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The immunomodulatory chemokine CCL28 in oesophageal disease progression

A dissertation submitted to the University of Dublin

for the degree of Doctor of Philosophy

by

Sarah Louise Picardo

under the supervision of Dr. Stephen Maher

Departmental head: Professor John V. Reynolds

at the

Department of Surgery, Institute of Molecular Medicine, St. James's Hospital, Dublin

August 2014

Declaration

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SPicardo

Sarah Picardo

August 2014

Summary

Barrett's oesophagus (BO) develops in response to bile acid reflux and confers increased risk of progression to oesophageal adenocarcinoma (OAC). During this disease progression, the immune microenvironment of the oesophageal tissue changes. Gene expression of the immunomodulatory chemokine *CCL28* has previously been shown to be increased in OAC; in this work, its role in oesophageal disease progression is examined.

Human normal and BO tissue explants, as well as oesophageal cell lines, representing various stages of oesophageal disease progression, were cultured with or without the bile acid deoxycholic acid (DCA), a major component of refluxate. Gene and protein expression of CCL28 was measured, along with expression of the pro-inflammatory cytokines IL-1β and TNF-α, known to regulate CCL28 expression in other tissue types. Transcription factor arrays were used to identify transcription factors regulating CCL28 expression in the oesophagus. Validation of the transcription factor NF-kB was performed in an OAC cell line by stable transfection of an overexpressing plasmid. The chemoattractant properties of CCL28 were examined using a transwell chemotaxis assay. Immunohistochemical staining for CCL28 was performed in oesophageal tissue microarrays.

BO tissue expressed increased CCL28, IL-1β and TNF-a compared with matched normal tissue, while DCA induced CCL28 expression in normal tissue but not in BO tissue. BO and OAC cell lines secreted CCL28 protein in response to DCA, via the pro-inflammatory cytokine IL-1β but not via TNF-α. IL-1 receptor neutralisation decreased DCA-induced CCL28 production. Using transcription factor arrays, the transcription factors NF-kB, HIF-1α, XBP1, PPARα and p53 were

identified as regulators of DCA-induced CCL28 expression in the oesophagus. Stably transfected oesophageal cell lines overexpressing NF-kB demonstrated increased DCA-induced CCL28 expression, validating NF-kB as a regulator of CCL28, while treatment with an NF-kB inhibitor decreased this expression. CCL28 secreted by oesophageal cells was demonstrated to be chemotactic for immune cells bearing the receptors CCR3 and CCR10. Immunohistochemical staining in normal, BO, dysplastic and OAC tissue demonstrated increased CCL28 in BO and low-grade dysplastic tissue compared with normal and OAC tissue. CCL28 expression correlated with lower tumour stage, previous diagnosis of BO and increased survival in OAC. CCL28 expression in BO tissue also correlated with increased risk of progression to high-grade dysplasia and OAC.

This study is the first to show expression of the chemokine CCL28 by BO and OAC tissue and cell lines. Expression is modulated by the bile acid DCA via the transcription factor NF-kB and the pro-inflammatory cytokine IL-1β. CCL28 secreted by the oesophagus was functional in chemoattracting CCR3 and CCR10 expressing lymphocytes. CCL28 expression correlates with increased survival in patients with OAC and correlates with risk of disease progression in patients with BO. DCA-induced CCL28 may alter the immune microenvironment of the oesophagus to affect disease progression during the metaplasia-dysplasia-adenocarcinoma sequence.

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I would initially like to acknowledge the generous funding I received from the Irish Cancer Society Research Scholarship (CRS10PIC), without which none of this work could have been carried out.

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Finally, I would like to thank my husband Sven. Words are not enough to thank you for everything you did for me during this PhD. Sorry for the many many

times I didn't spend time with you because of this thesis, I love you, and we have the rest of our lives together.

Dedication

I would like to dedicate this work to my grandfather, Adolph Dias.

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Abbreviations

AMP – adenosine monophosphate

APS - ammonium persulfate

AWD – alive with disease

BCA – bicinchoninic acid

BCM – Barrett's conditioned medium

BEBM - Bronchial Epithelial Cell Basal Medium

BO – Barrett's oesophagus

BSA – bovine serum albumin

CCK – cholecystokinin

cDNA – complementary deoxyribonucleic acid

CK8 – cytokeratin 8

CO2 – carbon dioxide

COX-2 – cyclooxygenase-2

CRT – chemoradiation therapy

Cu – copper

DAB - 3, 3'-diaminobenzidine

dATP – deoxyadenosine triphosphate

DC – dendritic cell

DCA – deoxycholic acid

dCTP – deoxycytidine triphosphate

dGTP – deoxyguanosine triphosphate

dH2O – distilled water

DHMEQ – dehydroxymethylepoxyquinomicin

DMSO - dimethyl sulfoxide

DNA – deoxyribonucleic acid

dNTP – deoxyribonucleotide triphosphate

DOD – died of disease

DTT – dithiothreitol

dTTP – deoxythymidine triphosphate

EDTA - ethylene diamine tetra-acetic acid

ELISA – enzyme-linked immunosorbent assay

ER – endoplasmic reticulum

ERK – extracellular signal-related kinase

FACS – fluorescence-activated cell sorting

FBS – foetal bovine serum

FFPE - formalin-fixed paraffin-embedded

FOXP3 – forkhead box P3

FXR – farsenoid X receptor

G418 – geneticin

GAPDH – glyceraldehyde 3-phosphate dehydrogenase

GFP – green fluorescent protein

GORD – gastro-oesophageal reflux disease

H_2SO_4 – sulphuric acid

HBSS - Hank's Balanced Salt Solution

HCl – hydrochloric acid

HGD – high-grade dysplasia

HIF – hypoxia inducible factor

HIV – human immunodeficiency virus

HRP – horseradish peroxidase

IFN – interferon

IgE – immunoglobulin-E

IkB – inhibitor of kappa B

IKK – inhibitor of kappa B kinase

IL-1R1 – interleukin 1 receptor 1

IL-1RA – interleukin-1 receptor antagonist

IL-1β – interleukin 1-β

IL-6 – interleukin-6

IL-8 – interleukin-8

IMC – intramucosal carcinoma

IPA – Ingenuity Pathway Analysis

JNK – c-Jun N-terminal kinase

KCl – potassium chloride

KLF4 – Kruppel-like factor-4

LDH – lactate dehydrogenase

LGD – low-grade dysplasia

LOS – lower oesophageal sphincter

LOX – lipooxygenase

MCP-1 – monocyte chemotactic protein-1

MeOH – methanol

$MgCl_2$ – magnesium chloride

MIP-1α – macrophage inflammatory protein-1α

MMP - matrix metalloproteinase

NADH – nicotinamide adenine dinucleotide

NF-kB – nuclear factor kappa-light-chain-enhancer of activated B cells

NK – natural killer

NLR – Nod-like receptor

OAC – oesophageal adenocarcinoma

OGJ – oesophagogastric junction

PBMC – peripheral blood mononuclear cells

PBS – phosphate buffered saline

PC – personal computer

PCR – polymerase chain reaction

PE – phycoerythrin

PGF – prostaglandin-F

PI – propidium iodide

PMSF – phenylmethylsulphonyl fluoride

POSTN - periostin

PPAR – peroxisome proliferator-activated receptor

PPI – proton pump inhibitors

PXR – pregnane X receptor

qPCR – quantitative polymerase chain reaction

rh – recombinant human

RNA – ribonucleic acid

RT°C – room temperature

RXR – retinoid X receptor

SCC – squamous cell carcinoma

SD – standard deviation

SDS – sodium dodecyl sulphate

SEM – standard error of the mean

siRNA – small interfering ribonucleic acid

STAT3 – signal transducer and activator of transcription-3

TAE - Tris-acetate ethylene diamine tetra-acetic acid

TAM – tumour-associate macrophage

TBS – Tris-buffered saline

TBST – Tris-buffered saline containing 0.1% Tween-20

TEMED - N, N, N', N'-tetramethylethylenediamine

TF – transcription factor

TFF1 – trefoil factor 1

TGF-β1 – transforming growth factor-β1

Th – 2 – T-helper – 2

Th-1 – T-helper – 1

TLR – Toll-like receptor

TMA – tissue microarray

TMB - 3, 3′,5,5′-Tetramethylbenzidine

TMEPAI – transmembrane prostate androgen-induced protein

TNF-R1 – tumour necrosis factor receptor 1

TNF-α – tumour necrosis factor α

TNM – tumour, node, metastases

TRADD – Tumour necrosis factor receptor type 1-associated DEATH domain protein

TRAF2 – tumour necrosis factor receptor-associated factor 2

Treg – T regulatory

TRG – tumour regression grade

TSP-1 – thrombospondin-1

uPA – urokinase-type plasminogen activator

UV – ultraviolet

VDR – vitamin D receptor

VEGF - vascular endothelial growth factor

VHL – von Hippel-Lindau

XBP-1 – X-box binding protein-1

YY1 – yin yang-1

βFGF – β-fibroblast growth factor

Units

bp	base pairs
°C	degrees Celsius
cm	centimetre
g	grams
× *g*	acceleration due to gravity
h	hour
IU	international units
kb	kilobase
kDa	kiloDalton
M	molar
mA	milliamp
mg	milligram
min	minute
mL	millilitre
mM	millimolar
mm	millimetre
µg	microgram
µL	microlitre
µM	micromolar
ng	nanogram
nM	nanomolar
nm	nanometre
nt	nucleotide

pg	picogram
s	seconds
U	units
V	volts
v/v	volume per volume
w/v	weight per volume
X	magnification

Publications, presentations and awards

Publications

Picardo SL, Maher SG, O'Sullivan JN, Reynolds JV "Barrett's to oesophageal cancer sequence: a model of inflammatory-driven upper gastrointestinal cancer" Dig Surg. 2012;29(3):251-60 Epub 2012 Aug 3 (review article)

Oral presentations

SARS Surgical Meeting, Dublin, Ireland, January 2011 "*Expression of the chemokine CCL28 and the effects of bile acid in Barrett's oesophagus and oesophageal adenocarcinoma"* (Patey Prize (plenary) session) Sarah L. Picardo, Gary Sommerville, Stephen G. Maher, John V. Reynolds

Trinity School of Medicine Postgraduate Research Day, Dublin, Ireland, September 2012 "*The immunomodulatory chemokine CCL28 in Barrett's oesophagus and oesophageal adenocarcinoma*" Sarah L. Picardo, Gary Sommerville, Stephen G. Maher, John V. Reynolds

Poster presentations

12th Annual Meeting of the Institute of Molecular Medicine, Dublin, Ireland, November 2009 *Bile acid exposure stimulates expression of the CCL28 chemokine in oesophageal cancer* Sarah L. Picardo, Gary Sommerville, Stephen G. Maher, John V. Reynolds

OESO 10th World Oesophageal Conference, Boston, USA, August 2010, *The chemokine CCL28 is increased by bile acid in esophageal adenocarcinoma* Sarah L. Picardo, Gary Sommerville, Stephen G. Maher, John V. Reynolds

13th Annual Meeting of the Institute of Molecular Medicine, Dublin, Ireland, November 2010 *The chemokine CCL28 is increased by bile acid in oesophageal adenocarcinoma* Sarah L. Picardo, Gary Sommerville, Stephen G. Maher, John V. Reynolds

5th National Barrett's Symposium, University College London, London, UK, April 2011, *Levels and importance of CCL28 in Barrett's oesophagus and oesophageal*

adenocarcinoma (Poster shortlisted in final 8) Sarah L. Picardo, Gary Sommerville, Jacintha O'Sullivan, John V. Reynolds, Stephen G. Maher

8th International Cancer Conference, Dublin, Ireland, September 2011 *Characterisation of the chemokine CCL28 in Barrett's oesophagus and oesophageal adenocarcinoma* Sarah Picardo, Stephen G. Maher, Jacintha O'Sullivan, John V. Reynolds

Tercentenary Symposium of the School of Medicine, Dublin, Ireland, November 2011, *Bile acid-induced regulation of the chemokine CCL28 in Barrett's oesophagus and oesophageal adenocarcinoma* Sarah L. Picardo, Stephen G. Maher, John V. Reynolds

UKCRF Network Conference, Dublin, Ireland, July 2012, "*Expression of the immunomodulatory chemokine CCL28 in an ex vivo model of Barrett's Oesophagus"* Sarah L. Picardo, John V. Reynolds, Stephen G. Maher

Irish Association for Cancer Research/European Association for Cancer Research Joint Conference on the Tumour Microenvironment, Dublin, Ireland, September 2012, *The immunomodulatory chemokine CCL28 in Barrett's oesophagus and oesophageal adenocarcinoma* Sarah L. Picardo, Ronan Feighery, Naoimh F. O'Farrell, Rory Casey, Gary Sommerville, Joanne Lysaght, Mary-Clare Cathcart, John V. Reynolds, Jacintha O'Sullivan, Stephen G. Maher

Awards

Irish Cancer Society Research Scholarship 2010 (Scholarship code *CRS10PIC*)

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Chapter 1

Introduction

1.1 The oesophagus

1.1.1 Anatomy of the oesophagus

The human oesophagus is an organ which is a part of the gastrointestinal tract, connecting the pharynx to the stomach. It is a muscular tube approximately 26 cm in length, the majority of which is found in the thoracic cavity. Embryologically, the oesophagus develops from foregut and is initially lined with ciliated epithelium, which is replaced by squamous epithelium at approximately 4 months gestation. The adult oesophagus has four main layers: an inner mucosa, consisting of a stratified squamous epithelium, the submucosa, which contains connective tissue in which lie blood vessels, lymphatics and lymphoid tissue, mucous glands and nerves, the muscularis propria consisting of an inner layer of circular muscle and an outer layer of longitudinal muscle, and finally an outer layer of adventitia. The muscular layers contract in waves called peristalsis, which push boluses of food material from the mouth down to the stomach for digestion. The muscle in the oesophagus is striated in the upper third of the organ, mixed in the middle third, and smooth in the lower third. Anatomically, the oesophagus commences at the level of the C6 cervical vertebra and continues into the thoracic cavity, where it passes firstly through the superior mediastinum and then into the posterior mediastinum. From here it passes inferiorly and anteriorly to the oesophageal hiatus in the diaphragm at the level of the T10 vertebra. In the abdomen, the oesophagus continues for approximately 1 cm until it reaches the cardia of the stomach at the gastrooesophageal junction (Figure 1.1). The arterial blood supply of the oesophagus varies throughout its course: the upper third receives blood from the inferior thyroid artery, the middle third from branches of the thoracic aorta, and the lower third from the left gastric branch of the coeliac trunk.

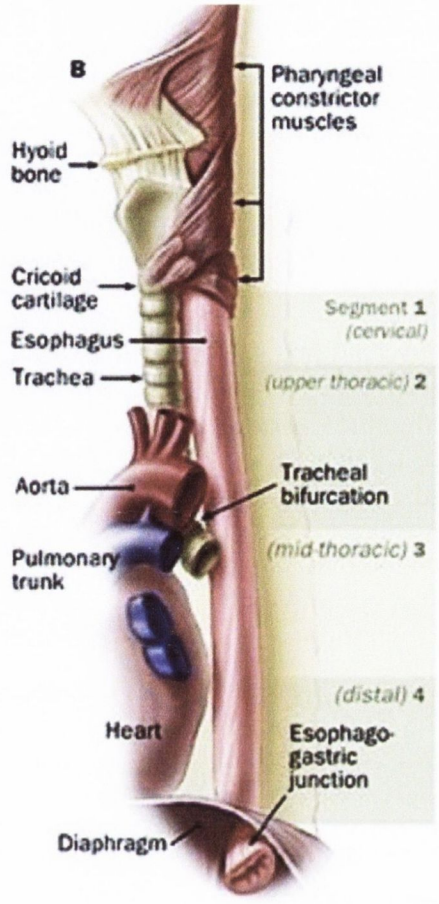


Figure 1.1. Anatomy of the oesophagus and oesophageal sphincters. The anatomy of the normal human oesophagus, showing the relations of the oesophagus to major thoracic structures. (Adapted from www.painneck.com)

The venous drainage from the upper and lower thirds of the oesophagus is to the azygous system, while the middle third drains to the left gastric vein, a part of the portal system. The oesophagus receives extrinsic motor nerve supply mainly from the vagus nerve, as well as parasympathetic supply via the vagus nerve and sympathetic supply from the cervical and thoracic sympathetic chain. Motility and secretion in the oesophagus is also regulated by intrinsic innervation; the myenteric plexus, or Auerbach's plexus, is a complex network of ganglia located between the circular and longitudinal muscular layers, while the submucous, or Meissner's plexus, is found within the submucosa. Sensory innervation of the oesophagus is via vagal and spinal afferent neurons; spinal afferent neurons mediate the pain sensation when the oesophageal lining is exposed to acid during gastro-oesophageal reflux.

1.1.2 The lower oesophageal sphincter

Where the oesophagus and stomach meet, an area of high pressure is located, known as the lower oesophageal sphincter (LOS). This sphincter regulates the entry of boluses of food into the stomach, and is highly regulated. There are two components to the sphincter, an intrinsic part consisting of smooth muscle from the oesophagus and stomach, and an extrinsic part which is the crural diaphragm encircling the oesophagus (Figure 1.2). The LOS is under reflex control – sensory afferents responding to distension or swallowing travel in the vagus nerve and cause relaxation of the muscle to allow passage of a bolus of food into the stomach. The LOS muscle has a higher basal tone than the rest of the oesophageal muscle tissue, and has a higher resting membrane potential.

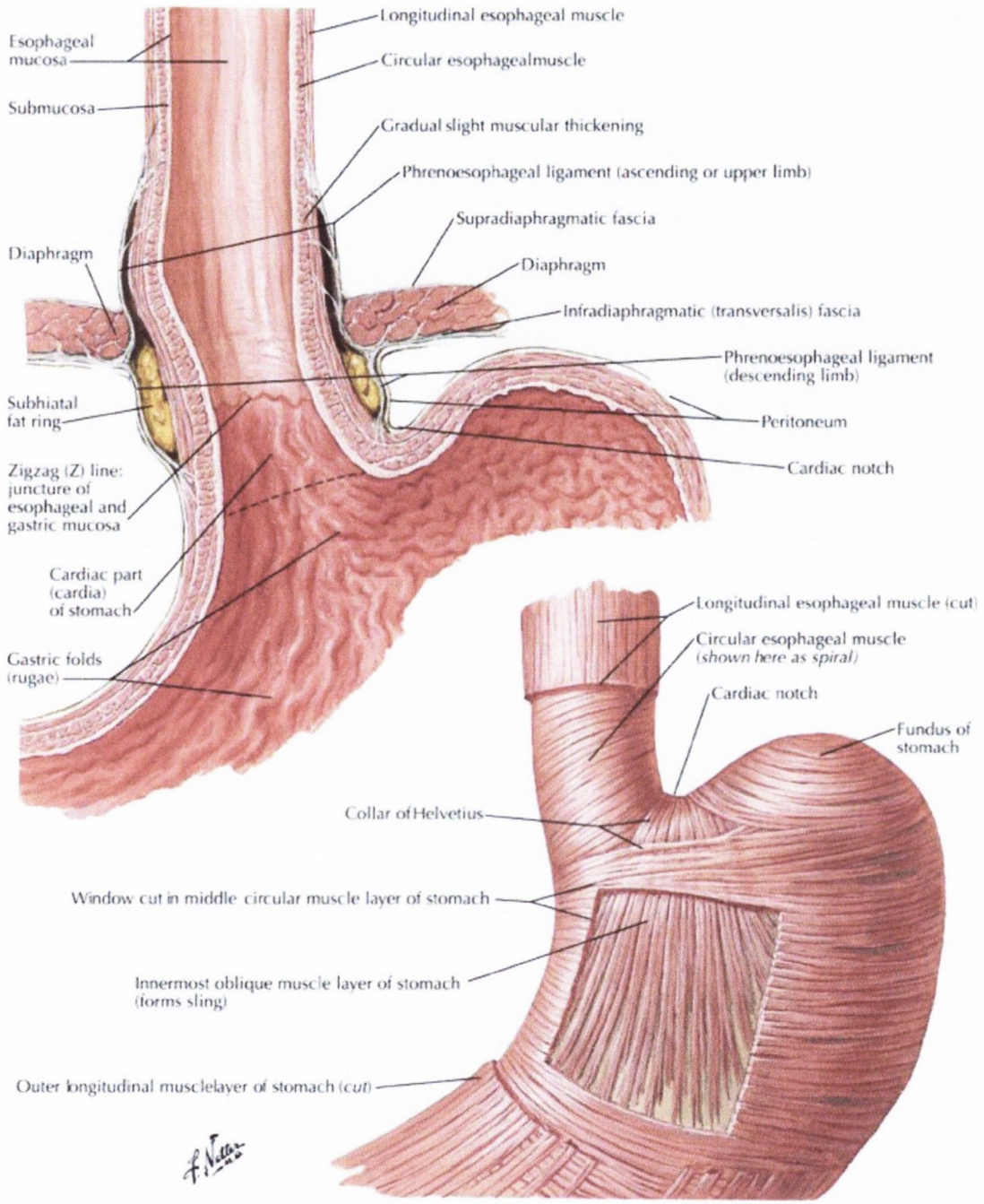


Figure 1.2. Anatomy of the lower oesophageal sphincter. Anatomy of the muscular fibres of the intrinsic and extrinsic lower oesophageal sphincters, demonstrating the diaphragmatic muscle, longitudinal and circular oesophageal muscles that make up this sphincter. (Source: Netter medical illustration)

Impairment of this highly regulated sphincter control mechanism is the cause of gastro-oesophageal reflux disease (GORD) and its complications. While it was previously thought that GORD was caused by a weakness of the LOS, the majority of cases of mild to moderate GORD are caused by inappropriate relaxation of the oesophageal and diaphragmatic components of the LOS, i.e. not in response to swallowing or peristalsis. It is now thought that most people have these transient relaxations of the lower oesophageal sphincter, (TLOSR) but that people who experience GORD have more reflux during these episodes.

1.2. Gastro-oesophageal reflux disease

GORD is the most common oesophageal disorder worldwide. It is estimated that 25-40% of healthy Americans experience symptomatic GORD. It is caused when acid and bile from the stomach refluxes into the lower oesophagus, causing the sufferer to experience a burning pain sensation in the middle of the chest, often described as 'heartburn'. However, many patients who complain of heartburn will not have erosive oesophagitis, do not respond to proton pump inhibitor (PPI) therapy and will not have abnormal findings on 24 h pH monitoring, suggesting that these patients do not have significant acid and bile exposure in their lower oesophagus. In most patients with symptomatic GORD confirmed on pH monitoring, TLOSR is the main cause for symptomatic episodes. However with more severe disease, lowering of the LOS tone and impairment of oesophageal contraction become more frequent. Symptomatic GORD is treated with PPIs; however up to 40% of patients do not respond to PPI treatment (Fass 2009).

1.2.1 Bile acids

Bile acids are a family of steroid molecules synthesised in the liver, where approximately 300 to 500 mg of cholesterol are converted to bile acids daily (Heaton 1979). They are produced as part of the metabolising of cholesterol, and consist of one cyclopentane and three cyclohexane rings, a carboxylic acid side chain and between 1 and 3 hydroxyl groups (Hofmann and Hagey 2008). The two primary bile acids made in the human liver are cholic acid and chenodeoxycholic acid, which are synthesised via the oxidation of cholesterol in the cytochrome P450 pathway. Three steps are involved in this pathway: firstly, insertion of a hydroxyl group into the 7α-position of cholesterol under the influence of the enzyme hydroxylase, secondly, a configurational change of the 3β-hydroxyl group to the 3α-position and finally, in the case of cholic acid, a 12α-hydroxylation and conversion of the 27-carbon cholesterol into a 24-carbon bile acid by oxidation and shortening of the side chain (Kauer and Stein 2010) (Figure 1.3).These primary bile acids are secreted into the lumen of the intestine, where intestinal bacteria dehydroxylate (7α-dehydroxylation) them to form the secondary bile acids, deoxycholic acid and lithocholic acid (Hofmann 1977). Bile acids can be reabsorbed into the bloodstream and returned to the liver via the enterohepatic circulation.

All bile acids can be conjugated to the amino acids glycine or taurine to form bile salts. The solubility of bile acids depends on a number of factors, including the pH of the solution they occur in, their pKa and the number of hydroxyl groups. Conjugation of bile acids to glycine or taurine decreases their pKa and allows them to become soluble in the acid pH of the intestinal lumen. The function of bile acids in the human intestine is to facilitate the absorption of lipids, as well as the fat-

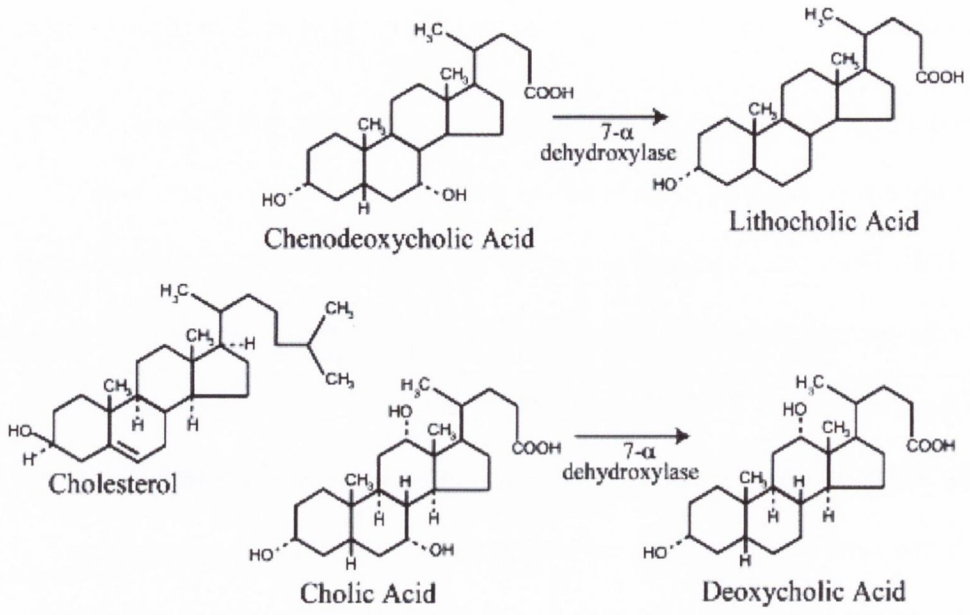


Figure 1.3. Formation of bile acids. Chenodeoxycholic acid and cholic acid are formed in the liver from cholesterol. The bacterial enzyme 7-α dehydroxylase converts chenodeoxycholic acid into lithocholic acid and converts cholic acid into deoxycholic acid (Bernstein, Bernstein et al. 2005)

soluble vitamins A, D, E and K, from the intestine (Russell et al, 2009). Since bile acids are amphipathic molecules, having hydrophobic and hydrophilic faces, they form micelles with lipids in the intestinal lumen, which allows absorption of the lipid molecules (Maldonado-Valderrama, Wilde et al. 2011). Bile acids also function in regulation of processes such as bile acid, lipid and glucose metabolism, by activating signalling pathways (Houten, Watanabe et al. 2006, Thomas, Pellicciari et al. 2008).

Bile acids can signal via a number of pathways. The nuclear receptor signalling pathways are activated by bile acids via the farsenoid X receptor (FXR), pregnane X receptor (PXR) and the vitamin D receptor (VDR) (Xie, Radominska-Pandya et al. 2001, Makishima, Lu et al. 2002, Wang, Chen et al. 2008). Bile acids can also activate kinase pathways including the JNK kinase, and can signal via G-protein coupled receptors including muscarinic receptors, formyl-peptide receptors and the receptor TGR5, which specifically binds bile acids (Pols, Noriega et al. 2011).

1.2.2 Bile acids in the oesophagus

Reflux of bile acids from the duodenum into the lower oesophagus has been hypothesised to be a causative factor in the development of Barrett's oesophagus and oesophageal adenocarcinoma. In animal models, exposure of the oesophagus to bile acids causes the animals to develop Barrett's metaplasia and oesophageal cancer; interestingly, in one rat model, acidic gastric juice appeared to protect the rats with bile acid reflux from developing oesophageal adenocarcinoma, while the rats who had bile acid reflux alone developed more frequent cancers (Ireland,

Peters et al. 1996, Nishijima, Miwa et al. 2004). This suggests that bile acids are the primary causative factors in refluxate that cause the progression of oesophageal disease, while also suggesting that perhaps the acid-suppressive treatments that are currently prescribed to patients with GORD are not the optimal treatment strategy.

The composition of duodenogastrooesophageal refluxate (DGOR) in patients with oesophageal disease has been the subject of much controversy since research into this topic began. A systematic review in 2011 showed that in five studies, patients with GORD had increased bile acids compared with asymptomatic controls (Iftikhar, Ledingham et al. 1993, Stein, Feussner et al. 1994, Iftikhar, Ledingham et al. 1995, Kauer, Peters et al. 1997, Nehra, Howell et al. 1998), while other studies demonstrated higher bile acids in reflux from patients with Barrett's oesophagus compared with GORD alone (McQuaid, Laine et al. 2011) (Figure 1.4). No consistent differences in the composition of the bile acids present were demonstrated between any of the groups. Increased number of patients in whom perfusion of the oesophagus was performed reported pain with perfusion of hydrochloric acid with bile salts compared with those perfused with hydrochloric acid alone (Bachir, Leigh-Collis et al. 1981), while another similar study demonstrated induction of pain with perfusion of acid alone and acid with bile acids, but not with bile acids alone (Bachir and Collis 1976).

While the relationship between GORD and oesophageal adenocarcinoma has been documented (Chow, Finkle et al. 1995, Lagergren, Bergstrom et al. 1999), the role of the components of refluxate is debated (Woodland and Sifrim 2010). Some studies demonstrate that both acid and bile exposure are increased in Barrett's patients compared with oesophagitis patients (Vaezi and Richter 1995,

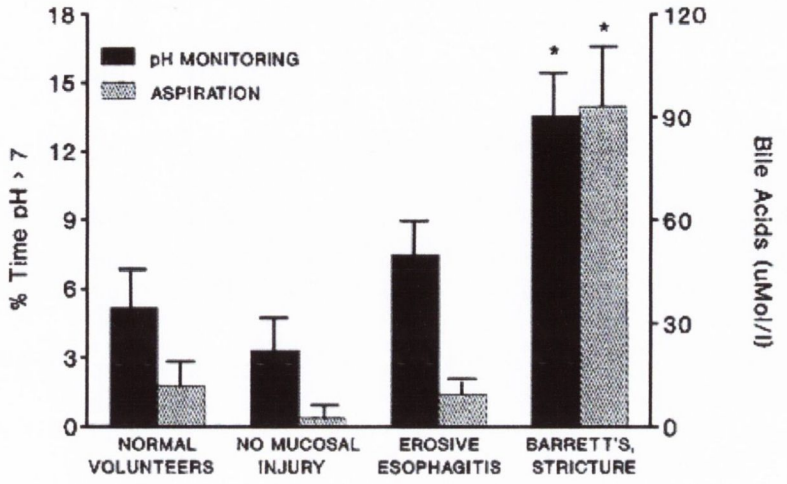


Figure 1.4. Bile acid concentrations and pH in the oesophagus of normal, oesophagitis and Barrett's patients. Total bile acid concentration in oesophageal reflux aspirates and the time that the pH was above seven on ambulatory 24-h oesophageal pH monitoring in normal volunteers ($n = 43$), patients with GERD and no mucosal injury ($n = 14$), patients with GERD and erosive oesophagitis ($n = 25$), and patients with GERD and stricture and/or Barrett's oesophagus ($n = 13$). *$p < 0.01$ vs. patients with no mucosal injury.

(Kauer and Stein, 2010)

Menges, Muller et al. 2001), with the latter study suggesting that Barrett's patients have higher gastric bile acid concentrations than control patients (Figure 1.5). The previously described animal models imply that duodenal reflux of bile acids is sufficient to induce the development of Barrett's and oesophageal adenocarcinoma, while another study suggests that acid alone may be the primary insulting factors in Barrett's oesophagus (Champion, Richter et al. 1994). Bile acids alone are certainly capable of inducing reflux oesophagitis in post-gastrectomy patients, who do not produce gastric acid (Yumiba, Kawahara et al. 2002).

1.3 Barrett's oesophagus (BO)

In a minority of patients with GORD, the normal squamous epithelial lining of the distal oesophagus is replaced by a specialised columnar type epithelium known as Barrett's epithelium (Badreddine and Wang 2010), named after Norman Rupert Barrett who first documented its existence in 1950 (Barrett 1950). This process in which one differentiated tissue is replaced by another is known as metaplasia, and since the columnar epithelium in Barrett's oesophagus is similar to that in the intestine, it is also known as intestinal metaplasia. Estimates of the prevalence of BO in the literature vary, as many cases go undiagnosed as diagnosis can only be made using upper gastrointestinal endoscopy. While population-based studies have shown prevalence of BO to be between 1.3% and 1.6% at endoscopy (Ronkainen, Aro et al. 2005, Zagari, Fuccio et al. 2008), Hayeck et al. estimate the prevalence of BO in the US to be 5.6% of the general population using a simulation model (Hayeck, Kong et al. 2010). The incidence of BO has been increasing in the Western world, a rise which cannot be explained by

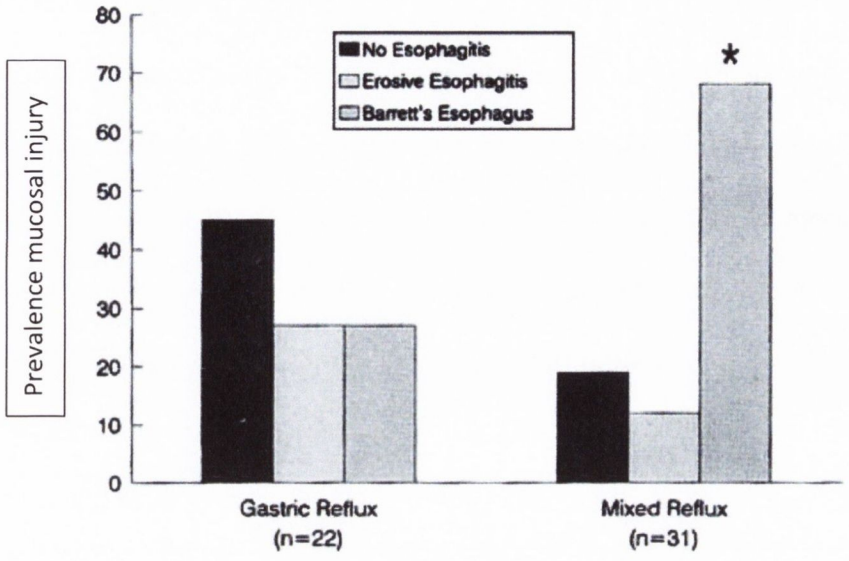


Figure 1.5. Oesophageal mucosal injury with gastric only and mixed gastric and duodenal refluxate. Prevalence of mucosal injury in patients with reflux of gastric juice only (gastric reflux) and in those with reflux of combined gastric and duodenal juices (mixed reflux) *$p < 0.005$ vs. gastric reflux.

(Kauer and Stein 2010)

increased rates of endoscopy (van Soest, Dieleman et al. 2005). Risk factors for the development of BO, apart from GORD, include male gender, Caucasian ethnicity, cigarette smoking, increasing age, obesity and a lack of *H. pylori* infection (Stein, El-Serag et al. 2005, Jacobson, Chan et al. 2009, Wang, Yuan et al. 2009, Cook, Shaheen et al. 2012). It is thought that the increases in obesity, which probably increases GORD (Gerson, Ullah et al. 2007), as well as a decrease in the rate of *H. pylori* infection, contribute to the increased incidence of BO, and indeed oesophageal adenocarcinoma (OAC) (Sonnenberg 2011). However GORD is the predominant risk factor for the development of BO. Reflux of both bile from the duodenum and acid from the stomach have been shown to contribute to BO (Champion, Richter et al. 1994, Vaezi and Richter 1996, Campos, DeMeester et al. 2001, Koek, Sifrim et al. 2008). However in animal studies, rats which underwent oesophago-jejunostomy to induce duodeno-oesophageal reflux of bile developed BO, despite the absence of gastric acid reflux (Nishijima, Miwa et al. 2004); the development of Barrett's oesophagus post gastrectomy has also been found in humans (Sinn, Kim et al. 2008, Matei, Dadu et al. 2010)

At endoscopic diagnosis, BO is seen as a reddish-coloured mucosa lining the distal oesophagus, a darker colour than the normal squamous mucosa (Figure 1.6). The length of segment of BO is measured from the proximal border of the gastric folds to the Z-line, or squamo-columnar junction, with a length >3 cm taken as long-segment BO, while a segment <3 cm is taken as short-segment BO. In Europe, a biopsy specimen is also necessary to confirm the diagnosis of BO – histology must show the presence of specialised intestinal metaplasia with columnar epithelial cells.

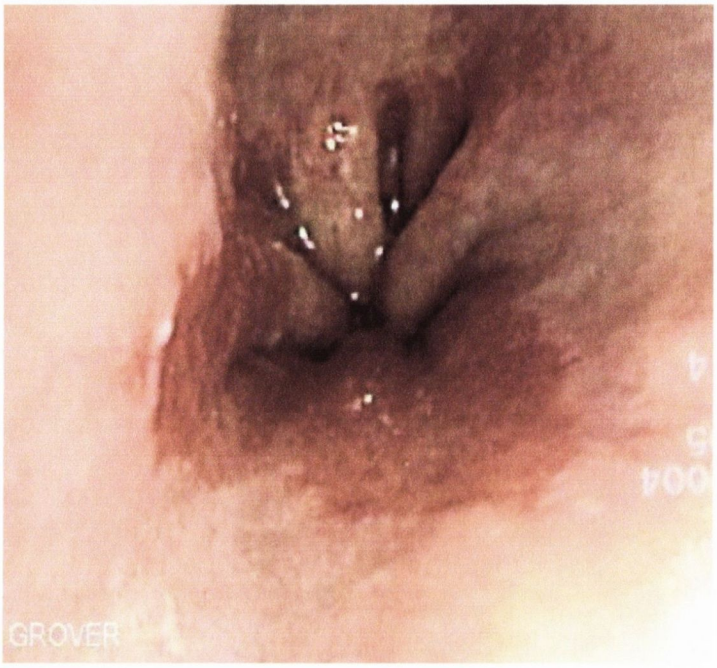


Figure 1.6. Barrett's oesophagus. Endoscopic image of Barrett's mucosa at the distal oesophagus demonstrating the reddish-coloured tongues above the gastric folds which represent metaplastic columnar epithelium (Source: www.deskarati.com)

1.4 Oesophageal cancer

Oesophageal cancers now represent one of the eight most common cancers worldwide, and ranks sixth in terms of cancer mortality (Kamangar, Dores et al. 2006). Oesophageal cancers are usually carcinomas, which can be subdivided into two main histological types, squamous cell carcinomas and adenocarcinomas, which are generally considered two separate diseases (Siewert and Ott 2007). Squamous cell carcinoma (SCC) of the oesophagus is the predominant subtype worldwide, accounting for 95% of cases of oesophageal cancer, and is usually located in the upper part of the oesophagus, arising from the squamous epithelial lining of the oesophagus. SCCs are most commonly found in Iran, Russia, China and Japan (Shibata, Matsuda et al. 2008), and risk factors for SCC of the oesophagus include cigarette smoking, alcohol consumption, betel quid chewing and genetic factors such as tylosis (Islami, Fedirko et al. 2011, Oze, Matsuo et al. 2012) (Iwaya, Maesawa et al. 1998, Shahabi, Noori Daloii et al. 2004, Chung, Lee et al. 2010, Steevens, Schouten et al. 2010). Adenocarcinoma of the oesophagus (OAC) is increasing in incidence in the Western world and is now the most common subtype of oesophageal cancer in the USA, while the incidence of SCC remains stable or appears to be decreasing (Bosetti, Levi et al. 2008, Cook, Chow et al. 2009, Jemal, Center et al. 2010). OAC arises from cells found in the lower oesophagus or at the gastrooesophageal junction (Figure 6). Risk factors for the development of OAC include male gender, obesity, diet, sedentary lifestyle, lack of *H. pylori* infection, gastrooesophageal reflux and BO (Solaymani-Dodaran, Logan et al. 2004, Kubo and Corley 2006, Abnet, Freedman et al. 2008, Balbuena and Casson 2009, Rubenstein and Taylor 2010, Vial, Grande et al. 2010,

Steevens, Schouten et al. 2011, Winberg, Lindblad et al. 2012, Yang, Sukocheva et al. 2012).

The reason that BO is the subject of research and surveillance is that the presence of Barrett's epithelium predisposes the patient to development of low grade dysplasia, high grade dysplasia and subsequently oesophageal adenocarcinoma (OAC) (Figure 1.7). OAC has demonstrated an increase in incidence of 400-600% over the last 30 years (Pohl and Welch 2005, Holmes and Vaughan 2007). The risk of developing OAC in a patient with BO is 30-125 times that of the general population (Wild and Hardie 2003).

1.4.1 Presentation and staging

OAC has an extremely poor prognosis, due predominantly to patients presenting in a late stage of disease, with a 5-year survival estimates ranging from 10-35% (Bonavina, Incarbone et al. 2003, Sundelof, Lagergren et al. 2008, Lagergren and Mattson 2012), although survival rates are slowly increasing. OAC usually presents with progressive dysphagia (difficulty swallowing), beginning with solids and progressing to liquids, and with weight loss. Other symptoms include odynophagia (pain on swallowing), cough, chest pain, and manifestations of metastatic disease. Staging of OAC is usually by the TNM staging system developed by the Union for International Cancer Control (IUCC), of which the most recent (7^{th}) edition was published in 2010 and allows good prognostic stratification of patients (Rice, Rusch et al. 2010, Talsma, van Hagen et al. 2012). This system gives OAC patients a stage from 0 to IV based on various parameters including pathological and histological tumour classification, histological grade, lymph node

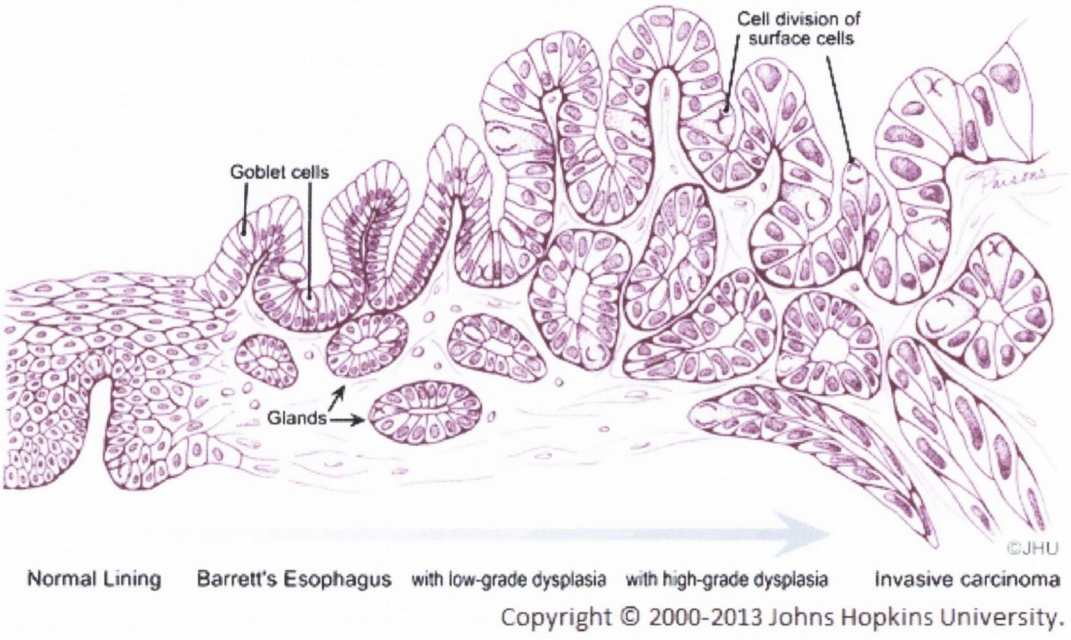


Figure 1.7. Progression of the oesophageal epithelium through the Barrett's – low-grade dysplasia – high-grade dysplasia – carcinoma sequence. This figure demonstrates the progression of the oesophageal epithelium as the columnar epithelium of Barrett's oesophagus develops from normal squamous epithelium. This can then progress to low-grade and high-grade dysplasia and finally to invasive carcinoma. (Source: John Hopkins University)

status and number of positive lymph nodes and presence of distant metastases (Table 1.1).

1.4.2 Treatment

Treatment for oesophageal cancer is difficult, as the majority of patients present at a late stage of disease; in an Irish high-volume centre for oesophageal cancer management, only 52% of presenting patients were treated with curative intent (Reynolds, McGillycuddy et al. 2011). For patients with advanced disease with metastases, often palliative treatment with oesophageal stenting is the only available option, improving the main symptom of dysphagia (Battersby, Bonney et al. 2012). Where curative therapy can be offered, the options are usually either surgery alone, surgery combined with chemoradiation therapy (CRT), or CRT alone.

Surgical treatment is usually offered to patients with stage I or II disease: oesophagectomy is a high-risk operation, with complications occurring in 61% of patients in one study, and an overall 5-year survival of 46.5%, although for stage I disease 5-year survival was 81% (Portale, Hagen et al. 2006). Other studies have demonstrated similarly poor complication rates and 5-year survival after oesophagectomy of 35-45% (Low, Kunz et al. 2007, Omloo, Lagarde et al. 2007, Leers, DeMeester et al. 2009). The outcome for patients postoperatively is related to the surgeon volume, with higher volume surgeons demonstrating decreased operative mortality (Birkmeyer, Siewers et al. 2002, Birkmeyer, Stukel et al. 2003). Histological tumour type may influence outcome, with adenocarcinoma having an increased postoperative survival rate in one study (Siewert, Stein et al. 2001),

Table 1.1. UICC staging for oesophageal cancer

Stage	T (Primary Tumour)	N (Regional Lymph Nodes)	M (Distant Metastases)
0	Tis	N0	M0
I	T1	N0	M0
IIA	T2	N0	M0
	T3	N0	M0
IIB	T1	N1	M0
	T2	N1	M0
III	T3	N1	M0
	T4	Any N	M0
IV	Any T	Any N	M1
IVA	Any T	Any N	M1a
IVB	Any T	Any N	M1b

UICC, International Union Against Cancer.

although a more recent study demonstrates no difference in survival after various treatment types including surgery between adenocarcinoma and squamous cell carcinoma (Chang, Chapman et al. 2009).

Neo-adjuvant CRT has become increasingly common in treatment of oesophageal adenocarcinoma; the patient is offered preoperative CRT in order to shrink down the tumour bulk prior to oesophagectomy, in the hope that curative resection will become possible. One meta-analysis has demonstrated that neo-adjuvant CRT confers a survival benefit by reducing the 3-year mortality rate and by downstaging the tumour prior to surgery; however it also increased the risk of postoperative mortality (Fiorica, Di Bona et al. 2004). The results from neo-adjuvant CRT are still controversial – only 19% of patients achieved a pathological complete response to therapy in one study, and these patients did not demonstrate a significant 5-year survival benefit (Reynolds, Muldoon et al. 2007), while another study demonstrated increased sepsis and respiratory failure in postoperative patients who had neo-adjuvant CRT who again did not gain any survival benefit (Reynolds, Ravi et al. 2006), while decreasing their preoperative health-related quality of life (Reynolds, McLaughlin et al. 2006). Perioperative chemotherapy, with a regimen of both pre- and post-operative chemotherapy, has been demonstrated to confer a survival benefit on patients with operable gastric and oesophageal tumours compared with surgery alone (Cunningham, Allum et al. 2006). These studies suggest that improvements to the treatment regime for oesophageal cancer are still needed, with randomised controlled trials comparing the various treatment modalities and combinations still required. The most recent recommendations for treatment strategy for oesophageal adenocarcinoma from the 1st St. Gallen Gastrointestinal Cancer Conference are for neo-adjuvant

chemoradiation therapy followed by oesophagectomy, with perioperative or neo-adjuvant chemotherapy considered an adequate alternative (Lutz, Zalcberg et al. 2012).

1.5 The immune profile of Barrett's oesophagus

While the immune profile of oesophagitis is primarily acute-phase and Th-1 dominant, the immune response in BO changes to a Th-2 dominant one. The cytokine profile of BO confirms a predominantly anti-inflammatory profile compared to that of oesophagitis.

O'Riordan et al. have shown that increased expression of NF-kB was much more frequent in patients with Barrett's than those with oesophagitis, with 60% of patients with Barrett's having upregulated NF-kB activation (O'Riordan, Abdel-latif et al. 2005). However in this same study, the levels of the pro-inflammatory cytokines IL-1β and IL-8 were similar in both oesophagitis and BO. In contrast to this, Fitzgerald et al. found markedly decreased levels of IL-1β and IL-8 in BO compared with oesophagitis, as well as decreased levels of IFN-γ, another pro-inflammatory mediator (Fitzgerald, Onwuegbusi et al. 2002). In the same study, Fitzgerald et al. found that there were increased levels of IL-4 in BO. This is a cytoprotective cytokine produced by T-cells and is important in the anti-inflammatory immune response by altering the Th-1 and Th-2 profiles. They also found that IL-10, another anti-inflammatory cytokine, was similarly increased in both oesophagitis and BO.

Another study by Fitzgerald et al. looked at the inflammatory gradient in Barrett's oesophagus from proximal (the new squamo-columnar junction) to distal (Fitzgerald, Abdalla et al. 2002). They found that at the proximal segment, the immune profile was more pro-inflammatory, with high levels of IL-1β and IL-8 and also greater numbers of immune cells. In contrast, the distal segment showed low levels of IL-1β and IL-8 but much higher levels of IL-10. IL-4 was increased throughout the Barrett's epithelium but there was no difference between the proximal and distal segments. The authors note the fact that the exposure to refluxate would be maximal at the distal segment; this indicates that Barrett's metaplasia is an adaptive response to a noxious stimulus.

IL-6 is another pleiotropic cytokine associated with the Th-2 response that has been previously shown to be involved in carcinogenesis in many other tumours e.g. ovarian and prostate cancers. It is thought to have anti-apoptotic and angiogenic actions, many of which are mediated through the STAT3 pathway. Dvorakova et al. have shown increased levels of IL-6 mRNA and protein in Barrett's tissues compared with normal squamous or duodenal epithelium from the same patients (Dvorakova, Payne et al. 2004). This study also showed higher levels of STAT3 and two anti-apoptotic proteins Mcl-1 and Bcl-xL in Barrett's epithelium. The authors speculate that perhaps a mutation in p53 (recognised to have a role in the Barrett's-dysplasia-neoplasia sequence) may cause upregulation of IL-6 expression and therefore suppress apoptosis in the Barrett's epithelial cells.

COX-2, an inducible enzyme involved in prostaglandin synthesis from arachidonic acid, has been shown to be involved in carcinogenesis. COX-2 is expressed in response to inflammation, thereby providing a potential link between inflammation and cancer. Ling et al. have shown a progressive increase in the

levels of COX-2 protein expression along the metaplasia-dysplasia-carcinoma sequence, with a very significant increase between squamous epithelium and Barrett's epithelium (Ling, Baldus et al. 2007). In this study a significant increase in active and chronic inflammatory reactions was noted in Barrett's compared with normal squamous epithelium.

Konturek et al. looked at Barrett's epithelium compared to normal squamous epithelium and found significant increases in NF-kB activity, IL-8 protein expression, COX-2 expression and hepatocyte growth factor (HGF), another growth factor implicated in carcinogenesis (Konturek, Nikiforuk et al. 2004). They also noted that Barrett's mucosa was positive for both CCK-2, a receptor for gastrin, and for gastrin itself, suggesting that this hormone could act as an autocrine promoter for carcinogenesis in the oesophagus; gastrin has been shown to stimulate COX-2 expression (Subramaniam, Ramalingam et al. 2008). Haigh et al had previously shown that gastrin could induce proliferation in Barrett's cells through the CCK-2 receptor, which was also expressed in a majority of patients with oesophagitis and with oesophageal carcinoma (Haigh, Attwood et al. 2003).

One of the ways in which chronic inflammation is thought to facilitate tumour development is in the switch from a cellular to a humoral inflammatory response. This may be associated with increased angiogenic potential and decreased immune surveillance. Moons et al. have shown that a predominantly humoral, Th-2 immune response is present in Barrett's tissue, compared with a cellular, Th-1 response in inflamed squamous epithelium (Moons, Kusters et al. 2005). They found greater numbers of plasma cells and mast cells and fewer CD8+ T cells and macrophages in BO compared with the squamous tissue. They also found that IgE

producing plasma cells and lymphoid follicles were more common in BO compared with reflux oesophagitis, fitting with the Th-2 profile.

1.5.1 Barrett's oesophagus, oesophageal adenocarcinoma and the inflammatory microenvironment

The link between cancer and the inflammatory microenvironment was described as early as 1863 by Rudolf Virchow, where it was hypothesised that tumours could arise in sites of chronic inflammation. Currently, cancer-related inflammation is classified as the "seventh hallmark of cancer" (Colotta, Allavena et al. 2009), in addition to the six delineated by Hanahan and Weinberg in 2000 (Hanahan and Weinberg 2000). Cancer is not only a disease of the tumour cells, but is dependent on its environment for development and progression (Gribben, Rosenwald et al. 2010, Allen and Louise Jones 2011). Many studies have now shown that tumours develop and progress in the context of chronic diseases.

Alterations in the tissue microenvironment occur in most tumours, coupled with an abnormal inflammatory response characterised by infiltration of immune cells, alterations in cytokines and chemokines, and changes in the vascular network. Therefore chronically inflamed tissues, in which these changes are often already present, are innately susceptible to tumour formation.

1.5.1.1 Immune cells in Barrett's oesophagus and oesophageal adenocarcinoma

Immune cells are found in the microenvironment of all stages of the progression from BO to dysplasia to OAC, although there is still a lack of significant research into the roles that these cells play in this disease progression model. DCs have been shown to be increased in Barrett's oesophageal tissue compared with normal oesophageal tissue, implying that these cells may have a role in the development of Barrett's metaplasia (Bobryshev, Tran et al. 2009); DCs may also play a role in the subsequent progression to cancer, as they are found at greater density in OAC than in BO. They are usually in clusters with T and B cells in the lamina propria of the tissue (Bobryshev, Tran et al. 2009). Although the role of DCs remains unclear, a fascinating idea has been proposed by Bobryshev et al. – that DCs in the oesophageal microenvironment may activate stem cells, identified by the stem cell marker Musashi-1, dormant in the epithelium, causing the development of BO and OAC (Bobryshev, Freeman et al. 2010).

T cells are key players in the immune environment of many tumours. In oesophageal carcinogenesis, various types of T cells may play different roles during progression. T helper cells are located in both normal squamous epithelium and in Barrett's epithelium, while precursor (CD7+) T cells are most frequently located in adenocarcinoma tissue (Berndt, Philipsen et al. 2010). This study also found increasing levels of NF-kB and caspase activity, as well as increasing apoptosis, in T cells in Barrett's and cancer tissue. This suggests that the immune response is compromised in malignant transformation; apoptosis in naive T cells was increased in Barrett's epithelium, implying that these cells never develop into mature effector T cells in order to mount an immune response against the

developing tumour. Indeed, intra-tumoural activated CD8+ T cell infiltration has been shown to correlate with improved disease-free survival in OAC (Schumacher, Haensch et al. 2001). Increased CD8+ T cells, along with increased FOXP3+ Treg cells, are associated with a lower stage of tumour (Zingg, Montani et al. 2010); dysregulation of the T cell response therefore seems to be important in progression of the tumour.

Moons et al. have shown that in BO, a predominantly humoral type immune response is seen, with significantly more plasma cells and mast cells than in reflux oesophagitis, which was characterised by a Th1 type immune profile (Moons, Kusters et al. 2005). They also found lymph follicles (segregated areas of T cells, B cells and dendritic cells) in a subset of Barrett's patients; however, these were not found in reflux oesophagitis patients, again demonstrating dysregulation of the immune cell profile in BO. Infiltration of eosinophils has been shown in the mucosa of a subset of BO patients, and was associated with basal cell hyperplasia (Ravi, Katzka et al. 2011). Macrophages, while found in similar number in reflux oesophagitis and BO (Moons, Kusters et al. 2005), are increased in OAC and produce both the angiogenic factor vascular endothelial growth factor (VEGF) (McDonnell, Bouchier-Hayes et al. 2003) and the matrix metalloproteinase MMP-12 (Salmela, Karjalainen-Lindsberg et al. 2001), the latter of which being increased in BO although not to the same extent as OAC.

1.5.1.2 Small molecules in Barrett's oesophagus and oesophageal adenocarcinoma

As an inflammatory-driven cancer, OAC has a similar array of small molecules that play a role in its development from BO (Figure 1.8). In a cohort of oesophageal cancer patients, IFN-γ, IL-1α, IL-8, IL-21 and IL-23 were found to be associated with a poor prognosis, with a particularly strong association in known Barrett's-derived cancers (Nguyen, Schetter et al. 2010). IL-6, a pleiotropic inflammatory cytokine, was found to be increased in transformed Barrett's cell lines (with activated Ras and p53 knock-down) compared with non-transformed lines, along with its regulator STAT3 (Zhang, Zhang et al. 2011). These trends have also been shown in tissue samples from BO and cancer (Dvorakova, Payne et al. 2004, Dvorak, Chavarria et al. 2007). This is a similar pattern to that seen in the progression from UC to CRC. IL-6 is known to inhibit apoptosis in other cancers (Lin, Juan et al. 2001, Wei, Kuo et al. 2001), providing a possible mechanism for its carcinogenic effects.

TGF-β1 is an anti-inflammatory and tumour-suppressive cytokine under normal conditions, but in an abnormal microenvironment it can promote tumourigenesis in many cancers (Hong, Lee et al. 2010). TGF-β1 is significantly increased in oesophageal tumour tissue compared with Barrett's tissue, and is associated with advanced tumour stage (von Rahden, Stein et al. 2006). TGF-β1 has been shown to increase migration and invasion in OAC cells by inducing extracellular matrix degrading enzymes, as well as by causing failure of cell-cycle arrest and thereby increasing proliferation (Onwuegbusi, Rees et al. 2007). TGF-β1 may also be involved in epithelial-to-mesenchymal transition (EMT) in the oesophagus, thought to play a role in carcinogenesis (Rees, Onwuegbusi et al.

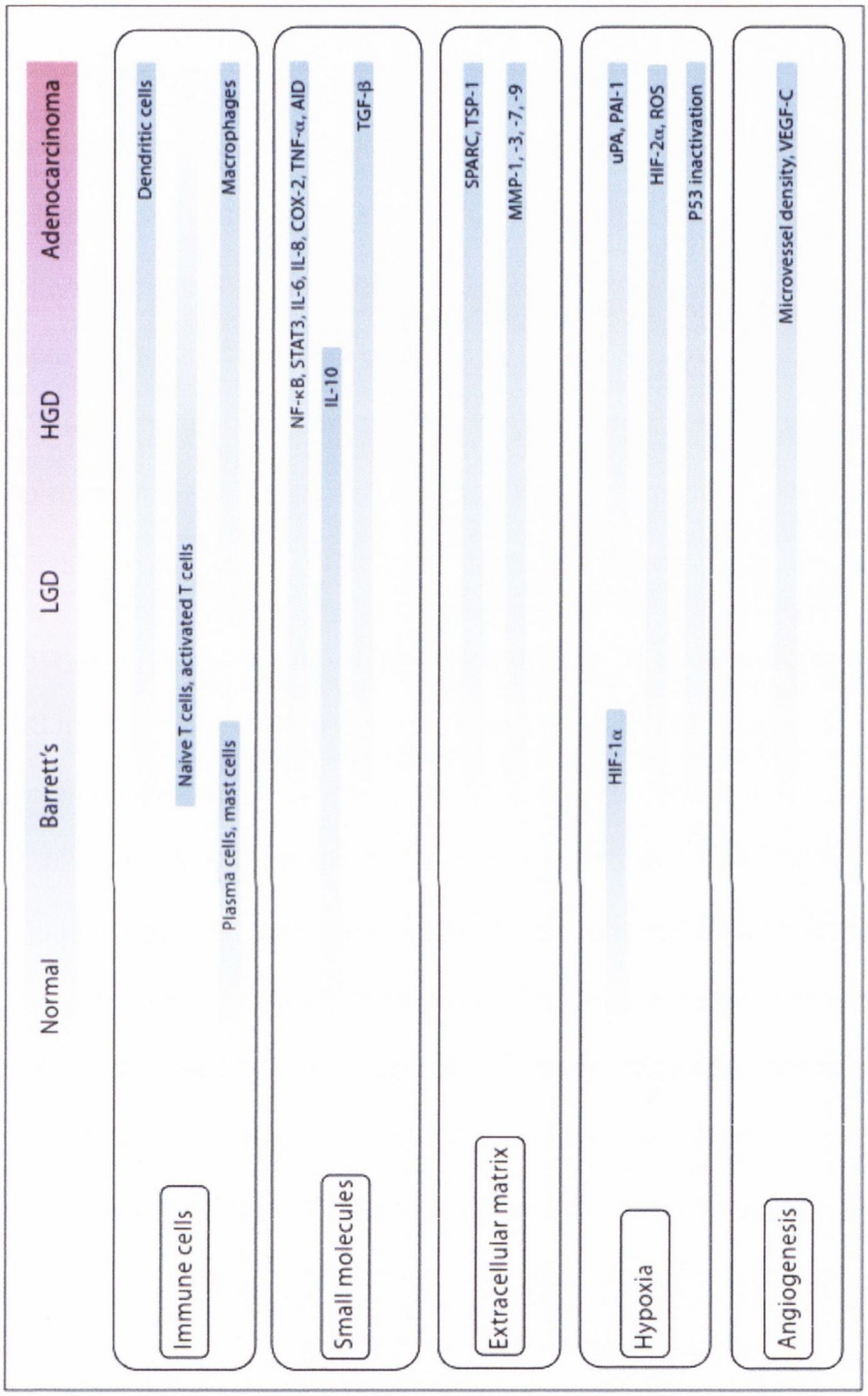


Figure 1.8. Factors involved in the progression of oesophageal disease. Immune cells, cytokines, chemokines and transcription factors and other factors that are either increased or decreased during the progression of oesophageal disease from normal oesophagus to Barrett's oesophagus to oesophageal adenocarcinoma.

2006). Another interesting study examining genes differentially expressed in the stromal compartment of oesophageal tissue found TGF-β-related genes TSP-1, POSTN and TMEPAI to be dysregulated across the sequence from metaplasia to dysplasia to carcinoma, in addition to other inflammatory mediators such as IL-6 and COX-2 (Saadi, Shannon et al. 2010). COX-2, itself a key inflammatory molecule implicated in many cancers, is upregulated in BO and further increased in dysplasia and adenocarcinoma (Morris, Armstrong et al. 2001, Lagorce, Paraf et al. 2003, Kuramochi, Vallbohmer et al. 2004), with increased COX-2 expression in oesophageal cancer associated with a worse prognosis (France, Drew et al. 2004).

The transcription factor NF-kB, which regulates a myriad of pathways in carcinogenesis, has been found to be upregulated along the sequence from BO to OAC in tissue samples, along with one of its many targets, the pro-inflammatory chemokine IL-8 (O'Riordan, Abdel-latif et al. 2005, Jenkins, Mikhail et al. 2007). NF-kB can also be activated by the bile acid deoxycholic acid, a component of refluxate, in oesophageal cells (Jenkins, Harries et al. 2004). NF-kB may be a major regulator of the neoplastic process in the oesophagus, as well as in many other cancers. Interestingly, Fitzgerald et al. found that the pro-inflammatory chemokine IL-8 was upregulated in the proximal segment of BO in patients compared with the distal segment, along with other pro-inflammatory cytokines like IL-1β. However, in the distal segment, where the majority of cancers develop, the anti-inflammatory cytokine IL-10 was increased, and was also higher in dysplastic oesophageal tissue (Fitzgerald, Abdalla et al. 2002). This suggests that IL-10, and the Th2 immune response may act as an immune escape mechanism in the development of tumours in the metaplastic Barrett's epithelium.

A novel way in which small molecules may modulate neoplastic progression in the oesophagus is via proteins secreted by adipose tissue. Given that obesity is a recognised risk factor for oesophageal adenocarcinoma, the effect of adiponectin and ghrelin, two cytokines that are decreased in obesity, on OAC cells was examined. It was observed that adiponectin increased apoptosis, while ghrelin decreased inflammation by lowering COX-2 and IL-1β production (Konturek, Burnat et al. 2008). The reduction in these two factors seen in obese patients may drive progression to cancer by decreasing apoptosis and promoting inflammation. Another study looking at visceral adipose tissue in oesophageal cancer found a large population of activated CD4+ and CD8+ T lymphocytes in omental fat, which produced abundant IFN-γ, further implicating adipose tissue in driving the chronic inflammation seen in OAC (Lysaght, Allott et al. 2011).

1.6 Transcription factors

Transcription factors (TFs) are key cellular components, comprising a group of proteins expressed by eukaryotic cells which bind to specific DNA sequences, tightly regulating the expression of specific genes and thereby controlling cellular processes. In the human genome, almost 1400 genomic loci encoding TFs have been identified, with another 216 loci possibly encoding TFs, suggesting that the human genome contains approximately 1700-1900 TF-coding genes (Vaquerizas, Kummerfeld et al. 2009), representing ~6% of all human genes. Many of these TFs remain uncharacterised; however, transcription factors have been found to be over-represented in cancer genes (Furney, Higgins et al. 2006) and in human developmental disorders (Boyadjiev and Jabs 2000). TFs bind to DNA sequences

in the promoter regions of specific genes in order to regulate transcription of those genes. Three families of TFs are predominant in the human genome: zinc finger, homeodomain and helix-loop-helix TFs together make up over 80% of TFs (Luscombe, Austin et al. 2000, Vaquerizas, Kummerfeld et al. 2009). The expression of TFs varies in tissue and organ types: organs with a large number of cell types and which express a large number of genes express a greater number of TFs. TFs can also act in combination to regulate gene expression, forming co-factor complexes and dimerisation complexes (Levine and Tjian 2003, Amoutzias, Veron et al. 2007). Some transcription factors are ubiquitously expressed and so regulation of particular genes may depend upon co-expression and binding of several TFs. Promoter regions of genes may contain binding sites for multiple TFs, and binding of two TFs may cause dimerisation and thus activate transcription of the gene.

