1.21286	0.144453	-8.39623	4.61E-17	8.46E-15
Trib2	4740.16696	-0.92211	0.11127	-8.28721	1.16E-16	2.10E-14
Cmah	2235.367179	-1.32201	0.159691	-8.27852	1.25E-16	2.22E-14
Ccr7	9690.604775	-0.80949	0.097868	-8.27124	1.33E-16	2.33E-14
Bhlhe40	9054.911145	0.81972	0.099873	8.207663	2.26E-16	3.86E-14
Gnptab	1800.481551	0.916791	0.1117	8.207646	2.26E-16	3.86E-14
Aars1	4977.495269	1.101601	0.134459	8.192864	2.55E-16	4.31E-14
Samhd1	32594.57183	-0.86688	0.106537	-8.13685	4.06E-16	6.76E-14
Gramd2b	2091.875659	-1.13345	0.140143	-8.0878	6.08E-16	1.00E-13
Gpr65	2022.758295	1.391007	0.172268	8.074653	6.77E-16	1.10E-13
Plcd1	574.5009087	1.403644	0.174423	8.047348	8.46E-16	1.36E-13
Chchd10	3082.938071	0.919603	0.11491	8.002797	1.22E-15	1.93E-13
Ppp1r3b	3274.965333	0.942649	0.11783	8.000056	1.24E-15	1.95E-13
Ank	931.913749	-0.98613	0.124047	-7.94962	1.87E-15	2.89E-13
Cyria	638.0419392	-1.06394	0.134298	-7.92221	2.33E-15	3.57E-13
Klf3	1848.554387	-1.11691	0.141145	-7.91316	2.51E-15	3.79E-13
Cd27	2059.466662	-1.11692	0.141226	-7.90873	2.60E-15	3.88E-13
Tgtp1	693.288904	-1.47124	0.186518	-7.88792	3.07E-15	4.53E-13
Gbp5	397.5032498	-1.28734	0.163728	-7.8627	3.76E-15	5.48E-13
Gbp2	1654.692821	-0.88585	0.11294	-7.84358	4.38E-15	6.32E-13
Atf3	502.3008976	1.190911	0.151913	7.839437	4.53E-15	6.46E-13
Ero1a	12987.79493	1.051617	0.134251	7.833197	4.76E-15	6.71E-13

Plin2	732.1663818	1.198636	0.153776	7.794675	6.46E-15	9.01E-13
Txk	1464.548946	-0.90736	0.116984	-7.75626	8.75E-15	1.21E-12
Cyb5r1	976.7966696	0.919344	0.118827	7.736805	1.02E-14	1.39E-12
Gbp11	270.7490552	-1.58681	0.205896	-7.70686	1.29E-14	1.73E-12
Tiam1	2113.707901	-1.08228	0.140435	-7.7066	1.29E-14	1.73E-12
Fam20a	174.6274018	-1.71129	0.222541	-7.68975	1.47E-14	1.95E-12
Chac1	197.5303007	2.971404	0.387303	7.672029	1.69E-14	2.22E-12
Tcf7	10763.97879	-0.7988	0.10414	-7.67047	1.71E-14	2.22E-12
Lyst	1553.718328	-1.49117	0.194742	-7.65714	1.90E-14	2.44E-12
Slamf6	947.3743033	-1.34547	0.176262	-7.63336	2.29E-14	2.91E-12
Nin	2694.634674	-1.02601	0.134701	-7.61697	2.60E-14	3.27E-12
Igtp	3635.328867	-0.84175	0.110837	-7.59451	3.09E-14	3.85E-12
Sbk1	1028.919829	-1.37408	0.182377	-7.53427	4.91E-14	6.06E-12
A930005H10Rik	557.2362993	-1.20049	0.159425	-7.53014	5.07E-14	6.20E-12
Foxp1	3535.553549	-0.78082	0.103807	-7.52182	5.40E-14	6.54E-12
Lilrb4a	796.534442	1.392712	0.185616	7.503194	6.23E-14	7.47E-12
Slc15a3	594.000541	1.078133	0.14394	7.490134	6.88E-14	8.18E-12
Igfbp4	3258.88208	-0.81656	0.109333	-7.46856	8.11E-14	9.55E-12
Aldh18a1	3451.34382	0.784843	0.105485	7.440332	1.00E-13	1.17E-11
Adgre5	5942.432235	-0.70637	0.095515	-7.39534	1.41E-13	1.63E-11
Slc43a1	82.58578646	-2.8922	0.391973	-7.37857	1.60E-13	1.83E-11
Aven	860.4764621	-0.91701	0.124424	-7.37006	1.71E-13	1.94E-11
Slc2a8	948.6526738	1.027451	0.140596	7.307816	2.72E-13	3.06E-11
Cd7	305.5306737	-1.63742	0.225526	-7.26042	3.86E-13	4.31E-11
Mxd1	4338.295363	-0.67931	0.093579	-7.25919	3.89E-13	4.31E-11
Hspa9	6058.332417	0.755199	0.104239	7.244896	4.33E-13	4.75E-11
Mtmr3	7640.282909	-0.62286	0.086272	-7.21969	5.21E-13	5.67E-11
Lef1	8821.690339	-0.73777	0.102665	-7.18612	6.67E-13	7.19E-11
Ahcyl2	7033.052206	-0.82754	0.115692	-7.15292	8.49E-13	9.09E-11
Map6	538.960723	-1.61389	0.225721	-7.14992	8.68E-13	9.21E-11
Dapl1	1191.573885	-0.82222	0.115028	-7.14802	8.80E-13	9.27E-11
Tafa3	327.4258976	-1.50838	0.211373	-7.1361	9.60E-13	1.00E-10
Fbln1	1440.205094	-0.99604	0.1397	-7.12984	1.00E-12	1.04E-10
Atp1a1	38762.47827	-0.64684	0.091072	-7.10258	1.22E-12	1.26E-10
Hilpda	1065.667469	0.805181	0.114308	7.043961	1.87E-12	1.90E-10
Pecam1	3346.832459	-0.68722	0.097631	-7.039	1.94E-12	1.96E-10
Rap1gap2	268.1713261	-1.69873	0.242189	-7.01409	2.31E-12	2.32E-10
Pik3r5	2659.200839	-0.8901	0.127094	-7.0035	2.50E-12	2.48E-10
Tbc1d31	1261.706245	0.76479	0.109351	6.993874	2.67E-12	2.64E-10
Klf2	4555.715328	-0.97234	0.139399	-6.97524	3.05E-12	2.99E-10
Psph	1808.248703	1.024183	0.147012	6.966688	3.24E-12	3.16E-10
Filip1l	1029.730253	-0.90899	0.130858	-6.94637	3.75E-12	3.62E-10
Gpm6b	810.9896487	0.995315	0.144135	6.905418	5.01E-12	4.80E-10
St8sia6	509.3514925	-1.28762	0.186598	-6.90052	5.18E-12	4.93E-10
Gm12250	388.8163225	-1.11958	0.16231	-6.8978	5.28E-12	4.99E-10
Ncf1	1209.501303	-1.07224	0.155553	-6.89306	5.46E-12	5.12E-10
Prss12	88.66457391	-2.26139	0.328136	-6.89162	5.52E-12	5.13E-10
Cyth1	4257.318891	-0.7274	0.105581	-6.8895	5.60E-12	5.17E-10

Slc15a1	182.8957631	1.878161	0.273786	6.859967	6.89E-12	6.32E-10
Baspl	419.2729491	1.671193	0.244612	6.832004	8.37E-12	7.62E-10
Kbtbd11	9673.901411	-0.84386	0.124201	-6.79427	1.09E-11	9.84E-10
N4bp1	1422.845375	-0.92991	0.137032	-6.78607	1.15E-11	1.03E-09
Izumolr	3989.979158	-0.67969	0.100909	-6.73571	1.63E-11	1.45E-09
Lilrb4b	970.0664562	1.28076	0.193181	6.629859	3.36E-11	2.98E-09
Tnfsf8	697.3282834	-1.18042	0.178965	-6.59582	4.23E-11	3.72E-09
Rbpj	6241.478815	0.842969	0.127851	6.593351	4.30E-11	3.76E-09
Fam53b	3075.950111	-0.91427	0.138912	-6.58163	4.65E-11	4.04E-09
Asb2	1160.066318	1.023781	0.156164	6.555812	5.53E-11	4.77E-09
Pde1a	133.4309805	1.7378	0.266054	6.531763	6.50E-11	5.56E-09
Resf1	1633.398948	-0.89372	0.137054	-6.52097	6.99E-11	5.94E-09
Lats2	894.4353216	-0.98983	0.151939	-6.51465	7.29E-11	6.15E-09
Patj	1351.317588	-1.11667	0.172305	-6.48078	9.13E-11	7.66E-09
Nav2	149.7173463	-1.88647	0.291686	-6.46748	9.96E-11	8.31E-09
Dnai4	618.119567	0.905404	0.140057	6.464533	1.02E-10	8.42E-09
Smim3	390.3517493	1.231251	0.19064	6.458519	1.06E-10	8.70E-09
Selenbp1	1694.182702	0.958523	0.148969	6.434379	1.24E-10	1.01E-08
Nfkbiz	1593.917131	-0.92978	0.144676	-6.42666	1.30E-10	1.06E-08
4930404I05Rik	249.380566	1.261937	0.19702	6.405131	1.50E-10	1.21E-08
Slc17a6	77.67356121	2.131436	0.332926	6.402133	1.53E-10	1.23E-08
Sesn2	504.5551002	0.945787	0.147742	6.401593	1.54E-10	1.23E-08
Iigp1	146.0577813	-1.5795	0.248302	-6.36119	2.00E-10	1.59E-08
Adpgk	822.4336744	-0.78112	0.122857	-6.358	2.04E-10	1.61E-08
Amigo2	153.1365636	-1.64304	0.258565	-6.35445	2.09E-10	1.64E-08
Tars1	2127.437459	0.719079	0.113297	6.34683	2.20E-10	1.71E-08
Ift80	2504.460305	-0.71822	0.113198	-6.34476	2.23E-10	1.72E-08
Il6ra	2056.410014	-1.23872	0.19553	-6.33517	2.37E-10	1.82E-08
Snn	781.2168406	-0.87899	0.138951	-6.32594	2.52E-10	1.92E-08
Phgdh	6595.626977	0.59215	0.09361	6.325698	2.52E-10	1.92E-08
Soat1	4795.09686	0.621351	0.098319	6.319756	2.62E-10	1.98E-08
Bcl2	8094.68818	-0.64507	0.102209	-6.31127	2.77E-10	2.08E-08
Serpine1	153.5998395	2.147099	0.340395	6.307667	2.83E-10	2.11E-08
Ms4a6b	6859.374652	-0.88878	0.14108	-6.29987	2.98E-10	2.21E-08
Abcb1b	1214.998065	0.828387	0.131969	6.277149	3.45E-10	2.54E-08
Ccr8	4181.773789	0.71403	0.113914	6.268165	3.65E-10	2.68E-08
Cd55	482.0512744	-0.98611	0.158868	-6.20711	5.40E-10	3.92E-08
NA	707.2080781	-1.07031	0.17244	-6.20685	5.41E-10	3.92E-08
Pik3cd	14916.6041	-0.75864	0.122264	-6.20494	5.47E-10	3.95E-08
Eif4ebp1	2236.221118	0.835739	0.134851	6.197495	5.74E-10	4.11E-08
Sntb1	193.4263995	-1.72698	0.278688	-6.19683	5.76E-10	4.11E-08
Eif2s2	4671.999227	1.080267	0.174805	6.179837	6.42E-10	4.55E-08
Rexo2	2873.475454	-0.66068	0.106942	-6.17789	6.50E-10	4.57E-08
Atp10a	3727.59585	-0.98284	0.159105	-6.1773	6.52E-10	4.57E-08
Tagap	1900.167409	-0.81341	0.13178	-6.17251	6.72E-10	4.69E-08
Gm867	94.27373922	2.169903	0.351925	6.165815	7.01E-10	4.87E-08
Card6	1746.255958	-0.85488	0.138794	-6.15933	7.31E-10	5.04E-08
Vars1	2879.507525	0.686203	0.111426	6.1584	7.35E-10	5.05E-08

H2-Q10	354.1252206	1.257123	0.204282	6.15386	7.56E-10	5.16E-08
Nid1	294.7519224	1.142599	0.186532	6.125497	9.04E-10	6.14E-08
Garin1a	182.9638431	1.435564	0.235294	6.101152	1.05E-09	7.12E-08
Itgae	518.7975713	-0.92368	0.151574	-6.09394	1.10E-09	7.41E-08
Lfng	3966.985136	-0.58711	0.096389	-6.09103	1.12E-09	7.50E-08
Tbc1d4	5656.987968	-0.61648	0.101687	-6.06258	1.34E-09	8.91E-08
Sema4a	1435.345094	-0.80268	0.132446	-6.06044	1.36E-09	8.98E-08
Vsir	3674.581813	-0.70353	0.116295	-6.04957	1.45E-09	9.56E-08
Cd9	1504.336673	-0.67249	0.111564	-6.0279	1.66E-09	1.09E-07
A630023P12Rik	634.2536573	-0.84985	0.141143	-6.02115	1.73E-09	1.13E-07
Socs3	2022.226579	-1.04826	0.174213	-6.01711	1.78E-09	1.15E-07
Slc2a3	18616.05699	0.661923	0.110508	5.989821	2.10E-09	1.36E-07
Usp6nl	789.2532898	-0.93991	0.157107	-5.98259	2.20E-09	1.41E-07
Gpnmb	100.4462162	2.183719	0.365507	5.974492	2.31E-09	1.47E-07
Rnf157	1977.50047	0.786039	0.132262	5.943053	2.80E-09	1.78E-07
Emp3	1988.627566	-0.6926	0.116583	-5.94086	2.84E-09	1.79E-07
Prxl2a	700.8476148	1.074897	0.18102	5.938005	2.89E-09	1.82E-07
Ddr1	782.1216446	-1.39626	0.235788	-5.92168	3.19E-09	2.00E-07
Adprm	1219.666725	0.828536	0.140028	5.916931	3.28E-09	2.04E-07
Haao	231.2498344	1.354631	0.229078	5.913411	3.35E-09	2.08E-07
Stat3	12429.29873	0.57078	0.096539	5.912408	3.37E-09	2.08E-07
Stk38	6434.067228	-0.53502	0.090594	-5.9057	3.51E-09	2.16E-07
Cacna2d4	354.9914444	-0.99003	0.168047	-5.89136	3.83E-09	2.34E-07
Slc35d3	102.023396	1.92078	0.326457	5.883709	4.01E-09	2.44E-07
Nupr1	55.24434081	2.625795	0.446406	5.882079	4.05E-09	2.45E-07
Ccdc141	184.1261692	-1.24042	0.211321	-5.86985	4.36E-09	2.63E-07
Ttc39c	1029.92013	0.830792	0.141562	5.868741	4.39E-09	2.63E-07
Got1	3311.040785	0.61969	0.105704	5.862504	4.56E-09	2.72E-07
Wars1	3550.939152	-0.56567	0.096532	-5.85991	4.63E-09	2.75E-07
S100a13	1850.061605	-0.70447	0.120899	-5.82692	5.65E-09	3.34E-07
Snx18	1258.648647	0.746501	0.128246	5.820851	5.85E-09	3.45E-07
Cebpb	381.3249281	1.119712	0.192476	5.817419	5.98E-09	3.50E-07
Lpxn	3269.119784	0.700456	0.121551	5.762661	8.28E-09	4.83E-07
Selenop	2480.786617	-0.8961	0.155625	-5.75808	8.51E-09	4.94E-07
Jaml	483.1519422	-1.12217	0.194959	-5.75595	8.62E-09	4.98E-07
Rcn1	726.6387077	-0.88713	0.154195	-5.75332	8.75E-09	5.04E-07
Ikzf3	2226.356017	-1.13988	0.199494	-5.71389	1.10E-08	6.33E-07
Rflnb	666.5165967	-0.88209	0.154646	-5.70394	1.17E-08	6.68E-07
Gvin1	270.9130288	-1.0871	0.190932	-5.69363	1.24E-08	7.07E-07
Syt11	1032.357686	-0.72908	0.128068	-5.69287	1.25E-08	7.07E-07
Sccpdh	188.2430502	1.348057	0.238019	5.663665	1.48E-08	8.34E-07
Khdrbs3	64.89339004	-2.53993	0.448553	-5.66249	1.49E-08	8.36E-07
Crip1	6213.291411	-0.71791	0.126815	-5.66107	1.50E-08	8.40E-07
Phf10	2501.870451	0.65591	0.115934	5.657608	1.53E-08	8.53E-07
Tgtp2	281.6816578	-1.09691	0.193935	-5.65608	1.55E-08	8.57E-07
Egr2	325.6248183	1.228607	0.217305	5.653846	1.57E-08	8.65E-07
Id1	307.3495963	-1.16083	0.205551	-5.64739	1.63E-08	8.94E-07
Trat1	932.4043267	-0.86505	0.153443	-5.63757	1.72E-08	9.42E-07

