CORA Cork Open Research Archive
Cartlann Taighde Oscailte Chorcaí

Title	Investigation of the role of interleukin-1 family members, IL-33 and IL-36, in the pathogenesis of colon cancer
Authors	O'Donnell, Charlotte
Publication date	2016
Original Citation	O'Donnell, C. 2016. Investigation of the role of interleukin-1 family members, IL-33 and IL-36, in the pathogenesis of colon cancer. PhD Thesis, University College Cork.
Type of publication	Doctoral thesis
Rights	© 2016, Charlotte O'Donnell. - http://creativecommons.org/licenses/by-nc-nd/3.0/
Download date	2026-10-31 07:43:40
Item downloaded from	https://hdl.handle.net/10468/3793

UCC
University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

Investigation of the Role of Interleukin-1 family members, IL-33 and IL-36, in the pathogenesis of colon cancer



Submitted to the National University of Ireland, Cork
in fulfilment of the
requirements for the degree of Doctorate of Philosophy.

Thesis presented by
Charlotte O'Donnell BSc,
Department of Medicine

Under the supervision of
Dr Aileen Houston and Dr Elizabeth Brint

Table of Contents

Declaration vii
List of publications viii
Abstract ix
Acknowledgements xi
Abbreviations xii
1.0 Introduction 1
1.1 Cancer 1
1.1.1 Colon cancer 1
1.1.2 Colon cancer development 3
1.1.3 CRC Staging 5
1.1.4 CRC Treatment 6
1.2 Inflammation and cancer 6
1.2.1 The Inflammatory component of tumours 7
1.2.2 Tumour associated macrophages 9
1.2.3 Tumour associated Neutrophils 11
1.2.4 Myeloid-derived suppressor cells 11
1.2.5 Tumour infiltrating lymphocytes 12
1.2.6 Regulatory T cells 12
1.2.7 Natural killer cells 13
1.3 The IL-1 cytokine 13
1.3.1 IL-1 and IL-1R family members 14
1.3.2 IL-1R family signalling 16
1.3.3 IL-1α, IL-1β and the IL-1R complex 16
1.3.4 The IL-18 subfamily 18
1.4 ST2, a member of the IL-1R subfamily 19
1.4.1 Regulation of ST2 expression 19
1.4.2 Expression of ST2 22
1.4.3 IL-33, the ligand of ST2L 22
1.4.4 Processing of IL-33 22
1.4.5 IL-33 expression 23
1.4.6 Nuclear IL-33 regulates gene expression 24
1.5 IL-33/ST2 signalling 25
1.5.1 Regulation of ST2L signalling 27

1.6 A physiological role for IL-33 29
1.6.1 A role for IL-33 in barrier function and epithelial wound healing..... 29
1.6.2 The role of IL-33/ST2 in immune cells 30
1.7 The Pathological functions of IL-33/ST2 32
1.7.1 IL-33/ST2 in inflammatory bowel disease..... 32
1.8 ST2 as a positive prognostic indicator 33
1.9 IL-33/ST2 in cancer 34
1.10 The IL-36 subfamily 36
1.10.1 Expression and function of IL-36 family members 37
1.10.2 IL-36 regulation 37
1.10.3 IL-36 signalling..... 38
1.11 Physiological functions of IL-36..... 40
1.12 Pathophysiological functions of IL-36..... 41
1.12.1 IL-36 in IBD 41
1.12.2 IL-36 in cancer..... 42
1.13 Aims:..... 44
2.0 Material and Methods 56
2.1 Materials 56
2.1.1 Cell lines and tissue 56
2.1.2 Mice 56
2.1.3 Reagents..... 56
2.2 Methods..... 58
2.2.1 Cell culture..... 58
2.2.1.2 Viability 58
2.2.2 Western blotting..... 58
2.2.2.1 Preparation of whole cell lysates 58
2.2.2.2 Quantitation of total protein concentration 59
2.2.2.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis and Western blotting . 59
2.2.2.4 Immunoblotting..... 60
2.2.3 Quantitative real-time polymerase chain reaction (qRT-PCR)..... 60
2.2.3.1 RNA extraction 60
2.2.3.2 cDNA preparation..... 61
2.2.3.3 qRTPCR..... 61
2.2.5 MTT assay 62
2.2.6 BrdU Assay..... 63
2.2.7 Immunohistochemistry 63

2.2.8 Enzyme Linked Immunoassay (ELISA) 64
2.2.9 Cell migration assay 64
2.2.10 RAW 264.7 migration assay 65
2.2.11 Invasion Assay 65
2.2.12 Caspase 3 and 7 Assay 66
2.2.13 Generation of ST2 knockdown cancer cells 66
2.3 *In vivo* studies 67
2.3.1 Treatment groups and ear marking 68
2.3.2 Monitoring body weight 68
2.3.4 Tumour sampling 68
2.3.5 Single cell suspension of tumour cells for flow cytometric analysis 69
2.3.6 Flow cytometry 69
2.4 Statistical analysis 70
Chapter 3 71
Characterisation of IL-33 and ST2 expression, signalling and function in colon cancer cells *in vitro.* 71
3.1 Introduction 72
3.2 Results 75
3.2.1 LPS induces ST2L expression in a murine macrophage cell line 75
3.2.2 Human colon cancer cell lines express both sST2 and ST2L. 75
3.2.3 Murine colon cancer cells express both sST2 and ST2L. 75
3.2.4 Colon cancer cell lines and macrophages express IL-33 mRNA, the ligand for ST2L 80
3.2.5 IL-33 protein expression was not effected by pro-inflammatory stimuli in colon cancer cells. 80
3.2.6 IL-33 inhibits proliferation of colon cancer cells 83
3.2.7 IL-33 induces migration, but not invasion of colon cancer cells 87
3.2.8 IL-33 induces limited expression of cytokines/chemokines in colon cancer cells 90
3.2.9 IL-33 activates MAPK, NFκB and AKT signalling pathways 91
3.3 Discussion: 98
3.3.1 sST2 expression was increased in colon cancer cells compared to ST2L expression 98
3.3.2 IL-33 suppressed cancer cell proliferation 99
3.3.3 IL-33 increased migration of colon cancer cells 100
3.3.4 IL-33 activated chemokine production by colon cancer cells 101
3.3.5 IL-33 activates signalling pathways common to other IL-1 family members 103
Chapter 4 107

The IL-33/ST2 axis modulates tumour growth and the tumour microenvironment *in vivo* 107

4.1 Introduction:..... 108

4.2 Results:..... 111

4.2.1 Generation of stable cell lines with suppressed ST2 expression by stable transfection with short hairpin RNA (shRNA) encoding plasmids. 111

..... 112

4.2.2 Knocking down ST2 expression in CT26-derived tumours increases tumour formation and growth *in vivo*. 114

4.2.3 Ki67 expression was unchanged in $CT26^{ST2\ shRNA\#1}$ tumours compared to $CT26^{scr\ shRNA\#1}$ tumours. 117

4.2.4 Generation and functional assessment of additional $ST2^{-/-}$ CT26 cells *in vitro* and *in vivo*. 117

4.2.5 Suppression of ST2 expression does not alter colon cancer cell proliferation *in vitro* in response to pro-inflammatory stimuli. 130

4.2.6 Suppression of ST2 expression in tumour cells results in reduced macrophage and $CD8^{+}$T cell recruitment *in vivo*. 130

4.2.7 Migration of macrophages towards tumour cell supernatant is increased by IL-33 stimulation of the tumour cells. 135

4.2.8 Serum levels of IL-33 are slightly decreased in CRC patients compared to healthy controls, while sST2 levels are unchanged. 139

4.2.9 Expression of ST2L in CRC is lower compared to adjacent non-tumour tissue..... 139

4.3 Discussion 143

4.3.1 A potential anti-tumorigenic role for IL-33 in cancer:..... 143

4.3.2 The role of TAMs in tumorigenesis:..... 145

4.3.3 Alternative pro-tumorigenic role for IL-33/ST2 axis:..... 147

Chapter 5 153

***Characterisation of the role of IL-36 in colon cancer* 153**

5.1 Introduction..... 154

5.2 Results..... 157

5.2.1 IL-36α and IL-36γ expression were increased in tumour tissue compared to adjacent tissue, while IL-36R expression was unchanged. 157

5.2.2 Characterisation of $IL\text{-}36R^{+}$ cells in colonic tumours. 168

5.2.3 Colon cancer cells express the IL-36R *in vitro*. 170

5.2.4 IL-36α, IL-36β and IL-36γ cytokines induce cellular proliferation in SW480 cells, whereas in HT29 cells only IL-36γ increases cellular proliferation. 172

5.2.5 IL-36 cytokines did not induce apoptosis in HT29 cells..... 172

5.2.6 IL-36 cytokines did not induce migration of HT29 colon cancer cells. 176

5.2.7 Chemokines CXCL-1, CCL2 and CCL20 were produced in response to IL-36α and IL-36γ stimulation in HT29 human colon cancer cell lines. 176
5.3 Discussion 181
5.3.1 IL-36 cytokines are increased in colon cancer: 181
5.3.2 IL-36R expression on tumour infiltrating immune cells: 183
5.3.3 IL-36 induces proliferation of colon cancer cells: 184
5.3.4 IL-36 induces chemokine and cytokine induction: 186
6.0 Final discussion and future perspectives: 189
Bibliography 196
Appendix 214
A. Table of Primers 214
B. Table of Antibodies 1 216
C. Table of Antibodies 2 217
D. Table of Reagents 218

Declaration

I hereby declare that this thesis is the result of my own work and has not been submitted in whole or in part elsewhere for any award. Any assistance and contribution by others in this work is duly acknowledged within the text.

Charlotte O'Donnell

List of publications

This work has been published in the following formats:

Journal article

Charlotte O'Donnell, Amr Mahmoud, Jonathan Keane, Carola Murphy, Declan White, Siobhan Carey, Micheal O'Riordain , Michael Bennett, Elizabeth Brint and Aileen Houston.

An anti-tumorigenic role for the IL-33 receptor, ST2L, in colon cancer, British Journal of Cancer 2016 January doi: 10.1038/bjc.2015.433. Epub 2015 Dec 17.

Oral Presentation

New Horizons in Medicine, Cork, Ireland. O'Donnell C., Mahmoud A., Murphy C, Brint E. and Houston A., (2014), The Potential Role of the ST2/IL-33 axis, in Colon Cancer.

Irish Society of Immunology, Dublin, Ireland. O'Donnell C., Mahmoud A., Murphy C, Brint E. and Houston A., (2014), Characterisation of the Role of ST2/IL-33 in Colon Carcinogenesis.

Poster Presentations

Cell Symposia: Cancer, Inflammation and Immunity, Sitges, Spain. O'Donnell C, Mahmoud A, Murphy C, Brint E and Houston A, **(2015)** The Role of IL-33 and ST2 in Colon Cancer

Irish Association of Cancer Research, Limerick, Ireland. O'Donnell C, Mahmoud A, Murphy C, Brint E and Houston A, (2015), The Potential Role of the IL-33/ST2 axis in Colon Cancer.

EMBO Symposium: Tumour microenvironment and signalling, O'Donnell C, Mahmoud A, Murphy C, Brint E and Houston A, (2014), Characterization of the Role of ST2 and its Ligand, IL-33, in Colon Cancer.

Irish Association of Cancer Research, Galway Ireland. O'Donnell C, Mahmoud A, Murphy C, Brint E and Houston A, **(2014),** Investigation of the role of ST2 and its ligand, IL-33, in Colon Cancer.

Irish Society of Immunology, Dublin, Ireland. O'Donnell C, Mahmoud A, Murphy C, Brint E and Houston A, **(2014),** The Potential Role of IL-33 and its Receptor ST2 in Colon Carcinogensis.

Abstract

The importance of inflammation in cancer is well established, with cytokines/chemokines playing an important role in carcinogenesis. IL-33 was recently identified as the ligand for ST2. ST2 is a member of the toll-like receptor/IL-1 receptor family. Three isoforms of ST2 exist: a trans-membrane receptor (ST2L), a secreted soluble form (sST2), and a variant form (ST2V). The IL-33/ST2 pathway has been implicated in inflammatory bowel disease, a major risk factor for colon cancer. The aim of the first part of my thesis was to investigate the role of IL-33 and ST2 in colon cancer. CT26 and HT29 colon cancer cells were found to express ST2 and IL-33 *in vitro*, with expression increased by inflammatory mediators (LPS, TNF-α and PGE_2). Stimulation with IL-33 increased the migration, but not proliferation, of colon tumour cells. Functional analyses showed that stimulation with IL-33 induced the expression of CXCL-1 by CT26 and CCL2 expression by HT29 cells. To investigate the role of ST2 *in vivo*, ST2 knockdown cells were generated using ST2-specific shRNA (CT26 ^{ST2shRNA}) and injected subcutaneously into BALB/C mice. Knockdown of ST2 in colon tumours resulted in enhanced tumour growth (2.3 fold increase compared to CT26 ^{scrshRNA}) *in vivo*. This was associated with alterations in immune cell infiltration, including an increase in macrophage infiltration. In contrast, characterisation of human colon tumours revealed that ST2L expression was increased in tumour cells relative to adjacent non-tumour cells, with no change in expression of total ST2. These results indicate that the IL-33/ST2 signalling axis may play an important role in colon carcinogenesis and merits further investigation.

The role of the IL-36R, a second IL-1R family member, in colon cancer was investigated in the second part of my thesis. Characterization of human colon tumours

e*x vivo* showed significantly increased expression of the IL-36 ligands, IL-36α and IL-36γ, compared to adjacent non-tumour tissue. *In vitro* colon cancer cell lines HT29 and SW480 were shown to express the IL-36R and IL-36 ligands. IL-36α and IL-36γ stimulation of HT29 cells also increased the expression of the chemokines CXCL-1, CCL2, CCL20, and IL-8. This suggests that IL-36 signalling may promote tumour-derived immune cell recruitment. This field requires further study to determine if the recruitment of immune cells by IL-36 signalling could be utilised to break tolerance against tumour antigens. This thesis has laid the basis for further studies to explore the role of IL-36 signalling in colon cancer.

Acknowledgements

I would like to thank my supervisors Aileen and Beth for all of their insight, guidance and dedication throughout the course of my PhD studies. I am very grateful to you both for all of your advice, positivity and hard work. I would also like to thank all of the members of the department of medicine both past and present for making work more fun along the way and of course for all of the cake breaks. A special thanks to Amruta, Ciara, Philana, Caitriona, and Declan for all of your technical and social assistance.

To my parents, none of this would have been possible without your love and support. I am very grateful to both of you. I know I can never repay all that you have sacrificed for me and I realise how lucky I am to have you both. A special thanks to Steve and Stef, you two are a constant motivation to me.

Last but not least, to Bryan the love of my life, I know that I would not have succeeded without all of the encouragement and support that you have given me. Thank you for letting me follow my dreams.

Abbreviations

AOM	azoxymethane
β-actin	beta-actin
BSA	bovine serum albumin
CAC	colitis associated cancer
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CD	Crohn's disease
cDNA	complementary DNA
c-FLIP	cellular FLICE-like inhibitory protein
CLR	C-type lectin receptor
CNS	central nervous system
COX	cyclooxygenase
CRC	colorectal cancer
CTL	cytotoxic T lymphocytes
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
DAMP	danger associated molecular pattern
DC	dendritic cells
DISC	death inducing signalling complex
DMEM	Dulbecco's modified Eagles Medium
DSS	dextran sodium sulphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinases
FCS	foetal calf serum

FSC	Forward Scatter
GF	germ free
H&E	haematoxylin and eosin
HRP	horseradish peroxidase
IBD	inflammatory bowel disease
IEC	intestinal epithelial cell
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
IP	immunoprecipitation
IRAKm	interleukin-1 receptor-associated kinase monocytes/macrophages
JNK	c-Jun N-terminal kinases
LBP	LPS binding protein
LPS	Lipopolysaccharide
mAB	monoclonal antibody
MAP	mitogen activated protein
M-CSF	monocyte macrophage colony stimulating factor
MCP-1	monocyte chemoattractant protein-1
MDSC	myeloid derived suppressor cells
MMP	matrix metalloprotease
mRNA	messenger RNA
MyD88	myeloid differentiation primary response gene
NF-κB	nuclear factor kappa b
NGS	normal goat serum
NK	natural killer
ODN	oligodeoxynucleotide
PAMP	pathogen associated molecular pattern

PBS	phosphate buffered saline
PGE_2	prostaglandin E2
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear leukocytes
PPAR-γ	peroxisome proliferator-activated receptor gamma
PRR	pathogen recognition receptor
RA	rheumatoid arthritis
RIPA	radio-immunoprecipitation assay
RNA	Ribonucleic acid
ROS	reactive oxygen species
RT	room temperature
RT	enzyme reverse transcriptase
s.c.	subcutaneous
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
shRNA	short hairpin RNA
SIGIRR	single Ig IL-1-related receptor
TAM	tumour associated macrophage
TAN	tumour associated neutrophil
TBS	tris buffered saline
Th	T helper
TIR	Toll/interleukin-1 (IL-1) receptor domain
TLR	toll-like receptor
TNF-α	tumour necrosis factor alpha
TOLLIP	toll interacting protein
TRAIL	TNF-related apoptosis-inducing ligand
Tregs	T regulatory cells

UC	ulcerative colitis
WT	wild type

1.0 Introduction

1.1 Cancer

Carcinogenesis is a multi-step process that develops through epigenetic changes and mutation of multiple genes, including loss of function of tumour suppressor genes and gain of function of oncogenes. These genetic changes in normal cells can each contribute a growth advantage, leading to the transformation of cells into cancer cells [1]. In 2000, Hanahan and Weinberg described a series of 6 biological capabilities or 'hallmarks' acquired by most types of cancers during the multi-step development of cancer (Figure 1). They are as follows; 'sustaining proliferative signalling', 'evading growth suppressors', 'activating invasion and metastasis', 'inducing angiogenesis', 'resisting cell death' and 'enabling replicative immortality' [2].

In 2011, Hanahan and Weinberg went on to describe two additional 'hallmarks' of cancer. These were 'deregulating cellular energetics' and 'avoiding immune destruction' (Figure 1). Acquisition of these capabilities is enabled by genomic instability, that is important for the generation of the genetic diversity that promotes their acquisition [3]. As well as genetic instability, a second enabling characteristic is tumour-promoting inflammation, which refers to the ability of the tumour to harness the inflammatory response to promote tumorigenesis.

1.1.1 Colon cancer

Adenocarcinoma of the colon and rectum (colorectal cancer (CRC)) is the third most common cancer worldwide, with over 1.4 million people diagnosed each year [4]. 1 in 5 CRCs have a familial or congenital gene mutation that compounds colon cancer

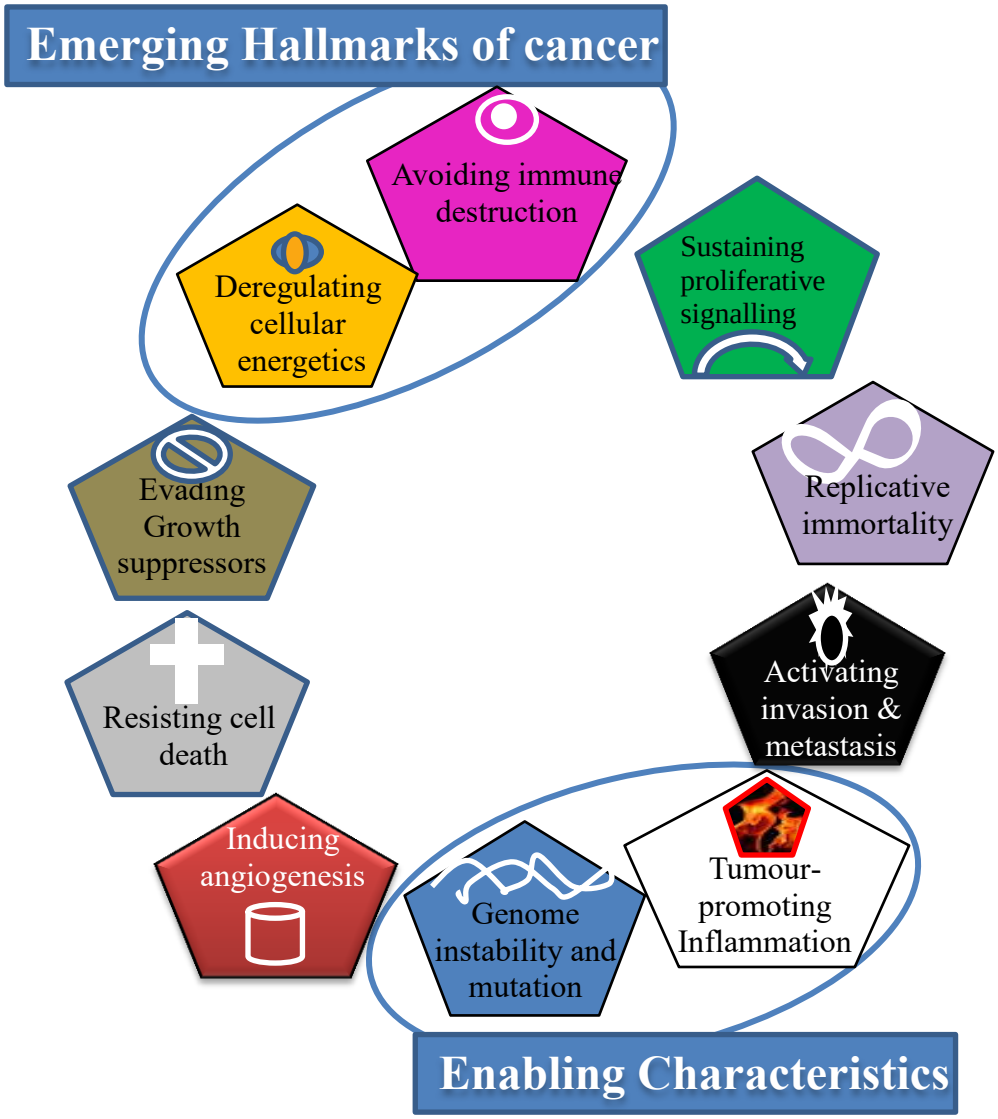


Figure 1: The 'hallmarks' of cancer. The hallmarks of cancer are biological functions acquired during the development of tumours. They include; sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis. The two most recently described hallmarks of cancer are reprogramming of energy metabolism and evading immune destruction. Enabling characteristics such as genomic instability allow cancer cells to generate mutations that enhance tumour progression. The immune response used to protect against pathogenic insult can facilitate tumour promotion by activating proliferation and metastasis. Figure adapted from D Hanahan and R. Weinberg, Hallmarks of cancer: the next generation, Cell 2011.

risk and these cancers usually become established at a young age. Hereditary non-polyposis colorectal cancer (HNPCC) accounts for approximately 5% of these cases and familial adenomatous polyposis (FAP) accounts for ~1%. The genetic mutations responsible for these two conditions lie in the mismatch repair genes and in the adenomatous polyposis coli (APC) gene, respectively. The remaining ~80% are sporadic, with no clear genetic origin. They usually occur at an advanced age. This suggests that environmental factors may cause genetic mutations that accrue over time.

The risk of developing cancer depends on many factors. Those with a parent, sibling or child with the disease have a 2-fold increased risk of developing the disease [5]. Other risk factors include family history, increasing age, the presence of colonic polyps, inflammatory bowel disease (IBD) and a previous history of colon, ovarian or breast cancer. Environmental factors such as smoking, alcohol, viral exposure, exogenous oestrogens, a low fibre diet and physical inactivity have also been identified as important risk factors for developing colon cancer.

1.1.2 Colon cancer development

Colon cancer development normally follows a measured gradient process, in which various genetic mutations gradually accumulate over time, inducing the transformation of healthy cells into cancerous cells. In the majority of cases, this malignant disease begins as a benign adenomatous polyp, which then develops into an advanced adenoma with high grade dysplasia. This is followed by progression to carcinoma and ultimately to metastasis (Figure 2). This process normally takes

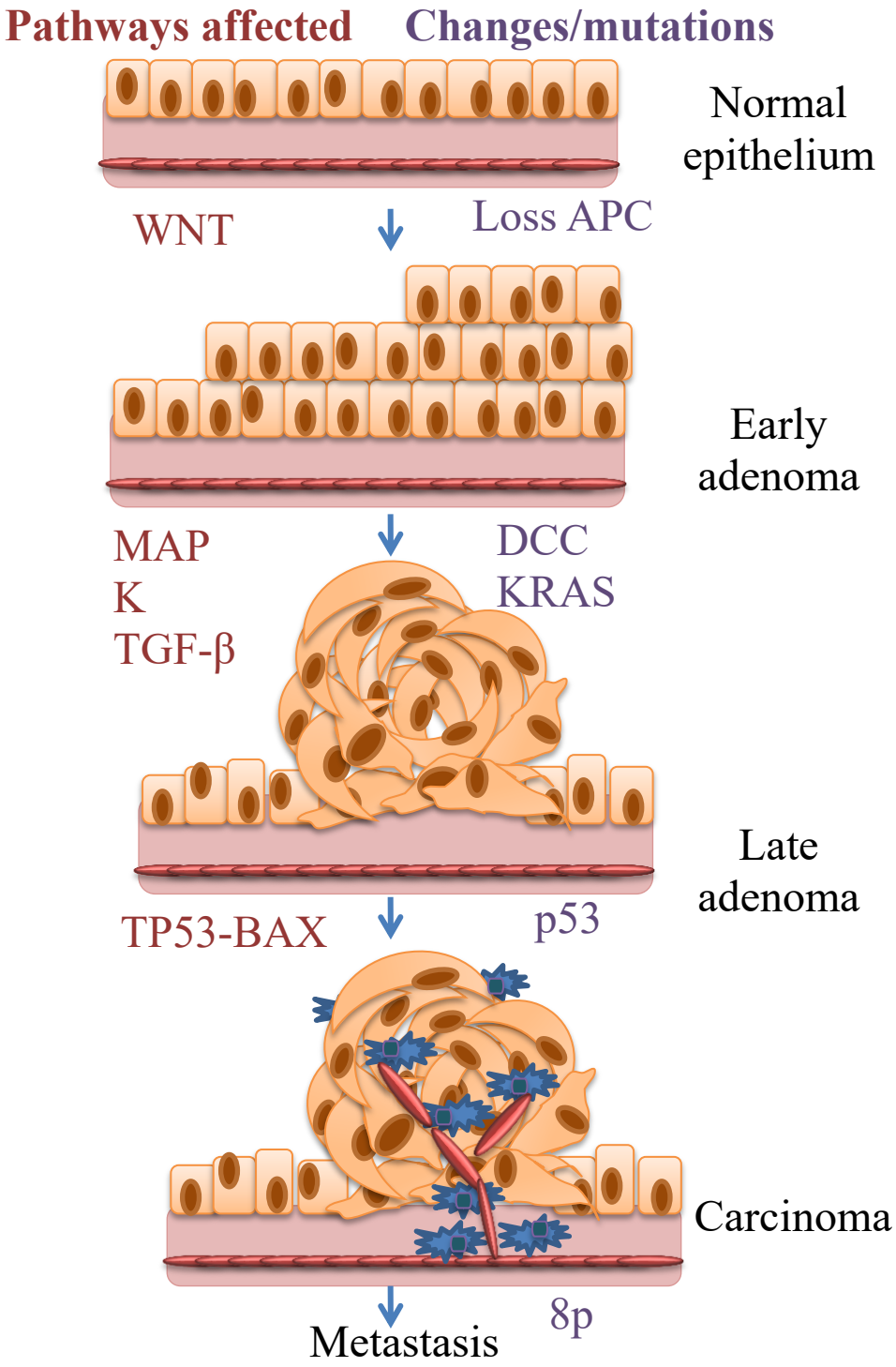


Figure 2: Sequential morphological changes involved in CRC development. Hyper-proliferation of the epithelium is observed in the early stages of adenoma development. Initially, small adenomas form. Once severe dysplasia occurs, this marks the progression from early to late adenoma. The next sequential step is the progression from late adenoma to malignant carcinoma. During this transition increased vascularization and recruitment of immune cell populations are observed. This is followed by metastasis. Common alterations in genes and pathways associated with each stage of the transformation of normal epithelium to carcinoma are illustrated.

decades (Figure 2) [6]. Different genetic mutations have been shown to be associated with the various stages of colon cancer development (Figure 2). One of the earliest changes associated with CRC development is inactivation of the adenomatous polyposis coli (APC) tumour suppressor gene. APC is inactivated in the majority of sporadic CRCs, and is the genetic mutation responsible for colon tumour development in FAP [7]. Silencing of APC drives genomic instability and promotes cell growth as the cells are no longer bound by normal cell cycle checkpoints [8]. In many cancers, the RAS-GTPase family proto-oncogene K-RAS also becomes activated. This is usually linked with the transition from early to intermediate adenoma. The gene called deleted in colon cancer (DCC) can also be lost. This commonly occurs in the transition from intermediate adenoma to late adenoma. Mutations of the tumour suppressor p53 are associated with the development of carcinoma. Loss of chromosome 8p is associated with carcinoma to metastasis transition [9]. Other genes or pathways that are frequently mutated in CRC include the cyclooxygenase (COX) signalling pathway, the signal transducer phosphatidylinositol 3-kinase (PI3K), the Wnt/beta-catenin signal transduction pathway and the transforming growth factor (TGF)-β-signalling pathway [10-12].

1.1.3 CRC Staging

Clinical staging of CRC is used to determine the extent of the cancer and involves the use of the Tumour/Node/Metastasis (TNM) staging system. The TNM system classifies the stages of colon cancer under the following headings: T-the degree of invasion of the intestinal wall; N-the degree of lymphatic node involvement; and M-the degree of metastasis. Stage I refers to CRCs confined to the mucosa or indicates lymph node metastasis and stage IV cancers are those that have metastasized to

distant organs. The liver and the lung are the two most common sites of CRC metastasis [13].

1.1.4 CRC Treatment

Surgery alone is used to treat individuals with stage I or stage II CRC. Radiation therapy or chemotherapy may be recommended in stage III post-surgery. Most commonly 5-Fluorouracil (5-FU), lucovorin and oxaliplatin combined, or cappecitabine and oxaliplatin combined chemotherapy regimens are employed. In stage IV disease, chemotherapy may be employed pre-surgery to shrink tumours and also post-surgery to remove any remaining cancer cells not removed by surgery. At this stage, targeted therapies may be employed alone or in combination with the previously mentioned chemotherapy regimens. Targeted therapies include Cetuximab and Regorafenib which target the EGFR and VEGFR respectively. Radiation therapy is also used in Stage IV to relieve symptoms [14]. The 5-year survival rate for patients with stage I colon cancer is approximately 92% and this drops dramatically to ~11% for patients with state IV or metastatic disease [15].

1.2 Inflammation and cancer

The link between inflammation and cancer is well established, with inflammation highlighted as one of the enabling characteristics in cancer development [3]. As early as 1863, Virchow indicated that cancer tended to occur at sites of chronic inflammation. Indeed many cancers are associated with chronic inflammation. Patients with chronic hepatitis caused by Hepatitis B and C infection are at increased risk of developing hepatocellular carcinoma [16], while infection with human papilloma virus (HPV) is linked to the development of cervical cancer [17, 18]. In the colon, inflammatory bowel disease (IBD)-associated inflammation increases the

risk of developing CRC [19]. Inflammatory bowel diseases (IBDs) are inflammatory disorders of the gastrointestinal tract that can be subdivided into two major disorders: ulcerative colitis (UC) and Crohn's disease (CD). Indeed, the extent and the duration of UC was found to directly correlate with the risk of CRC development [20]. Mortality is also increased in patients who develop CRC following UC [21], suggesting that the inflammatory processes observed in this disease may result in a more aggressive tumour phenotype.

1.2.1 The Inflammatory component of tumours

Tumours are composed of both tumour cells and non-tumour cells, with the tumour microenvironment describing the non-cancerous cells present in the tumour. In this microenvironment tumour cells interact with surrounding cells, including cancer-associated fibroblasts, endothelial cells, adipocytes and immune cells. Immune cells present in the tumour microenvironment have been shown to have wide ranging effects. They can influence cellular proliferation signals, angiogenesis and tissue remodelling in ways that can either promote or suppress tumour progression (Figure 3) [22].

Immune cells are recruited into the tumour microenvironment by soluble chemo-attractants produced by cancer cells and stromal cells [23-25]. Chemokines are small (8-11 kDa), secreted proteins that regulate the number and the phenotype of immune cells recruited by tumours. However, chemokines can also be exploited by the tumour to promote tumour growth, survival, angiogenesis and tumour cell migration [26, 27]. For instance, the chemokine CXCL1 can function as both a growth factor

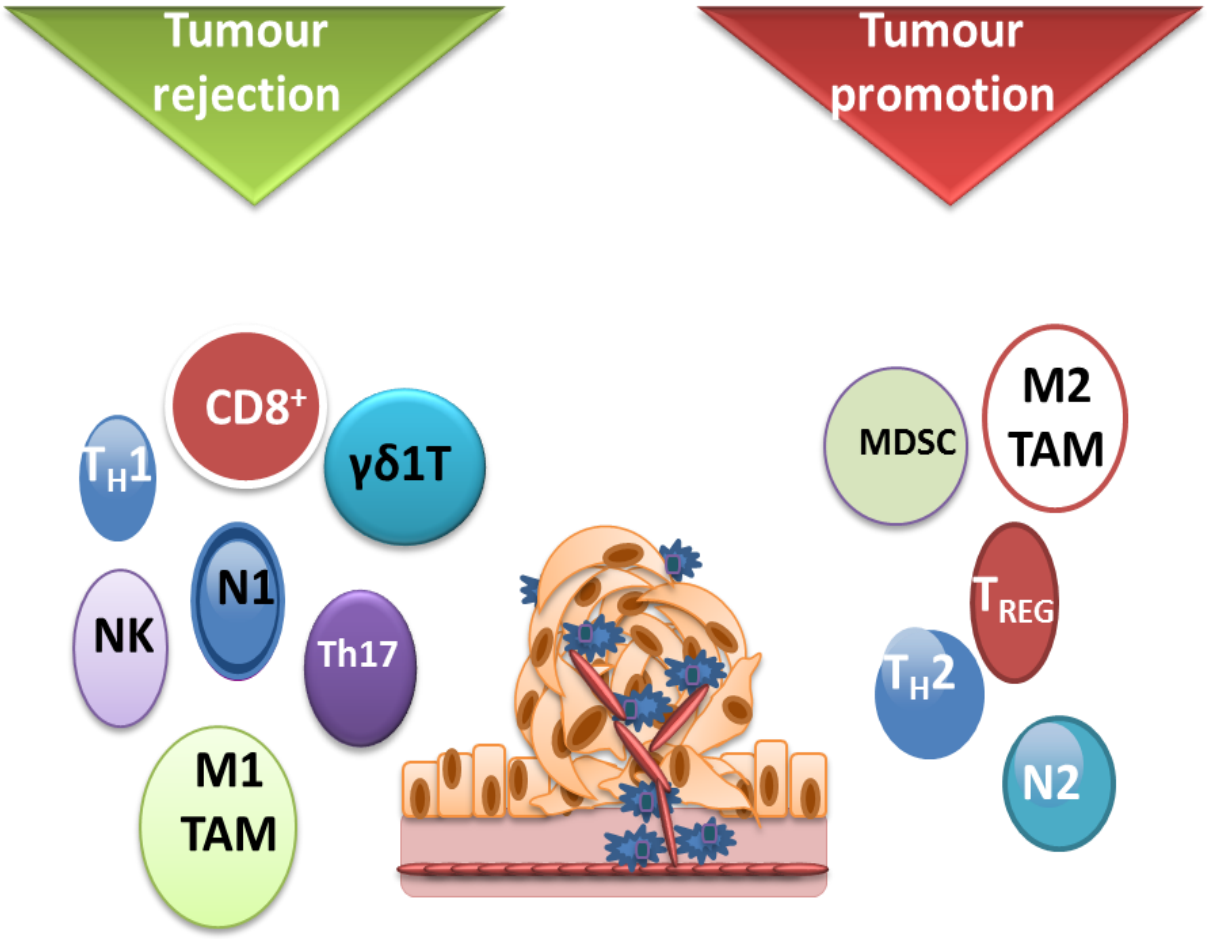


Figure 3: Tumour-infiltrating immune cells and tumorigenesis. Upon recruitment to tumours, immune cells are exposed to various tumour- or immune-derived factors. These factors can skew the function of the immune cell towards an anti-tumour or pro-tumour response. Immune cells associated with pro-tumour activities include M2 macrophages, N2 neutrophils, myeloid-derived suppressor cells (MDSCs) and T-reg cells. Immune cells associated with tumour rejection include M1 macrophages and N1 neutrophils. Anti-tumour activity is also displayed by various lymphocyte subsets, such as NK cells, $CD8^+$ T cells, γδ1 T cells and Th1 cells. These leukocytes are usually cytotoxic and produce cytokines that can promote tumour rejection.

for cancer cells and as an angiogenic factor that regulates angiogenesis, which is critical for tumour growth and progression [28, 29].

Cytokines are low molecular weight protein mediators that facilitate cell-to-cell communication. Multiple cell types in the tumour microenvironment express these inflammatory mediators, including immune cells and stromal cells such as fibroblasts and endothelial cells. Cytokines perform numerous functions including the regulation of cellular proliferation, migration and cell death as well as immune cell activation. Depending on the tumour microenvironment, cytokines can promote a pro- or anti-tumour immune response. This is dependent on a number of factors, including the balance of pro- and anti-inflammatory cytokines, their comparative concentrations and cytokine receptor expression by the immune cells [30].

1.2.2 Tumour associated macrophages

Macrophages are key components of the immune infiltrate found in tumours [31]. Two main macrophage phenotypes have been identified (Figure 4) [32]. Classically activated M1 macrophages are regarded as anti-tumorigenic, while M2 macrophages are pro-tumorigenic (Figure 4) [33, 34]. M1 macrophages can stimulate an anti-tumour immune response via production of pro-inflammatory cytokines such as IL-12, IL-23 and TNFα [35]. M2 macrophages promote an immunosuppressive microenvironment by production of cytokines such as IL-10, can be immunosuppressive and inhibit the activity of Th1 cells and NK cells [36]. Moreover, this immune suppressed environment can be exploited by the tumour to invade surrounding tissue and metastasize to distant organs [37]. M1 and M2 chemokine profiles can vary significantly, resulting in the recruitment of distinct immune cells. M1 macrophages are known to chiefly produce CXCL9 and CXCL10,

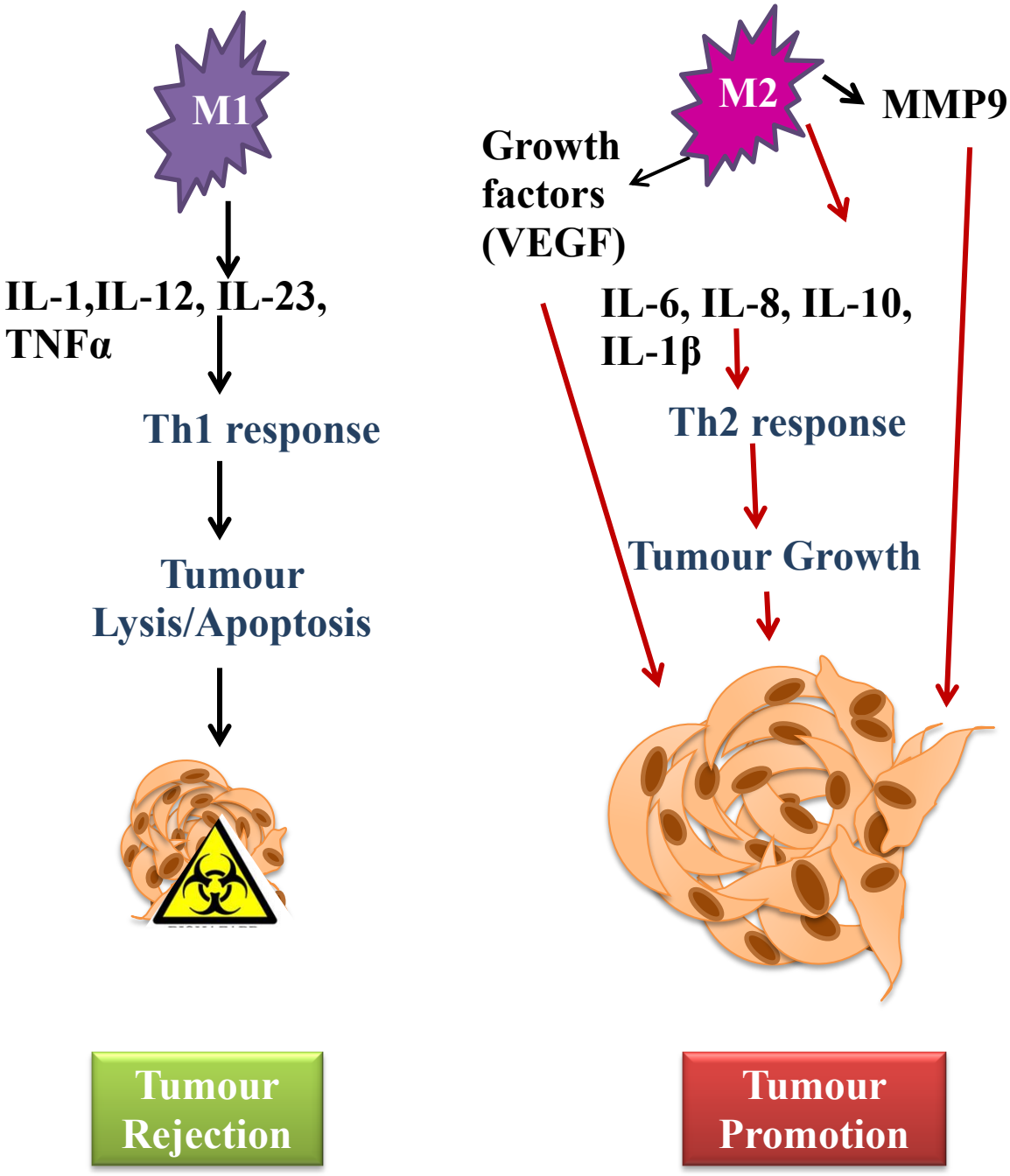


Figure 4: M1 versus M2 macrophages in tumorigenesis. Upon recruitment to the tumour, macrophages are exposed to factors derived from the tumour microenvironment that can skew their function. TAMs polarized towards an M1 phenotype play a vital role in tumour rejection, while TAMs polarized towards an M2 phenotype can drive tumour progression. M1 macrophages stimulate a tumour suppressing response via production of immunosuppressive cytokines, such as IL-1, IL-12 and TNFα. M2 macrophages activate a tumour promoting response by promoting angiogenesis, metastasis and invasion via a Th2 response. M2-polarized macrophages also produce potent pro-survival molecules. These molecules regulate gene expression in neoplastic cells by altering cell-cycle progression and increasing tumour cell survival. Together these processes circumvent immuno-surveillance and tumour-reactive immunity.

which are chemotactic factors for T-helper 1 (Th1) and cytotoxic T-cells (CTLs), while M2 macrophages mainly secrete CCL17 and CCL22, which recruit regulatory T cells (T-reg) and T-helper type (Th2) subsets [38, 39]. Studies have shown that tumour associate macrophages (TAMs) are phenotypically more like M2 macrophages [40, 41]. M2 TAMs can contribute to tumorigenesis through several mechanisms, such as release of PGE_2 and IL-10 which suppresses the anti-tumour immune response [42]. Alternatively activated TAMs facilitate tumour growth by secreting pro-angiogenic factors [43]. Although TAMs are predominantly associated with poor prognosis in many cancers, the role of TAMs in CRC still remains unclear. Indeed, numerous studies have shown that TAMs can positively influence CRC patient survival [44-46].

1.2.3 Tumour associated Neutrophils

Although not as well studied as TAMs, tumour associated neutrophils (TANs) can have either a pro-tumorigenic (N1) or an anti-tumorigenic (N2) phenotype. N1 neutrophils are cytotoxic to tumour cells and express increased levels of the pro-inflammatory cytokines IL-12, VEGF, TNF-α, and IL-1β [199]. N1 TANS also produce tumouricidal factors such as proteases [47], while N2-neutrophils secrete arginase to suppress T cell effector functions, comparable to M2 like TAMs. N2 TANS can also influence angiogenesis [48] through the production of mediators such as oncostatin M, which stimulate the production of VEGF by malignant cells [49].

1.2.4 Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are $CD11b^+Gr1^+$ immature myeloid cells that have been shown to be capable of suppressing immune responses. In addition,

these cells become pathologically activated and can directly support tumour progression. MDSCs suppress immune function in a number of ways including the expression of arginase, inducible NOS (iNOS) and COX2 [50].

1.2.5 Tumour infiltrating lymphocytes

Low numbers of lymphocytes are found in tumours, these cells predominantly localize to the invasive margin [51]. T cells can express either CD8 glycoprotein and are called $CD8^+$ T cells (cytotoxic) or CD4 glycoprotein and are called CD4 cells, but are also known as T helper (Th) cells. The two main subsets of $CD4^+$ cells are Th1 and Th2 cells. These cells differ in their cytokine secretion patterns and functions [52]. Th1 cells are involved in the maturation of B cells and the activation of macrophages. Th2 cells play a key role in immunological tolerance [53]. Although both $CD4^+$ and $CD8^+$ T cells can infiltrate tumours, $CD4^+$ T-cells are unable to recognise cancer cells directly [54], as opposed to $CD8^+$ T cells, which can directly kill cancer cells. Indeed, intraepithelial $CD8^+$ TILs were found to be a positive predictor of survival in CRC [55, 56]. Similarly low $CD8^+$ T cell infiltration is indicative of therapy resistance and poor prognosis in many human malignancies [57, 58].

1.2.6 Regulatory T cells

Regulatory T cells (T-regs) are $CD4^+CD25^+Foxp3^+$ cells involved in maintaining self-tolerance [59]. One of the ways in which they promote immune suppression in CRC is through production of TGF-β and IL-10. [60]. TGF-β can directly suppress effector T cell signalling [61]. However, the role of T reg cells in CRC is

controversial as numerous studies have shown that increased T reg cells are indicative of an improved prognosis [60, 62].

1.2.7 Natural killer cells

Natural killer (NK) cells are cytotoxic cells that target and kill both virally infected cells and cancer cells [63]. NK cells initiate the innate immune response [64] and can promote powerful anti-tumour cytotoxicity *in vitro.* Their cytotoxicity is dependent on the level of activation of the NK cells by the presence of surface markers, as tumours express both activating and inhibiting receptors [65]. Transformed cells may express reduced levels of MHC class I molecules in combination with other ligands. This enables them to evade being targeted by $CD8^+$ T cells and CTLs. However, this makes them more vulnerable to attack by NK cells [66].

1.3 The IL-1 cytokine

The cytokine, IL-1, has been the subject of copious research since it was first identified. This pro-inflammatory cytokine is known to play a dichotomous role in disease by inducing pathogenesis of auto-inflammatory disorders, while simultaneously defending against invading pathogens [67]. IL-1 is implicated in the development of numerous inflammatory diseases including ulcerative colitis [68]. This has resulted in the mechanism behind IL-1 signalling becoming the subject of much interest. Likewise, novel members of both the IL-1 family of cytokines and the IL-1 receptor (IL-1R) family are also the focus of intense research in order to determine their involvement in the host response to disease. I will now discuss these cytokines and receptors in more detail, focusing specifically on the IL-33/ST2 and IL-36/ IL-36R pathways as these are the subject of this thesis.

1.3.1 IL-1 and IL-1R family members

The IL-1R/ toll-like receptor (TLR) superfamily consists of two subgroups: the IL-1R family whose members contain three extracellular immunoglobulin domains (Ig) and the Toll-like receptor subgroup whose members contain extracellular leucine-rich repeats. All family members share a similar intracellular Toll-IL1R (TIR) domain, which is required for binding of adaptor proteins. The IL-1 family is divided into three subfamilies based on the length of the N-terminal domain (Table 1). The IL-1 subfamily consists of IL-1α, IL-1β, IL-1Ra and IL-33. The IL-18 subfamily is composed of IL-18 and IL-37, and finally the IL-36 subfamily contains IL-36α, IL-36β, IL-36γ, IL-36RN and IL-38.

The IL-1R family members are as follows; the type I IL-1 receptor (IL-1R1) [69], which binds IL-1α and IL-1β and was the first member of the family to be identified, the type II IL-1 receptor (IL-1R2) which is a decoy receptor [70], ST2 (IL-1RL1) which binds IL-33 [71-74], and the IL-1Rrp2 (IL-36R), which binds three agonistic ligands, IL-36α, IL-36β and IL-36γ (Figure 5) [75]. Other IL-1R family members include the IL-1 receptor accessory protein (IL-1RAcP) [76], which acts as a co-receptor for IL-1R, ST2 and IL-36R, the IL-18 receptor (IL-18Rα) [77, 78] and its accessory protein (IL-18Rβ), and TIGIRR-2 (IL-1 receptor accessory protein like) [79, 80] and the three Ig domain-containing IL-1 receptor-related (TIGIRR-1) [79, 81] . Single Ig-domain containing Il-1 receptor related (SIGIRR) [82] is also a family member although it only possesses one Ig-domain extracellularly, similar to the IL-

Previously called	Name	Receptor/ coreceptor	Property	Synthesized as precursor	Processing required for activity
IL-1F1	IL-1α	IL-1R1/ IL-1RAcP	Proinflammatory	Yes	No
IL-1F2	IL-1β	IL-1R1/ IL-1RAcP	Proinflammatory	Yes	Yes
IL-1F3	IL-1Ra	IL-1R1	Antagonist for IL-1α,β	No	No
IL-1F4	IL-18	IL-18α/ IL-18β	Proinflammatory	Yes	Yes
IL-1F5	IL-36Ra	IL-1Rrp2	Antagonist for IL-36	Yes	Yes
IL-1F6	IL-36α	IL-1Rrp2/ IL-1RAcP	Proinflammatory	Yes	Yes
IL-1F7	IL-37	IL-18Rα,IL-18BP	Anti-inflammatory	Yes	Yes
IL-1F8	IL-36β	IL-1Rrp2/ IL-1RAcP	Proinflammatory	Yes	Yes
IL-1F9	IL-36γ	IL-1Rrp2/ IL-1RAcP	Proinflammatory	Yes	Yes
IL-1F10	IL-38	IL-1Rrp2	?Antagonist	Yes	?No
IL-1F11	IL-33	ST2/IL-1RAcP	Proinflammatory	Yes	No

Table 1: IL-1 family ligands and receptors The IL-1 family is divided into three subfamilies based on the length of the N-terminal pro-pieces. The IL-1 subfamily shown in green consists of IL-1α, IL-1β, IL-1Ra and IL-33. The IL-18 subfamily is composed of IL-18 and IL-37 shown in blue, and finally the IL-36 subfamily contains IL-36α, IL-36β, IL-36γ, IL-36Ra and IL-38 (purple). The TIR domain of each receptor chain comes together and recruits MyD88. IL-36Ra binds to IL-1Rrp2 but fails to form a complex with IL-1RAcP. Thus, IL-36Ra prevents the binding of IL-36α, IL-36β, or IL-36γ to IL-1Rrp2, and thus is the natural IL-36R antagonist.

18BP [83]. SIGIRR is considered an orphan receptor, and it also has regulatory activity [84].The IL-1 family of cytokines share a core tetrahedron-like structure composed of 12 β-strands [85]. This family consists of 11 members, of which seven are agonistic (i.e. IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ) and four are antagonistic (IL1Ra, IL-36Ra, IL-37 and IL-38). The genes encoding the IL-1 family cytokines cluster on human chromosome 2, with the exception of IL-33 and IL-18, which are located on chromosome 9 and 11, respectively [86]. IL-1 family members form heterodimeric transmembrane receptor complexes and trigger signalling pathways that include the adaptor molecule MYD88, IRAK kinase family members and the ubiquitin ligase TRAF6.

1.3.2 IL-1R family signalling

The receptor complex for each IL-1 family cytokine is a heterodimer with a specific receptor and a common subunit, usually the IL-1RAcP. Once the complex is formed, following interaction with its cognate ligand, the TIR domain of each receptor chain come together and recruit MyD88, followed by phosphorylation of IL-1R–associated kinases (IRAKs) and activation of TRAF6 (Figure 5). This pathway diverges to activate mitogen activated protein kinases (MAPKs) such as ERK, JNK and p38 or to activate TAK1, which subsequently results in the translocation of NF-κB to the nucleus. However, not all IL-1 family receptors activate these pathways; e.g. SIGIRR is an inhibitory receptor, inhibiting IL-1, IL-18 and IL-33 signalling [87].

1.3.3 IL-1α, IL-1β and the IL-1R complex

The IL-1R1 is the best characterised receptor in the IL-1R superfamily. This 80kDa glycoprotein binds the agonists IL-1α and IL-1β, which have similar biological properties. However, there are some differences between these cytokines. The IL-1α

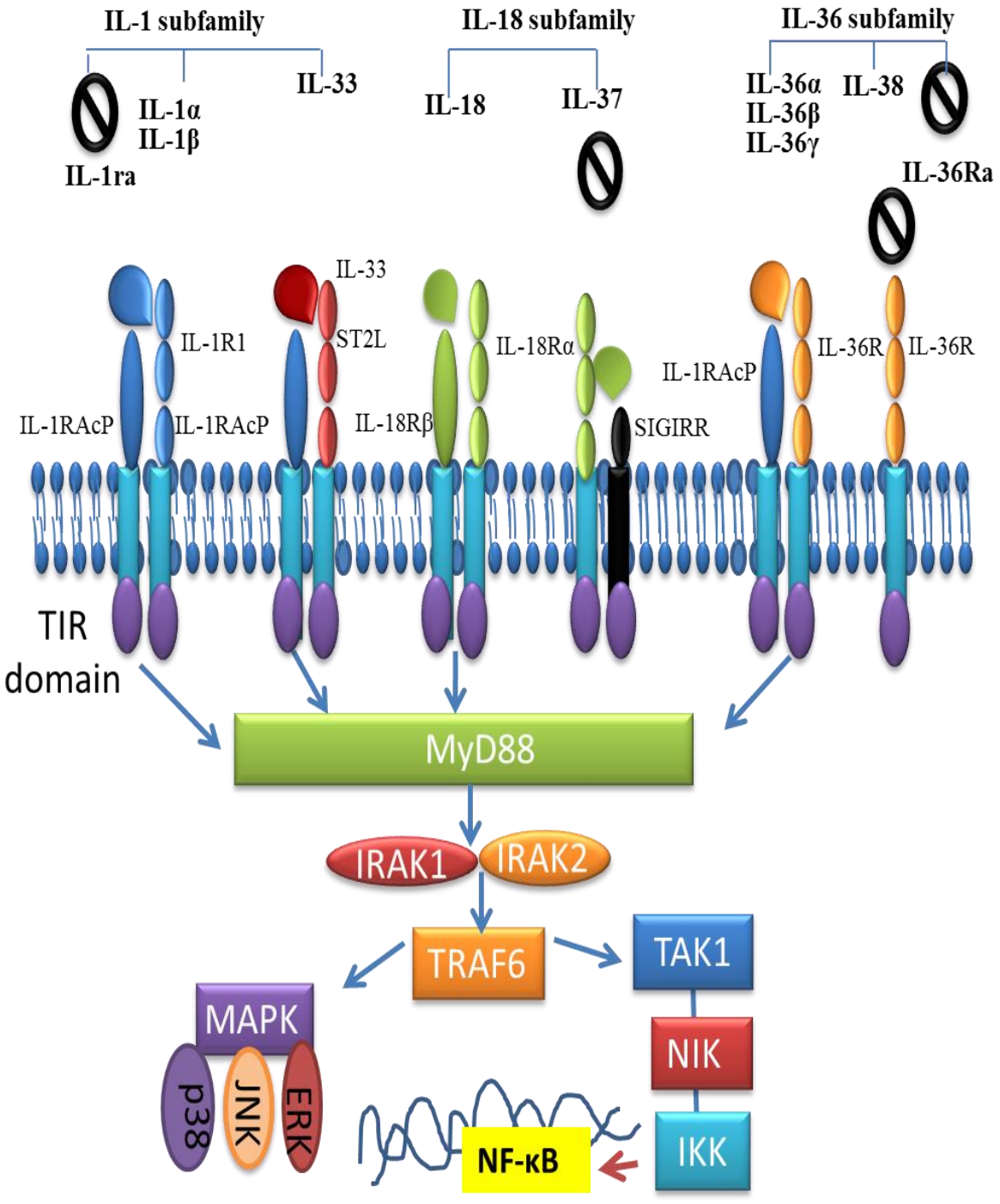


Figure 5: IL-1R family signalling activates MAPK and NF-κB. The IL-1 subfamily consists of IL-1α, IL-β, IL-1Ra, and IL-33. The IL-18 subfamily is composed of IL-18 and IL-37. While IL-36α, IL-36β, IL-36γ, IL-36Ra and IL-38 all belong to the IL-36 subfamily. The receptor for each IL-1 family cytokine is a heterodimer composed of a specific receptor and a common co-receptor. The TIR domain of each receptor chain align and recruit MyD88, followed by phosphorylation of IL-1R–associated kinases (IRAKs), activation of TRAF6, and this pathway activates mitogen activated protein kinases (MAPKs). TAK1 can also be activated this results in the translocation of NF-κB to the nucleus. However, not all IL-1R family members activate these pathways. SIGIRR contains an altered TIR domain and inhibits IL-18 signalling.

precursor is fully active and found in epithelial layers throughout the gastrointestinal tract. It is released upon necrotic cell death to function as a cellular “alarmin” [88, 89]. IL-1α also contains a nuclear localization signal and localizes to the nucleus. In contrast, IL-1β is produced by haematopoietic cells such as macrophages and must be cleaved by caspase 1 to become active. Moreover, it has never been observed in the nucleus. In tumorigenesis another variation between IL-1α and IL-1β has been observed. IL-1β-deficient mice develop fewer tumours compared to IL-1α-deficient mice [90]. IL-1β has also been shown to induce angiogenesis and enhance metastatic spread [91]. The IL1R antagonist (IL-1Ra) functions to block both IL-1α and IL-1β activity. The type II IL-1 R contains a truncated cytoplasmic domain lacking the TIR domain and therefore is unable to transduce a signal upon IL-1 ligation. It functions as a decoy receptor to negatively regulate IL-1 signalling [92].

1.3.4 The IL-18 subfamily

The cytokine IL-18 was first recognised in 1995 as a potent inducer of IFN-γ [93]. The receptor for this cytokine, the IL-18R, was identified as a member of the IL-1R family due to its homology to the IL-1R1, IL-1RAcP and ST2 [77]. The IL-18R shares the common conserved sequences found in all members of the IL-1R family. Similar to IL-1β, IL-18 is synthesized as an inactive precursor and requires activation by caspase-1 cleavage. The IL-18 precursor is constitutively expressed in most human and murine cells [94]. Once activated, IL-18 binds to the IL-18 α-chain (IL-18Rα) forming a signalling complex. The accessory protein IL-18R β-chain (IL-18Rβ) then associates to form a heterodimeric complex. The IL-18 binding protein (IL-18BP) is located in the extracellular compartment and regulates IL-18 activity by binding to mature IL-18 and preventing it from activating the IL-18R. IL-37 is also a

member of the IL-18 family. The IL-18 precursor is structurally similar to the IL-37 precursor and IL-37 also binds to the IL-18R to inhibit signalling. IL-37 is an anti-inflammatory cytokines, similar to IL-10. IL-37 suppresses innate inflammation and immune responses in a number of ways, including by inhibiting DC activation at the cellular level [95].

1.4 ST2, a member of the IL-1R subfamily

ST2 is a member of the IL-1R subfamily and was identified in 1989 [72, 73, 96]. The ST2 gene is located on chromosome 2q12 in humans and contains 13 introns and spans 40 kilobases (kb) [97]. In humans three splice variants exist, ST2L, sST2 and ST2V (Figure 6a). ST2L is a transmembrane bound receptor and as such specifically belongs to the Toll/IL-1R (TIR) superfamily and shows homology to the intracellular domain of IL-1R1 [97-99]. sST2 is a soluble protein with no transmembrane sequence, it is excreted extracellularly and binds to IL-33. Both ST2L and sST2 contain three identical Ig extracellular domains, although sST2 lacks the transmembrane sequence. Instead sST2 contains an additional 9 amino acids at the C terminus. sST2 is thought to act as a decoy receptor sequestering IL-33 away from the transmembrane bound receptor ST2L [100]. ST2V is a membrane-bound receptor that contains a hydrophobic tail [101]. It contains two Ig domains and is expressed in the gut [102]. The function of ST2V has not been fully elucidated [103].

1.4.1 Regulation of ST2 expression

All ST2 isoforms are produced from the IL-1RL1 gene as a result of alternative splicing under the control of two distinct promoters, the distal and the proximal promoter (Figure 6b) [104, 105]. Early ST2 expression studies were carried out on

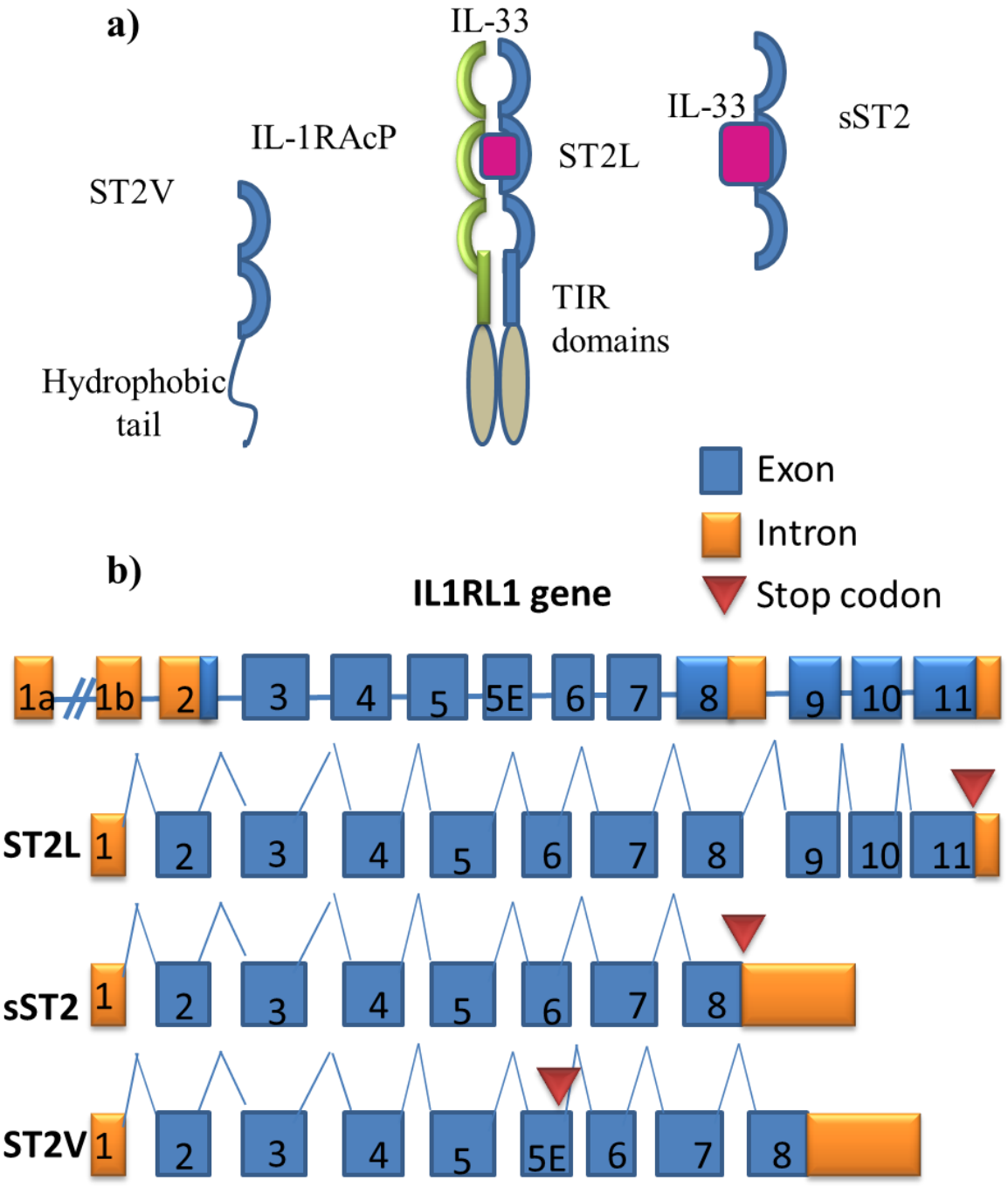


Figure 6: There are three human ST2 isoforms- ST2L, sST2 and ST2V. ST2 is encoded by the IL1RL1 gene. In humans three splice variants exist, ST2L, sST2 and ST2V. (a) ST2L is a transmembrane receptor that binds to IL-33. sST2 is a soluble protein with no transmembrane sequence and can also bind to IL-33. ST2V is a membrane-bound receptor that contains two Ig domains and a hydrophobic tail. (b) All ST2 isoforms are produced from the IL-1RL1 gene as a result of alternative splicing under the control of two distinct promoters, i.e. the distal and the proximal promotor.

the rat ST2 gene, known as *Fit1*. The ST2 gene contains two discrete promoters. It was originally suggested that the sST2 and ST2L mRNA isoforms were transcribed from different promoters resulting in alternative splicing to generate two discrete 3' coding sequences [106]. Further investigations demonstrated that both the human and murine ST2 genes similarly contain both promoters, a distal and a proximal promoter [104]. However, in contrast to the initial study, it was shown that the two isoforms can be transcribed from either promoter, with the choice of promoter governed by the cell type. For instance, the human leukaemic cell line, UT-7 can transcribe both ST2 isoforms using either the distal or proximal promoter, but predominantly use the distal promoter. In contrast, most ST2 expression in the human TM12 fibroblastic cell line is initiated from the proximal promoter [105]. Similarly, it was found that mast cells utilised the distal promoter, while fibroblasts employed the proximal promoter, suggesting that promoter usage is dependent on the cell type and is not transcript specific [104].

