Selenium modulation of gut epithelial cell stress responses

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Abstract

Selenium (Se) is an essential micronutrient necessary for human health. In humans, Se deficiency has been associated with inflammatory bowel disease (IBD) and increased risk of certain cancers, including colorectal cancer. Se has well established antioxidant and anti-inflammatory properties which are mediated, in part, though the actions of the selenoproteins, in which Se is present in the form of the amino acid selenocysteine (Sec). The cells of the gastrointestinal tract are exposed to stresses from pro-oxidative and hypoxic conditions, which have been suggested to be involved in the pathogenesis and pathology of IBD. Further characteristics of IBD are inappropriate immune responses of the gut epithelial cells to the gut microbiota. Thus, to help explain the roles of Se in IBD, it is important to understand the modulatory effects of Se on the cell innate immune responses following challenge of intestinal epithelial cells with pathogen-associated molecular patterns (PAMPs), as well as oxidative and hypoxic stresses. The present work aimed to assess the roles of Se and the selenoproteins, SelH and TR1, in the responses of Caco-2 cell, modelling the gut epithelium, to hypoxia and infection, the latter replicated by challenge with *S. typhimurium* flagellin.

To investigate the responses of gut cells to low Se and PAMPs, undifferentiated Caco-2 cells with either supplemented with Se (40 nM selenite) or depleted of Se for 72 h before challenging with flagellin (F) (500 ng/mL). The gene expression of the pro-inflammatory cytokines IL-8 and TNF-α were measured in addition to the genes encoding the antimicrobial peptides (AMPs) hBD1 and hBD2. Data showed that Se depletion significantly affected hBD1 expression (0.88-fold increase, $P < 0.05$), but that Se depletion plus F significantly increased the induced expression of all genes (IL-8: 1.68-fold, $P < 0.001$; TNF-α: 0.71-fold, $P < 0.001$; hBD2: 1.74-fold, $P < 0.001$) compared with the Se supplemented cells. F and Se depletion were also associated with a significant increase in expression of TR1 (F: 1.68-fold, $P < 0.001$; Se depletion: 0.33-fold, $P < 0.01$) and GPX2 (F: 3-fold, $P < 0.001$; Se depletion: 11-fold, $P < 0.001$), but a significant decrease due to Se depletion in SelH (62 %, $P < 0.001$) and GPX1 (47 %, $P < 0.001$).

The selenoprotein TR1 is an antioxidant enzyme and the primary regulator of the thioredoxin system (TXN), which has previously been shown to regulate immune responses. Knockdown of TR1 expression resulted in the reduced flagellin-induced expression of IL-8 (40 %, $P < 0.001$), TNF-a (45 %, $P < 0.01$), hBD1 (40 %, $P < 0.01$) and hBD2 (45 %, $P < 0.001$). These data suggested that Se, through TR1, is involved in regulating the expression of flagellin-induced immune effectors. The selenoprotein SelH has also been suggested to have antioxidant functions. Knockdown of SelH was associated with the increased expression of the oxidative stress-associated genes NQO1 (0.41-fold, $P < 0.001$), and HMOX1 (1.78-fold, $P < 0.001$), supporting a role for SelH in the expression of oxidative stress-associated genes. The role of Se, through SelH and oxidative stress, in regulating the gut responses to flagellin, has been discussed.

The Caco-2 cell model is more representative of intestinal epithelial cells in vivo, when the cells are differentiated and placed in a gaseous environment reflecting the oxygen gradient of the gut. Thus the F challenge experiments using differentiated Caco-2 cells were repeated using a dualoxic environment. Interestingly, no potentiation of gene expression relating to the pro-inflammatory agents IL-8 and TNF-α, and the defensins hBD1 and hBD2 was observed. These data suggested that the dualoxic environment completely diminished the effects of Se depletion on the expression of immune effectors IL-8, TNF-α, hBD2 and hBD1, following flagellin challenge. These data suggested the effects of Se in more physiologically relevant intestinal epithelial cell models, more representative of the in vivo state, are required.

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Acronyms

18S	18S ribosomal RNA.
ACTB	β-actin.
AMP	antimicrobial peptide.
AP-1	activator protein-1.
ARE	antioxidant response element.
ATF2	activating transcription factor 2.
ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, β polypeptide.
B2M	β2-microglobulin.
Bach1	BTB Domain and CNC Homolog 1.
BME	β-mercaptoethanol.
BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3.
BSA	bovine serum albumin.
CD	Crohn's disease.
cDNA	complementary DNA.
cJUN	complementary JUN.

COX-2	cyclooxygenase-2.
CRE	cAMP responsive element.
Cu	copper.
DIO1	iodothyronine deiodinase type I.
DIO2	iodothyronine deiodinase type II.
DMEM	Dulbecco's modified Eagle's medium.
DNA	deoxyribonucleic acid.
dNTP	deoxyribose nuclear triphosphates.
DSS	dextran sulfate sodium.
EFSec	Sec-specific elongation factor.
EIF4A2	eukaryotic initiation factor 4A-II.
ELISA	enzyme-linked immunosorbent assay.
ER	endoplasmic reticulum.
ERK	extracellular signal–regulated kinase.
FBS	Foetal Bovine Serum.
FIH	factor inhibiting HIF.
GALT	gut-associated lymphoid tissue.
GAPDH	glyceraldehyde 3-phosphate dehydrogenase.
GPX	glutathione peroxidase.
GPX1	glutathione peroxidase 1.

GPX2	glutathione peroxidase 2.
GPX3	glutathione peroxidase 3.
GPX4	glutathione peroxidase 4.
GSH	glutathione.
GST	glutathione S-transferase.
GSTP1	glutathione S-transferase P 1.
GTC	guanidine thiocyanate.
hBD1	human beta-defensin 1.
hBD2	human beta-defensin 2.
hBD3	human beta-defensin 3.
HDAC	histone deacetylase.
HDAC-1	histone deacetylase-1.
HIF	hypoxia-inducible factor.
HIF-1β	hypoxia-inducible factor-1 beta.
HIF-1	hypoxia inducible factor.
HIF-1α	hypoxia-inducible factor-1 alpha.
HMOX1	heme oxygenase 1.
HRE	hypoxia response element.
IBD	inflammatory bowel disease.
IEC	intestinal epithelial cell.

IFN-γ	interferon-γ.
IKK	IκB kinase.
IL-1α	Interleukin-1α.
IL-6	Interleukin-6.
IL-2	interleukin-2.
IL-17	interleukin-17.
IL-8	Interleukin-8.
iNOS2	inducible nitric oxide synthase-2.
JNK	c-Jun N-terminal kinases.
LDS	lithium dodecyl sulphate.
LPS	lipopolysaccharide.
MAPK	mitogen-activated protein kinases.
MES	2-(N-morpholino)ethanesulfonic acid.
MeSA	methylseleninic acid.
MKK4	mitogen-activated protein kinase kinase 4.
Mn	manganese.
MRE	metal response element.
mRNA	messenger RNA.
MsrB1	methionine-R-sulfoxide reductase 1.
MyD88	myeloid differentiation primary response gene 88.

NADPH	nicotinamide adenine dinucleotide phosphate.
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells.
NOD	nucleotide-binding oligomerisation domains.
NOD1	nucleotide-binding oligomerisation domain-containing protein 1.
NOD2	nucleotide-binding oligomerisation domain-containing protein 2.
NPC	Nutritional Prevention of Cancer.
NQO1	NAD(P)H dehydrogenase (quinone 1).
NRF	NF-κB repressing factor.
Nrf1	nuclear factor (erythroid-derived 2)-like 1.
Nrf2	nuclear factor (erythroid-derived 2)-like 2.
Nrf3	nuclear factor (erythroid-derived 2)-like 3.
PAGE	polyacrylamide gel electrophoresis.
PAMP	pathogen-associated molecular pattern.
PBS	Phosphate buffered saline.
PCR	polymerase chain reaction.
PHD	prolyl hydroxylase.
PHD1	prolyl hydroxylase 1.
PHD2	prolyl hydroxylase 2.
PHD3	prolyl hydroxylase 3.

PI3K	phosphoinositide 3-kinase.
pO_2	partial pressure of O_2.
PP2A	protein phosphatase 2A.
PPARγ	peroxisome proliferator-activated receptor γ.
PPARα	peroxisome proliferator-activated receptor α.
PRDX1	peroxiredoxin 1.
PRR	pattern recognition receptor.
pVHL	von Hippel–Lindau tumour suppressor.
RNA	Ribonucleic Acid.
RNS	reactive nitrogen species.
ROS	reactive oxygen species.
RPL13A	ribosomal protein L13a.
RT-qPCR	Real-time quantitative polymerase chain reaction.
SART3	Squamous cell carcinoma antigen recognized by T-cells 3.
SBP2	SECIS-binding protein 2.
SDS	sodium dodecyl sulphate.
Se	Selenium.
Sec	selenocysteine.
SECIS	selenocysteine insertion sequence.

SELECT	Selenium and Vitamin E Cancer Prevention Trial.
SELENBP1	selenium-binding protein 1.
SelH	selenoprotein H.
SelK	selenoprotein K.
SelM	selenoprotein M.
SelN	selenoprotein N.
SelO	selenoprotein O.
SelS	selenoprotein S.
SelT	selenoprotein T.
SelW	selenoprotein W.
SEM	standard error of the mean.
Sep15	15 kDa selenoprotein.
SePP1	selenoprotein P.
siRNA	small interfering RNA.
SOD1	superoxide dismutase 1.
SOD2	superoxide dismutase 2.
SOD3	superoxide dismutase 3.
TBE	Tris-borate EDTA.
TER	trans-epithelial resistance.
TGR	thioredoxin glutathione reductase.

TIR	Toll/interleukin-1 receptor.
TLR	toll-like receptor.
TLR2	Toll-like receptor 2.
TLR3	Toll-like receptor 3.
TLR4	Toll-like receptor 4.
TLR5	Toll-like receptor 5.
TNF-α	Tumour necrosis factor-α.
TOP1	topoisomerase I.
TR	thioredoxin reductase.
TR1	thioredoxin reductase 1.
TR2	thioredoxin reductase 2.
TR3	thioredoxin reductase 3.
TRIF	TIR-domain-containing adaptor-inducing interferon-β.
tRNA	transfer ribonucleic acid.
$tRNA^{Sec}$	Sec-specific transfer RNA.
TXN	thioredoxin.
UBC	ubiquitin C.
UC	ulcerative colitis.
UTR	untranslated region.
UV	ultraviolet.

VDR	vitamin D receptor.
YWHAZ	tyrosine 3-monooxygenase.
Zn	zinc.

Chapter 1

Introduction

1.1 Selenium: An essential micronutrient for human health

Selenium (Se) is an essential micronutrient for both animal and human health [1, 2]. During the first half of the 20^{th} century, the original focus for Se research, in relation to health and disease, centred around the toxicity of Se compounds in farm animals [3]. The latter half of that century saw the research focus shift from the harmful effects of Se to elucidating the roles of Se as a nutrient, which is a requirement for good health, as it was shown to prevent death from Se deficiency-associated diseases in many animals [4]. In humans, dietary intake of Se is partly determined by individual dietary habits. However, the actual Se content of foods derived from vegetables and meats are, in part, determined by the Se content of the soil on which livestock or crops are raised [5]. Thus, it has been observed that certain regions with low Se soil content exhibit a higher prevalence of endemic pathologies associated with Se deficiency. For instance, Keshan disease is a cardiomyopathy, which is endemic within regions of China that have a low soil Se content. When individuals in these Se-deficient regions were supplemented with Se, the prevalence of Keshan disease was greatly reduced [6]. Furthermore, a plethora of studies have suggested an important role for Se-deficiency in a number of various pathologies, including Kaschin-Beck disease, inflammatory bowel disease (IBD) and a number of different cancers [1, 7]. Such discoveries produce insight into how either Se deficiency or supplementation can modulate the pathogenesis and treatment of a number of nutritionally-related diseases.

The relationship between Se status and cancer risk has received much attention. Yet despite a wealth of ecological, cohort, case-controlled studies and randomised controlled trials, the link between low Se status and increased cancer risk is still uncertain [8]. One issue with the interpretation of data from such studies is that such studies have used different biomarkers, such as plasma levels of selenoprotein P (SePP1) or glutathione peroxidase (GPX), to assess Se status [8].

Two large scale supplementation studies have investigated the effects of Se status and supplementation on cancer risk: the Nutritional Prevention of Cancer (NPC) trial [9, 10]

and Selenium and Vitamin E Cancer Prevention Trial (SELECT) [11, 12]. The NPC trial, performed over a mean of 7.7 years, supplemented 1,312 individuals, of varying baseline Se status, with 200 μg organic Se per day, in the form of selenised bakers yeast. This trial indicated a decreased risk of prostate and colorectal cancers in individuals with low baseline Se status who were supplemented with Se [13, 14]. However, an increased risk of total non-melanoma skin cancer was reported in supplemented groups [10]. Additionally, it was reported that individuals that already exhibited higher baseline levels of Se, showed increased risk of prostate cancer [15]. The SELECT trial also supplemented individuals with 200 μg organic Se, but in the form of selenomethionine, as well as some receiving an additional supplementation of Vitamin E. Due to concerns regarding increased risk of the development of Type II diabetes with Se supplementation, the trial was terminated early [8]. In contrast to the NPC trial, the SELECT trial provided evidence that suggested that Se supplementation was associated with a slightly increase risk of colorectal, prostate and lung cancers [16, 17]. The reason for the contradictory conclusions between the two trials is not fully understood, but has been reviewed in depth [8]. Briefly, one possibility that has been argued is that the baseline dietary Se intake in the SELECT trial was already optimal [18]. Thus, in the Se-adequate population, one would not expect to observe benefits from additional Se intake, and may instead introduce the possibility of causing harm due to excessive Se intake. However, it has also been argued that the differences in baseline Se intake in both of the trials was so small that it could not account for the contradictory conclusions that the two studies reached [8]. Another fundamental difference between the two trials may relate to the study populations used in each trial. The NPC trial used a study population consisting of patients diagnosed with non-melanoma skin cancer, whereas the SELECT trial used a population of healthy volunteers, albeit restricted to males. Additionally, a relatively recent study in a large European population reported that a low baseline Se status was significantly correlated with an increased risk of rectal cancer in women [19]. Nonetheless, the effects of Se supplementation on cancer risk remains poorly understood and controversial and more work is needed on understanding how Se affects the gut epithelium.

1.1.1 Selenium to selenoproteins

One of the main mechanisms by which Se is thought to exert many of its biological functions is via the incorporation into selenoproteins, in the form of the 21st amino acid, selenocysteine (Sec). The incorporation of Sec into selenoproteins requires a highly conserved and multi-step mechanism in which Sec is coded for by a UGA stop codon present in selenoprotein messenger RNA (mRNA) (Figure 1.1) [20]. The synthesis of Sec occurs on a Sec-specific transfer ribonucleic acid (tRNA), tRNA^{Sec}, which forms a supramolecular complex with SECIS-binding protein 2 (SBP2) and Sec-specific elongation factor (EFSec) [21, 22]. The supramolecular complex then binds to a stem-loop structure, known as the selenocysteine insertion sequence (SECIS), which is present in the 3'untranslated region

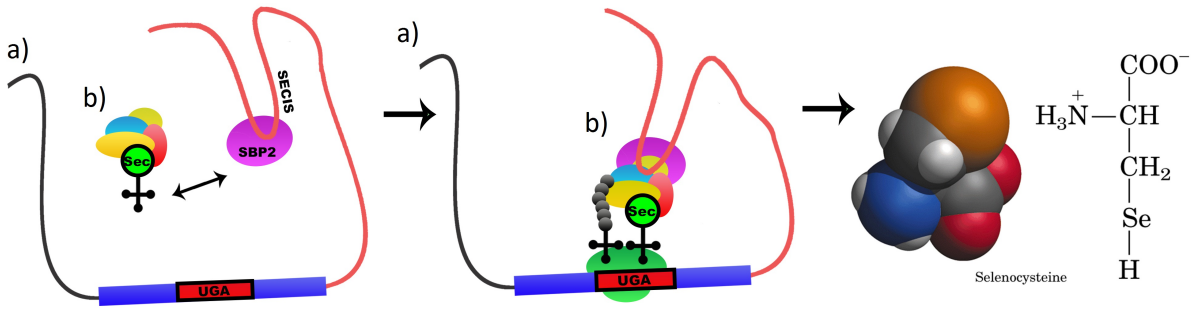

Figure 1.1. Insertion of the amino acid, selenocysteine (Sec), into selenoproteins. Sec is encoded by a UGA codon and a SECIS present in the 3' UTR of the selenoprotein mRNA is necessary (a). The SECIS element has a unique stem-loop structure to which SBP2 and a complex surrounding a Sec-specific tRNA can bind. The assembly of all of these factors on selenoprotein mRNA can permit the decoding of the UGA codon to Sec (b).

(UTR) of selenoprotein mRNAs [23]. Recent data have suggested that, for a number of selenoproteins, the successful insertion of Sec into selenoproteins can be highly dependent upon the position of the UGA stop codon but for other selenoproteins, merely possessing a UGA stop codon and SECIS element is sufficient for successful incorporation of Sec [24].

There are a total of 25 selenoproteins that have found to be expressed in humans but not all have been relatively well characterised (Table 1.1) [2]. Among the few selenoproteins that have been extensively studied are the GPX family of selenoproteins, which have been identified primarily as antioxidant enzymes [25]. Additionally, the thioredoxin reductase (TR) family of selenoproteins have been shown to exhibit antioxidant functions and indicated to be involved in redox signalling [26].

The iodothyronine deiodinase family of selenoproteins have also been well characterised and found to be involved in thyroid hormone metabolism [25]. The rest of the selenoproteome is less well characterised and functions are only starting to be fully understood. Current research has indicated a range of potential functional roles, including protein folding at the endoplasmic reticulum (ER) and roles in antioxidant defence [25].

1.1.2 Selenoprotein H (SelH)

Selenoprotein H (SelH) is a 14 kDa selenoprotein that is reported to be moderately expressed in many tissues. Interestingly, higher expression of SelH in certain cancers including lung, stomach and liver cancers [27]. Se status is a strong regulator of SelH expression in both *in vivo* and *in vitro* studies [28–31]. SelH possesses a thioredoxin-like 'CXXU' motif, as does SelW, SelT, SelM and SelV, thus indicating a possible oxidoreductase role [27, 32–34]. However, SelH shares no significant homology with any other functionally characterised proteins [35]. Additionally, a nuclear-localisation signal is present in the peptide sequence of SelH, which is consistent with reports that SelH protein has been found to reside in the nucleus and nucleolus [27, 36].

Selenoprotein	Symbol	Description
Glutathione peroxidase 1	GPX1	Reduction of cytosolic lipid hydroperoxides
Glutathione peroxidase 2	GPX2	Reduction of cytosolic lipid hydroperoxides in the gut
Glutathione peroxidase 3	GPX3	Reduction of plasma lipid hydroperoxides
Glutathione peroxidase 4	GPX4	Reduction of phospholipid hydroperoxides
Glutathione peroxidase 6	GPX6	Reduction of olfactory lipid hydroperoxides
Iodothyronine deodinase 1	DIO1	Regulation of thyroid hormone activity
Iodothyronine deodinase 2	DIO2	Regulation of tissue-specific thyroid hormone
Iodothyronine deodinase 3	DIO3	Regulation of tissue-specific thyroid hormone
Thioredoxin reductase 1	TR1	Reduction of cytosolic thioredoxin
Thioredoxin reductase 2	TR2	Testis-specific thioredoxin reduction
Thioredoxin reductase 3	TR3	Reduction of mitochondrial thioredoxin and glutaredoxin
Methionine-R-sulphoxide reductase	MSRB1	Reduction of oxidized methionine residues
Selenophosphate synthetase 2	SPS2	Involved in synthesis of selenoproteins
Selenoprotein W	SelW	Redox regulation of 14-3-3 protein
Selenoprotein T	SelT	Regulation of pancreatic B-cell function and glucose homeostasis
Selenoprotein H	SelH	Regulation of GSH synthesis and phase II detoxification enzymes
Selenoprotein V	SelV	Unknown function
Selenoprotein I	SelI	Unknown function
15 kDa selenoprotein	Sep15	Putative role in quality control of protein folding in the ER
Selenoprotein M	SelM	Rearrangement of disulphide bonds in the ER-localised proteins
Selenoprotein K	SelK	Putative role in endoplasmic reticulum-associated degradation
Selenoprotein S	SelS	Putative role in endoplasmic reticulum-associated degradation
Selenoprotein O	SelO	Unknown function
Selenoprotein N	SelN	Putative role during muscle development
Selenoprotein P	SelP	Selenium transport

Table 1.1. List of the 25 known human selenoproteins and their functions. Adapted from Labunskyy and Hatfield, 2014 [40]

It has also been reported in the literature that SelH is highly responsive to the redox status of the cell [37, 38]. Additionally, under conditions of oxidative stress, it was found that overexpression of SelH resulted in enhanced antioxidant capacity of cells. This may have been facilitated by a potential gene regulatory function of SelH attributed to a proposed AT-binding hook, which could bind to deoxyribonucleic acid (DNA) and regulate gene expression [37]. Interestingly, under resting conditions, overexpression of SelH was reported to have little effect on the antioxidant capacity of cells [37]. However, the direction of the SelH response to stress is suspected to be dependent upon the nature of stress. For example, one study reported that mouse embryonic CGR8 cells stressed with inorganic arsenic resulted in decreased SelH expression [38], while another reported that SelH expression was negatively regulated by heat stress [39].

SelH expression has been found to be regulated by metals, such as zinc and copper. This regulation is proposed to be mediated by the presence of a metal response element (MRE) in the transcribed region of SelH [41, 42]. Interestingly, the effect of zinc treatment has been observed to both increase and decrease SelH expression depending upon which cell-type is examined. Treatment of HEK-293T kidney cells with zinc resulted in decreased SelH mRNA expression, whereas zinc treatment of WISH amniotic epithelial cells resulted in increased expression [41]. MREs also exist within the promoter regions of the selenoproteins glutathione peroxidase 4 (GPX4) and selenoprotein W (SelW) [43–45]. A transcription factor involved in the regulation of cell cycle progression, known as delta-lactoferrin, has also been suggested to regulate the expression of SelH [46, 47]. Furthermore, the activity of this transcription factor was found to be directly responsible for SelH overexpression in the MDA-MB-231 breast cancer cell line [46]. Additionally, SelH has been reported to be down-regulated by treatment with lead, but the mechanism of such regulation is unknown [48].

Relatively little is known about the physiological functional roles of SelH in different tissues. A number of studies have reported that SelH can promote neuronal cell survival by ameliorating ultraviolet (UV)-induced damage by limiting the formation of superoxide [49–52]. Some studies have suggested that the protective effects of SelH may be mediated by involvement in nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathways, as well as mitochondrial biogenesis and glutathione synthesis [37, 41, 50, 51, 53].

Previous work in the laboratory has explored the potential roles for SelH in Caco-2 cells. A microarray analysis after knockdown of SelH using SelH-specific small interfering RNA (siRNA) in the Caco-2 gut epithelial cell model suggested an involvement in a number of stress response pathways, including the Nrf2 and hypoxia signalling pathways (Gautrey H, unpublished data).

1.1.3 Thioredoxin reductase 1 (TR1) and the thioredoxin (TXN) antioxidant system

The TR family of selenoproteins exist in three isoforms in mammals: the cytoplasmic thioredoxin reductase 1 (TR1), the mitochondrial thioredoxin reductase 2 (TR2) and thioredoxin reductase 3 (TR3), an isoform expressed only in specialised tissues [54]. They each contain a Sec residue, which acts as the functional site for the main activities of the TR proteins [55, 56]. These TR proteins are homodimeric flavoproteins and an essential component of the thioredoxin (TXN) antioxidant system, which comprises three components: nicotinamide adenine dinucleotide phosphate (NADPH), TR and TXN. Together, these components form a major disulphide reduction system, which provides free electrons to a range of enzymes critical for responses to various forms of stress. Indeed, the activity of a number of transcription factors, including NF-κB, Nrf2 and hypoxia-inducible factor-1 alpha (HIF-1α), are modulated by the TXN system [26, 57]. Furthermore, TR1 is the primary regulator of the TXN system by using NADPH to reduce TXN [58, 59].

The TXN system is an essential regulator of the redox system of the cell and contributes to a number of cellular processes, including cell proliferation and viability [60, 61], protein folding and signal transduction [62, 63] and defence against oxidative stress [64, 65]. The reduction of TXN allows it to modulate the activity of a number of pathways by either directly interacting with redox enzymes or by donating an electron to them [61, 65]. Indeed, the TXN system is an important regulator of redox events within the cell, as well as being regulated itself by redox events [66–71]. The translocation of TXN to the nucleus allows it to control gene expression by regulating the activation of a number of transcription factors, including NF-κB, Nrf2, activator protein-1 (AP-1), HIF-1α and p53 [72–76].

TXN has been suggested to be a regulator of NF-κB activity. One study has reported activation of NF-κB via redox control of the mitogen-activated protein kinases (MAPK), mitogen-activated protein kinase kinase 4 (MKK4), by TXN [77]. Additionally it has been shown that TXN can mediate the S-nitrosylation of the NF-κB subunit, p65, which inhibits NF-κB DNA binding activity [78]. TXN has previously been suggested to be important in the reduction of disulphide bonds of antimicrobial peptides to increase their potency [79–81].

TR1 has also been suggested to be a potent regulator of the Nrf2 antioxidant defence system. The selenoproteome and Nrf2 have a well documented relationship, as a down-regulation of the selenoproteome by Se-depletion or by knockout of Sec-specific transfer RNA (tRNA^{Sec}) has been reported to induce activation of the Nrf2 signalling pathway [82–84]. The Nrf2 signalling pathway is up-regulated in response to oxidative stress and is involved in phase II detoxification and antioxidant defence, and initiates the transcription of genes containing an antioxidant response element (ARE) in their promoter, as discussed in further detail in Section 1.3.1. TR1 contains an ARE in its promoter and is thus able to

be up-regulated in response to oxidative stress by Nrf2 [85]. However, an inter-dependent relationship between TR1 and Nrf2 has previously been reported in which the activities of each protein are able to regulate each other [86, 87]. For instance, inhibition or down-regulation of TR1 has been shown to result in activation of the Nrf2 pathway [82, 88, 89]. As TR1 is a key regulator of the TXN system and thus regulates redox control, TR1 inhibition can result in oxidative stress, which can then activate the Nrf2 system [88]. However, previous studies have also reported that knockout of TR1 had no effect on the activities of the TXN system and did not induce oxidative stress but still resulted in activation of the Nrf2 signalling pathway [89–91]. These data suggest that inhibition of TR1 activity can cause an activation of the Nrf2 pathway via a mechanism independent of oxidative stress. Additionally, TR1 induction and Nrf2 activation do not always occur at the same time. For instance, treatment of cells with compounds such as sulforaphane, isothiocyanates and other Nrf2 activators can activate Nrf2 but inhibit expression and activity of TR1 [92–96]. Thus, a complex relationship exists between the Nrf2 pathway and TR1 where the cross-activation of both components appears to be stimulus- and situation-dependent.

1.2 Innate intestinal immunity, the microbiota and the modulatory effects of selenium

1.2.1 Innate immunity of the gut and the microbiota

The complex network of the enteric microbiota, mucus, secreted factors, epithelial cells and signalling receptors cooperate in the intestinal tract to protect the host from enteric infection. In addition to a physical barrier, they also contribute to maintaining immune homeostasis of the intestine as well as extra-intestinal tissues. Loss of this barrier function has been shown to contribute to the pathogenesis and pathology of intestinal inflammation, and chronic disease states, such as inflammatory bowel disease [97].

The gut microbiota consists of a huge range of species of bacteria, protozoa, fungi and viruses [98]. Together, the microbiota can assist in digestion, provide nutrients and vitamins to host cells and prevent the colonisation of the intestinal tract by pathogenic species [99, 100]. The luminal layer of mucus, secreted by Goblet cells, is another component of the intestinal barrier. This protective layer also contains a number of different antimicrobial agents, such as the defensins, mucins and trefoil factors, which help prevent colonisation of the intestine by pathogens [98]. In addition to their antimicrobial function, these factors can also act to promote epithelial reconstitution and regulate susceptibility to colonic inflammation [101, 102].

The gut epithelium plays a critical role in maintaining the intestinal barrier and is composed of a number of different cell types, each of which plays an essential role in maintaining the barrier and regulating the immune responses of the gut [103]. These cell

types include enterocytes (or intestinal epithelial cells (IECs)), M cells, Goblet cells and Paneth cells [98]. The most numerous of these cell types are the enterocytes, through the formation of tight junctions, prevent the contents of the lumen from accessing the underlying tissues [104]. Additionally, the IEC layer expresses a variety of receptors including the toll-like receptor (TLR), pattern recognition receptor (PRR) and nucleotide-binding oligomerisation domains (NOD) families. These are able to detect and respond to microbial stimuli to coordinate and regulate the immune cells of the gut accordingly in either a tolerogenic or immunogenic manner, depending upon the perceived threat of the stimulus [103]. For instance, IECs can be stimulated by a number of microbial factors, such as flagellin and lipopolysaccharide (LPS) binding to cell surface receptors, such as TLRs. This can result in the immediate activation of immune signalling pathways, such as the NF-κB pathway, which results in the secretion of cytokines, such as Interleukin-8 (IL-8) and antimicrobial peptides (AMPs), such as the defensins, in order to coordinate local immune responses to deal with the perceived threat [105–108].

AMPs are small peptides of less than 50 amino acids, which include defensin and cathelicidin families, which can kill or inhibit the growth of microorganisms [109]. The AMPs which have been investigated in the present work are the human β-defensins, human beta-defensin 1 (hBD1) and human beta-defensin 2 (hBD2) [110]. The defensins are primarily secreted by Paneth cells, epithelial cells and immune cells of the gut. They are cysteine-rich cationic proteins which can bind to negatively charged phospholipids on the surface of microorganisms, which results in their antimicrobial pore-forming activity on cell membranes [110]. hBD1 is a constitutively expressed AMP in the human colonic epithelium and is regulated by peroxisome proliferator-activated receptor γ (PPARγ) [111]. The potency of the antimicrobial activity of hBD1 is increased after the reduction of three disulfide bonds, which can occur via the activity of TXN and TR1 [79–81]. Unlike the constitutively expressed hBD1, hBD2 is an inducible defensin which can be induced by stimulation with pro-inflammatory cytokines, such as interleukin-17 (IL-17) and Tumour necrosis factor-α (TNF-α), via the activities of NF-κB and AP-1 pathways [112, 113]. Additionally, hBD2 can be induced by flagellin in a colon carcinoma cell line and its expression could be significantly reduced by inhibitors of NF-κB, MAPK p38 and c-Jun N-terminal kinases (JNK) but not ERK1/2 [114]. Previous human studies have reported higher levels of hBD2 expression in patients with active ulcerative colitis, but not in patients with Crohn's disease [115–117]. hBD2 has also been suggested to have a role in regulating the innate immune response by acting as a chemoattractant for Mast cell migration [118]. AMPs in humans are thought to play an important role in maintaining immune homeostasis in the gut during early childhood development of the gut microbiota [119]. The gut microbiota is also thought to play an important role in the transcriptional regulation of some AMPs, such as hBD2 [120]. Furthermore, AMPs can be subject to post-translational regulation and can be activated in response to redox changes in their environment. For example, conditions of low oxygen have been shown to increase the

antimicrobial potency of hBD1 [121].

1.2.2 Immunomodulatory effects of selenium and selenoproteins, with a focus on intestinal inflammation

Se has a well-established, yet poorly understood, role in the modulation of inflammatory pathways. Generally, the micronutrient has been reported to exhibit anti-inflammatory properties, both *in vitro* and *in vivo* [122, 123]. Moreover, clinical trials investigating the effects of Se supplementation in critically ill patients with systemic inflammation [124], and patients suffering from other forms of inflammation [125], report a positive impact of Se on slowing disease progression. In addition to the reported activities of individual selenoproteins as mediators of the immunological effects of Se, the metabolites of Se have previously been suggested to have chemopreventative effects, which may indicate a role for Se metabolites in immune response pathways [126, 127]. Moreover, Se supplementation in farm animals has demonstrated a positive role for Se in the development of a healthy immune system in poultry [128], as well as the recovery of innate and humoral immune responses following disease in sheep [129]. A study in horses suggested that different immunomodulatory effects of Se are observed depending upon whether or not the dietary source of Se is organic or inorganic [130]. Furthermore, in mice, selenoprotein mRNAs were differentially expressed in gut-associated lymphoid tissue (GALT) in response to Se deficiency. The largest decreases in expression were observed in SelW, glutathione peroxidase 1 (GPX1), SelH and selenoprotein M (SelM) mRNA, which coincided with a decrease in expression of mRNAs associated with inflammatory responses initiated by stimulation with TNF-α and interleukin-2 (IL-2) [29]. On the other hand, activation of immune cell signalling can induce cell-type dependent changes in expression levels of the selenoproteome. For example, in mouse macrophages stimulated with LPS, increases in the expression of TR1 mRNA and protein, as well as the ER-localised selenoproteins selenoprotein K (SelK) and selenoprotein S (SelS), have been reported [122, 131]. In humans neutrophils stimulated with TNF-α, a reactive oxygen species (ROS)-dependent increase in GPX4 expression has also been reported previously [132]. A Se supplementation study which followed up supplemented and non-supplemented patients vaccinated against influenza showed an increase in SelS mRNA in response to vaccination, with higher levels reported in those supplemented with Se [133].

IBD is a chronic disease, that commonly presents as either Crohn's disease (CD) or ulcerative colitis (UC). A phenotype associated with patients with IBD are exacerbated immune responses to commensal bacteria [134]. It has also been previously suggested that Se deficiency may be involved in the pathogenesis or pathology of IBD due to the potential absence of proposed anti-inflammatory effects of Se. The aetiology of IBD and its subtypes is not very well understood but over 150 genes, microbiome composition and environmental factors, such as nutrition, have been identified as risk factors [135–137]. In particular, micronutrients, such as Vitamin D and Se have been investigated

in human IBD as well as associated animal models [138–140]. Previous studies focusing on the importance of Se in IBD have reported that low Se status has been associated in patients with CD [141–143]. However, a further study of CD patients who had undergone an enterectomy found that the length of small intestine that was resected was inversely correlated to Se status [144]. Additionally, a study on patients suffering from short bowel syndrome found a correlation between short bowel length and Se uptake [145]. Thus, these reports suggest that Se deficiency occurs as a result of CD and not as a cause of CD. Furthermore, two studies have noted that low Se status occurs early in CD is not associated with the progression of the disease and hence does not support involvement of low Se status in the disease pathology [146, 147]. Clinical studies have indicated less involvement of Se in UC than in CD with one study reporting lower serum Se in UC patients that that have recently been diagnosed and in remission [148]. However, other studies have found no significant change in Se in UC patients compared with healthy controls [142, 149]. This may be attributed to the fact that CD primarily affects the small intestine and thus inhibits the absorption of Se.

Se supplementation in experimental colitis has found to be beneficial in a number of different rodent models [140, 143, 150, 151], with Se deficiency associated with worsened inflammation [150], decreased survival and increased weight loss [140], as well as increased microscopic and macroscopic colonic damage [143, 151]. Many of the biological activities of Se are mediated by the expression and activities of selenoproteins. As discussed previously, the expression levels of different selenoproteins change in response to Se deficiency - some selenoproteins increase in expression whereas others decrease, depending upon where they lie in the selenoprotein hierarchy. In the gut, SelH, GPX1, SelM and SelW are particularly responsive to Se intake [7]. Additionally, Se intake has an effect on a number of genes involved in the regulation of inflammatory responses [152]. In rodent experimental colitis models, glutathione peroxidase 2 (GPX2) mRNA is consistently highly expressed when compared with controls [153]. On the other hand, SePP1 expression in the colon is down-regulated in experimental colitis, which was suggested to be via the induction of inducible nitric oxide synthase-2 (iNOS2) [154].

Another mechanism by which Se may affect colitis risk or progression is through interactions with the gut microbiota. A number of dietary and specific nutritional components have previously been identified to affect to gut microbiome and consequently the immune response in the gut [136]. *In vivo* murine studies reported that the Se deficiency had a greater impact on reducing the expression of selenoproteins in plasma, liver and intestine in germ-free mice compared with mice with gut microbiota. This effect was not observed in mice, which were fed adequate levels of Se suggesting that under Se deficiency conditions, the microbiota may be able to compete for limited Se supplies [155]. Another study reported that Se deficiency reduced the diversity of the gut microbiota and affected the relative proportion of certain phyla of bacteria in mice [156]. Reduced diversity of the gut microbiota has previously been associated with inflammatory disorders in the gut and

there is evidence of involvement of the gut microbiota in the pathogenesis of CD [156].

Evidence suggests that selenoproteins can be regulated in response to activation of the innate immune system. SelS has been reported to contain a binding site for NF-κB in its promoter region and can be up-regulated by NF-κB activity [157, 158]. Pro-inflammatory cytokine, TNF-α, was reported to induce expression of GPX4 in immune cells [159]. On the other hand, TNF-α treatment was shown to inhibit the mRNA expression in FRTL5 cells [160]. iodothyronine deiodinase type II (DIO2), however, has been reported to be NF-κB responsive and can be induced by NF-κB activity [161]. As described in Section 1.5, there is a lot of cross-regulatory mechanisms between oxidative stress, hypoxia and immune signalling. Thus, any selenoproteins which are regulated in response to oxidative stress and hypoxia transcription factors, such as Nrf2 and hypoxia inducible factor (HIF-1) transcription factors, for example, may have their expression modulated in response to NF-κB signalling as a consequence of cross-regulatory mechanisms.

Overall, there is good evidence to indicate that a link exists between inflammation and Se in the intestine. However, whether or not lower Se status associated with inflammatory conditions is causative or a consequence of disease remains unknown. The mechanism by which Se exerts its anti-inflammatory properties is currently not well understood. However, studies have suggested that Se has a modulatory effect on NF-κB signalling pathway [31, 162], which is discussed in further detail in Section 1.2.4.

1.2.3 The role of TLR5/flagellin signalling in intestinal epithelial cells

IECs express a range of PRR molecules on the cell surface, as well as intracellularly, in order to recognise pathogen-associated molecular patterns (PAMPs) and activate downstream signalling pathways that lead to the induction of an innate immune response. The TLR family of receptors are an essential member of PRR family in the gut and are important in responding to pathogens, helping to coordinate adaptive and innate immune responses, and maintaining barrier integrity [163]. The present work focused on Toll-like receptor 5 (TLR5), which has only one known natural agonist, flagellin, a bacterial monomer component of flagella [164]. TLR5 is expressed predominantly in the colon, but has also been found to be expressed in the small intestine [163, 165, 166]. In IECs isolated from patients with IBD, TLR5 expression was found to be lower when compared with healthy controls [165–168]. The expression levels of TLR5, as well as Toll-like receptor 2 (TLR2), Toll-like receptor 3 (TLR3) and Toll-like receptor 4 (TLR4), have also been found to be induced by commensal bacteria [169].

TLRs can recruit utilise Toll/interleukin-1 receptor (TIR) domain-containing adaptor proteins, such as myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adaptor-inducing interferon-β (TRIF), to induce pro-inflammatory gene expression (Figure 1.2). All TLRs use MyD88 recruitment to activate NF-κB and

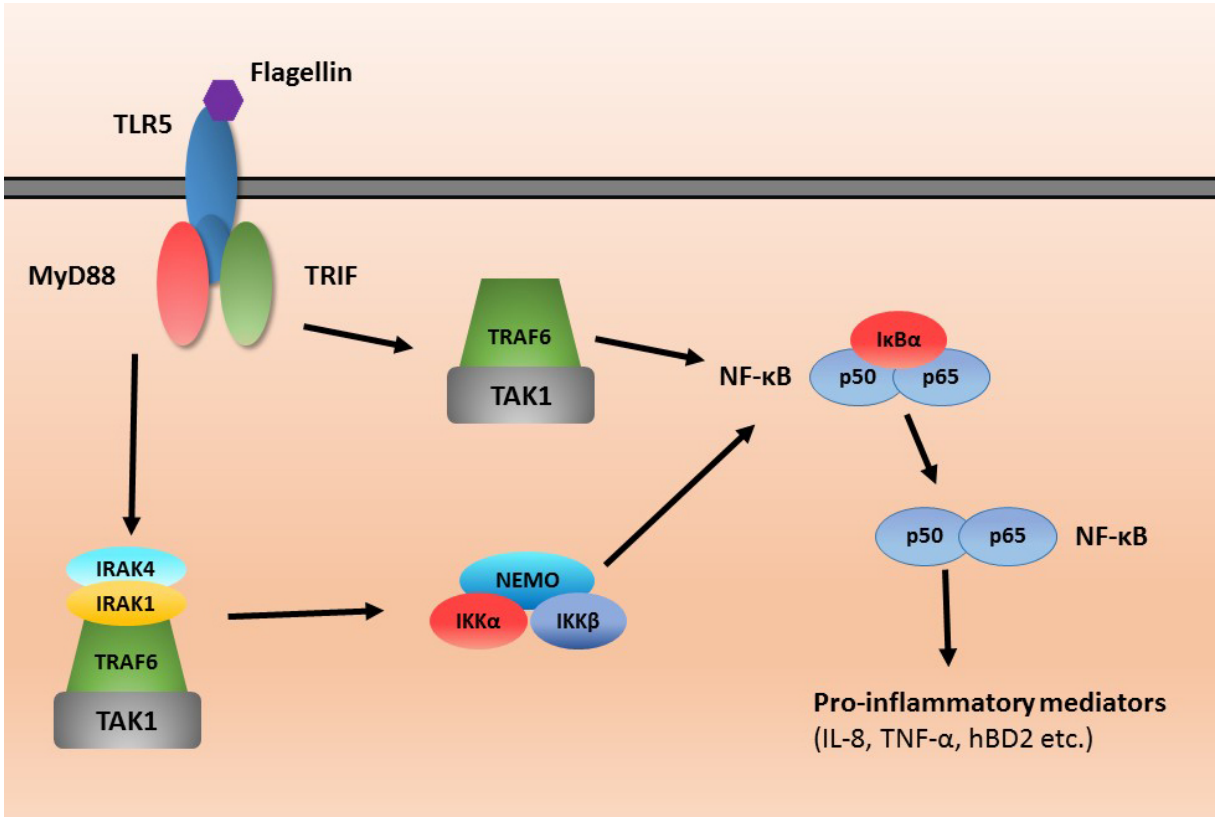

Figure 1.2. Schematic overview of activation of NF-κB signalling by TLR5 activation. TLR5 bound by flagellin results in activation of NF-κB mediated by MyD88 and TRIF adaptor proteins associated with the TLR5 receptor. MyD88 forms a complex with IRAK kinases, resulting in the activation of TRAF6 and TAK1 by IRAK1. TRIF activation also results in the activation of TRAF6 and TAK1, which subsequently result in dissociation of IκBα from the NF-κB complex. The NF-κB complex then binds to responsive elements in the promoters of pro-inflammatory mediators, such as IL-8, TNF-α and hBD2.

