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**Identification of the biochemical signalling
mechanisms underlying CD40 killing in colorectal
cancer cells**

Albashir M A Mohamed

**A thesis submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy**

**The University of Huddersfield
School of Applied Sciences**

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Abstract

CD40 is a member of the tumour necrosis factor receptor (TNFR) superfamily and ligation by membrane-presented CD40 ligand (mCD40L), but not soluble agonists, causes extensive apoptosis in malignant epithelial cells, including colorectal carcinoma (CRC) cells. This thesis aimed to unravel the precise cell signalling pathways responsible for mCD40L-mediated apoptosis in CRC cells.

This study has provided evidence that CRC cell death by mCD40L is rapid. mCD40L activated MOMP, cytochrome c release from mitochondria and induction of Bak/Bax within <6 hours post ligation. The pro-apoptotic role of Bax was confirmed by shRNA-mediated Bax knockdown as this attenuated apoptosis and decreased caspase 3/7 activity. mCD40L triggered rapid TRAIL induction and a caspase-dependent pathway that involved caspase-10 (but not caspase-8) and caspase-9 to cause CRC cell death. Thus CD40 cross-talks with the extrinsic pathway by inducing TRAIL-mediated, caspase-10 activation, mitochondrial disruption, tBid activation, Bak/Bax induction, and activation of caspase-9 and -3/7 to cause CRC cell death.

When the signalling pathways triggered by CD40 were studied further, we found that CD40 induced both p-JNK and p-p38 in CRC cells which is necessary for apoptosis, and that JNK might be acting downstream of p38. p38 and JNK directly regulated Bak/Bax and TRAIL induction at the transcriptional level. We also showed that TRAF1, -3, and 6 were induced in CRC cells as early as 1.5 hours post ligation. Our studies not only demonstrated a novel pattern of TRAF regulation in CRC cells but revealed for the first time that TRAF3 has an essential role in CD40-mediated CRC cells death. TRAF3 is central in the induction of apoptosis as its knockdown attenuates apoptosis, by abrogating p38 and JNK activation, induction of Bak/Bax and caspase-3/7 activation. Therefore, despite the existence to a dual apoptotic pathway being engaged in CRC cells, TRAF3 appears to be central in both signalling axes.

ROS are rapidly induced in CRC cells by CD40 in a Nox-dependent fashion and this plays an important role in CD40-mediated killing. More specifically, CD40 activation appears to result in TRAF3-dependent p40phox activation. CD40 also regulates directly ROS scavenging mediators, as we detected reduction in Trx-1 expression. Moreover, CD40 triggered activation of the Trx-regulated pro-apoptotic kinase ASK-1, which provided direct molecular explanation for the importance of ROS in CD40 signalling and downstream activation of MKKs and p38/JNK. Thus, the mCD40L-CD40-TRAF3-NOX axis utilises ROS for the activation of ASK-1/MKK/p38/JNK pro-apoptotic pathways in CRC cells.

Based on observations in this thesis and more recent findings following completion of this work, we hypothesise that at some point the MAPK/p38/JNK pathway diverges to drive on one hand transcriptional upregulation of TRAIL, activation of tBid and cross talk to the mitochondria, whilst the other p38/JNK pathway directly induces Bak/Bax to also induce MOMP and mitochondrial death, the latter being more reminiscent of CD40-mediated cell death in UCC cells. However, unlike UCC cells where the operation of only the latter pathway takes place means apoptosis requires a minimum of 24-36 hours to occur, in CRC cells there is rapid amplification of the apoptotic signal and quick induction of death. To our knowledge, this is the first demonstration of such extensive and rapid carcinoma cell apoptosis triggered by CD40 ligation.

Overall, this study has identified the intracellular signalling cascade triggered by CD40 ligation and results in extensive apoptosis in CRC cells. It has identified a TRAF3-Nox-ROS-ASK1-MKK-p38/JNK pathway (that activates caspase-10 and caspase-9) as the driving force that triggers both a TRAIL-associated extrinsic as well as the intrinsic apoptotic pathways. Thus, in CRC cells CD40 induces apoptosis by pathway cross talk which permits strikingly rapid apoptosis. These findings not only provided novel observations on the mechanisms of apoptosis triggered by the TNFRF member CD40, and also reinforced the importance of the quality of CD40 signal in determining functional outcome, but they have also raised interesting hypotheses for further biological studies. Equally importantly, the findings have also assisted in the formulation of a novel combinatorial therapeutic approach that may exploit CD40 for anticancer therapy.

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There is a person of great importance in my life, who is with me and supporting me all the time by the strength and energy available to it. This is **my wife**, my wonderful and virtuous wife.

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Finally, if I mention the name of this ultimately it is rather because he is my life, the base and the spring of all my actions, the author of the realization of my dreams, and my mission on earth. One who is always there when everything seems to leave me. He is my Lord **ALLAH**

DEDICATION

This work is dedicated to my father and mother who I missed them and my oldest brother Omer who always encouraged me to complete my study

Albashir Mohamed

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Abbreviations

µg.....	Micro gram
µl.....	Micro litre
µM.....	Micro molar
aa.....	Amino acids
ACD.....	Accidental cell death
AIF.....	Apoptosis inducing factor
AIP.....	Inhibitor of apoptosis
AML.....	Amyloid leukaemia
ANT.....	Adenyle nucleotide Transporter
AP-1.....	Activator protein 1
Apaf-1.....	Apoptosis protease-activating factor 1
APC.....	Antigen presenting cell
ASK1.....	Apoptosis signalling kinase 1
Bad.....	Bcl2 antagonist of cell death BCL2 binding protein
BAD.....	a pro-apoptotic member of the Bcl-2 protein family
BAFF.....	B cell activating factor belonging to the TNF family
Bak.....	Bcl2 antagonist killer
Bax.....	Bcl2 associated X protein
BAX.....	BCL2-associated X protein
BCL-2.....	B-cell lymphoma 2
Bcl-w.....	Bcl2 like 2 proteins (Apoptosis regulator Bcl-w)
Bcl-xL.....	B-cell lymphoma-extra large
Bcl-XS.....	Bcl2 related protein (short isoform)
bFGF.....	basic Fibroblast Growth factor
Bid.....	BH3 interacting domain death agonist p22 BID
BID.....	BH3 interacting domain death agonist
BIK.....	Bcl-2 Interacting Killer
BIM (BOD).....	BCL-2 interacting mediator of cell death
BLyS.....	B-lymphocyte Stimulation protein
BOK/MTD.....	Bcl-2 related ovarian killer/ Matador
BOO/DIVA.....	Bcl-2 homolog of <u>O</u> vary
BrdU.....	5-bromo-2-dedoxyuridine
BSA.....	Bovine Serum Albumine
CAD.....	Caspase activated DNase
CARD.....	Caspase activation and recruitment domain
Caspase.....	Cysteiny aspartic acid-protease
CD154.....	Cluster of Differentiation 154
CD28.....	Cluster of Differentiation 28
CD40R.....	CD40 receptor (Cluster of differentiation 40)
CDK.....	Cyclin dependant kinase
cDNA.....	Complementary DNA
c-FLIP.....	FLICE-inhibitory protein
CMV.....	Cytomegalovirus
CN.....	Calcinneurin
CO ₂	Carbone dioxide
Cox-2.....	Cyclooxygenase-2
CRC.....	Colorectal cancer cells
Cyto-c.....	Cytochrome C
dATP.....	2'-deoxyadenosine triphosphate
DC.....	Dendritic cell
DD.....	Death domain
DED.....	Death effector domain
DEDAF.....	Death effector domain- Associated Factor

DEM.....	Diethyl Maleate
DFF40.	DNA Fragmentation Factor 40/CAD
DFF45.	DNA Fragmentation Factor 45/ICAD
DISC.	Death inducing signalling complex
DMEM.	Dulbecco's modified Eagle's medium
DMSO.	Dimethyl Sulphoxide
DNA.....	Deoxyribonucleic acid
DNase.....	Deoxyribonuclease
DPI.	Diphenylene iodonium
DR.....	Death receptor
DR4.....	Death receptor 4
DR5.....	Death receptor 5
EDTA.	Ethylineamide
ELISA.	Enzyme-linked immunosorbent assay
Endo G.....	Endonuclease G
ERK1, 2.	Extracellular signal regulated Kinase1, 2
FAD.	Flavine adinine dinucleotide
FADD.	Fas-associated death domain
FBS.	Foetal Bovine Serum
GM-CSF.	Granulocyte Macrophage – Colony Stimulating Factor
H2DCFDA.	6-carboxy-2,7 dichloro dihydrofluorescein diacetate
H ₂ SO ₄	Sulphuric acid
HIGM.	Hyper IgM syndrome
HSP27, 70, 90.....	Heat shock proteins 27, 70, 90
hTERT.....	Telomerase catalytic sub-unit
Htr A2.....	High temperature requirement protein-A2
IAP.....	Inhibitor of Apoptosis Proteins
ICAM-1.	Intracellular adhesion molecule-1
ICD.....	Intracellular domain
IFN.....	Interferon
Ig.....	Immunoglobulin
IgA.	Immunoglobulin A
IgE.	Immunoglobulin E
IgG.	Immunoglobulin G
IKK.....	I-κB kinase
IL.....	Interleukin
IL-2,4,6,8,12.....	Interleukin-2,4,6,8,10,12
IκB.....	Inhibitor of NF-κB
JAK3.....	Janus Kinase 3
JNK.....	C-jun N-terminal kinase
KDa.....	Kilo Dalton
KGD domain.....	Lysine - Aspartic acid - Glycine domain
LFA-1.....	Lymphocyte function Antigen-1
LG.....	L-glutamine
LMP1.....	Latent membrane protein-1
Mac-1.....	Macrophage-1 antigen
MAPK.....	Mitogen activated Protein kinase
mCD40L.....	Membrane CD40 ligand
MCL-1.....	Myeloid cell leukaemia-1
MCP-1.....	Monocyte Chemo-attractant protein-1
MEKK-1.....	MAP Kinase kinase-1
MHC-1.....	Histocompatibility Complex-1
MHC-2.....	Histocompatibility Complex-2
mM.....	Millimolar
MM.....	Malignant melanoma

MMC.....	Mitomycine c
MMP-1, 2, 3.....	Metalloproteinase Matrix 1, 2, 3
MOMP.....	Mitochondrial outer membrane permeabilisation
mRNA.....	Messenger Ribonucleic acid
NAC.....	N-acetyl cysteine
NF- κ B.....	nuclear factor kappa-B
NIK.....	NF- κ B-inducing kinase
NK.....	Natural killer
NO.....	Nitric Oxide
Nox.....	NADPH oxidase
NR.....	Near infrared
OMM.....	Outer mitochondrial membrane
PBS.....	Phosphate buffer saline
PCD.....	Programmed cell death
PVDF.....	Poly Vinylidene difluoride membrane
RANTES	Regulated on Activation Normal T cell Expressed and Secreted
Redox.....	Reduction-oxidation
RFU.....	Relative Fluorescent unit
RIP.....	Receptor-interacting protein
RLU.....	Relative Luminescence Unit
RNA.....	Ribonucleic acid
RNase.....	Ribonuclease
ROS	Reactive oxygen Species
RPMI Medium.....	Roswell Park Memorial Institute medium
RT.....	Rome Temperature
SAPK.....	Stress Activated Protein Kinase
sCD40L.....	Soluble CD40 ligand
shRNA.....	Short hairpin RNA
Smac/DIABLO.....	Second mitochondrial activator of caspases/direct IAP binding protein with low PI
TC	T-cells (T-lymphocyte)
TCR.....	T-cell receptor
TGF- β	Transforming Growth factor- β
TIM.....	TRAF interacting motif
TNF	Tumour necrosis factor
TNFR.....	Tumour necrosis factor receptor
TNFR-I.....	Tumour necrosis factor receptor I
TNFRSF.....	Tumour necrosis factor receptor superfamily
TNF- α	Tumour necrosis factor alpha
TRADD.....	Tumour necrosis factor receptor associated death domain
TRAF.....	Tumour necrosis factor receptor associated factor
TRAP.....	Tumour Necrosis Factor-related Activation Protein
UV.....	Ultra violet
VDAC.....	Voltage Dependent Anion Channel
XAF-1.....	XIAP- associated factor-1
xiAP.....	X-linked inhibitor of apoptosis protein

CHAPTER 1: INTRODUCTION

1.1 Apoptosis

1.1.1 General

Apoptosis or Programmed Cell Death (PCD) refers to a physiological type of cell death involving an active process. The two terms refer to distinct and well defined physiological processes, however often they are used interchangeably.

PCD is a term originally used to describe cells that die at a specific time and place during development. Cell death is a genetically programmed natural phenomenon which eliminates unnecessary cells (Pereira and Amarante-Mendes, 2011). Physiologically, there is a close balance between apoptosis and the production of new cells which prevents excessive cell proliferation and achieves homeostatic control of overall cell numbers in the body. When apoptosis is not working cells can multiply uncontrollably and thus dysregulation of apoptosis can be associated with cancer. Apoptosis is distinct from necrosis, which in general is a pathological cell death; however more recent research has provided evidence for the existence of controlled necrosis which will not be discussed here (Edinger and Thompson, 2004).

In 1972, Kerr's team showed that, in several cell types, death is preceded by a condensation of the nucleus and cytoplasm, fragmentation of the cell contents in apoptotic bodies (membrane structures containing cytoplasmic and nuclear debris) and elimination of the last components by phagocytosis (see Figure 1.1) (Kerr et al., 1972). In addition, this type of cell death induced no inflammatory response and did not affect neighbouring cells. Necrosis, by contrast, is in general referred to as "accidental" cell death, which occurs while cells are exposed to extreme conditions that differ from physiological situations, such as hypothermia, hypoxia or tissue injury. In necrosis, the cell swells and bursts the cell membrane, discharging the cell contents into the surrounding tissue and causing a local inflammation. This inflammation is caused by the presence of inflammatory substances and enzymes present in the cytoplasm. Organelles (including the mitochondria and the nucleus) remain intact throughout the process (Weedon et al., 1979). Since the characteristics observed by Kerr contrasted with those of necrotic cells (see Figure 1.1), this group of researchers proposed the term apoptosis, which in Greek describes the fall of leaves in autumn, to identify this type of PCD (Kerr et al., 1972).

Currently, it is recognised that cell apoptosis is mainly characterised by cell shrinkage, condensation of chromatin, fragmentation of the nucleus, degradation of nuclear DNA and the formation of vesicles containing the cell debris. From a biochemical point of

view, generation of DNA fragments corresponding to multiples of 160-200bp has long been a characteristic associated with apoptosis (Martin et al., 1994).

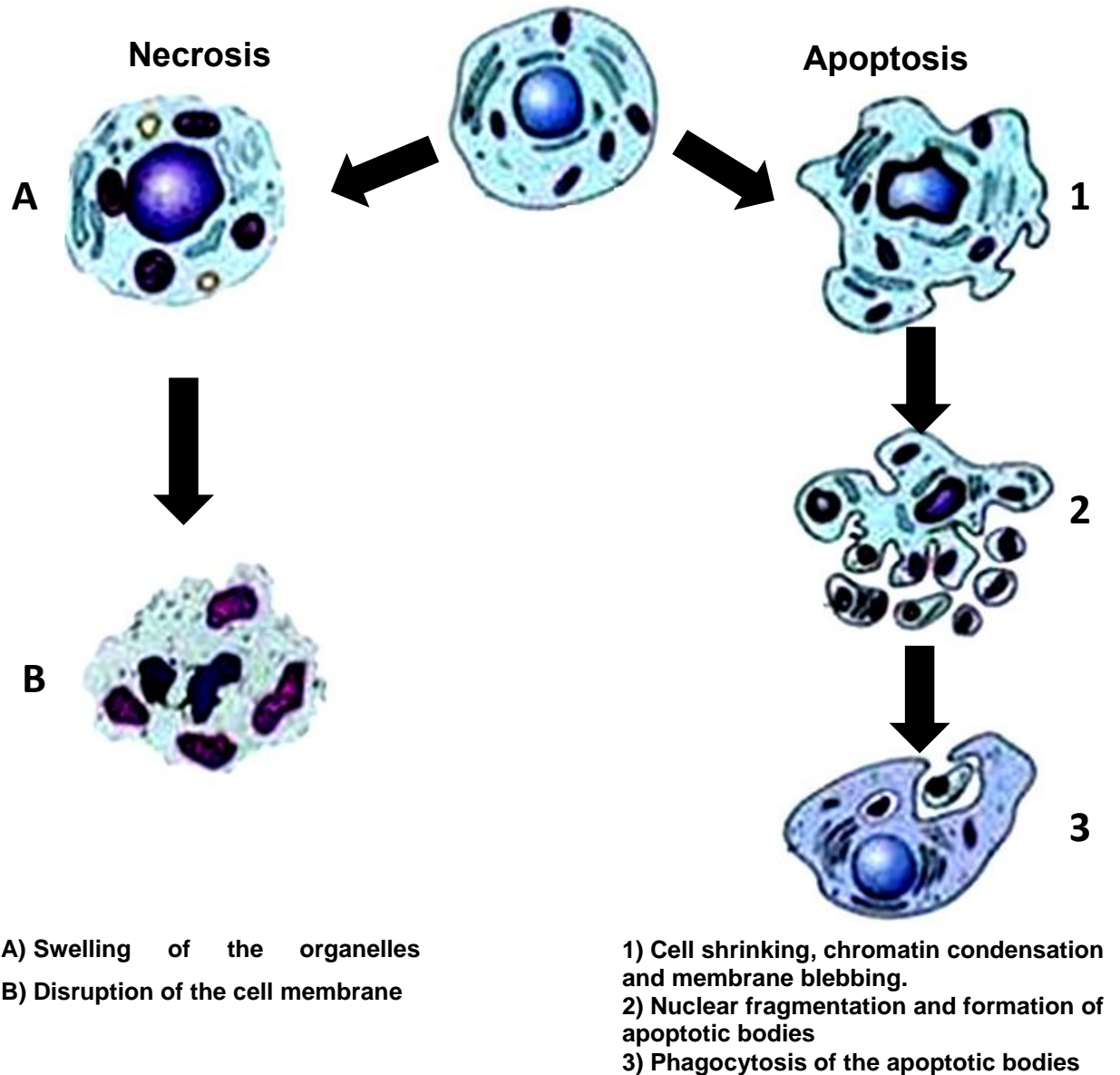


Figure 1. 1 Apoptosis *versus* necrosis

This diagram shows the characteristics of different morphological changes in the process of cell death by apoptosis versus necrosis) is characterized by loss of plasma membrane integrity, flocculation of chromatin, cell lysis followed by swelling with leakage of intracellular content and disintegration of organelles. While the apoptotic process involves alteration of membrane permeability, chromatin condensation, cell shrinkage, formation of apoptotic bodies without disintegration of organelles (Weedon et al., 1979).

1.1.2 Physiological roles of apoptosis

Apoptosis plays an important role in embryogenesis (and in this case it is correctly referred to as PCD, in the morphological changes in cellular homeostasis, in atrophy in tissue repair, in tumour regression, and in immunity. Apoptosis is critically important during various developmental processes. For instance, both the nervous and immune system arises during overproduction of cells. This early overproduction is then followed by the death of those cells that were unsuccessful in establishing functional synaptic connections in the nervous system or productive antigen specificities in the immune system. Apoptosis plays a critical role in T cell function, and for instance in the removal of T cells during the last phase of the immune response. In addition, during the development of the central nervous system in vertebrates, approximately 50% of neurons degenerate during the perinatal period by PCD. This phenomenon is controlled by specific trophic factors (Raff et al., 1993).

1.1.3 Pathological aspects of apoptosis

Dysregulation of apoptotic cell death is involved in the pathogenesis of many diseases. In healthy tissue, there is a regulated balance between cell division and cell death. This balance is disturbed in cancer cells, not only by unrestrained cell division but also by a dysregulation of programmed cell death. In addition to cancer, dysfunction of apoptosis can also lead to diseases such as neurodegeneration or autoimmune diseases (Thompson, 1995). Increased rates of apoptosis can lead to degenerative diseases such as stroke, atherosclerosis, diabetes, Parkinson's disease and Alzheimer's disease, while inhibition of apoptosis pathways may lead to proliferative diseases such as autoimmune diseases or cancer (MacFarlane and Williams, 2004).

1.2. Molecular components of apoptosis

1.2.1 Caspases

Caspases are cysteine proteases that can be involved in the process of cell death upon stimulation of cells by chemical factors such as physicochemical signals (UV, gamma rays) or the deprivation of growth factors. Caspases can have a key role in the initiation and execution of apoptosis. In the term 'caspase' (proposed by Alnemri et al., 1996), the 'C' is the active site cysteine while 'aspase' defines the strict specificity of cleavage of the substrates of this family of proteases after an aspartic acid residue. These enzymes are initially synthesised as inactive pro-enzymes (zymogens) that upon both conformational changes and proteolytic cleavage becomes activated (as detailed in following sections). Of note, in addition to caspases, another known protease with the same specificity as caspases

(cleavage at aspartate residue) is Granzyme B, a serine protease contained in the granules of cytotoxic cells (e.g. cytotoxic T lymphocytes, CTL) that initiates the apoptotic death of target cells (Alnemri et al., 1996).

1.2.1.1 Structure and activation of caspases

The first caspase, caspase-1 or ICE, has been demonstrated in mammals by homology with the pro-apoptotic protein Ced-3 identified in *Caenorhabditis elegans* (*C. elegans*) (Miura et al., 1993). Fourteen different caspases have been identified so far (Lippens et al., 2003). All caspases have a conserved structure and are synthesised as inactive precursors or zymogens (Parrish et al., 2013). Caspases comprise a pro-domain of variable size and sequence located in the amino-terminal portion of the protein, a large subunit (20kDa) in the middle of the molecule and a small subunit (10kDa) localised in the carboxy terminal part. Some members of the caspase family have a binding domain between the large and small subunit. The N-terminal domain appears to play a role in protein-protein interactions and, thus, in regulating the activation of these enzymes (Thornberry, 1998) (Figure 1.2).

Caspase activation involves the proteolytic cleavage of the zymogen form at two consensus sites for cutting the pro-domain and separating the two subunits. Caspases can activate other caspases or substrates to form an enzymatic cascade to amplify and integrate pro-apoptotic signals (Thornberry and Lazebnik, 1998, Thornberry, 1998). The two sites have different consensus cleavage caspases but they always occur after the Asp-X bond (Figure 1.2). Although the large subunit contains the catalytic domain, its activity requires binding to the small subunit. Indeed, crystallography studies indicated that the active forms of caspase tetramers are formed by the association of two heterodimers containing two independent catalytic sites (Wilson et al., 1994).

Caspase activation tends to be an early event occurring during apoptosis and inhibition of caspases by specific proteins (caspase-specific peptides) prevents the occurrence of the morphological characteristics of apoptosis; conversely, the administration of recombinant caspases induces apoptosis (Thornberry et al., 2000).

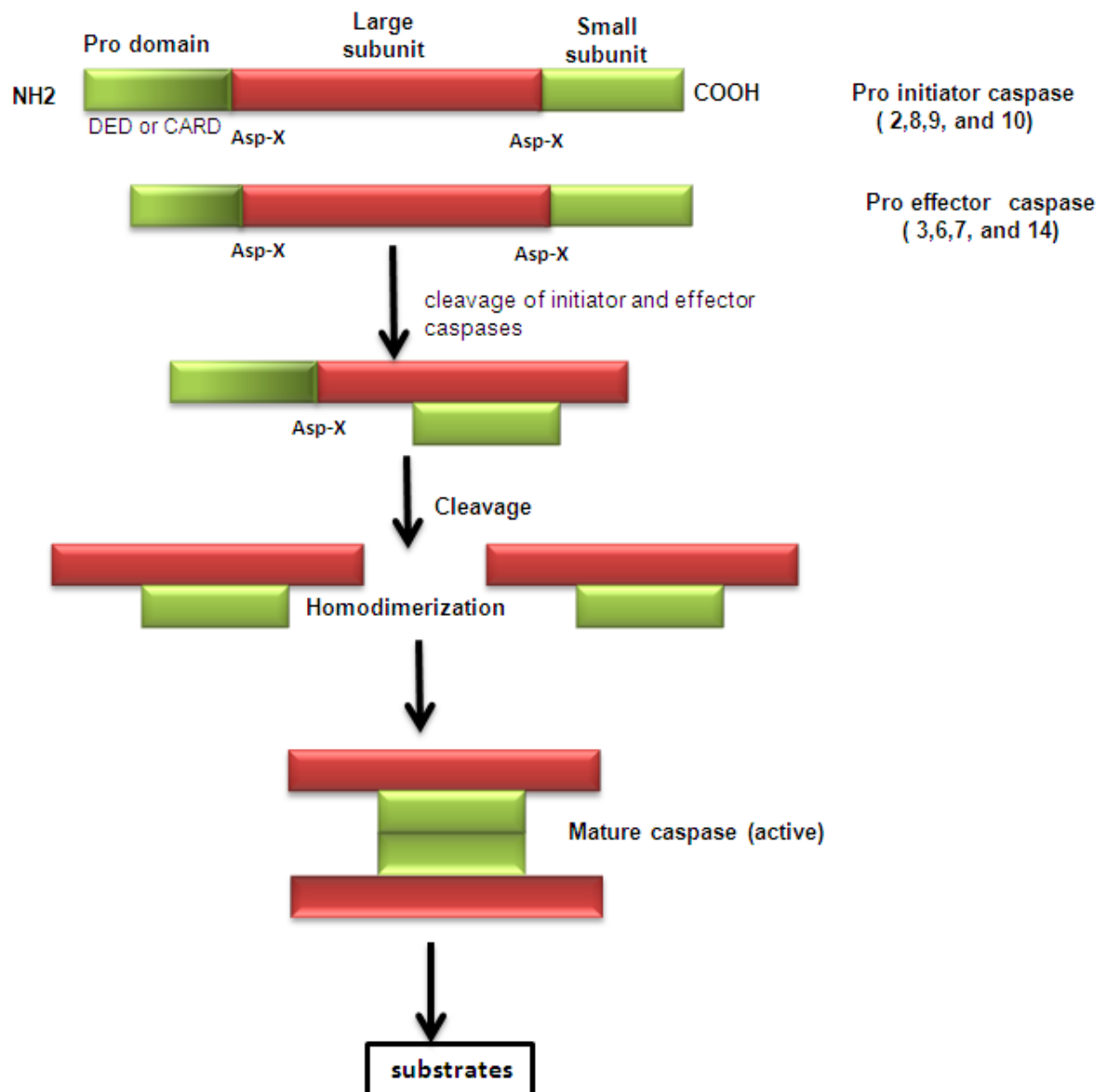


Figure 1. 2 Activation of caspases

Caspases are produced as inactive precursors containing a pro-domain and two subunits of approximately 20 and 10 kDa (p20 and p10, p18 and p12, respectively, in the examples shown). The pro-domain of initiator caspases is longer and contains domains (such as Death Effector Domain, or DED) important for interaction with adapter proteins. The arrows show the order of cleavage of pro-caspases. After cleavage, the subunits associate to form the active structure, having two active sites (Amarante-Mendes and Green, 1999).

1.2.1.2 The various classes of caspases

Not all caspases act as direct, effector molecules of the dying process. Indeed, only caspase-3, -6, -7 and -14, which have a short pro-domain, are directly involved in the execution of apoptosis. In fact, caspase-2, -8, -9, and -10, which have a long pro-domain, are initiators or regulatory molecules of apoptosis (Figure 1.2). These function as signalling molecules; they are recruited to affect protein complexes via their pro-domain and are capable of self-activation by the transducing signal to activate effector caspases. It is now known that caspase-8 and -10 are activated by the death receptor pathway (see following sections), whereas caspase-9 is activated by the mitochondrial apoptotic pathway (Amarante-Mendes and Green, 1999, Gupta, 2003). This difference in the role of caspases relates to the presence of units of protein-protein interactions, such as areas of death effector domain (DED) (for caspase -8 and -10) or caspase recruitment domain (CARD) (for caspase -1, -2, -4 and -9) at the pro-domain. Indeed, caspase-8 and -10 contain DEDs capable of binding in a homophilic fashion to other DEDs present in adaptor signalling molecules. The CARD domain in pro-caspase -1, -2, -4 and -9 is allowed in the same way to join other caspases or other adaptor molecules. Some caspases such as caspase-1, -4, -5, -11, -12 and -13 appear to be involved as inflammation caspases probably by inducing cleavage of the pro-inflammatory cytokines such as Interleukin-1 and Interleukin-18 (Oyadomari et al., 2002).

1.2.1.3 Main substrates of caspases and their cleavage

Several substrates of caspases have been identified and one of the most studied mechanisms of activation is the nucleases leading to DNA fragmentation. These nucleases cleave genomic DNA between nucleosomes to generate fragments of ~180 base pairs. These nucleases are named DFF for DNA fragmentation factor in humans and CAD for caspase-activated DNase in mice. They exist in the cell as inactive complexes because they are associated with an inhibitory subunit. Nuclease DFF40 is complexed with the inhibitory protein DFF45 in humans and the mouse CAD is complexed with ICAD. Normally CAD exists as an inactive complex with ICAD (inhibitor of CAD). During apoptosis, ICAD is cleaved by caspases such as caspase 3 to release CAD then rapid fragmentation of DNA (Figure 1.2). The endonuclease so released will generate DNA fragments of 180 base pairs (Samejima et al., 2001).

However, it appears that, in some forms of apoptosis, this internucleosomal DNA fragmentation is replaced by a fragmentation of high molecular weight (Kaufmann et al., 2000). Some members of the Bcl-2 family (see following sections) may also be cleaved to be activated; the best example of this is the Bid protein, which is cleaved by caspase -8 in

certain conditions (Li et al., 1998, Luo et al., 1998) (Figure1.2). The loss of cellular morphology is probably the consequence of cleavage of cytoskeletal proteins such as gelsolin and nuclear proteins such as nuclear laminins (Kothakota et al., 1997), and the occurrence of budding membranes appears to be caused by cleavage of the p21-activated kinase (PAK-21) at the regulatory subunit and at the catalytic subunit, allowing activation (Rudel and Bokoch, 1997) (Figure1.2). Nearly 100 caspase substrates have been reported previously, indicating the complexity of death by apoptosis (Hengartner, 2000).

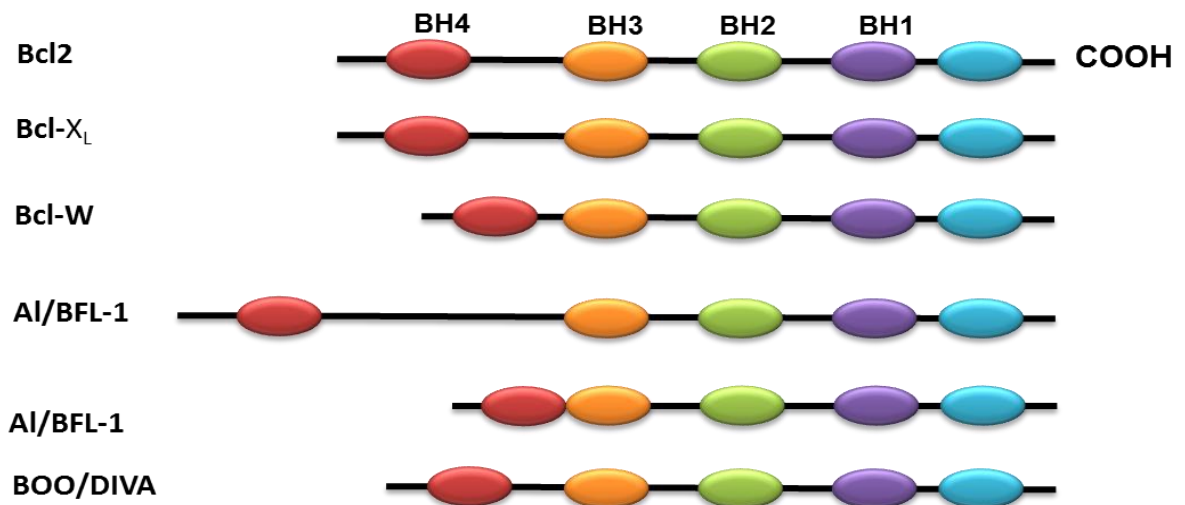
1.3 Members of the Bcl-2 family

1.3.1 Structure

Members of the Bcl-2 family (B-cell leukaemia/lymphoma 2-like proteins) are important regulators of apoptosis. This family, containing about 15 members, can be divided into two groups according to their activity: proteins with anti-apoptotic activity and proteins with pro-apoptotic activity. These two groups differ in their structure but they comprise four common and conserved regions; the BH domains for "Bcl-2 homology". The regions BH1, 2 and 3 form the hydrophobic pocket capable of binding a BH3 domain from another protein; the BH3 domain is an amphipathic α helix (Gross et al., 1999).

Members of the Bcl-2 family that are anti- apoptotic, such as Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1/BFL-1 and BOO/DIVA, contain domains BH1, 2, 3 and 4 (Figure 1.3). The pro-apoptotic members are divided into two subgroups; the first contains three domains (BH1, 2, and 3) such as Bax, Bak, Bok/MTD, while the second group contains those with only the BH3-domain such as Bid, Bad, Bik/NBK, BLK, HRK, and BIM (BOD), also called "BH3 proteins". The BH3 region seems to be heavily involved in the pro-apoptotic activity (Figure 1.3). The BH4 and the near region sequences present in only the anti-apoptotic proteins can be phosphorylated. By forming complexes with other proteins, such as calcineurin (CN), connections with other pathways of apoptosis can be initiated (Shibasaki et al., 1997). All the proteins of the Bcl-2 family contain a hydrophobic carboxy-terminal domain of 20 amino acids for their attachment in intracellular membranes, mostly in the mitochondria, but also in the endoplasmic reticulum (Krajewski et al., 1993).

Anti-apoptotic proteins



Pro-apoptotic proteins

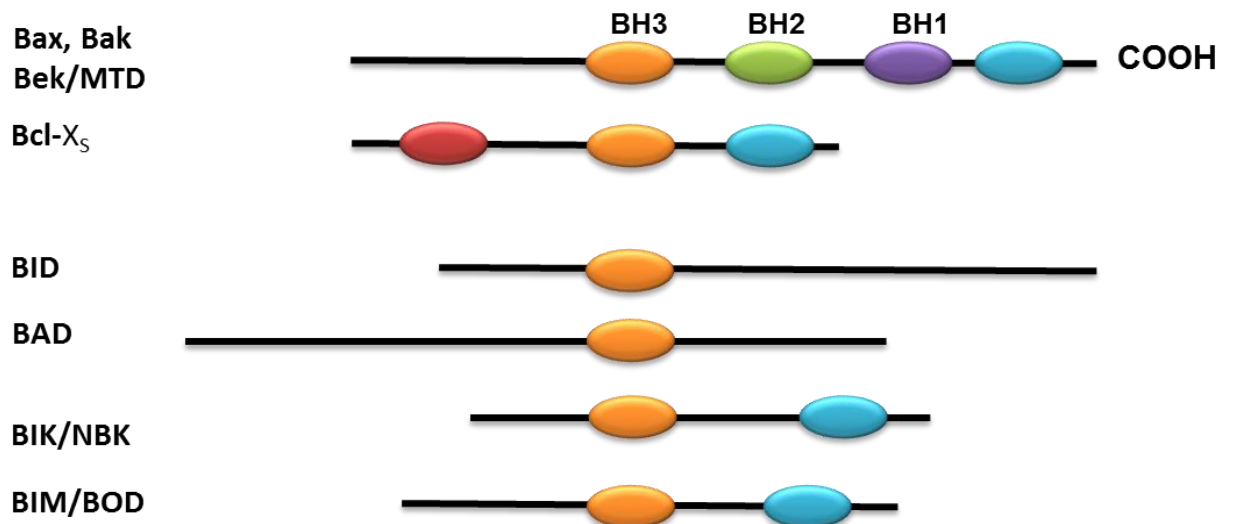


Figure 1. 3 Members of the Bcl-2 family

Classification of Bcl-2 family members, only some representative members are shown, and they are found in mammals, unless otherwise stated. The BH domains are indicated (Adapted from (Gross et al., 1999)).

1.3.2 Regulation of the Bcl-2 members

Members of the Bcl-2 are regulated transcriptionally and post-translationally by cytokines or survival factors and death. In addition to the dimerisation process allowing proteins to activate (homodimerisation) or to inhibit (heterodimerisation), other phenomena such as post-translational modifications (phosphorylation or proteolysis) can regulate the activity of certain members of this family. Hyper-phosphorylation of Bcl-2 appears to alter its anti-apoptotic activity in certain cells (Chang et al., 1997). Kinases may phosphorylate Bcl-2, an example being the c-Jun N-terminal kinase (JNK) which is activated by stress (Chang et al., 1997).

Bad is a protein that can bind to Bcl-XL and inhibit its anti-apoptotic activity. This connection is only allowed if the binding site of Bad is dephosphorylated. Several kinases can phosphorylate the Bad protein. This is the case with the Akt/PKB/RAC protein, which is a serine threonine kinase acting upstream kinase of phosphatidyl inositol triphosphate (PIP-3) (Zha et al., 1996) and cAMP dependent kinase (PKA) (Harada et al., 1999).

The Bid protein (22 kDa) is a substrate of caspase-8 and as demonstrated more recently of caspase-10 and has to be cleaved to be active. The C-terminal 15 kDa fragment generated by proteolysis (truncated Bid, tBid) can thus be inserted at the level of the mitochondrial membrane, and it allows the activation of the mitochondrial pathway, thus amplifying the signal initiated by the death receptor pathway (Li et al., 1998, Luo et al., 1998, Milhas et al., 2005).

Bim can interact with Bcl-2 and promote the induction of apoptosis after certain stimuli. The Bim is a protein localised at the microtubule complex in intact cells. After induction of death by certain death signals, it dissociates from the complex and translocates to the mitochondria without being cleaved (Puthalakath et al., 1999).

1.3.3 Mechanism of action

Most of these small Bcl-2 proteins can dimerise; therefore, this often occurs between pro-apoptotic proteins and anti-apoptotic proteins. The regulation of apoptosis by these proteins therefore results from the delicate balance in the expression level of pro- *versus* anti-apoptotic proteins; cells expressing more pro-apoptotic proteins are susceptible to death, while cells that do not are resistant (Hengartner, 2000).

The main function of these regulators is to control the release of pro-apoptotic factors, such as cytochrome c, from the mitochondrial intermembrane space to the cytosol. Indeed, the addition of pro-apoptotic proteins is sufficient to induce the release of cytochrome c; in

contrast, the anti-apoptotic proteins prevent release of the cytochrome c (Antonsson and Martinou, 2000, Gross et al., 1999). In addition, caspase inhibitors do not alter the release of cytochrome c, which implies that caspases are not involved in these events. Bcl-2 appears to be very often linked to mitochondrial membrane proteins, while others such as Bax, Bid, Bad and Bim translocate from the cytosolic areas to the mitochondria during apoptosis. These proteins play an important role in transducing the signal from the cytosol to the mitochondria. The translocation of these proteins is controlled by post-translational modifications, such as Bad de-phosphorylation or cleavage of Bid (Gross et al., 1999).

Bax is first translocated from cytosol to mitochondria. Its conformation is then modified and the outsourcing of its amino-terminal domain allows its oligomerisation and its insertion in the outer mitochondrial membrane (Jurgensmeier et al., 1998). This insertion capacity appears to be related to the structural homology of family members Bcl-2 with certain bacterial toxins, allowing them to form pores at the mitochondrion. This insertion is indeed quickly followed by the release of cytochrome c. Other studies suggest that the capacity for insertion is facilitated by the interaction with the voltage-dependent anion channel (VDAC). These changes in the conformation of Bax appeared to be favoured by Bid interaction (Eskes et al., 2000). These changes can also be reduced or prevented by Bcl-2 or Bcl-xL, as these anti-apoptotic proteins can act by interactions with Bax (Desagher et al., 1999). Unexpectedly, Bcl-2 also fails to inhibit Bax-induced cytochrome c release, although it co-localises with Bax to mitochondria. Rosse and colleagues reported that cells overexpressing both Bcl-2 and Bax show no signs of caspase activation and survive with important amounts of cytochrome c in the cytoplasm. Also, reported that Bcl-2 can interfere with Bax killing downstream of and independently of cytochrome c release (Rosse et al., 1998).

Interestingly, members of the Bcl-2 family can modulate the cell cycle; indeed, Bcl-2 can promote the entry of cells in the G₀ phase, and block and delay their entry into the cell cycle (Linette et al., 1996, Mazel et al., 1996). This effect is probably separate from its anti-apoptotic activity as an alteration of its structure at the non-conserved domain allows cells to continue the cell cycle but does not change its anti-apoptotic activity; (Uhlmann et al., 1996).

1.4 Role of mitochondria in apoptosis

1.4.1 General

The mitochondrion plays a fundamental role in the cell, producing a large part of the energy required by the cell, participating in calcium homeostasis, maintaining the redox potential and intracellular pH, and playing a key role in the modulation of calcium homeostasis and oxidative stress (Adams, 2003). This means that a major mitochondrial dysfunction can result in programmed cell death. A change in the electron transport may be sufficient to increase the production of oxygen free radicals and acidify the cytoplasm. Under these conditions, electrons are no longer produced in sufficient quantity and reduction of the synthesis of ATP results in the accumulation of lactate by stimulating glycolysis. Moreover, the electrons released from the mitochondria can reduce oxygen superoxide ions, which are highly reactive oxygen free radicals (Adams, 2003).

1.3.4 Role of Bcl-2 family members in the regulation of the cell cycle

1.4.2 Channel opener mechanisms

During apoptosis, water and various solutes enter the mitochondria, causing its swelling and the release of the various constituents of the inter-membrane space into the cytosol, while the matrix components are retained in the mitochondria through the inner membrane and remain intact. Several models have been proposed to explain the underlying mechanism of substance release from the mitochondria to the cytoplasm during the effector phase of apoptosis (illustrated in Figure 1.4) and these will be discussed below.

1.4.3 The rupture of the outer mitochondrial membrane

The first model involves hyperpolarisation of the internal membrane prior to the release of cytochrome c in some systems. This hyperpolarisation results from the failure of the exchange between cytoplasmic ADP and mitochondrial ATP (Heiden et al., 1999). This exchange is normally carried out by the voltage-dependent anion channels (VDAC) localised in the outer membrane of the mitochondria and the carrier of adenylic nucleotide (ANT), which is located in the inner membrane. This lack of exchange appears to inhibit the activity of the F₁F₀-ATPase, which prevents the return of H⁺ ions to the matrix and therefore contributes to the hyperpolarisation. Such an increase in mitochondrial membrane potential may cause the osmotic swelling of the matrix, resulting in the rupture of the outer mitochondrial membrane (Heiden et al., 1999) (Figure 1.4a).

1.4.4 The mitochondrial permeability transition pore

This model involves a second mega-channel, the mitochondrial permeability transition pore (or MPTP). The MPTP is a non-selective channel with "high conductance", which may be formed by affixing transmembrane proteins residing in the inner membrane and the outer membrane of the mitochondrion (Crompton, 1999). Different studies show that the pore is mainly formed by the association of the ANT of VDAC and cyclophilin D (Figure 1.4b). The opening of the pore can be induced by various physiological effectors such as calcium, a decrease in the concentration of adenine nucleotide or inorganic phosphate, the production of oxygen free radicals or a change in pH (Crompton, 1999), and by the presence of Bax protein.

The opening of the pore increases the permeability of the inner membrane of the mitochondria. This leads to dissipation of the mitochondrial membrane potential (proton-dependent), a chemical imbalance between the cytoplasm and the mitochondrial matrix and an uncoupling of oxidative phosphorylation, thus causing an osmotic swelling which can lead to rupture of the outer membrane. Importantly, it has been suggested that the amount of ATP available after the opening of the pore is a key factor in the induction of death by necrosis or apoptosis. In addition, members of the Bcl-2 family can regulate the opening of the pore. Bcl-2 can prevent this opening (Kroemer et al., 1997, Shimizu et al., 1998); Bax, on the other hand, causes a drop in membrane potential mitochondrial and promotes the opening (Marzo et al., 1998) (Figure 1.4d).

1.4.5 Pore formation by members of the Bcl-2 family

It remains unclear, however, what the chronology of events is, i.e. whether the pore opening is the cause or the consequence of the release of cytochrome c. Indeed, several studies have shown that the release of cytochrome c can occur in the absence or before the collapse of mitochondrial membrane potential (Bossy-Wetzel et al., 1998, Goldstein et al., 2000). One explanation of this phenomenon is that the reversible (transient) MPTP opening may affect the permeability of the mitochondrial outer membrane, while allowing restoration of the mitochondrial membrane potential. In addition, the opening of the pore may be a consequence of the inhibition of electron transport due to the release of cytochrome c, resulting in a fall of the mitochondrial membrane potential and the level of ATP, or the consequence of the activation of caspases (Marzo et al., 1998). Indeed, caspase inhibitors can prevent the collapse of mitochondrial membrane potential without blocking the release of cytochrome c (Bossy-Wetzel et al., 1998). Opening caspase-dependent MPTP could amplify the loop through which the early release of cytochrome c induces changes at the mitochondrial level. This model would seem to reinforce the observations made, i.e. the

release of cytochrome c followed by a drop in mitochondrial membrane potential. Rupture of the outer mitochondrial membrane explains the massive release of pro-apoptotic factors or soluble inner mitochondrial membrane proteins (SIMP) contained in the mitochondria (AIF, caspases...) (Bossy-Wetzel et al., 1998).

Nevertheless, many studies indicate that these changes would be a consequence rather than a cause of the release of cytochrome c. Therefore, another mechanism must allow the release of cytochrome c. The assumption of a channel capable of passing the protein has been studied. It could be formed by some members of the Bcl-2 in view of the strong homology of Bcl-xL with the subunit of diphtheria toxin capable of forming a membrane pore. It has been suggested that of the Bcl-2 family such as Bax can insert themselves, following appropriate conformational change (see previous sections), in the outer mitochondrial membrane (Figure 1.4c). Whether these proteins, consisting of a hydrophobic region and a helix alpha (α) surrounded by five amphipathic helices (Schendel et al., 1998), can be inserted into the lipid bilayer and oligomerised to form a channel that is large enough to pass small proteins remains to be demonstrated. It has been shown that these proteins could form a functional ion channel in synthetic lipid vesicles. These channels are pH-dependent, have voltage and show low ionic selectivity. The properties of the channels formed by proteins, pro-or anti-apoptotic, differ significantly (Schlesinger et al., 1997).

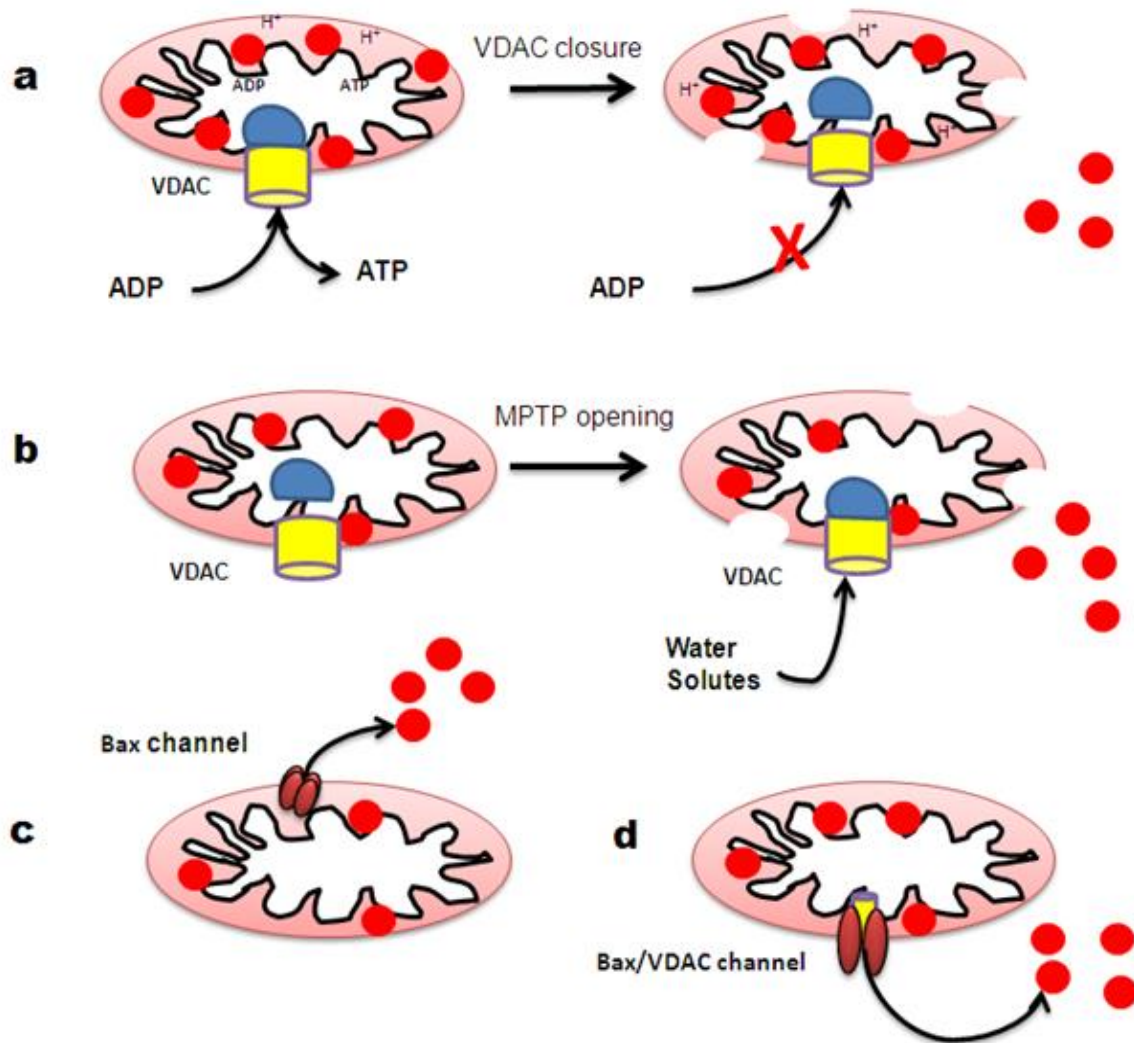


Figure 1. 4 Mechanisms of channel opening explaining the release of cytochrome c

Mechanisms of mitochondrial membrane permeabilization are regulated by the pro-apoptotic Bcl-2 family and other proteins. (a) Increase in mitochondrial membrane potential may cause the osmotic swelling of the matrix, resulting in the rupture of the outer mitochondrial membrane. (b) Opening of the MPTP (which includes among others VDAC, ANT and cyclophilin D) causes the ingress of water which leads to the bursting of the outer membrane. (c) Formation of channels by Bax or Bak. (d) Formation of chimeric channels, such as Bax / VDAC (from (Desagher and Martinou, 2000)).

1.5 Pathways for the induction of apoptosis

The initiation stage of apoptosis is a reversible phenomenon in which the apoptotic signal (-intra or -extra-cellular) is transmitted by the initiator caspases following recruitment of adaptor molecules (Reed, 2000).

There are two main caspase-dependent signalling pathways of apoptosis: the death receptor pathway (also known as the Extrinsic pathway) and the mitochondrial pathway (or Intrinsic pathway) (Reed, 2000). These pathways appear to be distinct, but the Death Receptor pathways can cross-talk with and cause apoptosis by the mitochondrial pathway through cleavage and activation of Bid as described above (Figure 1.5). In addition to these two classical pathways, more recently the apoptotic pathway that involves the endoplasmic reticulum-dependent caspase-12 has been demonstrated (Oyadomari et al., 2002), and another caspase-independent apoptotic pathway is can be initiated by the mitochondria through the release of an apoptosis-inducing factor (AIF) (Elmore, 2007).

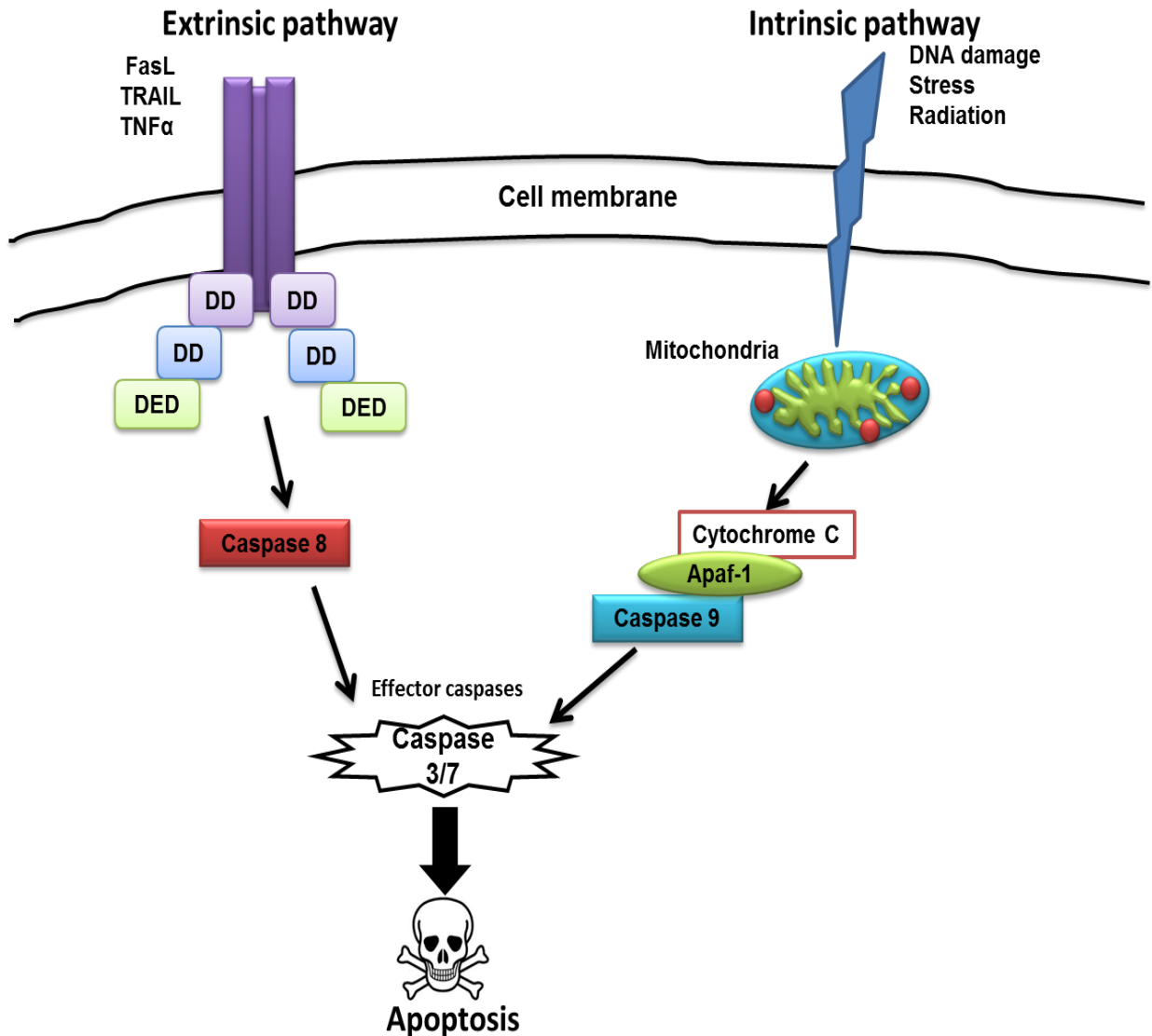


Figure 1. 5 The extrinsic and intrinsic pathways of apoptosis

The receptor-mediated (extrinsic) pathway is triggered by members of the TNFR subfamily known as the Death Receptors (such as CD95/Fas/Apo-1). Receptor-specific ligands mediate receptor aggregation and formation of a death-inducing complex, which recruits pro-caspases via death domain proteins associated with the receptor. The mitochondrial pathway is often activated in response to DNA damage, involving the activation of a pro-apoptotic member of the Bcl-2 family (Bax, Bid). Pro- and anti-apoptotic Bcl-2 family members regulate the release of cytochrome c from the inner mitochondrial membrane. Released cytochrome c associates with Apaf-1, dATP and procaspase-9 forming the apoptosome. Subsequently effector caspases are activated, resulting in the cleavage of specific substrates and cell death (Adapted from (Reed, 2000)).

1.5.1 The death receptor pathway (the extrinsic apoptotic pathway)

The ligands, which are members of the family of tumour necrosis factor (TNF) and are responsible for the activation of their cognate receptors, play an important role in cell proliferation, differentiation, apoptosis, modulation of the immune response and the induction of inflammation (Pitti et al., 1996). More than sixteen members of the TNF ligand family have been identified, including TNF α , FasL, TRAIL (APO- 2L), lymphotoxin α , lymphotoxin β , CD27L, CD30L, CD40L, CD137L, OX40L, RANKL, LIGHT, TWEAK, APRIL, and TL1 BAFF. Most ligands are synthesised as transmembrane precursors before their extracellular domains are cleaved by the action of metalloproteinases to form soluble forms. The ligands are produced in the form of trimers and bind to receptors of the TNF receptor family (the TNFRs), which are transmembrane proteins characterised by a cysteine-rich extracellular pattern (on the extracellular region) and a death domain (DD) in their cytoplasmic tail. Therefore, cell death initiated by these ligands requires receptor trimerisation (Pitti et al., 1996).

Not all of these ligands induce cell death. Indeed, CD27L, CD30L and CD40L can be involved in cell survival, unlike ligands TNF α , FasL or TRAIL. Death induced by the members of the TNF family of receptors leads to the activation of caspases and is dependent (Longthorne and Williams, 1997).

1.5.1.1 Fas (CD95, Apo -1) and Fas ligand (CD178, FasL)

Glycoprotein Fas (CD95, Apo-1) is a transmembrane receptor constitutively expressed in the lymphocytes, while the transmembrane ligand FasL (CD95L, CD178, Apo-1L) is induced after activation. The Fas receptor is also expressed on the surface of many cell types. The ligand can be released from the cell surface as soluble protein, and it also has the ability to bind with the receptor. The steps in the apoptotic pathway mediated by Fas are shown in Figure 1.6. Binding of the ligand to its receptor results in receptor trimerisation (and multimerisation) and activation. The cytoplasmic domain of Fas has no intrinsic enzymatic activity but contains a death domain (DD) of 80 amino acids, enabling binding of an adaptor protein, Fas-associated death domain (FADD). FADD also has, in addition to its DD, a death effector domain (DED), allowing it to bind with pro-caspase-8 (FLICE or "FADD - like ICE") or pro-caspase-10. The formation of this complex, called Death-Inducing Signalling Complex (DISC), initiates the enzymatic activation of apoptosis. Active caspases are released from the complex and will activate other pro-caspases such as pro-caspase -3, -6, and -7. These effector caspases induce activation of different substrates, causing cellular changes characteristic of apoptosis (Gupta, 2003).

1.5.1.2 The TNF/TNF-R pathway

TNF- α is secreted primarily by activated macrophages and lymphocytes in response to infection. This factor acts by binding to receptors of type 1 and 2 (TNF-R1 and TNF-R2) and activates several signalling pathways. Both receptors are transmembrane receptors which differ in their cytoplasmic domain. These two receptors can induce a cell survival signal, but TNF-R1 can also cause a death signal by DD domain. The binding of TNF- α to its receptor can lead to activation of the transcription factors NF- κ B and (anti-apoptotic) (Figure 1.6) and AP-1 (apoptotic) (Hsu et al., 1995).

The binding of TNF α causes trimerisation of TNF-R1 and allows binding of the adaptor protein TRADD (TNF-R Associated death domain); this adaptor will in turn recruit FADD through its DD domain. Similarly, as in the apoptosis induced by the Fas receptor, caspase -8 or -10 will be activated by the DISC TNF-R1 / TRADD / FADD to act on effector caspases -3, -6 -7 (Figure 1.7) (Boldin et al., 1996).

However, TNF-R1 can also activate an independent pathway of FADD via the receptor interacting protein (RIP), although this route is less common than the FADD-dependent pathway. TRADD has a field that can be associated with the RIP protein. The latter is associated with the protein RAIDD (RIPK1 domain containing adapter with DD) which has a CARD domain (Recruiting caspase domain); this domain also has caspases-3, -9 and -2. Although the activation of caspase-8 and -10 is FADD dependent, activation of caspase-2 is independent of FADD and is done through the DISC TNF-R1/TRADD/RIP/RAIDD, as shown in Figure 1.6 (Karin and Lin, 2002).

TNF α can also induce a cell survival signal through two types of adaptor proteins, TNFR-associated factor-2 (TRAF-2) and RIP. TRAF-2 and RIP can induce cell survival by activation of the MAP kinase pathway and the NF- κ B, respectively. TNF-R2 receptor does not have a cytoplasmic domain (DD), but TNF α binding to TNF-R2 leads to the interaction of TRAF-1 and -2 in the cytoplasmic tail of TNF-R2. It has been reported that TNF-R2 has an important role that induces anti-apoptotic and inflammatory responses by TNF-R1 (Declercq et al., 1998).

The DR3 receptor resembles TNF-R1, and it induces apoptosis in the same way through the proteins TRADD, FADD and caspase-8. The ligand of this receptor, Apo3L, is close to the TNF but is synthesised constitutively in all tissues, unlike TNF which is synthesised after activation of macrophages and lymphocytes (Choi et al., 2008, Janeway et al., 2001).

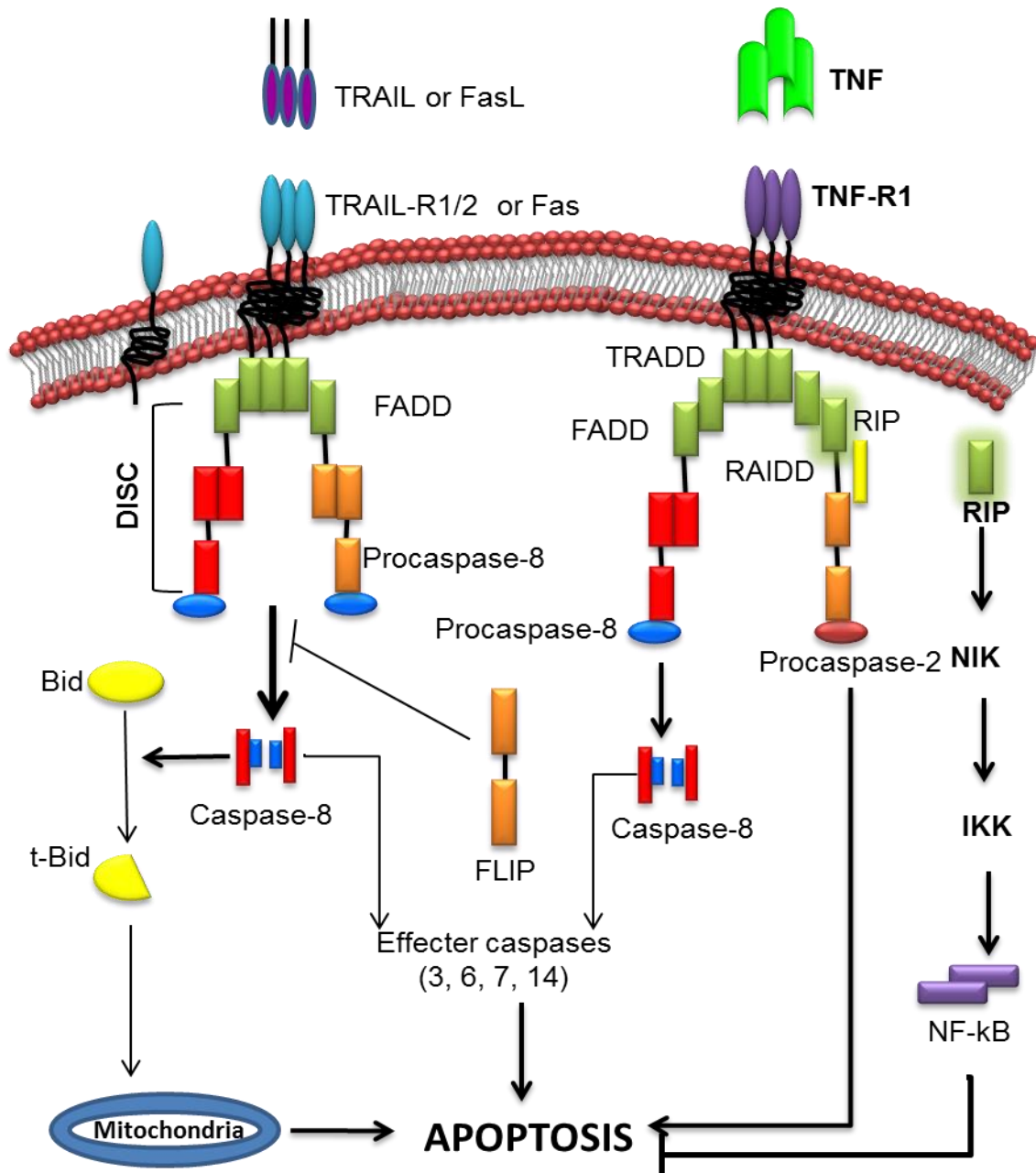


Figure 1. 6 The death receptor pathway (extrinsic pathway)

Regulation of apoptosis by death receptors, Fas signalling induces apoptosis, while TNF-R1 can induce or represses death. The case of Fas provides the simplest model of triggering apoptosis by death receptors. In the presence of ligand (FasL), Fas trimerises and recruits, via its cytoplasmic domain DD (death domain), the FADD (Fas Associated Death Domain) adapter with a DD-DD interaction. FADD also contains a DED and in turn recruits pro-caspases-8 interaction domains between homologous DED. This activates pro-caspase-8 by means of induced proximity and leads to the release of active caspase-8. But TNF- α binding to TNF-R1 can also result in survival signals when TRADD binds other mediators. The RIP (Receptor Interactive Protein) is essential for the activation of the anti-apoptotic NF-kB by TNF-R1 and interferes with the binding between TRADD and FADD (which leads to the activation of pro-caspase -8) by associating transiently TRADD. Blocking or inhibition of the activation of NF-kappa B plays an important role in regulating signal (survival or death) induced by TNF-R1. (Adapted from (Gupta, 2003).

1.5.1.3 The TRAIL (Apo-2L) receptor pathway

Similar to TNF ligands, tumour necrosis factor-related apoptosis-inducing ligand (TRAIL or Apo2L) is a transmembrane protein of approximately 34 kDa, which can form trimers as with other members of the TNF family. The interaction between the TRAIL and TRAIL-R1 receptors (DR4) and/or TRAIL/R2 (DR5, Apo-2, TRICK2) quickly induces cell death in target cells and particularly in tumour cells (Pitti et al., 1996, Mariani et al., 1997). TRAIL ligands and their receptors are constitutively expressed in many tissues (Pitti et al., 1996, Mariani et al., 1997), which implies the existence of a control mechanism of apoptosis induced by TRAIL. Ligand-binding to the receptor allows the interaction of this complex with adaptor proteins such as FADD or TRADD (previously described in section 1.5.1).

The progress-induced signalling by the receptors TRAIL-R1 and TRAIL-R2 is similar to that induced through FasL/Fas (Figure 1.7). Indeed, procaspase-8 is activated by interaction of DED present in the adapter proteins as well as procaspase-8. Three other receptors belonging to the family of TRAIL receptors have been identified: TRAIL-R3 (DcR1, TRID, LIT), TRAIL-R4 (DcR2, TRUNDD) and osteoprotegerin (OPG). Their function as modulators interferes with the activity of death receptors because they do not have their own cytoplasmic domain. For this reason they are considered to be non-apoptotic and represent a control mechanism of TRAIL-induced apoptosis (Figure 1.7). Osteoprotegerin has been described as a soluble receptor capable of binding to TRAIL and inhibiting its action (Emery et al., 1998).

1.5.1.4 Regulation of the Extrinsic apoptotic pathway

Apoptosis mediated by the extrinsic pathway is regulated in particular at the DISC complex assembly or its activation. The FLIP protein (FLICE- inhibitory protein) is an isoform of the caspase-8 containing two DED but no areas of the catalytic site. It acts by competing with caspase-8 and -10 and preventing their recruitment at the DISC (Figure 1.6). Two FLIP isoforms have been identified, the long (FLIP_L) and the short (FLIP_S) cellular form. They were presented to bind to the CD95 DISC and thus inhibit activation of caspase 8. Both are capable of inhibiting the induction of apoptosis induced several death receptors (CD95, TNF-R1, DR3, and DR4), suggesting that these receptors use similar signalling pathway (Krueger et al., 2001). FLIP over-expression induces resistance to receptor-mediated apoptosis. Furthermore, it has been shown that FLIP could induce the activation of the transcription factor NF- κ B and the Extracellular signal-Regulated protein Kinase (ERK) signalling pathway (Kataoka et al., 2000). Therefore, these proteins act as anti-apoptotic mediators (Krueger et al., 2001).

Another mode of regulation relates to the receptor itself. Most of the TNFRs also exist in a soluble form following alternative splicing or by proteolysis. These soluble forms, therefore, compete vis-a-vis the transmembrane form of the receptor with the ligand, thus blocking the recruitment of adaptor proteins and, therefore, activation of pro-caspases initiators. In addition, these soluble forms have the PLAD domain (pre-ligand assembly domain) necessary for the trimerisation of these receptors, but distinct from the ligand-binding domain. Two studies have shown that a Fas trimer could be assembled independently of ligand binding (Papoff et al., 1999, Siegel et al., 2000).

1.5.1.5 Amplification of the death receptor pathway

The classical death receptor pathway occurs in cells expressing caspase-8, but in other cell types this route can be amplified via cross-talk with the mitochondrial pathway through the activation of Bid by caspase-8 (Figure 1.6). Indeed, caspase-8 cleaves Bid, a member of the Bcl-2, N-terminal to the exposure of its BH3 domain level. The rapid translocation of the truncated form of Bid from the cytosol to the mitochondrial membrane suggests a similar association of the ligand to a specific receptor mechanism (Wang et al., 1996). The exposure of the BH3 domain allows Bid to fit into the mitochondrial membrane and bind Bax or other pro-apoptotic proteins. Bid causes the release of cytochrome c and induces the activation of caspase-9 before activating caspase-3 (Wang et al., 1996).

Another protein forming the junction between the two channels has been identified. This is the BAR protein (Bifunctional Apoptosis Regulator), a regulatory protein capable of associating with anti-apoptotic molecules Bcl-2/Bcl-xL by a SAM domain (Sterile Alpha Motif) and caspase-8 by the DED domain (Zhang et al., 2000). Another signalling pathway of Fas independent of caspase-8 has been proposed showing the involvement of serine-threonine kinase RIP (receptor-interacting protein) (Pitti et al., 1996).

1.5.2 The mitochondrial pathway (the intrinsic apoptotic pathway)

1.5.2.1 The caspase-dependent mitochondrial pathway

Many stimuli, such as chemotherapeutic agents, UV radiation, cellular stress (e.g. lack of attachment to substrate or 'anoikis') and lack of growth factors ('death by neglect'), appear to induce apoptosis via the mitochondrial pathway independent of a death receptor pathway. The mitochondrion is an organelle comprising an outer membrane, a transmembrane area, an inner membrane and a matrix. The inner membrane has several proteins such as ATP synthase, the electron transport chain and the Adenylic Nucleotide Transporter (ANT) (Zoratti and Szabò, 1995). Under normal physiological conditions, these

three proteins allow the formation of an electrochemical gradient (membrane potential) by the respiratory chain. The inner membrane space contains cytochrome c, some pro-caspases (-2, -3, and -9), the Smac /Diablo protein, Apoptosis-Inducing Factor protein (AIF) and endonuclease G. The outer membrane has a voltage-dependent anion channel (VDAC), which creates a major pathway for ATP/ADP, cytochrome c and other mitochondrial metabolites through the mitochondrial outer membrane (MOM) (Shoshan-Barmatz and Gincel, 2003). Then causes the release of these proteins into the cytoplasm, and the permeabilisation of the inner membrane causes a change of the mitochondrial membrane potential as well as the release of cytochrome c, which is one of the major steps in the induction of apoptosis by the mitochondria (Figure 1.7) (Ravagnan et al., 2001).

1.5.2.2 Cytochrome c

Cytochrome c is encoded by a nuclear gene and is synthesised as a precursor that is unable to participate in the induction of apoptosis. The precursor is imported into the mitochondria where it is processed. The protein becomes globular due to cytochrome c heme lyase (CCHL). Apo-cytochrome c, the precursor of cytochrome c, has relatively little detectable secondary structure and lacks covalently attached heme compared to its mature counterpart holocytochrome c (Fisher et al., 1973). It is a nuclear gene product which is synthesized on free cytoplasmic ribosomes and then released into a cytoplasmic pool. Its import into mitochondria is mediated by specific binding sites (Zimmermann et al., 1981). During import, heme is covalently attached via thioether linkages to cysteine residues, near the amino terminus of the apo-cytochrome c precursor, in a reaction which is catalysed by the enzyme cytochrome c heme lyase (CCHL) (Taniuchi et al., 1983). The process requires heme in the reduced state (19) and is coupled to the transport of cytochrome c across the outer mitochondrial membrane. Cytochrome c is sequestered in the mitochondrial intermembrane space where it exerts its physiological function of electron transport between complexes III and IV of the respiratory chain (Ravagnan et al., 2002). In 1996, Liu and colleagues showed that cytochrome c was required for activation of caspase-3 (Liu et al., 1996a). Other studies have confirmed this by showing that the release of cytochrome c and caspase activation was blocked by the anti-apoptotic protein Bcl-2 (Kluck et al., 1997, Yang et al., 1997).

It is now well-established that cytochrome c released into the cytosol is the origin of the formation of the apoptosome. Most recently, the knockout of the gene-encoding cytochrome c confirmed the crucial importance of this protein in apoptosis (Vempati et al., 2007). These studies show that no other cellular protein can replace cytochrome c for the oligomerisation of Apaf-1 (Apoptotic protease activating factor-1) and activation of caspase-

3 induced by cellular stress agents or agents targeting the mitochondria cellular stress or a mitochondrial targeting agent. (Li et al., 2000). In most cases, the release of cytochrome c is dependent on caspase activity (Bossy-Wetzel et al., 1998).

The mechanism by which cytochrome c is released, as well as its release kinetics, has been the subject of much controversy. The release of cytochrome c seems to be dependent on the presence of Bax or Bak in the outer mitochondrial membrane. Regarding the kinetics of the release of cytochrome c, work by Douglas Green's team has demonstrated that it occurs rapidly and at once (Goldstein et al., 2000). It appears that the mitochondrion integrates different signals, and once the threshold is reached, the entire cytochrome c is released in one step. However, this observation cannot be generalised. According to the apoptotic stimulus, depending on the cell type used and the level of polarisation of the mitochondrial membrane, in certain cases only a proportion of cytochrome c from a few mitochondria is released in an amount sufficient to induce caspase activation. This observation suggests that there is a link between the release of cytochrome c and the collapse of mitochondrial membrane potential (MMP). Goldstein et al. (2000) showed that the release of cytochrome c was done before the fall of MMP, while Heiskanen et al. (1999) showed that these two events occur simultaneously (Goldstein et al., 2000, Heiskanen et al., 1999). However, if the release of cytochrome c occurs before the fall of MMP, this implies that the mitochondrion maintains its MMP with a group of cytochrome c which remained associated with the respiratory chain. There are therefore two groups in mitochondrial cytochrome c; a large quantity of cytochrome c would be in free form in the intermembrane space and would allow a small amount to ensure the release of mitochondrial respiration in the first group so as to maintain production of ATP required for the formation of the apoptosome (Martinou et al., 2000).

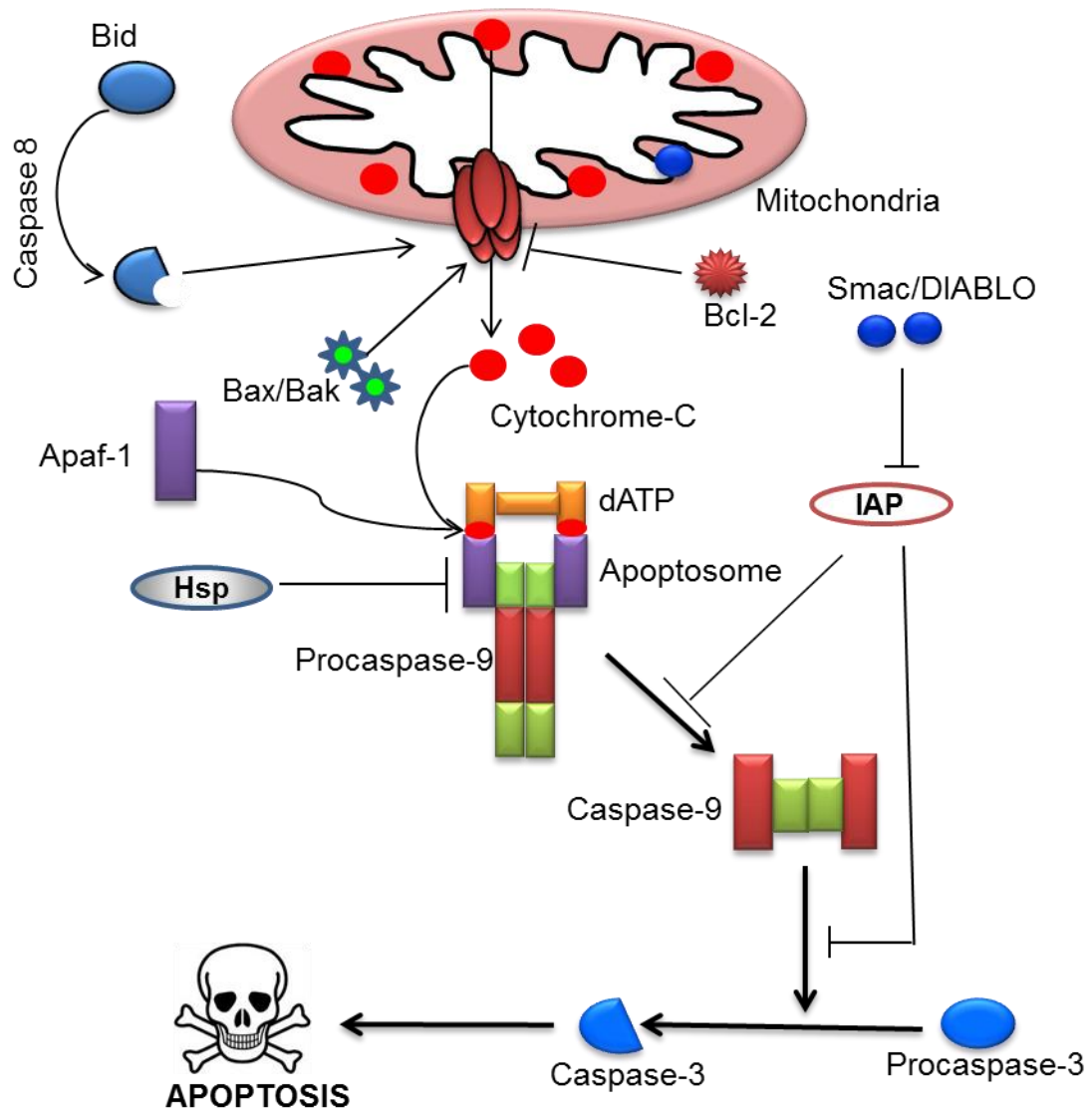


Figure 1. 7 The mitochondrial pathway of apoptosis

Many pro-apoptotic signals converge on mitochondria leading to the release of many of the intermembrane space proteins under the control of proteins of the Bcl2 family. Cytochrome c (cyt c) induces caspase activation execution (caspases-3 and -7) via the apoptosome. Smac/Diablo and Omi/HtrA2 neutralise caspase inhibition induced by the IAP (inhibitor of apoptosis protein) execution. AIF (apoptosis inducing factor) and endoG (endonuclease G) are involved in the degradation of DNA execution independent of caspase activity (adapted from (Ravagnan et al., 2002).

1.5.2.3 The protein Apaf-1

Apaf-1 (Apoptotic protease for activating factor-1) is a protein of approximately 130kDa comprising a caspase recruitment domain (CARD domain) in the amino terminal part, a region having high homology to Ced-4 and C-terminal domain containing several WD40 repeats (WD40 domains) that are involved in protein-interactions proteins (Cain et al., 2002). The WD40 repeats are necessary for binding to cytochrome c, but they are not sufficient. The WD domain also plays an important role in the recruitment of pro-caspase-3. The CARD domain of Apaf-1 is not exposed in normal conditions and therefore cannot interact with caspase-9; however, in the presence of ATP and cytochrome c, Apaf-1 changes conformation and can interact with caspase-9 through the exposure CARD domain as shown in Figure 1.8 (Li et al., 1997). In 2000, previous years, several Apaf-1 isoforms have been identified in tumor cell lines, but their expression in tissues and ability to activate procaspase-9 remain poorly characterized. Several studies have reported that Apaf-1 has at least six splice isoforms in human cells (Li et al., 1997). Also, some studies have been reported to have at least six splice isoforms in human cells. Apaf-1XL and Apaf-1L have the ability to cleave procaspase-9, binding with cytochrome C. Apaf-1 cDNAs cloned from Hela cells, Apaf-1M, and Apaf-1S, and Apaf-1 mRNA in normal tissues including prostate also has been reported (Walke and Morgan, 2000, Fu et al., 2001, Perkins et al., 2000).

1.5.2.4 The formation of the apoptosome

The apoptosome involved in apoptosis induced by mitochondria consists of Apaf-1, cytochrome c, and procaspase-9, which is a protein complex of ~700kDa in size (Figure 1.8). In the cytosol, cytochrome c interacts with the carboxy terminal domain of Apaf1 (Hu et al., 1998b) and allows the presence of nucleotides to unmask the key areas of Apaf1, the WD40 domains and CARD domain. The WD40 domains allow multimerization of Apaf-1 and the CARD domain can recruit the initiator caspase pro-caspase-9 via its own CARD domain (Cain et al., 2001). The three-dimensional structure of the apoptosome has been solved by cryo-electron microscopy (Acehan et al., 2002). The apoptosome is formed by seven Apaf1 molecules that interact with each other at their N-terminus to form a wheel-like structure (the “wheel of death”). The CARD domains of Apaf1 monomers are at the center of the apoptosome that is, as the DISC, an inducer of proximity used to locally concentrate pro-caspase-9. Association between Apaf-1 and pro-caspase-9 forms a holoenzyme complex resulting in active caspase-9 by self-dimerization and proteolytic cleavage and also increases its enzymatic activity (Rodriguez and Lazebnik, 1999). Caspase-9 has been described as capable of cleaving in both the cytoplasm and in the apoptosome, but the active caspase-9 is actually the one related to the apoptosome (Rodriguez and Lazebnik, 1999).The

autoproteolytic cleavage of caspase-9 is at its residue D315, although it should be noted that this cleavage is not required for its activity but it is a reflection of its activation (Rodriguez and Lazebnik, 1999). Once activated, caspase-9 can then cleave executionercaspases like caspase-3 and -7 (Acehan et al., 2002).

