Investigation of the biological mechanisms activated by CD40 in prostate cancer cells

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Abstract

CD40 is a prominent member of the TNFR family due to its ability to be expressed by and regulate the fate of not only immunocytes, but also non-lymphoid cells. Previous studies have demonstrated that CD40 ligation by cell-surface presented agonists, and in particular membrane CD40L (mCD40L), caused extensive apoptosis specifically in a variety of malignant epithelial cells (including bladder and colorectal). By contrast, soluble CD40 agonists are weakly pro-apoptotic and only become significantly pro-apoptotic by pharmacological intervention. Recent work from our laboratory has shed light onto the tumour-specificity of CD40 as well as the differences in soluble versus membrane-presented agonists in terms of pro-apoptotic capacity. As the role of CD40 in prostate cancer remains unknown, the main aim of this study was to investigate the hypothesis that the CD40/CD40L dyad regulates prostate carcinoma (PCa) cell fate and to explore the mechanisms of this in a panel of well-characterised human PCa lines.

In order to achieve CD40 ligation by mCD40L, a co-culture in vitro model was used, whereby target PCa cells were co-cultured with third-party (murine fibroblasts 3T3CD40L (engineered to express mCD40L). This mode of ligation was compared to agonistic CD40 antibody. Flow cytometry allowed detection of CD40 expression in a panel of PCa lines, comprising DU145, LNCaP and PC-3 cells. Apoptosis was detected using several assays, focusing on classical hallmarks of apoptosis (loss of cell membrane integrity, caspase activation, and DNA fragmentation). ELISA assays were employed for detection of pro-inflammatory cytokine secretion and spectrophotometry and flow cytometry were used for detection of ROS. Immunoblotting techniques were also standardised and utilised for the accurate and sensitive detection of intracellular proteins involved in CD40 signalling. Experiments using retroviruses were also employed to engineer CD40 expression in negative PCa cells.

Ligation of CD40 caused apoptosis in DU145 cells and LNCaP cells. By contrast, CD40-ve cells PC-3 were refractory to CD40 ligation. Restoration of CD40 expression restores susceptibility to CD40 apoptosis. Importantly, receptor ligation by mCD40L, and not soluble agonist, could cause cell death, as soluble agonist (cross-linked G28-5 mAb) was not pro-apoptotic. mCD40L, but not G28-5, induced rapid secretion of pro-inflammatory cytokines IL-6, IL-8 and GM-CSF, thus CD40 killing was pro-inflammatory. CD40 induced apoptosis as evident by membrane integrity loss and DNA fragmentation, both hallmarks of apoptotic death. Yet, it was found that CD40 triggers a death type that is caspase-independent. The work showed that CD40 in PCa cells triggers death that does not involve cross-talk with the extrinsic pathway, but via a direct signal that involved the mitochondrial pathway as indicated by the induction of Bak and Bax proteins. mCD40L triggered rapid induction of TRAF1 and TRAF3 whilst TRAF2 expression was downregulated. ASK1 was activated which was subsequently followed by MKK7 but not MKK4 activation and this was followed by JNK phosphorylation. Functional inhibition experiments showed that both JNK/AP-1 and p38 are important for death induction. ROS production could not be detected upon CD40 activation and functional inhibition experiments showed ROS is not critical for CD40 mediated death in PCa cells, observations raising the possibility of ROS-independent ASK1 activation. Finally, preliminary experiments using prostate cancer stem cells (CSC), well-established ‘drivers’ of PCa, showed that CSCs were CD40+ve, however, within the time constraints of this project, it was not possible to assess whether CD40 ligation could induce CSC-targeted cell death.

These findings have not only generated novel observations in terms of the ability of CD40 to induce PCa cell death, but have also added to our knowledge of the intriguingly multifaceted effects of CD40 in carcinoma cells. These fascinating observations imply that CD40, whilst engaging signalling pathways with some common intracellular mediators, its precise death pathways can differ both in their exact nature and their exact features. Moreover, in addition to providing biological evidence for the mechanisms of CD40 apoptosis, these observations may represent a promising targeted approach for PCa therapy as the ability to lead to extensive apoptosis in PCa cells. Equally importantly, by efficiently killing PCa cells and causing rapid pro-inflammatory cytokine secretion, whilst at the same time targeting what is potentially the cellular driver of carcinogenesis (CSCs), CD40-mediated killing represents a very promising potential therapeutic tool for PCa therapy in the near future.

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DEDICATION

This work is dedicated to the patients who suffering from cancer disease.

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Abbreviations

ACD	Accidental cell death
AIF	Apoptosis inducing factor
AML	Amyloid leukaemia
ASM	Acid sphingomyelinase
AP-1	Activator protein-1
Apaf-1	Apoptosis protease-activating factor-1
ASK1	Apoptosis signalling kinase 1
APRIL	A proliferation-inducing ligand
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)
Bid	BH3 interacting domain death agonist p22 BID
BID	BH3 interacting domain death agonist
BIK	Bcl-2 Interacting Killer
BIM	BCL-2 interacting mediator of cell death
BAFF	B-cell activating factor belonging to the TNF family
BrdU	5-bromo-2-dedoxyuridine
BSA	Bovine Serum Albumin
CSCs	Prostate cancer stem cells
CRC	Colorectal cancer cells
CAD	Caspase activated DNase
CLL	Chronic lymphocytic leukaemia
CARD	Caspase activation and recruitment domain
Caspase	Cysteine aspartic acid-protease

c-FLIP	FLICE-inhibitory protein
Cyto-c	Cytochrome C
CDK	Cyclin dependant kinase
cDNA	Complementary DNA
CD	Cluster of Differentiation
CD40	CD40 receptor
DIABLO	Direct IAP binding protein with low PI
dATP	2'-deoxyadenosine triphosphate
DFF40	DNA Fragmentation Factor 40/CAD
DFF45	DNA Fragmentation Factor 45/ICAD
DISC	Death inducing signalling complex
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DD	Death domain
DR	Death receptor
DcR	decoy receptor
DR4	Death receptor 4
DR5	Death receptor 5
DED	Death effector domain
DIF	Differentiation-inducing factor
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl Sulphoxide
DPI	Diphenylene iodonium
Endo G	Endonuclease G
ERK1/2	Extracellular signal regulated Kinase1, 2
EDTA	Ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
FAD	Flavine adinine dinucleotide
FADD	Fas-associated death domain
Fas	Fibroblast-associated cell-surface
FBS	Foetal Bovine Serum
GM-CSF	Granulocyte Macrophage – Colony Stimulating Factor
H_2DCFDA	6-carboxy-2,7 dichloro dihydrofluorescin diacetate

H_2SO_4	Sulphuric acid
HVEM	Herpes-virus entry mediator
IAP	Inhibitor of Apoptosis Proteins
ICAM-1	Intracellular adhesion molecule-1
ICD	Intracellular domain
IFN	Interferon
IFN-γ	Interferon-gamma
IU	International unit
IL	Interleukin
IκB	Inhibitor of NF-κB
JNK	C-jun N-terminal kinase
kDa	Kilo Dalton
LT	Lymphotoxin
LIGHT	Lymphotoxin-like exhibits Inducible expression and competes with herpes simplex virus Glycoprotein D for HVEM, HVEM being a receptor expressed on T lymphocytes
LTβR	Lymphotoxin beta receptor
Mac-1	Macrophage-1 antigen
MAPK	Mitogen activated Protein kinase
MEKK-1	MAP Kinase kinase-1
MHC-1	Histocompatibility Complex-1
MHC-2	Histocompatibility Complex-2
MMP	Metalloproteinase Matrix
MOMP	Mitochondrial outer membrane permeabilisation
mRNA	Messenger Ribonucleic acid
MMC	Mitomycine c
mg	Milligram
mM	Millimolar
NF-κB	Nuclear factor kappa B
NAC	N-acetyl cysteine
NIK	NF-κB-inducing kinase
NK	Natural killer cells
NO	Nitric Oxide
Nox	NADPH oxidase

NGF	Nerve growth factor
OMM	Outer mitochondrial membrane
PBS	Phosphate buffer saline
PVDF	Polyvinylidine difluoride membrane
RANTES	Regulated on Activation Normal T cell Expressed and Secreted
Redox	Reduction-oxidation
(PLAD)	per-ligand binding assembly domain
PG	propyl gallate
PCD	programmed cell death
RFU	Relative Fluorescent unit
RLU	Relative Luminescence unit
RIP	Receptor-interacting protein
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen Species
RT	Room Temperature
RANK	Receptor activator of nuclear factor-kB
SAPK	Stress Activated Protein Kinase
sCD40L	Soluble CD40 ligand
sLIGHT	Soluble LIGHT
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
Smac	Second mitochondrial activator of caspases
TIM	TRAF interacting motif
TNF	Tumour necrosis factor
TNFSF	TNF superfamily
TNFL	TNF ligand
TNFR	TNF receptor
TNFRSF	Tumour necrosis factor receptor superfamily
TNFR-I	Tumour necrosis factor receptor
TNFR-II	Tumour necrosis factor receptor I
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL-R	TRAIL receptor

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
TL1A	TNF-like molecule 1A
TF	Transcription factor
CTLs	Cytotoxic T lymphocytes
UCC	urothelial cancer cells
UV	Ultra violet
µl	Microlitre
µM	Micromolar
µg	Micro gram
VDAC	Voltage Dependent Anion Channel
XAF-1	XIAP- associated factor-1
xIAP	X-linked inhibitor of apoptosis protein

Chapter 1: **Introduction**

1.1. Cell death: overview

Various diseases can be underpinned by deregulation of the process of cell death. In most cases that may be too much or too little cell death. Normal tissue homeostasis can be disrupted when cell death and cell division are insufficiently balanced (Igney and Krammer, 2002). Eukaryotic cells undergo different types of cell death including, but not limited to necrosis, autophagy, cornification and apoptosis. The biochemical and morphological changes behind these various types of cell death are better characterised (Table 1) (Kroemer et al., 2008; Kroemer et al., 2009).

As discussed by Golstein and Kroemer (2007), catabolic mechanisms and signal transduction pathways could be factors that cause necrosis or cell death, but this had been widely believed to be uncontrolled and accidental (Golstein and Kroemer, 2007). Inflammation driven by the immune system could be caused by loss of intracellular contents to the extracellular environment, plasma membrane rupture, swelling of organelles and gains in cell volumes, which are morphologically characterised by necrosis (Festjens et al., 2006).

Unlike programmed cell death (PCD) type I or apoptosis, accumulation of autophagic vacuoles is referred to as type II of PCD or autophagic cell death. In this case, it could be the result of a massive destruction of cellular components due to autophagic process exacerbated by removal of elements essential for the functioning of the cell. Autophagy can also participate in the process of cell death, or as parallel and different pathways of apoptosis, or share features with apoptosis by activating or amplifying the signals' death (Scarlatti et al., 2009). Autophagy has a quality control function in the cytoplasm, because it maintains cellular homeostasis, and is an important role, so that for cell survival, this process is favourable and defined as a catabolic process that involves the engulfment of proteins and entire organelles within double membrane vesicles (Rubinstein and Kimchi, 2012). Indeed, it prevents the accumulation of structures (organelles and proteins), and autophagy can be considered favourable for cell survival through its role in innate immunity by removing microorganisms, and in adaptive immunity in generating peptides, which can be presented at the cell surface as the antigen (Levine and Deretic, 2007).

As discussed by Elmore (2007), many cancer types, autoimmune disease, ischemic damage and neurodegenerative disease can be presented as conditions in humans when the process of apoptosis is not appropriate. Apoptosis is also important for many

processes of the immune system, in tissue development and normal cell turnover, and in embryonic development (Elmore, 2007). As it relevant to this study, the initiation, execution and regulation, of apoptosis via specific signalling pathways will be discussed in depth in the following sections.

Table 1.1: The Features of the different types of cell death

	Apoptosis (type 1 PCD)	**Autophagy cell death (type 2 PCD)**	**Necrosis**
Mode of cell death	Programmed	Programmed	Programmed
Trigger and induction	Death receptor and non-death receptor, UV, chemotherapy drugs and pathogens infection	Nutrient starvation, hypoxia and infectious pathogens	Toxins, inflammation and pathogen infection
Inflammation	Non- inflammatory	Non-inflammatory	Pro-inflammatory
Morphological characteristics	Cell shrinkage, cell membrane blabbing and DNA fragmentation	Formation of autophogolysosome and self-digesting	Plasma membrane rupture and leak of cell content
Pathway	Extrinsic and intrinsic pathway	Caspase independent Caspase-independent autophagosome formation Lysosomal protease	Extrinsic, can be initiated by TNFα and TRAIL

This table shows the characteristics of different types of cell death (apoptosis, necrosis and autophagy). The apoptotic process involves alteration of membrane permeability, chromatin condensation, cell shrinkage, formation of apoptotic bodies without disintegration of organelles. Whereas, 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. Vacuolization, degradation of cytoplasmic contents which is the main characteristic for autophagy (de Almagro and Vucic, 2015; Rubinstein and Kimchi, 2012).

1.2. Apoptosis

Apoptosis was first reported by Kerr et al. (1972) as a distinct type of cell death with the term derived from the Greek language literally referring to ‘autumn leaves or petals falling’. Apoptosis is a process that can involve cellular signalling that can indirectly eliminate DNA damage and defends the human body against external insults and viruses. Apoptosis is also responsible for replacing all cells over a time period, and it

is occurs throughout the body affecting cells on developing stages (Elmore, 2007; Tait and Green, 2010).

1.2.1. Physiological roles of apoptosis

As a physiological process, apoptosis preserves tissue integrity and induces cell destruction via regulation of various anti-apoptotic genes or pro-apoptotic genes, and often the activation of enzymes, executed in a highly regulated fashion. It is essential to the remodelling, maintenance of tissue homeostasis (Evan and Littlewood, 1998) and critical in the functioning of the immune system (for instance negative selection of lymphocytes). It allows, in fact, to select and eliminate potentially dangerous cell for the body: the unwanted and dysfunctional cells. Insufficient apoptosis may be the cause of autoimmune diseases, such as systemic lupus erythematous (Su et al., 2015). The target cells of the cell-mediated immunity also die by apoptosis. Natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) recognise cells that are infected for instance. By contrast, overactive or high levels of apoptosis resulted in several diseases such as ischemic and neurodegenerative diseases . The apoptosis process could last between 12 and 24 hours, but this is normally determined by cell type and stimulus (Saraste, 1999).

Exposure of ‘factors’ that trigger phagocytosis (engulfing of an apoptotic cell body) by surrounding cells or macrophages (phosphatidyl serine exposure), cellular DNA fragmentation, disruption of the cytoskeletal structure, condensation of chromatin and condensation of the plasma and nuclear membrane are some of the classical morphological characteristics of apoptosis. Apoptosis differs from necrosis, because the contents of cells release is prevented by the apoptotic bodies that are formed, so that inflammatory responses are either not triggered (or are minimal), and compared with necrosis, this is an ‘immunologically silent’ process of cell death. Cell remains are normally ingested by phagocytes and macrophages during the process of apoptosis (Saraste, 1999).

1.2.2. Pathological roles of apoptosis

In various diseases, pathogenesis is associated with apoptotic cell death dysregulation, because cell death and cell division are delicately balanced in healthy tissue. However, cancer disturbs this balance by programmed cell death dysregulation and unrestrained cell division, whilst autoimmune diseases and neurodegeneration are also associated with apoptosis dysfunction (Thompson, 1995). Therefore, cancer, can

be caused by inhibition of apoptosis pathways, whereas, autoimmune diseases and other proliferative diseases, Alzheimer's disease, Parkinson's disease, diabetes, atherosclerosis, stroke and other degenerative diseases could be associated with higher rates of apoptosis as reviwed by (MacFarlane and Williams, 2004).

1.3. Molecular components of apoptosis

1.3.1. Caspases

When growth factors are restricted or following exposure to gamma rays, UV or ultra violet rays and other physicochemical signals that are chemical factors, apoptosis can be mediated via activation of cysteine proteases and these are called caspases (Li and Yuan, 2008). In terms of their mechanism of action, these enzymes show cleavage specificity in the context of an aspartic acid (Asp) residue and a cysteine (C) residue in their active site, hence the term caspase; these enzymes often play key role in the execution of apoptosis.

These enzymes are initially synthesised as inactive pro-enzymes (zymogens) and upon both conformational changes and proteolytic cleavage become activated (detailed below). 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 (cytotoxic T lymphocytes, CTL) that initiate the apoptotic death of target cells (Alnemri et al., 1996).

1.3.1.1. Structure and activation of caspases

The first caspase, caspase-1 or ICE, was isolated in mammals by homology with the pro-apoptotic protein Ced-3 identified in C. elegans (Miura et al., 1993). Fourteen different caspases have been identified so far (Lippens et al., 2003). 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. Their activation is regulated when protein-protein interactions are influenced by the N-terminal domain (Parrish et al., 2013; Thornberry, 1998).

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, 1998; Thornberry and Lazebnik, 1998). The two sites have different consensus cleavage caspases, but they always occur after the Asp-X bond. The activity of the large subunit needs binding to the small subunit, but the catalytic domain is contained within the large subunit. Two independent catalytic sites are contained within the association of two heterodimers that form active forms of caspase tetramers based on findings from crystallography studies (Wilson et al., 1994).

1.3.1.2. Classes of caspases

As shown in Figure 1.1, caspases that are regulatory or initiator have a long pro-domain, such as caspase-10, caspase-9, caspase-8 and capase-3, so only some caspases are associated with cell death processes as effector and direct molecules. Apoptosis execution directly involves caspases that have a short pro-domain, such as caspase-14, caspase-7, caspase-6 and caspase-3 (Li and Yuan, 2008). 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 has been reported that caspase-9 is activated by the mitochondrial apoptotic pathway, but the death receptor pathway activates caspase-10 and caspase-8, which is explained further in later sections (Amarante-Mendes and Green, 1999; Gupta, 2003).

Due to the existing of a CARD (caspase activation and recruitment domain) or DED (death effector domain) within initiator caspases's structuers which allow initiator caspases to bind to activation complexes such as to bind death inducing signaling complex (DISC) in the case of caspases 8,10 involved in extrinsic pathway . but in caspases-2 and -9 that involved in internsic pathway actvated by mitochondrial damage, the dimerisation of procaspase-9 occuers The apoptosome binds to it and then activated , therefore activates downstream effector caspases (Degterev et al., 2003; Li and Yuan, 2008; Oyadomari et al., 2002).

1.3.1.3. 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. It has been shown that DNA fragments rapidly when CAD is released by caspase-3 when caspases cleave ICAD during apoptosis, although under normal conditions CAD is an inactive complex, and ICAD inhibits CAD. Therefore, 180 base pairs of fragmented DNA are generated when the endonuclease is released (Fischer et al., 2003; Talanian et al., 1997; Timmer and Salvesen, 2007).

DNA fragmentation with a high molecular weight replaces the internucleosomal DNA fragmentation in some types of apoptosis (Kaufmann et al., 1999). It has also been reported that in certain conditions, caspase-8 cleaves the Bid protein, and to be activated, some Bcl-2 family proteins are also cleaved by caspase-8 (Li et al., 1998; Luo et al., 1998). Cellular morphology loss 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). Nearly 100 caspase substrates have been reported previously, indicating the complexity of death by apoptosis (Hengartner, 2000; Mihov, 2008). The general structural and functional characteristics of the caspases are diagrammatically illustrated in Figure 1.1.

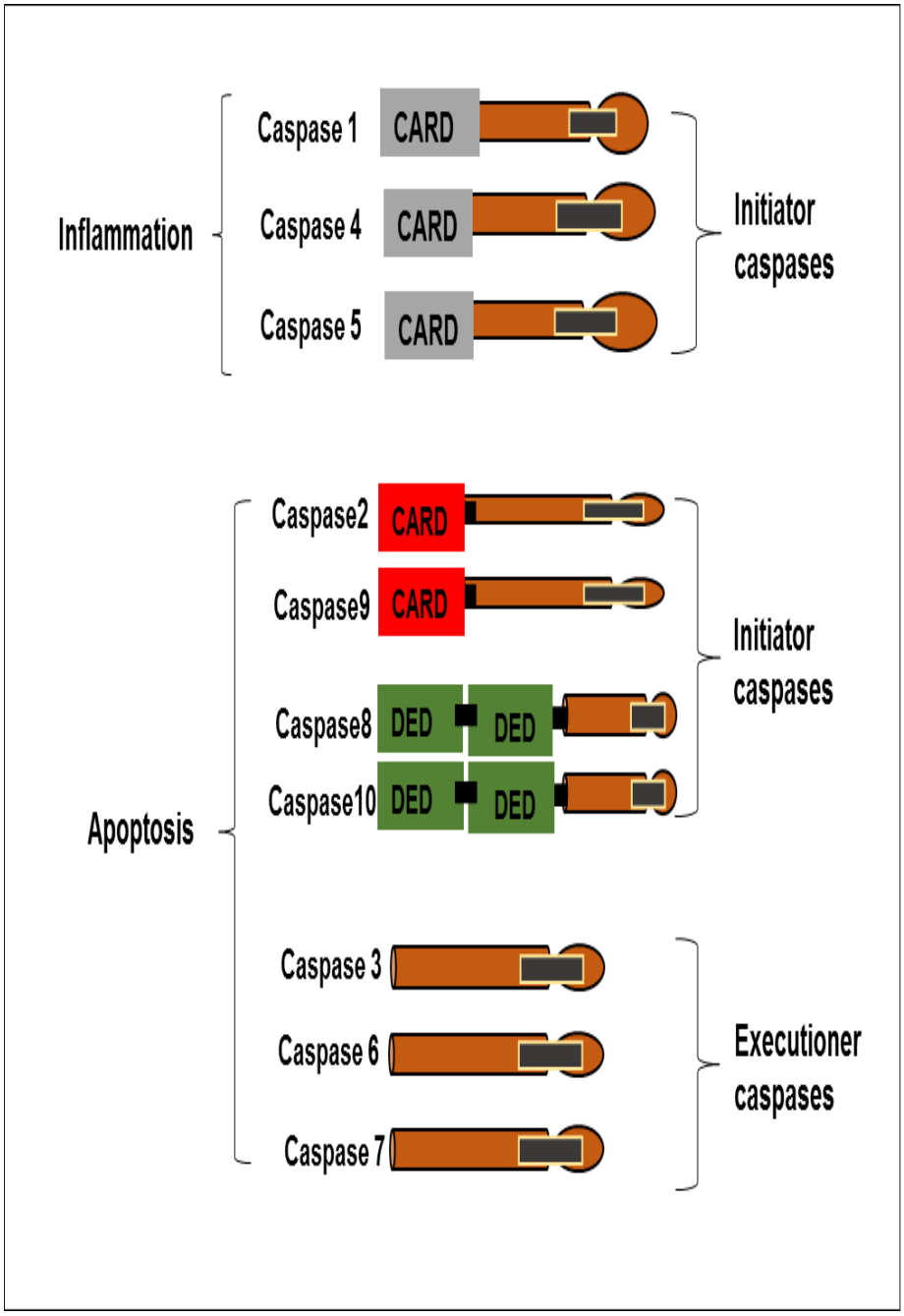


Figure 1.1: The structural and functional features of caspases

Each caspase consist of different sub-units with large, small, DED and CARD sub-units sharing structural homology. Caspases-2, 3, 6, 7, 8, 9, and 10 are known to be mainly involved in mediating cell death signalling transduction. Whereas 1, 4, 5, with others not mentioned, are termed pro-inflammatory caspases that regulate cytokine maturation during inflammation. Adapted from (Li and Yuan, 2008)

1.4. The Bcl-2 protein family

1.4.1. Protein structure

Apoptosis is regulated by B-cell lymphoma 2-like proteins, which are Bcl-2 family members. This family, containing about 15 members, can be divided into two groups according to their activity: proteins with anti-apoptotic activity and 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; Levine et al., 2008; Martinou and Youle, 2011; Ola et al., 2011).

Figure 1.2 shows anti-apoptotic Bcl-2 family members, such as BOO/DIVA, A1/BFL-1, Mcl-1, Bcl-w, Bcl-xL and Bcl-2 that have domains BH4, BH3, BH2 and BH1. There are two subgroups that divide the pro-apoptotic members with Bok/MTD, Bak and Bax within BH3, BH2 and BH1 domains, and second subgroup have BH3 proteins that containing BOD, BIM, HRK, BLK, Bik/NBK, Bad and Bid with the BH3 domain. The BH3 region seems to be heavily involved in the pro-apoptotic activity. 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), whilst a hydrophobic carboxy-terminal domain of 20 amino acids available in Bcl-2 family proteins permits attachment within the endoplasmic reticulum and within mitochondrial membranes (Krajewski et al., 1993; Ola et al., 2011; Petros et al., 2004).