MAPKs, and induce pro-inflammatory gene expression [170]. TLR5 recruits and utilises both the MyD88 and TRIF adaptor proteins to induce pro-inflammatory gene expression [171, 172]. When a MyD88-recruiting TLR is activated, such as when TLR5 is bound by flagellin, MyD88 forms a complex with members of the IRAK kinase family, which results in the release of IRAK1 from the complex [173, 174]. IRAK1 then induces activation of TRAF6 and subsequently TAK1 [175, 176], which then can result in the activation of the IKK complex. This results in NF-κB pathway activation and consequently the expression of pro-inflammatory cytokines. Additionally, TAK1 activation can result in activation of MAPKs, such as JNK, p38 and ERK1/2. These can, in turn, mediate activation of the AP-1 pathway which has been shown be required for maximal gene expression of certain pro-inflammatory cytokines, such as IL-8 [177–186].

1.2.4 The NF-κB pathway and the modulatory effects of selenium

In addition to mediating immune responses, the NF-κB family of transcription factors also play an essential role in the cellular proliferation, differentiation and survival [187]. The NF-κB family consists of five monomer subunits in humans: p65/RelA, RelB, c-Rel, p50 and p52. These subunits can form homodimers or heterodimers which can translocate to the nucleus to bind to DNA and initiate transcription of target genes [188, 189]. The NF-κB family of transcription factors are controlled by two regulatory pathways: the canonical pathway and noncanonical pathway. Both of these pathways control NF-κB pathway activity by either the degradation of IκB inhibitor proteins, processing NF-κB precursor proteins or by the expression of NF-κB subunits [190, 191]. NF-κB pathway activation results in the transcription of pro-inflammatory mediators, such as IL-8, TNF-α and hBD2 [187, 192]. Previous studies have reported that Se-supplementation suppresses the activation of NF-κB via the inhibition of MyD88- and TRIF- signalling pathways [193, 194]. Furthermore, Se-depletion has been reported to increase the luciferase activity of a reporter gene assay in undifferentiated Caco-2 cells [31]. Additionally, it has been shown that Se supplementation at pharmacological doses leads to inhibition of NF-κB binding to DNA and modify the cysteine residues of NF-κB [195–197].

1.3 Oxidative stress in the gut and the effects of selenium

Oxidative stress is the consequence of an inadequate antioxidant capacity to process a certain level of pro-oxidants, such as ROS or reactive nitrogen species (RNS). The inability of a cell to deal with pro-oxidants can have a profound impact on the normal function of a cell via oxidative damage of cell components. Lipid peroxidation, as one example of oxidative damage, can initiate a chain reactions, which can result in the generation of more free radicals [198]. The modulatory effects of lipid peroxidation has been observed in core cellular processes such as altered gene expression and cell proliferation [198, 199]. Furthermore, excessive lipid peroxidation can result in pathophysiological changes resulting in increased membrane permeability and inflammation [200]. Additionally, the products of lipid peroxidation can react with DNA and studies have suggested that these DNA modifications may contribute to the pathogenesis of certain cancers, including prostate and colorectal cancers [201, 202]. Proteins can also be subject to oxidative damage which causes structural changes and subsequent impairment the specificity or activity of enzymes, including polymerases involved in DNA repair and replication [203]. Additionally, proteins with oxidative damage can be poorly targeted for ER degradation and, as a result, can accumulate inside cells and potentially contribute a variety of pathologies [203–205]. Finally, DNA can also be directly damaged by oxidation, leading to errors in both DNA transcription and methylation. An example of DNA oxidative damage is the generation

of 8-oxodG in DNA resulting in G-T transversions during DNA replication [202, 206]. Alternatively, a metabolic product of 8-oxodG, known A 8-oxoGTP can be formed, leading to A-C transversions [207]. Oxidative damage of DNA can also lead to epigenetic changes via the oxidation of DNA at CpG sites, causing changes in DNA methyltransferase to cytosine residues, leading to hypomethylation of DNA and thus altering the regulation of genes by DNA methylation [206].

Originally, ROS and RNS were investigated in relation to their ability to cause damage within cells. However, they are increasingly being recognised as having a major regulatory role as intracellular signalling molecules [208]. For instance, ROS have been implicated in the regulation of a number of biological pathways, including NF-κB and MAPK signalling pathways [209–211]. However, in pathophysiological conditions where ROS levels are in excess against antioxidant enzymes, oxidative damage within the cell can lead to inflammation, uncontrolled proliferation and apoptosis [208, 212].

Oxidative stress has been suggested to play an important role in the pathogenesis and pathology of IBD. Activated immune cells produce large amounts of ROS, such as superoxide and nitric oxide [213]. Hence, this increase in ROS levels can further exacerbate inflammation and tissue injury [214]. Thus, it has been suggested that an increase in ROS or a decrease in antioxidant capacity in the gut may be involved in the pathology and pathogenesis of IBD [215, 216]. Furthermore, IEC injury as a result of ROS has been reported in studies on patients with IBD [217, 218]. As discussed earlier, one of the intracellular consequences of increased ROS is oxidative damage to DNA, which can result in DNA lesions [219]. There is evidence to suggest that oxidative damage to DNA may play a role in the pathogenesis of IBD and also in the associated increase risk of cancer during inflammation-associated tumourigenesis [220–222]. Animal models of experimental colitis have reported an increase in oxidative stress markers when compared with healthy controls [223, 224]. Previous studies in patients with IBD have also found increased levels of ROS compared with healthy controls [225]. Furthermore, patients with IBD have been observed to have decreased antioxidant capacities compared with healthy controls [218, 226]. In particular, IBD patients have also been found to exhibit increased GPX activity [227, 228]. This is relevant to Se research as GPX selenoenzymes are induced in response to oxidative stress [229]. Additionally, patients with IBD have been observed to have increased levels of oxidative damage to DNA in their blood and mucosa [230].

The GPX and TR family of selenoproteins have very well characterised antioxidant functions [2, 231]. There have also been studies which suggest that SelK, SelM, selenoprotein N (SelN) and SelH may also possess redox functions [232]. Interestingly, there is also evidence to suggest that Se-containing compounds may also possess pro-oxidant capacities within the cell. For example, the excessive production of the metabolic products of hydrogen selenide and methylselenol has been linked with pro-oxidant effects [233]. Additionally, there is evidence that both organic and inorganic Se compounds can enhance the levels of intracellular oxygen free radicals [234, 235]. The general consensus in the

literature, however, is that the overall effects of Se at physiological levels is antioxidant in nature, but the pro-oxidant capacity of Se at higher concentrations requires further investigation [236].

1.3.1 The Nrf2 pathway in antioxidant defence and the modulatory roles of selenium

The Nrf2 pathway is a defence mechanism that is activated in response to specific stimuli, such as oxidative stress and xenobiotics [237]. The Nrf2 protein is a member of a cap 'n' collar family of transcription factors, which also include nuclear factor (erythroid-derived 2)-like 1 (Nrf1), nuclear factor (erythroid-derived 2)-like 3 (Nrf3) and p45 NF-E2 [238, 239]. Activation of the Nrf2 pathway initiates transcription of cytoprotective genes necessary for protection against oxidative stress. Gene transcription is initiated by the binding of Nrf2 to AREs in the promoter regions of cytoprotective genes. Previous studies have reported that consistently high expression levels of Nrf2 contributes to chemoresistance and promotes carcinogenesis [240]. Conversely, Nrf2 has also been suggested to have a role in inhibiting tumourigenesis and chemoresistance [241].

Upon activation, Nrf2 induces transcription of various genes involved in oxidative stress defence and phase II detoxification [242, 243]. During resting states, cells minimise protein levels of Nrf2 by sequestering Nrf2 via an inhibitory protein, Keap1, in the cytoplasm. The Nrf2/Keap1 complex is directed to CUL E3 ligase for ubiquitylation, which then targets the complex for proteasomal degradation, thus keeping levels of Nrf2 low during resting conditions [244, 245]. Under conditions of oxidative stress, the cysteine residues of Keap1 become oxidised and allow dissociation of Nrf2 from the inhibitory complex [246]. The activated Nrf2 protein then translocates to the nucleus where it heterodimerises with proteins, such as small Maf proteins or complementary JUN (cJUN), to help facilitate gene transcription [247–250]. This Nrf2 transcriptional complex then binds to AREs, a *cis*-acting enhancer, located in the promoter regions of Nrf2 target genes, such as heme oxygenase 1 (HMOX1) and NAD(P)H dehydrogenase (quinone 1) (NQO1) [237, 251, 252]. Bach1 is an example of a negative regulator of the Nrf2 pathway, which competes with Nrf2 by also binding to ARE in Nrf2 target genes [253]. A further level of Nrf2 regulation is by modulation of the nuclear export and proteasomal degradation of negative regulators of Nrf2. This reduces inhibition of Nrf2 nuclear import and thus has a positive effect on Nrf2 target gene induction [254–256]. Induction of Nrf2 is thought to be mediated by a number of kinases that can phosphorylate Nrf2, leading to its dissociation from the inhibitory complex with Keap1 [257–259]. Additionally, Nrf2 can induce the transcription of itself via the presence of an ARE in its promoter region, which facilitates the rapid induction of Nrf2 in response to cellular stress [237].

A number of studies have reported a link between Se status and Nrf2 pathway activity and in particular, Se deficiency has shown to be linked with up-regulated Nrf2 activity in both *in vitro* and *in vivo* models [82, 261, 262]. Certain selenoproteins have also been reported

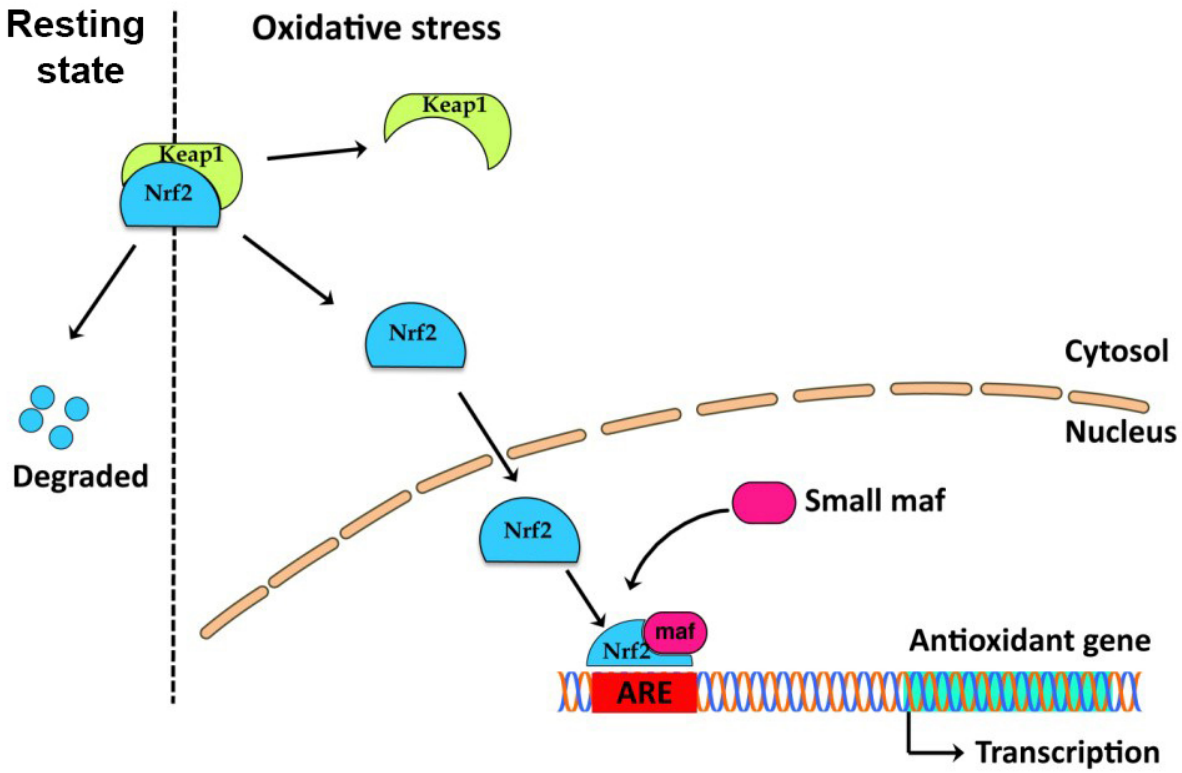

Figure 1.3. Schematic illustration of gene activation by Nrf2 signalling. Nrf2 acts as a transcription factor under conditions of oxidative stress by associating with small Maf proteins in the nucleus and binding to genes containing an antioxidant response element (ARE) in the promoter region. Under oxidative stress, Keap1 dissociates from Nrf2 in the cytoplasm. allowing Nrf2 to translocate to the nucleus, associate with small Maf proteins and initiate transcription of target genes. Adapted from Bhatia, 2013 [260]

to be regulated by Nrf2 pathway activity, including GPX2 and TR1 [263, 264]. Thus, there is evidence for cross-regulation between selenium, selenoproteins and the Nrf2 pathway, but the mechanisms are not fully understood and the data are inconsistent. Previous studies using cells with a deletion of the *Trsp* gene, which encodes for $tRNA^{Sec}$, reported inconsistent results with regards to the Nrf2 pathway. One study which performed this deletion in liver cells and mouse macrophages, reported an up-regulation of ARE-regulated genes [265]. However, another study utilising a *Trsp* knockdown in mice reported no induction of ARE-regulated genes [266]. Interestingly, both studies reported a regulation of HMOX1 which was suggested by both to be independent of Nrf2 activity. In the former study, HMOX1 was up-regulated in response to Nrf2 knockout in *Trsp*-null cells, whereas other ARE-containing genes were down-regulated [265]. In the second study, HMOX1 failed to be induced in response to *Trsp* knockout despite the other ARE-containing genes being up-regulated [266]. These data suggest Nrf2-independent regulatory mechanisms for HMOX1 associated with altered expression levels of selenoproteins.

1.4 Hypoxia in the gut and the responses of intestinal epithelial cells

In the gut there exists a steep oxygen gradient across the epithelium, with the oxygen concentration at the apical IEC surface at less than 2 % and the intestinal tissue with approximately 8 %, when compared to the partial pressure of O_2 (pO_2) of arterial blood [267]. However the luminal pO_2 is subject to regular fluctuations. Food intake increases the pO_2 of the intestinal lumen whereas periods of fasting decrease the pO_2; these fluctuations in luminal pO_2 are physiological and have been referred to as physoxia [268, 269].

It has previously been described that a constant hypoxia-associated low-level of inflammation is the physiological norm for the gastrointestinal tract, in which the mucosal immune response is essential in the response to this inflammation [270]. However, conditions in which there is excessive and uncontrolled inflammation are characteristic of IBDs such as Crohn's Disease and Ulcerative Colitis [271]. Thus, the levels of inflammation in the gut must be carefully controlled and one level of control is suggested to be via cellular responses to hypoxia and the hypoxia signalling [272].

1.4.1 The role of the HIF-1 transcription factor in the responses of intestinal epithelial cells to hypoxia

One of the primary responses to hypoxic stress is mediated by the transcription factor, HIF-1. HIF-1 is able to bind to hypoxia response elements in the promoters of several genes involved in the cellular response to hypoxic stress [273, 274]. The HIF-1 transcription factor is a heterodimer, which consists of two subunits: an α subunit which is responsive to oxygen status, and a constitutively expressed β subunit [275]. The HIF-1α protein is highly regulated by cellular pO_2. During normoxia, there are physiological pO_2 levels and therefore a hypoxia response is not needed and thus HIF-1α is rapidly targeted for degradation. During these periods of normoxia, prolyl hydroxylase (PHD) proteins, which are oxygen- and iron- dependent, hydroxylate two proline residues on HIF-1α [276]. This allows von Hippel–Lindau tumour suppressor (pVHL) to bind to it and targets HIF-1α for degradation. However, under hypoxic conditions, oxygen-dependent hydroxylation of HIF-1α by PHD is inhibited and results in the rapid accumulation of HIF-1α inside the cell [277]. Another layer of HIF-1α regulation is provided for by factor inhibiting HIF (FIH), which hydroxylates asparaginyl residues in HIF-1α, resulting in protein interactions between HIF-1α transactivation domains and coactivators in the transcriptional complex being inhibited. This inhibition by FIH is diminished during hypoxia [278]. During periods of hypoxia, PHD and FIH hydroxylation activity is reduced, allowing the nuclear translocation of the HIF-1 complex, where it can interact with co-activators and bind hypoxia response element (HRE) in the promoters of target genes [278, 279].

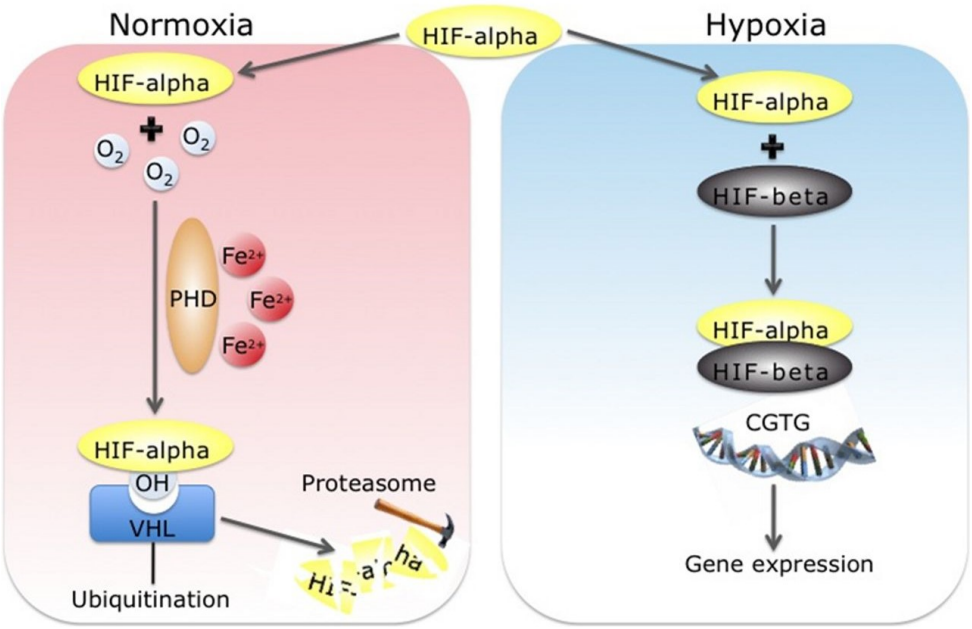

Figure 1.4. Schematic illustration of the regulation of HIF-α by hypoxia. During periods of normoxia, the presence of oxygen and iron allows PHD proteins to hydroxylate HIF-1α and target it for pVHL-mediated degradation. During periods of hypoxia, oxygen-dependent hydroxylation of HIF-1α is inhibited and allows HIF-1α to accumulate. HIF-1α dimerises with the HIF-1β subunit, translocates to the nucleus and regulates the transcription of hypoxia-responsive genes. Adapted from Petousi, 2014 [280]

Inflammation and infection within tissues can increase cellular oxygen demand through the recruitment of oxygen consuming immune cells, such as neutrophils and macrophages, which can induce a localised state of hypoxia [270, 281, 282]. IECs obtained from patients with IBD, were found to have higher expression levels of HIF-1α compared with healthy controls [283]. The role of HIF-1α has been suggested to be protective within the gut by improving barrier function and preventing apoptosis of IECs [284, 285]. In murine epithelial cells, a conditional knockout or reduced expression of HIF-1α diminished barrier function and exacerbated colitis symptoms [286, 287]. Furthermore, inhibiting the degradation of HIF-1α by use of inhibitors reduces the severity of colitis symptoms [285, 287]. Additionally, treatment of a murine experimental model of colitis with a HIF-1α agonist resulted in an increase in the innate immune response of the intestinal epithelium [288].

1.4.2 The cross-regulatory mechanisms between selenium and hypoxia signalling

The literature supports a complex level of cross-regulation between selenium, selenoprotein biosynthesis and responses to hypoxia. A number of *in vivo* studies in a variety of species have suggested that supplementation with Se, in both inorganic and organic forms, has a protective effect against hypoxic stress [289–291]. Furthermore, *in vitro* studies have suggested that this effect may be due to modulatory effects of Se on mRNA and protein expression, as well as the transcriptional activity, of HIF-1α [292–300]. However, the ac-

tual regulation of HIF-1α *in vitro* appears to be dependent upon the dose of Se supplied. For instance, high doses of Se supplementation, at 5 μM and greater, have been shown to have an inhibitory effect on the mRNA and protein levels of HIF-1α [293–295, 297]. In contrast, physiological levels of Se, between 100 and 145 nM, have shown a protective effect of Se against hypoxic stress and raise the level of HIF-1α protein [296, 298].

Hypoxia has been shown to have a modulatory effect on some of the members of the selenoprotein biosynthesis machinery, thereby regulating the expression of different selenoproteins [301]. For instance, hypoxia had a suppressive effect on the mRNA and protein levels of SePP1, TR1 and also the enzyme activities of iodothyronine deiodinase type I (DIO1) and GPX [290, 301, 302]. However, hypoxia has also been shown to increase the mRNA and protein levels of GPX1, GPX4 and selenium-binding protein 1 (SELENBP1) [301, 303]. Furthermore, previous studies have suggested that SELENBP1 is directly regulated by HIF-1 during hypoxia [303, 304]. Additionally, SELENBP1 has also been suggested to regulate the expression of HIF-1α but the mechanism is currently unknown [303, 305]. Interestingly, the regulation of selenoproteins by hypoxia have been described to occur via a mechanism that is independent of HIF-1 [290, 301].

1.5 Roles for Se in the cross-talk between oxidative stress, hypoxia and innate immune signalling

1.5.1 Oxidative Stress and innate immune signalling

Evidence indicates that a significant amount of cross-talk exists between inflammatory and oxidative stress signalling pathways. Caco-2 cells depleted of Se for 72 h have been reported to have raised levels of intracellular ROS [31], which have been previously been reported to modulate the activity of the NF-κB pathway by a number of mechanisms. These include the suppression of protein phosphatase 2A (PP2A) IκB kinase (IKK) dephosphorylation [306, 307], and histone deacetylase (HDAC)-mediated down-regulation of NF-κB transcription of target genes [308, 309]. On the contrary, pre-exposure to ROS has also been shown to inhibit NF-κB activation [310, 311].

Increased intracellular ROS activates the Nrf2 signalling pathway. Both the Nrf2 and NF-κB signalling pathways have downstream effectors involved in carcinogenesis and inflammation, and both pathways have been reported to cross-regulate each other [312, 313]. In fact, inducers of the Nrf2 pathway, including Se-depletion, have previously been reported to inhibit NF-κB pathway activation [312, 314–316]. However, the mechanisms of interaction between the two pathways are unclear and relevant studies have provided contradictory evidence, suggesting both positive and negative regulation between the two pathways [317].

Oxidative stress has also been reported to up-regulate the AP-1 pathway, which has been shown to be mediated through a number of pathways, including JNK, MAPK and p38

signalling pathways [318]. The AP-1 pathway is a regulator of cell homeostasis, and is an important modulator of cell proliferation and apoptotic signalling cascades [319–321]. Se has been reported to be involved in the regulation of AP-1 and cJUN [322–327]. Furthermore, roles for AP-1 and cJUN have previously been reported in the regulation of the NF-κB pathway [177–186].

Se has been shown to modulate the Nrf2 and AP-1 pathways and both of these pathways are regulators of each other and the NF-κB pathway [82, 261, 262, 265, 312, 313, 322, 323, 328]. The modulation of the NF-κB response by Se is well documented but the pathways by which this modulation occurs, is not well understood.

1.5.2 Hypoxia and innate immune signalling

The hypoxia-inducible factor (HIF) family of transcription factors are the main regulators of the cellular response to hypoxia [329]. A number have studies have shown that HIF-1α is induced in a number of cell types in response to pathogenic infection by NF-κB [330], and is also associated with a number of inflammatory diseases [331].

Hypoxia is a well characterised inducer of HIF-1, but is also able to activate the NF-κB pathway in a TAK1-dependent manner [332]. Additionally, during periods of hypoxia and inflammation, NF-κB has been reported to directly regulate the expression of HIF-1 [333, 334]. On the other hand, HIF-1 has previously been shown to inhibit the transcriptional activity of NF-κB [335, 336].

Se has been shown to regulate the activity of both the HIF pathway [296, 298] and the NF-κB pathway [31, 193, 194]. However, the involvement of Se in the cross-talk mechanisms between hypoxia and immune signalling pathways has not yet been been investigated.

1.6 Intestinal epithelial cell models

The Human colonic adenocarcinoma cell model, Caco-2, was originally established in 1974 by Jorgen Fogh [337]. The usefulness of this cell line as an intestinal cellular model was recognised when it was observed that Caco-2 cells spontaneously exhibit enterocytic differentiation within 20 days of passaging [338, 339]. Caco-2 cells, once differentiated, become functionally polarised, featuring apically located microvilli and tight junctions between adjacent cells, as well as expressing characteristic membrane transporters and enzymes [340, 341]. The differentiation process in Caco-2 cells results in down-regulation of Squamous cell carcinoma antigen recognized by T-cells 3 (SART3), a Ribonucleic Acid (RNA) binding protein, which is involved in the splicing of pre-mRNA. Phenotypically, Caco-2 cells initially exhibit a coloncyte phenotype but upon differentiation, start to display properties of small intestine enterocytic phenotype with properties more similar to foetal, rather than adult, ileal enterocytes [339, 342, 343]. Consequently, the Caco-2 cell model has been extensively used for *in vitro* models to investigate a host of intestinal

properties, including the study of gut physiology and nutrient absorption in the gut [344, 345].

The Caco-2 cell model, either on its own or in co-cultures with other cell types found in the intestinal mucosa, has also been used extensively as a model for innate immunity in the gut [346–350]. Similar to intestinal epithelial cells *in vivo*, Caco-2 cells also express receptors for PAMPs, such as TLR5. Accordingly, they also express and secrete immune mediators such as antimicrobial peptides, such as hBD1 and hBD2, as well as pro-inflammatory mediators, such as Interleukin-6 (IL-6), IL-8 and TNF-α, in response to various PAMPs [345, 351, 352]. However, caution must be taken when inferring properties of undifferentiated Caco-2 cells to differentiated Caco-2 cells, as alluded to above.

1.7 The aim of current research

1.7.1 Gaps in knowledge

Although the anti-inflammatory effects of Se in a number of species have been previously discussed in Section 1.3, the role of Se in the responses of *in vitro* models of IECs have received less attention. One study has reported anti-inflammatory effects of Se in undifferentiated IECs [31], but the effects of Se in differentiated IECs, which are considered more physiologically relevant, have not been investigated prior to the submission of the present work.

The effects of low Se status on altered expression of oxidative stress and hypoxia associated genes have been reported in a number of cell types (Sections 1.3.1 and 1.4). However, the effects of IEC differentiation on the regulation of oxidative stress and hypoxia associated genes by Se-depletion has not received much attention. As a significant amount of cross-talk exists between immune, oxidative stress and hypoxia signalling pathways (Section 1.5), it is important to investigate how the effects of Se on pathways which affect immune responses in more physiologically relevant IEC models.

In order to more accurately model the conditions of IECs *in vivo*, it is important to consider the steep oxygen gradient which exists in the gut, with the apical membranes of IECs exposed to hypoxic conditions and the basolateral membranes exposed to normoxic conditions. At the time of submission of the present work, the effects of a steep oxygen gradient on the responses of IECs in an *in vitro* IEC model has not yet been investigated.

1.7.2 Research aims and objectives

The aim of the work presented in this thesis was to investigate the effects of Se-depletion, and the selenoproteins TR1 and SelH, in the Caco-2 model of IECs on responses to flagellin, oxidative stress and hypoxia. Furthermore, changes in the effects of Se-depletion were investigated in more physiologically relevant conditions, including differentiated Caco-2 cells and with the presence of a steep oxygen gradient.

In order to achieve these aims, the following objectives were set:

1. Investigate the effects of Se-depletion, and knockdown of expression of the selenoprotein TR1, on responses to flagellin in both differentiated and undifferentiated Caco-2 cells.

2. Investigate the effects of Se-depletion, and knockdown of expression of the selenoproteins SelH and TR1, on the expression of oxidative stress-associated genes in both differentiated and undifferentiated Caco-2 cells.

3. Investigate the effects of Se-depletion, and knockdown of expression of the selenoprotein SelH, on the expression of hypoxia-associated genes in both differentiated and undifferentiated Caco-2 cells in response to hypoxia.

4. Investigate the effects of Se-depletion on responses to flagellin, under conditions of normoxia, hypoxia and a novel dualoxic cell culture chamber, in differentiated Caco-2 cells.

Chapter 2

Materials and Methods

2.1 Human intestinal cellular model

2.1.1 Cell culture maintenance, routine passaging and Caco-2 cell polarisation

All cell culture operations were performed in a Envair Bio2+D Microbiological Class II safety hood. Routine culture of Caco-2 cells (ATCC® HTB-37™) was performed in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose and 0.58 g/L L-glutamine (D5796, Sigma Aldrich UK). To this basal medium, 10% (*v/v*) Foetal Bovine Serum (FBS), 1% (*w/v*) non-essential amino acids, 100 units/mL penicillin and 100 μg/mL streptomycin was added. All cell culture reagents were obtained from Sigma Aldrich UK, unless otherwise stated. Cells were grown in an atmosphere of 5% CO_2 at 37°C. Cell media was routinely replenished every 48-72 h.

Cells were passaged when 70-100% confluent. Passaging was performed by washing cells three times with 1X Phosphate buffered saline (PBS) (D8662, Sigma Aldrich UK), incubating for 10 minutes with 5 mL 1X Trypsin-EDTA (T3924, Sigma Aldrich UK) at 37°C to detach the cell monolayer from the cell culture flask, inactivating the trypsin by adding 13 mL DMEM culture medium to the cell suspension, centrifuging at 1500 rpm for 4 minutes (Centrifuge B 4i, Jouan SA), after which the cell supernatant was decanted from the cell pellet. The cell pellet was re-suspended in 10 mL of routine DMEM cell culture medium and 1 mL cell suspension was transferred to a 75 cm^2 flask or 2.5 mL to a 175 cm^2 flask.

To culture polarised differentiated Caco-2 cells, 1.5 x 10^5 cells were seeded on to 6-well Transwell permeable inserts (Corning UK) for 18 - 21 days in complete DMEM media, as detailed in Section 2.1.1. The media was changed every 3 - 4 days and prior to changing the media, the trans-epithelial resistance (TER) between the apical and basal environments was measured using an epithelial volt-ohmmeter (World Precision Instruments Inc.). The cells were considered polarised and differentiated when the TER values plateaued after approximately 18 days. When cell polarisation was confirmed by TER assay, the cells

	Sense	Antisense
siSELH1	CCGUUGUUGUUAUCGAGCAUUG	CAAUGCUCGAUAACAACGGTC
siSELH2	GAGGCGACCGUUGUUAUCGAGCAUU	AAUGCUCGAUAACAACGGUCGCCUC
siTR1	CACGUGCUUGUGGACAUCAUU	UGAUGUCCACAAGCACGUGUU

Table 2.1. List of SelH-specific and TR1-specific siRNA sequences for transient transfection into Caco-2 cells. Three distinct siRNA sequences were used to knockdown SelH and TR1 gene expression in Caco-2 cells, siSELH1, siSELH2 and siTR1 The sense and antisense sequences are indicated.

were used for further experiments.

2.1.2 Selenium starvation and supplementation

In Se depletion and supplementation experiments, serum-containing medium was replaced with serum-free DMEM medium containing 1% (*w/v*) non-essential amino acids, 100 units/mL penicillin and 100 μg/mL streptomycin, human insulin (I9278, Sigma UK), apo-Transferrin (T5391, Sigma UK). Where appropritae, this was supplemented with 7 nmol/L sodium selenite (S9133, Sigma UK), which is equivalent to 40 nM Se in Se-supplemented media. Caco-2 cells were cultured in Se-deficient (NoSe) or Se-supplemented (Se) medium for 72 h before their responses to different stimuli were measured.

2.1.3 Transfection of SELH-specific or TR1-specific siRNA into Caco-2 cells

Knockdown of gene expression using siRNA was performed using Viromer BLUE (Lipocalyx), a polymer-based transfection reagent. The knockdown of SelH and TR1 expression was performed using two distinct SelH-specific sequences: siSELH1, a HP Custom siRNA (Qiagen UK); siSELH3, a pre-designed Stealth RNAi^{TM} siRNA (ThermoFisher UK); and siTR1, a *Silencer*® siRNA (Ambion UK). The sense and antisense sequences forming the siRNA duplexes for each sequence are shown in Table 2.1.

Caco-2 cells were plated at a seeding density of 3.5 x 10^5 cells per well, in a 6-well plate format in antibiotic-free DMEM media containing 10% FBS, 1% (*w/v*) non-essential amino acids, 100 units/mL penicillin and 100 μg/mL streptomycin. Cell media were replaced 24 h later with 2 mL Opti-MEM Reduced Serum Medium (ThermoFisher UK), supplemented with 5% FBS, 1% (*w/v*) non-essential amino acids. A cocktail of transfection complexes with a total volume of 200 μL was prepared consisting of 186 μL Buffer F (Lipocalyx), 2.5 μL Viromer BLUE (Lipocalyx) transfection reagent, 11.5 μL of siRNA duplexes from 20 μM stock. The transfection cocktail was incubated for 10 minutes at room temperature and added to the Caco-2 cell media. The cell media was changed to the standard DMEM culture media, as described in Section 2.1.1, at 24 h post-transfection. At 24- and 48-h post-transfection, cells were lysed for RNA and protein analysis as detailed in Section

2.2.1 and Section 2.3.2, respectively.

2.1.4 Hypoxic challenge of Caco-2 cells

Caco-2 cells were subjected to a hypoxic environment within a sealed Invivo$_2$ 400 (Baker Ruskinn) hypoxia chamber. The hypoxic environment was maintained at an ambient temperature of 37 °C with a gaseous composition of 1% O_2, 5% CO_2 and 94% N_2.

To ensure the DMEM media had the appropriate hypoxic gaseous composition, it was left exposed inside the hypoxia chamber overnight. This ensured that the gases dissolved inside the liquid media had reached equilibrium with the hypoxic environment. Caco-2 cells were left for 3 or 7 h inside the hypoxia chamber. Lysates were collected for RNA and protein analysis as described in Section 2.2.1 and Section 2.3.1, respectively.

2.1.5 NF-κB activation of Caco-2 cells via TLR5-specific agonist, flagellin

Caco-2 cells, both undifferentiated and differentiated, were challenged with 500 ng/mL *S. typhimurium* flagellin (FLA-ST) (Invivogen) for 6 and 16 h. After the challenge period, cell lysates and media were collected for RNA and protein analysis, respectively.

2.1.6 Use of a novel dual-environment cell culture chamber to challenge Caco-2 cells with a TLR5 agonist, flagellin, in a more physiologically relevant environment

Studies that use the differentiated Caco-2 cell culture model use non-physiological 20% O_2 aerobic or 1% anaerobic environments to study immune responses. A novel dual-environment cell culture chamber was used in order to investigate if Se has a modulatory role which is dependent upon the levels of oxygen present in the apical and basal culture media (Figure 2.1).

To assess the effects of culture environment on the modulatory effects of Se, Caco-2 cells were first grown until polarised and differentiated, as described in Section 2.1.1. Caco-2 cells were incubated at 37 °C in either a dual-environment (apical: 0% O_2, 10% CO_2; basal: 20% O_2, 5% CO_2), hypoxic environment (1% O_2, 5% CO_2) or aerobic environment (20% O_2, 5% CO_2) for 4 h, with their media changed to Se or NoSe media (Section 2.1.2), which has been equilibrated to the appropriate environment for 24 h, at the 0 h time-point. After 4 h, the apical and basal media were treated with flagellin to a final concentration of 500 ng/mL for 6 h. Cell lysates and apical/basal media samples were collected for RNA and protein analysis, respectively.

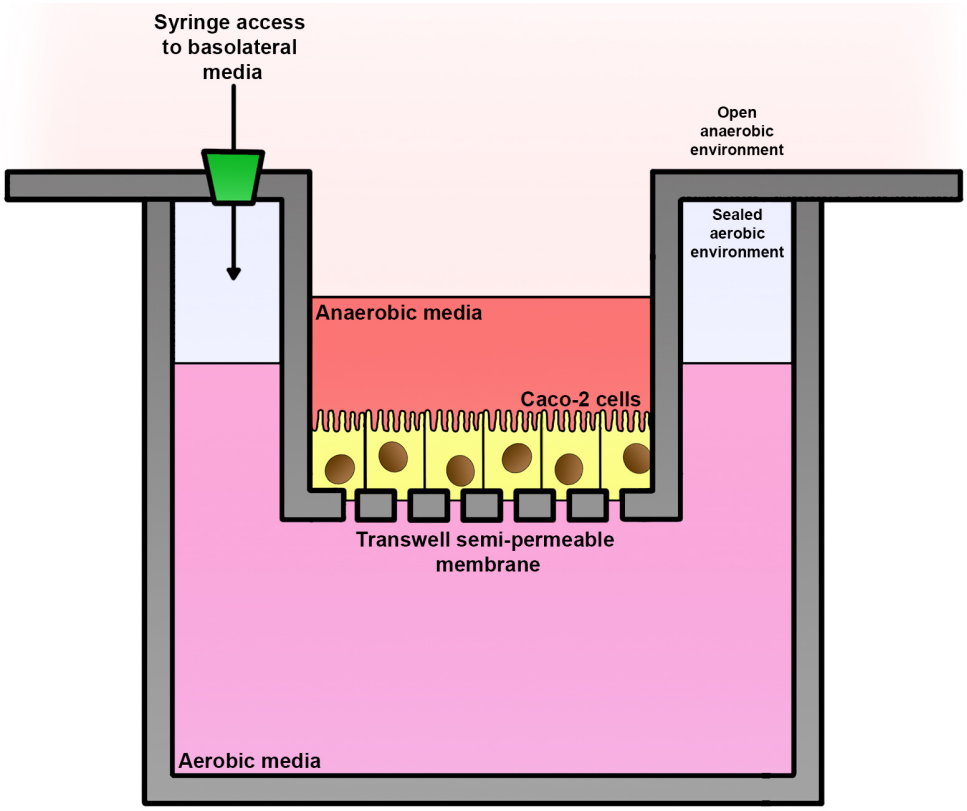

Figure 2.1. The dual-environment cell culture chamber. The apical pole of the cells were exposed to an anaerobic environment (0% O_2, 10% CO_2, 90% N_2) maintained by an anaerobic cell culture hood. The basal pole of the cells were exposed to an aerobic environment (20% O_2, 5% CO_2, 75% N_2), maintained by the air-tight seal of the dual-environment cell culture chamber and the the tight junctions formed between the Caco-2 cells. Access to the basal media for the treatment of the Caco-2 cells with flagellin was via a syringe access port.

2.2 Real-time quantitative PCR to quantify gene expression

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed to quantify gene mRNA expression.

2.2.1 Total RNA extraction and purification

Total RNA extractions were performed using SV Total RNA Isolation extraction kit (Promega UK) as per the manufacturer protocol. Briefly, Caco-2 cells were lysed in 0.01 M Tris RLA lysis buffer (Promega UK) containing 4 M guanidine thiocyanate (GTC) and 0.97% β-mercaptoethanol (BME), and cell lysates passed through a 20-gauge needle. Proteins present in the lysates were precipitated from solution using RNA Dilution Buffer (Promega UK) and incubating at 70 °C. Cell lysates were diluted with 95% ethanol and filtered through spin columns containing a silica-membrane based system. Bound RNA was washed with RNA Wash Solution (Promega UK), containing 162.8 mM potassium acetate and 27.1 mM Tris-HCl (pH 7.5), treated for 15 minutes with DNase I enzyme

(Promega UK) followed by a DNA Stop Solution (Promega UK) containing 2 M GTC, 4 mM Tris-HCl and 57% ethanol. Two more washes with RNA Wash Solution were performed and the bound RNA eluted using 100 μL nuclease-free water. The RNA concentrations of samples, as well as purities, 260/280 and 260/230 ratios, were measured using a NanoDrop 1000 (Thermo Scientific USA). Isolated RNA samples were stored at -80 °C until needed for the synthesis of complementary DNA (cDNA) via reverse transcription, as described in Section 2.2.2.

2.2.2 cDNA synthesis via reverse transcription

cDNA synthesis was performed by incubating 0.5 μg of isolated total RNA with 500 ng of random hexamers (GE Biosciences) at 65 °C for 5 minutes and incubation on ice for 2 minutes to prevent secondary structure formation. The samples were incubated with 0.5 mM of deoxyribose nuclear triphosphates (dNTP) mixture (Promega UK), 1X M-MLV Buffer (Promega UK), 100 U M-MLV Reverse Transcriptase and 20 U Recombinant RNasin® Ribonuclease Inhibitor (Promega UK) in a final volume of 20 μL at 42 °C for 120 minutes. Samples underwent a enzyme-inactivation step by incubating at 70 °C for 1 minute. For each batch of reverse transcription reactions, three negative controls were included: a control including only RNA and water, a control containing the normal reaction mixture minus reverse transcriptase and a water-only control. cDNA samples were diluted by a factor of 1 in 10 and stored at -20 °C until required.