Isy1	6340.66292	0.500656	0.089034	5.623206	1.87E-08	1.02E-06
Anxa4	750.4857632	0.825618	0.147359	5.602754	2.11E-08	1.14E-06
Mybbp1a	2158.680778	-0.64243	0.114979	-5.58741	2.30E-08	1.24E-06
Nolc1	1119.064779	-0.68739	0.12328	-5.57589	2.46E-08	1.32E-06
Ltbp1	95.03861805	-2.01331	0.36158	-5.56809	2.58E-08	1.38E-06
Add3	9619.904285	-0.52786	0.094829	-5.56646	2.60E-08	1.38E-06
Nlrc5	1078.425479	-0.8758	0.157878	-5.54732	2.90E-08	1.54E-06
Tuba8	101.3732303	1.616688	0.291581	5.544558	2.95E-08	1.56E-06
Ncs1	372.0360613	0.961438	0.17365	5.536647	3.08E-08	1.62E-06
Asap1	2120.489365	-0.62517	0.113202	-5.52257	3.34E-08	1.75E-06
Pim1	11483.18671	-0.6971	0.126387	-5.51561	3.48E-08	1.81E-06
Clint1	6715.461321	-0.51204	0.0933	-5.48808	4.06E-08	2.11E-06
Gm9892	618.7901474	1.123064	0.204716	5.48597	4.11E-08	2.13E-06
Mif4gd	2464.633232	0.592327	0.108052	5.481851	4.21E-08	2.17E-06
Mat2a	2605.922458	-0.67804	0.123829	-5.4756	4.36E-08	2.24E-06
Dgka	19282.45583	-0.48539	0.08869	-5.47285	4.43E-08	2.27E-06
Vwa1	450.1161732	0.92766	0.169867	5.461084	4.73E-08	2.41E-06
Rasgrp4	925.9496077	0.728151	0.133472	5.455458	4.88E-08	2.48E-06
Phlda1	1727.38994	0.642734	0.117886	5.452161	4.98E-08	2.52E-06
Adcy3	518.1142079	-0.88052	0.161886	-5.43916	5.35E-08	2.70E-06
F2rl1	215.6612793	-1.12125	0.20642	-5.43187	5.58E-08	2.80E-06
Nsg2	1015.663926	-0.63404	0.116761	-5.43026	5.63E-08	2.81E-06
Dpfl	251.6500735	1.218223	0.225224	5.408945	6.34E-08	3.15E-06
Lars1	3259.818348	0.611531	0.113153	5.404479	6.50E-08	3.22E-06
Nr4a2	106.2530115	1.56884	0.290893	5.393187	6.92E-08	3.42E-06
Il17ra	4238.988008	-0.60768	0.112922	-5.38139	7.39E-08	3.64E-06
Ier5l	179.7341262	1.221617	0.227369	5.372834	7.75E-08	3.79E-06
Aldh1l2	49.31962221	2.734636	0.509008	5.372477	7.77E-08	3.79E-06
Lyl1	88.03770846	1.660966	0.30982	5.361069	8.27E-08	4.02E-06
Myo6	21902.67039	-0.73384	0.136959	-5.35813	8.41E-08	4.07E-06
Ctsw	634.0169222	-0.80575	0.150412	-5.35694	8.46E-08	4.09E-06
Gm9199	100.860147	1.611466	0.301488	5.345044	9.04E-08	4.35E-06
Nrros	1788.475324	-0.55931	0.104697	-5.34214	9.19E-08	4.40E-06
Entpd1	2544.158679	-0.55364	0.103653	-5.34131	9.23E-08	4.40E-06
Tmem64	10032.97907	-0.69143	0.129912	-5.3223	1.02E-07	4.87E-06
Gramd4	2341.870575	-0.59066	0.110992	-5.32165	1.03E-07	4.87E-06
Sptbn1	13579.72281	-0.71255	0.134275	-5.30668	1.12E-07	5.27E-06
D630039A03Rik	2029.579212	-0.57769	0.108975	-5.3011	1.15E-07	5.41E-06
Sypl1	1355.968133	0.839027	0.158664	5.288081	1.24E-07	5.79E-06
Ctla4	19291.66954	0.53393	0.101027	5.28502	1.26E-07	5.87E-06
B630019A10Rik	108.2664663	-1.91881	0.364076	-5.27034	1.36E-07	6.33E-06
Rasgrp1	8195.239824	-0.60191	0.114379	-5.26243	1.42E-07	6.59E-06
Herpud1	1826.321776	0.611348	0.116282	5.25747	1.46E-07	6.75E-06
Aebp2	19306.97027	0.591563	0.11257	5.255076	1.48E-07	6.81E-06
Il6st	2665.911309	-1.06218	0.202254	-5.25172	1.51E-07	6.91E-06
Ptp4a3	2389.166272	-0.6107	0.116331	-5.24966	1.52E-07	6.96E-06
Mpzl2	235.7094667	-1.57979	0.301289	-5.24343	1.58E-07	7.18E-06
Nfe2l1	3210.593677	0.768081	0.146637	5.237965	1.62E-07	7.37E-06

Ptger4	1465.529758	-0.57415	0.109678	-5.23485	1.65E-07	7.46E-06
Slc39a4	1159.401464	0.580011	0.110979	5.226297	1.73E-07	7.79E-06
Eprs1	4497.107164	0.515977	0.098811	5.221847	1.77E-07	7.95E-06
Unc13a	1847.969418	0.951586	0.182634	5.210361	1.88E-07	8.43E-06
Fut7	255.9858952	-0.95896	0.184095	-5.20906	1.90E-07	8.46E-06
Col6a3	826.4382535	-0.85011	0.163289	-5.20619	1.93E-07	8.56E-06
Lta	1325.99932	-0.85892	0.16552	-5.18921	2.11E-07	9.35E-06
Clic4	2750.294822	0.530375	0.10226	5.18653	2.14E-07	9.45E-06
Abcc4	482.0431257	-0.88983	0.171865	-5.1775	2.25E-07	9.89E-06
Cnp	7256.931772	-0.49512	0.095662	-5.17569	2.27E-07	9.95E-06
Ehd3	1558.923925	-0.66786	0.129232	-5.16792	2.37E-07	1.03E-05
Bivm	544.2686616	0.935529	0.181185	5.163396	2.43E-07	1.06E-05
Col15a1	131.9580437	-1.29375	0.250781	-5.1589	2.48E-07	1.08E-05
Arl14ep	1428.852964	0.728305	0.141213	5.15749	2.50E-07	1.08E-05
Itih5	3107.350112	-0.79018	0.153246	-5.15627	2.52E-07	1.09E-05
Eif3c	13132.21511	0.451289	0.087632	5.149842	2.61E-07	1.12E-05
Wdr82	6245.615362	-0.49142	0.095453	-5.14826	2.63E-07	1.12E-05
Smtn	571.1576875	-1.03163	0.200402	-5.14782	2.64E-07	1.12E-05
Itga6	2562.432304	-0.79567	0.15486	-5.13803	2.78E-07	1.18E-05
Alox12e	21.14103295	-4.61467	0.898962	-5.13333	2.85E-07	1.21E-05
Crim1	1727.874232	-0.65606	0.128039	-5.12387	2.99E-07	1.26E-05
Lrrc8a	1138.360021	-0.6889	0.134565	-5.11944	3.06E-07	1.29E-05
Slc28a2	992.1658637	-0.61265	0.119753	-5.11592	3.12E-07	1.31E-05
Elovl5	5854.93061	-0.50015	0.097792	-5.11447	3.15E-07	1.32E-05
Cxcr3	308.6901541	-1.05085	0.205701	-5.10864	3.24E-07	1.35E-05
Vav3	1173.214162	0.637292	0.124898	5.102498	3.35E-07	1.39E-05
Atp1b1	905.5672952	-0.73777	0.144594	-5.10236	3.35E-07	1.39E-05
Kdm3b	5057.357445	-0.61918	0.121427	-5.0992	3.41E-07	1.41E-05
Sco1	884.9108719	0.945501	0.185541	5.0959	3.47E-07	1.43E-05
Epas1	2423.968644	0.781373	0.153733	5.082677	3.72E-07	1.53E-05
Add1	12874.29454	-0.55501	0.109607	-5.06363	4.11E-07	1.68E-05
Gm17767	168.1850406	-1.11739	0.220861	-5.05925	4.21E-07	1.72E-05
Fkbp1a	9841.69676	-0.52387	0.103571	-5.05805	4.24E-07	1.72E-05
Nrp1	1030.397173	-0.8194	0.162085	-5.05539	4.30E-07	1.74E-05
Lsp1	12739.11666	-0.50965	0.100874	-5.05234	4.36E-07	1.76E-05
Il2rb	23012.70175	-0.55507	0.110089	-5.04202	4.61E-07	1.85E-05
Sgk3	159.5532167	-1.18079	0.234246	-5.0408	4.64E-07	1.86E-05
Fry	192.097348	-1.36172	0.271913	-5.00792	5.50E-07	2.20E-05
Atp10d	2301.070119	-0.62843	0.125509	-5.00705	5.53E-07	2.20E-05
Crem	9870.086216	0.837178	0.167802	4.989089	6.07E-07	2.41E-05
Arhgef40	702.1420504	0.696211	0.139713	4.983133	6.26E-07	2.48E-05
Fryl	3538.367293	-0.79921	0.160425	-4.98184	6.30E-07	2.49E-05
Cdc25b	11404.15494	-0.51985	0.10437	-4.98079	6.33E-07	2.49E-05
She	184.0817642	-1.23984	0.249049	-4.97832	6.41E-07	2.52E-05
P4hb	13332.54466	0.49018	0.098549	4.973977	6.56E-07	2.57E-05
Slc12a6	4110.327492	-0.67168	0.135246	-4.9664	6.82E-07	2.66E-05
Dusp16	481.7346335	0.747838	0.150904	4.955727	7.21E-07	2.80E-05
Ddit4	8521.757202	0.815962	0.164681	4.954815	7.24E-07	2.81E-05

Scml4	1041.523006	-0.62316	0.125887	-4.95016	7.42E-07	2.87E-05
Fos	909.5773842	-0.82251	0.16629	-4.94622	7.57E-07	2.92E-05
Tgfb2	5397.350685	-0.45288	0.091578	-4.94528	7.60E-07	2.92E-05
Samd11	228.8116307	1.248234	0.2525	4.94349	7.67E-07	2.94E-05
Myh7b	145.2420847	1.540853	0.311887	4.940421	7.80E-07	2.98E-05
Bach2	3834.245458	-0.67979	0.138051	-4.92418	8.47E-07	3.23E-05
Vegfa	1680.040298	0.551811	0.112167	4.91956	8.67E-07	3.29E-05
Upp1	150.3152638	1.583988	0.322619	4.909777	9.12E-07	3.45E-05
Pkp2	424.4885018	0.858781	0.175143	4.90331	9.42E-07	3.56E-05
Ablim1	16076.81786	-0.5902	0.120542	-4.89622	9.77E-07	3.68E-05
Gbe1	4248.987754	0.672137	0.13731	4.895025	9.83E-07	3.69E-05
Cebpg	1657.900881	0.635497	0.129918	4.891524	1.00E-06	3.75E-05
Egln3	5327.461187	0.575847	0.117881	4.884979	1.03E-06	3.86E-05
Trib1	1129.664125	-0.64047	0.131259	-4.87939	1.06E-06	3.96E-05
Prkar1b	58.98834155	-2.41871	0.496294	-4.87355	1.10E-06	4.07E-05
Casp4	199.0543025	1.192417	0.244955	4.867904	1.13E-06	4.17E-05
Myli	363.1031611	-0.94169	0.193461	-4.86761	1.13E-06	4.17E-05
Slc35a2	1280.376398	0.567762	0.116678	4.866037	1.14E-06	4.19E-05
Piwil2	283.7359646	1.030052	0.211942	4.860055	1.17E-06	4.30E-05
Hivep2	1478.32296	-0.68875	0.1419	-4.85375	1.21E-06	4.43E-05
Pparg	69.47049522	1.752194	0.362045	4.839711	1.30E-06	4.74E-05
Cysltr2	135.6159338	1.346265	0.278428	4.835228	1.33E-06	4.83E-05
Rassf4	98.66248622	-1.41649	0.29296	-4.83512	1.33E-06	4.83E-05
Krt83	229.4298615	1.055129	0.21833	4.832727	1.35E-06	4.87E-05
Pld4	960.4673353	0.649925	0.134576	4.829416	1.37E-06	4.93E-05
Cyth3	810.5683516	-0.70679	0.146353	-4.82937	1.37E-06	4.93E-05
Ltbp3	554.0765433	0.988274	0.204691	4.828133	1.38E-06	4.94E-05
Glcc1	926.1664425	-0.67705	0.14029	-4.82605	1.39E-06	4.98E-05
Hacd3	2454.184007	0.541483	0.112215	4.82539	1.40E-06	4.98E-05
Ankrd55	358.3249639	-0.9707	0.20118	-4.82504	1.40E-06	4.98E-05
Mettl27	620.9908838	-0.70415	0.146046	-4.82147	1.43E-06	5.05E-05
Cdk6	309.1468453	-1.09071	0.226242	-4.82099	1.43E-06	5.05E-05
Ugcg	2648.482217	-0.52829	0.109941	-4.80527	1.55E-06	5.45E-05
Rab8b	10952.71039	0.582279	0.12119	4.804687	1.55E-06	5.45E-05
Angptl6	110.24426	1.395783	0.290554	4.803861	1.56E-06	5.46E-05
Cd8a	273.9020122	-1.00481	0.209239	-4.80222	1.57E-06	5.49E-05
Ckb	683.3105285	0.65016	0.135464	4.799494	1.59E-06	5.55E-05
Pepd	1094.548855	-0.56121	0.116992	-4.79703	1.61E-06	5.60E-05
Frmpd1	121.0852626	-1.29841	0.271208	-4.78748	1.69E-06	5.86E-05
Plekhh3	154.0336308	1.151778	0.240638	4.786343	1.70E-06	5.86E-05
Sesn1	2018.395807	-0.53544	0.111874	-4.78614	1.70E-06	5.86E-05
Mkl	257.8337482	1.044716	0.218298	4.785744	1.70E-06	5.86E-05
Plk2	597.424618	0.736547	0.154043	4.781433	1.74E-06	5.97E-05
Tespa1	636.5386392	0.72495	0.15165	4.780423	1.75E-06	5.98E-05
Ly6a	2265.84354	0.944299	0.197545	4.780172	1.75E-06	5.98E-05
Gimap3	24618.97354	-0.43067	0.090158	-4.77686	1.78E-06	6.06E-05
Smap2	5936.863955	-0.57972	0.121458	-4.77303	1.81E-06	6.16E-05
Gpr83	330.9872149	-1.01694	0.213373	-4.76604	1.88E-06	6.36E-05