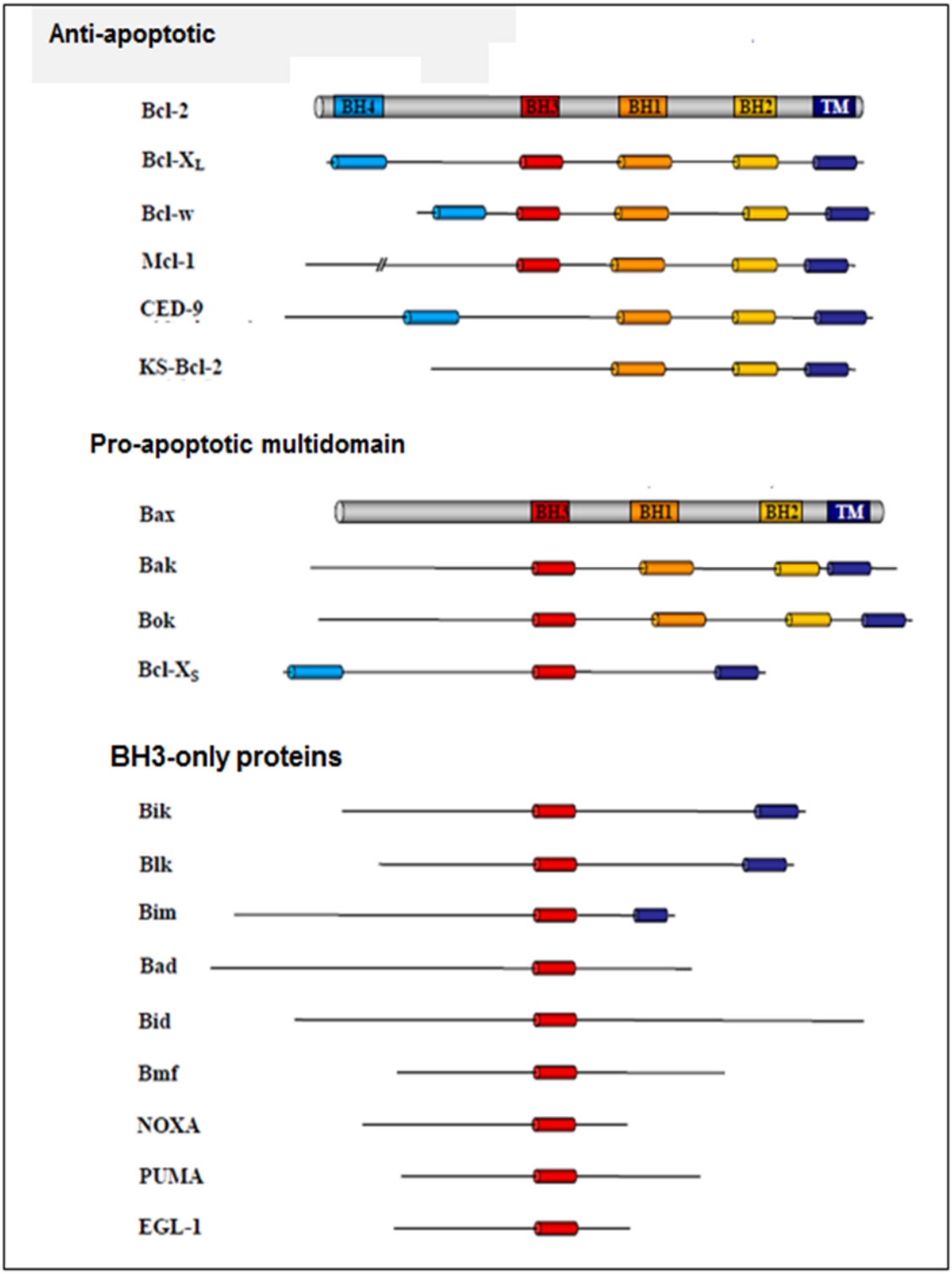


Figure 1.2: Classification of members of the Bcl-2 family.

Bcl-2 homology (BH) domains and transmembrane segment (TM) domains are represented. Bcl-2 family has been divided to three main groups; anti-apoptotic proteins, pro-apoptotic protein and BH3-only protein. Adapted from (Martinou and Youle, 2011).

1.4.2. Regulation

Death, survival factors or cytokines regulate Bcl-2 members transcriptionally and post-translationally; specific Bcl-2 family members are regulated by proteolysis or phosphorylation due to post-translational modifications, which is in addition to heterodimerisation when proteins are inhibited and homodimerisation when proteins are activated as a result of the dimerisation process. However, in some cells, the anti-apoptotic activity of Bcl-2 seems to change due to hyper-phosphorylation (Chang et al., 1997). Stress activates the N-terminal kinase (JNK) or (c-Jun), which is one example indicating that Bcl-2 is phosphorylated by kinases (Burlacu, 2003; Chang et al., 1997; Chipuk et al., 2010; Ola et al., 2011).

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 phosphatidylinositol triphosphate (PIP-3) (Zha et al., 1996) and cAMP dependent kinase (PKA) (Harada et al., 1999; Ola et al., 2011).

The Bid protein (22 kDa) is a substrate of caspase-8 and, as demonstrated more recently also of caspase-10, that has to be cleaved to be active. Several studies report that the death receptor pathway initiates the signal that is then amplified when the mitochondrial pathway is activated, when the C-terminal 15 kDa fragment is inserted in the mitochondrial membrane following cleavage into truncated Bid (tBid) or proteolysis (Li et al., 1998; Luo et al., 1998; Milhas et al., 2005).

Following specific stimuli, apoptosis is also induced when Bcl-2 and Bim interact, and for intact cells, the Bim protein is found within the microtubule complex. This dissociates from the complex after specific death signals induce death, so that without cleaving, it translocates to the mitochondria (Puthalakath et al., 1999; Youle and Strasser, 2008).

1.4.3. Mechanism of action

Dimerisation is observed frequently between anti-apoptotic proteins and pro-apoptotic proteins, so that small Bcl-2 proteins mostly dimerise. Therefore, cells that do not express more pro-apoptotic proteins are resistant to death, but those that do are vulnerable to death, and these proteins regulate apoptosis based on levels of

expression of pro-apoptotic proteins and anti-apoptotic proteins, as part of a delicate balance (Hengartner, 2000; Youle and Strasser, 2008).

The release of cytochrome c is a key event in apoptosis induction (to be discussed further in subsequent sections) and this is controlled by these regulators, with cytochrome c being prevented from release by anti-apoptotic proteins, and cytochrome c is encouraged to be released by pro-apoptotic proteins (Antonsson and Martinou, 2000; Gross et al., 1999). These events do not involve caspases, as the release of cytochrome c is not changed by caspase inhibitors. During apoptosis, Bim, Bad, Bid and Bax translocate to the mitochondria from cytosolic areas, so there is often a link between mitochondrial membrane proteins and Bcl-2 (Gross et al., 1999; Youle and Strasser, 2008).

Bax is first to be translocated from the cytosol to the 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 (Jürgensmeier et al., 1998). This insertion capacity appears to be related to the structural homology of the family members of Bcl-2 with certain bacterial toxins, allowing them to form pores in the mitochondrion. Cytochrome c is quickly released after this insertion. The VDAC or voltage-dependent anion channel is suggested by other studies to interact and facilitate the insertion capacity, so that Bid interaction is favoured due to changes in Bax conformation (Eskes et al., 2000). Other findings indicate that as Bax and anti-apoptotic proteins interact, such as Bcl-xL or Bcl-2 can inhibit (prevent or reduce) these changes (Desagher et al., 1999). Bcl-2 and Bax co-localise to the mitochondria, but Bax-induced cytochrome c release is not inhibited by Bcl-2, which is unexpected. Another study reported that within the cytoplasm, cells that overexpress Bax and Bcl-2 can survive with significant quantities of cytochrome c, but without evidence of caspase activation. Also, Bax medaited killing can be influenced by Bcl-2 that is independent and downstream of cytochrome c release (Martinou and Youle, 2011; Rossé et al., 1998).

Various studies have reported that cells entering the cell cycle can be delayed and blocked by Bcl-2, and in some cases cells enter the G0 phase (Linette et al., 1996; Mazel et al., 1996). The anti-apoptotic activity of cells is not changed when cells continue to the cell cycle (Martinou and Youle, 2011; Tsujimoto, 2003; Uhlmann et al., 1996).

1.5. The role of the mitochondria in apoptosis

1.5.1. General

The mitochondrion influences the modulation of oxidative stress and calcium homeostasis, maintains intracellular pH and redox potential, and mostly produces the energy needed by cells, so it has critically important roles for cells (Adams, 2003). But it is now clear that as a result of this major mitochondrial dysfunction is directly linked with the induction of programmed cell death. A change in 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 resulting in the synthesis of ATP results and the accumulation of lactate by stimulating glycolysis. The electrons released from the mitochondria can reduce oxygen superoxide ions, which are highly reactive oxygen free radicals (Adams, 2003).

1.5.1.1. Mechanisms: Opening Channels

The mitochondria retain the matrix components that remain intact within the inner membrane, while different constituents are released into the cytosol from the inter-membrane space when swelling is caused by various solutes and water that enters the mitochondria during apoptosis as illustrated in Figure 1-3. During the apoptosis effector phase, component release from the mitochondria to the cytoplasm is important in terms of the underlying mechanisms of apoptosis in various models, and these are discussed in the following sections.

1.5.1.2. Outer Mitochondrial Membrane (OMM) rupture

When exchanges between the mitochondrial ATP and the cytoplasmic ADP fail, this causes hyperpolarisation of the internal membrane before cytochrome c is released, (Vander Heiden et al., 1999). This mechanism of exchange is located within the inner membrane of the mitochondria for the carrier of the ANT or adenylic nucleotide and within the outer membrane for the voltage-dependent anion channels (VDAC). 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. The outer mitochondrial membrane can be ruptured as a result of osmotic swelling of the matrix caused by increased mitochondrial membrane potential (Kroemer et al., 2007; Vander Heiden and Thompson, 1999).

1.5.1.3. Mitochondrial Permeability Transition Pore

Within the mitochondrial outer membrane and inner membrane there are affixed transmembrane proteins that form the mitochondrial permeability pore (MPTP) that has high conductance and is a channel that is non-selective, which involves another mega channel for this model. Different studies show that the pore is mainly formed by the association of the ANT of VDAC and cyclophilin D. Presence of the Bax protein, change in pH or production of oxygen free radicals, reduced concentration of inorganic phosphate or adenine nucleotide, calcium and other different physiological effectors are shown to induce the opening of the pore (Crompton, 1999; Kinnally and Antonsson, 2007).

The mitochondria inner membrane experiences increased permeability when the pore is opened, which results in outer membrane rupture caused by osmotic swelling due to uncoupling of oxidative phosphorylation and a chemical imbalance between the mitochondrial matrix and the cytoplasm due to the dissipation of the proton dependent mitochondrial membrane potential. Apoptosis or necrosis that induces death is shown to be influenced by the available amount of ATP following the opening of the pore, and the pore opening is regulated by Bcl-2 family members. The opening is promoted and the membrane potential of the mitochondrial drops as a result of Bax (Marzo et al., 1998), but the opening is prevented by Bcl-2 (Kroemer et al., 1997; Kroemer et al., 2007; Shimizu et al., 1998).

1.5.1.4. Bcl-2 Family Members: Pore Formation

There is insufficient understanding as to whether cytochrome c is released as a consequence or is the cause of opening the pore, so that event chronology lacks clarity. Some findings indicate that cytochrome c release occurs before the collapse of the mitochondrial membrane potential or in the absence of the mitochondrial membrane potential (Bossy-Wetzel et al., 1998; Goldstein et al., 2000). However, transient or reversible MPTP opening could restore the mitochondrial membrane potential and could influence the permeability of the outer membrane of the mitochondria. Also, the level of ATP and the mitochondrial membrane potential fall due to cytochrome c being released from the inhibition of electron transport that is a consequence of opening the pore, or as a consequence of caspases activation (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). At the mitochondrial level, changes can be induced by cytochrome c being released early when the loop is amplified by opening the caspase-dependent MPTP. Mitochondrial membrane potential drops when cytochrome c is released, and this observation validates this model. Within the mitochondria, soluble inner mitochondrial membrane proteins (SIMP), such as caspases and apoptosis inducing factor (AIF), are released in large numbers when the outer mitochondrial membrane ruptures (Bossy-Wetzel et al., 1998; Forte and Bernardi, 2006; Shamas-Din et al., 2013).

However, various researchers have challenged the proposition that cytochrome c release causes these changes, as these could be a consequence of these changes. Another mechanism that is suggested involves a membrane pore that is formed by a subunit of diphtheria toxin from the strong homology of Bcl-xL, so that some Bcl-2 members could form a channel that could pass the protein when cytochrome c is released. Bax proteins can insert themselves, following appropriate conformational change (see previous sections), in the outer mitochondrial membrane. 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,however 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).

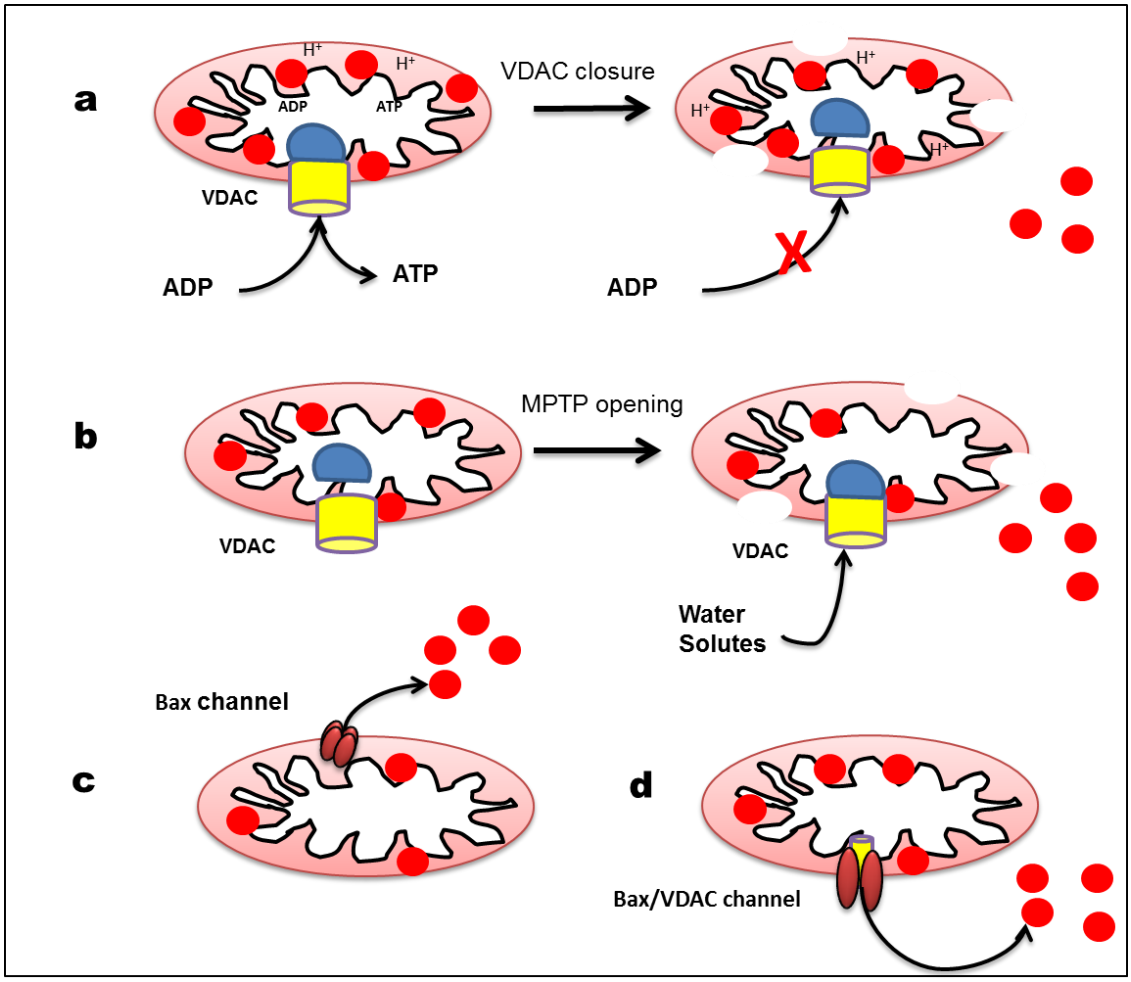


Figure 1.3: Mechanisms of mitochondrial channel opening explaining the release of cytochrome c.

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. Adapted from (Martinou et al., 2000).

1.6. Pathways 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). The intrinsic pathway or mitochondrial pathway and the extrinsic pathway or death receptor pathway form the main apoptosis caspase-dependent signalling pathways (Reed, 2000). The death receptor pathway can also be responsible for Bid activation and cleavage and apoptosis via the

mitochondrial pathway due to cross talk, but the two pathways seem to be distinctly different. However, more recently the apoptotic pathway that involves the endoplasmic reticulum-dependent caspase-12 has been demonstrated (Oyadomari et al., 2002). Apoptosis-inducing factor (AIF) is released when the mitochondria initiates a caspase-independent apoptotic pathway. The perforin/granzyme pathway, as well as the intrinsic apoptotic pathway and the extrinsic apoptotic pathway, all lead to programmed cell death. The perforin/granzyme pathway only occurs in multi-cellular organisms, and is associated with an organism's immune responses, for instance it can be mediated by lymphocytes in humans (see below) (Brunner et al., 2003; Elmore, 2007; Pardo et al., 2008).

1.6.1. The Perforin/granzyme Pathway

This apoptotic cell death pathway is mediated by two different immune cell types, more specifically Cytotoxic T cells (CTLs) and Natural Killer (NK) cells. CTLs are able to eradicate target cells by interaction between FasL/Fas and extrinsic pathway (Elmore, 2007). However, they are also able to kill antigen-bearing cells (tumour or virus-infected cells) in the absence of the extrinsic pathway, via a pathway that involves secretion of the transmembrane pore-forming molecule perforin with a subsequent release of cytoplasmic granules through the pore and into the target cell. Perforin binds to the extracellular membrane of the target cell and initiates a pore-forming complex resulting in the release of cytoplasmic granules into the target cell (Trapani and Smyth, 2002) The granules released are granzyme A and granzyme B Granzyme A acts in a caspase-independent apoptotic pathway through inducing DNA damage, and destruction of the nuclear envelope as well as the rapid loss of cellular membrane integrity. Granzyme A also inhibits the function of the protein complex Ape1,which is responsible for the cellular response in protection against DNA damage (Golstein and Kroemer, 2007). Granzyme B is a serine protease that can cleave several downstream proteins in the apoptotic pathway. It can cleave pro-caspase-10, as well as the protein complex ICAD resulting in the activation of several DNases (Goldstein et al., 2000). This activity combined with the inhibition of Ape1 from granzyme A, leads to eventual cell death. The perforin/granzyme pathway is shown in Figure 1.4.

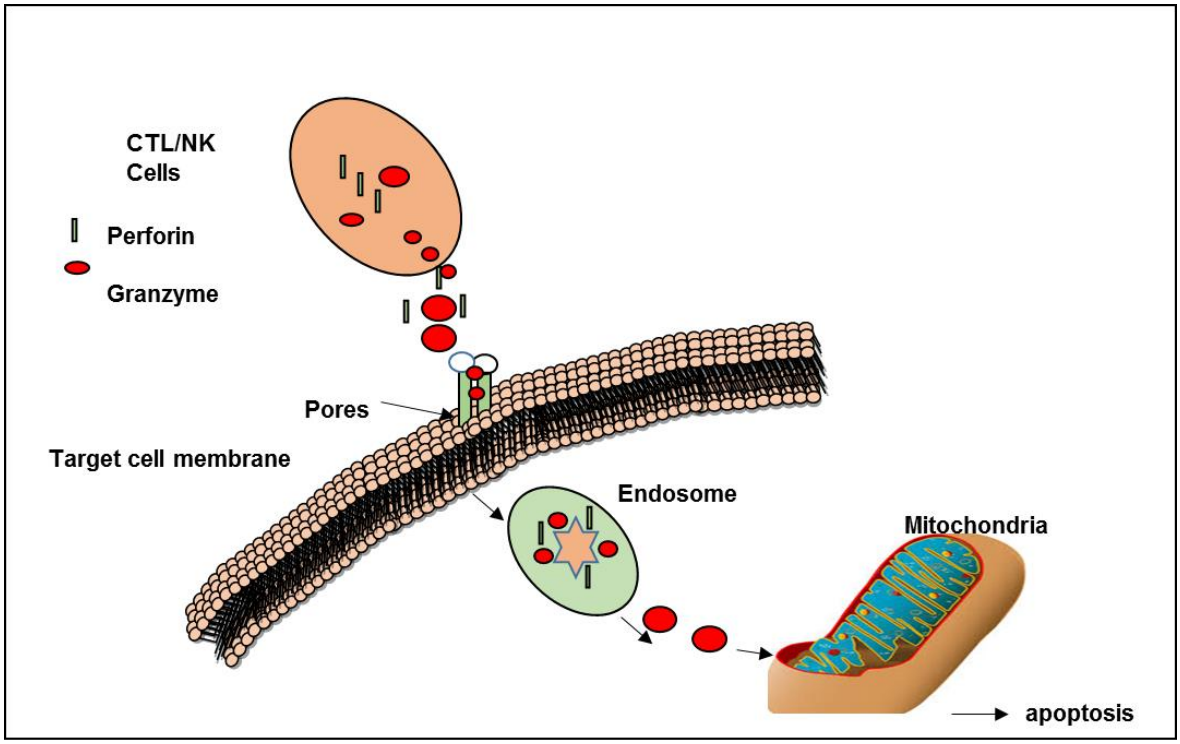


Figure 1.4: The Perforin/granzyme pathway.

Cytotoxic T lymphocytes (CTL) and Natural killer (NK) cells are effector lymphocytes that share common cytotoxic pathway that are necessary for defence against virus-infected or transformed cells. Upon antigen recognition, Cytoplasmic granule toxic a membrane-disrupting protein known as perforin. Subsequently, a family of serine protease (granzyme A or B) are secreted by CTL & NK and induced apoptosis via two distinct mechanisms. Granzyme A targets nuclear proteins and causing DNA fragmentation and the induction of apoptosis. Whereas, Granzyme B targets Bid for cell death executed by the intrinsic pathway of cell death. Adapted from (Trapani and Smyth, 2002).

1.6.2. The Extrinsic Apoptotic Pathway

The extrinsic pathway is initiated by the activation (ligation) of members of the Tumour Necrosis Factor (TNF) Receptor (TNFR) superfamily of receptors and their cognate ligands. TNF ligands are in general involved with the induction of inflammation, modulation of immune responses, apoptosis and cell differentiation and proliferation (Pitti et al., 1996; Wajant, 2002). TL1 BAFF, APRIL, TWEAK, LIGHT, RANKL, OX40L, CD137L, CD40L, CD30L, CD27L, lymphotoxin β, lymphotoxin α, TRAIL (APO- 2L), FasL and TNFα form the main identified members of the TNF ligand family. Soluble TNF ligands are formed by metalloproteinase action, resulting in cleavage of the extracellular domains (most ligands are synthesised as transmembrane precursors). Within the cytoplasmic tail, a death domain (DD) and extracellular region or cysteine-rich extracellular pattern characterise the transmembrane proteins that form the TNF

receptor family, and ligands bind to these receptors in the form of trimers. Therefore, receptor trimerisation is one of the requirements for these ligands to initiate cell death (Albarbar et al., 2015; Locksley et al., 2001; Pitti et al., 1996). Not all of these ligands induce cell death; for instance the death receptor (DR) subfamily and their ligands, TRAIL, FasL and TNF*a*, can be involved in cell survival, as well as CD40L, CD30L and CD27L. Caspases are in most cases activated by the TNFR family members that induce death. The commonality point between the, extrinsic and intrinsic pathway performed the same end goal which led to programed cell death, by activating a serine enzyme known as caspase-3 (Gaur and Aggarwal, 2003; Longthorne and Williams, 1997).

1.6.2.1. Fas Ligand (FasL) and CD95/Apo-1/Fas

Expression of CD95L (CD178/Apo-1L) or transmembrane ligand FasL is inducible and in lymphocytes where the CD95 (Apo-1) or glycoprotein Fas transmembrane receptor is constitutively expressed. Various types of cells show Fas expression, and the ligand can bind with the receptor and becomes a soluble protein when released from the cell surface. Receptor activation, receptor trimerisation and multimerisation occur following receptor binding with ligand. Subsequently, Fas-associated death domain (FADD) that binds with Fas, which is enabled by 80 amino acids contained within the death domain (DD) of its cytoplasmic domain, although no intrinsic enzymatic activity is evident. Pro-caspase-10 and FADD like ICE (FLICE) also known as pro-caspase-8 can bind with FADD, which also has a death effector domain (DED) and a DD. Apoptosis is initiated by enzymatic activation as a result of death-inducing signalling complex (DISC) that is a complex that is formed (see figure 1.5). Pro-caspase-7, pro-caspase-6, pro-caspase-3 and other pro-caspases are activated when the complex releases active caspases. The characteristic of apoptosis-associated cellular changes that are caused by the activation of various substrates are induced by the effector caspases (Gupta, 2003).

1.6.2.2. The TNF-α/TNF-R pathway

Signals including infection initiate responses of activated lymphocytes and activated macrophages which then secrete TNF-α, and this causes the activation of various signalling pathways. Ligand mediated receptor activation engages TNF-R1 and TNF-R2 receptors. Both are transmembrane receptors, which differ in their cytoplasmic domain. The DD domain can initiate a death signal induced by TNF-R1, but cell survival signals are also induced by both receptors. Activation of apoptotic transcription factor

AP-1, and anti-apoptotic transcription factor NF-kB can be caused by binding of the receptor to TNF-α (Hsu et al., 1995). The signalling events responsible for apoptosis activation by TNF- α and TNFR1 are illustrated in Figure 1.5.

