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Characterisation of the role of the IL-36 family of cytokines in the pathogenesis of colon cancer

Thesis presented by

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for the degree of

Doctor of Philosophy

University College Cork

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College of Medicine and Health Science

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.

Signed:  .....

Kevin Baker

List of publications

This work has been published in the following formats;

Journal articles

Baker KJ, O'Donnell C., Bendix M. *et al*. IL-36 signalling enhances a pro-tumorigenic phenotype in colon cancer cells with cancer cell growth restricted by administration of the IL-36R antagonist. Oncogene 41, 2672–2684 (2022). https://doi.org/10.1038/s41388-022-02281-2

Baker KJ, Houston A, Brint E. IL-1 Family Members in Cancer; Two Sides to Every Story. Front Immunol 2019; 10: 1197-1197.

Byrne J, **Baker KJ**, Houston A, Brint E. IL-36 cytokines in inflammatory and malignant diseases: not the new kid on the block anymore. Cellular and molecular life sciences : CMLS 2021; 78: 6215-6227.

Baker KJ, Brint E, Houston A. Cancer cell directed IL-36R signalling in CRC; a transcriptomic analysis. *Manuscript under peer review*. 2022

Oral Presentations

University Hospital Waterford Research Meeting, Waterford, Ireland. **Baker KJ***, O'Donnell C, Bendix M, Keogh S, Byrne J, O'Riordain M *et al* (2022). IL-36 signalling enhances a pro-tumorigenic phenotype in colon cancer cells with cancer cell growth restricted by administration of the IL-36R antagonist.

Irish Association for Cancer Research Annual Meeting, Ireland. **Baker KJ***, O'Donnell C, Bendix M, Keogh S, Byrne J, O'Riordain M *et al* (2021). IL-36 signalling in Colorectal Cancer.

Poster presentations

American Association for Cancer Research, New Orleans, LO, U.S.A. **Baker KJ***, O'Donnell C, Bendix M, Keogh S, Byrne J, O'Riordain M *et al* (2022). IL-36 signalling enhances a pro-tumorigenic

phenotype in colon cancer cells with cancer cell growth restricted by administration of the IL-36R antagonist.

Irish Association for Cancer Research Annual Meeting, Cork, Ireland. **Baker KJ***, O'Donnell C, Bendix M, Keogh S, Byrne J, O'Riordain M *et al* (2022). IL-36 signalling enhances a pro-tumorigenic phenotype in colon cancer cells with cancer cell growth restricted by administration of the IL-36R antagonist.

Irish Association for Cancer Research Annual Meeting, Galway, Ireland. **Baker KJ***, O'Donnell C, O'Riordain M, Houston A, Brint E. (2020). Investigation of the role of IL-36 cytokines in colon cancer

Irish Society for Immunology, Ireland. **Baker KJ***, O'Donnell C, O'Riordain M, Houston A, Brint E. (2020). Investigation of the role of IL-36 cytokines in colon cancer

Irish Association for Cancer Research Annual Meeting, Belfast, Ireland **Baker KJ***, O'Donnell C, O'Riordain M, Houston A, Brint E. (2019). Charachterisation of the IL-36 cytokines in colon cancer cell lines.

Irish Society for Immunology, Dublin, Ireland. **Baker KJ***, O'Donnell C, O'Riordain M, Houston A, Brint E. (2019). Charachterisation of the IL-36 cytokines in colon cancer cell lines.

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Abstract

The IL-36 cytokines are a recently described subset of the IL-1 family of cytokines. These cytokines have now been identified to play a role in the pathogenesis of many inflammatory diseases and are increasingly being implicated in tumourigenesis. Given the pluripotent nature of other IL-1 family members and the relationship between inflammation and tumorigenesis, here we have investigated the effects of IL-36 signalling in colorectal cancer.

In this study we demonstrate that expression of IL-36 family member mRNA and protein is significantly increased in colorectal cancer tissue compared to adjacent colonic non-tumour tissue. Colon cancer cell lines express IL-36 family genes differentially, and these genes are inducible with Toll-like Receptor ligands and pro-inflammatory cytokines. Following stimulation with IL-36 agonists, colon cancer cell lines increase expression of pro-inflammatory genes, especially genes involved in myeloid cell chemotaxis. Colon cancer cells lines are more responsive to IL-36β and IL-36γ in comparison to IL-36α. *In vitro* assays showed stimulation of colon cancer cell lines with IL-36 agonists augmented several pro-tumorigenic phenotypes such as cellular migration, invasion and proliferation in both 2D and 3D models.

In pre-clinical models of colon cancer, intraperitoneal injection of the IL-36 Receptor antagonist (IL-36Ra) significantly reduced tumour burden using the subcutaneous CT26 tumour model in syngeneic Balb/mice. This was associated with a decrease in Ki-67 expression by tumour cells in the IL-36Ra-treated group relative to untreated control tumours, suggesting the inhibition of the pro-proliferative signalling of IL-36 agonists resulted in the decreased tumour size. Moreover, colon cancer cells lacking the IL-36R also

showed reduced tumour growth and reduced Ki-67 expression *in vivo*. IL-36 agonist administration also resulted in a tumour reduction in mice, although this was not as effective as IL-36Ra administration and did not alter Ki67 expression levels in tumour tissue but rather acted through immune infiltration of tumours. Taken together, this data suggests that targeting IL-36R signalling may be a useful targeted therapy for colorectal cancer patients with IL-36R^{+} cancer cells.

In order to further understand the effects of IL-36 cytokine signalling in the context of immune cells, co-cultures of macrophages and colon cancer cells were completed *in vitro*. The THP-1 model of macrophages showed minimal changes in response to IL-36 agonist stimulation. M1 macrophage cells significantly reduced spheroid formation of HT29 cells, with addition of IL-36 agonists facilitating recovery of spheroid size back to untreated size, indicating colon cancer cells are more responsive to IL-36 stimulation than macrophages when in co-culture in this model. Preliminary work using HL-60 cells as models of neutrophils showed IL-36 can augment cancer-cell induction of neutrophil NETosis, potentially contributing to immune evasion and metastasis.

Transcriptomic analysis of publicly available patient cohorts revealed increased expression of IL-36 family members in malignant intestinal tissue in comparison to paired healthy tissue. Moreover, IL-36R expression is associated with poorer patient survival rates in colon cancer. Our DEG analysis of tumours expressing high levels of IL-36R mRNA revealed a possible role for the IL-17/IL-22/IL-23 signalling axis in colon cancer involving IL-36 signalling. Together, this study demonstrates that IL-36 signalling in colon cancer may contribute to disease progression and that inhibition of this signalling, in subgroups of

patients stratified according to cancer cell expression of the IL-36R, may benefit survival rates, as shown in our *in vivo* pre-clinical models of colon cancer.

Abbreviations

5-FU	5-Fluorouracil
AC	Adjuvant chemotherapy
AJCC	American Joint Committee on Cancer
AMP	Antimicrobial protein
AOM	azoxymethane
AP-1	Activator Protein
APC	Adenomatous polyposis coli
BMDM	Bone marrow-derived macrophage
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
C. albicans	Candida albicans
C. rodentium	Citrobacter rodentium
CAC	Colitis-associated cancer
$CaCL_2$	Calcium Chloride
CAF	Cancer associated fibroblast
CANTOS	Canakinumab Anti-Inflammatory Thrombosis Outcome Study
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CD	Cluster of Differentiation
CD	Crohn's Disease
cDC	conventional Dendritic Cell
cDNA	Complimentary DNA
CESC	Cervical Squamous Cell Carcinoma/Endocervical Adenocarcinoma
CIMP	CPG Island Methylation Phenotype
CIN	Chromosomal Instability
CMS	Clinical Molecular Subtype
CNS	Central nervous system
CO_2	Carbon dioxide
COAD	Colon adenocarcinoma
COPD	Chronic obstructive pulmonary disease
COX	cyclooxygenase
CRC	Colorectal cancer
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CT	Cycle threshold
ctDNA	Circulating Tumour DNA
CTL	Cytotoxic T lymphocyte
CTRL/CTL	Control
CTSG	Cathepsin G
CTSS	Cathepsin S
CV	Crystal violet

CXCL	C-X-C Chemokine ligand
CXCR	C-X-C Chemokine receptor
DAMP	Damage associated molecular pattern
DC	Dendritic Cell
DEG	Differentially expressed gene
DITRA	Deficiency of IL-36Ra
dM1	Differentiated to M1 phenotype
dM2	Differentiated to M2 phenotype
DMEM	Dulbecco's modified Eagle medium
dMMR	deficient mismatch repair
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonucleic acid nuclease
DSS	Dextran sodium sulphate
ECD	Extracellular domain
ECD	Ectodomain
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGC	Enteric glial cell
EGF	Epidermal Growth Factor
EGFR	EGF receptor
ELANE	Neutrophil Elastase
ELISA	Enzyme labelled immunosorbent assay
ERK	Extracellular signal-regulated kinase
EV	Extracellular vesicle
FBS	Foetal bovine serum
FCS	Foetal calf serum
fl	flox
FLA	Flagellin
FSC	Forward scatter
G0	gap 0
G1	gap 1
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPP	Generalized pustular psoriasis
gRNA	Guide RNA
GSH	Glutathione synthetase
HCC	Hepatocellular carcinoma
HDR	Homology derived recombination
HNPCC	Hereditary nonpolyposis colorectal cancer
HNSC/HNSCC	Head and Neck squamous cell carcinoma
Hr	Hour
HS	hidradenitis suppurativa
hsCRP	high-sensitivity C-reactive protein
HSV	Herpes Simplex Virus
i.p.	Intraperitoneal

IBD	Inflammatory bowel disease
ICE	Inference of CRISPR Edits
ICI	Immune Checkpoint Inhibitor
IEC	Intestinal epithelial cell
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IL-1RAcP	Interleukin-1 receptor accessory protein
ILC	Innate lymphoid cell
Indels	Insertion–deletion mutations
iNOS	inducible nitric oxide synthase
IP	Immunoprecipitation
IRF	Interferon regulatory factor
IκBζ	Inhibitor of nuclear factor kappa B zeta
JNK	c-Jun N-terminal kinase
KEGG	Kyoto Encyclopedia of Genes and Genomes
KM	Kaplan Meier
KO	Knock-out
L. pneumophilia	Legionella pneumophilia
LCN2	Lipocalin 2
LOH	Loss of heterozygosity
LPS	Lipopolysaccharide
LV	Leucovorin
M. tuberculosis	Mycobacterium tuberculosis
mAb	Monoclonal Antibody
MAPK	Mitogen Activated Protein kinase
MCP	Microenvironment Cell Populations
MCP-1	Monocyte chemoattractant protein 1
mCRC	Metastatic colorectal cancer
MDDC	Monocyte derived dendritic cell
MDSC	Myeloid derived suppressor cell
MEK	mitogen-activated protein kinase kinase 1
MHC	Major histocompatibility complex
Min	Minute
MMP	Matrix metalloprotease
MNAse	Micrococcal nuclease
mRNA	messenger RNA
MSC	Mesenchymal stromal cell
MsC	Mesenchymal stem cell
MSI	Microsatellite instability
mTORC1	mammalian target of rapamycin complex 1
MyD88	Myeloid differentiation primary response gene
N/A	Not available
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa b

NGS	Normal goat serum
NHEJ	Non-homologous end joining
NK	Natural Killer
NLRP3	NLR family pyrin domain containing 3
NOS	Nitric oxide synthase
NSCLC	Non-small cell lung cancer
OV	Oncolytic virus
P. aeruginosa	Pseudomonas aeruginosa
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death protein ligand 1
PGE2	Prostaglandin E2
PMA	phorbol 12-myristate 13-acetate
pMMR	proficient mismatch repair
PMN	Polymorphonuclear
Poly(I:C)	Polyinosinic:polycytidylic acid
PPI	Protein Protein interaction
PRNT3	Proteinase 3
PRR	Pathogen recognition receptor
Puro	Puromycin
qRT-PCR	Quantitative reverse transcriptase PCR
RA	Rheumatoid arthritis
REG3A	Regenerating Family Member 3 Alpha
RFP	Red fluorescent protein
RIPA	Radio-immunoprecipitation assay
RNA	Ribonucleotide
RNAseq	RNA sequencing
ROS	Reactive oxygen species
RPM	Revolutions per minute
RT	Room temperature
RT	Reverse transcriptase
S. aureus	Staphylococcus aureus
S. epidermis	Staphylococcus epidermidis
s.c.	Subcutaneous
SARS -CoV	severe acute respiratory syndrome–related coronavirus
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SIGIRR	Single Immunoglobulin IL-1 related receptor
SMA	Smooth muscle actin
Sonic Hedgehog	SHH
SSC	Side scatter
STAD	Stomach adenocarcinoma
STAT	Signal transducer and activator of transcription
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins

TAK	Transforming growth factor-β (TGF-β)-activated kinase 1
TAM	Tumour associated macrophage
TAN	Tumour associated neutrophil
T-bet	T-box expressed in T cells
TBS	Tris buffered saline
TCGA	The cancer genome atlas
TCR	T cell receptor
Tfh	Follicular helper T
TGF	Transforming growth factor
Th	T helper
TIDE	Tracking of Indels by DEcomposition
TIGIRR	Interleukin 1 Receptor Accessory Protein Like 2
TIL	Tumour infiltrating lymphocyte
TIMER	Tumor Immune Estimation Resource
TIR	Toll/IL-1 receptor domain
TLO	Tertiary lymphoid organ
TLR	Toll like receptor
TLS	Tertiary lymphoid structure
TME	Tumour microenvironment
TMN	tumour-node-metastasis
TNF	Tumour necrosis factor
TOLLIP	toll interacting protein
TP53	Tumour protein 53
TRAF	TNF Receptor Associated Factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	T regulatory
TRP53	transformation related protein 53
TS	Thymidylate Synthase
UC	Ulcerative colitis
UICC	Union for international Cancer Control
VEGF	Vascular Endothelial Growth Factor
VEGFR	VEGF receptor
WRT	With respect to
WT	Wild type
β-actin	Beta-actin
µl	Microliter
µM	Micromolar

Chapter 1

Introduction

Results of this chapter were published in the following manuscripts;

- **Baker, K. J.,** Houston, A., & Brint, E. (2019). IL-1 Family Members in Cancer; Two Sides to Every Story. *Frontiers in immunology, 10*, 1197-1197. doi:10.3389/fimmu.2019.01197
- Byrne, J., **Baker, K.J.,** Houston, A., & Brint, E. (2021). IL-36 cytokines in inflammatory and malignant diseases: not the new kid on the block anymore. *Cell Mol Life Sci, 78*(17-18), 6215-6227. doi:10.1007/s00018-021-03909-4

1.1. Cancer

Descriptions of cancer incidence in humans can be found as far back as 3500 years ago, with observations written on papyri describing breast cancer to be untreatable when tumours were bulging and had spread [1]. Over 1000 years later, The Hippocratic Corpus was the first medical text to use the words “karkinos” and “karkinoma” to describe tumour appearances which had crab-like features [2]. Our understanding since these early observations has grown exponentially in the past century, with cancer now well understood to be a genetic disease known to stem from a series of molecular events/insults that alter the fundamental properties of normal cells leading to uncontrolled cellular division [3]. Inheritable germline mutations contribute to risk of cancer development however somatic mutations are considered to be much more influential in this process [4].

1.2. The Hallmarks of Cancer

The processes that contribute to carcinogenesis were first thoroughly discussed by Hanahan & Weinberg in 2000, who presented these as the hallmarks of cancer [5]. This described six biological abilities obtained by cancer cells which facilitates their growth and survival. These included evading programmed cell death to sustain proliferation, vascularisation of tumour tissue which can counteract toxic metabolite build-up in tumour tissue and unrestricted cell division. Additionally, other hallmarks include tissue invasion and metastasis to allow spread of disease, insensitivity to negative growth feedback signals and self-sufficiency to induce cellular division and sustain cellular proliferation. In 2011, two further hallmarks were added to this list, consisting of deregulating cellular energetics and avoiding immune destruction [6]. Two enabling characteristics were also added to these descriptions, namely sustained tumour promoting inflammation and genomic instability, both of which contribute to previously described hallmarks. A very recent

update of these descriptive processes and characteristics has been released, describing the continually growing body of work completed on describing features of cancer [7]. New additions to these hallmarks included phenotypic plasticity and cellular senescence. Two further enabling characteristics were also added which included non-mutational epigenetic reprograming and polymorphic microbiomes. This description of cancer has greatly helped summarise the heterogeneous collection of diseases that is cancer (Fig. 1.1).

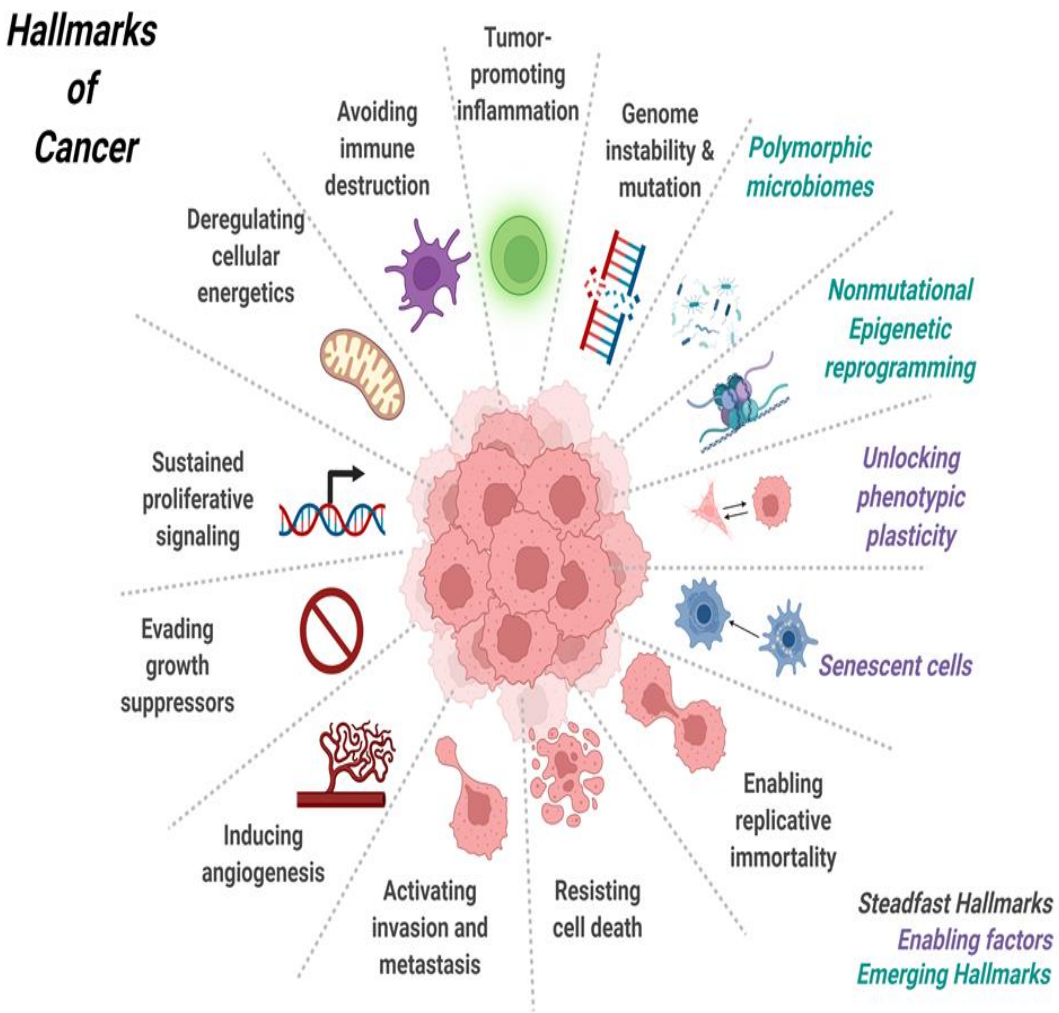


Figure 1.1: The Hallmarks of Cancer. A proposed collections of properties which characterise the cancer phenotype. These include established/steadfast hallmarks, enabling characteristics and emerging hallmarks, as described by Hanahan and Weinberg, 2022.

1.3. Colon cancer

Colorectal cancer describes colon and rectal cancers as a collective. This is due to shared traits such as origin from one organ, the intestine, their shared histological features of mucosa, muscular layer and serosa and finally the similar functions of both tissue types [8]. CRC poses a highly substantial health and economic burden within society. Globally, it ranks as the third most incident type of cancer and has the second highest associated mortality [9]. Development of CRC can be influenced by many cultural and social factors [10]. Factors such as dietary choice, inactive lifestyles, tobacco smoking and alcohol consumption may all contribute to development of colon cancer [11]. There has been a worrying trend of increased incidence of CRC in patients under the age of 50 years, suggesting screening programmes should be changed to detect more of these cases [12]. The cause of this increase has not been discretely defined, with multiple factors believed to be at play here including the increased prevalence of obesity, with weight gain previously shown to be associated with increased risk of CRC development [13].

1.4. Colon cancer development

1.4.1. Genomic instability

Development of colorectal cancer has been linked with multiple pathways, each of which are influenced by somatic mutations but may also be influenced by germline mutations [4]. Furthermore, epigenetic mutations have also been shown to contribute to CRC development [14]. As previously mentioned here and described by Hanahan and Weinberg, genetic instability plays a key role in colon cancer development. There are three core pathways involved in this instability and thereby pathogenesis of CRC. Chromosomal Instability (CIN) describes the significant gain or loss of sections or entire chromosomes

resulting in both activation of oncogenes genes and deactivation of tumour suppressor genes, as well as a loss of heterozygosity (LOH) contributing to CRC development [15]. Microsatellite instability (MSI) is a hallmark of Familial CRC, accounting for more than 95% of cases of hereditary nonpolyposis colorectal cancer (HNPCC) [16]. MSI consists of mismatch repair gene mutation resulting in the reduced ability of cells to repair DNA replication errors. CpG island methylator phenotype (CIMP) pathways contribute to gene silencing of tumour suppressor genes by hypermethylation [17]. These pathways contribute to the transition and transformation of tissue from normal intestinal epithelial tissue to dysplastic and malignant tissue over time.

1.4.2. Adenoma-Carcinoma Sequence

This gradual transition, which can take decades, is known as the adenoma-carcinoma sequence and was first described by Vogelstein et al in 1990 [18] (Fig 1.2). The most common and most studied sporadic cancer involves increasing mutation accumulation resulting in the transformation of each tissue type. This sequence may vary between patients, but commonly reoccurring mutations have characterised this process. This often begins with normal epithelial mutation of the tumour suppressor gene, Adenomatous polyposis coli (APC), this dysfunctional mutation leads to unrestricted Wnt signalling followed by stabilisation of the β-catenin protein which enters the nucleus and induces transcription of several Wnt target genes involved in cellular proliferation [19]. This leads to early adenoma development which can then be followed by activating KRAS mutations, which is a downstream regulator of EGFR, a growth hormone receptor which induces cellular proliferation in cells [20]. This is followed by intermediate adenoma development and Smad2/4 inactivation, resulting in TGF-β response inactivation which prevents the anti-

proliferative signal from TGF-β being applied to epithelial cells [21]. Finally, loss of function mutations of p53 results in inhibition of cell programmed death but also increased cellular proliferation which gives way to carcinoma development [22]. Further mutations, such as BRAF, may then significantly contribute to CRC metastasis to poor prognostic sites such as distant lymph nodes[23].

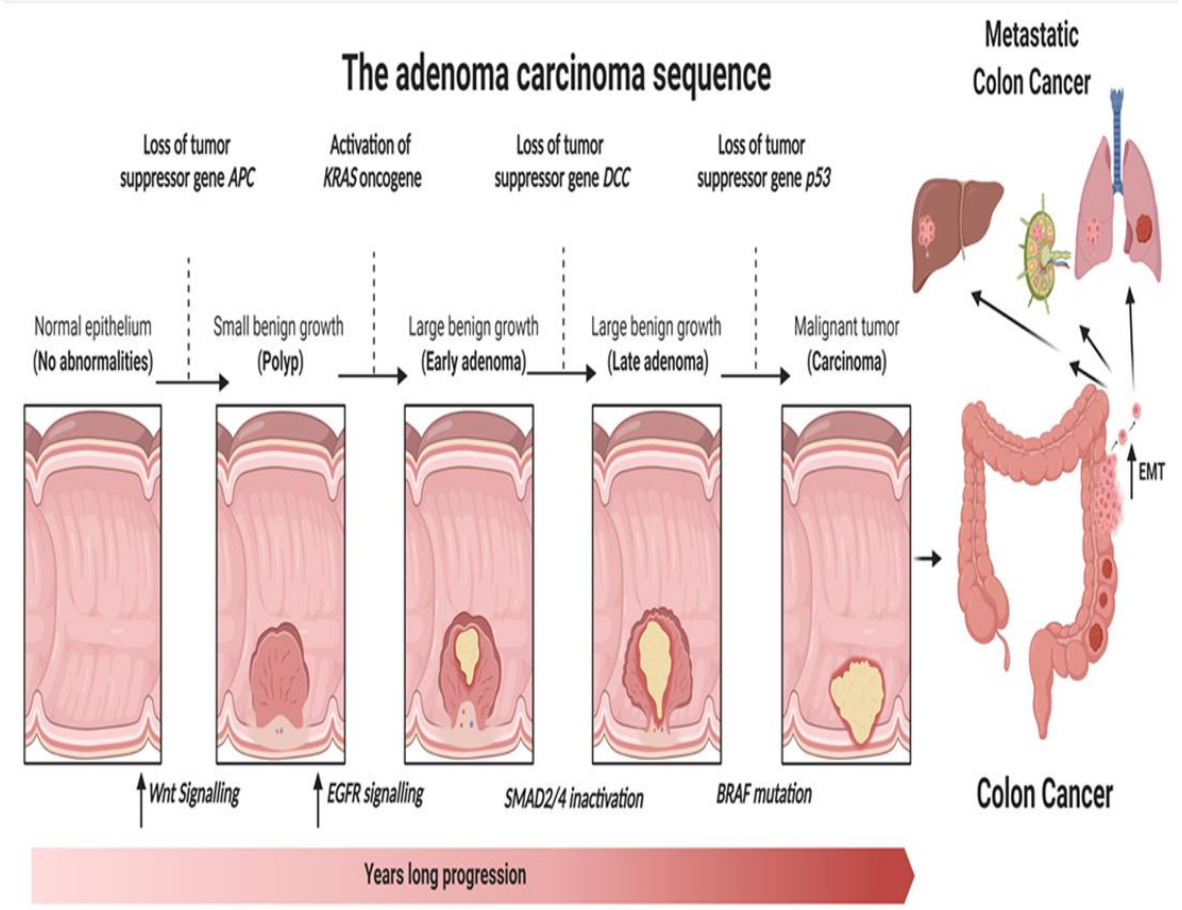


Figure 1.2: **The adenoma carcinoma sequence in CRC**. The most common and well characterised sequence of events that results in CRC. Normal epithelium transforms to a polyp by loss of APC tumour suppressor gene expression, followed by mutation accumulation leading to neoplastic tissue and malignancy. Over decades this may result in the eventual metastasis of the primary cancer to sites such as draining lymph nodes, the liver and the lungs.

1.4.3. Hereditary/Familial CRC

Colon cancer development may also arise from hereditary germline mutations, although this accounts for less than 10% of cases. These include Familial adenomatous polyposis and hereditary nonpolyposis colorectal cancer, which arise from APC mutation and genetic alterations of DNA mismatch repair genes, respectively [24]. Other hereditary mutations accounting for CRC development account for approximately 25% of cancers, with positive family histories identified without identification of the exact mutation which gives way to CRC development [25].

1.4.4. Colitis associated Cancer (CAC)

Furthermore, CRC may develop from chronic inflammation in patients suffering from intestinal disease such as Ulcerative Colitis and less so, Crohn's Disease. Whilst this is a significant development in these patients, accounting for one sixth of all deaths in UC patients, this does however account for <1% of all CRC cases [26]. This pathway is discrete from sporadic CRC in that it is more frequent in younger patients, arises from flat dysplasia with indistinct margins surrounded by inflamed tissue, fibrosis and pseudopolyposis which can reduce detection efficacy of endoscopic screening [27]. Given the diverse genetic background that is associated with each type of colon cancer, careful consideration has been taken in the development further characterisation.

1.5. Colon cancer staging and characterisation

1.5.1. TMN (AJCC/UICC)

In order to standardise and assist therapeutic regimens for patients, tumour-node-metastasis (TMN) staging (both AJCC and UICC) is used internationally. Since its inception

in 1977, the AJCC TNM staging has undergone repeated revision to improve grouping of tumours for therapy selection by physicians [28]. This staging system describes cancers according to the following: T – the degree of cancer invasion of the intestinal wall; N- the extent of cancer cell migration to lymph nodes; and M – the degree of metastasis to local and distant sites. Revisions have added further subheadings to each of these groups. Stage I cancer cells are limited to the mucosa or the large intestine without any invasion or metastasis to lymph nodes or other organs. Stage IV can be divided into several groups (A, B and C) with each showing distant metastasis to another organ. The 8th edition of this staging is currently in use, with the addition of further subgrouping of metastasis in Stage IV cancers to account for peritoneal metastases with our without metastasis of other organs [29]. As well as determining the most appropriate treatment regimens, this has highlighted the deadliness of CRC in stage IV disease, with a 5 year survival from 90% to <15% in Stage I to Stage IV disease, respectively [30].

1.5.2. Consensus Molecular Subtype

With advancements in genome wide transcriptomics so too have more molecular approaches of CRC characterisation for understanding of cancers. The consensus molecular subtype has helped describe cancers according to their gene expression signatures which have since been categorised into four subtypes [31]. CMS1 describes tumours that are hyper-mutated displaying MSI with strong immune activation. CMS2 describes canonical, epithelial tumours with marked WNT and MYC signalling. CSM3 describes a metabolically dysregulated class of tumours and CMS4 describes a mesenchymal rich tumour, with extensive TGF-β activation, stromal invasion and angiogenesis.

1.5.3. Immunoscore

The past decade has seen the immune system come to the forefront of cancer therapeutic design and treatment, but this has also taken place for tumour characterisation. Galon and Lanzi have recently described the translational benefits of using Immunoscore to assess $CD3^+$ and $CD8^+$ T cell subsets in CRC patient tumours by an IHC and digital pathology-based assays which showed improved prognostic value than conventional AJCC/UICC grading in stage I-III patients [32]. This may become standardised protocol for physicians to determine therapeutic regimens for patients.

1.6. Treatment of colorectal cancer

1.6.1. Early Stage CRC

Current standards of treatment vary according to whether the cancer is at an early (Stage I-III) or late (stage IV) stage of disease [33]. Stage I CRC is highly treatable by curative surgery alone, with 5 year survival rates of >90%, without requirements for adjuvant chemotherapy [34]. Stage II cancer is also treated by curative surgery, with adjuvant chemotherapy (AC) sparingly used based on clinicopathological features of tumours [35]. Guidance for use of chemotherapy has been a contentious issue, however circulating tumour DNA (ctDNA) detection can assist in clinical decision making as observed in the DYNAMIC-II trial. This reported ctDNA detection being able to reduce AC exposure by >50% without a significant reduction in recurrence free survival (92.4% vs 91.7%) when compared to standard management of stage II CRC [36]. Stage III cancer also involves surgery but this is combined with AC in the form of 5-FU (targets DNA replication in rapidly dividing cells by inhibition of cellular thymidylate synthase) and oxaliplatin (platinum based DNA crosslinking to inhibit

DNA replication of rapidly dividing cells). AC in stage III CRC can increase the 5 year disease free rate to 70% in comparison to surgery alone (45-50%).

1.6.2. Late Stage CRC

1.6.2.1. Chemotherapy and growth factor targeted therapies

Patients with locally advanced cancer which is inoperable, or metastatic disease, are recommended to receive systemic chemotherapy. 5-Fluorouracil, is readily taken up by cancer cells, as these cells have a preference for uracil uptake. Once metabolised by cells, 5-FU, and other fluoropyrmidines, act as anti-metabolites to inhibit thymidylate synthase (TS) and thereby disrupt intracellular pools of deoxynucleotides required for DNA replication [37]. Alternatively or in combination, oxaliplatin may be used to induce cytotoxic lesions which involve formation of intra-strand and inter-strand platinum-DNA adducts. These large adducts inhibit mismatch repair enzyme complex formation with nucleotides and inhibits DNA replication [38]. Chemotherapy selection is based on availability, patient-dependent toxicity concerns, patient chemotherapy history and the goals of treatment (i.e. tumour shrinkage to facilitate surgery or palliation)[39]. Leucovorin (LV) is given in combination with 5-Fluoropyrmidines to limit side effects and can also improve patient survival and increase tumour response rates [40].Targeted therapy may also be used which include anti-EGFR and anti-VEGF(R) monoclonal antibodies to inhibit cellular proliferation and angiogenesis, respectively. Through multiple clinical trials analysis, it has been proposed that anti-EGFR treatment be used in RAS-wild type mCRC with primary tumours on the left side of the colon. However, targeted therapies have been shown to be rendered ineffective by acquired resistance in cancer cells over time [41]. Continual research is ongoing in targeted therapy development, such as colon cancer stem

cell screens which have produced new bi-specific antibodies such as MCLA-158 which triggers degradation of EGFR in cancer stem cells without toxic effects on local LGR5+ healthy stem cells [42].

1.6.2.2. Immunotherapy

Targeted immune checkpoint inhibition (ICI) therapy has also been used in the treatment of mCRC, although only 12 to 15% of patients (MSI-H or with dMMR) actually stand to benefit from ICI therapy [43]. The ICIs currently in use augment fatigued T cells located locally and systemically in order to drive the adaptive anti-tumour response. This is achieved by mAb binding to PD-1 (Pembrolizumab/Nivolumab) or CTLA-4 (Ipilimumab) to prevent immunosuppression of T cells (Fig. 1.3). ICI therapy is now recommended as the preferred treatment of unresectable advanced or mCRC in these suitable patients ahead of chemotherapy with or without targeted monoclonal antibodies to growth factors or tyrosine kinase inhibitors [43]. Furthermore, recent NCCN guidelines have outlined that Nivolumab +/- Ipilimumab or Pembrolizumab can be used as a preoperative neoadjuvant therapy for resectable MSI-H/dMMR mCRC [44]. A very recent clinical trial in dMMR rectal cancer showed a 100% clinical response in stage II/III in response to PD-1 ICI therapy, with no evidence of tumours in 14 patients. Remarkably, these patients did not require surgery or radio-chemotherapy for treatment [45].

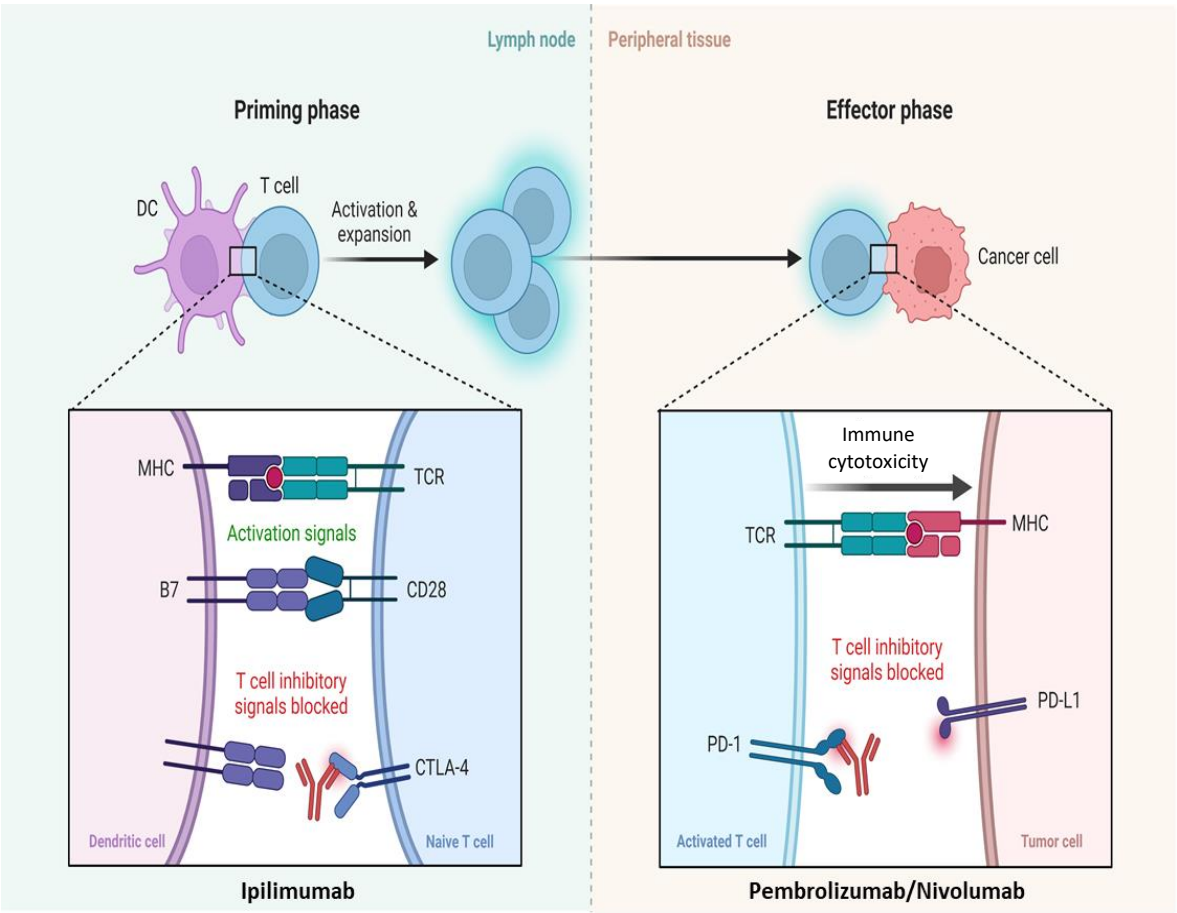


Figure 1.3: **Immune checkpoint inhibitors in CRC**. Immune checkpoint inhibitors are now becoming more commonly used in the treatment of stage II/III/IV CRC. ICIs bind to inhibitory proteins in the priming and effector phases of T cell development and activation. Inhibiting this interaction allows for sustained signalling by effector T cells to carry out tumour cell targeted cytotoxicity.

1.7. Inflammation and Cancer

The modern recognition of the relationship between inflammation and cancer dates back to 1858, when Rudolf Virchow acknowledged that neoplastic lesions emerge from sites of persistent irritation. This was soon after developed into the observation of the casual link between cancer and inflammation [46]. Over 100 years later, Dvorak recognised the similarities between tumour stroma generation and wound healing by describing cancer as "a wound that does not heal" [47]. It has been recognised that this may result from much

stronger evolutionary pressure for wound healing and tissue regeneration in comparison to tumour development which often occurs at post-reproductive age [48]. Tissue damage or microbial infection can result in abhorrent epithelial signalling to recruit innate immune cells which secrete growth hormones and cytokines. This restores epithelial barriers by inducing proliferation of epithelial cells, which can progress to tumourigenesis if functional restoration is not achieved [49]. This chronic inflammation induced risk of tumourigenesis can be seen in inflammatory bowel disease, where ulcerative colitis patients are at an increased risk of developing CRC [50]. Somatic mutations may be acquired in tumour suppressor genes during chronic inflammation as increased levels of mutagenic metabolites build up and induce mutagenic DNA lesions and double stranded breaks which, if not repaired correctly, may contribute to tumourigenesis [51, 52]. Although chronic inflammation can induce tumourigenesis and mediate tumour progression, new interest has arisen in the past 15 years to harness this pro-inflammatory state to induce tumour rejection by means of immune checkpoint inhibition. In order to achieve this, careful consideration is required for the cancer cells, stromal cells, immune cells and extracellular matrix within the tumour [53]. Together these make up what is called the Tumour Microenvironment.

1.8. The Tumour Microenvironment

The tumour microenvironment is a complex and dynamic collection of cancer, immune, stromal cells and extracellular matrix [54]. The composition of the tumour tissue changes according to cytokine signalling between cells (Fig. 1.4). This signalling can direct tumour progression by recruitment of anti-inflammatory immune cells such as M2 macrophages, N2 tumour associated neutrophils, myeloid derived suppressor cells and T regulatory cells

[55]. Alternatively, tumour-rejection may be achieved by increased infiltration of cytotoxic T cells, NK cells and M1 macrophages [55]. Furthering this complexity, several cell types such as macrophages and neutrophils are capable of undergoing polarisation between pro-tumour and anti-tumour states, depending on local cytokine signalling [56-58]. The complexity of this microenvironment is also influenced by several stromal cell types including fibroblasts, mesenchymal stem cells and endothelial cells [59, 60]. Furthermore, extracellular matrix proteins in the TME are becoming more implicated as immunomodulatory components which can contribute to tumour progression [61]. Tumours which are immunologically infiltrated with favourable anti-tumour immune cells have been described as 'hot' tumours, whilst those that are immune-desert or immunosuppressive have been described as 'cold', with recent additions to this description including altered-excluded, altered-immunosuppressed to further stratify differences in tumours [62]. The array of cells involved in this dichotomy will be discussed in the context of colon cancer; however the genetic instability and cellular composition of each TME varies greatly according to cancer type and individual [63].

1.8.1. Immune cell populations in the TME

1.8.1.1. Tumour associated macrophages (TAMs)

Tumour associated macrophages exist in a heterogeneous spectrum which can be directed to what is considered their classically activated, pro-inflammatory state (M1, anti-tumour) or their alternatively activated, anti-inflammatory state (M2, pro-tumour) (Fig. 1.4). This polarisation is dictated by local chemokine secretion from local cells as well as the presence of microbial metabolites and surface molecules such as LPS [64].

1.8.1.1.1. M1 TAMs

M1 TAMs are pro-inflammatory myeloid cells which can be activated by other cancer-cell targeting immune cells such as $CD8^+$ T cells and NK cells which secrete high concentrations of IFNy [65]. Activated M1 TAMS in turn can recruit and augment $CD8^+$ T cells and NK cells in the TME [66]. This is achieved by M1 TAM secretion of cytokines and metabolites such as IL-12, IL-6, and TNFα, IL-1β. M1 TAMs can also directly kill tumour cells by toxic metabolite production (NOS and ROS) and antibody-dependent cell mediated cytotoxicity [67]. Furthermore, M1 TAMs act as a bridge between the innate and adaptive immune response by phagocytosis of cancer cells and antigen presentation for initiation of the adaptive immune response against cancers [68]. Cancer cells may prevent phagocytic capacities of M1 TAMs by expression of CD47 and may also polarise these cells toward an M2 phenotype by secretion of soluble factors such as the sonic hedgehog (SHH) [69]. Furthermore, the hypoxic conditions found within the TME may also induce polarisation toward an M2 TAM phenotype [57]. Increased infiltration of M1 TAMs is correlated with improved outcomes in colon cancer patients in a stage dependent manner [70].

1.8.1.1.2. M2 TAMs

In contrast to M1 TAMs, M2 TAMs promote the wound healing phenotype of cancers. These cells are polarised to this state by immunosuppressive hypoxia found in the TME, and cancer cell-secreted soluble factors such as M-CSF, TGF-β, IL-10 and EGF [71, 72]. Other polarising factors include cytokines such as IL-4 and IL-13 which are secreted by several immune cells to negatively regulate inflammation [73]. M2 TAMs can contribute to tumour progression by chemokine-mediated induction of cancer cell proliferation [74], migration [75] and invasion [76]. Furthermore, these cells can support neovascularisation of tumour

tissue [77] which is further assisted by extensive matrix remodelling within the TME by these cells [78]. A high M2/M1 ratio in the TME is correlated with increased liver metastases in CRC patients [79]. Next generation immunotherapy strategies are investigating means to convert the TAM population, which is mostly M2 macrophages, to a M1 phenotype to drive immune-mediated tumour rejection [80].

1.8.1.2. Dendritic cells

Dendritic cells (DCs), similar to M1 macrophages, comprise a heterogeneous population of cells which are involved in antigen capture, processing and presentation in order to link the innate and adaptive arms of the immune response [81]**.** Conventional DCs (cDCs) are integral cross-presenting tumour-associated antigen cells [82]. cDC1cells can be activated by TLR3 agonists and then cross present $CD8^+$ T cells in the TME to enhance cancer cell directed cytotoxicity [83]. cDC1 cells cross-present to $CD4^+$ T cells and also induce cellular proliferation of these lymphoid cells. $CD4^+$ T helper cells may then support of $CD8^+$ mediate cytotoxicity [84]. These cells also mediate T cell trafficking in the TME by cytokine/chemokine secretion as well as priming and activation of $CD8^+$ T cells via co-stimulatory molecules and soluble factors [85]. cDC1s have been shown to induce NK cell production of IFNy by IL-12 secretion to enhance the anti-tumour immune response [86]. Cancer cell secretions and surface antigens such as CD47 can impede the anti-tumour surveillance of cDCs by prevention of tumour mitochondrial DNA detection via the CD47-SIRPα axis [87]. Plasmacytoid dendritic cells and Monocyte Derived Dendritic Cells have also been reported to have CTL augmentative effects, but also immunosuppressive roles in the CRC TME [88-90].

1.8.1.3. Tumour associated neutrophils

Neutrophils which migrate towards and integrate into the TME or around it are known as Tumour associated neutrophils (TANs). Neutrophils are majorly implicated as driving tumour progression. However, like macrophages, these cells are divided according to their function in the TME as N1 (Anti-tumourigenic) or N2 (Pro-tumourigenic)(Fig. 1.4) [91].

1.8.1.3.1. N1 TANs

Polarisation of neutrophils towards an N1 phenotype is achieved by inhibition of TGF-β signalling and increased IFN-β signalling [92]. These cells can secrete cytokines to augment anti-tumour immune cells such as cytotoxic T cells, NK cells and induce dendritic cell maturation to further propagate this response [93]. N1 TANs also possess direct cytotoxic effects on cancer cells by secretion of granzyme B and release of NO, reducing tumour growth and metastases [94, 95]. Increased N1 TAN infiltration to CRC tumours in stage III patients has been shown to be associated with high responsiveness to 5-FU chemotherapy [96]. It was hypothesis that this may result from increased sensitivity of cancer cells to neutrophil anti-tumour properties following 5-FU treatment, although a definitive explanation remains aloof.

1.8.1.3.2. N2 TANs

Neutrophil recruitment into the TME is achieved by tumoural secretion of CXC chemokines such as CXCR1 and CXRC2, two receptors highly expressed by N2 TANS [97]. Increased TGFb signalling, derived from cancer cells, leads to polarisation of neutrophils to the N2 phenotype [92]. N2 cells have been highly implicated in several pro-tumourigenic processes [98]. Neutrophils may directly induce colon cancer cell proliferation [99], invasion [100],

angiogenesis [101, 102], metastasis [103] and immune evasion[104]. In contrast to extensive reporting for TANs to be highly pro- tumourigenic, TAN-based prognosis remains unclear, with papers reporting both positive and negative outcomes for patients with high TAN infiltration. A limitation of these studies are the methods of TAN quantification, with issues pertaining to unique cell markers to differentiate the N1, N2 and MDSCs [102]. This should be clarified in the near future by spatial transcriptomic analysis of CRC patient cohorts.

1.8.1.4. Myeloid derived suppressor cells (MDSCs)

MDSCs are another type of innate immune cell which shares similarities to other myeloid cells in their heterogeneity and rapid expansion in infection, inflammation and cancer [105]. MDSCs may also be subdivided into two cell subtypes proposed by murine data, monocyte (Mo-MDSCs) and polymorphonuclear (PMN-MDSCS), although these are generally described as one, heterogeneous population in human disease. Colon cancer cells secrete CXC chemokines to induce migration of these cells into the TME as a means of immunosuppression [106]. This assists cancer cell immune-evasion by suppression of effector T cell proliferation but also by stimulation of fellow immunosuppressive cells, T regulator cells [107]. Furthermore, these cells may directly contribute to cancer cell proliferation and metastasis by production of soluble factors such as CCL7 and TGFb [106, 108]. Circulating MDSCs have been reported to be a poor prognostic marker in CRC patients [109].

1.8.1.5. TILS

Tumour infiltrating lymphocytes (TILs), all stemming from lymphoid progenitor cells, have been reported as major indicators of patient prognosis and predict responsiveness to immunotherapy across many cancer types [32, 110]. These cells are diverse in their phenotypes and functions. Roles exist for these cells in the innate and adaptive immune arms of the immune system as well as cells capable of bridging these arms. Furthermore, cells of the adaptive immune response are described in both cellular and humoral immunity to sustain anti-tumour responses in patients [111]. As with other cells types in the TME, some of these TILs can also contribute to tumour progression [112].

1.8.1.5.1. Innate lymphoid cells/NK cells

Innate lymphoid cells play an important role in the maintenance of the gut epithelium by immune-surveillance for pathogen or damage associated molecular patterns (PAMPS/DAMPS) as more often tissue-resident immune cells [113]. These cells can be subdivided into three categories, with distinct but overlapping phenotypes which mirror properties of adaptive T cells (ILC1/2/3). ILC2/ILC3 cells play both pro-tumourigenic and anti-tumourigenic roles in CRC due to their plasticity [112]. ILC1 and NK cells share properties which are reflective of cytotoxic T cells [112]. These cells are activated by intestinal epithelial cell-derived (IEC) IL-18 and by dendritic cell-derived IL-12 [114]. ILC1 cells secrete cytotoxic molecules such as IFN-y and GznB which have direct cytotoxic effects on colon cancer cells [115]. These cells have been shown to highly infiltrate the colon cancer TME [116]. NK cells act by eliminating cancer stem cells in a similar manner to ILC1 cells. Low levels of MHC-1 expression, as well as high levels of NK cell receptor ligand expression, activates this cytotoxicity, however tumour cells can overcome this by

increased MHC-I expression [115]. Additionally, NK cells can influence the adaptive immune response via cytokine signalling by promoting DC recruitment and maturation in a feedback loop between the two cell types, as previously described [117]. NK cells also display some adaptive capacities themselves, with evidence of immune memory [118]. Cancer cells may compromise NK cell function by increased expression of PD-1, the immunosuppressive regulatory protein which acts as an immune checkpoint [119]. Despite strong anti-tumourigenic functions of NK cells in CRC, it has been shown that NK cell infiltration alone does not correlate with patient outcomes in CRC, but the presence of both $CD8^+$ cytotoxic T cells and NK cells together displays improved patient outcomes [120, 121].

1.8.1.5.2. $CD8^+$ Cytotoxic T cells

$CD8^+$ T cells are cytotoxic lymphocytes considered to be the most prolific effector cells of anti-tumour immunity. $CD8^+$ T cells mature in the thymus from $CD4^+/CD8^+$ naïve T cells following extensive T cell receptor recombination following exposure to antigens by thymic epithelial cells. These cells then undergo clonal expansion and eventually migrate to peripheral sites and can become further activated by local cytokines such as IL-2, IL-12, IL-15 and IFN-y produced by M1 TAMs, dendritic cells and $CD4^+$ T helper cells [65, 85, 122]. These cells have similar cytotoxic effects as described for NK cells previously, with production of substances such as perforin, granzyme, FAS-L and TNF-α to induce cell death in cancer cells [123].

The prognostic value of CTLs is well characterised, with increased CTL infiltration associated with improved patient outcomes in CRC [32, 124]. Furthermore, CTLs have been reported to be the most important immune cell type in the TME to positively influence patient outcomes [125]. This increased CTL infiltration has been shown to be closely associated

with MSI-H tumours in CRC [126]. These tumours generate increased numbers of neo-antigens due to mismatch repair of DNA making a variety of irregular proteins (neo-antigens) and thereby tumours are more immunogenic. Indeed, MSI is used as response predictor for ICIs [43].

1.8.1.5.3. $CD4^+$ T Helper cells

$CD4^+$ Helper T lymphocytes are a subset of cells which support other effectors cells, including T cells and B cells, in the TME to mediate immune signalling. Like other cell types, these cells can be categorised into several different subsets according to phenotypes and functions. These include T helper 1 cells (Th1), T helper 2 cells (Th2), T helper 17 cells (Th17) , T helper 22 cells (Th22) , follicular T helper cells (Tfh, discussed later) and regulatory T cells (Tregs) [127].

1.8.1.5.3.1. Th1/Th2 cells

Th1 cells are key support cells for cancer cell destruction. These cells are differentiated by dendritic cell-derived IL-12 which drives TBET expression, an important transcription factor for type I responses [128]. These cells function as mediators of cellular immunity. After lymphoid tissue maturation and expansion, re-exposure of $CD4^+$ helper cells to its complimentary TCR antigen and IL-12 results in further maturation to a Th1 phenotype with high levels of IFN-y production [129]. This may act in a paracrine manner on CTLs or in an autocrine manner to drive STAT1-mediated constitutive expression of TBET. Th1 infiltration in CRC is has been reported to be a positive for patient outcomes [130]. Th2 cells secrete IL-4 and IL-10, which may induce TAM phenotypes toward wound healing states [65].Conflicting reports have reported both good and bad prognoses for Th2 infiltration across several cancer types [127].

1.8.1.5.3.2. Th17/Th22 cells

Th17 cells recruit myeloid lineage cells to sites of inflammation and stimulate phagocytosis by these cells [131]. Differentiation of $CD4^+$ T cells to a Th17 phenotype is driven by TGFb production and interleukin-6 [132]. These cells play a dichotomous role in cancer, as they can recruit CTLs and dendritic cells to the TME to drive a type 1 immune response [133], but also attract myeloid cells resulting in a more immunosuppressive TME leading to cancer cell immune-evasion and poorer prognosis [134]. Th17 cytokines reflect the dichotomous nature of these cells, having both pro-tumourigenic and anti-tumourigenic effects [135]. Th22 cells are closely related to Th17 cells and have been heavily implicated as driving tumour progression in CRC [136]. The IL-22 receptor is exclusively expressed on epithelial cells, including colon cancer cells [137]. Th22-derived IL-22 can directly stimulate colon cancer cell proliferation and plays a key role in tissue repair and tumourigenesis in the intestines [27, 138]. Th22 cell infiltration into the TME is associated with poor patient outcomes [139].

1.8.1.5.3.3. T regulatory cells (Treg cells)

T regulatory cells (Tregs) act as important mediators of inflammation and the anti-tumour immune response. Tregs develop into their mature phenotype by TGF-β stimulation. They can also be induced from naïve $CD4^+$ T cells due to chronic exposure to antigens [140, 141]. Treg cells function in several ways to dampen the anti-tumour immune response, including inhibition of Th1 IFN-y production and impeding CTL function by PD-1 expression [142, 143]. Cancer cells can induce Treg migration into the TME by enhanced CCL2 expression, and they can stabilise Treg phenotypes through TGF-β secretion [144, 145]. Contradicting the convention of sustained ‘hot’ tumours leading to better patient outcomes, Treg cell

infiltration in CRC has been reported to be associated with improved patient survival [146, 147]. However, contrasting studies have reported Tregs to indeed be associated with poor patient outcomes in CRC [148].

1.8.1.5.4. Other immune cells

Memory T cells are long lasting T cells with similar properties to helper and effector cells according to subclass. These cells can become tissue resident cells with strong cytotoxic capacities in CRC [149-151]. γδ T cells are a recently described subset of peripheral blood lymphocytes ($CD4^-/CD8^-$) which can infiltrate the CRC TME, and this cell type is one of the most favourable infiltrating immune cell types across multiple cancers for patient outcomes[152, 153]. However, cancer cells can convert the anti-tumour γδ T cell phenotype to an immune-tolerant one [154]. Mast cells are highly granular cells associated with histamine release in type 1 hyper-sensitivity. Mast cells have also been implicated in the progression of CRC by contributing to carcinogenic chronic inflammation [155, 156]. Enteric glial cells (EGCs) are a unique subset of neuronal cells found in the intestines which show immune-mediating properties similar to that of other immune cells. These cells have been largely implicated in CRC tumourigenesis and progression, especially during earlier stages of tumour progression [157, 158].

1.8.1.5.5. Tertiary Lymphoid structures

TLSs are discrete regions of the TME consisting of a T cell zone (Th1, CTL, DCs and fibroblastic reticular cells) as well as the B cell zone which resembles other lymphoid germinal centres consisting of memory B cells and plasma cells (Fig 1.4) [159]**.** TLS structure formation has been described as a multi-step process which may occur spontaneously in

tissues with non-resolving chronic inflammation, or can be induced by exogenous administration of pathogens or inflammatory molecules [160]. The location of spontaneously formed TLSs varies according to local chemokine induction with these structures capable of localising intratumourally [161], peri-tumourally [162] or a combination of both [163]. Chemokine secretion further dictates the structural composition of these lymphoid structures [164]. Functions of TLS can include augmentation of the cellular-mediated anti-tumour response by enhanced DC maturation and subsequent activation of CTLs against tumour cells [165]. Furthermore, humoral immunity may be promoted by T follicular helper cell secretion of CXCL13 to promote germinal centre B cell expansion resulting in plasma cell production to contribute to anti-body mediated cytotoxicity of TILs [163, 166]. Intratumoural TLS formation is associated with improved patient outcomes, with a significantly lower risk of disease recurrence [167]. It has also been recently shown that increased density of TLSs in CRC is favourable for patient outcomes [168].

1.8.2. Mesenchymal Stromal Cells (MSCs) in the TME

In addition to immune cell infiltration of the TME, which largely dictate the prognosis of CRC, MSC cell populations are also highly influential in the TME as these cells can make up as much as 50% of the primary tumour mass [169]. These cell populations include several different cell types all of which undergo crosstalk with cancer cells, the immune infiltrate and other MSCs. These cells may be tissue resident cells or recruited to the TME [170].

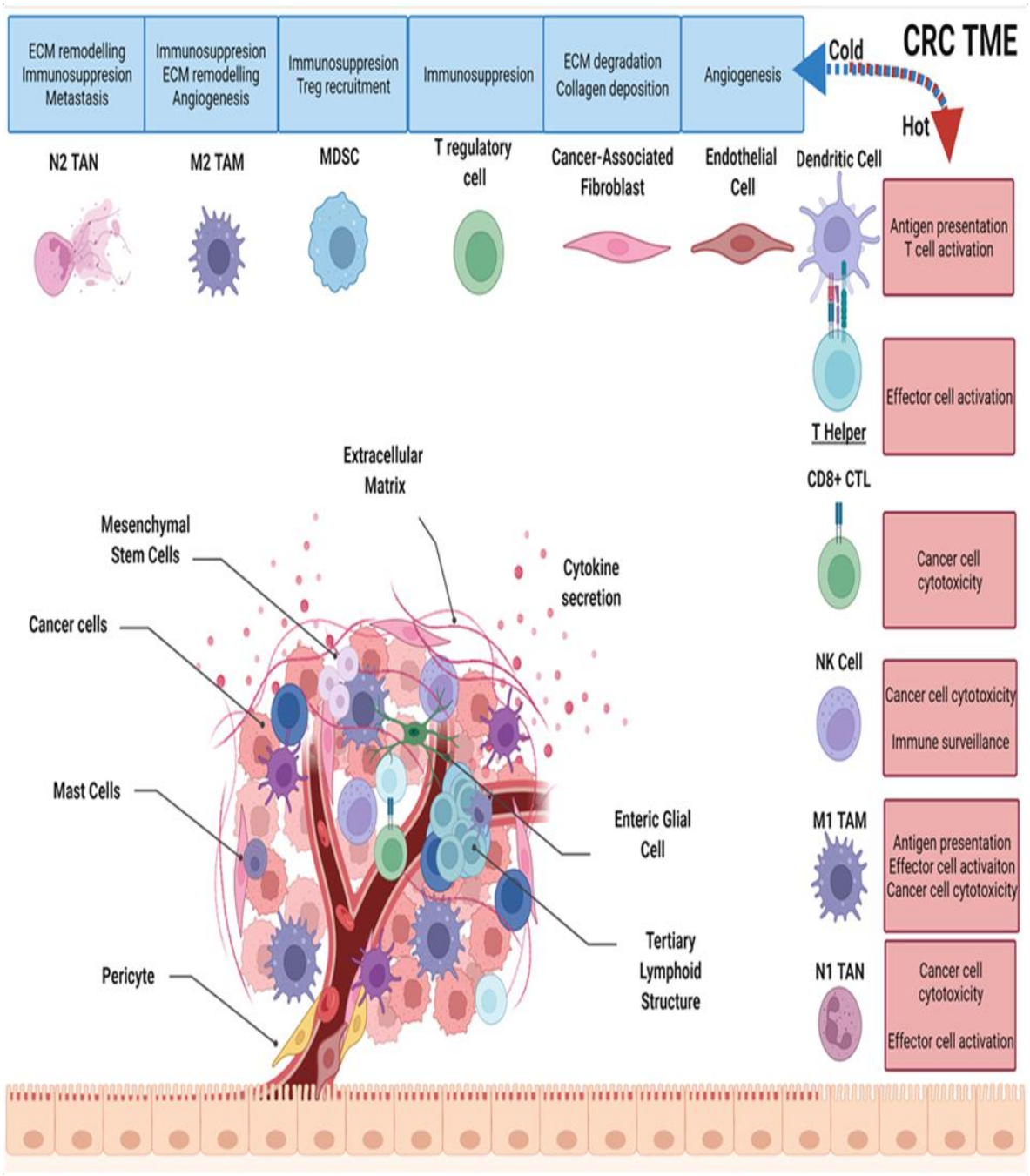


Figure 1.4: **The CRC tumour microenvironment**. Diverse and complex in cellular makeup and trafficking, the CRC TME can be simplified as to containing tumour promoting and tumour suppressing cell populations, often termed as 'cold' and 'hot', respectively. Many other components influence the TME including TLSs, Enteric Glial cells and the ECM. Cytokine secretion between all of these cells and cancer cells dictate the trajectory of tumour rejection or progression.

1.8.2.1. Cancer Associated Fibroblasts (CAFs)

CAFs have been recognised as the primary cells to construct and maintain the structural architecture of the TME and are therefore highly influential in patient outcomes [171]. CAFs are a heterogeneous population of matrix-manufacturing cells with most of these cells originating from connective tissues and bone marrow [172]. Colon cancer cell secretion of TGF-β can convert normal resident tissue fibroblasts toward a classical CAF phenotype with increased αSMA expression [173]. Many other sources of CAF origin have been described by Deng *et al* , including mesenchymal stem cells and endothelial cells [172]. CAFs play many roles in CRC tumourigenesis [171]. CAFS may contribute to matrix remodelling by protein degradation and matrix synthesis [174]. Soluble factor secretion by CAFs can directly contribute to cancer cell proliferation [175]. Furthermore these cells can disrupt the anti-tumour immune response by metabolic shifts [176], suppressor cell chemoattractant secretion [177] as well as collagen synthesis which is associated with immune cell dampening [178]. Other pro-tumourigenic features of CAFs include roles in angiogenesis via IL-6 secretion [179], stemness [180], targeted therapy resistance [181] and induction of cancer cell invasion [182]. Multi-cohort IHC analysis of primary CRC tumours has shown increased CAF proportions to be associated with poorer patient outcomes [183].

1.8.2.2. Endothelial Cells

Endothelial cells function to line blood and lymphatic vessels in order to regulate several transport properties such as vascular permeability, vessel growth and maintenance of blood flow [184]. Tumour associated endothelial cells may stem from progenitor endothelial cells, but also from colon cancer stem cells to support angiogenesis via VEGFR2 [185]. Other roles for endothelial cells in the CRC TME include cancer cell metastasis [186]

and suppression of the immune response by increased expression of PD-L1 and downregulation of leukocyte adhesion molecules [60]. In terms of endothelial cells and prognosis, it has been reported that increased circulating endothelial cells is a poor prognostic marker in mCRC [187].

1.8.2.3. Other MSCs in the CRC TME

Mesenchymal stem cells (MSCs) are progenitor cells which may differentiate into several cell types that are found in the CRC TME. MSCs have been reported to be an additional dichotomous cell type in the CRC TME [188]. MSCs are recruited from the bone marrow into the TME and undergo differentiation to different cell types but predominantly CAFs [189]. Adipocytes are specialised cells used as storage sites of fat within lipid droplets. It has been shown that these cells undergo crosstalk with colon cancer cells in the TME which may contribute to several pro-tumourigenic processes [190]**.** Pericytes are endothelium-associated cells which assist in the maintenance of blood vessel and in the CRC TME. These cells can contribute to neovascularisation [191], metastasis [192] and other properties previously described upon differentiation into CAFs [172]. Many of these cells contribute to extracellular matrix production, which is being increasingly implicated as an immunomodulatory feature of the TME and is extensively altered in CRC relative to normal tissue[193-195].

1.9. IL-1 cytokines

The importance of inflammation in cancer is now well established [49, 66, 196, 197]. In some cancers, the inflammatory conditions precede the development of malignancy, e.g., chronic bronchitis is a major risk factor for lung cancer. Alternatively, aberrant signalling due to oncogenic mutations in tumours can result in a chronic inflammatory state developing both proximal to, and within, the tumour. This chronic inflammation acts to inhibit the anti-tumourigenic immune response, normally mediated by cells such as M1 macrophages, NK cells and $CD8^+$ T cells. Tumour cells themselves can also directly induce an immunosuppressive microenvironment through recruitment and activation of specific immune cell subtypes such as myeloid derived suppressor cells (MDSC), M2 macrophages and T regulatory cells, thereby further promoting tumourigenesis [6, 7, 198]. Understanding the complexity of immunomodulation by tumours is important for the development of effective immunotherapies [127]. Cytokines, such as Interleukin-1 (IL-1) are central mediators of the interactions between cells in the inflammatory tumour microenvironment [199]. The IL-1 family now includes seven ligands with pro-inflammatory activity (IL-1α and IL-1β, IL-18, IL-33, IL-36α, IL-36β, IL-36γ) as well as anti-inflammatory cytokines (IL-37, IL-38). Several members of this family, such as IL-1β and IL-18, have been extensively investigated in cancer with both pro- and anti-tumourigenic functions ascribed to these cytokines [196]. In contrast, far less is currently understood concerning the role of more recently identified members of this family in cancer such as IL-33, IL-36 and IL-37, although such data that is available also indicates that these may have both pro- and anti-tumourigenic effects [199].

1.9.1. IL-1 cytokines in cancer

The IL-1 superfamily is a diverse family of cytokines, most of whose members have been shown to have dual functions in tumourigenesis with both pro- and anti-tumourigenic roles being ascribed [196]. The role of these cytokines varies greatly depending on the tissues and organs involved, the inflammatory background and the stage of the cancer [199]. Levels of the cytokines present within the tumour microenvironment have a role in determining the outcome of the effect on tumourigenesis. For example, when the pro-inflammatory cytokines are produced at persistent, chronic low levels, often by the tumour itself, this can play a role in shaping an immune-suppressive tumour microenvironment, facilitating a chronic inflammatory state and enabling tumour growth and spread [196, 200-202]. In line with this, results from the CANTOS trial imply that suppressing such chronic inflammatory effects of these cytokines over an extended period may have significant benefits in terms of limiting cancer development and progression [203]. In contrast, exogenous administration of high levels of these cytokines has been shown to often have a potent anti-tumourigenic effect due to recruitment and activation of Type 1 immune responses [204]. Further insights into IL-1 cytokine concentrations, tailored to each cancer type, will allow for determining efficacy of inhibiting or enhancing IL-1 signalling to combat tumourigenesis.

1.10. The IL-36 cytokine subfamily

1.10.1. Genetics and homology

The IL-36 cytokines were identified by multiple groups on the turn of the 21st century as discrete cytokines that were homologous to other IL-1 family members [205-207]. Rapid expansion of nucleotide sequencing allowed for prediction of these novel cytokines to have

similar functions to IL-1 family based on conserved sequences within these genes [205]. Sequence homology between the three IL-36 agonists (IL-36α, IL-36β and IL-36γ) further shows greater similarities between IL-36α and IL-36γ, than with IL-36β. Direct inhibitory antagonists of these cytokines have been identified which include the IL-36 receptor antagonist and IL-38. IL-36 agonists and antagonists are encoded within chromosome 2 in humans. Close chromosomal proximity and sequence conservation of IL-36 cytokines suggests that these cytokines arose as a result of gene duplications during eukaryotic evolution [208]. IL-36 signalling takes place via IL-36 agonist interaction with their specific heterodimeric receptor which consists of IL-36R and the universal IL-1 receptor accessory protein, IL-1RAcP. Receptor antagonism is achieved through binding of IL-36Ra or IL-38 to the IL-36R, preventing dimerization of this receptor protein with IL-1RAcP [209]. These events were characterised in the early 2000's in IL-36 transgenic mouse studies, which sequentially revealed each family member, each an orphan ligand at the time, to be part of the IL-36 family. This was then further described by refined biochemical assays showing IL-36 agonists and IL-36Ra to indeed be agonists and antagonists of the IL-36 receptor [210].

1.10.2. IL-36 cytokine secretion

Unlike other cytokines, IL-1 family member genes do not encode a signal peptide to mediate protein trafficking to the endoplasmic reticulum or Golgi apparatus. Furthermore, IL-36 cytokines processing is inflammasome-independent, an activation process used by other IL-1 cytokines such as the proforms of IL-1β and IL-18 resulting in their processing by caspase-1 followed by their membrane pore release mediated by gasdermin D [211, 212]. Cell death mediated release of IL-36 cytokines has been reported, however these genes also do not encode caspase cleavage sites therefore suggesting it to be unlikely that this is

the primary mechanism of IL-36 release from cells [213]. Macrophage mediated release of IL-36γ in microparticles and exosomes has been reported in response to bacterial stimulation of cells [214]. Very recent work has reflected this in the release of IL-36α from keratinocytes which have undergone PRR stimulation followed by mechanical disruption of cells [215].

1.10.3. IL-36 Proteolytic Activation

Full length IL-36 cytokines initially show very low levels of activity after synthesis and it was first shown in 2011 that these cytokines require N-terminal removal for induction of full cytokine activity [210]. This was first shown by N-terminal deletion and later further characterised by enzymatic processing of these cytokines by several proteases derived from myeloid cells. IL-36α is cleaved by Cathepsin G (CTSG) or neutrophil elastase (ELANE) at different amino acid residues (>1000 fold activation) [210]. IL-36γ can be cleaved by ELANE, and also less effectively by Proteinase 3 (PRTN3) and Cathepsin S (CTSS) [216]. IL-36β is also cleaved and activated by CTSG. The pro-form of IL-36Ra also undergoes N-terminal proteolytic cleavage by ELANE, and PRTN3/CTSG to a lesser extent [217]. A type of neutrophil cell death, NETosis, can result in the extrusion of protease-studded chromatin which can also activate IL-36 cytokines with epithelial cell derived CTSS shown to cleave IL-36 in a neutrophil-independent manner [218]. Moreover, IL-36γ can be cleaved by pathogen derived proteases [215]. Similarly, IL-38 N-terminal truncation increases its inhibitory capacities across several IL-36 driven pathologies [219].

1.10.4. IL-36R Signalling

The IL-36R protein is located as a transmembrane protein, with classical features of IL-1 receptor family members including an extracellular immunoglobulin-like domain, a single-pass transmembrane domain and an intracellular-cytoplasmic Toll/interleukin-1 receptor (TIR) domain (Fig. 1.5) [220, 221]. Glycosylation of several asparagine residues in the ectodomain of the protein is required for cell trafficking of the IL-36R, with disruption of this capable of significantly reducing surface expression of the protein [222]. Furthermore, in the absence of agonist binding, the IL-36R is endocytosed and recycled back to the plasma membrane. The IL-36R may also be endocytosed upon agonist binding and accumulate in lysosomes. This is stabilised by Tollip recruitment which may then lead to polyubiquitination or recycling of IL-36R to the cell surface [223]. The extracellular IG domain (ECD) of the IL-36R may interact with IL-36 agonists at different residues, for example a mutation of C42A in ECD1, a component of the ECD, resulted in reduced IL36α and IL-36γ signalling, but not IL-36β [222]. Furthermore, crystallisation studies of the IL-36R have shown residues Asp150, Asn148 and Ala162 are necessary for hydrogen bonding formation and IL-36α, β, γ binding, respectively [224].

In contrast with other IL-1 receptor complexes, it was proposed that the IL-36R and IL-1RAcP may dimerize before ligand binding and that subsequent cytokine binding to this complex may then lead to conformational changes of the IL-36R/IL-1RAcP/agonist complex resulting in downstream phosphorylation cascades along the TIR domain [222]. Further studies investigating all possible binding conformations could not detect IL-36R with IL-1RacP binding before agonist binding took place. However, Fc-linkage of IL-36R to IL-36RAcP showed that IL-36α had a higher binding affinity to this preformed complex than

the IL-36R alone. It was also shown that IL-36Ra displayed a much stronger binding affinity to IL-36R than other IL-36 agonists. Notably, these studies were completed using recombinant, purified cytokines and keratinocyte cells lines which may vary to the *in vivo* setting and several binding affinities between proteins were estimated due to detection limitations [225].

Following cytokine, receptor and accessory protein interaction, complex formation or conformational changes leads to phosphorylation of the TIR domain [225]. Adaptor proteins, such as MyD88, are subsequently recruited to the TIR domain upon TIR-TIR interaction, leading to IRAK-1/4 translocation to the TIR domain. Autophosphorylation of IRAK1 results in its activated release which is then followed by TAB2 interaction, an adaptor protein for TAK1 in the MAPK signalling pathway. This allows for TRAF6/TAK1/TAB1 interactions, leading to downstream phosphorylation of IκBζ resulting in NF-κB and p38/JNK pathway activation. This ultimately leads to eventual downstream pro-inflammatory gene transcription in stimulated cells [226].

Unsurprisingly, IL-36R signalling and IL-1R signalling, when investigated under similar conditions, have been shown to share common transcription factors and also show extensive downstream gene expression overlap in keratinocytes as assessed by RNAseq [227]. This suggested that IL-36 signalling may differ from IL-1R signalling according to spatial localisation of IL-36R, tissue expression of IL-36 cytokines and availability of proteolytic enzymes [227].

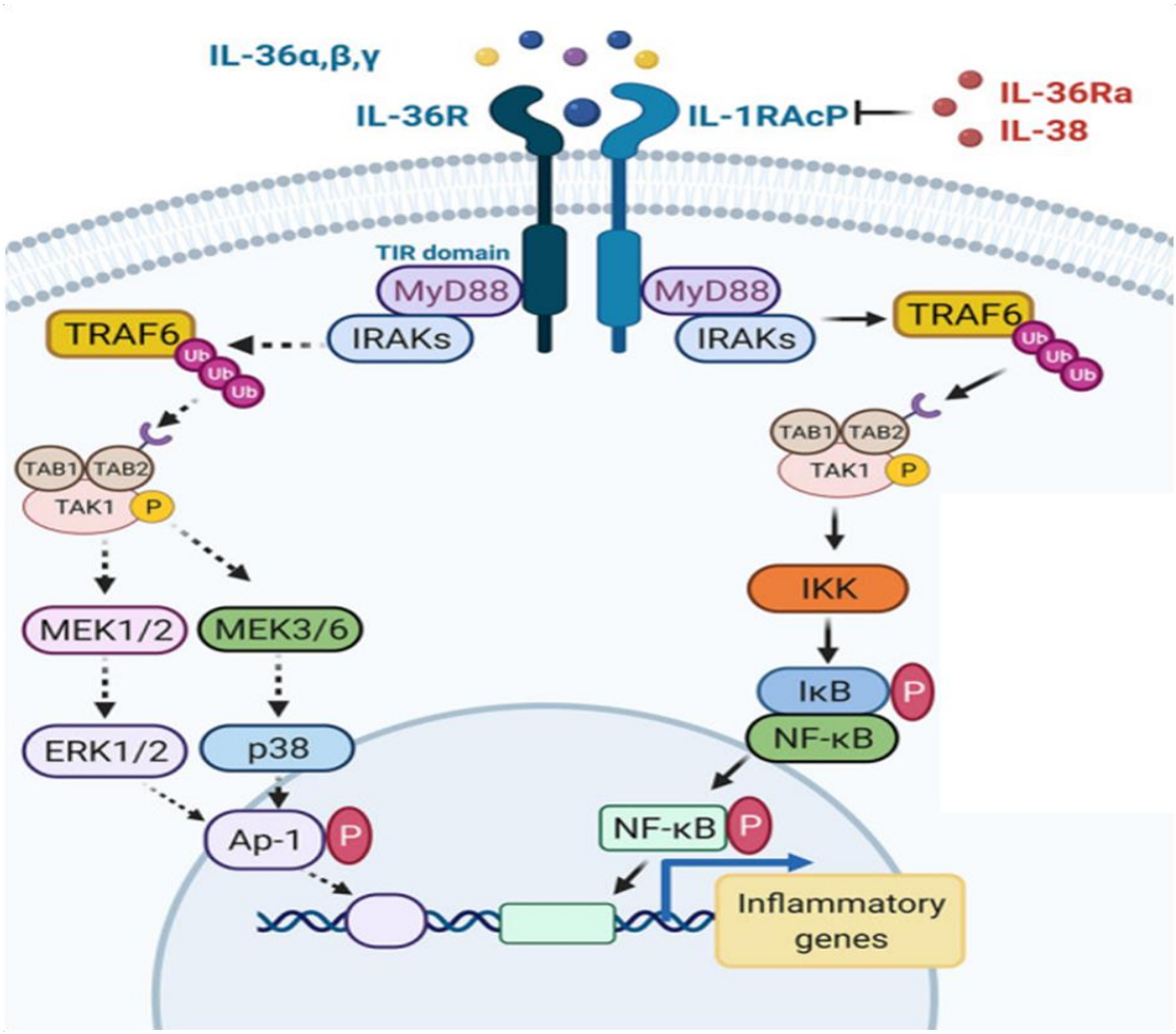


Figure 1.5: The IL-36 signalling cascade. IL-36 cytokines bind to the IL-36R and dimerize with the IL1-RAcP to induce a phosphorylation cascade in the TIR domain. This leads to recruitment of the MyD88 adapter protein which can then lead to downstream activation of MEK/ERK/p38 signalling as well as IκB signalling. This results in the induction of many downstream genes being expressed to induce a Th1 and Th17 immune response in cells, as well as multiple intrinsic processes.

1.10.5. IL-36 Regulation

IL-36Ra and IL-38 have been shown to negatively regulate the IL-36 signalling pathway. IL-36Ra was the initial IL-36 cytokine discovered and it shares 44% sequence homology with IL-1Ra [228]. IL-36Ra exhibits antagonistic functions by competitively binding to IL-36R, suppressing IL-36 agonist recognition and IL-1RAcP recruitment, thus inhibiting activation of the receptor by the agonist members of this family [229]. Blocking IL-36R-mediatied

activation thereby prevents signalling of MAP kinases and NF-κB pathways. Unlike IL-1Ra, which is purely inhibitory, the IL-36Ra can itself induce the expression of cytokines in glial cells, with IL-36Ra shown to induce upregulation of IL-4 mRNA/protein expression through recruitment of the IL-1 orphan receptor SIGIRR/TIR8 *in vitro* [230]. IL-38 shares similar biochemical structural characteristics with IL-36Ra as shown by Zhou and Todorovic [213]. *In vitro* administration of IL-38 was observed to suppress IL-36γ biological activity in human peripheral blood mononuclear cells [231]. The exact means by which IL-38 inhibits IL-36 signalling is yet to be determined, whether it is derived from antagonistic activities by blocking IL-1RAcP/IL36R dimerization or by recruiting an inhibitory receptor from the IL-1 superfamily such as SIGIRR, TIGIRR1 or TIGIRR2 [231].

1.10.6. IL-36 Functions

IL-36R and IL-36 cytokine expression is most active at barrier tissues at the surface of the skin, lung and intestines. It is therefore presumed that IL-36R signalling has primarily evolved to act as a regulator of these barriers, in unison with other innate signalling receptors, in response to environmental cues. This is clear from the roles of IL-36 cytokines in epithelial barrier maintenance, response to cellular damage and the response to infection across these barrier tissues. Mesenchymal cells and immune cells can also highly express IL-36 family members e.g. dendritic cells [232], NK cells [233], $CD4^+$ T helper cells [234], Cytotoxic T cells [235], macrophages [236], neutrophils [237], fibroblasts [238], endothelial cells [239], glial cells [240] as well as many forms of epithelial cells across multiple organs [63, 238, 241-243].

1.10.6.1. IL-36 as a key regulator of epithelial barriers

IL-36 cytokines play an important role in the regulation of epithelial barrier turnover, permeability and resistance to infection and across multiple tissue sites. It has been speculated that IL-36 family members may have emerged from their common ancestor, IL-1, as a means of overcoming pathogen resistance to the IL-1-mediated pathogen response [244]. The importance of these cytokines in regulating epithelial homoeostasis is further highlighted by the plethora of pathologies that derive from excessive or insufficient IL-36 cytokines at these sites to maintain healthy tissue.

1.10.6.1.1. IL-36 signalling at the epidermal barrier

The identification of IL-36α as an important inducible inflammatory molecule came about from studies investigating HSV infection of keratinocytes [245]. This has since been further examined with strong evidence supporting IL-36 cytokines as important mediators in infection following PAMP and DAMP by TLRs at the skin barrier [246-248]. This results in downstream Th1 and Th17 responses to regulate this process [249-251]. Indeed it has been shown that IL-36 signalling is important in clearance of bacterial, fungal and viral infections including HSV, *S. aureus* and *C. albicans* [248, 250, 252]. Furthermore, it has been shown that IL-36 signalling can act as an important identifier of commensal microbes and pathogens [253].

Following on from epithelial cell damage by infections or mechanical stress, IL-36 cytokines have been shown to be important in the regenerative capacity of these tissues. Keratinocytes, and several other cells associated with the skin barrier, express the IL-36 receptor [236]. This suggests a direct role for IL-36 signalling on these cells under homeostatic conditions or to quickly respond to insult. Following injury, TLR3 detection of

RNA from damaged keratinocytes results in downstream expression of IL-36γ which subsequently induces expression of the antimicrobial peptide REG3A to promote wound healing [254]. IL-36γ-induced REG3A can directly induce keratinocyte proliferation by inhibition of differentiation genes [255]. Furthermore, it has been shown that IL-36γ can directly stimulate human epidermal keratinocyte proliferation [256]. However, it has also been reported that IL-36 cytokines can delay wound healing and are not directly involved in keratinocyte hyper-proliferation [257, 258].

1.10.6.1.2. IL-36 signalling at the pulmonary barrier

Reflective of the immunoregulatory role of IL-36 at environmental sensing organs, the lungs also have extensive roles for IL-36 cytokines in the response to infection, regulation of barrier permeability and epithelial cell turnover. IL-36γ deficient mice show an increased influenza viral titre as well as reduced apoptosis of virus-infected lung epithelial cells [259, 260]. Furthermore, IL-36γ has recently been used as a vaccine adjuvant for the development of influenza vaccines, as well as HIV and Zika virus vaccines. Additionally, IL-36 has been shown to be heavily involved in the Th1 response to Sars-Cov-2 infection [261, 262]. IL-36 cytokines also play an important role in TLR-mediated bacterial infections in the lungs, controlling and clearing M*. tuberculosis* growth, although it has been reported that IL-36R expression is dispensable in clearance of this infection [263-266]. IL-36γ also mediates macrophage polarisation for clearance of *P. aeruginosa, S. pneumonia* and *L. pneumophilia* [267-269].It has also been shown that IL-36α and IL-36γ expression is upregulated in response to cigarette smoke compounds, suggesting a potential protective role for IL-36 signalling in the lung barrier [270].

1.10.6.1.3. IL-36 signalling at the intestinal barrier

IL-36R expression has been shown to be required for clearance of the enteropathogenic bacterium *C. rodentium*. In this mouse model of colitis, IL-36R deficiency resulted in impairment of the IL-17/IL-22/Anti-microbial peptide pathway, with a substantially increased bacterial burden found in the intestinal mucosal wall. IL-36 signalling can also influence commensal colonisation, with this shown by IL-36γ facilitating growth of the commensals *Bacteroidetes* by the Th17 priming of the immune response to *Klebsiella pneumonia* in the intestines [271].

As a means of regulating barrier turnover, IP administration of IL-36α and IL-36γ (combined 3μg total) has been shown to induce the proliferation of intestinal epithelial cells [238]. Moreover, IL-36α/β/γ could individually increase Ki67 expression in mouse-derived colonic organoids, with IL-36β and IL-36γ more potent at inducing this effect. This same group also showed IL-36 signalling to directly induce proliferation of intestinal fibroblasts and increased gene expression for pathways involved in wound healing. In agreement with this work, IL-36R deficient mice are inefficient at wound healing compared to WT mice [272]. This phenotype was resolved by inducing IL-22 expression in IL-36R deficient mice. Furthermore, reduced IL-36 signalling can prevent IL-23/IL-22/AMP mediated wound healing, and this pathway may indeed play a role in maintenance of intestinal barrier permeability via tight junction components such as occludin and claudin 2 [273]. More recently, it was reported that IL-36Ra deficient mice have an improved regulation of intestinal barrier permeability, which was concomitant with increased mucus production in intestinal crypts [274]. Together these studies show, especially in the case of the

intestinal barrier, that IL-36 cytokines play an important role of pathogen clearance and barrier homeostasis.