Tlr7	385.6647816	-0.92117	0.193546	-4.75947	1.94E-06	6.56E-05
Tmtc2	544.1654587	0.769749	0.161873	4.755271	1.98E-06	6.68E-05
Akap13	7447.001619	-0.79971	0.168382	-4.7494	2.04E-06	6.86E-05
Irgm2	813.2555215	-0.59118	0.124621	-4.7438	2.10E-06	7.03E-05
Ptpn5	140.7688037	1.539172	0.325295	4.731625	2.23E-06	7.45E-05
Actn1	707.7296565	-0.77926	0.164892	-4.72592	2.29E-06	7.64E-05
Xdh	670.8449349	-1.30915	0.277446	-4.71856	2.38E-06	7.90E-05
Jdp2	723.2925632	0.920327	0.195235	4.713956	2.43E-06	8.06E-05
Rnasel	1166.3483	-0.60891	0.130256	-4.67469	2.94E-06	9.74E-05
Cdkn1a	1632.6998	0.646079	0.138441	4.666832	3.06E-06	0.000101
Rab19	971.1587134	0.653126	0.140059	4.663236	3.11E-06	0.000102
Gbp3	254.6499852	-0.90799	0.194884	-4.65911	3.18E-06	0.000104
Utrn	3097.192448	-0.79117	0.170527	-4.63954	3.49E-06	0.000114
Hip1	528.7705036	0.894387	0.192798	4.638994	3.50E-06	0.000114
Socs2	3501.881956	-0.54149	0.11677	-4.63723	3.53E-06	0.000115
Aqp1	30.71286795	-2.6424	0.570156	-4.63452	3.58E-06	0.000116
Syne2	525.401165	-1.01016	0.218118	-4.63124	3.63E-06	0.000118
Pmel	448.3694252	-0.86083	0.18606	-4.62659	3.72E-06	0.00012
Tubb6	2212.015725	0.669852	0.145077	4.617234	3.89E-06	0.000125
Cep97	1492.047641	-0.52456	0.113614	-4.61707	3.89E-06	0.000125
Camk1d	958.102167	-0.68914	0.149413	-4.61232	3.98E-06	0.000128
P2rx7	4968.647109	-0.46492	0.100843	-4.61033	4.02E-06	0.000129
Gadd45b	325.9371186	-0.86075	0.187012	-4.60263	4.17E-06	0.000133
1700025G04Rik	1657.530577	-0.47287	0.102841	-4.59804	4.26E-06	0.000136
Fam169b	2369.545418	-0.46463	0.101232	-4.58976	4.44E-06	0.000141
Gzfl	1453.330231	0.588711	0.128324	4.587679	4.48E-06	0.000142
Pgpep1l	51.40788082	-2.80787	0.61281	-4.58196	4.61E-06	0.000146
Slc12a7	1533.806843	-0.77137	0.168435	-4.57962	4.66E-06	0.000147
Faah	5998.613184	-0.58063	0.126861	-4.57693	4.72E-06	0.000148
Stx11	1071.114578	0.607377	0.132954	4.568322	4.92E-06	0.000154
Bcl9l	989.0001461	-1.03064	0.225633	-4.56776	4.93E-06	0.000154
Pik3ip1	1316.901093	-0.49476	0.108325	-4.56743	4.94E-06	0.000154
Fosl2	6563.487636	0.583597	0.128107	4.555555	5.22E-06	0.000163
Il17rb	134.0326721	-1.4001	0.30748	-4.55346	5.28E-06	0.000164
Ikzf4	1073.055427	-0.91946	0.202139	-4.54864	5.40E-06	0.000167
Ltb	6398.454658	-0.439	0.09664	-4.54259	5.56E-06	0.000172
S100a6	236.9329399	-1.29845	0.286215	-4.53661	5.72E-06	0.000176
Zbp1	2017.544701	-0.58507	0.129156	-4.52999	5.90E-06	0.000182
Spred3	210.4136265	1.250139	0.276206	4.526101	6.01E-06	0.000185
Cbarp	889.7957292	0.63693	0.141203	4.510738	6.46E-06	0.000198
Macf1	8376.258335	-0.90667	0.201076	-4.50911	6.51E-06	0.000199
Lgals7	94.4660617	-1.29215	0.286955	-4.50299	6.70E-06	0.000204
Tnfaip8l1	833.3078493	-0.56146	0.124975	-4.49261	7.04E-06	0.000214
Tcp1	11313.52578	0.61061	0.136038	4.488521	7.17E-06	0.000218
Mbd5	558.1799268	-0.74351	0.165701	-4.48706	7.22E-06	0.000219
Hif1a	28001.31657	0.674225	0.150806	4.470823	7.79E-06	0.000235
Mgst2	258.4942108	-1.00872	0.225796	-4.46741	7.92E-06	0.000239
Snhg5	791.7867968	0.929349	0.208472	4.457903	8.28E-06	0.000249

Fscn1	64.11106158	1.817144	0.40861	4.447138	8.70E-06	0.000261
Ifi209	660.3695571	-0.61041	0.137291	-4.44614	8.74E-06	0.000262
C1qtnf6	491.9446582	-0.75389	0.169586	-4.44546	8.77E-06	0.000262
Ccr2	1008.251868	-0.56542	0.127238	-4.44377	8.84E-06	0.000263
Ifrd1	1287.975914	0.676212	0.15247	4.435035	9.21E-06	0.000274
Lman2l	468.0366784	0.727337	0.164076	4.432926	9.30E-06	0.000276
Cst7	1188.083185	0.520447	0.117448	4.43129	9.37E-06	0.000277
Cacna1c	110.7811384	-1.41669	0.31976	-4.43049	9.40E-06	0.000277
Ghdc	286.8488654	0.797292	0.179961	4.430372	9.41E-06	0.000277
Sh2d3c	3312.939843	-0.62001	0.140107	-4.42528	9.63E-06	0.000283
Dst	373.0506161	-1.36079	0.308725	-4.40776	1.04E-05	0.000306
Inf2	2718.342233	-0.68869	0.15639	-4.40366	1.06E-05	0.000311
Gata1	306.5259763	-0.75613	0.171748	-4.40257	1.07E-05	0.000312
Hspa4	7986.253594	0.42366	0.096246	4.401844	1.07E-05	0.000312
Prkd2	5392.251036	-0.53352	0.121283	-4.39901	1.09E-05	0.000316
Arhgef3	5196.837334	-0.47006	0.10704	-4.39146	1.13E-05	0.000326
Gpr171	2680.375148	0.693516	0.157969	4.390201	1.13E-05	0.000327
Krtcap2	2725.330012	0.650163	0.148205	4.386918	1.15E-05	0.000332
Maged2	665.1676548	0.608598	0.139172	4.373011	1.23E-05	0.000353
Insl6	180.3752334	1.089294	0.249417	4.367358	1.26E-05	0.000361
Tatdn2	1729.933498	-0.6254	0.143302	-4.36423	1.28E-05	0.000366
Stk32c	94.82069452	1.278369	0.29328	4.358875	1.31E-05	0.000373
Tmem205	162.7536965	1.077364	0.247166	4.358862	1.31E-05	0.000373
Hsp90ab1	75969.65369	0.365921	0.083986	4.356956	1.32E-05	0.000375
Gm19422	166.0777167	-1.48873	0.341792	-4.35565	1.33E-05	0.000377
Xrn2	4916.204111	-0.46053	0.105781	-4.35364	1.34E-05	0.000379
Il7r	6727.879839	0.592369	0.136264	4.347234	1.38E-05	0.00039
Slc2a6	2514.001257	-0.71815	0.165327	-4.34379	1.40E-05	0.000395
Focad	1697.31308	0.496568	0.114404	4.340475	1.42E-05	0.0004
S1pr4	5098.688187	-0.42415	0.097786	-4.33752	1.44E-05	0.000405
Sqor	623.2270589	-0.66418	0.153146	-4.33687	1.45E-05	0.000405
Arhgef9	537.5343813	0.692807	0.159818	4.334981	1.46E-05	0.000408
Lrrc32	2627.439123	-0.82073	0.189488	-4.33133	1.48E-05	0.000414
Lonpl	8036.327988	0.512859	0.118426	4.330623	1.49E-05	0.000414
Hip1r	1271.343571	-0.57268	0.132423	-4.32461	1.53E-05	0.000425
Tnrc6b	2386.77941	-0.76119	0.176383	-4.31555	1.59E-05	0.000441
Zfp608	246.313041	-0.98167	0.227524	-4.31459	1.60E-05	0.000442
Nt5e	5256.212448	-0.45586	0.105808	-4.30837	1.64E-05	0.000454
Ikzf2	415.5934032	-0.79879	0.185477	-4.3067	1.66E-05	0.000457
Pde4b	3956.479357	0.472179	0.10974	4.30271	1.69E-05	0.000464
Gcsh	376.6741522	-0.75092	0.174898	-4.29347	1.76E-05	0.000483
Bambi-ps1	142.3450196	-1.0863	0.253208	-4.29015	1.79E-05	0.000489
Ppp1r13b	1380.323236	-0.4725	0.110179	-4.28845	1.80E-05	0.000491
Gna13	8374.728663	0.393969	0.091992	4.282667	1.85E-05	0.000503
Ctsl	2404.957744	-0.48777	0.113914	-4.28194	1.85E-05	0.000504
Cycs	1908.094763	-0.42944	0.100486	-4.27362	1.92E-05	0.000522
Sorl1	561.0438532	-0.92594	0.216872	-4.26953	1.96E-05	0.000531
AB124611	667.8107796	-0.63826	0.149681	-4.26411	2.01E-05	0.000542

Celsr1	1002.901097	-0.81927	0.192225	-4.26203	2.03E-05	0.000546
Zdhhc15	449.4375105	-0.71798	0.168999	-4.24841	2.15E-05	0.000579
Scrt1	273.3197003	0.971272	0.228737	4.24624	2.17E-05	0.000584
Slc25a3	8354.338597	0.405523	0.095603	4.241758	2.22E-05	0.000594
Rbms1	1945.015994	-0.54882	0.129514	-4.23753	2.26E-05	0.000604
Ikbke	5197.10109	-0.44456	0.105072	-4.23094	2.33E-05	0.000621
Hey1	55.07434691	1.642515	0.388357	4.2294	2.34E-05	0.000624
Adamtsl5	71.04698342	1.404299	0.332317	4.225783	2.38E-05	0.000633
Wdr46	1476.733424	-0.66023	0.156773	-4.21135	2.54E-05	0.000673
Gvin3	1411.525078	-0.82765	0.196574	-4.21034	2.55E-05	0.000675
E2f2	5604.648953	-0.48091	0.114369	-4.20493	2.61E-05	0.00069
Actr3b	129.7188108	-1.1989	0.285534	-4.1988	2.68E-05	0.000707
Jun	1076.550127	-0.75679	0.180304	-4.1973	2.70E-05	0.000711
Mcm4	8308.005806	-0.35766	0.085225	-4.19668	2.71E-05	0.000711
Dgcr8	1750.643929	-0.47937	0.114298	-4.19409	2.74E-05	0.000718
Marcks	1747.267965	0.525898	0.125452	4.19202	2.76E-05	0.000723
Spin4	192.9265367	0.974952	0.232661	4.190429	2.78E-05	0.000726
Dynlt3	1221.570558	0.794202	0.189544	4.190061	2.79E-05	0.000726
Pde7b	130.2000136	1.072783	0.256218	4.186992	2.83E-05	0.000735
Gemin8	836.068391	0.6556	0.156735	4.182857	2.88E-05	0.000747
Sult5a1	52.85466389	-1.6258	0.388987	-4.17957	2.92E-05	0.000756
Gstp3	1811.38574	0.459068	0.109854	4.178907	2.93E-05	0.000757
Cacna1s	96.5273433	1.581316	0.378563	4.17716	2.95E-05	0.000761
Bltp3a	694.8656302	0.719446	0.172282	4.175979	2.97E-05	0.000763
Txnip	6695.618244	0.429696	0.102948	4.173899	2.99E-05	0.000767
Zhx2	462.3574394	-0.62956	0.150842	-4.17364	3.00E-05	0.000767
Dock5	276.4687059	0.948462	0.227261	4.173444	3.00E-05	0.000767
Padi2	1887.275575	0.485036	0.116236	4.172865	3.01E-05	0.000768
Pitpnc1	2254.571262	-0.42691	0.102322	-4.17219	3.02E-05	0.000769
Plekhb2	4076.967484	0.412319	0.098882	4.169823	3.05E-05	0.000775
Rap2a	983.8605159	0.55571	0.133343	4.167522	3.08E-05	0.000781
Ncald	51.607786	1.618915	0.388619	4.165817	3.10E-05	0.000786
Dnah7a	71.5708908	-1.62382	0.390001	-4.16362	3.13E-05	0.000792
Pou6f1	327.5004698	-0.71905	0.172737	-4.1627	3.15E-05	0.000793
Akr1c12	181.6475315	-1.18981	0.28637	-4.15481	3.26E-05	0.00082
Zfas1	1281.931876	0.549111	0.132226	4.152815	3.28E-05	0.000825
Prdx5	3333.243773	0.516391	0.124434	4.14992	3.33E-05	0.000834
Trafd1	1447.364895	0.461845	0.111342	4.147968	3.35E-05	0.00084
Trgc4	36.30702879	-2.05315	0.495269	-4.14553	3.39E-05	0.000847
Blcap	365.2951838	0.888013	0.214466	4.140569	3.46E-05	0.000864
Serpinf1	94.08130925	1.359107	0.328586	4.136226	3.53E-05	0.000878
Glul	1092.696557	-0.56441	0.136495	-4.13506	3.55E-05	0.000881
Gadd45g	738.1930927	-0.64196	0.155377	-4.13166	3.60E-05	0.000892
Gcnt1	138.7551241	0.986251	0.238734	4.131181	3.61E-05	0.000892
NA	208.7205936	0.855085	0.207001	4.130829	3.61E-05	0.000892
Rpl22l1	4950.083421	0.804926	0.194987	4.12811	3.66E-05	0.000901
Pja1	2211.472549	-0.42693	0.103487	-4.12545	3.70E-05	0.00091
Foxk1	4045.711852	-0.76564	0.185674	-4.12354	3.73E-05	0.000916