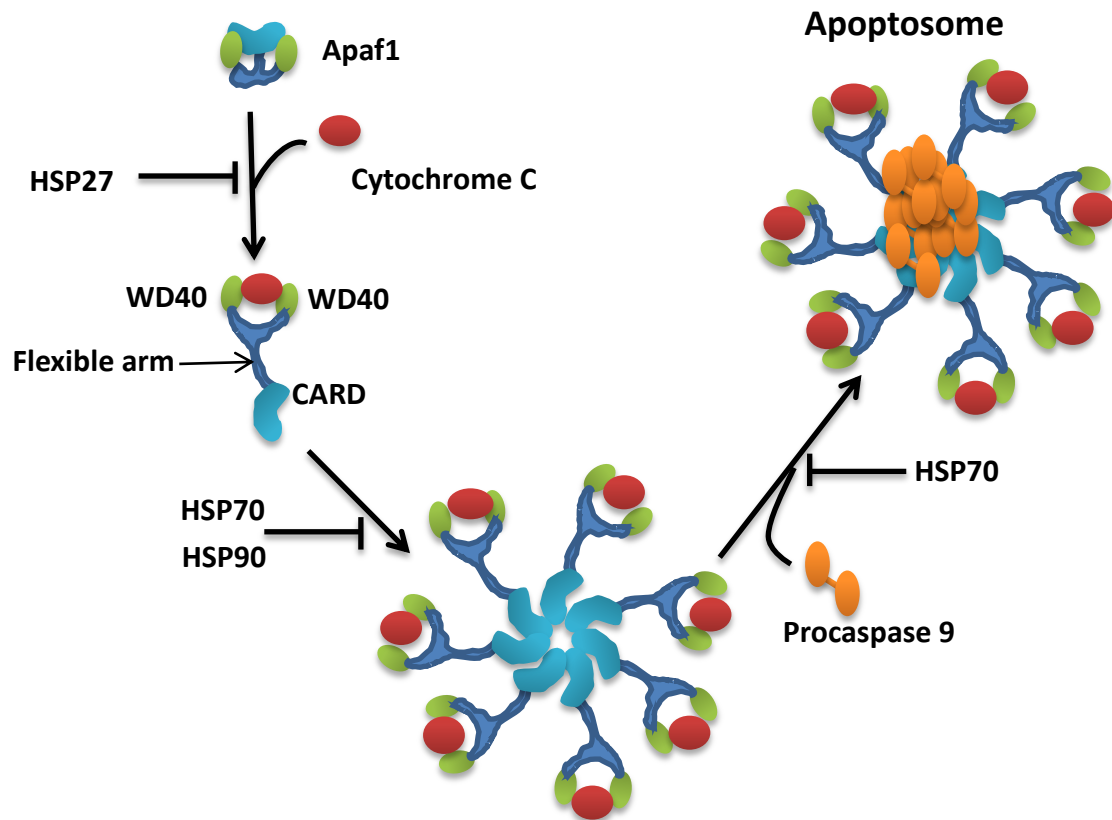


Figure 1. 8 The formation of the apoptosome and its inactivation

In the cytoplasm, cytochrome c interacts with the WD40 domain of the protein Apaf1, causing a conformational change that allows the oligomerization of Apaf1 as heptamer. The CARD domain of Apaf1 molecules are at the center of the apoptosome and will be able to recruit procaspases 9. This arrangement of Apaf1 CARD domains can concentrate procaspases 9, which promotes their activation by dimerization. The various stages of forming apoptosome are inhibited by several antiapoptotic proteins. HSP27 inhibits binding cytochrome c to Apaf1. HSP70 and 90 inhibit multimerization of Apaf1. HSP70 and inhibits the recruitment of procaspase 9. Adapted from (Acehan et al., 2002).

1.5.2.5 Role of ATP in apoptosis

The integrity of the mitochondrial membrane is often altered during apoptosis induced by the mitochondrion, which causes a reduction in ATP production accompanied by the release of apoptotic factors in the cytosol. Furthermore, apoptosis is a process requiring energy, including the apoptosome formation (Figure 1.8) for the condensation of the chromatin, for the fragmentation of DNA but also to transport molecules to the nucleus (Kass et al., 1996, Richter et al., 1996).

1.5.3 Regulation of caspase-dependent pathways

1.5.3.1 The inhibitors of apoptosis proteins (IAPs)

The IAPs are proteins that inhibit cell death by preventing cleavage of caspases and therefore their activity (Fesik and Shi, 2001) (Figure 1.8). They were originally described as inhibitors of viral, but unlike the other two viral proteins, which are CrmA bovine smallpox virus and baculovirus p35 protein, the IAPs are the only ones with homologous proteins in mammals. The IAPs comprise one or more BIR (baculoviral IAP repeat) domains that are essential for their anti-apoptotic activity and allow binding to caspases. Each BIR domain has distinct functions and a binding specificity to caspases (Verhagen et al., 2001). The BIR2 domain inhibits caspase -3 and -7, while the BIR3 domain inhibits the activity of caspase -9 (Ekert et al., 2001).

XIAP (X-linked inhibitor-of-apoptosis protein) is one of the best known of this family of molecules and it can inhibit the activation of the initiator caspase-9 as well as the effector caspases -3 and -7. Indeed, it can bind to active caspase -9 and has the ability to act on caspase-3 or caspase-7, thus blocking the apoptotic pathway (Deveraux et al., 1999, Wei et al., 2008).

1.5.3.2. Inhibitors of IAPs

Smac (Second Mitochondria-derived Activator of Caspase) and its counterpart DIABLO (Direct IAP Binding protein with Low pI) and have similarities with the Grim Reaper and HID Drosophila proteins. These block the anti-apoptotic activity of the IAP, as does Smac/DIABLO in mammals (Du et al., 2000, Verhagen et al., 2000) (Figure 1.8). Smac/DIABLO is highly expressed in the heart, liver, kidneys, spleen and in several cancer cell lines. It is synthesised in the cytoplasm as a precursor of 239 amino acids and is exported into the mitochondrion by means of its 55 amino acid N-terminal mitochondrial localisation signal (MLS). Once in the new compartment, the localisation signal is cleaved and the protein acquires its pro-apoptotic activity by homo-dimerising (Chai et al., 2000). Its

release from the mitochondria is induced by many apoptotic stimuli and controlled by members of the Bcl-2 family (Adrain et al., 2001). Smac/DIABLO was the first identified protein to directly inhibit the functions of IAPs; it is also linked to the death receptor pathway (Srinivasula et al., 2001). Smac/DIABLO interacts by binding IAPs with the third BIR domain (BIR3) of XIAP; in this way it prevents the binding of IAPs to caspase -3, -7 and -9. XAF-1 (XIAP associated factor 1) is a protein capable of activating other caspases by inhibiting IAPs (Figure 1.8). Unlike Smac/DIABLO, XAF-1 is a continuously active nuclear protein which has a zinc finger domain allowing it to interact directly with XIAP. Its expression seems to be reduced in some cancer cell lines (Liston et al., 2001).

Smac and XAF-1 are not the only inhibitory proteins known to target IAPs. More recently Omi/HtrA2 (high temperature requirement protein A2) was identified as a new inhibitory protein (Martins et al., 2002, Verhagen et al., 2001) (Figure 1.8). The precursor HtrA2 is 50kDa protein in which the N-terminal part contains the MLS. This is cleaved after being imported into the mitochondria and then generates a protein of 36 kDa. HtrA2 belongs to the family of serine proteases that are conserved during evolution. In normal human cells, HtrA2 is contained in the mitochondrial intermembrane space, but following apoptosis induction with various agents such as staurosporine, TRAIL, or UV irradiation, it is released into the cytosol. In the cytosol, HtrA2 binds to XIAP and IAPs in the same manner as Smac /DIABLO and facilitates caspase activation. HtrA2 induces apoptosis by two different mechanisms, the first by inhibition of IAPs to caspase activation, and the second by the serine protease activity independent of caspase (Hegde et al., 2002, Suzuki et al., 2001).

1.5.3.3 Other regulatory proteins

Other complex proteins involved in the regulation of the apoptotic signal exist in other "strategic sites" of the cell, such as the endoplasmic reticulum, the Golgi apparatus, the lysosomes or the nucleus (Ferri and Kroemer, 2001). Like the activity of the XAF-1 protein recently described, the nucleus also has a role in the regulation of apoptosis and it does not suffer the events used to remove the cell. Another nuclear protein DESD (DED-containing DNA-binding protein) exerts its anti-apoptotic activity by inhibiting the activation of caspase-6 or by blocking transcription. This protein seems to be modulated by another protein called DEDAF (DED- associated factor), which is also able to bind to FADD and caspase -8 or -10 and promote the formation of the DISC (Zheng et al., 2001).

Members of the Bcl-2 family (Bcl-2, Bax, Bid, Bak) can also regulate the mitochondrial pathway by acting on the release of cytochrome c (Hu et al., 1998a). Hu and his colleagues have demonstrated that Bcl-xL interacts with caspase-9 and Apaf-1, and also in that study

showed that Bcl-xL inhibits the maturation of caspase-9 that was mediated by Apaf-1, a process that was conserved from nematodes to humans (Hu et al., 1998a).

In addition to that, the heat shock proteins or Hsp are also both inducers and inhibiting factors of apoptosis (Figure 1.9). Various studies show that the Hsp-70 and Hsp-90 can bind to the CARD domain of Apaf-1, thus preventing the oligomerisation and activation of pro-caspase-9 (Pandey et al., 2000, Saleh et al., 2000). Hsp-27 blocks the oligomerisation of Apaf-1 by binding to cytochrome c (Bruey et al., 2000).

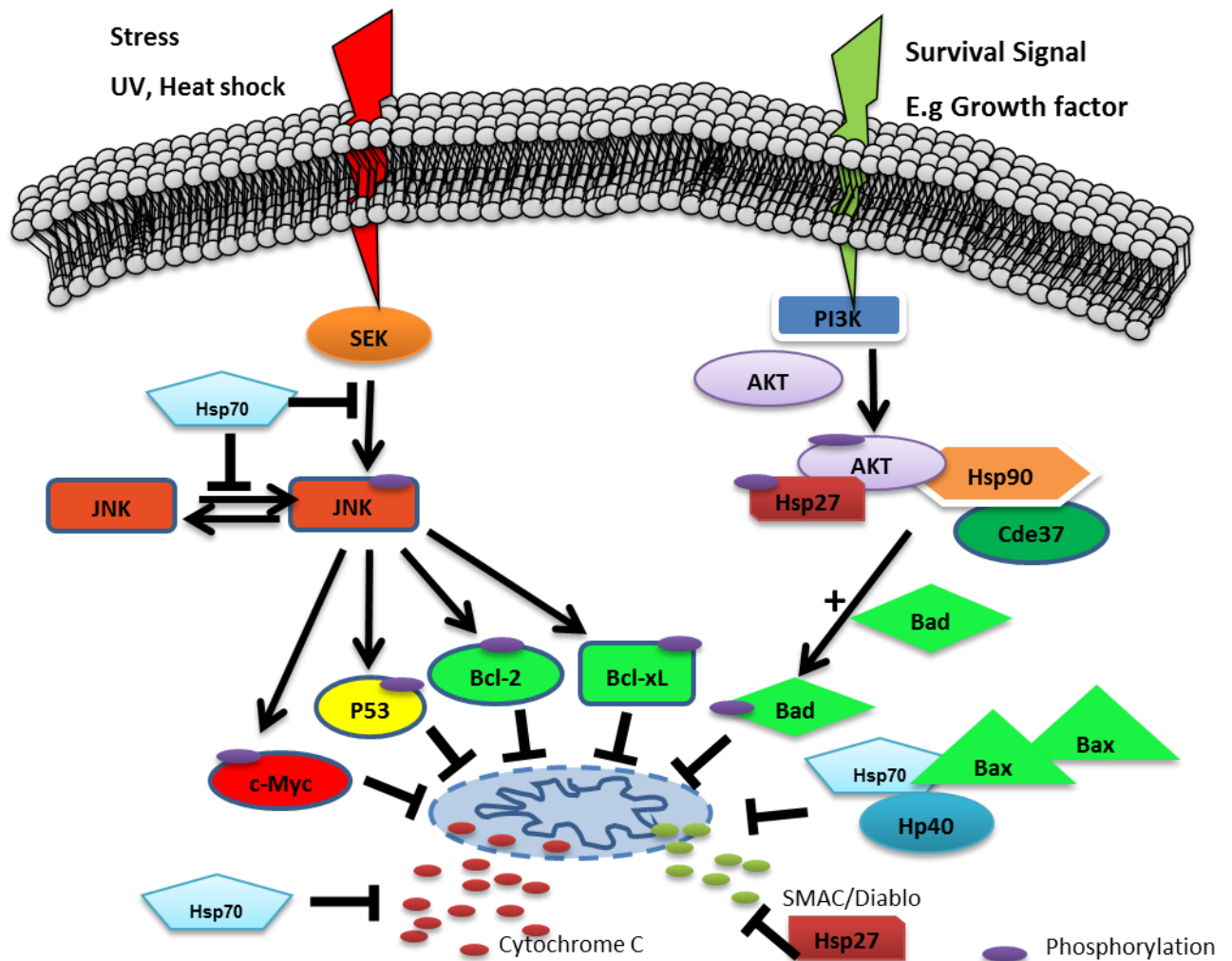


Figure 1. 9 Events regulated by Heat shock proteins (HSPs) in the mitochondrial pathway

Extracellular signals (Survival signals) or stresses (Death signals) congregate to control the mitochondria mediated pathway to caspase activation and apoptosis. HSPs intervene at multiple points within this pathway; upstream of the associated mitochondrial changes to regulate the engagement and/or progression of apoptotic events. HSP-mediated potentiation of a signalling pathway is depicted as a direct interaction between the HSP and its target (+) Adapted from (Beere, 2004).

1.5.4 The caspase-independent mitochondrial pathway

Several proteins in the intermembrane space can induce apoptosis directly without activation of caspases. This is the case in the induction of apoptotic factor (AIF) and Endonuclease G (Endo G) which, when released from mitochondria, translocate to the nucleus causing chromatin condensation and cleavage of DNA thus generating large fragments of DNA (Figure 1.7) Lorenzo et al., 1999).

1.5.4.1 The AIF

The AIF factor was identified several years ago by Guido Kroemer's team (Susin et al., 1999, Cande et al., 2002). The AIF gene is located on chromosome X and encodes a protein of 57kDa. AIF is a flavoprotein consisting of three domains: the MLS sequence at the amino-terminal side, a sequence of 27 amino acids and a domain with oxidoreductase activity of carboxy-terminal 485 amino acids (Lorenzo et al., 1999). The precursor of the AIF (67 kDa) is synthesised in the cytoplasm and then imported into the mitochondria (Susin et al., 1999). Once in the intermembrane space, the MLS sequence is cleaved and the protein changes its conformation while incorporating the prosthetic group FAD (Flavin adenine dinucleotide). This is probably a bifunctional protein with an oxidoreductase activity and pro-apoptotic role (Ye et al., 2002).

After cell exposure to pro-apoptotic stimuli, AIF translocates from the intermembrane space into the cytosol and to the nucleus (Susin et al., 2000). This usually precedes the release of cytochrome c. The mechanism by which the AIF translocates to the cytosol is still unknown. Transport to the nucleus could be due to a nuclear localisation sequence. The effect of the AIF as an apoptogenic molecule has been studied both *in vitro* and *in vivo*. *In vitro* it has been shown that the AIF translocates through the cytoplasm to the nucleus, the place where it leads to chromatin condensation and DNA degradation into large fragments of 50 kbp condensations; this is a direct interaction with DNA without sequence specificity (Ye et al., 2002). This interaction is mainly by its carboxy-terminal domain, and it may be modulated by the level of translocation to the nucleus and is greater during the phase of condensation during the late phase of formation of apoptotic bodies (Ye et al., 2002).

In addition, the effects of AIF are negated by over-expression of Bcl-2 and are the same in cells with or without caspase activities: the action of AIF is thus independent of caspases. AIF can also be inhibited by an endogenous protein, Hsp70 (Ravagnan et al., 2001). This has been shown *in vitro* by chromatin condensation and *in vivo* by the nuclear and mitochondrial level overexpression of Hsp70. The action of Hsp70 is due to apoptosis by firstly inhibiting the formation of the apoptosome as Hsp70 binds to Apaf-1. But

overexpression of Hsp70 in cells not expressing caspase also prevents cell death; this suggests that Hsp70 can bind to other proteins such as AIF. The binding of Hsp70 to Apaf-1 and AIF appears to be by the binding domain of Hsp70 ATP present, without intervention of Hsp70 chaperone activity (Ravagnan et al., 2001).

Many aspects of the function of AIF are still unknown, including its mode of action on DNA, its oxidoreductase activity and its signal transduction. The DNA condensation observed during apoptosis may be explained by the direct interaction of the DNA- AIF (Ye et al., 2002). Indeed, this interaction could alter the structure of chromatin and promote nucleases such as topoisomerase II or cyclophilin, which also generate fragments of 50kb similar to those obtained after induction of AIF. These fragments correspond to the loop-like structures at the level of chromatin (Widlak and Garrard, 2009, Samejima et al., 2001).

1.5.4.2 Endonuclease G

Endonuclease G is a highly conserved non-specific nuclease (Li et al., 2001). It is encoded by a nuclear gene and probably involved in the replication of the mitochondrial genome. During apoptosis, endonuclease G passes through the outer membrane of the mitochondria and translocated into the nucleus (van Loo et al., 2001). Endonuclease G can act with exo-nuclease and the DNase I in the nucleus to generate DNA fragments of higher molecular weight (Widlak et al., 2001), but it can also generate oligonucleosomal fragments (Samejima et al., 2001).

1.6 Reactive oxygen species (ROS)

ROS represent an intensive area of current research due to their role as signalling molecules in pathways controlling an array of aspects of cellular physiology. This concept has been there ever since it was essentially brought out in the 1990s when it was found that hydrogen peroxide was mandatory in cytokine, hormone (such as insulin), growth factor, as well as AP-1 and NF- κ B signalling (Finkel, 1998).

Not before long, many reports pointed out that H_2O_2 may endorse phosphatase inactivation using cysteine oxidation and gave reasonable biochemical mechanisms in which ROS could be capable of imposing on signalling routes (Rhee et al., 2000). Simultaneous with these preliminary findings on ROS signaling, the common concept of the mitochondrion being the “powerhouse of the cell” was contested with the unearthing that letting loose of cytochrome c regulates apoptosis (Liu et al., 1996b). The notion that a mitochondrial protein that is involved in oxidative phosphorylation similarly takes a major part in signalling pathway, unlocked the likelihood that metabolism-related signaling is a rather common but not unique system. Even though it was evident that metabolism was controlled by cellular signalling pathways, it was however not greatly valued that metabolism could react to control signalling. Cytochrome c's two-fold role implied a cross-talk between mitochondrial metabolism and signaling pathways. It could possibly be that the release of mitochondrial ROS was a different way of this cross-talk (Liu et al., 1996b).

The first studies to check this likelihood brought out that mitochondria emit H_2O_2 under physiological hypoxia to initiate the transcription factor hypoxia inducible factor1 (HIF-1), which is essential for metabolic adaptation in low oxygen (Chandel et al., 1998). After that, mitochondrial release of H_2O_2 appeared to initiate c-Jun N-terminal kinase1 (JNK1), p53, and NF- κ B (Chandel et al., 2000, Nemoto et al., 2000). There have been a lot of reports that brought out the significance of ROS-dependent signaling in numerous systems (Collins et al., 2012). When brought together, this information proposed that the release of ROS represents an important mode of communication (amid mitochondrial role) and other cellular processes to sustain homeostasis and encourage adaptation to constant stress (Collins et al., 2012).

1.6.1 Mitochondria-generated ROS

ROS are superoxide molecules that are formed from oxygen (O_2) and can easily oxidize other molecules. The majority of the intracellular ROS originate from superoxide ($O_2^{\cdot-}$), which is made by the one electron reduction of O_2 . Superoxide is changed into hydrogen peroxide (H_2O_2) by superoxide dismutases (SODs) (Collins et al., 2012).

There are several location that have been identified to generate $O_2^{\cdot-}$ in mitochondria (Brand, 2010); although exactly how ROS levels are regulated *in vivo* is still unclear. What is interesting to note is that whereas these ROS generating locations can deposit $O_2^{\cdot-}$ into the mitochondrial matrix, merely place IIIQo (on complex III) and glycerol-3-phosphatedehydrogenase can emit $O_2^{\cdot-}$ in to the inter membrane space. The inter membrane space has higher chances of accessing the cytosol because all ROS have to do is traverse the external mitochondrial membrane but the matrix ROS has to traverse the internal and the external mitochondrial membranes (Muller et al., 2004). This then means that $O_2^{\cdot-}$ which is generated from site IIIQo and glycerol-3-phosphate dehydrogenase could have an advantage when it comes to signalling ability in the cytosol. Despite all this knowledge, more research is required to determine which places/sites of ROS production are physiologically significant (Muller et al., 2004).

1.6.2 Mitochondrial ROS are tightly regulated

Very tight regulation of ROS levels is essential especially because the quantities of ROS can deduce specificity and role when it comes to their capability to engage in physiological cell signaling (Figure 1.10). This then means that signaling ability of ROS is maintained on numerous levels (Muller et al., 2004).

To begin with, antioxidant enzymes are able to remove ROS. As mentioned earlier, SODs transform $O_2^{\cdot-}$ to H_2O_2 . While SOD1 is found in the mitochondrial inter membrane space and cytoplasm, SOD2 is found in the mitochondrial matrix, whereas SOD3 is tied to the extracellular matrix. Peroxiredoxin enzymes (PRXs), glutathione peroxidases enzyme (GPXs) together with catalase enzymes, have the ability to remove H_2O_2 . Mammalian cells express six PRX isoforms together with PRX3 and PRX5 in the mitochondria. PRXs perform this by going through oxidation by H_2O_2 at a functional site cysteine and thereafter reduction by thioredoxin, thioredoxin reductase, and NADPH. The available eight GPXs are oxidized by H_2O_2 and then subsequently reduced by glutathione (GSH). Catalases are located in peroxisomes. How quick these antioxidant enzymes react with H_2O_2 (rateconstant, k) and the

concentration of H_2O_2 and enzyme, concentrations of H_2O_2 remain poorly characterised. On the other hand, peroxiredoxins have elevated rate constant and this is the reason they are believed to be behind scavenging nanomolar levels of H_2O_2 linked with signalling. GPXs too have the same rate constants however, they are only vital when there is higher intracellular concentration of H_2O_2 , and this is when GPXs are able to start to compete with PRXs for substrate (Winterbourn and Hampton, 2008). This shows that PRXs are important for switching ROS signaling off as GPXs are important for cushioning elevated levels of ROS to a level which the cell cannot be destroyed and also stimulate signaling stress responses. Catalases have very low affinity for hydrogen peroxide and are limited to peroxisomes. The control of actions and expression levels of these antioxidants take place in many ways and in different operation so as to deal with ROS levels (Winterbourn and Hampton, 2008).

Besides control of ROS scavenging, controlling ROS generation could modify the signaling ability of ROS. The factors that control electron transport chain ETC production of ROS *in vivo* are not completely comprehended. Most of what is currently understood is as a result of research that involved mitochondria and cells (Murphy, 2009). It has been discovered that what largely regulates ROS generation is the redox state of the ETC. For instance, hindering ETC electron carriers results in them being decreased, as a result more of superoxide is produced. An additional significant cause of ROS generation is the proton motive force (pmf) which is made up of an electrical gradient (ψ mitochondrial membrane potential) and chemical gradient (pH) through the internal mitochondrial membrane. The pmf is produced when protons are removed out of the matrix into the inter membrane space by complexes I, III, and IV as electrons are shifted across the ETC. When there is pmf is increased it results in more ROS being generated (Echtay et al., 2002).

Finally, the signalling ability of ROS could be modified by mitochondrial localization. ROS are molecules that have a short life span and thus one way to improve their competence is by co-localisation of their site of generation and their site of signalling role. In theory, the quantity of mitochondria in the cell has the ability to modify the quantity of ROS generated and as a result, also modify their intracellular function. Nevertheless, the mitochondrial biogenesis factor increases expression of antioxidant enzymes to upfold redox (St-Pierre et al., 2006).

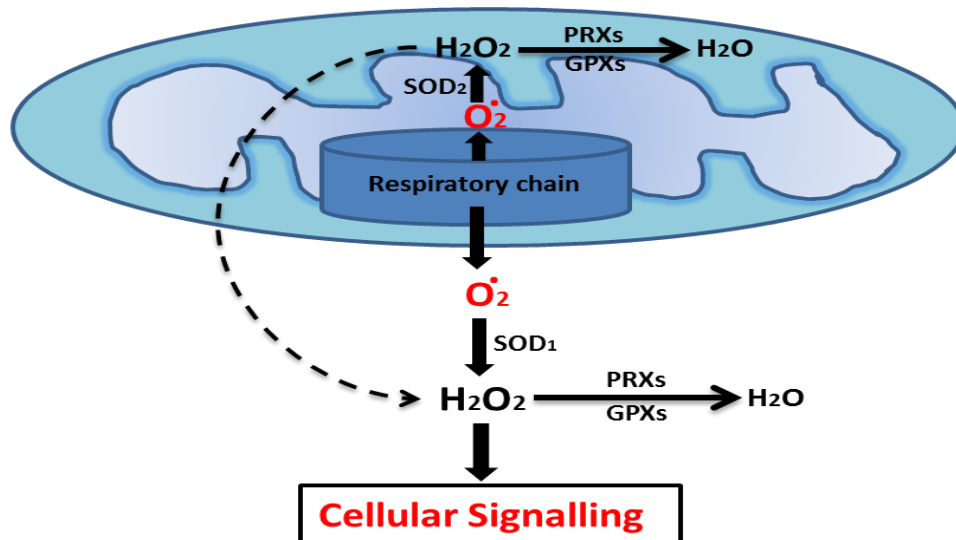


Figure 1. 10 Mitochondria Produce ROS for signalling

Superoxide ($\text{O}_2^{\bullet -}$) is generated at the mitochondrial respiratory chain and emitted into both the matrix and intermembrane space. Matrix superoxide is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase 2 (SOD2); this hydrogen peroxide can diffuse through both inner and outer mitochondrial membranes to access the cytosol or be converted to water (H_2O) by glutathione peroxidases (GPX) or peroxiredoxins (PRX). Inter-membrane space superoxide can exit the mitochondria through voltage-dependent anion channels (VDAC) and be converted to hydrogen peroxide in the cytosol by superoxide dismutase 1 (SOD1). The cytosol also possesses glutathione peroxidases and peroxiredoxins that can reduce hydrogen peroxide to water. Cytosolic hydrogen peroxide is believed to be the primary form of signalling ROS in the cell, where it can oxidize protein thiol residues (Winterbourn and Hampton, 2008).

1.6.3 Physiological targets of ROS

There is a lot of evidence that ROS can result in reversible post translational protein modifications to precisely control signaling pathways. H_2O_2 can oxidise thiol groups (-SH) on cysteine residues to develop sulphenic acid (-SOH), which is able to bond with GSH to turn it into a glutathionylated form (-SSG), with neighboring thiols to create a disulphide bond (-SS-), it could also with amides to create amide (-SN-) (Finkel, 2012). All these alterations could have an effect on the actions of the target protein thus changing its operation in a signaling pathway. Phosphatases seem to be vulnerable to control by ROS because they have a reactive cysteine which is their active site. It prevents their de-phosphorylation activity (Rhee et al., 2000). PTP1b, PTEN, and MAPK phosphatases are a good example of phosphatases that can be controlled in such a way (Rhee et al., 2000).

1.6.4 Mechanisms of ROS generation and their role as signalling molecules

The NADPH oxidase (Nox) protein family contains several isoforms, namely NOX1, NOX2, NOX3, NOX4, and NOX5 also, in parallel, two members appointed DUOX1 and DUOX2 (dual oxidase) (De Deken et al., 2000, Dupuy et al., 1999). These enzymes share the ability to transport electrons through the plasma membrane to produce superoxide and other reactive oxygen species (ROS) (Genestra, 2007).

The NOX produce ROS by electron transfer from NADPH to O_2 through an FAD heme group which then results into $O_2^{\cdot-}$. The reactive oxygen species or ROS (Reactive Oxygen Species) are small molecules including oxygen-derived oxygen radicals: superoxide anions ($O_2^{\cdot-}$), hydroxyl radicals (OH^{\cdot}), hydroperoxyl (HOO^{\cdot}) peroxy (RO_2^{\cdot}) and alkoxy (RO^{\cdot}) but also non-radical as hypochlorous acid (HOCl) compounds, ozone (O_3), singlet oxygen (1O_2) and hydrogen peroxide (H_2O_2) (Genestra, 2007). $O_2^{\cdot-}$ is changed to H_2O_2 by superoxide dismutase (SOD) and when H_2O_2 reacts with metal ions it creates water or OH^{\cdot} or otherwise could be altered to H_2O via glutathione peroxidase catalase, as shown in Figure 1.11 (Winterbourn and Hampton, 2008, Paletta-Silva et al., 2013, Terada, 2006).

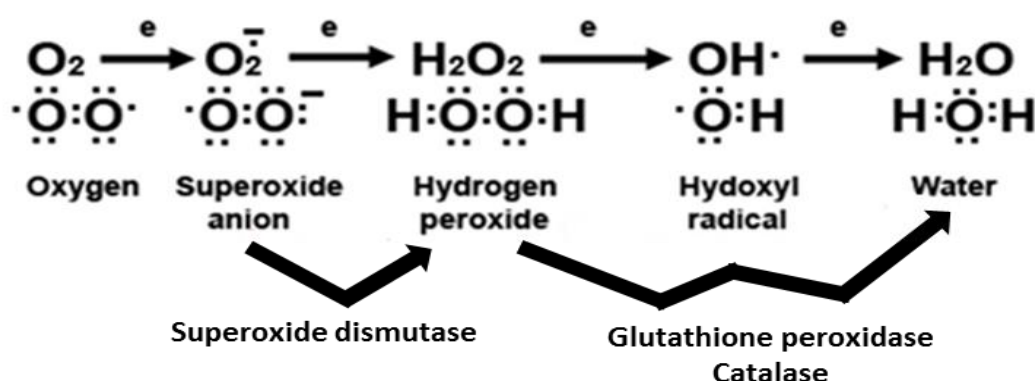


Figure 1. 11 The formation and elimination of ROS

The oxygen approves an electron; it generates superoxide anion which is very reactive. Superoxide anion is changed to hydrogen peroxide via superoxide dismutase (SOD). Hydroxyl radicals are created when hydrogen peroxide receives electrons. The hydroxyl radicals are converted to water by means of Glutathione peroxidase catalase. Figure adopted from (Terada, 2006).

ROS intracellular concentration seems to control stress survival or apoptotic responses. There is evidence to suggest that low levels of ROS means that they are being utilised inside the cell as signaling mediators for natural homeostasis (frequently proliferation); moderate levels (even though linked to proliferation) could trigger a stress response, making cells to adapt through the expression of anti-oxidants, while high levels of ROS at a pro-apoptotic limit, primarily harm cellular structural elements and dynamically

prompt pathways of apoptosis - see Figure 1.12 (Jiang et al., 2011, Sena and Chandel, 2012, Terada, 2006).

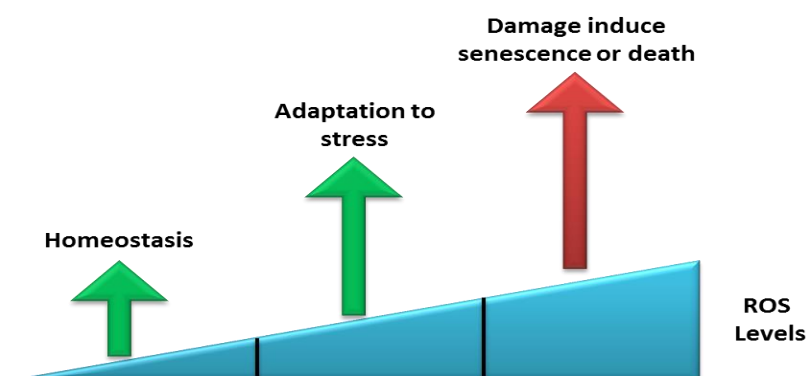


Figure 1. 12 Balance shifts in ROS levels alter cellular homeostasis

The generation of low ROS in ordinary throughout cell homeostasis while the elevated volume resulted into cellular adaptation like growth in anti-oxidant expression through NF- κ B and Ref-1 pathways. Elevated levels of ROS / oxidative stress trigger pathways of apoptosis or cell senescence found in p38 or JNK pathways (Adopted from Terada, 2006).

1.6.5 ROS and malignant transformation

The ability of tumor cells to utilise oncogene activation and tumor suppressor mutation to positively control tumour development appears to be associated with ROS-linked signalling (Dang, 2012). Recent evidence has indicated that *de novo* activation of the oncogenes epidermal growth factor receptor (EGFR) HER-1 and -2 receptors (Trachootham et al., 2006) and H-Ras elevates the *in vitro* division rate of tumour cells which coincides with an abundance of high ROS concentrations (Choudhary et al., 2010, Choudhary et al., 2011, Choudhary and Wang, 2009). Additionally, the over-expression of the leukotriene B4 receptor 2 (LTB4R2) gene amplifies NOX-1 and -4 expression, and produces elevated levels of oxidative stress *in vitro* and *in vivo* (Kim et al., 2009, Shimada et al., 2009). In agreement with these results, the *in vivo* attenuation of ROS stimulating genes NOX-1 and alkylated DNA healing protein alkB homolog 8 (ALKBH8) lessen oxidative stress (Shimada et al., 2009). Mitochondrial cytochrome B gene (MT-CYB) increases oxidative stress, NF- κ B activation, Cyclin-D1 and type IV collagenase, which enhances the exceedingly malignant properties of tumour cells *in vivo* (Dasgupta et al., 2008). This shows that there is a very

strong connection between oncogene activation and the generation of oxidative stress in epithelial cells (Shimada et al., 2009).

The elevated energy requirements that is vital for extreme cell division after oncogene activation makes the mitochondria generate ROS as a natural secondary product which results in oxidative stress (Chung et al., 2009, Trachootham et al., 2009, Trachootham et al., 2006). The extra ROS triggers additional DNA damage (mutations) as well as triggering cell survival and proliferation pathways via NF- κ B and Ref-1, which repeatedly endorse carcinogenesis (Angkeow et al., 2002, Li et al., 2009, Weinberg et al., 2010). To add on to that, it has been proposed that oxidative stress could leaves the cells in the state of H₂O₂ and on the positive side, alter the tumor micro-environment for sustained growth (Schmielau and Finn, 2001). Thus, uncontrolled ROS generation could encourage numerous elements that are hallmarks of cancer development (Schmielau and Finn, 2001).

However, in spite of the advantages provided by ROS-mediated signalling, the sustained oxidative stress in response to hyper proliferative signaling simultaneously results in the cell's disadvantage because it can be 'pushed' toward ROS-related pro-apoptosis. In fact, it has been revealed that *de novo* oncogene expression stimulates normal cells following cisplatin or H₂O₂ treatment and that this is due to increased JNK signaling mediated by a ROS level rise (Benhar et al., 2001, Trachootham et al., 2006).

While they get used to the increased metabolic requirements, cancer cells have to control their basal ROS concentrations by over-expression of antioxidants so as to inhibit or prevent cell death. Evidence for this has been provided by experiments that demonstrated a significant effect for the compound β -phenyl ethyl isothiocyanate (PITC). This is a chemical which interrupts the cell's key antioxidant protein Glutathione (GSH). The pharmacological inhibitor PITC, when used in combination with cisplatin, has been shown to be highly effective in more aggressive tumors over cisplatin treatment alone, because more aggressive tumour cells are highly reliant on the elimination of metabolic ROS via the essential GSH antioxidant defense (Trachootham et al., 2006). The disparity in ROS between normal cells and malignant cells throughout carcinogenesis provides a novel therapeutic target as the biochemical modification of ROS, the high levels of which are necessary for tumor cell growth, might represent a tumour-specific therapeutic opportunity (Wang and Choudhary, 2011).

1.6.6 Cancer cell antioxidant defence against ROS

1.6.6.1 General

Tumour cells acquire resistance to chemotherapeutic drugs because they alter their intracellular environment (Shannon et al., 2003). Given that ROS-regulated oxidative stress is vital to cancer development, tumour cells adapt by increasing the expression of the antioxidant protein families Glutathione (GSH) and Thioredoxin (Trx), thus upholding intracellular ROS concentrations that are lower than the critical pro-apoptotic limit that pushes them in the direction of cell death (Raffel et al., 2003, Godwin et al., 1992). This increased expression protects cancer cells from the activation of oxidative stress receptive ASK1 kinase, the continued activation of JNK (Liu and Min, 2002), and the opening of PTPs in the mitochondria, as every single one of these could potentially activate apoptotic death (Damdimopoulos et al., 2002). To add on to that, Trx and GSH are located in the mitochondria, in which they stabilize mitochondrial levels of oxidative stress and inhibit cell apoptosis beside the antioxidant enzymes like cytochrome c (Aon et al., 2012). The important roles of Trx and GSH in regulating oxidative stress and ROS-induced cell apoptosis, and thus their impact on cancer development, are becoming increasingly appreciated (Damdimopoulos et al., 2002).

1.6.6.2 Glutathione (GSH)

GSH is stimulated by the combinations of Glutathione S-transferases (GSTps), together which are described as excessively expressed in numerous malignancies (Pljesa-Ercegovac et al., 2011). The elevated GSH and GSTp proteins function so as to shield cancer cells from ROS releasing agents that include chemotherapy, radiotherapy and oxidative stress-inducing cancer drugs (Kato et al., 2000). This renders the GSH system a target for pharmacological intervention which may permit the differential targeting of tumour cells by ROS-mediated cell death, as they are operating at conditions of high oxidative stress in comparison to their normal counterparts (Estrela et al., 2006, Trachootham et al., 2006). In view of the fact that GSTp is a biological inhibitor of JNK and ASK1 (Simic et al., 2009) it has been proposed that GSH reduction may also alter the responses of tumour cells to TNFR mediated apoptosis (Estrela et al., 2006); nevertheless, there have not been any clinical experiments that have employed this method. Overall, however, GSH inhibition in combination with tumour apoptosis-stimulating molecules represents a new possibility of cancer therapies (Ortega et al., 2011).

JNK activation is based on its release from its biological inhibitor protein GSTp as ROS results in disulphide bond creation and dimerization (Adler et al., 1999) as described in

Figure 1.13. *In vivo* research has confirmed that genetic knockout of GSTp weakens JNK initiation and in accordance with this, mice deficient in GSTp show constitutive initiation of JNK (and thereafter AP-1), which is what is behind the elevated expression of antioxidant enzymes, so as to protect the cell from oxidative stress (Elsby et al., 2003). The elevated expression of GSTp has been seen in carcinomas, such as that of the bladder (UCC); it has been suggested that increased GSTp expression could inhibit the initiation of apoptotic pathways and thus result in tumours that are unaffected by ROS-inducing chemotherapeutic drugs (Simic et al., 2009). To add on to that, bladder cancer cells also exceedingly express superoxide dismutase (SOD), which as described above is an enzyme which enables the conversion of superoxide anion to hydrogen (Hempel et al., 2009).

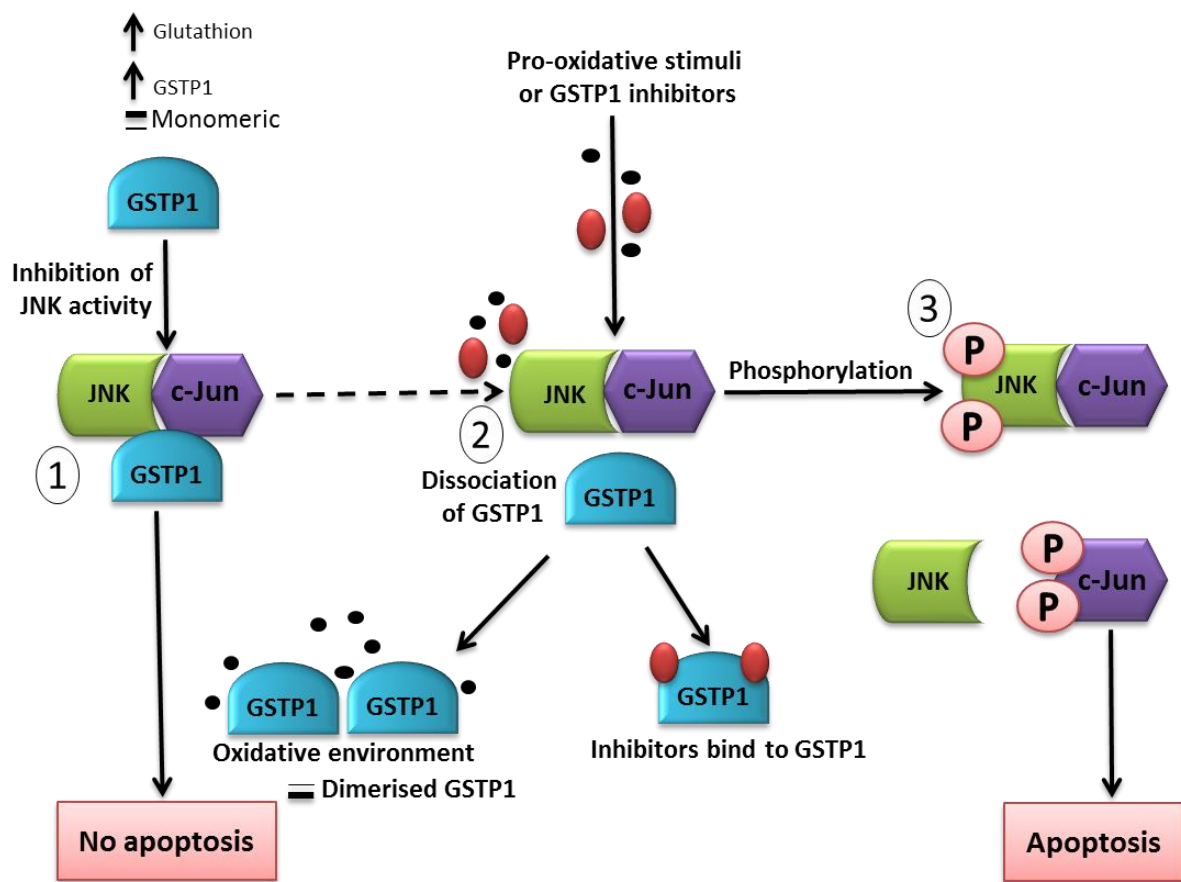


Figure 1. 13 The regulation of JNK by GSTp

Monomeric GSTp prevents JNK actions and therefore the activation of c-Jun. Elevated oxidative stress which also means increased ROS results in GSTp to detach from JNK giving way for it to initiate c-Jun activation and provoke cellular apoptosis (for more explanation see 5.3.2). The figure was redrawn from (Simic et al., 2009).

1.6.6.3 Thioredoxin (Trx)

The human Thioredoxin (Trx) system includes Trx and Trx reductase which function as antioxidants to regulate cellular redox reactions. It has been shown that Trx can translocate from the cytoplasm to the nucleus when oxidative stress occurs to enable initiation/activation of transcription factors NF- κ B, AP-1 and p53 (Hirota et al., 1999, Ueno et al., 1999). Oxidative stress just as it is similar to other outer stresses, results in activation of ROS sensitive MAPK signalling pathways which coordinate the apoptotic response (Apel and Hirt, 2004) and the most noticeable target associated with is mitogen activated protein kinase-kinase-kinase (MAPKKK) apoptosis signaling kinase-1 (ASK1). Under normal conditions, reduced Trx binds to and inhibits ASK-1. When ROS levels increase, Trx is oxidized by ROS (oxidative stress), it is released from the N-terminus of ASK1 and after this ASK1 homo-oligomerises and auto-phosphorylates at residue Thr845 (Liu and Min, 2002) as shown in Figure 1.14 .

Clinically, excessive expression of Trx has been found for instance in colon tumours and this has been associated with more aggressive tumours, diminishing sensitivity to chemotherapy and general diminished survival rates (Raffel et al., 2003). This reduces patient's prognosis since it triggers the generation of VEGF and Ref-1 which are associated with angiogenesis and cell survival (Noike et al., 2008). It has been shown that cancer patients have Trx blood plasma levels and this is also linked with more generation of VEGF. Therefore, preventing Trx by employing novel pharmacological Trx inhibitors like, such as PX-12, has been suggested as a cancer therapeutic agent that aims at reducing Trx and VEGF in cancer patients (Baker et al., 2006). Clinical testing has revealed some success following intravenous infusions of PX-12 (Ramanathan et al., 2009) although this required significant lengths of infusion time (Ramanathan et al., 2011). It has been recommended that new research should aim at new generation Trx inhibitors if these are ever to be utilized as mono-therapies (Ramanathan et al., 2011).

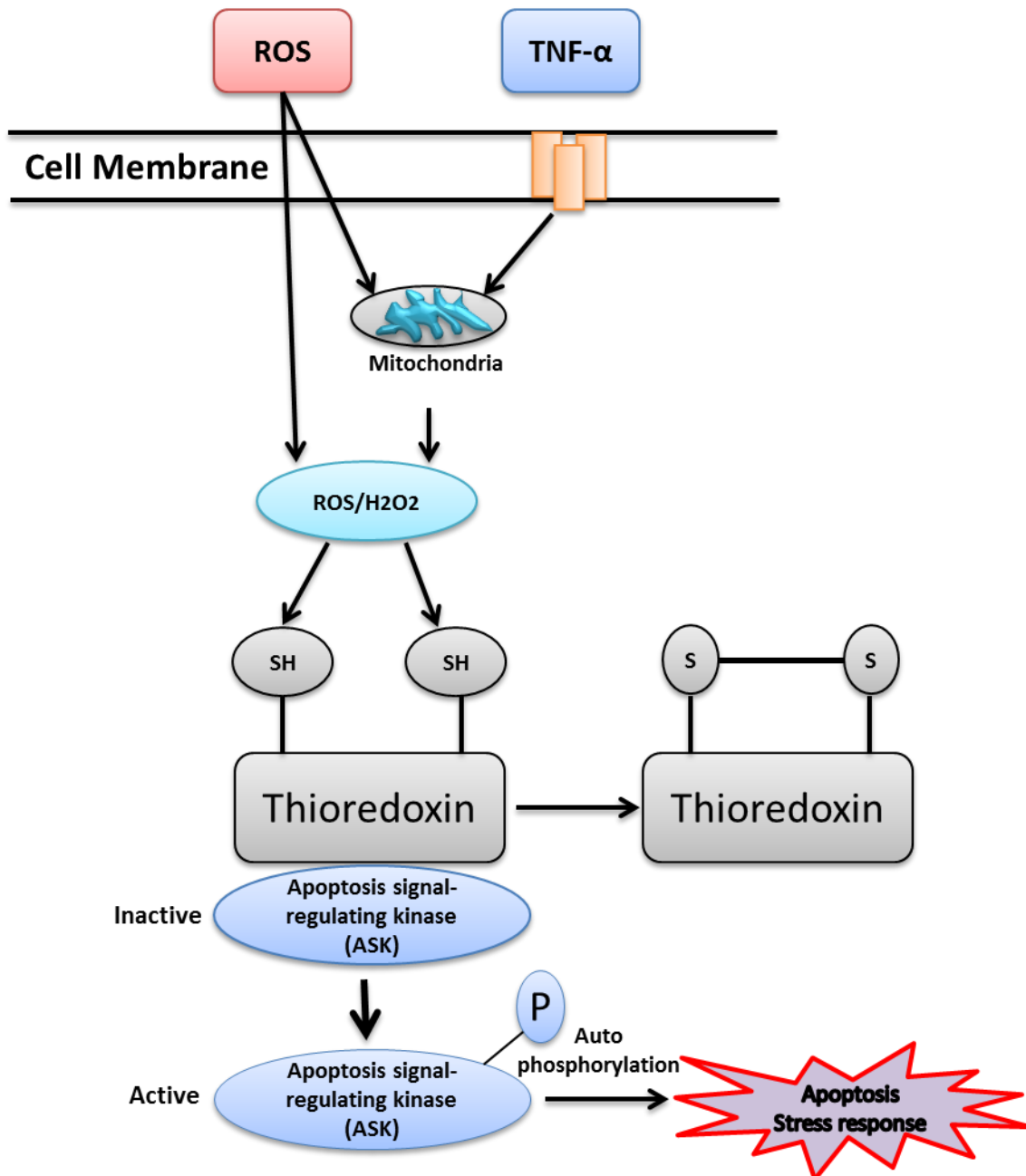


Figure 1. 14 The regulation of ASK1 by thioredoxin (Trx)

Upon oxidative stress ROS oxidizes thioredoxin that ensures release from ASK1. ASK1 is then auto phosphorylated and drives apoptosis or cell stress responses. The figure is redrawn from (Biswas et al., 2006).

1.7 CD40 and its ligand CD154 (CD40L)

In 1984, the CD40 antigen was first identified by an antibody raised against a urinary bladder carcinoma that was also found to bind B cells (Karmann et al., 1995, Koho et al., 1984, Paulie et al., 1984). In 1985 and 1986, it was detected by a monoclonal antibody (mAb) interacting with carcinomas and B cells (mAb S2C6, antigen p50 (Paulie et al., 1985)), and an antibody showing co-stimulatory effects for B lymphocyte proliferation (mAb G28-5, antigen Bp50 (Clark and Ledbetter, 1986, Paulie et al., 1985). This antigen was labelled CDw40 in 1986 at the Third International Workshop on Leukocyte Antigens in Oxford, and in 1989 at the Fourth Workshop in Vienna it was documented as CD40. Stamenkovic and colleagues isolated a cDNA clone encoding CDw40 from a mammalian cell expression library (Stamenkovic et al., 1989). The cDNA encodes a polypeptide with structural similarities to several growth factor receptors and an extensive similarity to nerve growth factor (NGF) receptor (Johnson et al., 1986, Radeke et al., 1987).

CD40 is known to be expressed on different cells in the immune system, such as B cells, dendritic cells, basophils, eosinophils and monocytes. CD40 can, furthermore, be detected on endothelial cells, keratinocytes, smooth muscle cells, epithelial cells and fibroblasts (Kooten and Banchereau, 1997, Schönbeck and Libby, 2001, van Kooten and Banchereau, 2000). Its cognate ligand, CD40 ligand (CD40L), also common known as CD154, is mainly transiently expressed on activated T-cells, but it can also be found on basophils, eosinophils, monocytes, macrophages, dendritic cells, NK cells, B lymphocytes, platelets, mast cells, endothelial cells, smooth muscle cells and epithelial cells (Schönbeck and Libby, 2001). A common feature of all these cells is that the CD154 expression is non-constitutive but can be rapidly induced upon activation. CD40 ligand (also known as gp39, T-BAM, or TRAP) was first described as a membrane protein on activated T cells (Armitage et al., 1992, Hollenbaugh et al., 1992).

1.7.1 Structure of CD40

CD40, the high-affinity receptor of CD40L, is a 48kDa Type I transmembrane protein belonging to the family of TNF receptors (Andre et al., 2002a). The CD40 gene is found in the region of chromosome 20, q12.13.2 and consists of 9 exons with a total length of 16.3 kb (Grimaldi et al., 1992). Exon I encodes the promoter sequence of the protein exons II-VI for the extracellular domain, exon VII for the transmembrane domain and exons VIII and IX encode the intracellular domain of CD40. The final total protein contains 255 amino acids; the majority (171 amino acids) forms the extracellular region of the molecule (Naismith and Sprang, 1998), with the C-terminal domain being located in the intracellular region and the N-

terminal domain found in the extracellular side. The extracellular region of CD40 is mainly composed of a repetitive sequence rich in cysteine residues (20 in total), which are divided into four areas, each comprising an arrangement of two subdomains of a total of four (A1, A2, B1 and B2) (Figure 1.15). This arrangement is typical of proteins belonging to the family of TNF receptors (van Kooten and Banchereau, 2000).

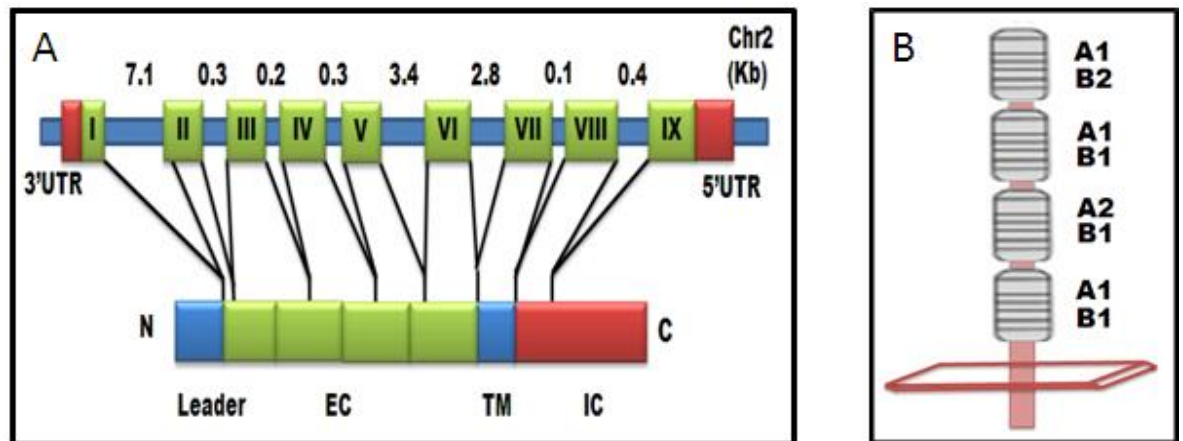


Figure 1. 15 Structure of the gene and the human CD40 protein

A) The CD40 gene contains 9 exons encoding the extracellular, transmembrane and intracellular domains of the protein (leader sequence= promoter; EC = Extracellular; TM= transmembrane; IC= intracellular). B) The CD40 protein is composed mainly of a long extracellular domain containing a sequence of 20 cysteine residues (horizontal lines) divided into four areas each comprising two sub-areas. Adapted from (van Kooten and Banchereau, 2000).

Some confusion as to the exact arrangement of multimeric CD40 present on the membrane surface still exists. Some investigators have reported the presence of CD40 as dimers, while others reported an assembly of three CD40 molecules (trimer) constitutively in trimeric form on the cell surface via PLAD, a pre-ligand-binding assembly domain. (Chan et al., 2000).

1.7.2 Structure of CD40L

CD40L (CD154) is a Type II transmembrane protein of 39kDa in size belonging to the TNF superfamily (Abou-Saleh et al., 2009). The gene of CD40L is located in the q26.3-27.1 region of chromosome X, a fragment with a length 13 kb (Chakrabarti et al., 2005). It consists mainly of five exons; exon I encodes the transmembrane and intracellular region of CD40L, whereas exons II -V encode the extracellular region of the molecule (Figure 1.16).

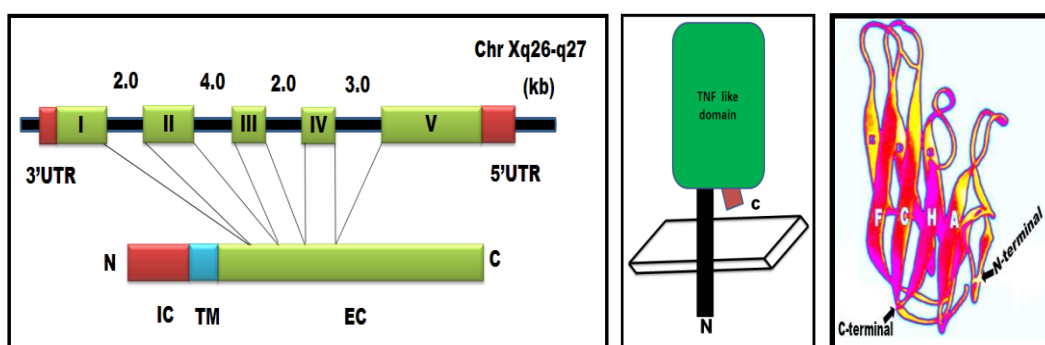


Figure 1. 16 Structure of the gene and protein of human CD40L

The gene encoding CD40L contains 5 exons coding for different regions of the protein (IC=intracellular, TM= transmembrane, extracellular=EC). Schematic structure (left), and crystallographic representation (right). Protein CD40L (monomer). The extracellular region of the molecule comprises the C-terminal domain, whereas the N-terminal domain is found in the intracellular region. This organisation is typical of proteins belonging to the TNF family. Adapted from (van Kooten and Banchereau, 2000).

CD40L protein consists of 261 amino acids and comprises a C-terminal domain located in the extracellular region and a N- terminal domain in the intracellular region of the CD40L molecule (Chakrabarti et al., 2007). Although CD40L is a type II transmembrane protein, it appears to form a multimeric complex composed of three monomers (trimer) associated with the cell membrane (Xia et al., 2010). This structure would facilitate its interaction with CD40, most probably as a trimer, to allow induction of intracellular signals. Besides the membrane form, there is also a form of soluble CD40L (sCD40L) circulating in the blood. This form is almost exclusively an enzymatic cleavage at the membrane following platelet activation and remains a functional trimer of 18kDa (Li et al., 2008).

1.8 Interaction of CD40 with CD40L

The structure of the extracellular part of CD154 has been resolved by X-ray crystallography (Karpusas et al., 1995). It consists of two beta sheets with jellyroll topology that forms a symmetric homotrimer. The crystal structure of CD154 and the CD40 model has been used together with site-directed mutagenesis to identify five CD40 residues, Y82, D84, N86, E74 and E117, and five CD154 residues, K143, Y145, Y146, R203 and Q220, which are important for the CD40-CD154 interaction (Bajorath, 1998, Bajorath et al., 1995, Bajorath and Aruffo, 1997). Thus, the CD154 binding site has been shown to be located in the second and third domains of CD40. It has been suggested that polar interaction between the basic residues on the CD154 and the acidic residues on CD40 plays an important role in this interaction (Singh et al., 1998). Currently, there are two models of possible interaction between CD40L and CD40 receptor. CD40 is a complex formed of either three molecules (trimer) constitutively associated with the membrane, or simply an assembly of three individual molecules (monomers) not associated with the ability to trimerise the following binding of CD40L. Thus, the first interaction model suggested that CD40L (always trimeric) promotes the formation of a homotrimeric complex of CD40 following its interaction with it (Figure 1.17A) (Anand et al., 2003). This would eventually troop the association of cytoplasmic adapter proteins and induction of intracellular signals. Furthermore, in the second model of interaction, CD40 is already found in the form of a trimer level of the membrane, thereby increasing its affinity for the stoichiometric CD40L, also a trimer (Figure 1.17B) (Anand et al., 2003). This last interaction hypothesis is currently the most commonly accepted in the literature, since it appears to be supported by a large amount of experimental data. Certainly the most compelling evidence in favour of the latter statistic is based on an elegant study demonstrating that all receptors associated with the TNF family, including CD40, are found as trimers pre-assembled at the plasma membrane via the PLAD domain (Chan et al., 2000), contrary to the original view of oligomerisation receptor upon binding of CD40L (Anand et al., 2003).

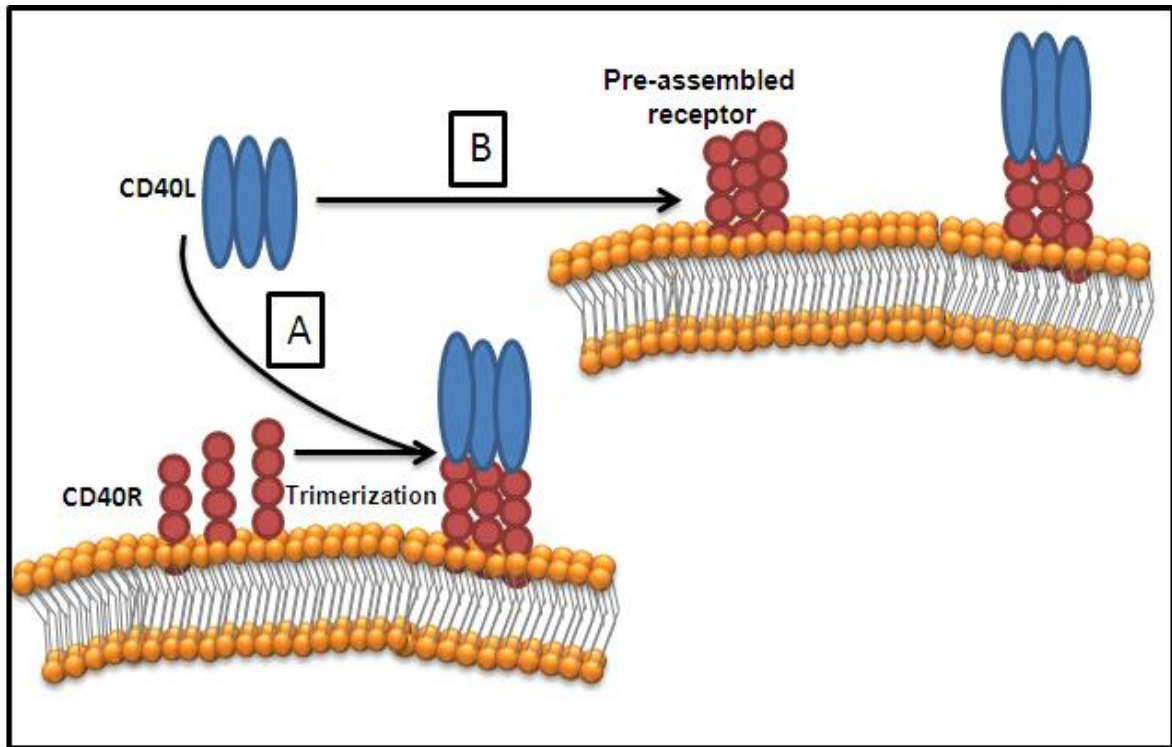


Figure 1. 17 The two models of interaction between CD40L and CD40

A) The first interaction model of CD40L (always trimeric) promotes the formation of a homotrimeric complex. B) CD40 is found already in the form of a trimer level of the membrane, thereby increasing its affinity for the stoichiometric CD40L. Adapted from (Anand et al., 2003).

1.8.1 Other receptors of CD40L

CD40 was long considered to be the only receptor for CD40L. However, three other receptors have been identified, in particular integrins $\alpha\text{IIb}\beta 3$, Mac-1 and $\alpha 5\beta 1$. Although CD40 remains the main high-affinity receptor of CD40L, these other partners seem to perform a very special function in various pathophysiological conditions. $\alpha\text{IIb}\beta 3$ integrin was identified as a receptor for CD40L on the platelet surface, and the interaction between these two seems to promote platelet activation and stabilisation of the platelet thrombus *in vivo* (Andre et al., 2002b, Prasad et al., 2003). The interaction is possible between CD40L and $\alpha\text{IIb}\beta 3$ through the KGD domain (Lysine-Aspartic acid-Glycine) of CD40L. Most integrin receptors $\alpha\text{IIb}\beta$, have at least one KGD and $\alpha\text{IIb}\beta$ contains a recognition domain for this pattern, allowing their interaction domain. Mac-1 is the major integrin involved in the adhesion of monocytes and neutrophils to the activated endothelium during inflammation and is also a receptor for CD40L. This interaction promotes the adhesion and transmigration of leukocytes at the atherosclerotic plaque, and it is involved in neointimal formation, a key component of atherosclerosis (Li et al., 2008). The exact residues involved in this interaction are still unknown, but it seems that CD40L interacts with Mac-1 in its active conformation. Finally, integrin $\alpha 5\beta 1$ is the main fibronectin receptor and was more recently identified as one of the other receptors of CD40L (Leveille et al., 2007).

1.9 Role of CD40/CD40L in immune system

In previous sections of this chapter, the function of CD40-CD40L in different cell types was introduced. In this section, a comprehensive discussion of the main physiological functions of the CD40/CD40L system will be undertaken, which will include details on its involvement in humoral immunity (antibody production), cellular immunity and regulation of inflammatory mediators, and more specifically its involvement in the induction of apoptosis (Ma and Clark, 2009).

1.9.1 Humoral immunity

Humoral immunity is associated with B cells and antibody production, which requires close involvement of T lymphocytes and APCs, such as dendritic cells. Together, these factors ultimately lead to the differentiation of B cells into plasma cells and the production of immunoglobulins (for example; IgA, IgE, IgG and IgM) (Ma and Clark, 2009). The importance of the CD40/CD40L axis in humoral immunity was demonstrated mainly by three approaches: first, by clinical manifestations and symptoms observed in patients with hyper IgM (HIM) syndrome, (Aruffo et al., 1993, Allen et al., 1993, Korthäuer et al., 1993); secondly, by the genetic approach (deletion of the genes for CD40 and CD40L) (Renshaw et al., 1994,

Castigli et al., 1994, Xu et al., 1994); and, finally, by the use of blocking peptides or antibodies directed against the complex CD40/CD40L (Foy et al., 1994, Van den Eertwegh et al., 1993). Each of these approaches, as highlighted by several studies, points to the same conclusion, i.e. the absence of the CD40/CD40L interaction leads to a severe defect in the production of the immunoglobulins IgG, IgA and IgE (that is to say, thymus-dependent T cells response) in response to a pathogenic infection, without affecting the T cell- independent immune response (Ma and Clark, 2009)

The CD40/CD40L complex occupies a critical role in humoral immunity through its involvement in the interactions between dendritic cells (or APC), T cells and B lymphocytes in the immune response cells (Figure 1.18). These cell interactions are characterised primarily by two important steps, namely activation of T cells via the APCs (dendritic cells in particular) and the activation and differentiation of B lymphocytes into plasma cells via their interaction with activated T cells and dendritic cells (Ma and Clark, 2009). Figure 1.18 illustrates the cellular interactions in humoral immunity and highlighting the importance of the CD40/CD40L complex in this process (Ma and Clark, 2009).

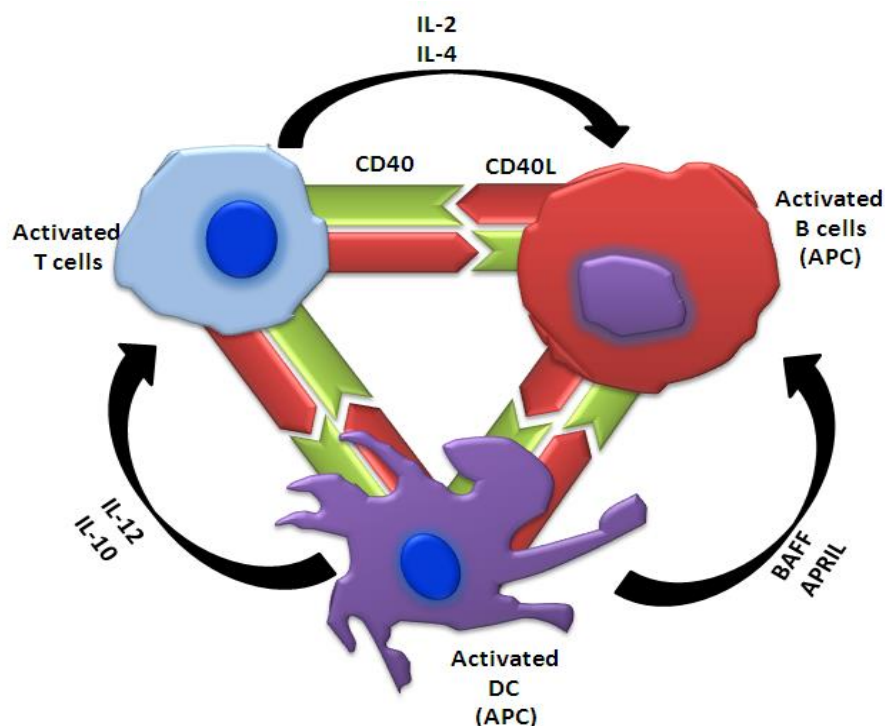


Figure 1. 18 Role of CD40/CD40L interactions in humoral immunity

The CD40/CD40L complex occupies a critical role in humoral immunity through its association in the interactions between dendritic cells (or APC), T-lymphocyte and B- lymphocytes in the immune response cells. Adapted from (Ma and Clark, 2009)

The exposure to a pathogenic agent (eg. bacterial infection) leads to activation of CD40 on the dendritic cells (Liang et al., 2009, Hellman and Eriksson, 2007). The dendritic cells were subsequently able to interact with CD40L-activated T cells, increasing the expression of co-stimulatory molecules CD80/CD86 and B7-1/B7-2, and promoting the release of IL-12 and IL-10 by dendritic cells (Cella et al., 1996, Caux et al., 1994, Ma and Clark, 2009). The cellular responses are intimately involved in the differentiation of T cells into Th1 effector cells, regulatory T and Th17 (Th1 IL-12 and IL-10, and regulatory T Th17) (Iezzi et al., 2009, Bettelli et al., 2006, Veldhoen et al., 2006). Subsequently, the interaction of CD40L on T cell effector CD40 B cells induces the release of IL-2 and IL-4 by T effector cells (Natural killer cells) effectives. Meanwhile, the CD40/CD40L complex is also involved in the interaction of dendritic cells with B lymphocytes. This interaction facilitates the secretion of Stimulator Protein of B lymphocytes ("B lymphocyte Stimulator protein", BLyS or BAFF) and a proliferation-inducing ligand (APRIL) by dendritic cells which, in conjunction with IL- 2 and IL-4 released by T lymphocytes, promotes differentiation of B lymphocytes into immunoglobulin-producing plasma cells (DeKruyff et al., 1993, Craxton et al., 2003).

It is important to note that it is difficult to put a precise chronological order to these events since, in reality; these interactions are bidirectional and may take place in concurrent ways. To conclude, the dendritic cells, T lymphocytes and B lymphocytes are able to interact simultaneously, indicating the need to consider these cellular responses as a whole and not as separate elements. In short, the CD40/CD40L axis is an integral element in the cooperation between the different elements in the humoral response and antibody production.

1.9.2 CD40/CD40L in cell mediated immunity

As mentioned above, the CD40/CD40L axis was originally discovered on cells involved in immunity, such as T and B lymphocytes. The CD40 receptor is expressed constitutively on professional antigen-presenting cells such as B cells (B-lymphocyte) (Valle et al., 1989, Clark, 1990, Stamenkovic et al., 1989), and it also expressed early and throughout development (Uckun et al., 1990). Thus, it activates T cells (both CD4⁺ or CD8⁺) (Ware et al., 1991, Indzhia et al., 1992), monocytes (van Kooten and Banchereau, 2000), macrophages/microglia (Gerritse et al., 1996), dendritic cells (van Kooten and Banchereau, 2000), platelets (Inwald et al., 2003) and is expressed on epithelial cells (Ruggiero et al., 1996), fibroblasts (See figure 1.19) (Yellin et al., 1995), endothelial cells and carcinomas (Schönbeck and Libby, 2001).

The CD40 receptor is frequently expressed in the form of a doublet consisting of protein 43 to 47kDa (Clark and Ledbetter, 1986, Braesch-Andersen et al., 1989). Although the form in which the receptor is expressed may depend on the cell type (BERG et al., 1996), it is certain that its expression is constitutive in most cell types. CD40 is found in lipid rafts (Kaykas et al., 2001, Pham et al., 2002). In addition, cytokines such as IFN- γ , IL-1, IL-3, IL-4, TNF- α and GM-CSF (granulocyte macrophage colony-stimulating factor) may increase its expression (reviewed in (Schönbeck and Libby, 2001), while transforming growth factor- β (TGF- β) inhibits the increase in the degradation of the mRNA of CD40 (Nguyen et al., 1998).

As mentioned above, the CD40 receptor and CD40 ligand were originally discovered in the cells involved in immunity, such as B lymphocytes and T lymphocytes (Armitage et al., 1992, Lederman et al., 1992). Indeed, this receptor and its ligand are present in most of cells of the immune system and they have an important role in inflammation in addition to a pivotal role in immune reactions. Table 1.1 summarises the expression of CD40 and CD40L in the various cells of the vascular cells (Yellin et al., 1995).

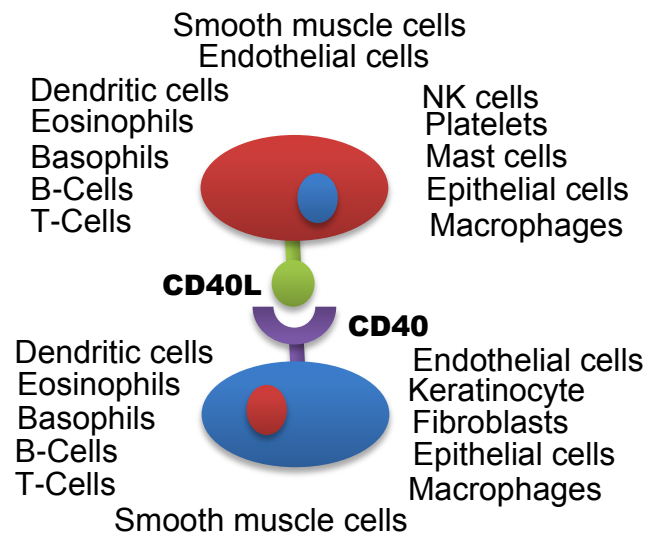


Figure 1. 19 Expression of CD40 and CD40 ligand on different cells types

Different cells have ability to express CD40 and its ligand. The interaction of CD40 with CD40L causes to express of different proteins and triggers the activation of different signalling patwys in different cells in the body (Gormand et al., 1999).

1.9.2.1 B Lymphocytes

The role of CD40/CD40L complex in B cells is crucial to the humoral immune response. B cells constitutively express CD40 interacting with the CD40L on T cells activated in the presence of antigens in any infection. This interaction, in the presence of cytokine released by T cells (IL-4, IL-2 and IL-10), induces the proliferation and differentiation of B lymphocytes into plasma cells and antibody production, as shown in Figure 1.20 (Aruffo et al., 1993). CD40/CD40L interaction seems sufficient by itself to induce the production of IgG and IgA antibodies, while a co-stimulation in the presence of IL-4 is necessary particularly for the production of antibody IgE (Armitage et al., 1993, Spriggs et al., 1992).

In the absence of the CD40/CD40L interaction, B cells produce only IgM, as observed in patients suffering from syndrome HIGM (Hyper IgM syndrome) (Hill and Chapel, 1993). Furthermore, in the activated B cell, the CD40/CD40L interaction induces the release of cytokines IL-6, IL-10 and TNF- α (Boussiotis et al., 1994), an increase in the intercellular adhesion molecule-1 (ICAM-1) associated with lymphocyte function- antigen-1 (LFA-1), vascular cell adhesion molecule-1 (VCAM-1) (Barrett et al., 1991, Rousset et al., 1991) and increased major histocompatibility protein complex-1 and-2 (MHC-I and MHC-II), (Khanna et al., 1997, Klaus et al., 1994), all of which facilitate the proliferation and differentiation of these cells into plasma cells. Interestingly, B lymphocyte' expression of CD40L also appears to be involved in a positive feedback loop, since CD40L expressed on one B cells can in turn interact with CD40 of another B cell, thereby facilitating activation and differentiation of B lymphocytes (Clodi et al., 1998, Grammer et al., 1995). CD40 activation would then be more involved in the differentiation of these cells into memory B cells (Gray et al., 1997, Pound and Gordon, 1997).

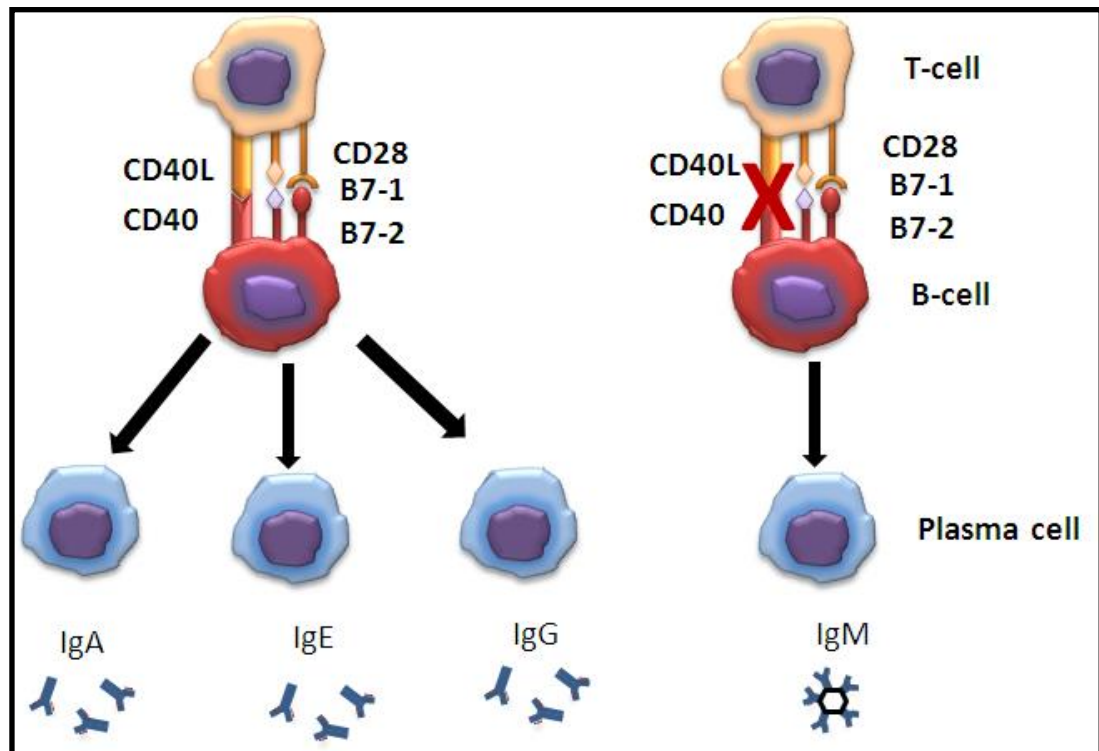


Figure 1. 20 Role of CD40/CD40L complex in the B lymphocytes and antibody production

CD40/CD40L interaction appears necessary by itself to induce the production of IgA, IgE and IgG antibodies. B) In the non-appearance of the CD40/CD40L interaction, B cells produce only IgM Adapted from (Lievens et al., 2009).

1.9.2.2 T Lymphocytes

T cells require the presence of two main signals in order to induce their full activation. The first signal (Signal 1) comes from the interaction of the T cell receptor (TCR) with peptide MHC molecules on antigen-presenting cells (APC). The second (Signal 2) is called co-stimulatory and is mainly via the CD28/B7 complex and CD40/CD40L – in fact the CD40/CD40L signal is sometimes referred to as Signal 3 (Lievens et al., 2009). This co-stimulatory signal is essential to the proliferation, differentiation and survival of T cells (van Essen et al., 1995, Grewal et al., 1995). The first signal induces the expression of CD40L in the T cell membrane, and it subsequently interacts with CD40 on APCs cells, such as B cells, macrophages or dendritic cells. This bidirectional interaction enhances the activation of T cells and promotes their proliferation and differentiation into effector (mature) T cells (van Essen et al., 1995). Activated T lymphocyte also expresses CD40, but its exact involvement in the function of these cells remains poorly characterised. On the other hand, it appears that CD40 is involved in the interaction of T lymphocytes with CD4⁺ and CD8⁺ cells. The interaction of CD40 with CD40L on the CD8⁺ and CD4⁺ allows differentiation of CD8⁺ lymphocytes into memory cells, as with B lymphocytes (Bourgeois et al., 2002).

1.9.2.3 Monocytes / Macrophages

The role of CD40/CD40L complex in the function of monocytes is well documented. Monocytes constitutively express CD40 and, as APC, they are able to interact with the CD40L of T cells (Cella et al., 1996, Schönbeck and Libby, 2001). This interaction is bidirectional, so that the monocyte promotes activation of T cells which, in turn, induce the activation and differentiation of monocytes into macrophages. Binding of CD40L on the T lymphocytes to CD40 on monocytes and macrophages leads to the release of several cytokines (IL-12, IL-1 β , IL-6, IL-8 and TNF- α) (Alderson et al., 1993, Wagner et al., 1994) and Metalloproteinase Matrix (MMPs), (MMP-1 MMP-2, MMP-3 and MMP-9) (Mach et al., 1997, Malik et al., 1996), an increase of co-stimulatory molecules ICAM-1, LFA-3, B7-1 and B7-2 (Kiener et al., 1995) and secretion of tissue factor and nitric oxide (NO) (Mach et al., 1997). These biological effects are essential to the immunity-related, inflammatory and angiogenic function of these cells. Most biological effects of CD40L on monocytes are due to its interaction with CD40, although other receptors on the surface of these cells are able to induce various cellular responses, especially Mac-1. Indeed, it was recently demonstrated that the interaction of CD40L/Mac-1 promotes the adhesion and migration of monocytes to the endothelium, and the release of myelo-peroxidases during the inflammatory response (Zirlik et al., 2007). Activation of monocytes increases membrane expression of CD40, as well as CD40L. The precise function of CD40L in these cells is still unknown, but it may be

involved in monocyte/monocyte interactions via CD40. This interaction would amplify the activation and differentiation of monocytes, increasing their secretory function (Suttles and Stout, 2009).

1.9.2.4 Dendritic cells

The dendritic cell (DC) occupies a central place in the immune system. The CD40-CD40L interaction on DC, mainly as a co-stimulatory factor, promotes the activation of T lymphocytes. Activation of DC, such as that observed in the presence of pathogens, causes a significant increase in DC cell surface CD40. This receptor is able to interact with the CD40L on activated T cells, increasing the expression of co-stimulatory molecules CD80/CD86 and B7-1/B7-2, and it promotes the release of the IL-12 by DC (Cella et al., 1996, Caux et al., 1994). These cellular responses are intimately involved in the differentiation of T lymphocytes for Th1 immune responses (Shepherd and Kerkvliet, 1999, Iezzi et al., 2009). In addition, CD40L is functionally expressed by in response DC-CD40 ligation (Pinchuk et al., 1996). DC CD40 ligation by T-cell-CD40L, primes DC cells to become effective antigen presenting cells (APC) via upregulation of MHC class II molecules and CD80/CD86 (Ma and Clark, 2009) and CD40-CD40L co-stimulation is essential for the activation of DC in response to pathogen associated molecular patterns (PAMPs) (Sacks and Noben-Trauth, 2002). Additionally, CD40L is functionally expressed by DC in response CD40 ligation (Pinchuk et al., 1996). In such manner, DC have been shown *in vitro* to utilise membrane CD40L (mCD40L) in order to mediate their cytotoxic effect effects towards urothelial cell carcinomas (UCC) and colorectal carcinomas (CRC), therefore, CD40 is not only essential for DC activation, but for their ability to induce CD40-mediated cell cytotoxicity (Hill et al., 2008b). DC also express CD40L, but in small quantities compared to CD40. Although the exact function of CD40L dendritic cells remains to be fully verified, it appears to be involved in bidirectional interaction between dendritic cells and B lymphocytes (Bergtold et al., 2005, Wykes and MacPherson, 2000).

1.9.2.5 Neutrophils

Neutrophils are among the first vascular cells recruited to inflammatory sites and are actively involved in the immune mechanisms of the body. Vanichakarn et al. have demonstrated that activated neutrophils express CD40 and this appears to play an important role in platelet/neutrophil interactions (Vanichakarn et al., 2008). In addition, platelets are able to activate neutrophils through the release of soluble CD40L that follows their stimulation. sCD40L induces secretion of ROS in neutrophils, which in turn promotes the activation of platelets reciprocally. Furthermore, the results of the study by Li et al indicated that the interaction of platelets with neutrophils increases expression of Mac-1 and high levels of sCD40L, and that neutrophils promote platelet aggregate formation and neointimal formation following vascular injury (Li et al., 2008).

1.9.2.4 Platelets

Platelets play a fundamental role in haemostasis but also actively participate in inflammatory reactions by inflammatory cytokines, growth factors and MMPs. The resting platelets constitutively express CD40, whereas CD40L is present in the membrane upon cell activation (Henn et al., 1998). The study by Henn et al. first demonstrated the importance of the CD40/CD40L complex function in platelets (Henn et al., 1998). They showed that CD40L on activated platelets is able to interact with the CD40 on endothelial cells and monocytes to induce a significant inflammatory reaction. In particular, this interaction increases the expression of some proteins such as Intercellular Adhesion Molecule-1 ICAM-1 (CD54), Vascular Cell Adhesion Molecule-1 VCAM-1 (CD106), and E-selectin protein (CD62E); it also promotes the release of the Monocyte chemo-attractant protein-1 (MCP-1/CCL2), IL-6, IL-8, and induces the production of MMP-9. Moreover, it was also demonstrated that platelet sCD40L is involved in stabilising the thrombus through its interaction with integrin $\alpha\text{IIb}\beta\text{3}$ (Andre et al., 2002b). Little information exists about the role of platelet CD40, but it seems that the stimulation of platelets by sCD40L induces the release of Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES) and the production of reactive oxygen species (ROS) (Chakrabarti et al., 2005, Danese et al., 2004).

1.9.2.6 Endothelial cells

CD40/CD40L occupies a prominent position in the activation of endothelial cells and smooth muscle cells. Endothelial cells express CD40 and CD40L. Activation of CD40 on endothelial cells contributes significantly to their inflammatory role (Karmann et al., 1995). In particular, this binding induces expression of adhesion molecules ICAM-1, VCAM-1 and E-

selectin, and it causes the release of cytokines such as IL-1, IL-6, IL-8, MCP-1, the macrophages inflammatory protein-1 α (MIP-1 α) and RANTES (Rizvi et al., 2008, Bavendiek et al., 2002). Endothelial cells are actively involved in the mechanisms of angiogenesis, and the CD40/CD40L axis seems to occupy a more important place in this phenomenon (Karmann et al., 1995).

First, the activation of endothelial cells by CD40L promotes the synthesis and secretion of MMP-1, MMP-2 and MMP-9 which are involved in the digestion of the extracellular matrix, a key step in angiogenesis (Mach et al., 1999). In addition, CD40 activation triggers the secretion of vascular endothelial growth factor (VEGF) by endothelial cells, which is essential to the initiation and progression of angiogenesis mechanisms (Melter et al., 2000). Additionally, the binding of CD40 on endothelial cells promotes the expression of cyclooxygenase-2 (COX-2), which has a pro-angiogenic activity via the induction of the basic fibroblast growth factor (bFGF) (Schonbeck et al., 1999). The CD40/CD40L complex is involved in the pro-coagulant function of endothelial cells. Consequently, it was demonstrated that ligation of CD40 via CD40L induces the synthesis and release of tissue factor from endothelial cells, (Bavendiek et al., 2002), which triggers the activation of the coagulation cascade and platelet activation (Schonbeck et al., 1999).