1.6.1 Transcription factors and cancer

TFs have been found to be involved in almost all cancer types, as their role in regulating gene expression makes them key players in the initiation and progression of cancer cell growth. Many major TFs have been demonstrated to be overexpressed in cancer. Hypoxia inducible factor (HIF) is a TF which mediates the cellular response to a hypoxic environment. In cancer, HIF has been found to be upregulated by many oncogenes, such as VHL, and to correlate with tumour growth and vascularisation, as well as patient mortality (Semenza 2010, Semenza 2012). HIF regulates transcription of many genes in cancerous states, including TGF-β in renal cell carcinoma and VEGF in colorectal and gastric cancers, as well

as MMPs and LOX factors, which promote invasion and metastasis (Forsythe, Jiang et al. 1996, Gunaratnam, Morley et al. 2003, Erler, Bennewith et al. 2006, Dallas, Fan et al. 2007, Wong, Gilkes et al. 2011); HIF inhibitors decrease cancer growth and metastasis in studies, and clinical trials are ongoing (Terzuoli, Puppo et al. 2010, Kummar, Raffeld et al. 2011, Wong, Zhang et al. 2012). The glioma-associated oncogene homolog (GLI) transcription factors, regulated via the sonic hedgehog signalling pathway (Carpenter and Lo 2012), are upregulated in breast cancer and pancreatic cancer (Nolan-Stevaux, Lau et al. 2009, Cao, Geradts et al. 2012). GLI1 expression increases cancer cell proliferation and development (Kimura, Stephen et al. 2005), angiogenesis (Hsieh, Ellsworth et al. 2011) and also correlates with tumour grade and lymph node status (ten Haaf, Bektas et al. 2009). The Janus kinase/signal transducer and activator of transcription (Jak/Stat) pathway, and in particular STAT3, is another important transcription factor pathway in cancer development. STAT3 is regulated via the cytokines IL-6 and IL-10 and is persistently activated in many malignancies (Levy and Inghirami 2006, Yu, Pardoll et al. 2009, Sansone and Bromberg 2012), promoting proliferation, angiogenesis and metastasis. Peroxisome proliferator-activated receptors (PPARs) are a family of ligand-activated TFs involved in lipid and glucose metabolising pathways; their expression is variable in different types of cancers, with increased expression of PPARβ/δ in ovarian and endometrial cancers compared with normal tissue, while other tumours such as colon tumours demonstrate unchanged or decreased PPARβ/δ (Tong, Tan et al. 2000, Peters, Hollingshead et al. 2008, Davidson, Hadar et al. 2009, Foreman, Chang et al. 2011). Agonists for PPARα cause inhibition of tumour growth in various cell types (Pozzi, Ibanez et al. 2007, Panigrahy, Kaipainen et al. 2008). p53 is a tumour suppressor TF that is

inactivated by mutation in over 50% of cancers, causing loss of genomic stability, and has been widely studied as it influences many cancer-related pathways including DNA repair, cell growth and proliferation and apoptosis (Talos and Moll 2010, Stegh 2012). However, the most widely studied TF in cancer, and which also relates cancer and inflammation, is NF-kB.

1.6.2 NF-kB

NF-kB was originally discovered in B cells in 1986 (Sen and Baltimore 1986) and was found to be homologous to an oncoprotein called c-Rel, and a similar protein being studied in *Drosophila* (Steward 1987). It was later discovered that it is ubiquitously expressed in almost all cells, and is activated by liberation from its inhibitor protein IkB by the IkB kinase (IKK) (Sen and Baltimore 1986, Baeuerle and Baltimore 1988). The proteins that make up the NF-kB family all contain the N-terminal Rel homology domain which allows DNA binding and dimerisation; the family is then divided into the NF-kB proteins, such as p100 and p105, and the rel proteins, cRel, RelA (p65) and RelB, all of which homodimerise and heterodimerise allowing varying combinations causing altered gene regulation (Hoffmann, Natoli et al. 2006, Gilmore and Wolenski 2012). NF-kB is primarily activated via Toll-like receptors (TLRs), of which 10 have been identified in humans. TLRs are receptors that span the cell membrane. Their extracellular domain binds various ligands, including bacterial pathogens and double-stranded RNA, and their intracellular domain activates a signalling pathway which includes NF-kB (Lu, Yeh et al. 2008). TLRs are very similar to the IL-1 receptors, having the same intracellular domain, but the IL-1 receptors have an immunoglobulin extracellular domain, allowing

binding of different ligands, but activating the same pathways (Medzhitov 2001, Garlanda, Anders et al. 2009). NF-kB can be activated via two pathways: the canonical pathway, which is activated by infectious agents and pro-inflammatory cytokines, causing activation of the IKKβ kinase and degradation of the IkB inhibitor, and the non-canonical pathway, involving the TNF family of cytokines and activation of another subunit, IKKα, and the NF-kB2 protein (Figure 1.9). The links between inflammation and cancer have been discussed in section 1.5.1.

1.7 IL-1β

1.7.1 IL-1β

IL-1β is an inflammatory cytokine that forms part of the interleukin 1 family, comprising 11 members, which includes IL-1α and the IL-1 receptor antagonist, as well as IL-18 and IL-33 (Dinarello 2010). It was discovered in the 1970s while researchers were investigating fever, and was originally named human leucocytic pyrogen (Dinarello, Renfer et al. 1977, Dinarello and Wolff 1977). Further research revealed the central role of this protein in the acute inflammatory response to infection and injury. IL-1β is initially expressed as a 31 kDa inactive precursor molecular, which is cleaved into its 17 kDa active form by the enzyme caspase-1 (also known as interleukin-1β-converting enzyme) intracellularly, or extracellularly, this cleavage can be performed by proteinases such as proteinase-3 (Kostura, Tocci et al. 1989, Fantuzzi, Ku et al. 1997). Caspase-1 itself is activated via the inflammasome, a complex of proteins which are formed in the cell in response to

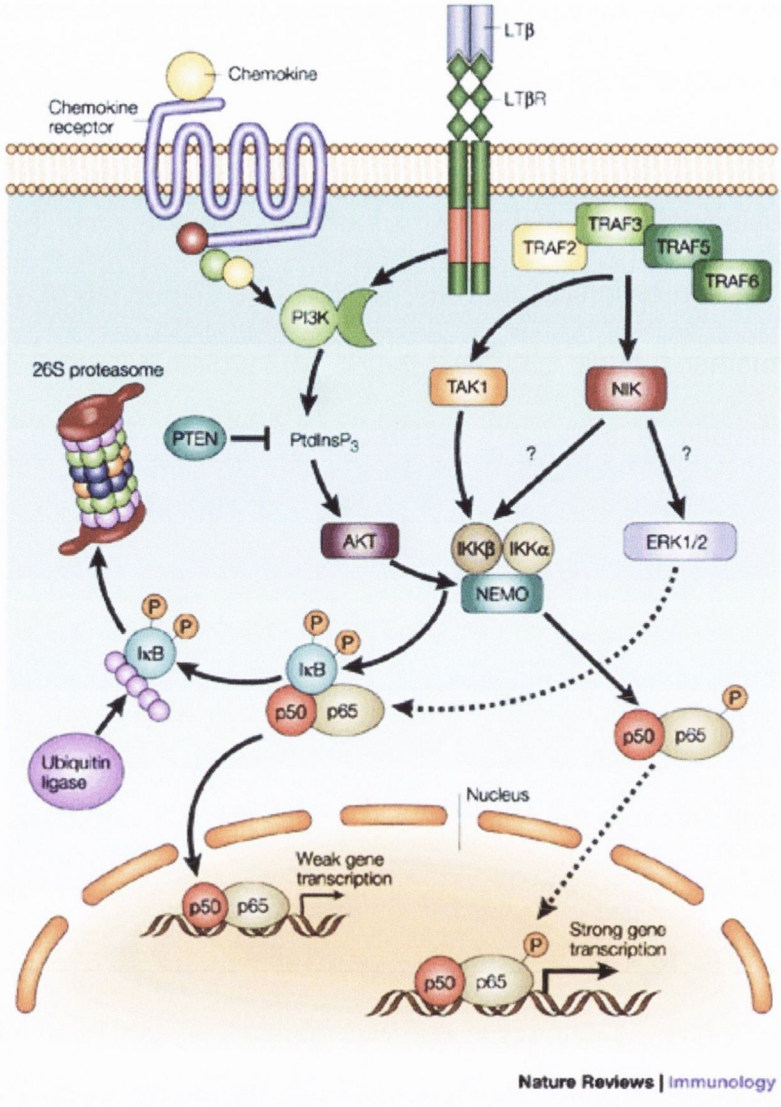


Figure 1.9. NF-kB activity in the cell. Phosphatidylinositol 3-kinase (PI3K) can activate AKT, which potentially affects the phosphorylation of both inhibitor of NF-κB (IκB) kinase (IKK; a complex of IKKα, IKKβ and NEMO) and p65 (RELA). In addition, linker proteins, such as tumour-necrosis factor (TNF)-receptor-associated factors (TRAFs), participate in the activation of NF-κB-inducing kinase (NIK), as well as transforming-growth-factor-β-activated kinase 1 (TAK1). NIK-mediated enhancement of NF-κB activity might be facilitated by its activation of mitogen-activated protein (MAP) kinases, such as extracellular-signal-regulated kinase 1 (ERK1) and ERK2. The activation of IKK leads to enhanced phosphorylation of IκB, followed by its ubiquitylation then degradation by the 26S proteasome. This frees the NF-κB p50 and p65 subunits to be transported to the nucleus. Nuclear NF-κB p50–p65 heterodimers that are not phosphorylated on the p65 subunit can only partially activate gene transcription, whereas if p65 is phosphorylated, the enhancement of transcription is greatly magnified. LTβ, lymphotoxin-β; LTβR, lymphotoxin-β receptor; NEMO, NF-κB essential modulator; $PtdInsP_3$, phosphatidylinositol-3,4,5-trisphosphate; PTEN, phosphatase and tensin homologue. (Ghosh 2008)

pathogens, which activate a NOD-like receptor (NLR) to induce formation of the inflammasome and activation of caspases (Martinon and Tschopp 2007).

IL-1β can drive its own production (Dinarello, Ikejima et al. 1987), and this may be the mechanism behind many autoimmune diseases, in which IL-1β is known to play a prominent role (Goldbach-Mansky, Dailey et al. 2006). IL-1β has also been demonstrated to play a role in many cancers, especially those with an inflammatory origin (Germano, Allavena et al. 2008, Mantovani, Allavena et al. 2008). The effects of IL-1β are myriad: IL-1β is a potent inducer of fever, as previously discussed. It can also affect vascular endothelium and has been implicated in atherosclerotic disease, by promoting thrombosis (Libby 2002, Tedgui and Mallat 2006). IL-1β has also been demonstrated to be essential for tumour angiogenesis and invasiveness (Voronov, Shouval et al. 2003, Nakao, Kuwano et al. 2005). IL-1β blockade has been demonstrated to be effective in many diseases including type 2 diabetes, gout and cancers such as multiple myeloma (Larsen, Faulenbach et al. 2007, So, De Smedt et al. 2007, Lust, Lacy et al. 2009, Cook, Savic et al. 2010, Dinarello 2011).

1.7.2 IL-1β in the oesophagus

IL-1β is thought to play a major role in oesophageal disease. Animal models of oesophagitis demonstrate increased release of IL-1β into the circulation compared with controls, as well as increased IL-1β with the development of Barrett's oesophagus and oesophageal adenocarcinoma (Majka, Rembiasz et al. 2010, Pawlik, Pajdo et al. 2011, Quante, Bhagat et al. 2012). It has also been demonstrated that bile acids can induce secretion of IL-1β in oesophageal cells,

which may drive the development of oesophageal disease (Souza, Huo et al. 2009), and anti-reflux surgery, which decreases bile acid exposure in the oesophagus, decreases IL-1β in Barrett's oesophageal mucosa (Babar, Ennis et al. 2010). IL-1β expression has been found to be maximal at the proximal segment in Barrett's oesophagus, along with the chemokine IL-8 (Isomoto, Nishi et al. 2007), although another study found no difference in IL-1β expression between normal and Barrett's mucosa (Fitzgerald, Abdalla et al. 2002). It has been found to increase along the inflammation – metaplasia – dysplasia - adenocarcinoma sequence, along with activation of the transcription factor NF-kB, with highest expression in oesophageal adenocarcinoma (O'Riordan, Abdel-latif et al. 2005). IL-1β expression also correlated with stage of disease in cancer patients in this study. The anti-inflammatory adipocytokine ghrelin has been demonstrated to decrease IL-1β expression in oesophageal adenocarcinoma cells; ghrelin expression is decreased in obese individuals, suggesting a mechanism, via IL-1β, through which obesity can influence the development or progression of oesophageal cancer (Konturek, Burnat et al. 2008). In oesophageal cancer patients, increased IL-1β expression in tumour tissue has been demonstrated compared with oesophageal tissue from healthy controls, and this correlated with systemic inflammation and reduced overall survival (Deans, Wigmore et al. 2006). IL-1β is also increased in tumour tissue compared with matched normal tissue in oesophageal adenocarcinoma patients (Abdel-Latif, O'Riordan et al. 2005); this study demonstrated that IL-1β expression was reduced in tumours that subsequently responded completely to neo-adjuvant chemoradiation therapy.

1.7.3 The IL-1 receptor

The discovery of the IL-1 receptor types I and II meant that researchers now had a better understanding of the signalling pathways of the IL-1 family of cytokines (Sims, March et al. 1988, McMahan, Slack et al. 1991). The type II receptor is a decoy receptor which does not activate signalling pathways as it has no cytosolic domain, while the type I receptor binds to the IL-1 receptor accessory protein when bound by a ligand, and activates many downstream pathways (Stylianou, O'Neill et al. 1992, Greenfeder, Nunes et al. 1995). It was also discovered that the IL-1 receptor shares a similar intracellular domain, called the TIR (Toll-IL-1 receptor) domain, with the Toll-like receptor family, leading to the grouping of the IL-1/TLR receptor superfamily, which is highly conserved across plant and animal species (Gay and Keith 1991, O'Neill and Greene 1998). The IL-1 receptors are characterised by having an immunoglobulin domain extracellularly, which is responsible for binding ligands, which, for the IL-1R1, are IL-1α and IL-1β, as well as the naturally occurring IL-1 receptor antagonist (IL-1RA) (Eisenberg, Brewer et al. 1991). When bound by an activating compound, the IL-1R1, with its accessory protein, binds to an adapter protein called MyD88, leading to a complex series of protein-binding steps, which ultimately lead to activation of NF-kB (Brikos, Wait et al. 2007, O'Neill 2008). The IL-1R1 has been demonstrated to be important in disease states; in a model of allergic asthma, mice deficient in the IL-1R1 had significantly decreased pulmonary inflammation and antibody responses compared with wild-type mice (Schmitz, Kurrer et al. 2003), and this receptor may provide a future therapeutic target in treating asthma (Lee, Wang et al. 2010). Interestingly, the balance between the activating ligands IL-1α/β and the IL-1RA may be of critical importance in preventing the development of disease; rabbits immunised

against their own IL-1RA develop colitis, while mice deficient in IL-1RA develop arthritis, and also develop tumours in response to carcinogens much more rapidly than wild-type mice (Ferretti, Casini-Raggi et al. 1994, Horai, Saijo et al. 2000, Krelin, Voronov et al. 2007).

1.8 TNF

1.8.1 TNF

Tumour necrosis factor (TNF) is a cytokine which is involved in the inflammatory response, especially in the acute phase response of inflammation. It was first discovered in 1975, when it was noticed that both bacterial lipopolysaccharide and the BCG strain of *Mycobacterium tuberculosis* had anti-tumour effects when injected into mice, but did not have the same effect in *in vitro* studies on tumour cells, leading to the identification of this serum factor, initially called TNF-α, which was toxic to tumour cells (Carswell, Old et al. 1975). However, the anti-tumour effects of bacterial isolates had been documented by the New York surgeon William Coley in 1893 (Coley 1910) (Coley, 1893). It was later revealed that TNF-α was identical to a previously identified protein called cachectin, which caused fever, shock and wasting in patients (Beutler and Cerami 1986). It is now recognised that TNF-α, now known as TNF, is a member of the TNF superfamily, of which 19 ligands have so far been identified.

TNF itself is predominantly expressed by macrophages, NK, T and B cells. Although it was initially thought to have potential as an anti-cancer agent, it was found to be systemically toxic when administered in phase I trials, causing fever,

chills, headache and fatigue (Feinberg, Kurzrock et al. 1988). More recently, isolated limb perfusion with TNF has been used to treat melanomas and sarcomas (Eggermont, de Wilt et al. 2003). TNF is essential for the immune response to infections, as it mediates various pathways in the inflammatory response such as B cell follicle formation and T cell activation, via transcription factors such as NF-kB and JNK (Fu and Chaplin 1999, Siegel, Chan et al. 2000, Chen and Goeddel 2002). It is now also recognised as playing a role in a multitude of other disease types, including cardiovascular, neurologic and respiratory diseases (Aggarwal, Gupta et al. 2012). Patients treated with anti-TNF therapies are more susceptible to infections such as tuberculosis, as well as developing neutropenia and thrombocytopenia (Gardam, Keystone et al. 2003, Vidal, Fontova et al. 2003, Azevedo, Silva et al. 2012, Mori and Sugimoto 2012). Indeed, anti-TNF monoclonal antibodies have been developed and are widely used in treating immune-mediated disorders such as rheumatoid arthritis, Crohn's disease and psoriasis. The role of TNF in cancer is more complex; while it was initially thought to be toxic to tumour cells, TNF has now been implicated in the development and progression of various cancers (Balkwill 2009). It has been demonstrated to promote cancer cell growth and to reduce response to chemotherapy in ovarian cancer patients (Maccio and Madeddu 2012). TNF can also promote the development of renal cell carcinoma, colorectal carcinoma, breast cancer and gastric cancer (Galban, Fan et al. 2003, Suganuma, Kuzuhara et al. 2006, Popivanova, Kitamura et al. 2008, Kamel, Shouman et al. 2012).

1.8.2 TNF in the oesophagus

In the oesophageal disease sequence from normal oesophagus to oesophageal adenocarcinoma, TNF appears to play a role. TNF has been demonstrated to be increased in Barrett's epithelium compared with normal squamous epithelium, and is further increased along the sequence to dysplasia and oesophageal adenocarcinoma tissue (Tselepis, Perry et al. 2002, Isomoto, Nishi et al. 2007, Menke, van Zoest et al. 2012). An interesting mechanism of action for TNF in oesophageal disease progression is proposed by Tselepis et al. The cell adhesion molecule e-cadherin acts as a tumour suppressor that can be downregulated by TNF (Perry, Tselepis et al. 1999) and binds to an associated anchoring protein called β-catenin. E-cadherin has been demonstrated to be downregulated across the oesophageal metaplasia-dysplasia-adenocarcinoma sequence (Bailey, Biddlestone et al. 1998), while β-catenin was translocated to the nucleus. β-catenin can activate oncogenes (Mann, Gelos et al. 1999), and when unbound by e-cadherin, is free to translocate to the nucleus to promote this transcription. In the oesophagus, it was found that TNF could induce expression of the oncogene c-myc, via β-catenin expression independently of NF-kB, suggesting a role for this pathway in oesophageal carcinogenesis. TNF can also induce the transcription factor Cdx1 in oesophageal cells, which is a regulator of intestinal development, but is not expressed in normal squamous oesophagus, and so may play a role in the development of Barrett's metaplasia and adenocarcinoma (Wong, Wilding et al. 2005). Several animal models have demonstrated increased TNF in oesophagitis compared with non-inflamed oesophageal mucosa, along with other inflammatory factors such as IL-1β, IL-6, MCP-1 and MIP-1α (Hamaguchi, Fujiwara et al. 2003, Pawlik, Pajdo et al. 2011). TNF may also provide one mechanism by

which obesity can be linked to the development of Barrett's oesophagus and adenocarcinoma; visceral adipose tissue can secrete TNF, which may lead to oesophageal disease development and carcinogenesis, while weight loss is associated with decreased cancer risk (Akiyama, Yoneda et al. 2011, Byers and Sedjo 2011).

1.8.3 The TNF receptor

The TNF superfamily bind to a group of 29 TNF receptors, of which TNF itself binds to two receptors: TNFR1 and TNFR2. TNFR1 is expressed by almost all cell types, while TNFR2 is expressed mainly by immune cells. Varying roles for the two receptors have been proposed: it has been suggested that TNFR1 binds the soluble form of TNF while TNFR2 binds the transmembrane form of the ligand, activating different pathways (Grell, Douni et al. 1995). Deletion of either receptor in macrophages blocked activation of NF-kB, JNK and MAPK by TNF, suggesting that both receptors are necessary for signalling (Mukhopadhyay, Suttles et al. 2001). When bound by TNF, the TNFR1 binds to the TNFR-associated death domain protein (TRADD), which can lead to several pathways being activated, including NF-kB, via ubiquitin-mediated degradation of its inhibitor IkBα, causing inflammation, and caspase-3, via caspase-8, causing apoptosis (Aggarwal 2003, Wajant, Pfizenmaier et al. 2003). When bound, the TNFR1 has been shown to translocate to cholesterol- and sphingolipid-enriched membrane microdomains, called lipid rafts, where it binds to TRADD and TNFR-associated factor 2 (TRAF2) and activates NF-kB, and is subsequently ubiquitylated and ultimately degraded by the proteasome. If the lipid raft is interfered with, the TNF signalling is switched to

apoptosis, providing a mechanism by which TNF can regulate both these pathways (Legler, Micheau et al. 2003).

1.9 Chemokines, immune cells and cancer

1.9.1 Chemokines

Chemokines are molecules that are among the most potent stimuli for leucocyte migration in the body and therefore are a fundamental part of the inflammatory process. They are small low-molecular-weight proteins with a large degree of structural homology, and are produced by many cells in the body in response to inflammatory triggers (Rossi and Zlotnik 2000, Zlotnik and Yoshie 2000). They are divided into subgroups based on their cysteine group – these are CXC, CC, C and CX3C groups (Figure 1.10). Each of the more than 50 known chemokines binds to a G-protein coupled receptor, of which 20 have been identified; however many chemokines may bind to one receptor, and equally one chemokine may bind to many receptors, suggesting a very high degree of complexity within this system. Chemokines are pleiotropic molecules, that is they have effects on many different types of cells. After binding to their receptors, they induce signalling in the cell which leads to alterations in cell motility as well as other functions such as angiogenesis and pro-inflammatory cytokine production.

Functionally chemokines can be divided into two groups – homeostatic and inflammatory. Homeostatic chemokines are constitutively expressed, usually by cells involved in maintaining the haematopoietic and immune defences in lymphoid tissue and in non-lymphoid sites such as skin and mucosal surfaces. These

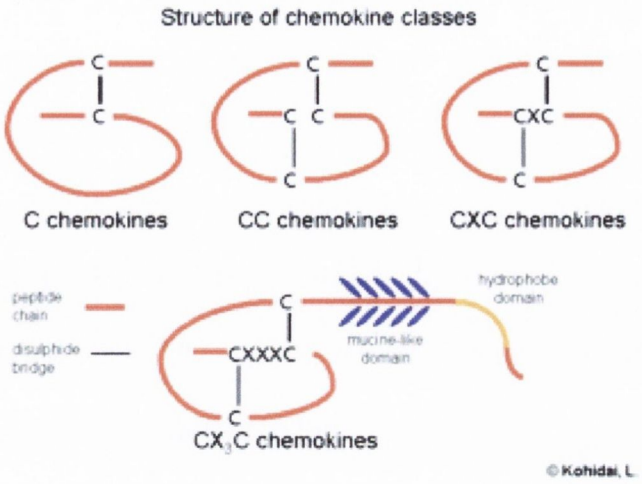


Figure 1.10. Chemokine structure. Chemokines are divided into subgroups based on the cysteine structure of each molecule, with four variations on the structure of the first two cysteine amino acids. (Source: wikipedia.org)

chemokines are involved in physiological positioning of immune cells, antigen processing and general surveillance. The inflammatory chemokines are inducible and are expressed in response to inflammatory stimuli and regulated by various cytokines and other factors. These chemokines then recruit inflammatory cells to the site of insult as part of the immune response. There is significant overlap between these two functional groups, with some chemokines acting in one situation as a homeostatic chemokine, but also capable of being induced in another situation. The binding of a chemokine to its receptor causes the leucocyte bearing that receptor to migrate across the endothelium of a blood vessel and into the surrounding tissue where it can contribute to the inflammatory response.

The functional abilities of chemokines, apart from having anti-tumour effects, are exploited by cancer cells in many ways; their multitude of actions are useful to the tumour in both evading the host immune system and in using the immune system for its own purposes (Figure 1.11). The cancer cells also utilise the chemokine system to aid in growth promotion, angiogenesis and in metastasis to distant sites (Barbieri, Bajetto et al. 2010)

1.9.2 Chemokines, cancer and Treg cells

While chemokines are produced to stimulate leucocyte migration towards an immunogenic stimulus, tumour cells may take advantage of this ability to help them to evade the host immune response. Cancer cells may decrease the production of chemokines themselves or chemokine receptors to decrease the host response and therefore decrease the immune infiltrate. Another method to evade the host anti-tumour response is to attract immunosuppressive inflammatory cells which

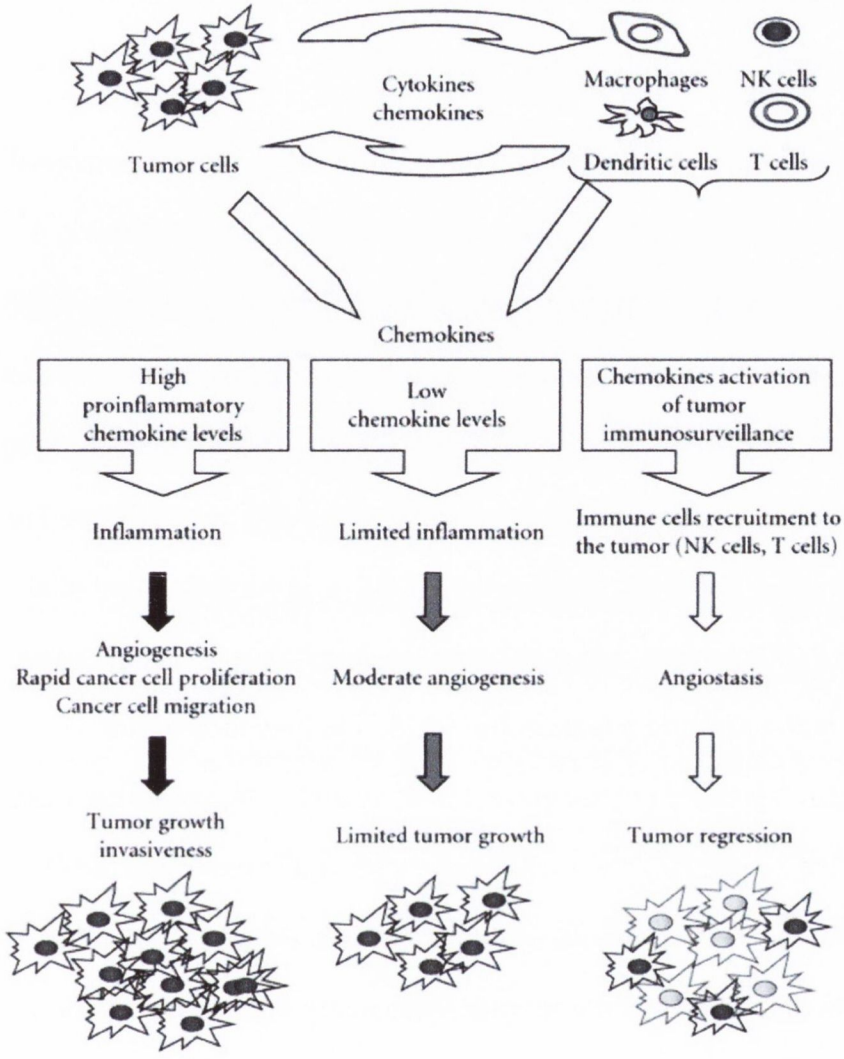


Figure 1.11. Role of chemokines in the tumour-specific immune response. The type and the amount of the chemokines secreted by tumour and inflammatory cells determine the extent and the effect of immune response leading to antitumour cytotoxic response, limited inflammatory, and vascular activation or potentiating tumour cell proliferation and neoangiogenesis

(Barbieri, Bajetto et al. 2010)

dampen down the host response. Cancers also attract immature forms of leucocytes which have impaired function.

T regulatory (Treg) cells are used by tumours to evade the anti-tumoural host immune response. In a recent study, lymphoid aggregates surrounding a primary breast tumour were shown to contain a high number of activated FOXP3+ Treg cells, which are functionally suppressive and may act by blocking activation of anti-tumoural T-cell activity (Gobert, Treilleux et al. 2009). The presence of Treg cells was found to correlate with increased risk of relapse and death. These Tregs may be chemoattracted by CCL22 through the CCR4 receptor. Mizukami et al. showed that Treg FOXP3+ cells also play a significant role in gastric cancer as they are found in both early and advanced tumours, and their localisation correlates with patient survival (Mizukami, Kono et al. 2008). In gastric tumours, the chemokines CCL17 and CCL22 were upregulated and chemoattracted Tregs, which had an immunosuppressive role on effector T cells (Mizukami, Kono et al. 2008). The authors speculate that the tumour microenvironment may induce production of CCL17 and CCL22 by macrophages and dendritic cells present in the peritumoural stroma. Also using gastric cancer cells as an example, Treg cells from gastric cancer patients decreased transendothelial migration of effector T cells, probably by decreasing production of T cell attracting chemokines (CXCL10, CXCL9, CXCL11, CCL22) during migration, as well as by direct action (Enarsson, Lundin et al. 2007). Increased numbers of circulating Treg cells were seen in gastric cancer patients compared to normal controls; similar increases were seen in colon cancer and lung cancer patients. This suggests that tumours can induce decreases in chemokine production to evade the immune response.

Ovarian cancer cells and tumour-associated macrophages in the tumour microenvironment have been shown to secrete the chemokine CCL22 (Curiel, Coukos et al. 2004), which attracts Treg cells positive for the CCL22 receptor CCR4. These Treg cells inhibited the induction of effector T cell proliferation by antigen-presenting cells such as myeloid dendritic cells, and also promoted tumour growth in mouse models of ovarian cancer with tumour-specific T cells. Treg cell content in human ovarian cancer specimens correlated negatively with patient survival.

Treg cells have also been shown to aid in allowing tumours to metastasise. In a mouse model of lung metastasis in breast cancer, tumour cells induced production of chemokines CCL17 and CCL22 in lung epithelial and stromal cells (Olkhanud, Baatar et al. 2009). These chemokines bind to the receptor CCR4; this receptor was found to be expressed on a proportion of mammary tumour cells, and also on Treg cells. When Treg cells were depleted, the mammary tumour cells were unable to metastasise to the lung, indicating that these cells are necessary for metastasis. The mechanism by which Treg cells facilitated metastasis appeared to be by inducing apoptosis in natural killer (NK) cells, which can inhibit metastasis via an IL-10-mediated mechanism (Zheng, Ojcius et al. 1996). Breast cancer cells prepare the target organ (lung) by inducing chemokine production which will then chemoattract both the tumour cells themselves and also the immune cells necessary to facilitate the metastasis.

1.9.3 Chemokines, cancer and dendritic cells

Fewer and more immature dendritic cells are found in the serum and tissue microenvironment of cancer patients compared with healthy controls (Gabrilovich 2004). Immature dendritic cells have been demonstrated to have poor antigen-presenting abilities in non-small cell lung cancer (Perrot, Blanchard et al. 2007). In a mouse colon cancer cell line, activation of a toll-like receptor by LPS induced production of CCL20 via activation of TLR4 signalling pathway (Wang, Liu et al. 2008). CCL20 recruited immature dendritic cells to the tumour, providing a chemokine-mediated mechanism for tumours to escape the host dendritic cell immune response. CCL11 (eotaxin), initially known as a homeostatic chemokine regulating eosinophil migration, has been shown to block dendritic cell maturation and endocytic ability by inducing expression of molecules called suppressor of cytokine signalling 1 (SOCS-1) and SOCS-3 (Stevenson, Addley et al. 2009), therefore functioning as an immune modulator. Ovarian carcinoma cells have been shown to overexpress receptors for CCL11 (CCR2, CCR3 and CCR5) and proliferated and became more invasive in the presence of recombinant CCL11 (Levina, Nolen et al. 2009). This chemokine was also found to be significantly higher in colorectal tumour tissue than in normal tissue, and was expressed by stromal cells and leucocytes surrounding the tumour cells (Wagsater, Lofgren et al. 2007).

CCL19/CCR7 interactions normally promote maturation and migration of dendritic cells; however in the presence of neuroblastoma cells, an aggressive tumour found in children, dendritic cells showed significantly decreased migration, despite normal expression of the chemokine and its receptor (Walker, Ogagan et al. 2006). Even with supersaturation of the dendritic cells with CCL19, they failed to

migrate, suggesting that the tumour cells may have caused dysfunction of the receptor, or may secrete another immunosuppressive molecule which impairs signalling.

Melanoma cells have been observed to secrete many chemokines including CXCL1, CXCL2 and CXCL3, which are also induced in fibroblasts in the peritumoural stroma (Navarini-Meury and Conrad 2009). CCL2 and CCL5 produced by melanoma cells not only activate monocytes/macrophages, but also induce metalloproteinase expression, leading to extracellular matrix breakdown and tumour invasion. Immature myeloid dendritic cells are found commonly in melanoma, possibly in response to CCL5, and these have been previously shown to induce T cell anergy and suppress the CD8+ cytotoxic anti-tumour response (Kusmartsev, Nagaraj et al. 2005, Nagaraj and Gabrilovich 2008).

1.9.4 Chemokines, cancer and tumour-associated macrophages

Tumour-associated macrophages (TAMs) have been noted in many cancers and appear to have a deleterious effect on invasiveness, metastasis and overall prognosis. Macrophages are overall the most abundant immune cell found in the peritumoural stroma (Allavena and Mantovani 2012). They are attracted by several chemokines, including CCL2, CCL5 and CXCL1, as well as by other growth factors such as VEGF and TGF-β. TAMs promote tumour growth and metastasis by producing growth factors and angiogenic factors e.g. IL-1 (Carmi, Voronov et al. 2009), by breaking down extracellular matrix and remodelling tissue and thereby facilitating invasion by producing MMP-9 and uPA (Hildenbrand, Dilger et al. 1995, Hiratsuka, Nakamura et al. 2002, Pollard 2004), and also by suppressing the anti-

tumour adaptive immune response by producing immunosuppressive cytokines and chemokines. Macrophages can be activated to either of two profiles; M1 is predominantly pro-inflammatory, while the M2 phenotype is involved in immune modulation, suppresses the immune system, is pro-angiogenic and promotes tissue repair (Mantovani, Sozzani et al. 2002). In tumours, the macrophages are predominantly activated to an M2 type which is promoted by chemokines and other factors produced by the tumour e.g. CCL2, IL-4, IL-10, M-CSF, TLRs (Sica, Schioppa et al. 2006).

Colon cancer cells and TAMs are mutually chemoattracted to each other; when macrophages were exposed to conditioned buffer from the tumour cells, they upregulated the chemokines CXCL2 and CXCL12, as well as pro-angiogenic factors such as VEGF, while when the colon cancer cells were exposed to buffer from macrophages, they upregulated CSF-1 (a cytokine that controls production and function of macrophages, and also acts as a chemoattractant for these cells), as well as the chemokines CCL2, CCL20, CXCL2 and CXCL20 (Green, Liu et al. 2009). This suggests that a positive feedback loop may exist between the tumour and TAMs, promoting macrophage infiltration which then promotes tumour growth, angiogenesis and matrix remodelling. CCL2 expression correlated with tumour stage in patients with colorectal adenocarcinoma, with rising numbers of infiltrating macrophages within the tumours, implying that accumulation of TAMs is a poor prognostic factor in colon carcinoma. These cells migrated actively towards CCL2 and this migration was attenuated by blocking CCL2. They also noted that high levels of CXCL8 (IL-8, a potent growth promoting chemokine) in tumours of all stages, which suggests that this chemokine may have an early role in tumour promotion. CCL2 has also been found to be produced by prostate cancer cells

(Loberg, Ying et al. 2007); in these tumours it appears to promote growth not only through chemotaxis of TAMs but also by direct effect on cancer cells.

A recent review on the chemokines CCL2 and CCL5 in breast cancer (Soria and Ben-Baruch 2008) suggests that these molecules induce an imbalance in leucocyte subsets attracted to the tumour microenvironment, including an excess of monocytes which give rise to TAMs. These two chemokines, not produced by normal breast epithelium, promote a tumour-supporting immune environment through chemotaxis. Breast cancer cells that expressed CCL2 have also been found to decrease the T cell response (measured by IFN-ɣ production) compared with tumour cells that did not express CCL2 (Vitiello, Shainheit et al. 2004). Therefore CCL2 could also play a role in evading the host immune response as well as in attracting pro-cancerous inflammatory cells. A positive correlation between stromal levels of CCL2 and the number of TAMs has been demonstrated in the breast tumour environment, and also between stromal CCL2 and relapse-free survival (Fujimoto, Sangai et al. 2009).

1.9.5 Chemokines, cancer and effector T cells

An interesting study (Sugasawa, Ichikura et al. 2008) on gastric cancer found that serum levels of CCL5 were significantly higher in cancer patients than in normal volunteers, and also that the levels of serum CCL5 correlated with disease progression and with survival. They found that in these patients, CD4+ infiltrating lymphocytes expressed CCL5 while the tumour cells expressed the CCL5 receptors CCR1, CCR3 and CCR5. CCL5 increased proliferation of the tumour cells, and CCL5-treated tumour cells upregulated CCL5 production by CD4+

lymphocytes while inducing apoptosis of CD8+ lymphocytes. Thus the gastric cancer cells exploit the host CD4+ cell production of CCL5 to promote tumour cell proliferation, and use the same mechanism to evade cytotoxic anti-tumour CD8+ lymphocytes.

CXCL16 and its receptor CXCR6 have been found to be co-expressed on prostate cancer cells, and levels of these correlated with grade and stage of the tumour (Darash-Yahana, Gillespie et al. 2009). Correlation was found between levels of CXCL16 and CXCR6 and the presence of reactive stroma. They found that CD4+ T cells in the stroma both expressed CXCL16 and proliferated in the presence of CXCL16, and that they expressed the receptor CXCR6. This shows that there may be both an autocrine role for this chemokine in tumour cell proliferation, and a role in recruitment of leucocytes that in turn promote growth.

Skinnider and Mak reviewed the production and chemoattractant abilities of chemokines in Hodgkin's lymphoma (HL); they found that Reed-Sternberg cells produced many cytokines and chemokines, including CCL17 (TARC), CCL22 (MDC) and CCL11 (eotaxin) (Skinnider and Mak 2002). These attract mainly Th2 leucocytes as well as eosinophils, which are commonly found associated with Hodgkin's lymphoma specimens. Some Th1-associated chemokines were also found to be produced in HL, including CXCL9, CXCL10, CCL2, CCL3 and CCL5. The predominant Th2 infiltrate is thought to activate NF-kB, which then causes production of growth promoting and angiogenic cytokines.

1.9.6 Chemokines, cancer and neutrophils

Neutrophils are normally the first leucocytes recruited to sites of acute insult or inflammation. They usually release cytokines and chemokines which then attract other cells to the area. However some cancers can use the pro-inflammatory activities of neutrophils to aid tumour growth and spread. In a mouse model of pancreatic cancer, neutrophils were found within the tumour while macrophages were found at the peripheries (Nozawa, Chiu et al. 2006). Both of these cell types expressed matrix-metalloproteinase 9 which could then liberate VEGF from the pancreatic cells themselves; MMP- 9 released from neutrophils is a potent stimulator of angiogenesis (Ardi, Kupriyanova et al. 2007). Ablating the neutrophils using a monoclonal antibody caused a significant reduction in angiogenic switching and in the number of VEGF:VEGF-receptor complexes on pancreatic cells.

Tumour-associated neutrophils have also been shown to enhance invasion through the basement membrane; an in vitro assay demonstrated that neutrophils from mice with tumours caused mammary tumour cells to penetrate a reconstituted basement membrane, while neutrophils from normal controls did not have the same effect (Welch, Schissel et al. 1989). Neutrophils, as well as mast cells and macrophages, have been found to be the primary source of MMP-9 in dysplastic skin and in skin carcinomas, and that this MMP-9 promoted carcinogenesis and invasion (Coussens, Tinkle et al. 2000). Another study used benign fibrosarcoma cells which converted to a malignant phenotype once implanted into mice; the predominant infiltrate into the implanted tumour was of neutrophils. When these neutrophils were knocked out or blocked, the tumour still developed but had greatly impaired metastatic ability (Tazawa, Okada et al. 2003).

An interesting study by Pahler et al. looked at the interplay between macrophages and neutrophils in cervical cancer (Pahler, Tazzyman et al. 2008). They found, as expected, that increased expression of CCL2 and CCR2 correlated with increased macrophage infiltrate, and with increased MMP-9. However, in CCR2-deficient mice, the tumour burden was unchanged and the expression of MMP-9 remained high; this MMP-9 was associated with an increased number of MMP-9+ neutrophils in the tumour. Macrophage-conditioned media ablated the normal chemotaxis of neutrophils towards CXCL-8. The authors suggest that macrophages usually inhibit neutrophil migration; in the absence of macrophages, neutrophils can take their place in the tumour, producing MMP-9 and promoting growth and invasion. This indicates that tumours can be flexible in the ways in which they exploit the host immune cells – if one leucocyte type is blocked, it can use another for a similar purpose.

Increased neutrophil infiltrate seen in myxofibrosarcomas correlated significantly with increases in microvessel density within the tumour (Mentzel, Brown et al. 2001). Neutrophil chemoattractants are produced by many tumour types; CXCL8 is produced by bronchioalveolar carcinoma, as well as by colon cancer and prostate cancer (Bellocq, Antoine et al. 1998). CXCL6 was found to be produced by melanoma cells and induced neutrophil infiltration and also promoted angiogenesis in a mouse model (Van Coillie, Van Aelst et al. 2001). CXCL5, another potent chemokine for neutrophils, is expressed in significantly higher levels in colorectal carcinoma tissue than in normal tissue (Dimberg, Dienus et al. 2007).

Signalling through a prostaglandin pathway (PGF-2-alpha) in endometrial carcinoma can stimulate the cancer cells to produce CXCL1, which acted as a chemoattractant for neutrophils which expressed its receptor CXCR2 (Wallace,

Sales et al. 2009). CXCL1 has been shown not only to recruit neutrophils, but also to induce release of VEGF which then promotes angiogenesis (Scapini, Morini et al. 2004). Colon carcinoma cells have also been found to express CXCL1, with levels of expression correlating with metastasis, proliferation and invasive potential (Li, Varney et al. 2004).

1.9.7 Chemokines, cancer and eosinophils

Persson-Dajotoy et al have described eosinophils expressing large amounts of the chemokine CXCL1 (GRO-alpha) in response to TNF-α (Persson-Dajotoy, Andersson et al. 2003); eosinophils themselves can produce TNF-α, thereby creating an autocrine loop (Costa, Matossian et al. 1993). The original isolation of CXCL1 showed that it stimulated growth in melanoma cells (Richmond and Thomas 1986), while it has also been found to be upregulated in colon cancer (Erreni, Bianchi et al. 2009). Production of CXCL1 was seen in eosinophils isolated from patient tumour samples of Hodgkin's lymphoma; in fact, eosinophilia in Hodgkin's lymphoma has been shown to correlate negatively with prognosis (von Wasielewski, Seth et al. 2000). The Reed-Sternberg cells of Hodgkin's lymphoma also express CCL28 (MEC) which is a chemoattractant for eosinophils via CCR3 and for plasma cells via CCR3 and CCR10. Hanamoto et al. found that expression of CCL28 correlated significantly with infiltration of these immune cells in lymphoma tumour samples (Hanamoto, Nakayama et al. 2004). CCL5 and its receptor CCR5 are found in Reed-Sternberg cells, again causing chemotaxis of eosinophils as well as T cells (Aldinucci, Lorenzon et al. 2008).

Cormier et al examined recruitment of eosinophils into melanoma tumours in mice; they found that eosinophils accumulated in the capsule and in necrotic areas of the tumour (Cormier, Taranova et al. 2006). Conditioned media from these cells was strongly chemotactic for eosinophils; the known chemoattractants CCL11 (eotaxin-1) and CCL24 (eotaxin-2) were not found in the media, suggesting that a different chemokine may be responsible for this migration. They found that the same chemotaxis was seen with conditioned media from lung cancer and rectal cancer cells. Murdoch et al. speculate that these eosinophils in hypoxic areas may release pro-angiogenic factors such as VEGF, TGF-β and MMP-9 from their secretory granules, thus promoting tumour growth (Murdoch, Muthana et al. 2008). Eosinophilic infiltrate has also been shown to correlate with a worse clinical outcome in patients with cervical carcinoma (van Driel, Hogendoorn et al. 1996); the authors suggest that tumour cells or other immune cells may produce chemoattractant molecules that chemotax eosinophils.

1.9.8 Chemokines, cancer and mast cells

Mast cells are immune cells that are derived from a unique bone marrow precursor; they are involved in the innate immune response and also in allergic and inflammatory processes. They have also been shown to be involved in angiogenic and pro-tumour activity (Crivellato, Nico et al. 2008). The granules of mast cells contain many factors that could promote carcinogenesis and tumour growth – VEGF, TGF-β, TNF-α, βFGF, tryptase and IL-8, as well as other chemokines such as MIP-1 alpha (CCL3), and matrix metalloproteinases such as MMP-2 and MMP-9 (Norrby 2002).

Mast cell infiltration has been described in pancreatic cancer (Soucek, Lawlor et al. 2007, Theoharides 2008); in response to activation of the Myc transcription factor (activated in many neoplasms), numerous chemokines were induced including CCL2, CXCL2 and CCL5, the latter being a potent chemoattractant for mast cells (Conti, Pang et al. 1997). Mast cells were recruited to the tumour within 24 hours of Myc activation. These cells were found to be essential for tumour expansion and for maintenance of established tumours, as blocking mast cell degranulation resulted in tumour cell death, endothelial cell death and loss of tumour expansion. Since during expansion, blocking mast cell degranulation resulted in hypoxia, it would appear that the contents of mast cell granules are essential for angiogenesis in these tumours. Sawatsubashi et al. found that the higher microvessel density in laryngeal carcinoma compared with normal laryngeal tissue was associated with expression of VEGF; on staining a significant correlation was seen between VEGF expression and mast cell infiltrate (Sawatsubashi, Yamada et al. 2000). A similar correlation between microvascular density and mast cell count was seen in renal cell carcinoma (Tuna, Yorukoglu et al. 2006), oral squamous cell carcinoma (Iamaroon, Pongsiriwet et al. 2003, Michailidou, Markopoulos et al. 2008), oesophageal squamous cell carcinoma (Elpek, Gelen et al. 2001) and gastric cancer (Yano, Kinuta et al. 1999), with significantly higher numbers of mast cells in tumour tissue than in normal tissue.

1.9.9 Chemokines, cancer and B cells

While research has not focused much on the functions of B lymphocytes in cancer, some evidence has emerged that they may play a key role in

carcinogenesis. De Visser et al. found that mice depleted of B cells did not develop chronic inflammation and therefore did not develop tumours (de Visser, Korets et al. 2005). Transfer of B cells or serum into these mice resulted in restoration of the immune response and of carcinogenesis. The authors suggest that activated B cells may produce factors such as chemokines e.g. CXCL1 (Hu, Dixit et al. 2004) which maintain chronic inflammation and then promote tumour formation.

1.10 CCL28

CCL28 or mucosa-associated epithelial chemokine (MEC) is a CC chemokine which binds the receptors CCR3 and CCR10 (Pan, Kunkel et al. 2000). The CCL28 gene is located on chromosome 5 where the coding sequence includes 4 exons separated by large introns, and encodes a 127 amino acid protein, measuring 14 kDa in size. It was first discovered in 2000, where it was found to share 83% amino acid identity with its mouse homologue, and shared the CCR10 receptor with the previously identified chemokine CCL27 (Pan, Kunkel et al. 2000, Wang, Soto et al. 2000); CCL28 displays approximately 40% amino acid identity with CCL27.

CCL28 functions as a chemokine by chemoattracting a wide range of immune cells. It has been demonstrated to be chemotactic for IgA-secreting plasma cells in many tissues, including in intestinal lymphoid tissues, suggesting a role in the mucosal immune system. It is also chemotactic for T regulatory cells, eosinophils, CD4+ and CD8+ T lymphocytes and dendritic cells (John, Thomas et al. 2005, Eksteen, Miles et al. 2006, Bourges, Meurens et al. 2008, Sundstrom, Lundin et al. 2008, Cha, Ko et al. 2011, Facciabene, Peng et al. 2011, Scanlon,

Hawksworth et al. 2011). Human CCL28 has also been shown to have a direct anti-microbial activity, killing various bacterial species including *Pseudomonas aeruginosa, Streptococcus pyogenes* and *Staphylococcus aureus*, as well as the fungus *Candida albicans* (Hieshima, Ohtani et al. 2003). It has been found in several cell types, such as airway and colonic epithelium, keratinocytes and cholangiocytes, to be increased by the pro-inflammatory cytokines IL-1β and TNF-α (Ogawa, Iimura et al. 2004, O'Gorman, Jatoi et al. 2005, Kagami, Saeki et al. 2006, Shibata, Maeda et al. 2010). A recent study examining haematopoietic stem cells found that CCL28 may have a direct stimulatory effect on stem cells, enhancing cell cycling and promoting cell survival via gene expression changes (Karlsson, Baudet et al. 2013). This function of CCL28 may lead to future discoveries with regard to its role in cancer promotion and development.

CCL28 is expressed in a range of human tissues, predominantly in columnar epithelial tissues such as colon, small intestine, salivary gland, airway epithelium, uterus and mammary gland (Ogawa, Iimura et al. 2004, O'Gorman, Jatoi et al. 2005, Bourges, Meurens et al. 2008, Cha, Ko et al. 2011); it is also found to be increased in inflammatory conditions such as inflammatory bowel disease, asthma, atopic dermatitis, gastritis and biliary cirrhosis (Eksteen, Miles et al. 2006, Hansson, Hermansson et al. 2008, Ezzat, Sallam et al. 2009, Arijs, De Hertogh et al. 2011).

CCL28 is expressed by the Reed-Sternberg cells found in Hodgkin's lymphoma, associated with accumulation of eosinophils and plasma cells (Hanamoto, Nakayama et al. 2004) It has also recently been shown to be linked to the development of colorectal cancer in the inflamed colon in a mouse model – loss of the transcription factor STAT3 leads to increased CCL28 expression which

recruits Treg cells and plays a role in tumourigenesis (Nguyen, Wu et al. 2013). Once the tumour has developed, the chemotactic activity of CCL28 may be less beneficial to the cancer tissue. Colorectal adenocarcinoma tissue has been found to have lower levels of CCR10+ expressing IgA plasma cells than normal tissue, which do not migrate towards CCL28 (Muthuswamy, Sundstrom et al. 2013). The authors hypothesise that the lack of IgA antibodies may decrease the colonic barrier and lead to further inflammation and tumour growth. A similar finding in tumours of the salivary glands, with reduced expression of CCL28 in adenomas and adenolymphomas compared with normal glands, may again reflect decreased mucosal or antitumour immunity (Liu, Lan et al. 2012).

CCL28 has been shown to be regulated via various transcription factors. The transcription factor NF-kB has most frequently been demonstrated to be a regulator of CCL28 expression, in colonic epithelium, airway epithelium and human and canine keratinocytes (Ogawa, Iimura et al. 2004, O'Gorman, Jatoi et al. 2005, Kagami, Saeki et al. 2006, Shibata, Maeda et al. 2010). This regulation by NF-kB is via the cytokines IL-1β and TNF-α in several of these studies. It has also been shown to be increased by hypoxia via HIF-1α in ovarian cancer cells, where it promoted Treg cell recruitment and induced tumour tolerance and angiogenesis (Facciabene, Peng et al. 2011). STAT3 may be a negatively regulating transcription factor for CCL28, as loss of STAT3 activity increased CCL28 expression in colonic epithelium in a mouse model (Nguyen, Wu et al. 2013).

Interestingly, CCL28 expression has been found to correlate with resistance to treatment and immune evasion in several cancers including breast, ovarian and oesophageal tumours (Maher, Gillham et al. 2009, Tsao, Chang et al. 2010, Facciabene, Peng et al. 2011, Krech, Scheuerer et al. 2012). CCL28 expression

may be increased in response to radiation therapy in rectal tumours (Supiot, Gouraud et al. 2013) In OAC, CCL28 gene expression was found to correlate significantly with a poor response to neoadjuvant chemoradiation therapy in oesophageal cancer patients prior to surgery (Maher, Gillham et al. 2009). CCL28 has never previously been shown to be expressed in normal squamous epithelium of the oesophagus.

1.11 Summary and hypotheses

Based on the information presented in the preceding sections, it is clear that chronic inflammation, and the multitude of cytokines, chemokines, transcription factors and immune cells associated with this state, can have a severely deleterious effect on the human body and may be involved in tumourigenesis in many cell types. A previous study from a group based in Trinity College Dublin identified CCL28 as one of the genes correlating with poor response to therapy in oesophageal adenocarcinoma, which was interesting as CCL28 expression had never been demonstrated in oesophageal tissue (Maher, Gillham et al. 2009). As CCL28 has been demonstrated to be regulated via the pro-inflammatory cytokines IL-β and TNF-α in other tissue types, it was hypothesised that this may also be the pathway by which CCL28 is induced in the oesophagus. CCL28 can be regulated via many transcription factors, including NF-kB which can be induced by IL-1β and TNF-α. As it is known that reflux of bile acids into the oesophagus is an important risk factor for the development of BO and OAC, and that bile acids can induce expression of IL-1β and TNF-α, it was also hypothesised that bile acids may induce CCL28 expression in the oesophagus via NF-kB and pro-inflammatory cytokines,

and therefore that CCL28 may be differentially expressed during the progression of oesophageal disease from BO to dysplasia to OAC.

Based on these data, the hypotheses of this study are as follows:

- CCL28 is differentially expressed during the progression of oesophageal tissue from normal tissue to Barrett's oesophagus to low-grade and high-grade dysplasia to oesophageal adenocarcinoma, in response to bile acids
- CCL28 expression in the oesophagus is regulated via the pro-inflammatory cytokines IL-1β and TNF-α through transcription factors induced by these cytokines
- CCL28 expressed by the oesophagus modulates the immune microenvironment of the oesophagus by recruiting CCR3+ and CCR10+ immune cells, thereby influencing oesophageal disease development, progression and response to therapy

1.12 Specific aims

1. To characterise the expression of CCL28 and the pro-inflammatory cytokines IL-1β and TNF-α in normal, pre-neoplastic and neoplastic oesophageal cell lines (in vitro) in response to bile acid; to characterise the expression of CCL28 in tissue explants (ex vivo) from Barrett's and oesophageal cancer patients.

2. To examine the mechanisms by which CCL28 is induced in the oesophagus via transcription factors, cytokines and reactive species; to investigate the effects that CCL28 has on chemotaxis of immune cells.

3. To retrospectively assess the expression of CCL28 and CCL28-driven immune infiltrate in tissue microarrays from Barrett's, dysplastic and carcinoma patients and correlate this data with disease progression and clinical status.

Chapter 2

Materials and Methods

2.1 Reagents and materials

All laboratory reagents and chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated, and were prepared and stored according to the manufacturer's specifications. Solid reagents were weighed using a Scout Pro electronic balance (Ohaus Corporation, Pine Brook, NJ, USA) or an Explorer Pro analytical electronic balance (Ohaus Corporation) and made up using distilled water (dH_2O), unless otherwise stated. The pH of solutions was measured using a pH 211 microprocessor pH metre (Hanna Instruments, Smithfield, RI, USA), calibrated with buffers at pH 4, 7 and 10. Solutions were stored at room temperature (RT°C), unless otherwise stated. Calibrated Gilson pipettes (Gilson Inc., Middleton, WI, USA) were used to transfer liquid volumes up to 1 mL. Electronic pipette aids (Drummond Scientific Company, Broomall, PA, USA) and disposable sterile Pasteur pipettes (Sarstedt Ltd., Wexford, Ireland) were used for volumes greater than 1 mL, and graduated cylinders were used for volumes greater than 10 mL.

2.1.1 Deoxycholic acid (DCA)

Deoxycholic acid was weighed as previously described and made up to the appropriate concentration in complete medium. This solution was then filtered using a 0.2 µm sterile filter to eliminate bacterial contamination and was stored at 4°C for use in cell culture treatments. All chemicals used in cell culture medium were sterile filtered in this manner.

2.1.2 Recombinant human cytokines

Recombinant human cytokines IL-1β and TNF-α were purchased from Prospec Bio (Prospec Bio, East Brunswick, NJ, USA), reconstituted in dH_2O and were aliquoted and stored at -20°C. Solutions were made up in cell culture medium immediately prior to inclusion in experiments.

2.2 Cell culture

All cell culture media was purchased from Lonza (Basel, Switzerland) and all cell culture plastics were purchased from Sarstedt Ltd. (Wexford, Ireland), unless otherwise stated. Cell lines were purchased from the American Collection of Cell Cultures (ATCC) (ATCC, Manassas, Virginia, USA), unless otherwise stated. Cell lines were maintained as monolayers in either 25 cm^2 or 75 cm^2 sterile vented flasks, in an incubator at 37°C in 95% humidified air containing 5% CO_2, unless otherwise stated. Cell culture was carried out in a dedicated cell culture room. Cell culture was carried out in a Grade II laminar air-flow cabinet using aseptic technique while wearing a clean Howie style laboratory coat with elasticated cuffs and disposable gloves. The hood and incubators were cleaned and sterilised every month. The cabinet and all reagents, media and plastics were cleaned with 70% (v/v) ethanol (Merck) in dH_2O before and after use.

2.2.1 Oesophageal cell lines

2.2.1.1 HET-1A cell line

The HET-1A cell line, which represents histologically normal squamous oesophageal tissue, was maintained in Bronchial Epithelial Cell Basal Medium (BEBM), supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS), as well as the hormonal cocktail BEGM® SingleQuots® (hereafter known as complete medium). This supplemented the BEBM media with bovine pituitary extract (BPE), insulin, hydrocortisone, GA-1000, transferrin, tri-iodo-thyronine (T3), epinephrine and human epidermal growth factor (EGF).

2.2.1.2 QH-TERT and GO-TERT cell lines

The QH-TERT cell line, representing metaplastic Barrett's oesophageal epithelium, and the GO-TERT cell line, representing high-grade dysplastic oesophageal epithelium, were kind gifts from Dr. Shane Duggan of Trinity College Dublin. These cell lines were maintained in BEBM supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin/streptomycin (50 U/mL penicillin, 50 U/mL streptomycin) solution, as well as the hormonal cocktail BEGM® SingleQuots® (complete medium). This supplemented the BEBM media with BPE, insulin, hydrocortisone, GA-1000, transferrin, T3, epinephrine and human EGF.

2.2.1.3 OE33, OE19 and JH-Eso-Ad1 oesophageal adenocarcinoma cell lines

The cell lines OE33, OE19 and JH-Eso-Ad1 represent oesophageal adenocarcinoma. These cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin/streptomycin (50 U/mL penicillin, 50 U/mL streptomycin) solution (hereafter known as complete medium).

2.2.2 Cell subculture

Cell lines were examined daily using an inverted phase contrast Nikon microscope (Nikon Corporation, Tokyo, Japan) and subcultured upon reaching 80-90% confluency. Cells were detached for subculturing by trypsinisation. Spent growth medium was decanted to waste and cells were washed with 5 mL warm, sterile phosphate buffered saline (PBS) (13.8 mM NaCl, 2.7 mM KCl, pH 7.4), to remove residual FBS, which inactivates trypsin. A volume of 1 mL trypsin – ethylene diamine tetra-acetic acid (EDTA) (0.05% (w/v) trypsin, 0.02% (w/v) EDTA) was added to the cells. Cells were incubated at 37°C in 95% humidified air containing 5% CO_2 for 1-10 min or until 90-100% of adherent cells were observed as detached from the surface of the flask. An equal volume of complete medium was then added to inactivate the trypsin and cells were seeded at appropriate densities in new tissue culture flasks. All cells were maintained in 5 mL or 13 mL complete medium in 25 cm^2 or 75 cm^2 sterile vented flasks, respectively.

2.2.3 Preparation of frozen cell stocks

Frozen stocks of cells at low passage were prepared from cell lines growing in the exponential phase and at 70-80% confluency. Cells were trypsinised as previously described (section 2.2.2) and 10 mL complete medium was added to inactivate the trypsin. Cells were centrifuged at 180 x *g* for 3 min, the supernatant was decanted and the cell pellet re-suspended in FBS containing 10% (v/v) dimethyl sulfoxide (DMSO) by drop-wise addition. DMSO prevents crystal formation during freezing of cells. The cell suspension was divided into 1 mL aliquots in sterile labelled 2 mL cryotubes. Cryotubes were placed in a container containing isopropanol and were then transferred to a -80°C freezer overnight, a procedure which lowers the temperature by 1°C per min. Cells were stored at -80°C for short term storage (up to 1 month), or for long term storage, cells were stored under liquid nitrogen.

2.2.4 Reconstitution of frozen cells

Frozen cells were removed from the freezer or liquid nitrogen and were immediately thawed at 37°C in a water bath for approximately 1 minute with regular, mild agitation. The cell suspension was added to 10 mL pre-warmed complete medium and centrifuged at 180 x *g* for 3 min. The supernatant was decanted and the cell pellet re-suspended in 5 mL complete medium and transferred to a 25 cm^2 flask.

2.2.5 Cell enumeration and viability assessment

Cells were quantified using a Bright Line haemocytometer (Hausser Scientific, Horsham, PA, USA). Cells were trypsinised as previously described (section 2.2.2), combined with 10 mL complete medium and centrifuged at 180 x *g* for 3 min. The supernatant was decanted and cell pellet re-suspended in 1 mL complete medium. A 20 µL volume of this cell suspension was added to 180 µL Trypan Blue solution (0.4% w/v) and incubated at RT°C for 1 min. Viable cells remain unstained due to their active exclusion of Trypan blue stain, whilst dead cells are unable to exclude the stain due to disruption of their cell membrane integrity, appearing blue, rather than clear, when viewed under a microscope. A 10 µL volume of this cell suspension was added to the counting chamber of the haemocytometer and viable cells in each of the four corner squares of the grid were counted under 40X magnification. Live cells crossing the top and left hand side of the grid were included, whilst live cells crossing the bottom and right hand sides were excluded from the count. The number of cells per mL in the suspension was calculated using the equation:

$(n/4) \times 10 \times 10^4$ = number of cells per mL

where,

n = Total number of cells counted

4 = number of fields counted

10 = dilution factor

10^4 = constant

As necessary, all densities were adjusted using complete medium, unless otherwise stated.

2.2.6 Mycoplasma testing

Upon receipt of a new cell line, and every three months thereafter, cell lines were tested for infection by mycoplasma. This was performed using a polymerase chain reaction (PCR) – based assay (Young, Sung et al. 2010). If the sample tested was contaminated with mycoplasma, a PCR product measuring 270 bp was detected. To do this test, cells were maintained and subcultured in complete medium for a minimum of 2 passages. A 1 mL volume of culture medium was taken from a confluent 75 cm^2 flask of each cell line to be tested and was stored at 4°C for testing. Tubes were centrifuged at 180 x *g* for 3 min to pellet any cells and debris present. The PCR reaction was then set up in a laminar flow cabinet. A master mix was made up to contain (per PCR reaction): 25 µL Green GoTaq (2X) (Promega, Madison, WI, USA), 1 µL forward and reverse primers (10 µM) and 22 µL AccuGENE molecular-grade H_2O. A 1 µL volume of cell culture supernatant was then added to each reaction to give a final volume of 50 µL per reaction.

A negative control was included, and supernatant from a cell line known to be contaminated with mycoplasma provided a positive control. Samples were then amplified in an automated DNA Thermal Cycler (MJ Research Inc., Watertown, MA, USA) using the conditions described in table 2.1. The products were analysed by agarose gel electrophoresis. A 2% w/v agarose gel was made by adding 3.0 g agarose powder (Bioline, London, UK) to 150 mL 1X Tris-acetate EDTA (TAE) buffer (40 mM Tris HCl, pH 8.0, 20 mM glacial acetic acid, 1 mM EDTA, pH 8.0).

Table 2.1 Forward and reverse primer sequences for mycoplasma PCR assay

Mycoplasma primer	Sequence
FWD 5' to 3'	TGCACCATCTGTCACTCTGTTAACCTC
REV 5' to 3'	GGGAGCAAACAGGATTAGATACCCT

The agarose was melted in the TAE buffer by boiling in a microwave for 2 min, then allowed to cool to 55-60°C. A 6 µL volume ethidium bromide (500 µg/mL) was added to the TAE solution, mixed thoroughly and was then poured into a gel electrophoresis rig mold. Two 16 well combs were inserted and bubbles removed from the gel using a pipette tip. The gel was allowed to set at RT°C for 30 min. The combs were then gently removed from the gel and the gel was submerged in 1X TAE buffer with the wells proximal to the anode. A 5 µL volume of 100 bp molecular weight DNA ladder (Promega) was loaded into the first well of the gel, and subsequently 18 µL of each PCR product to be tested was loaded into each well. Electrophoresis was carried out using a MAXI Horizontal electrophoresis rig (Sigma-Aldrich) keeping the voltage constant at 125 V for ~45 min, or until the molecular weight marker dye front was ~3/4 down the length of the gel. The PCR products were then visualised under UV light using a Biospectrum Imaging System (Ultra Violet Products, Cambridge, UK). Images were taken using the Vision Works LS software programme. Positive samples demonstrated a band at the 270 bp level, while negative samples had no band at this level.

2.2.7 DCA and cytokine treatments

Cell lines were treated with varying concentration of DCA (0 - 500 µM) made up in complete medium as previously described (2.1.1). Cells were treated with DCA for 24 h, unless otherwise stated. Cells were treated with recombinant human cytokines (IL-1β and TNF-α) at 10 ng/mL in complete medium for 24 h. Cells were seeded into 6-well plates at appropriate density and allowed to adhere overnight at 37°C in 95% humidified air containing 5% CO_2. Cells were then washed once with

PBS warmed to 37°C and the treatment was applied. Treated cells were then incubated for the appropriate time period at 37°C in 5% CO_2/95% humidified air.

2.2.8 Cell viability following DCA treatment

Following 24 h DCA treatment, oesophageal cell lines were assessed for cell viability using the Trypan blue exclusion method described previously in section 2.3.6. Cells were seeded at appropriate density (3 x 10^5 cells/well) in 6-well plates, allowed to adhere overnight, and were then treated with varying concentrations of DCA (0 - 500 µM) for 24 h, except for the Het-1A cell line, which was treated with concentrations varying from 0 - 100 µM for 1 h. Cells were washed with PBS warmed to 37°C and then trypsinised as previously described (section 2.2.2). Cells were centrifuged at 180 x *g* for 3 min and the cell pellet re-suspended in 1 mL complete medium. A 100 µL volume of this cell suspension was then added to 100 µL Trypan blue and allowed to incubate at RT°C for 1 min. Viable cells (which exclude Trypan blue) and non-viable cells (which include Trypan blue) were counted and recorded. Each count was performed at least 5 times for each concentration of DCA used. The number of non-viable cells was expressed as a percentage of the total number of cells.