The distal ST2 promoter contains two GATA consensus sites which enable binding of the transcription factors GATA1 and GATA2, both of which are involved in the regulation of ST2 gene expression. These transcription factors bind upstream of the transcription start site and regulate ST2 gene transcription. The ST2 promoter is transactivated by GATA-2 and repressed by GATA-1 in mast cells and basophils [107]. In mast cells and Th2 cells, ST2L expression is regulated by transcription factors GATA-3 and STAT5 [108, 109], while the transcription factor Gfi1 was found to upregulate surface expression of ST2L in innate lymphoid cells [110]. IL-33, the ligand for ST2, has been found to downregulate sST2 and ST2L mRNA in pancreatic cancer cells [111].

1.4.2 Expression of ST2

The kidney, lung, placenta and stomach all express high levels of sST2 and ST2L. In addition, many endothelial cells from lung, bronchus, coronary artery and umbilical cord express both ST2L and sST2 mRNA [112]. The expression levels of the isoforms vary, with ST2L expression found to be higher in the spleen, heart, testis and colon than sST2, whilst sST2 expression levels are higher in the brain and liver [97]. sST2 is induced by serum. ST2L is also expressed on the surface of fibroblasts and hematopoietic cells such as T helper type 2 (Th2) lymphocytes and mast cells [113, 114], and has recently been found to be expressed by Th1 cells, $CD8^{+}$T cells, NK cells and NKT cells [115-118].

1.4.3 IL-33, the ligand of ST2L

IL-33 was originally named nuclear factor from high endothelial venules (NF-HEV), as it was first found to be expressed in the nucleus of quiescent endothelial cells [119-121]. Later, in 2005, IL-33 was identified as a member of the IL-1 family of cytokines and as the ligand for ST2L [122]. Unlike other IL-1 family members, which are located on chromosome 2, the IL-33 gene is located on chromosome 9 at 9p24.1. It spans ~16 kb and contains seven exons and contains two alternative promoters. Human IL-33 comprises 270 amino acids (aa), while murine IL-33 consists of 266 aa [85].

1.4.4 Processing of IL-33

Different IL-33 splice variants have been observed in human tissues. IL-33 is generated as a full-length protein [123-127], and contains a caspase-1 cleavage site [122]. It was originally proposed that full-length IL-33 required cleavage by caspase-

1 [123, 125], similar to IL-1β and IL-18, to generate the mature biologically active form of the cytokine [125-127]. However, further studies demonstrated that full-length IL-33 does not require activation by caspase-1 to be active [128]. Although IL-33 is not activated by caspase-1, IL-33 can be processed by caspase-3 and caspase-7 to yield biologically inactive fragments [125-127].

Processing by proteolytic enzymes may play a vital part in modulating IL-33 activity during inflammation. Un-cleaved full-length IL-33 is released from the nucleus of barrier tissue during necrosis and functions as a danger signal or 'alarmin'. However, the alarmin signal can be amplified by cleavage of macrophage, neutrophil, or mast cell-derived proteases, such as elastase, which cleaves full-length IL-33 into the IL-$33_{95\text{-}270}$, IL-$33_{99\text{-}270}$, and IL-$33_{109\text{-}270}$ mature forms. These isoforms are between 18 and 21 kDa in human and 20 kDa in mouse, i.e. IL-$33_{102\text{-}266}$. These mature forms of IL-33 have up to 30-fold increased biological activity compared to the full length uncleaved, IL-33 [129]. In this way a small number of alarmin molecules can have a potent local effect upon proteolytic cleavage of the N-terminus. Bioactivity is lost upon cleavage of the core IL-1 family structure by chymase or other proteases secreted by mast cells, thus silencing the alarmin signal [130]. A second mechanism of silencing IL-33 has also been documented. This involves the formation of two disulphide bridges, which inhibits binding of IL-33 to ST2L, thus abrogating the ability of IL-33 to transduce a signal [131].

1.4.5 IL-33 expression

IL-33 is expressed by a diverse range of cells in many organs, such as activated leukocytes, especially innate immune cells (e.g. mast cells, macrophages and DCs), endothelial cells, epithelial cells, keratinocytes, fibroblasts, fibrocytes and smooth

muscle cells [132]. IL-33 can be induced by immune stimuli. For instance, pro-inflammatory stimuli or pathogen recognition receptor (PRRs), especially TLRs, induce IL-33 expression in immune cells [133]. Similarly, in human keratinocytes IL-33 expression is constitutively weak but is strongly induced during inflammation [134]. In contrast, although IL-33 is also constitutively expressed in mouse epithelial cells of various origins, expression is lost during acute inflammation [134]. However, this is thought to be due to its release to perform its alarmin function.

IL-33 exhibits two diverse functions. IL-33 can function as a cytokine that binds to ST2L and regulates the immune response, epithelial repair and activates Th2 cells. IL-33 can also function as a nuclear factor involved in maintaining barrier function through gene regulation. However, once the barrier is breached, IL-33 is released and functions as an 'alarmin'. The structure of IL-33 is vital to its multiple functions. IL-33 shares the IL-1 family C-terminal core tetrahedron structure, and similar to other IL-1 cytokine members, IL-1β and IL-18, IL-33 is translated without a signal sequence for secretion [135].

1.4.6 Nuclear IL-33 regulates gene expression

Under homeostatic conditions IL-33 predominantly localizes to the nucleus as it possesses a nuclear localization signal within its N terminus [136]. Here it functions as a nuclear factor, binding directly to chromatin in the nucleus. Chromatin interaction is enabled by a conserved homeodomain-like helix-turn-helix motif located in the N-terminal domain [136, 137]. Nuclear IL-33 regulates gene expression in numerous ways. IL-33 binds to the nucleosome acidic patch in histone H2A-H2B dimers and regulates chromatin structure and by default, gene expression [136-138]. IL-33 also activates histone deacetylase-3 (HDAC3) activity, indicating a

potential role for IL-33 in modulating epigenetic regulation [139]. IL-33 may also bind to the transcriptional repressor, histone methyltranserase SUV39H1 [136]. Additionally, nuclear IL-33 has been reported to directly bind to NF-κB, suppressing its activity [140]. IL-33 has been shown to alter expression of the p65 NF-κB subunit by binding to the p65 promoter [141].

1.5 IL-33/ST2 signalling

IL-33 functions as a cytokine once it has been secreted into the extracellular environment. Similar to other IL-1 family members, it binds to its specific plasma membrane receptor, ST2L. Once IL-33 has bound to ST2L, the receptor undergoes a conformational change, recruiting the IL-1RAcP, thus bringing the two intracellular TIR domains together [85]. Signalling occurs as previously described in section 1.2 (IL-1 family signalling). Similar to other IL-1 signalling pathways, IL-33 signal transduction may result in the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2, p38 MAPK and JNKs (Figure 7) [122]. IL-33 may therefore also activate the transcription factor AP-1 independently of NF-κB activation [142]. Indeed, although TRAF6 appears to be required for IL-33-mediated NF-κB activation, IL-33-mediated ERK activation may be TRAF6-independent [143]. This indicates a level of variation between the IL-33/ST2 pathway and other IL-1R signalling pathways.

In certain cells, such as mast cells, IL-33/ST2L signalling appear to be more complex as the IL-1RAcP has been shown to be complexed to c-Kit, a receptor tyrosine kinase (RTK). ST2L cross-activates c-Kit, and this RTK regulates IL-33 signalling in mast cells [144]. c-Kit is activated by its ligand, stem cell factor (SCF). As well as mast cells, c-Kit is also expressed by haemopoiectic stem cells, progenitor

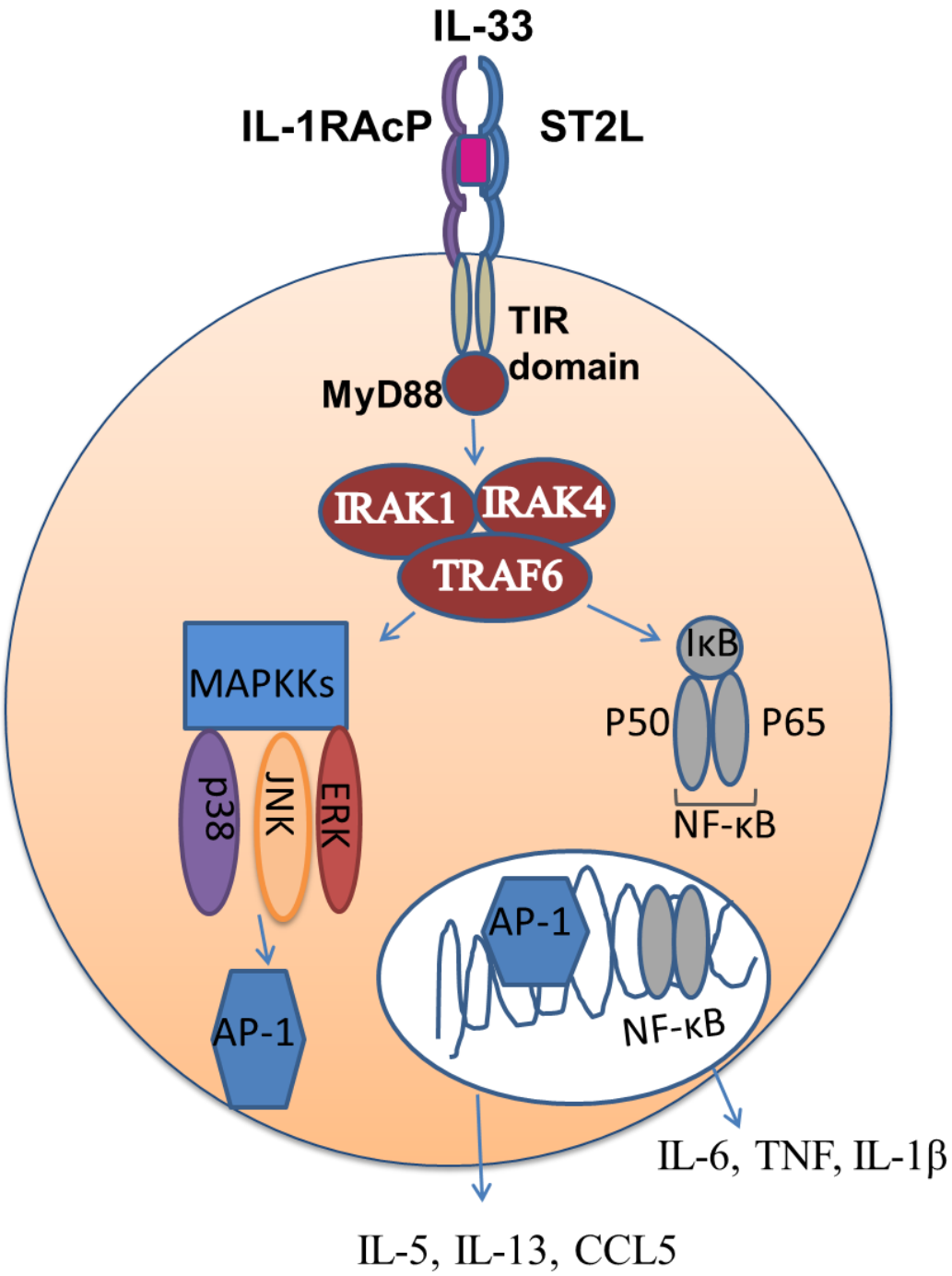


Figure 7: IL-33/ST2 signalling in immune cells. IL-33 binds to the ST2L receptor and once the IL-1RAcp is recruited, the TIR domain of IL-1RAcp aligns with the ST2L TIR domain. The heterodimeric complex acts as a scaffold for the recruitment of MyD88, IRAK-1 and IRAK4. This results in the phosphorylation of IκB which activates the transcription factor NF-κB. AP-1 has also been shown to be activated through phosphorylation of the MAPK signalling pathway. This activates a pro-inflammatory response via the induction of cytokines and chemokines such as IL-6, IL-1β, TNF and CCL5.

cells, innate lymphoid cells and neoplastic cells. It is unclear whether other RTKs associate with the IL-33/ST2L complex, and are involved in attenuating IL-33 responses [145].

Downstream of IL-33 signalling, activation of this pathway has been shown to have far reaching effects in many different cell types. The number of genes or proteins modified varies depending on the cell type being examined. In RAW 264.7 murine macrophages, IL-33 stimulation altered the expression of over 670 proteins [146]. In CCD-18Co, a subepithelial myofibroblast cell line, IL-33 upregulated 700 transcripts and downregulated 650 transcripts [147]. In human umbilical vein endothelial cells (HUVECs), IL-33 regulated over 300 genes [148]. Further research on the response of cells to stimulation with IL-33 alone and combined with other cytokines may provide an insight into the *in vivo* crosstalk which occurs during inflammation.

1.5.1 Regulation of ST2L signalling

Given the potent immune reactions that occur following IL-33-induced ST2 signalling it is essential that this pathway is tightly regulated. Activation of ST2L by IL-33 results in activation of focal adhesion kinase (FAK) and glycogen synthase kinase-3β (GSK-3β) (Figure 8) [149]. Activated GSK-3β binds to ST2L at Ser446 and phosphorylates it at Ser442. This results in the swift internalization of ST2L [150]. Transmembrane emp24 domain- containing (TMED1) is a protein involved in the vesicular trafficking of proteins. TMED1 co-precipitates with ST2L [151]. Once internalized, ST2L is polyubiquitinated by the E3 ubiquitin ligase FBXL19, [149], leading to proteasomal degradation of ST2L. IL-33 and ST2L signalling can also be regulated in other ways. Unlike other IL-1 family members, there is no known

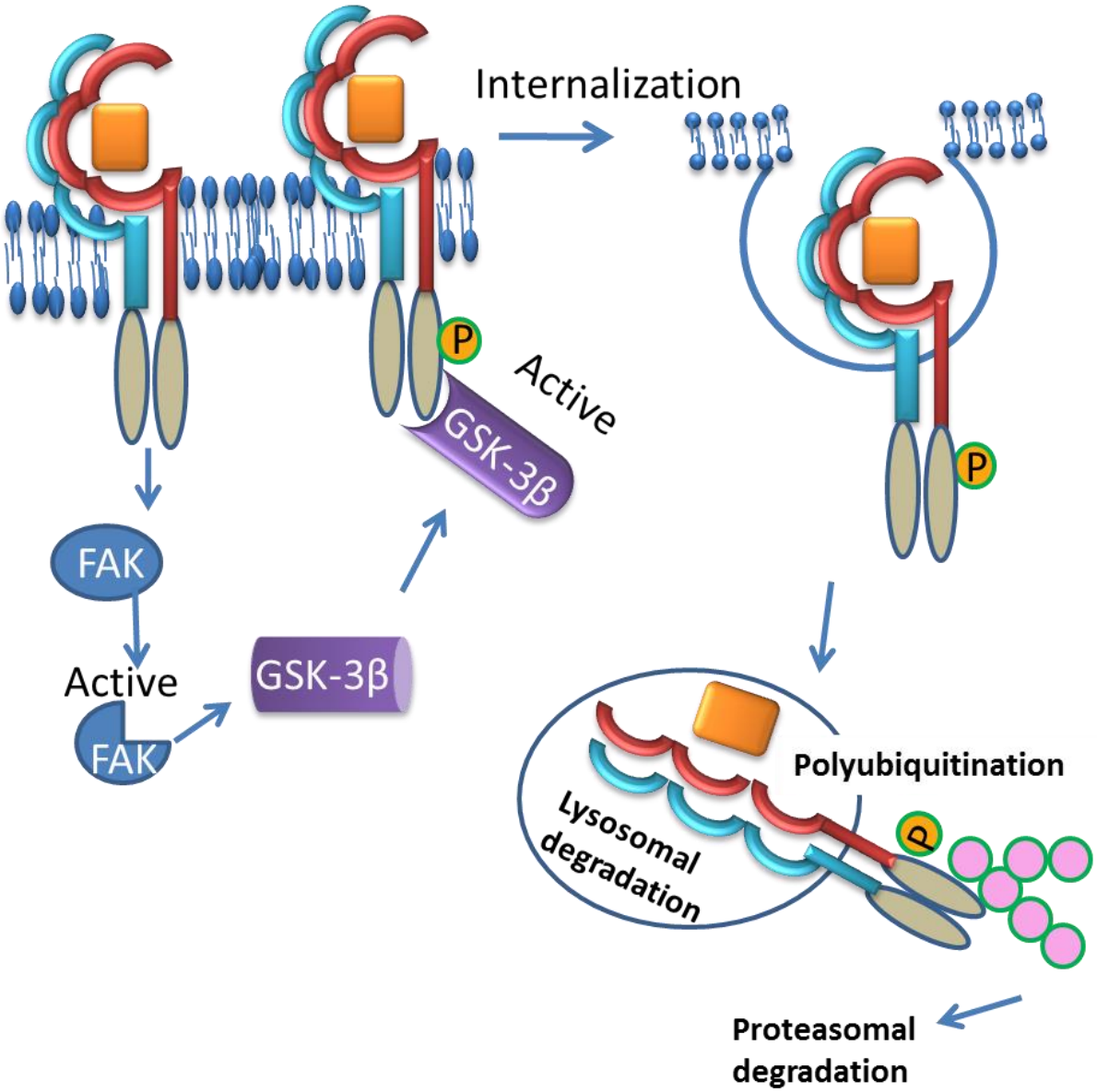


Figure 8: Degradation of the IL-33 signalling complex.. Internalization and degradation of the IL-33 signalling complex occurs through the activation of FAK, which in turn activates GSK-3β. GSK-3β phosphorylates ST2L and once labelled ST2L is rapidly internalized. The IL-33R complex is internalized and extracellular regions are digested. The cytoplasmic region of ST2L is polyubuitquitinated and sent for proteasomal degradation. This figure was adapted from N. Martin *et al*., IL- 33 is a guardian of barriers and a local alarmin, 2015.

antagonistic ligand for ST2L. However, SIGIRR is an IL-1R family member that associates with ST2L. It is known to negatively regulate IL-1R-mediated immune responses [152], and may interact with ST2L to inhibit IL-33/ST2L signalling. Evidence of this comes from SIGIRR-/- mice where IL-33 has been shown to induce a greater inflammatory response in the absence of SIGIRR [153, 154]. The heterodimer IL-1RAcP that makes up the IL-33/ST2 signalling complex is constitutively expressed at low levels and does not appear to be subject to notable modulation.

1.6 A physiological role for IL-33

1.6.1 A role for IL-33 in barrier function and epithelial wound healing

IL-33 is constitutively expressed by cells involved in the maintenance of mechanical barriers, including keratinocytes, lung and gut epithelial cells, fibroblasts and smooth muscle cells [145]. In these cells IL-33 is localized to the nucleus and mediates gene transcription. IL-33 is believed to maintain a quiescent state as it is only produced in barrier cells when they are senescent. Indeed, downregulation of IL-33 has been linked to the initiation of cellular proliferation in barrier cells [145]. Once a barrier is breached, nuclear IL-33 is released and functions as an “alarmin” or damage associate molecular pattern (DAMP) [155]. This extracellular IL-33 binds to ST2L expressing cells, such as mast cells, DCs, macrophages or group 2 innate lymphoid cells to activate a primary acute immune response. Th2 and mast cells constitutively express ST2L on the surface and rapidly respond to IL-33 [156]. Mast cells secrete pro-inflammatory cytokines, typically resembling a Th2 response (i.e. IL-4 and IL-5), thus promoting the migration of basophils and eosinophils to the site of the barrier breach. Proteases are also secreted which disrupt connective tissue to allow

the influx of immune cells. These enzymes cleave the full-length IL-33 alarmin into N-terminally truncated forms, which can amplify the bioactivity up to 30-fold [131, 157, 158]. Activated mast cells also actively secrete IL-33, leading to high local levels of IL-33.

At the same time, recent data has shown IL-33 can trigger wound healing responses, to rapidly repair the damaged barrier by directly activating surrounding fibroblasts [159-161]. DCs are activated in this inflammatory environment and phagocytose pathogens. The pathogenic antigens are transported to nearby lymph nodes to activate naïve T helper cells, thus activating the appropriate immune response. Eventually, IL-33 bioactivity is extinguished upon destruction of the IL-1 family core structure, by chymase produced by activated mast cells [130, 158].

Consistent with its role in maintaining barrier function, IL-33 is emerging as a significant mediator of colonic mucosal wound healing. Indeed, administration of IL-33 was shown to promote wound healing in a surgical incision model by increasing re-epithelialisation and extracellular matrix (ECM) deposition [162]. Following damage to the intestinal barrier, an initial inflammatory phase occurs. IL-33 is significantly upregulated during this phase. The increased levels of IL-33 activate local innate immune cells. Subsequently, a proliferative phase occurs, during which collagen is laid down and angiogenesis occurs. This is followed by a remodelling stage. During this time, inflammation is reduced and intestinal homeostasis is re-established. Finally, fibrosis takes place [163].

1.6.2 The role of IL-33/ST2 in immune cells

Given the high level of expression of ST2 on Th2 cells, a vast amount of literature has explored the role of IL-33/ST2 signalling in these cells and the effects of this

pathway on the cell populations. ST2L modulates effector functions in infectious, allergic and autoimmune disorders [164]. ST2L is expressed by polarized Th2 cells and can induce production of Th2 cytokines such as IL-4, IL-5 and IL-13 [122]. This is in contrast to Th1 cells, which produce mainly interferon (IFN)-γ, IL-2 or TNF-β [113]. Treatment of mice with recombinant IL-33 induced a Th2-mediated immune response. Increased IL-5, IgE and eosinophil recruitment was also observed, accompanied by goblet cell hyperplasia and increased mucus production [165].

Recently innate lymphoid cell (ILC) populations have been shown to strongly express ST2 and as such, much recent focus concerns this pathway in ILC2s. Increased ILC populations are found at barrier surfaces such as the skin, lung and gut [166, 167]. ILCs are involved in regulating the immune response from initiation to resolution of inflammation. These cells produce many pro-inflammatory and immunoregulatory cytokines in response to cytokine or microbial stimuli [166-168]. This cell population can be divided into three groups depending on their expression of surface markers, transcription factors and cytokines [167]. Group 2 ILCs (ILC2s) respond to IL-33, as well as thymic stromal lymphopoietin (TSLP) and IL-25. In the lung, following damage to the epithelial barrier, the 'alarmin' IL-33 is released and ILC2s become activated [169]. Depletion of ILC2s in mice reduced repair of the airway epithelium in influenza infected mice. [169]. In an intestinal murine model, ILC2s were shown to support eosinophil development and survival in the intestine via IL-5 production. These cells also modulate tissue-resident eosinophils by secretion of IL-13 and subsequent eotaxin production [170]. As well as regulating innate immunity, ILCs have also been implicated in the regulation of adaptive immunity [166-168], as studies have suggested that ILC2s may be involved in crosstalk with other cells expressing ST2L, such as T-regs [171-173].

1.7 The Pathological functions of IL-33/ST2

As IL-33/ST2 is such a potent inducer of inflammation, it is not surprising that dysregulation of the pathway has been linked to the pathogenesis of several autoimmune disorders. In asthma, IL-33 was shown to be a key driver of lung inflammation, as lymphocyte and eosinophil recruitment and general lung inflammation were reduced in the absence of IL-33. This suggests that the IL-33/ST2 axis could be a potential therapeutic target in asthma [174]. IL-33 is also implicated in rheumatic diseases. Indeed, IL-33 was detected in the serum of rheumatoid arthritis (RA) patients, while being completely absent in healthy controls [175]. The level of circulating IL-33 correlated with IgM and RA-related antibodies. Research to date also suggests that IL-33 has a pro-inflammatory effect in various rheumatological diseases, activating synovial fibroblasts and mast cells in the joints [153]. IL-33 was also increased in the skin lesions of patients with atopic dermatitis compared to non-inflamed skin [176], thus indicating a role for IL-33 in psoriatic pathology.

1.7.1 IL-33/ST2 in inflammatory bowel disease

IL-33 and ST2 are also implicated in the pathogenesis of inflammatory bowel disease (IBD). Both IL-33 mRNA and protein were found to be upregulated in inflamed ulcerative colitis (UC) and Crohn's disease (CD) [177, 178]. It has been reported that IL-33 expression was increased in the epithelium of mild to moderate UC. As the intestinal epithelium becomes damaged and mucosal lesions form in severe UC, IL-33 becomes increased in myofibroblasts, where it functions as an alarmin [179, 180]. The expression pattern of ST2 was also altered in IBD. ST2 expression becomes depleted in intestinal epithelial cells (IECs), and is prominent in

the lamina propria of IBD patients. The expression of ST2 isoforms is also altered in UC, ST2L becomes reduced [177], while sST2 is increased compared to CD and control tissue [177, 181]. Circulating levels of both IL-33 and sST2 were also increased in UC patients compared to controls [177]. Expression of IL-33 and ST2 were found to be regulated by TNF, as anti-TNF therapy reduced IL-33 levels and increased sST2 expression in UC patients [177].

Mouse models of IBD have shown a complex role for IL-33 [177, 182] and ST2 [183] in this disease. Loss of IL-33 or ST2 improved symptoms and reduced intestinal inflammation in the early stage of dextran sodium sulphate (DSS) induced colitis. Contrastingly, however, a further study reported delayed resolution of DSS dependent tissue damage in IL-$33^{-/-}$ mice [174]. Consistent with this finding, treatment with exogenous IL-33 ameliorated chronic DSS colitis [184]. Therefore, IL-33 may have dual roles in intestinal pathology as well as in the maintenance of intestinal homeostasis.

1.8 ST2 as a positive prognostic indicator

ST2 has been investigated in many diseases including obesity, atherosclerosis, and cardiovascular disease. Studies to date indicate that sST2 may be useful as a possible prognostic biomarker in cardiovascular disease [185-187], as sST2 levels are associated with heart failure severity and poor outcome [185, 188]. Circulating levels of sST2 also appear to decline with improved prognosis in patients with heart failure [189, 190]. Thus, sST2 was recently included in current AHA guidelines for determining risk in acute and chronic heart failure patients [191]. However, concerns were raised regarding the specificity of sST2 as it is also increased in other disease types such as autoimmune disorders.

1.9 IL-33/ST2 in cancer

A link between the IL-33/ST2 signalling axis and tumorigenesis has recently been identified. Immune infiltrates within tumours can positively or negatively influence patient mortality by altering angiogenesis, metastasis and response to therapeutics [3, 192]. Therefore, it is vital to determine the effect of specific cytokines on tumorigenesis. Initially the link between IL-33/ST2 and cancer was identified in breast cancer and most research to date has focused on this cancer type. Early studies utilising $ST2^{-/-}$ mice demonstrated that ST2 deletion inhibited breast cancer progression and increased the intra-tumoural accumulation of both innate i.e. NK cell and acquired immune cells i.e. $CD8^{+}$ T-cells and Th1/Th17 cytokines [193], indicating a lack of IL-33 signalling through ST2L promotes a Th1 response. In addition, suppressing sST2 reduced ErbB2-induced cell motility in breast cancer cells. Moreover, breast cancer patients with metastatic disease showed increased levels of circulating sST2 compared to patients with primary tumours [194]. Further studies in breast cancer also showed significantly higher levels of both IL-33 and sST2 in the serum of patients with ER positive breast cancer relative to healthy controls [195]. Moreover, administration of IL-33 to breast cancer-bearing mice showed accelerated tumour growth and increased metastasis. The proposed mechanism responsible for the enhanced tumour growth was the increase in the number of infiltrating immunosuppressive immune cells and innate lymphoid cells, providing further evidence of the role of IL-33 in driving carcinogenesis [196].

Since the link between IL-33/ST2 in carcinogenesis was first identified in breast cancer, this pathway has now been examined in numerous cancer types. Consistent with a role for IL-33 and ST2 in promoting tumour metastasis and invasion,

inhibition of IL-33 and ST2 in glioma cells resulted in reduced tumour growth and colony formation *in vitro,* and reduced tumour size *in vivo* [197]. In contrast, an anti-tumorigenic role for IL-33 has been reported in other studies, with IL-33 reduced in the serum of non-small cell lung cancer patients relative to controls [198], and circulating IL-33 negatively correlating with tumour stage in multiple myeloma patients [199]. Over-expression of IL-33 in breast and melanoma tumour cells was also observed to result in reduced tumour growth *in vivo*. Moreover, IL-33 overexpression induced recruitment of $CD8^+$ cells and NK cells to the site of the tumour [200]. Tumour metastasis was similarly attenuated in metastatic models of B16 melanoma and Lewis lung carcinoma cells transplanted into transgenic mice overexpressing IL-33. The mechanism proposed was through the activation of NF-κB signalling, which induced the proliferation, activation and recruitment of $CD8^+$ cells and NK cells, which in turn attenuated pulmonary metastasis in melanoma and lung carcinoma models [201].

In relation to CRC, the IL-33/ST2 pathway has recently been investigated in a number of studies, with these studies being published during the course of my PhD studies. IL-33 was shown to be increased in the epithelium and stroma of CRC as compared to adjacent normal tissue and healthy volunteers [147, 202]. Expression of both IL-33 and ST2 was increased in intestinal adenomas. However, expression of both proteins was lower in the colon tumour cells compared to the intestinal adenoma cells [203]. A recent study by Mertz *et al.* demonstrated that IL-33 and ST2 expression decreased with increasing tumour grade [202]. *In vitro* studies have demonstrated that IL-33 stimulation enhanced invasion of primary CRC cells [204]. Moreover, overexpression of ST2 followed by stimulation with IL-33 further increased invasion in a dose dependent manner. Correspondingly, reducing IL-33 and

ST2 expression using shRNA targeting both proteins inhibited the increased invasion observed upon IL-33 stimulation. Overexpression of IL-33 by SW620 cells in a nude mouse model reduced survival time. This was reversed using shRNA targeting IL-33 [204]. Furthermore, $ST2^{-/-}$ mice treated with AOM and DSS showed a reduced tumour load compared to WT mice. This is a model of colitis-associated cancer [202]. In an APC^{Min} mouse model IL-33, sST2 and ST2L were increased in polyps compared to normal intestinal mucosa of WT mice. Knocking out IL-33 in the APC Min model reduced polyp number [147]. Therefore, this data suggests that IL-33 may be active during polyp development. The majority of studies to date in CRC point to a pro-tumorigenic role for IL-33/ST2 signalling in CRC.

1.10 The IL-36 subfamily

The genes of the IL-36 family members are located in a cluster on chromosome 2 [205]. All three activating ligands, IL-36α, IL-36β and IL-36γ share the classic IL-1 β-trefoil structure, and lack a signal peptide. IL-36α, IL-36β, and IL-36γ lack a signal sequence, and thus cannot be transported to the endoplasmic reticulum. Similar to IL-1β and IL-18, the N-terminus must be cleaved for full agonist or antagonist bioactivity of each of the IL-36 cytokines [206]. The protease responsible for this cleavage has not yet been elucidated. It is not thought to be a caspase as the site does not resemble a caspase cleavage site [207]. These three agonists induce proinflammatory cytokines, chemokines and other stimulatory molecules, thus promoting the infiltration of many immune cells such as DCs and neutrophils, activation of Th1 and IL-17-producing T cells, and keratinocyte proliferation [208, 209].

1.10.1 Expression and function of IL-36 family members

IL-36R ligands are principally expressed by epithelial cells, keratinocytes, brain tissue and macrophages [210-212]. However, T cells have also been shown to express IL-36α and IL-36β [27]. Variation in cellular expression of the ligands has also been observed. For instance, IL-36α is constitutively expressed by keratinocytes while IL-36γ is induced upon stimulation with TNFα [213]. *In vitro,* monocytes were found to specifically express IL-36γ following LPS treatment [214]. IL-36α is secreted by BMDMs upon treatment with LPS, indicating that IL-36α can be externalized in response to a stimulus comparable to IL-1β [215].

IL-36 cytokines can regulate the immune response by influencing antigen presenting cells (APCs), such as macrophages and DCs. Indeed, the IL-36R is expressed by both monocyte and plasmacytoid derived DCs. Both of these APCs increase expression of CD83 in response to IL-36β and IL-36γ stimulation. In particular, IL-36β can induce secretion of IL-12 and IL-18 production by monocyte derived DCs. These cytokines can then activate IFN-γ producing T-cells [216]. $CD11^+$ cells stimulated with IL-36α produce TNFα, CD40, CXCL1 and CXCL2 which activate proliferation of $CD4^+$ T cells. The IL-36R is also expressed on naïve T-cells, enabling IL-36 agonists to stimulate the proliferation of T-cells and induce IL-2 secretion by naïve T cells [217]. Indeed, IL-36R signalling has also been shown to activate a Th1 and a Th17 response [209, 212].

1.10.2 IL-36 regulation

To date, few studies have investigated the regulation of IL-36 signalling. The IL-36R and the IL-1RAcP have been shown to be recycled in the absence of agonists.

However, in the presence of agonists both receptors accumulated in higher abundance in lysosomes [218]. Toll-interacting protein (Tollip) is a protein that is central to the regulation of Toll-like receptor (TLR) signalling pathways. Tollip also regulates IL-36 trafficking by elevating the levels of IL-36R and IL-1RAcP. In addition, Tollip has been shown to stabilise the IL-36R once the agonist has bound. This is unlike IL-1R signalling where Tollip has been shown to target the ligand-bound IL-1R for degradation [218]. Proteases produced by immune cells such as lymphocytes and neutrophils have been found to enhance the bioactivity of IL-33 and may also have similar effects on IL-36R agonists [129, 219].

Unlike IL-33, IL-36 cytokines require proteolytic processing to become activated. Proteases produced by immune cells such as lymphocytes and neutrophils increase the bioactivity of IL-36 cytokines. IL-36α, IL-36β, and IL-36γ are distinctively processed and activated by neutrophil granule-derived proteases cathepsin G, elastase, and proteinase-3 [220]. In this manner neutrophil-derived proteases can increase inflammation through the regulation of IL-36 cytokines.

1.10.3 IL-36 signalling

Few studies to date have investigated the role of the IL-36R signalling axis in the intestine. Similar to other IL-1R family members, the co-receptor IL-1RAcP is recruited to the IL-36R upon binding by one of the three agonistic ligands. This stabilizes ligand binding [207]. Conformational changes are induced in the TIR domains of both heterodimers. The IL-36R adaptor protein, MyD88, is subsequently recruited. Similar to other IL-1R family members, IL-36R signalling has been shown to activate MAPK, ERK1/2, JNK (Figure 9) [221]. Indeed, studies investigating IL-

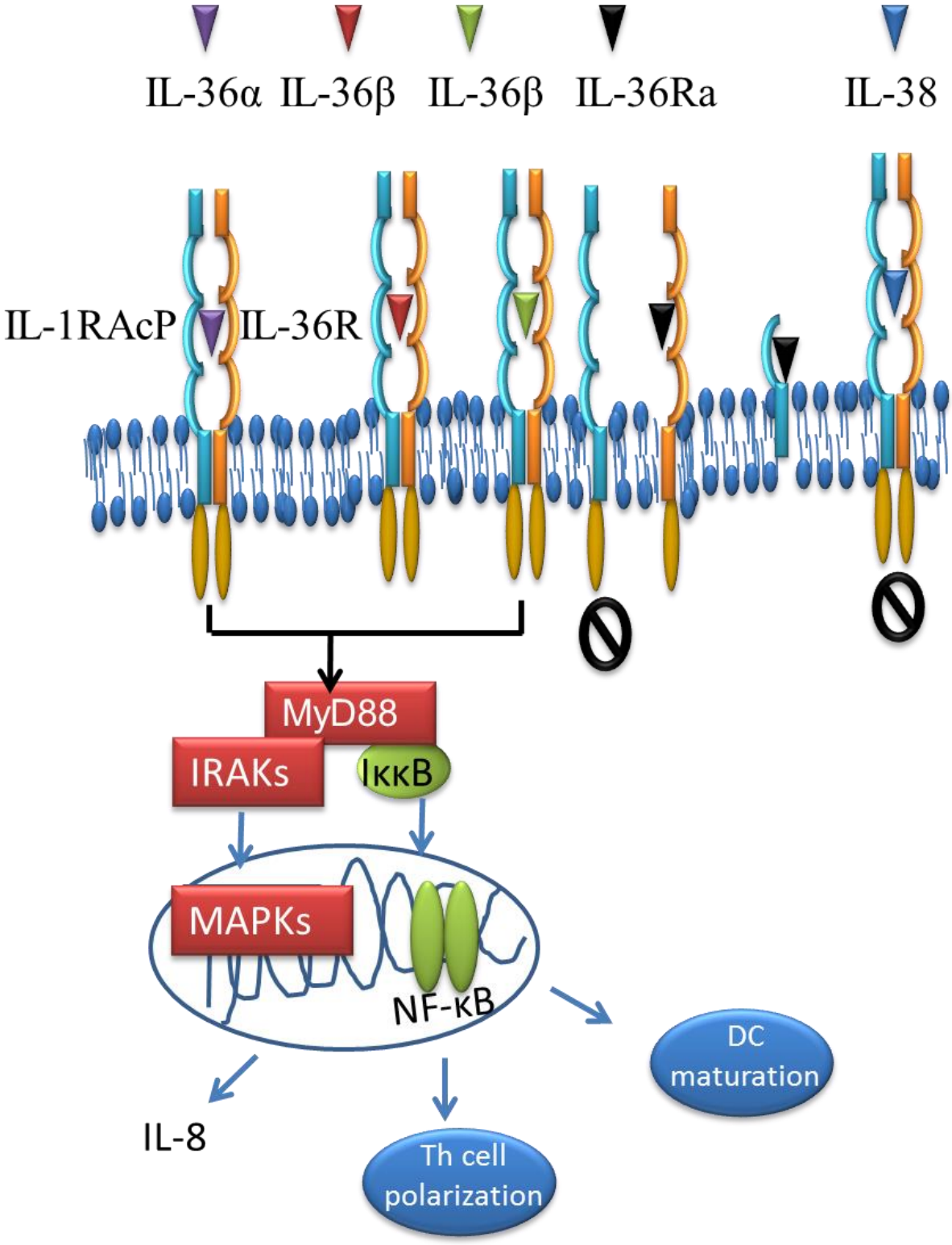


Figure 9: The IL-36 family members and signal transduction. The IL-36R is a member of the IL-1R family. IL-36α, IL-36β and IL-36γ exert their actions by binding to the IL-36R. Ligand binding enables the recruitment of the IL-1RAcP. This leads to signal transduction through MYD88-dependent pathways, and activation of NF-κB and MAPKs. The IL-36R antagonist, IL-36Ra, also binds to the IL-36R, but fails to recruit the IL-1RAcp. Moreover, it also prevents other agonist ligands from binding to the receptor. IL-38 is also thought to act as an antagonist for this receptor.

36 signalling in both HT29 and Widr colon cancer cells have shown that stimulation with IL-36α and IL-36γ resulted in the recruitment of MyD88, TRAF6, IRAK1 and TAK1 adaptor proteins [222]. This adaptor complex induced the activation of NF-κB, the phosphorylation of the MAPKs and AP-1 activation. Similar to IL-33, stimulation of the cells with IL-36 cytokines upregulated CXC chemokines (such as CXCL1, CXCL2, CXCL3) in intestinal epithelial cells [222]. Consistent with a role for the MAPK pathway and NF-κB in IL-36R signalling, activation of the IL-36R by IL-36γ was reduced in the presence of MAPK inhibitors and siRNAs specific for NF-κB and AP-1 [223]. In addition to the induction of chemokines, IL-6 was strongly induced in response to IL-36R stimulation in colonic fibroblasts [224]. IL-6 has a broad range of functions in the colon. This cytokine has been implicated in a pro-inflammatory response, but it is also thought to play a role in mucosal healing of mucosal lesions [225]. The antagonist IL-36Ra shares homology with IL-1Ra. It binds to the IL-36R preventing the formation of the heterodimer with IL-1RAcP. However, IL-36Ra can induce IL-4 production in glial cells [207].

1.11 Physiological functions of IL-36

Similar to IL-33, IL-36R ligands are involved in maintaining intestinal homeostasis. Analogous to IL-33, IL-36γ is localised to the nuclei of intestinal epithelial cells [226]. The presence of IL-36γ in the nucleus may be due to its role as an “alarmin”.

In murine models of intestinal damage such as the DSS colitis model and also in mechanical mucosal injury model, IL-36γ was released by the intestinal epithelial cells and shown to enhance mucosal healing [224]. The effects of IL-36γ on colonic fibroblasts have been examined in a recent study [224]. It was demonstrated that IL-36γ induced proliferation of murine fibroblasts to effect closure of wounds. IL-36γ

also promoted mechanical wound repair. This is a complex task in which numerous pathways participate including Wnt ligand signals, cytokines and growth factors [227-229].

1.12 Pathophysiological functions of IL-36

Studies to date have demonstrated a role for IL-36 in many diverse diseases with an inflammatory component. Given the high expression of these ligands and the receptor on keratinocytes, much early work focussed on the role of IL-36 cytokines on cutaneous diseases. IL-36 is known to play a pathogenic role in chronic psoriatic disorders [208, 217, 230]. IL-36 was demonstrated to mediate crosstalk between keratinocytes and DCs that was vital for controlling the IL-23/IL-17/IL-22 axis during psoriatic development [231]. IL-36α was also found to be increased in the joints of psoriatic and rheumatoid arthritis patients [232]. IL-36 has also been implicated in pulmonary diseases such as asthma and chronic obstructive pulmonary disease (COPD) [233]. Indeed, plasma from acute COPD patients had lower IL-36α and IL-36RN levels than healthy controls [234]. IL-36 may also play a role in joint disease, as IL-36β is expressed in human articular chondrocytes and stimulation with IL-36β induces proinflammatory cytokine production [235]. The IL-36 family have also been implicated in obesity [236]. IL-36α is present in adipose tissue resident macrophages, and both IL-36α and IL-36γ promote inflammatory gene expression in mature adipocytes [237]. Consistent with this, IL-36Ra was demonstrated to be downregulated in pre-adipocytes [236].

1.12.1 IL-36 in IBD

The role of IL-36 in IBD has recently been the focus of several studies. IL-36 is known to be dysregulated in psoriasis and these patients are at increased risk of

developing IBD and vice versa. Therefore, this suggests IL-36 dysregulation may act through a common mechanism in these diseases [238, 239]. Some studies suggest that IL-36 plays a pro-inflammatory role in IBD, while others have suggested that IL-36 promotes wound healing. Initial studies have shown that IL-36α and IL-36γ are upregulated in the colonic mucosa of UC patients [222, 240, 241]. IL-36RN was reduced in these patients, which may contribute to increased activation of IL-36 signalling [240]. DSS-induced colitis models have shown delayed wound healing in IL-36$^{-/-}$ mice. This was accompanied by a reduction in the barrier protective cytokine IL-22 and a reduction in the number of infiltrating neutrophils [241]. Interestingly, IL-36γ was found to be the most strongly upregulated gene on inflammatory macrophages that infiltrate the colon after DSS treatment [209]. As well as epithelial cells and macrophages, human colonic subepithelial myofibroblasts (SEMFs) were also shown to express IL-36γ in response to IL-1β [223]. Evidence in support of IL-36 promoting wound healing was shown in an IL-36R$^{-/-}$ mice, which demonstrated delayed wound healing in DSS-induced colitis. This was potentially due to a reduction in neutrophil recruitment [241]. This suggests there may be a very fine balance required in the level of neutrophils recruited. Low levels of neutrophil recruitment may lead to poor wound healing, while high levels of neutrophil recruitment may lead to prolonged inflammation and enhancement of IBD.

1.12.2 IL-36 in cancer

The link between IL-36 and cancer has only recently been investigated. To date two papers have investigated the role of IL-36 family members in cancer. IL-36α expression was found to correlate with mortality of hepatocellular carcinoma (HCC) patients [242]. These authors examined expression of IL-36α in a cohort of 345

patients by IHC and found IL-36α was expressed by nearly half of all HCC patients examined. IL-36α was found to be predominantly expressed in the cytoplasm of normal hepatocytes and well-differentiated HCC cells. Low expression of IL-36α was associated with increased tumour volume and increased TNM stage. Survival analysis showed that reduced expression of IL-36α was indicative of a poor prognosis for HCC patients, suggesting a possible anti-tumorigenic role for IL-36α in HCC. Cancers expressing high levels of IL-36α contained higher populations of intra-tumoral $CD3^+$ and $CD8^+$ tumour-infiltrating lymphocytes (TILs), but not $CD4^+$ TILs [242]. This suggests that IL-36α can attract $CD3^+$ and $CD8^+$ TILs and promote an adaptive T-cell immune response, which can impact the prognosis of HCC patients.

The second study injected B16 melanoma cells and 4T1 breast cancer cells overexpressing murine IL-36γ into WT mice and found tumour growth to be reduced in both models compared to vector controls. In the B16 melanoma model, the total number of $CD8^+$ and $CD4^+$ TILS and the percentage of NK and γδ T cells were increased in IL-36γ-expressing tumours compared to vector controls. Higher percentages of $Foxp3^+$ $CD4^+$ T cells, most likely T reg cells, were also detected in the IL-36γ-expressing tumours. Cells which have been shown to promote tumour growth such as type 1 lymphocytes and B cells were reduced in B16-IL-36γ compared to WT tumours [243, 244]. Overall these results suggest that a type 1 immune response was activated in B16-IL-36γ. This may have been regulated by the increased numbers of T reg cells observed [245]. These studies have identified a link between IL-36 agonists and tumorigenesis. Further studies are required to determine the role of additional IL-36 family members in other cancer types.

1.13 Aims:

Therefore the aims of this study were to;

1. Characterise expression of IL-33 and ST2 in colon cancer cells *in vitro* and investigate the effect of IL-33 on colon cancer cells *in vitro* (Chapter 3).
2. Evaluate the role of tumour cell-expressed ST2 in colon carcinogenesis *in vivo* (Chapter 4).
3. Investigate the expression and function of IL-36 in colon cancer (Chapter 5).

2.0 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, HT29, HCT116, SW480, THP1s, U266B1, JJN3 and RAW 264.7 cells were obtained from the American Type Culture Collection (MD, USA).

2.1.2 Mice

Six week old female Balb/C mice were obtained from Harlan (Oxon, UK) and maintained in the animal facility of University College Cork. Standard housing and environmental conditions were maintained (temperature 21°C, 12 hrs light and 12 hrs 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, the mice were afforded an adaptation period of at least 7 days.

2.1.3 Reagents

Anti-fade fluorescent mounting media GM-304 (Dako, Glostrup, Denmark)

Bovine Serum Albumin (BSA) A9418 (Sigma Aldrich, Dublin, Ireland)

BCA Protein Assay Reagents A and B 23223 and 23224 (Thermo Fisher Scientific, MA, USA)

Dulbecco's Modified Eagle Medium D5796 (Sigma Aldrich)

Collagenase dispase solution 10269638001 (Roche Diagnostics, Basel, Switzerland)

Congo red C6277 (Sigma Aldrich)

Crystal Violet Dye C3886 (Sigma Aldrich)

Dimethyl sulphoxide D2650 (Sigma Aldrich)

DPX mounting medium 44581 (Sigma Aldrich)

Eosin 45260 (Sigma Aldrich)

Human recombinant epidermal growth factor 585506 (Biolegend)

Murine recombinant epidermal growth factor PMG8044 (Thermo Fisher Scientific)

Normal Goat serum X0907 (DAKO Diagnostics)

Heat-inactivated foetal calf serum (FBS) F2442 (Sigma Aldrich)

Haematoxylin 51260 (Sigma Aldrich)

Human recombinant IL-33 CYT-655 (Prospec, NJ, USA)

Murine recombinant IL-33 CYT-425 (Prospec)

Human recombinant IL-36α 551602 (Biolegend)

Human recombinant IL-36β CYT-159 (ProspecBio)

Human recombinant IL-36γ 711602 (Biolegend)

Immobilon Western Chemiluminiscent HRP substrate WBLUF0500 (Merck Millipore, MA, USA)

Ultra-pure LPS tlrl-pb5lps (Invivogen, CA, USA)

Matrigel® Basement Membrane Matrix 356234 (Corning, NY, USA)

Thiazolyl blue tetrazolium bromide (MTT) sigma aldrich

Penicillin/Streptomycin P4333 (Sigma Aldrich)

Phorbol 12-myristate 13-acetate (PMA) P1585 (Sigma Aldrich)

Phosphate Buffered Saline D8662 (Sigma Aldrich)

Polyoxyethylene (20) sorbitan monolaurate (TWEEN) P1379 (Sigma Aldrich)

Polybrene sc-134220 (Santa Cruz, Heidelberg, Germany)

Prostaglandin E2 14010 (PGE_2) (Cayman Chemical, MI, USA)

Protease inhibitor cocktail I 539131 (Merck Millipore)

Puromycin P8833 (Sigma Aldrich)

Normal rabbit serum R9133 (Sigma Aldrich)

Red cell lysis buffer R7757 (Sigma Aldrich)

Resazurin powder R7017 (Sigma Aldrich)

RNAlater R0901 (Sigma Aldrich)

RPMI-1640 R8758 (Sigma Aldrich)

Tumour necrosis factor alpha 300-01A (PeproTech, NJ, USA)

Triton X T8787 (Sigma Aldrich)

2.2 Methods

2.2.1 Cell culture

Cells were cultured in 75cm^2 flasks at 37°C in 5 % $C0_2$ in DMEM (Sigma Aldrich) supplemented with 10% FBS 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 pre-warmed PBS and subsequent incubation at 37°C with 3 ml of Trypsin to detach cells from the flask wall.

2.2.1.2 Viability

10µl of cell suspension was added to 90µl of Trypan Blue (Sigma aldrich) and mixed well by pipetting. 20µl was transferred to a haemocytometer. The number of stained cells was counted, in addition to the total number of cells and percentage viability assessed.

2.2.2 Western blotting

2.2.2.1 Preparation of whole cell lysates

Cells were seeded at 1x10^5 cells/ml into 6 well plates and then cultured until 70% confluent. Cells were then treated 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). Samples being examined for expression of the heavily glycosylated ST2L were lysed in sample buffer containing 50 mM Tris-HCl (pH 7.5), 150mM NaCl, 0.1% NP40, 0.05% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPs), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1x protease inhibitor cocktail (Merck Millipore). Cells were then scraped and transferred to a 1.5ml eppendorf. Lysates were centrifuged at 12000 rpm at 4°C for 15 min. The resulting pellet containing cellular debris was discarded and lysate was stored at -20°C.

2.2.2.2 Quantitation of total protein concentration

Protein standards were prepared using BSA (Thermo Fisher Scientific) (0, 2.5, 5, 7.5, 10, 15, 20µg/ml) and added to a 96 well plate. 2µl of each sample was added to the plate followed by 38µl of distilled H_20. Both standards and samples were analysed in triplicate. 160µl of BCA Protein Assay Reagent (Thermo Fisher Scientific) 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).

2.2.2.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis and Western blotting

The appropriate amount of cell supernatant containing 50µg of protein was mixed with 4 x Polyacrylamide Gel Electrophoresis (PAGE) loading buffer (125mM Tris, 2% SDS, 20% Glycerol, and 20 mM DTT) and lysis buffer containing 50 mM Tris-HCl (pH 8.0) to a final volume of 20µl. Lysates were boiled for 5 min before loaded onto a separating and stacking SDS gel. A 19-180 kilo Dalton (kDa) molecular weight marker (Sigma Aldrich) 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. The following morning, membranes were stained with Ponceau to ensure even transfer of proteins.

2.2.2.4 Immunoblotting

Ponceau was removed with distilled water and Tris buffered Saline (TBS)-0.1% TWEEN (TBST) before membranes were blocked using 5 % (w/v) 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 expression were determined after normalising the band intensity of each lane to that of β-actin.

2.2.3 Quantitative real-time polymerase chain reaction (qRT-PCR)

2.2.3.1 RNA extraction

RNA was isolated from cultured cells using the GenElute total mRNA kit (Sigma Aldrich). Cells were lysed in 700 µl of RNA lysis buffer and RNA extracted and stored at -80^{o}C. RNA concentration and quality was determined spectrophotometrically using the Nanodrop 1000 (Thermo Fisher Scientific).

2.2.3.2 cDNA preparation

1 µg 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). This was added to 1µl of Oligo (Dt), and 1µl of 10Mm dNTP and heated to 65°C for 10 mins. Following 5 mins on ice, 4µl of 5x Reverse Transcriptase Buffer, 1µl of RNase inhibitor, 0.25µl of Reverse Transcriptase, and 2.75µl 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 and chilling samples on ice.

2.2.3.3 qRTPCR

1 µl of cDNA template was amplified in a 25 µl total reaction volume as per Applied Biosystems standard PCR protocol using appropriate probes and TaqMan Gene Expression Master Mix (Applied Biosystems, UK). Thermal cycling was performed in a PCR machine (Applied Biosystems), with general conditions as follows: 50 °C for 2 mins; 95 °C for 10 mins; 60 cycles at 95 °C and 60 °C for 1 min.

Other samples were run on the LightCycler® 480 (Roche) in a 10 µl total reaction volume. The reaction mix contained a final concentration of 400nM of each primer and 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 at 40^{o}c for 30 seconds. All results were analysed using the ΔΔ ct 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. Grubbs test was used to remove any outliers and the results achieved were then interpreted using GraphPad Prism software.

2.2.4 Resazurin Assay

Resazurin powder (Sigma Aldrich) was hydrated with PBS under sterile conditions to make a 10 x stock solution (440µM). The solution was filter sterilized using a 0.22µm filter and stored in a foiled covered container at 4°C until use. The media was aspirated from the 6-well plates and each well was washed once with PBS. 2ml of prewarmed 1x Resazurin solution was added to each well before measuring fluorescence at 535-590nm on a GenIOS fluorometer. Fluorescence readings were taken at a variety of time points (5-55 mins) to create a standard curve of fluorescence against time. Readings were subsequently taken during the log phase of the reaction.

2.2.5 MTT assay

The MTT assay depends on the ability of viable cells to reduce MTT to a coloured formazan product. MTT was dissolved at a final concentration of 5mg/ml in complete culture medium. The solution was then filter sterilized using a 0.22µm filter and stored in a foiled covered container at 4°C until use. 100 µl of the MTT solution was added per well to a 96-well plate. Cells were incubated at 37^{o}C, and 5% CO_2 for 2 hours. Medium was aspirated and the MTT-formazan precipitate was dissolved with 100 µl of DMSO per well. The plate was further incubated for 15 min at room temperature and the optical density (OD) of the wells was determined at a wavelength of 560nm using the Glomax multi-detection system. Changes in MTT reduction were normalised relative to untreated cells.

2.2.6 BrdU Assay

BrdU incorporation assay was performed using a Cell Proliferation ELISA System (Roche) according to the manufacturer's instructions. 2,000 cells per well were seeded in a 96 well plate, and serum starved overnight in 0.5% serum. Cells were treated with 15, 30 or 60ng/ml IL-33 or 100 ng/ml of EGF for 24 h. Following incubation the cells were treated for with 10μM of BrdU labelling solution and re-incubated in at 37^{o}C for 2h. The cells were fixed and denatured in one step using the FixDenat solution. An anti-BrdU-POD antibody was then added for 90 mins at room temperature. Following three wash steps substrate solution was added for 30 mins at room temperature. Stop solution was then added and the BrdU incorporation was measured at 450nm using the Glomax multi-detection system (Promega).

2.2.7 Immunohistochemistry

Formalin fixed and paraffin embedded human and murine colonic sections were deparaffinised and rehydrated using Xylene and an ethanol gradient (100%, 95%, 70% ethanol and then distilled water). The sections were then boiled with sodium citrate (pH 8) in order to induce epitope retrieval. Sections were washed in Tris Saline solution (1% Tris Saline (0.05M) and 0.001% Saponin), hereafter known as wash solution, and blocked in 3% hydrogen peroxide. Following a further wash in 1% normal goat serum (NGS) or normal rabbit serum (NRS) Wash solution, a blocking step in 5% NGS Tris Saline and a final wash, sections were incubated with primary antibody in 1% NGS Tris Buffer over night at 4°C. The following morning, sections were washed with wash solution before incubation for 45 mins at room temperature with 0.5% biotinylated anti-Rabbit IgG or anti-goat IgG (Vector Laboratories, Inc CA, USA). A five minute wash step followed, after which the sections were incubated with ABC Elite complex (Vector Laboratories) for 45 mins

at room temperature. Sections were then washed and DAB substrate added (Vector Laboratories) according to manufacturer's instructions. Finally sections were washed in tap water and counterstained with Mayer's haematoxylin (Sigma Aldrich), then washed in tap water again before being mounted with crystal mount (Sigma Aldrich). The specificity of the reaction was proven by staining with a normal rabbit IgG or normal goat IgG isotype control.

2.2.8 Enzyme Linked Immunoassay (ELISA)

CXCL-1 levels in CT26 cell supernatant treated with IL-33 for 24h were quantified using the murine CXCL-1 Quantikine ELISA (R&D Systems (Minneapolis, MN) according to the manufacturers' instructions. Three independent experiments were performed. The concentration of IL-33 and ST2 in serum was determined using commercially available IL-33 (Biolegend Inc.,) and ST2 (R&D Systems) ELISAs according to the manufacturers' instructions. All samples were analysed in duplicate.

2.2.9 Cell migration assay

HT29, SW480 or CT26 cells were plated in the top chambers of 8μm-pore transwells (Merck Millipore) in media containing 0.5% serum at a concentration of $1x10^5$ cells/insert. Increasing concentrations of IL-33 as indicated in the figure legend were added to the cells. DMEM with 10% FCS was added to the lower chamber to serve as the chemoattractant. After 16 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 min and stained with 0.5% crystal violet for 20 min. The dye was eluted from the membrane using 10% acetic acid, and crystal violet absorbance was measured at 560 nm. 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 migration was assessed relative to migration of untreated cells towards DMEM with 10% serum.

2.2.10 RAW 264.7 migration assay

For the RAW 264.7 migration assay, 1.2 x 10^5 cells were plated on the top of 5μm-pore transwells in media containing 0.5% serum. Supernatant derived from CT26 cells either untreated or treated with 15ng/ml IL-33 for 24h served as a chemoattractant. To block CCL2-induced migration, anti-CCL2 antibody (R&D Systems) was added to the lower chamber as indicated. The % change in migration was assessed relative to migration towards supernatant derived from untreated CT26 cells.

2.2.11 Invasion Assay

The transwells were coated with 100 μl of Matrigel® (Corning) diluted in serum free media to a final concentration of 4mg/ml. The Matrigel® was incubated at 37^{o}C for 45 mins to set. Once the Matrigel® had set, CT26 and HT29 cells were then seeded in media containing 0.5% serum +/- IL-33 on top of the Matrigel® at a density of 1x10^5 cells/well into the upper chamber of an 8μm pore size transwell. 750μl of media containing 10% serum was added to the bottom chamber of the transwell and served as the chemoattractant. Following 48 h 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).

2.2.12 Caspase 3 and 7 Assay

HT29 cells were plated in white 96 well plates at $2x10^4$ cells per well. The cells were then incubated with 100ng/ml of IL-36α, IL-36β and IL-36γ for 4.5 hours. Staurosporine (1μM) was used as the positive control to induce apoptosis (Sigma Aldrich). 100 μl of the Apo-One® Caspase 3/7 detection reagent (Promega) was added to a final volume of 200μl and the contents were gently mixed using a plate shaker. The plate was incubated at room temperature and readings were taken at 1, 2 and 3 hours. Fluorescence was recorded using a BioTek Flx800 spectrofluorometer at an excitation wavelength of 485nm and an emission wavelength of 530nm. Background readings were determined from wells containing culture medium without cells.

2.2.13 Generation of ST2 knockdown cancer cells

Lentiviral particles were used to deliver shRNA encoding plasmids into the cells. The lentiviral particles enter the CT26 cells where the shRNA is transcribed. The shRNA is then cleaved by the Dicer enzyme into small interfering RNAs (siRNAs) which are short duplexes of 19-21 nucleotides, including hairpin, with two nucleotide 3' overhangs on each strand. The siRNAs are then assembled into endoribonuclease containing complexes known as RNA induced silencing complexes (RISCS), unwinding in the process. Activated RISC's subsequently bind to complementary transcripts by base pairing interactions between the siRNA anti-sense strand and the complementary mRNA. The bound mRNA is cleaved and sequence specific degradation of mRNA results in gene silencing. Control shRNA lentiviral particles encode a scrambled shRNA sequence that does not lead to the specific degradation of any known cellular mRNA.

Prior to transfection with lentivirus, the optimal concentration of the transfection reagent polybrene and the selection antibiotic puromycin (Sigma Aldrich) was determined for CT26 cells. Polybrene is a polycation that neutralizes charge interactions to increase binding between the pseudoviral capsid and the cellular membrane. The optimum concentration of polybrene was determined to be 4μg/ml. To select for positive clones following transfection with shRNA particles, the antibiotic puromycin was used. The lowest concentration of puromycin that resulted in 100% cell death was determined to be 6 ng/ml. CT26 cells were seeded 24 hours prior to viral infection. Polybrene, at a final concentration of 6 μg/ml was added to the media. Cells were transfected with lentiviral particles containing target-specific shRNA against ST2 (sc-40036-V) or control lentiviral particles containing scrambled shRNA (sc-108080) (Santa Cruz Biotechnology), according to the manufacturers' instructions. Cells were seeded in 12-well plates at a concentration of 7.5 $x10^4$ cells/ml. Cells were infected 24hrs later with lentiviral particles in the presence of 4 μg/ml polybrene and cultured in selection medium containing 6-8 μg puromycin until resistant clones could be identified. Resistant clones were selected by limiting dilution. Knockdown of ST2 expression was determined by Western blotting and functional analysis.

2.3 *In vivo* studies

For tumour induction $2x10^5$ cells in 200ul of PBS was injected subcutaneously into the flank of the mice. For the initial *in vivo* study, female Balb/c mice were injected with either $CT26^{scrshRNA\#}1$ (n=4) or $CT26^{ST2shRNA\#1}$ cells (n=4) and tumour growth monitored over a period of 21 days. A separate control group were injected with PBS only (n=3). The subsequent investigation compared two $CT26^{ST2shRNA}$ groups each with a control $CT26^{scrshRNA}$ group and again another control group were injected with

PBS only (n=3). The viability of cells used for inoculation was more than 95% as determined by Trypan Blue Dye Exclusion. Following tumour establishment, the health parameters which can lead to the endpoints were carefully monitored three times per week: cachexia (acute weight loss), lack of activity and loss of appetite. Weight was measured and general behaviour as well as body condition was assessed. Tumour size was carefully monitored to ensure that it doesn't exceed maximal allowed sized of 2 cm^3. Tumour volume was measured using Vernier calipers and calculated according to the formula 1/2 (length x $width^2$). At the end of the experiment animals were euthanized by CO_2 inhalation.

2.3.1 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.

2.3.2 Monitoring body weight

Mice were weighed thrice weekly to monitor changes in body weight.

2.3.3 Tumour formation

Mice were subcutaneously injected into the right flank with CT26 cells suspended in 200µl of PBS. Tumour formation was monitored thrice weekly by palpation at the site of injection. The width (w) and length (l) of the tumours was measured using Vernier calipers and the mean tumour volume calculated using the formula:

$$½ (l \times w^2).$$

2.3.4 Tumour sampling

Following euthanasia by CO_2 inhalation and 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 in liquid nitrogen, formalin and RNAlater (Sigma Aldrich). In addition, tissue was placed in an

eppendorf containing 1 ml of a working solution of collagenase/dispase (Roche) in PBS on ice.

2.3.5 Single cell suspension of tumour cells for flow cytometric analysis

Tumours in collagenase/dispase solution were incubated at 37^{o}C for 1hr with shaking. Tumour tissue was placed into a petri dish, 10ml of serum free DMEM was added and cells were then passed through a cell strainer (Fisher). Cells were pelleted, washed in PBS and resuspended in 2ml of Red Cell Lysis Buffer. Following incubation at room temperature for 10 mins, 10% FCS DMEM media was added to stop the lysis reaction. Finally, a cell count was performed to obtain a final concentration of 1 x 10^{6} cells/ml.

2.3.6 Flow cytometry

Single cell suspensions from tumour tissue were prepared. 200,000 cells per tumour were re-suspended in 200µl of cell staining buffer and samples were incubated with antibodies to LY6G, CD4, CD8, CD25, CD49b, Siglecf or F4/80 for 30 minutes at 4^{o}C in the dark. Cells were washed with cell staining buffer, centrifuged at 350 x g for 5 min and resuspended in 0.5ml of cell staining buffer. 2.5ug/ml of ViViD viability staining solution (Life Technologies) was added per million cells and samples were incubated on ice for 15 min in the dark. 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 using 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

FACSLSRII 5 laser (UV/violet/blue/yellowgreen/red) cytometer and BD Diva software (Becton, Dickinson).