1.9.2.7 Smooth muscle cells

The role of the CD40/CD40L dyad in smooth muscle cells is less well documented, but it appears that CD40L is able to trigger the activation of signalling pathways involved in the mitogenic activity of these cells. It also particularly activates the Src tyrosine kinase pathway, which leads to the secretion of cytokines such as MCP-1 and IL-8 (Hermann et al., 2002, Mukundan et al., 2004). In addition, the activation of the CD40 receptor of this cell leads to the degradation of collagen through the interstitial matrix and also via MMPs, which have the ability to promote proliferation and migration of these cells (Horton et al., 2001, Newby, 2007).

1.9.2.8 Epithelial cells

Young and colleagues first discovered CD40 expression on epithelial cells of human nasopharynx, tonsil and ectocervical tissue by using immunohistochemical analysis, as well as cultured epithelial cells and several epithelial cell lines (Young et al., 1989), while CD40 ligand (CD154) expression has been observed on epithelial cells of the glomerulus as well as the proximal tubule. Yellin and colleagues reported initially that CD40 also expressed in normal kidney, and expressed on parietal epithelial cells (Yellin et al., 1995). Cruickshank and co-workers reported that intestinal epithelial cells, which encounter enteric antigens, express CD40 together with other co-stimulatory molecules; however, these cells were unable to promote mitogen- or antigen-driven activation of CD4⁺ T cells (Yellin et al., 1995).

The presence of CD40 on diseased cells and its absence on unaffected epithelium as well as its inducibility by pro-inflammatory cytokines, e.g., IFN- α , indicated a role for epithelial CD40 in the development of carcinomas/epithelial neoplastic at sites of chronic inflammation (Stamenkovic et al., 1989), a hypothesis also supported by the later finding that a large majority of nasopharyngeal carcinoma cells expressed CD40 (Zong et al., 1991). More studies of CD40 distribution in the human thymus revealed that cortical and medullary thymic epithelial cells express this receptor *in situ* and, inducible through pro-inflammatory cytokines such as IL-1, TNF- α , or IFN- γ , also *in vitro* (Galy and Spits, 1992, Patel et al., 1995). Further studies established that CD40 expressed on these thymic epithelial cells offers co-stimulation for clonal expansion of CD4⁺ thymocytes (Briscoe et al., 1998). In addition to neoplastic and thymic epithelial cells, normal human bronchial epithelial cells constitutively express CD40 *in situ* (Gormand et al., 1999).

Table 1. 1 Expression of CD40/CD40L axis in the cells of the immune vascular system

Cell Type	CD40		CD40L		Form of CD40L
	Active	Inactive	Active	Inactive	
B lymphocytes	+++	+++	++	+++	Membrane
T lymphocytes	-	+++	-	+++	Membrane, soluble
Monocytes / Macrophages	+	+++	+	+++	Membrane
Platelets	++	+++	-	+++	Membrane, soluble
Endothelial cells	+	+++	+	+	Membrane
Smooth Muscle Cells	+++	ND	+	ND	Membrane
Dendritic cells	-	+++	+	+	Membrane
Neutrophils	+	+++	+	+	Membrane

- Non expressed; +, weakly expressed; ++, moderately expressed, +++, strongly expressed, ND, undocumented.

1.10 Intracellular signalling pathways induced by CD40

Since CD40 lacks intrinsic signalling activity, adapter molecules and TNF receptor associated factors (so-called TRAFs) are required for signal transduction by a CD40-CD154 interaction (Schönbeck and Libby, 2001). The TRAF- family consists of six members, five of which (TRAF 1, 2, 3, 5, 6) depend on cell type and function to bind CD40 (Zapata, 2003). In endothelial cells, the association of TRAF2 and CD40 causes activation of pro-inflammatory signalling pathways (Mukundan et al., 2004).

The specificity of the CD40 signal transduction is influenced not simply by the interaction with the different TRAFs. Rather, in this case, the localisation of the receptor plays an important role. CD40 is located in and on special membrane micro domains called "lipid rafts". After CD40 CD154 interaction, the majority of CD40 in the lipid rafts are translocated, where the receptor is then associated with the various TRAFs (Arron et al., 2002). The lipid raft-dependent association of TRAF2 with CD40 leads to the formation of pro-inflammatory cytokines (such as MCP-1) that the arteriosclerosis (Arron et al., 2002, Chen et al., 2006).

The various cellular responses mediated by CD40/CD40L interaction are related to the activation of specific intracellular signalling pathways. CD40 is the main receptor promoting signalling, and there is still relatively little information about the precise signalling pathways triggered following activation by CD40L especially in epithelial cells (Georgopoulos et al., 2006). The binding of CD40 with CD40L causes the recruitment of adapter proteins, called TRAFs, in the cytoplasmic tail of the CD40 molecule (Bishop et al., 2007). Cellular relocation of TRAFs and their interaction with CD40 triggers the activation of several signalling pathways, which include the main pathway of NF-KB, the MAPK pathway (p38, JNK and Akt), the PI3 kinase pathway, and others (Davies et al., 2005, Elgueta et al., 2009). Any intracellular signalling induced by CD40 depends almost exclusively on TRAFs, but can be independent of TRAFs signalling pathways, such as the pathway of STAT5 resulting from the direct association of Janus kinase 3 (JAK3) with CD40 (Säemann et al., 2003, Säemann et al., 2002). Figure 1.21 summarises the main signalling pathways that result from the activation of CD40 as well as the function of the different members of the family of TRAFs in the activation of different signalling pathways triggered (Elgueta et al., 2009).

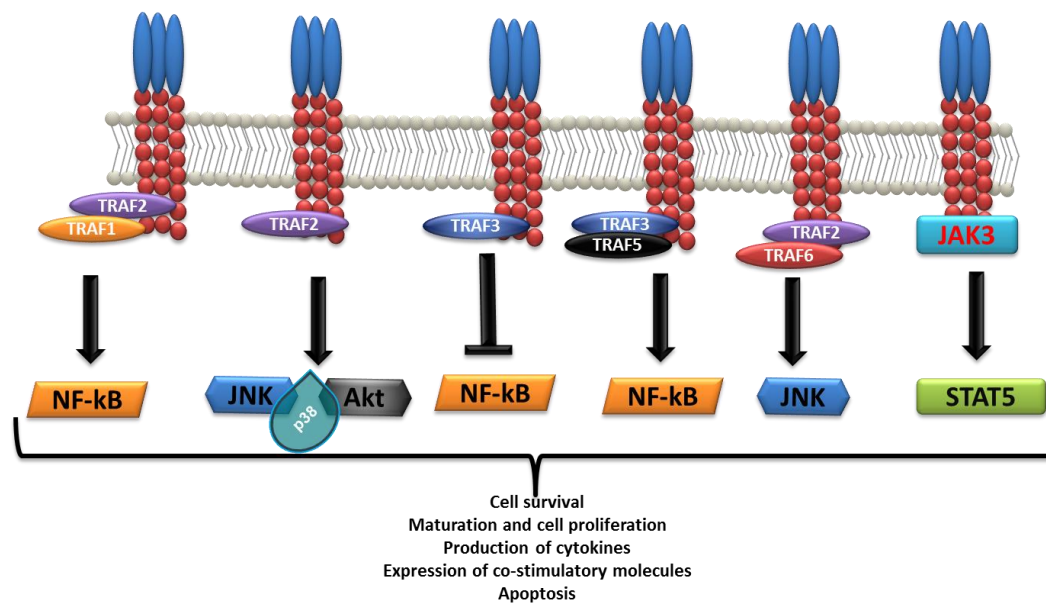


Figure 1. 21 The different signalling pathways induced by CD40

Each member of the family of TRAFs has a separate function, but they sometimes overlap. The majority of signalling pathways of interaction between CD40 and CD40L depends on the activation of TRAFs, but there are also TRAF independent channels, such as the STAT5 pathway (Elgueta et al., 2009).

1.10.1 Structure of TRAFs

The TRAFs family consists of six members, named TRAF1 to TRAF6. The TRAFs consist of a C-terminal carboxyl named TRAF-C, a rich domain leucine zipper, TRAF-N, and two areas rich in zinc motifs, named zinc fingers and zinc ring, with the exception of TRAF-1 which is devoid of both zinc areas (Figure 1.22) (Arch et al., 1998). The TRAF-C domain is involved in binding to TRAFs of CD40, whereas the N-TRAF domain is responsible for the homo- and hetero-dimerisation interactions of TRAFs (for example TRAF2/TRAF3 interaction). The zinc-rich domains are involved in the recruitment and identifying additional proteins, such as kinases and transcriptional factors (Arch et al., 1998).

Following the binding of CD40L to CD40, the TRAFs are, directly or indirectly via other TRAF members, recruited to the cytoplasmic tail of the CD40 molecule. The protein sequence involved in the interaction of TRAFs with CD40 differs from one member to another; thus, there is no consensus on the sequence responsible for this interaction, although some sequences have been found to overlap between some members, such as TRAF2 and TRAF3 (McWhirter et al., 1999, Ni et al., 2000).

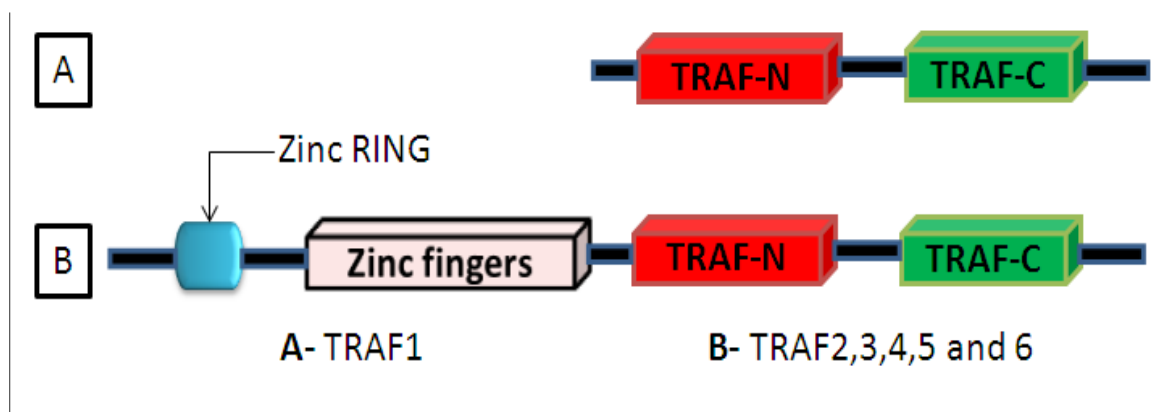


Figure 1. 22 Structures of TRAFs

The schematic represents the structural organisation of the TRAFs. Currently six different TRAFs are known. All TRAFs are characterized by a C-terminally-located TRAF domain. TRAF proteins have a highly conserved C-terminus, through which they bind to the receptors and can interact with other TRAFs. The TRAFs family consists of six members: A) TRAF1, which is devoid of both zinc areas (Zinc fingers and Zinc ring) B) TRAF2 to TRAF6 which contain those domains.

1.10.2 TRAF1

Following the activation of CD40, TRAF1 protein expression is significantly increased (Zapata et al., 2000, Schwenzer et al., 1999). Since TRAF1 has no motif of a zinc-rich domain, it seems to be involved in regulating the signalling of other members of TRAFs (Bishop et al., 2007). The binding site of TRAF1 on CD40 overlaps with that of TRAF2 and TRAF3, and it appears that TRAF1 is only able to interact weakly with CD40, in the absence of the involvement of TRAF2 (Pullen et al., 1998). At the level of B lymphocytes and APCs, TRAF1 deficiency causes a decrease in the recruitment of TRAF2 to the CD40 and an increase in the enzymatic degradation of the receptor (Xie et al., 2006, Arron et al., 2002). In addition, it appears that the recruitment of TRAF1 and TRAF2 is required for complete activation of the NF- κ B pathway, since the dual genetic deletion of both members generates a stronger attenuation of the activation of NF- κ B, compared to the individual gene deletion of one or any of these members (Xie et al., 2006).

1.10.3 TRAF2

TRAF2 is one of the most studied members and its main role is the activation of p38 (MAPK), Akt, JNK and ERK1/2. Hostager et al. (2003) and Yeh et al. (1997) demonstrated that, in fibroblasts and B cells of the embryo in TRAF2-deficient mice, the activation of these signalling pathways was significantly inhibited after the binding of CD40L to CD40 (Hostager et al., 2003, Yeh et al., 1997). In addition, the recruitment of TRAF2 induces recruitment of protein kinase mitogen-activated kinase-1 (MEKK1) to the cytoplasmic tail of CD40 after CD40 ligation, which is an essential step in the activation of ERK1/2 proteins and activation of kinases JNK and p38 (Gallagher et al., 2007). At the level of B lymphocytes, TRAF2 also seems to participate in the activation of NF- κ B, in collaboration with TRAF6. On the other hand, the interaction of one or the other with CD40 seems sufficient to induce activation of NF- κ B. This was confirmed by results showing that the deficiency of TRAF2 or TRAF6 does not cause abnormality in the activation of NF- κ B, but the double deletion of these two members induced severe inhibition of this pathway at the level of B lymphocytes. TRAF2 also seems to participate in the activation of NF- κ B, in collaboration with TRAF6. However, the interaction of one or the other with the CD40 seems sufficient to induce the activation of NF- κ B. This was confirmed by studies showing that the deficiency of TRAF2 or TRAF6 does not cause abnormality in activation of NF- κ B, but the double deletion of these two members causes severe inhibition of this pathway (Hsing et al., 1997, Rothe et al., 1995, Yeh et al., 1997).

Despite its important role in the activation of signalling pathways induced by CD40, TRAF2 also appears to have a negative regulatory function (Gardam et al., 2008). Its

involvement as a negative regulator of signalling via CD40 comes from its constitutive interaction with TRAF3 (Figure 1.23). The interaction of TRAF2/TRAF3 allows proteins cIAP1 and cIAP2 to degrade NIK protein, which inhibits activation of the NF- κ B and promotes apoptosis (Vallabhapurapu et al., 2008, Zarnegar et al., 2008). Moreover, the removal of an accumulation of TRAF2 generates NIK in B cells (Vince et al., 2007). Following stimulation with CD40L, this protein complex is destabilized and TRAF2/TRAF3 is recruited to the CD40, which allows TRAF2 to activate NF- κ B. Degradation of TRAF2 (Self degradation) and TRAF3 (via cIAP1 / 2), has a role in the releasing and allow NIK to activate NF- κ B. In short, cIAP1/2 occupy a dual function, that of degradation of NIK in the basal condition and the degradation of TRAF3 upon binding of CD40L. Meanwhile, TRAF2 also performs a dual function, i.e. that of its inhibitory role of NF- κ B activation via its interaction with TRAF3, and its activator role of the NF- κ B following its interaction with CD40 and its subsequent degradation (Bishop et al., 2007, Brown et al., 2002, Zarnegar et al., 2008).

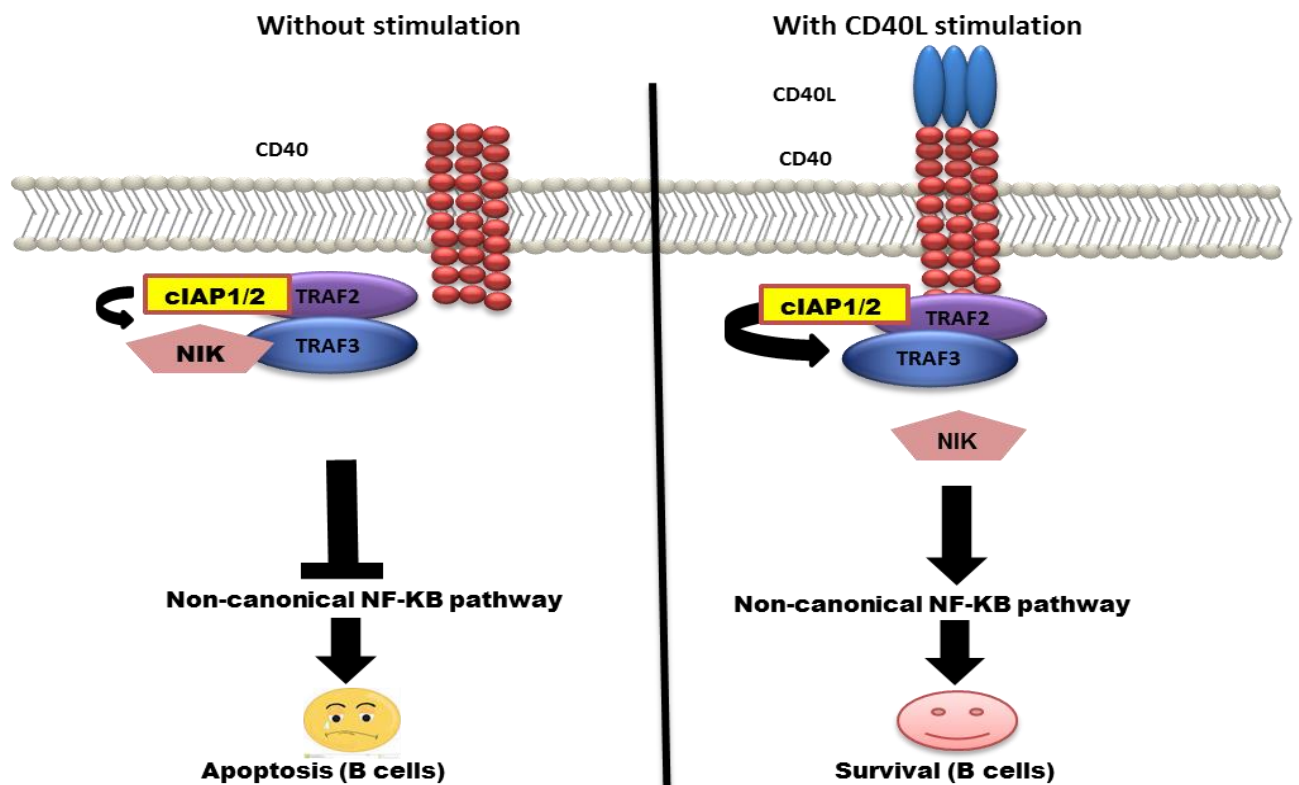


Figure 1. 23 Illustration of the dual function of TRAF2 in the regulation of NF- κ B

TRAF2 functions A) Inhibitory role of NF- κ B activation via its interaction with TRAF3, B) Activator role of the NF- κ B following its interaction with CD40 and its subsequent degradation (Adapted from (Elgueta et al., 2009)).

1.10.4 TRAF3

TRAF3 was first known as an adaptor molecule that interacts with the cytoplasmic tail of CD40 and the EBV oncoprotein LMP-1 (latent membrane protein-1) (Cheng et al., 1995, Mosialos et al., 1995). Nevertheless, Inoue et al. reported that TRAF3 associates with other TNF-Receptor superfamily proteins such as CD27, CD30, 4-1BB, OX40, LT- β R, ATAR, AITR, and RANK (Inoue et al., 2000); it has also been demonstrated that TRAF3 plays different roles for various receptor functions in *in vitro* culture systems (Hostager and Bishop, 1999).

TRAF3 blocks the activation of NF- κ B induced by CD40 and TNFR2 overexpression (Rothe et al., 1995). TRAF3 negatively regulates CD40-stimulated antibody secretion, however, whether the inhibition of NF- κ B mediates this response is not clear (Hostager and Bishop, 1999). This implication is supported by experiments showing that removal of the protein in B cells increases the activation of NF- κ B and JNK following stimulation by CD40L (He et al., 2007, Xie et al., 2004). In addition, mice deficient in TRAF3 demonstrate an intracellular accumulation of the protein NIK (Xie et al., 2004, Vallabhapurapu et al., 2008, Zarnegar et al., 2008). However, it was demonstrated that overexpression of TRAF3 in epithelial cells promotes activation of the NF- κ B pathway, contrary to B cells (Propst et al., 2002), while Urbich et al. suggest that TRAF3 has dichotomous functions depending on the cell type (Urbich et al., 2001).

1.10.5 TRAF4

TRAF4 was initially known as a protein localised in the nuclei of breast cancer cells, and it has also been detected in the cytoplasm of the cells (Régnier et al., 1995, Glauner et al., 2002). TRAF4 is barely expressed by vascular cells. It is more important in neuronal cell physiology and during embryogenesis (Masson et al., 1998, Regnier et al., 1997). Moreover, TRAF4 seems unable to interact with CD40 (Krajewska et al., 1998). Paradoxically, TRAF4 has been implicated in promoting apoptotic pathways mediated by p53, yet has been observed to inhibit Fas-mediated cell death (Sax and El-Deiry, 2003, Fleckenstein et al., 2003). Expression of TRAF4 in T cells is dependent on stimulators of the NF- κ B pathway (Glauner et al., 2002).

1.10.6 TRAF5

Very little information exists regarding the role of TRAF5 in CD40-mediated signalling. TRAF5 is unable to interact directly with CD40, but it appears to form a heterodimer with TRAF3 to facilitate activation of the NF- κ B (Bishop et al., 2007). This was demonstrated in B cells where TRAF5 deficiency by the use of small interfering RNA (siRNA) significantly reduces the activation of the NF- κ B, resulting in the reduction of expression of co-stimulatory molecules and antibody production by these cells (Hauer et al., 2005, Nakano et al., 1999).

1.10.7 TRAF6

TRAF6 plays an important role in the signalling pathways triggered by CD40 and also has a specific binding site for this function (Bishop et al., 2007) and can have several (often opposing) functions. Recently, TRAF6 has been shown to induce apoptosis via interaction with caspases and its activation by a RING domain-dependent mechanism (He et al., 2006). Interestingly, however, Benson and colleagues have shown that CD40 can defend B cells from CD95-mediated apoptosis by inhibition of caspase activation via TRAF6 and the PI3K/Akt pathway (Benson et al., 2006).

In human epithelial cells treated with small specific interfering RNA for TRAF6, activation of NF- κ B pathways, p38, JNK and Akt is significantly reduced or even completely inhibited following stimulation with CD40L, demonstrating the fundamental importance of this TRAF member (Davies et al., 2005). TRAF6 interacts directly with TRAF2 to regulate the activation of NF- κ B. This close collaboration between TRAF2 and TRAF6 does not seem to depend on the interaction with the CD40 receptor since, even in the presence of the deletion of the binding domain of TRAF6 to CD40, TRAF6 is still able to interact indirectly with CD40 through its direct interaction with TRAF2 and activate several important signalling pathways (Rowland et al., 2007). Under these conditions (where the binding domain is removed from CD40), activation of CD40 leads to expression of CD80 receptor and activation of the JNK pathway, in contrast to the total deficiency of TRAF6 protein in B cells (Rowland et al., 2007). This suggests that an important function of TRAF6 is connected to its indirect interaction with CD40, via its binding to TRAF2. One of the other important functions of TRAF6 lies in the PI3K pathway activation and subsequent activation of Akt, which protects against apoptosis (Arron et al., 2001, Davies et al., 2004).

1.11. The mitogen activated protein kinases (MAPKs)

MAPKs are serine threonine kinase activated by phosphorylation at threonine and tyrosine residues. These proteins are part of phosphorylation cascades that have been described in many species including mammals (L'Allemain, 1994). Many stimuli activate the MAPK cascades including hormones, growth factors and stressors. MAPK cascades are involved in signaling pathways leading to mitosis, proliferation, differentiation, cell growth, gene expression and cell death in response to extracellular signals (Johnson and Lapadat, 2002a, Zhang and Liu, 2002).

1.11.1 General structure

The MAPK family comprises 5 groups of kinases. Three groups have been widely studied, which include: Extracellular signal regulated kinases 1 and 2 (ERK), c-Jun N-terminal kinases (JNK) 1, 2 and 3 (or stress-activated protein kinase, SAPK)) and the p38MAPK α , β , γ and δ . Two other groups are less well known and their roles in cell signaling remains unclear; these are Extracellular signal regulated kinases the 3 and 4 and Extracellular signal regulated kinase 5 (Coulombe and Meloche, 2007). Downstream, the phosphorylated MAPK activate transcription factors such as c-myc, c-Jun or ATF2 but can also activate other kinases upstream and downstream of the MAPK cascade, thus refining the regulation of signaling pathways (Whitmarsh, 2007).

Upstream signaling pathways activating the MAPK are numerous. To a large extent, they are activated by small G proteins of the Ras superfamily that activate MAPK kinase kinases such as Raf proteins (Chong et al., 2003). The MAPKKKs phosphorylate serine and threonine residues at the patterns Ser-xxx-Ser/Thr MAPK kinases such as MEK. Finally, MAPKK MAPKs phosphorylate at xxx-Tyr-Thr residues which in turn phosphorylate Pro-xxx-Ser/Thr pattern transcription factors or other proteins of the signaling pathways (Biondi and Nebreda, 2003).

1.11.2 ERK 1/2

Extracellular signal regulated kinase (ERK) 1 and 2, which are 42 and 44kDa in size, respectively, were the first MAPK characterised and are the most studied. They are activated by growth factors, hormones, osmotic shock, cytokines, GPCR (G-protein coupled receptors heterotrimeric) and phorbol esters. They play a major role in cell proliferation and differentiation (Kang and Sucov, 2005, Kim et al., 2007). Activation of the ERK pathway leads to induction of genes leading to cellular hypertrophy, increased protein synthesis and the formation of sarcomeres (Aoki et al., 2000, Kim et al., 2007).

1.11.3 JNK 1/2 (SAPK)

Three isoforms of c-Jun N-terminal kinase (JNK) or stress-activated MAP kinase (SAPK) have been characterised and are activated in response to many stimuli such as physical stress (heat, UV, osmotic shock), chemical factors (pH, ROS), metabolic factors (ischemia), biological factors (bacterial proteins, cytokines). The JNK cascade follows the traditional pattern of MAPK activation with MAPKKK (MEKK1-4) that activate MAPKK (MKK4, MKK7) activating the MAPK (Figure 1.24). As with ERK, JNK activation leading to gene expression by phosphorylation of transcription factors including c-Jun, ATF-2, Elk-1, MEF-2. JNK pathways predominate in cellular responses initiated by adrenergic G-protein coupled receptors and tyrosine kinase receptors such as the receptors for growth factors. The JNKs are involved in the activation of apoptosis, but apart from their pro-apoptotic role, they can also have anti-apoptotic functions depending on the cell type and the nature of the stimulation. Their roles in cell physiology are numerous and depend on the area studied. They are involved in the development of the nervous system as in the regulation of insulin and obesity but also in hypertrophy and heart failure (Brancho et al., 2003, Derijard et al., 1995).

1.11.4 The p38/MAPK

Four isoforms of the p38 serine threonine kinases are activated by the MAPK signalling pathways, namely p38 α /Mpk2/CSBP, p38 β , p38 δ /SAPK4 and p38 γ /SAPK3. Stimulation of p38 shows similarities to JNK. At first, these two MAPKs were grouped under the term SAPK. Stimuli such as osmotic shock, or UV irradiation, oxidative stress and anticancer agents can activate the cascade of p38 phosphorylation. Different stimuli can activate MAPKKK (ASK, TAK, PTKs), which activate MAPKK (MEK3, MEK6 MAPKK) but also other cascades (such as MEK4, MEKK1, 2 and 5, ASK), allowing them to phosphorylate p38. As with other MAPKs, p38 kinases are activated by phosphorylation of threonine and tyrosine residues of their activation loop TGY by MAPKK kinases MKK3, 4 and 6 (Brancho et al., 2003, Derijard et al., 1995). The MAPKKs involved in the p38 pathway are TAK1 ASK1/MAPKKK5, MUK/DLK/ZPK, MEKK4 and MLK3 (Zarubin and Jiahuai, 2005). The p38 pathway plays an important role in the induction of genes involved in the inflammatory response (Kontoyiannis et al., 2001) and in the neuronal differentiation of myoblasts (Puri et al., 2000). The p38 pathway is also involved in apoptosis induced by Fas (Juo et al., 1997), by the loss of cell anchorage (Cardone et al., 1997) and may play a synergistic role with the JNK pathway in the induction of apoptosis (Xia et al., 1995).

The pathway of MAPK1/3 is composed of a multi-module complex where the protein kinases Raf, MEK and MAPK1/3 are activated in a cascade by sequential phosphorylation.

The stimulation of Ras coupled membrane receptors, such as growth factor receptors, activates the MAPK1/3 pathway, by a complex mechanism involving both phosphorylation and dephosphorylation. MAPK1/3 kinases transmit the signal generated by the receptor by phosphorylating a variety of substrates in different subcellular compartments, which leads to the execution of various biological functions such as cell proliferation, cell differentiation and cell migration (Katz et al., 2007).

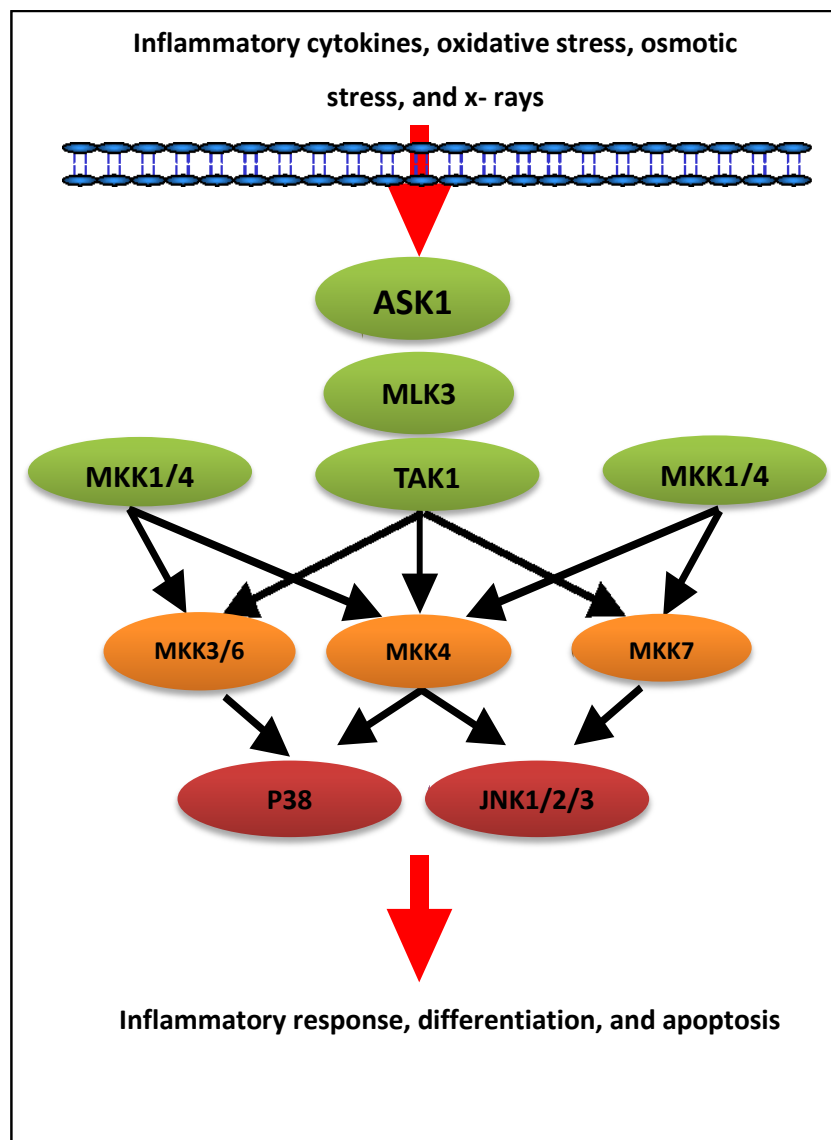


Figure 1. 24 The stress kinase pathway JNK/p38 MAPK

Signalling pathways triggered by the JNK and p38 kinases which belong to the family of SAPK (for stress-activated protein kinase). JNK and p38 pathways are activated by various extracellular stress and inflammatory cytokines. JNK/p38MAPK kinase pathways are composed of a multi-module complex having various protein kinases activated by phosphorylation in a hierarchical order: MKKKK activate MKK MAPK which ultimately activate JNK and/or p38 (Brancho et al., 2003).

1.13 Induction of apoptosis by the CD40

A plethora of studies have demonstrated the importance of the CD40/CD40L system in apoptosis induction. However, the effect of CD40 ligation appears to be cell type and context-specific, as it induces either a pro-apoptotic or an anti-apoptotic effect (cell survival), depending on the cell type and/or its state. In general, activation of CD40 in B cells and T cells induces an anti-apoptotic signal and cell survival (Banchereau et al., 1991, Lomo et al., 1997). Meanwhile, activation of several tumour cell lines, including B lymphoblastic, causes cellular apoptosis (Henriquez et al., 1999, Baker et al., 1998).

As discussed above, the binding of CD40 in B cells generates the proliferation, differentiation and survival of these cells. Indeed, the blocking of CD40/CD40L interaction, by genetic or pharmacological approaches, prevents differentiation of B lymphocytes into plasma cells and causes cell death (apoptosis) (Clark and Ledbetter, 1986, Klaus et al., 1997). The anti-apoptotic effects observed following activation of CD40 in B cells are primarily via an increase in anti-apoptotic proteins Bcl-xL, A20, Bfl-1, Mcl-1, and cFLIP (Figure 1.25). These factors protect against cell apoptosis in response to certain extrinsic apoptotic agents such as IgMs, FasL (Fas ligand) and TNF, and intrinsic agents, such as mitochondrial damage (Zhang et al., 1996).

Unlike normal B cells, activation of CD40 on B lymphoblastoid and some tumour cells cause significant anti-proliferative and apoptotic effects. In response to CD40L, Bax, Bak and Bik apoptotic proteins are found to increase, thereby promoting the activation of caspase-3 (Figure 1.25) (Szocinski et al., 2002, Tong et al., 2001). Furthermore, the activation of CD40 on tumour cells also promotes the binding of FasL and TNF to their respective receptors, resulting in the increase of caspase 8 and cell apoptosis (Garrone et al., 1995, Wingett et al., 1998).

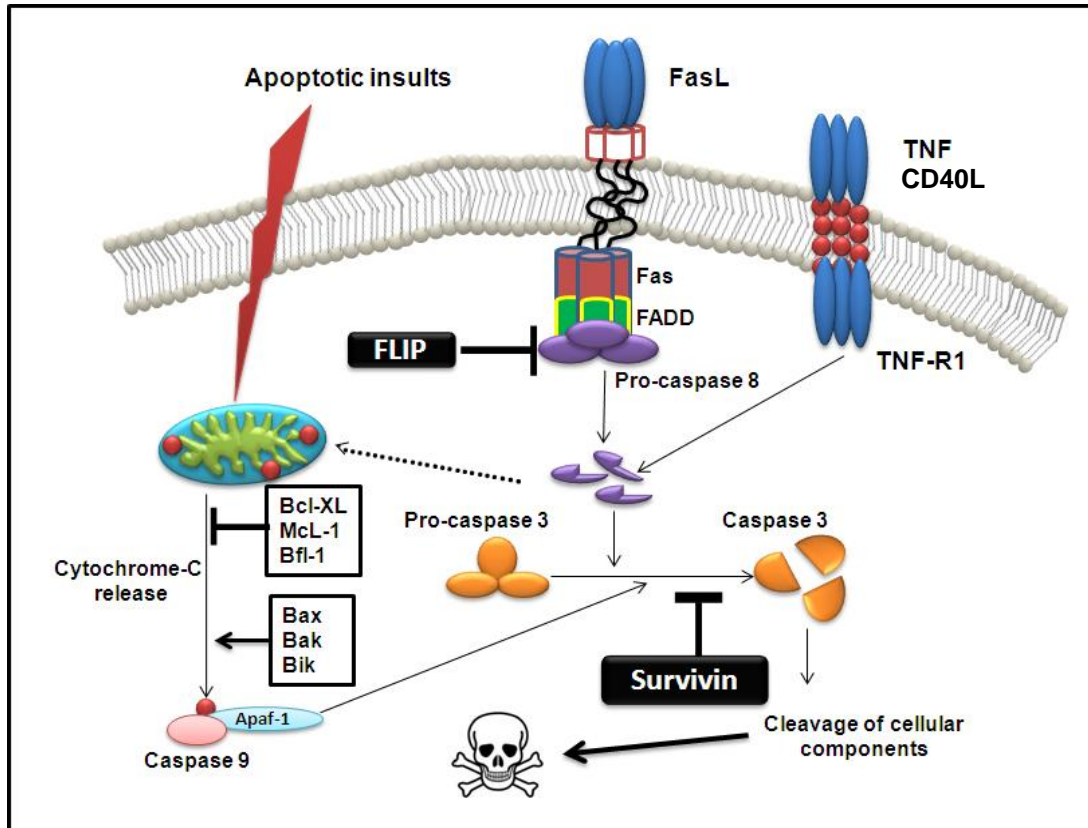


Figure 1. 25 Mechanisms involved in apoptotic and anti-apoptotic effects mediated by the activation of CD40.

Binding of CD40 in B cells prevents apoptosis through the extrinsic and intrinsic pathways. The intrinsic pathway comprises an increase in Bcl-xL anti-apoptotic factors, Mcl-1 and Bfl-1, which inhibits the activation of cytochrome c/Apaf-1/caspase-9 complex and the activation of caspase-3. Activation of CD40 also increases the apoptosis inhibitor protein survivin and also inhibits caspase-3. The extrinsic pathway of activation is from protein FLIP and their inhibitory effects on the activation of the TNF receptors and FasL, respectively. Unlike B cells, activation of CD40 on tumour cells causes the activation of the apoptotic proteins Bax, Bak and Bik, and promotes the binding of FasL and TNF to their receptors, while promoting cell death. Adapted from (Dallman et al., 2003).

1.13.1 Role of CD40/CD40L in apoptosis of the tumour

As discussed in Section 1.5.3 of this chapter, the activation of CD40 on several tumour cell lines induces pro-apoptotic signals, causing cell death. For example, stimulation of B lymphoma cells, multiple myeloma cells and Burkitt's lymphoma cells with CD40L significantly reduces cell survival and proliferation, both *in vitro* and *in vivo* (in mice) (Henriquez et al., 1999, Baker et al., 1998). These anti-proliferative effects were also demonstrated in solid tumours, such as carcinomas of the bladder, ovary and skin. Georgopoulos and colleagues demonstrated that membrane CD40 ligand (mCD40L) can induce apoptosis via a direct mechanism, while soluble CD40L did not induce apoptosis (Georgopoulos et al., 2006). Moreover, Georgopoulos and colleagues demonstrated that activation of the CD40 receptor on colorectal cancer cells *in vitro* via mCD40L causes high levels of death (Georgopoulos et al., 2007). Indeed, the treatment of carcinomas by a recombinant form of CD40L inhibits malignant cell proliferation and significantly increases the lifespan in mice (Tong et al., 2001, Eliopoulos et al., 1996, Ghamande et al., 2001, von Leoprechting et al., 1999) according to these observations we endeavour to understand the mechanism of CD40 ligation in colorectal cancer in order to develop effective cancer therapy.

1.13.2 Cancer

At first glance, the CD40/CD40L complex appears to facilitate the development and progression of cancer, as noted by the high levels of expression of these proteins on the surface of many tumour cells (Tong et al., 2001, Baxendale et al., 2005, Van den Oord et al., 1996). For example, it was demonstrated that tumour cell non-Hodgkin's lymphoma, chronic lymphocytic leukaemia and burkitt lymphoma exploit the CD40/CD40L system as a tool of survival and cell growth, primarily through signage of NF- κ B (Challa et al., 2002). In addition, there is a positive correlation between the expression of CD40L levels on certain tumour cells and the degree of aggressiveness and evolution of these cells (Van den Oord et al., 1996). However, after a review of the function of this complex in the pathophysiology of cancer, one arrives at the conclusion that the CD40/CD40L axis occupies a negative role in the pathogenesis of cancer. Admittedly, the most definitive finding supporting this observation comes from the symptoms observed in patients with XHIM syndrome (a genetic mutation causing a severe abnormality of the interaction of CD40L with CD40), in which there is a high frequency of several lymphomas and carcinomas (Hayward et al., 1997). Indeed, many tumour cells seek to deregulate the CD40/CD40L system in order to maintain their growth and survival, such as CD4⁺ T lymphocytes of patients with chronic lymphocytic leukaemia trying to suppress their expression membrane CD40L, or by enzymatic cleavage or by a defect in membrane mobilisation following the activation (Cantwell et al., 1997).

These compensatory mechanisms serve to avoid the anti-tumour effects of cytotoxic T lymphocytes and natural killer cells (NK) in the immune response. In addition, Hock et al. reported that patients with acute myelogenous (or myeloid) leukaemia (AML) and multiple myeloma have high levels of soluble receptor CD40 (sCD40) in their circulation, thus reducing the cellular response to CD40L and correlating with a poor prognosis in these patients (Cantwell et al., 1997).

The mechanisms by which the CD40/CD40L axis contributes negatively to tumour progression appear to be well characterised, and they include indirect and direct effects on the tumour cell. First, the CD40/CD40L complex is intimately linked to the production of cytotoxic T lymphocytes and NK cells that act directly on tumour cells to induce their cell death (indirect effects) (Loskog and Eliopoulos, 2009). Moreover, the binding of CD40 on tumour cells triggers a pro-apoptotic signal, which promotes cell death and leads to increased adhesion molecules and membrane proteins, such as the transporter antigen peptide-1 (TAP-1) (Cromme et al., 1994). This connection thus facilitates the recognition and interaction of anti-tumour immune cells (direct effects) (Loskog and Eliopoulos, 2009).

1.13 Colorectal Cancer

1.13.1 General

The colon is the name given to the last part of the intestine, which has a length of about 1.80 meters (6 feet) and is located between the small intestine and the rectum. The colon, also called the large intestine, ends in the rectum (Figure 1.26). Colon cancer and rectal cancer, commonly called colorectal cancer, is the third form of cancer most common cancers worldwide (Pisani et al., 1999, Fodde, 2002, Midgley and Kerr, 1999). In 2012, the World health Organisation (WHO) reported that “Colorectal cancer is the third most common cancer in men (746,000 cases, 10.0% of the total) and the second in women (614,000 cases, 9.2% of the total) worldwide. Almost 55% of the cases occur in more developed regions <http://www.cssanz.org/index.php/news>.

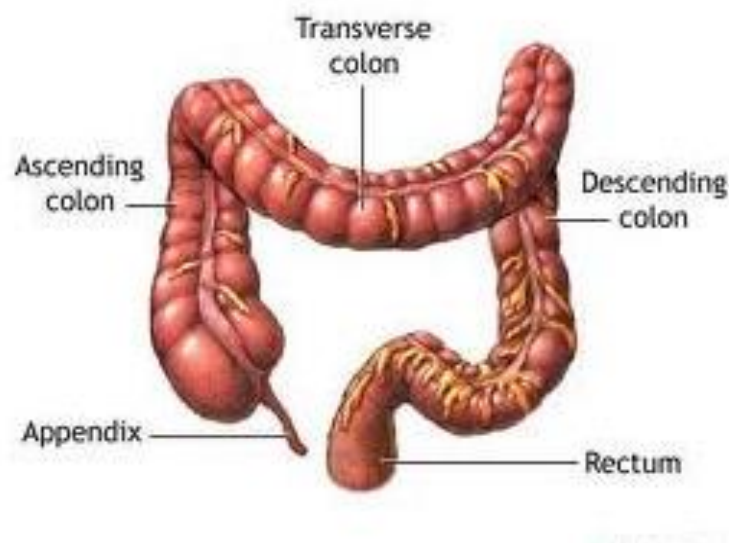


Figure 1. 26 shows structure of the large intestine.

<http://www.nlm.nih.gov/medlineplus/ency/imagepages/19220.htm>

1.13.2 Causes

The causes of cancer are still not well established. In some cases, genes that determine the activity of a cell mutate and begin to give instructions that cause the uncontrolled growth of tissue. The main risk factor, which is the most obvious warning signal, is the presence of colorectal polyps. These benign growths that are occur within the colon.

Polyps in the shape of a small ball placed on a rod which forms a protrusion in the inner wall of the colon (Levin et al., 2008). They may cause rectal bleeding, but the blood is generally masked in the stool. Each new polyp has a 2.5% risk of becoming cancerous in the first 5 years and 24% after 20 years. Larger polyps are more likely to become malignant as well as some people exhibit more than one polyp (Ham et al., 2010) (Figure 1.27). There are different stages of development of colorectal cancer; these stages are distinguished, depending on the degree of spread of cancer; Stage 0: at this stage The tumour is in situ, which means it is very superficial and does not invade the sub-mucosa, lymph nodes are not achieved and that there is no metastasis remotely. Stage I: the tumour invades the second layer (sub-mucosa) or the muscle layer (muscular) in the wall of the colon or rectum, lymph nodes are not affected and there is no distant metastasis. Stage II: The cancer cells through several layers of the wall of the colon or rectum, but no node is reached and there is no metastasis. Stage III: Cancer cells have spread to the lymph nodes near the tumour, and spread beyond the colon or rectum or spread to neighbouring organs such as the liver and lung (Shelton, 2002).

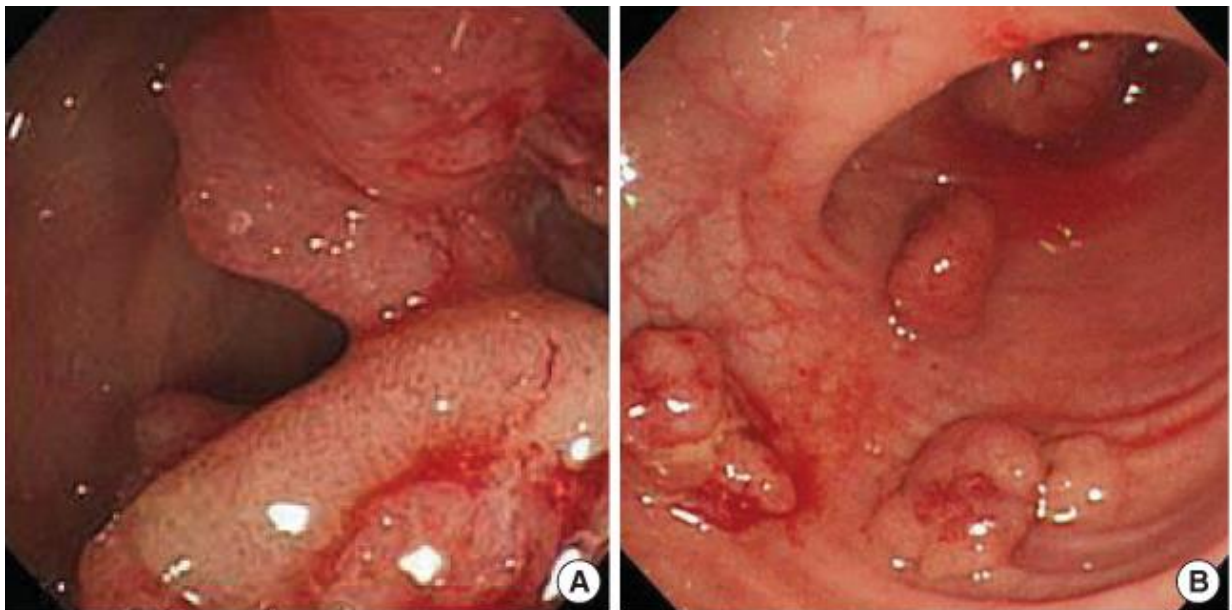


Figure 1. 27 Colonoscopy of CRC with multiple polyps

This figure shows images from a colonoscopy demonstrating colon and rectal cancer with multiple polyps: (A) Rectum (B) Multiple polyps in sigmoid colon – adopted from (Ham et al., 2010)).

1.13.3 Treatment and Prevention

There are three forms of treatment possible in case of colon cancer: surgery, radiation and chemotherapy. Surgery is usually recommended treatment to cure colon cancer, but it is only suitable for people suffering from these disease stages 1-3. If the disease is diagnosed at stage 3, radiotherapy or chemotherapy is then associated with surgery.

Immunogenic therapy may be a new therapeutic option for colon cancer and distant organ metastasis. Immunogenic therapy using immuno-stimulatory molecules to enhance anti-tumoral immunity may be effective at treating cancer.

The CD40 ligand (CD40L) is a type II membrane protein that belongs to the tumour necrosis factor (TNF) family, is predominantly expressed on CD4⁺ T cells, and binds the CD40 receptor on the membrane of antigen-presenting cells (APCs) (Roy et al., 1993, Grewal and Flavell, 1998). The interaction between CD40L and CD40 plays a crucial role in the activation of APC and in the initiation of both humoral and cellular immune responses (Bennett et al., 1998, Schoenberger et al., 1998). Therefore, gene transfer of CD40L has been proposed as an effective means of treating malignancies. Moreover, CD40 ligation triggers TNF, TRAIL and FasL induction in tumour cells which result in tumour cell death. It has also been reported that tumour specific T cells can be activated by CD40 ligation. Furthermore, CD40 ligation stimulates antigen presenting cells and natural killer cells to mediate cytotoxicity in tumour cells. In addition, cytokine secretion such as TNF α , cytokines, T helper cell activation, major histocompatibility induction and antigen presentation are other outcomes of CD40 ligation (Eliopoulos and Young, 2004). Collectively, because of its ability and both enhance immune responses and at the same time because of its capacity to induce direct cytotoxic effects on tumour cells Cd40 represents as very promising therapeutic target, for a variety of cancers involving CRC (Georgopoulos et al., 2007).

1.14 Aims

The main aim of this project was to for the first time provide a comprehensive understanding of the mechanisms of CD40 killing in CRC cells by unravelling the signaling events that underpin the capability of membrane CD40 ligand to stimulate CD40-mediated apoptosis in CRC cells.

More specifically:

- **Chapter 3:** Utilize a co-culture system to induce mCD40L-mediated CD40 ligation in CRC cells and optimise a number of assays for the detection of CD40 mediated apoptosis.
- **Chapter 4:** Using apoptosis detection assays, immunoblotting, pharmacological inhibitors and retrovirus-mediated shRNA-based RNA interference (RNAi) to study for the first time the regulation of key intracellular signalling components involved in pro-apoptotic mCD40L-induced CD40 signalling in CRC cells and provide functional evidence for a role of these components in apoptosis.
- **Chapter 5:** Provide a detailed understanding of the involvement of the ROS pathway in CD40 apoptosis by examining the extent of ROS generation, activation of NADPH oxidases as mediators of ROS generation and CD40-mediated apoptosis and potential CD40-mediated modulation of antioxidant pathways to influence the activation of downstream apoptotic signaling pathways.

CHAPTER 2: MATERIALS AND METHODS

2. Material and Methods

2.1 General

All practical work was carried out in the School of Applied Sciences, at the University of Huddersfield in the X1/17 and XB/07 laboratories.

2.2 Suppliers

Commercial suppliers and manufacturers are indicated at the first mention of the reagent or equipment in the text. A comprehensive list of all suppliers is provided in Appendix I.

2.3 Disposable plasticware

Sterile and non-sterile plasticware was obtained from different suppliers (Sarstedt, Fisher Scientific, Greiner Bio-One or Alpha Laboratories). Non-sterile, disposable plasticware was sterilised by autoclaving in a Prior Clave/London Autoclave at 121°C under pressure (2 Bar) for 15 minutes and then left to dry at room temperature.

2.4 Stock solutions

All chemical reagents were either of analytical or tissue culture grade as appropriate for the experiment and were supplied by Sigma Aldrich unless otherwise stated. General laboratory stock solutions were prepared in the laboratories with deionised water (dH₂O). All solutions which were used in tissue culture were prepared with ultra-pure water from a LabStar Ultra Violet purification unit. Heat stable solutions were sterilised by autoclaving at 121°C (1 bar) for 15 minutes. Otherwise to ensure lack of contamination, solutions were also filter sterilised using Acrodisc (VWR) low-protein binding Tuffryn® HT syringe filters with a pore size of 0.2µm.

2.5 Reagents

2.5.1 Primary antibodies

Primary antibodies used in this study are illustrated in the following Table 2.1. These antibodies were aliquoted in eppendorf tubes (10µl) and stored as recommended by the manufacturer until required. Working stock antibody solutions were prepared by diluting stock antibodies with TBS-Tween buffer (Tween20; Sigma Aldrich). This was dependent on the type of antibody.

Table2. 1 Primary antibodies

Antigen	Catalogue no/ Clone	Host	Supplier (product of)	Dilution	Application	Molecular weight (kDa)
Human CD40L	AF617	Rabbit	R&D systems	1:500 in TBS Tween20 0.1%	FC, WB	37
TRAF3	sc-949 / c20	Rabbit	Insight Bio (Santa Cruz)	1:250 in TBS Tween20 0.1%	WB, IP, IF, FCM and ELISA	65
TRAF1	sc-7186 / h-186	Rabbit	Insight Bio (Santa Cruz)	1:500 in TBS Tween20 0.1%	WB, IP, IF and ELISA	52
TRAF6	sc-8409	Mouse	Insight Bio (Santa Cruz)	1:500 in TBS Tween20 0.1%	WB, IP, IF, IHC(P) and ELISA	60
Phospho- SEK1/MKK4 (Ser457)	#4514 (C36C11)	Rabbit	New England Biolabs (NEB)/Cell Signalling Technology (CST)	1:1000 in TBS, 5% w/v BSA, 0.1% Tween20	WB, FC	44
Phospho-MKK7 (Ser271/Thr275)	#4171	Rabbit	NEB (CST)	1:1000 in TBS, 5% w/v BSA, 0.1% Tween20	WB	48
JNK/SAPK	#9258	Rabbit	NEB (CST)	1:1000 in TBS, 5% w/v BSA, 0.1% Tween20	WB	46, 54
Phospho-ASK1 (Ser967)	#3794	Rabbit	NEB (CST)	1:1000 in TBS, 5% w/v BSA, 0.1% Tween20	WB	155
Phospho-JNK/SAPK (Thr183/Tyr185)	255 (G9)	Mouse	NEB (CST)	1:500 in TBS, 5% w/v non-fat dry milk, 0.1% Tween20	WB, IP IF, FC	46 (phospho- JNK1) 54 (Phospho-JNK2/3)
Cytochrome C (H- 104)	sc-7159	Mouse	Insight Bio (Santa Cruz)	1:500 in TBS Tween20 0.1%	WB, IP, IF and ELISA	15
ASK1	#3762	Rabbit	NEB (CST)	1:1000 in TBS, 5% w/v BSA, 0.1% Tween20	WB	155
BAX	2282-MC-100 (YTH- 2D2)	Mouse	R&D systems (Trevigen)	1:500 in TBS 0.1% Tween20	WB, IP	23
Bcl-2	2291-MC-100 (YTH- 8C8)	Mouse	R&D systems (Trevigen)	1:500 in TBS 0.1% Tween20	WB, IP	25
BAK	AF816	Rabbit	R&D systems	1:500 in TBS 0.1% Tween20	WB	28
Phospho-p40phox (Thr154)	#4311	Rabbit	NEB (CST)	1:500 5% w/v BSA, 0.1% Tween20	WB	40

Human Thioredoxin	#2285S	Rabbit	NEB (CST)	1:500 5% w/v BSA, 0.1% Tween20	WB	12
β -actin Clone AC15	A5441 - 2ML	Mouse	Sigma	1:20,000 in 0.1% Tween20	WB	42
CD40	Sc-13128/ (H	Mouse	NEB(CST)	1:500 in 0.1% Tween20	WB	43
CK18	C8541	Mouse	Sigma-Aldrich	1:2000 in 0.1% Tween20	WB	45
CK18	081213	Mouse	Invitrogen	1:1000 in 0.1% Tween20	WB	45
TRAIL	3219	Rabbit	NEB (CST)	1:500 in 0.1% Tween 20	WB	28
FasL	4273	Rabbit	NEB (CST)	1:500 in 0.1% Tween 20	WB	26, 40
CK8/18	889257A	Mouse	Invitrogen	1:1000 in 0.1% Tween 20	WB	52/48
Phospho-P38	4511	Mouse	NEB (CST)	1:500 in 0.1% Tween 20	WB	40
DR5	8074	Rabbit	NEB (CST)	1:500 in 0.1% Tween 20	WB	40, 48
Bid (Human specific)	2002	Rabbit	NEB (CST)	1:250 0.1% tween 20	WB	15-22

A table listing all primary antibodies used in this study, their catalogue number, host origin, supplier or manufacturer, optimal dilution, type of blocking buffer and the range of their applications is shown here. (Abbreviations - WB: Western blotting, IP: Immunoprecipitation, IF: Immunofluorescence, FC: Flow cytometry).

2.5.2 Secondary antibodies

For detection of all monoclonal antibodies the Molecular probes Alexa Fluor® 680 Goat anti-mouse IgG (H+L) antibody was used (Invitrogen Cat # A21057). Detection of all polyclonal antibodies was achieved using the Goat anti-Rabbit IgG IRDYE800 antibody (Tebu-bio Cat # 039611-132-122). Fluorochrome conjugated secondary antibodies were titrated prior to use and are listed in Table 2.2.

Table 2. 2 Secondary antibodies

Antigen	Catalogue no/ Clone	Host	Supplier (product of)	Dilution	Application
Mouse IgG	A21057	Rabbit	Invitrogen	1:10,000 in TBS 0.1% Tween20	WB
Rabbit IgG	039611-132-122	Goat	Tebu-bio	1:10,000 in TBS 0.1% Tween20	WB
Goat IgG Alexa 680	A-21084	Donkey	Invitrogen	1:10,000 in TBS 0.1% Tween20	WB

All secondary antibodies, which used in the current study, their catalogue number, host origin, supplier or manufacturer, optimal dilution, type of blocking buffer and the range of their applications. (Symbols - WB: Western blotting). Fluorescence detection of antibodies at wavelengths 680nm and 800nm was performed using the Licor Odyssey Infra-red imaging system. When not in use antibodies were stored in the dark at 4°C.

2.5.3 Agonists & antagonists

Pharmalogical agonists and antagonists (Table 2-3) were reconstituted in either tissue culture grade dimethyl sulphoxide (DMSO; Sigma) or sterile distilled water (dH2O) according to the manufacturer's instructions. This was stored in single use aliquots at -20°C as recommended. Prior to use, all compounds were titrated using the cell viability assay (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay; Promega, UK, Cat # G3581) to determine the effective and non-toxic dosage. All reagents used in this study were purchased from the indicated supplier.

Table2. 3 Agonists & antagonists

Compound	Target	Supplier	Stock concentration	Effective concentration
N-acetyl L-cysteine (NAC)	ROS	Sigma	30mM (culture media)	30mM
DPI	NADPH Oxidase	Sigma	10mM (DMSO)	0.5µM
SP600125	JNK	Enzo	20mM (DMSO)	5µM
NDGA	AP-1	Sigma	20mM (DMSO)	5µM
SB202190	p38	Sigma	20mM (DMSO)	5µM
PX-12	Thioredoxin	Sigma	20mM (DMSO)	1-3µM
Diethyl Maleate	Gluthathione	Sigma	6.45 Molar	75µM
Staurosporine	Protein Kinases	Sigma	100µM	1-10µM
Caspase-8 Inhibitor	Caspase-8	R&D systems	20mM	25µM
Caspase-9 Inhibitor	Caspase-9	R&D systems	20mM	100µM
Caspase-10 Inhibitor	Caspase-10	R&D systems	20mM	100µM
General caspase Inhibitor (Z-VAD)	Caspases	R&D systems	20mM	100µM
Hydrogen peroxide	N/A	Sigma	9.79M	100µM-3200µM
G28-5	CD40	N/A	1.1mg/ml	10µg/ml
NOK1	FasL (CD95L)	Gift of Prof Yagita, Japan	1mg	100µg
RIK2	TRAIL	Gift of Prof Yagita, Japan	1mg	100µg

Agonists and antagonists used in this study, their target molecule, the supplier, the stock and effective concentrations are shown.

2.6 Tissue culture

2.6.1 General

All tissue culture work was undertaken using aseptic techniques within a HEPA filtration CellGard class II biological safety cabinet (NUAIRE). Prior to and after use, internal working areas within the hood were disinfected using 70% (w/v) ethanol (Fisher). To do so 99% Ethanol was diluted appropriately (150ml: 350ml) with purified autoclaved dH₂O. Internal hood spillages were disinfected using Mikrozid® (Gompel Healthcare Cat# 32644) and this was also used for a monthly routine sterilisation. Any unwanted cells, exhausted media or solutions were aspirated into a large conical flask containing 10% (w/v) Virkon and were then left for a minimum of 30 minutes before being decanted and washed into sewage.

All cell culture reagents were of tissue culture grade, and were a product of Sigma unless otherwise stated. To separate cells from solution, cell suspensions were spun for 5 minutes at 1500 RPM (210 RCF) using a Hettich Zentrifugen Universal 320 bench top centrifuge. Cell counts were performed from cell suspensions using a Marienfield Neubauer improved bright line haemocytometer before cells were seeded at the required cell density. When cells were not being manipulated they were kept in an Iso class 5 Nuaire Autoflow direct heat CO₂ incubator with a HEPA filtration system at 37°C in a 5% CO₂ humidified atmosphere (incubator contained dH₂O supplemented with Sigma clean (Sigma cat# S5525-40Z). Cultured cells were routinely observed by phase contrast microscopy using an EVOS XL (PeqLab) inverted microscope at x100 magnification.

2.6.2 Cryo-preservation and recovery of cell lines

Cells were cryo-preserved and kept in liquid nitrogen in a Statebourne storage dewar at -196°C. For cryopreservation of cell lines, cultures were collected as for passaging (as explained in section 2.6.3) and collected by centrifugation. The cell pellet was re-suspended in the appropriate ice-cold growth medium supplemented with 10% (v/v) FBS and 10% (v/v) dimethylsulphoxide (DMSO) at a cell density not less than 1x10⁶ cells/ml. Cells were aliquoted in a total of 1-1.5ml to polypropylene cryovials (Sarstedt) and then transferred to an ice-cold Nalgene “Mr Frosty” (Fisher) containing 250ml of isopropanol (Fisher) to control the cooling rate to 1°C per minute. Cells were then placed within a -80°C freezer for 4-6 hours prior to transfer to liquid nitrogen.

Cells were recovered by thawing rapidly at 37°C, before 5-10ml of pre-warmed growth medium was added. Cells were centrifuged at 1500RPM/210g for 5 minutes and then seeded to tissue culture flasks as required.

2.6.3 Carcinoma cell culture

In this study three colorectal cancer cell lines were used; HCT116 which naturally expresses CD40 receptor on the cell membrane (as shown in Chapter 3), SW480 were used as negative control (as they do not express CD40) and SW480-CD40, which is an SW480 isogenic derivative that was transduced with a CD40-expressing retrovirus to express CD40 (Georgopoulos et al., 2007, Hill et al., 2008a). The malignant bladder carcinoma cell line was also often used as a positive control for CD40 ligation studies as previously (Georgopoulos et al., 2007, Hill et al., 2008a).

HCT116 was initially grown in complete DMEM 10% FBS but was then adapted in D:R medium with 1% L-Glutamine, supplemented with 5% FBS (adaptation of cells is shown in Chapter 3). All colorectal cancer cells were maintained in a 50:50 (v/v) mixture of Dulbecco's modified eagle medium (DMEM Sigma cat # D6546-6X500ML) and Roswell Park Memorial Institute 1640 (RPMI Sigma cat # R0883-6X500ML) (referred to as D:R medium). This medium was supplemented with 5% fetal calf serum (FCS Biosera cat # S1810/500) and 1% L-Glutamine (Sigma cat #G7513-100ML). The SW480-CD40 cell line was grown in D:R medium with 1% L-glutamine, supplemented with 10% FBS, and 1.0mg/ml G418, while the original line SW480 was cultured in D:R medium without antibiotic (G418). All cell lines were maintained in T75 flasks with 12-14ml medium or T25 flasks in 5ml medium and were incubated at 37°C under 5% CO₂. All cell lines were sub-cultured every 2 to 3 days, when they were 80-95% confluent. At all times cell lines were cultured in the above mentioned medium and incubated at 37°C in 5% CO₂ unless otherwise stated.

For routine passaging, cells were collected by washing with 0.1% (w/v) EDTA in phosphate buffered saline (PBS) (without Ca and Mg) free (Invitrogen Cat# 14200-067) for 5 minutes and then addition of Trypsin-EDTA (Sigma Cat# T41474-20ml) in Calcium and Magnesium free Hanks-balanced salt solution (HBSS, Sigma Cat# H9394-6X500ML) until cells detached from culture flask. Trypsin was inactivated by the re-addition of the respective serum-supplemented culture medium when cells were re-suspended.

2.6.4 Murine fibroblast (3T3) cell culture

The mouse fibroblast cell line NIH3T3 has been previously stably transfected with two expression plasmids, the first one bearing the sequences coding for CD40L and Neomycin resistance gene (3T3-CD40L cells) and the second with Neomycin resistance alone (3T3-Neo cells) as described in Bugajska et al. (2002). During routine culture, these 3T3 derivatives were maintained in D:R supplemented with 10% FCS, 1% L-Glutamine (DR: 10%FCS/1% L-

G) and 0.5µg/ml G418 (Invitrogen Cat# ant-gn-1; supplied by Source BioScience LifeSciences) to ensure cells maintained transgene expression. At all times cell lines were cultured in the mentioned medium and incubated at 37°C in 5% CO₂ unless otherwise stated. 3T3 cells were harvested and passaged as carcinoma cells with the exemption of a very short 0.1% (w/v) EDTA in PBS treatment, as extended periods risked cell detachment.

2.7 Molecular Biology

2.7.1 shRNA design

Short hairpin RNA (shRNA) sequences were designed using the Invitrogen siRNA design tool (<http://rnaidesigner.invitrogen.com/rnaiexpress>) and incorporating *Bam*HI and *Eco*R1 overhangs as well as an internal *Mlu*I restriction site to aid in selection of positive clones. A minimum of two shRNA oligos were created improving probability of successful knockdown. Some designed shRNAs were based using published literature and some created using the online design tool from Invitrogen.

2.7.2 Cloning

Molecular cloning, transformation of competent *E.coli*, and purification of plasmid DNA was previously performed by Dr Nik Georgopoulos at the Leeds Institute of Molecular Medicine (LIMM), Cancer Research UK, St James Hospital (University of Leeds). A range of shRNAs were created using the designed oligonucleotides (supplied by Eurofins, former MWG Biotech) shown in Table 2-4.

Table2. 4 shRNA Oligonucleotides

TARGET mRNA	Complete sequence (<u>target</u>) of Oligonucleotides (orientated 5' to 3')	Design source
TRAF3	GATCCGAGTCAGGTTCCGATGATCTTCAAGAGAGATCATCGGAACCTGACTCTTTTTTACGCGTG (TRAF3siR1-for) AATTCACGCGTAAAAAAGAGTCAGGTTCCGATGATCTCTCTTGAAGATCATCGGAACCTGACTCG (TRAF3siR1-rev)	Liao et al., 2004
TRAF3	GATCCGCCCCACTGGAGAGATGAATTTCAAGAGAATTCATCTCTCCAGTGGGCTTTTTTACGCGTG (TRAF3siR2-for) AATTCACGCGTAAAAAAGCCCACTGGAGAGATGAATTCTCTTGAATTCATCTCTCCAGTGGGCG (TRAF3siR2-rev)	Invitrogen design tool
BAX	GATCCGCATGGAGCTGCAGAGGATGTTCAAGAGACATCCTCTGCAGCTCCATGTTTTTACGCGTG (BaxsiR1-for) AATTCACGCGTAAAAAACATGGAGCTGCAGAGGATGTCTCTTGAACATCCTCTGCAGCTCCATGCG (BaxsiR1-rev)	Ray and Almasan 2003
BAX	GATCCGGTGCCGGAACCTGATCAGATTCAAGAGATCTGATCAGTTCCGGCACCTTTTTTACGCGTG (BaxsiR2-for) AATTCACGCGTAAAAAAGGTGCCGGAACCTGATCAGATCTCTTGAATCTGATCAGTTCCGGCACCG (BaxsiR2-rev)	Bidere et al., 2003

The table shows the forward and reverse oligonucleotides for the construction of shRNA delivery vectors and expression by retroviral transduction using the pSIREN RetroQ system.

2.7.3 RNAi delivery plasmid

RNAi ready pSIREN RetroQ (Clontech) is a self-inactivating retroviral expression vector designed to express shRNAs via a U6 promoter. The vector encodes a 5' long terminal repeat (LTR) containing a cytomegalovirus type 1 (CMV) enhancer region and a mouse sarcoma virus (MSV) promoter to drive transcription of the RNA packaging signal Ψ^+ , shRNA of interest and Puromycin resistance cassette in eukaryotic cells. When expressed in the packaging cell line PT67, the plasmid will produce infectious but replication-incompetent viral particles which lack the structural genes necessary for virus formation and can infect a wide host range but cannot replicate. Insertion of the target shRNA sequence is via *Bam*HI and *Eco*RI restriction sites (Figure 2.1).

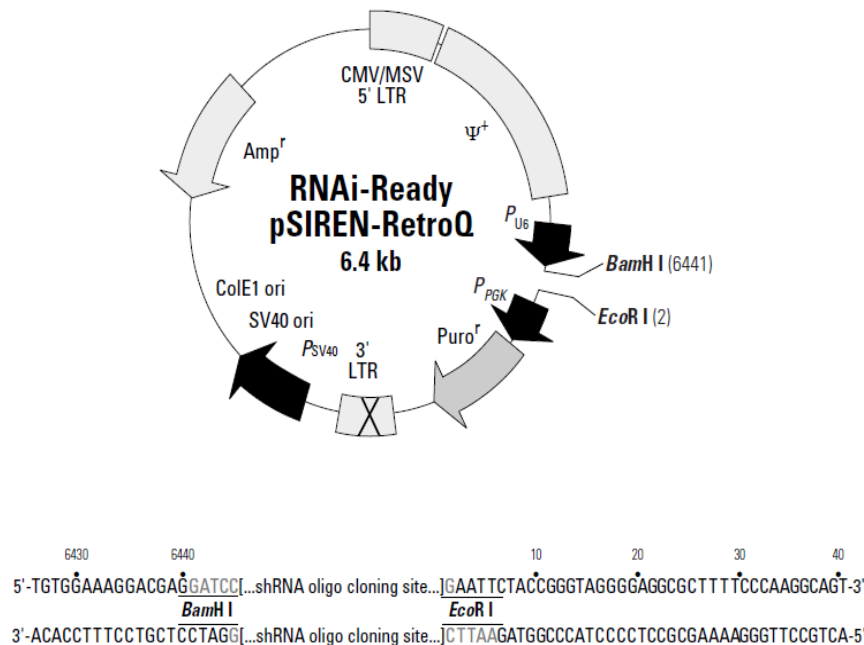


Figure 2. 1 pSIREN RetroQ plasmid vector

pSIREN RetroQ plasmid is based on the Moloney mouse leukemia virus (MMLV) and can be used for targeted gene silencing using RNAi. Oligonucleotides encoding short-hairpin RNA sequences can be cloned between the *Bam*HI and *Eco*RI restriction enzyme sites. Plasmid is then propagated in competent *E.coli* bacteria using the ColE1 origin of replication (ColE1^{ORI}) and successfully transformed cells are selected via expression of the ampicillin resistance gene (*Amp*^r). Upon transfection into the packaging cell line, PT67, the RNA packaging signal (Ψ^+), shRNA of interest and Puromycin resistance cassette (*Puro*^r) is transcribed via a 5' long terminal repeat (LTR) containing a cytomegalovirus type 1 (CMV) enhancer region and a mouse sarcoma virus (MSV) promoter. During reverse transcription of the retroviral RNA, the 3' LTR is copied and replaces the 5' LTR, resulting in inactivation of the 5' LTR CMV enhancer sequences. Vector is then packaged and the resulting retroviral particles can be utilised in a variety of mammalian cells. In target cells, expression of the short hairpin RNA and *Puro*^r is driven via a U6 promoter, an RNA polymerase III-dependant promoter. Imaged reproduced from the Clontech pSIREN RetroQ manual PT3737-5.

2.8 Methodologies for induction of CD40 ligation

CD40 receptor ligation for the experiments described in this study was carried out by membrane CD40L. Delivery of membrane-presented CD40L (mCD40L) was achieved by co-culture of 3T3CD40L cells (mCD40L) with CD40-positive target epithelial cells. As a negative control, epithelial cells were co-cultured with equal numbers of 3T3Neo cells (Controls). mCD40L and Control cells were growth arrested by treatment with 10 µg/ml of Mitomycin C (Sigma) for two hours in D:R before they were washed, harvested and seeded into 96 well plates at 1×10^4 cells/well or in 10cm² culture dishes at 3×10^6 /dish, for apoptosis detection assays and preparation of protein lysates, respectively. After mCD40L and Control cells were attached to either both 96 wells plates or dishes (in most cases following overnight incubation), epithelial cells were added at a ratio of 0.8 or 1.0 of epithelial cells, as optimised in this study (and detailed in Chapter 3). More specifically, 1×10^4 epithelial cells were seeded into 3T3 cell-containing 96 well plates and 3×10^6 cells were seeded into 10cm² culture dishes, respectively.

2.9 Detection of cell growth, death (apoptosis) and reactive oxygen species (ROS) production

2.9.1 General

Previously published guidelines regarding the use and interpretation of assays for monitoring cell death (Galluzzi et al., 2009) have recommended that a minimum of two assays are utilised for the detection of cell apoptosis. The current research made use of a cell proliferation assay (MTS) in addition to four apoptosis detection-specific assays; a) CytoTox-Glo b) caspase 3/7 activation c) DNA fragmentation and d) the loss of cell membrane integrity were all used as markers for apoptosis.

These assays were based on measurement of absorbance, fluorescence or luminescence. 96 well Nunc white, tissue treated culture plates (Fisher cat # TKT-186-010C) were used for luminescence and fluorescent based assays, 96 well Costar transparent tissue treated culture plates (Fisher Cat # TKT-186-010C) for absorbance, and 96 well ELISA microplates (Greiner bio one Cat # 655101) for ELISA. For most assays epithelial cells were co-cultured with either 3T3-CD40L (mCD40L) or 3T3-Neo (Control) cells. To calculate cell death background fluorescence and luminescence readings were

subtracted pairwise as appropriately, (e.g. “mCD40L/EJ – mCD40L” and “Control/EJ – Control” readings). The exemption was DNA fragmentation as this was unnecessary due to the pre-labelling of target epithelial cells. Finally, in all experiments blank controls were included as appropriate.

2.9.2 Detection of cell growth (biomass)

Detection of cell viability via determination of cell biomass was assessed using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation assay. The CellTiter 96® AQ_{ueous} One Solution Cell Proliferation assay involves the use of the MTS tetrazolium (yellow) which is reduced to a formazan derivative (brown Colour) by respiring cells. The observed change in colour is proportional to the total number of viable/proliferating cells. Epithelial cells were plated into 96 well plates with 6 replicate wells and then left to adhere overnight before the addition of culture medium containing pharmacological agonists or inhibitors. 20µl of CellTiter 96® AQ_{ueous} One Solution was added to appropriate wells and plates were incubated at 37°C in 5% CO₂ for a total of four hours. Total levels of formazan formation/cell viability were assessed using a FLUOstar OPTIMA (BMG Labtech) plate reader at a wavelength of 492nm and data was acquired using MARS software (BMG Labtech) and assessed using Microsoft Excel. Cell viability was calculated as percentage viability in comparison to controls using the following formula: (T/C) x100, where T= treated cells and C= controls cells.

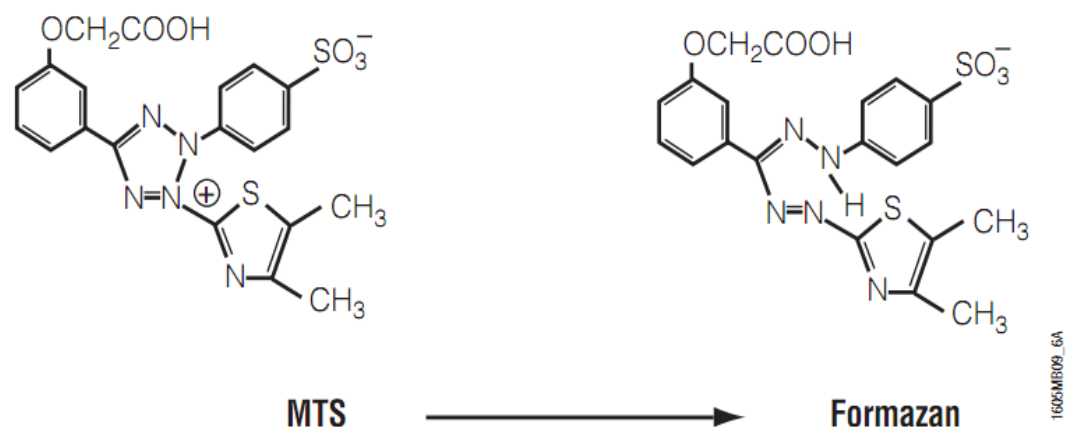


Figure 2. 2 The structure of MTS tetrazolium and its reaction product

This assay is a colorimetric method containing only one solution reagent consist of a new tetrazolium compound includes 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt: MTS and also contains phenazine ethosufate (PES), which uses as an electron coupling reagent that can bind with MTS to form a constant solution. This reagent can be reduced by cells into coloured product which is soluble in the medium which is known as formazan. The number of living/viable cells proportional with the absorption resulting of the interaction (formazan colour).

2.9.3 Detection of apoptosis using caspase3/7 assays

The activation of Caspases 3/7 is a well-established feature of cell apoptosis. Caspases 3 and 7 target a specific amino acid sequence located on many proteins which leads to overall cell demise by an organised apoptotic event. The activation of Caspases 3/7 was determined using the SensoLyte® Homogenous AFC Caspase-3/7 substrate (Anaspec Cat # 71114, supplied by Cambridge Bioscience), or the caspase3/7-Glo substrate assay (Promega Cat # g8091).

2.9.3.1 SensoLyte® Homogenous AFC Caspase-3/7 assay

The assay utilises the cleavage of the recognition sequence of caspase -3 and -7 that is Asp-Glu-Val-Asp (DEVD). The SensoLyte® Homogeneous AFC Caspase-3/7 assay kit uses Ac-DEVD-AFC as the fluorogenic indicator for assaying caspase-3/7 activity. Upon caspase-3/7-mediated cleavage, Ac-DEVD-AFC generates the AFC fluorophore which has bright blue fluorescence and can be detected at Excitation / Emission = 380nm/500nm. The degree of production of the strongly fluorescent fluorophore is relative to total levels of caspase-3/7 activation as shown in figure 2.3.

Epithelial cells were treated with membrane CD40 agonist in 96 well plates as described in section 2.8 before the addition of 50µl SensoLyte® Homogenous AFC Caspase-3/7 substrate. Fluorescence was measured using a FLUOstar OPTIMA (BMG Labtech) plate reader using Excitation/Emission 355nm/520nm filters, following calibration of the reader using the Gain function on the MARS software to ensure the measurements were taken within the dynamic range of the instrument. Plates were kept away from light and left at room temperature (RT) overnight after which fluorescence measurements were taken. To account for background created by fibroblasts, these were cultured alone and their relative fluorescent units (RFU) subtracted from the representative co-culture in a pair wise fashion (as explained in Section 2.9.1).

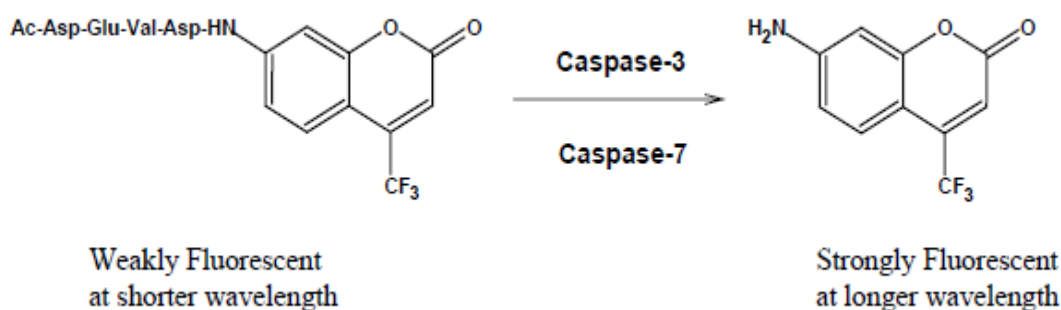


Figure 2. 3 Proteolytic cleavage of Ac-DEVD-AFC substrate

The amount of fluorescent product generated is proportional to the amount of caspase-3/7 cleavage activity present in the sample. Cleavage of the weakly-fluorescent caspase-3/7 Ac-DEVD-aminoluciferin substrate Z-DEVD by Caspase-3/7 to create the strongly fluorescent compound.

2.9.3.2 Caspase-Glo® 3/7 Assay

The assay utilises a pro-luminescent caspase-3/7 DEVD-aminoluciferin substrate and a thermostable luciferase in a reagent optimized for caspase-3/7 activity, luciferase activity and cell lysis. As the cells are lysed *in situ*, the luminescence can be detected immediately after substrate addition.

Epithelial cells were treated with membrane CD40 agonist as described in section 2.8 before the addition of 50µl caspase 3/7-Glo substrate. Luminescence was detected using a FLUOstar OPTIMA (BMG Labtech) plate reader, following calibration of the reader using the Gain function on the MARS software to ensure the measurements were taken within the dynamic range of the instrument. Plates were kept away from light and left at RT for 15 minutes before measurements were taken. Data was acquired using MARS software (BMG Labtech) and analysed by Microsoft Excel.

2.9.4 Detection of cell death using the CytoTox-Glo™ assay

The CytoTox-Glo assay is based on detection of the activity of a proprietary, specific protease normally present inside cells. During apoptosis, as the cell membrane is compromised the protease is released and it cleaves the AAF-Glo™ substrate thus generating a luminescence signal (Figure 2.4). The intensity of the luminescence signal indicates the degree of apoptotic cells in a population. Before the experiment, all reagents

were thawed at room temperature and all components mixed to ensure homogeneity. The CytoTox-Glo™ cytotoxicity reagent was prepared by transferring the contents of one bottle of assay buffer to the AAF-Glo™ substrate bottle, and then mixed to ensure homogeneity. The Lysis reagent was prepared by transferred 33 µl from digitonin to 5ml assay buffer.

Epithelial cells were treated with membrane CD40 agonist as described in section 2.8 before the addition of 50µl CytoTox-Glo substrate. Luminescence was detected using a FLUOstar OPTIMA (BMG Labtech) plate reader, following calibration of the reader using the Gain function on the MARS software to ensure the measurements were taken within the dynamic range of the instrument. Plates were kept away from light and left at RT for 15 minutes before measurements were taken. Data was acquired using MARS software (BMG Labtech) and analysed by Microsoft Excel. In cases where the percentage of dead cells was calculated the detergent Digitonin was added to lyse all cells according to the manufacturer's recommendations and a further reading was taken 15 minutes initial readings taken. To calculate percentage cell death the equation used was: (Background-corrected RLU before digitonin / background-corrected RLU after digitonin) x100, where RLU indicates relative luminescence units.

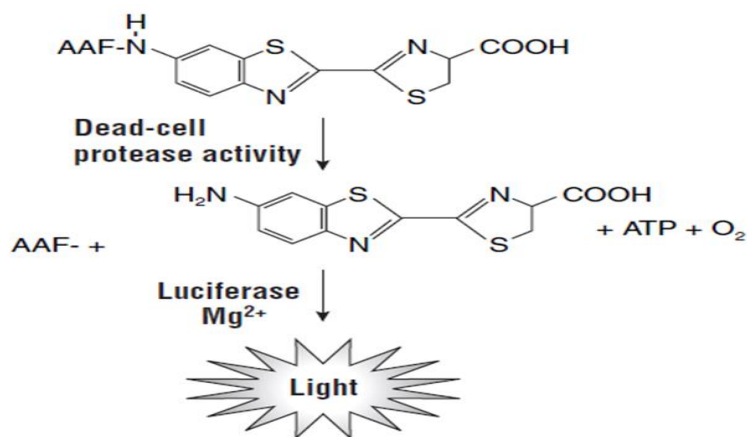


Figure 2. 4 The principle of the CytoTox-Glo™ assay

The first cleavage of luminogenic AAF-Glo™ substrate occurs by dead-cell protease activity and the second cleavage, and a substrate for luciferase (aminoluciferin) is released resulting in the luciferase-mediated production of light.

2.9.5 Detection of apoptosis using the DNA fragmentation ELISA

The fragmentation of DNA is often a hallmark of apoptosis and the DNA fragmentation ELISA assay uses 5-bromo-2'-deoxyuridine (BrdU) specific antibodies to detect BrdU-labelled fragments of DNA. Greater amounts of fragmented DNA labelled with BrdU represent a greater number of cells that have undergone apoptosis (the principle of the assay is schematically illustrated in Figure 2.5).