2.3 Gene expression

2.3.1 RNA isolation from cell lines

RNA was isolated from cell lines using TRI-reagent® RNA isolation reagent (Molecular Research Centre Inc., Cincinnati, OH, USA). TRI-reagent combines

phenol and guanidine thiocyanate to facilitate the inhibition of RNAse activity. Samples are lysed in TRI-reagent and addition of bromochloropropane (BCP) separates the lysate into organic and aqueous phases. RNA partitions to the uppermost aqueous phase, DNA to the interphase and protein to the lowest organic phase. RNA can then be precipitated from the aqueous phase.

Growth medium was decanted from cells and the cells were washed using PBS warmed to 37°C. Cells were then lysed directly in the flask or culture vessel by addition of 1 mL or 0.5 mL TRI-reagent in 75 cm^2 or 25 cm^2 flasks, respectively. Cells were scraped from the surface of the flask using a plastic cell scraper. The TRI-reagent/cell suspension was then pipetted up and down three times to aid lysing of cells, and transferred to a 1.5 mL eppendorf tube. Samples were stored immediately at -80°C.

To isolate total RNA, samples were allowed to thaw and incubated at RT°C for 10 min to allow complete dissociation of nucleoprotein complexes. A volume of 100 µL BCP per 1 mL TRI-reagent was added to the sample, vortexed for 15 s and then stored at RT°C for 10 min. Samples were then centrifuged at 13,400 x *g* for 15 min at 4°C. During centrifugation, samples separate into a lower pink phenol-chloroform phase, a white interphase and an upper clear aqueous phase. The upper aqueous phase was pipetted into a new 1.5 mL eppendorf tube and RNA was precipitated by the addition of 0.5 mL isopropanol per 1 mL TRI-reagent used. Samples were stored for 5 min at RT°C and then centrifuged at 13,400 x *g* for 8 min at 4°C. The supernatant was decanted to waste using a pipette and the remaining RNA pellet was washed by vortexing with 1 mL ethanol 75% (v/v) (Merck). Samples were centrifuged at 7,500 x *g* for 5 min at 4°C, the ethanol wash was removed and the pellet was allowed to air-dry for 3-4 min. The RNA pellet was

re-suspended in 30 - 50 µL molecular-grade H_2O, depending on the relative size of the pellet. Isolated RNA was stored at -80°C.

2.3.2 RNA quantification

RNA was quantified spectrophotometrically using a Nanodrop 1000 spectrophotometer v3.3 (Thermo Scientific, Wilmington, DE, USA). The instrument was cleaned before use, using 1 µL molecular-grade H_2O, and then blanked using 1 µL molecular-grade RNAse-free H_2O. A 1 µL volume of the RNA sample being quantified was loaded onto the Nanodrop pedestal. RNA was measured in ng/µL. DNA contamination was assessed by the 260/280 ratio; a ratio above 1.8 indicates a relatively pure yield of RNA. The 260/230 ratio determines the level of phenol contamination; a ratio above 1.7 indicates that the sample is phenol free.

2.3.3 Polymerase chain reaction (PCR)

2.3.3.1 Reverse transcription reaction

RNA was reverse transcribed to cDNA using the random primers method. This uses random hexamer oligodeoxyribonucleotides to prime mRNA for cDNA synthesis. A volume of 1 µL of random hexamers (Promega) was added to total RNA (1 µg) and the volume was adjusted to a total volume of 11 µL using RNAse-free H_2O. This mixture was incubated at 70°C for 10 min to denature the RNA. The samples were then placed on ice immediately for at least 2 min. For reverse transcription, a master mix was made up to contain (per reaction): 0.5 µL RNAsin (40 U/µL) (Promega), 0.5 µL 10 mM dNTPs (stock prepared as a 1:1:1:1 ratio of

dATP, dCTP, dGTP and dTTP) (Promega), 4 µL reverse-transcription reaction buffer (250 mM Tris HCl, 375 mM KCl, 15 mM $MgCl_2$) (Invitrogen, Carlsbad, CA, USA), 2 µL 0.1 M dithiothreitol (DTT) (Invitrogen), 0.5 µL Superscript II (200 IU/µL) (Invitrogen) and 0.5 µL RNAse-free H_2O. This master mix was added to the RNA/random hexamer reaction and mixed well by pipetting. Samples were pooled by centrifugation. The reaction mixture was incubated at 37°C for 1 h. cDNA samples were then stored at -20°C for further use.

2.3.3.2 Quantitative real-time PCR

cDNA reverse transcribed from total RNA was used as a template for quantitative real-time PCR. A PCR master mix was made up to contain (per sample to be amplified): 10 µL 2X TaqMan® Universal Master Mix (Applied Biosystems, Foster City, CA, USA), 8.0 µL nuclease-free H_2O, 1 µL TaqMan® gene expression assay for the gene of interest (Applied Biosystems). cDNA (1 µL) from each sample was added in triplicate to a 96-well optical reaction plate (ABI Prism®, Applied Biosystems). A no-template control using 1 µL H_2O in place of cDNA template was included on each plate. A 19 µL volume of the PCR master mix was then added to each well, to give a final reaction volume of 20 µL. The plate was sealed using an optically clear adhesive plastic cover (Applied Biosystems) and was centrifuged briefly to pool reagents and eliminate air bubbles. Real-time PCR detection was performed using an ABI 7900HT real-time thermal cycler (Applied Biosystems), using the standard thermal cycling programme outlined in Table 2.2.

2.3.3.3 Quantitative real-time PCR analysis

Quantitative real-time PCR analysis was carried out using SDS version 2.3 and SDS RQ Manager version 1.2 relative quantification software. A visual inspection of triplicate expression values was initially performed and any outliers were excluded. The threshold cycle (Ct) value for each well was calculated and the expression values of target genes were normalised to expression levels of the endogenous control gene, 18S. Analysis of target gene expression data was then carried out using the $2^{-\Delta\Delta Ct}$ relative quantification method, which describes the change in expression of a particular gene in a sample relative to the expression in a reference sample, such as an untreated control.

2.4 Transcription factor arrays

2.4.1 Expressed Transcription Factor Knockdown Transcriptome PCR Arrays

The expressed transcription factor knockdown transcriptome PCR array (SABiosciences, Qiagen, Hilden, Germany) identifies those transcription factors that regulate the expression of a particular gene. In each well of the array plate is a cDNA sample that was synthesized from an MCF-7 breast cancer cell sample treated with a unique siRNA for one of 270 transcription factors. This allows the screening of 270 different siRNA treatments with a single qPCR reaction (Figure 2.1). The real-time PCR assay can be for any gene of interest. A change in expression of the gene of interest in a specific well reveals that the corresponding transcription factor is a regulator of that gene; if the

gene of interest is downregulated in a sample, then the transcription factor knocked down in that sample is a positive regulator of the gene, while if the gene of interest is upregulated in a sample, that transcription factor is a negative regulator of the gene. The array consists of three 96-well plates that are pre-loaded with cDNA. A master mix was made up containing (per reaction well) 10 µL Gene Expression Mastermix (Applied Biosystems), 9 µL molecular-grade H_2O and 1 µL primer probe for CCL28 (Applied Biosystems). A 20 µL volume of master mix was loaded into each well of the array plate, the plate was covered with an optically clear adhesive film cover and centrifuged to pool the reagents. Real-time PCR detection was performed using an ABI 7900HT real-time thermal cycler (Applied Biosystems), using the programme outlined in table 2.2.

2.4.1.1 Transcription factor knockdown array analysis

Data was analysed using Excel-based data analysis software from SABiosciences for the Transcriptome PCR Array. This software automatically performed $^{\Delta\Delta Ct}$-based fold-change calculations from raw threshold cycle data for the gene-specific real time-PCR assays. A threshold of 1.5 fold change in CCL28 gene expression was set; any transcription factor that altered CCL28 gene expression above this threshold was considered a regulator of its expression.

Transcription factors were identified as positive and negative regulators of CCL28 gene expression using the Transcriptome PCR Array. Altered transcription factors were then filtered by identifying those transcription factors that had binding

Table 2.2 Thermal cycling programme for quantitative real-time PCR

Number of cycles	Time (min)	Temperature (°C)
1	2	50
1	10	95
40	0.25	95
	1	60

sites within the CCL28 promoter region, using Genomatix software (www.genomatix.de) (Genomatix Software GmBH, Munich, Germany).

2.4.2 Transcription factor activation profiling array

A transcription factor activation profiling array was purchased from Signosis (Signosis Inc., Sunnyvale, CA, USA). This array can measure the activation of multiple transcription factors simultaneously. A series of biotin-labelled probes are made based on the consensus sequences of transcription factor DNA-binding sites. When the probe mix incubates with nuclear extracts, individual probes will bind to their corresponding transcription factor and form complexes. The bound probes are detached from the complex through spin column purification and hybridised on a plate, in which each well is specifically pre-coated with complementary sequences of the probes. The captured DNA probe is further detected with streptavidin-HRP, and luminescence is measured on a microplate luminometer.

2.4.2.1 Nuclear extract preparation

Buffer A and Buffer C were made up prior to beginning nuclear extraction (table 2.3). The OE19 oesophageal adenocarcinoma cell line was treated with 500 μM DCA for 3 h in a 75 cm^2 vented flask at 37°C in 95% humidified air with 5% CO_2. Untreated cells provided a control. Cells were washed with PBS warmed to 37°C and trypsinised as previously described (section 2.2.2). Trypsin was

neutralised with 10 mL complete medium and centrifuged at 180 x *g* for 3 min. The supernatant was decanted and cells were washed with 10 mL PBS and centrifuged

Table 2.3 Composition of Buffer A and Buffer C for nuclear extraction

Buffer A	**Buffer C**
10 mM HEPES (pH 7.8)	20 mM HEPES (pH 7.8)
10 mM KCl	420 mM NaCl
0.1 mM EDTA	5 mM EDTA
2 mM DTT	5 mM DTT
1 mM PMSF	1 mM PMSF
	10% glycerol

at 180 x *g* for 3 min. The supernatant was decanted, 400 µL Buffer A was added to the cell pellet and the pellet was re-suspended by pipetting. The tube was placed on ice for 15 min to allow swelling of cells to occur. After this time period, 25 µL 10% Igepal-CA630 (Sigma Aldrich) was added to the tube, causing the cell membranes to burst. The tube was vortexed vigorously for 10 s and then centrifuged at 8,000 x *g* for 30 s. The supernatant was removed to a new eppendorf tube and stored at -80°C for future use; this represents the cytoplasmic component of the cells. To the remaining pellet, 50 µL Buffer C was added. The tube was placed on a shaker at 4°C for 30 min and then centrifuged at 8,000 x *g* for 10 min. The supernatant was transferred to a new eppendorf tube and the pellet was discarded. The supernatant was stored at -80°C for future use.

2.4.2.2 Confirmation of integrity of nuclear extract

Protein concentration in nuclear and cytoplasmic extracts was measured using the BCA assay, described in section 2.5.3.2. Nuclear and cytoplasmic extracts were run on a 12% separating SDS-PAGE gel to confirm quality and differential protein expression. Protein (30 µg) from each extract was loaded onto a 12% separating gel and protein electrophoresis was run for 90 min until the dye front had reached the bottom of the gel. The gel was removed from the electrophoresis rig and was stained with Coomassie blue stain (50% MeOH (v/v), 10% glacial acetic acid (v/v), 0.25% Coomassie brilliant blue G250 (w/v), made up to 100 mL with dH_2O), on a shaker (Bibby Sterilin Ltd., Staffordshire, UK) for 1 h. The gel was then destained (50% MeOH (v/v), 10% glacial acetic acid (v/v)) until the protein bands were clearly visible and background staining had been fully

eliminated. The de-stained gel was photographed. This stain allows for the visualisation of proteins for the assessment of protein degradation/integrity and loading.

2.4.2.3 Transcription factor activation profiling array

Hybridisation and wash buffers were prepared according to the manufacturer's instructions (Signosis) prior to the assay being performed. In an eppendorf tube, the following were mixed per reaction: 15 µL binding buffer mix, 4 µL transcription factor probe mix, a volume containing 15 µg nuclear protein extract, a volume of molecular grade H_2O to bring total volume to 30 µL. Two reaction mixtures were made up for the untreated and treated OE19 nuclear extracts. These were incubated at RT°C for 30 min to allow formation of the DNA-probe complexes. The reaction mix was added to the centre of a pre-equilibrated isolation column and incubated on ice for 30 min to separate the bound transcription factors and probes. A 500 µL volume of cold filter wash buffer was added to the column, incubated on ice for 2 min and then centrifuged at 4000 x *g* for 1 min at 4°C and the flow-through discarded. This wash step was repeated 4 times using 500 µL cold filter wash buffer for each wash. A 100 µL volume of elution buffer was added to the centre of the isolation column and incubated at RT°C for 5 min. The column was placed in a 1.5 mL eppendorf tube and centrifuged at 11800 x *g* for 2 min at RT°C to elute the bound probes. The eluted probes were then denatured at 98°C for 5 min and then transferred to 500 µL pre-chilled molecular grade H_2O and placed on ice. The sealing films were removed from the two hybridisation plates. A 600 µL volume of denatured probe mix was

added to 10 mL pre-warmed hybridisation buffer, gently mixed, and 100 µL added to each well of the hybridisation plate. Plates were sealed with foil pressed down firmly to prevent evaporation and incubated at 42°C overnight. Following this, the plate contents were decanted by inversion and the plates were washed three times with 200 µL hybridisation plate wash buffer per well. Complete removal of liquid after each wash was ensured by tapping the plate against clean paper towels on a bench. A 200 µL volume of blocking buffer was added to each well and incubated at RT°C for 15 min with gentle agitation. Blocking buffer was removed and 95 µL streptavidin-HRP conjugate (diluted 1:500 in blocking buffer) was added per well. Plates were incubated at RT°C for 45 min with gentle agitation. The plate was washed 3 times with 200 µL wash buffer for 5 min with gentle agitation. The substrate solution was prepared (1 mL Substrate A, 1 mL Substrate B, 8 mL Substrate Dilution Buffer) and 95 µL was added to each well. The plate was read after 5 min on a luminometer (Promega) with integration time set to 1 min and no filter.

2.4.2.4 Transcription factor activation profiling array analysis

Analysis was performed using Excel-based software. Relative light unit measurement from the luminometer reading was compared between the untreated and treated nuclear extract. A change in transcription factor level of greater than 1.4 fold was taken as the threshold level for activation by DCA. Transcription factors activated by DCA in the OE19 cell line were identified. These transcription factors were then filtered by identifying those transcription factors that had binding

sites within the CCL28 promoter region, using Genomatix software (Genomatix Software GmBH).

2.4.3 Ingenuity Pathway Analysis

Network and pathway analysis was carried out on the transcription factors identified in the Activation Profiling Array, the Transcription Factor Knockdown Array and the Genomatix program using Ingenuity Pathway Analysis™ (IPA) (Ingenuity Systems Inc., Redwood City, CA, USA) software. The IPA software allows analysis of signalling and metabolic pathways, molecular networks, and biological processes involved in the data inputted. It can also help to visualise downstream effects of the genes of interest and to build interactive pathways representing the key genes, chemicals, and processes in experimental systems of interest. The Build/Grow function within the software allows selection of genes of interest, i.e. CCL28, IL-1β and TNF-α, and growth of upstream and downstream pathways from these genes. The values from the dataset inputted into the programme from arrays can then be overlayed on these pathways. Pathways are linked to the relevant PubMed citations. Data from the transcription factor arrays was formatted into IPA compatible layouts using template files downloaded from IPA website and uploaded into the IPA program. Analysis was carried out using IPA, inputting the transcription factor data and including the factors CCL28, IL-1β and TNF-α in the pathway maps.

2.5 Protein expression

2.5.1 Enzyme-linked immunosorbent assay (ELISA)

Soluble protein secretion of CCL28, IL-1β and TNF-α was measured using enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA). This technique binds the soluble protein of interest to a specific capture antibody bound to the wells of a 96-well plate. The bound protein is then detected using a detection antibody specific for the protein of interest and a colourimetric system for quantification of the bound protein.

A high-binding ELISA plate (Nunc, Thermo Fisher Scientific, IL, USA) was coated with 50 µL capture antibody diluted to the appropriate concentration in PBS, covered with an adhesive cover film and incubated at RT°C overnight, to allow binding of the antibody to the well base. Wells were then washed 3 times using wash buffer consisting of PBS containing 0.05% Tween. Following this, the wells were blocked using 150 µL of a solution of 1% bovine serum albumin (BSA) (Sigma Aldrich) (w/v) in PBS for 1 h at RT°C. A standard curve was constructed using a range of concentrations of recombinant protein. The range was chosen so that the samples being measured would fall within the standard curve. Standards were made up in PBS containing 0.1% BSA (w/v). Samples and standards (50 µL) were added in duplicate to the wells and incubated for 2 h at RT°C, following which, the wells were washed 3 times using wash buffer as previously described above. The detection antibody (50 µL) was diluted to the appropriate concentration in PBS containing 0.1% BSA (w/v), added to the wells and incubated for 2 h at RT°C, following which the plate was washed 3 times as previously described. A solution of streptavidin conjugated with horseradish peroxidase diluted in a 1:200 ratio in PBS

containing 0.1% BSA (w/v) was added to each well (50 µL) and incubated at RT°C in the dark for 20 min. The plate was washed 3 times with wash buffer and 50 µL 3,3′,5,5′-Tetramethylbenzidine (TMB) (Sigma Aldrich) substrate was added to each well and incubated in the dark for ~10-30 min at RT°C or until the colour change had developed sufficiently. The colour change reaction was stopped using a stop solution of 1 M H_2SO_4 (25 µL). Bubbles were eliminated from wells and the plate was read on an Alpha Fluor spectrophotometer (Tecan Trading AG, Switzerland) at a frequency of 450 nm.

The standard curve was constructed and the protein concentration in samples tested was interpolated from the standard curve. The R value of the standard curve determined the accuracy of the curve; an R value of greater than 0.95 ensured accurate measurement. Protein concentration was measured in pg/mL.

2.5.2 Protein multiplex assay

For measurement of multiple soluble proteins in media, a protein multiplex assay was purchased from Meso Scale Discovery (Meso Scale Discovery, Maryland, USA). This assay can measure up to ten proteins on one plate. Capture antibodies for the proteins to be assayed are pre-coated on a single "spot" in a particular orientation on a 96-well plate. The sandwich immunoassay technique is then used to measure concentrations of the proteins in a sample.

The plate was blocked using 150 µL of the supplied blocking solution per well, sealed with an adhesive film and incubated for 1 h at RT°C with vigorous shaking. The calibration (standard) curve was prepared using serial dilutions of the supplied

stock solution. A 25 µL volume of calibrator or sample was added to each well, the plate was sealed and incubated for 2 h at RT°C with vigorous shaking. After this, 25 µL detection antibody solution was added to each well, the plate sealed and incubated for 2 h at room temperature with vigorous shaking. The plate was washed 3 times with 1X PBS containing 0.05% Tween-20 and dried by blotting on clean paper towels. A 150 µL volume of 2X Read Buffer was added to each well and the plate was read on a MSD Sector® Imager (Meso Scale Discovery).

2.5.3 Western blotting

2.5.3.1 Protein isolation

RIPA protein lysis buffer was made up prior to protein isolation (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium doxycholate, 0.1% SDS, 1 mM PMSF, 1mM protease inhibitor cocktail (5 µg/mL aprotinin, 5 µg/mL leupeptin)). Protein was isolated from cell lines cultured in a 75 cm^2 flask. Cell culture medium was decanted and cells were washed using cold PBS (pre-chilled on ice). Flasks were kept on ice at all times. A 10 mL volume of cold PBS was added to the flask and a plastic cell scraper was used to scrape cells into the liquid. The liquid was transferred to a 15 mL tube and centrifuged at 180 x *g* for 3 min. The supernatant was decanted and the pellet was re-suspended in 50-100 µL protein lysis buffer, depending on the size of the pellet. This mixture was transferred to a 1.5 mL eppendorf tube and allowed to rest on ice for 30 min. The solution was then sheared using a 29 gauge needle and centrifuged at 4,000 x *g* for 2 min to eliminate insoluble protein. The supernatant was transferred to a new 1.5 mL eppendorf tube and stored at – 80°C.

2.5.3.2 Protein quantification using the bicinchoninic acid (BCA) assay

Protein was quantified using the commercially available Pierce Bicinchoninic Protein Assay Kit (Thermo Scientific, IL, USA). The bicinchoninic acid (BCA) assay allows the quantification of total protein concentration in a sample. This assay uses a colorimetric technique to measure the protein contained in a sample compared with a standard curve of known protein concentrations on the same plate. In the BCA assay, protein in a sample reduces Cu^{2+} to Cu^{1+} in an alkaline medium. Then colorimetric detection of the cuprous cation (Cu^{1+}) is carried out using bicinchoninic acid.

Standards of known protein (bovine serum albumin) concentration were made up in RIPA buffer (or other solvent if samples to be quantified were in another solvent) and stored on ice. The BCA working reagent was made up using the ratio 50 parts reagent A (Na_2CO_3, $NaHCO_3$, bicinchoninic acid, $Na_2C_4H_4O_6$ (sodium tartrate) in 0.1 M NaOH): 1 part reagent B (containing 4% $CuSO_4$), with 200 µL working reagent per well required. A 10 µL volume of each sample or standard was added to each well of a standard flat-bottomed 96-well plate. Samples were assayed in triplicate. A 200 µL volume of working reagent was then added to each well and the plate incubated at 37°C for 30 min. Absorbance was then measured using an Alpha Fluor Plus spectrophotometer (Tecan Trading AG, Switzerland) at a frequency of 562 nm. The standard curve was plotted and used to determine the protein concentration of the unknown samples.

2.5.3.3 Denaturing polyacrylamide gel (SDS-PAGE)

A 12% SDS-PAGE gel was used for resolution of proteins smaller than 100 kDa, while a 15% SDS-PAGE gel was used for resolution of proteins smaller than 30 kDa. A 12% separating gel was prepared as follows for a 30 mL gel mould: 9.2 mL 30% acrylamide:bisacrylamide (37.5:1), 4.5 mL 1.875 M Tris-HCl (pH 8.8), 8.3 mL H_2O, 176 µL 10% (w/v) SDS, 120 µL 10% (w/v) fresh ammonium persulfate (APS), 8 µL N, N, N', N'-tetramethylethylenediamine (TEMED). A 15% separating gel was prepared as follows: 11.25 mL 30% acrylamide:bisacrylamide (37.5:1), 4.5 mL 1.875 M Tris-HCl (pH 8.8), 6.3 mL H_2O, 220 µL 10% (w/v) SDS, 150 µL 10% (w/v) fresh ammonium persulfate (APS), 10 µL N, N, N', N'-tetramethylethylenediamine (TEMED). The solution was gently mixed and the gels were cast between upright glass plates (Bio-Rad laboratories, Hercules, CA, USA) cleaned with isopropranol prior to use. Isopropanol was gently layered on top of the gels to exclude air and aid polymerisation. This was removed once polymerisation had occurred. A 5% (w/v) stacking gel was prepared containing: 1.7 mL 30% (w/v) acrylamide:bisacrylamide (37.5:1), 2 mL 0.6 M Tris-HCl (pH 6.8), 6 mL H_2O, 100 µL 10% (w/v) SDS, 150 µL 10% (w/v) fresh APS, 10 µL TEMED. The stacking gel was layered on top of the polymerised separating gel and a 12 well comb was inserted before allowing the gel to set.

2.5.3.4 Protein electrophoresis

Appropriate volumes of protein samples were transferred to eppendorf tubes and a corresponding volume of 2X Laemmli buffer (2 mL Tris-HCl pH 6.8, 5 mL 10% (w/v) SDS, 1 mL 2-β-mercaptoethanol, 2 mL glycerol, 0.05 g (w/v) bromophenol

blue) was added. Samples were denatured by heating to 95°C for 10 min, centrifuged to pool liquid and placed on ice. Gels were placed into an electrophoresis rack filled with electrode buffer (50 mM Trizma base, 384 mM glycine, 0.1% (w/v) SDS) and the comb was removed. Denatured samples were loaded into wells, along with 8 µL of a tri-chrom pre-stained protein marker (Pierce, Rockford, IL, USA). Samples were separated by electrophoresis at 50 mA per gel for 70-90 min, or until the blue dye front reached the bottom of the gel.

2.5.3.5 Protein transfer

Separated proteins were transferred onto a 0.45 µM polyvinylidene fluoride (PVDF) membrane (Pall Corp., FL, USA) using a wet Mini-Trans Blot cell (Bio-Rad Laboratories, CA, USA). A pre-activated (1 min in 100% MeOH) PVDF membrane and Whatman filter paper (Whatman Laboratory Division, Kent, UK) were soaked in cold transfer buffer (0.15 M glycine, 20 mM Trizma base, 0.1% (w/v) SDS, 20% (v/v) MeOH), prior to use. The cassette was then placed in a cooled electrode tank and topped up with cold transfer buffer and run at 100 V and 400 mA for 1 h.

2.5.3.6 Antibody probing of membranes

Following the transfer of proteins, membranes were blocked with 5% (w/v) non-fat dried milk (Marvel), which was reconstituted in 1X Tris-buffered saline (25 mM Tris-HCl (pH 7.6), 150 mM NaCl) containing 0.1% Tween-20 (TBST) for 1 h at room temperature. The membrane was incubated in the primary antibody in 5% Marvel TBST on a shaker overnight at 4°C unless otherwise specified. Membranes

were washed twice for 15 min each in TBST, before incubation in the appropriate species-specific horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,000 dilution in 5 % Marvel TBST) (Dako, Glostrup, Denmark) for 1 h at room temperature. The membrane was washed with 1X TBST over a period of six 5 min washes. The Supersignal West Pico Chemiluminescent substrate kit (Pierce), was used to detect bound antibody complexes. Working reagent was prepared just prior to use by mixing equal volumes of reagents A and B and was applied to the membrane for 1-5 min. Membranes were then exposed to scientific imaging X-ray film (Fuji Photo Film Co. Ltd., Tokyo, Japan), which was developed using a medical film processor (Agfa-Gevaert, Mortsel, Belgium). Exposure times ranged from 30 s to 30 min, depending on the signal intensity.

2.5.3.7 Stripping of membranes

In order for the membranes to be re-probed with a different antibody the blots were stripped. The membrane was incubated in 10 mL of Restore ™ western blot stripping buffer (Pierce) at room temperature on a shaker. This was removed after 15 min and the membrane washed twice for 10 min in TBST and stored at 4°C. Stripped membranes were re-probed as described previously (section).

2.5.3.8 Densitometry

Densitometric analysis was carried out using TINA (version 4.0) software (Raytest, Straubenhardt, Germany). Values were represented as a ratio of sample band intensity to that of the corresponding β-actin control.

2.6 Flow cytometry

Flow cytometry allows analysis of individual cells in suspension. Cells are allowed to flow through flow chambers; hydro dynamic focussing organises cells into a single cell stream. Cells can be analysed for single or multiple parameters using laser beams. Flow cytometry can detect both intracellular and cell surface factors.

2.6.1 IL-1R1 and TNF-R1 receptor staining

Oesophageal cell lines were assessed for the IL-1R1 and the TNF-R1 receptors using flow cytometry. Antibodies for these receptors were obtained from R&D Systems (R&D Systems, Minneapolis, USA). The antibody for IL-1R1 was conjugated with phycoerythrin (PE) and the antibody for TNF-R1 was conjugated with allophycocyanin (APC). In order to optimise staining for these antibodies, varying volumes of the antibodies were used to stain oesophageal cell lines. Cells were seeded in a 6-well plate at appropriate density and allowed to adhere overnight. Treatments of DCA and/or neutralising antibody were carried out as appropriate. For DCA treatments, cells were treated with varying concentrations of DCA (0-500 μM) for 24 h at 37°C. The IL-1R1 neutralising antibody (R&D Systems, Minneapolis, USA) was added to cell culture growth medium at varying concentrations (0-50 μg/mL) for 1 h. After 1 h, DCA was added to the same medium to reach an appropriate final concentration, so the neutralising antibody remained on the cells simultaneously with the DCA treatment. After treatments, growth medium was removed and cells were washed with PBS. Cells were detached from the wells using 10 mM EDTA solution (trypsin was not used in this experiment in order not to disrupt the cell surface receptors being measured). Cells were incubated in EDTA solution

for 10-20 min until visibly detached, and were then transferred into labelled FACS tubes containing 1 mL FACS buffer (PBS with 2% FBS, 0.1% (w/v) sodium azide) . These were centrifuged at 180 x *g* for 3 min and the supernatant poured off. A 1 mL volume of blocking buffer (50% FACS buffer, 50% FBS) was added to each tube, vortexed for 10 s and allowed to rest at room temperature for 10 min. Cells were washed once with FACS buffer and centrifuged at 180 x g for 3 min. Supernatant was poured off and 1-10 µL antibody was added to tubes. Tubes were vortexed and then incubated at 4°C in the dark for 30-40 min. Cells were washed once with FACS buffer, centrifuged and supernatant was poured off. Cells were re-suspended in 500 µL FACS buffer and then analysed on a FACS Caliber flow cytometer (BD Biosciences), using a gate to exclude cellular debris. Data analysis was carried out using CellQuest software (BD Biosciences) by an experienced user of the software. Instrumental controls were set up in parallel with experimental samples. Controls included unstained cells and single-stained cells with each stain.

2.6.2 Propidium iodide staining

Cells were also assessed for viability using propidium iodide (PI) staining. PI enters cells when the membrane integrity is compromised, as occurs in late apoptotic and necrotic cells, and intercalates with double stranded DNA. Therefore positive staining with PI identifies cells undergoing late apoptosis and/or necrosis, while cells which exclude PI are viable. Cells were processed as described in section 2.11.1, without the addition of the primary antibody. Immediately before analysis, 500 µL PI (1:4000 dilution of 1 mg/mL stock in FACS buffer) was added to each tube

to be analysed and vortexed. Cells were analysed on the FACSCalibur flow cytometer (BD Biosciences), using a gate to exclude cellular debris.

2.7 Patient samples

2.7.1 Patient recruitment

Following ethical approval from the St. James's Hospital and Adelaide, Meath and National Children's Hospital Institutional Review Board, a Barrett's biobank was set up. Written informed consent was obtained from patients attending St. James's Hospital endoscopy unit for routine surveillance endoscopy for previously confirmed Barrett's oesophagus. Blood samples and diagnostic biopsy specimens were taken from these patients, for inclusion in the departmental bioresource. Patients were included in this study if they fulfilled the following criteria: previously histologically confirmed Barrett's oesophagus, no chronic infectious or autoimmune disease, including HIV, hepatitis B or hepatitis C, no cancer at the time of endoscopy other than oesophageal cancer. Patients were also excluded if they had Barrett's segment length less than 3 cm.

2.7.2 Serum collection

Whole blood was collected in Z clot activator serum vacuette tubes (Greiner Bio-one) and tubes were allowed to stand at room temperature for 20 min to facilitate clotting. Samples were centrifuged at 960 x *g* for 10 min at 4°C. The uppermost serum layer was then transferred to a labelled 2 mL screw-top tube and stored at -80°C.

2.7.3 Biopsy collection

Patients who had consented to the study underwent routine surveillance endoscopy, as per hospital protocol. At the time of their endoscopy, additional biopsies were taken for the purpose of the study. Normal squamous epithelial biopsies were taken at least 5 cm proximal to the proximal border of Barrett's epithelium. Barrett's biopsies were taken for the study, and immediately adjacent tissue was taken for histologic confirmation, which was performed using routine haematoxylin and eosin staining by a consultant histopathologist. Tissue biopsies were placed on saline soaked gauze and were immediately transferred to the laboratory (within 10 min of endoscopy).

2.7.4 Explant culture

Biopsies were removed from the saline gauze using a forceps and placed into a well of a 24-well plate containing 1 mL of M199 medium (Lonza, Maryland, USA), supplemented with FBS (10%), penicillin/streptomycin (1%) and insulin (1 µg/mL). Two normal and two Barrett's biopsies were used from each patient, and were either incubated in medium alone or in medium supplemented with 100 µM DCA (Sigma Aldrich, Missouri, USA) for 24 h at 37°C in 95% humidified air with 5% CO_2. After 24 h, biopsies were removed from incubation medium, and medium (hereafter known as conditioned medium) was transferred to a 2 mL tube and immediately stored at -80°C. Biopsies were placed in RNA-*later* (Ambion, Warrington, UK) and refrigerated for 24 h, before removal of RNA-*later* and storage at -80°C.

2.7.5 Lactate dehydrogenase (LDH) assay

To confirm that explant culture and DCA treatment did not affect tissue viability, a lactate dehydrogenase (LDH) assay was performed. LDH is a soluble cytosolic enzyme which is released when cell membrane integrity is compromised, as occurs during apoptosis or necrosis. Therefore release of LDH into culture medium provides a useful measure of cytotoxicity. In this colourimetric assay, LDH in the tissue culture medium or in homogenised tissue catalyses the reduction of NAD^+ to NADH and H^+ by oxidation of lactate to pyruvate. Subsequently, diaphorase uses this NADH and H^+ to catalyse the reduction of a tetrazolium salt (INT) to coloured formazan and absorbance can be measured.

Normal and Barrett's biopsies were cultured with or without 100 µM DCA for 24 h, as previously described 2.7.4. The medium was then removed (hereafter known as conditioned medium), transferred to an eppendorf and stored on ice until further use. Biopsies were homogenised in a 2 mL tube in PBS containing 0.1% Triton-X using a tissue lyser (RetSch & Co GmbH, Haan, Germany) to disrupt the cell membranes of the tissue and release the LDH contained in the entire biopsy sample. The tissue lyser was set at a frequency of 25 agitations/s for 10 min and a 5 mm stainless steel bead (Qiagen) was added to each tube to aid homogenisation. The lysate was removed to a new eppendorf and centrifuged at 10,000 x *g* for 3 min to remove any debris, and the supernatant was removed to a new eppendorf.

The LDH assay (Caymenchem, Ann Arbor, MI, USA) was performed in a 96-well plate. The reaction solution was prepared according to the manufacturer's instructions. LDH standards were prepared and stored on ice. A 100 µL volume of

standards or samples were pipetted into each well of the plate, and then 100 µL reaction solution was added to each well. The plate was incubated at room temperature for 30 min with gentle agitation to allow colour development. Absorbance was read at 490 nm on an Alpha Fluor Plus spectrophotometer (Tecan Trading AG, Switzerland).

2.7.6 Confirmation of columnar epithelium using PCR

The presence of columnar Barrett's epithelium in biopsies from Barrett's oesophagus was confirmed using PCR and agarose gel electrophoresis. Primers were purchased for the columnar markers CK8 and TFF1, as well as the housekeeping gene GAPDH (Metabion). A 1.4% agarose gel was prepared as described in section 2.3.7. cDNA samples prepared from RNA extracted from normal and Barrett's biopsies were loaded onto the gel along with a 100 base pair DNA ladder (Promega Corporation, Madison, WI, USA) and electrophoresis was carried out at 100 volts in an EC-360 Maxicell gel system (EC Apparatus Corp., FL, USA), using 1X TAE as a running buffer. The bands were visualised and photographed under UV light using a BioSpectrum Imaging System (Ultra Violet Products, Cambridge, UK).

2.7.7 RNA extraction from biopsies

RNA was extracted from biopsies using the Qiagen RNEasy MiniKit (Qiagen Inc., CA, USA). Biopsies were removed from RNA-later and weighed to ensure not more than 30 mg of tissue was used for RNA extraction. β-mercaptoethanol was

added to the RLT buffer supplied with the kit and 600 µL RLT buffer was added to a 2 mL screw-top tube containing the biopsy sample. A 5 mm stainless steel bead (Qiagen Inc., CA, USA) was washed with ethanol 70% (v/v) in distilled H_2O and placed into the tube. The biopsy was homogenised using a tissue lyser (RetSch & Co GmbH, Haan, Germany) set at 25 agitations/s for 10 min, or until the tissue was completely homogenised. The bead was removed and samples were centrifuged at 12,000 x *g* for 3 min at 4°C. The supernatant was transferred to a new labelled tube. An equal volume of ethanol 70% was added to the supernatant and pipetted to mix. Up to 700 µL of this was transferred to an RNeasy spin column placed in a 2 mL plastic collection tube and centrifuged at 8000 × *g* for 15 s at RT°C. The flow-through was discarded, and successive aliquots were centrifuged in the same RNeasy spin column, discarding the flow through, until the entire sample volume had been centrifuged. A 700 µL volume of Buffer RW1 was added to the spin column and centrifuged at 8,000 x *g* for 15 s, and the flow-through was discarded. A 500 µL volume of Buffer RPE was added to the spin column and centrifuged at 8,000 x *g* for 15 s and the flow-through discarded. A 500 µL volume of Buffer RPE was added again to the spin column and centrifuged at 8,000 x g for 2 min to completely dry the spin column membrane. The spin column was placed in a new 1.5 mL collection tube and 30 µL molecular grade H_2O was added to the column. This was centrifuged at 8,000 x *g* for 1 min to elute the RNA. The spin column was discarded and RNA immediately stored at -80°C for future use.

2.8 Stable Transfection

2.8.1 Geneticin kill curve

The p65/dsRed1-N1 plasmid contains a neomycin/G418 resistance gene, which infers geneticin (G418) antibiotic resistance. Killing curves were set-up in OE19 cells using G418 (Sigma Aldrich) to establish the optimum concentration of the antibiotic required to kill the cells within 7-10 days. Cells at a density of 5 x 10^4 cells/well were seeded into 6-well plates and allowed to adhere overnight at 37°C. The cells were allowed to grow until they reached 60-70% confluency, at which point they were treated with G418 at a range of concentrations (0-1500 µg/mL). G418 was added directly to the culture medium, and both culture medium and the antibiotic were replaced every 3-4 days. The lowest concentration of antibiotic that resulted in complete cell death in 10 days was recorded, and used for future transfection experiments.

2.8.2 Transfection

Transfection was set up according to the Lipofectamine™ 2000 (Invitrogen, Life Technologies Corp., NY, USA) protocol in a ratio of 2:1. Lipofectamine™ 2000 is a lipid-based transfection reagent which facilitates transfection by complexing into aggregates with the negatively charged nucleic acid complexes. These nucleic acid-transfection reagent complexes are then efficiently taken up by cells. Cells were seeded at 5 x 10^4/well in antibiotic-free media in 6-well plates and allowed to reach 90% confluency. DNA and Lipofectamine™ 2000 were diluted separately in 100 µL aliquots of Optimem (Invitrogen, Life Technologies Corp., NY, USA) in a 1:2

(DNA:Lipofectamine) ratio. After 5 min incubation at RT°C, the diluted DNA and Lipofectamine™ 2000 were combined and incubated for a further 30 min at RT°C. The complex was added to cells in a drop-wise manner and the 6 well plates rocked to ensure proper distribution. Cells were incubated in media containing DNA/ Lipofectamine™ 2000 mix overnight. It was then replaced with complete medium containing G418 at previously optimised concentration.

Cells were examined daily for cell death and colony growth, as well as for red fluorescence. The cells were supplemented with fresh complete medium every 4 days. Once colonies were visible (approximately 3 weeks post transfection), single colonies were removed from the plate using a pipette tip and transferred to a well of a 24-well plate. Once these single clones had reached 80% confluency, clones were transferred to a 12-well plate, subsequently to a 6-well plate, a 25 cm^2 flask and then a 75 cm^2 flask. Once sufficient cells were available, transfected cell lines were frozen down as previously described (section 2.2.3)

2.8.3 Confirmation of p65 overexpression in stably transfected cell lines

Overexpression of the p65 subunit of NF-kB was confirmed by using Western blotting. 30 µg of protein lysate from OE19 cell line and from 2 overexpressing clones was separated on a 12% SDS-PAGE gel as described in section 2.6.3 and transferred to PVDF membrane. This was probed for the p65 subunit of NF-kB using a mouse anti-human antibody (Cell Signalling Technologies Inc., Danvers, MA, USA).

2.8.4 Expression of CCL28 in stably transfected cell lines

Stably transfected cell lines were treated with DCA for 24 h at 37°C as previously described in section 2.2.7. Supernatant was removed and soluble CCL28 protein was quantified using ELISA.

2.8.5 Inhibition of NF-kB in stably transfected cell lines

DHMEQ, a potent inhibitor of the translocation of the p65 subunit of NF-kB to the nucleus, was a kind gift from Dr. Kathy Gately. Cells were grown to 70% density in a 24-well plate and were treated with DHMEQ at 1 µg/mL or 5 µg/mL and 200 µM DCA for 24 h at 37°C. Supernatant was removed and soluble CCL28 protein was quantified using ELISA as previously described (section 2.5.1). Cells were assessed for cell viability after DHMEQ treatment using propidium iodide staining as described in section 2.6.2.

2.9 Chemotaxis assay

2.9.1 Isolation of PBMCs

Peripheral blood mononuclear cells (PBMCs) are used commonly in research; they consist of lymphocytes, monocytes, macrophages, basophils and dendritic cells extracted from whole blood. PBMCs do not include eosinophils, neutrophils or erythrocytes. For isolation of PBMCs, the Ficoll method was used, which separates whole blood into layers containing various components of the blood.

Whole blood was obtained from consented healthy volunteers. Volunteers were excluded if they had any current infection to ensure normal immune cell number and function. An 18 mL volume of blood was taken in EDTA-Vacutainers™. Blood was transferred to a 50 mL tube and diluted 1:1 with phosphate buffered saline (PBS). Ficoll-Paque (GE Healthcare Life Sciences, Buckinghamshire, UK) was added to a new 50 mL tube in a volume equal to 75% of the volume of the total diluted blood. This tube was tilted to almost horizontal and the diluted blood was carefully layered on top of the Ficoll-Paque using a transfer pipette. The final ratio was 3:4 Ficoll:diluted blood. This tube was centrifuged at 400 x g for 30 min at room temperature, after which the blood was seen to separate into several layers: an uppermost yellow layer representing plasma, an intermediate thin white layer containing the PBMCs, a clear layer which is the Ficoll-Paque, and finally the erythrocytes and granulocytes which were in a pellet at the base of the tube. The plasma was discarded using a transfer pipette. The layer containing the PBMCs was transferred carefully to a new 50 mL tube and washed twice with 25 mL Hank's Balanced Salt Solution (HBSS) (Table 2.4) for 6 min at 600 x *g* at 4°C. After the second wash, the cell pellet containing PBMCs was re-suspended in 3 mL HBSS solution and cells were counted using a haemocytometer as previously described (section 2.2.5).

2.9.2 Isolation of T cells

T cells are a subset of lymphocytes which can be isolated from the PBMC population of immune cells. A Pan T Cell Isolation Kit was purchased from Miltenyi Biotec (Miltenyi Biotec, Cologne, Germany). This kit uses a negative selection

Table 2.4 Composition of Hank's Buffered Salt Solution (HBSS)

0.137 M NaCl
5.4 mM KCl
0.25 mM Na_2HPO_4
0.1g glucose
0.44 mM KH_2PO_4
1.3 mM $CaCl_2$
1.0 mM $MgSO_4$
4.2 mM $NaHCO_3$

technique to isolate T lymphocytes. This technique involves magnetically labelling all non-T cells in the cell population. Non-target cells, i.e., monocytes, neutrophils, eosinophils, B cells, stem cells, dendritic cells, NK cells, granulocytes, or erythroid cells are labelled using a cocktail of biotin-conjugated antibodies. The cocktail contains antibodies against CD14, CD15, CD16, CD19, CD34, CD36, CD56, CD123, and CD235a (Glycophorin A). Subsequently, non-target cells are magnetically labelled with the Pan T Cell MicroBead Cocktail. Once labelled with the Micro-beads, the non-T cells are then depleted by retention on a magnetic column in a MACS separator, while unlabelled T cells pass through the column and are collected.

Buffer for T cell isolation was prepared (0.5% (w/v) BSA, 2 mM EDTA in PBS). PBMCs were isolated as previously described (section 2.9.1), counted, and 1 x 10^7 PBMCs were used for T cell isolation. PBMCs at a density of 1 x 10^7 were re-suspended in 40 µL buffer and a 10 µL volume of biotin-antibody cocktail was added. This mixture was incubated at 4°C for 10 min. A 30 µL volume of buffer was added, and then 20 µL anti-biotin microbeads cocktail was added and the mixture incubated at 4°C for 10 min. The total volume was then adjusted up to 500 µL and magnetic separation was carried out. This was done either on the autoMACS ProSeparator (using the "depletes" programme) or by using LS columns on a manual MACS separator.

2.9.3 Confirmation of T cell purity

Purity of the isolated T cell population was confirmed using CD3 staining. Cells were blocked and stained for CD3 as described in section 2.6.1 and analysed

on the FACSCalibur flow cytometry (BD Biosciences). Cells positive for CD3 were expressed as a percentage of the total number of cells.

2.9.4 Staining for CCR3, CCR10 and CD3

Antibodies for CCR3 and CCR10 were obtained from Biolegend (Biolegend, San Diego, CA, USA) conjugated to fluorescent labels APC and PE, respectively. An antibody for CD3 was obtained from Miltenyi Biotec (Miltenyi Biotec, Cologne, Germany), conjugated to the VioBlue fluorescent label. T cells were isolated as previously described (section 2.9.2). Cells were processed for FACS as described in section 2.6.1. Following blocking, cells were washed in FACS buffer and then incubated with the primary antibody at an appropriate volume for 30 min in the dark at 4°C. They were then washed with FACS buffer, re-suspended in FACS buffer and analysed on a FACSCalibur flow cytometer (BD Biosciences) as described in section 2.6.1.

2.9.5 Transwell chemotaxis assay

Transwell membrane inserts for a 24-well plate were purchased from Corning (Corning Inc. Life Sciences, Amsterdam, The Netherlands). Membranes were polycarbonate with a 5.0 μm pore size to allow passage of immune cells. The Transwell system allows the study of chemotaxis of cells from the upper chamber in the insert to the bottom chamber of the 24-well plate. The chemoattractant is placed into the bottom chamber and cells from the upper chamber can migrate through the pores in the membrane towards the chemoattractant.

T cells were isolated from blood of a healthy donor, as previously described (section 2.9.2). T cells, at a density of 2 x 10^5 were added to the top chamber of the transwell system. A 600 µL volume of medium was added to the bottom chamber. The 24-well plate was incubated at 37°C in 95% humidified air with 5% CO_2 for 2 h to allow chemotaxis to occur. After this time period, the insert was removed from the well. Cells in the bottom chamber were counted from 4 random fields of view using an inverted microscope with a digital camera at 20X magnification. Cells from the bottom chamber were then collected and stained for CCR3, CCR10 and CD3 as described in section 2.9.4. They were analysed on a FACSCalibur flow cytometer (BD Biosciences).

2.9.6 Neutralisation of CCL28

2.9.6.1 Optimisation of neutralising antibody

A neutralising/blocking antibody for CCL28 was purchased from R&D Systems (R&D Systems, Minneapolis, USA). The neutralising concentration of the antibody was optimised using recombinant CCL28. Complete medium containing 3 ng/mL rhCCL28 was made up and divided into four 400 µL aliquots in eppendorf tubes. Varying concentrations of neutralising antibody were added to each aliquot (0 – 50 µg/mL) and incubated with gentle agitation for 30 min at 4°C. A 20 µL volume of Protein G Agarose Beads (Santa Cruz, Dallas, Texas, USA) was added to each eppendorf and incubated with gentle agitation overnight at 4°C. Eppendorfs were centrifuged at 1000 x g for 10 min and supernatant was removed to a fresh labelled tube. The concentration of CCL28 in these samples was

measured using ELISA, as described in section 2.5.1 and the optimum concentration of neutralising antibody determined.

2.9.6.2 Neutralisation of CCL28 in conditioned medium

Conditioned media known to contain CCL28 was divided into 2 screw-top 2 mL tubes and each made up to a volume of 600 µL. One aliquot was treated with the appropriate concentration of neutralising antibody for 30 min with gentle agitation at 4°C. The two aliquots of conditioned media were then used in the bottom chambers of the transwell assay, as described in section 2.9.5.

2.10 Immunohistochemistry

2.10.1 Immunohistochemical staining of full face sections

Staining for CCL28, CCR3 and CCR10 was optimised on full face sections of colon and oesophageal tissue, prior to staining tissue microarrays. Oesophageal tissue samples from normal, Barrett's and adenocarcinoma patients and from colon tumours were fixed in 10% formalin overnight and embedded in paraffin wax on a Leica EG 1140H embedder (Laboratory Instruments and Supplies, Dublin, Ireland), in order to maintain the shape and architecture of the tissue. Tissue sections 5 µm in thickness were then cut using a Microm HM325 microtome (Thermo Fisher Scientific, IL, USA) and floated onto Superfrost Plus poly-L-lysine coated glass slides (Thermo Fisher Scientific, IL, USA). Cut sections were baked overnight at 37°C in a tissue-drying oven (Binder, Tuttlingen, Germany) and processed immediately.

De-paraffinisation and antigen retrieval was carried out using Trilogy™ Reagent. Vectastain Elite kits (Vector Labs, Burlingame, CA, USA) were used for all immunohistochemical staining. Slides were placed in 200 mL 1X Trilogy reagent (Cell Marque™ Corporation, Rocklin, CA, USA) (10 mL 20X Trilogy reagent, 190 mL distilled H2O) and were heated in a Princess DYB350 programmable pressure cooker at low pressure for 10 min. Slides were immediately transferred to a new container of fresh hot Trilogy reagent and allowed to stand for 5 min. Slides were washed for 5 min in distilled water on a shaker and then placed in a 3% hydrogen peroxide solution made up in methanol for 30 min in the dark. This quenched endogenous peroxidase activity in the tissue. Slides were washed in PBS for 5 min on a shaker and then blocked using serum from the animal in which the secondary antibody was raised. This solution was made up in PBS and slides were blocked for 30 min at room temperature. The primary antibody solution was made up in PBS at appropriate dilutions. Blocking serum was poured off slides and the primary antibody was applied for 1 h at room temperature. Sections were washed in PBS on a shaker three times for 5 min, and then were incubated with the secondary antibody (made up in PBS with normal serum from the animal the secondary antibody was raised in) for 30 min at room temperature. Slides were washed three times for 5 min in PBS and then incubated in the avidin-biotin complex for 30 min. Slides were washed three times in PBS as before and were then incubated with 3,3'-diaminobenzidine (DAB) for 1-15 min in the dark, depending on the level of colour change. DAB was washed off with distilled water and counterstaining was carried out using haematoxylin solution for 30 s. Slides were immediately rinsed under gently running tap water for 5 min and then transferred to a fume hood. Here they were dipped in two containers of 100% methanol (10 times up and down in

each container), and then placed in 2 containers of 100% xylene for 5 min each. Finally, they were placed in a container of 100% xylene for at least 2 h. Slides were removed and coverslips (VWR International, West Chester, PA, USA) were applied using DPX mountant (B.D.H. Ltd., Poole, Dorset, UK) and allowed to dry in the fume hood. Images were taken using Image Pro-Plus v4.1 software (Media Cybernetics, Gelichen, Germany). All immunohistochemical staining included positive and negative controls.

2.10.2 Tissue microarrays (TMAs)

Tissue microarrays were constructed using archival formalin-fixed and paraffin embedded tissue from normal oesophagus, Barrett's oesophagus, dysplastic and adenocarcinoma oesophageal tissue. Areas of interest on tissue sections were identified by a consultant histopathologist. TMA maps were constructed. Cores (either 2 mm or 0.6 mm in diameter) were taken from the marked areas of interest and were transferred to a recipient block. Sections (4 μm) were cut from the TMA blocks and mounted onto Superfrost Plus slides (Thermo Fisher Scientific, IL, USA). Cut sections were baked overnight at 37°C and stored at 4°C until use. TMA sections were stained as described previously (section 2.10.1). Once mounted and dried, sections were scanned in order to be graded.

2.10.3 Grading of immunohistochemical staining

Grading was carried out by two independent individuals. Cores were graded for intensity (0-3), with a grade of 0 corresponding to no staining, a grade of 1

corresponding to weak staining, a grade of 2 corresponding to moderate staining and a grade of 3 corresponding to strong staining. Cores were also graded for the percentage of positive staining (0%, 10%, 25%, 50%, 75%, 90%, 100%). Averages were taken from cores taken from a single patient/sample. The overall score was obtained by averaging the scores from the two independent individuals grading the TMAs. Scores were then correlated with clinical and pathological parameters and statistical analysis carried out as described in section 2.11.

2.11 Statistical analysis

Standard deviation (SD) describes the dispersion of observed values in a data set and is calculated by determining the square root of the variance. Alternatively, the standard error of the mean (SEM) is the standard deviation of the distribution of sample mean. As sample size increases, the SEM decreases. When the SEM is small, it indicates that the distribution of sample means has less error estimating the true mean. SEM is calculated as the SD of the original sample divided by the square root of the sample size. Here, the data are expressed as the mean ± SEM, which improves the appearance of the data when graphed, as opposed to the mean ± SD. Expressing the data as either the mean ± SEM or mean ± SD does not affect statistical significance in any way. Significance was determined via the student *t*-test. A probability of (p) ≤ 0.05 was considered to represent a significant difference between the groups. In cases where data was paired (i.e. untreated vs. treated), a paired students *t*-test was used for statistical analysis. Otherwise, an unpaired *t*-test was used. For patient samples, where samples were matched (i.e. matched normal and Barrett's samples from the same patient), the Wilcoxon signed-rank test was

used for statistical analysis. Where samples were not matched, the Mann-Whitney U test was used for analysis. Probabilities of survival were analysed using the Kaplan-Meier method. In order to carry out the analysis, data was split into two groups: median survival ≥ median value and median survival < median value. The resulting two sets of data were used to generate survival curves. Statistical analysis was carried out by the Gehan-Breslow-Wilcoxon test. Statistical analysis was performed using GraphPad Instat v3 (GraphPad Software Inc., La Jolla, CA, USA) software on a PC.

Chapter 3

Characterisation of CCL28 expression in response to bile acid in oesophageal cell lines

3.1 Introduction

The tissue microenvironment, and in particular the immune component of this microenvironment, is increasingly being recognised as playing a major role in the development of pre-neoplastic and neoplastic conditions; indeed cancer-related inflammation is now considered to be one of the "hallmarks of cancer" as originally described by Hanahan and Weinberg (Hanahan and Weinberg 2000, Colotta, Allavena et al. 2009, Hanahan and Weinberg 2011). Barrett's oesophagus (BO) is a metaplastic change in the epithelial lining of the oesophagus from normal squamous epithelium to a columnar epithelium resembling the small intestinal lining. BO is considered to be a pre-malignant condition, which predisposes to the development of oesophageal adenocarcinoma (OAC). During progression of the oesophagus from normal oesophagus to BO to dysplasia and to OAC, the immune microenvironment of the tissue changes, with altered expression of cytokines, chemokines and growth factors, leading to altered infiltration of immune cells (Poehlmann, Kuester et al. , Kavanagh, O'Sullivan et al. 2013).

Gastro-oesophageal reflux disease (GORD) is a common condition in which bile and acid from the stomach and duodenum are regurgitated into the lower oesophagus, causing damage to the epithelial lining. Chronic GORD is known to be a risk factor for the development of BO. Specifically, the reflux of bile acids is considered to play a major role in this progression and in the development of OAC; in animal models, oesophageal exposure to bile acid reflux, either by direct perfusion or by gastrectomy followed by oesophago-jejunal anastomosis, causes development of oesophagitis, BO and OAC (Nishijima, Miwa et al. 2004, Miyashita, Ohta et al. 2006, Miyashita, Shah et al. 2008). Bile acids have been demonstrated

to induce various inflammatory factors, including chemokines and cytokines, such as IL-8 and COX-2, from squamous oesophageal epithelium and Barrett's epithelium (Jenkins, Harries et al. 2004, Song, Guha et al. 2007, Capello, Moons et al. 2008), and to disrupt tight junctions in oesophageal epithelium (Chen, Wu et al. 2012). They are found at higher concentrations in the refluxate of patients with erosive GORD and BO (Nehra, Howell et al. 1999), and infusion of bile acids causes symptoms of heartburn and chest pain (Siddiqui, Rodriguez-Stanley et al. 2005).

Deoxycholic acid (DCA) is a secondary bile acid produced in the human intestine by bacteria in the lumen causing dehydroxylation of the primary bile acid cholic acid. DCA is one of the predominant bile acids found in human gastro-oesophageal refluxate and is found at concentrations varying from 0 μM to greater than 1 mM, as well as comprising ~22% of biliary bile acids (Fracchia, Pellegrino et al. 1999). It is known to be toxic to oesophageal epithelial cells (Jenkins, D'Souza et al. 2007), via induction of reactive oxygen species and DNA damage, and can also activate the pro-inflammatory and pro-carcinogenic transcription factor NF-kB, in a manner which is not replicated by other bile acids (Huo, Juergens et al. 2011). Therefore, DCA may be a major candidate for the role of primary carcinogen in gastro-oesophageal refluxate.

CCL28, or mucosae-associated epithelial chemokine (MEC), is a CC-chemokine composed of 127 amino acids, which is known to be expressed by columnar epithelium in the colon, small intestine, salivary glands and airway (Hieshima, Kawasaki et al. 2004, Ogawa, Iimura et al. 2004, O'Gorman, Jatoi et al. 2005, Liu, Lan et al. 2012). It has been demonstrated to modulate the immune

environment of tissues by chemoattracting immune cells expressing the receptors CCR3 and CCR10 (Pan, Kunkel et al. 2000, Hieshima, Ohtani et al. 2003, Eksteen, Miles et al. 2006). While expression of CCL28 has never previously been demonstrated in the oesophagus, a study from our laboratory identified *CCL28* expression as being increased in a subset of oesophageal tumours with a poor response to chemoradiation therapy compared with normal oesophagus (Maher, Gillham et al. 2009).

CCL28 expression has been demonstrated to be regulated by the pro-inflammatory cytokines IL-1β and TNF-α in airway epithelium and in human and canine keratinocytes (O'Gorman, Jatoi et al. 2005, Kagami, Saeki et al. 2006, Shibata, Maeda et al. 2010), and also via IL-1β in human cholangiocytes (Eksteen, Miles et al. 2006). Both IL-1β and TNF-α are increased in BO and OAC in animal and human models, with some studies demonstrating the induction of these cytokines by bile acids (Nicholson and Jankowski , O'Riordan, Abdel-latif et al. 2005, Isomoto, Nishi et al. 2007, Majka, Rembiasz et al. 2010, Menke, van Zoest et al. 2012, Quante, Bhagat et al. 2012). This suggests the possibility that CCL28 in the oesophagus may be induced by bile acids via IL-1β and TNF-α.

The cytokines IL-1β and TNF-α are released as soluble proteins from cells into the microenvironment, where they can act either locally or can be transported in the vasculature to distant sites (Balkwill 2009, Dinarello 2010). These cytokines activate inflammatory signalling pathways in cells by binding to their respective receptors on the cell surface. IL-1β signals through the IL-1 receptor; two receptors for IL-1β have been identified (O'Neill 2008). The IL-1 receptor type 1 is thought to transmit the inflammatory signal as it has a large cytoplasmic domain, while the

type 2 receptor has a much smaller cytoplasmic domain and so probably competes with the type 1 receptor for binding without transmitting any inflammatory signal (Kuno and Matsushima 1994). TNF-α binds to the members of the TNF receptor family, of which the main inflammatory mediator is the TNF receptor type 1. This receptor activates a multitude of downstream pathways, including the pro-inflammatory NF-kB and pro-apoptotic caspase 8 and caspase 10 (Barnhart and Peter 2003).

In this chapter, the expression of CCL28 by human oesophageal cells is characterised for the first time; *CCL28* had previously been demonstrated to be expressed by oesophageal tumours, and therefore its expression along the metaplasia-dysplasia-adenocarcinoma sequence is investigated here. The expression of this chemokine in response to the bile acid DCA, known to play a major role in the development of BO and OAC, is examined, as are the roles in regulation of CCL28, IL-1β and TNF-α, along with their respective receptors.

3.2 Aims and Objectives

To investigate the role of CCL28 in oesophageal disease progression, the aim of this chapter was to characterise the expression of CCL28 in oesophageal cell lines representing normal, Barrett's, dysplastic and cancerous oesophagus. The bile acid deoxycholic acid is known to be a major component of gastro-oesophageal refluxate and many studies have used DCA in studies simulating reflux. CCL28 is also known in other tissue types to be regulated via the pro-

inflammatory cytokines IL-1β and TNF-α. Thus, this chapter aims to characterise the expression of CCL28 and the roles of bile acid and cytokines in the regulation of this expression.

Specific objectives:

- Determine if CCL28 is expressed in oesophageal cell lines representing the stages of progression to adenocarcinoma

- Determine if the bile acid deoxycholic acid plays a role in inducing CCL28 in oesophageal cell lines

- Determine the role of the cytokines IL-1β and TNF-α and their receptors in regulation of CCL28 expression in oesophageal cell lines

3.3 Results

3.3.1 Mycoplasma screening of cell lines

All cell lines used in the course of this study were routinely tested for mycoplasma contamination as described in section 2.2.6. Mycoplasma infection of cells is a common occurrence in cell culture facilities and may be introduced via several sources, such as other contaminated cell lines, contaminated surfaces and apparatus in cell culture suites, laboratory personnel, especially from laboratory

coats and skin surfaces, and from contaminated reagents. Mycoplasma is resistant to antibiotics commonly used in cell culture medium such as penicillin and streptomycin, and unlike other microbial contaminants, infection with mycoplasma is not visible in the cell culture medium. Mycoplasma infection can cause various effects in cell lines, including changes in biochemistry, metabolism, growth, viability, morphology and gene and protein expression. Infection with mycoplasma therefore may have deleterious effects on experiments carried out on contaminated cell lines, leading to inaccurate and unreliable results. During the course of this work, the HET-1A, QH-TERT, GO-TERT and OE19 cell lines were tested for mycoplasma routinely every 3-4 months and no contamination was detected using the sensitive PCR-based mycoplasma test (Figure 3.1).

3.3.2 Optimisation of deoxycholic acid concentrations for cell line treatments

Deoxycholic acid (DCA) is a secondary bile acid known to induce DNA damage and apoptosis in tissue and cell lines. In order to treat oesophageal cell lines with DCA, it was necessary to ensure that cells were not killed by the concentrations of DCA being used. Kill curves for varying concentrations of DCA were constructed after 24 h treatment in the QH-TERT, GO-TERT and OE19 cell lines, and after 1 h in the HET-1A cell line. A 1 h time point was chosen for the HET-1A cell line as it was much more sensitive to the effects of DCA than the other cell lines. Concentrations varied from 0 – 500 μM DCA for the QH-TERT, GO-TERT and OE19 cell lines, and from 0-10 μM DCA for the HET-1A cell line. Cell death was measured by Trypan blue exclusion; dead cells were expressed as a

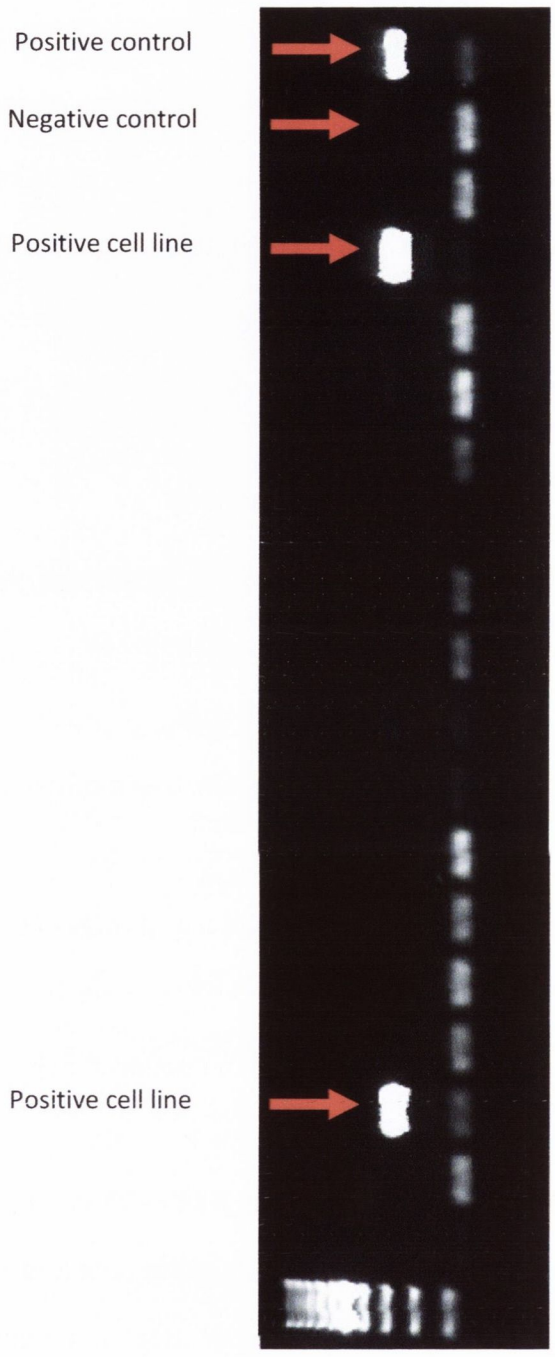


Figure 3.1 PCR-based assay for mycoplasma contamination in cell lines. Cell lines used in the cell culture facility during the course of this work were routinely tested for mycoplasma contamination using a PCR-based assay. Samples of supernatant from cells to be tested were run on a 2% agarose gel following PCR. Cell lines that were positive for mycoplasma infection demonstrated a band at 270 bp. A positive control of a cell line known to be contaminated with mycoplasma is shown in this representative image, along with a negative control and two cell lines which tested positive for mycoplasma infection on this test. All oesophageal cell lines used in this study tested negative for mycoplasma contamination.

percentage of the total number of cells. DCA induced less than 10% cell death in the normal squamous HET-1A cell line at concentrations up to 10 μM DCA after 1 h treatment (Figure 3.2 A). DCA induced similar proportions of cell death in the BO cell line QH-TERT at concentrations up to 500 μM DCA after 24 h treatment (Figure 3.2 B). Similar results were seen in the dysplastic GO-TERT cell line and the adenocarcinoma cell line OE19 (Fig 3.2 C, D).

3.3.3 CCL28 expression is induced in response to DCA treatment in oesophageal cell lines

Having established the concentrations of DCA that induced less than 10% cell death in oesophageal cell lines, protein expression of the chemokine CCL28 in response to DCA treatment was examined after 24 h in the QH-TERT, GO-TERT and OE19 cell lines, and after1 h in the HET-1A cell line. This was performed using ELISA on cell culture supernatants, as described in section 2.5.1.