2.4 Statistical analysis

Experiments were performed a minimum of three times in triplicate. Results were statistically evaluated using One-way Anova with Tukeys post-test, or by students paired t test. 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

Characterisation of IL-33 and ST2 expression, signalling and function in colon cancer cells *in vitro*.

3.1 Introduction

Expression of both IL-33 and ST2 has been examined in many cell types, both *in vitro* and *in vivo*. IL-33 is known to be robustly expressed by high endothelial venules in human tonsils, lymph nodes and Peyer's patches [246]. Its ligand, ST2L, is expressed on the surface of macrophages, mast cells and Th2 cells [122, 247, 248], while sST2, in turn, is expressed by fibroblasts and both endothelial and epithelial cells [106, 249]. In terms of expression in the colon, *in vivo* studies have found both IL-33 and ST2L to be expressed by intestinal epithelial cells [177, 181], as well as macrophages and lymphocytes infiltrating the intestine [173, 177]. More specifically, IL-33 was shown to be localized to both the nucleus and cytoplasm of intestinal epithelial cells [177]. The decoy receptor, sST2, was found to be particularly strongly expressed in intestinal stromal cells and infiltrating regulatory T-cells [173, 250]. Consistent with the *in vivo* findings demonstrating epithelial expression of IL-33, HT29 colon cancer cells were shown to express IL-33 mRNA, although the transcript was not detected in CaCo2 colon cancer cells. Subepithelial myofibroblasts were also shown in *in vitro* studies to have low basal levels of IL-33, with expression enhanced by TNFα stimulation [178]. In contrast, few, if any studies have examined ST2 expression in intestinal cells *in vitro*.

Studies investigating the physiological functions of the IL-33/ST2 signalling axis demonstrated that IL-33 plays a key role in maintaining the mucosal barrier in the gut. Under healthy conditions, constitutively produced, intracellular IL-33 participates in maintaining barrier function by regulating gene expression as a nuclear factor. Upon damage to the barrier, for instance as a result of helminth

infection, nuclear IL-33 is released and functions as an “alarmin”, promoting the gut inflammatory response to induce parasitic clearance [251]. Once released, IL-33 has dual roles, initially functioning as a potent driver of Th2 immunity at the site of infection, and later as a T-cell-independent proinflammatory cytokine [252]. A second immune related pathology associated with the intestine that, in turn is also associated with increased IL-33 expression, is Inflammatory Bowel Disease (IBD). IBD encompasses two main disorders, ulcerative colitis (UC) and Crohn’s disease (CD). Studies examining expression of IL-33 and ST2 showed that IL-33 expression is upregulated in active UC and CD patients [177, 178]. In severe UC, as the epithelium becomes damaged and mucosal lesions form, IL-33 is secreted, and functions as an alarmin [179]. The pattern of ST2 expression is also altered in IBD. ST2 becomes depleted in intestinal epithelial cells, and is prominent in the lamina propria of IBD patients. Not only is the pattern of expression changed, but the expression of the ST2 isoforms is also modified in UC; ST2L is reduced [177], while sST2 is increased compared to CD and control tissue [177, 181]. The increased levels of IL-33 are thought to contribute to the sustained inflammatory state present in active IBD. SAMP1/YitFc (SAMP) mice are a murine model of CD used to investigate the pathogenesis of chronic intestinal inflammation. In SAMP mice IL-33 expression was found to positively correlate with the severity of disease [177]. Inflammation has recently been described as an emerging hallmark of cancer. Consistent with this, individuals with IBD are at increased risk of developing colorectal cancer. This risk is specifically linked to the duration and extent of the inflammation [253]. However, whether IL-33 and ST2 signalling promotes inflammation and colon carcinogenesis is unclear.

Colon cancer represents the third most common cancer type worldwide, and is a major cause of morbidity and mortality. Thus, identifying factors that promote colon cancer is important. Whilst IL-33 and ST2 are known to be expressed by IECs, no studies to date have comprehensively investigated their pattern of expression in malignant IECs or colon cancer cells, nor have they characterised their cancer promoting abilities (e.g. capacity to induce migration and invasion), or factors that regulate or alter their expression in colon cancer cells. As there is an emerging focus on these proteins in cancer, with pro-tumorigenic roles for IL-33/ST2 reported in breast cancer *in vivo* [196], and IL-33 shown to promote migration and invasion of gastric cancer cells *in vitro* [254], the aim of this chapter is to investigate the potential role of IL-33 and ST2 in colon carcinogenesis.

3.2 Results

3.2.1 LPS induces ST2L expression in a murine macrophage cell line

Prior to investigating expression of the membrane bound form of ST2 (ST2L) in colon cancer, endogenous ST2L expression was examined in RAW 264.7, a murine macrophage cell line. This cell line was selected as a positive control, as macrophages are known to express high levels of ST2L in response to LPS stimulation [255]. Whilst ST2L expression was not observed at basal levels, it was seen to be upregulated upon stimulation with LPS (Figure 1).

3.2.2 Human colon cancer cell lines express both sST2 and ST2L.

As the ST2 antibody appeared to be able to detect ST2L, I subsequently examined expression levels of both the transmembrane (ST2L), and the soluble (sST2) forms of ST2 in human colon cancer cells. For this, the cell lines HT29 and SW480 were selected. These cells were stimulated with the pro-inflammatory stimuli LPS, TNFα and PGE_2, for 24, 48 and 72 hours. Basal expression of ST2L was observed to be low in both cell lines examined, but was upregulated in response to pro-inflammatory stimuli, LPS, TNFα and PGE_2 (Figure 2 and 3). In contrast, sST2 levels remain unchanged by pro-inflammatory stimuli in both of the human colon cancer cell lines (Figure 2 and 3).

3.2.3 Murine colon cancer cells express both sST2 and ST2L.

As I had shown that human colon cancer cells express both sST2 and ST2L (Figure 2 and 3), protein levels of both of these ST2 variants was next examined in murine colon cancer cells. Similar to findings in human colon cancer cell lines, both ST2L and sST2 were detected in resting CT26 cells (Figure 4), with sST2 expression

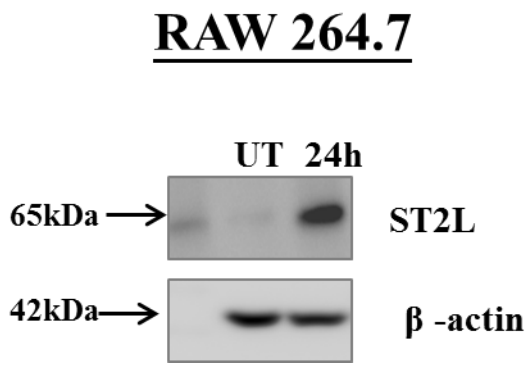


Figure 1: Murine macrophage cell line RAW 264.7 express ST2L in response to LPS stimulation. Macrophages were treated for 24h with 100ng/ml LPS, prior to being lysed and probed with anti-ST2 antibody or anti-β-actin specific antibodies as indicated. Images shown are representative of three independent experiments.

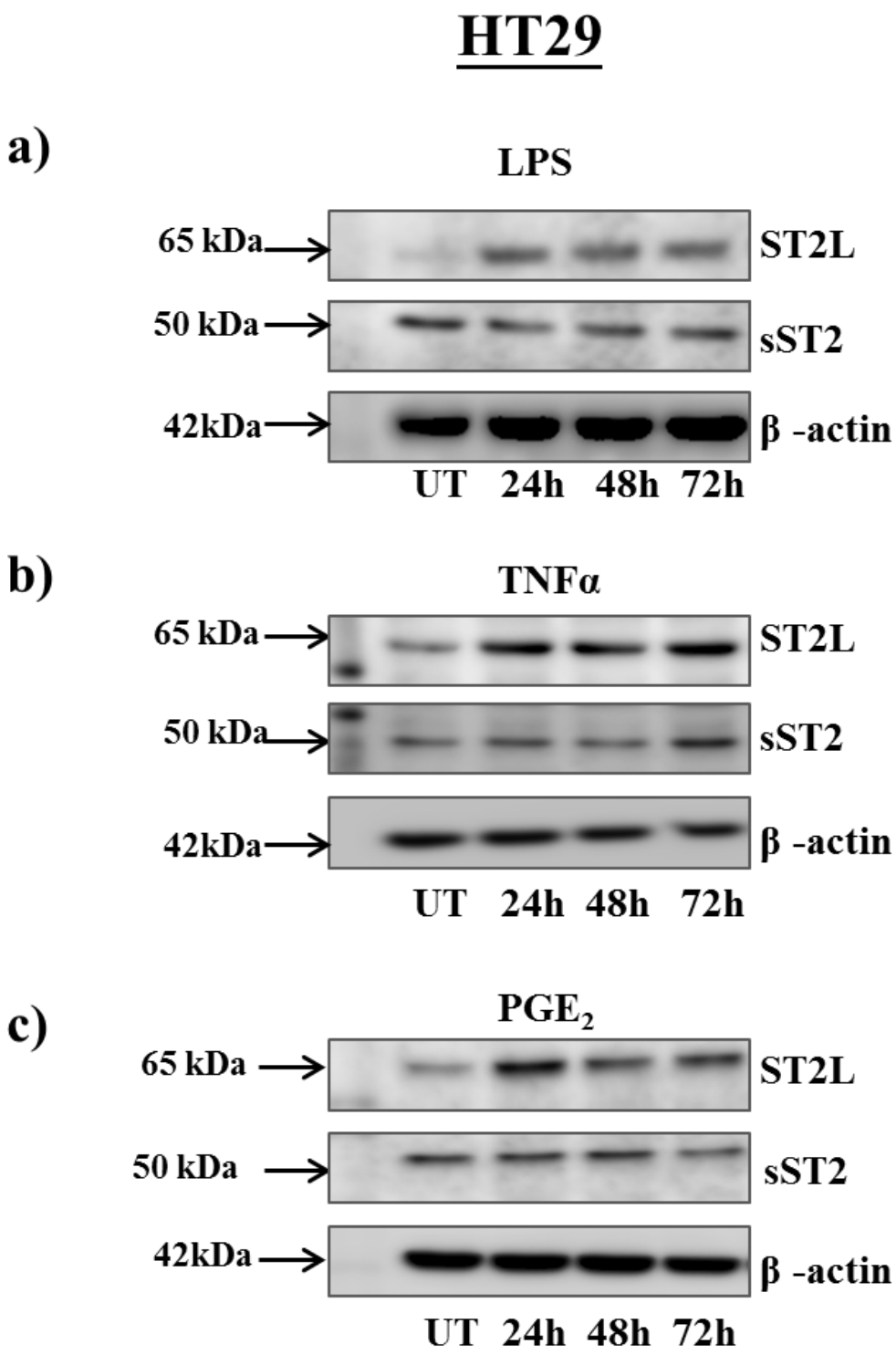


Figure 2: ST2L expression was increased in HT29 colon cancer cells stimulated with pro-inflammatory stimuli. Colon cancer cell lines HT29 were stimulated with (a) LPS (100ng/ml), (b) TNFα (10ng/ml) and (c) PGE_2 (1μM) for 24, 48 and 72h. Cell lysates were separated by SDS-PAGE and probed with anti-ST2 or anti-β actin specific antibodies as indicated. Images shown are representative of three independent experiments.

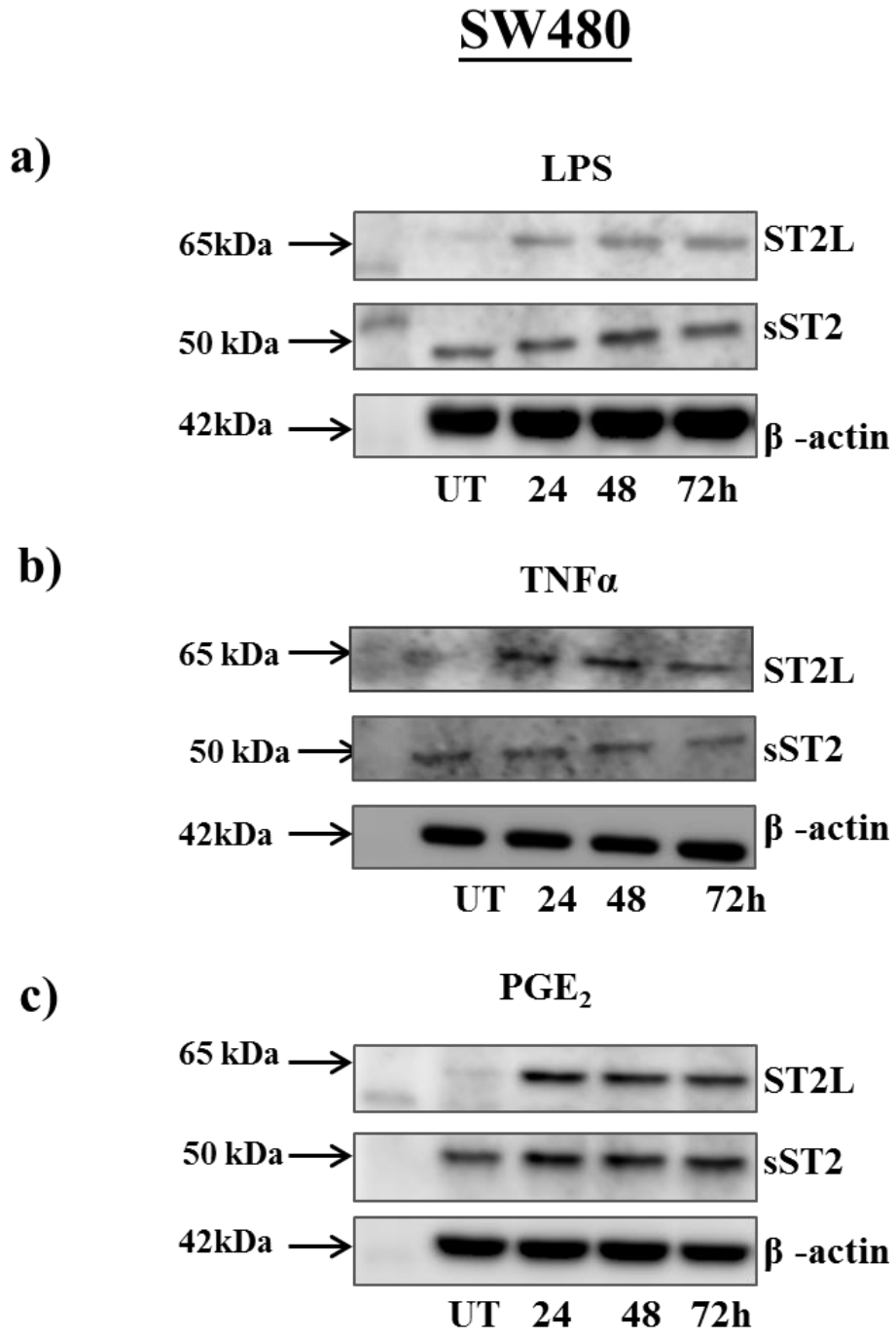


Figure 3: ST2L expression was increased in SW480 cells stimulated with pro-inflammatory stimuli. SW480 cells were stimulated with (a) LPS (100ng/ml), (b) TNFα (10ng/ml) and (c) PGE_2 (1μM) for 24, 48 and 72h. Cell lysates were separated by SDS-PAGE on a 10% gel and probed with anti-ST2 or anti-β actin specific antibodies as indicated. Data shown are representative of three independent experiments.

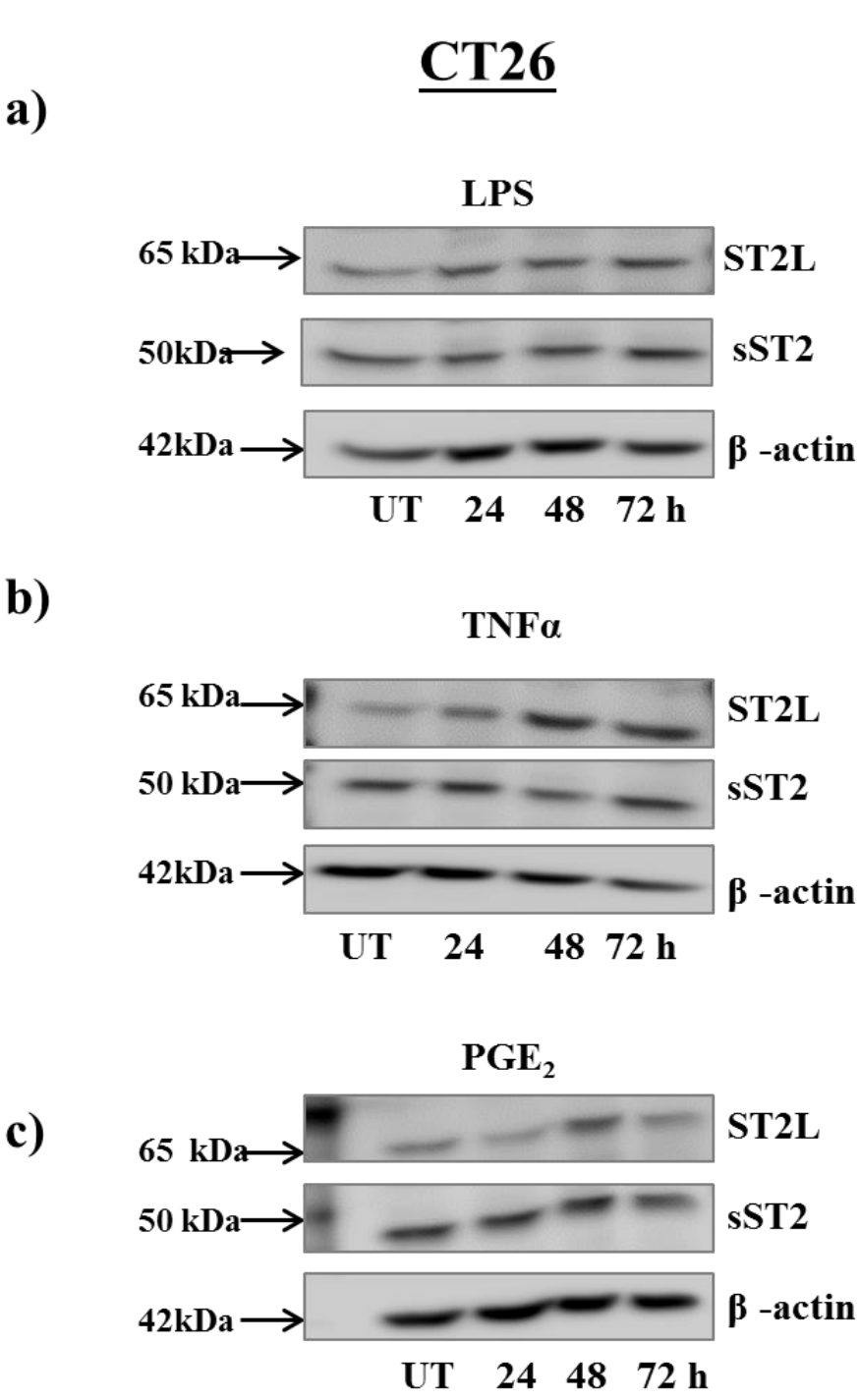


Figure 4: **ST2L expression was increased in CT26 cells stimulated with pro-inflammatory stimuli**. CT26 murine colon cancer cells were stimulated for 24, 48 and 72 hours with (a) LPS (100 ng/ml), (b) TNFα (10ng/ml) and (c) PGE_2 (1μM) for 24, 48 and 72h. Cell lysates were separated by SDS-PAGE and probed with anti-ST2 or anti-β actin specific antibodies as indicated. Data shown are representative of three independent experiments.

unchanged by stimulation with LPS, TNFα or PGE_2 (Figure 4). Similarly murine ST2L was upregulated by LPS, TNFα or PGE_2 (Figure 4). However, in murine cells the induction of ST2L expression by TNFα and PGE_2 occurred slightly later (48h), than in the human colon cancer cell lines (24h) (Figure 3).

3.2.4 Colon cancer cell lines and macrophages express IL-33 mRNA, the ligand for ST2L

As both human and murine colon cancer cells were found to express the ST2L receptor, the expression of its ligand, IL-33, was subsequently investigated in these cell lines. Similar to experiments performed in 3.1, the murine macrophage cell line RAW 237.4 was initially used as a positive control for confirmation of detection of IL-33 expression (Figure 5a), as macrophages have previously been shown to produce IL-33 [256]. Expression of IL-33 mRNA was seen to be increased 5000-fold in RAW 264.7 cells, following LPS stimulation and 22-fold in RAW 264.7 cells following PGE_2 stimulation (Figure 5a). IL-33 mRNA expression in both human colon cancer (HT29) and murine colon cancer (CT26) was also examined. Basal levels of IL-33 mRNA were detected in human and murine colon cancer cells. In HT29 cells, IL-33 expression was increased 2-fold in response to LPS and 1.5-fold by PGE_2 stimulation (Figure 5b). In CT26 cells, IL-33 was upregulated 1.5-fold in response to LPS, and 1.8 fold upon stimulation with PGE_2 (Figure 5c). Basal levels of IL-33 in all three cell lines were compared. Expression was similar in HT29 and RAW 237.4, while IL-33 expression in CT26 was higher ~10 fold (Figure 5d).

3.2.5 IL-33 protein expression was not effected by pro-inflammatory stimuli in colon cancer cells.

As IL-33 mRNA expression had been detected in colon cancer cells (Figure 5), with no significant changes observed upon stimulation of cells, the induction of IL-33

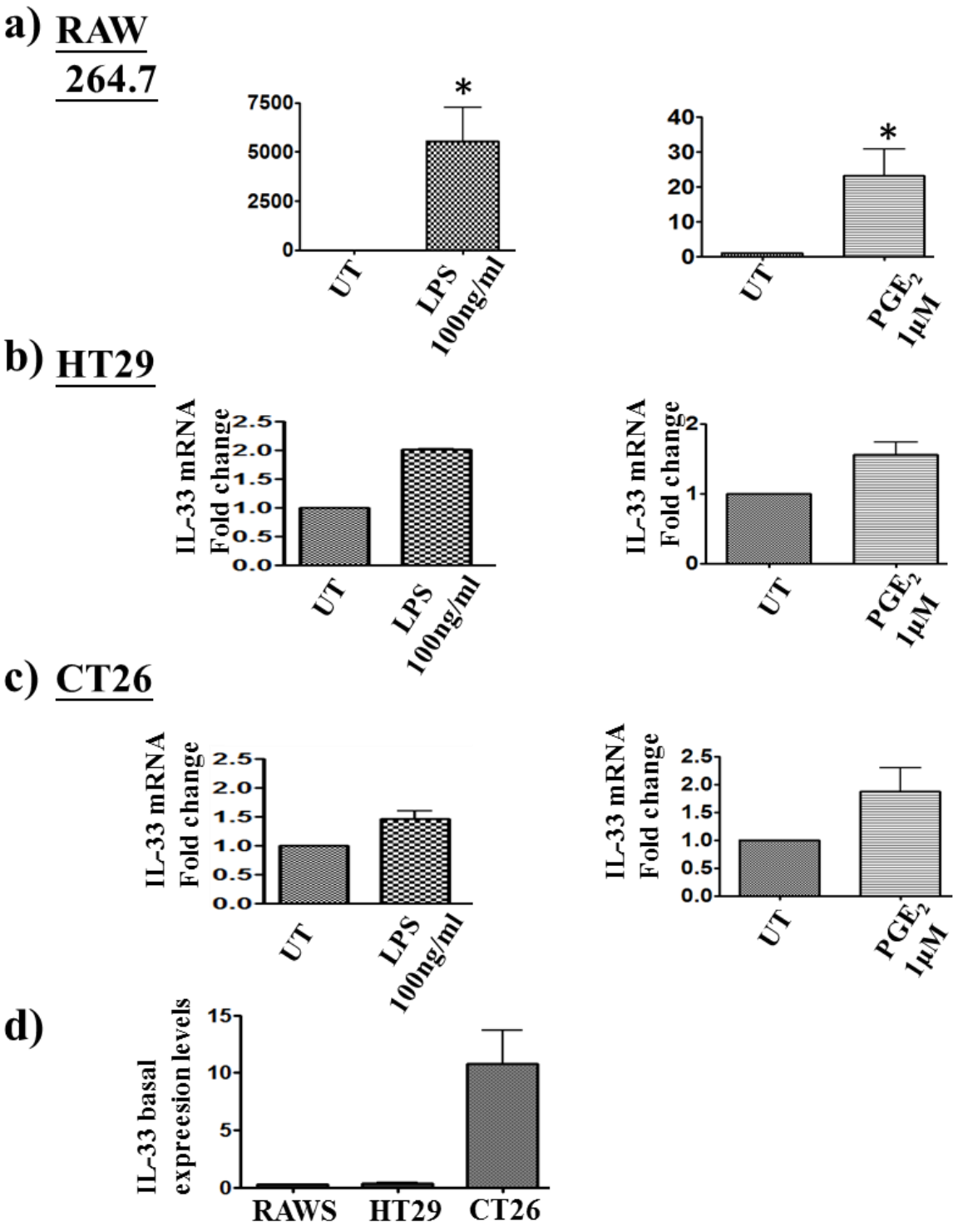


Figure 5: Stimulation with pro-inflammatory stimuli induced the expression of IL-33 mRNA in colon cancer cells and macrophages. IL-33 mRNA expression in (a) RAW 264.7, (b) HT29, and (c) CT26 cell lines (d) shows basal IL-33 expression levels in unstimulated cells. Colon cancer cells were stimulated for 4 hours with 100ng/ml of LPS or PGE_2 at 1µM. Total RNA was extracted and IL-33 mRNA levels were measured by qPCR. Data shown are the mean +/- SEM. Statistical analysis was carried out using a student t test, n=3.

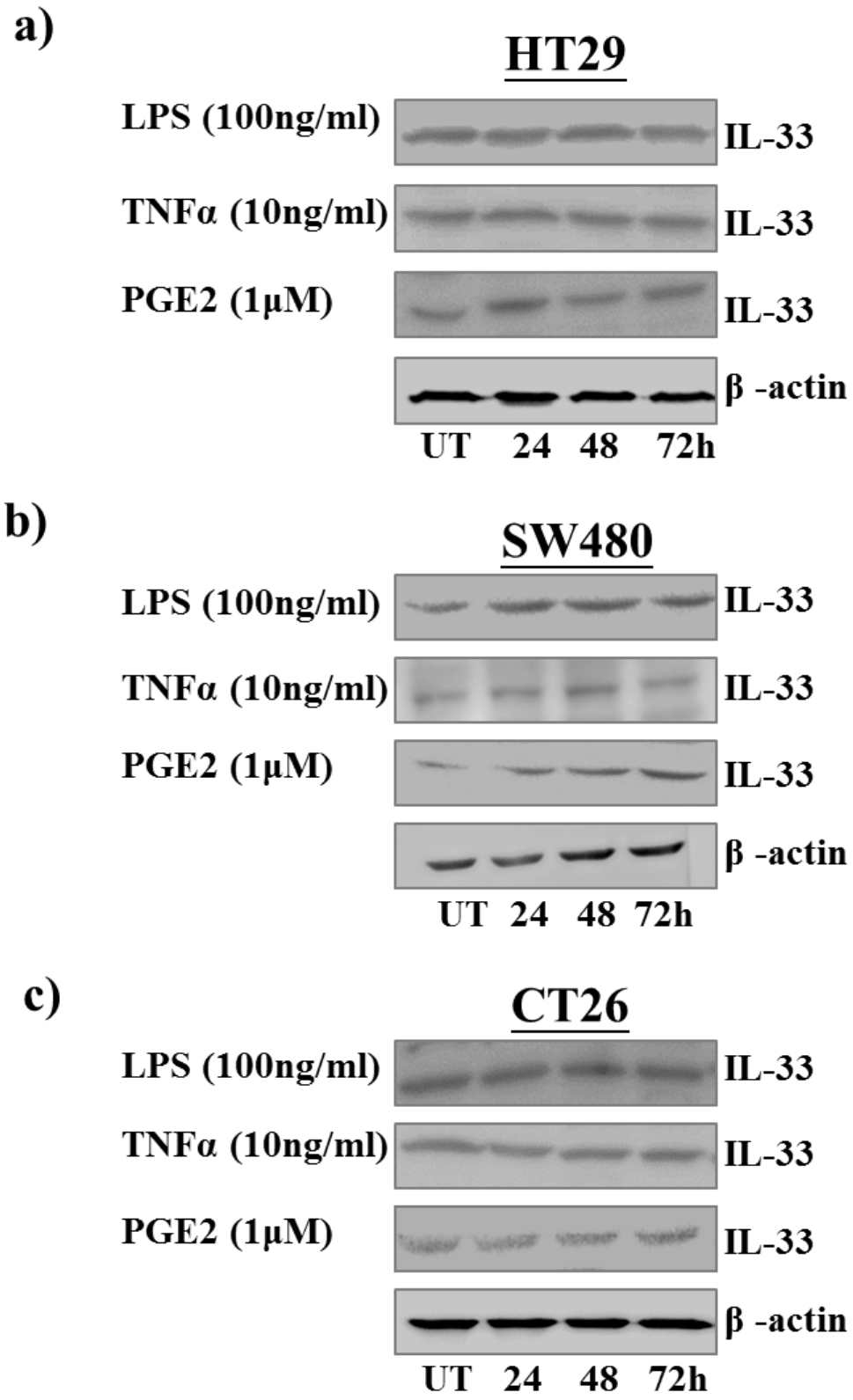


Figure 6: IL-33 expression was unchanged in colon cancer cells stimulated with pro-inflammatory stimuli. IL-33 expression was examined in (a) HT29, (b) SW480 and (c) CT26 cells by Western blotting analysis. Colon cancer cells were stimulated for 24, 48 and 72 hours with pro-inflammatory stimuli as indicated. Cell lysates were separated by SDS-PAGE and probed with anti-IL-33 or anti-β-actin specific antibodies. Data shown are representative of three independent experiments.

protein was next determined by Western blot analysis. Western blotting for IL-33 confirmed that HT29, SW480 and CT26 cells express basal levels of IL-33 (Figure 6). These levels were unchanged by treatment with LPS, TNFα or PGE_2, in all cell lines (Figure 6).

3.2.6 IL-33 inhibits proliferation of colon cancer cells

As I had demonstrated that colon cancer cells express sST2, ST2L and IL-33, I next wished to determine the functional role of these proteins in colon cancer cells. I initially investigated cellular proliferation, as sustained proliferative signalling is a key hallmark of cancer progression, which increases tumorigenicity [2]. Four colon cancer cell lines, HT29, SW480, HCT116 and CT26, were stimulated with increasing concentrations of IL-33 for 24, 48 hours, and changes in proliferation detected using the MTT assay. IL-33 was seen to suppress proliferation of all of these cells, with the exception of SW480 cells at 24h (Figure 7). As IL-33 did not increase cellular proliferation, cells were stimulated with epidermal growth factor (EGF), a growth factor known to increase tumour cell growth. Consistent with its ability to increase tumour cell growth, HT29 cells showed a 75% increase in proliferation when stimulated with EGF (Figure 8). SW480 cells showed a 30% increase in proliferation (Figure 8), while CT26 cells showed a 25% increase in cellular proliferation (Figure 8). This indicated that although the colon cancer cells did not proliferate in response to IL-33, they do proliferate in response to EGF.

Although the MTT assay can be used as an indicator of proliferation, this assay specifically detects changes in the metabolic activity of the cell. Therefore, any impact on metabolism such as cytotoxicity, apoptosis or necrosis can also influence the assay. Thus, it can be difficult to distinguish between cell viability and

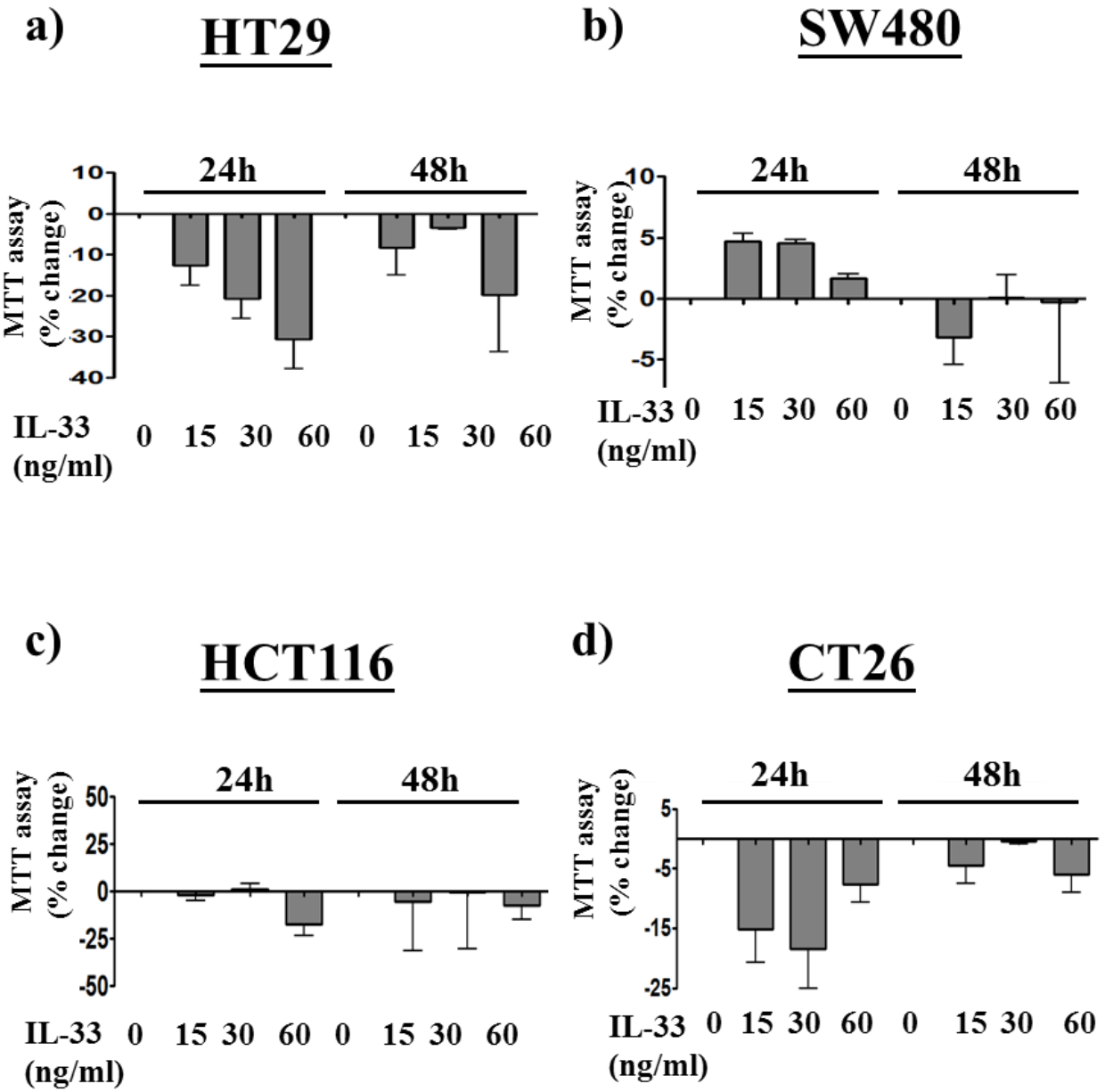


Figure 7: IL-33 suppressed proliferation of human colon cancer cells. Thiazolyl Blue Tetrazolium Bromide (MTT) analysis was used for evaluation of cell proliferation. (a) HT29, (b) SW480 (c) HCT116 and (d) CT26 were stimulated with increasing concentrations of IL-33 as indicated. After 24hr or 48hrs MTT solution was added to each well, 2hrs later, the media was removed, and dimethyl sulfoxide was added to each well to dissolve the MTT formazan crystals. Data shown are mean +/- SEM (n=3).

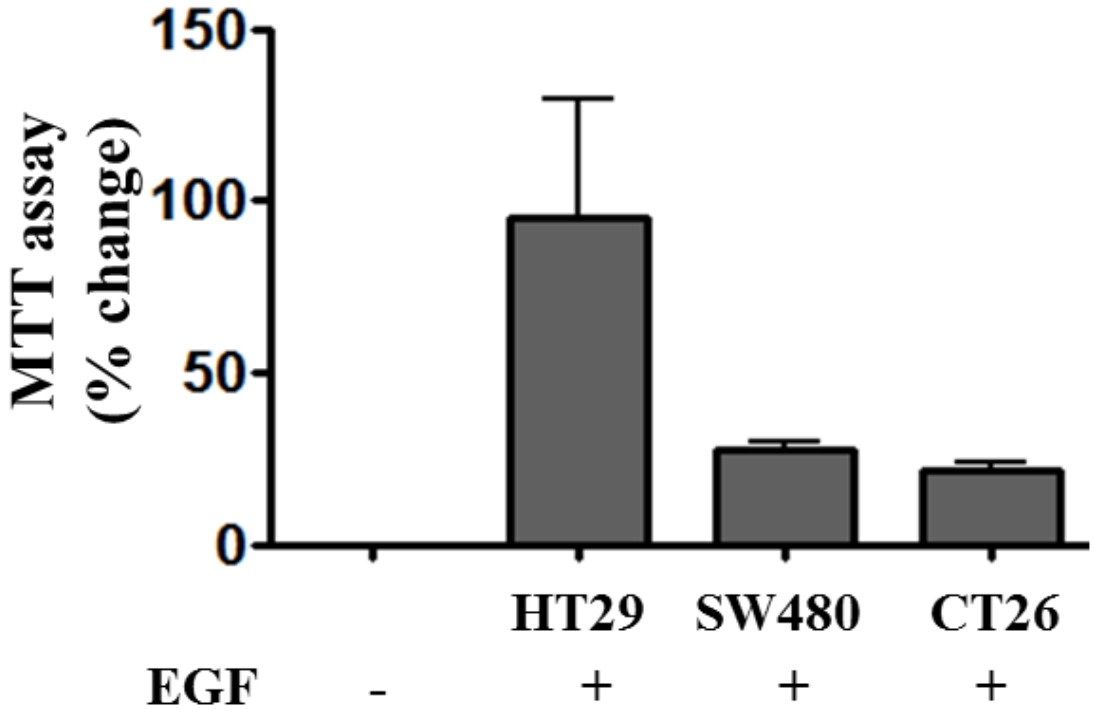


Figure 8: EGF induced proliferation of human colon cancer cells. Thiazolyl Blue Tetrazolium Bromide (MTT) analysis was used for evaluation of cell proliferation. HT29, SW480 and CT26 cells were stimulated with EGF 10 ng/ml as indicated, for 24 hrs. MTT solution was added to each well, 2hrs later, the media was removed, and dimethyl sulfoxide was added to each well to dissolve the MTT formazan crystals. Data shown are mean +/- SEM (n=3).

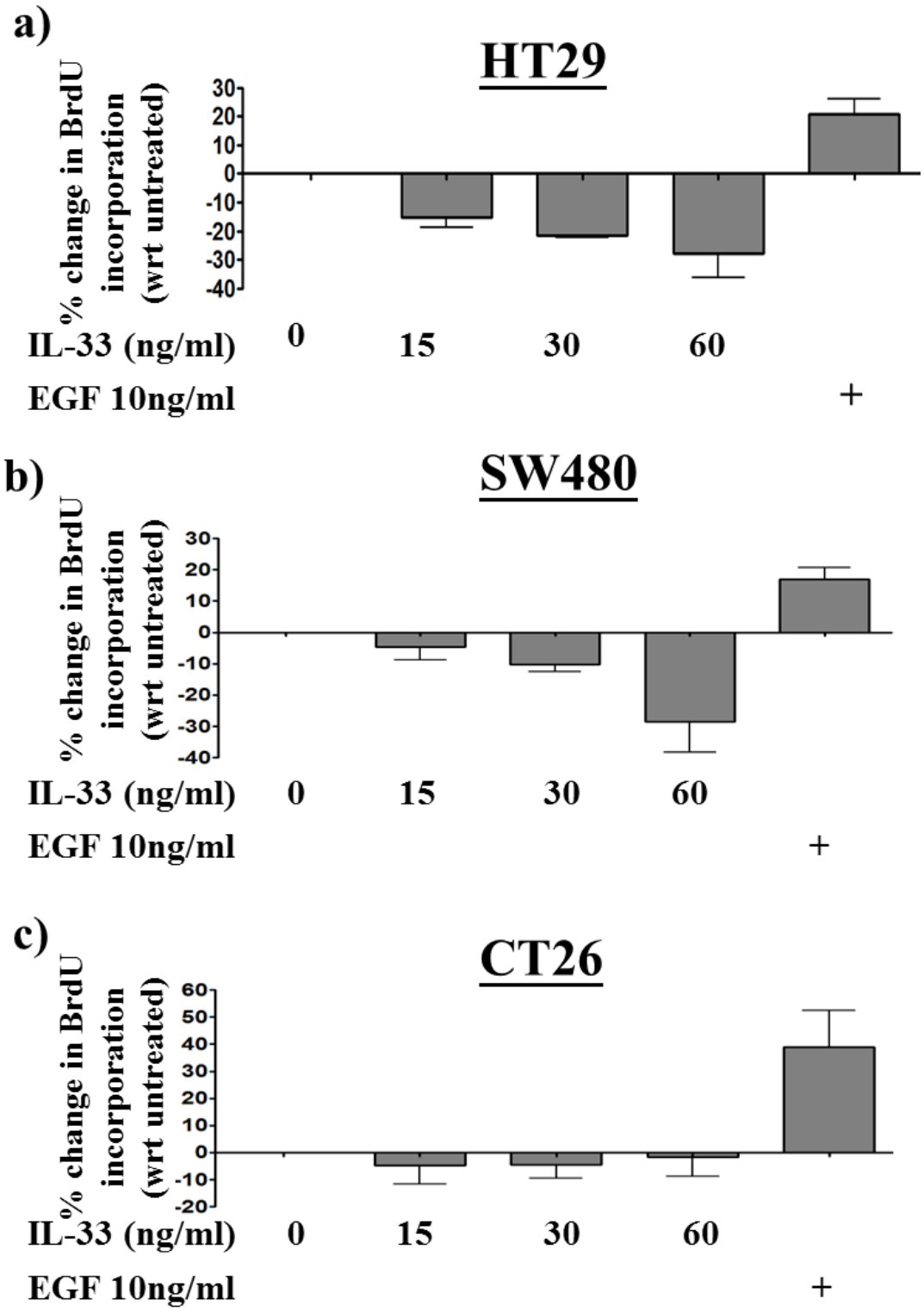


Figure 9: IL-33 does not induce proliferation of colon cancer cells.
Cellular proliferation was assessed by BrdU incorporation with EGF used as a positive control. (a) HT29, (b) SW480 and (c) CT26 cells were stimulated with varying concentrations of IL-33 as indicated, and 24 hrs later the cells were labelled with BrdU after 2hrs of incubation, the media was removed and the cells were fixed and denatured, and absorbance was measured. Data shown are mean +/- SEM (n=3).

proliferation using this assay. To ensure that results I have observed using the MTT assay were truly a measurement of proliferation, I verified these results by doing a second specific proliferation assay, examining BrdU incorporation into cellular DNA during cell proliferation. The results were similar to those observed with the MTT assay. Incorporation of BrdU was seen to be suppressed by IL-33 stimulation in HT29 (Figure 9a), SW480 (Figure 9b) and CT26 (Figure 9c). Overall, the data suggests that IL-33 may suppress colon cancer cell proliferation.

3.2.7 IL-33 induces migration, but not invasion of colon cancer cells

As IL-33 had no effect on cellular proliferation, the ability of IL-33 to effect cellular migration, invasion was next investigated. Indeed the main cause of cancer death is due to metastasis of the primary tumour to a new site. Most commonly colon cancer metastasizes to the liver [257], and as such cellular migration is an important pro-tumorigenic mechanism, required by cancer cells for sustained growth and progression. Cells were stimulated with increasing doses of IL-33 as indicated and changes in migration assessed using a modified Boyden chamber assay (Figure 10a). IL-33 significantly enhanced migration of HT29 cells, in a dose dependent manner, towards media containing 10% serum above migration observed by unstimulated cells, p value >0.01 (Figure 10b). IL-33 also increased migration of SW480 cells, but this was not significant (Figure 10c). Finally, migration was also significantly increased by IL-33 in CT26 cells, p-value >0.01 (Figure 10d).

As IL-33 increased the migration of colon cancer cells towards 10% serum, in a dose dependent manner, the ability of IL-33 to induce invasion in colon cancer cells was next investigated. Invasion is an essential function underlying cancer cell metastasis. Invasive cells travel through basement membranes and extracellular matrices into

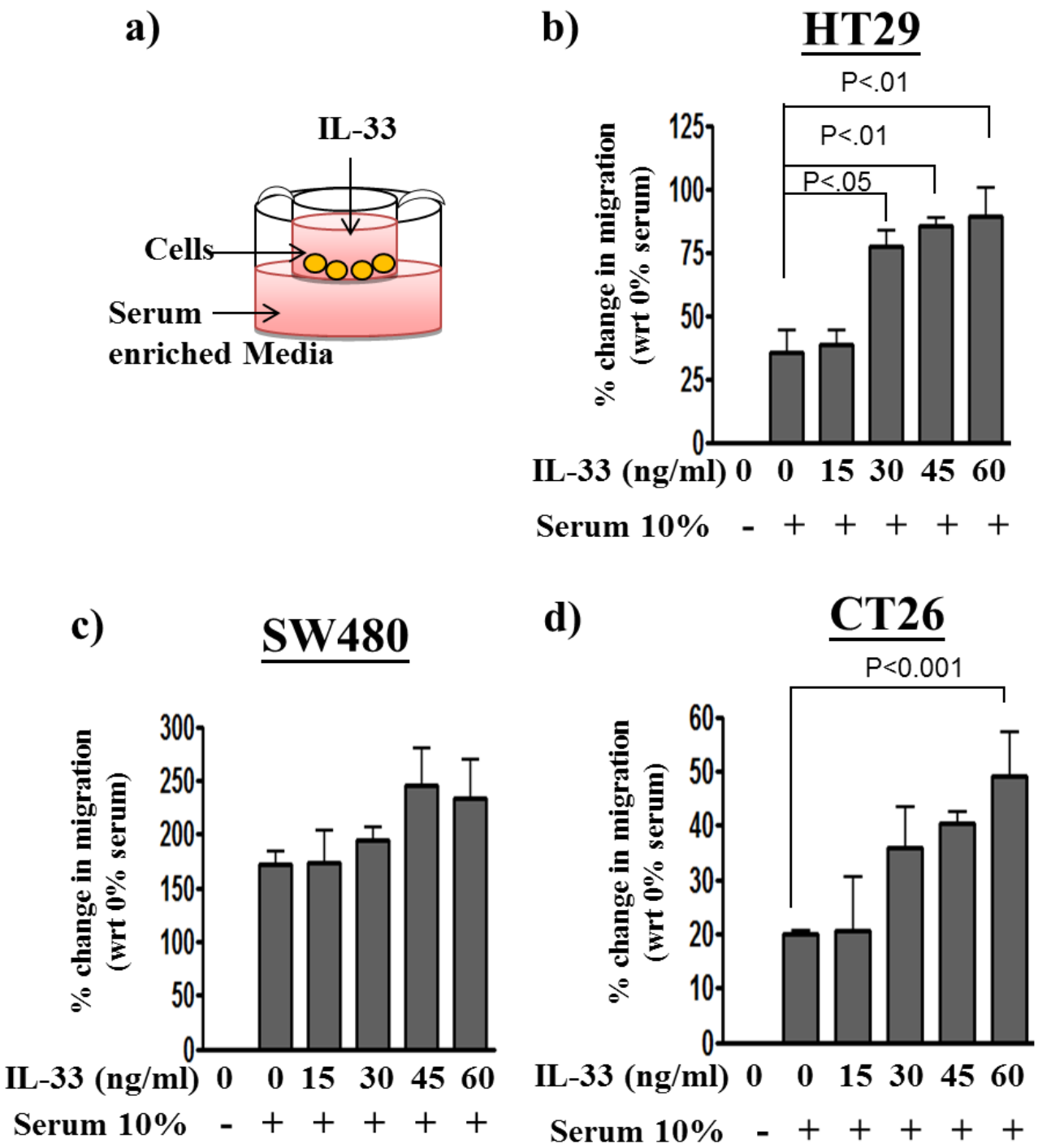


Figure 10: IL-33 induced migration of colon cancer cells towards 10% serum at increasing doses. (a) Changes in cellular migration were assessed using a modified Boyden chamber assay. (b) HT29, (c) SW480 and (d) CT26 cells were stimulated with increasing doses of IL-33 (0, 15, 30, 45 and 60 ng/ml). Data shown are mean +/- SEM (n=3). Statistical analysis were determined using a one-way ANOVA

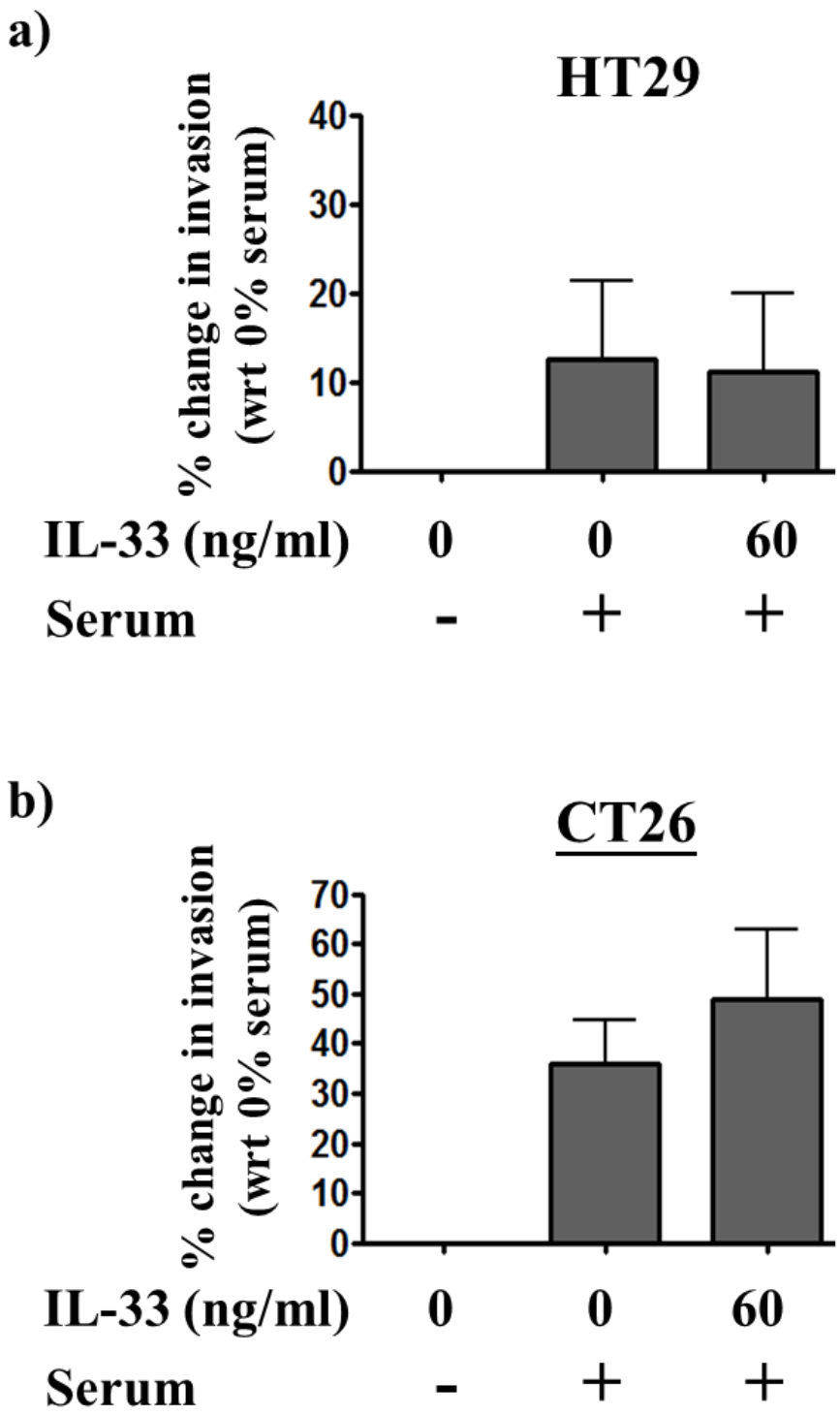


Figure 11: IL-33 did not induce cellular invasion of colon cancer cells towards 10% serum. (a) HT29 and (b) CT26 cells were stimulated with 60ng/ml of IL-33. Changes in cellular invasion were assessed using a modified Boyden chamber coated with 100 µl of 4mg/ml Matrigel. The cells were incubated for 48 hours at 37^{o}C. Data shown are mean +/- SEM (n=3).

neighbouring tissues in a complex process that involves adhesion, extracellular matrix degradation, proteolysis and migration. To assess the ability of IL-33 to induce cellular invasion, colon cancer cells were seeded on Matrigel coated transwells. As seen in Figure 13, IL-33 did not affect invasion of HT29 (Figure 11a) or CT26 cells (Figure 11b).

3.2.8 IL-33 induces limited expression of cytokines/chemokines in colon cancer cells.

The link between colon cancer and chronic inflammation is well established. Communication between the immune system and malignant cells can play an important role in tumorigenesis. This cell to cell interaction is usually via cytokines or chemokines, and can have far reaching effects, from recruiting immune cells that supply growth factors or pro-angiogenic factors, to increasing proliferative signalling and limiting cell death. To date, the effect of IL-33 stimulation on colon cancer cells, in terms of cytokine production, has not been comprehensively investigated. To address this, and to further our extensive characterisation of IL-33 and ST2 in colon cancer cells, a number of cells were stimulated with IL-33, and a panel of cytokines and chemokines examined (i.e. TGF-β, LTα, COX-2, IL-6, IL-8, IL-13, CXCL-8, VEGF, CCL2 and CXCL-1). This panel was selected for a number of reasons; TGF-β expression is indicative of poor prognosis in CRC [258], while increased COX-2 expression is associated with poor survival in CRC [259]. IL-6 is a key regulator in CRC development [260], while IL-8 is associated with migration and angiogenesis of colon cancer cells [261] and both have previously been shown to be activated by IL-33 [262, 263]. A number of chemokines were also selected (i.e. CXCL-1, CXCL-8, IL-13 and CCL2), as IL-33 has been shown to recruit immune cells [264].

In HT29 and SW480 cells, CCL2 was the only cytokine/chemokine to be induced. CCL2 was induced 7 fold in HT29 (Figure 12a), and 70 fold in SW480 (Figure 12b). Whilst several genes were not induced in the CT26 cells (i.e. IL-13, TGF-β and LTα), a slightly broader number of genes were found to be induced by IL-33 in these cells. IL-33 induced a 2 fold induction of VEGF, a 10 fold induction of CXCL1 and a 40 fold induction of CCL2 in CT26 cells (Figure 13). The CXCL-1 induction was further confirmed using increasing doses of IL-33, and was seen to occur in a dose dependent manner at both the mRNA (Figure 14a) and the protein level (Figure 14b).

To ensure that the lack of changes in the transcription of the selected panel of cytokines and chemokines was due to the concentration of IL-33 used being too low, a dose response of 0 to 60 ng/ml was employed. TGF-β was selected as the output, as this had previously shown a lack of induction in response to IL-33 stimulation at 15ng/ml in HT29 cells (Figure 12a). IL-33 did not increase transcription of TGF-β in HT29 (Figure 15a) or CT26 cells (Figure 15b) at any dose, indicating that the lack of transcription of the panel seen in (Figure 12 and 13) was unlikely due to the dose of IL-33 being too low.

3.2.9 IL-33 activates MAPK, NFκB and AKT signalling pathways

Whilst the majority of cytokines were not activated by IL-33 stimulation, CCL2 was activated in all cell lines. As such, I hypothesized that the activation of signalling pathways downstream of IL-33 may be responsible for the restricted cytokine induction observed. To examine this, I next investigated the ability of IL-33 to activate the canonical transcription factors and kinases, activated by other IL-1R/TLR superfamily members in colon cancer cell lines, such as NF-κB and MAPKs. Phosphorylation of the AKT pathway was also examined, as AKT is

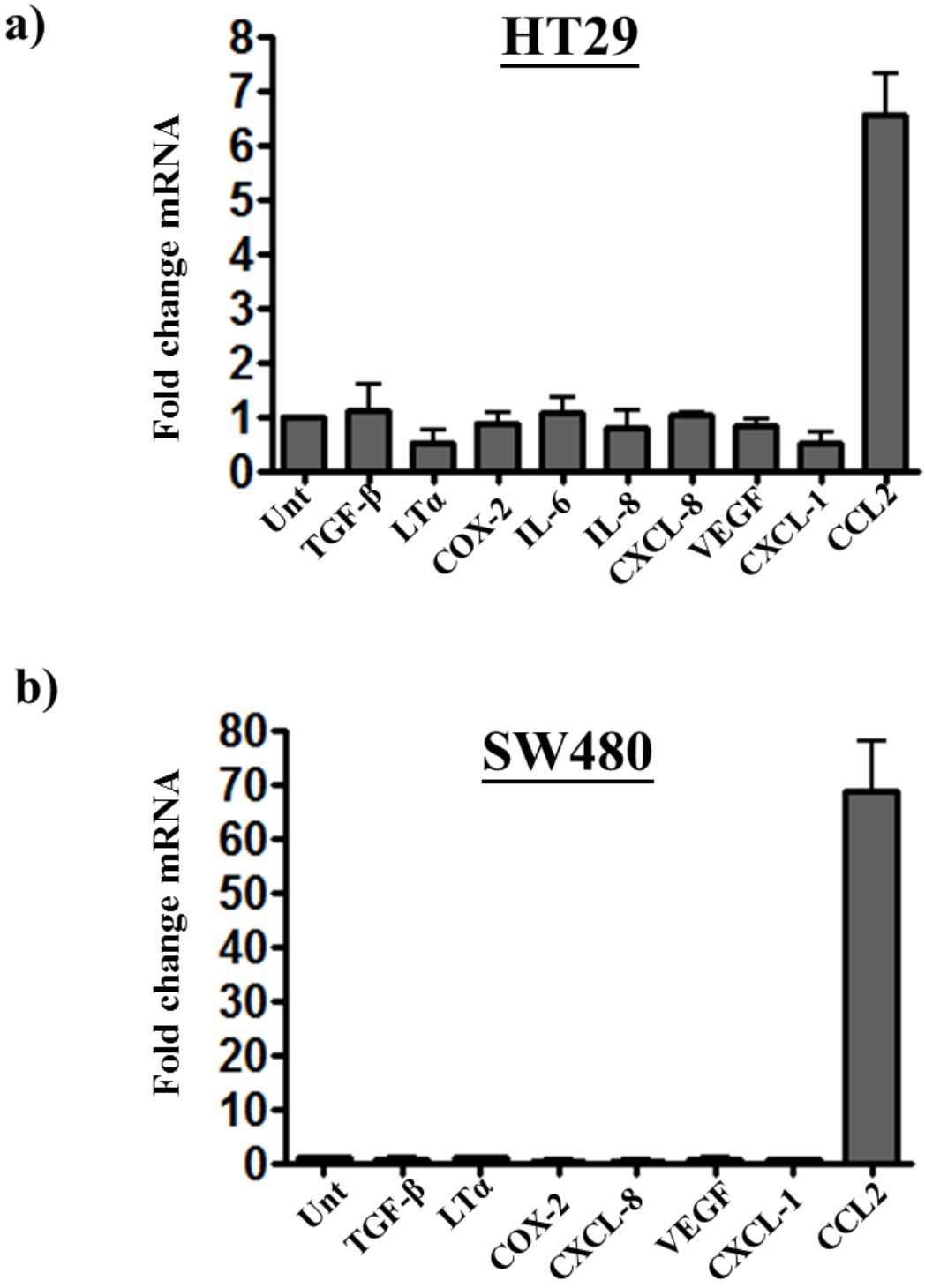


Figure 12: Stimulation of human colon cancer cells with IL-33 increased expression of CCL2. (a) HT29 and (b) SW480 cells were stimulated with 15ng/ml of IL-33 for 4 hours . CCL2 was induced by IL-33 stimulation, while it had no effect on transcription of a panel of other factors (i.e. TGFβ, VEGF, LTα, COX-2, IL-6, IL-8, CXCL-8 or CXCL-1). Data shown are mean +/- SEM (n=3).

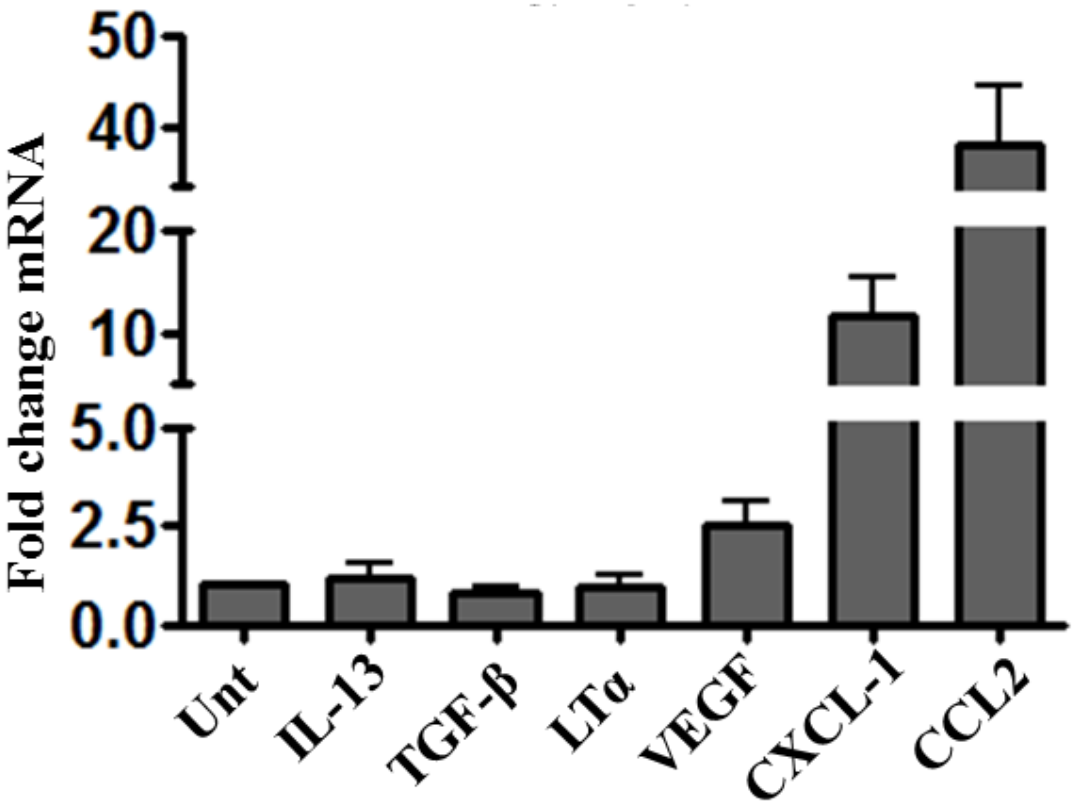


Figure 13: IL-33 induces the expression of VEGF, CXCL-1 and CCL2 in murine cancer cells. CT26 cells were stimulated with 15ng/ml of IL-33 for 4 hrs. RNA was extracted and changes in cytokine/chemokine expression were detected by qRT-PCR. RNA was extracted and changes in expression of VEGF, CXCL-1, and CCL2 were detected by qRT-PCR. Data shown are mean +/- SEM (n=3).