Tmcc1	863.8077715	-0.5279	0.12804	-4.12293	3.74E-05	0.000917
Mid1ip1	1441.442273	0.46935	0.114085	4.114021	3.89E-05	0.000951
Gprc5b	128.3556496	-1.7952	0.436452	-4.11316	3.90E-05	0.000953
Tdrp	982.9765992	-0.61837	0.150359	-4.11262	3.91E-05	0.000953
Slfn1	4057.575445	-0.53741	0.130687	-4.11216	3.92E-05	0.000953
Mt1	903.9036677	0.977706	0.237833	4.110895	3.94E-05	0.000957
Ghitm	9059.244814	0.501386	0.122069	4.107395	4.00E-05	0.000969
Ier5	1623.851622	-0.55293	0.134782	-4.10239	4.09E-05	0.000989
Il12rb1	1839.887367	0.73165	0.178577	4.097101	4.18E-05	0.001009
Cyp2s1	1739.93095	0.471632	0.115119	4.096924	4.19E-05	0.001009
Cgas	395.9922241	-0.66186	0.16165	-4.09441	4.23E-05	0.001018
Akr1c13	2207.859068	-0.48477	0.118567	-4.08858	4.34E-05	0.001042
Gpat4	4137.263529	-0.37314	0.091327	-4.0858	4.39E-05	0.001052
Inpp4b	4087.806282	-0.76679	0.187706	-4.08503	4.41E-05	0.001054
Nop56	2608.192307	-0.43386	0.106272	-4.08258	4.45E-05	0.001063
Ctse	7690.533524	-0.4019	0.098549	-4.07822	4.54E-05	0.001081
Nipal3	2249.542463	-0.53726	0.131859	-4.07449	4.61E-05	0.001096
Plod3	1159.024696	0.482598	0.118463	4.073829	4.62E-05	0.001098
Slc35b4	1462.437602	-0.50616	0.124345	-4.07058	4.69E-05	0.001111
Cd247	6634.741842	-0.4454	0.10965	-4.06208	4.86E-05	0.00115
Plscr1	952.5006558	0.583219	0.143618	4.06092	4.89E-05	0.001154
Megf8	154.3637817	0.988009	0.243356	4.059926	4.91E-05	0.001156
Atp8a1	739.4345587	-0.62586	0.154179	-4.05929	4.92E-05	0.001157
Eif1	12703.54739	0.54634	0.1347	4.055976	4.99E-05	0.001172
Pcgf5	1364.784284	0.611797	0.15088	4.054864	5.02E-05	0.001175
Gm20367	12.68551542	6.082674	1.502631	4.048015	5.17E-05	0.001208
Usp28	1031.138733	-0.58486	0.144566	-4.04562	5.22E-05	0.001218
Gart	1321.799041	-0.47151	0.116569	-4.04495	5.23E-05	0.001219
Mgll	783.0650307	-0.62654	0.154965	-4.04307	5.28E-05	0.001227
Rxra	1245.647759	-0.55596	0.137569	-4.04133	5.31E-05	0.001234
Dtx3l	2805.357963	-0.47599	0.117897	-4.03736	5.41E-05	0.001253
Adgrl1	1560.929166	-0.90318	0.223771	-4.0362	5.43E-05	0.001257
Hsd17b7	1871.984851	0.49469	0.122729	4.030739	5.56E-05	0.001284
Srm	382.675218	-0.68577	0.170171	-4.02989	5.58E-05	0.001286
Gimap6	7850.318741	-0.37894	0.094087	-4.02756	5.64E-05	0.001295
Capn3	389.9441341	-0.73985	0.183703	-4.02742	5.64E-05	0.001295
Pacs1	2884.34973	-0.58307	0.144833	-4.02579	5.68E-05	0.001302
Nsd3	6127.597655	-0.48965	0.121781	-4.02069	5.80E-05	0.001328
Evi2a	690.0855865	0.701167	0.174538	4.017262	5.89E-05	0.001345
Dido1	1995.319966	-0.58144	0.144797	-4.01557	5.93E-05	0.001352
Tbxa2r	781.217528	-0.54926	0.136912	-4.01179	6.03E-05	0.00137
Insl3	571.0731807	0.780509	0.194559	4.011679	6.03E-05	0.00137
Npm1	24726.73038	0.53986	0.134605	4.01071	6.05E-05	0.001373
Wdr13	1313.828468	0.451531	0.112643	4.00852	6.11E-05	0.001384
Ccs	1054.95102	-0.63521	0.158548	-4.00643	6.16E-05	0.001393
Gls2	368.3588263	0.756066	0.188751	4.005627	6.19E-05	0.001396
Adarb1	547.3080432	0.739249	0.18475	4.00136	6.30E-05	0.001419
Frmd6	977.7686118	-0.54627	0.136606	-3.99885	6.37E-05	0.001431

AI506816	183.416916	-0.94217	0.235833	-3.99505	6.47E-05	0.001452
Alox15	21.64928168	-3.89178	0.97478	-3.99247	6.54E-05	0.001465
Fbxo17	1351.116597	-0.52015	0.130541	-3.98459	6.76E-05	0.001512
Prmt2	471.4107582	0.615355	0.154635	3.979395	6.91E-05	0.001543
Frmd4b	230.5370851	0.886095	0.222827	3.976612	6.99E-05	0.001558
Ms4a4c	72.09529474	-1.44083	0.36237	-3.97613	7.00E-05	0.001559
C9orf72	406.4229833	0.712203	0.179157	3.975299	7.03E-05	0.001561
Pou2f2	635.2568252	-0.65256	0.164221	-3.97364	7.08E-05	0.00157
Flna	21727.51292	-0.61614	0.155091	-3.97276	7.10E-05	0.001573
Il21	31.7363645	2.155908	0.54286	3.971389	7.15E-05	0.001579
Rbm4b	399.7964028	-0.62804	0.158204	-3.96979	7.19E-05	0.001587
Dhx58	568.7860307	0.645793	0.162833	3.965985	7.31E-05	0.00161
Xxylt1	705.0671926	-0.52846	0.133293	-3.96466	7.35E-05	0.001616
Il17f	58.55663767	1.787214	0.451025	3.962558	7.42E-05	0.001627
Rras2	1980.069971	-0.47034	0.118723	-3.96169	7.44E-05	0.001631
Prkca	2247.548098	-0.53046	0.133922	-3.96101	7.46E-05	0.001631
Trem1	32.14572977	-2.26241	0.571207	-3.96074	7.47E-05	0.001631
Asnsd1	2605.881183	0.628543	0.158705	3.960449	7.48E-05	0.001631
Arl5c	720.7655723	-0.54923	0.138702	-3.9598	7.50E-05	0.001632
Ecm1	10211.67066	-0.53283	0.13464	-3.95742	7.58E-05	0.001646
Galnt10	584.5031549	-0.54235	0.137185	-3.95345	7.70E-05	0.001671
Hipk2	930.8330447	-1.03343	0.261938	-3.94534	7.97E-05	0.001725
Dennd2d	2643.684527	0.388197	0.098497	3.941227	8.11E-05	0.001752
Pgpep1	434.5787309	0.609446	0.154722	3.938972	8.18E-05	0.001766
Hormad2	120.1042664	1.16437	0.295973	3.934046	8.35E-05	0.001798
Mthfd1	964.2554928	-0.47756	0.121399	-3.93377	8.36E-05	0.001798
Tor2a	947.1943993	0.491148	0.125042	3.927878	8.57E-05	0.00184
Rassf3	2238.837309	-0.49237	0.125485	-3.92375	8.72E-05	0.001869
Laptm4b	137.6477988	1.085618	0.276858	3.921204	8.81E-05	0.001885
Aldh2	1347.612613	-0.84756	0.216552	-3.91389	9.08E-05	0.00194
Phlpp1	1935.883668	-0.46815	0.119656	-3.91244	9.14E-05	0.001945
Gab2	185.8602523	0.921045	0.235419	3.912368	9.14E-05	0.001945
Kmt2c	1755.573714	-0.77615	0.198398	-3.91206	9.15E-05	0.001945
Sorcs2	748.7238261	-0.63362	0.162146	-3.90773	9.32E-05	0.001977
Sidtl	1093.415751	-0.59711	0.152864	-3.90614	9.38E-05	0.001987
Coq3	277.2106267	0.715334	0.183256	3.90347	9.48E-05	0.002003
Usp18	172.3189786	0.871946	0.22338	3.903424	9.48E-05	0.002003
Apbb1ip	2441.502562	-0.39709	0.101771	-3.90183	9.55E-05	0.002013
Rpa1	7621.445849	-0.33407	0.085639	-3.90084	9.59E-05	0.002014
Ttyh3	3411.217344	-0.59777	0.153243	-3.90084	9.59E-05	0.002014
Mir22hg	287.0070584	0.972444	0.249337	3.900113	9.61E-05	0.002017
Cldn12	123.5715475	1.053713	0.270223	3.899421	9.64E-05	0.00202
Celf5	98.14216976	1.126532	0.289027	3.897672	9.71E-05	0.002031
Rab33a	95.58738749	1.628211	0.417856	3.896586	9.76E-05	0.002037
En2	13.69009056	-3.90509	1.003937	-3.88978	0.0001	0.002091
Cblb	6629.534058	-0.51722	0.133199	-3.88303	0.000103	0.002147
Aldoa	84363.35892	0.413143	0.10646	3.880731	0.000104	0.002164
Nek7	4923.753795	-0.45395	0.117207	-3.87307	0.000107	0.002227

Fli1	1728.519462	-0.49456	0.1277	-3.87287	0.000108	0.002227
Pla2g4f	197.5818011	-1.06988	0.276384	-3.87099	0.000108	0.002241
Ift43	92.67543129	1.296082	0.335334	3.865046	0.000111	0.002293
Pik3cg	3755.788215	0.434511	0.112432	3.864647	0.000111	0.002293
Hsf2bp	109.8967368	1.115809	0.288768	3.864034	0.000112	0.002295
3110082I17Rik	231.4667279	-0.98503	0.255061	-3.86195	0.000112	0.002311
Aqp9	136.6705869	1.149075	0.298015	3.855765	0.000115	0.002366
Polr1c	1391.666573	0.474886	0.123318	3.850907	0.000118	0.00241
Rhoq	1468.212223	0.703728	0.182787	3.849982	0.000118	0.002415
Klf13	7398.11155	-0.52845	0.137458	-3.84447	0.000121	0.002466
Cd8b1	362.482967	-0.66477	0.173235	-3.83742	0.000124	0.002534
Ndr3	2613.567639	-0.41873	0.109294	-3.8312	0.000128	0.002595
Nipal1	553.1907167	-0.5927	0.154841	-3.82777	0.000129	0.002627
Nup210l	117.6722971	-1.05417	0.275692	-3.82372	0.000131	0.002662
Acs1l	387.4971095	-0.71113	0.185979	-3.82371	0.000131	0.002662
BC106179	46.66090257	-1.90943	0.499688	-3.82124	0.000133	0.002685
Runde3b	1081.811618	0.450087	0.117868	3.818562	0.000134	0.00271
Ggt7	568.8602159	0.520891	0.136435	3.817867	0.000135	0.002713
Skap2	737.0797047	0.480612	0.125941	3.816171	0.000136	0.002728
Nherf1	6766.271284	-0.40735	0.106755	-3.81572	0.000136	0.002728
Pank4	1665.58804	-0.42713	0.111962	-3.81495	0.000136	0.002732
Il4ra	33680.37898	-0.41885	0.109885	-3.8117	0.000138	0.002764
Ranbp10	2428.035171	-0.55762	0.14634	-3.81043	0.000139	0.002771
Lrrc27	98.38128457	1.126718	0.295697	3.810378	0.000139	0.002771
Stra6	796.488094	0.854429	0.224535	3.805333	0.000142	0.002823
Dclk1	86.1777812	1.612516	0.424135	3.80189	0.000144	0.002857
Map1a	83.81516511	-1.3475	0.354451	-3.80165	0.000144	0.002857
Myb	743.5359981	0.499009	0.131519	3.794203	0.000148	0.002939
Rasa2	1660.089739	0.453548	0.119576	3.792962	0.000149	0.002949
Stk39	3602.982584	0.384657	0.101466	3.790984	0.00015	0.002967
Itga3	519.7164882	1.161407	0.306383	3.790704	0.00015	0.002967
Impa2	3365.228695	0.399714	0.105541	3.787297	0.000152	0.002998
Cyb561a3	1339.948821	0.39981	0.105567	3.787254	0.000152	0.002998
4930581F22Rik	234.6139983	-0.82636	0.218211	-3.78697	0.000152	0.002998
Irag2	6546.086135	0.356552	0.094195	3.785247	0.000154	0.003015
Pak1	337.2729336	0.726629	0.192005	3.784434	0.000154	0.00302
Orai2	1902.648056	-0.66209	0.174978	-3.78381	0.000154	0.003023
D430041D05Rik	117.0536287	-1.077	0.285062	-3.77812	0.000158	0.003083
Rapgef6	6814.799798	-0.43421	0.114928	-3.77812	0.000158	0.003083
Ufsp2	1385.619504	0.470891	0.124679	3.776832	0.000159	0.003095
Trgc2	67.02303332	-1.49049	0.395416	-3.76942	0.000164	0.003183
Cenpf	2850.916772	-0.48019	0.127515	-3.76577	0.000166	0.003225
Hsd1l	1965.869582	-0.48271	0.128268	-3.76333	0.000168	0.003252
Osbp15	221.0141526	-0.87123	0.231596	-3.76184	0.000169	0.003266
Adam19	4475.229387	-0.5557	0.147736	-3.76148	0.000169	0.003266
Gabrr2	41.73838911	-1.77647	0.472353	-3.7609	0.000169	0.003269
4932438H23Rik	618.593951	0.739731	0.197188	3.751402	0.000176	0.00339
Tspoap1	384.8822803	0.663121	0.176885	3.748882	0.000178	0.003419

Tnfsf14	556.0134735	0.584403	0.156247	3.740262	0.000184	0.003533
Gvin2	162.91396	-0.90922	0.243122	-3.73978	0.000184	0.003535
Ccr5	106.985865	-1.01956	0.272738	-3.73823	0.000185	0.003551
Dhrs3	465.656733	-0.68794	0.184223	-3.73426	0.000188	0.003602
Dach2	31.36771463	1.879641	0.503416	3.733769	0.000189	0.003604
Plek	83.83891875	1.264454	0.338745	3.732763	0.000189	0.003611
Stx1a	549.6600972	-0.59471	0.159333	-3.73251	0.00019	0.003611
Gm20559	98.79990598	-1.16867	0.313682	-3.72565	0.000195	0.003705
Sptan1	9390.337305	-0.52989	0.14229	-3.72401	0.000196	0.003724
Iah1	555.8687953	0.526567	0.141412	3.723645	0.000196	0.003724
Csrp1	3758.792735	-0.37427	0.100524	-3.72324	0.000197	0.003724
Mmp14	126.373363	1.089535	0.292976	3.718855	0.0002	0.003784
Chp1	4640.791344	0.364472	0.09806	3.716837	0.000202	0.003809
Dmbt1	34.64927915	-1.9637	0.52953	-3.70839	0.000209	0.003932
Stmn1	8858.612859	-0.55997	0.151022	-3.70785	0.000209	0.003935
Nabp1	3301.365954	0.459371	0.123903	3.707505	0.000209	0.003935
Gse1	1987.840442	-0.48858	0.132038	-3.70031	0.000215	0.004042
Tle6	104.4249209	1.104123	0.29847	3.69927	0.000216	0.004053
Pacsin1	1101.381517	-0.76673	0.207335	-3.69803	0.000217	0.004067
Plgrkt	1287.598199	0.586586	0.158716	3.695815	0.000219	0.004096
Mcm2	5498.686629	-0.46134	0.124874	-3.69444	0.00022	0.004112
Sec24a	1280.645449	-0.59638	0.16154	-3.69181	0.000223	0.004149
Cish	20095.47249	-0.39877	0.108028	-3.69133	0.000223	0.004151
Bcl11b	5623.031068	-0.46413	0.125831	-3.68856	0.000226	0.00419
6430571L13Rik	542.1199501	-0.54826	0.148761	-3.68551	0.000228	0.004235
Gpr146	3359.151975	-0.37795	0.102658	-3.68168	0.000232	0.004293
Cdin1	434.6222457	0.682769	0.18566	3.677527	0.000236	0.004357
Parp1	4671.248418	-0.3733	0.101536	-3.6765	0.000236	0.004368
Itpr1p2	579.3461867	0.62123	0.169088	3.674007	0.000239	0.004405
Slc38a2	5006.918796	0.389391	0.106105	3.669855	0.000243	0.00447
Gyg1	1452.630744	0.486044	0.132514	3.667867	0.000245	0.004499
Rps27a-ps3	2201.32109	0.642534	0.175499	3.661186	0.000251	0.004611
Kif3b	1975.404575	-0.42384	0.115791	-3.66037	0.000252	0.004619
Tnfaip3	4455.351006	0.369975	0.101101	3.65946	0.000253	0.004629
Sla2	1055.837145	-0.45016	0.12314	-3.65564	0.000257	0.004692
Pkib	243.2217417	0.87242	0.23868	3.655195	0.000257	0.004693
Sh2d2a	8022.830789	-0.4061	0.111225	-3.65116	0.000261	0.004761
Rap1gds1	5907.187705	0.454429	0.124726	3.643417	0.000269	0.0049
Atp2b1	3714.907902	-0.47361	0.130019	-3.6426	0.00027	0.004908
Pik3ap1	122.1006993	0.929054	0.255119	3.641641	0.000271	0.004913
Sfxn3	1903.696264	-0.46986	0.129024	-3.64161	0.000271	0.004913
Slfn3	459.3683967	-0.72014	0.197792	-3.64091	0.000272	0.00492
Slc35g1	910.8484839	-0.56505	0.155276	-3.63903	0.000274	0.004949
Slc25a45	590.1311724	-0.54058	0.148615	-3.63744	0.000275	0.004969
Rhov	18.09000207	-2.78817	0.766565	-3.63723	0.000276	0.004969
Adss1	296.9968335	0.681167	0.187537	3.632185	0.000281	0.005061
Tcf20	2777.674426	-0.52767	0.145311	-3.63133	0.000282	0.00507
Cd52	9976.88625	-0.3482	0.096023	-3.62624	0.000288	0.005164