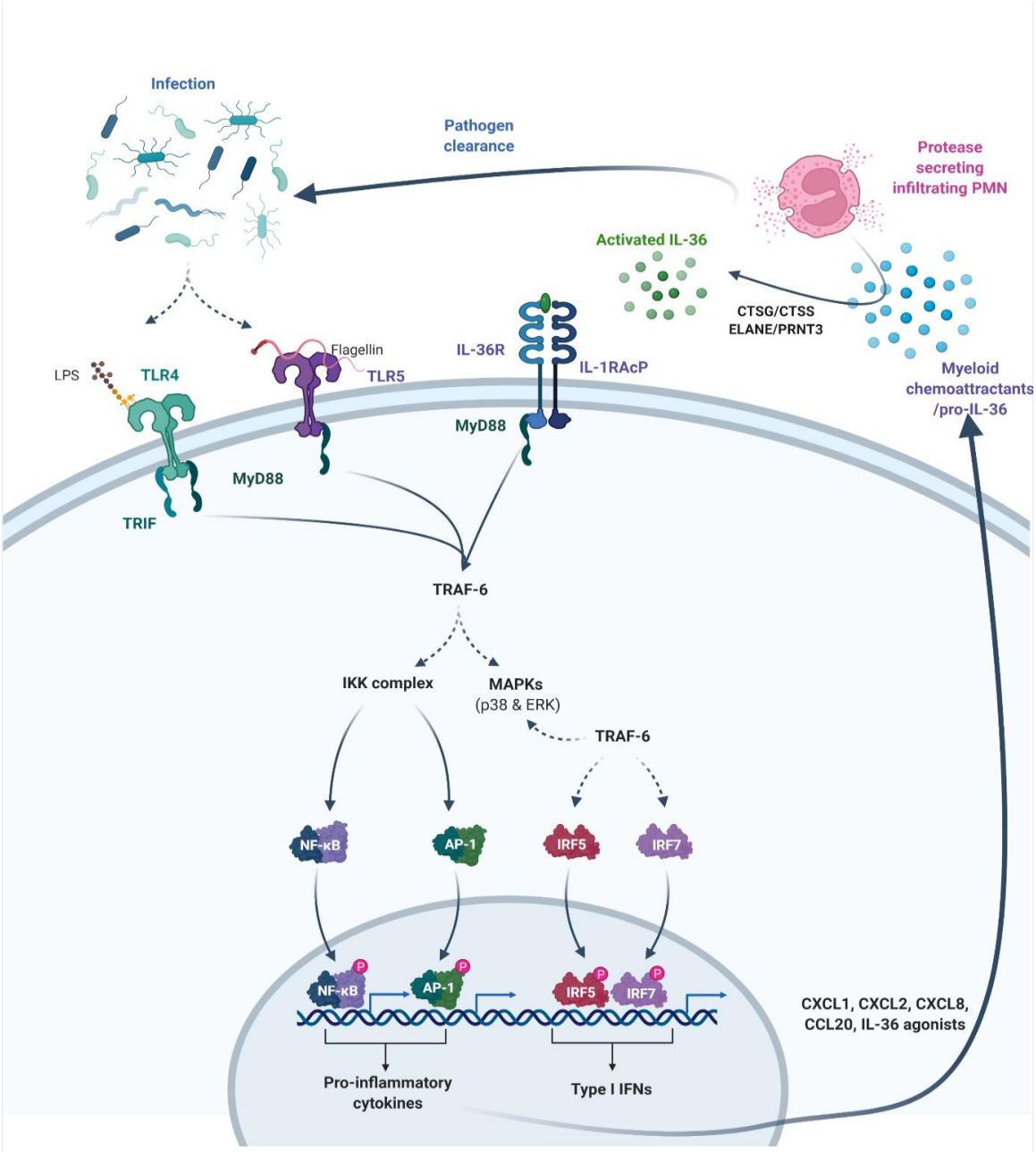


Figure 1.6: Model of IL-36 signalling during epithelial barrier tissue regulation. Activation of Pathogen Recognition Receptors, such as TLRs, leads to downstream induction of pro-inflammatory gene expression in epithelial cells such as myeloid cell chemoattractants and IL-1 family members, including the IL-36 family members. This leads to secretion of these proteins, which are activated by neutrophil-derived proteases. Cleaved IL-36 cytokines are activated, bind to epithelial IL-36R and the perpetuate the inflammatory process to clear infection or lead to chronic inflammation at these sites as shown in Psoriasis, IBD and COPD.

1.10.6.1.4. IL-36 signalling at other tissue barriers

Similar patterns of infection prevention and tissue homeostasis have been reported across multiple other barrier sites in mammalian systems. This includes the oral mucosa [215, 253, 275-279], the female and male reproductive tracts [280-282], and mammalian corneal barriers [283]. Across each of these barrier sites, IL-36 signalling once more responds to infection with rapid anti-microbial gene expression as well as epithelial cell signalling for induction of cellular damage repair (Fig. 1.6).

1.10.6.2. IL-36 signalling in immune cell differentiation, activation and expansion

IL-36 cytokines, as described previously, are potent stimulators of cytokine production across many epithelial barrier tissues in response to tissue damage and infections. Several immune cell and stromal cell populations have been reported to be sources of IL-36 cytokines, but IL-36 signalling cytokines have also been shown to be highly important proteins in the direct differentiation, maturation, activation and proliferation of multiple cell types in both homeostasis and response to disease. Multiple studies, across multiple different anatomical locations, have reported fibroblasts to express the IL-36R and to respond to stimulation by increasing pro-inflammatory cytokine expression, augmenting the fibroblast wound healing phenotype and increasing proliferation of these cells [238, 284-289].

Innate and adaptive immune system-bridging cells have been shown to be highly influenced by IL-36 signalling. Dendritic cell maturation and activation has been reported to be an effect of IL-36β stimulation of monocyte derived DCs (MDDCs) which resulted in downstream induction of $CD3^+$ T cell proliferation [273, 290, 291]. Moreover, bone marrow derived DCs have also been shown to upregulate pro-inflammatory chemokine expression

in response to IL-36 stimulation [292] with plasmacytoid dendritic cells also able to respond to IL-36 stimulation by potentiating TLR9 activation and IFN-a production [293]. Foster *et al* have also shown IL-36 signalling to induce pro-inflammatory gene expression in circulating monocytes, myeloid dendritic cells and MDDCs [294]. Furthermore, these IL-36-stimualted MDDC could induce the proliferation of $CD4^+$ T cells and these $CD4^+$ T cells can, in turn, directly respond to IL-36 stimulation resulting in increased cellular proliferation [295]. In further agreement with IL-36 activation of macrophages, IL-36 can directly contribute to the polarisation of macrophages from an M2 to M1 phenotype [236].

IL-36 signalling has also been reported to influence multiple lymphocyte populations. Th1 and Th2 differentiated lymphocytes were shown to express higher levels of IL-36R, with both of these cell types shown to respond to IL-36 cytokines with increases in IFN-γ, IL-4 and to a lesser extent, IL-17, with further work showing polarisation of these cells to a stronger Th1 phenotype [292]. Moreover, it was confirmed that IL-36β synergistically acts with IL-12 to promote Th1 polarisation of naïve T cells and proliferation of T cells [296]. This was also shown by Russel *et al*, with IL-36α reported to drive Th1 polarisation whilst inhibiting Th17 polarisation of naïve T cells [297]. In the context of the gut, the $CD4^+$ T cell population response to IL-36 cytokine stimulation has been investigated with increased Tbet/IFN-γ expression observed whilst IL-36 stimulation reduced Th17 and Treg differentiation [234]. Treg cell inhibition by IL-36 has also been shown to skew $CD4^+$ T cells to a Th9 phenotype [298]. $CD8^+$ T cells have also been reported to directly respond to IL-36 stimulation, with IL-36β and IL-36γ capable of inducing cytotoxic gene expression by mTORC1 induction [235, 299].

1.10.7. The role of IL-36 in the pathogenesis of inflammatory diseases

It is now well understood that IL-36 cytokines are potent drivers of chronic inflammation across several different types of inflammatory pathologies. These pathologies typically begin with innate immune signalling in response to pathogens or tissue damage at tissue barriers, followed by TLR-driven expression of IL-36 cytokines which act to augment the innate immune response to clear infection and resolve cellular damage. In certain cases, the innate immune response can overexpress IL-36 cytokines which, without effective inhibition/resolution, leads to severe and chronic inflammation.

1.10.7.1. IL-36 in skin disorders

Indeed there are several different types of inflammatory skin disorders which reflect the aforementioned IL-36 driven chronic inflammation observed at many other barrier tissues [226]. This TLR/IL-36 axis is seen frequently in the skin in response to bacterial and fungal pathogens. This has been clearly shown in cancer patients receiving EGFR/MEK inhibitors [300]. Here, the skin commensal C. acnes drives TLR2-mediated IL-36γ expression which is also simultaneously induced by EGFR/MEK inhibition. Two separate transcription factors, NF-κB and KLF4, then synergistically overexpress IL-36γ leading to severe skin toxicities driven by Th17 cutaneous neutrophilia. The importance of tight IL-36 signalling regulation is further highlighted by delayed epidermal wound healing in IL-36Ra deficient mice, where excessive IL-36 signalling prevents wound closure capacities due to excessive myeloid cell infiltration [257].

IL-36γ is the primary IL-36 cytokine involved in psoriasis, with this cytokine recognised as the most influential in disease pathogenesis when directly compared to IL-36α and IL-36β [301]. IL-36 cytokine signalling in psoriasis is mediated by the IL-17/IL-23 axis, with IL-17

inducing IL-36 cytokine expression in a feed-forward inflammatory loop [302, 303]. This loop results in reduced keratinocyte differentiation with characteristic epidermis thickening and inflammation caused by IL-36-driven Wnt signalling [256]. The importance of regulating this unrestricted IL-36 signalling is echoed in work showing that IL-38 expression is decreased in psoriasis and correlates with disease severity [304]. This study also complemented *in vitro* work which showed that IL-36Ra and IL-38 could indeed inhibit IL-36γ effects on keratinocytes, as well as corresponding *in vivo* work demonstrating pre-emptive administration of IL-36 antagonists could reduce disease severity of imiquimod-induced psoriasis and prevent the pathological phenotype [305].

Further highlighting the importance of inhibiting IL-36 signalling to control skin inflammation is the role of IL-36Ra in generalised pustular psoriasis (GPP), the most severe form of psoriasis. This can result from IL-36Ra gene mutation leading to a disease now known as Deficiency of IL-36Ra (DITRA). Individuals with this mutation generate biochemically instable IL-36Ra proteins with inefficient IL-36R receptor binding, resulting in excessive IL-36 signalling [306, 307]. This disease is characterised by acanthosis, hyperkeratosis and excessive myeloid infiltration of epidermal tissue due to unrestricted IL-36 signalling [293, 308]. This cutaneous neutrophilia is common to each form of psoriasis and has been reported to perpetuate chronic skin inflammation by excessive myeloid chemoattractant secretion [237, 309]. Moreover, increased concentrations of cutaneous myeloid chemoattractants can induce NETosis, a form neutrophil cell death resulting in the release of neutrophil chromatin coated in IL-36 cleaving proteases, further augmenting the inflammatory loop in psoriasis [237].

Many other skin conditions are driven by IL-36 cytokine overexpression such as atopic dermatitis, allergic dermatitis, folliculitis, pustual folliculitis and hidradenitis suppurativa (HS) [310-313]. Blistering autoimmune diseases such as bullous pemphigoid, pemphigus vulgaris and dermatitis herpetiformis have also been reported to be propagated by IL-36 overexpression [314]. IL-36 driven skin disorders can result in further autoimmune complications such as epicutaneous *S.aureus* infection resulting in hypersensitive lung disease which stems from IL-36α overexpression [315]. Furthering this, very recently a case has been reported of SARS -CoV-2 infection resulting in DITRA flare up, showing this IL-36 skin-lung inflammation can be bidirectional [316].

Imsidlomab, an anti-IL36R humanised mAb, is in phase 2 study for GPP and HS. Spesolimab, another anti-IL36R antibody, is in phase two trials for HS, AD and has recently reported for the findings in a GPP phase II trial. This trial showed Spesolimab treatment to result in a higher incidence of lesion clearance at 1 week than the placebo group, however, this was also associated with increased infections and systemic drug reactions [317]. Improvements of latent infections incidence to advance these trials may be achieved by earlier administration of IL-36R targeting antibodies as well as development of bi-specific antibodies to improve stabilisation of drugs within the intended anatomical milieu [318].

1.10.7.2. IL-36 in pulmonary disease

Extensive work has been completed to date on the role of IL-36 in multiple pulmonary pathologies, as well as the homeostatic functions of IL-36 in innate immunity to control infection, mechanical injury and epithelial barrier renewal.

IL-36 cytokines have been shown to be significantly upregulated in COPD patient tissues and sputum samples with this increased expression associated with pulmonary

neutrophilia and disease severity [270, 319]. Reflecting this, IL-36Ra expression is significantly decreased in COPD patients [320] whilst smoke exposure, the leading cause of COPD, increases IL-36 cytokine expression [270]. Moreover, smokers with and without COPD also express increased levels IL-36 cytokines. In agreement with this, IL-36R deficient mice are protected against tobacco smoke and influenza virus induced inflammation, with a significant reduction neutrophil driven inflammation. Neutrophils were identified as the primary source of IL-36 in these tissues, which, in combination with GM-CSF and Poly(I:C), could drive fibroblast and macrophage inflammation [321]. Given neutrophils and macrophages express IL-36 activating proteases, this work strongly suggests a propagating loop of IL-36 inflammation exists in COPD.

The pathological role of IL-36 signalling during and after viral infection has been elucidated with the recent rapid research advancements into respiratory viruses such as SARS-COV-2. Serum expression of IL-36α in COVID-19 patients has been shown to be associated with increased disease severity, whilst IL-38 expression was reported to be associated with shorter hospitalisation times [322]. Further implicating IL-36 in COVID pathogenesis, IL-36γ has been reported to contribute to COVID pathogenesis in a CASP11 mediated pathway [261]. IL-36γ was shown to be associated with pathogenesis of lung inflammation rather than viral clearance, given CASP11 gene deletion did not alter viral titre but did alter lung histopathology. Additionally, IL-36γ is induced in lung epithelial cells during SARS-COV-2 infection, although these authors did not determine if this is more effective at viral clearance or whether this contributed more to severe lung disease [262]. Further attesting to the post-infection pathology induced by IL-36, it was shown that IL-36R expression is dispensable for clearance of *Mycobacterium bovis* however, IL-36R deficient mice display a significantly less severe lung pathology to WT animals [266].

IL-36 has also been shown to play a role in several allergy associated pathologies. Serum IL-36Ra is reduced in asthma and administration of recombinant IL-36Ra resulted in alleviation of the associated lung pathology with a reduction in airway hypersensitivity and a significantly reduced immune infiltrate in lung tissue [323]. Moreover, another form of asthma, House Dust Mite-induced asthma, could be dampened by administration of IL-38 to mice. This resulted in significant alterations in the immune infiltrate including increase Treg populations in the lung, spleen and lymph nodes [324].

Many of these lung pathologies show significant neutrophil tissue infiltration. Given that neutrophils also contain the proteases necessary for IL-36 activation, it is evident that a pathological loop may exist with respect to IL-36 action in the lung, whereby lung damage upregulates expression of the IL-36R and IL-36 cytokines, resulting in the recruitment of neutrophils, and the subsequent enhancement of IL-36 activation, which then contributes to the pathological condition**.**

1.10.7.3. IL-36 in inflammatory bowel disease (IBD)

Similar to other barrier tissues, IL-36 signalling plays an important role in the innate and adaptive immune response in the intestines which, if dysregulated, can result in severe, chronic disease. It has been reported that IL-36 cytokine expression is increased in patient IBD colonic tissue, and indeed especially increased in ulcerative colitis patients [238, 272, 287, 289, 297]. These studies describe roles for IL-36α and IL-36γ primarily, although roles for IL-36β in intestinal epithelial cell proliferation have also been reported [238]. In order to further elucidate the role of IL-36 in IBD, several *in vitro* and *in vivo* studies have examined the effects of IL-36 signalling in pre-clinical models of intestinal inflammation.

Early in vitro work by two separate groups identified intestine-derived cell populations, including isolated colonic subepithelial myofibroblasts and colon adenocarcinoma cells, to be responsive to IL-36 cytokine signalling and that fibroblast stimulation could be synergistically enhanced by co-stimulation IL-17a or TNF-α [287, 288]. Furthering these findings, Russell *et al* reported that IL-36R deficient mice showed a significantly reduced intestinal pathology in a DSS-induced model of colitis. This was associated with a decrease in overall immune cell infiltrate as well a reduced proportion of neutrophils and macrophages infiltrating the colonic lamina propria. Moreover, IL-36R deficient mice once more have a reduced innate immune infiltrate in a *C. rodentium* model of colitis. These mice also showed an enhanced Th17 response and reduced Th1 responses, indicating that intestinal IL-36 signalling also regulates the adaptive immune response in IBD. In further agreement with this work, Scheibe *et al* have reported that IL-36α and IL-36γ were significantly upregulated in active human cases of IBD and in experimental models of colitis [238]. Paradoxically, IL-36R deficient mice exhibited increased intestinal disease following DSS-induced colitis, along with increased bacterial burden in the colonic mucosal wall. It was later shown that IL-36 signalling could contribute to intestinal wound healing by fibroblast activation and intestinal epithelial cell proliferation. These findings were complemented by other groups, showing IL-36R signalling to be beneficial for wound healing in acute DSS-induced colitis, with IL-22 signalling implicated as integral to this recovery process [272, 273].

In more long term models of intestinal inflammation, IL-36R deficient mice and mice administered with anti-IL-36R antibodies exhibit less severe colitis and fibrosis following DSS-induced or TNBS-induced colitis [289]. Reflecting these findings, an oxazolone model of ulcerative colitis showed significantly reduced inflammation in both IL-36R and IL-36γ

deficient mice, with this effect acting primarily through regulation of Treg-Th9 cell balance, giving way to uncontrolled inflammation [298]. Furthermore, two other individual chronic DSS-induced and oxazolone-induced models of colitis showed DITRA-like mice were highly susceptible to chronic inflammation and that this could be ameliorated by administration of an IL-36R blocking antibody. Similarly to the observations of Harusato *et al*, a T cell-driven model of colitis model showed IL-36R expression was required for the infiltration of Th cells into the colon which subsequently contributed to chronic intestinal inflammation [234].

IL-38 expression is increased in inactive UC patients and IL-36 cytokine expression is increased in active cases of UC, suggesting inhibition of IL-36R inhibition can enhance colitis resolution [325]. Furthermore, recombinant IL-38 expression can suppress intestinal inflammation DSS-induced model of colitis, likely to result from BMDM and macrophage suppression, two important sources of IL-36 cytokines [326]. IL-36 family members are differentially expressed in different phases of UC across multiple cell types, suggesting cells may temporally orchestrate colitis, which is reflective of the previous acute and chronic models [325]. Clinical trials are currently underway for patients with moderate sever disease ulcerative colitis to inhibit IL-36R signalling as an alternative to anti-TNFα therapy (NCT03482635).

1.10.7.4. IL-36 and fibrosis

IL-36 signalling has also been implicated across many tissues in driving pathological processes of fibrosis. It was reported that IL-38 expression is increased in ERK inhibitor induced lung injury and idiopathic pulmonary fibrosis, although this was likely to be expressed in resolution of lung inflammation. Very recently it has been reported that IL-36

cytokines (IL-36α and IL-36γ) are increased idiopathic pulmonary fibrosis and have their expression correlates with disease severity [327]. It has indeed been previously reported that IL-36γ can directly induce the fibroblasts to drive pathological pulmonary fibrosis [284]. Moreover, cystic fibrosis bronchial brushing samples have shown IL-36β to be upregulated with the reduction of IL-36Ra, suggesting IL-36 signalling does contribute to pulmonary fibrosis [328].

Intestinal fibrosis has shown to be driven by IL-36 cytokines also. Fibrostenotic Crohn's disease patients were found to have increased IL-36α, but not IL-36β or IL-36γ, in intestinal tissues which correlated with increases of activated myofibroblasts [289]. Moreover, this study showed multiple cellular sources of IL-36 cytokines in the gut involved in fibrosis such as myofibroblasts, intestinal fibroblasts, IECs and macrophage populations. RNAseq analysis has shown, by pathway enrichment analysis, that IL-36 stimulated fibroblasts increase gene expression in fibrosis-associated genes such as collagen IV, as well as proliferation and inflammation [289]. IL-36R neutralisation by monoclonal antibodies was shown to successfully impede colitis-induced fibrosis [289]. Furthermore, Weinstein et al reported IL-36γ expression to be associated with markers of fibrosis in CRC tissues [329]. IL-36 signalling can also drive renal tubulointerstitial fibrosis, with inhibition of IL-36 signalling/ NLRP3 activation capable of ameliorating this renal pathology [330].

Systemic sclerosis (SS) is an autoimmune connective tissue disease often resulting in multi-organ fibrosis followed by eventual organ failure in severe disease [331]. IL-36α expression in sera was elevated relative to healthy control samples, with neutrophil elastase was also elevated in these sera [332]. This warrants further investigations into SS given the extensive roles for IL-36 in both fibrotic and autoimmune disease.

1.10.7.5. IL-36 and other chronic inflammatory pathologies

In addition to barrier tissues and fibrotic disease, IL-36 cytokines have been shown to play a role in multiple other tissue pathologies associated with chronic inflammation. The role for IL-36 signalling in virus-induced chronic hepatitis is well established [333-335]. Inhibition of IL-36 signalling can protect against chronic inflammatory liver damage, however this requires tight regulation as complete loss of IL-36R signalling may also result in dysregulated proinflammatory T cell signalling without Treg inhibition, leading to hepatocyte necrosis [333, 335-338]. This delicate balance of liver injury vs recovery is highlighted in murine acetaminophen-induced liver injury, where increased IL-36γ and IL-36Ra expression could result in acute and chronic inflammation, respectively [339]. Other IL-36 associated chronic pathologies include chronic renal disease [291] , neurological disease [240, 340-344], obesity/diabetes [274, 345-349], cardiovascular disease [350-354], autoimmune diseases [238, 355-361], as well as arthritis and joint disorders [219, 285, 361-366]. This diversity of influence of IL-36 cytokines is also reflected in malignant tissue, with IL-36 cytokines reported to play a role in both tumour rejection and tumour progression across many of the sites where IL-36 chronic inflammation takes place.

1.10.8. IL-36 and cancer

As inflammation is well recognised as a hallmark of cancer, IL-36 signalling is being increasingly investigated in tumourigenesis and tumour eradication due to the pluripotent nature of these cytokines. However, there remain many unanswered questions on the role of IL-36 signalling in tumourigenesis, especially given the dichotomy of other IL-1 family members in malignancy [196]. Given the extensive role, as shown in this chapter, for IL-36 cytokines in both the innate and adaptive arms of the immune system, as well as being

important in bridging these, IL-36 signalling has become a highly investigated topic in cancer immunology. This work has shown both pro and anti-tumour effects, which will be discussed here.

1.10.8.1. Anti-tumorigenic effects of IL-36

The role of IL-36 signalling in tumourigenesis has been mostly reported to drive tumour rejection by enhancing the Type I immune response to increase anti-cancer cytotoxicity. IL-36 cytokine expression is decreased in multiple cancer tissues relative to normal paired tissues, with the loss of expression shown to correlate with poor clinical outcomes in Hepatocellular carcinoma, pancreatic adenocarcinoma, colon adenocarcinoma, melanoma, and epithelial ovarian cancer [233, 299, 367-371]. More relevant to this thesis, two separate studies reported reduced IL-36α expression in colon cancer to be associated with poorer patient outcomes, although decreased IL-36γ expression was associated with improved patient survival [369, 370].

In line with this, tumoral expression of IL-36R antagonists, such as IL-36Ra and IL-38, has been positively correlated with poorer patient outcomes. Weinstein *et al* reported increased IL-36Ra expression is also associated with increased expression of immune checkpoint genes such as PD-1, PD-L1 and CTLA4 in CRC tissue [239]. Moreover, retrospective analysis of microarray data reported IL-36Ra as a useful prognostic marker in bladder, breast, lung, colon and ovarian cancer [372]. Reflecting the influence reduced IL-36 signalling may play in cancer development, increased IL-38 expression is associated with multiple poor prognostic markers, including PD-L1 expression [373]. This was further reflected in a subsequent study by the same group reporting IL-38 expression to be an independent negative predictor of $CD8^+$ TIL infiltration in lung adenocarcinoma [374].

These expression studies therefore strongly implicate a protective role for IL-36 signalling in the prevention and reduction of cancer ultimately by augmentation of a sustained type I response to target cancer cells [367, 375].

Furthering these findings, several studies have investigated the mechanisms of IL-36-driven cancer cell rejection *in vitro* by culturing isolated immune cells and studying their behaviour in response to IL-36 stimulation. This work has identified IL-36 signalling to influence lymphocyte infiltration and activation during tumourigenesis. Pan *et al* reported IL-36α overexpressing HCC cells to increase $CD3^+$ and $CD8^+$ T cell chemotaxis, with this likely to benefit HCC outcomes [367]. Indeed it has been reported that IL-36β can directly drive $CD8^+$ T cell activation, expansion, and effector cytokine secretion [235]. This was further reported by Li *et al* who demonstrated IL-36β stimulation to directly activate $CD8^+$ T, resulting in increased IFN-γ and IL-2 secretion by downregulation of the miRNA let-7c-5p [233]. Similarly, IL-36γ can stimulate $CD8^+$ T cells, NK cells and γδ T cells synergistically with TCR signalling and/or IL-12 stimulation [299]. This co-stimulation has previously been described in the context of aerobic glycolysis, a process often lost in the TME due hypoxic conditions. Here it was described that IL-2 stimulated effector cells upregulate IL-36R expression and are then highly responsive to IL-36 stimulation, although this capacity was lost during anaerobic metabolism. These studies identified IL-36 as potent stimulant of lymphocytes.

In order to further elucidate these in vitro findings, many of the above studies and others have investigated the role of IL-36 signalling in pre-clinical models of cancer. One of the first groups to do this reported IL-36α-overexpressing HCC cells to contain increased TILs and thereby a reduced tumour burden in a subcutaneously injected mouse model of colon

cancer [367]. Shortly afterwards, another group reported IL-36 gene therapy (which cytokine was not specified by authors) to reduce the tumour burden in mouse models of fibrosarcoma [375]. This same group then later showed that IL-36β could synergistically stimulate $CD4^+$ T cells to produce IFN-γ and thereby enhance CTL cytotoxicity against melanoma cancer cells [232]. Indeed, intratumoural injection of IL-36β expressing adenoviral vectors increases infiltration of NK cells, γδ T cells and $CD8^+$ CTLs in a subcutaneous mouse model of pancreatic cancer [376].

Following these findings, Wang *et al* assessed the role of IL-36γ in tumour immunity [299]. Intra-dermally injected IL-36γ-overexpressing melanoma and breast cancer cells showed significantly lower tumour burdens and metastases in comparison to WT control cells in immune-competent mice. Moreover, these tumours contained increased numbers of anti-tumour TILs (including Tregs) as well as decreased MDSCs. This study also reported cancer cell expression of IL-36γ to enhance cancer vaccine efficacy and reduce tumour burden. The role of IL-36 signalling in the T cell response was then further highlighted by Tsurutani *et al*, whereby anti-CD134 and anti-CD-137 co-stimulation of mice bearing B16 melanoma tumours resulted in significant increases of IL-36γ and IL-36R expression as well as reductions in tumour burden [377]. Reflecting these T cell driven findings, an *in vivo* model of IL-36γ plasmid co-delivery in chemotherapy-containing micelles was shown to significantly enhance IFN production by $CD4^+$ and $CD8^+$ T cells whilst decreasing MDSC infiltration in subcutaneous tumours and lung metastases [378]. This same group later applied a similar approach to counteract Treg infiltration in tumours driven by IL-36γ [379]. Here, anti-CTLA4 antibodies effectively reduced Treg tumour composition whilst facilitating IL-36γ to augment effector cell function in the TME. Moreover, IL-36 expressing oncolytic viruses (OVs) show exceptionally enhanced capacities of tumour clearance in comparison

to OV controls. IL-36γ expression increased tumour-antigen specific $CD4^+$ and $CD8^+$ TILs, whilst reducing MDSC and M2 macrophage composition of tumours. Blocking of IL-36 signalling in the TME, by IL-38 overexpression from LLC cells, can inhibit $CD8^+$ effector cell infiltration in a subcutaneous mouse model of lung cancer. These studies collectively exemplify the potent role for IL-36 signalling in enhancing the adaptive anti-tumour immune response.

Further contributing to this anti-tumour response is the role described for IL-36 in the formation and sustenance of tertiary lymphoid structures, which facilitate the bridging of the innate and adaptive arms of immunity within the TME itself. This was first reported by Weinstein et al, where injection of Tbet expressing DCs into IL-36 deficient mice could not inhibit tumour growth, unlike WT mice [380]. This was also shown by co-administration of IL-36Ra, which resulted in the same susceptibility to tumour formation. Furthermore, it was then shown that intratumoural injection of IL-36γ overexpressing DCs could effectively reduce tumour burden with promotion of TLS formation within subcutaneous colon cancer cell tumours. Weinstein et al later reported that IL-36γ expression was associated with $CD4^+$ central memory T cell infiltrate and increased B cell density in TLS structures in colon cancer patient biopsies [329]. Reflecting this, recent work has shown intratumoural STING agonist administration results in tumour burden decreases as well as DC induction of TLS-associated genes, including IL-36 cytokines, in a subcutaneous model of melanoma [381]. These studies show IL-36 signalling can influence improved durable immune response to reduce tumour burdens via TLS formation in multiple models of cancer.

IL-36 signalling can also provide anti-tumorigenic functions in other capacities. IL-36α overexpression inhibits ovarian epithelial cell proliferation, migration and invasion *in vitro*

as well as inhibiting tumour formation in xenograft models of ovarian cancer in nude mice [371]. IL-36α can also reduce tumour growth by inhibiting VEGFA-mediated angiogenesis in nude mice subcutaneously injected with IL-36α overexpressing NSCLS cell lines [382]. Furthermore, IL-36 signalling drives many different immune populations toward a pro-tumorigenic phenotype, therefore it is expected more roles for IL-36 signalling in the TME will be come to light in the near future [236, 292, 296].

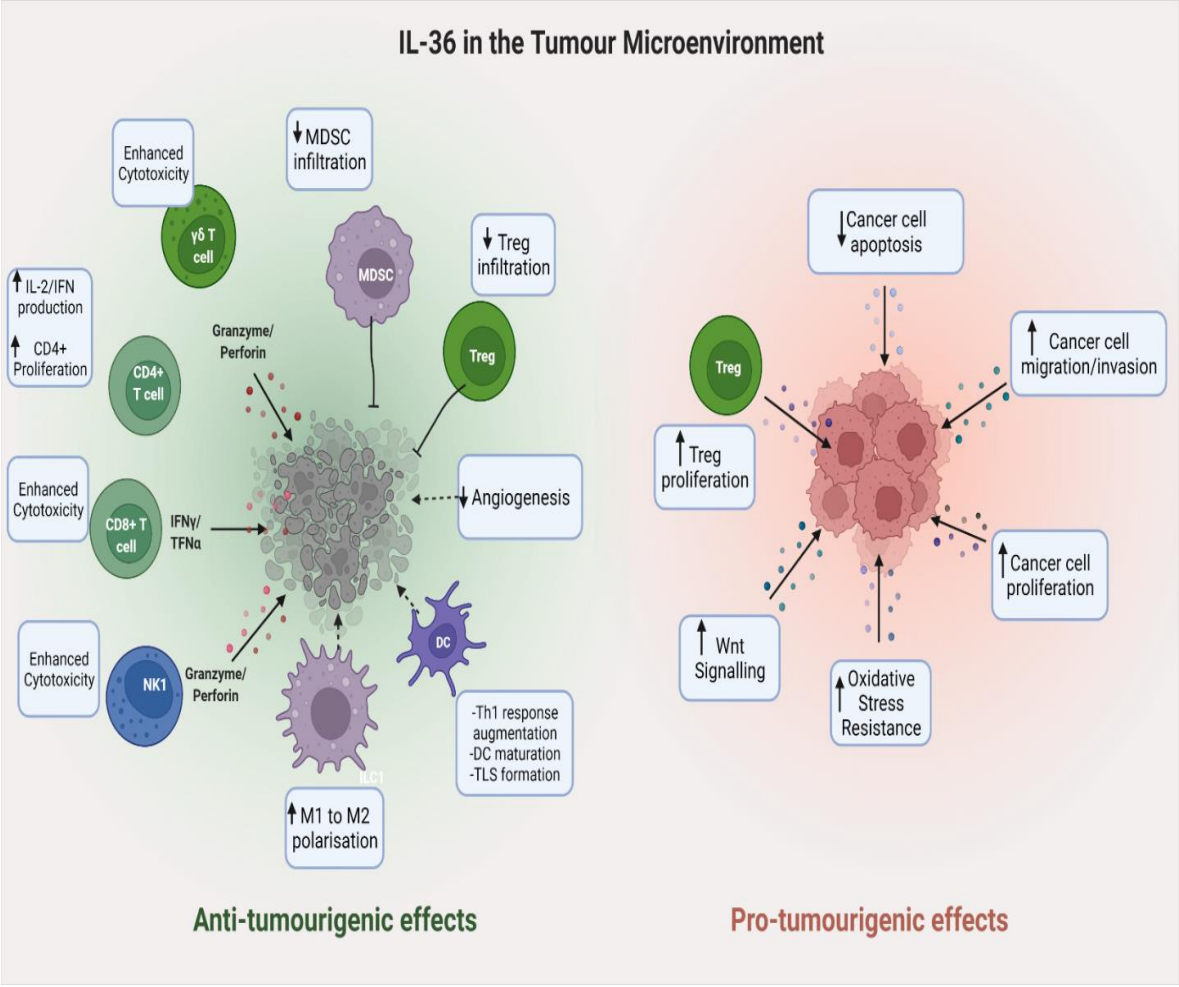


Figure 1.7: The role of IL-36 in the TME. IL-36 signalling plays a dichotomous role in cancer. IL-36 signalling has largely been reported to drive the Th1 immune response in the TME to augment tumour rejection through functional enhancement of cytotoxic effector cells such as NK cells, $CD8^+$ T cells and γδ T cells. In contrast to this, IL-36 signalling may also influence tumour cells themselves by induction of several intrinsic properties of cancers such as cellular proliferation, migration and invasion to promote tumour progression.

1.10.8.2. Pro-tumorigenic effects of IL-36

Multiple studies are now emerging showing IL-36 cytokines to also play a pro-tumorigenic role in certain contexts. Of note, the majority of these studies have only very recently been published. Indeed, at the time of starting this thesis, there was no reported pro-tumorigenic role for IL-36 cytokines in any cancer type.

Similar to identifying anti-tumorigenic roles for IL-36 cytokines, expression studies have also shown pro-tumorigenic associations for IL-36 signalling in different types of cancer. In colon cancer, increased IL-36γ expression is associated with significantly worse overall patient survival rates [369]. The accessory protein for IL-36R signalling, IL1RAcP, is increased in stomach adenocarcinoma (STAD) tissue relative to adjacent normal tissue, with this increased expression reported to be associated with poorer disease free survival and five year survival [383]. Another study analysing TCGA STAD data reported IL-36γ and IL-36R to both be increased in tumour tissue relative to adjacent normal tissue [243]. Furthermore, increased expression of IL-36γ and IL-36R mRNA was shown to be associated with poorer overall survival rates in this dataset, although IL-36Ra also showed the same trend. Increased IL-38 expression is associated with improved clinical outcomes in CRC and the expression of this endogenous IL-36R inhibitor is approximately 95% decreased in CRC tissue compared to normal adjacent tissue. Furthermore, this expression is strongly correlated with CRC differentiation, suggesting a protective role for this IL-36R inhibitory protein in intestinal barrier homeostasis [384]. These studies suggest roles for IL-36 signalling inhibition may indeed benefit patient outcomes.

Pre-clinical models of tumourigenesis have gained insight into how IL-36R can indeed disrupt tumour progression in multiple cancer types. Very recently published in preclinical models of lung cancer and colon cancer by the same group has highlighted this inhibitory effect. Extensive *in vivo* modelling showed that IL-36Ra and IL-36γ reciprocally regulate colon inflammation and tumourigenesis, with this work also indicating IL-36γ as an important driver of cancer cell proliferation *in vivo* [385]. This work used different models of carcinogenesis such as AOM/Vil-Cre; Trp53fl/fl mice also bearing IL-36γ or IL-36Ra gene knockout. Furthermore these authors also generated ApcMin/+ mice with gene knockout of IL-36γ or IL-36Ra. These studies showed that IL-36γ signalling significantly contributes to colon cancer carcinogenesis by directly stimulation colon cancer cells with alterations in cell–matrix adhesion pathways and the Wnt signalling pathway. Furthermore, this study showed that inhibition of IL-36γ proteolytic cleavage, or direct neutralisation by polyclonal anti-IL-36γ administration, could significantly reduce tumour burden. Similarly in a preclinical model of non-small cell lung cancer, this group showed IL-36γ and IL-36Ra to reciprocally regulate tumourigenesis by alterations in Glutathione (GSH) homeostasis and regulation of oxidative stress-induced cell death [386]. Moreover, IL-38 can inhibit colon cancer tumourigenesis by downregulation of ERK signalling to inhibit proliferation and migration of cancer cells, resulting in cancer cell apoptosis in pre-clinical murine models of colon cancer [387]. Two separate groups have recently reported IL-36 signalling to contribute to gastric cancer [243, 383]. Gastric cancer cell lines were shown to be responsive to IL-36γ stimulation by induction of intrinsic cancer properties including cellular migration, invasion and proliferation *in vitro* [243]. Furthermore, knockdown of the IL-1RAcP, can inhibit stomach carcinoma tumourigenesis both *in vitro* and *in vivo* by inhibition of cancer cell proliferation, migration, invasion, although this likely inhibited

other IL-1 family member signalling pathways [383]. These *in vitro* and *in vivo* studies present IL-36 signalling as having a dichotomous role in cancer, although there is clearly currently a far better understanding of the anti-tumourigenic role of this family of cytokines.

1.11. Aims

Therefore the aims of this study were to;

A. Characterise IL-36 family member expression and signalling in colon cancer cell lines.
B. To compare induction of inhibition of IL-36R signalling of tumours models *in vivo* by exogenous IL-36 receptor agonists/antagonist administration and by gene editing.
C. To investigate how IL-36 cytokines may influence interplay between colon cancer cells and the innate immune cells, macrophages and neutrophils.
D. To use large patient cohort transcriptomic datasets to gain further insights into the dichotomous role of IL-36 signalling in colon cancer.

Chapter 2

Materials and Methods

Material and Methods

2.1. Materials

All reagents were stored and prepared according to the manufacturer's guidelines.

2.1.1. Cell lines and tissue

CT26, CMT-93, HT29, HCT116, SW480, SW620, THP-1, HL-60 and NK92 cells were obtained from the American Type Culture Collection (ATCC) (MD, USA).

2.1.2. Mice

Six week old female Balb/C mice were obtained from Envigo UK (Bicester, U.K.) and maintained in the Biological Services Unit animal facility of University College Cork. Standard housing and environmental conditions were maintained (temperature 21°C, 12 hours light and 12 hours darkness with 50% humidity). Animals were fed a standard pellet diet and water *ad libitum*. Animal husbandry and experimental procedures were approved by the University College Cork Animal Experimentation Ethics Committee (AEEC). Prior to initiation of experiments, mice were afforded an adaptation period of at least 7 days.

2.1.3. Patient Study populations

The study protocol, including all procedures and study populations has been previously described [388]. In brief, the study was approved by the University College Cork Clinical Research Ethics Committee of the Cork Teaching Hospitals (ECM (3) P 3 September 2013). For patient cohort 1, 24 fresh samples of human colon cancer and paired normal colon tissues were collected in RNAlater (Sigma Aldrich) and stored at −20°C until further processing. For patient cohort 2, 66 samples were fixed in formalin and embedded in

paraffin wax for subsequent immunohistochemistry (IHC) analyses. All samples were obtained during surgery at Mercy University Hospital, Cork following informed consent. Details on patient demographics are outlined in Table 3.1.

2.1.4. Reagents/Materials

Reagent/Material	Catalogue Number	Manufacturer
1 kb Plus DNA Ladder	N3200	New England Biolabs, U.S.A.
100x DNAse I	DN25	Merck Millipore, U.S.A.
20x collagenase II	C6885	Merck Millipore, U.S.A.
Bovine Serum Albumin (BSA)	A9418	Sigma-Aldrich, U.S.A.
CFSE cell trace dye	C34554	Thermo Fisher Scientific, U.S.A.
Crystal Violet Dye	C3886	Sigma-Aldrich, U.S.A.
DEPC treated water	95284	Sigma-Aldrich, U.S.A.
Dimethyl sulphoxide (DMSO)	D2650	Sigma Aldrich, U.S.A.
Dulbecco's Modified Eagle Medium	D5796	Sigma Aldrich, U.S.A.
Foetal Bovine Serum (FBS)	F7524	Sigma Aldrich, U.S.A.
Ficoll Paque Plus	17-1440-02	Cytiva, U.S.A.
Fluoromount™ Aqueous Mounting Medium	F4680	Sigma-Aldrich, U.S.A.
Hematoxylin Solution, Mayer's	MHS1	Sigma-Aldrich, U.S.A.
Human recombinant CXCL1	574402	Biolegend, U.S.A.
Human recombinant CXCL2	582002	Biolegend, U.S.A.
Human recombinant CXCL5	573406	Biolegend, U.S.A.
Human recombinant IL-13	571102	Biolegend, U.S.A.
Human recombinant IL-1β	rcyec-hil1b	Invivogen, U.S.A
Human recombinant IL-2	130-097-744	Miltenyi Biotech, Germany
Human recombinant IL-36α	551604	Biolegend, U.S.A.
Human recombinant IL-36β	761104	Biolegend, U.S.A.
Human recombinant IL-36γ	711602	Biolegend, U.S.A.
Human recombinant IL-36Ra	760904	Biolegend, U.S.A.
Human recombinant IL-4	574002	Biolegend, U.S.A.
Human recombinant IL-8	574202	Biolegend, U.S.A.
Immobilon Western Chemiluminescent HRP substrate	WBKLS0500	Merck Millipore, U.S.A.
ImmunoHistoMount	sc-45086	Santa Cruz Biotechnology, Inc., U.S.A.
Lipofectamine 3000	L3000001	Thermo Fisher Scientific, U.S.A.
MACS® Tissue Storage Solution buffer	130-100-008	Miltenyi Biotech, Germany
MagicMark™ XP Western Protein Standard	LC5602	Thermo Fisher Scientific, U.S.A.

Matrigel® Basement Membrane Matrix	356234	Corning, U.S.A.
Mem-PERTM Plus® Membrane Protein Extraction Kit	89842	Thermo Fisher Scientific, U.S.A.
Micrococcal Nuclease (MNase)	88216	Thermo Fisher Scientific, U.S.A.
Mouse recombinant IL-1β	211-11B	Peprotech, U.S.A.
Mouse recombinant IL-36α	555904	Biolegend, U.S.A.
Mouse recombinant IL-36β	554504	Biolegend, U.S.A.
Mouse recombinant IL-36γ	552804	Biolegend, U.S.A.
Mouse recombinant IL-36Ra	760804 /2714-ML-025/CF	Biolegend, U.S.A./R & D Systems, U.S.A.
Myelocult H5100	P05150	StemCell Technologies, Canada
Normal Goat serum	X0907	Agilent Technologies, U.S.A.
OneComp eBeads™ Compensation Beads	01-1111-41	Thermo Fisher Scientific, U.S.A.
Opti-mem I	31985062	Thermo Fisher Scientific, U.S.A.
PD90859	P215	Sigma-Aldrich, U.S.A.
Penicillin/Streptomycin	P4333	Sigma Aldrich, U.S.A.
Phorbol 12-myristate 13-acetate (PMA)	P1585	Sigma-Aldrich, U.S.A.
Phosphatase Inhibitor Cocktail (100X)	5870	Cell signalling technology, U.S.A
Phosphate Buffered Saline	D8662	Sigma Aldrich, U.S.A.
Phosphate Buffered Saline (without $CaCl_2$ and $MgCl_2$)	D8537	Sigma Aldrich, U.S.A.
Poly-L-lysine solution	P4832	Sigma Aldrich, U.S.A.
Ponceau S solution	P7170	Sigma-Aldrich, U.S.A.
Prostaglandin E_2	P0409	Sigma-Aldrich, U.S.A.
Protease inhibitor cocktail I	539131	Merck Millipore, U.S.A.
Puromycin	P8833	Sigma-Aldrich, U.S.A.
Recombinant human IFN-γ	rcyec-hifng	Invivogen, U.S.A
Red blood cell lysis buffer 10X	420301	Biolegend, U.S.A.
Reparixin	SML2655	Merck Millipore, U.S.A.
RNA later	R0901	Sigma Aldrich, U.S.A.
RPMI	R8758	Sigma Aldrich, U.S.A.
RPMI Phenol (without phenol red and sodium bicarbonate)	R8755	Sigma Aldrich, U.S.A.
Sidestep Lysis Buffer	400900	Agilent Technologies, U.S.A.
SYBR™ Safe DNA Gel Stain	S33102	Sigma-Aldrich, U.S.A.
SYTOX™ Green Nucleic Acid Stain	S7020	Thermo Fisher Scientific, U.S.A.
Trypan Blue	T8154	Sigma-Aldrich, U.S.A.
Trypsin-EDTA solution	T4049	Sigma Aldrich, U.S.A.
Ultrapure flagellin	tlrl-epstfla-5	Invivogen, U.S.A
Ultra-pure LPS	tlrl-pb5lps	Invivogen, U.S.A
Wortmannin	W1628	Sigma-Aldrich, U.S.A.

2.2. Methods

2.2.1. *In vitro* methods

2.2.1.1. Cell line maintenance

HT29, SW480, SW620, CT26 and CMT93 monoculture cells were cultured in 75cm^2 flasks at 37°C in 5% CO_2 in DMEM (Sigma Aldrich, U.S.A.) supplemented with 10% FBS (Sigma Aldrich, U.S.A.) and 10,000 units/ml penicillin and 10mg/ml streptomycin. Cells were routinely grown to 70-80% confluence before being passaged three times per week. Passaging involved washing the cells with 10mL pre-warmed PBS (Ca^{2+} and $MgCl^{2+}$ free) and subsequent incubation at 37°C with 3mL of Trypsin-EDTA (Sigma Aldrich, U.S.A.) to detach cells from the flask wall. Trypsin was inactivated by addition of pre-warmed media to a final volume of 10mL. Suspension cells were treated similarly but grown in RPMI media (Sigma Aldrich, U.S.A.) supplemented with 10% Heat-inactivated FBS, 10,000 units/ml penicillin and 10mg/ml streptomycin in upright 25cm^2 flasks at 37°C in 5% CO_2. Passaging involved centrifugation at 300g for 5 minutes, followed by 1:10 dilution of cells in fresh media. NK92 cells were cultured as per ATCC guidelines in MyeloCult™ H5100 (StemCell Technologies cat # 05150) containing 1 vial of IL-2 IS (10 µg), premium grade (Miltenyi cat # 130-097-744). Co-culture of cells was completed using RPMI media supplemented with 10% Heat-inactivated FBS, 10,000 units/ml penicillin and 10mg/ml streptomycin. Heat-inactivation of FBS was achieved by thawing of frozen serum in a 56°C water bath immersed to fully cover the level of serum, for 30 minutes. Serum bottles were swirled regularly to ensure distribution of heating to prevent protein coagulation and evenly inactivate complement in serum.

2.2.1.2. THP-1 differentiation

2 x 10^5/mL THP-1 cells were cultured in the above mentioned RPMI media in 24 well plate format with 5 ng/mL PMA for 48 hours in a final volume of 500µL. Cells were isolated from media and cells were polarised to M1 or M2-like macrophage phenotypes for 24 hours as follows; LPS (200 ng/mL) and IFN-γ (20 ng/mL)) were added to RPMI for M1-like THP-1 differentiation, whilst IL-4 (20 ng/mL)) and IL-13 (20 ng/mL) were added to RPMI for M2-like THP-1 differentiation (Figure 5.1).

2.2.1.3. HL-60 differentiation

5 x 10^5/mL HL-60 cells were cultured in the above mentioned RPMI media in 24 well plate format in a final volume of 500 µL. Cells were cultured with differing DMSO concentrations (%volume/volume), as indicated in figure legends (Fig 5.9), for a total of 5 days. Media was replaced on day 3 of differentiation.

2.2.1.4. Polymorphonuclear cell isolation from whole blood

3 mL of whole blood was taken from healthy donors and collected in EDTA-vacuette (Fisher Scientific, U.S.A) tubes. 2 mL of this blood was then added to 2mL of PBS (Ca^{2+} and $MgCl^{2+}$ free) to a final volume of 4 mL. 3 mL of Ficoll Paque Plus (Cytiva, U.S.A.) was added to a 15 mL conical tube. Diluted whole blood was gently added to this tube on top of the ficoll layer. This tube was then centrifuged at 400g for 30 minutes at room temperature with centrifuge breaks deactivated. The upper layers of plasma, mononuclear cells and ficoll paque media were decanted with the thin white polymorphonuclear cell (PMN) layer then exposed above the RBC sediment. This PMN layer was collected and resuspended in red blood cell (RBC) lysis buffer (Biolegend, CA, USA) for 5 minutes at room temperature to a

final volume of 10 mL in sterile deionized water. The sample was then centrifuged, checked for RBC contamination (previous step was repeated if so), and resuspend in media. Cells were then counted for cell viability and further analysis was completed.

2.2.1.5. Cell viability

10 µL of cell suspension was added to 90 µL of Trypan Blue (Sigma Aldrich, U.S.A.) and mixed by pipetting. 20 µL was transferred to a haemocytometer. The stained cells were counted, in addition to the total number of cells and percentage viability was assessed (number of live cells/total number of cells).

2.2.1.6. Mycoplasma Testing

100 µl of cell culture supernatant was removed from a dense culture (80-100% confluent) into a 1.5 mL tube. Samples were heated for 5 minutes at 95°C and centrifuged for 2 minutes at 14'000 RPM. The following PCR reaction was then completed per sample; 2.5 µL 10X PCR buffer, 2 µL 25 mM $MgCl^{2+}$, 1 µL 10 mM dNTPs, 1 µL Forward primer mix (10 uM of each), 1 µL Reverse primer mix (10 µM each), 2 µL heated cell supernatant, 0.2 µL Taq polymerase, 15.2 µL DPEC treated water. The total reaction volume was 25 µL.

A PCR programme was then used consisting of the following steps; one cycle initial denaturation (95°C, 2 minutes), 5 cycle low specificity step (94°C for 30s, 50°C for 30s, 72°c for 35s), 30 cycles higher specificity (94°C for 15s, 56°C for 15s, 72°C for 30s) and then samples were cooled for storage. Samples were then analysed on a 1.5% agarose gel by electrophoresis and bands of approx. 500bp in length indicated mycoplasma contaminated samples. Positive controls used were supernatant frozen from previously confirmed mycoplasma-contaminated cell lines.

2.2.1.7. Western Blotting

2.2.1.7.1. Preparation of whole cell lysates and cell lysate fractions

Cells were seeded at a density of $1x10^5$ cells/mL into 6 well plates and then cultured until 70% confluent. Cells were serum-starved for 24 hours prior to stimulation in DMEM or RPMI media supplemented with 0.5% FBS and antibiotics as described previously (section 2.2.1.1). Cells were then stimulated as specified in the figure legends. Following treatment, cells were washed with ice-cold PBS and then lysed on ice for 1 hr with 100 µL of lysis buffer containing 50 mM Tris-HCl (pH 8.0), supplemented with 1x protease inhibitor cocktail (Merck Millipore) and 1x phosphatase inhibitor (Merck Millipore) when applicable. Cells were then scraped, lysates were passed through a 20G needle (Becton, Dickinson, U.S.A.) and transferred to a 1.5 mL Eppendorf. Lysates were centrifuged at 12'000 RPM at 4°C for 15 min. The resulting pellet containing cellular debris was discarded and lysate was stored at -20°C. Samples being examined for expression of transmembrane receptor proteins were lysed using Mem-PERTM Plus® Membrane Protein Extraction Kit (Thermo Scientific, U.S.A.) and processed as per manufacturer's guidelines. Membrane fractions were used for subsequent immunoblotting.

2.2.1.7.2. Quantitation of total protein concentration

Protein standards were prepared using BSA (Thermo Fisher Scientific, U.S.A.) (0, 2.5, 5, 7.5, 10, 15, 20 µg/ml) and added to a 96-well plate. 2 µL of each sample was added to the plate followed by 38 µL of distilled H20. Both standards and samples were analysed in triplicate. 160 µL of BCA Protein Assay Reagent (Thermo Fisher Scientific, U.S.A.) was added to each well, the plate agitated and then left at 37°C for 30 min before the absorbance was read at 560 nm on the Glomax multi-detection system (Promega, WI, USA). A standard curve of

BSA concentration was then used to determine protein concentration of cell lysates (µg/mL).

2.2.1.7.3. SDS-PAGE and Western blotting

The appropriate amount of cell supernatant containing 50µg of protein was mixed with 4 x Polyacrylamide Gel Electrophoresis (PAGE) loading buffer (125 mM Tris, 2% SDS, 20% Glycerol, and 20 mM DTT) and lysis buffer containing 50 mM TrisHCl (pH 8.0) to a final volume of 20 µL. Lysates were boiled for 5 minutes before being loaded onto a separating and stacking SDS gel. A 19-180 kilo Dalton (kDa) molecular weight marker (Sigma Aldrich, U.S.A.) was run alongside the samples. Proteins were separated by electrophoresis at 50 mA. Proteins were then transferred overnight onto an Immobilon–P® polyvinylidene diflouride membrane (Merck Millipore) at 40V and 4°C using a wet transfer method. Once overnight transfer was completed, membranes were stained with Ponceau (Sigma Aldrich, U.S.A.) to ensure even transfer of proteins. Ponceau staining was used to control loading for IL-36R protein immunoblotting.

2.2.1.7.4. Immunoblotting

Ponceau was removed with distilled water and Tris buffered Saline (TBS)-0.1% TWEEN (TBST) before membranes were blocked using 5 % (weight/volume) milk powder or BSA in TBST (henceforth referred to as blocking buffer) for 1 hr with rocking. Membranes were washed for 5 mins in TBST before the appropriate primary antibody was added diluted in blocking buffer and stored overnight at 4°C. Membranes were washed 3 times with TBST for 5 mins at a time, and then incubated for 1 hr at room temperature with the appropriate secondary antibody. Finally, membranes were washed 3 times with TBST for 5 mins at a

time before detection using an Immobilon Western Chemiluminescent HRP substrate (Merck Millipore) according to manufacturer's instructions. Protein bands were analysed using ImageJ (National Institutes of Health, Bethesda, MA, USA, http://imagej.nih.gov/ij/, 1997- 2012.). Changes in protein abundance were determined after normalising the band intensity of each lane to that of β-actin or Ponceau staining when detecting IL-36R protein expression. Antibodies were used as indicated in Appendix Table 1.

2.2.1.8. Quantitative real-time polymerase chain reaction (qRT-PCR)

2.2.1.8.1. RNA extraction

RNA was isolated from cultured cells using the GenElute total mRNA kit (Sigma Aldrich, U.S.A.) as per manufacturer's guidelines. Cells were lysed in 250 µL of RNA lysis buffer, washed, centrifuged, eluted and extracted RNA was then stored -80^{o}C. RNA concentration (ng/uL) and quality (260/280nm absorbance ratio) was determined spectrophotometrically using the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, U.S.A.).

2.2.1.8.2. cDNA preparation

500-1000 ng of total RNA was used as the template for cDNA synthesis using a commercially available cDNA synthesis kit according to the manufacturer's instructions (Bioline, UK). RNA concentration was normalised and diluted with DEPC-treated water per run. This was added to 1 µL of anchored oligo (dT) primers, and 1 µL of 10 mM dNTP and heated to 65°C for 10 minutes. Following 5 minutes on ice, 4 µL of 5x Reverse Transcriptase Buffer, 1 µL of RNase inhibitor, 0.25 µL of Reverse Transcriptase, and 2.75 µL of DEPC treated water was added to each sample before incubation at 37°C for 30 mins. The reaction was terminated

by a final incubation at 70°C for 15 mins followed by cDNA dilution with DEPC treated water (1:1). Samples were stored at -20°C.

2.2.1.8.3. qRT-PCR

2µl of diluted cDNA template was amplified on the LightCycler® 480 (Roche) in a 10µl total reaction volume. The reaction mix contained a final concentration of 400nM of each primer, 200nM of the appropriate Universal Probe Library probe (Roche) and 1x Sensifast Probe Lo-Rox kit (Bioline). Conditions for the LightCycler® 480 were 95°C for 10 mins; 45 cycles of 95°C for 10 seconds, 60°C for 30 seconds, followed by a final cooling cycle at 40°c for 30 seconds. All results were analysed using the ΔΔct method and the gene of interest was normalized to the corresponding housekeeping gene results. Data were expressed as fold induction relative to untreated cells. In the case of human tumours versus adjacent tissue, a pool was generated of all samples and values were normalised to this pool. Data were expressed as fold induction relative to the pool. Parameters investigated were completed in technical duplicates, with a minimum of three biological replicates completed.

2.2.1.9. Enzyme Linked Immunoassay (ELISA)

Cells were seeded at a density of 1 x 10^6/mL in 6 well plates and allowed adhere for 24 hours. Media was then changed and stimulants were added, as outlined per figure legend, for 24 hours. Cell culture supernatant was removed, centrifuged at 10'000RPM for 10 minutes with supernatant then decanted into a fresh tube and stored at -80°C. Protein concentration was quantified according to the manufacturer's instructions. CXCL1 (human and murine), CCL2 (murine) and CCL20 (human and murine) (R&D Systems, Minneapolis,

Minnesota, USA) and IL-1β (human) (Immunotools, Germany) were all quantified with similar approaches as per manufacture's guidelines. Parameters investigated were completed in technical triplicates, with a minimum of three biological replicates completed.

2.2.1.10. Arginase Activity Assay

Cell lysates of differentiated THP-1 cells, stimulated as indicated in figure legends, were prepared as per manufacturer guidelines (Assay Genie, Ireland). Arginase activity was then determined colorimetrically using the ChromaDazzle Arginase Activity Assay Kit (Assay Genie, Ireland) as per kit protocol. Parameters investigated were completed in technical duplicates, with a minimum of three biological replicates completed.

2.2.1.11. Resazurin Assay

Resazurin powder (Sigma Aldrich, U.S.A.) was hydrated with PBS under sterile conditions to make a 10X stock solution (440µM). The solution was filter sterilized using a 0.22 µm filter and stored in a foil-covered container at 4°C until use. Cell seeding density was determined per cell line for 96-well plate format. Cells were serum starved in 0.5% FCS-supplemented media for 24 hours prior to stimulation. Media was aspirated from the wells of 96-well plate and cells were stimulated in 0.5% FCS-supplemented media as indicated in figure legends. Media was aspirated once more and cells were washed once with PBS. 250 µL of pre-warmed media containing diluted Resazurin solution (44µM) was added to each well before measuring fluorescence at 535-590nm on a GenIOS fluorometer at several different time points. Parameters investigated were completed in technical triplicates, with a minimum of three biological replicates completed.

2.2.1.12. Bromodeoxyuridine (BrdU) Assay

BrdU incorporation assay was completed using a Cell Proliferation ELISA System (BrdU Cell Proliferation Assay Kit #6813, Cell Signalling Technology, MA, U.S.A.) according to the manufacturer's instructions. Optimally determined cell density of cells per well were seeded in a 96-well plate, and serum starved overnight in 0.5% FCS-supplemented media. Cells were treated with ligands, as outlined in figure legends, for 24 hours. Following incubation the cells were treated with 10µM of BrdU labelling solution and incubated in at 37°C for 24 hours. The cells were fixed and denatured in one step using the FixDenat solution provided in the kit. An anti-BrdU-POD antibody was then added for 90 mins at room temperature, diluted as per protocol guidelines. Following three wash steps with PBS, substrate solution was added for 30 mins at room temperature. Stop solution, to inhibit substrate-enzyme reduction by pH alteration, was then added and the BrdU incorporation was measured at 450nm using the Glomax multi-detection system (Promega, U.S.A.). Parameters investigated were completed in technical triplicates, with a minimum of three biological replicates completed.

2.2.1.13. Transwell Migration assay

Cells were seeded at an optimally determined density in the top chambers of 8µm-pore Transwell inserts (Merck Millipore) in media containing 0.5% FBS in a final volume of 250 µL. Ligands were added to the upper well of the chamber as indicated in figure legends. 700 µL DMEM with 10% FBS was added to the lower chamber to serve as the chemoattractant. After 24 hrs of incubation, cells in the top chamber were removed, and cells at the bottom of the filter were fixed in 100% ice cold methanol for 5 minutes and

stained with 0.5% crystal violet solution (CV) (weight/volume) for 20 min. Cells were washed with PBS and the CV was eluted from the membrane using 10% acetic acid. CV absorbance was measured at 560 nm using the Glomax multi-detection system (Promega). Alternatively, membranes were excised after fixation and staining, then mounted. Cells were counted per high power magnification with averages of 5 random fields taken. Duplicate wells were used per condition in each experiment. A minimum of three independent experiments for each cell line were completed. The percentage change in migration was assessed relative to migration of untreated cells towards DMEM with 10% FBS.

2.2.1.14. Wound Scratch assay

Cell migration was determined by wound scratch assay using IBIDI culture inserts (IBIDI GmbH). 70 µl of a 4×10^5 cells/mL solution were added into the two reservoirs of the same insert and incubated at 37°C/5% CO^2 overnight. The insert was gently removed creating a gap of ~500 µm and images of cell migration were taken daily until the 500 µm gap had been filled. Media was changed every 72 hours with fresh stimulant added as indicated in figure legends. Duplicate wells were used per condition in each experiment. A minimum of three independent experiments for each cell line was performed.

2.2.1.15. Transwell Invasion Assay

The Boyden chamber inserts were coated with 100µL of Matrigel® (Corning) diluted in FBS-free media to a final concentration of 300 µg/ml. The Matrigel® was incubated at 37°C for 45 minutes to set. Once the Matrigel® had set, CT26 and HT29 cells were then seeded in media containing 0.5% FCS ± indicated ligands on top of the Matrigel® at the optimally

determined cell density into the upper chamber of an 8 µm pore size Transwell insert at a final volume of 250 µL. 750 µL of media containing 10% FCS was added to the bottom chamber of the transwell and served as the chemoattractant. Following 24h incubation at 37^{o}C, the insert was washed twice in PBS and fixed in ice cold methanol for 5 mins. Inserts were then stained in 0.1% crystal violet in 0.1M borate pH 9.0 and 2% ethanol for 20 mins. Inserts were then washed twice in PBS and membranes removed from the transwell and placed into a 24 well plate containing 200µl of acetic acid to elute the stain. Absorbance was read at 560nm on the Glomax plate reader (Promega). Alternatively, membranes were excised after fixation and staining, then mounted and cells were counted per high power magnification with averages of 5 random fields taken. Duplicate wells were used per condition in each experiment. A minimum of three independent experiments for each cell line was performed. The percentage change in invasion was assessed relative to invasion of untreated cells towards DMEM supplemented with 10% FBS.

2.2.1.16. 3D Spheroid Generation

3D spheroid generation was completed using CT26 cells (2.5 x 10^^{3}/mL) or HT29 cells (5 x 10^^{3}/mL). 200 µL of these cell suspensions were added to agar-coated wells (60 µL, 1% agarose (Sigma Aldrich, U.S.A.)) of flat-bottom 96-well plates and subsequently centrifuged for 20 minutes at 300g and incubated in a humidified incubator at 37°C. Cells were allowed to aggregate for 72 hours and only wells containing one single spheroid were then proceeded with for experimentation. 100 µL of media was then removed and replaced with 100 µL of fresh media with or without stimulant, as indicated, every 72 hours. Images were obtained using an inverted microscope with an Olympus EP50 digital camera (Olympus Corporation, Japan). Images were quantified using the SpheroidJ plugin in the ImageJ

software suite (National Institutes of Health, Bethesda, MD; http://imagej.nih.gov/ij/, 1997-2012). Six technical replicates were completed per parameter measured, with a minimum of three biological replicates completed. For RNA extraction of spheroids, three spheroid replicates were combined and subsequently lysed for RNA extraction as described previously for suspension cells.

2.2.1.17. 3D spheroid co-culture assays – Supernatant

HT29 spheroids were cultured as described above. On day three of spheroid formation, supernatant derived from THP-1 differentiation was centrifuged for ten minutes at 14'000 RPM. 100µL of spheroid supernatant was removed and replaced with 100µL of THP-1 supernatant, where indicated. This was repeated every three days of spheroid culture. Six technical replicates were completed per parameter measured, with a minimum of three biological replicates completed.

2.2.1.18. 3D spheroid co-culture assays – Cell co-culture

Adherent THP-1 differentiated cells (cell type was added as indicated) were gently scraped, counted and added in equal density to a HT29 cell suspension (750 cells of each cell type). 1500 cells of this suspension was then added to wells of a 96-well plate as previously described for mono-culture spheroid generation and allowed to form spheroids. Six technical replicates were completed per parameter measured, with a minimum of three biological replicates completed.

2.2.1.19. NETosis detection by fluorescence multi-well plate reader

5 x 10^4 differentiated HL-60 cells were cultured for one hour in phenol-red free RPMI to allow cells to adhere to wells of a flat-bottom 96 well plate. Indicated stimulants were then

added to cells for 4 hours in wells of a flat bottom 96 well plate. Centrifuged cancer cell line supernatant stimulation was added at 1:1 ratio of RPMI at the beginning of cell stimulation. Cells were then cultured with 1 unit of MNAse to remove extracellular traps from cells for 20 minutes. MNase was inactivated by addition of 5mM EDTA. Supernatant was transferred from wells to individual tubes and centrifuged for 10 minutes at 10'000 RPM. NET-containing supernatant was then stained with 167nM Sytox green solution and added to Nunc™ MicroWell™ 96-Well, Nunclon Delta-Treated, Flat-Bottom Microplate (Thermo Fisher Scientific, U.S.A.). Wells were then read using an FLx800 fluorescence plate reader (Agilent Technologies, U.S.A.) at 490nm. Samples were all completed in duplicate, with a minimum of 3 biological replicates completed.

2.2.1.20. NETosis detection by Fluorescence Microscopy

5 x 10^4 differentiated HL-60 cells or isolated PMNs were seeded onto Poly-L-Lysine-treated coverslip in wells of a 24 well plate. Cells were stimulated as described above. After 4 hours of incubation with NETosis-inducing substances, cells were fixed using 2% PFA. Cells were then stained using 167nM Sytox green (Thermo Fisher Scientific, U.S.A.) and images were subsequently taken using a fluorescence microscope. Assays were all completed in duplicate, with a minimum of 3 biological replicates completed.

2.2.1.21. NETosis detection by FACS

Isolated PMNS were seeded and stimulated as described above, with stimuli indicated per figure legend. After 4 hours, cells were fixed with 2% PFA and stained with 5nM Sytox green. Cells were resuspended in FACS buffer (2% FBS in PBS) and analysed by FACS using a Guava 8HT Flow cytometer (Merck, Germany). Incyte Software (Merck, Germany) was

used to analyse collected data. Up to 5000 events were collected for each sample. Samples were all performed in duplicate, with a minimum of 3 biological replicates completed.

2.2.1.22. Immunohistochemical Analysis

Formalin-fixed, paraffin-embedded tissues were deparaffinized in xylene and rehydrated prior to analysis. Antigen retrieval was performed by microwave irradiation in 0.01 M citrate buffer, pH 6.0. Slides were washed twice for 5 min in a wash buffer containing 50 mM Tris-Cl, pH 7.6; 50 mM NaCl; 0.001% saponin. Endogenous peroxidase was quenched with 3.0% hydrogen peroxide in methanol for 10 min. Slides were washed as before, except that the wash buffer for this and all subsequent steps included 1% normal goat serum. Nonspecific binding was blocked using 5% normal goat serum in wash buffer for 1 h. Sections were incubated overnight at 4°C with primary Ab or normal rabbit IgG (sc-2027). Antibody binding was localized using a biotinylated secondary antibody contained within the VECTASTAIN Elite ABC detection kit (Vector Laboratories, Burlingame, CA). Slides were counterstained with hematoxylin. A parallel negative control was also performed, using rabbit IgG instead of the primary Ab. Details of antibodies used are outlined in Appendix Table 1.

2.2.1.23. CT26 puromycin kill curve

1 x 10^6/mL CT26 cells were seeded into wells of a 24 well plate with concentrations of puromycin as indicated in figure legends in a final volume of 500µL. Cell viability was assessed as previously outlined over the indicated days.

2.2.1.24. CT26 IL-36R KO generation/control cell line generation.

2.2.1.24.1. Transfection of CT26 cells

Cells were transfected using an adjusted manufacturer protocol completed in 24 well plate format. Cells were transfected with equivalent ratios of HDR and NHEJ DNA (1 µg total) (Santa Cruz Biotechnologies) using lipofectamine 3000 (Thermo Fisher Scientific, U.S.A.) as per manufacturer guidelines. 1 x 10^6/mL CT26 cells were seeded overnight into individual wells of a 24 well plate and grown to confluency. For transfection, a total 1µg of DNA (IL-36R targeting combined plasmids, CRISPR control plasmid or puromycin control plasmid) was co-incubated with 1 µL of P3000 reagent in 25 uL of Opti-mem I medium (Thermo Fisher Scientific, U.S.A.) per well. In a separate tube, 25 µL of Opti-mem I medium was mixed with 1.5 µL of lipofectamine 3000 reagent. These tubes were then combined and incubated at room temperature for 15 minutes. This mixture was then added drop-wise to confluent wells of CT26 cells and incubated for 24 hours. Transfection of cells was confirmed by Flow cytometry for transient GFP expression.

2.2.1.24.2. Cell sorting/serial dilution

After transfection of cells or after puromycin selection of cells and expansion, cells were trypsinised and cells were sorted based on RFP or GFP fluorescence, depending on plasmid transfection by FACS Aria II cell sorter (BD). Cells with the highest mean fluorescence intensity were selected for isolation. Alternatively, non-fluorescent puromycin control cells were serially diluted and seeded into 96 well plates with each well examined for the presence of a lone cell. These cells were then expanded to confluency in puromycin-supplemented (5 µg/mL) media.

2.2.1.24.3. Sidestep lysis buffer

Cell lysis of CT26 cells was completed using Sidestep Lysis Buffer (Roche, Switzerland) as per manufacturer's guidelines. Two-step qRT-PCR was completed to analyse RNA expression of CT26 cells. A 1:100 dilution of lysate was used to generate cDNA which was then synthesised as previously described. Approximately 200 cell equivalents were used with 0.1µL of lysate used in each cDNA reaction.

2.2.1.24.4. Genomic DNA extraction and PCR amplification

Genomic DNA extraction was completed using Qiagen DNeasy Blood & Tissues (Qiagen, Helden) spin column extraction kits as per manufacturer guidelines from a minimum of 1 x 10^6 expanded clone cells. Extracted clone DNA was then amplified using primers described in Appendix Table 4 using the following PCR programme: 30 cycles (94°C x 1 minute, 55°C x 2 minutes, 72°C for 3 minutes). PCR products were then analysed by 1% agarose gel electrophoresis.

2.2.1.24.5. Sanger Sequencing

Amplicons were isolated from agarose gels by manual excision and processed using Qiaquick PCR purification Kit (Qiagen, Helden) as per manufactures guidelines. 15 µL of isolated DNA was then mixed with 2 µL of forward primer (10 µM) with a final volume of 17 µL. Sample sequencing was completed by Eurofins Tubeseq label services (Eurofins, Luxembourg). Pherogram results were then analysed by TIDE (Bas van Steensel lab) and ICE (Synthego, USA) analysis to compare clones sequences with wild type sequences at guide RNA site regions.

2.2.1.24.6. Quantitative PCR (gel-based)

RNA extraction, cDNA generation, PCR amplification and DNA amplification was analysed as previously described. Primers used are outlined in Appendix Table 3.

2.2.2. *In vivo* studies

2.2.2.1. Treatment groups and ear marking

Female mice weighing 18-25g and 6 weeks of age were randomly divided into experimental groups. Mice were ear clipped for identification purposes.

2.2.2.2. Monitoring body weight

Mice were weighed a minimum of three times weekly to monitor changes in body weight.

2.2.2.3. Tumour induction and measurement

Single-cell cancer cells suspended in 200 µL PBS, as indicated in each study, were subcutaneously injected into the rear right flank of mice. Tumour formation was monitored three times weekly by palpation at the site of injection. The width (w) and length (l) of the tumours were measured using Vernier callipers and the mean tumour volume calculated using the formula: ½ ($l \times w^2$). Upon excision, tumours were weighed and volume was measured.

2.2.2.4. Tumour sampling

Following euthanasia by cervical dislocation, tumours were excised from mice and dissected for subsequent analysis using the clock face dissection method. This method ensured that all stored tissue incorporated tumour tissue from the core to the leading tumour edge. Tissue was stored for short term in formalin or RNAlater (Sigma Aldrich,

U.S.A.) until further processing. In addition, tissue was placed in a MACS® Tissue Storage Solution buffer for subsequent FACS analysis (Miltenyi Biotech, Germany).

2.2.2.5. Single cell suspension of tumour cells for flow cytometric analysis

Tumours were mechanically minced using scissors and scalpels into fine pieces (<1 mm^3). Tumour tissue was then centrifuged at 400 RPM for 5 minutes at room temperature and resuspended in 4.7mL serum-free pre-warmed DMEM. 250 µL of 20X collagenase II (Sigma Aldrich, U.S.A.) and 50 µL of 100X DNAse I (Sigma Aldrich, U.S.A.) were added to final concentrations of 1 mg/mL and 100 Kunitz/mL, respectively. Cells were incubated at 37°C in a shaking incubator for 60 minutes. The homogenous suspension was then filtered through a 70 µM cell strainer (Corning, U.S.A.). Cells were washed with FACS buffer, and resuspend in 5 mL RBC lysis buffer (Biolegend, U.S.A) for 5 minutes on ice. Cells were washed once more and then counted as described for cell viability checks.

2.2.2.6. Flow cytometry of tumour single cell suspension

Single cell suspensions from tumour tissue were prepared. 200,000 cells per tumour were re-suspended in 200 µL of cell staining buffer, blocked using a 1:200 dilution purified anti-mouse CD16/32 antibody (Biolegend, U.S.A.) for 20 minutes at 4°C and then resuspend for staining with antibodies listed in Appendix Table 1 at the corresponding dilutions in FACS buffer. A separate aliquot of cells was also used for intracellular staining for T-regulatory cell detection which was completed using True-NuclearTM Mouse Treg Flow Kit (Biolegend, CA, USA) as per manufacturer's guidelines. Cells were gated as outlined in kit instructions. Cells were stained with indicated viability marker (Thermo Fisher Scientific, U.S.A.) and fixed with Fixation Buffer (Biolegend, U.S.A.). Unstained controls, viability controls, Isotype

controls and antibody-binding beads (Ultracomp® beads, Thermo Fisher Scientific, U.S.A.) were prepared for each run. For each sample, 10,000 to 20,000 events were recorded. The percentage of cells labelled with each antibody was calculated in comparison with cells stained with the isotype control antibody. Analysis gates for each antibody were set by FMO (fluorescence minus one) controls with a threshold below 1%. The results represent the percentage of positively stained cells in the total cell population with a signal exceeding the background staining signal. Relative fluorescence intensities were measured using a BD LSRII flow cytometer (Becton, Dickinson, U.S.A.) and BD Diva software (Becton, Dickinson, U.S.A.). Analysis was completed using FlowJo Software (Becton, Dickinson, U.S.A.).

2.2.3. *In silico* studies

2.2.3.1. Pan-cancer and normal tissue gene expression

Gene expression comparison of IL1RL2/IL-36R expression was assessed using the 'Cancer Exploration' suite of the TIMER2.0 web tool [389].

2.2.3.2. Sample stratification and survival analysis

Samples were stratified according to their quartile range, with values in the 1st quartile (top 25%) designated as 'High' and values in the 4th quartile (bottom 25%) designated as 'Low'. Incomplete samples were not included in survival analysis. The R package "survival" and ''survminer'' were utilized to visualise the Kaplan–Meier survival curves and perform log rank testing using the TCGA-COAD dataset.

2.2.3.3. Immune cell infiltration assessment

The microenvironment cell populations (MCP)-counter was scored by using MCPcounter R package [390]. MCP-counter facilitates robust quantification of abundance of a total of

eight immune cell and two stromal cell populations from microarray data. Samples were stratified as previously described.

2.2.3.4. Differential Gene expression determination

GEO2R, a data processing tool on GEO was used to identify differentially expressed genes (DEGs) [391]. Four datasets were used to compare colon cancer tissue and normal colonic tissue, with cancer samples separated into two groups; A) IL-36R High (Upper 25% of IL-36R expressing cancer samples) and B) IL-36R Low (Lower 25% of IL-36R expressing tumours). Upregulated genes (FC >1.5 and p value < 0.05) were compared across datasets and only genes that were present in all four were proceeded with. Downregulated genes (FC <1.5 and p value <0.05) were compared similarly. IL-36R High and IL-36R Low upregulated genes were then compared, with genes exclusive to either group then proceeded with for subsequent analysis (e.g. 12 genes upregulated in IL-36R high tumours, 4 genes upregulated in IL-36R Low tumours, figure 6.7). The same was completed for downregulated genes in both tumour groups.

2.2.3.5. Protein-Protein interaction, clustering and module analysis

Search Tool for the Retrieval of Interacting Genes (STRING) [392] database was used to investigate protein-protein interactions of DEGs. PPI networks were visualised using Cytoscape software [393]. The Molecular Complex Detection (MCODE) plug-in was then used to identify gene modules by Markov clustering [394]. Modules identified were then separated as individual networks and STRING enrichment was performed for functional annotation of gene clusters with top Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) annotations used.

2.2.3.6. Correlation Matrix

A correlation matrix of genes from the KEGG *'IL-17 signalling pathway'* and IL-36 family members was made with GSE39582 and the R package 'corrplot'.

2.2.3.7. Gene expression correlation in normal and cancer tissue

The web tool 'AnalyzeR' was used to investigate gene expression of CXCL1 and LCN2 in normal and cancerous intestinal tissue [395].

2.2.3.8. Statistical analysis

GraphPad Prism 4 (GraphPad Software, U.S.A.) or the R 'stats' package was used for statistical analysis of experiments. Experiments were performed a minimum of three times in triplicate. Results were statistically evaluated using One-way Anova with Dunnett's post-hoc test, students paired t test or as outlined in figure legend if otherwise. Values of $p < 0.001$ are indicated by three asterisks (***). Values of $p < 0.01$ are indicated by two asterisks (**). Values of $p < 0.05$ are indicated by one asterisk (*). Grubbs test was used to detect outliers.

Chapter 3

In vitro characterisation of IL-36 signalling in colon cancer cell lines

Results of this chapter were published in the following manuscript;

Baker KJ, O'Donnell C, Bendix M, Keogh S, Byrne J, O'Riordain M *et al*. IL-36 signalling enhances a pro-tumorigenic phenotype in colon cancer cells with cancer cell growth restricted by administration of the IL-36R antagonist. Oncogene 2022

Charlotte O'Donnell contributed to Figure 3.1 of this chapter

3.1. Aim

The IL-36 cytokines are a recently described subset of the IL-1 family of cytokines, shown to play a role in the pathogenesis of chronic inflammatory diseases such as Inflammatory Bowel Disease (IBD) [396]. However, little is known regarding their role in the pathogenesis of Colorectal Cancer (CRC). Given the link between IBD and colon cancer development [397-399], as well as the involvement of other IL-1 family members in intestinal tumourigenesis [196, 202, 400], the aim of this chapter is to characterise IL-36 family member expression and signalling in colon cancer cell lines.

3.2. Introduction

The IL-36 cytokines are a subset of the IL-1 family of cytokines [221, 401]. The three agonistic members of this family, IL-36α, IL-36β and IL-36γ, all share the same receptor complex, which is composed of the IL-36 receptor (IL-36R/IL1RRP2/IL1RL1) and the IL-1 Receptor accessory protein. A biological inhibitor to this complex has also been identified, the IL-36R antagonist (IL-36Ra). The IL-36 cytokines and their receptor are expressed in several tissues, particularly the lung, skin and colon, as well as by immune cells such as monocytes, macrophages, dendritic cells and T cells [402, 403]. Similar to other IL-1 family members, IL-36 cytokines are important activators of the inflammatory response, stimulating both innate and adaptive immune responses [402, 403]. These cytokines have been shown to play an essential role in autoimmune diseases, in particular in the pathogenesis of psoriasis [402, 404], IBD and respiratory diseases [405].

The pluripotent nature of IL-36 family members has become apparent from *in vitro* and *ex vivo* assays showing numerous different roles for these cytokines across various cell types, including the induction of pro-inflammatory cytokine expression, as well as promoting a variety of intrinsic cellular processes [406]. For instance, IL-36 agonists have been shown to be key drivers in the cellular proliferation of immune cell populations, keratinocytes and intestinal fibroblasts [238, 379, 407]. As inflammation is now well recognised as a hallmark of cancer, IL-36 signalling in tumourigenesis and tumour eradication are becoming increasingly investigated due to the pluripotent nature of these cytokines.

IL-36-focussesd cancer research to date has primarily focused on these cytokines and the immune infiltrate in the TME but several studies have investigated tumoural IL-36 expression in patient cohorts. Oncomine analysis by Wang *et al.* has previously reported IL-36γ expression to be decreased in metastatic melanoma tissue in comparison to primary

melanoma and melanoma pre-cursor tissue [299]. Similarly, Hu *et al.* have observed reduced IL-36γ expression in hepatocellular carcinoma (HCC) tissue in comparison to paired adjacent normal tissue samples, whilst Pan *et al.* reported reduced expression of IL-36α in moderately and poorly differentiated HCC patient tissues [367, 368]. These studies showed that loss of IL-36 expression was associated with poorer prognostic outcomes for patients. A colon cancer study also reported loss of expression of each IL-36 agonist in colon cancer tissue when compared to adjacent healthy tissue [369]. A reduction in expression of IL-36α was associated with poorer patient survival whilst a reduction of IL-36γ expression was associated with improved patient survival. Despite various groups investigating IL-36 agonist expression across cancer types [406], fewer have investigated IL-36R expression and the direct effect of IL-36 signalling on cancers cells themselves. The aim of this chapter was to characterise IL-36 family member expression and subsequently investigate the effects of IL-36R signalling on colon cancer cells *in vitro.*

3.3. Results

3.3.1. IL-36 family member expression is increased in CRC

Previous investigations in the laboratory have clearly shown an increase in expression of IL-36 family members in CRC relative to adjacent healthy colonic tissue (Fig. 3.1). Using a cohort of 24 patient samples (patient cohort 1), gene expression was first examined by qRT-PCR. Expression of all family members, with the exception of the IL-36R, was found to be significantly upregulated in tumour tissue to adjacent non-tumour tissue (Fig. 3.1). Protein abundance of these family members was analysed by IHC (patient cohort 2) in both tumour and adjacent non-tumour tissue. IL-36α and IL-36γ were detected at a very low level in the adjacent normal epithelial cells and stromal cells, with a higher protein abundance detected

in the cancer cells. IL-36β was only detected in the stromal compartment. IL-36R was detectable in both the epithelial and stromal compartments in both adjacent normal and tumour tissue. To provide a semi-quantitative analysis of the changes in IL-36 family member expression, tumour cell and adjacent normal epithelial cell expression of IL-36α, IL-36γ and the IL-36R were scored based on intensity of staining. Findings were reflective of the qRT-PCR data, showing an increase in expression of these IL-36 family members relative to adjacent normal expression. Expression did not correlate with stage or grade. Given that IL-36 family members are differentially expressed in patient tumour samples, an important next step was to assess cancer cell lines as models for studying the functional effects of IL-36 family members *in vitro*.

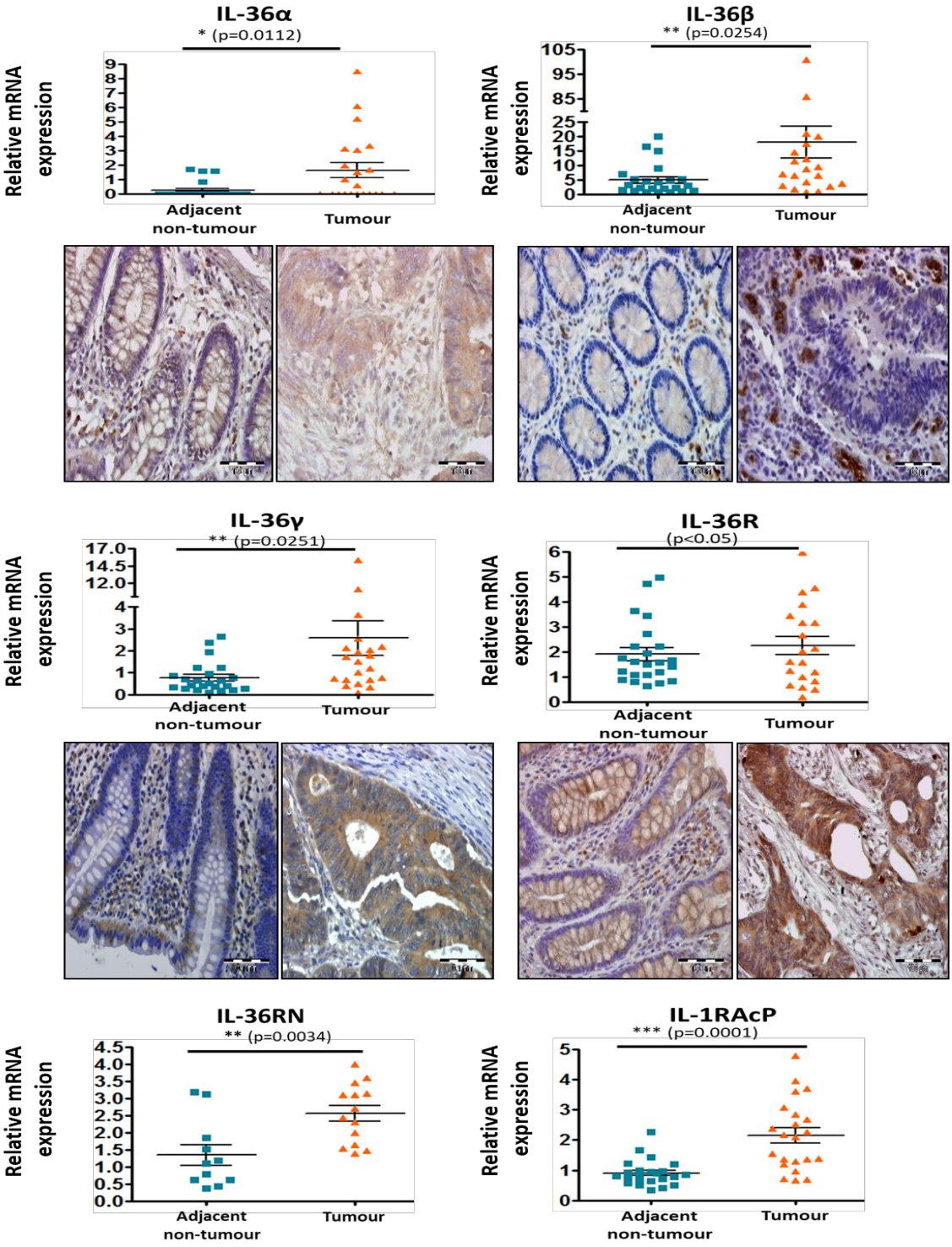


Fig. 3.1: IL-36 family members are more highly expressed in CRC tumour tissue in comparison to adjacent normal colon tissue. Total RNA was extracted from paraffin embedded tissue biopsies and expression of IL-36 family members was detected by qRT-PCR. IHC was completed to confirm mRNA findings and localise expression. Data indicates all family members, excluding IL-36R, are upregulated relative to healthy tissue. Representative images are shown (mag = 40X; Scale 100 µm). Statistical analysis was performed by Student T Test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Given the heterogeneity of cancer and the increasing importance of phenotyping/genotyping tumours for therapeutic regimen choice, a panel of colon cancer cell lines was selected to investigate the role of IL-36 cytokines in CRC. HCT116 and HT29 human cell lines were chosen for their relative aggression and less aggressive phenotype, respectively [408]. SW480 and SW620 cell lines were chosen as models of colon cancer progression as these cell lines were derived from the primary tumour and metastatic derivative resected from the same patient, respectively [409]. Finally, CT26 and CMT-93 murine cell lines were simply chosen as appropriate candidates for future use for *in vivo* models of disease.