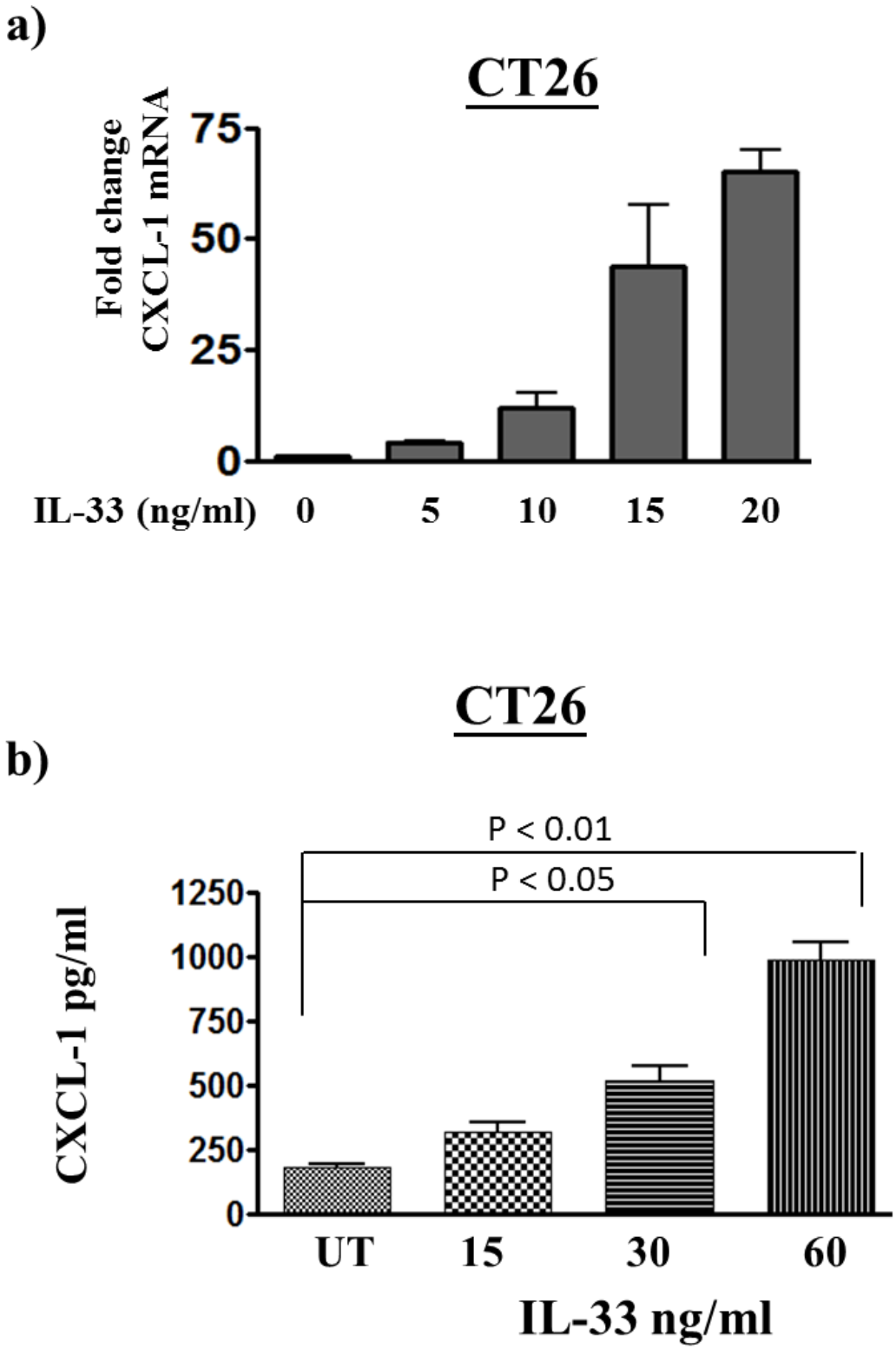


Figure 14: CT26 murine colon cancer cell produce CXCL-1 in response to IL-33 in a dose dependent manner. CT26 cells treated with IL-33 for 4 hours (a) and IL-33 mRNA expression was examined. CT26 cells were treated with IL-33 for 24 hours, (b) supernatants were removed and examined for CXCL-1 expression by ELISA. Data shown are mean +/- SEM (n=3). Statistical analysis was performed by one way ANOVA.

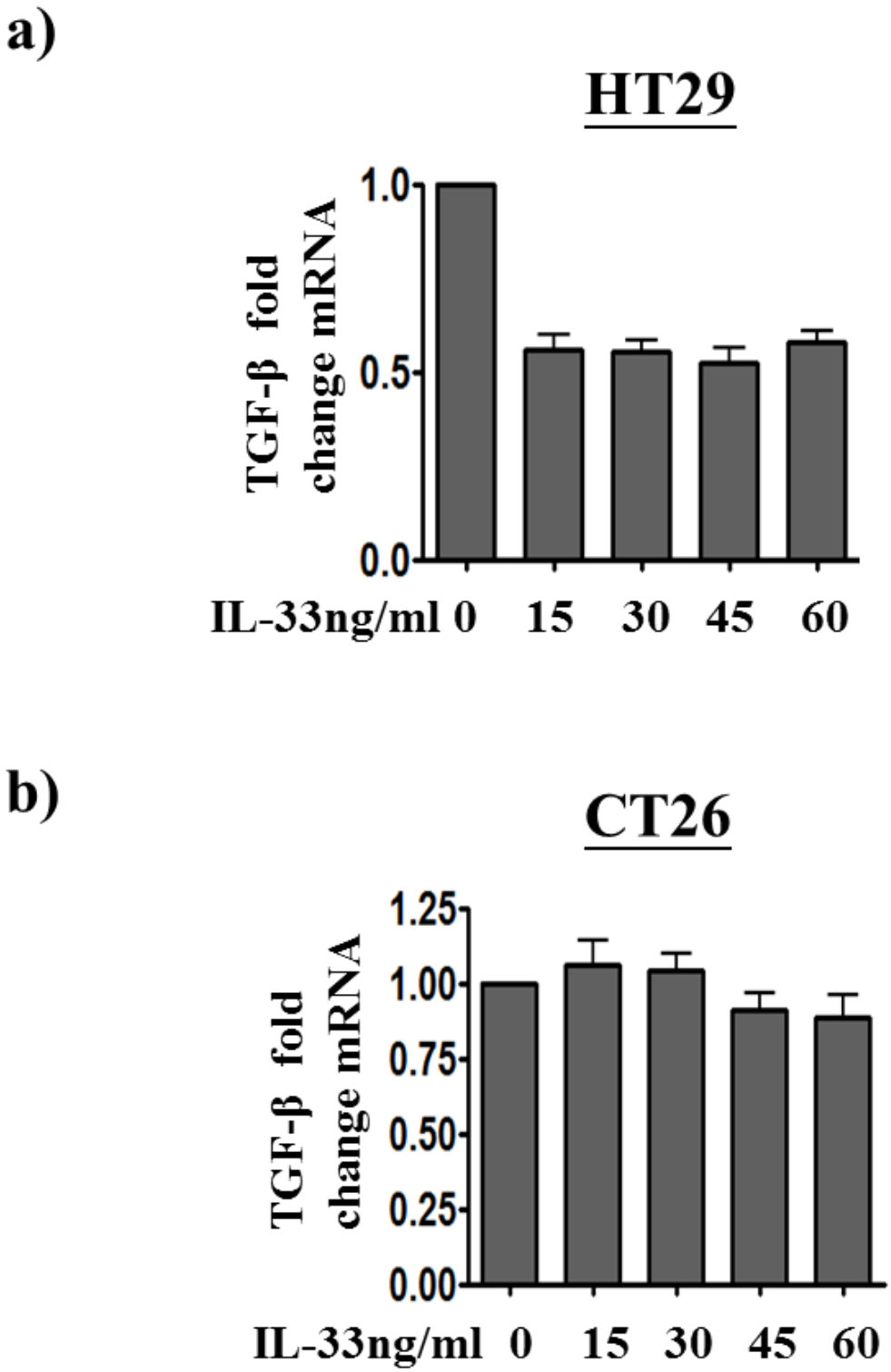


Figure 15: Increasing doses of IL-33 did not increase expression of TGF-β expression. Cells were stimulated with increasing doses of IL-33 as indicated. TGF-β mRNA expression was examined in (a) HT29 and (b) CT26. Data shown are mean +/- SEM (n=3).

strongly activated in many human cancers [265]. To investigate whether IL-33 was able to induce activation of NF-κB, phosphorylation of the NF-κB subunit p65 was examined in HT29 cells. p65 phosphorylation was observed 30 minutes after IL-33 stimulation, and this was sustained until the final time point of 60 minutes (Figure 16a), with no change in total p65 observed. To investigate MAPK activation, phospho-specific antibodies to JNK, ERK and p38 were used, with IL-33 stimulation seen to phosphorylate all of these. Phospho-ERK was observed at all-time points, however, the greatest phosphorylation of ERK was observed between 30 and 60 mins (Figure 16b). Maximal phosphorylation of p38 was observed between 30 and 60 mins (Figure 16b). IL-33 stimulation caused phosphorylation of JNK between 5-30 mins (Figure 16b). AKT phosphorylation in response to IL-33 treatment was observed at 45 and 60 min (Figure 16c). These results indicate that IL-33 can activate NFκB, MAPK and AKT subfamilies.

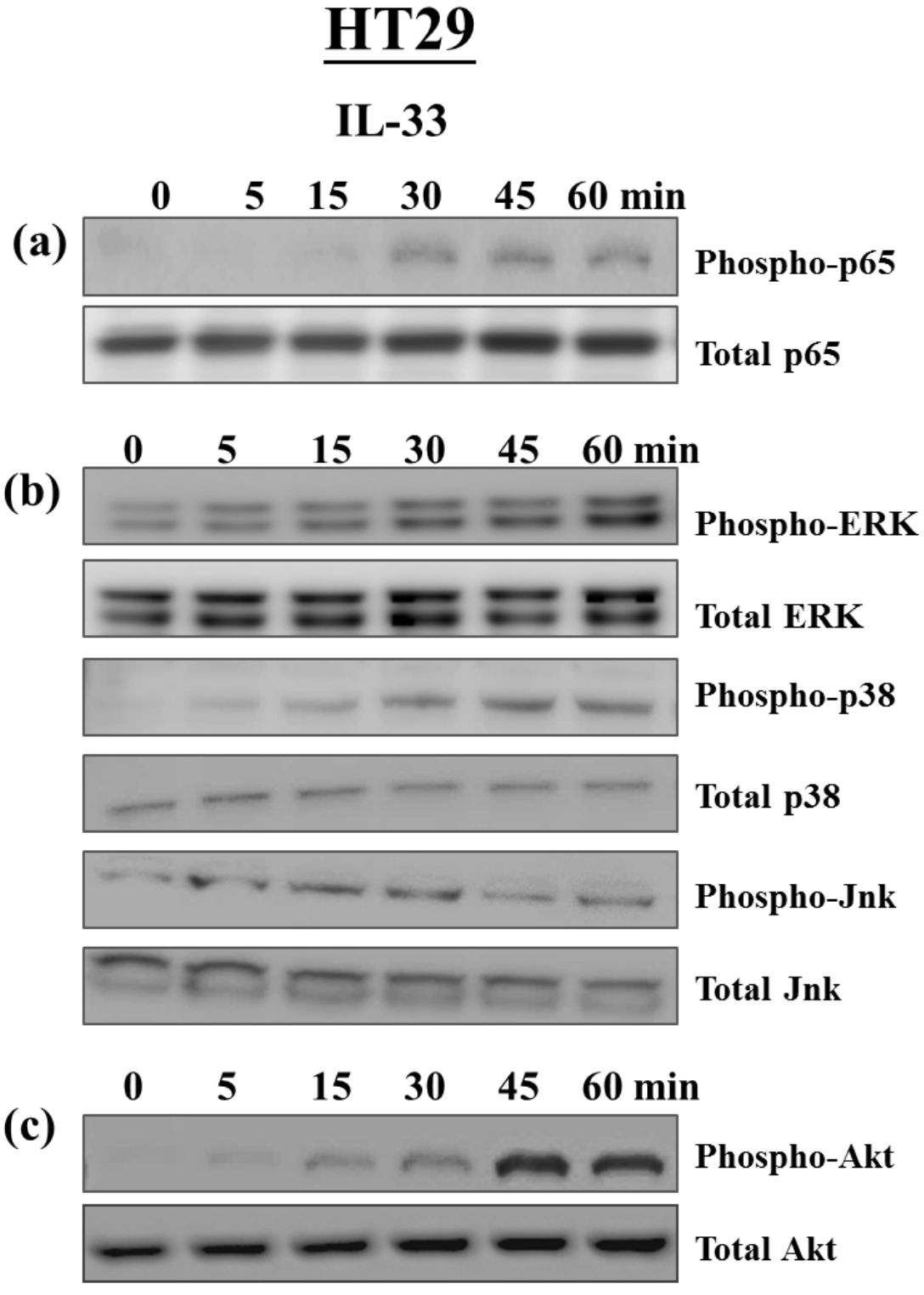


Figure 16: HT29 cells phosphorylate the NFκB p65 subunit p65 at 30 to 60 minutes. HT29 cells were treated for between 0-60 mins with IL-33. Cell lysates were separated by SDS-PAGE and probed with anti-Phospho-65 anti-p65, anti-Phospho-ERK, anti-ERK, anti-Phospho p38, anti-p38, anti-phospho Jnk, anti-Jnk and anti Phospho-Akt or anti-Akt specific antibodies as indicated. Data shown are representative of three independent experiments.

3.3 Discussion:

Here I have demonstrated that colon cancer cells express ST2L, sST2 and IL-33 *in vitro*. In terms of tumorigenic processes, stimulation with IL-33 suppressed cellular proliferation, increased migration, but did not affect invasion of colon cancer cells. IL-33 induced a very limited number of cytokines and chemokines in colon cancer cells, in particular only inducing expression of CCL2 in human cells, and CXCL-1, VEGF and CCL2 in murine cells. However, IL-33 activates multiple signalling pathways such as AKT, MAPK and NF-κB in colon cancer cells.

3.3.1 sST2 expression was increased in colon cancer cells compared to ST2L expression

In this chapter, I have comprehensively examined the expression of key components of the IL-33/ST2 axis in colon cancer cells. I have shown that colon cancer cells express both sST2 and ST2L isoforms. The basal level of sST2 was observed to be generally higher than that of ST2L in these cells. Both ST2 isoforms are transcribed from the same ST2 gene (IL1RL1). However, unlike ST2L, sST2 lacks the transmembrane and cytoplasmic domains [71, 72, 98]. ST2 gene expression is controlled by two discrete promoters, a distal and a proximal promoter [104]. Promoter usage is cell type specific, not transcript specific, as fibroblasts initiate transcription of ST2 (both sST2 and ST2L) at the proximal promoter, while acute myeloid leukemic cells are dependent on the distal promoter mainly for ST2L, with less sST2 transcribed [105]. Thus, the variation in the levels of ST2 isoforms that I have observed in colon cancer cells is unlikely to be due to differential promoter usage, with the exact mechanism responsible for variation between ST2L expression and sST2 expression being unclear. One possibility is that the different levels of ST2 isoforms detected are due to various regulatory processes, such as

transcriptional regulation [114]. In a study investigating the raised levels of sST2 produced by cardiac cells following myocardial infarction, stress or stress-activated cytokine release were found to be responsible for the sST2 induction [266]. However, further studies are required to clarify the exact mechanism of regulation of ST2 isoforms in colon cancer cells.

3.3.2 IL-33 suppressed cancer cell proliferation

IL-33 is an important mediator of intestinal barrier homeostasis. IL-33 is thought to maintain a quiescent state in barrier cells, as it is only produced when cells are senescent. Downregulation of IL-33 has been linked to initiation of cell proliferation in intestinal epithelial cells [145]. Here, I have shown that IL-33 stimulation did not enhance cellular proliferation of colon cancer cells *in vitro*. Indeed, similar to the findings for IL-33 in the normal intestine, these data demonstrate that IL-33 may, in fact, play an inhibitory role in colon cancer cell proliferation and may be downregulated during cellular proliferation, although changes in IL-33 expression during proliferation were not examined. Differences in the ability of IL-33 to suppress proliferation were observed between the cell lines examined, with HCT116 cells showing less suppression of proliferation by IL-33 than HT29 cells. HT29 cells are isolated from a stage II adenocarcinoma, while HCT116 are stage IV. This suggests that cancer cells may become less responsive to the anti-proliferative effects of IL-33, as they increase in cancer stage. Other studies investigating the ability of IL-33 to alter cell proliferation demonstrated that IL-33 played opposing roles in cellular proliferation depending on the confluency of the cells. When IL-33 was added to confluent murine embryonic fibroblast cells (NIH-3T3), proliferation of the cells was suppressed. Conversely, when IL-33 was added after the start of cellular

proliferation, it enhanced cellular growth [267]. The opposing effects observed may be due to the role that IL-33 plays in maintaining intestinal homeostasis. When cell density is low, IL-33 enhances cellular proliferation, while when cells are confluent IL-33 suppresses proliferation. The effect of IL-33 on cell proliferation has been examined in other cancer cell types. In pancreatic stellate cells, IL-33 stimulation did not induce proliferation [268]. Although, in contrast to this, in ovarian cancer cells, IL-33 stimulation increased cellular proliferation *in vitro,* whilst *in vivo* IL-33 expression positively correlated with Ki-67 expression [269]. The variation in IL-33-induced-proliferation between cancer cells suggests that the function of IL-33 may vary depending on the cancer type [269].

3.3.3 IL-33 increased migration of colon cancer cells

In contrast to the inhibition of proliferation, IL-33 was seen to enhance cellular migration of colon cancer cells *in vitro,* in a dose dependent manner, in all three cell lines examined. This ability of IL-33 to induce cellular migration *in vitro* suggests that IL-33 may play a role in metastasis *in vivo*. However, we only observed robust migration of colon cancer cells at very high doses of IL-33, which may not be physiologically relevant, as IL-33 levels in the serum of cancer patients is typically in the low pg/ml concentration [270]. Similarly, IL-33 induced migration of gastric and ovarian cancer cells [254, 269]. Furthermore, migration of ovarian cancer cells was reduced in response to transfection with IL-33 siRNA [269]. Contrastingly, transgenic expression of IL-33 was shown to attenuate tumour metastasis in the B16 melanoma and Lewis lung carcinoma metastatic models [201]. However, the amount of IL-33 produced by these cells was not quantified and therefore it is difficult to determine if the differences observed were due to large variations in IL-33

concentration. Therefore, this suggests, that similar to the differences we observed in the role of IL-33 in colon cancer cell proliferation, IL-33 may play a dichotomous role in cancer cell migration depending on the origin of the cancer.

Distinct from migration, I found that IL-33 did not promote invasion of colon cancer cells *in vitro*. In contrast to our findings in colon cancer cells, IL-33 has previously been shown to enhance the invasion of gastric cancer cells [271] and human CRC primary cells. Following transfection with either an IL-33 or an ST2 expression vector, human CRC primary cells extracted from surgical samples showing enhanced invasion compared to untransfected cells [204]. One explanation for the differences seen may be due to variations in the concentration of IL-33. Transgenic ST2 expression may also have increased the responsiveness of the cells, which could also account for the increase in invasiveness observed in the primary cells. The overall contribution of IL-33 to tumour progression remains to be directly assessed, and the mechanisms by which IL-33 contributes to tumour initiation and progression remain unexplored.

3.3.4 IL-33 activated chemokine production by colon cancer cells

Cytokines allow the rapid dissemination of signals between cells. However, cytokines produced in the tumour microenvironment can influence tumour development and progression in a number of ways, one of which is through the recruitment of immune cells to the tumour site. Most tumour associated macrophages are derived from monocytes recruited from the blood by the chemokine CCL2 [272]. Of the cytokines and chemokines examined, CCL2 was the only one consistently induced across all cell lines by IL-33. CXCL-1, a neutrophil chemotactic factor, was also induced by IL-33 in murine colon cancer cells. It is well established that IL-33

has the ability to promote inflammation, through the recruitment of immune cells [264]. Thus, it was not surprising that IL-33 induced the production of two immune cell chemoattractants. This profile of chemokine induction has also been demonstrated in other disease models. In a murine model of mucositis, IL-33 treatment combined with a chemotherapeutic agent (CPT-11), increased CCL2 and CXCL-1 expression in WT mice, above levels seen in $ST2^{-/-}$ mice [273]. Similarly, in a fibrosis mouse model, $ST2^{-/-}$ mice showed decreased levels of CXCL-1 and CCL2 in lung tissue compared to WT mice [274]. CXCL-1 is a known neutrophil chemoattractant and indeed, mice treated with IL-33 displayed neutrophil and eosinophil infiltration in the colonic mucosa [153]. In primary lung endothelial cells, CCL2 was also produced in response to IL-33 stimulation, sustaining chronic inflammation of the asthmatic airway [112]. Therefore, the increased production of CCL2 by colon cancer cells in response to IL-33 may lead to increased recruitment of macrophages to the tumour site. Macrophage infiltration is generally considered to be a poor prognostic indicator in cancer, although this can vary widely depending on the cancer types. For instance, increased macrophage recruitment is indicative of a poor prognosis in breast cancer [275]. However, in colon cancer it is currently unclear whether high macrophage infiltration is beneficial to the patient, as some studies have reported that TAMs appear to have anti-tumour activity and are associated with improved disease free survival [45], while other research has shown that increased macrophage infiltration positively correlates with tumour progression and aggressiveness of this disease [25, 276]. Similarly, neutrophils can also significantly influence the tumour microenvironment in a pro-tumorigenic manner, as they have been shown to induce angiogenesis and metastasis [277]. However other reports suggest an anti-tumour neutrophil phenotype can be generated *in vivo*

[278]. Therefore, further investigation is required to determine the downstream effects of increased chemokine production, activated in response to IL-33 stimulation, in the tumour cells.

3.3.5 IL-33 activates signalling pathways common to other IL-1 family members

As I have shown that IL-33 activates CCL2 and CXCL-1, I next sought to determine how these chemokines were activated. The CCL2 promoter contains binding sites for multiple transcription factors such as NF-κB subunits, AP-1, Sp-1 and the STAT family [279]. The CXCL-1 promoter is also regulated by numerous factors, e.g. NF-κB subunits, poly (ADP-ribose) polymerase (PARP) and CCAAT displacement protein (CDP) [280]. While selecting which signalling pathways to examine, I considered that, as IL-33 is a member of the IL-1 receptor family, and common to other family members, IL-33 signalling occurs through dimerization of the TIR domain of the receptor (i.e. ST2L in this case) with the co-receptor IL1RAcP. MyD88 is then recruited and IRAK1/2 activates TNF receptor associated factor 6 (TRAF6), leading to activation of the MAPK pathways [281]. Therefore, I examined the ability of IL-33 to activate the following pathways; the MAPK pathways, due to the IL-1 family connection; NF-κB, as this is known to activate both CCL2 and CXCL1; and the PI3K/AKT pathway, due to its increased activation in human cancer. I observed activation of multiple signalling pathways by IL-33 in colon cancer cells, including activation of the PI3K/AKT and MAPK pathways (JNK, ERK and p38). Similar to my findings, JNK was also found to be activated by IL-33 in gastric cancer cells [271], while both ERK and JNK were activated in ovarian cancer cells. P13K/Akt signalling was not investigated in that study [271]. Although IL-33 has previously been shown to activate PI3K/AKT signalling in endothelial

cells [282], this has not been shown to date in cancer cells. I have also demonstrated that IL-33 activated the NF-κB subunit, p65. There have been contrasting findings in the literature regarding the ability of IL-33 to activate NF-κB, with some studies showing that IL-33 can drive NF-κB activation [283, 284], while others have shown that IL-33 dampens NF-κB activity. For instance, nuclear IL-33 was shown to sequester nuclear NF-κB and suppress NF-κB activity, thereby reducing pro-inflammatory signalling [285]. NF-κB activation has previously been shown to mediate expression of CXCL1 and CCL2 in ovarian cancer cells [286, 287]. Similarly, CCL2 was upregulated via NF-κB and JNK pathways in prostate cancer cells [288]. Therefore, a combination of the pathways I examined may be responsible for activation of CXCL-1 and CCL2, i.e. MAPK, NF- κB and Akt/P13K.

Interestingly, I found very few other cytokines were induced by IL-33. We investigated a panel of ten cytokines, of which only three were seen to be robustly induced by IL-33. A similar absence of cytokine induction by IL-33 was observed in pancreatic and cardiac cells. In particular there was a lack of IL-6, IL-8 or CCL2 induction [249, 268]. Moreover, in a murine model of sepsis, treatment with IL-33 lowered the levels of the pro-inflammatory cytokines, TNF-α, IL-6 and CXCL2 in the blood, while no increase in IL-4, IL-10 or IL-13 was detected in lung tissue post IL-33 treatment [289]. In the intestine, IL-33 is a known regulator of barrier function and homeostasis. Under homeostatic conditions IL-33 is localized to the nucleus of epithelial cells and has been shown to suppress pro-inflammatory gene expression [285]. IL-33 is thought to maintain intestinal homeostasis by limiting inflammation [290]. However, upon cellular damage or necrosis IL-33 is released from the nucleus and functions as an alarmin, inducing a pro-inflammatory response. Therefore, IL-33 can have both pro- and anti-inflammatory functions. Indeed, under the conditions

examined in this chapter IL-33 may be acting to limit inflammation and this may explain the lack of cytokine induction observed. Given the ability of IL-33 to activate the MAPK, AKT and NF-κB pathways in colon cancer cells, the lack of cytokine induction is unclear. One possible explanation is that IL-33 also activates the transcription of inhibitors, such as SIGGIR [291], which may in turn dampen IL-33 signalling, reducing cytokine output. This is a potential mechanism whereby such limited cytokine expression may be accomplished, although such specificity being achieved through targeted inhibition has not been extensively reported. Another possible mechanism whereby limited cytokine expression could occur in colon cancer cells in response to IL-33 is through modification of the genes by epigenetic mechanisms. IL-33 expression is linked to histone deacetylase 3 (HDAC3) enzyme, which regulates gene expression [139]. IL-33 was also shown to be specifically regulated by HDAC inhibitors compared to other IL-1 members [139]. Epigenetic modification may explain why so few cytokines were produced in response to IL-33 stimulation, especially as transcription factors and pathways examined (i.e. NF-κB, MAPK, and AKT), were activated by IL-33. Therefore, it seems likely that the level of repression of transcription in response to IL-33 may be occurring through direct modification at the epigenetic level of inflammatory genes.

In conclusion, this chapter has characterised expression of IL-33 and ST2 in colon cancer cells, identifying an anti-proliferative role for IL-33/ST2 in colon cancer, and showing IL-33 enhanced migration while invasion was unaltered. Furthermore, this research has identified that colon cancer cells stimulated with IL-33 produce potent chemokines and angiogenic factors, which could recruit immune cells, specifically macrophages and neutrophils, which can influence the immune infiltrate in the tumour microenvironment and the vascularisation of the tumour. Therefore, further

studies are required to determine whether manipulation of this pathway represents a potential therapeutic target in colon cancer.

Chapter 4

The IL-33/ST2 axis modulates tumour growth and the tumour microenvironment *in vivo*

4.1 Introduction:

Current research has primarily focused on the role of IL-33 and ST2 in chronic disorders, with IL-33 and ST2 playing a role in the pathogenesis of several human inflammatory diseases including IBD [177]. On commencing this thesis there were only two papers pertaining to the role of the IL-33/ST2 axis in cancer, both of which examined this signalling axis in breast cancer [193, 194]. The first study utilised $ST2^{-/-}$ mice and demonstrated that deletion of ST2 suppressed breast cancer progression and metastasis, and increased the number of activated natural killer (NK) cells. Furthermore, ST2 deletion increased the cytotoxic activity of NK cells and $CD8^{+}$ T cells and systemic Th1/Th7 cytokines [193]. A second study specifically implicated sST2 in metastasis, as knocking down sST2 reduced ErbB2-induced cell motility in breast cancer cells, while cells from metastatic breast tumours secreted increased levels of sST2 relative to cells from primary tumours. Moreover, the authors demonstrated that patients with metastatic breast cancer had higher levels of serum sST2 compared to patients with primary tumours, leading to the conclusion that in breast cancer ST2 may serve as a biomarker of disease severity or progression [194]. Therefore, in breast cancer the IL-33/ST2 pathway appears play a pro-tumorigenic role. Subsequent studies in breast cancer also showed significantly higher levels of both IL-33 and sST2 in the serum of patients with ER positive breast cancer relative to healthy controls [195]. Moreover, administration of recombinant IL-33 to breast cancer-bearing mice resulted in accelerated tumour growth and enhanced metastasis. This was thought to be due to increased accumulation of immunosuppressive immune cells and innate lymphoid cells within the tumours, further supporting a role for IL-33

in promoting tumorigenesis [196]. More recently, the role of the IL-33/ST2 signalling axis has been investigated in other cancer types. Consistent with a role for IL-33 and ST2 in promoting tumour cell invasion and metastasis, inhibition of IL-33 and ST2 in glioma cells resulted in reduced tumour growth, migration and colony formation *in vitro*, and smaller tumours *in vivo* [197].

Other studies, however, have shown somewhat divergent effects, with IL-33 reduced in the plasma of non-small cell lung cancer patients relative to controls [198], and IL-33 negatively correlating with tumour stage in multiple myeloma patients [199]. Furthermore, over-expression of IL-33 potently inhibited tumour growth and metastasis in both B16 melanoma and 4T1 breast cancer models. NK cells and $CD8^+$ T cell numbers were increased [200]. Similarly, transgenic expression of IL-33 reduced tumour metastasis in a Lewis lung carcinoma and B16 melanoma model. Both the number and the cytotoxicity of $CD8^+$ T cells and NK cells were increased in response to IL-33 expression [201].

In the previous chapter, I demonstrated that stimulation of colon cancer cells with IL-33 induced the expression of the chemokines CCL2 and CXCL-1. Thus, IL-33/ST2 signalling in colon cancer may contribute to the chronic inflammatory microenvironment thereby promoting tumorigenesis. CXCL-1 is a neutrophil chemotactic factor, while CCL2 is a macrophage chemotactic factor. Recruitment of such immune cells can have a potent influence on the tumorigenic process. For instance, depending on activating signals, macrophages can be polarised towards either an anti-tumorigenic M1 phenotype or a tumour-promoting M2 phenotype. Consistent with

tumour-associated macrophages (TAMs) being skewed towards an M2 phenotype, increased numbers of macrophages can indicate a poor prognosis in certain cancers such as breast [292], gastric [293] and ovarian [293]. However, the role of TAMs in colon cancer seems to be more complex, with both tumour-suppressive and tumour-promoting effects being reported. Similarly, both pro- and anti-inflammatory effects have been described for tumour-associated neutrophils. Therefore, a cytokine such as IL-33 with the ability to influences immune cell recruitment may powerfully influence tumorigenesis.

Given the variation in the expression levels of IL-33 and ST2 in CRC reported in the literature, combined with the IL-33 induced chemokine production we had observed in colon cancer cells, the aim of this chapter was to characterise IL-33 and ST2 expression in CRC. Furthermore, I wished to specifically examine expression of the different isoforms of ST2 in human colon cancer tissue.

4.2 Results:

4.2.1 Generation of stable cell lines with suppressed ST2 expression by stable transfection with short hairpin RNA (shRNA) encoding plasmids.

In order to further investigate the role of ST2 in colon cancer, stable cell lines with suppressed ST2 expression were generated (Figure 1). CT26 mouse colon cancer cells were selected as these cells grow in immune competent BALB/c mice. Moreover, I had previously shown that these cells express ST2L, sST2 and IL-33 (Chapter 3, figure 4 and 6). CT26 cells were transfected with lentivirus particles containing shRNA encoding plasmids designed to target the murine ST2 gene (Figure 2). In parallel, CT26 cells were transfected with lentiviral plasmids containing a scrambled sequence showing no homology to any known mammalian gene. As can be seen in Figure 2a, a CT26 clone transfected with shRNA against ST2, hereafter referred to as $CT26^{ST2\ shRNA\#1}$ was generated that showed a significant reduction in ST2 expression (*** $P < 0.001$) relative to cells transfected with the scrambled control, $CT26^{scr\ shRNA\#1}$ cells, by Western blot. Although a number of clones were generated, this clone was selected as it showed the lowest expression of ST2 by Western blot. To further characterise knock down of ST2 expression, a functional assay was performed. In chapter 3, I showed that CXCL-1 is produced by CT26 cells in response to IL-33 stimulation. Thus, CXCL-1 production in response to IL-33 stimulation was selected to confirm that the ST2 receptor was knocked down. IL-33 stimulation led to a 27-fold increase in CXCL-1 production in $CT26^{scr\ shRNA\#1}$ cells (Figure 2b). In contrast, there was approximately a 4-fold reduction in CXCL-1 production by $CT26^{ST2\ shRNA\#1}$ cells upon IL-33 stimulation, relative to $CT26^{scr\ shRNA\#1}$ cells (Figure 2b). To determine

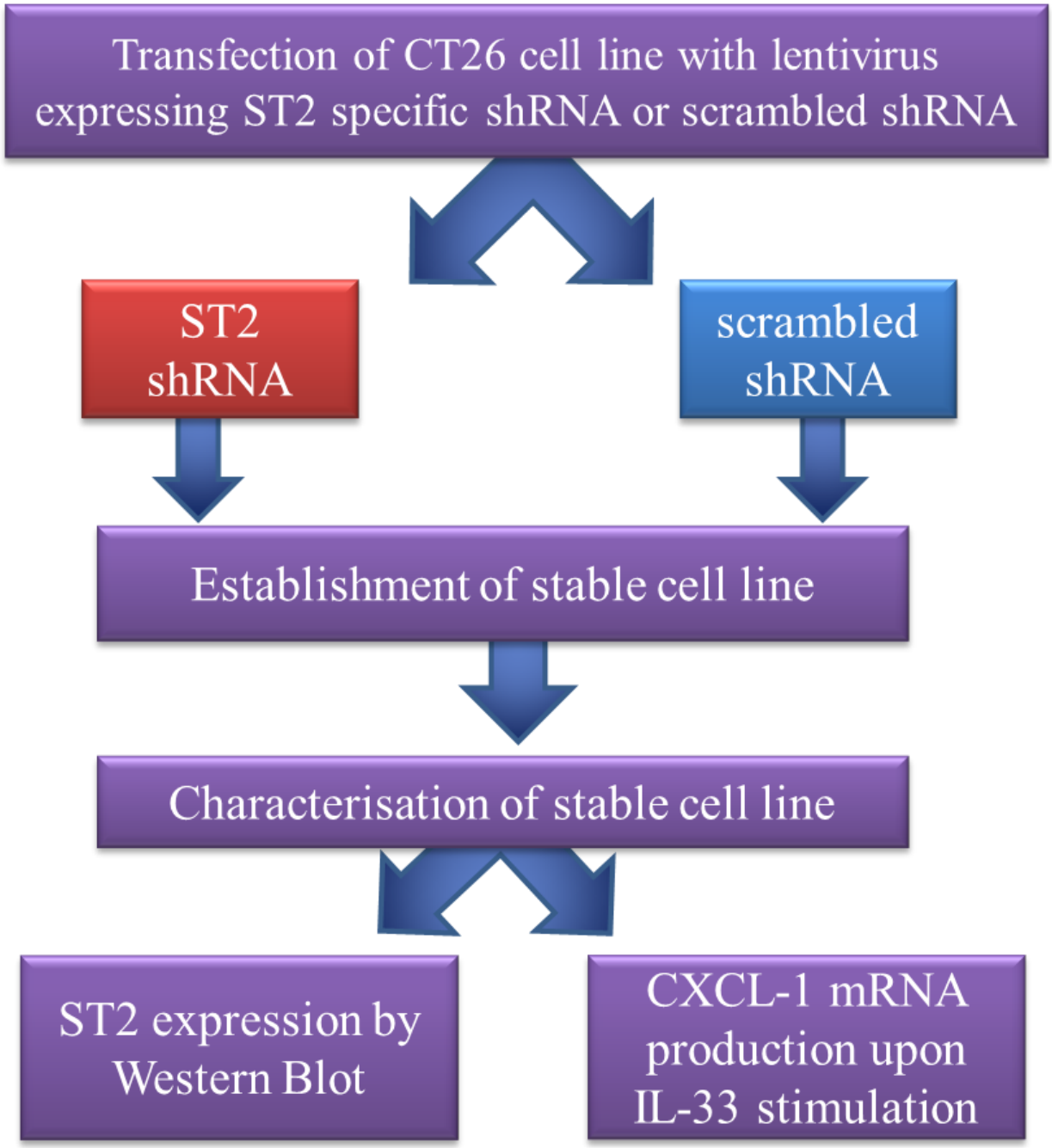


Figure 1: Experimental outline of the generation of CT26 stable cell lines with suppressed expression of ST2. CT26 cells were stably transfected with lentiviral particles containing either ST2-specific or scrambled control shRNA expressing plasmids. ST2 knockdown cells were characterised by Western blot and qRT-PCR.

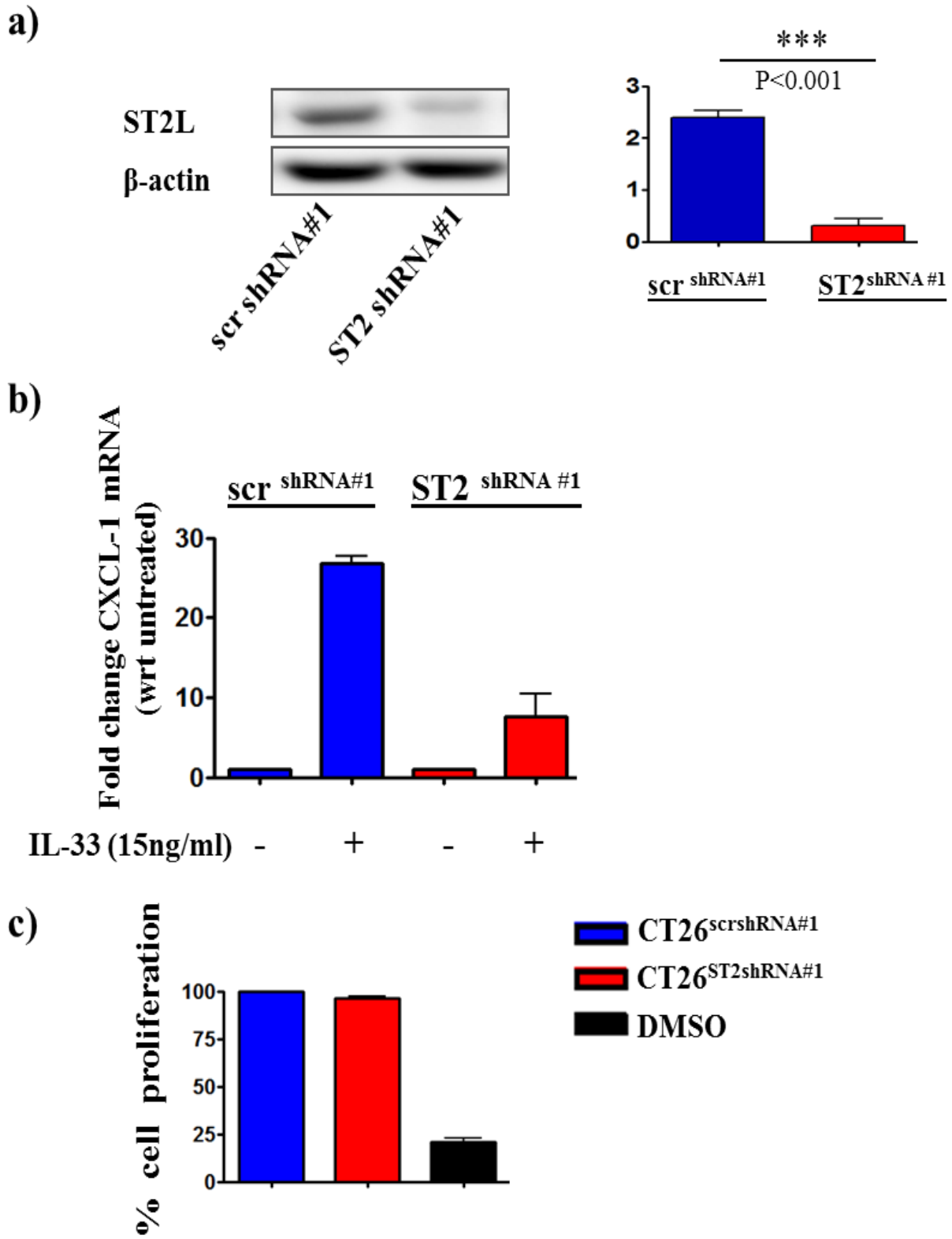


Figure 2: Characterisation of stable cell lines generated following transfection of CT26 cells with shRNA targeted against ST2 or scrambled control. CT26 cells were transfected with lentiviral particles containing either ST2-specific or scrambled control shRNA expressing plasmids in the presence of polybrene. Following puromycin selection, stable clones were generated and knockdown of ST2 expression was determined by (a) Western blotting with subsequent quantification by densitometry. (b) Changes in CXCL-1 production were determined by qRT-PCR. (c) Cell proliferation was measured by resazurin reduction. DMSO was added to ensure the assay could detect changes in proliferation. Values are plotted as Mean +/- S.E.M. n=3. *** $P < 0.001$. Results were statistically evaluated using One-way Anova with Tukeys post-test, or by students paired t test.

whether knocking down ST2 affected cellular proliferation, a resazurin reduction assay was utilised to examine the proliferation of the CT26 cell lines generated. Suppression of ST2 in CT26 cells ($CT26^{ST2\ shRNA\#1}$ cells) did not alter the proliferation of these cells relative to control cells ($CT26^{ST2\ scr\ shRNA\#1}$) (Figure 2c). This indicated that reducing ST2 expression in murine colon cancer cells does not affect the proliferation of these cells *in vitro*. In contrast, when treated with DMSO, an amphipathic molecule, that is known to induce apoptosis [294], cell proliferation was substantially reduced (~75%), relative to untreated cells.

4.2.2 Knocking down ST2 expression in CT26-derived tumours increases tumour formation and growth *in vivo*.

In order to assess the biological effect of reducing ST2 expression on tumour growth, we utilised the well-characterised murine model of CT26 colon cancer cells subcutaneously injected into the right flank of BALB/c mice. I first performed a preliminary study, whereby immunocompetent female Balb/c mice were subcutaneously injected with either $CT26^{scr\ shRNA\#1}$ cells (n=4) or $CT26^{ST2\ shRNA\#1}$ cells (n=4), and tumour growth was monitored over a period of 20 days. A separate control group were injected with PBS only (n=3) (Figure 3a). To determine whether tumour cell injection or cancer growth impacted on feeding capability, body weight and food consumption were measured throughout the course of the experiment. No differences in food consumption were observed between the $CT26^{scr\ shRNA\#1}$ and $CT26^{ST2\ shRNA\#1}$ groups (Figure 4a). Moreover, no significant differences in body weight were detected between the groups (Figure 4b).

a)

Group name	Number in group	Cell type inoculated
1	4	$CT26^{scr\ shRNA\#1}$
2	4	$CT26^{ST2\ shRNA\#1}$
3	3	PBS

b)

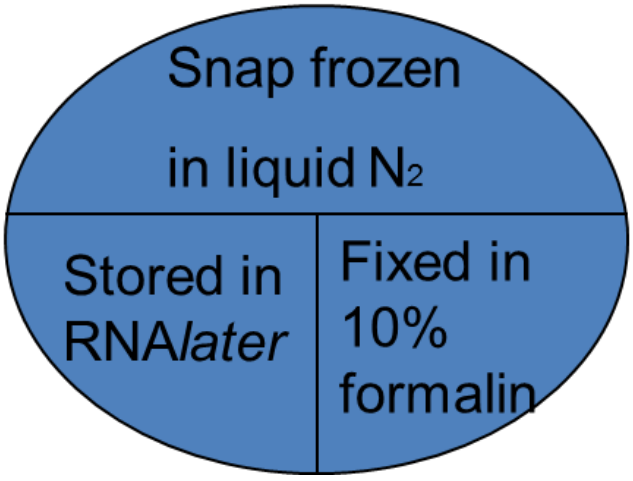


Figure 3: Outline of preliminary *in vivo* study design and tumour dissection. (a) Balb/c immunocompetent female mice were subcutaneously (s.c.) injected with 4 x 10^4 cells in 200µl of PBS in the right flank. The control group were injected with 200µl of PBS only. (b) Animals were sacrificed after 20 days, and harvested tumours were dissected as illustrated.

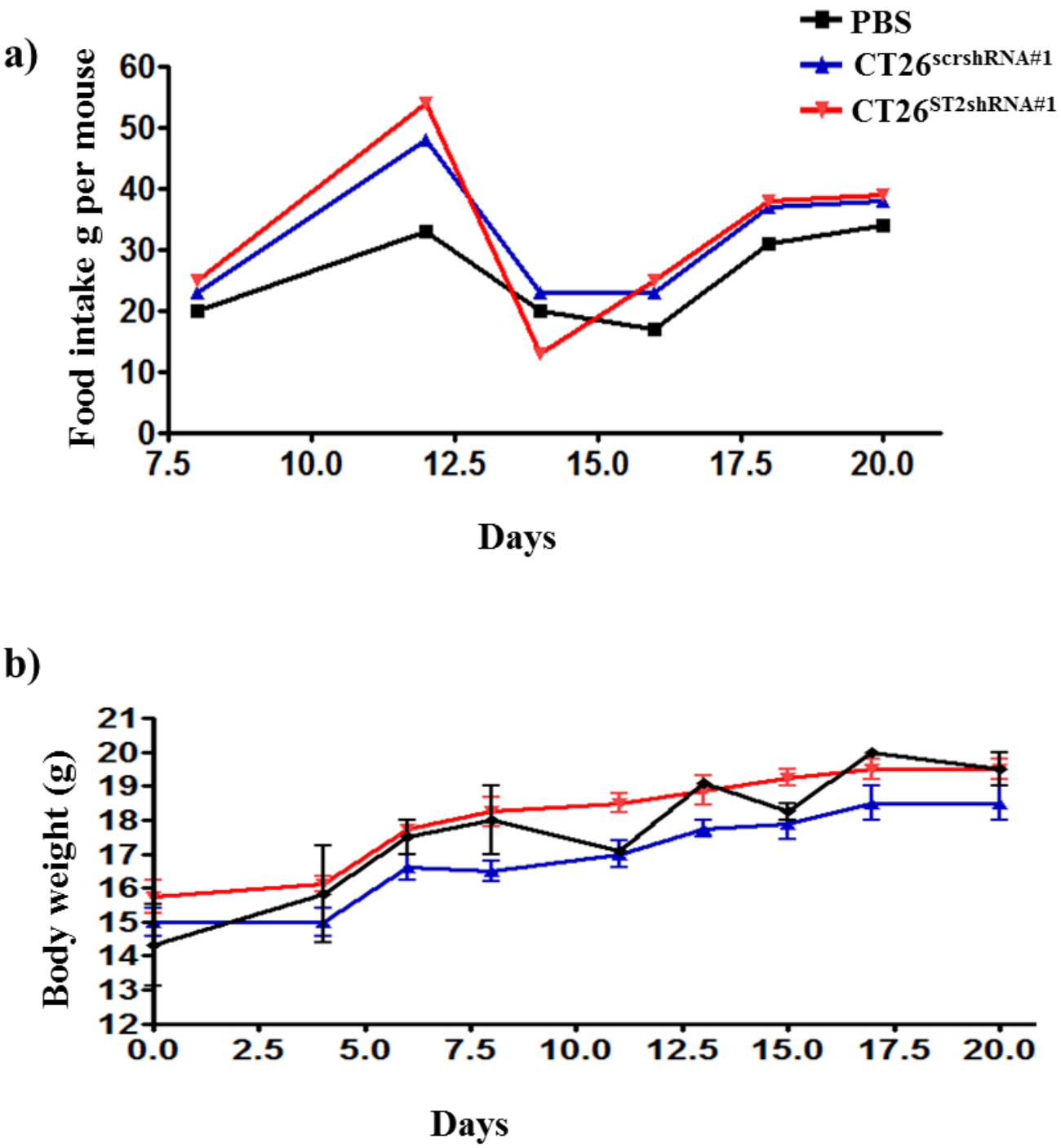


Figure 4: Food consumption and body weight was monitored, with no differences observed between the groups. (a) Food consumption for each group was measured twice weekly post injection of tumour cells. Values are displayed as average food consumption per mouse. (b) The weight of each animal in the study groups was monitored three times per week. Values are plotted as Mean ± S.E.M.

Tumour development was monitored over the course of the investigation. By day 12, all eight mice injected with either $CT26^{ST2\ shRNA\#1}$ or $CT26^{scr\ shRNA\#1}$ cells had developed palpable tumours (Figure 5a). However, in contrast to the *in vitro* findings, whereby suppressing ST2 expression did not alter tumour growth (Figure 2c), *in vivo* tumour growth was increased when ST2 expression was suppressed. The cancer cells with suppressed ST2 expression formed tumours that grew at a faster rate in mice, than those injected with $CT26^{scr\ shRNA\#1}$ cells. Once the tumours had reached the maximum size ($2cm^3$), all mice were sacrificed. On the day of termination, the final tumour volume was increased by ~85% in tumours derived from $CT26^{ST2\ shRNA\#1}$ cells relative to those derived from $CT26^{scr\ shRNA\#1}$ cells ($p=0.026$) (Figure 5b).

4.2.3 Ki67 expression was unchanged in $CT26^{ST2\ shRNA\#1}$ tumours compared to $CT26^{scr\ shRNA\#1}$ tumours.

Tumour tissue was excised and dissected according to figure 3b. Formalin-fixed paraffin-embedded tissue sections were stained using haematoxylin and eosin (Figure 6a). General tumour morphology was examined and no differences in tumour architecture were observed. As larger tumours resulted from $CT26^{ST2\ shRNA\#1}$ cells, compared to $CT26^{scr\ shRNA\#1}$ cells, sections were also immunostained with a Ki67 antibody, a marker of cellular proliferation. Ki67 staining was unchanged in $CT26^{ST2\ shRNA\#1}$ compared to $CT26^{scr\ shRNA\#1}$ (Figure 6b). Therefore, from these results I can conclude that both groups contained actively proliferating tumour cells.

4.2.4 Generation and functional assessment of additional $ST2^{-/-}$ CT26 cells *in vitro* and *in vivo*.

In order to confirm the findings of the initial study and to power the study

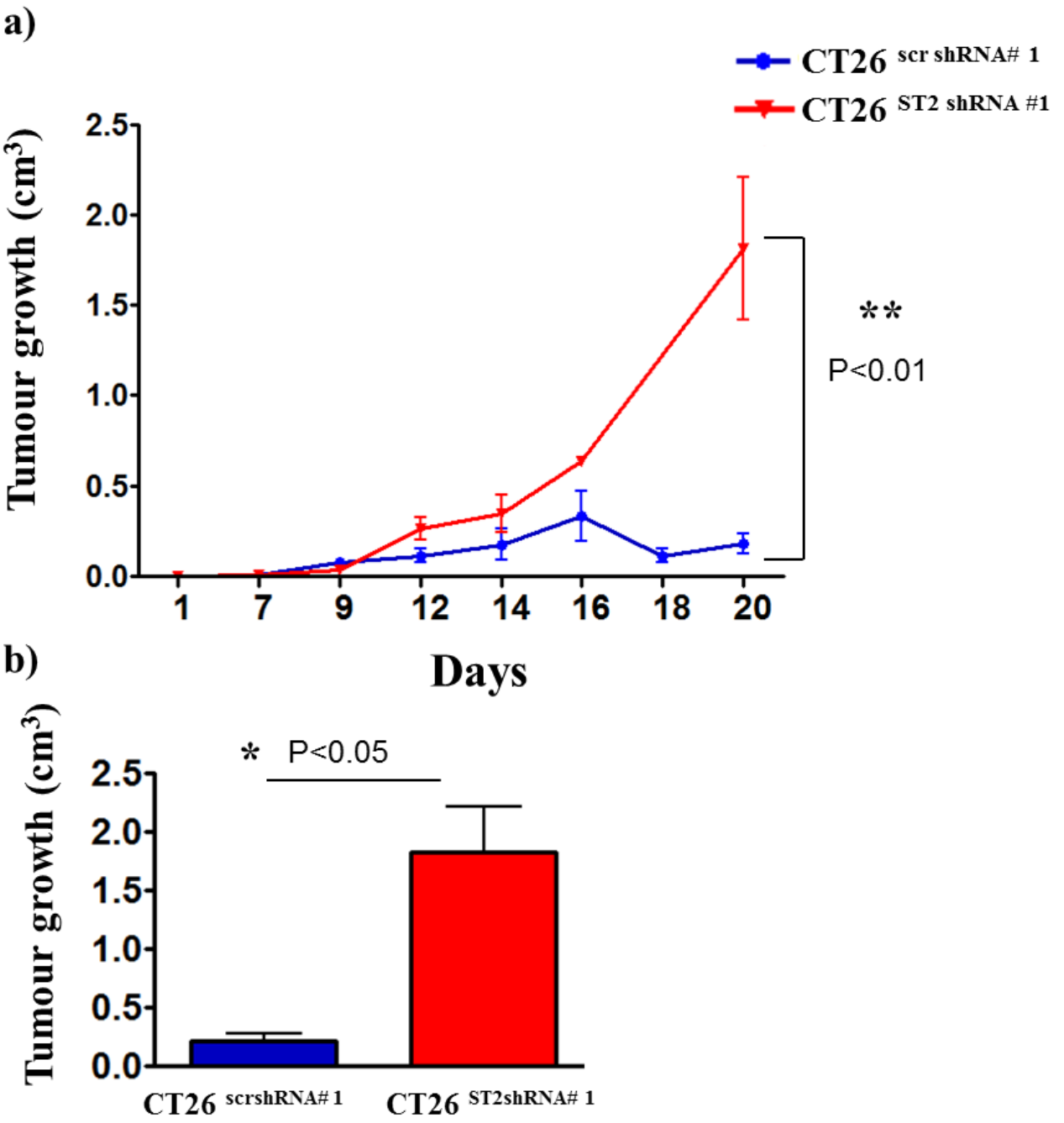


Figure 5: Suppression of ST2 expression in colon cancer cells results in increased tumour growth *in vivo*. (a) Female Balb/c immune-competent mice were s.c. injected with 4 x 10^4 cells into the right flank. The appearance of palpable tumours was determined by monitoring tumour development. Tumour growth was monitored at least twice weekly by measurement of tumour length (a) and width (b) using Vernier calipers. Tumour volume was calculated as ½ (axb^2). (b) 20 days after tumour injection, animals were sacrificed and the final tumour volume recorded. Values plotted as mean +/- S.E.M. n =4. * $P < 0.5$, ** $P < 0.01$. Results were statistically evaluated using a students paired t test.

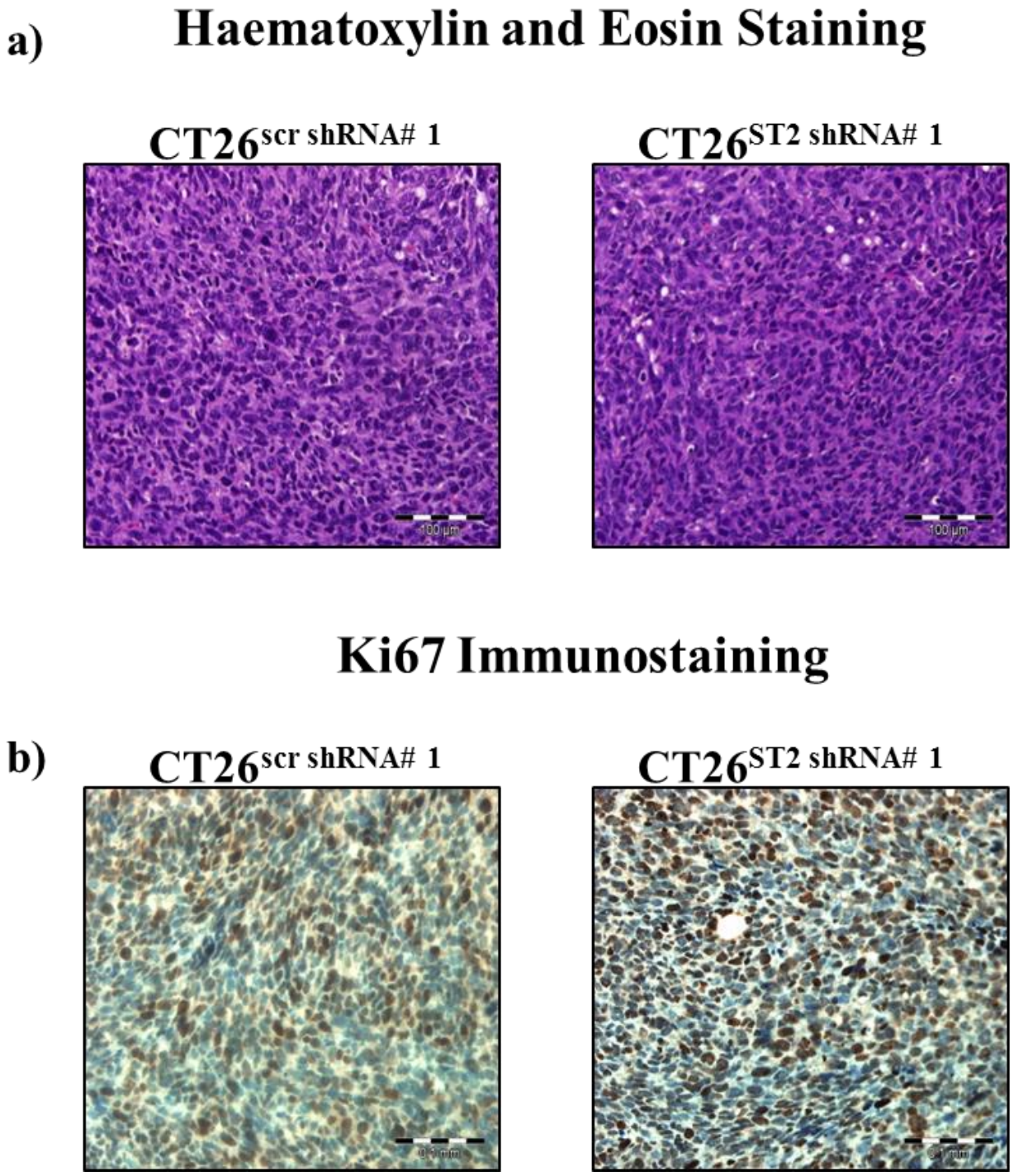


Figure 6: Ki67 expression was unchanged in CT26$^{ST2\ shRNA}$ tumours compared to CT26$^{scr\ shRNA}$ tumours. Tumour tissues were excised, formalin-fixed, paraffin-embedded and stained with (a) haematoxylin and eosin or (b) Ki67 antibody for histochemical analysis. Scale bar = 100µm. Images shown are representative of the findings obtained.

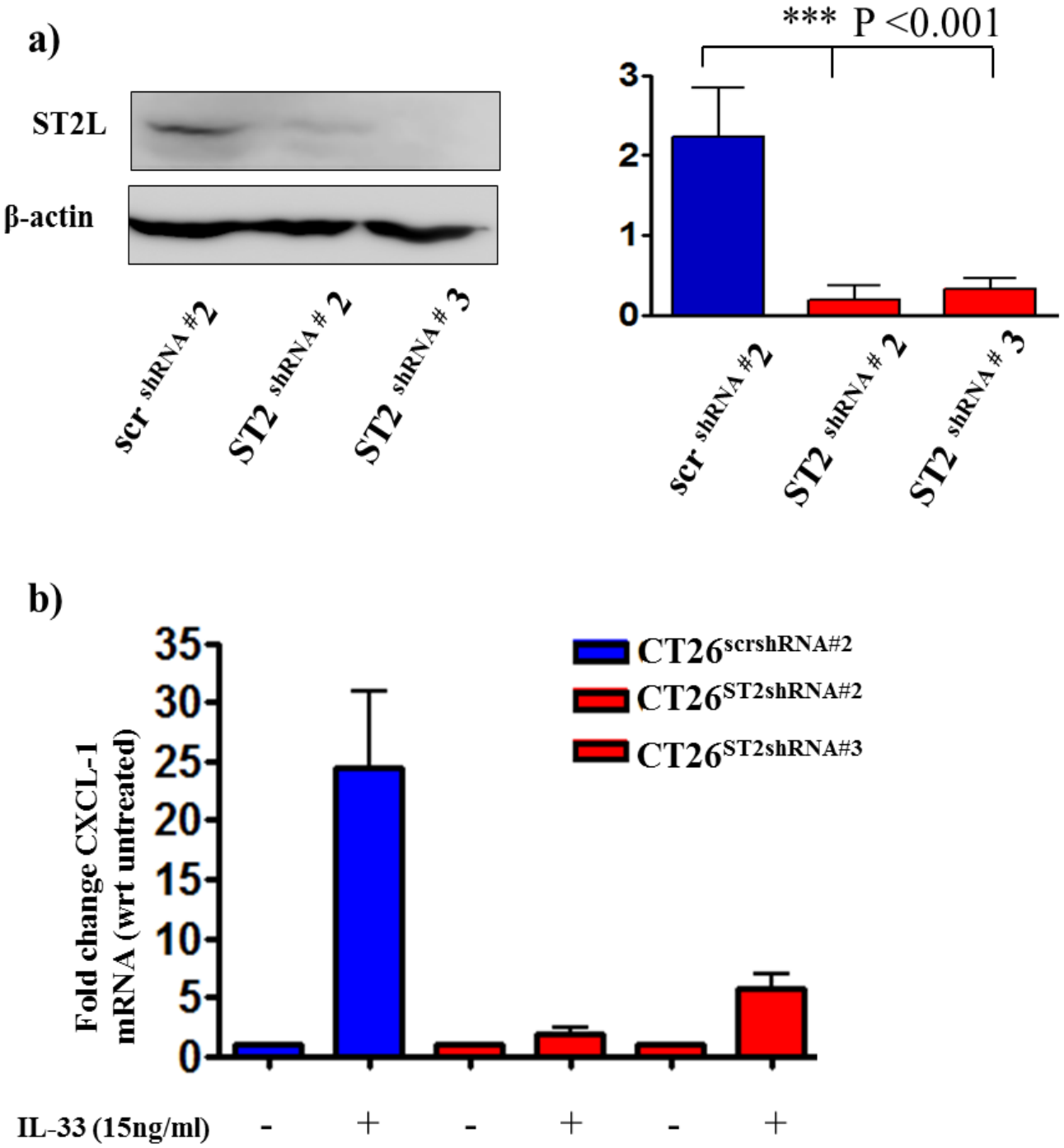


Figure 7: Characterisation of ST2 suppressed stable cell lines generated following transfection of CT26 cells with shRNA targeted against ST2 or scrambled control. (a) CT26 cells were transfected with lentivirus containing plasmids encoding ST2 or scrambled control shRNA. Knockdown of ST2 expression was determined by Western blotting and quantified by densitometry. (b) Changes in CXCL-1 production were determined by qRT-PCR. Values are plotted as Mean +/- S.E.M. n=3. *** $P < 0.001$.

sufficiently for statistical analysis, a second *in vivo* investigation using the same subcutaneous murine model of colon cancer was employed. To ensure the results observed in the preliminary *in vivo* study were not just a clonal effect, two new stable ST2 suppressing clones were generated ($CT26^{ST2\ shRNA\#2}$ and $CT26^{ST2\ shRNA\#3}$) in parallel with $CT26^{scrshRNA\#2}$ cells. Similar to the preliminary study, ST2 knockdown clones were selected using puromycin. A number of clones were generated. $CT26^{ST2\ shRNA\#2}$ and $CT26^{ST2\ shRNA\#3}$ clones were selected as they showed a significant reduction in ST2 expression, as assessed by Western blot analysis ($p<0.001$), relative to $CT26^{scr\ shRNA\#2}$ cells (Figure 7a). Knock down of ST2 expression was further characterised by a functional assay. IL-33 treatment led to ~30-fold increase in CXCL-1 production in $CT26^{scr\ shRNA}$ cells (Figure 7b). In contrast, IL-33 stimulation of $CT26^{ST2\ shRNA\#2}$ and $CT26^{ST2\ shRNA\ \#3}$ cells led to just a 2 and 4-fold increase in CXCL-1 production, respectively (Figure 7b).