2.2.3 Primer design for Real-Time qPCR

Primers were designed using the Primer3 search algorithm at the NCBI Primer-BLAST portal. The search identified primer sequences that spanned exon-exon junctions to prevent amplification of possible contaminant genomic DNA in the RT-qPCR reaction. Additionally, the search was constrained to retrieve primer sequences generating a PCR product size of between 70 bp and 150 bp. Primer sequences used for Real-Time qPCR are presented in Table 2.2.

The primer pairs for IL-8, TNF-α were bought as a pre-designed primer pair from PrimeTime Premade qPCR library (Integrated DNA Technologies UK) with the product codes Hs.PT.56a.38869678.g and Hs.PT.58.45380900, respectively. The housekeeping genes β2-microglobulin (B2M), eukaryotic initiation factor 4A-II (EIF4A2) and TOP1 were obtained from PrimerDesign as part of the GeNorm package, as detailed in Section 2.2.5. The optimal annealing temperatures for the geNorm primer pairs were set at 60 °C, as per manufacturer instructions. The optimal annealing temperatures of the primer pairs which were designed using Primer3, which can be found in Table 2.2, and those obtained from the PrimeTime Premade qPCR library were determined by thermal-gradient endpoint-polymerase chain reaction (PCR) as discussed in Section 2.2.4.

Target	Forward Primer	Reverse Primer	Product (bp)	Temp°C
SELH	CTTCGAGGTGACGCTGCT	CTTGAGGCTCAGGGAATTTG	101	59
HMOX1	CCTTCTTCACCTTCCCCAAC	CTCTTCTATCACCCTCTGCC	113	59
HIF1a	CCAGCAGACTCAAATACAAGAACC	TGTATGTGGGTAGGAGATGGAGAT	138	59
HERPUD1	GGTCCTGGTTTCTCCGGTTAC	TGGTTGTGAATAGGGGCTGGA	188	59
cJUN	TGCCTCCAAGTGCCGAAAAA	TGACTTTCTGTTTAAGCTGTGCC	143	59
NQO1	ATGTATGACAAAGGACCCTTCC	TCCCTTGCAGAGAGTACATGG	88	59
TR1	GACAGTTCGTACCAATTAAAGTTGAAC	GCCAGCATCACCGTATTATATTC	124	60
GPX1	TATCGAGAATGTGGCGTCCC	TCTTGGCGTTCTCCTGATGC	143	60
GPX2	GTCCTTGGCTTCCCTTGC	TGTTCAGGATCTCCTCATTCTG	67	60
GPX3	AAGAGCTTGCACCATTCGGT	CCTGGTCGGACATACTTGAGG	115	60
GPX4	GCCATCAAGTGGAACTTCACC	CCTTCTCTATCACCAGGGGC	119	60
BNIP3	TGGACGGAGTAGCTCCAAGA	CTTCCTCAGACTGTGAGCTGT	131	60
PHD3	CTTGGCATCCCAATTCTTGT	ATCGACAGGCTGGTCCTCTA	186	60
DEC1	CCTTGAAGCATGTGAAAGCA	TTCAGGTCCCGAGTGTTCTC	202	60
GLUT3	CAATGCTCCTGAGAAGATCATAA	AAAGCGGTTGACGAAGAGT	172	60
hBD1	GATGGCCTCAGGTGGTAACT	CGGGCAGGCAGAATAGAGAC	100	60
hBD2	CAGCCATCAGCCATGAGGGT	CCACCAAAAACACCTGGAAGAGG	83	58

Table 2.2. List of primer pairs designed for RT-qPCR. Optimal annealing temperatures (Temp°C), as determined using gradient endpoint PCR, are indicated. Primers in this table were designed using Primer 3 from the NCBI Primer BLAST portal as described in Section 2.2.3 and were synthesised by Eurofins MWG Operon.

2.2.4 Determining optimal annealing temperatures by endpoint-PCR and agarose gel electrophoresis

An TC-5000 thermal cycler (Techne) was used to perform amplification of genome DNA target sequences by PCR. Each 20 μL PCR reaction mixture consisted of 1.5 μL of an equal mixture of a primer pair (final concentration 1.0 μM), 5.0 μL of 5X GoTaq® Green PCR buffer (Promega UK), 2.5 μL of a 10X dNTP mixture (Promega UK), 1.25 U GoTaq® Polymerase enzyme (Promega UK), 9.8 μL nuclease-free water and 1.0 μL of undiluted template cDNA, generated as described in Section 2.2.2. A gradient-PCR temperature program was used for amplification. This included an initial 3 minute activation step at 95 °C, followed by 35 cycles of denaturing step at 95 °C for 30 seconds, annealing at a range of temperatures from 50 °C to 64 °C for 30 seconds and a final extension step at 72 °C for 30 seconds. Samples then underwent a final extension cycle for 10 minutes at 72 °C before incubation at 4 °C.

Electrophoresis of 20 μL of the above-mentioned endpoint-PCR product and 5 μL of HyperLadder IV (BioLine) was performed on a 2% agarose/1X Tris-borate EDTA (TBE) gel for 1 h at 5 V per cm of gel length. Products were visualised and imaged on a Bio-Rad™ Gel Doc XR system to ensure that they were of the expected product size as stated in Table 2.2.

2.2.5 Using GeNorm to determine appropriate housekeeping genes for Real-Time qPCR

For accurate RT-qPCR, it was necessary to identify suitable housekeeping genes which were stably expressed in both the cell line used and experimental condition being manipulated. geNorm is a script which calculates the gene-stability measures of candidate housekeeping genes in a given set of samples. The script discards the worst-scoring gene and recalculates the gene-stability measures for the remaining genes until two or more housekeeping genes with optimal gene-stability measures have been identified. The genes from the Human geNorm reference gene kit (Primer Design Ltd UK) were analysed for measures of gene-stability in differentiated Caco-2 cells under the conditions of exposure to 7 h hypoxic stress and 16 h of hypoxic challenge. The genes examined included β-actin (ACTB), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ubiquitin C (UBC), B2M, tyrosine 3-monooxygenase (YWHAZ), 18S ribosomal RNA (18S), ribosomal protein L13a (RPL13A), EIF4A2, topoisomerase I (TOP1) and ATP synthase, H+ transporting, mitochondrial F1 complex, β polypeptide (ATP5B).

2.2.6 RT-qPCR using Roche LightCycler 480

The Roche LightCycler® 480 was used to perform RT-qPCR with SYBR green and the relative quantification of target gene mRNA expression was calculated using a relative standard curve method [353]. RT-qPCR reactions were performed in duplicate with a

total reaction mixture of 10 μL. Each sample comprised out 2.0 μL template cDNA, 5.0 μL of SYBR Green I Master mix (Roche UK), 0.5 μL of a 10 μM mixture of primer pairs and 2.5 μL of nuclease-free water. The RT-qPCR reaction mixture underwent a thermal cycling program, first consisting of an initial denaturing step at 95 °C for 5 minutes. This was followed by 45 cycles of a thermal cycling program, which consisted of a further denaturing step at 95 °C for 10 seconds, incubation at the optimal annealing temperature for the primer pair as shown in Table 2.2 and Section 2.2.3 for 15 seconds, and an extension step at 72 ° for 5 seconds. Finally, this was followed by a melt-curve analysis and cooling to 4 °C. For each RT-qPCR run, three reactions were performed for the three negative controls described in Section 2.2.2.

2.2.7 Analysis and Normalisation of RT-qPCR data

The binding of the primer pair to the template cDNA forms a double stranded DNA amplicon with a minor groove to which the SYBR Green I Master mix (Roche UK) can intercalate with. This intercalation reaction generates fluorescence which increases exponentially with each cycle of RT-qPCR reaction. The point during thermal cycling at which this fluorescence reaches a pre-defined threshold of exponential growth is known as the C_T value. As the C_T values are proportional to the exponential accumulation of the DNA amplicon, the relative concentrations of a target gene in the original cDNA template can be calculated using the standard curve method [353]. Standard curves for every primer pair were generated using a dilution series of undiluted cDNA to assess amplification efficiencies at different dilutions. Each RT-qPCR reactions were performed in duplicate and the standard error of the technical replicates were calculated. If the standard error of a replicate exceeded 10% then that sample was not used in further analysis. Data are presented as average or normalised relative concentration of the experimental group ± standard error of the mean (SEM). All statistical analysis was performed using GraphPad (Instat USA) and these are detailed in figure legends and results sections.

RT-qPCR data of target genes were normalised against the geometric mean of two appropriate housekeeping genes for the experimental conditions being manipulated. The method by which these housekeeping genes were identified was discussed in Section 2.2.5.

2.3 Western blotting to quantify protein abundance in cell lysates

2.3.1 Total protein extraction

Caco-2 cells cultured in 6-well plates were washed twice with PBS and harvested by scraping into 150 μL of protein lysis buffer (Table 2.3). Protein lysates were sonicated using a Bioruptor (Diagenode) and stored at -80 °C before further analysis by Western Blotting.

Component	Concentration
HEPES	60 mM
NaCl	154 mM
KCl	3 mM
EDTA	5 mM
Triton X-100	1 %
SDS	0.1 %

Table 2.3. List of lysis buffer components for use in total protein extraction

2.3.2 Total protein concentration determination by Bradford assay

Total protein concentrations of the cell lysates were determined using Bradford assay (BIO-RAD). The assay was performed in a 96-well plate format (Greiner). The Bradford reagent was diluted 1:4 with deionised water and a standard calibration curve was generated using bovine serum albumin (BSA) (Promega) concentrations ranging from 1 mg/mL to 16 mg/mL. For each standard, sample or water control, 1 μL of each was added in duplicate to 200 μL of diluted Bradford reagent . The contents of the wells were mixed thoroughly by aspiration before the absorbance at 595 nm was read using a FluoStar Omega Plate Reader (BMG Labtech Germany). The sample concentration for each lysate was calculated using the calibration curve from the BSA standard curve.

2.3.3 SDS-PAGE electrophoresis

Protein collected from cell lysates were separated using sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) performed on 4-12 % Novex NuPAGE Bis-Tris Pre-Cast gels (Life Technologies). Protein samples were incubated at 70 °C in a mix of 4X NuPAGE lithium dodecyl sulphate (LDS) Sample Buffer and 10X NuPAGE Reducing Agent (Life Technologies). The protein samples were loaded onto a gel and electrophoresed at 200 V for 50 minutes in NuPAGE 2-(N-morpholino)ethanesulfonic acid (MES) SDS running buffer (50 mM MES, 50 mM Tris Base, 0.1 % SDS, 1 mM EDTA at pH 7.3) before use in Western Blot analysis.

2.3.4 Western Blot

Proteins separated by SDS-PAGE gel electrophoresis were transferred to nitrocellulose membrane (Bio-Rad) or PVDF membrane (Immobilon-P Transfer Membrane; IPVH00010) for 90 minutes by semi-dry transfer, after which, the membrane was blocked in 4% Marvel/TBS-T (0.1% TWEEN-20) for 1 h at room temperature. Membranes were washed 3 times with TBS-T before overnight incubation at 4 °C with the primary antibody anti-SELH (1:1000, Santa Cruz Biotechnology), anti-HIF1a (1:1000, Cell Signalling) or for 1 h at room temperature with anti-ACTIN (1:2000, Cell Signalling). Membranes were

washed for a further 3 times in TBS-T and incubated with the appropriate HRP-tagged secondary antibody (1:1000 in 4% Marvel/TBS-T) for 1 h at room temperature. Membranes were developed using ECL (Thermofisher) as per manufacturer's instructions on X-ray film using a X-ray film processor (Xograph Compact X4).

2.4 Enzyme-linked immunosorbent assay to quantify protein abundance of interleukin-8 in cell media extracts

Following stimulation of Caco-2 cells with *S. typhimurium* flagellin for 6 and 16 h, as described in Section 2.1.5 and Section 2.1.6, cell media was collected to measure the quantity of IL-8 present in the media. The enzyme-linked immunosorbent assay (ELISA) was performed using the Human IL-8 ELISA Ready-SET-Go! 2nd Generation (Affymetrix eBioscience) kit, as per manufacturer protocol. Briefly, 96-well plates (NUNC Maxisorp) were coated with IL-8 capture antibody overnight. Each well was washed the recommended number of times with wash buffer and 100 μL of sample, IL-8 standard or negative control was added to the appropriate wells overnight. Following further washes, 100 μL or detection antibody, Avidin-HRP, TMB Solution was added sequentially, with washes in between. Finally, 50 μL of Stop Solution was added to each well before reading the plate at 450 nm.

2.5 Statistical Analyses

Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, USA). One-way or two-way ANOVA were performed to compare the means of data sets, after which, multiple comparisons were performed using Tukey's post hoc tests to compare each treatment mean to every other treatment mean.

Chapter 3

Innate immunity of the gut and the importance of selenium

3.1 Introduction

3.1.1 The role of the microbiota and intestinal epithelial cells in host innate immunity

The gastrointestinal tract hosts an epithelium with the largest surface area of the body and is colonised by more than 100 trillion microorganisms, consisting mostly of bacteria but also includes fungi, viruses and parasites. Collectively, these microorganisms are commonly referred to as the microbiota. The microbiota have a mutualistic relationship with the host organism, digesting and fermenting dietary carbohydrates, producing vitamins and helping to prevent the colonisation of the gut by pathogens. In return, the host provides nutrients to the microbiota and a niche in which the microbiota can thrive [354].

Beneath the layer of microbiota is a continuous layer of IECs, which function to absorb nutrients from the diet, and protect of the host from invasion by potential pathogenic microorganisms that enter the gastrointestinal tract. The tight barrier of IECs includes a variety of cell types, including Paneth cells, Goblet cells and enteroendocrine cells, each possessing a unique distinct function in the gut. The most numerous of the IECs, however, are the enterocytes that function in nutrient absorption and the local immune responses that help protect the host from infection [103].

3.1.2 Dysbiosis, oxygen and chronic inflammation - breakdown of the barrier function and inappropriate immune responses to commensal microbiota

Although the relationship between the microbiota and host is mutualistic, under certain conditions, dysbiosis can occur. Dysbiosis occurs when there are imbalances, and hence changes, in the phyla of the microbiota [354]. Furthermore, changes in the gut microbiota

have been associated with the pathogenesis of certain inflammatory diseases, such as in IBD [355]. However, it is currently under debate whether the changes in the microbiota are a cause or consequence of IBD.

Observations have been made that the phyla of microbiota that colonise a particular location, either by their proximity to the epithelial cell wall, or longitudinally along the gut axis, are dependant upon the concentration of oxygen that the microbiota are exposed to [356–358]. Furthermore, it has been hypothesised that chronic inflammation, such as seen in IBD, can increase the oxygen concentration of the affected area of the gut and thus cause dysbiosis [359].

Chronic inflammation of the gut wall results in breakdown of the barrier function of the IECs, allowing bacterial antigens, such as flagellin, to leak through from the lumen into the host mucosa and initiate innate and adaptive immune responses [360]. Flagellin is the only known agonist for the cell-surface receptor, TLR5, which is known to induce a pro-inflammatory response in IECs [361, 362]. This response to flagellin results in release of pro-inflammatory cytokines, such as IL-8 and TNF-α, as well as defensins, such as hBD2. Furthermore, it has previously been reported that flagellin is one of the dominant antigens in Crohn's disease, demonstrating its potential importance in the pathogenesis and pathology of IBD [363, 364].

Se has primarily received much research interest due to its anti-oxidative and anti-inflammatory functions, in addition to its potential role in cancer prevention [1, 2]. Additionally, it was reported that low Se status was associated with increased prevalence of Crohn's disease [141]. In a number of rodent studies, it has been shown that Se deficiency correlates with exacerbated disease severity in experimental colitis models [140, 150], and furthermore, it has been shown that supplementation with Se has provided beneficial effects in a range of experimental settings of colitis in rodents [140, 143, 150, 151].

The mechanisms by which Se may modulate inflammatory responses are poorly understood. In mice, the composition of the gut microbiota was shown to affect the levels of Se in the host [155]. Conversely, alterations in the levels of dietary Se have been shown to affect the composition of the microbiota in mice [156]. A study using murine models of colitis found consistent up-regulation of GPX2 mRNA. Furthermore, a study in rodents found that a double knockout of the selenoproteins, GPX1 and GPX2, resulted in ileocolitis [365]. Additionally, knockout of glutathione peroxidase 3 (GPX3) expression in a dextran sulfate sodium (DSS)-induced colitis model was observed to exacerbate colitis symptoms in mice [366]. *In vitro* studies in cultured human cells suggest that modulation of the inflammatory response of IECs may partly be attributed to the regulation of NF-κB signalling through the activity of GPX selenoproteins [31, 162]. A role for the selenoprotein, TR1, in the regulation of the NF-κB-mediated inflammatory response has also been observed in an epithelial cell line [367]. Thus, there is evidence that the regulation of inflammation by Se may be mediated through altered expression of selenoproteins in host cells.

3.1.3 Research aims

The aim of the research reported in this chapter was to assess the effects of Se supplementation or depletion on the response of differentiated and undifferentiated Caco-2 cells to challenge with *S. typhimurium* flagellin. Furthermore, the potential roles of the selenoprotein TR1 in these responses to flagellin were also investigated.

3.2 Results

3.2.1 Effect of selenium depletion on the expression of immune effectors and selenoproteins in response to flagellin challenge in differentiated Caco-2 cells

Caco-2 cells were grown on Transwell semi-permeable inserts for 18 days to allow polarisation and differentiation (Section 2.1.1). The cells were then depleted of Se (NoSe), or supplemented with Se (Se) for 72 h (Section 2.1.2).

Following the 72 h depletion or supplementation period, the cells were challenged with bacterial flagellin (Section 2.1.5) and RNA isolated (Section 2.2.1). The mRNA expression level of the immune effectors IL-8, hBD2, TNF-α and hBD1 were measured after 6 h and 16 h flagellin treatment by RT-qPCR. mRNA levels relative to the geometric mean of housekeeping genes TOP1 and EIF4A2 are shown in Figure 3.1. After 6 h of flagellin treatment, mRNA expression of all of the immune effectors IL-8, hBD2, TNF-α and hBD1 was induced due to flagellin treatment compared with unchallenged controls ($P < 0.01$). There was a 0.21-fold increase in the flagellin-induced expression levels of hBD2 in the Se-depleted cells (NoSe) compared with those that were supplemented with Se ($P < 0.01$) (Figure 3.1B). After 16 h of flagellin treatment, there was an induction in the mRNA expression of IL-8 ($P < 0.001$), hBD2 ($P < 0.01$) and TNF-α ($P < 0.01$) but this induction was reduced compared to values observed for these targets after 6 h of flagellin treatment. At the 16 h time-point, there was a 0.7-fold increase in the flagellin-induced mRNA expression of hBD2 ($P < 0.001$; Figure 3.1B), as well as a 0.42-fold increase in induced TNF-α expression ($P < 0.05$; Figure 3.1C). There was no effect due to Se-depletion or flagellin on hBD1 expression at the 16 h time-point (Figure 3.1D) and no effect of Se-depletion on IL-8 expression in the flagellin challenged cells (Figure 3.1A).

The level of secreted IL-8 protein was measured by ELISA in spent media from Caco-2 cells after 6 and 16 h of flagellin challenge (Figure 3.2). At both the 6 h and 16 h time-points, flagellin challenge was found to induce the secretion of IL-8 into both the apical and basolateral media by differentiated Caco-2 cells. After 6 h of flagellin challenge, there was a 0.1-fold increase in the amount of flagellin-induced IL-8 secreted basolaterally by differentiated Caco-2 cell that were depleted of Se (NoSe), compared with those that were supplemented with Se ($P < 0.05$; Figure 3.2A-ii). There was no statistically significant changes observed in the IL-8 levels detected in the apical media after 6 h (Figure 3.2A-ii). After 16 h of flagellin challenge, there was a 0.2-fold increase in the flagellin-induced IL-8 secreted apically by differentiated Caco-2 cells depleted of Se (NoSe) (Figure 3.2B-i; $P < 0.01$). In the basolateral media, there was no significant change in flagellin-induced IL-8 secretion in Se-depleted cells (Figure 3.2B-ii).

The mRNA expression of the selenoproteins of TR1, SelH, GPX1 and GPX2 was measured in response to Se depletion and stimulation with *S. typhimurium* flagellin for 6 and 16

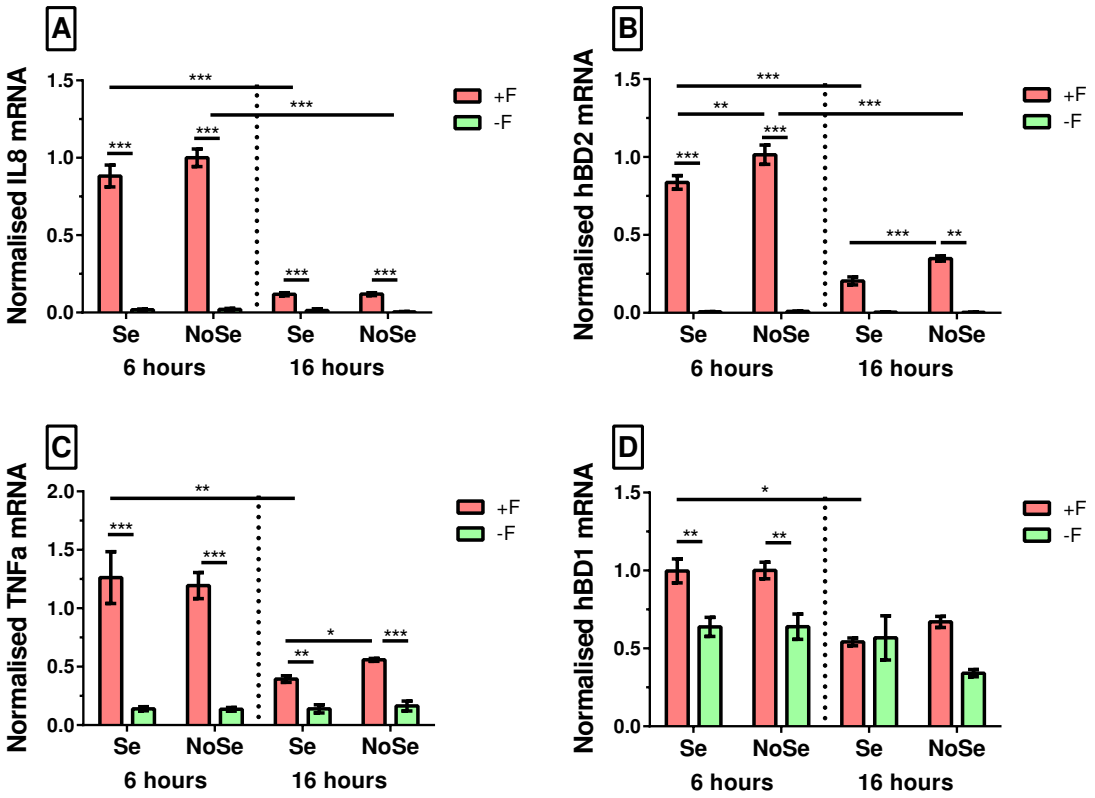

Figure 3.1. Effect of 72 h Se supplementation (Se) or selenium depletion (NoSe) on the mRNA expression of the innate immune effectors IL-8 (A), hBD2 (B), TNF-α (C) and hBD1 (D) in response to 6 and 16 h flagellin (+F) treatment in differentiated Caco-2 cells. All data were normalised to the housekeeping genes TOP1 and EIF4A2. Data were expressed as a proportion of the mean of the NoSe flagellin treated cells in order to combine 3 distinct biological replicates, each consisting of 3 technical replicates (n = 9). Data were analysed statistically using two-way ANOVA with a Tukey post-test for multiple comparisons. The data are displayed as mean ± standard error of the mean (SEM). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

h in differentiated Caco-2 cells (Figure 3.3). After 6 h of flagellin stimulation, there was an induction in the mRNA expression of TR1 in both the Se-supplemented (Se) and Se-depleted differentiated Caco-2 cells (Figure 3.3A; $P < 0.001$). After 16 h of flagellin treatment, there was no induction of TR1 mRNA but a 67 % and 58 % decrease in expression from the 6 h time-point was observed in the Se-supplemented and Se-depleted cells challenged with flagellin, respectively ($P < 0.001$). After 6 h of flagellin stimulation, there was a 41 % reduction in SelH mRNA expression in the Se-depleted differentiated Caco-2 cells (Figure 3.3B; $P < 0.05$), but not in the Se-supplemented cells. There was no effect of Se treatment or flagellin treatment on SelH mRNA expression at the 16 h time-point.

At the 6 h time-point, there was a decrease in GPX1 mRNA expression in both the flagellin-treated and untreated cells due to Se depletion by 50 % and 54 %, respectively (Figure 3.3C; $P < 0.001$). At the 16 h time-point, there was a decrease of 49 % observed in the mRNA expression of GPX1 in the untreated cells ($P < 0.01$), but not in the flagellin-

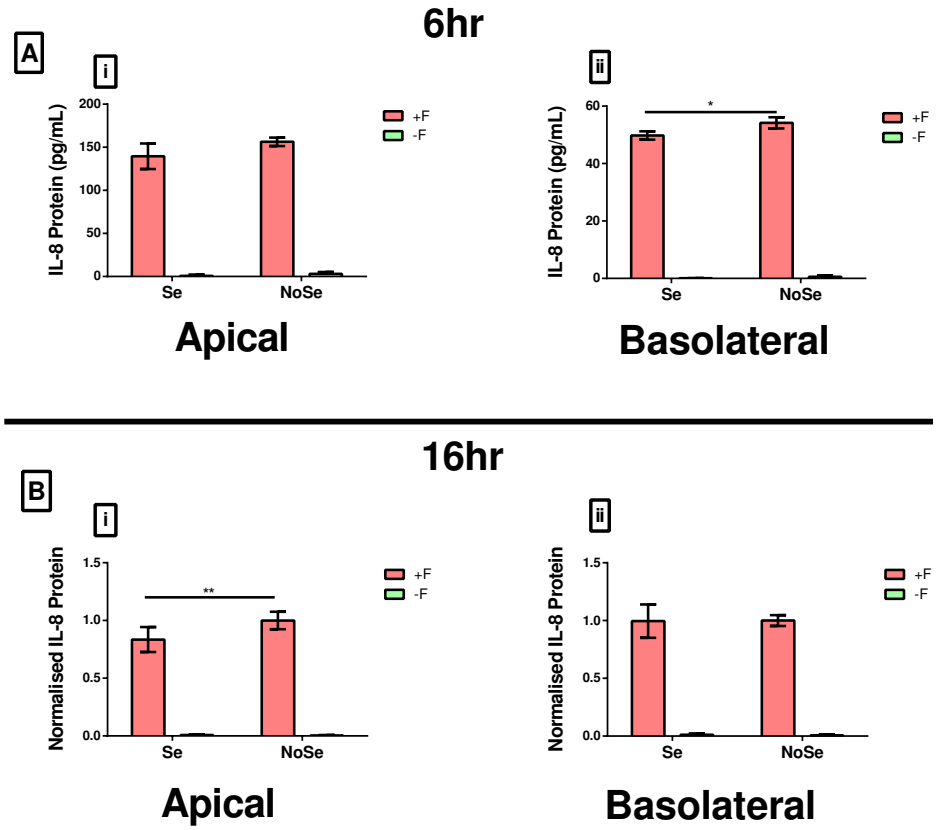

Figure 3.2. Effect of 72 h Se supplementation (Se) or selenium depletion (NoSe) on the secretion of IL-8 by Caco-2 cells in response to 6 and 16 h flagellin (+F) treatment in differentiated Caco-2 cells. The figures denoted by the letters A and B represent data from 6 h and 16 h, respectively, from both the apical (i) and basolateral (ii) secreted media. Data from the 16 h time point were expressed as a proportion of the mean of the NoSe flagellin treated cells in order to combine 2 distinct biological replicates, with each data point consisting of 4 technical replicates (n = 8). Data were analysed statistically using two-way ANOVA with a Tukey post-test for multiple comparisons. The data are displayed as mean ± standard error of the mean (SEM). * $P < 0.05$; ** $P < 0.01$.

treated cells. There were no significant changes in GPX2 mRNA as a result of any of the experimental conditions (Figure 3.3D). However, there was a trend towards increased GPX2 observed as a result of flagellin treatment.

3.2.2 Effect of selenium depletion on the expression of immune effectors and selenoproteins in response to flagellin challenge in undifferentiated Caco-2 cells

Caco-2 cells were grown on 6-well plates to confluency (Section 2.1.1) before being depleted (NoSe) of Se, or supplemented with Se (Se) for 72 h (Section 2.1.2).

Following 72 h depletion (NoSe) or supplementation (Se) of confluent but undifferentiated Caco-2 cells, the mRNA expression of the immune effectors IL-8, hBD2, TNF-α and hBD1 were measured in response to 6 h and 16 h flagellin treatment (Figure 3.4). At the 6 h

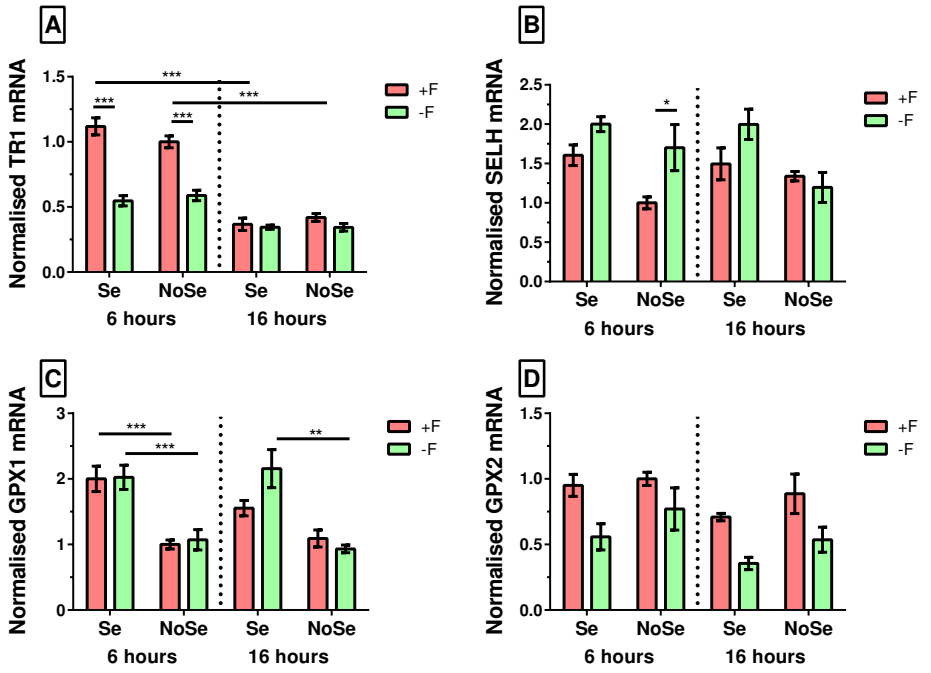

Figure 3.3. Effect of 72 h Se supplementation (Se) or selenium depletion (NoSe) on the mRNA expression of the selenoproteins TR1 (A), SelH (B), GPX1 (C) and GPX2 (D) in response to 6 and 16 h flagellin (+F) treatment in differentiated Caco-2 cells. All data were normalised to the housekeeping genes TOP1 and EIF4A2. Data were expressed as a proportion of the mean of the NoSe flagellin treated cells in order to combine 3 distinct biological replicates, each consisting of 3 technical replicates (n = 9). Data were analysed statistically using two-way ANOVA with a Tukey post-test for multiple comparisons. The data are displayed as mean ± standard error of the mean (SEM). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

time-point, there was flagellin-induced expression of IL-8 (Se: $P < 0.01$; NoSe: $P < 0.001$), hBD2 (Se: $P < 0.05$; NoSe < 0.001) and TNF-α ($P < 0.001$) in both the Se-supplemented and Se-depleted cells was observed. However, there was only induction of hBD1 mRNA at the 6 h time-point in the Se-depleted cells (NoSe: $P < 0.01$). The cells which were Se-depleted and challenged with flagellin for 6 h increased the flagellin-induced mRNA expression of IL-8 by 1.68-fold (Figure 3.4A; $P < 0.001$), hBD2 by 1.74-fold (Figure 3.4B; $P < 0.001$), TNF-α by 0.71-fold (Figure 3.4C; $P < 0.001$) and hBD1 by 0.88-fold (Figure 3.4D; $P < 0.001$), compared with those that were supplemented with Se and challenged with flagellin. At the 16 h time-point there was no detected induction of mRNA of any target gene due to flagellin challenge. Se-depletion resulted in an increase in expression of hBD1 in the cells treated with flagellin (Figure 3.4D; $P < 0.001$) and the untreated cells (Figure 3.4D; $P < 0.05$). When compared with flagellin challenged cells at the 6 h time-point there was markedly lower expression of IL-8 (Se: $P < 0.05$; NoSe: $P < 0.001$) and TNF-α ($P < 0.001$). Interestingly, there was an increase in hBD2 mRNA in the untreated Se-depleted cells from 6 h to 16 h ($P < 0.01$).

ELISAs were performed on spent media from undifferentiated Caco-2 cells after 6 and

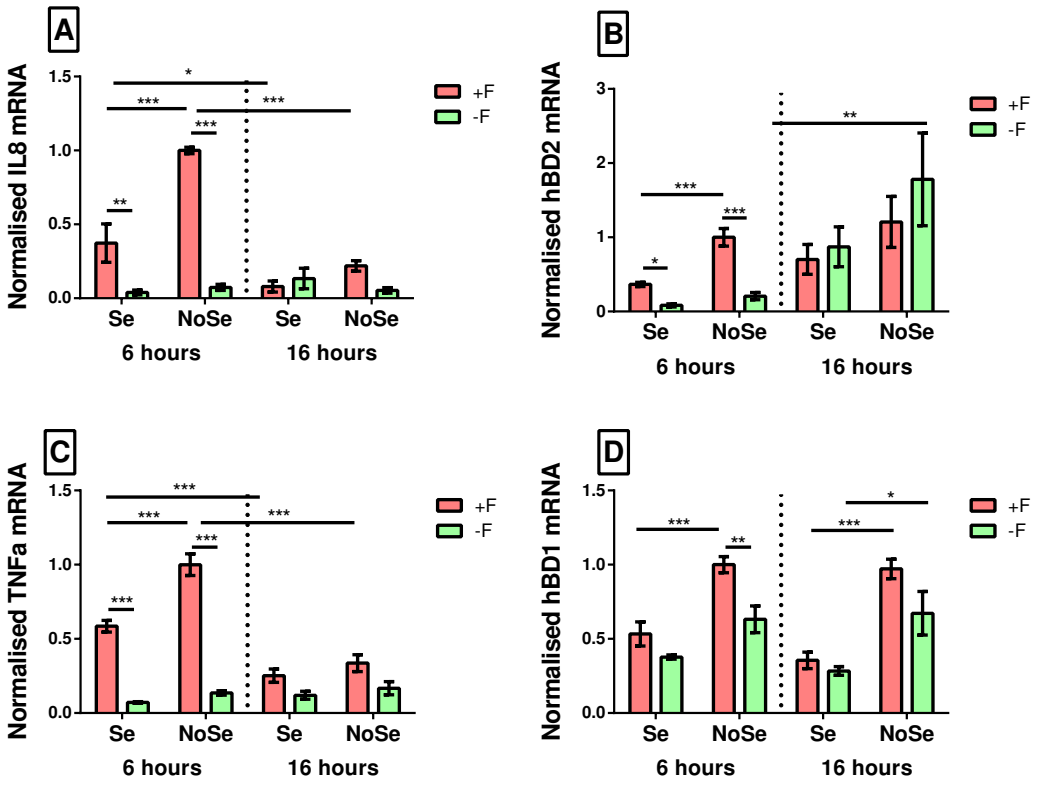

Figure 3.4. Effect of 72 h Se supplementation (Se) or selenium depletion (NoSe) on the mRNA expression of the innate immune effectors IL-8 (A), hBD2 (B), TNF-α (C) and hBD1 (D) in response to flagellin (+F) treatment after 6 and 16 h in undifferentiated Caco-2 cells. All data were normalised to the housekeeping genes TOP1 and EIF4A2. Data were expressed as a proportion of the mean of the NoSe flagellin treated cells in order to combine 3 distinct biological replicates, each consisting of 3 technical replicates (n = 9). Data were analysed statistically using two-way ANOVA with a Tukey post-test for multiple comparisons. The data are displayed as mean ± standard error of the mean (SEM). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

16 h of flagellin challenge in two distinct biological replicates (Figure 3.5). At the 6 h time-point (Figure 3.5A), there was induction of IL-8 in the cells treated with flagellin ($P < 0.001$). In the flagellin-treated cells which were depleted of Se (NoSe), there was a 0.4-fold increase in the amount of IL-8 detected in the media ($P < 0.001$) compared with Se-supplemented cells. At the 16 h time-point (Figure 3.5A), there was induction of IL-8 in the cells treated with flagellin ($P < 0.001$). In the flagellin-treated cells that were depleted of Se (NoSe), there was an 1.01-fold increase in the amount of IL-8 detected ($P < 0.001$) compared with Se-supplemented cells.

The mRNA expression of the selenoproteins of TR1, SelH, GPX1 and GPX2 was measured in response to Se depletion and stimulation with *S. typhimurium* flagellin for 6 and 16 h in undifferentiated Caco-2 cells is presented in Figure 3.6. The mRNA expression of TR1 (Figure 3.6A) was increased following flagellin challenge at the 6 h time-point in both the Se-supplemented cells (1.68-fold; $P < 0.001$) and Se-depleted cells (0.41-fold; $P < 0.01$). Furthermore, Se-depletion caused a reduction in flagellin-induced mRNA expression by

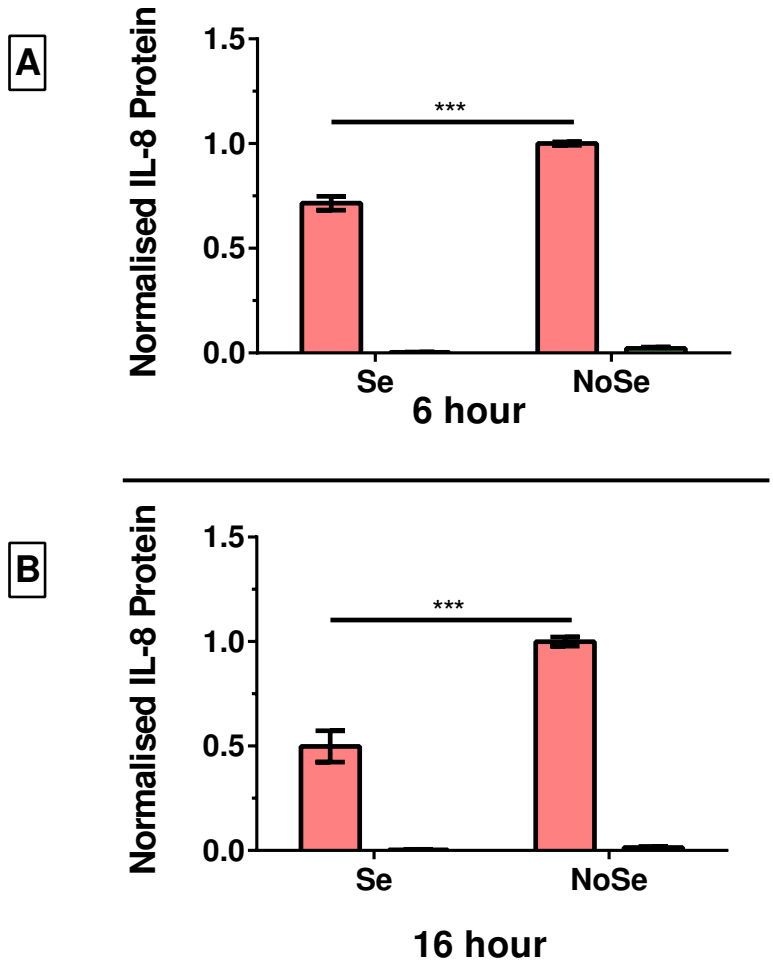

Figure 3.5. Effect of 72 h Se-supplementation (Se) or Se-depletion (NoSe) on the secretion of IL-8 into the media by undifferentiated Caco-2 cells as detected by ELISA. A: 6 h flagellin treatment. B: 16 h flagellin treatment. The data shown represents two biological replicate with 4 repeats (n = 8) for each time-point. Data were analysed statistically using two-way ANOVA with a Tukey post-test for multiple comparisons. The data are displayed as mean ± standard error of the mean (SEM). *** $P < 0.001$.

18 %, compared with that of the flagellin-induced mRNA in the Se-supplemented cells ($P < 0.05$). In the unchallenged cells, however, there was a 0.33-fold increase in TR1 mRNA compared with the Se-supplemented cells ($P < 0.01$). There was no induction due to flagellin challenge observed for TR1 at the 16 h time-point. However, an increase due to Se-depletion was observed in both the challenged and unchallenged cells by 0.29-fold ($P < 0.05$) and 0.53-fold ($P < 0.01$). TR1 mRNA expression levels were decreased at the 16 h time-point in the challenged Se-supplemented cells and the unchallenged Se-depleted cells when compared with their counterparts at the 6 h time-point ($P < 0.001$).

At the 6 h time-point, there was no induction of SelH mRNA observed following flagellin challenge (Figure 3.6B). However, there was a decrease due to Se-depletion observed in both the challenged and unchallenged cells by 62 % and 53 %, respectively ($P < 0.001$). There was also no induction due to flagellin challenge observed for SelH at the 16 h

time-point. A decrease in expression of 51 % due to Se-depletion was observed for the challenged cells ($P < 0.01$), but not for the unchallenged cells.

At the 6 h time-point, GPX1 showed a 0.48-fold induction in mRNA expression in the Se-supplemented cells ($P < 0.01$), but not in the Se-depleted cells (Figure 3.6C). A decrease in GPX1 mRNA expression due to Se-depletion was observed in the challenged and unchallenged cells by 47 % and 38 %, respectively ($P < 0.001$ and $P < 0.05$, respectively). There was no induction of GPX1 mRNA due to flagellin observed at the 16 h time-point but a decrease due to Se-depletion was observed in both the challenged and unchallenged cells by 70 % and 67 %, respectively ($P < 0.01$). There was also an increase in GPX1 mRNA detected at the 16 h time-point in both the challenged and unchallenged cells compared with their counterparts at 6 h ($P < 0.05$ and P¡< 0.001, respectively).