Itgb7	20548.87872	-0.32312	0.089131	-3.6252	0.000289	0.005178
Ankrd46	2314.481795	0.367733	0.101562	3.620786	0.000294	0.005259
Ibtk	2447.757097	-0.5056	0.139777	-3.61721	0.000298	0.005325
Gimap8	5417.813669	-0.34726	0.096028	-3.61622	0.000299	0.005335
Prnp	6089.771884	-0.37139	0.102707	-3.61599	0.000299	0.005335
Impdh2	5075.568426	0.347111	0.096015	3.615188	0.0003	0.005344
Ubal2	2546.066729	0.396047	0.109709	3.609975	0.000306	0.005444
Thra	1033.843418	-0.51047	0.141416	-3.60966	0.000307	0.005444
Mcm5	9998.420689	-0.38812	0.107592	-3.60731	0.000309	0.005486
Per2	61.54788347	-1.46344	0.405927	-3.60517	0.000312	0.005524
Dock2	9777.242803	-0.47309	0.131302	-3.60306	0.000314	0.005562
Dnajc12	170.9839279	0.924621	0.256693	3.602046	0.000316	0.005576
St8sia1	3291.851228	-0.46791	0.130041	-3.59817	0.00032	0.005652
Smox	1851.266761	0.524121	0.145714	3.596923	0.000322	0.005671
Thada	939.55642	-0.44287	0.123172	-3.59554	0.000324	0.00568
Ptpn1	5513.396689	-0.40449	0.112499	-3.59551	0.000324	0.00568
Kremen2	120.5898595	1.175757	0.327014	3.595436	0.000324	0.00568
Mier1	5530.530653	0.511296	0.142244	3.5945	0.000325	0.005693
Adamts6	640.5118365	-0.51784	0.144089	-3.59386	0.000326	0.005699
Faf1	1945.569231	0.653979	0.181989	3.593506	0.000326	0.005699
Mthfr	990.8356524	0.536319	0.149289	3.592492	0.000328	0.005714
Acadl	1375.660981	0.458947	0.127777	3.591796	0.000328	0.005716
Prrc2b	9087.937736	-0.59505	0.165673	-3.59169	0.000329	0.005716
Cnksr3	116.4623533	-1.09165	0.304024	-3.59068	0.00033	0.00573
Prkra	420.5886858	0.584439	0.163034	3.584761	0.000337	0.005854
Pnpla7	795.2733799	-0.44745	0.124889	-3.58276	0.00034	0.005891
Grn	2911.070857	0.366296	0.102349	3.578882	0.000345	0.005968
Csrnp1	4107.848516	-0.49939	0.139548	-3.57863	0.000345	0.005968
Fsd1l	271.5874519	-0.97631	0.272905	-3.57746	0.000347	0.005987
Prr5l	234.8200551	-0.80867	0.22638	-3.57216	0.000354	0.006102
Pla2g12a	1335.662723	0.535442	0.149985	3.569964	0.000357	0.006145
Igfbp7	231.56458	-0.74968	0.210071	-3.56872	0.000359	0.006166
Mapk12	99.884259	1.180081	0.330776	3.567615	0.00036	0.006183
Slc25a19	792.5804173	0.476523	0.133725	3.563453	0.000366	0.006274
Ocel1	498.4973098	-0.54667	0.153428	-3.56301	0.000367	0.006276
Mfhas1	1915.640058	-0.44717	0.125591	-3.56049	0.00037	0.006328
Hnrnpa1	20931.12972	0.33852	0.095108	3.559342	0.000372	0.006342
Pdk2	259.3465455	-0.7775	0.218447	-3.55922	0.000372	0.006342
Ftsj3	909.8709969	-0.6841	0.192238	-3.55859	0.000373	0.006349
Gtf2e2	1698.894871	0.508899	0.143224	3.553175	0.000381	0.006472
Mthfd1l	6049.013129	0.320348	0.090229	3.550387	0.000385	0.006533
Arid1b	1992.298659	-0.41997	0.118323	-3.54933	0.000386	0.00655
Dnajc1	1170.043919	0.412539	0.11628	3.547818	0.000388	0.006579
Acadv1	3432.352463	0.352769	0.099448	3.547289	0.000389	0.006584
Plod2	1668.235873	0.542053	0.152874	3.545749	0.000391	0.006614
Syne3	1595.187235	-0.67349	0.190232	-3.54039	0.0004	0.006741
Dctn4	2869.607465	0.464264	0.131202	3.538551	0.000402	0.006779
Irf8	1209.586065	0.558212	0.157965	3.53376	0.00041	0.006894

Rrm2	8147.682648	-0.30016	0.084975	-3.53232	0.000412	0.006922
Gpr174	1811.847472	0.491574	0.139295	3.52901	0.000417	0.007
Rorc	446.9062244	0.636399	0.180387	3.527969	0.000419	0.007019
Mfge8	688.9991389	-0.50013	0.14191	-3.5243	0.000425	0.007108
Jarid2	2854.934312	0.378212	0.107334	3.523689	0.000426	0.00711
Pgm1	5960.94152	0.378122	0.107314	3.523514	0.000426	0.00711
NA	160.3238032	0.819102	0.232576	3.521863	0.000429	0.007145
Setd7	2022.605242	-0.45864	0.130308	-3.5197	0.000432	0.007194
Hax1	1279.931685	0.465686	0.13235	3.518597	0.000434	0.007215
Herc2	1662.020327	-0.72025	0.204748	-3.51777	0.000435	0.007227
Dusp4	10900.30998	0.497284	0.141376	3.517459	0.000436	0.007227
Gm8995	8809.438087	-0.46235	0.13163	-3.51251	0.000444	0.007354
Col11a2	490.0420827	-0.80247	0.22859	-3.51052	0.000447	0.007399
Acbd7	55.22538315	1.450941	0.413784	3.506518	0.000454	0.007494
Tnfrsf26	884.521837	-0.58992	0.168239	-3.50646	0.000454	0.007494
Ttc12	23.62826541	2.293414	0.654186	3.505752	0.000455	0.007504
Tnip2	594.8129342	0.517618	0.147821	3.50165	0.000462	0.007611
Lsm6	948.6394252	-0.45876	0.131187	-3.49697	0.000471	0.007736
Prrc1	1367.001168	0.410094	0.11733	3.495233	0.000474	0.007776
Zfyve16	746.0534605	0.67489	0.193114	3.494783	0.000474	0.00778
Bend3	100.2222947	-1.11769	0.319949	-3.49334	0.000477	0.007812
Shisa5	21326.21412	-0.28798	0.082619	-3.48561	0.000491	0.008031
Cept1	3287.635179	0.570757	0.163852	3.483362	0.000495	0.008088
Jak1	14991.61329	-0.31952	0.091806	-3.48037	0.000501	0.008169
Rgmb	65.66639678	-1.20438	0.346245	-3.47841	0.000504	0.00821
Fasn	6820.28763	-0.69503	0.199816	-3.47835	0.000505	0.00821
Copz2	41.99447375	1.610395	0.463118	3.477288	0.000507	0.008232
Fabp5	643.1685735	-0.58596	0.168636	-3.47468	0.000511	0.008302
Mid1	73.7537342	-1.11991	0.322403	-3.47363	0.000513	0.008324
Map1b	58.63111518	1.561513	0.449826	3.471372	0.000518	0.008383
Cdipt	3362.809824	-0.34106	0.098267	-3.47069	0.000519	0.008392
Slc25a51	2919.975604	0.371238	0.106976	3.470295	0.00052	0.008392
Plagl2	3417.843675	-0.42733	0.123148	-3.47009	0.00052	0.008392
Tnnt3	82.36268383	-1.03314	0.298219	-3.46436	0.000532	0.008562
Pigh	279.4472253	-0.76711	0.221472	-3.46369	0.000533	0.008572
Pgbd1	230.1289592	0.693116	0.200227	3.461653	0.000537	0.008621
Plxnd1	1734.106548	0.573301	0.165622	3.461498	0.000537	0.008621
P2ry1	262.9883968	-0.64443	0.186196	-3.46102	0.000538	0.008626
Avpr1a	29.38956518	2.393685	0.691826	3.459955	0.00054	0.008649
Sf3b3	4975.351535	-0.31854	0.092099	-3.45868	0.000543	0.008678
Aspm	2209.835421	-0.57469	0.166174	-3.45837	0.000543	0.008678
Acss1	1117.490951	-0.57467	0.166214	-3.45737	0.000545	0.0087
Cluh	728.6612772	-0.51514	0.14909	-3.45525	0.00055	0.008758
Marcksl1	507.2393802	-0.57936	0.167699	-3.45474	0.000551	0.008763
Gpat3	269.3732789	0.718904	0.208136	3.454019	0.000552	0.008776
Iqgap2	2609.695918	-0.51515	0.149165	-3.45353	0.000553	0.008781
Ahnak	4445.673567	-0.59425	0.172112	-3.4527	0.000555	0.008797
Chd3	9370.782677	-0.69621	0.201879	-3.44863	0.000563	0.00892

Kdelr2	1628.493823	0.431269	0.125069	3.448243	0.000564	0.008922
Cystm1	96.96388297	1.116727	0.3239	3.447754	0.000565	0.008922
Heatr5a	938.7755413	-0.5131	0.148829	-3.44757	0.000566	0.008922
Adora2a	831.2785509	-0.45974	0.13339	-3.4466	0.000568	0.008943
Spty2d1	1642.423889	0.421842	0.122513	3.443247	0.000575	0.009044
Prex1	5129.745426	-0.5644	0.164026	-3.44092	0.00058	0.009111
Trpc4ap	6531.330674	-0.29414	0.085525	-3.43924	0.000583	0.009156
Stat5a	5384.922498	-0.31404	0.091436	-3.43454	0.000594	0.009288
BE692007	270.2896241	-0.82387	0.239888	-3.43439	0.000594	0.009288
Gpr68	1379.261415	-0.38345	0.111651	-3.43438	0.000594	0.009288
Crybg1	5870.513371	-0.34103	0.099364	-3.43209	0.000599	0.009355
Zfand1	527.7584559	0.529167	0.15422	3.431245	0.000601	0.009373
Tmem71	3007.700804	-0.48309	0.140874	-3.42923	0.000605	0.009431
Myo5a	916.4905954	-0.54894	0.160223	-3.42613	0.000612	0.009528
Trp53bp2	952.6427247	-0.43432	0.126791	-3.4255	0.000614	0.009539
Mdn1	281.4762908	-0.79785	0.23302	-3.42395	0.000617	0.009582
Zfp810	295.3002854	0.681179	0.199049	3.422165	0.000621	0.009633
Rpl35a	1488.205024	0.376378	0.110071	3.419406	0.000628	0.00972
Abcb1a	445.0528389	0.628131	0.183724	3.418889	0.000629	0.009726
Slc14a1	48.18320927	-1.45892	0.426927	-3.41726	0.000633	0.009773
Uhrf1	9222.92101	-0.38801	0.113638	-3.41448	0.000639	0.009843
Spes3	15850.64791	0.476998	0.139709	3.414225	0.00064	0.009843
Snapc1	416.1478549	0.539569	0.15804	3.414134	0.00064	0.009843
Aff1	1878.1675	-0.48264	0.141377	-3.41385	0.000641	0.009843
Osgepl1	378.3194032	0.708484	0.207544	3.413665	0.000641	0.009843
Map3k5	1449.241008	-0.45181	0.132377	-3.41306	0.000642	0.009843
Slc66a2	437.8438279	0.558576	0.16366	3.413018	0.000642	0.009843
Fen1	3456.413393	-0.34372	0.10075	-3.41157	0.000646	0.009884
Tamalin	80.33394988	-1.17138	0.343412	-3.41101	0.000647	0.009892
Tpcn1	3359.712253	-0.63596	0.186527	-3.40948	0.000651	0.009932
Polr1a	852.4716394	-0.46835	0.137376	-3.40926	0.000651	0.009932
F2rl3	176.815163	-0.75956	0.222843	-3.40849	0.000653	0.009948
Kcnqlot1	107.6317759	-1.41686	0.415722	-3.40819	0.000654	0.009948
Vdac1	11214.09185	0.40989	0.120367	3.405324	0.000661	0.010041
Zbtb4	983.598982	-0.6919	0.203203	-3.40499	0.000662	0.010042
Zc3hc1	886.4922965	0.500348	0.147063	3.402263	0.000668	0.01013
Rbm38	5309.125978	-0.31147	0.091585	-3.40093	0.000672	0.010156
Dhcr7	2326.197072	-0.34482	0.101391	-3.40092	0.000672	0.010156
Lactb	573.5213514	0.506771	0.149095	3.398975	0.000676	0.010217
Nrip1	2087.045964	-0.71015	0.20897	-3.39832	0.000678	0.010223
Serpini1	107.7934988	-1.17303	0.345196	-3.39817	0.000678	0.010223
Lmna	466.9810353	-0.5968	0.175663	-3.39742	0.00068	0.010239
Bcl6	214.6866601	0.721202	0.212346	3.396355	0.000683	0.010265
Dnajc10	4728.110375	0.406683	0.119751	3.396075	0.000684	0.010265
Ccnd2	7764.597868	-0.45748	0.134721	-3.39574	0.000684	0.010266
Pde4dip	967.0725138	0.471472	0.138958	3.392901	0.000692	0.010361
Paqr8	225.7751717	-0.6611	0.195063	-3.38916	0.000701	0.010491
Tpd52	1204.36916	0.4666	0.137719	3.388059	0.000704	0.010521

Dnm1	202.1521577	0.913289	0.269587	3.387729	0.000705	0.010521
Arhgap31	409.5192275	-0.6797	0.20084	-3.3843	0.000714	0.010638
Cep83	1575.253631	0.396529	0.117175	3.384069	0.000714	0.010638
Hook1	952.351159	0.418134	0.123704	3.380113	0.000725	0.010768
Hspe1	3104.934075	0.467116	0.138209	3.379778	0.000725	0.010768
Msn	24019.57762	-0.38662	0.114393	-3.37977	0.000725	0.010768
Anp32e	9517.724057	-0.36966	0.109388	-3.37931	0.000727	0.010773
Bmyc	614.530233	0.53261	0.15775	3.376286	0.000735	0.01088
Rundc3a	77.12701968	-1.49843	0.443922	-3.37545	0.000737	0.010901
Slc30a4	408.2965615	-0.72095	0.213611	-3.37504	0.000738	0.010904
Fut4	207.5096524	0.713051	0.211328	3.374138	0.00074	0.01092
Rabggtb	1759.568173	0.502104	0.148816	3.373987	0.000741	0.01092
Nrgn	171.7671019	0.770442	0.228368	3.373686	0.000742	0.01092
Eef1g	27577.08547	0.287297	0.085234	3.370669	0.00075	0.011028
Vmp1	2562.337517	0.517269	0.153493	3.369977	0.000752	0.011043
mt-Rnr1	14839.8452	0.623967	0.185403	3.365466	0.000764	0.011212
Ppm1h	1258.87633	-0.54651	0.162479	-3.36359	0.000769	0.011266
AA467197	149.2866644	1.093926	0.325232	3.36353	0.00077	0.011266
Ppp2r5a	4158.431394	-0.32514	0.096699	-3.3624	0.000773	0.011299
Tbrg1	2935.642737	0.33286	0.099044	3.360737	0.000777	0.011354
Brpf3	614.0878281	-0.50288	0.149678	-3.35976	0.00078	0.011381
Gdi1	4490.381053	0.394104	0.117333	3.358858	0.000783	0.011406
Ldlrap1	1809.416636	-0.36494	0.108782	-3.35475	0.000794	0.011556
Alcam	223.9104738	0.883905	0.26349	3.354608	0.000795	0.011556
Atg9b	687.2013585	0.49229	0.146817	3.353083	0.000799	0.011596
Espn	3348.157593	0.391521	0.116767	3.353023	0.000799	0.011596
Rab11fip2	258.8653167	-0.71067	0.212202	-3.34905	0.000811	0.011751
Gm8615	1164.156666	0.508521	0.151991	3.345731	0.000821	0.011879
Ptpa	2135.43275	-0.34056	0.101808	-3.34513	0.000822	0.011891
Dkk1	245.2509324	0.753217	0.225264	3.343709	0.000827	0.011939
Oas3	795.6745982	0.520162	0.155737	3.340006	0.000838	0.012085
Smad5	1032.249298	-0.41428	0.124136	-3.33728	0.000846	0.012191
Adcy6	961.4649797	-0.57363	0.171957	-3.33591	0.00085	0.012227
Macroh2a1	14758.85685	-0.34408	0.103147	-3.33583	0.00085	0.012227
Frzb	61.81440014	1.426524	0.427753	3.334925	0.000853	0.012253
Fasl	1162.679479	0.443946	0.133162	3.33387	0.000856	0.012286
Gm14718	139.8395726	1.044231	0.313326	3.332733	0.00086	0.012323
Huwe1	6614.613887	-0.66647	0.200051	-3.33153	0.000864	0.012363
Vat1	931.6445525	0.455976	0.136887	3.331036	0.000865	0.012371
Dbp	445.5021417	-0.66778	0.200573	-3.32936	0.00087	0.012431
Asf1b	3221.366436	-0.36609	0.110076	-3.32582	0.000882	0.012576
Nup153	3950.349573	-0.40344	0.121329	-3.32516	0.000884	0.012592
Tube1	1903.883561	-0.35475	0.106776	-3.32236	0.000893	0.012705
Als2cl	3765.680609	-0.55939	0.16844	-3.32103	0.000897	0.01274
Il1r2	64.79024652	1.548242	0.466225	3.320801	0.000898	0.01274
Actg1	76397.45458	0.564627	0.170034	3.320672	0.000898	0.01274
Tmx3	3185.750722	0.527437	0.15886	3.320141	0.0009	0.01275
Sun2	7826.474478	-0.41578	0.125265	-3.31919	0.000903	0.01278