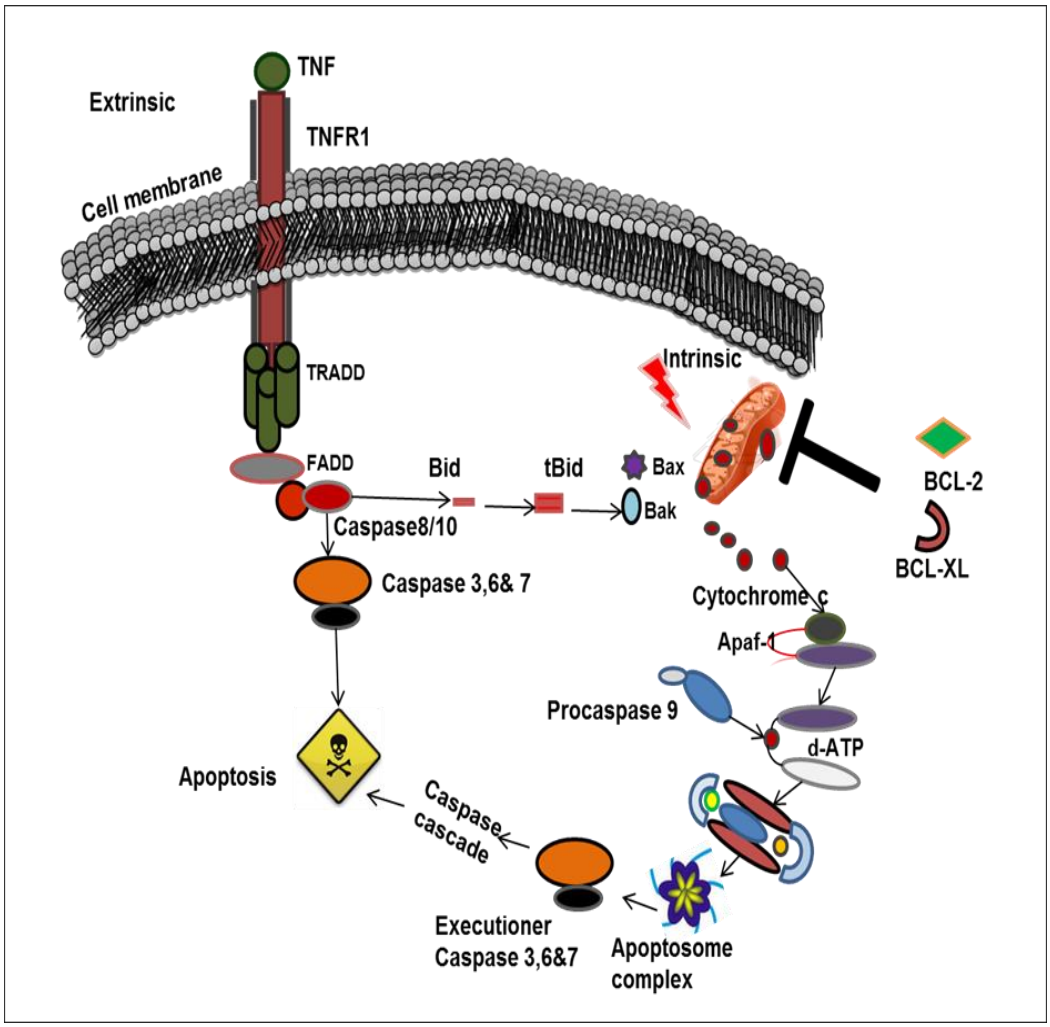


Figure 1.5: Extrinsic and intrinsic apoptosis pathways.

The main apoptotic pathways are intrinsic and extrinsic. The extrinsic pathway mediated by induction of death receptor such as TNFR1 that is expressed on the cell surface after ligation with its ligand TNFα, leading to recruitment of adaptor molecule TRADD, which facilitates binding of FADD to the receptor complex and then activate caspase 8. The intrinsic pathway is activated by mitochondrial disruption with subsequent cytochrome c release. This pathway is initiated by various signals, including UV irradiation and cytotoxic drugs. The formation of apoptosome is by interaction of cytochrome c, Apaf-1, d-ATP and procaspase-9, therefore initiation of caspase cascade. Both, BCL-XL and BCL-2 act as anti-apoptotic proteins. In addition, cross talk can occur between the extrinsic and intrinsic pathways via caspase 8/10-mediated cleavage of the Bid protein. Subsequently, pro-apoptotic BCL-2 family proteins Bax and Bak located on the mitochondria are binding with active form tBid, therefore leading to cytochrome c release. Thus, Bid acts as bridge between the extrinsic and intrinsic pathways.

Binding of TNF-R associated death domain (TRADD) that is an adaptor protein is allowed following trimerisation of TNF-R1 by TNFα binding, and also through its DD

domain this adaptor recruits FADD. Similarly, as in apoptosis induced by Fas, caspase -8 or -10 will be activated by the DISC TNF-R1 / TRADD / FADD to then mediate activation of effector caspases -3, -6, -7 (Boldin et al., 1996; Hsu et al., 1996).

Notably, TNF-R1 can also activate a FADD-independent pathway via the receptor interacting protein (RIP), although this route is less common than the FADD-dependent pathway. TRADD is associated with the RIP protein. The latter is associated with the protein RAIDD (RIPK1 domain containing adapter with DD), which has a CARD domain (caspase recruiting domain); this domain also has caspases-3, -9 and -2. The DISC TNF-R1/TRADD/RIP/RAIDD is responsible for the FADD dependent activation of caspase-8 and caspase-10, but caspase-2 activation occurs independently of FADD (Karin and Lin, 2002).

RIP and TNFR-associated factor-2 (TRAF-2) are adaptor protein types that receive cell survival signals induced by TNFα. NF-kB activation can result in cell survival induced by RIP, and MAP kinase pathway activation can result in cell survival induced by TRAF-2. Within the cytoplasmic tail of TNF-R2, interaction of TRAF-1 and TRAF-2 is caused as a result of TNF-R2 binding to TNFα, but there is no DD or cytoplasmic domain within the TNF-R2 receptor. Findings suggest that TNF-R1 produces inflammatory responses and anti-apoptotic responses via TNF-R2 involvement (Declercq et al., 1998).

The process of apoptosis induced by caspase-8, FADD and TRADD proteins is similar to the process of the DR3 receptor, which has similar characteristics to TNF-R1. Apo3L is the ligand of this receptor, which differs to the TNF, as following lymphocyte activation and macrophage activation, it is synthesised, but also it is synthesised constitutively across all tissues, which is similar to the TNF (Choi et al., 2008; Janeway Jr et al., 2001).

1.6.2.3.Apo-2L and TRAIL

Tumour necrosis factor-related apoptosis-inducing ligand (Apo2L or TRAIL) is a 34 kDa transmembrane protein with similarity to TNF ligands (Walczak and Krammer, 2000). In tumour cells and other target cells, cell death is induced quickly due to the interactions between DR5, Apo-2 and TRICK or TRAIL/R2 and TRAIL, and between DR4 or TRAIL-R1 receptor and TRAIL (Mariani et al., 1997; Pitti et al., 1996). Ligand-binding to the receptor allows the interaction of this complex with adaptor proteins such as FADD or TRADD (described in last section).

As in the case of FasL/Fas, caspase activation is central in the signalling pathways induced by TRAIL-R2 and TRAIL-R1 receptors. Indeed, caspase-8 is activated by interaction of DED present in the adaptor proteins. OPG or osteoprotegerin, CdR2, TRUNDD or TRAIL-R4 and DcR1, TRID, LIT or TRAIL-R3 are identified as receptors that form part of the TRAIL receptor family. 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. Osteoprotegerin can bind to TRAIL, and as soluble receptor, it can also inhibit its action (Emery et al., 1998).

1.6.2.4. Extrinsic Apoptotic Pathway Regulation

The DISC complex activation or the DISC complex assembly shows regulation of the extrinsic pathway that mediates apoptosis. The FLIP protein (FLICE-inhibitory protein) is an isoform of 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 Two FLIP isoforms have been identified, the long ($FLIP_L$) and the short ($FLIP_S$) cellular form. Activation of caspase-8 is inhibited when presented to bind to the DISC. Both receptors appear to regulate signalling pathways shared by DR3 (TRAIL-R1), DR4 (TRAIL-R2), TNF-R1 and CD95 death receptors (Krueger et al., 2001) and their over-expression induces resistance to receptor-mediated apoptosis. In addition, activation of the extracellular signal-regulated protein kinase signalling pathway (ERK) and the transcriptional factor (NF-κB) can induce FLIP (Kataoka et al., 2000), which is one way via which they can perform their anti-apoptotic mediator function (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. To achieve trimerisation, the soluble forms of these receptors have a pre-ligand assembly domain or PLAD domain, which is different to a ligand-binding domain. It has been reported for instance that in a ligand-independent fashion, a Fas trimer can be independently assembled (Papoff et al., 1999; Siegel et al., 2000).

1.6.2.5. DR pathway amplification via cross-talk to the mitochondrial pathway

As shown in Figure 1.5, active caspase-8 can activate Bid; as a result of this mitochondrial pathway cross-talk, the death signal can be amplified in some cells. A specific receptor mechanism association with the ligand is similar to the cytosol to mitochondrial membrane Bid rapid translocation in a truncated for (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. Before activating caspase-3, Bid is responsible for inducing activation of caspase-9 and releasing cytochrome c (Wang et al., 1996).

Another protein forming the junction between the two apoptotic pathways has been identified. This is the BAR protein (Bifunctional Apoptosis Regulator), which uses caspase-8 in the DED domain and Bcl-2/Bcl-xL in a sterile alpha motif domain (SAM domain) to associate with anti-apoptotic molecules (Zhang et al., 2000). Findings suggest that serine-threonine kinase receptor-interacting protein (RIP) is involved in another Fas signalling pathway, which is separate to the influence of caspase-8 (Pitti et al., 1996).

1.6.3. The Intrinsic (mitochondrial) apoptotic pathways

1.6.3.1. Caspase-dependent Mitochondrial Pathway

Separate to the DR pathway, the mitochondrial apoptosis pathway is induced by ‘death by neglect’ (lack of growth factors), anoikis (no attachment to substrate) and various types of cellular stress, UV radiation, chemotherapeutic agents and other stress-inducing stimuli (Kroemer et al., 2007). The mitochondrion is an organelle comprising an outer membrane, a transmembrane area, an inner membrane and a matrix. Adenylic nucleotide transporter (ANT), the electron transport chain and ATP synthase are some of the proteins within the inner membrane (Szabó et al., 1995). Under normal physiological conditions, these three proteins allow the formation of an electrochemical gradient (membrane potential) by the respiratory chain. Endonuclease G, apoptosis-inducing factor protein (AIF), the Smac/DIABLO protein, pro-caspase-2, pro-caspase-3 and pro-caspase-9, and cytochrome c are contained within the inner membrane space. The mitochondrial outer membrane (MOM) has a main components called apoptogenic factors such as, cytochrome c and ATP/ADP created by its VDAC or

voltage-dependent anion channel (Shoshan-Barmatz and Gincel, 2003). Figure 1.6 shows the steps involved when the mitochondria induces apoptosis when cytochrome c is released and the mitochondrial membrane potential is changed due to permeabilization of the inner membrane, following release of the proteins into the cytoplasm (Ravagnan et al., 2001).

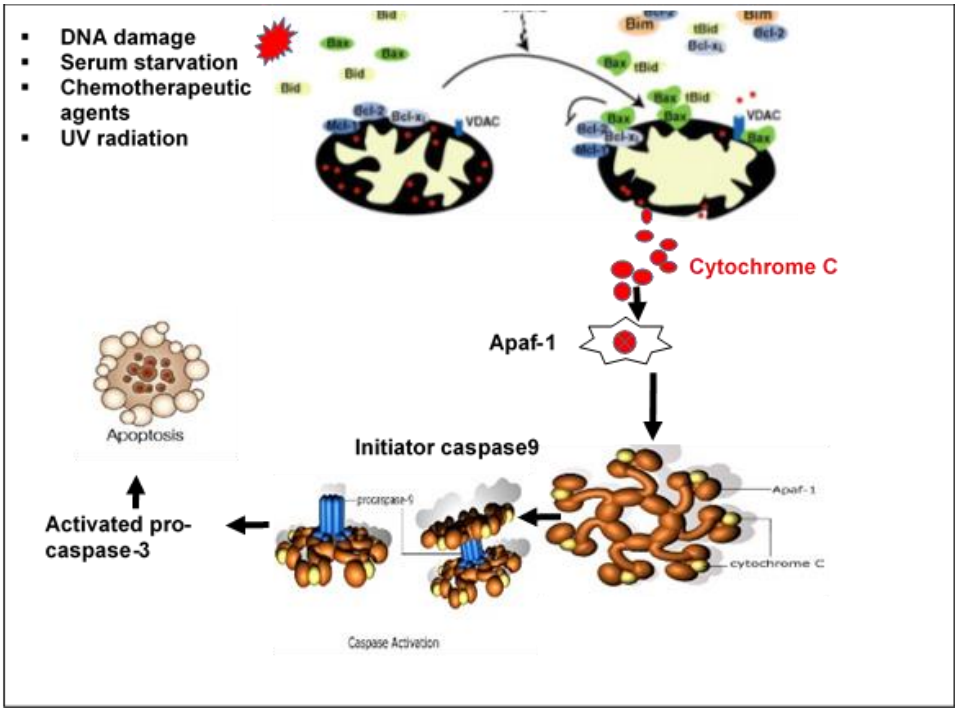


Figure 1.6: The intrinsic apoptotic pathway.

The intrinsic apoptotic pathway is stimulated by a variety of stresses. These stimuli lead to the localization and oligomerization of proteins Bax and Bak at the mitochondria. These proteins result in the release of pro-apoptotic factors from the inter-membrane space, which activate a caspase pathway resulting in apoptosis. Adapted from (Golstein and Kroemer, 2007).

1.6.3.2.Cytochrome c

This protein synthesised as a precursor, encoded by a nuclear gene; when the precursor moves to the mitochondria, it reacts to CCHL or cytochrome c heme lyase and becomes globular. When compared with the mature counterpart holocytochrome c, the precursor of cytochrome c, apo-cytochrome c lacks a covalently attached heme and has a secondary structure that is difficult to detect (Fisher et al., 1973; Guo et al., 2004). This nuclear gene product is released into a cytoplasmic pool after being synthesised on free cytoplasmic ribosomes, and certain binding sites mediate its import into the mitochondria (Zimmermann et al., 1981). Close to the amino terminus

of the apocytochrome c precursor, and through thio-ether linkages to cysteine residues, heme is covalently attached during import, and then catalysed by CCHL enzyme or cytochrome c heme lyase (Taniuchi et al., 1983). This process is associated with outer mitochondrial membrane transport of cytochrome c, and needs heme in its reduced state. The physiological function of electron transport of cytochrome c is exerted between the respiratory chain complex III and complex IV, after being sequestered in the mitochondrial intermembrane space (Ravagnan et al., 2002). As reported by Liu et al, activation of caspase-3 requires cytochrome c, and other studies also report that the anti-apoptotic protein Bcl-2 blocks caspase activation and cytochrome c release (Kluck et al., 1997; Yang et al., 1997).

It is widely reported that the formation of the apoptosome originates from release of cytochrome c into the cytosol and knockout of the gene encoding cytochrome c has confirmed the crucial importance of this protein in apoptosis (Vempati et al., 2007). For agents targeting the mitochondria via induction of cellular stress or cellular stress agents inducing activation of caspase-3 and for oligomerisation of apoptotic protease activating factor-1 (Apaf-1), cytochrome c cannot be replaced by any other cellular protein (Li et al., 2000). Normally, caspase activity is required for cytochrome c release (Bossy-Wetzel et al., 1998). However, some studies have challenged the findings regarding the release mechanisms of cytochrome c and its release kinetics, as within the outer mitochondrial membrane, Bak or Bax needs to be present for cytochrome c to be released. Goldstein et al. (2000) cite the research by Green et al, who claim that cytochrome c release kinetics occur at once and rapidly (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. In some cases, caspase activation can be induced when a small amount of cytochrome c is released from a few mitochondria, so that polarisation level of the mitochondrial membrane and the cell type used determines the apoptotic stimulus. Therefore, the MMP or mitochondrial membrane potential collapse and cytochrome c release could be linked. Findings suggest that MMP collapse and cytochrome c release occur simultaneously (Heiskanen et al., 1999), but other findings report that cytochrome c release occurs before MMP collapse (Goldstein et al., 2000). This raises an implication that MMP is maintained by the mitochondrion and remains linked with the respiratory chain within a group of cytochrome c, if cytochrome c is released before MMP collapse. Therefore, mitochondrial cytochrome c appears to

have two groups. Within the intermembrane space, cytochrome c is in a large quantity in a free form, and in the first group a small amount would support release of mitochondrial respiration, so that apoptosome formation is assured by maintaining ATP production (Dejean et al., 2005; Martinou et al., 2000).

1.6.3.3. Apaf-1

Apoptotic protease for activating factor-1 (Apaf-1) is a 130 kDa protein and for interaction with other proteins it comprises several WD40 domains (WD40 repeats) contained within the C-terminal domain and a region with high homology to Ced-4; in the amino terminal part, it has a CARD (caspase recruitment domain). Binding to cytochrome c requires WD40 repeats (Cain et al., 2002). The Apaf-1 CARD domain initially cannot interact with caspase-9, because it is not exposed, but following exposure of the CARD domain, Apaf-1 interacts with caspase-9 and changes conformation, which is shown in Figure 1.6 (Li et al., 1997). Tumour cell lines have indicated the presence of various Apaf-1 isoforms, but remain insufficiently characterised in terms of ability to activate pro-caspase-9 and in expression in tissues. Several studies have reported that Apaf-1 has at least six splice isoforms in human cells (Li et al., 1997). 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 have been reported (Fu et al., 2001; Perkins et al., 2000; Walke and Morgan, 2000).

1.6.3.4.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. The carboxy terminal domain of Apaf-1 and cytochrome c interact within the cytosol (Hu et al., 1998), so that the CARD domain, WD40 domains and important areas of Apaf-1 are unmasked. The CARD domain recruits caspase-9 the initiator caspase through its own domain and Apaf-1 multimerisation is enabled by the WD40 domains (Cain et al., 2001). Cryo-electron microscopy of the apoptosome has revealed its 3D structure (Acehan et al., 2002), which demonstrated a wheel-like structure (‘wheel of death’) formed by 7 Apaf-1 molecules at their N-terminus interacting to form the apoptosome. At the centre of the apoptosome are the Apaf-1 monomer CARD domains, so that the complex locally concentrates pro-caspase-9 by using inducer of proximity. A

holoenzyme complex is formed when pro-caspase-9 and Apaf-1 associate, which increases its enzymatic activity, and proteolytic cleavage and self-dimerisation produces active caspase-9 (Rodriguez and Lazebnik, 1999). Active caspase-9 is related to the apoptosome, but often caspase-9 is reported to be able to cleave in the apoptosome and in the cytoplasm (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 executioner caspases like caspase-3 and -7 (Acehan et al., 2002; Jiang and Wang, 2000).

1.6.3.5. Regulation of caspase-dependent pathways

1.6.3.5.1. Inhibitors of Apoptosis Proteins (IAPs)

These proteins inhibit cell death by preventing cleavage of caspases and therefore their activity (Fesik and Shi, 2001). They were originally described as viral inhibitors, but unlike the other two viral proteins, which are CrmA bovine smallpox virus and baculovirus p35 protein, the IAPs are the mammalian homologues. Baculoviral IAP repeat (BIR) domains are important in the function of IAPs, as BIRs enable binding to caspases and are important for anti-apoptotic activity, and each BIR domain has binding specificity to caspases and specific functions (Verhagen et al., 2001). Activity of caspase-9 is inhibited by the BIR3 domain, and caspase-3 and caspase-7 are inhibited by the BIR2 domain (Ekert et al., 2001).

Within this family of molecules, the protein most reported is X-linked inhibitor-of-apoptosis protein (XIAP), as it inhibits activation of effector caspase-3 and caspase-7 and initiator caspase-9. XIAP blocks the apoptotic pathway by acting on caspase-3 and caspase-7, and can bind to active caspase-9 (Deveraux et al., 1999; Wei et al., 2008).

1.6.3.5.2. IAP Inhibitors

These are HID Drosophila protein and Grim Reaper protein are similar to Direct IAP Binding protein with Low pI or DIABLO, and its counterpart Second Mitochondria-derived Activator of Caspase or Smac. Findings report that Smac/DIABLO inhibits the anti-apoptotic activity of IAP in mammals, (Du et al., 2000; Verhagen et al., 2000). Various cancer cell lines, the spleen, kidneys, liver and heart all present high

expression of Smac/DIABLO. The mitochondrial localisation signal (MLS) uses its 55 amino acids to export the synthesis of the protein in the cytoplasm to the mitochondrion with a precursor of 239 amino acids. Once in the new compartment, the localisation signal is cleaved and the protein acquires its pro-apoptotic activity by homodimerization (Chai et al., 2000). Bcl-2 family members control the release from the mitochondria when induced by various apoptotic stimuli (Adrain et al., 2001). Smac/DIABLO is associated with the death receptor pathway and inhibits directly the functions of IAPs (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. Unlike Smac/DIABLO, XAF-1 is a continuously active nuclear protein, which has a zinc finger domain allowing it to interact directly with XIAP and 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. Another inhibitory protein is high temperature requirement protein A2 or Omi/HtrA2 (Martins et al., 2002; Verhagen et al., 2001), and this precursor is a protein with 50 kDa and the MLS is contained within the N-terminal part. When imported to the mitochondria, it is cleaved and this produces a 36 kDa protein. HtrA2 is conserved during evolution as a serine protease family member. HtrA2 is released into the cytosol when UV irradiation, TRAIL, staurosporine or other agents induce apoptosis, but it is normally contained within the mitochondrial intermembrane space for human cells that are normal. Similar to Smac/DIABLO, HtrA2 binds to IAPs and XIAP in the cytosol, which encourages caspase activation. There are two mechanisms for apoptosis to be induced by HtrA2, where the serine protease activity is independent of caspase, and the second due to inhibition of IAPs to caspase activation (Hegde et al., 2002; Suzuki et al., 2001).

1.6.3.5.3. Other regulatory proteins

The nucleus, lysosomes, the Golgi apparatus and the endoplasmic reticulum (ER) are some of the strategic sites of cells where regulation of apoptotic signals involves complex proteins (Ferri and Kroemer, 2001). The nucleus does not experience the events used to remove the cell and has a role in apoptosis regulation, and is similar to XAF-1 protein activity. DED-containing DNA-binding protein or DESD is a nuclear protein that blocks transcription or inhibits activation of caspase-6 to demonstrate its

anti-apoptotic activity. 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). When Bak, Bid, Bax and Bcl-2 that are Bcl-2 family members release cytochrome c, they regulate the mitochondrial pathway (Hu et al., 1998). Therefore, when mediated by Apaf-1, Bcl-xL inhibits maturation of caspase-9, and also interacts with Apaf-1 and caspase-9, in a process simmalar to that in nematodes (Hu et al., 1998).

The heat shock proteins or Hsp are also both inducers and inhibiting factors of apoptosis. Pro-caspase-9 activation and oligomerization are prevented when Hsp-90 and Hsp-70 bind to the Apaf-1 CARD domain (Pandey et al., 2000; Saleh et al., 2000). In addition, Hsp-27 binds to cytochrome c, which blocks oligomerization of Apaf (Bruey et al., 2000).

1.6.3.6. The caspase-independent mitochondrial pathway

Mitochondria relay signals for both caspase-dependent and caspase-independent death pathways (Cheung et al., 2005). Several proteins in the intermembrane space can induce apoptosis directly without activation of caspases. Large fragments of DNA are generated when chromatin condensation and cleavage of DNA occurs after AIF the induction apoptotic factor and Endonuclease G (Endo G) are released from the mitochondria and relocated to the nucleus (Candé et al., 2002; Kroemer and Martin, 2005; Lorenzo et al., 1999; Norberg et al., 2010). Cytoplasmic reticulum organelles also can trigger apoptosis in response to cell stress by release of Ca^{2+} in cytosol (Sano and Reed, 2013), as showed in Figure 1.7.

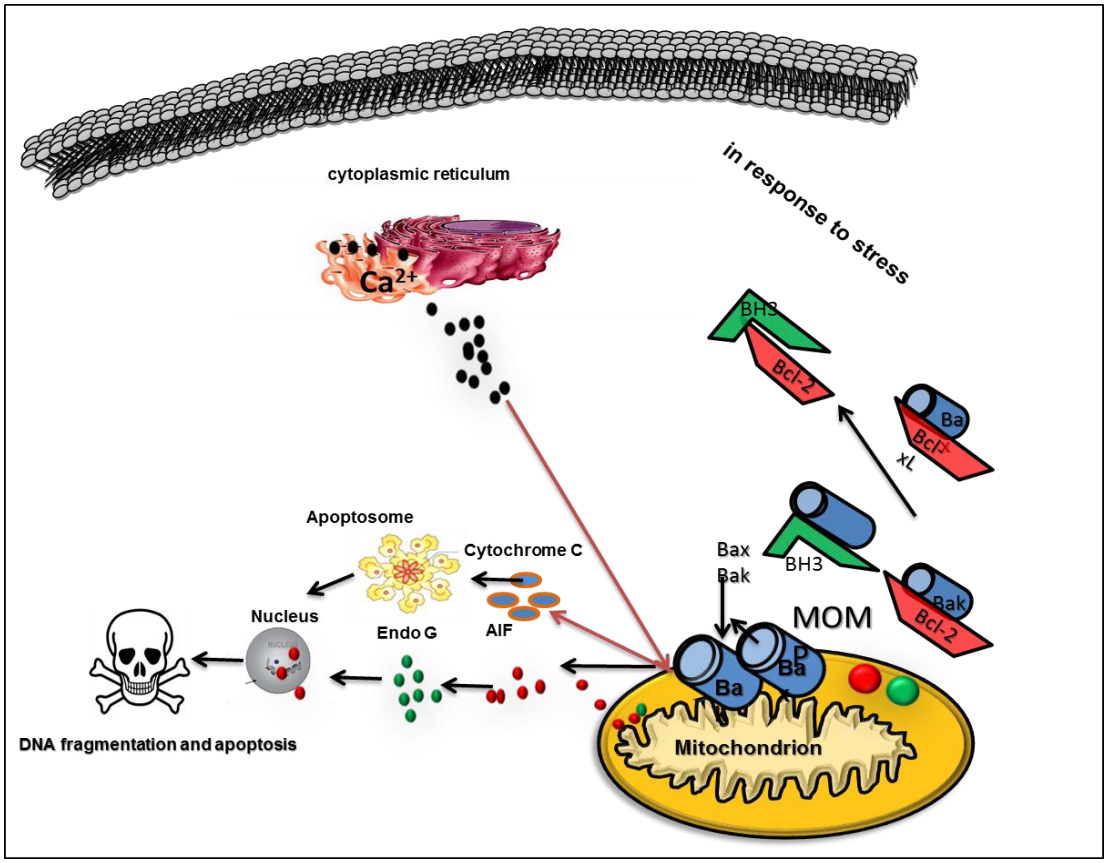


Figure 1.7: The caspase-independent mitochondrial pathway.