Exponentially growing epithelial cells were loaded with the DNA labelling agent BrdU for 2 hours at a concentration of 10 μ M according to the manufacturer's instructions. Cells were then treated with mCD40L by co-culture as described in section 2.8. An ELISA plate was coated with an anti-DNA antibody and then blocked to remove any non-specific binding sites. After washing of the ELISA plate to remove any blocking buffer, supernatants from cell cultures were added; these may contain DNA fragments pulsed with BrdU. The labelled fragments of DNA stick to the plate via the anti-DNA antibody and then a secondary, enzyme-linked antibody that specifically recognises BrdU was added. Finally, an enzyme substrate was added which is converted into a blue colour by the secondary, enzyme-linked antibody. The increased amount of colour change is relative to the amount of secondary antibody bound to BrdU labelled fragments of DNA. Diluted sulphuric acid (H₂SO₄) was used to stop the reaction after sufficient colour change, and following this, a deep yellow colour represented the degree of cell apoptosis. The plates were used to measure absorbance at using a 455-10nm filter on a FLUOstar OPTIMA (BMG Labtech) plate reader. Data was acquired using MARS software and analysed by Microsoft Excel. Although background controls were included to ensure the accuracy of measurements taken, no 3T3 cell alone background controls were necessary for this assay, as only epithelial cells were pulsed with the DNA labelling agent BrdU. Staurosporine was used as positive control (5 μ M).

$$\% \text{ of apoptotic cells} = \frac{(\text{Target cells} + \text{Killer cells})}{(\text{Target cell} + \text{Staurosporine})} \times 100$$

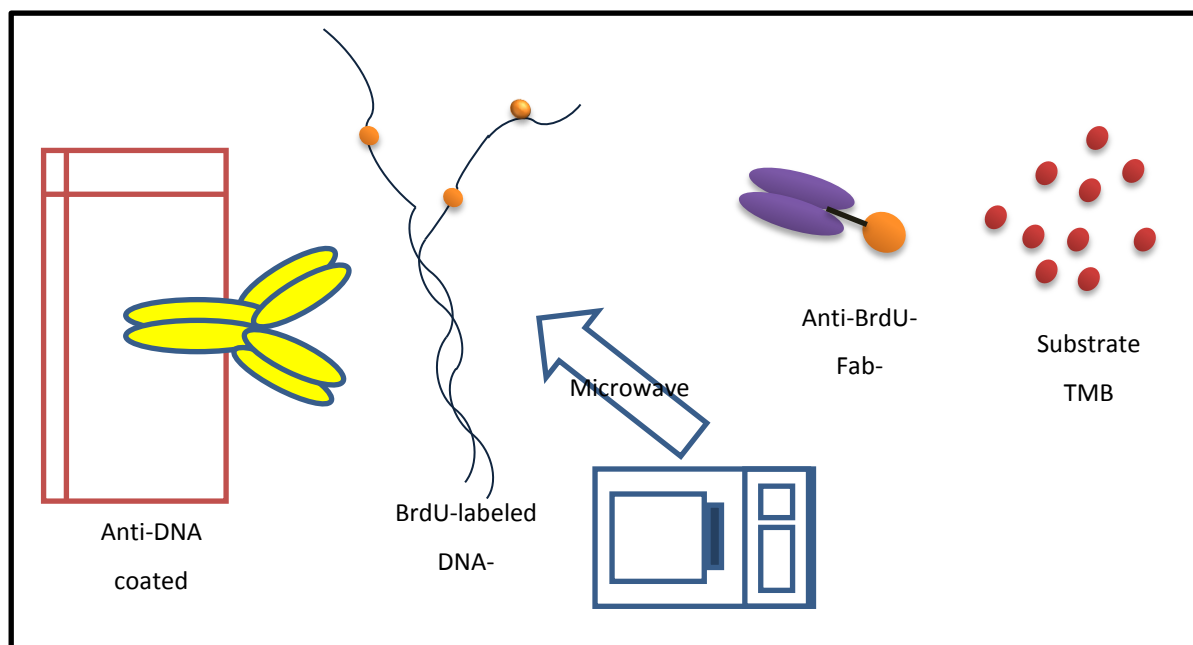


Figure 2. 5 Principle of DNA fragmentation assay.

Microtiterplate was coated with anti DNA antibody, BrdU-labelled fragments of DNA from co-culture supernatants which are fixed and denaturing by using microwave at 500watt for 5 minutes this was followed by the secondary antibody (anti-BrdU-Fab). Plate was incubated 90 minutes at room temperature and substrate was added. Once yellow colour was developed, sulphuric acid was added to stop the reaction.

2.9.6 Detection of Reactive oxygen species using H₂DCFDA

6-carboxy-2', 7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) (Invitrogen Cat # c2938) is a cell permeable, chemically-reduced, acetylated form of fluorescein used as an indicator for reactive oxygen species (ROS) in cells. This non-fluorescent molecule is readily converted to a green-fluorescent form when the acetate groups are removed by intracellular esterases and ROS-associated oxidation within the cell. As it is oxidation sensitive, H₂DCFDA was reconstituted in oxygen-free conditions (in a nitrogen environment) before aliquoting and storage at -80°C, as recommended by the manufacturer.

Epithelial cells were treated with membrane CD40 agonist as described in section 2.8. Notably, however, when co-cultures were performed, 3T3 fibroblasts used in this instance were not growth-arrested using Mitomycin C. Following co-culture, cells were first washed with PBS to remove any culture medium and were then treated with 1µM of H₂DCFDA in pre warmed (37°C) PBS for 30 minutes 37°C in 5% CO₂. The reduced forms of the substrate lack any fluorescence until acetyl groups are removed by intracellular esterases and oxidation is occurring in the cell. When this occurs the charge of the molecules makes it much less likely to leave the cell and it also emits detectable fluorescence. Following treatment with H₂DCFDA for the indicated time periods, fluorescence was measured on a FLUOstar OPTIMA (BMG Labtech) plate reader at Excitation 485nm/Emission 520nm, following calibration of the reader using the Gain function on the MARS software to ensure the measurements were taken within the dynamic range of the instrument.

2.10 SDS-PAGE and Immunoblotting (Western Blotting)

2.10.1 General

Western blotting is a powerful technique widely used in different fields in biological research to detect specific proteins throughout a cell lysate. Proteins from within a cell lysate can be gently extracted and maintained in buffers to ensure their integrity. Under denaturing conditions multiple proteins are size fractionated using SDS-PAGE, a form of gel electrophoresis. As electrophoretic any technique enables the SDS-PAGE separation of the particles made according to their electrical charge and to identical loads according to their size.

SDS-PAGE separation was performed under denaturing conditions due to the addition of SDS (sodium dodecyl sulfate). SDS is a strong detergent having a long hydrophobic hydrocarbon tail and a negatively charged end. It interacts with the protein in its hydrocarbon portion binder their hydrophobic regions. By binding to the protein, SDS prevents its folding and imparts a net negative charge. The native structure of the protein is denatured and an apparent negative charge is imparted to the protein. In the presence of SDS, the protein will thus have a negative apparent charge, so they all will migrate towards the anode. This means that only the molecular weight of the protein is factor separation. The proteins having a small molecular weight will be retained within the pores of the polyacrylamide gel and therefore will migrate farther than larger. Indeed, electrophoresis utilises the electrophoretic mobility of proteins to run them down a gel and specific gel pores separate them by size and charge. Proteins separated by this method are then transferred toward a transfer membrane which has high binding affinity for them. Stably bound to a membrane, size fractionated and denatured proteins are detected using epitope specific primary antibodies. A near-infrared (NR) fluorophore conjugated secondary antibody raised against the primary antibody is then added to the membrane and the membrane is scanned using an infrared scanner.

2.10.2 Co-culture and treatment to investigate intracellular signalling

3T3-Neo and 3T3-CD40L cell which express their CD40L surface were treated with MMC as explained in section (2.8). Cells were cultured in dish 10cm² at 3x10⁶ cells/. (Duplicate dishes for every cell line 5ml each) Figure2.6. All dishes were incubated at 37°C and 5% CO₂ for 24hours. The medium was replaced by SW480-CD40 (5 ml; 3.0 x10⁶ cells/dish) and dishes were incubated at 37°C and 5% CO₂ for the required times (6, 12 and 24hrs). Upon completion of incubation time lysates were made as explained in section (2.10.3). Protein concentration was measured in every lysates as explained in section (2.12.3). Lyastes were also used in immuonoblotting to detect intracellular protein expression in target cell (CRC cells).

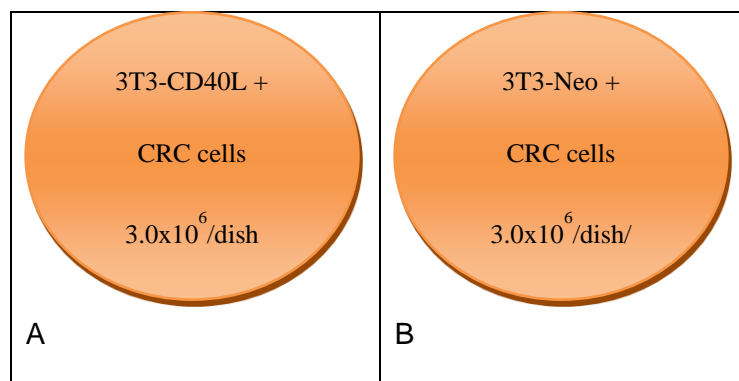


Figure 2. 6 Co-culture of CRC cells with 3T3 fibroblasts; 3T3-CD40L (A) and 3T3Neo

2.10.3 Protein extraction

Co-cultures of mCD40L and Control cells with target epithelial cells were grown in 10cm² culture dishes and lysed *in situ*. In some cases (where stated) cultures were maintained in the presence of pharmacological agonist/antagonists as indicated. Culture medium was aspirated and cell sheets were washed 2x in ice-cold D-PBS to remove any excess proteins. 20µl of ice-cold 2x sodium dodecyl sulphate (SDS) buffer (Appendix II) containing 2mg/ml DTT and 0.2%(v/v) protease inhibitor cocktail set 3 (Calbiochem) was pipetted onto the cell monolayer and the cells were scraped using a cell scraper (Fisher Cat# FB55199) into a lysate solution. The solution was then transferred to a chilled micro-centrifuge tube kept on ice. Samples were sonicated using an ultrasonic probe (Sonics Vibra cell) for 10-second bursts until lysate resembled froth like consistency it was then cooled on ice for 30 minutes. The lysates were centrifuged at 12,000-14000g, 4°C for 30 minutes to pellet the insoluble material, before aliquoting the supernatant and storing at -20°C.

2.12.4 Protein Quantification

The protein concentration of each sample was determined using a Coomassie protein reagent assay kit (Pierce cat# PN23236). Samples were diluted 4:46 in dH₂O and 10µl was aliquoted in quadruplicates into a transparent 96-well flat bottomed plate. A seven point standard curve of 0-1mg/ml (0, 25, 125, 250, 500, 750 100µg/ml) BSA (Pierce Cat# PN23208) was included on each plate. 200µl of ambient temperature Coomassie reagent was added to each well and mixed gently by pipetting. The absorbance was then measured

using a FLUOstar OPTIMA (BMG Labtech) plate reader at Abs 595nm against a dH₂O Control. MARS analysis software 2.0 (BMG Labtech) was used to plot a standard curve for the BSA and to estimate the protein concentration for each lysate (Appendix III).

2.10.5 SDS-Polyacrylamide gel Electrophoresis (SDS-PAGE)

20-40µg of protein lysate was made up to 13µl with dH₂O then this was totalled to 20µl by the addition of 5µl 4x lithium dodecyl sulphate sample buffer (LDS; Invitrogen Cat# NP0007) and 2µl of 10x reducing agent (500mM Dithiothreitol) (Invitrogen Cat#NP0009). The sample was denatured by heating 10 minutes in a 70°C water bath. 10-well NuPAGE™ Novex electrophoresis pre-cast gels (Invitrogen Cat# NP0321) were placed into an Xcell Surelock™ mini-cell upright electrophoresis tank (Invitrogen). 200ml and 600ml of 1x NuPAGE™ MES SDS running buffer (Invitrogen Cat# NP0002) was poured into the inner and outer chambers, respectively. 200µl of NuPAGE™ antioxidant (Invitrogen Cat # NP0005) was added to the inner chamber prior to loading of the samples. 5µl of All-Blue Precision Plus Protein™ standard (Bio-Rad #161-0373) was loaded alongside the samples as a marker of protein size (Figure 2.7) and gel was run at 200V for 35 minutes.

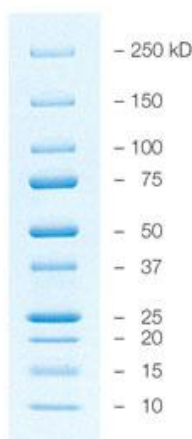


Figure 2. 7 Precision plus Protein standard

All Blue standards are a mixture of ten blue-stained recombinant proteins (10–250 kD), including three reference bands (25, 50, and 75 kDa).

2.10.6 Electrophoretic membrane transfer

Electrophoretically-separated proteins were transferred onto Immobilon-FL™ polyvinylidene difluoride membrane (PVDF; Millipore) using an Xcell II™ blot module (Invitrogen). PVDF membranes were dipped in methanol, rinsed in dH₂O and then soaked in 0.5x “Towbin” transfer buffer with 20% (v/v) methanol along with the required number of blotting pads and Whatman™ filter paper (Fisher). The gel membrane sandwich was assembled cathode to anode as follows; 2x blot pads, filter paper, gel, PVDF membrane, filter paper and 2x blot pads (as shown in figure 2.7). The blot module was secured into the Xcell SureLock™ Mini-Cell and filled with transfer buffer. The outer chamber was filled with ice-cold dH₂O and the transfers were performed on ice at 25V for 2 hours.

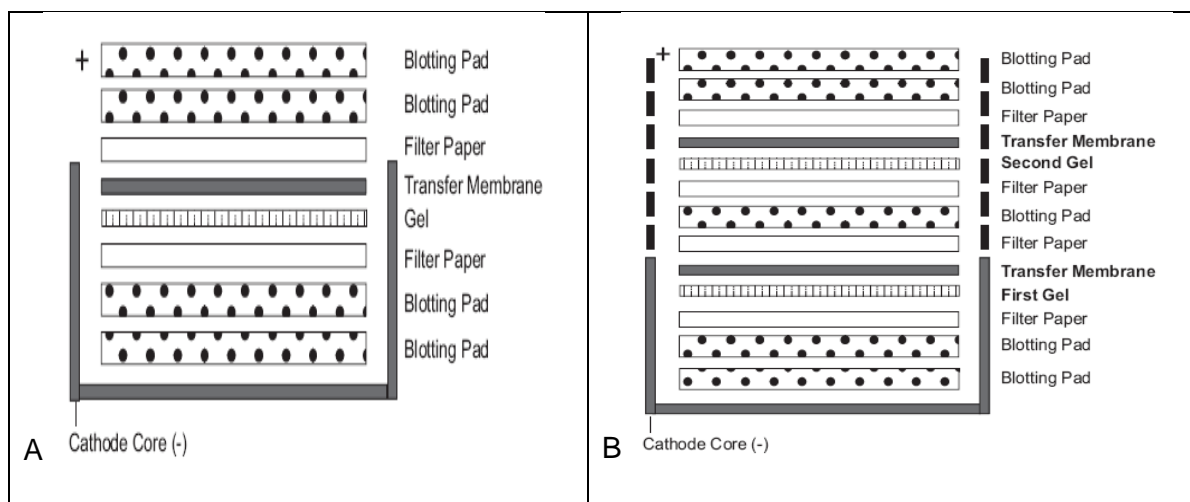


Figure 2. 8 A and B. Single sandwich and double sandwich.

The diagram illustrates protein transfer during immunoblotting

2.10.7 Membrane immunolabelling and visualisation using the Li-Cor Odyssey system

To minimise non-specific binding, membranes were blocked in 50:50 (v/v) Odyssey blocking buffer (Li-Cor Cat# 927-4000):10mM TBS pH 7.6 at ambient temperature on a plate rocker for 1 hour. Membranes were then probed with 5-8ml of pre-titrated primary antibody diluted in TBS+0.1% (v/v) Tween-20. All primary antibody incubations were performed on a rocking platform overnight at 4°C. Membranes were then washed 3 x 5 minutes in TBS+0.1% (v/v) Tween-20 prior to addition of 10ml appropriate infra-red secondary antibodies (Table 2-2) for 1 hour at ambient temperature on a rocker in.

Membranes were washed as for primary antibody and then washed 1x for 5 minutes with TBS prior to visualization using an OdysseyTM Infra-red Imaging system (Li-Cor). Where available, a positive control lysate known to express the protein of interest was included. Equal loading of epithelial lysate was verified using an antibody raised against the house keeping genes Cytokeratin 8 or 18 (or both, depending on the specificity of the antibody used). Densitometry was performed using Odyssey V3.1 software (Li-Cor) and protein expression was normalised relative to Cytokeratin 8/18, cytokeratin 8 and cytokeratin 18.

2.11 Flowcytometry

2.11.1 Background

Flow cytometry is used to analyse the chemical and physical characteristics of particles a technology, and interestingly used in immunology to highlight and count molecules or dead or alive cells by making them pass through a laser. The particles move at high speed in the laser beam, and a computer analysis of their physical characteristics and their number. Using flow cytometry to study cell morphology is determined and used to highlight any cell defects. This technique uses widespread in haematology, oncology and immunology.

2.11.2 Flow Cytometric Detection of CD40

To detect CD40 receptor on CRC cell surface, one monolayer about 80-85% confluent cells was harvested from flask by trypsinisation, spin to collect the cells and suspended again with 5 ml of DR medium. The cells were counted by using haemocytometer, then 1×10^6 , cells were collected and re-suspended in 400µl of FACS buffer (1X PBS/1% FBS). Then divided to four tubes 100µl was added to each tube; two for non-stain (NS), one for PE (Phycoerythrin) and one for CD40 antibody. 10 µl of diluted PE stain was added to the one tube used as negative control (diluted 1/10 in FACS buffer), and 15 µl of CD40 antibody (direct without dilution) added to the Ab tube to detect CD40, and the other two tubes without addition for non-stain (To detect live and death cells). Then all tubes incubated 25 minutes at 4°C. All tubes were washed with 700µl of FACS buffer then centrifuged at 15 RPM for 5 minutes in 25°C. Then 400µl of FACS buffer was added to each tube and all tubes mexed very well and read using flow cytometry, and data analysed by in Cyte 26 guava software (Millipore).

2.12 Separation of subcellular fractions for Western Blotting

2.12.1 Nuclear fractionation

In order to determine the localisation of certain proteins in response to CD40 ligation, the nuclei of cells were isolated using the Fisher Nuclear extraction kit (Fisher cat# PN78833) as instructed by the manufacturer. Nuclear pellets from co-cultures carried out in 10cm² dishes (3x10⁶ 3T3 cells and 3x10⁶ epithelial cells) were lysed with the provided lysis buffer and were processed in the same manner as for normal cell lysates and immunoblotting (Section 2.12). The kit also allows the isolation of the Cytoplasmic proteins which are used to compare the localisation of proteins between the nucleus and cytoplasm. Nuclear fractions and cytoplasmic fractions of cell cultures were screened for the presence of CD40, which is cytoplasmic only, to validate separation of the two cellular compartments. The required primary and secondary antibodies were added and then membranes were scanned as normal for western blotting techniques.

2.12.2 Mitochondrial fractionation

Part of the intrinsic pathway that executes cell death involves release of Cytochrome C from the mitochondrial matrix. In order to examine this phenomenon the mitochondria were isolated from co-cultures performed in 10cm² dishes (3x10⁶ 3T3 cells and 2.7x10⁶ epithelial cells) using a Dounce homogeniser (Fisher 11582443) and centrifugation as instructed by the manufacturers of the Millipore mitochondrial isolation kit (Cat # MT1000). Mitochondrial pellets were then lysed as instructed and processed the same way as for normal cell lysate and Western Blotting. The kit also allows the isolation of the Cytoplasmic proteins which are used to compare the localisation of proteins. Mitochondrial fractions of cell cultures were screened for the presence of Bcl-2 a protein located in the mitochondrial membrane and Cytochrome c a protein found within the mitochondrial matrix. Cytoplasmic fractions were also tested for Cytochrome C to determine any release and also for the cytoplasm-specific protein GAPDH. All antibodies for related proteins were supplied along with the kit along with recommended dilution factors. Appropriate primary and secondary antibodies were added and then membranes were scanned as normal for western blotting techniques. 2.15 Mycoplasma testing *Mycoplasma spp.* contamination is a huge problem in eukaryotic cell culture and can lead to unreliable experimental results (Capes-Davis et al., 2010). All cell lines were routinely tested for intracellular bacteria *Mycoplasma spp.* using the MycoProbe™ Mycoplasma detection assay (R&D systems Cat # CUL001B) which is

designed for screening of cultured cells. This assay detects Mycoplasma 16S ribosomal RNA (rRNA) using a colorimetric signal amplification system with sensitivity comparable to PCR. The assay was performed as recommended by the manufacturer's instructions which involved sample preparation in 96 well plates, and an ELISA based detection of 16S Ribosomal RNA and signal detection at absorbance 492nm measured on a FLUOstar OPTIMA (BMG Labtech) plate reader. Results were compared to positive control samples that were included in the kit.

2.13 Statistical analysis

Data analysis was carried out using Excel® (Microsoft) and as the mean of all replicates (minimum 5-6), with error bars representing \pm the standard error of mean (S.E.M.). Statistical analysis was performed using Minitab 15 statistical software. Two tailed, paired or unpaired t-tests were used to compare two sample means with levels of significance cited in the text. Comparisons were assumed to be biologically significant where $P < 0.05$.

CHAPTER 3: Optimization of experimental techniques to investigate CD40-mediated apoptosis in CRC cells

3.1 Background

CD40, a member of the tumour necrosis factor receptor (TNFR) family, is expressed in a variety of epithelial cells. Furthermore CD40 can be detected on endothelial cells, keratinocytes, smooth muscle cells, and fibroblast cells (Kooten and Banchereau, 1997, Schönbeck and Libby, 2001, van Kooten and Banchereau, 2000). Though its cognate ligand CD40L, which also is known as CD154, is mainly expressed on activated T-cells, it can also be found on basophils, eosinophils, monocytes, macrophages, dendritic cells, neutral killer cells, B lymphocytes, platelets, mast cells, endothelial cells, smooth muscle cells and in some cases epithelial cells (Schönbeck and Libby, 2001).

Previous studies have reported that CD40 engagement by CD40 agonists has the potential to regulate tumour cell growth (Eliopoulos and Young, 2004, Tong and Stone, 2003). Although a number of studies have shown that soluble CD40 agonists can induce growth inhibition in carcinoma cells of various origins (as reviewed by (Vonderheide, 2007, Tong and Stone, 2003), these agonists are mainly growth inhibitory or weakly pro-apoptotic and only become significantly pro-apoptotic by pharmacological intervention (Hess and Engelmann, 1996, Afford et al., 2001, Ahmed-Choudhury et al., 2003, Bugajska et al., 2002). By contrast, previous work in our laboratory has demonstrated that unlike soluble agonists (soluble trimeric CD40L or agonistic anti-CD40 antibody) which are weakly pro-apoptotic (Bugajska 2002; Georgopoulos 2007), membrane-presented CD40L is a highly pro-apoptotic signal (Shaw et al 2005; Georgopoulos et al 2006; Georgopoulos et al 2007; Hill et al 2008) that induces extensive apoptosis in malignant cells but not their normal epithelial counterparts (Bugajska 2002; Shaw et al 2005).

In order to achieve CD40 ligation by membrane CD40 ligand (mCD40L), epithelial (target) cells are co-cultured with third-party (effector) cells engineered to express membrane CD40L. At the beginning of this research, and following the move of the Georgopoulos laboratory to Huddersfield University, it was essential to ensure the reproducibility of the co-culture system and to establish and optimise a series of experimental techniques for the detection of apoptosis for use with this co-culture system, that would permit accurate and reliable detection and quantification of mCD40L-mediated apoptosis based primarily on 96-well plate format assays. In addition, optimisation of immunoblotting techniques for use in the co-culture system for CRC cells was equally essential.

3.2 Co-culture model for CD40 ligation by mCD40L

For the ligation of CD40 on target cells, a membrane CD40 ligand signal was delivered by their co-culture with pharmacologically growth-arrested effector cells (See section 2.10). The effector cells (killer cells) are murine fibroblasts (NIH3T3) that have been genetically manipulated to express membrane CD40L (Bugajska et al., 2002). Throughout this thesis effector cells displaying CD40L on their membrane were termed “3T3CD40L” cells. To ensure that 3T3CD40L cells retained CD40L expression (due to possible culture-related genetic drift) continuous culture in the presence of G418 antibiotic (0.5mg/ml) was performed, as a neomycin resistance gene was co-transfected as part of the CD40L gene expression construct. Homologous NIH3T3 cells with a gene cassette conferring G418 resistance only were used as negative (background) controls and therefore from this point will be termed “3T3Neo” cells throughout this study.

3.3 Objectives

The objectives of this chapter were:

- To demonstrate the expression of CD40 ligand by the effector (killer) cells and expression of CD40 receptor on the target cells (CRC cells) employed in this study for use in the co-culture system.
- To use this *in vitro* co-culture system and optimise its use for a variety of experimental techniques for the detection of mCD40L-mediated apoptosis in colorectal cancer (CRC)
- To perform optimisation of immunoblotting techniques for epithelial cell protein detection using the co-culture system.

3.4 Confirmation of CD40 and CD40L expression

For the purpose of performing co-cultures as described above, it was essential to ensure that effector and target CRC cells expressed CD40L and CD40, respectively. CD40 on CRC cells lines and CD40L on 3T3 fibroblasts were detected by Western blotting. This study confirmed CD40 was expressed on CRC cells HCT116. SW480 cells are CD40-negative in agreement with previous findings (Georgopoulos 2007), however retrovirus-transduced derivatives expressing *de novo* CD40 as previously described were CD40-positive (Figure 3.1), as was the positive control UCC line EJ as previously (Bugaska 2002; Georgopoulos 2007; Hill 2008). CD40 expression on these cells was also confirmed by flow cytometry, as shown in Figure 3.2.

CD40L expression on 3T3 fibroblasts (3T3Neo and 3T3CD40L) was detected by western blotting. CD40L was detected in 3T3CD40L cells, while 3T3Neo showed no detectable of CD40L expression as shown in Figure 3.3.

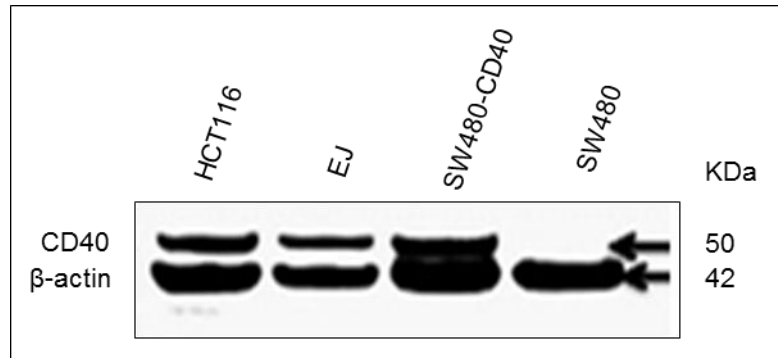


Figure 3. 1 Expression of CD40 detected by Western blotting

Western blot analysis for CD40 expression in CRC cells (HCT116, SW480 and SW480-CD40 cells) as well as the UCC line EJ). Total amount of protein loading was 20µg/well. The membranes were incubated with primary antibody (CD40 H-10 mouse monoclonal IgG diluted 1:500). Secondary antibody used was goat-anti mouse IgG, Alexa 680 dilution 1:10000. β-actin (AC-15-A5441) was used as specificity and loading control, the membrane was incubated with the antibody diluted at 1:50000 and secondary antibody goat-anti mouse IgG Alexa 680 diluted 1:10000. Membranes were scanned on Licor Odyssey Infra-Red Imaging System and images are shown in black and white.

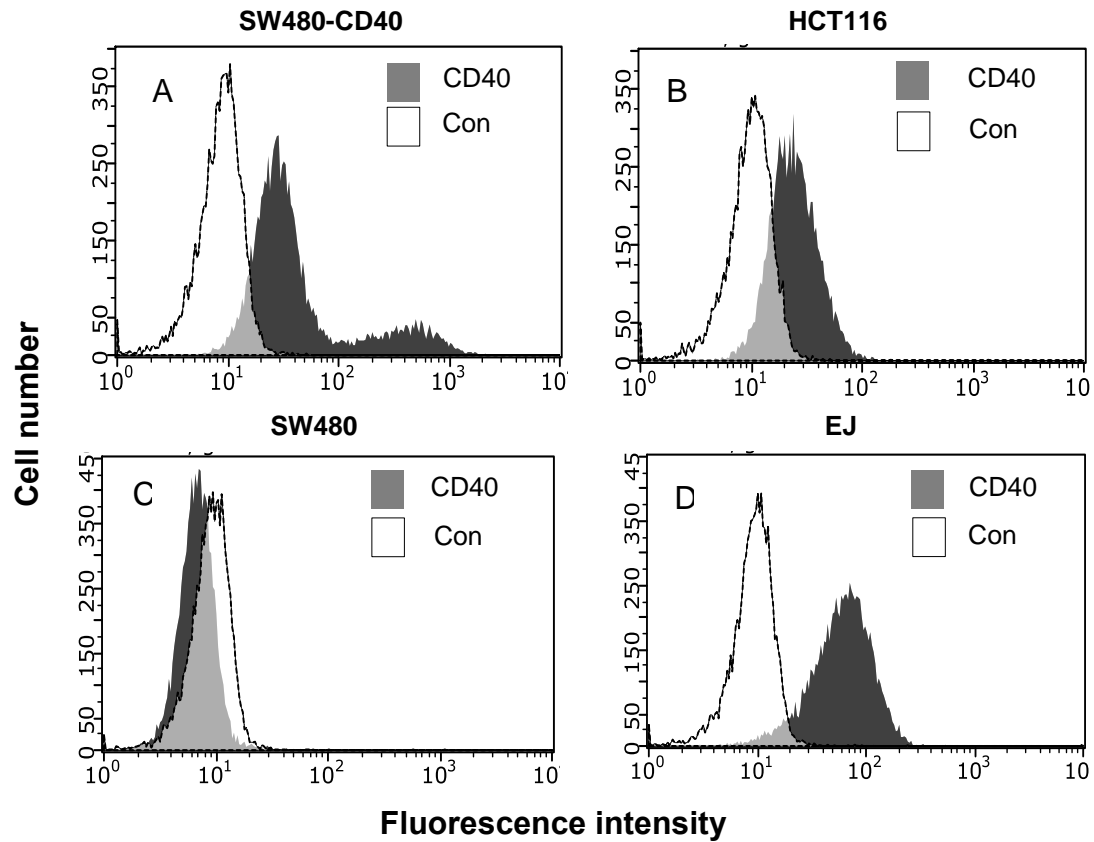


Figure 3. 2 Flow cytometric analysis of CD40 expression

Expression of CD40 receptor on the cell surface of CRC and EJ cells was detected by flow cytometry (section 1.12). Cells were cultured till approximately 80% confluent and were harvested by trypsinisation. Cells were counted and adjusted at 0.25×10^6 cells/100 μ l of FACS buffer. Cells were incubated with PE-conjugated mouse anti-human CD40, and a control PE-conjugated isotype-matched control Ab was also used. Cells were acquired on a Guava EasyCyte flow cytometer and results analysed using GuavaSoft software. A and B overlay histograms show SW480-CD40 and HCT116, which are positive for CD40 expression. C histogram show SW480 negative control cells for CD40 expression whereas, EJ cells in histogram D represent a positive control for CD40 expression.

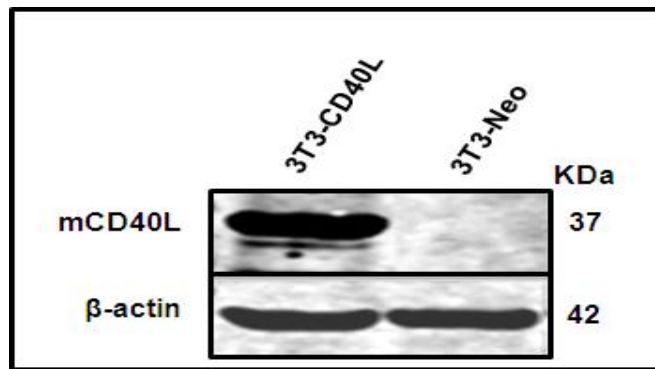


Figure 3. 3 Expression of membrane CD40 ligand (mCD40L) on 3T3 effector cells by western blotting

Western blot analysis for CD40L expression in 3T3s (3T3-Neo and 3T3CD40L). Total amount of protein loading was 20µg/well. The membranes were incubated with primary antibody (CD40 ligand/TNFSFS antibody monoclonal Anti human was diluted 1:500). Secondary antibody used was donkey-anti goat IgG, Alexa 680 dilution 1:10000. β-actin (AC-15-A5441) was used as specificity and loading control, the membrane was incubated with the antibody diluted at 1:50000 and secondary antibody goat-anti mouse IgG Alexa 680 diluted 1:10000. Membranes were scanned on Licor Odyssey Infra-Red Imaging System and images are shown in black and white.

3.5 Optimisation of experimental techniques (apoptosis assays) for the detection of mCD40L-mediated cell death

According to previously published guidelines regarding the use and understanding of assays for detecting cell death (Galluzzi et al., 2009), it is recommended that at least two independent assays are used for the detection and demonstration of apoptotic cell death. Moreover, previous studies into CD40-mediated apoptosis by the Georgopoulos group (Georgopoulos et al., 2007, Bugajska et al., 2002, Georgopoulos et al., 2006, Shaw et al., 2005, Hill et al., 2008b) generally have used assays that a) involved use of radioactive precursors (such as the JAM test of DNA fragmentation, b) did not permit high-throughput 96-well plate-based analysis (Annexin V/PI), and c) were not very sensitive (thus had a short linear dynamic range).

Therefore, one of the aims of this study was to employ assays that would address these issues and weaknesses. The apoptosis assays optimised for the detection of CD40-mediated cell death in this study were mainly the commercially available CytoTox-Glo cytotoxicity assay and the Sensolyte Homogenous Caspase3/7 assay (Materials and Methods). In addition, the DNA fragmentation ELISA assay was also investigated for its efficacy and practicality in this study.

3.5.1 Detection of mCD40L-induced apoptosis using the CytoTox-Glo assay

3.5.1.1 Optimization of apoptosis assays – detection of cell death and determination of optimal cell densities for the detection of CD40-mediated apoptosis

The CytoTox-Glo assay was tested for its ability to detect CD40-mediated apoptosis using the co-culture system. Co-cultures were carried out and the assay was performed as detailed in Section 2.10. An important consideration for these experiments was that the CytoTox-Glo assay does not distinguish specifically between dead epithelial cells and effector (3T3CD40L and 3T3Neo) cells during co-culture. In order to account for background RLU attributable to 3T3CD40L and 3T3Neo cells, in addition to experimental replicates that contained co-cultured cell populations, 3T3CD40L and 3T3Neo cultures alone were included, and their luminescence measured following substrate addition. Therefore, pair-wise subtraction of background readings was performed for each type of co-culture. 3T3CD40L alone readings were subtracted from the appropriate co-culture RLU; e.g.

$[(3T3CD40L/HCT116 - 3T3CD40L)]$ allowing the deduction of background-corrected RLU. For the control (3T3Neo) was treated in the same way to calculate the background corrected RLU, i.e.. $[(3T3Neo/HCT116 - 3T3Neo)]$.

It has been demonstrated previously by our group that CD40 ligation by membrane CD40L can induce apoptosis in carcinoma cells particularly in colorectal cancer cells (Georgopoulos 2007). In this study, different CRC cell numbers were used to optimise cell death via CD40 ligation. The target cells were co-cultured by seeding onto fibroblasts in white plates at different densities (8×10^3 , 1×10^4 , and 1.2×10^4 cells/well) with 3T3CD40L and 3T3Neo. As mentioned in the Materials and Methods chapter 3T3s cell were treated with mitomycin C (MMC) to avoid artifacts of cell overgrowth; they were initially seeded at 1×10^4 cells/well as previously determined (Georgopoulos et al., 2006, Georgopoulos et al., 2007). In this study, co-culture with 3T3CD40L resulted in high levels of death in HCT116 cells with 1×10^4 cells/well, whilst lower level of death was detected with others (8×10^3 and 1.2×10^4 cells/well), as shown in figure 3.4 where results are presented as background corrected RLU (A) and also presented as fold change (B).

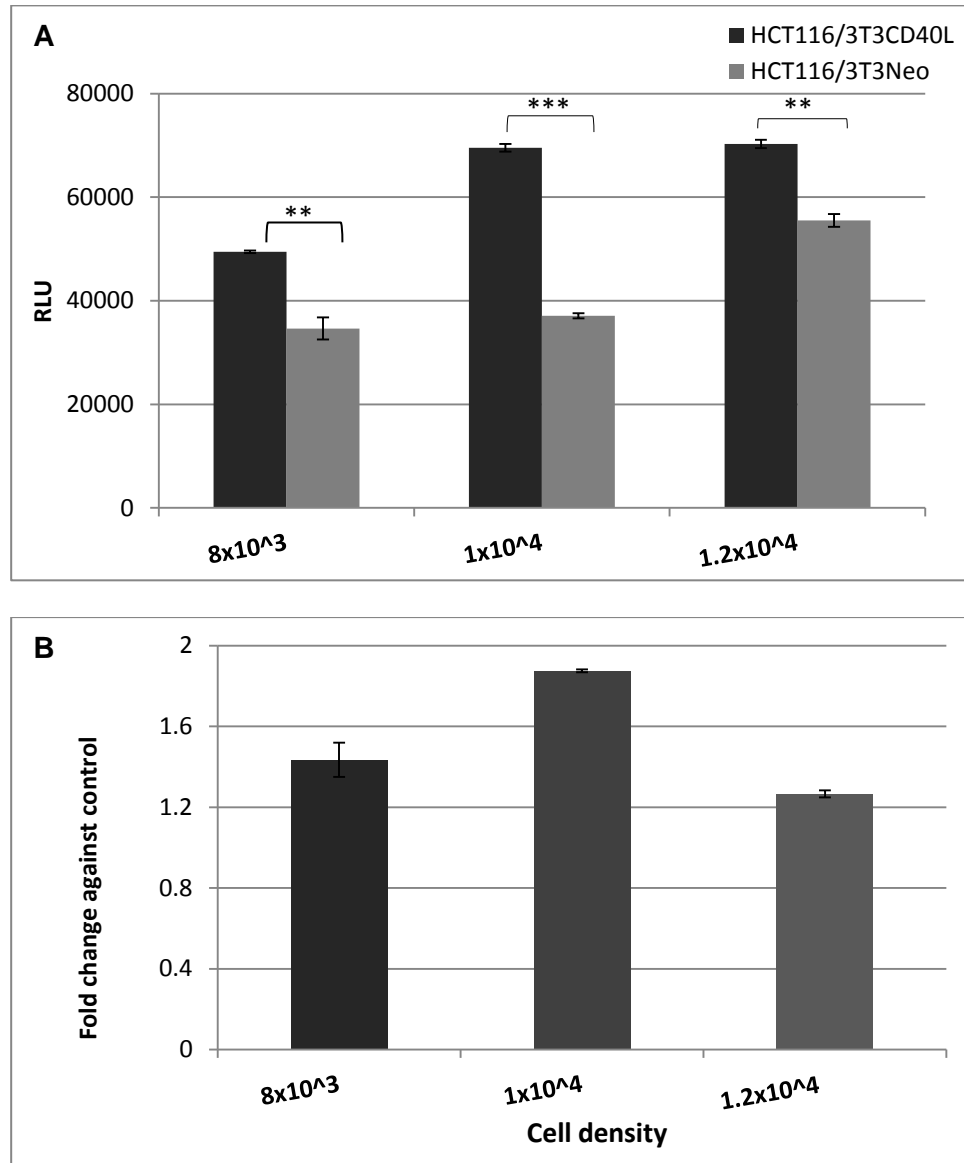


Figure 3. 4 Optimization of apoptosis assay using different cell number of target cells for detection of CD40-mediated apoptosis

1×10^4 cells/well of mouse fibroblast (3T3Neo and 3T3CD40L) were co-cultured in 96 well white plates with colorectal cancer cells (HCT116) seeded at different number of cells 8×10^3 , 1×10^4 and 1.2×10^4 in DR medium supplemented with 5% FCS and 1% L-glutamine. Plates were incubated for 24 hours at $37^\circ\text{C}/5\% \text{CO}_2$. 50 μl of CytoTox-Glo substrate were added and then plates were incubated 15 minutes and luminescence was measured by a FLUOStar Optima plate reader and background-corrected RLU readings deduced as described in the Materials and methods. Bars correspond to mean values of 4-6 replicates \pm SD, ***p-value ≤ 0.001 /**p-value ≤ 0.01 . A) Background corrected RLU readings of CytoTox-Glo for HCT116/3T3CD40L and HCT116/3T3Neo. B) Fold change against control of background corrected RLU readings of CytoTox-Glo for HCT116.

3.5.1.2 CD40 ligation by mCD40L for 24 hours causes extensive CRC cell apoptosis

Whilst performing further explorative experiments, it became apparent that the CytoTox-Glo assay was extremely sensitive and that background RLU arising from low level spontaneous *in vitro* cell death of target (epithelial) cells was significantly interfering with assay sensitivity. To reduce such 'noise', it was ensured that target cells were harvested only during their log phase of growth. This use of 'healthier' epithelial cells led to significant reductions in background RLU and allowed the cell apoptosis induced by 3T3CD40L (mCD40L) to be better detected (not shown).

Another important consideration related to the effector 3T3 cells (3T3CD40L and 3T3Neo). As these cells were treated with Mitomycin C to induce growth arrest, it was essential to ensure that this was efficient and they showed non-mitotic properties whilst remaining metabolically active (See section 2.10). If this treatment was insufficient, it could result in the continued growth of effector cells and such culture overgrowth, increased levels of spontaneous death and would interfere with the assay output. It was found that cell cycle arrest in combination with minimal cell toxicity was best achieved when 3T3CD40L or 3T3Neo cells were MMC treated during their log phase of growth and at approximately 50% confluency (data not shown).

Collectively, incorporation of these modifications and improvements in the experimental methodologies, led to a dramatic improvement in the results obtained using this assay, in comparison to earlier findings. Following these improvements, the results obtained following background correction of relative luminescence unite (RLU) revealed a high level of death in both HCT116 and SW480-CD40 as shown in Figure 3.5 a and b. In addition, results are shown as fold change (from raw RLU data in Figure 3.5) by comparing 3T3CD40L/HCT116 or 3T3CD40L/SW480-CD40 *versus* 3T3Neo/HCT116 or 3T3Neo/SW480-CD40 co-cultures shown in Figure 3.6.

Georgopoulos and colleagues have previously demonstrated mCD40L-mediated apoptosis in bladder carcinoma (EJ) and colorectal cancer cells (HCT116 and Colo320) (Georgopoulos et al., 2007, Georgopoulos et al., 2006, Bugajska et al., 2002) by also using Annexin V/PI and flow cytometry to demonstrate that maximal CD40-induced apoptosis occurred approximately 72 hours post CD40 ligation. Annexin V/PI is based on the detection of % cell apoptosis relying on the translocation of phosphatidylserine to the cell membrane,

while CytoTox-Glo depends on the exposure of substrate to a protease released during the loss of cell membrane integrity as shown previously in section 2.9.4 figure 2.4. Whilst both events (phosphatidylserine translocation and disruption of membrane integrity) are integral to apoptosis, it was important to determine the optimal time-point post CD40 ligation to perform CytoTox-Glo assays and whether perhaps an earlier time point might be more suitable. Although apoptosis by the Cytotox Glo assay was still detectable at 48 (or even 72) hours (data not shown), in this study results showed that mCD40L resulted in rapid cell death detected optimally at 24 hours post-ligation.

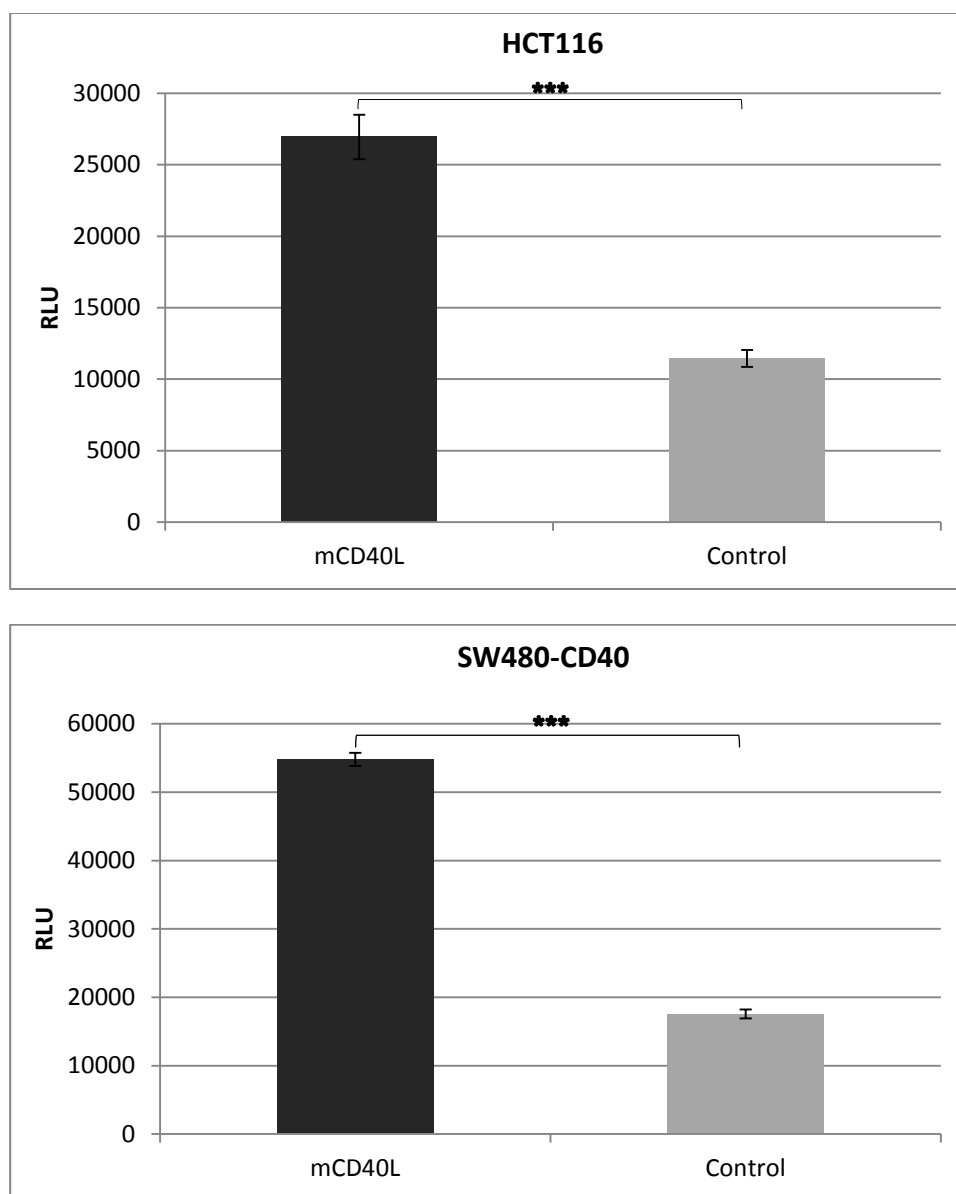


Figure 3. 5 mCD40L mediated apoptosis detection using CytoTox-Glo

1×10^4 CRC cells (HCT116 and SW480-CD40) were co-cultured with 1×10^4 MMC-treated mouse fibroblast cells 3T3CD40L (mCD40L) or 3T3Neo (Control) in DR medium supplemented with 5% FCS and 1%LG in white 96-well plates. 50 μ l CytoTox-Glo substrate was added after 24 hours incubation at 37°C/5% CO₂. After 15 minutes incubation at room temperature, luminescence was measured by plate reader and background-corrected. RLU readings deduced as described in material and methods. Bars correspond to mean values of 4-6 replicates \pm SD, ***p-value<0.001. The figure shows background corrected RLU readings of CytoTox-Glo for HCT116/mCD4L and HCT116/Control, and the background corrected RLU readings of CytoTox-Glo for SW480-CD40.

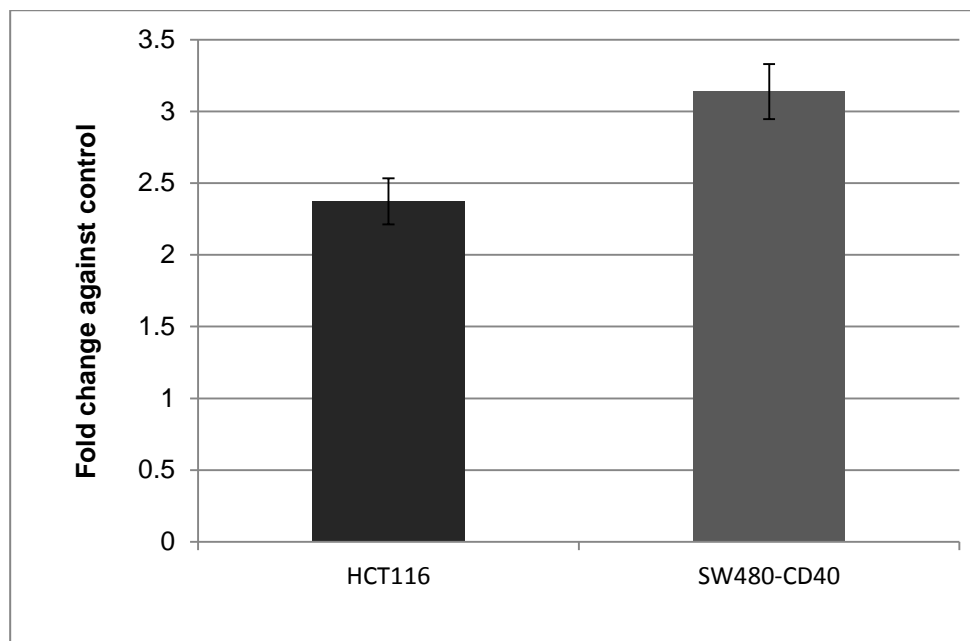


Figure 3. 6 Detection of mCD40L-mediated apoptosis by Cytotox Glo (expressed as fold change against control).

Results from Figure 3.5 are presented as fold increase for 3T3CD40L co-cultures in comparison to controls (3T3Neo).

3.5.1.3 Determination of percentage of cell death

In addition to using the detection of luminescence by the CytoTox-Glo assay as a surrogate marker of apoptosis, the assay may also be used to determine the percentage of apoptosis. When percentage of cell death was calculated (as detailed in Section 2.9.4) it was found that in line with previous results by our group (Georgopoulos et al., 2006, Georgopoulos et al., 2007) mCD40L results in approximately 75-85% cell death, which corresponds to a fold change of ~2.5-3.0 (Figure 3.7).

Membrane-CD40L can stimulate apoptosis in malignant cells (Bugajska et al., 2002, Georgopoulos et al., 2007, Georgopoulos et al., 2006). Previous studies by Georgopoulos et al have been reported that co-culture of urothelial carcinoma cells (UCC) with membrane-CD40L resulted in at least 60-70% apoptosis of the tumor cells at 48-72hrs as detected using Annexin V-FITC/PI and flow cytometry (Georgopoulos et al., 2006). In the present study, co-culture of SW480-CD40 with 3T3CD40L resulted in ~84.50% death for 3T3CD40L/HCT116 compared with control 35.74% as shown in figure 3.7a. As for the other cell line SW480-CD40, the percentage of cell death was 76.48% compared with control 33.83% as shown in figure 3.7b. For these experiments the Cyto Tox-Glo assay was used and percentage of death was calculated as described in the Materials and methods.

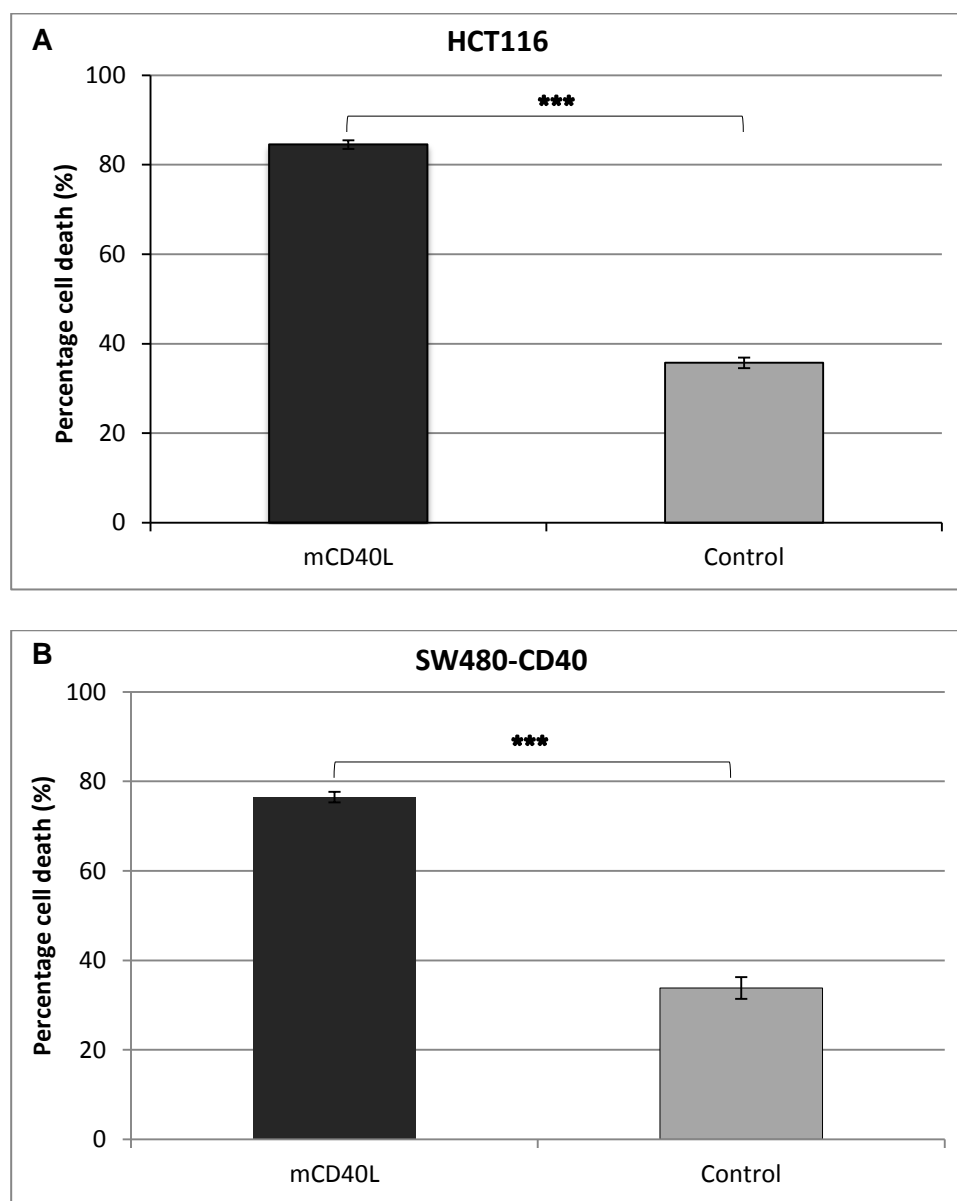


Figure 3. 7 Percentage of mCD40L-mediated cell death (based on the Cytotox Glo assay)

1×10^4 colorectal cancer cells (HCT116 and SW480-CD40) were co-cultured with 1×10^4 MMC treated 3T3CD40L (mCD40L) and 3T3Neo (control) in white 96 well plates in DR medium supplemented with 5% FBS and 1% L-glutamine. After 24 hours of co-culture, CytoTox-Glo test was used to detect apoptosis in colorectal cancer cells (HCT116) as described in chapter 2 (Materials and Methods). Percentage cell death was calculated for A) HCT116 co-cultures and B) SW480-CD40 co-cultures at 24 hours post-ligation. Bars correspond to mean values of 4-6 replicates \pm SD, ***p-value ≤ 0.001 .

3.5.2 Detection of apoptotic cell death using the SensoLyte caspase-3/7 assay

For optimisation of the caspase detection assay, staurosporine was used as a positive control. Staurosporine is a DMSO soluble compound derived from *Streptomyces Staurosporeus* that inhibits a range of protein kinases essential for normal cell function. Staurosporine is often used as a positive control to induce apoptosis by the intrinsic pathway via MOMP and caspase-3/7 activation, as previously described by our group (Chopra et al., 2009) and others (Zhang et al., 2004). As shown in HCT116 cells treated with staurosporine, the compound caused the activation of caspase-3/7 activity in a dose-dependent manner (see appendix IV).

Most recent studies indicate that caspases 3 and 7 have some overlapping, but also some distinct roles in programmed cell death (apoptosis), which includes the extrinsic and intrinsic (mitochondrial) pathway (Lakhani et al., 2006). Caspase 3 has an important role in driving DNA fragmentation and morphological changes of apoptosis, while caspase 7 plays a minor role in these processes (Lakhani et al., 2006). In this study we measured the caspase 3/7 activity in colorectal carcinoma cells after co-culture for 24, 48, and 72 hours. The results demonstrated that interaction between mCD40L (3T3-CD40L) and the target cells HCT116 and SW480-CD40 induced caspase 3/7 activity in CRC cells (see below). For the analysis of results from these assays, the same principle, as in the CytoTox-Glo assays (Section 3.4.1), was employed, which involved a) appropriate calculations for background 3T3 cell-related readings and b) the optimisation of target cell 'health' and effector cell MMC treatment (Section 3.4.1.1 & 3.4.1.2).

Georgopoulos et al (2006) had previously shown using FAM FLICA caspase detection assays and flow cytometry that Caspase-3 becomes active within 48 hours post CD40 ligation, which also coincided with the detection of DNA fragmentation (Georgopoulos et al., 2006), a direct downstream consequence of caspase activation during apoptosis. In this study the detection of caspase 3/7 activity was carried out at 24, 48 and 72 hours post ligation in 3T3CD40L/HCT116 or 3T3CD40L/SW480-CD40 co-cultures compared with 3T3Neo/HCT116 or 3T3Neo/SW480-CD40 ones. At 24 hours post CD40 ligation only little caspase 3/7 activity could be detected (Figure 3.8). However, by 48 hours there was a marked increase in caspase activity (Figure 3.9) which was sustained at even slightly higher levels even at 72 hours of co-culture (Figure 3.10), with an approximately 2.5-3.5 fold increase consistently detectable.

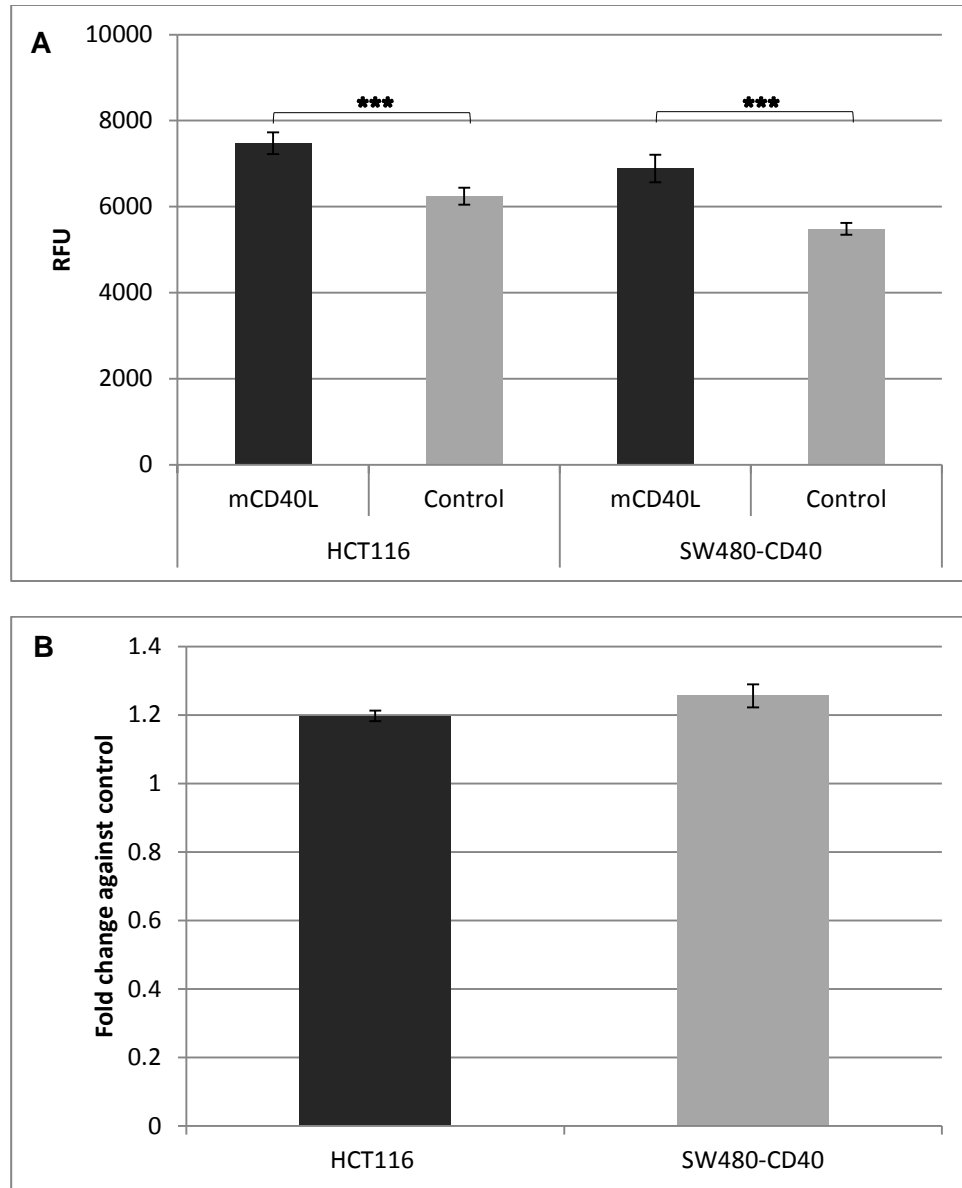


Figure 3. 8 Detection of CD40-induced caspase 3/7 activation after 24hours post-ligation

1×10^4 cells/well colorectal carcinoma cells (HCT116 and SW480-CD40) were co-cultured with 1×10^4 cells/well of mouse fibroblasts (3T3CD40L and 3T3Neo) in 96 well plates in DR medium supplemented with 5% FCS and 1% L-glutamine. Plates were incubated for 24 hours at $37^\circ\text{C}/5\% \text{CO}_2$. 50 μl substrate of the Anaspec assay was added to each well and then plates were incubated 60 minutes in the dark and fluorescence was measured and background-corrected RFU (Relative Fluorescence Units) calculated as described in the Materials and methods. Bars correspond to mean values of 4-6 replicates \pm SD ***p-value ≤ 0.001 . A) Background corrected RFU readings of caspase 3/7 activity for HCT116 and SW480-CD40/3T3CD4L and HCT116 and SW480-CD40/3T3Neo. B) Fold change against control was calculated by using background corrected RFU readings of caspase 3/7 activity for HCT116 and SW480-CD40.

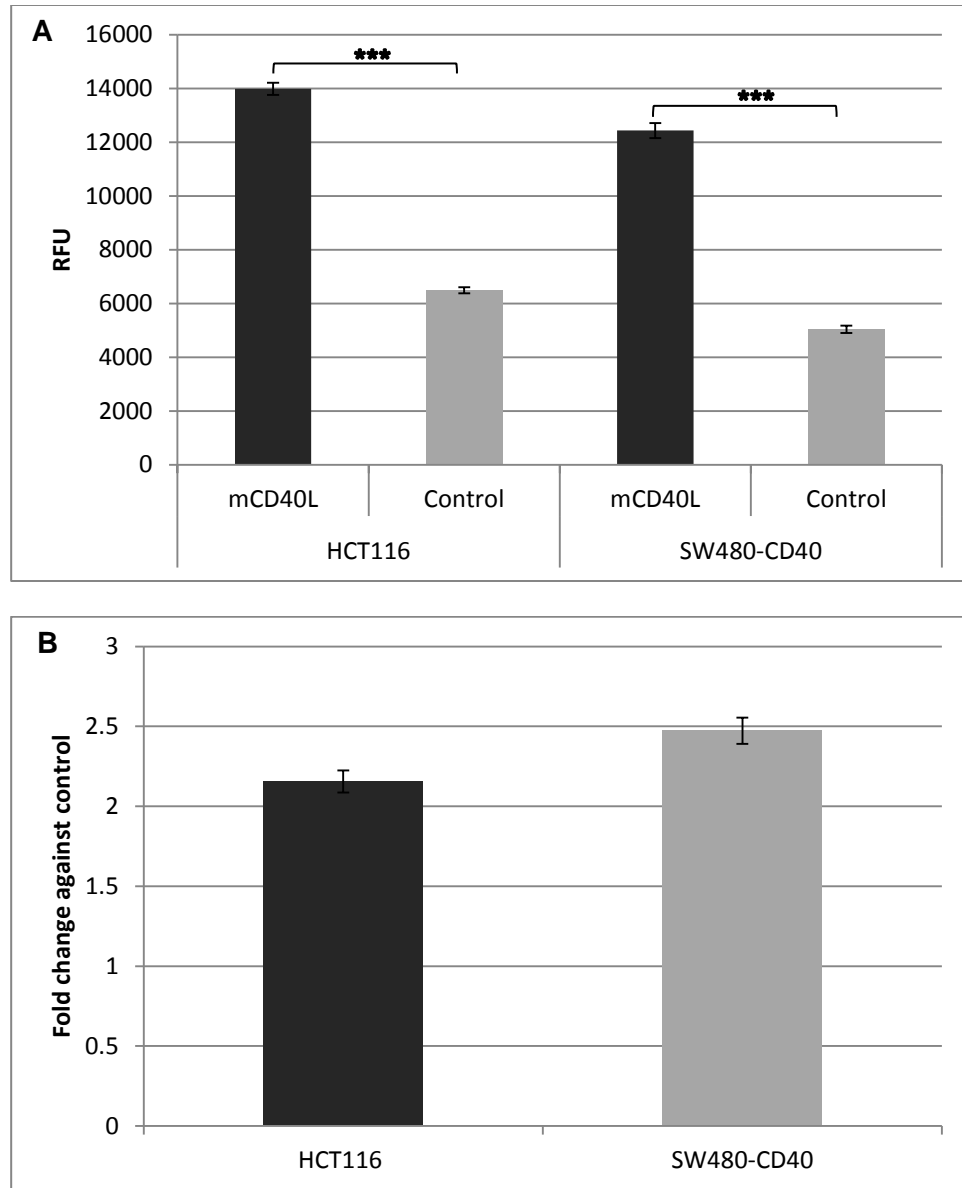


Figure 3. 9 Detection of CD40 induced caspase 3/7 activation after 48hours co-culture

1×10^4 cells/well colorectal carcinoma cells (HCT116 and SW480-CD40) were co-cultured with 1×10^4 cells/well of mouse fibroblasts (3T3CD40L and 3T3Neo) in 96 well plates in DR medium supplemented with 5% FCS and 1% LG. Plates were incubated for 48 hours at $37^\circ\text{C}/5\% \text{CO}_2$. 50 μl substrate of the Anaspec assay was added to each well and then plates were incubated 60 minutes in the dark and fluorescence was measured and background-corrected RFU (Relative Fluorescence Units) calculated as described in the Materials and methods. Bars correspond to mean values of 4-6 replicates \pm SD ***p-value ≤ 0.001 . A) Background corrected RFU readings of caspase 3/7 activity for HCT116 and SW480-CD40/3T3CD4L and HCT116 and SW480-CD40/3T3Neo. B) Fold change against control was calculated by using background corrected RFU readings of caspase 3/7 activity for HCT116 and SW480-CD40.

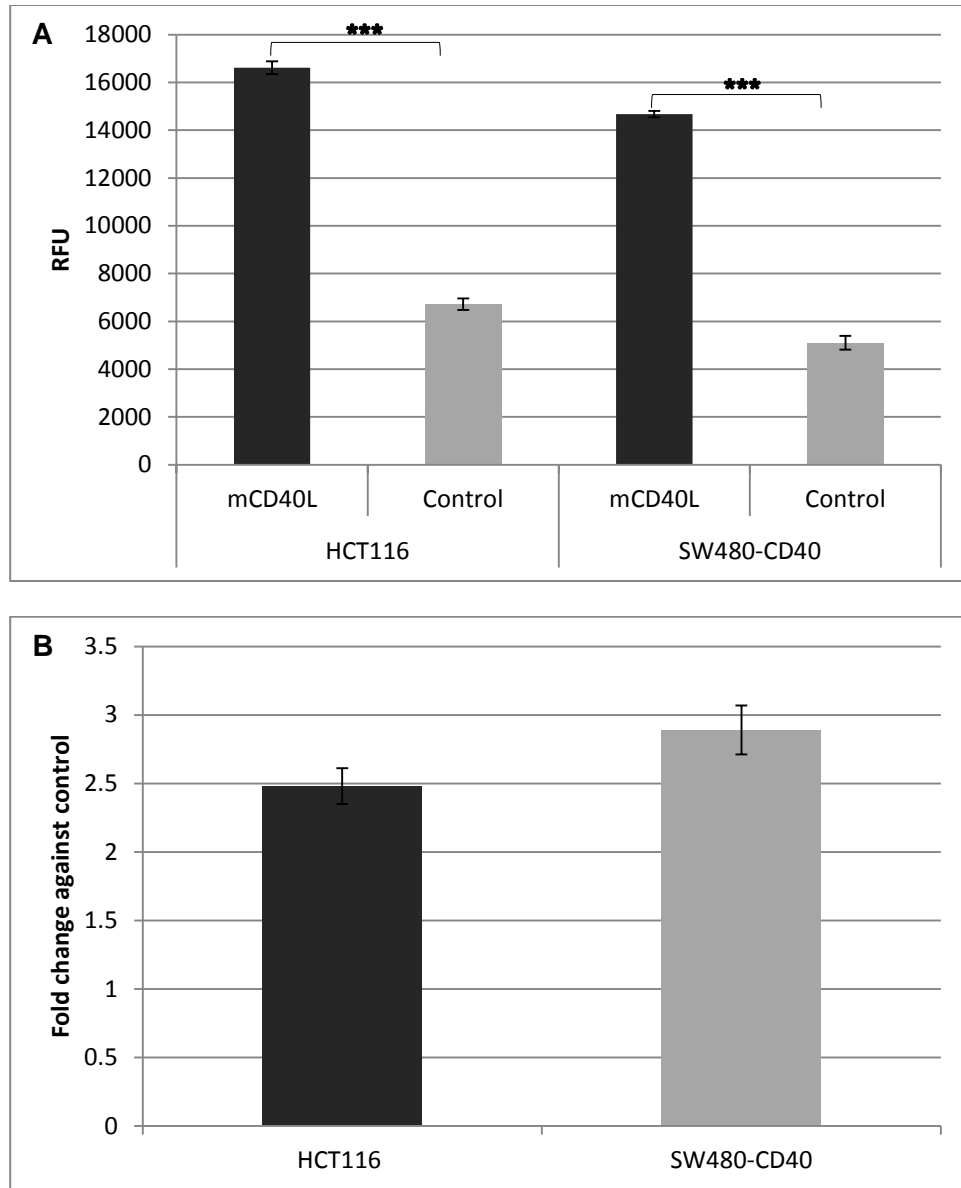


Figure 3. 10 Detection of CD40 induced caspase 3/7 activation after 72hours co-culture

1×10^4 cells/well colorectal carcinoma cells (HCT116 and SW480-CD40) were co-cultured with 1×10^4 cells/well of mouse fibroblasts (3T3CD40L and 3T3Neo) in 96 well plates in DR medium supplemented with 5% FCS and 1% LG. Plates were incubated for 72 hours at $37^\circ\text{C}/5\% \text{CO}_2$. 50 μl substrate of the Anaspec assay was added to each well and then plates were incubated 60 minutes in the dark and fluorescence was measured and background-corrected RFU (Relative Fluorescence Units) calculated as described in the Materials and methods. Bars correspond to mean values of 4-6 replicates \pm SD ***p-value ≤ 0.001 . A) Background corrected RFU readings of caspase 3/7 activity for HCT116 and SW480-CD40/3T3CD4L and HCT116 and SW480-CD40/3T3Neo. B) Fold change against control was calculated by using background corrected RFU readings of caspase 3/7 activity for HCT116 and SW480-CD40.

3.5.3 DNA fragmentation detection following CD40 ligation by mCD40L in CRC cells

Previous studies have demonstrated that membrane-CD40L can induce extensive cell death in CRC cells (60-70%) which were confirmed in this study. In addition to the detection of apoptosis by Cytotox-Glo (as a surrogate marker of loss of membrane integrity) and SensoLyte/Anaspec (for the detection of caspase activity), cell death (apoptosis) was also measured by means of a DNA fragmentation ELISA assay (see section 2.9.5). Results obtained from such experiments indicated that a high level (and high percentage) of death based on DNA fragmentation was observed in HCT116 cells (80% compared with 35.5% for controls) (Figure 3.11a/b), similar to results for SW480-CD40 cells (83.70% in comparison to 33.50% for controls) (Figure. 3.12a/b).

Although the DNA fragmentation assay used is a reliable, well characterised method to assess cell death (Bugajska et al., 2002, Georgopoulos et al., 2006, Shaw et al., 2005), it detects only one of the hallmarks of apoptosis. Moreover, apoptosis activated by members of the TNFR superfamily is not always accompanied by DNA fragmentation, as previous studies have demonstrated in colorectal cells by other groups (Wilson and Browning, 2002) and in bladder cancer cells in our laboratory (Steele et al., 2006). More importantly, for this work, although the DNA fragmentation test gave similar results to the CytoTox-Glo assay, it was however less cost-effective, and though reliable, was particularly laborious in comparison to the other assays. Therefore, for the majority of apoptosis detection experiments carried out in this work (following chapters), the Cytotox-Glo and SensoLyte/caspase-detection assays were mainly used (and confirmation using DNA fragmentation performed where appropriate).

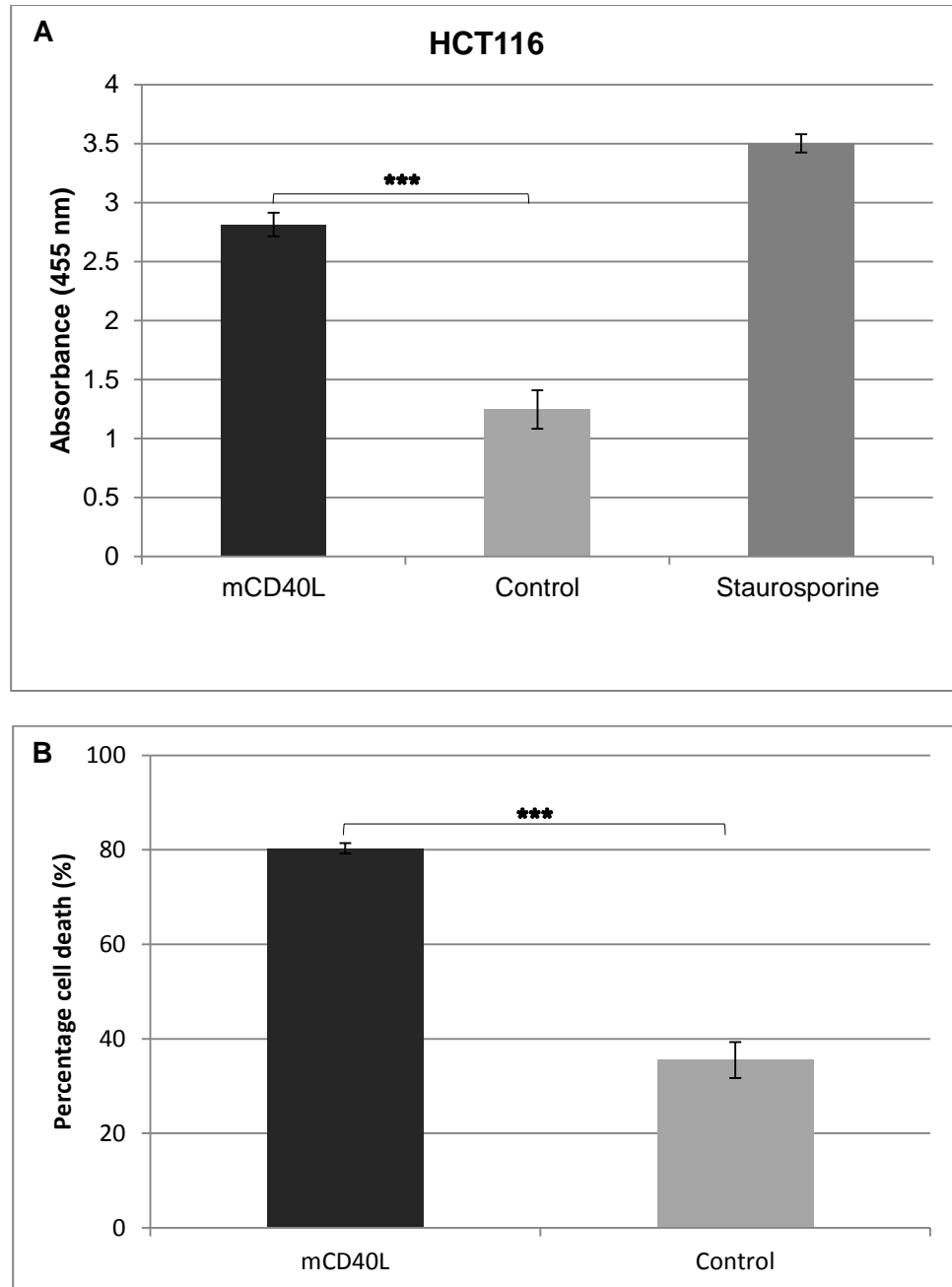


Figure 3. 11 DNA fragmentation assay for the detection of apoptosis in HCT116 cells mediated by mCD40L

1X10⁴ BrdU labeled CRC cells (HCT116) was co-cultured with 1x10⁴/well 3T3CD40L (mCD40L) or 3T3Neo (Control) (5μM staurosporine was used as positive control for cells alone). Absorbance (455-10nm) was measured by a plate reader and percentage was calculated as described in Chapter 2 (Materials and Methods). Data are represented means ± S.D for two to three experiments. ****P* ≤ 0.001 DNA fragmentation of co-cultures of CRC cells with 3T3CD40L was statistically significantly higher (in comparison to controls) in all cell lines. Raw data are presented in A and percentage cell death in B (calculated as described in the Materials and Methods).

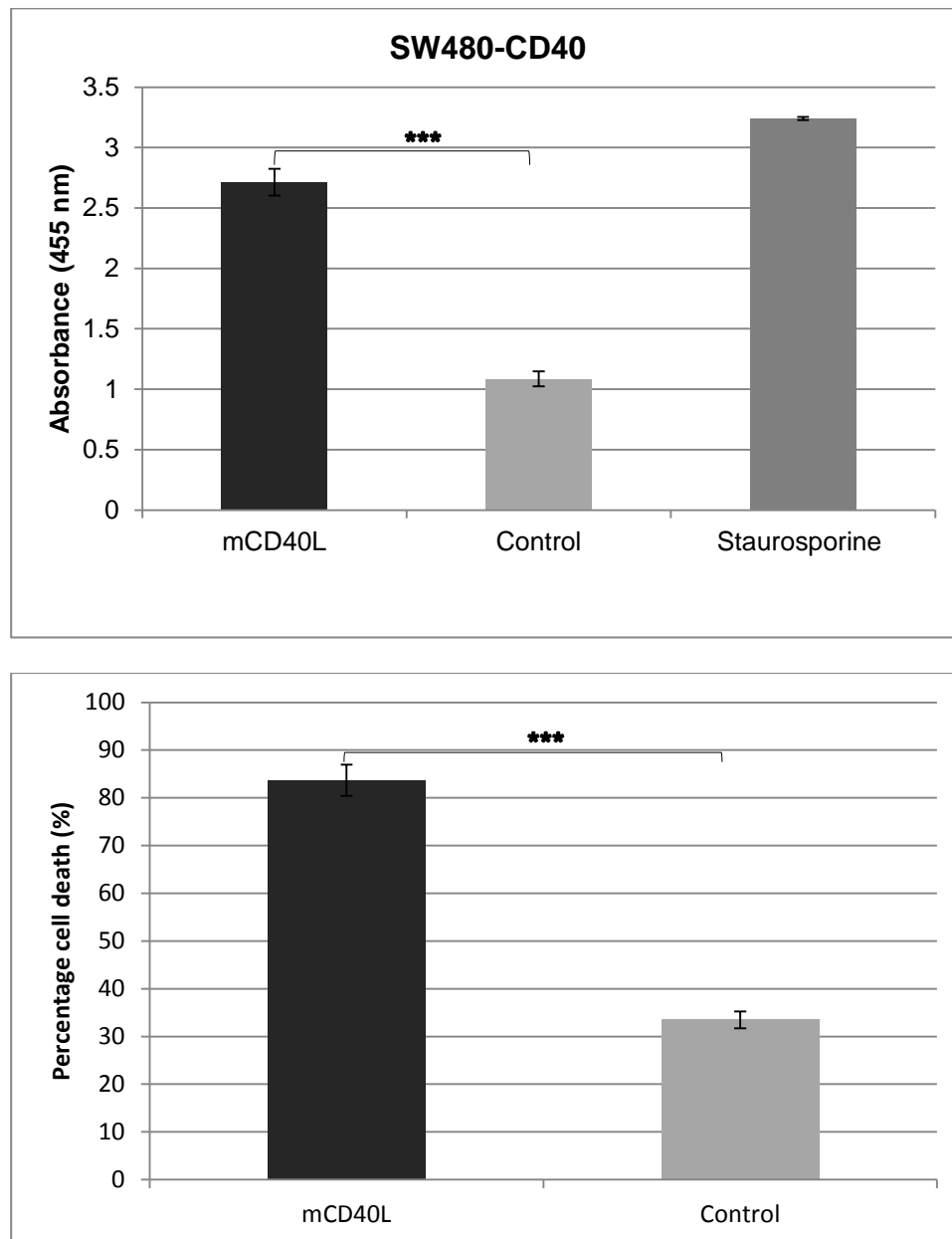


Figure 3. 12 DNA fragmentation assay for detection of apoptosis in SW480-CD40 cells mediated by mCD40L

1X10⁴ BrdU labeled CRC cells (SW480-CD40) was co-cultured with 1x10⁴/well 3T3CD40L (mCD40L) or 3T3Neo (Control) (5μM staurosporine was used as positive control for cells alone). Absorbance (455-10nm) was measured by a plate reader and percentage was calculated as described in Chapter 2 (Materials and Methods). Data are represented means ± S.D for two to three experiments. ****P* ≤ 0.001 DNA fragmentation of co-cultures of CRC cells with 3T3CD40L was statistically significantly higher (in comparison to controls) in all cell lines. Raw data are presented in A and percentage cell death in B (calculated as described in the Materials and Methods).

3.6 Optimisation of immunoblotting (Western blotting) for protein detection using co-cultures

As described previously for urothelial cell-3T3 fibroblast co-cultures (Georgopoulos et al 2006), preparation of whole cell lysates from co-cultures and performing Western blotting poses a challenge, as the lysate contains a mixture of proteins from both effectors (fibroblasts) and target (epithelial) cells. For this purpose, and as detailed previously (Georgopoulos et al 2006), expression of epithelial-specific markers (cytokeratins) has been employed to ensure equal loading based on cytokeratin (CK) expression.

Routine immunoblotting experiments that were performed to detect CK expression showed that CRC cell lines HCT116 and SW480-CD40 undergo rapid and extensive apoptosis, evident by the progressive loss of epithelial lysate in co-culture protein extracts (indicated by the reduction in expression of epithelial marker Cytokeratin CK8, 18, and 8/18 within less than 12 hours post-CD40 ligation, with severe loss of CK8 and CK8/18 observed by 24 hours as shown in Figures 3.13 and 3.14, respectively. Moreover, it was clear that as 3T3Neo co-cultures progressed, there was a progressive increase in epithelial lysate in such control cultures. Therefore, it was essential to ensure that equal loading could be achieved for both 3T3Neo and 3T3CD40L co-cultures. For this purpose, densitometric analysis (using LiCor Odyssey Infra-Red imaging software) was carried out for all prepared lysates from such co-cultures. This allowed the correction (following cytokeratin band intensity-based normalisation) of gel loading for all subsequent experiments. As shown in Figures 3.15a and 3.15b, equal expression of CK in HCT116 and SW480-CD40 co-cultures was achieved.