Clinical Characteristics		Cohort 1 (n=24)	Cohort 2 (n=66)
Gender	Male	12	44
	Female	12	22
Age	>60	18	41
	<60	6	25
Site	Left	18	42
	Right	6	26
Tumour Differentiation	MD	24	58
	PD	0	9
	WD	0	1
Tumour Stage	I	7	14
	II	5	21
	III	11	20
	IV	1	13
T classification	T1	1	8
	T2	9	12
	T3	12	25
	T4	2	23
N Classification	pN0	12	35
	pN1	12	33

Table 3.1: Patient cohort demographics. Demographics and tumour classification of patients used for qRT-PCR (cohort 1) analysis and IHC analysis (Cohort 2)

Expression of IL-36 family members was determined by qRT-PCR (Table 3.1). Of the human cell lines investigated, levels of mRNA transcripts for target genes showed variability between cell lines. The HT29 cell line expressed all IL-36 family members with varying levels (e.g. CT values of 31.68 for IL-36γ, and 37.64 for IL-36β). The IL-36R gene was the highest expressed gene in HT29 cells, with an average CT value of 28.21. The HCT116 cell line only expressed IL-36γ, with an average CT value of 34.13. SW480 and SW620 cell lines, derived from the same patient, showed differing basal expression patterns, with an increase in IL-36β and IL-36γ expression in the metastatic SW620 cell line. None of the target gene transcripts were detected in the murine cell line, CMT-93. The other murine cell line, CT26, showed expression of the IL-36R gene, which was highly expressed when compared to other cell lines, with an average CT value of 27.92. Both human and murine cell lines showed minimal expression of the IL-36Ra gene. Given that IL-36 cytokines are highly involved in the innate immune response at barrier tissues [406], we next investigated if soluble factors and pathogen-associated ligands could induce expression of these genes.

	IL-36α	IL-36β	IL-36γ	IL-36R	IL-36Ra	β-actin
HT29	36.49 ±1.62	37.64 ±0.76	31.68 ±1.22	28.21 ±0.57	35.31 ±0.68	18.72 ±0.63
HCT116	40 ±0	40 ±0	34.13 ±1.35	40 0±	40 ±0	18.81 ±0.36
SW480	34.46 ±2.788	36.46 ±1.07	32.04 ±1.20	30.04 ±0.43	37.79 ±1.11	15.69 ±0.65
SW620	37.25 ±0.26	33.11 ±0.27	28.81 ±0.10	33.94 ±3.03	40 ±0	15.48 ±0.24
CMT93	40 ±0	40 ±0	40 ±0	40 ±0	40 ±0	19.18 ±0.55
CT26	40 ±0	40 ±0	40 ±0	27.92 ±0.55	40 ±0	16.29 ±0.13

Table 3.2: Colon cancer cells differentially express IL-36 family members. Total RNA was extracted from colon cancer cells and expression of IL-36 family members was detected by qRT-PCR. Values are plotted as averages of respective CT values. N=3 biological replicates.

3.3.2. Cytokines and TLR-ligands alter IL-36 gene expression in CRC cell lines

Several pro-inflammatory ligands were chosen to investigate the changes in basal gene expression of IL-36 family cytokines. These included lipopolysaccharide (LPS) (100 ng/mL), flagellin (FLA) (100ng/mL), prostaglandin E_2 (PGE_2) (1.0μM) and IL-1β (10ng/mL). Cells were stimulated for 4 hours and harvested for RNA extraction. Changes in mRNA expression were determined by qRT-PCR. As was seen with basal expression of IL-36 family genes, the change of gene expression in cells post-stimulation also varied greatly between cell lines. HT29 cells were particularly responsive to LPS stimulation, with significant increases in the expression of IL-36α, IL-36β and IL-36Ra (Fig 3.2) compared to its unstimulated control (UT). FLA was a potent inducer of IL-36β expression in HT29 cells, with a 12-fold increase in transcription following stimulation, but had no effect on other genes. An induction of IL-36γ was observed with PGE_2 and IL-1β compared to the untreated control;

however, this induction was smaller compared to other IL-36 family members and stimulants. PGE_2 was shown to significantly decrease the expression of IL-36Ra. IL-1β stimulation of HT29 cells increases all IL-36 agonist genes; however this induction was noticeably smaller, with only two genes (IL-36α and IL-36γ) shown to be significantly increased. The IL-36R gene was unaltered by cell stimulation, although FLA significantly decreased its expression (0.80 fold reduction).

LPS and FLA, similar to HT29 cells, had the strongest effect on IL-36 gene member expression in SW480 cells (Fig 3.2), inducing a significant increase in transcription of IL-36β and IL-36γ compared to the untreated control. The IL-36Ra gene was shown to be significantly induced by both LPS and IL-1β, although these increases were smaller than other IL-36 family member gene expression changes. In contrast to the findings observed in the SW480 cells, none of the IL-36 family genes were induced following stimulation of SW620 cells, although significant reductions in gene expression were observed for IL-36α and IL-36R in response to FLA and LPS, respectively (Fig 3.2).

Given that CMT93 cells do not express the IL-36R, CT26 cells were investigated for changes in IL-36 family members in response to inflammatory factors. The only change detected was a small but significant increase in IL-36R gene expression in response to LPS and IL-1β (Fig 3.2). Given that IL-36R expression was not inducible across cell lines, it was decided to investigate one human and one murine cell line with the highest expression of IL-36R for intrinsic cellular responses to IL-36 cytokine stimulation.

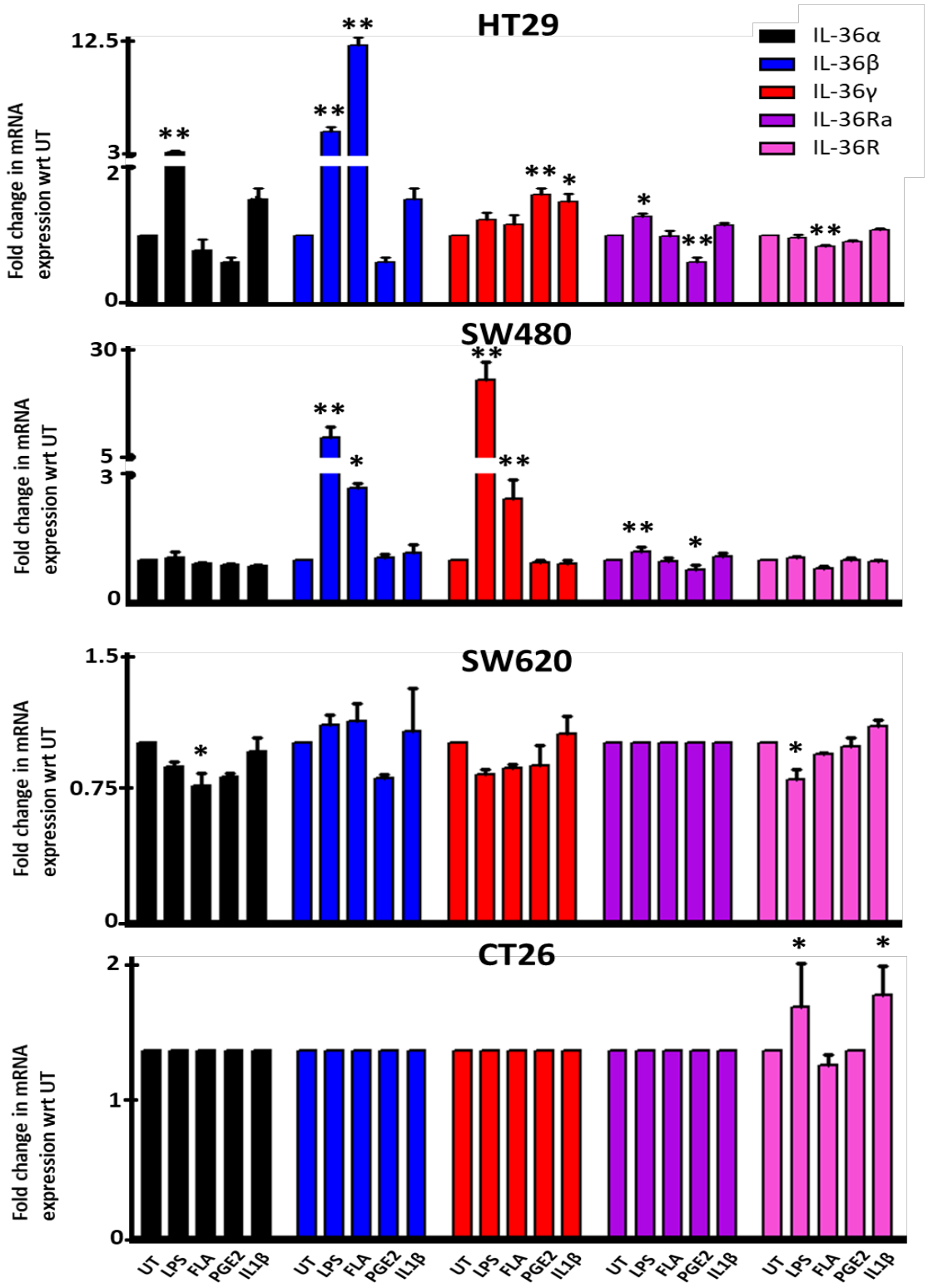


Figure. 3.2: Soluble factors can alter IL-36 family member gene expression in colon cancer cell lines. Cells were stimulated with pro-inflammatory ligands for 4 hours followed by total RNA extraction, cDNA synthesis and gene expression detection by qRT-PCR. Graphs are representative of independent, biological replicates (N=3). Data shown is the mean +/- SEM of experimental duplicate. Statistical analysis was completed by ANOVA with Dunnett's Multiple Comparison Test (*$p < 0.05$, **$p < 0.01$).

3.3.3. IL-36R signalling alters cytokine gene transcription in CRC cell lines

To investigate the effect of IL-36 cytokines on the expression of several inflammation-associated genes, cell lines were stimulated with individual IL-36 cytokines for 4 hrs and changes in gene expression were detected by qRT-PCR. Given the expression profile determined in the previous section for each cell line, of the human cell lines, HT29 cells were selected for further investigations due to expression levels of the IL-36R. Furthermore, the murine cell line CT26 was selected as these cells also express the IL-36R. Cells were stimulated with increasing concentrations of IL-36 cytokines (50-150 ng/mL) in order to determine whether cells were responsive to stimulation and whether this occurred in a concentration-dependent manner.

HT29 stimulation with IL-36α increased transcription of several cytokines and chemokines compared to the unstimulated control (0 ng/ml) (Fig. 3.3). However, when compared to the other IL-36R agonists, this induction was relatively small. Indeed, TNF-α was the only gene significantly increased following stimulation with 100 ng/mL of IL-36α (2.5 fold increase) when compared to the control.

In contrast, stimulation of cells with IL-36β resulted in large increases in gene expression, ranging from a 7.5 fold (CCL5) to a 700 fold (CXCL-1) increase, depending on the target gene (Fig. 3.3). IL-36β stimulation of cells with 50 ng/mL resulted in a large increase in gene expression, with 100 ng/mL and 150 ng/mL showing a further increase in gene transcription, indicating a dose-dependent induction. A similar dose-dependent transcription induction was observed with CCL20, IL-1β and TNF-α. However, CXCL1 and CXCL8 gene transcription were maximally upregulated at a

concentration of 100 ng/mL with no further increase at 150 ng/mL. Similar to IL-36β, IL-36γ stimulation of the cells resulted in large increases in pro-inflammatory gene expression compared to the unstimulated control (Fig 3.3). Once more, a concentration of 50 ng/mL resulted in large increases in gene expression, which was further increased at 100 ng/mL of IL-36γ. CCL20 was the only gene observed to increase in transcription at a concentration of greater than 100 ng/mL, with other genes appearing to plateau in response.

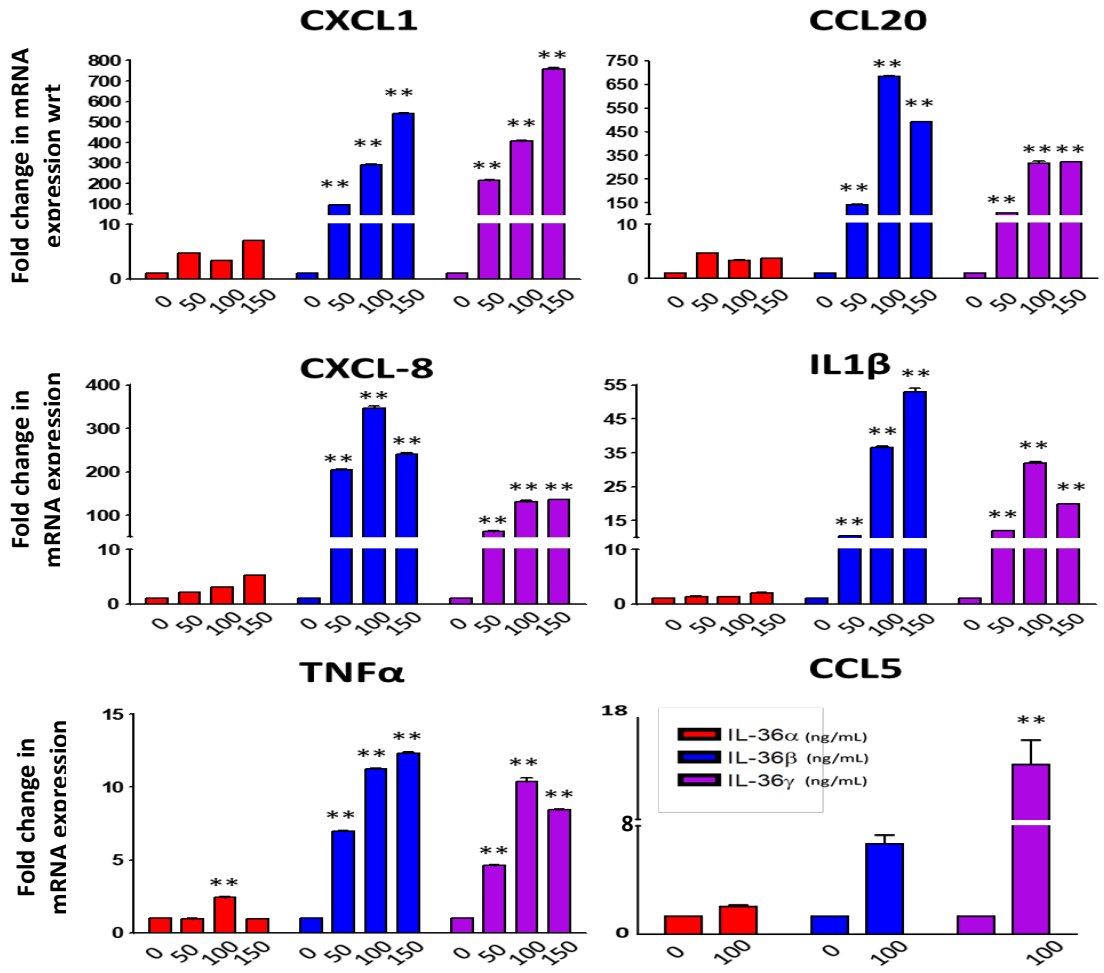


Figure 3.3: Cytokine mRNA expression is upregulated in HT29 cells in response to IL-36R stimulation in a dose-dependent manner. HT29 cells were stimulated with IL-36 agonists as indicated for 4 hours and gene expression changes were later detected by qRT-PCR. Values are plotted as averages of relative expression to untreated samples. Graphs are representative of independent, biological replicates (N=3). Data shown is the mean +/- SEM of experimental duplicate. Statistical significance was determined by ANOVA with post-hoc Dunnett's test (**p = <0.01, ***p = <0.001)

The response of murine CT26 cells to IL-36R stimulation was also investigated (Fig. 3.4). As before, cells were stimulated for 4 hrs and gene expression changes were detected by qRT-PCR. The response of CT26 cells to IL-36 cytokines was similar to that seen in HT29 cells; albeit the overall induction in gene expression was much lower. IL-36α stimulation of cells resulted in a small but significant increase in CCL2 and TGF-β transcription. No significant change in gene transcription was observed for CXCL-1 or CCL5. Similar to the findings seen with the HT29 cells, IL-36β stimulation resulted in much larger increases in gene expression compared to the unstimulated samples. This induction of gene transcription occurred in a dose-dependent manner across all genes. CXCL1 and CCL2 induction were found to be the most potently induced genes, followed by CCL5 and TGF-β. IL-36γ stimulation of the cells also resulted in large increases in gene expression. The largest increases of pro-inflammatory gene expression was observed when cells were stimulated with the highest concentration of IL-36γ. Together, these results suggest IL-36β and IL-36γ are the most potent IL-36 agonists, at least in terms of gene expression response in colon cancer cells.

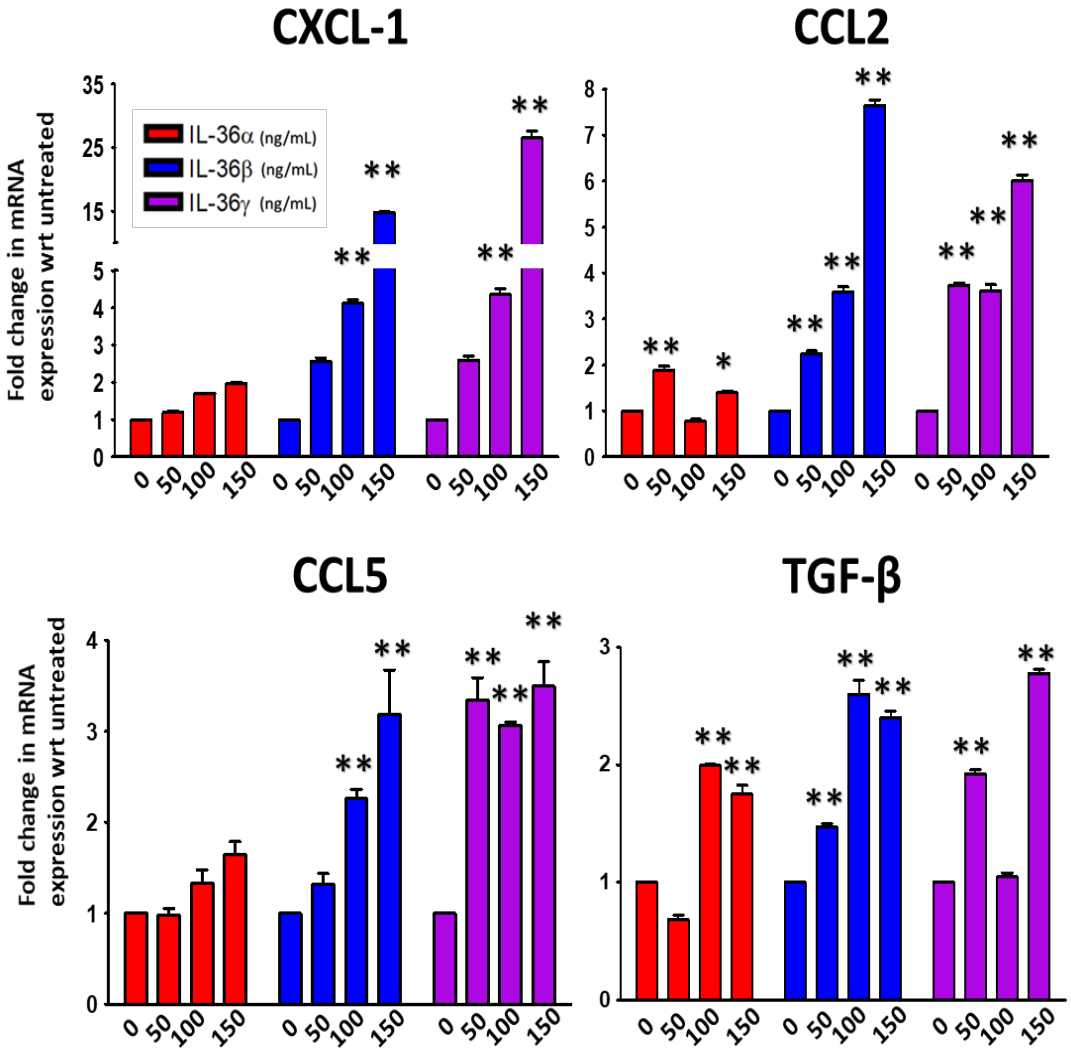


Figure 3.4: Cytokine mRNA expression is upregulated in CT26 cells in response to IL-36R stimulation in a dose-dependent manner. CT26 cells were stimulated with IL-36 agonists as indicated for 4 hours and cytokine gene expression changes were later detected by qRT-PCR. Graphs are representative of independent, biological replicates (N=3). Data shown is the mean +/- SEM of experimental duplicate. Statistical significance was determined by ANOVA with post-hoc Dunnett's test (* p= <0.05, **p = <0.01)

3.3.4. IL-36R signalling increases chemokine secretion in CRC cell lines

In order to confirm findings from the mRNA transcript analysis, changes in protein secretion were detected by ELISA. For the HT29 cells, the two most potently stimulated genes were chosen, CXCL1 and CCL20 (Fig. 3.5). Cells were stimulated for 24 hours with IL-36 cytokines (0 – 200 ng/ml). Stimulation of the cells with IL-36α had no effect on the secretion of either CXCL1 or CCL20. In contrast, secretion of both chemokines was significantly increased following stimulation with IL-36β and IL-36γ, supporting the mRNA data. Moreover, these increases in CXCL1 and CCL20 occurred in a concentration-dependent manner.

The mRNA findings of the CT26 cells were also confirmed by protein secretion from cells (Fig 3.5). CXCL1 and CCL2 were chosen as the target secreted proteins due to the fold induction of mRNA detected by qRT-PCR. As per the HT29 cells, CT26 cells were stimulated for 24 hours with IL-36 cytokines, and changes in secretion detected by ELISA. Stimulation of the cells with IL-36α did not result in any significant changes in CXCL1 or CCL2 secretion. IL-36β stimulation resulted in large increases in CXCL1 and CCL2 protein secretion. At 50ng/mL of IL-36β, the secretion of CXCL1 peaked compared to the other concentrations and the unstimulated control (Fig. 3.5), whilst increasing concentrations of IL-36β resulted in a similarly large increase in CCL2 secretion. Stimulation of the cells with IL-36γ also significantly increased secretion of both CXLC1 and CCL20, although higher concentrations were required to induce a significant increase compared to the untreated cells (Fig. 3.5). These findings confirmed IL-36 cytokine stimulation, particularly IL-36β and IL-36γ, could induce protein secretion of pro-inflammatory chemokines from human and murine colon cancer cells.

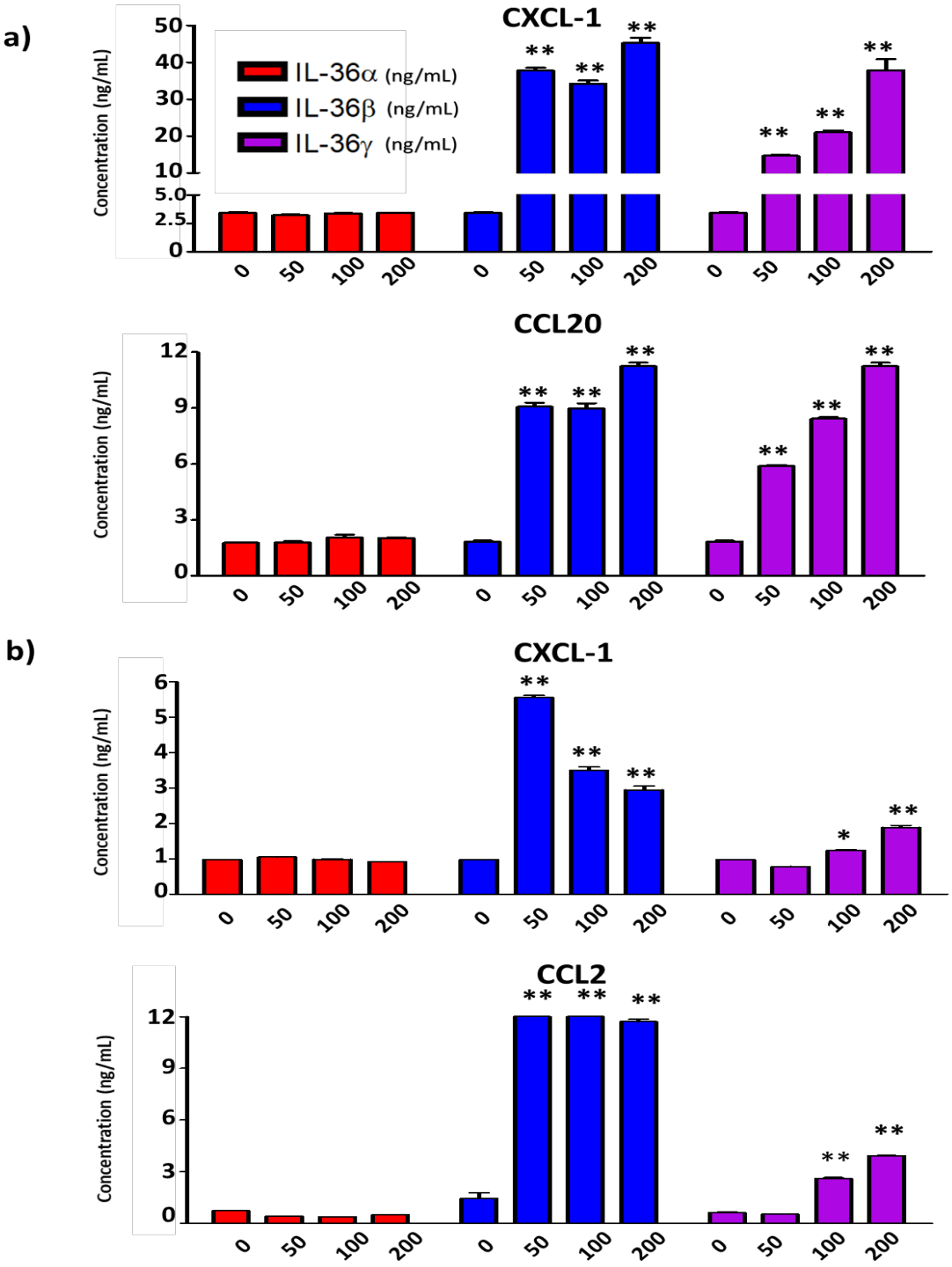


Figure 3.5: Cytokine expression increases a) HT29 cells and b) CT26 cells in response to IL-36R stimulation. Cells were stimulated with IL-36 agonists as indicated for 24 hours. Cell culture supernatant was decanted and changes in protein secretion detected by ELISA. Graphs are representative of independent, biological replicates (N=3). Data shown is the mean +/- SEM of experimental duplicate. Statistical significance was determined by ANOVA with post-hoc Dunnett's test (* p= <0.05, **p = <0.01)

3.3.5. IL-36R signalling induces cellular proliferation in CRC cell lines

To investigate whether IL-36 enhances the proliferative capacity of the cells, cellular proliferation assays were performed. To allow for variability in the basal rate of proliferation of the cell lines, both assays were optimised individually for the HT29 and CT26 cells. For the resazurin reduction assay (Fig. 3.6), the initial cell seeding density was determined using three different cell seeding concentrations, with 5 x 10^5 cells/mL identified as optimal for both the HT29 cells and the CT26 cells. Samples were analysed over a 24 hour time period to determine the optimal readout time and 8 hrs was chosen for all subsequent resazurin reduction assays (Fig 3.6).

The BrdU assay was also optimised (Fig. 3.6) for these cell lines. In general, shorter incubation times with BrdU is required for rapidly proliferating cells, with a longer incubation period for slow-growing cells. For both HT29 and CT26 cells, 1 x 10^5 cells/mL with a 24 hour BrdU incubation period yielded the optimal conditions for subsequent investigations (Fig. 3.6).

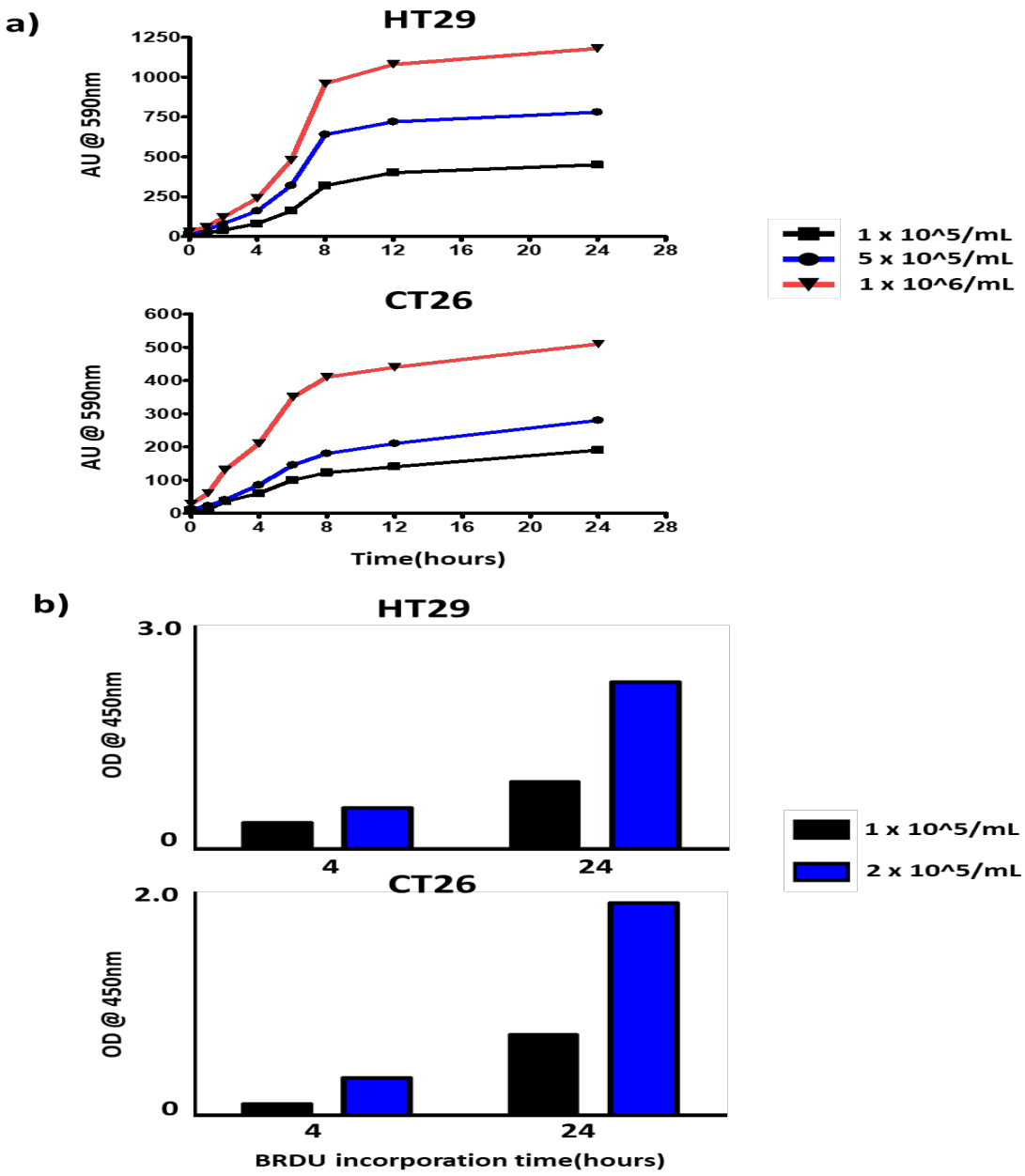


Figure 3.6: Optimisation of proliferation assays Resazurin Reduction and BRDU Incorporation. **a)** Resazurin assay optimisation was performed to determine the appropriate cell plating concentrations and readout times. **b)** BRDU assay optimisation required the determination of optimal cell seeding concentration and the optimal BRDU incubation time with the cells.

Prior to stimulation with the IL-36 ligands, cells were serum-starved for 24 hrs in order to synchronise the cell cycle of the cells to the G0/G1 phase. Cells were then stimulated with IL-36R ligands or left untreated for 24 hours, with changes in proliferation detected by Resazurin reduction and BrdU incorporation. All three IL-

36 cytokines significantly increased the proliferation rate of the HT29 cells compared to the control, as assessed by Resazurin reduction, with IL-36α, IL-36β and IL-36γ stimulation of cells increasing cell proliferation by 15, 40 and 69%, respectively (Fig. 3.7a(i)). Although the resazurin reduction assay has been shown to be effective in proliferation quantification [410], a second assay was used to confirm these findings. Cells were stimulated with individual IL-36 cytokines and BrdU incorporation was measured 24 hours later. Significant increases in cellular uptake of labelled-nucleoside were detected in IL-36β and IL-36γ-treated cells, with a trend of increased proliferation observed in IL-36α. Once more, IL-36β and IL-36γ were the most potent of the IL-36 cytokines, with both proliferation assays showing this effect (Fig. 3.7a (ii)).

The effect of IL-36 cytokines CT26 cellular proliferation was also investigated. A significant increase in resazurin reduction following stimulation with IL-36β and IL-36γ was detected. IL-36β was shown to be the most potent inducer of resazurin reduction, with an average increase of 73%, with IL-36α and IL36γ both inducing average increases of 22% and 31%, respectively (Fig. 3.7b(i)). A significant increase in BrdU incorporation was also observed in response to stimulation with IL-36β and IL-36γ (Fig. 3.7b (ii)). IL-36α once more showed a trend of increased BrdU incorporation, although this was deemed insignificant. IL-36β induction of CT26 cells was shown to be the largest in both resazurin reduction and BrdU assays. These results once more highlight IL-36β and IL-36γ as the most potent of the IL-36 cytokines in altering cellular processes.

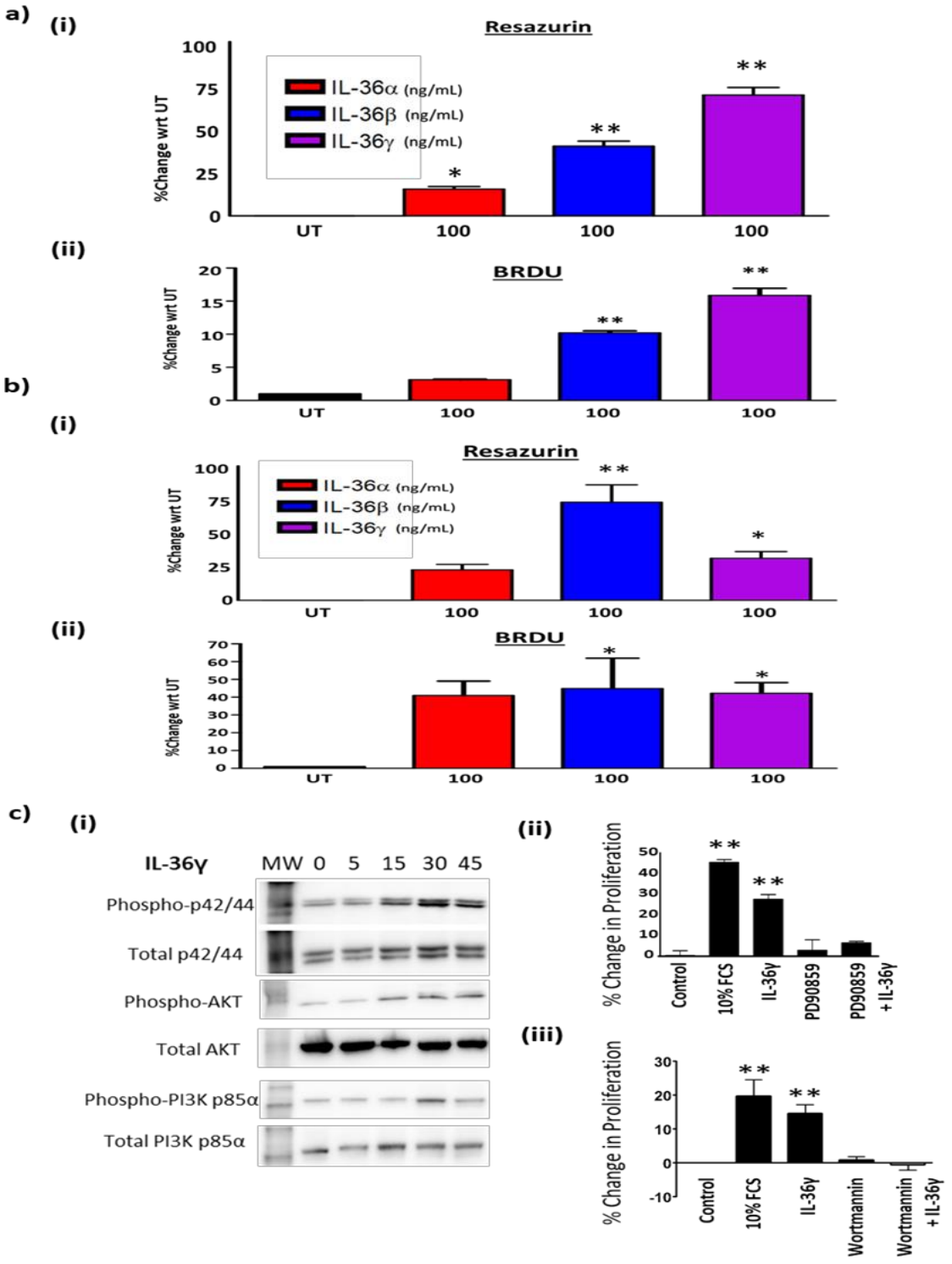


Figure 3.7: Cellular proliferation is increased in a) HT29 cells and b) CT26 cells in response to IL-36R stimulation *in vitro* c) via p42/44, AKT and PI3K pathways. **a/b**) Changes in proliferation were detected by **i)** resazurin reduction and **ii)** BrdU incorporation**. c) i)** Cell signalling pathways were assessed by time-course stimulation of cells followed by western blotting for protein detection**.** Cells were stimulated with IL-36γ for 24 h with/without **ii)** PD90859 (10 µM) or **iii)** Wortmannin (0.5 µM) pre-treatment and cellular proliferation was measured by resazurin reduction. Graphs are representative of independent, biological replicates (N=3). Data shown is the mean +/- SEM of experimental triplicate. Statistical significance was determined by ANOVA with post-hoc Dunnett's test (* p= <0.05, **p = <0.01)

In order to examine through which signalling pathway IL-36 may be mediating cellular proliferation, Western blotting was performed on HT29 cells stimulated with IL-36γ, given that it was the family member that consistently drove cellular proliferation to the greatest extent in HT29 cells. Both the p42/44 MAPK pathway and the PI3K/AKT pathway were activated in response to IL-36γ stimulation with IL-36R agonist-induced cellular proliferation seen to be inhibited upon incubation of cells with the inhibitors of these pathways PD98059 and Wortmannin, respectively (Figure 3.7c). These findings indicated IL-36 cytokine signalling could induce cellular proliferation of colon cancer cells through activation of both PI3K/AKT and p42/44 MAPK pathways.

3.3.6. IL-36R signalling induces colon cancer cell migration

A key step in tumour cell metastasis involves the invasion and migration of cancer cells into surrounding tissues via the circulatory system and draining lymph nodes [411]. This requires the chemotactic migration of cancer cells. Thus, the effect of IL-36 cytokines on cellular migration was investigated using two different assays, a Transwell Migration assay and Wound Scratch Assay. Transwell migration assays detect the migration of cells across an FCS gradient through an 8µm membrane. This method required optimisation of cell seeding concentrations and migration times for each cell line. Both parameters were determined simultaneously for each cell line. For HT29 cells, 5 x 10^5 cells/mL and 24 hours migration were determined as optimal. For CT26 cells, 2.5 x 10^5 cells/mL and 24 hours migration were determined as optimal (Fig 3.8).

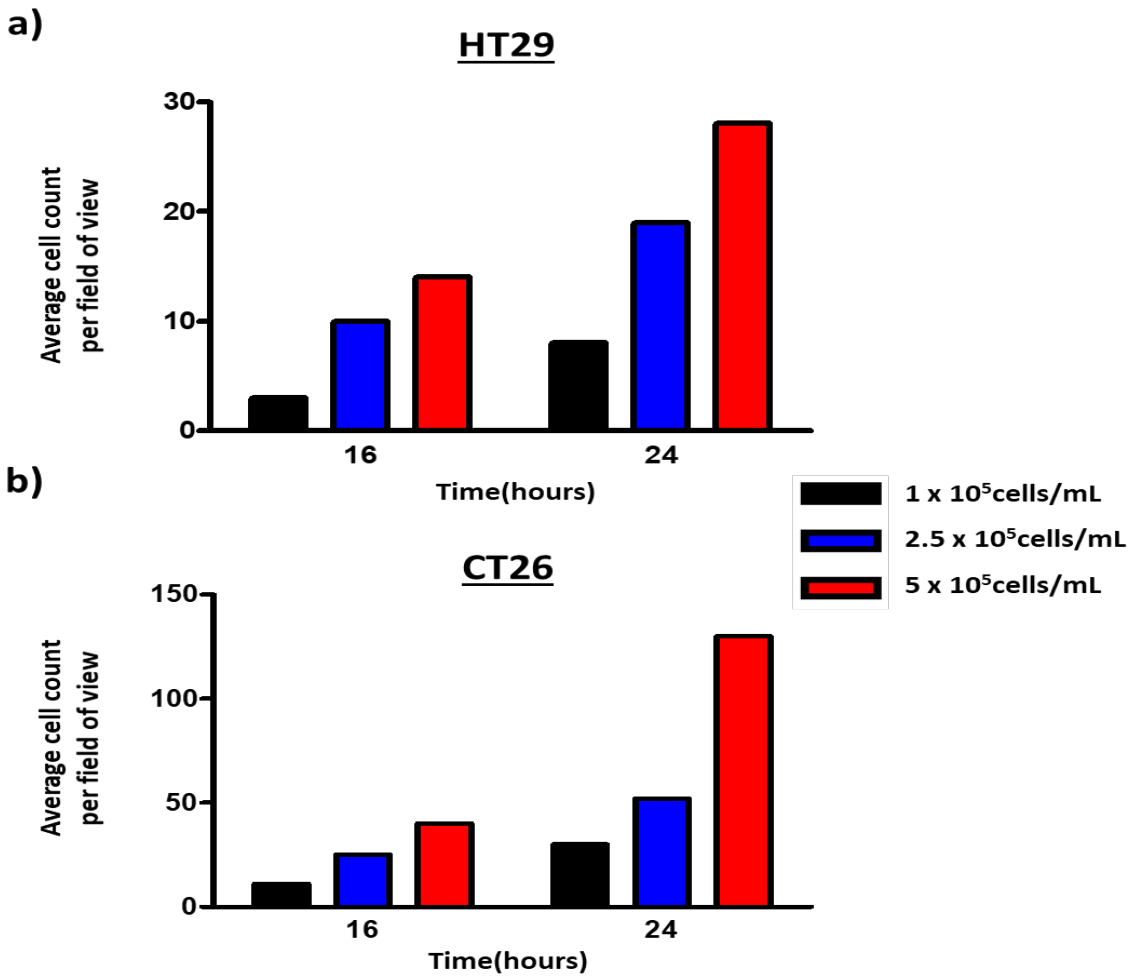


Figure 3.8: Optimisation of transwell migration assays. Transwell migration assays were optimised in order to achieve assay output for samples within an appropriate quantifiable range for **a)** HT29 and **b)** CT26 cells. The cell seeding concentration in the upper chamber was optimised as well as the assay incubation time.

Changes in cellular migration of HT29 cells were first investigated using the Transwell Migration assay (Fig. 3.9a-i). IL-36β stimulation, followed by IL36γ-stimulated cells, showed the largest increases in cellular migration, with both stimulations proving to be statistically significant. In contrast, IL-36α-stimulated cells only showed a low increase in cellular migration and this was determined not to be statistically significant. Cell migration was next assessed using the Wound Scratch assay (Fig. 3.9a-ii). The ability of cells to migrate into a pre-defined 500 µm space was investigated with or without stimulation with IL-36 cytokines. In the case

of the HT29 cells, at the 96hr time point, the IL-36R ligand-stimulated cells (100ng/mL) occupied the gap between monolayers quicker than unstimulated cells. Unstimulated samples averaged a 57% occupancy of the 500µm gap at 96hr, whilst IL-36α-, IL-36β- and IL36γ-stimulated cells averaged 59%, 67% and 64%, respectively. When comparing to UT cells, no difference was detected. Notably, the long incubation time required for wound closure could allow for cellular proliferation to contribute to migration in the wound scratch model.

CT26 cells were similarly assessed (Fig. 3.10b). Findings were first investigated by transwell migration assay (Fig. 3.10b-i). Cells showed a similar increase in migration as was seen in wound scratch assays. IL-36β and IL-36γ once more showed the most potent effect on cellular migration, with large increases in migrating cells across the 8µm membrane. Moreover, as was seen with the Transwell migration assay for the HT29 cells ((Fig. 3.9a-i), IL-36α had no significant effect. In the case of the wound scratch assay, IL-36β and IL-36γ showed potent stimulation of cellular migration, with cells rapidly migrating across the gap between the two cell monolayers (Fig. 3.9b-ii). At the 36hr time point of the assay, unstimulated samples averaged a 36% occupancy of the 500µm gap, whilst IL-36α-, IL-36β- and IL36γ-stimulated cells averaged 37%, 59% and 56% occupancy of the preformed gap, respectively. Agreeing with the previous data, these results indicated the multi-functional nature of the IL-36 cytokines and that IL-36β and IL-36γ are the most potent in inducing cellular processes in colon cancer cells.

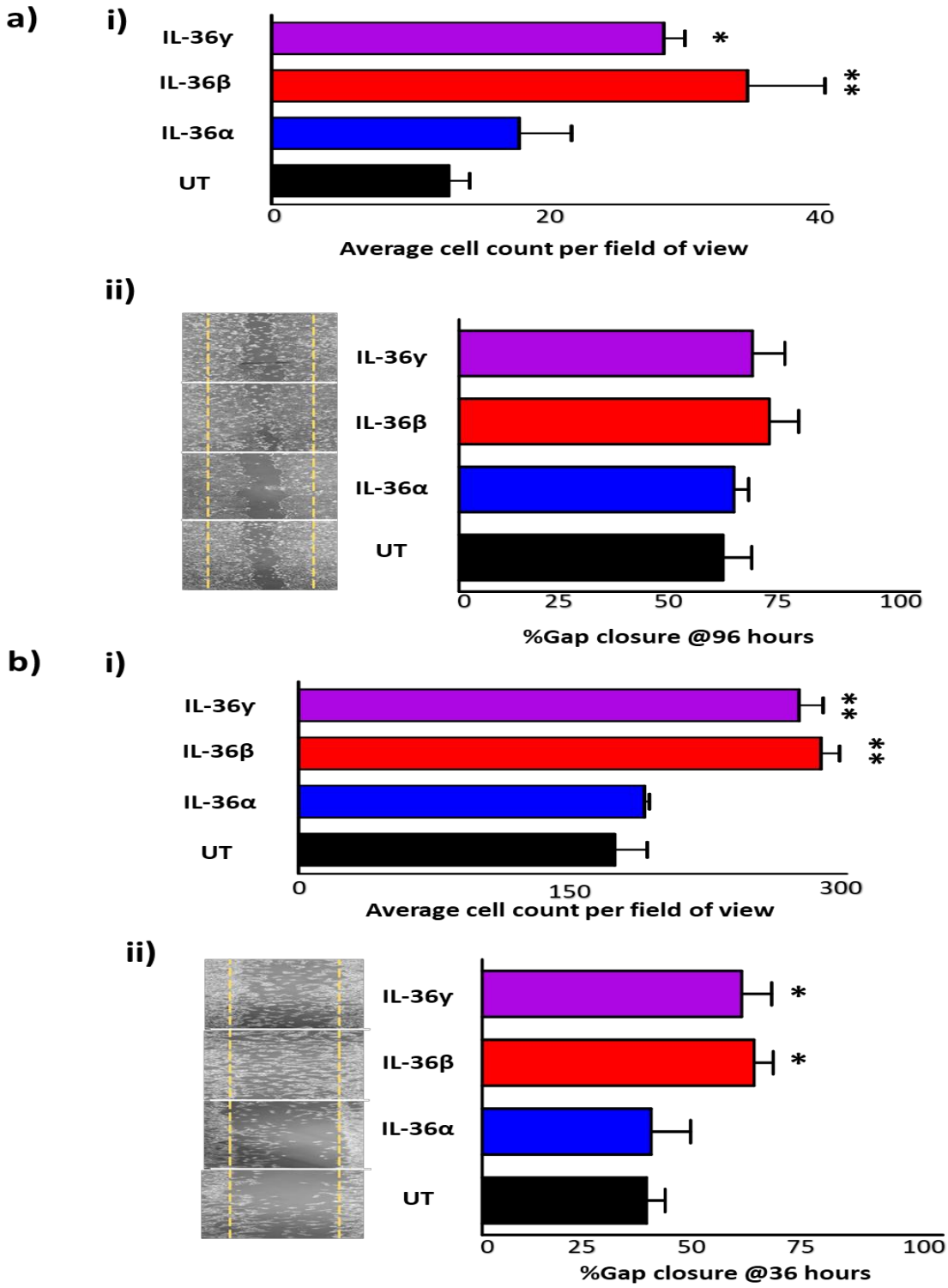


Figure 3.9: IL-36β and IL-36γ induce colon cancer cell migration. Cells were stimulated with IL-36 agonists, as indicated, during migration assay run time. Changes in migration were detected in **a)** HT29 and **b)** CT26 cells by **i)** transwell migrations assays and **ii)** wound scratch assays. Graphs are representative of independent, biological replicates (n=3). Errors bars shown are mean +/- SEM of technical duplicates. Statistical analysis for both assays was completed by one-way ANOVA and Dunnett's Multiple Comparison Test. (* p= <0.05, **p = <0.01)

3.3.7. IL-36R signalling induces colon cancer cell invasion *in vitro*

Another measurement to determine aggressiveness in cancer is cellular invasion. Therefore, cellular invasion assays were performed in order to further investigate the effect of IL-36 cytokines in a processes important for the metastatic spread of cancer cells. This was achieved by repeating the Transwell chamber assay, but in this case adding a solubilised basement membrane preparation (Matrigel®) which the cells must enzymatically degrade prior to migration through an 8µm pore membrane. This method required optimisation of both cell seeding concentration and Matrigel concentration (Fig. 3.10a). Both parameters were determined simultaneously for each cell line. An optimal cell and matrigel concentration was determined for HT29 cells and CT26 cells, which allowed for reproducible quantification of cells using imaging of membranes and analysis with ImageJ software. For both cells, 5 x 10^5 cells/mL and 300ug/mL matrigel were determined as optimal.

IL-36 cytokine stimulation of HT29 cells showed no significant change in cellular invasion. An average of 25 cells per field of view in unstimulated cells was counted. A trend of increased cellular invasion was observed in cells stimulated with IL-36β and IL-36γ, with averages of 48 and 52 cells per field observed, respectively (Fig 3.10b). CT26 cells were also stimulated with IL-36 cytokines and changes in cellular invasion assessed (Fig 3.10b). CT26 cells stimulated with IL-36β and IL-36γ cytokines showed an increase in cellular invasion, although IL-36γ-induced cellular invasion was determined to not be statistically significant. Unstimulated cells invaded through the matrigel and 8uM pore barriers with an average of 20 cells per field of

view. IL-36α-stimulated cells showed a similar pattern of invasiveness as unstimulated samples, with an average invasion of 21 cells per field of view. Only IL-36β-stimulated cells were shown to increase the cell count per field of view significantly, with increases of averages to 39 cells per field of view. These findings once more suggested IL-36 cytokines to have different potencies in inducing intrinsic cellular process in both human and murine colon cancer cells.

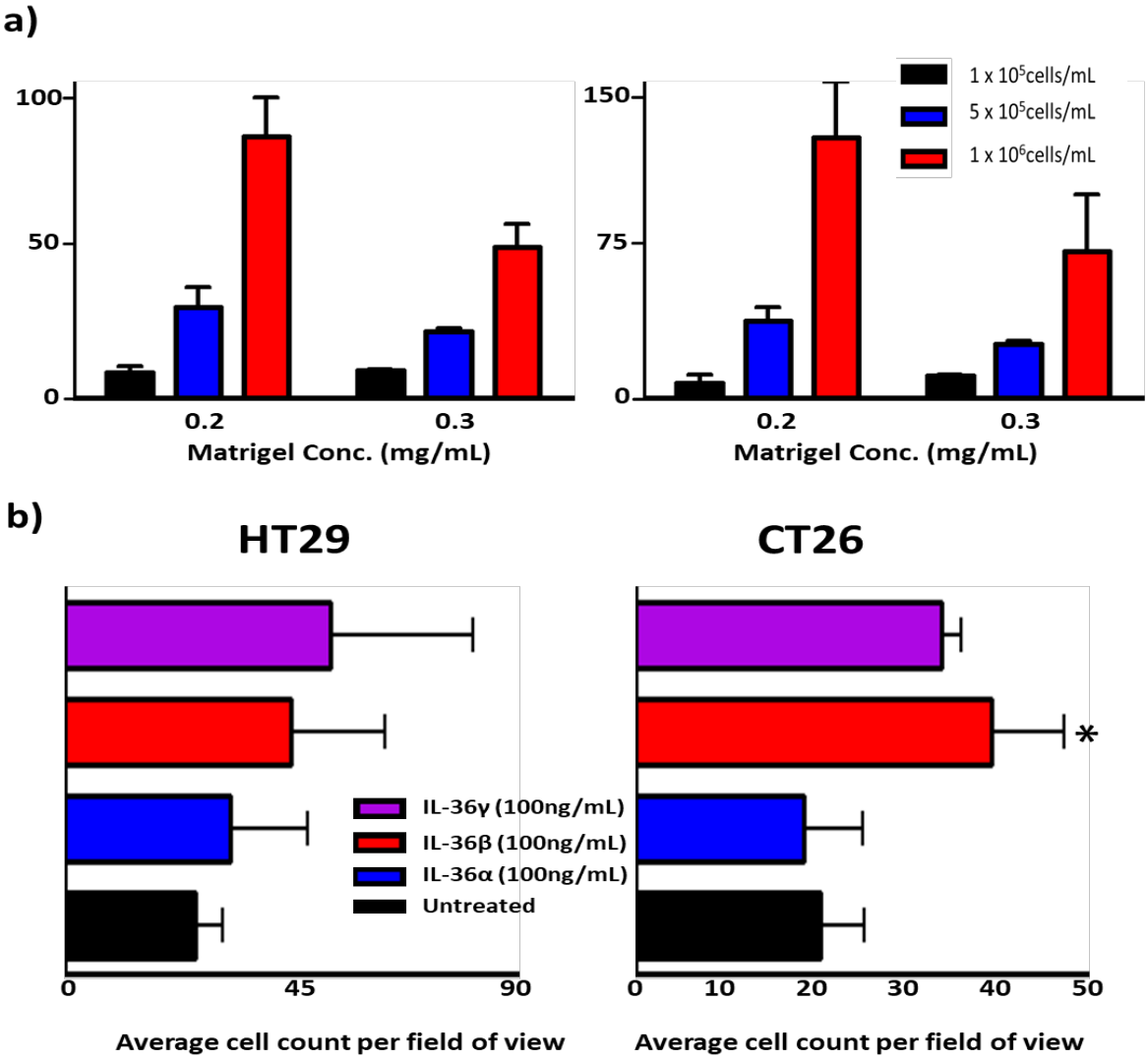


Figure 3.10: IL-36R stimulation increases cellular invasion of CT26 cells but not HT29 cells. a) Cell lines were optimised for cell concentration and matrigel concentration. **b)** Cells were stimulated with IL-36 agonists, as indicated, during invasion assay run time. Changes in invasion were counted by microscopic examination of 5 random fields of view. Graphs are representative of independent, biological replicates (n=3). Errors bars shown are mean +/- SEM of technical duplicates. Statistical significance was determined by one-way ANOVA and Dunnett's Multiple Comparison Test (* p=<0.05)

3.3.8. IL-36R signalling induces 3D cancer cell spheroid formation *in vitro*

In order to more closely recreate primary tumours *in vitro*, single-mass spheroids were generated and measured for changes in size in response to continued stimulation with an IL-36R agonist cocktail containing equal amounts of all three cytokines. Firstly, spheroid generation was optimised to ensure rapid formation of spheroids with continued cell growth for up to 10 days (Fig. 3.11). A starting cell concentration was chosen which displayed the best reproducibility and remained within camera diameter limitations after 10 days. It was determined that 1000 cells per well and 500 cells per well were most optimal for HT29 and CT26 cells, respectively. Additionally, several spheroids were combined and RNA was extracted to compare IL-36 gene expression in 3D culture in comparison to cell monolayers. Spheroids were also treated with LPS as a proinflammatory control for these cell lines.

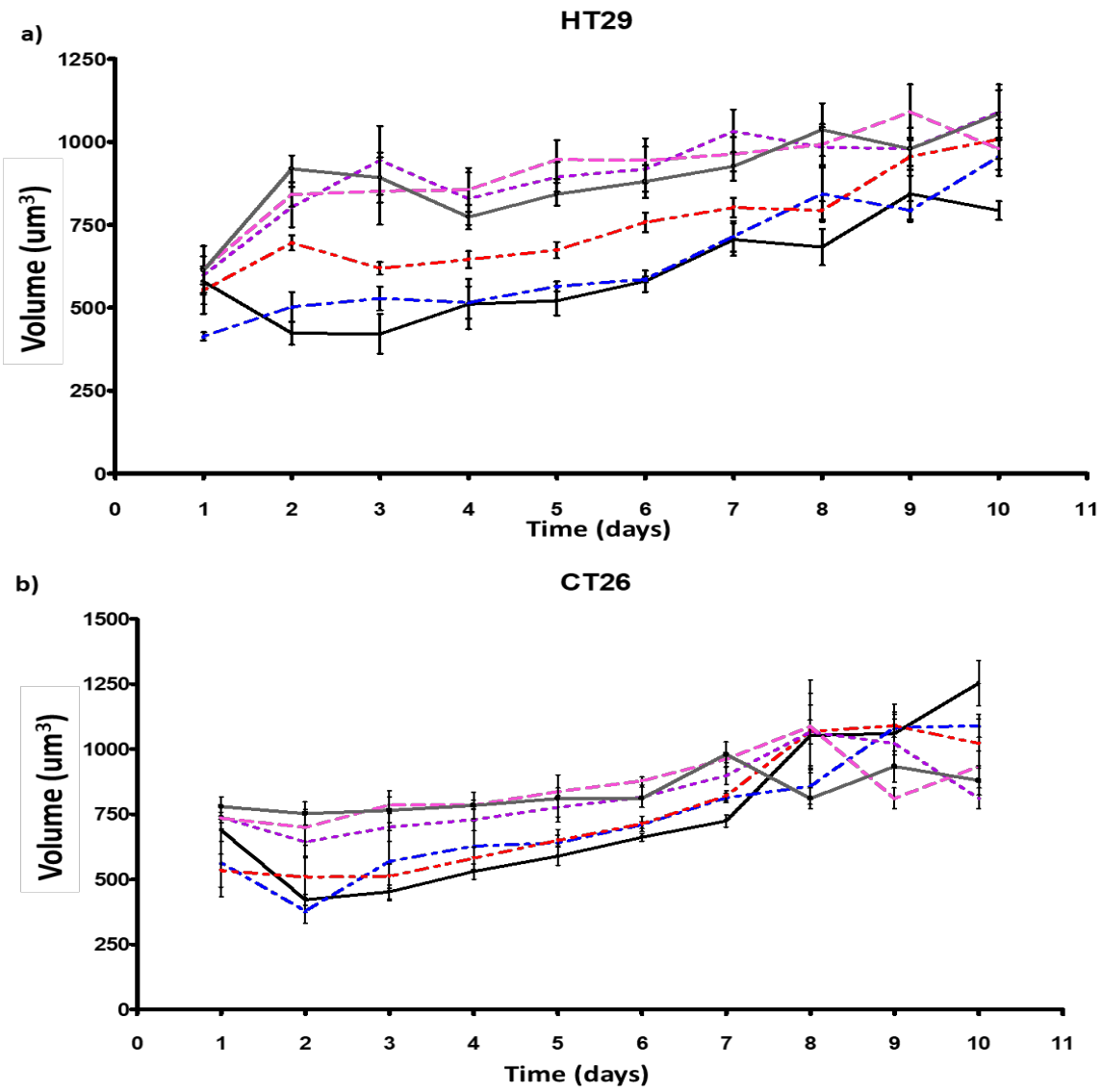
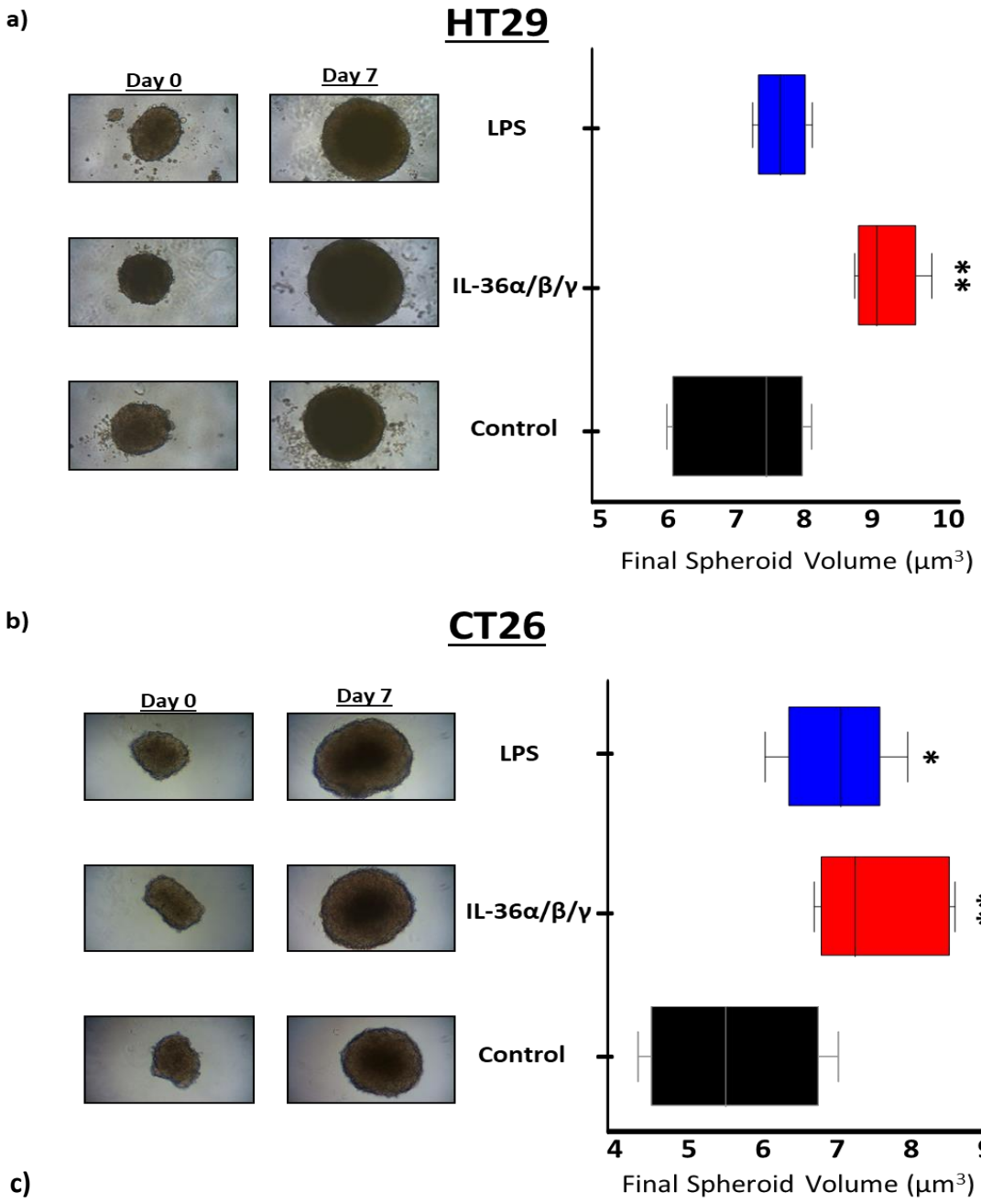


Figure 3.11: Optimisation of colon cancer cell spheroid growth. a) HT29 and **b)** CT26 cell seeding densities were optimised for spheroid formation over 10 days to ensure rapid formation and steady growth of spheroids until limitations of microscope and camera image diameter were reached.

HT29 spheroid formation was highly responsive to IL-36 treatment, with significant increases in spheroid diameter detected in comparison to non-treated spheroids and LPS-treated spheroids (Fig. 3.12a). A similar pattern was observed in CT26 spheroids when treated with the IL-36 cytokine cocktail (Fig. 3.12b). These results validated previous findings and further suggested an anti-cancer benefit from inhibiting IL-36R signalling on tumours cells *in vivo.* Additionally, it was shown that IL-36R expression remains

constitutively high during 3D culture of colon cancer cell lines (Figure 3.12c). A large increase in IL-36β expression was detected in spheroids in comparison to monolayer cultures of HT29 cells (Fig. 3.12c), suggesting endogenous IL-36β expression may contribute to 3D spheroid formation. Similarly, an increase in IL-36γ average CT values was shown in HT29 spheroids in comparison to monolayer growth expression values.

Collectively, this *ex vivo* and *in vitro* data strongly presents IL-36 cytokines as potent agonists of inducing intrinsic processes of cancer cell progression and can induce pro-inflammatory chemokine expression. Therefore, inhibition of this signalling pathway in vivo may be capable of reducing tumour progression by preventing intrinsic processes such as cellular proliferation and migration.



	IL-36α	IL-36β	IL-36γ	IL-36R	IL-36RN	B-actin
HT29 Monolayer	36.49 ±1.62	37.64 ±0.76	31.68 ±1.22	28.21 ±0.57	35.31 ±0.68	18.72 ±0.63
HT29 Spheroid	40 ±0	32.13 ±1.13	28.42 ±0.55	28.88 ±0.76	40 ±0	18.89 ±0.22

Figure 3.12: IL-36R stimulation drives 3D spheroid formation in colon cancer cells. a) HT29 cells were shown to increase in spheroid diameter in response to IL-36 (100 ng/mL) agonist stimulation**. b)** CT26 cells were shown to respond in growth to IL-36 (100 ng/mL) stimulation and LPS (100 ng/mL) stimulation. **c)** IL-36 gene expression was compared between HT29 monolayer growth and HT29 spheroid growth, with a significant increase observed in IL-36β observed as well as changes in IL-36γ and IL-36Ra expression. Graphs are representative of 3 biological replicates, consisting of 6 technical replicates per group and analysed by One-way ANOVA with Dunnetts post hoc test. *$p < 0.05$, **$p < 0.01$. Data shown are mean ± SEM. ($n = 3$).

3.4. Discussion

To date the focus of IL-36 pathogenesis-related research has centred on barrier tissue disease, such as psoriasis and IBD, with a more recent, increasing interest emerging in the role of IL-36 signalling in tumourigenesis [412, 413]. A number of these studies examining IL-36 signalling in tumourigenesis have utilised the potent pro-inflammatory nature of IL-36 cytokines to activate the immune response in order to enhance tumour rejection [299, 380, 413, 414]. However, there remain many unanswered questions on the role of the IL-36R in tumourigenesis, especially given the dichotomous roles of other IL-1 family members in malignancy [196]. In order to address this knowledge gap, the role of IL-36R signalling in the tumourigenic process was characterised *in vitro*. It was shown that IL-36R signalling can induce a pro-tumourigenic phenotype in colon cancer cell lines across several hallmarks of cancer.

This work demonstrates IL-36 family member expression is increased in colon cancer tissue relative to adjacent healthy normal tissue (Fig. 3.1). Furthermore, many colon cancer cell lines, including human and murine, differentially express IL-36 family members, with the IL-36R being constitutively expressed by many of these cell lines (Table 3.2). Moreover, pro-inflammatory factors including bacterial products such as lipopolysaccharide (LPS) and flagellin (FLA) may induce expression of IL-36 family members (Fig 3.2). This work also demonstrates that colon cancer cells produce a strong, differential pro-inflammatory response to IL-36 cytokines with large increases in myeloid-lineage associated chemokine expression (Figures 3.3-3.5). Finally, pro-tumourigenic properties such as cellular proliferation, migration and invasion were induced by IL-36R signalling in human and

murine cell lines (Figures 3.7-3.10). This proliferative effect of IL-36 cytokines was also shown in cells grown as 3D spheroids (Fig. 3.12).

IL-36 expression has become well characterised in barrier tissue disease with altered expression patterns in malignancies now becoming more apparent [406]. Several studies have now shown differential expression of IL-36 cytokines may have prognostic implications in several cancer types including ovarian cancer, hepatocellular carcinoma (HCC), CRC and melanoma. For instance, IL-36γ has been shown to be decreased in metastatic melanoma relative to precursor and primary melanoma tissue [299]. Similarly, IL-36γ has been shown to be reduced in HCC in comparison to adjacent tissue, whilst IL-36α expression is reduced in moderately and poorly differentiated HCC tissue [367, 368]. Decreased IL-36α expression has also been shown to be associated with epithelial ovarian cancer [371]. Little has been reported on IL-36 expression in colon cancer, with one group reporting IL-36α, IL-36β and IL-36γ expression to be decreased relative to adjacent normal tissue, although high levels of IL-36α and IL-36γ showed improved and poorer outcomes for patients in univariate survival analysis, respectively [369]. A second group has reported similar findings with decreased IL-36α expression detected in CRC tissue relative to adjacent normal tissue and this predicted a poor prognosis in these patients [370]. In contrast, our findings show that IL-36 family expression is increased in patient-derived colonic tumour samples as well as high expression levels of IL-36γ and IL-36R seen in several colon cancer cell lines. In order to better evaluate these discrepancies of IL-36 expression in cancer types, it may be of benefit to complete analysis of larger patient cohort datasets that are available such as The Cancer Protein Atlas or RNAseq datasets available on the Gene Expression Omnibus portal [415, 416]. Larger datasets, such as normalised multi-

cohort datasets, should contribute to the improved understanding of whether these cytokines are increased, decreased or unaltered in cancer tissue.

The data presented here highlights the differential expression of IL-36 family genes in colon cancer cell lines (Table 3.2), although IL-36 receptor gene expression was consistently expressed in most cell lines investigated. This pattern of expression suggests a conserved function for IL-36R signalling in colon tumourigenesis. IL-36γ expression was also highly expressed across all human cell lines, further suggesting a favourable role for this cytokine in colon cancer for tumourigenesis. IL-36γ cleavage of its proform can be achieved by cathepsin-s, which has been shown to be highly produced by epithelial cells [218]. Thereby, this uniquely facilitates IL-36γ production and activation by epithelial cells alone, unlike other IL-36R binding cytokines which require a multi-cellular process for secretion and activation [210]. Together, this suggests tumour IL-36R signalling may rely on both epithelial and stromal cells as sources of IL-36 cytokines and activating proteases. Furthermore, this data suggests that IL-36R-based therapy is likely to be responsive in most colonic tumours given its constitutive expression across many cell lines.

Microbial components have been shown to be key inducers of IL-36 family member expression [280]. For instance, exposure of several cell types located in the lung to TLR3 and TLR4 ligands resulted in dramatic increases in IL-36γ expression in these cells [321]. Similar findings have been found in other lung-inflammation studies, with IL-36α and IL-36γ expression also upregulated in murine BAL fluid and lung tissue homogenates following LPS stimulation [268]. Sinonasal epithelial cells have also been shown to increase expression of all IL-36 cytokines in response to TLR3, TLR4 and TLR5 agonists [417]. Furthermore, it has been reported that intestinal expression of IL-36 family members

increases in a microbial-mediated manner [396]. Our data compliments these studies, showing that intestinal IL-36 cytokine expression can be upregulated by microbial-derived molecules and inflammatory proteins associated with the innate immune response (Fig. 3.2). This suggests that epithelial cells are key IL-36 signalling regulators and that intestinal dysbiosis is a possible trigger in IL-36-driven intestinal disease.

The immune dialogue in the TME is integral to either the rejection of tumours or facilitating their growth and metastasis [418]. Here we show IL-36 cytokines can potently induce colon cancer cells to produce several chemokines and cytokines, which are predominantly myeloid-lineage chemoattractants (Figures 3.3-3.5). Research to date has indicated that IL-36 cytokines are capable of manipulating the TME to augment the anti-tumour response via stimulating infiltrating immune cells. This effect has been reported to result from indirect and direct interaction of IL-36 cytokines with IL-36R-expressing cells such as cytotoxic lymphocytes (CTLs), resulting in their activation and inducing the proliferation of $CD8^+$ T cells and natural killer (NK) cells [235, 299, 376, 380, 414, 419]. Little has been reported on the direct effect of IL-36 signalling on cancer cells and resulting chemokine production, although this has been studied in chronic inflammatory disease [226]. Previous work investigating HT29 cells as a simple model of IBD showed that cell stimulation with IL-36α and IL-36γ resulted in a large induction of chemokines in a dose and time-dependent manner [287]. Although our data reflected this for IL-36γ stimulation of cells, our findings demonstrate that IL-36β is equally potent, with IL-36α stimulation resulting in much lower levels of gene induction of chemokines relative to other IL-36 agonists (Fig 3.4). This heterogeneity of response to IL-36 family members is reflected in the literature, with IL-36α and IL-36γ typically being the cytokines most studied in this context [406]. IL-36β and IL-36γ consistently outperformed IL-36α as a potent stimulant of pro-inflammatory gene

transcription in colon cancer cells of both human and mouse origin. These differences in potency may be dependent on two key factors; 1. IL-36 cytokines show differential binding affinities to the IL-36 receptor and therefore result in differential activation of downstream adapter proteins of gene transcription [225] and 2. IL-36 cytokines have been shown to be cleaved by several different proteases [210]. This explains the tissue and cytokine dependent effect observed which varies according to surrounding immune cell composition; which provide proteases for IL-36 cytokine activation [216, 218].

CXCL-1 was one of the most highly upregulated cytokines expressed by colon cancer cells in response to IL-36R signalling, even with low concentrations of IL-36β and IL-36γ capable of inducing large increases in chemokine transcription (Fig 3.3-3.5). CXCL-1, a myeloid cell chemoattractant, has been highly implicated in the pathogenesis of CRC, with this cytokine playing a role in the adenoma-adenocarcinoma sequence (77% of cases), angiogenesis and recruitment of immunosuppressive cells such as tumour-associated neutrophils (TANs) and myeloid-derived suppressor cells (MDSCs) [420, 421].

IL-8, the product of the CXCL8 gene and neutrophil chemoattractant, was also highly upregulated in response to IL-36R signalling (Fig. 3.3). IL-8 has been implicated in the pathogenesis of CRC with direct effects via CXCR2-expressing cancer cells resulting in increased angiogenesis and metastatic potential of these cancer cells [422]. Well-characterised IL-36-dependent pathologies, such as psoriasis, consistently show neutrophilia in inflamed tissues [423]. Neutrophils are the primary source of activating proteases for pro-IL-36 cytokines [216] and this influx of neutrophils to tissue sites may induce a potent feedback loop by increased IL-36 cleavage [424]. This IL-36 driven neutrophilia has not yet been characterised in colon cancer and may be worth investigating

given the findings of this work and the direct effects of IL-8 on both colon cancer cells and neutrophils.

In addition to CXCL-1, CCL2 and TGF-β expression were both increased in murine cancer cells (Fig. 3.4). CCL2 has been extensively characterised as a pro-tumourigenic cytokine with a role in tumour growth and metastasis via TAM recruitment and MDSC recruitment [425]. The subsequent infiltration of immunosuppressive cells can alter the TME to a 'wound-healing' phenotype and contribute to tumour progression by conversion of the TME to a mesenchymal type, with poorer prognoses' for patients [426]. Similarly, TGF-β was shown to be significantly upregulated by this IL-36 stimulation in CT26 cells, with this cytokine also reported to be strongly influential in mesenchymal-classed tumours in CRC [427]. Collectively this data indicates IL-36-stimulation of colon cancer cells results in downstream gene transcription of chemokines which can contribute to tumour progression. Collectively these findings show IL-36 cytokines possess different potencies in inducing gene transcription in colon cancer cells; resulting in myeloid-lineage chemoattractant (CXCL1, CCL2, IL-8, CCL20) expression which is likely to change the TME to an immunosuppressive environment.

Proliferation represents one of the hallmarks of cancer. Cytokines have long been implicated as pluripotent signalling molecules, with many having growth factor-like properties in cancer [428]. The pluripotent nature of IL-1 cytokines is exemplified by IL-36R signalling and its ability to induce cellular proliferation across many types of immune and stromal cell populations [406]. Stimulation of peripheral $CD4^+$ T cells and naïve $CD4^+$ T cells with IL-36 can result in their rapid proliferation [295, 296, 429]. IL-36 cytokines may also induce cellular proliferation of $CD8^+$ T cells and NK cells [299]. Thus, IL-36 signalling has

been suggested as a possible adjuvant in immune checkpoint blockade (ICB) [379]. However, IL-36 cytokines can also induce the proliferation of regulatory T cells, which may represent a negative feedback loop, suppressing the immune response [379]. Moreover, IL-36 signalling also drives cellular proliferation in several stromal cell types such as intestinal fibroblasts, which may contribute to mesenchymal-type CRC [289, 430], and also in cancer cells [243]. Consistent with our findings in colon cancer (Fig. 3.9 and Fig. 3.11), a very recent study demonstrated that IL-36 cytokines promotes proliferation in gastric cancer cell lines [243]. Furthermore, two studies have also reported a pro-proliferative role for IL-36 signalling in colon cancer cells, with IL-36R signalling inhibition capable of reducing colon cancer cell proliferation as shown by IL-38, a natural IL-36R antagonist, shown to inhibit this phenotype [431]. Given that IL-36 signalling has been shown to have a clear beneficial effect in the augmentation of the anti-tumour response [380, 413, 414], the emergence of a pro-tumourigenic role for IL-36 signalling on tumour cells calls for the careful consideration of IL-36 cytokine adjuvant therapy in cancer. Patient stratification based on IL-36R expression would allow IL-36R negative tumours to benefit from IL-36 cytokine immune augmentation without the risk of proliferative bystander effects on cancer cells [379]. This could also be inhibited by an anti-IL36R antibody such as Spesolimab or by development of IL-36Ra or IL-38 as next generation chemokines [432].

Cellular migration, as well as being a hallmark of cancer, remains one of the most important factors of clinical outcomes for CRC patients, with stage IV cancers characterised by detection of distant metastases to the lung or liver. The five-year survival rate drops dramatically as cases progress to stage IV, with survival rates of 93% and 8% occurring in stage I and stage IV cancers, respectively [433]. Our *in vitro* data indicates that IL-36 cytokines, primarily IL-36β and IL-36γ, are potent inducers of cellular migration and cellular

invasion. To date, essential processes in the migration and invasion of cells involves remodelling of the extracellular matrix, focal complex formation followed by invadopodia and cytokine feedback loops which sustain this invasive phenotype of cancer cells [434]. It has been previously reported that IL-36 cytokines can directly stimulate the migration of cells, as observed with trophoblast migration in *vitro* [435] as well as inducing gastric cancer cell migration/invasion, contributing to their pro-tumourigenic phenotype [243]. Contrasting this, IL-36α can markedly supress epithelial ovarian cancer cell migration and invasion *in vitro* [371]. IL-36 cytokines have also been implicated as major driver of intestinal fibrosis [289], with this work showing IL-36-driven upregulation of matrix-remodelling genes, a mechanism integral to cancer cell metastasis [434]. In agreement with this, IL-36γ and IL-36Ra can regulate colon tumourigenesis by modulation of the cell-matrix adhesion network and WNT signalling, all of which contribute to the metastatic potential of primary tumours [436]. Moreover, IL-38 can inhibit colon cancer cell migration through downregulation of ERK signalling on cancer cells [431]. Collectively, our findings, in agreement with other studies, demonstrate that IL-36 cytokines may play an important role in cancer cell migration and invasion. In order to prevent the metastatic potential of early stage colon cancer in patients with multiple tumours which are not entirely resectable, it may be of benefit to use targeted IL-36R neutralizing therapies to prevent the pro-migration/invasion effect of IL-36 cytokines on primary cancer cells.

In conclusion, this chapter has shown IL-36 cytokines to influence a variety of cancer cell processes *in vitro*. The data presented here demonstrates IL-36 expression to vary between cells lines, although IL-36R expression was consistently highly expressed which suggests that this signalling pathway is greatly beneficial to cancer cell survival and disease progression. The upregulation of IL-36 agonist expression in HT29 cells, as well as the

constitutive expression of IL-36R in cancer cell lines, suggests that epithelial cell IL-36 signalling is a key component which has the potential for autocrine signalling, especially within the context of colon cancer given the associated infiltration of neutrophils [103]. Furthermore, sustained IL-36 signalling in colon cancer cells may then proceed to contribute to the migratory and invasive capacity of these cells. Given the interest in IL-36 cytokines as an adjuvant therapy in cancer to augment the immune response for long-term tumour rejection, this chapter calls for the careful consideration of this therapy, given potential bystander effects on the cancer cells themselves. Finally, this chapter proposes IL-36R inhibition may be an effective therapy to reduce the pro-tumourigenic phenotype of cancer cells.

Chapter 4

In vivo characterisation of IL-36 signalling in pre-clinical models of colon cancer

Results of this chapter were published in the following manuscript;

Baker KJ, O'Donnell C, Bendix M, Keogh S, Byrne J, O'Riordain M *et al*. IL-36 signalling enhances a pro-tumorigenic phenotype in colon cancer cells with cancer cell growth restricted by administration of the IL-36R antagonist. Oncogene 2022

4.1. Aim

Until recently, *in vivo* investigations of IL-36 cytokine signalling in cancer have primarily focussed on the role these cytokines can play in the anti-tumour immune response [199]. Given the pro-tumourigenic phenotype characterised from our *in vitro* findings shown in chapter 3 of this thesis and the dichotomous role of other IL-1 family members in cancer [196, 400], the aim of this chapter is to compare induction, by IL-36 agonist administration, or suppression, by IL-36 antagonist administration or gene editing, of IL-36R signalling in colon cancer tumour models *in vivo.*

4.2. Introduction

The IL-36 family has been studied across many different cells and tissues in order to elucidate their homeostatic role and contribution to, or resolution of, disease [226]. These studies have involved patient cohort data as well as *in vitro* characterisation assays and *in vivo* functional assessment. IL-36 signalling has been particularly studied in the pathogenesis of inflammatory diseases such as psoriasis, chronic lung inflammation, IBD and rheumatoid arthritis [406]. Through *in vivo* modelling, including gene-editing and gene-knockout mice studies, the understanding of IL-36 cytokines in such diseases has significantly improved with targeted therapies subsequently developed [196, 396, 432].

A good example of how *in vivo* models have contributed to enhanced understanding of the role of IL-36 can be seen in the field of IBD. Pre-clinical models have elucidated the pluripotent nature of IL-36 cytokines with effects shown on immune infiltrate and stromal cells involved in chronic intestinal inflammation [396]. Intraperitoneal (IP) injection of IL-36 cytokines has demonstrated that IL-36R ligands can drive intestinal fibroblast activity, with these researchers also showing that IL-36RKO mice have reduced mucosal inflammation compared to heterozygous control mice [238, 289]. Moreover, blockade of IL-36R signalling using an anti-IL-36R mAb successfully reduced intestinal inflammation and fibrosis in separate models of chronic intestinal inflammation including Chronic DSS and Chronic 2,4,6-Trinitro Benzene Sulfonic Acid Colitis [289]. Several other studies have also used *in vivo* models to further elucidate the roles of IL-36 cytokines in IBD, which include intestinal cell activation and proliferation, as well as augmentation of immune cell trafficking to disease sites [234, 238]. Such *in vivo* studies, amongst others, have laid the foundation for clinical trials of Spesolimab, a neutralising anti-IL-36R mAb originally developed for

treatment of psoriasis, but now being trialled for long term treatment of patients with moderate-to severe ulcerative colitis (NCT03648541).

Similarly, the use of *in vivo* preclinical models has greatly enhanced our understanding of the role of IL-36 signalling in cancer. To date, such models have predominately focussed on harnessing IL-36 signalling to promote anti-tumour immunity [199]. This has been investigated by genetic modification of tumour cells to overexpress IL-36 cytokines and investigating the subsequent physiological effects on tumour growth. Using lentiviral transfection, IL-36β overexpression has been shown to reduce tumour burden in mice by increasing the levels of $CD8^+$ T cells and NK cells in models of pancreatic cancer [376]. Similar trends have also been observed in mouse melanoma models, with IL-36γ-overexpressing B16 melanoma tumours shown to have increased $CD8^+$ T cell and NK cell infiltration which reduced the tumour burden in these models [299]. This work was also repeated in metastatic breast cancer models with similar findings reported [299]. Furthermore, adenoviral transduction of dendritic cells to overexpress IL-36γ has been shown to beneficially mediate the formation of Tertiary Lymphoid Structures (TLSs) in tumours, which are positive prognostic markers for long-term clinical responses, especially in the context of immunotherapy [380]. Although these efforts have looked promising as potential future therapeutics, no pre-clinical models during the writing of this chapter had investigated the direct roles of IL-36R signalling on cancer cells themselves using *in vivo* models.