In response to cell stress c-Jun N-Terminal kinase transcriptionally regulate pro-apoptotic Bcl-2 members for the induction of apoptosis. In this example, BH3-only proteins facilitate Bax to the outer mitochondrial membrane (OMM) whilst also removing anti-apoptotic Bcl-2 from Bak. This causes Bak and Bax to create pores in the OMM a process known as MOMP, which, apoptosis inducing factor (AIF) and Endo G and directly cause DNA fragmentation and apoptosis in a caspase-independent manner. During ER-stress-mediated apoptosis, release of high levels of Ca^{2+} in the cytosol from ER is required for mitochondria to release cytochrome c and induce apoptosome formation and contribute to large of scale DNA fragmentation. This diagram is drawn based on information from (Di Sano et al., 2006; Norberg et al., 2010).

1.6.3.6.1.Apoptosis inducer factor (AIF)

AIF is a 57 kDa flavoprotein, the gene of which is located on chromosome X and consists 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 (Norberg et al., 2010; Ye et al., 2002).

AIF relocates from the intermembrane space to the nucleus and into the cytosol following cell exposure to pro-apoptotic stimuli (Susin et al., 2000), and before cytochrome c is released, but how the AIF relocates to the cytosol in terms of the mechanism is insufficiently understood. However, nuclear location sequences could explain the relocation to the nucleus. *In vivo* and *in vitro* experiments have been undertaken to discover how the AIF could function as an apoptogenic molecule. There appears to be a direct interaction between AIF and DNA with no sequence specificity, as AIF is transported to the nucleus through the cytoplasm, and when reaching the nucleus, chromatin condensation occurs and DNA degrades into 50 kbp fragments revealed from *in vitro* experiments (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 by firstly inhibiting the formation of the apoptosome as Hsp70 binds to Apaf-1. However, overexpression of Hsp70 in cells not expressing caspase also prevents cell death; this suggests that Hsp70 may bind to other proteins such as AIF (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 (Norberg et al., 2010; Samejima et al., 2001; Widlak and Garrard, 2009).

1.6.3.6.2. Endonuclease G

Endonuclease G is possibly involved in mitochondrial genome replication, encoded by a nuclear gene, and is a non-specific nuclease (Li et al., 2001). Endonuclease G translocates to the nucleus after passing through the mitochondria outer membrane during apoptosis (van Loo et al., 2001). Endonuclease G can act in concert with exonucleases and the DNase I in the nucleus to generate DNA fragments of higher molecular weight (Widlak and Garrard, 2005; Widlak et al., 2001), but it can also generate oligonucleosomal fragments (Samejima et al., 2001).

1.6.3.6.3. Calcium Ca2+

The endoplasmic reticulum (ER) functions as the cellular site of polypeptide folding and adjustment. The unfolded protein response (UPR) is activated as a response to obstruction to one of these processes. Furthermore, when the damage is impossible to repair, apoptotic cells death is triggered, in particular in mammalian cells. As a consequence, mobilization of ER calcium (Ca^{2+}) stores sensitizes mitochondria to direct pro-apoptotic stimuli (Di Sano et al., 2006; Sano and Reed, 2013).

1.7. The Tumour Necrosis Factor superfamily (TNFSF)

TNFSF was first identified in 1984 as a cytokine with anti-tumour effects *in vivo* and in *vitro*. The TNFSF includes cytokines that are implicated in a wide variety of diseases including tumorigenesis, septic shock, viral replication, diabetes, and other inflammatory diseases. As explained in previous sections, the family includes a number of ligands that bind with their cognate receptors which are homologous in their extracellular domain. The primary function of TNFSF is to regulate differentiation, proliferation, apoptosis or necrosis (Albarbar et al., 2015; Gaur and Aggarwal, 2003). The TNFSF members including ligands and their cognate receptors are detailed in table 1.2.

1.7.1. Tumour necrosis factor receptors (TNFRs)

TNFRs are a family consisting of approximately 40 members characterized by their cysteine-rich repeat extracellular sequence homology; many of these members are activated by specific ligands (Table 1.2). The TNFRSF can be divided into three groups, on the base of both structure of cytoplasmic region and the signaling generated by interaction with downstream signalling mediators.

The first group are the death receptors (DR) and includes six members that comprise a DD in their cytoplasmic region; Fas, TNFR-1, TRAIL-1, TRAIL-2, TRAMP and DR6. The second group of TNFRs includes 19 members characterized by the presence of a TRAFs interacting motif (TIM) domain whilst lacking a DD, and they include CD40, lymphotoxin receptors and others. The third group called decoy receptors, includes TRAIL-R3, TRAIL-R4 and osteoprotegerin (OPG), this group, is incapable of activate downstream pathways of cell death or cell survival because of absence of intracellular domains. However, these decoy receptors possess an important role, as they may compete with the other two groups of TNF-Rs for the same ligands and thus can cause signal attenuation (Albarbar et al., 2015; Russo et al., 2010).

Table 1.2: TNFSF members of ligands with their cognate receptors (Albarbar et al., 2015).

Ligand	Receptor
TNFα	TNFRI and II
LTα	TNFRI and II
FasL	Fas
VEGL	DR3
TRAIL	DR4, DR5
CD27L	CD27
CD30L	CD30
4-IBBL	4-IBB
TWEAK	Fn14
LIGHT	LT-βR, HVEM
CD40L	CD40
OX40L	OX-40
RANKL	RANK
APRIL	TACI
BAFF	TACI,BCMA
GITRL	GITR
EDA-A1	EDAR
EDA-A2	XEDAR

1.7.2. CD40 and its ligand CD154 (CD40L)

CD40 was first identified in 1984 by using an antibody raised against a urinary bladder carcinoma cell antigen also capable of binding to B lymphocytes (Karmann et al., 1995; Koho et al., 1984; Paulie et al., 1984). Subsequent studies reported a monoclonal antibody (mAb) interacting with carcinoma and B cells (mAb S2C6, antigen p50), and an antibody showing co-stimulatory effects for B lymphocyte proliferation (mAb G28-5, antigen Bp50) (Clark and Ledbetter, 1986; Paulie et al., 1985). The product of the cloned CD40 cDNA showed nerve growth factor (NGF) receptor similarity (Johnson et al., 1986; Radeke et al., 1987). CD40 is expressed by a number of immune cells (e.g. monocytes, eosinophils, basophils, dendritic cells and B cells), as well as by fibroblasts, epithelial cells, smooth muscle cells, keratinocytes and endothelial cells (Schönbeck and Libby, 2001; van Kooten and Banchereau, 1997; van Kooten and Banchereau, 2000). The cognate ligand that binds to CD40 is known as CD40 ligand (CD40L) or CD154, and is expressed on mast cells, platelets, B lymphocytes, NK cells, dendritic cells, macrophages, monocytes, eosinophils and basophils, smooth muscle cells, endothelial cells, whilst CD40L is transiently expressed on activated T-cells (Schönbeck and Libby, 2001). A common feature of all these cells is that CD154 expression is non-constitutive, but can be rapidly induced upon activation (e.g. on activated T-cells) (Armitage et al., 1992; Hollenbaugh et al., 1992).

1.7.3. Structure of CD40

CD40 expression is type I transmembrane protein and is expressed at molecular weight (MW) 48kDa, and binds with high affinity to CD40L (André 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 (van Kooten and Banchereau, 2000). The extracellular region of the molecule is formed by 171 amino acids, from a total of 255 amino acids contained within the final total protein, and the intracellular region is the location of the C-terminal domain (Naismith and Sprang, 1998). 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) (van Kooten and Banchereau, 2000). The exact arrangement of CD40 present on the

membrane surface remains unclear. The presence of a pre-ligand binding assembly domain or PLAD (Wajant, 2015) may permit a pre-assembled trimeric form with 3 CD40 molecules, whereas other studies report CD40 pre-assembled in dimers (Chan et al., 2000). The structure of CD40 is shown in Figure 1.8.

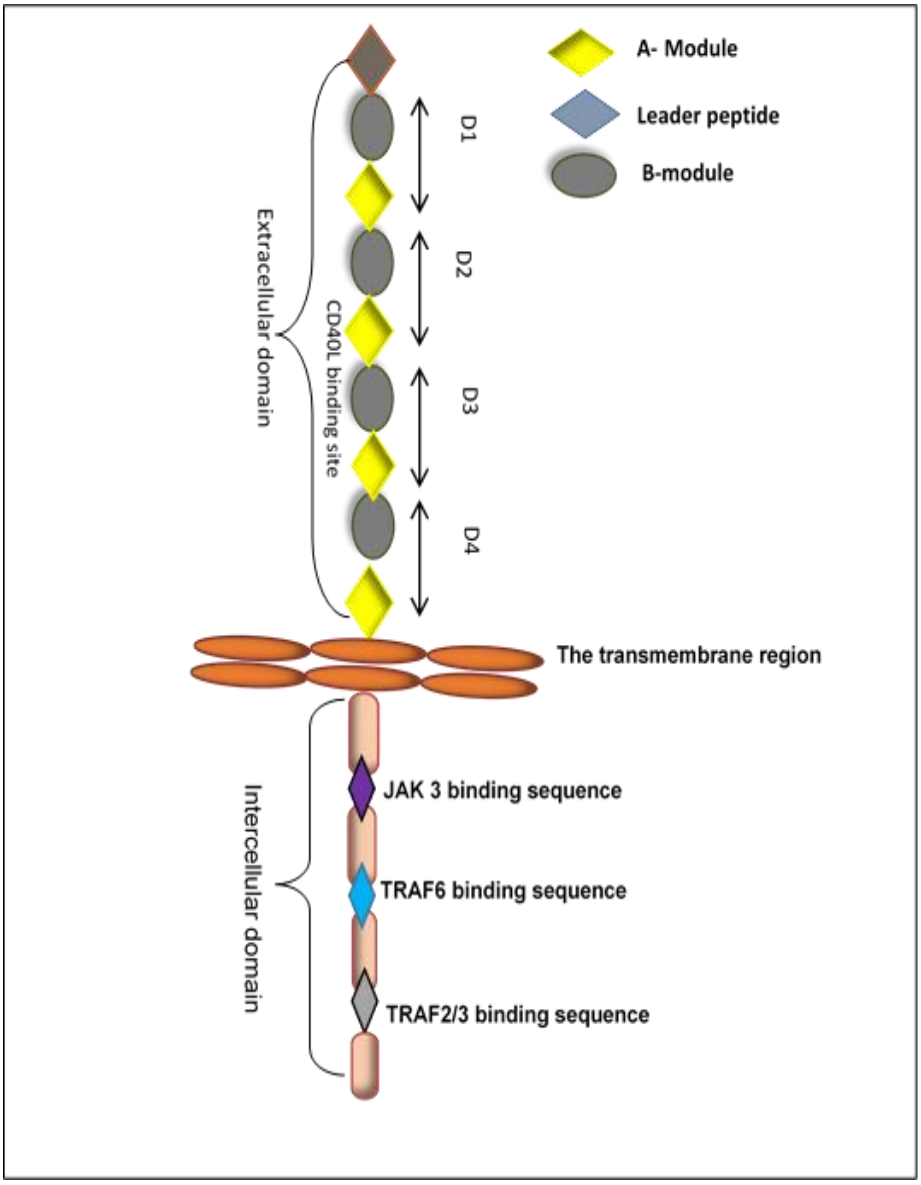


Figure 1.8: Schematic diagram of CD40 functional domains.

The cytoplasmic part of CD40 consists of TRAF 6, 2 and 3 binding site. The extracellular parts of CD40 are compose four domains each one consist of two modules. The 2nd & 3rd domains are the specific binding sites for CD40 ligand. Adapted from (Schönbeck and Libby, 2001).

1.7.4. CD40L structure

CD40L is a type II transmembrane protein of MW 39kDa (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 1.9).

CD40L comprises an extracellular region contains a C-terminal domain, 261 amino acids that form the CD40L protein and a N-terminal domain (Chakrabarti et al., 2007). On the cell membrane, three ligand monomers form a trimeric complex, (Xia et al., 2010). This structure facilitates interaction with its cognate receptor 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) that can be found, under certain circumstances, circulating in the bloodstream and this form is almost exclusively the result of an enzymatic cleavage at the membrane following platelet activation and is a functional trimer of 18kDa in size (Li et al., 2008).

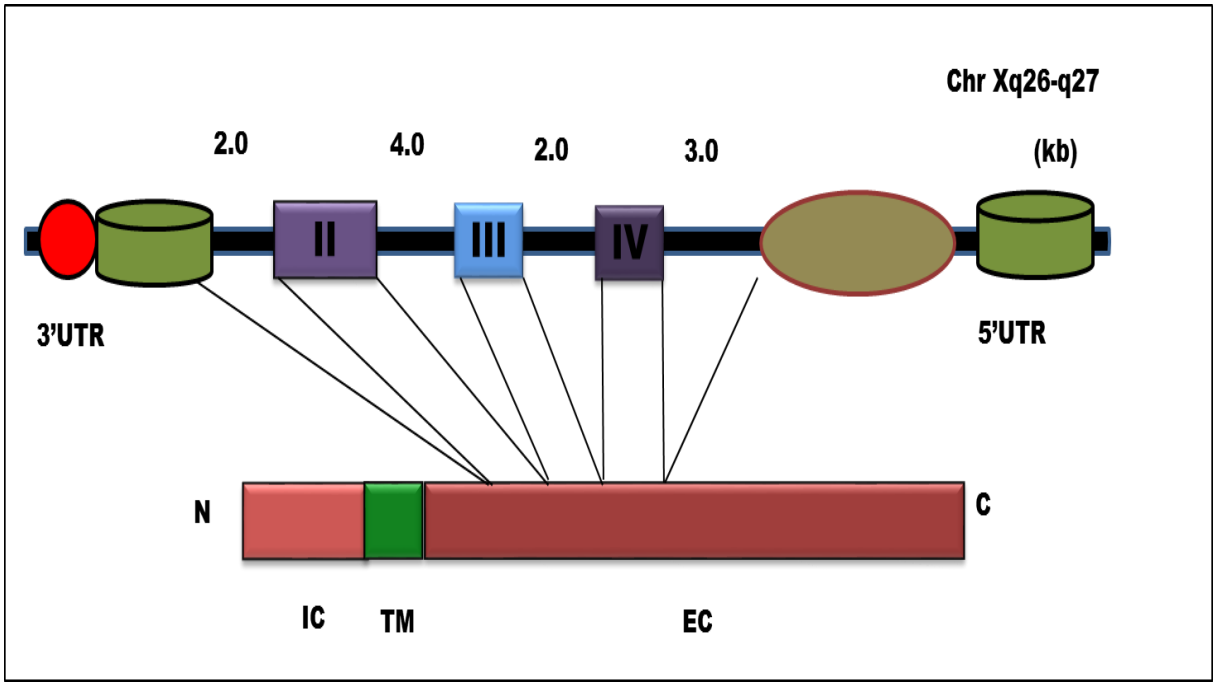


Figure 1.9: Structure of the gene and protein of human CD40L.

The gene encoding CD40L contains 5 exons coding for different regions of the protein (IC=intracellular TM= transmembrane, extracellular=EC). Schematic structure 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. Figure adapted from (van Kooten and Banchereau, 2000).

1.7.5. Interaction of CD40 with CD40L

The structure of the extracellular part of CD40L has been described by X-ray crystallography (Karpusas et al., 1995). It consists of two beta sheets with jellyroll topology that forms a symmetric homotrimer. CD40 and CD40L interaction requires Q220, R203, Y146, Y145 and K143 that are CD40L residues, and E117, E74, N86, D84 and Y82 that are CD40 residues that are identified by site-directed mutagenesis by the crystal structure of the CD40 and CD40L models (Bajorath, 1998; Bajorath and Aruffo, 1997; Bajorath et al., 1995). Thus, the CD40L binding site has been shown to be located in the second and third domains of CD40. It has been suggested that the polar interaction between the basic residues on the CD40L 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 (Singh et al., 1998; van Kooten and Banchereau, 2000). Thus, the first interaction model suggested that CD40L (always trimeric) promotes the formation of a homotrimeric complex of CD40 following its interaction with it (Anand et al., 2003). This would eventually troop the association of cytoplasmic adapter proteins and induction of intracellular signals. 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 (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 model is based on an elegant study demonstrating that most receptors belonging to the TNFRSF, 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 only upon binding of CD40L (Anand et al., 2003; Wajant, 2015).

1.7.6. Other receptors of CD40L

CD40 was long considered to be the only receptor for CD40L (André et al., 2002b; Léveillé et al., 2007). However, three other receptors have been identified, in particular integrins αIIbβ3, Mac-1 and α5β1 (Hassan et al., 2009). Although CD40 remains the main high-affinity receptor of CD40L, these other partners seem to perform a very special function in various pathophysiological conditions (Alturaihi et al., 2015). In studies of the platelet thrombus in vivo, platelet stabilisation and activation are promoted by the interaction of CD40L and αIIbβ3 integrin, which is a receptor on the platelet surface (André et al., 2002a; Prasad et al., 2003). The CD40L Lysine-Aspartic acid-Glycine (KGD) domain is responsible for CD40L and αIIbβ3 interaction. Most integrin receptors αIIbβ, have at least one KGD and αIIbβ contains a recognition domain for this pattern. During inflammation, neutrophils and monocytes adhere to the activated endothelium due to the integrin Mac-1, which also acts as a receptor for CD40L. Atherosclerosis is partly caused by neointimal formation, and at the atherosclerotic plaque, leukocytes are shown to transmigrate and adhere as a result of this interaction (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 α5β1 is the main fibronectin receptor and was more recently identified as one of the other receptors of CD40L (Alturaihi et al., 2015; Léveillé et al., 2007).

1.7.7. The Role of PLAD in CD40

It has been observed that some of members of TNF-R family assemble constitutively in the absence of their cognate ligand. Papoff and co-workers first demonstrated that ligand-independent oligomerisation of CD95 depends on the most distal extracellular domain CRD1, but not on the death domain (Papoff et al., 1999). Whereas the soluble form of the receptor homo-oligomerises, and hetero-oligomerises with the membranous form, this is not the case for a truncated form lacking the 42 N-terminal residues that compose most of the CRD1. Capacity to interact with CD95L and to initiate signalling when overexpressed at the membrane was conserved by this truncated form. Finally, a soluble truncated form of CD40 made of the 49 N-terminal amino acids is necessary and sufficient to mediate oligomerisation of the receptor (Papoff et al., 1999). Similarly, TNF-R1 and R2 self-associate in the absence of TNF-α as seen by fluorescence resonance energy transfer on living cells (Chan et al., 2000; Wajant, 2015) The putative N-terminal functional domain that mediates ligand-

independent receptor association was named per-ligand binding assembly domain (PLAD) (Wajant, 2015). The PLAD region is responsible for preformed receptor oligomerisation prior to ligand binding, and is not sufficient for initiation of intracellular signals, but is necessary for the interaction with the cognate ligand. In TNF-R1 signalling, PLAD was targeted in the treatment of inflammation (Deng et al., 2005). (Deng et al., 2005). These observations provide explanations for the resistance of lymphocytes from patients with autoimmune lymph proliferative syndrome to Fas-induced apoptosis (Siegel et al., 2000). A study demonstrated that mutations either in the ligand–binding domain or in the death domain still oligomerised with a wild type receptor, or formed mixed trimers. These defective molecules thus dominantly interfere with apoptosis induced by CD95L, but not with apoptosis mediated by agonistic anti-CD95 mAb (Siegel et al., 2000; Wajant, 2015).

1.7.8. The importance of PLAD in CD40 signalling

Although the mechanism of CD40-induced interaction is not fully understood, the simplest ligand-induced oligomerisation model is not sufficient to explain initiation of downstream pathways (Hager et al., 2003). Increases in the avidity of the receptor-ligand interaction, and/or changes in the membrane arrangement of the receptor that allows binding of adaptor molecules and activation upon ligation of the receptor, are certainly implicated (Hager et al., 2003). CD40, as well as TRAIL-R1 (DR4), are also shown to specifically pre-associate at the membrane in the absence of ligand (Deng et al., 2005; Wajant, 2015). This confirms earlier observations of the CD40 oligomeric state in unstimulated B cells (Braesch-Andersen et al., 1989). Malmborg, Hager and Ellmark have further studied the ligand-independent oligomerisation of CD40 (Hager et al., 2003) and they suggest that CD40L binding to its receptor requires the CARD1 of CD40, where PLAD might be located based on homology studies; although this domain does not contain any of the residues important for CD40L ligation. CD40 signalling is shown to be independent of the CARD1, since ligation of engineered proteins lacking part of their extracellular domain by a peptide tag is sufficient to rescue anti-IgM-induced apoptosis of WEHl cells (Ellmark and Borrebaeck, 2003).

1.7.9. CD40 signalling

1.7.9.1. Membrane lipid rafts

The outer leaf plasma membrane of many cell types contains microdomains of approximately 50nm of diameter, and called lipid rafts (Hayashi et al., 2006). These microdomain structures move within the fluid bilayer and constitute functional platforms for the formation of signalosomes (Lingwood and Simons, 2010). They are enriched in sphingolipids and cholesterol that self-aggregate and segregate from bulk unsaturated glycerophospholipids, and form a thick, liquid-ordered phase. This is highly resistant to solubilisation in non-ionic detergent, but can be isolated from low density fractions after buoyancy in a discontinuous sucrose gradient (Lingwood and Simons, 2010). There is insufficient understanding of the functions and composition of cholesterol-rich rafts that are similar within the plasma membrane inner leaflet (Hayashi et al., 2006). These microdomains are described as essential structure platforms for a variety of functions; for example, lipid rafts are shown to be crucial for T cell apoptosis mediated by Fas following T-cell receptor complex (TCR) stimulation. Modulation of TNF-R family protein location into lipid rafts might dynamically regulate the efficiency and outcomes of signalling by these receptors (Hayashi et al., 2006; Muppidi and Siegel, 2004).

1.7.9.2. CD40 is localised into lipid raft microdomains

Ligand-independent pre-associated CD95 complexes are preferentially found within lipid rafts (Muppidi and Siegel, 2004). Hostager et al. reported that a significant level of CD40 is constitutively found in these lipid microdomains in unstimulated mouse B cell lines (Hostager et al., 2000). CD40 engagement also induces the nearly complete translocation of CD40 form detergent-soluble to detergent-insoluble fractions of plasma membrane, and independently of downstream signalling molecules. Similar observations were reported in human dendritic cells (DCs) in which integrity and reorganisation of membrane rafts are required for engagement of signalling from CD40 (Vidalain et al., 2000). However, Malapati et al. (2001) described that CD40 functions outside the lipid rafts during B cell receptor (BCR) signalling in murine B lymphoma (Malapati and Pierce, 2001). The majority of published reports provide consistent data showing that co-engagement of BCR and CD40 brings their signalling complexes into close proximity within raft microdomains, allowing crosstalk between them (Haxhinasto and Bishop, 2004).

Interestingly, in B cell lymphomas, autonomous cell growth and constitutive activation of NF-kB are thought to be associated with unstimulated non-Hodgkin's lymphoma B cells. Within membrane lipid rafts, it appears that CD40L is co-localised with CD40 and constantly 'firing' in a CD40 signalosome complex constantly transmitting proliferation signals (Pham et al., 2002). Targeting of CD40 C-terminal signalling domain to lipid rafts and artificial trimerisation are shown to synergistically initiate cell signalling, suggesting that both localisation within lipid rafts and trimerisation of the receptor are important for activation of CD40-induced signalling. Finally, binding of CD40L may induce a conformational change in the CD40 transmembrane domain, which allows it to interact with ceramide of the membrane rafts, and link allosteric movements engaged by CD40L to locations within lipid rafts (Bollinger et al., 2005).

1.7.9.3.Translocation of CD40 into Lipid Rafts

CD40 clustering is shown to be dependent on acid sphingomyelinase (ASM) translocation from intracellular stores onto the outer leaflet of the cell membrane in the same manner as with CD95 (Grassmé et al., 2002; Grassmé et al., 2001). Primary stimulation via CD40 induced activation and membrane translocation of ASM then co-localises with the receptor and mediates release of ceramide. ASM accumulates in pre-existing sphingolipid-rich rafts and triggers the formation of larger ceramide-enriched platforms, bringing together receptor and signalling molecules into close contact (Figure 1.10). CD40-clustering also causes stabilisation of the ligand-receptor interaction leading to sustained signalling (Gulbins and Grassmé, 2002).

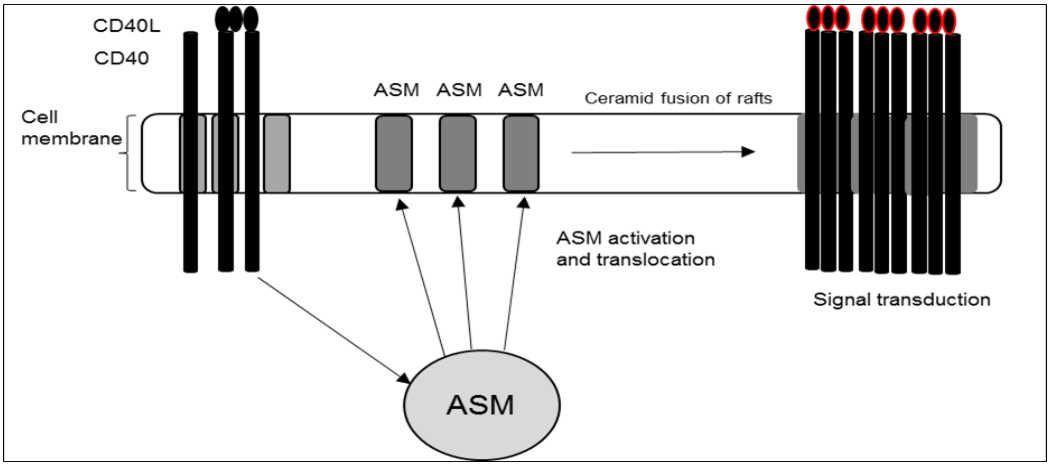


Figure 1.10: CD40 reorganisation into lipid rafts is dependent on ASM.