The normal squamous HET-1A oesophageal cell line did not express CCL28 soluble protein basally. In response to increasing concentrations of DCA up to 10 μM, it expressed only very minimal amounts of CCL28 (10.6 pg/mL) at maximum (Fig. 3.3 A). The BO metaplastic QH-TERT cell line did not express CCL28 protein at a basal level; however, it expressed CCL28 protein in response to DCA treatment, with maximal CCL28 induction at a concentration of 5 μM DCA after 24 h, which was significantly increased compared to the untreated control (147.2 ± 44.88 pg/mL vs. 0 pg/mL, $p = 0.0305$). The high-grade dysplastic GO-

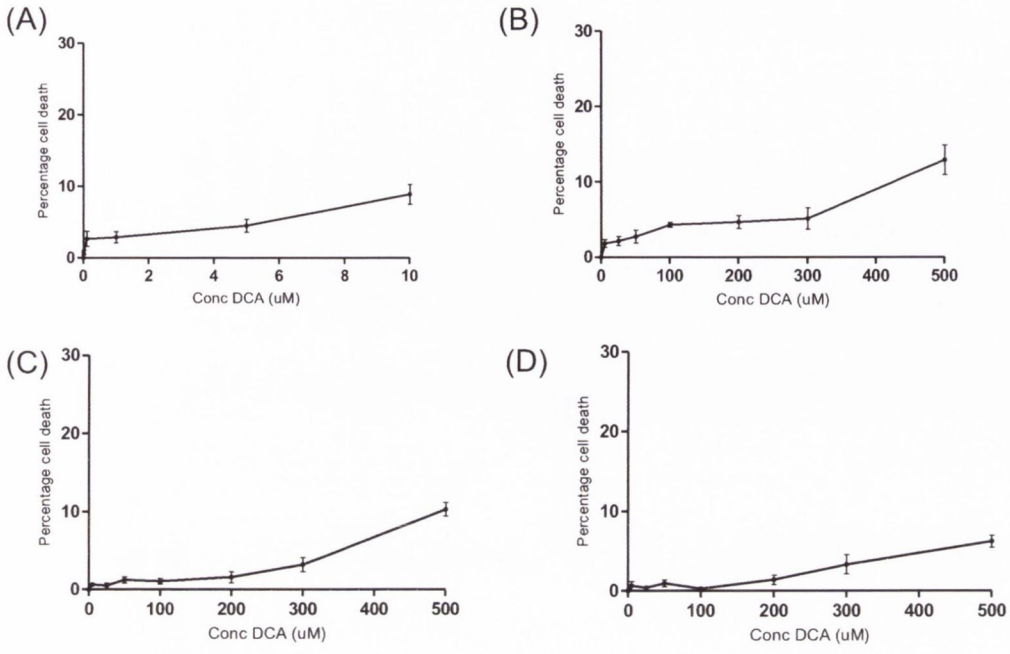


Figure 3.2 Treatment of normal, pre-neoplastic and neoplastic oesophageal cell lines with varying concentrations of deoxycholic acid does not induce significant cell death. Normal (HET-1A) squamous oesophageal cell line was treated with varying concentrations (0 – 10 μM) of the bile acid deoxycholic acid (DCA) for 1 h. Barrett's (QH-TERT), high-grade dysplastic (GO-TERT) and adenocarcinoma (OE19) oesophageal cell lines were treated with varying concentrations (0 – 500 μM) of DCA for 24 h. Cell death was measured after treatment using Trypan blue exclusion. Dead cells were expressed as a percentage of the total number of cells. The HET-1A cell line (A) demonstrates <10% cell death after 1 h treatment with 10 μM DCA. The QH-TERT (B), GO-TERT (C) and OE19 (D) cell lines demonstrate <15% cell death after 24 h treatment with 500 μM DCA. Data are expressed as mean ± SEM.

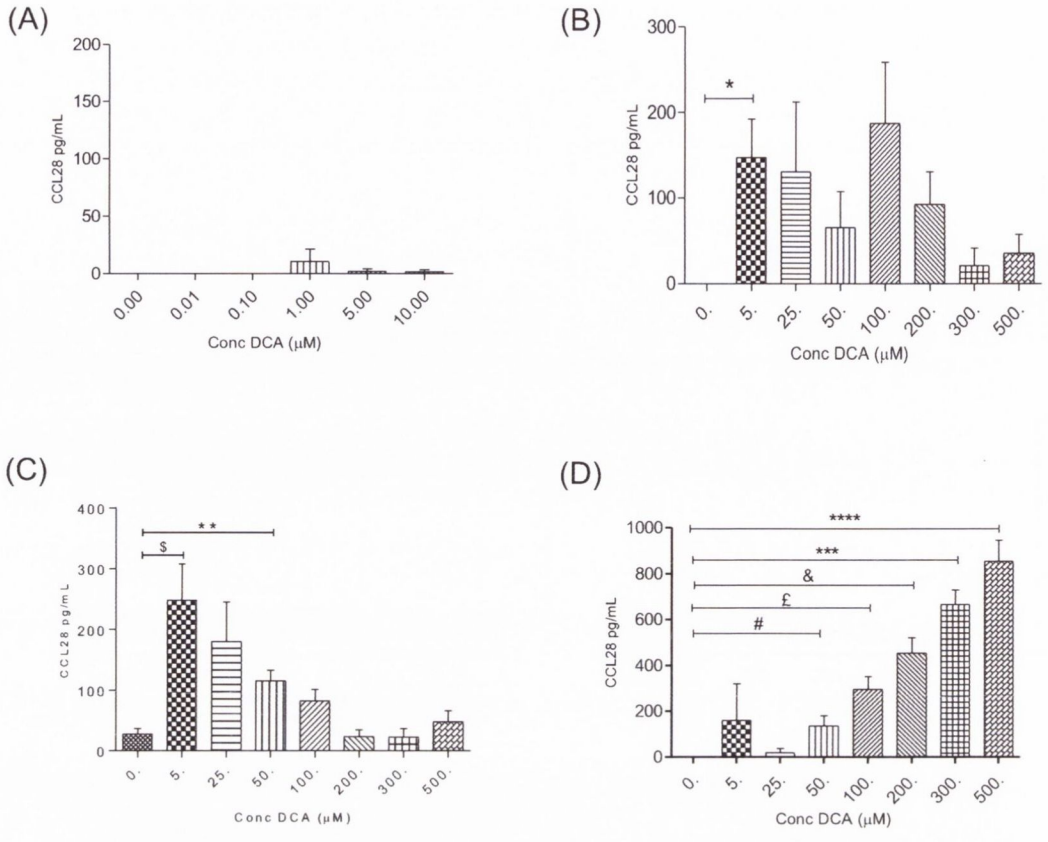


Figure 3.3 Pre-neoplastic and neoplastic oesophageal cell lines secrete CCL28 protein in response to treatment with the bile acid deoxycholic acid. Normal (HET-1A) squamous oesophageal cell line was treated with varying concentrations (0 – 10 μM) of the bile acid deoxycholic acid (DCA) for 1 h. Barrett's (QH-TERT), high-grade dysplastic (GO-TERT) and adenocarcinoma (OE19) oesophageal cell lines were treated with varying concentrations (0 – 500 μM) of DCA for 24 h. Soluble CCL28 protein was measured in cell culture supernatants using ELISA. (A) The normal squamous HET-1A cell line did not express significant amounts of CCL28 protein either basally or in response to DCA treatment. (B) The Barrett's metaplastic QH-TERT cell line did not express CCL28 protein basally but secreted CCL28 protein in response to treatment with DCA, with maximal induction of CCL28 protein with 5 μM DCA treatment. (C) The high-grade dysplastic GO-TERT cell line expressed a small amount of CCL28 protein basally, which was increased with DCA treatment. The GO-TERT cell line expressed maximal CCL28 protein with 5 μM DCA treatment. (D) The adenocarcinoma cell line OE19 did not express CCL28 protein at a basal level, but CCL28 protein secretion was induced by DCA in a dose-response fashion, with maximal CCL28 expression with 500 μM DCA treatment. Data are expressed as mean ± SEM and are the result of at least 5 independent experiments. Analysis was performed using the paired student's *t*-test. *p = 0305 vs. 0 μM DCA, $p = 0.0146 vs. 0 μM DCA, **p = 0.0022 vs. 0 μM DCA, #p = 0.0411 vs. 0 μM DCA, £p = 0.0063 vs. 0 μM DCA, &p = 0.0024 vs. 0 μM DCA, ***p = 0.0005 vs. 0 μM DCA, ****p = 0.0008 vs. 0 μM DCA.

TERT cell line expressed a small amount of soluble CCL28 protein basally (27.4 ± 9.37 pg/mL), which was increased by 24 h DCA treatment, with maximal induction of CCL28 at a concentration of 5 μM DCA (248.0 ± 59.69 pg/mL vs. 27.40 ± 9.37 pg/mL, p = 0.0146). CCL28 was also significantly increased by 50 μM DCA treatment compared with the untreated control (115 ± 18.07 pg/mL vs. 27.4 ± 9.37 pg/mL, p = 0.0022). The adenocarcinoma cell line OE19 demonstrated no basal CCL28 secretion, but a dose-response curve of CCL28 expression with increasing DCA concentrations was demonstrated, with a maximal induction of CCL28 at 500 μM DCA treatment, the maximal concentration tested (854 ± 92.22 pg/mL vs. 0 pg/mL, p = 0.0008).

3.3.4 Treatment with recombinant human IL-1β, but not recombinant human TNF-α, induces CCL28 expression in Barrett's and adenocarcinoma oesophageal cell lines.

The pro-inflammatory cytokines IL-1β and TNF-α are known to induce CCL28 expression in other tissue types such as columnar airway epithelium and cholangiocytes in the liver (O'Gorman, Jatoi et al. 2005, Eksteen, Miles et al. 2006). To determine if these cytokines regulate CCL28 expression in the oesophagus, the Barrett's, dysplastic and adenocarcinoma cell lines QH-TERT, GO-TERT and OE19 were treated with recombinant human cytokines IL-1β and TNF-α and CCL28 expression was measured. At an mRNA level, rhIL-1β, but not TNF-α, induced CCL28 gene expression in the QH-TERT (fold change 1.71 ± 0.07, p = 0.0105) and in the OE19 cell lines (fold change 115.10 ± 26.89), but not in the

GO-TERT cell line (Figure 3.4). At a protein level, similarly, rhIL-1β, but not rhTNF-α, induced CCL28 protein secretion from the QH-TERT (12.78 ± 6.39 pg/mL vs. 0 pg/mL, p = 0.1) and OE19 cell lines (71.27 ± 16.96 pg/mL vs. 0 pg/mL, p = 0.0085) (Figure 3.5). No induction of CCL28 protein expression was seen in the GO-TERT cell line. This suggests that in the Barrett's metaplasia cell line QH-TERT and in the adenocarcinoma cell line OE19, CCL28 expression is regulated via IL-1β, but in the dysplastic GO-TERT cell line, CCL28 expression is not regulated via this cytokine.

3.3.5 Oesophageal cell lines express the IL-1R1 and TNF-R1 receptors

Since oesophageal cell lines vary in their responses to rhIL-1β and rhTNF-α, expression of the receptors for these cytokines, the IL-1 receptor 1 and the TNF receptor 1 respectively, were investigated, in order to determine whether a lack of response to a cytokine may be due to a lack of the receptor for that cytokine. Initially, optimisation of the volume of fluorescent-labelled antibody against the IL-1R1 and the TNF-R1 needed to measure the receptor levels was carried out, and staining was confirmed by using the antibodies on peripheral blood mononuclear cells, which demonstrated clear positive populations (Figure 3.6).

Once the antibodies to be used had been optimised, levels of expression of the IL-1R1 and the TNF-R1 were measured using flow cytometry in the QH-TERT, GO-TERT and OE19 cell lines. All three cell lines examined expressed low levels of both the IL-1R1 compared with unstained controls (Fig 3.7) (QH IL-1R1: 0.01 ±

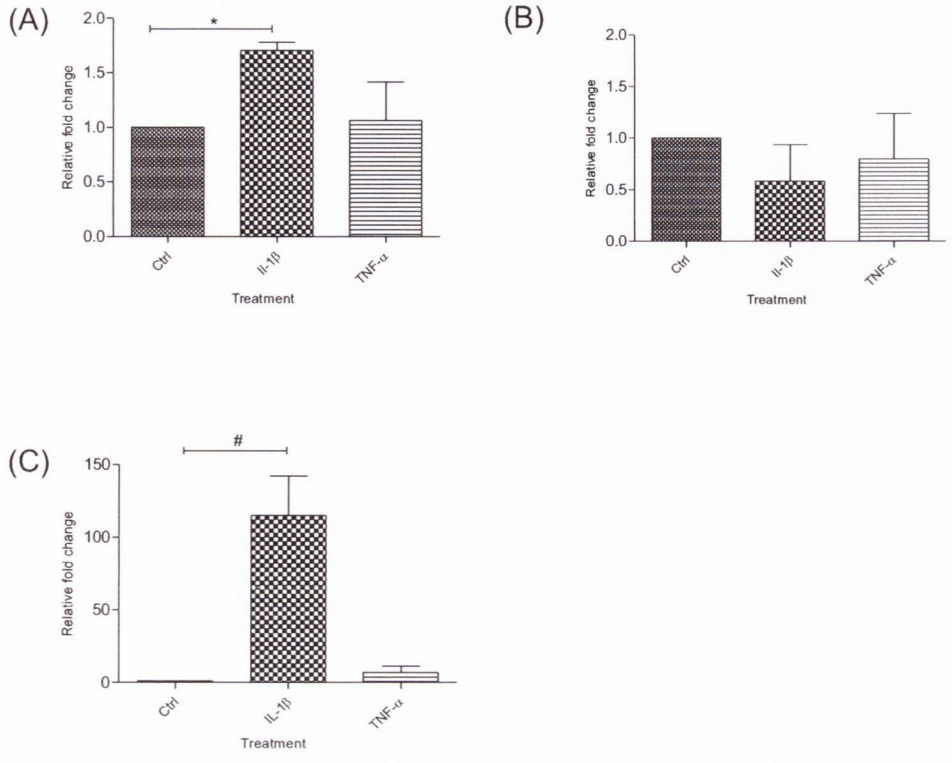


Figure 3.4 Treatment with recombinant human IL-1β, but not recombinant human TNF-α, induces CCL28 gene expression in Barrett's and adenocarcinoma oesophageal cell lines. Barrett's (QH-TERT), high-grade dysplastic (GO-TERT) and adenocarcinoma (OE19) oesophageal cell lines were treated with recombinant human (rh) cytokines IL-1β and TNF-α at a concentration of 10 ng/mL for 24 h. RNA was isolated from cells and gene expression of CCL28 was measured using quantitative real-time PCR. (A) The Barrett's cell line QH-TERT demonstrates increased CCL28 gene expression in response to treatment with rhIL-1β, but not in response to rhTNF-α. (B) The high-grade dysplastic cell line GO-TERT does not demonstrate increased CCL28 gene expression in response to rhIL-1β or rhTNF-α treatment. (C) The adenocarcinoma cell line OE19 demonstrates increased CCL28 gene expression in response to rhIL-1β treatment, but not in response to rhTNF-α treatment. Data are expressed as mean ± SEM and are the result of at least 3 independent experiments. Analysis was performed using the paired student's *t*-test. *p = 0.0105 vs. Ctrl, #p = 0.0513 vs. Ctrl.

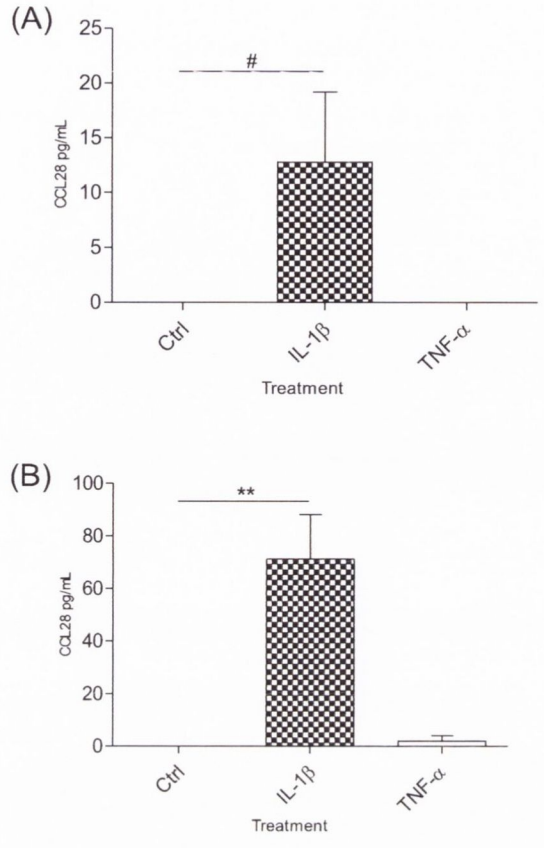


Figure 3.5 Treatment with recombinant human IL-1β, but not recombinant human TNF-α, induces CCL28 protein secretion in Barrett's and adenocarcinoma oesophageal cell lines. Barrett's (QH-TERT), high-grade dysplastic (GO-TERT) and adenocarcinoma (OE19) oesophageal cell lines were treated with recombinant human (rh) cytokines IL-1β and TNF-α at a concentration of 10 ng/mL for 24 h. Protein secretion of CCL28 into cell conditioned medium was measured using ELISA. (A) The Barrett's cell line QH-TERT demonstrates a trend towards increased CCL28 protein expression in response to treatment with rhIL-1β, but not in response to rhTNF-α. (B) The adenocarcinoma cell line OE19 demonstrates increased CCL28 protein expression in response to rhIL-1β treatment, but not in response to rhTNF-α treatment. The GO-TERT high-grade dysplasia cell line did not express any CCL28 in response to either IL-1β or TNF-α treatment. Data are expressed as mean ± SEM and are the result of at least 3 independent experiments. Analysis was performed using the paired student's *t*-test. $^{\#}p = 0.1$ vs. Ctrl, $^{**}p = 0.0085$ vs. Ctrl.

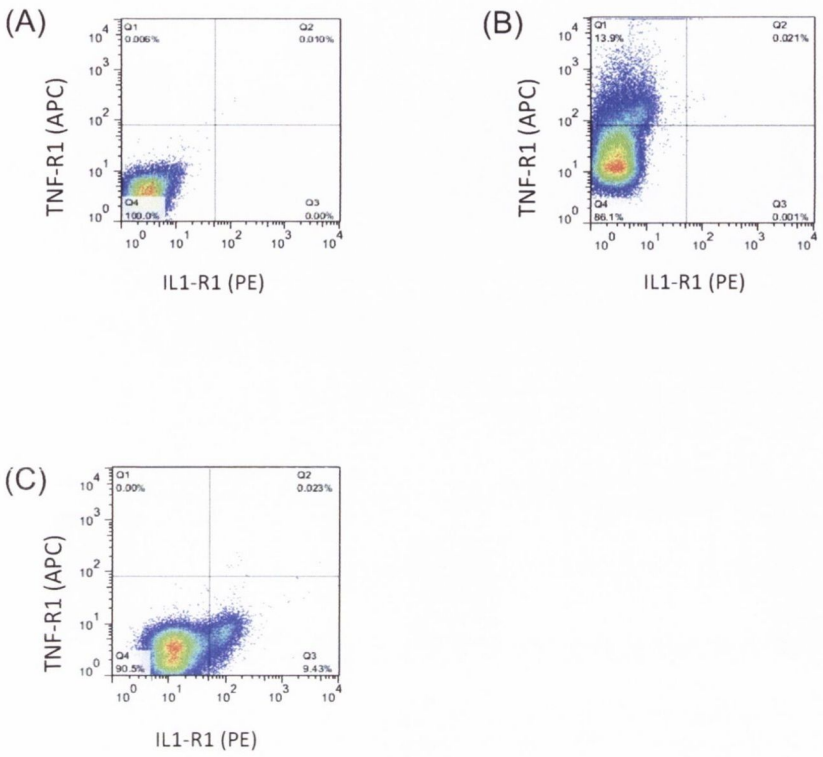


Figure 3.6 Optimisation of IL-1R1 and TNF-R1 staining on peripheral blood mononuclear cells. Peripheral blood mononuclear cells were isolated from a healthy donor and stained for the IL-1R1 and TNF-R1 receptors using fluorescent-labelled antibodies which were detected using flow cytometry. The IL-1R1 was labelled with PE and the TNF-R1 was labelled with APC. (A) The unstained cell population demonstrates no PE or APC fluorescence. (B) The APC-stained cells demonstrate a discrete population of TNF-R1-expressing cells. (C) The PE-stained cells demonstrate a discrete population of IL-1R1-expressing cells.

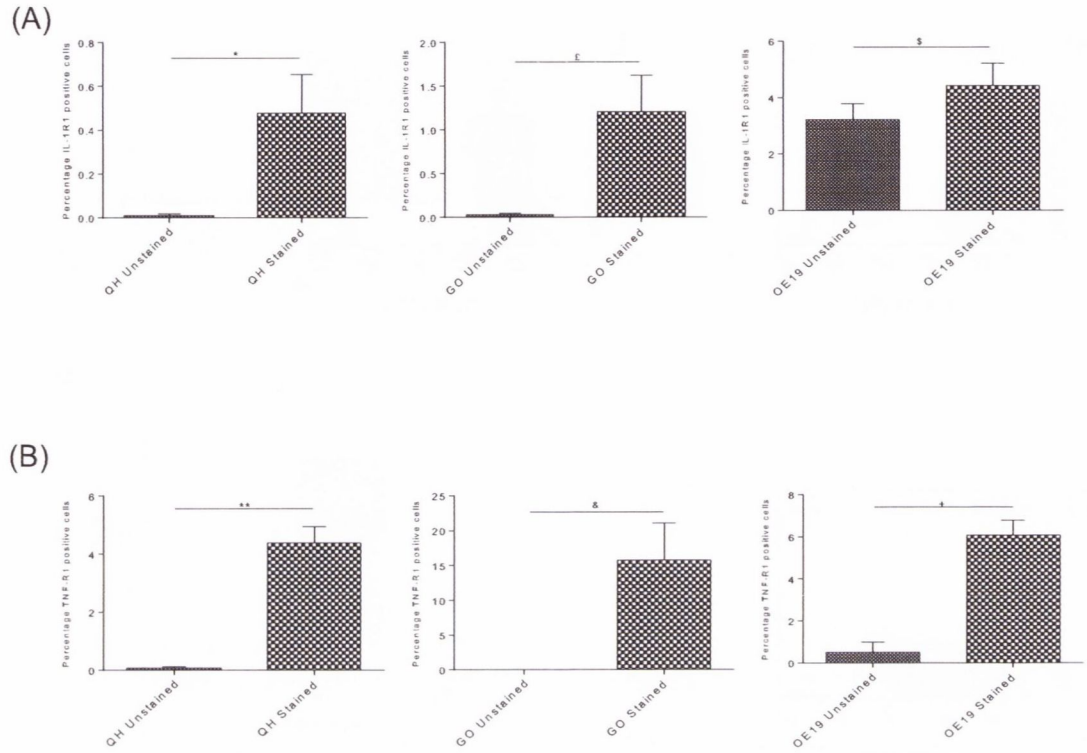


Figure 3.7 FACS staining for the IL-1R1 and TNF-R1 receptors in the QH-TERT, GO-TERT and OE19 oesophageal cell lines. The Barrett's cell line QH-TERT, high-grade dysplasia cell line GO-TERT and oesophageal adenocarcinoma cell line OE19 were stained for the receptors IL-1R1 and TNF-R1 using flow cytometry. (A) Staining for the IL-1R1 demonstrated that the receptor was expressed by the QH-TERT, GO-TERT and OE19 cell lines. (B) Staining for the TNF-R1 demonstrated that the receptor was expressed by the QH-TERT, GO-TERT and OE19 cell lines. Data are expressed as mean ± SEM and are the result of at least 3 independent experiments. Analysis was performed using the paired student's *t*-test. *p = 0.0398 vs. QH Unstained, $^{£}$p = 0.0460 vs. GO Unstained, $^{\$}$p = 0.0326 vs., OE19 Unstained, **p = 0.002 vs. QH Unstained, $^{\&}$p = 0.0322 vs. GO Unstained, $^{+}$p = 0.0123 vs. OE19 Unstained.

0.01 % vs. 0.48 ± 0.17 %, p = 0.0398 vs. Unstained; GO IL-1R1: 0.03 ± 0.01 % vs. 1.21 ± 0.42 %, p = 0.046 vs. Unstained; OE19 IL-1R1: 3.22 ± 0.57 % vs. 4.42 ± 0.79 %, p = 0.0326 vs. Unstained) and the TNF-R1 compared with unstained controls (QH TNF-R1: 0.08 ± 0.04 % vs. 4.38 ± 0.56 %, p = 0.002 vs. Unstained; GO TNF-R1: 0.04 ± 0.02 % vs. 15.77 ± 5.35 %, p = 0.0322 vs. Unstained; OE19 TNF-R1: 0.50 ± 0.49 % vs. 6.08 ± 0.70 %, p = 0.0123 vs. Unstained). Since these cell lines express both the IL-1 and TNF receptors but express CCL28 only in response to IL-1β, this suggests that TNF does not regulate expression of CCL28 in oesophageal cell lines, despite expressing the receptor.

3.3.6 Deoxycholic acid does not affect expression of the IL-1R1 or TNF-R1 receptors in oesophageal cell lines

Having established that pre-neoplastic and neoplastic oesophageal cell lines express the receptors IL-1R1 and TNF-R1, expression of these receptors in response to DCA treatment was examined, in order to determine whether DCA may upregulate the receptors, leading to an increase in response to the cytokines IL-1β and TNF-α. Cells were treated with DCA and receptor expression was measured and compared with receptor levels on untreated cells. DCA did not induce IL-1R1 or TNF-R1 expression in the QH-TERT, GO-TERT or OE19 cell lines (Fig. 3.8).

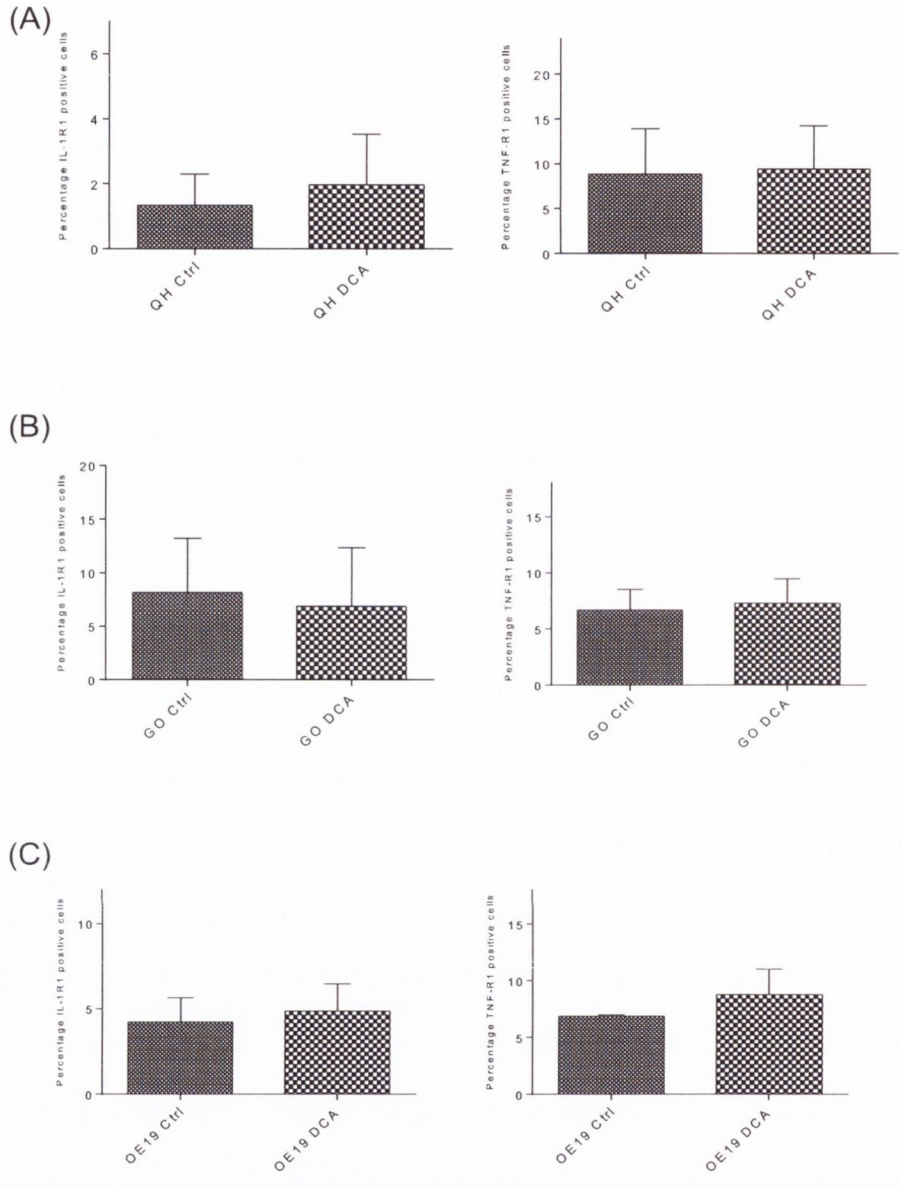


Figure 3.8 Deoxycholic acid does not affect expression of the IL-1R1 or TNF-R1 receptors in oesophageal cell lines. The oesophageal cell lines QH-TERT, representing Barrett's metaplasia, GO-TERT, representing high-grade dysplasia, and OE19, representing oesophageal adenocarcinoma, were treated with deoxycholic acid (DCA) at concentrations of either 200 μM (QH-TERT and GO-TERT) or 500 μM (OE19) for 24 h. After treatment, expression of the IL-1R1 and TNF-R1 receptors was measured using flow cytometry. (A) In the QH-TERT cell line, DCA does not affect expression of the IL-1R1 or the TNF-R1. (B) In the GO-TERT cell line, DCA does not affect expression of the IL-1R1 or the TNF-R1. (C) In the OE19 cell line, DCA does not affect expression of the IL-1R1 or the TNF-R1. Data are expressed as mean ± SEM and are the result of at least 3 independent experiments. Analysis was performed using the paired student's *t*-test.

3.3.7 Neutralising the IL-1R1 in the QH-TERT and OE19 oesophageal cell lines abrogates deoxycholic acid-induced CCL28 expression

Since the QH-TERT and OE19 oesophageal cell lines respond to rhIL-1β by secreting CCL28 protein, neutralisation of the receptor for IL-1β, IL-1R1 was carried out. Concentrations of the IL-1R1 neutralising antibody were optimised in order to determine the optimum concentration to neutralise the receptor (Fig. 3.9). A concentration of 10 μg/mL of neutralising antibody was determined to be the optimum concentration to use for both the QH-TERT and OE19 cell lines.

After optimising the concentration of the neutralising antibody to be used, the QH-TERT and OE19 oesophageal cell lines were treated with the neutralising antibody for 1 h, followed by treatment with DCA in the same medium for 24 h. This pre-blocked the IL-1R1 receptor prior to stimulation with DCA. Treatment with the IL-1R1 neutralising antibody alone had no effect. Treatment with DCA for 24 h induced CCL28 expression, as previously demonstrated. Pre-treatment with the IL-1R1 neutralising antibody before DCA stimulation decreased CCL28 protein secretion back to baseline levels (Fig. 3.10) in both the QH-TERT (20.08 ± 18.22 pg/mL, p = ns vs. Ctrl) and the OE19 (15.34 ± 8.19 pg/mL, p = ns vs. Ctrl) cell lines.

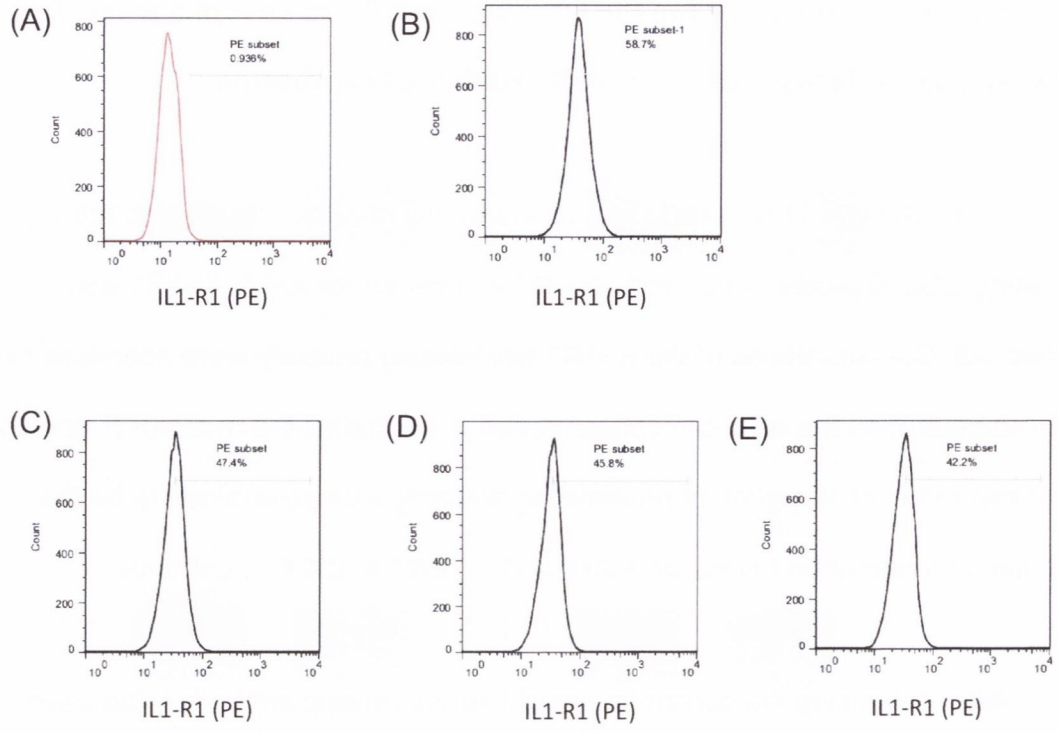


Figure 3.9 Optimisation of neutralising antibody for the IL-1R1 receptor in the QH-TERT oesophageal cell line. The QH-TERT Barrett's cell line was used to optimise the concentration of neutralising antibody for the IL-1R1 receptor to be used. The cell line was treated for 1 h with varying concentrations (0 – 20 µg/mL) of neutralising antibody and was then stained for the IL-1R1 using flow cytometry. (A) The unstained control demonstrates 0.936% expression of the IL-1R1 as measured by flow cytometry. (B) The untreated control stained for the IL-1R1 demonstrates that 57.6% of cells express the IL-1R1. (C) Pre-treatment of the QH-TERT cell line with 5 µg/mL of neutralising antibody for 1 h decreases the percentage of cells on which IL-1R1 was detectable to 47.4%. (D) Pre-treatment of the QH-TERT cell line with 10 µg/mL of neutralising antibody for 1 h decreases the percentage of cells on which IL-1R1 was detectable to 45.8%. (E) Pre-treatment of the QH-TERT cell line with 20 µg/mL of neutralising antibody for 1 h decreases the percentage of cells on which IL-1R1 was detectable to 42.4%.

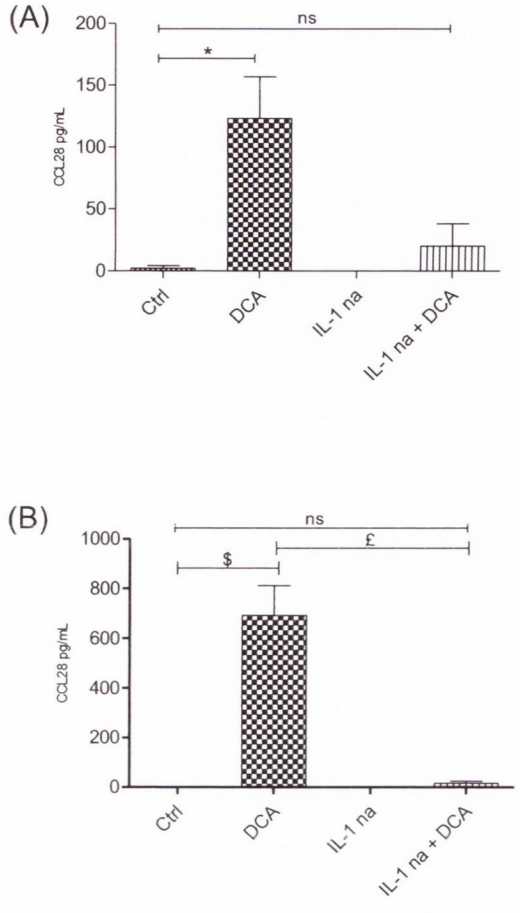


Figure 3.10 Neutralising the IL-1R1 in the QH-TERT and OE19 oesophageal cell lines abrogates deoxycholic acid-induced CCL28 expression. The Barrett's cell line QH-TERT and the oesophageal adenocarcinoma cell line OE19 were pre-treated with an IL-1R1 neutralising antibody for 1 h before 24 h treatment with the bile acid deoxycholic acid (DCA). Cells were also treated with DCA alone and the IL-1R1 neutralising antibody (IL-1 na) alone. CCL28 protein secretion into the conditioned culture medium was measured using ELISA. (A) In the QH-TERT cell line, DCA induces CCL28 protein secretion. This secretion is not induced by IL-1 na alone, and is decreased to basal levels by pre-treatment with the IL-1 na followed by DCA treatment. (B) In the OE19 cell line, DCA induces CCL28 protein secretion. This secretion is not induced by IL-1 na alone, and is decreased to basal levels by pre-treatment with the IL-1 na followed by DCA treatment. Data are expressed as mean ± SEM and are the result of at least 3 independent experiments. Analysis was performed using the paired student's *t*-test. *$p = 0.0393$ vs. Ctrl, $^{\$}p = 0.0285$ vs. Ctrl, $^{£}p = 0.0292$ vs. DCA.

3.3.8 Deoxycholic acid induces low concentrations of IL-1β protein in oesophageal cell lines

Since the neutralisation experiment suggested that CCL28 expression in response to DCA is regulated via the IL-1R1 receptor, the expression of IL-1β protein in response to DCA was examined. The HET-1A, QH-TERT, GO-TERT and OE19 oesophageal cell lines were treated with varying concentrations of DCA for 24 h, except for the HET-1A cell line which was treated with DCA for 1 h. The HET-1A cell line did not secrete any IL-1β soluble protein either basally or in response to DCA (Figure 3.11). The QH-TERT cell line secreted small amounts of IL-1β protein in response to 200 μM and 300 μM DCA treatment (200 μM DCA: 9.54 ± 2.23 pg/mL vs. 0 pg/mL, $p = 0.0129$ vs. 0 μM; 300 μM DCA: 4.85 ± 1.73 pg/mL vs. 0 pg/mL, $p = 0.0488$ vs. 0 μM), but did not express any IL-1β at a basal level. The GO-TERT cell line secreted no IL-1β basally, but expressed a very small amount of IL-1β protein in response to DCA treatment, with a maximum of 1.25 ± 0.82 pg/mL IL-1β protein in response to 500 μM DCA treatment. The OE19 cell line demonstrated a small amount of basal IL-1β protein secretion (5.04 ± 3.47 pg/mL) and also demonstrated increased IL-1β in response to 5 μM and 500 μM DCA treatment (5 μM DCA: 13.98 ± 3.53 pg/mL vs. 5.04 ± 3.47 pg/mL, $p = 0.089$ vs. 0 μM; 500 μM DCA: 18.8 ± 0.84 pg/mL vs. 5.04 ± 3.47 pg/mL, $p = 0.0221$ vs. 0 μM).

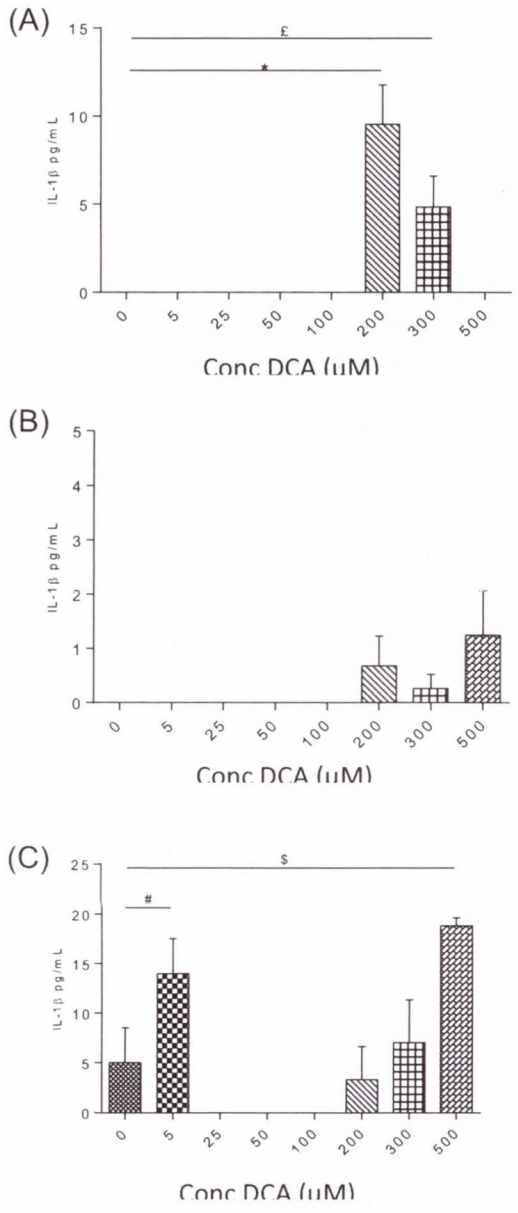


Figure 3.11 Deoxycholic acid induces low concentrations of IL-1β protein in oesophageal cell lines. The Barrett's cell line QH-TERT, high-grade dysplasia cell line GO-TERT and oesophageal adenocarcinoma cell line OE19 were treated with varying concentrations of the bile acid deoxycholic acid (DCA) for 24 h. IL-1β soluble protein secretion into the conditioned culture medium was measured using ELISA. (A) The QH-TERT cell line demonstrated induction of IL-1β protein secretion with 200 μM and 200 μM DCA treatments. (B) The GO-TERT cell line demonstrated secretion of very small amounts of IL-1β protein with 200-500 μM DCA treatments. (C) The OE19 cell line demonstrated induction of IL-1β protein secretion with 5 μM DCA treatment and 200-500 μM DCA treatment. Data are expressed as mean ± SEM and are the result of at least 3 independent experiments. Analysis was performed using the paired student's *t*-test. *p = 0.0129 vs. 0 μM QH-TERT, £p = 0.0488 vs. 0 μM QH-TERT, #p = 0.089 vs. 0 μM OE19, $p = 0.0221 vs. 0 μM OE19.

3.4 Discussion

The tissue microenvironment in pre-neoplastic conditions is increasingly being recognised as playing a vital role in promoting progression to cancer. BO is known to be a risk factor for the development of OAC and so is considered to be a pre-malignant change in the oesophageal epithelium. The risk of developing OAC in patients with BO was thought to be 5-10 cases per 1000 patient years (Thomas, Abrams et al. 2007, Yousef, Cardwell et al. 2008, Sikkema, de Jonge et al. 2010); however, a more recent study has estimated a much lower risk of 1.2 cases per 1000 patient years (Hvid-Jensen, Pedersen et al. 2011). This is a much higher incidence of OAC than in the general population, with a relative risk of 29.8 for patients with BO (Solaymani-Dodaran, Logan et al. 2004). The majority of patients who develop BO never progress to OAC. Therefore, identifying factors that may help to identify the patients who may progress to OAC is of great importance. Factors in the tissue microenvironment, such as cytokines and chemokines, are known to be altered during the progression from normal oesophagus to BO to OAC, and may function to promote this disease progression (Kavanagh, O'Sullivan et al. 2013). Furthermore, the exposure of the oesophagus to gastro-oesophageal reflux of bile and acid from the stomach and duodenum is thought to play a major role in causing the development of BO and OAC: gastro-oesophageal reflux disease (GORD) is a well-described risk factor for OAC (Chow, Finkle et al. 1995, Lagergren, Bergstrom et al. 1999). In this study, the chemokine CCL28 is studied in oesophageal cell lines representing various stage of oesophageal disease.

Here, oesophageal cell lines representing normal squamous epithelium, BO, high-grade dysplasia (HGD) and OAC were examined for expression of the

chemokine CCL28, both basally and in response to the bile acid deoxycholic acid (DCA). DCA is known to be a major component of gastro-oesophageal reflux (Fracchia, Pellegrino et al. 1999, Nehra, Howell et al. 1999). Initially it was established that DCA did not cause significant cell death at concentrations used in experimental conditions. Unsurprisingly, the squamous oesophageal cell line HET-1A was more sensitive to the effects of DCA than the pre-neoplastic and neoplastic oesophageal cell lines. This was an expected finding, as the metaplastic change from squamous to columnar epithelium, as occurs in BO, is thought to be an adaptive mechanism in response to the damaging effects of exposure to bile acid refluxate (Badreddine and Wang 2010).

Since CCL28 is normally expressed by columnar epithelial cells (e.g. colon and airway epithelium) (Ogawa, Iimura et al. 2004, O'Gorman, Jatoi et al. 2005), it was unsurprising that the squamous oesophageal cell line HET-1A did not express CCL28, either basally or in response to DCA stimulation. The columnar epithelial cell lines QH-TERT, representing BO, GO-TERT, representing HGD, and OE19, representing OAC, all secreted CCL28 protein in response to DCA treatment. These cell lines, progressing through the disease sequence from metaplasia to carcinoma, respond to stimulation with bile acid to produce this chemokine, suggesting that during disease progression, when the oesophageal epithelium is exposed to gastro-oesophageal reflux, CCL28 may be produced, causing alterations in the tissue microenvironment of the oesophagus, as has been demonstrated with other inflammatory molecules (Fitzgerald, Onwuegbusi et al. 2002, Moons, Kusters et al. 2005). Interestingly, the amount of CCL28 produced in response to DCA increased in the cell lines from BO to HGD to OAC; this suggests

that the response to DCA stimulation becomes stronger as the oesophageal cells become increasingly neoplastic. The concentration of DCA needed to induce maximal CCL28 was also increased in the OAC cell line, suggesting that this cell line is more adapted to exposure to DCA. This correlates with clinical findings, with patients with BO having higher concentrations of bile acids in refluxate than those without BO (Iftikhar, Ledingham et al. 1993).

CCL28 has previously been shown to be regulated via the pro-inflammatory cytokines IL-1β and TNF-α in tissues such as airway epithelium and cholangiocytes in the liver (O'Gorman, Jatoi et al. 2005, Eksteen, Miles et al. 2006). Here, it is demonstrated that in oesophageal cell lines, CCL28 is induced by IL-1β, but not by TNF-α. Indeed, the results shown here are similar to those seen in airway epithelium – IL-1β induced significantly more CCL28 in airway cells than TNF-α (O'Gorman, Jatoi et al. 2005). In the oesophagus, it appears that CCL28 is induced solely via IL-1β. This concentration of cytokine (10 ng/mL) was chosen as it has previously been used in similar experiments (O'Gorman, Jatoi et al. 2005, Eksteen, Miles et al. 2006). The GO-TERT cell line, representing HGD, did not respond to recombinant IL-1β protein. This surprising result may indicate that this pathway is disrupted in this cell line, or could suggest that the receptor is inactive.

Although it seems likely that DCA induces IL-1β, which subsequently induces CCL28, ELISA was unable to detect significant levels of IL-1β protein secreted by oesophageal cells in response to DCA after 24 h treatment, with levels less than 10 pg/mL detected. However, it may be that IL-1β protein reached peak levels at an earlier time point after DCA treatment, and then degrades and so is not detected by the ELISA assay. A previous study has demonstrated that DCA can

induce IL-1β in oesophageal cells (Fitzgerald, Onwuegbusi et al. 2002); so it was surprising that this study did not detect these proteins at significant levels. The study by Fitzgerald et al. did demonstrate that in BO tissue, IL-1β was induced by a 1 h pulsed treatment of bile acid, so this may be the optimal bile acid stimulation profile for this cytokine. It is also possible that very low levels of IL-1β protein are sufficient to induce a response in oesophageal cells – although recombinant cytokines were used at a concentration of 10 ng/mL, it may be that the much lower concentrations of cytokine detected in these experiments are sufficient to induce secretion of CCL28 in oesophageal cell lines.

It was hypothesised that the reason TNF-α does not induce CCL28 in oesophageal cell lines and also IL-1β does not induce CCL28 in the GO-TERT cell line is because they lack the receptors for these cytokines. The cell lines QH-TERT, GO-TERT and OE19 were assessed for expression of the receptors for IL-1β and TNF-α, the IL-1R1 receptor and the TNF-R1 receptor. All three cell lines expressed both the IL-1R1 and the TNF-R1. This suggests that CCL28 is not regulated via TNF-α stimulation – the cells express the receptor for TNF-α but do not respond by expressing CCL28. In the GO-TERT cell line, the pathway in which IL-1β induces CCL28 in the other oesophageal cell lines may be dysregulated or dysfunctional.

DCA may affect the expression of receptors on the cell surface. If DCA increased expression of the IL-1R1, this could be one explanation for the increased expression of CCL28 in response to DCA treatment. The expression of the receptors IL-1R1 and TNF-R1 were examined basally and in response to DCA. However, stimulation with DCA did not induce expression of the IL-1R1 or TNF-R1

receptors. Therefore, DCA increases CCL28 expression probably via increasing expression of IL-1β, without increasing expression of the IL-1R1 receptor. In order to confirm that DCA induced CCL28 by stimulating IL-1β to act via the IL-1R1, the IL-1R1 receptor was neutralised and then cell lines stimulated with DCA. When the IL-1R1 was neutralised, DCA-induced CCL28 expression was decreased back to levels similar to basal levels of expression. This suggests that DCA indeed acts to stimulate CCL28 expression via the IL-1R1.

This chapter demonstrates for the first time that oesophageal cell lines representing BO, HGD and OAC express the chemokine CCL28 in response to the bile acid DCA, while a cell line representing normal squamous oesophageal epithelium does not demonstrate CCL28 expression. Since bile acid stimulation is known to promote oesophageal disease progression, DCA-stimulated CCL28 may play a role in this progression. CCL28 expression in oesophageal cell lines is regulated via IL-1β acting through the IL-1R1 receptor, while TNF-α, which plays a role in regulation of CCL28 in other tissue types, does not appear to regulate CCL28 expression in the oesophagus. IL-1β is known to increase across the oesophageal disease sequence (O'Riordan, Abdel-latif et al. 2005, Quante, Bhagat et al. 2012) and so this may correlate with increasing CCL28 expression in response to exposure to gastro-oesophageal reflux of bile acid in the human oesophagus. In the next chapter the expression of CCL28 in *ex vivo* human oesophageal tissue will be examined.

Chapter 4

Characterisation of CCL28 expression basally and in response to bile acid in oesophageal tissue

4.1 Introduction

The establishment of a biobank is an invaluable resource for researchers in a translational research facility. With the increasing availability of human samples for research worldwide, it becomes of great importance to establish bioresources. In these biobanks, samples from patients are collected, processed, labelled and stored in a standardised manner according to a protocol, with full informed consent obtained from each patient. Establishing a biobank requires a large amount of planning, including obtaining ethical approval and designing and implementing protocols. Protocols needed for each biobank include which samples should be included and how they should be obtained, and how and where samples should be stored (Ennis, Pidgeon et al. 2010). A designated biobank allows correlation of biomarkers and other molecular characteristics of samples with clinical data which is recorded in parallel with sample collection. Biobanks for various disease and specimen types have been established worldwide, including such diverse conditions as Marfan's syndrome, paediatric inflammatory myopathies, schizophrenia and lung cancer (Agota, Agg et al. 2012) (Martin, Krol et al. 2011) (Inczedy-Farkas, Benkovits et al. 2010) (Li, Qiu et al. 2009); with increasingly stringent standardisation of protocols for sample processing, biobanks can establish international collaborations and sharing of samples and data, thereby providing increased resources for translational research to groups undertaking work in similar areas, although this can create legal and ethical issues (Kiehntopf and Krawczak 2011). Problems encountered during the running of a biobank include ethical issues surrounding informed consent, technical difficulties involving storage and extraction of appropriate biomolecules and collection of clinical data, and administrative problems, such as funding, inadequate personnel to run the

biobank and competition for use of samples (Gaffney, Madden et al. 2012). The issue of informed consent is a complex one for bioresource managers; however, studies consistently demonstrate that, in general, the public view biobanks as a resource primarily for public good, and that patients are willing to participate in biobanks primarily out of altruistic interests in the beneficiaries from the biobank, while privacy and confidentiality are less important factors in their consideration (Kaufman, Bollinger et al. 2012, Pullman, Etchegary et al. 2012).

While immortalised human epithelial cell lines provide an excellent model in order to examine the response of these cells to treatments and experimental conditions, monolayers of epithelial oesophageal cells do not fully replicate the complex microenvironment of the oesophageal tissue itself. This environment comprises not only the epithelial lining, but the underlying stroma, containing cells such as immune cells and fibroblasts, as well as blood and lymph vessels. Additionally the extracellular space contains growth factors, cytokines and chemokines, all of which are biologically active in modulating the tissue environment (Saadi, Shannon et al.). An improved model is provided by the *ex vivo* tissue explant model, which has been used in many tissue types, including oesophageal tissue, to more faithfully replicate the three-dimensional, physiological conditions found in human tissue, retaining the complex cell-cell and cell-cytokine relationships found *in vivo* (Muthuswamy, Berk et al. , Fitzgerald, Omary et al. 1996, Tchorzewski, Qureshi et al. 1998, Fitzgerald, Abdalla et al. 2002, Marsman, Buskens et al. 2004, Mariette, Piessen et al. 2008). The explant model has proven so effective that it has even been used in adenoviral transduction experiments in oesophageal tissue (Marsman, Buskens et al. 2004). The establishment of a biobank provides an excellent resource via which samples for use in an explant

culture model are obtained in a standardised manner from consented patients; it also allows correlation of molecular data obtained from the explants with clinical and epidemiological data on the patients used in the study.

The tissue biopsies used in explant culture must remain viable in the culture medium during the period of culture and treatments used must not adversely affect tissue viability, in order to obtain reliable results. The release of the enzyme lactate dehydrogenase (LDH) is frequently used as a measure of tissue viability in explant culture models (Audette, Greenwood et al.) (Aranibar, Borys et al.), as LDH leaks out of tissue when cell membranes are disrupted. This method has also been used in oesophageal tissue explant models (Fitzgerald, Omary et al. 1996, Kaur, Ouatu-Lascar et al. 2000).

The chemokine CCL28 has never previously been investigated in patients with oesophageal disease; however, serum levels of CCL28 have been examined in patients with a variety of other conditions including atopic dermatitis, bullous pemphigoid, chronic obstructive pulmonary disease and human immunodeficiency virus (Chen, Song et al. , Kagami, Kakinuma et al. 2005, Castelletti, Lo Caputo et al. 2007, Ezzat, Sallam et al. 2009), and have been found to be increased compared with the levels found in healthy controls. These conditions are all inflammatory diseases; chemokines modulate the immune cells infiltrating tissue and therefore may modulate the inflammatory cells chemoattracted to the tissue by distribution in the vasculature.

As chemokines play a role in chemotaxis of cells to a particular tissue, their expression within a localised tissue environment may be of greater importance than their concentrations in serum. The presence of cytokines and chemokines in

the oesophageal tissue microenvironment is widely recognised to play a major role in the progression of oesophageal disease (Poehlmann, Kuester et al.). Cytokines, such as IL-4, IL-6, IFN-γ, IL-1β and TNF-α, as well as chemokines, such as IL-8, eotaxin-1, -2 and -3, MIP-1α and MCP-1, have all been demonstrated to be expressed by human oesophageal tissue at various stages of disease (Altomare, Ma et al. , Cheng, Zhang et al. , Rieder, Biancani et al. , Zhong, Lin et al. , Corrado, Zicari et al. 1999, Deans, Wigmore et al. 2006, Isomoto, Wang et al. 2008, Babar, Ennis et al. 2010, Quante, Bhagat et al. 2012). As discussed in Chapter 3, exposure of the oesophagus to bile acids is thought to be a salient factor in the development of BO and OAC, and bile acids have been demonstrated to induce the expression of cytokines and chemokines in oesophageal cells. Therefore, understanding the relationship between bile acids and CCL28 in oesophageal tissue may provide insight into the role of this chemokine in oesophageal disease.

4.2 Aims and objectives

Chemokines play a role in altering the tissue microenvironment by modulating the immune cells infiltrating the tissue. In order to further elucidate the role of the chemokine CCL28 in oesophageal disease, this chapter aims to investigate the expression of CCL28 in human tissue explants, providing a model that includes both oesophageal epithelium and stroma that better represents the microenvironment of the oesophagus. The role of bile acid in this system is also examined.

Specific objectives:

- Establish a biobank for Barrett's oesophagus patient samples.
- Construct a tissue explant system for oesophageal biopsies.
- Determine if CCL28, along with the pro-inflammatory cytokines IL-1β and TNF-α, are expressed by normal, Barrett's and tumour tissue explants.
- Determine if the bile acid DCA influences CCL28, IL-1β and TNF-α expression in normal, Barrett's and tumour tissue explants.

4.3 Results

4.3.1 Barrett's biobank

A biobank of material from patients with BO was set up during the course of this project. The location of the laboratory facilities used for this work, in proximity to a major adult hospital, provided ideal conditions to set up this resource. Having received ethical approval (Appendix 1, Appendix 2), consultant gastroenterologists and gastrointestinal surgeons were informed regarding the protocol for taking biopsies from consented patients with BO. Patients with BO were identified from the list of patients undergoing endoscopy, consented, bloods were taken by the consenting clinician and biopsies were taken by the doctor performing the endoscopy from normal and Barrett's epithelium as described in section 2.7. Over 80 patients were recruited during the first year of the BO biobank; however, due to constraints of endoscopy, not all of these patients had biopsies taken for the biobank, resulting in a total of 66 patients being enrolled into the biobank during this year. The characteristics of this cohort of patients are outlined in Table 4.1.

Table 4.1 Characteristics of patients in Barrett's biobank in year 1

		Number (%)
Patients		66 (100)
Gender:	**Male**	49 (74.24)
	Female	17 (25.76)
Average age (years)		60.98
Age range (years)		27 - 86
Confirmed Barrett's on histology		57 (86.36)
On PPI:	**Yes**	46 (69.7)
	No	9 (13.63)
	Unknown	11 (16.67)

*PPI: proton pump inhibitor

4.3.2 CCL28 is differentially expressed in serum from patients with varying stages of oesophageal disease

Sera were obtained from consented patients with various stages of oesophageal disease including normal oesophagus, Barrett's metaplasia, low-grade and high-grade dysplasia, intramucosal carcinoma and invasive adenocarcinoma. Characteristics of this patient cohort are shown in Table 4.2. Serum levels of CCL28 were significantly higher in patients with Barrett's metaplasia than those with low-grade or high-grade dysplasia ($p = 0.0036$ vs. LGD and $p = 0.0023$ vs. HGD respectively) (Fig.4.1). Serum CCL28 trended towards an increase in patients with Barrett's metaplasia compared with those with normal oesophagus ($p = 0.15$ vs. Normal). CCL28 in the sera of patients with invasive oesophageal adenocarcinoma was also significantly higher than in the sera of patients with normal oesophagus ($p = 0.036$ vs. Normal) low-grade dysplasia ($p = 0.0063$ vs. LGD), high-grade dysplasia ($p = 0.0074$ vs. HGD) and intramucosal carcinoma ($p = 0.0415$ vs. IMC). No difference was observed in serum levels of CCL28 between patients with Barrett's metaplasia and invasive adenocarcinoma.

4.3.3 Confirmation of Barrett's columnar epithelium in biopsy samples

Before analysis of biopsies in the explant system could be performed, it was necessary to confirm that the biopsies taken were indeed from columnar Barrett's epithelium. Biopsies were taken immediately adjacent to the study biopsies during endoscopy and were examined by a consultant histopathologist (Dr. Cian Muldoon) to confirm the presence of columnar epithelium. Biopsies that did not contain columnar epithelium as confirmed by a histopathologist were excluded

Table 4.2 Characteristics of patient cohorts (sera and explant culture)

Sample	No. of male patients (%)	No. of female patients (%)	Average age in years (range)
Sera			
Normal	6 (60)	4 (40)	65.8 (44-81)
Barrett's metaplasia	15 (65.2)	8 (34.8)	62.7 (19-83)
Low grade dysplasia	12 (75)	4 (25)	60.9 (49-82)
High grade dysplasia	13 (93)	1 (7)	64 (45-77)
Intramucosal carcinoma	8 (80)	2 (20)	61.2 (40-76)
Invasive carcinoma	15 (83)	3 (17)	64.3 (44-81)
Explants			
Barrett's metaplasia	13 (65)	7 (35)	62.7 (39-79)

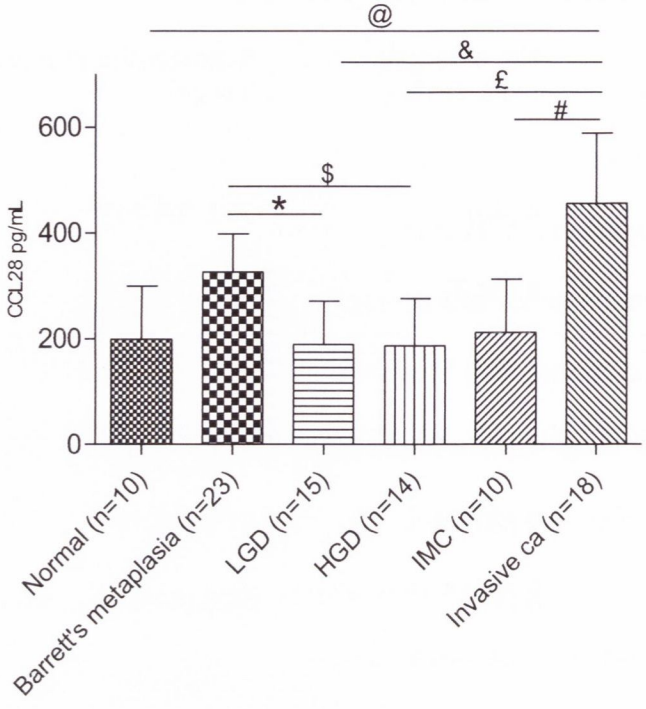


Figure 4.1 CCL28 is differentially expressed in serum from patients with varying stages of oesophageal disease. CCL28 protein was measured by ELISA in serum samples from patients with Barrett's metaplasia (n=23), low-grade dysplasia (LGD) (n=15), high-grade dysplasia (HGD) (n=14), intramucosal carcinoma (IMC) (n=10) and invasive adenocarcinoma (n=18). Serum CCL28 was significantly increased in serums from patients with Barrett's metaplasia compared with serums from patients with LGD or HGD. Serum CCL28 was also significantly increased in serums from patients with invasive adenocarcinoma compared with serums from patients with LGD, HGD or IMC. There was no difference in serum CCL28 between patients with Barrett's metaplasia and invasive adenocarcinoma. Data are presented as mean ± SEM. Analysis was performed using the unpaired Mann-Whitney U test. $^{*}p=0.0036$ vs. Barrett's metaplasia, $^{\$}p=0.0023$ vs. Barrett's metaplasia, $^{@}p=0.0361$ vs. Normal, $^{\&}p=0.0063$ vs. LGD, $^{£}p=0.0074$ vs. HGD, $^{\#}p=0.0415$ vs. IMC.

from the study. Biopsies that were confirmed histologically as containing Barrett's epithelium were analysed using PCR for the columnar markers CK8 and TFF1, as described in section 2.7.6. All Barrett's biopsies used in this study expressed these markers, while all normal biopsies used in this study were negative for these columnar markers. Representative agarose gels are shown in Figure 4.2.

4.3.4 Confirmation of tissue viability in explant culture system

Before analysis of biopsies used in the explant culture system, it was necessary to confirm that tissue biopsies were viable after 24 h explant culture, and also that 24 h treatment with 100 μM deoxycholic acid (DCA) did not significantly affect tissue viability. Matched normal and Barrett's biopsies (n=5) were obtained and cultured for 24 h in either medium alone or medium containing 100 μM DCA. Biopsies were homogenised and LDH released into the culture medium was expressed as a percentage of the total LDH in the biopsy sample. LDH released into culture medium was less than 30% for both normal and Barrett's biopsies (Figure 4.3). After 24 h treatment with 100 μM DCA, there was no significant increase in LDH released from either normal or Barrett's biopsies. This indicates that biopsies remained viable in the explant culture model and that DCA did not affect tissue viability.

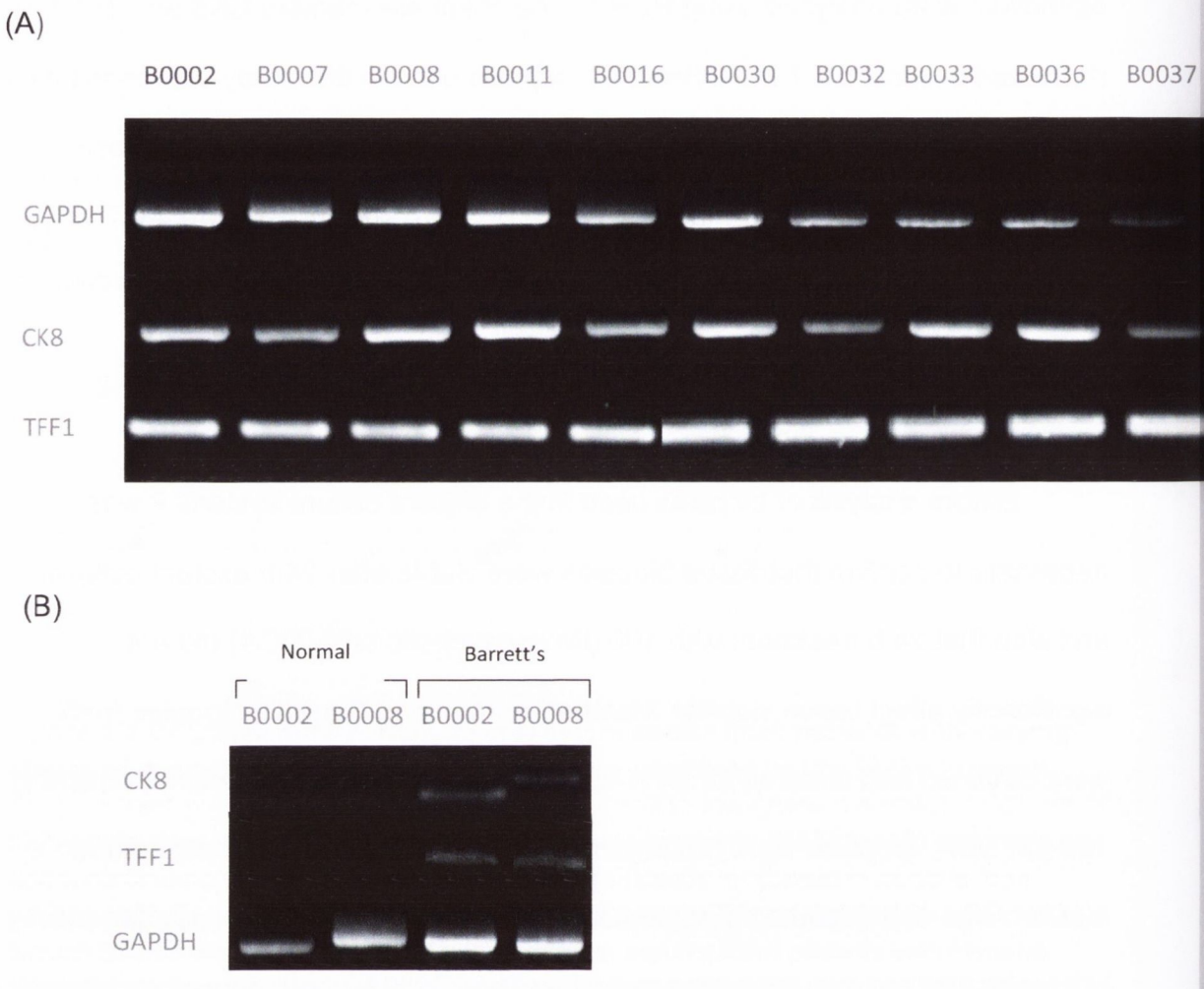


Figure 4.2 Barrett's biopsies, but not matched normal biopsies, express the columnar markers CK8, TSPAN and TFF1. Expression of the columnar markers CK8 and TFF1, along with the housekeeping gene GAPDH, was measured in normal and Barrett's biopsies by RT-PCR. Representative agarose gel images are shown from (A) Barrett's biopsies and (B) matched normal and Barrett's biopsies.