As tumour growth of CT26 cells was enhanced following suppression of ST2 expression *in vivo* in the preliminary study, and IL-33 suppressed proliferation *in vitro* in colon cancer cells (Chapter 3, figure 9), basal cellular proliferation was examined in the freshly generated clones. BrdU incorporation was measured and no significant changes were observed in the basal level of proliferation between the $CT26^{scr\ shRNA\#2}$, $CT26^{ST2\ shRNA\#2}$ and $CT26^{ST2\ shRNA\ \#3}$ cells over a 48-hour period (Figure 8a). As I had previously shown that migration was enhanced by IL-33 stimulation of CT26 cells (Chapter 3, figure 10), migration of the freshly generated clones towards 10% serum was also examined. However, no significant changes in

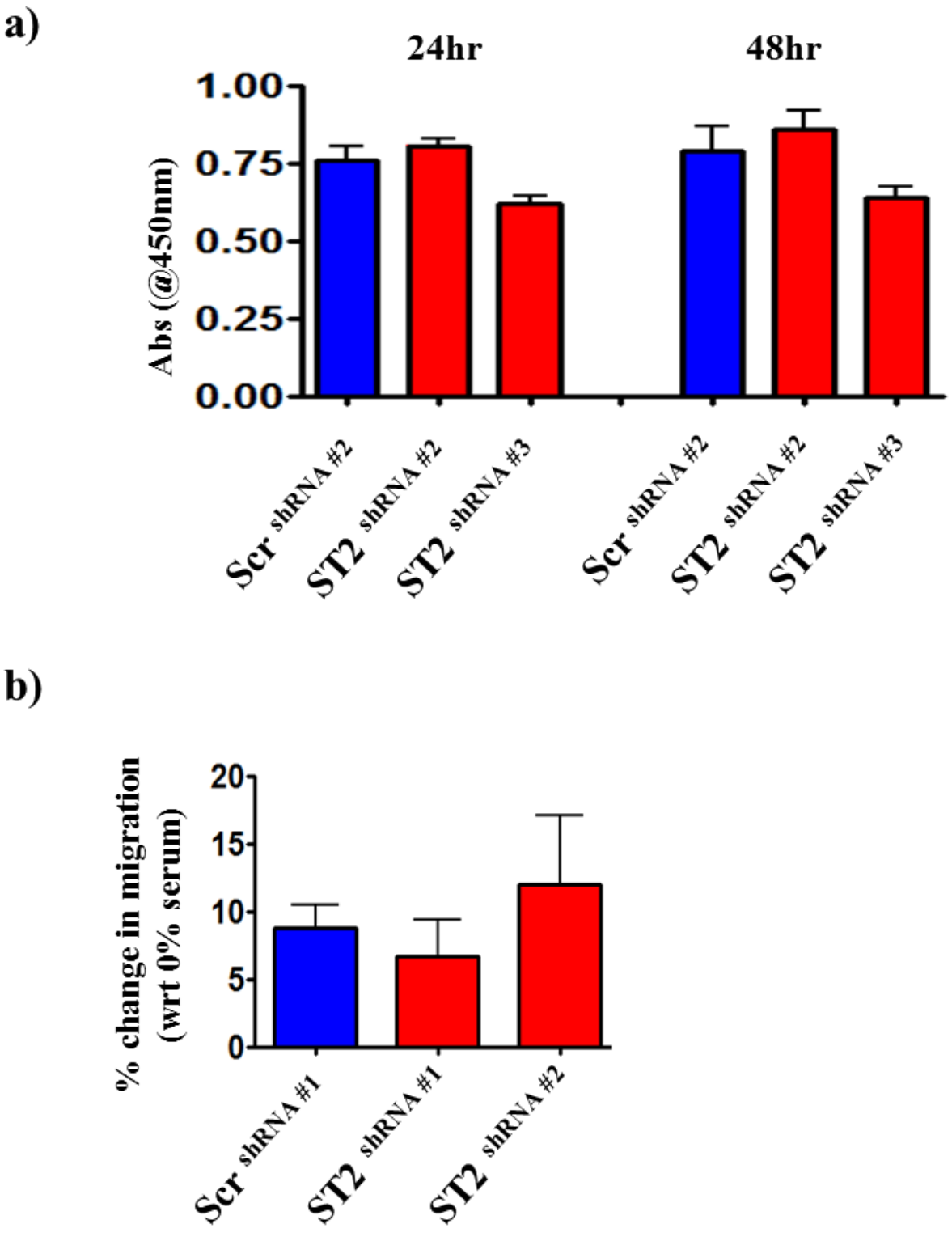


Figure 8: Suppressing ST2 expression did not alter proliferation or migration of colon cancer cells *in vitro.* (a) Cell proliferation of CT26$^{\text{scr shRNA\#2}}$, CT26$^{\text{ST2 shRNA\#2}}$ and CT26$^{\text{ST2 shRNA\#3}}$ cells was measured by BrdU assay. (b) Migration of stably transfected cells was measured using a modified Boyden Chamber assay. Values are plotted as Mean +/- S.E.M. n=3. Results were statistically evaluated using One-way Anova with Tukeys post-test.

basal migration were observed between the $CT26^{scr\ shRNA\#2}$, $CT26^{ST2\ shRNA\#2}$ and $CT26^{ST2\ shRNA\ \#3}$ cells towards 10% serum (Figure 8b).

In order to confirm the findings of the initial study, a second *in vivo* study using the same murine model of CRC was performed. Immunocompetent BALB/c mice were injected subcutaneously with $CT26^{ST2\ shRNA\#2}$ $CT26^{ST2\ shRNA\#3}$ and $CT26^{scr\ shRNA\#2}$ cells. To sufficiently power the study for statistical analysis, a larger number of animals were required in each group, i.e. n=6, except for the PBS injected control group, which contained n=3 (Figure 9a). To investigate if tumour cell injection and growth impacted on feeding capability, food consumption was measure twice weekly throughout the course of the experiment, and remained constant among all groups (Figure 10a). Each mouse was weighed twice per week and no significant difference in body weight was detected between the tumour cell injected groups and those animals injected with PBS alone (Figure 10b). Tumour development was monitored over the course of the investigation and by day 13, palpable tumours began to appear in all groups (Figure 11a). $CT26^{ST2\ shRNA\#3}$ derived tumours grew at a fastest rate than the other two groups and resulted in the largest tumours. $CT26^{ST2\ shRNA\#2}$ and $CT26^{scr\ shRNA\#2}$ showed similar growth rates up until day 20, at this point tumour growth continued to increase in $CT26^{ST2\ shRNA\#2}$ tumours, while $CT26^{scr\ shRNA\#2}$ tumours remain static (Figure 11a). The final tumour volume was two-fold greater in tumours derived from $CT26^{ST2\ shRNA\#2}$ cells and three-fold greater in $CT26^{ST2\ shRNA\#3}$ ($p=0.0425$) derived tumours, relative to those derived from $CT26^{scr\ shRNA\#1}$ cells (Figure 11b). This second independent study using two newly generated clones was consistent with the findings of the preliminary

a)

Group name	Number in group	Cell type inoculated
1	6	$CT26^{scr\ shRNA\ \#2}$
2	6	$CT26^{ST2\ shRNA\ \#2}$
3	6	$CT26^{ST2\ shRNA\ \#3}$
4	3	PBS

b)

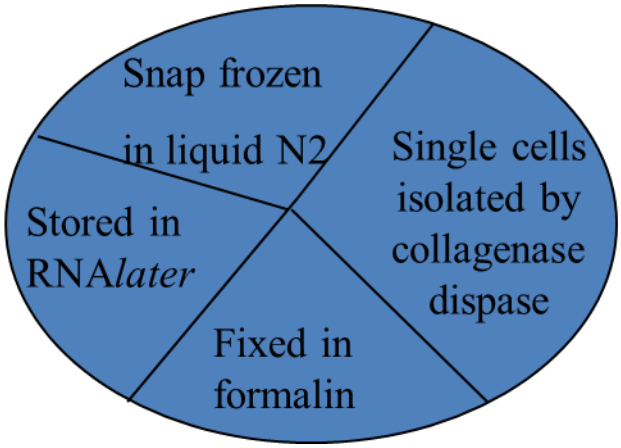


Figure 9: Outline of second *in vivo* study design. (a) Immunocompetent female Balb/c mice were s.c. injected with $4x10^4$ tumour cells in the right flank. The control group were injected with 200µl of PBS only. (b) Tumours were dissected using a "clock" dissection method for subsequent processing as indicated.

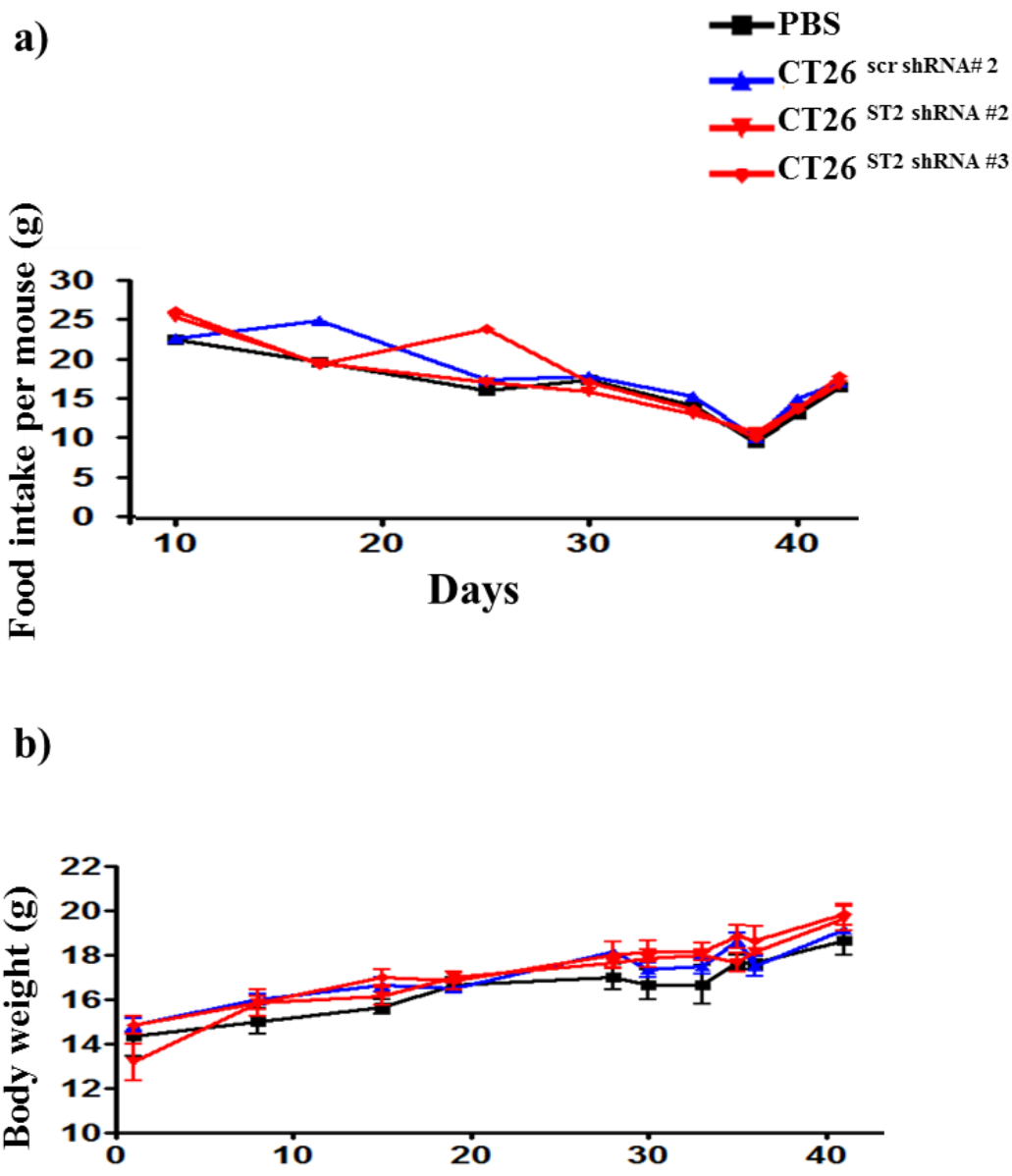


Figure 10: Food consumption and body weight was monitored, with no change observed between groups. (a) Food consumption for each group was measured twice per week. (b) The weight of each animal in the study groups was also monitored twice per week. (a) Values are plotted as g per mouse. (b) Values are plotted as mean +/- SEM.

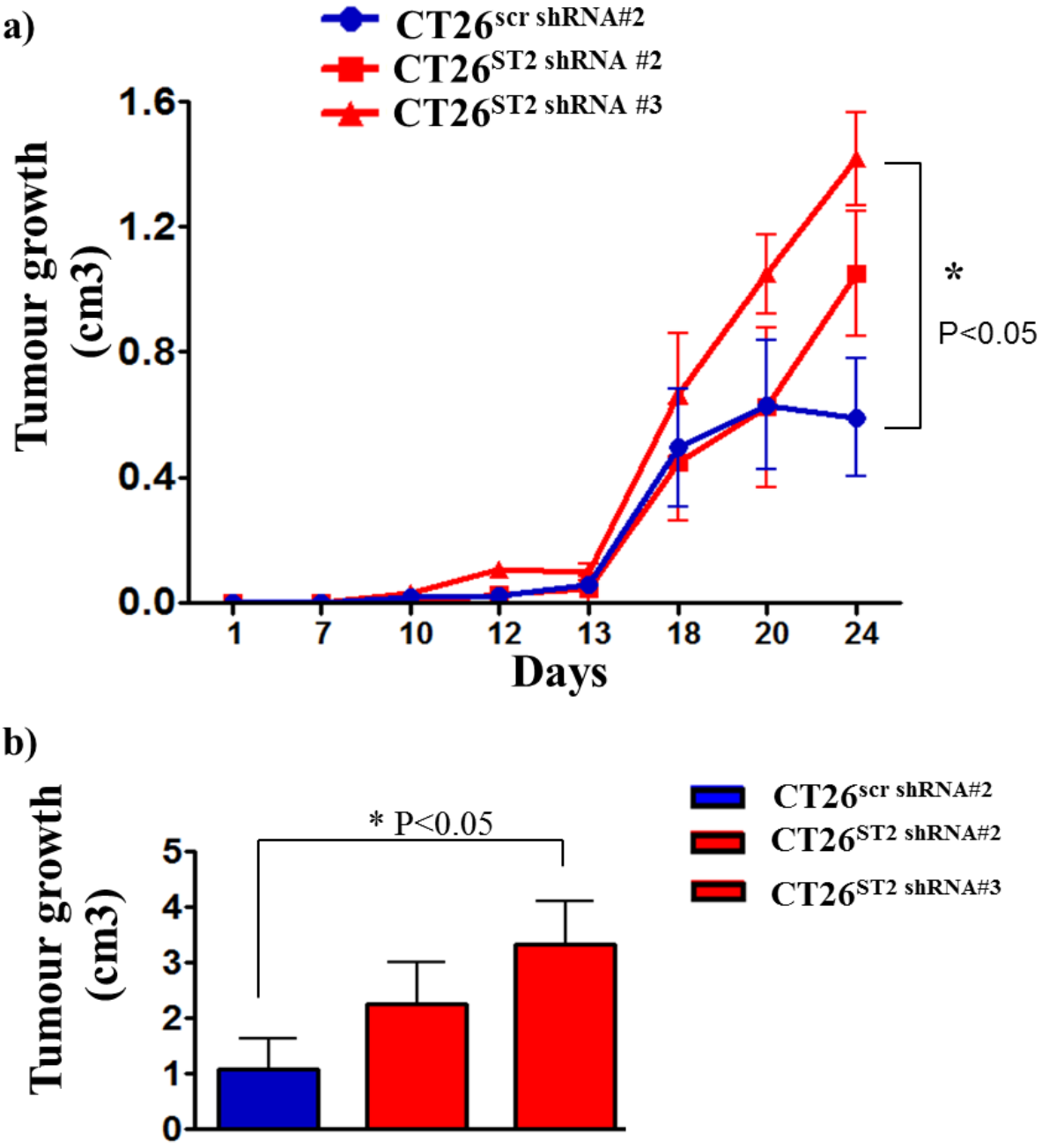


Figure 11: Suppression of ST2 expression in colon cancer cells results in increased tumour growth *in vivo*. (a) Female Balb/c immunocompetent mice were s.c. injected with 4 x10^4 cells into the right flank. The appearance of palpable tumours was determined by monitoring tumour development. Tumour growth was monitored at least twice weekly by measurement of tumour length (a) and width (b) using Vernier calipers. Tumour volume was calculated as ½(axb^2). (b) 24 days after tumour injection, animals were sacrificed and the final tumour volume recorded. Values plotted as mean +/- S.E.M. n =6. * $p < 0.05$. Results were statistically evaluated using One-way Anova with Tukeys post-test.

study, suggesting that a reduction in ST2 signalling leads to increased tumour growth *in vivo*.

Upon completion of the study, the murine tumour tissue was excised, and dissected as indicated in Figure 9b. As the ST2 suppressed tumours were increased in size compared to the scrambled control, Ki67 expression was also examined. This was found to be unchanged in $CT26^{ST2\ shRNA\ \#2}$ and $CT26^{ST2\ shRNA\ \#3}$ tumours compared to $CT26^{scr\ shRNA\#2}$ tumours (Figure 12a), indicating that all tumours contained cells that were actively proliferating. In addition, expression of IL-33 was examined in the murine tumours. IL-33 was found to be markedly reduced in the $CT26^{ST2\ shRNA\#2}$ and $CT26^{ST2\ shRNA\ \#3}$ tumours compared to $CT26^{scr\ shRNA\#2}$ tumours (Figure 12b). While the underlying mechanism for this decrease in IL-33 expression *in vivo* is unclear, it may be caused by a lack of positive regulation of IL-33 by ST2L signalling.

In the previous chapter I had observed that IL-33 stimulation can alter VEGF expression in CT26 cancer cells *in vitro* (Chapter 2, figure 13). Therefore, I wished to examine if differential expression of the pro-angiogenic factor, VEGF, was responsible for the alterations observed in tumour growth following suppression of ST2 expression. Expression of the pro-angiogenic mediator VEGF was found to be increased in $CT26^{ST2\ shRNA\#3}$ cells both *in vitro* (Figure 13a) and *in vivo* (Figure 13b) as compared to $CT26^{scr\ shRNA\ \#2}$ cells. However, this was not significant. Moreover, although, $CT26^{ST2\ shRNA\#2}$ cells also showed increased tumour growth VEGF expression was unchanged compared to $CT26^{scr\ shRNA\ \#2}$ cells. This suggests that increased VEGF expression is not responsible for the increased tumour

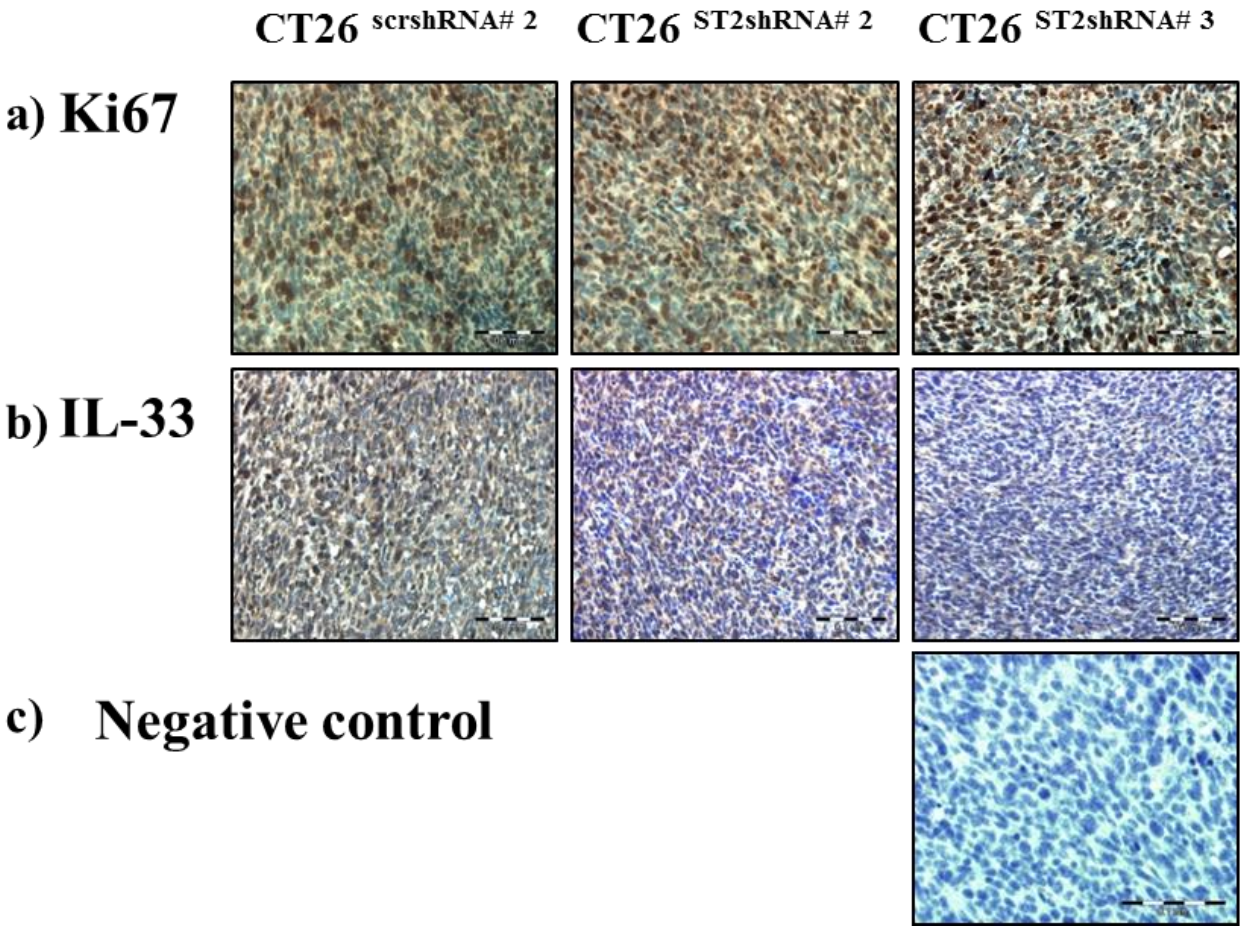


Figure 12: IL-33 expression is reduced in colon cancer tumours with reduced ST2 expression, while Ki67 expression is unchanged by suppressing ST2 expression. Tumour tissues were excised, formalin fixed and paraffin embedded. Murine tumour sections were immunostained using (a) anti-Ki67 or (b) anti-IL-33. (c) Primary antibody was omitted in the negative control. Scale bar: 100 μm. Images shown are representative of the findings obtained.

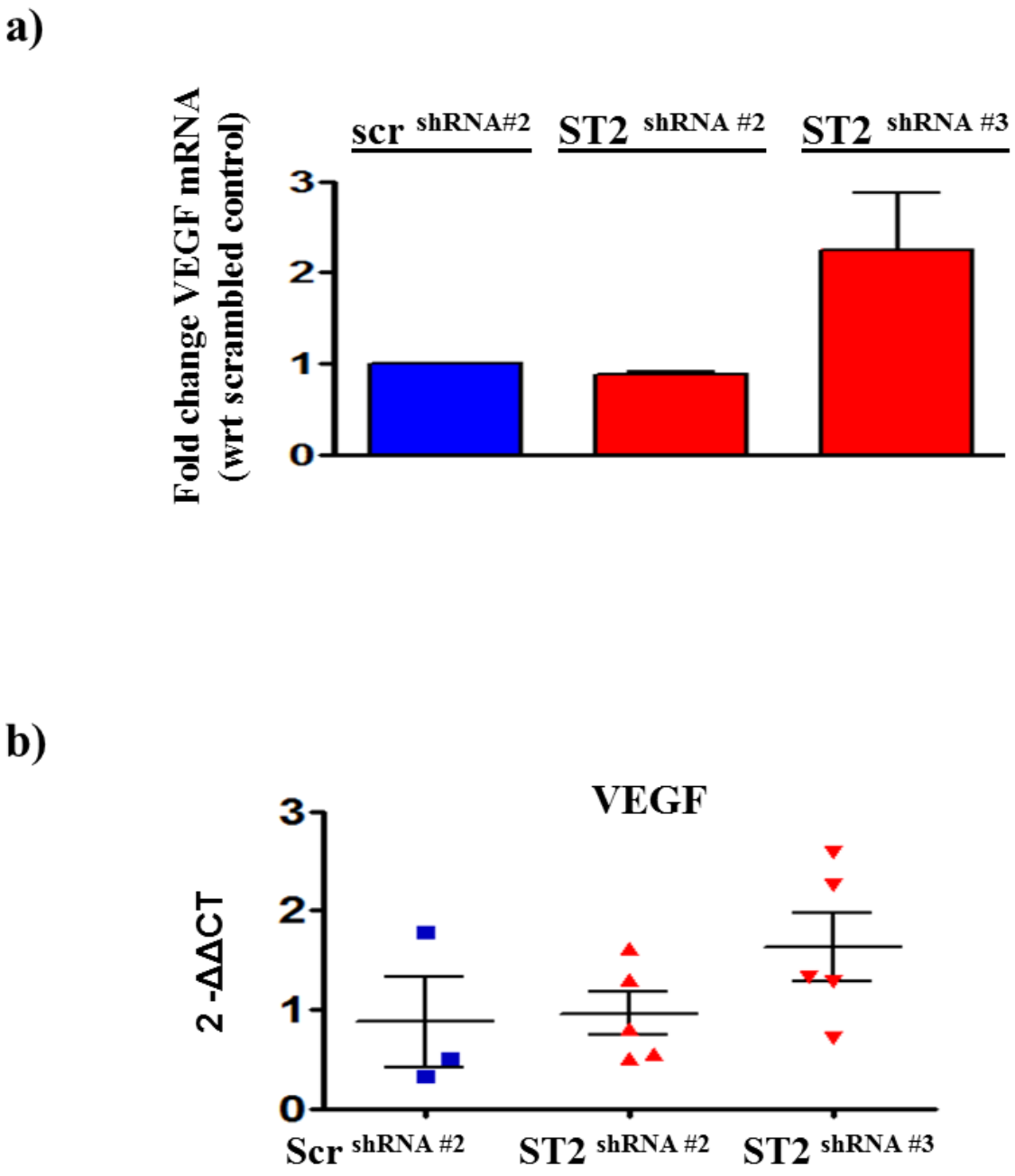


Figure 13: VEGF expression is increased in CT26$^{ST2\ shRNA\ \#3}$ cells *in vitro* and *in vivo.* (a) Total RNA was extracted from CT26$^{scr\ shRNA\ \#2,}$ CT26$^{ST2\ shRNA\ \#2}$ and CT26$^{ST2\ shRNA\ \#3}$ cells and changes in VEGF were examined by qRT-PCR. Data shown are mean +/- SEM (n=3). (b) Total RNA was extracted from excised tumours and expression of VEGF was examined by qRT-PCR. Results were statistically evaluated using One-way Anova with Tukeys post-test.

growth seen following suppression of ST2 expression.

4.2.5 Suppression of ST2 expression does not alter colon cancer cell proliferation *in vitro* in response to pro-inflammatory stimuli.

Suppression of ST2 in colon cancer cells did not alter the proliferation of unstimulated cells *in vitro* (Figure 2c and 8a), but did result in increased tumour cell growth *in vivo* (Figure 5 and 11). To investigate if pro-inflammatory factors in the tumour microenvironment may be driving proliferation of the ST2 suppressed cells, cells were stimulated with PGE_2, a pro-inflammatory prostaglandin that is commonly upregulated in colon cancer (Figure 14a), and LPS (Figure 14b), a potent pro-inflammatory cytokine that has been shown to activate MAPK pathways, which in turn influences proliferation [295]. Changes in proliferation were assessed by BrdU incorporation. No significant differences were observed between the $CT26^{ST2\ shRNA\#2}$ and $CT26^{ST2\ shRNA\#3}$ cells and the $CT26^{scr\ shRNA\#2}$ control cells, indicating that neither of the pro-inflammatory stimuli examined were responsible for the increase in proliferation observed *in vivo*.

4.2.6 Suppression of ST2 expression in tumour cells results in reduced macrophage and $CD8^+$T cell recruitment *in vivo*.

As ST2 suppressed cells did not show an increase in proliferation in response to pro-inflammatory mediators *in vitro.* I next examined the immune infiltrate in ST2 suppressed tumours. Tumours were excised and immune cell infiltration analysed by flow cytometry. Representative dot plots for the FACS analysis are shown in Figure 15. A number of immune cell populations were examined, including macrophages, neutrophils,

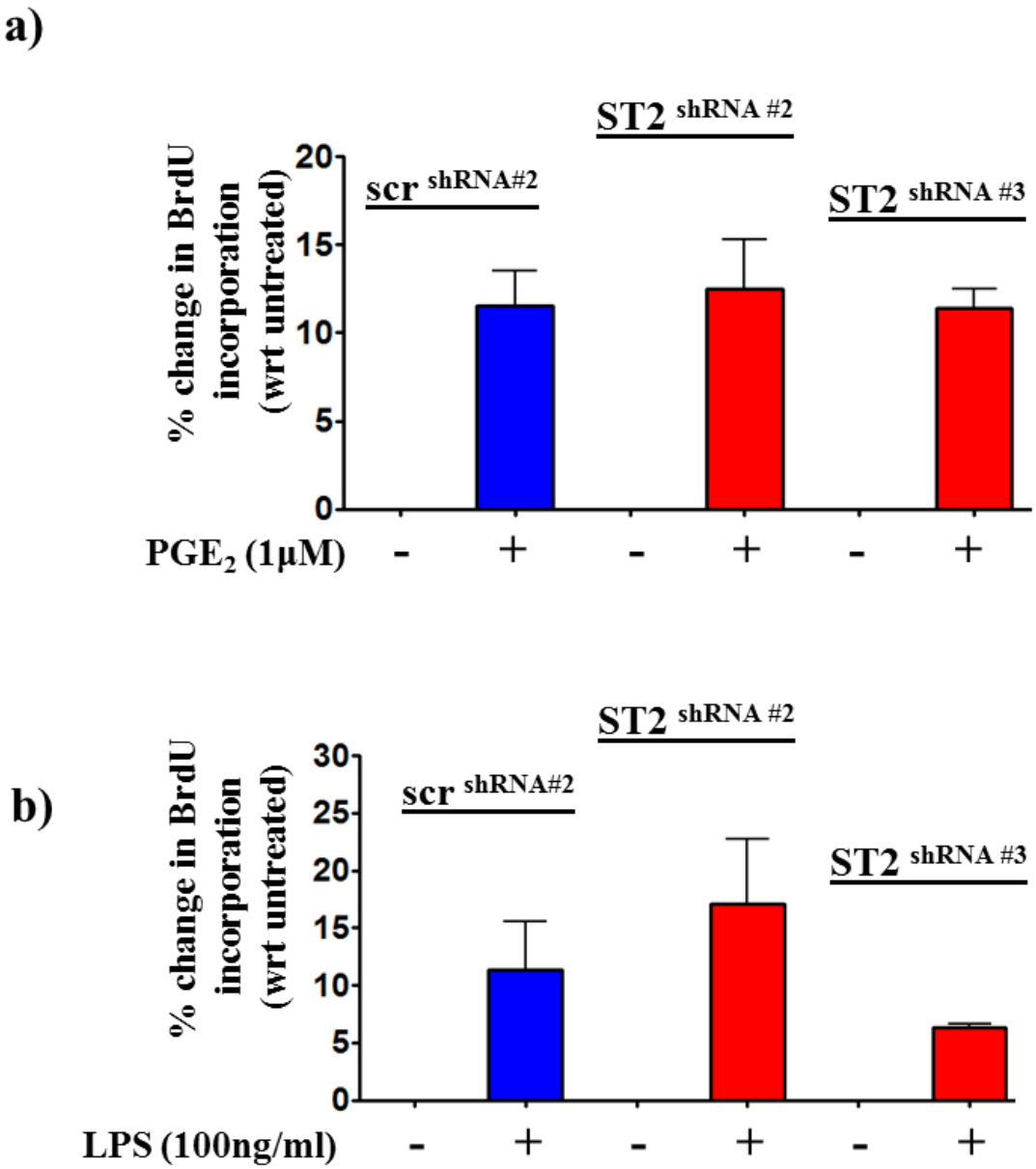


Figure 14: Suppression of ST2 expression in colon cancer cells does not alter their proliferation *in vitro* in response to pro-inflammatory stimuli. Cells were stimulated with (a) PGE_2 and (b) LPS as indicated and changes in proliferation assessed by BrdU incorporation. Data shown are mean +/- SEM (n=3). Results were statistically evaluated using One-way Anova with Tukeys post-test.

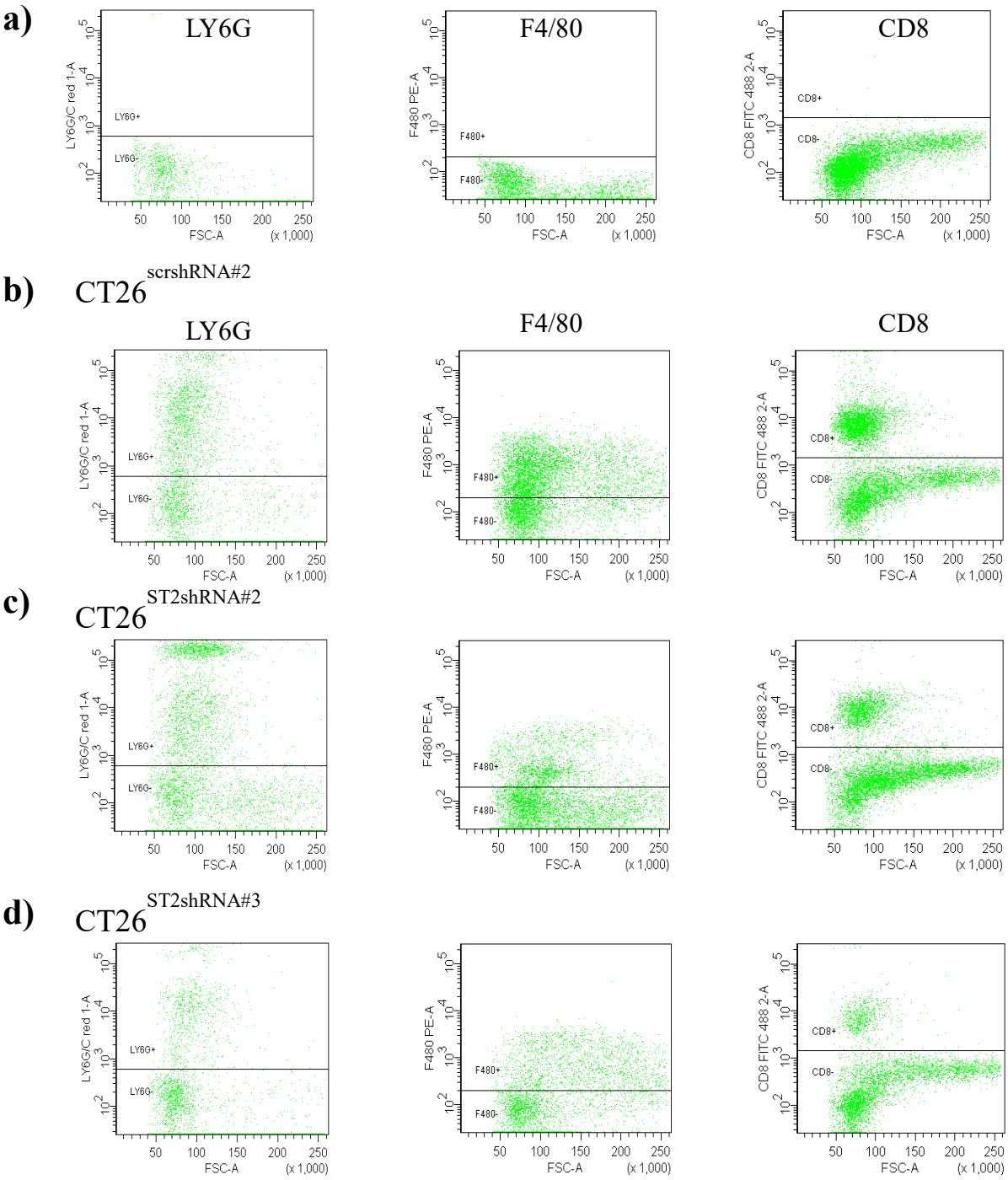


Figure 15: Tumour cells were stained with fluorochrome-labelled mAb and analysed by flow cytometry. (a) Dotplots showing the fluorescence minus one controls, which were used to set the threshold gates for the data presented in (b-d). Representative dot plots showing the percentage of $LY6G^{+}$ neutrophils, $F4/80^{+}$ macrophages and $CD8^{+}$ T cells in the viable tumour cell population from (b) $CT26^{scr\ shRNA\#2}$, (c) $CT26^{ST2\ shRNA\ \#2}$, and (d) $CT26^{ST2shRNA\ \#3}$ derived tumours. 20,000 to 50,000 events were recorded,

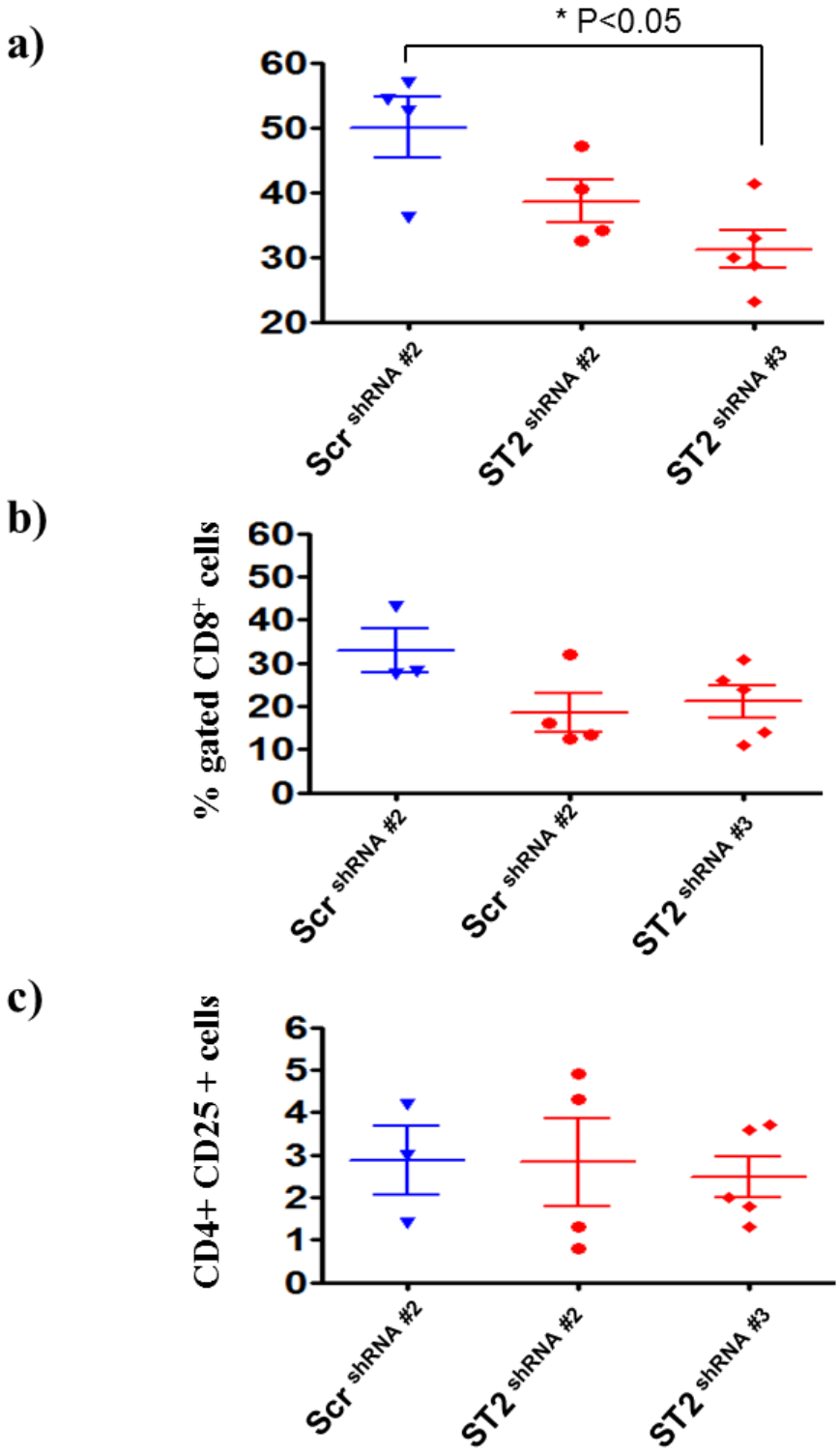


Figure 16: F4/80^{+} cells and CD8^{+} cells were decreased in the CT26$^{ST2\ shRNA\#2}$ and CT26$^{ST2\ shRNA\#3}$ tumours compared to the CT26$^{scr\ shRNA\#2}$ tumours, while the level of CD4^{+}CD25^{+} cell infiltration is unchanged. Tumours were excised and digested by collagen/dispase. Single cell suspensions were isolated from each tumour and surface stained with antibodies against **(a)** F4/80, **(b)** CD8, **(c)** CD4 and CD25. The percentages of each cell subset were assessed by gating on forward scatter (FSC) populations using an Accuri C6 Flow Cytometer, and analysis was carried out using CFlow software. Values are plotted as Mean ± S.E.M. Each point represents tumour cells isolated from a single mouse. * $P < 0.05$. Results were statistically evaluated using One-way Anova with Tukeys post-test.

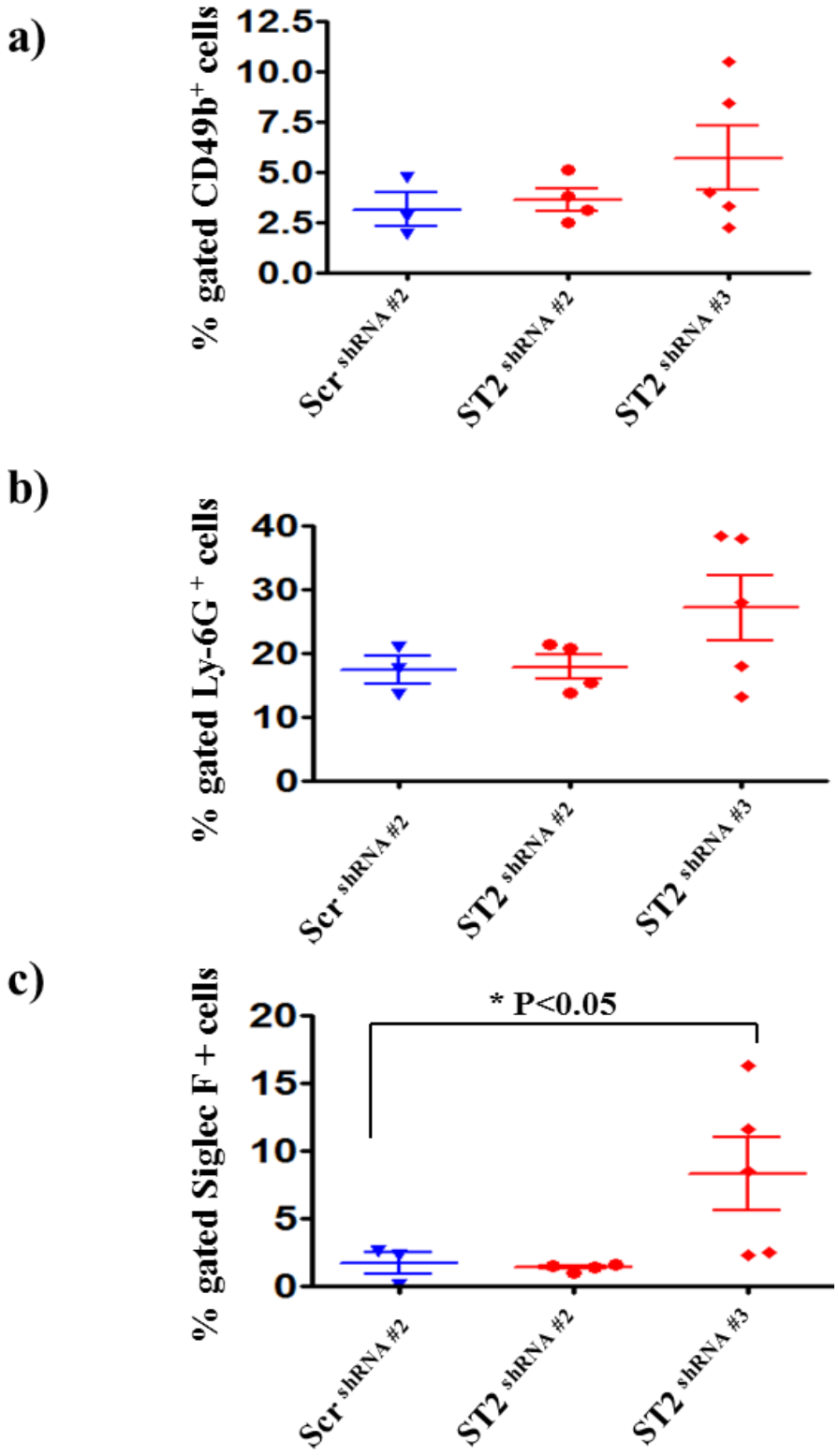


Figure 17: Eosinophils are increased in CT26$^{ST2\ shRNA\#3}$ tumours compared to the CT26$^{scrshRNA\#2}$, with no change seen in Ly-6G^{+} and CD49b^{+} cells. Tumours were excised and digested with collagen/dispase. Single cell suspensions were isolated from each tumour and surface stained with antibodies against **(a)** CD49b, **(b)** Ly-6G, **(c)** SiglecF. The percentages of each cell subset were assessed by gating on FSC populations using an Accuri C6 Flow Cytometer, and analysis was carried out using CFlow software. Values are plotted as Mean ± S.E.M. Each point represents a single mouse. * $P < 0.05$. Results were statistically evaluated using One-way Anova with Tukeys post-test.

eosinophils, $CD8^+$ T cells and NK cells. Macrophage recruitment was reduced in $CT26^{ST2\ shRNA\#2}$ and $CT26^{ST2\ shRNA\#3}$ tumours compared to $CT26^{scr\ shRNA\#2}$ tumours, with a significant reduction seen in $CT26^{ST2\ shRNA\#3}$-derived tumours, $p<0.05$ (Figure 16a). Suppressing ST2 also led to a decrease in the recruitment of $CD8^+$ T-cells (Figure 16b), while $CD4^+$ $CD25^+$ cell infiltration was unchanged (Figure 16c). NK cell infiltration was also unchanged (Figure 17a). Although I had previously shown that IL-33 stimulation increased the neutrophil chemokine CXCL-1 (Chapter 3, figure 13) suppressing ST2 did not affect neutrophil recruitment (Figure 17b). In contrast, eosinophil infiltration was increased in tumours derived from $CT26^{ST2\ shRNA\#3}$ cells, but not in $CT26^{ST2\ shRNA\#2}$-derived tumours, compared to the $CT26^{scr\ shRNA\#2}$ tumours (Figure 17c).

4.2.7 Migration of macrophages towards tumour cell supernatant is increased by IL-33 stimulation of the tumour cells.

Given the reduction in macrophage infiltration observed in tumours derived from $CT26^{ST2\ shRNA\#2}$ and $CT26^{ST2\ shRNA\#3}$ cells, and the ability of IL-33 stimulation to induce CCL2 production (Chapter 3, figure 13), I next investigated whether there was an increase in macrophage migration towards supernatant from IL-33-stimulated tumour cells, relative to untreated tumour cell supernatant. This experiment was performed using the modified Boyden chamber assay, as illustrated in figure 18a. There was ~25% increase in migration of macrophages as compared to unstimulated CT26 cell supernatant (Figure 18b). Consistent with the induction of CCL2 by IL-33, a blocking antibody to CCL2 reduced macrophage migration towards the supernatant of IL-33-stimulated CT26 cells (* $P < 0.05$) (Figure

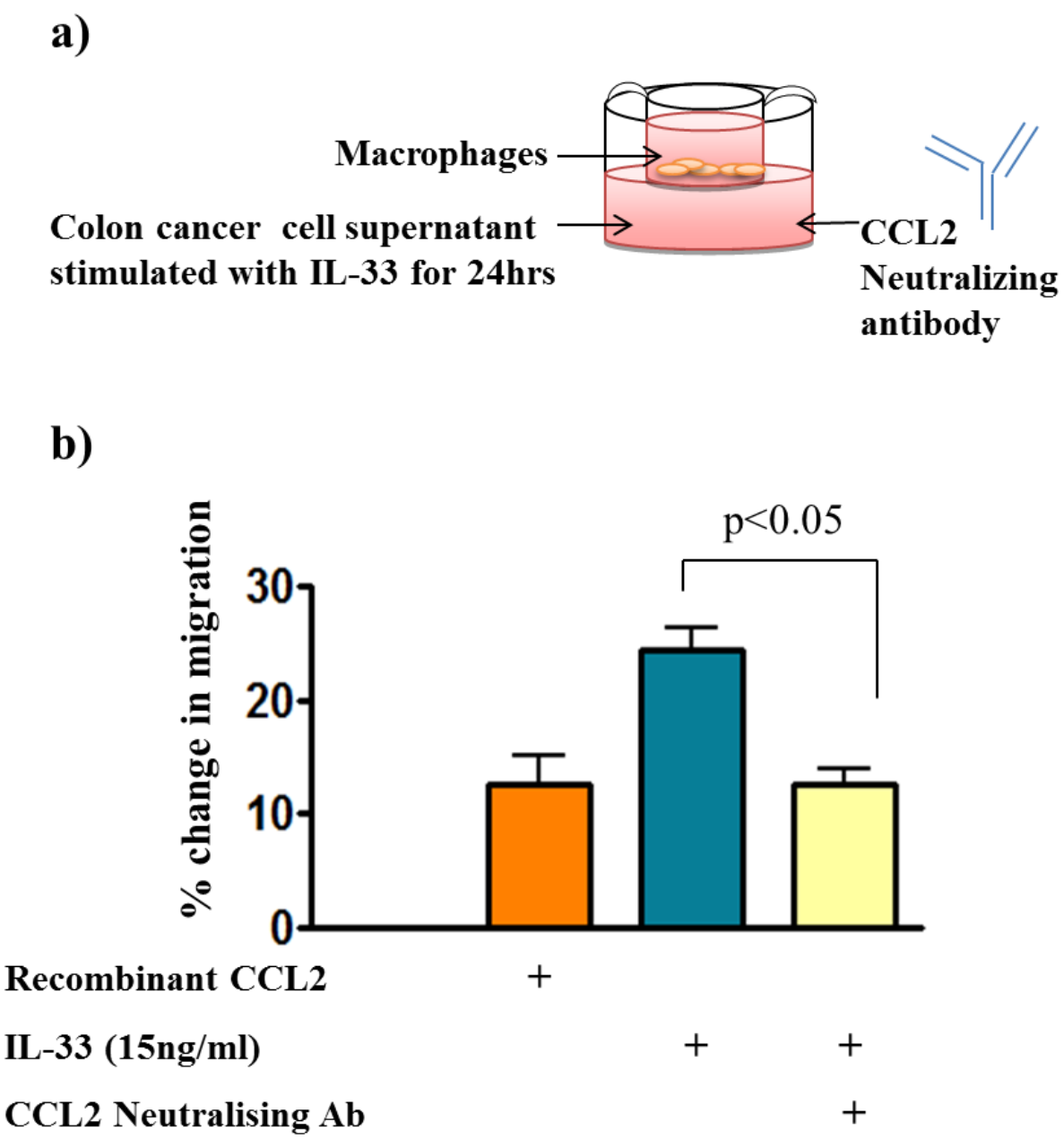


Figure 18: Supernatant from IL-33-stimulated tumour cells enhances macrophage migration, relative to supernatant from unstimulated cells. CT26 colon cancer cells were stimulated for 24hrs with IL-33 (15ng/ml). (a) The supernatant was isolated from unstimulated or IL-33-stimulated CT26 cells and placed in the lower chamber of the modified Boyden chamber assay. (b) Migration of RAW 264.7 macrophages towards the cell culture supernatant was assessed. Neutralising CCL2 antibody was added to the supernatant as indicated. Data shown are mean +/- SEM (n=3). Results were statistically evaluated using One-way Anova with Tukeys post-test.

Clinical Characteristics		Cohort 1 (n=24)
Gender	Male	12
	Female	12
Age	>60	18
	<60	6
Site	Left	18
	Right	6
Tumour Differentiation	MD	24
	PD	0
	WD	0
Tumour Stage	I	7
	II	5
	III	11
	IV	1
T classification	T1	1
	T2	9
	T3	12
	T4	2
N Classification	pN0	12
	pN1	12
Chemotherapy	Yes	8
	No	16

Table 1. **CRC cohort 1 patient demographics**. A cohort of 24 patients with CRC was recruited from the Mercy University Hospital Cork. The study protocol, including all procedures and study populations was approved by the University College Cork Clinical Research Ethics Committee of the Cork Teaching Hospitals (ECM (3) P 3/9/2013). All samples were collected with patients' informed consent.

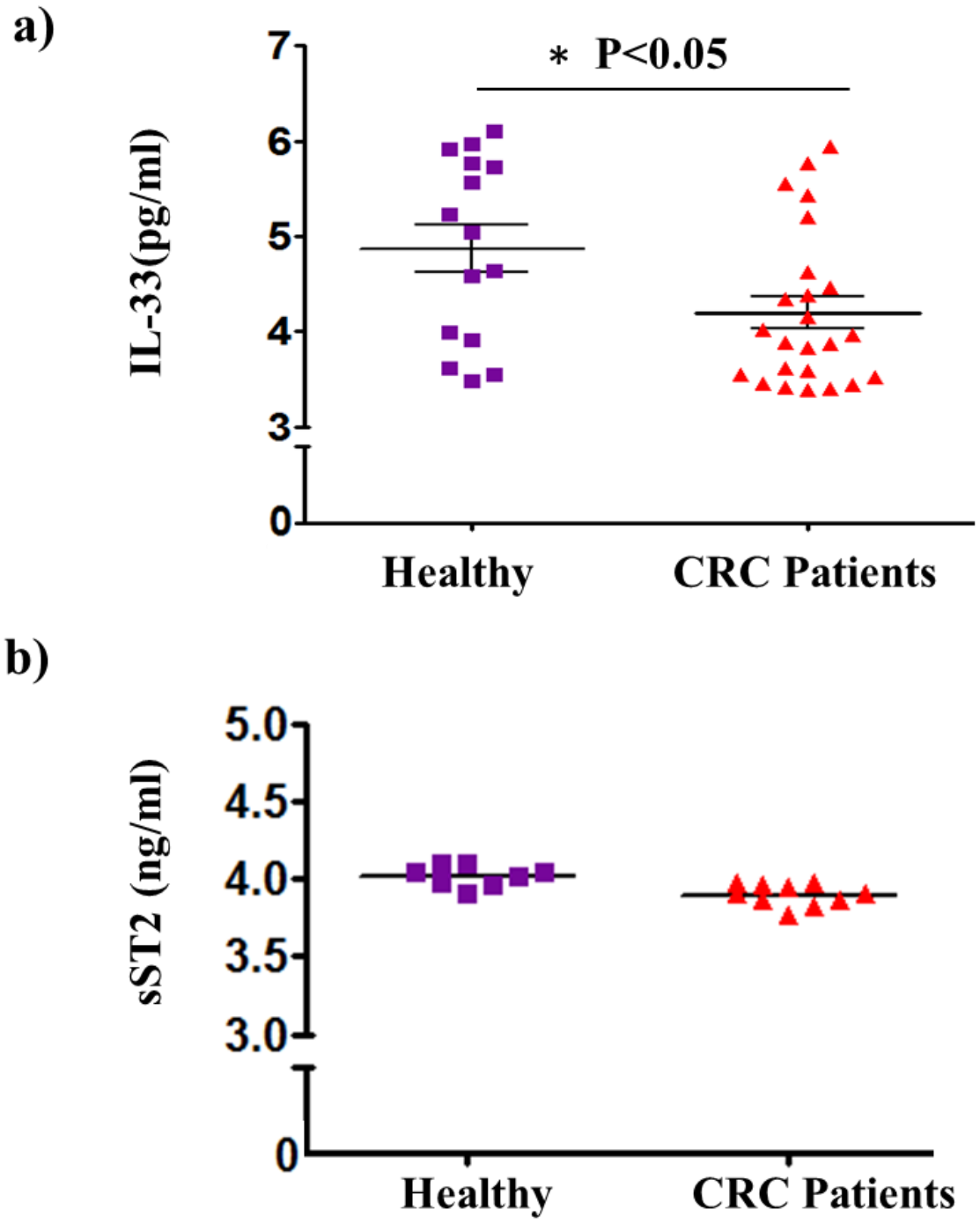


Figure 19: IL-33 serum levels were lower in CRC patients than in healthy controls, while sST2 was unchanged. Serum was obtained from healthy individuals and patients with CRC, and (a) IL-33 and (b) sST2 levels determined by ELISA. Statistical analysis was determined using a Students paired t test. * $P < 0.05$.

18b).

4.2.8 Serum levels of IL-33 are slightly decreased in CRC patients compared to healthy controls, while sST2 levels are unchanged.

As a clear biological effect was observed by suppressing ST2 in murine tumours, I next wished to determine if there was a correlation between the murine findings and human patient samples. A cohort of 24 patients with CRC was recruited from the Mercy University Hospital Cork in collaboration with my clinical collaborators, Dr. Amr Mahmoud and Dr. Michael Bennett. The demographics pertaining to these patients are contained in Table 1. Eight of these patients had received chemotherapy prior to surgery. I first measured the level of IL-33 and sST2 in the serum of CRC patients (n=24) and healthy individuals (n=15) by ELISA. There was a slight reduction in the level of IL-33 in the serum of CRC patients compared to the controls, (* P=0.0252) (Figure 19a), with no alteration seen in the circulating sST2 levels (Figure 19b).

4.2.9 Expression of ST2L in CRC is lower compared to adjacent non-tumour tissue

Although serum IL-33 and sST2 levels were not vastly altered in CRC patients, I next wished to examine expression of both IL-33 and its receptor ST2L in human CRC. Initially, I examined expression of IL-33 and ST2 by qRT-PCR. Similar to most previous reports examining expression levels of ST2 at the mRNA level, I initially utilised a qRT-PCR primer to the extracellular region of ST2, which detects both the soluble (sST2) and the transmembrane (ST2L) form of the receptor. There was no change in the

level of transcription of either IL-33 or total ST2 between CRC and paired adjacent non-tumour tissue (Figure 20a and b). I subsequently utilised a primer specific to the intracellular portion of ST2 to examine changes in expression of ST2L in colon cancer. The level of ST2L mRNA was significantly reduced (** P=0.0067) in CRC tissue as compared to adjacent non-tumour tissue (Figure 20c). Alterations in expression were confirmed at the protein level by IHC, using antibodies directed against IL-33 and both the extracellular portion of ST2 (total ST2) and the intracellular region (ST2L). ST2L was consistently decreased in the CRC tumours compared to the adjacent non-tumour (Figure 21c). There was no change in the levels of either IL-33 or total ST2 between CRC and adjacent non-tumour tissue (Figure 21a and b).

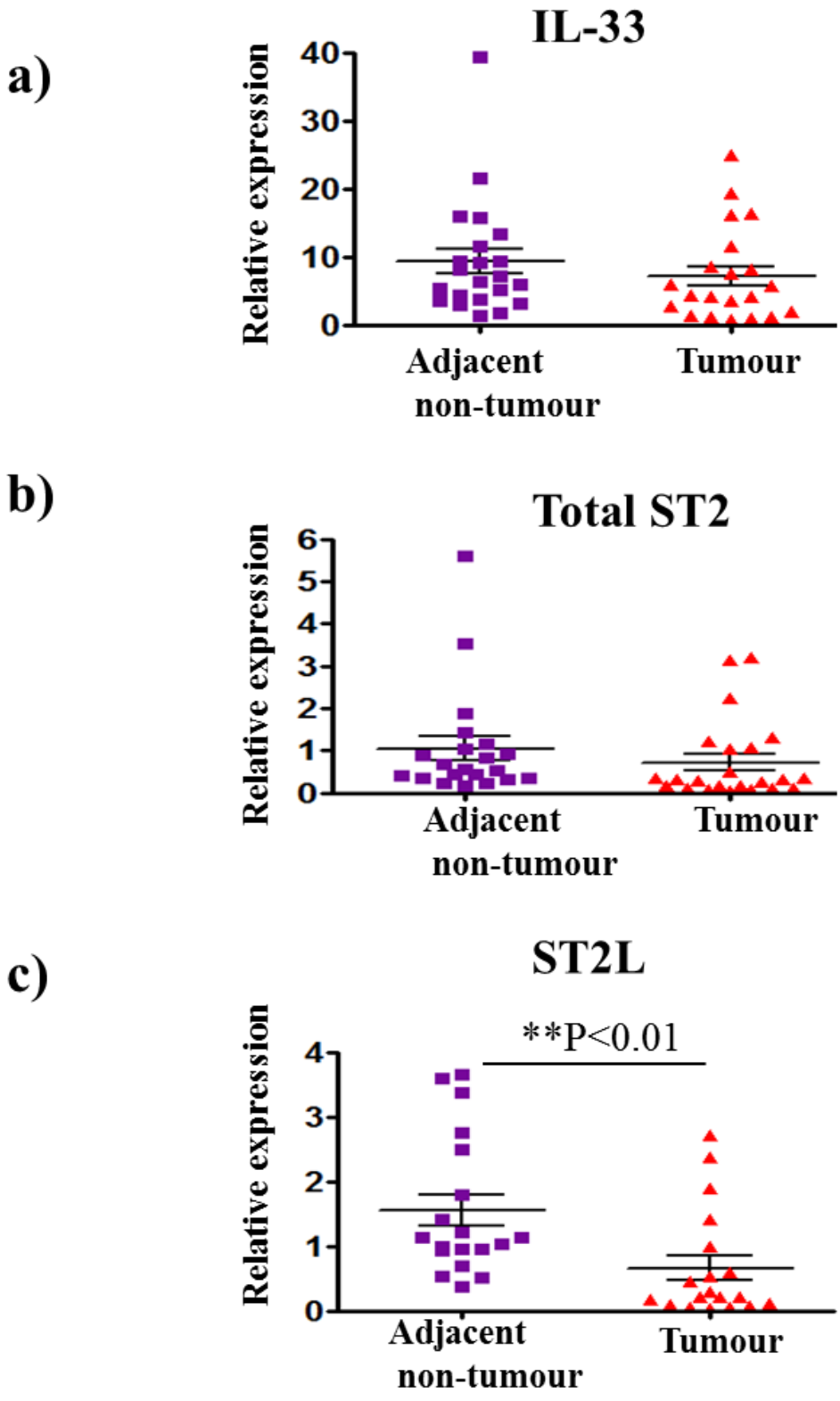


Figure 20: ST2L expression is lower in colorectal cancer tissue relative to adjacent non-tumour tissue. Total RNA was extracted and changes in (a) IL-33, (b) total ST2 and (c) ST2L were detected by qRT-PCR. Statistical analysis were determined using a Students paired t test. ** $P < 0.01$.

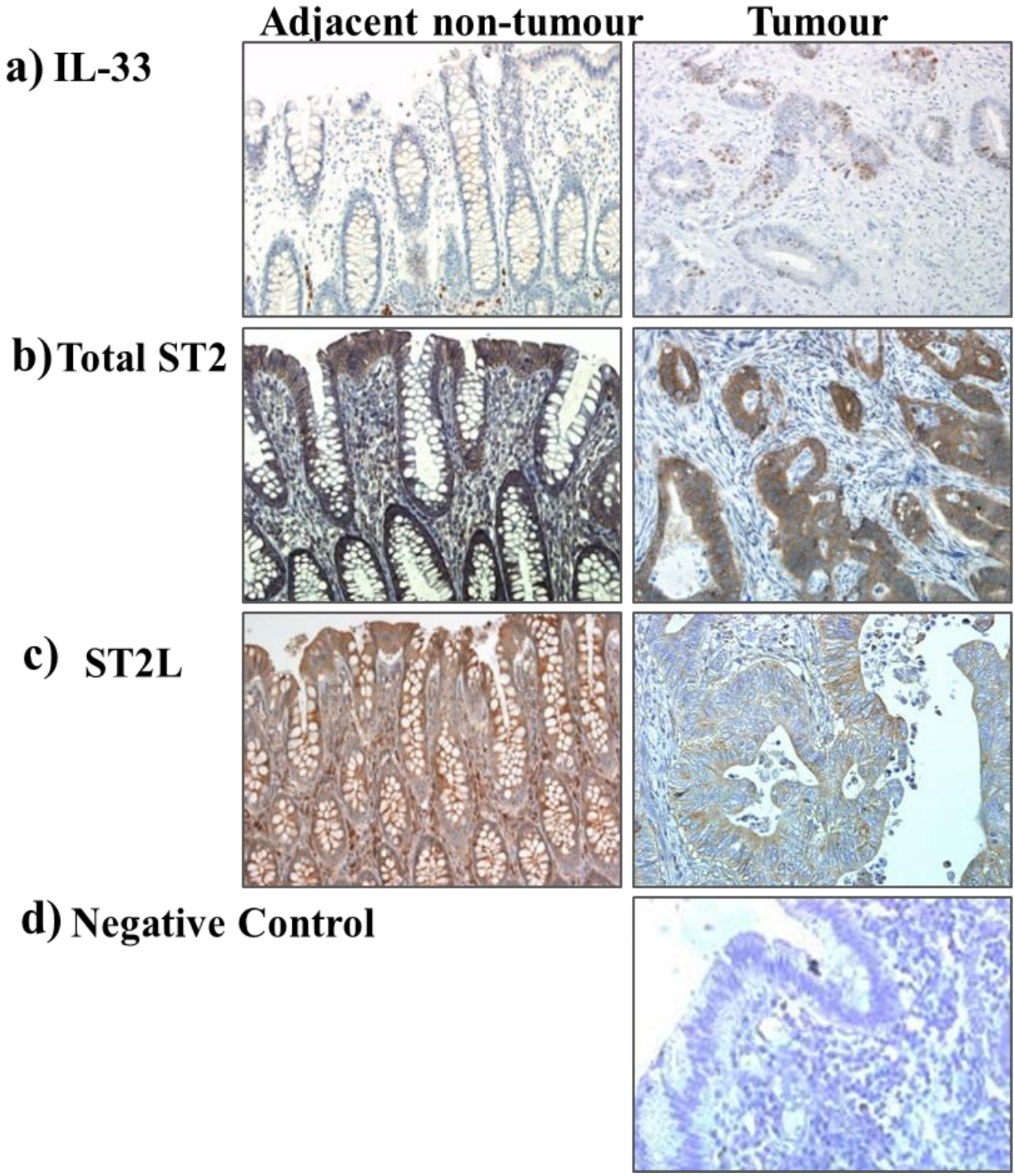


Figure 21: ST2L expression is lower in colorectal cancer tissue relative to adjacent non-tumour tissue, while IL-33 and total ST2 expression were unchanged. Immunohistochemical staining for (a) IL-33, (b) total ST2 and (c) ST2L was performed on paraffin-embedded tumour sections, (d) Primary antibody was omitted as a negative control. Original magnification: 20X. Images shown are representative of the findings obtained.