Given the pro-tumourigenic phenotype induced by IL-36R signalling on cancer cells and the lack of *in vivo* models investigating the direct role of IL-36 signalling on cancer cells, the aim

of this chapter was to investigate and compare IL-36 cytokine signalling and antagonism in pre-clinical colon cancer models.

4.3. Results

4.3.1. Administration of IL-36Ra or IL-36β reduces tumour growth *in vivo*

Given the *in vitro* findings presented in chapter 3 and the prominence of anti-tumourigenic functions ascribed to IL-36 signalling in the literature [199, 376, 379, 413], the role of IL-36R signalling in tumourigenesis using *in vivo* murine models was investigated. Initially, a syngeneic mouse model of colon cancer cells grown in immunocompetent Balb/c mice was selected to further characterise both IL-36 agonist and antagonist effects. Mice were subcutaneously injected with CT26 colon cancer cells and then intraperitoneally (IP) injected with PBS, IL-36Ra (antagonist) or IL-36β (agonist) twice-weekly; IL-36β was chosen for this experiment as this IL-36 cytokine had demonstrated the most potent induction of pro-tumourigenic phenotypes *in vitro* (Fig 3.4). IP injection of treatments began once the majority of mice had subcutaneously palpable tumours. Mice were culled on day 28 after injection of CT26 cells or upon reaching at least one humane endpoint (Fig 4.1a). As expected, PBS control-treated tumours rapidly increased in size. IL-36β-treated mice showed a reduced tumour burden in mice throughout the study after IP injections had commenced (Fig.4.1b). IL-36Ra-treated mice showed the smallest tumour burden when compared to both PBS control mice and IL-36β-treated mice (Fig. 4.1c). This data suggested that IL-36β could reduce tumour burden in mice, but IL-36Ra treatment of mice could more effectively reduce the tumour burden in this *in vivo* model of tumourigenesis.

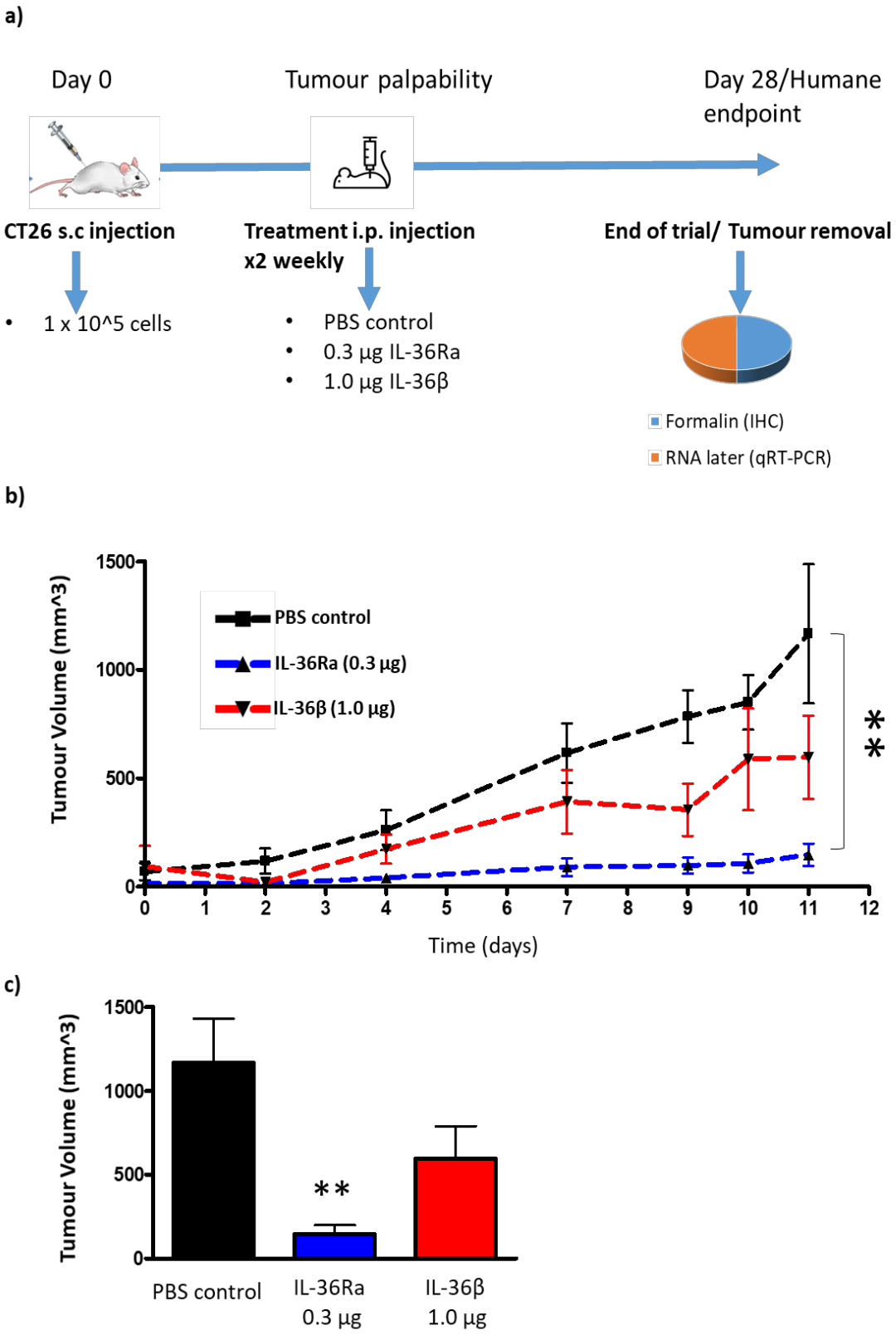


Figure 4.1 Alterations in IL-36 signalling can reduce tumour burden in mice. **a)** Study design of mouse model**. b)** Tumour growth curves over time for PBS control, IL-36Ra treated group and IL-36β treated group (n=7 mice per group). **c)** Final average tumour volumes for groups. One-way ANOVA with post-hoc Dunnett's test is annotated by the number of stars (**: p-value <0.01)

4.3.2. IP administration of IL-36β increases tumoural cytokine transcription and increases intratumoural CD8$^+$ cell infiltration

In order to assess the previously reported immune-associated functions of IL-36 cytokines in the TME, bulk RNA was extracted from excised tumours, cDNA synthesised and gene expression of several cytokines was assessed by qRT-PCR (Fig. 4.2). No significant changes were observed when comparing IL-36Ra and PBS treated groups although a trend of reduced CXCL-1 expression was shown (P >0.05). In contrast, IL-36β treated mice showed an increase in transcription of several genes such as CCL2, IFN-γ and Granzyme-B (GzmB) as compared to the PBS group (Fig 4.2).

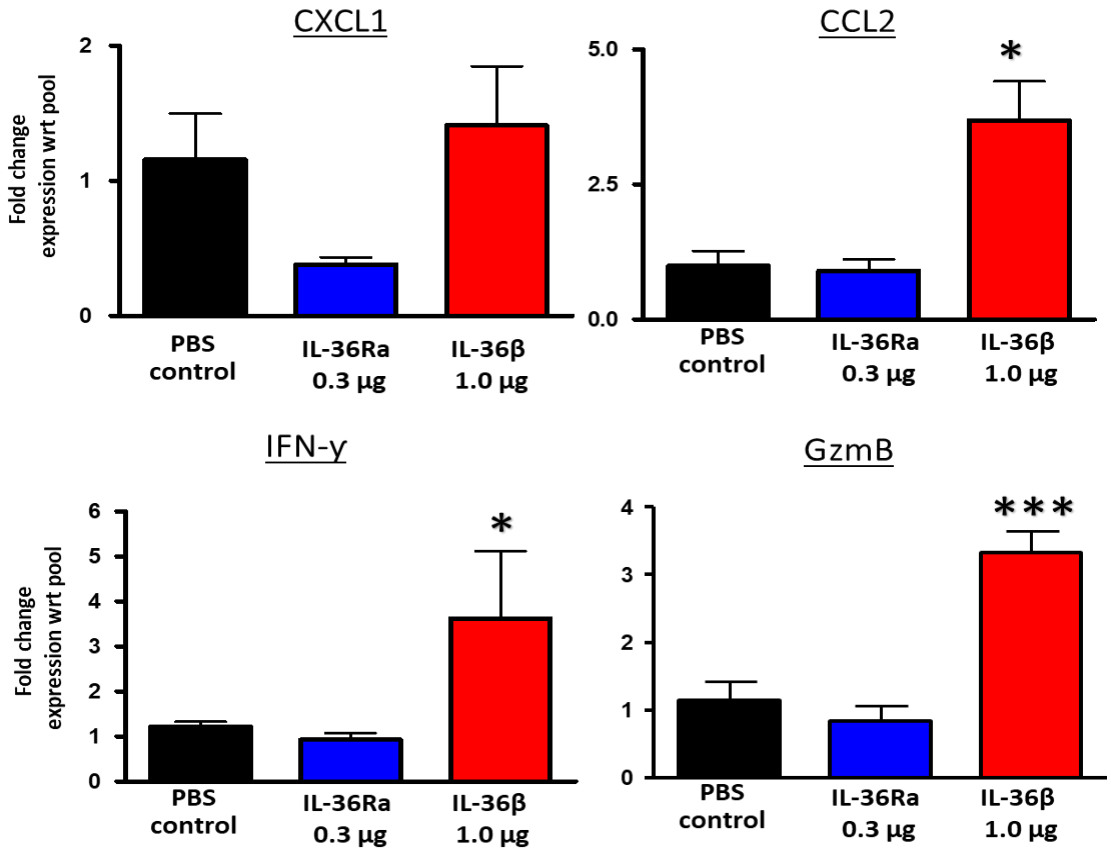


Figure 4.2: IL-36β significantly changes transcriptome of CT26 s.c. tumours when compared to IL-36Ra and PBS-treated controls. Tumour RNA was extracted with cDNA subsequently synthesised and qRT-PCR gene expression analysis performed on the genes indicated above. One-way ANOVA with post-hoc Dunnett's test is annotated by the number of stars (*: p-value < 0.05; ***: p-value <0.001).

Tumour samples were also processed for IHC analysis which similarly revealed no significant changes in immune infiltration between PBS control mice tumours and IL-36Ra-treated mice tumours (Figure 4.3a). However, tumours from mice treated with IL-36β showed an increase in $CD8^+$ T cell infiltration, although this was not statistically significant ($P > 0.05$) (Fig. 4.3b). This data suggested that IL-36β mediated tumour burden reduction could be, at least in part, via augmentation of the immune response against colon cancer cells.

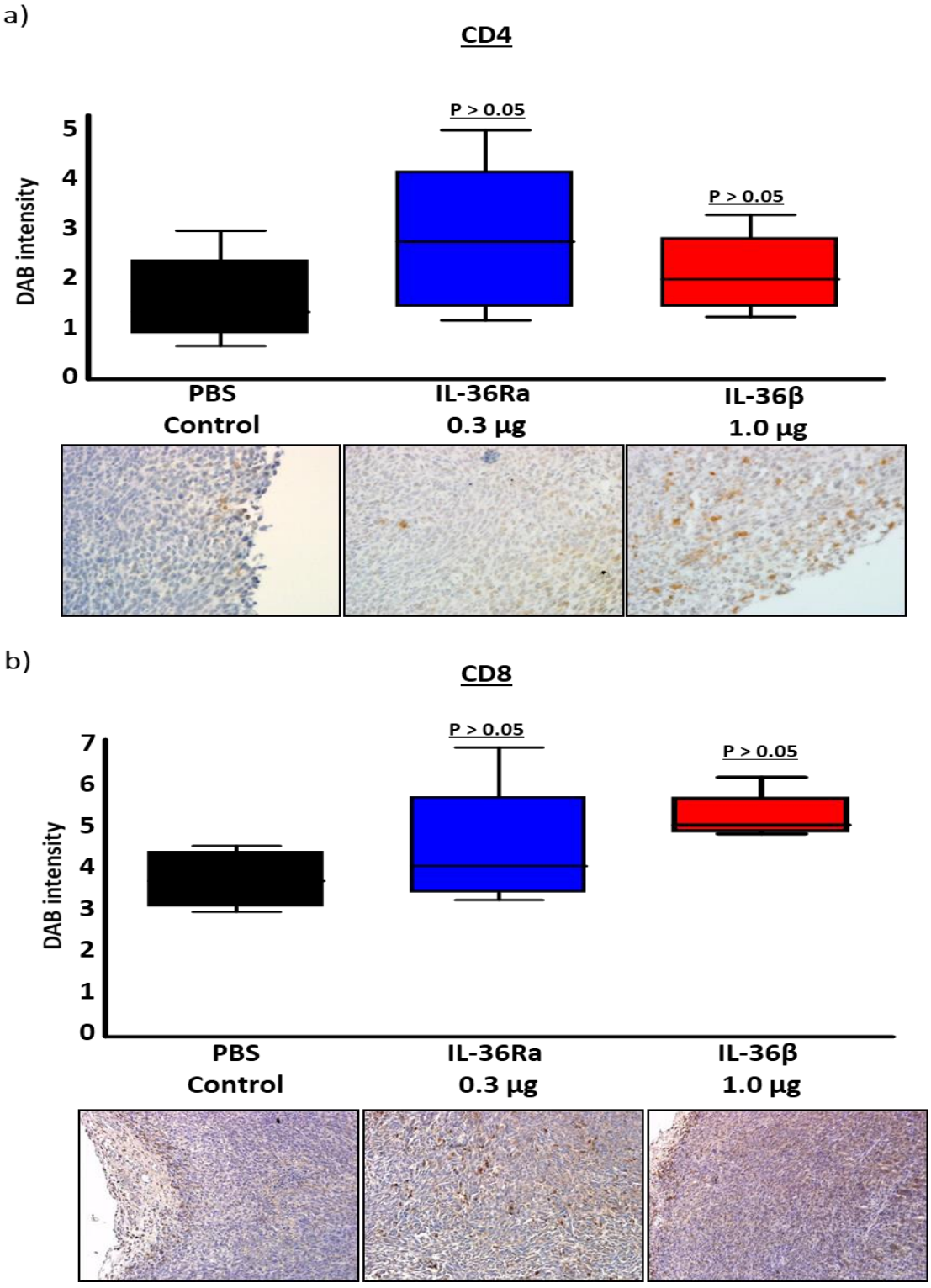


Figure 4.3: IL-36β and IL-36Ra treated mice showed no significant changes in immune population infiltration, although a trend of increased CD8$^+$ T cell infiltration was observed. Excised tumours were processed for IHC analysis for helper T cell and cytotoxic T cells markers, CD4 and CD8, respectively. Images were quantified by ImageJ based on DAB intensity. One-way ANOVA with post-hoc Dunnett's test was used to determine statistical significance.

4.3.3. IP administration of IL-36Ra reduces tumoural Ki67 expression

Given the proliferative effects of IL-36 cytokines previously reported in the literature [406], as well as the potent effects observed during *in vitro* assays described in chapter 3, further IHC analysis was performed to assess the levels of cellular proliferation in the tumours for each of the treated groups. No significant difference was observed between PBS control and IL-36β treated mice groups. However a significant reduction in Ki67-expressing cells was observed in IL-36Ra treated mice ($P < 0.05$) (Fig. 4.4). This suggested that the reduced tumour burden in IL-36Ra-treated mice could be, at least in part, as a result of suppressed pro-proliferative signalling induced by IL-36R signalling.

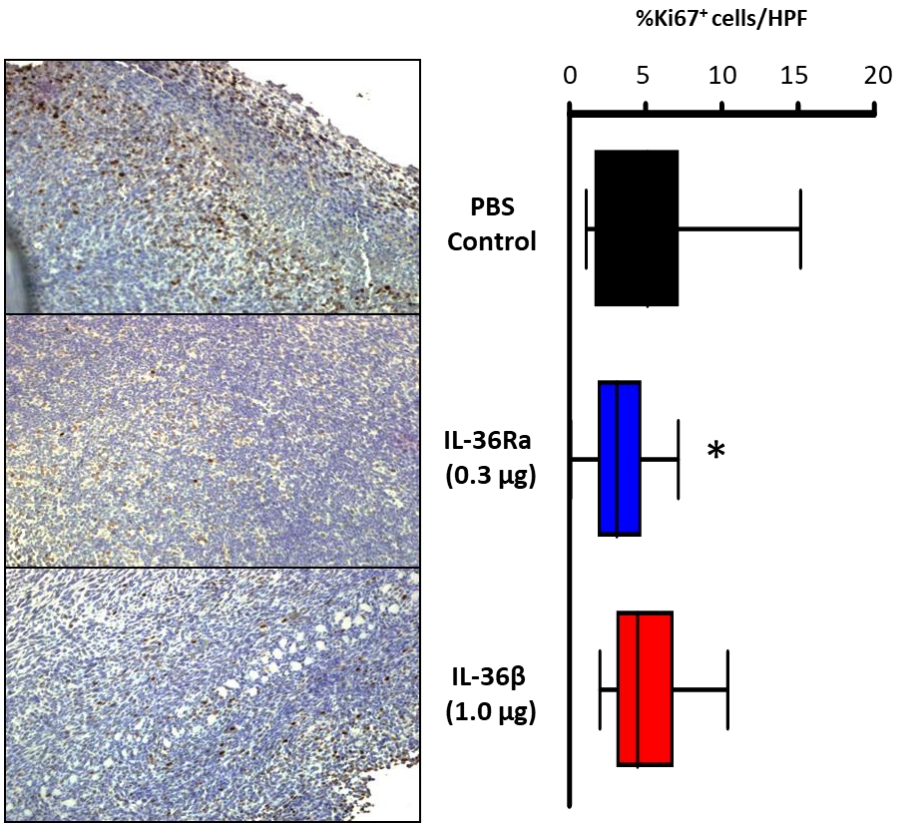


Figure 4.4: IL-36Ra treated mice tumours contain fewer Ki67 expressing cells. Excised tumours were processed for immunohistochemical analysis the proliferative cell marker, Ki67. Images were quantified as percentage $Ki67^+$ cells per high power field. . One-way ANOVA with post-hoc Dunnett's test was used to determine statistical significance (*: p-value < 0.05).

4.3.4. IP administration of IL-36Ra can more effectively reduce tumour burden in mice than IL-36 agonist administration

Although our previous *in vitro* assays clearly indicated that IL-36 signalling on tumours cells could significantly induce a pro-tumourigenic phenotype on CT26 cells, at the time of completion of this mouse model there had been no previous reports in the literature indicating that modification of IL-36R signalling would reduce tumour burden in mice. In order to further investigate this inhibitory effect, another Balb/c syngeneic model of tumourigenesis was completed using CT26 cells. However in this instance, groups were treated with the IL-36 receptor antagonist (0.3 μg or 1.0 μg) or a cocktail of the three IL-36 agonists (to better represent our previous findings of increased IL-36α, IL-36β and IL-36γ expression in colon tumours). In contrast to the previous *in vivo* trial, treatments were administered before tumours became palpable to investigate prophylactic administration capacities of IL-36 signalling/inhibition in the prevention of tumour growth, rather than as a curative treatment (Fig 4.5a). Using this approach, IL-36Ra treatment of mice again resulted in a significantly smaller tumour burden when compared to PBS control treated mice and IL-36 agonist treated tumours. IL-36 agonist treated mice also showed a significant reduction in tumour volume, although this was less effective in reducing tumour burden than IL-36Ra treatment (Fig 4.5b). This was particularly evident in the average tumour volume of groups once tumour tissues were excised from the rear flanks of mice (Fig 4.5c). This suggested IL-36Ra administration may be a more useful therapeutic to develop for more effective colon cancer growth inhibition.

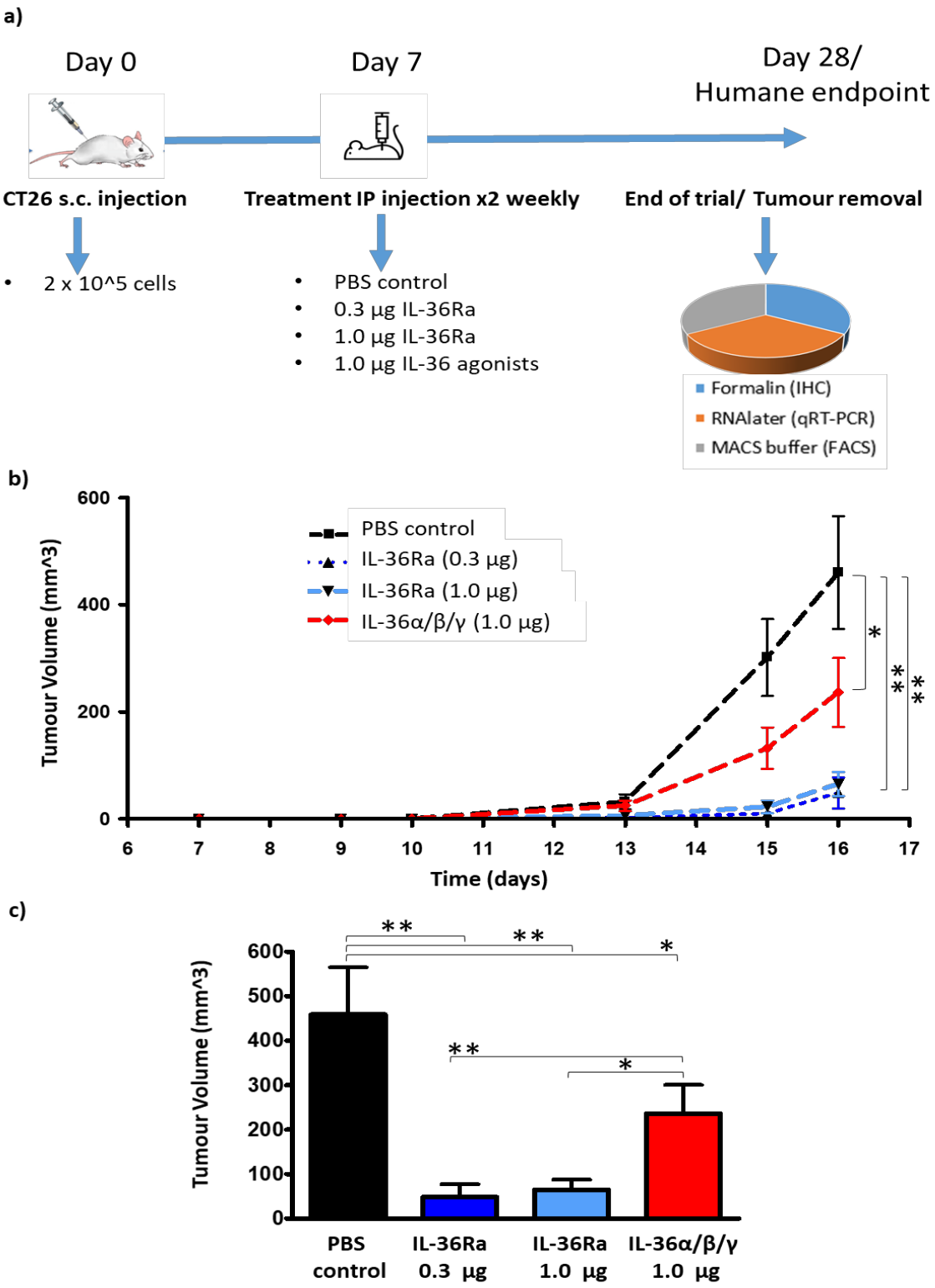


Figure 4.5 Inhibition of IL-36 signalling can more effectively reduce tumour burden in mice than IL-36 signalling augmentation. **a**) Study design of mouse model. **b**) Tumour growth curves over time for PBS control, IL-36Ra treated groups and IL-36 agonist cocktail (equal amounts of IL-36α/β/γ) treated group (n=7 mice per group). **c)** Final average tumour volumes for groups. One-way ANOVA with post-hoc Dunnett's test or Tukey's Test is annotated by the number of stars (*: p-value < 0.05; **: p-value <0.01)

4.3.5. IP administration of the IL-36 agonist cocktail alters tumoural cytokine transcription and intratumoural immune cell composition

Similar to the previous mouse model, bulk RNA was extracted from tumour tissue and gene expression was analysed by qRT-PCR. Tumours from IL-36 agonist-treated mice showed differential gene expression of cytokines and proteases including CCL2 and GzmB, as well as a trend of increased IFN-γ gene expression. Tumours from mice treated with the IL-36Ra showed no significant changes in gene transcription in comparison to PBS control mice; although a trend towards decreased CCL2 expression was observed between the two doses of IL-36Ra, with the higher dose showing lower levels of CCL2 transcription (Fig 4.6).

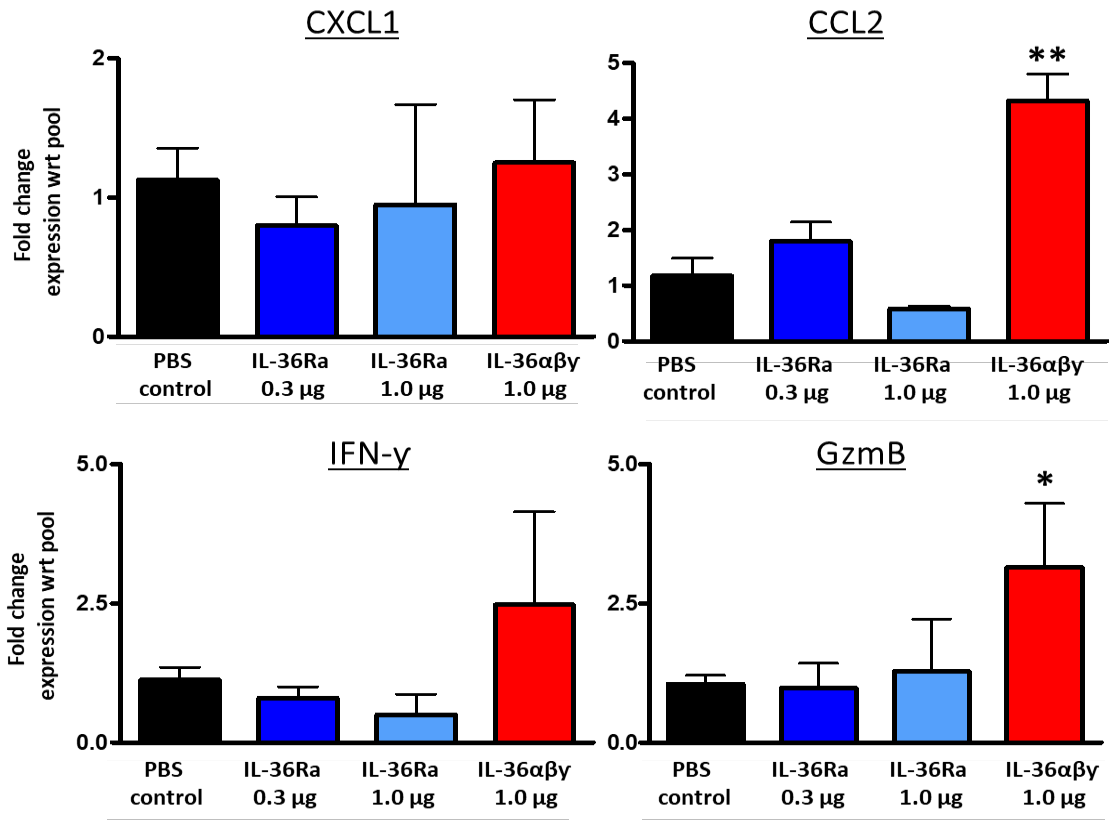


Figure 4.6 IL-36 agonist signalling significantly changes the transcriptome of CT26 subcut tumours when compared to IL-36Ra and PBS-treated controls. Tumour RNA was extracted with cDNA subsequently synthesised and qRT-PCR gene expression analysis performed on genes outlined above. One-way ANOVA with Dunnetts post hoc test is annotated as; *: p-value < 0.05; **: p-value <0.01)

IHC analysis revealed a significant decrease in $CD4^+$ cell infiltration in IL-36 agonist treated mice compared to PBS control mice (Fig 4.7a). As observed in the previous mouse model, a trend of increased $CD8^+$ T cell infiltration was detected in IL-36 agonist treated mice in comparison to the PBS control mice (Fig. 4.7b). This change in immune infiltrate indicated that the observed reduction in tumour burden likely resulted from IL-36 agonist-induced alterations in the TME composition.

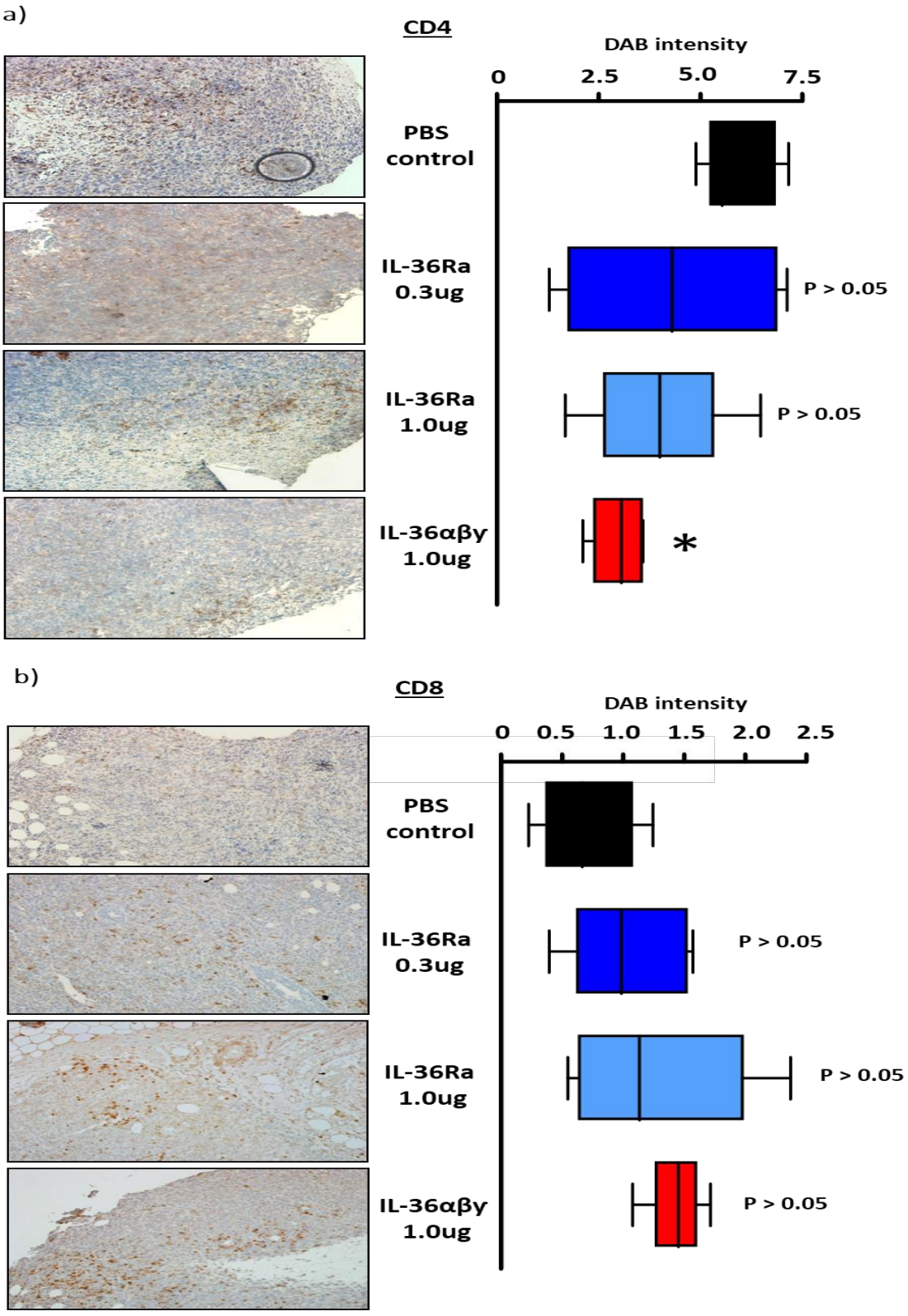


Figure 4.7 IP injection of IL-36 agonist cocktail significantly reduces CD4$^+$ cell infiltrate in tumours with a trend of increased in CD8$^+$ cell infiltration. Excised tumours were processed for IHC analysis for helper cell and cytotoxic t cells markers **a)** CD4 and **b)** CD8, respectively. Images were quantified by ImageJ based on DAB intensity. One-way ANOVA with Dunnett's post hoc test is annotated by the number of stars (*: p-value < 0.05).

4.3.6. IP injection of IL-36Ra dose-dependently decreases Ki67 expression in s.c. tumours

As per the previous murine trial, Ki67 expression was assessed by IHC as a marker of cellular proliferation. Both doses of IL-36Ra reduced the number of $Ki67^+$ cells in tumours in a dose dependant manner, further implicating IL-36Ra as an inhibitor of cellular proliferation (Fig. 4.8). Furthermore, no significant reduction in $Ki67^+$ cells was detected in IL-36 agonist treated tumours, further indicating that IL-36 agonist tumour growth reduction may be more associated with modification of the immune response, as indicated by changes in cytokine transcription of bulk tumour RNA and the changes of immune infiltrate composition (Figures 4.6 and 4.7).

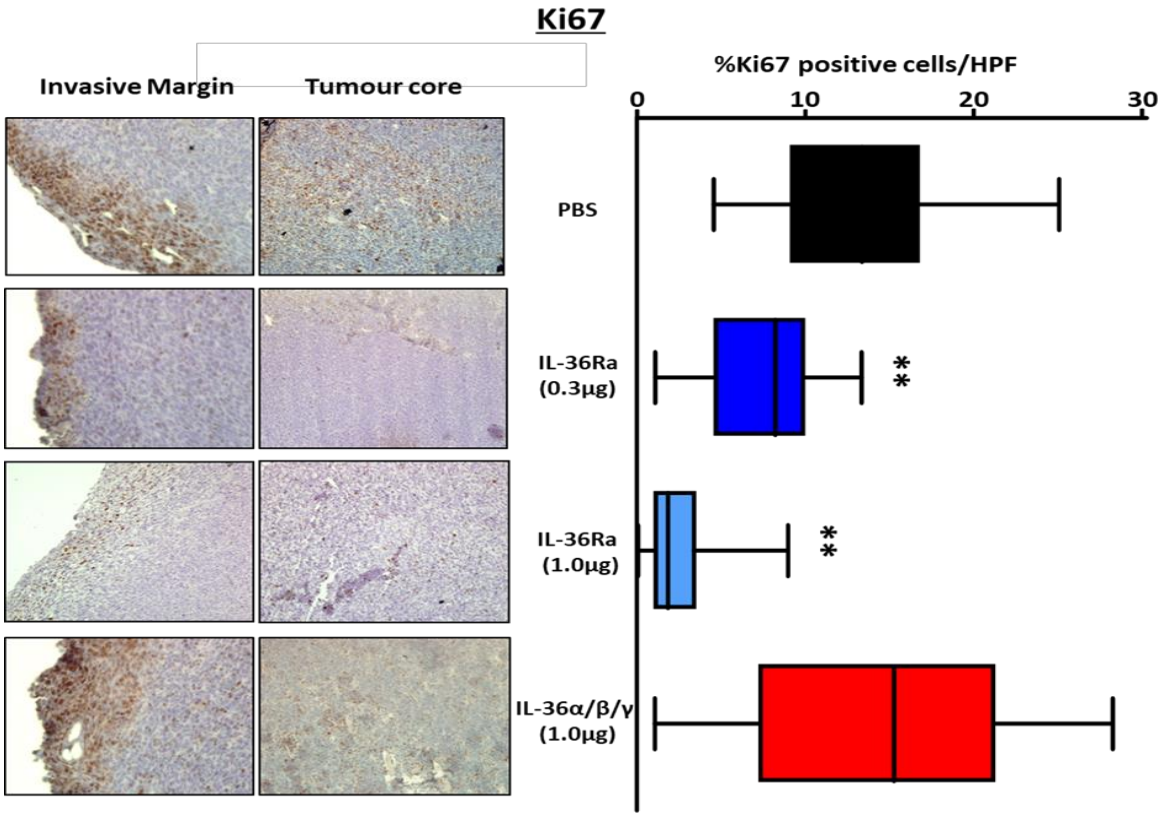


Figure 4.8: IP injection of IL-36Ra significantly decreases tumoural Ki67 expression. Excised tumours were processed for IHC analysis for the proliferation marker, Ki67. Images were quantified as percentage $Ki67^+$ cells per high power field. One-way ANOVA with Dunnett's post hoc test is annotated by the number of stars (**: p-value <0.01)

4.3.7. IL-36 agonist treated murine tumours contain increased $CD4^+$ cell and $CD8^+$ cell infiltrate

To gain further insight into the differences between IL-36Ra and IL-36 agonist-mediated tumour burden reduction, FACS analysis was completed on excised tumour tissue from mice flanks. Tumour tissue was mechanically and enzymatically dissociated then separately analysed for surface and intracellular immune cell markers. The only significant differences detected from tumour tissues were increases in both $CD4^+$ and $CD8^+$ T cell infiltration in IL-36 agonist treated tumours when compared to PBS controls (Fig. 4.9). No significant differences were observed in $LY6G^+$ (neutrophils), $F4/80^+$ (macrophages) or $CD3^+/CD25^+/Foxp3^+$ (Treg) cell populations between groups. This data suggests that IL-36 agonist administration may have a greater effect on altering the adaptive immune response in this context, despite previously having been shown to upregulate myeloid cell chemoattractant gene transcription (Figures 4.2 and 4.6).

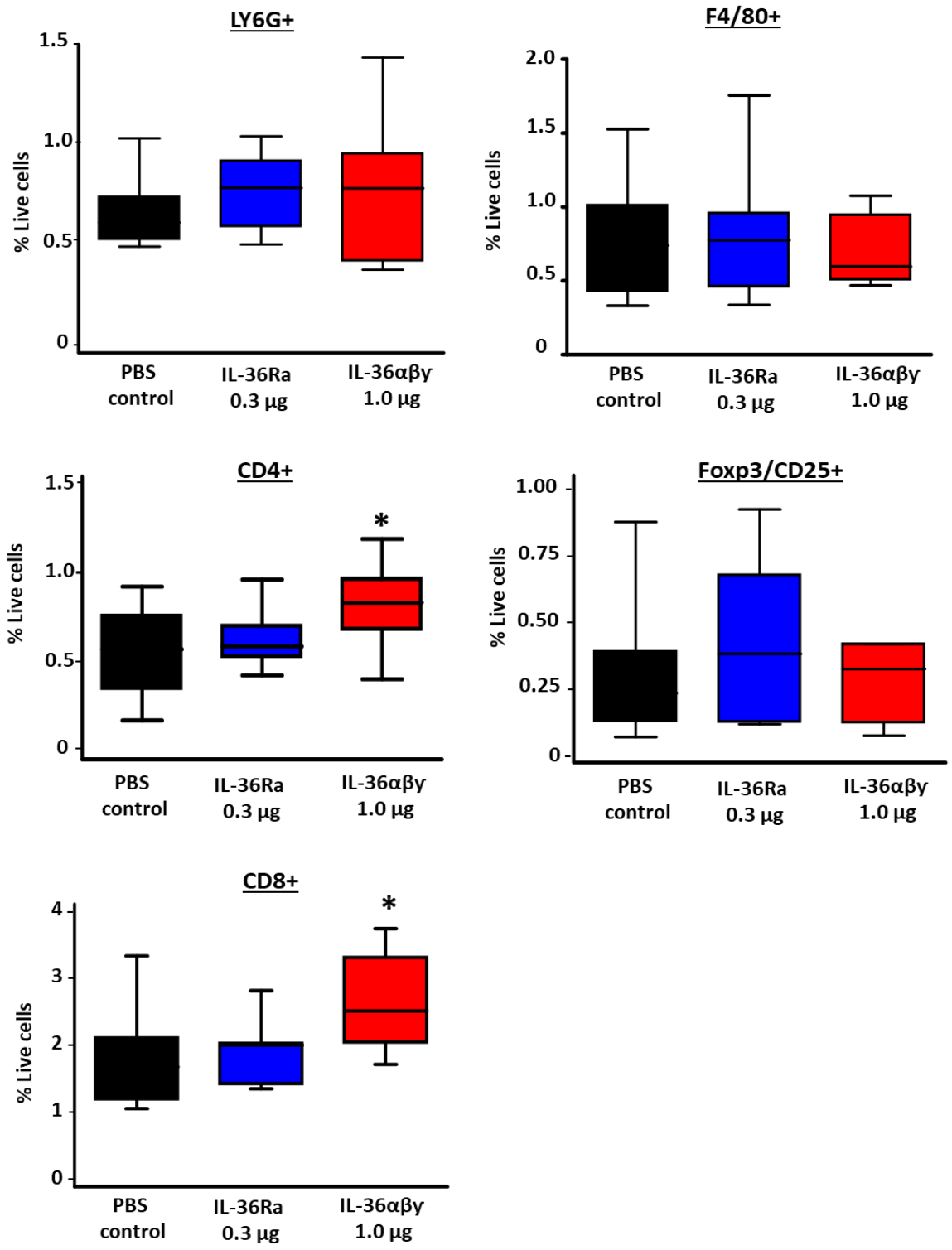


Figure 4.9: IL-36 agonists increase helper T cell and cytotoxic T cell infiltration into s.c. tumours. Excised tumours were enzymatically dissociated and stained, intracellularly and extracellularly for several immune cell markers. Increased $CD4^+/CD8^-$ cells and $CD4^-/CD8^+$ cells were detected in tumours of IL-36 agonist treated mice. One-way ANOVA with Dunnett's post hoc test is annotated by the number of stars (*: p-value < 0.05).

4.3.8. Generation and characterisation of CRISPR-Cas9 gene-editing control cells

Given that the reduction in tumour burden was observed following systemic administration of IL-36Ra, which would have the ability to affect multiple cell types, it was next of interest to investigate whether the IL-36Ra was acting directly on tumour cells to inhibit their proliferation. It was decided to achieve this by generating CT26 cells that did not express a functional IL-36R protein using CRISPR-Cas9 technology. To control for off-target effects of CRISPR-Cas9 transfection and puromycin selection of cells, two separate control cell clones were generated. These cell lines were generated simultaneously to the IL-36R KO cells, but for purposes of clarity, control cell line generation will first be described in this thesis. Firstly, a puromycin kill curve was completed using various concentrations of puromycin to determine which would be appropriate to kill all puromycin-sensitive cells during *in vitro* culture, gradually over 8 days. 5 µg/mL was determined to be the optimal concentration for this (Fig. 4.10).

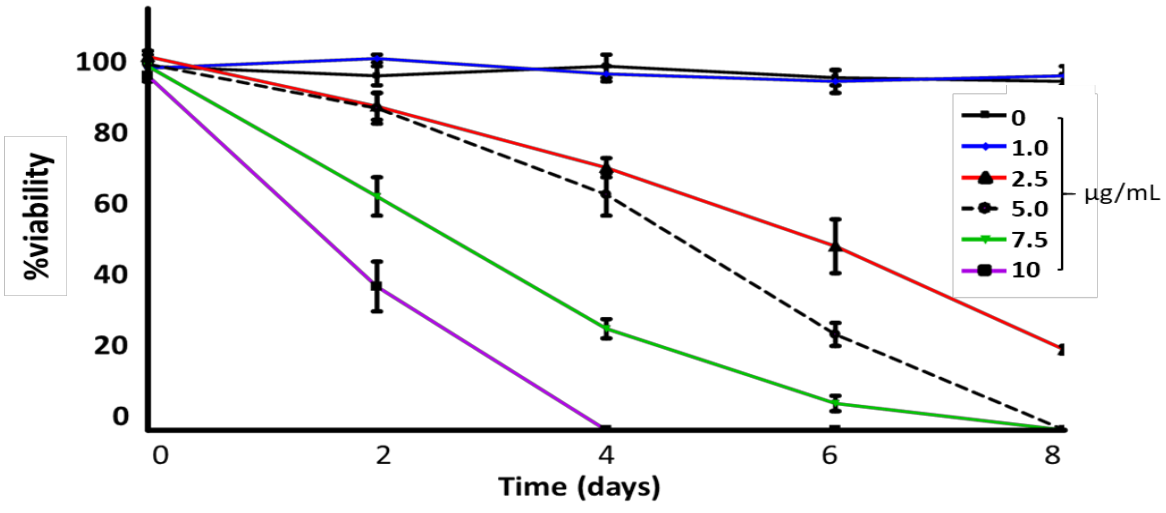


Figure 4.10: Puromycin kill curve optimisation for CT26 cells. CT26 cells were incubated in normal culture media containing varying concentrations of puromycin (0 – 10 µg/mL) to determine the minimum concentration required to kill all cells after 8 days. Cells were stained and counted to calculate %viability at each of indicated time points.

CT26 cells were transfected with the puromycin resistance plasmid (pcDNA3.1/Puro-CAG) and cultured in 5 µg/mL puromycin media to ensure antibiotic selection would not alter clone responsiveness to IL-36 treatment (Fig 4.11a). Puromycin-resistant cells were confirmed to be responsive to IL-36 cytokine stimulation and shown to have a similar response as WT CT26 cells in terms of CCL2 mRNA induction and cellular proliferation (Fig. 4.11b). Additionally, this process did not alter baseline levels of cellular proliferation as it was shown to be similar between WT cells and puromycin resistant control cells (Fig. 4.11b).

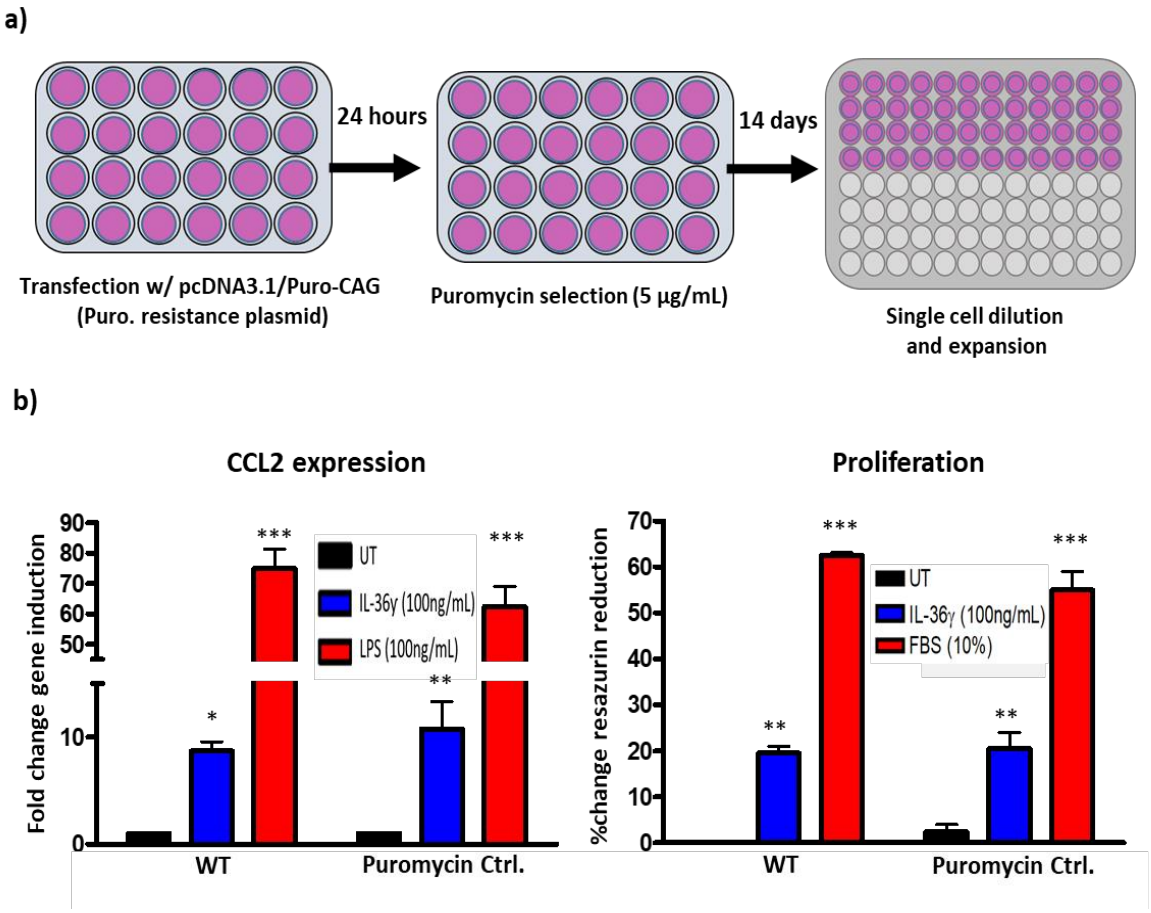


Figure 4.11: Puromycin selection does not alter puromycin resistant CT26 cellular response to IL-36 stimulation or baseline proliferation of cells. a) CT26 cells were transfected with puromycin-resistance plasmid and grown in media containing 5 µg/mL puromycin followed by single cell dilution and expansion. **b)** Puromycin control cells were stimulated with IL-36γ (100ng/mL) and CCL2 gene induction and cellular proliferation were measured by qRT-PCR and resazurin reduction, respectively. One-way ANOVA with Dunnett's post hoc test is annotated by the number of stars (*: p-value < 0.05, **: p-value < 0.01, ***: p-value < 0.001)

Additionally, a CRISPR-Cas9 control cell line was generated using Santa Cruz control plasmid sc-418922 (Fig 4.12a-i). This ensured transfection of cells with CRISPR-Cas9 technology did not have off-target effects. Following transfection of cells with the control plasmid, single cells were isolated by FACS based on transient GFP expression derived from successful plasmid transfection (Fig. 4.12a-ii). These cells were sequenced at guide RNA sites (as later described for IL-36RKO clones) and confirmed to share identical sequence homology to wild type CT26 cells at key indel locations, showing scramble control transfection did not genetically edit cells as later shown for IL-36R-KO plasmids. Cells were confirmed to function as wild type cells in response to IL-36 cytokine stimulation in terms of CCL2 gene induction and proliferation augmentation (Fig. 4.12b). Once more, this process did not alter baseline proliferation levels; as control cells showed a similar level of proliferation as WT cells (Fig 4.12b). This showed the process of cell line generation did not have any off-target effects on cells resulting in changes to their genotype or phenotype.

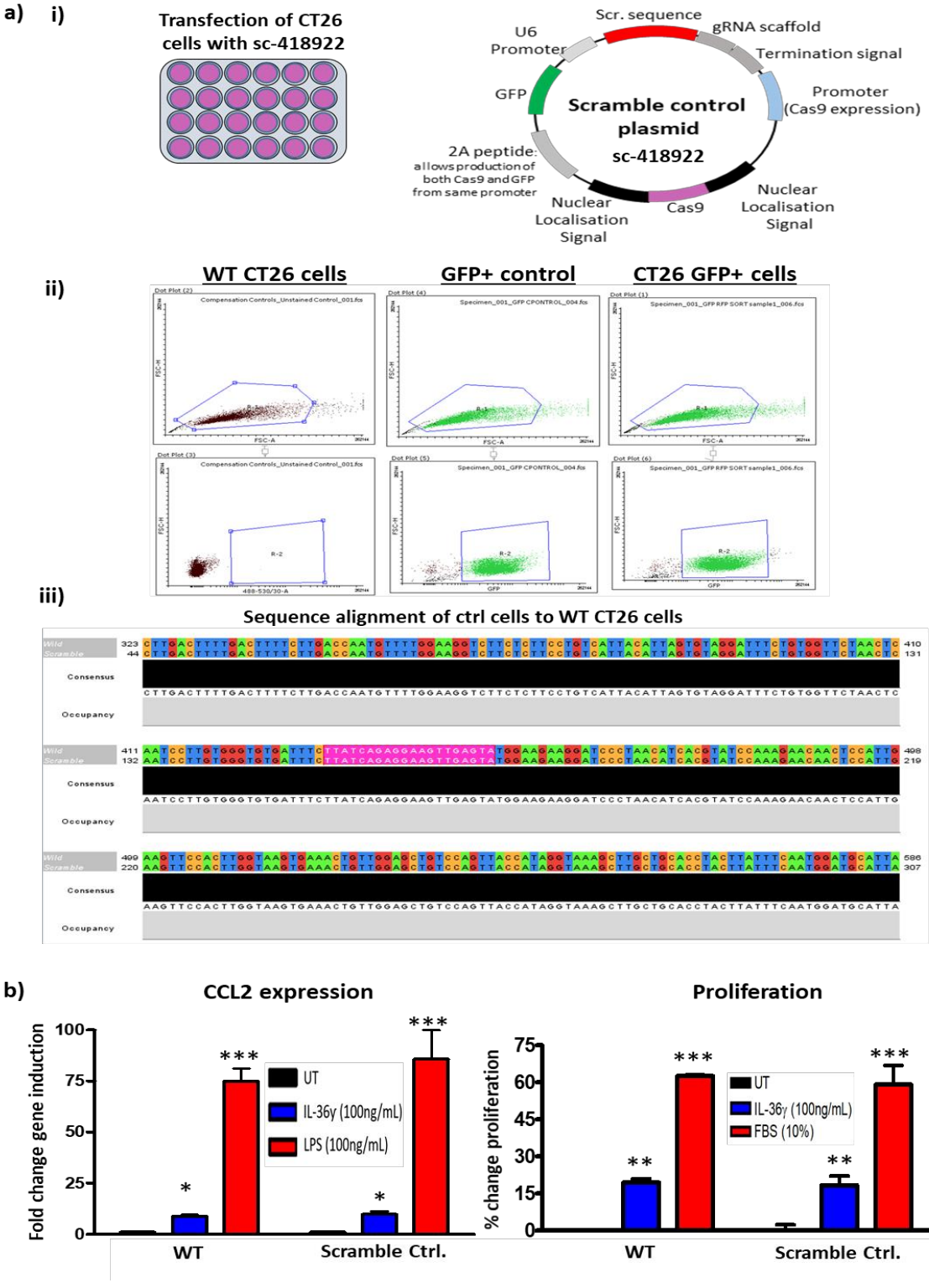


Figure 4.12: CRISPR-Cas9 scramble control cell lines generation. a) Work-flow of control cell line generation showing **i)** Transfection of cells in 24 well plate with control plasmid **ii)** GFP single cell sorting and **iii)** Single cell clone gRNA-site sequences were amplified by PCR then sequenced. Sequences were aligned with WT CT26 sequences and shown to be identical. **b)** Control cell response to IL-36γ stimulation was measured by CCL2 gene induction by qRT-PCR and resazurin reduction assay. One-way ANOVA with Dunnett's post hoc test is annotated by the number of stars (*: p-value < 0.05, **: p-value < 0.01, ***: p-value < 0.001).

4.3.9. CRISPR-Cas9 generation of IL-36R-KO clones cells

A combination of CRISPR-Cas9 plasmids were used to generate IL-36R KO CT26 cells, with the workflow of the experiment outlined in Figure 4.13. CT26 cells were transfected with a combination of HDR (Homology Derived Recombination) and NHEJ (Non-homologous end joining) plasmids targeting multiple locations within IL-36R exons (Fig 4.13a). HDR allowed for the insertion of a puromycin-resistance gene into transfected cells, which facilitated their identification based on their growth in puromycin-supplemented media (Fig 4.13b). Successfully transfected cells were cultured in 24 well plates in Puromycin containing media, as previously outlined. HDR also introduced a red fluorescent protein gene which facilitated cell sorting based on fluorescence. After puromycin selection had taken place, single cell clones were isolated prior to clonal expansion. Cells which expressed the highest levels of RFP were selected for and isolated, given the increased fluorescence was likely to reflect increased HDR plasmid insertion and thereby increase multi-allele edited clone detection probability (Fig. 4.13c). Single-cell isolated clones underwent clonal expansion and were characterised genotypically and phenotypically for IL-36R gene knockout (Fig 4.13d).

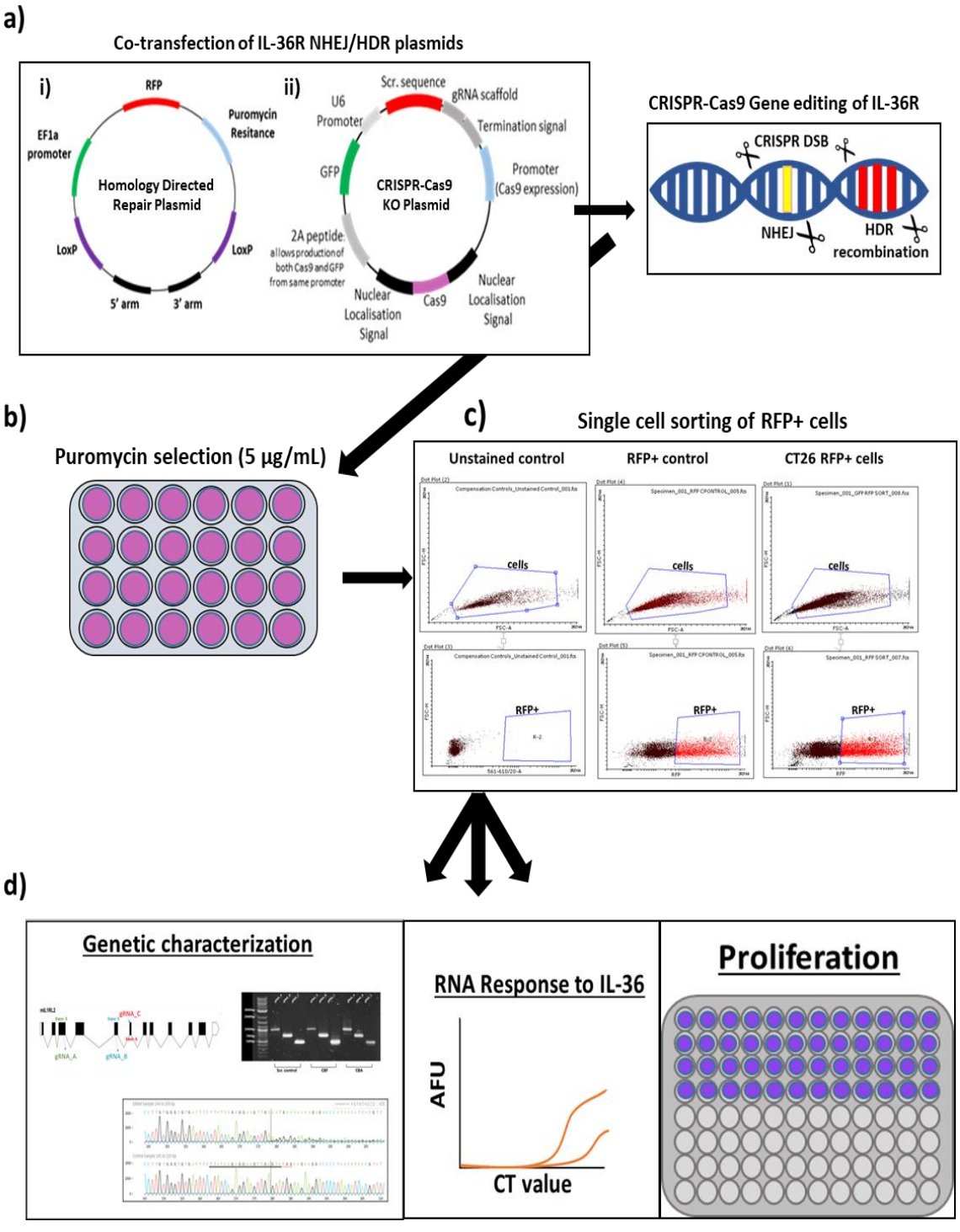


Figure 4.13: Workflow for generation of IL-36R KO CT26 cells using CRISPR-Cas9 technology. **a)** CT26 cells were co-transfected with NHEJ and HDR plasmids, **b)** cultured in Puromycin (5 µg/mL) and **c)** isolated by FACS based on RFP expression. **d)** Single cell clones were expanded then characterised genotypically and phenotypically.

4.3.10. Functional characterisation of IL-36R-KO clones cells in response to IL-36 stimulation

A subset of expanded clones that were generated, as per the workflow shown in figure 4.13, were next tested for responsiveness to IL-36 stimulation (n= 18). This preliminary screen was performed in a 96-well plate format using Sidestep Lysis buffer for high-throughput RNA extraction of clones, with CCL2 expression then assessed in response to IL-36 stimulation by qRT-PCR. Clones C8A and C8F were selected for further characterisation based on CCL2 expression in response to IL-36 stimulation (non-inducible) and LPS stimulation (Inducible) (Fig 4.14a). A six well plate format using increased numbers of cells was then used to stimulate selected clones with IL-36γ or LPS, with CCL2 mRNA expression analysed by qRT-PCR. This was done to confirm preliminary screen findings. Clones C8A and C8F were both shown to be indeed non-responsive to IL-36γ but still responsive to LPS in the form of increased CCL2 gene expression (Fig. 4.14b). Additionally, these two clones were examined for baseline proliferation and proliferative response to IL-36γ in comparison to control cells. This showed baseline proliferation levels of cells to be similar, but also showed the loss of proliferation induction in response to IL-36γ stimulation, whilst maintaining induction of cellular proliferation in response to 10% FCS (Fig 4.14c).

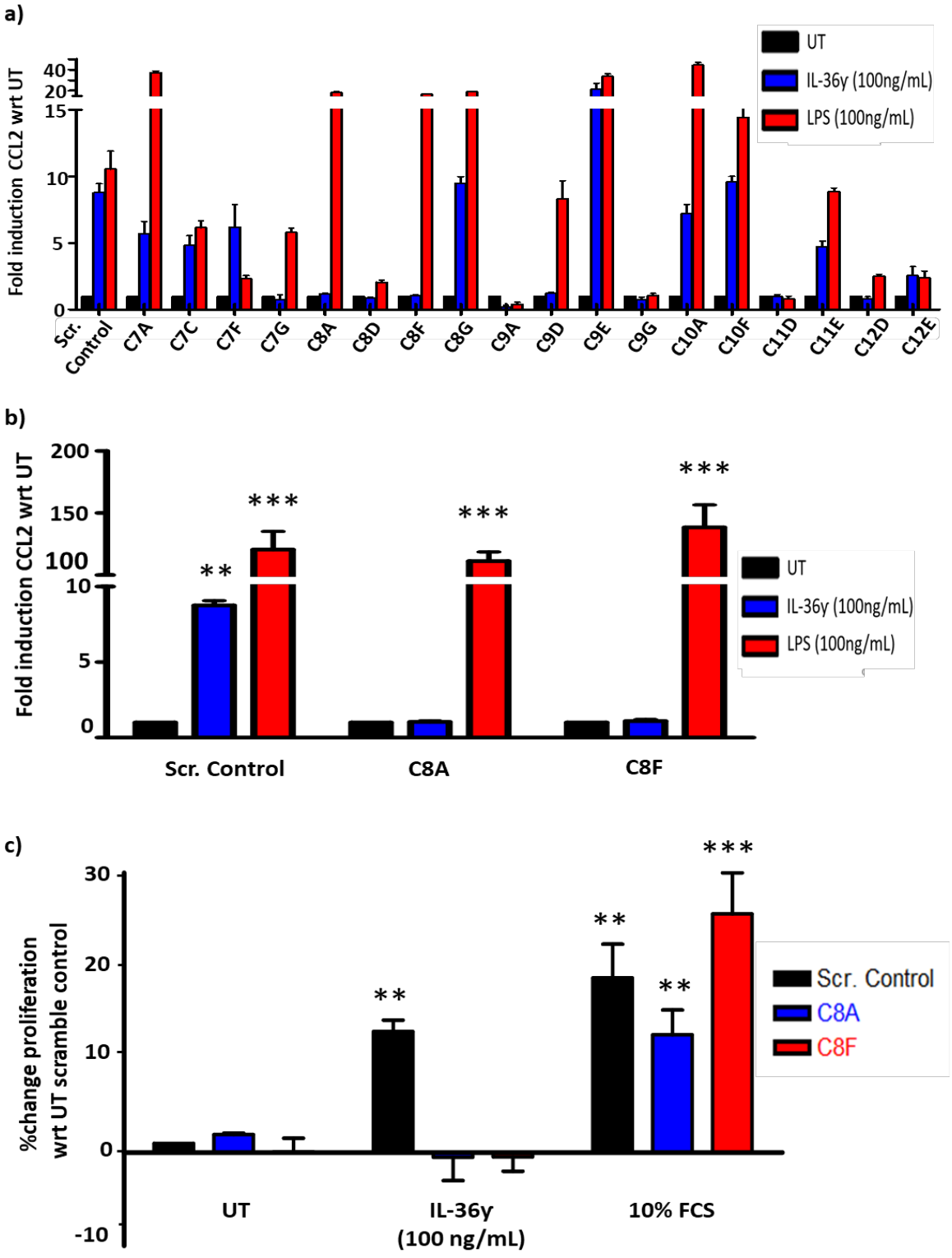


Figure 4.14: Confirmation of functional knockout of IL-36R in CT26 cells. a) Clones were screened for response to LPS or IL-36γ by CCL2 gene expression detection by qRT-PCR. **b)** Single cell clones were expanded and stimulation was confirmed in 6 well plate format by CCL2 induction measured by qRT-PCR following stimulation of cells with LPS or IL-36γ. **c)** Baseline proliferation of control and clones was assessed by resazurin reduction. Proliferative response to IL-36γ was also assessed. One-way ANOVA with Dunnett's post hoc test is annotated by the number of stars (**: p-value < 0.01, ***: p-value < 0.001).

4.3.11. Characterisation of IL-36RKO clones show NHEJ and HDR editing in Exon 5/6

After confirming the functional KO of C8F and C8A, it was next necessary to characterise and confirm the genetic alterations which occurred during the CRISPR-Cas9 gene editing. Firstly, protein detection was completed by Western blot to show loss of protein abundance of the IL-36R protein. Protein abundance was normalised by BCA assay as the membrane component extracted contained little-to-no β-actin as a loading control. Control cells showed normal levels of IL-36R whilst clone C8F showed a reduction in IL-36R protein abundance, although not entirely diminished. Clone C8A showed normal protein abundance also, despite functional KO confirmation being achieved (Fig 4.15a). Given the functional loss of response to IL-36 stimulation and the near-complete loss of IL-36R protein abundance, the clone C8F underwent further genetic characterisation clone.

To further understand the alterations made by CRISPR-Cas9 gene-editing, regions surrounding guide RNA bindings sites were amplified by PCR (Fig. 4.15b/c). PCR amplicons were then separated by gel electrophoresis, extracted from agarose gels, sequenced and analysed by TIDE (Fig. 4.15d-i/ii) and ICE analysis (Fig 4.15d-iii); two pherogram-reading programmes which can infer extent of gene knockout in cells. ICE and TIDE analysis revealed no trace of WT sequence to be found at gRNA site B, located in exon 5, showing IL-36R alleles to have undergone differential NHEJ by insertions and/or deletions. To investigate this further, qPCR primers were designed to target the WT sequence transcript in Exon 5 of the IL-36R gene, with only scramble control cells showing this transcript to still be present (Fig 4.15e). This showed that NHEJ had altered the sequence of exon 5 of the IL-36R gene, resulting in a decreased quantity and dysfunctional IL-36R protein being produced by cells.

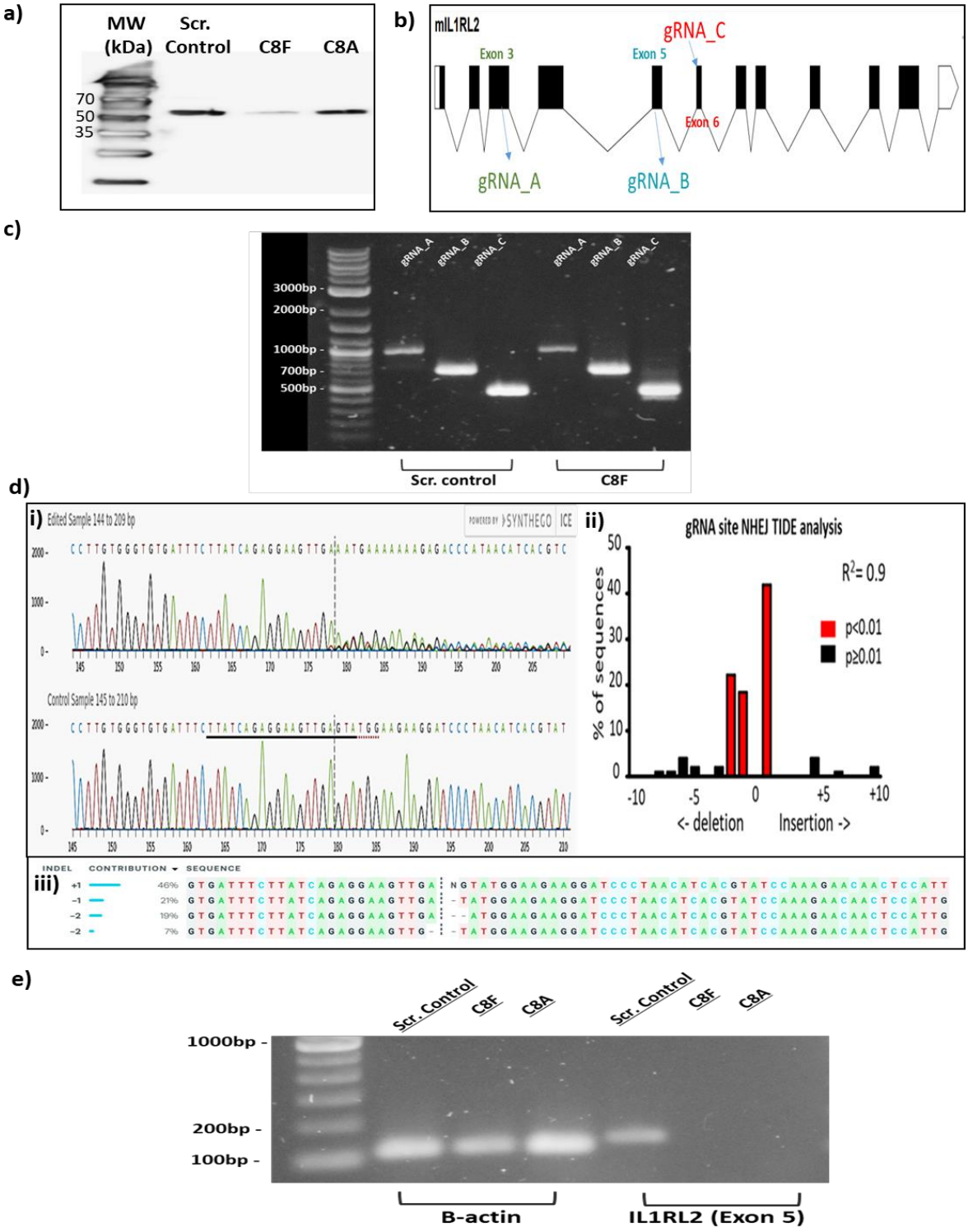


Figure 4.15: IL-36R KO CRISPR-Cas9 Clone generation characterisation shows NHEJ in exon 5. a) Western blotting was completed to detect IL-36R protein in clones. **b)** IL-36R gene map with guide RNA target sites indicated within exons. **c)** gRNA target sites were amplified by PCR, isolated and sequenced. **d)** Nucleotide sequence pherograms of amplicons were analysed by ICE and TIDE analysis, showing non homologous end joining recombination**. e)** RNA was extracted from control cells and clones and qPCR was completed to amplify WT exon 5 transcripts.

Furthermore, given that cells were indeed shown to be puromycin resistant and RFP^+ (Fig 4.13b/c), it was important to find the HDR insertion location in these cells. Primers were designed around guide RNA sites to detect insertion locations of HDR arms using a forward WT primer (upstream of gRNA sites) and reverse HDR insertion primer (located in the first LoxP site of the plasmid)(Fig 4.16a/b). HDR insertion amplicons were detected in exon 6 of IL-36R KO clones, showing that exon 6 of the IL-36R gene had undergone HDR gene editing in these cells (Fig. 4.16c). This work thereby characterised the clone C8F as having undergone NHEJ gene editing in Exon 5 (gRNA oligonucleotide B) and HDR in Exon 6 (gRNA oligonucleotide C) in the IL-36R gene, with complete functional knockout of the IL-36R protein.

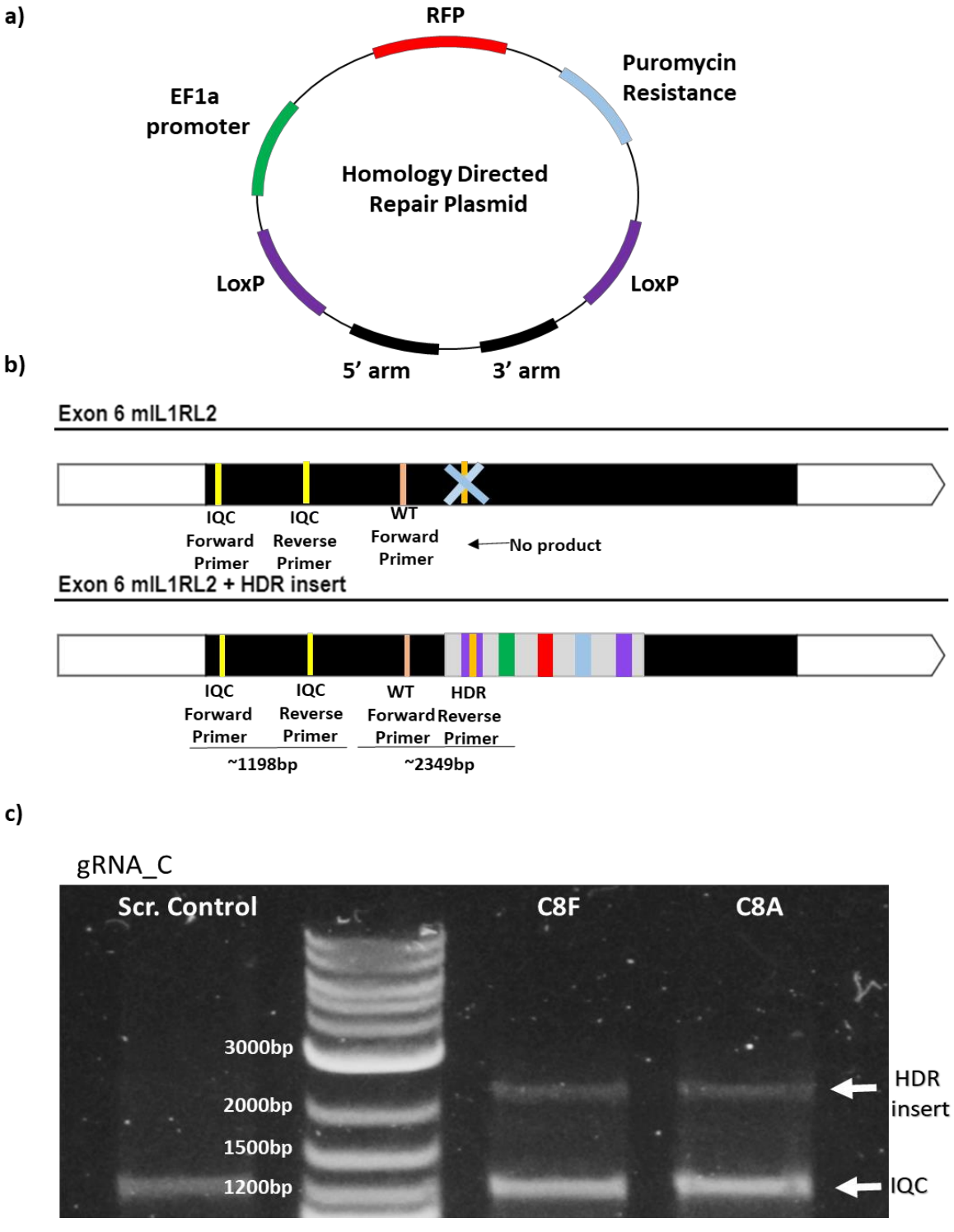


Figure 4.16: Genotyping of CT26 IL-36RKO clones shows HDR recombination in exon 6. a) HDR plasmid map showing key sequence regions. **b)** Primers were designed against WT sequence (Forward) upstream of gRNA site in exon 6 and within the LoxP site of HDR insert for HDR insertion detection. Internal control primers (IQC) were also designed upstream of gRNA site (forward and reverse) in exon 6. **c)** DNA was extracted from control cells and clones. PCR amplification using HDR insert primers and IQC primers as a multiplex to detect HDR insertion in clones.

4.3.12. Injection of CT26$^{IL\text{-}36RKO}$ cells results in lower tumour burden in Balb/c mice in a subcutaneous model of tumourigenesis compared to scramble control cells

Once characterisation of the IL-36R KO clone C8F, hereafter known as CT26$^{IL\text{-}36RKO}$**,** had been completed, the cells were expanded and subsequently used in a syngeneic, subcutaneous tumour model of colon cancer. Scramble controls cells or CT26^{IL36RKO} were injected into the rear-flank of Balb/c mice and tumour growth was monitored (Fig 4.17a). In agreement with our previous *in vivo* models, inhibition of IL-36R signalling, by gene editing in this case, resulted in a significantly decreased tumour burden in mice. An average reduction of 61% in tumour burden was observed in mice injected with CT26$^{IL\text{-}36RKO}$ in comparison to scramble control cells (P = 0.0190) (Fig. 4.17b/c). This data further suggested that direct inhibition of cancer cell IL-36R signalling can reduce cellular proliferation of cancer cells *in vivo.*

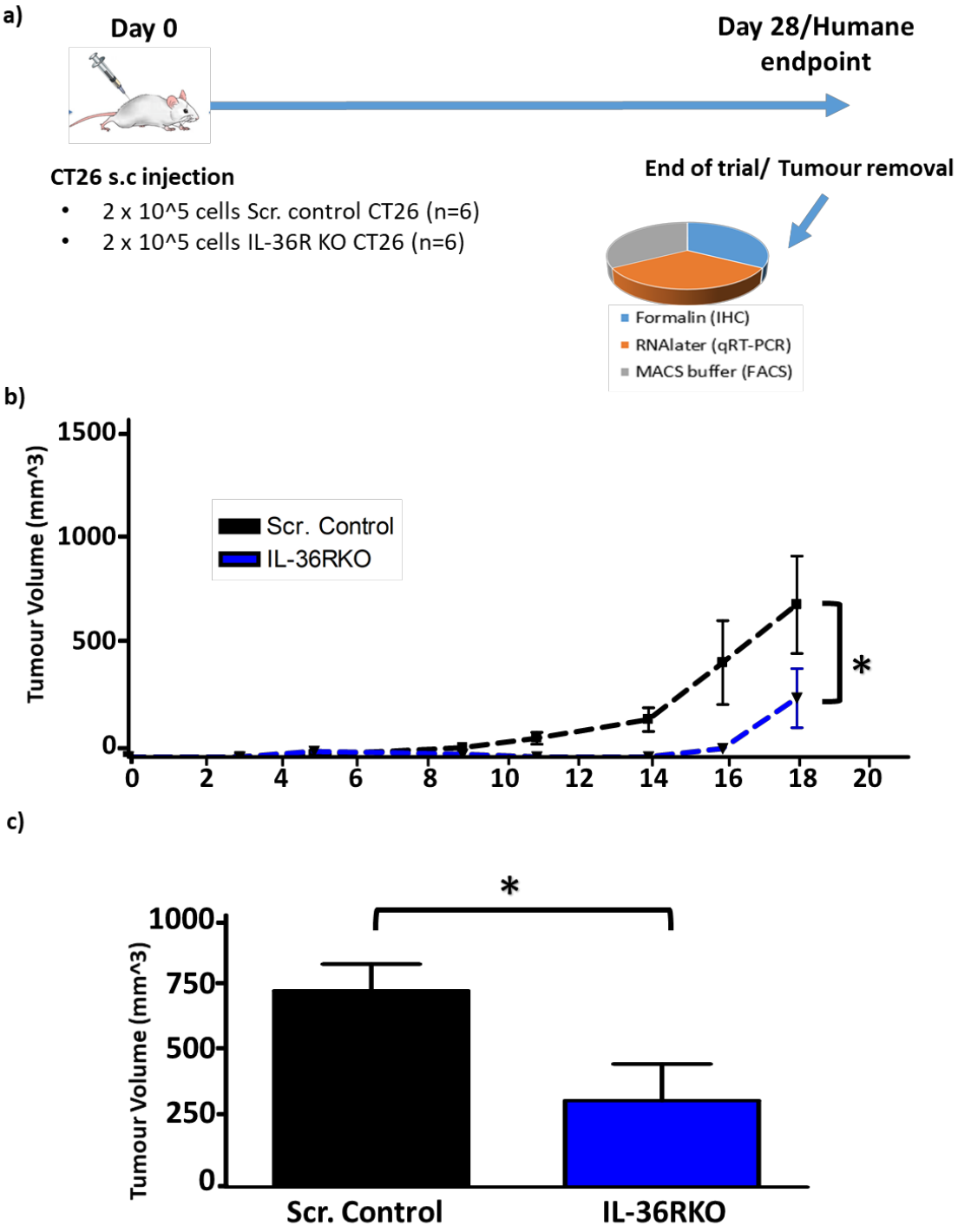


Figure 4.17: IL-36R KO in CT26 cells significantly reduces tumour burden in comparison to control cells. **a)** Study design of mouse model **b)** Tumour growth curves over time for scramble control group and IL-36R KO group **c)** Final average tumour volumes for groups. One-way ANOVA with Dunnett's post hoc test is annotated by the number of stars (*: p-value < 0.05).

4.3.13. CT26$^{IL\text{-}36RKO}$ cell tumours show decreased cytokine expression in s.c. tumours in comparison to control cells

In order to further investigate the mechanism behind the tumour reduction seen with the CT26^{IL36RKO} cells, qRT-PCR analysis was completed on bulk tumour RNA as previously described. The only significant change in gene expression detected was reduced CCL2 expression in tumours, consistent with previous IL-36R blockade by IL-36Ra administration (Fig. 4.18). Trends of decreased TGF-β, ARG1 and IFN-γ were also observed, but were not statistically significant.

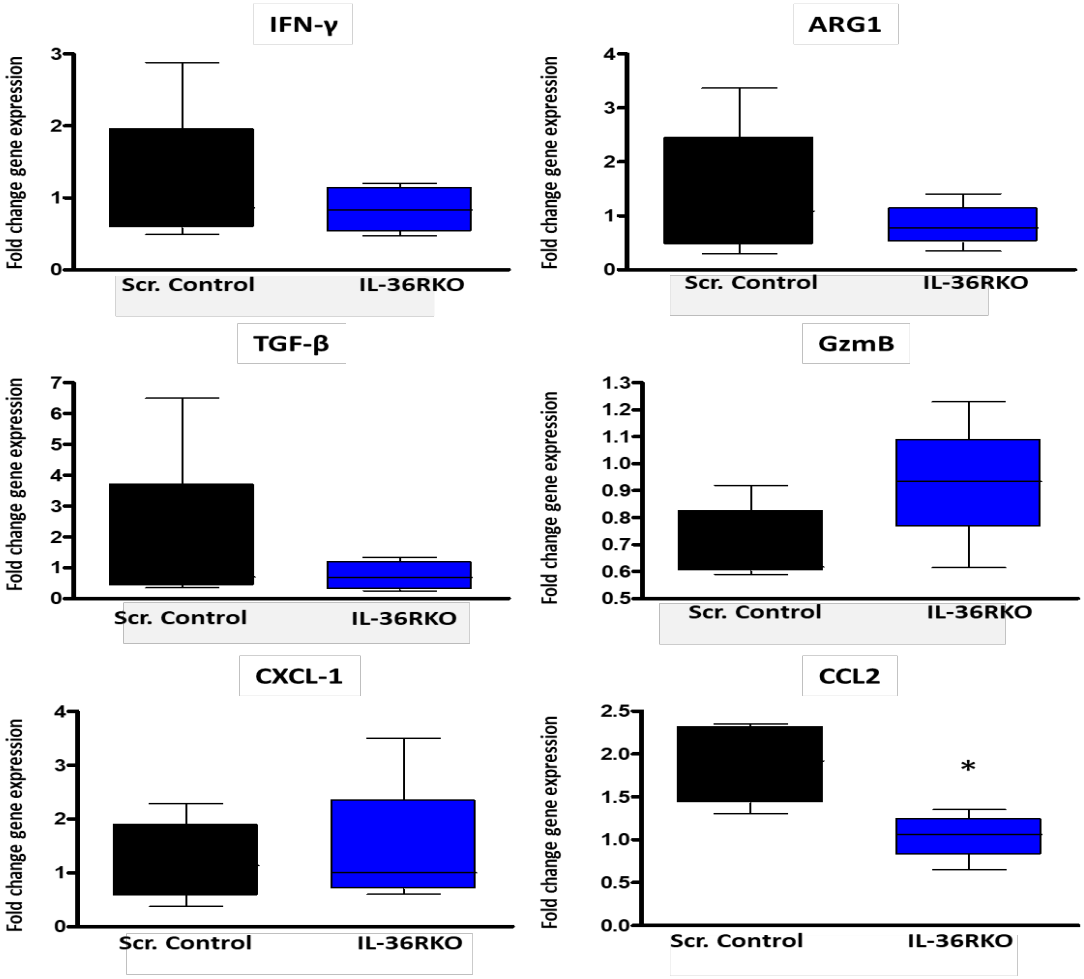


Figure 4.18: IL-36RKO significantly reduces tumoural CCL2 expression in s.c. tumours. Tumour RNA was extracted with cDNA subsequently synthesised and qRT-PCR gene expression analysis performed on genes as indicated above. Student's T-test is annotated by the number of stars (*: p-value < 0.05)

FACS analysis showed that CT26$^{IL\text{-}36RKO}$ cells contained an increased number of CD8^{+} T cells in comparison to scramble control cell tumours. In addition, there was a trend of decreased CD4^{+} cell infiltration observed in CT26$^{IL\text{-}36RKO}$ tumours (Fig. 4.19). This was unexpected given previous models had not shown any significant changes in immune cell infiltrate following suppression of IL-36R signalling with IL-36Ra administration. No other significant changes were observed in immune cell population infiltration in CT26$^{IL\text{-}36RKO}$ tumours (Fig 4.19).