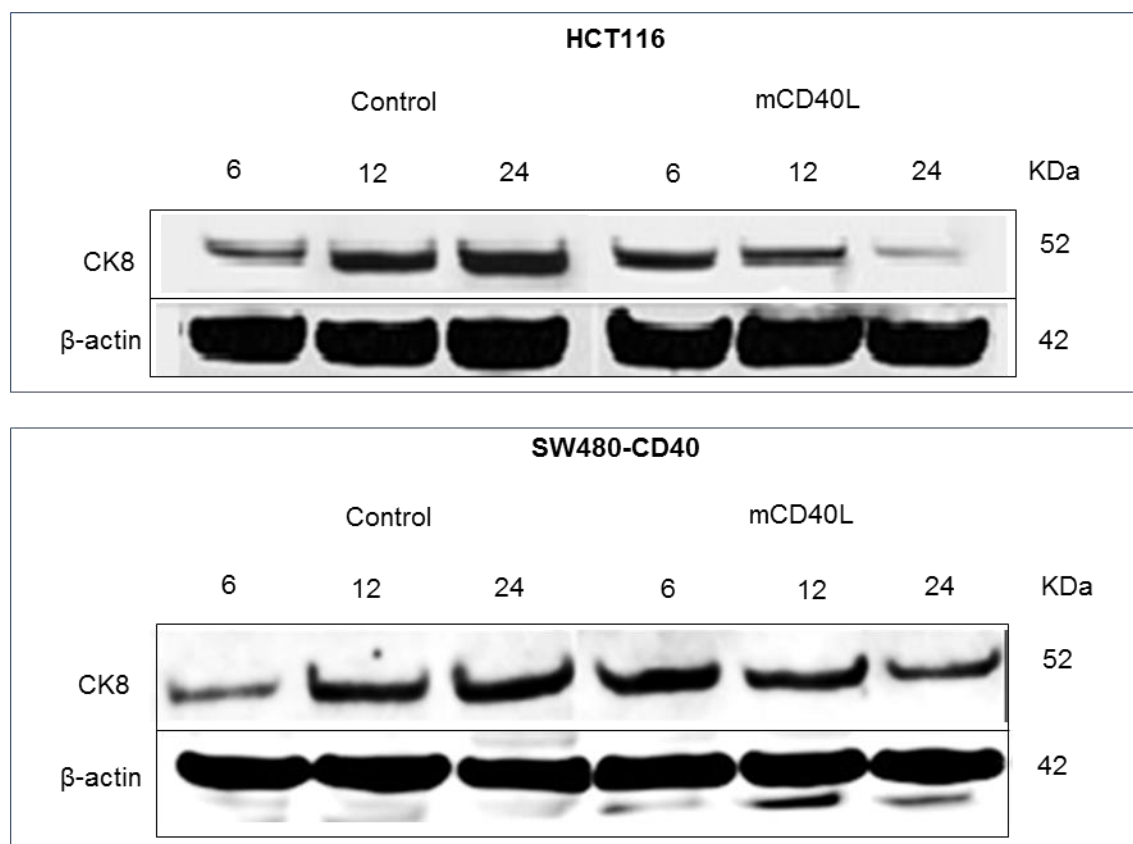


Figure 3. 13 Detection of protein expression based on CK8 in CRC cells following co-culture

Western blot analysis of cytokeratin 8 expression in CRC cells (SW480-CD40 and HCT116) after incubation with 3T3 Neo and 3T3CD40L. Total amount of protein was loaded was 20µg/well. The membranes were incubated with primary cytokeratin 8 monoclonal mouse monoclonal IgG (diluted 1:1000) and secondary antibody goat-anti mouse IgG, Alexa 680 (dilution 1:10000). β-actin AC-15-A5441 was used as specificity and loading control, membranes were incubated with primary monoclonal antibody (diluted 1:20000) and secondary antibody goat-anti mouse IgG Alexa 680 (diluted 1:10000). The membranes were scanned and images shown in black and white.

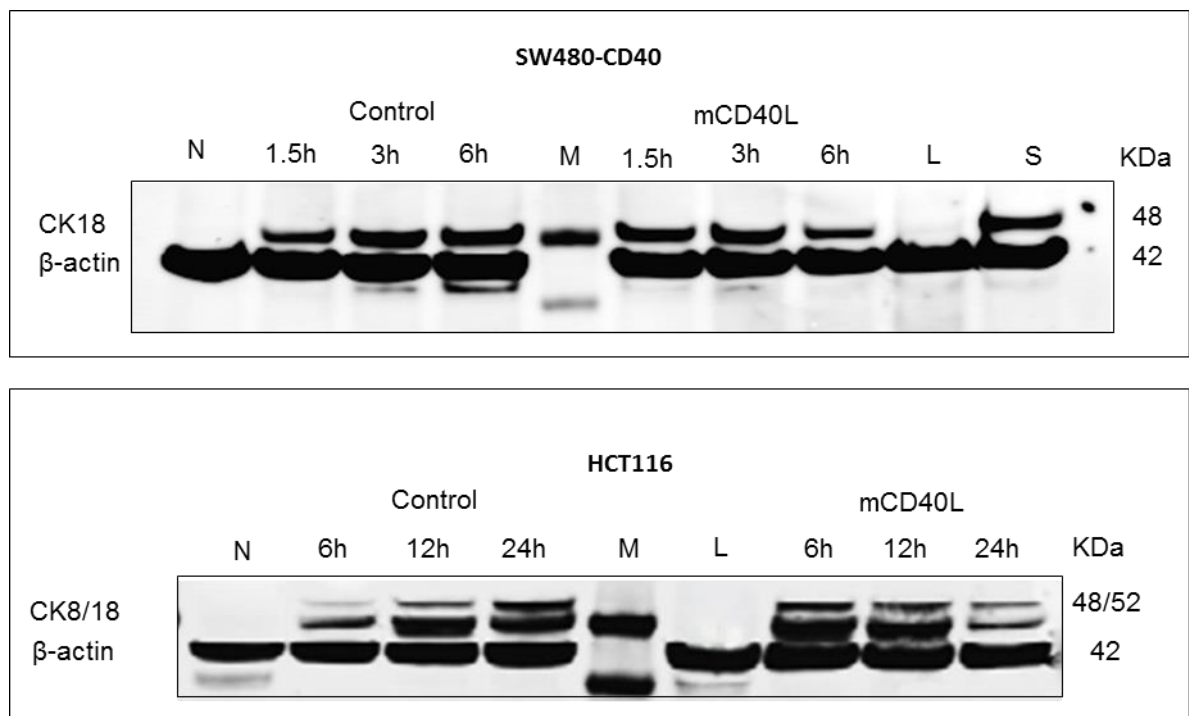


Figure 3. 14 Detection of protein expression based on CK8/18 in CRC cells following co-culture

Western blot analysis of cytokeratin 8 and 18 expression in CRC cells (SW480-CD40 and HCT116) after incubation with 3T3 Neo and 3T3CD40L. Total amount of protein was loaded was 20µg/well. The membranes were incubated with primary cytokeratin 8/18 for HCT116 and CK18 of SW480-CD40 monoclonal mouse monoclonal IgG (diluted 1:1000) and secondary antibody goat-anti mouse IgG, Alexa 680 (dilution 1:10000). β-actin AC-15-A5441 was used as specificity and loading control, membranes were incubated with primary monoclonal antibody (diluted 1:20000) and secondary antibody goat-anti mouse IgG Alexa 680 (diluted 1:10000). The membranes were scanned and images shown in black and white.

Keys: N: 3T3Neo

L: 3T3CD40L

S: SW480-CD40

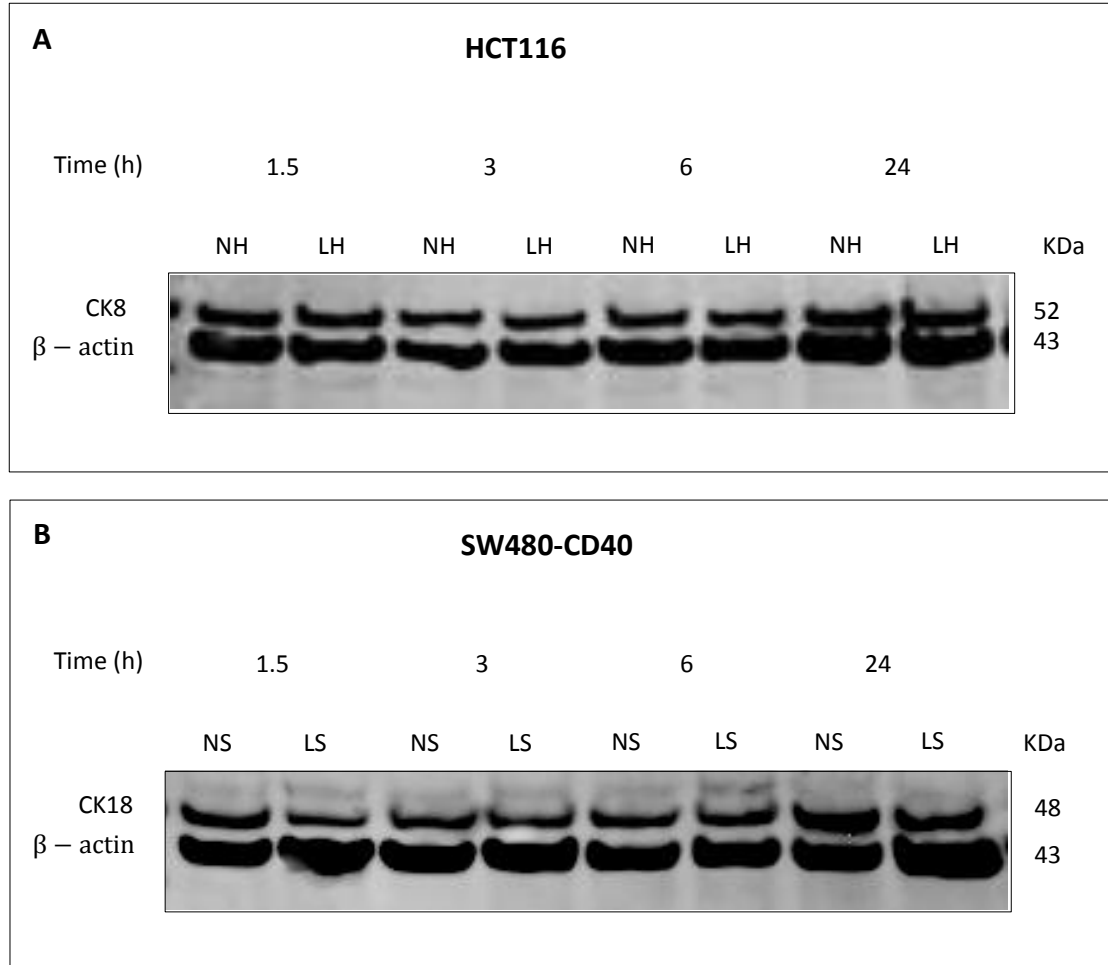


Figure 3. 15 Detection of 8 and CK18 after normalise the amount of protein expression in CRC cells

Western blot analysis of cytokeratin 8 and 18 expression in CRC cells (SW480-CD40 and HCT116) after incubation with 3T3 Neo and 3T3CD40L. Amount of normalize lysates were loaded was 20 μ g/well following correction by using densitometry of the band of cytokeratin in CRC cells before correction. The membranes were incubated with primary cytokeratin 8 for HCT116 and CK18 of SW480-CD40 monoclonal mouse monoclonal IgG (diluted 1:1000) and secondary antibody goat-anti mouse IgG, Alexa 680 (dilution 1:10000). β -actin was used as specificity and loading control, membranes were incubated with primary monoclonal antibody (diluted 1:20000) and secondary antibody goat-anti mouse IgG Alexa 680 (diluted 1:10000). The membranes were scanned and images shown in black and white. An A and B show the equal loading of CK8 and CK18 for both HCT116 and SW480-CD40.

Keys: NH: 3T3Neo co-cultured with HCT116
 NS: 3T3Neo co-cultured with SW480-CD40

LH: 3T3CD40L cocultured with HCT116
 LS: 3T3CD40L cocultured with SW480-CD40

3.7 Summary:

- The main objective of this chapter was to confirm that the fidelity of co-culture system for stimulation of CD40 in CRC cells in vitro and establish a number of cell cytotoxicity and caspase activity detection assays.
 - Two main death assays were used for the detection of mCD40L-mediated death:
 - The CytoTox-Glo assay to detect loss of cell membrane integrity and subsequent apoptosis
 - The Sensolyte Homogenous caspase-3/7 assay to detect caspase 3/7 activity in apoptotic cells.
 - The DNA fragmentation test was also used to detect cell death and provide an additional confirmatory assay.
- The results have confirmed that CRC (target) cells expressed CD40 by western blotting; this was also confirmed by using Flow cytometry.
- Immunoblotting also confirmed that 3T3CD40L cells (killer/effector cells) expressed CD40 ligand compared with 3T3Neo, which were used as a negative isogenic control.
- The results indicated for the first time that CD40-mediated CRC cell death happened very rapidly (within less than 24 hours post CD40 ligation, approximately between 6 and 12 hours post ligation).
- Routine immunoblotting experiments performed to detect cytokeratin (CK) expression also provided indirect evidence that CD40+ve CRC cell lines undergo rapid and extensive apoptosis evident by the progressive loss of epithelial lysate in co-culture protein extracts. In particular, the results demonstrated rapid and extensive loss of CK8 and CK8/18 observed at 12 and even more at 24 hours in CRC cells.
- Densitometric analysis, normalisation and subsequent lysate loading correction to confirm equal amount of CRC cells protein (based on CK8 or CK18 band intensity) permitted the establishment of the appropriate methodologies in order to follow CD40-mediated signalling in CRC cells.

CHAPTER 4: Investigation into the activation and functional involvement of key intracellular mediators in CD40-mediated apoptosis in CRC cells

4.1 Objectives

The specific aims of this chapter were:

- To optimise immunoblotting methods for the correct and sensitive detection of intracellular proteins associated with CD40-mediated apoptosis.
- To perform retrovirus-mediated transduction of CRC cell lines and optimise the selection of carcinoma cells expressing virally-transduced shRNAs for RNAi-mediated protein knockdown.
- To utilize RNAi, immunoblotting and cell death assays to determine the functional role of key intracellular mediators, for instance whether TRAF3 regulates a) JNK phosphorylation, b) Bak and Bax induction and c) CD40-mediated apoptosis overall.
- To employ specific pharmacological inhibitors to determine whether JNK/p38 regulate a) Bak and Bax expression and b) CD40-mediated apoptosis.
- To delineate the precise nature of the molecular pathways of cell death through
 - a) RNAi-mediated reduction of Bax, immunoblotting and cell death assays, and
 - b) loss of mitochondrial membrane permeability by cell fractionation (cytoplasm and mitochondria).

4.2 Expression of TRAF -1, -3 and -6 following CD40 ligation in CRC cells

The mechanisms of CD40 signalling, particularly the role of TRAF adaptor molecules, have been mainly studied in the context of CD40-mediated B cell activation (Bishop, 2004); however, a lot less is known with regards to CD40 signalling in epithelial cells. In fact, relatively little is known in terms of the role of the TRAFs as proximal signalling activators in non-lymphoid cells at all. Depending on the cell context, the TRAFs orchestrate a diverse range of cell responses such as proliferation, differentiation or apoptosis (Bishop, 2004).

Despite the ability of CD40 to induce extensive and rapid apoptosis in CRC cells, as shown previously (Georgopoulos et al., 2007, Hill et al., 2008a) and extensively characterised in this thesis using multiple assays, no investigations have so far examined which of the TRAFs may be regulated by CD40 and play a functional role in apoptosis. It has been previously demonstrated in urothelial carcinoma cells lines (UCC) lines that CD40 ligation by mCD40L (but not soluble agonists) specifically promotes the expression of TRAF1, TRAF2 and TRAF3 (Georgopoulos et al., 2006), but how mCD40L triggers CD40 TRAF signalling in CRC cells, remain unexplored. Of note, due to the inability of soluble agonists (e.g. agonistic antibody G28-5) to induce any apoptosis in CRC cells (Georgopoulos et al., 2007, Hill et al., 2008a), this project focused on ligation of CD40 by membrane ligand (mCD40L).

This study screened only for TRAFs which have been implicated in CD40 signalling. To do so, CRC cell lines were co-cultured with control and mCD40L expressing effector cells and TRAF adaptor protein expression was investigated by immunoblotting using human specific antibodies. Following co-culture with mCD40L-effectors, both HCT116 and SW480-CD40 CRC cell lines showed rapid and dramatic increases in TRAF1 expression after 6, 12 and 24 hours compared with co-culture with the control cells as shown in Figure 4.1. Immunoblotting also showed the induction of TRAF3, which was markedly and rapidly increased as early as 1.5 hour, with further increases at 3, 6, 12 and maximal expression occurring 24 hours post CD40 ligation in both HCT116 and SW480-CD40 as shown in Figure 4.2. Interestingly, there was also rapid but very transient induction of TRAF6 expression with high expression levels observed after 1.5 hours in HCT116 cells but the expression disappeared at 3, 6, and 24 hours.

Similar trends of TRAF expression were observed in the transduced SW480-CD40 cells. In fact, there was a near identical pattern of TRAF1 (Figure 4.1) and TRAF3 (Figure 4.2) regulation to that observed in HCT116 cells. However, the pattern of TRAF6 protein expression showed some difference, as in SW480-CD40 cells, TRAF6 expression after the 3 hour time point did not disappear but showed marked reduction as shown in Figure 4.3. Although an explanation for such minor differences in TRAF expression could not be provided, a good degree of similarity was observed between naturally CD40 expressing (HCT116) and engineered, de novo CD40 expressing (SW480-CD40) CRC cell lines, suggesting that the proximal signalling events triggered following CD40 by mCD40L appear to be similar.

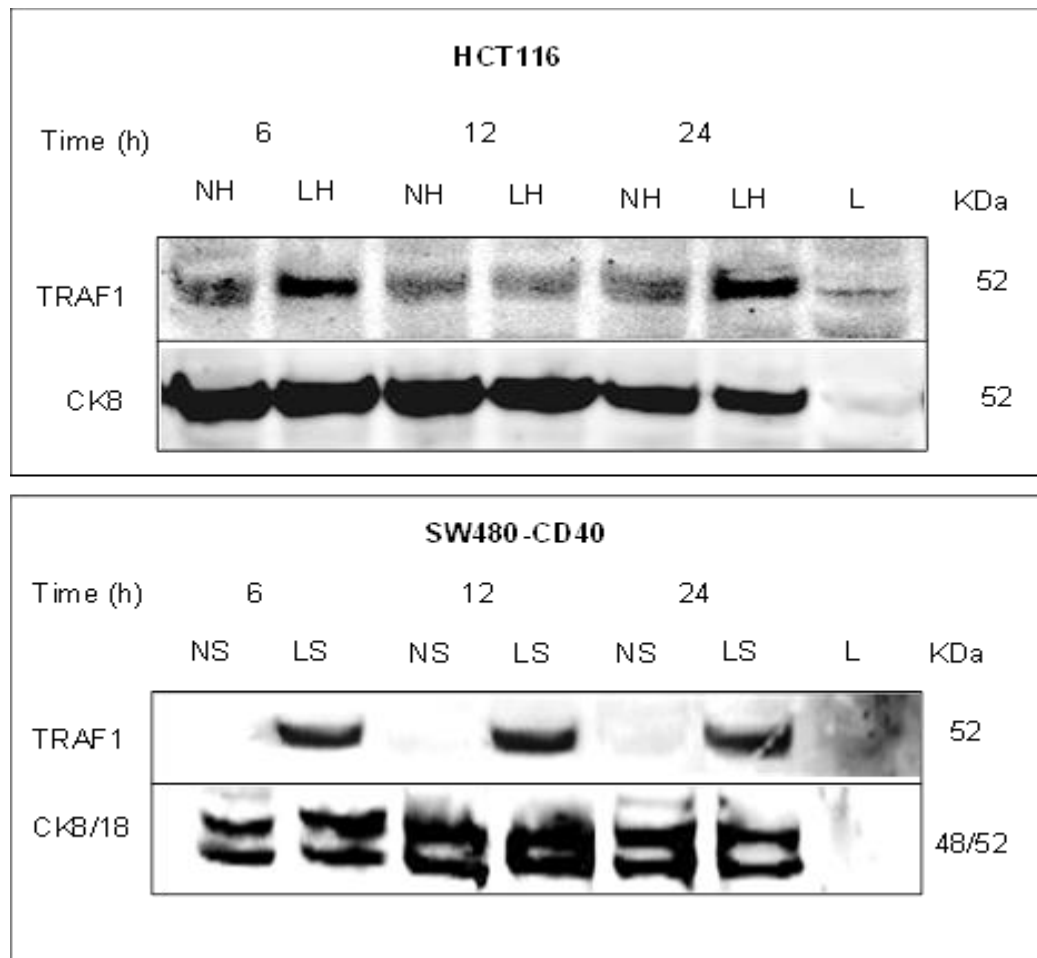


Figure 4. 1 The regulation of TRAF1 expression by CD40 ligation

HCT116 (H) and SW480-CD40 (S) cells were seeded at 3×10^6 cells /dish, and co-cultured with 3×10^6 cells /dish MMC treated fibroblast cells 3T3-Neo (N) or 3T3-CD40L (L). Co-cultured cells were incubated for various times, 3, 6, 12 and 24 hours in DR 5% supplemented with 5% of FCS and 1% L-glutamine in 10cm^2 culture dishes. After each of incubation time period, cells were lysed by using 2x SDS-lysis buffer and protein concentration determined. Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were separated under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membranes. The membrane was probed for overnight at 4°C with primary polyclonal antibody (anti-TRAF1) in TBS/Tween 0.1% (1:250 dilution) and then with an anti CK8 and anti-CK18 antibody in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one hour with secondary antibody [goat anti-rabbit IgG IRDye 800nm (1:10000 dilution)] for TRAF1 detection and with goat anti-mouse antibody Igg IRDye 680 (1:10000 dilution) for CK8 and CK8/18. Antibody binding was visualised at 700nm using an OdysseyTM Infra-red Imaging system (CK8/18 and CK8 were used as loading controls).

Key: NH: 3T3Neo co-cultured with HCT116 LH: 3T3CD40L cocultured with HCT116
 NS: 3T3Neo co-cultured with SW480-CD40 LS: 3T3CD40L cocultured with SW480-CD40
 L: 3T3CD40L

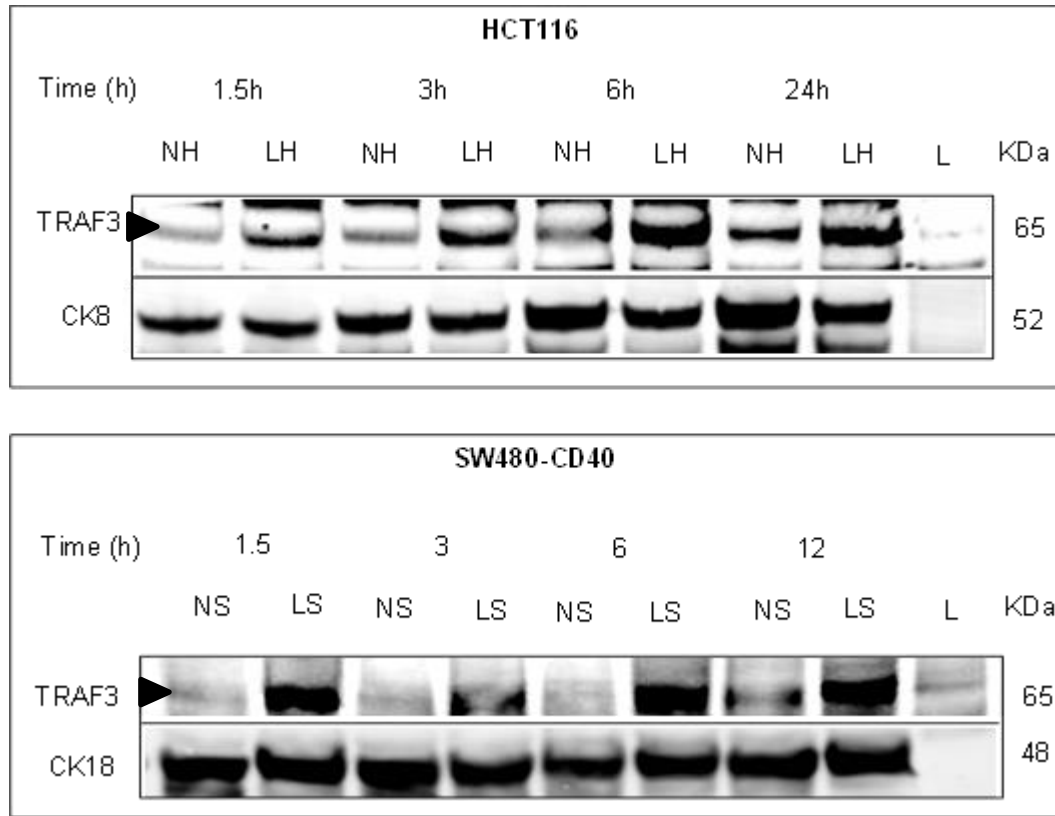


Figure 4. 2 The regulation of TRAF3 expression by CD40 ligation

HCT116 (H) and SW480-CD40 (S) cells were seeded at 3×10^6 cells/dish, and co-cultured with 3×10^6 MMC treated fibroblasts 3T3-Neo (N) or 3T3-CD40L (L). Co-cultures were incubated for the indicated time points (1.5, 3, 6, 12 and 24 hours) in DR 5% supplemented with 5% of FCS and 1% L-glutamine in 10 cm^2 culture dishes. After each of incubation time, cells were lysed using 2x SDS-lysis buffer and protein concentration determined. Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were separated under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membranes. The membrane was probed for overnight at 4°C with primary polyclonal antibody (anti-TRAF3) in TBS/Tween 0.1% (1:250 dilution) and then with an anti-CK8 and anti-CK18 antibody in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one hour with secondary antibody goat anti-rabbit IgG IRDye 800 (1:10000 dilution) for TRAF3 detection and with goat anti-mouse antibody IgG IRDye 680 (1:10000 dilution) for CK8 and CK18. Antibody binding was visualised at 700nm and 800nm using an OdysseyTM Infra-red Imaging system (CK8 and CK18 were used as loading controls).

Key: NH: 3T3Neo co-cultured with HCT116 LH: 3T3CD40L cocultured with HCT116
 NS: 3T3Neo co-cultured with SW480-CD40 LS: 3T3CD40L cocultured with SW480-CD40
 L: 3T3CD40L

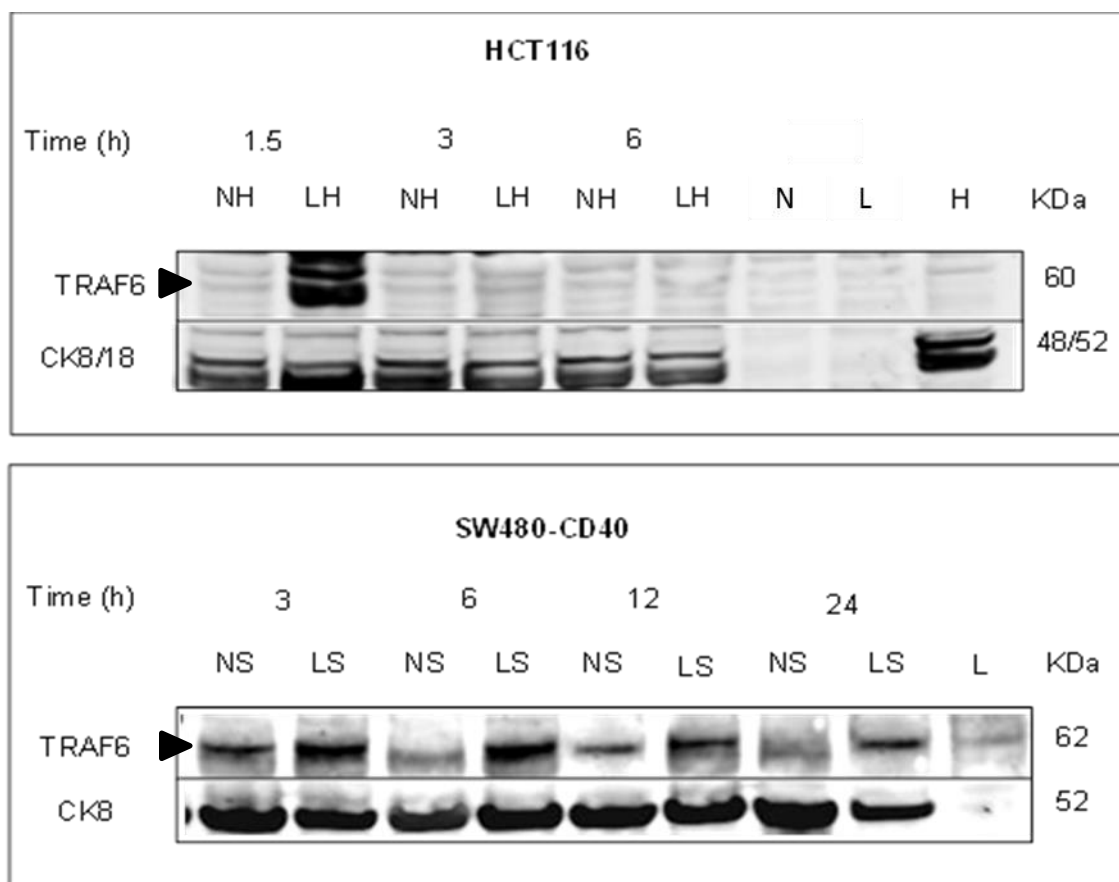


Figure 4. 3 The regulation of TRAF6 expression by CD40 ligation

HCT116 (H) and SW480-CD40 (S) cells were seeded at 3×10^6 cells /dish, and co-cultured with 3×10^6 cells /dish MMC treated fibroblast cells 3T3-Neo (N) or 3T3-CD40L (L). Co-cultured cells were incubated for various times, 1.5, 3, 6, 12, and 24 hours in DR 5% supplemented with 5% of FCS and 1% L-glutamine, in 10cm^2 culture dishes. After each of incubation time, cells were lysed by using 2x SDS-lysis buffer and protein concentration determined. Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were separated under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membranes. The membrane was probed for overnight at 4°C with primary monoclonal antibody (anti-TRAF6) in TBS/Tween 0.1% (1:250 dilution) and then with an anti CK8/18 and anti-CK8 antibody in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one hour with secondary antibody [goat anti-mouse IgG IRDye 680nm (1:10,000 dilution)] for TRAF6 detection and with goat anti-mouse antibody IgG IRDye 680 (1:10000 dilution) for CK8 and CK8/18. Antibody binding was visualised at 700nm using an OdysseyTM Infra-red Imaging system (CK8/18 and CK8 were used as loading controls).

Key: NH: 3T3Neo co-cultured with HCT116 LH: 3T3CD40L cocultured with HCT116
 NS: 3T3Neo co-cultured with SW480-CD40 LS: 3T3CD40L cocultured with SW480-CD40
 L: 3T3CD40L H: HCT116

4.3 Expression of MKK4 and MKK7 during CD40-mediated apoptosis in CRC cells

The regulation of pro-apoptotic transcriptional activators, such as JNK and p38, by upstream MAPKKs (and particularly MKK4 and MKK7) is well documented (Wagner and Nebreda, 2009). The MAPK pathway is an important key signal transduction regulating apoptosis, cell proliferation and differentiation. This is a successive complex phosphorylation cascade involving a large number of proteins. The MAPK pathway may be separated into three groups of proteins: MAPKKK (MAP kinase kinase kinase), the MAPKKs (MAP kinase kinase) and MAPK (MAP kinase). The principle of their activation is simple: activated MAPKKKs activate MAPKK by loop phosphorylation of two seryl residues. The MAPKKs turn trigger the MAPK (JNK and p38) phosphorylation by a tyrosyl and threonyl phosphorylation. MAPKKs proteins include MKK-3, -4, 6, and -7 as well as MEK1/2. MKK3 activates p38 MAPK alpha and beta (p38- α , and - β) while MKK6 can activate both isoforms equally. CD40-mediated MKK3/6 and p38 activation have been shown important for IL-6 and IL-10 monocyte secretion during inflammation (Inoue et al., 2004). Furthermore, it has been shown that MKK4 is mainly activated by environmental stress and MKK7 by cytokines; MKK4 and MKK7 activate JNK in response to external stimuli, whilst it has been reported that MKK4 and MKK7 phosphorylate members of the family of p38 in vitro when overexpressed. (Davis, 2000). No studies have investigated or demonstrated a link between CD40-mediated MKK -4 or -7 JNK or p38 activation, although activation of JNK in CD40-mediated apoptosis has been reported by our laboratory and others (Elmetwali et al., 2010b, Georgopoulos et al., 2006).

This work for the first time aimed to examine whether MKK4 and/or MKK7 are active during CD40 signalling. Immunoblotting using human phospho-specific antibodies demonstrated that MKK4 was activated in response to mCD40L as shown by its phosphorylation at Ser257 (Figure 4.4); phosphorylation of MKK4 was detected within 3 hours in both HCT116 and SW480-CD40. MKK4 expression further increased 6 hours post CD40 interaction but then decreased after 12 hours while completely disappearing at 24 hours, indicating a relatively transient mode of activation (Figure 4.4). Interestingly this pattern of expression was essentially identical in both HCT116 and SW480-CD40 cells.

Previous studies have been reported that both MKK4 and MKK7 are required for full activation of JNK in vitro (Fleming et al., 2000, Kishimoto et al., 2003). Also some studies showed that loss of MKK7 in fibroblasts cells leads to increase proliferation suggesting a

role for this kinase in negatively regulating growth (Wada and Penninger, 2004). Similarly to MKK4, phospho-MKK7 dramatically increased within 1.5 hours post CD40 ligation in both CRC cell lines. MKK7 expression was readily detectable in SW480-CD40 at 3, 6, 24 hours post CD40 ligation, while in the HCT116 cells it decreased 6 hours post CD40 ligation (Figure 4.5). Therefore these findings demonstrate for the first time that CD40 ligation induces activation of both MKK4 and MKK7 in CRC cells.

4.4 Expression of JNK and p38 MAPK during CD40-mediated apoptosis in CRC cells

MAP kinases are activated through phosphorylation by MAP kinase kinases (MKK or MAP2K) which themselves are stimulated by MAP kinase kinase kinase (MAP3K) located most upstream (see Figure 1.24); for example, JNK is primarily activated by two upstream kinases MKK4 and MKK7 (Weston and Davis, 2002, Dhanasekaran and Reddy, 2008, Kyriakis and Avruch, 2012).. Activated JNK stimulates the transcription factor c-Jun, which can then form the complex of transcription factors AP-1 (complex transcription-factor activator protein) by homodimerization or heterodimerization by partnering with another family member factors Jun and Fos transcription. AP-1 has a ubiquitous distribution and controls, the expression of metalloproteinases (MMP), inflammatory cytokines etc. The same activation cascades exist for the other two MAPK: MEK1 and MEK2 activate ERK1 and ERK2 as MKK3 / 6 activate p38 MAP kinase. The activation of MAPK is closely controlled (temporally and spatially) in each cell and its inactivation is dependent on serine / threonine phosphatase, tyrosine phosphatase, and dual specificity phosphatases (dual-specificity phosphatases; DUSP).

As mentioned above, CD40 ligation by mCD40L has been shown to activate JNK in UCC cells (Georgopoulos et al., 2006), whilst a number of previous studies have reported that activation of the JNK pathway is usually associated with the regulation of cell death (Sabapathy and Wagner, 2004, Johnson and Lapadat, 2002b). Furthermore, activation of the p38 pathway is also generally associated with the activation of transcription factors and protein kinases involved in the regulation of differentiation and inflammatory response and also cell death (Zhang and Liu, 2002). This study aimed to decipher whether JNK and/or p38 (collectively known as stress activated protein kinases) were activated during CD40 mediated apoptosis in CRC cells. Immunoblotting demonstrated that JNK phosphorylation

occurs within 1.5 hours post CD40 ligation for HCT116 then this expression was attenuated after 6 hours (Figure 4.6). By contrast, in SW480-CD40 cells there was rapid JNK phosphorylation occurring within 1.5 hours, which was sustained until peaking at 12 hours post mCD40L treatment (Figure 4.6). Also by immunoblotting, expression of activated p38 MAPK was shown in both CRC cell lines. The detection of p-p38 following CD40 ligation was rapid, increasing in HCT116 and SW480-CD40 within 1.5 hours after mCD40L-CD40 interaction, and in both cases reaching maximal expression at 12 hours (Figure 4.7).

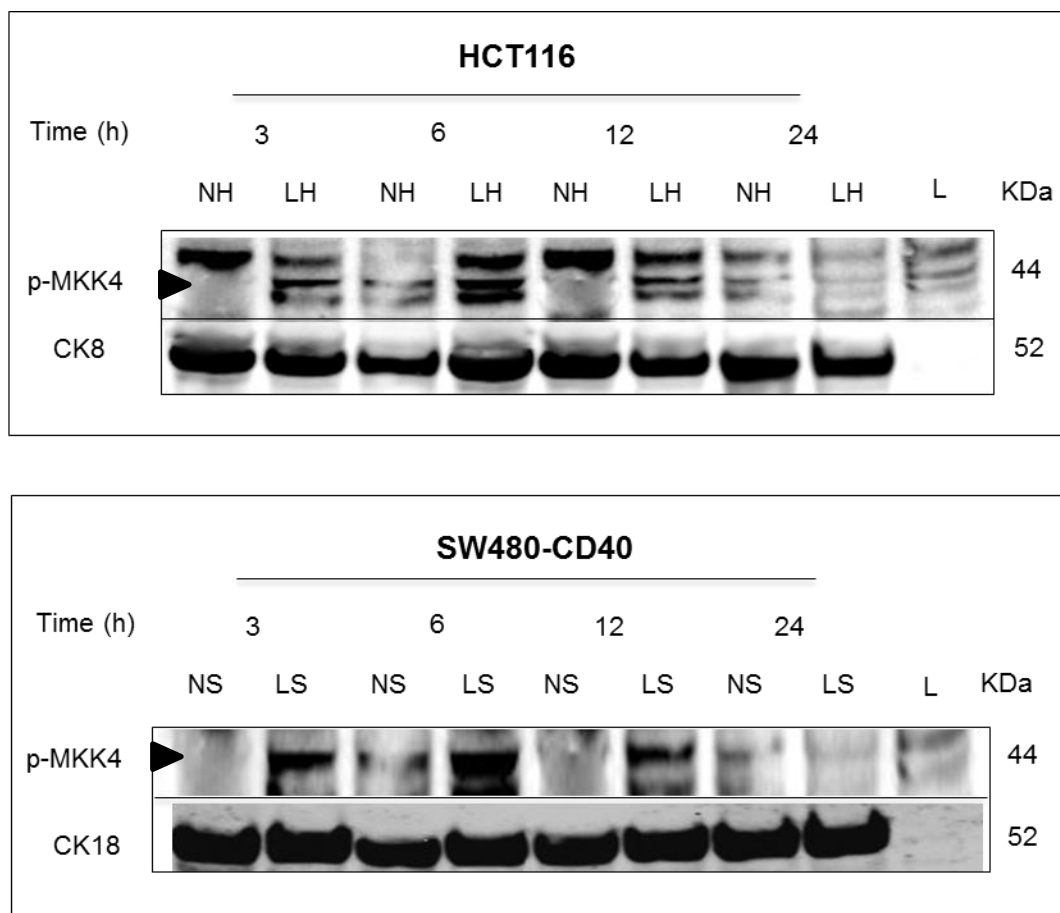


Figure 4. 4 The activation of MKK4 following CD40 ligation

HCT116 (H) and SW480-CD40 (S) cells were seeded at 3×10^6 cells /dish and co-cultured with 3×10^6 MMC treated fibroblasts 3T3-Neo (N) or 3T3-CD40L (L). Co-cultures were incubated for the indicated time points (3, 6, 12 and 24 hours) in DR 5% supplemented with 5% of FCS and 1% L-glutamine, in 10cm^2 culture dishes. After each of incubation time, cells were lysed by using 2x SDS-lysis buffer and protein concentration determined. Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were separated under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membranes. The membrane was probed for overnight at 4°C with primary polyclonal antibody (anti-p-MKK4) in TBS/Tween 0.1% (1:500 dilution) and then with an anti-CK8 and anti-CK18 antibody in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one hour with secondary antibody [goat anti-rabbit IgG IRDye 680 (1:5000 dilution)] for MKK4 detection and with goat anti-mouse antibody IgG IRDye 680 (1:10000 dilution) for CK8 and CK18. Antibody binding was visualised at 700nm and 800nm using an OdysseyTM Infra-red Imaging system (CK8 and CK18 were used as loading controls).

Key: NH: 3T3Neo co-cultured with HCT116 LH: 3T3CD40L cocultured with HCT116
 NS: 3T3Neo co-cultured with SW480-CD40 LS: 3T3CD40L cocultured with SW480-CD40
 L: 3T3CD40L

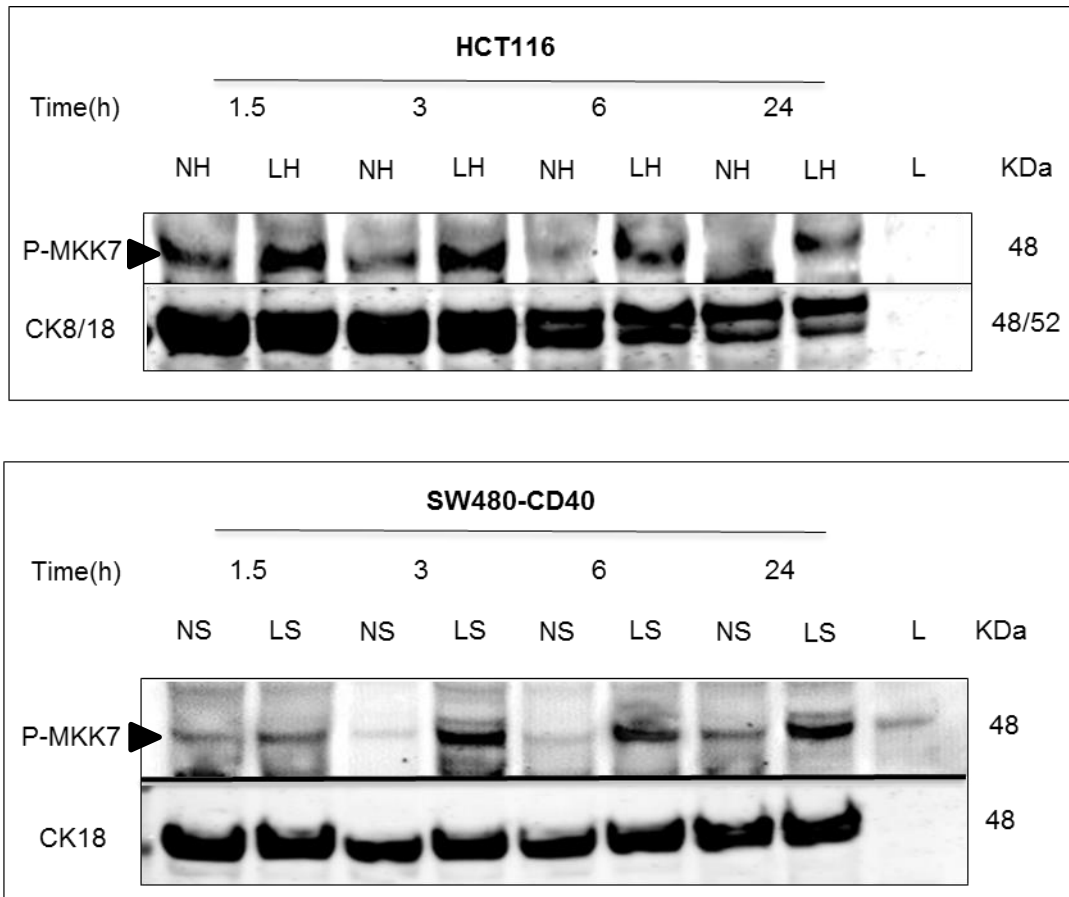


Figure 4. 5 The activation of MKK7 following CD40 ligation

HCT116 (H) and SW480-CD40 (S) cells were seeded at 3×10^6 cells /dish and co-cultured with 3×10^6 MMC treated fibroblasts 3T3-Neo (N) or 3T3-CD40L (L). Co-cultures were incubated for the indicated time points (3, 6, 12 and 24 hours) in DR 5% supplemented with 5% of FCS and 1% L-glutamine, in 10 cm^2 culture dishes. After each of incubation time, cells were lysed by using 2x SDS-lysis buffer and protein concentration determined. Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were separated under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membranes. The membrane was probed for overnight at 4°C with primary polyclonal antibody (anti-p-MKK7) in TBS/Tween 0.1% (1:500 dilution) and then with an anti-CK8/18 and anti-CK18 antibody in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one hour with secondary antibody [goat anti-mouse IgG IRDye 680 (1:10,000 dilution)] for MKK7 detection and with goat anti-mouse antibody IgG IRDye 680 (1:10000 dilution) for CK8/18 and CK18. Antibody binding was visualised at 700nm and 800nm using an OdysseyTM Infra-red Imaging system (CK8/18 and CK18 were used as loading controls).

Key: NH: 3T3Neo co-cultured with HCT116 LH: 3T3CD40L cocultured with HCT116
 NS: 3T3Neo co-cultured with SW480-CD40 LS: 3T3CD40L cocultured with SW480-CD40
 L: 3T3CD40L

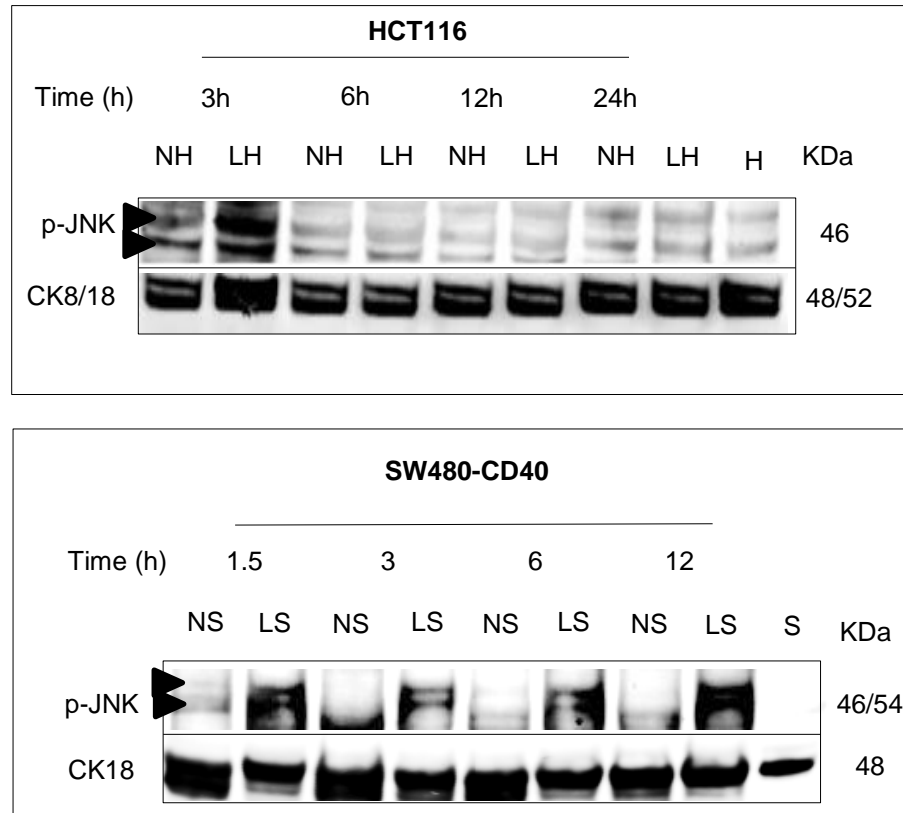


Figure 4. 6 The activation of JNK following CD40 ligation

HCT116 (H) and SW480-CD40 (S) cells were seeded at 3×10^6 cells /dish and co-cultured with 3×10^6 MMC treated fibroblasts 3T3-Neo (N) or 3T3-CD40L (L). Co-cultures were incubated for the indicated time points (1.5, 3, 6, 12 and 24 hours) in DR 5% supplemented with 5% of FCS and 1% L-glutamine, in 10 cm^2 culture dishes. After each of incubation time, cells were lysed by using 2x SDS-lysis buffer and protein concentration determined. Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were separated under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membranes. The membrane was probed for overnight at 4°C with primary monoclonal antibody (anti-p-JNK) in TBS/Tween 0.1% (1:500 dilution) and then with an anti-CK18 and anti-CK8/18 antibody in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one hour with secondary antibody [goat anti-mouse IgG IRDye 680nm (1:10,000 dilution)] for p-JNK detection and with goat anti-mouse antibody IgG IRDye 680 (1:10000 dilution) for CK18 and CK8/18. Antibody binding was visualised at 700nm using an OdysseyTM Infra-red Imaging system (CK8/18 and CK8 were used as loading controls).

Key: NH: 3T3Neo co-cultured with HCT116 LH: 3T3CD40L cocultured with HCT116
 NS: 3T3Neo co-cultured with SW480-CD40 LS: 3T3CD40L cocultured with SW480-CD40
 S: SW480-CD40

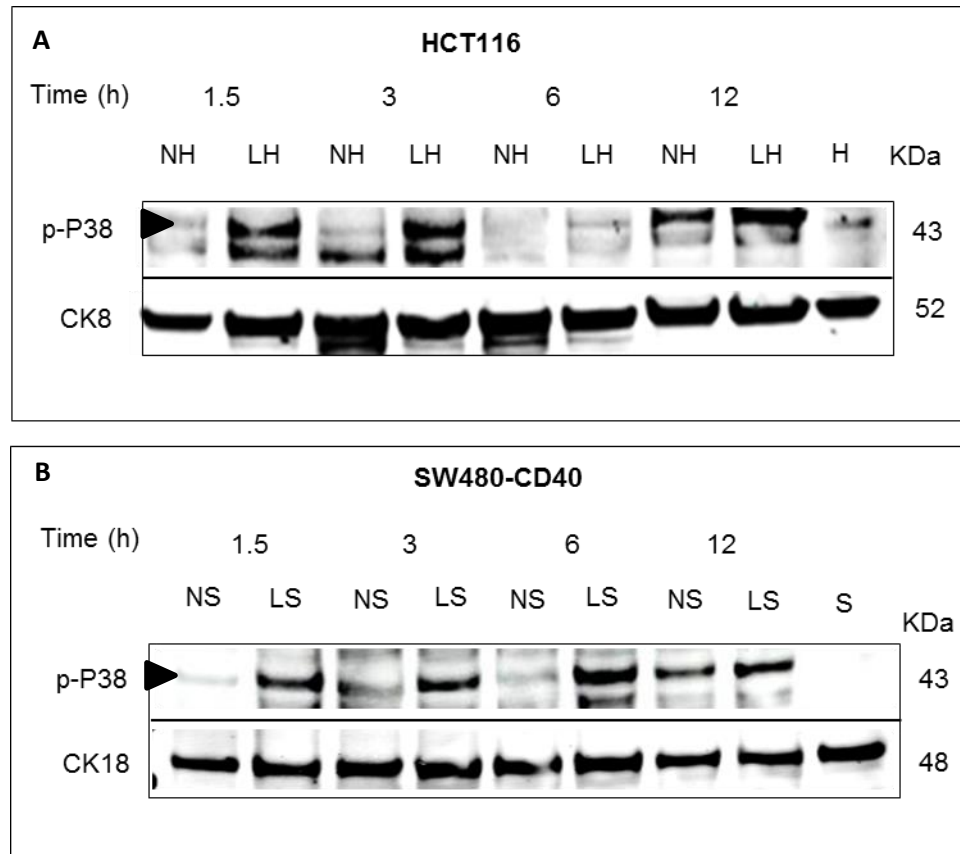


Figure 4. 7 The activation of p38 following CD40 ligation

HCT116 (H) and SW480-CD40 (S) cells were seeded at 3×10^6 cells /dish and co-cultured with 3×10^6 MMC treated fibroblasts 3T3-Neo (N) or 3T3-CD40L (L). Co-cultures were incubated for the indicated time points (1.5, 3, 6, and 12 hours) in DR 5% supplemented with 5% of FCS and 1% L-glutamine in 10cm^2 culture dishes. After each of incubation time, cells were lysed by using 2x SDS-lysis buffer and protein concentration determined. Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were separated under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membranes. The membrane was probed for overnight at 4°C with primary monoclonal antibody (anti-p-p38) in TBS/Tween 0.1% (1:500 dilution) and then with an anti-CK8 and anti-CK18 antibody in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one hour with secondary antibody [goat anti-rabbit IgG IRDye 800nm (1:10,000 dilution)] for p-p38 detection and with goat anti-mouse antibody IgG IRDye 680 (1:10000 dilution) for CK8 and CK18. Antibody binding was visualised at 700nm and 800nm using an OdysseyTM Infra-red Imaging system (CK8 and CK18 were used as loading controls).

Key: NH: 3T3Neo co-cultured with HCT116 LH: 3T3CD40L cocultured with HCT116
 NS: 3T3Neo co-cultured with SW480-CD40 LS: 3T3CD40L cocultured with SW480-CD40
 L: HCT116 S: SW480-CD40

4.5 mCD40L-induces activation of the extrinsic pathway of apoptosis: induction of the TRAIL pathway

Earlier studies have reported that CD40-mediated apoptosis of some types of carcinoma cells may involve up-regulation of death receptors and ligands, specifically FasL and TRAIL (Eliopoulos et al., 2000), thus implying a CD40 signalling cross-talk with the extrinsic pathway for the induction of cell death in some cell types. Yet, no such finding was reported in UCC cells where a direct intrinsic pathway of apoptosis has previously been reported (Georgopoulos et al., 2006). Immunoblotting experiments showed that CD40 ligation mediates rapid induction of the death ligand TRAIL within 1.5 hours in CRC cell lines. This expression of TRAIL continued at 3, 6, and 12 hours post ligation (Figure 4.8). In contrast to the TRAIL observation, a FasL specific antibody was unable to detect induction of FasL expression for the same series of time points (Figure 4.9). Interestingly, when TRAIL receptor expression was also examined, TRAIL-RII (DR5) was found to be upregulated at 3 and 6 hours in both CRC cell lines (Figure 4.10).

As these findings implied a cross-talk mechanism, the cleavage of Bid was also investigated. To detect the cleavage of Bid *in vitro*, a specific polyclonal antibody was used, and the results showed that, when HCT116 cells were treated with mCD40L to induce apoptosis, Bid was cleaved to t-Bid a truncated form known to induce apoptosis by mitochondrial cross-talk and by facilitating Bak/Bax activation. The cleavage of Bid occurred in the early stage of apoptosis in HCT116 (1.5 hours) (Figure 4.11). For unknown reasons SW480-CD40 showed no presence of t-Bid (not shown), however the functional significance of Bid cleavage was explored and confirmed in HCT116 cells, following the completion of this thesis, using a Bid-specific shRNA (see general Discussion).

4.5.1 mCD40L-mediated induction of TRAIL and FasL in CRC cells does not occur in a paracrine/juxtacrine fashion

As TRAIL was detected to be upregulated, its functional significance in CD40-mediated apoptosis was addressed. For this purpose, CytoTox-Glo assays were performed with CRC cells treated with mCD40L in the presence of antagonistic antibodies RIK2 and NOK-1, which block TRAIL and FasL, respectively. The blocking antibodies had no effect on mCD40L-induced apoptosis at 10µg/ml in both cell lines and levels of apoptosis remained significant ($p>0.05$) (Figure 4.12). A previous study by Steele et al from our laboratory showed that the RIK2 antibody efficiently blocks TRAIL-mediated apoptosis when soluble preparations of TRAIL are applied to carcinoma cells (Steele et al., 2006). The lack of any reduction in CD40-mediated apoptosis in the presence of RIK2 implies that during CD40-mediated apoptosis, TRAIL is possibly cytotoxic via an autocrine, but not paracrine/juxtacrine mechanism. In support of this, we confirmed also lack of surface TRAIL expression (following the completion of this thesis, using flow cytometry – Chris Dunnill unpublished observations). Following the completion of this thesis we also used a TRAIL siRNA to fully decipher whether TRAIL ligand was playing a functional role in CD40 mediated apoptosis (see general discussion). As for blocking of FasL by using NOK1 monoclonal antibody, co-culture with mCD40L cells and FasL blocking antibody (NOK1) as would be expected did not block apoptosis in HCT116 and SW480-CD40 ($p>0.05$) as shown in Figure 4.12.

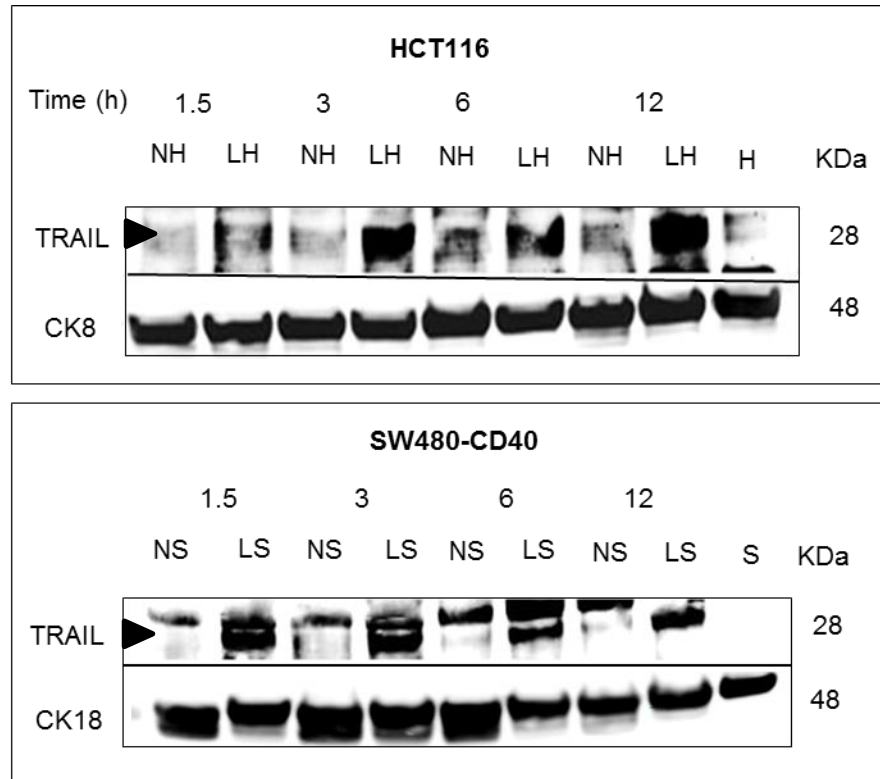


Figure 4. 8 Induction of TRAIL expression by CD40 ligation

HCT116 (H) and SW480-CD40 (S) cells were seeded at 3×10^6 cells/dish, and co-cultured with 3×10^6 MMC treated fibroblasts 3T3-Neo (N) or 3T3-CD40L (L). Co-cultures were incubated for the indicated time points (1.5, 3, 6, and 12 hours) in DR 5% FCS and 1% LG in 10cm^2 culture dishes. After each of incubation time, cells were lysed using 2x SDS-lysis buffer and protein concentration determined. Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were separated under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membranes. The membrane was probed for overnight at 4°C with primary polyclonal antibody (anti-TRAIL) in TBS/Tween 0.1% (1:500 dilution) and then with an anti-CK8 antibody for HCT116 and anti-CK18 for SW480-CD40 in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one hour with secondary antibody goat anti-rabbit IgG IRDye 800 (1:10000 dilution) for TRAIL detection and with goat anti-mouse antibody IgG IRDye 680 (1:10000 dilution) for CK8 and CK18. Antibody binding was visualised at 700nm and 800nm using an OdysseyTM Infra-red Imaging system (CK8 and CK18 were used as loading controls).

Key: NH: 3T3Neo co-cultured with HCT116 LH: 3T3CD40L cocultured with HCT116
 NS: 3T3Neo co-cultured with SW480-CD40 LS: 3T3CD40L cocultured with SW480-CD40
 L: HCT116 S: SW480-CD40

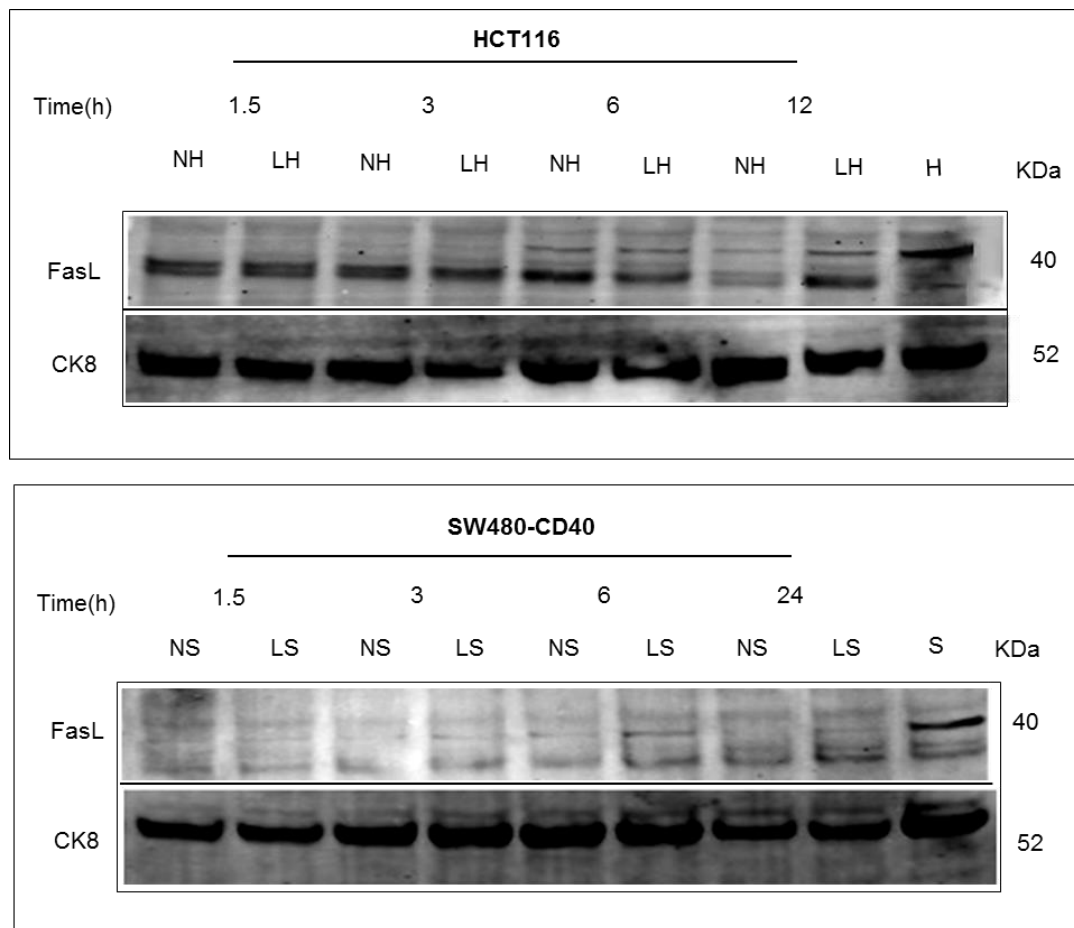


Figure 4. 9 Induction of FasL expression by CD40 ligation

HCT116 (H) and SW480-CD40 (S) cells were seeded at 3×10^6 cells/dish and co-cultured with 3×10^6 MMC treated fibroblasts 3T3-Neo (N) or 3T3-CD40L (L). Co-cultures were incubated for the indicated time points (1.5, 3, 6, and 12 hours) in DR 5% FCS and 1% LG in 10 cm^2 culture dishes. After each of incubation time, cells were lysed using 2x SDS-lysis buffer and protein concentration determined. Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were separated under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membranes. The membrane was probed for overnight at 4°C with primary polyclonal antibody (anti-FasL) in TBS/Tween 0.1% (1:500 dilution) and then with an anti-CK8 antibody for HCT116 and SW480-CD40 in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one hour with secondary antibody goat anti-rabbit IgG IRDye 800 (1:10000 dilution) for FasL detection and with goat anti-mouse antibody IgG IRDye 680 (1:10000 dilution) for CK8. Antibody binding was visualised at 700nm and 800nm using an OdysseyTM Infra-red Imaging system (CK8 was used as loading control).

Key: NH: 3T3Neo co-cultured with HCT116 LH: 3T3CD40L cocultured with HCT116
 NS: 3T3Neo co-cultured with SW480-CD40 LS: 3T3CD40L cocultured with SW480-CD40
 L: HCT116 S: SW480-CD40

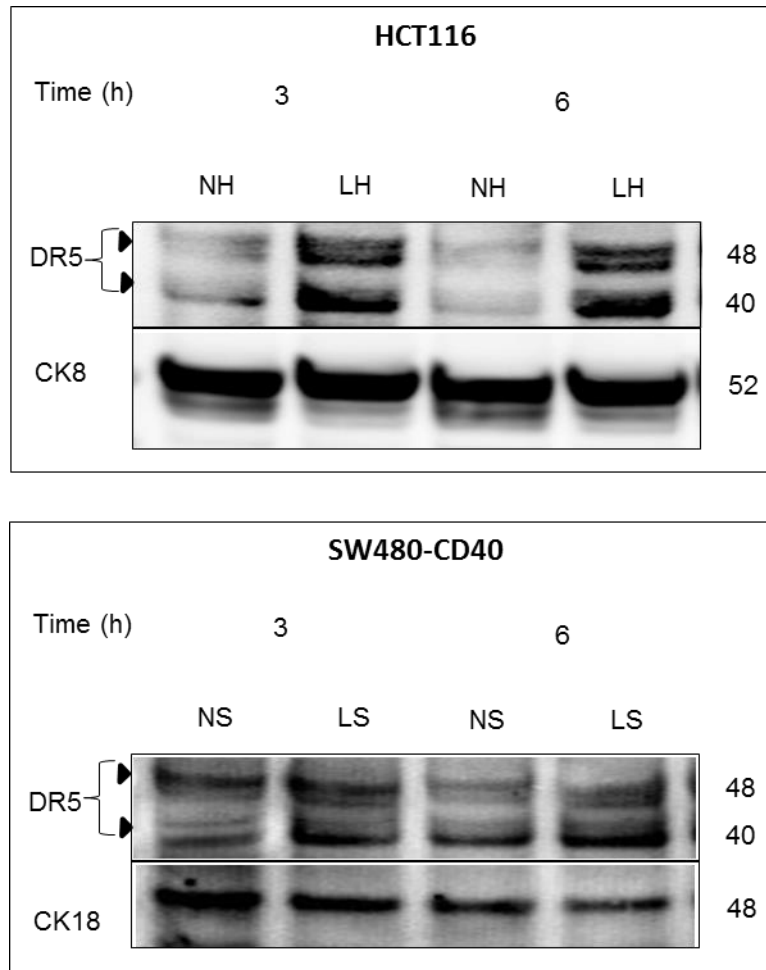


Figure 4. 10 Induction of TRAIL-R2 (DR5) expression in CRC Cells by CD40 ligation

HCT116 (H) and SW480-CD40 (S) cells were seeded at 3×10^6 cells/dish and co-cultured with 3×10^6 MMC treated fibroblasts 3T3-Neo (N) or 3T3-CD40L (L). Co-cultures were incubated for the indicated time points (1.5, 3, 6, and 12 hours) in DR 5% FCS and 1% LG in 10 cm^2 culture dishes. After each of incubation time, cells were lysed using 2x SDS-lysis buffer and protein concentration determined. Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were separated under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membranes. The membrane was probed for overnight at 4°C with primary polyclonal antibody (anti-DR5) in TBS/Tween 0.1% (1:500 dilution) and then with an anti-CK8 and CK18 for HCT116 for SW480-CD40 antibody in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one hour with secondary antibody goat anti-rabbit IgG IRDye 800 (1:10000 dilution) for TRAIL-R2 (DR5) detection and with goat anti-mouse antibody IgG IRDye 680 (1:10000 dilution) for CK8. Antibody binding was visualised at 700nm and 800nm using an OdysseyTM Infra-red Imaging system (CK8 and CK18 were used as loading controls).

Key: NH: 3T3Neo co-cultured with HCT116 LH: 3T3CD40L cocultured with HCT116
 NS: 3T3Neo co-cultured with SW480-CD40 LS: 3T3CD40L cocultured with SW480-CD40

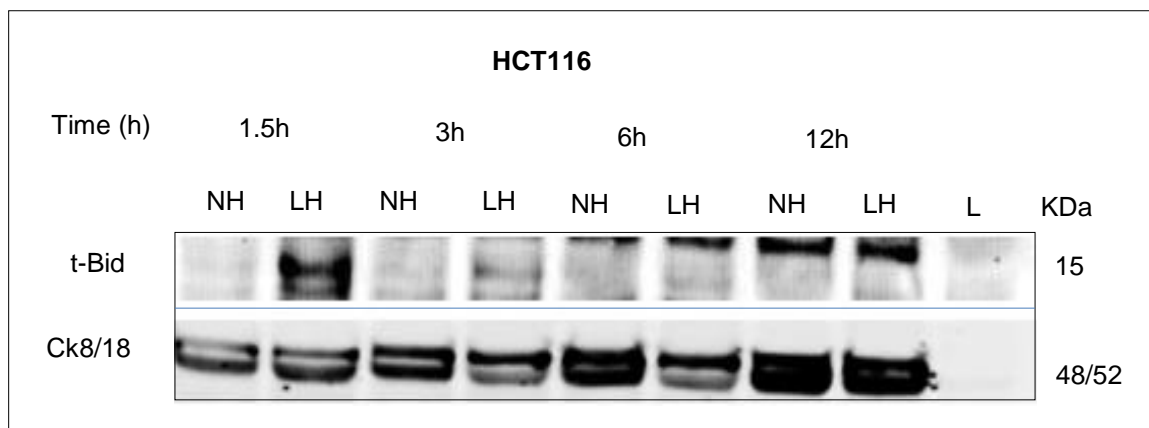


Figure 4. 11 Induction of t-Bid expression by CD40 ligation

HCT116 (H) cells were seeded at 3×10^6 cells/dish and co-cultured with 3×10^6 MMC treated fibroblasts 3T3-Neo (N) or 3T3-CD40L (L). Co-cultures were incubated for the indicated time points (1.5, 3, 6, and 12 hours) in DR 5% FCS and 1% LG in 10cm^2 culture dishes. After each of incubation time, cells were lysed using 2x SDS-lysis buffer and protein concentration determined. Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were separated by under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membranes. The membrane was probed for overnight at 4°C with primary polyclonal antibody (anti-t-Bid) in TBS/Tween 0.1% (1:500 dilution) and then with an anti-CK8 in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one hour with secondary antibody goat anti-rabbit IgG IRDye 800 (1:10000 dilution) for t-Bid detection and with goat anti-mouse antibody IgG IRDye 680 (1:10000 dilution) for CK8. Antibody binding was visualised at 700nm and 800nm using an OdysseyTM Infra-red Imaging system (CK8 was used as loading control).

Key: NH: 3T3Neo co-cultured with HCT116
L: 3T3CD40L

LH: 3T3CD40L cocultured with HCT116

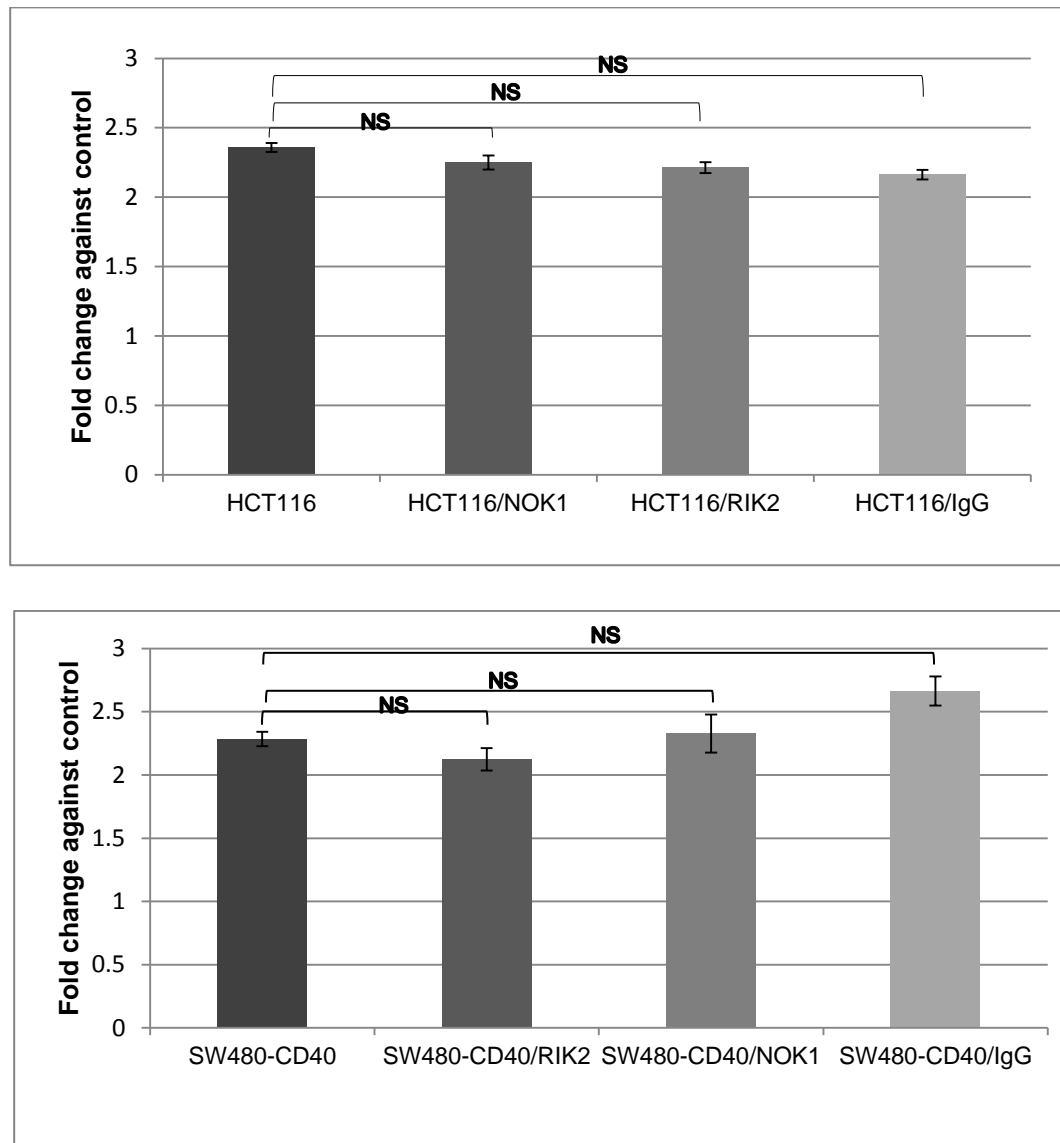


Figure 4. 12 Effect of blocking antibodies NOK1 and RIK2 on CD40-mediated apoptosis

1×10^4 CRC cells (HCT116 and SW480-CD40) were co-cultured with 1×10^4 MMC treated 3T3CD40L and 3T3Neo fibroblasts in 96 well plates in DR medium supplemented with 5% FCS and L-glutamine $\pm 10 \mu\text{g/ml}$ of NOK1 and RIK2 (or isotype control IgG mAb) to block FasL and TRAIL respectively. After 24 hour, apoptosis was measured by CytoTox-Glo assay. 50 μl of substrate was added, luminescence measured (as described in the Materials and methods) and background-corrected relative luminescence unit (RLU) readings deduced by pair-wise subtraction of mCD40L and Control cell from the respective Co-cultures. Panel A and B show fold change against control and antibodies were blocked the ligands (TRAIL and FasL) in co-cultures for both HCT116 and SW480-CD40 compared with control. Data shows no significant differences in both cell lines (HCT116 and SW480-CD40). Bars show mean \pm SD of 4-5 replicates and results are representative of three experiments. Results are presented as fold change and calculated as described in chapter 2. (Non-significant; $p > 0.05$)

4.6 CD40-mediated apoptosis involves both intrinsic and extrinsic mechanisms

Previous studies in UCC cells reported that CD40 ligation activates apoptosis via activation of caspase-9 but not caspase-8 (Georgopoulos et al., 2006). In this project, functional investigations of caspases were performed using biochemical caspase inhibitors. CytoTox-Glo apoptosis assays were performed in mCD40L-treated HCT116 cells in the presence of caspase-8, -9, -10 inhibitors (Z-IETD, Z-LEHD, and Z-VEVD respectively) as well as pan-caspase inhibitor (z-VAD). Notably, inhibition of caspase-9 resulted in dramatic attenuation of apoptosis. Apoptosis of CRC cells (HCT116) following co-culture with mCD40L cells was also nearly completely blocked by the pan-caspase inhibitor z-VAD confirming that CD40-mediated apoptosis in CRC cells is caspase-dependent. More specifically, inhibitors of caspases 9 and 10 (Z-LEHD and Z-VEVD, respectively) caused less marked yet highly significant reductions in apoptosis (Figure 4.13) whereas a caspase-8 inhibitor (Z-IETD) had no effect on cell death in HCT116 at all (Figure 4.14).

It is well established that Bak and Bax regulate apoptotic cell death by facilitating MOMP, subsequent cytochrome c release and activation of caspase-9 during the intrinsic pathway (Kroemer et al., 2007). Because of this, we examined expression of Bak and Bax following CD40 ligation in CRC cells. In what would support an intrinsic mediated mechanism of cell apoptosis, it was found that CD40 engagement caused the marked and rapid induction of Bak within 3, 6, 12, and 24 hours in HCT116. In SW480-CD40 this occurred even faster than 3 hours (1.5 hours) and continued gradually increased expression at 6 to 12 hours (Figure 4.15). Also Bax protein expression was detected in HCT116 within 6 hours post interaction with mCD40L and this continued at 12 and 24 hour time points. In contrast with SW480-CD40 has rapid expression of Bax at 1.5 and this gradually increased following CD40 ligation (Figure 4.16).

We also next examined whether apart from an increase in Bak/Bax expression there was induction of MOMP and release of cytochrome c. mCD40L-treated HCT116 and SW480-CD40 cells tested 6 hours post-ligation (as this is when optimal Bak and Bax expression was detected – see figures 4.15 and 4.16) and then the mitochondrial and cytosolic fractions were separated as described in section 2.13.2 in the Methods. Further, fractions for control and mCD40L treated cells were screened for mitochondrial cytochrome

c, mitochondrial Bcl-2 and cytoplasmic GAPDH through immunoblotting (Figure 4.17). mCD40L caused CRC cells to induce MOMP and cytochrome c release into their cytoplasm thus confirming a role for the mitochondria in the induction of CD40-mediated apoptosis. This is in agreement with previous studies in UCC cells that demonstrated that CD40 engagement by mCD40L induces expression of the pro-apoptotic molecules Bak and Bax, whilst down-regulating anti-apoptotic Bcl-2 protein (Bugajska et al., 2002).

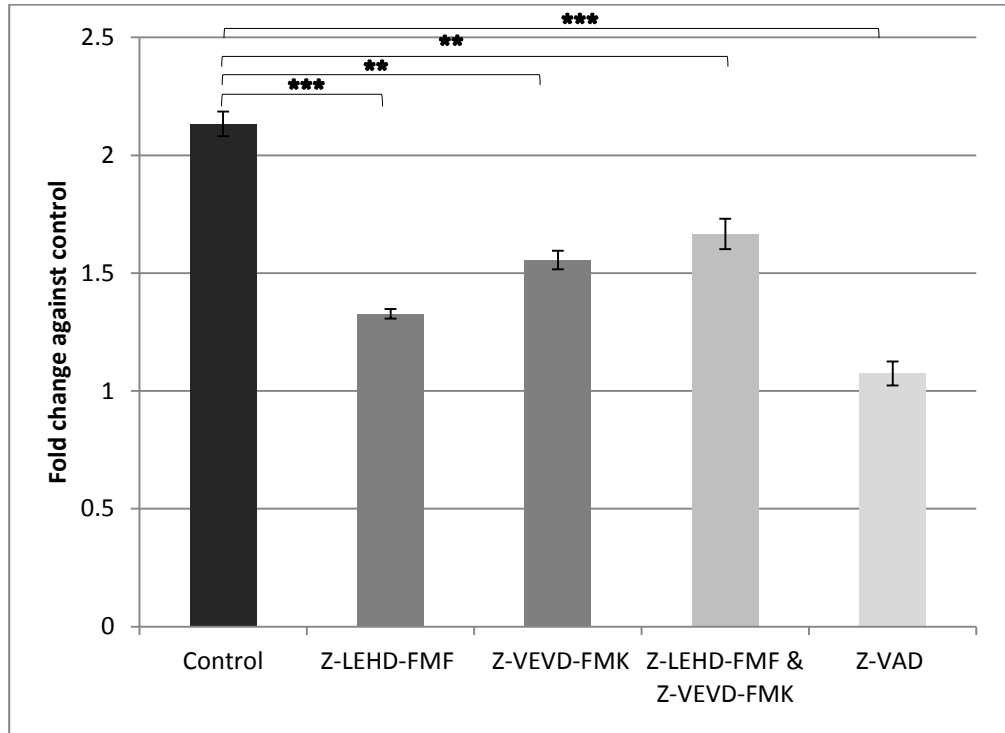


Figure 4. 13 Effect of caspase -9 and -10 inhibition on CD40 ligation by mCD40L

1×10^4 cells/well CRC cells (HCT116) were co-cultured with 1×10^4 cells/well MMC treated 3T3CD40L and 3T3Neo fibroblasts in 96 well plates in DR /5% FCS / L-glutamine \pm 100 μ M of caspase -9 (Z-LEHD-FMF) or -10 (Z-AEVD-FMK) inhibitor and general caspases inhibitor (z-VAD). After 24 hour at 37°C / 5% CO₂, apoptosis was measured by CytoTox-Glo assay. 50 μ l of substrate was added, luminescence measured (as described in the Materials and methods) and background-corrected relative luminescence unit (RLU) readings deduced by pair-wise subtraction of mCD40L and Control cell from the respective Co-cultures. Figure shows fold change against control and caspase -9 and -10 inhibitor inhibit apoptosis in HCT116. Bars show mean fold change of apoptosis \pm SD of 4-5 replicates and results are representative of three experiments. Stats: ** P<0.01, paired student t-test, HCT116 compared with control cells.

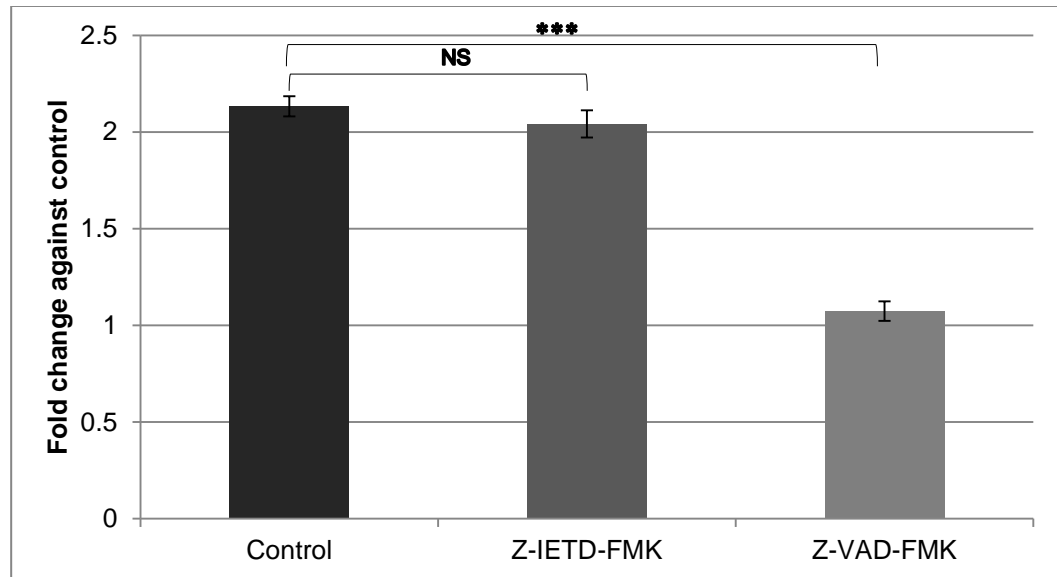


Figure 4. 14 Effect of caspase -8 inhibition on CD40 ligation

1×10^4 cells/well CRC cells (HCT116) were co-cultured with 1×10^4 cells/well MMC treated 3T3CD40L and 3T3Neo fibroblasts in 96 well plates in DR /5% FCS / L-glutamine \pm 100 μ M of caspase -8 inhibitor (Z-IETD-FMK) inhibitor and general caspases inhibitor (z-VAD) used as positive control. After 24 hour at 37°C / 5% CO₂, apoptosis was measured by CytoTox-Glo assay. 50 μ l of substrate was added, luminescence measured (as described in the Materials and methods) and background-corrected relative luminescence unit (RLU) readings deduced by pair-wise subtraction of mCD40L and Control cell from the respective Co-cultures. Results show fold change against control and caspase -8 inhibitor did not inhibit apoptosis and there is no significant difference between control and caspase -8 inhibitor. Bars show mean fold change of apoptosis \pm SD of 4-5 replicates and results are representative of three experiments. Stats: NS $p > 0.07$ / *** $p < 0.001$, paired student t-test, HCT116 compared with control cells.