Mrtfa	3249.384652	-0.48882	0.147567	-3.31254	0.000925	0.013073
Lmnbl	8413.173484	-0.3578	0.108054	-3.31127	0.000929	0.013118
Ephx1	823.9162555	-0.44578	0.134648	-3.31074	0.00093	0.013128
Ski	4988.27903	-0.32388	0.097922	-3.3075	0.000941	0.013267
Myo10	656.9918129	0.667803	0.201939	3.30696	0.000943	0.013277
Tafazzin	1321.893545	0.373948	0.11309	3.306652	0.000944	0.013277
Nxpe3	4292.858915	-0.29814	0.090196	-3.30548	0.000948	0.013319
Stard10	348.3299589	0.539803	0.163341	3.304759	0.000951	0.013338
Gem	104.5884003	0.945978	0.286454	3.302374	0.000959	0.013432
Ubtf	4708.514958	-0.38032	0.115183	-3.30189	0.00096	0.013432
Mcm3	11156.64216	-0.28828	0.08732	-3.30146	0.000962	0.013432
Pou2f1	805.3602807	-0.67536	0.204568	-3.3014	0.000962	0.013432
Trabd	3341.347602	0.329131	0.099698	3.301265	0.000962	0.013432
Acads	1489.283318	0.414665	0.125692	3.299062	0.00097	0.013523
Clspn	3120.050614	-0.45303	0.137419	-3.29669	0.000978	0.013611
Smurf1	1595.041808	-0.52105	0.158055	-3.29662	0.000979	0.013611
Gprin3	242.5134843	-1.00077	0.303607	-3.29626	0.00098	0.013611
Limd2	13335.92572	-0.39436	0.119648	-3.29603	0.000981	0.013611
Amz1	70.21193154	1.194615	0.362517	3.295336	0.000983	0.013626
Kctd14	128.6066174	0.865592	0.26269	3.295114	0.000984	0.013626
Ppme1	3850.260676	0.327318	0.099387	3.293356	0.00099	0.013684
Smc5	2935.764787	0.496861	0.150881	3.293064	0.000991	0.013684
Tmem131	4344.162244	-0.45359	0.137743	-3.29301	0.000991	0.013684
Scd4	19.44943735	-2.15451	0.654909	-3.28979	0.001003	0.013827
Mov10	1016.315538	-0.70437	0.214318	-3.28657	0.001014	0.013971
Ung	854.9314908	-0.40828	0.124294	-3.2848	0.001021	0.014044
Sugt1	2424.557767	0.504542	0.153708	3.282469	0.001029	0.014136
Dock10	3558.936315	-0.42452	0.129335	-3.28236	0.001029	0.014136
NA	5999.216805	0.315613	0.096172	3.281762	0.001032	0.01415
Mki67	19007.75174	-0.44576	0.135859	-3.28101	0.001034	0.014173
Tlcd2	189.9787842	0.773302	0.235744	3.280264	0.001037	0.014181
Prrc2c	9135.610966	-0.53743	0.163836	-3.28026	0.001037	0.014181
Spef2	164.6954359	0.731149	0.222982	3.278953	0.001042	0.014231
Fmc1	259.804195	0.618247	0.188676	3.276764	0.00105	0.014327
Cbx2	560.2919518	-0.50739	0.154939	-3.27475	0.001058	0.014414
Trmu	186.0196833	0.718798	0.219576	3.27358	0.001062	0.014458
Col20a1	82.2884629	-1.04242	0.318952	-3.26827	0.001082	0.014717
Mmp10	17.34021148	2.3857	0.730337	3.266574	0.001089	0.01479
Psmc6	2771.963296	0.572902	0.175456	3.26522	0.001094	0.014845
Mphosph6	744.0581484	0.506212	0.155088	3.264035	0.001098	0.014891
Arhgap1	4258.619053	-0.36668	0.112453	-3.26072	0.001111	0.015051
Scn2b	384.2180451	-0.66924	0.205284	-3.26005	0.001114	0.01507
Ccni	17463.336	0.475198	0.145837	3.258419	0.00112	0.015141
Gatm	408.8878506	0.595158	0.182723	3.257161	0.001125	0.015193
Rnf128	169.1170827	0.789519	0.242517	3.255517	0.001132	0.015265
Odc1	2592.009296	0.319509	0.098178	3.254382	0.001136	0.01531
Traf3ip3	4788.938864	-0.34265	0.10535	-3.25247	0.001144	0.015397
Wsb1	3872.242821	0.416707	0.12821	3.250192	0.001153	0.015505

Serpinb9	800.1834027	0.741749	0.228491	3.2463	0.001169	0.015702
Hnrnp3	347.4254125	0.542867	0.167285	3.245155	0.001174	0.015748
Cd200	557.9318758	0.57212	0.176414	3.243059	0.001183	0.015848
Gdi2	19465.32516	0.45685	0.140901	3.242347	0.001185	0.015871
Sfrp2	48.63378376	-1.44868	0.447262	-3.23899	0.0012	0.016043
Cdhr3	155.0188182	-1.01561	0.313632	-3.23822	0.001203	0.016069
Axin2	601.8696087	-0.56399	0.17424	-3.23686	0.001209	0.016129
Fam162a	3789.279235	0.415967	0.128568	3.235384	0.001215	0.01619
Cdc6	2513.538111	-0.34494	0.106621	-3.2352	0.001216	0.01619
Prr13	4767.793832	-0.31287	0.096727	-3.23454	0.001218	0.01621
Ccr9	79.31424954	-1.14992	0.355665	-3.23317	0.001224	0.016272
Rhob	337.8026637	0.55871	0.17302	3.229153	0.001242	0.016472
Ccdc126	334.6756017	-0.57091	0.176803	-3.22908	0.001242	0.016472
Eif3d	3291.17588	0.320409	0.09924	3.228615	0.001244	0.016482
Slc35c2	904.4357021	0.472975	0.146602	3.226261	0.001254	0.016601
Mtus1	84.90008071	-1.40411	0.435292	-3.22566	0.001257	0.016618
Prdx2	5173.92865	0.402749	0.124965	3.222903	0.001269	0.016762
NA	50.60293132	-1.72927	0.53802	-3.21414	0.001308	0.017264
4933434E20Rik	1304.781212	0.377044	0.117334	3.213424	0.001312	0.017279
Tmem67	282.5236775	0.641483	0.199634	3.213299	0.001312	0.017279
Klhl5	905.1606454	-0.47605	0.148184	-3.21255	0.001316	0.017281
Hook2	514.0415139	0.482378	0.150166	3.212298	0.001317	0.017281
Suox	898.0659341	-0.41351	0.128737	-3.21207	0.001318	0.017281
Hmgcl	1394.666682	0.389279	0.121202	3.211813	0.001319	0.017281
Sv2c	40.66928186	1.873434	0.583298	3.211796	0.001319	0.017281
Ajuba	41.90696027	1.512312	0.470981	3.21098	0.001323	0.017313
Atp8b2	5796.333422	-0.36696	0.114317	-3.21003	0.001327	0.017352
Pdcd5	674.9957839	0.446301	0.13916	3.207093	0.001341	0.017511
Dars1	3875.367044	0.534573	0.166699	3.206821	0.001342	0.017511
Rab34	195.680283	1.007956	0.314467	3.205281	0.001349	0.017588
Nhsl2	48.92966203	-1.30589	0.40753	-3.20441	0.001353	0.017623
Tmem263	1228.308519	0.5379	0.167899	3.203705	0.001357	0.017648
Cth	36.93175498	1.95646	0.610994	3.202094	0.001364	0.017729
Id3	557.300162	-0.50353	0.157264	-3.20181	0.001366	0.017729
Rnf145	5774.50154	-0.30656	0.095766	-3.20113	0.001369	0.017753
Ikzf1	12229.87795	-0.30116	0.094123	-3.19961	0.001376	0.017812
Golph3	5652.091632	-0.40194	0.125623	-3.19959	0.001376	0.017812
Eif4a2	8383.847491	0.494912	0.154732	3.198502	0.001381	0.017858
Adamts20	18.45817682	2.815364	0.880278	3.198267	0.001383	0.017858
Tex9	704.9055986	0.434676	0.135929	3.197812	0.001385	0.017868
9930111J21Rik1	363.5782492	-1.03414	0.323681	-3.19495	0.001399	0.018028
Nmnat2	36.09114562	-1.62052	0.507261	-3.19465	0.0014	0.018029
Slc17a9	1215.931525	-0.37704	0.118148	-3.19127	0.001416	0.018223
Rtn	180.7608227	0.786024	0.246967	3.18271	0.001459	0.018751
Pign	1114.632084	0.457427	0.143926	3.178216	0.001482	0.019025
Fas	862.3776899	-0.4535	0.142707	-3.1778	0.001484	0.019034
Vps54	3287.575528	0.420899	0.132687	3.172121	0.001513	0.019378
Kif20a	2407.118971	-0.43803	0.138094	-3.17199	0.001514	0.019378

Armcx6	145.2725968	0.755955	0.238342	3.171723	0.001515	0.019378
Sat1	3715.348892	0.498103	0.157136	3.169877	0.001525	0.019482
Penk	275.5715823	0.658452	0.207816	3.168446	0.001533	0.019559
Snx5	7515.520351	0.499095	0.157609	3.166676	0.001542	0.019659
Pyroxd2	34.2562191	-1.88613	0.595847	-3.16546	0.001548	0.019704
Arhgap18	2063.379613	0.387758	0.122497	3.165442	0.001548	0.019704
Amz2	1739.801837	0.43665	0.137978	3.164632	0.001553	0.019739
Abcf2	1980.028586	-0.35381	0.111837	-3.16365	0.001558	0.019786
Cdc42se2	5959.522808	-0.29068	0.0919	-3.16295	0.001562	0.019814
Ccnd3	7649.438096	-0.31301	0.098984	-3.16222	0.001566	0.019844
Zfp811	25.5983113	-1.83521	0.580549	-3.16116	0.001571	0.019897
Sms	1590.583493	0.546397	0.172906	3.160079	0.001577	0.019951
Hadh	2501.243327	0.412376	0.130522	3.159437	0.001581	0.019976
Phc3	4413.185629	-0.47055	0.149079	-3.15639	0.001597	0.020166
Prpf31	1412.141601	-0.39759	0.125995	-3.1556	0.001602	0.020201
Kdelr3	37.20834536	1.775742	0.563115	3.153428	0.001614	0.020331
Gm5547	576.4224609	0.609699	0.193405	3.152444	0.001619	0.02038
Fem1a	840.3133764	-0.44516	0.141375	-3.14879	0.00164	0.020596
Neto2	82.87363099	-0.98937	0.314227	-3.1486	0.001641	0.020596
Ache	115.3964686	0.901486	0.286322	3.1485	0.001641	0.020596
Zfhx2	424.6724807	-0.75504	0.239891	-3.14743	0.001647	0.020639
A630001O12Rik	40.74676464	-1.51299	0.480723	-3.14732	0.001648	0.020639
Znrf3	589.5241188	-0.76787	0.244374	-3.14221	0.001677	0.020982
Snhg1	1532.148034	0.575105	0.183068	3.141477	0.001681	0.020995
Rftn1	3178.890717	-0.32398	0.103131	-3.14147	0.001681	0.020995
Cd53	23073.93424	0.54696	0.174255	3.138856	0.001696	0.021162
Snrpd3	1524.930426	-0.45604	0.145301	-3.13856	0.001698	0.021163
Dag1	1132.067076	-0.66998	0.213492	-3.13821	0.0017	0.021168
Ep300	3523.580301	-0.66799	0.213112	-3.13447	0.001722	0.021419
Twf2	3067.56902	-0.38971	0.124394	-3.13283	0.001731	0.021518
Ccr4	10029.58703	0.325122	0.103931	3.128232	0.001759	0.021836
F2rl2	317.6682371	0.514065	0.164474	3.125499	0.001775	0.022017
Hibch	607.2465521	0.529837	0.169535	3.12524	0.001777	0.022017
Zechc9	992.6645329	0.60394	0.19347	3.121621	0.001799	0.022268
D930048N14Rik	190.6932526	0.671189	0.215061	3.12093	0.001803	0.022299
Stag3	146.5316964	-1.07033	0.343007	-3.12043	0.001806	0.022315
Coq9	924.6421064	0.446563	0.14314	3.119772	0.00181	0.022338
Plcl2	2547.28943	-0.32851	0.105307	-3.11956	0.001811	0.022338
Kif14	1814.48927	-0.37613	0.120616	-3.11837	0.001819	0.022407
Bcar1	196.6929255	0.678485	0.217704	3.116549	0.00183	0.022524
Aff3	2443.728835	-0.47277	0.1518	-3.11439	0.001843	0.022668
Il1rl1	388.5868019	-0.54702	0.17568	-3.11372	0.001847	0.022698
Rab26	135.1477493	0.795003	0.255395	3.112833	0.001853	0.022745
Mri1	558.1927252	0.51267	0.164769	3.111447	0.001862	0.02283
Nfya	2137.691254	-0.36506	0.117403	-3.10943	0.001874	0.022964
Dusp1	455.1670518	-0.48381	0.155615	-3.10903	0.001877	0.022973
Hnrnpab	11832.98476	-0.42025	0.135284	-3.10646	0.001893	0.023152
Mast4	177.0529703	-0.75526	0.243343	-3.10369	0.001911	0.023328