At the 6 h time-point, there was an induction of GPX2 mRNA due to flagellin in both the Se-supplemented and Se-depleted cells by 3-fold and 0.44-fold, respectively ($P < 0.01$ and $P < 0.001$, respectively). Se-depletion resulted in increased GPX2 mRNA expression in both challenged and unchallenged cells by 3.4-fold and 11-fold, respectively (Figure 3.6D; $P < 0.001$). At the 16 h time-point there was a 0.6-fold induction due to flagellin challenge in the Se-depleted cells ($P < 0.001$), but no induction observed in the Se-supplemented cells. There was also an increase in GPX2 mRNA detected in the Se-depleted cells that were both challenged and unchallenged ($P < 0.001$) but these expression levels were reduced compared to their counterparts at the 6 h time-point ($P < 0.05$).

3.2.3 Effects of TR1 knockdown in undifferentiated Caco-2 cells on the expression of immune effectors in response to flagellin challenge

As there was consistent flagellin-induced expression of the selenoprotein TR1 in both the differentiated and undifferentiated Caco-2 cells, the gene was selected for further study. Knockdown of TR1 mRNA expression, as described in Section 2.1.3, was achieved using a TR1-specific siRNA in undifferentiated Caco-2 cells to assess the effect of reduced TR1 expression on the ability of Caco-2 cells to respond to challenge with *S. typhimurium* flagellin for 6 h. Following transfection with TR1-specific siRNA, the level of TR1 mRNA measured 48 h post-transfection and showed an 85% reduction in TR1 mRNA expression compared with cells transfected with negative control (NC) siRNA (Figure 3.7). There was no effect of flagellin challenge on the expression level of TR1 in either the cells transfected with TR1-specific siRNA or negative control siRNA.

After 6 h of flagellin challenge, the expression of IL-8 mRNA increased in the cells transfected with TR1-specific and negative control (NC) siRNA ($P < 0.001$) (Figure 3.8). However, the cells transfected with TR1-specific siRNA exhibited a flagellin-induced increase of IL-8 mRNA but this was 40% reduced compared with cells transfected with negative control siRNA ($P < 0.001$). This effect was also observed in the concentration of secreted IL-8 protein, with a 32 % reduction in IL-8 secreted IL-8 protein (Figure 3.9). After 6 h of

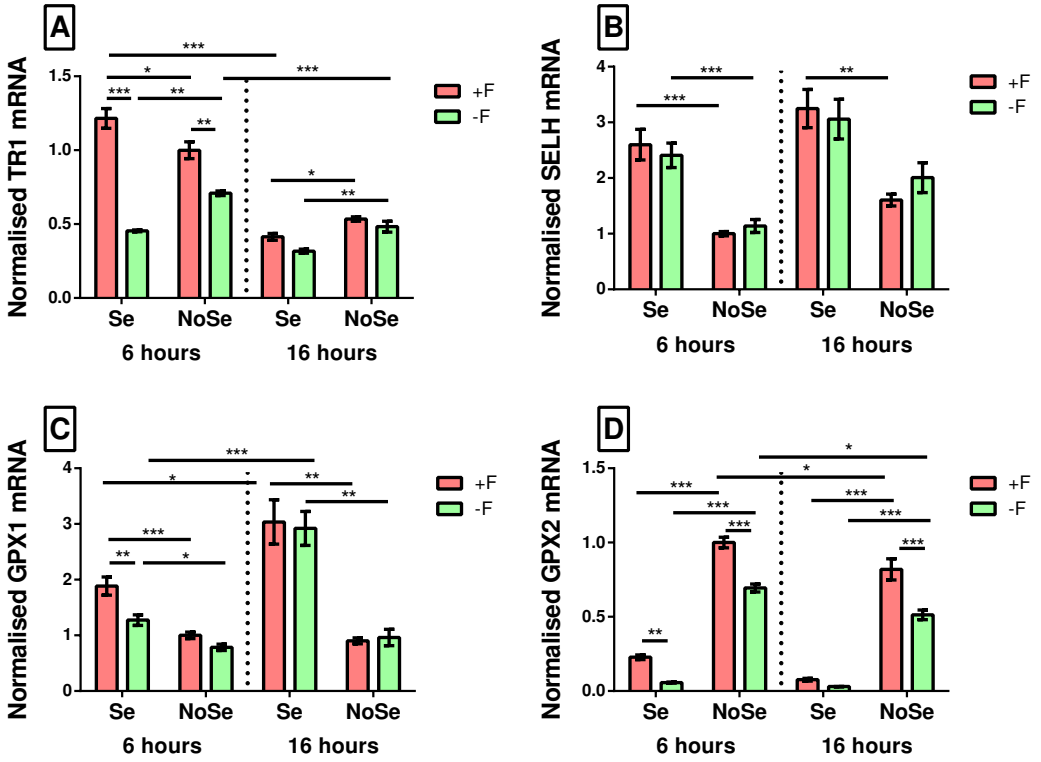

Figure 3.6. Effect of 72 h Se-supplementation (Se) or Se-depletion (NoSe) on the mRNA expression of the selenoproteins TR1 (A), SelH (B), GPX1 (C) and GPX2 (D) in response to 6 and 16 h flagellin (F) treatment in undifferentiated Caco-2 cells. All data were normalised to the housekeeping genes TOP1 and EIF4A2. Data were expressed as a proportion of the mean of the NoSe flagellin treated cells in order to combine 3 distinct biological replicates, each consisting of 3 technical replicates (n = 9). Data were analysed statistically using two-way ANOVA with a Tukey post-test for multiple comparisons. The data are displayed as mean ± standard error of the mean (SEM). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

flagellin challenge, the expression of hBD2 mRNA increased in the cells transfected with TR1-specific and negative control (NC) siRNA ($P < 0.01$ and $P < 0.001$, respectively). However, this was 45% reduced in the siTR1 transfected cells compared with the control ($P < 0.001$). After 6 h of flagellin challenge, the expression of TNF-α mRNA increased in the cells transfected with TR1-specific and negative control (NC) siRNAs ($P < 0.05$ and $P < 0.001$, respectively). However, this was 45% reduced in the siTR1 transfected cells compared with the control ($P < 0.01$). After 6 h of flagellin challenge, the expression of hBD1 mRNA increased in the cells transfected with negative control (NC) siRNA ($P < 0.05$), but not in the cells transfected with TR1-specific siRNA. However, the cells transfected with TR1-specific siRNA exhibited a increase in the challenged and unchallenged cells of hBD1 mRNA which was approximately 40% smaller than that of cells transfected with negative control siRNA ($P < 0.01$ and $P < 0.05$, respectively).

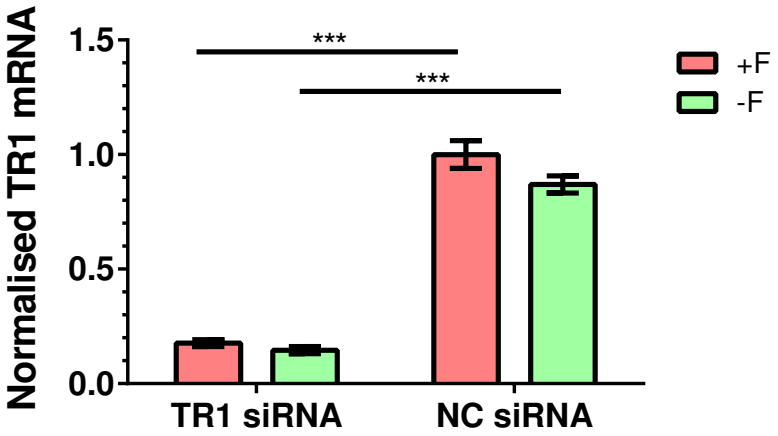

Figure 3.7. TR1 mRNA expression level 48 h post-transfection with TR1-specific siRNA or negative control (NC) siRNA, followed by 6 h challenge with *S. typhimurium* flagellin (+F) or water negative control (-F). All data were normalised to the housekeeping genes TOP1 and B2M. Data were expressed as a proportion of the mean of the NC siRNA transfected cells challenged with flagellin in order to combine 2 distinct biological replicates, each consisting of 3 technical replicates (n = 6). Data were analysed statistically using two-way ANOVA with a Tukey post-test for multiple comparisons. The data are displayed as mean ± standard error of the mean (SEM). *** $P < 0.001$.

3.3 Discussion

The roles of Se in the innate defences of the gut are conflicting but the anti-inflammatory effects of Se have been well-documented in a number of *in vivo* models as well as a range of different cell types in *in vitro* models [193, 368–371]. In this chapter, the anti-inflammatory effects of Se were investigated in Caco-2 cells after stimulation with *S. typhimurium* flagellin. These *in vitro* experiments were performed to assess the effects of Se supplementation or depletion on the responses of differentiated and undifferentiated Caco-2 cells, modelling the gut epithelium, to the bacterial PAMP flagellin. Effectors measured are involved in innate immunity and focused on the pro-inflammatory cytokines IL-8 and TNF-α, and the antimicrobial killing agents, hBD1 and hBD2.

Se-depletion was found to stimulate the expression of the immune effectors IL-8, hBD2, TNF-α and hBD1, induced by stimulation with *S. typhimurium* flagellin in Caco-2 cells. The effect of Se-depletion on the induction of immune effectors was much larger in undifferentiated Caco-2 cells (Figure 3.4). Differentiated Caco-2 cells were more resistant to the effects of Se-depletion and, in response to flagellin treatment, exhibited a higher-fold induction of target mRNA (Figure 3.1) and secreted IL-8 protein (Figure 3.2). The effect of flagellin treatment on selenoprotein mRNA expression was measured and showed a consistent induction of TR1 mRNA expression by flagellin in both the differentiated and undifferentiated Caco-2 cell models. Therefore, a knockdown of TR1 was performed

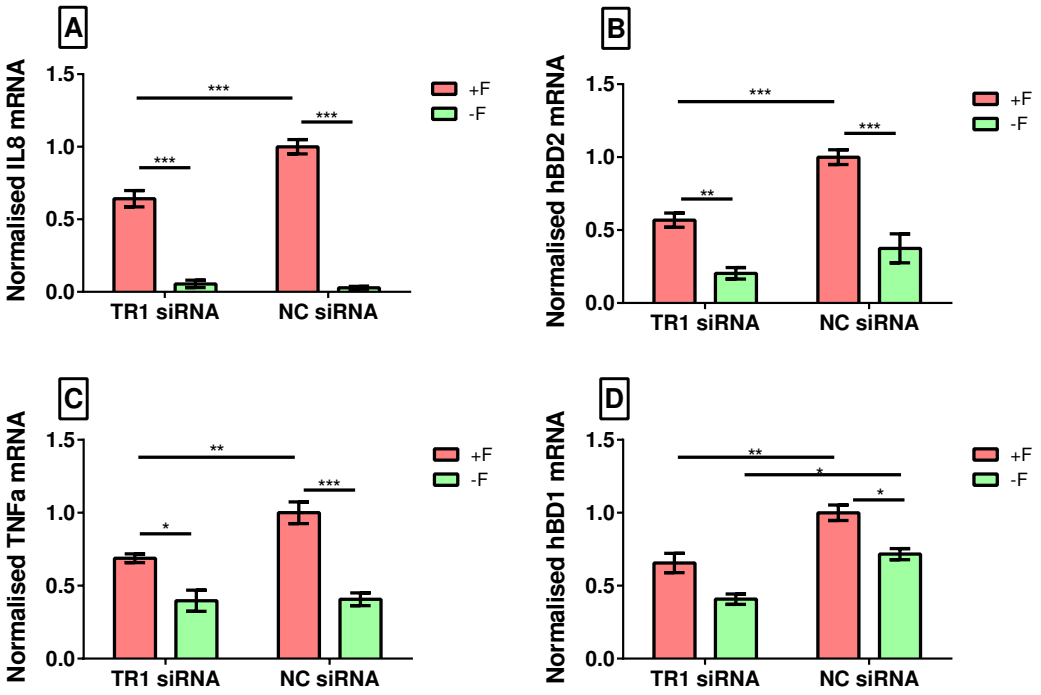

Figure 3.8. Effects of TR1 knockdown on the expression of the inflammatory mediators IL-8 (A), hBD2 (B), TNF-α (C) and hBD1 (D) at 48 h post-transfection with TR1-specific siRNA or negative control (NC) siRNA, and 6 h challenge with S. typhimurium flagellin (+F) or water negative control (-F). All data were normalised to the housekeeping genes TOP1 and B2M. Data were expressed as a proportion of the mean of the NC siRNA transfected cells challenged with flagellin in order to combine 2 distinct biological replicates, each consisting of 3 technical replicates (n = 6). Data were analysed statistically using two-way ANOVA with a Tukey post-test for multiple comparisons. The data are displayed as mean ± standard error of the mean (SEM). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

(Figure 3.7) which indicated that reduced expression of TR1 mRNA resulted in a decrease in the induction of immune effectors by flagellin in undifferentiated Caco-2 cells (Figure 3.8).

The Caco-2 cell line has extensively been used as an *in vitro* model of IECs to study the effects of Se on various pathways and morphologies under a variety of experimental conditions. Some of these studies were performed in undifferentiated Caco-2 cells [31, 229, 372–389], whereas others were performed in Caco-2 cells that had been polarised and fully differentiated [154, 390–397]. Caco-2 cell polarisation and differentiation have previously been shown to result in changes in a number of cellular processes, including cell cycle regulation and apoptosis [398–400], inflammatory responses and signalling [400, 401], cell metabolism [402], nutrient uptake [403–406], responses to oxidative stress [407, 408], global transcriptomic and proteomic changes [398, 405, 409], as well as changes in which reference genes can be used for analysis of RT-qPCR [410]. The work mentioned above indicates that the polarisation and differentiation of Caco-2 cells represent the phenotypic change of the cell line from a tumorigenic phenotype to a one more similar to

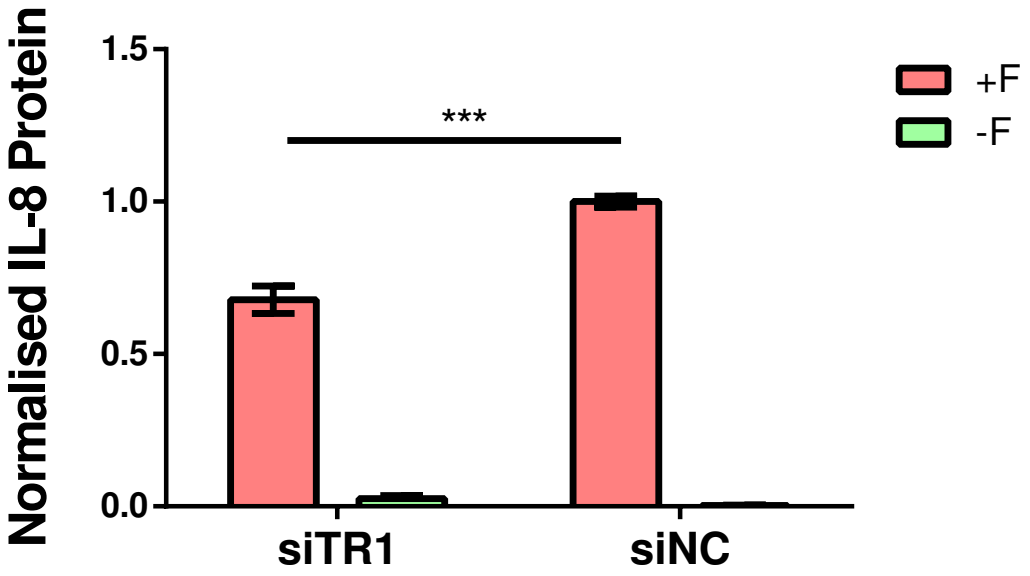

Figure 3.9. Effects of treatment of Caco-2 cells with TR1-specific siRNA (siTR1) or negative control (NC) siRNA, and 6 h treatment with *S. typhimurium* flagellin (+F) or water negative control (-F) on secreted IL-8 concentration by undifferentiated Caco-2 cells as detected by ELISA. The data shown represents 2 biological replicate with 3 repeats (n = 6) for each time-point. Data were analysed statistically using two-way ANOVA with a Tukey post-test for multiple comparisons. The data are displayed as mean ± standard error of the mean (SEM). *** $P < 0.001$.

that of a differentiated enterocytic intestinal cell. Therefore, it was important to study the responses of differentiated and undifferentiated Caco-2 cells to changes in Se when faced with various stressors.

The effect of selenium on the induction of immune effectors by flagellin stimulation in undifferentiated and differentiated Caco-2 cells

An induction in the immune targets IL-8, hBD2, TNF-α and hBD1 was observed in response to the challenge of Caco-2 cells with *S. typhimurium* flagellin in both differentiated and undifferentiated cells (Figure 3.1 and Figure 3.4, respectively).

In the undifferentiated Caco-2 cells, Se-depletion resulted in an increase in the induction of IL-8 mRNA after 6 h of flagellin treatment (Figure 3.4), and secreted IL-8 protein after 6 and 16 h of flagellin treatment (Figure 3.5). Previous research that assessed the effects of Se supplementation on undifferentiated Caco-2 cells to immune stressors found no effect of Se on responses to flagellin challenge, but did find it modulated responses to challenge with TNF-α [31]. There was no effect of Se-depletion on the mRNA levels of IL-8 in differentiated Caco-2 cells (Figure 3.1). However, Se-depletion resulted in increased induction of secreted IL-8 protein in the basolateral media after 6 h, and apical and basolateral media after 16 h of flagellin challenge (Figure 3.2). Interestingly, there was

a decrease in IL-8 basolaterally-secreted protein observed in one biological replicate after 16 h due in differentiated Caco-2 cells which were Se-depleted. The effects of Se-depletion on the induction of IL-8 has not been reported in differentiated Caco-2 cells prior to the work described in this thesis.

There are a number of potential mechanisms by which Se may repress the expression of the IL-8 gene, *CXCL8*, at the transcriptional level. The gene *CXCL8* contains a 200 nucleotide promoter region that contains various motifs and response elements necessary for transcriptional regulation of IL-8 [411]. The binding of NF-κB to its response element, and *CXCL8* promoter activity in general, can be repressed by the deacetylation of histones. Previous studies have found that the inhibition of histone deacetylase-1 (HDAC-1) caused the derepression of *CXCL8*, leading to the hyperacetylation of histones and chromatin remodelling, which led to the reduced repression of *CXCL8* [412, 413]. Inhibition of histone deacetylation has also been reported to prevent the activation of NF-κB in undifferentiated Caco-2 cells [414]. With regards to the effects of Se, one study reported that treatment of prostate cancer cells with 1.5 μM selenite resulted in a significant inhibition of histone deacetylase activity [415]. Although this study did not focus specifically on NF-κB activation, the results suggest that Se treatment may enhance expression of IL-8 via inhibition of histone deacetylation. However, this does not agree with the data presented in this chapter. The dose of selenite used in Xiang et al [415] was over 35-fold higher and hence a pharmacological dose, compared with what is generally considered physiological or nutritionally-relevant concentrations from 10 nM to 100 nM [397, 416]. The importance of histone deacetylation in the differentiation of intestinal cells, including Caco-2 cells, is well documented in the literature [417–421]. Although the role of histone deacetylation in the context of Se is poorly understood, changes in histone deacetylation due to differentiation could offer a potential explanation as to why Se-depletion resulted in exacerbation of IL-8 induction in undifferentiated, but not differentiated Caco-2 cells. Other mechanisms by which the *CXCL8* promoter is known to be repressed is via the binding of Oct-1 and NF-κB repressing factor (NRF) to regulatory elements in the *CXCL8* promoter [177, 422]. It is not currently known whether Se has a regulatory role in those mechanisms of transcriptional repression.

A key transcription factor in terms of inducing transcription of *CXCL8* in response to challenge with *S. typhimurium* flagellin is NF-κB, which has been reported to be essential for the transcription of *CXCL8* [423–425]. However, there are a number of binding sites in the promoter of *CXCL8* to which a number of different transcription factors can bind that act to enhance the expression of IL-8 [426], including the AP-1 binding site. AP-1 is a homodimer or heterodimer which is composed of the subunits cJUN, Jun-B, Jun-D, Atf-2, Fra-1, Fra-2 and c-Fos [427, 428]. The AP-1 pathway is activated by MAPK in response a variety of stimuli, including growth factors, chemokines and environmental stressors [429] and all three MAPK pathways, extracellular signal–regulated kinase (ERK), JNK and p38 MAPK, have been shown to regulate *CXCL8* gene expression [423]. Although not

essential for CXCL8 expression, the AP-1 pathway has been suggested to be required for maximal gene expression [177–186]. Additionally, much like NF-κB, JNK is essential for *CXCL8* gene expression, most likely through activation of cJUN [430]. Interestingly, AP-1 regulation of hBD2 [192, 431, 432] and TNF-α [433] expression in response to microbial challenge has also been reported.

There is no consensus in the literature on the direction of the regulation of AP-1 pathway and the AP-1 subunit cJUN by Se. In the data presented in this thesis, both differentiated and undifferentiated Caco-2 cells observed raised levels of cJUN mRNA under conditions of Se-deficiency (Figure 4.5 and Figure 4.2). A study in rats indicated that Se deficiency resulted in a decrease in NF-κB activity that was accompanied with a increase in AP-1 pathway activity [322]. In a human breast cancer cell line, it was observed that selenite treatment in the dose range of 250 nM to 2.5 μM reduced AP-1 binding to DNA. Furthermore, one study reported that human whole blood treated with 100 μM of Se-containing compounds resulted in reduced NF-κB and AP-1 pathway activity [323]. Interestingly, in a study using physiologically relevant concentrations of selenite, in the ranges of 10 nM to 100 nM, it was found that NF-κB binding to DNA was inhibited in a human T cell line supplemented with Se, but it was accompanied by an increase in AP-1 transactivation [324]. In agreement with the data presented here, a study in human HEK-293 cells reported that Se supplementation at 100 nM resulted in decreased cJUN luciferase reporter activity, as well as an inhibition of JNK in response to UV stress [434]. Additionally, a study also found that Se-supplementation of rat neurons, at a concentration of 700 nM, resulted in a reduction in cJUN mRNA and protein. However, there have been three studies from Shalini et al, that have reported that Se-deficiency resulted in a decrease in cJUN mRNA [325–327]. The direction of regulation of AP-1 and cJUN is poorly understood and seems to vary in different cell types and at different concentrations of Se treatment. However, there is a strong indication for a role for Se in the regulation of AP-1 and cJUN and this role needs further study in intestinal cell lines, such as Caco-2. Given that there is a well documented regulation of NF-κB by AP-1 and cJUN, there is a possibility that the effect of Se on the responses to flagellin presented in this chapter may be occurring through modulation of AP-1 and cJUN. This is supported by the data observed in undifferentiated Caco-2 cells where there was a significant affect of Se-depletion on all immune targets tested (Figure 3.4) accompanied by an increase in the level of cJUN mRNA detected (Figure 4.5).

hBD2 is an AMP which can form pores in the membranes of pathogens in order to neutralise them. Along with hBD1 and human beta-defensin 3 (hBD3), hBD2 has been found to be expressed in the gastrointestinal tract [435]. Previous studies have indicated that hBD2 can be induced by probiotic bacteria and increase the barrier function of the gut [436, 437]. However, increased expression of hBD2 has been associated with inflammation in CD and UC [116, 438]. hBD2 has been induced *in vitro* in response to challenge with a number of PAMPs, including flagellin, in various cell types including IECs [114, 432].

The promoter region of hBD2 contains two NF-κB binding sites [436] and it has previously been suggested that intact function of NF-κB is required for hBD2 expression [439]. Furthermore, hBD2 expression has previously been suggested to be regulated by a number of pathways in IECs, including PI3K/Akt, p38 MAPK, JNK, AP-1, nucleotide-binding oligomerisation domain-containing protein 1 (NOD1) and nucleotide-binding oligomerisation domain-containing protein 2 (NOD2) [114, 440–443]. Much like IL-8, hBD2 is a pro-inflammatory mediator which often is co-expressed in response to microbial challenge. However, previous studies have reported that hBD2 can be differentially regulated to IL-8. One study infected undifferentiated Caco-2 cells with *P. aeruginosa* and reported an inhibition of IL-8 expression, but an induction of hBD2 [442]. Another study using an IEC model reported that supplementation of cells with vitamin D resulted in a down-regulation of IL-8, but an up-regulation of hBD2 [440], which is consistent with previous reports that vitamin D receptor (VDR) signalling plays and important role in defensin regulation [444, 445]. The present work reports that Se-depletion results in an increase in flagellin-induced hBD2 expression in both differentiated and undifferentiated Caco-2 cells (Figure 3.1B and Figure 3.4B, respectively). This consistent effect of Se-depletion on hBD2 in both the differentiated and undifferentiated Caco-2 cells, which was not observed for the other immune effectors, may reflect the involvement of pathways other than NF-κB that hBD2 may be more responsive to during Se-depletion. Nonetheless, the present work reports that hBD2 expression is responsive to Se status in IECs. Thus, further study is warranted to investigate a role for altered hBD2 expression in the pathogenesis of UC in patients with low Se status [116, 142, 149, 438].

TNF-α plays an essential role in both the innate and adaptive immune responses and is widely expressed in a number of different cell types [446, 447]. The present work suggests that flagellin-induced TNF-α mRNA is up-regulated in response to Se-depletion in differentiated Caco-2 cells after 16 h flagellin challenge (Figure 3.1C), and undifferentiated Caco-2 cells after 6 h flagellin challenge (Figure 3.4C). These data indicated that TNF-α may be modulated by Se status at a transcriptional level. Transcription of TNF-α can be induced by a number of different stimuli, including hypoxia [448], oxidative stress [449] and PAMPs, including flagellin [450, 451]. The TNF-α promoter contains a cAMP responsive element (CRE) [452, 453] to which transcription regulators, such as activating transcription factor 2 (ATF2) and cJUN can bind [454–456]. This suggests that Se modulation of TNF-α may occur via changes in cJUN transcriptional activity, which is supported by a previous study which found that *de novo* expression of cJUN and ATF2 in *Drosophila* cells resulted in activation of a TNF-α reporter gene [454]. Data reported later in this thesis found that Se modulated cJUN expression levels in differentiated and undifferentiated Caco-2 cells (Figure 4.2 and Figure 4.5, respectively), which supports that altered TNF-α expression may occur as a result of increased cJUN transcriptional activity. Additionally, the TNF-α promoter contains a number of NF-κB binding sites to which NF-κB is known to bind following TLR5 stimulation by flagellin [457–459]. As discussed

in Section 1.2.4, Se modulates NF-κB signalling, thus providing a possible mechanism by which TNF-α expression may have increased in response to Se-depletion.

hBD1 is an AMP which, similarly to hBD2, is able to form pores in the membranes of pathogens in order to kill them [460]. Unlike hBD2, hBD1 has been reported to be constitutively expressed in a number of different cell types, including the IEC cell lines, Caco-2 and HT29 [461, 462]. However, hBD1 has been found to be inducible by LPS and TNF-α in monocytes [463], as well as TLR3 agonists in uterine epithelial cells [464]. A previous study using undifferentiated Caco-2 cells reported that hBD2, but not hBD1, mRNA was inducible by Interleukin-1α (IL-1α), interferon-γ (IFN-γ), LPS and *Salmonella dublin* infection [461], while flagellin has only been reported to induce hBD1 mRNA *in vivo* when applied to the corneal epithelial cells of mice [465]. Interestingly, the data presented in the present work indicated that hBD1 gene expression is inducible in both differentiated and undifferentiated Caco-2 cells (Figure 3.1D and Figure 3.4D, respectively). This is surprising as a previous study in undifferentiated Caco-2 cells was unable to induce hBD1 mRNA in response to a number of microbial challenges, including live infection with enteroinvasive and flagellated bacteria which would normally induce a TLR5 response [461]. However, the concentration of flagellin used in the present work was relatively high and would only bind TLR5, whereas live infection with flagellated bacteria would trigger responses from a number of different PAMP receptors. This indicates that the hBD1 response of Caco-2 cells to flagellin was specific to TLR5. In the undifferentiated Caco-2 cells, Se-depletion increased the expression of hBD1 in the challenged and unchallenged cells at both time-points (Figure 3.4D). The mechanism of hBD1 regulation by Se is not currently known but operates within 6 h and is thus fast-acting. Previous studies have suggested that HDAC-1 is able to regulate the expression of hBD1 in lung epithelial cells [462]. Inhibition of histone deacetylation has also been reported to prevent the activation of NF-κB in undifferentiated Caco-2 cells [414]. With regards to the effects of Se, one study reported that treatment of prostate cancer cells with 1.5 μM selenite resulted in a significant inhibition of histone deacetylase activity [415]. Although this study wasn't looking specifically at NF-κB activation, the results would suggest that selenite treatment may enhance expression of hBD1 via inhibition of histone deacetylation, which would not agree with the data presented in this chapter. However, the dose of selenite used in that study was over 35-fold higher and would be considered a pharmacological dose, compared with what is generally considered physiological or nutritionally-relevant concentrations [397, 416]. Additionally, it has been reported that the NF-κB pathway inhibitors, peroxisome proliferator-activated receptor α (PPARα) and PPARγ inhibit the expression of hBD1 after LPS stimulation [466]. Previous studies have suggested that Se-depletion exerts pro-inflammatory effects by suppressing PPARγ and thereby increasing NF-κB signalling [467, 468]. Hence, the modulation of PPARγ activity by Se may be a potential mechanism by which Se regulates the expression of hBD1.

The effect of selenium on the expression of selenoproteins after flagellin stimulation in undifferentiated and differentiated Caco-2 cells

The data presented in this chapter indicated that Se-depletion and flagellin treatment, both on their own and in combination, had different effects on the expression of the selenoproteins TR1, SelH, GPX1 and GPX2 depending on the differentiation status of the Caco-2 cells.

Thioredoxin reductase 1 (TR1)

TR1 is a selenoprotein which plays an essential role in the TXN antioxidant system, which comprises of three components: NADPH, TR and TXN. Together, these components form a major disulfide reduction system, which provides free electrons to a range of enzymes critical for responses to various forms of stress. Indeed, the activity of a number of transcription factors, including NF-κB, Nrf2 and HIF-1α, are modulated by the TXN system [26, 57]. TR exists in three isoforms which consist of the cytoplasmic TR1, mitochondrial TR2 and testis-specific thioredoxin glutathione reductase (TGR) [65].

In the differentiated Caco-2 cells, the mRNA expression of TR1 increased approximately two-fold in response to treatment with *S. typhimurium* flagellin after 6 h but exhibited no change in expression in response to Se-depletion (Figure 3.3). In the undifferentiated Caco-2 cells, there was still an induction of TR1 mRNA due to flagellin after 6 h, but the induction was significantly lower in the Se-depleted cells. Additionally, Se-depletion resulted in a greater expression level of TR1 in unchallenged cells at the 6 h time-point, and in both the challenged and unchallenged cells at the 16 h time-point (Figure 3.6). These data suggested a regulation of TR1 mRNA by both flagellin and Se-depletion, which is dependent upon the differentiation status of the Caco-2 cell. Interestingly, the majority of studies in rodent and human *in vivo* and *in vitro* models report decreases in thioredoxin reductase activity accompanied by decreased TR1 mRNA due to Se-depletion, although the increase in mRNA tend to be small or has no change [229, 469–475]. However, there is one *in vivo* study in chickens that reported a decrease in thioredoxin activity due to Se-deficiency but an increase in TR1 mRNA. Furthermore, the down-regulation of TR1 by siRNA or inhibitor, has previously been reported to not change thioredoxin redox status [476–478]. Instead, it has been suggested that the glutathione (GSH) system serves as a backup system to reduce thioredoxin [65]. The role of flagellin in the induction of TR1 mRNA has not previously been investigated.

TR1 was the only selenoprotein observed to be consistently up-regulated in response to treatment with *S. typhimurium* flagellin in both differentiated and undifferentiated Caco-2 cells. Therefore, it was decided that TR1 was most suitable for further investigation into its role in the modulation of the responses to flagellin challenge. Undifferentiated Caco-2 cells were treated with a TR1-specific siRNA for 48 h in order to knockdown expression of TR1 and this was successful with 80 % knockdown achieved (Figure 3.7). The cells which were pre-treated with TR1-specific siRNA exhibited an approximately

40 - 50% reduction in the flagellin-induced mRNA expression of IL-8, hBD2, TNF-α and hBD1 (Figure 3.8), as well as a significant decrease in the secretion of IL-8 protein into the media in response to flagellin challenge (Figure 3.9). Studies investigating the involvement of TR1 in the NF-κB pathway have reported a regulatory role for TR1 in the binding of NF-κB to DNA, most likely through changes in the TXN system as a whole [479–481]. It is well documented that TXN is a known regulator of NF-κB activity by reducing a cysteine residue in NF-κB DNA binding domain [482, 483]. Additionally, TXN has been suggested to be an important factor in the potency of antimicrobial activity of the defensin AMP family [79–81]. Thus, the regulation of TXN activity by TR1 may serve as a potential explanation for how decreased expression levels of TR1 mRNA attenuated the flagellin-induced expression of IL-8, hBD2, TNF-α and hBD1.

Selenoprotein H (SelH)

SelH is a small 14 kDa selenoprotein expressed in many tissues, with elevated expression in certain cancers, including colorectal cancer [27]. Se status has been reported to regulate SelH expression both *in vivo* and *in vitro* [28–31]. SelH possesses a TXN-like 'CXXU' motif, thus indicating a possible oxidoreductase role [27, 32–34]. However, SelH shares no significant homology with any other functionally characterised proteins [35]. Additionally, a nuclear-localisation signal is present in the peptide sequence of SelH, which is consistent with reports that the protein resides in the nucleus and nucleolus [27, 36]. Relatively little is known about the physiological roles of SelH, but studies have suggested that the potential protective effects of SelH are mediated through its roles in the Nrf2 and NF-κB pathways, mitochondrial biogenesis, and GSH synthesis [41, 50, 51, 53].

In the differentiated Caco-2 cells, SelH mRNA expression was found to be down-regulated in response to flagellin treatment under conditions of Se depletion (Figure 3.3) but there was no response to flagellin in the undifferentiated Caco-2 cells (Figure 3.6). Interestingly, SelH was found to be unresponsive to Se status in the differentiated Caco-2 cells, but decreased more than 2-fold in response to Se-depletion in undifferentiated Caco-2 cells. The effects of Se-depletion on SelH expression have never been investigated in differentiated Caco-2 cells but similar results have been observed in undifferentiated Caco-2 cells previously [31]. This may be to compensate for redistribution of scarcer supplies of Se during Se-depletion.

Glutathione peroxidase 1 (GPX1)

GPX1 is the most abundantly expressed member of the glutathione peroxidase family and is present in cytoplasmic, mitochondrial and peroxisomal compartments. It is a selenoprotein that has the essential antioxidant function of preventing harmful accumulation of intracellular hydrogen peroxide [484]. *In vitro* studies have demonstrated potential regulatory roles for GPX1 in NF-κB pathway activation. Overexpression of GPX1 in a breast cancer cell line resulted in inhibition of IκBα degradation and reduced NF-κB (p50) translocation to the nucleus, the effects of which have been reversed by Se-depletion

[162, 485]. GPX1 has also been found to be very responsive to Se status in *in vitro* cell models, including in undifferentiated Caco-2 cells [31, 376].

In the present work, the mRNA expression of GPX1 was found to be unresponsive to challenge with *S. typhimurium* flagellin in differentiated Caco-2 cells (Figure 3.3). However, an induction by flagellin treatment was observed in undifferentiated Caco-2 cells, which were supplemented with Se (Figure 3.6). As described previously, a potential regulatory role has been suggested between GPX1 and NF-κB activation [162, 485]. Given that GPX1 expression is highly regulated by Se status [31, 376], these data may indicate that GPX1 may have a regulatory role but only if there is adequate levels of intracellular Se.

Glutathione peroxidase 2 (GPX2)

GPX2 has similar peroxidase activity as GPX1, but its expression is mainly limited to IECs and thus is it is thought to be important in protection against gut pathogens and inflammation [365, 486, 487]. It has previously been shown that GPX2 knockout mice are prone to severe intestinal inflammation [150, 365, 486]. Furthermore, it has been suggested that GPX2 may suppress intestinal inflammation by inhibition of the expression or activity of a pro-inflammatory cytokine, COX-2 [488, 489]. Similar to GPX1, GPX2 is a selenoprotein, but it responds differently to Se-depletion. In fact, expression of GPX2 mRNA has been reported in increase in response to Se-depletion [373]. In undifferentiated Caco-2 cells, it has been reported that GPX2 mRNA is unresponsive to Se-depletion but protein levels of GPX2 decrease with Se-depletion [229], and that GPX2 mRNA levels increased with Se-depletion. Thus, the link between Se status and GPX2 expression level is contradictory and not completely understood.

There was no consistent induction of GPX2 due to *S. typhimurium* flagellin treatment in differentiated Caco-2 cells (Figure 3.3). In undifferentiated Caco-2 cells, induction was observed in both Se groups at the 6 h time-point and interestingly, only in the Se-depleted Caco-2 cells at the 16 h time-point (Figure 3.4). In fact, GPX2 was the only selenoprotein mRNA induced by flagellin treatment at the 16-h time point. These data suggest a potential role for GPX2 in the later phases of the Caco-2 cell response to flagellin. Se-depletion had no effect on GPX2 mRNA in differentiated Caco-2 cells, but resulted in approximately 4-fold increases in mRNA expression in undifferentiated Caco-2 cells. These data suggest that the selenoprotein hierarchy may change as the Caco-2 cell differentiates or that Caco-2 cells may become less sensitive to shortages in Se supply as they differentiate.

Summary

Se-depletion exacerbated the mRNA expression of pro-inflammatory cytokines and defensins in response to flagellin challenge in Caco-2 cells, with Se observed to have a greater

effect in undifferentiated Caco-2 cells. Both Se-depletion and flagellin challenge modulated the expression of selenoproteins that have been previously described to be involved in responses to oxidative stress, as discussed further in Section 4. Thus, it is important to determine the effect of Se-depletion on oxidative stress to determine if Se-depletion has similar effects on both immune and oxidative stress targets.

Chapter 4

Selenium, selenoproteins and oxidative stress

4.1 Introduction

4.1.1 Oxidative stress, damage and the cellular defences against it

Oxidative stress is the consequence of an inadequate antioxidant capacity to process a certain level of pro-oxidants, such as ROS or RNS. The inability of a cell to deal with pro-oxidants can have a profound impact on the physiological functions of the cell via the oxidation of a variety of compounds within the cell, including lipids, proteins and DNA [198, 202, 203]. Although ROS and RNS are considered harmful in excess, they are essential in cell signalling and act as second messengers for many physiological processes [490]. As such, ROS have been implicated in the regulation of a number of biological pathways, including the NF-κB, PI3K/Akt and MAPK pathways [209–211]. However, in pathophysiological conditions where ROS levels are in excess, the oxidative damage within the cell can lead to inflammation, uncontrolled proliferation or apoptosis [208, 212].

There are a number of multiple defence mechanisms within the cell to ensure that the levels of ROS are kept within the physiological range. Firstly, the level of oxidative stress in the cell can be modulated by altering either the level of oxygen transport, or by the expression of iron-binding proteins [208, 491]. The secondary response to oxidative stress involves the induction of antioxidant defence enzymes via a number of pathways, such as the Nrf2, AP-1 and HIF-1 [492, 493]. Antioxidant defence enzymes neutralise ROS and RNS and contain a number of selenoproteins. Finally, repair enzymes can be used to repair oxidative damage to lipids, proteins and nucleic acids [208].

4.1.2 The antioxidant properties of selenium and selenoproteins

Se-containing compounds have been found to be essential in multiple antioxidant defence systems. It has previously been reported that Se treatment improves oxidative stress

status in rodent with sepsis and ovalbumin-induced asthma [370, 494, 495]. There is also evidence to suggest that Se-containing amino acids, such as Sec and selenomethionine, are able to scavenge both single and double electron oxidants [496, 497], as well as repair or recycle oxidised Se species [498, 499]. Additionally, a role for Se-containing compounds have been reported in binding metal ions involved in oxidative stress homeostasis [500, 501].

Se has also been shown to be involved in the response to oxidative express through its incorporation into a number of selenoproteins in the form of the amino acid, Sec. Sec, catalytically active residue in the redox-active selenoproteins. The families of selenoproteins, which have very well characterised antioxidant functions are the GPXs the the TRs [2, 231]. Additionally, potential antioxidant roles for other selenoproteins, including methionine-R-sulfoxide reductase 1 (MsrB1) [502], 15 kDa selenoprotein (Sep15) [503], SelM [504], selenoprotein O (SelO) [35], selenoprotein T (SelT) [505] and SelH [35] have previously been suggested.

4.1.3 The cross-talk between oxidative stress, the Nrf2 and AP-1 pathways and the NF-κB pathway

Evidence suggests a significant amount of cross-talk and inter-dependence between inflammatory and oxidative stress signalling pathways. Se-depletion of Caco-2 cells for 72 h has previously been shown to significantly increase the amount of ROS present in the cell [31]. ROS have been shown to have a modulatory role on the NF-κB pathway in either a positive or negative direction via mechanisms which are not yet fully understood. It has been reported, for instance, that pre-exposure to ROS prolongs the activation of the NF-κB pathway through mechanisms, including suppression of PP2A IKK dephosphorylation [306, 307] and HDAC-mediated down-regulation of NF-κB gene transcription [308, 309]. However, ROS pre-exposure has also been shown to inhibit NF-κB activation [310, 311].

The Nrf2 pathway is also up-regulated in response to ROS. Furthermore, the Nrf2 and NF-κB signalling pathways, which both have downstream effectors that are involved in carcinogenesis and inflammation, show cross-talk between each other [312, 313]. In fact, inducers of the Nrf2 pathways include Se-depletion, have previously been shown to have an inhibitory effect on the activation of the NF-κB pathway [312, 314–316]. However, the mechanisms of interaction between the two pathways are unclear and relevant studies have provided contradictory evidence, suggesting both positive and negative regulation between the two pathways [317].