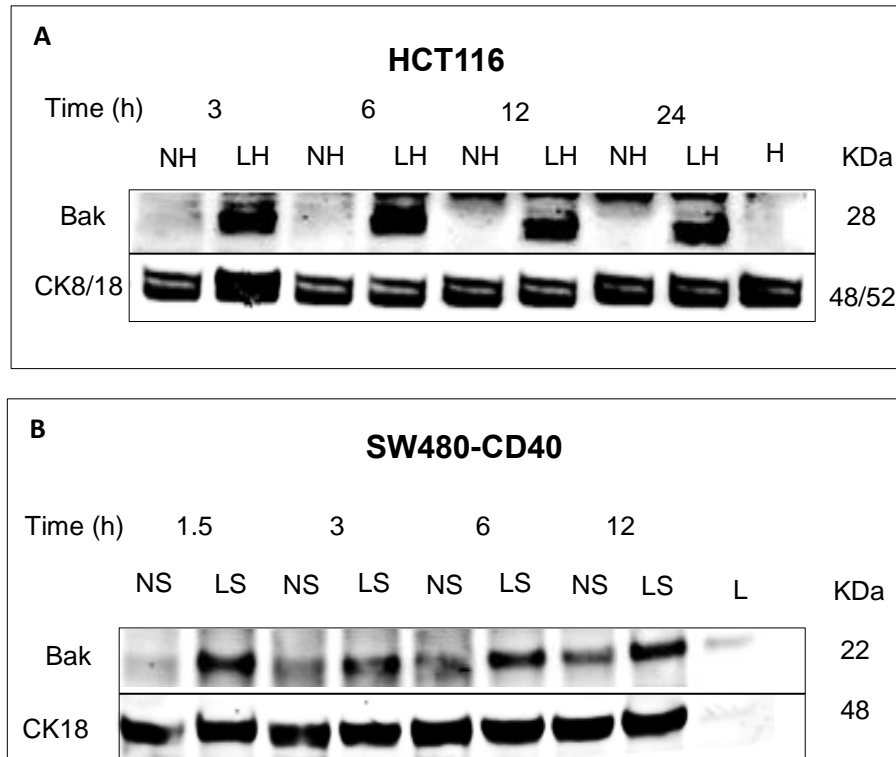


Figure 4. 15 The activation of Bak following CD40 ligation

HCT116 (H) and SW480-CD40 (S) cells were seeded at 3×10^6 cells/dish and co-cultured with 3×10^6 MMC treated fibroblasts 3T3-Neo (N) or 3T3-CD40L (L). Co-cultures were incubated for the indicated time points (1.5, 3, 6, 12 and 24 hours) in DR / 5%FCS / 1%L-glutamine in 10cm^2 culture dishes. After each of incubation time, cells were lysed using 2x SDS-lysis buffer and protein concentration determined. Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were separated under denaturing conditions by SDS-PAGE using 4 -12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membrane. The membrane was probed for overnight at 4°C with primary monoclonal antibody (anti-Bak) in TBS/Tween 0.1% (1:500 dilution) and then with an anti-CK8/18 and anti-CK18 antibody in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one hour with secondary antibody goat anti-rabbit IgG IRDye 800 (1:10000 dilution) for Bak detection and with goat anti-mouse antibody IgG IRDye 680 (1:10000 dilution) for CK8/18 and CK18. Antibody binding was visualised at 700nm and 800nm using an OdysseyTM Infra-red Imaging system (CK8/18 and CK18 were used as loading controls).

Key: NH: 3T3Neo co-cultured with HCT116 LH: 3T3CD40L cocultured with HCT116
 NS: 3T3Neo co-cultured with SW480-CD40 LS: 3T3CD40L cocultured with SW480-CD40
 L: 3T3CD40L

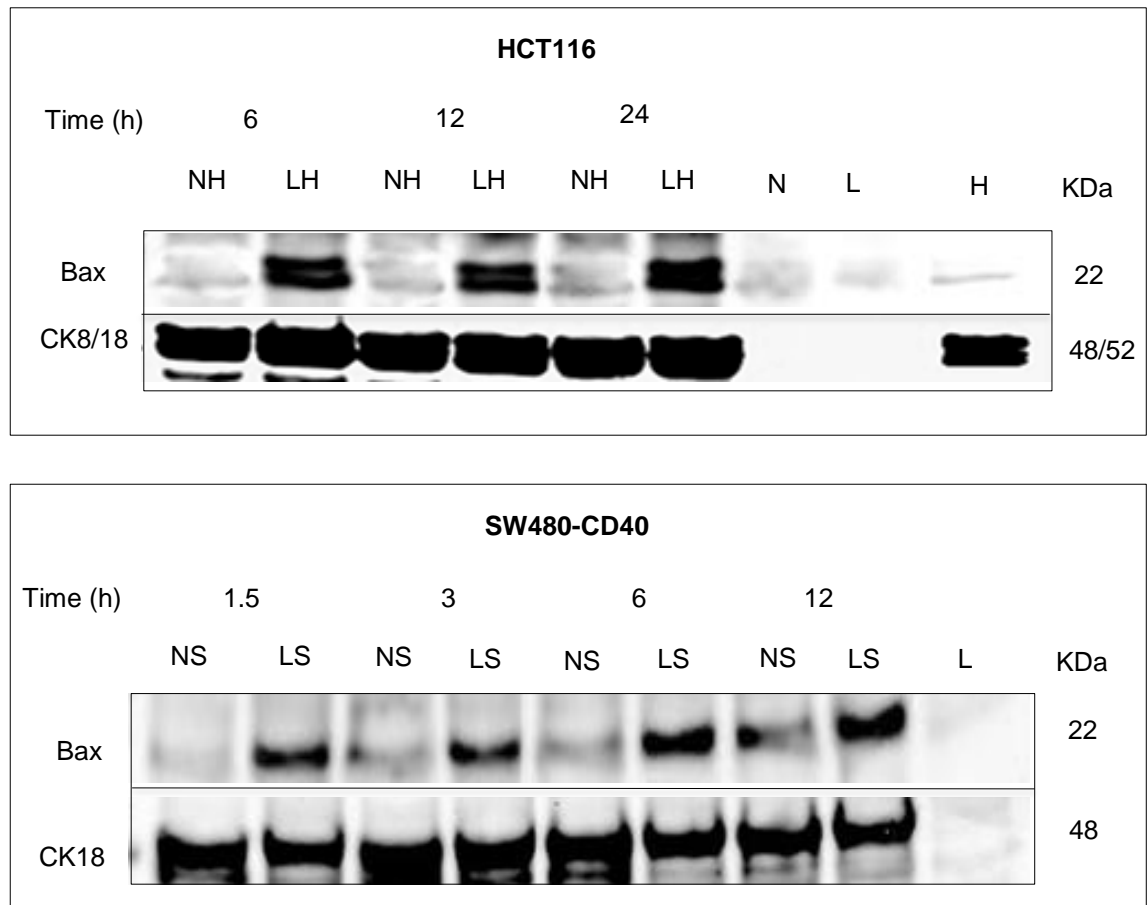


Figure 4. 16 The activation of Bax following CD40 ligation

HCT116 (H) and SW480-CD40 (S) cells were seeded at 3×10^6 cells/dish and co-cultured with 3×10^6 MMC treated fibroblasts 3T3-Neo (N) or 3T3-CD40L (L). Co-cultures were incubated for the indicated time points (1.5, 3, 6, 12 and 24 hours) in DR / 5%FCS / 1%L-glutamine in 10cm² culture dishes. After each of incubation time, cells were lysed using 2x SDS-lysis buffer and protein concentration determined. Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were separated under denaturing conditions by SDS-PAGE using 4 -12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membrane. The membrane was probed for overnight at 4°C with primary monoclonal antibody (anti-Bax) in TBS/Tween 0.1% (1:500 dilution) and then with an anti-CK8/18 and anti-CK18 antibody in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one hour with secondary antibody goat anti-mouse IgG IRDye 680 (1:10000 dilution) for Bax detection and with goat anti-mouse antibody IgG IRDye 680 (1:10000 dilution) for CK8/18 and CK18. Antibody binding was visualised at 700nm using an Odyssey™ Infra-red Imaging system (CK8/18 and CK18 were used as loading controls).

Key: NH: 3T3Neo co-cultured with HCT116 LH: 3T3CD40L cocultured with HCT116
 NS: 3T3Neo co-cultured with SW480-CD40 LS: 3T3CD40L cocultured with SW480-CD40
 L: 3T3CD40L

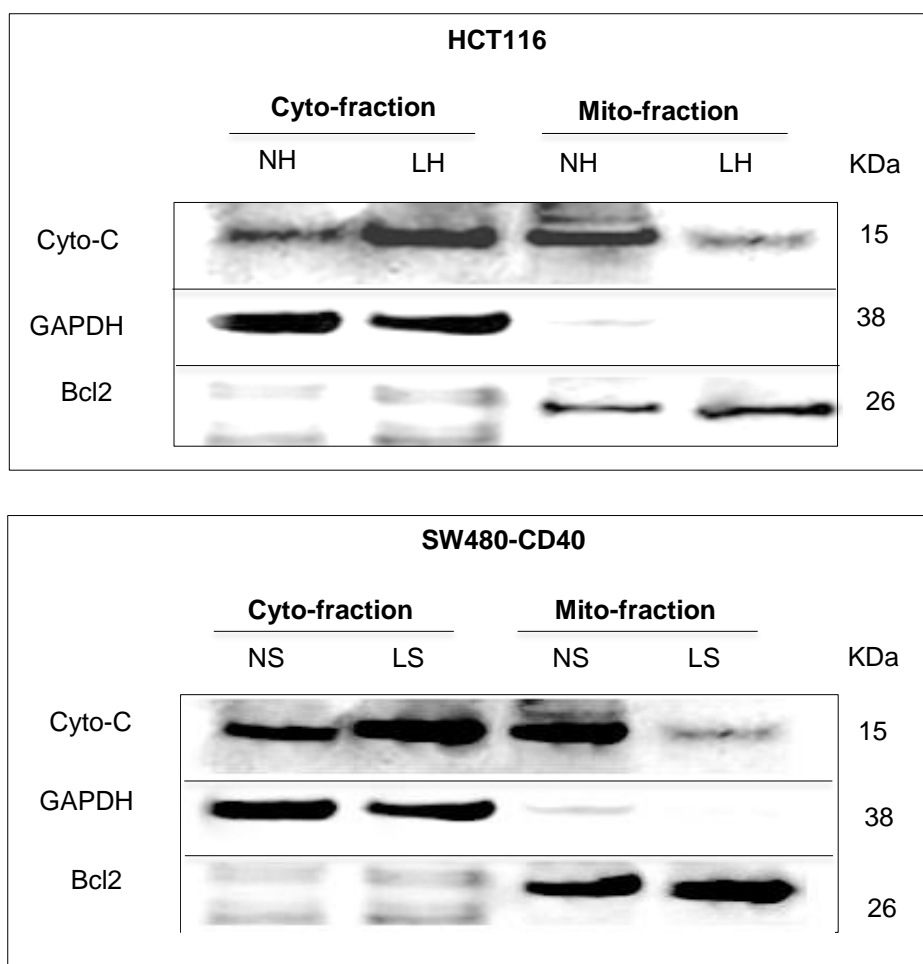


Figure 4. 17 Induction of MOMP by mCD40L in CRC cells

HCT116 cells (H) and SW480-CD40 (S) cells were seeded at 3×10^6 cells/dish and co-cultured with 3×10^6 cells/dish MMC treated fibroblast cells 3T3-Neo (N) or 3T3-CD40L (L). Co-cultured cells were incubated for 6 hours, in DR /5% FCS and 1% LG, in 10cm^2 culture dishes. After incubation, cells were harvested by trypsinisation and processed accordingly to the manufacturer's protocol (Merck mitochondrial isolation kit see section 2.11.2). After that protein concentration determined. Cytoplasmic and mitochondrial fraction were separated under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membrane. The membrane was probed for overnight at 4°C with primary polyclonal antibody; an anti-Cytochrome C) in TBS/Tween 0.1% (1:500 dilution), anti-GAPDH antibody in TBS/Tween 0.1% (1:1000 dilution) and anti-Bcl-2 antibody in TBS/Tween 0.1% (1:500 dilution). The membrane was then incubated for one hour with goat anti-rabbit IgG Alexa 800 (1:10000 dilution) to detection of Cytochrome C and one hour with goat anti mouse to detect both GAPDH and Bcl2 (loading controls for cytosolic and mitochondrial fractions respectively). Antibody binding was visualised at 700nm and 800nm using an Odyssey™ Infra-red Imaging system.

Key; NH: 3T3Neo co-cultured with HCT116 LH: 3T3CD40L co-cultured with HCT116
 NS: 3T3Neo co-cultured with SW480-CD40 LS: 3T3CD40L co-cultured with SW480-CD40

4.7 Use of shRNA-based RNAi by retrovirus-mediated delivery to study the functional role of intracellular mediators in CD40-mediated apoptosis

Short-hairpin RNAs (shRNAs) are a form of RNA interference (RNAi) and are used as a mechanism to specifically knockdown proteins of interest (Rao et al., 2009). A panel of retroviral shRNA expression vectors were prepared (Section 2.7.1) for the stable knockdown of proteins of interest for functional studies, including TRAF3 (below) and Bax. Replication-incompetent retroviral particles were used to transduce the target CRC cell line HCT116 (as explained in section 2.8.4). For selection of stable shRNA HCT116 expressers, puromycin antibiotic selection was used, as the viral expression cassette comprises a puromycin resistance gene (section 2.7.2).

The CellTiter 96® AQ_{ueous} One Solution Cell Proliferation assay (section 2.9.2) was used to perform titration experiments to determine adequately cytotoxic concentrations of puromycin for selection of antibiotic-resistant cell populations, following viral transductions. This proliferation assay involves the use of MTS tetrazolium (yellow) which is reduced to a formazan derivative (brown) by respiring cells. The increased absorbance at 492nm occurs due to the colour change induced by viable cells as they produce electron coupling reagents such as NADH that reduce a formazan substrate (yellow-brown). Following the combination of results from such pre-titration experiments (Figure 4.18) and routine phase contrast microscopic examination during antibiotic treatment, it was found that a concentration of 0.25µg/ml of puromycin concentration effectively perished non-transduced control HCT116 cells after several days, whilst allowing the proliferation of their virally-transduced counterparts. Figure 4.19 shows representative results for experiments involving HCT116 transduction with a retrovirus expressing TRAF3 shRNA (to be discussed in detail in subsequent sections).

4.7.1 The role of TRAF3 in CD40-mediated apoptosis

After retroviral transduction for the expression of an shRNA for knockdown of TRAF3 (Table 2.4), HCT116 cells were selected in puromycin as previously described (section 4.1.1); for the purposes of this thesis, HCT116 derivatives bearing the TRAF3 shRNA will be termed TRAF3-KD cells. The ability of the designed shRNAs to knockdown TRAF3 protein expression in TRAF3-KD cells was confirmed by immunoblotting (Figure 4.20). Densitometric analysis was also used to confirm that, the expression of TRAF3 was reduced after 1.5 and 3 hours CD40 ligation compared with control as illustrated in Table 4.1 and Figure 4.21.

As TRAF3 protein is induced only when CD40 was engaged by mCD40L to give a pro-apoptotic signal in UCC cells (Georgopoulos et al., 2006) it was next determined whether this is the case in CRC cell lines. Results demonstrated that TRAF3-KD cells had a significant reduction ($P<0.05$) of apoptosis compared with HCT116 as determined by CytoTox-Glo assays (Figure 4.22). This was further confirmed using the caspase-3/7 activation-based apoptosis assay, where TRAF3 knockdown led to a significant reduction ($P<0.05$) also in caspase-3/7 activity (Figure 4.23).

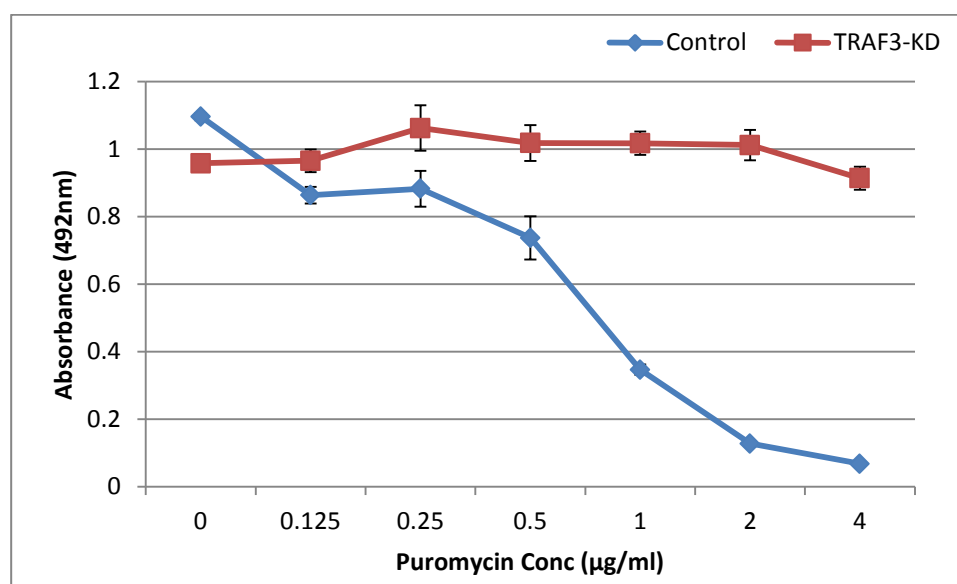


Figure 4. 18 HCT116 and TRAF3-KD cell proliferation following treatment with a range of puromycin concentrations

1×10^4 HCT116-TRAF3-KD and HCT116 cells were seeded in 96 well plates and then treated with the indicated concentrations of puromycin antibiotic in DR/5%FCS/1%L-G for 24 hours. 20μl of CellTiter 96® AQ_{ueous} One Solution was added to appropriate wells and plates were incubated at 37°C in 5% CO₂ for a total of four hours. To assess cell viability, total levels of formazan formation were measured using a FLUOstar OPTIMA (BMG Labtech) plate reader at a wavelength of 492nm. Bars correspond to mean absorbance values of 5-6 technical replicates \pm SEM and results are representative of three independent experiments.

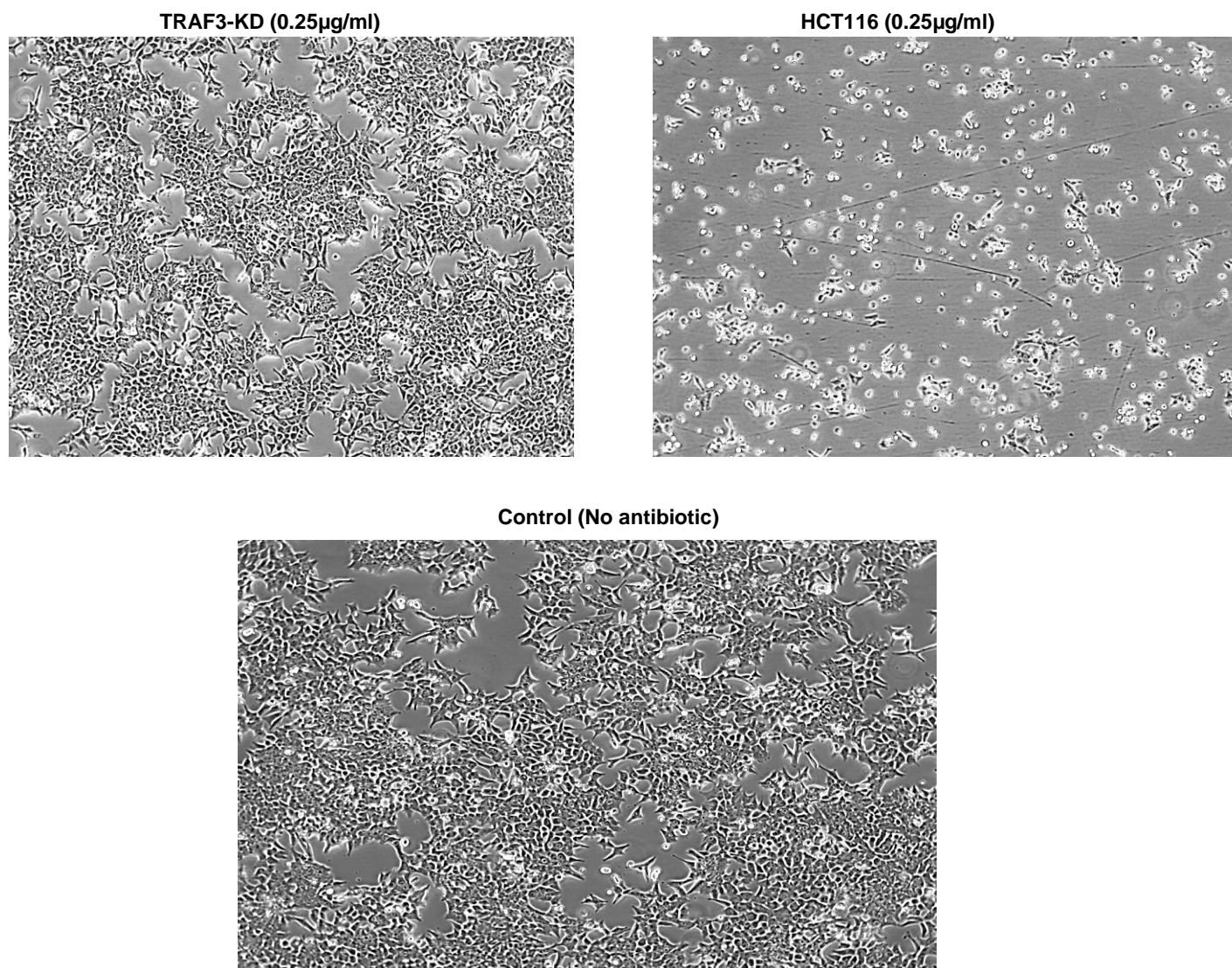


Figure 4. 19 Antibiotic (Puromycin) selection of transduced HCT116 cells with TRAF3 shRNA-expressing retrovirus

Transduced and non-transduced HCT116 cells were cultured in DR/5%FCS/1%L-G supplemented with 0.25µg/ml puromycin compared with non-treated cells as control (the transduced cells shown here are cells in which an anti-TRAF3 shRNA is expressed, denoted TRAF3-KD). HCT116 were also cultured with DR/5%FCS/1%L-G alone to demonstrate their normal growth pattern (Control). Note non-resistant HCT116 perish from the flask within 24 hours, however resistant cell populations continue to proliferate. Images are representative phase contrast micrographs (taken at 100x magnification).

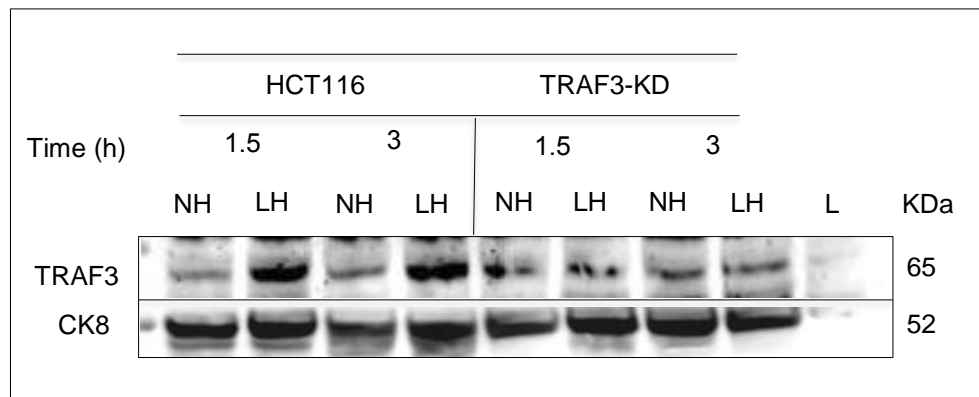


Figure 4. 20 The effect of TRAF3 knockdown on the regulation of TRAF3 expression following CD40 ligation

3×10^6 control HCT116 cells (H) and HCT116 cells expressing TRAF3 shRNA (TRAF3-KD) were co-cultured with 3×10^6 MMC treated mCD40L (L) and Controls (N) for 1.5 and 3 hours in DR / 5%FCS / 1%L-glutamine in 10cm^2 culture dishes in order to lyse with 2X SDS-lysis buffer. Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were separated under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membrane. The membrane was probed overnight at 4°C with primary polyclonal antibody (anti-TRAF3) in TBS/Tween 0.1% (1:500 dilution) and then with an anti-CK8 antibody in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one hour with secondary antibody [goat anti-rabbit IgG IRDye 800nm (1:10000 dilution)] for TRAF3 detection and with goat anti-mouse antibody IgG IRDye 680 (1:10000 dilution) for CK8 (used as loading control).

Key: NH: 3T3Neo co-cultured with HCT116
L: 3T3CD40L

LH: 3T3CD40L cocultured with HCT116

	HCT116				TRAF3-KD			
Time (h)	1.5		3		1.5		3	
Co-cultures	NH	LH	NH	LH	NH	LH	NH	LH
Background band intensity	625.12	1737.21	712.15	1816.21	650.31	823.19	613.23	721.2
CK8 BC band intensity	2324.54	2421.28	2098.55	2331.95	1989.94	2347.25	2468.24	2319.75
Fold change	2.78		2.55		1.27		1.18	

Table 4. 1 Densitometric analysis to calculate fold change in TRAF3 protein expression

Densitometry was used to normalise data using Cytokeratin 8 as the loading Control. Readings represent band intensity values generated by the LiCor Odyssey analysis software. Data was normalised against densitometry values for Controls and then fold change was calculated by the following equation: mCD40L/HCT116 ÷ Control/HCT116. BC - blank corrected. The table above is representative of the values obtained from the blot shown in Figure 4.20.

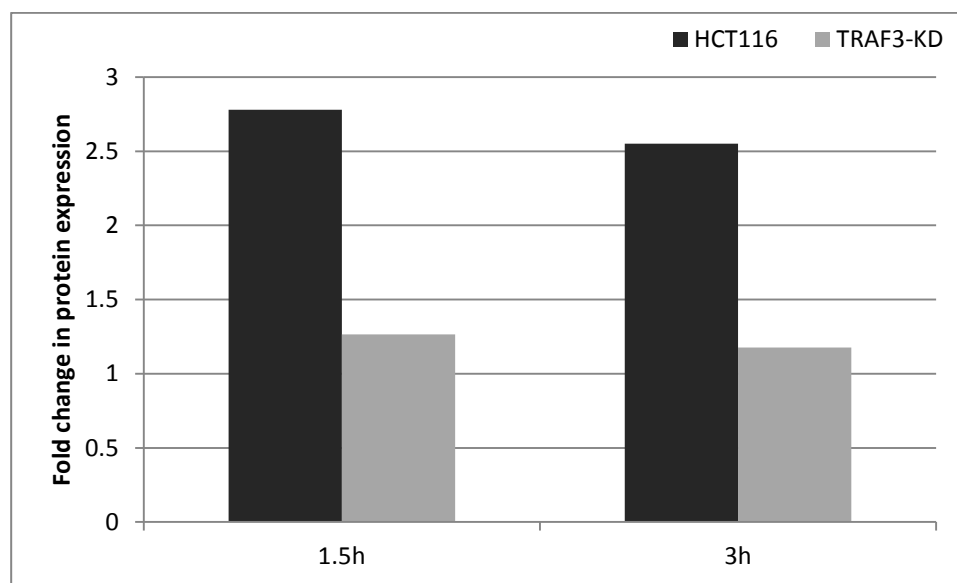


Figure 4. 21 Densitometric analysis to calculate fold change in TRAF3 protein expression following shRNA-mediated knockdown

Densitometry was used to normalise data using Cytokeratin 8 as the loading Control. Readings represent band intensities values generated by the LiCor Odyssey analysis software. Data was normalised against densitometry values for Controls and then fold change was calculated by the following equation (mCD40L/HCT116 ÷ Control/HCT116). BC - blank corrected. The table above is representative of values shown in Figure 4.20.

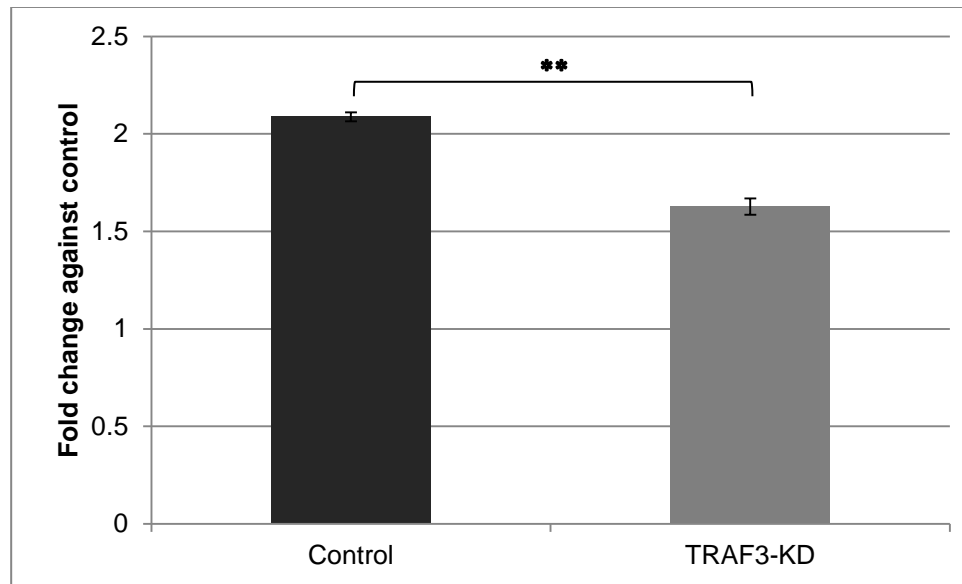


Figure4. 22 Effect of shRNA-mediated TRAF3 knockdown on CD40-induced apoptosis

1×10^4 colorectal cancer cells (HCT116 and TRAF3-KD cells) were cocultured with 1×10^4 MMC treated 3T3CD40L (mCD40L) and 3T3Neo (control) in white 96 well plates in DR medium supplemented with 5% FBS and 1% L-glutamine. After 24 hours of coculture, CytoTox-Glo test was used to detect apoptosis in colorectal cancer cells (HCT116) as described in chapter 2 (Materials and Methods). Background corrected RLU readings deduced by subtraction of mCD40L from coculture [(3T3CD40L + HCT116) – 3T3CD40L] and control [(3T3Neo + HCT116) - 3T3Neo]. Bars show mean fold change of apoptosis \pm S.D of 4 to 6 replicates and results are representative of three experiments. Results show significant differences between HCT116/3T3CD40L versus TRAF3-KD/3T3CD40L indicated by ** $p < 0.01$ for HCT116 cells and TRAF3-KD cells.

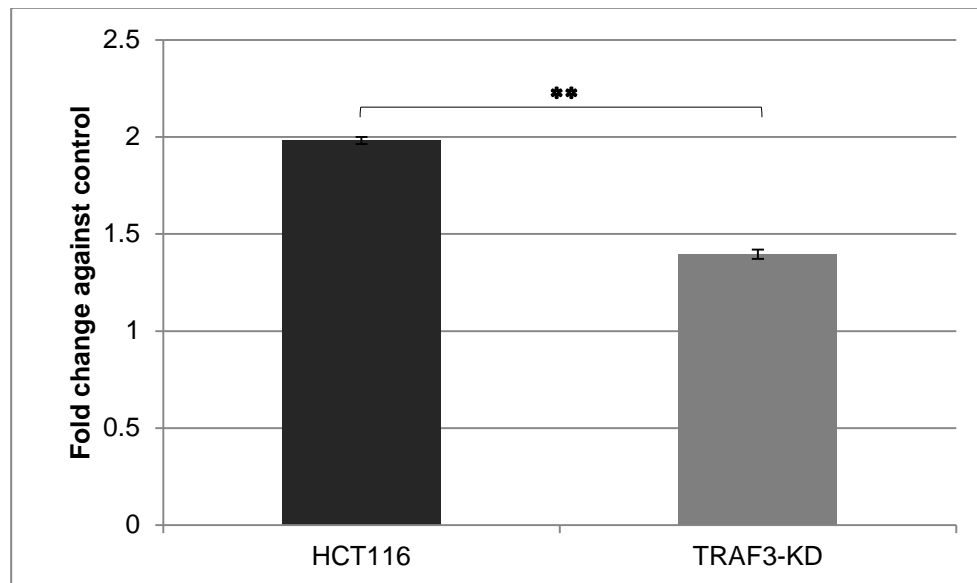


Figure 4. 23 Effect of TRAF3 protein knockdown on caspase-3 and -7 activation during CD40-mediated apoptosis

1×10^4 HCT116 and TRAF3-KD cells were co-cultured with 1×10^4 MMC treated mCD40L or Controls in DR medium supplemented with 5% FCS and 1% L-glutamine in white 96-well plates. 50 μ l of SensoLyte 3/7 substrate was added after 48 hours, fluorescence measured (as described in the Materials and methods) and background-corrected relative fluorescence unit (RFU) readings deduced by pair-wise subtraction of mCD40L and Control values from respective co-cultures. Bars show mean fold change of caspase 3 and 7 activity \pm S.D of 4 to 6 replicates and results are representative of three experiments. Stats: ** $p < 0.01$, paired student t-test, HCT116 compared with knockdown cells.

4.7.2 Role of TRAF3 in the induction of JNK and p38 MAPK

Previous studies on CD40-induced p38 (Sutherland et al., 1996, Salmon et al., 1997) and JNK (Elmetwali et al., 2010b, Georgopoulos et al., 2006, Li et al., 1996) activation indicated that CD40 may induce TRAF3-mediated activation of the kinase activities of these MAPKs protein. Moreover, TRAF3 appeared to play a critical role in CD40-induced up-regulation of a variety of signal transduction mediators.

TRAF3-KD cells were first used to investigate whether TRAF3 regulated downstream JNK activation during CD40-mediated apoptosis. As shown in Figure 4.24, TRAF3-KD cells had reduced activation of JNK within 3 and 6 hours post CD40 ligation compared with HCT116. Also, the results showed that TRAF3 regulates the expression of p38 at 6 hours post CD40 ligation as TRAF3 knockdown abolished the p38 phosphorylation normally observed in the HCT116 cells (Figure 4.25). These findings demonstrated for the first time a critical role for TRAF3 in the activation of p38 and particularly JNK.

4.7.3 Role of TRAF3 in the expression of Bax

As CD40 ligation by mCD40L causes the expression of Bax and because UCC cells following TRAF3 knockdown have decreased mCD40L susceptibility (Georgopoulos et al., 2006), it was hypothesised that TRAF3 stabilisation may also regulate Bax expression. TRAF3-KD cells were used to explore this possibility. Results demonstrated conclusively that TRAF3-mediated signalling regulates the expression of pro-apoptotic regulator Bax, as TRAF3 knockdown completely abrogated the Bax expression that is normally seen in HCT116 (Figure 4.26). The complete inhibition of CD40-mediated Bax protein induction by TRAF3 knockdown suggested that loss of TRAF3 interferes with the induction of apoptosis in CRC cells.

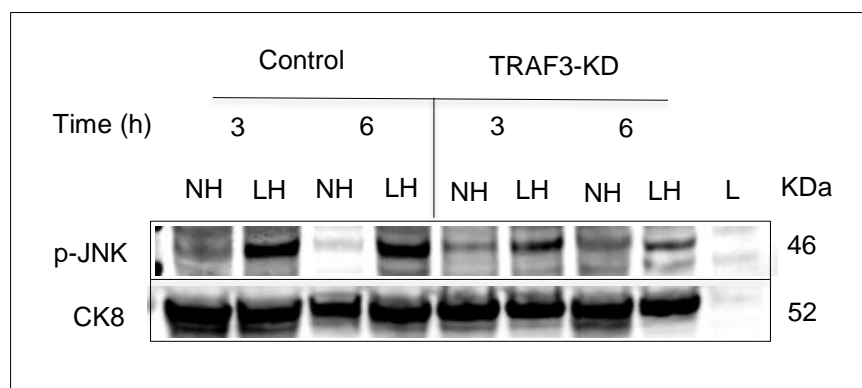


Figure 4. 24 The role of TRAF3 knockdown on p-JNK following CD40 ligation

3×10^6 CRC cells [HCT116 (H)] and HCT116 cells expressing TRAF3 sh RNA (TRAF3-KD) were co-cultured with 3×10^6 MMC treated mCD40L (L) and Controls (N) for 3 and 6 hours in DR / 5%FCS / 1%LG in 10cm^2 culture dishes in order to lyse with 2X SDS-lysis buffer. Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were separated under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membrane. The membrane was probed for overnight at 4°C with primary monoclonal antibody/goat anti-mouse antibody (anti-p-JNK) in TBS/Tween 0.1% (1:500 dilution) and then with an anti-CK8 antibody in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one hour with secondary antibody [goat anti-mouse IgG IRDye 680 (1:10000 dilution)] for p-JNK detection and membrane was incubated with goat anti-mouse antibody IgG IRDye 680 (1:10000 dilution) for CK8 (used as loading control).

Key: NH: 3T3Neo co-cultured with HCT116
L: 3T3CD40L

LH: 3T3CD40L cocultured with HCT116

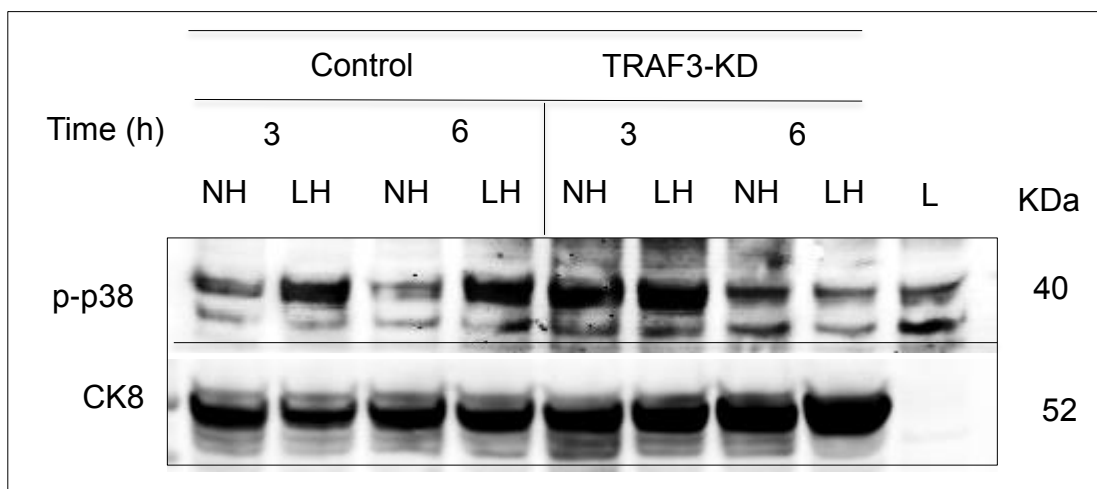


Figure 4. 25 The role of TRAF3 knockdown on p-p38 expression following CD40 ligation

3×10^6 control HCT116 cells (H) and HCT116 cells expressing TRAF3 shRNA (TRAF3-KD) were co-cultured with 3×10^6 MMC treated mCD40L (L) and Controls (N) for 3 and 6 hours in DR / 5%FCS / 1%L-glutamine in 10cm^2 culture dishes in order to lyse with 2X SDS-lysis buffer. Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were separated under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membrane. The membrane was probed overnight at 4°C with primary polyclonal antibody (anti-p-p38) in TBS/Tween 0.1% (1:500 dilution) and then with an anti-CK8 antibody in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one hour with secondary antibody [goat anti-rabbit IgG IRDye 800nm (1:10000 dilution)] for p-p38 detection and with goat anti-mouse antibody IgG IRDye 680 (1:10000 dilution) for CK8 (used as loading control).

Key: NH: 3T3Neo co-cultured with HCT116
L: 3T3CD40L

LH: 3T3CD40L cocultured with HCT116

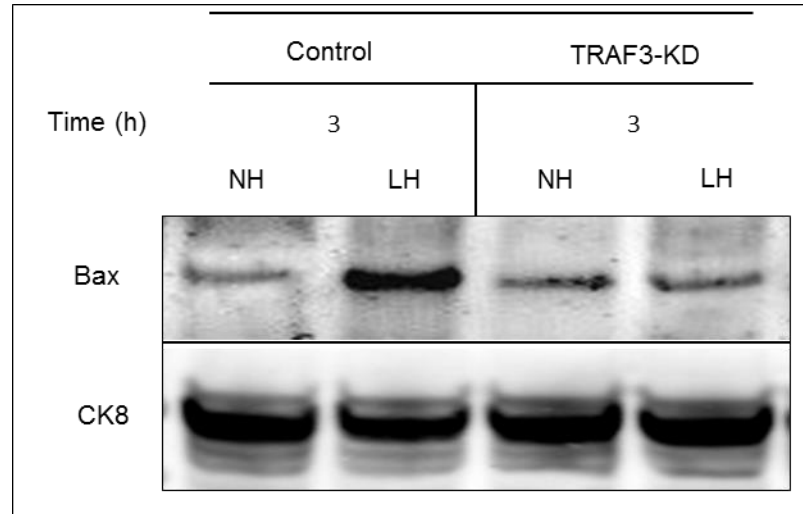


Figure 4. 26 Effect of shRNA-mediated TRAF3 knockdown on Bax induction during CD40-mediated apoptosis

3×10^6 HCT116 and TRAF3-KD and cells were co-cultured with 3×10^6 MMC treated mCD40L and Controls for 3 hours in DR/5%FCS/1%L-G and 10cm^2 culture dishes prior to lysis with 2X SDS-lysis buffer. Whole lysates, following normalisation on the basis of CK8 expression (see chapter 3) were under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membrane. The membrane was probed overnight with an anti-Bax antibody (1:500 dilution) and then with an anti-CK8 antibody (1:2000 dilution). The membrane was incubated for one hour with goat anti-mouse IgG IRDye 680 (1:10000 dilution) for the detection of CK8 (loading control) and Bax. Antibody binding was visualised at 700nm using an OdysseyTM Infra-red Imaging system.

Key: NH: 3T3Neo co-cultured with HCT116

LH: 3T3CD40L cocultured with HCT116

4.8 Role of JNK and p38 MAPK in CD40-mediated apoptosis in CRC cells

In light of the finding above that JNK and p38 are activated in response to CD40 ligation by mCD40L and that TRAF3 regulates apoptosis and the expression of both p38 and particularly JNK, it was determined whether these phosphoproteins have a direct role on CD40-mediated apoptosis. Inhibition of both JNK by JNK inhibitor (SP600125) and p38 by p38 MAPK inhibitor (SB202190) led to a significant reduction ($P < 0.001$) of apoptosis in HCT116 and SW480-CD40 as determined by CytoTox-Glo death detection assays as shown in Figures 4.27 and 4.28. In addition to this, JNK inhibition significantly reduced ($p < 0.001$) caspase-3/7 activity in both HCT116 and SW480-CD40 (Figures 4.29). Also the p38 inhibitor reduced caspase 3/7 activity during CD40-mCD40L ligation as determined by caspase 3/7 activity assay in both cell lines HCT116 and SW480-CD40 (Figure 4.30). The results showed a significant ($p < 0.001$) reduction of caspase 3/7 activity of SB202190 treated cells compared with non-treated during CD40 activation.

4.8.1 Effects of JNK and p38 inhibitors on JNK and p38 expression in CRC cells

To find out the events that occur when the inhibition of JNK and p38 MAPK proteins in the CRC cells after mCD40L ligation by using JNK and p38 inhibitor (SP600125 and SB202190 respectively), immunoblotting experiment was used with specific human antibodies to detect JNK and p38 expression. Results demonstrated that JNK inhibition by SP600125 reduced p-JNK expression, but did not stop p38 MAPK expression in CRC cells (HCT116 and SW480-CD40) within 3 and 6 hours post CD40 ligation as shown in Figures 4.31 and 4.32. By contrast, interestingly, inhibition of p38 by SB202190 clearly blocked not only p38 activation but also expression of p-JNK within 3 and 6 hours post CD40L ligation in both cell line compared with positive control as shown in Figures 4.31 and 4.32. The significance of these findings remains unknown, however this result suggests that p38 may function upstream of JNK.

4.8.2 Role of JNK and p38 MAPK in the induction of TRAIL

A possible role for JNK and/or p38 in TRAIL expression was also investigated using their respective inhibitors. Immunoblotting showed that CD40-mediated induction of TRAIL proteins in HCT116 and SW480-CD40 cells was completely inhibited by pharmacological inhibition of both JNK (SP600125) and p38 (SB202190). These findings imply that the expression of TRAIL is blocked by both inhibitors within 3 and 6 hours post CD40 ligation (Figure 4.33), and thus TRAIL is most likely to be transcriptionally regulated by the action of JNK and p38. These results suggest that SAPKs play a key role in regulation of many downstream pro-apoptotic events in CRC CD40 mediated apoptosis.

4.8.3 Role of JNK and p38 MAPK in the induction of mitochondrial pathway pro-apoptotic proteins Bak and Bax

Specific pharmacological inhibitors of JNK (SP600125) and p38 MAPK (SB202190) were used to inhibit apoptosis, and it was determined whether this would also prevent the expression of Bak and Bax in CRC cells treated with mCD40L. Both inhibitors JNK and p38 inhibitors (SB600125 and SP202190) completely reduced Bak protein expression at 3 and 6 hours in CRC cells as shown in Figure 4.34. Additionally, inhibition of JNK and p38 MAPK by SP600125 and SB202190 respectively, also significantly reduced Bax expression in HCT116 after CD40 ligation (Figure 4.35a). During inhibition of JNK and P38, only moderate inhibition of Bax protein was detected by immunoblotting in SW480-CD40 compared with HCT116 as shown in Figure 4.35b. Collectively however, results indicated that p38 and JNK play functional roles in regulation of Bak and Bax and this is in line with previous results showing that they also directly regulate apoptosis in this context.

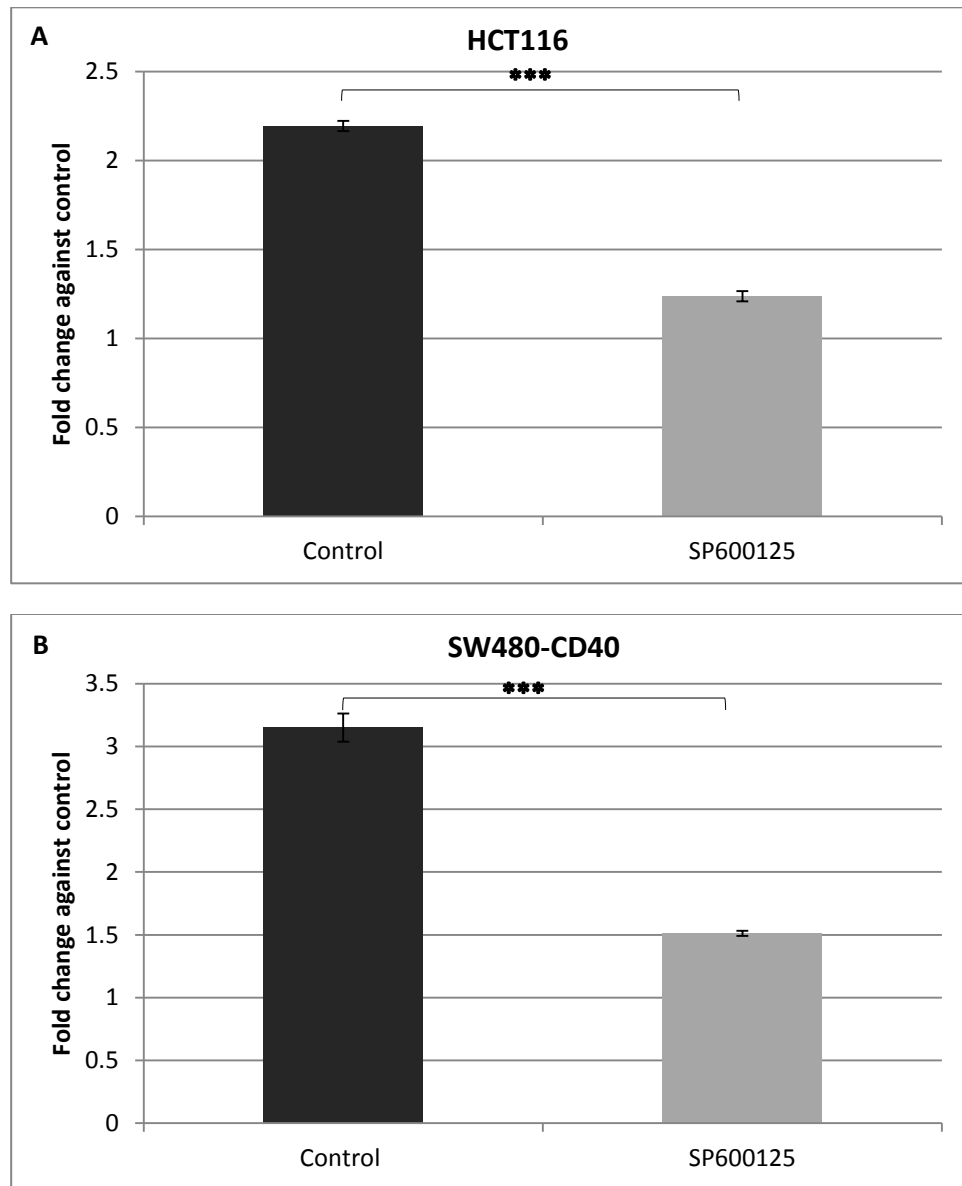


Figure 4. 27 The effect of pharmacological inhibition of JNK on CD40-mediated apoptosis

1×10^4 cells/well CRC cells (HCT116 and SW480-CD40) were co-cultured with 1×10^4 cells/well MMC treated (3T3CD40L and 3T3Neo) fibroblasts in 96 well plates in DR medium supplemented with 5% FCS and L-glutamine \pm 5 μ M JNK inhibitor (SP600125). After 24 hour at 37°C/ 5%CO₂, apoptosis was measured using the CytoTox-Glo assay. After incubation, 50 μ l of substrate was added to each well, and luminescence was measured. Background corrected RLU readings deduced by pair-wise subtraction of mCD40L and Control values from respective co-cultures. Panels A and B show fold change against control in HCT116 and SW480-CD40 cells, respectively. Bars show mean \pm S.D for 4-5 technical replicates ($p < 0.001$) expressed as fold activity in mCD40L-treated cells versus negative control. Results were calculated as described in Chapter 2 (Materials and Methods).

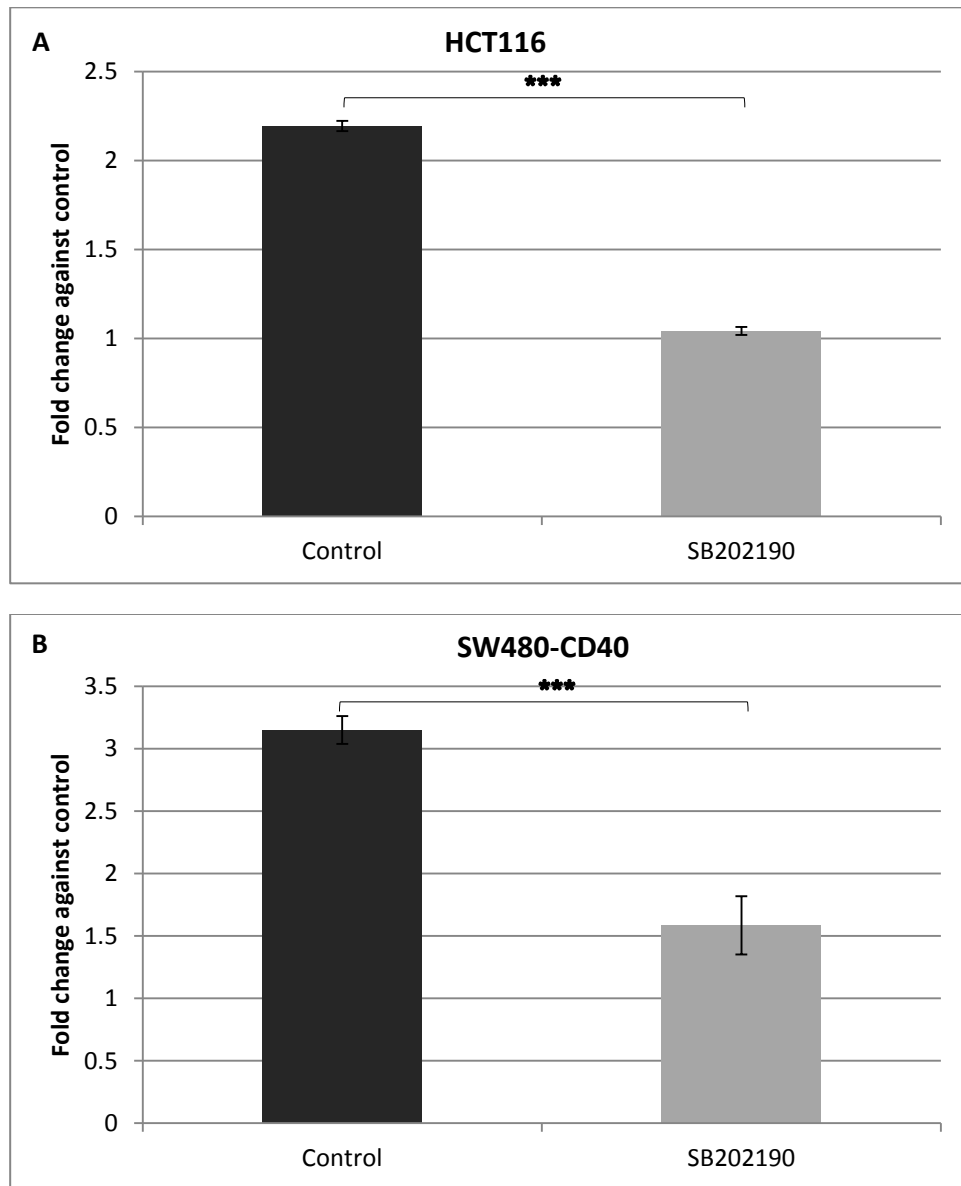


Figure 4. 28 The effect of pharmacological inhibition of p38 on CD40-mediated apoptosis

1×10^4 cells/well CRC cells (HCT116 and SW480-CD40) were co-cultured with 1×10^4 cells/well MMC treated (3T3CD40L and 3T3Neo) fibroblasts in 96 well plates in DR medium supplemented with 5% FCS and L-glutamine \pm 5 μ M p38 inhibitor (SB202190). After 24 hour at 37°C/ 5%CO₂, apoptosis was measured using the CytoTox-Glo assay. After incubation, 50 μ l of substrate was added to each well, and luminescence was measured. Background corrected RLU readings deduced by pair-wise subtraction of mCD40L and Control values from respective co-cultures. Panels A and B show fold change against control in HCT116 and SW480-CD40 cells, respectively. Bars show mean \pm S.D for 4-5 technical replicates ($p < 0.001$) expressed as fold activity in mCD40L-treated cells versus negative control. Results were calculated as described in Chapter 2 (Materials and Methods).

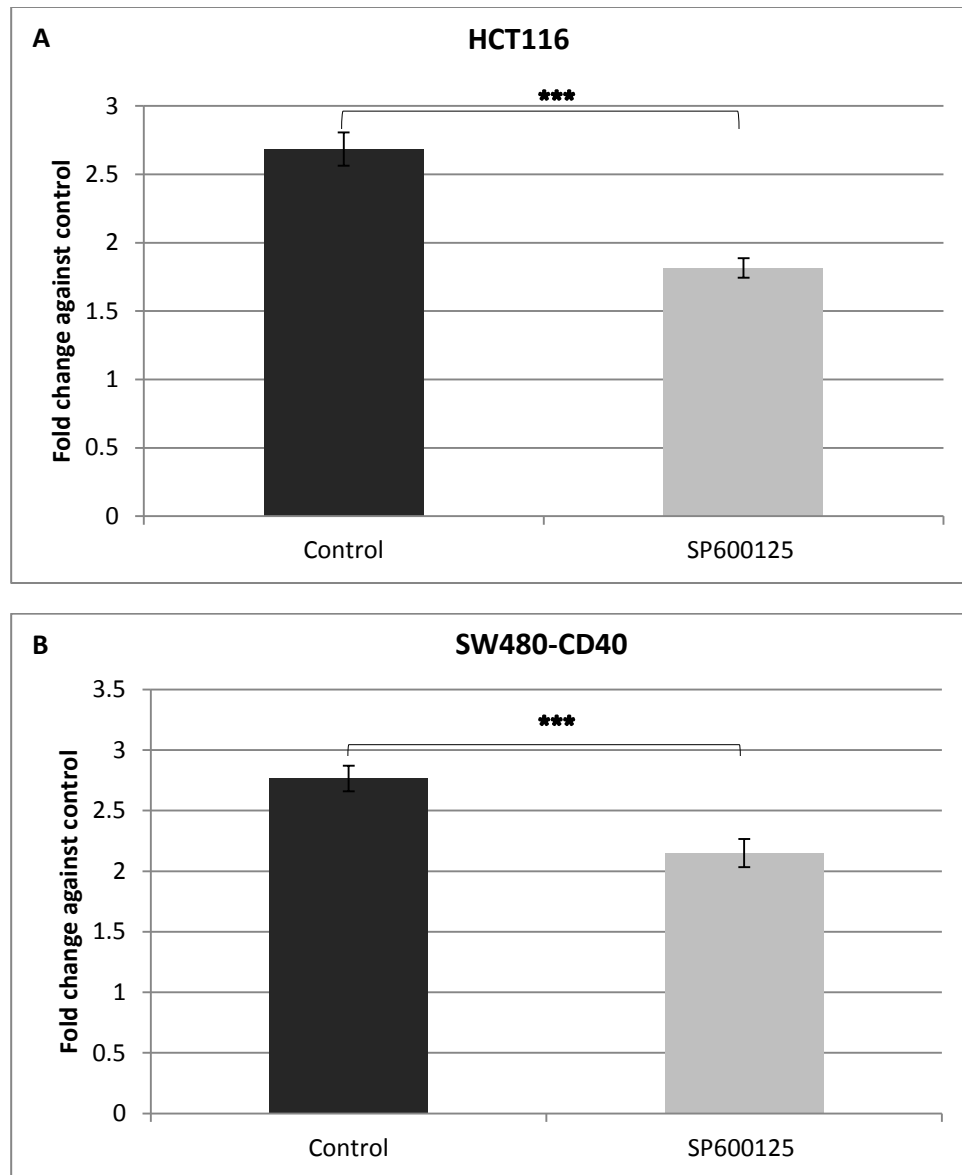


Figure 4. 29 The effect of pharmacological inhibition of JNK on CD40-induced caspase-3/7 activation

1×10^4 cells/well CRC cells (HCT116 and SW480-CD40) were co-cultured with 1×10^4 cells/well MMC treated 3T3CD40L and 3T3Neo fibroblasts in 96 well plates in DR medium supplemented with 5% FCS and L-glutamine \pm 5 μ M JNK inhibitor (SP600125). After 48 hour at 37°C/5%CO₂, 50 μ l of SensoLyte 3/7 substrate was added, fluorescence measured (as described in the Materials and methods) and background-corrected relative fluorescence unit (RFU) readings deduced by pair-wise subtraction of mCD40L and Control cell from the respective co-cultures. Bars show mean fold change of caspase 3/7 activity \pm SD of 4-5 technical replicates and results are representative of three experiments. Stats: ** P<0.01, paired student t-test, HCT116 compared with control cells.

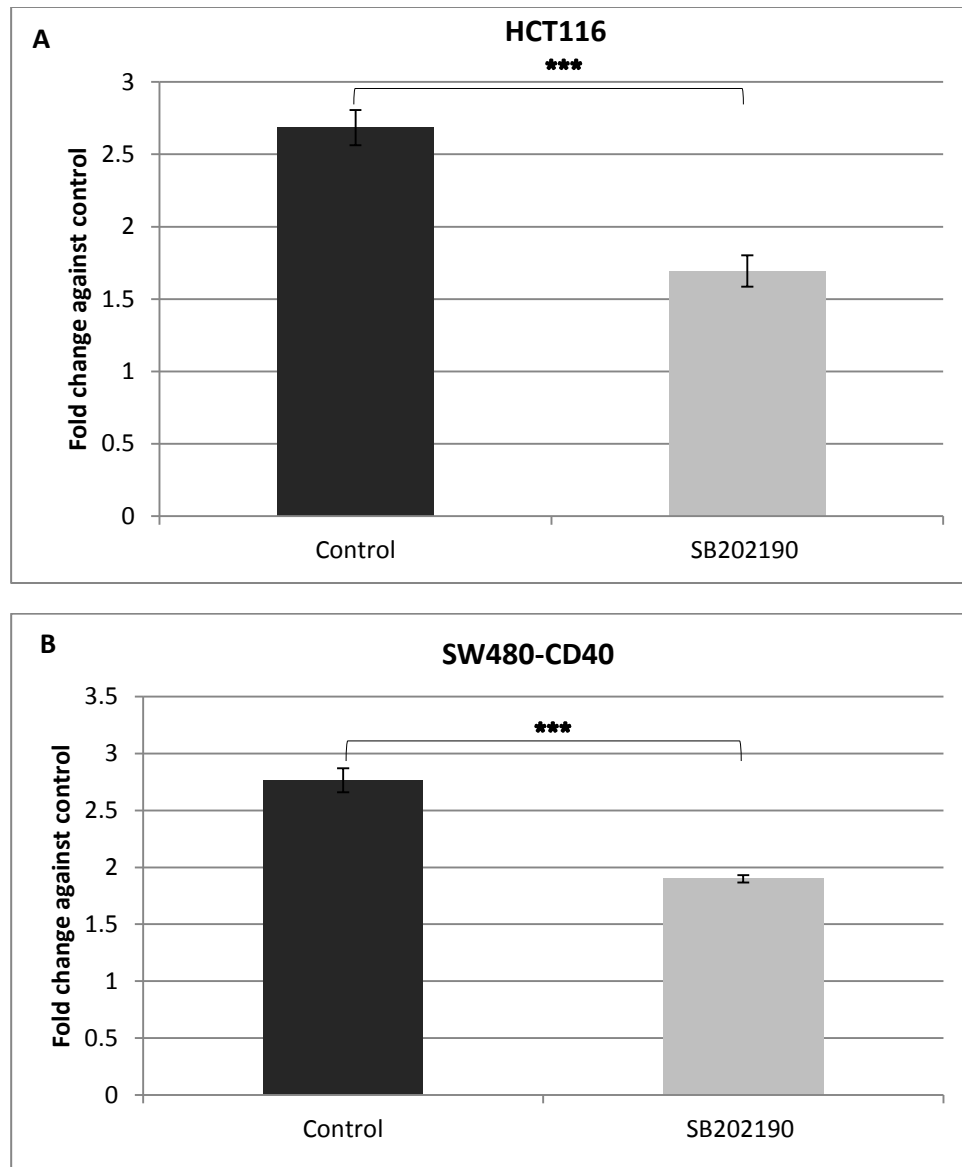


Figure 4. 30The effect of pharmacological inhibition of p38 MAPK on CD40-induced caspase-3/7 activation

1x10⁴ cells/well CRC cells (HCT116 and SW480-CD40) were co-cultured with 1x10⁴ cells/well MMC treated 3T3CD40L and 3T3Neo fibroblasts in 96 well plates in DR medium supplemented with 5% FCS and L-glutamine ± 5μM p38 inhibitor (SB202190). After 48 hour at 37°C/5%CO₂, 50μl of SensoLyte 3/7 substrate was added, fluorescence measured (as described in the Materials and methods) and background-corrected relative fluorescence unit (RFU) readings deduced by pair-wise subtraction of mCD40L and Control cell from the respective co-cultures. Bars show mean fold change of caspase 3/7 activity ± SD of 4-5 replicates and results are representative of three experiments. Stats: ** P<0.01, paired student t-test, HCT116 compared with control cells.

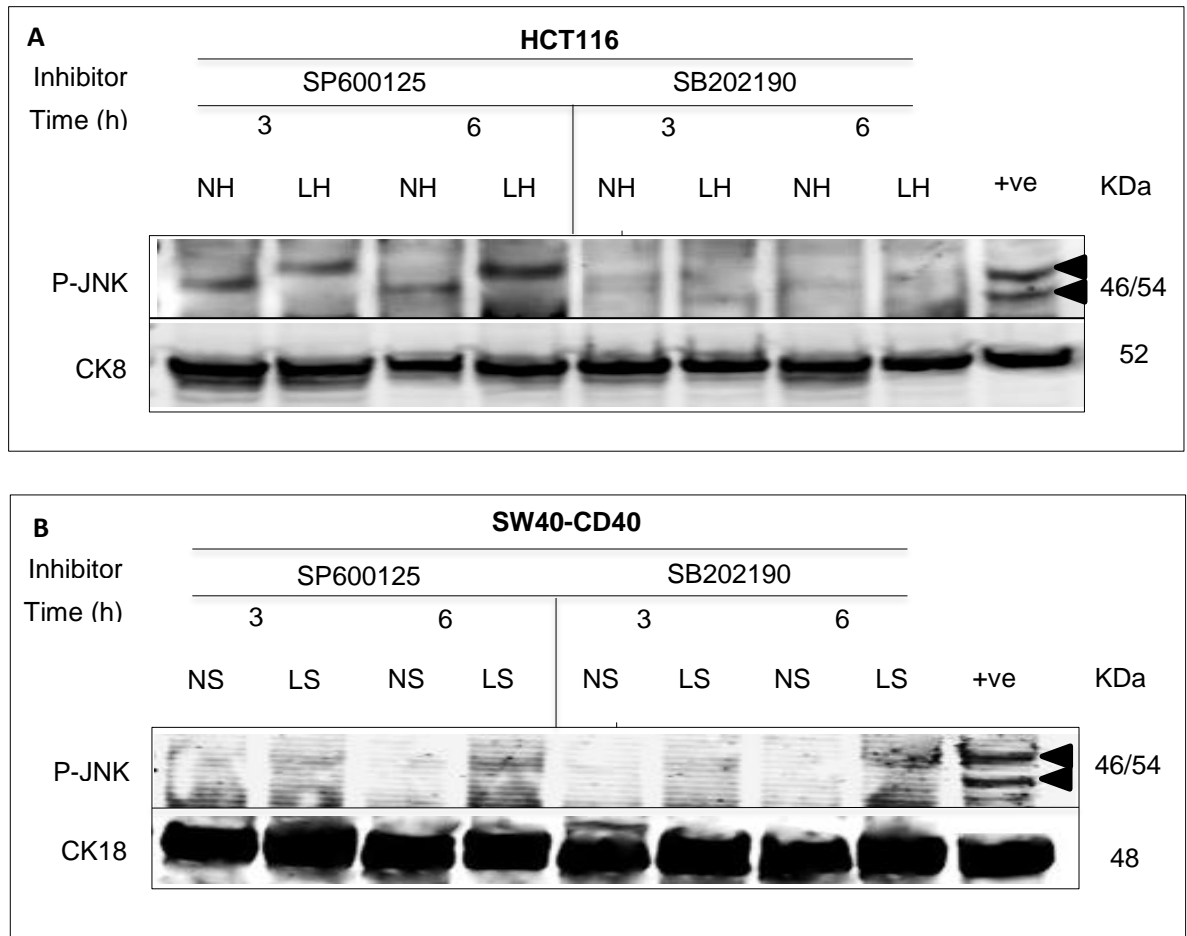


Figure 4. 31 Effect of pharmacological inhibitors of JNK and p38 on JNK phosphorylation following CD40 ligation

HCT116 (H) and SW480-CD40 (S) cells were seeded at 3×10^6 cells/dish and co-cultured with 3×10^6 MMC treated fibroblasts 3T3-Neo (N) or 3T3-CD40L (L). Co-cultures were incubated for the indicated time periods (3, 6 hours) in DR 5% supplemented with 5% of FCS and 1% L-glutamine \pm 5 μ M SP600125 (JNK inhibitor) and 5 μ M SB202190 (p38 inhibitor) in 10cm² culture dishes. After incubation, cells were lysed using 2x SDS-lysis buffer and protein concentration was determined. Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were separated under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membrane. The membrane was probed overnight at 4°C with primary monoclonal antibody (p-JNK) in TBS/Tween 0.1% (1:250 dilution) and then with an anti-CK8 antibody for HCT116 and CK18 for SW480CD40 in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one hour with secondary antibody goat anti-mouse IgG IRDye 680 (1:10000 dilution) for p-JNK detection and with goat anti-mouse IgG Alexa 680 (1:10,000 dilution) for the detection of CK18. A shows HCT116, B shows SW480-CD40 co-culture experiments. Antibody binding was visualised at 700nm using an OdysseyTM Infra-red Imaging system (CK8 and CK18 were used as loading controls in A and B, respectively).

Key: NH: 3T3Neo co-cultured with HCT116 LH: 3T3CD40L cocultured with HCT116
 NS: 3T3Neo co-cultured with SW480-CD40 LS: 3T3CD40L cocultured with SW480-CD40
 +ve: 3T3CD40L cocultured with CRC cells (no inhibitor)

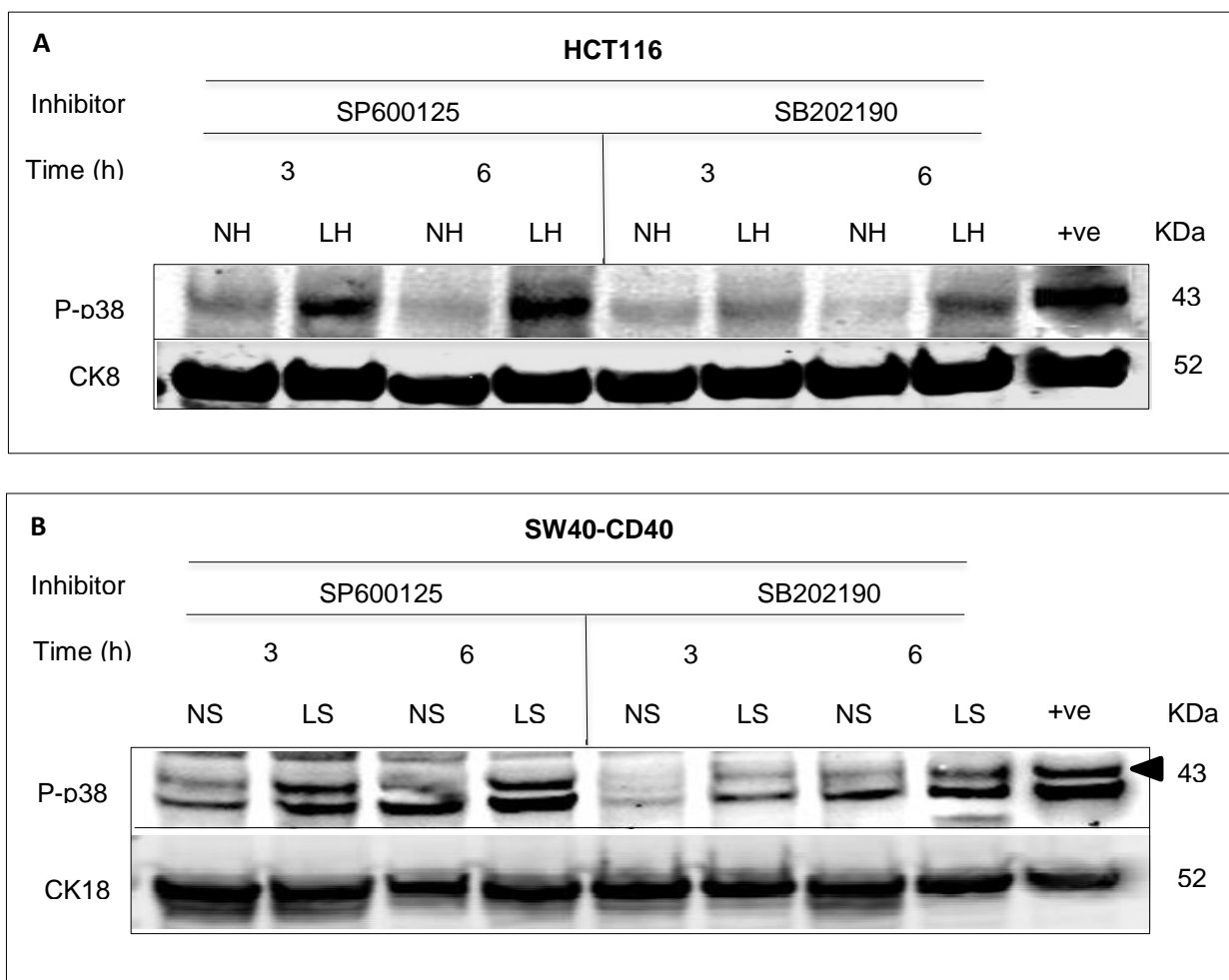


Figure 4. 32 Effect of pharmacological inhibitors of JNK and p38 on p38 phosphorylation following CD40 ligation

HCT116 (H) and SW480-CD40 (S) cells were seeded at 3×10^6 cells/dish and co-cultured with 3×10^6 MMC treated fibroblasts 3T3-Neo (N) or 3T3-CD40L (L). Co-cultures were incubated for the indicated time periods (3, 6 hours) in DR 5% supplemented with 5% of FCS and 1% L-glutamine \pm 5 μ M SP600125 (JNK inhibitor) and 5 μ M SB202190 (p38 inhibitor) in 10cm² culture dishes. After incubation, cells were lysed using 2x SDS-lysis buffer and protein concentration was determined. Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were separated under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membrane. The membrane was probed overnight at 4°C with primary monoclonal antibody (p-p38) in TBS/Tween 0.1% (1:250 dilution) and then with an anti-CK8 antibody for HCT116 and CK18 for SW480CD40 in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one hour with secondary antibody goat anti-rabbit IgG IRDye 800 (1:10000 dilution) for p-p38 detection and with goat anti-mouse IgG Alexa 680 (1:10,000 dilution) for the detection of CK18. A shows HCT116, B shows SW480-CD40 co-culture experiments. Antibody binding was visualised at 700nm and 800nm using an OdysseyTM Infra-red Imaging system (CK8 and CK18 were used as loading controls in A and B, respectively).

Key: NH: 3T3Neo co-cultured with HCT116 LH: 3T3CD40L cocultured with HCT116
 NS: 3T3Neo co-cultured with SW480-CD40 LS: 3T3CD40L cocultured with SW480-CD40
 +ve: 3T3CD40L cocultured with CRC cells (no inhibitor)

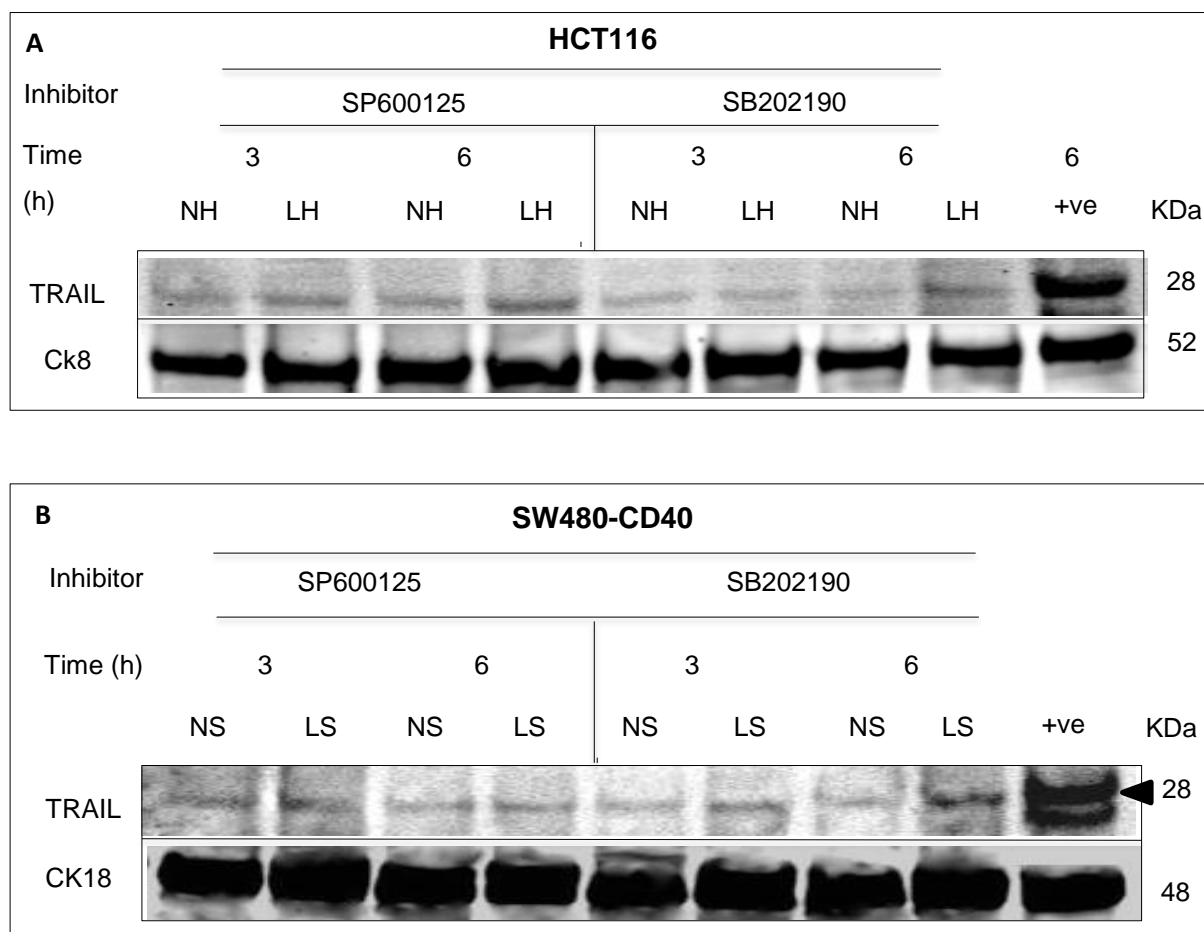


Figure 4. 33 Effect of pharmacological inhibitors of JNK and p38 on TRAIL expression during CD40-mediated apoptosis

HCT116 (H) and SW480-CD40 (S) cells were seeded at 3×10^6 cells/dish and co-cultured with 3×10^6 MMC treated fibroblasts 3T3-Neo (N) or 3T3-CD40L (L). Co-cultures were incubated for the indicated time periods (3, 6 hours) in DR 5% supplemented with 5% of FCS and 1% L-glutamine \pm 5 μ M SP600125 (JNK inhibitor) and 5 μ M SB202190 (p38 inhibitor) in 10cm² culture dishes. After incubation, cells were lysed using 2x SDS-lysis buffer and protein concentration was determined. Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were separated under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membrane. The membrane was probed overnight at 4°C with primary polyclonal antibody (anti-TRAIL) in TBS/Tween 0.1% (1:500 dilution) and then with an anti-CK8 antibody for HCT116 and CK18 for SW480-CD40 in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one hour with secondary antibody goat anti-rabbit IgG IRDye 800 (1:10000 dilution) for TRAIL detection and with goat anti-mouse IgG Alexa 680 (1:10,000 dilution) for the detection of CK8 and CK18. A shows HCT116, B shows SW480-CD40 co-culture experiments. Antibody binding was visualised at 700nm and 800nm using an Odyssey™ Infra-red Imaging system (CK8 and CK18 were used as loading controls in A and B, respectively).

Key: NH: 3T3Neo co-cultured with HCT116 LH: 3T3CD40L cocultured with HCT116
 NS: 3T3Neo co-cultured with SW480-CD40 LS: 3T3CD40L cocultured with SW480-CD40
 +ve: 3T3CD40L cocultured with CRC cells (no inhibitor)

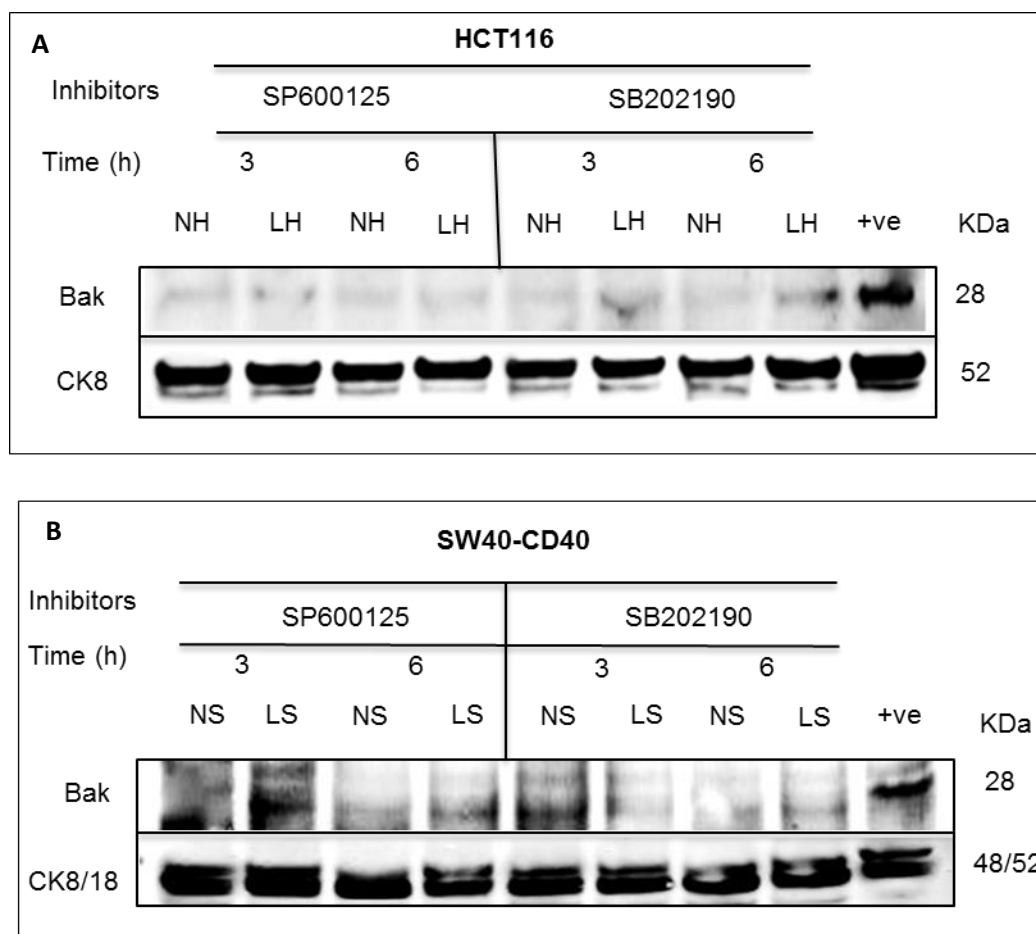


Figure 4. 34 Effect of pharmacological inhibitors of JNK and p38 on Bak induction during CD40-mediated apoptosis

HCT116 (H) and SW480-CD40 (S) cells were seeded at 3×10^6 cells/dish and co-cultured with 3×10^6 MMC treated fibroblasts 3T3-Neo (N) or 3T3-CD40L (L). Co-cultures were incubated for the indicated time periods (3, 6 hours) in DR 5% supplemented with 5% of FCS and 1% L-glutamine \pm 5 μ M SP600125 (JNK inhibitor) and 5 μ M SB202190 (p38 inhibitor) in 10cm² culture dishes. After incubation, cells were lysed using 2x SDS-lysis buffer and protein concentration was determined. Whole lysates, following normalisation on the basis of CK8 or CK18 expression (see chapter 3) were separated under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membrane. The membrane was probed overnight at 4°C with primary polyclonal antibody (anti-Bak) in TBS/Tween 0.1% (1:500 dilution) and then with an anti-CK8 antibody for HCT116 and CK8/18 for SW480-CD40 in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one hour with secondary antibody goat anti-rabbit IgG IRDye 800 (1:10000 dilution) for Bak detection and with goat anti-mouse IgG Alexa 680 (1:10,000 dilution) for the detection of CK8/18. A shows HCT116, B shows SW480-CD40 co-culture experiments. Antibody binding was visualised at 700nm and 800nm using an OdysseyTM Infra-red Imaging system (CK8 and CK8/18 were used as loading controls in A and B, respectively).