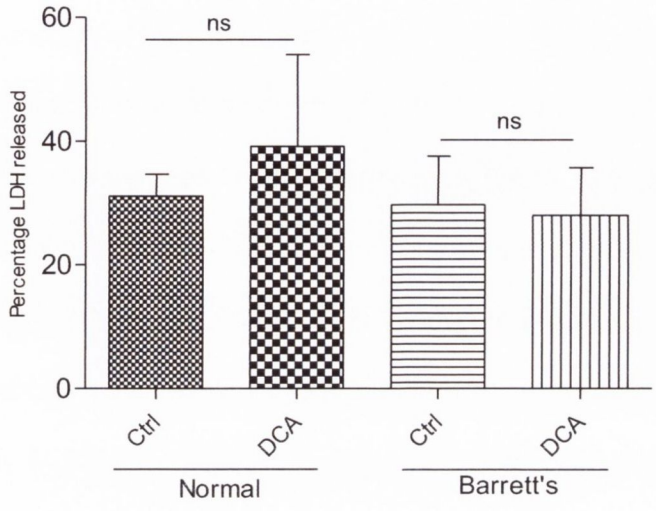


Figure 4.3 Treatment with 100 μM DCA for 24 h does not affect explant tissue viability in normal or Barrett's biopsies. Normal and Barrett's tissue biopsies were cultured for 24 h in either medium alone or in medium containing 100 μM DCA. LDH released into the culture medium was measured and expressed as a percentage of total LDH in the tissue biopsy. There were no significant differences between LDH released from biopsies cultured in medium alone and biopsies cultured in medium with DCA. Data are presented as mean ± SEM. Analysis was performed using the paired Students *t*-test.

4.3.5 Gene expression of *CCL28, IL-1β* and *TNF-α* is significantly increased in Barrett's tissue compared with matched normal tissue

Having established that biopsies were viable in culture and contained columnar Barrett's epithelium, analysis of gene expression in biopsies was carried out. Endoscopic biopsies from matched normal and BO tissue were obtained from 20 patients with BO who had consented to be part of the Barrett's biobank. Characteristics of these patients are shown in Table 4.2. After 24 h culture, biopsies were stored in RNA-*later*™ and RNA was extracted as described in section 2.7.7. cDNA was synthesised and gene expression of *CCL28, IL-1β* and *TNF-α* was measured by qPCR. Gene expression in BO tissue was measured as relative fold change compared with matched normal tissue. Gene expression of *CCL28* was significantly upregulated in BO tissue compared with matched normal tissue ($p < 0.0001$ vs. Normal) (mean relative fold change 15.68 +/- 2.97) (Figure 4.4). Gene expression of *IL-1β* was also significantly increased in Barrett's tissue compared with matched normal tissue ($p < 0.0001$ vs. Normal) (mean relative fold change 21.87 +/- 5.91), as was *TNF-α* (*TNF-α* $p = 0.0419$ vs. Normal) (mean relative fold change 3.58 +/- 1.04).

4.3.6 Barrett's tissue conditioned medium contains increased CCL28, IL-1β and TNF-α soluble protein compared with normal tissue conditioned medium

Having examined CCL28, IL-1β and TNF-α at a gene expression level, these molecules were then investigated at a protein level, along with the additional inflammatory cytokines IL-6 and IFN-γ. As cytokines and chemokines are released as soluble proteins into the extracellular space and circulation, soluble protein

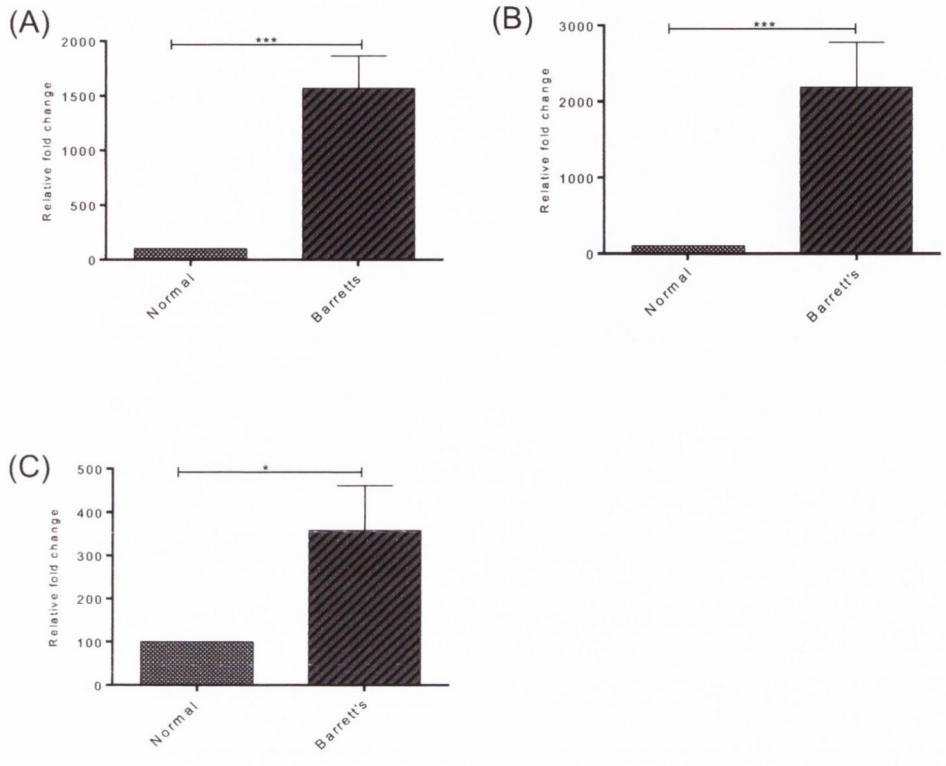


Figure 4.4 Gene expression of *CCL28, IL-1β* and *TNF-α* is significantly increased in Barrett's tissue compared with matched normal tissue (n=20). Gene expression of *CCL28, IL-1β* and *TNF-α* was measured using qPCR in matched normal and Barrett's tissue obtained at endoscopy from 20 patients with Barrett's oesophagus. Gene expression of (A) *CCL28*, (B) *IL-1β* and (C) *TNF-α* was significantly upregulated in Barrett's tissue biopsies compared with matched normal tissue biopsies. Data are expressed as mean ± SEM. Analysis was performed using the Wilcoxon signed-rank *t*-test. *p = 0.0419 vs. Normal, ***p < 0.0001 vs. Normal.

expression was measured from the cultured explants. Matched normal and BO biopsies from 20 patients with BO were cultured in medium for 24 h to generate conditioned media, and soluble protein levels of CCL28, IL-1β, TNF-α, IL-6 and IFN-γ were measured using ELISA or protein multiplex assay as described in sections 2.5.2 and 2.5.3.

It was found that CCL28 protein showed a trend towards increased expression in Barrett's conditioned media compared with matched normal conditioned media ($p = 0.13$ vs. Normal) (mean protein concentration 0.45 +/- 0.23 pg/μg vs. 0.06 +/- 0.03 pg/μg) (Figure 4.5). IL-1β protein was significantly increased in Barrett's conditioned media compared with normal conditioned media ($p = 0.0002$ vs. Normal) (mean protein concentration 3.27 +/- 0.66 pg/μg vs. 0.21 +/- 0.16 pg/μg), as was TNF-α protein ($p = 0.0054$ vs. Normal) (mean protein concentration 2.55 +/- 0.38 pg/μg vs. 1.23 +/- 0.47 pg/μg).

The inflammatory cytokines IL-6 and IFN-γ were also measured on the same multiplex protein assay as IL-1β and TNF-α. IL-6 protein was significantly increased in Barrett's conditioned media compared with normal conditioned media ($p = 0.0001$ vs. Normal) (mean protein concentration 21.59 +/- 8.01 pg/μg vs. 1.14 +/- 0.46 pg/μg), as was IFN-γ protein ($p = 0.0001$ vs. Normal) (mean protein concentration 0.40 +/- 0.12 pg/μg vs. 0.03 +/- 0.01 pg/μg) (Figure 4.6).

4.3.7 CCL28 protein expression correlates with IL-1β and TNF-α protein expression in Barrett's conditioned medium

It has previously been demonstrated that CCL28 expression is regulated by the pro-inflammatory cytokines IL-1β and TNF-α in other tissue types (O'Gorman,

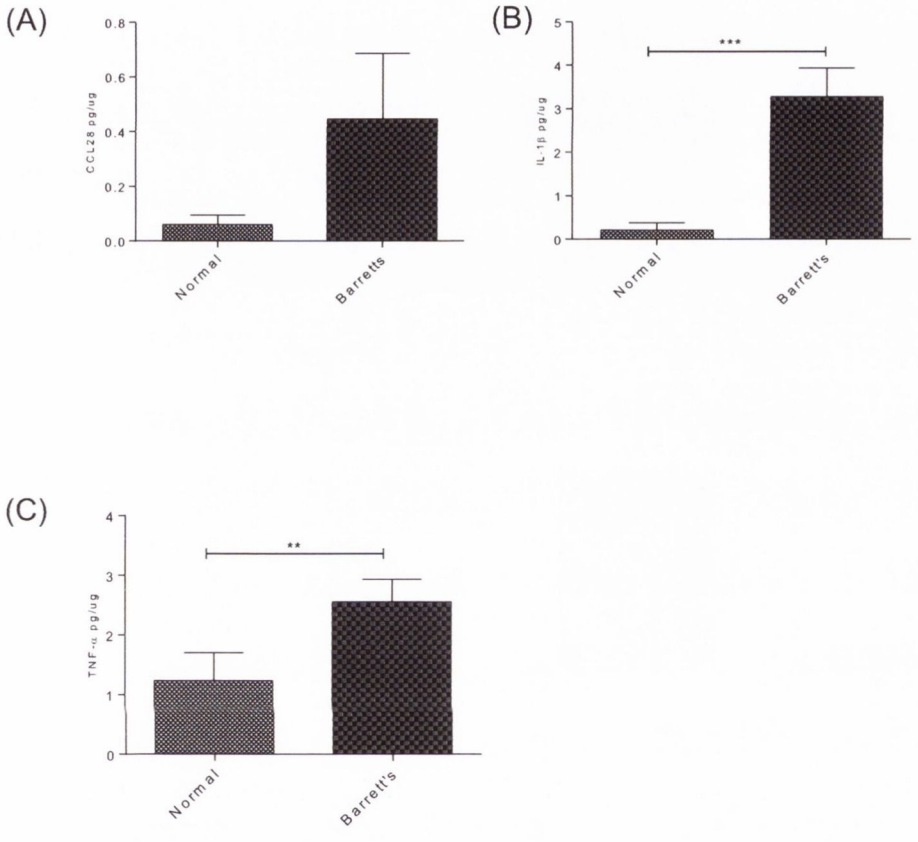


Figure 4.5 Barrett's tissue conditioned medium contains increased CCL28, IL-1β and TNF-α soluble protein compared with normal tissue conditioned medium. Matched normal and Barrett's biopsies from 20 patients with Barrett's oesophagus were cultured in medium for 24 h to generate conditioned medium. Protein concentration of CCL28, IL-1β and TNF-α was measured using ELISA or protein multiplex assay. (A) CCL28 soluble protein demonstrated a trend towards increased expression in Barrett's conditioned medium compared with normal conditioned medium ($p = 0.13$ vs. Normal). (B) IL-1β and (C) TNF-α soluble protein was significantly increased in Barrett's conditioned medium compared with normal conditioned medium. Data are expressed as mean ± SEM. Analysis was performed using the Wilcoxon signed-rank *t*-test. **$p = 0.0054$ vs. Normal, ***$p = 0.0002$ vs. Normal.

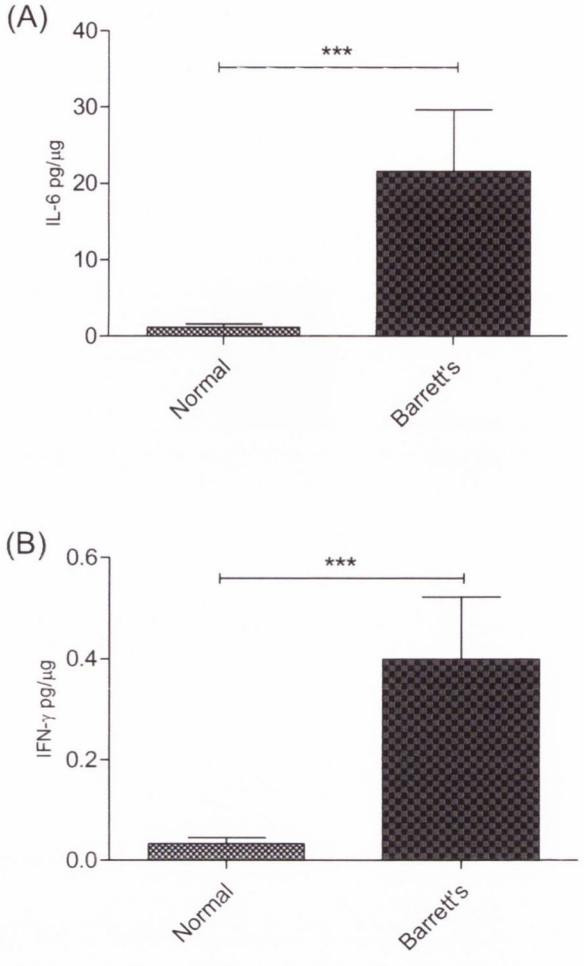


Figure 4.6 Barrett's conditioned medium contains increased IL-6 and IFN-γ protein compared with matched normal conditioned medium (n=20). Matched normal and Barrett's biopsies from 20 patients with Barrett's oesophagus were cultured in medium for 24 h to generate conditioned medium. Protein concentration of IL-6 and IFN-γ was measured using a protein multiplex assay. (A) IL-6 soluble protein secretion was increased in Barrett's conditioned medium compared with normal conditioned medium. (B) IFN-γ soluble protein was significantly increased in Barrett's conditioned medium compared with normal conditioned medium. Data are expressed as mean ± SEM. Analysis was performed using the Wilcoxon signed-rank *t*-test. ***p = 0.0001 vs. Normal.

Jatoi et al. 2005, Eksteen, Miles et al. 2006, Shibata, Maeda et al. 2010). Therefore it was hypothesised that this may be the case in the oesophagus. Protein concentrations of CCL28 secreted from BO tissue biopsies were correlated with the protein concentrations of IL-1β and TNF-α from the same biopsies. CCL28 protein secretion from BO tissue was found to correlate with secretion of both IL-1β and TNF-α, with a stronger correlation noted with IL-1β (IL-1β $R=0.8446$, $p<0.001$, TNF-α $R=0.5415$, $p=0.0137$) (Figure 4.7).

4.3.8 Deoxycholic acid treatment increases gene expression of *CCL28, IL-1β* and *TNF-α* in normal tissue but not in matched Barrett's tissue

The effect of DCA on gene expression of *CCL28*, *IL-1β* and *TNF-α* was also examined in normal and BO tissue explants. DCA increased gene expression of *CCL28, IL-1β* and *TNF-α* in normal tissue *(CCL28* p=0.0239, fold change 3.45 +/- 1.35, *IL-1β* p=0.054, fold change 3.14 +/- 0.96, *TNF-α* p=0.003, fold change 3.01 +/- 0.63) (Figure 4.8). Treatment with DCA had no effect on gene expression in matched BO tissue.

4.3.9 Protein secretion of CCL28 and IL-1β is increased by deoxycholic acid treatment in normal tissue conditioned medium, but not in Barrett's tissue conditioned medium

The effect of DCA treatment on soluble CCL28, IL-1β and TNF-α protein secretion was measured. DCA increased IL-1β protein secretion in normal tissue (p=0.002, 0.74 +/- 0.16

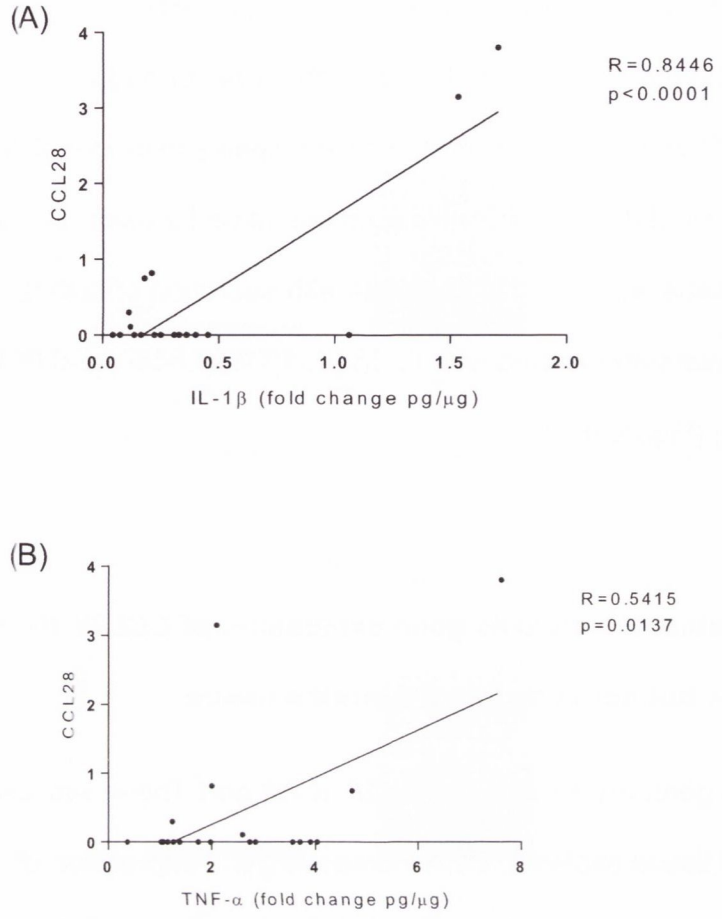


Figure 4.7 CCL28 protein expression correlates with IL-1β and TNF-α protein expression in Barrett's conditioned medium. Barrett's tissue biopsies from 20 patients with Barrett's oesophagus were incubated in medium for 24 h to generate Barrett's conditioned medium. Protein expression of CCL28, IL-1β and TNF-α was measured in this medium using ELISA and protein multiplex assay. (A) CCL28 protein expression correlates with IL-1β protein expression in Barrett's conditioned medium. (B) CCL28 protein expression correlates with TNF-α protein expression in Barrett's conditioned medium. Analysis was performed using the Pearson correlation test.

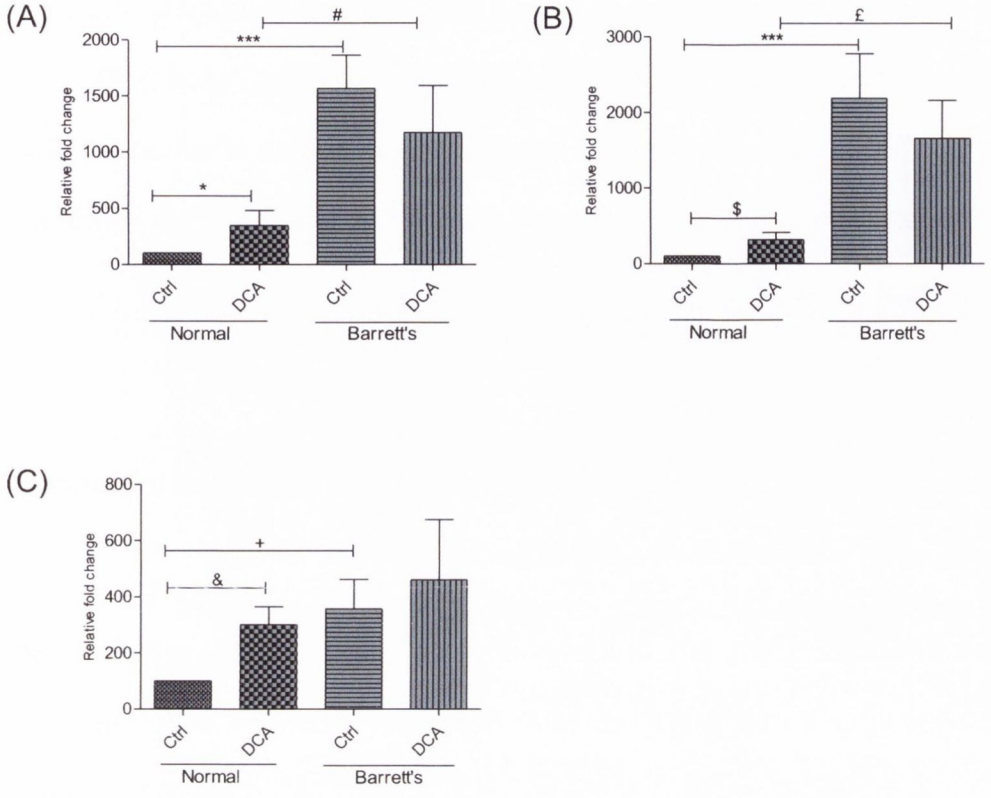


Figure 4.8 Deoxycholic acid treatment increases gene expression of *CCL28, IL-1β* and *TNF-α* in normal tissue but not in matched Barrett's tissue (n=20). Matched normal and Barrett's tissue biopsies from 20 patients with Barrett's oesophagus were incubated for 24 h in either medium alone or medium containing 100 μM deoxycholic acid (DCA). RNA was isolated and gene expression of *CCL28, IL-1β* and *TNF-α* was measured using qPCR. (A) Gene expression of *CCL28* in DCA-treated normal tissue was increased compared with untreated normal tissue. No difference in *CCL28* gene expression was observed between untreated and DCA-treated Barrett's tissue. *CCL28* gene expression was increased in DCA-treated Barrett's tissue compared with DCA-treated normal tissue. (B) Gene expression of *IL-1β* in DCA-treated normal tissue was increased compared with untreated normal tissue. No difference in *IL-1β* gene expression was observed between untreated and DCA-treated Barrett's tissue. *IL-1β* gene expression was increased in DCA-treated Barrett's tissue compared with DCA-treated normal tissue. (C) Gene expression of *TNF-α* in DCA-treated normal tissue was increased compared with untreated normal tissue. No difference in *TNF-α* gene expression was observed between untreated and DCA-treated Barrett's tissue. Data are expressed as mean ± SEM. Analysis was performed using the Wilcoxon signed-rank *t*-test. *$p = 0.0239$ vs. Normal Ctrl, ***$p < 0.0001$ vs. Normal Ctrl, $^{\#}p = 0.0166$ vs. Normal DCA, $^{\$}p = 0.0545$ vs. Normal Ctrl, $^{£}p = 0.0069$ vs. Normal DCA, $^{\&}p = 0.0030$ vs. Normal Ctrl, $^{+}p = 0.0419$ vs. Normal Ctrl.

pg/μg vs. 0.21 +/- 0.16 pg/μg) (Figure 4.9). A trend towards increased CCL28 protein expression in normal tissue treated with DCA was also noted (p=0.1, 0.47 +/- 0.22 pg/μg vs. 0.06 +/- 0.03 pg/μg). However, DCA had no effect on CCL28 or IL-1β protein secretion from BO tissue, and also had no effect on TNF-α protein secretion in either tissue type.

4.3.10 Gene expression of *CCL28, IL-1β* and *TNF-α* is increased in tumour tissue compared with matched normal tissue

Matched normal and tumour tissue biopsies from 5 patients with OAC who had consented to be part of this study were incubated for 24 h in either medium alone or medium containing 100 μM deoxycholic acid (DCA). After 24 h culture, biopsies were stored in RNA-*later*™ and RNA was extracted as described in section 2.7.7. cDNA was synthesised and gene expression of *CCL28, IL-1β* and *TNF-α* was measured using qPCR. Gene expression in tumour tissue was measured as relative fold change compared with matched normal tissue. Gene expression of *CCL28* in tumour tissue trended towards an increase compared with matched normal tissue (p = 0.0775 vs. Normal) (Mean relative fold change 3.20 ± 0.9309) (Figure 4.10). No alterations in *CCL28* gene expression were observed with DCA treatment of normal or tumour tissue. Gene expression of *IL-1β* also trended towards increased expression in tumour tissue compared with matched normal tissue (p = 0.1250 vs. Normal), but no difference in *IL-1β* gene expression was observed with DCA treatment. *TNF-α* demonstrated a trend towards increased gene expression in tumour tissue compared with matched normal tissue (p = 0.0774 vs. Normal) (Mean relative fold

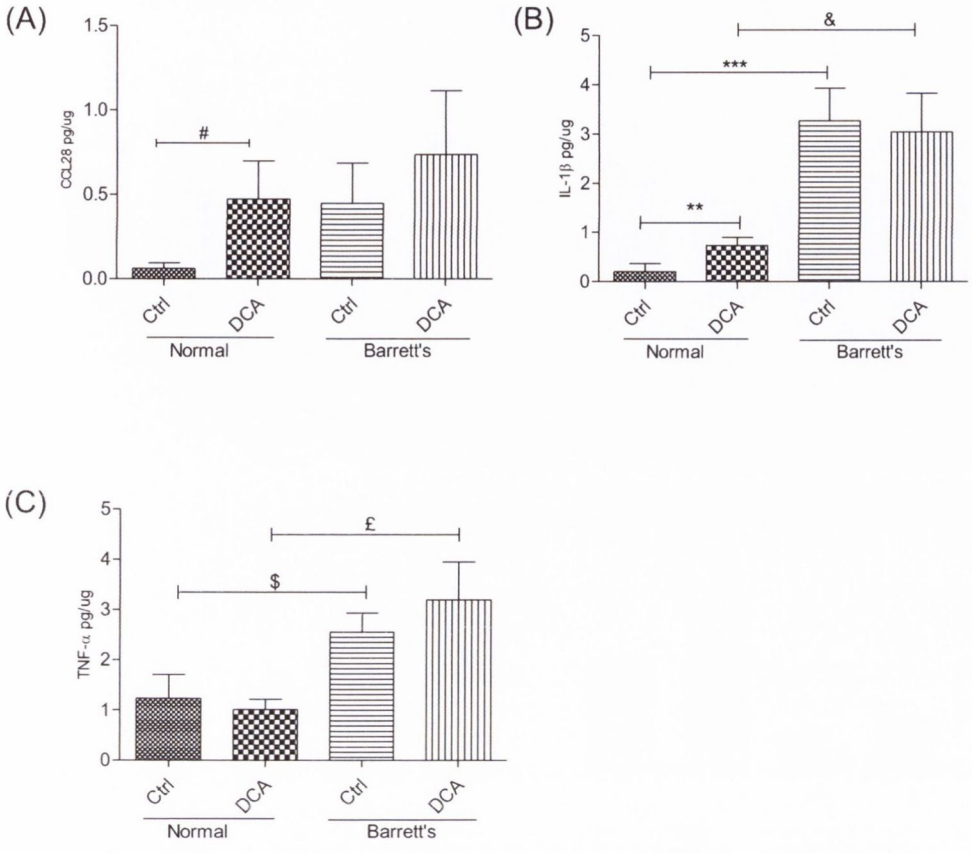


Figure 4.9 Protein secretion of CCL28 and IL-1β is increased by deoxycholic acid treatment in normal tissue conditioned medium, but not in Barrett's tissue conditioned medium (n=20). Matched normal and Barrett's tissue biopsies from 20 patients with Barrett's oesophagus were incubated in either medium alone or medium containing 100 μM deoxycholic acid (DCA) for 24 h in order to generate conditioned medium. Protein expression of CCL28, IL-1β and TNF-α was measured in the conditioned medium using ELISA or protein multiplex assay. (A) Protein secretion of CCL28 from normal tissue was increased by treatment with DCA. DCA treatment had no effect on CCL28 protein secretion from Barrett's tissue. (B) Protein secretion of IL-1β from normal tissue was increased by treatment with DCA. DCA treatment had no effect on IL-1β protein secretion from Barrett's tissue. (C) DCA treatment did not alter TNF-α protein secretion from either normal or Barrett's tissue. TNF-α protein secretion was increased from DCA-treated Barrett's tissue compared with DCA-treated normal tissue. Data are expressed as mean ± SEM. Analysis was performed using the Wilcoxon signed-rank *t*-test. $^{\#}p = 0.1094$ vs. Normal Ctrl, $^{**}p = 0.0021$ vs. Normal Ctrl, $^{***}p = 0.0002$ vs. Normal Ctrl, $^{\&}p = 0.0008$ vs. Normal DCA, $^{\$}p = 0.0054$ vs. Normal Ctrl, $^{£}p = 0.0006$ vs. Normal DCA.

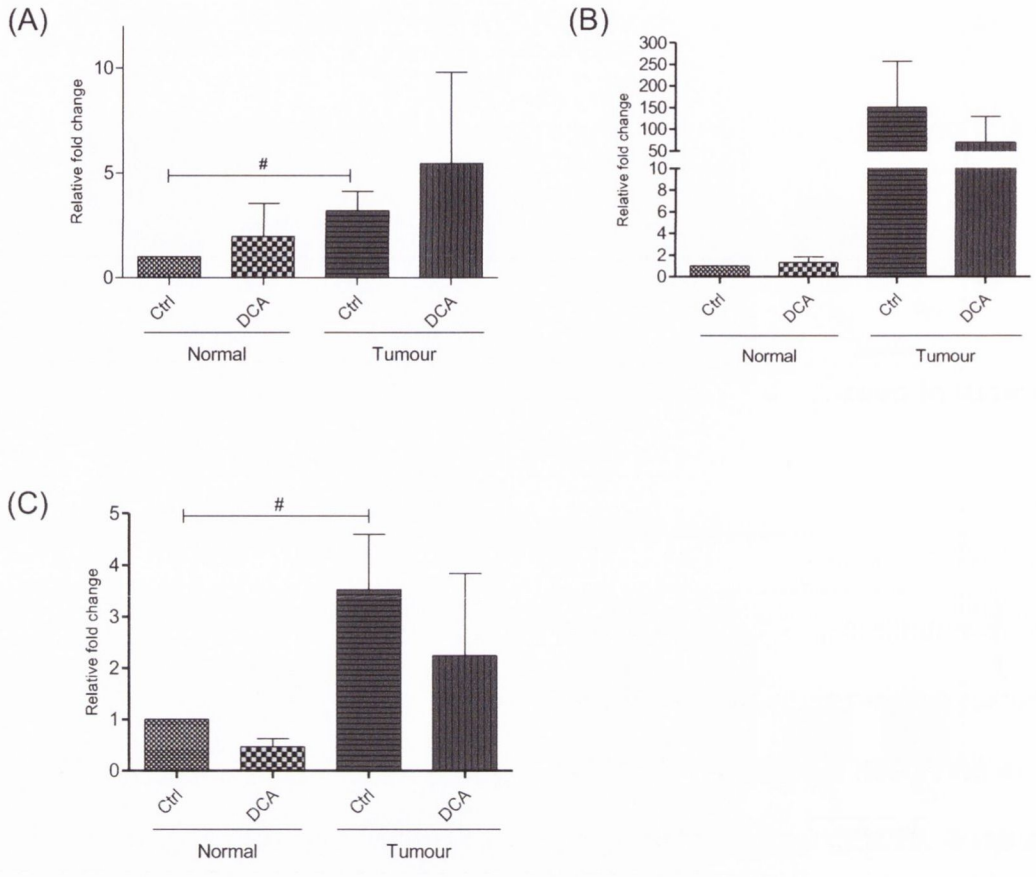


Figure 4.10 Gene expression of *CCL28, IL-1β* and *TNF-α* is increased in tumour tissue compared with matched normal tissue (n=5). Matched normal and tumour tissue biopsies from 5 patients with oesophageal adenocarcinoma were incubated for 24 h in either medium alone or medium containing 100 μM deoxycholic acid (DCA). RNA was isolated and gene expression of *CCL28, IL-1β* and *TNF-α* was measured using qPCR. (A) Gene expression of *CCL28* in tumour tissue was increased compared with matched normal tissue. No difference in *CCL28* gene expression was observed with DCA treatment of normal or tumour tissue. (B) Gene expression of *IL-1β* demonstrated a trend towards increased expression in tumour tissue compared with matched normal tissue. No difference in *IL-1β* gene expression was observed between untreated and DCA-treated normal or tumour tissue. (C) Gene expression of *TNF-α* in tumour tissue was increased compared with matched normal tissue. No difference in *TNF-α* gene expression was observed with DCA treatment in either normal or tumour tissue. Data are expressed as mean ± SEM. Analysis was performed using the Wilcoxon signed-rank *t*-test. $^{\#}p = 0.0775$ vs. Normal.

change 3.53 ± 1.07), but again, DCA did not affect *TNF-α* expression in either normal or tumour tissue.

4.3.11 CCL28 protein secretion from matched normal and oesophageal adenocarcinoma tumour tissue demonstrates no significant differences, either basally or in response to deoxycholic acid treatment

Matched normal and tumour tissue biopsies from 5 patients with OAC were incubated for 24 h in either medium alone or medium containing 100 μM deoxycholic acid (DCA). Protein secretion of CCL28 into the conditioned medium was measured using ELISA. There was no significant difference between CCL28 protein expression in normal conditioned medium and in tumour conditioned medium (Figure 4.11). Treatment with DCA did not significantly affect CCL28 protein expression in either normal or tumour tissue.

4.4 Discussion

Biobanking is increasingly being recognised as an area of research that requires much input and planning, both from a clinical and a scientific perspective. The establishment of a biobank for a particular tissue type involves obtaining appropriate ethical approval for the collection of specimens, and involvement of the clinical staff, in this case in the St. James's Hospital Endoscopy Unit, the largest of its kind in Europe. Once this has been achieved, a protocol for collection and processing of specimens must be made and distributed to the appropriate individuals, in order to standardise the procedure and ensure consistent practices

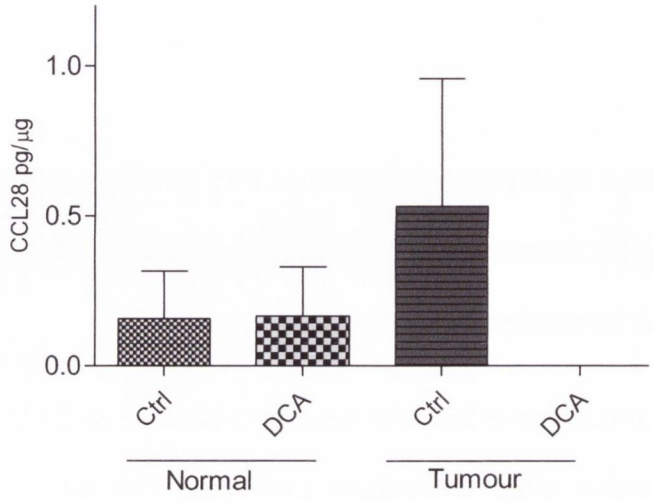


Figure 4.11 CCL28 protein secretion from matched normal and oesophageal adenocarcinoma tumour tissue demonstrates no significant differences, either basally or in response to deoxycholic acid treatment. Matched normal and tumour tissue biopsies from 5 patients with oesophageal adenocarcinoma were incubated for 24 h in either medium alone or medium containing 100 µM deoxycholic acid (DCA). Protein secretion of CCL28 into the conditioned medium was measured using ELISA. There was no significant difference between CCL28 protein expression in normal conditioned medium and in tumour conditioned medium. Treatment with DCA did not affect CCL28 protein expression in either normal or tumour tissue. Data are expressed as mean ± SEM.

and results. The BO biobank was set up during the course of this study. Once ethical approval had been obtained from the hospital ethical board, the staff of the Endoscopy Unit were informed about the procedure for collection of specimens. A consent form and patient information leaflet were drawn up and printed, and patients were identified from the database who had previously been diagnosed with BO. When these patients returned for their routine endoscopy, they were consented for the study and enrolled into the BO biobank. Biopsy specimens were collected immediately after endoscopy and were immediately transferred to the laboratory on saline-soaked gauze for experimental work. Samples were processed within 30 min for RNA work.

Characteristics of the patients recruited to the BO biobank in the first year are similar to those of other BO patient populations in the literature (Gaddam, Singh et al. 2013, Solaymani-Dodaran, Card et al. 2013). The majority (75%) of patients were male and had an average age of 60.98 years, which are consistent with other reported studies on demographics in BO patients. The majority of patients were on proton pump inhibitors to decrease gastro-oesophageal reflux, which is a standard treatment for patients with BO.

Chemokines are released from tissue into the tissue microenvironment where they exert their chemotactic effects. Therefore, in order to fully study a chemokine, the cell culture system of oesophageal cells is insufficient, as this is a pure population of epithelial cells only. To examine the chemokine CCL28 using a method more representative of the actual oesophageal microenvironment, a tissue explant culture system was established in this chapter. This system has been recognised as a more appropriate way to study cytokines and chemokines which may be secreted from and may exert their effects on the entire oesophageal

microenvironment, not just on the epithelial cells. Previous studies have used the explant culture system both in the oesophagus (Fitzgerald, Omary et al. 1996, Mariette, Piessen et al. 2008) and in other tissue types (Hackett, Scarci et al. 2010, Eigeliene, Elo et al. 2012, Muthuswamy, Berk et al. 2012) to mimic the environment of a tumour. In this study, we used tissue explants from patients with BO – matched normal and BO tissue were taken at routine surveillance endoscopy. We also obtained tissue explants from tumour tissue, taking matched normal and tumour biopsies. These biopsies were confirmed to be viable after 24 h tissue culture and were confirmed to be BO or tumour tissue both histologically and using columnar PCR markers.

Initially levels of CCL28 in the sera of patients from varying stages of oesophageal disease progression were examined. Interestingly, CCL28 in serum was increased in patients with BO and in patients with invasive OAC, compared with those with LGD, HGD and intramucosal carcinoma. Normal patients had lower serum CCL28 than patients with BO, although this did not reach significance ($p = 0.15$), and had a significantly lower serum CCL28 than patients with invasive OAC. Many chemokines and cytokines have been found to be increased in serums of patients with tumours compared with normal controls, including IL-1, IL-6 and IL-8 (Kaminska, Nowacki et al. 2005, Kohga, Tatsumi et al. 2012, Xiao, Chen et al. 2013). These levels are often, but not always found to correlate with levels in the tissue microenvironment of the tumour (Khar, Muralikrishna et al. 1997, Hartmann, Dwyer et al. 2011). It was interesting that serum CCL28 was lower in patients with LGD, HGD and IMC – these patients are in an intermediate group between the patients with BO, which, being a metaplastic, adaptive state, do not have neoplastic tissue, and those with invasive OAC, which is a neoplastic tissue.

Patients in the pre-neoplastic stages of LGD and HGD may secrete smaller amounts of CCL28 protein into the vasculature, suggesting that the patients who progress to LGD and HGD have a different immune profile, expressing lower CCL28, than patients who have Barrett's metaplasia only. As it is known that BO may be derived from a number of small clonal populations and that therefore BO tissue is mosaical in nature (Nicholson, Graham et al. 2012) , it may be that, in LGD and HGD, clonal populations that secrete lower levels of CCL28 predominate, while cell types that secrete increased CCL28 are involved in invasive OAC. Alternatively, these patients with LGD and HGD may produce CCL28, but the chemokine may exert its effects only within the oesophageal microenvironment, and so may not be released into the circulation. Since chemokines are released from cells and exert a local chemotactic effect in the tissue in which they are released, it is hypothesised that, while circulating levels of CCL28 in these patient groups vary, the levels of CCL28 specifically within the oesophageal tissue microenvironment may be of more importance and more reflective of the role played by this chemokine. CCL28 may be expressed locally in the oesophageal microenvironment, and may exert its effect within the tissue, in an altered manner to its systemic effects by dispersion throughout the vasculature. This was examined using the *ex vivo* explant culture system.

CCL28 expression in oesophageal tissue biopsies was examined using an *ex vivo* explant culture model in order to examine the entire oesophageal microenvironment, including the epithelium and the stroma. Interestingly, protein secretion of CCL28 was seen from normal tissue, although at low levels. This was an unexpected finding, as CCL28 is usually expressed in columnar tissue at mucosal surfaces. This may be because the normal tissue obtained from patients

with BO may not be "normal" squamous tissue, as the oesophagus from these patients has been chronically exposed to refluxate and the more distal parts of the oesophagus have already undergone metaplastic change to columnar Barrett's epithelium. Capello et al (2008) have previously found that squamous epithelium from Barrett's patients is dissimilar to that of healthy patients in terms of various markers, including bile acid receptors, such as FXR (Capello, Moons et al. 2008); therefore it may be that the normal tissue biopsies in this study were more responsive to bile acid treatment since they were obtained from patients with BO. CCL28 could also be produced by immune cells within the stroma of the normal tissue biopsies, as it has been shown to be expressed by dendritic cells (Khan and Grayson 2010).

BO tissue expressed greater *CCL28* mRNA and showed a trend towards increased CCL28 protein secretion than matched normal tissue. This suggests that CCL28 expression increases during the progression from squamous oesophageal tissue to columnar BO tissue. Since CCL28 is an immunomodulatory chemokine, it is hypothesised that during progression to BO, CCL28 alters the immune microenvironment of the oesophagus, possibly promoting this progression. Within the cohort of BO tissue explants examined, not all of these expressed CCL28 soluble protein. This may identify a subset of patients, expressing CCL28, who may be at higher risk of progression to OAC, as we know from a previous study from our group that expression of CCL28 in OAC confers a worse prognosis (Maher, Gillham et al. 2009). This subset of patients may have an altered immune environment as CCL28 may chemoattract a different population of immune cells.

It is known that in other tissue types, CCL28 expression is regulated by the pro-inflammatory cytokines IL-1β and TNF-α. It was hypothesised that this might

be the case in the oesophagus, and that expression of these cytokines may correlate with expression of CCL28. In this explant culture model, BO tissue expressed more IL-1β and TNF-α than matched normal tissue, at both a gene and a protein level, and also expressed more IL-6 and IFN-γ protein. It was also found that in BO tissue, expression of CCL28 correlated with expression of IL-1β and TNF-α protein, suggesting that these cytokines may be involved in regulation of CCL28, as they are in airway epithelium and in cholangiocytes (O'Gorman, Jatoi et al. 2005, Eksteen, Miles et al. 2006).

When the response of tissue explants to DCA treatment was examined, it was found that in normal tissue, DCA increased expression of *CCL28, IL-1β* and *TNF-α* at a gene level, and increased CCL28 and IL-1β protein secretion. However, in BO tissue DCA had no effect on expression of these factors, at either a gene or a protein level. This suggests that normal tissue from these patients is more susceptible to the effects of DCA, while columnar BO tissue, thought to be an adaptive mechanism in response to exposure to refluxate, is more resistant to the effects of DCA. While DCA appears to increase the expression of CCL28 and IL-1β in normal tissue, it may be that in BO tissue, the response to DCA is saturated and the tissue cannot produce increases in CCL28 or IL-1β. It is known that exposure to DCA plays a major role in the progression to BO (Burnat, Majka et al. 2010); therefore this data suggests that in normal tissue, DCA induces CCL28, possibly regulated by IL-1β and TNF-α. Secretion of CCL28 may modulate the immune microenvironment of the oesophagus to promote progression to BO, which is more resistant to the effects of DCA exposure.

In tumour tissue, the number of patient samples obtained limits conclusions that can be drawn. However *CCL28* does appear to be increased at a gene

expression level in tumour tissue compared with matched normal tissue, while at a protein level CCL28 secretion was seen, although no significant differences were seen either basally or with DCA treatment. The levels of CCL28 protein secretion from BO and tumour tissue appear to be similar, again suggesting that CCL28 expression may be an early change in oesophageal disease progression.

In summary, this chapter demonstrates the expression of CCL28 by normal, BO and oesophageal tumour tissue from human oesophageal biopsies, using an *ex vivo* explant culture model. CCL28 expression is increased in BO tissue compared with matched normal tissue at both a gene and a protein level. CCL28 expression correlates with expression of the cytokines IL-1β and TNF-α, supporting the hypothesis that CCL28 may be regulated via these cytokines. The bile acid deoxycholic acid (DCA) induces expression of CCL28, as well as IL-1β and TNF-α, in normal oesophageal tissue, suggesting that gastro-oesophageal reflux may induce CCL28 as an early event, possibly even before the development of BO. Serum CCL28 levels vary between stages of oesophageal disease progression, with the intermediate stages of low-grade and high-grade dysplasia having lower serum CCL28 than patients with BO and invasive OAC. This may suggest that patients who progress from BO have a different immune profile to those who do not, having a lower serum CCL28 than those who have intestinal metaplasia only.

In the next chapter, the functional properties of CCL28 in the oesophagus are examined, in order to further elucidate the regulation and role of CCL28 expression in oesophageal disease.

Chapter 5

Regulation and function of CCL28 in the oesophagus

5.1 Introduction

Chemokines are molecules that are among the most potent stimuli for leucocyte migration in the body and therefore are a fundamental part of the inflammatory process. Each of the more than 50 known chemokines binds to a G-protein coupled receptor, of which 20 have been identified; however, many chemokines may bind to one receptor, and equally one chemokine may bind to many receptors, suggesting a very high degree of complexity within this system (Rossi and Zlotnik 2000, Zlotnik and Yoshie 2000). Chemokines are pleiotropic molecules, having effects on many different types of cells. After binding to their receptors, they induce signalling cascades in the cell, which lead to alterations in cell motility, as well as other functions, such as angiogenesis and proinflammatory cytokine production (Carmi, Voronov et al. 2009, Barbieri, Bajetto et al. 2010, Facciabene, Peng et al. 2011). Infiltration of immune cells into tissues has the potential to exert tissue damage; therefore, the induction and regulation of chemokine expression is of importance in controlling when and where the chemokine is secreted in order to exert its immunomodulatory effects (Bailey, Negus et al. 2007, Muthuswamy, Berk et al. 2012).

Inflammation is a complex process involving the activation of many key transcription factors, which in turn increase or decrease various signalling pathways to promote inflammatory processes, such as expression of cytokines, chemokines and other factors such as matrix metalloproteinases (MMPs) and reactive oxygen species (Deans, Wigmore et al. 2006, Ardi, Kupriyanova et al. 2007, Germano, Allavena et al. 2008, Sethi, Shanmugam et al. 2012). In the oesophageal disease sequence, various transcription factors have been identified as being upregulated during the progression to OAC, some directly in response to

bile acid exposure, including NF-kB, Cdx-1 and -2, hepatocyte nuclear factors, RUNX3 and KLF4 (Eda, Osawa et al. 2002, Lin, Miller et al. 2002, Moons, Bax et al. 2004, Schulmann, Sterian et al. 2005, Kazumori, Ishihara et al. 2006, Piessen, Jonckheere et al. 2007, Chen, Fang et al. 2011, Dvorak, Goldman et al. 2011, Kazumori, Ishihara et al. 2011). DCA itself is a potent activator of transcription factors, such as Cdx-2 and NF-kB (Burnat, Majka et al. 2010, Huo, Juergens et al. 2011, McAdam, Haboubi et al. 2012, Tamagawa, Ishimura et al. 2012). NF-kB is considered to be a primary inflammatory transcription factor as it is activated by pro-inflammatory cytokines, such as IL-1β and TNF-α, via the classical pathway, as well as by mitogens, lipopolysaccharide, bacteria and viruses (Wong and Tergaonkar 2009, Madge and May 2011). It also plays a major role in the tumourigenic process, as it promotes cell survival and proliferation via its downstream targets, which number over 400 and include genes involved in inflammation, immunoregulation, tumour cell genesis and proliferation, angiogenesis, chemoresistance, invasion and metastasis (Wong and Tergaonkar 2009, Li and Sethi 2010, Mantovani 2010, Sethi, Shanmugam et al. 2012). CCL28 has previously been demonstrated to be regulated via NF-kB in other tissue types (O'Gorman, Jatoi et al. 2005, Kagami, Saeki et al. 2006), as well as by the transcription factor HIF-1α, which is induced by hypoxia (Facciabene, Peng et al. 2011).

Chemokines function as small secreted proteins that are released into extracellular spaces and into the vasculature (Rossi and Zlotnik 2000). As they are secreted, a gradient is created, which chemoattracts immune cells bearing the appropriate receptors towards the source of chemokine production. When bound to their receptors, all of which are G-protein-coupled receptors, chemokines trigger

intracellular pathways causing chemotaxis, degranulation and alterations in integrin expression on cell surfaces (Conti, Pang et al. 1997, Hieshima, Kawasaki et al. 2004, Bailey, Negus et al. 2007). CCL28 binds to the G-protein-coupled receptors CCR3 and CCR10, which are found on several types of immune cells, including CD4+ and CD8+ T cells, Treg cells, IgA and IgE plasma cells, dendritic cells, eosinophils and basophils (Pan, Kunkel et al. 2000, Morteau, Gerard et al. 2008, Scanlon, Hawksworth et al. 2011, Sisirak, Vey et al. 2011). It has been demonstrated to be chemotactic in various tissue types, including ovarian, gastric and liver tissue, for cells bearing these receptors (Eksteen, Miles et al. 2006, Hansson, Hermansson et al. 2008, Facciabene, Peng et al. 2011), via activation of the α_4-β_7-integrin molecule expressed on lymphocytes causing adhesion to mucosal addressin cell adhesion molecule-1 (MAdCAM-1) on vascular endothelium (Bourges, Wang et al. 2004, Miles, Liaskou et al. 2008). Assessment of the functionality of chemokines is frequently performed using a transwell chemotaxis system, where the chemotaxis of immune cells in an upper chamber through a porous membrane towards a chemokine in a lower chamber is measured (Scanlon, Hawksworth et al. 2011, Lee, Kim et al. 2012, Maeda, Ohno et al. 2012, Zhou, Wang et al. 2012).

Having demonstrated that CCL28 is induced in oesophageal cell lines by the bile acid DCA in Chapter 3, here the transcription factors that may regulate DCA-induced CCL28 in oesophageal cells are investigated. Transcription factors may provide an alternative therapeutic target in order to neutralise the effects of a downstream target, such as CCL28. It is also examined whether the CCL28 secreted by oesophageal cells and tissue is functional, i.e. whether it is

chemotactic for immune cells and can therefore modulate the immune environment of the oesophagus.

5.2 Aims and objectives

Having established that CCL28 is expressed both in vitro by oesophageal cell lines and ex vivo by oesophageal tissue explants, this chapter explores the transcription factors by which CCL28 is regulated and also whether the CCL28 expressed by oesophageal cells and tissue is functional. Since CCL28 is induced by deoxycholic acid (DCA), the transcription factors induced by DCA which may regulate CCL28 are explored and validated. Chemokines play a role in chemotaxis of immune cells, and so the immune cells responding to CCL28 expressed by the oesophagus are examined.

Specific objectives:

- Identify transcription factors activated by DCA, which may regulate CCL28
- Validate transcription factors in oesophageal cells
- Examine immune cells chemoattracted by CCL28 expressed by the oesophagus

5.3 Results

5.3.1 Nuclear extract from OE19 adenocarcinoma cell lines

The oesophageal adenocarcinoma cell line OE19 was grown in a 75 cm^2 vented flask and treated with 500 µM DCA for 3 h at 37°C in an incubator, while an untreated flask provided a control. The time period of 3 h was chosen as previous studies had demonstrated upregulation of transcription factors including NF-kB at time points between 0 and 4 h (Jenkins, Harries et al. 2004, Rafiee, Nelson et al. 2009, Green, Huang et al. 2011). The OE19 cell line was used as it had previously demonstrated secretion of CCL28 protein in response to DCA treatment in a dose-response manner. Cells were then removed and nuclear extract was isolated as described in section 2.4.2.1. In order to confirm that nuclear extract isolated was of high quality, the extract was run on a 12% separating SDS-PAGE gel along with the corresponding cytoplasmic extract from the same cells. The gel was then stained with Coomassie blue and de-stained to reveal bands of protein. The nuclear extract demonstrates clear discrete bands of protein, confirming protein integrity (Figure 5.1). If the protein had degraded, the gel would show a "smear" of protein instead of discrete bands. The nuclear extract also demonstrates a differing pattern of bands to the cytoplasmic extract, confirming that these extracts have been separated. Having confirmed integrity of protein, this nuclear extract was then applied to the transcription factor activation array.

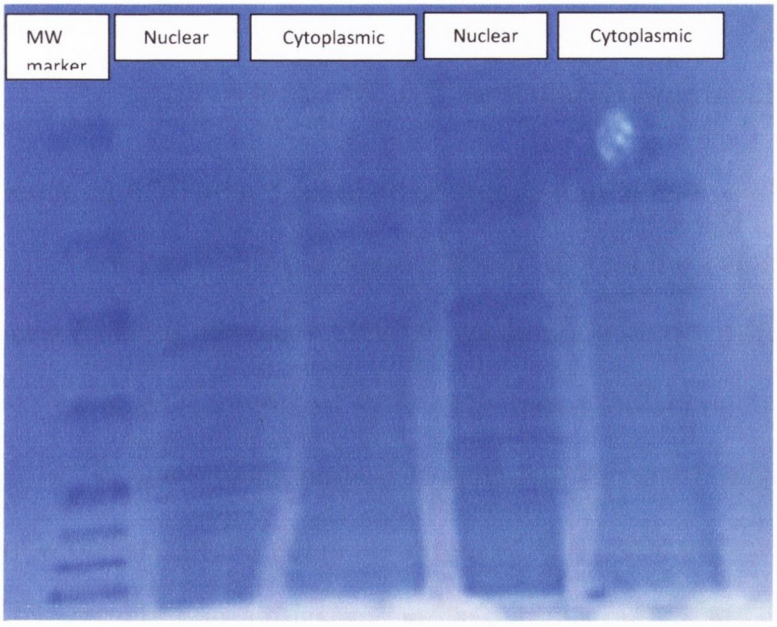


Figure 5.1 Confirmation of integrity of nuclear extract from OE19 oesophageal adenocarcinoma cell line by protein separation on SDS-PAGE gel. The OE19 oesophageal adenocarcinoma cell line was grown in a 75 cm2 flask. Nuclear and cytoplasmic protein was extracted from the cells using the method described in section 2.4.2.1. In order to confirm quality of protein, the extracts were separated by gel electrophoresis on a 12% SDS-PAGE gel for ~80 min. The gel was removed from the electrophoresis apparatus and incubated overnight with gentle shaking in Coomassie blue stain. It was then de-stained for 1 h to visualise bands. Representative image shown here demonstrates discrete bands of protein and altered patterns of bands between nuclear and cytoplasmic extracts.

5.3.2 Transcription factors are activated by deoxycholic acid treatment in the OE19 oesophageal adenocarcinoma cell line.

Given that CCL28 has been demonstrated to be inducible by DCA treatment in Chapter 3, nuclear extracts from the OE19 cell line and the OE19 cell line treated with DCA for 3 h were run on a transcription factor activation profiling array in order to identify transcription factors activated by DCA treatment. This array measures the activation of 96 transcription factors simultaneously. The transcription factors activated by 500 μM DCA treatment in the OE19 cell line were measured on this array. Of the 96 transcription factors measured on the array, 71 were activated to some degree by DCA treatment (Figure 5.2). A change in transcription factor level of greater than 1.4 was taken as the threshold level for activation by DCA, as NF-kB, known to be activated by DCA in this cell type, reached this threshold (Debruyne, Witek et al. 2006). Once these transcription factors had been identified, this list was filtered by identifying those transcription factors that had binding sites within the CCL28 promoter region, using Genomatix software. The transcription factors that were activated by DCA, which had binding sites in the CCL28 promoter region, are shown in Table 5.1 including p53, NF-kB, HIF-1α, PPARα, XBP-1 and RXR.

5.3.3 Transcription factors regulating CCL28 gene expression are identified using the MCF-7 transcription factor knockdown array.

The Expressed Transcription Factor Knockdown Transcriptome PCR Array (SABiosciences) identifies which transcription factors regulate the expression of a particular constitutively expressed gene. Each well of the array contains a cDNA

0
200000
400000
600000
800000
1000000
1200000
AP1
AP2
AR
ATF2
Brn-3
C\EBP
CAR
CBF
CDP
CREB
E2F-1
EGR
ER
Ets
FAST-1
GAS/ISRE
GATA
GR/PR
HIF
HNF4
IRF
MEF2
Myb
Myc-Mas
NF-1
NFAT
NF-E2
NFkB
38261
p53
Pax-5
Pbx1
Pit
PPAR
PXR
SMAD
Sp1
SRF
SATB1
Stat1
Stat3
Stat4
Stat5
Stat6
TCF/LEF
YY1
TR
TFIID
AP3
AP4
COUP
ELK
FOXA1
FoxC1
FOXD3
FOXG1
FOXO1
FREAC-2
GFI-1
Gli-1
HEN(NSCL-1)
HNF-1
HOX4C
HoxA-5
HSF
KLF4
MEF-1
MEF-3
MyoD
MZF
Nkx2-5
Nkx3-2
NRF-1
NRF2(A)
37165
Pax2
Pax3
Pax8
PIT1
Prox1
RNUX
ROR(RZR)
RXR
SF-1
SMUC
Snail
SOX-2
SOX9
SOX18
TFE3
TFEB
USF-1
VDR
WT1
XBP-1
Ctrl
500uM DCA

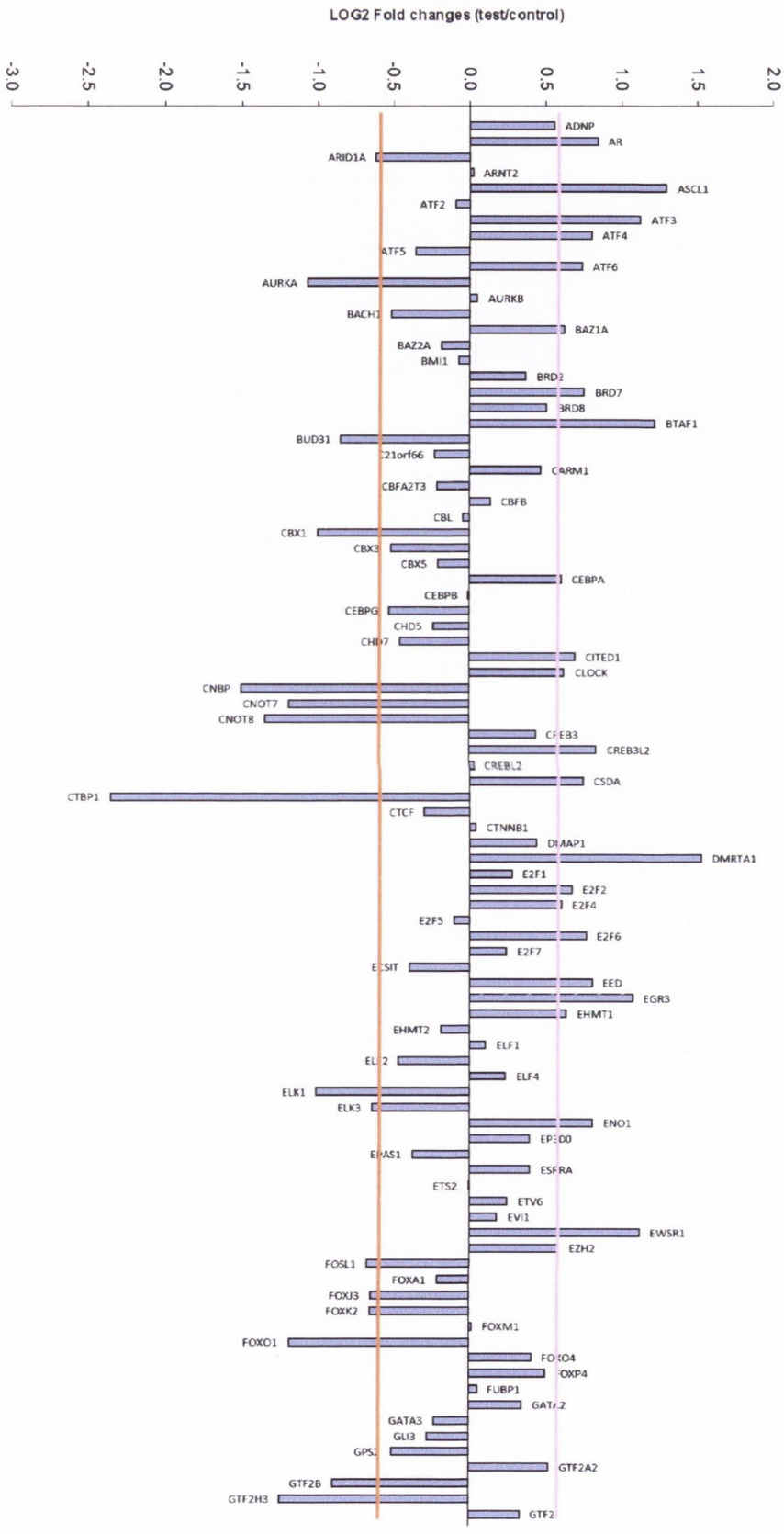
Figure 5.2 Transcription factors activated by deoxycholic acid treatment in the OE19 oesophageal adenocarcinoma cell line. The OE19 oesophageal adenocarcinoma cell line was treated with 500 µM deoxycholic acid (DCA) for 3 h. Nuclear extract was prepared from treated cells and from untreated cells as described in section 2.4.2.1 and integrity of proteins was confirmed as demonstrated in Figure 5.1. Nuclear extract was run on a transcription factor activation profiling array in order to identify transcription factors activated by DCA treatment. This graph displays the transcription factors activated by 500 µM DCA treatment. 96 transcription factors were measured on the array, of which 71 were activated to some degree by DCA treatment.

Table 5.1 Transcription factors increased by DCA with binding site in CCL28 promoter region

Transcription factor	Fold change
XBP-1	6.191583
RXR	2.692674
p53	2.373086
NFAT	2.17773
YY1	2.135231
IRF	2.090122
NF-1	2.038668
NRF2(A)	1.96881
OCT	1.908097
Sp1	1.69669
HIF	1.598491
EGR	1.575343
PPAR	1.506932
KLF4	1.475478
NF-kB	1.47025

sample that was synthesized from an MCF-7 breast cancer cell sample treated with a unique siRNA for one of 270 transcription factors. This allows the screening of 270 different siRNA treatments with a single qPCR reaction for a gene of interest, in this case, CCL28. Before running the array, constitutive expression of CCL28 in the MCF-7 cell line was confirmed using quantitative PCR. Gene expression of CCL28 was then measured in each of the siRNA-transfected lines on the array using quantitative real-time PCR to determine if CCL28 was under the control of any of these 270 transcription factors. Potential positive regulators of CCL28 demonstrate a negative fold change, as knock down of the transcription factor causes a decrease in CCL28 expression, while potential negative regulators of CCL28 demonstrate a positive fold change. The fold change threshold was set at 1.5. The transcription factors which altered CCL28 expression are shown in Figure 5.3. Once these transcription factors had been identified, this list was filtered by identifying those transcription factors that had binding sites within the CCL28 promoter region, using Genomatix software. The transcription factors that were identified as positive or negative regulators of CCL28 expression, which had binding sites in the CCL28 promoter region, are shown in Table 5.2. After filtering, 10 transcription factors were identified as positive regulators and 8 as negative regulators of CCL28 expression. As CCL28 is induced by DCA treatment, transcription factors which positively regulated CCL28 expression were focused on for the purpose of this study.