The AP-1 pathway is also up-regulated in response to an imbalance of ROS. This up-regulation of the AP-1 pathway has been shown to be mediated through a number of pathways, including JNK, MAPK and p38 signalling pathways [318]. The AP-1 pathway is a regulator of cell homeostasis, and is an important modulator of cell proliferation and apoptotic signalling cascades [319–321]. Previous studies have described potential roles

for Se in the regulation of AP-1 and cJUN [322–327]. Furthermore, roles for AP-1 and cJUN have previously been reported in the regulation of the NF-κB pathway [177–186].

Se has been shown to modulate the Nrf2 and AP-1 pathways and both of these pathways are regulators of each other and the NF-κB pathway [82, 261, 262, 265, 312, 313, 322, 323, 328]. The modulation of the NF-κB response by Se is well documented but the pathways by which this modulation occurs, is not well understood.

4.1.4 Research aims

The aim of the experiments reported in this chapter was to assess the effects of Se supplementation or depletion on the expression of oxidative stress-associated target genes in differentiated and undifferentiated Caco-2 cells. Furthermore, using gene knockdown techniques, the potential roles of the selenoproteins SelH and TR1 in the expression of these oxidative stress-associated target genes was also investigated.

4.2 Results

4.2.1 Effects of selenium depletion on mRNA expression of oxidative stress-associated targets in differentiated Caco-2 cells

Differentiated Caco-2 cells were grown on Transwell semi-permeable inserts for 18 days, as described in Section 2.1.1, and were depleted of Se (NoSe), or supplemented with Se (Se), for 72 h as described in Section 2.1.2. Following this 72 h Se depletion or supplementation period, RNA was extracted from the cells and the mRNA expression levels of Nrf2 downstream effectors were analysed.

After 72 h of either Se-depletion or Se-supplementation, the mRNA levels of downstream effector genes known to be involved in responses to oxidative stress and including NQO1, HMOX1, superoxide dismutase 2 (SOD2), peroxiredoxin 1 (PRDX1) and glutathione S-transferase P 1 (GSTP1) were measured by RT-qPCR and expressed relative to housekeeping genes, as described in Section 2.2 (Figure 4.1). Se-depletion resulted in a 0.12-fold increase in expression of NQO1 ($P < 0.05$) but there was no change in mRNA expression levels of either HMOX1, SOD2, PRDX1 or GSTP1. Additionally, the levels of the upstream target cJUN, involved in the transcriptional regulation of genes involved in oxidative stress, was analysed. Se-depletion resulted in a 0.38-fold increase in cJUN mRNA expression (Figure 4.2; $P < 0.001$).

The levels of expression of selenoproteins are presented in Figure 4.3. Se-depletion for 72 h resulted in a reduction in GPX1 and SePP1 mRNA by 50 % ($P < 0.001$) and 32 % ($P < 0.01$), respectively. Additionally, a trend towards a decreased response to Se-depletion was observed for SelH mRNA, but this was not statistically significant ($P = 0.057$). Se-depletion resulted in a 0.38-fold increase in GPX2 mRNA ($P < 0.001$). No effect of Se-depletion was observed for the selenoproteins GPX4 and TR1.

4.2.2 Effect of selenium depletion on oxidative stress-associated targets in undifferentiated Caco-2 cells

Undifferentiated Caco-2 cells were grown on 6-well plates to confluency, as described in Section 2.1.1 and were depleted of Se (NoSe), or supplemented with Se (Se), for 72 h as described in Section 2.1.2.

The mRNA expression levels of the downstream effector genes known to be involved in responses to oxidative stress and including NQO1, HMOX1, SOD2, PRDX1 and GSTP1 were measured (Figure 4.4). Se-depletion resulted in a 1.09-fold increase in the mRNA expression of NQO1 ($P < 0.001$); a 0.44-fold increase of SOD2 ($P < 0.01$); a 0.25-fold increase of PRDX1 ($P < 0.001$) and a 0.45-fold increase of GSTP1 ($P < 0.001$). Additionally, the levels of an upstream target, cJUN, involved in the transcriptional regulation

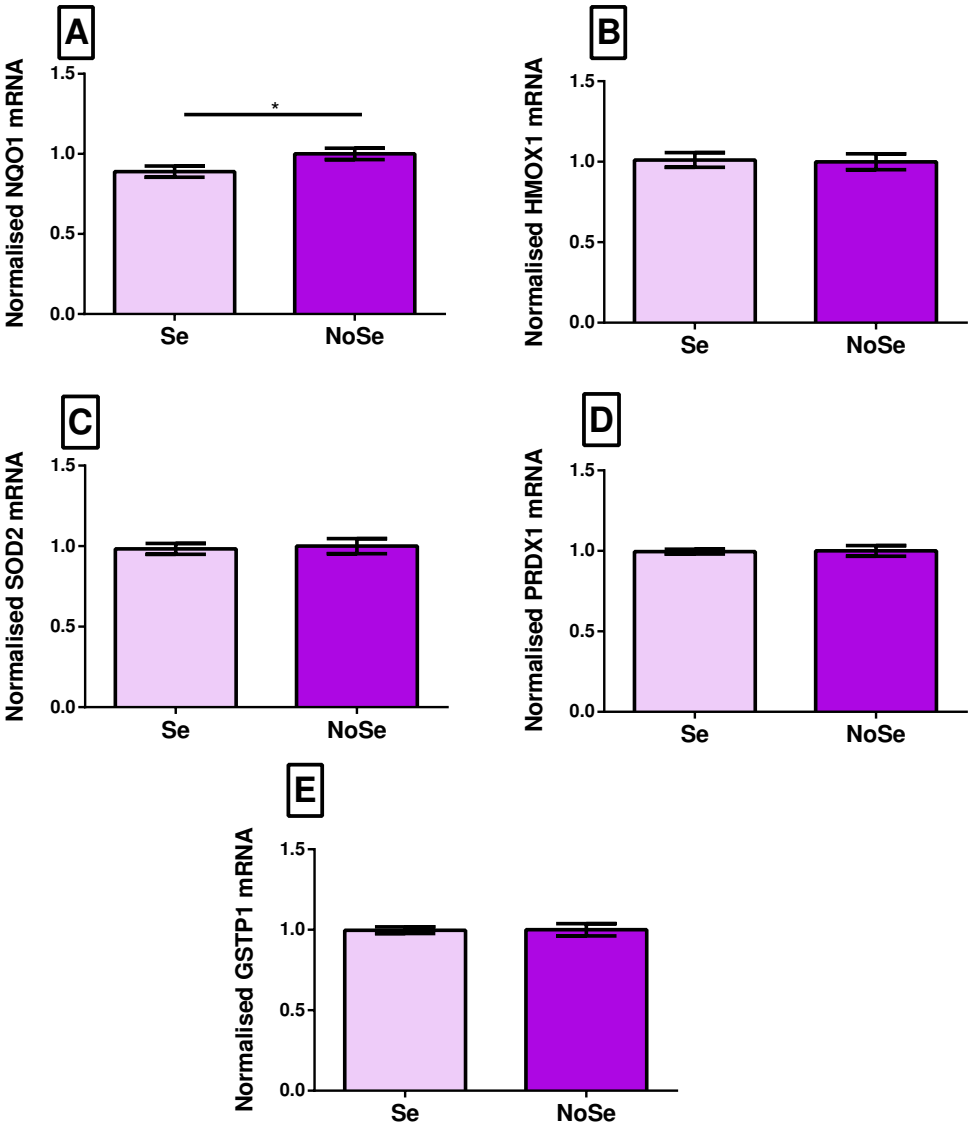

Figure 4.1. Effect of 72 h selenium starvation or supplementation on the mRNA expression of Nrf2 targets in differentiated Caco-2 cells. All data were normalised to the housekeeping genes TOP1 and EIF4A2. Data were expressed as a proportion of the mean of the NoSe in order to combine 3 distinct biological replicates, each consisting of 4 technical replicates (n = 12). Data were analysed statistically using a two-tailed unpaired t-test. The data are displayed as mean ± standard error of the mean (SEM). * $P < 0.05$.

of genes involved in oxidative stress, increased by 2-fold after Se-depletion (Figure 4.5; $P < 0.001$).

Selenoprotein mRNAs were also examined in response to Se-depletion or Se-supplementation for 72 h was examined in undifferentiated Caco-2 cells (Figure 4.6). Se-depletion for 72 h resulted in a reduction in the mRNA expression of GPX1 by 62 % ($P < 0.001$), GPX4 by 21 %, SePP1 by 30 % ($P < 0.001$) and SelH by 61 % ($P < 0.001$). Additionally, Se-depletion resulted in an increase in the mRNA of GPX2 by 10.6-fold ($P < 0.001$) and TR1 by 0.45-fold ($P < 0.001$).

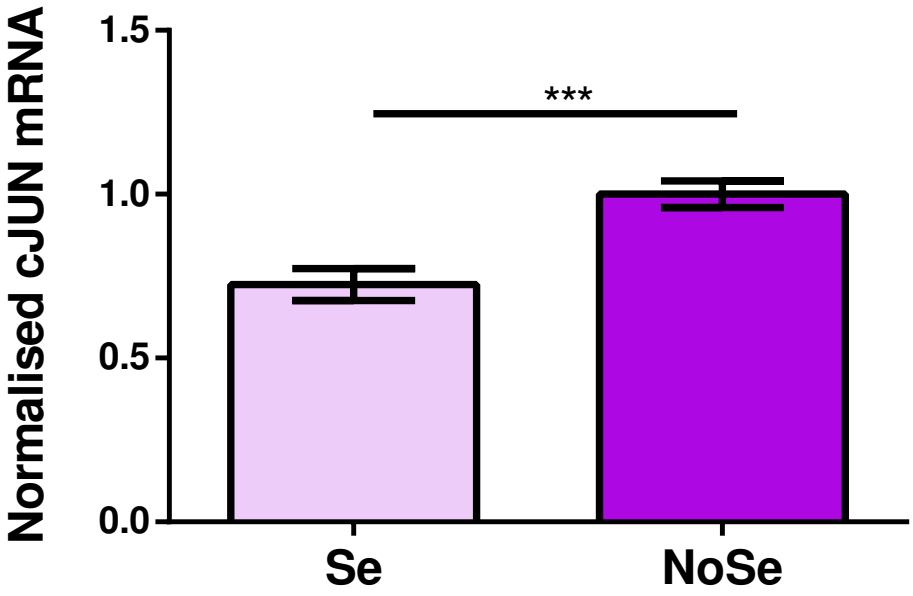

Figure 4.2. Effect of 72 h selenium starvation or supplementation on the mRNA expression of cJUN mRNA in differentiated Caco-2 cells. All data were normalised to the housekeeping genes TOP1 and EIF4A2. Data were expressed as a proportion of the mean of the NoSe in order to combine 3 distinct biological replicates, each consisting of 4 technical replicates (n = 12). Data were analysed statistically using a two-tailed unpaired t-test. The data are displayed as mean ± standard error of the mean (SEM). *** $P < 0.001$.

4.2.3 Effects of SelH knockdown on the mRNA expression of oxidative stress-associated genes in undifferentiated Caco-2 cells

The data presented in Figure 3.6B suggested that SelH expression was regulated at the mRNA level by Se status. Additionally, previous microarray data from the laboratory indicated that reduced expression of SelH in Caco-2 cells by siRNA modulated the expression of oxidative stress-associated genes via the Nrf2 pathway (Gautrey, Hall, Hesketh unpublished data). Thus, the effect of a knockdown of SelH expression on expression of specific oxidative stress-associated genes was assessed at 48 h and 72 h post-transfection.

The transfection of two SelH-specific siRNAs, siSELH1 and siSELH2, into undifferentiated Caco-2 cells, the level of SelH mRNA expression was measured after 48 h and 72 h post-transfection in order to assess the efficiency of knockdown. At 48 h post-transfection, the mRNA expression of SelH was reduced by 79 % in cells transfected with siSELH1 ($P < 0.001$) and by 71 % in cells transfected with siSELH2 ($P < 0.001$), compared with the expression levels of their respective negative controls. There was no statistically significant difference detected between the extent of knockdown with the two siRNAs at 48 h post-transfection. At 72 h post transfection, the mRNA expression of SelH was reduced by 93

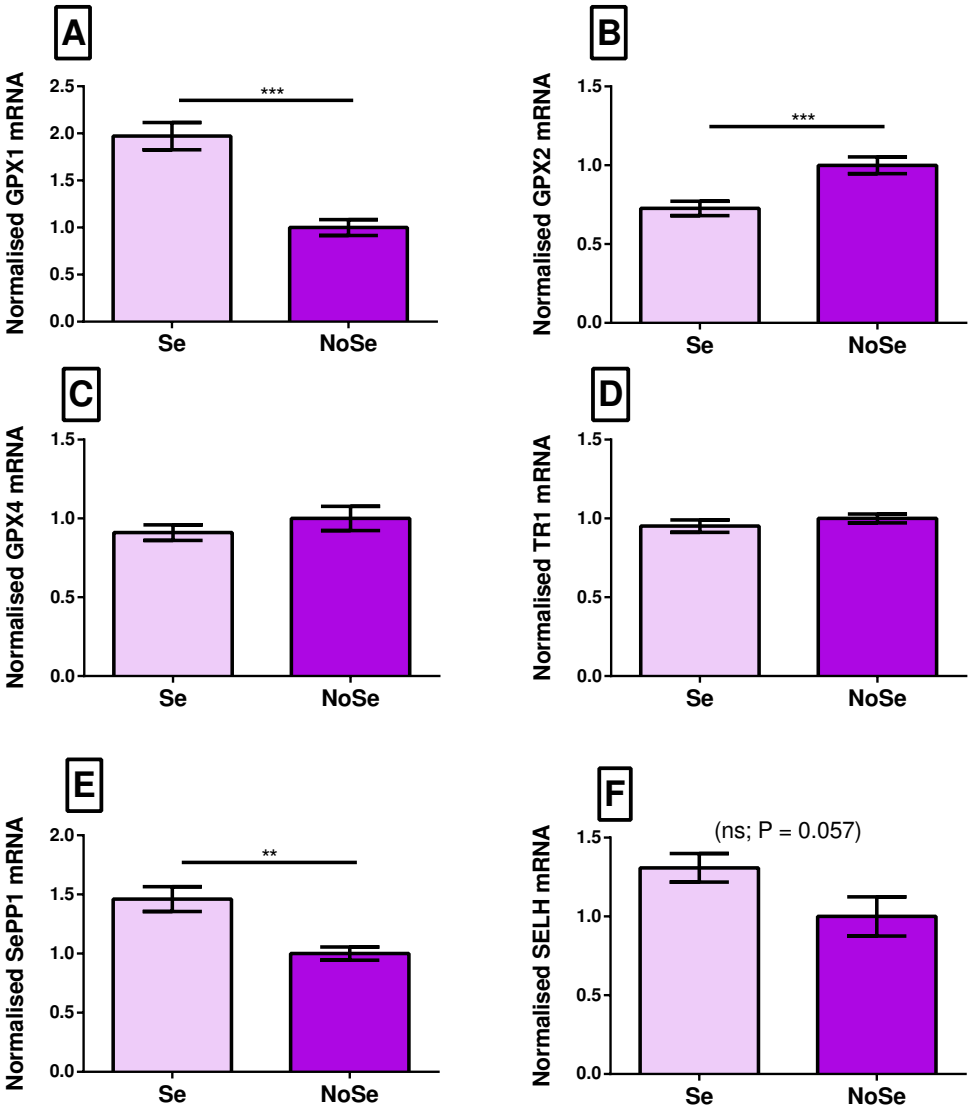

Figure 4.3. Effect of 72 h Se-supplementation or Se-depletion (NoSe) on the mRNA expression of the selenoproteins GPX1, GPX2, GPX4, TR1, SePP1 and SelH in differentiated Caco-2 cells. All data were normalised to the housekeeping genes TOP1 and EIF4A2. Data were expressed as a proportion of the mean of the NoSe in order to combine 3 distinct biological replicates, each consisting of 4 technical replicates (n = 12). Data were analysed statistically using a two-tailed unpaired t-test. The data are displayed as mean ± standard error of the mean (SEM). ** $P < 0.01$; *** $P < 0.001$.

% in cells transfected with siSELH1 ($P < 0.001$) and by 88 % in cells transfected with siSELH2 ($P < 0.001$). There was no statistically significant difference detected between the level of SelH knockdown by either of the SelH-specific siRNAs.

At 48 h post-transfection, the mRNA expression level of NQO1 was increased by 0.41-fold when transfected with siSELH1 ($P < 0.001$), and by 0.24-fold when transfected with siSELH2 ($P < 0.05$) compared with treatment with their negative control siRNA. There was no difference between siSELH1 and siSELH2 treatment in the expression levels of NQO1 at 48 h post-transfection. However, at 72 h post-transfection, siSELH1 or siSELH2

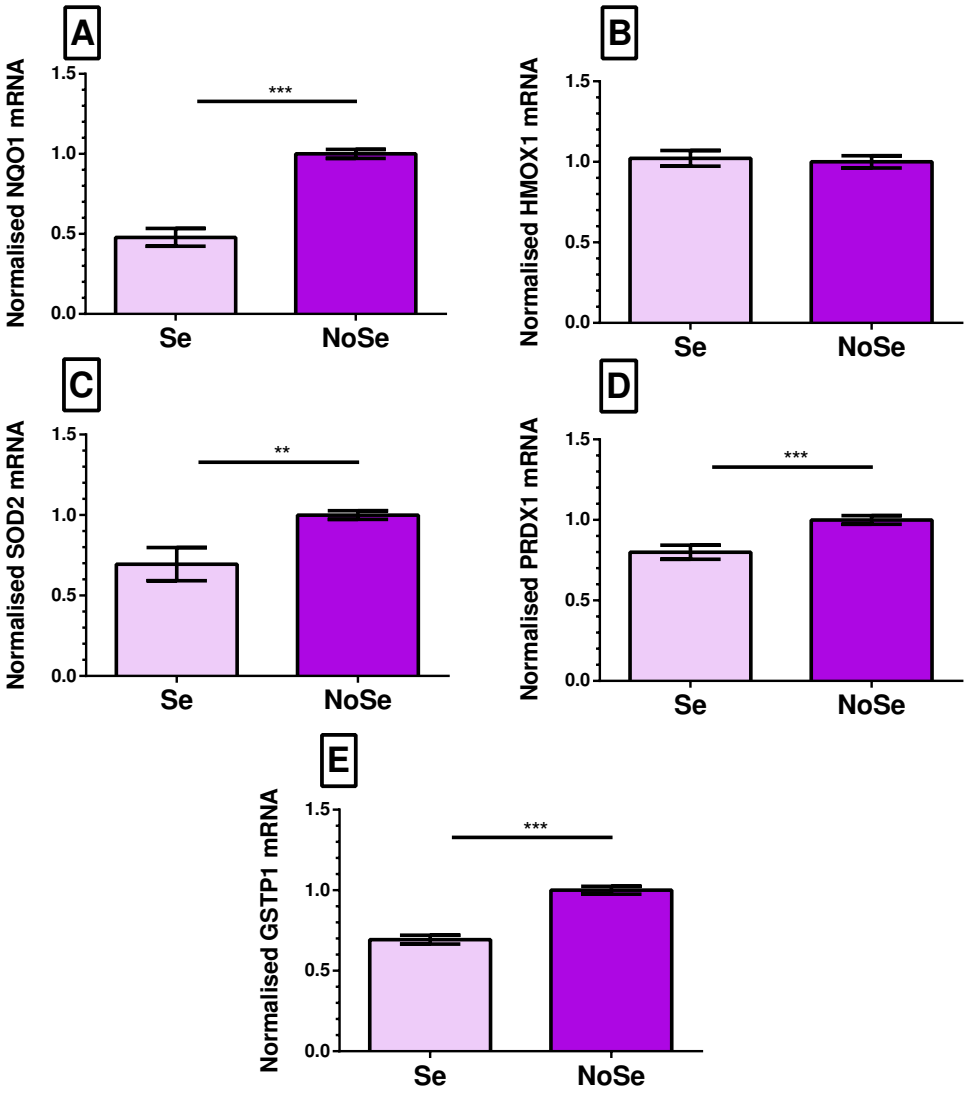

Figure 4.4. Effect of 72 h Se-supplementation or Se-depletion (NoSe) on the mRNA expression of the oxidative stress-associated genes NQO1, HMOX1, SOD2, PRDX1 and GSTP1. All data were normalised to the housekeeping genes TOP1 and EIF4A2. Data were expressed as a proportion of the mean of the NoSe in order to combine 3 distinct biological replicates, each consisting of 4 technical replicates (n = 12). Data were analysed statistically using a two-tailed unpaired t-test. The data are displayed as mean ± standard error of the mean (SEM). ** $P < 0.01$; *** $P < 0.001$.

treatment resulted in no change in NQO1 mRNA and there was no difference detected in the expression of NQO1 between siSELH1 and siSELH2.

After 48 h post-transfection there was an increase in HMOX1 mRNA by 1.78-fold when treated with siSELH1 ($P < 0.001$) and by 0.95-fold when treated with siSELH2 ($P < 0.01$), relative to the cells treated with negative control siRNA. Additionally, the induction of HMOX1 mRNA by siSELH1 treatment was significantly greater than the induction by siSELH2 ($P < 0.01$). At 72 h post-transfection, there was an increase in HMOX1 mRNA of 1.97-fold in cells transfected with siSELH1 ($P < 0.001$) but no significant change in

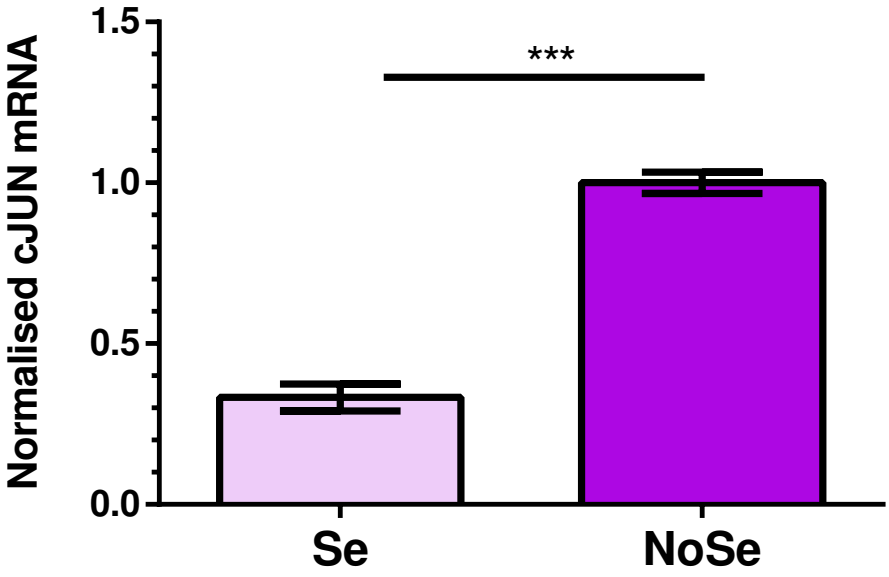

Figure 4.5. Effect of 72 h selenium starvation or supplementation on the mRNA expression of cJUN mRNA in undifferentiated Caco-2 cells. All data were normalised to the housekeeping genes TOP1 and EIF4A2. Data were expressed as a proportion of the mean of the NoSe in order to combine 3 distinct biological replicates, each consisting of 4 technical replicates (n = 12). Data were analysed statistically using a two-tailed unpaired t-test. The data are displayed as mean ± standard error of the mean (SEM). *** $P < 0.001$.

cells treated with siSELH2.

mRNA expression of selenoproteins, GPX1, GPX2, GPX4 and TR1 were also measured to assess their responses to SelH knockdown. Treatment with either siSELH1 or siSELH2 resulted in no significant change of GPX1 mRNA at either 48 or 72 h post-transfection. Transfection with siSELH1 resulted in increases in GPX2 mRNA by 0.93-fold at 48 h post-transfection ($P < 0.001$), and by 0.38-fold at 72 h post-transfection, but no change in GPX2 mRNA was observed due to treatment with siSELH2. There was no change in GPX4 mRNA due to either siRNA at 48 h post-transfection but an increase of 0.17-fold was detected at 72 h post-transfection with siSELH1 compared with the negative control ($P < 0.05$). Similarly, there was no change observed in TR1 mRNA at 48 h post-transfection, but an increase of 0.49-fold was observed after 72 h post-transfection with siSELH2 compared with negative control ($P < 0.01$).

4.2.4 Effects of TR1 knockdown on the expression of oxidative stress-associated genes in undifferentiated Caco-2 cells

TR1 was shown to have an effect on the mRNA expression of immune effectors in response to flagellin challenge (Section 3.2.3). Additionally, previous studies have suggested a role for TR1 in the regulation of oxidative stress-associated genes[60, 63, 506]. There exists a

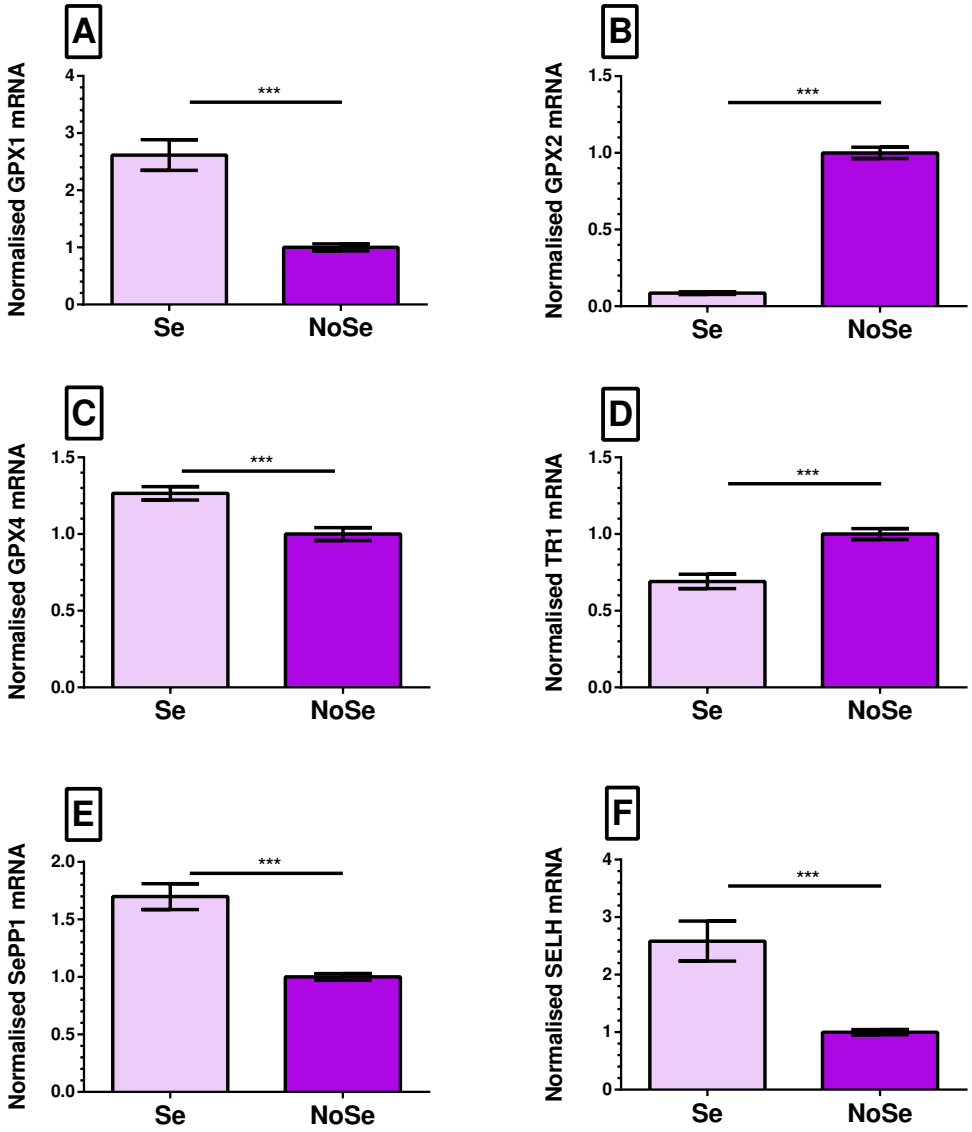

Figure 4.6. Effects of 72 h Se-supplementation or Se-depletion (NoSe) on the mRNA expression of the selenoproteins GPX1, GPX2, GPX4, TR1, SePP1 and SelH in undifferentiated Caco-2 cells. Data were expressed as a proportion of the mean of the NoSe in order to combine 3 distinct biological replicates, each consisting of 4 technical replicates (n = 12). Data were analysed statistically using a two-tailed unpaired t-test. The data are displayed as mean ± standard error of the mean (SEM). *** $P < 0.001$.

wealth of evidence in the literature of cross-talk between immune response and oxidative stress pathways [82, 261, 262, 265, 312, 313, 322, 323, 328]. Thus, it was hypothesised that TR1 may exert the immunomodulatory effects observed in Section 3 by modulation of oxidative stress-associated genes. To test this a knockdown of TR1 mRNA expression was carried out in undifferentiated Caco-2 cells and gene expression of NQO1, HMOX1, GPX2 and SelH assessed.

Following the transfection of a TR1-specific siRNA, siTR1, into undifferentiated Caco-2 cells, the level of TR1 mRNA expression was measured 48 h post-transfection to assess

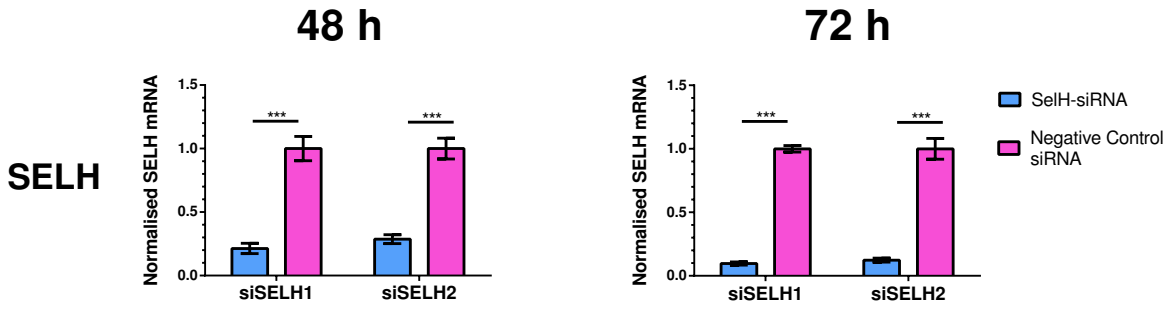

Figure 4.7. Effects of treatment of undifferentiated Caco-2 cells with SelH-specific siRNAs on mRNA expression of SelH at 48 h and 72 h post-transfection. All data were normalised to the housekeeping genes B2M and TOP1. Data were expressed as a proportion of the mean of the NoSe in order to combine 2 distinct biological replicates, each consisting of 4 technical replicates (n = 8). Data were analysed statistically using two-way ANOVA with a Tukey post-test for multiple comparisons. The data are displayed as mean ± standard error of the mean (SEM). *** $P < 0.001$.

the efficiency of knockdown (Figure 4.10). mRNA expression of TR1 was reduced by 83 % in cells transfected with siTR1 ($P < 0.001$), compared with cells transfected with negative control siRNA.

mRNA expression data relating to the oxidative stress-associated genes NQO1 and HMOX1 are shown in Figure 4.11. Transfection of Caco-2 cells with siTR1 resulted in no statistically significant change in the expression of NQO1. However, Caco-2 cells treated with siTR1 exhibited a 0.63-fold increase in HMOX1 mRNA compared with cells treated with negative control siRNA ($P < 0.001$).

Treatment of Caco-2 cells with siTR1 resulted in no statistically significant change in the expression of GPX2 compared with cells treated with negative control siRNA, while an 8 % decrease in SelH mRNA was detected (Figure 4.12; $P < 0.05$).

4.3 Discussion

The data presented in this chapter suggest that Se-depletion increases the expression of oxidative stress-associated genes in undifferentiated Caco-2 cells but not in differentiated Caco-2 cells, while cJUN expression was affected by Se-depletion in both differentiated and undifferentiated Caco-2 cells. Knockdown of SelH and TR1 expression also resulted in modulation of oxidative stress-associated gene expression but not in a manner consistent with the effects of Se-depletion.

Se-depletion was found to increase the mRNA expression of oxidative stress-associated targets in undifferentiated Caco-2 cells, although HMOX1, a target which is highly associated with Nrf2 activation, exhibited no change in expression. Interestingly, Se-depletion

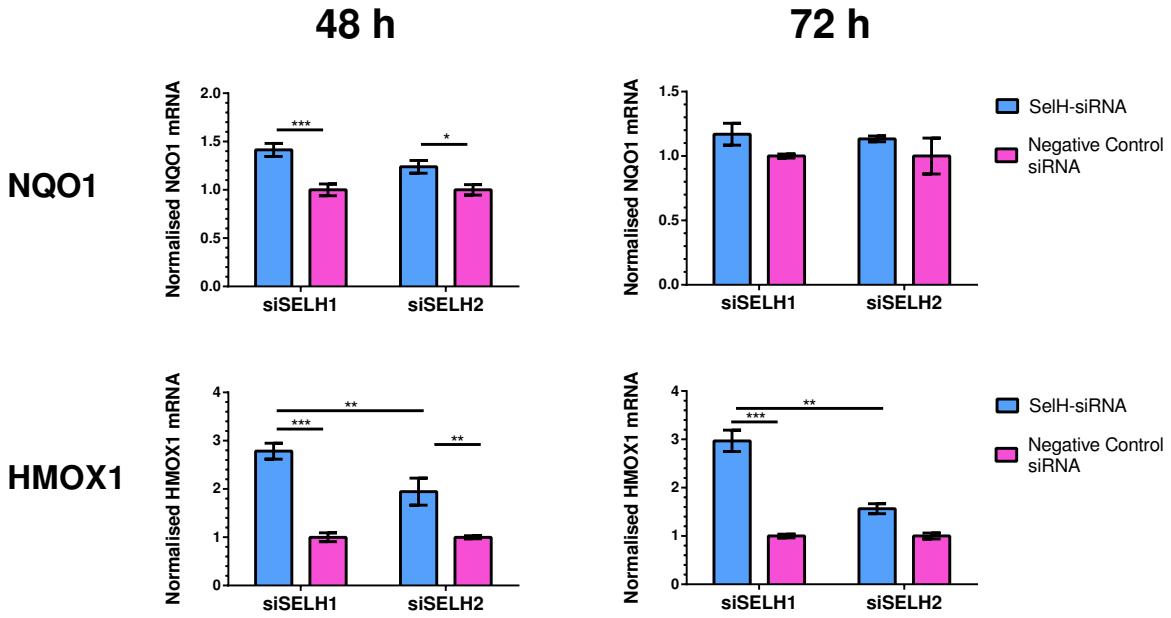

Figure 4.8. Effects of treatment of undifferentiated Caco-2 cells with SelH-specific siRNAs on mRNA expression of oxidative-stress associated targets, NQO1 and HMOX1 at 48 h and 72 h post-transfection. All data were normalised to the housekeeping genes B2M and TOP1. Data were expressed as a proportion of the mean of the NoSe in order to combine 2 distinct biological replicates, each consisting of 4 technical replicates (n = 8). Data were analysed statistically using two-way ANOVA with a Tukey post-test for multiple comparisons. The data are displayed as mean ± standard error of the mean (SEM). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

resulted in the increased expression of the AP-1 and Nrf2 transcription factor subunit, cJUN, suggesting a potential role for cJUN in the regulation of the oxidative stress-associated targets.

Knockdown of SelH was performed in undifferentiated Caco-2 cells, which resulted in the increase in expression of NQO1 and HMOX1. Additionally, a knockdown of TR1 was performed which resulted in the increase in expression of HMOX1, but not NQO1. Thus, these data indicated that, in undifferentiated Caco-2 cells, Se-depletion increases the expression of oxidative stress-associated genes and the expression of some of these targets is modulated by knockdown of either SelH or TR1. However, the link between these selenoproteins and that the expression of oxidative stress targets is not clear. Importantly, the response of oxidative stress targets to Se-depletion was markedly different in differentiated Caco-2 cells, with there only being a relatively small increase in NQO1 observed and no changes measured in the other targets.

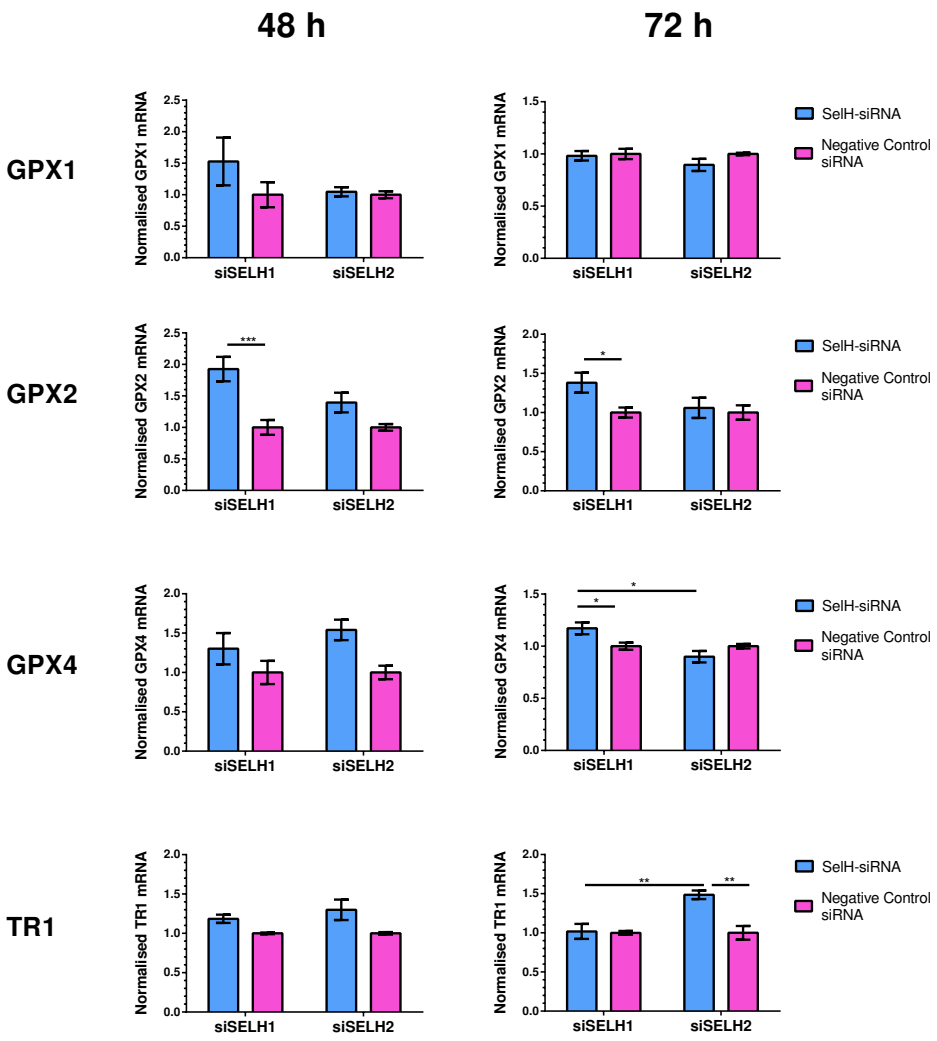

Figure 4.9. Effects of treatment of undifferentiated Caco-2 cells with SelH-specific siRNAs on mRNA expression of selenoproteins, GPX1, GPX2, GPX4 and TR1 at 48 h and 72 h post-transfection. All data were normalised to the housekeeping genes B2M and TOP1. Data were expressed as a proportion of the mean of the NoSe in order to combine 2 distinct biological replicates, each consisting of 4 technical replicates (n = 8). Data were analysed statistically using two-way ANOVA with a Tukey post-test for multiple comparisons. The data are displayed as mean ± standard error of the mean (SEM). * $P < 0.05$; ** $P < 0.01$.

4.3.1 The effect of selenium depletion on the expression of oxidative stress-associated genes and selenoprotein mRNAs in undifferentiated Caco-2 cells

NAD(P)H dehydrogenase quinone 1 (NQO1)

NQO1 is a homodimeric flavoprotein that catalyses the reduction reaction of quinones to hydroquinones and is a key antioxidant enzyme in the oxidative stress defence system [507, 508]. It has previously been reported that NQO1 is involved in cellular senescence [508, 509] and exhibits increased expression in a number of cancers, including colon cancer [510–512]. NQO1 has also been suggested to have a protective effect against excessive pro-

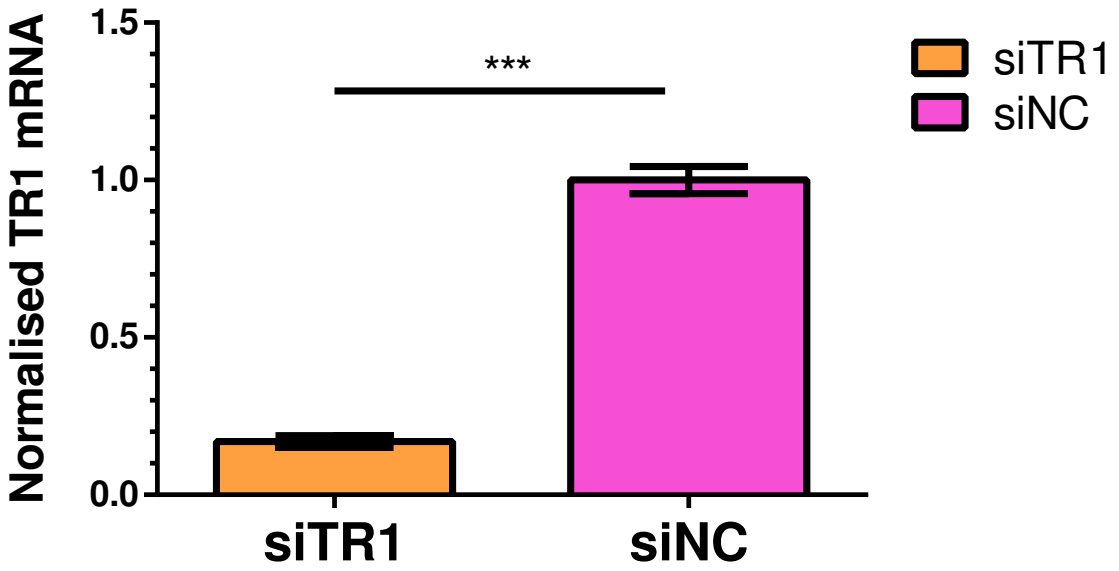

Figure 4.10. Effects of treatment of undifferentiated Caco-2 cells with TR1-specific siRNA (siTR1) on mRNA expression of TR1 at 48 h post-transfection. All data were normalised to the housekeeping genes B2M and TOP1. Data were expressed as a proportion of the mean of the NoSe in order to combine 2 distinct biological replicates, each consisting of 3 technical replicates (n = 6). Data were analysed statistically using a two-tailed unpaired t-test. The data are displayed as mean ± standard error of the mean (SEM). *** $P < 0.001$.

inflammatory responses by suppressing NF-κB induction of pro-inflammatory cytokines [513, 514].