Key: NH: 3T3Neo co-cultured with HCT116 LH: 3T3CD40L cocultured with HCT116
 NS: 3T3Neo co-cultured with SW480-CD40 LS: 3T3CD40L cocultured with SW480-CD40
 +ve: 3T3CD40L cocultured with CRC cells (no inhibitor)

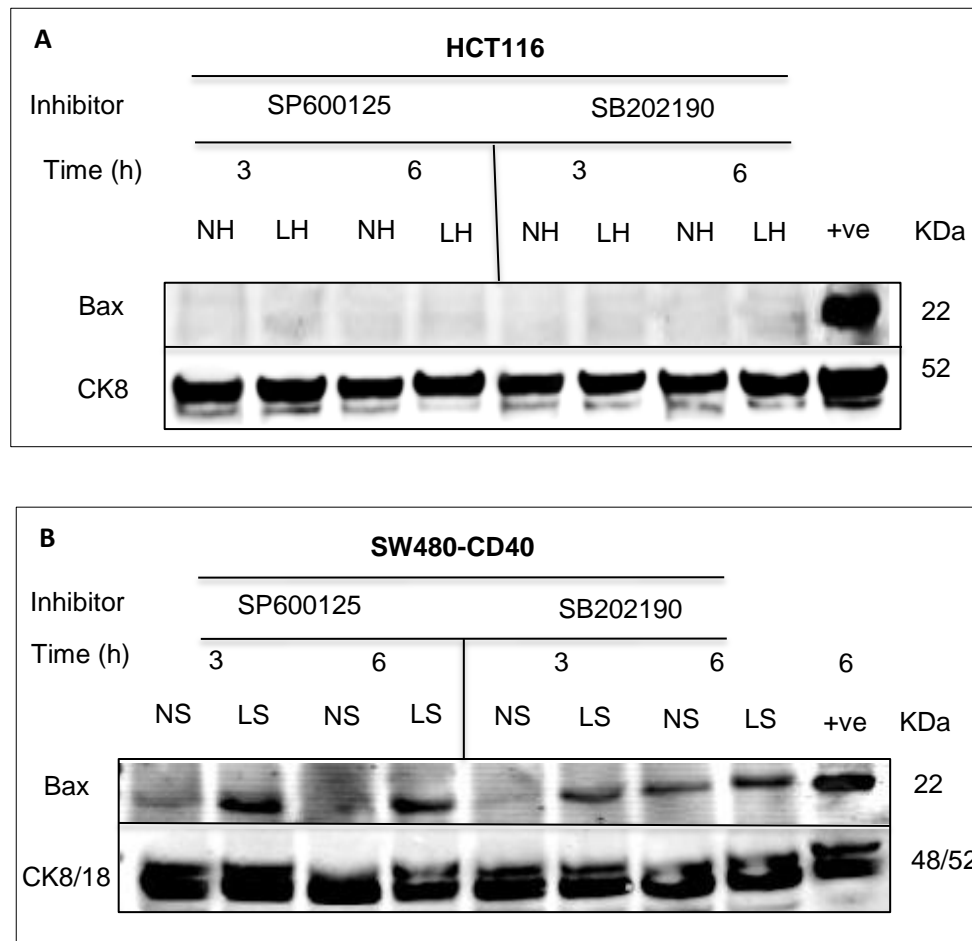


Figure 4. 35 Effect of pharmacological inhibitors of JNK and p38 on Bax induction during CD40-mediated apoptosis

HCT116 (H) and SW480-CD40 (S) cells were seeded at 3×10^6 cells/dish and co-cultured with 3×10^6 MMC treated fibroblasts 3T3-Neo (N) or 3T3-CD40L (L). Co-cultures were incubated for the indicated time periods (3, 6 hours) in DR 5% supplemented with 5% of FCS and 1% L-glutamine $\pm 5 \mu\text{M}$ SP600125 (JNK inhibitor) and $5 \mu\text{M}$ SB202190 (p38 inhibitor) in 10cm^2 culture dishes. After incubation, cells were lysed using 2x SDS-lysis buffer and protein concentration was determined. Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were separated under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membrane. The membrane was probed overnight at 4°C with primary monoclonal antibody (anti-Bax) in TBS/Tween 0.1% (1:500 dilution) and then with an anti-CK8 antibody for HCT116 and CK8/18 for SW480-CD40 in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one hour with secondary antibody goat anti-mouse IgG IRDye 680 (1:10000 dilution) for Bax detection and with goat anti-mouse IgG Alexa 680 (1:10000 dilution) for the detection of CK8/18. A shows HCT116, B shows SW480-CD40 co-culture experiments. Antibody binding was visualised at 700nm and 800nm using an OdysseyTM Infra-red Imaging system (CK8 and CK8/18 were used as loading controls in A and B, respectively).

Key: NH: 3T3Neo co-cultured with HCT116 LH: 3T3CD40L cocultured with HCT116
 NS: 3T3Neo co-cultured with SW480-CD40 LS: 3T3CD40L cocultured with SW480-CD40
 +ve: 3T3CD40L cocultured with CRC cells (no inhibitor)

4.9 The role of Bax in CD40-mediated apoptosis

4.9.1 The knockdown of Bax using shRNA-mediated RNAi

The functional role of the pro-apoptotic mitochondrial protein Bax in CD40-mediated apoptosis was investigated through shRNA-mediated RNAi. HCT116 cells were transduced with a retrovirus expressing Bax-specific shRNA (see Table 2.4). Following the combination of results from pre-titration experiments (Figure 4.36) and routine phase contrast microscopic examination during antibiotic treatment, it was found that a concentration of 0.25µg/ml of puromycin concentration effectively perished non-transduced control HCT116 cells after 72 hours of treatment. These derivatives will be referred to as Bax-KD.

Of note, an interesting observation during the preparation of the Bax knockdown cell line (HCT116) was that following initial selection in antibiotic (Puromycin) and establishment in culture, the functional knockout cells demonstrated increased growth rates in comparison to control cells. The increased growth rates appeared to be due to attenuation of spontaneous, culture stress-related apoptosis *in vitro* (Figure 4.37).

4.9.2 Bax knockdown attenuates CD40-mediated apoptosis

Having established the Bax-KD cells, experiments were performed to examine whether the induction of Bax was of functional importance in CD40 killing. The ability of the designed shRNAs to knockdown Bax protein expression in Bax-KD cells was confirmed by immunoblotting (Figure 4.38). Densitometric analysis was also used to confirm that, the expression of Bax was reduced after 6 hours CD40 ligation compared with control as illustrated in Table 4.2 and Figure 4.39.

Using CytoTox-Glo experiment assays, it was demonstrated that Bax knockdown significantly ($p < 0.01$) reduced the apoptosis in CRC cells (HCT116) compared with control (Figure 4.40). Moreover, the results also showed that Bax inactivation significantly ($p < 0.001$) abolished the activation of caspase 3/7 activity during CD40 mediated apoptosis (Figure 4.41); these findings suggested that Bax plays an important role during CD40-mediated apoptosis and thus may also be used as downstream marker of apoptosis mediated by JNK and TRAF3.

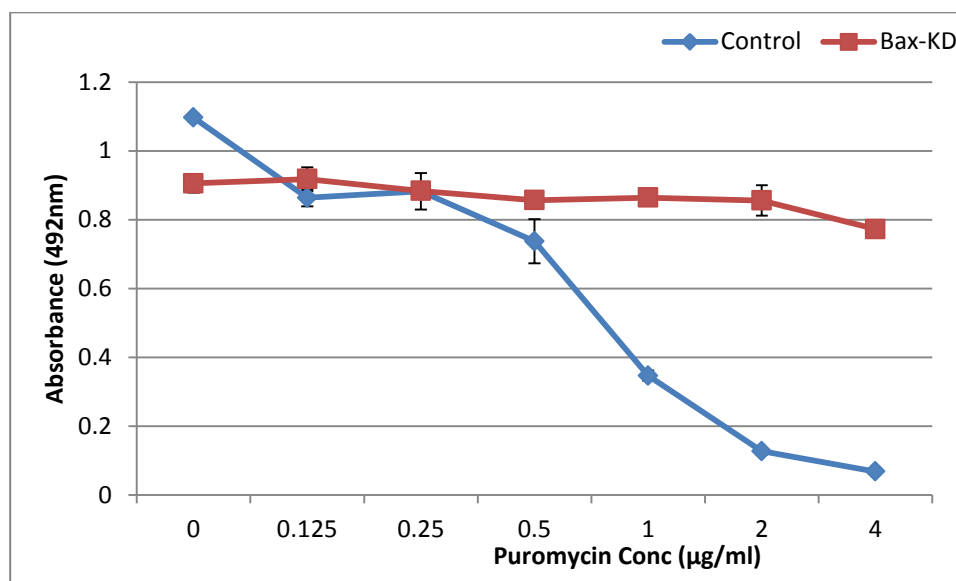


Figure 4. 36 HCT116 and Bax-KD cell proliferation following treatment with a range of puromycin concentrations

1×10^4 HCT116-Bax-KD cells/ well and HCT116 cells/well were seeded in a transparent TC treated 96 well plate and then treated with the indicated concentrations of puromycin antibiotic in DR/5%FCS/1%L-G for 24 hours. 20µl of CellTiter 96® AQueous One Solution was added to appropriate wells and plates were incubated at 37°C in 5% CO₂ for a total of four hours. To assess cell viability, total levels of formazan formation were measured using a FLUOstar OPTIMA (BMG Labtech) plate reader at a wavelength of 492nm. Bars correspond to mean absorbance values of 5-6 technical replicates \pm SEM and results are representative of three independent experiments.

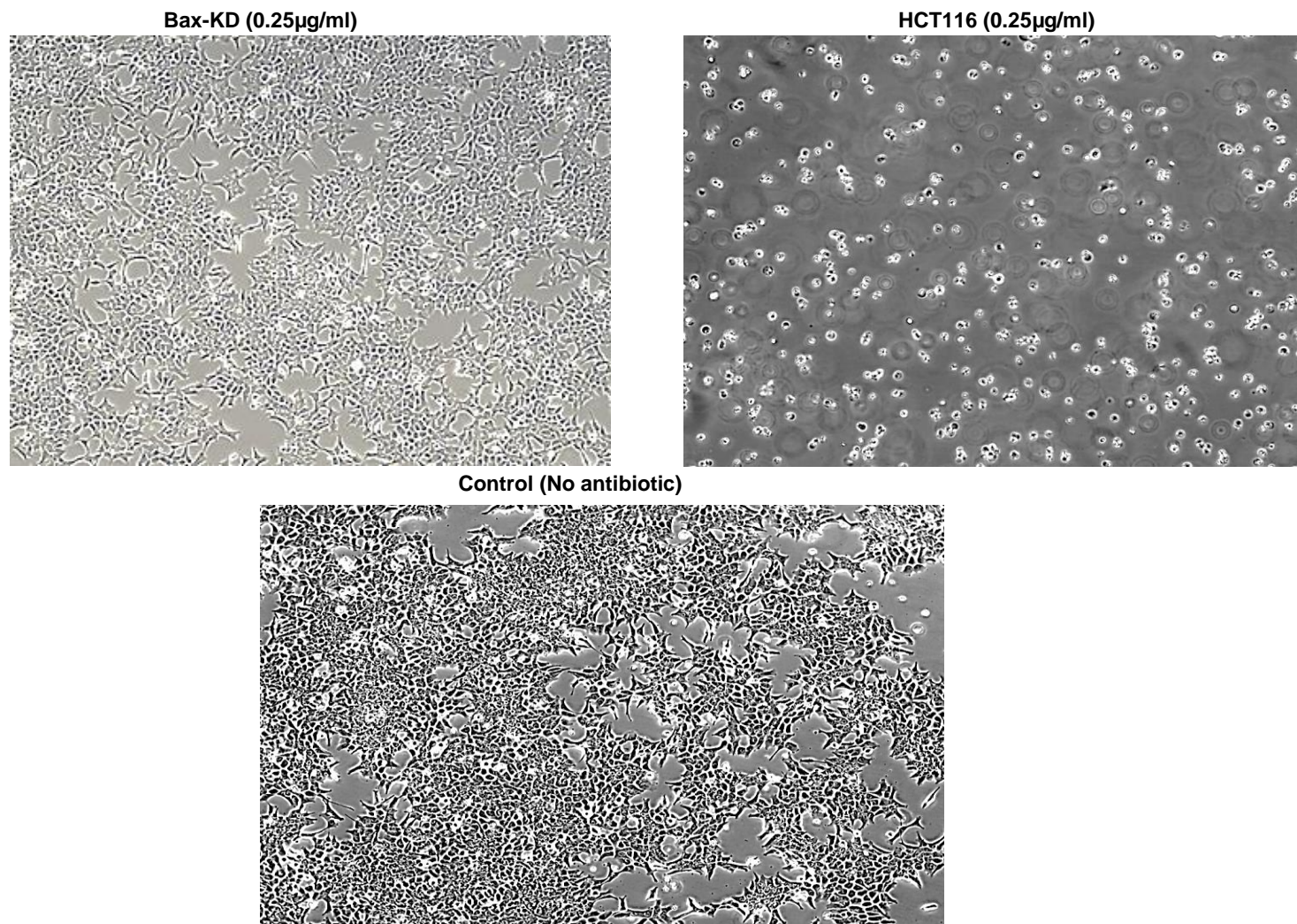


Figure 4. 37 Antibiotic (Puromycin) selection of transduced HCT116 cells with Bax shRNA-expressing retrovirus

Transduced and non-transduced HCT116 cells were cultured in DR/5%FCS/1%L-G supplemented with 0.25µg/ml puromycin compared with non-treated cells as control (the transduced cells shown here are cells in which an anti-Bax shRNA is expressed, denoted Bax-KD. HCT116 were also cultured with DR/5%FCS/1%L-G alone to demonstrate their normal growth pattern (Control). Note non-resistant HCT116 perish from the flask within 24 hours, however resistant cell populations continue to proliferate. Images are representative phase contrast micrographs (taken at 100x magnification).

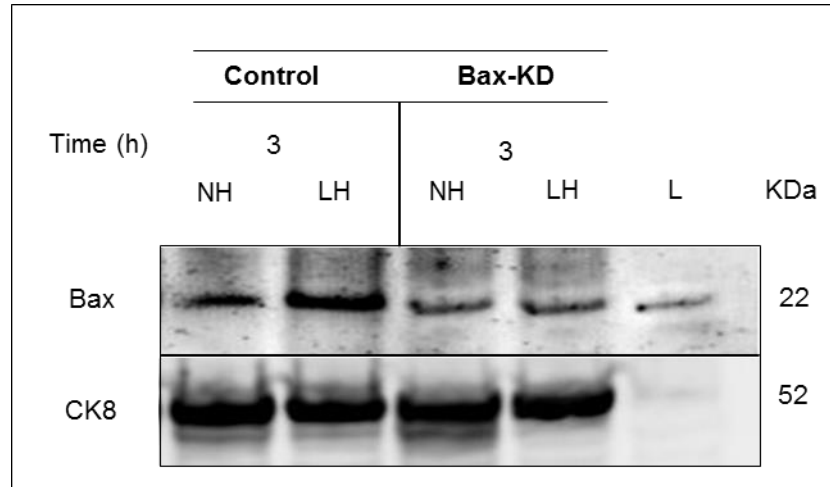


Figure 4. 38 The role of Bax knockdown in the regulation of Bax during CD40-mediated apoptosis.

3×10^6 control HCT116 cells (H) and HCT116 cells expressing Bax shRNA (Bax-KD) were co-cultured with 3×10^6 MMC treated mCD40L (L) and Controls (N) for 3 hours in DR / 5%FCS / 1%L-glutamine in 10cm^2 culture dishes in order to lyse with 2X SDS-lysis buffer. Whole lysates, following normalisation on the basis of CK8 expression (see chapter 3) were separated under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membrane. The membrane was probed overnight at 4°C with primary monoclonal antibody (anti-Bax) in TBS/Tween 0.1% (1:500 dilution) and then with an anti-CK8 antibody in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one hour with secondary antibody [goat anti-rabbit IgG IRDye 800nm (1:10000 dilution)] for Bax detection and with goat anti-mouse antibody IgG IRDye 680 (1:10000 dilution) for CK8 (used as loading control).

Key: NH: 3T3Neo co-cultured with HCT116
L: 3T3CD40L

LH: 3T3CD40L cocultured with HCT116

	HCT116		Bax-KD	
Time (h)	3		3	
Co-culturs	NH	LH	NH	LH
Background band intensity	1010.23	1968.45	856.14	958.14
CK8 BC band intensity	2245.45	2301.29	2014.58	2289.35
Fold change	1.94852		1.11914	

Table 4. 2 Densitometric analysis to calculate fold change in Bax protein expression

Densitometry was used to normalise data using Cytokeratin 8 as the loading Control. Readings represent band intensity values generated by the LiCor Odyssey analysis software. Data was normalised against densitometry values for Controls and then fold change was calculated by the following the equation (mCD40L/HCT116 ÷ Control/HCT116). BC - blank corrected. The table above is representative of the values obtained from the blot shown in Figure 4.38.

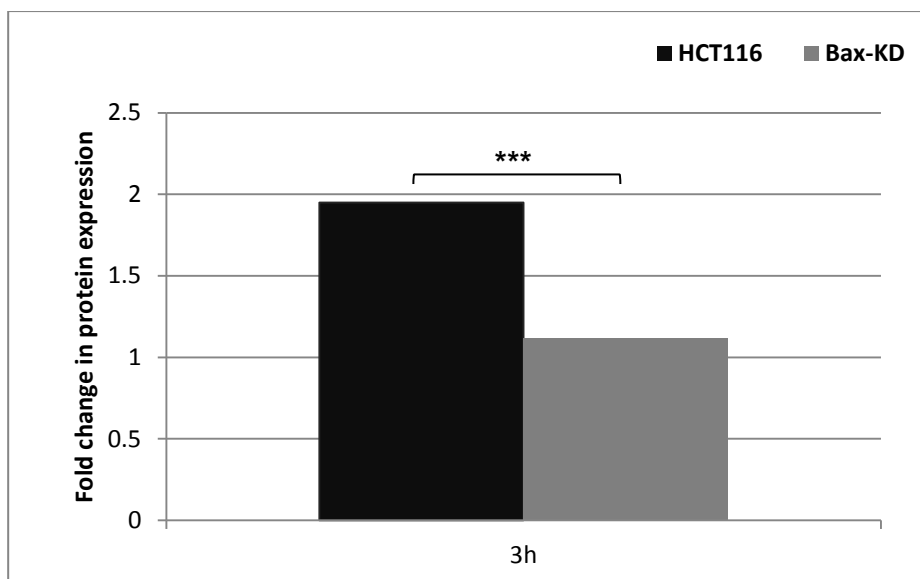


Figure 4. 39 Densitometric analysis to calculate fold change in Bax protein expression following shRNA-mediated knockdown

Densitometry was used to normalise data using Cytokeratin 8 as the loading Control. Readings represent band intensities values generated by the LiCor Odyssey analysis software. Data was normalised against densitometry values for Controls and then fold change was calculated by the following equation (mCD40L/HCT116 ÷ Control/HCT116). BC - blank corrected. The table above is representative of values shown in Figure 4.38.

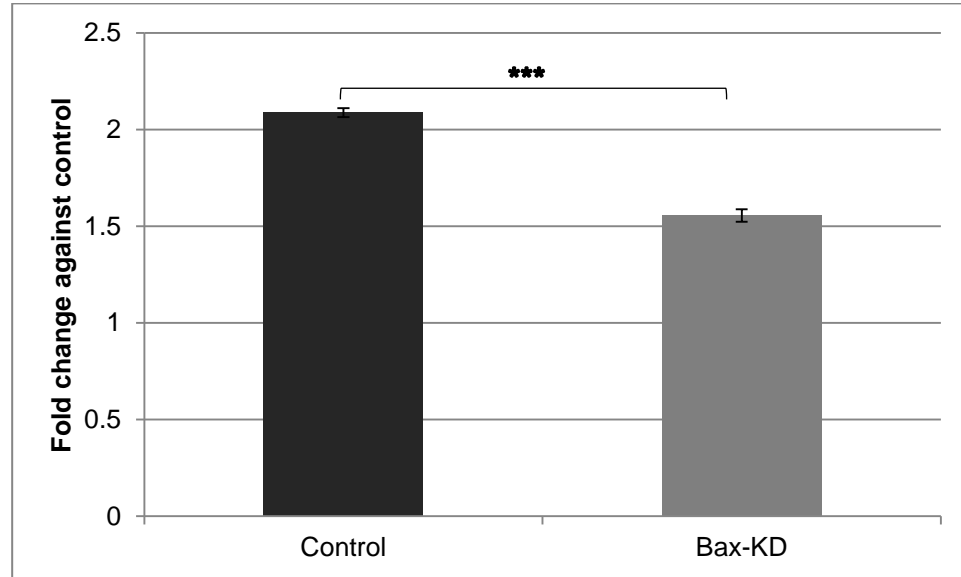


Figure 4. 40 Effect of shRNA-mediated Bax knockdown on CD40-induced apoptosis

1×10^4 colorectal cancer cells/well (HCT116 and Bax-KD cells) were cocultured with 1×10^4 MMC treated 3T3CD40L (mCD40L) and 3T3Neo (control) in white 96 well plates in DR medium supplemented with 5% FBS and 1% L-glutamine. After 24 hours of co-culture, the CytoTox-Glo assay was used to detect apoptosis in colorectal cancer cells (HCT116) as described in Chapter 2. Background corrected RLU readings deduced by subtraction of mCD40L from coculture [(3T3CD40L + HCT116) – 3T3CD40L] and control [(3T3Neo + HCT116) - 3T3Neo]. Bars show mean fold change \pm S.D of 4 to 6 replicates and results are representative of three experiments. Results show significant differences between HCT116/3T3CD40L versus Bax-KD/3T3CD40L indicated by ***p-value ≤ 0.001 for HCT116 cells and Bax-KD cells.

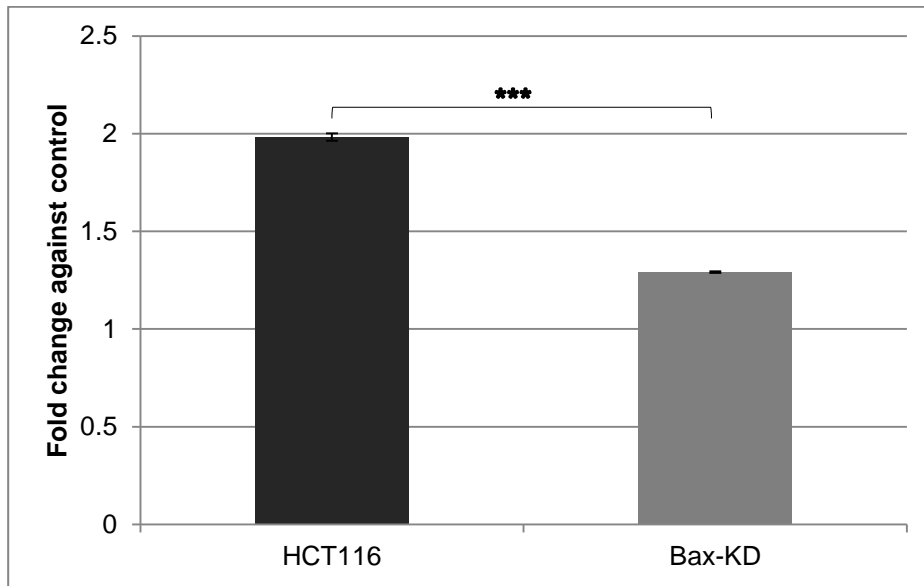


Figure 4. 41 Effect of Bax knockdown on caspase-3 and 7 activation during CD40-mediated apoptosis

1×10^4 HCT116 and Bax-KD cells were co-cultured with 1×10^4 MMC treated mCD40L or Controls in DR medium supplemented with 5% FCS and 1% L-glutamine in white 96-well plates. 50 μ l of SensoLyte 3/7 substrate was added after 48 hours, fluorescence measured (as described in the Materials and methods) and background-corrected relative fluorescence unit (RFU) readings deduced by pair-wise subtraction of mCD40L and Control values from respective co-cultures. Bars show mean fold change of caspase 3 and 7 activity \pm S.D of 4 to 6 replicates and results are representative of three experiments. Stats: *** $p < 0.001$, paired student t-test, HCT116 compared with Bax knockdown cells.

4.10 Summary:

- The main objective of this chapter was to identify the intracellular signalling mediators involved in CD40-induced signalling in CRC cells
- Western blotting results demonstrated induction of TRAF1 in CRC cells at 6, 12 and 24 hours following mCD40L-mediated CD40 ligation.
- Dramatic and rapid induction of TRAF3 expression was observed at 1.5 hours with further increases at 3, 6, 12 and maximal expression occurring 24 hours post CD40 ligation in CD40+ve CRC cell lines.
- Interestingly, mCD40L-CD40 interaction caused rapid induction of TRAF6 at 1.5 hours in CRC cell lines, however, the increase in TRAF6 expression was only temporary and TRAF6 expression was rapidly lost, thus indicating a transient effect.
- Western blot experiments demonstrated that pro-apoptotic MAP kinases MKK4 and MKK7 were activated in response to mCD40L-CD40 ligation in CRC cell lines.
- JNK activation (phosphorylation) occurred rapidly following CD40 ligation within 1.5 hours in CRC cell lines.
- Detection of activated p-p38 following CD40 ligation was also rapid, being detectable in both HCT116 and SW480-CD40 cells within 1.5 hours.
- The results have demonstrated that proapoptotic CD40 ligation in CRC cells induced both Bax and Bak expression.
- By using pharmacological inhibitors, the results also documented that JNK and p38 MAPK induced cell death involves the mitochondrial (intrinsic) pathway, requiring the pro-apoptotic Bax member of the Bcl-2 family to activate caspase-9.
- The results have provided direct evidence that CD40-mediated cell death is dependent on activation of caspase-9 but also caspase-10, but not caspase-8, thus implying cross talk with an extrinsic, caspase-10 associated pathway. Apoptosis of

CRC cells was completely blocked by the pan-caspase inhibitor z-VAD (which confirmed that CD40-mediated apoptosis in CRC cells is entirely caspase-dependent), as well as by specific inhibitors of caspases -9 and -10.

- It was found that Bid was cleaved to t-Bid and cleavage of Bid occurred in the early stage of apoptosis in CRC cell lines at 1.5 hours, thus confirming the existence of a cross talk with an extrinsic apoptotic pathway.
- Immunoblotting experiments showed that CD40 ligation mediated rapid induction of TRAIL expression within 1.5 hours of activation in CRC cell lines and it was sustained even at 3, 6, and 12 hours.
- Induction of TRAIL receptor expression was also observed as a consequence of CD40 ligation, with TRAIL-RII (DR5) detected through immunoblotting at 3 and 6 hours in both CRC cell lines.
- This is the first ever report that p38 and JNK are induced by CD40 which in turn activates TRAIL to induce a caspase-10-dependent pathway.
- The results have demonstrated that cell death is caspase-dependent and that CD40 triggers both intrinsic as well as extrinsic apoptotic cascades, as apoptosis involved
 - a) TRAIL/TRAIL-R induction and caspase-10 activation
 - b) Bak/Bax up-regulation, cytochrome c release and caspase-9 activation.
- The results indicated that in CRC cells mCD40L triggers rapid induction of apoptosis in less than 12 hours. Interestingly, CD40-induced, TRAIL-associated death appears to take place not by paracrine (juxtacrine) signalling but in an endogenous (autocrine) fashion (as indicated by well established, blocking antibodies).
- Stable, retrovirus mediated TRAF3 knockdown caused significant reduction in apoptosis as determined by CytoTox-Glo assays and reduced the activity of caspase -3/-7 post CD40 ligation in CRC cells.

- Western blot results documented that TRAF3 KD abrogates JNK and p38 MAPK phosphorylation and completely abrogate Bax expression.
- In short, the results have indicated that uniquely in CRC cells, CD40 ligation triggers rapid and dramatic cytotoxicity via a novel, cross-talk mechanism with the extrinsic apoptotic pathway.

CHAPTER 5: Reactive oxygen species and CD40-mediated apoptosis in CRC cells

5.1 Rationale for the study of ROS and antioxidant defence in CD40 signalling

As discussed in Chapter 1, ROS are natural secondary products of mitochondrial oxidative metabolism, however they act as messengers to homeostatically stabilise cell proliferation and survival (Ray et al., 2012, Terada, 2006). ROS are elevated when reacting to extracellular stress for example cytokines, bacterial invasion and xenobiotics as to alert the cell that it is necessary to adapt (Jiang et al., 2011). In situations when ROS are not adequately 'managed' by antioxidants, a cellular state forms that is called 'oxidative stress' which often results in macromolecular damage (Sharma et al., 2004). At the same time its chronic persistence is involved in malignant transformation because it harms the DNA and, as a result, mediating an elevated rate of oncogene and/or tumor suppressor mutations (Valko et al., 2004). The controlling significance of oxidative stress is clinically demonstrated by an upsurge in cancer risks by way of mutation in the cellular antioxidants GSH and Trx. Yet, paradoxically, a lot of more advanced cancers over-express antioxidants in order to guard themselves against oxidative stress taking place due their extraordinary great energy generation demands (Halliwell, 2007). It has thus been hypothesised that tumours use continuous oxidative stress to initialize cell signaling pathways that support malignant transformation.

Nonetheless, it is similarly well known that ROS initialization performs significantly in pro-apoptotic responses (Biswas et al., 2006, Circu and Aw, 2010) and first and foremost those that involve mitochondrial pathways. Thus, collectively, ROS activation embodies a 'double-edged sword' (Pan et al., 2009), supporting increased proliferation ability at early stages of malignant transformation yet increasing sensitivity to pro-apoptotic signals due to oxidative stress 'pushing' cells towards a 'pro-apoptotic threshold'. In other words, it has been suggested that tumour cells could be pushed over a pro-apoptotic 'ROS limit' when compared to the other normal cells (Raj et al., 2011, Wang and Choudhary, 2011). Even though there are numerous factors to bear in mind in cellular redox management, there exists overall agreement that: a) diminished/low levels of ROS are a benchmark for normal cell homeostasis, b) elevated ROS levels initiate stress-responsive cell signaling pathways such as NF- κ B and JNK, and lastly c) elevated ROS/oxidative stress can initiate cell apoptosis (D'Autreaux and Toledano, 2007, Terada, 2006).

Most of the members of the TNFR superfamily use ROS to control cell survival and apoptosis by initializing oxidative stress responsive MAPK signaling pathway (Shen and

Pervaiz, 2006). The generation of is most of the time as a result of receptor-TRAF interaction with NADPH oxidases (Li et al., 2005, Ha and Lee, 2004, Chandel et al., 2001), which perform a critical role in ROS production (Jiang et al., 2011). A limited amount of evidence has suggested that CD40 ligation produces ROS through the 5-lipoxygenase pathway (Ha et al., 2011) and a TRAF3-NADPH oxidase association has in the past been suggested (Ha and Lee, 2004). B-cells research reveals that low levels of CD40 cross-linking produces low levels of ROS and initiate NF- κ B, however on the other hand enhanced receptor cross-linking produces elevated oxidative stress, prompting the initiation of JNK (Ha et al., 2011).

Oxidative stress is associated with the positive regulation of apoptotic pathways as it directly activates responsive proteins (Circu and Aw, 2010). As discussed in the Introduction, oxidative stress results in activation of ASK1 by means of Thioredoxin release and auto phosphorylation (Liu and Min, 2002) and the initiation of MAPKs by means of their release from dual-specificity MAPK phosphatases (DS-MKPs) (Bermudez et al., 2010). Correspondingly, oxidative stress permits the initiation of JNK via GSTp discharge (Simic et al., 2009) and enhances AP-1 activity (Biswas et al., 2006), and there is evidence that reveals that pro-apoptotic members of the Bcl-2 family, like Bak/Bax are also altered by oxidative stress (Steckley et al., 2007, Tomiyama et al., 2006), as it is the same with enzymatic initiators of cell death, the caspases (Circu and Aw, 2010). In light of this evidence, it was hypothesised that ROS-associated signaling could possibly have a huge impact on CD40-mediated cell death in CRC cells.

Up to this day, there are no comprehensive exploration functions of CD40-mediated ROS stimulation in apoptosis in cells of an epithelial origin. Because of this reason, the co-culture system (Figure 3.1) together with apoptosis assays and immunoblotting methods were employed to examine in order, the likely stimulation of ROS by mCD40L and to understand the exact function of ROS production in the outcome of CD40 ligation in CRC cells.

5.2 Objectives

The specific aims of this chapter are:

- To optimise assays for the detection of ROS generation and investigate whether ROS are elevated as a result of CD40 ligation in CRC cells.
- To utilize immunoblotting and cell death assays as well as pharmacological inhibitors to examine whether NADPH oxidases could be the crucial mediator of ROS generation and CD40-mediated apoptosis.
- To define whether CD40 ligation modulates antioxidant pathways to influence the activation of ASK1 and downstream apoptotic signaling pathways.

5.3 The optimisation of ROS detection in CRC cells

As explained above, this study aimed to provide detailed and functional evidence that mCD40L-induced apoptosis is dependent on production of ROS. To investigate the possibility of ROS production in CRC cell death the ROS detection fluorescent marker 6-carboxy-2', 7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) was used.

The oxidation of 2', 7' dichlorofluorescein (H₂DCF) to 2'-7'-dichlorofluorescein (DCF) has been used rather widely for the quantitation of H₂O₂. The di-acetate form, H₂DCFDA and its acetomethyl ester H₂DCFDA-AM are taken up by cells where nonspecific cellular esterases act upon it to cleave off the lipophilic groups, causing in a charged compound believed to be trapped inside the cell. Oxidation of H₂DCF by ROS converts the molecule to 2', 7' dichlorodihydrofluorescein (DCF), which is highly fluorescent (Figure 5.1). The described wavelengths for the measurement of DCF fluorescence are 498 nm for excitation and 520 nm for emission. Initially, DCF was thought to be specific for hydrogen peroxide (H₂O₂), but current evidence has shown that other ROS such as nitrate and hypochlorous acid can oxidise H₂DCF (Hoffman et al., 2008). Most importantly is the fact that H₂O₂-dependent oxidation of H₂DCF requires ferrous iron (Rothe and Valet, 1990). In addition, as H₂DCF is no longer ionic it is not precluded from migrating out of the cell and accumulating in the media, where it is free to interact with oxidants.

In initial experiments the two CRC cell lines HCT116 and SW480-CD40 were used to determine the concentration of H₂DCFDA that would sensitively determine levels of intracellular ROS and discriminate between the different cell lines (as excessive amounts of reagent resulted in misleadingly high fluorescence levels in these cell lines) (Figure 5.2). By accounting for fluorescence background arising from differences in cell line dependent metabolism and division rate (which affects confluency) relative fluorescent values derived from treatment with H₂DCFDA were optimised based on natural 'auto-fluorescence' of the two cell lines HCT116 and SW480-CD40. During a long series of pre-titration experiments for the optimisation of conditions including a) H₂DCFDA concentration, b) incubation periods and c) ROS activators as positive controls (staurosporine), it was found that the concentration of 0.5µM H₂DCFDA sensitively and consistently discriminated between intracellular concentrations of ROS (Figure 5.3); these optimised methods for ROS detection were applied for all experiments.

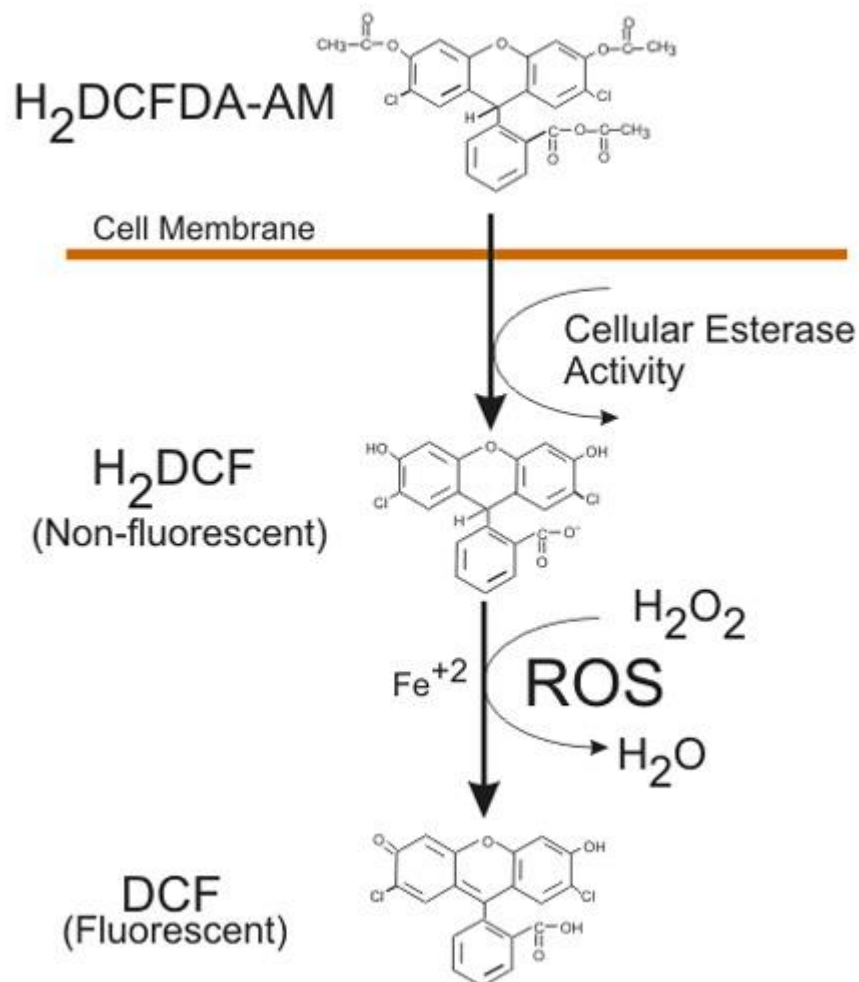


Figure 5. 1 The principle of ROS detection using H_2DCFDA

6-carboxy-2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) is a derivative of reduced fluorescein that has cell permeability. The reduced forms of fluorescein lack any fluorescence until acetyl groups are removed by intracellular esterase(s) and oxidation is occurring within the cells. When this occurs the charge of the molecules makes it much less likely to leave the cell and also emits detectable fluorescence. The levels of fluorescence intensity deduced are therefore an indication of the intracellular concentration of ROS. Adapted from (Held, 2010)

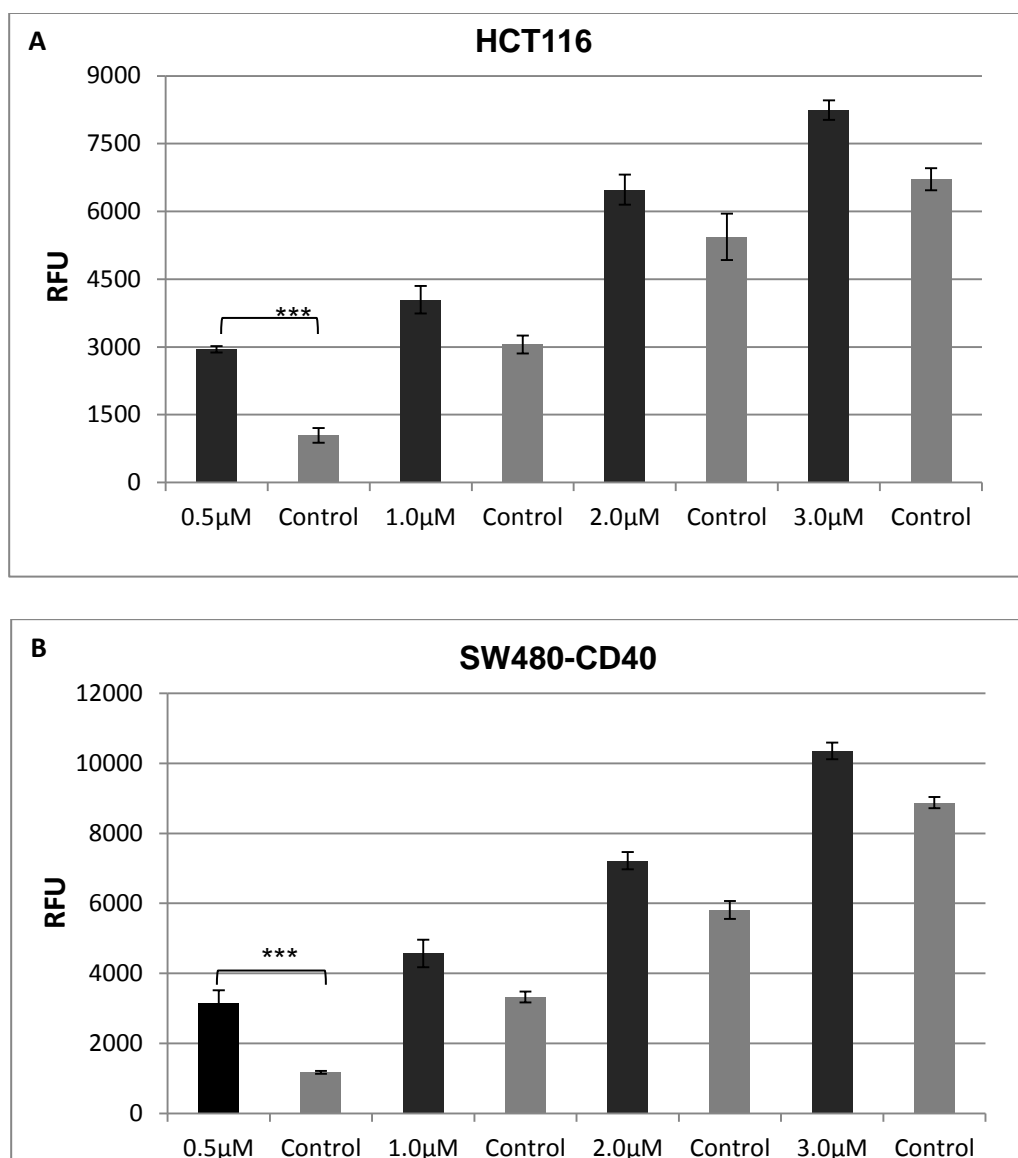


Figure 5. 2 Optimisation experiments for the measurement of ROS production using H₂DCFDA

1x10⁴ CRC cells/well (HCT116 and SW480-CD40) were plated for 24 hours in DR/5%FCS/1%L-G in white 96-well plates. The next day all wells were washed with PBS and the reagent at different concentrations was added and an incubation of 30 minutes at 37°C in 5%CO₂ was carried out. Fluorescence was measured by using a plate reader at Excitation 485nm/Emission 520nm in the presence of PBS. Bars represent mean RFU values for 6-8 technical replicates ± S.D and results are representative of three independent experiments.

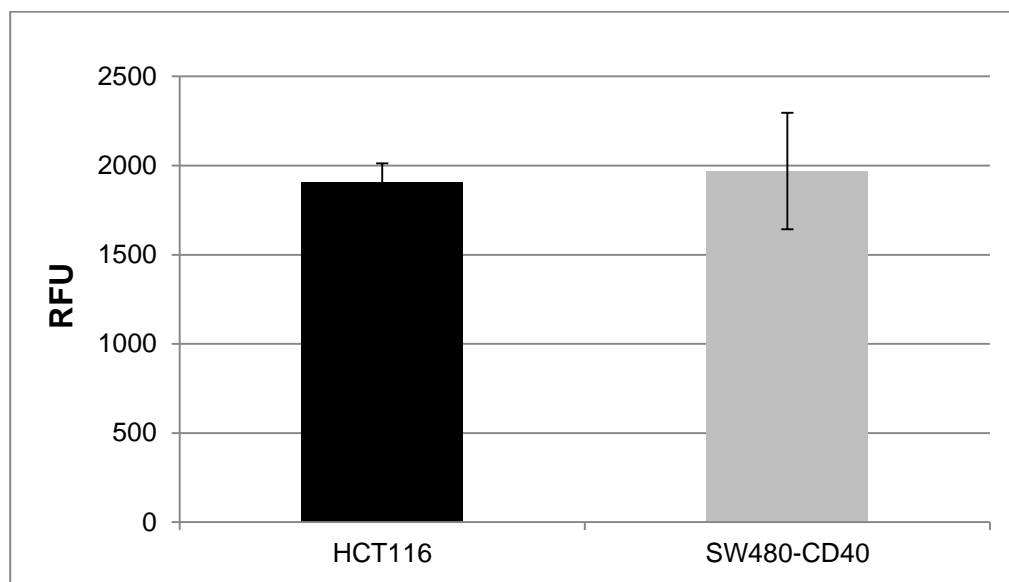


Figure 5. 3 Optimisation experiments for measurement of ROS activation in CRC cells (measurement of auto-fluorescence)

1×10^4 CRC cells/well (HCT116 and SW480-CD40) were plated for 24 hours in DR/5%FCS/1%L-G in white 96-well plates. The next day all lines were assessed for their relative autofluorescence at Excitation 485nm/Emission 520nm in the presence of PBS. Bars represent mean RFU values for 6-8 technical replicates \pm S.D and results are representative of three independent experiments.

5.4 The induction of ROS by mCD40L in CRC cells

This study further determined whether the optimised system for ROS detection could be used to demonstrate ROS induction as a consequence of mCD40L-CD40 engagement, as reported by others (Bhogal et al., 2012, Ha and Lee, 2004, Ha et al., 2011). Target cells (HCT116 / SW48CD40) were first screened for ROS elevation using the co-culture methods for CD40-mCD40L interaction (section 3.1.1) following fluorescence (RFU) 3T3 cell-associated background correction, as described previously for cell death assays (section 3.4.1.1). Preliminary experiments showed optimal detection of ROS at 2.5 hours post CD40 ligation, significantly greater ($p < 0.05$) degree of ROS production compared with the controls (Figure 5.4). ($p < 0.001$) However, the earlier time points of 1.5, 2 hour ligation were investigated.

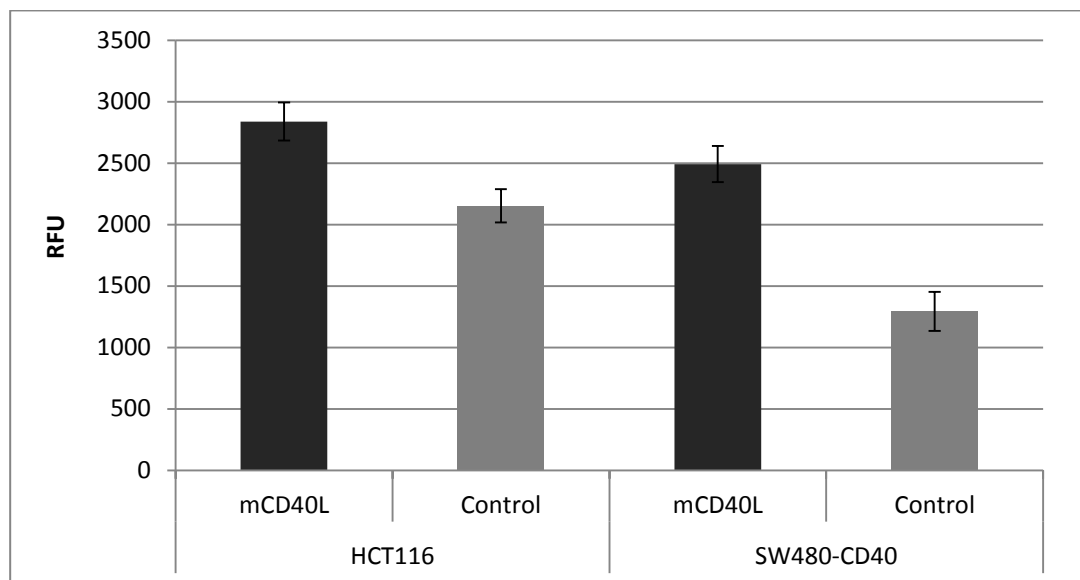


Figure 5. 4 mCD40L-mediated ROS induction in CRC cells

1×10^4 cells/well HCT116 or SW480-CD40 were co-cultured with 1×10^4 3T3-CD40L (mCD40L) or 3T3-Neo (Controls) in DR/5%FCS/1%L-G in white 96-well plates. Cells were first washed with PBS to remove any culture medium and were then treated with $0.5 \mu\text{M}$ of H_2DCFDA in pre warmed (37°C) PBS for 30 minutes 37°C in 5% CO_2 . Background-corrected RFU readings were deduced by pair-wise subtraction of mCD40L and Control cells from the respective co-culture. Bars represent mean RFU values for 5-6 technical replicates \pm SEM and results are representative of three independent experiments.

5.5 The role of ROS in CD40-mediated cell death

To determine whether ROS-mediated oxidative stress is essential to mCD40L-mediated apoptosis, the aim was to induce CD40 activation in the CRC cell lines HCT116 and SW480-CD40 by co-cultures as previously (Figure 3.1) in the presence of the antioxidant/ROS scavenger N-acetyl L-cysteine (NAC).

To ensure that the optimal concentration of NAC was used so that it did not interfere with cell viability, pre-titration experiments were carried out using the CellTiter 96® AQueous One Solution Cell Proliferation assay to determine an effective dose for both the effector (killer) cells (3T3-CD40L and 3T3-Neo controls) (Figure 5.5) and target (CRC) cells (HCT116 and SW480-CD40) (Figure 5.6). It was observed that doses of 2.5 and 5mM were well tolerated by CRC cells; however, higher concentrations of NAC appeared toxic to CRC cells even though they were well tolerated by the effector cells.

Based on these optimisation experiments, addition of 2.5mM and 5mM NAC during co-culture completely and significantly ($p < 0.001$) attenuated mCD40L-mediated apoptosis as determined by CytoTox-Glo assays (Figure 5.7).

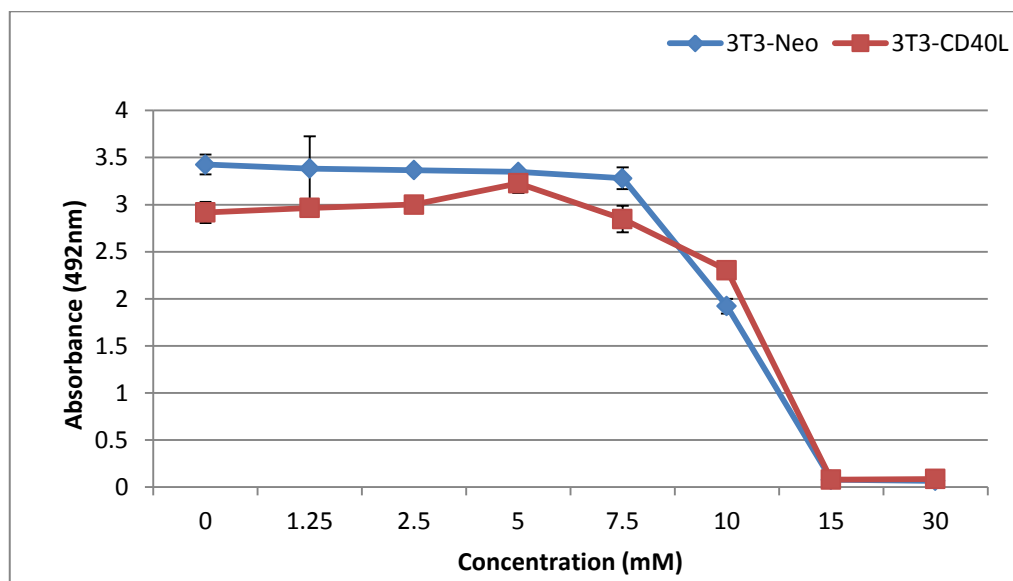


Figure 5. 5 Determination of effective dose of NAC on effector (killer) cells (3T3- CD40L / 3T3-Neo)

1×10^4 cells/well were seeded in 96 well plate in DR medium supplemented with 5% FBS and 1%LG. Cells were incubated overnight in 37°C / 5% CO_2 . Then the cells were treated with different concentrations of NAC. After 24hours incubation, 20 μl of CellTiter 96® AQueous One Solution was added to appropriate wells and plates were incubated at 37°C in 5% CO_2 for a total of four hours. By using a plate reader at a wavelength of 492nm, absorbance was measured to know. Bars correspond to mean absorbance values of 5-6 technical replicates \pm SEM and results are representative of three independent experiments.

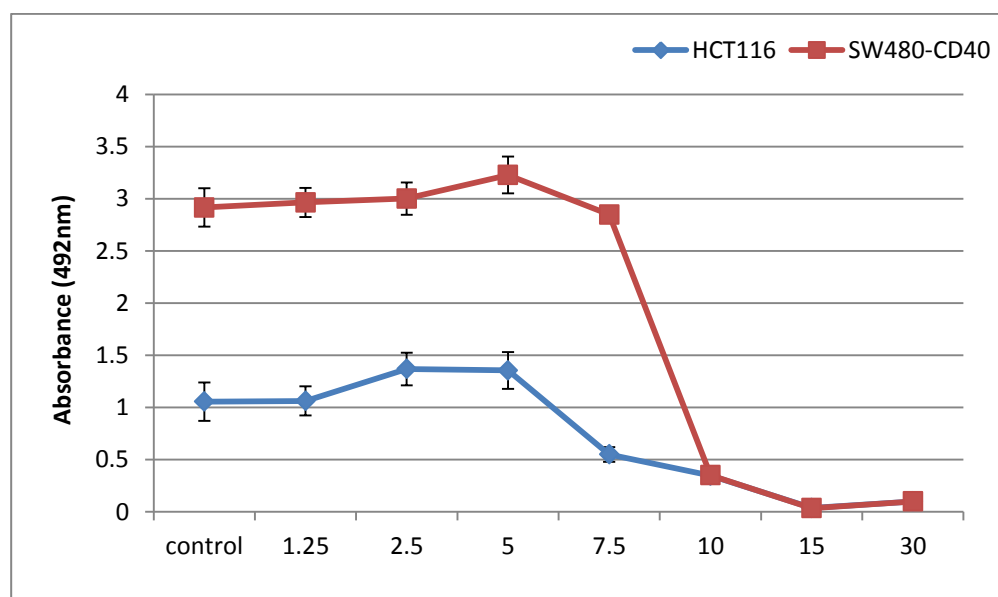


Figure 5. 6 Determination of effective dose of NAC on CRC cells (HCT116/SW480-CD40)

1×10^4 cells/well were seeded in 96 well plate in DR medium supplemented with 5% FBS and 1%LG. Cells were incubated overnight in 37°C / 5% CO_2 . Then the cells were treated with different concentrations of NAC. After 24hours incubation, $20\mu\text{l}$ of CellTiter 96® AQ_{ueous} One Solution was added to the appropriate wells and plates were incubated at 37°C in 5% CO_2 for a total of four hours. By using a plate reader at a wavelength of 492nm, absorbance was measured. Bars correspond to mean absorbance values of 5-6 technical replicates \pm SEM and results are representative of three independent experiments.

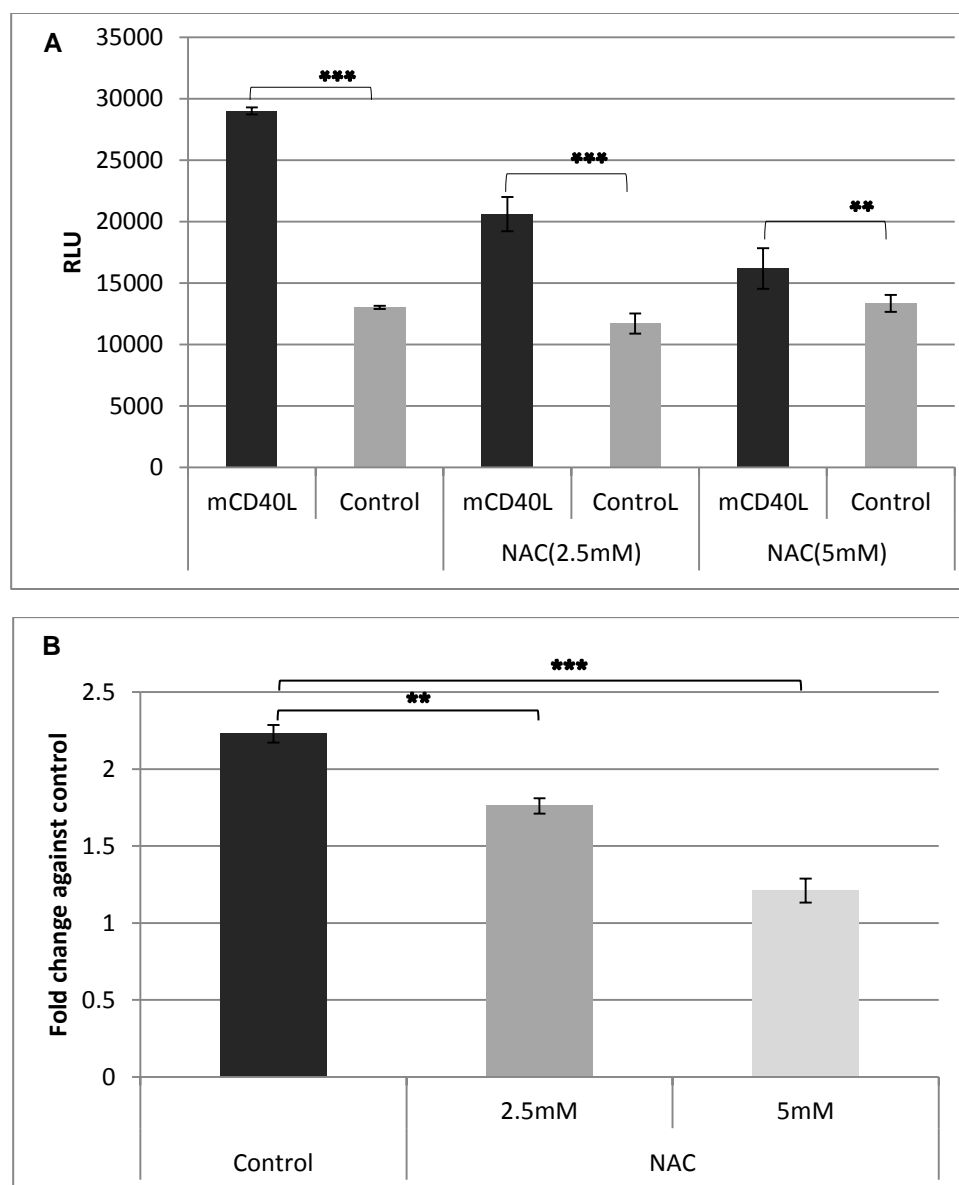


Figure 5. 7 Effect of the antioxidant NAC on CD40-mediated apoptosis

CRC cells (HCT116) were co-cultured at density 1×10^4 cells/well with either 1×10^4 cells/well MMC treated fibroblast cells (3T3CD40L and 3T3Neo) in DR medium supplemented with 5%FCS/1% L-glutamine. 2.5mM and 5mM of NAC was added to co-culture in 96 well plates and incubated for 24 hours at 37°C and 5% CO_2 . After the incubation time, apoptosis was measured by using CytoTox-Glo. 50 μM of substrate was added and then luminescence was measured by plate reader then background-corrected RLU reading deduced by pair-wise subtraction of mCD40L and control cell from the respective co-culture (Materials and methods). Bars presented mean RLU value for 5-6 replicates \pm S.D and results are representative of three experiments; ***p-value ≤ 0.001 and **p<0.01. A and B show background corrected raw data and fold change, respectively.

5.6 The role of NADPH oxidase in CD40-mediated cell death

As it has been reported that CD40 may generate ROS in malignant B-cells via TRAF3 and Nox (Ha and Lee, 2004) it was assumed that this may also happen in epithelial CRC carcinoma cells.

Following initial pre-titration experiments (using the Cell Titer 96® AQueous One Solution Cell Proliferation assay) to determine an effective dose, as shown in Figure 5.8 for killer cells (3T3-CD40L and 3T3-Neo) and Figure 5.9 for target cells (HCT116 and SW480-CD40), the highly specific Nox inhibitor DPI was added during in co-culture 3T3 and CRC cell co-cultures. The addition of DPI completely abrogated mCD40L-induced cell death in comparison to the control, as determined by CytoTox-Glo death detection assays in both HCT116 and SW480-CD40 cells (Figures 5.10 and 5.11). In addition to a significant reduction in apoptosis ($p < 0.001$), DPI significantly reduce the activity of caspase-3/7 in CRC cells (HCT116 and SW480-CD40) (Figures 5.12 and 5.13) (p -value < 0.05). These data suggest for the first time that CD40-mediated death by mCD40L in carcinoma cells is dependent on ROS induction by Nox.

Immunoblotting was used to determine whether ROS production may be linked to the association of TRAF3 with the specific Nox subunit p40-phox as observed by others (Ha and Lee, 2004). Results implied that CD40 engagement generates ROS through p40-phox as this was phosphorylated by 1.5, 3, 6, 12 hours post CD40 ligation, however no changes were seen in the control (Figure 5.14). Although strictly no direct interaction of TRAF3 and p40-phox was demonstrated here, the results strongly imply that a similar mechanism for ROS production by CD40-mediated recruitment of TRAF3/Nox may occur in both malignant B-cells (Ha and Lee, 2004) and carcinoma (CRC) cells.

Previous studies have indicated that the possible mechanism(s) for the CD40 induced production of ROS following activation of TRAF3 (Ha and Lee, 2004). The immunoblotting results on TRAF3-KD cells lysate co-culture with 3T3CD40L COMPARED WITH CONTROL (HCT116) also demonstrate that CD40-linked TRAF3 probably mediates the activation of NADPH oxidase via the recruitment of p40phox. As shown in Figure 5.15, TRAF3-KD completely abrogated p40phox expression subsequent to CD40 ligation. This result suggest that CD40 ligation produces ROS by the TRAF3 dependent motivation of NADPH oxidase (NOX) , and that ROS link the CD40 ligation to promote signalling events.

Interestingly, when CD40 ligation was initiated in CRC cells in the presence of the Nox inhibitor DPI, the activation of TRAF3 was severely diminished. (Figure 5.16) thus implying a role for ROS in the stabilisation of TRAF3 in the first place.

In addition to a significant reduction in apoptosis ($p < 0.001$), DPI also prevented expression one of the pro-apoptotic protein Bax, as determined by western blotting (Figure 5.17). These results suggested that CD40-mediated death by membrane CD40L ligand in CRC cells is dependent on ROS induction triggered by NOX.

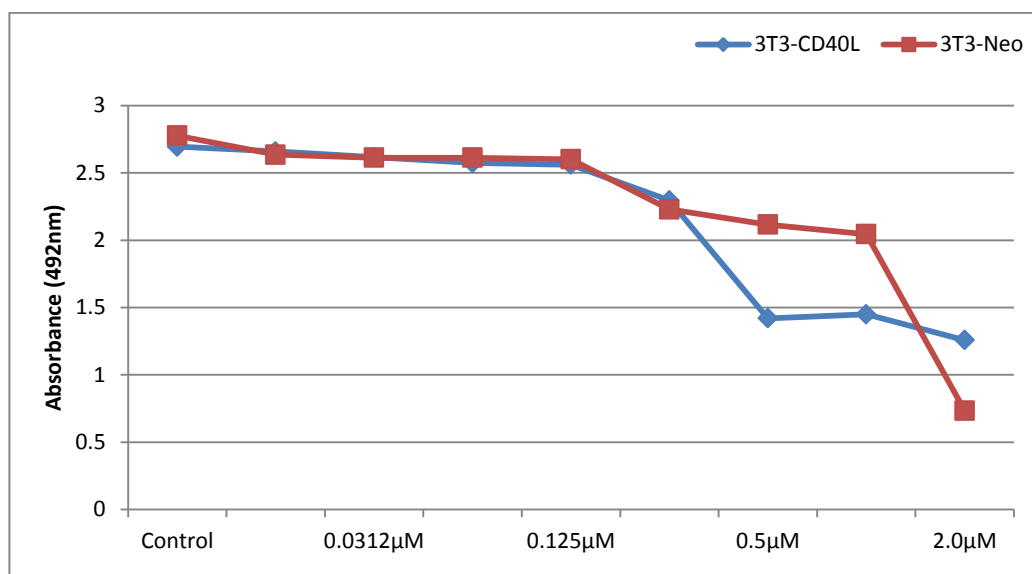


Figure 5. 8 Determination of effective dose of DPI on effector (killer) cells (3T3-CD40L/ 3T3Neo)

1×10^4 cells/well were seeded in 96 well plates in DR medium supplemented with 5% FBS and 1%LG. Cells were incubated overnight in 37°C / 5% CO_2 . Then the cells were treated with different concentrations of DPI compared with control (Untreated cells). After 24hours incubation, 20µl of CellTiter 96® AQ_{ueous} One Solution was added to the appropriate wells and plates were incubated at 37°C in 5% CO_2 for a total of four hours. By using a plate reader at a wavelength of 492nm, absorbance was measured. Bars correspond to mean absorbance values of 5-6 technical replicates \pm SEM and results are representative of three independent experiments.

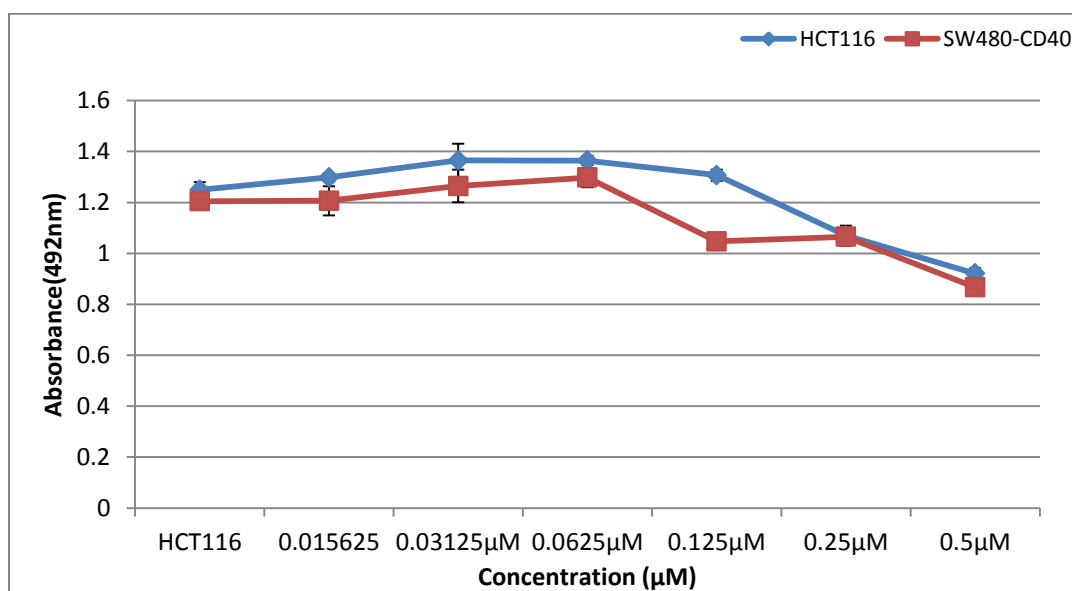


Figure 5. 9 Determination of effective dose of DPI on target cells (HCT116/ SW480-CD40)

1x10⁴ cells/well were seeded in 96 well plate in DR medium supplemented with 5% FBS and 1%LG. Cells were incubated overnight in 37°C/ 5% CO₂. Then the cells were treated with different concentrations of DPI compared with control (Untreated cells). After 24hours incubation, 20μl of CellTiter 96® AQ_{ueous} One Solution was added to the appropriate wells and plates were incubated at 37°C in 5% CO₂ for a total of four hours. By using a plate reader at a wavelength of 492nm, absorbance was measured. Bars correspond to mean absorbance values of 5-6 technical replicates ± SEM and results are representative of three independent experiments.

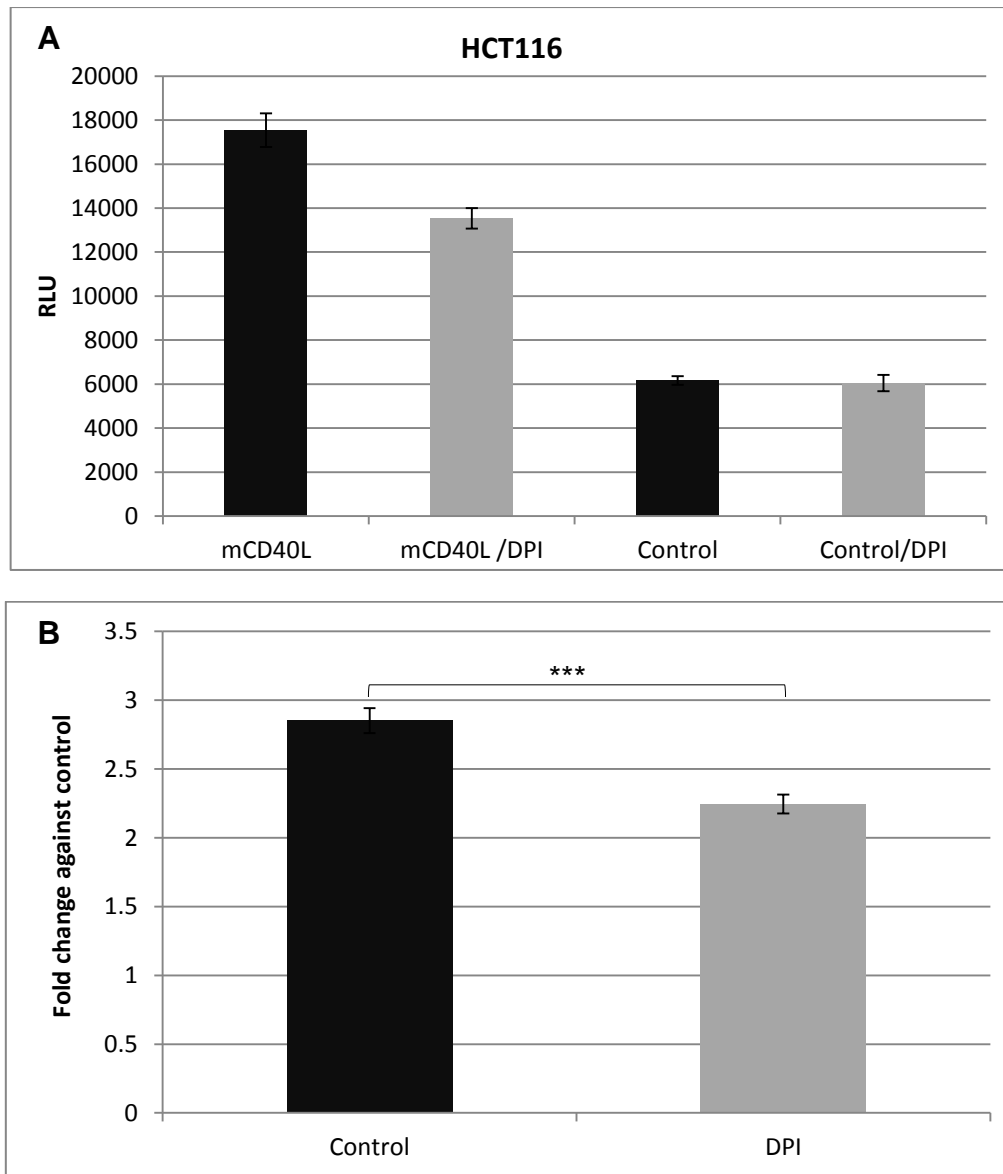


Figure 5. 10 NADPH oxidase (Nox) inhibition by DPI reduces CD40-mediated apoptosis in HCT116 cells

CRC cells (HCT116) were co-cultured at density 1×10^4 cells/well with 1×10^4 cells/well MMC-treated fibroblasts (3T3CD40L and 3T3Neo) in DR medium supplemented with 5%FCS/ 1% L-glutamine. $0.125 \mu\text{M}$ of DPI was added to the cultures in 96 well plates and incubated for 24 hours at 37°C and $5\% \text{CO}_2$. After the incubation time, apoptosis was measured by using CytoTox-Glo by adding $50 \mu\text{M}$ of substrate then luminescence was measured by a plate reader then background-corrected RLU reading deduced by pair-wise subtraction of mCD40L and control cells from the respective co-culture (Materials and methods). Bars represent mean RLU value for 5-6 replicates and results representative of three experiments; $p\text{-value} \leq 0.001$. A and B show background corrected readings and fold change in luminescence, respectively, following CD40 ligation in the HCT116 cell line.

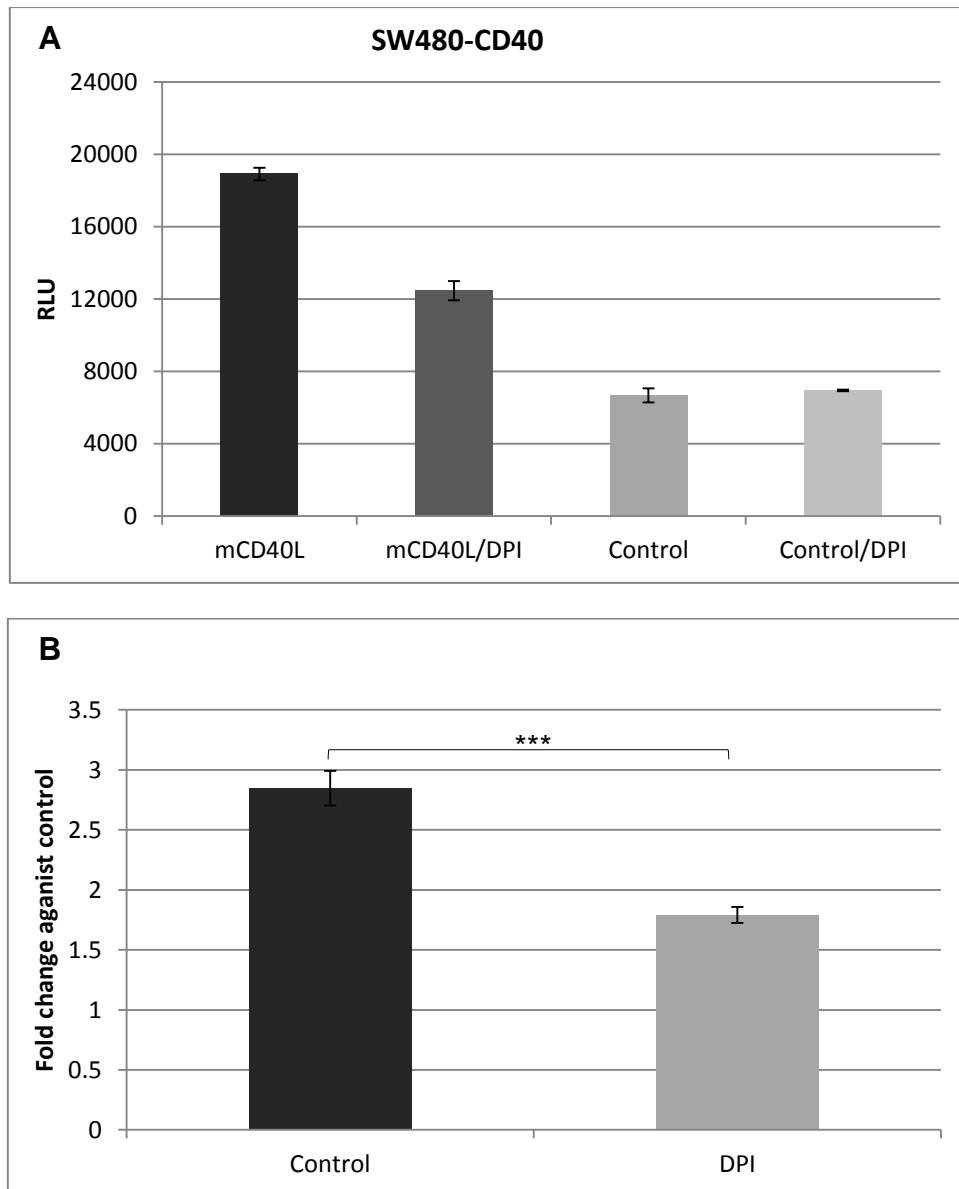


Figure 5. 11 NADPH oxidase (Nox) inhibition by DPI reduces CD40-mediated apoptosis in SW480-CD40 cells

CRC cells (SW480-CD40) were co-cultured at density 1×10^4 cells/well with 1×10^4 cells/well MMC-treated fibroblasts (3T3CD40L and 3T3Neo) in DR medium supplemented with 5%FCS/ 1% L-glutamine. $0.125 \mu\text{M}$ of DPI was added to the cultures in 96 well plates and incubated for 24 hours at 37°C and $5\%\text{CO}_2$. After the incubation time, apoptosis was measured by using CytoTox-Glo. $50 \mu\text{M}$ of substrate was added and then luminescence was measured by a plate reader then background-corrected RLU reading deduced by pair-wise subtraction of mCD40L and control cell from the respective co-culture (Materials and methods). Bars represent mean RLU value for 5-6 replicates and results representative of three experiments; $p\text{-value} \leq 0.001$. A and B show background corrected readings and fold change in luminescence, respectively, following CD40 ligation in the SW480-CD40 cell line.

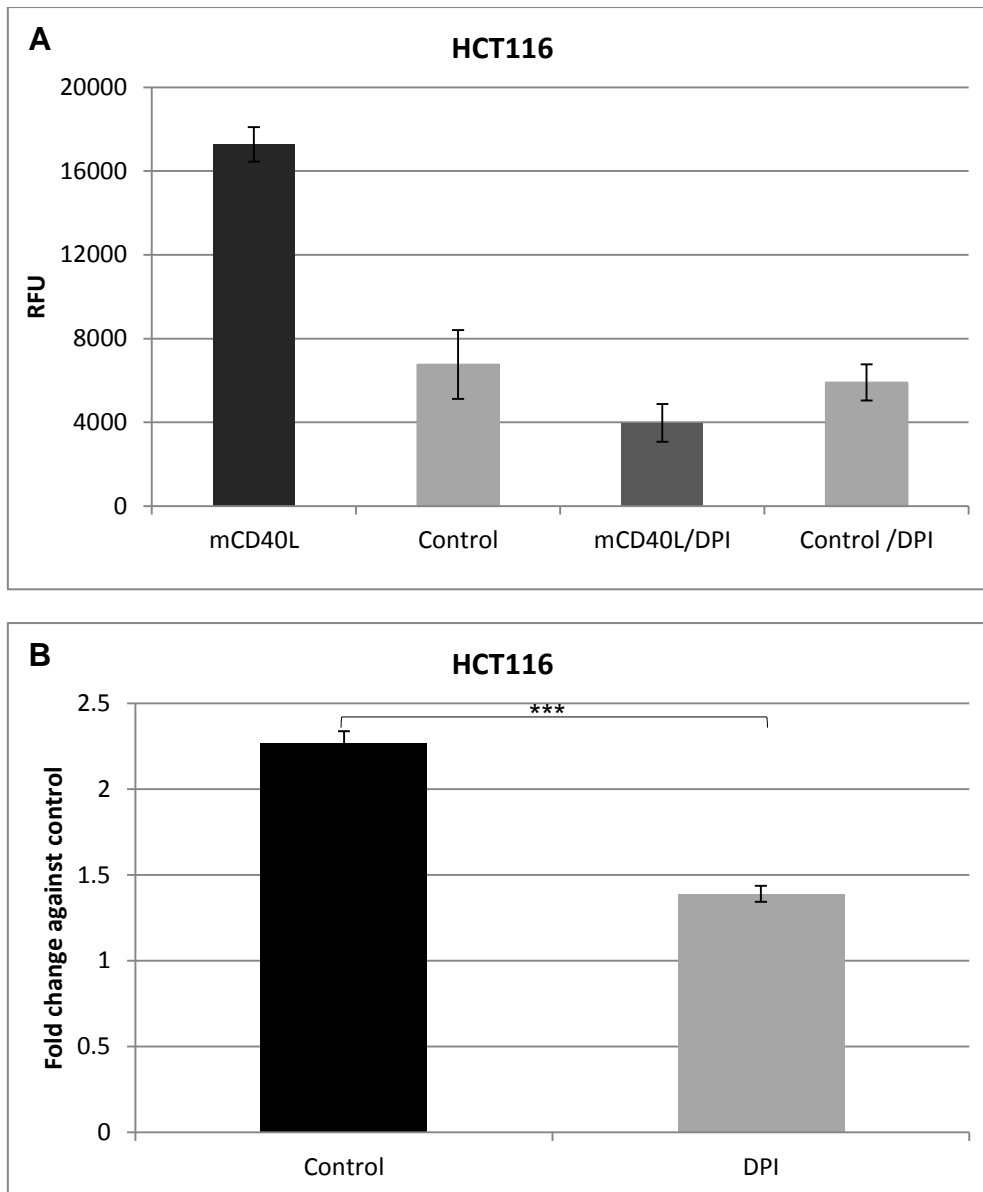


Figure 5. 12 NADPH oxidase (Nox) inhibition reduces caspases 3/7 activity in the HCT116 cell line following CD40 ligation

CRC cells (HCT116) were co-cultured at density 1×10^4 cells/well with either 1×10^4 cells/well MMC treated fibroblast cells (3T3CD40L and 3T3Neo) in DR medium supplemented with 5%FCS/ 1% L-glutamine. $0.125 \mu\text{M}$ of DPI was added to the cultures in 96 well plates and incubated for 24 hours at 37°C and $5\% \text{CO}_2$. After the incubation time, caspase 3/7 activity was assessed by using SensoLyte 3/7 reagent was added after 48 hours, fluorescence measured (Materials and methods) then background-corrected RFU reading deduced by pair-wise subtraction of mCD40L and control cells from the respective co-culture. Bars represent mean RFU value for 5-6 replicates and results representative of three experiments; $p\text{-value} \leq 0.001$. A and B show background corrected and fold change respectively for caspase 3/7 activity of NADPH oxidase inhibition by antioxidant following CD40 ligation.

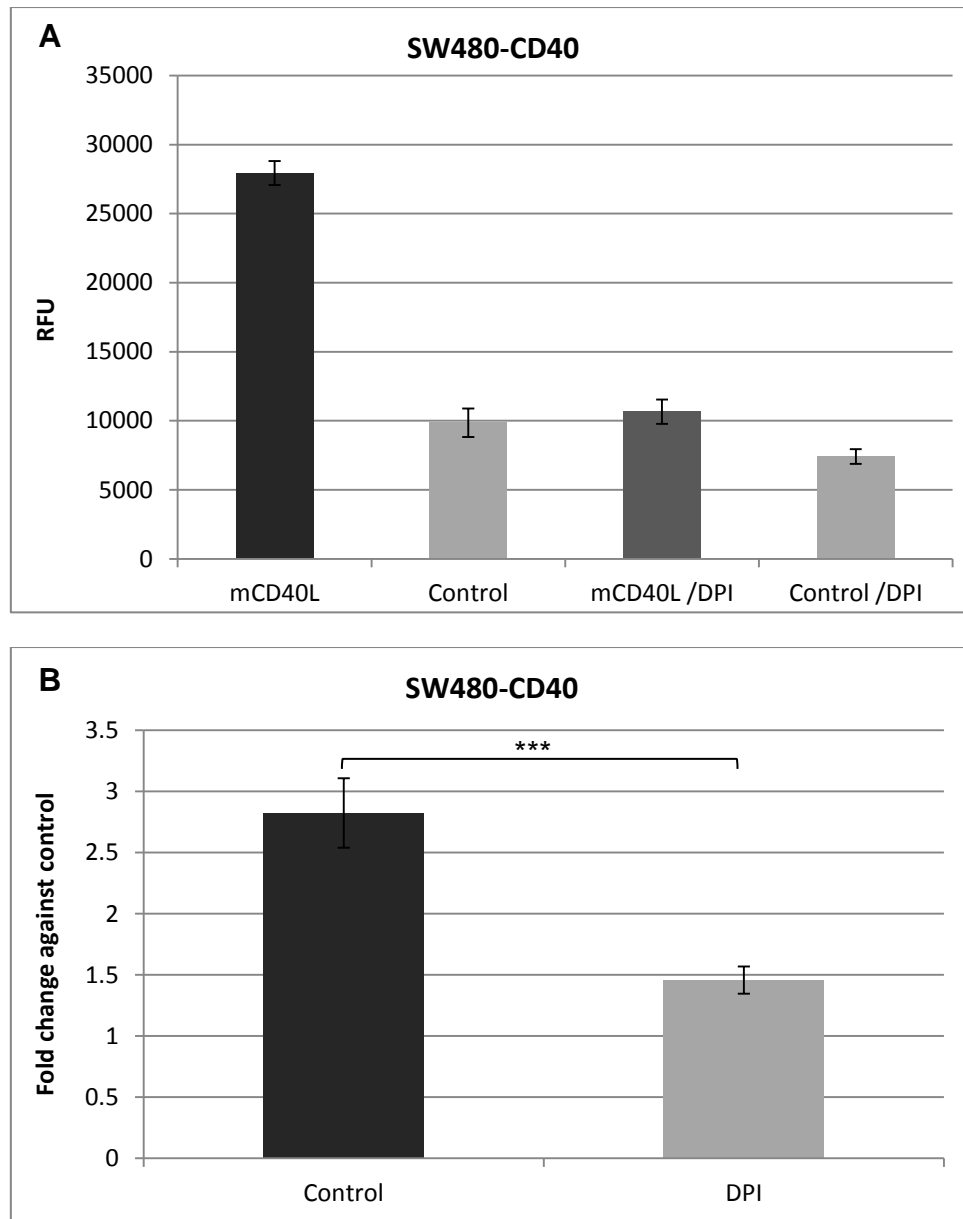


Figure 5. 13 NADPH oxidase (Nox) inhibition reduces caspases 3/7 activity in the SW480-CD40 cell line following CD40 ligation

CRC cells (SW480-CD40) were co-cultured at density 1×10^4 cells/well with 1×10^4 cells/well MMC-treated fibroblasts (3T3CD40L and 3T3Neo) in DR medium supplemented with 5%FCS/ 1% L-glutamine. 0.125 μ M of DPI was added to the cultures in 96 well plates and incubated for 48 hours at 37°C and 5%CO₂. After the incubation time, caspase 3/7 activity was assessed by using SensoLyte 3/7 reagent that was added after 48 hours, fluorescence measured (Materials and methods) then background-corrected RFU readings deduced by pair-wise subtraction of mCD40L and control cells from the respective co-culture. Bars represent mean RFU value for 5-6 replicates and results representative of three experiments; p-value ≤ 0.001 . A and B show background corrected and fold change respectively for caspase 3/7 activity of NADPH oxidase inhibition following CD40 ligation.