Plate 1: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ATF6
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CARM1
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CBFB
CBL
CBX1
CBX3
CBX5
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CEBPB
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CHD7
CITED1
CLOCK
CNBP
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CNOT8
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ELF4
ELK1
ELK3
ENO1
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ESRRA
ETS2
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EWSR1
EZH2
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FOXJ3
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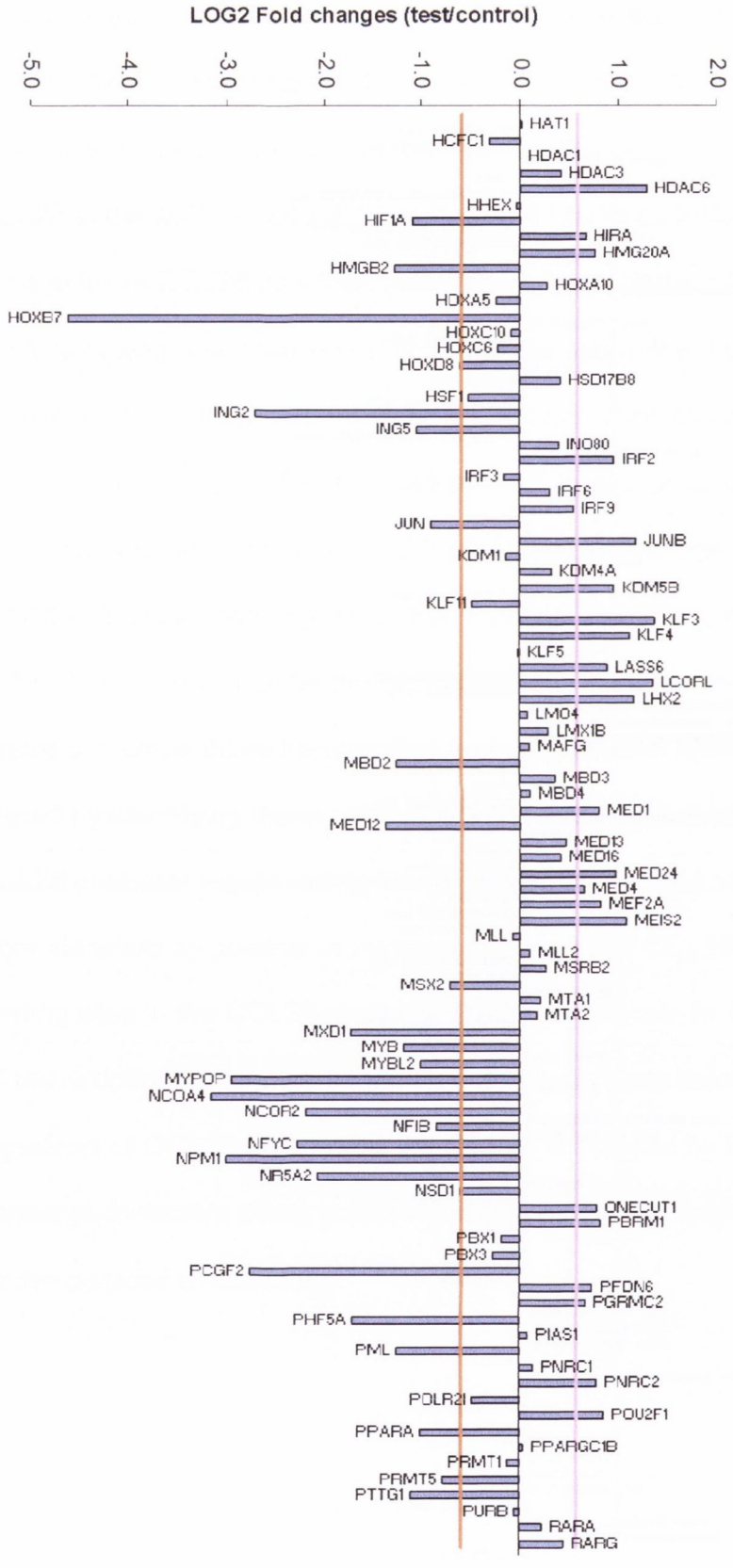
GTF2H3

Plate 2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HIRA
HMG20A
HMGB2
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HOXA5
HOXB7
HOXC10
HOXC6
HOXD8
HSD17B8
HSF1
ING2
ING5
INO80
IRF2
IRF3
IRF6
IRF9
JUN
JUNB
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KLF3
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KLF5
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LHX2
LMO4
LMX1B
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MBD2
MBD3
MBD4
MED1
MED12
MED13
MED16
MED24
MED4
MEF2A
MEIS2
MLL
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MSRB2
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PBRM1
PBX1
PBX3
PCGF2
PFDN6
PGRMC2
PHF5A
PIAS1
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PNRC1
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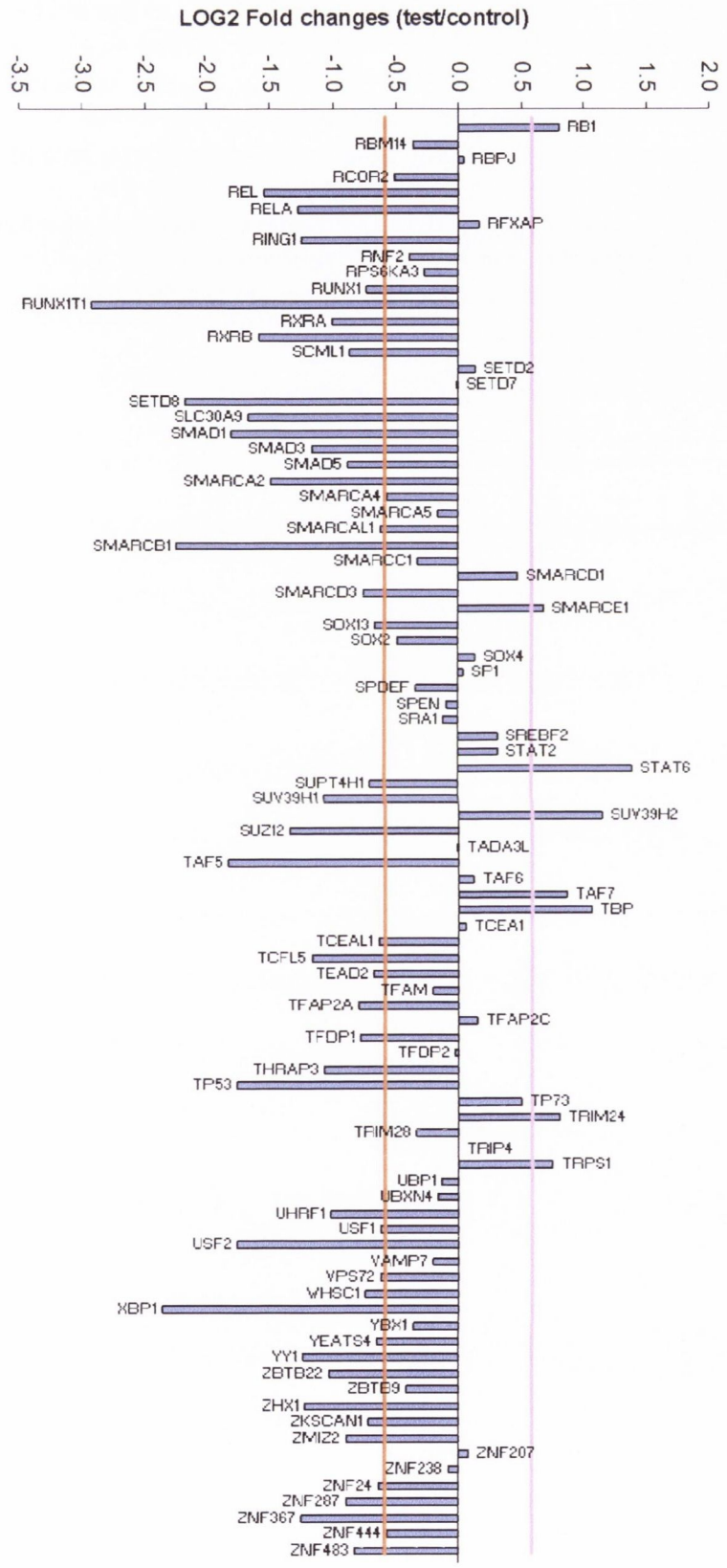
PTTG1
PURB
RARA
RARG

Plate 3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RBM14
RBPJ
RCOR2
REL
RELA
RFXAP
RING1
RNF2
RPS6KA3
RUNX1
RUNX1T1
RXRA
RXRB
SCML1
SETD2
SETD7
SETD8
SLC30A9
SMAD1
SMAD3
SMAD5
SMARCA2
SMARCA4
SMARCA5
SMARCAL1
SMARCB1
SMARCC1
SMARCD1
SMARCD3
SMARCE1
SOX13
SOX2
SOX4
SP1
SPDEF
SPEN
SRA1
SREBF2
STAT2
STAT6
SUPT4H1
SUV39H1
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SUZ12
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TAF6
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TRPS1
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USF2
VAMP7
VPS72
WHSC1
XBP1
YBX1
YEATS4
YY1
ZBTB22
ZBTB9
ZHX1
ZKSCAN1
ZMIZ2
ZNF207
ZNF238
ZNF24
ZNF287
ZNF367
ZNF444
ZNF483

Figure 5.3 Transcription factors regulating CCL28 gene expression in the MCF-7 transcription factor knockdown array. The MCF-7 breast cancer cell line was transfected with siRNA in order to knock down 270 different transcription factors and cDNA from these transfected lines were loaded onto three 96-well plates. Gene expression of CCL28 was measured in each of these transfected lines using quantitative real-time PCR to determine if CCL28 was under regulation by any of these 270 transcription factors. Potential positive regulators of CCL28 demonstrate a negative fold change, as knock down of this transcription factor causes a decrease in CCL28 expression, while potential negative regulators of CCL28 demonstrate a positive fold change. The fold change threshold was set at 1.5.

Table 5.2 Positive and negative regulators of CCL28 gene expression identified on the transcription factor knockdown array that have binding sites on the CCL28 promoter region

Transcription factor	Name	Alternative names	Fold change in CCL28 expression
Positive regulators			
GTF2B	General transcription factor IIB	TF2B, TFIIB	-1.85574422
HOXB7	Homeobox B7	HHO.C1, HOX2, HOX2C, Hox-2.3	-24.64244712
HIF1A	Hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	HIF-1alpha, HIF1, HIF1-ALPHA, MOP1, PASD8, bHLHe78	-2.125408461
PPARA	Peroxisome proliferator-activated receptor alpha	MGC2237, MGC2452, NR1C1, PPAR, hPPAR	-2.015371954
XBP1	X-box binding protein 1	TREB5, XBP2	-5.127892436
SMAD1	SMAD family member 1	BSP1, JV4-1, JV41, MADH1, MADR1	-3.499280949
TP53	Tumour protein p53	FLJ92943, LFS1, TRP53, p53	-3.38897149
REL	V-rel reticuloendotheliosis viral oncogene homolog (avian)	C-Rel	-2.918054847
RELA	V-rel reticuloendotheliosis viral oncogene homolog A (avian)	MGC131774, NFKB3, p65	-2.417787582
YY1	YY1 transcription factor	DELTA, INO80S, NF-E1, UCRBP, YIN-YANG-1	-2.344188364
Negative regulators			
ASCL1	Achaete-scute complex homolog 1 (Drosophila)	ASH1, HASH1, MASH1, bHLHa46	2.457962022
CREBL2	CAMP responsive element binding protein-like 2	MGC117311, MGC138362	1.789333897
ATF4	Activating transcription factor 4 (tax-responsive enhancer element B67)	CREB-2, CREB2, TAXREB67, TXREB	1.743410888
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha	C, EBP-alpha, CEBP	1.523522308
KLF3	Kruppel-like factor 3 (basic)	BKLF, MGC48279	2.591497272
KLF4	Kruppel-like factor 4 (gut)	EZF, GKLF	2.165913121
IRF2	Interferon regulatory factor 2	DKFZp686F0244, IRF-2	1.946831975
TBP	TATA box binding protein	GTF2D, GTF2D1, MGC117320, MGC126054, MGC126055, SCA17, TFIID	2.084747994

5.3.4 Ingenuity pathway analysis demonstrates interactions between identified transcription factors from arrays and CCL28.

Network and pathway analysis was carried out on the transcription factors identified in the Activation Profiling Array, the Transcription Factor Knockdown Array and the Genomatix programme using Ingenuity Pathways Analysis™ (IPA). The IPA software allows analysis of signalling and metabolic pathways, molecular networks, and biological processes involved in the data inputted (Thomas and Bonchev 2010). Data from the transcription factor arrays was formatted into IPA compatible layouts using template files downloaded from IPA website and uploaded into the IPA programme. Analysis was carried out using IPA, inputting the transcription factor data and including the factors CCL28, IL-1β and TNF-α in the pathway maps. Interactions between CCL28, IL-1β, TNF-α and transcription factors identified from arrays is shown in figure 5.4. It can be seen that CCL28 is linked to the transcription factors NF-kB, p53, PPAR-α and HIF-1α via the cytokine IL-1β, while it is also linked to the transcription factor XBP1 via TNF.

As CCL28 is known to be regulated via NF-kB in other cell types, and as DCA is known to induce NF-kB activity, it was decided to focus further on the role of NF-kB in regulation of CCL28 expression in the oesophagus.

5.3.5 Optimisation of conditions for stable transfection in the OE19 oesophageal adenocarcinoma cell line

Transfection conditions were optimised using Lipofectamine 2000™ in varying ratios with a green fluorescent protein (GFP) plasmid. Ratios of 1:1, 2:1

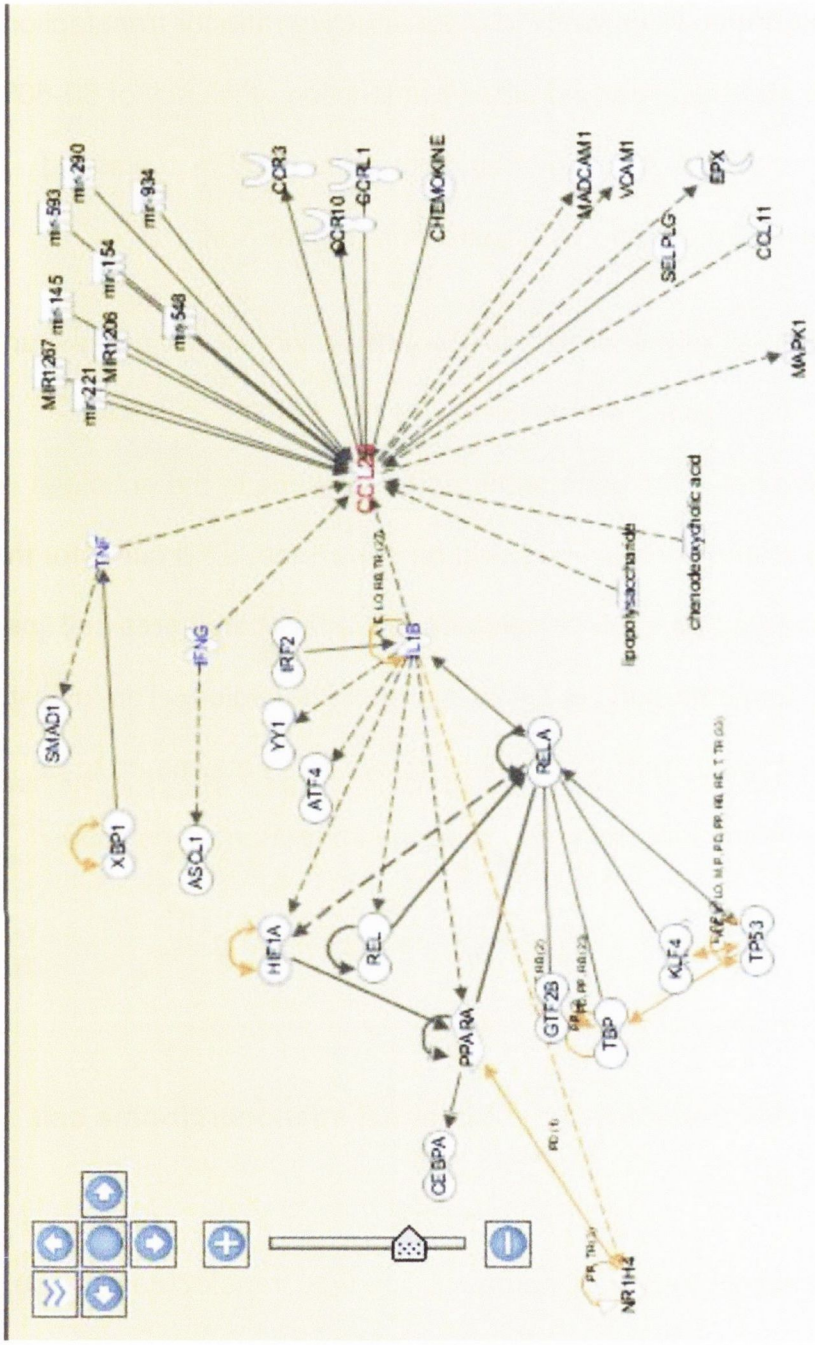


Figure 5.4 Ingenuity Pathway Analysis software demonstrates interactions between identified transcription factors from arrays and CCL28. The transcription factors identified from the transcription factor activation profiling array and the transcription factor knockdown array were analysed using Ingenuity Pathway Analysis software to visualise pathways and interactions between CCL28, IL-1β, TNF-α and identified transcription factors.

and 3:1 were used to determine which was the most effective ratio for transfection. Since stable transfection was being carried out, a transfection efficiency of 50-60% was desired for this experiment. A 2:1 ratio of Lipofectamine 2000™ to plasmid was determined to be the optimum ratio for transfection (Figure 5.5).

A plasmid containing a gene encoding for overexpression of the p65 subunit of NF-kB was obtained. This plasmid also encoded for the dsRed fluorescent protein, and incorporated a selection gene conferring resistance to the antibiotic G418 (geneticin). Before stable transfection could be performed, a kill curve for the antibiotic G418 was carried out. The OE19 oesophageal adenocarcinoma cell line was treated with varying concentrations of G418 in complete medium. The lowest concentration of G418 that killed 100% of cells in 10 days was determined to be 200 ng/mL G418. This concentration was then used in subsequent transfection experiments.

5.3.6 Generation of a stably transfected oesophageal adenocarcinoma cell line overexpressing NF-kB

The OE19 oesophageal adenocarcinoma cell line was transfected with the dsRed/p65 plasmid. Clones which had been transfected with the plasmid demonstrated red fluorescence due to the presence of the dsRed fluorescent protein (Figure 5.6A). In order to confirm the overexpression of the p65 subunit of NF-kB, Western blotting was performed on protein extracted from OE19 cells and from the two clones selected, p65-OE1 and p65-OE2. Western blotting demonstrated increased p65 expression in the clones p65-OE1 and p65-OE2

(A) (B)

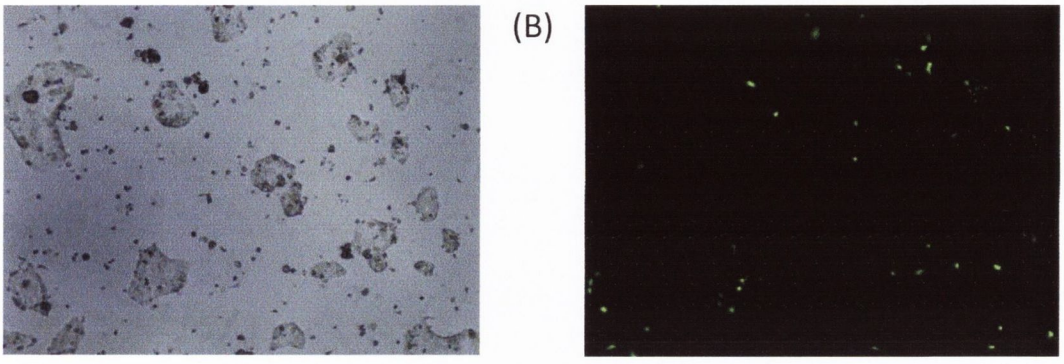


Figure 5.5 Optimisation of transfection reagent ratios. The OE19 oesophageal adenocarcinoma cell line was incubated with varying ratios of the lipid-based transfection reagent Lipofectamine 2000™ and a green fluorescent protein (GFP) plasmid. Lipofectamine 2000™ and GFP were combined in ratios 1:1, 2:1 and 3:1, incubated in serum-free medium for 30 min to allow complexes to form and were then applied to cells in a 6-well plate and incubated at 37°C overnight. Cells were then examined under a fluorescent microscope to determine which was the optimal ratio for transfection. A 2:1 ratio of Lipofectamine 2000™ to plasmid was determined to be the optimum ratio to give a 50% transfection efficiency in this cell line. (A) Light microscopy of transfected OE19 cells. (B) Fluorescent imaging of transfected OE19 cells.

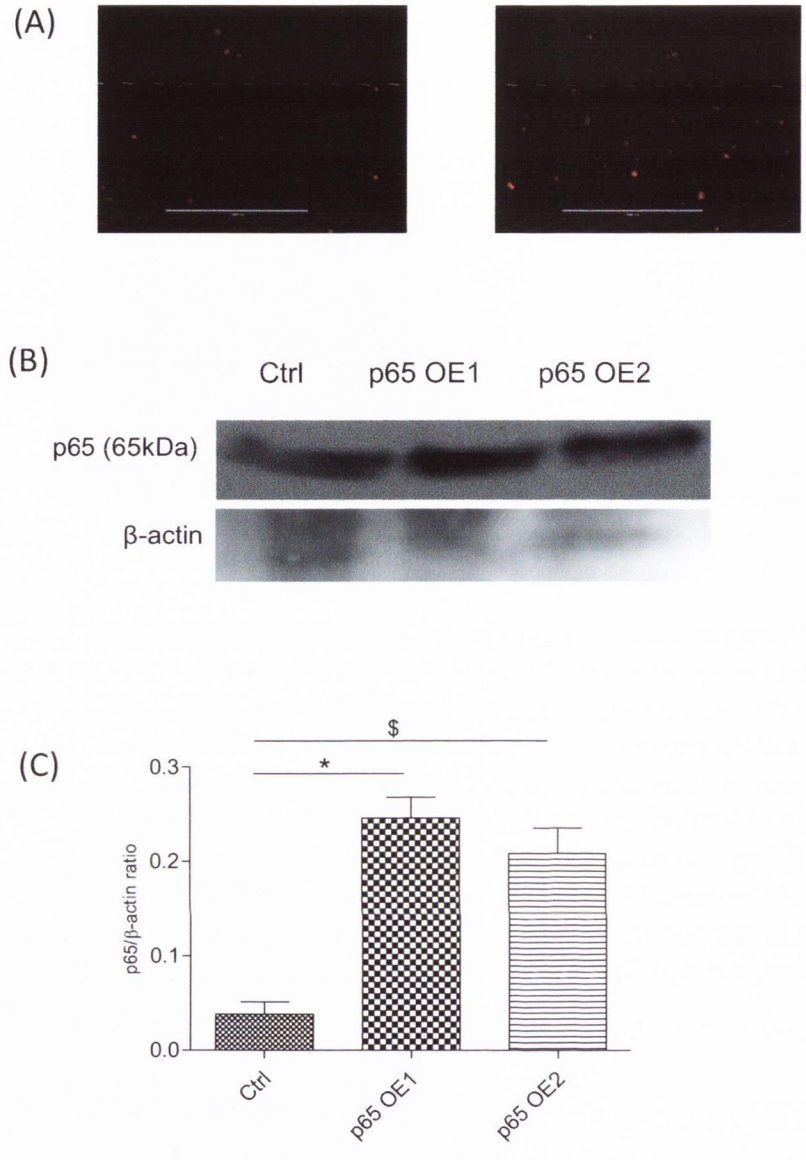


Figure 5.6 OE19 oesophageal adenocarcinoma cells transfected with the p65/dsRed plasmid demonstrate red fluorescence and overexpress the p65 subunit of NF-kB. The OE19 oesophageal adenocarcinoma cell line was stably transfected with a dsRed fluorescent plasmid overexpressing the p65 subunit of NF-kb and stable clones were selected out using G418. (A) Transfected OE19 cells demonstrate red fluorescence when viewed under a fluorescent microscope. (B) A representative Western blot demonstrates overexpression of p65 in transfected OE19 cell lines compared with the control (untransfected) OE19 cell line. The band for p65 is shown at 65 kDa on the protein ladder, and β-actin bands are shown below. (C) Densitometric analysis confirms overexpression of p65 in transfected OE19 cell lines compared with the control (untransfected) OE19 cell line. Data are presented as mean ± SEM and are the results of 3 independent experiments. Analysis was performed using the paired student's *t*-test. *p = 0.0249 vs. Ctrl, $^{\$}$p = 0.0479 vs. Ctrl.

compared with the untransfected OE19 cell line (Figure 5.6B). Densitometric analysis confirmed overexpression of p65 in these two clones (Figure 5.6C).

5.3.7 Oesophageal adenocarcinoma cell lines overexpressing NF-kB demonstrate increased CCL28 expression in response to deoxycholic acid treatment compared with the parent cell line

The parent OE19 oesophageal adenocarcinoma cell line and the two NF-kB-overexpressing cell lines p65-OE1 and p65-OE2 were treated with varying concentrations of deoxycholic acid (DCA) for 24 h. CCL28 protein in the cell culture supernatant was measured by ELISA. The OE19 parent cell line demonstrated increased CCL28 secretion in response to 200 μM DCA treatment (56.14 ± 23.88 pg/mL vs. 0 pg/mL Untreated, $p = 0.0655$) and 500 μM DCA treatment (404.8 ± 101.7 pg/mL vs. 0 pg/mL Untreated, $p = 0.01$), as did the p65-OE1 cell line (200 μM DCA 682.3 ± 220.7 pg/mL vs. 37.59 ± 17.87 pg/mL Untreated, $p = 0.0260$; 500 μM DCA 907.6 ± 89.69 pg/mL vs. 37.59 ± 17.87 pg/mL Untreated, $p = 0.0002$) and the p65-OE2 cell line (200 μM DCA 756.7 ± 224.2 pg/mL vs. 29.91 ± 26.72 pg/mL Untreated, $p = 0.0171$; 500 μM DCA 306.5 ± 145.9 pg/mL vs. 29.91 ± 26.72 pg/mL Untreated, $p = 0.133$) (Figure 5.7). The overexpressing cell lines p65-OE1 and p65-OE2 demonstrated significantly increased CCL28 in response to 200 μM DCA treatment compared with the parent OE19 cell line (p65-OE1 682.3 ± 220.7 pg/mL vs. 56.14 ± 23.88 pg/mL, $p = 0.0182$; p65-OE2 756.7 ± 224.2 pg/mL vs. 56.14 ± 23.88 pg/mL, $p = 0.0111$). The overexpressing cell line p65-1 also demonstrated increased CCL28 in response to 500 μM DCA treatment compared with the parent OE19 cell line (907.6 ± 89.69 pg/mL vs. 404.8 ± 101.7 pg/mL, $p = 0.0041$). These

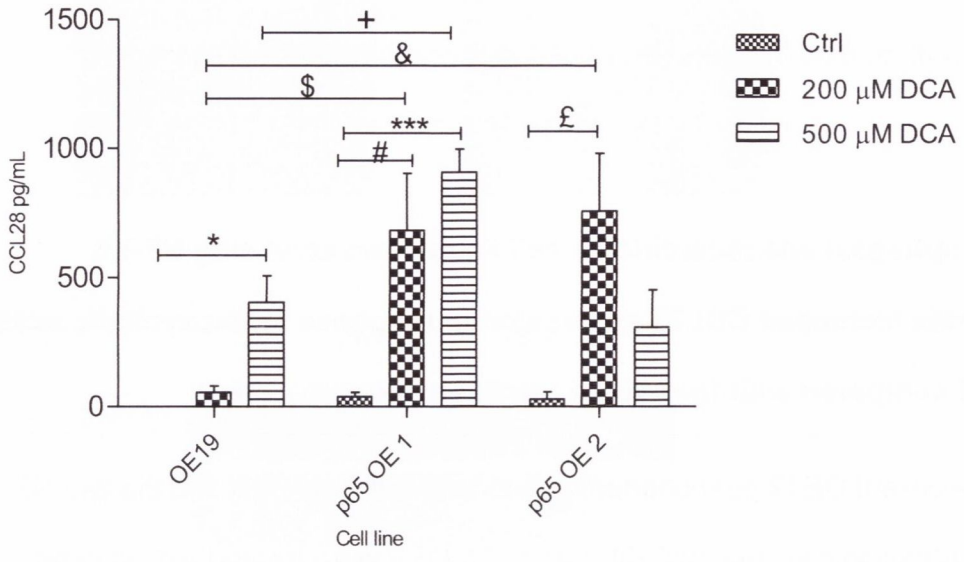


Figure 5.7 The stably transfected oesophageal adenocarcinoma cell lines overexpressing NF-kB demonstrate increased CCL28 expression in response to deoxycholic acid treatment compared with the parent untransfected cell line. The OE19 oesophageal adenocarcinoma cell line was transfected with a plasmid encoding for overexpression of the p65 subunit of NF-kB. Two overexpressing clones were selected out (p65-OE1 and p65-OE2) and were grown up. The parent cell line and the two overexpressing cell lines were treated with varying concentrations of deoxycholic acid (DCA) for 24 h. CCL28 protein in the cell culture supernatant was measured using ELISA. All three cell lines demonstrated increased CCL28 secretion in response to DCA treatment. The overexpressing cell lines p65-OE1 and p65-OE2 demonstrated increased CCL28 in response to 200 μM DCA treatment compared with the parent OE19 cell line. The overexpressing cell line p65-1 also demonstrated increased CCL28 in response to 500 μM DCA treatment compared with the parent OE19 cell line. Data are presented as mean ± SEM and are the results of 3 independent experiments. Analysis was performed using the paired students t-test for matched values, and the unpaired t-test for unmatched values. *p = 0.0105 vs. OE19 Ctrl, #p = 0.0260 vs. p65-OE1 Ctrl, ***p = 0.0002 vs. p65-OE1 Ctrl, £p = 0.0171 vs. p65-OE2 Ctrl, $p = 0.0182 vs. OE19 200 μM, &p = 0.0111 vs. OE19 200 μM, +p = 0.0041 vs. OE19 500 μM.

data indicate that CCL28 expression is induced by DCA via the transcription factor NF-kB.

5.3.8 The NF-kB inhibitor DHMEQ inhibits induction of CCL28 secretion by deoxycholic acid in the OE19 oesophageal adenocarcinoma cell line and in OE19 cell lines overexpressing NF-kB

The parent OE19 cell line and the two NF-kB-overexpressing cell lines p65-OE1 and p65-OE2 were treated with 200 μM deoxycholic acid (DCA) for 24 h, and also with a combination of 200 μM DCA and varying concentrations of the NF-kB inhibitor DHMEQ (1-5 μg/mL) (Saisho, Hirose et al. 2008, Kozakai, Kikuchi et al. 2012). CCL28 protein in the cell culture supernatant was measured by ELISA as before. All three cell lines demonstrated increased CCL28 secretion in response to DCA treatment (Figure 5.8) (OE19 200 μM DCA 235.9 ± 46.05 pg/mL vs. 11.48 pg/mL Untreated, p = 0.0143; p65-OE1 200 μM DCA 427.3 ± 105.2 pg/mL vs. 11.67 pg/mL, p = 0.0216; p65-OE2 200 μM DCA 1132 ± 333 pg/mL vs. 14.42 pg/mL, p = 0.0397). All three cell lines demonstrated a significant decrease in CCL28 secretion in response to DCA with the NF-kB inhibitor DHMEQ. The OE19 cell line demonstrated a decrease in CCL28 with 5 μg/mL DHMEQ treatment (42.79 ± 25.97 pg/mL vs. 235.9 ± 46.05 pg/mL 200 μM DCA, p = 0.0141). The p65-OE1 cell line demonstrated a decrease in CCL28 with both 1 μg/mL DHMEQ (164 ± 140 pg/mL vs. 427.3 ± 105.2 pg/mL 200 μM DCA, p = 0.0165) and 5 μg/mL DHMEQ (29.96 ± 15.66 pg/mL vs. 427.3 ± 105.2 pg/mL 200 μM DCA, p = 0.0246). The p65-OE2 cell line demonstrated similar decreases with both 1 μg/mL DHMEQ (245.2 ± 163.5 pg/mL vs. 1132 ± 333 pg/mL pg/mL 200 μM DCA, p = 0.0911) and

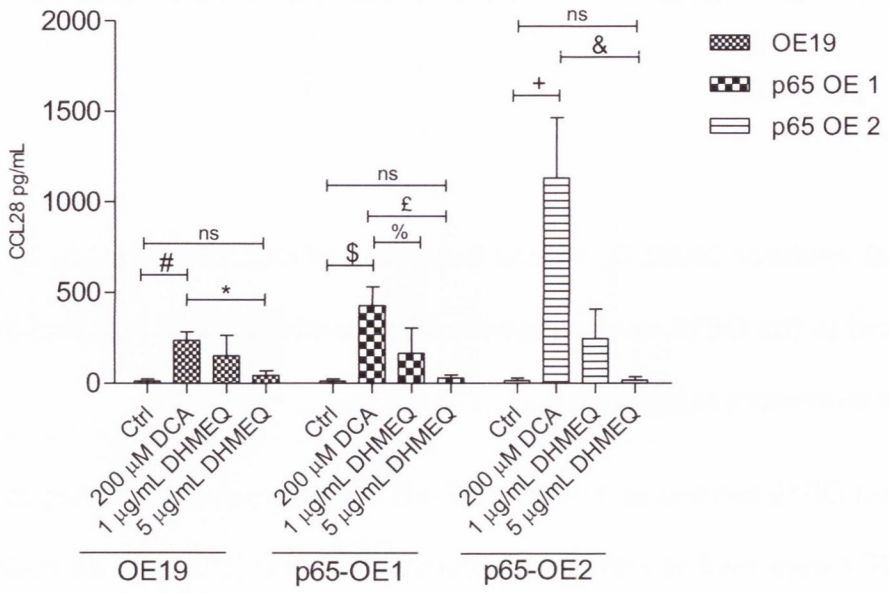


Figure 5.8 The NF-kB inhibitor DHMEQ inhibits induction of CCL28 secretion by deoxycholic acid in the parent OE19 oesophageal adenocarcinoma cell line and in OE19 cell lines overexpressing NF-kB. The OE19 oesophageal adenocarcinoma cell line was transfected with a plasmid encoding for overexpression of the p65 subunit of NF-kB. Two overexpressing clones were selected out (p65-OE1 and p65-OE2) and were grown up. The parent cell line and the two overexpressing cell lines were treated with 200 µM deoxycholic acid (DCA) for 24 h, and also with a combination of 200 µM DCA and varying concentrations of the NF-kB inhibitor DHMEQ (1-5 µg/mL). CCL28 protein in the cell culture supernatant was measured using ELISA. All three cell lines demonstrated increased CCL28 secretion in response to DCA treatment. All three cell lines demonstrated a significant decrease in CCL28 secretion in response to DCA with the NF-kB inhibitor DHMEQ. Data are presented as mean ± SEM and are the results of at least 3 independent experiments. Analysis was performed using the paired student's t-test for matched values, and the unpaired t-test for unmatched values. $^{\#}p = 0.0143$ vs. OE19 Ctrl, $^{*}p = 0.0141$ vs. OE19 200 µM DCA, $^{\$}p = 0.0216$ vs. p65-OE1 Ctrl, $^{\%}p = 0.0165$ vs. p65-OE1 200 µM DCA, $^{£}p = 0.0246$ vs. p65-OE1 200 µM DCA, $^{+}p = 0.0397$ vs. p65-OE2 Ctrl, $^{\&}p = 0.0390$ vs. p65-OE2 200 µM DCA.

5 μg/mL DHMEQ (17.96 ± 17.96 pg/mL vs. 1132 ± 333 pg/mL 200 μM DCA, p = 0.0390). These data indicate again that DCA-induced CCL28 expression is regulated via the NF-kB pathway, as inhibition of this pathway decreases DCA-induced CCL28 expression.

5.3.9 Optimisation of conditions for chemotaxis assay

Peripheral blood mononuclear cells (PBMCs) were isolated from a healthy donor. Fluorescently-labelled antibodies for the receptors CCR3 and CCR10 were obtained, labelled with APC and PE, respectively. PBMCs were stained with varying concentrations of antibody (0-20 μL). The optimum volume of antibody was determined to be 5 μL for both stains. Discrete populations of cells positive for CCR3 and CCR10 were observed upon flow cytometric analysis (Figure 5.9).

It was decided to use T cells only for the chemotaxis assay as this was a purer population with more predictable chemotaxis. After isolation of PBMCs, T cells were isolated using a pan T-cell isolation kit (negative selection). Once isolated, T cells were stained using a CD3 antibody to confirm purity of T cell population. CD3 staining confirmed 99% purity of T cells as measured by CD3 positivity (Figure 5.10).

5.3.10 T cells migrate in response to RANTES and FBS in transwell chemotaxis assay

T cells were placed into the upper chamber of a transwell chemotaxis system. The bottom chamber contained either medium alone (control), medium

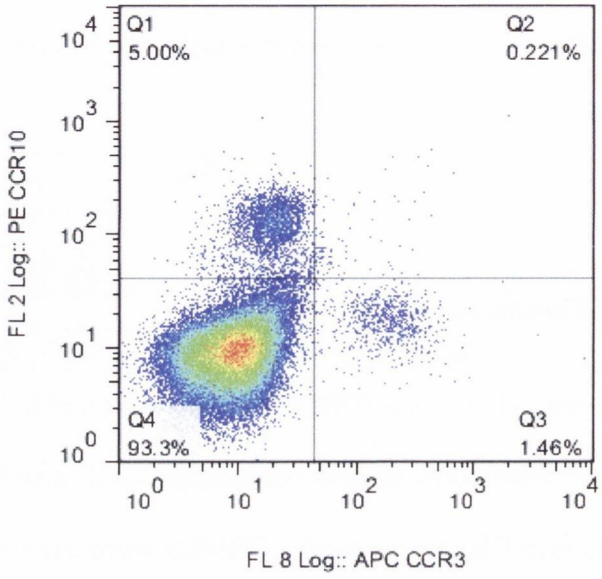


Figure 5.9 Optimisation of staining for CCR3 and CCR10 in human peripheral blood mononuclear cells demonstrates populations of CCR3 and CCR10 positive cells. Peripheral blood mononuclear cells (PBMCs) were isolated from a healthy donor. Fluorescent-labelled antibodies for the receptors CCR3 and CCR10 were obtained, labelled with APC and PE respectively. PBMCs were stained with varying concentrations of antibody (0-20 µL). 5 µL was determined to be the optimum volume of antibody for both stains.

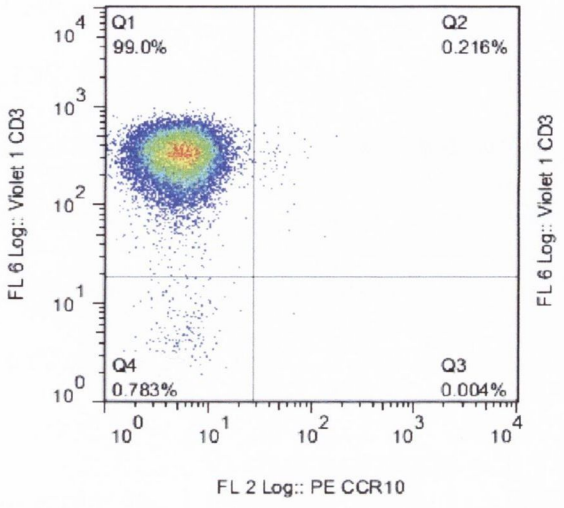


Figure 5.10 CD3 staining after T cell isolation demonstrates 99% purity. After isolation of PBMCs, T cells were isolated using a pan T-cell isolation kit. Once isolated, T cells were stained using a CD3 antibody to confirm purity of T cell population. CD3 staining confirmed 99% purity of T cells as measured by CD3 positivity.

containing recombinant human CCL28, recombinant human RANTES (CCL5), 20% foetal bovine serum (FBS), 10% FBS, or conditioned medium from DCA-treated OAC (OE19) cells. RANTES was used as a control chemokine known to induce chemotaxis (Salsman, Chow et al. 2011, Chan, Burke et al. 2012). After 2 h chemotaxis, T cells that had migrated to the bottom chamber were counted in 5 random fields of view using an inverted microscope. Recombinant human RANTES (CCL5) and 20% FBS stimulated chemotaxis of increased numbers of T cells compared with medium alone (RANTES 20.38 ± 1.281 vs. 13.75 ± 1.306 Ctrl, $p = 0.0028$; 20% FBS 29.13 ± 2.083 vs. 13.75 ± 1.306 Ctrl, $p < 0.0001$) (Figure 5.11). Medium containing 20% FBS stimulated increased T cell migration compared with medium containing 10% FBS (29.13 ± 2.083 vs. 16.00 ± 2.198, $p = 0.0029$) and all cell culture conditioned media (OE19: 15.25 ± 1.652 vs. 29.13 ± 2.083, $p = 0.0015$, p65-OE1: 16.25 ± 0.629 vs. 29.13 ± 2.083, $p = 0.0018$, p65-OE2: 19.75 ± 1.652 vs. 29.13 ± 2.083, $p = 0.0154$). These data indicate that RANTES and FBS stimulate chemotaxis of T lymphocytes in the transwell chemotaxis assay.

5.3.11 Conditioned media from oesophageal adenocarcinoma cells treated with deoxycholic acid is chemotactic for T cells expressing the CCR3 and CCR10 receptors

T cells were placed into the top chamber of a transwell chemotaxis insert in a 24-well plate. The bottom chamber of the wells contained either medium alone, medium containing recombinant human CCL28 (rhCCL28), recombinant human RANTES (rhCCL5), 20% foetal bovine serum (FBS), or conditioned medium from

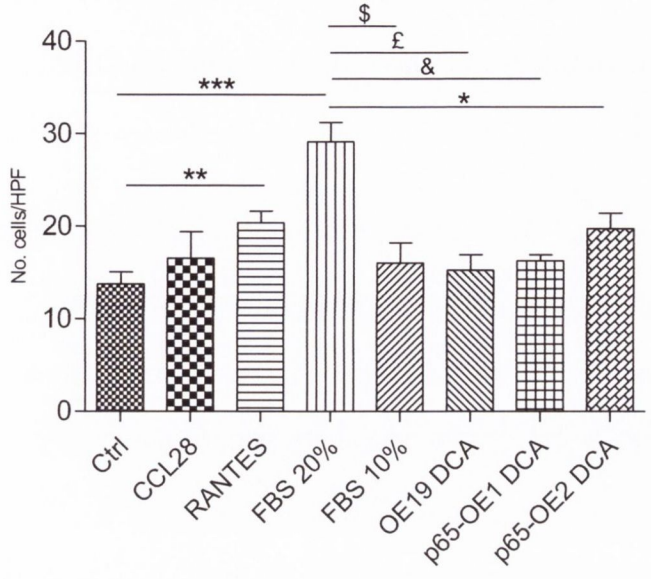


Figure 5.11 Recombinant human RANTES and 20% foetal bovine serum chemoattract increased numbers of T cells compared with medium alone. T cells were placed into the upper chamber of a transwell chemotaxis system. The bottom chamber contained either medium alone, medium containing recombinant human CCL28, recombinant human RANTES, 20% foetal bovine serum (FBS), 10% FBS, or conditioned medium from deoxycholic acid-treated oesophageal adenocarcinoma cells. The transwell system was incubated at 37°C for 2 h and then the top chamber was removed. T cells that had migrated to the bottom chamber were counted in 5 random fields of view using an inverted microscope. Recombinant human RANTES and 20% FBS caused migration of increased numbers of T cells compared with medium alone. 20% FBS caused increased T cell migration compared with 10% FBS and all cell culture conditioned media. Data are presented as mean ± SEM and are the results of at least 3 independent experiments. Analysis was performed using the two-tailed unpaired t-test. **p = 0.0028 vs. Ctrl, ***p <0.0001 vs. Ctrl, $p = 0.0029 vs. FBS 20%, £p = 0.0015 vs. FBS 20%, &p = 0.0018 vs. FBS 20%, *p = 0.0154 vs. FBS 20%.

oesophageal adenocarcinoma cells treated with deoxycholic acid (DCA) - the OE19 oesophageal adenocarcinoma cells and the p65-OE1 and p65-OE2 oesophageal adenocarcinoma cells.

Staining for CCR3 on T cells alone demonstrated that CCR3-positive cells made up 2.0% of all T cells (Figure 5.12). Of cells in the bottom chamber containing rhCCL28, CCR3-positive cells made up only 1.7%. Of cells in the bottom chamber containing rhCCL5 (RANTES), known to bind to the CCR3 receptor, CCR3-positive cells made up 11.5%, suggesting that rhCCL5 chemoattracts CCR3-positive cells. The positive control, medium containing 20% FBS, chemoattracted T cells containing 11.5% CCR3-positive cells. Cell conditioned medium from OE19 OAC cells treated with DCA, known to contain CCL28, chemoattracted T cells containing 14.1% CCR3-positive cells. Cell conditioned medium from p65-OE1 and p65-OE2 OAC cells treated with DCA, known to contain significantly higher levels of CCL28 than the parent OE19 cell line, chemoattracted T cell populations containing 18.6% and 13.9% CCR3-positive cells respectively. This suggests that the CCL28 secreted by OAC cells in response to DCA is functional in chemoattracting CCR3-positive T cells.

Staining for CCR10 on T cells alone demonstrated that CCR10-positive cells made up 2.7% of all T cells (Figure 5.13). Of cells in the bottom chamber containing rhCCL28, CCR10-positive cells made up only 1.4%. Of cells in the bottom chamber containing rhCCL5 (RANTES), known to bind to the CCR3 receptor, CCR10-positive cells made up 2.9%, suggesting that, as expected, rhCCL5 does not chemoattract CCR10-positive cells. The positive control, medium containing 20% FBS, chemoattracted T cells containing 2.9% CCR10-positive cells. Cell conditioned medium from OE19 OAC cells treated with DCA, known to

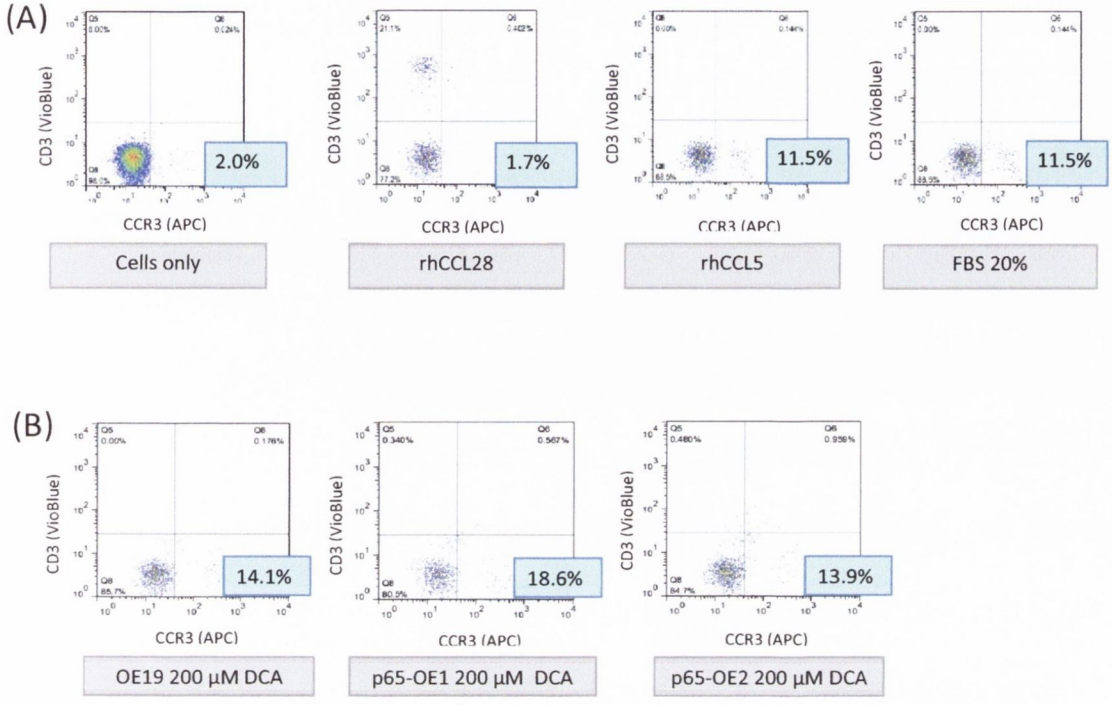


Figure 5.12. Conditioned media from oesophageal adenocarcinoma cells treated with deoxycholic acid is chemotactic for T cells expressing the CCR3 receptor (n=1). T cells were isolated from a healthy donor. Cells were placed into the top chamber of a transwell chemotaxis insert in a 24-well plate. The bottom chamber of the wells contained either medium containing recombinant human CCL28 (rhCCL28), recombinant human RANTES (rhCCL5), 20% foetal bovine serum (FBS), or conditioned medium from oesophageal adenocarcinoma cells treated with deoxycholic acid (DCA). These cells were the OE19 oesophageal adenocarcinoma cells and the p65-1 and p65-2 oesophageal adenocarcinoma cells, which overexpress the p65 subunit of NF-kB. (A) CCR3-positive cells make up 2.0% of all T cells. Of cells in the bottom chamber containing rhCCL28, CCR3-positive cells make up only 1.7%. Of cells in the bottom chamber containing rhCCL5, known to bind to the CCR3 receptor, CCR3-positive cells make up 11.5%, suggesting that rhCCL5 chemoattracts CCR3-positive cells. The positive control, medium containing 20% FBS, chemoattracted cells containing 11.5% CCR3-positive cells. (B) Cell conditioned medium from OE19 oesophageal adenocarcinoma cells treated with DCA, known to contain CCL28, chemoattracts cells containing 14.1% CCR3-positive cells. Cell conditioned medium from p65-1 and p65-2 oesophageal adenocarcinoma cells treated with DCA, known to contain CCL28, chemoattracts cells containing 18.6% and 13.9% CCR3-positive cells respectively.

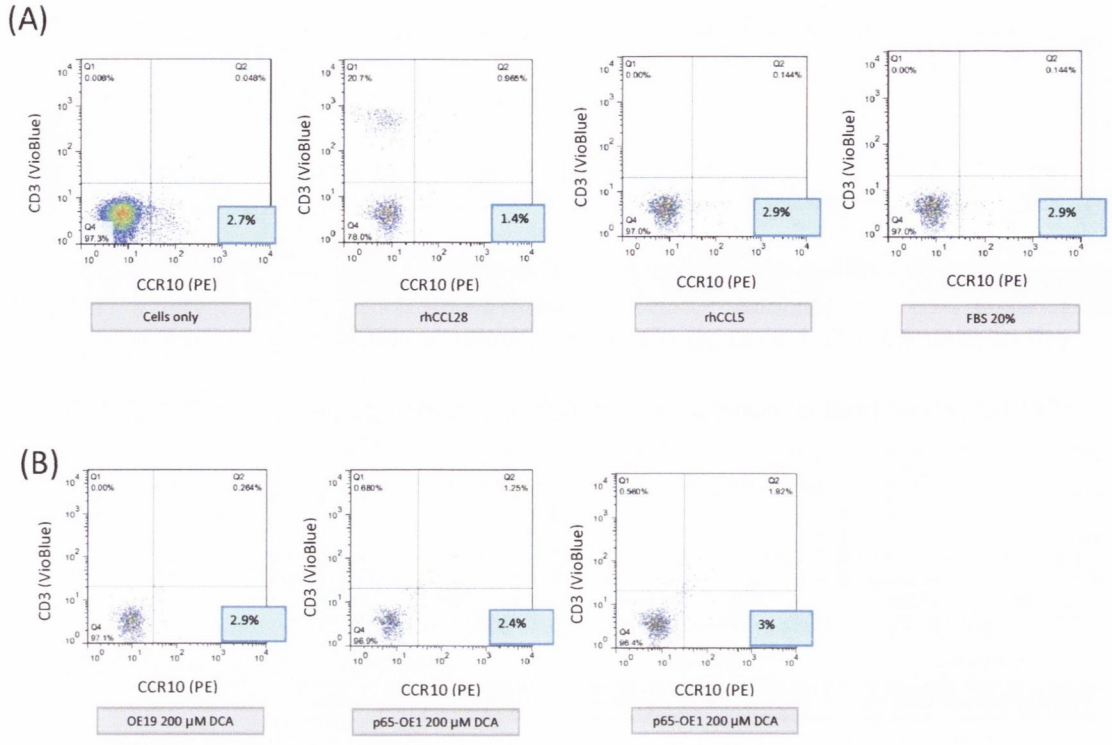


Figure 5.13. Conditioned media from oesophageal adenocarcinoma cells treated with deoxycholic acid is not chemotactic for T cells expressing the CCR10 receptor (n=1). T cells were isolated from a healthy donor. Cells were placed into the top chamber of a transwell chemotaxis insert in a 24-well plate. The bottom chamber of the wells contained either medium containing recombinant human CCL28 (rhCCL28), recombinant human RANTES (rhCCL5), 20% foetal bovine serum (FBS), or conditioned medium from oesophageal adenocarcinoma cells treated with deoxycholic acid (DCA). These cells were the OE19 oesophageal adenocarcinoma cells and the p65-1 and p65-2 oesophageal adenocarcinoma cells, which overexpress the p65 subunit of NF-kB. (A) CCR10-positive cells make up 2.7% of all T cells. Of cells in the bottom chamber containing rhCCL28, CCR10-positive cells make up 1.4%. Of cells in the bottom chamber containing rhCCL5, known to bind to the CCR3 receptor, CCR10-positive cells make up 2.9%. The positive control, medium containing 20% FBS, chemoattracted cells containing 2.9% CCR10-positive cells. (B) Cell conditioned medium from OE19 oesophageal adenocarcinoma cells treated with DCA, known to contain CCL28, chemoattracts cells containing 2.9% CCR10-positive cells. Cell conditioned medium from p65-1 and p65-2 oesophageal adenocarcinoma cells treated with DCA, known to contain CCL28, chemoattracts cells containing 2.4% and 3% CCR10-positive cells respectively.

contain CCL28, chemoattracted T cells containing 2.9% CCR10-positive cells. Cell conditioned medium from p65-OE1 and p65-OE2 OAC cells treated with DCA, known to contain CCL28, chemoattracted T cell populations containing 2.4% and 3% CCR10-positive cells respectively. This suggests that the CCL28 secreted by oesophageal adenocarcinoma cells in response to DCA does not chemoattract CCR10-positive T cells.

5.3.12 Neutralisation of CCL28 in conditioned medium abrogates chemotaxis of CCR3 and CCR10-expressing T cells

T cells isolated from a healthy donor were placed into the top chamber of a transwell chemotaxis insert in a 24-well plate. The bottom chamber of the wells contained either medium alone, Barrett's conditioned medium (BCM) from Barrett's tissue explants which had been cultured for 24 h, or the same BCM samples in which CCL28 had been neutralised using an anti-CCL28 neutralising antibody (previously optimised as described in section 2.9.6.1). The BCM samples used in this experiment were known to contain measurable amounts of CCL28, measured previously using ELISA.

BCM was demonstrated to chemoattract increased percentages of CCR3+ T cells compared with medium alone ($p = 0.1$ vs. Ctrl) (Figure 5.14). When CCL28 in the BCM was neutralised, this decreased the percentage of CCR3+ T cells chemoattracted to the bottom chamber ($p = 0.22$). Similarly, BCM was chemotactic for increased percentages of CCR10+ T cells compared with medium alone ($p = 0.1$ vs. Ctrl), while neutralisation of CCL28 in this BCM significantly decreased the percentage of CCR10+ T cells in the bottom chamber ($p = 0.0235$ vs. BCM

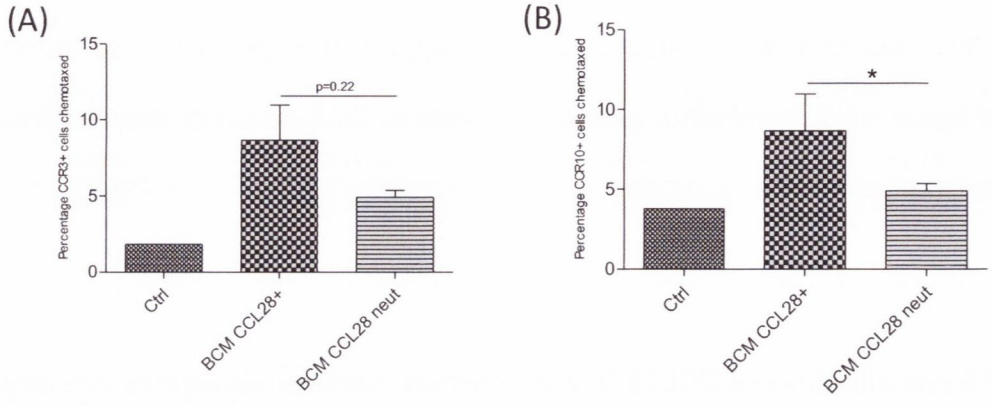


Figure 5.14. Barrett's explant conditioned media is chemotactic for CCR3+ and CCR10+ T cells; this chemotaxis is decreased by neutralisation of CCL28. T cells were isolated from a healthy donor. Cells were placed into the top chamber of a transwell chemotaxis insert in a 24-well plate. The bottom chamber of the wells contained either medium alone, Barrett's conditioned medium (BCM CCL28+) from cultured Barrett's tissue explants which was known to contain measurable amounts of CCL28 protein, or the same Barrett's conditioned medium in which CCL28 had been neutralised using an anti-CCL28 neutralising antibody (BCM CCL28 neut). (A) BCM is chemotactic for increased CCR3+ T cells compared with medium alone, while neutralisation of CCL28 in this BCM decreases chemotaxis of CCR3+ T cells. (B) BCM is chemotactic for increased CCR10+ T cells compared with medium alone, while neutralisation of CCL28 in this BCM decreases chemotaxis of CCR10+ T cells. Statistical analysis was performed using the paired student's t-test. *p = 0.0235 vs. BCM CCL28+.

CCL28+). This suggests that it is specifically the CCL28 protein in Barrett's conditioned medium that is chemotactic for CCR3- and CCR10-expressing immune cells, since neutralisation of CCL28 abrogates chemotaxis of these cells.

5.4 Discussion

Having demonstrated for the first time that the chemokine CCL28 is expressed by oesophageal tissue and by oesophageal cell lines derived from varying stages of oesophageal disease progression, the mechanisms by which CCL28 is regulated in oesophageal tissue were explored in further detail. The induction of CCL28 by the bile acid DCA suggested that transcription factors activated by DCA would play a major role in regulating CCL28 expression.

Transcription factors make up complex systems with a multitude of interactions. The transcription factor activation profiling array allowed examination of the activation of 96 transcription factors by DCA in the OE19 OAC cell line. The observation that 71 of the 96 transcription factors assayed were induced to some degree by DCA was unsurprising, as it is widely known that DCA is a potent activator of many intracellular pathways via the receptors FXR, PXR and the vitamin D receptor (Xie, Radominska-Pandya et al. 2001, Makishima, Lu et al. 2002, Capello, Moons et al. 2008). The DCA-induced activation of transcription factors such as NF-kB, ERK, AKT, and AMP-activated protein kinases can in turn activate downstream pathways (Hoffmann, Natoli et al. 2006, Wu, Gong et al. 2008, Wong and Tergaonkar 2009, Lee, Crawley et al. 2010). Indeed, NF-kB is known to be a "master regulator" of inflammatory pathways and many of the transcription factors assayed on the array in this study are under regulation by NF-

kB. Therefore it was unsurprising that NF-kB was one of the key transcription factors that was activated on the array, along with the transcription factors XBP1, KLF4, SMAD1 and YY1, which are all regulated by or regulators of NF-kB (Graham, Odero-Marah et al. 2009, Haberzettl, Vladykovskaya et al. 2009, Liao, Sharma et al. 2011, Siednienko, Maratha et al. 2011, Ye, Li et al. 2012). p53 is another key "master regulator" transcription factor, and again it was unsurprising that this was one of the key transcription factors identified in the activation profiling array. p53 interacts with several other transcription factors identified, including NFAT, IRF and XBP1 (Mori, Anazawa et al. 2002, Dornan, Eckert et al. 2004, Shinmen, Koshida et al. 2009, Dioufa, Chatzistamou et al. 2012).

On the transcription factor knockdown array, a number of transcription factors emerged as potential positive and negative regulators of CCL28. This array, while based on knockdown of transcription factors in the breast cancer cell line MCF-7, allowed screening of 270 transcription factors in one array to determine which of these regulated expression of CCL28. This meant it provided an invaluable tool to immediately screen this large number of factors, which otherwise would take an excessive amount of time to screen individually. The MCF-7 cell line constitutively expresses CCL28, and so screening this cell line would allow identification of definite regulators of CCL28 expression, which could then be validated in oesophageal cells. Many of the transcription factors identified as regulators of CCL28 in this array are modulated by NF-kB and p53, and six key transcription factors were identified in common by both arrays – they were both activated by DCA and positive regulators of CCL28 expression. These transcription factors were NF-kB, p53, XBP1, PPAR-α, YY1 and HIF-1α.

NF-kB has previously been demonstrated to regulate CCL28 expression in many tissue types, including airway epithelium and keratinocytes (O'Gorman, Jatoi et al. 2005, Kagami, Saeki et al. 2006), and as previously described, NF-kB can modulate, and be modulated by, many other transcription factors via downstream pathways and feedback loops, including XBP1 and PPAR-α (Lim, Ng et al. 2013, Remels, Gosker et al. 2013). NF-kB is also known to be induced by the cytokines IL-1β and TNF-α, and indeed in airway epithelial cells, CCL28 is induced by these cytokines via NF-kB (O'Gorman, Jatoi et al. 2005). It has also been demonstrated to be increased in BO, by DCA in OAC and across the oesophageal metaplasia-dysplasia-adenocarcinoma sequence (Jenkins, Harries et al. 2004, Konturek, Nikiforuk et al. 2004, O'Riordan, Abdel-latif et al. 2005, Debruyne, Witek et al. 2006). HIF-1α has also recently been identified as a regulator of CCL28 expression in ovarian epithelium as it is induced by tumour hypoxia in ovarian tumours (Facciabene, Peng et al. 2011). While hypoxia is the main stimulus for HIF-1α, it is also induced to a lesser extent by growth factors and cytokines, including IL-1β and TNF-α (Bhawal, Ito et al. 2012, Sartori-Cintra, Mara et al. 2012, Ghosh, Paul et al. 2013). p53 is a key tumour suppressor transcription factor which regulates over 100 genes; it is often mutated in neoplastic and pre-neoplastic conditions (Stegh 2012), which leads to loss of its tumour suppressor functions. p53 expression has been found to be increased in OAC and in BO compared with controls (Murray, Sedo et al. 2006). XBP1 is involved in endoplasmic reticulum (ER) stress – on accumulation of unfolded proteins in the ER, the XBP1 gene is processed to an active form and then activates the unfolded protein response. It can be regulated by NF-kB and p53, and is induced by IL-1β and IFN-γ (Miani, Colli et al. 2012), and regulates other chemokines including IL-8 and MCP1 and CXCL3 (Gargalovic,

Gharavi et al. 2006). DCA is known to induce Golgi fragmentation, affecting protein synthesis downstream from the ER, in the colon (Byrne, Foran et al. 2010). Finally, PPAR-α is a nuclear receptor which heterodimerises with the retinoid X receptor, which binds to bile acids. PPAR-α expression has been found to be increased in BO compared with both normal oesophagus and OAC (De Gottardi, Hadengue et al. 2008).

Here, for the first time, it is demonstrated that DCA-induced expression of the chemokine CCL28 is regulated in the oesophagus via the transcription factor NF-kB. NF-kB was chosen to validate in an oesophageal cell line as it is a key player in the inflammatory process and has also been shown to regulate CCL28 expression in other tissue types (Ogawa, Iimura et al. 2004, O'Gorman, Jatoi et al. 2005). Stable overexpression of the active p65 subunit of NF-kB in an oesophageal cell line allowed multiple experiments to be carried out in a controlled environment. The response to DCA had previously been noted to be variable in oesophageal cell lines, which suggested that transient transfection of the p65-overexpressing plasmid would introduce even more variability into this experimental setup. Treatment of the p65-overexpressing oesophageal cell lines with DCA induced increased CCL28 protein secretion compared with the parent untransfected cell line. This supports the hypothesis that CCL28 is regulated via NF-kB in response to DCA stimulation.

In further support of this hypothesis, treatment of these cell lines with the NF-kB inhibitor DHMEQ decreased DCA-induced expression of CCL28 almost to basal levels. Interestingly, treatment with DHMEQ completely decreased DCA-induced CCL28 expression, suggesting that inhibiting NF-kB completely inhibits expression of CCL28 in response to bile acids. This suggests that the other

transcription factors identified in our arrays, XBP1, PPARα, HIF-1α and p53, may be under regulation via NF-kB. NF-kB is known to regulate these transcription factors, as demonstrated in the Ingenuity Pathway Analysis, and so inhibiting NF-kB as the "master" transcription factor may inhibit the other factors downstream. While NF-kB has now been validated as a regulator of CCL28, the other transcription factors may potentially provide more specific targets in the future for manipulation of CCL28 expression, as they may be more specific for regulation of CCL28 expression, without affecting as many other cellular processes as NF-kB.

In order to fully examine the role of the chemokine CCL28 in the oesophagus, it is necessary to demonstrate that it is functional in its role as a chemokine; CCL28 is known to chemoattract immune cells expressing the receptors CCR3 and CCR10, including eosinophils, CD4+ and CD8+ effector T cells, T-regulatory cells and plasma cells (Pan, Kunkel et al. 2000, Hieshima, Ohtani et al. 2003, John, Thomas et al. 2005, Eksteen, Miles et al. 2006, Hansson, Hermansson et al. 2008, Facciabene, Peng et al. 2011, Scanlon, Hawksworth et al. 2011). To investigate this, the transwell chemotaxis system was used. This is the standard method of examining chemotaxis, in which cells in a top chamber are allowed to migrate in response to a factor in the bottom chamber through a membrane in which pores have been created of a specific size, in this case 5 μm in diameter. Foetal bovine serum (FBS) contains a multitude of growth factors, cytokines and chemokines and therefore medium containing 20% FBS was used as a positive control for chemotaxis assays, while medium without FBS was used as a negative control. Recombinant human CCL5 (RANTES) is known to bind to the CCR3 receptor, and as expected it was chemotactic for increased numbers of T cells, as was medium containing 20% FBS, compared with medium alone.

rhCCL5 also chemoattracted an increased proportion of CCR3+ T cells, which again was an expected result. However, rhCCL28 did not chemoattract either CCR3 or CCR10-expressing T cells. This may be due to the recombinant protein being dysfunctional, or having degraded. Interestingly, cell culture conditioned medium from OAC cells treated with DCA, which was known to contain CCL28 produced by the cells, was chemotactic for CCR3+ T cells, chemoattracting increased percentages of CCR3+ cells compared with the positive control containing 20% FBS. However, the same increases were not seen in the chemotaxis of CCR10+ cells. The chemotaxis of CCR3+ cells demonstrates that the CCL28 protein secreted by the oesophageal cells is functional. One limitation to this experiment is that the DCA in the cell conditioned medium was moderately toxic to the T cells which migrated to the bottom chamber, and therefore a lower number of cells was collected for flow cytometric analysis from these chambers. This would also explain why lower numbers of cells were counted in the bottom chambers of these wells compared with the positive control containing 20% FBS.

The chemotaxis assay was carried out using BO conditioned medium, which was generated by culturing tissue explants from patients with BO in medium for 24 h. Medium from the BO tissue explants used was assayed using ELISA and was found to contain measurable amounts of CCL28 protein. This conditioned medium was chemotactic for CCR3+ and CCR10+ T cells compared with medium alone, and this chemotaxis was abrogated by neutralisation of CCL28 in the Barrett's conditioned medium. This suggests that it is the CCL28 alone in the conditioned medium that is responsible for the chemotaxis of the CCR3+ and CCR10+ T cells towards the bottom chamber of the assay. This is notable, as it demonstrates for the first time that the CCL28 expressed by human oesophageal tissue is

immunomodulatory, by chemoattracting immune cells bearing the receptors CCR3 and CCR10.

This chapter has further investigated the mechanisms via which CCL28 expression is regulated in the oesophagus, and has also demonstrated that CCL28 secreted by the oesophagus is capable of modulating the immune environment of the tissue. In the next chapter, we examine expression of CCL28 *in vivo* during the metaplasia-dysplasia-adenocarcinoma sequence of oesophageal disease progression in order to investigate further the role of this chemokine.

Chapter 6

CCL28 in oesophageal tissue microarrays

6.1 Introduction

The oesophageal disease sequence progresses with exposure of the oesophagus to gastro-oesophageal refluxate, from normal squamous epithelium to Barrett's metaplastic epithelium, to low-grade and high-grade dysplasia and then to OAC. As the majority of patients with BO do not progress to dysplasia or OAC, it is of vital importance to investigate biomarkers that could identify the subgroup of patients with BO whose disease will progress (di Pietro, Peters et al. 2008, Ong, Lao-Sirieix et al. 2010). To do this, tissue can be obtained from patients with various stages of the disease, and also from patients who have progressed or regressed in the disease sequence, to investigate whether a biomarker varies during this time. Identification of biomarkers can lead to development of targeted drugs. Biomarkers can also help to identify patients who have a better or worse prognosis once cancer has been diagnosed, or to stratify response to various therapies.