The induction of NQO1 expression is primarily controlled via the binding of Nrf2/small Maf protein heterodimers to ARE in the promoter region of NQO1 [259]. However, the ARE of NQO1 also contains AP-1 recognition elements to which other transcription factors, such as Nrf1, Nrf2, c-Fos, cJUN, BTB Domain and CNC Homolog 1 (Bach1) and small Maf proteins can bind [259]. Thus, the transcriptional regulation of NQO1 in response to oxidative stress has the potential to be controlled by a number of different transcription factor complexes. The transcription of NQO1 can also be repressed. A previous study has found that c-Fos is able to repress the transcription of NQO1 by binding to the ARE in the NQO1 promoter [515]. Furthermore, overexpression of c-Fos has been shown to downregulate the transcription of ARE-containing genes, NQO1 and glutathione S-transferase (GST) genes, such as GSTP1 [516]. It is not currently understood if c-Fos forms a complex to repress ARE-mediated gene transcription. However, there is evidence that suggests cJUN protects c-Fos from degradation as overexpression of cJUN results in accumulation of phosphorylated c-Fos [517].

The effects of Se status on the expression of NQO1 is not currently well understood. Three clinical trials in humans have reported that low Se or supplementation with Se had

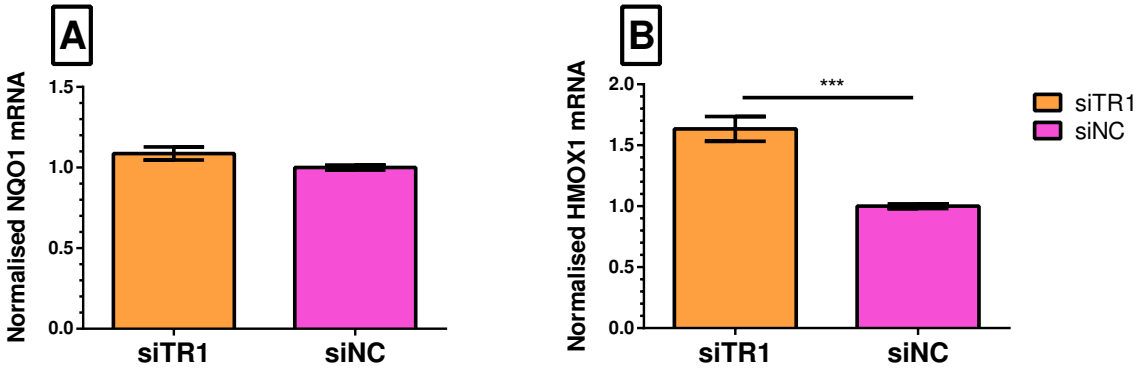

Figure 4.11. Effects of treatment of undifferentiated Caco-2 cells with TR1-specific siRNA on mRNA expression of oxidative stress-associated targets, NQO1 and HMOX1, at 48 h post-transfection. All data were normalised to the housekeeping genes B2M and TOP1. Data were expressed as a proportion of the mean of the NoSe in order to combine 2 distinct biological replicates, each consisting of 3 technical replicates (n = 6). Data were analysed statistically using a two-tailed unpaired t-test. The data are displayed as mean ± standard error of the mean (SEM). *** $P < 0.001$.

no effect on NQO1 gene expression [518–520] but toxic levels of Se were found to increase the levels of NQO1 protein in one study [521]. A number of rodent studies have found that Se deficiency resulted in an up-regulation of NQO1 mRNA expression [328, 522, 523]. On the other hand, some rodent studies have found no correlation between Se status and NQO1 gene expression or activity [524, 525]. Surprisingly, there is a distinct lack of *in vitro* studies that have investigated both Se status and NQO1 gene expression.

The data presented in this chapter indicated that 72 h Se-depletion in both differentiated and undifferentiated Caco-2 cells induced the expression of NQO1 mRNA, with a much larger effect of Se-depletion observed in undifferentiated Caco-2 cells (Figure 4.1 and Figure 4.4, respectively). Interestingly, NQO1 was the only oxidative stress-associated target, which increased in expression due to Se-depletion in the differentiated Caco-2 cells (Figure 4.1). As NQO1 exhibited the largest increase in expression due to Se-depletion in the undifferentiated cells (Figure 4.4), this suggests that NQO1 is particularly sensitive to changes in Se status and hence why it was the only oxidative stress-associated target to increase in expression in differentiated Caco-2 cells. There are no previous reports examining the effect of Se status on NQO1 expression in Caco-2 cells. However, NQO1 was found to be inducible in undifferentiated Caco-2 cells by oxidative stress and Nrf2 pathway activators [526, 527]. Interestingly, basal NQO1 protein levels and activity have previously found to be undetectable in undifferentiated Caco-2 cells but the mRNA levels of NQO1 were not tested [528]. In differentiated Caco-2 cells, NQO1 has been found to be induced by homogenised broccoli, possibly via phytochemical-induced Nrf2 activation [529]. There is lack of agreement between the present data and *in vivo* studies in humans, which showed no increase in NQO1 expression in response to reduced Se status [518–521].

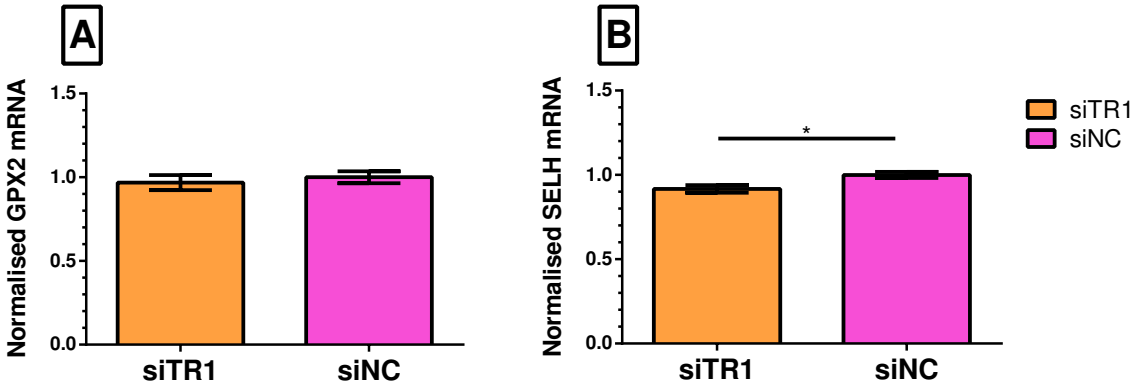

Figure 4.12. Effects of treatment of undifferentiated Caco-2 cells with TR1-specific siRNA on mRNA expression of selenoproteins, GPX2 and SELH, at 48 h post-transfection. All data were normalised to the housekeeping genes B2M and TOP1. Data were expressed as a proportion of the mean of the NoSe in order to combine 2 distinct biological replicates, each consisting of 3 technical replicates (n = 6). Data were analysed statistically using a two-tailed unpaired t-test. The data are displayed as mean ± standard error of the mean (SEM). * $P < 0.05$.

NQO1 is inducible in both differentiated and undifferentiated Caco-2 cells, but the effect of Se-depletion is greater in undifferentiated Caco-2 cells (Figure 4.1A and Figure 4.4A, respectively). One explanation for the lack of observed effect of Se status *in vivo*, may be the fact that the effect of Se-depletion is more pronounced *in vitro*.

To dissect the mechanisms by which Se-depletion may be inducing oxidative stress-associated target genes, knockdown of the siRNA of the selenoprotein SelH was performed. SelH was chosen because it showed a decrease in both differentiated and undifferentiated Caco-2 cells (Figure 4.3 and Figure 4.6, respectively). Additionally, previous microarray data from the laboratory showed that SelH knockdown in Caco-2 cells had a modulatory effect on genes involved in the Nrf2 pathway (unpublished data). Moreover, SelH has been suggested to have a protective role with in the cell during stress, which may be mediated by Nrf2 and NF-κB pathway activity [37, 41, 50, 51]. At the time of publication, there are no other studies known to have studied the effects of SelH on the expression of NQO1. Rodent studies have reported a down-regulation of SelH mRNA due to Se deficiency with a concurrent up-regulation of NQO1 mRNA [328, 522, 523], although one rodent study has observed a decrease in SelH mRNA with no change in NQO1 activity [525]. Despite no evidence for the direct effects of SelH on the expression of NQO1, these rodent studies provide some correlative evidence that further supports an association between SelH expression levels and NQO1 expression or activity. The present data indicates an increase in NQO1 mRNA as a result of SelH knockdown at only 48 h but, unlike HMOX1, there was no increase at 72 h. The reason behind this result is unclear. One explanation is that HMOX1 is more sensitive to the effects of SelH knockdown than NQO1. However, if SelH mediates the modulatory effects of Se on the expression of oxidative stress-associated targets, SelH knockdown would be expected to affect NQO1 expression, but not HMOX1

expression.

As previously discussed in Chapter 3, the data suggest that TR1 is a mediator of the modulatory effects of Se-depletion on the responses of Caco-2 cells to flagellin. TR1 is a key enzyme in one of the prominent redox regulatory systems in mammalian cells, the TXN system, which is important for cellular defences against oxidative stress and signal transduction [60, 63, 506]. Several studies have shown that knockdown or inhibition of TR1 leads to activation of the Nrf2 signalling pathway, leading to the hypothesis that TR1 is a potent regulator of the Nrf2 signalling pathway [506]. Additionally, there is also evidence that suggests that TR1 itself is regulated by the Nrf2 signalling pathway as it contains an ARE in its promoter [530]. Interestingly, TR1 knockdown did not result in any change in NQO1 mRNA which is surprising because a previous study has reported that inhibition of TR1 resulted in increased levels of NQO1 mRNA. In future work, it would be important to verify that TR1 protein levels and activity were reduced in response to TR1 knockdown.

Haem oxygenase-1 (HMOX1)

HMOX1 is an enzyme which catabolises haem into carbon monoxide, biliverdin and iron [531], and has also been reported to possess antioxidant functions [532–535]. The induction of HMOX1 is important in the protection against a diverse variety of states of cellular stress, including hypoxia and oxidative stress [536]. Additionally, it has been reported to be a potent anti-inflammatory enzyme, which is an inhibitor of NF-κB pathway activity [537]. There is also evidence that HMOX1 plays a role in the pathogenesis of a number of different malignancies [538].

The expression of HMOX1 is controlled at the transcriptional level [539–542] and it has been shown to be induced by a variety of stimuli, including haem, hypoxia, UV radiation, hydrogen peroxide and LPS [543–548]. Transcriptional control of HMOX1 has been shown to be mediated by binding of a number of transcription factors, including AP-1 and Nrf2 [549–560]. AP-1 and Nrf2 have been shown to be able to regulate the expression of HMOX1 by the binding of their transcription factor complexes to an AP-1 binding site and a core ARE in the HMOX1 promoter sequence, where both binding sites can overlap and both transcription factors can bind [253]. Although Nrf2 is commonly described as the major transcription factor which binds to AREs to induce HMOX1 expression [561], there is also evidence that HMOX1 can be induced in Caco-2 cells in a manner which is independent of Nrf2 activity [562].

It is well documented that Se deficiency activates the Nrf2 pathway, and induces the expression of Nrf2 target genes [82, 261, 328, 520, 563–565]. One well-characterised Nrf2 target is HMOX1, which is induced by Nrf2 pathway activation [566, 567]. Therefore, it was expected that the activation of the Nrf2 pathway by Se deficiency would induce HMOX1 expression. A number of studies on the effects of Se deficiency in a number of species have found a mixture of results. For instance, Se deficiency in chickens has been

linked to an increase in HMOX1 kidney protein [568], but no change in HMOX1 mRNA in either kidney cells [568, 569] or lymphocytes [570]. In rodent studies, Se deficiency was observed to increase HMOX1 mRNA in the gut and, as well as both mRNA and protein in the liver [83, 328]. However, it has also been reported that there was no change in HMOX1 mRNA due to Se deficiency in rat liver cells [571]. The effects of Se deficiency on the expression of HMOX1 in humans is sparse. However, one study reported that individuals in China with excessively high Se status exhibited high levels of HMOX1 mRNA and protein, compared with individuals with normal Se status [572].

The data presented in this chapter found no induction of HMOX1 mRNA in response to Se-depletion in either differentiated (Figure 4.1) or undifferentiated (Figure 4.4) Caco-2 cells. Interestingly, HMOX1 was the only oxidative stress-associated target tested that was not up-regulated due to Se-depletion in undifferentiated Caco-2 cells. These data agree with a previous study which has described no effect of Se on HMOX1 mRNA in undifferentiated Caco-2 cells. However, depleting cells of Se for 24 h may not be long enough to induce a stress response in Caco-2 cells [389]. As all targets tested contain an ARE and have been previously reported to be induced by Nrf2 activation, this might indicate a repressive mechanism of transcriptional regulation of HMOX1, which acts in addition to ARE-mediated transcriptional activation.

To dissect the mechanisms by which Se-depletion induced oxidative stress-associated target genes, knockdown of the siRNA of the selenoprotein SelH was performed. SelH was chosen because 1) it showed a decrease in both differentiated and undifferentiated Caco-2 cells (Figure 4.3 and Figure 4.6, respectively) 2) as discussed above, previous unpublished microarray data indicate that SelH knockdown in Caco-2 cells had a modulatory effect on genes involved in the Nrf2 pathway (unpublished data), and 3) SelH has previously been suggested to have a protective role with during stress, which may be mediated by Nrf2 and NF-κB pathway activity [37, 41, 50, 51].

The present work suggested that SelH is involved in the transcriptional regulation of HMOX1 as knockdown of SelH siRNA resulted in an increase in HMOX1 mRNA at 48 h and 72 h post-transfection in undifferentiated Caco-2 cells(Figure 4.8). However, SelH was not the mediator of HMOX1 transcription regulation following Se-depletion as Se-depletion did not result in any change in HMOX1 mRNA in undifferentiated Caco-2 cells (Figure 4.6). There are a number of possible explanations for this result. One possibility is that the level of SelH knockdown, which was 90 % (Figure 4.7), was much lower than the 61 % reduction in SelH mRNA observed in response to Se-depletion. Hence the modulatory effects of SelH on HMOX1 transcription may be more pronounced at lower levels of SelH expression. However, it is not known how the protein levels of SelH correspond to changes in mRNA levels and therefore it would be useful to determine the protein levels of SelH by Western blotting, after both Se-depletion and SelH knockdown.

A selenoprotein hierarchy also exists in the cell, by which each selenoprotein is differentially up-regulated or down-regulated in response to Se-deficiency, depending on where

they lie in the hierarchy [382, 469], and many of the 25 selenoproteins in humans are suggested to exhibit antioxidant functions [40]. Therefore, as Nrf2 and MAPK activity can be directly activated by oxidative stress [573, 574], it is feasible that the differential expression of the selenoproteome contributes to the transcriptional regulation of HMOX1 and that it cannot be attributed to the expression level of SelH alone.

The data presented in Section 3 suggest that TR1 was a mediator of the modulatory effects of Se-depletion on the responses of Caco-2 cells to flagellin. In undifferentiated Caco-2 cells, Se-depletion resulted in a 0.45-fold increase in TR1 mRNA (Figure4.6A), which occurred simultaneously to an increase in oxidative stress-associated targets, but not HMOX1 (Figure 4.4). An 83 % knockdown of TR1 siRNA in undifferentiated Caco-2 cells (Figure 4.10) resulted in a 0.63-fold increase in HMOX1 mRNA, but not NQO1 mRNA (Figure 4.11). Thus, much like with SelH, there were no modulatory effects of Se-depletion on the expression of HMOX1, but there were effects of TR1 knockdown. Thus, the effects of Se-depletion of oxidative stress-associated genes are probably not mediated through TR1 alone, but through a complex interaction between many members of the selenoproteome.

Superoxide dismutase 2 (SOD2)

The superoxide dismutase family of antioxidant enzymes catalyse the dismutation of oxygen free radicals to hydrogen peroxide, which is then further detoxified by catalase to oxygen and water [575]. There exists three isoforms of the superoxide dismutase family - superoxide dismutase 1 (SOD1), SOD2 and SOD3. SOD1 is primarily localised to the cytosol and peroxisomes, and is dependent upon incorporated copper (Cu) and zinc (Zn) for its redox activities. SOD2, which will be discussed further in this section, is localised to the mitochondrial matrix and is dependent upon manganese (Mn). Finally, superoxide dismutase 3 (SOD3) is an extracellular isoform that relies on Cu and Zn for its redox activities [576]. The expression of each isoform correlates with levels of intracellular ROS [577]. SOD2, in particular, has been reported to be responsive to oxidative stress and has been suggested to be pivotal in the protection against oxidative stress [578–580].

The transcriptional regulation of the SOD2 promoter can be modulated by a number of different redox-sensitive transcription factors, including NF-κB, AP-1 and Nrf2 [581]. Members of the NF-κB family are able to bind to a confirmed NF-κB binding site in the SOD2 [582]. Previous studies have shown that NF-κB is able to induce SOD2 gene expression in response to oxidative stress [583–585]. However, the p50 subunit of the NF-κB family has previously been suggested to negatively regulate the transcription of SOD2 [586]. SOD2 has also been shown to contain an AP-1 binding site in its promoter region by which SOD2 can be regulated in response to oxidative stress [581] via two signalling pathways involving JNK and p38 MAPK via AP-1 [587]. Additionally, the SOD2 promoter also contains an ARE to which Nrf2 can modulate its expression in response to oxidative stress [581].

In vivo studies in rats and humans reported that Se supplementation resulted in increased SOD2 expression [588, 589], whereas one study in chickens reported no increase [590]. A study in humans reported an inverse correlation between SOD2 expression and Se status [520]. On the contrary, one *in vitro* study in macrophages reported that Se supplementation caused a decrease in SOD2 gene expression [591]. However, caution is needed as none of the studies mentioned here compared the effects of Se deficiency or depletion, whereas the data reported in the present chapter present a direct comparison between Se supplemented and depleted status.

The data presented in this chapter suggested no effect of Se-depletion on SOD2 expression in differentiated Caco-2 cells (Figure 4.1). However, Se-depletion resulted in a 0.43-fold increase in SOD2 mRNA in undifferentiated Caco-2 cells (Figure 4.6). These data do not agree with the previously mentioned studies that found an increase in SOD2 expression with Se supplementation [588, 589]. However, these studies did not examine the effects of Se deficiency and only the effects of Se supplementation, and furthermore were carried out in a cell type (macrophages) in which ROS have a specialised function. Additionally, at high concentrations, Se has been reported to have pro-oxidant effects which may explain the increase in SOD2 expression in these studies [233]. These data do agree, however, with one study looking at the effect of Se status, both low and high, which reported that low Se status correlates with increased expression of SOD2. However, further study is needed to elucidate the mechanisms of transcriptional regulation that occurs in the SOD2 promoter at varying levels of Se.

Peroxiredoxin 1 (PRDX1)

Peroxiredoxins are a family of TXN-dependent antioxidant peroxidase enzymes, of which PRDX1 is a major member. PRDX1 has been reported to play a role in defence against oxidative stress, cell proliferation and apoptosis [592]. Additionally, PRDX1 has been reported to regulate of the NF-κB pathway [593, 594].

The transcriptional regulation of PRDX1 can occur via the action of a number of transcription factors. It has previously been reported that accumulation of ROS induced by hypoxia resulted in increased expression of PRDX1 via the Nrf2 pathway [595, 596]. Furthermore, it has been suggested that PRDX1 regulated the expression of HMOX1 via the activation of the NF-κB pathway [595]. However, the role of NF-κB in the transcriptional regulation of HMOX1 is currently debated [597–600]. An *in vivo* study performed in rodents and a study in humans have reported that Se deficiency is associated with an increase in expression of PRDX1 [328, 520]. The data presented in this chapter using undifferentiated Caco-2 cells agrees with the results from these studies as PRDX1 mRNA expression was found to be increased in response to Se-depletion (Figure 4.4D). However, Se-depletion in differentiated Caco-2 cells do not agree with the results from these *in vivo* studies as no change in PRDX1 expression was observed in response to Se-depletion (Figure 4.1D).

Glutathione S-transferase P 1 (GSTP1)

GSTP1 belongs to the GST family of phase II detoxification enzymes, which are involved in the neutralisation of free radicals [601]. GSTP1 in particular has been suggested to be involved in cell proliferation and has been observed to be highly expressed in proliferating epithelial cells [602]. Basal and inducible expression of GSTP1 has been suggested to be partly mediated by AP-1 transcription factors [603–605]. GSTP1 mRNA expression has also been shown to be up-regulated directly by NF-κB in response to hydrogen peroxide treatment [606]. As Se-depletion has been associated with increased NF-κB activity, the increase in expression of GSTP1 observed in undifferentiated Caco-2 cells in the present work (Figure 4.4D) may be mediated by the NF-κB pathway.

However, the GSTP1 promoter contains a number of AREs, to which Nrf2 can bind and initiate gene transcription [607, 608]. A study in human epidermal cells reported that AREs play no role in the transcriptional regulation of GSTP1 but it is not currently known if this ARE silencing is cell-type specific [609]. A previous study observed that undifferentiated Caco-2 cells exhibit higher mRNA and protein expression of GSTP1 compared with differentiated Caco-2 cells [407]. Although absolute quantification of mRNA was not measured in the present work, Se-depletion resulted in increased expression of GSTP1 in the undifferentiated Caco-2 cells but not the differentiated Caco-2 cells (Figure 4.4D and Figure 4.1D, respectively). Thus, an increase of GSTP1 expression in undifferentiated Caco-2 cells relative to differentiated Caco-2 cells may contribute to the increased responsiveness of GSTP1 to Se-depletion in undifferentiated Caco-2 cells in the present work. Consistent with the present work, low Se status has also correlated with higher GSTP1 mRNA expression in peripheral blood leukocytes in a previous study [520].

4.3.2 The effect of Caco-2 cell differentiation on the modulatory effects of selenium on the expression of selenoprotein mRNAs

Glutathione peroxidase 1 (GPX1)

In both the differentiated and undifferentiated Caco-2 cells, Se-depletion resulted in a decrease in GPX1 mRNA (Figure 4.3 and Figure 4.6, respectively). These data agree with previous studies that have also shown that GPX1 is particularly sensitive to Se status as it is particularly low on the selenoprotein hierarchy [31, 376]. In addition to being regulated in response to Se, GPX1 mRNA expression can be up-regulated in response to oxidative stress. Previous reports have indicated that GPX1 expression can be induced via the binding of p53 to a p53 binding site in the GPX1 promoter [610, 611]. Additionally, GPX1 has previously been shown to be upregulated in response to oxidative stress via the NF-κB and AP-1 transcription factors binding to their respective binding sites in the GPX1 promoter [612]. The Nrf2 or Nrf1 transcription factors have not previously been reported to directly induce the expression of GPX1, but GPX1 expression has been reported to correlate with Nrf1 activity [613]. The data from these studies show a decrease in GPX1

mRNA expression in response to Se-depletion in both differentiated and undifferentiated Caco-2 cells (Figure 4.3 and Figure 4.6, respectively). As described in Section 1.3.1, Se-depletion has been observed to exacerbate oxidative stress and up-regulate antioxidant enzymes. However, despite GPX1 being well characterised as an antioxidant enzyme, Se-depletion resulted in a down-regulation of GPX1 expression. As the selenoproteins GPX2 and TR1 were up-regulated, this may indicate a common method of transcriptional regulation between GPX2 and TR1, discussed below, that GPX1 does not possess.

Glutathione peroxidase 2 (GPX2)

GPX2 has similar peroxidase activity as GPX1, but has a widely different tissue distribution as it is mainly expressed in IECs and is thought to be important in protection against gut pathogens and inflammation [365, 486, 487]. Previous studies have reported that GPX2 knockout mice are viable, but exhibit severe intestinal inflammation [150, 365, 486]. Furthermore, it has been suggested that GPX2 is able to help suppress intestinal inflammation by inhibiting the expression or activity of cyclooxygenase-2 (COX-2) [488, 489]. Unlike GPX1, GPX2 mRNA was upregulated in the present work in response to Se-depletion in both differentiated and undifferentiated Caco-2 cells (Figure 4.3 and Figure 4.6, respectively). This is consistent with a previous study in undifferentiated Caco-2 cells, which found an increase of GPX2 mRNA in response to Se-depletion [376]. Another study in undifferentiated Caco-2 cells found no change in GPX2 mRNA in response to Se-depletion, but did report an increase in protein levels [229]. One mechanism by which GPX2, but not GPX1, may be upregulated in response to Se-depletion is via the up-regulation of GPX2 by the Nrf2 transcription factor. Unlike GPX1, GPX2 mRNA has been shown to directly regulated by Nrf2 [229, 264, 614], which may act using a core ARE in the GPX2 promoter [252, 615].

Glutathione peroxidase 4 (GPX4)

GPX4, much like GPX1 and GPX2, exhibits peroxidase activity and has been suggested to play an important role in the reduction of lipid peroxides and the protection of cells from lipid hydroperoxides [616]. The GPX4 gene consists of seven exons and is alternately spliced into cytoplasmic, mitochondrial and sperm nuclear-specific isoforms [617]. GPX4 has been reported to be up-regulated in response to hydrogen peroxide treatment, although the transcriptional regulatory mechanisms for GPX4 are not currently well understood [470, 618]. GPX4 was down-regulated in response Se-depletion, but only in the undifferentiated Caco-2 cells (Figure 4.6C). This is consistent with previous studies which have reported that GPX4 is regulated by Se status, but is less sensitive than GPX1 [619, 620].

Thioredoxin reductase 1 (TR1)

TR1 is an important antioxidant defence enzyme which is a regulator of the TXN system, as discussed in further detail in Section 1.1.3. There is a well documented relationship

between TR1 and the Nrf2 antioxidant defence pathway, where TR1 and Nrf2 have been reported to regulate the expression and activity of the other [82–84]. TR1 contains an ARE in its promoter, which means that its expression can be induced by Nrf2 directly. Se-depletion is associated with increased oxidative stress, which activates the Nrf2 pathway. As TR1 contains an ARE in its promoter, this may act as a mechanism by which TR1 is upregulated in response to Se-depletion [85]. The present work observed an upregulation of TR1 in response to Se-depletion, but only in the undifferentiated Caco-2 cells (Figure 4.3D). Other studies in undifferentiated Caco-2 cells have not reported any responsiveness of TR1 to Se [229, 389, 621]. One potential explanation for the differences between results is that the above mentioned studies did not starve Caco-2 cells of Se for as long as 72 h.

Selenoprotein P (SePP1)

SePP1 is a unique selenoprotein containing ten Sec residues, rather than the single Sec residue that most selenoproteins possess [622]. Due to the high number of Se-containing Sec residues and the observation that SePP1 is secreted extracellularly into the blood stream, SePP1 has been suggested to be primary form of Se transport around the body [622]. However, SePP1 has also been reported to have antioxidant functions via an N-terminal Sec that is involved in catalysing the oxidation of GSH by hydrogen peroxide [623, 624]. SePP1 has also been suggested to be important in the pathogenesis of colitis as rodent experimental colitis models with a macrophage-specific SePP1 knockout exhibit worsened inflammatory injury [625]. Additionally, one study observed that SePP1 was the most highly induced gene in pro-inflammatory and tumour-conditioned macrophages, indicating an important role for SePP1 in macrophage activity [626]. The present work observed a decrease in SePP1 mRNA in both the differentiated and undifferentiated Caco-2 cells (Figure 4.3E and Figure 4.6E, respectively). This agrees with a previous study in differentiated Caco-2 cells, which found that SePP1 protein levels are responsive to Se status [397].

Selenoprotein H (SelH)

SelH is a nuclear-localised selenoprotein which exhibits oxidoreductase activity, as discussed in further detail in Section 1.1.2. The present work observed a trend towards decreased SelH expression in the differentiated Caco-2 cells (Figure 4.3F) and a significant reduction in SelH expression in the undifferentiated Caco-2 cells (Figure 4.6F). This was consistent with previous studies, which have reported that SelH expression was highly regulated by Se status, in a number of cell types including undifferentiated Caco-2 cells [28–31]. Se-depletion is associated with increased oxidative stress [82, 261, 262, 265, 328]. SelH has previously been suggested to mediate cytoprotective functions via modulation of pathways involved in protection against oxidative stress [37, 41, 50, 51, 53]. The down-regulation of the nuclear-localised SelH by Se-depletion coupled with the up-regulation of Nrf2 target genes in the present work indicates that SelH exerts some cytoprotective

function by associating with transcription factors in the nucleus to help facilitate the transcription of target genes.

4.3.3 Summary

Similarly to the effects of Se-depletion observed the responses of Caco-2 cells to flagellin (Section 3), Se-depletion resulted in the induction of oxidative-stress associated targets but only in the undifferentiated Caco-2 cells, with NQO1 being the exception. This indicates that the effects of Se-depletion on oxidative stress and responses to flagellin may have a common mechanism, as differentiation of Caco-2 cells diminishes the effects of Se in both cases. Knockdown of SelH and TR1 also had an effect on the expression of oxidative-stress associated genes but the effects were not in line with the effects of Se-depletion. This suggests that the effects of Se-depletion may be mediated through the actions of SelH and TR1 but other mechanisms of modulation are likely to be involved.

Chapter 5

Selenium as an important factor affecting the response of gut cells to hypoxic stress

5.1 Introduction

A tissue is described as hypoxic when the pO_2 falls below levels that are considered physiological and which lead to hypoxic stress [627]. Hypoxia has both physiological and pathological roles, with roles ranging from angiogenesis to the pathogenesis of several diseases, such as several cancers and gastrointestinal disorders [329].

5.1.1 Physiological and pathological hypoxia in the gut

In the gut there exists a steep oxygen gradient across the epithelium, with the oxygen concentration at the apical IEC surface at less than 2 %, and the intestinal tissue with approximately 8 %, as a percentage of the pO_2 of arterial blood [267]. However the luminal pO_2 is subject to regular fluctuations in pO_2. Food intake increases the pO_2 of the intestinal lumen whereas periods of fasting decreases the pO_2 of the intestinal lumen; these fluctuations in luminal pO_2 are physiological and have been referred to as physoxia [268, 269].

It has previously been described that a constant hypoxia-associated low-level of inflammation is the physiological norm for the gastrointestinal tract, in which the mucosal immune response of IECs are essential in the response to this inflammation [270]. However, conditions in which there is excessive and uncontrolled inflammation are characteristic of IBDs such as Crohn's Disease and Ulcerative Colitis [271]. Thus, the levels of inflammation in the gut must be carefully controlled and one level of control is suggested to be via cellular responses to hypoxia and the hypoxia signalling [272].

5.1.2 The role for HIF-1a in the hypoxia signalling pathway

One of the primary responses to hypoxic stress is mediated by the transcription factor, HIF-1. HIF-1 is able to bind to hypoxia response elements in the promoters of several genes involved in the cellular response to hypoxic stress [273, 274]. The HIF-1 transcription factor is a heterodimer which consists of two subunits: an α subunit which is responsive to oxygen status, and a constitutively expressed β subunit [275]. Thus, it is HIF-1α which is recognised as the primary modulator of the cellular responses to hypoxic stress.

The HIF-1α protein is highly regulated by cellular pO_2. During normoxia, there are physiological pO_2 levels and therefore a hypoxia response is not needed. Thus, the HIF-1α protein is rapidly targeted for degradation. During these periods of normoxia, PHD proteins, which are oxygen- and iron- dependent, hydroxylate two proline residues on HIF-1α [276]. This allows pVHL to bind to it and targets HIF-1α for degradation. However, under hypoxic conditions, oxygen-dependent hydroxylation of HIF-1α by PHD is inhibited and results in the rapid accumulation of HIF-1α inside the cell [277]. Thus, this allows the heterodimerisation of HIF-1α and hypoxia-inducible factor-1 beta (HIF-1β) which can then go on to regulate the transcription of genes involved in the cellular response to hypoxia.

5.1.3 The link between selenium and cellular responses to hypoxia

The literature suggests that a complex level of cross-regulation between selenium, selenoprotein biosynthesis and responses to hypoxia exists. A number of *in vivo* studies in a variety of species have suggested that supplementation with Se, in both inorganic and organic forms, has a protective effect against hypoxic stress [289–291]. Furthermore, *in vitro* studies have suggested that this effect may be due to modulatory effects of Se on the mRNA and protein expression, as well as the transcriptional activity, of HIF-1α [292–300]. However, the specific direction of the regulation of HIF-1α *in vitro* appears to be dependent upon the dose of Se supplied. For instance, high doses of Se supplementation, at 5 μM and greater, have been shown to have an inhibitory effect on the mRNA and protein levels of HIF-1α [293–295, 297]. On the other hand, levels of Se closer to physiological levels, between 100 and 145 nM, have shown a protective effect of Se against hypoxic stress and raises the level of HIF-1α protein [296, 298].

Hypoxia has been shown to have a modulatory effect on some of the members of the selenoprotein biosynthesis machinery, thereby regulating the expression of different selenoproteins [301]. For instance, previous studies have shown that hypoxia has a suppressive effect on the mRNA and protein levels of SePP1, TR1 and also a suppressive effect on the enzyme activities of DIO1 and GPX [290, 301, 302]. On the contrary, hypoxia has been shown to increase the mRNA and protein levels of GPX1, GPX4 and

SELENBP1 [301, 303]. Furthermore, previous studies have suggested that SELENBP1 is directly regulated by HIF-1 during hypoxia [303, 304]. Additionally, SELENBP1 has also been suggested to regulate the expression of HIF-1α but the mechanism is currently unknown [303, 305]. Interestingly, the regulation of some selenoproteins by hypoxia have been described to occur via a mechanism which is independent of HIF-1 [290, 301].

5.1.4 Research aims

As introduced above, a modulatory role of HIF-1 signalling in the NF-κB pathway has been well described in the literature. As there is evidence that suggests Se is able to regulate cellular responses to hypoxia, this may present a possible mechanism by which Se may modulate Caco-2 cell responses to flagellin, as reported in Section 3, via changes in HIF-1 signalling.

The aim of the research reported in this chapter is to assess the effects of Se supplementation or depletion on the expression of genes downstream of HIF-1 signalling in differentiated and undifferentiated Caco-2 cells. Furthermore, the potential role for SelH on the expression of these hypoxia-associated genes is also investigated.

5.2 Results

5.2.1 Effect of selenium depletion on responses to hypoxia in differentiated Caco-2 cells

Caco-2 cells were grown on Transwell semi-permeable inserts for 18 days in order to for them to completely polarise and differentiate, as described in Section 2.1.1. They were then depleted (NoSe) of Se, or supplemented with Se (Se), for 72 h as described in Section 2.1.2. Following this 72 h Se-depletion or supplementation period, cells were exposed to hypoxia or normoxia for 7 h and then mRNA was extracted and the expression of hypoxia-associated target genes analysed in response to 7h hypoxia or normoxia. The levels of HIF-1α mRNA are shown in Figure 5.1. Treatment of differentiated Caco-2 cells with hypoxia for 7 h resulted in no change in the mRNA expression of HIF-1α in either the Se-supplemented or Se-depleted groups. Se-depletion for 72 h (NoSe) resulted in no change in HIF-1α mRNA expression in either the normoxic or hypoxic treatment groups. However, when data from normoxic and hypoxic treatment groups were combined, there was an overall effect of Se depletion in reducing the expression of HIF-1α ($P < 0.05$).

After 72 h of either Se-depletion or Se-supplementation, the mRNA levels of the HIF-1 downstream effectors, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) and prolyl hydroxylase 3 (PHD3) were measured in response to 7 h normoxia or hypoxia (Figure 5.2). Treatment of Se-supplemented differentiated Caco-2 cells with hypoxia for 7 h resulted in a 13-fold increase in BNIP3 mRNA ($P < 0.001$) and a 2.8-fold increase in PHD3 mRNA ($P < 0.05$), compared with the mean for Se-supplemented cells during

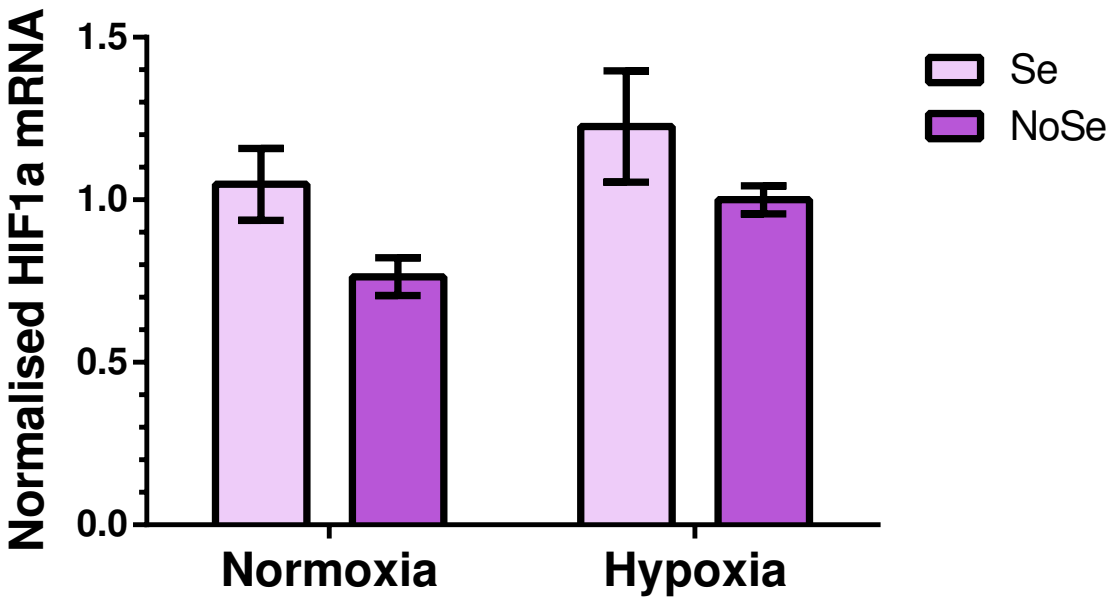

Figure 5.1. Effect of 72 h Se-depletion or supplementation on the mRNA expression of HIF-1α after treating differentiated Caco-2 cells with 7 h hypoxia. All data were normalised to the housekeeping genes TOP1 and EIF4A2. Data were expressed as a proportion of the mean of the Se-depleted cells treated with hypoxia in order to combine 3 distinct biological replicates, each consisting of 4 technical replicates (n = 12). Data were analysed statistically using two-way ANOVA with a Tukey post-test for multiple comparisons. The data are displayed as mean ± standard error of the mean (SEM). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

normoxia. There was no significant increase in the expression of BNIP3 or PHD3 in the Se-depleted cells due to hypoxia. There was no change in BNIP3 or PHD3 mRNA expression in the normoxic cells due to Se-depletion. In the differentiated Caco-2 cells treated with hypoxia, however, Se-depletion resulted in a decrease in the expression of BNIP3 and PHD3 by 58 % and 69 %, respectively ($P < 0.05$).

After 72 h of either Se-depletion or Se-supplementation, the mRNA levels of the selenoproteins GPX1, GPX2, TR1 and SelH, were measured in response to 7h normoxia or hypoxia (Figure 5.3). Treatment of Se-supplemented differentiated Caco-2 cells with hypoxia for 7 h resulted in a 35 % decrease in GPX1 mRNA ($P < 0.05$) but not change in the expression of other targets, compared with the mean for Se-supplemented cells during normoxia. There was no significant change in the expression of GPX1, GPX2, TR1 or SelH in the Se-depleted cells due to hypoxia. In the differentiated Caco-2 cells treated with normoxia, Se-depletion resulted in a 64 % decrease in GPX1 mRNA ($P < 0.001$), a 68 % decrease in SelH mRNA ($P < 0.01$) and a 0.57-fold increase in GPX2 mRNA ($P < 0.05$). In the differentiated Caco-2 cells treated with hypoxia, Se-depletion resulted in a 57 % decrease in GPX1 mRNA ($P < 0.01$) and a 0.75-fold increase in GPX2 mRNA ($P < 0.01$). There was no effect of Se-depletion or hypoxia treatment on the expression levels

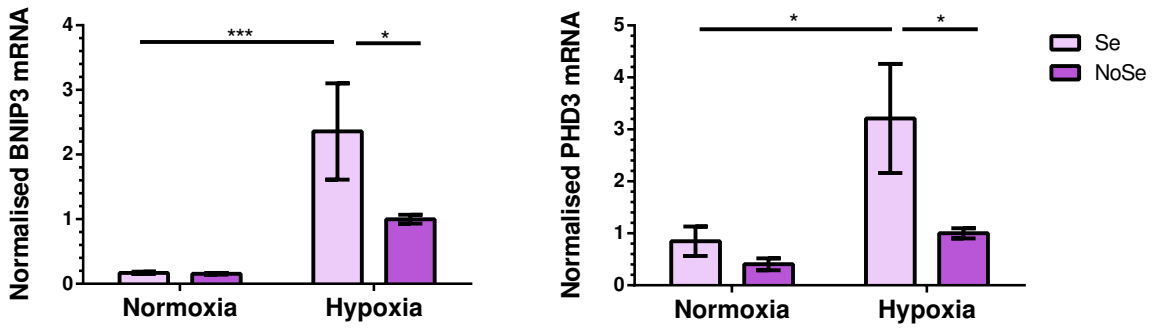

Figure 5.2. Effect of 72 h selenium starvation or supplementation on the mRNA expression of HIF-1 target genes, BNIP3 and PHD3 after treating differentiated Caco-2 cells with 7 h hypoxia or normoxia. All data were normalised to the housekeeping genes TOP1 and EIF4A2. Data were expressed as a proportion of the mean of the Se-depleted cells treated with hypoxia in order to combine 3 distinct biological replicates, each consisting of 4 technical replicates (n = 12). Data were analysed statistically using two-way ANOVA with a Tukey post-test for multiple comparisons. The data are displayed as mean ± standard error of the mean (SEM). * $P < 0.05$; *** $P < 0.001$.

of TR1.