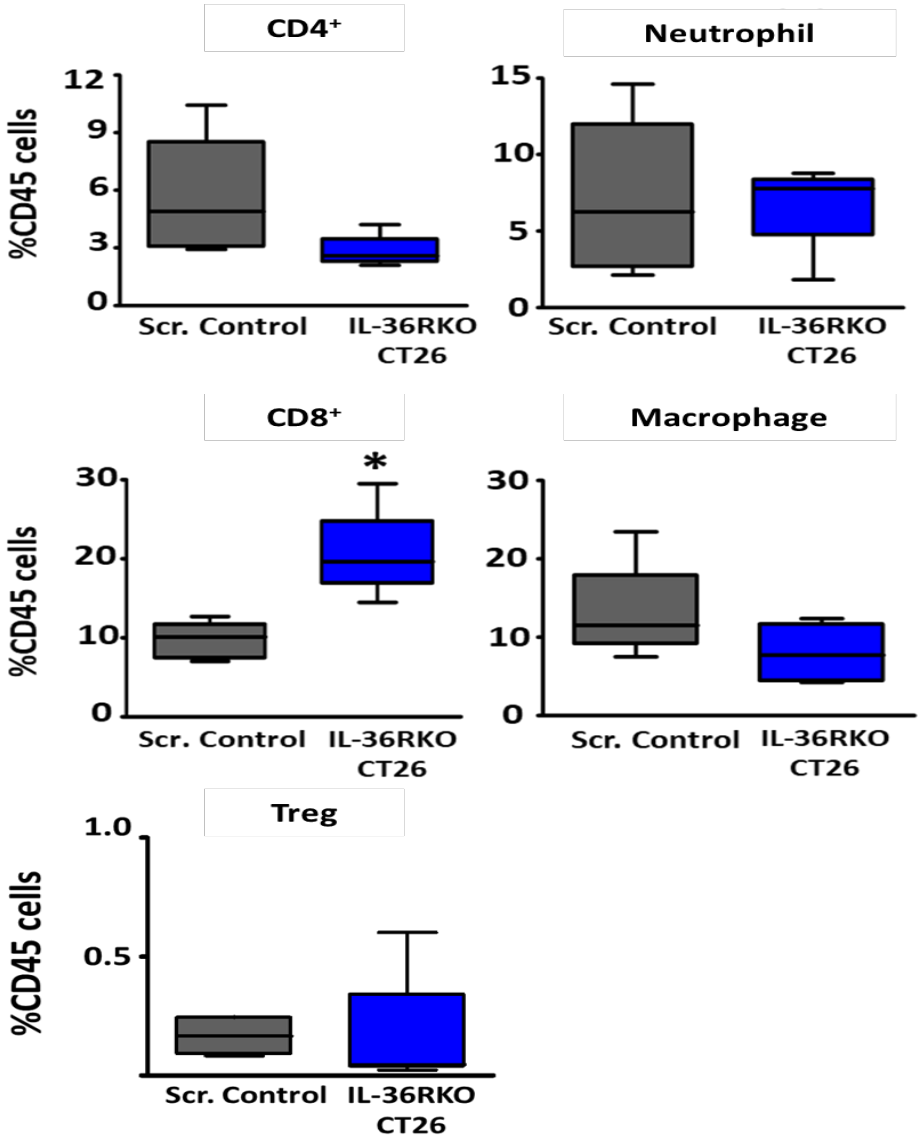


Figure 4.19: IL-36R KO tumours contain increased cytotoxic T cells in comparison to control cell tumours. Excised tumours were enzymatically dissociated and stained, intracellularly and extracellularly for several immune cell markers. Student's test is annotated by the number of stars (*: p-value < 0.05)

4.3.14. CT26$^{IL\text{-}36RKO}$ tumours show decreased Ki67 expression *in vivo*

To further understand the direct role of IL-36R signalling on tumour cells, it was investigated whether knockout of IL-36R expression on tumour cells could reduce cellular proliferation *in vivo,* as seen previously with administration of the IL-36Ra. CT26$^{IL\text{-}36RKO}$ cells displayed a significant decrease in Ki67 expressing cells in tumours in comparison to scramble control cell tumours (Fig 4.20). This data further implicates that inhibition of cancer cell IL-36R signalling, by knockout of the IL-36R gene or inhibition by IL-36Ra, can reduce the proliferative capacity of cancer cells in an *in vivo* model of colon cancer.

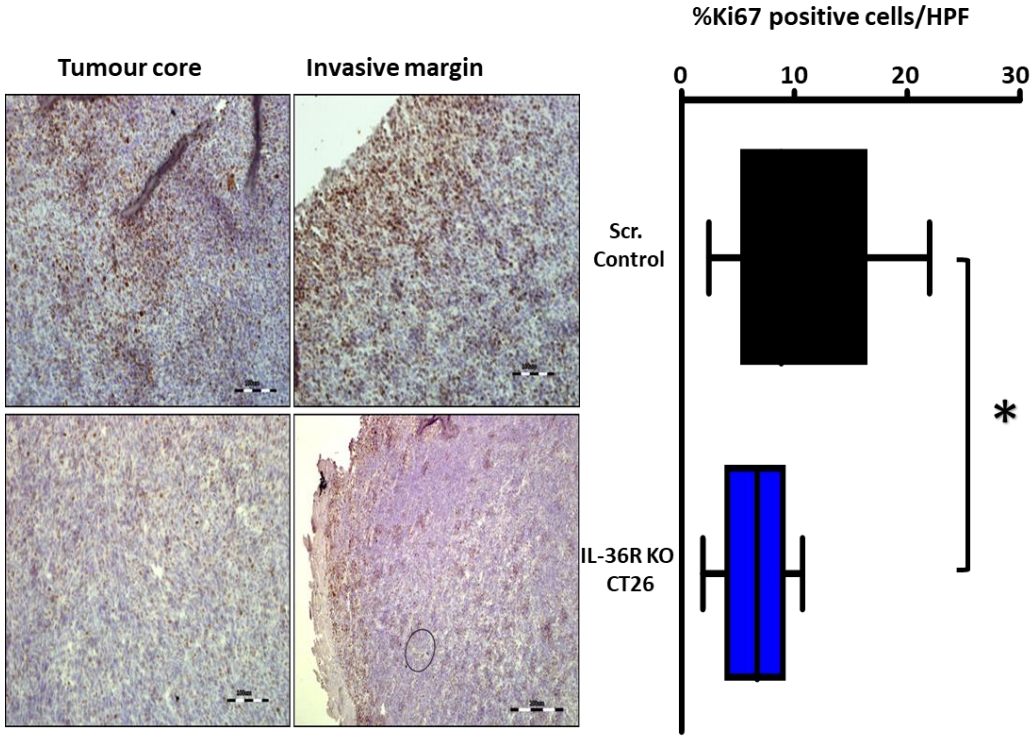


Figure 4.20: IL-36RKO CT26 cells tumours contain fewer Ki67 expressing cells in comparison to control cell tumours. Excised tumours were processed for IHC analysis and stained for detection of the proliferative cell marker, Ki67. Images were quantified as percentage Ki67^{+} cells per high power field. Student's test is annotated by the number of stars (*: p-value < 0.05)

4.4. Discussion

When experimentation for this chapter was commenced, *in vivo* studies investigating IL-36 signalling in cancer had focussed exclusively on the anti-tumourigenic properties of IL-36 cytokines on immune cells resulting in tumour rejection [199, 406]. Across many pathologies, IL-36 cytokines have been shown to be pluripotent, with several cell types shown to express the IL-36 receptor and respond to IL-36 signalling [226]. Given the pro-tumourigenic effects of IL-36 signalling on cancer cells which were identified in the previous chapter using *in vitro* assays, as well as the well-characterised ability of IL-1 family cytokines to play dichotomous roles in cancer [400], the aim of this chapter was to further investigate the role of IL-36R signalling in cancer cells using pre-clinical models of colon cancer.

In addition to investigating IL-36 cytokine signalling in colon cancer to develop cancer therapies, it was also important to characterise the role of IL-36 signalling in colon cancer to progress knowledge of this cytokine family to contribute to the broader field of cytokine research. In order to confirm that the effect of IL-36Ra activity was indeed via inhibiting IL-36R signalling on tumour cells, CRISPR-Cas9 mediated knockout of IL-36R expression in CT26 cells was completed. This allowed for the comparison of CT26 control cells, expressing functional and normal levels of IL-36R protein, directly to $CT26^{IL\text{-}36RKO}$ cells. Previous studies on IL-36 signalling have used the overexpression of these cytokines in cells to investigate the effects of this in the TME. Plasmid insertion of IL-36γ into 4T1 and B16 cancer cells resulted in reduced tumour burden in mice, with another study later performed investigating IL-36β overexpression in cancer cells reporting similar findings [235, 299]. Similarly, another group has used adenoviral vectors to overexpress IL-36γ in BM-derived dendritic cells, with intratumoural injection of these cells shown to reduce tumour burden

and improve TLO formation in WT mice but not IL-36R KO mice [380]. Furthermore, lentiviral derived IL-36β overexpressing pancreatic cancer cells reduce tumour burden with increased $CD8^+$ T cell and NK infiltration into xenograft tumours. This same group also administered IL-36β-overexpressing adenovirus which also resulted in increased TIL infiltration [376]. Although these studies were effective in highlighting IL-36 signalling in the TME, these were limited in investigating the direct effect of IL-36 signalling on cancer cells. IL-36 family gene germline knockout mouse models have also been used in tumourigenesis studies [242, 272, 273, 380, 386, 437]. Although these models have been effective in interrogating the role of IL-36 signalling in cancer, they answer different, albeit complimentary, questions to the research presented here in this thesis. As previously described, the germline knockout of IL-36γ and IL-36Ra have been used to elucidate the role of IL-36 signalling in colon cancer tumourigenesis. Although this method was effective in highlighting IL-36γ to play a pro-tumourigenic role in pre-clinical models of colon cancer, the complete knockout of IL-36γ or IL-36Ra will alter TME signalling across many cell types which express the IL-36 receptor. No reports to date have investigated IL-36R gene editing of cancer cells in order to interrogate the direct effect of IL-36R signalling on these cells in pre-clinical models. Here we show successful knockout of IL-36R signalling in CT26 cells and that this specific knockout did not result in phenotypic changes of TLR responsiveness or baseline cellular proliferation, indicating this to be a useful model of IL-36R signalling in colon cancer progression, which could be applied to other cell lines to model multiple cancer types. By use of subcutaneous injection of $CT26^{IL\text{-}36RKO}$cells, we were able to show that IL-36R signalling on cancer cells plays a role in tumourigenesis.

An obvious attraction of the CRISPR-Cas9 approach is the precision of gene editing available by targeting highly specific regions of genes in order to introduce NHEJ and/or insertion of

genes by HDR [438]. This is a major advantage over germline knockout, with the ability to generate novel cell lines by 'knockout' or 'knock in' of genes into cellular genomic DNA. In this study, we took advantage of NHEJ and HDR in order to improve the probability of detecting full IL-36R knockout cells. The CT26 cell line is a well-characterised cell line, with a high mutational burden and large copy number variation across chromosomes [439]. It has been estimated that CT26 cells display tetraploidy across chromosome 1, where the IL-36R gene is located. Highly efficient knockout of the IL-36R gene is therefore required to alter each allele [440]. For this reason, combined approaches of NHEJ and HDR insertion were used in order to maximise allele editing in cells. A limitation of our IL-36R knockout is the lack of thorough characterisation by sequencing at each of the guide RNA target sites. The tetraploidy present at these sites would likely yield unreadable pherograms with excessive background noise, as a result of multiple different types of indels and HDR insertions at each site. TIDE and ICE analysis have helped to overcome this issue as both software can infer the spectrum of indels and their frequencies by comparison of pherograms from Sanger-sequencing of wild type and CRISPR-edited cells [441, 442]. In order to overcome this study limitation, high throughput sequencing of expanded single cell clones would be required to detect identical mutations at each guide RNA site of CRISPR-editing for a complete genotyping of edited clones.

Our study showed a signification reduction in tumour burden of mice by inhibiting IL-36R signalling. This was achieved by systemic IL-36Ra administration in mice or by knockout of IL-36R expression in tumour cells. In both models, reduced IL-36R signalling by IL-36Ra or IL-36R gene knockout resulted in tumour burden reduction, which was accompanied by a significant reduction in Ki67 expression, an important marker for cell proliferation. Very recently, coinciding with the publication of our findings, it has been reported that IL-36Ra

and IL-36γ reciprocally regulate colon inflammation and tumourigenesis, with this work also indicating IL-36γ as an important driver of cancer cell proliferation *in vivo* [385]. Unlike our use of subcutaneous injection of tumour cells in syngeneic mice, this work used different models of carcinogenesis such as AOM/Vil-Cre; $Trp53^{fl/fl}$ mice also bearing IL-36γ or IL-36Ra gene knockout. Furthermore these authors also generated $Apc^{Min/+}$ mice with gene knockout of IL-36γ or IL-36Ra. These studies complemented our findings as they showed that IL-36γ signalling significantly contributes to colon cancer carcinogenesis. Furthermore, this study showed that inhibition of IL-36γ proteolytic cleavage, or direct neutralisation by polyclonal anti-IL-36γ administration, could significantly reduce tumour burden. The mechanism by which this was achieved was reported to be via inhibition of the cell–matrix adhesion pathways and the Wnt signalling pathway. This group have also recently shown a similar pro-tumourigenic role for IL-36γ signalling in Non-Small Cell Lung Cancer (NSCLC), although in this instance, they identified the mechanism of carcinogenesis to be a product of Glutathione (GSH) homeostasis and regulation of oxidative stress-induced cell death [386]. Cellular proliferation, however, remains the cornerstone of tumourigenesis, with this proliferative activity routinely targeted in later stage disease by different therapeutic strategies such as 5-FU and anti-EGFR therapy to inhibit cancer cell proliferation [41, 443]. Our findings offer an alternative approach to limiting cancer cell proliferation signalling. Single-target therapy often results in the natural selection of therapy-resistance cancer cells [41, 444], with dual-therapy approaches able to overcome this resistance by targeting multiple pathways in cancer cells [41]. Taken together, our findings identify that IL-36R signalling in cancer cells significantly contributes to carcinogenesis, and that IL-36Ra administration may be useful as a therapeutic or prophylactic treatment to reduce tumour formation *in vivo*.

In agreement with previous studies [199], we have also shown that IL-36 agonists influence the anti-tumour immune response and reduce tumour burden in pre-clinical models of tumourigenesis. The advent of immunotherapy has rapidly transformed cancer therapeutic options, with this approach now being used across many tumour types, in the forms of adoptive cell therapy, immune checkpoint inhibitors, cancer vaccines and cytokine administration [445]. Next generation cytokine administration therapies are rapidly being developed, with major improvements in the half-life, site-directed specificity and binding affinity of these cytokine approaches [445]. A desirable consequence of immunotherapy is to generate a long-term immunological response to improve patient survival outcomes. This is achieved by improving tumour cell recognition, antigen presentation, cytotoxic T cell expansion and activation [204, 445]. Previous work has shown that IL-36 cytokines stimulate dendritic cell maturation resulting in increased type 1 anti-tumour immunity [380]. Furthermore, cytotoxic cells such as NK cells and $CD8^+$ T cells have been shown to be activated and proliferate in response to IL-36 signalling [413]. Additionally, roles for IL-36 signalling have been demonstrated in TLS maintenance to sustain long-term anti-tumour immunity, a key prognostic marker of improved patient outcomes [239, 299, 380]. Our results are in agreement with these findings, showing induction of the anti-tumour response to result in decreased tumour size relative to PBS controls with increased $CD8^+$ T cell infiltration. The potential of IL-36 cytokine treatment in conjunction with patient stratification according to cancer cell IL-36R expression may further enhance this treatment option to reduce bystander effects of IL-36 cytokines on cancer cells. Further experimentation by IP injection of IL-36 cytokines into mice bearing $CT26^{IL\text{-}36RKO}$ tumours could exemplify the usefulness of these cytokines to augment the immune response without by-stander effects on cancer cells themselves.

Surprisingly, whilst observing no immune changes in tumours of mice injected with IL-36Ra, there was a significant increase in $CD8^+$ T cell infiltration in $CT26^{IL\text{-}36RKO}$ tumours. It may be possible that given the lack of IL-36R expression on cancer cells, which are the major cell type in the subcutaneous tumours, more IL-36 cytokines in the TME were available to immune populations in order to stimulate their activation and proliferation as previously reported [296, 379]. However, it has also previously been reported that tumour size alone can influence the immune cell composition of subcutaneous tumours, and therefore the immune cell composition changes observed following IL-36Ra administration and in $CT26^{IL\text{-}36RKO}$ tumours may result from the decreased tumour burden alone [446]. This is reflected in the other recent papers investigating IL-36 in lung cancer and colon cancer tumourigenesis, with IL-36γ and IL-36Ra knockout mice shown to have the largest changes in gene expression in non-immune infiltrate associated pathways. In these studies, expression analysis was completed on bulk RNA from tumours with significant gene expression changes observed in genes associated with GSH homeostasis, oxidative stress induced cell death, cell matrix adhesion and ECM-receptor interaction pathways [385, 386]. Although epithelial tumour cells can be significantly induced by IL-36 cytokines to secrete large increases of myeloid chemoattractants and other pluripotent IL-1 family members [287], this work suggests that alteration of IL-36 signalling in the TME is more influential in other cellular processes.

In order to optimise treatment approaches for utilising either IL-36Ra or IL-36 agonists as therapies in cancer, it may be important to consider the stage of disease. Given the repeated implication of IL-36 signalling in IBD [396, 437] and the association of colon cancer with chronic inflammation [397, 399], IL-36Ra administration may prove to be of most use in the prevention of malignant transformation. This may be of greatest benefit

prophylactically for high risk patients such as FAP and HNPCC, or curatively for primary tumour reduction as an alternative to, or in combination with, current targeted therapies when curative surgery is insufficient. An anti-IL-36R mAb, Spesolimab, is already in phase II/III clinical trials for the treatment of severe ulcerative colitis in patients, and this may prove to be effective in reducing IBD disease severity as well as carcinogenesis-risk in these patients [201]. It may be that early stage colon cancer may have greater benefits of combined surgical resection and IL-36Ra administration in order to limit tumour growth before metastasis. In the event of later stage detection, IL-36 agonist treatment may be favourable in order to achieve a systemic, sustained anti-tumour response to improve tumour rejection at local and distant metastatic sites [299, 380, 413, 414]. Our studies have shown that IL-36Ra can be used as a therapeutic to inhibit tumourigenesis, but also to act as a prophylactic inhibitory treatment. An important consideration is whether this effect would be sustained over time, or if tumour cells would become resistant to this therapy, with IL-36 agonist treatment more likely to achieve long-term immunity, given the adaptive immune-mediated response we and others have shown it being capable of augmenting [199]. To further investigate this question, long term models of tumourigenesis would be required, with treatments to include either IL-36Ra or IL-36 agonists over time. In the event of clearance of tumour burden, it would then be of interest to re-challenge mice with tumours and measure tumour growth to detect if long-term adaptive immunity had developed in mice that received IL-36Ra or IL-36 agonist treatment.

In the context of colon cancer, tumours may be divided into four molecular subtypes, according to transcriptome gene signatures [31, 418]. These have been further characterised to show unique immune cell compositions as well as different mutational background, with this being highly indicative of patient prognosis [32]. Given the previously

shown functions of IL-36 cytokines in the TME and the findings of this thesis, administration of IL-36 cytokines may benefit CSM2/3 tumours the most, given their relative lack of immune infiltrate or the lack of activation of the immune populations. Of course, given the bystander effects of IL-36 signalling in tumourigenesis reported here and by other recent papers, patient stratification according to IL-36R expression may stand to benefit patients to maximise CTL-directed effects of IL-36 signalling. However, a major drawback of cytokine treatment has been observed in the severe toxicity reported in patients [447]. Recent advancements in cytokine delivery have attempted to overcome this issue by improving cytokine conjugation to antibodies for site specificity and mutation of cytokines to reduce binding affinity [447]. Given the pluripotent effects of IL-36 signalling on anti-tumour immune cell populations, development of IL-36 cytokines as a next generation immunotherapy treatments would be useful, but site-directed specificity will be required to limit toxicities. Patient IL-36 expression characterisation of both immune and tumour cell populations by multiplex IHC could identify patients who may benefit most from IL-36 agonist treatment. From our findings and the literature, a patient with an anti-tumour immune infiltrate which expresses IL-36R, as well as cancer cells which do not express IL-36R, may be most suitable for this treatment. Contrasting this, a patient with cancer cells expressing high levels of IL-36R are likely to benefit from IL-36Ra administration to limit tumour growth.

In conclusion, the work in this chapter has further highlighted the usefulness of IL-36 cytokines and IL-36R inhibition in colon cancer tumourigenesis. We show that IL-36R inhibition, by administration of the natural antagonist or gene-editing, can reduce the tumour burden in pre-clinical models of colon cancer by the prevention of proliferative pathway signalling in tumour cells. Furthermore, we show further evidence that IL-36

cytokine signalling can reduce tumour in pre-clinical models of colon cancer, with this mechanism shown to be immune-mediated targeting of tumours cells. This work reiterates that careful consideration is required for the future use of IL-36 cytokines in colon cancer therapy, given the bystander effects of IL-36 cytokines on cancer cells, although this may be overcome by patient stratification by cancer cell IL-36R expression.

Chapter 5

Investigation of IL-36 signalling in co-cultures of myeloid immune cells and colon cancer cells *in vitro*

5.1. Aim

IL-36 studies have presented IL-36 signalling to play key roles in both innate and adaptive immunity, as well as bridging these two arms of the immune response. However, the majority of IL-36-related cancer research has largely focussed on the ability of these cytokines to augment the adaptive immune response to reduce tumour burden. Macrophages and neutrophils are well characterised, heterogeneous innate immune cells which may contribute to tumour rejection or progression. These cells have also been highly implicated in IL-36 driven disease. Given our findings indicating IL-36 signalling to contribute to tumour progression and the lack of research regarding IL-36 cytokines and macrophages/neutrophils in the TME, the aim of this chapter is to investigate how IL-36 cytokines may influence the interplay between colon cancer cells and these innate immune cells.

5.2. Introduction

The tumour microenvironment is a complex and dynamic collection of cancer, immune and stromal cells, as well as a multi-component extracellular matrix [54]. The composition of the tissue changes according to cytokine signalling between cells. This signalling dictates tumour progression by recruitment of immune cell populations such as M2 macrophages, Tumour Associated Neutrophils, Myeloid Derived Suppressor Cells and T Regulatory cells [55]. Alternatively, tumour rejection may be achieved by increased infiltration of Cytotoxic T cells, NK cells and M1 macrophages [55]. Furthering this complexity, several cell types such as macrophages and neutrophils are capable of undergoing polarisation between pro-tumour and anti-tumour states, depending on local cytokine signalling [56-58]. The innate immune cells, macrophages and neutrophils, can strongly influence whether the TME is directed toward tumour rejection or tumour progression [102, 448]. These cells have been heavily implicated as sources, activators and responders of IL-36 signalling in chronic inflammatory diseases, suggesting further roles may exist for IL-36 and these cells in the TME. The IL-36 cytokines reflect the overall complexity of the TME, with many cells of the TME shown to express the IL-36 receptor and respond to these cytokines including cancer cells, $CD8^+$ T cells, $CD4^+$ T cells and Treg cells [235, 386]. Our findings presented in chapter 4 clearly highlight the complex roles of IL-36 cytokines within the TME, as we demonstrated reduced tumour growth following administration of the IL-36 agonists, but also by administration of the IL-36R antagonist. Unravelling the anti-tumourigenic function of IL-36 signalling on immune cells within the TME and the pro-tumourigenic function of IL-36 cytokines acting on cancer cells will greatly aid in our understanding of the role of these cytokines in tumourigenesis and thereby enable the development of therapeutic interventions.

IL-36 cancer-related research to date has focussed on the augmentation of adaptive immune response to enhance tumour rejection. Indeed, increased expression of IL-36γ can enhance tumoural $CD8^+$ T cell and NK cell infiltration in melanoma, pancreatic cancer, breast cancer and other cancers [80, 233, 378, 379, 419]. Bridging the innate and adaptive arms, increased IL-36γ expression in dendritic cells can beneficially mediate the formation of Tertiary Lymphoid Structures (TLSs) in tumours, which are positive prognostic markers for long-term clinical responses, especially in the context of immunotherapy [380]. Only a handful of other findings have been reported for IL-36 cytokines and innate immune cells in the TME, with macrophages reported to act as an important source of IL-36γ in the TME for TLS formation [414], and that IL-36γ overexpression by cancer cells can increase the NK cell composition of tumours *in vivo* [299]. Extensive studies have been completed showing either production or stimulation of IL-36 from/of macrophages can play a role in enhanced macrophage activation [236, 290, 294, 325] and phagocytosis [242, 267, 449], as well as induction of macrophage-fibroblast crosstalk [321, 450]. IL-36 cytokines can indeed influence macrophage polarisation from inflammatory (M1) to wound healing (M2) phenotypes, which is a key determinant of cancer rejection or progression [236]. Neutrophils act as the primary source of for IL-36 cytokine activating proteases [216, 218, 424, 451, 452], as well as being an important producer of IL-36 cytokines themselves [452]. Furthermore, it has been shown that several different epithelial tissues (skin, intestinal, and lung) are highly responsive to IL-36 stimulation, all of which respond with large upregulation of neutrophil chemotactic chemokine gene expression [287, 321, 453]. A specific type of neutrophil cell death, NETosis, can result in the increased activation of local IL-36 cytokines. This chemokine-induced cell death results in the release of modified chromatin decorated with granules including key serine proteases, which thereby cleave

and activate local IL-36 cytokines [454]. Furthermore, this neutrophil cell death has been implicated in multiple pro-tumourigenic processes including immune evasion, induction of cancer cell proliferation and metastasis [104, 455, 456]. These studies have propagated the understanding of IL-36 driven disease, although this work in the context of malignancies remains underdeveloped.

Given that macrophages and neutrophils have been consistently highlighted in IL-36-driven disease, and the extensive roles of macrophages [448] and neutrophils [102] in tumour rejection and tumour progression, the aim of this chapter is to investigate how IL-36 may influence crosstalk between colon cancer cells and these innate immune cells in the TME.

5.3. Results

5.3.1. THP-1 differentiated M1 and M2-like macrophages show increased IL-36γ and IL-36R gene expression.

Our findings from chapter 3 showed the strong gene induction of monocyte/macrophage chemoattractant expression, such as CCL2 and CCL5, in IL-36 stimulated colon cancer cells *in vitro* (Figures 3.3 and 3.4). Furthermore, CCL2 gene expression was upregulated in CT26 tumours of IL-36 agonist-treated Balb/c mice (Figures 4.2 and 4.6). Given these findings, and previous studies in the literature reporting monocyte/macrophage chemokine production from IL-36 stimulated epithelial cells [294], as well as direct effects of IL-36 cytokines on macrophage polarisation [236], we hypothesised that co-culture of macrophages and colon cancer cells in the presence of IL-36 could alter the phenotypes of these immune cells and thereby promote and/or inhibit colon cancer spheroid growth.

THP-1 cells were selected as a macrophage cell model for this investigation as these experiments were performed during the period of COVID-19 pandemic restrictions, and it was not possible to collect whole blood samples at this time in order to isolate primary monocytes. THP-1 cells were differentiated into M0-like cells (dM0), with cells then further polarised to M1 (dM1) and M2-like (dM2) states (Fig. 5.1a). To confirm maturation of these cells, CD11b expression was analysed by FACS. This showed increased expression across each of the cell states relative to undifferentiated cells (Fig 5.1b).

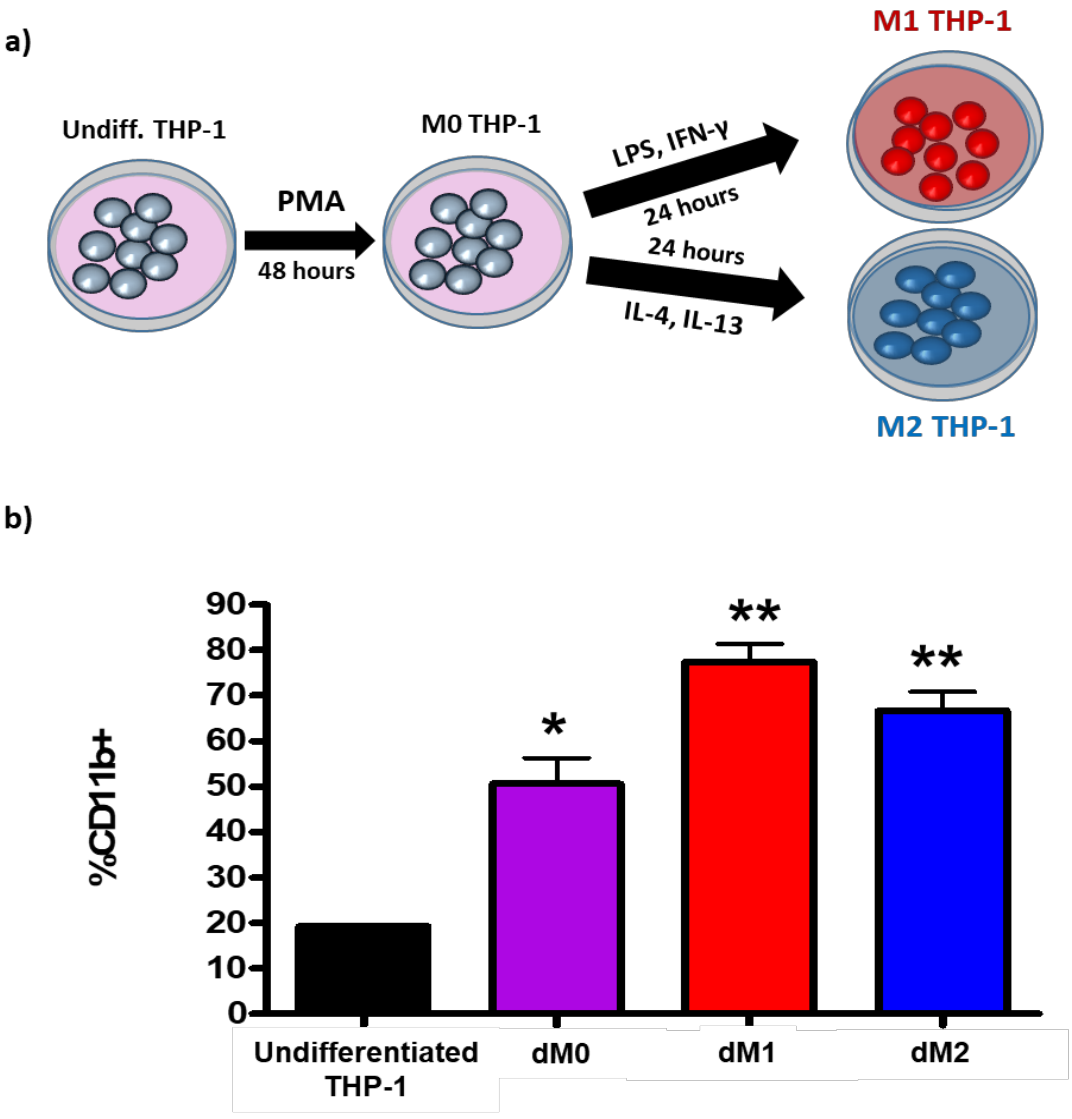


Figure 5.1: Differentiation of THP-1 macrophages and detection of CD11b expression. a) THP-1 cells were differentiated in 5 ng/mL PMA for 48 hours then further differentiated to M1 (200 ng/mL LPS, 20 ng/mL IFN-y) or M2 (20 ng/mL IL-4, 20 ng/mL IL-13) cells. **b)** CD11b expression was quantified by FACS analysis after differentiation had been completed. Graph is representative of three independent, biological replicates. Data shown is the mean +/- SEM of experimental duplicate. One-way ANOVA with post-hoc Dunnett's test is annotated as; * p= <0.05, **p = <0.01.

Furthermore, RNA was extracted from dM0, dM1 and dM2 cells, cDNA was synthesised and relative expression of classic M1 and M2 macrophage-associated genes [457] was quantified by qRT-PCR (Fig 5.2a). Relative to dM0 cells, dM1 cells showed increased

expression of both M1 and M2 macrophage-associated genes. Relative to dM0 cells, dM2 cells showed a significant increase in M2-associated genes as well as down regulation of M1-associated genes, with the exception of IL-12, which was increased. These changes in gene expression were confirmed by ELISA for IL-1β protein and enzyme activity for Arginase-1 (Fig 5.2b). This data showed that dM0 cells could be polarised to M1 and M2 macrophage-like cells, although dM1 cells upregulated M2-associated genes in addition to classically activated macrophages associated genes. This unexpected increase in M2 genes may have resulted from basal levels of anti-inflammatory factors found in FBS-supplemented media.

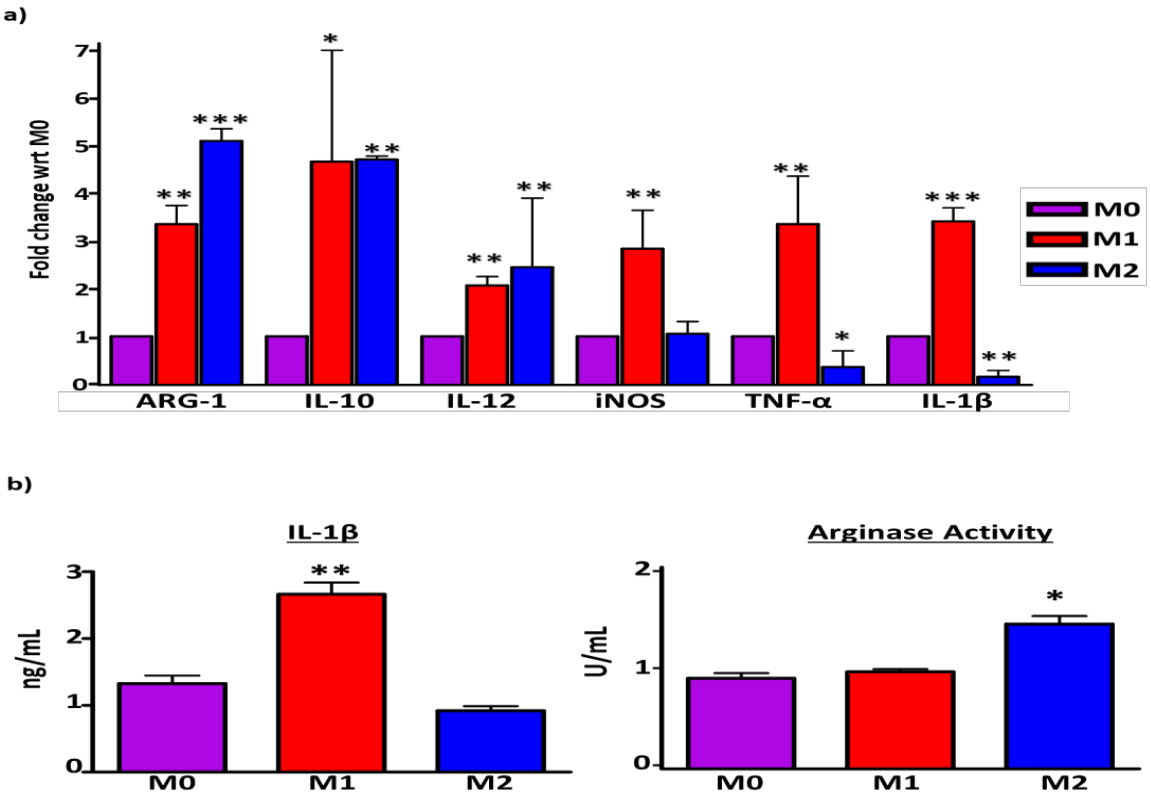


Figure 5.2: Characterisation of differentiated THP-1 M0, M1 and M2 cells by cytokine production and arginase activity. a) Differentiated cell gene transcription of key cytokines produced by M1 and M2 macrophages was detected by qRT-PCR. **b)** Changes in M0, M1 and M2 cells IL-1β protein secretion and arginase activity were detected by ELISA and Arginase enzyme activity assay, respectively. Graphs are representative of three independent, biological replicates. Data shown is the mean +/- SEM of experimental duplicate. One-way ANOVA with post-hoc Dunnett's test is annotated as; * p= <0.05, **p = <0.01, ***p = <0.001.

IL-36 family member gene expression was also assessed in these cells by qRT-PCR (Figure 5.3). dM1 cells showed a significant increase in IL-36γ and IL-36R expression, with IL-36Ra expression becoming undetectable. dM2 cells showed a significant increase in IL-36α, IL-36γ and IL-36R expression relative to dM0 cells.

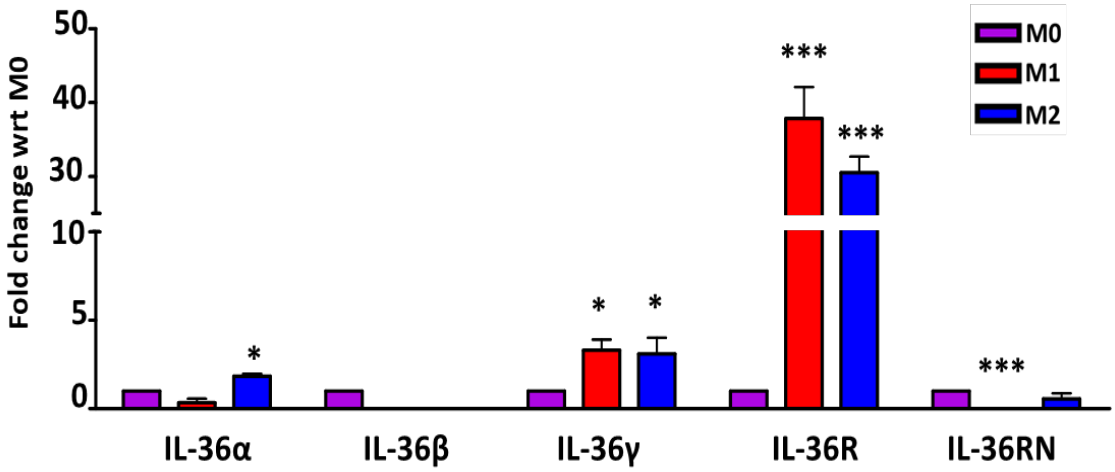


Figure 5.3: M1 and M2 differentiated THP-1 cells increase IL-36 family gene expression upon differentiation. M1 and M2 differentiated THP-1 cells were analysed by qRT-PCR for gene expression of IL-36 family members. Graphs are representative of independent, biological replicates (N=3). Data shown is the mean +/- SEM of experimental duplicate. One-way ANOVA with post-hoc Dunnett's test is annotated as; * p= <0.05, ***p = <0.001.

5.3.2. IL-36β and IL-36γ stimulation of dM2 cells significantly reduces IL-1β expression and secretion.

Following differentiation of THP-1 cells as outlined in figure 5.1a, cells were analysed for changes in gene expression in response to IL-36 stimulation. Cells were exposed to IL-36 cytokines for a further 4 hours after complete polarisation, and gene expression changes quantified by qRT-PCR for several macrophage polarisation associated genes (Fig 5.4). dM0 cells showed a trend of increased dM2-associated gene expression, although this was not statistically significant (Fig 5.4a). dM1 cells were unresponsive to IL-36 agonist stimulation

for both M1 and M2-associated genes (Fig 5.4b). dM2 cells stimulated with IL-36β and IL-36γ showed a significant reduction of IL-1β gene expression (Fig 5.4c).

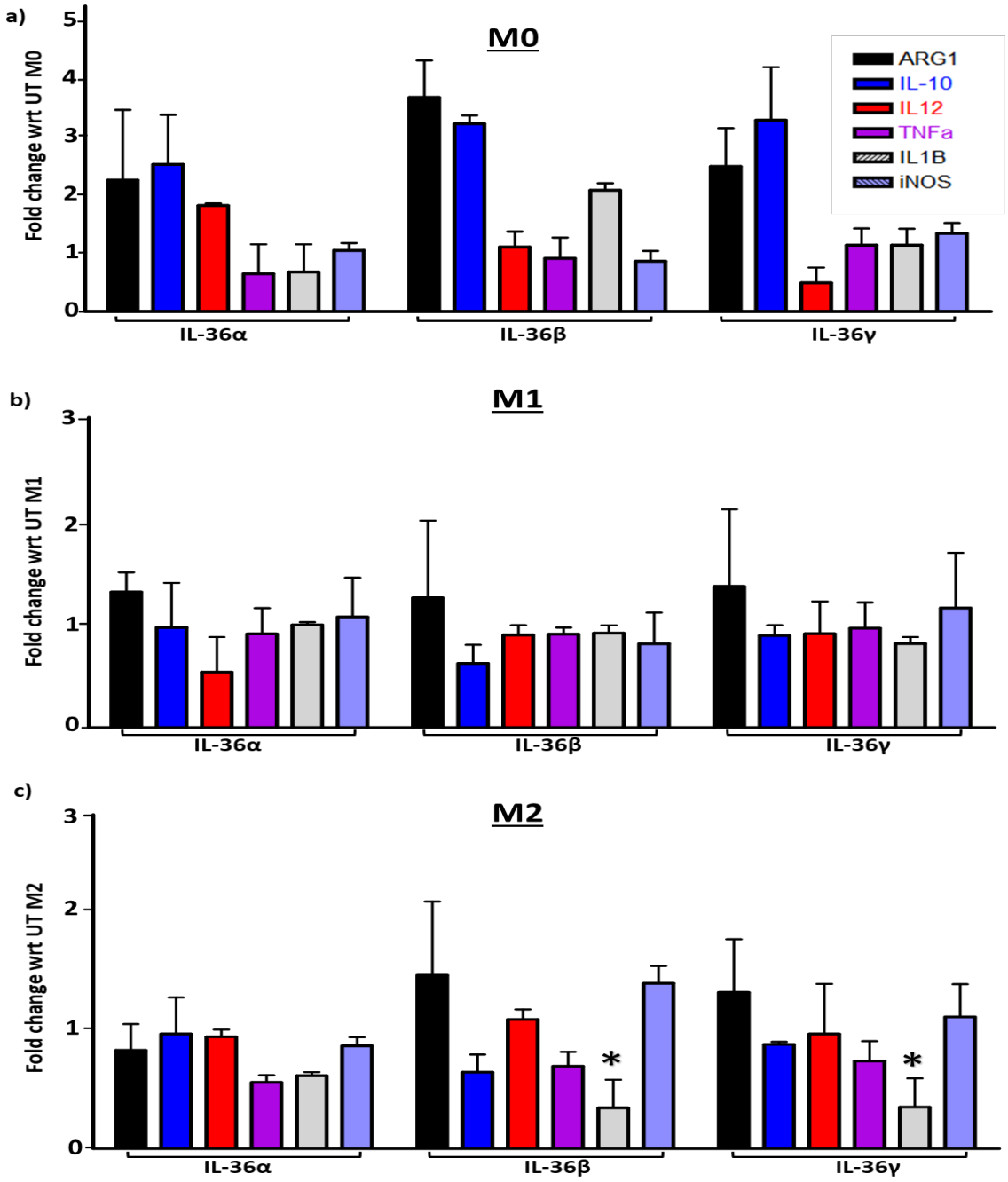


Figure 5.4: IL-36β and IL-36γ stimulation of M2 differentiated THP-1s cells significantly reduces IL-1β gene expression. a) M0, **b)** M1 and **c)** M2 dTHP-1 cells were stimulated with IL-36 agonists (100 ng/mL) for four hours after differentiation had been completed. Changes in gene expression were detected by qRT-PCR. Graphs are representative of independent, biological replicates (N=3). Data shown is the mean +/- SEM of experimental duplicate. One-way ANOVA with post-hoc Dunnett's test is annotated as; * p= <0.05.

Gene expression changes in dM2 cells were confirmed by IL-1β ELISA following stimulation of these cells with IL-36γ (Fig. 5.5). This was an unexpected response to observe in dM2 cells, given it has been previously reported that IL-36 stimulation of PBMC M2 cells results in their stimulation to a more pro-inflammatory state [236]. This IL-1β down regulation may have acted as a regulatory mechanism to the high concentration of IL-36 cytokines.

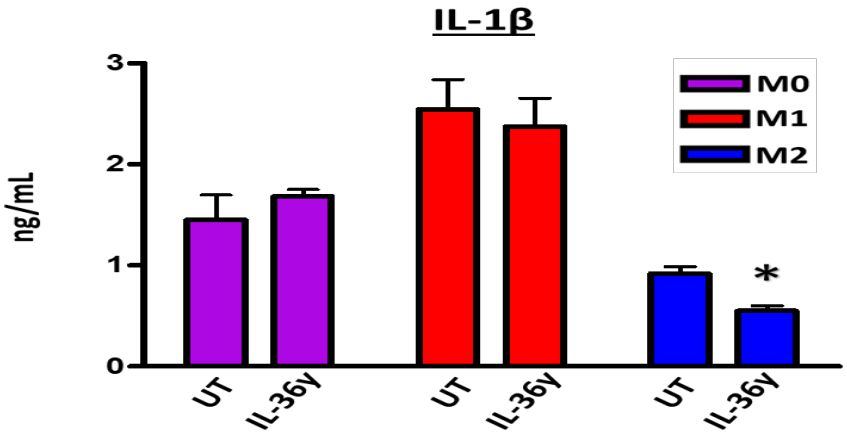


Figure 5.5: IL-36γ (100 ng/mL) stimulation of M2 differentiated THP-1s cells significantly reduces protein secretion of IL-1β. M2 dTHP-1 cell protein secretion of IL-1β was analysed by ELISA. Graphs are representative of independent, biological replicates (N=3). Data shown is the mean +/- SEM of experimental duplicate. One-way ANOVA with post-hoc Dunnett's test is annotated as; * p= <0.05.

5.3.3. dM1 cell supernatant significantly inhibits HT29 spheroid growth, with IL-36γ stimulation of dM1 and dM2 cells shown to have no effect on spheroid growth.

Although no large changes in chemokine expression were observed in previous experiments, only a subset of genes were examined. In order to detect possible changes in chemokine expression or metabolic activity of by-products of cells not previously observed by qRT-PCR, IL-36γ stimulated dM1 or dM2 supernatant was added to HT29 spheroids to

study the effects this may have on cancer cell growth. A schematic outline of this experimental plan is detailed in Figure 5.6a. The growth of HT29 spheroids was significantly reduced following culture in dM1 cell supernatant. However, IL-36γ stimulation of the dM1 cells did not affect this inhibition (Fig 5.6b). Neither dM2 nor IL-36γ-stimulated dM2 cell supernatant resulted in significant changes in HT29 spheroid growth (Fig 5.6b). This indicated that IL-36γ stimulation of dM1 and dM2 could not alter the ability of these cell types to inhibit/promote HT29 spheroid formation by protein/metabolite secretions alone from these cells.

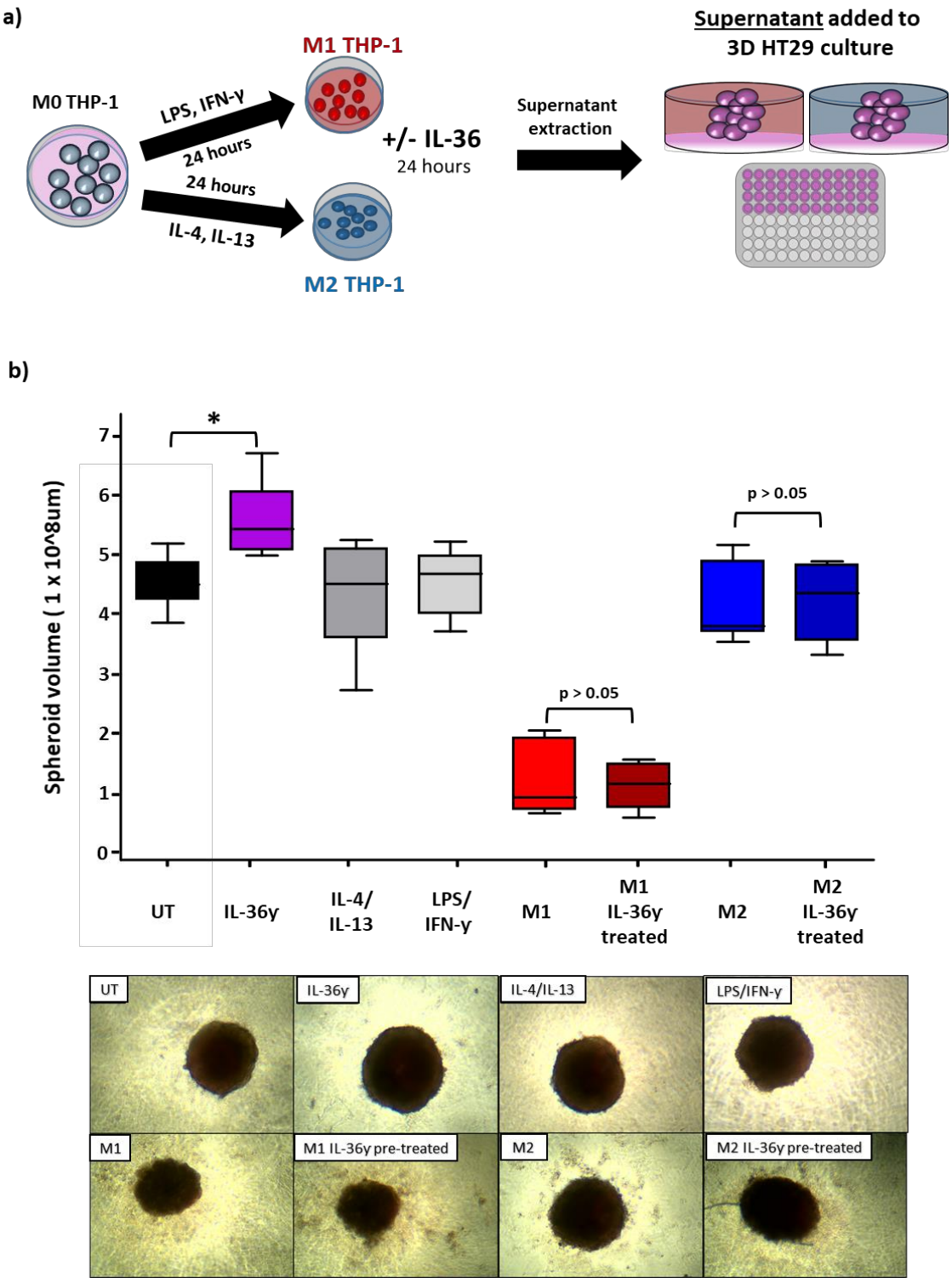


Figure 5.6: IL-36γ stimulation of M1 and M2 dTHP-1 cells does not alter their supernatant effect on cancer cell spheroid growth. a) THP-1 cells were differentiated to M1 and M2 types followed by 24 hour stimulation with IL-36γ (100 ng/mL). Cell supernatant was then removed and added to preformed HT29 cell spheroids. **b)** Spheroid diameter was monitored using live-microscopy imaging. Graphs are representative of independent, biological replicates (N=3). Data shown is the mean +/- SEM of six technical replicates. One-way ANOVA with post-hoc Dunnett's test is annotated as; * p= <0.05.

5.3.4. dM1 co-culture with HT29 cells inhibits spheroid growth, with IL-36γ stimulation of dM1 cells shown to have no effect on this inhibition of spheroid growth.

As the above experiment had shown no ability of the supernatant/secretome from IL-36γ-stimulated M1/M2 cells to affect cancer cell growth, we hypothesised that direct cell-cell contact might be required. As such, co-culture of cancer cell spheroids with IL-36γ stimulated dM1 or dM2 was investigated next. This was also to allow for direct cell contact between dM1, dM2 and cancer cells for investigation of properties such as macrophage phagocytosis/efferocytosis and localised delivery of cytotoxic metabolites such as iNOS and ROS which can induce cancer cell apoptosis [56]. A schematic of this experimental plan is outlined in Figure 5.7a. Similar to Figure 5.6, co-culture of dM1 and HT29 cells inhibited spheroid growth, with IL-36γ pre-treatment of dM1 cells did not alter this effect (Fig 5.7b). Similarly, IL-36γ pre-treatment of dM2 cells did not alter cell spheroid growth in co-culture when compared to dM2 cells. Moreover, co-culture of dM2 cells with HT29 cells did not significantly alter spheroid growth (Fig 5.7b). These findings further indicated that IL-36 did not significantly alter differentiated THP-1 phenotypes.

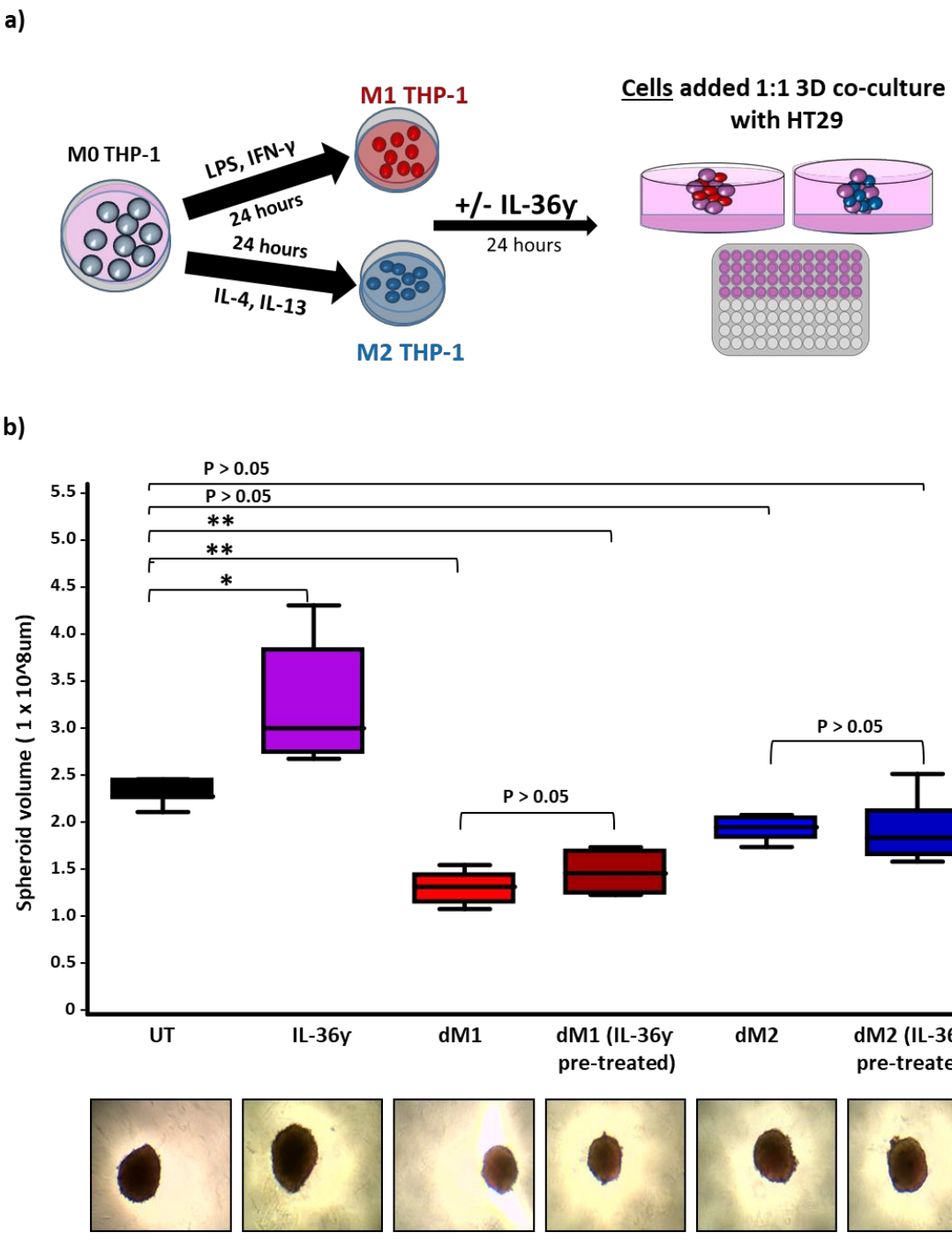


Figure 5.7: Co-culture of dM1 cells reduces spheroid formation but IL-36 pre-treatment of M1 or M2 dTHP-1s does not influence spheroid formation. a) THP-1 cells were differentiated to M1 and M2 cell types, stimulated with IL-36γ (100 ng/mL) for 24 hours then added to HT29 cells (1:1) and grown in 3D culture together. **b)** Spheroid diameter was monitored using live-microscopy imaging. Graphs are representative of independent, biological replicates (N=3). Data shown is the mean +/- SEM of six technical replicates. One-way ANOVA with post-hoc Dunnett's test is annotated as; ** p= <0.01, *** p= <0.001.

5.3.5. M1 dTHP-1 cell inhibition of HT29 spheroid formation can be recovered by addition of IL-36γ to co-cultures.

IL-36γ, as shown in chapter 3 of this thesis, can significantly contribute to HT29 spheroid formation *in vitro* and induce myeloid chemokine secretion. Cancers cells, particularly in CRC, can drive the pro-tumourigenic conversion of macrophage phenotypes [57]. Given this, we next investigated if IL-36 cytokines may contribute to this crosstalk in a co-culture of HT29 cancer cells and polarised macrophages. Similarly to the previous experiment, dM1 or dM2 cells were added in equal cell numbers to HT29 cells and seeded for spheroid formation. IL-36γ was then periodically added to culture supernatant to observe effects on both cancer cells and dM1/dM2 cells (Fig 5.8a). As previously shown, IL-36γ could drive increased HT29 spheroid growth (Fig. 5.8B, Lane 1 vs 2, $p = 0.0149$). Introduction of either dM1 or dM2 cells to HT29 spheroid cultures resulted in the inhibition of this IL-36-mediated augmentation of spheroid formation (Lane 2 vs 4/6, $p = 0.0018$ and 0.0499, respectively). Addition of IL-36γ to HT29:dM1 co-culture resulted in the return of spheroids to basal spheroid volume, indicating IL-36γ could overcome inhibitory effects of dM1 cells (Lane 3 vs 4, $p = 0.0290$). This was similarly shown in HT29:dM2 cultures, with IL-36γ treatment increasing the spheroid growth of this co-culture beyond basal levels (Lane 5 vs 6, $p = 0.0109$). This thereby indicated that IL-36γ more strongly influences cancer cells than dM1/dM2 cells. To gain further insight into this, the effects of IL-36 stimulated cancer cell supernatant on polarised macrophages should be investigated. This work does, however, indicate that IL-36 cytokines induce a more potent cellular response in cancer cells than macrophages. This would ideally be completed using peripheral blood monocytic cells. Whole blood sample limitations due to COVID-19 restrictions meant this component of the

project was progressed to the other aim of this chapter, IL-36 signalling and neutrophils in colon cancer.

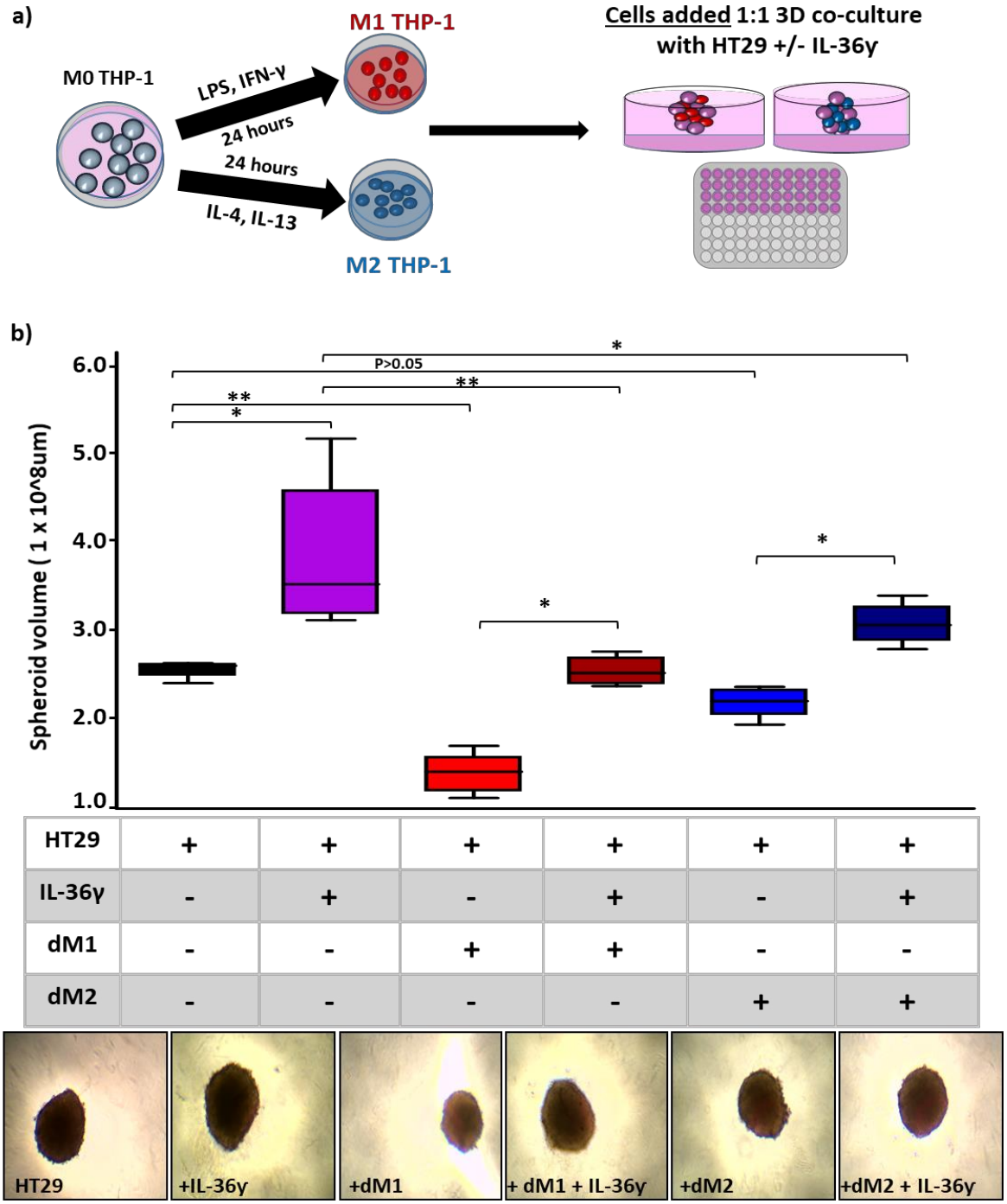


Figure 5.8: In co-culture, cancer cells are more responsive to IL-36γ stimulation than M1 or M2 dTHP-1 cells. a) THP-1 cells were differentiated to M1 and M2 cell types then mixed to equal numbers of HT29 cells (1:1) and grown in 3D culture together with repeated stimulation with IL-36γ (100 ng/mL). **b)** Spheroid diameter was measured using live-microscopy imaging. Graphs are representative of independent, biological replicates (N=3). Data shown is the mean +/- SEM of experimental duplicate. One-way ANOVA with post-hoc Dunnett's test is annotated as; * p= <0.05, ** p = < 0.01.

5.3.6. Differentiation of HL60 cells matures cells toward neutrophil-like cells with significantly increased expression of IL-36γ and chemokine receptors.

Our findings in chapter 3 strongly indicated that IL-36 stimulation of colon cancer cells results in a large induction of neutrophil chemoattractants including CXCL1, CCL20 and CXCL8 (Fig. 3.3). Furthermore, this induction has been previously reported in IL-36-driven psoriasis, with epithelial cell-derived chemokine production resulting in an influx of neutrophils to inflamed sites, with eventual induction of neutrophil NETosis [237]. Recently, it has been reported that neutrophil NETosis can contribute to cancer progression by immune evasion through coating cancer cells in NETs, thereby inhibiting innate killer cell contact with cells, significantly reducing their cytotoxic capacity [104]. We therefore hypothesised that IL-36 driven chemokine induction by intestinal cancer cells may contribute to neutrophil NETosis in the TME and thereby inhibit the cytotoxic effects of innate surveillance cells such as NK cells (Fig. 5.18).

In order to investigate whether IL-36γ-stimulated HT29 supernatant could induce NETosis in neutrophils, cells from the promyeoloblast cell line, HL-60, were differentiated into mature neutrophil-like cells. As above, HL-60 cells were selected as these experiments were performed during the period of COVID-19 pandemic restrictions, and it was not initially possible to collect blood at this time to isolate primary neutrophils. Cells were differentiated with varying concentrations of DMSO and assessed for CD11b expression (maturation marker), as well as cell viability, to determine the optimal conditions for future assays (Fig 5.9a). A concentration of 1.25-1.5% was determined to be optimal for HL-60 maturation of cells without excessive loss of viable cells. Cells were fixed (4% PFA), stained (167nM Sytox Green) and imaged by fluorescence microscopy to observe cellular morphology (Fig. 5.9B), which showed both mature and immature cells.

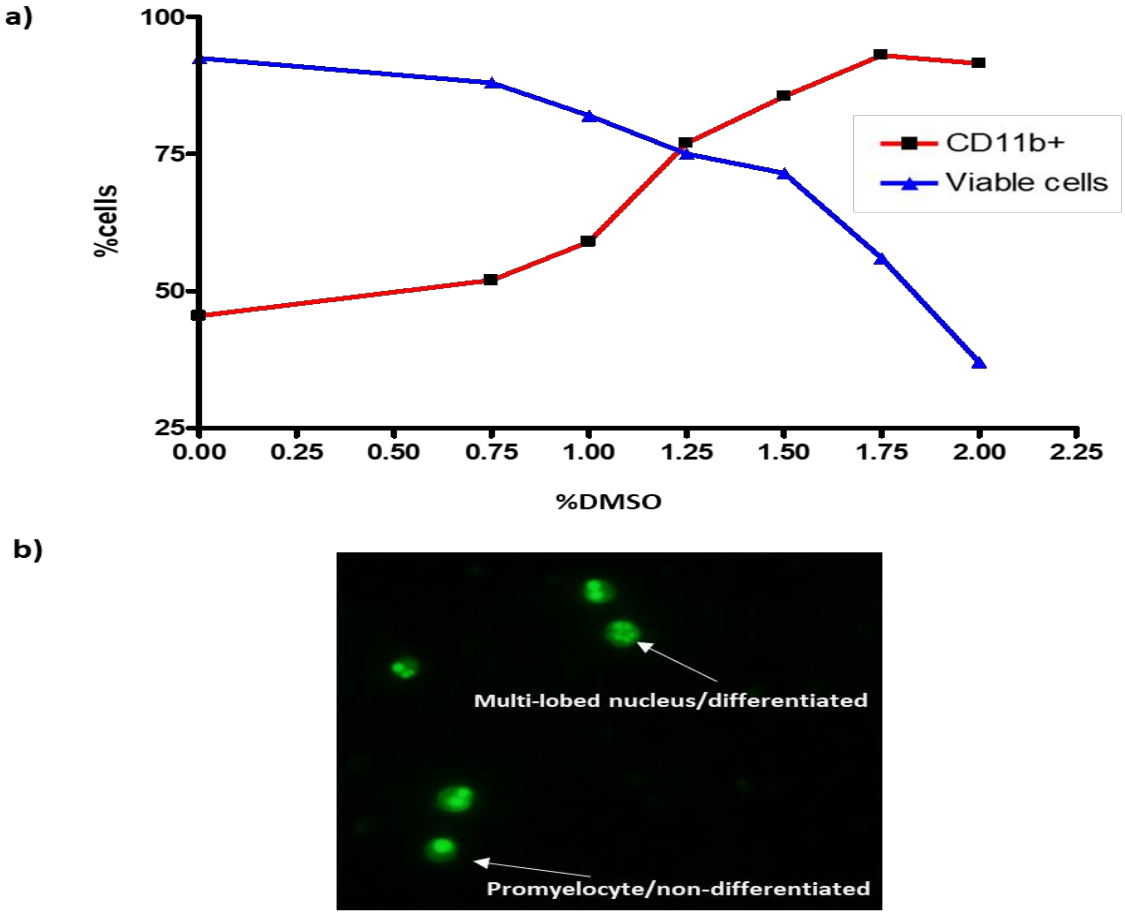
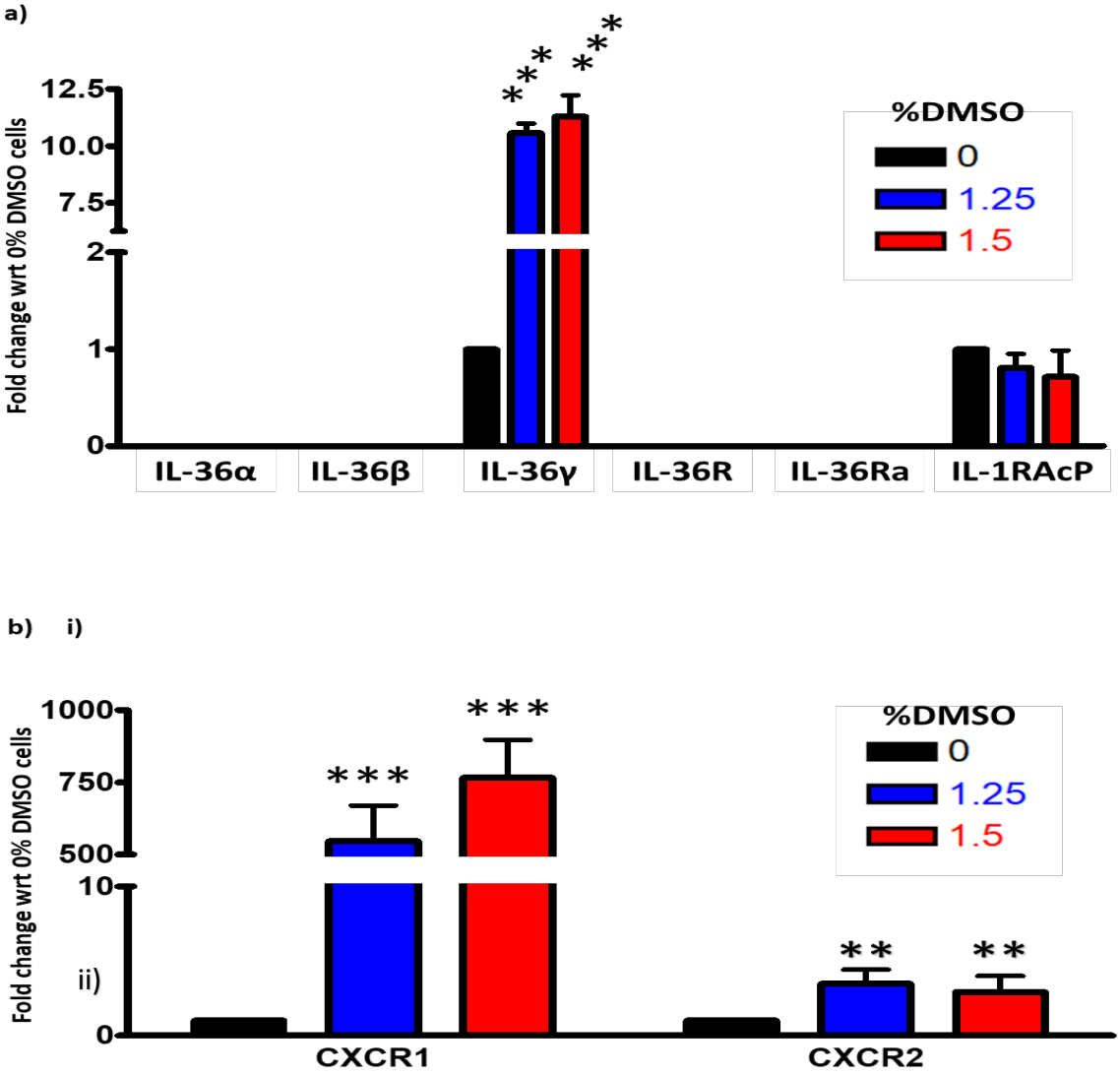


Figure 5.9: Culture of HL-60 cells in 1.25–1.5% DMSO optimally differentiates cells to neutrophil-like cells. a) CD11b expression in viable cells and cell viability were measured by FACS analysis to determine optimal differentiation concentrations of DMSO for HL-60 differentiation. Graph is representative of two biological replicates, completed in technical duplicate **b)** Differentiated HL-60 cells were fixed and then stained with nuclear dyes to examine nuclear morphology to indicate cell maturation.

Furthermore, differentiated cells significantly upregulated IL-36γ expression, with no other IL-36 family member genes shown to be expressed (Fig 5.10a). Moreover, expression of the chemokine receptors, CXCR1 and CXCR2, was significantly upregulated by DMSO differentiation, which is further indicative of neutrophil-like maturation (Fig 5.10b). Given the improved viability and similar gene expression profiles observed, HL-60 cells were differentiated using 1.25% DMSO thereafter.



%DMSO cells	CXCR1	CXCR2	β-actin
0	40 ± 0	31.51 ± 0.66	18.84 ± 0.42
1.25	27.96 ± 1.15	29.04 ± 0.87	17.29 ± 0.88
1.5	26.78 ± 0.54	28.39 ± 0.331	18.55 ± 0.46

Figure 5.10: Differentiation of HL-60 cells increases IL-36γ, CXCR1 and CXCR2 expression in cells. HL-60 cells were differentiated with the indicated DMSO concentration and gene expression of **a)** IL-36 family members and **b) i)** chemokine receptors was analysed by qRT-PCR. **ii)** Chemokine receptor CT values shown with SEM. Graphs are representative of three biological replicates. Error bars indicate SEM. One-way ANOVA with post-hoc Dunnett's test is annotated by the number of asterisks (**: p-value <0.01; ***: p-value <0.001).

5.3.7. dHL-60 cells can be stimulated by PMA to induce NETosis.

NETosis detection assays were optimised for PMA-induced dHL-60 NETosis. Two methods of detection were employed, fluorescence microscopy and a fluorescence plate reader assay (Fig 5.11a). The optimum PMA concentration (Pos. control) and MNAse activity was determined and used for subsequent experiments (Fig. 5.11b). 1 unit of MNase was shown to be sufficient to digest NETs for detection. Cells were highly responsive to various concentrations of PMA, with a concentration of 25-50 nM identified and used in subsequent experiments (Fig. 5.11c).

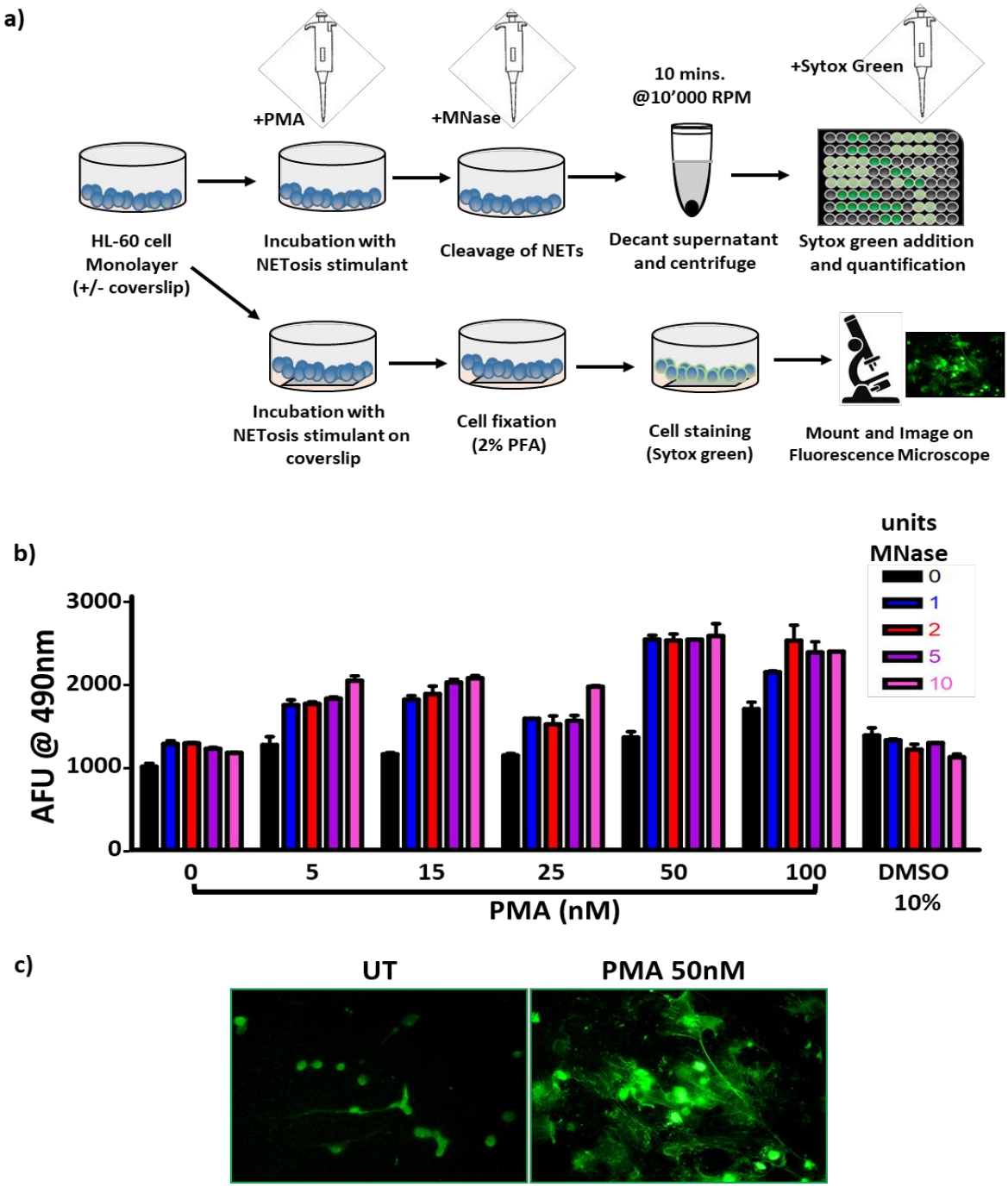


Figure 5.11: NETosis assay optimisation. a) Schematic of plate reader assays to detect NETosis in HL-60 cells. Cells were allowed adhere to the well of a 96 well plate, PMA (0-100 nM) was added followed by MNase (0-10 units) digestion of NETs. Cell supernatants were centrifuged, aliquoted, stained with Sytox green (167 nM) then read on a fluorescent plate reader at 490 nm. Alternatively, cells were grown on a poly-L-lysine treated coverslip, cells were stimulated, fixed, stained and mounted for fluorescence microscopy **b)** MNase and PMA concentration for subsequent assays was optimised as described above. **c)** Representative images of HL-60 cell netosis induced by 50 nM PMA.

5.3.8. HT29 cell supernatant can induce dHL-60 NETosis, with IL-36y stimulation of HT29 cells capable of further increasing this effect.

In order to determine whether HT29 protein secretion could indeed induce NETosis in dHL-60 cells, dHL-60 cells were cultured in HT29 cell supernatant. dHL-60 cell NETosis was detected as outlined in figure 5.11a. HT29 cell culture supernatant significantly induced NETosis in dHL60 cells (Fig 5.12a/b). Prior stimulation of HT29 cells with IL-36y was capable of a much more significant induction of NETosis in dHL60 cells as compared to unstimulated cells. In contrast, direct stimulation of dHL-60 cells with IL-36y did not induce NETosis in the cells. These results indicated that colon cancer cell secretions could indeed induce NETosis in dHL60 cells and that IL-36 stimulation of colon cancer cells resulted in further induction of NETosis in dHL60 cells.

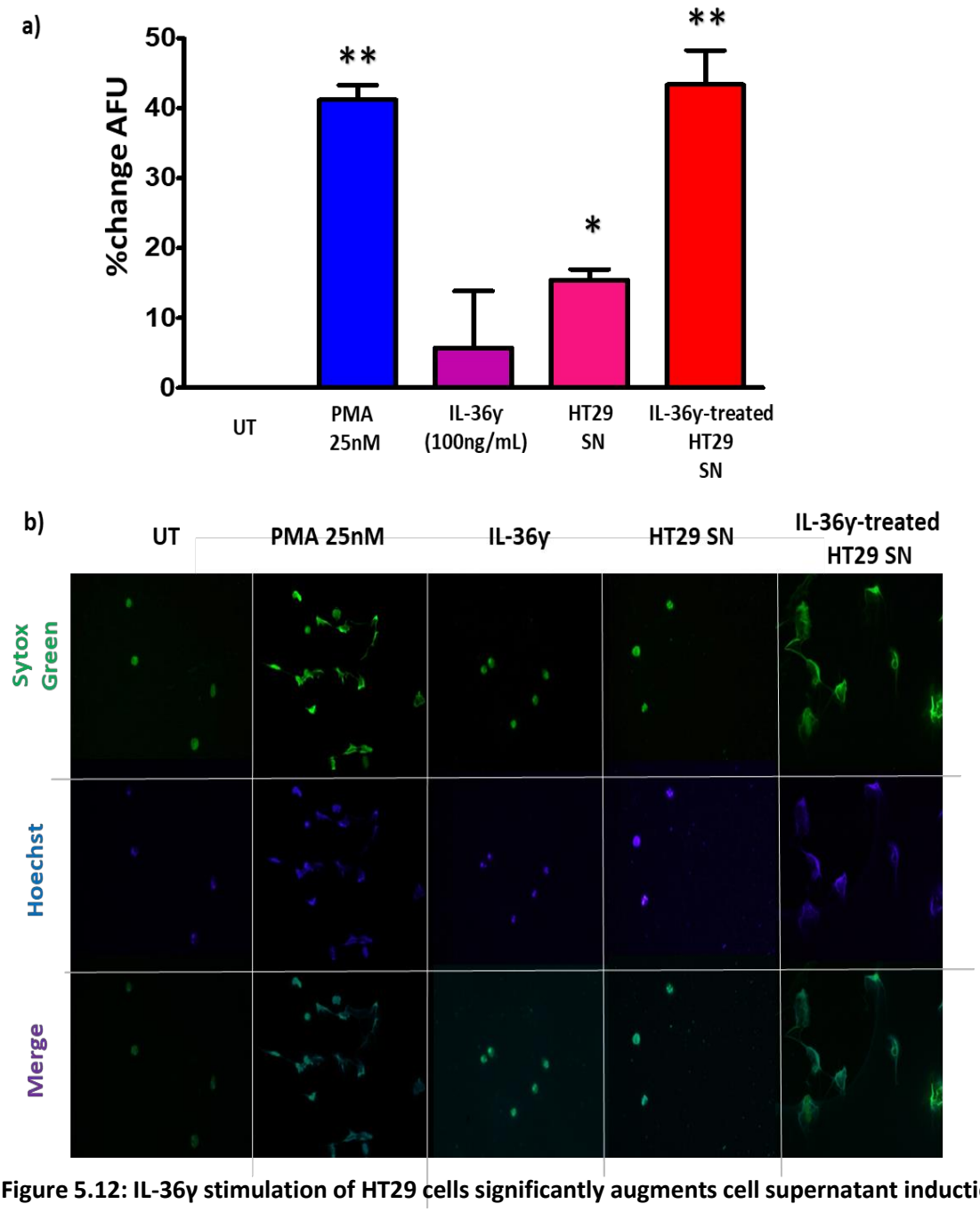


Figure 5.12: IL-36γ stimulation of HT29 cells significantly augments cell supernatant induction of HL-60 NETosis. a) HT-29 cells were stimulated with IL-36γ (100 ng/mL) for 24 hours and supernatant was then directly added to a HL-60 monolayer for four hours. NETosis was quantified by plate reader assay. **b)** Individual and merged fluorescence microscopy images of HL-60 cells treated with indicated stimulants. All experiments were repeated as technical duplicates, with a minimum of 3 biological replicates completed. One-way ANOVA with post-hoc Dunnett's test is annotated as; * p= <0.05, ** p = < 0.01.

5.3.9. Isolated polymorphonuclear cells show varying IL-36 family member expression per donor, with constitutively high chemokine receptor expression.