The use of the tissue microarray (TMA), in which multiple tissue samples are analysed on one block, was first described in 1998 (Kononen, Bubendorf et al. 1998), and provided a method by which samples from large numbers of patients in various stages of disease could be investigated for potential prognostic and predictive biomarkers. The most commonly used TMA type is the formalin-fixed paraffin-embedded (FFPE) type; previously fixed tissue is histologically analysed, and appropriate cores of the tissue type needed are transferred into a paraffin block in a pre-determined grid pattern (Vogel 2009, Warren, Chan et al. 2011) (Figure 6.1). TMAs can be categorised by their application: to establish markers that predict response to therapy, to validate biomarkers that have been identified in gene or protein extracts, prognostic TMAs to correlate a marker with clinical data,

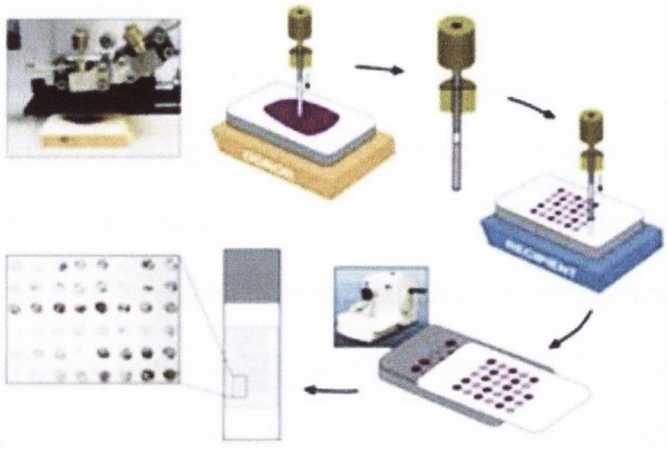


Figure 6.1. Construction of tissue microarrays. The arrays are assembled by taking core needle "biopsies" from specific locations in pre-existing paraffin-embedded tissue blocks and re-embedding them in an arrayed "master" block, using techniques and an apparatus developed by Konenen et al. In this way, tissue from many specimens can be represented in a single paraffin block. Source: Yale School of Medicine

and TMAs that predict progression. The number of cores from each tissue type used in the TMA needs to be considered; studies have shown that taking three cores from a particular tissue sample correlates well with the whole tissue sample (Hoos, Urist et al. 2001, O'Grady, Flahavan et al. 2003). Collection of appropriate clinical data is also of importance. Tissue microarray technology has been used previously for analysis of BO and OAC tissue, in order to try to identify biomarkers for disease progression (Hu, Bandla et al. 2011, Dimaio, Kwok et al. 2012, Streppel, Vincent et al. 2012, Vega, May et al. 2012).

The survival rates for oesophageal cancer are extremely poor, with recent 5-year survival figures of 10.3% for squamous cell carcinoma and 14.6% for adenocarcinoma (Lagergren and Mattson 2012), although survival rates for OAC are improving, with 5-year survival doubling in the last 20 years in the Netherlands (Dikken, Lemmens et al. 2012), and also increasing in Sweden. Survival rates for OAC are much higher with earlier tumour stage (Mariette, Taillier et al. 2004, Wang, Yuan et al. 2009), with patients undergoing surgery for OAC having a 2-year disease-free survival of 85.6% for stage I disease, compared with 26.3% for stage IV disease (Reid, Davies et al. 2012). However, the poor overall prognosis for OAC is primarily because patients generally present at a late stage of disease: in the above study by Reid et al., only 8.3% of the total number of patients undergoing surgery for OAC had stage I disease, while 67% had stage III disease and 59% had nodal disease, with other studies reporting similar rates (Schlansky, Dimarino et al. 2006). Since BO is recognised as being a risk factor for development of OAC, with estimates of progression to OAC of 0.6% per year (Yousef, Cardwell et al. 2008), patients in Ireland with histologically confirmed BO are offered routine surveillance endoscopy, with 2-yearly endoscopy and biopsies

of the lower oesophagus. The cost-effectiveness of this surveillance is comparable to many other cancer screening strategies, with an estimated cost of $12,140 per life-year gained compared with no screening (Gerson, Groeneveld et al. 2004). As demonstrated by the survival figures for stage of OAC, early diagnosis is vital to improve treatment outcomes and survival (Bird-Lieberman and Fitzgerald 2009), and OAC diagnosed from BO surveillance presents at an earlier stage than those presenting spontaneously (Schlansky, Dimarino et al. 2006).

Treatment for OAC is controversial. Surgical resection, or oesophagectomy, is considered to be the standard treatment option for operable tumours, with either the transthoracic or transhiatal approach used (Hulscher, van Sandick et al. 2002, Mariette, Piessen et al. 2007). Surgical outcome is heavily dependent on surgeon volume, with the recommendation that all oesophagectomies should be carried out in high-volume centres of expertise (Birkmeyer, Siewers et al. 2002, Verhoef, van de Weyer et al. 2007). Interestingly, a recent study compared definitive chemoradiation therapy (CRT) with surgery for oesophageal cancer and found similar disease-free survival rates in stages III and IV disease, although rates were higher for surgical treatment in earlier stages (Reid, Davies et al. 2012). Current recommendations, however, are that all patients with operable oesophageal tumours should have neoadjuvant CRT except for those with exceptionally early stage (T1) tumours (Walsh 2011, Wolf, Stahl et al. 2011), with a recent meta-analysis demonstrating a clear survival benefit for neoadjuvant CRT with surgery over surgery alone (Sjoquist, Burmeister et al. 2011). However, the main drawback of neoadjuvant CRT is that only a subgroup of patients have a response to CRT, with an even smaller subgroup demonstrating a complete pathological response (Reynolds, Muldoon et al. 2007), with this group of patients having an improved

disease-free and overall survival (Donington, Miller et al. 2003, Schneider, Baldus et al. 2005, Swisher, Hofstetter et al. 2005).

Given the continuing poor survival statistics and the controversy over treatment options for patients with OAC, it is of vital importance to identify those patients who are at increased risk of progression from BO to OAC, as these patients may be offered either therapeutic options or else increased surveillance compared to the overall BO population. Identifying OAC patients at an early stage would dramatically improve outcomes, given the statistics for stage I disease previously described. It would also be of benefit to identify the OAC patients whose tumours would respond to CRT, thus allowing maximum benefit for these patients, while allowing patients whose tumours would not respond to escape the deleterious side effects of CRT.

In this chapter we further investigate the protein expression of CCL28 in vivo in oesophageal tissue from each stage of the oesophageal disease sequence, and also in oesophageal cancer tissue in order to correlate CCL28 expression with various clinical parameters including history of Barrett's oesophagus, lymph node status and CRT.

6.2 Aims and objectives

The chemokine CCL28 has been demonstrated to be expressed in both *in vitro* and *ex vivo* models. Here its expression is examined *in vivo* using paraffin-embedded tissue from patients with varying stages of oesophageal disease in order to further elucidate its role in oesophageal cancer development.

Specific objectives:

- Examine immunohistochemical staining for CCL28 in tissue from patients with varying stages of oesophageal disease from normal oesophagus to oesophageal adenocarcinoma and correlate with clinical data
- Examine immunohistochemical staining for CCL28 in tissue from patients with oesophageal cancer and correlate with clinical data including overall survival
- Examine immunohistochemical staining for CCL28 in pre-treatment tissue from patients with oesophageal adenocarcinoma undergoing neo-adjuvant chemoradiation therapy and correlate with tumour regression grade.

6.3 Results

6.3.1 Optimisation of immunohistochemical staining for CCL28, CCR3 and CCR10 on full-face sections.

Antibodies for CCL28, CCR3 and CCR10 were purchased and optimised on full-face sections. Sections from paraffin-embedded colon tissue were cut and mounted onto glass slides. Slides were de-paraffinised, antigen retrieval performed and staining for each antigen was optimised using three concentrations of the appropriate antibody. A negative control was included in each optimisation (appendix 3). The anti-CCL28 antibody was used at dilutions of 1/40, 1/80 and 1/120. The 1/40 dilution was deemed to produce optimal staining (Figure 6.2(A)). The anti-CCR3 antibody was used at dilutions of 1/100, 1/150 and 1/300. The 1/150 dilution was deemed to produce optimal staining (Figure 6.2(B)). The anti-

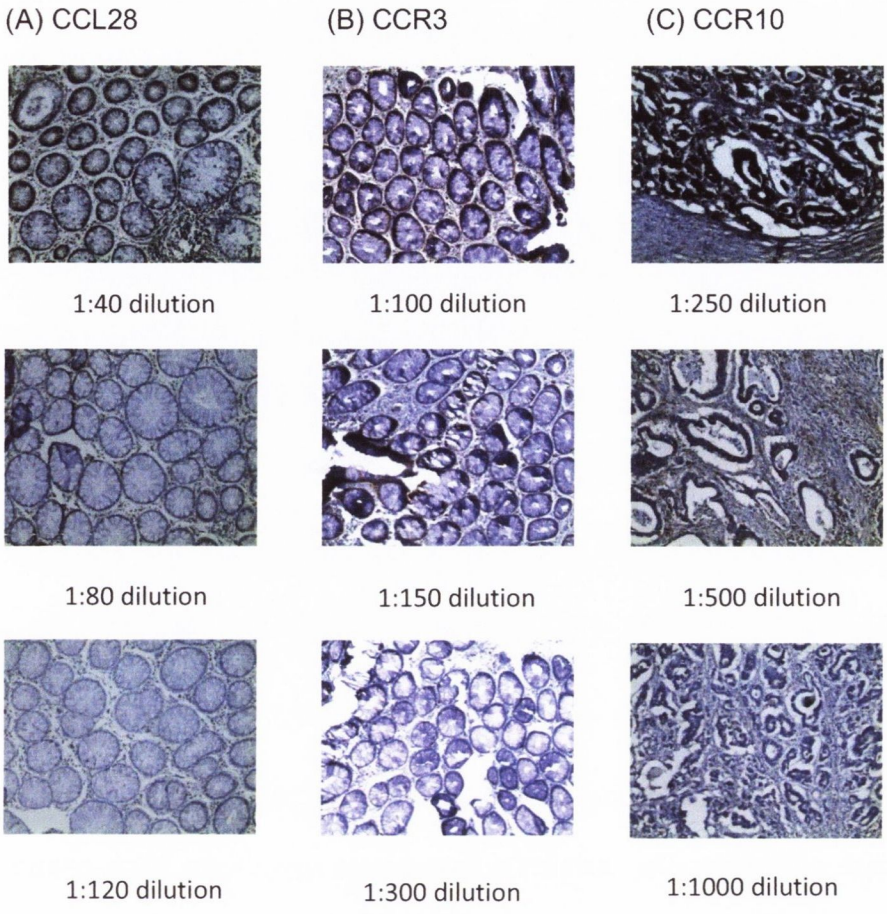


Figure 6.2 Optimisation of immunohistochemical staining for CCL28, CCR3 and CCR10 on full-face sections. Antibodies for CCL28, CCR3 and CCR10 were purchased and optimised on full-face sections. Sections from paraffin-embedded colon tissue were cut and mounted onto glass slides. Slides were de-paraffinised, antigen retrieval performed and staining for each antigen was optimised using three concentrations of the appropriate antibody. A negative control was included in each optimisation (shown in Appendix 3). (A) The anti-CCL28 antibody was used at dilutions of 1/40, 1/80 and 1/120. The 1/40 dilution was deemed to produce optimal staining. (B) The anti-CCR3 antibody was used at dilutions of 1/100, 1/150 and 1/300. The 1/150 dilution was deemed to produce optimal staining. (C) The anti-CCR10 antibody was used at dilutions of 1/250, 1/500 and 1/1000. The 1:500 dilution was deemed to produce optimal staining.

CCR10 antibody was used at dilutions of 1/250, 1/500 and 1/1000. The 1:500 dilution was deemed to produce optimal staining (Figure 6.2(C)).

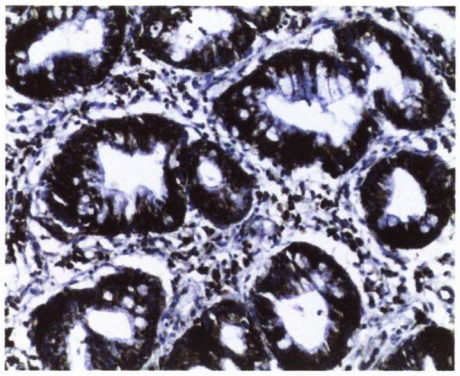
6.3.2 Immunohistochemical staining on full-face sections of Barrett's tissue for CCL28, CCR3 and CCR10

Full-face sections of Barrett's oesophageal tissue were stained for CCL28, CCR3 and CCR10 using the previously optimised technique. CCL28 staining was found to correlate with CCR3 and CCR10 staining in Barrett's tissue sections (Figure 6.3).

6.3.3 Immunohistochemical staining of CCL28 in tissue microarrays containing normal, oesophagitis, Barrett's, low-grade dysplasia, high-grade dysplasia and oesophageal adenocarcinoma

Tissue microarrays (TMAs) were constructed using cores of tissue from normal, oesophagitis, Barrett's (BO), low-grade dysplasia (LGD), high-grade dysplasia (HGD) and oesophageal adenocarcinoma (OAC) from 81 patients. Demographic data for patients used in these TMAs are shown in Table 6.1. TMAs were stained for CCL28 using the previously optimised antibody. Slides were scanned and staining was graded for percentage positive staining and intensity of staining in both epithelium and stroma by two independent graders and results were averaged.

(A)



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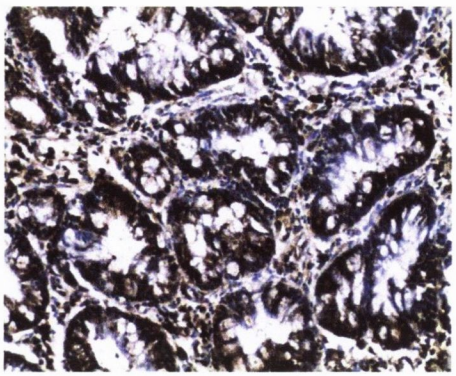


(C)

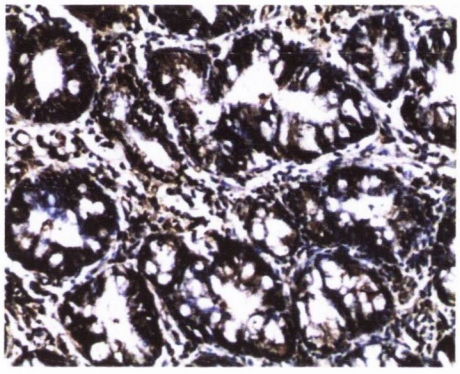


Figure 6.3 Immunohistochemical staining for CCL28, CCR3 and CCR10 on full-face sections of Barrett's oesophageal tissue. Full-face sections of Barrett's oesophageal tissue were stained for CCL28, CCR3 and CCR10 using the previously optimised technique. (A) CCL28 staining demonstrated positive staining in Barrett's epithelium and in stroma. (B) CCR3 staining demonstrated positive staining in the same section of Barrett's epithelium and in stroma. (C) CCR10 staining demonstrated positive staining in the same sections of Barrett's epithelium and stroma.

Table 6.1 Characteristics of patients used in Barrett's tissue microarrays

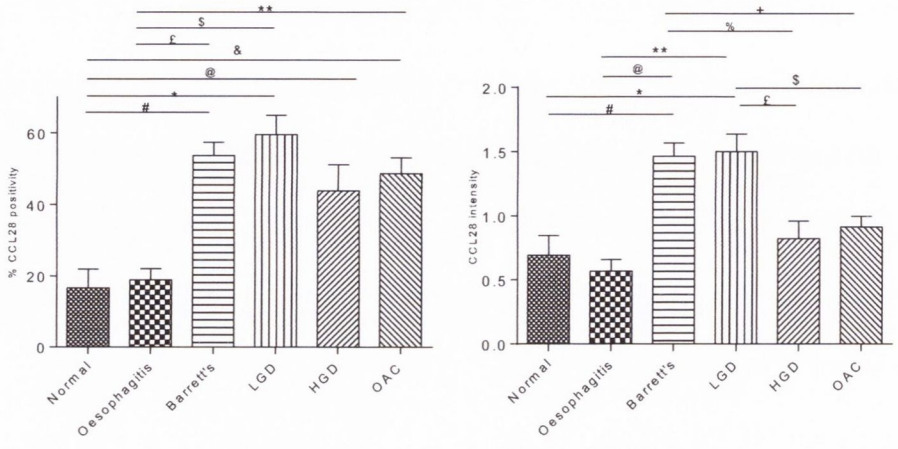
			Female	Male
Gender			33	48
		Total	Female	Male
Age		57.75	55.13333	58.97656
Per histology		Male	Female	Total
	Normal	9	8	17
	Oesophagitis	19	22	41
	Barrett's	50	16	66
	LGD	26	6	32
	HGD	8	4	12
	OAC	16	4	20
		Age (total)	Age (male)	Age (female)
Per histology				
	Normal	42.41176	43.77778	40.875
	Oesophagitis	51.34146	51.52632	51.18182
	Barrett's	59.63636	58.26	63.9375
	LGD	60.28125	59.96154	61.66667
	HGD	63.33333	66.25	57.5
	OAC	70.3	73.375	58

*LGD – low-grade dysplasia, HGD – high grade dysplasia, OAC – oesophageal adenocarcinoma

The percentage of epithelium staining positive for CCL28 was increased in Barrett's tissue compared with normal and oesophagitis tissue (53.66 ± 3.752 vs. 16.54 ± 5.290, p = 0.0001 vs. Normal; 53.66 ± 5.117 vs. 18.88 ± 3.108, p < 0.0001 vs. Oesophagitis) (Figure 6.4(A)). It was also increased in LGD tissue compared with normal and oesophagitis tissue (59.55 ± 5.413 vs. 16.54 ± 5.290, p < 0.0001 vs. Normal; 59.55 ± 5.413 vs. 18.88 ± 3.108, p < 0.0001 vs. Oesophagitis), as well as in HGD tissue compared with normal and oesophagitis tissue. CCL28 epithelial percentage staining was increased in OAC compared with normal and oesophagitis tissue (48.64 ± 4.450 vs. 16.54 ± 5.290, p = 0.0002 vs. Normal; 48.64 ± 4.450 vs. 18.88 ± 3.108, p < 0.0001 vs. Oesophagitis).

The intensity of epithelial CCL28 staining was increased in Barrett's and LGD tissue compared with normal tissue (Barrett's: 1.462 ± 0.1053 vs. 0.6923 ± 0.1546, p = 0.0018 vs. Normal; LGD: 1.500 ± 0.1359 vs. 0.6923 ± 0.1546, p = 0.0007 vs. Normal) (Figure 6.4(B)). CCL28 intensity was also increased in Barrett's and LGD tissue compared with oesophagitis tissue (Barrett's: 1.462 ± 0.1053 vs. 0.5696 ± 0.0877, p < 0.0001 vs. Oesophagitis; LGD 1.500 ± 0.1359 vs. 0.5696 ± 0.0877, p < 0.0001 vs. Oesophagitis). Decreased CCL28 staining intensity was also demonstrated in HGD and OAC tissue compared with Barrett's tissue (HGD: 0.8235 ± 0.1366 vs. 1.462 ± 0.1053, p = 0.0039 vs. Barrett's; OAC: 0.9143 ± 0.0807 vs. 1.462 ± 0.1053, p = 0.0013 vs. Barrett's) and LGD tissue (HGD: 0.8235 ± 0.1366 vs. 1.500 ± 0.1359, p = 0.0010 vs. LGD; OAC: 0.9143 ± 0.080 vs. 1.500 ± 0.1359, p = 0.0002 vs. LGD).

When stromal staining of CCL28 was examined, the percentage of stroma staining positive for CCL28 was increased in Barrett's and LGD tissue compared with normal tissue (Barrett's: 24.95 ± 3.847 vs. 9.167 ± 5.782, p = 0.0116 vs.



(A) epithelial percentage positivity (B) epithelial intensity

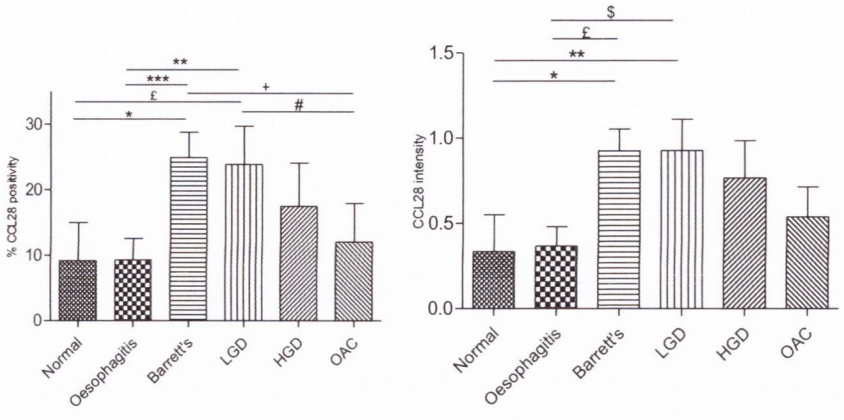
Figure 6.4 Immunohistochemical epithelial staining of CCL28 demonstrates increased percentage positivity and intensity in Barrett's tissue and low grade dysplasia compared with normal and oesophagitis tissue. Tissue microarrays (TMAs) were constructed using cores of tissue from normal, oesophagitis, Barrett's (BO), low-grade dysplasia (LGD), high-grade dysplasia (HGD) and oesophageal adenocarcinoma (OAC) from 81 patients. TMAs were cut, mounted onto slides and stained for CCL28 using the previously optimised antibody. Slides were scanned and epithelial staining was graded for percentage positive staining and intensity of staining by two independent graders and results were averaged. (A) The percentage of epithelium staining positive for CCL28 was increased in Barrett's, LGD, HGD and OAC tissue compared with normal and oesophagitis tissue. Data are presented as mean ± SEM. Statistical analysis was performed using the Mann-Whitney U test. $^{\&}p = 0.0002$ vs. Normal, $^{\#}p = 0.0001$ vs. Normal, @p = 0.024 vs. Normal, *p < 0.0001 vs. Normal, $^{£}p < 0.0001$ vs. Oesophagitis, $^{\$}p < 0.0001$ vs. Oesophagitis, **p < 0.0001 vs. Oesophagitis. (B) The intensity of epithelial CCL28 staining was increased in Barrett's and LGD tissue compared with normal and oesophagitis tissue. CCL28 intensity was also increased in BO and LGD tissue compared with HGD and OAC tissue. Data are presented as mean ± SEM. Statistical analysis was performed using the Mann-Whitney U test. $^{\#}p = 0.0018$ vs. Normal, *p = 0.0007 vs. Normal, @p < 0.0001, **p < 0.0001 vs. Oesophagitis, $^{£}p = 0.001$ vs. LGD, $^{\$}p = 0.0002$ vs. LGD, %p = 0.0039 vs. Barrett's, +p = 0.0013 vs. Barrett's.

Normal; LGD: 23.92 ± 5.753 vs. 9.167 ± 5.782, p = 0.0115 vs. Normal) and also compared with oesophagitis tissue (Barrett's: 24.95 ± 3.847 vs. 9.271 ± 3.276, p = 0.0009 vs. Oesophagitis; LGD: 23.92 ± 5.753 vs. 9.271 ± 3.276, p = 0.0016 vs. Oesophagitis) (Figure 6.5(A)). It was also increased in Barrett's and LGD compared with OAC tissue (Barrett's: 24.95 ± 3.847 vs. 12.06 ± 5.842, p = 0.0788 vs. OAC; LGD: 23.92 ± 5.753 vs. 12.06 ± 5.842, p = 0.0840 vs. OAC).

The intensity of stromal CCL28 staining was increased in Barrett's and LGD tissue compared with normal tissue (Barrett's: 0.9253 ± 0.1284 vs. 0.3333 ± 0.2164, p = 0.0105 vs. Normal; LGD: 0.9263 ± 0.1845 vs. 0.3333 ± 0.2164, p = 0.008 vs. Normal) and also compared with oesophagitis tissue (Barrett's: 0.9253 ± 0.1284 vs. 0.3667 ± 0.1131, p = 0.0011 vs. Oesophagitis; LGD: 0.9263 ± 0.1845 vs. 0.3667 ± 0.1131, p = 0.0021 vs. Oesophagitis) (Figure 6.5).

6.3.4 CCL28 immunohistochemical staining in tissue microarrays comparing normal tissue from patients with Barrett's oesophagus, low-grade dysplasia and oesophageal adenocarcinoma

Tissue microarrays (TMAs) were constructed using cores of tissue from normal, oesophagitis, Barrett's (BO), low-grade dysplasia (LGD), high-grade dysplasia (HGD) and oesophageal adenocarcinoma (OAC). TMAs were stained for CCL28 using the previously optimised antibody. Slides were scanned and staining was graded for percentage positive staining and intensity of staining in both epithelium and stroma by two independent graders and results were averaged. Tissue cores were reviewed by a consultant histopathologist and it was found that certain cores contained only normal tissue from patients known to have BO, LGD



(A) stromal percentage positivity (B) stromal intensity

Figure 6.5 Immunohistochemical stromal staining of CCL28 demonstrates increased percentage positivity and intensity in Barrett's tissue and low grade dysplasia compared with normal and oesophagitis tissue. Tissue microarrays (TMAs) were constructed using cores of tissue from normal, oesophagitis, Barrett's (BO), low-grade dysplasia (LGD), high-grade dysplasia (HGD) and oesophageal adenocarcinoma (OAC) from 81 patients. TMAs were cut, mounted onto slides and stained for CCL28 using the previously optimised antibody. Slides were scanned and stromal staining was graded for percentage positive staining and intensity of staining by two independent graders and results were averaged. (A) The percentage of stroma staining positive for CCL28 was increased in Barrett's and LGD tissue compared with normal and oesophagitis tissue. It was also increased in Barrett's and LGD compared with OAC tissue. Data are presented as mean ± SEM. Statistical analysis was performed using the Mann-Whitney U test. $^{\#}p$ = 0.0840 vs. LGD, ^{+}p = 0.0788 vs. Barrett's, *p = 0.0116 vs. Normal, $^{£}p$ = 0.0115 vs. Normal, **p = 0.0016 vs. Oesophagitis, ***p = 0.0009 vs. Oesophagitis. (B) The intensity of stromal CCL28 staining was increased in Barrett's and LGD tissue compared with normal and oesophagitis tissue. Data are presented as mean ± SEM. Statistical analysis was performed using the Mann-Whitney U test. *p = 0.0105 vs. Normal, **p = 0.0080 vs. Normal, $^{£}p$ = 0.0011 vs. Oesophagitis, $^{\$}p$ = 0.0021 vs. Oesophagitis.

or OAC. These normal cores were compared with normal tissue from healthy patients.

The percentage of epithelium staining positive for CCL28 was increased in normal tissue from patients with BO, LGD and OAC compared with normal tissue from patients with normal oesophagus (BO: $p = 0.0006$ vs. Normal, LGD: $p = 0.0004$ vs. Normal, OAC: $p = 0.0001$ vs. Normal). There was no difference observed between normal tissue and abnormal tissue from patients with BO, LGD or OAC (Figure 6.6(A)).

The intensity of epithelial CCL28 staining was increased in normal tissue from patients with BO, LGD and OAC compared with normal tissue from patients with normal oesophagus (BO: $p = 0.007$ vs. Normal, LGD: $p = 0.0023$ vs. Normal, OAC: $p = 0.035$ vs. Normal). There was no difference observed between normal tissue and abnormal tissue from patients with BO, LGD or OAC (Figure 6.6(B)).

6.3.5 Logistic regression analysis correlating CCL28 staining with progression from BO to LGD, HGD and OAC.

Tissue microarrays (TMAs) were constructed using cores of tissue from Barrett's (BO) tissue. TMAs were cut, mounted onto slides and stained for CCL28 using the previously optimised antibody. Slides were scanned and epithelial staining was graded for percentage positive staining and intensity of staining by two independent graders and results were averaged. Patients with Barrett's oesophagus were stratified into groups based on whether they had progressed to

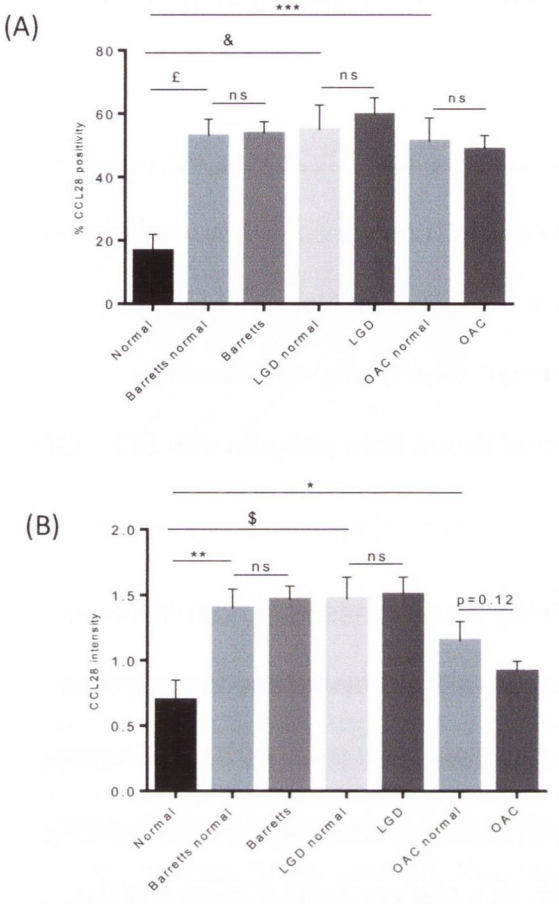


Figure 6.6 Immunohistochemical epithelial staining of CCL28 demonstrates increased percentage positivity and intensity in normal tissue from patients with BO, LGD and OAC compared with normal tissue from normal patients. Tissue microarrays (TMAs) were constructed using cores of tissue from normal, oesophagitis, Barrett's (BO), low-grade dysplasia (LGD), high-grade dysplasia (HGD) and oesophageal adenocarcinoma (OAC) from 81 patients. TMAs were cut, mounted onto slides and stained for CCL28 using the previously optimised antibody. Slides were scanned and epithelial staining was graded for percentage positive staining and intensity of staining by two independent graders and results were averaged. (A) The percentage of epithelium staining positive for CCL28 was increased in normal tissue from patients with BO, LGD and OAC compared with normal tissue from patients with normal oesophagus. There was no difference observed between normal tissue and abnormal tissue from patients with BO, LGD or OAC. Data are presented as mean ± SEM. Statistical analysis was performed using the Mann-Whitney U test. $^{£}p = 0.0006$ vs. Normal, $^{\&}p = 0.0004$ vs. Normal, ***p = 0.0001 vs. Normal. (B) The intensity of epithelial CCL28 staining was increased in normal tissue from patients with BO, LGD and OAC compared with normal tissue from patients with normal oesophagus. There was no difference observed between normal tissue and abnormal tissue from patients with BO, LGD or OAC. Data are presented as mean ± SEM. Statistical analysis was performed using the Mann-Whitney U test. **p = 0.007 vs. Normal, $^{\$}p = 0.0023$ vs. Normal *p = 0.035 vs. Normal.

LGD, HGD or OAC during follow up. Results were analysed using logistic regression analysis.

Mean and maximum values for CCL28 intensity and percentage positivity correlated with progression from BO to LGD, HGD or OAC, reaching significance for the maximal intensity of CCL28 staining in BO tissue (p=0.021) (Figure 6.7, Figure 6.8). Mean and maximum values for CCL28 intensity and percentage positivity correlated with progression from BO to HGD or OAC, reaching significance for the mean and maximal intensity and mean percentage positivity of CCL28 staining in BO tissue (mean intensity p = 0.017, maximal intensity p = 0.023, mean percentage positivity p = 0.037). Mean and maximum values for CCL28 intensity and percentage positivity correlated with progression from BO to OAC, reaching significance for the mean and maximal intensity and mean percentage positivity of CCL28 staining in BO tissue (mean intensity p = 0.027, maximal intensity p = 0.032, mean percentage positivity p = 0.039).

6.3.6 CCL28 immunohistochemical staining in tissue microarrays containing tissue from oesophageal cancer patients

Tissue cores from 125 patients with oesophageal cancer were taken and used to construct tissue microarrays (TMAs). Demographic data on patients included in the TMAs are shown in Table 6.2. TMAs were stained for CCL28 using the previously optimised antibody. Slides were scanned and staining was graded for percentage positive staining and intensity of staining by two independent graders and results were averaged.

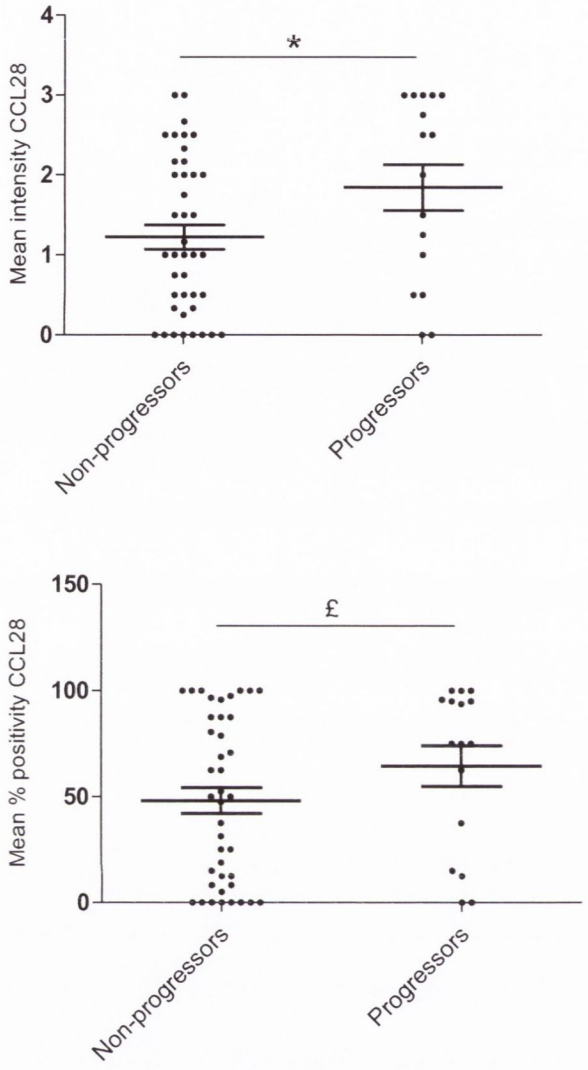


Figure 6.7 Logistic regression analysis demonstrates that mean CCL28 intensity and percentage positivity correlates with progression to OAC. Tissue microarrays (TMAs) were constructed using cores of tissue from Barrett's (BO) tissue. TMAs were cut, mounted onto slides and stained for CCL28 using the previously optimised antibody. Slides were scanned and epithelial staining was graded for percentage positive staining and intensity of staining by two independent graders and results were averaged. Patients with Barrett's oesophagus were stratified into groups based on whether they had progressed to OAC during follow up. Results were analysed using logistic regression analysis. (A) Mean values for CCL28 intensity correlated with progression to OAC. (B) Mean values for CCL28 percentage positivity correlated with progression to OAC. *p = 0.027, £p = 0.039

(A)	*LGD - HGD - OAC progressors*			
Measure	**Statistic**	**% Progressors**	**Odds ratio**	**p-value**
CCL28 Intensity	Mean	31.5%	1.415	0.061
	Max	*31.5%*	*1.496*	***0.021***
CCL28 % positivity	Mean	31.5%	1.009	0.057
	Max	*31.5%*	*1.008*	*0.076*

(B)	*HGD - OAC progressors*			
Measure	**Statistic**	**% Progressors**	**Odds ratio**	**p-value**
CCL28 Intensity	Mean	24.8%	1.617	**0.017**
	Max	*24.8%*	*1.532*	***0.023***
CCL28 % positivity	Mean	24.8%	1.011	**0.037**
	Max	*24.8%*	*1.099*	*0.071*

(C)	*OAC only progressors*			
Measure	**Statistic**	**% Progressors**	**Odds ratio**	**p-value**
CCL28 Intensity	Mean	21.2%	1.6	**0.027**
	Max	*21.2%*	*1.528*	***0.032***
CCL28 % positivity	Mean	21.2%	1.011	**0.039**
	Max	*21.2%*	*1.01*	*0.063*

Figure 6.8 Logistic regression analysis demonstrates that CCL28 intensity and percentage positivity correlates with progression to LGD, HGD and OAC based on both mean and maximum values. Tissue microarrays (TMAs) were constructed using cores of tissue from Barrett's (BO) tissue. TMAs were cut, mounted onto slides and stained for CCL28 using the previously optimised antibody. Slides were scanned and epithelial staining was graded for percentage positive staining and intensity of staining by two independent graders and results were averaged. Patients with Barrett's oesophagus were stratified into groups based on whether they had progressed to LGD, HGD or OAC during follow up. Results were analysed using logistic regression analysis. (A) Mean and maximum values for CCL28 intensity and percentage positivity correlated with progression to LGD, HGD or OAC, reaching significance for the maximal intensity of CCL28 staining in BO tissue. (B) Mean and maximum values for CCL28 intensity and percentage positivity correlated with progression to HGD or OAC, reaching significance for the mean and maximal intensity and mean percentage positivity of CCL28 staining in BO tissue. (C) Mean and maximum values for CCL28 intensity and percentage positivity correlated with progression to OAC, reaching significance for the mean and maximal intensity and mean percentage positivity of CCL28 staining in BO tissue.

Table 6.2 Characteristics of patients used in oesophageal cancer tissue microarrays

Gender			
Male	74		
Female	51		
Age at diagnosis (years)	Mean	Range	
Male	64.78378	37-84	
Female	66.92157	26-84	
BMI at diagnosis			
Male	24.75257	18.5-34.7	
Female	22.80333	16.7-38	
Days follow up	684.954	27-3276	
Histology			
Adenocarcinoma	87	Male	66
		Female	21
Adenosquamous carcinoma	2	Male	2
		Female	0
Squamous cell carcinoma	36	Male	6
		Female	30

There were no differences in CCL28 staining between male and female patients (35.77 ± 6.916 vs. 31.50 ± 5.636), patients with body mass index (BMI) less than and greater than 25 (28.52 ± 6.462 vs. 27.39 ± 7.640) and patients whose age at diagnosis was less than and greater than 70 years (35.04 ± 6.227 vs. 32.72 ± 7.066) (Figure 6.9).

Tissue from patients with oesophageal adenocarcinoma (OAC) with a history of Barrett's oesophagus (BO) demonstrated increased CCL28 staining than tissue from patients with OAC with no history of BO (38.37 ± 11.09 vs. 23.89 ± 6.708, p = 0.0256 vs. BO negative) (Figure 6.10(A)). Tissue from patients whose tumour histology was squamous cell carcinoma (SCC) had increased CCL28 staining compared with those whose tumour histology was OAC (39.24 ± 7.177 vs. 32.60 ± 5.996, p = 0.0249 vs. OAC) (Figure 6.10(B)). Tissue from patients whose tumour was classified as oesophagogastric junction (OGJ) type I demonstrated increased CCL28 staining compared with tissue from patients with OGJ type II or OGJ type III tumours (OGJ II: 57.31 ± 13.17 vs. 20.37 ± 13.00, p = 0.011 vs. OGJ II; OGJ III: 57.31 ± 13.17 vs. 22.03 ± 6.673, p = 0.0071 vs. OGJ III) (Figure 6.10(C)).

Clinical T staging correlates with CCL28 staining, with a trend towards increased CCL28 expression in clinical T stages 1-2 compared with clinical T stages 3-4 (48.21 ± 13.03 vs. 24.09 ± 4.607, p = 0.0739 vs. T3-4) (Figure 6.11(A)). Pathological T staging correlates with CCL28 staining, with increased CCL28 expression in pathological T stages 0-2 compared with pathological T stages 3-4 (39.07 ± 8.088 vs. 30.09 ± 5.654, p = 0.0135 vs. T3-4) (Figure 6.11(B)).

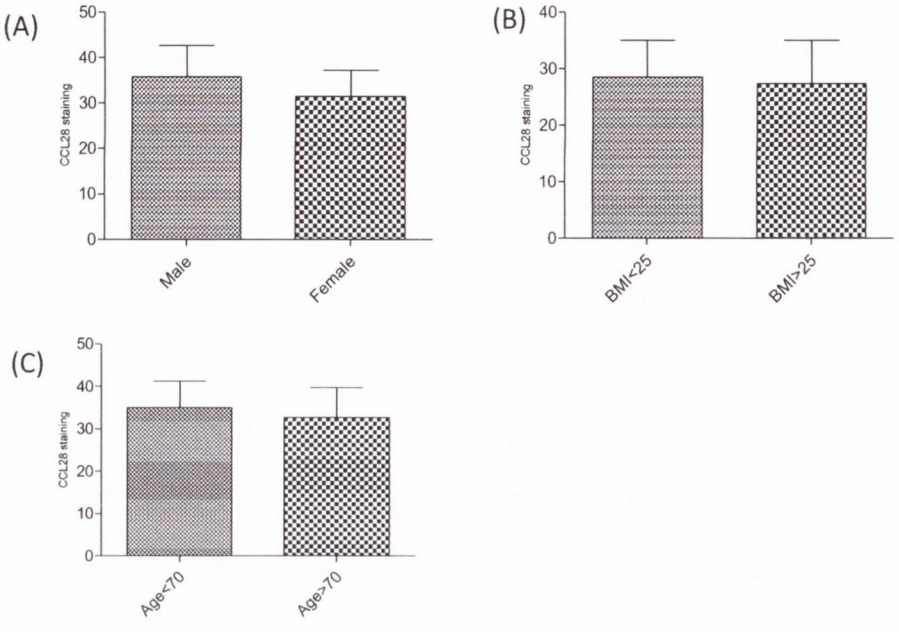


Figure 6.9 CCL28 expression in oesophageal cancer patients: demographics. Tissue cores from 125 patients with oesophageal cancer were taken and used to construct tissue microarrays (TMAs). Three cores were taken from each patient. TMAs were cut, mounted onto slides and stained for CCL28 using the previously optimised antibody. Slides were scanned and staining was graded for percentage positive staining and intensity of staining by two independent graders and results were averaged. There were no differences in CCL28 staining between (A) male and female patients, (B) patients with body mass index (BMI) less than and greater than 25 and (C) patients whose age at diagnosis was less than and greater than 70 years. Data are presented as mean ± SEM. Statistical analysis was performed using the Mann-Whitney U test.

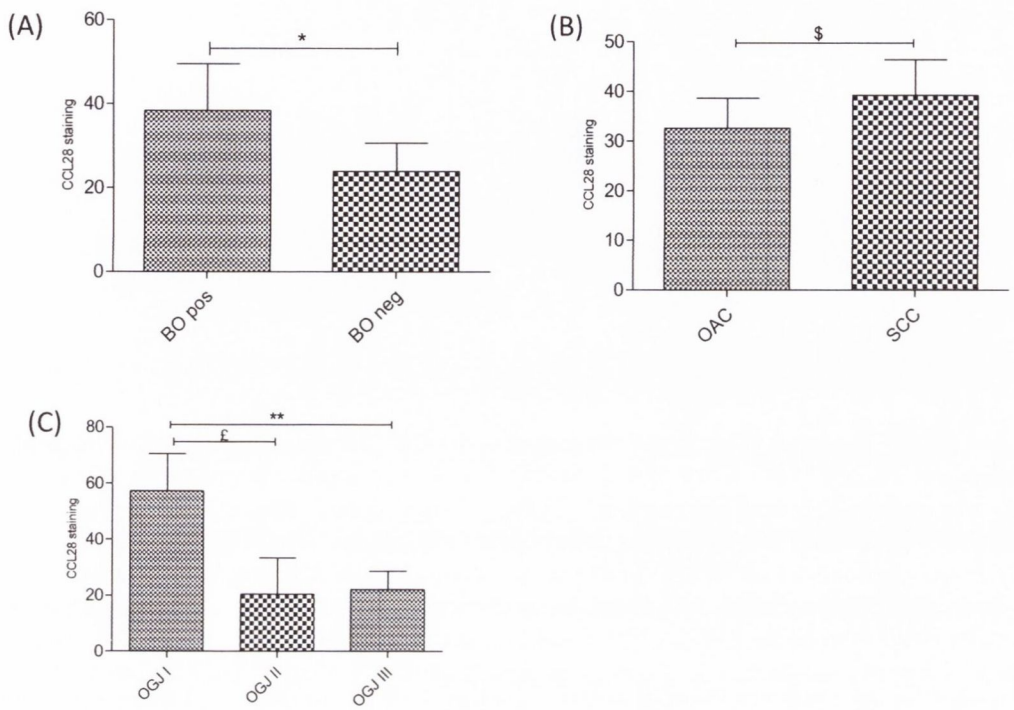


Figure 6.10 History of Barrett's oesophagus, tumour histology and oesophagogastric junction site correlate with CCL28 staining in oesophageal tumours. Tissue cores from 125 patients with oesophageal cancer were taken and used to construct tissue microarrays (TMAs). Three cores were taken from each patient. TMAs were cut, mounted onto slides and stained for CCL28 using the previously optimised antibody. Slides were scanned and staining was graded for percentage positive staining and intensity of staining by two independent graders and results were averaged. (A) Tissue from patients with oesophageal adenocarcinoma (OAC) with a history of Barrett's oesophagus (BO) demonstrated increased CCL28 staining than tissue from patients with OAC with no history of BO. (B) Tissue from patients whose tumour histology was squamous cell carcinoma (SCC) had increased CCL28 staining compared with those whose tumour histology was OAC. (C) Tissue from patients whose tumour was classified as oesophagogastric junction (OGJ) type I demonstrated increased CCL28 staining compared with tissue from patients with OGJ type II or OGJ type III tumours. Data are presented as mean ± SEM. Statistical analysis was performed using the Mann-Whitney U test. *p = 0.0256 vs. BO neg, $p = 0.0249 vs. OAC, £p = 0.0110 vs. OGJ II, **p = 0.0071 vs. OGJ III.

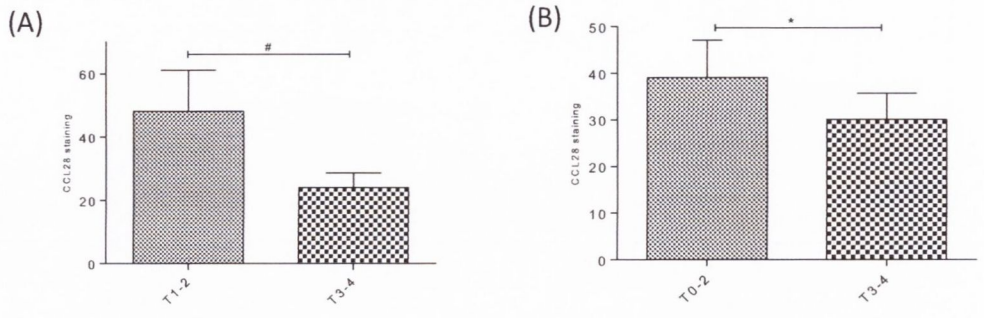


Figure 6.11 Tumour (T) stage correlates with CCL28 staining in oesophageal tumours. Tissue cores from 125 patients with oesophageal cancer were taken and used to construct tissue microarrays (TMAs). Three cores were taken from each patient. TMAs were cut, mounted onto slides and stained for CCL28 using the previously optimised antibody. Slides were scanned and staining was graded for percentage positive staining and intensity of staining by two independent graders and results were averaged. (A) Clinical T staging correlates with CCL28 staining, with a trend towards increased CCL28 expression in clinical T stages 1-2 compared with clinical T stages 3-4. (B) Pathological T staging correlates with CCL28 staining, with increased CCL28 expression in pathological T stages 0-2 compared with pathological T stages 3-4. Data are presented as mean ± SEM. Statistical analysis was performed using the Mann-Whitney U test. $^{\#}p = 0.0739$ vs. T1-2, $^{*}p = 0.0135$ vs. T0-2.

6.3.7 Kaplan-Meier survival curves correlating CCL28 intensity and percentage positivity with overall survival of patients with oesophageal cancer.

Intensity and percentage positivity of CCL28 staining on oesophageal cancer TMAs were correlated with overall survival of the 125 patients used on these TMAs. It was found that neither intensity of CCL28 staining nor percentage positivity of CCL28 staining correlated with overall survival in all oesophageal cancer patients (Figure 6.12). Intensity of CCL28 staining did not correlate with overall survival in the subgroup of patients with oesophageal adenocarcinoma. However, percentage positivity of CCL28 staining correlated with overall survival in oesophageal adenocarcinoma patients, with higher percentage positivity of CCL28 staining correlating with increased survival (p=0.0242).

6.3.8 Correlation of history of Barrett's oesophagus in oesophageal adenocarcinoma patients with survival and T stage.

Demographics, medical history and tumour characteristics for patients used on the oesophageal cancer TMAs were recorded and analysed. Patients with oesophageal adenocarcinoma who had a history of Barrett's oesophagus had increased survival compared with those who did not have a history of Barrett's oesophagus. (p=0.0322 vs. Barrett's No) (Figure 6.13). Tumours in patients with oesophageal adenocarcinoma who had a history of Barrett's oesophagus had a lower pathological T stage than tumours in patients with no history of Barrett's oesophagus (2.261 ± 0.1568 vs. 2.759 ± 0.1022, p = 0.0086 vs. Barrett's No).

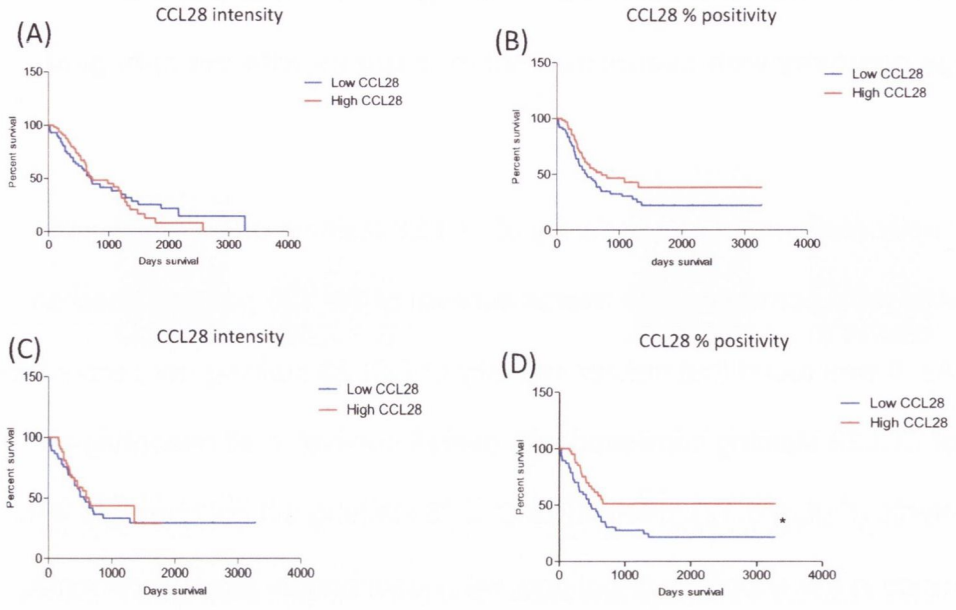


Figure 6.12 Kaplan-Meier survival curves correlating CCL28 intensity and percentage positivity with overall survival of patients with oesophageal cancer. Tissue cores from 125 patients with oesophageal cancer were used to construct tissue microarrays (TMAs). Three cores were taken from each patient. TMAs were cut, mounted onto slides and stained for CCL28 using the previously optimised antibody. Slides were scanned and staining was graded for intensity of staining and percentage positive staining by two independent graders and results were averaged. Intensity and percentage positivity of CCL28 staining were correlated with overall survival. (A) Intensity of CCL28 staining does not correlate with overall survival in all oesophageal cancer patients. (B) Percentage positivity of CCL28 staining does not correlate with overall survival in all oesophageal cancer patients. (C) Intensity of CCL28 staining does not correlate with overall survival in oesophageal adenocarcinoma patients. (D) Percentage positivity of CCL28 staining correlates with overall survival in oesophageal adenocarcinoma patients, with higher percentage positivity of CCL28 staining correlating with increased survival. Data was analysed using the Gehan-Breslow-Wilcoxon test. *p=0.0242.

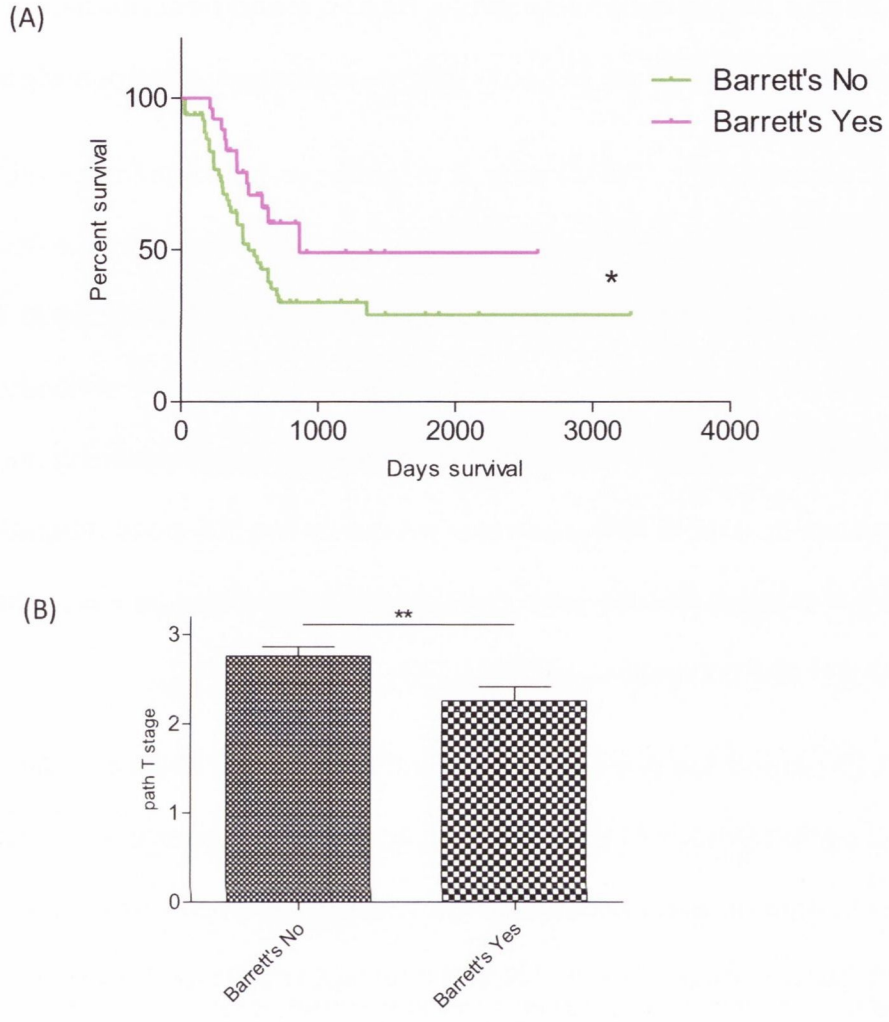


Figure 6.13 History of Barrett's oesophagus in oesophageal adenocarcinoma patients correlates with increased survival and lower pathological T stage. Tissue cores from 125 patients with oesophageal cancer were taken and used to construct tissue microarrays (TMAs). Demographics, medical history and tumour characteristics for each patient were recorded and analysed. (A) Patients with oesophageal adenocarcinoma who have a history of Barrett's oesophagus have increased survival compared with those who did not have a history of Barrett's oesophagus. Survival data was analysed using the Gehan-Breslow-Wilcoxon test. (B) Tumours in patients with oesophageal adenocarcinoma who had a history of Barrett's oesophagus had a lower pathological T stage than tumours in patients with no history of Barrett's oesophagus. Data are presented as mean ± SEM. Statistical analysis was performed using the Mann-Whitney U test. *p=0.0322 vs. Barrett's No, **p = 0.0086 vs. Barrett's No.

6.3.9 CCL28 staining in matched normal, Barrett's and oesophageal adenocarcinoma tissue from patients with oesophageal adenocarcinoma

Tissue microarrays (TMAs) were constructed using cores from matched normal, Barrett's (BO), low oesophageal adenocarcinoma (OAC) tissue from 39 patients. Demographic data for patients used in these TMAs are shown in Table 6.3. TMAs were stained for CCL28 using the previously optimised antibody. Slides were scanned and staining was graded for percentage positive staining and intensity of staining in both epithelium and stroma by two independent graders and results were averaged. Results were correlated with histology, gender, lymph node (LN) status and chemoradiation therapy (CRT) history.

CCL28 percentage positivity was found to be similar between matched normal and Barrett's tissue in OAC patients, while a trend towards a decrease in OAC tissue compared with matched Barrett's tissue was seen ($p = 0.16$ vs. Barrett's) (Figure 6.14(A)). CCL28 staining intensity was similar between matched normal and Barrett's tissue in OAC patients, while again there was a trend towards decreased CCL28 intensity in OAC tissue compared with matched Barrett's tissue ($p = 0.1$ vs. Barrett's) (Figure 6.14 (B)).

Combined CCL28 staining scores in normal tissue trended towards an increase in male OAC patients compared with female OAC patients ($p = 0.11$ vs. Female normal) (Figure 6.14(C)). There were no differences between male and female patients in CCL28 staining in Barrett's tissue and OAC tissue. Female patients demonstrated a trend towards increased CCL28 staining in Barrett's tissue compared with matched OAC tissue ($p = 0.125$ vs. Female OAC). Patients who did not undergo neo-adjuvant CRT demonstrated no differences in CCL28 staining

Table 6.3 Characteristics of patients used in matched normal/Barrett's/OAC TMAs

Gender		
Female	10	
Male	29	
Age at diagnosis	Mean	Range
Total	66.16	(42-85)
Female	69.54	(55-75)
Male	65.00	(42-85)
Neo-adjuvant CRT		
Yes	16	
No	23	
Lymph node positive at resection		
Yes	15	
No	24	

*OAC – oesophageal adenocarcinoma; CRT – chemoradiation therapy

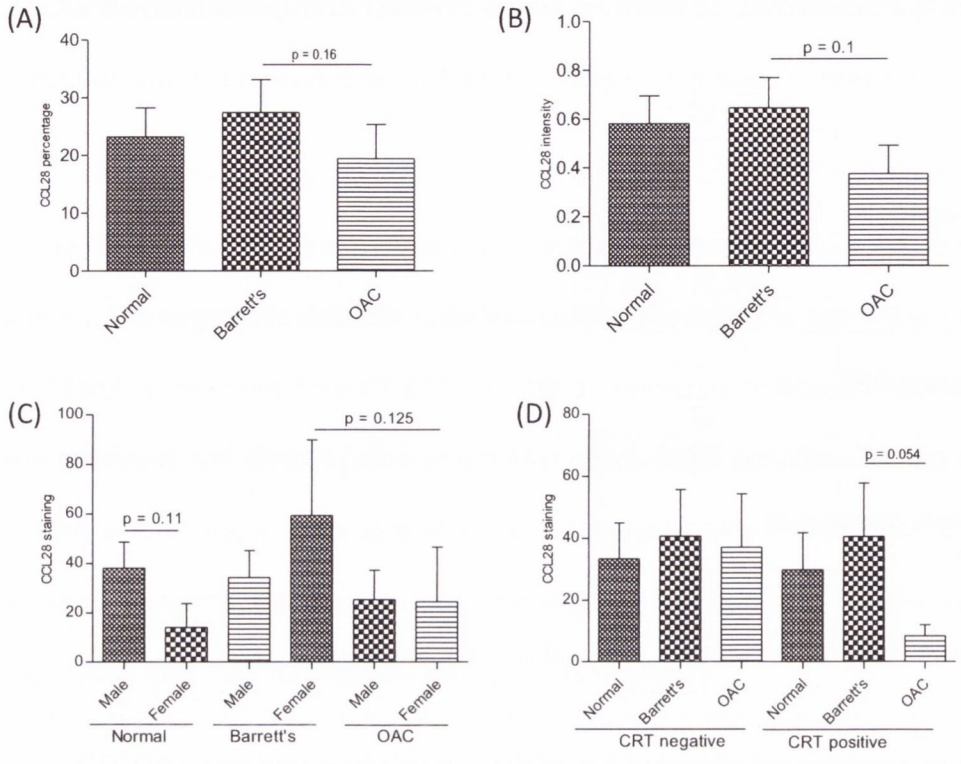


Figure 6.14 Matched normal, Barrett's and oesophageal adenocarcinoma tissue from patients with oesophageal adenocarcinoma demonstrate similar CCL28 immunohistochemical staining; chemoradiation therapy decreases CCL28 staining in OAC tissue. Tissue microarrays were constructed using tissue cores from matched normal, Barrett's and adenocarcinoma (OAC) tissue from 39 patients with OAC. Three cores were taken from each tissue sample. TMAs were cut, mounted onto slides and stained for CCL28 using the previously optimised antibody. Slides were scanned and staining was graded for intensity of staining and percentage positive staining. CCL28 staining was correlated with histology, gender and history of neo-adjuvant chemoradiation therapy (CRT). (A) CCL28 percentage positivity is similar between matched normal and Barrett's tissue in OAC patients, while it demonstrates a trend towards decrease in OAC tissue compared with matched Barrett's tissue ($p = 0.16$). (B) CCL28 intensity is similar between matched normal and Barrett's tissue in OAC patients, while there is a trend towards decreased CCL28 intensity in OAC tissue compared with matched Barrett's tissue ($p = 0.1$). (C) CCL28 staining in normal tissue trends towards an increase in male OAC patients compared with female OAC patients ($p = 0.11$). (C) There were no differences between male and female patients in CCL28 staining in Barrett's tissue and OAC tissue. Female patients demonstrate a trend towards increased CCL28 staining in Barrett's tissue compared with matched OAC tissue ($p = 0.125$). (D) Patients who did not undergo neo-adjuvant CRT demonstrates no differences in CCL28 staining between matched normal, Barrett's and OAC tissue. Patients who underwent neo-adjuvant CRT demonstrate decreased CCL28 in OAC tissue compared with matched Barrett's tissue ($p = 0.054$). Statistical analysis was performed using the Wilcoxon signed-rank test for paired data and using the Mann-Whitney U test for unpaired data

between matched normal, Barrett's and OAC tissue (Figure 6.14(D)). Patients who underwent neo-adjuvant CRT demonstrated decreased CCL28 staining in OAC tissue compared with matched Barrett's tissue (p = 0.054 vs. Barrett's).

Normal tissue from patients who had a complete response to CRT demonstrated a trend towards increased CCL28 expression post-therapy compared with patients who did not have a complete response (p = 0.0527 vs. Non-responders), while Barrett's tissue from responders and non-responders demonstrated no difference in CCL28 expression (Figure 6.15).

When OAC tissue was divided into groups that either stained positively or negatively for CCL28, patients with no CCL28 staining in their tumours were more likely to have lymph node involvement than patients whose tumours were positive for CCL28, although this did not reach statistical significance (p=0.083) (Figure 6.16).

6.3.10 Correlation of CCL28 staining with response to neo-adjuvant chemoradiation therapy in oesophageal adenocarcinoma

Tissue microarrays (TMAs) were constructed using cores from pre-treatment tumour tissue from 23 patients with OAC who underwent neo-adjuvant chemoradiation therapy (CRT) subsequent to the biopsy being taken. Demographic data for patients used in these TMAs are shown in Table 6.4. TMAs were stained for CCL28 using the previously optimised antibody. Slides were scanned and staining was graded for percentage positive staining and intensity of staining in both epithelium and stroma by two independent graders and results

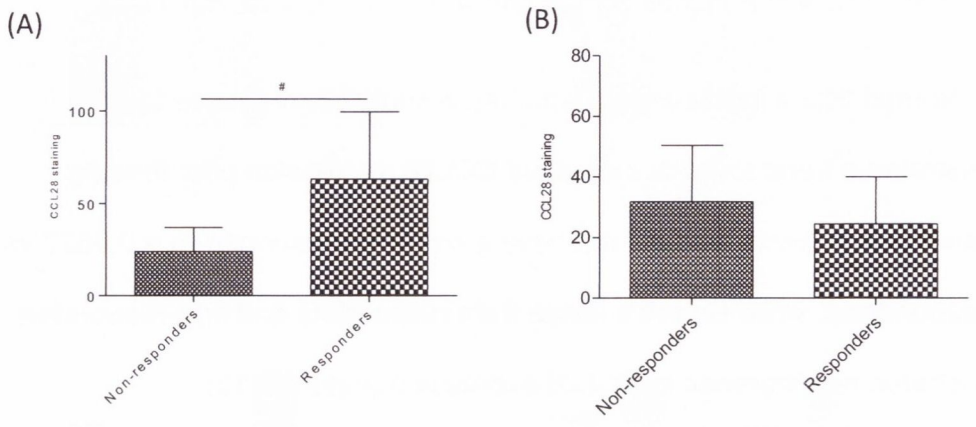


Figure 6.15 CCL28 trends towards increased expression in normal tissue of responders to neo-adjuvant chemoradiation therapy post-therapy compared with non-responders, while Barrett's tissue does not demonstrate a similar increase. Tissue microarrays were constructed using tissue cores from matched normal, Barrett's and adenocarcinoma (OAC) tissue from 39 patients with OAC. Three cores were taken from each tissue sample. TMAs were cut, mounted onto slides and stained for CCL28 using the previously optimised antibody. Slides were scanned and staining was graded for intensity of staining and percentage positive staining. CCL28 staining was correlated with response to neo-adjuvant chemoradiation therapy (CRT) in patients who had undergone CRT, with responders classified as those who had no residual tumour after neo-adjuvant CRT. (A) Normal tissue from patients who had a complete response to CRT demonstrated a trend towards increased CCL28 expression post-therapy compared with patients who did not have a complete response ($^{\#}p = 0.0527$ vs. Non-responders). (B) Barrett's tissue from responders and non-responders demonstrated no difference in CCL28 expression. Statistical analysis was performed using the Mann-Whitney U test.

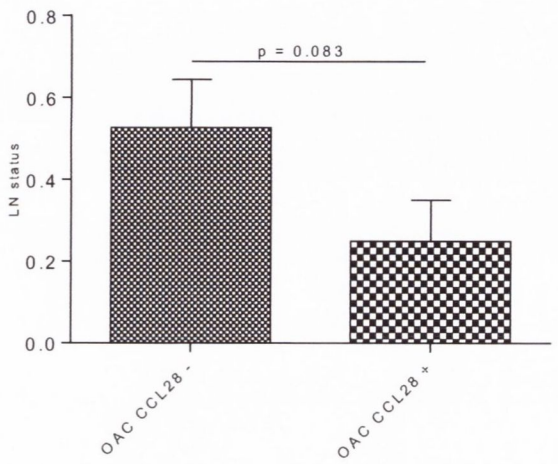


Figure 6.16 Oesophageal adenocarcinoma patients whose tumours are positive for CCL28 staining are less likely to have lymph node involvement than those whose tumours are negative for CCL28. Tissue microarrays were constructed using tissue cores from matched normal, Barrett's and adenocarcinoma (OAC) tissue from 39 patients with OAC. Three cores were taken from each tissue sample. TMAs were cut, mounted onto slides and stained for CCL28 using the previously optimised antibody. Slides were scanned and staining was graded for intensity of staining and percentage positive staining. CCL28 staining was correlated with lymph node (LN) involvement at resection. Patients whose tumours were completely negative for CCL28 staining were more likely to have lymph node involvement than patients whose tumours were positive for CCL28 staining ($p = 0.083$). Statistical analysis was performed using the Mann-Whitney U test.

Table 6.4 Demographics of patients in pre-treatment biopsy TMAs

Gender		
Male	16	
Female	7	
Age at diagnosis (years)	Mean	Range
Male	61.49	45.14-73.41
Female	60.64	44.81 - 71.43
	Mean	Range
Days follow up	838.1	150 - 2591
Alive with disease	1159	975-1262
Died of disease	605.6	294 - 1136
Patient status		
Alive with disease	3	
Died of disease	13	
Other cause/post-operative death	4	
No evidence disease	3	
Tumour regression grade		
1	2	
2	5	
3	8	
4	5	
5	3	

were averaged. Results were correlated with the tumour regression grade (TRG), graded from 1 to 5, with TRG 1 representing a pathological complete response to CRT, while TRG 5 represented no response to CRT. CCL28 staining was compared between patients who had a complete response to neo-adjuvant chemoradiation therapy (TRG 1) and those who had either a partial or absent response to therapy (TRG 2-5). CCL28 staining and TRG were also correlated with patient status at most recent follow up – either alive with disease (AWD) or died of disease (DOD).