5.2.2 Effect of selenium depletion on responses to hypoxia in undifferentiated Caco-2 cells

Caco-2 cells were grown on 6-well plates to confluency, as described in Section 2.1.1 before being depleted (NoSe) of Se, or supplemented with Se (Se), for 72 h as described in Section 2.1.2. Following this 72 h Se-depletion or supplementation period, mRNA was extracted from cells and the expression levels of hypoxia-associated target genes were analysed in response to 7h hypoxia or normoxia.

After 72 h of either Se-depletion or Se-supplementation, the mRNA levels of HIF-1α were measured after 7 h of normoxia or hypoxia (Figure 5.4). Treatment of undifferentiated Caco-2 cells with hypoxia for 7 h resulted in no change in the mRNA expression of HIF-1α in either the Se-supplemented or Se-depleted groups. Se-depletion for 72 h (NoSe) resulted in no change in HIF-1α mRNA expression in either the normoxic or hypoxic treatment groups, although a trend towards decreased expression was observed in the hypoxic group ($P = 0.08$). However, in the context of both the normoxic and hypoxic treatment groups, there was an overall effect of Se-depletion in reducing the expression of HIF-1α ($P < 0.01$).

After 72 h of either Se-depletion or Se-supplementation, the mRNA levels of the HIF-1 downstream effectors, BNIP3 and PHD3 were measured in response to 7 h normoxia or hypoxia (Figure 5.5). Treatment of Se-supplemented undifferentiated Caco-2 cells with hypoxia for 7 h resulted in a 27-fold increase in BNIP3 mRNA ($P < 0.001$) and a 2.3-fold

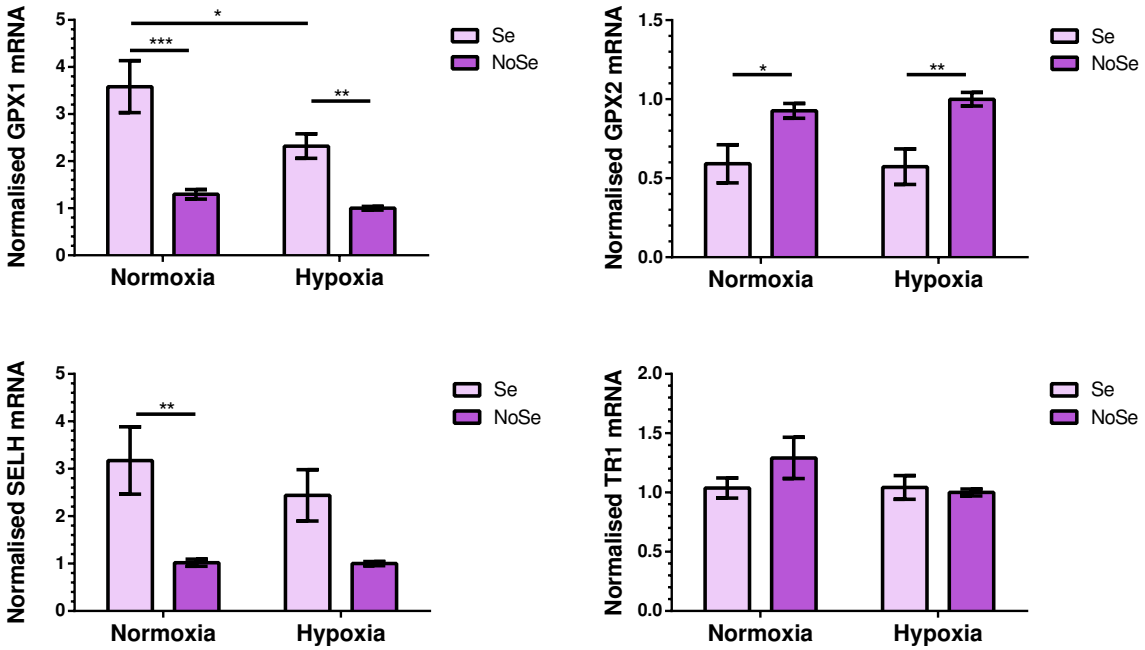

Figure 5.3. Effect of 72 h selenium starvation or supplementation on the mRNA expression of the selenoproteins GPX1, GPX2, TR1 and SelH after treating differentiated Caco-2 cells with 7 h hypoxia or normoxia. All data were normalised to the housekeeping genes TOP1 and EIF4A2. Data were expressed as a proportion of the mean of the Se-depleted cells treated with hypoxia in order to combine 3 distinct biological replicates, each consisting of 4 technical replicates (n = 12). Data were analysed statistically using two-way ANOVA with a Tukey post-test for multiple comparisons. The data are displayed as mean ± standard error of the mean (SEM). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

increase in PHD3 mRNA ($P < 0.01$), compared with the mean for Se-supplemented cells during normoxia. There was no significant increase in the expression of BNIP3 or PHD3 in the Se-depleted cells due to hypoxia. In the undifferentiated Caco-2 cells treated with hypoxia, Se-depletion resulted in a decrease in the expression of BNIP3 and PHD3 by 86 % and 84 %, respectively ($P < 0.05$).

After 72 h of either Se-depletion or Se-supplementation, the mRNA levels of the selenoproteins GPX1, GPX2, TR1 and SelH, were measured in response to 7h normoxia or hypoxia (Figure 5.6). Treatment of Se-supplemented undifferentiated Caco-2 cells with hypoxia for 7 h resulted in no change in the expression of any targets, compared with the mean for Se-supplemented cells during normoxia or hypoxia. In the undifferentiated Caco-2 cells treated with normoxia, Se-depletion resulted in a 69 % decrease in GPX1 mRNA ($P < 0.001$), an 80 % decrease in SelH mRNA ($P < 0.001$) and a 7.6-fold increase in GPX2 mRNA ($P < 0.001$). In the undifferentiated Caco-2 cells treated with hypoxia, Se-depletion resulted in a 71 % decrease in GPX1 mRNA ($P < 0.01$), a 77 % decrease in SelH mRNA ($P < 0.01$) and a 7.3-fold increase in GPX2 mRNA ($P < 0.01$).

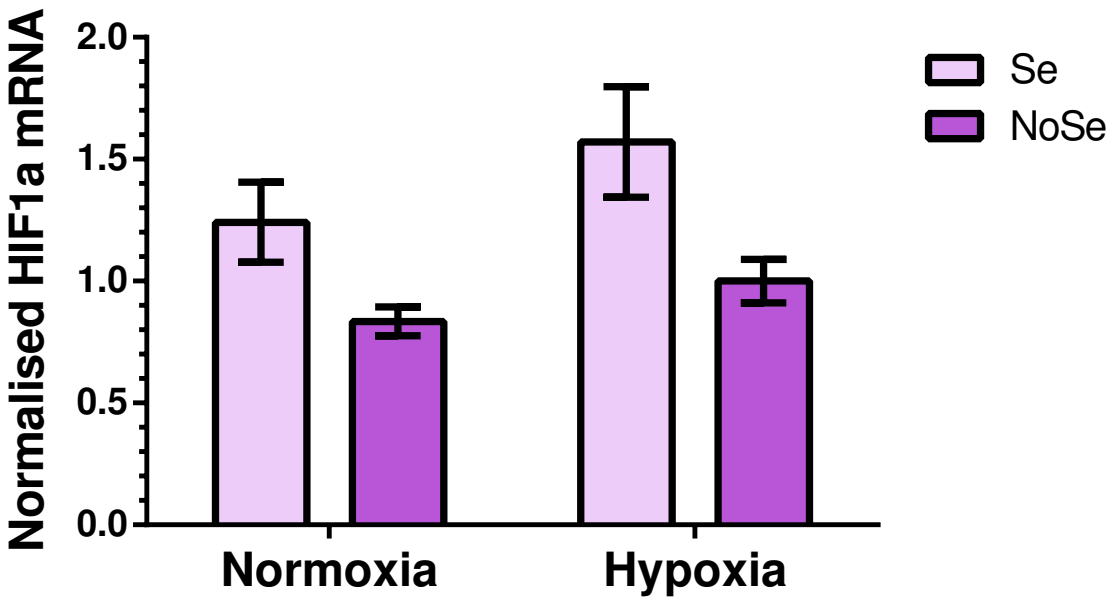

Figure 5.4. Effect of 72 h selenium starvation or supplementation on the mRNA expression of HIF-1α after treating undifferentiated Caco-2 cells with 7 h hypoxia. All data were normalised to the housekeeping genes TOP1 and EIF4A2. Data were expressed as a proportion of the mean of the Se-depleted cells. Data were analysed statistically using two-way ANOVA with a Tukey post-test for multiple comparisons. The data are displayed as mean ± standard error of the mean (SEM).

5.2.3 Effect of SelH knockdown on responses to hypoxia in undifferentiated Caco-2 cells

The data presented in this chapter suggest that SelH are regulated by Se status (Figure 5.3 and Figure 5.6). Additionally, previous unpublished data from the laboratory by Hannah Gautrey, where the expression SelH was reduced by knockdown in Caco-2 cells, indicated a potential role for SelH in the expression of hypoxia-associated genes. Therefore experiments were carried out to investigate the effects of SelH gene silencing under hypoxic conditions. Knockdown of SelH expression was performed by transfection of SelH-specific siRNAs into undifferentiated Caco-2 cells, as described in Section 2.1.3, and the mRNA expression of hypoxia-associated genes was measured at 72 h post-transfection, after 7 h of treatment with normoxia or hypoxia.

Following the transfection of either of two SelH-specific siRNAs, siSELH1 and siSELH2, into undifferentiated Caco-2 cells separately, the level of SelH mRNA expression was measured after 72 h post-transfection in order to assess the efficiency of knockdown, as discussed in Section 4.7. The cells were then treated with 7 h normoxia or hypoxia and the mRNA expression of HIF-1α was then measured (Figure 5.7A). Knockdown of SelH expression using siSELH1 resulted in a 40 % decrease in HIF-1α mRNA ($P < 0.001$) and treatment with siSELH2 resulted in a 0.27-fold increase in HIF-1α mRNA ($P < 0.05$),

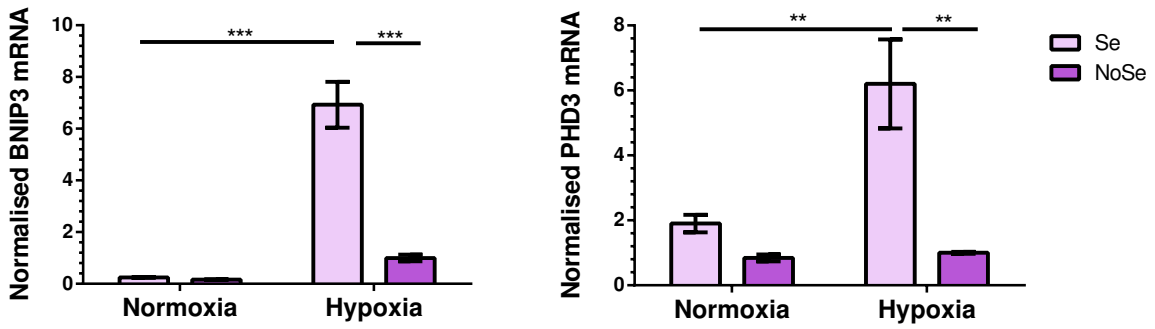

Figure 5.5. Effect of 72 h selenium starvation or supplementation on the mRNA expression of HIF-1 target genes, BNIP3 and PHD3 after treating undifferentiated Caco-2 cells with 7 h hypoxia or normoxia. All data were normalised to the housekeeping genes TOP1 and EIF4A2. Data were expressed as a proportion of the mean of the Se-depleted cells treated with hypoxia. Data were analysed statistically using two-way ANOVA with a Tukey post-test for multiple comparisons. The data are displayed as mean ± standard error of the mean (SEM). ** $P < 0.01$; *** $P < 0.001$.

compared with cells treated with negative control siRNA. There was a significant difference detected between the level of HIF-1α expression after treatment with siSELH1 and siSELH2 ($P < 0.001$). Additionally, the level of HIF-1α protein was measured following treatment with 3 h and 7 h hypoxia, after 72 h post-transfection with siSELH1 (Figure 5.7B). Treatment with siSELH1 resulted in an approximate 45 % reduction in band intensity of HIF-1α protein after 3 h and 7 h hypoxia after normalisation against β-actin band intensity.

At 72 h post-transfection with the SelH-specific siRNAs, siSELH1 and siSELH2, the cells were then treated with 7 h normoxia or hypoxia and the mRNA expression of HIF-1 targets, BNIP3 and PHD3, were then measured (Figure 5.8). Knockdown of SelH expression using siSELH1 resulted in a 50 % decrease in BNIP3 mRNA ($P < 0.001$) but treatment with siSELH2 resulted in no change in BNIP3 mRNA ($P < 0.05$), compared with cells treated with negative control siRNA. There was a significant difference detected between the level of BNIP3 expression after treatment with siSELH1 and siSELH2 ($P < 0.01$). Knockdown of SelH expression with siSELH1 or siSELH2 resulted in no change in PHD3 expression in undifferentiated Caco-2 cells treated with 7 h hypoxia.

5.3 Discussion

Se-depletion was found to have a significant inhibitory effect on the expression of downstream HIF-1α targets in both differentiated and undifferentiated Caco-2 cells (Figure 5.2 and Figure 5.4, respectively). Interestingly, Se-depletion had no effect on the mRNA expression of HIF-1α in either the differentiated or undifferentiated Caco-2 cells within each hypoxic or normoxic treatment group but Se-depletion did reduce the level of HIF-

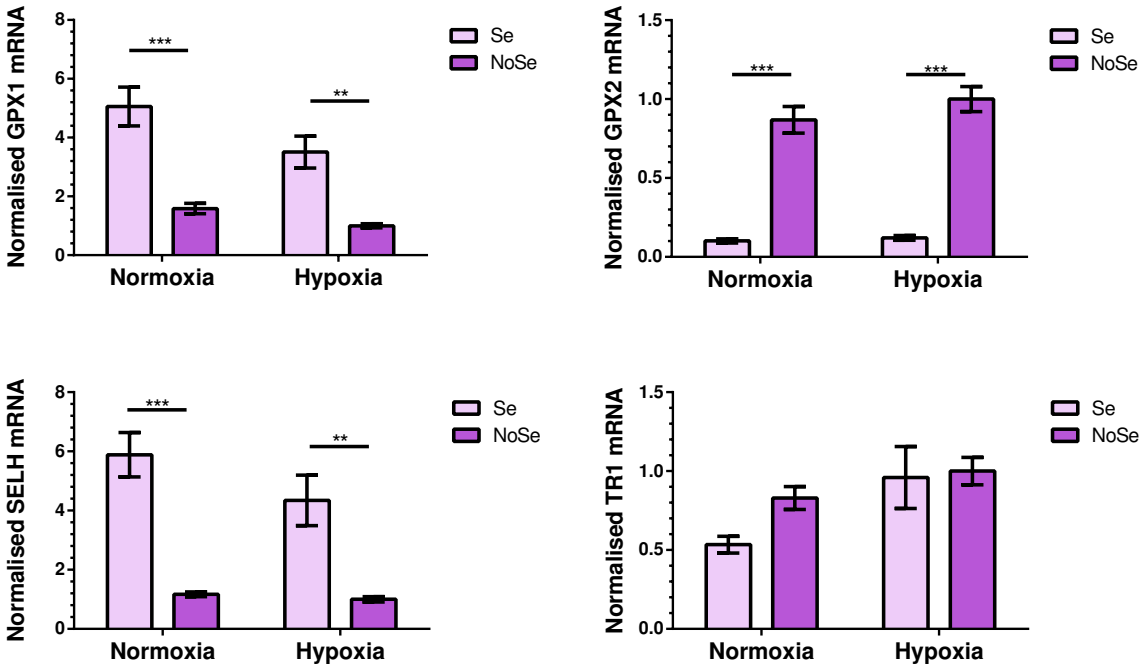

Figure 5.6. Effect of 72 h selenium starvation or supplementation on the mRNA expression of the selenoproteins GPX1, GPX2, TR1 and SelH after treating undifferentiated Caco-2 cells with 7 h hypoxia or normoxia. All data were normalised to the housekeeping genes TOP1 and EIF4A2. Data were expressed as a proportion of the mean of the Se-depleted cells treated with hypoxia. Data were analysed statistically using two-way ANOVA with a Tukey post-test for multiple comparisons. The data are displayed as mean ± standard error of the mean (SEM). ** $P < 0.01$; *** $P < 0.001$.

1α mRNA in the context of both treatment groups combined (Figure 5.1 and Figure 5.4). Hypoxia itself did not appear to modulate the mRNA expression of many selenoproteins, with only GPX1 being down-regulated in the differentiated Caco-2 cells (Figure 5.3). Previous microarray data from the laboratory suggested a role for SelH in hypoxia signalling and thus SelH knockdowns were performed using two different SelH-specific siRNAs, siSELH1 and siSELH2, but the effects on HIF-1α expression and HIF-1 target genes were found to be inconsistent between the two siRNAs (Figure 5.7 and Figure 5.8, respectively). Thus, the present work suggests a role for selenium in hypoxia signalling in Caco-2 cells but the role for SelH remains poorly understood.

5.3.1 The effect of Caco-2 cell differentiation on the modulatory effects of selenium on Caco-2 cell responses to hypoxia and potential roles for SelH

HIF-1α is the hypoxia-regulated subunit of the heterodimeric transcription factor, HIF-1, which is one of the primary regulators of cellular responses to hypoxia [275]. HIF-1 activation induced by hypoxia has previously been demonstrated in both differentiated and undifferentiated Caco-2 cells [628, 629]. HIF-1α is regulated post-translationally by

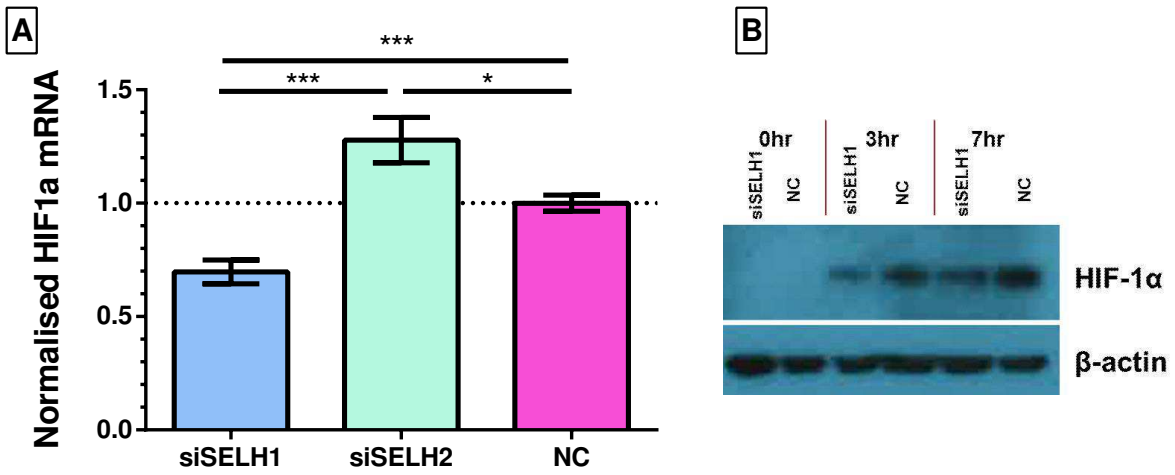

Figure 5.7. A: Effect of 72 h treatment with SelH-specific siRNAs, siSELH1 and siSELH2, or negative control siRNA on the mRNA expression of HIF-1α after treating undifferentiated Caco-2 cells with 7 h hypoxia. All mRNA data were normalised to the housekeeping genes TOP1 and EIF4A2. Data were expressed as a proportion of the mean of the Se-depleted cells. Data were analysed statistically using one-way ANOVA with a Tukey post-test for multiple comparisons. The data are displayed as mean ± standard error of the mean (SEM). * $P < 0.05$; *** $P < 0.001$.

hypoxia and thus the mRNA expression level of HIF-1α does not necessarily change when placed in hypoxia [630], although it has been previously demonstrated that HIF-1α mRNA expression can decrease in Caco-2 cells over long periods of hypoxia due to the action of miRNA-155 [631]. The present results are consistent with these findings as there was an induction of hypoxia targets as a result of hypoxia but there was no change in HIF-1α mRNA in either the differentiated or undifferentiated Caco-2 cells (Figure 5.1 and Figure 5.4, respectively).

Although no significant effect of Se-depletion on the mRNA expression of HIF-1α was detected within normoxic and hypoxic groups, a global effect Se-depletion on reducing HIF-1α mRNA expression was detected in both differentiated and undifferentiated Caco-2 cells. These data agree with previous studies that used physiological levels of Se and found Se-depletion to result in lower levels of HIF-1α protein [296, 298]. However, other studies that have treated cells with concentrations of Se greater than 5 μM, which are regarded as inducing apoptosis [632, 633], have found that Se has an inhibitory effect of HIF-1α mRNA and protein [293–295, 297]. Although HIF-1α protein levels were not measured in the present experiments on Se-depleted cells, HIF-1α protein was measured in response to knockdown of SelH expression using a SelH-specific siRNA (Figure 5.7). The results here suggest that a decrease in HIF-1α mRNA by 30 % resulted in an approximate 45 % reduction in HIF-1α protein. Thus, Se may be able to regulate the level of HIF-1α protein via changes in transcription or mRNA stability. In any future studies, the protein levels of HIF-1α should be measured in order to assess whether or not the decrease in mRNA

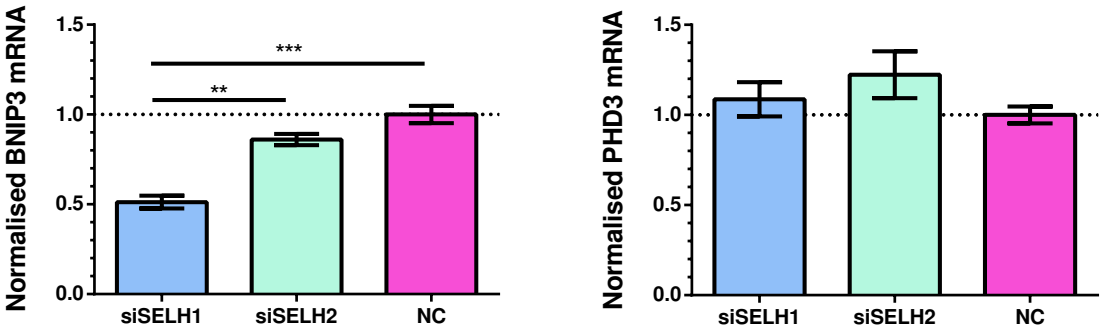

Figure 5.8. Effect of 72 h treatment with SelH-specific siRNAs, siSELH1 and siSELH2, or negative control siRNA on the mRNA expression of HIF-1 target genes, BNIP3 and PHD3, after treating undifferentiated Caco-2 cells with 7 h hypoxia. All mRNA data were normalised to the housekeeping genes TOP1 and EIF4A2. Data were expressed as a proportion of the mean of the Se-depleted cells. Data were analysed statistically using one-way ANOVA with a Tukey post-test for multiple comparisons. The data are displayed as mean ± standard error of the mean (SEM). ** $P < 0.01$; *** $P < 0.001$.

of HIF-1α results in a significantly decreased protein level of HIF-1α. However, it is not currently known to what extent the effect of SelH knockdown had on the transcriptional regulation of HIF-1α.

The mechanism by which Se modulates the expression level of HIF-1α is currently not well understood. One study has reported that high doses of Se, the in form of methylseleninic acid (MeSA), inhibited the expression and activity of HIF-1α and does not affect the expression of HIF-1β in prostate cancer cells [295]. HIF-1α has also been shown to be induced by the phosphoinositide 3-kinase (PI3K) pathway in a number of cell types [634–636]. The PI3K pathway has also been reported to be modulated by Se [637, 638]. Thus, this may be a potential mechanism by which Se can modulate responses to hypoxia. However, further study is required to determine how signal transduction of the PI3K pathway during hypoxia is modulated by Se. Additionally in this earlier study, treatment of prostate cancer cells with MeSA resulted in decreased DNA binding of HIF-1α and down-regulation of HIF-1 target genes. On the other hand, treatment with high doses of selenite was not reported to result in any significant changes in HRE transcriptional activity. In another study, treatment of a renal cancer cell line with high doses of Se in the form of methylselenocysteine resulted in inhibition of HIF-1α protein synthesis via a post-translational mechanism dependent upon the action of prolyl hydroxylase 2 (PHD2) [297].

In the present work, in order to assess whether Se-depletion had an effect of the expression of downstream HIF-1 targets, the expression levels of BNIP3 and PHD3 were measured. BNIP3 is a hypoxia-inducible gene which can be regulated at the transcriptional level by HIF-1α by the binding of it to a HRE in the promoter of BNIP3 [639, 640]. Inter-

estingly, the NF-κB subunit, p65, has previously been reported to bind to the BNIP3 promoter and repress its transcription [641]. The down-regulation of BNIP3 by NF-κB signalling is discussed in Chapter 6. PHD3, along with prolyl hydroxylase 1 (PHD1) and PHD2, functions as an oxygen-sensor which is able to hydroxylate HIF-1α and target it for pVHL-mediated proteasomal degradation [642]. compared with PHD1 and PHD2, PHD3 has comparatively better hydroxylating activity during hypoxia and is more highly induced by hypoxia [643–647]. In Caco-2 cells and IECs, PHD3 has also been reported to have a protective effect on barrier function by stabilising occludin [648]. The PHD3 promoter region has been found to contain a HRE to which HIF-1α can bind and induce expression of PHD3 in response to hypoxia [649]. PHD3 has also been reported to be a negative regulator of NF-κB transcriptional activity in a hydroxylase-dependent manner [650, 651]. The data presented in this chapter suggest that Se-depletion represses the mRNA expression of BNIP3 and PHD3 in both differentiated and undifferentiated Caco-2 cells (Figure 5.2 and Figure 5.5, respectively). This may be due to the down-regulation of HIF-1α mRNA by Se-depletion (Figure 5.1 and Figure 5.4), resulting in lower HIF-1α protein levels and thus lower transcription of hypoxia-responsive genes with a HRE in their promoter.

5.3.2 A potential role for SelH in the regulation of the responses to hypoxia

Treatment of Caco-2 cells with siSELH1 and siSELH2 had opposite effects on HIF-1α mRNA levels (Figure 5.7A), despite resulting in similar size knockdown of SelH mRNA (Figure 4.7). This inconsistent effect on SelH mRNA knockdown between the two siRNAs could be explained due to an off-target effect by one or both siRNA sequences. However, the probability of both distinct sequences observing an off-target effect that affected HIF-1α mRNA is unlikely. As SelH has previously been suggested to be able to bind directly to DNA [37], it may be possible that differences in the protein level of SelH, which were not assessed in the present work, may account for the differences in regulation of HIF-1α mRNA. Furthermore, if SelH has a modulatory role in the transcriptional regulation of HIF-1α, the relationship between SelH protein levels and transcriptional regulation may not exhibit a non-linear behaviour, an effect which has been described for some other transcription factors [652, 653].

The present data showed an effect of SelH knockdown using siSELH1, but not siSELH2, on the expression level of BNIP3. As siSELH1 treatment reduced both HIF-1α mRNA and protein, the effect of knockdown of SelH on BNIP3 may be due to decreased transcriptional activity of HIF-1α on the promoter of BNIP3. Furthermore, SelH has previously been suggested to have redox functions [37, 38] which can affect the ability of HIF-1α to bind to DNA and thus affect its ability to regulate the transcription of target genes [654]. However, there was no effect of SelH knockdown on the mRNA expression of PHD3 which may suggest that SelH modulation of hypoxia-associated targets may not be through altered

expression of HIF-1α. Instead, it may be possible that SelH is able to bind to a select group of HIF-1 targets using its AT-binding hook and regulate gene expression directly. On the other hand, it may also be possible that hypoxia-induced expression PHD3 reaches a maximum at low levels of HIF-1α and thus is less sensitive to changes in HIF-1α protein levels. This is supported by previous studies which have reported that PHD3 is highly responsive to hypoxia relative to the other prolyl hydroxylases [643–647].

Chapter 6

The effect of different gaseous environments on the modulatory effects of selenium of differentiated Caco-2 cell responses to flagellin

6.1 Introduction

The responses of IECs to microbial challenges have previously been reported to be altered by the pO_2 of the environment within which the cells are present [284]. Most studies, however, are performed in a normoxic environment containing 20 % oxygen or hypoxic environment containing 1 % oxygen.

The situation *in vivo* is quite different as the cells are exposed to a steep oxygen gradient - from a relatively normoxic (aerobic) basolateral side to an anoxic (anaerobic) apical side, but this is a technically challenging system to model *in vitro*. A novel cell culture chamber system has been developed, which allows the cells to be treated in a dualoxic environment where the apical membrane of cells is exposed to an anoxic environment and the basolateral membrane of cells are exposed to a normoxic environment (Figure 2.1), as described in Section 2.1.6.

Both *in vitro* and *in vivo* studies have indicated that hypoxia activates the NF-κB signalling pathway [655]. However, during inflammation, hypoxia signalling via HIF-1 was found to be able to repress NF-κB activity [331]. Thus, it has been suggested that there are a number of complex interactions between hypoxia and the inflammatory responses [656].

In particular, HIF-1α has been suggested to have a protective role in the defence against intestinal pathogens via the promotion of IEC barrier function and inhibiting IEC apoptosis [285, 656]. In a murine colitis model, knockout of HIF-1α in IECs was found to exacerbate symptoms and reduce barrier function of the intestinal epithelium [286]. In

contrast,, HIF-1α has been found to be highly expressed in IECs in patients with IBD [283], and the use of inhibitors to inhibit the degradation of HIF-1α reduced the mucosal damage caused by inflammation [285, 287]. Thus, a very complex cross-talk exists between cellular responses to hypoxia, HIF-1 signalling and NF-κB activation.

6.1.1 Research aims

The data reported in Chapter 3 and Chapter 5, have suggested that Se modulates the responses of Caco-2 cells to both flagellin and hypoxia when cells are grown under normoxic or hypoxic conditions. However *in vivo*, IECs form a barrier across a steep oxygen gradient in the gut and therefore it is important to explore the modulation of Caco-2 cell responses to Se when the cells are grown in a more physiological dualoxic environment. Experiments were performed to investigate the modulatory role of Se in the responses of Caco-2 cells to flagellin in differentiated cells grown under conditions where the apical surface was in anoxic conditions, and the basolateral surface was in normoxic gaseous conditions.

6.2 Results

6.2.1 Effect of a different gaseous environments and a novel dualoxic cell culture chamber on the modulatory effects of selenium on the induction of immune effectors by flagellin in differentiated Caco-2 cells

Differentiated Caco-2 cells were grown on Transwell semi-permeable inserts for 18 days, as described in Section 2.1.1. They were then depleted of Se (NoSe), or supplemented with Se (Se), for 72 h as described in Section 2.1.2.

Following the 72 h depletion (NoSe) or supplementation (Se) period, the cells were placed in either a normoxic, hypoxic or dualoxic environment for 6 h, followed by a 6 h challenge with or without flagellin, after which the mRNA expression level of the immune effectors IL-8, hBD2, TNF-α and hBD1 were measured Additionally, the protein level of secreted IL-8 was measured in spent culture medium.

After 6 h treatment with or without flagellin, the level of IL-8 mRNA expression was measured in differentiated Caco-2 cells under conditions of normoxia, hypoxia and dualoxia (Figure 6.1A). In a normoxic environment, flagellin treatment induced a 23-fold increase in IL-8 mRNA expression in the Se-supplemented cells ($P < 0.001$) and a 28-fold increase in the Se-depleted cells ($P < 0.001$). Additionally, the Se-depleted cells exhibited a 2.4-fold increased level of induced IL-8 mRNA compared with the Se-supplemented cells ($P < 0.01$).

In the Caco-2 cells treated in a hypoxic environment, flagellin treatment induced a 13-fold increase in IL-8 mRNA expression in the Se-supplemented cells ($P < 0.01$) and a

42-fold increase in the Se-depleted cells ($P < 0.01$). There was no statistically significant difference in induced IL-8 mRNA levels detected due to Se-depletion in the hypoxic cells.

In the Caco-2 cells treated in a dualoxic environment, flagellin treatment induced an increase in IL-8 mRNA expression of 14-fold in the Se-supplemented cells ($P < 0.001$) and of 8.9-fold in the Se-depleted cells ($P < 0.001$). There were no statistically significant differences in induced IL-8 mRNA levels detected due to Se-depletion in the dualoxic cells. The Se-depleted cells treated with flagellin in dualoxia had reduced expression of IL-8 when compared with the Se-depleted cells treated with flagellin in hypoxia ($P < 0.01$).

Secreted IL-8 protein induced by flagellin was also measured from differentiated Caco-2 cells under conditions of normoxia, hypoxia and dualoxia (Figure 6.1B). In the Caco-2 cells treated in a normoxic environment, flagellin treatment induced an increase in secreted IL-8 protein of 97-fold in the Se-supplemented cells ($P < 0.001$) and of 79-fold in the Se-depleted cells ($P < 0.001$). Additionally, the Se-depleted cells exhibited a 0.14-fold increased level of induced IL-8 protein compared with the Se-supplemented cells ($P < 0.05$).

In the Caco-2 cells treated in a hypoxic environment, flagellin treatment induced an increase in secreted IL-8 protein of 89-fold in the Se-supplemented cells ($P < 0.001$) and of 13-fold in the Se-depleted cells ($P < 0.001$). There was no change in induced IL-8 protein detected due to Se-depletion.

In the Caco-2 cells treated in a dualoxic environment, flagellin treatment induced an increase in secreted IL-8 protein of 21-fold in the Se-supplemented cells ($P < 0.01$) and of 21-fold in the Se-depleted cells ($P < 0.05$). There was no change in induced IL-8 protein levels detected due to Se-depletion. The dualoxic environment resulted in decreased secreted IL-8 protein for both the Se-supplemented and Se-depleted cells compared with the cells treated in normoxia ($P < 0.05$ and $P < 0.001$, respectively). When compared with Se-depleted cells treated in hypoxia, the dualoxic environment Se-depleted cells exhibited decreased secreted IL-8 protein levels ($P < 0.001$). Additionally, the normoxic Se-supplemented cells exhibited an increased induced IL-8 protein level compared with the challenged Se-supplemented cells in hypoxia ($P < 0.05$).

After 6 h treatment with or without flagellin, the level of hBD2 mRNA expression was measured in the differentiated Caco-2 cells under conditions of normoxia, hypoxia and dualoxia (Figure 6.2). In the Caco-2 cells treated in a normoxic environment, flagellin treatment induced a increase in hBD2 mRNA expression of 8.4-fold in the Se-supplemented cells ($P < 0.001$) and of 4.6-fold in the Se-depleted cells ($P < 0.001$). The Se-depleted cells showed a similar level of induced hBD2 mRNA compared with the Se-supplemented cells ($P = 0.21$).

In the hypoxic environment, flagellin treatment induced a increase in hBD2 mRNA expres-

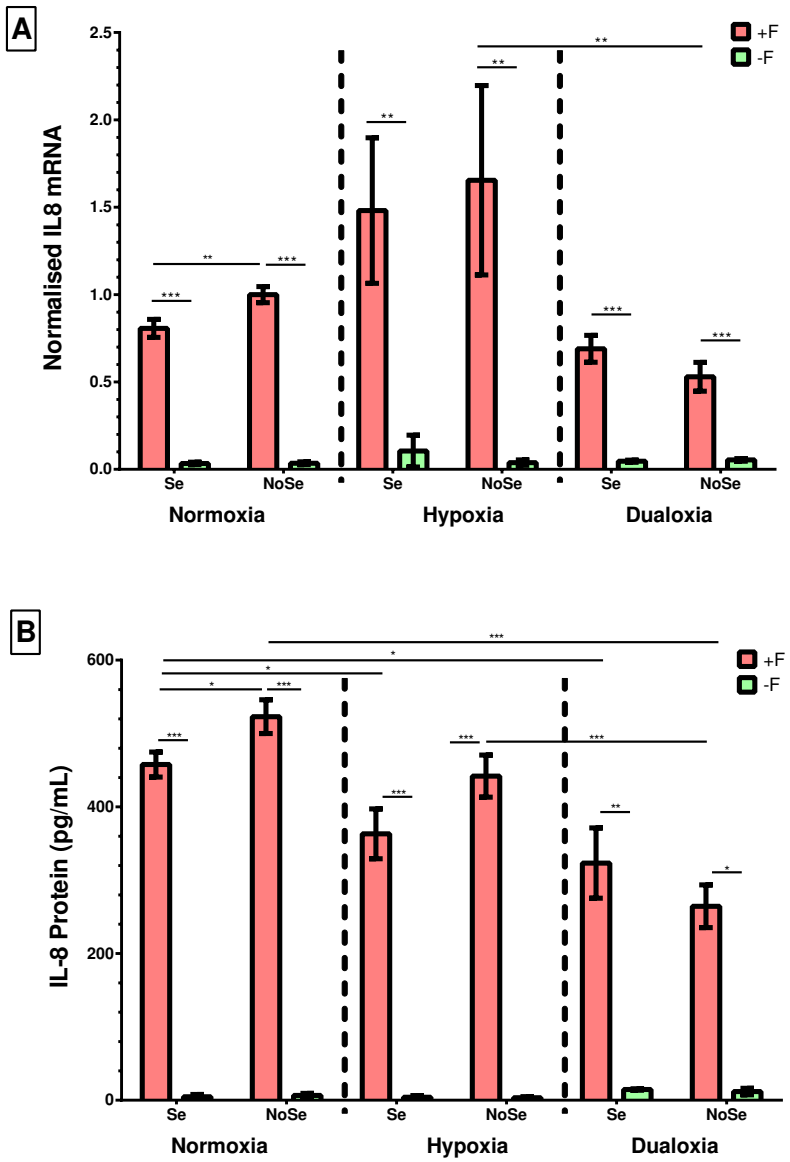

Figure 6.1. Effect of 72 h Se supplementation (Se) or selenium depletion (NoSe) on the mRNA (A) and protein (B) expression of IL-8 in response to 6 h flagellin (+F) treatment differentiated Caco-2 cells. All mRNA data were normalised the housekeeping genes TOP1 and EIF4A2. Data were expressed as a proportion of the mean of the NoSe flagellin treated cells in order to combine 2 distinct biological replicates, each consisting of 3 technical replicates (n = 6) for cells treated with normoxia and hypoxia. Caco-2 cells under dualoxia consisted of 1 biological replicate with 5 technical replicates (n = 5). Data were analysed statistically using two-way ANOVA with a Tukey post-test for multiple comparisons. The data are displayed as mean ± standard error of the mean (SEM). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

sion of 13-fold in the Se-supplemented cells ($P < 0.001$) and of 14-fold in the Se-depleted cells ($P < 0.001$). The Se-depleted cells were found to have a similar level of induced hBD2 mRNA compared with the Se-supplemented cells.

In the dualoxic environment, flagellin treatment did not induce an increase in hBD2 mRNA expression. The normoxic Se-supplemented and Se-depleted Caco-2 cells chal-

lenged with flagellin exhibited increased hBD2 mRNA when compared with their counterparts in the dualoxic environment ($P < 0.001$). Additionally, the hypoxic Se-supplemented and Se-depleted Caco-2 cells challenged with flagellin exhibited increased hBD2 mRNA when compared with their counterparts in the dualoxic environment ($P < 0.001$ and $P < 0.05$, respectively).

Figure 6.2 the level of TNF-α mRNA expression in differentiated Caco-2 cells under conditions of normoxia, hypoxia and dualoxia. In a normoxic environment, flagellin treatment induced a increase in TNF-α mRNA expression of 9.2-fold in the Se-supplemented cells ($P < 0.001$) and of 8.9-fold in the Se-depleted cells ($P < 0.001$). The Se-depleted cells showed no significant increase in level of induced TNF-α mRNA compared with the Se-supplemented cells. In a hypoxic environment, flagellin treatment induced a increase in TNF-α mRNA expression of 11.2-fold in the Se-supplemented cells ($P < 0.001$) and of 9.2-fold in the Se-depleted cells ($P < 0.001$). The Se-depleted cells showed no significant increase in level of induced TNF-α mRNA compared with the Se-supplemented cells. In a dualoxic environment, flagellin treatment induced an increase in TNF-α mRNA expression of 1.9-fold in the Se-supplemented cells ($P < 0.001$) and of 1.7-fold in the Se-depleted cells ($P < 0.001$). The Se-depleted cells treated in dualoxia exhibited a greater level of induced TNF-α mRNA expression compared with Se-supplemented cells ($P < 0.05$). The normoxic Se-supplemented Caco-2 cells challenged with flagellin exhibited increased TNF-α mRNA when compared with their counterparts in the hypoxic environment ($P < 0.01$). Additionally, the normoxic Se-supplemented and Se-depleted Caco-2 cells challenged with flagellin exhibited increased TNF-α mRNA when compared with their counterparts in the dualoxic environment ($P < 0.01$ and $P < 0.001$, respectively). Furthermore, the hypoxic Se-supplemented and Se-depleted Caco-2 cells challenged with flagellin exhibited increased TNF-α mRNA when compared with their counterparts in the dualoxic environment ($P < 0.001$).