Mxi1	4661.656645	0.330994	0.106647	3.103651	0.001911	0.023328
Zfp710	392.8566324	-0.67518	0.217621	-3.10257	0.001918	0.023392
S100a4	24.25207679	-2.70417	0.872066	-3.10088	0.001929	0.023503
Gm5122	13.83025722	2.662782	0.858977	3.099946	0.001936	0.023555
Parp8	1281.945334	0.504119	0.162692	3.098613	0.001944	0.023639
Furin	2563.860525	-0.49605	0.160125	-3.09788	0.001949	0.023675
St6galnac4	343.3791773	0.530699	0.171353	3.097113	0.001954	0.023714
Entpd5	1620.425065	-0.34436	0.111318	-3.09344	0.001978	0.023987
Tial1	4396.784953	0.32628	0.105532	3.091774	0.00199	0.024099
Ifi47	2675.597681	-0.54963	0.178125	-3.08564	0.002031	0.024577
Alpl	24.88899787	-2.63209	0.853085	-3.08538	0.002033	0.024577
Tdrkh	676.1431241	0.457671	0.14836	3.084857	0.002037	0.024597
Gfi1	2178.842809	0.481712	0.156185	3.084234	0.002041	0.024625
NA	92.79956334	-1.26624	0.410636	-3.08362	0.002045	0.024653
Itm2a	3207.469835	0.487537	0.158221	3.081376	0.00206	0.024816
Rabac1	3879.885989	0.306536	0.099524	3.080018	0.00207	0.024906
Gas2	156.8114558	0.727623	0.236313	3.079058	0.002077	0.024964
Cryz	467.5104975	0.468548	0.152272	3.077056	0.002091	0.025108
Slc25a23	1363.415955	-0.52627	0.171202	-3.07396	0.002112	0.025346
Cdc42ep2	31.26310365	1.856397	0.604567	3.070624	0.002136	0.025607
Nphp3	377.6087002	0.504541	0.16436	3.06974	0.002142	0.02565
Aim2	2413.061011	0.391067	0.127401	3.069567	0.002144	0.02565
Zfp281	4231.276893	-0.38674	0.126018	-3.06892	0.002148	0.025682
Dusp2	1733.302653	-0.31188	0.101641	-3.06846	0.002152	0.025696
4930431P03Rik	352.6813065	-0.57655	0.187912	-3.0682	0.002154	0.025696
Prkx	822.9784018	-0.46725	0.152392	-3.06613	0.002168	0.025851
Ctla2b	873.6920146	0.634834	0.207073	3.065749	0.002171	0.02586
Arell	2779.884554	-0.36097	0.117837	-3.06332	0.002189	0.026046
Rnf130	1081.033649	0.43079	0.140739	3.060922	0.002207	0.026232
Scd1	13326.16129	0.361737	0.1182	3.060388	0.002211	0.02624
Mxd3	1065.635792	-0.46471	0.151852	-3.06027	0.002211	0.02624
Hbegf	283.1250605	-0.61358	0.200614	-3.05852	0.002224	0.026369
Sema4f	1117.256099	0.389274	0.127295	3.058032	0.002228	0.026388
Strbp	1486.538661	0.410929	0.134438	3.056645	0.002238	0.026486
Acot13	461.8282654	0.468329	0.153265	3.055675	0.002246	0.026547
Atp2b4	5461.366888	-0.52488	0.171829	-3.05468	0.002253	0.026612
Zmynd19	431.6792825	-0.48023	0.157229	-3.05435	0.002255	0.026616
Tcf19	3503.958286	-0.34635	0.113619	-3.0483	0.002301	0.027133
Tbc1d16	326.8251021	-0.54254	0.178036	-3.04738	0.002308	0.027172
Lrrc8c	3258.35006	-0.34313	0.1126	-3.04732	0.002309	0.027172
Cul2	1920.35636	0.496913	0.163132	3.046081	0.002318	0.027259
Fbxl6	1454.88378	0.32775	0.107761	3.041464	0.002354	0.027655
Prkcb	3304.57697	-0.46398	0.152574	-3.04103	0.002358	0.027669
Chaf1b	1372.269429	-0.35091	0.115427	-3.04009	0.002365	0.027731
Sdf4	19805.27639	0.308651	0.101542	3.039628	0.002369	0.027748
Asap2	110.5238756	-0.99749	0.328363	-3.03776	0.002383	0.027877
Elmod2	329.3595319	0.633321	0.208488	3.037686	0.002384	0.027877
Fam111a	3105.565618	-0.30276	0.099713	-3.03629	0.002395	0.027981

Piwi4	62.79968057	1.074579	0.353951	3.035958	0.002398	0.027986
Nup43	550.499143	0.534374	0.176032	3.03566	0.0024	0.027988
Plekhg3	66.79367017	-1.29376	0.426249	-3.03522	0.002404	0.028003
Eif5	8325.247783	0.462141	0.152282	3.034769	0.002407	0.02802
Urb1	297.3593776	-0.61402	0.202381	-3.03398	0.002414	0.028068
Gde1	1743.855581	0.513616	0.16932	3.033407	0.002418	0.028096
Rps23rg1	877.1415804	0.39605	0.130615	3.032199	0.002428	0.028183
Dnajb4	441.4968998	0.751911	0.248106	3.030599	0.002441	0.028307
1700017B05Rik	2605.093451	-0.3502	0.115631	-3.02863	0.002457	0.028467
Il18rap	330.5105135	-0.59091	0.195155	-3.02788	0.002463	0.028511
Noxo1	208.644875	0.606179	0.200258	3.026989	0.00247	0.02857
Trav15n-1	22.16040107	-1.8823	0.62203	-3.02605	0.002478	0.028633
Rab28	962.8520756	0.392066	0.129668	3.023619	0.002498	0.028785
Col6a4	16.09930803	-2.10472	0.696103	-3.02357	0.002498	0.028785
Gm11579	60.57971466	-1.23766	0.40938	-3.02326	0.002501	0.028785
Gmcl1	1519.7934	0.379321	0.125474	3.023111	0.002502	0.028785
Stk38l	523.7900219	0.47149	0.155963	3.023092	0.002502	0.028785
Clcf1	281.7287732	-0.73615	0.243723	-3.02044	0.002524	0.029012
D16Ertd472e	5008.203188	0.361848	0.119894	3.018065	0.002544	0.029214
Cdon	935.0093842	-0.4975	0.164884	-3.0173	0.00255	0.029262
Nars2	378.9006052	0.564685	0.187219	3.016177	0.00256	0.029344
Cnih1	1256.834371	0.626971	0.207989	3.014447	0.002574	0.029486
Slc7a1	2587.938408	0.530686	0.176082	3.01386	0.002579	0.029489
Zfpm1	1305.768735	-0.34436	0.114263	-3.01374	0.00258	0.029489
Il12a	29.36459542	1.582651	0.525171	3.013594	0.002582	0.029489
Shmt1	387.7372131	-0.50591	0.16789	-3.01334	0.002584	0.029489
Sec1	20.62218871	-1.83451	0.608887	-3.01289	0.002588	0.029506
Tle4	1206.539453	-0.36779	0.122122	-3.01167	0.002598	0.029599
Ccl5	48.64902883	1.3413	0.445753	3.009067	0.002621	0.029827
Tmbim4	1277.99241	0.418478	0.139154	3.007312	0.002636	0.029942
Sv2a	312.9874959	0.513443	0.170734	3.007275	0.002636	0.029942
Smarchb1	2418.508109	0.340982	0.113393	3.007088	0.002638	0.029942
Tmem209	1477.294984	0.331289	0.110197	3.006343	0.002644	0.029989
Tmem120a	525.1539635	0.420341	0.139854	3.005567	0.002651	0.030039
Slc4a11	74.76096767	1.169767	0.389238	3.005275	0.002653	0.030042
Rgs2	951.9867317	0.501224	0.166834	3.004337	0.002662	0.030108
Pced1b	1239.403479	-0.35637	0.118631	-3.00401	0.002664	0.030114
Il10ra	2084.404003	-0.38331	0.12773	-3.00096	0.002691	0.030391
Mrtfb	568.4069871	-0.55302	0.184311	-3.00045	0.002696	0.030414
Susd6	2294.700406	-0.347	0.11579	-2.99683	0.002728	0.03075
Hemgn	178.3031179	-0.70557	0.235577	-2.99506	0.002744	0.030869
Sra1	1901.865841	0.358129	0.119578	2.994947	0.002745	0.030869
Rcsd1	6110.455126	-0.32225	0.107599	-2.99486	0.002746	0.030869
Cdk8	1137.367187	0.432701	0.144508	2.994312	0.002751	0.030897
Dtx4	268.1260938	-0.65677	0.219445	-2.99289	0.002764	0.031015
Bcl2l14	46.07518875	1.250098	0.418145	2.98963	0.002793	0.03132
NA	304.210146	-0.83506	0.279641	-2.9862	0.002825	0.031646
Prkcsh	4852.920774	0.286809	0.096086	2.984915	0.002837	0.031748

Ankle1	567.8259613	-0.61586	0.206342	-2.98468	0.002839	0.031748
Siah2	407.3331549	0.478982	0.160533	2.983693	0.002848	0.031801
Babam2	1748.617898	0.446667	0.149706	2.983636	0.002848	0.031801
Polr3e	327.1673527	-0.53273	0.178655	-2.98187	0.002865	0.031957
Tbc1d9b	5156.272558	0.322677	0.108225	2.981538	0.002868	0.031964
Edaradd	29.05256738	1.91474	0.642425	2.980487	0.002878	0.032046
Slc30a7	1795.672592	-0.34211	0.114837	-2.97912	0.002891	0.032162
Ccn6	97.65643684	-1.38625	0.465421	-2.97849	0.002897	0.032201
Ndufs2	4434.379322	0.308389	0.103601	2.976684	0.002914	0.032362
Hivep3	451.0224664	-0.99467	0.334213	-2.97614	0.002919	0.032389
Zfyve1	1299.196616	-0.36753	0.123502	-2.9759	0.002921	0.032389
Bcl7a	176.7247702	-0.68923	0.231687	-2.97482	0.002932	0.032475
C3	274.8865589	0.785794	0.264201	2.974234	0.002937	0.032509
Srcap	3500.040456	-0.59607	0.200436	-2.97387	0.002941	0.03252
Ndfip1	9013.821942	0.345594	0.116317	2.97113	0.002967	0.032783
Mafg	493.283498	0.475169	0.160068	2.968538	0.002992	0.033033
Ankrd17	3118.591455	-0.43859	0.147838	-2.96669	0.00301	0.033203
S100a10	8345.604884	-0.32408	0.109271	-2.96587	0.003018	0.033264
Tjp2	263.7398712	-0.74084	0.250142	-2.96167	0.00306	0.033691
Abtb3	57.51938711	-1.26322	0.426563	-2.96139	0.003063	0.033693
Sh3kbp1	9537.224372	0.304665	0.10289	2.961065	0.003066	0.0337
Adcy9	66.93818353	-1.21112	0.409078	-2.96061	0.00307	0.033721
Tspan32	4241.257834	-0.32218	0.108915	-2.95812	0.003095	0.033946
Acer2	216.7475268	-0.58305	0.197108	-2.95803	0.003096	0.033946
Gzma	21.93521048	1.926068	0.651207	2.957689	0.0031	0.033955
Plxnb2	474.2421844	0.608445	0.20574	2.957353	0.003103	0.033958
Katna1	1818.634358	0.457177	0.154601	2.957139	0.003105	0.033958
Plrg1	2118.272874	0.320521	0.108425	2.956142	0.003115	0.034039
Appl2	1506.81677	0.346054	0.117081	2.95567	0.00312	0.034053
Ell2	609.7388653	0.612477	0.207249	2.955267	0.003124	0.034053
Prcp	648.524134	0.443791	0.150171	2.955227	0.003124	0.034053
Slc30a2	144.5796508	0.733178	0.248132	2.954791	0.003129	0.034072
Gm15501	978.5120485	0.476488	0.161318	2.95371	0.00314	0.034162
Slc25a40	1409.712964	0.427773	0.144885	2.952503	0.003152	0.034267
Lrrfip2	654.7906025	0.444914	0.150819	2.949982	0.003178	0.034519
Rnh1	3327.707104	0.338647	0.114835	2.948974	0.003188	0.034602
Selplg	7105.917033	-0.28768	0.097595	-2.94767	0.003202	0.034702
Snx14	1501.254002	0.365517	0.124006	2.947568	0.003203	0.034702
Cdv3	3584.410938	0.312442	0.106025	2.946874	0.00321	0.03475
Gpr141	13.66274408	2.48638	0.844022	2.945869	0.00322	0.034834
Eif3i	6260.417861	0.440633	0.149634	2.944731	0.003232	0.034933
Tomm40l	735.7987827	0.405963	0.137907	2.943756	0.003243	0.034997
Optn	292.4880304	0.521766	0.177252	2.943642	0.003244	0.034997
Tnfrsf23	85.62444904	-1.1993	0.407512	-2.94298	0.003251	0.035043
Arl2	502.7453731	0.479074	0.163004	2.939042	0.003292	0.035461
Slk	3865.285298	0.322166	0.10965	2.938123	0.003302	0.035537
Snord35b	15.92409711	-2.70291	0.920235	-2.93719	0.003312	0.035614
Tnfrsf4	12979.10878	0.323684	0.110312	2.934272	0.003343	0.03592

Cacfd1	890.1207257	0.366148	0.124841	2.932927	0.003358	0.036046
Lix1l	197.83227	0.704806	0.240738	2.927684	0.003415	0.036629
Nol8	468.4806697	-0.48475	0.165593	-2.92733	0.003419	0.03664
Ankrd50	1743.186582	-0.38941	0.133084	-2.926	0.003433	0.036766
Kank3	158.5057678	0.653326	0.223396	2.924518	0.00345	0.036911
Pla2g6	514.0873256	0.432643	0.14797	2.92385	0.003457	0.03696
Zfp821	393.8779774	0.468138	0.160311	2.92018	0.003498	0.037367
Cdh24	194.5924106	-0.61092	0.209234	-2.91981	0.003503	0.037368
Eif2a	3690.989722	0.44236	0.151511	2.919652	0.003504	0.037368
Cwc27	520.0749567	0.443221	0.151834	2.919113	0.00351	0.037384
Slc4a7	1808.560768	-0.45859	0.157108	-2.91895	0.003512	0.037384
Ighm	5257.278492	-0.44318	0.151841	-2.91874	0.003515	0.037384
Micall2	26.3942045	1.726824	0.591847	2.917685	0.003526	0.03748
Evl	5111.489147	-0.44688	0.153247	-2.91605	0.003545	0.037646
Zfp219	1997.917755	0.419659	0.144023	2.913841	0.00357	0.037882
Spidr	1161.795845	0.329831	0.113219	2.913219	0.003577	0.037912
Tincr	53.28500586	-1.26764	0.435155	-2.91307	0.003579	0.037912
H2ac18	52.58612049	-1.13216	0.388748	-2.91231	0.003588	0.037973
Kif20b	2191.237402	-0.32267	0.110805	-2.91201	0.003591	0.037979
H2-Ob	54.93890182	-1.28787	0.442746	-2.90882	0.003628	0.038313
Cdca5	1827.433291	-0.28978	0.099623	-2.90876	0.003629	0.038313
Stk10	8082.432555	-0.42613	0.146641	-2.90592	0.003662	0.038589
E2f7	559.0455251	-0.52827	0.181801	-2.90577	0.003663	0.038589
Mbp	5354.462013	-0.3013	0.10369	-2.90574	0.003664	0.038589
Ptpcrap	3959.258999	-0.32566	0.112093	-2.90526	0.003669	0.038617
Nts	11.69891068	2.757297	0.949492	2.903971	0.003685	0.038744
Parp16	292.2559886	0.537245	0.185049	2.903259	0.003693	0.038801
C2cd3	2099.198982	-0.57302	0.19739	-2.90297	0.003696	0.038804
Itpr2	2216.868475	-0.60644	0.208954	-2.90228	0.003705	0.038858
Vdac2	6300.986006	0.395909	0.136505	2.90033	0.003728	0.039069
Dnaaf5	1350.762351	-0.35196	0.121425	-2.89856	0.003749	0.039258
Qars1	6757.407144	0.265914	0.091768	2.897677	0.003759	0.039337
Mpp1	1352.460094	-0.32274	0.111476	-2.89511	0.00379	0.039629
Zfp949	1440.323493	-0.39087	0.135101	-2.89318	0.003814	0.03984
Plpp2	472.0066006	0.469645	0.162401	2.89188	0.003829	0.039972
Niban1	1826.802654	-0.4039	0.139691	-2.8914	0.003835	0.040001
Elk4	992.8976686	-0.609	0.210662	-2.8909	0.003841	0.040028
H2bc18	62.53769992	-1.0096	0.34926	-2.89068	0.003844	0.040028
Tnfrsf25	1469.245068	-0.38949	0.134758	-2.89026	0.003849	0.040049
Mei4	109.4482845	1.089564	0.377063	2.889606	0.003857	0.0401
Alad	749.1349469	0.371767	0.128716	2.888262	0.003874	0.040239
Rbm39	10851.80416	0.397471	0.137751	2.885433	0.003909	0.04057
Ifi27l2a	4072.650382	-0.30747	0.106685	-2.88207	0.003951	0.040972
Clybl	931.4639028	0.538769	0.186986	2.881329	0.00396	0.041035
Arhgap4	8106.842518	-0.38949	0.13519	-2.88107	0.003963	0.041036
Sec22a	403.1793496	0.524449	0.182098	2.880035	0.003976	0.041126
Rab6a	2883.742432	0.365922	0.127062	2.879869	0.003978	0.041126
Tnfrsf22	170.9161133	-0.67569	0.234764	-2.87817	0.004	0.041315