Primary CD40 engagement by its ligand induces activation and translocation to the plasma membrane. Acid sphingomyelinase (ASM) induces fusion of rafts microdomains and oligomerisation of the CD40 molecule that is an essential step for CD40L/CD40 induced signal transduction. Adapted from (Grassmé et al., 2002).

1.7.10. Role of CD40/CD40L in the immune system

1.7.10.1. 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. IgM, IgG, IgE, IgA and other immunoglobulins are produced and B cells are differentiated into plasma cells due to these factors (Ma and Clark, 2009). The importance of the CD40/CD40L axis in humoral immunity is demonstrated mainly by three approaches: first, by clinical manifestations and symptoms observed in patients with hyper IgM (HIM) syndrome. Another approach involves deletion of CD40L and CD40 genes (the genetic approach) (Allen et al., 1993; Aruffo et al., 1993; Korthäuer et al., 1993). The third approach used blocking peptides or antibodies directed towards the CD40L/CD40 complex (Foy et al., 1994; Van den Eertwegh et al., 1993). Each of these approaches highlighted the same conclusion, i.e. that the absence of the CD40/CD40L interaction leads to a severe defect in the production of immunoglobulins IgG, IgA and IgE or a thymus-dependent T cells response in response to a pathogenic infection, without affecting T-cell independent immune responses (Ma and Clark, 2009).

1.7.10.2.B Lymphocytes

Humoral immune responses are closely associated with B cells and the influence of CD40/CD40L complex. When antigens are present in any infection, T cells are activated when CD40L interacts with B cells that constitutively express CD40. Antibodies and plasma cells are produced due to the proliferation and subsequent differentiation of B lymphocytes that are induced by IL-10, IL-2 and IL-4 and helper T cell interactions (Aruffo et al., 1993). The antibody IgE is produced when IL-4 is present as a co-stimulation, but IgA and IgG antibodies are produced with CD40/CD40L interaction only (Figure1.11) (Armitage et al., 1993; Spriggs et al., 1992).

Patients that present Hyper IgM (HIGM) syndrome show that only IgM is produced by B cells, as CD40/CD40L interaction is absent (Hill and Chapel, 1993). TNF-α, IL-10 and IL-6 cytokines are released as a result of CD40/CD40L interaction within the activated B cell (Boussiotis et al., 1994), and vascular cell adhesion molecule-1 (VCAM-1), lymphocyte function- antigen-1 (LFA-1), and intercellular adhesion molecule-1 (ICAM-1) associated with these are increased (Barrett et al., 1991; Rousset et al., 1991). Expression of both major histocompatibility protein complex-1 and -2 (MHC-I and MHC-II) is also increased (Khanna et al., 1997; Klaus et al., 1994), so that these cells differentiate and proliferate into plasma cells. B lymphocyte differentiation and activation is facilitated by CD40L expression of B cells interacting with CD40 in a different B cell, which highlights a positive feedback loop for B lymphocyte expression of CD40L (Clodi et al., 1998; Grammer et al., 1995). Therefore, memory B cell differentiation is more involved with CD40 activation (Gray et al., 1997; Pound and Gordon, 1997).

1.7.10.3. Other CD40/CD40L system functions in cell-mediated immunity

B and T lymphocytes function and related APC interactions during immune responses are directly influenced by the function of the CD40/CD40L system. DC and activated T cell interactions cause B lymphocytes to differentiate and become activated into plasma cells represents one step, whilst DC/APCs causing T cell activation forms another step for these cell interactions (Ma and Clark, 2009).

The exposure to a pathogenic agent or bacterial infection leads to activation of CD40 on DCs (Hellman and Eriksson, 2007; Liang et al., 2009). DCs release IL-10 and IL-12 when activated via B7-1/B7-2 and CD80/CD86 co-stimulatory molecule activity increases due to the interaction of CD40L-activated T cells and dendritic cells (Caux

et al., 1994; Cella et al., 1996; Ma and Clark, 2009). Regulatory T cells Th17, IL-10, Th1 IL-12 that forms Th17, regulatory T and Th1 effector cells are differentiated from T cells as a result of the intimate involvement of these cellular responses (Bettelli et al., 2006; Iezzi et al., 2009; Veldhoen et al., 2006). NK cells or T effector cells release IL-4 and IL-2 that is triggered by T cell effector CD40 B cell and CD40L interaction. B lymphocyte and DC interaction in the context of the CD40/CD40L complex 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 that in conjunction with IL- 2 and IL-4 released by T lymphocytes, promotes differentiation of B-lymphocytes into immunoglobulin-producing plasma cells (Craxton et al., 2003; DeKruyff et al., 1993). There is no precise chronological order to these events, because interactions are bidirectional and may take place in concurrent ways.

To conclude, DCs, T 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. Hence, due to its vital role in the functioning of the immune responses, this is why the CD40/CD40L system has been previously described as being at the ‘centre of the immune universe’ (Grewal et al., 1997).

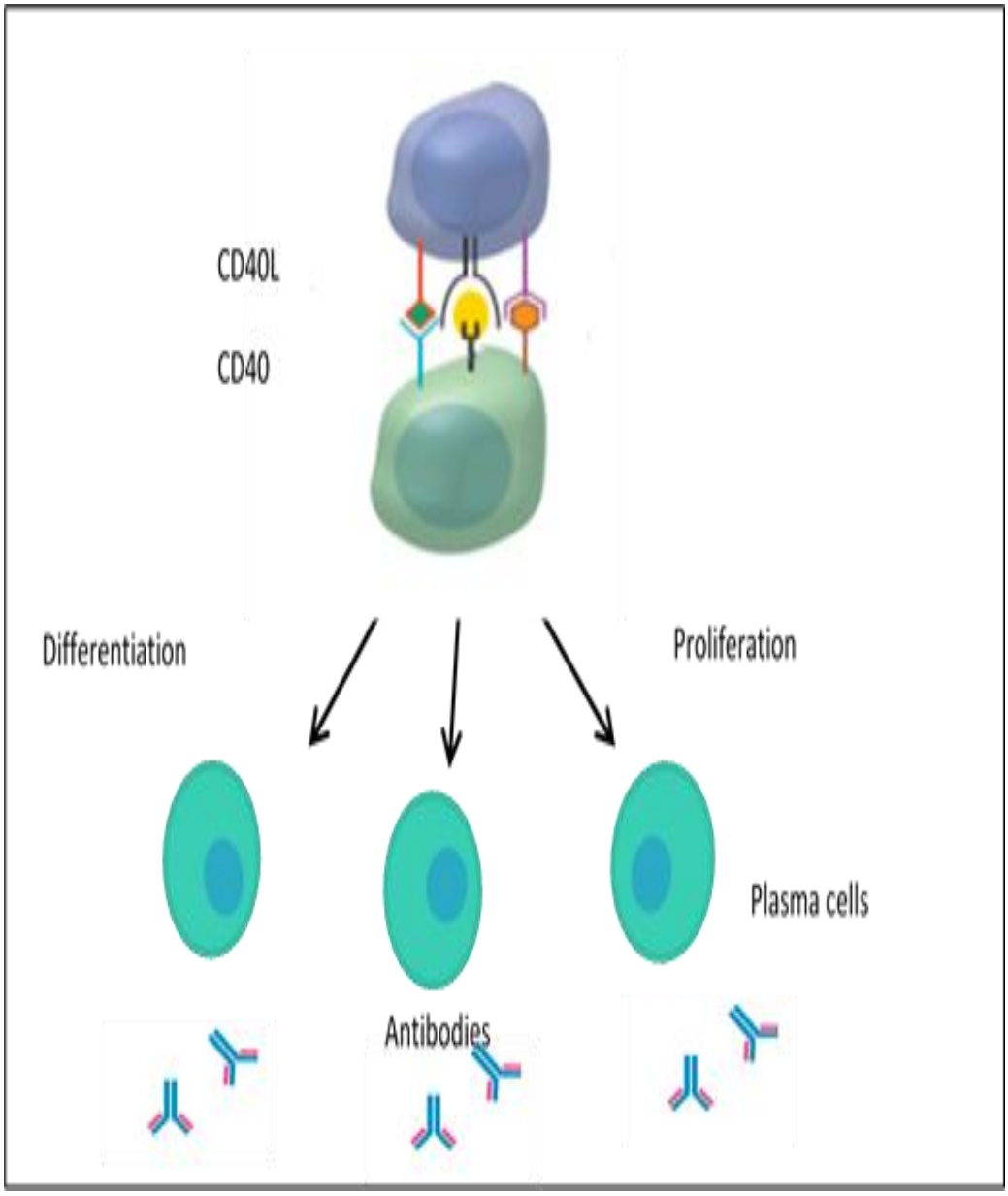


Figure 1.11: Role of the CD40/CD40L system in B cells.

CD40/CD40L interaction upon B cell resulting in proliferation, differentiation to plasma cells which is produce antibody against specific infection, memory cells for the secondary infection. Adapted from (Hassan et al., 2014).

1.7.10.4. T Lymphocytes

Full activation of T cells is induced when two main signals are present in T cells. On APC peptide-MHC molecules and T cell receptors (TCRs) interact, which corresponds to signal 1. CD28/B7 interaction is known as co-stimulatory and represents signal 2; an additional interaction sometimes referred to as signal 3 relates to CD40/CD40L (Lievens et al., 2009). This third co-stimulatory signal is essential to the proliferation, differentiation and survival of T cells (Grewal et al., 1995; van Essen et al., 1995). Dendritic cells, macrophages, B cells and other APC cells are the locations of interactions with CD40, when the T cell membrane shows expression of CD40L induced by signal 1. Mature or effector T cells differentiate and proliferate, and T cells are activated as a result of this bidirectional interaction (van Essen et al., 1995). Activated T lymphocytes express CD40, and both CD4+ and CD8+ cell T lymphocyte development, do appear to involve CD40. CD8+ lymphocytes differentiate into memory cells, as a result of CD40L and CD40 interaction on CD8+ and CD4+ (Figure 1.12) (Bourgeois et al., 2002).

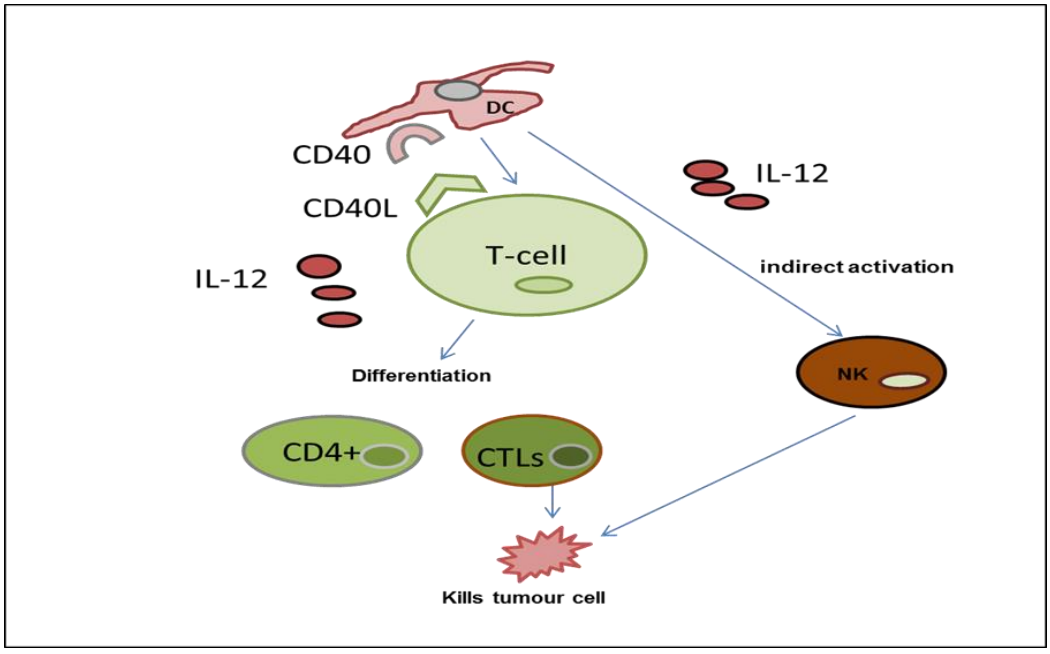


Figure 1.12: The role of CD40 in the immune system.

Interactions between T-cell and professional APC dendritic cell (DC), resulting in increased CD80/CD86 expression, and induction of IL-12 production, leading to T cell activation and differentiation to $CD4^+$ and $CD8^+$. NK cell could be activated indirect way in the presence of proinflammatory cytokines (IL-12), which is produced by DC. Adapted from (Hassan et al., 2014).

1.7.10.5. Monocytes / Macrophages

When monocytes present as APC, they can interact with T cell CD40L, and constitutively express CD40 (Cella et al., 1996; Schönbeck and Libby, 2001). This interaction is bidirectional, so that the monocyte promotes activation of T cells that induces the activation and differentiation of monocytes into macrophages. TNF-α, IL-8, IL-6, IL-1β and IL-12 cytokines are released as a result of CD40 activation on macrophages and monocytes following binding of CD40L on the T lymphocytes to CD40 (Alderson et al., 1993; Mach et al., 1997; Wagner et al., 1994). In addition, B7-2, B7-1, LFA-3 and ICAM-1 co-stimulatory molecules are increased (Kiener et al., 1995), alongside an increase in the secretion of matrix metalloproteinase MMP-1, -2, -3 and -9 (Mach et al., 1997; Malik et al., 1996). These cells have various functions, such as pro-inflammatory and angiogenesis roles, and although Mac-1 and other surface receptors can induce cellular responses, the interaction of CD40 and CD40L is critical to monocyte biological outcomes. 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 myeloperoxidases 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).

1.7.10.6. Dendritic cells

The dendritic cell (DC) occupies a central place in the immune system. The CD40-CD40L interaction on DCs, mainly as a co-stimulatory factor, promotes the activation of T lymphocytes. Activation of DCs, such as that observed in the presence of pathogens, causes a significant increase in DC surface CD40. The DC releases IL-12 as a result of B7-1/B7-2 and CD80/CD86 co-stimulatory molecule expression being increased and via activated T cell-mediated interaction of CD40L and its receptor (Caux et al., 1994; Cella et al., 1996). These cellular responses are intimately involved in the differentiation of T lymphocytes for Th1 immune responses (Iezzi et al., 2009; Shepherd and Kerkvliet, 1999). CD80/CD86 and MHC class II molecule upregulation results in mature APCs forming from DCs when primed by T-cell-CD40L causing DC CD40 ligation (Ma and Clark, 2009). Thus, when pathogen associated molecular

patterns (PAMPs) are presented, dendritic cells need to be activated to respond to PAMPs through CD40-CD40L co-simulation (Sacks and Noben-Trauth, 2002).

Additionally, CD40L is functionally expressed by DC in response to CD40 ligation (Pinchuk et al., 1996). In such a manner, DCs have been shown *in vitro* to utilise membrane CD40L (mCD40L) in order to mediate their cytotoxic effect towards urothelial cell carcinoma (UCC) and colorectal carcinoma (CRC) cells; therefore, CD40 is not only essential for DC activation, but for the ability to induce CD40-mediated cell cytotoxicity (Hill et al., 2008). DCs also express CD40L, but in smaller quantities compared to CD40. B lymphocyte and dendritic cell bidirectional interaction seems to have a significant involvement, but it is not possible to completely verify the full functions of CD40L dendritic cells (Bergtold et al., 2005; Wykes and MacPherson, 2000).

1.7.10.7. Neutrophils

Neutrophils are among the first immune cells recruited to inflammatory sites and are actively involved in the immune response. Neutrophils and platelet interactions seem to be closely related to activate neutrophils that express CD40 (Vanichakarn et al., 2008). In addition, following stimulation, soluble CD40L is released when neutrophils are activated by platelets. There is a reciprocal activation of platelets promoted by reactive oxygen species (ROS) being secreted within neutrophils that is induced by sCD40L. Another study has reported that after vascular injury, neointimal formation and platelet aggregate formation are promoted by neutrophils when these interact with platelets, as this is shown to increase high levels of sCD40L and expression of Mac-1 (Li et al., 2008).

1.7.10.8. Platelets

Platelets play a fundamental role in haemostasis, but they also actively participate in inflammatory reactions by inflammatory cytokines, growth factors and MMPs. Resting platelets constitutively express CD40, whereas CD40L is present on the membrane upon cell activation (Henn et al., 1998). Within platelets, CD40/CD40L complex function is shown to be important (Henn et al., 1998), because inflammatory reactions that are significant are induced when monocytes and endothelial cells are influenced by the interaction of CD40 with CD40L on activated platelets. Therefore, production of MMP-9 is induced, release of IL-8, IL-6, MCP-1/CCL2 or monocyte chemo-attractant protein-1 is promoted, and expression of CD62E or E-selectin protein, CD106 or

Vascular Cell Adhesion Molecule-1 VCAM-1, CD54 or Intercellular Adhesion Molecule-1 (ICAM-1) and other proteins is increased by this interaction. It has also been demonstrated that platelet sCD40L is involved in stabilising the thrombus through its interaction with integrin αIIbβ3 (André et al., 2002a). ROS are produced and RANTES ('regulated on activation normal T cell expressed and secreted') is released when induced by sCD40L stimulating platelets, but the functions of CD40 remain insufficiently understood (Chakrabarti et al., 2005; Danese et al., 2004).

1.7.10.9. Endothelial cells

There is a significant inflammatory influence associated with endothelial cells when CD40 is activated, and these cells also express CD40L and CD40, hence smooth muscle cell and endothelial cell activation is significantly regulated by the CD40/CD40L system (Karmann et al., 1995). In addition, RANTES, macrophage inflammatory protein-1α (MIP-1α), IL-8, IL-6, IL-1 and other cytokines are released as a result of expression of E-selectin, VCAM-1 and ICAM-1 adhesion molecules that are induced by this binding (Bavendiek et al., 2002; Rizvi et al., 2008). Endothelial cells are actively involved in the mechanisms of angiogenesis, and the CD40/CD40L axis seems to occupy an important place in this phenomenon (Karmann et al., 1995). In angiogenesis, one important stage is the digestion of the extracellular matrix that is influenced by MMP-9, MMP-2 and MMP-1 secretion and synthesis promoted when CD40L activates endothelial cells (Mach et al., 1999). Mechanisms of angiogenesis, such as progression and initiation, are influenced when endothelial cells secrete vascular endothelial growth factor (VEGF) when triggered by CD40 activation (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 basic fibroblast growth factor (bFGF) (Schönbeck et al., 1999). The CD40/CD40L complex is functionally involved in the pro-coagulant function of endothelial cells. It has been shown that ligation of CD40 via CD40L induces the synthesis and release of tissue factor that played a vital role in clot formation vascular from endothelial cells (Bavendiek et al., 2002), which triggers the activation of the coagulation cascade and platelet activation (Schönbeck et al., 1999).

1.7.10.10. Smooth muscle cells

Smooth muscle cell mitogenic activity is influenced by signalling triggered by CD40L, but there is insufficient understanding as to precisely how these cells are regulated by

CD40/CD40L signalling. IL-8 and MCP-1 secretion is produced when the Src tyrosine kinase pathway is activated (Hermann et al., 2002; Mukundan et al., 2004) whilst smooth muscle cells migrate and proliferate due to degradation of collagen via MMPs and via the interstitial matrix, as a result of CD40 receptor activation (Horton et al., 2001; Newby, 2007).

1.7.11. Epithelial cells

1.7.11.1. Effects of CD40 on normal and carcinoma cells

In addition to being expressed on a variety of cells of the immune system, CD40 is expressed by a large variety of malignant epithelial cells and by some normal epithelial cell types. The first report of CD40 expression relates to a study of human ectocervical, tonsil and nasopharynx tissue, which also included several epithelial cell lines, cultured epithelial cells and immunohistochemistry analysis to evaluate CD40 expression (Young et al., 1989). CD40 (and CD154) expression by the proximal tubule and glomerulus epithelial cells and parietal kidney epithelial cells has been reported (Yellin et al., 1995). Also, intestinal epithelial cells cannot promote activation of CD4+ T cells when antigen-driven or mitogen-driven, but do express CD40 with other co-stimulatory molecules when encountering enteric antigens (Yellin et al., 1995). Other studies report that IFN-gamma, TNF-α and lL-1 pro-inflammatory cytokines are induced, and medullary and cortical thymic epithelial cells express CD40 within the human thymus *in vitro* and *in situ*. In addition, CD4+ thymocyte clonal expansion involves co-stimulation due to thymic epithelial cells as a result of CD40 expression (Briscoe et al., 1998). A further study reports that CD40 is constitutively expressed by human bronchial epithelial cells and thymic epithelial cells (Gormand et al., 1999).

CD40 expression by a variety of carcinoma cells has been reported and such cell types include bladder (urothelial), colorectal, ovarian carcinoma cells (Bugajska et al., 2002; Georgopoulos et al., 2006).

A number of studies have demonstrated that soluble CD40 ligand (sCD40L) combined with protein synthesis inhibitors can be cytotoxic to CD40-transfected cervical, lung and ovarian carcinoma cells (Eliopoulos et al., 2000). By contrast, membrane-presented CD40 ligand (mCD40L) was highly cytotoxic independently of pharmacological intervention as shown previously (Hess and Engelmann, 1996) and more recently by Georgopoulos and colleagues (Bugajska et al., 2002; Georgopoulos et al., 2006). It has been reported that sCD40L required protein synthesis inhibition to

kill CD40-transfected cervical carcinoma cells (Eliopoulos et al., 2000). However, in ovarian cell lines both apoptotic and non-apoptotic responses have been demonstrated (Gallagher et al., 2002). Furthermore, the most unique feature of CD40/CD40L appears to be its ability to kill malignant cells and not normal epithelial cells (Bugajska et al., 2002).

Previous studies have reported that CD40 engagement may lead to apoptosis by cross-talk with ligands of DRs, as expression of TNF-α, Fas and TRAIL was demonstrated in some cell types, which resulted in apoptosis via the extrinsic cell death pathway (Eliopoulos et al., 2000). On contrary, studies from our laboratory (and by others) have challenged this, as it has been shown that CD40 mediated apoptosis in carcinoma but not in normal cells and this is regulated by direct signalling pathways (Dunnill et al., 2016; Elmetwali et al., 2010b; Georgopoulos et al., 2006).

The activation of CD40 can induce downstream signalling pathways of pro-apoptotic proteins depending on the type of tumour cells (Elgueta et al., 2009; Tong and Stone, 2003). The subsequent studies showed that, the effect induced by CD40 has appeared to depend not only on the type of cells, but on the strength of the signal transmitted by the ligand. Strong signals which is generated mCD40L induce apoptosis of cancer cells, whereas low signal stimulate cancer growth (Dunnill et al., 2016; Georgopoulos et al., 2006; Georgopoulos et al., 2007; Huang et al., 2012; Korniluk et al., 2014). Moreover the results obtained by Gerogopoulos et al, indicate that the interaction between CD40/CD40L on colorectal cancer cells leads to the formation of a strong and rapid pro-apoptotic signal (Georgopoulos et al., 2007). In agreement with these findings are previous observations that activated DCs can mediate direct killing of tumour cells which involves interactions between mCD40L expressed on the DCs and CD40 on the surface of bladder and colorectal cancer cells but not normal epithelial cells or fibroblasts (Hill et al., 2008). The effects of CD40 ligation in epithelial cells are complex, with the quality of the CD40 signal being central in influencing the physiological outcome. Low levels of CD40 ligation promote either cell survival or proliferation, whereas high levels induce growth arrest or apoptosis (Dunnill et al., 2016; Elmetwali et al., 2010a; Georgopoulos et al., 2006).

CD40 ligation can stimulate IL-8 and -6 production from ovarian, colorectal carcinoma cells. This suggests that the pathway mediating the expression and secretion of these cytokines may be involvied the endogenous stimulation of the CD40 pathway

(Gallagher et al., 2002; Georgopoulos et al., 2007). Studies in 2011 have shown that CD40-mediated tumour cell killing suggested that TRAF2 is a master regulator of the antitumour functions of CD40 in malignant epithelial cells (Knox et al., 2011). However, more extensive studies carried out by our laboratory have provided evidence in both colorectal and bladder carcinoma cells that apoptosis by CD40 following activation by mCD40L involves a novel intracellular signalling pathway that is driven by TRAF3-mediated signalling (Dunnill et al., 2016).

1.7.11.2. CD40 in prostate cancer

Expression of CD40 in prostate cancer (PCa) has been investigated by Palmer et al, where it was reported that the degree of CD40 expression varies and is associated with the grade of the PCa tumour. As shown by immunohistochemistry, CD40 expression was evident in basal cells in normal tissue but little/or CD40 expression was shown in invasive prostate cancers (Moghaddami et al., 2001; Palmer et al., 2004). It has been reported that agonistic anti-CD40 mAb G28-5 was able to inhibit the growth of PCa lines naturally expressing CD40 and in transfected cell lines engineered to express CD40, but it did not induce apoptosis in these cell lines (Rokhlin et al., 1997). However, CD40/CD40L interactions on PCa cell lines to induce cytotoxicity have not been studied and therefore the role of CD40 in prostate cancer remains unknown.

1.7.12. CD40 intracellular signalling

CD40 activation by CD40L can stimulate a variety of outcomes, such as kinase activation, gene expression, antibody production, cytokine secretion and the protection from or promotion of apoptosis (Bishop et al., 2007). CD40 has no death domain in its cytoplasmic tail unlike other members of the TNFR family and has no intrinsic kinase activity. CD40-mediated downstream signalling is mediated via its interaction with specific TNFR associated factors (TRAFs), and within the cytoplasmic tail of CD40 various binding motifs have been identified. Inflammation, stress responses, cell survival and apoptosis signals can be triggered via recruitment of six TRAF proteins and the ability of several of these TRAFs to interact with CD40 to regulate its cellular signals has been described. The C-terminal domain is needed for receptor binding and multimerisation of TRAFs to TNFR family members, and shares homology with TRAF molecules (Bishop et al., 2007).