In a normoxic environment, flagellin treatment induced a increase in hBD1 mRNA expression of 0.41-fold in the Se-supplemented cells ($P < 0.01$) and of 0.56-fold in the Se-depleted cells (Figure 6.2; $P < 0.001$). The Se-depleted cells showed no significant increase in level of induced hBD1 mRNA compared with the Se-supplemented cells. In the hypoxic environment, flagellin treatment induced a increase in hBD1 mRNA expression of 2.3-fold in the Se-supplemented cells ($P < 0.001$) and of 1.9-fold in the Se-depleted cells ($P < 0.01$). The Se-depleted cells showed no significant increase in level of induced hBD1 mRNA compared with the Se-supplemented cells. In the Caco-2 cells treated in a dualoxic environment, flagellin treatment induced a increase in hBD1 mRNA expression of 0.72-fold in the Se-supplemented cells ($P < 0.05$) and of 0.51-fold in the Se-depleted cells ($P < 0.05$). The Se-depleted cells showed no significant increase in level of induced hBD1 mRNA compared with the Se-supplemented cells. The normoxic Se-supplemented Caco-2 cells that were not challenged with flagellin exhibited an increased level of hBD1 mRNA compared with the cells in hypoxia ($P < 0.05$).

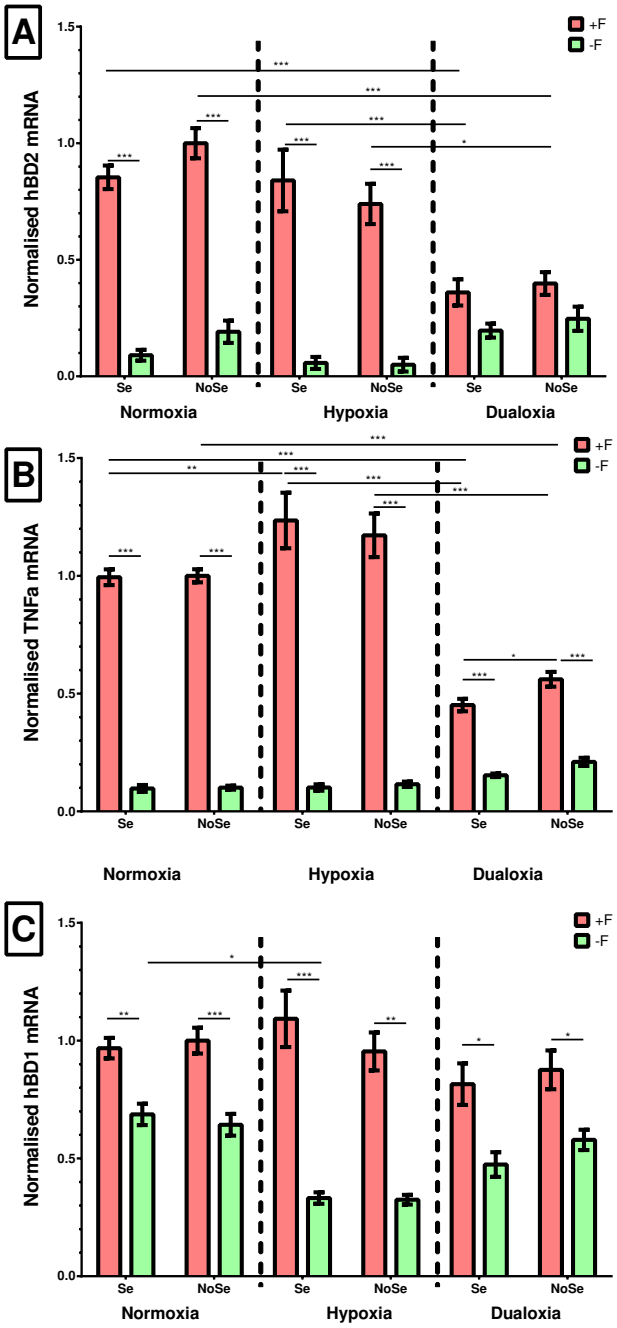

Figure 6.2. Effects of 72 h Se supplementation (Se) or selenium depletion (NoSe) on the mRNA expression of hBD2 (A), TNF-α (B) and hBD1 (C) mRNA in response to challenge with or without flagellin (+F or -F, respectively) for 6 h in differentiated Caco-2 cells. All mRNA data were normalised the housekeeping genes TOP1 and EIF4A2. Data were expressed as a proportion of the mean of the NoSe flagellin treated cells in order to combine 2 distinct biological replicates, each consisting of 3 technical replicates (n = 6) for cells treated with normoxia and hypoxia. Caco-2 cells under dualoxia consisted of 1 biological replicate with 5 technical replicates (n = 5). Data were analysed statistically using two-way ANOVA with a Tukey post-test for multiple comparisons. The data are displayed as mean ± standard error of the mean (SEM). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

6.2.2 Effect of a different gaseous environments and a novel dualoxic cell culture chamber on the modulatory effects of selenium on the induction of selenoproteins by flagellin in differentiated Caco-2 cells

After 6 h treatment with or without flagellin, SelH, TR1 and SePP1 mRNA expression were also measured in differentiated Caco-2 cells following normoxia, hypoxia and dualoxia (Figure 6.3). In the normoxic environment, flagellin treatment resulted in a 21 % decrease in SelH mRNA expression in the Se-supplemented cells. The normoxic Se-depleted cells treated with and without flagellin resulted in decreased SelH mRNA expression compared with Se-supplemented cells ($P < 0.001$). In the hypoxic environment, flagellin treatment had no effect on the mRNA expression of SelH, but Se-depletion cells resulted in decreased SelH mRNA expression in both the flagellin-challenged and unchallenged cells ($P < 0.001$). In the Caco-2 cells treated in a dualoxic environment, neither Se nor flagellin treatments affected the mRNA expression of SelH. The normoxic Se-supplemented cells that were treated with flagellin exhibited an increased SelH mRNA expression level when compared with their dualoxic counterparts ($P < 0.001$). The normoxic Se-supplemented cells that were not treated with flagellin exhibited an increased SelH mRNA expression level when compared with their counterparts in hypoxia and dualoxia ($P < 0.01$ and $P < 0.001$, respectively). The normoxic Se-depleted cells that were not treated with flagellin exhibited an increased SelH mRNA expression level when compared with their counterparts dualoxia ($P < 0.05$). The normoxic Se-supplemented cells, both treated and untreated, exhibited an increased SelH mRNA expression when compared with their dualoxic counterparts ($P < 0.05$ and $P < 0.001$, respectively).

As shown in Figure 6.3B, treatment of Caco-2 cells with flagellin in a normoxic environment resulted in an increase in TR1 mRNA expression of 0.84-fold in the Se-supplemented cells ($P < 0.001$). The normoxic Se-depleted cells treated with flagellin resulted in decreased TR1 mRNA expression compared with its Se-supplemented counterpart ($P < 0.05$). On the contrary, the normoxic Se-depleted cells that were not treated with flagellin exhibited increased TR1 mRNA when compared with the Se-supplemented cells ($P < 0.05$). In the hypoxic environment, flagellin treatment resulted an increase in TR1 mRNA expression of 0.74-fold in the Se-supplemented cells ($P < 0.001$) and of 0.9-fold in the Se-depleted cells. The hypoxic cells exhibited no change in TR1 mRNA due to Se-depletion. In the dualoxic environment, there were no effects detected due to treatment with flagellin. However, Se-depletion resulted in decreased TR1 mRNA in the cells treated with flagellin ($P < 0.05$). The normoxic Se-supplemented cells that were treated with flagellin exhibited an increased TR1 mRNA expression level when compared with their counterparts in a dualoxic environment ($P < 0.001$). The normoxic Se-supplemented cells, treated with and without flagellin, exhibited increased TR1 mRNA expression when compared with their counterparts in dualoxia ($P < 0.001$ and $P < 0.01$, respectively). The hypoxic Se-supplemented cells that were treated with flagellin exhibited increased TR1

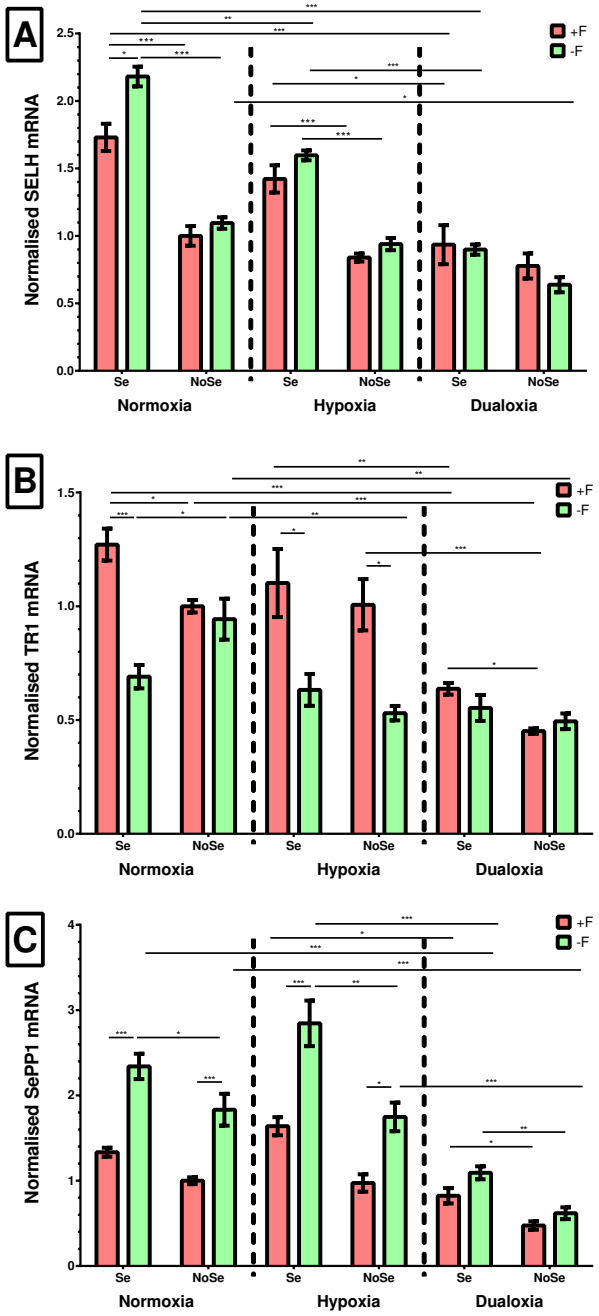

Figure 6.3. Effects of 72 h Se supplementation (Se) or selenium depletion (NoSe) on the mRNA expression of hBD2 (A), TNF-α (B) and hBD1 (C) mRNA in response to challenge with or without flagellin (+F or -F, respectively) for 6 h in differentiated Caco-2 cells. All mRNA data were normalised the housekeeping genes TOP1 and EIF4A2. Data were expressed as a proportion of the mean of the NoSe flagellin treated cells in order to combine 2 distinct biological replicates, each consisting of 3 technical replicates (n = 6) for cells treated with normoxia and hypoxia. Caco-2 cells under dualoxia consisted of 1 biological replicate with 5 technical replicates (n = 5). Data were analysed statistically using two-way ANOVA with a Tukey post-test for multiple comparisons. The data are displayed as mean ± standard error of the mean (SEM). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

mRNA expression compared with their counterparts in dualoxia ($P < 0.01$). Additionally, the hypoxic Se-depleted cells that were treated with flagellin exhibited an increased TR1 mRNA expression level when compared with their counterparts in dualoxia ($P < 0.01$).

After 6 h treatment with or without flagellin, SePP1 mRNA expression was measured in differentiated Caco-2 cells under conditions of normoxia, hypoxia and dualoxia (Figure 6.3C). In the normoxic environment, flagellin treatment resulted a 43 % decrease in SePP1 mRNA expression in the Se-supplemented cells ($P < 0.001$) and 45 % in the Se-depleted cells ($P < 0.001$). The normoxic Se-depleted cells treated without flagellin resulted in decreased SePP1 mRNA expression compared with its Se-supplemented counterpart ($P < 0.05$). In the hypoxic environment, flagellin treatment resulted a 42 % decrease in SePP1 mRNA expression in the Se-supplemented cells ($P < 0.001$) and 44 % in the Se-depleted cells ($P < 0.05$). The hypoxic Se-depleted cells in absence of flagellin resulted in decreased SePP1 mRNA expression compared with its Se-supplemented counterpart ($P < 0.01$). In the dualoxic environment, there were no effects detected due to treatment with flagellin. However, the Se-depleted cells, both with and without flagellin, exhibited decreased SePP1 mRNA compared with the Se-supplemented counterparts ($P < 0.05$ and $P < 0.01$, respectively). The normoxic Se-supplemented and Se-depleted cells that were not treated with flagellin exhibited increased SePP1 mRNA expression when compared with their counterparts in dualoxia ($P < 0.001$). The hypoxic Se-supplemented cells, treated with flagellin, exhibited an increased SePP1 mRNA expression level when compared with their counterparts in dualoxia ($P < 0.05$). The hypoxic Se-supplemented and Se-depleted cells treated without flagellin, exhibited an increased SePP1 mRNA expression level when compared with their counterparts in dualoxia ($P < 0.001$).

6.3 Discussion

The data presented in previous chapters suggest that Se-depletion has modulatory effects in Caco-2 cell responses to flagellin, oxidative stress and hypoxia. As discussed previously, these pathways have been reported to be highly inter-dependent and cross-regulate each other. Thus, it is important to determine how Se modulates the Caco-2 cell responses to flagellin in traditional cell culture environments, compared with a more physiologically relevant, dualoxic environment. The data presented in this chapter suggest that a Caco-2 cells treated in a physiologically-relevant dualoxic environment (anoxic apical membrane surface conditions and normoxic basolateral membrane conditions) respond differently to flagellin compared with their responses in normoxic and hypoxic environments. Additionally, selenoprotein mRNA changed when placed in a dualoxic environment as well as selenoprotein expression levels in response to flagellin may change in different gaseous environments.

However, the results described in this chapter need to be interpreted and compared with the results from Chapter 3 with caution as working with the dualoxic environment required a fundamental change to the experimental protocol when adding flagellin to the media. In Chapter 3, the media was changed once after 72 h Se supplementation or depletion to add the media with or without flagellin. In contast, the use of the dualoxic environment required cells to be placed in dualoxia for 4 h prior to flagellin treatment and as replenishment of the basolateral media was not possible in the dualoxic environment due to technical constraints, flagellin was added directly to the basolateral, and apical media after 4 h dualoxia. In order for the experiments within the present chapter to be comparable, the experiments performed in normoxia and hypoxia followed the same media replenishment and flagellin treatment protocols as were performed in dualoxia. A previous study in retinal pigment epithelial cells reported that changing the media can induce gene expression of a number of genes including HMOX1, JunB and FosB, all of which can modulate the NF-κB pathway [657]. The results from the present chapter should be compared tentatively with results from Chapter 3 as there is evidence to suggest that the change in protocol may change the responses of Caco-2 cells to Se and flagellin challenge. Additionally, caution must be observed as the dualoxic data consists of only a single biological replicate, thus more biological replicates are needed to increase the validity of results.

In Chapter 3, IL-8 mRNA expression and secreted protein were increased in response to treatment with flagellin under conditions of normoxia, hypoxia and dualoxia (Figure 6.1). However, in the analyses reported in this chapter, the level of flagellin-induced IL-8 mRNA was up-regulated in response to Se-depletion in the normoxic environment. This anomaly may be due a difference in the methodology, as discussed above, which may have induced gene expression of a number of genes including HMOX1, JunB and FosB, all of which can have modulatory effects on the NF-κB pathway [657]. The mRNA levels of IL-8

were unchanged between hypoxic and normoxic cells. However, IL-8 mRNA expression and secreted protein levels of IL-8 were decreased in the Se-depleted cells treated with flagellin, compared with relevant cells in hypoxia. Additionally, IL-8 secreted protein concentrations were decreased from normoxia, to hypoxia and then on to dualoxia (Figure 6.1B). This agrees with findings from a previous study showing that during inflammation, hypoxia signalling via HIF-1 was found to be able to repress NF-κB activity [331].

hBD2, TNF-α and hBD1 all showed similar responses to Se-depletion after Se-depletion (Figure 6.2). However, TNF-α exhibited an increase in flagellin-induced mRNA expression in the dualoxic environment, but not the normoxic and hypoxic environments. hBD2 expression in the flagellin-challenged was significantly diminished compared with counterparts in normoxia and hypoxia. Additionally, there was no induced hBD2 expression in dualoxia. These observations may be due to the altered TLR5 signalling resulting from the steep oxygen-gradient across the epithelium. Although hBD2 expression has never been measured in a dualoxic environment, hypoxia was found to induce hBD2 expression in human macrophage cells [658]. In contrast, the data from present work showed no effect of hypoxia on the expression of hBD2 compared with experiments performed in normoxia.

Interestingly, hBD1 expression remained relatively constant for all treatment groups between the different gaseous environments (Figure 6.2C). However, there was a significant decrease in expression in the hypoxic unchallenged Se-supplemented cells compared with the normoxic counterparts, as well as a similar trend observed towards a decrease in the Se-depleted cells. Both TNF-α and hBD2 expression were diminished in the dualoxic environment compared with the normoxic and hypoxic environments. However, as hBD1 expression was unaffected by dualoxia, this indicated that hBD2 and TNF-α may have a common regulatory pathway by which dualoxia diminishes their expression. As hBD1 is constitutively expressed and is not as readily as inducible as hBD2 by PAMPs via the NF-κB pathway [114]. This may suggest that the NF-κB pathway may be mediating the diminished expression of TNF-α and hBD2 in the dualoxic environment.

Interestingly, in the dualoxic environment Se-depletion had no effect on SelH mRNA expression, which has previously been reported to be very responsive to Se status [28–31]. Hypoxia has been suggested to have modulatory effects on selenoprotein biosynthesis machinery [659]. However, the effects of hypoxia on SelH expression in response to Se-depletion was similar to that in normoxia. Thus, the steep oxygen gradient that exists in the dualoxic environment may have a greater effect on selenoprotein biosynthesis than the hypoxic environment. Furthermore, the changes of TR1 and SePP1 observed in normoxia are different than in dualoxia. However, the dualoxia experiment needs to be repeated as a single biological replicate is insufficient for reliable conclusions.

6.3.1 Summary

The data presented in this chapter suggest that a dualoxic environment reduces the expression of the immune mediators, IL-8, hBD2 and TNF-α and that the modulatory effects of Se of IL-8 and hBD2 in particular, are diminished in a dualoxic environment. Furthermore, a dualoxic environment down-regulated expression of the selenoprotein mRNA for SelH and TR1 and Se-depletion had no effect on the expression of SelH in dualoxia. However, as the data obtained from dualoxia in this chapter are from only one biological replicate, the experiment needs to be repeated to increase the reliability of the conclusions drawn in this chapter.

Chapter 7

Final Discussion

The data presented in this thesis suggest a model for how Se status modulates the pro-inflammatory response of undifferentiated Caco-2 cells to flagellin, a PAMP involved in gut infection and inflammation (Figure 7.1). This model integrates Se modulation of oxidative stress-associated genes (Chapter 4) and hypoxia signalling (Chapter 5), and directs a putative model by which Se modulates the response of undifferentiated Caco-2 cells to flagellin (Chapter 3). Each chapter is discussed separately below, followed by a discussion of how they are linked. Overall, however, the data presented in this thesis has shown that the effects of Se-depletion in differentiated Caco-2 cells, as well as in the physiological dualoxic environment, suggest that the effects of Se on cell stress pathways are reduced in more physiologically-relevant IEC models. These data therefore support further investigation on how Se status affects the responses of differentiated Caco-2 to cellular stress.

7.1 Se and pro-inflammatory responses to flagellin

The anti-inflammatory properties of Se have been previously well documented [122, 123], and low Se status has also been indicated to play a role in the pathogenesis of IBD, which is a condition characterised by exaggerated immune responses to the microbiota [141–143]. However, the role of Se in the responses of *in vitro* models of IECs to microbial challenge has received less attention. One study reported the effects of Se on the responses of undifferentiated Caco-2 cells to microbial challenge [31], but there are no data in differentiated Caco-2 cells, which are considered a more realistic model of IECs [339, 342, 343]. The work presented in Chapter 3 reported that Se-depletion resulted in a higher expression of the pro-inflammatory mediators, IL-8, hBD2, TNF-α and hBD1, in undifferentiated Caco-2 cells (Figure 3.4), suggesting that Se, functioning through the selenoprotein, TR1, modulates the expression of pro-inflammatory mediators.

Previous studies have reported that Se can reduce the activity of the TLR5 adaptor proteins, MyD88 and TRIF, and also inhibit the binding of NF-κB to the DNA of target genes [193, 194], but this has not been verified in Caco-2 cells. In this research, knockdown of

the selenoprotein TR1 was found to reduce to expression of pro-inflammatory mediators in flagellin-challenged Caco-2 cells (Figure 3.8). These data imply that increased expression of TR1 is associated with increased expression of pro-inflammatory mediators, which agrees with data from undifferentiated Caco-2 cells that shows that TR1 expression is elevated following Se-depletion (Figures 3.6 and 4.6). Hence, work presented in this thesis provides a putative mechanism with TR1 involved in mediating the pro-inflammatory effects of low Se status, possibly through the TXN system.

7.2 Se and oxidative stress

Oxidative stress in the gut has been associated with the pathology of IBD, as well as the pathogenesis of cancers, such as colorectal cancer [208, 660]. Se has been very well characterised due to its antioxidant properties [2], which may explain the association between low Se status and increased risk of cancer, including colorectal cancer [19].

The results presented in Chapter 4 showed that low Se status resulted in increased expression of genes associated with oxidative stress, which is in agreement with previous studies [82, 261, 262]. As the genes affected by Se-depletion in Figure 4.4 all contained an ARE, this indicated that the Nrf2 transcription factor is involved in mediating the induction of these genes, which agrees with previous studies that link low Se status with increased Nrf2 activity [82, 261, 262]. Interestingly, the expression of HMOX1, which is well characterised as a Nrf2 downstream effector [661], was unaffected (Figure 4.4). This indicated that HMOX1 is either regulated independently of Nrf2 in Se-depleted Caco-2 cells or that alternate mechanisms of regulation, such the AP-1 pathway, are involved. In fact, the increased expression of cJUN, a member of the AP-1 family, in response to Se-depletion (Figures 4.2 and 4.5), also supported the potential involvement of AP-1 in the regulation of oxidative stress-associated genes in response to Se-depletion.

Knockdown of SelH and TR1 expression in undifferentiated Caco-2 cells modulated the expression of the oxidative stress-associated targets, NQO1 and HMOX1 (Figures 4.8 and 4.11), but this was inconsistent with data from Se-depletion in undifferentiated Caco-2 cells as HMOX1 was unaffected by Se status (Figure 4.4). However, knockdown data for both SelH and TR1 were consistent with previous studies that have reported the regulation of oxidative stress-associated genes by these two selenoproteins [53, 82, 88, 89]. As Se-depletion impacts on the expression of all 25 selenoproteins according to their place in the selenoprotein hierarchy, and as many have antioxidant functions [2], it is likely that the effects of Se-depletion on the expression of oxidative stress-associated genes is a consequence of the altered expression of a number of different selenoproteins. Thus, it is not surprising that the knockdown of the individual selenoproteins, SelH and TR1, did not mirror the effects of Se-depletion. These data do, however, support the hypothesis proposed in present work that altered SelH and TR1 expression contributes to the effects of Se-depletion on the expression of oxidative stress-associated genes.

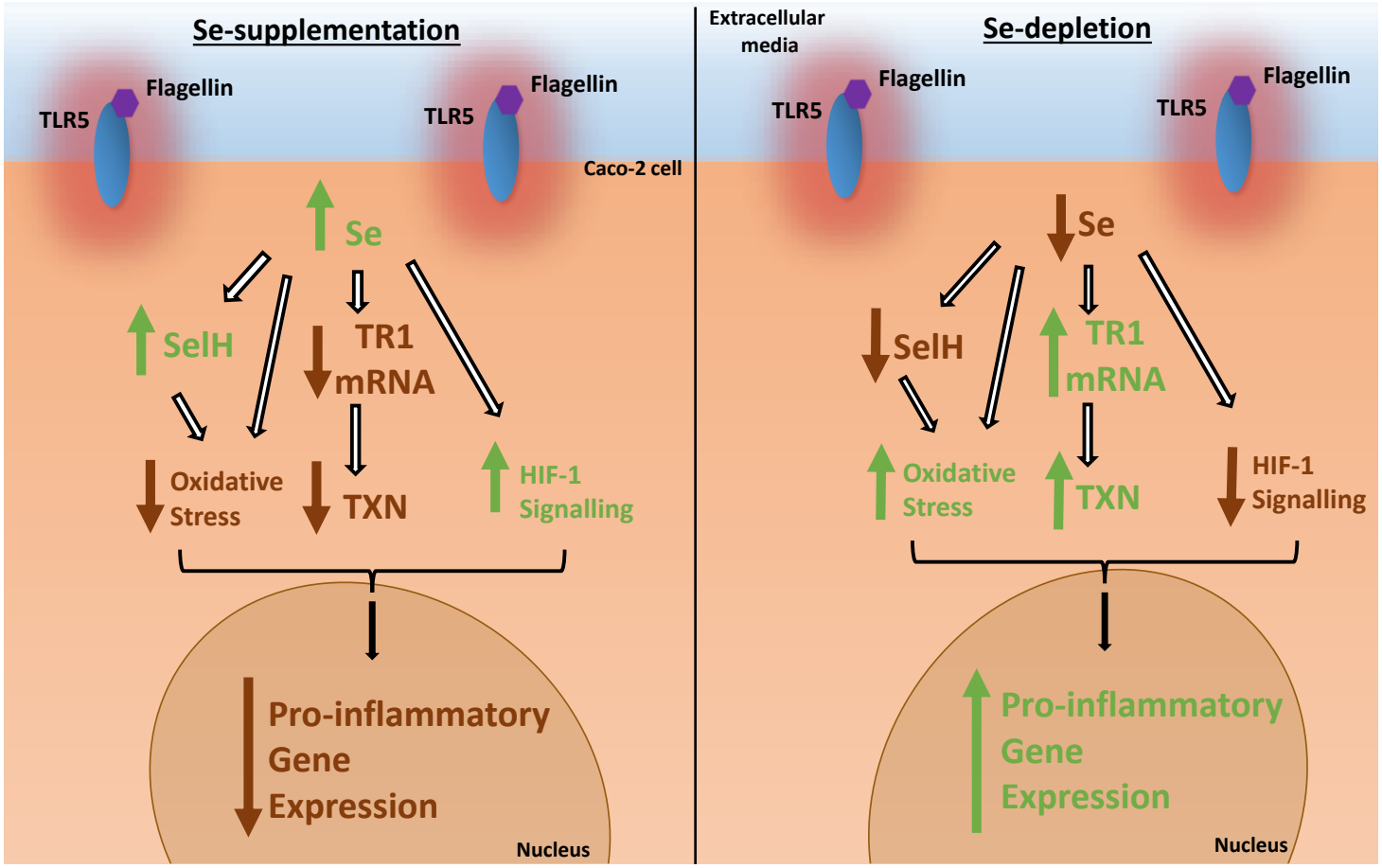

Figure 7.1. Schematic overview of the mechanism of action of Se-depletion on pro-inflammatory gene expression in a flagellin-challenged cell. Low Se status results in a decrease in SelH expression and an increase in ROS associated with oxidative stress, leading to an amplification of pro-inflammatory gene expression through increased NF-κB activity. Additionally, low Se increased expression of TR1 and subsequently and increase in TXN activity, which is associated with increased NF-κB transcriptional activity of pro-inflammatory genes. Finally, low Se is associated with a decrease in HIF-1 signalling, which is associated with an increase in the activity of NF-κB and thus, increased pro-inflammatory gene expression.

7.3 Se and responses to hypoxia

The gut has a constant hypoxia-associated low-level of inflammation [270]. However, conditions in which there is excessive and uncontrolled inflammation are characteristic of IBDs such as Crohn's Disease and Ulcerative Colitis [271], thus making the transcription factor associated with hypoxia, HIF-1, a potential therapeutic target in IBD [662].

The literature suggests the existence of a complex level of cross-regulation between selenium, selenoprotein biosynthesis and responses to hypoxia. The data presented in Chapter 5 agreed with previous studies that also used physiological levels of Se-supplementation, and which reported raised HIF-1α expression following supplementation [296, 298]. Furthermore, this regulation of HIF-1 signalling by Se was supported by Se-depletion being followed by decreased expression of the downstream effectors, BNIP3 and PHD3, in both differentiated and undifferentiated Caco-2 cells. This indicated a down-regulation of HIF-1 transcriptional activity.

Hypoxia has been shown to have a modulatory effect on members of the selenoprotein biosynthesis machinery, thereby regulating the expression of different selenoproteins [301]. However, the work reported in this thesis indicated that hypoxia had little effect on the expression on selenoproteins in both the differentiated and undifferentiated Caco-2 cells (Figures 5.3 and 5.6, respectively). Interestingly, Se-depletion had similar effects in both the differentiated and undifferentiated Caco-2 cells on the response of downstream effector expression to hypoxia. Further work is needed to confirm that protein synthesis of the HIF-1 downstream effectors actually reflects changes in mRNA for both cell types. Given the previously discussed links between HIF-1 signalling and IBD, the effects of Se on HIF-1 pathway activity in the present work shows promise as a potential route of investigating Se as a therapeutic target in IBD.

Previous work in the laboratory has explored potential roles for SelH in Caco-2 cells. Microarray analysis following knockdown of SelH using SelH-specific siRNA in the Caco-2 gut epithelial cell model suggested its involvement in the hypoxia signalling pathway (Gautrey H, unpublished data). Based on these data, SelH was selected and experiments performed to elucidate if SelH mediated the modulation of HIF-1 signalling by Se. Knockdown of SelH expression using two independent siRNAs was followed by modulation of HIF-1α mRNA expression and protein synthesis (Figure 5.7). However, the data were conflicting as each siRNA regulated the mRNA differently. The reason for these contradictory data is not clear, however, it may have been due to the differences in the size of the knockdown of mRNA achieved by each siRNA (Figure 4.7). Hence, it would be important in future work to perform Western blotting to assess the protein abundance of SelH after transfection with each siRNA. As SelH has been suggested to bind to DNA, possibly in association with transcription factors [37], the level of SelH protein might be an important determinant of the direction of regulation of target genes. Overall, further work is needed to characterise the actual mechanisms by which SelH regulates the transcription of HIF-1

target genes.

7.4 Proposed mechanism of Se modulation of pro-inflammatory gene expression in Caco-2 cells

Figure 7.1 shows a schematic overview of the proposed mechanism by which Se helps modulate the expression of pro-inflammatory mediators. As discussed in Section 1.5, oxidative stress, hypoxia and immune signalling have been reported to cross-regulate the activities of one another, and each have been reported in the present work to be affected by Se-depletion and hence, regulated by Se.

Firstly, Se has been shown to directly affect the signal transduction of NF-κB signalling via the inhibition of MyD88 and TRIF signalling [193, 194], as well as Se supplementation at pharmacological doses inhibiting NF-κB binding to DNA [195–197]. Furthermore, the increase in expression of pro-inflammatory mediators following Se-depletion observed in the present work (Figure 3.4) agrees with data in a previous study using undifferentiated Caco-2 cells that reported that Se-depletion increased NF-κB transcriptional activity and ROS levels [31]. Although there is evidence that suggests Se directly modulates the transcription factor activity of NF-κB, the cross-talk observed between oxidative stress and NF-κB signalling may offer an additional level of pro-inflammatory mediator regulation by Se-depletion.

An indirect mechanism by which Se-depletion exacerbated the pro-inflammatory response to flagellin was by increasing ROS. As shown in Figure 7.1, a decrease in Se status was followed by an increase in the expression of oxidative stress-associated genes in unpolarised Caco-2 cells (Figure 4.4). This followed an increase in the levels of intracellular ROS, which was reported in a previous study investigating Se-depletion in undifferentiated Caco-2 cells [31]. Additionally, a decrease in Se was followed by a decreased expression of SelH (Figure 4.6). SelH has previously been suggested to have a protective effect on cells and to be involved in antioxidant defence [49–52]. Thus, decreased SelH expression may impair the ability of cells to reduce ROS levels during oxidative stress. As discussed in Chapter 4, oxidative stress and associated pathways can amplify pro-inflammatory gene expression through mechanisms which are not fully understood, but may involve the Nrf2 and AP-1 pathways [177–180, 312, 313]. Thus, as a consequence, the combination of oxidative stress and decreased SelH expression may have contributed to the increased pro-inflammatory gene expression in flagellin-challenged Caco-2 cells observed in the present work.

Se-depletion also resulted in increased expression of TR1 (Figure 4.6), previously reported to increase the activity of TXN. Additionally, decreased expression of TR1 by knockdown was followed by the reduced expression of pro-inflammatory mediators in response to flagellin challenge (Figure 3.8). Interestingly, the majority of studies in rodent and human *in vivo* and *in vitro* models report decreases in thioredoxin reductase activity accompanied by decreased TR1 mRNA due to Se-depletion, although the increase in mRNA tend to be

small or has no change [229, 469–475]. However, there is one *in vivo* study in chickens that reported a decrease in thioredoxin activity due to Se-deficiency but an increase in TR1 mRNA. Furthermore, the down-regulation of TR1 by siRNA or inhibitor, has previously been reported to not change thioredoxin redox status [476–478]. Increased TXN activity has been reported to activate the NF-κB pathway via redox control of MAPKs [77]. Thus, TR1 may be a potential therapeutic target in IBD as it is the primary regulator of TXN, which has been reported to be involved in experimental models of IBD [663].

Finally, Se-depletion down-regulated the expression of downstream effectors of HIF-1α signalling in both differentiated and undifferentiated Caco-2 cells (Figures 5.2 and 5.5), which may be explained by a decrease in HIF-1α transcription factor activity. Previous studies have reported that HIF-1α activity inhibits the NF-κB pathway [664], thus it is possible that the inhibition of pro-inflammatory gene expression by Se-depletion may be mediated by decreased HIF-1 signalling.

7.5 The effects of Se in more physiological models of intestinal epithelial cells

The data obtained from undifferentiated Caco-2 cells in the present work indicated that Se is a modulator of the responses to flagellin, oxidative stress and hypoxia. However, when the same experiments were performed in differentiated Caco-2 cells, which are considered to be a more physiologically relevant model [339, 342, 343], there were no observed effects of Se on responses to flagellin and expression of genes associated with oxidative stress. The model of Se-depletion that was used in the experiments performed in the present work was developed in rat hepatoma cells [665] and further validated undifferentiated Caco-2 cells [376]. At the time of submission, this model of Se-depletion has not been used previously in differentiated IECs. As the effects of Se-depletion were almost negligible in differentiated Caco-2 cells, then previous work using Se-depletion in undifferentiated IEC models, which are less representative of IECs *in vivo*, should be interpreted with caution. Moreover, further work is required to understand how the effects of Se-depletion are muted in differentiated Caco-2 cells. The differentiated Caco-2 cells may be more resistant to Se-depletion for a number of reasons, including: 1) increased stores of intracellular Se; 2) extracellularly secreted Se, possibly incorporated into SePP1 [397]; 3) altered selenoprotein hierarchy; 4) increased resistance to oxidative stress [407, 408].

Additionally, a dualoxic environment was used to grow differentiated Caco-2 cells in a gaseous environment that better represents the steep oxygen gradient observed *in vivo* (Figure 2.1 and Chapter 6). Interestingly, the dualoxic environment resulted in the reduced expression of pro-inflammatory mediators and were resistant to the effects of Se-depletion (Figure 6.2). These data may reflect what occurs *in vivo*, as the immune response may need to be suppressed to prevent the immune system over-reacting to bacteria present in food, as well as the microbiota, as seen in IBD. Thus, future studies using *in*

vitro models of the gut should take into consideration the effects of the gaseous environment of the *in vivo* situation that is being modelled as the present work suggests that a dualoxic environment modulates the responses to flagellin and Se-depletion. This is important for studies using *in vitro* IEC models to investigate the immunomodulatory effects of factors, such as diet and pharmacological compounds, on the responses of IECs, which may be considered relevant to IBD.

7.6 Summary

The overall aims of this thesis was to investigate the effects of Se-depletion, and the selenoproteins TR1 and SelH, in the Caco-2 model of IECs on responses to flagellin, oxidative stress and hypoxia. This aim was to be achieved by fulfilling four objectives for both differentiated and undifferentiated Caco-2 cells. However, data were not consistent between the two differentiation states and thus, the success of objective completion will initially be discussed for the undifferentiated Caco-2 cells for the first three objectives.

The first objective was to investigate the effects of Se-depletion and knockdown of the expression of the selenoprotein TR1, on responses to flagellin. In the undifferentiated Caco-2 cells, Se-depletion exacerbated the mRNA expression of pro-inflammatory cytokines in response to challenge with flagellin. TR1 mRNA was found to be induced by both flagellin and Se-depletion and was thus considered to be an important selenoprotein in the response of undifferentiated Caco-2 cells to flagellin. Therefore, a knockdown of TR1 mRNA by siRNA was performed. In the Caco-2 cells that had reduced TR1 expression, there was a significant reduction in the mRNA expression of pro-inflammatory cytokines. In summary, TR1 was suggested to have an important role in the pro-inflammatory effect of Se-depletion on target mRNA.

The second objective was to investigate the effects of Se-depletion, and knockdown of expression of the selenoproteins SelH and TR1, on the expression of oxidative stress-associated genes. In the undifferentiated Caco-2 cells, Se-depletion resulted in increased mRNA expression of genes associated with oxidative stress. The knockdown of mRNA expression of SelH in Caco-2 cells resulted in increased expression of oxidative stress-associated genes that had been observed to change with Se-depletion. This was not observed when TR1 expression was reduced by knockdown. Thus, SelH was suggested to play an important role in the effect of Se-depletion on oxidative stress-associated gene expression.

The third objective was to investigate the effects of Se-depletion, and knockdown of expression of the selenoprotein SelH, on the expression of hypoxia-associated genes in response to hypoxia. In the undifferentiated Caco-2 cells, Se-depletion reduced the expression of hypoxia-associated genes in response to hypoxic stress. However, knockdown of SelH did not result in consistent changes in hypoxia-associated gene expression. Thus,

Se-depletion was suggested to reduce the ability of Caco-2 cells to express the mRNA of genes associated with responses to hypoxic stress.

Interestingly, when the first three objectives were investigated in differentiated Caco-2 cells, there was no change in the majority of the stress-associated targets that were investigated. This suggested that the differentiated Caco-2 cells were more resistant to the effects of Se-depletion than the undifferentiated Caco-2 cells. As differentiated Caco-2 cells have been considered to be more physiologically representative of IECs than undifferentiated Caco-2 cells, this suggested that further investigation is required into IEC models of Se deficiency.

The fourth and final objective was to investigate the effects of Se-depletion on responses to flagellin, under conditions of normoxia, hypoxia and a novel dualoxic cell culture chamber in differentiated Caco-2 cells. The Caco-2 cell responses to flagellin were similarly not affected by Se-depletion in either gaseous environment. Interestingly, the expression of the inducible pro-inflammatory cytokines were diminished in the more physiologically relevant dualoxic environment. Thus, further work is required to investigate the effects the Se-depletion under conditions *in vitro*, that are more representative of the *in vivo* conditions.

Thus, in summary, the data presented in this thesis suggest that, in undifferentiated Caco-2 cells, Se-depletion exacerbated the mRNA expression of pro-inflammatory cytokines in response to challenge with flagellin, as well as genes associated with responses to oxidative stress. Additionally, the data suggested that Se-depletion down-regulated the mRNA expression of genes associated with responses to hypoxia. However, the data were not consistent when the experiments were performed in differentiated Caco-2 cells, which suggested that differentiation of Caco-2 cells may have increased their resistance to Se-depletion. Additionally, the data were not consistent when experiments were performed on Caco-2 cells that were cultured in a more physiologically-representative, dualoxic environment. This outlined the requirement for further work to verify that the common IEC model for Se-depletion is representative of how IECs respond to Se-depletion when under stress *in vivo*.

7.7 Future Work

The data presented in this thesis provided a hypothetical mechanism by which Se modulated the expression of genes associated with inflammation, oxidative stress and hypoxia. However, on the whole, the current work was limited to mRNA expression levels of target genes which, for upstream targets, was not confirmed at the protein level.

TR1 mRNA was found to be downregulated in response to Se-supplementation and was suggested to play an important role in the response of Caco-2 cells to flagellin (Chapter 3). However, previous work has found increased TR1 mRNA and protein levels following Se-supplementation [229, 469–475]. Thus, it would be useful for future work to measure the

protein level of TR1 following supplementation and depletion of Se by Western blotting. If, for instance, the protein level of TR1 decreased in response to Se-supplementation, this would agree with the decrease in mRNA expression of TR1 observed in the present work, as well as fit the proposed mechanism of action in Section 7.4.

The changes in the mRNA expression of the 25 members of the selenoproteome were limited to just measuring GPX1, GPX2, GPX4, TR1, SePP1 and SelH. Previous studies have shown that altering Se supply differentially affects the expression of the entire selenoproteome. Additionally, the treatment of Caco-2 cells with flagellin and hypoxia are also likely to affect other members of the selenoproteome, and multiple selenoproteins may also be involved in coordinating responses to flagellin and hypoxia. Thus, future work could involve examining an array of selenoprotein mRNA expression, so that all selenoproteins are analysed in response to stress. Furthermore, it would also be useful to measure the protein concentrations of selenoproteins, in order to investigate that changes on the mRNA level reflect the changes in protein concentrations. Selenoproteome analyses could be performed using laser ablation-inductively coupled plasma mass spectrometry (LA-ICP MS), which is a semi-quantitative method for measuring the protein levels of all 25 selenoproteins [666]. This would provide insight into how the selenoproteome changes as a whole in response to Se-depletion and other challenges.

The use of a novel dualoxic cell culture chamber allowed differentiated Caco-2 cells to be cultured in a more physiologically-relevant environment. Although it was considered more physiological, there is little evidence that this novel *in vitro* model is more representative of IECs than undifferentiated or differentiated Caco-2 cells cultured using normal cell culture protocols. In addition to repeating the experiments performed in Chapter 6, future work should involve performing the same experiments in other IEC cell lines, such as HT-29 cells, in order to verify the effects of Se in other human immortalised differentiated cell lines in a dualoxic environment. If the data are consistent, then *in vivo* experiments could be performed in a rodent experimental model.

Chapter 8

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