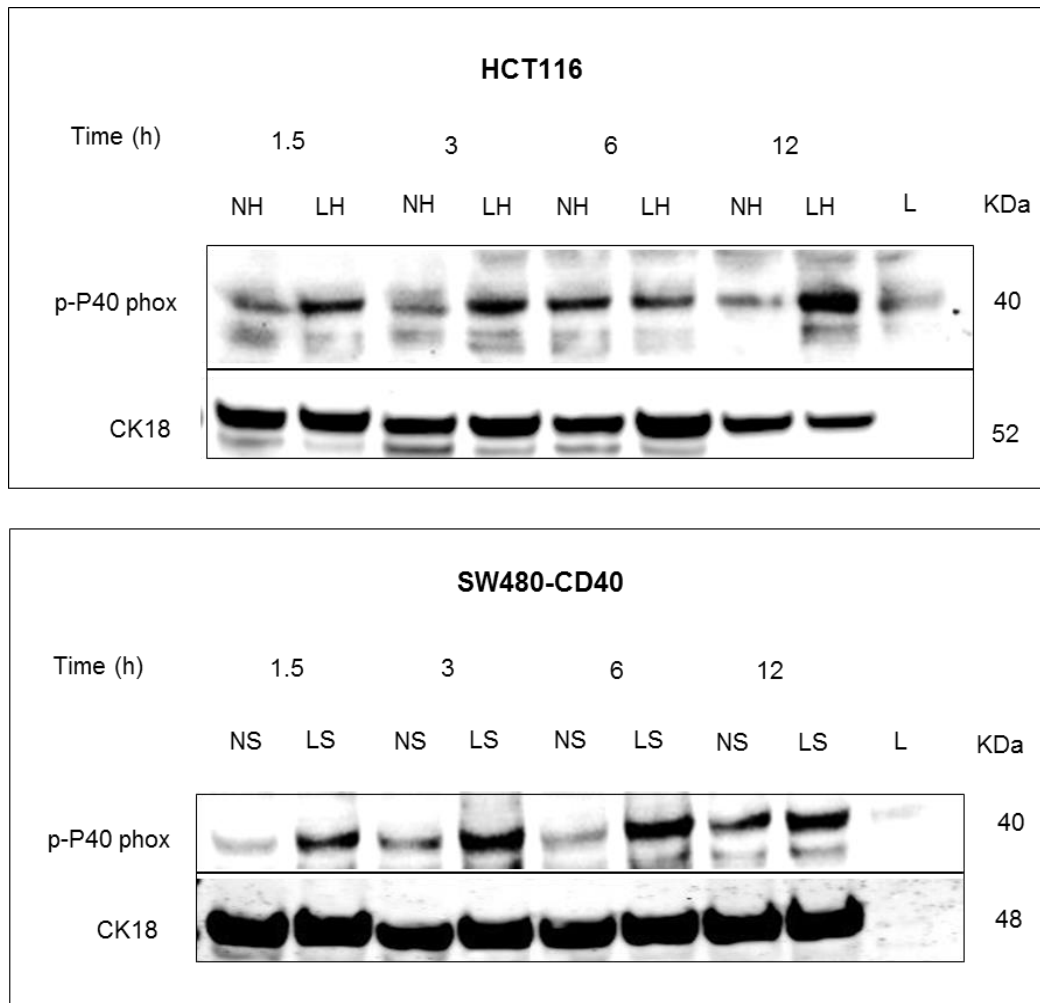


Figure 5. 14 p40-phox phosphorylation induced by mCD40L

HCT116 (H) and SW480-CD40 (S) cells were seeded at density 3×10^6 cells/dish, and co-cultured with 3×10^6 MMC-treated 3T3-Neo (N) or 3T3-CD40L (L), as indicated. Co-cultured cells were incubated for various times 1.5, 3, 6, 12 hours in DR5% supplemented with 5% of FCS and 1% L-glutamine in 10 cm^2 culture dishes. After each incubation time, cells were lysed by using 2x SDS-lysis buffer. After that protein concentration was determined. Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were separated by western blotting under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membrane. The PVDF membrane was probed overnight at 4°C with primary monoclonal antibody (anti-p40phox) in TBS/Tween 0.1% (1:250 dilution) and then with an anti-CK8 antibody for HCT116 and CK18 antibody for SW480-CD40 in TBS/Tween 0.1% (1:2000 dilution). The membranes were then incubated for one hour with secondary antibody [goat anti-rabbit IgG IRDye 800 (1:10000 dilution)] for p40phox detection and with goat anti-mouse antibody IgG IRDye 680 (1:10000 dilution) for CK8 and CK18. Antibody binding was visualised at 700nm and 800 using an Odyssey™ Infra-red Imaging system.

Keys: NH: 3T3Neo co-cultured with HCT116 LH: 3T3CD40L co-cultured with HCT116
 NS: 3T3Neo co-cultured with SW480-CD40 LS: 3T3CD40L co-cultured with SW480-CD40
 L: 3T3CD40L

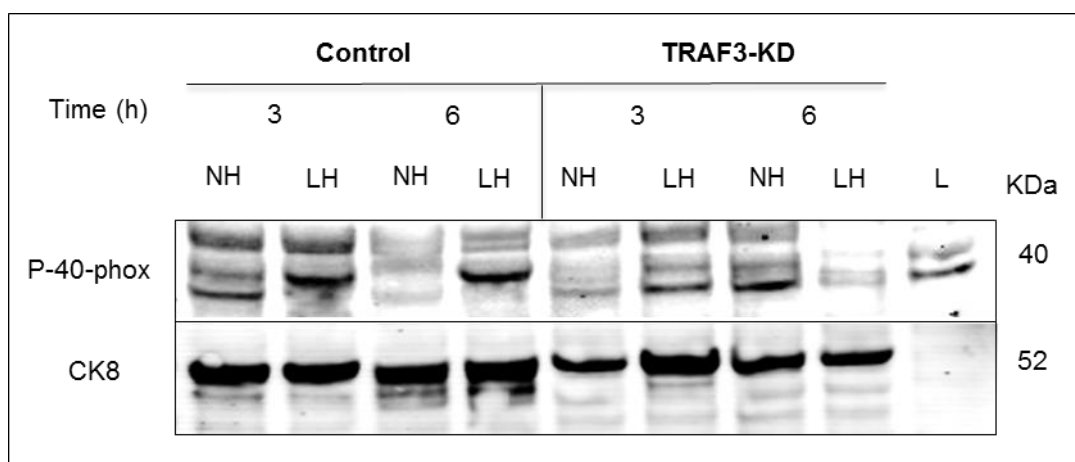


Figure 5. 15 The role of TRAF3 knockdown on p40phox following CD40 ligation

3×10^6 CRC cells [HCT116 (H)] and HCT116 cells expressing TRAF3 sh RNA (TRAF3-KD) were co-cultured with 3×10^6 MMC treated mCD40L (L) and Controls (N) for 3 and 6 hours in DR / 5%FCS / 1%LG in 10cm^2 culture dishes in order to lyse with 2X SDS-lysis buffer. Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were separated by western blotting under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membrane. The membrane was probed for overnight at 4 °C with primary monoclonal antibody/goat anti-mouse antibody (anti-p40phox) in TBS/Tween 0.1% (1:520 dilution) and then with an anti-CK8 antibody in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one hour with secondary antibody [goat anti-mouse IgG IRDye 680 (1:10000 dilution)] for p- p40phox detection and membrane was incubated with goat anti-mouse antibody IgG IRDye 680 (1:10000 dilution) for CK8 (used as loading control).

Keys: NH: 3T3Neo co-cultured with HCT116

LH: 3T3CD40L co-cultured with HCT116

L: 3T3CD40L

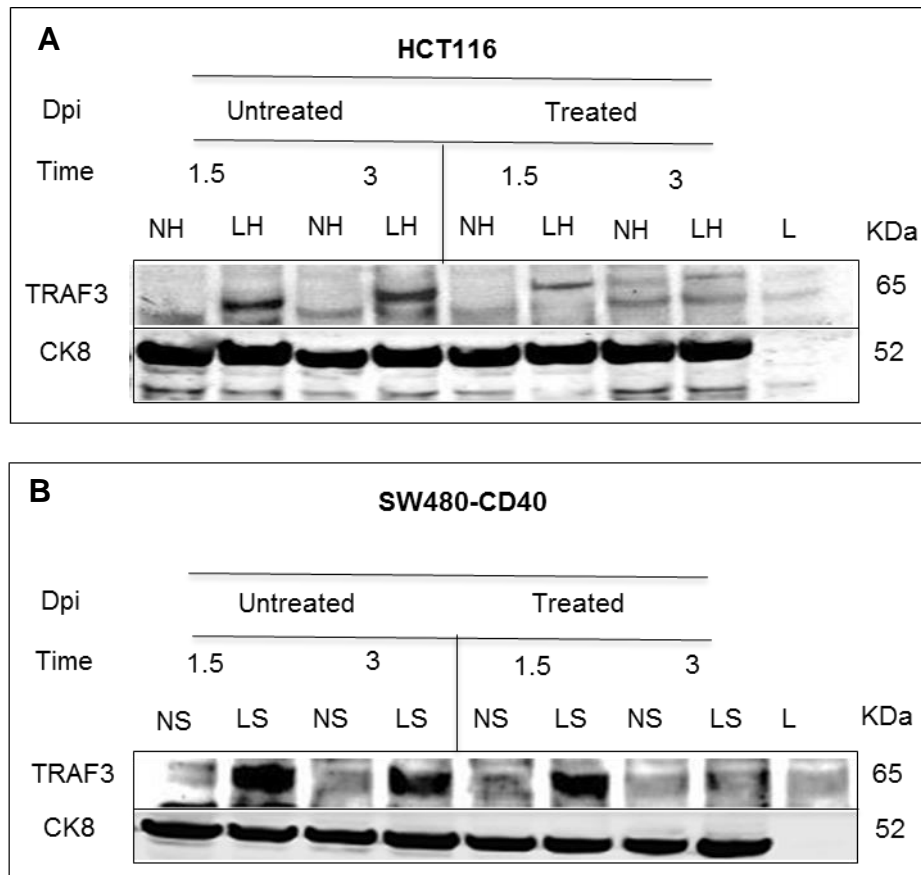


Figure 5. 16 The Nox inhibitor DPI prevents the induction of TRAF3 following mCD40L-mediated CD40 ligation

3×10^6 MMC-treated 3T3-Neo (N) and 3T3-CD40L (L) were co-cultured with either 3×10^6 of HCT116 cells (H) and SW480-CD40 (S). Co-cultured cells were incubated with $0.125 \mu\text{M}$ DPI for 1.5 and 3 hours. In control experiments, cells were co-cultured in the absence of DPI. Cell lysates were prepared and whole lysates, following normalisation on the basis of CK8 expression (see chapter 3) were loaded by western blotting. Cell proteins were separated under denaturing conditions by SDS-PAGE using 4-12% (W/V) Bis-Tris gels and then immunoblotted onto a PVDF membrane. The PVDF membrane was probed overnight at 4°C with primary polyclonal antibody (anti-TRAF3 antibody) in TBS/Tween 0.1% (1:500) and then with an anti-CK8 antibody for HCT116 [A] and CK18 for SW480-CD40 [B] (1:2000 dilution). The membrane was incubated for one hour with goat anti-rabbit IgG Alexa 800 (1:10000) for TRAF3 detection and one hour with goat anti-mouse IgG Alexa 680 (1:10000) for CK8 detection. Antibody binding was visualised at 700nm and 800nm using an OdysseyTM infra-red Imaging system.

Keys: NH: 3T3Neo co-cultured with HCT116
 NS: 3T3Neo co-cultured with SW480-CD40
 L: 3T3CD40L

LH: 3T3CD40L co-cultured with HCT116
 LS: 3T3CD40L co-cultured with SW480-CD40
 DPI: Diphenyleneiodonium

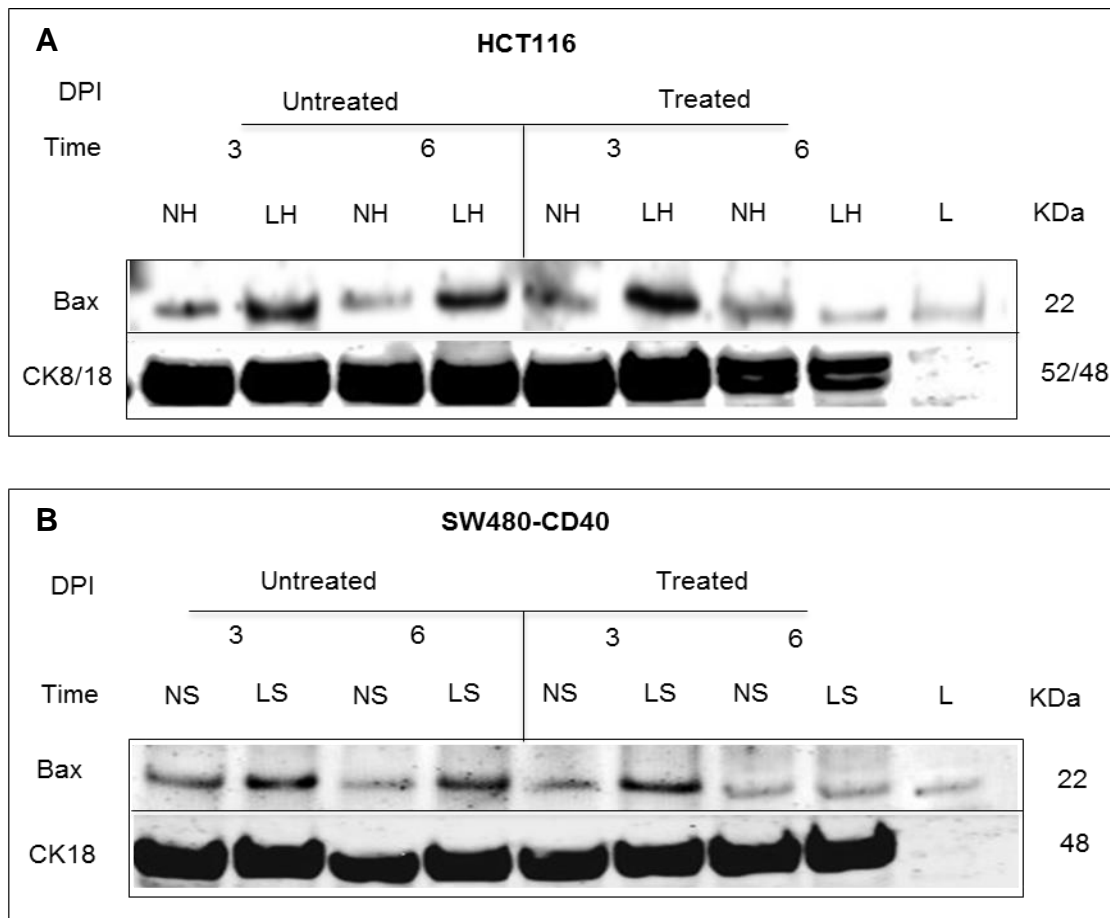


Figure 5. 17 Nox inhibitor DPI prevents mCD40L-mediated induction of Bax

3×10^6 MMC-treated 3T3-Neo (N) and 3T3-CD40L (L) were co-cultured with either 3×10^6 of HCT116 cells (H) and SW480-CD40 (S). Co-cultured cells were incubated with $0.125 \mu\text{M}$ DPI for 3 and 6 hours. In control experiment, cells were co-cultured in the absence of DPI. Cell lysates were prepared and whole lysates, following normalisation on the basis of CK18 and CK8/18 expression (see chapter 3) were resolved by western blotting. Cell proteins were separated under denaturing conditions by SDS-PAGE using 4-12% (W/V) Bis-Tris gels and then immunoblotted onto a PVDF membrane. The PVDF membrane was probed overnight at 4°C with primary monoclonal antibody (anti-Bax antibody) in TBS/Tween 0.1% (1:500) and then with an anti-CK8/18 antibody for HCT116 as shown in A, while CK18 was used for SW480-CD40 as shown in B (1:2000 dilution). The membrane was incubated for one hour with goat anti-mouse IgG Alexa 680 (1:10000) for the detection Bax, CK8/18 and CK18. Antibodies binding were visualised at 700nm and 800nm using an Odyssey™ infra-red Imaging system.

Keys: NH: 3T3Neo co-cultured with HCT116

NS: 3T3Neo co-cultured with SW480-CD40

L: 3T3CD40L

LH: 3T3CD40L co-cultured with HCT116

LS: 3T3CD40L co-cultured with SW480-CD40

5.7 The role of ASK1 in CD40-mediated cell death

Apoptosis signal-regulating kinase 1 (ASK1) is a member of the MAP3K (MAPKKK) family, which activates the MAPKK family, including MKK4 and MKK7 and MAPK such as JNK and p38 pathways (Takeda et al., 2011, Ichijo et al., 1997). ASK1 is specifically activated in response to different types of stress, such as ROS, TNF- α , lipopolysaccharide (LPS), and ER stress, and has pivotal roles in a wide variety of cellular responses, including apoptosis, differentiation, and inflammation (Ichijo et al., 1997, Nishitoh et al., 2002). Therefore, the extreme stimulation and dysregulation of ASK1 are closely linked to various diseases such as cancer and arteriosclerosis.

In this project, the role of ASK1 in apoptosis following CD40-CD40L interaction was investigated. Immunoblotting showed that the activation of CD40 on CRC cells by its membrane ligand activated the expression of ASK1 within 1.5 hours post CD40 ligation (Figure 5.18). Moreover, inhibition of apoptosis by using DPI (0.125 μ M) attenuated ASK1 activation as shown in Figure 5.19

Previous studies (Ha and Lee, 2004) have showed that ROS generation is triggered by CD40 ligation in B cells and recent work in our laboratory (Chris Dunnill, PhD thesis) in bladder (UCC) cells has demonstrated that ROS is important for early CD40-mediated signalling events that lead to the activation of JNK. In particular, Nox-generated ROS appear to be involved in the activation of JNK as inhibition of ROS production and subsequent JNK activation was achieved using DPI treatment. Reducing elevation of ROS by pre-treating HCT116 and SW480-CD40 cells with 0.125 μ M DPI significantly reduced CD40 ligation-induced phosphorylation of JNK (Figure 5.20). Thus implying that CD40 induces TRAF3 and NOX in a co-ordinated manner to activate ROS release and trigger subsequent ASK1 and JNK activation.

5.8 Effect of CD40 ligation on Thioredoxin (Trx) expression

As mentioned above, it was shown that CD40-mediated apoptosis triggers the activation of ASK1. ASK1 is regulated by Trx which under physiological ROS levels physically binds ASK1 and inhibits its auto-phosphorylation and thus activation. During oxidative stress, Trx scavenges ROS, however in doing so it releases ASK1 which undergoes activation via auto-phosphorylation at Thr845 (Soga et al., 2012).

Based on the findings shown in this and the previous chapter, it was hypothesised that ROS elevation mediates Trx/ROS-'occupancy' for rapid release of ASK1 within a few hours post-ligation. Moreover, recent findings in our laboratory in bladder (UCC) cells have

demonstrated that CD40 ligation can efficiently down-modulate Trx-1 expression during the course of CD40 ligation (Chris Dunnill, PhD thesis). Thus, immunoblotting was used to examine the expression of Trx over a 24 hour time window. Interestingly, it was found that Trx expression appeared to be moderately reduced by CD40 ligation particularly in HCT116 cells. Notably also, there was a gradual increased expression of Trx in the Controls (HCT116 and SW480-CD40/ 3T3-Neo) (Figure 5.21) suggesting that during *in vitro* cell culture, tumour cells progressively increase Trx possibly as a cyto-protective mechanism against their sustained oxidative stress associated with proliferation signaling.

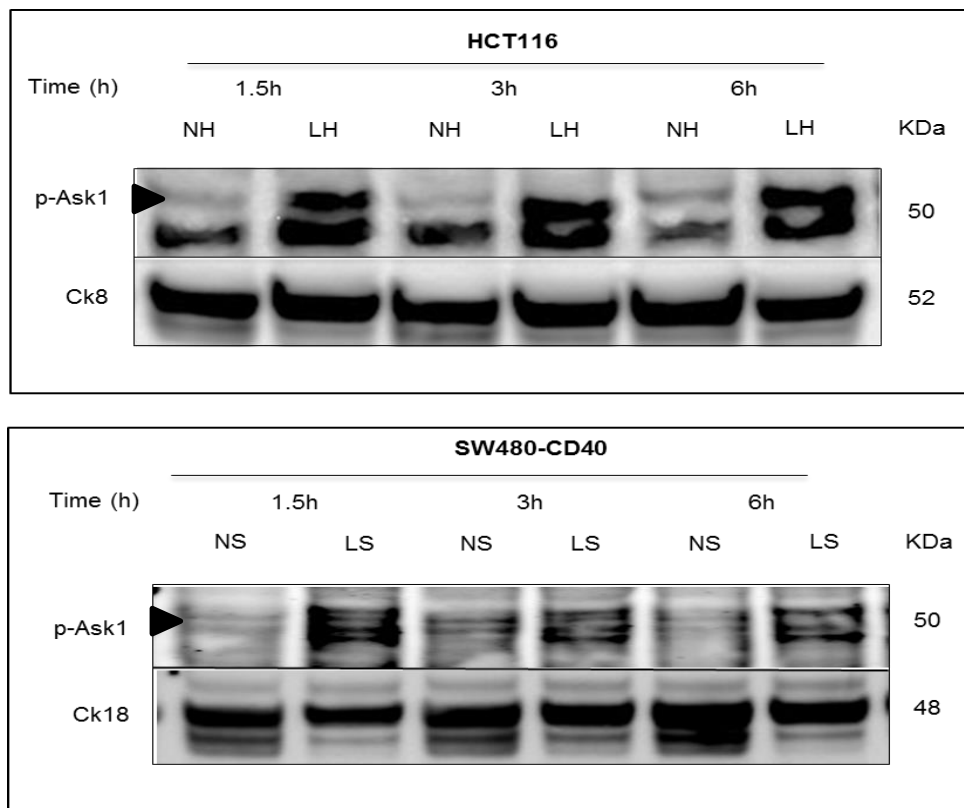


Figure 5. 18 Activation of ASK1 evident by activatory Thr845 residue phosphorylation in response to mCD40L

HCT116 (H) and SW480-CD40 (S) cells were seeded at density 3×10^6 cells /dish, and co-cultured with 3×10^6 cells/dish MMC-treated 3T3-Neo (N) or 3T3-CD40L (L), as indicated. Co-cultures were incubated for 1.5, 3, and 6 hours in DR 5% supplemented with 5% of FCS and 1% L-glutamine, using 10cm^2 culture dishes. After each of incubation time, cells were lysed by using 2x SDS-lysis buffer and protein concentration was determined. Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were separated by western blotting under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membrane. The PVDF membrane was probed for overnight at 4°C with primary polyclonal antibody (anti-p-Ask1) in TBS/Tween 0.1% (1:250 dilution) and then with an anti-CK8 and CK18 antibody in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one hour with secondary antibody [goat anti-rabbit IgG IRDye 800 (1:10000 dilution)] for p-Ask1 detection. Also, membranes were incubated with [goat anti-mouse IgG IRDye 680 (1:10000 dilution)] to detect CK8 and CK18. Antibody binding was visualised at 700nm and 800nm using an OdysseyTM Infra-red Imaging system. CK8/18 and CK8 were used as loading controls for HCT116 and SW480-CD40.

Keys: NH: 3T3Neo co-cultured with HCT116

NS: 3T3Neo co-cultured with SW480-CD40

LH: 3T3CD40L co-cultured with HCT116

LS: 3T3CD40L co-cultured with SW480-CD40

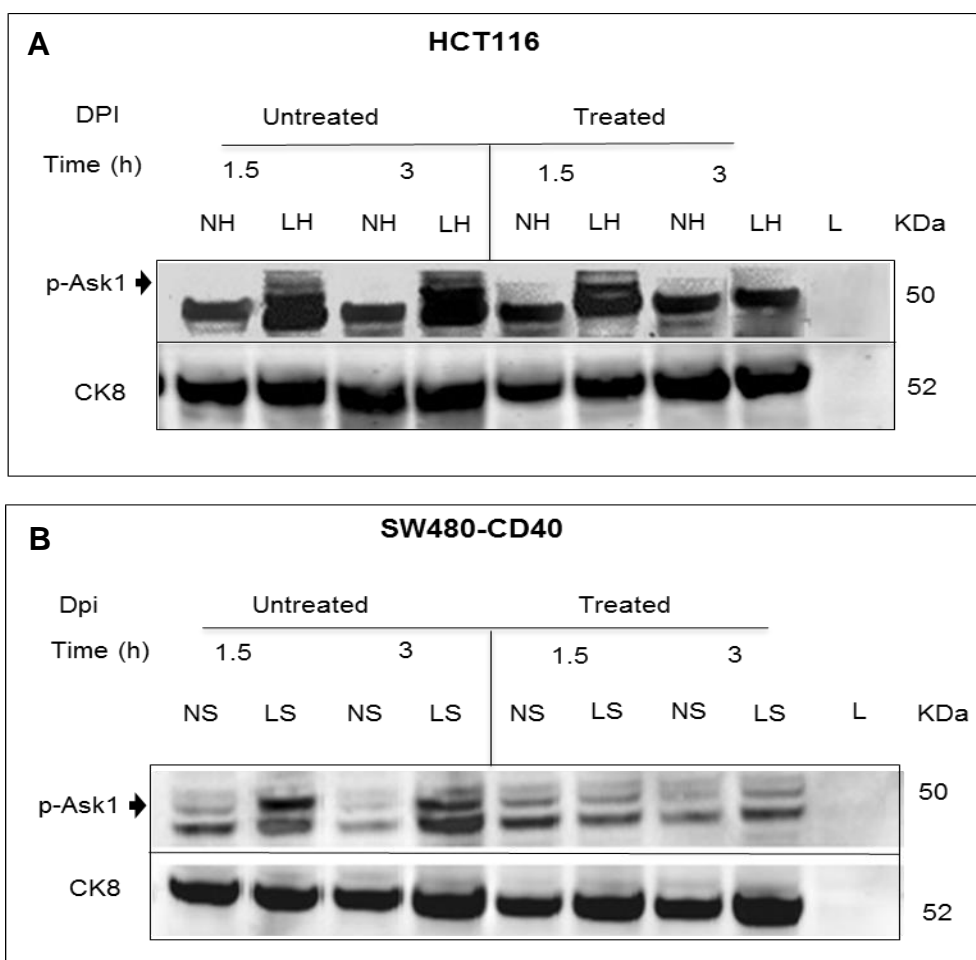


Figure 5. 19 The Nox inhibitor DPI blocks mCD40L-mediated ASK1 induction

3×10^6 MMC-treated 3T3-Neo (N) and 3T3-CD40L (L) were co-cultured with both 3×10^6 of HCT116 cells (H) or SW480-CD40 (S) shown in panels A and B, respectively. Co-cultures were incubated with $0.125 \mu\text{M}$ DPI for 3 and 6 hours. In control experiments, cells were co-cultured in the absence of DPI. Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were separated under denaturing conditions by SDS-PAGE using 4-12% (W/V) Bis-Tris gels and then immunoblotted onto a PVDF membrane. The PVDF membrane was probed overnight at 4°C with primary polyclonal antibody (anti-ASK1 antibody) in TBS/Tween 0.1% (1:500) and then with an anti-CK8/18 antibody for HCT116 as shown in A, while CK18 was used for SW480-CD40 as shown in B (1:2000 dilution). The membrane was incubated for one hour with goat anti-mouse IgG Alexa 680 (1:10000) for the detection of ASK1, CK8/18 and CK18. Antibody binding was visualised at 700nm and 800nm using an OdysseyTM infra-red Imaging system.

Keys: NH: 3T3Neo co-cultured with HCT116
 NS: 3T3Neo co-cultured with SW480-CD40
 L: 3T3CD40L

LH: 3T3CD40L co-cultured with HCT116
 LS: 3T3CD40L co-cultured with SW480-CD40
 DPI: Diphenyleneiodonium

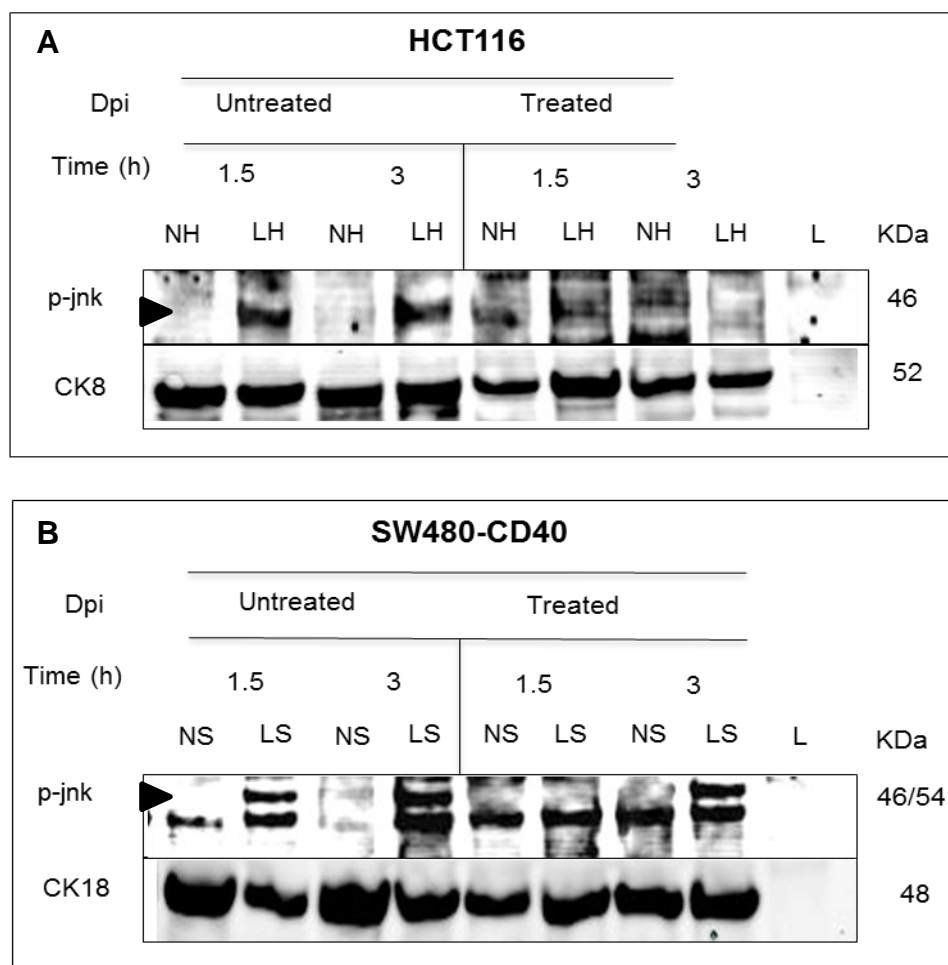


Figure 5. 20 The effect of DPI on mCD40L-mediated JNK activation

3×10^6 MMC treated mouse fibroblasts cells (3T3-Neo (N) and 3T3-CD40L (L) were co-cultured with either 3×10^6 of HCT116 cells (H) and SW480-CD40 (S) shown in panels A and B, respectively. Co-cultured cells were incubated with $0.125 \mu\text{M}$ DPI for 1.5 and 3 hours. In control experiment, cells were co-cultured in the absence of DPI. Cell lysates were prepared by using lysis buffer and then Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were loaded by western blotting. Cell proteins were separated under denaturing conditions by SDS-PAGE using 4-12% (W/V) Bis-Tris gels and then immunoblotted onto a PVDF membrane. The PVDF membrane was probed overnight at 4°C with primary monoclonal antibody (anti-phospho-JNK antibody) in TBS/Tween 0.1% (1:500) and then with an anti-CK8 antibody for HCT116 [A] and CK18 for SW480-CD40 [B] (1:2000 dilution). The membrane was incubated for one hour with goat anti-mouse IgG Alexa 680 (1:10000) for p-JNK, CK8 and CK18. Antibody binding was visualised at 700nm and 800nm using an OdysseyTM infra-red Imaging system.

Keys: NH: 3T3Neo co-cultured with HCT116 LH: 3T3CD40L co-cultured with HCT116
 NS: 3T3Neo co-cultured with SW480-CD40 LS: 3T3CD40L co-cultured with SW480-CD40
 L: 3T3CD40L DPI: Diphenyleneiodonium

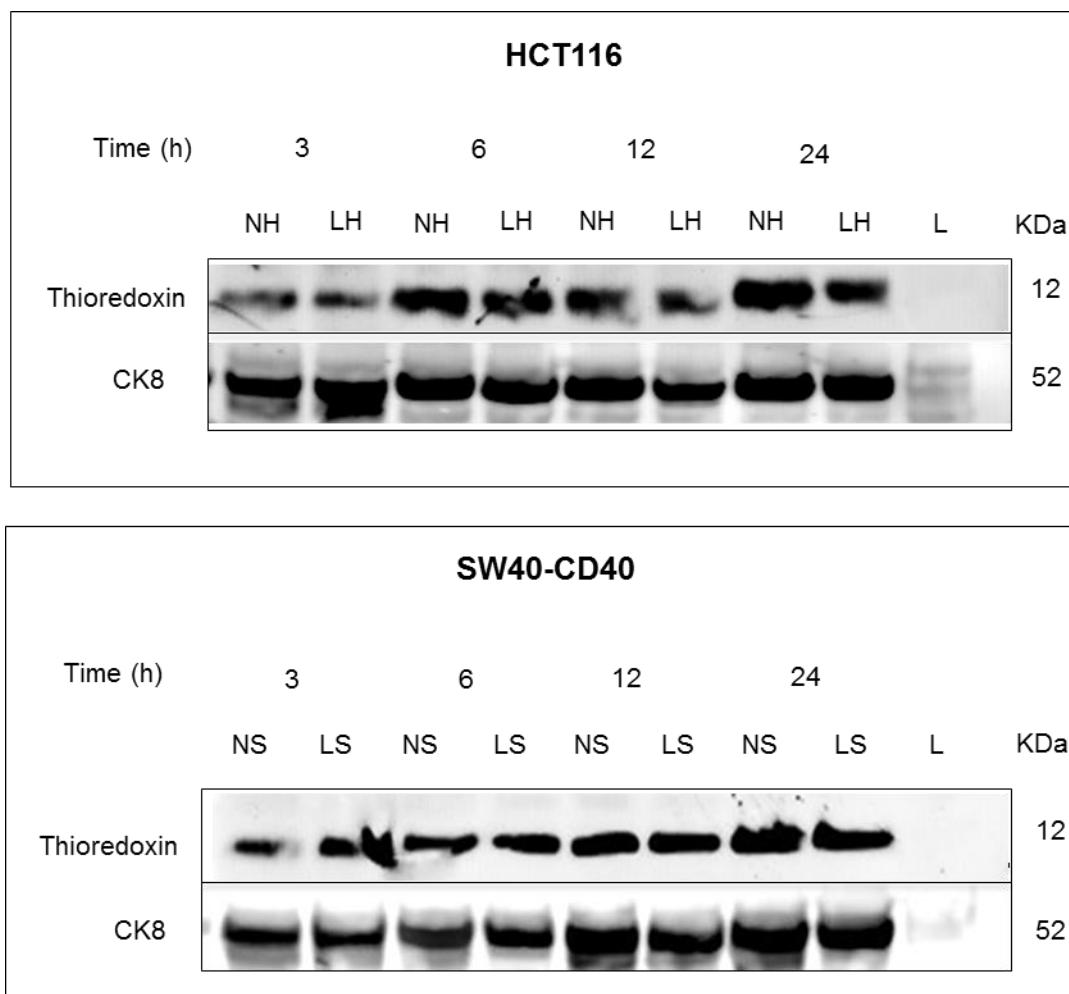


Figure 5. 21 The effect of CD40 ligation on thioredoxin-1 (Trx-1) expression

HCT116 (H) and SW480-CD40 (S) cells were seeded at density 3×10^6 cells /dish, and co-cultured with 3×10^6 MMC-treated 3T3-Neo (N) or 3T3-CD40L (L) as indicated. Co-cultures were incubated for 3, 6, 12 and 24 hours in DR 5% supplemented with 5% of FCS and 1% L-glutamine, in 10cm^2 culture dishes. After each of incubation time, cells were lysed by using 2x SDS-lysis buffer and protein concentration was determined. Whole lysates, following normalisation on the basis of CK8 and CK18 expression (see chapter 3) were separated by western blotting under denaturing conditions by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and then immunoblotted onto a PVDF membrane. The PVDF membrane was probed for overnight at 4°C with primary polyclonal antibody (anti-thioredoxin-1) in TBS/Tween 0.1% (1:500 dilution) and then with an anti-CK8 antibody in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one hour with secondary antibody [goat anti-rabbit IgG IRDye 800 (1:10000 dilution)] for thioredoxin detection. Antibody binding was visualised at 700nm and 800nm using an Odyssey™ Infra-red Imaging system CK8/18 and CK8 were used as loading control for HCT116 and SW480-CD40, respectively.

Keys: NH: 3T3Neo co-cultured with HCT116

NS: 3T3Neo co-cultured with SW480-CD40

L: 3T3CD40L

LH: 3T3CD40L co-cultured with HCT116

LS: 3T3CD40L co-cultured with SW480-CD40

5.9 Summary:

The results presented in this chapter provided a series of observations on the mechanisms of mCD40L-mediated apoptosis:

- The objective of this chapter was to further characterise the intracellular signalling pathway responsible for CD40-induced apoptosis in CRC cells by studying the role of ROS and associated signalling mediators.
- CD40 ligation promoted rapid generation of reactive oxygen species in CRC cells. ROS production after CD40 ligation in CRC cells was detected with H₂-DCFDA at 30 min post ligation.
- The results demonstrated that the ability of the NADPH oxidase inhibitor DPI and the ROS scavenger NAC to prevent mCD40L-induced apoptosis is linked to their ability to prevent the induction of ROS and thus oxidative stress.
- DPI decreased the level of caspase -3 and -7 activity in CRC cells post CD40 ligation thus providing direct evidence for the role of the Nox family in CD40-mediated death.
- Immunoblotting showed that mCD40L induced rapid phosphorylation of Nox enzyme subunit p40phox.
- Collectively, the results provided evidence that mCD40L-mediated ROS generation is dependent on the induction of the p40phox sub-unit.
- mCD40L induced TRAF3-mediated p40phox activation, which was detected rapidly at 1.5 hours post CD40 ligation. Hence TRAF3 may trigger ROS production via its interaction with p40phox.
- TRAF3 KD prevented the expression of p40phox after 3 hours post CD40 ligation.
- Inhibition of NOX by pharmacological drug (DPI) abrogated TRAF3 induction at 3 hours following mCD40L ligation thus indicating the importance of Nox-mediated ROS generation in potential stabilisation of TRAF3.
- Inhibition of NOX by DPI also abrogated Bax induction in CRC cells.

- For the first time ASK1 phosphorylation in response to mCD40L-mediated CD40 ligation was also demonstrated and it occurred at 1.5 hours which is the point when maximal ROS elevation was detected.
- Inhibition of NOX by DPI abrogated ASK1 induction at 3 and 6 hours as determined by immunoblotting in CRC cells.
- Inhibition of Nox reduced JNK expression in CRC cells post CD40 ligation.
- The results also demonstrated that Thioredoxin-1 (Trx) expression appeared to be abrogated by mCD40L-CD40 ligation beginning with low expression at 6 hours with a further gradual decrease over 12, and 24 hours intervals.
- The results demonstrated that NOX has an important role in regulation cell apoptosis in response to the mCD40L-CD40 ligation.
- Results suggested that for the first time CD40-mediated death by membrane CD40 ligand in carcinoma cells is dependent on ROS induction by NOX and it involves activation of ASK-1 which in turns would be expected to trigger MKK activation and subsequent phosphorylation of JNK and p38 (as reported in the previous chapter).

CHAPTER 6: Discussion

6.1 Thesis background and rationale

It has been widely reported that CD40 ligation by CD40 agonists has the potential to regulate tumour epithelial cell growth (reviewed by (Eliopoulos and Young, 2004, Tong and Stone, 2003). However, accumulating evidence suggests that the 'quality' of the CD40 signal is very important in the functional outcome of CD40 ligation. In fact, although a number of studies have shown that soluble CD40 agonists can induce growth inhibition in carcinoma cells of various origins (reviewed by (Tong and Stone, 2003, Vonderheide, 2007), these agonists are at best weakly pro-apoptotic and only become significantly pro-apoptotic by pharmacological intervention (Bugajska et al., 2002, Afford et al., 2001, Ahmed-Choudhury et al., 2003, Eliopoulos et al., 2000, Hess and Engelmann, 1996). By contrast, work from our laboratory has demonstrated that CD40 ligation by cell-surface presented agonists and membrane CD40L (mCD40L) induces extensive apoptosis in carcinoma cells (Bugajska et al., 2002, Georgopoulos et al., 2007, Georgopoulos et al., 2006, Hill et al., 2008a). whilst sparing their normal epithelial counterparts (Lievens et al., 2009, Shaw et al., 2005, Bugajska et al., 2002). The necessity to deliver the signal in membrane, rather than soluble form, to achieve maximal pro-apoptotic capacity has also been supported by more recent work by Young and colleagues (Elmetwali et al., 2010a, Elmetwali et al., 2010b).

Previous work from our laboratory has provided evidence that mCD40L, but not soluble agonist, constitutes a strong pro-apoptotic signal in CRC cells (Georgopoulos et al., 2007, Hill et al., 2008a); however the mechanisms responsible for the induction of apoptosis remain unknown. In order to achieve CD40 ligation by membrane ligand (mCD40L) experimentally, target, epithelial cells are co-cultured with third-party cells engineered to express CD40L. At the beginning of this project, and following the move of the Georgopoulos laboratory to Huddersfield University a) it was essential to ensure the reproducibility of the co-culture system and thus b) part of the work aimed to establish and optimise a series of experimental techniques, for use with this co-culture system, that would permit accurate and reliable detection and quantification of mCD40L-mediated apoptosis based on 96-well plate format assays.

6.2 The mechanisms of mCD40L-mediated apoptotic death in CRC cells

The main aim of this thesis was to elucidate the molecular nature of CD40 signalling, i.e. the precise signalling pathways responsible for the activation of apoptosis in CRC cells. Whilst building on previous studies in our laboratory, this work has for the first time provided functional evidence for a TRAF3-Nox-ASK1-MKK4-JNK/p38 signalling pathway that drives CD40-induced CRC cell death and shown for the first time that unlike for instance urothelial cells, in CRC cells CD40 induces both intrinsic and extrinsic apoptotic mechanisms. The work has thus provided an explanation for the rapid nature of CD40-mediated death in CRC cells in comparison to other types of carcinoma cells. Of note, although previous work in our laboratory has studied the role of CD40 ligation in normal epithelial cells, as normal human colonic epithelial cells are CD40-negative both in vivo and in vitro (Gelbmann et al., 2003, Cruickshank et al., 2004), this study focused on the effect of CD40 ligation on malignant (CRC) cells. The following sections will discuss these observations in more detail.

Besides the novelty of the observations presented here relating to CD40 and more generally the TNFR field, it is important to emphasise that unlike the vast majority of available studies reporting on the role of critical pro-apoptotic mediators, such as MKKs and p38/JNK and their interactions in apoptotic signalling, the present study has analysed the expression and functional roles of such signalling mediators in a 'natural' system. This is based on endogenous protein expression and not engineered over-expression, which is the case with virtually all TNFR (and CD40) studies reported in the literature.

6.3 Membrane CD40L (mCD40L) induces rapid activation of both intrinsic and extrinsic pathways of apoptosis

This study has provided for the first time evidence that unlike the mechanism of CD40-mediated apoptosis in malignant urothelial (UCC) cells that is relatively slow (apoptosis requires over 36 hours to be detectable) (Bugajska et al., 2002) and appears to involve purely the intrinsic apoptotic pathway (Georgopoulos et al., 2006), CRC cell death triggered by mCD40L is rapid and occurs within less than 12 hours and it involves both apoptotic pathways, i.e. both the intrinsic and extrinsic pathways.

It is well established that the BCL-2 family of proteins, and particularly pro-apoptotic Bak and Bax can form oligomers and insert themselves stably into the mitochondrial outer membrane permeabilisation to induce MOMP and subsequently apoptosis (Chipuk and Green, 2008). Furthermore, previous studies have suggested that mCD40L can induce the

expression of Bak and Bax proteins (Bugajska et al., 2002, Georgopoulos et al., 2006). Moreover, recent studies in our laboratory have investigated the precise regulation and functional role of Bax and Bak in mCD40L-mediated apoptosis in UCC cells (Dunnill et al, in preparation). Those studies showed that Bak and Bax are activated by 24 hours post CD40 ligation and are critical in CD40 killing in UCC cells. Interestingly, in agreement with these recent findings, in this project we confirmed that mCD40L-CD40 interaction activated MOMP, release of cytochrome c from mitochondria and induction of Bax and Bak, but this occurred far more rapidly (within less than 6 hours). In addition, the current study has confirmed the pro-apoptotic role of Bax by shRNA-mediated protein knockdown as Bax knockdown attenuated CRC cell apoptosis mediated by CD40, and decreased caspase 3/7 activity.

Previous studies have indicated that CD40-mediated apoptosis in hepatocytes requires induction of the Fas pathway (Afford et al., 2001, Afford et al., 1999). whilst a report using carcinoma cells showed that CD40 ligation may induce up-regulation of death receptor and ligand, specifically FasL and TRAIL (Eliopoulos et al., 2000). TRAIL and FasL are members of TNF-superfamily trigger apoptosis by binding to Fas (CD95), the DR4 (TRAIL-R1) and/or DR5 (TRAIL-R2) respectively. Upon binding, death receptors recruit death domain of Fas associated with the adaptor molecule (FADD), caspase-8 and triggering of the protease in an apoptosis inducing signalling complex death (DISC), which in turn triggers the apoptotic pathway (Sayers, 2011). Due to the rapid nature of cell death in CRC cells we hypothesised that apoptosis might be amplified by engaging an additional pathway, in light of the speed by which for instance TRAIL induces apoptosis in carcinoma cells, as shown previously by our group (Steele et al., 2006).

This study found that mCD40L-mediated CD40 ligation triggered rapid TRAIL induction (but not FasL upregulation) in CRC cells. Furthermore, TRAIL receptor expression (TRAIL-RII (DR5)) was also induced. Studies carried out following the completion of this work involving siRNA-mediated knockdown of TRAIL have suggested a partial role for TRAIL in apoptosis (unpublished observations). Moreover, this work suggested that TRAIL activates caspase-10 (but not caspase-8) and caspase-9 to cause extensive CRC cell death. Although classically TNF ligands activate mainly a caspase-8 associated extrinsic pathway, TRAIL-mediated caspase-10 (and not caspase-8) activation to induce caspase-3/7 and cell death has previously been reported (Sprick et al., 2002, Sprick et al., 2000, Engels et al., 2005). Interestingly also, CD40-induced TRAIL-associated death appears to take place not by paracrine signalling (as anti-TRAIL blocking antibody did not affect apoptosis) but in endogenous/autocrine fashion, which is in agreement with our previous observation for a role of 'intracellularly-signalling' TRAIL ligand in carcinoma cells (Steele et al., 2006).

These findings are suggestive for the first time of a signalling cross-talk between extrinsic and intrinsic apoptosis pathways in CRC cells, a finding supported by the observation that Bid is rapidly cleaved into t-Bid following CD40 ligation. Importantly, recent studies carried out following the completion of this work that have involved shRNA-mediated Bid knockdown demonstrated that complete knockdown of Bid results in 50% reduction of CD40 mediated apoptosis (unpublished observations). Collectively, our findings suggest that CD40 cross-talks with the extrinsic apoptotic pathway by inducing TRAIL-mediated, caspase-10 (but not caspase-8) activation, mitochondrial disruption, tBid activation, Bak/Bax induction, and activation of caspase-9 and caspase-3/7 to cause extensive and rapid CRC cell death. However, TRAIL-induced apoptosis appears to be partially driving CD40-mediated death (findings presented here and unpublished data mentioned above).

Despite the current findings on CD40-mediated apoptosis, it would be interesting to further investigate the role of mitochondria in apoptosis. Of interest would be the involvement of other mitochondrial death-related mediators, such as BH3-only proteins, or to study whether release of AIF or Endo G or SMAC/DIABLO (Norberg et al., 2010) may take place during apoptosis. SMAC/DIABLO shRNA-expressing retroviruses are available in our laboratory and could be used to produce HCT116 derivatives for future investigations. A good candidate for BH3-only protein exploration would be PUMA as it has been shown that it is important in p53-mediated induction of intrinsic apoptosis and it activates Bax during oxidative stress to induce apoptosis (Steckley et al., 2007).

6.4 Unravelling the precise signalling pathways of pro-apoptotic CD40 signalling: role of JNK and p38

The 'stress' MAPK signaling pathways, and particularly those driven by p38 and JNK, can play an important role in the control of cell death. c-Jun N-terminal kinase/ stress kinase (JNK) can play a critical role in activating apoptosis (Shen and Pervaiz, 2006). JNK can be activated by several different stimuli including growth factors (Walsh et al., 2002), cytokines (Raingeaud et al., 1995) and stress factors (Nishitani and Matsumoto, 2006). JNK kinases are activated by phosphorylation of threonine and tyrosine residues of their activation loop by MAPKK kinases and particularly MKK4 and MKK7, which are themselves activated as several MAPKK kinases (such as ASK-1) (Kyriakis and Avruch, 2001, Kyriakis and Avruch, 2012). On the other hand, p38 kinase isoforms can be activated by phosphorylation of threonine and tyrosine residues of their activation loop by MAPK kinases MKK3, 4 and 6 (Brancho et al., 2003, Derijard et al., 1995). The MAPKKs involved in the p38 and JNK pathway are mainly ASK1 and MLK3 (Zarubin and Jiahuai, 2005).

We showed here for the first time that CD40 induced rapid upregulation of both p-JNK and p-p38 in CRC cells. Although the activation of JNK is in agreement with mCD40L-mediated JNK activation in UCC cells (Georgopoulos et al., 2006), in this work interaction of mCD40L with CD40 on CRC cells caused both JNK and p38 activation and we also showed that this activation is necessary for apoptosis, whilst our functional blocking studies implied that JNK might be acting downstream of p38 in the CD40 signalling axis (p38 activates JNK, which in turn induces apoptosis, while JNK does not appear to activate p38) – although further experiments would be required to definitively clarify this.

JNK and p38 MAPK activation mediated by mCD40L directly regulated Bax and Bak expression hence these pro-apoptotic mediators appear to be induced transcriptionally rather than being stabilised at the protein level. Similarly, p38 and JNK appeared to be responsible for TRAIL induction in a similar fashion. We have also shown that cytochrome c was released during CD40-mediated apoptosis, so we speculate that this protein in turn creates a complex (apoptosome) using Apaf1 and pro-caspase-9, and that this complex then proceeds to activate caspase-3/7 (and CAD) through the mitochondrial pathways of cell death (Shiozaki et al., 2002).

6.5 Insights into the regulation (and functional role) of TRAFs and MAP2Ks in mCD40L-induced CRC cell apoptosis

Having identified for the first time that CD40-mediated signalling in CRC cells engages both extrinsic and intrinsic pathways with p38 and JNK being responsible for induction of apoptosis by Bak and Bax, which is triggered at least in part by TRAIL induction, t-Bid mediated cross-talk to mitochondria, and induction of caspase-10 and caspase-9 dependent death, the study sought to identify the precise, more receptor-proximal (early), CD40-induced signalling events.

As described previously (Introduction), the signals induced by CD40 are partly mediated through a family of proteins. These molecules include members of the TRAF family (TRAF-1, -2, -3, -4, -5, -6) (Aggarwal, 2000). Several studies have demonstrated that ligation of CD40 leads to quick recruitment of these TNFR-associated factors (TRAFs) molecules from the cytoplasm to the receptor's cytoplasmic domain. TRAF1, TRAF-2, TRAF5, and TRAF6 tend to act as positive regulators of CD40L-CD40 signalling, whereas TRAF3 has been implicated as a negative regulator (Hauer et al., 2005). However virtually all such studies, with the exception of TRAF regulation by CD40 in normal and malignant urothelial cells reported by our laboratory (Georgopoulos et al 2006; Dunnill et al, manuscript in

preparation), are reports on CD40-mediated effects in lymphoid cells (Bishop et al., 2002, Bishop, 2004, Bishop et al., 2007).

Here we show for the first time that TRAF1, -3, and 6 were induced by mCD40L-CD40 interactions in CRC cells. Our findings documented that the regulation of TRAF3 and -6 by mCD40L was rapid as TRAF induction was observed as early as 1.5 hour post receptor ligation. Our studies have not only demonstrated a novel pattern of TRAF regulation in CRC cells but have also revealed for the first time that TRAF3 has an essential role in CD40-mediated CRC cells death as shown by stable TRAF3 knockdown experiments.

TRAF3 is a CD40-associated adaptor protein which relays signals for the activation of MAPKs. In general, such signals can generate multifaceted cell responses ranging from epithelial cell death to cell growth and survival (Häcker et al., 2011). TRAF3 has been shown in some instances to trigger growth inhibition, yet in others apoptotic signals in epithelial based models, although the mechanistic explanations for this are largely unknown (Eliopoulos et al., 1996, Dadgostar and Cheng, 2000). The ability of TRAF3 to mediate apoptotic signals is likely to be mediated by lipid raft formation (Dadgostar and Cheng, 2000) with more recent evidence suggesting that this process may be directly dependent on ROS-mediated receptor clustering via acid sphingomyelinase activation and ceramide production (Zhang et al., 2007, Zhang et al., 2006).

Based on expression detection analysis and stable shRNA-mediated knock-down experiments, this study has provided evidence for the first time that TRAF3 plays a critical role in CD40-mediated apoptosis (see Chapter 4) and has shown that mCD40L-CD40 interactions cause TRAF3 induction in CRC cell lines that naturally express CD40 or are engineered to express the receptor. Although the study focused exclusively on mCD40L as a ligation signal, soluble CD40 agonists do not stabilise TRAF3 as effectively as does membrane agonist, further supporting the notion that CD40 cross-linking is a key factor for high TRAF3 expression (Elmetwali et al., 2010b, Georgopoulos et al., 2006). Moreover, we have shown that TRAF3 is central in the induction of apoptosis as not only does it attenuate apoptosis, but it also specifically abrogates p38 and JNK activation, induction of Bak/Bax and caspase-3/7 activation. Therefore, despite the existence to a dual apoptotic pathway being engaged in CRC cells, TRAF3 appears to be central in both of these signalling axes. These findings have therefore provided novel insights into a molecule the function of which in both normal and malignant epithelial cells has remained relatively unknown; they are also in agreement with a central role for TRAF3 in CD40-mediated UCC cell death (Georgopoulos et al 2006; Dunnill et al, manuscript in preparation), even though the precise molecular pathways driving CD40-mediated apoptosis in UCC and CRC cells appear to be distinct.

Finally, we also showed that CD40 ligation induced activation of MAPKKs MKK4 and MKK7 as shown by immunoblotting. These results are also in agreement with our recent findings in UCC cells (Dunnill et al, manuscript in preparation) where we showed that MKK4 and MKK7 are induced by CD40 in a TRAF3-dependent fashion, with MKK4 being critical in apoptosis.

It should be noted that because of the previously reported important role of TRAF3 in CD40 killing in UCC cells (Georgopoulos et al 2006; Dunnill et al, manuscript in preparation) this study did not examine the functional role of other TRAFs in CD40-mediated apoptosis. Of interest would particularly be the role of TRAF6. Notably, we have previously reported that CD40 ligation does not appear to induce TRAF6 in UCC cells although it does induce TRAF1 (Georgopoulos et al., 2006), yet TRAF1 induction appears to be a 'consequence' of CD40 ligation (our unpublished observations). Identifying whether TRAF6 is induced at the transcriptional or post-transcriptional level should form part of future work, whilst it would be important that such studies identify whether TRAF6 loss regulates CD40-mediated signalling and thus apoptosis in CRC cells.

Therefore, collectively, we have demonstrated for the first time that CD40 triggers a signalling pathway that activates TRAF3 and in turn that appears to be responsible for activation of MKK4 and MKK7. And although future studies should also address whether it is MKK4 or MKK7 (or both) that are responsible for downstream signalling, it is tempting to speculate that one of these or both (as in UCC cells) trigger downstream activation of p38 and JNK to activate pro-apoptotic CD40 signalling. Interestingly, these findings did not reveal precisely how TRAF3 is linked with/activates the MKK4/7-p38/JNK signalling axis, i.e. which MAP3K is responsible for triggering MKK and subsequently p38/JNK activation. We believe that our findings in Chapter 5 have provided the molecular basis for this and identified the missing MAP3K (discussion below).

6.6 The role of ROS and Nox in CD40-mediated pro-apoptotic signalling during CRC cell apoptosis

ROS were originally thought to be molecules that caused cell damage, through lipid, protein and DNA oxidation. Yet, it is now widely recognised that they also play a crucial, secondary messenger role in activating intracellular signalling pathways (Terada, 2006). In fact, ROS are implicated in most of the hallmarks of cancer proposed by (Hanahan and Weinberg, 2011) Hanahan and Weinberg (2011) due to their diverse effects on cellular molecules and processes such as angiogenesis, invasion and proliferation (Paletta-Silva et al., 2013).

ROS are molecules derived from oxygen often produced by the NADPH oxidase (NOX) proteins, the family of 'professional' ROS producers (Jiang et al., 2011). The family of NADPH oxidases in humans is composed of 7 members: NOX1 to NOX5, DUOX1 and DUOX2 (De Deken et al., 2000, Dupuy et al., 1999). Although the structures of the various isoforms of NOX are highly homologous, their tissue distributions, cellular and subcellular and their activation mechanisms, and therefore their physiological functions, are very different (Bedard and Krause, 2007). Nox may induce ROS in response to chemotherapeutic agents and members of the TNF-superfamily such as TNF- α or lymphotoxin (such as lymphotoxin- β receptor) (LT β R) (You et al., 2006), and this may be used to specifically target tumours cell due to their already raised oxidative stress state (Benhar et al., 2001, Jin et al., 2008). Moreover, CD40 engagement by soluble CD40 agonist (sCD40L) has been reported to trigger the production of ROS in B-cells and normal hepatocytes, which is mediated through the action of NOX (Bhogal et al., 2012, Ha and Lee, 2004, Wheeler and DeFranco, 2012).

As shown in Chapter 5, ROS are rapidly induced in CRC cells by CD40 in what appears to be a Nox-dependent phenomenon and this plays an important role in CD40-mediated killing. More specifically, CD40 activation appears to result in TRAF3-dependent p40phox activation as shown by p-p40phox immunoblotting experiments and functional, shRNA-mediated TRAF3 knockdown. We also show for the first time that CD40 regulates directly ROS scavenging mediators, as we detected modest yet clear reduction in Trx-1 expression. In agreement with the observations, CD40 triggered activation of the Trx-regulated pro-apoptotic kinase ASK-1, which provided direct molecular explanation for the importance of ROS in CD40 signalling and downstream activation of MKKs and p38/JNK.

Although an unequivocal role for TRAF3 in the recruitment of Nox has not been provided, the work shown here has strongly implicated a TRAF3-Nox interaction in CD40-killing, which is in agreement with a previous study in malignant B cells (Ha and Lee, 2004). In addition to performing co-immunoprecipitation studies to investigate whether TRAF3 and Nox indeed interact during mCD40L-induced signalling, the possible role of ROS in lipid raft formation should equally be explored in future work, as it appears that Nox activity shown in this thesis in CRC cells and ROS induction in UCC cells (Dunnill and Georgopoulos, unpublished observations) are necessary for TRAF3 stabilisation.

Through stable, shRNA-mediated TRAF3 knockdown it has been shown by this study that mCD40L-induced phosphorylation of p38 and JNK and apoptosis is TRAF3 driven. As mentioned, it has been suggested that TRAF3 signalling may be dependent on lipid raft formation and its cell membrane interaction with Nox to generate ROS (Bhogal et al., 2012,

Ha and Lee, 2004, Wheeler and DeFranco, 2012). Thus, in light of the importance of ROS in apoptosis, the current study determined that a TRAF3-Nox functional link may underpin CD40-signalling responses, which is in agreement with our recent observations in malignant urothelial (UCC) cells (Dunnill et al, manuscript in preparation).

It has been shown that CD40's intracellular domain recruits TRAF3 for NOX interaction via one of several sub-units, namely p40phox (Ha and Lee, 2004). These findings regarding TRAF3-Nox interactions were reported in B-cells via their overexpression. This study, and without being based on over-expression, suggests that this phenomenon may also occur in epithelial cells. Also in this study, through use of the Nox inhibitor DPI, it was shown that NOX inhibition prevents ASK-1 activation, expression of pro-apoptotic Bcl-2 members Bax, and mCD40L-induced apoptosis suggesting that most probably p40phox regulates CD40-mediated cell death via ROS production and ASK-1 mediated p38/JNK activation. In support of this there are more recent reports that phospho-p40phox has a specific role of ROS production in endothelial cells (Fan et al., 2009), thus enforcing this common mechanism between Nox mediated ROS production. Finally, and despite not demonstrating a direct physical interaction between Nox and TRAF3, the importance of the ROS pathway in CD40 apoptosis is further supported by the observation that stable TRAF3 knockdown attenuated p40phox phosphorylation by CD40 ligation.

Although DPI is not a specific inhibitor of the p40phox Nox subunit, but a universal Nox inhibitor, clearly DPI prevented CD40-induced CD40-mediated death (shown here) as well as ROS generation (subsequently demonstrated following completion of the current thesis – Dunnill et al, in preparation). This has provided evidence that mCD40L-CD40-TRAF3-NOX interaction utilises ROS for the activation of ASK-1/MKK/p38/JNK pro-apoptotic pathways in CRC cells. Future work should target p40-phox specifically through shRNA knockdown in order to firmly establish its role in CD40-mediated apoptosis.

ASK1 is a MAP3K that 'sits' at the top of the MKK4/JNK signalling pathway responding primarily to oxidative cell stress mediated by reactive oxygen species (ROS). ASK1 is normally suppressed by a redox protein known as thioredoxin (Trx) making it an inactive protein kinase. Trx binds ASK1 through a reduced intramolecular disulphide bridge, however the bonds are broken through oxidation, and in turn ASK1 auto-phosphorylates at Thr845 to become an active signalling kinase (Soga et al., 2012). Our findings that CD40 induced TRAF3 dependent p40phox activation, caused Trx down-regulation, and triggered ASK-1 phosphorylation are in agreement with our recent observations in UCC cells (Dunnill et al, in preparation) where we also showed that stable, retrovirus mediated ASK-1 protein

knockdown fully blocked CD40 apoptosis. It would be important that similar studies are carried out to study whether this is the case in CRC cells.

The above observations combined with the results presented in this study, provide novel mechanistic explanations on the molecular nature of the CD40 signalling 'blackbox'. It appears therefore that, despite the partial differences in the downstream signalling components and exact apoptotic pathways (intrinsic and/or extrinsic) that mediate apoptosis, induction of ROS by Nox, down-regulation of Trx and activation of ASK-1 may represent the core signalling component that drives CD40-mediated apoptosis. In UCC cells, abrogation of ASK1 via shRNA mediated knockdown not only prevented mCD40L-induced apoptosis, but also abrogated the phosphorylation of JNK (Dunnill et al, in preparation). Future studies in CRC cells could provide support to these observations and may confirm the universality of these mechanisms.

Also such experiments will help clarify the exact point in the CD40 signalling axis where the MAPK pathways diverge. More specifically, as tBid and TRAIL knockdown appear to partially abrogate apoptosis (unpublished observations), we hypothesise that at some point the MAPK/p38/JNK pathway diverges to drive on one hand transcriptional upregulation of TRAIL, activation of tBid and cross talk to the mitochondria whilst the other p38/JNK pathway directly induces Bak/Bax to also induce MOMP and mitochondrial death, the latter being more reminiscent of CD40-mediated cell death in UCC cells (Dunnill et al, in preparation). However, unlike UCC cells where the operation of only the latter pathway takes place means apoptosis requires a minimum of 24-36 hours to take place, in CRC cells there is rapid amplification of the pro-apoptotic signal and quick induction of cell death. To our knowledge, this is the first demonstration of such extensive and at the same time rapid carcinoma cell apoptosis triggered by CD40 ligation.

6.7 Future directions: utilising the CD40 pathway for therapeutic purposes

The ability of CD40 ligation to induce such extensive and rapid apoptosis in CRC cells has clear therapeutic promise. However, the requirement to use membrane presented ligand poses significant therapeutic obstacles if CD40 is to be used as a therapeutic target in colorectal cancer therapy. We have previously shown that soluble CD40 agonist has little pro-apoptotic activity in CRC cells (Georgopoulos et al., 2007); Dunnill and Georgopoulos, unpublished observations) therefore a therapeutic approach that can render a soluble CD40 agonist functionally equivalent to mCD40L would be an ideal therapeutic approach.

However, whilst this project was taking place, work in our laboratory has led to a new combinatorial approach that involved treatment of carcinoma cells with soluble CD40 agonist ('biologic') in combination with a pharmacological inhibitor of Trx (pharmaceutic) that in combination a) elevated reactive oxygen species (ROS) and b) blocked anti-apoptotic Thioredoxin (Trx)-mediated cyto-protection (against ROS) – this formed part of the “Combinatorial use of Tumour Necrosis Factor Receptor agonists with pharmacological inhibitors of Thioredoxin proteins for carcinoma therapy”, patent application # 1301928.6.

Based initially on the urothelial model, work in our laboratory showed that pharmacological Trx inhibition sensitised tumour cells to soluble CD40 agonists, that when normally used alone are non-cytotoxic to UCC cells (Bugajska et al., 2002). Notably, the soluble-agonist/Trx inhibitor combinatorial treatment was functionally equivalent to mCD40L-induced CD40 ligation. Importantly, following completion of this thesis, it was found that CRC cell lines were even more sensitive to this combinatorial therapy than were UCC cells. This observation is interesting as Trx inhibitors have been suggested to hold promise for the treatment of CRC over other epithelial tumours (Lincoln et al., 2003). Therefore the administration of Trx inhibitors combined with soluble CD40 agonists merits further pre-clinical testing to determine its universal efficacy not only against UCC, but also in CRC. In fact, we are currently testing the efficacy of this combinatorial approach in SW480-CD40 versus SW480 mouse xenografts in immunodeficient mice in collaboration with Prof Alan Melcher (LICAP, University of Leeds).

6.8 Concluding remarks

This study has for the first time identified the intracellular signalling cascade that is triggered by CD40 ligation and results in extensive apoptosis in CRC carcinoma cells summarised schematically in figure 6.1. It has identified a TRAF3-Nox-ROS-ASK1-MKK-p38/JNK pathway (that activates caspase-10 and caspase-9) as being the driving force that triggers both a TRAIL-associated extrinsic as well as the intrinsic apoptotic pathways. Thus in CRC cells CD40 demonstrates ability to induce apoptosis by pathway cross talk which permits strikingly rapid apoptosis. These findings have not only provided novel observations on the mechanisms of apoptosis triggered by the TNSRF member CD40 and also reinforced the importance of the quality of CD40 signal in determining functional outcome, but they have also raised interesting hypotheses for further biological studies. Equally importantly, the findings have also assisted in the formulation of a novel therapeutic avenue that may exploit CD40 for anticancer therapy.

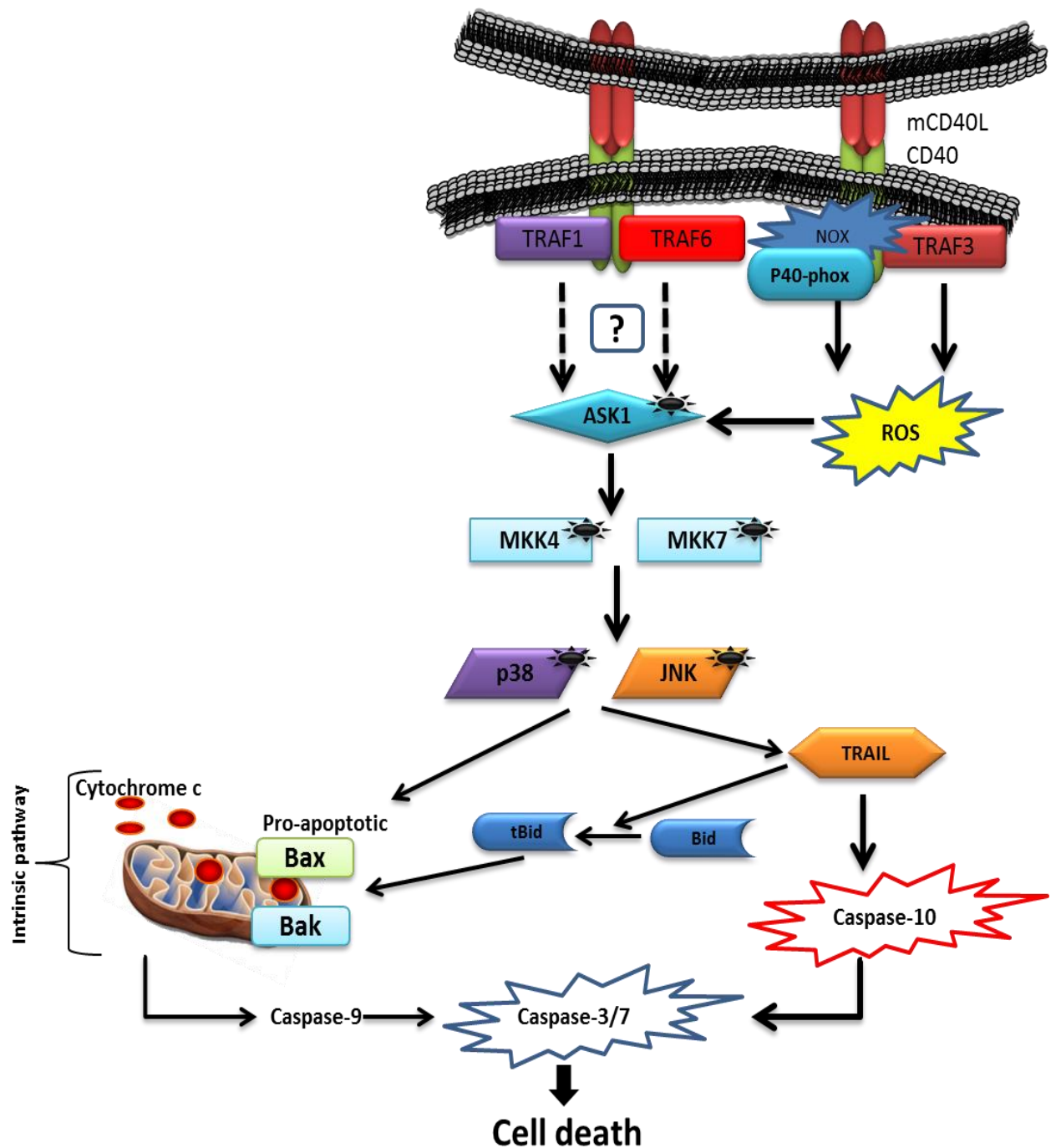


Figure 6. 1 Fintracellular signalling triggered by CD40 ligation in CRC

Intracellular signalling triggered by CD40 ligation and results in extensive apoptosis in CRC carcinoma cells. It has identified a TRAF3-Nox-ROS-ASK1-MKK-p38/JNK pathway (that activates caspase-10 and caspase-9) as being the driving force that triggers both a TRAIL-associated extrinsic as well as the intrinsic apoptotic pathways. Thus, in CRC cells CD40 demonstrates the ability to induce apoptosis by pathway cross talk which permits strikingly rapid apoptosis.

Appendix I

List of Suppliers

Supplier	Webpage/Address
Alpha labs	www.alphalabs.co.uk
Autogen Bioclear UK Ltd	Holly Ditch Farm, Mile Elm,
BD Biosciences	www.bdbiosciences.com/eu
BDH	Supplied by VWR
Bio-Rad Laboratories Ltd	www.bio-rad.com
Biosera	www.biosera.com
Calbiochem	Supplied by Merck
Cambridge Bioscience	www.bioscience.co.uk
Clontech (Takara Bio Inc)	www.clontech.com
ENZO	www.enzolifesciences.com
Falcon	Supplied by VWR
Fisher Scientific UK Ltd	www.fisher.co.uk
Gompels healthcare	www.gompels.co.uk
Greiner Bio-one Ltd	www.greinerbioone.com/en/england/start/
Insight Biotechnology Ltd	www.insightbio.com
Invitrogen Ltd	www.invitrogen.com
Invivogen	www.invivogen.com
Jencons-PLS	Supplied by VWR International
Li-Cor Biosciences UK Ltd	www.licor.com
Merck	www.merck.co.uk
Microsoft Corporation	www.microsoft.com
Millipore	www.merckmillipore.co.uk
Molecular Probes	Supplied by Invitrogen
Nalgene Europe Ltd	Supplied by Fisher Scientific
New England Biolabs (UK)	www.neb.uk.com
Pierce	Supplied by Thermo Scientific
Promega UK Ltd	www.promega.com
Qiagen Ltd	www.qiagen.com
Roche Diagnostics Ltd	www.roche.co.uk/portal/uk/diagnostics
Santa Cruz Biotechnology	Supplied by Insight Biotechnology Ltd
Sigma-Aldrich Company	www.sigmaaldrich.com
Starstedt Ltd	www.sarstedt.com
Statebourne Cryogenics	www.statebourne.com
Sterilin Ltd	Supplied by Fisher
Tebu-bio	www.tebu-bio.co.uk
ThermoFisher Scientific	www.fisher.com
VWR international	www.vwr.com

Appendix II

Reagents

1- Phosphate Buffer Saline (PBS) (Invitrogen 14200-67)

Compound	Concentration
137mM	Sodium Chloride NaCl
2.7mM	Potassium Chloride KCl
3.2mM	Disodium hydrogen phosphate Na ₂ HPO ₄
147mM	Potassium hydrogen phosphate KH ₂ PO ₄
pH 7.2 dissolved in autoclaved dH ₂ O. Prepared from 10X solution	

2- 2x SDS lysis buffer:

Amount(volume or mass)	Material
10 ml (2% W/V)	Glycerol (Sigma)
1 gram (2% W/V)	SDS
6.25 ml (Stock 1M)	Tris-HCl
0.42g /200mM	Sodium fluoride (NaF)(Sigma)
0.446g	Sodium pyrophosphate tetrabasic
2mM	Sodium Orthovanadate
Up to total volume of 50ml	Deionised water

Chemicals used in preparation of SDS sample buffer. To dissolve all chemicals, use magnetic heat block to and magnetic flea then store at -20C in 2.5ml or aliquots.

3- Standards: Cat# PN23208

Standards	Concentrations µg/ml BSA/ volume
bovine serum albumin (BSA)	125, 250, 500, 750, 1000, 1500, 2000/ 3.5ml each
Diluted in 0.9% saline and preserved with 0.05% sodium azide	

4- Tris Buffered Saline (TBS)

Volume or Weight	Chemical
1.21g	Tris (Hydroxy methyl amino methan)
8.18g	Sodium hydrochloride (NaCl) (Sigma)
Made up to 1 L by deionised water pH 7.4	

5- TBS-Tween

Volume or Weight	Chemical
1.21g	Tris (Hydroxy methyl amino methane)
8.18g	Sodium hydrochloride (NaCl) (Sigma)
Made up to 1 L by deionised water, and pH was adjusted (pH 7.4) then 1ml of Tween-20 was added.	

6- Transfer Buffer

Volume or Weight	Chemicals
1.45g	Tris(12mM)
7.2g	Glycine(96mM)
200ml	Methanol (20%)
Make up to 1 litter by deionised water (*made fresh on day of use*)	

7- The CellTiter 96® Cell proliferation assay

Volume	Chemicals
100ml CellTiter 96	Cell Proliferation Assay 5,000 assays CellTiter 96® AQueous One Solution G3581
Ready to use / 20µl/well- store the reagent at -20C for long term storage or for short up to 6 week at 4C protected from the light	

(www.promega.com)

8- Caspase 3/7 activity reagents

Components	Volume	Store components
Caspase 3/7 substrate	270 µl	-20 °C
AFC, Fluorescence reference standard	20µl (in 10mM DMSO)	-20 °C
Ac-DEVD-CHO a Known caspase 3/7 inhibitor	5 mM DMSO solution 15 µl	-20 °C
Assay buffer	30ml	4 °C
DTT	1ml	-20 °C
Lysis buffer	20ml	4 °C

The chemical compound used in the measurement of caspase 3/7 activity.

(www.anaspec.com)

9- CytoTox-Glo™ Cytotoxicity reagents

Component	Bottle/Size	Storage	Cat #
Assay Buffer	5 x10ml	-20 °C	G9291
AAF-Glo™ substrate(Alanlyle alanyl-phenylal alanyl-amino luciferin	5 bottles		
Digitonin	175 µl		

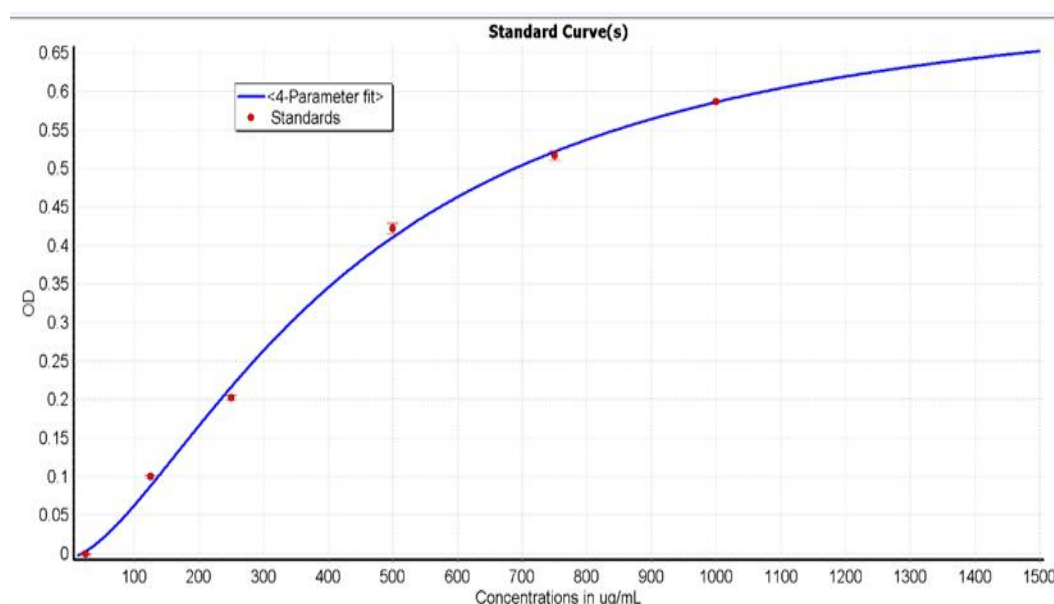
(www.promega.com)

Appendix III

The concentrations of protein for all lysates; (3T3-CD40, 3T3-neo, and SW480-CD40) are shown in the Table.

	3T3Neo	3T3CD40L	SW480-CD40
µg/ml	8010.849	6871.365	4819.18
µg/µl	8.01	6.87	4.82
Volume with 20µg	2.50	2.91	4.15
dH₂O	10.50	10.09	8.85
Sample volume	13	13	13
Reducing Agent	2	2	2
LDS Sample buffer	5	5	5
Total (µl)	20	20	20

Protein concentration in different cell lysates. The table also indicates the amount of other reagents added to the lysates preparation for western blotting.

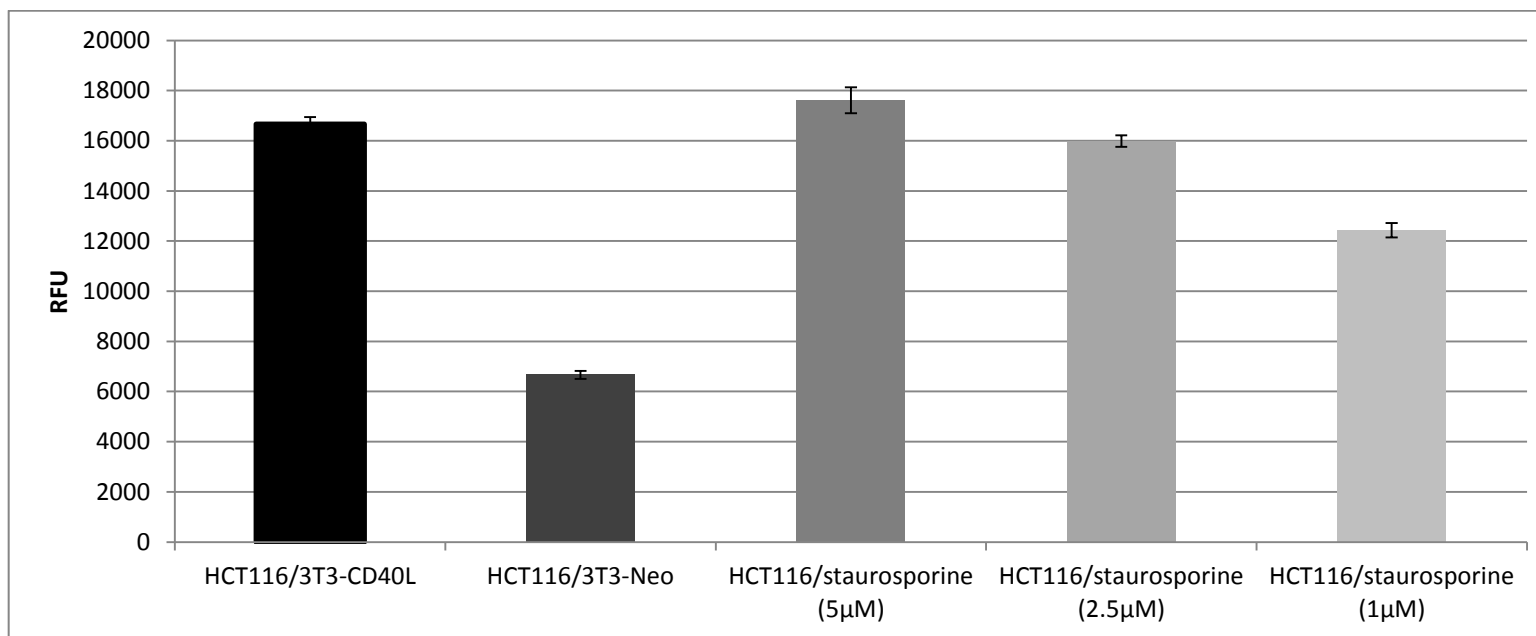


Standard curve (4-parameter-fit to measure protein concentration in different cell lines (3T3-CD40L, 3T3-Neo, and SW480-CD40).

Appendix IV

Caspase 3/7 activity with different concentration of staurosporine

HCT116/3T3-CD40L	HCT116/3T3-Neo	HCT116/staurosporine(5)	HCT116/staurosporine(2.5)	HCT116/staurosporine (1)	HCT116/3T3-Neo
16571	6779	17471	16210	12994	6446
16422	6862	17222	15534	12400	6709
16433	6680	18643	16065	12118	6506
17233	6344	17123	16141	12215	6314
16664.75	6666.25	17614.75	15987.5	12431.75	6493.75
284.125	161.125	514.125	226.75	281.125	113.75



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