Interaction of CD40 with several of TRAFs to trigger signal transduction has been demonstrated which is also supported by work relating to signalling “lipid rafts” that contain actively-signalling CD40. Receptor activation is rapidly followed by translocation of the majority of CD40 receptors into lipid rafts, where the receptor is then associated with various TRAF adaptor proteins (Arron et al., 2002).

1.7.12.1. The TRAF Family

TNFR associated factors (TRAFs) are signalling adaptor proteins ranging from 409 to 567 amino acids in size and have no intrinsic enzymatic activity. The TRAF family is a conserved group of scaffold proteins that link TNFR receptors to downstream signalling pathways (Nishina et al., 1997). As mentioned earlier, most TRAF family members have a conserved C-terminal domain termed TRAF domain, which in turn is divided into a highly homologous C-terminal region termed TRAF-C and a region termed TRAF-N. TRAF-C possesses a coiled-coil domain that allows interactions of TRAF proteins with other proteins found in the TRAF receptor complex. In addition, TRAF-C is essential for the interactions of TRAFs with the cytoplasmic domain of their receptors (see Figure 1.13) (Grech et al., 2000). All TRAF proteins have an N-terminal ring finger except TRAF1, and all consist of one (TRAF1) or several (TRAF2-6) zinc fingers. These structural motifs are important for downstream signalling (Wajant et al., 2001; Wang et al., 2010).

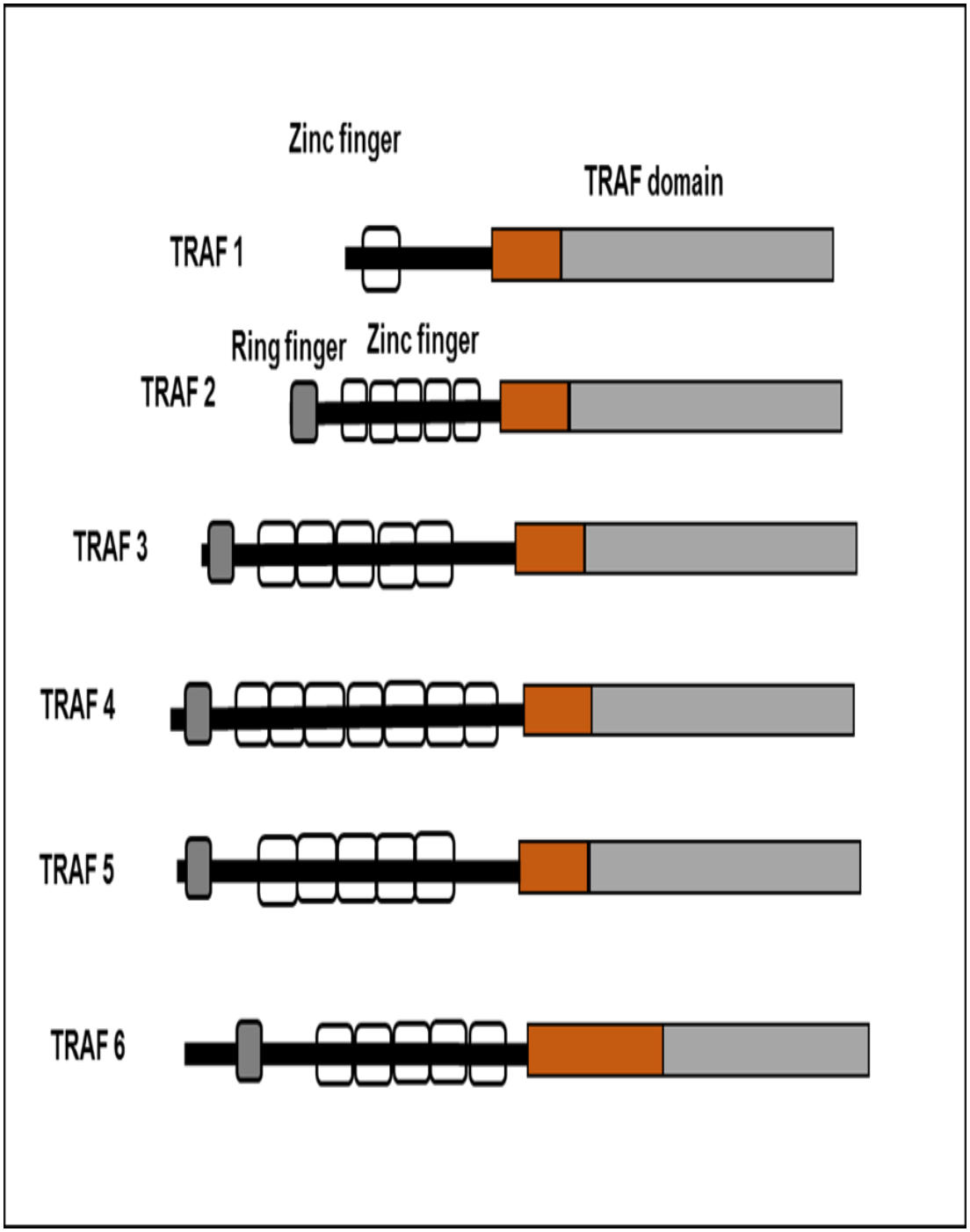


Figure 1.13: The TRAF family of adaptor proteins.

Schematic representation of the TRAF family of adaptor proteins and their functional domains. Adapted from (Wajant et al., 2001).

1.7.12.2.TRAF interactions with with CD40

TRAF-CD40 interactions are mediated by specific sites on both proteins. Distinct binding sites for TRAFs 1, 2, 3, 5 and 6 are described, with TRAFs interacting directly (TRAF2, 3 and 6) or indirectly (TRAF1 and 5) with CD40 (Ishida et al., 1996a). Since both TRAF2 and TRAF3 are detected by immunoprecipitation using anti-CD40 mAb in human B cells, the recruitment of both TRAF proteins at the same time is possible (Bishop and Hostager, 2001; Georgopoulos et al., 2006).

Nevertheless, specific TRAF have distinct contribution to receptor signalling. In DCs, IL-12 production and JNK and p38 activation only occurs with TRAF6, but DC maturation occurs with TRAF2, TRAF3, TRAF5 and TRAF6 (Mackey et al., 2003). In addition, during B cell differentiation and development, stages of signalling are transduced by different TRAFs, which suggests that TRAF2 and TRAF3 are essential for class switching; whereas, TRAF 6 plays a crucial role in infinity maturation and long-lived plasma cells (Ahonen et al., 2002; Jabara et al., 2002).

The accepted fate of the TRAFs is that TRAF proteins remain in the cytosol until activation of the receptor. After that, TRAFs are recruited to the membrane, then, activated and released to the cytoplasm for the propagation of the signal by interacting with diverse kinases and adaptors that regulate various signalling pathways (see Figure 1.14). Activation of TRAF proteins can occur by post- transcriptional modification, for instance BCR engagement alone or in combination with anti-CD40 mAb is shown to enhance phosphorylation of TRAF2 (Wajant et al., 2001).

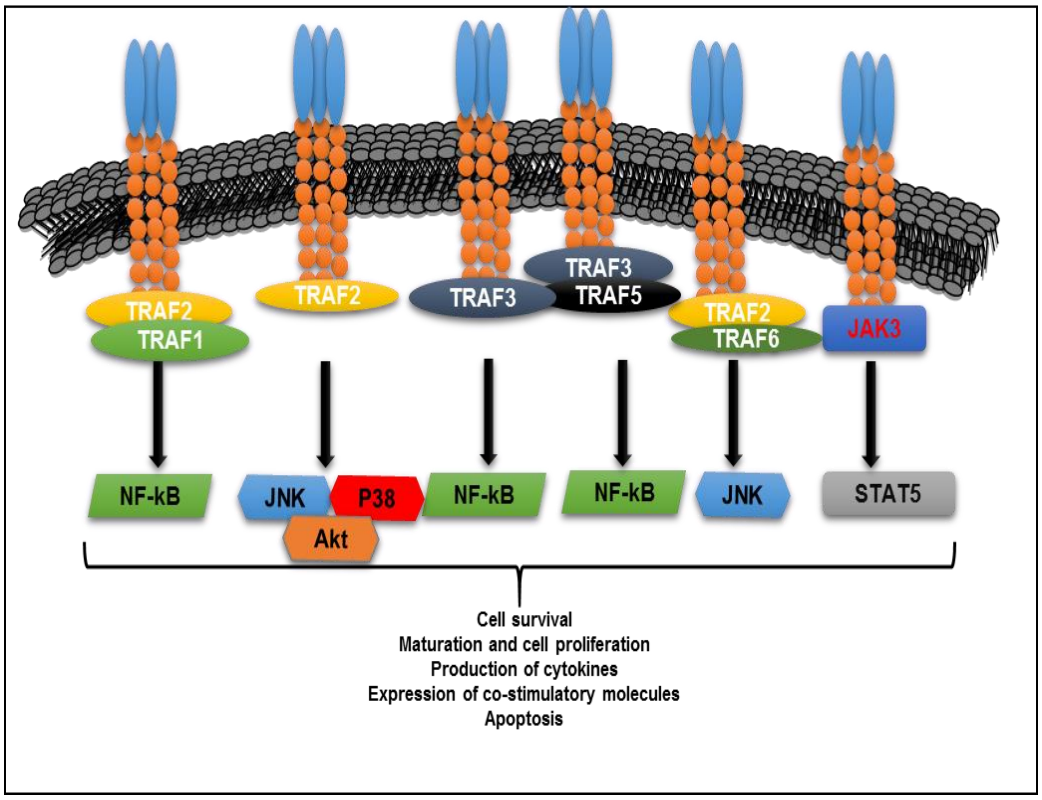


Figure 1.14: The different signalling pathways induced by CD40.

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. This diagram drown based on information from (Elgueta et al., 2009).

1.7.12.3.TRAF family members and their functions

1.7.12.3.1. TRAF1

TRAF1 interacts only weakly with CD40 in the absence of TRAF2, and cooperates with TRAF2 in order to promote some CD40-induced pathways (Xie et al., 2006). It is also proposed that the stoichiometry of each protein in TRAF1-TRAF2 complexes determine their ability to engage signalling downstream of CD40 (Fotin-Mleczek et al., 2004). At the level of B lymphocytes and APCs, TRAF1 deficiency causes a decrease in the recruitment of TRAF2 to CD40 and an increase in the enzymatic degradation of the receptor (Arron et al., 2002; Xie et al., 2006). The activation of CD40 in B cells, resulted in upregulation of TRAF1 and proteasome-mediated degradation of TRAF2 and 3 (Brown et al., 2001; Brown et al., 2002). Furthermore, TRAF1 protein upregulation as a result transcriptional and post-transcriptional mechanisms has been demonstrated (Georgopoulos et al., 2006).

1.7.12.3.2. TRAF2

TRAF2 has a positive role in B-cell differentiation, with opposing effects entrained by TRAF3, and can therefore lead to NF-κB activation when CD40 is engaged (Georgopoulos et al., 2006; Hostager and Bishop, 1999). It has been shown that MAP3K8TPI/COT1 is recruited to the CD40 receptor complex by its interaction with TRAF2 and TRAF6, resulting in activation of inhibiter of KB (IKB) kinase (IKK) (Chan and Reed, 2005). It also has the ability to stimulate NF-κB, as well as interact with MAP3K (Chan and Reed, 2005). TRAF2 also seems to participate in the activation of NF-κB, in collaboration with TRAF6, however, the interaction of one or the other with CD40 seems sufficient to induce the activation of NF-κB (Chan and Reed, 2005). This is confirmed by studies showing that the deficiency of TRAF2 or TRAF6 does not cause abnormality in the activation of NF-κB, but the double deletion of these two members causes severe inhibition of this pathway (Hsing et al., 1997; Rothe et al., 1995b; Yeh et al., 1997).

1.7.12.3.3.TRAF3

TRAF3 overexpression has been shown to block TRAF2-dependent NF-κB induction (Georgopoulos et al., 2006). The CD40- mediated degradation of TRAF3 is inhibited in B cells that lackTRAF2 expression (Moore and Bishop, 2005). It has been shown that association between TRAF3 and 2 can occur, and this interaction involves the TRAF-c domain for TRAF3 and zinc finger of TRAF2, TRAF3/TRAF2 hetero-trimerisation inhibits the TRAF2 induced NF-κB pathways, but not activation of AP-1 (Hostager et al., 2003). TRAF3 is shown to regulate both the classical p50 and alternative p52 NF-κB pathways induced by TRAF2/5. An important role for TRAF3 in CD40-mediated apoptosis in cancer cells has been described by work in our laboratory, by demonstration of activation of the JNK/AP-1 pathway and activation of caspase 9 (Georgopoulos et al., 2006); more recent work by Georgopoulos and colleagues demonstrated that TRAF3 knockdown abolished CD40-mdiated activation of ASK1, following phosphorylation of MKK4 and JNK, and the induction of Bak and Bax expression and attenuated apoptosis, thus, providing an unequivocal evidence about its role in defining the outcome of CD40 signalling (Dunnill et al., 2016).

1.7.12.3.4. TRAF4

TRAF4 is shown to be expressed in breast carcinoma, but not in normal tissue, and located mainly in the nucleus (Régnier et al., 1995). To date, no studies have described defined roles for TRAF4 in CD40 signalling, in spite of a complex between CD40-TRAF4 being detected (Aizawa et al., 1997).

1.7.12.3.5. TRAF5

Cells from TRAF5 mice$^{+/-}$ were used to investigate the role of TRAF5 in CD40 signalling, and the studies showed that receptor–mediated lymphocyte activation was substantially impaired (Nakano et al., 1999), yet cells displayed normal CD40-mediated NF-κB and JNK activation (Nakano et al., 1999). This suggests that TRAF5 compensation mechanisms might be efficient to some extent (Nakano et al., 1999). TRAF5 can show self-association in the cytoplasm of HeLa cells (Xu et al., 1996), and interacts with either CD40 directly (Ishida et al., 1996a) or by heterotrimerisation with TRAF3 (Pullen et al., 1998). Thus, the latter case is suggested to explain why CD40-induced NF-κB mediated by TRAF5 is enhanced in the presence of TRAF3 (Leo et al., 1999). Finally, like TRAF2, TRAF5 is important for NF-κB activation via both the classical and alternative pathways that are regulated by TRAF3 (Dejardin, 2006).

1.7.12.3.6. TRAF6

For macrophage and monocyte CD40-mediated NF-κB activation and CD40-mediated IKK activation, TRAF6 is closely implicated (Mukundan et al., 2005). Inflammatory cytokine production requires TRAF6, and CD40 signalling in B cells is also implicated with TRAF6. TRAF6 is shown to induce apoptosis through interaction with caspase and its activation by a ring domain-dependent mechanism (He et al., 2006). Interestingly, CD95 was unable to mediate apoptosis in B cells due to inhibition of caspase activation via TRAF6 and the P13/Akt pathway which activated by CD40 (Benson et al., 2006). In human epithelial cells treated with small specific interfering RNA for TRAF6, activation of NF-κB pathways, p38, JNK and Akt, is significantly reduced or even completely inhibited following stimulation with CD40L, demonstrating the importance of this TRAF member in these pathway (Davies et al., 2005); whilst more recent studies have demonstrated that CD40-mediated apoptosis requires attenuation of TRAF6 activity for TRAF3-mediated apoptosis to occur following CD40 ligation (Elmetwali et al., 2010a).

1.8. The mitogen-activated protein kinases (MAPK) family

1.8.1. Overview

Evidence for the existence of the MAPK family was found for the first time about twenty years ago (reviewed by (Avruch, 2007)). At that time, it was shown that insulin and other mitogens promote intracellular phosphorylation by protein kinases (Avruch, 2007). These substances are also known as "mitogens", they are known triggers of mitosis. The MAPKs represent one of the main pathways along which extracellular signals are transmitted within cells to mediate intracellular effects, and a variety of MAPKs are triggered by different extracellular stimuli (Herlaar and Brown, 1999). These include, among others, cytokines, growth factors and cellular stress. There are at least four different cascades of protein kinases in the MAPK family. The best characterised ones are the extracellular signal-related kinases (ERK) 1 and 2, the C-Jun N-terminal kinases (JNK) 1, 2 and 3, the p38 MAP kinases, and ERK5 (Chang and Karin, 2001; Chen et al., 2001). JNK and p38 MAPK are also referred to as stress-activated protein kinases (SAPKs) (Chen et al., 2001).

Activation of the MAPK pathways is carried out in three main steps and this applies to all members of the MAPK family. The extracellular signal first activates a MAPK kinase kinase (MAPKKK or MAP3K) by phosphorylation (Figure 1.15). At first sight this principle appears relatively simple and linear, however different cross-connections exist between the individual MAPK families (these cross-links are also referred to as ‘cross-talk’) (Cuschieri and Maier, 2005 307). The three MAPKs ERK1/2, JNK and p38-MAPK will be discussed in more detail in subsequent sections.

1.8.2. ERK1/2

ERK1 and ERK2 are related protein-serine/threonine kinases, known as Extracellular signal regulated kinase (ERK1/2), with expected molecular weight 42kDa and 44kDa, respectively (Roskoski, 2012). They participate in the Ras-Raf-MEK-ERK signal transduction cascade that is activated via a variety of stimuli such as growth factors, hormones, osmotic shock, cytokines, GPCR (G-protein coupled receptors). The signal cascade generated plays major roles in cell fate, including cell adhesion, cell cycle progression, cell migration, cell survival, differentiation, metabolism and mainly proliferation; however is some rare cases ERK activity can be involved in the induction of cell death/apoptosis (Kang and Sucov, 2005; Kim et al., 2007).

1.8.3. JNK1/2 (SAPK)

The c-Jun NH2-terminal kinases (JNKs) or also termed as stress-activated MAP kinase (SAPK), members of the mitogen-activated protein kinase (MAPK) family, regulate gene expression in response to a variety of physiological and environmental stimuli. It was first identified and isolated from mouse livers treated with cycloheximide (Kyriakis and Avruch, 1990). JNK consist of three isoforms that are JNK1 (SAPKγ), JNK2 (SAPKα) and JNK3 (SAPKβ), and each is divided into α and β with all having similar substrate specificity (Kyriakis and Avruch, 2012). Apart from anther JNK isoforms, only JNK3 presents in the brain and heart, whereas JNK1, JNK2 are expressed ubiquitously (Kyriakis and Avruch, 2001; Roux and Blenis, 2004). Furthermore, JNKs appear at two MWs, a short 46kDa form and a long 54kDa form (Pulverer et al., 1991).Various cellular stressors can activate the JNK, such as physical stress (heat, UV, osmotic shock), chemical factors (pH, ROS), metabolic factors (ischemia), biological factors (bacterial proteins, cytokines). These are, for example, cytokines of the TNF family, including TNFα or TGF-β (Kyriakis and Avruch, 2001; Weston and Davis, 2007). Receptor tyrosine kinases mediate the further activation of the signal cascade (Davis, 2000) and cascade initiation follows the traditional pattern of MAPK activation with MAPKKK (MEKK1-4) activating MAPKK (MKK4, MKK7), then activating MAPKs. A large number of MAP3Ks can initiate the activation of JNK. Which MAP3K is activated depends on the cell type and the type of stimulus. The strongest known activator is MEKK1. Among the MAP3Ks, the transforming growth factor-β activated kinase-1 (TAK-1) is a well characterised trigger. It has been shown that TAK-1 is necessary for the activation of JNK by inflammatory cytokines, antigen receptors, and TLR receptors. (Sato et al., 2005). The MAP3Ks activate MAP2Ks, in the JNK pathway these are MKK4 and MKK7. These two kinases are again responsible for the activation of the JNK (Figure 1.15) (Karin and Gallagher, 2005). JNK has a wide range of substrates, which include components of the cell membrane, elements of the cytoplasm and the cytoskeleton as well as substrates in the cell nucleus, and particularly main elements of transcriptional regulation. These include, in particular, c-Jun, but also other transcription factors such as ATF-2, STAT3, MEF2C or HSF-1 (Avruch, 2007; Chen et al., 2001; Karin and Gallagher, 2005). In this way the JNK exerts many physiological and pathophysiological functions. JNK plays a role in tumour cells with both oncogenic and tumour-suppressive implicated (Weston and Davis, 2007), hence why JNK and AP-1 downstream of it have been referred to as a double-edged sword in carcinogenesis

(Eferl and Wagner, 2003). Other pathophysiological processes in which the JNK has been implicated so far are insulin resistance, autoimmune diseases such as type I diabetes mellitus and a mouse model of multiple sclerosis, stroke and myocardial infarction (Tran et al., 2006; Weston and Davis, 2007).

On the one hand, the JNK plays an important role in the regulation of apoptosis. This is mediated by the AP-1 mediated induction of FasL, a transmembrane protein that can induce apoptosis in interaction with its receptor (Kyriakis and Avruch, 2001). It has been reported that ROS oxidized thioredoxin to disaggregate from ASK1 for its activation, and eventually resulted in JNK activation (Son et al., 2011). Furthermore, it is well known that ROS act as an important molecular in TNFR1-mediated JNK activation, and the sustained JNK activation constitutes one of the key events in TNF-induced cell death including both apoptotic and necrotic cell death(Shen and Pervaiz, 2006). Cryptotanshinone (CPT), a natural compound isolated from the plant induction of oxidative stress activates p38/JNK and inhibits Erk1/2, leading to caspase-independent cell death in tumor cells (Chen et al., 2012). Moreover, on the level of hematopoietic cells (humoral B-cell responses), CD40 also functions in cooperative interaction with other receptors (BCR and TLR7) to activate JNK, leading to the secretion of IL-6 (Bush and Bishop, 2008). Several studies have highlighted the significance of JNK in CD40-induced carcinoma cell death without the requirement for p38 or ERK activation (Eliopoulos et al., 2000; Elmetwali et al., 2010a; Georgopoulos et al., 2006), and more recently (Dunnill et al., 2016; Elmetwali et al., 2016), consequently, indicating that p38 and ERK are not necessary in this context. It has been suggested that JNK activation induced apoptosis via the regulation of pro-apoptotic Bcl-2 members (Bax and Bak) during the intrinsic (mitochondrial) pathway of cell death (Georgopoulos et al., 2006).

1.8.4. p38-MAPK

The p38-MAPK is a serine threonine kinase phosphorylated in response to stimuli such as UV radiation, osmotic stress, pro-inflammatory cytokines and hypoxia and anticancer agents (Raman et al., 2007). These elements can activate the p38 pathways to four isoforms, namely p38α/Mpk2/CSBP, p38β, p38γ/SAPK3 and p38δ/SAPK4. MAPKK kinases MKK3, 4 and 6 are able to activate p38 kinases by phosphorylation of threonine and tyrosine residues of their activation loop TGY

(Brancho et al., 2003). Upstream of these MKKs a variety of MAP3Ks can be found, which lead to the activation of p38 (Figure 1.14) (Zarubin and Jiahuai, 2005).

The various substrates of p38 include transcription factors, including the activating transcription factor (ATF) family, p53 and HMGB1. AP-1, whose component is c-Jun, can also be regulated by p38. For example, ATF-2, which is a substrate of p38, forms heterodimers with proteins of the Jun family and thus gains access to the AP-1 binding site and there is evidence that p38 also directly affects c-Jun. The pro inflammatory effect of the p38 pathway is well documented (Kontoyiannis et al., 2001). The p38-MAPK plays a central role in the expression of cytokines such as IL-1β, TNF-α and IL-6. In addition, p38 has pro apoptotic activity and is involved in regulating the cell cycle (Zarubin and Jiahuai, 2005). The stress kinase p38 activation, resulted in a critical role in cell death of rat fetal brown adipocytes triggered by TNF-α treatment (Valladares et al., 2000). Also, in human gingival fibroblasts (Kim et al., 2010). However, p38 MAPK plays a dual role as a regulator of cell death and this be can occur dependent on the types of stimulus or in a cell type-specific manner in a variety of cancer patients such as prostate, breast, bladder, liver, and lung cancer (Koul et al., 2013). Furthermore, studies carried out in our laboratory demonstrated that both LTβR and CD40-mediated apoptosis activated p38 and blocking the function of p38 attenuated death in CRC cells (Mohammed and Georgopoulos, manuscript in preparation) (Albarbar and Georgopoulos, manuscript in preparation).

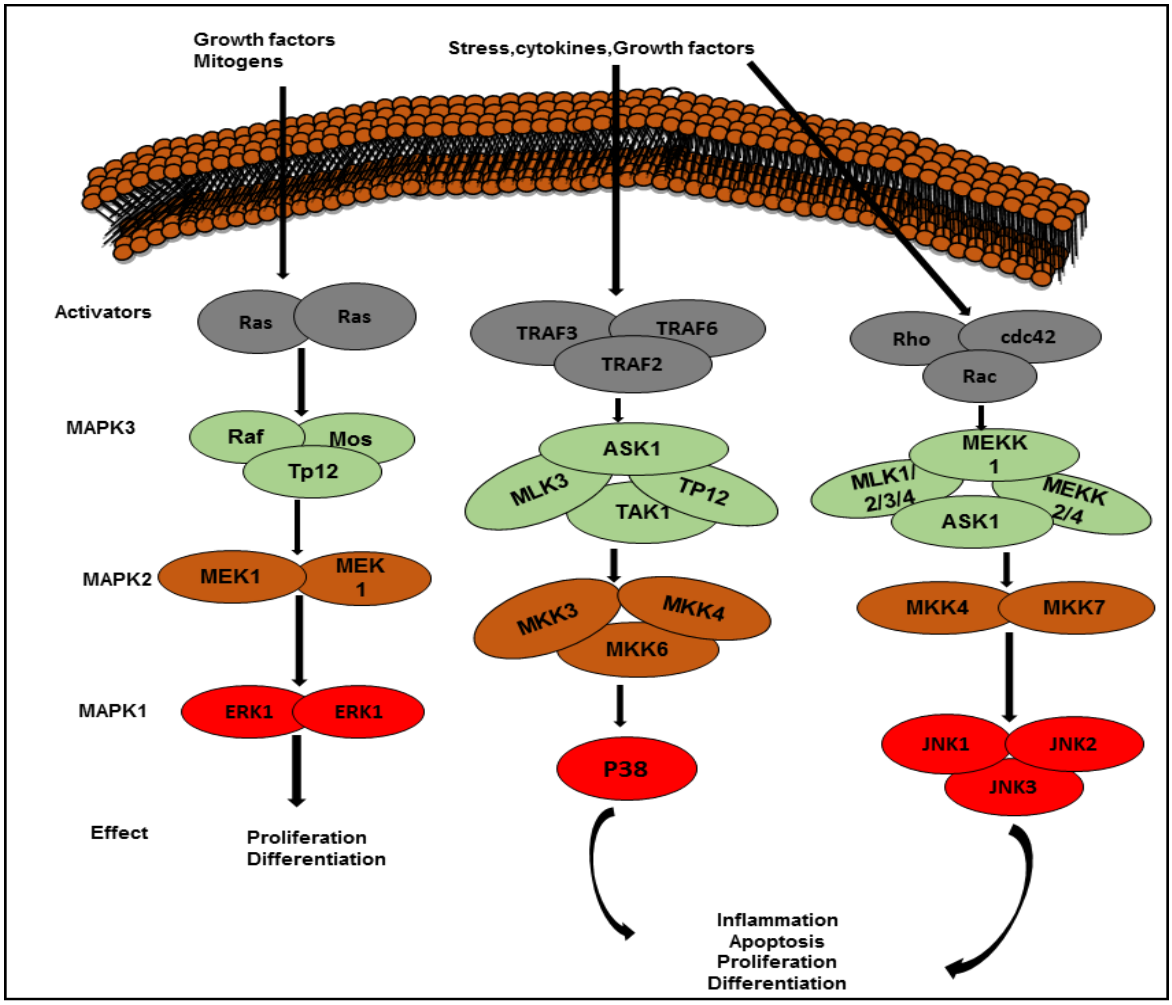


Figure 1.15: The MAPK signalling cascades.