4.3 Discussion

In this chapter, I have shown that suppressing ST2 expression in murine colon cancer cells resulted in significantly increased tumour growth in BALB/c mice *in vivo*. This was associated with alterations in immune cell infiltration, in particular a reduction in macrophage and $CD8^+$ T cell infiltration. Furthermore, migration of macrophages towards tumour cell supernatant was increased by IL-33 stimulation of the tumour cells *in vitro*, while macrophage migration was reduced by antagonising CCL2 *in vitro*. These findings were consistent with the induction of CCL2 by IL-33 in colon cancer cells *in vitro* and the reduction in macrophage populations observed in ST2 suppressed tumours *in vivo*. In human CRC patient samples, ST2L expression by neoplastic cells was reduced in human colon tumours *in vivo*, as compared to adjacent non-tumour tissue. Working concurrently, my clinical collaborator Dr. Amr Mahmoud found that protein expression of ST2L was lower in human tumours relative to adjacent non-tumour tissue, and that the higher the grade of the tumour, the lower the expression of ST2L. Taken together this data suggests an anti-tumorigenic role for the IL-33/ST2 axis in colon carcinogenesis.

4.3.1 A potential anti-tumorigenic role for IL-33 in cancer:

In line with findings in this chapter, numerous recent reports have revealed an anti-tumorigenic role for IL-33 and ST2. In these studies, IL-33 was shown to promote the recruitment of immune cells known to activate an anti-tumorigenic immune response, such as $CD8^+$ T cells [118, 200, 201]. $CD8^+$ T cells mediate a vital role in the defence against cancer by targeting

cells compromised by oncogenic transformation, as well as targeting virally infected cells [296, 297]. IL-33 has been observed to be increased in response to viral infection and to be important for the eradication of a viral insult, as it can differentiate CTLs into anti-viral $CD8^+$ T cells [298]. IL-33 has also been shown to synergize with IL-12 to promote $CD8^+$ T cell effector function, thereby protecting the host [118]. Additional evidence supporting a pro-tumorigenic role for IL-33 has been shown utilising both the B16 melanoma and the 4T1 breast cancer models. In these models, overexpression of IL-33 potently inhibited tumour growth and metastasis through the recruitment of increased numbers of NK cells and tumour antigen specific $CD8^+$ T cells. These NK and $CD8^+$ T cells showed increased IFN-γ production [200]. IFN-γ and perforin were important in the anti-tumorigenic effect, as they were increased in B16 tumours overexpressing IL-33. In addition, when cells overexpressing IL-33 were transplanted into IFN-$\gamma^{-/-}$ mice, growth inhibition was partially reversed [200]. In a further study utilising both lung and melanoma tumour models, both the number and the cytotoxicity of $CD8^+$ T cells and NK cells were increased in response to transgenic IL-33 expression in both a Lewis lung carcinoma and a B16 melanoma model [201]. These authors again reported that the increased number of $CD8^+$ T cells and NK cells was associated with reduced tumour metastasis [201]. Moreover, depletion of $CD8^+$ T cells and NK cells reversed the suppression of metastasis that was observed in response to transgenic IL-33 expression [201]. Thus, an increase in IL-33 either systemically, or directly in the tumour cells, resulted in decreased tumour growth and metastasis. Therefore, from these studies, it appears that

IL-33 influences tumour growth and metastasis by altering the tumour microenvironment, in particular by increasing $CD8^+$ cell and NK cell infiltration. This is consistent with my findings in which I observed a reduction in $CD8^+$ cell infiltration and increased tumour growth in ST2 suppressed tumours.

Similar to our findings, a recent publication demonstrated that both IL-33 and ST2 expression were decreased with increasing tumour stage, in a large cohort of 713 CRC patients [202]. Other cancer types have shown similar patterns of circulating IL-33, as IL-33 was reduced in the plasma of non-small cell lung cancer patients relative to controls [198], and IL-33 negatively correlating with tumour stage in multiple myeloma patients [199]. Our data suggests that ST2 expression may be lost as colon cancer progresses. If ST2 plays an anti-tumorigenic role as the data suggests and promotes anti-tumour immunity, loss of ST2 as the tumour progresses may result in increased growth and tumorigenesis.

4.3.2 The role of TAMs in tumorigenesis:

Tumour associated macrophages (TAMs) have been shown to increase the proliferation of tumour cells through the secretion of growth factors and by promoting neovascularization [299]. *In vitro* findings presented here (Chapter 3) show that IL-33 induces CCL2 expression in colon tumour cells, which is a known macrophage chemotactic factor. We also demonstrated that suppressing ST2 expression by tumour cells results in decreased macrophage infiltration associated with a concomitant increase in tumour size. These findings suggest that a reduction in CCL2 production in

the ST2 suppressed cells could be responsible for the reduction in macrophage recruitment seen *in vivo.* To date three studies have reported on the impact of the IL-33/ST2 signalling axis on tumour-associated macrophage populations in tumour-bearing mice [196, 200, 300]. Similar to my findings, overexpression of IL-33 by fibrosarcoma cells resulted in an increase in F4/80^{+} TAMs *in vivo*. These TAMs were further classified and M2 subpopulations were significantly increased in IL-33 overexpressing tumours compared to cells containing the vector alone. Indeed, the same study went on to examine TAM polarization of IL-33 positive cells on an ST2$^{-/-}$ background. Tumours from ST2$^{-/-}$ mice showed reduced M2 markers compared to tumours grown in WT mice [300]. Although I attempted to I was unable to assess the phenotype of the TAMs in our studies, unfortunately this did not prove possible due to the low numbers of TAMs isolated from excised tumours. However, in contrast to our findings, two studies have reported that infiltration by TAMs was unchanged by manipulation of the IL-33 and ST2 pathway. The first study overexpressed IL-33 in melanoma cells and reported that TAM numbers were unaltered compared to WT [201]. In a breast cancer model, the number of TAMs was also unchanged by systemic IL-33 administration. However, these tumours had increased numbers of alternatively activated M2 macrophages [196]. This correlates with IL-33 being a known inducer of macrophage polarization, promoting the development of alternatively activated M2 macrophages [301]. Unlike our study, which suppressed ST2, these studies either overexpressed IL-33 or administered exogenous IL-33. Therefore, the levels of IL-33 present could vary quite significantly. This may be an

important factor as other models examining immune responses induced by IL-33, particularly in hepatitis, have shown that higher doses of IL-33 induced strong type 1 immune responses, while reduced IL-33 doses have led to immune tolerance [302, 303]. Therefore, the immune phenotype induced by IL-33 may be influenced by the dose or level of IL-33 present. Overall, these findings suggest that suppressing ST2 signalling alters the tumour environment through the loss of cells that promote tumour eradication, such as macrophages and $CD8^+$ T cells. It has also been suggested that IL-33 could be potentially useful in immunotherapy, by initiating anti-tumorigenic inflammation at the tumour site to enhance the response rate of therapy in colon cancer by increasing the immunogenicity of the tumour [304].

4.3.3 Alternative pro-tumorigenic role for IL-33/ST2 axis:

To date, most studies investigating the role of IL-33 in tumorigenesis have focused on breast cancer. In contrast to our findings in the colon, the IL-33/ST2 signalling axis has been shown to promote breast cancer, as deletion of ST2 in BALB/c mice reduced tumour growth in a 4T1 breast cancer model [305]. Necrosis of the tumour was increased upon ST2 deletion and associated with a reduction in VEGF and was, therefore, proposed as the mechanism responsible for suppressing tumour growth. Necrosis may be associated with an increase in the anti-tumour immune response as necrotic cells facilitate maturation of antigen presenting cells. Consistent with this, administering IL-33 to mice accelerated the growth of 4T1 breast cancer cells by suppressing anti-tumour immunity and promoting angiogenesis

[193]. A pro-tumorigenic role for IL-33 was also recently reported in lung cancer. IL-33 was shown to enhance lung carcinogenesis by promoting cell death in low metastatic cells *in vitro*, thus, favouring the growth of more metastatic cells that did not express ST2L [304]. The differences between these findings and my results may potentially be explained through the different models employed, as these studies focused on IL-33 whereas our study did not look at IL-33 directly. Therefore, as IL-33 was not suppressed in my model and could still function as nuclear factor. It may be through this function as a nuclear factor that IL-33 exerted its pro-tumorigenic effect.

The role of IL-33/ST2 in CRC is an area of ongoing research, which has resulted in several reports being published during the course of my PhD studies. Certain of these have yielded conflicting findings to our data, and have indicated a pro-tumorigenic role for this axis in the development of CRC [147, 202-204]. Some of the contrasting findings concern the expression levels of IL-33 and ST2 being reported in CRC. In our study, expression of total ST2 was unchanged compared to adjacent non-tumour tissue, while ST2L expression was significantly lower in human tumours P=0.01 (n=24). Cui *et al.* reported however, that total ST2 expression was shown to be higher in adenoma samples relative to normal control samples [203]. These levels then decreased from adenoma to tumour, as detected by both qRT-PCR and IHC. However, these authors investigated total ST2 expression, and did not distinguish between the different ST2 variants [203]. Therefore, although we did not examine ST2 levels in adenoma patients, the CRC data presented by Cui *et al.* are in agreement with our

findings, whereby total ST2 expression was unaltered between normal adjacent tissue and CRC tissue. Also, similar to our findings, a second study showed no increase in total ST2 expression in CRC compared to normal tissue [147]. In contrast a third study, reported total ST2 to be increased in CRC samples relative to adjacent normal tissue [204]. The differences observed between this study and ours may be due to sample ethnicity or lack of discrimination between ST2 isoforms, as our observed decrease in ST2 expression was confined to the transmembrane bound signalling active L-isoform (ST2L). Indeed, to the best of my knowledge, no other study has distinctly examined the expression of ST2L in CRC.

I found that the level of IL-33 expression was low in both CRC tissue and non-tumour tissue (n=24). Similarly, my clinical collaborator, Dr. Amr Mahmoud examined IL-33 expression in a cohort of 66 CRC patients and found IL-33 expression to be low in tumour and adjacent non-tumour tissue. In contrast, all of the CRC studies published to date have reported an increase in IL-33 levels in CRC as compared to adjacent normal tissue and healthy volunteers [147, 202-204]. However, between these publications there were variations in the level of IL-33 being reported in CRC. IL-33 expression levels were low in two of the studies [203, 204], with any differences in IL-33 levels predominantly due to alterations in the level of IL-33 in the tumour stroma. In contrast to the low expression levels reported in the first two studies, further studies detected high levels of IL-33 expression [147, 202]. It is worth noting that, one of these consisted of a cohort of patients that were receiving 5-FU-based chemotherapy [147]. IL-33 is well known to be released upon cellular damage. It is possible that the

chemotherapeutic regimes directly affected the levels of IL-33 in these patients. Indeed, the low level of IL-33 seen in my study may be reflective of the lack of prior chemotherapy in our cohorts. A second study (n=713) found that IL-33 was highly expressed in low-grade tumours compared to high-grade tumours. Indeed, numerous studies have reported that IL-33 expression is low in healthy colonic tissue [178, 306], consistent with its role as an alarmin IL-33 only induces robust inflammation when released by necrotic tissue [307].

Using the AOM/DSS model of intestinal tumorigenesis, Mertz *et al.* reported that tumour growth was decreased in $ST2^{-/-}$ mice compared to WT [202]. Tumour load, tumour grade and the number of tumours were also reduced in the absence of ST2. The combination of AOM with DSS is used as a model of colitis-associated cancer. DSS damages the intestinal monolayer inducing intestinal inflammation creating an inflammatory environment. This model examines the early development of adenoma through to carcinoma. The use of this highly inflammatory AOM/DSS model of colon cancer may account for some of the differences in these results, as our model shows much lower levels of inflammation by comparison. An additional study demonstrated that inhibition of the IL-33/ST2 signalling pathway in the $APC^{Min/+}$ mouse model of intestinal tumorigenesis inhibited tumour growth, induced apoptosis and suppressed angiogenesis in adenomatous polyps. Inhibition of the IL-33/ST2 axis reduced both tumour number, tumour size and mast cell infiltration into polyps and suppressed the expression of mast cell derived cytokines and proteases that promote polyposis. The authors propose that IL-33 derived

from the tumour epithelium promotes polyposis through the activation of stromal cells and the formation of a protumorigenic microenvironment [147]. The APC^{MIN} mouse develops polyps in the intestine and these benign growths transform and become malignant. These studies suggest that IL-33 and ST2 may play a role during the initial oncogenic transformation of cells which would have been an event excluded from our choice of tumour model. An additional CRC study, in nude mice, demonstrated that overexpression of IL-33 in human CRC cells increased tumour growth, while reducing IL-33 expression inhibited growth [204]. However, as nude mice are unable to mount a T-cell mediated response, this suggests that T-cells could be an important factor in our model to reduce tumour growth. T-cells can be either tumour suppressive or tumour promoting, as determined by their effector functions [308]. Many factors can affect the polarization of Th1/Th2 cells, including the cytokine milieu in the local environment. Th1 cells can directly kill tumour cells, while Th2 cells can promote tumour growth [309]. BALB/c mice are known to generate strong Th2, but weak Th1 responses [244]. Therefore, if a Th1 response induces the pro-tumorigenic IL-33 response, but is absent in our model this could explain the variation observed between results. Other possible explanations for the disparity between results may be due to the specific levels of these proteins present in the microenvironment. In the current study, I suppressed ST2 expression in cancer cells, while other studies employed knockout mice, but then introduced tumours cells expressing IL-33/ST2. IL-33 has been described as a double-edged sword; as it is known to have dual pro- and anti-inflammatory functions and therefore, at different levels it may drive

either pro- or anti-inflammatory immunity.

Taken together, this chapter has shown a potential anti-tumorigenic role for the IL-33/ ST2 pathway in colon cancer. The data suggests that IL-33 mediates tumour growth through the recruitment of immune cells into the tumour microenvironment, in particular through recruiting $CD8^+$ T cells and macrophages. Therefore, targeting ST2L could provide potential therapeutic benefit for the treatment of colon cancer by priming the anti-tumour immune response. However, given that IL-33 and ST2 appear to have diverging and opposing effects, depending on the cancer type examined further research investigating the role of IL-33 and ST2 in cancer is required.

Chapter 5

Characterisation of the role of IL-36 in colon cancer

5.1 Introduction

The IL-36 cytokines are a recently described subset of the IL-1 family of cytokines. The three agonistic members of the IL-36 family, IL-36α, IL-36β and IL-36γ, all share a common receptor complex, which is composed of the IL-36 receptor (IL36R/IL1RRP2/IL1RL1) and the IL1 Receptor accessory protein (IL1RAcP). A biological inhibitor to this complex has also been identified, the IL-36R antagonist (IL-36RN). Similar to other IL-1 family members, IL-36 cytokines are important activators of the immune response. Recent emerging evidence has demonstrated a role for these cytokines in autoimmunity with dysregulated responses indicated in the pathogenesis of psoriasis [310], asthma [311] and obesity [236].

The function of IL-36 family members in intestinal pathogenesis was, until recently, largely unknown. However, it has lately become the focus of many studies and is currently an area of active research. Numerous studies have recently identified IL-36 cytokines as being upregulated in IBD [222, 224, 240]. In particular, IL-36α and IL-36γ have been highlighted as being increased in active IBD [222, 224, 240]. The expression pattern of these cytokines differed as IL-36γ was expressed in the nucleus of intestinal epithelial cells and IL-36α was expressed in the cytoplasm of $CD14^+$ macrophages [224]. The IL-36RN was also found to be decreased in UC patients compared to normal tissue [240]. Additional evidence for a novel role for IL-36 family members in the intestine has been demonstrated in various murine models. In a DSS-induced model of acute colitis, $IL\text{-}36R^{-/-}$ mice showed reduced disease severity and decreased innate inflammatory cell infiltration to the colonic lamina propria compared to WT mice [240]. In the absence of the IL-36R, however, bacterial colonization by the pathogenic strain *Citrobacter rodentium* was increased

and a reduced Th1 response was observed. This suggests that IL-36 signalling is required for protection against pathogenic invasion, but that this response may be skewed in intestinal pathogenesis such as IBD.

Recent investigations have shown that the IL-36R exerts its effect in colonic fibroblasts by inducing cytokine and chemokine activation which regulates the recruitment of immune cells to the inflamed colon [224]. Consistent with a role in maintaining barrier function, an additional study demonstrated that DSS-treated IL-36R$^{-/-}$ mice showed diminished wound healing [224]. IL-22, the barrier protective cytokine, was also decreased in the colon of IL-36R$^{-/-}$ mice. This was accompanied by a reduction in IL-22 producing colonic neutrophils at the wound site. This suggests that signalling through the IL-36R promotes wound healing of the intestinal mucosa via IL-22 production and neutrophil infiltration [241]. While some papers suggest that IL-36 plays a pro-inflammatory role in IBD as IL-36 ligands are increased in IBD, others suggest that IL-36 promotes wound healing, as IL-36R$^{-/-}$ mice show delayed wound healing due to reduction in neutrophil recruitment. Thus, this normally protective response may be skewed in intestinal pathogenesis such as IBD, and therefore may also be implicated in colon carcinogenesis.

Given the involvement of other IL-1 family members in the tumorigenic process, it is highly likely that these novel IL-36 cytokines also play a role in cancer. A recent study investigating the role of these proteins in cancer identified a potential anti-tumorigenic role for IL-36γ in breast and melanoma tumorigenesis [244]. Tumoral expression of IL-36γ reduced tumour growth and metastasis in both a 4T1 breast cancer model and a B16 melanoma model. The proposed mechanism responsible for this anti-tumorigenic effect was through the activation of a potent type 1 immune response, as increased infiltration of CD8^{+} T-cells, NK cells, γδ T-cells and T-regs

cells were observed in the tumour microenvironment. Both IL-12 and IFN-γ were also greatly increased in the IL-36γ expressing tumours. Therefore, it was proposed that these cytokines worked in combination with IL-36γ to induce the anti-tumour response observed. Moreover, IL-36γ expression was shown to inversely correlate with the progression of melanoma. These authors, however, did not examine the involvement of the other IL-36 cytokines in tumorigenesis. A second study identified a positive correlation between IL-36α expression and the mortality of hepatocellular carcinoma patients (HCC) [242]. These authors demonstrated that low expression of IL-36α was associated with increased tumour volume and increased TNM stage. Survival analysis showed that reduced expression of IL-36α was indicative of a poor prognosis for HCC patients. Increased tumoral expression of IL-36α was associated with higher infiltration of $CD3^+$ and $CD8^+$ T cells [242]. Given the recent identification of a role for IL-36 in IBD, and the recently described link between IL-36 agonists and cancer, the aim of this chapter is to investigate the role of the IL-36 family in colon cancer.

5.2 Results

5.2.1 IL-36α and IL-36γ expression were increased in tumour tissue compared to adjacent tissue, while IL-36R expression was unchanged.

In order to investigate the role of the IL-36R in CRC, IL-36 mRNA expression was initially examined by qRT-PCR in the same cohort of CRC patients utilised in the previous chapter. The demographics pertaining to these patients (termed CRC cohort 1) are contained in Chapter 4 Table 1. IL-36α expression was detected in 4 out of the 24 adjacent non-tumour tissue samples, while 11 out of 24 CRC tumours expressed IL-36α. Of those samples that expressed IL-36α, expression was significantly higher in tumour tissue compared to adjacent non-tumour tissue ($p=0.0112$) (Figure 1a). Alterations in IL-36α expression was further validated using a second cohort of 46 patients (Table 1). IL-36α expression was investigated at the protein level and was found to be low in adjacent healthy tissue, but was increased in the tumour cells of CRC (Figure 1b). IL-36α expression was further analysed in this second cohort ($n=46$) and samples were stratified according to cancer stage by IHC (Figure 2). Representative images are shown in Figure 2a. A scoring system was developed, with the staining intensity of IL-36α in tumour epithelium categorised as negative, weak, moderate or strong. No correlation was observed between IL-36α expression and tumour stage (Figure 2b).

IL-36β expression was next investigated and quantitative qRT-PCR analysis showed that IL-36β was expressed in colon cancer tissues and normal adjacent tissue. IL-36β was also found to be significantly increased in tumour tissue compared to adjacent non-tumour tissue ($p=0.0474$) (Figure 3a). These changes in IL-36β mRNA expression were not replicated at the protein level as both adjacent non-tumour tissue

Clinical Characteristics		Cohort 2 (n=46)
Gender	Male	30
	Female	16
Age	>60	31
	<60	15
Site	Left	29
	Right	17
Tumour Differentiation	MD	41
	PD	5
	WD	0
Tumour Stage	I	8
	II	13
	III	13
	IV	12
T classification	T1	4
	T2	6
	T3	18
	T4	18
N Classification	pN0	23
	pN1	23
Chemotherapy	Yes	15
	No	31

Table 1. **CRC cohort 2 patient demographics**. A cohort of 46 patients with CRC was recruited from the Mercy University Hospital Cork. The study protocol, including all procedures and study populations was approved by the University College Cork Clinical Research Ethics Committee of the Cork Teaching Hospitals (ECM (3) P 3/9/2013). All samples were collected with patients' informed consent.

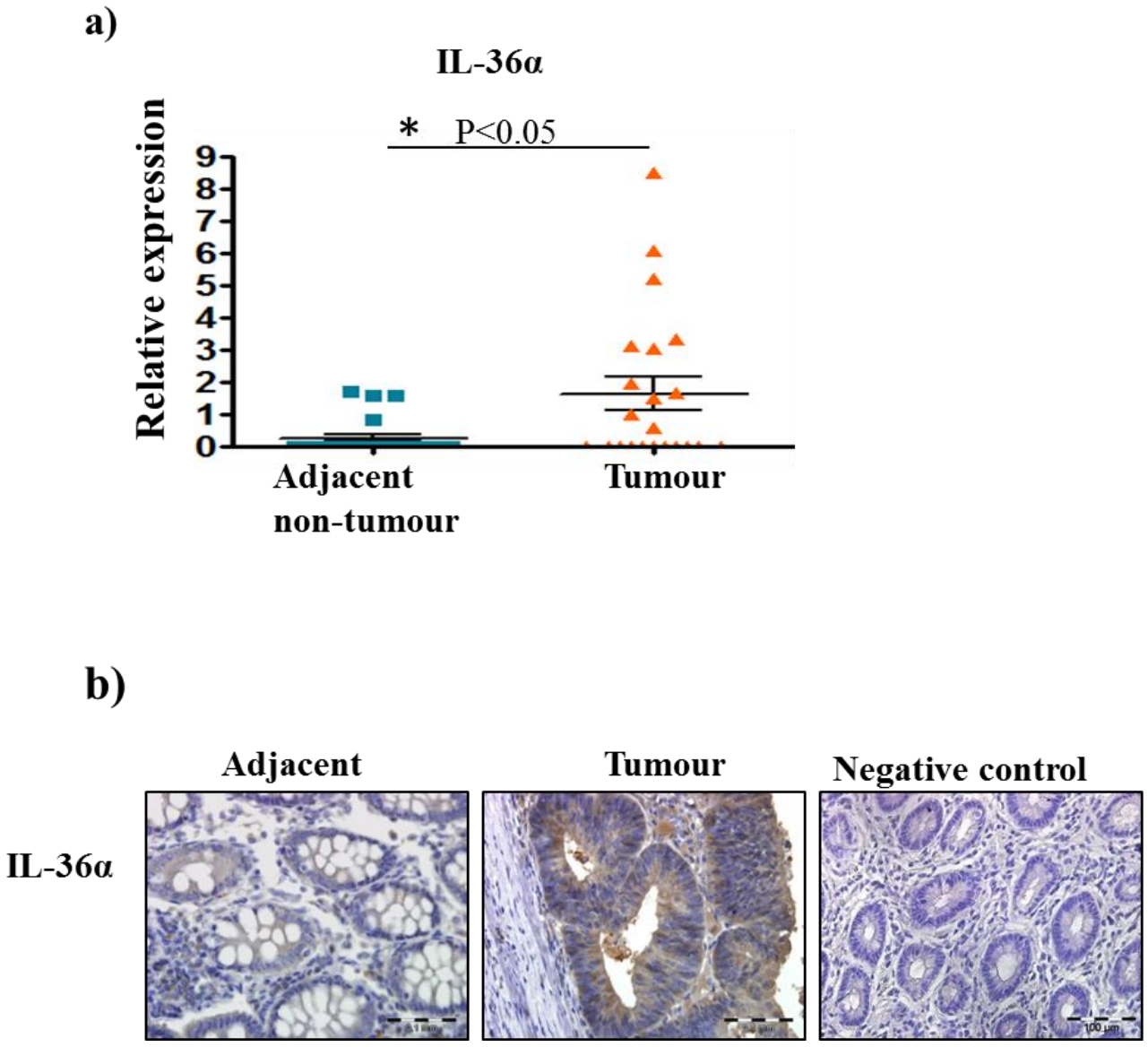
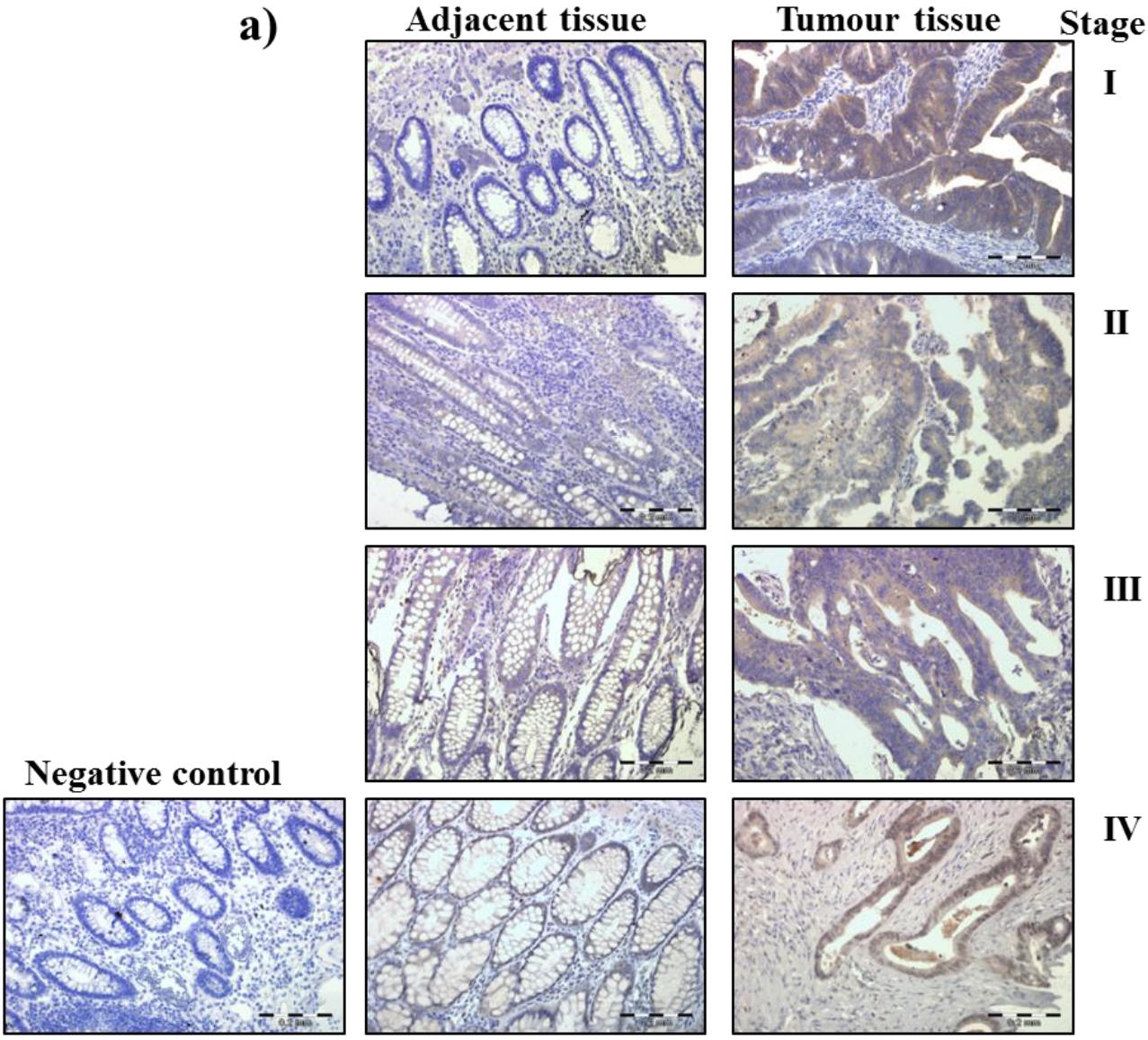


Figure 1: IL-36α expression was significantly higher in human CRC, compared to adjacent non-tumour tissue. (a) IL-36α mRNA expression was higher in human CRC tumours compared to adjacent non-tumour tissue. Total RNA was extracted and changes in expression were detected by qRT-PCR. Statistical analysis was carried out using a student t test. P=0.0034. (b) IL-36α protein expression was examined by immunohistochemistry. Immunohistochemical staining was performed on paraffin-embedded human tumour sections. Primary antibody was omitted as a negative control. Scale bar = 100μm. Images shown are representative of the findings obtained.

a)



b)

CRC stage	Total	% Negative /Weak	% Moderate /Strong
Stage I	5	80%	20%
Stage II	6	67%	33%
Stage III	8	63%	38%
Stage IV	10	70%	30%

Figure 2: No correlation between IL-36α expression and tumour stage was observed. (a) Stage I to IV paraffin-embedded human CRC tumours were examined for IL-36α expression by immunohistochemistry No correlation was observed between IL-36α protein expression and CRC stage. Primary antibody was omitted as a negative control. Scale bar = 100μm. Images shown are representative of the findings obtained. (b) Table of IL-36α expression by CRC stage. Tumour sections were scored as negative, weak, moderate or strong, according to the intensity of IL-36α staining. No correlation was observed between IL-36α protein expression and CRC stage.

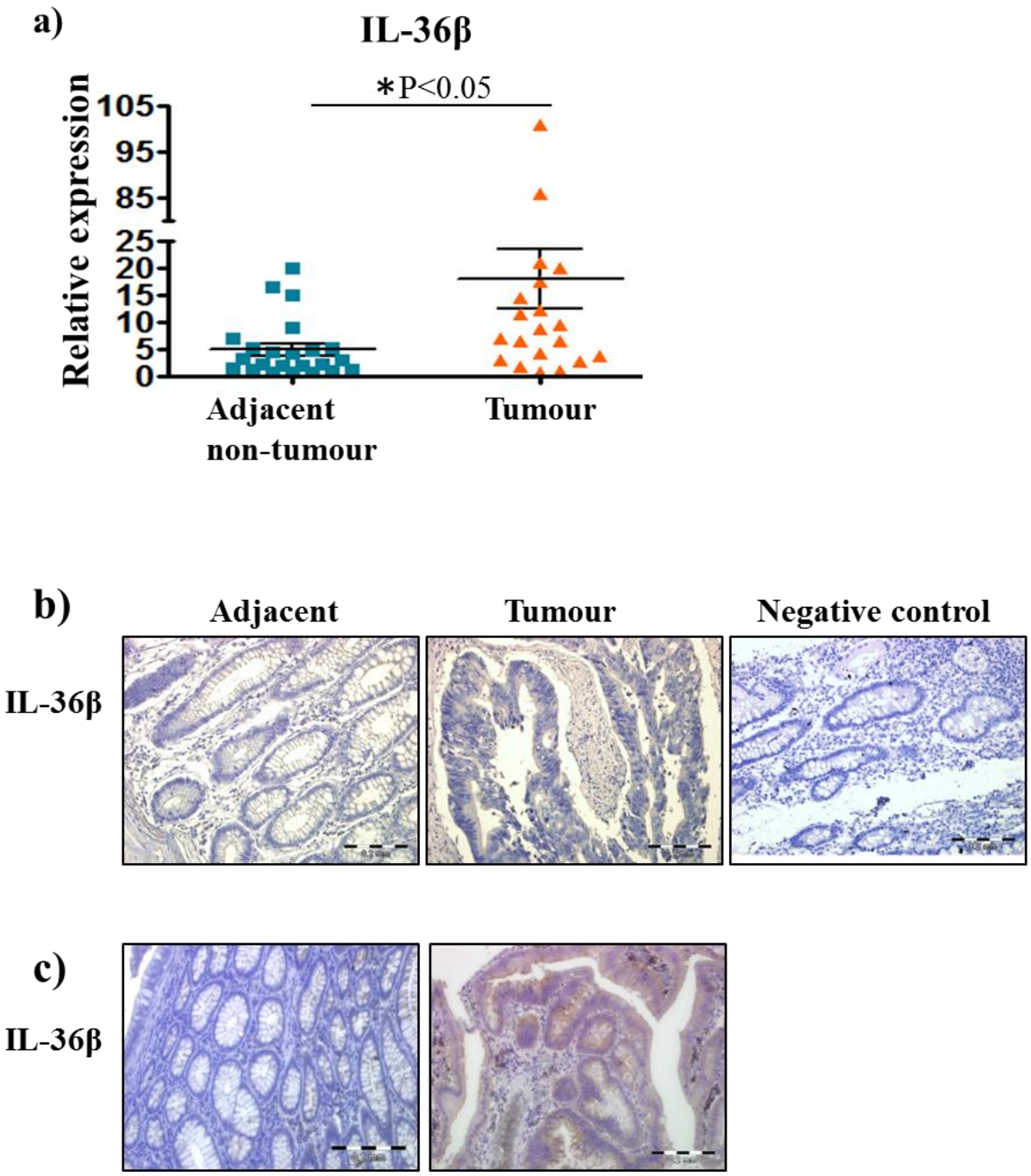


Figure 3: IL-36β mRNA expression was significantly higher in human CRC, compared to adjacent non-tumour tissue. However, IL-36β protein expression was unchanged. (a) IL-36β mRNA expression was higher in human CRC tumours compared to adjacent non-tumour tissue. Total RNA was extracted and changes in expression were detected by qRT-PCR. Statistical analysis was carried out using a student t test, P=0.0474. (b) IL-36β protein expression was examined by immunohistochemistry. Immunohistochemical staining was performed on paraffin-embedded human tumour sections. Scale bar = 200μm. Images shown are representative of the findings obtained. Primary antibody was omitted as a negative control. (c) The majority of tumours were negative for IL-36β expression, however 2 tumours showed strong IL-36β expression in the epithelium and stromal cells.

and tumour tissue showed no positive staining (Figure 3b). There were some exceptions to this, as 2/46 tumours showed strong stromal expression and some epithelial expression of IL-36β (Figure 3c). However, there was no unifying link between these tumours as they were different stages, and one patient had received chemotherapy while the other had not.

IL-36γ expression was also examined, with transcription of IL-36γ significantly increased in CRC tumours compared to adjacent non-tumour tissue ($p=0.0056$) (Figure 4a). Alterations in expression were confirmed at the protein level by IHC, using an antibody directed against IL-36γ (Figure 4b). IL-36γ was found to be strongly expressed in the colonic tumour cells of CRC tumours compared to adjacent non-tumour tissue. We further analysed IL-36γ expression in relation to CRC stage in our second cohort of patient samples and did not observe any correlation between IL-36γ expression and CRC stage (Figure 5). Representative images are presented.

We also investigated expression of the IL-36 receptor antagonist (IL-36RN), which showed significantly higher mRNA expression in tumour tissue compared to adjacent non-tumour tissue ($p=0.0034$) (Figure 6a). Similarly, the IL-1RAcP was also significantly increased in tumour tissue compared to adjacent non-tumour tissue (Figure 6b). However, it is worth noting that the IL-1RAcP is also an accessory protein for other members of the IL-1R family such as ST2L. I subsequently examined IL-36R mRNA expression. Transcription of the IL-36R was unchanged in tumour tissue compared to adjacent non-tumour tissue, as determined by qRT-PCR (Figure 7a). Consistent with the detection of expression of IL-36R mRNA, IL-36R protein was expressed in epithelial tumour tissue and expression was unchanged between tumour tissue and adjacent non-tumour tissue (Figure 7b). Similar to the

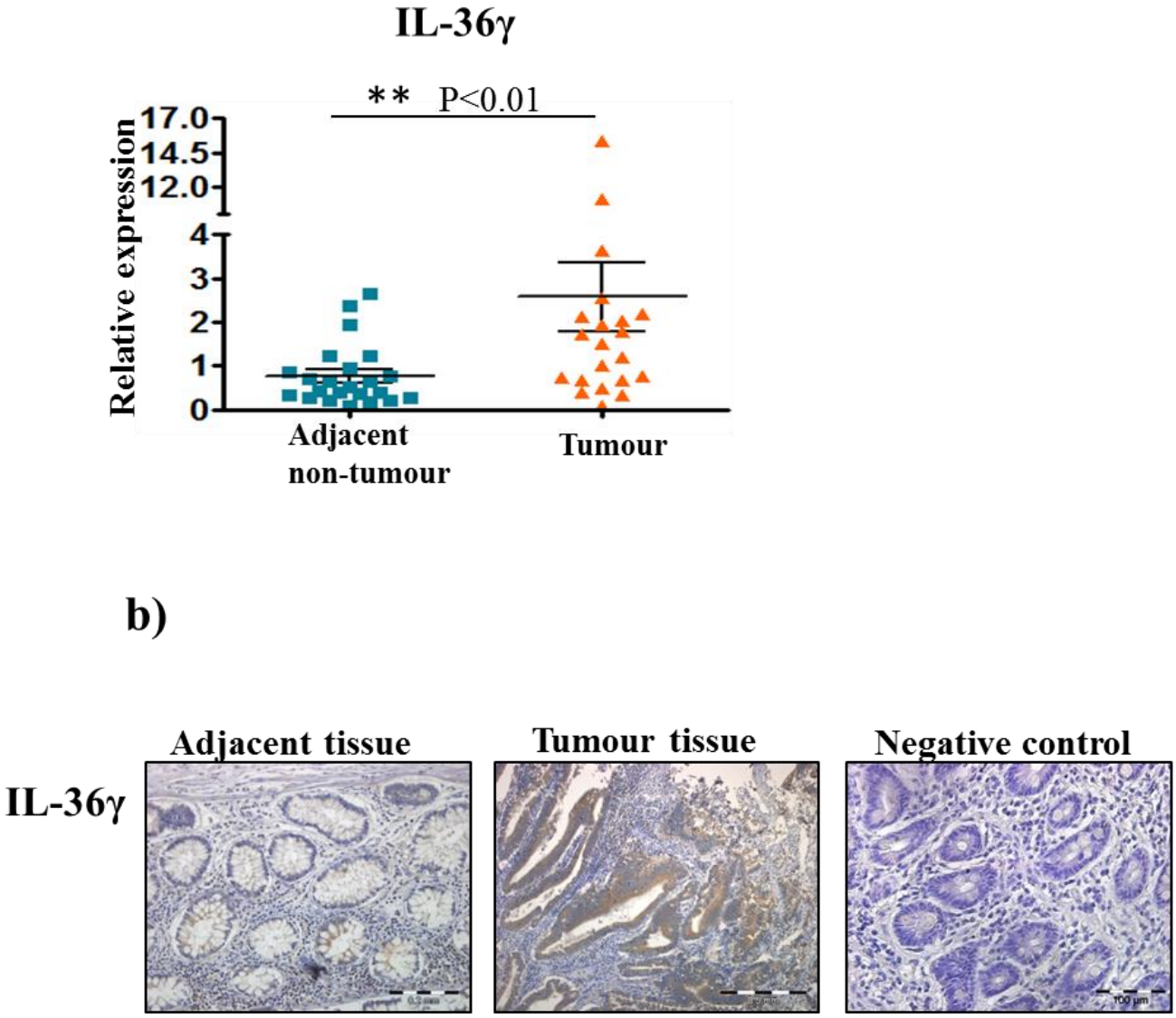
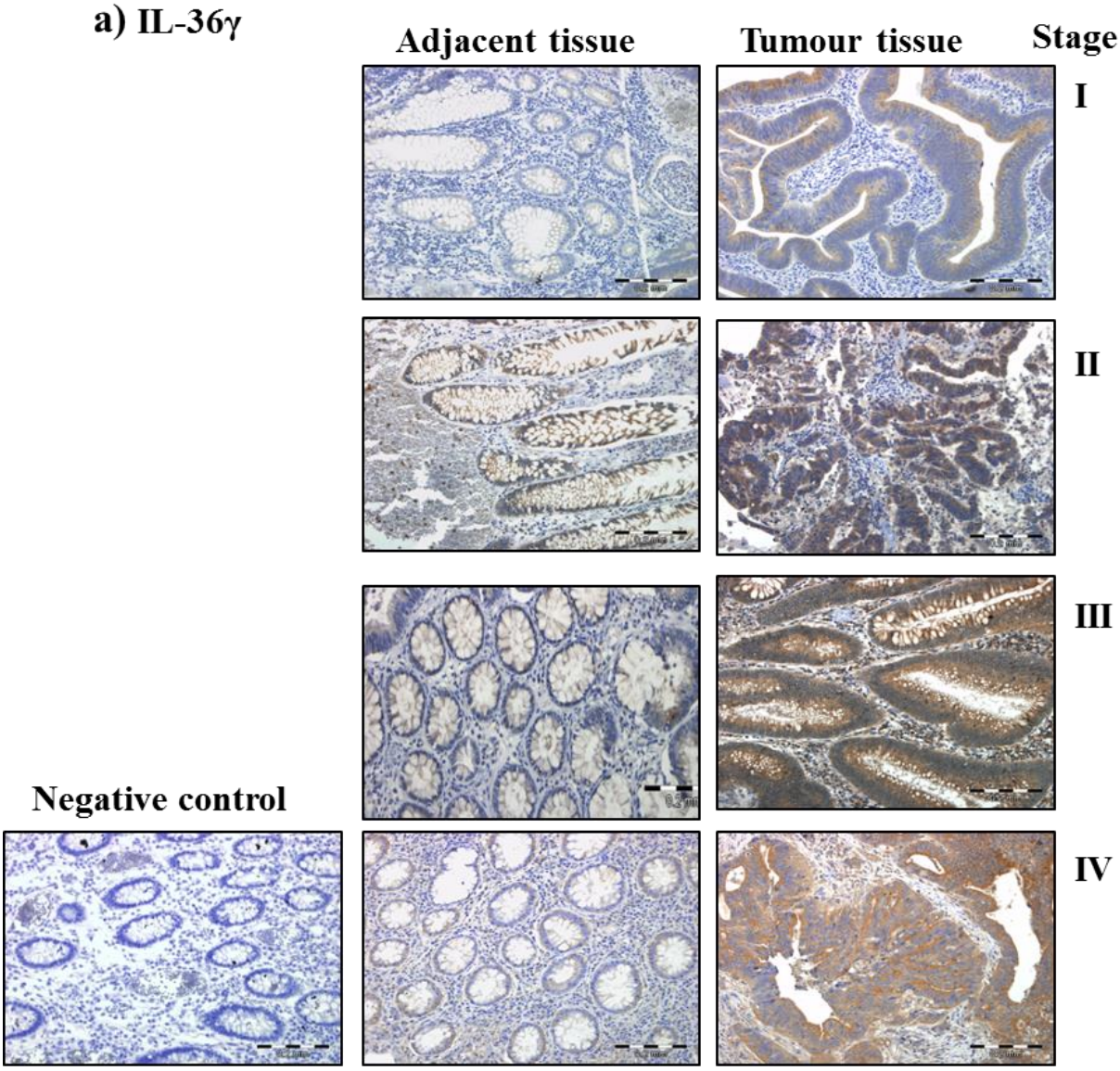


Figure 4: IL-36γ expression was significantly higher in human CRC, compared to adjacent non-tumour tissue. (a) IL-36γ mRNA expression was higher in human CRC tumours compared to adjacent non-tumour tissue. Total RNA was extracted and changes in expression were detected by qRT-PCR. Statistical analysis was carried out using a student t test p=0.0056. (b) IL-36γ protein expression was higher in tumour tissue compared to adjacent tissue. Paraffin-embedded human CRC sections were immunostained with anti-IL-36γ antibody. Primary antibody was omitted as a negative control.

a) IL-36γ



b)

CRC stage	Total	% Negative /Weak	% Moderate /strong
Stage I	10	50%	50%
Stage II	13	23%	77%
Stage III	11	45%	55%
Stage IV	12	50%	50%

Figure 5: IL-36γ expression was increased in tumour tissue compared to adjacent tissue, but did not correlate with tumour stage. (a) Paraffin-embedded human CRC tumours, stage I to IV were immuno-stained with IL-36γ. No correlation between CRC grade and IL-36γ expression was observed. (b) Tumour sections were scored as negative, weak, moderate or strong, according to the intensity of IL-36γ staining.

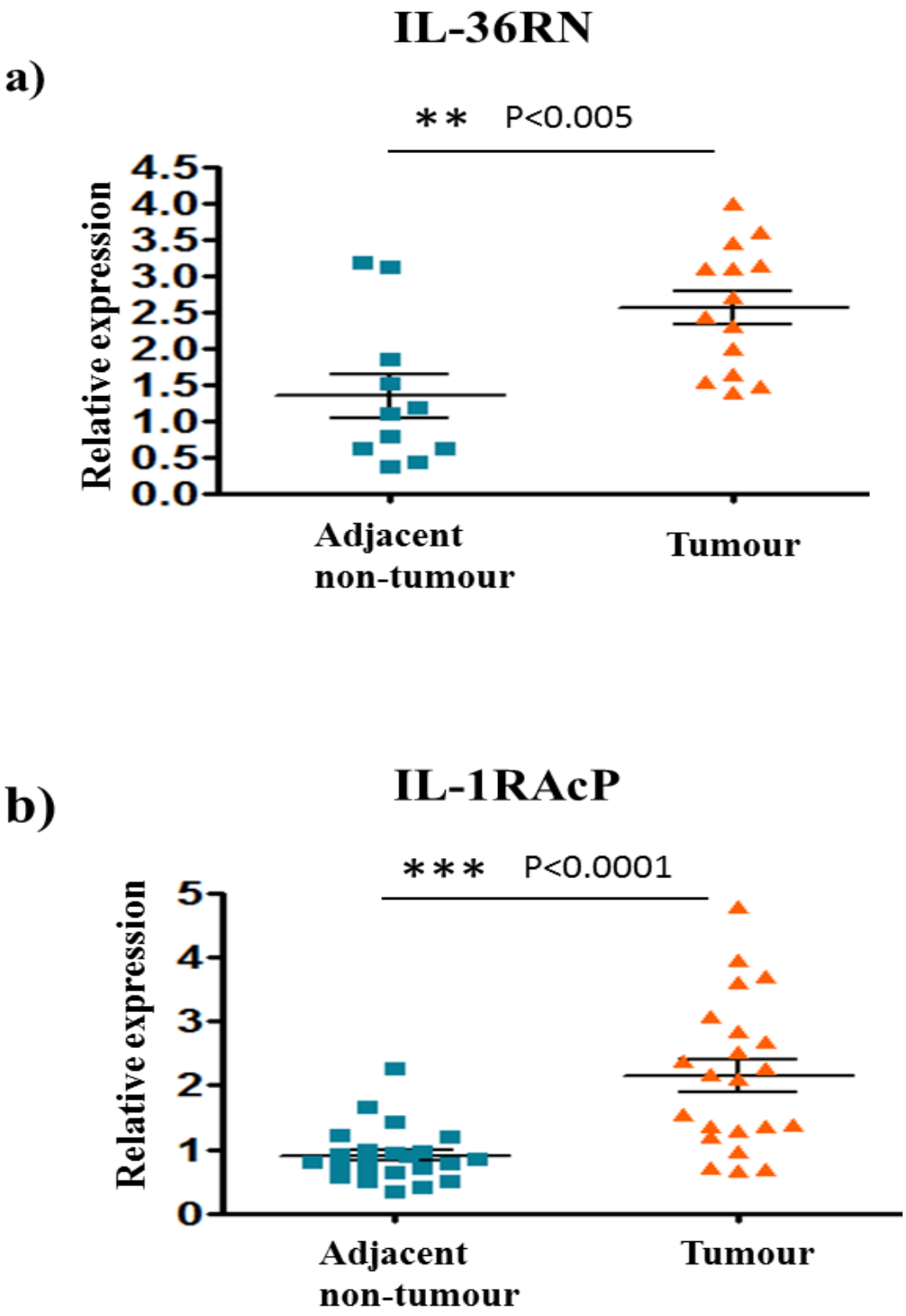
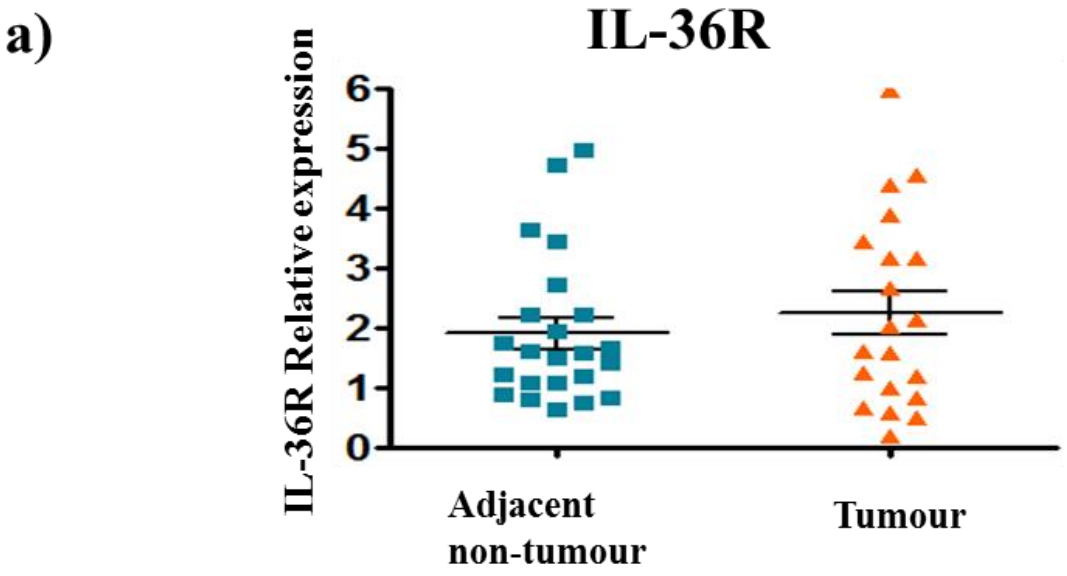


Figure 6: IL-36Ra and IL-1RAcP mRNA expression was significantly higher in human CRC, compared to adjacent non-tumour tissue. (a) IL-36RN and (b) IL-1RAcP mRNA expression was significantly higher in human CRC tumours compared to adjacent non-tumour tissue p=0.0034 and p=0.0001 respectively. Total RNA was extracted and changes in expression were detected by qRT-PCR. Statistical analysis was carried out using a student t test.



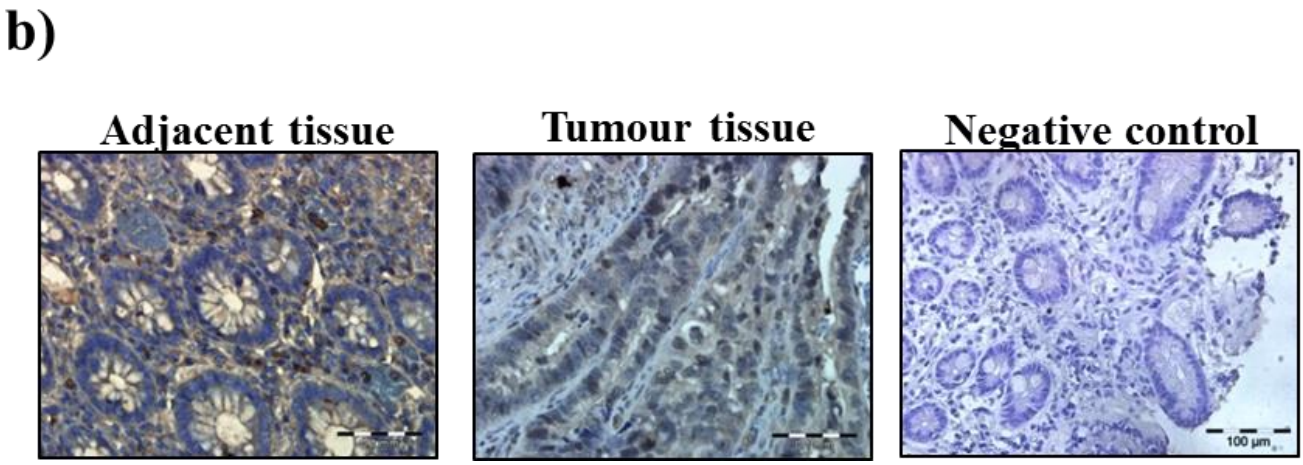
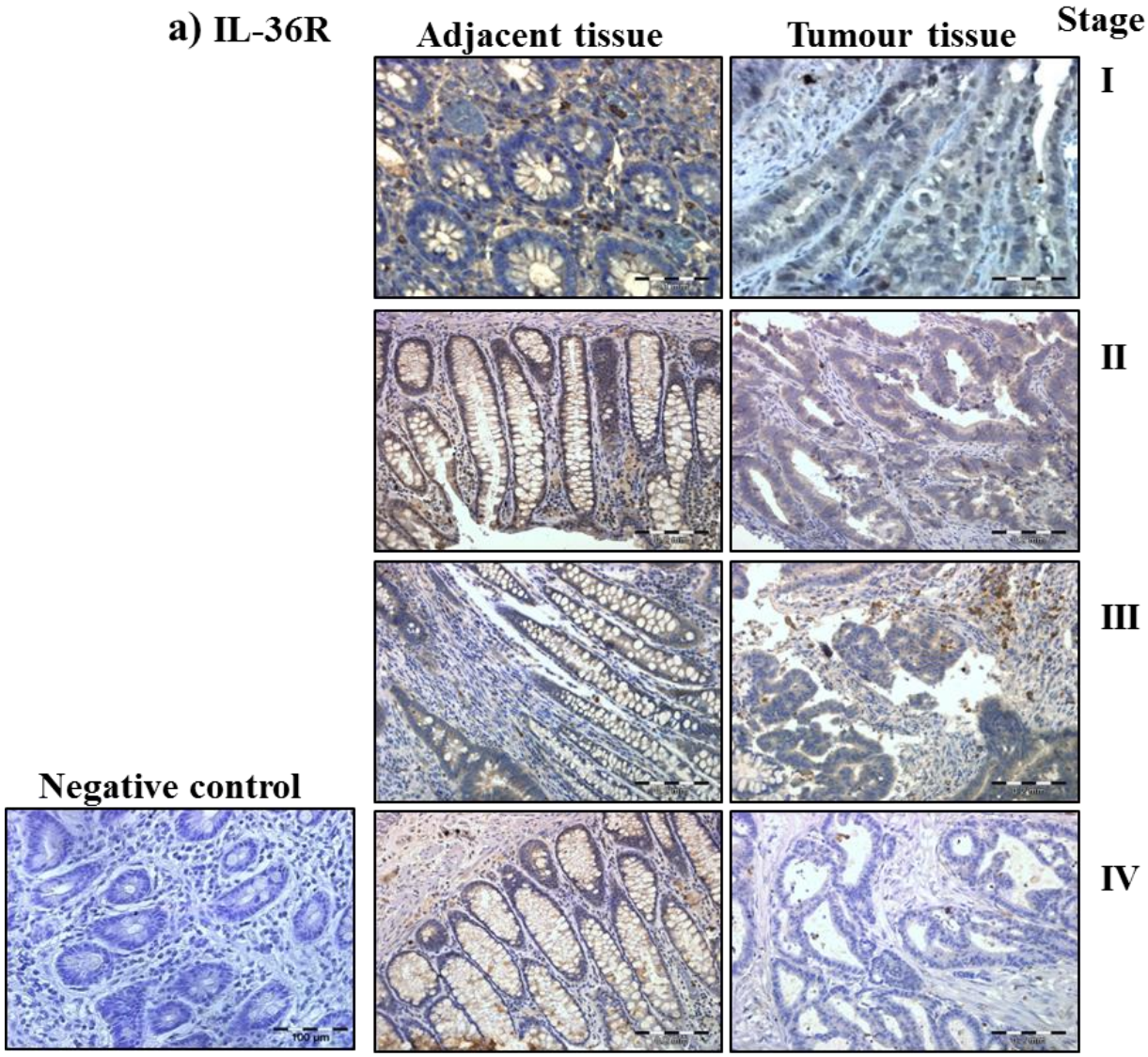


Figure 7: IL-36R expression was unchanged in human CRC tissue compared to adjacent tissue. (a) IL-36R mRNA expression was unchanged in human CRC tumours compared to adjacent non-tumour tissue. Total RNA was extracted and changes in expression were detected by qRT-PCR. Statistical analysis was carried out using a student t test. p=0.0474. (b) IL-36R protein expression was unchanged in CRC compared to adjacent tissue. Immunohistochemical staining for IL-36R, in human CRC was performed on paraffin-embedded tumour sections. Primary antibody was omitted as a negative control. Scale bar = 100µm. Images shown are representative of the findings obtained.



b)

CRC stage	Total	% Negative /Weak	% Moderate /strong
Stage I	10	50%	50%
Stage II	13	23%	77%
Stage III	11	45%	55%
Stage IV	12	50%	50%

Figure 8: IL-36R expression was unchanged in tumour tissue compared to adjacent tissue. IL-36R expression did not correlate with changes in tumour grade (a) Paraffin-embedded human CRC tumours, stage I to IV were immuno-stained with IL-36γ. No correlation between CRC grade and IL-36R expression was observed. (b) Tumour sections were scored as negative, weak, moderate or strong, according to the intensity of IL-36R staining.

findings obtained with IL-36α and IL-36γ, expression of the IL-36R did not alter with tumour stage when assessed in 46 patients (Figure 8). Collectively, these findings suggest that although the IL-36R is not increased in tumour tissue, two of its agonistic ligands (IL-36α and IL-36γ) and the IL-36RN are significantly upregulated in tumour tissue, indicating that the IL-36R pathway may be relevant in colon cancer.

5.2.2 Characterisation of IL-36R$^+$ cells in colonic tumours.

Unlike IL-36α and IL-36γ, which showed localization to the tumour cells in the tumour tissue, the IL-36R was also strongly expressed in other cells in the tumour microenvironment. To identify and characterise these cells that strongly expressed the IL-36R, consecutive tissue sections were stained initially for CD45 (a protein marker common to all immune cell subtypes) to determine whether these cells were of immune origin. Positivity for both CD45 and IL-36R expression was observed in overlapping areas of tissue (Figure 9a). This confirmed that the IL-36R positive cells in the tumour microenvironment were immune derived. Macrophages have been shown to express the IL-36R [312]. I next examined expression of CD68, a human macrophage marker (Figure 9b). Indeed, some overlap in staining patterns was observed, indicating that some of the IL-36R positive cells in the tumour were macrophages. However, some cells that were IL-36R positive did not stain positive for CD68 expression, indicating that these IL-36R positive cells were not just macrophages.

I next investigated expression of CD4 and found a significant overlap between IL-36R positive immune cells and CD4$^+$ cells (Figure 9c). Although I had initially suspected macrophages to be the enlarged IL-36R positive cells present in the

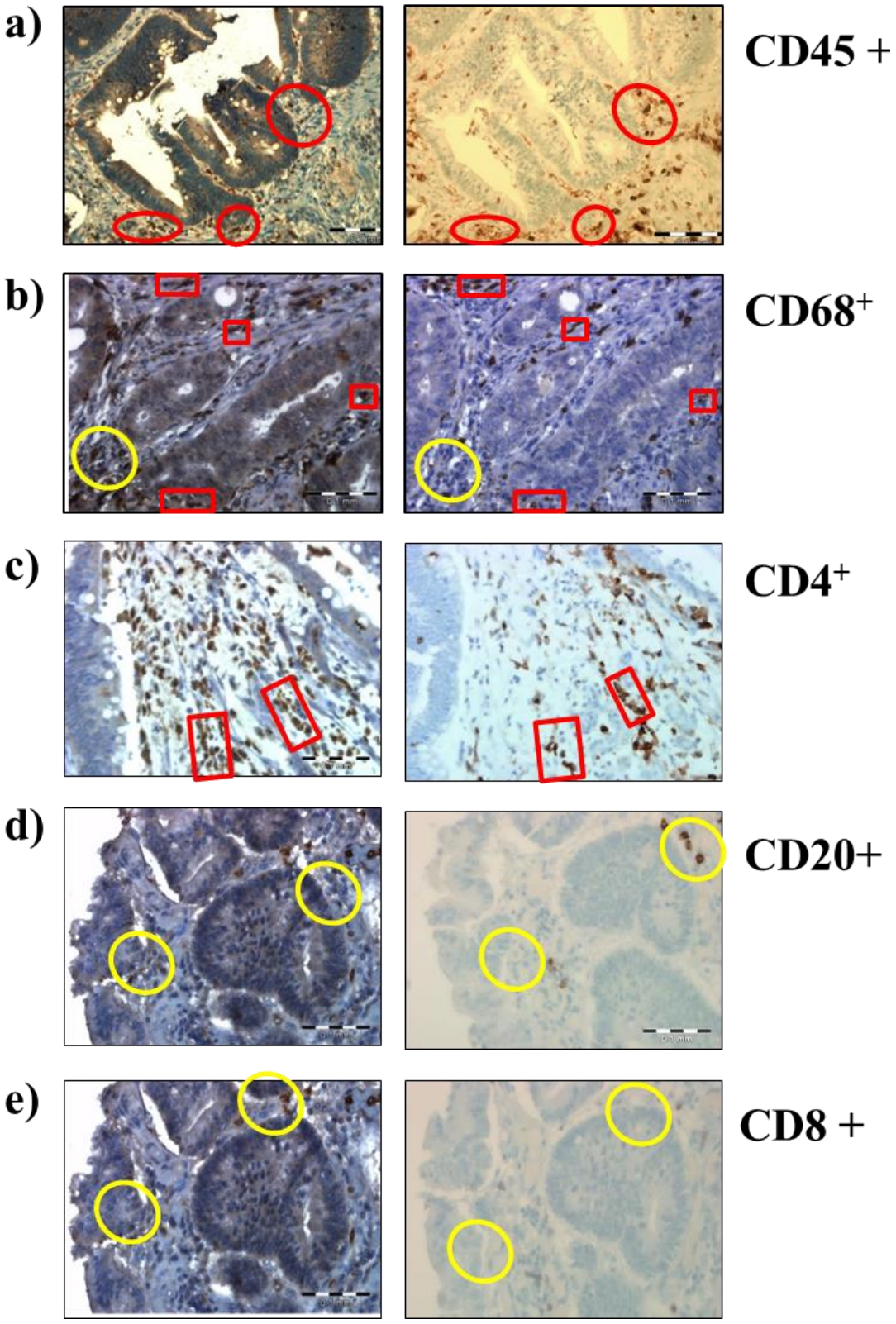


Figure 9: IL-36R+ cells also stain positive forCD45, CD68 and CD4 expression in human CRC tissue. Consecutive CRC tissue sections were stained for the IL-36R and (a) CD45+, (b) CD68, (c) CD4, (d) CD20, (e) CD8 expression. Immunohistochemical staining was performed on paraffin-embedded human tumour sections. Areas of common staining are highlighted in red, areas of no co-expression are labelled in yellow. Scale bar = 100µm. Images shown are representative of the findings obtained.

tumour microenvironment, T cells stimulated with IL-36γ have been shown to enlarge upon activation, which could explain the larger size of these immune cells [244]. To determine if the IL-36R positive cells were B lymphocytes, I next examined expression of CD20. Fewer CD20 positive cells were present in the tumours than either macrophages or $CD4^+$ T cells, and those that were CD20 positive did not overlap with those cells that were IL-36R positive, indicating that B cells do not express IL-36R, at least in intestinal tissue (Figure 9d). As IL-36γ has been shown to activate $CD8^+$ cells in a melanoma model, I next examined expression of CD8 and found very few $CD8^+$ cells present in these tumours (Figure 9e). These data suggest that the strongly positive IL-36R cells in the lamina propria comprise both $CD68^+$ macrophages and $CD4^+$ T cells, but not B cell or $CD8^+$ T cells. Dendritic cells have also been shown to express the IL-36R and may also be positive for IL-36R expression. However markers for dendritic cells were not examined.