Given the above findings, and a change in COVID-19 pandemic regulations meaning that we were now in a position to collect blood samples, it was next decided to investigate this effect using whole blood sample derived PMNs. PMNs were isolated from EDTA anti-coagulated whole blood samples from individual donors (Fig. 5.13a). PMNs were fixed (4% PFA) and stained (167 nM Sytox green) to examine cell morphology and the purity of isolated cells. The majority of isolated cells consisted of neutrophils, with the typical multi-lobed nuclei present in these cells (Fig 5.13b). Furthermore, isolated PMNs were analysed by FACS for cell viability and CD11b expression, with both viability and CD11b expression typically >90% (Fig 5.13c). Once isolation of PMNs was optimised, RNA was extracted from these cells, cDNA generated, and gene expression of IL-36 family and chemokine receptors determined by qRT-PCR (Fig. 5.14a). IL-36γ was highly expressed by all donors, as was CXCR1 and CXCR2. Other genes varied per donor, although IL-36Ra expression was not detected in any PMNs. Pooled donor cDNA relative expression of CXCR1 and CXCR2 was compared with dHL-60 cells, with both receptors much more highly expressed in PMNs isolated from donor blood (Fig. 5.14b)

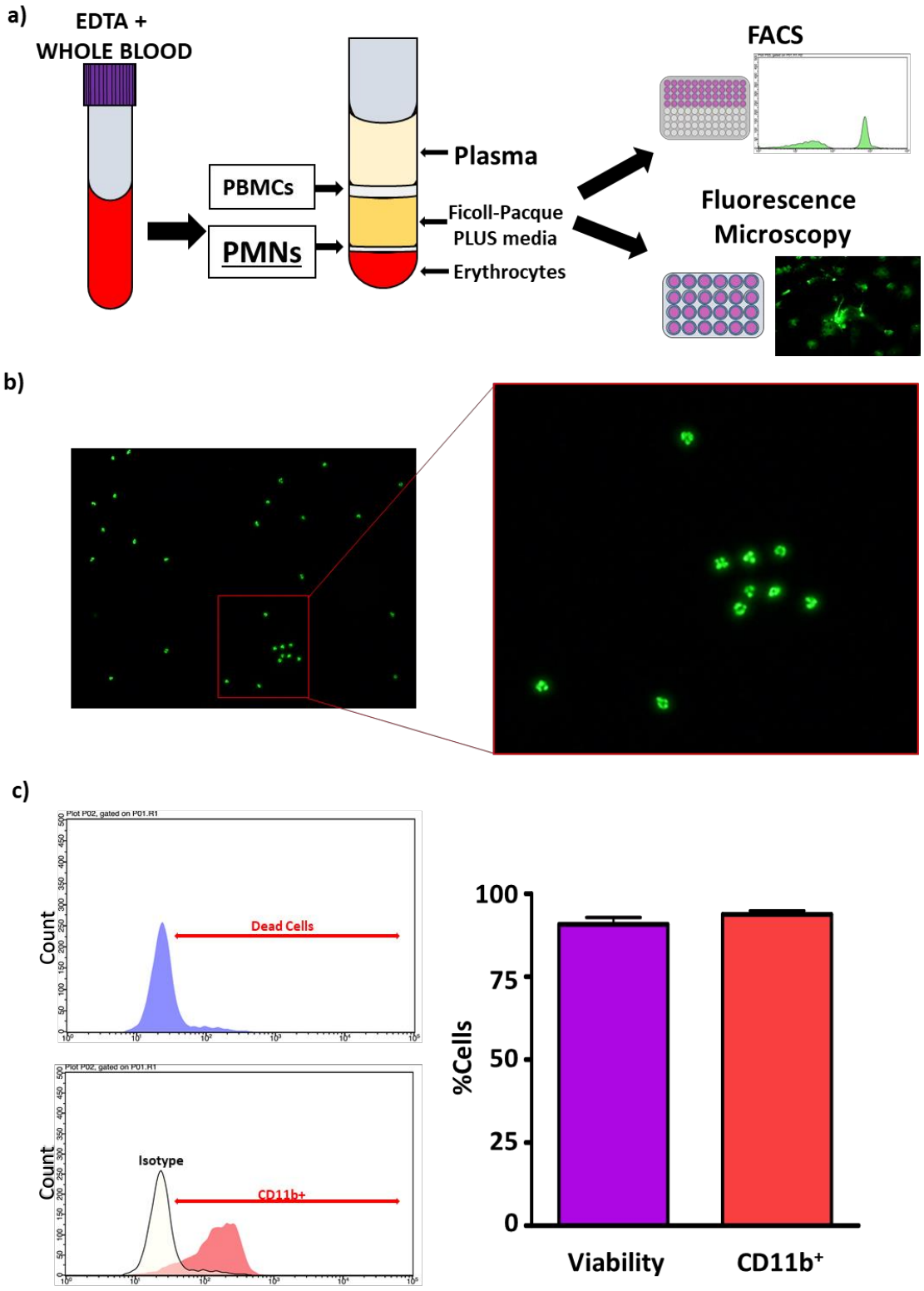


Figure 5.13: Isolation of PMNs from peripheral blood samples. a) Whole blood was collected in EDTA vacuum tubes then separated by gradient density into blood components. The granulocyte cell layer was removed from density-separated samples and treated with RBC lysis buffer and then used for subsequent analysis. **b)** Isolated PMNs were fixed and stained with Sytox green to assess cell morphology and purity. **c)** Isolated PMNs were assessed for CD11b expression and viability by FACS analysis.

a)

Gene	N=1	N=2	N=3	Average CT
IL-36α	40	35.84	40	38.613 ± 1.38
IL-36β	40	29.87	31.92	33.93 ± 3.09
IL-36γ	29.77	26.9	27.69	28.12 ± 0.85
IL-36R	40	28.27	40	36.09 ± 3.91
IL-36RN	40	40	40	40 ± 0
CXCR1	27.87	25.82	28.37	27.353 ± 0.78
CXCR2	28	25.77	29.31	27.693 ± 1.03
B-actin	23.28	22.04	24.61	23.31 ± 0.742

b)

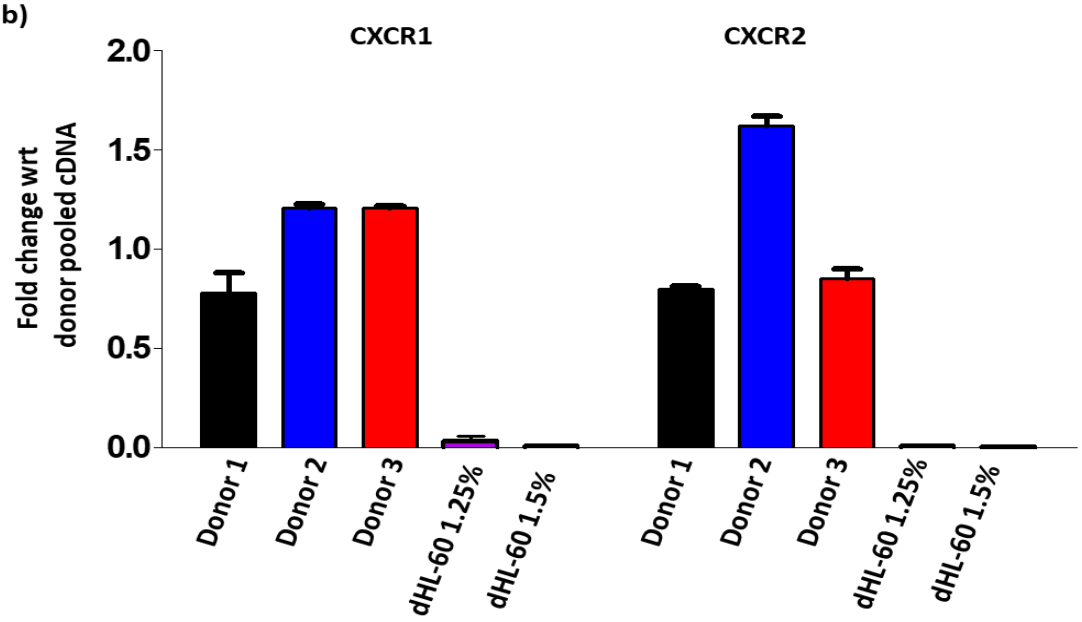


Figure 5.14: IL-36 expression in PMNs varies per donor and CXCR1/2 expression is higher in PMN's when compared to dHL60 cells. a) PMNs were isolated from whole blood sample donors and IL-36 family member gene expression was quantified by qRT-PCR (N=3). SEM is indicated in average CT value column. **b)** CXCR1 and CXCR2 expression was compared between dHL-60 cells and PMN's isolated from healthy donors (n=3)

5.3.10. CXCL1, CXCL5 and IL-8 can induce NETosis in PMNs via CXCR1/CXCR2 binding.

Initial control experiments were performed to confirm that PMN NETosis could be induced by chemokines that were highly expressed in response to IL-36 stimulation of colon cancer cells, as previously shown in chapter 3 (Fig 3.3). Cells were stimulated with a series (200 ng/mL) or combination (200 ng/mL combined total) of chemokines as indicated (Fig. 5.15a). CXCL1 and IL-8 were the most potent inducers of NETosis, although CXCL5 could also significantly induce NETosis in PMNs. Stimulation of PMNs with a combination of chemokines showed the largest induction of NETosis. Reparixin, a chemokine receptor (CXCR1/CXCR2) inhibitor, successfully inhibited chemokine induced NETosis in cells, without the inhibition of PMA-induced NETosis. This confirmed that chemokines could induce, albeit variably, NETosis in isolated PMNs. Images were also taken and used to confirm these results using fluorescence microscopy (Fig 5.15b). It was also confirmed, similar to dHL-60 cells, that direct IL-36 stimulation of PMNs did not induce NETosis in cells (Fig 5.16). These findings confirmed chemokines, and not IL-36 cytokines, could induce NETosis in PMNs

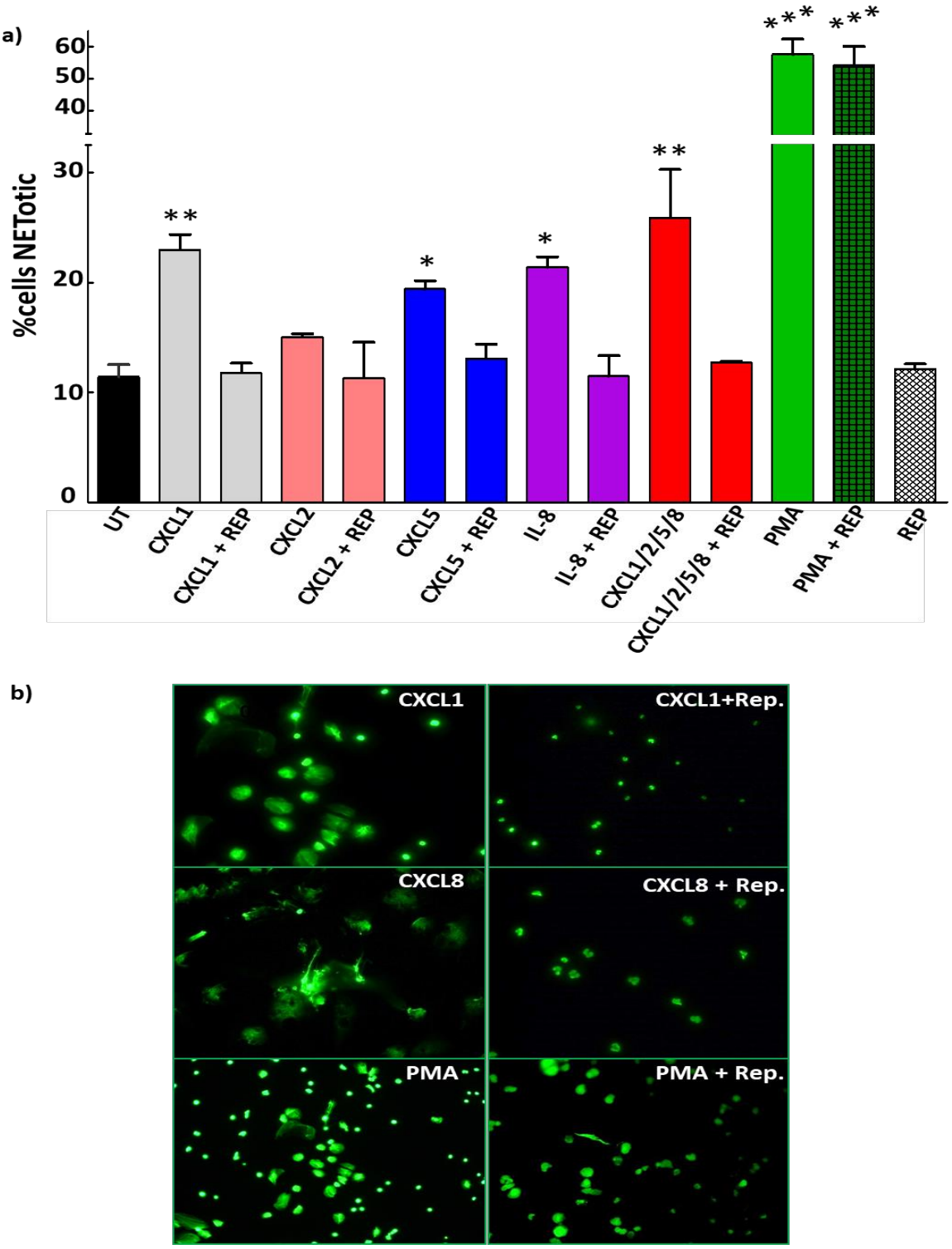


Figure 5.15: Chemokines can directly induce NETosis in PMNs via CXCR1/CXCR2. a) Isolated PMNs were pre-treated with 5 nM reparixin and then treated with various chemokines (200 ng/mL) to induce NETosis, which was detected by FACS analysis of Sytox green positive cells. **b)** Representative fluorescence microscopy images of chemokine induced NETosis and inhibition by reparixin. One-way ANOVA with post-hoc Dunnett's test is annotated as; * p= <0.05, ** p = < 0.01, *** p =< 0.001.

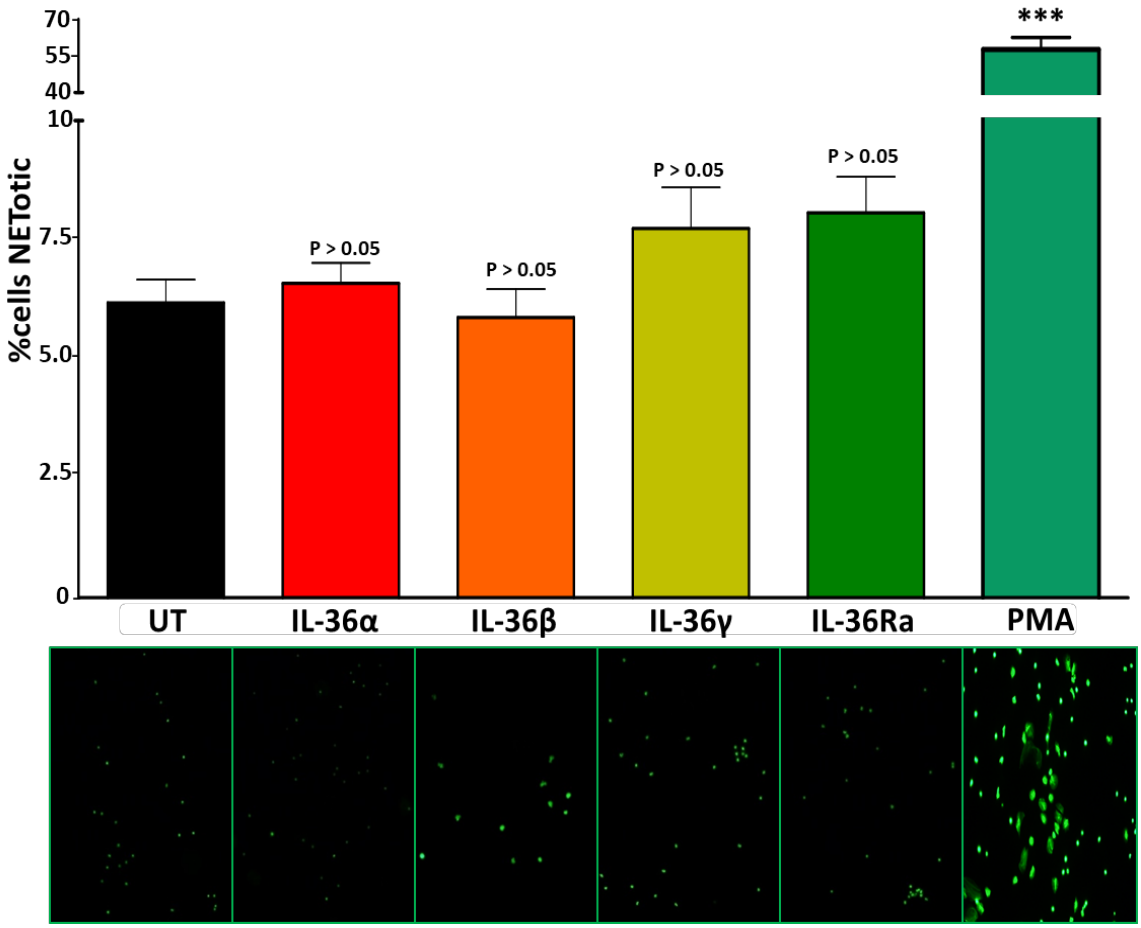


Figure 5.16: IL-36 cytokines do not directly induce NETosis in PMNs. Isolated PMN's were cultured with individual IL-36 cytokines (100 ng/mL), IL-36Ra (100 ng/mL) or PMA (50 nM). Cells were then processed for FACS analysis or fluorescence microscopy. Graphs are representative of three biological replicates. Error bars indicate SEM. One-way ANOVA with post-hoc Dunnett's test is annotated as; ***: p-value <0.001).

5.3.11. Stimulation of isolated PMNs with IL-36 cytokine-treated supernatant does not induce NETosis, but does induce migration of PMNs.

Given that PMN NETosis could be induced by stimulation of cells with chemokines, which were previously shown to be highly inducible by IL-36 stimulation of colon cancer cells, it was next of interest to investigate cancer cell-derived induction of PMN NETosis. HT29 cells

were stimulated with IL-36 ligands (100 ng/mL) for 24 hours, the supernatant was then centrifuged to remove debris and then added to adherent PMNs for 4 hours. No significant increase in PMN NETosis was detected following incubation of the cells with either the HT29 cell culture supernatant or IL-36 agonist-stimulated HT29 cell culture supernatant. IL-8 or PMA stimulation of cells continued to show significant increases in PMN netosis (Fig 5.17a). Given there was no detectable change in NETosis, cells were examined for phenotypic changes by live-cell imaging, where it was observed that addition of HT29 supernatant did alter the phenotype of PMNs to an elongated, migratory state (Fig. 5.17b). This migratory state could be inhibited with the addition of reparixin. This suggested that cancer cell chemokines could indeed influence PMNs but concentrations may not have been sufficient for NETosis induction in this model.

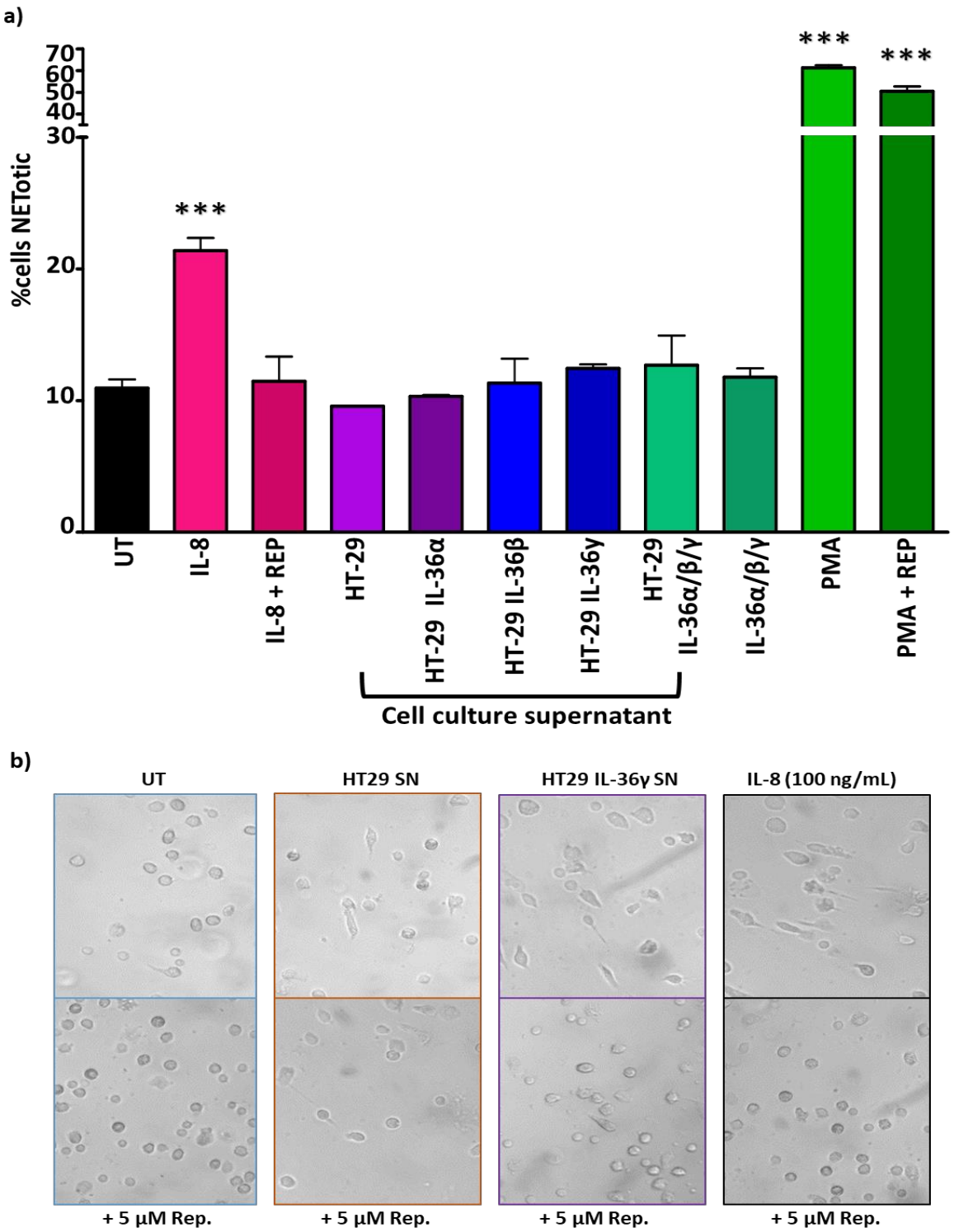


Figure 5.17: IL-36 treated HT29 cell supernatant could not induce NETosis in PMNs but did induce a migratory phenotype in cells. Isolated PMNs were stimulated with HT29 and IL-36 treated HT29 supernatants (stimulated individual IL-36 cytokines or combined [100 ng/mL]) for 4 hours. **a)** PMNs were then analysed by FACS for changes in NETosis. Graphs are representative of three biological replicates. Error bars indicate SEM. One-way ANOVA with post-hoc Dunnett's test is annotated as; ***: p-value <0.001. **b)** Brightfield images (20X obj. lens) of isolated PMNs were taken after 4 hours under the indicated conditions.

5.3.12. Discussion

IL-36 signalling has been extensively studied across many pathologies, with these cytokines most often associated with the innate immune response or bridging the innate-adaptive immune response [273, 458]. In contrast to this, the field of IL-36 cancer research, although expanding rapidly, has focussed predominantly on the role of IL-36 signalling in the adaptive immune response [199, 379, 413]. This is understandable given that adaptive anti-tumour immunity has been shown to be at the core of tumour rejection and improvement of survival outcomes for patients [459, 460]. Despite myeloid cells being frequently implicated in driving tumourigenesis [461], roles for the innate arm of immunity in IL-36 cancer research remain relatively unexplored. Given this research gap and our previous findings indicating IL-36 signalling strongly induces the expression of numerous myeloid cell chemoattractants, the aim of this chapter was to investigate the role IL-36 signalling plays in cancer cell crosstalk with macrophages and neutrophils.

Macrophages, both M1 and M2, have been implicated as an important source of IL-36 in the TME [414]. Furthermore, it has been shown, albeit in carotid plaques, that M2 macrophages display higher cathepsin S activity than M1 macrophages, a key proteolytic enzyme for IL-36 activation [462], suggesting a role in IL-36 in tumour associated macrophage-mediated disease progression. It was decided to investigate macrophages and IL-36 signalling in the TME by differentiating the human leukaemia monocytic cell line, THP-1, given the whole blood sample limitations at the time due to pandemic restrictions. Our work here showed that THP-1 cells could indeed be polarised into M1 (dM1) and M2-like (dM2) macrophages *in vitro*; however further IL-36 stimulation of cells did not show significant changes in gene expression, with the exception of further downregulation IL-1β

in dM2 cells, which could be due to a negative feedback loop to regulate inflammation. IL-36 stimulation of macrophages has been previously reported to promote their activation [236, 290, 294, 325]. The lack of responsiveness reported here may result from the excessive stimulation of cells *in vitro*, with cells cultured continually with high concentrations of M1 and M2 polarising soluble factors, thus limiting further polarisation. It has previously been shown that THP-1 dM0 cells do not respond to IL-36β or IL-36γ, due to a lack of IL-36R expression [290]. However, this is the first time, to the best of our knowledge, that dTHP-1 cells have been reported to be non-responsive to IL-36 stimulation despite a significant increase of IL-36R expression in both cell types. In further contrast to our findings, it has also been shown that M2 macrophages may be polarised toward a more pro-inflammatory phenotype, with IL-36β inducing IL-6 secretion in these cells. However, this study used PBMCs, which are likely to be more reflective of IL-36 macrophages *in vivo* [236]. It may be possible that IL-36 stimulation of these dTHP-1 cells may require co-stimulation for synergistic augmentation of cells, as seen with IL-17a, IL-22 and TNF-α in combination with IL-36 cytokines to stimulate keratinocytes [463].

Previous work has investigated the reliability of polarised THP-1 cells as models of classically or alternatively activated macrophages, with M2 polarised THP-1 cells shown to be less reflective of PBMC polarised M2 cells [464]. These lack of changes reflect our findings, especially in the context of supernatant culture with spheroids which showed similar growth patterns as untreated colon cancer cells. However, the same study did show that other functional properties did differ between polarised cells types, such as dM1 and dM2 internalisation of beads, reflecting phagocytosis capacities of these cells [464]. Although this work suggests a limited capacity of THP-1 derived macrophages to model IL-36 interactions, it is also important to consider heterogeneity of recruited and tissue

resident macrophages, with isolated tumour-derived macrophages the best models of cytokine-macrophage interaction in the TME [465].

Although the response of differentiated THP-1 cells to IL-36 in terms of chemokine production was minimal, it was still of interest to examine the influence dM1 and dM2 cells may have on colon cancer cell growth given the limited scope of gene expression changes assessed. Other considerations include cancer cell-macrophage crosstalk, given the ability of tumour cells to convert macrophages between M1 and M2 phenotypes. Furthermore, macrophage ingestion of tumour-cellular debris by phagocytosis or efferocytosis can dictate the pro-inflammatory or anti-inflammatory profile of macrophages, thereby influencing tumour cell growth [466]. Co-culture of dM1 cells with HT29 cells significantly reduced spheroid formation, although IL-36 pre-treatment of dM1 cells before co-culture did not alter this inhibition. Similarly, pre-treatment of dM2 cells did not alter their effect on spheroid culture, with M2 cells shown neither to inhibit nor promote spheroid growth. In agreement with M1 and M2 macrophage classical functions, this work showed dM1 cells had an inhibitory effect on colon cancer cell growth, potentially due to secretion of iNOS, resulting in toxic metabolite production which can slow tumour growth [467]. This is reflected in work previously showing dM1 co-culture with A549 cells to increase susceptibility to etoposide treatment in these lung cancer cells, with co-culture of M1 macrophages shown to limit cancer cell growth [67, 468, 469]. dM2 cells showed little influence on HT29 spheroid growth, possibly given the weaker polarisation of these cells in comparison to dM1, as reported here and by others [464]. Notably, simultaneous stimulation of dM1 and HT29 co-culture could recover the significant reduction of spheroid growth. This recovery was limited in that it did not reproduce, in full, the increase in growth of HT29 cells in response to IL-36γ stimulation alone. However, the co-culture spheroids

consisted of equal cell numbers of dM1s and HT29 cells (750 cells each), and thus the proliferative growth capacity of HT29 cells (1500 cells) treated was unlikely to reproduce that of IL-36 treated cancer cells in monoculture. A further possibility for this apparent diminished proliferative effect may be the expression of the IL-36R in these unresponsive dM1 cells, which thereby 'soak up' free IL-36 cytokine and prevent binding to IL-36R-expressing cancer cells. Overall, this work shows that IL-36 signalling is likely to have more potent effects on IL-36R^{+} expressing cancer cells more so than IL-36R^{+} macrophage populations. Whilst we did measure the influence of dM1 and dM2 cells and supernatants on colon cancer cell growth, we did not investigate the role of cancer cell supernatant on dM0, dM1 an dM2 cells. It has been previously shown that colon cancer cell line supernatant can alter THP-1 phenotypes towards a mixed population of M1 and M2 phenotype cells, and this therefore may have influenced outcomes of our co-culture studies [470]. IL-36 may contribute to other key macrophage anti-tumour functions such as antigen cross presentation with CD8^{+} T cells, however, this would require more sophisticated *in vitro* models to study [68]. In conclusion, our findings, with this THP-1 model of macrophages, indicated that IL-36 may alter influence M2 macrophage gene expression but ultimately in a co-culture with colon cancer cells, IL-36 cytokines more potently drive pro-tumourigenic properties of cancer cells.

Research to date has reported both anti and pro-tumourigenic roles for neutrophils, although this research suggests these cells mostly contributing to tumour progression [103] . These roles include genotoxicity [471], tumour-proliferation induction [472], angiogenesis [101], immunosuppression [473] and pro-tumour feedback loops of inflammation [474]. Furthermore, NETosis has now become highly implicated in several processes in cancer progression such as immune evasion [104], cancer cell proliferation induction [455] and

direct induction of cancer cell metastasis [456]. Here we show that cancer cell induction of NETosis is augmented by IL-36 stimulation of cancer cells. We, and others, have previously reported significant induction of epithelial cell secretion of CXCR1/CXCR2 binding chemokines in response to IL-36 stimulation of these cells [287, 294, 475]. Furthermore, it has been reported that this IL-36 driven chemokine secretion in tissues results in significant increases in neutrophil infiltration and subsequent NETosis of these cells upon reaching these chemokine-rich local sites of inflammation [237]. Additionally, it has been well documented that chemokines are chronically upregulated in both local tissues and systemically in colon cancer patients [476]. It is therefore of little surprise that colon cancer cell culture supernatant induces dHL-60 NETosis, with IL-36 stimulated colon cancer cell supernatant increasing this further. We, at the time of writing this chapter, are the first to report NETosis induction by IL-36 stimulated cancer cell culture supernatant.

A recent study has shown that cancer-cell derived chemokine secretion can induce neutrophil NETosis and thereby assist in immune evasion of cancer cells from surveillance cells by cells such as NK cells [104]. We could reproduce this induction of NETosis in the HL-60 model of neutrophils and show that IL-36γ treatment of cancer cells could further induce NETosis. However, we could not reproduce this work using whole blood derived PMNs. We showed that induction of NETosis in isolated PMNs was possible with several chemokines and that this effect could be blocked using a CXCR1/CXCR2 inhibitor, reparixin. However, HT-29 supernatant, IL-36γ-stimulated or otherwise, did not induce PMN NETosis. It has been previously reported that neutrophil migration and induction of NETosis are governed by the chemokine gradients present in circulation. Cells migrate closer to chemokine-rich inflamed tissues, then undergo NETosis to provide their homeostatic function of pathogen-arrest and degradation of NETs to control infection [477]. We have shown that the

concentration of CXCL-1 in unstimulated and IL-36-stimulated HT29 cell culture supernatant to be approximately 2 ng/ml and 10-50 ng/mL, respectively [475]. The concentrations of chemokines used in this work were higher than those used previous studies, which would therefore be expected to result in increased NETosis. However, we did not see this effect, which suggests that our assays may not have been sufficiently sensitive in detection of NETosis [104, 477]. However, it was observed that the neutrophil phenotype did alter upon incubation with HT-29 supernatant, with substantial changes showing elongation of cells to a migratory phenotype. This suggests that the concentration of chemokines, in our model, was sufficient for migration induction, but not NETosis induction of these cells. Furthermore, it may be possible that HL-60 cell NETosis induction may have occurred as a result of TNF-α/ GM-CSF secretion by HT-29 cells, both of which may be induced by IL-36 signalling [478, 479]. Although significant induction of CXCR1/2 expression was enhanced by DMSO differentiation of these cells, the relative expression of these receptors was much lower than that seen in PMNs, further suggesting an alternative pathway may have contributed to this induction of NETosis.

As described by previous groups investigating IL-36 in psoriasis [237], and based on our preliminary model of IL-36-stimulated colon cancer cell induction, we further propose that an IL-36 mediated cancer cell and neutrophil bidirectional feedback loop may contribute to CRC pathogenesis. It has been well recorded, and shown in this thesis, that IL-36 cytokines can significantly induce the secretion of neutrophil chemoattractants by tumour cells [287, 475], and that IL-36 is a critical amplifier in neutrophil-driven inflammation [321]. Furthermore, an increasing chemokine gradient can change neutrophils from migratory to NETotic cells [477]. Our preliminary work here demonstrates this by showing induction of neutrophil NETosis by colon cancer cell culture supernatant *in vitro*, with IL-36 stimulation

of cancer cells enhancing this process even further. Importantly, it has also been shown that neutrophils are the primary sources of IL-36 cleaving proteases and proteomic analysis of NETs has shown these proteases account for ~9% of the total protein associated with the NETs released from cells [216, 309, 480]. In addition to this, it has been shown that NETs may interact directly with CCDC25 expressing colon cancer tumour cells and enhance cellular migration, with these properties also closely associated with IL-36 stimulation of colon cancer cells [385, 456, 475]. It is therefore plausible that neutrophil-cancer cell crosstalk via NETosis may be readily amplified by IL-36 signalling which may contribute to colon cancer progression by several pathways including immune evasion, cancer cell metastasis and induction of cancer cell proliferation (Fig. 5.18). Although these findings are preliminary, the ability of IL-36 cytokines to drive NETosis and contribute to cancer progression warrants further investigation.

In conclusion, this chapter has investigated the role of IL-36 signalling in the TME with respect to myeloid lineage cells. Our co-culture model of dM1 and dM2 macrophages suggests that in the TME, IL-36R-expressing cancer cells are more responsive to IL-36 stimulation; however, these proliferative effects may be diminished with large infiltration of macrophages. Further modelling using tumour-derived macrophages would be required to answer this question more definitively. Our *in vitro* co-culture of neutrophil-like cells has shown the potential for a feedback loop to exist in colon cancer that may strongly contribute to the pre-metastatic niche of cancer cells by perpetual IL-36 signalling and neutrophil-cancer cell crosstalk. Overall, this chapter has highlighted potential roles for IL-36 signalling in innate immune cell to cancer cell crosstalk in the CRC TME.

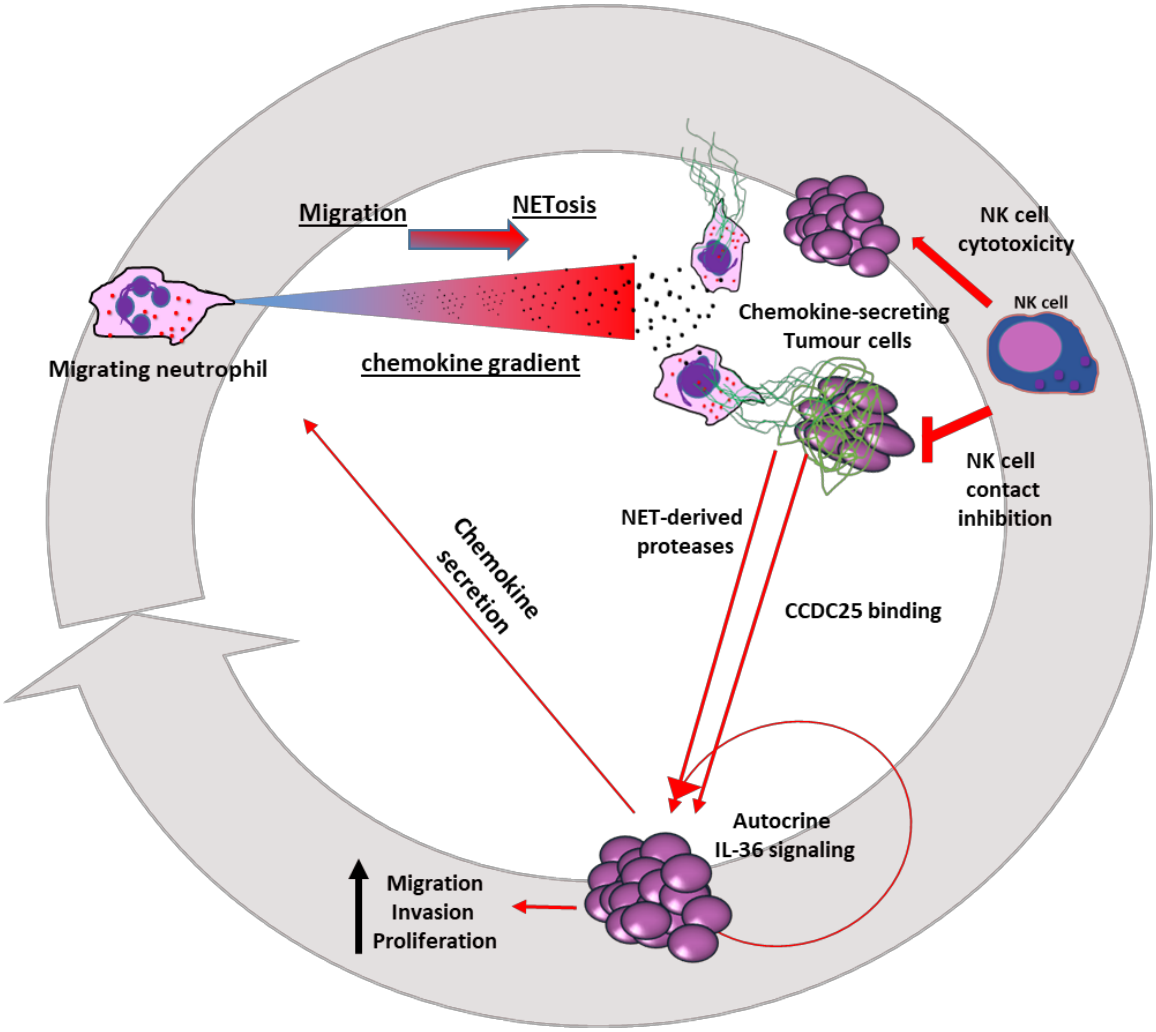


Figure 5.18: Proposed neutrophil positive feedback loop in CRC resulting in cancer progression. IL-36 stimulation of cancer cells results in a large augmentation of neutrophil chemoattractant expression. This results in a chemokine gradient for neutrophils to migrate towards the TME. If sufficient, neutrophils may undergo NETosis resulting in NET-coated cancer cells which avoid immune surveillance cell contact. Furthermore, NETosis releases IL-36 activating proteases which can perpetuate this feedback loop enhancing IL-36-driven cancer cell migration, invasion, proliferation and further chemokine secretion.

Chapter 6

Transcriptomic analysis of IL-36 signalling in colorectal cancer

Results of this chapter have been submitted for publication;

Baker KJ, Brint E, Houston A.

Cancer cell directed IL-36R signalling in CRC; a transcriptomic analysis.

6.1. Aim

Research in the field of IL-36 signalling in tumorigenesis has expanded rapidly in recent years with novel findings reported across multiple different cancer types [199]. Initially, the majority of this research reported IL-36 signalling to be highly beneficial to the enhancement of the anti-tumour immune response, thereby facilitating immune-mediated tumour rejection [235, 299, 378-380, 414]. More recent findings have highlighted the bystander effects of IL-36 signalling in the TME, with direct stimulation of tumour cells shown to induce pro-tumorigenic responses [243, 385, 386, 431, 475, 481]. This dichotomous effect of IL-36 signalling is particularly evident in colon cancer, with both anti-tumorigenic and pro-tumorigenic roles now reported. The aim of this chapter is to use large patient cohort transcriptomic datasets to gain further insights into the role of IL-36 signalling in colon cancer.

6.2. Introduction

Research investigating IL-36 signalling in multiple cancers has expanded greatly in the past decade [199]. Initially, the majority of this research reported IL-36 signalling to be anti-tumorigenic via augmentation of the immune response in order to drive immune-mediated tumour rejection [235, 299, 378-380, 414]. Complementing this work was evidence of decreased IL-36 expression in multiple cancer types, including colon cancer [369, 371]. This loss of expression was proposed to result in a reduction in stimulation of type-1 mediated immune responses, which could be subsequently rescued by overexpression of IL-36 expression in the TME, resulting in increased infiltration of anti-tumour immune populations, as well as the increased formation and sustenance of tertiary lymphoid structures [235, 299, 419]. The complexities of IL-36 signalling are further highlighted by IHC analysis performed in multiple patient cohorts revealing contrasting findings as to whether IL-36 family member expression is indeed increased or decreased in cancer tissue. Wang *et al.* have previously reported IL-36 cytokine expression to be lost in colon cancer tissue, whilst reporting increased IL-36α and IL-36γ expression to be associated with poorer and better overall survival, respectively [369]. Contrasting this, our group has previously reported IL-36 cytokine expression to be increased in colon cancer tissue relative to adjacent normal tissue [475]. Recent studies are now reporting that IL-36R^{+} tumour cells are able to potently respond to IL-36 cytokine stimulation; resulting in the induction of pro-tumorigenic phenotypes of these cells. This has been shown most recently in gastric cancer cells *in vitro*, in NSCLC *in vivo* and colon cancer cells, both in this thesis and recently by an independent research group *in vivo* [243, 385, 386, 431, 475, 481]. Therefore, the role of IL-36 signalling in the context of cancer remains unclear, with evidence of divergent pro-

and anti- tumorigenic phenotypes being reported, similar to that seen with other IL-1 family members [196].

Given the multi-faceted role of IL-1 family members in cancer, which can influence many contributing and mitigating factors of carcinogenesis, further insights into several aspects of the colon cancer TME such as immune cell composition, differential gene expression analysis of tumours and survival analysis of patient cohorts based on IL-36 family gene expression are required. Investigating the molecular basis of cancers has helped to develop targetted-therapies based on the molecular profile of the tumour, e.g., the EGFR status of tumours [459]. Moreover, transcriptomic analysis of colon cancers has provided insight into the immune composition, mutational background and prognosis of tumours based on gene signatures alone by clinical molecular subtyping as well as other more recent methods [31, 426, 430, 482]. Given the diverse and vast information which can be provided by transcriptomic data and then applied to pre-clinical models to enhance therapies, the aim of this chapter is to analyse transcriptomic data in order to provide new insights into the role of IL-36 in tumour progression and/or rejection in colon cancer and validate these findings using *in vitro* and *ex vivo* methods for colon cancer cell lines and CRC patient cohort samples, respectively.

6.3. Results:

6.3.1. IL-36 family member expression is altered across the adenoma-carcinoma sequence

To further investigate our previous findings in this thesis, as well as the mounting evidence that IL-36R signalling plays an important role in colon cancer tumorigenesis [385, 406, 475], it was decided to investigate IL-36 family member expression in the adenoma-carcinoma sequence of CRC. The dataset GSE68468 contains RNA expression data from bulk tissue samples including primary colon cancer, polyps, metastases, and matched normal mucosa (obtained from the margins of the resection). The expression of all IL-36 family members assessed, with the exception of IL-36Ra (the gene encoding the IL-36Ra), showed a trend of increased expression from normal colonic tissue through to colonic adenocarcinoma and subsequent lung and liver metastases (Fig. 6.1). The largest increases in gene expression were observed for the IL-36R ($p = 0.0019$) and the IL-1RAcP ($p < 0.001$). Of note, the largest increases in IL-36 family member gene expression were detected in lung metastases when compared to normal colonic tissue. In addition, IL-36Ra gene expression showed a significant decrease in expression from normal colonic tissue to adenocarcinoma ($p < 0.001$) and subsequent metastases ($p < 0.001$). As this dataset did not include IL-36β, it is unknown whether this cytokine would also have been increased during CRC development.

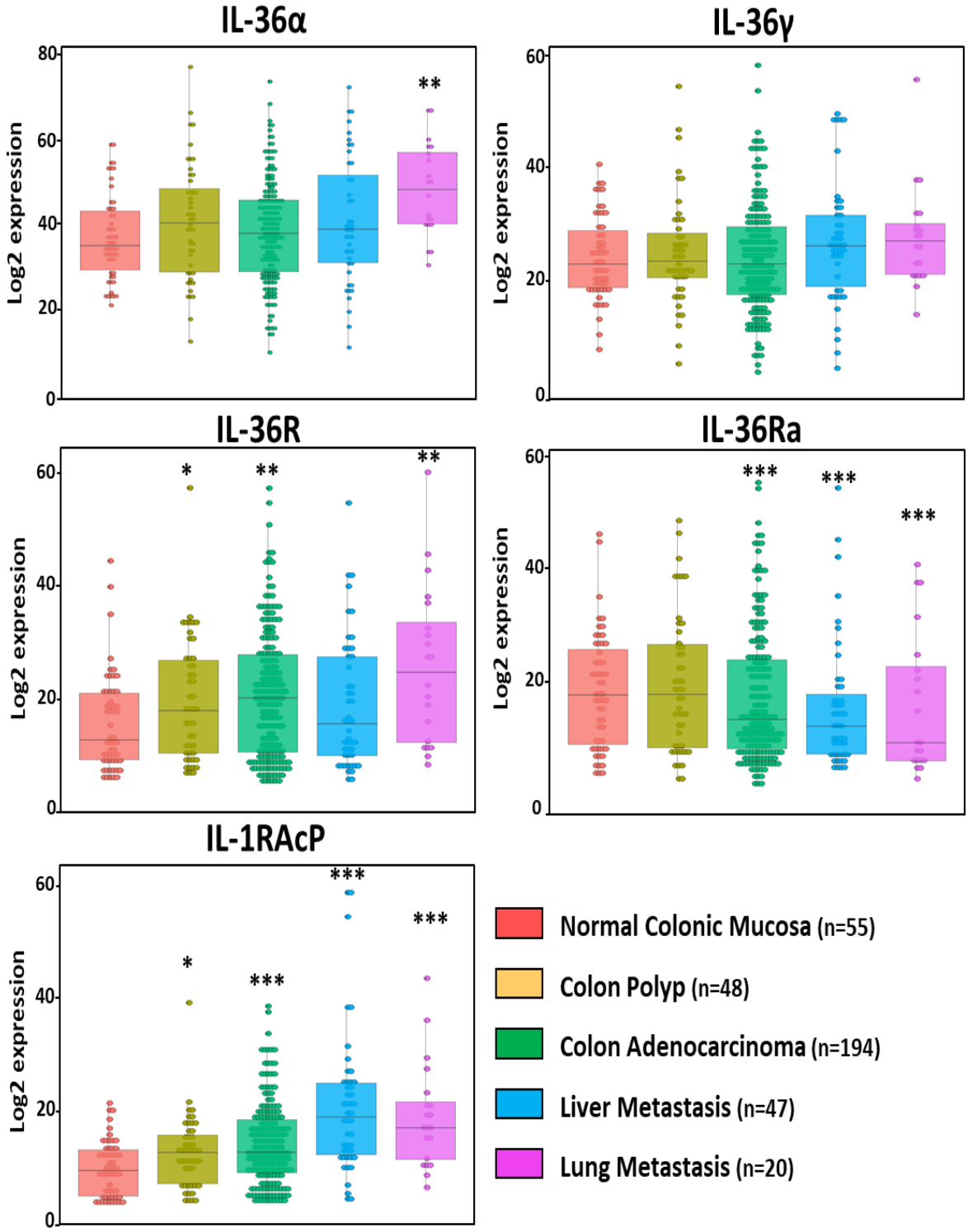
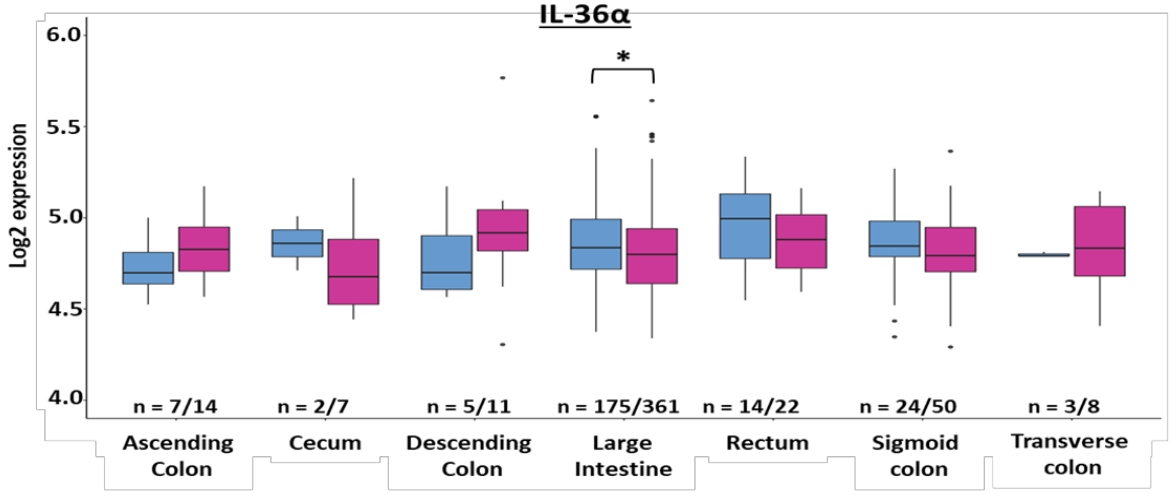
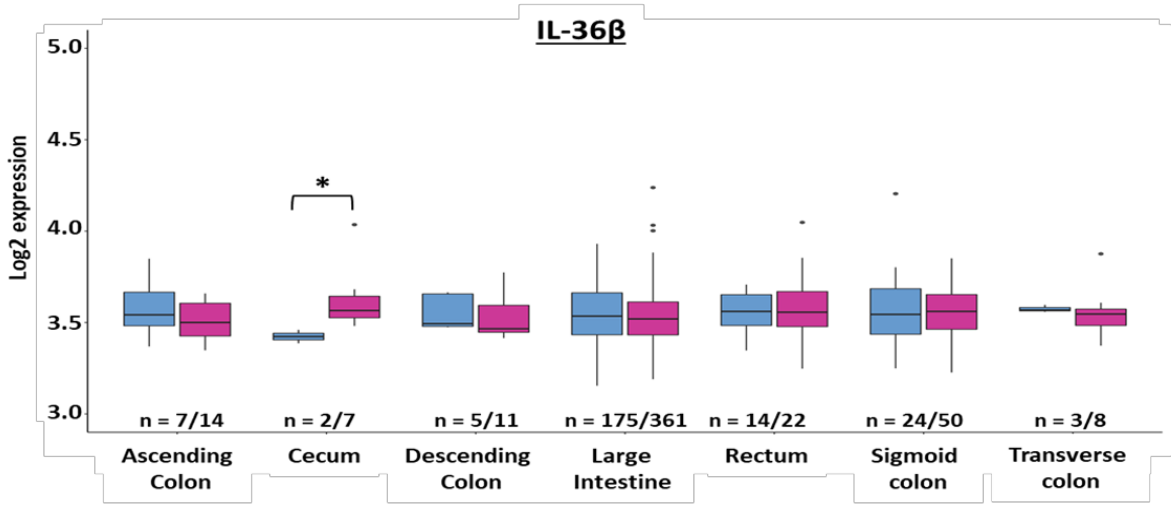


Figure 6.1: IL-36 family member expression is altered in CRC disease development. Expression of IL-36 family members (IL-36α, IL-36γ, IL-36R, IL-36RN and IL-1RAcP) were investigated across tissue types in CRC progression using the dataset GSE68468. Dunnett's post hoc analysis is annotated by the number of asterisks (*: p-value < 0.05; **: p-value <0.01; ***: p-value <0.001)

6.3.2. IL-36R expression is raised across sites in CRC anatomical sites of primary tumours associated with a poorer prognosis

To further the above findings concerning IL-36 expression in the adenoma-carcinoma sequence, a meta-dataset [483] was next used to investigate the expression of IL-36 genes in different anatomical sites of the colon in both normal and neoplastic tissue (Fig. 6.2). Patient demographics are outlined in Appendix Table 6. IL-36γ (ascending colon p=0.0073) and IL-36β (cecum, p=0.037) were both shown to be increased in the right side of the colon, which is associated with poorer patient prognosis. The only changes in IL-36α (p=0.024) and IL-36Ra (p=0.016) expression that were detected, was an increase and decrease in expression in the 'large intestine', respectively. This site was annotated for undescribed anatomical locations of the colon. This increased expression in the 'large intestine' was also seen in IL-36γ gene expression. IL-36R expression was significantly increased in all the tumour tissue sites, with the exception of the rectum and cecum. This increase in expression was seen in both right sided locations such as the ascending (p =0.0031) and the transverse (p=0.011), which is a site particularly associated with a poor patient survival rate (25). Increases were also seen in left-sided locations including the transverse colon and the sigmoid colon. Additionally, the gene accessory protein for IL-36R receptor dimerization, IL-1RAcP, was shown to be significantly increased in malignant tissue across all sites. This data indicates IL-36 signalling to play a role in malignant tissue transformation.


IL-36α
Log2 expression
6.0
5.5
5.0
4.5
4.0
*
n = 7/14
n = 2/7
n = 5/11
n = 175/361
n = 14/22
n = 24/50
n = 3/8
Ascending Colon
Cecum
Descending Colon
Large Intestine
Rectum
Sigmoid colon
Transverse colon


IL-36β
Log2 expression
5.0
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3.5
3.0
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n = 2/7
n = 5/11
n = 175/361
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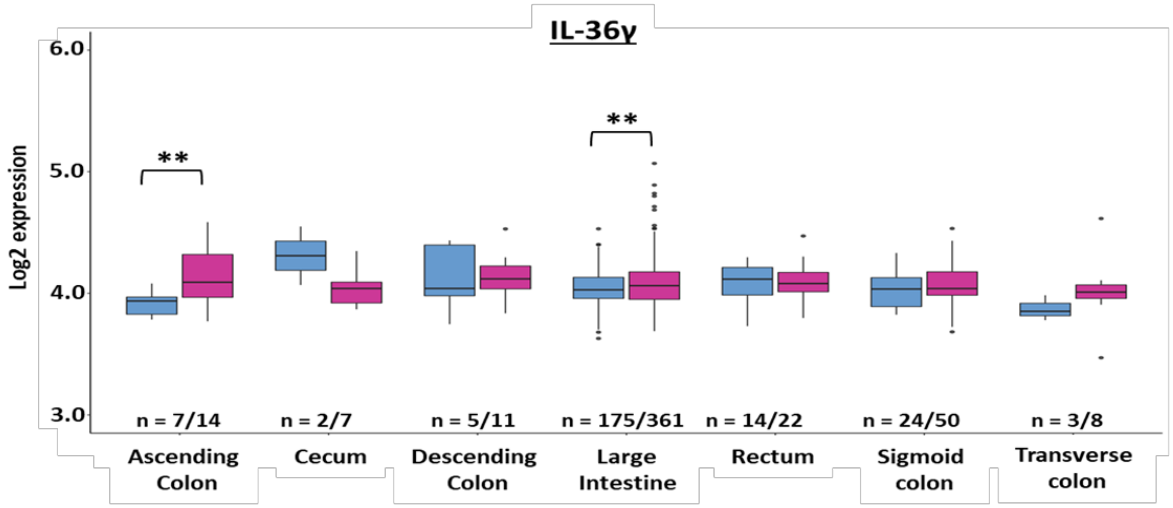
n = 3/8
Ascending Colon
Cecum
Descending Colon
Large Intestine
Rectum
Sigmoid colon
Transverse colon


IL-36γ
Log2 expression
6.0
5.0
4.0
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**
n = 7/14
n = 2/7
n = 5/11
n = 175/361
n = 14/22
n = 24/50
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Ascending Colon
Cecum
Descending Colon
Large Intestine
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Sigmoid colon
Transverse colon

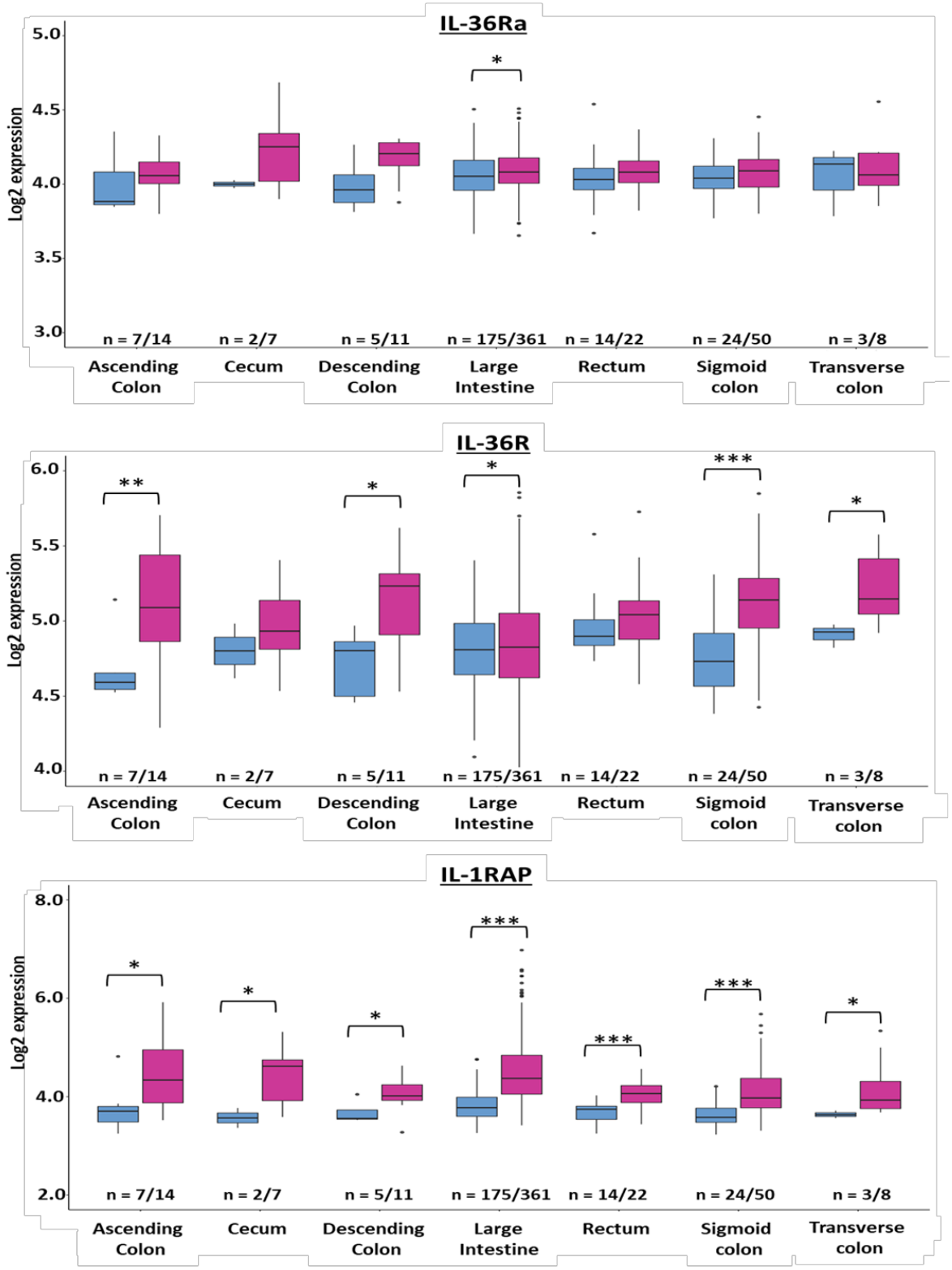


Figure 6.2: IL-36 family member expression is increased in neoplastic tissue across several intestinal anatomical locations. Changes in IL-36 family gene expression in anatomical sites of the colon/rectum were investigated in normal and malignant tissue (n = number of normal specimens/number of malignant specimens) using a meta-dataset published by Rohr *et al*. Wilcoxon test is annotated by the number of asterisks (*: p-value < 0.05; **: p-value <0.01; ***: p-value <0.001).

6.3.3. Increased tumoral IL-36R expression is associated with decreased 5 year survival rates.

In order to investigate the clinical outcome of increased or decreased IL-36 signalling in CRC tumours, the five year survival rate of patients with tumours containing high versus low expression of IL-36 family members was investigated (Fig. 6.3). No significant difference in the five year survival rate was observed for any family member with the exception of the IL-36R. Tumours which highly expressed IL-36R were associated with a significantly reduced five year survival rate ($p = 0.025$), particularly in the first four years of disease. Demographics of these patients (Sex, Age and AJCC staging) can be found in Table 6.1, showing similar demographics between the IL-36R high and low groups. IL-36α was investigated using a multi-cohort dataset as expression of IL-36α was not available in the TCGA-COAD dataset used for the other genes.

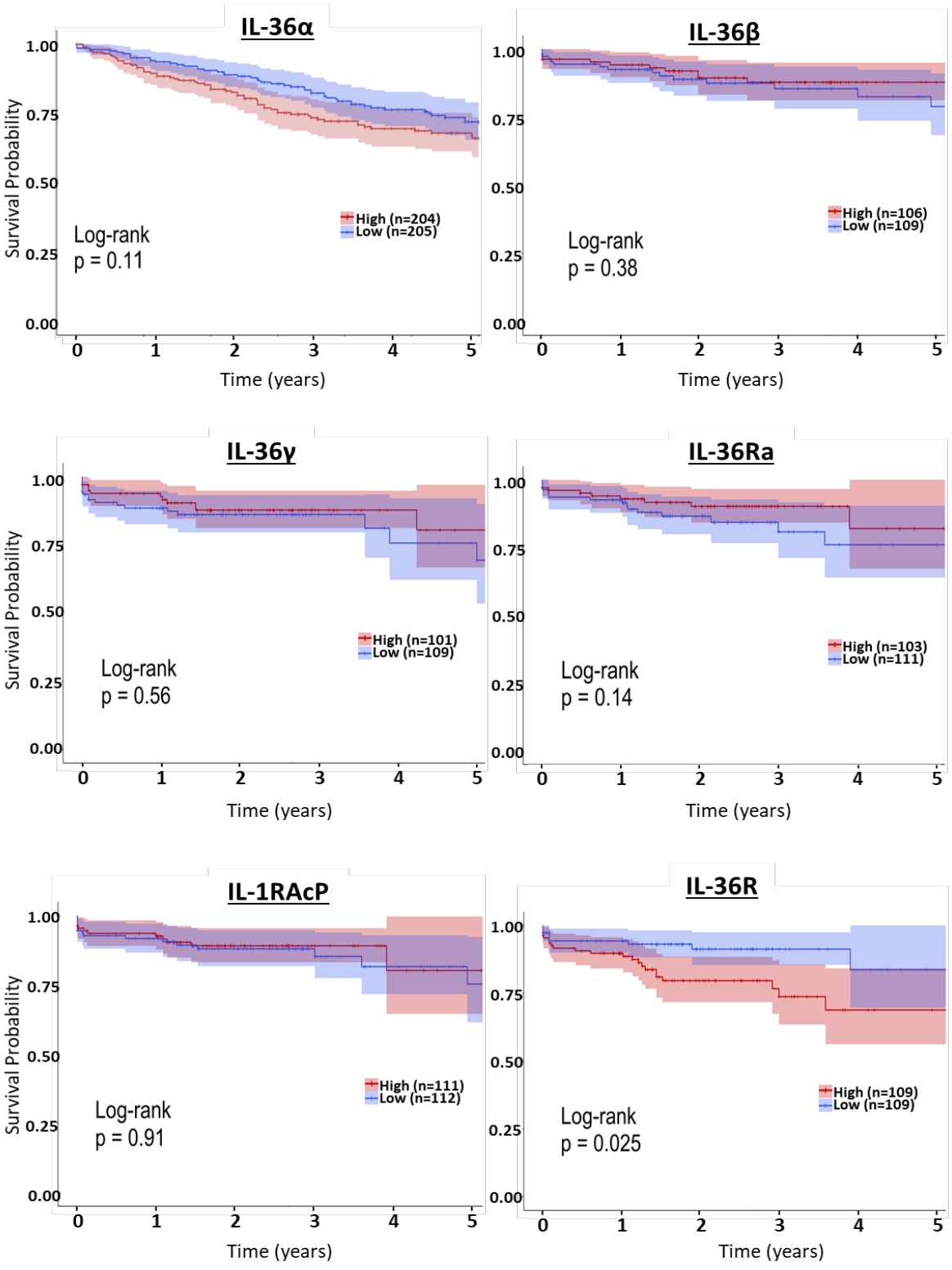


Figure 6.3: High expression of IL-36R is associated with a decreased 5 year survival rate. IL-36 family gene expression in TCGA-COAD (IL-36α data was analysed by multi-cohort data, see Appendix Table 5) was analysed for patient survival outcomes comparing top (high) and bottom (low) quartiles. Log rank testing was completed to assess statistical significance, indicated as a p value in each graph.

Variables		IL-36R Low (N=109)*	IL-36R High (N = 109)
Sex	**Female**	54	55
	Male	55	54
Age	**<70**	57	59
	>70	50	50
	NA	2	0
AJCC Stage	**NA**	2	3
	I	20	21
	II	10	4
	IIA	27	31
	IIB	7	11
	III	7	3
	IIIA	3	0
	IIIB	13	12
	IIIC	11	12
	IV	6	9
	IVA	3	3

Table 6.1: Patient sex, age and AJCC staging for IL-36R high and low expressing tumours shown in 5 year survival Kaplan Meier plot. Patient stratification of TCGA COAD according to IL-36R expression showed a similar distribution of multiple demographics.

6.3.4. Increased IL-36R expression is associated with TP53 mutation and with mismatch repair proficiency

Given the association between high expression of the IL-36R and decreased patient survival probability, patient samples were stratified based on genetic and epigenetic alterations

commonly associated with CRC development and IL-36R expression was assessed (Fig. 6.4). The TCGA COAD dataset for survival analysis did not contain sufficient patient clinical details to assess this, therefore GSE39582 was used to investigate these genotypes. Patient demographics are outlined in Appendix Table 6. Increased IL-36R expression was strongly associated with mutation in the TP53 gene (p=0.00045). In contrast, IL-36R expression was shown to be significantly decreased in samples derived from patients with the mismatch-repair deficiency (dMMR) genotype (p= 0.016). This data suggests a possible relationship between IL-36R expression and genetic mutations commonly found in CRC; although both positive (dMMR) and negative (TP53 mutation) prognostic markers were associated with increased IL-36R expression.

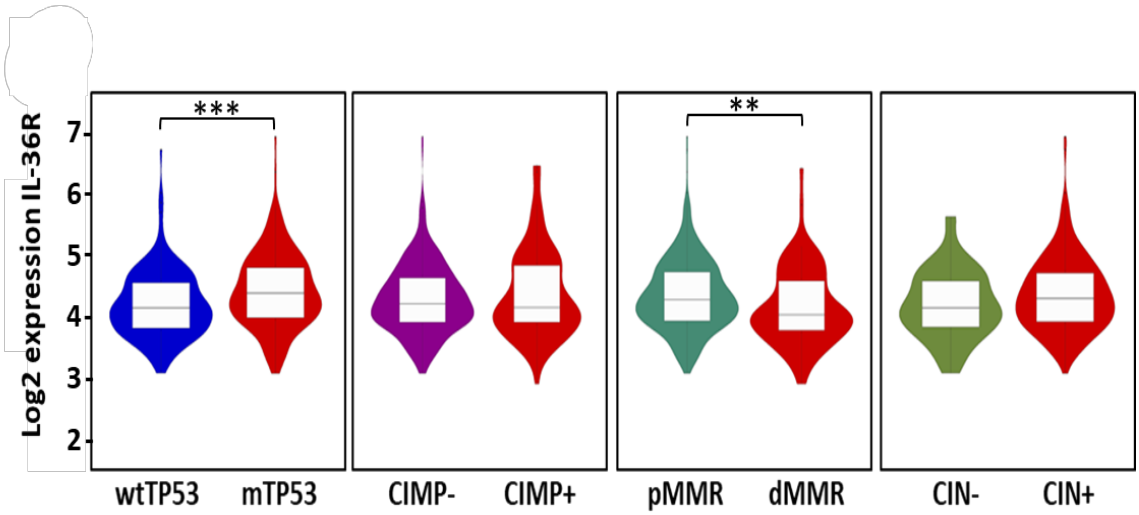


Figure 6.4: Tumoural IL-36R expression is associated with key CRC molecular pathways. Specimen data was stratified according to key genetic and epigenetic markers in GSE39582 and IL-36R expression was compared. Wilcoxon test is annotated by the number of asterisks; (**: p-value <0.01; ***: p-value <0.001).

6.3.5. Increased IL-36 cytokine expression is associated with increased NK cell infiltration

Given the associated changes in survival between IL-36R high versus low expressing tumours, and the key roles of IL-36 signalling in both innate and adaptive immunity [226],

it was decided to investigate the immune cell signatures present in tumours expressing high versus low levels of IL-36 family members. Microarray gene expression levels were used to estimate the immune infiltrate composition of colonic tumours and to assess whether differences were observed in tumours with high and low levels of IL-36 family member gene expression (Fig. 6.5). The R package 'MCPcounter' allows for the robust quantification of the absolute abundance of several immune cell populations in heterogeneous tissues using gene signatures from transcriptomic data [390]. The large dataset GSE39582 was used to its size, standardised sample collection protocols and its previous use for CMS characterisation of colon cancers [31]. Increased NK cell infiltration was associated with increased expression of IL-36α ($p=0.0021$), IL-36β ($p=0.0045$), IL-36γ ($p=0.0154$) and IL-36Ra ($p=0.0012$). Additionally, increased IL-36β expression was associated with a decrease in B lineage cells ($p=0.0322$). Most notably, decreased IL-36R expression was associated with a statistically significant increase in the cytotoxic lymphocyte (CTL) gene signature ($p=<0.0001$). Given our observations of improved survival associated with reduced IL-36R expression in the TCGA-COAD dataset, and increased immune cell infiltration of CTLs, it was decided to further investigate these findings regarding the IL-36R by examining differentially expressed genes in colonic tumours compared to normal colonic tissue.

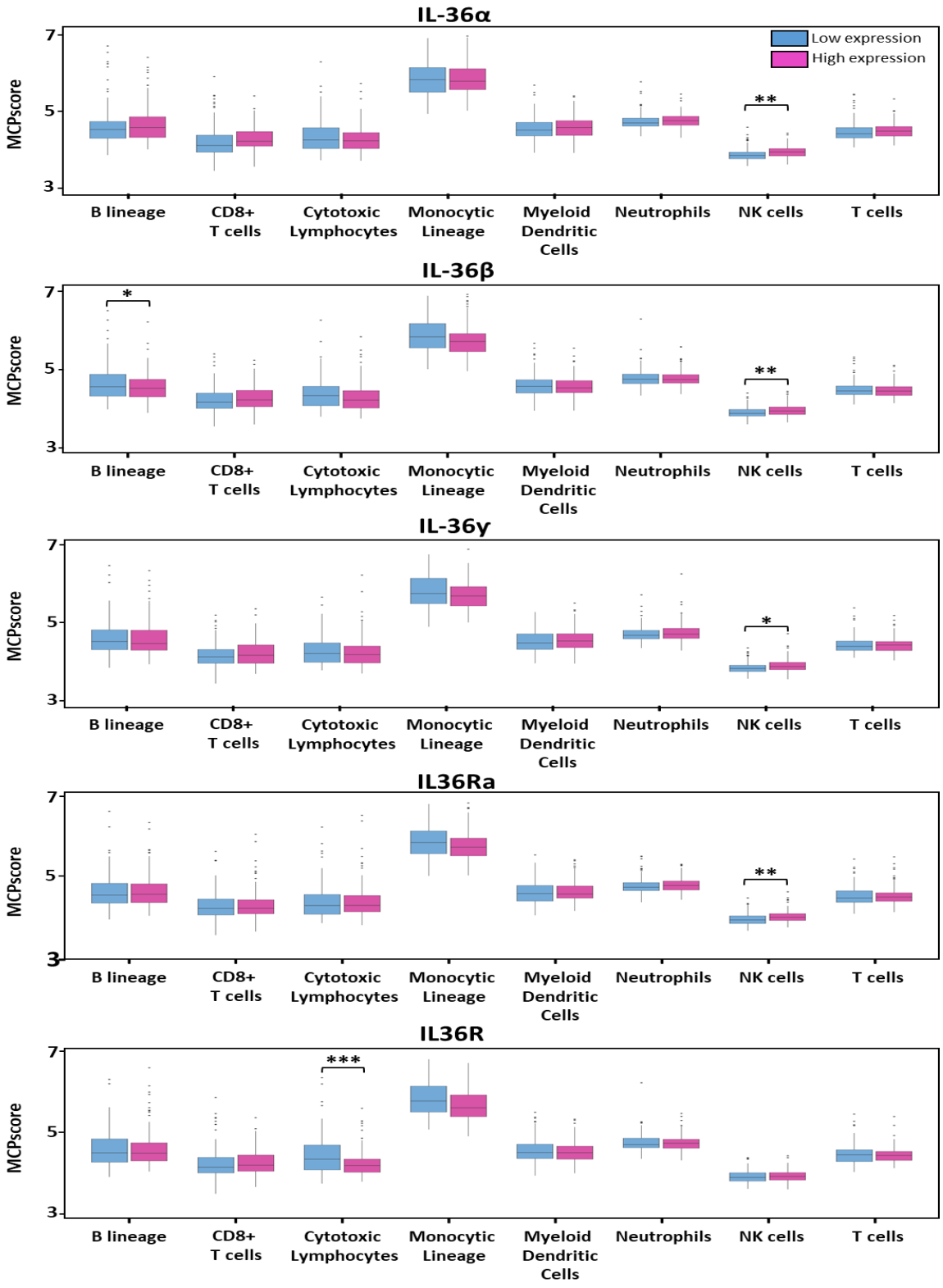


Figure 6.5: Changes in IL-36 family member expression is associated with alterations in TME cellular composition. Immune infiltrate signatures were assessed according to IL-36 family member gene expression in the dataset GSE39582 using the R package 'MCPcounter'. Wilcoxon test is annotated by the number of asterisks (*: p-value < 0.05; **: p-value <0.01; ***: p-value <0.001).

6.3.6. DEG analysis of IL-36R high expressing tumours reveals a role for the IL-17 signalling pathway in IL-36 TME signalling

A total of four datasets (comparing normal colonic tissue with tumour tissue) were selected to identify DEGs in tumours expressing high and low levels of the IL-36R gene in comparison to normal healthy tissue (Fig 6.6). These datasets were chosen based on microarray platform, cohort size and the availability of paired tumour and normal tissue samples. Dataset details are outlined in Appendix Table 8. Default settings in GEO2R were used for DEG identification. Significantly altered genes (Log fold change < 1.5 and adj. p value < 0.05) were checked for overlap across datasets.

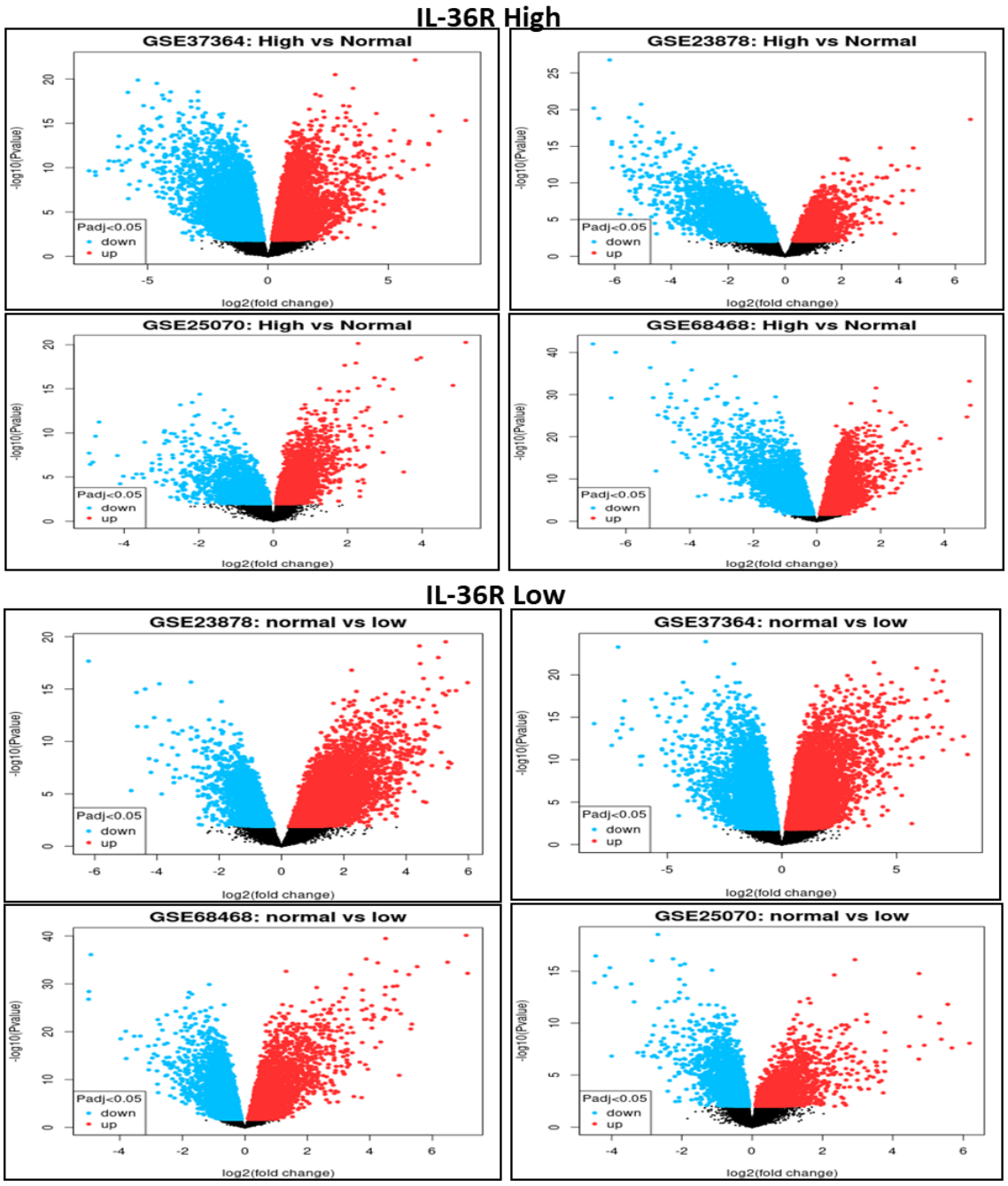
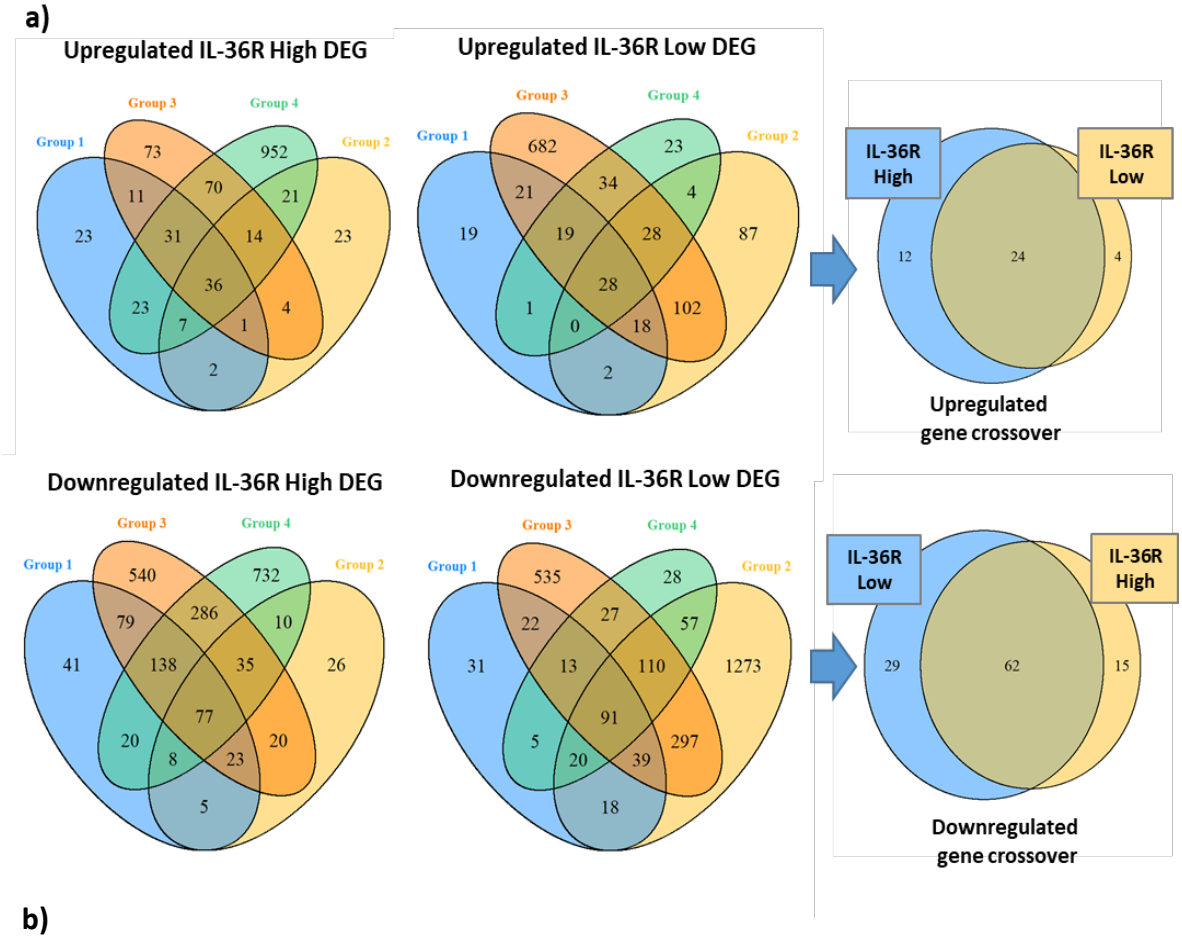


Figure 6.6: DEG analysis of colon cancer tissue versus normal adjacent by GEO2Analyzer. DEG analysis was performed on the four datasets (GSE68468, GSE37364, GSE23878 and GSE25070) to identify significantly altered gene expression in comparison to normal healthy colonic tissue.

A total of 12 up-regulated and 15 down-regulated genes were identified, which were unique to IL-36R high expressing tumours, when compared to normal colon tissue (Fig. 6.7a, see methods for further details). The gene expression data of IL-36R low tumours was not investigated further given that these patients showed similar survival rates to all other patients in our survival data, and that it was IL-36R high tumours that were associated with worse survival rates compared to all other gene subpopulations. The top 10 largest fold changes are presented here, with additional genes then used for further analysis (Figure 6.7b).

a)



b)

Top 10 Upregulated genes in IL-36R-High tumours

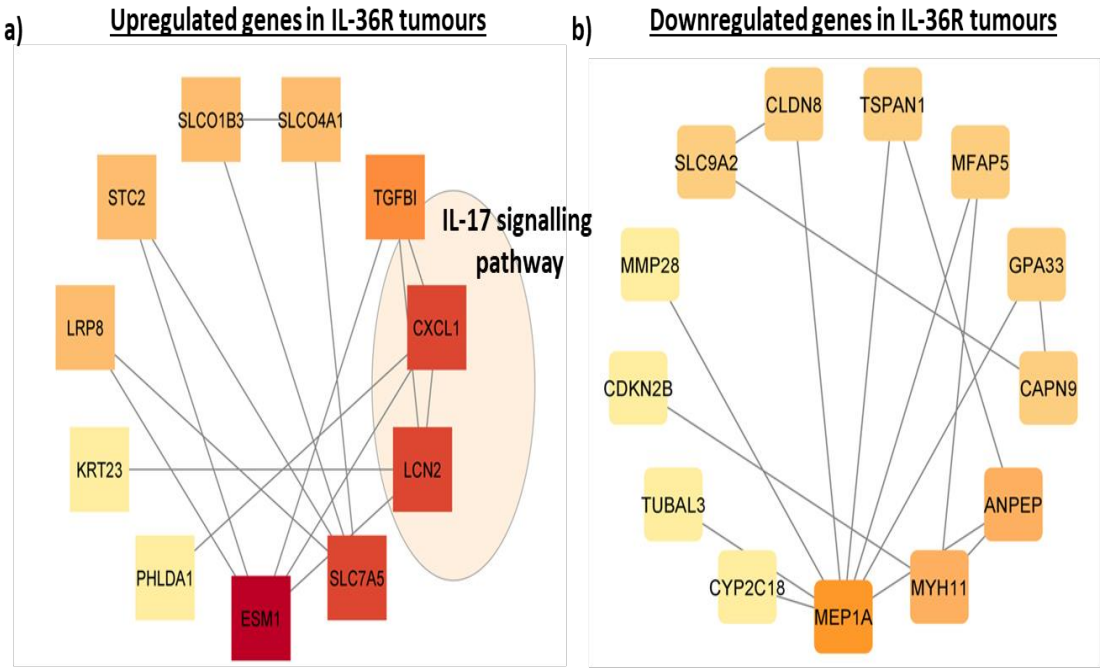
Gene	Adj. P value	Log FC
KRT23	4.01E-06	3.051357
ESM1	2.46E-07	2.942161
CXCL1	0.001398	2.622718
LCN2	0.01237	2.29319
SLCO4A1	1.41E-06	2.241063
SLC7A5	1.83E-06	2.238802
STC2	1.07E-05	2.12938
LRP8	0.000173	2.057757
PHLDA1	0.002366	2.045397

Top 10 Downregulated genes in IL-36R-High tumours

Gene	Adj. P value	Log FC
CLDN8	3.38E-03	-4.40372
MMP28	1.1E-07	-3.12676
ANPEP	0.00798	-2.89038
CDKN2B	0.000603	-2.74814
MFAP5	0.000124	-2.60605
TUBAL3	6.61E-05	-2.44673
MYH11	0.001538	-2.42304
MEP1A	0.00118	-2.40705
C1orf115	8.1E-05	-2.11833

Figure 6.7: DEG validation to find common trends amongst different datasets. a) DEG analysis was performed on the four datasets (GSE68468, GSE37364, GSE23878 and GSE25070) to identify significantly altered gene expression in comparison to normal healthy colonic tissue. DEG overlap in upregulated and downregulated genes was examined in IL-36R high expression and IL-36R low expression tumours. **b)** Top 10 significantly upregulated and downregulated genes according to fold change across all datasets which were unique to IL-36R high expressing tumours. P values were determined by Benjamini & Hochberg false discovery rate method.

Both sets of genes underwent STRING Protein-Protein interaction (PPI) analysis in order to identify clusters of associated genes using default settings, which showed a network for 11 upregulated genes (Fig. 6.8a) and 13 downregulated genes (Fig. 6.8B) using default settings. Functional enrichment revealed only one small cluster in the upregulated genes, consisting of LCN2 and CXCL1; two genes involved in IL-17 signalling. No significant clusters were identified in downregulated genes in IL-36R high tumours. This data suggests that upregulated IL-17 signalling may be an associated factor contributing to the pathogenesis of CRC, particularly in IL-36R high expressing tumours.