The cascade of the MAP kinases triggered by different extracellular stimuli such as inflammatory cytokines, growth factors and anti-. They including extracellular signal-related kinases (ERK) 1 and 2, the C-Jun N-terminal kinases (JNK) 1, 2 and 3, the p38 MAP kinases. JNK and p38 MAPK are also referred to as stress-activated protein kinases (SAPK). This diagram drawn based on information from (Kyriakis and Avruch, 2012).

1.9. Cancer Immunotherapy: CD40

CD40 is shown to be a tumour target antigen and a therapeutic target in immune cell activity, because it is expressed constitutively on various tumour cells and antigen presenting cells including DCs and B cells. Various cellular responses occur when CD40 is stimulated on APCs, such as stimulation of antigen specific T cells, induction of antigen presentation following stimulation of CD40 on B cells, and maturation of DCs and secretion of cytokines, which demonstrate its immune regulation involvement (Vonderheide and Glennie, 2013). Chronic lymphocytic leukaemia (CLL), Non-Hodgkin lymphoma (NHL), Hodgkin lymphoma and other mature B cell tumours show significant

CD40 expression (Banchereau et al., 1994; O'Grady et al., 1994; Wang et al., 1997). CD40 expression is also reported to be present with ovary, prostate, neck, breast and melanoma tumours that represent solid tumours, so that this is not restricted to B cell malignancies (Ottaiano et al., 2002; Pellat-Deceunynck et al., 1994).

Various types of cancer have recently been reported an anti-cancer therapy with immune system stimulation (Gao et al., 2013).The immune system often presents CD40 expression across various cells and malignancies, and immunotherapy is a very promising treatment for cancer. Anti-tumour immunity is closely associated with M1 (classic and alternative activation) macrophages, NK cells, CD8+ cytotoxic T lymphocytes and other DCs when CD40 stimulation leads to releasing effector immune cells, which appear to be alink between CD40 stimulation, anti-tumour activity and immune responses (Loskog and Eliopoulos, 2009).

In combination with chemotherapy or on its own CD40, anti-tumour efficacy and tumour growth inhibition have been repoprted for agonistic CD40 antibodies (Khong et al., 2013; Vardouli et al., 2009). In addition, in various murine tumour models, post-operative cancer metastasis and recurrence are inhibited (Khong et al., 2013).

It has been also demonstrated that chemotherapy agents mediated amplifying AAV2-mediated gene transduction resulted in CD40 ligand-based immunotherapy expression in breast cancer cells, which ultimately led to activation DCs immune cells confirmed by cytokine 12 realised (Koppold et al., 2006).

In addition, the interaction between chemotherapy and sCD40L (immunotherapy) resulted in immunogenic tumor cell death, as reviewed by (Schlom et al., 2013). also demonstrated that the morphological changes in the tummour cells due to chemotherapuitic agents, renders these cells more targeted and sensetive to T-lymphocytes. Thus all these affects induced by some chemotherapeutic agents (Schlom et al., 2013; Zitvogel et al., 2010). Recent study also demonstrated that although soluble receptor agonist is a weak pro apoptotic molecule alone (Georgopoulos et al., 2006), however combinatorial treatment with pharmacological inhibition of Trx-1 (Px-12) renders CD40 agonist function as the same as mCD40L, and provided promising approach for cancer treatment (Dunnill et al., 2016).

CD40 antibody anti-tumour mechanisms are explained by two hypotheses. One is based on how antibody-dependent phagocytosis of tumour cells are triggered by the CD40 antibody, and inhibition of the CD40L/CD40-pathway that disrupts the proliferation of the tumour. The other is based on anti-tumour immunity being mediated by effector immune cells being released, and immune system CD40 stimulation (Moran et al., 2013). Currently, CD40 agonistic antibodies are combined with chemotherapeutic drugs, so that anti-tumour activity is synergised, or CD40 is used alone as an anti-tumour drug that is effective and safe in phase 1 studies (Beatty et al., 2013; Hussein et al., 2010). The following table 1.3 lists some agonistic CD40 antibodies that are used in the clinical research.

Table 1.3: Agonistic anti-CD40 antibodies used in clinical research as potential antitumor agents in different cancer type.

Some type of CD40 antibodies	Different cancer treatment	References
1- Chi Lob 7/4	Advanced solid tumors and Lymphoma (ongoing research)	(Vonderheide and Glennie, 2013)
2- CP-870,893	Advanced pancreatic ductaladenocarcinoma (solid tumour)	(Beatty et al., 2013)
3- FGK45	AB1-HA mesothelioma tumour in mice	(Khong et al., 2013)
4- ADX40	Murine B-cell lymphoma model in mice	(Beatty et al., 2013)
5- Dacetuzumab (SGN-40)	Non-Hodgkin's lymphoma (NHL)	(Lewis et al., 2011)
6- G28-5	Lymphoma xenografted mice	(Vonderheide and Glennie, 2013)

1.10. Prostate cancer (PCa)

Prostate cancer originates from the uncontrolled proliferation of epithelial cells lines of the prostate gland, which multiply anarchically and form a malignant tumour (Kaestner, 2016). Prostate cancer may be in the form of a localized tumour, which remains circumscribed to the prostate (Mottet et al., 2017). The cancer at this stage can be also termed as localized or intracapsular cancer. The cancerous cells can then migrate out of the prostate, leading mainly to lymph node and bone metastases which identified as non-localized or extra-capsular cancer (Mottet et al., 2017). Prostate cancer has a slow growth and evolves over several years, for most men, the slow progress of the

tumour and its localized character do not lead to clinical signs or symptoms in their lifetime. Approximately of 80% of prostate cancers are discovered at an early stage and localized, without clinical signs, during a screening or a control examination. In a large majority of cases, urinary disorders attributed to the prostate are due to a prostatic adenoma and not to cancer. Adenoma and cancer, however, can coexist in the prostate (Heidenreich et al., 2014; Kaestner, 2016; Mottet et al., 2017).

1.10.1. The classification of prostate cancer

The evolution and prognosis of prostate cancer are related to tumour volume, the level of a blood marker called PSA ,the stage and degree of histological differentiation of the tumor at the time of diagnosis (Heidenreich et al., 2014; Kaestner, 2016).

1.10.2. Gleason Score

The vast majority of prostate cancers are adenocarcinomas. There are rapidly metastatic forms (transitional cell adenocarcinoma or small cell carcinoma) but they are very rare. Non-epithelial primary tumours (sarcomas) can also be found (Kaestner, 2016).

The diagnosis of adenocarcinoma is made by histological examination of prostate tissue taken during biopsies. Prostatic carcinomas can take a variety of forms, ranging from very well differentiated carcinomas consisting of tumour glands close to the normal glands to very poorly differentiated carcinomas that no longer have the morphology or secretory functions of the prostatic glands (Mottet et al., 2017). The degree of differentiation of cells and the morphological diversity of carcinomas are defined by the Gleason score, established in 1974 (Heidenreich et al., 2014; Kaestner, 2016).

Gleason's classification has 5 grades of differentiation ranging from grade 1(well differentiated carcinoma) at grade 5 (anaplastic carcinoma). The Gleason score, scored from 2 to 10, is the sum of the two ranks most frequently represented in the tumour analysed. It has a significant prognostic value. Patients with low grade cancer with a Gleason score of 6 have the same life expectancy as the general population. It is halved when the Gleason score is between 8 and 9 (Kaestner, 2016).

1.10.3. D'Amico classification

It distinguishes 3 levels of risk of biochemical recurrence (re-increase in PSA level) 10 years after local treatment, and indicates whether there is a low, intermediate or high risk of "relapse" of cancer (Kaestner, 2016). This risk is defined according to the digital rectal examination, the PSA value and the Gleason score. The classification of D'Amico has a prognostic value. It applies to patients with localized tumors who have not reached the seminal vesicles or other organs (T1- to T3a-tumors, M0 and N0 according to the TNM classification) (Heidenreich et al., 2014).

1.10.4. TNM classification

The different clinical stages of prostate cancer are described by the TNM classification (T for Tumor, N for Lymph Nodes and M for Metastasis) ,which indicates the degree of local cancer extension and eventual involvement surrounding tissues and other organs (Heidenreich et al., 2014).

T1: the tumor is not perceptible on palpation during digital rectal examination.

T2: the tumor is palpable in digital rectal examination, but it has not crossed the limits of the gland.

T2a: reaching half of at least one lobe.

T2b: involvement of more than half of one lobe without involvement of the other lobe.

T2c: attack of both lobes.

T3: the tumor has passed the capsule that surrounds the prostate.

T3a: extra-capsular extension.

T3b: extension to seminal or unilateral vesicles.

T4: the tumor has spread to the neighborhood (bladder, rectum, muscles) and may have spread to the lymph nodes and created metastases (bone, lungs, liver, brain) Lymph nodes.

N0: no lymph nodes affected.

N +: lymph nodes affected metastases.

M0: no distant metastasis.

M +: distant metastases (bone involvement in almost all cases).

1.10.5. Treatment of prostate cancer

Depending on the severity of the tumour, the localized or metastatic nature of the cancer, the tumour volume and the life expectancy of the patient, the therapeutic management will be different (Heidenreich et al., 2014). In a large number of cases, prostatic tumours have a slow evolution and in some elderly patients treatment may be reduced to simple active surveillance (Heidenreich et al., 2014). Radical prostatectomy involves surgically removing the prostate and seminal vesicles, it is indicated primarily in patients under 65 years of age with localized cancer. It causes significant sexual side effects. External radiotherapy leads to the destruction of tumour tissue by radiation emission centred on the tumour (Heidenreich et al., 2014). Brachytherapy consists in permanently implanting small grains of radioactive iodine 125 into the prostate to destroy the tumour. It is offered to young men, it sometimes has easily treatable side effects of inflammation. Finally, hormone therapy is used as a palliative treatment for advanced tumours. It helps stop the proliferation of cancer cells but its effect is limited by an escape phenomenon after 18 to 24 months of treatment, In the long run, she can also benefit to mild and non-metastatic forms but leads to osteoporosis (Heidenreich et al., 2014; Kaestner, 2016; Mottet et al., 2017).

Also it has been shown that *in vivo*, PC-specific oncolytic adenovirus (Ad-PL-PPT-E1A) designed with fusion gene of prostate-specific antigen and CD40 ligand. CD40 only function as immune system enhancer but to cause a direct killing effect. Resulted in apoptosis induction in particular PC cells. Also in mouse models, Ad-PL-PPT-E1A treatment, clearly indicate that increase cancer patient life due to enhance the survival and inhibited the tumours growth which provided a promising approach for gene therapy of advanced PC (Yang et al., 2014).

1.11. Research hypothesis and aims of study

The hypothesis of this study is that the activation of CD40 by ligation with mCD40L will trigger apoptosis in PCa cell lines. The aim of this study was to investigate the biological activity and functional outcome of CD40 ligation in PCa cells, as well as the mechanisms underpinning these effects.

The specific aims were:

- **Chapter 3:** Using a variety of techniques (flow cytometry and western blotting), determine whether PCa cell lines, in particular DU145, PC-3 LNCaP and express CD40 the prostate cancer stem cells (CSC). Furthermore, only for PCa cell lines, whether this expression can be regulated by cytokines. Using several assays for the detection of cell death, caspase activity, DNA fragmentation, optimise and utilise a co-culture system to activate CD40 by membrane CD40L (mCD40L) on target cells.

- **Chapter 4:** Using the co-culture system for induction of mCD40L-mediated cell death, perform immunoblotting and functional inhibition experiments (using specific pharmacological inhibitors) to identify the key events that involved in mCD40L associated signalling and/or cell death.

- **Chapter 5:** Based on the co-culture system experiments, using an ELISA assays in order to measure pro-inflammatory cytokine secretion following CD40 ligation by mCD40L (compared with soluble agonists) in PCa cell lines.

Chapter 2: **Materials and methods**

2.1. General

In throughout the study, the experimental work was performed in hudersfield university, at School of Applied Sciences in the JP/68 and JP/70 laboratories.

2.1.1. Suppliers

All the reagent material and equipments that been used in this study are indicated their Commercial suppliers and manufacturers in the text.

2.1.2. Disposable plasticware

The autoclaving in a Prior Clave/London Autoclave at 121°C under pressure (1 Bar) aws used in order to sterilise the non-sterile plasticware which was obtained from different suppliers (Sarstedt, Fisher Scientific, Greiner Bio-One or Alpha Laboratories).

2.1.3. Stock solutions

The autoclaving at 121°C (1 bar) for 15 minutes was also used to sterilise all solutions that used in cell cutture, in addition they were prepared with ultra-pure water from a LabStar Ultra Violet purification unit in otder to ensure non contamination crossed. and also, eat stable solutions were sterlised using filter sterilised using Acrodisc (VWR) low-protein binding Tuffryn® HT syringe filters with a pore size of 0.2µm following their sterlised with the same mention machine(The autoclaving at 121°C (1 bar). Further, the deionised water (dH2O) was used for preparation of general laboratory stock solutions that have been prepared in n the laboratories. (Mohammed and Georgopoulos, manuscript in preparation) (Albarbar and Georgopoulos, manuscript in preparation).

2.2. Reagents

2.2.1. Primary antibodies

The antibodies that are used in this study are stored in Appropriate condition as recommended by supplier(manufacturer) following its aliquoted in Eppendorf tubes (10µl) to be ready as working solation; which will prepared by diluting antibody stock with TBS-Tween buffer (Tween20; Sigma Aldrich). The primary antibodies are illustrated in the following table 2.1. And also as shown in unpublished data. (Mohammed and Georgopoulos, manuscript in preparation) (Albarbar and Georgopoulos, manuscript in preparation).

Table 2.1 Primary antibodies

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, Immunofluorescence, FC: Flow cytometry).

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	SANTA CRUZ	1:250 in TBS Tween20 0.1%	WB	65
TRAF1	sc-7186 / h-186	Rabbit	SANTA CRUZ	1:500 in TBS Tween20 0.1%	WB	52
TRAF6	sc-8409	Mouse	SANTA CRUZ	1:500 in TBS Tween20 0.1%	WB	60
TRAF2	Sc-876/c-20	Rabbit	SANTA CRUZ	1:1000 in TBS Tween 20 0.1%	WB	50
BAX	2282-MC-100 (YTH-2D2)	Mouse	R&D systems (Trevigen)	1:500 in TBS 0.1% Tween20	WB	23
BAK	AF816	Rabbit	R&D systems	1:500 in TBS 0.1% Tween20	WB	28
-actin Clone AC15	A5441 - 2ML	Mouse	Sigma	1:20,000 in0.1% Tween20	WB	42
CD40	Sc-13128/ (H	Mouse	NEB(CST)	1:500 in0.1% Tween20	WB	43
CK18	081213	Mouse	Invitrogen	1:1000 in 0.1% Tween20	WB	45
CK8/18	889257A	Mouse	Invitrogen	1.1000 in 0.1% Tween 20	WB	52/48
P38	4511	Rabbit	Cell signalling	1:1000 in 5% BSA TBS Tween 20 0.1%	WB	43-40
JNK	255 (G9)	Mouse	Cell signalling	1:1000 5% Milk and TBS Tween 20	WB	54-46
ASK-1	555589	Rabbit	Cell signalling	1:1000	WB	155
Phospho-MKK7	#4171	Rabbit	Cell signalling	1:1000 in TBS, 5% w/v BSA, 0.1% Tween20	WB	48

Phospho-SEK1/MKK4	#4514 (C36C11)	Rabbit	Cell signalling	1:1000 in TBS, 5% w/v BSA, 0.1% Tween20	WB	44
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
CK18	C8541	Mouse	Sigma-Aldrich	1:2000 in0.1% Tween20	WB	45

2.2.2. Secondary antibodies

Both secondry antibodies polyclonal and monoclonal antibodies that used in this study were detected using the Goat anti-Rabbit IgG IRDYE800 antibody (Tebu-bio Cat # 039611-132-122) or he Molecular probes Alexa Fluor® 680 Goat anti-mouse IgG (H+L) antibody (Invitrogen Cat # A21057) respectively as shwon in (Mohammed and Georgopoulos, manuscript in preparation) (Albarbar and Georgopoulos, manuscript in preparation).

Table 2.2: Secondary antibodiesTable

The table below illustrated that all secondary antibodies and their host reigin, catalogue number and supplier or manufacturer as well as the ptimal dilution, type of blocking buffer and the range of their applications.

Antigen	Catalogue no/ Clone	Host	Supplier	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	1.10,1000 in TBS 0.1% Tween20	WB

2.2.3. Agonists & antagonists

According to instruction of manufacturer the agonists and antagonists (Table 2-3) that used in this study were dissolved in sterile distilled water (dH20) or tissue culture grade dimethyl sulphoxide (DMSO; Sigma). in addition, to ensure non-toxic dosage and deterime the optimal and effectve dosage; all compounds were assaed Utilising the cell viability assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay; Promega, UK, Cat # G3581). (Mohammed and Georgopoulos, manuscript in preparation) (Albarbar and Georgopoulos, manuscript in preparation).

Table 2.3: Agonists & antagonists

The table below illustrates that agonists and antagonists utilized in this study and their Guidance uses.

Reagents	Cat	Target	Supplier	Stock conc.	Optimal conc.
IFN-γ Human Recombinant	167300-02-B	IFN-γ receptor	Tebu-bio	$20x10^6$ Unit	180U/mL
TNF-α Human Recombinant	167300-01A-B	TNFRI and TNFRII	Tebu-bio	$20x10^6$ Unit	100U/mL
Agonistic Anti CD40 mAb	G28-5 clone	CD40	N/A	1.1mg/mL	10µg/mL

2.3. Pharmacological inhibitors

The general caspases inhibitors that used in this study either (z-VAD) or CAS-BIND™ Pro Pan were purchased from R&D and Vergent Bioscience Systems, respectively. Further, 2.3. Pharmacological specific inhibitors for MAPK kinase including JNK, p-38, MEK and NF-□B that reconstituted in tissue culture grade dimethyl sulphoxide DMSO and to ensure there is no toxicity related to it the solvent control was considered in all experiments. The specific ROS inhibitors utilised in this study were NAC and Propyl gallate PG and were purchased from Sigma-Aldrich. pre-titration experiments were performed the CellTiter 96® AQueous One Solution Cell Proliferation assay in order to determine the optimal concentrations for all the chemical inhibitors as shown in an appendix and elsewhere (Mohammed and Georgopoulos, manuscript in preparation) (Albarbar and Georgopoulos, manuscript in preparation).

Table 2.4: Pharmacological inhibitors used in this study

Inhibitors	Cat	Target	Supplier	Stock conc.	Optimal conc.
CAS-BIND™ Pro Pan	90101-1	All caspases	Vergent Bioscience	10mM	1-10µM
z-VAD-FMK	FMK001	All caspases	R&D systems	20mM	50-100µM
SP600125	sc-200635	JNK	Santa Cruz	100mM	5-10µM
SB202190	sc-202334B	p38	Santa Cruz	200mM	25µM
U0126	sc-222395A	MEK/ERK	Santa Cruz	100mM	10-20µM
NF-κB Activation inhibitor III	sc-204818	NF-κB	Santa Cruz	100mM	5µM
NAC	A7250-5g	ROS	Sigma-Aldrich	20mM	1.25-2.5mM
DPI	D2926-10MG	NADPH oxidase	Sigma-Aldrich	30mM	0.01562-0.03125µM
Quercetin	Q4951-100G	ROS	Sigma	100mM	2.5-5mM
Propyl gallate	02370-100G	ROS	Sigma	10µM	0.1-0.05 µM
Dantrolene sodium salt	D9175-100MG	Ca^{2+}	Sigma	100mM	6-8µM
Bbapta, cell permeant chelator	B1205	Ca^{2+}	Fisher	10mM	6-8µM

2.4. Tissue culture

2.4.1. General

All tissue culture experiments were carried out in sterilised condition, using cell Gard class II biological safety cabinet (NUAIRE). The provided areas for the internal work within the hood were decontaminated utilising 70% within the hood were that provided from Fisher, the preparation of this by diluted 99% ethanol (150mL: 350mL) with deionised water autoclaved dH20. The prepared ethanol solution was used when spillages occurs inside the hood after were disinfected using Mikrozid® (Gompel Healthcare Cat# 32644). In addition, the hood was a monthly routine sterilisation using both Mikrozid® followed by 99% ethanol. Further, to ensure no contamination occurs, the useless cells and or any exhausted solutions or media were aspirated using aspiration system which containing 10% (w/v) Virkon for 30 minutes.

2.4.2. Reagents

All the reagents that used in cell culture were provided a product of Sigma and classified as tissue culture grade. the Hettich Zentrifugen Universal 320 bench top centrifuge was used in order to isolate cells from solution by spun the cells in cell suspensions for 5 minutes at 1500 RPM (210 RCF). Also, the Marienfield Neubauer improved bright line haemocytometer was used for cell counts after they diluted in cell suspensions following cells seeded. in addition when cells are not used for experiment, they were incubated 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). The phase contrast microscopy using an EVOS XL (PeqLab) inverted microscope at x100 magnification was routinely utilised to examine cultured cells. (Mohammed and Georgopoulos, manuscript in preparation) (Albarbar and Georgopoulos, manuscript in preparation).

2.4.3. Cryo-preservation and recovery of cell lines

The Statebourne storage Dewar was used to keep cells stock t in liquid nitrogen in after cryo-preserved. To do so (cryopreservation of cell lines),cultures cells were taken as for passaging (see section 2.6.3) and then, after freezing medium was prepared supplemented with 10% (v/v) FBS and 10% (v/v) dimethylsulphoxide (DMSO) at a cell density not less than 1x106 cells/mL using centrifugation, then the pellet was re-

suspended using freezing medium. Then using cryovials (Sarstedt) cells were divided 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. Then before cells were stored to liquid nitrogen Dewar they were located within a -80°C freezer for 4-6 hours. Thus, when the cells needed again, they were thawing rapidly using about 10mL pre-warmed growth medium then cells were isolated using centrifuged at 1500RPM/210g for 5 minutes and then seeded to tissue culture flasks as required. (Mohammed and Georgopoulos, manuscript in preparation) (Albarbar and Georgopoulos, manuscript in preparation).

2.4.4. Carcinoma cell culture

For all experiments, the human prostate carcinoma cell lines LNCaP, DU-145 and PC-3 from the American Type Culture Collection (ATCC) were used. In addition, both the malignant bladder carcinoma (EJ) and colorectal cancer (HCT116) cell lines were also often used as a positive control for CD40 ligation in previous studies (Dunnill et al., 2016). These were obtained from the Heidelberg laboratory Cell line Services (CLS). All cells were tested before use to confirm lack of Mycoplasma contamination.

2.4.4.1. LNCaP

The LNCaP cell line isolated from a left-sided supraclavicular lymph node metastasis of a 50-year-old man with prostate carcinoma (1977). LNCaP are androgen-dependent cells that show a slow growth pattern with an average doubling time of 72h. They form prostate-specific antigen and prostate-specific acid phosphatase. The desmosomes visible in the electron microscope indicate an epithelial character (Russell and Kingsley, 2003).

2.4.4.2. DU-145

The epithelial cell line DU145 comes from the CNS metastasis of a 69-year-old man with prostate carcinoma (1975).The cell line is not androgen dependent, only weakly positive for prostate-specific acid phosphatase and does not express any prostate-specific antigen (Russell and Kingsley, 2003).

2.4.4.3. PC-3

These are prostate carcinoma cells derived from the metastasis of a lumbar vertebral body of a 62-year-old Caucasian man with an oesophageal metastatic prostate carcinoma after androgen suppression therapy. The cell line PC-3 grows with an

average doubling time of 33 h and is epithelial differentiation. The cells are androgen-independent (Russell and Kingsley, 2003).

2.4.5. Cell maintenance

The prostate cell line DU145 was initially grown in complete EMEM 10% FBS, whereas LNCaP was grown in RPMI and PC-3 in DMEM: Ham's but all cell lines were then adapted in D: R medium with 1% L-Glutamine, supplemented with 5% FBS, while, the PC3-CD40 cell line was grown in D:R medium with 1% L-glutamine, supplemented with 5% FBS, and 5.0mg/mL G418. All prostate 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% fatal calf serum (FCS Biosera cat # S1810/500) and 1% L-Glutamine (Sigma cat #G7513-100ML).

when the cells reach confluence they undergo to subculture passages to avoid reduced mitotic index, in specific for cells that frequently used. To do so, firstly 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' incubation time followed by addition of Trypsin-EDTA (Sigma Cat# T41474-20mL) that prepared in Hanks-balanced salt solution free from Calcium and Magnesium free (HBSS, Sigma Cat# H9394-6X500ML). when cells are detached from culture flask, the Trypsin reaction was inactivated by addition of the respective serum-supplemented culture medium when cells were re-suspended (Dunnill et al., 2016).