5.2.3 Colon cancer cells express the IL-36R *in vitro*.

As IL-36α and IL-36γ were found to be upregulated in CRC tumour tissue, I next wished to determine their role in tumorigenesis. Before further investigations were performed, I first wished to investigate if colon cancer cell lines expressed IL-36 family members *in vitro*. HT29, SW480 and SW620 cells were all found to express the IL-36R (Figure 10a), albeit at different levels, while differential expression of IL-36 cytokines was observed (Figure 10b-d). HT29 cells expressed all three agonistic cytokines. SW480 cells expressed IL-36β and IL-36γ, while none of the three agonistic IL-36 cytokines were detected in SW620 cells. Caco2 and HCT116 colon cancer cells were also examined for IL-36R mRNA expression. However, IL-36R

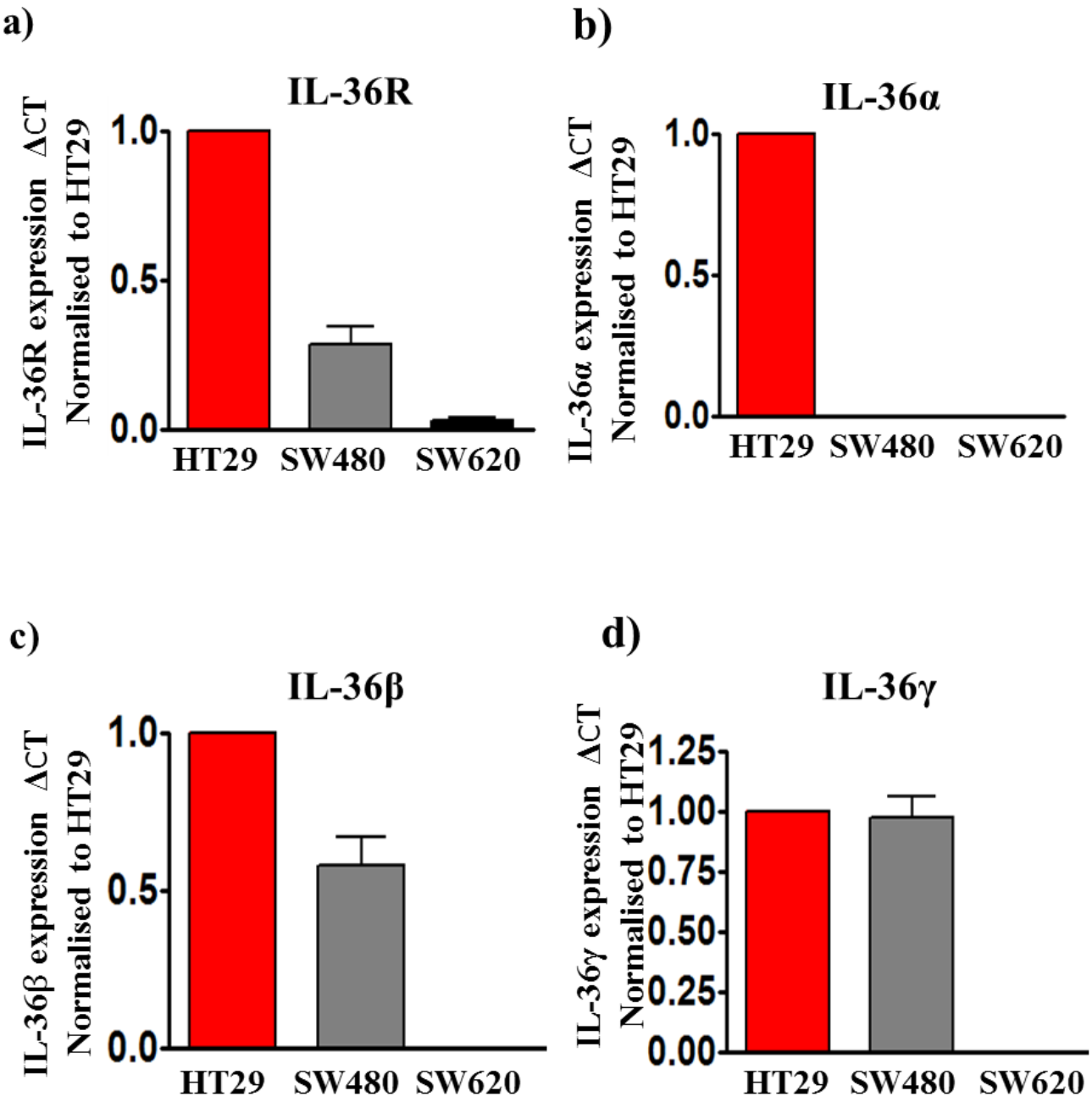


Figure 10: HT29, SW480 and SW620 express IL-36R mRNA, IL-36α mRNA is expressed by HT29 cells , IL-36β and IL-36γ mRNA is expressed by HT29 and SW480 cells. (a) HT29, SW480 and SW620 cells all express IL-36R. (b) IL-36α mRNA was only detected in HT29 cells. (c) IL-36β and (d) IL-36γ were detected in HT29 and SW480 cells. Total RNA was extracted and following cDNA synthesis, expression levels of IL-36 family members was determined by qRT-PCR. For comparison ΔCT values were normalised to expression in HT29 cells. Data shown are mean +/- SEM (n=3).

mRNA was not detected in these cell lines and they were excluded from further investigations.

5.2.4 IL-36α, IL-36β and IL-36γ cytokines induce cellular proliferation in SW480 cells, whereas in HT29 cells only IL-36γ increases cellular proliferation.

Once I had determined which colon cancer cell lines expressed the IL-36R, I next assessed the ability of IL-36 cytokines to induce cellular proliferation in colon cancer cell lines *in vitro*, using a BrdU incorporation assay. Stimulation with IL-36γ increased proliferation of HT29 cells above that seen in untreated cells, while stimulation with IL-36α and IL-36β had no effect (Figure 11a). In SW480 cell lines all three agonistic IL-36 cytokines strongly increased cellular proliferation in a dose-dependent manner, with a ~50 fold increase in BrdU incorporation observed for each IL-36 cytokine (Figure 11b). SW620 cells are derived from the same patient as SW480 cells. SW480 cells are derived from the primary tumour, while SW620 cells are their lymph node metastatic derivatives. Unlike SW480 cells, stimulation with IL-36 cytokines did not induce an increase in cellular proliferation in the more metastatic SW620 cells (Figure 11c).

5.2.5 IL-36 cytokines did not induce apoptosis in HT29 cells.

As I had observed a reduction in proliferation in response to IL-36α and IL-36β in HT29 cells and a reduction in proliferation in SW620 by all three IL-36 cytokines, I wished to determine if stimulation with IL-36 cytokines induced apoptosis of cells which could account for the reduction in proliferation seen. Therefore, I next examined the ability of IL-36 cytokines to induce apoptosis. To this end, I investigated the ability of IL-36 cytokines to activate cysteine aspartic acid-specific protease (caspase) 3 and caspase 7 activity in HT29 cells. Caspases 3 and 7 play key

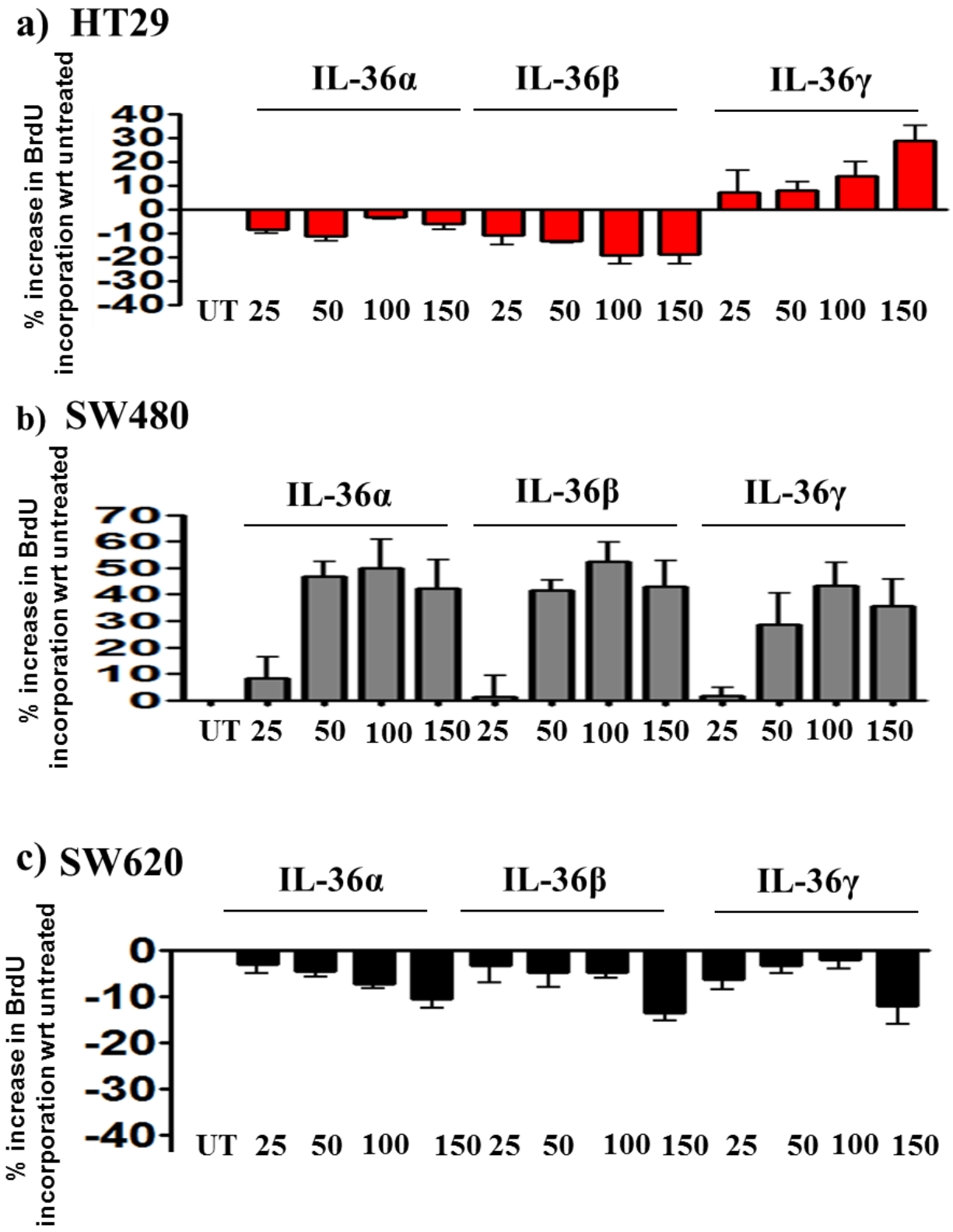


Figure 11: IL-36γ increased proliferation of HT29 cells. IL-36α, IL-36β and IL-36γ increased proliferation of SW480 cells *in vitro*. In SW480 cells IL-36α, IL-36β and IL-36γ did not increase proliferation. (a) Cellular proliferation was assessed by BrdU incorporation (a) HT29, (b) SW480 and (c) SW620 cells were stimulated with 25 to 150 ng/ml of IL-36α, IL-36β or IL-36γ, 24h later the cells were labelled with BrdU. Following 16 hrs of incubation, the media was removed and the cells were fixed and denatured, and absorbance was measured. Data shown are mean +/- SEM (n=3).

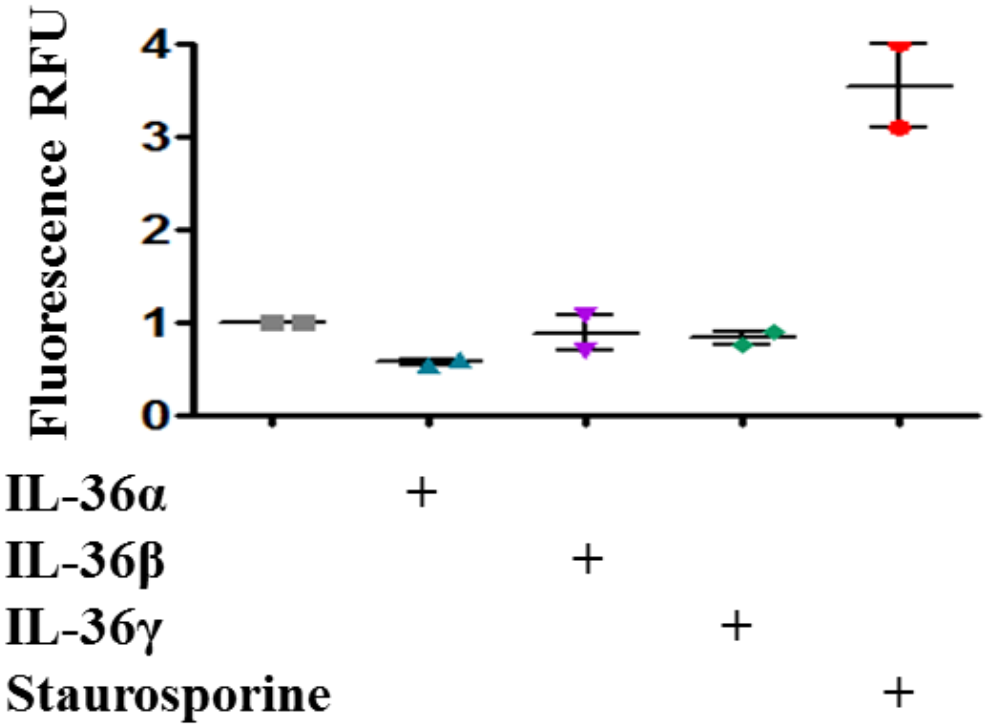


Figure 12: Stimulation of HT29 cells with IL-36α, IL-36β and IL-36γ did not increase caspase 3 and 7 activity compared to untreated cells. (a) HT29 cells were stimulated with IL-36 ligands at 150ng/ml for 4.5 hours at 37^{O}C. Staurosporine (1μM) was used as a positive control for apoptosis. Caspase activity was measured fluorescently and readings were taken at 1hr, 2hr and 3hr. IL-36 cytokines did not increase apoptosis of HT29 cells. This data was obtained from two independent experiments. Data shown are mean +/- SEM (n=2).

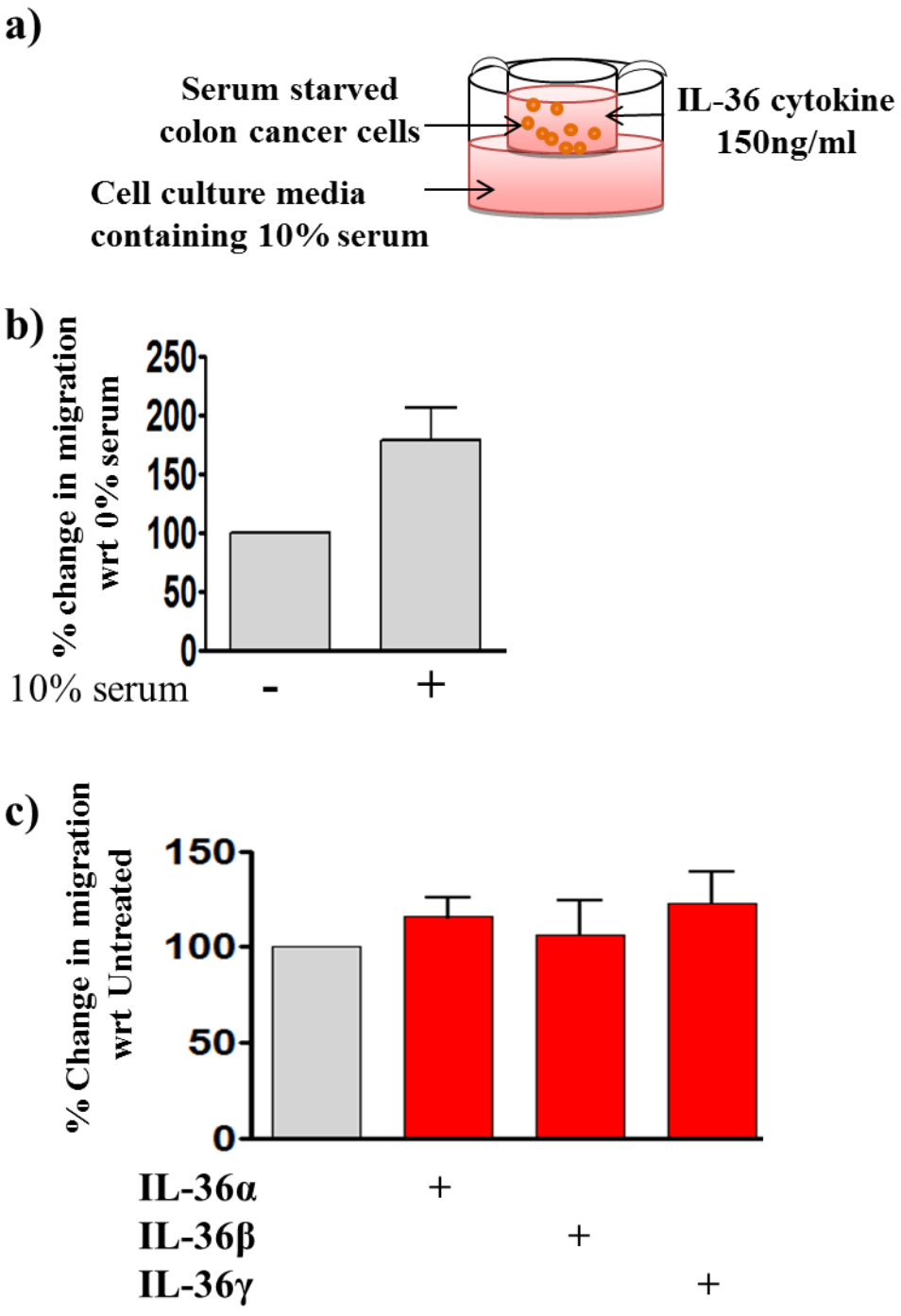


Figure 13: Stimulation of cells with IL-36α, IL-36β and IL-36γ did not significantly increase migration of colon cancer cells towards 10% serum.
Utilising a modified Boyden chamber assay, serum starved HT29 cells were seeded in the upper chamber and stimulated with IL-36α, IL-36β and IL-36γ at 150ng/ml for 16 hours. Cells migrate towards the 8 μm membrane, attracted by the 10% serum enriched media. (b) Migration of cells towards 10% serum was utilised as a positive control. Data shown are mean +/- SEM (n=2).

effector roles in apoptosis in mammalian cell lines [313]. No increase in caspase 3/7 activity was observed in response to IL-36 stimulation. Staurosporine was utilised as a positive control (Figure 12). This suggests that IL-36 does not influence colon cancer cell line growth through the induction of apoptosis.

5.2.6 IL-36 cytokines did not induce migration of HT29 colon cancer cells.

To investigate whether IL-36 cytokines have the ability to promote cell migration, a key step in tumour metastasis, I next performed a migration assay to determine whether IL-36 cytokines have the ability to promote the migration of colon cancer cells *in vitro*. HT29 colon cancer cells were stimulated with IL-36 cytokines as indicated and changes in migration were assessed using a modified Boyden chamber assay (Figure 13a). Migration towards 10% serum was examined as a positive control (Figure 13b). IL-36 cytokines did not enhance the migration of colon cancer cells (Figure 13c). Therefore, it appears IL-36 cytokines do not directly induce migration of colon cancer cells.

5.2.7 Chemokines CXCL-1, CCL2 and CCL20 were produced in response to IL-36α and IL-36γ stimulation in HT29 human colon cancer cell lines.

As the immune cells in the tumour microenvironment can be potent drivers of either pro-tumorigenic or anti-tumorigenic immune responses, which can either promote or inhibit tumour growth, I next wished to investigate whether IL-36 cytokines could alter the tumour microenvironment through the recruitment of immune cells. Therefore I investigated the ability of IL-36 cytokines to induce chemokine and cytokine production by colon cancer cells. As SW480, SW620 and HT29 cells been shown to express the IL-36R (Figure 10a), they were stimulated with IL-36α, IL-36β and IL-36γ at increasing doses and changes in transcription of cytokines and

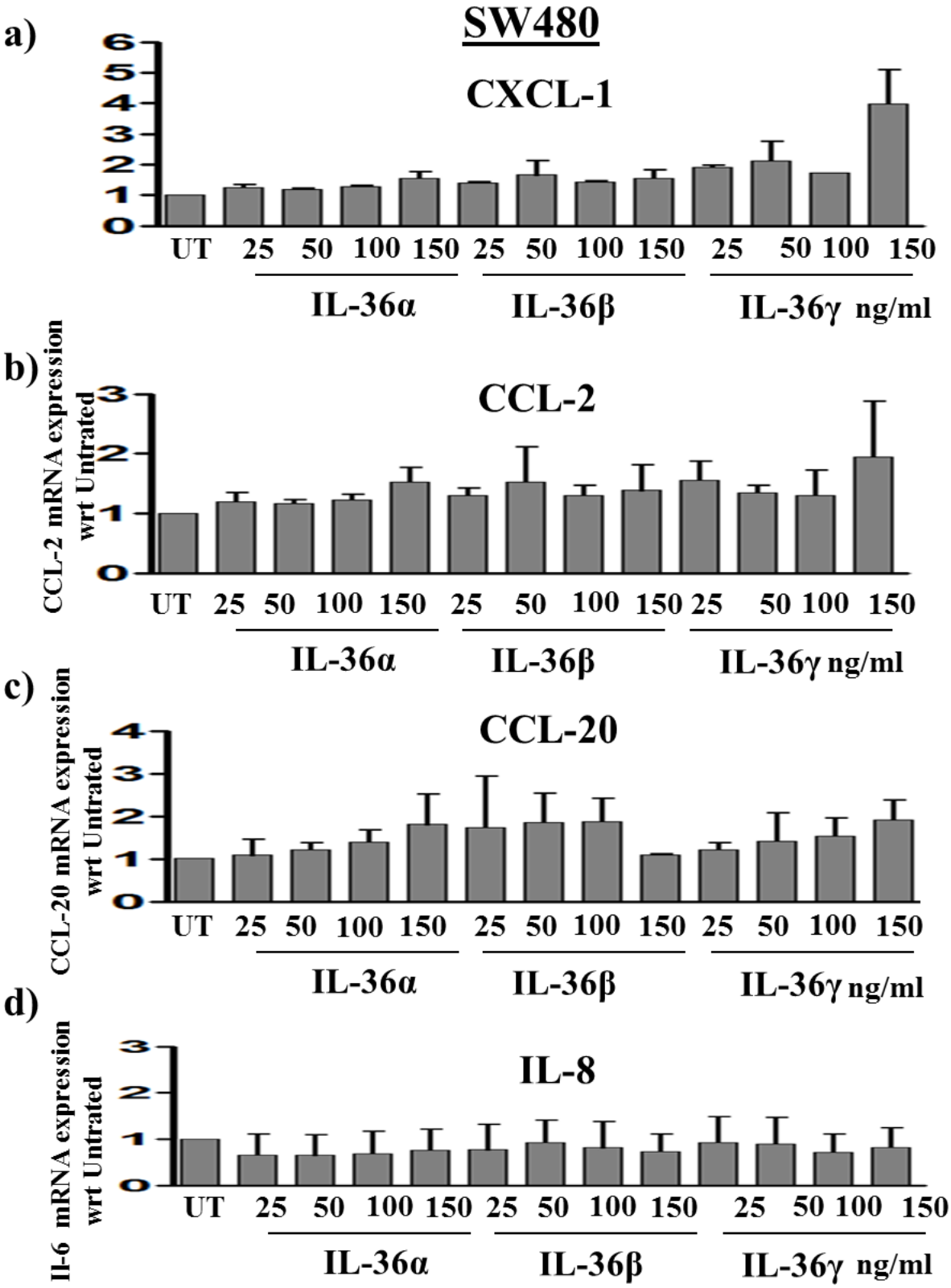


Figure 14: Neither IL-36α, IL-36β or IL-36γ increased expression CXCL-1, CCL-2, CCL-20 or IL-8 in SW480 cells. SW480 cells were stimulated with increasing doses of IL-36α, IL-36β and IL-36γ cytokines for 4h. Total mRNA was then extracted and following cDNA synthesis expression of the cytokines (a) CXCL-1, (b) CCL-2, (c) CCL20 and (d) IL-8 were examined. IL-36 cytokines did not induce CXCL-1, CCL2, CCL20 or IL-8 activation in SW480 cells. Data shown are mean +/- SEM (n=2).

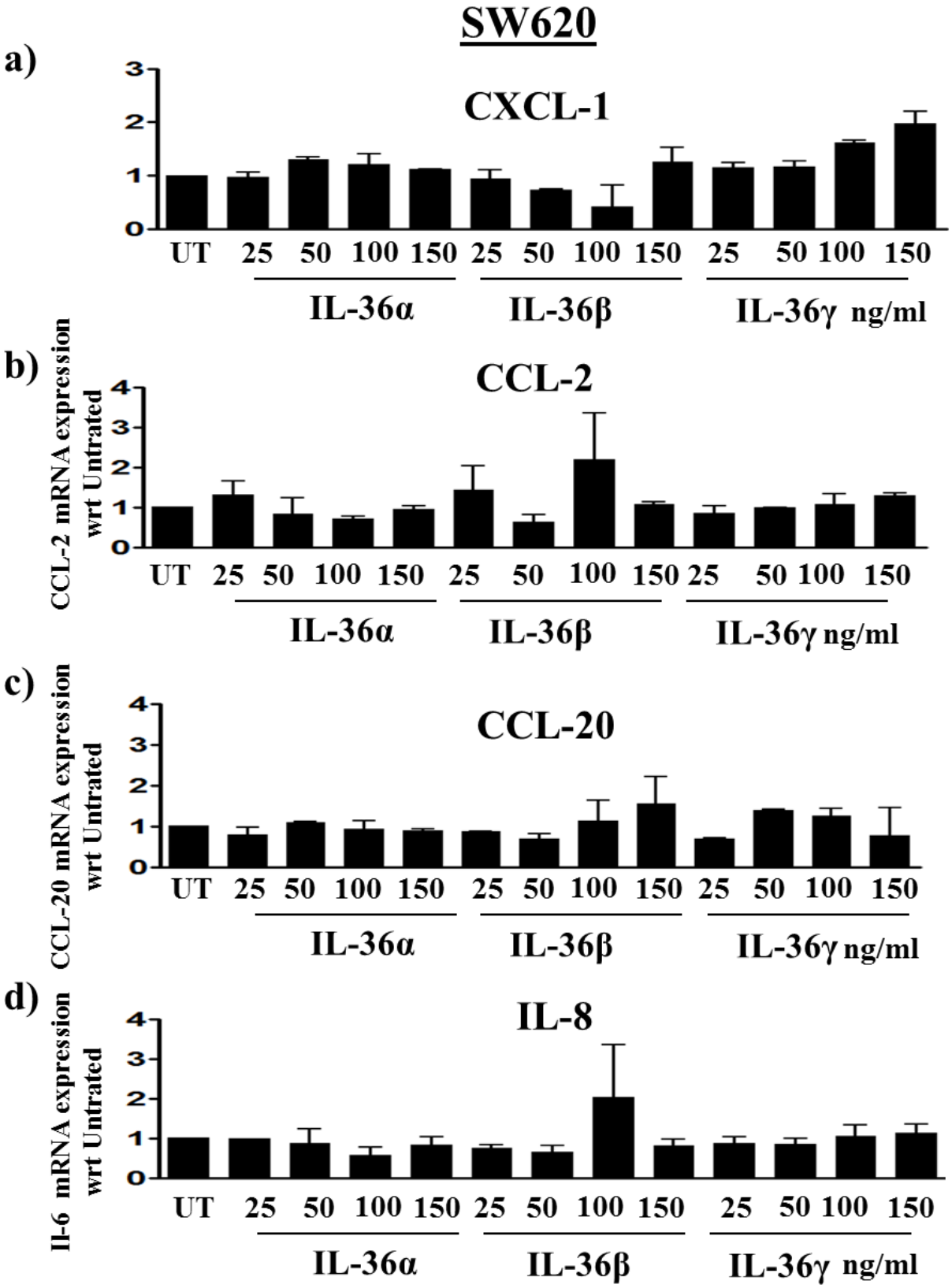


Figure 15: Neither IL-36α, IL-36β or IL-36γ increased production of CXCL-1, CCL2, CCL-20 or IL-8 in SW620 cells. SW620 cells were stimulated with increasing doses of IL-36α, IL-36β and IL-36γ cytokines for 4h. Total mRNA was then extracted and following cDNA synthesis expression of the cytokines (a) CXCL-1, (b) CCL-2, (c) CCL20 and (d) IL-8 were examined. IL-36 cytokines did not induce chemokines or IL-8 activation in SW620 cells. Data shown are mean +/- SEM (n=2).

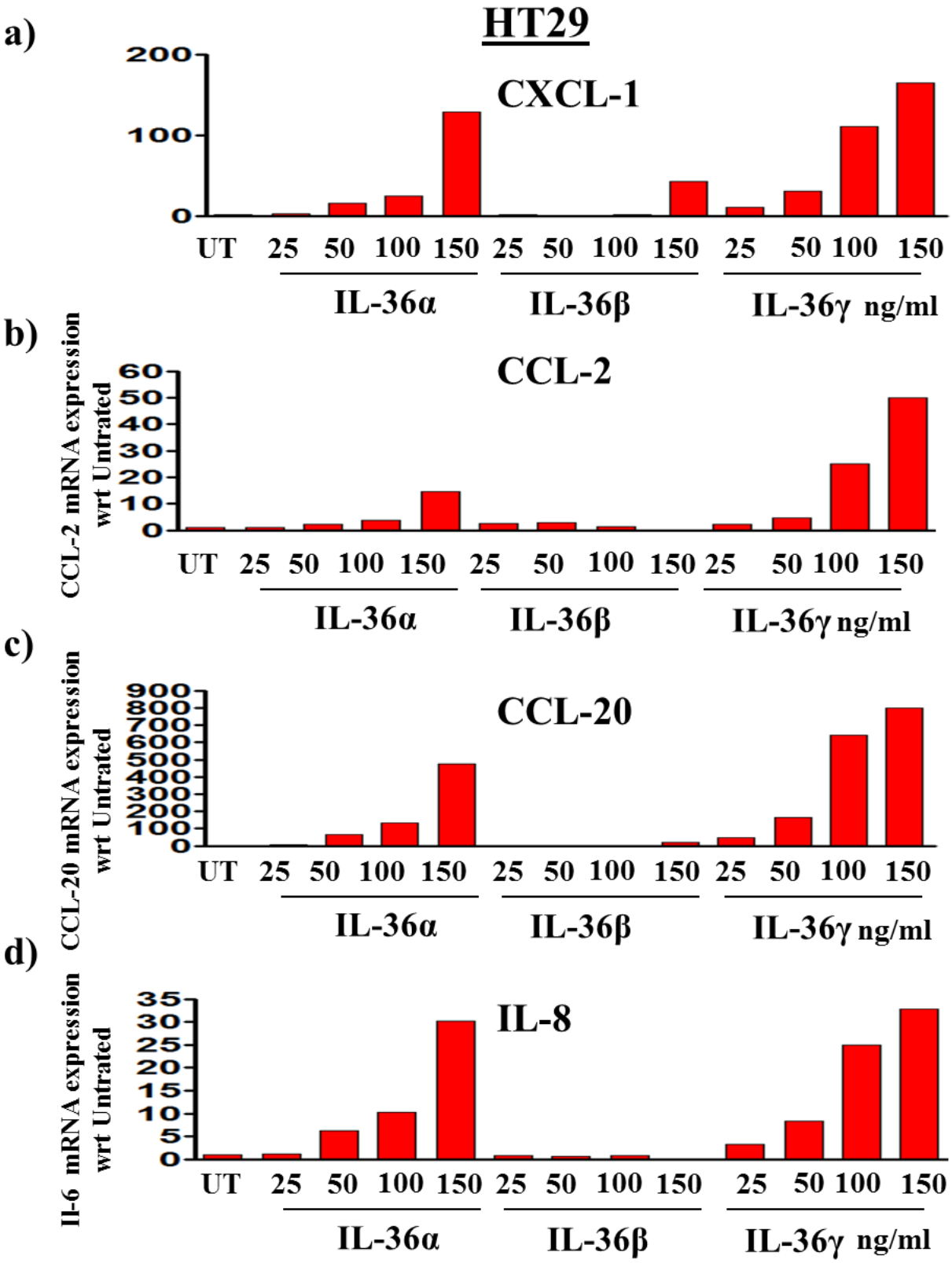


Figure 16: IL-36α and IL-36γ increased expression of CXCL-1, CCL2, CCL20 and IL-8 in HT29 cells in a dose dependent manner. HT29 cells were stimulated with increasing doses of IL-36α, IL-36β and IL-36γ cytokines for 4h. Total mRNA was then extracted and following cDNA synthesis expression of the cytokines (a) CXCL-1, (b) CCL-2, (c) CCL20 and (d) IL-8 were examined. Data shown are mean +/- SEM (n=2).

chemokines were examined by qRT-PCR. IL-36 cytokines failed to induce expression of CXCL1, CCL2, CCL20 or IL-8 mRNA in SW480 and SW620 cells (Figure 14a-d and 15a-d). In contrast, IL-36α and IL-36γ, but not IL-36β induced CXCL-1 (Figure 16a), CCL2 (Figure 16b), CCL20 (Figure 16c) and IL-8 (Figure 16d) in HT29 cells. This is similar to findings recently published by Nishida *et al.* in which they found increased secretion of CXCL-1, CXCL-2, CXCL-3, CXCL-6 and CXCL-8 by HT29 cells in response to IL-36α and IL-36γ stimulation, but not by IL-36β stimulation [314]. It is worth noting that although all three colon cancer cell lines expressed the IL-36R, SW480 and SW620 cells expressed the receptor at much lower levels compared to HT29. Indeed, SW480 cells expressed ~3-fold lower IL-36R expression compared to HT29 cells, while SW620 cells expressed ~10-fold less IL-36R mRNA expression compared to HT29 (Figure 10a).

Taken together, this data suggests that IL-36α and IL-36γ expression may be upregulated in colonic tumours. These ligands in turn may induce chemokine production by the tumour cells, thereby recruiting immune cells. This pro-inflammatory response may alter the immune populations in the tumour microenvironment, either promoting or inhibiting tumour growth.

5.3 Discussion

The current work was initiated after the recent surge in studies describing the role of IL-36 cytokines in IBD. Moreover, a study by Wang *et al.* has recently shown that IL-36γ expression by tumour cells reduces both melanoma and breast tumour growth [244]. As no studies to date have investigated the role of IL-36 in CRC, I first analysed human CRC tumour samples for expression of IL-36 cytokines. I found both mRNA and protein for IL-36α and IL-36γ were increased in CRC compared to adjacent normal tissue. In contrast, IL-36R expression was unchanged at both the protein and the mRNA level. IL-36R expression was shown to be strongly positive on infiltrating $CD4^+$ and $CD68^+$ immune cells. To investigate whether IL-36 cytokines influenced the growth of colon cancer cells, we examined *in vitro* cellular proliferation and found all three IL-36 cytokines increased proliferation of SW480 cells. In addition, IL-36γ also strongly induced proliferation of HT29 colon cancer cells. Finally, we demonstrated that IL-36α and IL-36γ induced strong expression of CXCL-1, CCL2, CCL20 and IL-8 in HT29 cells in a dose dependent manner. Taken together, these data show that certain IL-36 cytokines are increased in colon cancer and that tumour cells may respond to IL-36 ligand stimulation in terms of an increase in proliferation and an induction of chemokines.

5.3.1 IL-36 cytokines are increased in colon cancer:

IL-36 cytokines and IL-36R are expressed by normal epithelial cell types such as keratinocytes and bronchial epithelial cells [231, 237, 315], however, they have not previously been investigated in colon cancer. Here we have demonstrated that IL-36α and IL-36γ mRNA expression were increased in tumour tissue compared to adjacent non-tumour tissue. Indeed, protein expression of these cytokines was

similarly increased in CRC tissue by IHC. Other studies investigating the role of IL-36α in cancer demonstrated that IL-36α is expressed in hepatocellular carcinoma (HCC), but also in normal hepatocytes (n=163). Poorly differentiated tumours showed lower IL-36α expression compared to well-differentiated tumours. Furthermore, IL-36α expression was shown to significantly correlate with tumour size and tumour stage. These authors suggest that decreased IL-36α expression may contribute to tumour progression [242]. However, we also examined IL-36α expression in relation to tumour stage, and no correlation was observed. The variations observed may be due to different tumour types, or the low numbers in our cohort.

I also observed an increase in IL-36β mRNA expression in colon tumours compared to normal adjacent tissue. However, when I attempted to confirm the increase in IL-36β by IHC, I failed to detect IL-36β expression in the majority of CRC tumours. There were some exceptions as 2/46 tumour samples which showed IL-36β expression in the epithelium and even stronger IL-36β expression was observed in surrounding stromal cells. These data confirmed that our antibody was working correctly and could detect IL-36β. Other studies have also reported that they were unable to detect IL-36β protein expression in colon tissue from IBD patients [224], although these studies were also unable to detect IL-36β mRNA [240]. Therefore, this data suggests that although IL-36β mRNA is expressed in CRC tumour tissue, it is not translated into protein. It is possible that IL-36β mRNA does get translated under inflammatory conditions, as a study by Boutet *et al.* detected IL-36β protein expression in patients with Crohn's disease (CD) [214]. Similarly, elevated levels of IL-36β have been detected in mice treated with DSS [224]. However, our data

suggest that the low level of inflammation in CRC is not sufficient to induce translocation of IL-36β mRNA.

We also showed that IL-36γ was expressed by the colonic tumour epithelium and was increased in tumour tissue compared to adjacent non-tumour tissue. Consistent with our findings, data available in online databases reported IL-36γ expression in the following cancer types; lung cancer, head and neck cancer, oesophageal cancer and CRC (Oncomine database) [244]. We also investigated if IL-36γ expression correlated with tumour progression and no association was observed (n=46). However, a recent study reported that IL-36γ expression was reduced in later stage lung cancer, compared to earlier stages of the disease [244]. Although our study was made up of primary tumours, expression of human IL-36γ available online from the NCBI GEO database was analysed by Wang *et al*. They reported that IL-36γ expression was lower in metastatic melanoma compared to primary tumours [244]. We may need to increase our study numbers and in particular examine IL-36γ expression in metastatic samples. However, this research did not compare IL-36γ expression levels in healthy tissue compared to tumour tissue as we did. Taken together, the two studies to date examining IL-36α and IL-36γ in cancer suggest that IL-36 cytokine expression is lost with tumour progression. This may suggest that IL-36α and IL-36γ expression is important in the early stages tumorigenesis. Further studies are required to identify whether IL-36 cytokine expression is also altered in other tumour types.

5.3.2 IL-36R expression on tumour infiltrating immune cells:

Unlike IL-36α and IL-36γ which were predominantly localised to the tumour epithelium, the IL-36R was expressed by both tumours cells and immune cells in the

tumour microenvironment. This staining pattern was also observed by Russell *et al*. in IBD where they describe the IL-36R$^+$ cells as lamina propria mononuclear cells (LPMCs) [240]. I wished to characterise these IL-36R$^+$ cells, and demonstrated that these (CD45$^+$) immune cells also expressed CD4 or CD68 markers, but not CD20 or CD8 protein markers. In support of our findings, Vigne *et al.* showed that the IL-36R was constitutively expressed in CD4$^+$ T cells, and macrophages, but not CD8$^+$ T cells or B cells [211]. Although we did not examine DCs they were also found to be positive for IL-36R expression in this study [211].

Current research has shown that murine CD4$^+$ cells constitutively express the IL-36R, and IL-36 cytokines have been shown to regulate CD4$^+$ T helper responses in mice [208, 209]. Macrophages were also found to express the IL-36R [211]. This is consistent with current research which demonstrates that macrophages express the IL-36R [211]. IL-36 signalling can influence TAMs in different ways depending on their phenotype, IL-36R mRNA was higher in M2 than M1, indicating that M2 polarized TAMs may be more responsive to IL-36 stimulation, with M2 macrophages shown to produce IL-6, IL-8 and TNF-α in response to IL-36 stimulation [312]. Our data suggests a subset of the IL-36R positive cells identified in our colon cancer sections are macrophages and CD4$^+$ T cells. Ideally to further investigate the phenotype of these IL-36R$^+$ immune cells I would obtain fresh human CRC biopsies to identify the immune cells by flow cytometry. However, as we were unable to access fresh human CRC biopsies our only alternative was formalin-fixed tissue. Without further analysis it is difficult to determine the exact phenotype of these cells. Taken together, this data suggests that CD4$^+$ T cells and macrophages in the colon tumour microenvironment express the IL-36R.

5.3.3 IL-36 induces proliferation of colon cancer cells:

We showed that IL-36γ induced proliferation of HT29 and SW480 cells *in vitro*. Consistent with this finding IL-36γ has been shown to induce proliferation in other cell types including keratinocytes and immune cells such as Th1 cells [209, 316]. Of the cell lines examined, SW480 cells were the most responsive to IL-36 cytokines as they showed an increase in proliferation in response to all three IL-36 cytokines. SW620 cells were the least responsive as cellular proliferation was not increased in response to any of the three IL-36 cytokines. SW480 and SW620 cells are derived from the same patient; SW480 cells were cultured from the primary tumour, while SW620 cells were derived from a lymph node metastasis. Metastatic cells can become less reliant on outside influences to induce proliferation as they can make many of their own growth factors [3]. It is also possible that genetic mutations and other inherent changes from primary to metastatic tumour may be responsible for the inability of IL-36 cytokines to induce proliferation in the SW620s. Moreover, SW620 cells showed ~5-fold lower IL-36R expression compared to SW480 cells. This may also make them less responsive to stimulation with IL-36 cytokines.

The effect of IL-36 cytokines on tumour proliferation *in vivo* was investigated in two recent studies [242, 244]. Tumours derived from hepatocellular carcinoma cells overexpressing IL-36α were shown to grow more slowly compared to controls *in vivo* [242]. Increased IL-36γ expression by both melanoma and breast cancer cells also reduced tumour growth *in vivo* [244]. The proposed mechanism responsible for this anti-tumorigenic effect was an increased inflammatory response. Increased infiltration of NK cells, γδ T cells, $CD8^+$ T cells and T-reg cells were observed, while the number of immunosuppressive MDSCs was reduced. In these studies, proliferation of IL-36γ expressing cells was measured *in vitro* and no alteration in

proliferation was observed compared to cells containing the vector alone. This is in contrast to our findings in which IL-36γ increased proliferation in two of the three colon cancer cell lines examined. Variations in results may be due to the different cancer types utilised. Similar to the B16-F10 melanoma cells, SW620 cells were not altered in response to stimulation with IL-36 cytokines. Both of these cell lines are highly metastatic and this may contribute to the lack of response observed. Our data combined with data from Wang *et al.* suggests that *in vitro* IL-36 cytokines do not directly suppress cellular proliferation [244]. However, *in vivo* IL-36 signalling may suppress tumour cell growth indirectly through the recruitment of anti-tumorigenic immune cells.

5.3.4 IL-36 induces chemokine and cytokine induction:

Studies have shown that IL-36R signalling promotes an inflammatory response through the recruitment of immune cells [244]. We showed that IL-36α and IL-36γ induced the production of cytokines and chemokines in HT29 colon cancer cells, with CXCL-1, CCL2, CCL20 and IL-8 being strongly induced in a dose dependent manner. Our findings were confirmed in a recent study by Nishida *et al.* who showed that HT29 secreted increased levels of CXCL-1, CXCL-2, CXCL-3, CXCL-6 and CXCL-8 in response to IL-36α and IL-36γ stimulation, but not IL-36β stimulation [314]. Stimulation of colonic subepithelial myofibroblasts (SEMFs) with IL-36α and IL-36γ also resulted in significantly increased CXCL-1 and CXCL-2 production [314]. These data suggest that it is not just tumour cells that respond to IL-36 cytokines, surrounding tissue can also respond. Intestinal epithelial cells and colonic explants stimulated with IL-36γ also induced expression of CXCL-1 and CXCL-2 neutrophil-recruiting chemokines [241]. This induction of neutrophil chemotactic

factors correlates with IL-36R$^{-/-}$ mice showing reduced neutrophil recruitment in DSS induced wounds [241]. This is also consistent with an overall reduction in immune cells observed in IL-36R$^{-/-}$ mice compared to WT mice [240].

In the tumour microenvironment increased chemokine production can induce the recruitment of immune cells, such as neutrophils by CXCL1, macrophages by CCL2, and lymphocytes by CCL20. As IL-36α and IL-36γ expression is increased in the tumour epithelium, this may signal through an autocrine loop, resulting in increased chemokine production by the tumour cells and increased immune cell recruitment to the tumour. However, these infiltrating immune cells, such as macrophages, neutrophils and lymphocytes can have both pro-tumorigenic and anti-tumorigenic activity as determined by the tumour microenvironment. Thus, the increase in immune cell infiltration caused by an increase in chemokines in response to IL-36 signalling could potentially be beneficial to the host and suppress tumour growth. Alternatively, in an immunosuppressive environment the anti-tumour activity of immune cells may be inhibited. This is consistent with loss of IL-36 cytokine expression as the tumour progresses [242, 244], thereby inhibiting recruitment of immune cells to the tumour microenvironment, contributing to an immunosuppressive phenotype.

An *in vivo* experimental model is essential to further advance our knowledge in this field, as this could provide an insight into the role of the IL-36R in colon cancer to clearly define whether it has pro- or anti-tumorigenic effects in CRC. It would also determine whether IL-36 represents a novel target for therapeutic intervention. To investigate the role of IL-36 in the immune cells an AOM/DSS model in IL-36R$^{-/-}$ murine model could be utilised to examine tumour growth in the absence of the IL-36R. To investigate the role of tumour-expressed IL-36R in tumorigenesis, colon

cancer cells in which expression of the IL-36R has been silenced could be injected into WT mice and compared to growth of WT cells. These experiments could determine if tumour growth is affected by a reduction in systemic IL-36 signalling or if tumour mediated IL-36 expression influences tumorigenesis. Alternatively, an inducible model could be utilised to show the effects of inhibiting IL-36 signalling once the tumour has developed. This model would be more representative of a therapeutic intervention. Finally, to characterise the role of the individual IL-36 cytokines in tumorigenesis, IL-36α, IL-36β and IL-36γ could be silenced individually in tumour cells and the growth of these tumour cells investigated. IL-36$\alpha^{-/-}$, IL-36$\beta^{-/-}$ and IL-36$\gamma^{-/-}$ mice have all been generated and an AOM/DSS model in each of these mice could be utilised to identify the role of each of these cytokines on immune cell recruitment [317]. These experiments would help to definitively clarify the role of IL-36 in colon cancer.

6.0 Final discussion and Future Perspectives:

6.1 Alternative roles of IL-1 family members in disease

IL-1 family cytokines are key pro-inflammatory mediators involved in the activation of inflammation, which can eliminate infection and repair damaged tissue. However, if this inflammatory response becomes dysregulated, these cytokines can contribute to the pathogenesis of many diseases, such as IBD and rheumatoid arthritis [318-320]. Various studies, however, support protective roles for several IL-1R family members in disease. Data presented in this thesis suggest that the cytokine IL-33, whilst normally pro-inflammatory and an important initiator of several inflammatory diseases, may play a protective role in colon cancer. Indeed, similar to data presented here, a divergent protective role for the normally highly pro-inflammatory cytokine IL-18 has also been shown in the intestine. IL-18 was shown to be upregulated in the intestine of patients with active Crohn's disease and it was initially suggested that blocking IL-18 may be beneficial for intestinal inflammation [321]. However, IL-18 was later found to be protective in intestinal epithelial cells and involved in maintaining epithelial integrity and protecting against bacterial translocation [322, 323]. Consistent with this, mice deficient in IL-18 and caspase-1 subunits are at increased risk of developing colitis in a model of acute experimental colitis [322, 323]. A protective role for IL-18 in the intestine was further demonstrated by the administration of IL-18, which rescued colitis in inflammasome-deficient mice [324]. Another IL-1 family member that has been found to display anti-inflammatory activity is IL-37. This cytokine has been shown to reduce inflammation associated with colitis [325]. This may have therapeutic implications for both IBD and CRC patients. In addition to activating an inflammatory response, protective roles for both

IL-33 and IL-36 have also been reported in intestinal epithelial repair, mucosal wound healing and intestinal homeostasis. IL-33 in particular has been shown to play a dual role in the gut, as it can polarize cells towards a Th2 immune response while also inducing Th1-mediated inflammation [326]. These studies highlight the divergent roles that IL-1 family members play in health and disease, with members appearing to mediate alternative functions depending on the system examined, and as such may explain why ST2 signalling mediated anti-tumorigenic effects in our colon tumour model.

Another potential explanation as to why we saw an anti-tumorigenic role for ST2 in cancer may have been the fact that we examined the role of these proteins in colon cancer. Colon cancer differs to most other tumour types, as colon cancer develops in the presence of the intestinal microbiota. Indeed, the composition of the intestinal microbiome is known to be altered in colon cancer [327]. Whether these alterations pre-dispose to the development of colon cancer however is unclear. It was initially suspected that intestinal pathogens may play a potential role in colon carcinogenesis [328]. These findings were later confirmed in CRC-predisposed mice [329]. This is similar to other cancers, such as gastric cancer, which has been linked to exposure of the pathogen, *Helicobacter pylori* [330]. In CRC, dysbiosis of the microbiota may promote carcinogenesis through remodelling of the microbiome to become pro-inflammatory, thereby promoting epithelial transformation and driving carcinogenesis [331]. A second theory proposes that intestinal ‘driver bacteria’ cause DNA damage and tumorigenesis, which enables the proliferation of ‘passenger bacteria’ in the tumour microenvironment [332, 333]. However, to date, it is not fully understood whether the dysbiosis observed in CRC is as a result of tumorigenesis or the cause of the CRC [331].

Both IL-33 and IL-36 cytokines are expressed at barrier tissues and are important factors in regulating the anti-microbial defence in the gut. For instance, IL-33 has been shown to be protective against the pathogen *S. typhimurium* [334]. This suggests that IL-33 and possibly IL-36 may play potential roles in regulating the epithelial immune response to specific microbes, thereby impacting on the composition of the intestinal microbiota. This could potentially favour the growth of specific bacteria that may be either pro- or anti-tumorigenic [335]. The gut microbiota in turn, has been implicated in regulating IL-36γ and IL-33 expression. Germ-free DSS-treated mice failed to express IL-36γ compared to conventionally-housed DSS-treated mice [241]. Similarly, the intestinal microbiota is implicated in driving IL-33 expression, as IL-33 expression is reduced in germ-free mice [336].The role of the microbiota in CRC is becoming an area of intense research and in the future may present a novel therapeutic approach for patients with this disease. Therefore, the involvement of both IL-33 and IL-36 in the interaction between the microbiota and colon tumour cells merits further investigation to determine if their manipulation could be of therapeutic benefit to patients. It is possible, therefore that interactions between the IL-1 cytokine family and the microbiota may determine whether these proteins exert either a protective or an inflammatory response.

6.2 Development of IL-1 family members as new therapeutic strategies in cancer

The immune system is inextricably linked to the pathogenesis of CRC, as supported by the number of retrospective examinations carried out on the immune infiltrates of resected CRC tumours [337-339]. Cancer immunotherapy has recently shown great potential as a novel anti-cancer therapeutic approach. As IL-1 family cytokines are key pro-inflammatory mediators, several studies have investigated whether

manipulation of IL-1R family signalling is of therapeutic benefit in inflammatory diseases such as cancer. IL-1 has been associated with pro-tumorigenic effects when expressed by tumour cells or in the tumour microenvironment [340]. However, administration of recombinant IL-1α and IL-1β resulted in anti-tumorigenic effects in experimental models [341-343]. This anti-tumour response was observed when the recombinant IL-1 was administered during the initial stages of tumour development. Both IL-1α and IL-1β reduced metastasis and increased overall survival [344]. However, IL-1 treatment resulted in systemic toxic effects *in vivo* [344]. To overcome these side effects other therapeutic approaches have utilised encapsulated IL-1α, which forms microspheres which are slowly released. This resulted in reduced tumour growth and increased survival [345]. However, as it appears there is a short window in which IL-1 can be of therapeutic benefit, this may not be clinically relevant to patients presenting with tumours.

Anti-tumour vaccines developed utilising irradiating tumour cells require an adjuvant to enhance a spontaneous anti-tumour response. IL-1α and IL-1β have also been investigated as to their efficacy to perform this function and were found to function as systemic adjuvants when injected within 10 days of the irradiated tumour cells. When IL-1 was administered following tumour cell vaccination, 70-100% of mice became tumour free, while mice that had received the vaccination alone were 0-20% tumour free [346]. IL-18 has also been shown to enhance the activity of adjuvants such as IL-12 [347].

IL-33 and IL-36 may also prove to have therapeutic potential in the treatment of cancer. IL-33 has been shown to be strongly expressed in many tumour types such as breast [348], head and neck [349] and colon [350]. However, when further investigated, increased IL-33 expression was found to be localised to the tumour

stroma in cells such as cancer-associated fibroblasts. Indeed, the cancer cells showed lower IL-33 expression when compared to normal tissue [348-350]. However, IL-33 is an 'alarmin', and thus a reduction in its expression in tumour tissue may be in part responsible for immune tolerance towards these tumour types [351]. This reduction could contribute to lower immunogenicity of cancer cells, while stromal cell expression of IL-33 may mediate immune suppression and tumour progression through the recruitment of MDSC and T reg cells [352]. This suggests that high doses of IL-33 could potentially reverse the immune-suppressive tumour microenvironment. This is consistent with our findings, which suggest that therapeutically administering IL-33 directly to the tumour cells would drive an anti-tumorigenic effect. In many cancers, systemic IL-33 is also increased [353-355]. Therefore, alternatively, reducing systemic IL-33 levels using anti-IL-33 antibodies to reduce systemic IL-33 could also be investigated as an anti-cancer therapy. Therefore, the manipulation of IL-33 could potentially vary depending on its expression levels in the tumour or systemically.

Regarding IL-36, only two studies to date have investigated the role of IL-36 in cancer, with both showing that IL-36 promotes anti-tumour immunity. In this study I have demonstrated that IL-36α and IL-36γ expression is increased in colon cancer compared to adjacent non-tumour tissue. Furthermore, stimulation of colon cancer cells *in vitro* with IL-36α and IL-36γ increased expression of chemokines responsible for recruiting immune cells into the tumour microenvironment. Future studies examining the role of IL-36 in colon cancer will determine if IL-36 family members are targets for future cancer therapy due to their ability to activate an anti-tumour immune response. As this receptor has three agonistic ligands, IL-36α, IL-36β and IL-36γ, these individual ligands may have different functions. In particular

IL-36β did not induce chemokine expression in colon cancer cells and may have an alternate role to the other two IL-36 ligands.

One of the findings of this thesis was the reduction in $CD8^+$ T cell recruitment observed *in vivo* when we suppressed ST2 expression in tumour cells. Other studies have shown that overexpression of IL-36α in hepatocellular carcinoma and IL-36γ in melanoma resulted in increased numbers of $CD8^+$ T cells recruited to the tumour site *in vivo*. T cells are central to anti-tumour immunity as they can recognize and target antigens expressed on the surface of tumour cells that have been altered due to genetic or epigenetic changes. In CRC tumours, $CD8^+$ T cells are associated with a better prognosis [356]. Increased numbers of $CD8^+$ T cells are strongly associated with decreased risk of recurrence of CRC and improved survival [357, 358]. The presence of these effector T-cells is more important than naıve T-cells in reducing the risk of relapse and improving survival. The prognostic significance of $CD8^+$ T cells and the ability of both IL-33 and IL-36 to alter the levels of these cells suggest that both of these proteins may have potential in future cancer immunotherapies. Existing immunotherapy regimens target checkpoint molecules such as PD-1 & CTLA4, but this requires a spontaneous anti-tumour immune response. Given the potential ability of IL-33 and IL-36 to modulate $CD8^+$ T cell levels, they could be utilised to increase the immunogenicity of tumours to encourage the breaking of immune tolerance to tumour antigens. To do this IL-36γ could be delivered directly to the tumour site through antibody-cytokine fusion or the use of an oncolytic virus. IL-36γ could also be utilised as an adjuvant to enhance tumour vaccination. Therefore, both IL-36 and IL-33 cytokines may represent a mechanism to break immune tolerance, reversing the immune suppression observed in many tumour microenvironments, thus enabling the hosts' immune response to target the cancer

cells. Further work in this field is necessary, however, to establish the therapeutic impact of IL-33 and IL-36. Studies such as these would enhance the findings of this thesis, which demonstrated that IL-33 may play a protective role in tumorigenesis and that both IL-33 and IL-36 signalling can increase chemokine production, which may have therapeutic implications for the treatment of colon cancer.

Bibliography

1. Nowell, P.C., *The clonal evolution of tumor cell populations.* Science, 1976. **194**(4260): p. 23-8.
2. Hanahan, D. and R. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**: p. 57 - 70.
3. Hanahan, D. and R. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**: p. 646 - 674.
4. Bray, F., et al., *Global estimates of cancer prevalence for 27 sites in the adult population in 2008.* Int J Cancer, 2013. **132**(5): p. 1133-45.
5. Butterworth, A.S., J.P. Higgins, and P. Pharoah, *Relative and absolute risk of colorectal cancer for individuals with a family history: a meta-analysis.* Eur J Cancer, 2006. **42**(2): p. 216-27.
6. Rao, C.V. and H.Y. Yamada, *Genomic instability and colon carcinogenesis: from the perspective of genes.* Front Oncol, 2013. **3**(130).
7. Powell, S.M., et al., *APC mutations occur early during colorectal tumorigenesis.* Nature, 1992. **359**(6392): p. 235-237.
8. Fodde, R., et al., *Mutations in the APC tumour suppressor gene cause chromosomal instability.* Nat Cell Biol, 2001. **3**(4): p. 433-438.
9. Fearon, E.R. and B. Vogelstein, *A genetic model for colorectal tumorigenesis.* Cell, 1990. **61**(5): p. 759-67.
10. Wood, L.D., et al., *The genomic landscapes of human breast and colorectal cancers.* Science, 2007. **318**(5853): p. 1108-13.
11. Barber, T.D., et al., *Chromatid cohesion defects may underlie chromosome instability in human colorectal cancers.* Proc Natl Acad Sci U S A, 2008. **105**(9): p. 3443-8.
12. Chittenden, T.W., et al., *Functional classification analysis of somatically mutated genes in human breast and colorectal cancers.* Genomics, 2008. **91**(6): p. 508-11.
13. Leufkens, A.M., et al., *Diagnostic accuracy of computed tomography for colon cancer staging: A systematic review.* Scandinavian Journal of Gastroenterology, 2011. **46**(7-8): p. 887-894.
14. *Colorectal cancer treating by stage.* American Cancer Society, 2016. **http://www.cancer.org/cancer/colonandrectumcancer/detailedguide/colorectal-cancer-treating-by-stage-colon**.
15. *Survival rates for colon cancer by stage.* American Cancer Society, 2016. **http://www.cancer.org/cancer/colonandrectumcancer/detailedguide/colorectal-cancer-survival-rates**.
16. Anzola, M., *Hepatocellular carcinoma: role of hepatitis B and hepatitis C viruses proteins in hepatocarcinogenesis.* J Viral Hepat, 2004. **11**(5): p. 383-93.
17. Boccardo, E., A.P. Lepique, and L.L. Villa, *The role of inflammation in HPV carcinogenesis.* Carcinogenesis, 2010. **31**(11): p. 1905-12.
18. Mangino, G., et al., *Inflammatory microenvironment and human papillomavirus-induced carcinogenesis.* Cytokine Growth Factor Rev, 2016. **18**(16): p. 007.
19. Farraye, F.A., et al., *AGA technical review on the diagnosis and management of colorectal neoplasia in inflammatory bowel disease.* Gastroenterology, 2010. **138**(2): p. 746-74, 774.e1-4; quiz e12-3.
20. McConnell, B.B. and V.W. Yang, *The Role of Inflammation in the Pathogenesis of Colorectal Cancer.* Curr Colorectal Cancer Rep, 2009. **5**(2): p. 69-74.
21. Richards, M.E., R.R. Rickert, and F.C. Nance, *Crohn's disease-associated carcinoma. A poorly recognized complication of inflammatory bowel disease.* Annals of surgery, 1989. **209**(6): p. 764-73.

22. Porta, C., et al., *Cellular and molecular pathways linking inflammation and cancer.* Immunobiology, 2009. **214**(9-10): p. 761-77.
23. Green, C.E., et al., *Chemoattractant Signaling between Tumor Cells and Macrophages Regulates Cancer Cell Migration, Metastasis and Neovascularization.* PLoS One, 2009. **4**(8): p. e6713.
24. Tokunaga, T., et al., *Vascular endothelial growth factor (VEGF) mRNA isoform expression pattern is correlated with liver metastasis and poor prognosis in colon cancer.* British Journal of Cancer, 1998. **77**(6): p. 998-1002.
25. Jedinak, A., S. Dudhgaonkar, and D. Sliva, *Activated macrophages induce metastatic behavior of colon cancer cells.* Immunobiology, 2010. **215**(3): p. 242-9.
26. Strieter, R.M., et al., *CXC chemokines in angiogenesis of cancer.* Semin Cancer Biol, 2004. **14**(3): p. 195-200.
27. Balkwill, F., *Cancer and the chemokine network.* Nat Rev Cancer, 2004. **4**(7): p. 540-50.
28. Arenberg, D.A., et al., *The role of CXC chemokines in the regulation of angiogenesis in non-small cell lung cancer.* J Leukoc Biol, 1997. **62**(5): p. 554-62.
29. Bolitho, C., et al., *The chemokine CXCL1 induces proliferation in epithelial ovarian cancer cells by transactivation of the epidermal growth factor receptor.* Endocr Relat Cancer, 2010. **17**(4): p. 929-40.
30. Zamarron, B.F. and W. Chen, *Dual roles of immune cells and their factors in cancer development and progression.* Int J Biol Sci, 2011. **7**(5): p. 651-8.
31. Chai, C.Y., et al., *Hypoxia-inducible factor-1alpha expression correlates with focal macrophage infiltration, angiogenesis and unfavourable prognosis in urothelial carcinoma.* J Clin Pathol, 2008. **61**(5): p. 658-64.
32. Sica, A., et al., *Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy.* Eur J Cancer, 2006. **42**(6): p. 717-27.
33. Kim, D.W., et al., *High tumour islet macrophage infiltration correlates with improved patient survival but not with EGFR mutations, gene copy number or protein expression in resected non-small cell lung cancer.* Br J Cancer, 2008. **98**(6): p. 1118-24.
34. Sica, A. and A. Mantovani, *Macrophage plasticity and polarization: in vivo veritas.* The Journal of Clinical Investigation. **122**(3): p. 787-795.
35. Chanmee, T., et al., *Tumor-Associated Macrophages as Major Players in the Tumor Microenvironment.* Cancers, 2014. **6**(3): p. 1670-1690.
36. Couper, K.N., D.G. Blount, and E.M. Riley, *IL-10: The Master Regulator of Immunity to Infection.* The Journal of Immunology, 2008. **180**(9): p. 5771-5777.
37. Qian, B.Z. and J.W. Pollard, *Macrophage diversity enhances tumor progression and metastasis.* Cell, 2010. **141**(1): p. 39-51.
38. Biswas, S.K. and A. Mantovani, *Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm.* Nat Immunol, 2010. **11**(10): p. 889-896.
39. Mantovani, A., et al., *The chemokine system in diverse forms of macrophage activation and polarization.* Trends Immunol, 2004. **25**: p. 677 - 86.
40. Mantovani, A., et al., *Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes.* Trends Immunol, 2002. **23**.
41. Mantovani, A., et al., *Role of tumor-associated macrophages in tumor progression and invasion.* Cancer Metastasis Rev, 2006. **25**(3): p. 315-22.
42. Makita, N., et al., *IL-10 enhances the phenotype of M2 macrophages induced by IL-4 and confers the ability to increase eosinophil migration.* Int Immunol, 2015. **27**(3): p. 131-41.

43. Zajac, E., et al., *Angiogenic capacity of M1- and M2-polarized macrophages is determined by the levels of TIMP-1 complexed with their secreted proMMP-9.* Blood, 2013. **122**(25): p. 4054-4067.
44. Algars, A., et al., *Type and location of tumor-infiltrating macrophages and lymphatic vessels predict survival of colorectal cancer patients.* Int J Cancer, 2012. **131**(4): p. 864-73.
45. Forssell, J., et al., *High macrophage infiltration along the tumor front correlates with improved survival in colon cancer.* Clin Cancer Res, 2007. **13**(5): p. 1472-9.
46. Lackner, C., et al., *Prognostic relevance of tumour-associated macrophages and von Willebrand factor-positive microvessels in colorectal cancer.* Virchows Arch, 2004. **445**(2): p. 160-7.
47. Di Carlo, E., et al., *The intriguing role of polymorphonuclear neutrophils in antitumor reactions.* Blood, 2001. **97**(2): p. 339-45.
48. Tazzyman, S., C.E. Lewis, and C. Murdoch, *Neutrophils: key mediators of tumour angiogenesis.* Int J Exp Pathol, 2009. **90**(3): p. 222-31.
49. Queen, M.M., et al., *Breast cancer cells stimulate neutrophils to produce oncostatin M: potential implications for tumor progression.* Cancer Res, 2005. **65**(19): p. 8896-904.
50. Marvel, D. and D.I. Gabrilovich, *Myeloid-derived suppressor cells in the tumor microenvironment: expect the unexpected.* The Journal of Clinical Investigation. **125**(9): p. 3356-3364.
51. Ohtani, H., *Focus on TILs: prognostic significance of tumor infiltrating lymphocytes in human colorectal cancer.* Cancer Immun, 2007. **7**: p. 4.
52. Mosmann, T.R., et al., *Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins.* J Immunol, 1986. **136**(7): p. 2348-57.
53. Miceli, M.C. and J.R. Parnes, *The roles of CD4 and CD8 in T cell activation.* Semin Immunol, 1991. **3**(3): p. 133-41.
54. Hadrup, S., M. Donia, and P. Thor Straten, *Effector CD4 and CD8 T cells and their role in the tumor microenvironment.* Cancer Microenviron, 2013. **6**(2): p. 123-33.
55. Morris, M., C. Platell, and B. Iacopetta, *Tumor-infiltrating lymphocytes and perforation in colon cancer predict positive response to 5-fluorouracil chemotherapy.* Clin Cancer Res, 2008. **14**(5): p. 1413-7.
56. Chiba, T., et al., *Intraepithelial CD8+ T-cell-count becomes a prognostic factor after a longer follow-up period in human colorectal carcinoma: possible association with suppression of micrometastasis.* Br J Cancer, 2004. **91**(9): p. 1711-7.
57. Halama, N., et al., *Localization and density of immune cells in the invasive margin of human colorectal cancer liver metastases are prognostic for response to chemotherapy.* Cancer Res, 2011. **71**(17): p. 5670-7.
58. Waldner, M., C.C. Schimanski, and M.F. Neurath, *Colon cancer and the immune system: the role of tumor invading T cells*. World J Gastroenterol. 2006 Dec 7;12(45):7233-8.
59. Sakaguchi, S., et al., *Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases.* J Immunol, 1995. **155**(3): p. 1151-64.
60. Kim, K.-J., et al., *Prognostic implications of tumor-infiltrating FoxP3+ regulatory T cells and CD8+ cytotoxic T cells in microsatellite-unstable gastric cancers.* Human Pathology, 2014. **45**(2): p. 285-293.
61. Marie, J.C., et al., *TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells.* J Exp Med, 2005. **201**(7): p. 1061-7.