Patients whose tumours demonstrated a TRG 1-2 response to therapy (complete or good response) demonstrated no significant difference in CCL28 expression in their pre-treatment biopsy compared with those whose tumours demonstrated TRG 3-5 (intermediate or poor response) (Figure 6.17).

Patients whose status at most recent follow up was AWD demonstrated increased CCL28 staining compared with those whose status was DOD ($p = 0.0529$ vs. DOD). Patients whose status at most recent follow up was AWD demonstrated a trend towards increased mean TRG compared with those whose status was DOD ($p = 0.14$ vs. DOD). This suggests that patients who have survived with disease to most recent follow up had a poorer response to CRT than those who died of their disease.

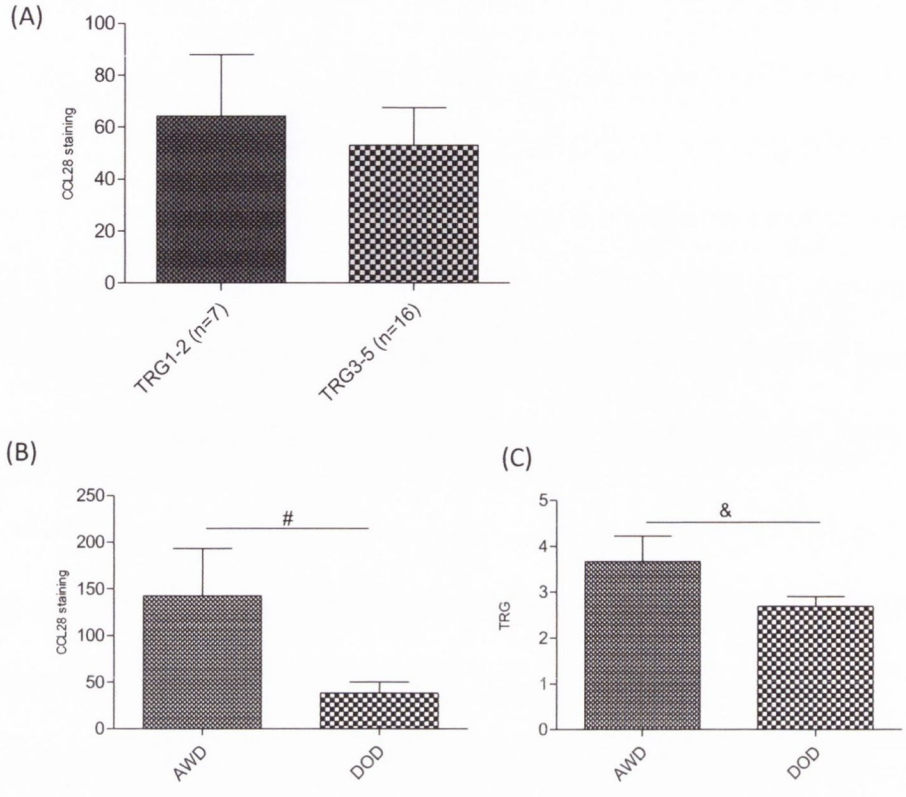


Figure 6.17. CCL28 staining correlates with response to neo-adjuvant chemoradiation therapy as measured by tumour regression grade and with patient status in pre-treatment biopsies of oesophageal tumours. Biopsies were obtained from the tumours of 23 oesophageal adenocarcinoma patients prior to commencing neo-adjuvant chemoradiation therapy (CRT). Three cores were taken from each tissue sample and tissue microarrays were constructed. TMAs were cut, mounted onto slides and stained for CCL28 using the previously optimised antibody. Slides were scanned and staining was graded for intensity of staining and percentage positive staining. CCL28 staining was correlated with response to CRT as measured by tumour regression grade (TRG) on a scale from 1 to 5, with TRG 1 being a complete pathological response to CRT and TRG 5 being no response to CRT. CCL28 staining was also correlated with patient status at latest follow up – either alive with disease (AWD) or died of disease (DOD). (A) CCL28 staining demonstrated no significant difference in the pre-treatment tumour tissue of patients with a response to CRT (TRG 1-2) compared with tissue from patients who had a partial or no response (TRG 3-5). (B) CCL28 staining was demonstrated to be increased in the pre-treatment tumour tissue of patients whose status was AWD compared with those whose status was DOD (C) TRG trended towards an increase in patients whose status was AWD compared with those whose status was DOD. Data are presented as mean ± SEM. Statistical analysis was performed using the Mann-Whitney U test. $^{\#}p = 0.0529$ vs. DOD, $^{\&}p = 0.14$ vs. DOD.

6.4 Discussion

The use of TMAs in analysis of biomarkers provides a quick and relatively easy method to quantify a protein of interest in many tissue types from many patients in one assay. Here we use TMAs from each stage of the oesophageal disease sequence, from normal oesophagus to BO to dysplasia to OAC. Of course, as previously described, the majority of patients with BO do not progress to dysplasia or OAC, and indeed in many patients with dysplasia, the disease regresses and these patients never develop OAC. In the Barrett's sequence TMAs, tissue from patients who demonstrated some form of progression of disease was examined for expression of CCL28.

Firstly it was found that, in full-face sections of BO tissue, CCL28 staining correlated well with staining for the receptors CCR3 and CCR10, both in the epithelium, and in the stroma. CCR3 and CCR10 staining appeared to be especially increased in the stroma of Barrett's tissue. This was an expected finding, but confirmed that CCL28 in the oesophagus is found in close proximity to cells bearing its known receptors, CCR3 and CCR10 (Pan, Kunkel et al. 2000, Rainone, Dubois et al. 2011, Sun, Zhang et al. 2013). Interestingly, epithelial cells appeared to express both CCL28 and its receptors. A study looking at the Reed-Sternberg cells in Hodgkin's lymphoma found that in cells that expressed CCL28, many of these also expressed the receptor CCR10, suggesting a possible autocrine effect, with CCL28 regulating its own expression (Hanamoto, Nakayama et al. 2004). Similarly, cells in adult T-cell leukaemia also co-express CCL28 and CCR10, which may play a role in invasiveness (Harasawa, Yamada et al. 2006).

We found that, in the epithelium, CCL28 was increased in both percentage of positive staining and intensity of staining in BO tissue and LGD tissue compared with normal and OAC tissue. This was interesting, as differential expression of CCL28 during the oesophageal disease progression suggests that CCL28 may be a potential biomarker for progression through the sequence from normal oesophagus to OAC. As our data shows that CCL28 is increased in BO tissue and LGD tissue compared with normal tissue, and then decreases with further disease progression to HGD and OAC, it may be that a decrease in CCL28 expression in BO or LGD tissue is a marker for progression to HGD and/or OAC. A similar finding in a mouse model by Nguyen et al. demonstrated that STAT3 inactivation increased CCL28 expression and CCR10-expressing Treg infiltration, and decreased progression from colonic inflammation to cancer, while mice with intact intestinal STAT3 expression, and therefore decreased CCL28 expression, were able to progress to tumour formation (Nguyen, Wu et al. 2013). Both percentage positivity and intensity of CCL28 staining were increased in BO tissue compared with normal and oesophagitis tissue; this may be due to the fact that BO and LGD epithelium both consist of columnar epithelium, resembling small and large intestinal epithelium, both of which express CCL28 constitutively (Pan, Kunkel et al. 2000, Shang, Thirunarayanan et al. 2009), while normal and oesophagitis tissue are both squamous epithelial tissues. Percentage positivity of CCL28 staining was higher in HGD and OAC tissue than in normal or oesophagitis tissue, suggesting that although the intensity of staining decreased, OAC tissue can still be differentiated from normal tissue and oesophagitis by CCL28 expression. The decrease in intensity of CCL28 expression in OAC compared with BO and LGD tissue may suggest that in OAC the columnar epithelial expression of CCL28 may

be somehow dysregulated and therefore decreased; indeed in the colon, inflamed colonic tissue has been shown to express increased CCL28, but colonic tumours in fact express decreased CCL28 compared with normal colon, which correlates with our results (Ogawa, Iimura et al. 2004, Dimberg, Hugander et al. 2006). The columnar epithelium in BO and LGD expresses CCL28, whereas the progression towards neoplasia decreases this expression similarly to the trend seen in colonic epithelium. Stromal trends in CCL28 expression were similar to those seen in the epithelium.

In a finding that has important implications for the prognostic value of CCL28 in oesophageal disease, logistic regression analysis of CCL28 expression in patients with BO demonstrates that increased levels of CCL28 correlate with the likelihood of progression to LGD, HGD and OAC, with both intensity and percentage positivity of CCL28 staining correlating with progression to HGD and OAC. This strongly suggests that CCL28 expression in BO tissue could be used as a biomarker for progression to oesophageal cancer – patients undergoing surveillance for BO who express high levels of CCL28 are more likely to have oesophageal disease progression and therefore should be monitored more closely, while those with lower levels could potentially have less frequent surveillance. In ulcerative colitis, an inflammatory condition of the colon which can progress to colonic carcinoma in a similar sequence to BO-OAC, research is ongoing to identify similar biomarkers in order to stratify patients into high-risk progressors versus low-risk progressors (Jawad, Direkze et al. 2011, May, Pan et al. 2011, Talero, Sanchez-Fidalgo et al. 2011, van Schaik, Oldenburg et al. 2012).

In the oesophageal cancer TMAs, a surprising result was that CCL28 expression was higher in SCC tissue than in OAC tissue. However, in the skin,

normal human keratinocytes, squamous epithelial cells, express very little CCL28, but lesional keratinocytes in skin conditions such as atopic dermatitis and psoriasis express increased CCL28; therefore the squamous cells in oesophageal SCC may be dysfunctional in a similar fashion and therefore express CCL28 (Kagami, Kakinuma et al. 2005, Kagami, Saeki et al. 2006). Unsurprisingly given the trend seen in the Barrett's TMAs, OAC patients with a history of BO demonstrated increased CCL28 expression compared with those who did not have a history of BO. Increased CCL28 expression also correlated with earlier pathological tumour (T) stage and with OGJ type I tumours, the majority of which are Barrett's-derived cancers. Therefore, it appears that Barrett's-derived OAC cancers, most of which are diagnosed at an earlier tumour stage, demonstrate increased CCL28 expression compared with those OAC tumours which have not arisen on a background of BO. In support of this, percentage positivity of CCL28 correlates with increased survival in OAC patients, and decreased CCL28 staining correlates with increased lymph node involvement.

A history of BO in OAC patients also correlated with increased survival, supported by the fact that patients with a history of BO are diagnosed at an earlier T stage than those with no history of BO, as has been previously reported in the literature (Schlansky, Dimarino et al. 2006, Cooper, Kou et al. 2009, Hayashi, Correa et al. 2012). Surveillance of patients with BO is a controversial subject, with studies and articles reporting that endoscopic surveillance of BO does more harm than good (Somerville, Garside et al. 2008, Knox 2011), while economically Barrett's surveillance is not cost-effective (Hirst, Gordon et al. 2011). However, other studies have reported that surveillance and early diagnosis of OAC provide both cost and survival benefits (Roberts, Harper et al. 2010, Gupta, Bansal et al.

2011, Gordon, Hirst et al. 2012). The results of our study support BO surveillance, since patients previously diagnosed with BO presented at an earlier tumour stage and demonstrated increased survival compared with those who had no history of BO.

When matched normal, BO and OAC tissue from patients with OAC were examined for CCL28 expression, the expression of CCL28 was found to be similar in normal and BO tissue, while OAC tissue demonstrated a trend towards a decrease in CCL28 expression compared with BO tissue. This was interesting, as these tissue samples were all derived from patients with OAC, suggesting that their normal, BO and OAC epithelial cells may all be similar. The fact that normal and BO epithelium in these patients express CCL28 indicates that these epithelia may resemble each other more than their differing epithelial cell type would suggest. A fascinating recent study looking at the cellular origin of Barrett's metaplasia found that in Barrett's patients, squamous epithelium and Barrett's columnar epithelium could in fact be derived from the same precursor stem cell, as they both contained the same mitochondrial mutation, even though they are different cell types (Nicholson, Graham et al. 2012). They found that the squamous epithelium was derived from stem cell progenitor cells that formed clonal patches that could expand laterally, and that Barrett's tissue formed in the same way from stem cells, which had previously been demonstrated (Leedham, Preston et al. 2008, Lorinc and Oberg 2012). This suggests that alterations in expression of factors such as chemokines may be a "field effect" in the oesophagus, as the squamous and columnar epithelia may be derived from the same or similar stem cell progenitors, and may help to explain why CCL28 expression is increased in Barrett's tissue compared with completely normal tissue in our Barrett's TMAs, but is similar in

normal and Barrett's tissue in these matched TMAs. This is again supported by our data examining "normal" tissue from patients with BO, LGD and OAC compared with "abnormal" tissue from these patients, which demonstrates that CCL28 protein levels are increased in the normal tissue from the diseased oesophagus compared with normal tissue from the healthy oesophagus, while the levels in "normal" tissue from BO, LGD and OAC patients are similar to the levels in the BO, LGD and OAC tissue.

The trend towards a decrease in CCL28 expression in OAC tissue may be because during the transition to neoplasia, the columnar cells become less differentiated and therefore resemble their cell of origin less. A confounding factor in these TMA tissue samples is that some of these patients had undergone neo-adjuvant CRT. When these groups were divided, it can be seen that in fact, patients who had not undergone CRT demonstrated no differences at all in CCL28 expression between matched normal, BO and OAC tissue, while neo-adjuvant CRT appears to decrease CCL28 expression in OAC tissue compared with matched BO tissue. It may be that one or all of the chemotherapeutic agents or the radiation therapy used for these patients decreases CCL28 expression in some way, affecting only the tumour tissue, which is the target of CRT. It has previously been demonstrated that neo-adjuvant CRT can decrease NF-kB activation, as well as the cytokine IL-1β and the chemokine IL-8, in OAC tumour tissue, which would correlate with our results; this suggests that neo-adjuvant CRT could downregulate NF-kB and therefore decrease CCL28 expression, which is modulated by NF-kB (Abdel-Latif, O'Riordan et al. 2005). A recent study examining patients with rectal cancer found that neoadjuvant radiation therapy increased gene expression of CCL28 (Supiot, Gouraud et al. 2013). However, this study only examined 6

patients, and also investigated gene expression only. The small study size may have affected results. Our laboratory had similarly identified gene expression of CCL28 as being increased by neoadjuvant chemoradiation therapy in oesophageal cancer (Maher, Gillham et al. 2009). Therefore it may be that post-transcriptional modifications affect the levels of CCL28 protein expressed after radiation therapy.

When the group of patients who received CRT were subdivided into those who had a complete pathological response (pCR) and those who did not, it can be seen that in the normal tissue of patients who achieved a pCR, CCL28 expression trends towards an increase compared with those who did not have a pCR, while in BO tissue this trend is not seen. OAC tissue was obviously not obtained from patients who had a pCR since there was no residual tumour tissue in the oesophagus of these patients. It may be that normal tissue in these patients expresses increased CCL28 in response to neo-adjuvant CRT as part of the inflammatory response to therapy, therefore chemoattracting immune cells, increasing the effect of CRT and shrinking the tumour, while in the tumour tissue, CRT has a deleterious effect on the tumour cells as intended. Other studies have identified factors post CRT which correlated with response to therapy and also to post-CRT disease-free survival, including immune factors (Saigusa, Inoue et al. 2013, Sprenger, Conradi et al. 2013) (Ko, Zehong et al. 2012).

When CCL28 expression was examined in biopsies of oesophageal cancer taken before CRT, CCL28 expression in patients with a good response to CRT (TRG 1-2) demonstrated no difference compared with patients who had an intermediate or poor response to CRT (TRG 3-5). This suggests that CCL28 may not play a role in response of oesophageal tumours to CRT as previously described, although it may alter the immune microenvironment of the oesophagus

to favour response to therapy in those with a good response. Again many studies have focused on identifying biomarkers that will predict which patients will respond to neo-adjuvant CRT in various cancers (Chen, Wu et al. 2012, Fuksa, Micuda et al. 2012, Grim, Jandik et al. 2012). Carruthers et al. have found that patients with rectal cancer with higher pre-CRT markers of systemic inflammation have a worse outcome post-CRT (Carruthers, Tho et al. 2012), suggesting that CCL28, by altering the immune response, may alter the course of disease after CRT.

In patients whose pre-treatment biopsies were examined, CCL28 staining correlated with their status at most recent follow up, with patients who were alive with disease having significantly higher CCL28 than those who had died of their disease. This was interesting, as it correlates with findings from previous TMAs in this study, suggesting that increased CCL28 expression predicts increased survival from oesophageal disease. Patients who were alive with disease also trended towards an increased TRG compared with those who had died of disease – this was surprising, as this suggests that patients who are alive had a worse response to CRT than those who had a good response. As this was a small patient cohort (36 patients), this suggests that perhaps this patient group was not representative of overall trends in the disease course of oesophageal cancer and further studies should be performed examining CCL28 in relation to response to CRT, possibly in a larger patient group. It also suggests that TRG is not predictive of outcome from OAC. As previously described, only a small subgroup of patients actually benefit from CRT, and Reynolds et al have previously published that TRG is not a strong predictor of outcome in a study of 243 patients (Reynolds, Muldoon et al. 2007). Indeed, some data suggests that there is no overall benefit to neo-adjuvant CRT (Hurmuzlu, Ovrebo et al. 2010), again supporting the research goal of identifying

which patients will respond to CRT. Other studies have suggested that TRG is predictive of outcome (Donington, Miller et al. 2003, Fareed, Al-Attar et al. 2010, Ajani, Correa et al. 2012) – however other factors may predict pathological complete response, including gender and histologic grade of tumour. In oesophageal squamous cell carcinoma, similar difficulties have been encountered in identifying molecular markers to predict response to CRT (Arsenijevic, Micev et al. 2012).

In summary, the data presented here demonstrates that CCL28 is differently expressed across the oesophageal disease sequence in patient tissue samples, with increased CCL28 expression in BO and LGD tissue compared with normal and OAC tissue. CCL28 expression correlates with earlier tumour stage in OAC and with overall survival. Importantly, CCL28 expression in BO appears to be predictive of progression to HGD and OAC. Further work is needed to elucidate the role of CCL28 in response to neo-adjuvant CRT, although differential expression was seen in a small cohort of patients in this study.

Chapter 7

Concluding discussion

7.1 Discussion

Barrett's oesophagus is becoming increasingly common in the Western world, often undiagnosed, and, as previously described, can progress to dysplasia and to oesophageal adenocarcinoma in a minority of patients (van Soest, Dieleman et al. 2005, di Pietro, Peters et al. 2008). Identifying the subgroup of patients with BO who progress to OAC remains an elusive prospect and is the subject of much research. At present, all patients diagnosed with BO in Ireland undergo surveillance endoscopy and biopsy of the oesophagus at regular intervals in order to monitor for disease progression. As most of these patients will never progress to OAC, many unnecessary procedures take place, causing discomfort and increasing risk of complications (Knox 2011). For patients who do develop OAC, the prognosis is poor, with many diagnosed at late stage of disease. The risk of developing OAC is greatly increased in patients with BO compared with the general population (Solaymani-Dodaran, Logan et al. 2004). Treatment for OAC is controversial, with many patients having disease too advanced for resection at diagnosis, while for those whose disease is potentially curable, the benefit of neo-adjuvant chemoradiation therapy is debated (Donington, Miller et al. 2003, Fiorica, Di Bona et al. 2004, Cunningham, Allum et al. 2006, Hurmuzlu, Ovrebo et al. 2010).

BO is thought to develop in the oesophagus in response to reflux of bile acids into the lower oesophagus (Iftikhar, Ledingham et al. 1993, Kauer and Stein 2010, Dvorak, Goldman et al. 2011). Bile acid reflux causes inflammation in normal oesophageal tissue, altering the immune microenvironment of the oesophagus during the development of BO (Capello, Moons et al. 2008, McQuaid, Laine et al. 2011, Kavanagh, O'Sullivan et al. 2013). It is known that chronic inflammation is

pro-tumourigenic, and therefore the "inflammatory" nature of BO is thought to predispose to the development of OAC (Konturek, Nikiforuk et al. 2004, O'Riordan, Abdel-latif et al. 2005).

A previous study from our laboratory identified a gene signature in patients with OAC which correlated with response to neo-adjuvant CRT (Maher, Gillham et al. 2009). Among the genes identified was *CCL28*, which codes for a 14 kDa CC-chemokine, never previously demonstrated to be expressed in the oesophagus. CCL28 has been demonstrated to be expressed in columnar epithelium in the stomach, small intestine and colon (Wang, Soto et al. 2000, Ogawa, Iimura et al. 2004, Hansson, Hermansson et al. 2008). As BO is a metaplastic columnar epithelium type, resembling intestinal cells, it was hypothesised that the expression of CCL28 may develop during the progression of oesophageal disease from normal squamous epithelium to columnar BO epithelium, and may play a role in the development of OAC. CCL28 has previously been demonstrated to be induced via the pro-inflammatory cytokines IL-1β and TNF-α in airway epithelium (O'Gorman, Jatoi et al. 2005). As bile acids are known to induce these cytokines in the oesophagus (Fitzgerald, Abdalla et al. 2002, Hamaguchi, Fujiwara et al. 2003), it was also hypothesised that bile acids may induce expression of CCL28 in the oesophagus, via IL-1β and TNF-α, during progression through the metaplasia-dysplasia-adenocarcinoma sequence. As CCL28 is a chemokine, potentially functioning to chemoattract immune cells (positive for the receptors CCR3 and CCR10), such as Treg cells, plasma cells and eosinophils, to its site of expression (Hanamoto, Nakayama et al. 2004, Eksteen, Miles et al. 2006, Hansson, Hermansson et al. 2008, Facciabene, Peng et al. 2011), it may modulate the

immune microenvironment of the oesophagus to influence disease progression and response to therapy.

In the first results chapter of this study, the expression of CCL28, IL-1β and TNF-α in oesophageal cell lines was examined. It was found that CCL28 can be induced by the bile acid DCA in BO, LGD and OAC cell lines, with the OAC cell line demonstrating the greatest induction, while CCL28 was not expressed in a cell line representing normal squamous oesophageal epithelium. This supports the hypothesis that CCL28 expression is increased as the oesophageal epithelium becomes columnar, as columnar intestinal epithelium is known to express CCL28 (Ogawa, Iimura et al. 2004, Shang, Thirunarayanan et al. 2009). These data also support the role of bile acid in this progression. CCL28 in the oesophagus was induced by the cytokine IL-1β, but not by TNF-α, even though the cells expressed the IL-1R1 and TNF-R1 receptors, which are the primary receptors for these cytokines (Chen and Goeddel 2002, O'Neill 2008). It was expected that these cells would express these receptors, as both IL-1β and TNF-α are known to induce signalling pathways in the oesophagus (Wong, Wilding et al. 2005). However, it appears that IL-1β is a much more potent inducer of CCL28 expression than TNF-α in oesophageal cell lines, a similar finding to that in airway epithelium (O'Gorman, Jatoi et al. 2005). This was confirmed by neutralising the IL-1R1, which decreased DCA-induced CCL28 expression. In our data, DCA induced low concentrations of IL-1β protein in oesophageal cell lines. IL-1β has previously been demonstrated to increase along the oesophageal disease sequence (O'Riordan, Abdel-latif et al. 2005). These data suggest that, as DCA-induced IL-1β increases, it induces CCL28 expression, during progression from BO to OAC.

In the next chapter, expression of CCL28 was examined in serum and in tissue explants from the human oesophagus. The explant model provides an excellent method with which to study chemokines and cytokines, as the explant includes both the epithelium and the microenvironment of the tissue being studied (Kaur, Ouatu-Lascar et al. 2000, Muthuswamy, Berk et al. 2012). CCL28 was differentially expressed in sera from patients with normal oesophagus, BO, LGD, HGD and OAC. Although these differences were significant, the circulating levels of a chemokine may not reflect the levels within the microenvironment it is secreted in, highlighting the importance of measuring chemokine levels in the tissue of importance (Khar, Muralikrishna et al. 1997, Hartmann, Dwyer et al. 2011). CCL28 expression was increased in BO tissue explants, at both gene and protein secretion levels. The levels of protein secretion are important, as chemokines exert their chemoattractant functions when secreted into the environment and vasculature (Rossi and Zlotnik 2000). Interestingly, DCA induced CCL28 expression in normal tissue, but not in BO tissue, suggesting that normal tissue may be more susceptible to the effects of DCA, while BO tissue is more resistant to its effects, as it is thought to develop as an adaptive mechanism in response to bile acid reflux (di Pietro, Peters et al. 2008, Chen, Fang et al. 2011). It may also suggest that CCL28 expression in BO tissue is constitutive, or that the level of expression has plateaued. These data may indicate that CCL28 expression is an early event in the progression of squamous epithelium to BO, as the data suggest that normal tissue can be induced by DCA to secrete a factor normally secreted by columnar epithelium only. BO tissue expressed increased IL-1β and TNF-α protein, the levels of which correlated with CCL28 protein secretion. DCA could also induce IL-1β and TNF-α in normal tissue but not in BO tissue, again suggesting a potential

role for these cytokines in regulation of CCL28, similar to the pathways seen in airway and liver epithelium (O'Gorman, Jatoi et al. 2005, Eksteen, Miles et al. 2006). Tumour tissue did not appear to demonstrate increased secretion of CCL28 protein compared with normal tissue, although sample size here was small, and this was explored further in tissue microarrays in later work.

The next chapter examined in more detail the regulation of CCL28 expression in the oesophagus via transcription factors, and also investigated functional aspects of CCL28 as a chemoattractant. Transcription factors were identified, using array technology, which were induced by bile acids and which were regulators of CCL28 expression. These were then filtered to include only transcription factors that had binding sites in the CCL28 promoter region. Altogether, six transcription factors were identified as being induced by DCA in an OAC cell line in the activation array, and as a regulator of CCL28 in the transcription factor knockdown array, which had binding sites in the CCL28 promoter region. Transcription factors identified as bile-acid induced regulators of CCL28 in the oesophagus included NF-kB and HIF-1α, both of which have previously been identified as regulators of CCL28 expression in other tissue types (O'Gorman, Jatoi et al. 2005, Kagami, Saeki et al. 2006, Facciabene, Peng et al. 2011). As NF-kB is known to be increased in expression during the metaplasia – dysplasia-adenocarcinoma sequence in the oesophagus (O'Riordan, Abdel-latif et al. 2005), it was chosen to be validated as a regulator of CCL28 in the oesophagus. This was achieved using stably transfected OAC cell lines over-expressing the p65 active subunit of NF-kB. These over-expressing cell lines demonstrated increased DCA-induced CCL28 expression compared with the parent cell line expressing only basal levels of NF-kB, while treatment with an NF-

kB inhibitor, DHMEQ, suppressed this increase. These data support NF-kB as an important regulator of CCL28 expression in the oesophagus, as it is in other tissue types. As DHMEQ completely suppressed DCA-induced secretion of CCL28, this suggests that the other transcription factors identified on the arrays may be under "master regulation" by NF-kB, as it is known to be involved in pathways with these factors, as demonstrated on the Ingenuity Pathway Analysis diagram (Hoffmann, Natoli et al. 2006, Wong and Tergaonkar 2009). These other transcription factors, which may therefore be downstream from NF-kB in DCA-induced inflammatory pathways, may provide more specific targets for future modulation of the oesophageal microenvironment.

CCL28 secreted by oesophageal cells was demonstrated to be functional as it was chemotactic for T cells bearing the CCR3 and CCR10 receptors, known to be the receptors for CCL28 (Pan, Kunkel et al. 2000). This was important, as this indicates that the CCL28 protein secreted by the oesophagus during disease progression is capable of modulating the immune milieu of the tissue by chemoattracting inflammatory cells, which may alter the course of the disease.

Finally, in the last chapter, CCL28 protein expression was examined using immunohistochemical techniques in tissue microarrays of tissue from patients with normal oesophagus, BO, LGD, HGD and OAC. CCL28 was co-expressed with CCR3 and CCR10 in full-face sections of BO tissue, suggesting that CCL28 is chemotactic for cells bearing these receptors. Interestingly, CCR3 and CCR10 were also expressed on epithelial cells in the oesophagus, suggesting that an autocrine loop may be present, with CCL28 regulating its own expression, as has been demonstrated in lymphoma and leukaemia cells (Hanamoto, Nakayama et al. 2004, Harasawa, Yamada et al. 2006).

CCL28 expression was increased in BO and LGD tissue compared with normal tissue and with HGD and OAC tissue, suggesting that during the intermediate stages of oesophageal disease progression, CCL28 is increased, and it may be that a decrease in CCL28 signals the dysregulatory process that leads to HGD and OAC, as has been found in colon cancer previously, where CCL28 is decreased in progression from normal colon to carcinoma (Dimberg, Hugander et al. 2006, Nguyen, Wu et al. 2013). In a logistic regression analysis of CCL28 expression in oesophageal disease progression, CCL28 expression in BO tissue was found to correlate with progression to HGD and OAC. This is an important finding, as it indicates that CCL28 may be used as a biomarker to identify patients with BO who are at risk of progression to cancer, the goal of research in BO (di Pietro, Peters et al. 2008, Ong, Lao-Sirieix et al. 2010, Chen, Fang et al. 2011). Patients with BO who have high expression of CCL28 may need more frequent surveillance compared with those with low expression of CCL28, who may require less frequent monitoring, thus improving cost-effectiveness and decreasing morbidity from endoscopy, which is a major issue with BO surveillance programmes (Gerson, Groeneveld et al. 2004, Hirst, Gordon et al. 2011).

CCL28 expression in OAC correlates with earlier tumour stage, previous history of BO and increased overall survival. As CCL28 was demonstrated to be increased in BO and LGD and then decrease in HGD and OAC, this supports the hypothesis that CCL28 expression decreases as columnar epithelium of the oesophagus becomes more dysplastic and abnormal as it progresses to OAC, and therefore that decreased CCL28 expression may indicate a more poorly differentiated tumour, with less resemblance to the well differentiated BO tissue. Another protein, HSP27, has demonstrated a similar trend, with a decrease in

expression in BO, and subsequently an increase as oesophageal disease progresses from dysplasia to OAC (Doak, Jenkins et al. 2004). As expected, patients with OAC with a previous history of BO were diagnosed at an earlier tumour stage and had improved overall survival, supporting the continuation of BO surveillance.

The role of CCL28 in response to CRT has previously been examined at a gene expression level, with increased CCL28 correlating with a poor response to CRT (Maher, Gillham et al. 2009). Data presented here suggests that CCL28 may not play a role in the response to CRT, with no significant differences in CCL28 expression seen between good responders and poor responders. However, a confounding factor in this study may be that the group of patients with a poor response to CRT appeared to have an increased survival compared with those with a good response. As it has previously been demonstrated that patients with OAC have improved survival with a pathological complete or good response to CRT (Donington, Miller et al. 2003, Fareed, Al-Attar et al. 2010), this indicates that the patient cohort included in this TMA may not be representative of overall trends in OAC patients.

In summary, data presented here demonstrates that the chemokine CCL28 is differentially expressed in the progression of oesophageal disease from normal squamous epithelium to BO, LGD, HGD and OAC. This chemokine is induced in the oesophagus by the bile acid DCA, possibly via the pro-inflammatory cytokine IL-1β, and is regulated by the transcription factor NF-kB, as well as other transcription factors which have not yet been validated. CCL28 protein secreted by the oesophagus is functional in chemoattracting immune cells positive for the receptors CCR3 and CCR10. Finally, CCL28 expression correlates with lower

tumour stage and increased overall survival in patients with OAC, and logistic regression analysis indicates that CCL28 expression in BO is predictive of progression to HGD and OAC, validating its potential use as a biomarker in this disease.

7.2 Clinical applications

Potential clinical applications of data presented here include

- Using CCL28 as a biomarker for identifying patients with BO at higher risk of progression to OAC
- Using CCL28 as a biomarker for identifying patients with OAC with decreased survival
- Using CCL28 and/or IL-1β as a potential therapeutic target to prevent development of BO in patients with gastro-oesophageal reflux disease
- Targeting of NF-kB or other transcription factors in order to modulate disease progression
- Targeting of CCR3 or CCR10 receptors in order to modulate disease progression

7.3 Future directions

There are many areas in this study which would warrant further research. These include

- Examining the effects of mixtures of bile acids on oesophageal cell lines and tissue explants, to more accurately reflect the composition of gastro-oesophageal refluxate in the human oesophagus
- Examining the role of other cytokines in the regulation of CCL28 in the oesophagus, as these were identified as being increased in BO conditioned media compared with normal conditioned media
- Further investigating the role of other transcription factors identified in this study in the regulation of CCL28 expression, as these may provide improved therapeutic targets for modulation of the immune environment in the oesophagus
- Further elucidating the types of CCR3- and CCR10-expressing immune cells found in the oesophageal microenvironment during various stages of disease progression and correlating this data with CCL28 expression and localisation
- Neutralising CCL28 in oesophageal tissue and in animal models and examining the effect on immune infiltrate and disease progression

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Appendix

Appendix 1: Barrett's biobank patient information sheet

PATIENT INFORMATION SHEET

Study title: Establishment of a Registry and Biobank of Irish Patients with Barrett's Oesophagus

Gastro-oesophageal reflux disease is one of the most common conditions affecting adults in the Western World. It starts as a mild disease, such as heartburn, with no damage to the lining of the oesophagus, however, if left untreated, the lining of the oesophagus can change to resemble that of the stomach or intestine, a condition called Barrett's oesophagus. In a very small number of Barrett's oesophagus cases, oesophageal cancer can develop.

The aim of this study is to establish a Barrett's Oesophagus Registry and Biobank, which will contain health information, blood and tissue samples for future research purposes. You are invited to donate samples (blood and/or tissue samples), which will be coded and stored in a -80 freezer indefinitely. Information about you will also be coded and stored on the Barrett's Oesophagus national registry. Nothing that will reveal your identity will be disclosed. Your doctor and/or nurse will discuss the study with you and allow you time to ask any questions. This patient information sheet is designed to help you understand what the study is about. Take time to read through the following information and discuss it with others if you wish. Please do not hesitate to ask if something is not clear.

What is the purpose of the study? To collect information from individuals with Barrett's oesophagus so we can better understand the development of Barrett's oesophagus. This study will also allow registration, surveillance and standardization of care of Barrett's Oesophagus patients and bring Ireland in line with other European Countries.

Why have I been chosen? As a patient with Barrett's oesophagus, you have been invited to participate in this study. We are collecting blood and/or tissue samples from all participating patients with this condition on a continual basis.

Do I have to take part? ***No, participation in the study is entirely voluntary.*** You will be given time to consider taking part in the study. Your standard of care and medical treatment will not be affected based on your decision. If you do decide to take part you will be asked to sign a consent form. You are free to withdraw at any time and without giving a reason. This will in no way affect your future care.

What will it involve if I decide to take part? We will ask you to donate small samples of blood (approximately two tablespoons) and/or tissue biopsies to be stored indefinitely for research purposes. You can agree to give just blood if you wish or both blood and tissue biopsies. These will be taken at the same time as your routine medical diagnostic tests or planned treatment. During your routine endoscopy the doctor will take some additional tissue samples for research to those taken for diagnostic testing. This procedure will happen at each surveillance visit. You can choose to give both blood and tissue, blood only, tissue only or none at each surveillance visit. During the endoscopy procedure to retrieve the additional biopsies, there is no added risk outside the normal risks explained to patients; risk of bleeding. Your diagnostic biopsies will be taken first, then the research biopsies. You will also be asked if we can store your information on a national registry for Barrett's Oesophagus. Your information will be coded and will not reveal your identity. If you have had previous diagnostic tissue samples taken, you may be asked if we can use them for future research studies.

What will happen to my samples and the information I provide? Your samples will be used to extract blood components and tissue components in order to help us look for new genes and proteins associated with Barrett's oesophagus. Your samples are coded and stored in our Biobank indefinitely. Your information will be coded and stored on a National Barrett's Oesophagus Registry. Samples will be used for new research projects subject to approval by the relevant hospitals' Ethics Committee.

What will happen if you change your mind? This can be done at any time by contacting the hospital or research team (contact details below). If you change your mind, all samples in storage will be destroyed in the way human tissue and bloods from hospitals are normally destroyed and the information we store about you will be deleted from the national registry.

What are the possible benefits of taking part? We cannot promise that this study will benefit you directly. However, by participating in this study you will be helping us to better understand the disease, search for factors that might help us identify risk factors and potential new drug targets. You will not benefit financially or otherwise for providing samples.

Will my taking part be confidential? Yes. All information that is collected about you during the course of the research will be kept strictly confidential. If you consent to take part in the research, the researchers conducting the study will abide by the Data Protection Act.

What will happen to the results of the research study? Results are likely to be published in medical and scientific journals, used for scientific presentations and may also be forwarded to health authorities worldwide. The confidentiality of all participants will be maintained. You will not be identified in any reports or publications resulting from the study. You will not receive individual scientific results. However, your care will be unaffected whether you participate in this study or not, and you will receive your clinical results as normal.

Who is funding the research? The study is funded by the Oesophageal Cancer Fund.

Genetic Research: Most biological research involves analysis of either DNA (contains genes) or RNA or Protein. As such this is sometimes referred to as Genetic Research. It is important you understand that the aim of this research is to understand the factors that influence disease and its treatment, and is ***NOT*** a form of genetic testing.

What if I have more questions or haven't understood something? Please feel free to ask any further questions of the doctors and nurses caring for you before deciding to take part in the study. If you would like more information please do not hesitate to contact a member of our research team at:

Departments of Gastroenterology and Surgery, St. James Hospital

Lead Investigator: Prof. John Reynolds

Collaborators: Dr. Dermot O'Toole, Mr. Narayanasamy Ravi, Dr. Jacintha O'Sullivan, Dr. Graham Pidgeon and Dr. Ronan Feighery.

Contact Numbers:

Prof. John Reynolds: (Tel: 01-416)

Dr. Dermot O'Toole: (Tel: 01-416)

Dr. Jacintha O'Sullivan (Tel: 01-416)

Dr. Ronan Feighery: (Tel: 01-4162274)

Thank you for reading this information sheet

Appendix 2: Barrett's biobank patient consent form

PATIENT CONSENT FORM

Title of study: Establishment of a Registry and Biobank of Irish Patients with Barrett's Oesophagus

Principle Investigators:

Centre Number: Patient Identification Number:

Please initial box to indicate agreement

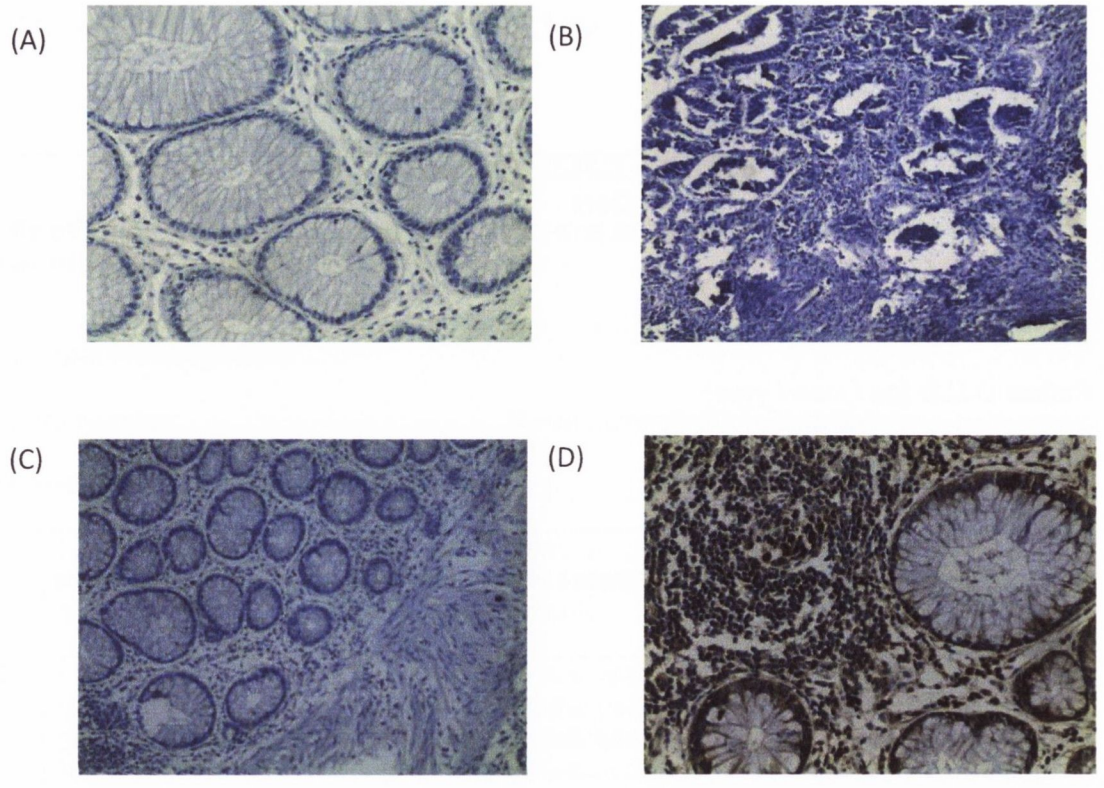
1.	I confirm that I have read and understand the patient information sheet for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
2.	I agree to donate blood ☐ and/or tissue biopsies ☐ (***tick where applicable)*** for health related research as described in the patient information sheet dated and give permission for storage and use of this and other information about me for health-related research. I give my permission to have my information coded and stored on a national registry ☐ ***(please tick for permission).***	
3	I give permission for any previous information and/or tissue samples taken for diagnostic purposes, which are applicable to this study, to be used for future research purposes.	
4.	I understand that my participation is entirely voluntary and that I am free at any time, without giving any reason and without my medical care or legal rights being affected to withdraw my permission for storage and distribution of my research donated samples providing they have not already been used in research.	
5.	I understand that relevant sections of my healthcare records and data collected during the study may be looked at by responsible individuals from research team, where it is relevant to my taking part in this research. Nothing that will reveal my identity will be disclosed. I give permission for these individuals to have access to my records.	
6.	I understand that I will not benefit financially or otherwise for providing samples. This includes research leading to development of a new treatment or medical test.	
7.	Consent for genetic research: I understand that this study and future research may include genetic research on the samples aimed at understanding the factors that influence cancer and its treatment but the results of these investigations are unlikely to have any implications for me personally	
8.	I agree to take part in the above study.	

Name of Patient	Date	Signature

Patient D.O.B (dd / mon / yyyy)

Name of Person taking consent (If different from Investigator)	Date	Signature

Appendix 3: Negative and positive controls in immunohistochemistry



Appendix 3. (A) Negative control (colon) used in CCL28 immunohistochemical staining. (B) Negative control (colon) used in CCR3 immunohistochemical staining. (C) Negative control (colon) used in CCR10 immunohistochemical staining. (D) Positive control (colon) used in CCL28 immunohistochemical staining (known to express CCL28 constitutively)

Publications

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Barrett's to Oesophageal Cancer Sequence: A Model of Inflammatory-Driven Upper Gastrointestinal Cancer

Sarah L. Picardo Stephen G. Maher Jacintha N. O'Sullivan
John V. Reynolds

Department of Surgery, Trinity Centre for Health Sciences, St James's Hospital, Dublin, Ireland

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Abstract
Cancer-related inflammation is considered the 'seventh hallmark of cancer'; many studies show that tumours develop and progress within inflammatory diseases. This review focuses on Barrett's oesophagus, a common condition in which chronic inflammation and resulting alterations in the stroma can lead to carcinogenesis, specifically oesophageal adenocarcinoma. Changes that occur in the tissue microenvironment during development of this disease are discussed. Infiltration of immune cells facilitates tumour development through production of factors that promote carcinogenesis and by enabling tumours to evade the host immune response. Small molecules including cytokines, chemokines and growth factors play key roles in both inflammation and cancer by promoting proliferation, angiogenesis and carcinogenesis and by recruiting immune cells. The extracellular matrix is altered in inflammation, and provides structural support to developing tumours. Hypoxia is a common state in cancers and inflamed tissues which causes DNA damage and induces tumourigenic factors. Finally, tissue vasculature is a vital part of its microenvironment, supplying oxygen, nutrients and growth factors to rapidly dividing cells, and providing a mechanism for metastatic spread. The cells and molecules outlined here represent potential targets for treatment of this cancer, especially in its pre-cancerous, inflammatory stage.

Introduction

The link between the tumour and its environment has been established and is hypothesised to be driven by chronic inflammation, considered now to be the seventh hallmark of cancer [1]. Tumour cells are dependent on their environment for development and progression [2, 3]. The inflammatory response in the tumour microenvironment is characterised by infiltration of immune cells, alterations in cytokines and chemokines, and vascular changes. Chronically inflamed tissues, in which these changes are often already present, are innately susceptible to tumour formation. Barrett's oesophagus (BO) may be an exemplar model of inflammation-associated cancer. It is characterised pathologically by specialised intestinal metaplasia and develops from chronic gastro-oesophageal reflux of acid and bile, and it represents the sole pathological precursor of oesophageal adenocarcinoma (OAC). The Barrett's to cancer spectrum progresses from specialised intestinal metaplasia through low-grade dysplasia (LGD) and high-grade dysplasia (HGD) to OAC. In this review, we describe various aspects of the oesophageal microenvironment which can

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John V. Reynolds
Department of Surgery
Trinity Centre for Health Sciences
St James Hospital, Dublin 8 (Ireland)
Tel. +351 ■■■ ■■■ ■■■, E-Mail ■■■@■■■.■■

promote progression to cancer. First, we examine immune cells that infiltrate the tissue in BO and during the progression to OAC. Second, we look at the cytokines and chemokines produced during this sequence. Third, we explore the role of the extracellular matrix (ECM) and associated proteins in promoting tumour development in the oesophagus. Fourth, hypoxia and oxidative stress in the oesophagus is examined, and how these factors affect disease progression. Finally, we look at angiogenesis and how this process is facilitated and also facilitates oesophageal carcinogenesis.

Barrett's Oesophagus

BO develops in response to chronic reflux of bile and acid from the stomach and duodenum. OAC, which can develop from BO, has increased 5-fold over the last 30 years [4, 5] and has an extremely poor 5-year survival. The risk of developing OAC in a patient with BO is approximately 30 times that of the general population [6]. It is thought that chronic inflammation in BO may drive progression to OAC; the risk of cancer development in BO has been shown to be decreased with the use of aspirin and other anti-inflammatory drugs [7–9], and it is suggested that non-steroidal anti-inflammatory drugs (NSAIDs) may act after the formation of BO but before OAC develops, implicating inflammation as a causative factor in OAC development.

Role of Immune Cells

Immune cells are found in all tumours and inflammatory conditions. They can facilitate tumour development through the production of factors that promote carcinogenesis and by helping the tumour to evade the host response by creating an immunosuppressive environment. Immune cell infiltration is observed during all stages of the progression from BO to dysplasia to OAC, although there is still a lack of significant research into the roles that they play in oesophageal disease. Dendritic cells (DCs) have been shown to be increased in BO tissue compared with normal oesophageal tissue [10]; DCs may also play a role in progression to cancer, as they are found at greater density in OAC than in BO, often in clusters with T and B cells in the lamina propria [11]. Although the role of DCs remains unclear, an intriguing proposal is that DCs in the oesophageal microenvironment may activate dormant stem cells, identified by the stem cell marker Musashi-1, causing the development of BO and OAC [12].

T-helper cells are located in both squamous epithelium and in Barrett's epithelium, while precursor (CD7+) T cells are most frequently located in adenocarcinoma tissue [13]. NF-κB activation, a marker of inflammation, as well as apotosis and caspase activity are observed in such T cells in Barrett's and OAC. Apoptosis in naive T cells was increased in BO, implying that these cells never develop into mature effector T cells. This suggests that the immune response is compromised in malignant transformation. Intratumoural activated CD8+ T-cell infiltration has been shown to correlate with improved disease-free survival in OAC [14]. Increased CD8+ T cells, along with increased FOXP3+ T_{reg} cells, are associated with a lower stage of tumour [15]; dysregulation of the T-cell response therefore seems to be important in OAC progression.

In BO, a predominantly humoral (Th2)-type immune response is seen, with increased plasma cells and mast cells. This contrasts with non-Barrett's with reflux oesophagitis (RO), which is characterised by a Th1-type immune profile which represents a more pro-inflammatory T-cell phenotype [16]. Lymph follicles (segregated areas of T cells, B cells and DCs) were observed in a subset of Barrett's patients; however, these were not found in RO patients. Infiltration of eosinophils has been shown in the mucosa of a subset of BO patients, associated with basal cell hyperplasia [17]. Macrophages, while found in similar number in RO and BO [16], are increased in OAC and produce the angiogenic factor vascular endothelial growth factor (VEGF) [18] and the matrix metalloproteinase MMP-12 [19], the latter of which is increased in BO, although not to the same extent as in OAC.

Role of Cytokines, Chemokines and Growth Factors

Small molecules, including cytokines, chemokines and growth factors, play key roles in the development of both inflammation and cancer, through the direct effects of promoting proliferation, angiogenesis and carcinogenesis as well as through the recruitment of immune cells. BO demonstrates a multitude of immune mediators in its microenvironment.

OAC has an array of small molecules that play a role in its development from BO. In a cohort of OAC patients, IFN-γ, IL-1α, IL-8, IL-21 and IL-23 were found to be associated with a poor prognosis, with a particularly strong association in known Barrett's-derived cancers [20]. IL-6 was increased in transformed Barrett's cell lines (with activated Ras and p53 knock-down) compared with non-transformed lines, along with its regulator STAT3

[21]. These trends have also been demonstrated in tissue samples from BO and cancer [22, 23]. IL-6 is known to inhibit apoptosis in other cancers [24, 25], providing a possible carcinogenic mechanism.

TGF-β_1 is an anti-inflammatory and tumour-suppressive cytokine under normal conditions, but, in an abnormal microenvironment, can promote tumourigenesis [26]. TGF-β_1 is significantly increased in OAC tissue compared with Barrett's tissue, and is associated with advanced tumour stage [27]. It has been shown to increase migration and invasion in OAC cells by inducing ECM-degrading enzymes, as well as by causing failure of cell-cycle arrest and thereby increasing proliferation [28]. TGF-β_1 may also be involved in epithelial-to-mesenchymal transition in the oesophagus, thought to play a role in carcinogenesis [29]. In the stromal compartment of oesophageal tissue, the TGF-β-related genes TSP-1, POSTN and TMEPAI were found to be dysregulated across the metaplasia-dysplasia-carcinoma sequence, in addition to inflammatory mediators including IL-6 and COX-2 [30]. BO is associated with upregulated COX-2, a key inflammatory molecule implicated in many cancers, and linked with NF-κB activation, and COX-2 is further increased in dysplasia and adenocarcinoma [31–33], with increased COX-2 expression in OAC associated with worse prognosis [34].

The transcription factor NF-κB has been found to be upregulated along the sequence from BO to OAC in tissue samples, along with one of its target molecules, IL-8 [35, 36]. NF-κB can also be activated by the bile acid deoxycholic acid, a component of refluxate, in oesophageal cells, as well as low pH [37–39] IL-8 was upregulated in the proximal segment of BO in patients compared with the distal segment, along with other pro-inflammatory cytokines like IL-1β. However, in the distal segment, where the majority of cancers develop, the anti-inflammatory cytokine IL-10 was increased, and was also higher in dysplastic oesophageal tissue [40]. This suggests that IL-10, and the Th2 immune response, may act as an immune escape mechanism in the development of tumours in Barrett's epithelium.

A novel way in which small molecules may modulate oesophageal neoplastic progression is via proteins secreted by adipose tissue. As obesity is a recognised risk factor for OAC, the effect of adiponectin and ghrelin, two cytokines decreased in obesity, on OAC cells was examined. It was observed that adiponectin increased apoptosis, while ghrelin decreased inflammation by lowering COX-2 and IL-1β production [41]. The reduction in these factors seen in obese patients may drive OAC progression by decreasing apoptosis and promoting inflammation. Visceral (omental) adipose tissue from OAC patients contain a large population of activated CD4+ and CD8+ T lymphocytes, which produces abundant IFN-γ, further implicating adipose tissue in driving the inflammation seen in OAC [42].

Role of the Extracellular Matrix

The ECM is an often-overlooked factor in the tumour microenvironment, comprised of a meshwork of structural proteins including collagen and fibronectin and matricellular proteins, as well as enzymes such as matrix metalloproteinases. The ECM must be modified in order to support an inflammatory-driven tumour with associated growth factors and immune cells.

Matricellular proteins are a unique class of proteins, the importance of which are now being explored. Secreted protein acidic and rich in cysteine (SPARC) is a protein with multiple effects, including counteradhesive and antiproliferative functions, as well as cell cycle modulation and matrix remodelling properties [43]. SPARC is increased in BO and OAC [44], while its expression is higher in the normal oesophagus of Barrett's and cancer patients compared with healthy patients, suggesting that this may be a field effect in the oesophagus [45, 46]. SPARC may be overexpressed in the tumour microenvironment in an attempt to inhibit tumour growth, while tumour cells themselves reduce SPARC. Another matricellular protein, thrombospondin-1 (TSP-1), has antiangiogenic effects and regulates TGF-β_1 [47]. TSP-1 is differentially expressed across the oesophageal carcinogenic sequence [30]. The matricellular protein osteopontin, which promotes metastasis in many tumours, has been found to be upregulated in OAC, along with the MET oncogene [48].

Another major component of the ECM is the matrix metalloproteinase (MMP)/tissue inhibitor of matrix metalloproteinase (TIMP) system, which is involved in turnover and remodelling of the ECM, as well as tissue growth and angiogenesis. In humans, over 23 MMPs have been identified. They are involved in the processes of inflammation and carcinogenesis, as many are produced by inflammatory and stromal cells, and so may have a role in inflammatory-driven cancers (table 1). In the oesophagus, MMP-1 has been found to be increased in BO and Barrett's-derived cancers and in OAC cell lines; it also correlates with lymph-node metastasis and poor prognosis [49–51]. Another study found that MMP-1,

Table 1. Overview of factors involved in the inflammatory-driven cancer that develops in the oesophagus

Category	Factor	Alteration	Authors
Immune cells	Naive T cells	Increased apoptosis in BO	Berndt et al., 2010
	Macrophages	Similar in RO and BO; produce VEGF in OAC and MMP-12 in BO-OAC	McDonnell et al., 2003; Salmela et al., 2001
	Dendritic cells	Increase in progression from BO-OAC; may activate stem cells	Bobryshev et al., 2009, 2010
Small molecules	NF-κB	Increases in progression from BO-OAC	Abdel-Latif et al., 2004
	IL-6	Increases in progression from BO-OAC, along with regulator STAT3	Dvorakova et al., 2004; Dvorak et al., 2007; Yu et al., 2009
	IL-8	Increased in progression from BO-OAC	O'Riordan et al., 2005; Jenkins et al., 2007
	TGF-β_1	Increased in progression from BO-OAC	Von Rahden et al., 2006
	COX-2	Increased in progression from BO-OAC	Morris et al., 2001
Extracellular matrix	SPARC	Increased in BO-OAC progression	Botelho et al., 2010
	TSP-1	Dysregulated in BO-OAC progression	Saadi et al., 2010
	Osteopontin	Increased in OAC	Miller et al., 2006
	MMP-1	Increased in BO-OAC progression	Grimm et al., 2010; Keld et al., 2010; Murray et al., 1998
	MMP-3	Increased in BO-OAC progression	Clemons et al., 2010; Lagarde et al., 2007
	MMP-7	Increased in BO-OAC progression	Salmela et al., 2001
	MMP-9	Increased in BO-OAC progression; increased in immature vessels in BO	Herszenyi et al., 2007; Auvinen et al., 2002
Hypoxia and oxidative stress	ROS	Increased in BO-OAC, increased with bile acid exposure	Clemons et al., 2007; Zhang et al., 2009; Feagins et al., 2008; Hong et al., 2010
	HIF-1α	Increased in BO compared with normal	Ling et al., 2009
	HIF-2α	Increased in dysplasia and OAC	Griffiths et al., 2007
Angiogenesis	Microvessel density (CD34)	Increased in BO-OAC progression	Couvelard et al., 2000; Mobius et al., 2003; Saad et al., 2005
	VEGF family	VEGF-C increased in BO-OAC, VEGF-A increased in BO	Couvelard et al., 2000; Auvinen et al., 2002

-3, -7 and -10 along with TIMP-1 were increased along the sequence from BO to OAC [19]. MMP-9 demonstrates a similar trend [52], suggesting that alterations in these proteinases may be early events in oesophageal carcinogenesis. MMP-1, -3, -7 and -9 have been found to be prognostic biomarkers for oesophageal cancer [53]. MMP-9 is increased in the immature blood vessels seen in BO and therefore may promote angiogenesis in OAC [54], while mean expression of MMP-9 by OAC cells is higher with increasing tumour stage [55]. Leptin, an adipocyte-derived cytokine, stimulates proliferation of oesophageal tumour cells via MMPs, as MMP inhibitors block this proliferation [56], again linking obesity, the tumour microenvironment and OAC development. There has been a clinical trial of an MMP inhibitor in OAC [57], although results were inconclusive. In another link between inflammation and tumourigenesis, TGF-β can induce the ECM-degrading proteinases urokinase-type

plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) in OAC cell lines; production of these molecules correlates with an invasive phenotype in migration and invasion assays [28].

Role of Hypoxia and Oxidative Stress

In inflammation and carcinogenesis, high metabolic demand from rapidly dividing cells causes greater demand for oxygen than the existing vasculature can supply, and hypoxia occurs. Although in normal tissues hypoxia is generally avoided due to healthy physiological regulation, the loss of these regulatory mechanisms and abnormal angiogenesis in tumours means that hypoxia is a common state. Hypoxia can cause significant DNA damage and induce tumourigenic factors including hypoxia-inducible factors 1 and 2 (HIF-1 and -2). Reoxygenation via the inefficient tumour vasculature can also cause significant oxidative stress through the production of reactive oxygen species (ROS) such as nitric oxide (NO) and hydrogen peroxide (H_2O_2).

HIF-1α is increased in BO compared with squamous epithelium, and correlates with the degree of acute and chronic inflammation. Expression was not further increased in dysplasia or OAC, implying that this may be an early change in neoplastic progression and in inflammation [58]. HIF-2α is increased in dysplasia and further increased in OAC, along with hypoxia-associated proteins VEGF and the erythropoietin receptor, while HIF-2α was not expressed in BO tissue, suggesting that this may be a later change in tumour development [59]. P53 is a key tumour suppressor gene induced by hypoxia and by ROS, and regulates cellular apoptosis. In chronic inflammation and in carcinogenesis, p53 is often mutated and dysfunctional, leading to inactivation of its tumour-suppressive function. In the oesophagus, p53 mutational inactivation correlates with progression from BO to OAC in patient samples [60–63], and overexpression of mutated p53 is seen in OAC [64, 65]. In a BO cell line, knocking down p53 and overexpressing the oncogene Ras caused malignant transformation [66]. Bile and acid treatment, which can induce ROS production in the oesophagus [67], increased p53 expression in BO cells initially, but this expression subsequently decreased with malignant transformation [68], suggesting that reflux may play a role in p53 alterations.

Chronic inflammation in the oesophagus producing oxidative stress is thought to increase tumour formation through DNA damage and increased mutational rate. Chronic inflammation in the oesophagus producing ROS is thought to increase tumour formation through DNA damage and increased mutational rate. Genomic instability, as evidenced by sister chromatid exchange, micronuclei and deletion at chromosomal fragile sites, is increased in BO compared with normal squamous epithelium [69, 70], and further increased in cancers [71]. In Barrett's tissue, dysplasia is associated with increased oxidative DNA damage [72]. Bile acids can induce DNA damage via ROS production [73] and NF-κB activation [74–76] (another link to the inflammatory process), with increases in DNA double-strand breaks and intracellular ROS levels [77, 78]. NO can increase invasiveness of dysplastic and cancerous cells via regulation of MMPs and TIMPs [79]. In BO and OAC cell lines, bile acids induce increased ROS and increased cell proliferation in a complex system, possibly by induction of PI-PLCγ2, ERK2 MAP kinase, and NADPH oxidase NOX5-S via the TGR5 receptor [80, 81]. Similar to findings in the colon, antioxidants such as reservatrol and vitamin C can block DCA-induced NF-κB expression [75], while people with high antioxidant intake have a 50% decreased risk of developing OAC compared with those with a low intake [82].

Role of Angiogenesis

The tumour vasculature provides oxygen, nutrients and growth factors to the rapidly dividing cells, and provides a mechanism for metastatic spread. The link between inflammation and angiogenesis has been well described [83, 84]. Endothelial cells in the vasculature facilitate the inflammatory response by modulating the influx of leucocytes via adhesion molecules and chemokines, and by forming new vessels [85, 86]. Expression of VEGF has been demonstrated in almost all cancers. This family of growth factors are potent stimulators of endothelial cell proliferation and migration.

In the oesophagus, microvessel density, as measured by CD34 staining, increases from BO to HGD to intramucosal carcinoma, which correlated with VEGF expression [87, 88]. Angiogenesis and VEGF have also been shown to correlate with lymph node metastases in OAC [89]. VEGF-A is expressed by goblet cells in BO, and its receptor is expressed on new vessels in the tissue [54]. The lymphangiogenic factor VEGF-C was increased from normal to BO to OAC tissue and microvessel density increased similarly along this sequence. Another marker for angiogenesis, endoglin or CD105, a member of the TGF-β_1 receptor complex, increased from LGD to HGD to OAC, while VEGF expression correlated with

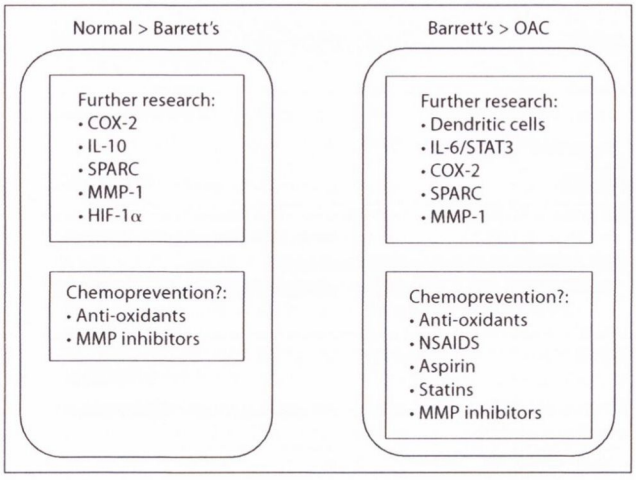

Fig. 1. Overview of potential targets in the oesophageal disease microenvironment which warrant further investigations and/or clinical trials. Further research: these molecules, previously demonstrated to be expressed in the tissue environment, may provide targets for prevention of development or progression of Barrett's oesophagus and oesophageal adenocarcinoma. Chemoprevention: therapeutic agents in this figure should be further investigated as to their potential role in treatment and/or prevention of oesophageal disease, based on previous research.

angiolymphatic invasion [90]. In an interesting link between angiogenesis and inflammation, COX-2 expression in OAC correlates with neovascularisation (CD31 staining) [91] and VEGF expression [92]; this is supported by evidence in other tissues that COX-2 inhibitors can suppress vessel growth [93]. In BO patients, CD34 vessel staining is associated with reflux symptoms and COX-2 expression, suggesting that bile and acid may induce angiogenesis via COX-2 expression [94].

Therapeutic Targets

The microenvironment in BO and oesophageal cancer provides an ideal target for interventions, since it plays a vital role in the development and progression of this tumour. The cells and molecules outlined in this review represent potential targets for treatment of this condition, especially in precancerous, inflammatory BO. The areas of research that may warrant further investigation and trials are outlined in figure 1. BO is often asymptomatic or the symptoms may be easily controlled, and so targeted medical treatment for BO is relatively under-researched, while endoscopic treatment is becoming more readily available and successful [95]. The most commonly prescribed treatment for BO is a proton pump inhibitor, which decreases acid production and therefore acid reflux into the oesophagus. In general, proton pump inhibitors have been found to have little or no effect on Barrett's tissue itself or on molecular markers such as p53 or COX-2, or DNA methylation [96–98]. Aspirin and NSAIDs have been found to have a protective association with the development of OAC, with aspirin having an especially beneficial effect [7]. Despite the role of COX-2 in OAC development, trials of a selective COX-2 inhibitor, celecoxib, found no protective effect on progression of BO [99]. Currently a large-scale randomised controlled trial, the AspECT trial, is underway in the UK, examining the chemopreventative effect of aspirin on OAC [100]. Other targets in the oesophagus include the ROS which are known to play a role in progression to cancer; one study examining dietary antioxidant intake has shown that patients with high antioxidant intake had a reduced risk of OAC (OR 0.57) and especially those with high vitamin C intake (OR 0.37), compared with those with low intake [101].

Concluding Remarks

Inflammation fuels almost every aspect of the carcinogenic process in the Barrett's to OAC paradigm – the production of growth factors, cytokines and chemokines that stimulate tumour cell development, the immune cells that both protect the developing tumour from the host response and also cause the oxidative stress which facilitates DNA damage and cancer development, angiogenesis that provides vasculature needed by the growing tumour, and the ECM needed by the tumour for structural support, invasion and metastasis. BO has an inflammatory origin, and the resulting tissue microenvironment provides the conditions needed for tumourigenesis. Central processes in this disease seem to be the activation of NF-κB and STAT3, as well as neovascularisation, oxidative damage and p53 inactivation due to chronic inflammation. These core changes seem to trigger a cascade of pathways involving various cytokines, especially IL-6 and TGF-β, and other molecules such as the MMPs and VEGF; these pathways facilitate the development and proliferation of cancerous cells and allow them to metastasise. Changes occurring in the inflammatory microenvironment of the oesophagus during disease progression are summarised in figure 2.

Although there has been an increase in research into tumour microenvironments, there remains much to do

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Fig. 2. Overview of factors involved in the inflammatory microenvironment during oesophageal disease progression. This figure outlines the main factors that increase or decrease during the progression of oesophageal tissue from normal squamous epithelium to Barrett's metaplasia to oesophageal adenocarcinoma, categorised by the aspects of the microenvironment described in this review: immune cells, small molecules, ECM, hypoxia and angiogenesis. LGD = Low-grade dysplasia; HGD = high-grade dysplasia.

before aspects of this area can be targeted therapeutically. Ubiquitous transcription factors, such as NF-κB and STAT3, are unlikely targets due to their multitude of downstream effects – more specific molecules, such as cytokines or MMPs, would have fewer side effects if neutralised, and would provide a more direct target. With continued research in this premalignant inflammatory disease, real therapeutic targets may emerge. Directing treatments at the inflammatory microenvironment means that cancer cells never gain the immune infiltrate, vasculature and ECM that they need to develop and survive. In this common condition, prevention of tumour development by targeting the carcinogenic tissue microenvironment is the ultimate aim.

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