Category	Description	FDR value	Genes	p-value
KEGG Pathways	IL-17 signalling pathway	0.0077	LCN2|CXCL1	2.28E-05

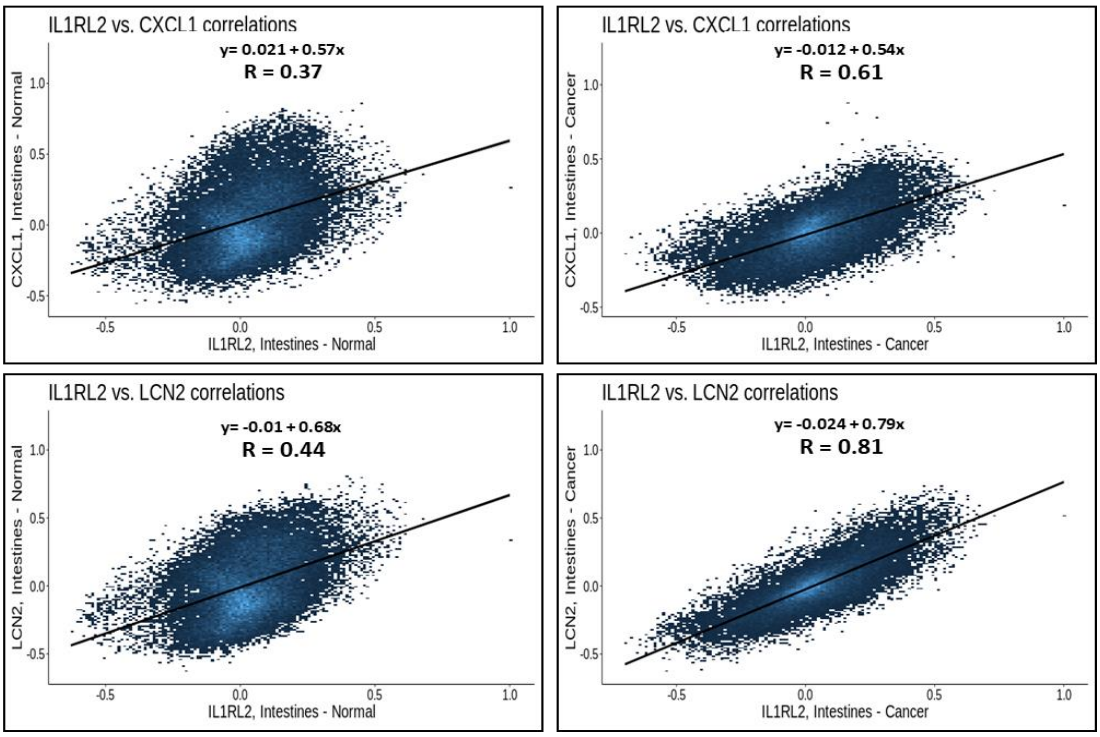
Category	Description	FDR value	Genes	p-value
N/A	No significant clusters	N/A	N/A	N/A

Figure 6.8: Upregulated genes in IL-36R high colon cancer tumours are associated with the IL-17 signalling pathway. PPI, Markov clustering and functional gene enrichment were used to identify and annotate gene clusters in **a)** upregulated and **b)** downregulated genes in IL-36R high tumours. Default parameters were used for each method.

6.3.7. IL-17 signalling genes show stronger correlative expression with the IL-36R gene in colon tumour tissue compared to healthy colon tissue

In order to further investigate the IL-36/IL-17 crosstalk in the context of CRC, the AnalyzeR web tool was used with default settings to investigate correlations between key genes identified from DEG analysis in both normal and malignant intestinal tissue (Fig 6.9a). CXCL-1 was shown to have a positive correlation with IL-36R (IL1RL2) expression in normal intestinal tissue (R = 0.37). Furthermore, this correlation was stronger in intestinal cancer tissue in comparison to normal intestinal tissue (R = 0.61). LCN2 gene expression was shown to have a similarly stronger correlation with IL-36R gene expression in intestinal cancer tissue (R = 0.81) in comparison to normal intestinal tissue (R = 0.44). A correlation matrix was also completed for IL-36 family members and IL-17 signalling annotated genes from KEGG pathway analysis. Hierarchal clustering showed a positive correlation to exist between several IL-17 family genes and IL-36 family members, as well as chemokines and cytokines involved in the IL-17 signalling pathway (Fig. 6.9b, red triangle).

a)



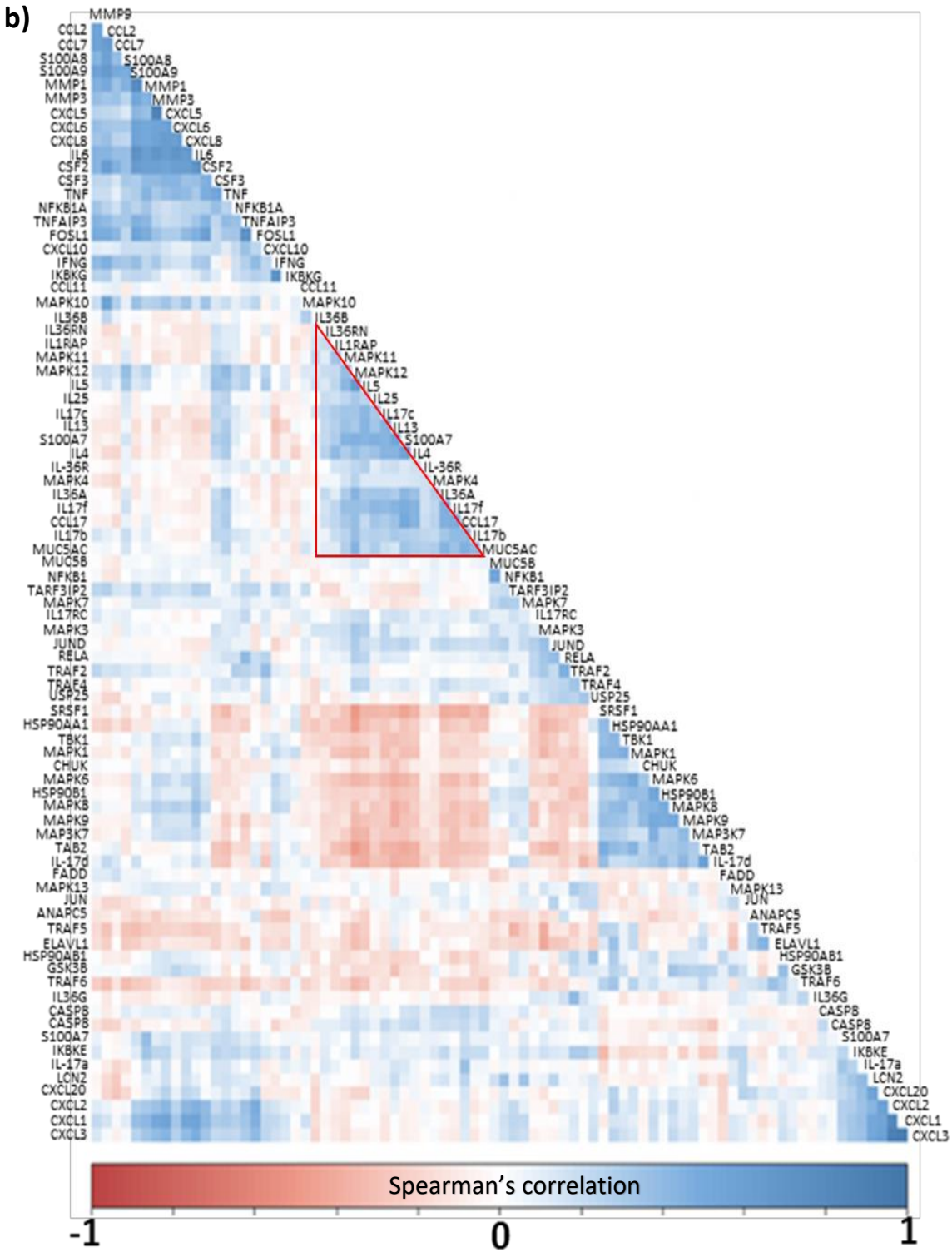


Figure 6.9: IL-36R and CXCL1/LCN2 expression is more strongly correlated in colon cancer tissue than normal colonic tissue. a) Correlation plots between cluster genes identified from DEG analysis and IL-36R expression were analysed using the AnalyzeR web tool in normal and malignant intestinal tissue. R values were calculated by Spearman's correlation. **b)** Correlation matrix was graphed comparing IL-36 family member gene expression and KEGG pathway 'IL-17 signalling pathway' using the dataset GSE39582.

6.3.8. IL-17 signalling genes are upregulated by IL-36 stimulation of colon cancer spheroids, which is reflected in CRC patient cohort biopsy gene expression

Given the pro-tumorigenic functions identified for CXCL1 in CRC [420, 421] and the PPI findings shown in Figure 6.8, CXCL-1 expression was further investigated in the context of expression of the IL-36R gene (IL1RL2). To further elucidate the relationship between IL-36R expression and IL-17 signalling, *in vitro* assays were performed. Human HT29 colon cancer cell spheroids were generated in culture in the presence of either IL-36γ or LPS (Fig 6.10A). IL-36γ significantly increased the expression of both CXCL-1 and IL-23 by the spheroids, two cytokines closely associated with the IL-17 pathway (Fig. 6.10a). Indeed, this increase in gene expression induced by IL-36γ was far greater than that seen with the LPS control (Fig. 6.10a). Moreover, expression of several other IL-17 signalling pathway genes are upregulated in patient-derived colonic tumour tissue in comparison to paired normal adjacent tissue (Fig. 6.10b).

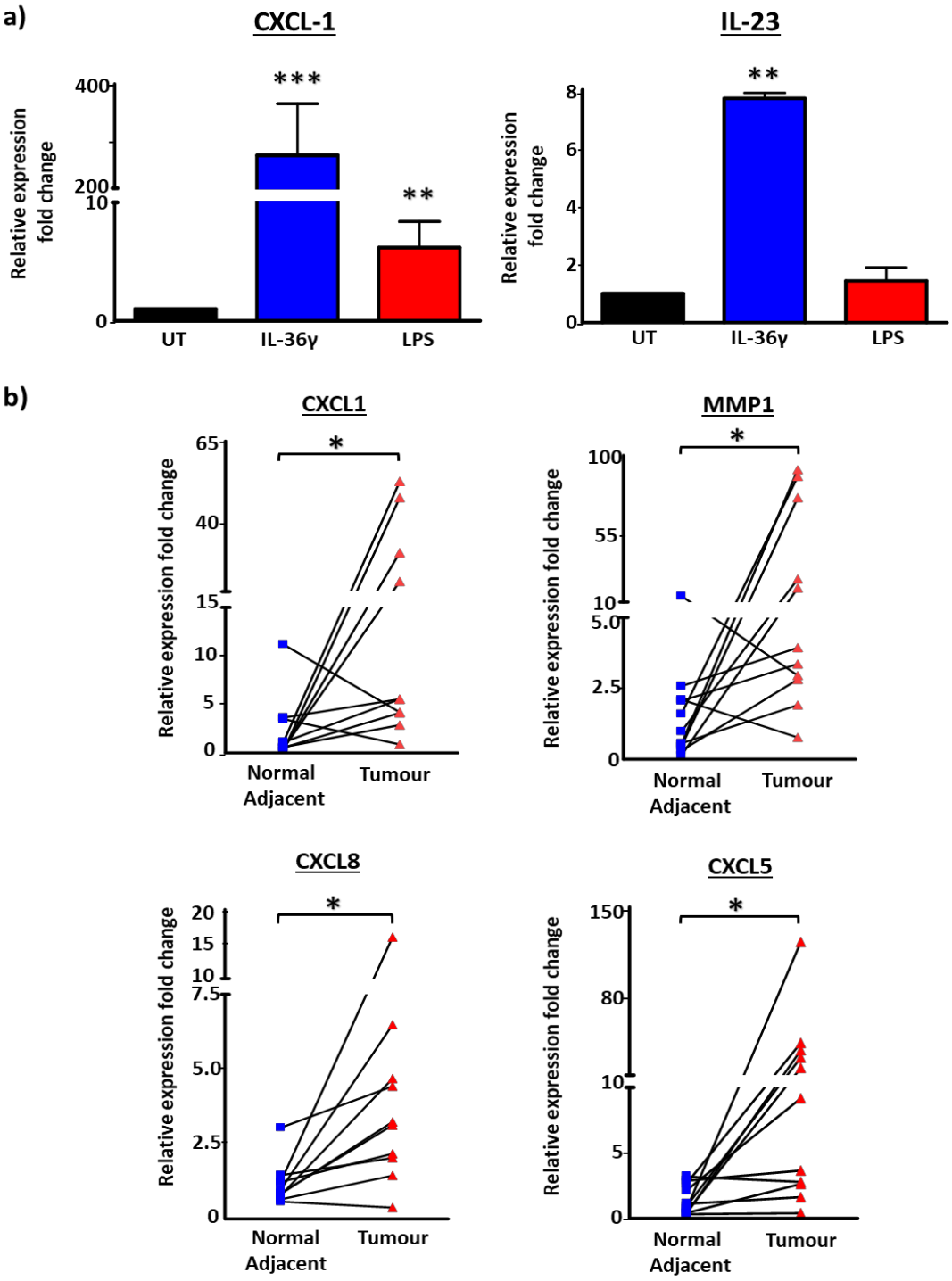
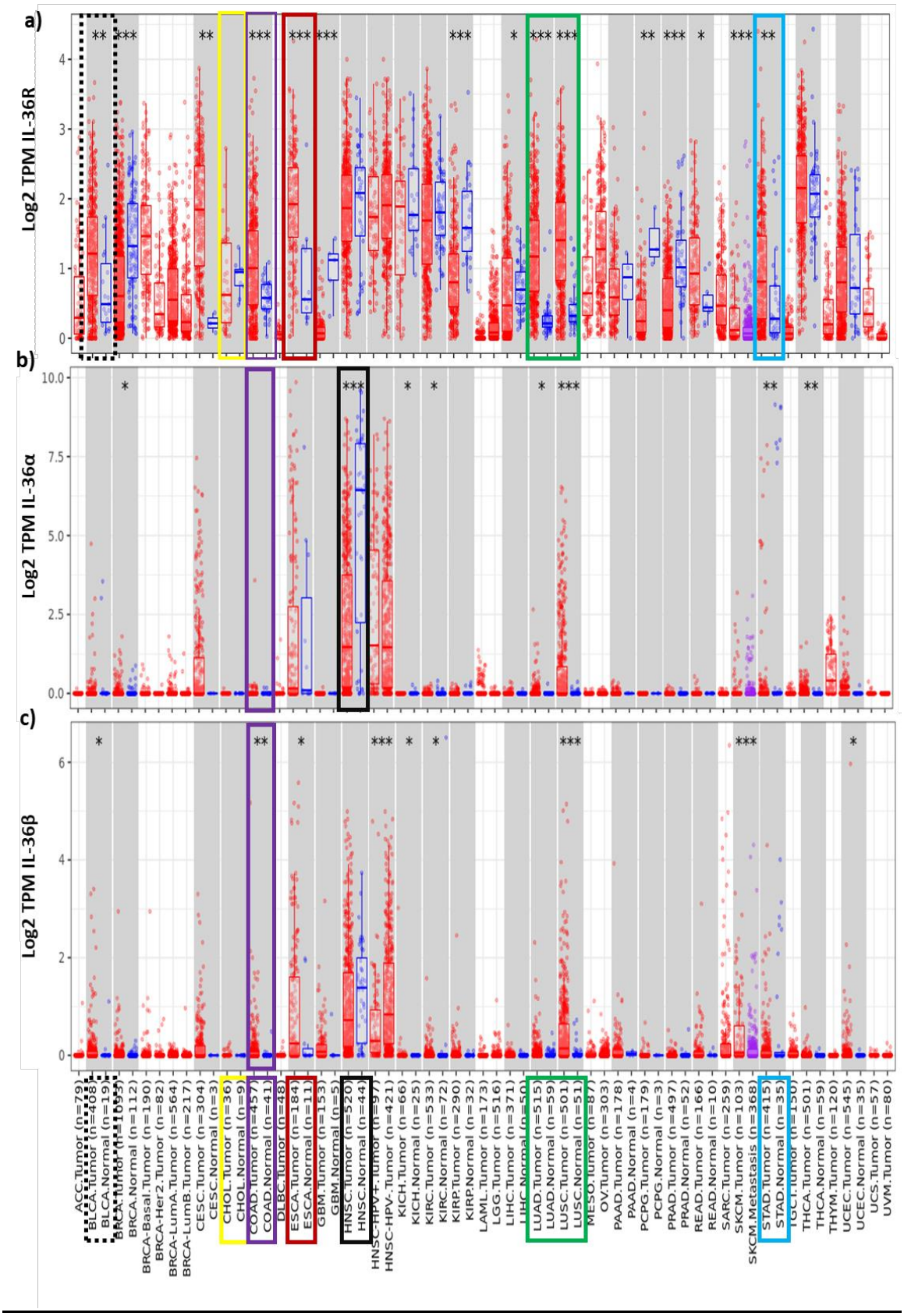


Figure 6.10: IL-17 signalling genes are upregulated by IL-36 stimulation of colon cancer spheroids, which is reflected with other IL-17 signalling genes in a CRC patient cohort. a) HT29 spheroids were grown in culture and stimulated with 100ng/mL IL-36γ or 10ng/mL LPS and CXCL-1/IL-23 gene expression changes were detected by qRT-PCR after 4 hours. Statistical analysis was completed by one-way ANOVA with Dunnett's Multiple Comparison Test (**: p-value <0.01; ***: p-value <0.001). **b)** Gene expression changes in human colon cancer and normal tissue were investigated in IL-17 signalling pathway genes. Paired Student's T-test is annotated by the number of stars (*: p-value < 0.05).

6.3.9. Other malignant tissues show similar patterns of increased IL-36 family member expression when compared to normal adjacent tissue

The expression of the IL-36R across various cancer types was examined and compared to normal adjacent tissue, using the web tool TIMER 2.0 [389]. Several cancer types showed increased expression of the IL-36R (Fig. 6.11a, boxplot with dot plot overlay cancer tissue in red, normal tissue in blue), especially in epithelium-originating cancers such as colon adenocarcinoma (COAD, purple), stomach adenocarcinoma (STAD, blue), Cervical Squamous Cell Carcinoma/Endocervical Adenocarcinoma (CESC, yellow), Urothelial Bladder Carcinoma (BLCA, dotted black) and NSCLC (LUAD/LUSC, green). Other IL-36 family genes were also assessed (Fig. 6.11b-f). Head and Neck cancer (HNSC, black) was the only dataset to show a decrease in IL-36 agonist expression (IL-36α), although this dataset did show an increase in IL-36γ expression in malignant tissue. Genes with the most frequent changes in malignant tissue expression were the genes encoding IL-36R and IL-1RAcP. This data indicates that changes in tumoral cell directed IL-36 signalling, and not IL-36 cytokine production, are more important to cancer cells in the TME.


a)
Log2 TPM IL-36R
b)
Log2 TPM IL-36α
c)
Log2 TPM IL-36β
ACC.Tumor (n=79)
BLCA.Tumor (n=408)
BLCA.Normal (n=19)
BRCA.Tumor (n=1093)
BRCA.Normal (n=112)
BRCA-Basal.Tumor (n=190)
BRCA-Her2.Tumor (n=82)
BRCA-LumA.Tumor (n=564)
BRCA-LumB.Tumor (n=217)
CESC.Tumor (n=304)
CESC.Normal (n=3)
CHOL.Tumor (n=36)
CHOL.Normal (n=9)
COAD.Tumor (n=457)
COAD.Normal (n=41)
DLBC.Tumor (n=48)
ESCA.Tumor (n=184)
ESCA.Normal (n=11)
GBM.Tumor (n=153)
GBM.Normal (n=5)
HNSC.Tumor (n=520)
HNSC.Normal (n=44)
HNSC-HPV+.Tumor (n=97)
HNSC-HPV-.Tumor (n=421)
KICH.Tumor (n=66)
KICH.Normal (n=25)
KIRC.Tumor (n=533)
KIRC.Normal (n=72)
KIRP.Tumor (n=290)
KIRP.Normal (n=32)
LAML.Tumor (n=173)
LGG.Tumor (n=516)
LIHC.Tumor (n=371)
LIHC.Normal (n=50)
LUAD.Tumor (n=515)
LUAD.Normal (n=59)
LUSC.Tumor (n=501)
LUSC.Normal (n=51)
MESO.Tumor (n=87)
OV.Tumor (n=303)
PAAD.Tumor (n=178)
PAAD.Normal (n=4)
PCPG.Tumor (n=179)
PCPG.Normal (n=3)
PRAD.Tumor (n=497)
PRAD.Normal (n=52)
READ.Tumor (n=166)
READ.Normal (n=10)
SARC.Tumor (n=259)
SKCM.Tumor (n=103)
SKCM.Metastasis (n=368)
STAD.Tumor (n=415)
STAD.Normal (n=35)
TGCT.Tumor (n=150)
THCA.Tumor (n=501)
THCA.Normal (n=59)
THYM.Tumor (n=120)
UCEC.Tumor (n=545)
UCEC.Normal (n=35)
UCS.Tumor (n=57)
UVM.Tumor (n=80)

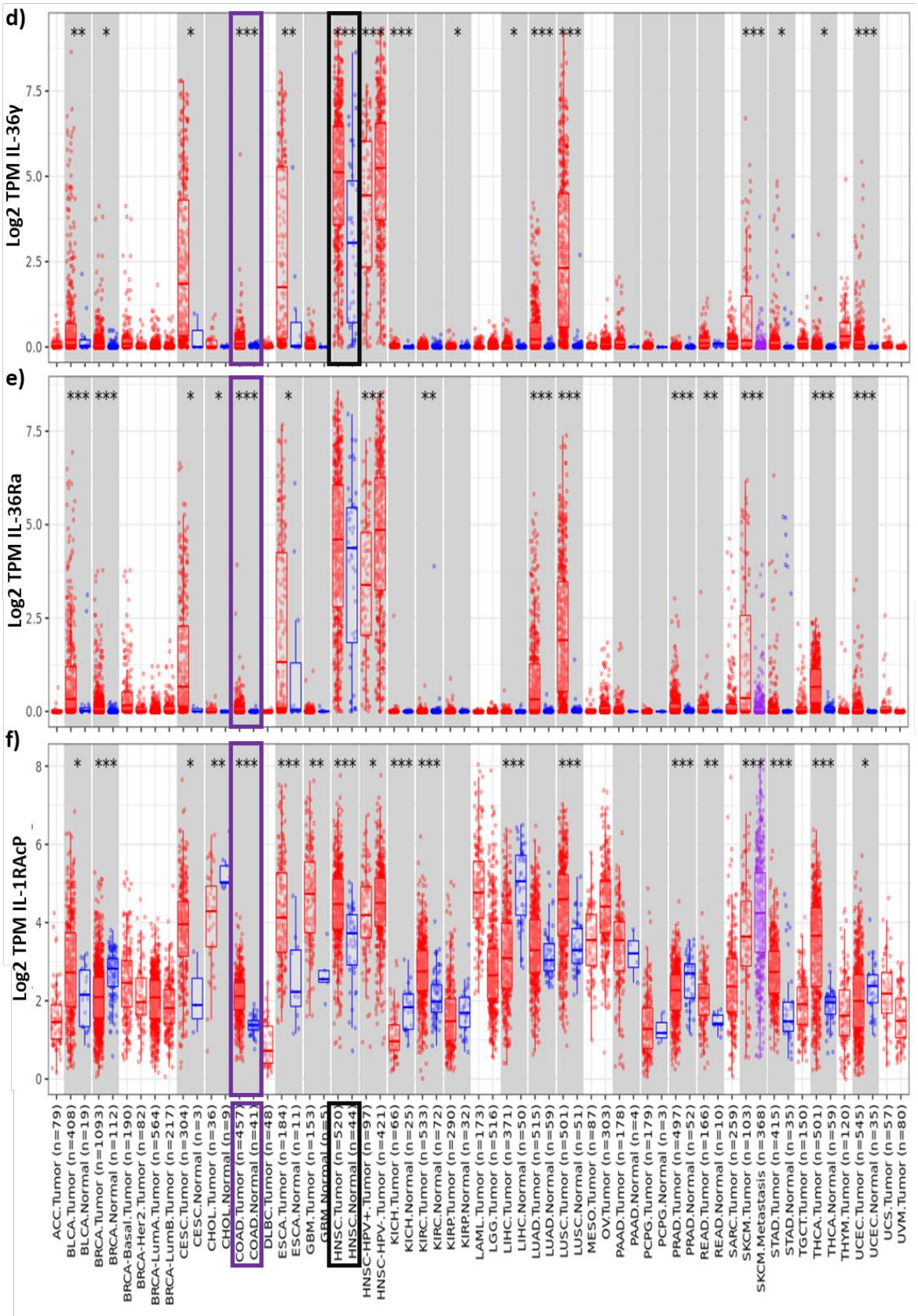


Figure 6.11: Expression of IL-36 family genes predominantly increased across several malignant tissues in comparison to normal adjacent tissue. IL-36 family gene expression was investigated using the 'Cancer Exploration' suite of the TIMER2.0 to examine differences in gene expression between normal healthy and malignant tissues in similar anatomical locations. Wilcoxon test is annotated by the number of stars (*: p-value < 0.05; **: p-value <0.01; ***: p-value <0.001).

6.4. Discussion

Given the recently described divergent roles for the IL-36 family in cancer [406], the aim of this chapter was to utilise transcriptomic analyses to identify further roles for IL-36 signalling in CRC. Here, this data shows a significant increase in IL-36 agonist and receptor gene expression from normal colonic mucosal tissue to tumour tissue to distant metastasis, with IL-36Ra expression showing the opposite. TCGA-COAD derived Kaplan Meier graphs showed an association between increased IL-36R expression and poorer patient outcomes. Furthermore, by DEG analysis, PPI analysis and Markov clustering, we have shown that tumours that express high levels of the IL-36R are associated with increased IL-17 signalling, which may contribute to disease pathogenesis. We have confirmed several of these *in silico* findings using both cell lines and a patient cohort. Collectively our analysis further indicates tumoral IL-36 signalling may be an important therapeutic target that warrants further investigation.

The work presented here strongly implicates tumoural IL-36R signalling in playing a significant pro-tumorigenic role in colon cancer. Previous transcriptomic and protein expression data has reported increased IL-36 expression to be beneficial to tumour rejection in melanoma, HCC and also CRC [239, 299, 367-369, 473]. Immunohistochemistry analysis of a CRC patient cohort reported that IL-36α expression was associated with improved patient CRC survival, although the same report demonstrated that lower levels of IL-36γ were also associated with improved patient survival [369]. In contrast to these previous findings, our transcriptomic analysis across multiple different platforms and cohorts strongly implicates IL-36R tumoral expression in playing a pro-tumorigenic role in colon cancer. Our analysis of the colorectal cancer dataset, GSE68468, showed that the

expression of the IL-36R and the IL-36Ra genes was increased and decreased throughout disease progression from normal colonic mucosal tissue to distant metastasis, respectively. This expression may not be cancer cell specific however and changes in IL-36 expression may result from alterations in TME infiltrate. However, this still suggests a pro-tumorigenic role for unrestricted tumoural IL-36 signalling in disease progression, which may then contribute to metastasis [437, 475]. Furthermore, analysis of IL-36 family members showed increased expression of IL-36R in nearly all anatomical colonic locations in malignant tissue in comparison to normal-adjacent, with poor prognosis right-sided colonic sites showing this increased IL-36R expression trend [484]. This was reflected in our survival curves analysis, where increased IL-36R expression was associated with poorer patient outcomes. This data, in contrast with previous work reporting loss of expression of IL-36 cytokines, shows a loss of IL-36Ra expression and an increase of IL-36R expression, which may facilitate unrestricted IL-36 signalling, may drive a pro-tumourigenic phenotype in intestinal epithelial cells.

CRC can broadly be described based on the genetic and epigenetic alterations that occur during pathogenesis [14]. TP53 acts as an important WT tumour suppressor gene which may become mutated in microsatellite instability (MSI) cancers, resulting in poor prognosis from dysregulation of the cell cycle and increased genomic instability [485]. Our data shows that increased IL-36R expression is associated with TP53 mutation in colon cancer, further supporting a role for IL-36R signalling in cancer cell proliferation. Previous reports have implicated the closely related cytokine IL-1β [227] to play a role in TP53 mutant tumours, with this cytokine also shown to be an important regulator of gene methylation and expression in CRC [486]. Knockout of IL-36y in TP53 floxed mice has been shown to attenuate tumour progression significantly, albeit in lung cancer, with our data suggesting

a relationship between IL-36 signalling and TP53 mutation may also exist in colon cancer (9). In support of this hyper-proliferative state, IL-36 signalling has previously been linked to induction of proliferation in the intestinal epithelium (40), reflective of our *in vitro* and *in vivo* experiments described in the previous chapters of this thesis. Moreover, our findings showed increased IL-36R expression is also associated with pMMR tumours, which is generally considered a negative prognostic indicator for patient survival as these tumours tend to be less immunologically active [487]. This further highlights the complexities of IL-36 signalling in the TME, and indicates that IL-36R signalling is associated with a hyper-proliferative phenotype, but also that IL-36R expression may be associated with poorer immune-modulation.

Analysis of immune infiltrate signatures in IL-36 high and low-expressing tumours did indeed show an improved NK infiltration into IL-36 agonist expressing tumours. IL-36 signalling has been previously shown to increase NK infiltration and proliferation in IL-36γ overexpressing tumours [299]. Our work also showed an increased NK cell signature in IL-36Ra high expressing tumours, which may be a negative feedback loop to control TME inflammation. Previous authors have reported immune inhibitory and IL-36Ra gene expression associations. The immune checkpoint proteins PD-L1 and CTLA-4 are strongly associated with poorer patient outcomes due to suppression of the anti-tumour immune response, and have been shown to be associated with IL-36Ra expression in tumours [239, 488]. Contrasting this, decreased IL-36R expression in tumours showed an associated increase in CTL infiltration. This may result from the decreased stimulation of tumor cells with IL-36 agonists, reducing MDSC chemoattraction and thereby improving CTL infiltration and activation [475]. These findings further highlight the complex nature of IL-36 signalling in the TME and warrants further investigation.

Direct signalling of IL-36 on cancer cells and crosstalk with other pathways has not been extensively investigated in colon cancer. Our DEG analysis of upregulated genes unique to tumours expressing high levels of IL-36R revealed a small gene cluster of IL-17 associated signalling proteins and hierarchal clustering showed positive correlations between IL-17 signalling genes and IL-36 family members. Several inflammatory diseases, especially psoriasis, have shown activation of the IL-36 signalling pathway to be associated with activation of the IL-17/IL-23 signalling pathway, an association which has not, to date, been reported in colon cancer [270, 273, 291, 412]. Indeed, it has been previously shown that both IL-17 and IL-36 may regulate one another in an inflammatory loop, and additionally that these cytokines may synergize to augment this feedback loop, with strong induction of IL-23 and IL-22 [489, 490]. Furthermore, IL-17a has also been shown to strongly induce IL-36, both of which have been shown to induce downstream cellular proliferation through ERK1/2 only or else through ERK1/2, STAT3 and NF-κB [491]. This feedback loop has successfully been targeted by anti-IL17a mAbs or by genetic deletion of IL-36R in keratinocytes, resulting in similar effects to global knockout of IL-36R in mice with reduced IL-17a, IL-23 and IL-22 expression [241, 491]. This suggests that epithelial cell directed IL-17/IL-36 crosstalk may be the most important axis amongst cell populations, given that global knockout of IL-36R was seen to be equivocal to keratinocyte IL-36R KO, although this study did only focus on this set of epithelial tissue and changes are likely to have occurred in intestinal and lung barrier tissues in global knockouts.

As a major part of IL-17/IL-36 signalling crosstalk, we show that colon cancer cell IL-23 expression is highly inducible by IL-36 stimulation. More recently, this feedback loop has been described in intestinal barrier disease, with IL-36γ/IL-36R signalling shown as central upstream regulators of the IL-23/IL-22/antibacterial peptide pathway during intestinal

injury and contribute to wound healing in the intestine [273]. This process of wound healing has come to light recently in the IL-36 and cancer research field as it has been shown that IL-36γ upregulates ECM and cell–matrix adhesion molecule expression. This subsequently facilitates Wnt signalling, which can be mitigated by IL-36Ra or IL-36γ neutralizing antibody administration *in vivo* [437]. IL-36γ expression was shown to positively correlate with extracellular matrix and β-catenin expression in human CRC biopsies. Reflective of this, IL-17a deficient mice have been shown to express reduced of Ki67 and β-catenin in models of colitis associated cancer [492], and TGF-β signalling in Th17 cells has been shown to promote IL-22 production and contribute to colitis-associated cancer progression [493]. Collectively, this suggests that combined inhibition of IL-17 and IL-36 signalling in colon cancer may benefit inhibition of tumour progression.

Importantly, the IL-17 signalling pathway is well characterised in the direct pathogenesis of CRC via STAT3 activation, resulting in the expression of genes such as matrix metalloproteases (MMPs) and anti-apoptotic genes with acquired chemoresistance (14). One of the two major genes implicated in IL-17 signalling from our DEG analysis of IL-36R high tumours, LCN2, has also been implicated in chemoresistance. Epigenetic induction of LCN2 can drive acquired 5-FU resistance, with another group showing LCN2-driven therapy resistance stemming from inhibition of ferroptosis in CRC [494]. LCN2, an antibacterial protein, has previously been shown to be potently induced by epithelial IL-36R signalling [454]. Our analysis of LCN2 and IL-36R expression suggests that LCN2-mediated chemoresistance may be influenced by IL-36R signalling in colon cancer, given their more significant co-expression in malignant intestinal tissue in comparison to normal intestinal tissue. Furthermore, high levels of LCN2 expression in other types of cancer have been associated with many recently described IL-36-driven pro-tumorigenic properties [243,

437, 475], such as increased cell proliferation [495], EMT-transition/invasion [496], metastasis [497] and tumoral angiogenesis [498]. Additionally, several IL-17 signalling associated genes are upregulated in colon cancer tissue relative to adjacent normal tissue, with this cohort previously demonstrated to increasingly express IL-36 family members in chapter 3 of this thesis. We additionally showed IL-36γ can strongly induce expression of the other IL-17 signalling gene from our IL-36R high tumour cluster, CXCL1. This expression is highly inducible in colon cancer monolayers and in 3D cultures as described in chapter 3 and here, respectively. In combination, our preliminary findings strongly implicate a role for the IL-36R/IL-17 axis in colon cancer, and that combined therapy inhibition of IL-36Ra and anti-IL-17a may improve efficacy of colon cancer treatment.

Given the findings of this thesis concerning the novel pro-tumorigenic role for IL-36 signalling in colon cancer, it was of interest to see if other cancer types shared similar expression trends as colon cancer in comparison to normal tissue in order to extend this knowledge and identify tumours that could potentially benefit from inhibition of this signalling. TIMER 2.0 analysis of TCGA datasets showed a striking upregulation of IL-36 family members in cancers. A total of 16 different types of cancer showed upregulation of at least one IL-36 agonist, whilst only Head and Neck squamous cell carcinoma (HNSCC) showed downregulation of IL-36α. IL-36γ was also shown to be upregulated in HNSCC. However, IL-36Ra expression was also shown to be vastly upregulated across multiple cancer types, which is likely to have resulted from negative feedback loops to inhibit IL-36R signalling. IL-36R and IL1RAcP gene expression showed much greater variation in regulation relative to normal tissue. This data indicates IL-36 signalling to play important roles across many different cancer types and warrants *in vitro* characterisation as completed in this thesis.

Of the cancers investigated, the lung squamous cell carcinoma (NSCLC) dataset was the only cancer type to show increased expression of all IL-36 family gene members. This dataset was also amongst the most upregulated IL-36R expressing tumours compared to their normal tissue. NSCLC has previously been shown to express much higher levels of IL-36γ protein relative to normal tissue [481] and that these lung cancers are driven by IL-36 stimulation [386]. Our transcriptomic analysis here agrees with this work, suggesting IL-36R inhibition in these tumours may benefit attenuation of tumour progression in NSCLC. Stomach adenocarcinoma was also shown to have a highly significant increase in IL-36R expression, with a recent paper showing IL-36 signalling on these cells to induce pro-tumorigenic effects [243]. These studies, in agreement with our findings, implicate increased tumoral IL-36R expression to benefit tumour progression (Fig. 6.11a). Two other cancer types also stood out for an increase in IL-36R expression, namely esophageal carcinoma (ESCA, crimson) and bladder urothelial cell carcinoma (BLCA, orange). Little has been reported on IL-36 in the bladder and the oesophagus, with only one study reporting IL-36γ to be expressed highly in esophageal squamous cells [206]. The large increase of IL-36R expression suggests that IL-36R inhibition by IL-36Ra may be beneficial to these cancers to prevent induction of pro-tumorigenic processes previously described in this thesis for colon cancer IL-36R$^+$ cells.

Several cancer types in this analysis showed little to no expression of IL-36R expression, indicating the possibility that these may benefit from receipt of next-generation IL-36 cytokine administration to augment the immune response and promote tumour rejection. These include glioblastoma, acute myeloid leukaemia, Lymphoid Neoplasm Diffuse Large B-cell Lymphoma, uveal melanoma, testicular Germ Cell Tumors and metastatic melanoma. Of these cancers, only melanoma has been investigated for the role of increased IL-36

expression in pre-clinical models [299]. Firstly, it was shown that IL-36γ expression does not directly alter tumour cell proliferation, unsurprisingly given the lack of IL-36R expression on these cells. Secondly, it was shown that B16–IL36γ overexpressing cells showed a significant reduction in tumour burden compared to vector control, with significant increases in Type 1 immune response cells such as $CD8^+$ T cells, NK cells, γδ T cells, as well as $CD4^+$ Helper T cells and Treg cells. This highlights the efficacy of IL-36 cytokine production in the TME to augment this immune response, and that development of next generation IL-36 cytokines targeting IL-36R^- cancer cells may be highly beneficial to augment the anti-tumour immune response. This should be further investigated in the listed tumours which express little to no IL-36R in order to develop effective immunotherapy options for these patients.

In conclusion, our analysis of transcriptomic datasets further supports our previous findings that IL-36R signalling is a novel therapeutic target in CRC. Our findings suggest that inhibition of this signalling pathway on cancer cells may benefit overall patient survival and that IL-36 interplay with the IL-17/IL-23 axis may contribute to colon carcinogenesis. Our pan-cancer analysis of IL-36 gene expression also proposes that certain cancer types may be favourable for IL-36Ra inhibition or IL-36 cytokines augmentation and that this is where further research should be completed.

Chapter 7

Final discussion and future perspectives

7.1. Final discussion and future perspectives

IL-1 cytokines are pluripotent signalling molecules across many tissues and influence a diverse array of mesenchymal and immune cells [196]. The potent effects of these cytokines make them highly effective in the acute response to infection, wound healing and tissue homeostasis [199, 499]. The efficiency of this response does however come at a cost, with dysregulated and unresolved signalling in these highly inflammatory pathways giving way to chronic inflammation which can eventually precipitate malignancies in multiple tissues [48, 500]. Therefore, an applied understanding of the spatial and temporal activation or inhibition of IL-36 signalling will greatly benefit the targeting of the IL-36 pathway in cancer.

7.2. Kicking the Can(akinumab) down the road – The future debt of effective barrier protection.

The importance of mammalian epithelial barriers in the clearance of pathogens from the body is evident from untreated acute, lethal infections resulting in fatal pathologies such as toxic shock syndrome, encephalitis and meningitis [501, 502]. These infections can result in rapid patient decline and mortality if the immune system does not gain a rapid hold of viral/bacterial replication. Therefore, it is imperative that immune surveillance of epithelial tissues be capable of a rapid and potent response to viral, bacterial and fungal pathogens. This selects for an immune system which carries a greater precedence in the organism to be capable of surviving potentially fatal disease during years of fertility, rather than preventing the development of malignancies at a much later stage where reproductive capacities of mammals may be lost.

The somewhat of a misnomer that is ‘survival of the fittest’ is a testament to this immune-precedence, which emphasises that natural selection is not a process of an individual organism’s longevity (i.e. survival), but rather the capacity of an organism to reproduce viable offspring [503]. This is strongly reflected in the evolution of the IL-1 family, whereby the potent immune response to PAMPs or DAMPs gives the organism’s immune system a clear advantage to eliminate potentially fatal infections and injuries [504]. The immune system may then protect the organism for many years up to, and beyond, sexual maturity, where reproduction is more likely to occur. Much later in life, the potencies of these cytokines may then take their toll, where decades-long activation of the immune response and ROS production can contribute to mutation accumulation in many important cancer-regulatory genes [505]. These mutations would not affect the ecological appropriateness of the organism in the context of their pathogen-dense environment during sexual maturity, but can compromise lifespan much later on in life. Inhibition of viral replication can also prevent virus-induced carcinogenesis in cervical, oesophageal and Non-Hodgkin’s lymphoma which can be considered an added benefit of strong barrier defences [506].

In addition to the use of IL-36 family members in clearing viral infections, they also play a clear role in many pathologies, inducing the proliferation of the epithelium in order to repair mechanical injury or the bystander cellular damage effects of acute/chronic inflammation [238, 254]. Impaired wound healing, as more acutely observed in failures of haemostasis, can have fatal outcomes [507]. Once more, the immediate advantages of blood clotting capacities and proliferation of epithelial barriers over uninhibited hyper-proliferative tissue, which may take years to become pathological, are clear.

This trade off of IL-1 family member mediated acute protection against pathogens versus chronic pathological inflammation is apparent in a phase III clinical trial (CANTOS) investigating the use of anti-IL-1β antibodies (Canakinumab) for preventing adverse cardiac events [203]. This large clinical study showed a primary finding of reduced incidence of myocardial infarction, stroke or cardiovascular death in treated patients versus placebo, as well as reduced hsCRP in a dose-dependent manner. Incidence of several chronic inflammatory diseases such as arthritis, osteoarthritis and gout were all decreased in Canakinumab-treated patients in comparison to the placebo group. Furthermore, targeting of IL-1β significantly reduced the lung cancer incidence in these treated patients, and moreover, the mortality of lung cancer was also reduced. This data exemplified the role IL-1β signalling in the pathogenesis of both chronic inflammation and cancer, however the cost was apparent in this study also. It was reported that the incidence of fatal infection or sepsis nearly doubled (0.18% vs 0.34% in placebo and 300mg Canakinumab patients, respectively). Overall, the use of anti-IL-1β treatment slightly reduced the incidence of death, irrespective of cause. Notably, the average participant age of this study was 61.1 ± 10.1 years, limiting the ability to assess how IL-1β inhibition may affect younger patients. This does however further highlight the importance of IL-1 family members in the prevention of lethal infections whilst implicating how central these cytokines are to chronic inflammation and malignancy.

Recently, a phase II clinical trial using an anti-IL-36R mAb (Spesolimab) was reported to have reduced the severity of GPP, the most severe form of psoriasis with periodic flare ups of painful skin lesions[317]. However, this too was shown to come at a significant cost, with the incidence of infections reported to be over triple that of the placebo group within the first week (17.1% vs 5.6%). Furthermore, the infections reported consisted of both viral and

bacterial pathogens, across multiple tissue sites. This study further exemplified the multi-faceted importance of IL-36 cytokines in the protection of tissue against infection. The size and duration of this trial were limited, therefore it may very well be possible that a longer study would yield similar reductions in the incidence of chronic inflammatory pathologies and cancer, as observed in the CANTOS trial.

As described in this thesis, IL-36 signalling is heavily involved in the protection against viral, bacterial and fungal pathogens in the skin, lungs, reproductive tract and intestines. Each of these sites has been heavily described to have pathologies which are prone to IL-36 driven inflammation and which may contribute to malignant transformation [406]. Given this, it would not be unreasonable to assume that long term inhibition of IL-36R signalling in these tissues may indeed reduce the incidence and mortality of malignant disease at these sites as observed in the CANTOS trial. Further studies into improved site-specific delivery and IL-36R signalling inhibition durability may successfully reduce the infection co-morbidities of this treatment whilst improving the inhibition of chronic inflammatory pathologies and preventing malignancy.

7.3. Effective targeting of IL-36R signalling in cancer prevention/therapy

With this knowledge of the conflicting role for IL-36 cytokines in the context of malignancy prevention and treatment, it is a question of when and how to use IL-36 signalling inhibitors and IL-36 agonists to provide the best outcomes for patients. Pre-clinical models of many chronic inflammatory diseases have culminated in the development of anti-IL-36R antibodies, two of which have undergone or are currently in phase II trials for severe forms of psoriasis and ulcerative colitis. It has been extensively shown in murine models of chronic inflammation that IL-36R deficient mice are indeed protected against chronic

inflammation and this has also been shown to be protective against cancer in IL-36R/IL-36γ KO mouse models[257, 385, 386, 475, 508]. These short terms models cannot account for the complexity and longevity of mutational accumulations in human cancer, but the immediate and direct effects of IL-36 signalling have shown that intrinsic capacities of cancer cells may be augmented by IL-36 signalling leading to increased cellular proliferation, migration and invasion [243]. This poses the question of how can this be targeted most effectively to reduce the pro-tumourigenic effects of endogenous IL-36 cytokines, and at what stage should this avenue of treatment be considered most appropriate?

It is universally accepted that cancer prevention is the best way to reduce malignancy mortality rates [509]. This suggests that the use of anti-IL36R antibodies could benefit groups of patients at highest risk of developing cancer, such as HNPCC and FAP patients [24]. Although these aetiologies account for a minority of CRC cases, these patients show a very high risk of CRC development, with 57.1% of HNPCC patients developing CRC by 75 years of age and a 100% lifetime risk of CRC development in FAP patients [510, 511]. These patients may therefore benefit from prophylactic low dose anti-IL-36R mAb treatment in combination with routine colonoscopy examinations given their increased risk of CRC development.

As discussed, optimisation of treatment strategies for anti-IL36R therapy is required to reduce the incidence of adverse events. One strategy to overcome the systemic immunosuppressive features of anti-IL-36R antibodies may be the development of bi-specific antibodies in order to localise treatment to target intestinal crypts to prevent off-target effects in other epithelial barriers which may result in impaired infection control at

these sites [42]. A further alternative measure could be the development of IL-36Ra and IL-38 cytokine administration to inhibit IL-36R signalling. The delivery of recombinant cytokines has been a complex journey with many off-target effects recorded including cytokine storm which can cause severe co-morbidities and fatalities in patients [447]. However, there are advantages in the use of endogenous proteins in comparison to anti-body treatments, such as preventing the generation of antibodies to the drug being used to treat patients [512]. Indeed, anti-drug antibodies were generated in 46% of patients in the Spesolimab phase II psoriasis trial, suggesting development of the natural IL-36Ra or IL-38 cytokines as a therapeutic may stand to reduce adverse reactions to treatment [203]. Moreover, next-generation cytokine engineering is evolving rapidly with multiple methods now being reported to improve specificity and durability of cytokines [447]. These strategies, which are used to more safely and efficaciously administer agonists, could theoretically be used for receptor antagonists given their homology to the corresponding agonists.

To further improve specificity of IL-36 signalling intervention and in line with the movement towards personalised medicine, it may be of benefit to generate an IL-36R profile for patients using high throughput single cell sequencing approaches in order to assess the IL-36R expressing cells in a tumour, especially given the mixed reports on whether cytotoxic cells express the IL-36 receptor [233, 235, 292]. This expression characterisation could then be used to predict IL-36R inhibition efficacy in preventing the IL-36 driven intrinsic hallmarks of cancer cell progression, but also predict bystander effects on anti-tumour immune cells. Should a patient show high levels of IL-36R expression on cancer cells, as well as lower levels of expression in the surrounding TME, this patient may stand to benefit

greatly from next generation IL-36R targeting therapy, whether that be a natural antagonist to the receptor or a monoclonal, bi-specific antibody.

7.4. Effective and safe use of IL-36R agonists in Immuno-oncology

True to the dichotomy of IL-1 family members, there is also the benefit of utilising IL-36 cytokines to effectively augment the immune response to facilitate tumour rejection. As described by others [199] and in chapter 5 of this thesis, IL-36 cytokines can augment the immune response through several cell types and processes contributing to a Th1 response for cancer cell clearance [413]. Briefly, IL-36 cytokines induce innate surveillance cells such as NK cells to become more cytotoxic in the clearance of tumours cells, mature dendritic cells to increase their ability to bridge the innate and adaptive arms of immunity and drive effector T cell activation directly and indirectly [233, 419]. Furthermore, this can lead to formation of TLSs which are important lymphoid structures for the generation of long-term immunity to cancer cells and thereby limit tumour progression [380]. Although pre-clinical models of this IL-36-driven augmentation are promising, the clinical translation carries several issues.

Cytokine storm is a well-characterised phenomenon which dates back to field-founding immunotherapy studies of Coley, where tumour inoculation with streptococci resulted in an 'Influenza-like syndrome' [513]. Since then, IL-2 administration to patients was shown to potently reduce tumour burden in patients but coincide with severe adverse events and mortalities in several clinical trials [514]. The systemic effects of cytokine administration, although beneficial with endogenous cytokines, poses a dangerous side effect and difficult hurdle to overcome. As reported with IL-2 administration to patients in multiple studies [515], IL-36 cytokines have also been reported to induced Treg proliferation which acts as

a regulatory mechanism to control excessive production of these cytokines in the body to prevent chronic inflammation or advance cancer [379]. Although this is ideal in the resolution of inflammatory pathologies, this is not desirable in cancer immunotherapy. Furthermore, there is also the issue of IL-36 cytokine bystander effects on tumour cells resulting in induction of their intrinsic pro-tumourigenic properties as seen in gastric, colon and lung cancer [243, 386, 437, 475].

Next generation cytokine engineering approaches are in development to overcome these limitations of cancer treatment with cytokines. These strategies are currently being developed for IL-2/15, IL-12, IL-21, IL-18 and IFNs, however, IL-36 cytokines may be an ideal candidate for future work [516]. Polymer conjugation has been shown to reduce non-specific binding of cytokines, and site specific PEGylation has also been shown to decouple the immune stimulatory and immune inhibitory properties of cytokines, allowing for selective interaction with immune-augmentative or immunosuppressive cell types [517]. The term 'immunocytokines' has recently been coined to describe the fusion of antibodies and cytokines, facilitating cell-specific targeting, as observed with anti-TRP1 fused IL-2 which directed IL-2 binding away from IL-2R$^+$ tumours and towards IL-R2$^+$ immune cells [518]. Moreover, masking moieties have been fused with cytokines to prevent their activation until in contact with proteases which are highly expressed in tumour tissue such as MMP2 and MMP4, although off-site targeting is still very possible given these proteases are expressed in many tissues [519]. These approaches, amongst others, can help overcome the systemic toxicities associated with multiple types of cytokines [447]. However, immunocytokines may be the most effective approach for IL-36 agonist anti-tumour efficacy in order to overcome the cancer cell bystander effects of IL-36 signalling in the TME.

Alternatively, oncolytic viral vaccination may also be used in the treatment of cancers, with work already completed on IL-36γ expressing vaccinia virus which showed significantly enhanced anti-tumour efficacy in multiple *in vivo* models of cancer [80]. Once more, several hurdles face clinical translation of this technology, such as patient response heterogeneity, clearance of OV's by the immune system and questions over biosafety on virotherapy [520]. Similarly, alternative cancer vaccination approaches may be viable options such as neo-antigen generation in the presence of IL-36γ which could effectively drive the anti-tumour Th1 response.

As described for determining the value of IL-36Ra treatment of patients, IL-36R expression should be used for determining the value of IL-36 cytokine treatment in order to predict the off-target effects of these cytokines on epithelial cells and tumour cells which can drive undesirable intrinsic cancer cell processes and Treg recruitment. In this case, an ideal candidate for IL-36 cytokine treatment would be a patient with minimal cancer cell expression of the IL-36R, in conjunction with high levels of anti-tumour immune cell IL-36R expression.

The future of immunotherapy is bright and ever-expanding. In line with this, targeting IL-36 signalling by augmentation or inhibition is a very viable option to be integrated into the current repertoire of immunotherapy strategies.

Bibliography

1. Lukong, K., *Understanding Breast Cancer – The long and winding road.* BBA Clinical, 2017. **7**.
2. Tsoucalas, G. and M. Sgantzos, *Hippocrates (ca 460-370 BC) on nasal cancer.* J buon, 2016. **21**(4): p. 1031-1034.
3. Stratton, M.R., P.J. Campbell, and P.A. Futreal, *The cancer genome.* Nature, 2009. **458**(7239): p. 719-24.
4. Li, Z., et al., *Germline and somatic mutation profile in Cancer patients revealed by a medium-sized pan-Cancer panel.* Genomics, 2021. **113**(4): p. 1930-1939.
5. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.
6. Hanahan, D. and Robert A. Weinberg, *Hallmarks of Cancer: The Next Generation.* Cell, 2011. **144**(5): p. 646-674.
7. Hanahan, D., *Hallmarks of Cancer: New Dimensions.* Cancer Discovery, 2022. **12**(1): p. 31-46.
8. Paschke, S., et al., *Are Colon and Rectal Cancer Two Different Tumor Entities? A Proposal to Abandon the Term Colorectal Cancer.* Int J Mol Sci, 2018. **19**(9).
9. Bray, F., et al., *Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries.* CA Cancer J Clin, 2018. **68**(6): p. 394-424.
10. Purnell, J.Q., et al., *Social and cultural factors are related to perceived colorectal cancer screening benefits and intentions in African Americans.* Journal of Behavioral Medicine, 2010. **33**(1): p. 24-34.
11. Aleksandrova, K., et al., *Combined impact of healthy lifestyle factors on colorectal cancer: a large European cohort study.* BMC Medicine, 2014. **12**(1): p. 168.
12. Saad El Din, K., et al., *Trends in the epidemiology of young-onset colorectal cancer: a worldwide systematic review.* BMC Cancer, 2020. **20**(1): p. 288.
13. Vuik, F.E., et al., *Increasing incidence of colorectal cancer in young adults in Europe over the last 25 years.* 2019. **68**(10): p. 1820-1826.
14. Hong, S.N., *Genetic and epigenetic alterations of colorectal cancer.* Intest Res, 2018. **16**(3): p. 327-337.
15. Markowitz, S.D. and M.M. Bertagnolli, *Molecular origins of cancer: Molecular basis of colorectal cancer.* The New England journal of medicine, 2009. **361**(25): p. 2449-2460.
16. Geiersbach, K.B. and W.S. Samowitz, *Microsatellite instability and colorectal cancer.* Arch Pathol Lab Med, 2011. **135**(10): p. 1269-77.
17. Magzoub, M.M., et al., *The impact of DNA methylation on the cancer proteome.* PLoS Comput Biol, 2019. **15**(7): p. e1007245.
18. Fearon, E.R. and B. Vogelstein, *A genetic model for colorectal tumorigenesis.* Cell, 1990. **61**(5): p. 759-67.
19. Fodde, R., *The APC gene in colorectal cancer.* Eur J Cancer, 2002. **38**(7): p. 867-71.
20. Markman, B., et al., *EGFR and KRAS in colorectal cancer.* Adv Clin Chem, 2010. **51**: p. 71-119.
21. Fleming, N.I., et al., *SMAD2, SMAD3 and SMAD4 mutations in colorectal cancer.* Cancer Res, 2013. **73**(2): p. 725-35.
22. Nakayama, M. and M. Oshima, *Mutant p53 in colon cancer.* J Mol Cell Biol, 2019. **11**(4): p. 267-276.
23. Lipsyc, M. and R. Yaeger, *Impact of somatic mutations on patterns of metastasis in colorectal cancer.* Journal of gastrointestinal oncology, 2015. **6**(6): p. 645-649.
24. Soravia, C., B. Bapat, and Z. Cohen, *Familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC): a review of clinical, genetic and therapeutic aspects.* Schweiz Med Wochenschr, 1997. **127**(16): p. 682-90.

25. Jasperson, K.W., et al., *Hereditary and familial colon cancer.* Gastroenterology, 2010. **138**(6): p. 2044-2058.
26. Kameyama, H., et al., *Genomic characterization of colitis-associated colorectal cancer.* World Journal of Surgical Oncology, 2018. **16**(1): p. 121.
27. Low, D., M. Mino-Kenudson, and E. Mizoguchi, *Recent advancement in understanding colitis-associated tumorigenesis.* Inflammatory bowel diseases, 2014. **20**(11): p. 2115-2123.
28. Yarbro, J.W., et al., *American Joint Committee on Cancer prognostic factors consensus conference.* Cancer, 1999. **86**(11): p. 2436-46.
29. Tong, G.-J., et al., *Comparison of the eighth version of the American Joint Committee on Cancer manual to the seventh version for colorectal cancer: A retrospective review of our data.* World journal of clinical oncology, 2018. **9**(7): p. 148-161.
30. Provenzale, D., et al., *NCCN Guidelines Insights: Colorectal Cancer Screening, Version 1.2018.* J Natl Compr Canc Netw, 2018. **16**(8): p. 939-949.
31. Guinney, J., et al., *The consensus molecular subtypes of colorectal cancer.* Nature medicine, 2015. **21**(11): p. 1350-1356.
32. Galon, J. and A. Lanzi, *Immunoscore and its introduction in clinical practice.* Q J Nucl Med Mol Imaging, 2020. **64**(2): p. 152-161.
33. Chakrabarti, S., et al., *Early stage colon cancer: Current treatment standards, evolving paradigms, and future directions.* World journal of gastrointestinal oncology, 2020. **12**(8): p. 808-832.
34. Osterman, E. and B. Glimelius, *Recurrence Risk After Up-to-Date Colon Cancer Staging, Surgery, and Pathology: Analysis of the Entire Swedish Population.* Dis Colon Rectum, 2018. **61**(9): p. 1016-1025.
35. Costas-Chavarri, A., et al., *Treatment of Patients With Early-Stage Colorectal Cancer: ASCO Resource-Stratified Guideline.* Journal of global oncology, 2019. **5**: p. 1-19.
36. Tie, J., et al., *Circulating Tumor DNA Analysis Guiding Adjuvant Therapy in Stage II Colon Cancer.* New England Journal of Medicine, 2022.
37. Vodenkova, S., et al., *5-fluorouracil and other fluoropyrimidines in colorectal cancer: Past, present and future.* Pharmacology & Therapeutics, 2020. **206**: p. 107447.
38. Comella, P., et al., *Role of oxaliplatin in the treatment of colorectal cancer.* Therapeutics and clinical risk management, 2009. **5**(1): p. 229-238.
39. Chiorean, E.G., et al., *Treatment of Patients With Late-Stage Colorectal Cancer: ASCO Resource-Stratified Guideline.* JCO Global Oncology, 2020(6): p. 414-438.
40. Thirion, P., et al., *Modulation of fluorouracil by leucovorin in patients with advanced colorectal cancer: an updated meta-analysis.* 2004. **22**(18): p. 3766-3775.
41. Xie, Y.-H., Y.-X. Chen, and J.-Y. Fang, *Comprehensive review of targeted therapy for colorectal cancer.* Signal Transduction and Targeted Therapy, 2020. **5**(1): p. 22.
42. Herpers, B., et al., *Functional patient-derived organoid screenings identify MCLA-158 as a therapeutic EGFR × LGR5 bispecific antibody with efficacy in epithelial tumors.* Nature Cancer, 2022. **3**(4): p. 418-436.
43. Zhang, X., et al., *Neoadjuvant Immunotherapy for MSI-H/dMMR Locally Advanced Colorectal Cancer: New Strategies and Unveiled Opportunities.* 2022. **13**.
44. Benson, A.B., et al., *Colon Cancer, Version 2.2021, NCCN Clinical Practice Guidelines in Oncology.* Journal of the National Comprehensive Cancer Network, 2021. **19**(3): p. 329-359.
45. Cercek, A., et al., *PD-1 Blockade in Mismatch Repair-Deficient, Locally Advanced Rectal Cancer.* N Engl J Med, 2022.
46. Virchow, R., *Vorlesungen über pathologie*. Vol. 1. 1862: Hirschwald.
47. Dvorak, H.F., *Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing.* N Engl J Med, 1986. **315**(26): p. 1650-9.
48. Greten, F.R. and S.I. Grivennikov, *Inflammation and Cancer: Triggers, Mechanisms, and Consequences.* Immunity, 2019. **51**(1): p. 27-41.

49. Aran, D., et al., *Widespread parainflammation in human cancer.* Genome Biol, 2016. **17**(1): p. 145.
50. Lucafò, M., et al., *Inflammatory Bowel Disease and Risk of Colorectal Cancer: An Overview From Pathophysiology to Pharmacological Prevention.* 2021. **12**.
51. Lonkar, P. and P.C. Dedon, *Reactive species and DNA damage in chronic inflammation: reconciling chemical mechanisms and biological fates.* International journal of cancer, 2011. **128**(9): p. 1999-2009.
52. Meyerson, M., S. Gabriel, and G. Getz, *Advances in understanding cancer genomes through second-generation sequencing.* Nat Rev Genet, 2010. **11**(10): p. 685-96.
53. Anderson, N.M. and M.C. Simon, *The tumor microenvironment.* Curr Biol, 2020. **30**(16): p. R921-r925.
54. Giraldo, N.A., et al., *The clinical role of the TME in solid cancer.* British Journal of Cancer, 2019. **120**(1): p. 45-53.
55. Zhao, H., et al., *Inflammation and tumor progression: signaling pathways and targeted intervention.* Signal Transduction and Targeted Therapy, 2021. **6**(1): p. 263.
56. Kashfi, K., J. Kannikal, and N. Nath, *Macrophage Reprogramming and Cancer Therapeutics: Role of iNOS-Derived NO.* Cells, 2021. **10**(11).
57. Boutilier, A.J. and S.F. Elsawa, *Macrophage Polarization States in the Tumor Microenvironment.* International journal of molecular sciences, 2021. **22**(13): p. 6995.
58. Giese, M.A., L.E. Hind, and A. Huttenlocher, *Neutrophil plasticity in the tumor microenvironment.* Blood, 2019. **133**(20): p. 2159-2167.
59. Calon, A., et al., *Dependency of colorectal cancer on a TGF-β-driven program in stromal cells for metastasis initiation.* Cancer cell, 2012. **22**(5): p. 571-584.
60. Chen, W.-Z., et al., *Endothelial cells in colorectal cancer.* World journal of gastrointestinal oncology, 2019. **11**(11): p. 946-956.
61. Rømer, A.M.A., M.L. Thorseth, and D.H. Madsen, *Immune Modulatory Properties of Collagen in Cancer.* Front Immunol, 2021. **12**: p. 791453.
62. Galon, J. and D. Bruni, *Approaches to treat immune hot, altered and cold tumours with combination immunotherapies.* Nature Reviews Drug Discovery, 2019. **18**(3): p. 197-218.
63. Kim, S., et al., *The tumor immune microenvironmental analysis of 2,033 transcriptomes across 7 cancer types.* Scientific Reports, 2020. **10**(1): p. 9536.
64. Wang, H., T. Tian, and J. Zhang, *Tumor-Associated Macrophages (TAMs) in Colorectal Cancer (CRC): From Mechanism to Therapy and Prognosis.* International journal of molecular sciences, 2021. **22**(16): p. 8470.
65. Mantovani, A., et al., *Tumour-associated macrophages as treatment targets in oncology.* Nat Rev Clin Oncol, 2017. **14**(7): p. 399-416.
66. Brigati, C., et al., *Tumors and inflammatory infiltrates: Friends or foes?* Clinical & Experimental Metastasis, 2002. **19**(3): p. 247-258.
67. Pan, Y., et al., *Tumor-Associated Macrophages in Tumor Immunity.* 2020. **11**.
68. Muntjewerff, E.M., L.D. Meesters, and G. van den Bogaart, *Antigen Cross-Presentation by Macrophages.* 2020. **11**.
69. Petty, A.J., et al., *Hedgehog signaling promotes tumor-associated macrophage polarization to suppress intratumoral CD8+ T cell recruitment.* J Clin Invest, 2019. **129**(12): p. 5151-5162.
70. Edin, S., et al., *The distribution of macrophages with a M1 or M2 phenotype in relation to prognosis and the molecular characteristics of colorectal cancer.* PLoS One, 2012. **7**(10): p. e47045.
71. Erreni, M., A. Mantovani, and P. Allavena, *Tumor-associated Macrophages (TAM) and Inflammation in Colorectal Cancer.* Cancer microenvironment : official journal of the International Cancer Microenvironment Society, 2011. **4**(2): p. 141-154.

72. Lian, G., et al., *Colon Cancer Cell Secretes EGF to Promote M2 Polarization of TAM Through EGFR/PI3K/AKT/mTOR Pathway.* Technol Cancer Res Treat, 2019. **18**: p. 1533033819849068.
73. Zhou, R., et al., *Interleukin-13 and its receptors in colorectal cancer (Review).* Biomedical reports, 2013. **1**(5): p. 687-690.
74. Zhao, P., et al., *Response gene to complement 32 expression in macrophages augments paracrine stimulation-mediated colon cancer progression.* Cell Death Dis, 2019. **10**(10): p. 776.
75. Yu, X., et al., *CXCL12/CXCR4 promotes inflammation-driven colorectal cancer progression through activation of RhoA signaling by sponging miR-133a-3p.* J Exp Clin Cancer Res, 2019. **38**(1): p. 32.
76. Xu, H., et al., *Six1 promotes colorectal cancer growth and metastasis by stimulating angiogenesis and recruiting tumor-associated macrophages.* Carcinogenesis, 2017. **38**(3): p. 281-292.
77. Spiller, K.L., et al., *The role of macrophage phenotype in vascularization of tissue engineering scaffolds.* Biomaterials, 2014. **35**(15): p. 4477-88.
78. Afik, R., et al., *Tumor macrophages are pivotal constructors of tumor collagenous matrix.* The Journal of experimental medicine, 2016. **213**(11): p. 2315-2331.
79. Cui, Y.L., et al., *Correlations of tumor-associated macrophage subtypes with liver metastases of colorectal cancer.* Asian Pac J Cancer Prev, 2013. **14**(2): p. 1003-7.
80. Yang, M., et al., *IL-36γ-armed oncolytic virus exerts superior efficacy through induction of potent adaptive antitumor immunity.* Cancer immunology, immunotherapy : CII, 2021. **70**(9): p. 2467-2481.
81. Subtil, B., et al., *The Therapeutic Potential of Tackling Tumor-Induced Dendritic Cell Dysfunction in Colorectal Cancer.* 2021. **12**.
82. Hildner, K., et al., *Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity.* Science, 2008. **322**(5904): p. 1097-100.
83. Jongbloed, S.L., et al., *Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens.* J Exp Med, 2010. **207**(6): p. 1247-60.
84. Binnewies, M., et al., *Unleashing Type-2 Dendritic Cells to Drive Protective Antitumor CD4(+) T Cell Immunity.* Cell, 2019. **177**(3): p. 556-571.e16.
85. Kim, C.W., K.D. Kim, and H.K. Lee, *The role of dendritic cells in tumor microenvironments and their uses as therapeutic targets.* BMB Rep, 2021. **54**(1): p. 31-43.
86. Mittal, D., et al., *Interleukin-12 from CD103(+) Batf3-Dependent Dendritic Cells Required for NK-Cell Suppression of Metastasis.* Cancer Immunol Res, 2017. **5**(12): p. 1098-1108.
87. Xu, M.M., et al., *Dendritic Cells but Not Macrophages Sense Tumor Mitochondrial DNA for Cross-priming through Signal Regulatory Protein α Signaling.* Immunity, 2017. **47**(2): p. 363-373.e5.
88. Gai, X.D., et al., *Potential role of plasmacytoid dendritic cells for FOXP3+ regulatory T cell development in human colorectal cancer and tumor draining lymph node.* Pathol Res Pract, 2013. **209**(12): p. 774-8.
89. Drobits, B., et al., *Imiquimod clears tumors in mice independent of adaptive immunity by converting pDCs into tumor-killing effector cells.* J Clin Invest, 2012. **122**(2): p. 575-85.
90. Stary, G., et al., *Tumoricidal activity of TLR7/8-activated inflammatory dendritic cells.* 2007. **204**(6): p. 1441-1451.
91. Masucci, M.T., M. Minopoli, and M.V. Carriero, *Tumor Associated Neutrophils. Their Role in Tumorigenesis, Metastasis, Prognosis and Therapy.* Frontiers in oncology, 2019. **9**: p. 1146-1146.

92. Fridlender, Z.G., et al., *Transcriptomic analysis comparing tumor-associated neutrophils with granulocytic myeloid-derived suppressor cells and normal neutrophils.* PLoS One, 2012. **7**(2): p. e31524.
93. Riise, R.E., et al., *TLR-Stimulated Neutrophils Instruct NK Cells To Trigger Dendritic Cell Maturation and Promote Adaptive T Cell Responses.* J Immunol, 2015. **195**(3): p. 1121-8.
94. Martin, A., et al., *Tumor-derived granzyme B-expressing neutrophils acquire antitumor potential after lipid A treatment.* Oncotarget, 2018. **9**(47): p. 28364-28378.
95. Finisguerra, V., et al., *MET is required for the recruitment of anti-tumoural neutrophils.* Nature, 2015. **522**(7556): p. 349-53.
96. Galdiero, M.R., et al., *Occurrence and significance of tumor-associated neutrophils in patients with colorectal cancer.* Int J Cancer, 2016. **139**(2): p. 446-56.
97. Gijsbers, K., et al., *GCP-2/CXCL6 synergizes with other endothelial cell-derived chemokines in neutrophil mobilization and is associated with angiogenesis in gastrointestinal tumors.* Exp Cell Res, 2005. **303**(2): p. 331-42.
98. Coffelt, S.B., M.D. Wellenstein, and K.E. de Visser, *Neutrophils in cancer: neutral no more.* Nature Reviews Cancer, 2016. **16**(7): p. 431-446.
99. Shang, A., et al., *Exosomal KRAS mutation promotes the formation of tumor-associated neutrophil extracellular traps and causes deterioration of colorectal cancer by inducing IL-8 expression.* Cell Commun Signal, 2020. **18**(1): p. 52.
100. Cools-Lartigue, J., et al., *Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis.* J Clin Invest, 2013. **123**(8): p. 3446-58.
101. Nozawa, H., C. Chiu, and D. Hanahan, *Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis.* Proc Natl Acad Sci U S A, 2006. **103**(33): p. 12493-8.
102. Mizuno, R., et al., *The Role of Tumor-Associated Neutrophils in Colorectal Cancer.* International journal of molecular sciences, 2019. **20**(3): p. 529.
103. Hedrick, C.C. and I. Malanchi, *Neutrophils in cancer: heterogeneous and multifaceted.* Nature Reviews Immunology, 2022. **22**(3): p. 173-187.
104. Teijeira, Á., et al., *CXCR1 and CXCR2 Chemokine Receptor Agonists Produced by Tumors Induce Neutrophil Extracellular Traps that Interfere with Immune Cytotoxicity.* Immunity, 2020. **52**(5): p. 856-871.e8.
105. Sieminska, I. and J. Baran, *Myeloid-Derived Suppressor Cells in Colorectal Cancer.* Frontiers in immunology, 2020. **11**: p. 1526-1526.
106. Ren, X., et al., *Inhibition of CCL7 derived from Mo-MDSCs prevents metastatic progression from latency in colorectal cancer.* Cell Death & Disease, 2021. **12**(5): p. 484.
107. Gabrilovich, D.I. and S. Nagaraj, *Myeloid-derived suppressor cells as regulators of the immune system.* Nat Rev Immunol, 2009. **9**(3): p. 162-74.
108. OuYang, L.-Y., et al., *Tumor-induced myeloid-derived suppressor cells promote tumor progression through oxidative metabolism in human colorectal cancer.* Journal of Translational Medicine, 2015. **13**(1): p. 47.
109. Toor, S.M., et al., *Increased Levels of Circulating and Tumor-Infiltrating Granulocytic Myeloid Cells in Colorectal Cancer Patients.* Front Immunol, 2016. **7**: p. 560.
110. Zou, Y., et al., *Efficacy and predictive factors of immune checkpoint inhibitors in metastatic breast cancer: a systematic review and meta-analysis.* Therapeutic advances in medical oncology, 2020. **12**: p. 1758835920940928-1758835920940928.
111. Paijens, S.T., et al., *Tumor-infiltrating lymphocytes in the immunotherapy era.* Cellular & molecular immunology, 2021. **18**(4): p. 842-859.
112. Huang, Q., et al., *Innate Lymphoid Cells in Colorectal Cancers: A Double-Edged Sword.* 2020. **10**.
113. Vivier, E., et al., *Innate Lymphoid Cells: 10 Years On.* Cell, 2018. **174**(5): p. 1054-1066.

114. Okazawa, A., et al., *Human intestinal epithelial cell-derived interleukin (IL)-18, along with IL-2, IL-7 and IL-15, is a potent synergistic factor for the proliferation of intraepithelial lymphocytes.* Clinical and Experimental Immunology, 2004. **136**(2): p. 269-276.
115. Tallerico, R., et al., *Human NK Cells Selective Targeting of Colon Cancer–Initiating Cells: A Role for Natural Cytotoxicity Receptors and MHC Class I Molecules.* The Journal of Immunology, 2013. **190**(5): p. 2381.
116. de Vries, N.L., et al., *High-dimensional cytometric analysis of colorectal cancer reveals novel mediators of antitumour immunity.* Gut, 2020. **69**(4): p. 691-703.
117. Pontrelli, P., et al., *The Role of Natural Killer Cells in the Immune Response in Kidney Transplantation.* Frontiers in immunology, 2020. **11**: p. 1454-1454.
118. Morvan, M.G. and L.L. Lanier, *NK cells and cancer: you can teach innate cells new tricks.* Nat Rev Cancer, 2016. **16**(1): p. 7-19.
119. Liu, Y., et al., *Increased expression of programmed cell death protein 1 on NK cells inhibits NK-cell-mediated anti-tumor function and indicates poor prognosis in digestive cancers.* Oncogene, 2017. **36**(44): p. 6143-6153.
120. Sconocchia, G., et al., *Tumor infiltration by FcγRIII (CD16)+ myeloid cells is associated with improved survival in patients with colorectal carcinoma.* Int J Cancer, 2011. **128**(11): p. 2663-72.
121. Sconocchia, G., et al., *NK cells and T cells cooperate during the clinical course of colorectal cancer.* Oncoimmunology, 2014. **3**(8): p. e952197.
122. Curtsinger, J.M., et al., *Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells.* J Immunol, 1999. **162**(6): p. 3256-62.
123. Golstein, P. and G.M. Griffiths, *An early history of T cell-mediated cytotoxicity.* Nat Rev Immunol, 2018. **18**(8): p. 527-535.
124. Nosho, K., et al., *Tumour-infiltrating T-cell subsets, molecular changes in colorectal cancer, and prognosis: cohort study and literature review.* J Pathol, 2010. **222**(4): p. 350-66.
125. Bruni, D., H.K. Angell, and J. Galon, *The immune contexture and Immunoscore in cancer prognosis and therapeutic efficacy.* Nat Rev Cancer, 2020. **20**(11): p. 662-680.
126. Zheng, Y., et al., *Neoantigen: A Promising Target for the Immunotherapy of Colorectal Cancer.* Disease markers, 2022. **2022**: p. 8270305-8270305.
127. Bai, Z., et al., *Tumor-Infiltrating Lymphocytes in Colorectal Cancer: The Fundamental Indication and Application on Immunotherapy.* 2022. **12**.
128. Lugo-Villarino, G., et al., *T-bet is required for optimal production of IFN-gamma and antigen-specific T cell activation by dendritic cells.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(13): p. 7749-7754.
129. Afkarian, M., et al., *T-bet is a STAT1-induced regulator of IL-12R expression in naïve CD4+ T cells.* Nature Immunology, 2002. **3**(6): p. 549-557.
130. Fridman, W.H., et al., *The immune contexture in cancer prognosis and treatment.* Nat Rev Clin Oncol, 2017. **14**(12): p. 717-734.
131. Weaver, C.T. and R.D. Hatton, *Interplay between the TH17 and TReg cell lineages: a (co-)evolutionary perspective.* Nat Rev Immunol, 2009. **9**(12): p. 883-9.
132. Mangan, P.R., et al., *Transforming growth factor-beta induces development of the T(H)17 lineage.* Nature, 2006. **441**(7090): p. 231-4.
133. Guéry, L. and S. Hugues, *Th17 Cell Plasticity and Functions in Cancer Immunity.* Biomed Res Int, 2015. **2015**: p. 314620.
134. Liu, J., et al., *IL-17 is associated with poor prognosis and promotes angiogenesis via stimulating VEGF production of cancer cells in colorectal carcinoma.* Biochem Biophys Res Commun, 2011. **407**(2): p. 348-54.
135. De Simone, V., et al., *Role of T(H)17 cytokines in the control of colorectal cancer.* Oncoimmunology, 2013. **2**(12): p. e26617-e26617.

136. Cui, G., *T(H)9, T(H)17, and T(H)22 Cell Subsets and Their Main Cytokine Products in the Pathogenesis of Colorectal Cancer.* Frontiers in oncology, 2019. **9**: p. 1002-1002.
137. Ziesché, E., et al., *The interleukin-22/STAT3 pathway potentiates expression of inducible nitric-oxide synthase in human colon carcinoma cells.* J Biol Chem, 2007. **282**(22): p. 16006-15.
138. Mizoguchi, A., *Healing of intestinal inflammation by IL-22.* Inflamm Bowel Dis, 2012. **18**(9): p. 1777-84.
139. Huang, Y.-H., et al., *Th22 cell accumulation is associated with colorectal cancer development.* World journal of gastroenterology, 2015. **21**(14): p. 4216-4224.
140. Chen, W., et al., *Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3.* J Exp Med, 2003. **198**(12): p. 1875-86.
141. Apostolou, I. and H. von Boehmer, *In vivo instruction of suppressor commitment in naive T cells.* J Exp Med, 2004. **199**(10): p. 1401-8.
142. Fujimoto, H., et al., *Deregulated Mucosal Immune Surveillance through Gut-Associated Regulatory T Cells and PD-1(+) T Cells in Human Colorectal Cancer.* J Immunol, 2018. **200**(9): p. 3291-3303.
143. Clarke, S.L., et al., *CD4+CD25+FOXP3+ regulatory T cells suppress anti-tumor immune responses in patients with colorectal cancer.* PLoS One, 2006. **1**(1): p. e129.
144. Xue, J., et al., *PIPKIγ Regulates CCL2 Expression in Colorectal Cancer by Activating AKT-STAT3 Signaling.* Journal of immunology research, 2019. **2019**: p. 3690561-3690561.
145. Olguín, J.E., et al., *Relevance of Regulatory T Cells during Colorectal Cancer Development.* Cancers, 2020. **12**(7): p. 1888.
146. Gunnarsson, U., et al., *Association between local immune cell infiltration, mismatch repair status and systemic inflammatory response in colorectal cancer.* J Transl Med, 2020. **18**(1): p. 178.
147. Frey, D.M., et al., *High frequency of tumor-infiltrating FOXP3(+) regulatory T cells predicts improved survival in mismatch repair-proficient colorectal cancer patients.* Int J Cancer, 2010. **126**(11): p. 2635-43.
148. Le Flahec, G., et al., *Mismatch repair-deficient colorectal cancer: a model of immunogenic and immune cell-rich tumor despite nonsignificant programmed cell death ligand-1 expression in tumor cells.* Hum Pathol, 2018. **72**: p. 135-143.
149. Toh, J.W.T., et al., *Cytotoxic CD8+ T cells and tissue resident memory cells in colorectal cancer based on microsatellite instability and BRAF status.* World journal of clinical oncology, 2021. **12**(4): p. 238-248.
150. Yang, R., et al., *Distinct epigenetic features of tumor-reactive CD8+ T cells in colorectal cancer patients revealed by genome-wide DNA methylation analysis.* Genome Biology, 2019. **21**(1): p. 2.
151. Pagès, F., et al., *Effector memory T cells, early metastasis, and survival in colorectal cancer.* N Engl J Med, 2005. **353**(25): p. 2654-66.
152. Gentles, A.J., et al., *The prognostic landscape of genes and infiltrating immune cells across human cancers.* Nat Med, 2015. **21**(8): p. 938-945.
153. Rong, L., et al., *Analysis of tumor-infiltrating gamma delta T cells in rectal cancer.* World J Gastroenterol, 2016. **22**(13): p. 3573-80.
154. Meraviglia, S., et al., *Distinctive features of tumor-infiltrating γδ T lymphocytes in human colorectal cancer.* Oncoimmunology, 2017. **6**(10): p. e1347742-e1347742.
155. Reynolds, J.L., et al., *Histamine content in colorectal cancer. Are there sufficient levels of histamine to affect lymphocyte function?* Eur J Surg Oncol, 1997. **23**(3): p. 224-7.
156. Zhao, P., et al., *Levels of circulating mast cell progenitors and tumour-infiltrating mast cells in patients with colorectal cancer.* Oncol Rep, 2022. **47**(5): p. 89.

157. Valès, S., et al., *Tumor cells hijack enteric glia to activate colon cancer stem cells and stimulate tumorigenesis.* eBioMedicine, 2019. **49**: p. 172-188.
158. Yuan, R., et al., *Enteric Glia Play a Critical Role in Promoting the Development of Colorectal Cancer.* 2020. **10**.
159. Domblides, C., et al., *Tumor-Associated Tertiary Lymphoid Structures: From Basic and Clinical Knowledge to Therapeutic Manipulation.* 2021. **12**.
160. Flavell, R.A., A. Kratz, and N.H. Ruddle, *The contribution of insulitis to diabetes development in tumor necrosis factor transgenic mice.* Curr Top Microbiol Immunol, 1996. **206**: p. 33-50.
161. Willis, S.N., et al., *The microenvironment of germ cell tumors harbors a prominent antigen-driven humoral response.* J Immunol, 2009. **182**(5): p. 3310-7.
162. Väyrynen, J.P., et al., *Characteristics and significance of colorectal cancer associated lymphoid reaction.* Int J Cancer, 2014. **134**(9): p. 2126-35.
163. Gu-Trantien, C., et al., *CD4⁺ follicular helper T cell infiltration predicts breast cancer survival.* J Clin Invest, 2013. **123**(7): p. 2873-92.
164. Kang, W., et al., *Tertiary Lymphoid Structures in Cancer: The Double-Edged Sword Role in Antitumor Immunity and Potential Therapeutic Induction Strategies.* 2021. **12**.
165. Schlößer, H.A., et al., *B cells in esophago-gastric adenocarcinoma are highly differentiated, organize in tertiary lymphoid structures and produce tumor-specific antibodies.* Oncoimmunology, 2019. **8**(1): p. e1512458.
166. Gu-Trantien, C., et al., *CXCL13-producing TFH cells link immune suppression and adaptive memory in human breast cancer.* JCI Insight, 2017. **2**(11).
167. Posch, F., et al., *Maturation of tertiary lymphoid structures and recurrence of stage II and III colorectal cancer.* Oncoimmunology, 2017. **7**(2): p. e1378844-e1378844.
168. Trajkovski, G., et al., *Tertiary Lymphoid Structures in Colorectal Cancers and Their Prognostic Value.* Open access Macedonian journal of medical sciences, 2018. **6**(10): p. 1824-1828.
169. Binnewies, M., et al., *Understanding the tumor immune microenvironment (TIME) for effective therapy.* 2018. **24**(5): p. 541-550.
170. Turley, S.J., V. Cremasco, and J.L.J.N.r.i. Astarita, *Immunological hallmarks of stromal cells in the tumour microenvironment.* 2015. **15**(11): p. 669-682.
171. Sahai, E., et al., *A framework for advancing our understanding of cancer-associated fibroblasts.* Nat Rev Cancer, 2020. **20**(3): p. 174-186.
172. Deng, L., et al., *The Versatile Roles of Cancer-Associated Fibroblasts in Colorectal Cancer and Therapeutic Implications.* 2021. **9**(2772).
173. Hawinkels, L.J., et al., *Interaction with colon cancer cells hyperactivates TGF-β signaling in cancer-associated fibroblasts.* Oncogene, 2014. **33**(1): p. 97-107.
174. Ma, H., et al., *Periostin Promotes Colorectal Tumorigenesis through Integrin-FAK-Src Pathway-Mediated YAP/TAZ Activation.* Cell Rep, 2020. **30**(3): p. 793-806.e6.
175. Li, Z., et al., *Nodal Facilitates Differentiation of Fibroblasts to Cancer-Associated Fibroblasts that Support Tumor Growth in Melanoma and Colorectal Cancer.* Cells, 2019. **8**(6).
176. Zhou, W., et al., *Oxidative stress induced autophagy in cancer associated fibroblast enhances proliferation and metabolism of colorectal cancer cells.* Cell Cycle, 2017. **16**(1): p. 73-81.
177. Stadler, M., et al., *Stromal fibroblasts shape the myeloid phenotype in normal colon and colorectal cancer and induce CD163 and CCL2 expression in macrophages.* Cancer Letters, 2021. **520**: p. 184-200.
178. Gieniec, K.A., et al., *Cancer-associated fibroblasts—heroes or villains?* British Journal of Cancer, 2019. **121**(4): p. 293-302.
179. Heichler, C., et al., *STAT3 activation through IL-6/IL-11 in cancer-associated fibroblasts promotes colorectal tumour development and correlates with poor prognosis.* Gut, 2020. **69**(7): p. 1269-1282.