62. Vlad, C., et al., *The prognostic value of FOXP3+ T regulatory cells in colorectal cancer.* J Buon, 2015. **20**(1): p. 114-9.
63. Topham, N.J. and E.W. Hewitt, *Natural killer cell cytotoxicity: how do they pull the trigger?* Immunology, 2009. **128**(1): p. 7-15.
64. Lanier, L.L., *Natural killer cell receptor signaling.* Curr Opin Immunol, 2003. **15**(3): p. 308-14.
65. Basse, P.H., et al., *Accumulation of adoptively transferred A-NK cells in murine metastases: kinetics and role of interleukin-2.* In Vivo, 1994. **8**(1): p. 17-24.
66. Smyth, M.J., et al., *Activation of NK cell cytotoxicity.* Molecular Immunology, 2005. **42**(4): p. 501-510.
67. Dinarello, C.A., *Interleukin-1 in the pathogenesis and treatment of inflammatory diseases.* Blood, 2011. **117**(14): p. 3720-3732.
68. Carter, M., et al., *Association of the interleukin 1 receptor antagonist gene with ulcerative colitis in Northern European Caucasians.* Gut, 2001. **48**(4): p. 461-467.
69. Sims, J.E., et al., *cDNA expression cloning of the IL-1 receptor, a member of the immunoglobulin superfamily.* Science, 1988. **241**(4865): p. 585-9.
70. McMahan, C.J., et al., *A novel IL-1 receptor, cloned from B cells by mammalian expression, is expressed in many cell types.* Embo J, 1991. **10**(10): p. 2821-32.
71. Klemenz, R., S. Hoffmann, and A.K. Werenskiold, *Serum- and oncoprotein-mediated induction of a gene with sequence similarity to the gene encoding carcinoembryonic antigen.* Proc Natl Acad Sci U S A, 1989. **86**(15): p. 5708-12.
72. Tominaga, S.-i., *A putative protein of a growth specific cDNA from BALB/C-3T3 cells is highly similar to the extracellular portion of mouse interleukin 1 receptor.* FEBS Letters, 1989. **258**(2): p. 301-304.
73. Werenskiold, A.K., S. Hoffmann, and R. Klemenz, *Induction of a mitogen-responsive gene after expression of the Ha-ras oncogene in NIH 3T3 fibroblasts.* Molecular and Cellular Biology, 1989. **9**(11): p. 5207-5214.
74. Born, T.L., et al., *Cloning of a novel receptor subunit, AcPL, required for interleukin-18 signaling.* J Biol Chem, 1998. **273**.
75. Lovenberg, T.W., et al., *Cloning of a cDNA encoding a novel interleukin-1 receptor related protein (IL 1R-rp2).* J Neuroimmunol, 1996. **70**(2): p. 113-22.
76. Greenfeder, S.A., et al., *Molecular cloning and characterization of a second subunit of the interleukin 1 receptor complex.* J Biol Chem, 1995. **270**(23): p. 13757-65.
77. Parnet, P., et al., *IL-1Rrp is a novel receptor-like molecule similar to the type I interleukin-1 receptor and its homologues T1/ST2 and IL-1R AcP.* J Biol Chem, 1996. **271**(8): p. 3967-70.
78. Torigoe, K., et al., *Purification and characterization of the human interleukin-18 receptor.* J Biol Chem, 1997. **272**(41): p. 25737-42.
79. Born, T.L., et al., *Identification and characterization of two members of a novel class of the interleukin-1 receptor (IL-1R) family. Delineation of a new class of IL-1R-related proteins based on signaling.* J Biol Chem, 2000. **275**(39): p. 29946-54.
80. Carrie, A., et al., *A new member of the IL-1 receptor family highly expressed in hippocampus and involved in X-linked mental retardation.* Nat Genet, 1999. **23**(1): p. 25-31.
81. Ferrante, M.I., et al., *IL1RAPL2 maps to Xq22 and is specifically expressed in the central nervous system.* Gene, 2001. **275**(2): p. 217-221.
82. Thomassen, E., B.R. Renshaw, and J.E. Sims, *Identification and characterization of SIGIRR, a molecule representing a novel subtype of the IL-1R superfamily.* Cytokine, 1999. **11**(6): p. 389-99.
83. Aizawa, Y., et al., *Cloning and expression of interleukin-18 binding protein.* FEBS Letters, 1999. **445**(2–3): p. 338-342.

84. Riva, F., et al., *TIR8/SIGIRR is an Interleukin-1 Receptor/Toll Like Receptor Family Member with Regulatory Functions in Inflammation and Immunity.* Frontiers in Immunology, 2012. **3**: p. 322.
85. Lingel, A., et al., *Structure of IL-33 and Its Interaction with the ST2 and IL-1RAcP Receptors—Insight into Heterotrimeric IL-1 Signaling Complexes.* Structure, 2009. **17**(10): p. 1398-1410.
86. Priestle, J.P., H.P. Schär, and M.G. Grütter, *Crystal structure of the cytokine interleukin-1 beta.* The EMBO Journal, 1988. **7**(2): p. 339-343.
87. Qin, J., et al., *SIGIRR inhibits interleukin-1 receptor- and toll-like receptor 4-mediated signaling through different mechanisms.* J Biol Chem, 2005. **280**(26): p. 25233-41.
88. Chen, C.J., et al., *Identification of a key pathway required for the sterile inflammatory response triggered by dying cells.* Nat Med, 2007. **13**(7): p. 851-6.
89. Rider, P., et al., *IL-1alpha and IL-1beta recruit different myeloid cells and promote different stages of sterile inflammation.* J Immunol, 2011. **187**(9): p. 4835-43.
90. Krelin, Y., et al., *Interleukin-1β–Driven Inflammation Promotes the Development and Invasiveness of Chemical Carcinogen–Induced Tumors.* Cancer Research, 2007. **67**(3): p. 1062-1071.
91. Carmi, Y., et al., *The role of IL-1beta in the early tumor cell-induced angiogenic response.* J Immunol, 2013. **190**(7): p. 3500-9.
92. Colotta, F., et al., *Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4.* Science, 1993. **261**(5120): p. 472-5.
93. Okamura, H., et al., *Cloning of a new cytokine that induces IFN-gamma production by T cells.* Nature, 1995. **378**(6552): p. 88-91.
94. Puren, A.J., G. Fantuzzi, and C.A. Dinarello, *Gene expression, synthesis, and secretion of interleukin 18 and interleukin 1beta are differentially regulated in human blood mononuclear cells and mouse spleen cells.* Proc Natl Acad Sci U S A, 1999. **96**(5): p. 2256-61.
95. Nold, M.F., et al., *IL-37 is a fundamental inhibitor of innate immunity.* Nat Immunol, 2010. **11**(11): p. 1014-1022.
96. Klemenz, R., S. Hoffmann, and A.K. Werenskiold, *Serum- and oncoprotein-mediated induction of a gene with sequence similarity to the gene encoding carcinoembryonic antigen.* Proc Natl Acad Sci USA, 1989. **86**.
97. Li, H., et al., *The Cloning and Nucleotide Sequence of Human ST2L cDNA.* Genomics, 2000. **67**(3): p. 284-290.
98. Yanagisawa, K., et al., *Presence of a novel primary response gene ST2L, encoding a product highly similar to the interleukin 1 receptor type 1.* FEBS Letters, 1993. **318**(1): p. 83-87.
99. Yanagisawa, K., et al., *Presence of a novel primary response gene ST2L, encoding a product highly similar to the interleukin 1 receptor type 1.* FEBS Lett, 1993. **318**(1): p. 83-7.
100. Trajkovic, V., M.J. Sweet, and D. Xu, *T1/ST2—an IL-1 receptor-like modulator of immune responses.* Cytokine & Growth Factor Reviews, 2004. **15**(2–3): p. 87-95.
101. Tominaga, S., et al., *Presence and expression of a novel variant form of ST2 gene product in human leukemic cell line UT-7/GM.* Biochem Biophys Res Commun, 1999. **264**(1): p. 14-8.
102. Tago, K., et al., *Tissue distribution and subcellular localization of a variant form of the human ST2 gene product, ST2V.* Biochem Biophys Res Commun, 2001. **285**: p. 1377 - 1383.
103. Tago, K., et al., *Tissue distribution and subcellular localization of a variant form of the human ST2 gene product, ST2V.* Biochem Biophys Res Commun, 2001. **285**.

104. Gachter, T., A.K. Werenskiold, and R. Klemenz, *Transcription of the interleukin-1 receptor-related T1 gene is initiated at different promoters in mast cells and fibroblasts.* J Biol Chem, 1996. **271**(1): p. 124-9.
105. Iwahana, H., et al., *Different promoter usage and multiple transcription initiation sites of the interleukin-1 receptor-related human ST2 gene in UT-7 and TM12 cells.* European Journal of Biochemistry, 1999. **264**(2): p. 397-406.
106. Bergers, G., et al., *Alternative promoter usage of the Fos-responsive gene Fit-1 generates mRNA isoforms coding for either secreted or membrane-bound proteins related to the IL-1 receptor.* Embo J, 1994. **13**(5): p. 1176-88.
107. Baba, Y., et al., *GATA2 Is a Critical Transactivator for the Human IL1RL1/ST2 Promoter in Mast Cells/Basophils: OPPOSING ROLES FOR GATA2 and GATA1 IN HUMAN IL1RL1/ST2 GENE EXPRESSION.* Journal of Biological Chemistry, 2012. **287**(39): p. 32689-32696.
108. Hayakawa, M., et al., *T-helper type 2 cell-specific expression of the ST2 gene is regulated by transcription factor GATA-3.* Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression, 2005. **1728**(1–2): p. 53-64.
109. Guo, L., et al., *IL-1 family members and STAT activators induce cytokine production by Th2, Th17, and Th1 cells.* Proc Natl Acad Sci U S A, 2009. **106**(32): p. 13463-8.
110. Spooner, C.J., et al., *Specification of type 2 innate lymphocytes by the transcriptional determinant Gfi1.* Nat Immunol, 2013. **14**(12): p. 1229-1236.
111. Schmieder, A., G. Multhoff, and J. Radons, *Interleukin-33 acts as a pro-inflammatory cytokine and modulates its receptor gene expression in highly metastatic human pancreatic carcinoma cells.* Cytokine, 2012. **60**.
112. Yagami, A., et al., *IL-33 mediates inflammatory responses in human lung tissue cells.* J Immunol, 2010. **185**(10): p. 5743-50.
113. Lohning, M., et al., *T1/ST2 is preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function.* Proc Natl Acad Sci USA, 1998. **95**: p. 6930 - 6935.
114. Lipsky, B.P., et al., *Deletion of the ST2 proximal promoter disrupts fibroblast-specific expression but does not reduce the amount of soluble ST2 in circulation.* Eur J Immunol, 2012. **42**(7): p. 1863-9.
115. Bourgeois, E., et al., *The pro-Th2 cytokine IL-33 directly interacts with invariant NKT and NK cells to induce IFN-gamma production.* Eur J Immunol, 2009. **39**(4): p. 1046-55.
116. Smithgall, M., et al., *IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells.* Int Immunol, 2008. **20**: p. 1019 - 1030.
117. Bourgeois, E.A., et al., *A natural protective function of invariant NKT cells in a mouse model of innate-cell-driven lung inflammation.* Eur J Immunol, 2011. **41**(2): p. 299-305.
118. Yang, Q., et al., *IL-33 synergizes with TCR and IL-12 signaling to promote the effector function of CD8+ T cells.* Eur J Immunol, 2011. **41**(11): p. 3351-60.
119. Onda, H., et al., *Identification of genes differentially expressed in canine vasospastic cerebral arteries after subarachnoid hemorrhage.* J Cereb Blood Flow Metab, 1999. **19**: p. 1279 - 1288.
120. Baekkevold, E., et al., *Molecular characterization of NF-HEV, a nuclear factor preferentially expressed in human high endothelial venules.* Am J Pathol, 2003. **163**: p. 69 - 79.
121. Kuchler, A., et al., *Nuclear interleukin-33 is generally expressed in resting endothelium but rapidly lost upon angiogenic or proinflammatory activation.* Am J Pathol, 2008. **173**: p. 1229 - 1242.

122. Schmitz, J., et al., *IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines.* Immunity, 2005. **23**: p. 479 - 490.
123. Hong, J., et al., *Identification of Constitutively Active Interleukin 33 (IL-33) Splice Variant.* Journal of Biological Chemistry, 2011. **286**(22): p. 20078-20086.
124. Hong, J., et al., *Identification of constitutively active IL-33 splice variant.* J Biol Chem, 2011.
125. Cayrol, C. and J. Girard, *The IL-1-like cytokine IL-33 is inactivated after maturation by caspase-1.* Proc Natl Acad Sci USA, 2009. **106**: p. 9021 - 9026.
126. Ali, S., et al., *Caspase 3 inactivates biologically active full length interleukin-33 as a classical cytokine but does not prohibit nuclear translocation.* Biochemical and Biophysical Research Communications, 2010. **391**(3): p. 1512-1516.
127. Luthi, A., et al., *Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases.* Immunity, 2009. **31**: p. 84 - 98.
128. Talabot-Ayer, D., et al., *Interleukin-33 is biologically active independently of caspase-1 cleavage.* J Biol Chem, 2009. **284**: p. 19420 - 19426.
129. Lefrancais, E., et al., *IL-33 is processed into mature bioactive forms by neutrophil elastase and cathepsin G.* Proc Natl Acad Sci U S A, 2012. **109**(5): p. 1673-8.
130. Lefrancais, E. and C. Cayrol, *Mechanisms of IL-33 processing and secretion: differences and similarities between IL-1 family members.* Eur Cytokine Netw, 2012. **23**(4): p. 120-7.
131. Cohen, E.S., et al., *Oxidation of the alarmin IL-33 regulates ST2-dependent inflammation.* Nat Commun, 2015. **6**.
132. Küchler, A.M., et al., *Nuclear interleukin-33 is generally expressed in resting endothelium but rapidly lost upon angiogenicor proinflammatory activation.* Am J Pathol, 2008. **173**.
133. Zhao, W.H. and Z.Q. Hu, *Up-regulation of IL-33 expression in various types of murine cells by IL-3 and IL-4.* Cytokine, 2012. **58**(2): p. 267-73.
134. Sundnes, O., et al., *Epidermal Expression and Regulation of Interleukin-33 during Homeostasis and Inflammation: Strong Species Differences.* J Invest Dermatol, 2015. **135**(7): p. 1771-80.
135. Zhao, W. and Z. Hu, *The enigmatic processing and secretion of interleukin-33.* Cell Mol Immunol, 2010. **7**(4): p. 260-2.
136. Carriere, V., L. Roussel, and N. Ortega, *IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo.* Proc Natl Acad Sci USA, 2007. **104**: p. 282-287.
137. Roussel, L., et al., *Molecular mimicry between IL-33 and KSHV for attachment to chromatin through the H2A-H2B acidic pocket.* EMBO Rep, 2008. **9**: p. 1006 - 1012.
138. Kalashnikova, A.A., et al., *The role of the nucleosome acidic patch in modulating higher order chromatin structure.* J R Soc Interface, 2013. **10**(82): p. 6.
139. Zhang, F., et al., *Expression of IL-33 and its epigenetic regulation in multiple sclerosis.* Annals of Clinical and Translational Neurology, 2014. **1**(5): p. 307-318.
140. Ali, S., A. Mohs, and M. Thomas, *The dual function cytokine IL-33 interacts with the transcription factor NF-kappaB to dampen NF-kappaB-stimulated gene transcription.* J Immunol, 2011. **187**: p. 1609-1616.
141. Ali, S., et al., *The Dual Function Cytokine IL-33 Interacts with the Transcription Factor NF-κB To Dampen NF-κB–Stimulated Gene Transcription.* The Journal of Immunology, 2011. **187**(4): p. 1609-1616.
142. Brint, E.K., et al., *Characterization of signaling pathways activated by the interleukin 1 (IL-1) receptor homologue T1/ST2. A role for Jun N-terminal kinase in IL-4 induction.* J Biol Chem, 2002. **277**(51): p. 49205-11.

143. Funakoshi-Tago, M., et al., *TRAF6 is a critical signal transducer in IL-33 signaling pathway.* Cell Signal, 2008. **20**(9): p. 1679-86.
144. Drube, S., et al., *The receptor tyrosine kinase c-Kit controls IL-33 receptor signaling in mast cells.* Blood, 2010. **115**(19): p. 3899-906.
145. Martin, N.T. and M.U. Martin, *Interleukin 33 is a guardian of barriers and a local alarmin.* Nat Immunol, 2016. **17**(2): p. 122-131.
146. Pinto, S.M., et al., *Quantitative phosphoproteomic analysis of IL-33-mediated signaling.* Proteomics, 2015. **15**(2-3): p. 532-44.
147. Maywald, R.L., et al., *IL-33 activates tumor stroma to promote intestinal polyposis.* Proc Natl Acad Sci U S A, 2015. **112**(19): p. 27.
148. Pollheimer, J., et al., *Interleukin-33 drives a proinflammatory endothelial activation that selectively targets nonquiescent cells.* Arterioscler Thromb Vasc Biol, 2013. **33**(2): p. 15.
149. Zhao, J., et al., *F-box protein FBXL19-mediated ubiquitination and degradation of the receptor for IL-33 limits pulmonary inflammation.* Nat Immunol, 2012. **13**(7): p. 651-8.
150. Zhao, J., et al., *Focal adhesion kinase-mediated activation of glycogen synthase kinase 3beta regulates IL-33 receptor internalization and IL-33 signaling.* J Immunol, 2015. **194**(2): p. 795-802.
151. Connolly, D.J., L.A. O'Neill, and A.F. McGettrick, *The GOLD domain-containing protein TMED1 is involved in interleukin-33 signaling.* J Biol Chem, 2013. **288**(8): p. 5616-23.
152. Garlanda, C., H.J. Anders, and A. Mantovani, *TIR8/SIGIRR: an IL-1R/TLR family member with regulatory functions in inflammation and T cell polarization.* Trends Immunol, 2009. **30**(9): p. 439-46.
153. Miller, A., *Role of IL-33 in inflammation and disease.* Journal of Inflammation, 2011. **8**(1): p. 22.
154. Bulek, K., et al., *The essential role of single Ig IL-1 receptor-related molecule/Toll IL-1R8 in regulation of Th2 immune response.* J Immunol, 2009. **182**: p. 2601 - 2609.
155. Haraldsen, G., et al., *Interleukin-33 - cytokine of dual function or novel alarmin?* Trends Immunol, 2009. **30**: p. 227 - 233.
156. Lunderius-Andersson, C., M. Enoksson, and G. Nilsson, *Mast Cells Respond to Cell Injury through the Recognition of IL-33.* Frontiers in Immunology, 2012. **3**: p. 82.
157. Lefrancais, E., et al., *Central domain of IL-33 is cleaved by mast cell proteases for potent activation of group-2 innate lymphoid cells.* Proc Natl Acad Sci U S A, 2014. **111**(43): p. 15502-7.
158. Roy, A., et al., *Mast cell chymase degrades the alarmins heat shock protein 70, biglycan, HMGB1, and interleukin-33 (IL-33) and limits danger-induced inflammation.* J Biol Chem, 2014. **289**(1): p. 237-50.
159. Kurokawa, M., et al., *Expression and effects of IL-33 and ST2 in allergic bronchial asthma: IL-33 induces eotaxin production in lung fibroblasts.* Int Arch Allergy Immunol, 2011. **1**: p. 12-20.
160. Bianchetti, L., et al., *IL-33 promotes the migration and proliferation of circulating fibrocytes from patients with allergen-exacerbated asthma.* Biochem Biophys Res Commun, 2012. **426**(1): p. 116-21.
161. Hayashi, H., et al., *IL-33 enhanced the proliferation and constitutive production of IL-13 and IL-5 by fibrocytes.* Biomed Res Int, 2014. **738625**(10): p. 13.
162. Yin, H., et al., *IL-33 promotes Staphylococcus aureus-infected wound healing in mice.* Int Immunopharmacol, 2013. **17**(2): p. 432-8.
163. Yin, H., et al., *IL-33 accelerates cutaneous wound healing involved in upregulation of alternatively activated macrophages.* Mol Immunol, 2013. **56**(4): p. 347-53.

164. Abbas, A.K., K.M. Murphy, and A. Sher, *Functional diversity of helper T lymphocytes.* Nature, 1996. **383**(6603): p. 787-93.
165. Palmer, G. and C. Gabay, *Interleukin-33 biology with potential insights into human diseases.* Nat Rev Rheumatol, 2011. **7**(6): p. 321-9.
166. Walker, J.A., J.L. Barlow, and A.N. McKenzie, *Innate lymphoid cells--how did we miss them?* Nat Rev Immunol, 2013. **13**(2): p. 75-87.
167. Spits, H., et al., *Innate lymphoid cells--a proposal for uniform nomenclature.* Nat Rev Immunol, 2013. **13**(2): p. 145-9.
168. Sonnenberg, G.F. and D. Artis, *Innate lymphoid cell interactions with microbiota: implications for intestinal health and disease.* Immunity, 2012. **37**(4): p. 601-10.
169. Monticelli, L.A., et al., *Innate lymphoid cells promote lung tissue homeostasis following acute influenza virus infection.* Nature immunology, 2011. **12**(11): p. 1045-1054.
170. Nussbaum, J.C., et al., *Type 2 innate lymphoid cells control eosinophil homeostasis.* Nature, 2013. **502**(7470): p. 245-8.
171. Vasanthakumar, A., et al., *The transcriptional regulators IRF4, BATF and IL-33 orchestrate development and maintenance of adipose tissue-resident regulatory T cells.* Nat Immunol, 2015. **16**(3): p. 276-285.
172. Burzyn, D., et al., *A Special Population of Regulatory T Cells Potentiates Muscle Repair.* Cell. **155**(6): p. 1282-1295.
173. Schiering, C., et al., *The alarmin IL-33 promotes regulatory T-cell function in the intestine.* Nature, 2014. **513**(7519): p. 564-8.
174. Oboki, K., et al., *IL-33 is a crucial amplifier of innate rather than acquired immunity.* Proc Natl Acad Sci USA, 2010. **107**: p. 18581 - 18586.
175. Mu, R., et al., *Elevated serum interleukin 33 is associated with autoantibody production in patients with rheumatoid arthritis.* J Rheumatol, 2010. **37**: p. 2006 - 2013.
176. Pushparaj, P., et al., *The cytokine interleukin-33 mediates anaphylactic shock.* Proc Natl Acad Sci USA, 2009. **106**: p. 9773 - 8.
177. Pastorelli, L., et al., *Epithelial-derived IL-33 and its receptor ST2 are dysregulated in ulcerative colitis and in experimental Th1/Th2 driven enteritis.* Proc Natl Acad Sci USA, 2010. **107**: p. 8017 - 8022.
178. Kobori, A., et al., *Interleukin-33 expression is specifically enhanced in inflamed mucosa of ulcerative colitis.* Journal of Gastroenterology, 2010. **45**(10): p. 999-1007.
179. Seidelin, J.B., G. Rogler, and O.H. Nielsen, *A role for interleukin-33 in T(H)2-polarized intestinal inflammation?* Mucosal Immunol, 2011. **4**(5): p. 496-502.
180. Sponheim, J., et al., *Inflammatory bowel disease-associated interleukin-33 is preferentially expressed in ulceration-associated myofibroblasts.* Am J Pathol, 2010. **177**: p. 2804 - 2815.
181. Beltran, C., et al., *Characterization of the novel ST2/IL-33 system in patients with inflammatory bowel disease.* Inflamm Bowel Dis, 2009.
182. Zhu, J., et al., *IL-33 Aggravates DSS-Induced Acute Colitis in Mouse Colon Lamina Propria by Enhancing Th2 Cell Responses.* Mediators Inflamm, 2015. **2015**: p. 913041.
183. Sedhom, M.A., et al., *Neutralisation of the interleukin-33/ST2 pathway ameliorates experimental colitis through enhancement of mucosal healing in mice.* Gut, 2013. **62**(12): p. 1714-23.
184. Grobeta, P., et al., *IL-33 attenuates development and perpetuation of chronic intestinal inflammation.* Inflamm Bowel Dis, 2012. **18**(10): p. 1900-9.

185. Januzzi, J., et al., *Measurement of the interleukin family member ST2 in patients with acute dyspnea: results from the PRIDE (Pro-Brain Natriuretic Peptide Investigation of Dyspnea in the Emergency Department) study.* J Am Coll Cardiol, 2007. **50**: p. 607 - 613.
186. Martinez-Rumayor, A., et al., *Soluble ST2 plasma concentrations predict 1-year mortality in acutely dyspneic emergency department patients with pulmonary disease.* Am J Clin Pathol, 2008. **130**(4): p. 578-84.
187. Weinberg, E., et al., *Expression and regulation of ST2, an interleukin-1 receptor family member, in cardiomyocytes and myocardial infarction.* Circulation, 2002. **106**: p. 2961 - 2966.
188. Martinez-Rumayor, A., et al., *Soluble ST2 plasma concentrations predict 1-year mortality in acutely dyspneic emergency department patients with pulmonary disease.* Am J Clin Pathol, 2008. **130**: p. 578 - 584.
189. Boisot, S., et al., *Serial sampling of ST2 predicts 90-day mortality following destabilized heart failure.* J Card Fail, 2008. **14**: p. 732 - 738.
190. Bayes-Genis, A., et al., *Soluble ST2 Monitoring Provides Additional Risk Stratification for Outpatients With Decompensated Heart Failure.* Rev Esp Cardiol, 2010. **63**: p. 1171 - 1178.
191. Yancy, C.W., et al., *2013 ACCF/AHA Guideline for the Management of Heart Failure: A Report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines.* Circulation, 2013. **128**(16): p. e240-e327.
192. Grivennikov, S.I., F.R. Greten, and M. Karin, *Immunity, inflammation, and cancer.* Cell, 2010. **140**(6): p. 883-99.
193. Jovanovic, I., et al., *ST2 deletion enhances innate and acquired immunity to murine mammary carcinoma.* Eur J Immunol, 2011. **41**.
194. Gillibert-Duplantier, J., et al., *Gene expression profiling identifies sST2 as an effector of ErbB2-driven breast carcinoma cell motility, associated with metastasis.* Oncogene, 2012. **31**(30): p. 3516-24.
195. Lu, D.-p., et al., *Serum soluble ST2 is associated with ER-positive breast cancer.* BMC Cancer, 2014. **14**(1): p. 1-8.
196. Jovanovic, I.P., et al., *Interleukin-33/ST2 axis promotes breast cancer growth and metastases by facilitating intratumoral accumulation of immunosuppressive and innate lymphoid cells.* International Journal of Cancer, 2014. **134**(7): p. 1669-1682.
197. Fang, K.M., et al., *Induced interleukin-33 expression enhances the tumorigenic activity of rat glioma cells.* Neuro Oncol, 2014. **16**(4): p. 552-66.
198. Barrera, L., et al., *Cytokine profile determined by data-mining analysis set into clusters of non-small-cell lung cancer patients according to prognosis.* Ann Oncol, 2015. **26**(2): p. 428-35.
199. Musolino, C., et al., *Reduced IL-33 plasma levels in multiple myeloma patients are associated with more advanced stage of disease*. Br J Haematol. 2013 Mar;160(5):709-10. doi: 10.1111/bjh.12146. Epub 2012 Dec 4.
200. Gao, X., et al., *Tumoral expression of IL-33 inhibits tumor growth and modifies the tumor microenvironment through CD8+ T and NK cells.* J Immunol, 2015. **194**(1): p. 438-45.
201. Gao, K., et al., *Transgenic expression of IL-33 activates CD8+ T cells and NK cells and inhibits tumor growth and metastasis in mice.* Cancer Letters, 2013. **335**(2): p. 463-471.
202. Mertz, K.D., et al., *The IL-33/ST2 pathway contributes to intestinal tumorigenesis in humans and mice.* OncoImmunology, 2016. **5**(1): p. e1062966.

203. Cui, G., et al., *Dynamics of the IL-33/ST2 network in the progression of human colorectal adenoma to sporadic colorectal cancer.* Cancer Immunology, Immunotherapy, 2015. **64**(2): p. 181-190.
204. Liu, X., et al., *IL-33/ST2 pathway contributes to metastasis of human colorectal cancer.* Biochem Biophys Res Commun, 2014. **453**(3): p. 486-92.
205. 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.
206. Gunther, S. and E.J. Sundberg, *Molecular determinants of agonist and antagonist signaling through the IL-36 receptor.* J Immunol, 2014. **193**(2): p. 921-30.
207. Towne, J.E., et al., *Interleukin-36 (IL-36) ligands require processing for full agonist (IL-36alpha, IL-36beta, and IL-36gamma) or antagonist (IL-36Ra) activity.* J Biol Chem, 2011. **286**(49): p. 42594-602.
208. 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.
209. 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.
210. Sims, J.E. and D.E. Smith, *The IL-1 family: regulators of immunity.* Nat Rev Immunol, 2010. **10**(2): p. 89-102.
211. Vigne, S., et al., *IL-36R ligands are potent regulators of dendritic and T cells.* Blood, 2011. **118**(22): p. 5813-5823.
212. Gresnigt, M.S., et al., *The IL-36 receptor pathway regulates Aspergillus fumigatus-induced Th1 and Th17 responses.* European Journal of Immunology, 2013. **43**(2): p. 416-426.
213. 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. 213-6.
214. 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.* Clinical & Experimental Immunology, 2016. **184**(2): p. 159-173.
215. Martin, U., et al., *Externalization of the Leaderless Cytokine IL-1F6 Occurs in Response to Lipopolysaccharide/ATP Activation of Transduced Bone Marrow Macrophages.* The Journal of Immunology, 2009. **183**(6): p. 4021-4030.
216. 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.
217. Johnston, A., et al., *IL-1F5, -F6, -F8, and -F9: a novel IL-1 family signaling system that is active in psoriasis and promotes keratinocyte antimicrobial peptide expression.* J Immunol, 2011. **186**(4): p. 2613-22.
218. Saha, S.S., et al., *Signal Transduction and Intracellular Trafficking by the Interleukin 36 Receptor.* J Biol Chem, 2015. **290**(39): p. 23997-4006.
219. Afonina, Inna S., et al., *Granzyme B-Dependent Proteolysis Acts as a Switch to Enhance the Proinflammatory Activity of IL-1α.* Molecular Cell, 2011. **44**(2): p. 265-278.
220. Henry, C.M., et al., *Neutrophil-Derived Proteases Escalate Inflammation through Activation of IL-36 Family Cytokines.* Cell Rep, 2016. **14**(4): p. 708-22.
221. Martin, U., et al., *Externalization of the leaderless cytokine IL-1F6 occurs in response to lipopolysaccharide/ATP activation of transduced bone marrow macrophages.* J Immunol, 2009. **183**(6): p. 4021-30.
222. 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.

223. Takahashi, K., et al., *Interleukin (IL)-1β Is a Strong Inducer of IL-36γ Expression in Human Colonic Myofibroblasts.* PLoS One, 2015. **10**(11): p. e0138423.
224. Scheibe, K., et al., *IL-36R signalling activates intestinal epithelial cells and fibroblasts and promotes mucosal healing in vivo.* Gut, 2016.
225. Choi, J.S., K.H. Kim, and L.F. Lau, *The matricellular protein CCN1 promotes mucosal healing in murine colitis through IL-6.* Mucosal Immunol, 2015. **8**(6): p. 1285-1296.
226. Lian, L.-H., et al., *The Double-Stranded RNA Analogue Polyinosinic-Polycytidylic Acid Induces Keratinocyte Pyroptosis and Release of IL-36γ.* Journal of Investigative Dermatology, 2012. **132**(5): p. 1346-1353.
227. Stappenbeck, T.S. and H. Miyoshi, *The role of stromal stem cells in tissue regeneration and wound repair.* Science, 2009. **324**(5935): p. 1666-9.
228. Neufert, C., et al., *Tumor fibroblast-derived epiregulin promotes growth of colitis-associated neoplasms through ERK.* J Clin Invest, 2013. **123**(4): p. 1428-43.
229. Pickert, G., et al., *STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing.* The Journal of Experimental Medicine, 2009. **206**(7): p. 1465-1472.
230. Blumberg, H., et al., *Opposing activities of two novel members of the IL-1 ligand family regulate skin inflammation.* J Exp Med, 2007. **204**(11): p. 2603-14.
231. Tortola, L., et al., *Psoriasiform dermatitis is driven by IL-36–mediated DC-keratinocyte crosstalk.* The Journal of Clinical Investigation. **122**(11): p. 3965-3976.
232. Frey, S., et al., *The novel cytokine interleukin-36alpha is expressed in psoriatic and rheumatoid arthritis synovium.* Ann Rheum Dis, 2013. **72**(9): p. 1569-74.
233. Parsanejad, R., et al., *Distinct regulatory profiles of interleukins and chemokines in response to cigarette smoke condensate in normal human bronchial epithelial (NHBE) cells.* Journal of Interferon and Cytokine Research, 2008. **28**(12): p. 703-712.
234. Chen, H., et al., *Alterations of plasma inflammatory biomarkers in the healthy and chronic obstructive pulmonary disease patients with or without acute exacerbation.* J Proteomics, 2012. **75**(10): p. 2835-43.
235. Magne, D., et al., *The new IL-1 family member IL-1F8 stimulates production of inflammatory mediators by synovial fibroblasts and articular chondrocytes.* Arthritis Research & Therapy, 2006. **8**(3): p. 1-11.
236. Do, M.-S., et al., *Inflammatory Gene Expression Patterns Revealed by DNA Microarray Analysis in TNF-α-treated SGBS Human Adipocytes.* Yonsei Med J, 2006. **47**(5): p. 729-736.
237. van Asseldonk, E.J.P., et al., *The Effect of the Interleukin-1 Cytokine Family Members IL-1F6 and IL-1F8 on Adipocyte Differentiation.* Obesity, 2010. **18**(11): p. 2234-2236.
238. Bernstein, C.N., A. Wajda, and J.F. Blanchard, *The clustering of other chronic inflammatory diseases in inflammatory bowel disease: a population-based study.* Gastroenterology, 2005. **129**(3): p. 827-36.
239. Weng, X., et al., *Clustering of inflammatory bowel disease with immune mediated diseases among members of a northern california-managed care organization.* Am J Gastroenterol, 2007. **102**(7): p. 1429-35.
240. Russell, S.E., et al., *IL-36[alpha] expression is elevated in ulcerative colitis and promotes colonic inflammation.* Mucosal Immunol, 2016.
241. Medina-Contreras, O., et al., *Cutting Edge: IL-36 Receptor Promotes Resolution of Intestinal Damage.* J Immunol, 2016. **196**(1): p. 34-8.
242. Pan, Q.Z., et al., *Decreased expression of interleukin-36alpha correlates with poor prognosis in hepatocellular carcinoma.* Cancer Immunol Immunother, 2013. **62**(11): p. 1675-85.
243. Balkwill, F., A. Montfort, and M. Capasso, *B regulatory cells in cancer.* Trends Immunol, 2013. **34**(4): p. 169-73.

244. Wang, X., et al., *IL-36gamma Transforms the Tumor Microenvironment and Promotes Type 1 Lymphocyte-Mediated Antitumor Immune Responses.* Cancer Cell, 2015. **28**(3): p. 296-306.
245. Wang, X., et al., *IL-36γ Transforms the Tumor Microenvironment and Promotes Type 1 Lymphocyte-Mediated Antitumor Immune Responses.* Cancer Cell. **28**(3): p. 296-306.
246. Baekkevold, E.S., et al., *Molecular characterization of NF-HEV, a nuclear factor preferentially expressed in human high endothelial venules.* Am J Pathol, 2003. **163**(1): p. 69-79.
247. Oboki, K., et al., *IL-33 and IL-33 receptors in host defense and diseases.* Allergol Int, 2010. **59**(2): p. 143-60.
248. Barlow, J.L. and A.N. McKenzie, *Nuocytes: expanding the innate cell repertoire in type-2 immunity.* J Leukoc Biol, 2011. **90**(5): p. 867-74.
249. Demyanets, S., et al., *Components of the interleukin-33/ST2 system are differentially expressed and regulated in human cardiac cells and in cells of the cardiac vasculature.* Journal of Molecular and Cellular Cardiology, 2013. **60**: p. 16-26.
250. Zhang, J., et al., *ST2 blockade reduces sST2-producing T cells while maintaining protective mST2-expressing T cells during graft-versus-host disease.* Sci Transl Med, 2015. **7**(308).
251. Wills-Karp, M., et al., *Trefoil factor 2 rapidly induces interleukin 33 to promote type 2 immunity during allergic asthma and hookworm infection.* J Exp Med, 2012. **209**(3): p. 607-22.
252. Humphreys, N., et al., *IL-33, a potent inducer of adaptive immunity to intestinal nematodes.* J Immunol, 2008. **180**: p. 2443 - 2449.
253. Farraye, F.A., et al., *AGA technical review on the diagnosis and management of colorectal neoplasia in inflammatory bowel disease.* Gastroenterology, 2010. **138**(2): p. 746-74.
254. Yu, X.X., et al., *IL-33 Promotes Gastric Cancer Cell Invasion and Migration Via ST2-ERK1/2 Pathway.* Dig Dis Sci, 2015. **60**(5): p. 1265-72.
255. Espinassous, Q., et al., *IL-33 enhances lipopolysaccharide-induced inflammatory cytokine production from mouse macrophages by regulating lipopolysaccharide receptor complex.* J Immunol, 2009. **183**: p. 1446 - 1455.
256. Chang, Y.J., et al., *Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity.* Nat Immunol, 2011. **12**(7): p. 631-8.
257. Patanaphan, V. and O.M. Salazar, *Colorectal cancer: metastatic patterns and prognosis.* South Med J, 1993. **86**(1): p. 38-41.
258. Calon, A., et al., *Stromal gene expression defines poor-prognosis subtypes in colorectal cancer.* Nat Genet, 2015. **47**(4): p. 320-329.
259. Wang, D. and R.N. Dubois, *The role of COX-2 in intestinal inflammation and colorectal cancer.* Oncogene, 2010. **29**(6): p. 781-8.
260. Waldner, M.J., S. Foersch, and M.F. Neurath, *Interleukin-6 - A Key Regulator of Colorectal Cancer Development.* International Journal of Biological Sciences, 2012. **8**(9): p. 1248-1253.
261. Ning, Y., et al., *Interleukin-8 is associated with proliferation, migration, angiogenesis and chemosensitivity in vitro and in vivo in colon cancer cell line models.* Int J Cancer, 2011. **128**(9): p. 2038-49.
262. Allakhverdi, Z., et al., *Cutting edge: The ST2 ligand IL-33 potently activates and drives maturation of human mast cells.* J Immunol, 2007. **179**: p. 2051 - 2054.
263. Cherry, W., et al., *A novel IL-1 family cytokine, IL-33, potently activates human eosinophils.* J Allergy Clin Immunol, 2008. **121**: p. 1484 - 1490.

264. Komai-Koma, M., et al., *IL-33 is a chemoattractant for human Th2 cells.* Eur J Immunol, 2007. **37**: p. 2779 - 2786.
265. Altomare, D.A. and J.R. Testa, *Perturbations of the AKT signaling pathway in human cancer.* Oncogene, 0000. **24**(50): p. 7455-7464.
266. Weinberg, E.O., et al., *Expression and regulation of ST2, an interleukin-1 receptor family member, in cardiomyocytes and myocardial infarction.* Circulation, 2002. **106**(23): p. 2961-6.
267. Tominaga, S.-i., et al., *Dual function of IL-33 on proliferation of NIH-3T3 cells.* Cytokine, 2015. **72**(1): p. 105-108.
268. Masamune, A., et al., *Nuclear expression of interleukin-33 in pancreatic stellate cells.* Am J Physiol Gastrointest Liver Physiol, 2010. **299**(4): p. 5.
269. Tong, X., et al., *Interleukin-33 predicts poor prognosis and promotes ovarian cancer cell growth and metastasis through regulating ERK and JNK signaling pathways.* Mol Oncol, 2015. **9**(15): p. 00128-3.
270. Zhang, P., et al., *Detection of interleukin-33 in serum and carcinoma tissue from patients with hepatocellular carcinoma and its clinical implications.* J Int Med Res, 2012. **40**(5): p. 1654-61.
271. Ye, X.L., et al., *IL-33-induced JNK pathway activation confers gastric cancer chemotherapy resistance.* Oncol Rep, 2015. **33**(6): p. 2746-52.
272. Goede, V., et al., *Induction of inflammatory angiogenesis by monocyte chemoattractant protein-1.* Int J Cancer, 1999. **82**(5): p. 765-70.
273. Guabiraba, R., et al., *IL-33 targeting attenuates intestinal mucositis and enhances effective tumor chemotherapy in mice.* Mucosal Immunol, 2014. **7**(5): p. 1079-93.
274. Li, D., et al., *IL-33 promotes ST2-dependent lung fibrosis by the induction of alternatively activated macrophages and innate lymphoid cells in mice.* Journal of Allergy and Clinical Immunology, 2014. **134**(6): p. 1422-1432.e11.
275. Zhang, Y., et al., *High-Infiltration of Tumor-Associated Macrophages Predicts Unfavorable Clinical Outcome for Node-Negative Breast Cancer.* PLoS One, 2013. **8**(9): p. e76147.
276. Barbera-Guillem, E., et al., *Vascular endothelial growth factor secretion by tumor-infiltrating macrophages essentially supports tumor angiogenesis, and IgG immune complexes potentiate the process.* Cancer Res, 2002. **62**(23): p. 7042-9.
277. Tazawa, H., et al., *Infiltration of neutrophils is required for acquisition of metastatic phenotype of benign murine fibrosarcoma cells: implication of inflammation-associated carcinogenesis and tumor progression.* Am J Pathol, 2003. **163**(6): p. 2221-32.
278. Jablonska, J., et al., *Neutrophils responsive to endogenous IFN-β regulate tumor angiogenesis and growth in a mouse tumor model.* The Journal of Clinical Investigation. **120**(4): p. 1151-1164.
279. Yadav, A., V. Saini, and S. Arora, *MCP-1: chemoattractant with a role beyond immunity: a review.* Clin Chim Acta, 2010. **411**(21-22): p. 1570-9.
280. Dhawan, P. and A. Richmond, *Role of CXCL1 in tumorigenesis of melanoma.* Journal of Leukocyte Biology, 2002. **72**(1): p. 9-18.
281. Kakkar, R. and R.T. Lee, *The IL-33/ST2 pathway: therapeutic target and novel biomarker.* Nat Rev Drug Discov, 2008. **7**(10): p. 827-40.
282. Choi, Y., et al., *Interleukin-33 induces angiogenesis and vascular permeability through ST2/TRAF6-mediated endothelial nitric oxide production.* Blood, 2009. **114**: p. 3117 - 3126.
283. Bouffi, C., et al., *IL-33 markedly activates murine eosinophils by an NF-kappaB-dependent mechanism differentially dependent upon an IL-4-driven autoinflammatory loop.* J Immunol, 2013. **191**(8): p. 4317-25.

284. Choi, Y.S., et al., *Nuclear IL-33 is a transcriptional regulator of NF-kappaB p65 and induces endothelial cell activation.* Biochem Biophys Res Commun, 2012. **421**(2): p. 305-11.
285. Ali, S., et al., *The dual function cytokine IL-33 interacts with the transcription factor NF-kappaB to dampen NF-kappaB-stimulated gene transcription.* J Immunol, 2011. **187**(4): p. 1609-16.
286. Kavandi, L., et al., *Progesterone and calcitriol attenuate inflammatory cytokines CXCL1 and CXCL2 in ovarian and endometrial cancer cells.* J Cell Biochem, 2012. **113**(10): p. 3143-52.
287. Xu, L., et al., *Hypoxia-induced elevation in interleukin-8 expression by human ovarian carcinoma cells.* Cancer Res, 1999. **59**(22): p. 5822-9.
288. Qian, D.Z., et al., *CCL2 is induced by chemotherapy and protects prostate cancer cells from docetaxel - induced cytotoxicity.* The Prostate, 2010. **70**(4): p. 433-442.
289. Alves-Filho, J., et al., *Interleukin-33 attenuates sepsis by enhancing neutrophil influx to the site of infection.* Nat Med, 2010. **16**: p. 708 - 712.
290. Waddell, A., et al., *IL-33 Signaling Protects from Murine Oxazolone Colitis by Supporting Intestinal Epithelial Function.* Inflamm Bowel Dis, 2015. **21**(12): p. 2737-46.
291. Bulek, K., et al., *The essential role of SIGIRR/TIR8 in regulation of Th2 immune response.* Journal of immunology (Baltimore, Md. : 1950), 2009. **182**(5): p. 2601-2609.
292. Gwak, J.M., et al., *Prognostic Value of Tumor-Associated Macrophages According to Histologic Locations and Hormone Receptor Status in Breast Cancer.* PLoS ONE, 2015. **10**(4): p. e0125728.
293. Zhang, Q.W., et al., *Prognostic significance of tumor-associated macrophages in solid tumor: a meta-analysis of the literature.* PLoS One, 2012. **7**(12): p. e50946.
294. Wang, C.C., et al., *Dimethyl sulfoxide promotes the multiple functions of the tumor suppressor HLJ1 through activator protein-1 activation in NSCLC cells.* PLoS One, 2012. **7**(4): p. 17.
295. Fang, J.Y. and B.C. Richardson, *The MAPK signalling pathways and colorectal cancer.* Lancet Oncol, 2005. **6**(5): p. 322-7.
296. Xie, Y., et al., *A novel T cell-based vaccine capable of stimulating long-term functional CTL memory against B16 melanoma via CD40L signaling.* Cell Mol Immunol, 2013. **10**(1): p. 72-7.
297. Prado-Garcia, H., et al., *Tumor-Induced CD8+ T-Cell Dysfunction in Lung Cancer Patients.* Clinical and Developmental Immunology, 2012. **2012**: p. 11.
298. Bonilla, W.V., et al., *The alarmin interleukin-33 drives protective antiviral CD8(+) T cell responses.* Science, 2012. **335**(6071): p. 984-9.
299. Tsutsumi, C., et al., *The critical role of ocular-infiltrating macrophages in the development of choroidal neovascularization.* J Leukoc Biol, 2003. **74**(1): p. 25-32.
300. Yang, Y., et al., *The PDGF-BB-SOX7 axis-modulated IL-33 in pericytes and stromal cells promotes metastasis through tumour-associated macrophages.* Nat Commun, 2016. **7**.
301. Kurowska-Stolarska, M., et al., *IL-33 amplifies the polarization of alternatively activated macrophages that contribute to airway inflammation.* J Immunol, 2009. **183**: p. 6469 - 6477.
302. Chen, J., et al., *Blockade of IL-33 ameliorates Con A-induced hepatic injury by reducing NKT cell activation and IFN-gamma production in mice.* J Mol Med, 2012. **90**(12): p. 1505-15.
303. Volarevic, V., et al., *Protective role of IL-33/ST2 axis in Con A-induced hepatitis.* J Hepatol, 2012. **56**(1): p. 26-33.

304. Akimoto, M., et al., *Interleukin-33 enhances programmed oncosis of ST2L-positive low-metastatic cells in the tumour microenvironment of lung cancer.* Cell Death Dis, 2016. **7**: p. e2057.
305. Milosavljevic, M.Z., et al., *Deletion of IL-33R attenuates VEGF expression and enhances necrosis in mammary carcinoma.* Oncotarget, 2016. **23**(10).
306. Seidelin, J., et al., *IL-33 is upregulated in colonocytes of ulcerative colitis.* Immunol Lett, 2010. **128**: p. 80 - 85.
307. Villarreal, D.O. and D.B. Weiner, *Interleukin 33: a switch-hitting cytokine.* Current Opinion in Immunology, 2014. **28**: p. 102-106.
308. Smyth, M.J., G.P. Dunn, and R.D. Schreiber, *Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity.* Adv Immunol, 2006. **90**: p. 1-50.
309. Ellyard, J.I., L. Simson, and C.R. Parish, *Th2-mediated anti-tumour immunity: friend or foe?* Tissue Antigens, 2007. **70**(1): p. 1-11.
310. Sims, J.E., et al., *IL-36: An Epithelial Cytokine Important in Psoriasis*, in *Cytokine Frontiers: Regulation of Immune Responses in Health and Disease*, T. Yoshimoto and T. Yoshimoto, Editors. 2014, Springer Japan: Tokyo. p. 199-214.
311. Ramadas, R.A., et al., *IL-1 Receptor antagonist as a positional candidate gene in a murine model of allergic asthma.* Immunogenetics, 2006. **58**(10): p. 851-855.
312. 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.
313. Walsh, J.G., et al., *Executioner caspase-3 and caspase-7 are functionally distinct proteases.* Proceedings of the National Academy of Sciences, 2008. **105**(35): p. 12815-12819.
314. Kanda, T., et al., *Interleukin(IL)-36α and IL-36γ Induce Proinflammatory Mediators from Human Colonic Subepithelial Myofibroblasts.* Frontiers in Medicine, 2015. **2**: p. 69.
315. 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.
316. Kurinna, S., et al., *Autocrine and Paracrine Regulation of Keratinocyte Proliferation through a Novel Nrf2-IL-36gamma Pathway.* J Immunol, 2016. **196**(11): p. 4663-70.
317. Bozoyan, L., et al., *Interleukin-36γ is expressed by neutrophils and can activate microglia, but has no role in experimental autoimmune encephalomyelitis.* Journal of Neuroinflammation, 2015. **12**(1): p. 1-10.
318. Luheshi, N.M., N.J. Rothwell, and D. Brough, *Dual functionality of interleukin-1 family cytokines: implications for anti-interleukin-1 therapy.* British Journal of Pharmacology, 2009. **157**(8): p. 1318-1329.
319. Lopetuso, L.R., S. Chowdhry, and T.T. Pizarro, *Opposing Functions of Classic and Novel IL-1 Family Members in Gut Health and Disease.* Front Immunol, 2013. **4**: p. 181.
320. Barksby, H.E., et al., *The expanding family of interleukin-1 cytokines and their role in destructive inflammatory disorders.* Clin Exp Immunol, 2007. **149**(2): p. 217-25.
321. Pizarro, T.T., et al., *IL-18, a novel immunoregulatory cytokine, is up-regulated in Crohn's disease: expression and localization in intestinal mucosal cells.* J Immunol, 1999. **162**(11): p. 6829-35.
322. Dupaul-Chicoine, J., et al., *Control of intestinal homeostasis, colitis, and colitis-associated colorectal cancer by the inflammatory caspases.* Immunity, 2010. **32**(3): p. 367-78.

323. Zaki, M.H., et al., *The NLRP3 inflammasome protects against loss of epithelial integrity and mortality during experimental colitis.* Immunity, 2010. **32**(3): p. 379-91.
324. Oficjalska, K., et al., *Protective role for caspase-11 during acute experimental murine colitis.* J Immunol, 2015. **194**(3): p. 1252-60.
325. McNamee, E.N., et al., *Interleukin 37 expression protects mice from colitis.* Proceedings of the National Academy of Sciences, 2011. **108**(40): p. 16711-16716.
326. Fehervari, Z., *IL-33 supports TH1 cells.* Nat Immunol, 2015. **16**(5): p. 447-447.
327. Sanapareddy, N., et al., *Increased rectal microbial richness is associated with the presence of colorectal adenomas in humans.* Isme j, 2012. **6**(10): p. 1858-68.
328. Weisburger, J.H., et al., *Germ-free status and colon tumor induction by N-methyl-N'-nitro-N-nitrosoguanidine.* Proc Soc Exp Biol Med, 1975. **148**(4): p. 1119-21.
329. Li, Y., et al., *Gut microbiota accelerate tumor growth via c-jun and STAT3 phosphorylation in APCMin/+ mice.* Carcinogenesis, 2012. **33**(6): p. 1231-8.
330. Handa, O., Y. Naito, and T. Yoshikawa, *Helicobacter pylori: a ROS-inducing bacterial species in the stomach.* Inflamm Res, 2010. **59**(12): p. 997-1003.
331. Gagnière, J., et al., *Gut microbiota imbalance and colorectal cancer.* World Journal of Gastroenterology, 2016. **22**(2): p. 501-518.
332. Sears, Cynthia L. and Wendy S. Garrett, *Microbes, Microbiota, and Colon Cancer.* Cell Host & Microbe. **15**(3): p. 317-328.
333. Tjalsma, H., et al., *A bacterial driver-passenger model for colorectal cancer: beyond the usual suspects.* Nat Rev Microbiol, 2012. **10**(8): p. 575-82.
334. Mahapatro, M., et al., *Programming of Intestinal Epithelial Differentiation by IL-33 Derived from Pericryptal Fibroblasts in Response to Systemic Infection.* Cell Rep, 2016. **15**(8): p. 1743-56.
335. Schwabe, R.F. and C. Jobin, *The microbiome and cancer.* Nat Rev Cancer, 2013. **13**(11): p. 800-12.
336. De Salvo, C., et al., *IL-33 Drives Eosinophil Infiltration and Pathogenic Type 2 Helper T-Cell Immune Responses Leading to Chronic Experimental Ileitis.* The American Journal of Pathology, 2016. **186**(4): p. 885-898.
337. Sinicrope, F.A., et al., *Intraepithelial effector (CD3+)/regulatory (FoxP3+) T-cell ratio predicts a clinical outcome of human colon carcinoma.* Gastroenterology, 2009. **137**(4): p. 1270-9.
338. Fridman, W.H., et al., *The immune contexture in human tumours: impact on clinical outcome.* Nat Rev Cancer, 2012. **12**(4): p. 298-306.
339. Deschoolmeester, V., et al., *Immune Cells in Colorectal Cancer: Prognostic Relevance and Role of MSI.* Cancer Microenvironment, 2011. **4**(3): p. 377-392.
340. Pantschenko, A.G., et al., *The interleukin-1 family of cytokines and receptors in human breast cancer: implications for tumor progression.* Int J Oncol, 2003. **23**(2): p. 269-84.
341. Malik, S.T., et al., *Effects of intraperitoneal recombinant interleukin-1 beta in intraperitoneal human ovarian cancer xenograft models: comparison with the effects of tumour necrosis factor.* British Journal of Cancer, 1992. **65**(5): p. 661-666.
342. Braunschweiger, P.G., et al., *Potentiation of interleukin 1 alpha mediated antitumor effects by ketoconazole.* Cancer Res, 1990. **50**(15): p. 4709-17.
343. Nakata, K., et al., *Augmented antitumor effect of recombinant human interleukin-1 alpha by indomethacin.* Cancer Res, 1988. **48**(3): p. 584-8.
344. Apte, R.N. and E. Voronov, *Interleukin-1--a major pleiotropic cytokine in tumor-host interactions.* Semin Cancer Biol, 2002. **12**(4): p. 277-90.
345. Cohen, S., L. Chen, and R.N. Apte, *Drug Delivery and Slow ReleaseControlled release of peptides and proteins from biodegradable polyester microspheres: an approach*

for treating infectious diseases and malignancies. Reactive Polymers, 1995. **25**(2): p. 177-187.
346. McCune, C.S. and D.M. Marquis, *Interleukin 1 as an Adjuvant for Active Specific Immunotherapy in a Murine Tumor Model.* Cancer Research, 1990. **50**(4): p. 1212-1215.
347. Eberl, M., et al., *IL-18 potentiates the adjuvant properties of IL-12 in the induction of a strong Th1 type immune response against a recombinant antigen.* Vaccine, 2000. **18**(19): p. 2002-2008.
348. Liu, J., et al., *Significance of interleukin-33 and its related cytokines in patients with breast cancers.* Front Immunol, 2014. **5**: p. 141.
349. Chen, S.F., et al., *The paracrine effect of cancer-associated fibroblast-induced interleukin-33 regulates the invasiveness of head and neck squamous cell carcinoma.* J Pathol, 2013. **231**(2): p. 180-9.
350. Maywald, R.L., et al., *IL-33 activates tumor stroma to promote intestinal polyposis.* Proc Natl Acad Sci U S A, 2015. **112**(19): p. E2487-96.
351. Matzinger, P., *The danger model: a renewed sense of self.* Science, 2002. **296**(5566): p. 301-5.
352. Lu, B., M. Yang, and Q. Wang, *Interleukin-33 in tumorigenesis, tumor immune evasion, and cancer immunotherapy.* J Mol Med (Berl), 2016. **94**(5): p. 535-43.
353. Ishikawa, K., et al., *Expression of interleukin-33 is correlated with poor prognosis of patients with squamous cell carcinoma of the tongue.* Auris Nasus Larynx, 2014. **41**(6): p. 552-7.
354. Bergis, D., et al., *High Serum Levels of the Interleukin-33 Receptor Soluble ST2 as a Negative Prognostic Factor in Hepatocellular Carcinoma.* Transl Oncol, 2013. **6**(3): p. 311-8.
355. Sun, P., et al., *Serum interleukin-33 levels in patients with gastric cancer.* Dig Dis Sci, 2011. **56**(12): p. 3596-601.
356. Oberg, A., et al., *Different occurrence of CD8+, CD45R0+, and CD68+ immune cells in regional lymph node metastases from colorectal cancer as potential prognostic predictors.* Int J Colorectal Dis, 2002. **17**(1): p. 25-9.
357. Pages, F., et al., *In situ cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer.* J Clin Oncol, 2009. **27**(35): p. 5944-51.
358. Galon, J., et al., *Type, density, and location of immune cells within human colorectal tumors predict clinical outcome.* Science, 2006. **313**(5795): p. 1960-4.

Appendix

A. Table of Primers

Gene	Species	Direction	Sequence (5' to 3')
β-actin	Human	F	ATTGGCAATGAGCGGTTC
		R	GGATGCCACAGGACTCCA
β-actin	Mouse	F	AAGGCCAACCGTGAAAAGAT
		R	GTGGTACGACCAGAGGCATAC
CCL2	Human	F	TCAAACTGAAGCTCGCACTCT
		R	GTGACTGGGGCATTGATTG
CCL2	Mouse	F	TCACTGAAGCCAGCTCTCTCT
		R	GTGGGGCGTTAACTG
COX2	Human	F	CTTCACGCATCAGTTTTTCAA
		R	TCACCGTAAATATGATTTAAGTCC
CXCL-1	Human	F	TCCTGCATCCCCCATAGTTA
		R	CTTCAGGAACAGCCACCAGT
CXCL-8	Human	F	GAGCACTCCATAAGGCAC
		R	ATGGTTCCTTCCGGTGGT
GAPDH	Human	F	AGCCACATCGCTCAGACAC
		R	GCCCAATACGACCAAATC
IL-1RAcP	Human	F	CCCTCTCAGCTTCCCAAGA
		R	GGGCAAGAGTGAGGCTTCTA
IL-6	Human	F	CAGGAGCCCAGCTATGAACT
		R	AGCAGGCAACACCAGGAG
IL-8	Human	F	AGACAGCAGAGCACACAAGC
		R	ATGGTTCCTTCCGGTGGT
IL-36RN	Human	F	GAGGAACAGGCAGACTCCAC
		R	CAATGCCGAGTCCTTCATTC
IL-13	Mouse	F	CCTCTGACCCTTAAGGAGCTTAT

		R	CGTTGCACAGGGGAGTCT
IL-33	Human	F	AGCAAAGTGGAAGAACACAGC
		R	CTTCTTTGGCCTTGTGTTGG
IL-36β	Human	F	TGAAGACATCATGAACCCACA
		R	TGTCGAGAATCACGAATAGCA
IL-36R	Human	F	GCGTGTCAAGCCATACTGAC
		R	CCTCCATATCCAGCTCTTTCTG
IL-36γ	Human	F	AAGTGACAGTGTGACCCCAGT
		R	GGATTCTGGATTCCCAAATAAA
LTα	Human	F	CAGCCCCGACCTAGAACC
		R	TTTATAGAGGAAGCGGCAGTG
LTα	Mouse	F	TTCGACTGAAACAGCAGCAT
		R	GGTGGTGTCATGGGGAGA
ST2	Human	F	TTGTCCTACCATTGACCTCTACAA
		R	GATCCTTGAAGAGCCTGACAA
ST2L	Human	F	GGGAGAGATATGCTACCTGGAGA
		R	CGCCTGCTCTTTCGTATG
TGF-β	Human	F	ACTACTACGCCAAGGAGGTCAC
		R	TGCTTGAACTTGTCATAGATTTCG
TGF-β	Mouse	F	CTCCGCTGACTCTCTTGG
		R	AGGTGGTCGCAAAAACGA
TNFα	Human	F	CGCTCCCCAAGAAGACAG
		R	AGAGGCTGAGGAACAAGCAC
VEGF	Human	F	TTAAACGAACGTACTTGCAGATG
		R	GAGAGATCTGGTTCCCGAAA
VEGF	Murine	F	AAAAACGAAAGCGCAAGAAA
		R	TTTCTCCGCTCTGAACAAGG

B. Table of Antibodies 1

Antibody against	Supplier	Cat no.	Monoclonal/ polyclonal	Clone	Reacts with	WB	IHC	Migration
IL-33	Enzo Life Sciences (Farmingdale, NY.)	ALX-804-840	M	Nessy-1	Human, mouse	1:500		
ST2L	Novus (Littleton, CO),	NBP1-85251	P		Human			
ST2	Abcam (Cambridge, UK),	ab25877	P		Human Mouse	1:500	1:400	
ST2V	Millipore	06-1116	P		Human			
CCL2	R&D Systems (Minneapolis, MN).	AF-479-NA	P		Mouse			1.5 ng/ml
Rabbit anti-mouse Ig/HRP	Dako Corp (Carpinteria, CA).	P0260	P		Mouse	1:10,000		
Goat anti-rabbit Ig/HRP	Dako Corp (Carpinteria, CA).	P0448	P		Rabbit	1:10,000		
β-actin	Sigma Aldrich (St Louis, MO)	A5441	M	AC-15	Human mouse	1:10,000		
IL-36R	Abcam	ab180894	P		Human mouse, rat		1:2000	
IL-36α	Abcam	Ab180909	P		Human		1:2000	
IL-36β	Novus	NBP1-83892	P		Human		1:100	
IL-36γ	LifeSpan BioSciences (Seattle)	LS-C338023	M	2F4	Human		1:300	

C. **Table of Antibodies 2**

Pre-diluted antibodies			
Antibody targeting	**Supplier**	**Catalogue number**	**Specificity**
CD4 (SP35)	Ventana (Arizona, USA)	7904423	Human
CD8 (SP57)	Ventana	790-4460	Human
CD20 (L26)	Ventana	760-2531	Human
CD45(RP2/18)	Ventana	760-2505	Human
CD68 (514H12)	Leica (Newcastle Upon Tyne, UK)	PA0273	Human

D. Table of Reagents

Reagent	Supplier	Catalogue number	Species	Concentration	
				WB	Migration
TNF-α	PeproTech (Rocky Hill, NJ),	300-01A	Human	10ng/ml	
LPS	Invivogen (San Diego, CA	O111:B4	Human	100ng/ml	
PGE_2	Cayman Company (Ann Arbor, MI	14810	Human	1 μM/ml	
IL-33	ProSpec (East Brunswick, NJ).	CYT-425	Human		15-60 ng/ml
IL-33	ProSpec	CYT-655	Mouse		15-60 ng/ml
IL-36α	Biolegend , Inc. (San Diego, CA).	551602	Human	25 -150 ng/ml	100ng/ml
IL-36β	ProSpec	CYT-159	Human	25 -150 ng/ml	100ng/ml
IL-36γ	Biolegend	711602	Human	25 -150 ng/ml	100mg/ml
CCL2	Biolegend	576502	Mouse		40 ng/ml