Zfp106	6867.461206	-0.37924	0.13178	-2.87782	0.004004	0.041328
Kctd20	1383.118667	-0.42425	0.147459	-2.87709	0.004014	0.04139
Tnfaip8	2090.355782	0.454911	0.158146	2.876532	0.004021	0.04143
Spag7	1188.254698	0.333521	0.116018	2.874737	0.004044	0.041633
Rdh10	210.2973031	-0.59847	0.208451	-2.87102	0.004091	0.042092
Ggact	494.3513145	-0.46503	0.162113	-2.86853	0.004124	0.042391
Ikbkb	3349.007732	-0.26482	0.09239	-2.86634	0.004152	0.042651
Senp6	3672.242586	0.312514	0.109066	2.865374	0.004165	0.042707
Mcl1	14686.16622	-0.2618	0.091369	-2.86527	0.004167	0.042707
L1cam	784.3366961	-0.50535	0.176378	-2.86517	0.004168	0.042707
Aak1	5876.972502	-0.4438	0.154964	-2.86391	0.004184	0.042842
Tha1	398.2380521	-0.52664	0.183915	-2.86349	0.00419	0.042855
Slc12a5	69.58696899	0.965414	0.33718	2.863201	0.004194	0.042855
Tmem176a	249.0590995	-0.56684	0.197985	-2.86306	0.004196	0.042855
Tcn2	743.1038878	-0.40648	0.142002	-2.86247	0.004203	0.0429
Hspa4l	962.5720096	0.44516	0.155589	2.861131	0.004221	0.043048
Stk17b	11976.56669	0.436671	0.152788	2.858012	0.004263	0.043439
Rcan3	3495.604253	-0.35872	0.125608	-2.85591	0.004291	0.043692
Fam234a	666.202237	-0.37126	0.13001	-2.85567	0.004295	0.043692
Samd3	26.4265893	-1.54924	0.542746	-2.85445	0.004311	0.043825
Tet3	3519.583872	-0.4672	0.163704	-2.85393	0.004318	0.043862
Zfp865	1162.465095	-0.35744	0.12526	-2.85356	0.004323	0.043878
Tnfrsf8	429.9260496	0.612677	0.214766	2.852762	0.004334	0.043954
Cep68	817.7740665	-0.41475	0.145428	-2.85193	0.004345	0.044034
Firre	296.4981092	0.552619	0.193828	2.851085	0.004357	0.044102
NA	234.0979909	0.787437	0.276202	2.850941	0.004359	0.044102
Cdc25a	1415.824133	-0.30478	0.10693	-2.85026	0.004368	0.044161
Sp2	1319.809818	-0.36367	0.127608	-2.8499	0.004373	0.044167
Trp53inp1	6186.797683	-0.36734	0.128905	-2.8497	0.004376	0.044167
Nop2	608.4824561	-0.48391	0.169827	-2.84943	0.00438	0.044167
Herc1	1840.701095	-0.56717	0.199067	-2.84912	0.004384	0.044167
Ermp1	705.9237477	-0.68951	0.242026	-2.84892	0.004387	0.044167
Bmf	68.3057263	1.079073	0.378792	2.848723	0.00439	0.044167
Zfp131	1158.641668	0.367383	0.129034	2.847179	0.004411	0.044347
Kcnmb1	41.78119109	-1.36757	0.48037	-2.84691	0.004415	0.04435
Spn	15004.2434	-0.30751	0.108156	-2.84322	0.004466	0.044832
1110032F04Rik	1635.826827	-0.42573	0.149793	-2.8421	0.004482	0.044954
Aox2	30.88411623	1.977237	0.696039	2.840699	0.004501	0.045117
Bzw2	3267.062796	-0.29281	0.10316	-2.83838	0.004534	0.045409
Jund	10711.10431	-0.31029	0.109328	-2.83814	0.004538	0.045409
Utp20	511.8841224	-0.48806	0.171996	-2.83763	0.004545	0.045446
Paxip1	2293.937117	-0.40471	0.142698	-2.83609	0.004567	0.04563
Rem2	40.42144081	1.212486	0.427666	2.835128	0.004581	0.045733
Cd101	884.4779631	-0.35584	0.125545	-2.83439	0.004591	0.045742
NA	7330.626682	0.311505	0.10991	2.834186	0.004594	0.045742
Mcm9	546.502681	-0.47139	0.166337	-2.83396	0.004598	0.045742
Fam78a	4442.899206	-0.3631	0.128127	-2.83391	0.004598	0.045742
Metap1	1325.090881	0.397713	0.140345	2.833821	0.0046	0.045742

Tent5c	1270.083994	-0.4049	0.142964	-2.83216	0.004624	0.045945
Tstd3	758.5103811	0.424458	0.149999	2.829749	0.004658	0.046257
Zbtb18	807.2667315	0.411304	0.145426	2.828269	0.00468	0.046435
Acp5	6650.873432	-0.29084	0.102863	-2.82745	0.004692	0.046493
Cast	5127.834713	-0.29411	0.104022	-2.82737	0.004693	0.046493
Isyna1	2727.494379	0.290475	0.10277	2.826465	0.004706	0.046582
Rtf1	4690.005711	-0.27895	0.098704	-2.82615	0.004711	0.046582
Leprotl1	5245.069691	0.345143	0.122134	2.825937	0.004714	0.046582
Fgfr1	51.91007044	-1.67653	0.593313	-2.82571	0.004718	0.046582
Tlnrd1	643.3529261	-0.36581	0.129467	-2.82553	0.00472	0.046582
Ifi203	898.9224095	-0.54395	0.192575	-2.82463	0.004733	0.046641
Vps13d	1433.96868	-0.5822	0.206116	-2.82461	0.004734	0.046641
Ppp1r9b	4663.601772	-0.46092	0.163195	-2.82438	0.004737	0.046641
Tbrg4	1042.80158	-0.32524	0.115212	-2.82301	0.004757	0.046806
Cuedc1	544.7133042	0.558382	0.197832	2.822511	0.004765	0.046817
Rab5c	4471.750886	-0.41387	0.146635	-2.82244	0.004766	0.046817
Cpne4	38.97286059	-2.24489	0.795891	-2.8206	0.004793	0.04705
Crebbp	1331.818198	-0.70973	0.25168	-2.81997	0.004803	0.047108
Ptms	4022.879223	-0.32416	0.114968	-2.81957	0.004809	0.04713
Arpp19	1334.483242	-0.34688	0.123044	-2.81917	0.004815	0.047153
Trbv1	2552.363833	-0.32131	0.114015	-2.81813	0.00483	0.047269
Rybp	460.4184714	-0.41635	0.147811	-2.81675	0.004851	0.047438
Themis	2417.636099	-0.34751	0.123389	-2.8164	0.004856	0.047452
Rbm15	865.6274073	-0.40586	0.14414	-2.8157	0.004867	0.047466
Eid1	1962.567527	0.485501	0.172429	2.815661	0.004868	0.047466
Unc5cl	255.1450291	-0.63232	0.224601	-2.81529	0.004873	0.047466
Irf4	8383.774978	-0.25635	0.091061	-2.81514	0.004876	0.047466
Smpdl3b	111.1887281	-0.87635	0.311305	-2.81509	0.004876	0.047466
Abca1	73.38314237	-1.20927	0.429665	-2.81445	0.004886	0.047525
Ptp4a2	14193.08082	0.420413	0.149406	2.813905	0.004894	0.047569
Fgf2	56.21874818	1.139889	0.405176	2.813321	0.004903	0.04762
Mms19	2938.576098	0.270414	0.096177	2.811637	0.004929	0.047833
Nlgn2	601.2784116	0.528891	0.188262	2.809328	0.004964	0.048141
Fam161a	109.1006317	0.866573	0.308491	2.809074	0.004968	0.048143
Tmem203	390.2379298	0.473588	0.168661	2.807919	0.004986	0.04828
Pdk3	2078.577793	0.439562	0.156566	2.807518	0.004992	0.048283
Cep89	630.5481171	-0.43916	0.15643	-2.80741	0.004994	0.048283
E2f8	1910.554018	-0.35734	0.127345	-2.80605	0.005015	0.048424
Ep400	4129.716087	-0.46967	0.167403	-2.80563	0.005022	0.048424
Sytl2	604.8258707	-0.44053	0.157022	-2.80554	0.005023	0.048424
Gvin-ps6	112.6506431	-2.54766	0.90814	-2.80536	0.005026	0.048424
Trrap	4118.392615	-0.55401	0.19749	-2.80526	0.005028	0.048424
Pabpc1	73235.92724	0.296011	0.10555	2.804455	0.00504	0.048509
Rplp1	34194.00375	0.402276	0.143479	2.803719	0.005052	0.048583
Akt3	1268.575994	-0.37721	0.134572	-2.803	0.005063	0.048655
Ifih1	165.2535876	0.73308	0.261603	2.802263	0.005075	0.04873
Ifi204	12.48756968	-2.76715	0.987569	-2.80198	0.005079	0.048736
Rassf1	1665.163241	0.331582	0.118356	2.801574	0.005085	0.048761

Ubr4	4179.53606	-0.60232	0.215081	-2.80041	0.005104	0.048901
Zfp945	205.4250708	-0.60252	0.215215	-2.79962	0.005116	0.048985
Edem1	6461.822275	0.302478	0.10806	2.799162	0.005124	0.049017
Dnajb2	365.9696736	0.532847	0.190559	2.796227	0.00517	0.049398
Slc37a2	333.0118246	0.505256	0.180695	2.796185	0.005171	0.049398
Trav14-1	153.964339	-0.73075	0.261452	-2.79496	0.005191	0.049549
Tmem70	869.5371944	0.500006	0.178962	2.793914	0.005207	0.049672
Irf2	1951.328619	-0.38183	0.136688	-2.79343	0.005215	0.049709
Klf16	598.8599193	-0.44162	0.158144	-2.79252	0.00523	0.049812
Hcfc2	865.5160345	0.400706	0.143516	2.792066	0.005237	0.049845

Table 8.1.5.2 – GSEA using gene sets from the MSigDB for iTregs primed $\pm$ 50 nmol L⁻¹ IGF-1 stimulated in PCa CM.

Name	NES	FDR q-val	Rank at max
hallmark_oxidative_phosphorylation	2.005832	0	5925
hallmark_unfolded_protein_response	1.943295	0.000786	1684
hallmark_mtorc1_signaling	1.811671	0.001379	4785
hallmark_hypoxia	1.613065	0.009442	4214
gobp_cholesterol_storage	2.053821	0.01103	451
hallmark_adipogenesis	1.560202	0.014288	5189
wp_electron_transport_chain	1.851349	0.025066	5782
gobp_response_to_interferon_beta	-1.86324	0.028797	2612
gobp_alpha_amino_acid_biosynthetic_process	1.92167	0.03904	1766
gobp_pirna_processing	1.927917	0.040692	3188
gomf_ligase_activity_forming_carbon_oxygen_bonds	1.931933	0.044344	1223
gobp_regulation_of_cholesterol_storage	1.935293	0.048829	42
gomf_pheromone_activity	1.938082	0.05803	3618
gobp_negative_regulation_of_cholesterol_storage	1.877693	0.066971	42
gobp_response_to_prostaglandin	-1.80497	0.068246	4708
wp_cytoplasmic_ribosomal_proteins	1.647893	0.069436	6409
gobp_amino_acid_activation	1.869451	0.070836	1653
wp_lung_fibrosis	1.611621	0.082455	3960
gomf_acyl_coa_binding	1.845276	0.087656	4791
wp_translation_factors	1.65391	0.088464	5731
gomf_protein_disulfide_reductase_activity	1.849459	0.08912	5474
hallmark_tnfa_signaling_via_nfkb	1.340979	0.095522	1737
wp_oxidative_phosphorylation	1.683479	0.102272	6054
gobp_regulation_of_mast_cell_activation	1.829062	0.105275	3561
gocc_inner_mitochondrial_membrane_protein_complex	1.814295	0.114149	5656
gocc_mitochondrial_protein_containing_complex	1.81625	0.119469	5687
wp_amino_acid_metabolism	1.522097	0.121106	4384

gobp_cytoplasmic_translation	1.805412	0.121611	5822
wp_glycolysis_and_gluconeogenesis	1.538994	0.123735	7018
hallmark_myc_targets_v1	1.258235	0.126835	7360
gomf_translation_initiation_factor_activity	1.798243	0.127022	5699
gomf_structural_constituent_of_ribosome	1.788279	0.135505	6571
hallmark_p53_pathway	1.235461	0.138322	4091
hallmark_cholesterol_homeostasis	1.262196	0.139196	1186
hallmark_glycolysis	1.268749	0.15109	5136
gobp_vascular_associated_smooth _muscle_cell_apoptotic_process	1.774781	0.155938	1415
gobp_establishment_of_lymphocyte_polarity	-1.75428	0.160594	4614
gobp_regulation_of_skeletal_muscle_cell_proliferation	1.763672	0.171266	3869
gomf_atp_dependent_protein_folding_chaperone	1.757794	0.17587	6454
gocc_cytosolic_ribosome	1.742049	0.179117	6571
gobp_amino_acid_biosynthetic_process	1.743353	0.183926	1766
hallmark_mitotic_spindle	-1.37233	0.185153	6026
gocc_nucleoid	1.748595	0.187796	5385
gobp_skeletal_muscle_cell_proliferation	1.744734	0.188515	5198
gocc_small_ribosomal_subunit	1.733224	0.191792	5687
gobp_negative_regulation_	1.724964	0.203295	2838
hallmark_epithelial_mesenchymal_transition	1.162227	0.20549	3008
gobp_cell_redox_homeostasis	1.719279	0.208722	2497
hallmark_apoptosis	1.167047	0.211707	4118
hallmark_e2f_targets	-1.29416	0.214344	6876
hallmark_protein_secretion	1.172151	0.220746	5389
hallmark_estrogen_response_early	-1.30889	0.221033	5583
hallmark_kras_signaling_dn	-1.39259	0.22149	2863
gobp_sodium_independent_organic_	1.706792	0.226338	5419
gomf_transferase_activity_	1.683201	0.230292	452
gobp_cytoplasmic_translational_initiation	1.707534	0.231636	5743
gobp_carbohydrate_phosphorylation	1.684663	0.232826	6596
gobp_amino_acid_metabolic_process	1.685198	0.237858	1994
gomf_intramolecular_oxidoreductase_ activity_transposing_c_c_bonds	1.698355	0.241214	2130
gobp_alanine_transport	1.675431	0.243368	2672
gocc_cytosolic_small_ribosomal_subunit	1.685445	0.243809	5558
gomf_disulfide_oxidoreductase_activity	1.693257	0.245533	5474
gocc_ribosomal_subunit	1.686514	0.247851	6571
wp_gpcrs_other	-1.64824	0.279936	2935

Chapter 9 - References

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