180. Huang, T.-X., X.-Y. Guan, and L.J.A.j.o.c.r. Fu, *Therapeutic targeting of the crosstalk between cancer-associated fibroblasts and cancer stem cells.* 2019. **9**(9): p. 1889.
181. Garvey, C.M., et al., *Anti-EGFR Therapy Induces EGF Secretion by Cancer-Associated Fibroblasts to Confer Colorectal Cancer Chemoresistance.* Cancers (Basel), 2020. **12**(6).
182. Wanandi, S.I., et al., *Cancer-associated fibroblast (CAF) secretomes-induced epithelial-mesenchymal transition on HT-29 colorectal carcinoma cells associated with hepatocyte growth factor (HGF) signalling.* 2021. **71**(2): p. S18-24.
183. Gu, J., et al., *Immunohistochemical detection of cancer-associated fibroblasts in gastrointestinal cancer as a potential prognostic biomarker of survival: meta-analysis.* 2020, 2020. **9**(11): p. 6629-6638.
184. Kawczyk-Krupka, A., et al., *Secretion of the angiogenic factor VEGF after photodynamic therapy with ALA under hypoxia-like conditions in colon cancer cells.* Photodiagnosis Photodyn Ther, 2018. **21**: p. 16-18.
185. Liu, Z., et al., *VEGFR2 regulates endothelial differentiation of colon cancer cells.* BMC Cancer, 2017. **17**(1): p. 593.
186. Ohga, N., et al., *Heterogeneity of tumor endothelial cells: comparison between tumor endothelial cells isolated from high- and low-metastatic tumors.* Am J Pathol, 2012. **180**(3): p. 1294-1307.
187. Rahbari, N.N., et al., *Prognostic value of circulating endothelial cells in metastatic colorectal cancer.* Oncotarget, 2017. **8**(23): p. 37491-37501.
188. Yuan, J., et al., *The Effects of Mesenchymal Stem Cell on Colorectal Cancer.* Stem cells international, 2021. **2021**: p. 9136583-9136583.
189. Koliaraki, V., et al., *Mesenchymal Cells in Colon Cancer.* Gastroenterology, 2017. **152**(5): p. 964-979.
190. Tabuso, M., et al., *Role of tissue microenvironment resident adipocytes in colon cancer.* World journal of gastroenterology, 2017. **23**(32): p. 5829-5835.
191. Falcón, B.L., et al., *Contrasting actions of selective inhibitors of angiopoietin-1 and angiopoietin-2 on the normalization of tumor blood vessels.* The American journal of pathology, 2009. **175**(5): p. 2159-2170.
192. Barlow, K.D., et al., *Pericytes on the tumor vasculature: jekyll or hyde?* Cancer Microenviron, 2013. **6**(1): p. 1-17.
193. Karlsson, S. and H. Nyström, *The extracellular matrix in colorectal cancer and its metastatic settling – Alterations and biological implications.* Critical Reviews in Oncology/Hematology, 2022. **175**: p. 103712.
194. Brauchle, E., et al., *Biomechanical and biomolecular characterization of extracellular matrix structures in human colon carcinomas.* 2018. **68**: p. 180-193.
195. Kuczek, D.E., et al., *Collagen density regulates the activity of tumor-infiltrating T cells.* Journal for immunotherapy of cancer, 2019. **7**(1): p. 68-68.
196. Baker, K.J., A. Houston, and E. Brint, *IL-1 Family Members in Cancer; Two Sides to Every Story.* Frontiers in immunology, 2019. **10**: p. 1197-1197.
197. Bent, R., et al., *Interleukin-1 Beta-A Friend or Foe in Malignancies?* Int J Mol Sci, 2018. **19**(8).
198. Mantovani, A., et al., *Cancer-related inflammation.* Nature, 2008. **454**(7203): p. 436-444.
199. Sun, R., et al., *The IL-1 family in tumorigenesis and antitumor immunity.* Semin Cancer Biol, 2022.
200. Diaz-Barreiro, A., A. Huard, and G. Palmer, *Multifaceted roles of IL-38 in inflammation and cancer.* Cytokine, 2022. **151**: p. 155808.
201. Neurath, M.F., *IL-36 in chronic inflammation and cancer.* Cytokine Growth Factor Rev, 2020. **55**: p. 70-79.
202. Mantovani, A., I. Barajon, and C. Garlanda, *IL-1 and IL-1 regulatory pathways in cancer progression and therapy.* Immunol Rev, 2018. **281**(1): p. 57-61.

203. Ridker, P.M., et al., *Effect of interleukin-1β inhibition with canakinumab on incident lung cancer in patients with atherosclerosis: exploratory results from a randomised, double-blind, placebo-controlled trial.* Lancet, 2017. **390**(10105): p. 1833-1842.
204. Carlsen, L., K.E. Huntington, and W.S. El-Deiry, *Immunotherapy for Colorectal Cancer: Mechanisms and Predictive Biomarkers.* Cancers, 2022. **14**(4): p. 1028.
205. Smith, D.E., et al., *Four new members expand the interleukin-1 superfamily.* J Biol Chem, 2000. **275**(2): p. 1169-75.
206. Busfield, S.J., et al., *Identification and Gene Organization of Three Novel Members of the IL-1 Family on Human Chromosome 2.* Genomics, 2000. **66**(2): p. 213-216.
207. Mulero, J.J., et al., *IL1HY1: A novel interleukin-1 receptor antagonist gene.* Biochem Biophys Res Commun, 1999. **263**(3): p. 702-6.
208. Rivers-Auty, J., et al., *Redefining the ancestral origins of the interleukin-1 superfamily.* Nature Communications, 2018. **9**(1): p. 1156.
209. Yuan, X., et al., *Role of IL-38 and its related cytokines in inflammation.* Mediators Inflamm, 2015. **2015**: p. 807976.
210. Towne, J.E., et al., *Interleukin-36 (IL-36) ligands require processing for full agonist (IL-36α, IL-36β, and IL-36γ) or antagonist (IL-36Ra) activity.* The Journal of biological chemistry, 2011. **286**(49): p. 42594-42602.
211. Martinon, F., K. Burns, and J. Tschopp, *The Inflammasome: A Molecular Platform Triggering Activation of Inflammatory Caspases and Processing of proIL-β.* Molecular Cell, 2002. **10**(2): p. 417-426.
212. Kayagaki, N., et al., *Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling.* Nature, 2015. **526**(7575): p. 666-671.
213. Zhou, L. and V. Todorovic, *Interleukin-36: Structure, Signaling and Function.* Adv Exp Med Biol, 2021. **21**: p. 191-210.
214. Kovach, M.A., et al., *IL-36γ is secreted in microparticles and exosomes by lung macrophages in response to bacteria and bacterial components.* J Leukoc Biol, 2016. **100**(2): p. 413-21.
215. Williams, D.W. and R.H. Kim, *Epithelial cells release IL-36α in extracellular vesicles following mechanical damage.* Biochemical and Biophysical Research Communications, 2022. **605**: p. 56-62.
216. Henry, C.M., et al., *Neutrophil-Derived Proteases Escalate Inflammation through Activation of IL-36 Family Cytokines.* Cell Reports, 2016. **14**(4): p. 708-722.
217. Macleod, T., et al., *Neutrophil Elastase-mediated proteolysis activates the anti-inflammatory cytokine IL-36 Receptor antagonist.* Scientific reports, 2016. **6**: p. 24880-24880.
218. Ainscough, J.S., et al., *Cathepsin S is the major activator of the psoriasis-associated proinflammatory cytokine IL-36γ.* Proceedings of the National Academy of Sciences of the United States of America, 2017. **114**(13): p. E2748-E2757.
219. de Graaf, D.M., et al., *Human recombinant interleukin-38 suppresses inflammation in mouse models of local and systemic disease.* Cytokine, 2021. **137**: p. 155334-155334.
220. O'Neill, L., *The Toll/interleukin-1 receptor domain: a molecular switch for inflammation and host defence.* Biochem Soc Trans, 2000. **28**(5): p. 557-63.
221. Dunn, E., et al., *Annotating genes with potential roles in the immune system: six new members of the IL-1 family.* Trends Immunol, 2001. **22**(10): p. 533-6.
222. Yi, G., et al., *Structural and Functional Attributes of the Interleukin-36 Receptor.* J Biol Chem, 2016. **291**(32): p. 16597-609.
223. Saha, S.S., et al., *Signal Transduction and Intracellular Trafficking by the Interleukin 36 Receptor.* The Journal of biological chemistry, 2015. **290**(39): p. 23997-24006.
224. Günther, S. and E.J. Sundberg, *Molecular Determinants of Agonist and Antagonist Signaling through the IL-36 Receptor.* The Journal of Immunology, 2014. **193**(2): p. 921.

225. Zhou, L., et al., *Quantitative ligand and receptor binding studies reveal the mechanism of interleukin-36 (IL-36) pathway activation.* The Journal of biological chemistry, 2018. **293**(2): p. 403-411.
226. Manzanares-Meza, L.D., R. Valle-Rios, and O. Medina-Contreras, *Interleukin-1 Receptor-Like 2: One Receptor, Three Agonists, and Many Implications.* J Interferon Cytokine Res, 2022. **42**(2): p. 49-61.
227. Swindell, W.R., et al., *RNA-Seq Analysis of IL-1B and IL-36 Responses in Epidermal Keratinocytes Identifies a Shared MyD88-Dependent Gene Signature.* Front Immunol, 2018. **9**: p. 80.
228. Dunn, E.F., et al., *High-resolution structure of murine interleukin 1 homologue IL-1F5 reveals unique loop conformations for receptor binding specificity.* Biochemistry, 2003. **42**(37): p. 10938-44.
229. Gresnigt, M.S. and F.L. van de Veerdonk, *Biology of IL-36 cytokines and their role in disease.* Semin Immunol, 2013. **25**(6): p. 458-65.
230. Costelloe, C., et al., *IL-1F5 mediates anti-inflammatory activity in the brain through induction of IL-4 following interaction with SIGIRR/TIR8.* J Neurochem, 2008. **105**(5): p. 1960-9.
231. van de Veerdonk, F.L., et al., *Biology of IL-38 and its role in disease.* Immunol Rev, 2018. **281**(1): p. 191-196.
232. Morales Del Valle, C., et al., *Costimulation Induces CD4 T Cell Antitumor Immunity via an Innate-like Mechanism.* Cell Rep, 2019. **27**(5): p. 1434-1445.e3.
233. Li, D., et al., *IL-36β promotes anti-tumor effects in CD8(+) T cells by downregulating micro-RNA let-7c-5p.* Annals of translational medicine, 2021. **9**(23): p. 1734-1734.
234. Leon, G., et al., *IL-36 cytokines imprint a colitogenic phenotype on CD4+ T helper cells.* Mucosal Immunology, 2022. **15**(3): p. 491-503.
235. Zhao, X., et al., *IL-36β Promotes CD8(+) T Cell Activation and Antitumor Immune Responses by Activating mTORC1.* Front Immunol, 2019. **10**: p. 1803.
236. Dietrich, D., et al., *Interleukin-36 potently stimulates human M2 macrophages, Langerhans cells and keratinocytes to produce pro-inflammatory cytokines.* Cytokine, 2016. **84**: p. 88-98.
237. Watanabe, S., et al., *Neutrophil extracellular traps are induced in a psoriasis model of interleukin-36 receptor antagonist-deficient mice.* Scientific Reports, 2020. **10**(1): p. 20149.
238. Scheibe, K., et al., *IL-36R signalling activates intestinal epithelial cells and fibroblasts and promotes mucosal healing in vivo.* Gut, 2017. **66**(5): p. 823.
239. Weinstein, A.M., et al., *Association of IL-36γ with tertiary lymphoid structures and inflammatory immune infiltrates in human colorectal cancer.* Cancer Immunol Immunother, 2019. **68**(1): p. 109-120.
240. Li, Q., et al., *Spinal IL-36γ/IL-36R participates in the maintenance of chronic inflammatory pain through astroglial JNK pathway.* Glia, 2019. **67**(3): p. 438-451.
241. Hernández-Santana, Y.E., et al., *Keratinocyte interleukin-36 receptor expression orchestrates psoriasiform inflammation in mice.* Life science alliance, 2020. **3**(4): p. e201900586.
242. Aoyagi, T., et al., *Interleukin-36γ and IL-36 receptor signaling mediate impaired host immunity and lung injury in cytotoxic Pseudomonas aeruginosa pulmonary infection: Role of prostaglandin E2.* PLoS pathogens, 2017. **13**(11): p. e1006737-e1006737.
243. Le, N., et al., *IL-36G promotes cancer-cell intrinsic hallmarks in human gastric cancer cells.* Cytokine, 2022. **155**: p. 155887.
244. Jensen, L.E., *Interleukin-36 cytokines may overcome microbial immune evasion strategies that inhibit interleukin-1 family signaling.* Sci Signal, 2017. **10**(492).
245. Kumar, S., et al., *Identification and initial characterization of four novel members of the interleukin-1 family.* J Biol Chem, 2000. **275**(14): p. 10308-14.

246. Gresnigt, M.S., et al., *The IL-36 receptor pathway regulates Aspergillus fumigatus-induced Th1 and Th17 responses.* 2013. **43**(2): p. 416-426.
247. Rana, A.A., et al., *Poly(I:C) induces controlled release of IL-36γ from keratinocytes in the absence of cell death.* Immunologic research, 2015. **63**(1-3): p. 228-235.
248. Liu, H., et al., *Staphylococcus aureus Epicutaneous Exposure Drives Skin Inflammation via IL-36-Mediated T Cell Responses.* Cell Host & Microbe, 2017. **22**(5): p. 653-666.e5.
249. Wang, P., A.M. Gamero, and L.E. Jensen, *IL-36 promotes anti-viral immunity by boosting sensitivity to IFN-α/β in IRF1 dependent and independent manners.* Nature Communications, 2019. **10**(1): p. 4700.
250. Braegelmann, J., et al., *Candida induces the expression of IL-36γ in human keratinocytes: implications for a pathogen-driven exacerbation of psoriasis?* 2018. **32**(11): p. e403-e406.
251. Nakagawa, S., et al., *Staphylococcus aureus Virulent PSMα Peptides Induce Keratinocyte Alarmin Release to Orchestrate IL-17-Dependent Skin Inflammation.* Cell host & microbe, 2017. **22**(5): p. 667-677.e5.
252. Milora, K.A., et al., *Interleukin-36β provides protection against HSV-1 infection, but does not modulate initiation of adaptive immune responses.* Scientific Reports, 2017. **7**(1): p. 5799.
253. Macleod, T., et al., *The Proinflammatory Cytokine IL-36γ Is a Global Discriminator of Harmless Microbes and Invasive Pathogens within Epithelial Tissues.* Cell Rep, 2020. **33**(11): p. 108515.
254. Jiang, Z., et al., *IL-36γ Induced by the TLR3-SLUG-VDR Axis Promotes Wound Healing via REG3A.* J Invest Dermatol, 2017. **137**(12): p. 2620-2629.
255. Lai, Y., et al., *The Antimicrobial Protein REG3A Regulates Keratinocyte Proliferation and Differentiation after Skin Injury.* Immunity, 2012. **37**(1): p. 74-84.
256. Wang, W., et al., *IL-36γ inhibits differentiation and induces inflammation of keratinocyte via Wnt signaling pathway in psoriasis.* International journal of medical sciences, 2017. **14**(10): p. 1002-1007.
257. Saito, K., et al., *IL-36 receptor antagonist deficiency resulted in delayed wound healing due to excessive recruitment of immune cells.* Scientific Reports, 2020. **10**(1): p. 14772.
258. Goldstein, J., et al., *P093/O16 IL-36 signaling in keratinocytes is mandatory in imiquimod-induced psoriasis in mice.* 2019. **78**(Suppl 1): p. A40-A41.
259. Wein, A.N., et al., *IL-36γ Protects against Severe Influenza Infection by Promoting Lung Alveolar Macrophage Survival and Limiting Viral Replication.* J Immunol, 2018. **201**(2): p. 573-582.
260. Liu, S., et al., *High Expression of IL-36γ in Influenza Patients Regulates Interferon Signaling Pathway and Causes Programmed Cell Death During Influenza Virus Infection.* Frontiers in immunology, 2020. **11**: p. 552606-552606.
261. Eltobgy, M.M., et al., *Caspase-4/11 exacerbates disease severity in SARS–CoV-2 infection by promoting inflammation and immunothrombosis.* 2022. **119**(21): p. e2202012119.
262. Chandrashekar, D.S., et al., *Comparative transcriptome analyses reveal genes associated with SARS-CoV-2 infection of human lung epithelial cells.* Sci Rep, 2021. **11**(1): p. 16212.
263. Ahsan, F., et al., *IL-36/LXR axis modulates cholesterol metabolism and immune defense to Mycobacterium tuberculosis.* Scientific Reports, 2018. **8**(1): p. 1520.
264. Ahsan, F., et al., *Role of Interleukin 36γ in Host Defense Against Tuberculosis.* The Journal of Infectious Diseases, 2016. **214**(3): p. 464-474.
265. Gao, Y., et al., *IL-36γ Promotes Killing of Mycobacterium tuberculosis by Macrophages via WNT5A-Induced Noncanonical WNT Signaling.* 2019. **203**(4): p. 922-935.
266. Segueni, N., et al., *Limited Contribution of IL-36 versus IL-1 and TNF Pathways in Host Response to Mycobacterial Infection.* PLoS One, 2015. **10**(5): p. e0126058.

267. Kovach, M.A., et al., *IL-36γ is a crucial proximal component of protective type-1-mediated lung mucosal immunity in Gram-positive and -negative bacterial pneumonia.* Mucosal Immunology, 2017. **10**(5): p. 1320-1334.
268. Aoyagi, T., et al., *Interleukin-36γ and IL-36 receptor signaling mediate impaired host immunity and lung injury in cytotoxic Pseudomonas aeruginosa pulmonary infection: Role of prostaglandin E2.* PLOS Pathogens, 2017. **13**(11): p. e1006737.
269. Nanjo, Y., et al., *Overlapping Roles for Interleukin-36 Cytokines in Protective Host Defense against Murine Legionella pneumophila Pneumonia.* 2019. **87**(1): p. e00583-18.
270. Kovach, M.A., et al., *IL-36 Cytokines Promote Inflammation in the Lungs of Long-Term Smokers.* Am J Respir Cell Mol Biol, 2021. **64**(2): p. 173-182.
271. Sequeira, R.P., et al., *Commensal Bacteroidetes protect against Klebsiella pneumoniae colonization and transmission through IL-36 signalling.* Nature Microbiology, 2020. **5**(2): p. 304-313.
272. Medina-Contreras, O., et al., *Cutting Edge: IL-36 Receptor Promotes Resolution of Intestinal Damage.* J Immunol, 2016. **196**(1): p. 34-8.
273. Ngo, V.L., et al., *A cytokine network involving IL-36γ, IL-23, and IL-22 promotes antimicrobial defense and recovery from intestinal barrier damage.* 2018. **115**(22): p. E5076-E5085.
274. Giannoudaki, E., et al., *Interleukin-36 cytokines alter the intestinal microbiome and can protect against obesity and metabolic dysfunction. Nat Commun 10: 4003*. 2019.
275. Huynh, J., et al., *IRF6 Regulates the Expression of IL-36γ by Human Oral Epithelial Cells in Response to Porphyromonas gingivalis.* J Immunol, 2016. **196**(5): p. 2230-8.
276. Heath, J.E., et al., *IL-36γ regulates mediators of tissue homeostasis in epithelial cells.* Cytokine, 2019. **119**: p. 24-31.
277. Scholz, G.M., et al., *Regulation of the Peptidoglycan Amidase PGLYRP2 in Epithelial Cells by Interleukin-36&#x3b3.* 2018. **86**(9): p. e00384-18.
278. Scholz, G.M., et al., *MEK-ERK signaling diametrically controls the stimulation of IL-23p19 and EBI3 expression in epithelial cells by IL-36γ.* Immunol Cell Biol, 2018. **96**(6): p. 646-655.
279. Kim, S., et al., *IL-36 Induces Bisphosphonate-Related Osteonecrosis of the Jaw-Like Lesions in Mice by Inhibiting TGF-β-Mediated Collagen Expression.* 2017. **32**(2): p. 309-318.
280. Murrieta-Coxca, J.M., et al., *Role of IL-36 Cytokines in the Regulation of Angiogenesis Potential of Trophoblast Cells.* International Journal of Molecular Sciences, 2021. **22**(1).
281. Murrieta-Coxca, J.M., et al., *Estrous Cycle and Gestational Age-Dependent Expression of Members of the Interleukin-36 Subfamily in a Semi-Allogeneic Model of Infected and Non-Infected Murine Pregnancy.* 2016. **7**.
282. Murrieta-Coxca, J.M., et al., *IL-36 Cytokines: Regulators of Inflammatory Responses and Their Emerging Role in Immunology of Reproduction.* International journal of molecular sciences, 2019. **20**(7): p. 1649.
283. Li, J.-M., et al., *IL-36α/IL-36RA/IL-38 signaling mediates inflammation and barrier disruption in human corneal epithelial cells under hyperosmotic stress.* The Ocular Surface, 2021. **22**: p. 163-171.
284. Chustz, R.T., et al., *Regulation and function of the IL-1 family cytokine IL-1F9 in human bronchial epithelial cells.* American journal of respiratory cell and molecular biology, 2011. **45**(1): p. 145-153.
285. Magne, D., et al., *The new IL-1 family member IL-1F8 stimulates production of inflammatory mediators by synovial fibroblasts and articular chondrocytes.* 2006. **8**(3): p. 1-11.
286. Frey, S., et al., *The novel cytokine interleukin-36α is expressed in psoriatic and rheumatoid arthritis synovium.* Ann Rheum Dis, 2013. **72**(9): p. 1569-74.
287. Nishida, A., et al., *Increased Expression of Interleukin-36, a Member of the Interleukin-1 Cytokine Family, in Inflammatory Bowel Disease.* Inflamm Bowel Dis, 2016. **22**(2): p. 303-14.

288. Kanda, T., et al., *Interleukin(IL)-36α and IL-36γ Induce Proinflammatory Mediators from Human Colonic Subepithelial Myofibroblasts.* 2015. **2**.
289. Scheibe, K., et al., *Inhibiting Interleukin 36 Receptor Signaling Reduces Fibrosis in Mice With Chronic Intestinal Inflammation.* Gastroenterology, 2019. **156**(4): p. 1082-1097.e11.
290. Mutamba, S., et al., *Expression of IL-1Rrp2 by human myelomonocytic cells is unique to DCs and facilitates DC maturation by IL-1F8 and IL-1F9.* Eur J Immunol, 2012. **42**(3): p. 607-17.
291. Chi, H.H., et al., *IL-36 Signaling Facilitates Activation of the NLRP3 Inflammasome and IL-23/IL-17 Axis in Renal Inflammation and Fibrosis.* J Am Soc Nephrol, 2017. **28**(7): p. 2022-2037.
292. Vigne, S., et al., *IL-36R ligands are potent regulators of dendritic and T cells.* Blood, 2011. **118**(22): p. 5813-5823.
293. Catapano, M., et al., *IL-36 Promotes Systemic IFN-I Responses in Severe Forms of Psoriasis.* J Invest Dermatol, 2020. **140**(4): p. 816-826.e3.
294. Foster, A.M., et al., *IL-36 promotes myeloid cell infiltration, activation, and inflammatory activity in skin.* Journal of immunology (Baltimore, Md. : 1950), 2014. **192**(12): p. 6053-6061.
295. Penha, R., et al., *IL-36 receptor is expressed by human blood and intestinal T lymphocytes and is dose–dependently activated via IL-36β and induces CD4+ lymphocyte proliferation.* Cytokine, 2016. **85**: p. 18-25.
296. Vigne, S., et al., *IL-36 signaling amplifies Th1 responses by enhancing proliferation and Th1 polarization of naive CD4+ T cells.* Blood, 2012. **120**(17): p. 3478-87.
297. Russell, S.E., et al., *IL-36α expression is elevated in ulcerative colitis and promotes colonic inflammation.* Mucosal Immunol, 2016. **9**(5): p. 1193-204.
298. Harusato, A., et al., *IL-36γ signaling controls the induced regulatory T cell-Th9 cell balance via NFκB activation and STAT transcription factors.* Mucosal Immunol, 2017. **10**(6): p. 1455-1467.
299. Wang, X., et al., *IL-36γ Transforms the Tumor Microenvironment and Promotes Type 1 Lymphocyte-Mediated Antitumor Immune Responses.* Cancer Cell, 2015. **28**(3): p. 296-306.
300. Satoh, T.K., et al., *IL-36γ drives skin toxicity induced by EGFR/MEK inhibition and commensal Cutibacterium acnes.* J Clin Invest, 2020. **130**(3): p. 1417-1430.
301. D'erme, A.M., et al., *IL-36γ (IL-1F9) is a biomarker for psoriasis skin lesions.* 2015. **135**(4): p. 1025-1032.
302. Pfaff, C.M., et al., *The psoriasis-associated IL-17A induces and cooperates with IL-36 cytokines to control keratinocyte differentiation and function.* 2017. **7**(1): p. 1-13.
303. Wang, W., et al., *IL-36γ inhibits differentiation and induces inflammation of keratinocyte via Wnt signaling pathway in psoriasis.* 2017. **14**(10): p. 1002.
304. Mercurio, L., et al., *IL-38 has an anti-inflammatory action in psoriasis and its expression correlates with disease severity and therapeutic response to anti-IL-17A treatment. Cell Death Dis 9: 1104*. 2018.
305. Hernández-Santana, Y.E., et al., *Keratinocyte interleukin-36 receptor expression orchestrates psoriasiform inflammation in mice.* 2020. **3**(4): p. e201900586.
306. Farooq, M., et al., *Mutation analysis of the IL 36 RN gene in 14 Japanese patients with generalized pustular psoriasis.* 2013. **34**(1): p. 176-183.
307. Onoufriadis, A., et al., *Mutations in IL36RN/IL1F5 are associated with the severe episodic inflammatory skin disease known as generalized pustular psoriasis.* 2011. **89**(3): p. 432-437.
308. Sugiura, K., et al., *The Majority of Generalized Pustular Psoriasis without Psoriasis Vulgaris Is Caused by Deficiency of Interleukin-36 Receptor Antagonist.* Journal of Investigative Dermatology, 2013. **133**(11): p. 2514-2521.
309. O'Donoghue, A.J., et al., *Global Substrate Profiling of Proteases in Human Neutrophil Extracellular Traps Reveals Consensus Motif Predominantly Contributed by Elastase.* PLOS ONE, 2013. **8**(9): p. e75141.

310. Suárez-Fariñas, M., et al., *RNA sequencing atopic dermatitis transcriptome profiling provides insights into novel disease mechanisms with potential therapeutic implications.* 2015. **135**(5): p. 1218-1227.
311. Hessam, S., et al., *Interleukin-36 in hidradenitis suppurativa: evidence for a distinctive proinflammatory role and a key factor in the development of an inflammatory loop.* 2018. **178**(3): p. 761-767.
312. Mattii, M., et al., *The balance between pro-and anti-inflammatory cytokines is crucial in human allergic contact dermatitis pathogenesis: the role of IL-1 family members.* 2013. **22**(12): p. 813-819.
313. Sato, S., et al., *Upregulation of IL-36 cytokines in folliculitis and eosinophilic pustular folliculitis.* Australas J Dermatol, 2020. **61**(1): p. e39-e45.
314. Żebrowska, A., et al., *Correlation between IL-36α and IL-17 and activity of the disease in selected autoimmune blistering diseases.* 2017. **2017**.
315. Patrick, G.J., et al., *Epicutaneous Staphylococcus aureus induces IL-36 to enhance IgE production and ensuing allergic disease.* J Clin Invest, 2021. **131**(5).
316. Bozonnat, A., et al., *SARS-CoV-2 infection inducing severe flare up of Deficiency of Interleukin Thirty-six (IL-36) Receptor Antagonist (DITRA) resulting from a mutation invalidating the activating cleavage site of the IL-36 receptor antagonist.* Journal of Clinical Immunology, 2021. **41**(7): p. 1511-1514.
317. Bachelez, H., et al., *Trial of Spesolimab for Generalized Pustular Psoriasis.* 2021. **385**(26): p. 2431-2440.
318. Roberts, K.J., et al., *Preclinical development of a bispecific TNFα/IL-23 neutralising domain antibody as a novel oral treatment for inflammatory bowel disease.* Scientific Reports, 2021. **11**(1): p. 19422.
319. Li, W., et al., *Elevated sputum IL-36 levels are associated with neutrophil-related inflammation in COPD patients.* Clin Respir J, 2021. **15**(6): p. 648-656.
320. Baker, J.R., et al., *IL-36? – a key mediator of neutrophilic inflammation in chronic obstructive pulmonary disease.* 2021. **58**(suppl 65): p. PA678.
321. Koss, C.K., et al., *IL36 is a critical upstream amplifier of neutrophilic lung inflammation in mice.* Communications biology, 2021. **4**(1): p. 172-172.
322. Gao, X., et al., *Interleukin-38 ameliorates poly(I:C) induced lung inflammation: therapeutic implications in respiratory viral infections.* Cell Death Dis, 2021. **12**(1): p. 53.
323. Liu, X.-G., et al., *Interleukin-36 receptor antagonist alleviates airway inflammation in asthma via inhibiting the activation of Interleukin-36 pathway.* 2020. **81**: p. 106200.
324. Sun, X., et al., *Anti-inflammatory mechanisms of the novel cytokine interleukin-38 in allergic asthma.* Cellular & molecular immunology, 2020. **17**(6): p. 631-646.
325. Fonseca-Camarillo, G., et al., *Differential Expression of IL-36 Family Members and IL-38 by Immune and Nonimmune Cells in Patients with Active Inflammatory Bowel Disease.* BioMed Research International, 2018. **2018**: p. 5140691.
326. Xie, C., et al., *Interleukin-38 is elevated in inflammatory bowel diseases and suppresses intestinal inflammation.* Cytokine, 2020. **127**: p. 154963.
327. Zhang, Q., et al., *Serum IL-36 cytokines levels in idiopathic pulmonary fibrosis and connective tissue disease-associated interstitial lung diseases.* Clin Chim Acta, 2022. **530**: p. 8-12.
328. Chen, K., et al., *Antiinflammatory effects of bromodomain and extraterminal domain inhibition in cystic fibrosis lung inflammation.* 2016. **1**(11).
329. Weinstein, A.M., et al., *Association of IL-36γ with tertiary lymphoid structures and inflammatory immune infiltrates in human colorectal cancer.* Cancer immunology, immunotherapy : CII, 2019. **68**(1): p. 109-120.
330. Yang, S.-R., et al., *NSC828779 Alleviates Renal Tubulointerstitial Lesions Involving Interleukin-36 Signaling in Mice.* Cells, 2021. **10**(11): p. 3060.

331. Bhattacharyya, S., et al., *Fibrosis in systemic sclerosis: common and unique pathobiology.* Fibrogenesis & tissue repair, 2012. **5**(Suppl 1): p. S18-S18.
332. O'Reilly, S., *Interleukin-36α is elevated in diffuse systemic sclerosis and may potentiate fibrosis.* Cytokine, 2022. **156**: p. 155921.
333. Gong, Y., et al., *Elevated production of IL-36α in chronic hepatitis B virus-infected patients correlates with viral load.* Microbial Pathogenesis, 2017. **113**: p. 412-415.
334. Mele, D., et al., *Monocytes inhibit hepatitis C virus-induced TRAIL expression on CD56bright NK cells.* Journal of Hepatology, 2017. **67**(6): p. 1148-1156.
335. Wang, H.-J., et al., *Elevated serum interleukin-38 level at baseline predicts virological response in telbivudine-treated patients with chronic hepatitis B.* World journal of gastroenterology, 2016. **22**(18): p. 4529-4537.
336. Peng, X., et al., *Protective effect of interleukin-36 receptor antagonist on liver injury induced by concanavalin A in mice.* Iranian journal of basic medical sciences, 2020. **23**(5): p. 623-628.
337. Shang, Y., et al., *Modulation of interleukin-36 based inflammatory feedback loop through the hepatocyte-derived IL-36R-P2X7R axis improves steatosis in alcoholic steatohepatitis.* **n/a**(n/a).
338. Wang, X., et al., *The Protective Role of IL-36/IL-36R Signal in Con A–Induced Acute Hepatitis.* 2022: p. ji2100481.
339. Scheiermann, P., et al., *Application of IL-36 receptor antagonist weakens CCL20 expression and impairs recovery in the late phase of murine acetaminophen-induced liver injury.* Scientific Reports, 2015. **5**(1): p. 8521.
340. Zhao, Z., et al., *Altered serum levels of IL-36 cytokines (IL-36α, IL-36β, IL-36γ, and IL-36Ra) and their potential roles in Guillain-Barré syndrome.* Medicine, 2020. **99**(52): p. e23832-e23832.
341. Alsahebfosoul, F., et al., *Serum level of interleukin 36 in patients with multiple sclerosis.* J Immunoassay Immunochem, 2018. **39**(5): p. 558-564.
342. Zarrabi, M., et al., *Elevated IL-38 serum levels in newly diagnosed multiple sclerosis and systemic sclerosis patients.* 2021. **30**(2): p. 146-153.
343. Zhang, Q.-X., et al., *Increased serum IL-36γ levels are associated with disease severity in myasthenia gravis patients.* BMC Neurology, 2020. **20**(1): p. 307.
344. Tomuschat, C., et al., *Altered expression of IL36γ and IL36 receptor (IL1RL2) in the colon of patients with Hirschsprung's disease.* Pediatric Surgery International, 2017. **33**(2): p. 181-186.
345. Hayran, Y., et al., *Serum IL-36α, IL-36β, and IL-36γ levels in patients with hidradenitis suppurativa: association with disease characteristics, smoking, obesity, and metabolic syndrome.* Arch Dermatol Res, 2020. **312**(3): p. 187-196.
346. Pournaras, N., et al., *Glucose Homeostasis in Relation to Neutrophil Mobilization in Smokers with COPD.* International journal of chronic obstructive pulmonary disease, 2022. **17**: p. 1179-1194.
347. Frühbeck, G., et al., *Increased Levels of Interleukin-36 in Obesity and Type 2 Diabetes Fuel Adipose Tissue Inflammation by Inducing Its Own Expression and Release by Adipocytes and Macrophages.* 2022. **13**.
348. de Graaf, D.M., et al., *Reduced concentrations of the B cell cytokine interleukin 38 are associated with cardiovascular disease risk in overweight subjects.* 2021. **51**(3): p. 662-671.
349. Maculewicz, E., et al., *The interactions between interleukin-1 family genes: IL1A, IL1B, IL1RN, and obesity parameters.* BMC Genomics, 2022. **23**(1): p. 112.
350. Kavanagh, D., G. Neag, and N. Kalia, *416Investigating the role of the IL-36/IL1Rrp2 pathway in the inflammatory response of normal and aged cardiac microcirculation.* Cardiovascular Research, 2018. **114**(suppl_1): p. S100-S101.

351. Luo, C., et al., *Deficiency of Interleukin-36 Receptor Protected Cardiomyocytes from Ischemia-Reperfusion Injury in Cardiopulmonary Bypass.* Medical science monitor : international medical journal of experimental and clinical research, 2020. **26**: p. e918933-e918933.
352. El-Awaisi, J., et al., *Targeting IL-36 improves age-related coronary microcirculatory dysfunction and attenuates myocardial ischemia/reperfusion injury in mice.* JCI insight, 2022. **7**(5): p. e155236.
353. Wei, Y., et al., *Interleukin-38 alleviates cardiac remodelling after myocardial infarction.* 2020. **24**(1): p. 371-384.
354. Xue, Y., et al., *Neutralization of interleukin-38 exacerbates coxsackievirus B3-induced acute myocarditis in mice.* Virology Journal, 2021. **18**(1): p. 220.
355. Wang, X.-R., J.-P. Xiao, and D.-G. Wang, *Elevated levels of serum IL-36α in patients with systemic lupus erythematosus.* Biomedical reports, 2021. **15**(3): p. 76-76.
356. Mohamed, A.E., et al., *Association of interleukin-36α gene expression in Egyptian patients with systemic lupus erythematosus with organ involvement and disease activity.* Egypt J Immunol, 2021. **28**(2): p. 75-84.
357. Mai, S.-z., et al., *Increased serum IL-36α and IL-36γ levels in patients with systemic lupus erythematosus: association with disease activity and arthritis.* 2018. **58**: p. 103-108.
358. Chu, M., et al., *Elevated Expression and Pro-Inflammatory Activity of IL-36 in Patients with Systemic Lupus Erythematosus.* 2015. **20**(10): p. 19588-19604.
359. Yuan, Z.-C., et al., *Biology of IL-36 Signaling and Its Role in Systemic Inflammatory Diseases.* 2019. **10**.
360. Majumder, S., et al., *Elevated urinary IL-36γ in patients with active lupus nephritis and response to treatment.* Lupus, 2021. **30**(6): p. 921-925.
361. Boutet, M.A., et al., *Distinct expression of interleukin (IL)-36α, β and γ, their antagonist IL-36Ra and IL-38 in psoriasis, rheumatoid arthritis and Crohn's disease.* Clin Exp Immunol, 2016. **184**(2): p. 159-73.
362. Li, T., et al., *TGF-β type 2 receptor–mediated modulation of the IL-36 family can be therapeutically targeted in osteoarthritis.* 2019. **11**(491): p. eaan2585.
363. Boutet, M.A., et al., *Interleukin-36 family dysregulation drives joint inflammation and therapy response in psoriatic arthritis.* Rheumatology (Oxford), 2020. **59**(4): p. 828-838.
364. Schmitt, V., et al., *Interleukin-36 receptor mediates the crosstalk between plasma cells and synovial fibroblasts.* 2017. **47**(12): p. 2101-2112.
365. Dietrich, D. and C.J.N.R.R. Gabay, *IL-36 has proinflammatory effects in skin but not in joints.* 2014. **10**(11): p. 639-640.
366. Wang, M., et al., *Detection of the novel IL-1 family cytokines by QAH-IL1F-1 assay in rheumatoid arthritis.* 2016. **62**(4): p. 31-34.
367. Pan, Q.-Z., et al., *Decreased expression of interleukin-36α correlates with poor prognosis in hepatocellular carcinoma.* Cancer Immunology, Immunotherapy, 2013. **62**(11): p. 1675-1685.
368. Hu, M., et al., *IL36 indicating good prognosis in human Hepatocellular Carcinoma.* Journal of Cancer, 2020. **11**(21): p. 6248-6255.
369. Chen, F., et al., *IL-36 s in the colorectal cancer: is interleukin 36 good or bad for the development of colorectal cancer?* BMC Cancer, 2020. **20**(1): p. 92.
370. Wang, Z.-S., et al., *Decreased expression of interleukin-36α predicts poor prognosis in colorectal cancer patients.* International journal of clinical and experimental pathology, 2014. **7**(11): p. 8077-8081.
371. Chang, L., R. Guo, and Z. Yuan, *IL-36α suppresses proliferation of ovarian cancer cells.* Tumor Biology, 2017. **39**(6): p. 1010428317706918.
372. Lv, Z., et al., *Integrative genomic analysis of interleukin-36RN and its prognostic value in cancer.* Mol Med Rep, 2016. **13**(2): p. 1404-1412.

373. Takada, K., et al., *Clinical implications of the novel cytokine IL-38 expressed in lung adenocarcinoma: Possible association with PD-L1 expression.* PLoS One, 2017. **12**(7): p. e0181598.
374. Kinoshita, F., et al., *Interleukin-38 promotes tumor growth through regulation of CD8+ tumor-infiltrating lymphocytes in lung cancer tumor microenvironment.* Cancer Immunology, Immunotherapy, 2021. **70**(1): p. 123-135.
375. Solahaye-Kahnamouii, S., et al., *The effect of interleukin 36 gene therapy in the regression of tumor.* Iranian journal of cancer prevention, 2014. **7**(4): p. 197-203.
376. Li, D., et al., *IL-36β promotes anti-tumor effects in CD8 + T cells by downregulating micro-RNA let-7c-5p.* 2021, 2021. **9**(23): p. 1734.
377. Tsurutani, N., et al., *Costimulation Endows Immunotherapeutic CD8 T Cells with IL-36 Responsiveness during Aerobic Glycolysis.* Journal of immunology (Baltimore, Md. : 1950), 2016. **196**(1): p. 124-134.
378. Chen, Y., et al., *Targeted codelivery of doxorubicin and IL-36γ expression plasmid for an optimal chemo-gene combination therapy against cancer lung metastasis.* Nanomedicine, 2019. **15**(1): p. 129-141.
379. Qu, Q., et al., *IL36 Cooperates With Anti-CTLA-4 mAbs to Facilitate Antitumor Immune Responses.* 2020. **11**(634).
380. Weinstein, A.M., et al., *Tbet and IL-36γ cooperate in therapeutic DC-mediated promotion of ectopic lymphoid organogenesis in the tumor microenvironment.* Oncoimmunology, 2017. **6**(6): p. e1322238.
381. Chelvanambi, M., et al., *STING agonist-based treatment promotes vascular normalization and tertiary lymphoid structure formation in the therapeutic melanoma microenvironment.* Journal for immunotherapy of cancer, 2021. **9**(2): p. e001906.
382. Xie, X., et al., *Interleukin-36α suppresses growth of non-small cell lung cancer in vitro by reducing angiogenesis.* FEBS Open Bio, 2021. **11**(5): p. 1353-1363.
383. Lv, Q., et al., *The Potential Role of IL1RAP on Tumor Microenvironment-Related Inflammatory Factors in Stomach Adenocarcinoma.* Technol Cancer Res Treat, 2021. **20**: p. 1533033821995282.
384. Chen, F., et al., *Interleukin-38 in colorectal cancer: a potential role in precision medicine.* Cancer Immunology, Immunotherapy, 2020. **69**(1): p. 69-79.
385. Yang, W., et al., *IL-36γ and IL-36Ra Reciprocally Regulate Colon Inflammation and Tumorigenesis by Modulating the Cell-Matrix Adhesion Network and Wnt Signaling.* Adv Sci (Weinh), 2022. **9**(10): p. e2103035.
386. Wang, P., et al., *IL-36γ and IL-36Ra Reciprocally Regulate NSCLC Progression by Modulating GSH Homeostasis and Oxidative Stress-Induced Cell Death.* Adv Sci (Weinh), 2021. **8**(19): p. e2101501.
387. Huang, L., et al., *Interleukin-38 Suppresses Cell Migration and Proliferation and Promotes Apoptosis of Colorectal Cancer Cell Through Negatively Regulating Extracellular Signal-Regulated Kinases Signaling.* 2021. **41**(10): p. 375-384.
388. O'Donnell, C., et al., *An antitumorigenic role for the IL-33 receptor, ST2L, in colon cancer.* British Journal of Cancer, 2016. **114**(1): p. 37-43.
389. Li, T., et al., *TIMER2.0 for analysis of tumor-infiltrating immune cells.* Nucleic Acids Res, 2020. **48**(W1): p. W509-w514.
390. Becht, E., et al., *Estimating the population abundance of tissue-infiltrating immune and stromal cell populations using gene expression.* Genome Biology, 2016. **17**(1): p. 218.
391. Barrett, T., et al., *NCBI GEO: archive for functional genomics data sets—update.* Nucleic Acids Research, 2013. **41**(D1): p. D991-D995.
392. Szklarczyk, D., et al., *The STRING database in 2021: customizable protein-protein networks, and functional characterization of user-uploaded gene/measurement sets.* Nucleic Acids Res, 2021. **49**(D1): p. D605-d612.

393. Shannon, P., et al., *Cytoscape: a software environment for integrated models of biomolecular interaction networks.* Genome Res, 2003. **13**(11): p. 2498-504.
394. Bader, G.D. and C.W.V. Hogue, *An automated method for finding molecular complexes in large protein interaction networks.* BMC Bioinformatics, 2003. **4**(1): p. 2.
395. Miller, H.E. and A.J.R. Bishop, *Correlation AnalyzeR: functional predictions from gene co-expression correlations.* BMC Bioinformatics, 2021. **22**(1): p. 206.
396. Leon, G., S. Hussey, and P.T. Walsh, *The Diverse Roles of the IL-36 Family in Gastrointestinal Inflammation and Resolution.* Inflamm Bowel Dis, 2021. **27**(3): p. 440-450.
397. Long, A.G., E.T. Lundsmith, and K.E. Hamilton, *Inflammation and Colorectal Cancer.* Curr Colorectal Cancer Rep, 2017. **13**(4): p. 341-351.
398. Van Der Kraak, L., P. Gros, and N. Beauchemin, *Colitis-associated colon cancer: Is it in your genes?* World journal of gastroenterology, 2015. **21**(41): p. 11688-11699.
399. Yashiro, M., *Ulcerative colitis-associated colorectal cancer.* World J Gastroenterol, 2014. **20**(44): p. 16389-97.
400. Gelfo, V., et al., *Roles of IL-1 in Cancer: From Tumor Progression to Resistance to Targeted Therapies.* International journal of molecular sciences, 2020. **21**(17): p. 6009.
401. Bassoy, E.Y., J.E. Towne, and C. Gabay, *Regulation and function of interleukin-36 cytokines.* Immunol Rev, 2018. **281**(1): p. 169-178.
402. Gabay, C. and J.E. Towne, *Regulation and function of interleukin-36 cytokines in homeostasis and pathological conditions.* J Leukoc Biol, 2015. **97**(4): p. 645-52.
403. Queen, D., C. Ediriweera, and L. Liu, *Function and Regulation of IL-36 Signaling in Inflammatory Diseases and Cancer Development.* 2019. **7**(317).
404. Uppala, R., et al., *"Autoinflammatory psoriasis"-genetics and biology of pustular psoriasis.* Cell Mol Immunol, 2021. **18**(2): p. 307-317.
405. McCombs, J.E. and J.K. Kolls, *Walking down the "IL": The Newfound Marriage between IL-36 and Chronic Obstructive Pulmonary Disease.* Am J Respir Cell Mol Biol, 2021. **64**(2): p. 153-154.
406. Byrne, J., et al., *IL-36 cytokines in inflammatory and malignant diseases: not the new kid on the block anymore.* Cell Mol Life Sci, 2021. **78**(17-18): p. 6215-6227.
407. Zhou, X., et al., *Advances in the pathogenesis of psoriasis: from keratinocyte perspective.* Cell Death & Disease, 2022. **13**(1): p. 81.
408. Richard, S. and V. Martinez Marignac, *Sensitization to oxaliplatin in HCT116 and HT29 cell lines by metformin and ribavirin and differences in response to mitochondrial glutaminase inhibition.* 2015. **11**(2): p. 336-340.
409. Hewitt, R.E., et al., *Validation of a model of colon cancer progression.* J Pathol, 2000. **192**(4): p. 446-54.
410. Borra, R.C., et al., *A simple method to measure cell viability in proliferation and cytotoxicity assays.* Braz Oral Res, 2009. **23**(3): p. 255-62.
411. Fares, J., et al., *Molecular principles of metastasis: a hallmark of cancer revisited.* Signal Transduction and Targeted Therapy, 2020. **5**(1): p. 28.
412. Yuan, Z.-C., et al., *Biology of IL-36 Signaling and Its Role in Systemic Inflammatory Diseases.* Frontiers in immunology, 2019. **10**: p. 2532-2532.
413. Chelvanambi, M., A.M. Weinstein, and W.J. Storkus, *IL-36 Signaling in the Tumor Microenvironment.* Adv Exp Med Biol, 2020. **1240**: p. 95-110.
414. Weinstein, A.M., et al., *Association of IL-36gamma with tertiary lymphoid structures and inflammatory immune infiltrates in human colorectal cancer.* Cancer Immunol Immunother, 2019. **68**(1): p. 109-120.
415. *Comprehensive molecular portraits of human breast tumours.* Nature, 2012. **490**(7418): p. 61-70.
416. Edgar, R., M. Domrachev, and A.E. Lash, *Gene Expression Omnibus: NCBI gene expression and hybridization array data repository.* Nucleic Acids Res, 2002. **30**(1): p. 207-10.

417. Joo, Y.H., et al., *Increased expression of interleukin 36 in chronic rhinosinusitis and its contribution to chemokine secretion and increased epithelial permeability.* Cytokine, 2020. **125**: p. 154798.
418. Roelands, J., et al., *Immunogenomic Classification of Colorectal Cancer and Therapeutic Implications.* International journal of molecular sciences, 2017. **18**(10): p. 2229.
419. Mao, D., et al., *Long Noncoding RNA GM16343 Promotes IL-36β to Regulate Tumor Microenvironment by CD8(+)T cells.* Technol Cancer Res Treat, 2019. **18**: p. 1533033819883633.
420. Wang, D., et al., *CXCL1 Is Critical for Premetastatic Niche Formation and Metastasis in Colorectal Cancer.* Cancer Res, 2017. **77**(13): p. 3655-3665.
421. le Rolle, A.-F., et al., *The prognostic significance of CXCL1 hypersecretion by human colorectal cancer epithelia and myofibroblasts.* Journal of translational medicine, 2015. **13**: p. 199-199.
422. Lee, Y.S., et al., *Interleukin-8 and its receptor CXCR2 in the tumour microenvironment promote colon cancer growth, progression and metastasis.* British Journal of Cancer, 2012. **106**(11): p. 1833-1841.
423. Madonna, S., et al., *The Significance of IL-36 Hyperactivation and IL-36R Targeting in Psoriasis.* International journal of molecular sciences, 2019. **20**(13): p. 3318.
424. Sullivan, G.P., et al., *Suppressing IL-36-driven inflammation using peptide pseudosubstrates for neutrophil proteases.* Cell death & disease, 2018. **9**(3): p. 378-378.
425. Itatani, Y., et al., *The Role of Chemokines in Promoting Colorectal Cancer Invasion/Metastasis.* International journal of molecular sciences, 2016. **17**(5): p. 643.
426. Sawayama, H., et al., *Investigation of colorectal cancer in accordance with consensus molecular subtype classification.* Annals of Gastroenterological Surgery, 2020. **4**(5): p. 528-539.
427. Fessler, E., et al., *TGFβ signaling directs serrated adenomas to the mesenchymal colorectal cancer subtype.* EMBO molecular medicine, 2016. **8**(7): p. 745-760.
428. Kartikasari, A.E.R., et al., *Tumor-Induced Inflammatory Cytokines and the Emerging Diagnostic Devices for Cancer Detection and Prognosis.* 2021. **11**.
429. Yao, Q.-M., et al., *Elevated Interleukin-36α And CD4(+)IL-36α(+)T Cells Are Involved in the Pathogenesis of Graves' Disease.* Frontiers in endocrinology, 2018. **9**: p. 591-591.
430. Khaliq, A.M., et al., *Redefining colorectal cancer classification and clinical stratification through a single-cell atlas.* bioRxiv, 2021: p. 2021.02.02.429256.
431. Huang, L., et al., *Interleukin-38 Suppresses Cell Migration and Proliferation and Promotes Apoptosis of Colorectal Cancer Cell Through Negatively Regulating Extracellular Signal-Regulated Kinases Signaling.* J Interferon Cytokine Res, 2021. **41**(10): p. 375-384.
432. Iznardo, H. and L. Puig, *Exploring the Role of IL-36 Cytokines as a New Target in Psoriatic Disease.* Int J Mol Sci, 2021. **22**(9).
433. Virk, G.S., et al., *Staging and survival of colorectal cancer (CRC) in octogenarians: Nationwide Study of US Veterans.* Journal of gastrointestinal oncology, 2019. **10**(1): p. 12-18.
434. Poltavets, V., et al., *The Role of the Extracellular Matrix and Its Molecular and Cellular Regulators in Cancer Cell Plasticity.* Frontiers in oncology, 2018. **8**: p. 431-431.
435. Coxca, J.M., et al., *Role of IL-36 Cytokines in the Regulation of Angiogenesis Potential of Trophoblast Cells.* International Journal of Molecular Sciences, 2020. **22**: p. 285.
436. Yang, W., et al., *IL-36γ and IL-36Ra Reciprocally Regulate Colon Inflammation and Tumorigenesis by Modulating the Cell–Matrix Adhesion Network and Wnt Signaling.* **n/a**(n/a): p. 2103035.
437. Yang, W., et al., *IL-36γ and IL-36Ra Reciprocally Regulate Colon Inflammation and Tumorigenesis by Modulating the Cell–Matrix Adhesion Network and Wnt Signaling.* Advanced Science, 2022. **n/a**(n/a): p. 2103035.

438. Yoshimi, K., et al., *Combi-CRISPR: combination of NHEJ and HDR provides efficient and precise plasmid-based knock-ins in mice and rats.* Hum Genet, 2021. **140**(2): p. 277-287.
439. Castle, J.C., et al., *Immunomic, genomic and transcriptomic characterization of CT26 colorectal carcinoma.* BMC Genomics, 2014. **15**(1): p. 190.
440. Xia, B., et al., *CRISPR-based engineering of gene knockout cells by homology-directed insertion in polyploid Drosophila S2R+ cells.* Nat Protoc, 2020. **15**(10): p. 3478-3498.
441. Brinkman, E.K., et al., *Easy quantification of template-directed CRISPR/Cas9 editing.* 2017: p. 218156.
442. Conant, D., et al., *Inference of CRISPR Edits from Sanger Trace Data.* Crispr j, 2022. **5**(1): p. 123-130.
443. Wigmore, P.M., et al., *Effects of 5-FU.* Adv Exp Med Biol, 2010. **678**: p. 157-64.
444. Meriggi, F., et al., *Anti-EGFR therapy in colorectal cancer: how to choose the right patient.* Curr Drug Targets, 2009. **10**(10): p. 1033-40.
445. Yang, L., Q. Ning, and S.-s. Tang, *Recent Advances and Next Breakthrough in Immunotherapy for Cancer Treatment.* Journal of Immunology Research, 2022. **2022**: p. 8052212.
446. Yu, J.W., et al., *Tumor-immune profiling of murine syngeneic tumor models as a framework to guide mechanistic studies and predict therapy response in distinct tumor microenvironments.* PloS one, 2018. **13**(11): p. e0206223-e0206223.
447. Xue, D., et al., *Next-generation cytokines for cancer immunotherapy.* Antibody Therapeutics, 2021. **4**(2): p. 123-133.
448. Liu, J., et al., *New insights into M1/M2 macrophages: key modulators in cancer progression.* Cancer Cell International, 2021. **21**(1): p. 389.
449. Tao, X., et al., *Interleukin 36α Attenuates Sepsis by Enhancing Antibacterial Functions of Macrophages.* J Infect Dis, 2017. **215**(2): p. 321-332.
450. Sommerfeld, S.D., et al., *Interleukin-36γ-producing macrophages drive IL-17-mediated fibrosis.* Sci Immunol, 2019. **4**(40).
451. Swindell, W.R., et al., *RNA-Seq Analysis of IL-1B and IL-36 Responses in Epidermal Keratinocytes Identifies a Shared MyD88-Dependent Gene Signature.* 2018. **9**.
452. Wang, H., et al., *The activation and function of IL-36γ in neutrophilic inflammation in chronic rhinosinusitis.* J Allergy Clin Immunol, 2018. **141**(5): p. 1646-1658.
453. Gardner, J.K. and M.M. Herbst-Kralovetz, *IL-36γ induces a transient HSV-2 resistant environment that protects against genital disease and pathogenesis.* Cytokine, 2018. **111**: p. 63-71.
454. Shao, S., et al., *Neutrophil Extracellular Traps Promote Inflammatory Responses in Psoriasis via Activating Epidermal TLR4/IL-36R Crosstalk.* Front Immunol, 2019. **10**: p. 746.
455. Cristinziano, L., et al., *Anaplastic Thyroid Cancer Cells Induce the Release of Mitochondrial Extracellular DNA Traps by Viable Neutrophils.* J Immunol, 2020. **204**(5): p. 1362-1372.
456. Yang, L., et al., *DNA of neutrophil extracellular traps promotes cancer metastasis via CCDC25.* Nature, 2020. **583**(7814): p. 133-138.
457. Orecchioni, M., et al., *Macrophage Polarization: Different Gene Signatures in M1(LPS+) vs. Classically and M2(LPS–) vs. Alternatively Activated Macrophages.* 2019. **10**.
458. Ngo Vu, L., et al., *IL-36R signaling integrates innate and adaptive immune-mediated protection against enteropathogenic bacteria.* Proceedings of the National Academy of Sciences, 2020. **117**(44): p. 27540-27548.
459. Zaborowski, A.M., D.C. Winter, and L. Lynch, *The therapeutic and prognostic implications of immunobiology in colorectal cancer: a review.* British Journal of Cancer, 2021. **125**(10): p. 1341-1349.
460. Hiam-Galvez, K.J., B.M. Allen, and M.H. Spitzer, *Systemic immunity in cancer.* Nature Reviews Cancer, 2021. **21**(6): p. 345-359.

461. Deng, J. and J.B. Fleming, *Inflammation and Myeloid Cells in Cancer Progression and Metastasis.* 2022. **9**.
462. Abd-Elrahman, I., et al., *Characterizing Cathepsin Activity and Macrophage Subtypes in Excised Human Carotid Plaques.* 2016. **47**(4): p. 1101-1108.
463. Carrier, Y., et al., *Inter-Regulation of Th17 Cytokines and the IL-36 Cytokines In Vitro and In Vivo: Implications in Psoriasis Pathogenesis.* Journal of Investigative Dermatology, 2011. **131**(12): p. 2428-2437.
464. Tedesco, S., et al., *Convenience versus Biological Significance: Are PMA-Differentiated THP-1 Cells a Reliable Substitute for Blood-Derived Macrophages When Studying in Vitro Polarization?* 2018. **9**.
465. Gordon, S., A. Plüddemann, and F. Martinez Estrada, *Macrophage heterogeneity in tissues: phenotypic diversity and functions.* Immunological Reviews, 2014. **262**(1): p. 36-55.
466. Lecoultre, M., V. Dutoit, and P.R. Walker, *Phagocytic function of tumor-associated macrophages as a key determinant of tumor progression control: a review.* Journal for ImmunoTherapy of Cancer, 2020. **8**(2): p. e001408.
467. Jiang, H., et al., *Tumor target-derived soluble factor synergizes with IFN-gamma and IL-2 to activate macrophages for tumor necrosis factor and nitric oxide production to mediate cytotoxicity of the same target.* J Immunol, 1992. **149**(6): p. 2137-46.
468. Ley, K., *M1 Means Kill; M2 Means Heal.* The Journal of Immunology, 2017. **199**(7): p. 2191.
469. Genin, M., et al., *M1 and M2 macrophages derived from THP-1 cells differentially modulate the response of cancer cells to etoposide.* BMC Cancer, 2015. **15**(1): p. 577.
470. Wu, T.-H., et al., *Culture supernatants of different colon cancer cell lines induce specific phenotype switching and functional alteration of THP-1 cells.* Cellular Immunology, 2014. **290**(1): p. 107-115.
471. Canli, Ö., et al., *Myeloid Cell-Derived Reactive Oxygen Species Induce Epithelial Mutagenesis.* Cancer Cell, 2017. **32**(6): p. 869-883.e5.
472. Houghton, A.M., et al., *Neutrophil elastase-mediated degradation of IRS-1 accelerates lung tumor growth.* Nat Med, 2010. **16**(2): p. 219-23.
473. He, G., et al., *Peritumoural neutrophils negatively regulate adaptive immunity via the PD-L1/PD-1 signalling pathway in hepatocellular carcinoma.* J Exp Clin Cancer Res, 2015. **34**: p. 141.
474. Katoh, H., et al., *CXCR2-expressing myeloid-derived suppressor cells are essential to promote colitis-associated tumorigenesis.* Cancer Cell, 2013. **24**(5): p. 631-44.
475. Baker, K., et al., *IL-36 signalling enhances a pro-tumorigenic phenotype in colon cancer cells with cancer cell growth restricted by administration of the IL-36R antagonist.* Oncogene, 2022.
476. Pączek, S., M. Łukaszewicz-Zając, and B. Mroczko, *Chemokines-What Is Their Role in Colorectal Cancer?* Cancer control : journal of the Moffitt Cancer Center, 2020. **27**(1): p. 1073274820903384-1073274820903384.
477. Teijeira, A., et al., *Differential Interleukin-8 thresholds for chemotaxis and netosis in human neutrophils.* European Journal of Immunology, 2021. **51**(9): p. 2274-2280.
478. Elias, M., et al., *IL-36 in chronic inflammation and fibrosis — bridging the gap?* The Journal of Clinical Investigation, 2021. **131**(2).
479. Keshari, R.S., et al., *Cytokines Induced Neutrophil Extracellular Traps Formation: Implication for the Inflammatory Disease Condition.* PLOS ONE, 2012. **7**(10): p. e48111.
480. Urban, C.F., et al., *Neutrophil Extracellular Traps Contain Calprotectin, a Cytosolic Protein Complex Involved in Host Defense against Candida albicans.* PLOS Pathogens, 2009. **5**(10): p. e1000639.
481. Liu, L., et al., *Association between interleukin-36γ and tumor progression in non-small cell lung cancer.* Oncol Lett, 2020. **19**(3): p. 2457-2465.

482. Müller, M.F., A.E. Ibrahim, and M.J. Arends, *Molecular pathological classification of colorectal cancer.* Virchows Arch, 2016. **469**(2): p. 125-34.
483. Rohr, M., et al., *A merged microarray meta-dataset for transcriptionally profiling colorectal neoplasm formation and progression.* Scientific data, 2021. **8**(1): p. 214-214.
484. Ahmed, M., *Colon Cancer: A Clinician's Perspective in 2019.* Gastroenterology research, 2020. **13**(1): p. 1-10.
485. Michel, M., et al., *The Role of p53 Dysfunction in Colorectal Cancer and Its Implication for Therapy.* Cancers, 2021. **13**(10): p. 2296.
486. Li, J., et al., *Temporal DNA methylation pattern and targeted therapy in colitis-associated cancer.* Carcinogenesis, 2020. **41**(2): p. 235-244.
487. Lizardo, D.Y., et al., *Immunotherapy efficacy on mismatch repair-deficient colorectal cancer: From bench to bedside.* Biochimica et biophysica acta. Reviews on cancer, 2020. **1874**(2): p. 188447-188447.
488. Leach, D.R., M.F. Krummel, and J.P. Allison, *Enhancement of antitumor immunity by CTLA-4 blockade.* Science, 1996. **271**(5256): p. 1734-6.
489. Pfaff, C.M., et al., *The psoriasis-associated IL-17A induces and cooperates with IL-36 cytokines to control keratinocyte differentiation and function.* Scientific Reports, 2017. **7**(1): p. 15631.
490. Goldstein, J.D., et al., *IL-36 signaling in keratinocytes controls early IL-23 production in psoriasis-like dermatitis.* Life Sci Alliance, 2020. **3**(6).
491. Mercurio, L., et al., *Interleukin (IL)-17/IL-36 axis participates to the crosstalk between endothelial cells and keratinocytes during inflammatory skin responses.* PLoS One, 2020. **15**(4): p. e0222969.
492. Hyun, Y.S., et al., *Role of IL-17A in the development of colitis-associated cancer.* Carcinogenesis, 2012. **33**(4): p. 931-936.
493. Perez, L.G., et al., *TGF-β signaling in Th17 cells promotes IL-22 production and colitis-associated colon cancer.* Nature Communications, 2020. **11**(1): p. 2608.
494. Chaudhary, N., et al., *Lipocalin 2 expression promotes tumor progression and therapy resistance by inhibiting ferroptosis in colorectal cancer.* Int J Cancer, 2021. **149**(7): p. 1495-1511.
495. Fernández, C.A., et al., *The matrix metalloproteinase-9/neutrophil gelatinase-associated lipocalin complex plays a role in breast tumor growth and is present in the urine of breast cancer patients.* Clin Cancer Res, 2005. **11**(15): p. 5390-5.
496. Nuntagowat, C., K. Leelawat, and R. Tohtong, *NGAL knockdown by siRNA in human cholangiocarcinoma cells suppressed invasion by reducing NGAL/MMP-9 complex formation.* Clin Exp Metastasis, 2010. **27**(5): p. 295-305.
497. Yang, J., et al., *Lipocalin 2 promotes breast cancer progression.* Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(10): p. 3913-3918.
498. Yang, J., et al., *Lipocalin 2 is a novel regulator of angiogenesis in human breast cancer.* Faseb j, 2013. **27**(1): p. 45-50.
499. Voronov, E. and R.N. Apte, *IL-1 in Colon Inflammation, Colon Carcinogenesis and Invasiveness of Colon Cancer.* Cancer Microenviron, 2015. **8**(3): p. 187-200.
500. Coussens, L.M. and Z. Werb, *Inflammation and cancer.* Nature, 2002. **420**(6917): p. 860-7.
501. Hans, D., et al., *Rapidly fatal infections.* Emergency medicine clinics of North America, 2008. **26**(2): p. 259-vii.
502. Wang, S., et al., *Pathogenic Triad in Bacterial Meningitis: Pathogen Invasion, NF-κB Activation, and Leukocyte Transmigration that Occur at the Blood-Brain Barrier.* 2016. **7**.
503. Gregory, T.R., *Understanding Natural Selection: Essential Concepts and Common Misconceptions.* Evolution: Education and Outreach, 2009. **2**(2): p. 156-175.
504. Dinarello, C.A., *Overview of the IL-1 family in innate inflammation and acquired immunity.* Immunological reviews, 2018. **281**(1): p. 8-27.

505. Waris, G. and H. Ahsan, *Reactive oxygen species: role in the development of cancer and various chronic conditions.* Journal of carcinogenesis, 2006. **5**: p. 14-14.
506. Liao, J.B., *Viruses and human cancer.* The Yale journal of biology and medicine, 2006. **79**(3-4): p. 115-122.
507. Caspers, M., et al., *How do external factors contribute to the hypocoagulative state in trauma-induced coagulopathy? – In vitro analysis of the lethal triad in trauma.* Scandinavian Journal of Trauma, Resuscitation and Emergency Medicine, 2018. **26**(1): p. 66.
508. Nishikawa, H., et al., *Knockout of the interleukin-36 receptor protects against renal ischemia-reperfusion injury by reduction of proinflammatory cytokines.* Kidney Int, 2018. **93**(3): p. 599-614.
509. Lewandowska, A.M., et al., *Cancer prevention - review paper.* Ann Agric Environ Med, 2021. **28**(1): p. 11-19.
510. Dominguez-Valentin, M., et al., *Cancer risks by gene, age, and gender in 6350 carriers of pathogenic mismatch repair variants: findings from the Prospective Lynch Syndrome Database.* Genet Med, 2020. **22**(1): p. 15-25.
511. Bussey, H.J., A.M. Veale, and B.C. Morson, *Genetics of gastrointestinal polyposis.* Gastroenterology, 1978. **74**(6): p. 1325-30.
512. Tovey, M.G. and C. Lallemand, *Immunogenicity and other problems associated with the use of biopharmaceuticals.* Therapeutic advances in drug safety, 2011. **2**(3): p. 113-128.
513. Coley, W.B.J.T.A.J.o.t.M.S., *The treatment of malignant tumors by repeated inoculations of erysipelas: With a report of ten original cases. 1.* 1893. **105**(6): p. 487.
514. Wrangle, J.M., et al., *IL-2 and Beyond in Cancer Immunotherapy.* J Interferon Cytokine Res, 2018. **38**(2): p. 45-68.
515. Wrangle, J.M., et al., *IL-2 and Beyond in Cancer Immunotherapy.* Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research, 2018. **38**(2): p. 45-68.
516. Holder, P.G., et al., *Engineering interferons and interleukins for cancer immunotherapy.* Advanced Drug Delivery Reviews, 2022. **182**: p. 114112.
517. Zhang, B., et al., *Site-specific PEGylation of interleukin-2 enhances immunosuppression via the sustained activation of regulatory T cells.* Nature Biomedical Engineering, 2021. **5**(11): p. 1288-1305.
518. Tzeng, A., et al., *Antigen specificity can be irrelevant to immunocytokine efficacy and biodistribution.* 2015. **112**(11): p. 3320-3325.
519. Hsu, E.J., et al., *A cytokine receptor-masked IL2 prodrug selectively activates tumor-infiltrating lymphocytes for potent antitumor therapy.* Nature Communications, 2021. **12**(1): p. 2768.
520. Cao, G.-d., et al., *The Oncolytic Virus in Cancer Diagnosis and Treatment.* 2020. **10**.

Appendix

Appendix Table 1 – Table of antibodies/ELISA kits/viability markers

Primary Antibodies					
Name	**CAT. NO**	**SPECIES REACTIVITY**	**DILUTION USED**	**COMPANY**	**Application**
Alexa Fluor® 488 anti-mouse FOXP3	320012	Human, Mouse, Rat	1 in 100	Biolegend, U.S.A.	FACS
Alexa Fluor® 700 anti-mouse CD45	103128	Mouse	1 in 50	Biolegend, U.S.A.	FACS
anti-mouse CD4 APC/CD25 PE Cocktail	320029	Mouse	1 in 100	Biolegend, U.S.A.	FACS
APC anti-mouse CD3	100236	Mouse	1 in 100	Biolegend, U.S.A.	FACS
APC/Cyanine7 anti-mouse CD8a	100714	Mouse	1 in 200	Biolegend, U.S.A.	FACS
BD Pharmingen™ PE Rat Anti-CD11b	557397	Human	1 in 200	BD Biosciences, U.S.A.	FACS
Brilliant Violet 421TM anti-mouse F4/80	123132	Mouse	1 in 50	Biolegend, U.S.A.	FACS
Brilliant Violet 605TM anti-mouse CD4	100548	Mouse	1 in 100	Biolegend, U.S.A.	FACS
FITC Annexin V	640906	Human, mouse , rat, others	1 in 50	Biolegend, U.S.A.	FACS
PE anti-mouse Ly-6G	127608	Mouse	1 in 50	Biolegend, U.S.A.	FACS
Purified anti-mouse CD16/32	101302	Mouse	1 in 100	Biolegend, U.S.A.	FACS
Anti-human IL-36α	ab180909	Human	1 in 2000	Abcam, U.S.A.	IHC
Anti-human IL-36β	ab180890	Human	1 in 100	Abcam, U.S.A.	IHC
Anti-human IL-36γ	LS-C338023	Human	1 in 300	Lifespan Biosciences, U.S.A.	IHC
Anti-human IL-36R	ab180894	Human, Mouse, rat	1 in 2000	Abcam, U.S.A.	IHC
Anti-mouse CD4	4SM95	Human, Mouse	1 in 200	Thermo Fisher	IHC

				Scientific, U.S.A.	
Anti-mouse CD8 alpha	EPR21769	Mouse	1 in 2000	Merck Millipore, U.S.A.	IHC
Anti-mouse Ki67	ab16667	Human, mouse, rat	1 in 200	Abcam, U.S.A.	IHC
IL-36R/IL1RL2	PA587629	Human, Mouse	1 in 500	Invitrogen, U.S.A.	Western Blotting
Anti-Phospho AKT	s473	Human, mouse , rat, others	1 in 1000	Cell Signalling, U.S.A.	Western Blotting
Anti Phospho p42/44	9102s	Human, mouse , rat, others	1 in 1000	Cell Signalling, U.S.A.	Western Blotting
Phospho PI3K p85a	PA5-105116	Human, mouse , rat, others	1 in 2000	Invitrogen, U.S.A.	Western Blotting
Total AKT	9272s	Human, mouse , rat, others	1 in 1000	Cell Signalling, U.S.A.	Western Blotting
Total p42/44	9102	Human, mouse , rat, others	1 in 1000	Cell Signalling, U.S.A.	Western Blotting
Total PI3K p85a	MAB2998	Human, Rat	1 in 2000	R&D Systems, U.S.A.	Western Blotting
β-actin	A5441	Human, Mouse	1:10000	Sigma-Aldrich, U.S.A.	Western Blotting
Secondary Antibodies					
Secondary biotinylated anti-Rabbit	E0432	Rabbit	1 in 500	Agilent, U.S.A.	IHC
Secondary biotinylated anti-Rat	BA-9400	Rat	1 in 400	Vector labs, U.S.A.	IHC
Goat anti-rabbit Ig/HRP	P0448	Rabbit	1 in 10000	Agilent, U.S.A.	Western
Rabbit anti-mouse Ig/HRP	P0260	Mouse	1 in 10000	Agilent, U.S.A.	Western
Isotype antibodies					
Alexa Fluor® 700 Rat IgG2b, κ Isotype Ctrl	400628	N/A	1 in 200	Biolegend, U.S.A.	FACS

Brilliant Violet 421TM Rat IgG2a, κ Isotype	400536	N/A	1 in 200	Biolegend, U.S.A.	FACS
PE Purified Rat IgG2b, κ	553989	N/A	1 in 200	BD Biosciences, U.S.A.	FACS
PE Rat IgG2a, κ Isotype Ctrl	400508	N/A	1 in 200	Biolegend, U.S.A.	FACS
Goat IgG Isotype Control	#02-6202	N/A	1 in 100 to 1 in 2000	Thermo Fisher Scientific, U.S.A.	IHC/WB
ELISA					
Human CCL20	DY360-05.	Human	1 in 100	R & D systems	ELISA
Human CXCL1	DY275-05.	Human	1 in 100	R & D systems	ELISA
Human IL-1B	31670019U1	Human	1 in 100	Immunotools GmbH, Germany	ELISA
Murine CCL2	DY479	Mouse	1 in 100	R & D systems	ELISA
Murine CXCL1	DY453-05.	Mouse	1 in 100	R & D systems	ELISA
Cell viability Markers					
LIVE/DEAD™ Fixable Green Dead Cell Stain Kit, for 488 nm excitation	L34970	Human, mouse , rat, others	1 in 100	Invitrogen, U.S.A.	FACS
LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit, for 633 or 635 nm excitation	L10119	Human, mouse , rat, others	1 in 100	Invitrogen, U.S.A.	FACS

Appendix Table 2 – qRT-PCR primers

Gene (human)	Forward Sequence	Reverse Sequence
ARG1	GTCTGTGGGAAAAGCAAGCG	CACCAGGCTGATTCTTCCGT
β-actin	ATTGGCAATGAGCGGTTC	GGATGCCACAGGACTCCA
CCL2	TCAAACTGAAGCTCGCACTCT	GTGACTGGGGCATTGATTG
CCL20	TTGCTCCTGGCTGCTTTGAT	AGTCAAAGTTGCTTGCTGCT
CCL5	ACAGGTCAAGGATGCCAAAG	GTTCTTTCGGGTGACAAAGC
CXCL1	TCCTGCATCCCCCATAGTTA	CTTCAGGAACAGCCACCAGT
CXCL8	GAGCACTCCATAAGGCAC	ATGGTTCCTTCCGGTGGT
CXCR1	CCTAGTGTTCCTGCTGAGCC	GGCCGACCCTGCTGTATAAG
CXCR2	ACCCTCTTTAAGGCCCACAT	AGGACGACAGCGAAGATGAC
FasL	ATTGGGCCTGGGGATGTTTC	TGTGCATCTGGCTGGTAGAC
IL-10	CATAAATTAGAGGTCTCCAAAATCG	AAAGGGCTGGGTCAGCTAT
IL-12	CCCTGACATTCTGCCTTCA	AGGTCTTGTCCGTGAAGACTCTA
IL-1β	CACAGACCGTGGGTTCTTCA	TGTAATGAAAGACGGCACACC
IL-1RAcP	CCCTCTCAGCTTCCCAAGA	GGGCAAGAGTGAGGCTTCTA
IL-23	GGCAGAGATTCCACCAGGAC	GCTCCCCAGCATCTTTTTGC
IL-36β	TGAAGACATCATGAACCCACA	TGTCGAGAATCACGAATAGCA
IL-36γ	AAGTGACAGTGTGACCCCAGT	GGATTCTGGATTCCCAAATAAA
IL-36R	GCGTGTCAAGCCATACTGAC	CCTCCATATCCAGCTCTTTCTG
IL-36RN	GAGGAACAGGCAGACTCCAC	R CAATGCCGAGTCCTTCATTC
iNOS	TAGAGGAGTTCCCGTCCCTG	AACCTGGGCTTCAGAATGGG
NCR1/NKp46	GACTAGAGAGCGAGCCAGCA	AAGAGTCTGTGTGTTCAGCCTTC
Perforin	TGCTTGGACTGAAGGGGTTC	GGGTTGGACAAGCTTGGTCT
TGFβ	ACTACTACGCCAAGGAGGTCAC	TGCTTGAACTTGTCATAGATTTCG
TNFα	CGCTCCCCAAGAAGACAG	AGAGGCTGAGGAACAAGCAC
Gene (mouse)	**Forward Sequence**	**Reverse Sequence**
ARG1	GGGACCTGGCCTTTGTTGAT	CACAGACCGTGGGTTCTTCA
CXCL1	AGCCTCTAACCAGTTCCAGC	CTGGGATCATGGTGCTGTGT
CCL2	TCACTGAAGCCAGCTCTCTCT	GTGGGGCGTTAACTG
CCL5	AATCCCCTACTCCCACTCGG	TCTTGGGTTTGCTGTGCAGA
β-actin	CTCCGCTGACTCTCTTGG	AGGTGGTCGCAAAAACGA
GznB	GCTGCTCACTGTGAAGGAAGT	TGGGGAATGCATTTTACCAT
IFN-γ	ATCTGGAGGAACTGCCAAAA	TTCAAGACTTCAAACAGTCTGAGG
TGF-β	CTCCGCTGACTCTCTTGGA	AGCTGCTCGCAAAAACGAT
Other primer formats		
Gene (human)	**Primer Code**	
IL-36α	137628 custom Taqman assay	
Gene (mouse)	**Primer Code**	
IL-36α	mm00457645_m1	
IL-36β	mm01337546_m1	
IL-36γ	mm00463327_m1	
IL-36R	Mm00519245_m1	
IL-36RN	Mm01333586_m1	

Appendix Table 3 – qPCR primers

Name	Forward Primers	Reverse Primers	Expected Band Size
Exon 5 IL-36R	TTGTGAACAACGTTGCTGTG	CCTTCTTCCATACTCAACTTCCTT	130bp
B-actin	ATTGGCAATGAGCGGTTC	GGATGCCACAGGACTCCA	111bp

Appendix Table 4 – Other PCR primers

IL-36R KO Clone generation			
Name	**Forward**	**Reverse**	**Expected Band Size (bp)**
gRNA site A	GGGGAAACACCCCAAATAAACA	TGGTGGGGCACGTATTTTCT	995
gRNA site B	TTTGGCTACCCGGCTTTACT	ACGACTTGGTTTAATTGCCCTC	715
gRNA site C	CATTTCCGCACAGTTCATAGGTT	AGGAGAGAAAAGTGAGCTGGTT	453
HDR A	CCCAACTTCTCGGGGACTG	CTCGCATCTCTCCTTCACGC	2113
HDR B	ATCGCAGATCCTTGCGGC	CTCGCATCTCTCCTTCACGC	2357
HDR C	CAACTTCTCGGGGACTGTGG	CTCGCATCTCTCCTTCACGC	2349
IQC C	GGCTTGCAATCCTGACAAAGG	TGTGGAGGAGCAGCAAACTA	1198
Mycoplasma Testing			
Myco-5-1	CGCCTGAGTAGTACGTTCGC	GCGGTGTGTACAAGACCCGA	~500
Myco-5-2	CGCCTGAGTAGTACGTACGC	GCGGTGTGTACAAAACCCGA	~500
Myco-5-3	TGCCTGAGTAGTACATTCGC	GCGGTGTGTACAAACCCCGA	~500
Myco-5-4	TGCCTGGGTAGTACATTCGC		~500
Myco-5-5	TGCCTGGGTAGTACATTCGC		~500
Myco-5-6	CGCCTGAGTAGTATGCTCGC		~500

Appendix Table 5 –Microarray/RNAseq datasets used for transcriptomic analysis

Dataset	Platform	Normalisation	Used in paper for;	Source
TCGA	Illumina HiSeq	Limma	Malignant vs normal tissue expression multiple cancer types	TIMER 2.0
GSE68468	Affymetrix Human Genome U133A Array (HG-U133A-GPL96)	RMA	Colon Tissue gene expression comparison, differential gene expression (DEG) analysis	NCBI GEO
Meta-dataset	Affymetrix Human Genome U133 Plus 2.0 Array	fRMA	Tissue comparison expression normal and neoplastic	Rohr *et al[483]*
GSE39582	Affymetrix Human Genome U133 Plus 2.0 Array	RMA	Molecular pathway gene expression, MCPcounter, correlation matrix	NCBI GEO
GSE86362	Affymetrix Human Genome U133 Plus 2.0 Array	fRMA	Immune cell gene expression	NCBI GEO
TCGA COAD	Illumina HiSeq	Limma	Survival analysis	TCGA
E-MTAB-10089[483]	Affymetrix Human Genome U133 Plus 2.0 Array	fRMA	Normal vs Neoplastic colonic tissue	ArrayExpress
GSE37364	Affymetrix Human Genome U133 Plus 2.0 Array	Mas5	DEG analysis	NCBI GEO
GSE23878	Affymetrix Human Genome U133 Plus 2.0 Array	PLIER	DEG analysis	NCBI GEO
GSE25070	Illumina HumanRef-8 v3.0	RSN	DEG analysis	NCBI GEO
Multi-cohort(GSE12945, GSE17536, GSE39582)	Affymetrix Human Genome U133 Plus 2.0 Array	fRMA	IL-36α Survival analysis	NCBI GEO

Appendix Table 6 – Multi-cohort patient details demographics used for Figure 6.2

Variables	Female (n=194)	Male (n=179)	N/A (n=332)
Age	66.19 ± 11.20	66.54 ± 11.72	n/a
Histology			
Normal	51	31	149
Adenoma	23	27	82
CRC	120	121	101
Staging			
AJCC			
I	-	-	10
II	48	42	37
III	-	-	23
IV	-	-	18
Dukes A/B	-	-	14
Low grade polyp dysplasia	-	-	16
High grade polyp dysplasia	-	-	13
Metastasis			
M0	58	65	68
M1	9	9	18
Recurrence	10	8	26
Primary location			
Cecum	4	5	0
Ascending Colon	9	4	8
Transverse Colon	1	8	2
Descending Colon	7	1	8
Sigmoid	21	21	32
Rectum	11	11	14

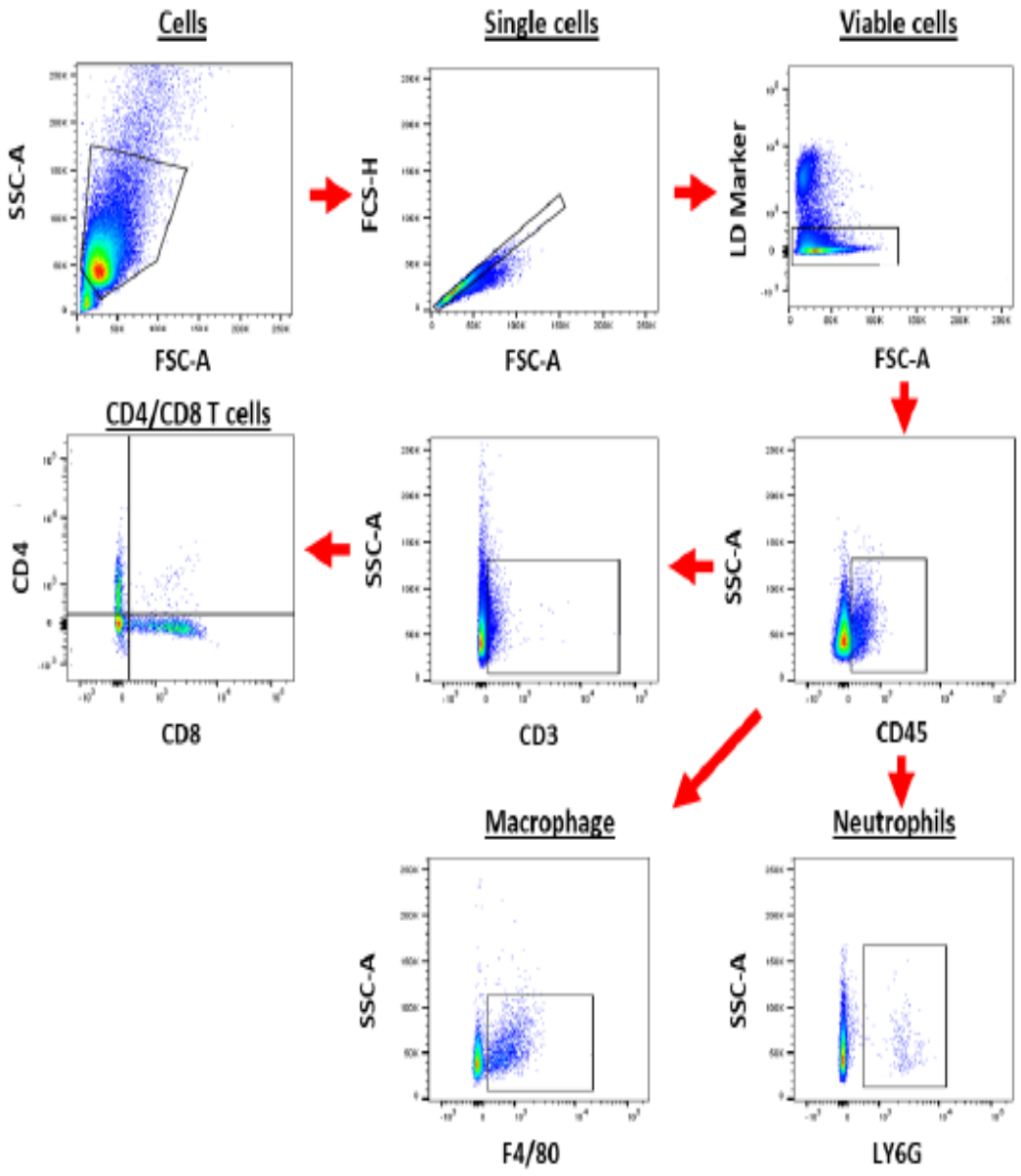
Appendix Table 7 – GSE39582 demographics used for figures 6.4, 6.5 and 6.9.

GSE39582	N=562		
Sex		**Chemotherapy received**	
Male	309	Y	233
Female	253	N	313
		N/A	16
Age		**MMR status**	
<70	300	dMMR	72
>70	261	pMMR	444
N/A	1	N/A	46
TNM		**TP53 mutation**	
0	4	WT	159
I	32	M	190
II	262	N/A	213
III	204	**KRAS mutation**	
IV	60	WT	325
T		M	216
T0	1	N/A	21
T1	11	**BRAF mutation**	
T2	44	WT	459
T3	364	M	49
T4	119	N/A	54
N/A	23		
N		**Location**	
N0	299	Proximal	220
N1	133	Distal	342
N2	98		
N3	4		
N/A	29		
M			
M0	479		
M1	61		
N/A	22		

Appendix Table 8 – Microarray datasets used for DEG analysis

Dataset	Sample Type	n	Assay	Platform	Sample type
GSE37364	Normal	38	Microarray	GPL570	Bulk tissue
	Adenoma	29			
	Low grade dysplasia	16			
	High grade dysplasia	13			
	CRC	27			
	Dukes A/B	14			
	Dukes C/D	13			
GSE23878	Normal	24	Microarray	GPL570	Bulk tissue
	CRC	35			
GSE25070	Normal	26	Microarray	GPL6883	Bulk tissue
	CRC	26			
GSE68468	Normal	55	Microarray	GPL96	Bulk tissue
	CRC	195			

Appendix Figure 1 – FACS Cell surface staining gating strategy



Appendix Figure 1: FACS cell surface markers gating strategy for dissociated tumour tissue from *in vivo* mouse models. Cells were gated based side (SSC-A) and forward scatter (FSC-A) area followed by single cell selection based on FSC-A versus forward scatter height. A fixable live/dead marker gate was used to exclude dead cells. Neutrophils were gated as $CD45^+/LY6G^+$, macrophages were gated as $CD45^+/F4/80^+$, CD4 and CD8 cells were gated as $CD45^+/CD3^+$ and $CD4^+$ or $CD8^+$, respectively.