2.4.6. Cancer stem cells (CSC)

Cells were kindly provided by Professor Normal Maitland and Dr Fiona Frame (Department of Biology, University of York). Csc were isolated from patients with different cancer stages, and maintained in complete KSFM free of serum and incubated at 37°C in 5% CO_2, as previously described (Maitland et al., 2011).

2.4.7. Murine fibroblast (3T3) cell culture

The effector cells that utilised to deliver the CD40L signal (mouse fibroblast cell line) NIH3T3 that has been engineered in order to stably express CD40Lthe process of transfection was with two expression plasmids, the first one bearing the sequences coding for CD40L and the Neomycin resistance gene (3T3-CD40L cells) and the

second with Neomycin resistance alone (3T3-Neo cells) that used as a control to ensure no virus related affect as described in Bugajska et al. (2002). Maintenance of cells in long-term or routinely culture for in experiments,, these 3T3 derivatives were maintained in DR supplemented with 10% FCS, 1% L-Glutamine (DR: 10%FCS/1% L-G) and 0.5µg/mL G418 (Invivogen Cat# ant-gn-1; supplied by Source BioScience LifeSciences) to prevent cells loss of transgene expression. cells were permanently cultured in previously mentioned medium and incubated at 37°C in 5% CO2 unless otherwise stated. fibroblast cells were routinely collected and passaged as previously mentioned in cell maintenance section. (Mohammed and Georgopoulos, manuscript in preparation) (Albarbar and Georgopoulos, manuscript in preparation).

Table 2.5: Epithelial and fibroblast cell lines used in this study.

Cell lines	**Tissue type**	**Cancer type**
PC-3	Epithelial	PCa/Adenocarcinoma
PC-3CD40	Epithelial	PCa/Adenocarcinoma
DU145	Epithelial	PCa /carcinoma
LNCaP	Epithelial	PCa/carcinoma
EJ	Epithelial	UCC
HCT116	Epithelial	CCs
CSCs	Epithelial	PCa/carcinoma
3T3CD40L	Fibroblast	N/A
3T3Neo	Fibroblast	N/A

2.5. Cell line transfections

2.5.1. Culture of Retropack^{TM} PT67 Packaging cell line

the NIH3 fibroblast derived cell line that termed also The Retropack PT67 cell line which has been genetically manipulated to steady express the retroviral gag, pol and env gene. after transfection of these cells,they gain the ability to produce a replication faulty retrovirus with a broad (amphotrophic) mammalian host range. the murin fibroblast cells were cultured in DMEM supplemented with 10% (v/v) FCS. Cells were split at a ratio of 1:5-1:15. and for more details for the transfection process as puplished in (Dunnill et al., 2016).

2.5.2. Retroviral transduction of Carcinoma cells

The manipulated PT67 cells which constantly transfected were cultured in its original medium till100% confluence after that DR/5% FCS/1% L-G was added and replaced with DR/10%/1% L-G that previously contained antibiotics and incubated for 16 hours. Following collection and filtering Conditioned virus-containing antibiotic-free medium through a 0.45µg/mL Tuffryn™ filter (Acrodisc, VWR) in order to take away any cellular debris. prior to start for infection, the 8µg/mL polybrene supplier from (Sigma) was added to virus conditioned medium. on the day before starting transfection of target cells (PC-3), this cells should grow to 60-80% confluence in T25 flask, then p33) was passaged 1:5 into two separate T25 flasks, then the following day the medium was removed before one flask of PC-3 cells were treated a) with 4mL of polybrene and virus containing conditioned medium or b) 4mL of DR/5% FCS/1%L-G to act as a negative control.

The transfected cells including both transduced and non-transduced PC-3 carcinoma cells were detached and then collected and passaged at split ratio of 1:5 into a new T25 tissue culture flasks in fresh medium supplemented with 0.8µg/mL G418 antibiotic, and the optimal concentration pre-determined by pre-titration experiment (see section 3.4.1). Transduced PC-3 and negative control PC-3 were then observed under the microscope until all negative control cells had perished from the flask. And then transduced cells were cultured in medium containing G418 concentration reduced to by half (0.4µg/mL) for transgene maintenance purposes (Mohammed and Georgopoulos, manuscript in preparation)(Dunnill et al., 2016).

2.6. Methodologies for induction of CD40 ligation

In order to activate the CD40 receptor which required its ligation with the main ligand CD154 (CD40L). Thus this was achieved by using the co-culture system, involving the fibroblast 3T3CD40L to delivery of membrane-presented CD40L when co-cultured with equal numbers of the CD40-positive target epithelial cells. Similarly, 3T3Neo cells were co-cultured with target epithelial cells to acts as a negative control. the 10 µg/mL of Mitomycin C (Sigma) was utilised as treatment for 2h incubation time to causes growth arrested for mCD40L-expressing and Control cells in D:R5% then, followed by washed, detached and cultured either into 96 well plates at $1x10^4$ cells/well or in $10cm^2$ culture dishes at $3x10^6$/dish which depends on the type of the experiments, for the purpose of detection of apoptosis assays or preparation of protein lysates to identify the

intracellular proteins , respectively.. After mCD40L and control cells were seeded to either both 96 wells plates or dishes and left overnight incubation time the target prostate epithelial cells were cultured at a different ratio of 0.8 , 1.0 and 1.2 of epithelial cells, as optimised in this study (and detailed in Chapter 3). (Mohammed and Georgopoulos, manuscript in preparation)(Dunnill et al., 2016).

2.6.1. Detection of cell growth, death (apoptosis)

2.6.1.1.General

In order to assays or monitoring cell death accurately, and based on the previously published guidelines concerning to e use and interpretation of assays for monitoring cell death (Galluzzi et al., 2009), they have recommended that at least of two assays are utilised for the detection of cell apoptosis. Therefore, this study had involved of a cell proliferation assay (MTS) as well as utilising of four apoptosis detection-specific assays; such as CytoTox-Glo. In addition of two assays were used as markers for apoptosis for instance b) caspase 3/7 activation c) DNA fragmentation. They measurement of these assays was different which is involved the absorbance, fluorescence or luminescence. Cells were cultured in 6 well Nunc white, tissue culture treated culture plates (Fisher cat # TKT-186-010C) for the measurement of luminescence and fluorescent based assay. however,96 well ELISA microplates (Greiner bio one Cat # 655101) for ELISA and 96 well Costar transparent tissue treated culture plates (Fisher Cat # TKT-186-010C) for absorbance were used. These assay were based on the co-cultured of epithelial cells 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/DU145 – mCD40L” and “Control/DU145 – Control” readings). With the exceptional of DNA fragmentation as this was unnecessary due to the pre-labelling of target epithelial cells. The blank control (medium only or mixed with reagent involved with the experiment) was included in all experiments. (Mohammed and Georgopoulos, manuscript in preparation)(Dunnill et al., 2016).

2.6.2. Detection of cell viability (cell biomass)

By utilising the CellTiter 96® AQueous One Solution Cell Proliferation assay, the detection of cell viability was assessed based on determination of cell biomass. This assay involves the use the solution of MTS tetrazolium (yellow) which only reduced by viable cells to a formazan derivative (brown Colour), however, non-proliferating or dying

cells are incapable to do so. The colour development is proportional to the total number of viable/proliferating cells. Following an addition of 20µl of CellTiter 96® AQueous One Solution to appropriate wells and plates were incubated at 37°C in 5% CO2 for a total of 3-4h, epithelial cells were seeded into 96 transparent well plates with 6 replicate wells and then left to adhere overnight. 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. Using the following formula Cell viability was calculated as percentage viability in comparison to controls (T/C) x100, where T= treated cells and C= controls cells. (Mohammed and Georgopoulos, manuscript in preparation).

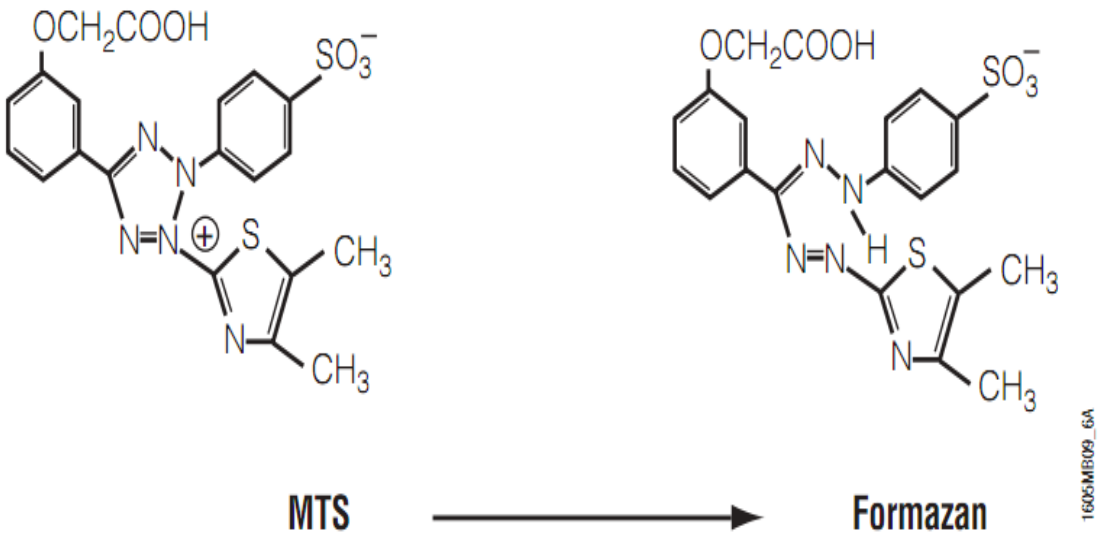


Figure 2.1: The structure of MTS tetrazolium and its reaction product.

This assay (MTS) is based on measuring mitochondrial (metabolic) activities, by adding a single reagent (a tetrazolium compound 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS)). Only viable cells are able, during a defined incubation time (3-4h), to reduce MTS to a water-soluble coloured formazan product. By contrast non-proliferating or dying cells lose their ability to do so file:///C:/Users/u0974455/Downloads/celltiter-96-aqueous-one-solution-cell-proliferation-assay-system-protocol.pdf.

2.6.3. Detection of apoptosis using caspase3/7 assays

The caspases 3/7 is one of the apoptosis hallmark. They played a crucial roles in executioner phase, targeting a specific amino acid sequence which leads to an organised apoptotic event. therefore, the activation of Caspases 3/7 was carried out utilising 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). (Mohammed and Georgopoulos, manuscript in preparation).

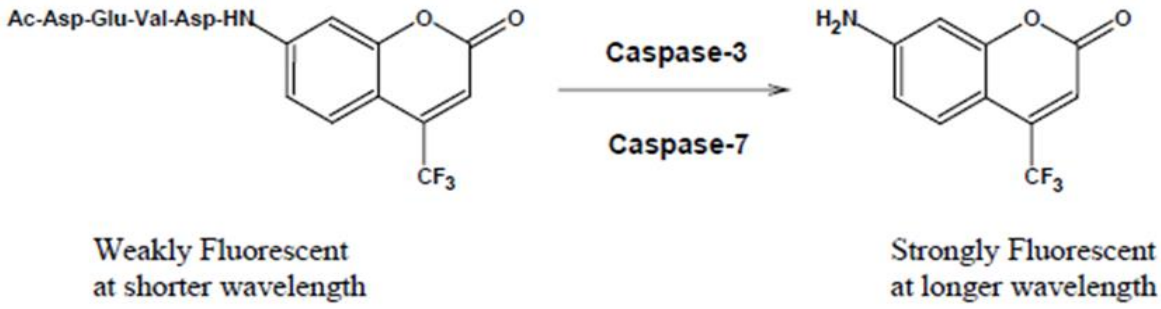


Figure 2.2: Proteolytic cleavage of Ac-DEVD-AFC substrate.

The diagram shown that as soon as the caspases become active which change from weakly-fluorescent caspase-3/7 Ac-DEVD-aminoluciferin substrate Z-DEVD to create the strongly fluorescent compound due to Cleavage process. https://www.promega.com/~/media/Files/Resources/Protocols/Technical%20Bulletins/101/Caspase-Glo%203%207%20Assay%20Protocol.pdf

2.6.3.1. Caspase-Glo® 3/7 Assay

The SensoLyte Homogenous Caspase 3/7 substrate (Ac-DEVD-AMC) utilises the cleavage recognition sequence of Caspase 3/7 for the in vitro detection of active Caspases 3/7. Before Caspase recognise and cleavage the substrate Ac-DEVD-AMC is a weakly fluorescent molecule. Nevertheless, as soon as caspases 3/7 recognise the sequence DEVD and cleave it, Ac-DEVD-AMC generates the AMC fluorophore, which releases bright blue fluorescence upon excitation at the appropriate wavelength. The bright blue fluorescence signal released is corresponding to Caspase 3/7 activity after in situ lysis .(Mohammed and Georgopoulos, manuscript in preparation).

2.6.4. Detection of cell death using the CytoTox-Glo™ assay

The CytoTox-Glo assay is a reliable and an appropriate assay involves the addition of a single reagent to cell cultures, which allows obtaining the results quickly by generating the analysis within 15-minutes after substrate addition. Unlike, others death assays, a such DNA fragmentation assay that multiple reagents and adequate time for preforming experiment, or caspase activation which is required frequent measurements to detect the optimal point for caspases activity.

The principle of this assay utilises the substrate (alanyl-alanyl-phenylalanyl-aminoluciferin; AAF-Glo™ Substrate). Only dead cells, which is lost their membrane integrity and release a specific intracellular protease during the cell death process, which is in turn, cleaved the AAF-Glo™ Substrate. On contrary, AAF-Glo does not able to penetrate the live cells intact membranes. In addition, Cleavage of the substrate *in situ* generates a luminescence signal (Figure 2-4) and the intensity of this signal is relative to the proportion of cell death in the population (Niles et al., 2007).

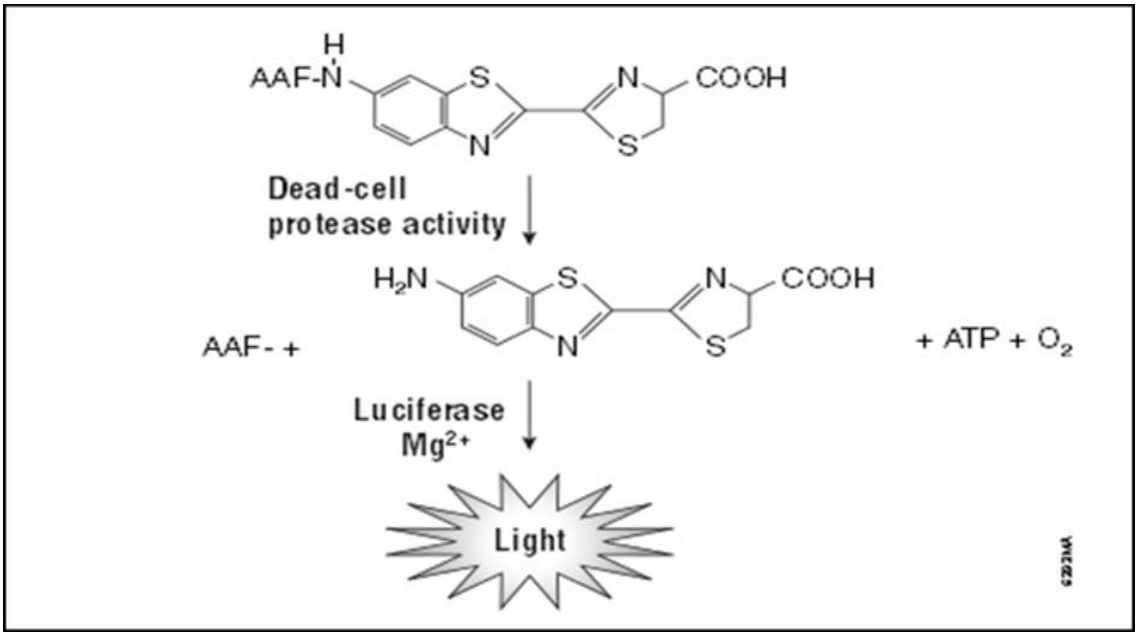


Figure 2-4: The principle of the CytoTox-Glo™ assay.

When employing the CytoTox-Glo™ assay, the compound (alanyl-alanyl-phenylalanyl-aminoluciferin; AAF-Glo™ Substrate) is a luminogenic peptide substrate used to detect dead cells, based on protease activity released from cells that have lost their membrane integrity. The AAF-Glo™ Substrate is incapable of penetrating live cells due to the integrity of their membranes. Consequently, there is no any appreciable signal generated from live cells.

file:///C:/Users/u0974455/Downloads/cytotox-glo-cytotoxicity-assay-protocol.pdf

2.6.5. Detection of apoptosis using the DNA fragmentation ELISA

The DNA fragmentation considered as one of the most characteristic that lead to define an event of apoptosis. This universal feature occurs particular in the in the development of multicellular organisms. Thus,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). The DNA labelling agent BrdU was used to stain the prostate growing culturing epithelial cells for 2h incubation time, and based on the manufacturer's instructions, at the concentration of 10μM. cells were then co-cultured with effectors cells as described in section 2.8. After that the supernatant was collected after 48h. An ELISA plate was used and coated with an anti-DNA antibody or capture antibody which bound to the plate, followed by blocked in order to remove any non-specific binding sites. After that plate was washed to remove any blocking buffer, followed by adding the samples from the co-culture which contain may contain DNA fragments pulsed with BrdU that will stick to the plate via the anti-DNA antibody then the additional probe is called the secondary antibody enzyme-linked antibody that specifically recognises BrdU labelled DNA was added. Then an enzyme substrate was added that resulted in yellow colure development as result of DAN fragmentation or apoptosis. Then the reaction of an enzyme substrate was stopped by added Diluted sulphuric acid (H2SO4) after sufficient colour change. 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. Doxorubicin was used as positive control at a concentration of 5μM.(Mohammed and Georgopoulos, manuscript in preparation).

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

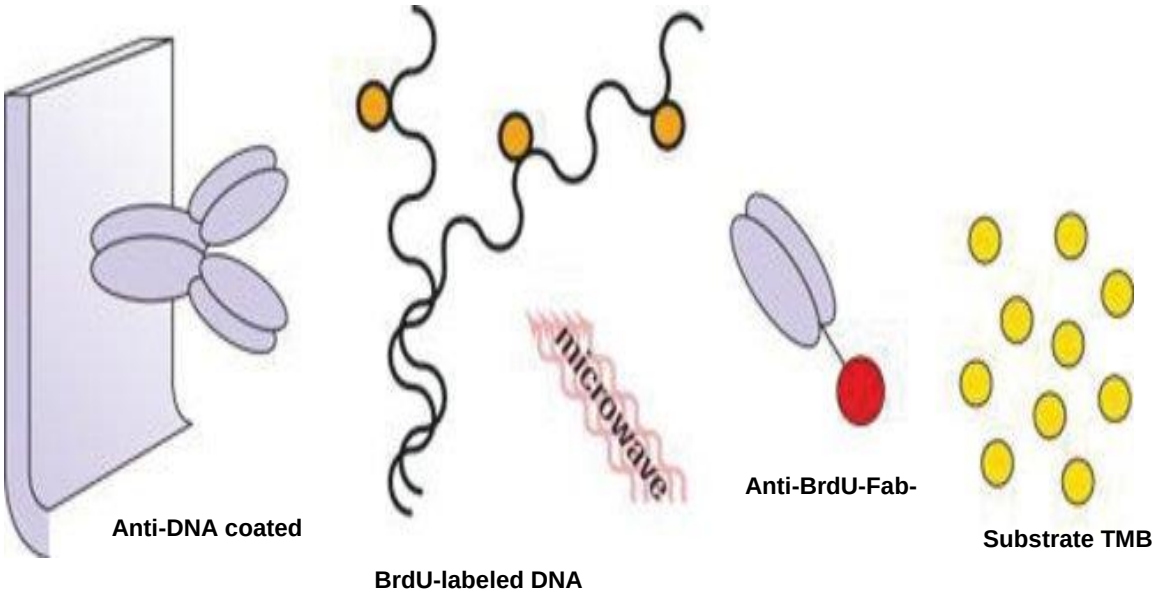


Figure 2.3: Principle of DNA fragmentation assay.

Micro-titter plates were coated with anti DNA antibody. BrdU-labelled fragments of DNA from co-culture supernatants were fixed and denatured by using microwave at 500watt for 5 minutes; this was followed by treatment with secondary antibody (anti-Brdu-Fab). Plates were incubated for 90 minutes at room temperature and substrate was added. Once yellow colour started to develope, sulphuric acid was added to stop the reaction.

2.7. SDS-PAGE and Immunoblotting (Western Blotting)

2.7.1. General

Western blotting is a technique allow for identification of a specific proteins in different fields in biological within a throughout a cell lysate. Also termed immunoblotting, due to utilising of an antibody to specifically detect their antigen. The extracted lysate was kept in buffer to prevent its integrity. Using SDS-PAGE, a form of gel electrophoresis, various proteins are size fractionated under specific denaturing conditions, thus, the electrophoretic technique enables the SDS-PAGE separation of the particles based on their charges and to identical loads according to their size. The SDS is an effective detergent acquiring and a negative charged end a long hydrophobic hydrocarbon tail. SDS interacts with the protein in their hydrocarbon portion linker their hydrophobic regions which prevents its folding and imparts a net negative charge. When the process of Proteins separated in a gel by electrophoresis is finished, then Protein transfer toward a solid support matrix (such as membrane PVDF) which has high binding affinity for them. When an electric field is applied, the proteins stably bound to the surface of the membrane, where the proteins, therefore, it is become tightly

attached. after that the proteins are detected using epitope specific primary antibodies followed by incubation of the membrane with conjugated secondary antibody raised against the primary antibody and the membrane is scanned using an infrared scanner (Mohammed and Georgopoulos, manuscript in preparation).

2.7.2. Co-culture and treatment to investigate intracellular signalling

As explained in section 2.6. Following MMC treated fibroblast effector cells (3T3-Neo and 3T3-CD40L cell which express their CD40L) were cultured in 10cm2 dishes at 3x106 cells/dish and left overnight (duplicate dishes for every cell line 10mL each) see Figure 2.6. On the next day early morning, the prostate cancer DU145 cells were co-cultured and the medium was replaced (5 mL; 3x106 cells/dish) and dishes were incubated at 37ºC and 5% CO2 for the required times (1.5, 3, 6, 12, 24, 36 and 48h). Further, the lysate were made as explained in section (2.10.3). And protein assay was performed in order to quantify Protein concentration in every lysates as explained in section (2.10.4). Lysates were also used in immunoblotting to detect intracellular protein expression in target cell (PCa cells)(Mohammed and Georgopoulos, manuscript in preparation).

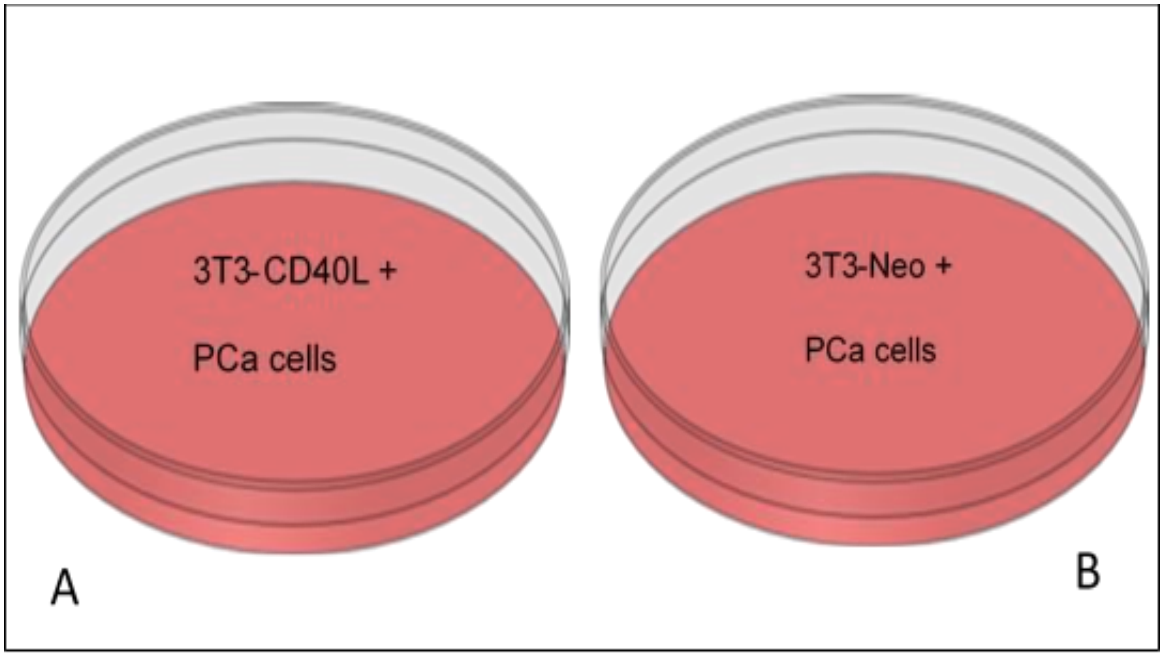


Figure 2.4: Co-culture of PCa cells with 3T3 fibroblasts; 3T3-CD40L (A) and (B) 3T3Neo.

2.7.3. Protein extraction

Transfected fibroblast cells (mCD40L and control cells) were co-cultured with target prostate epithelial cells and left for grown in 10cm2 culture dishes and lysed in situ. in some functional experiments where pharmacological agonist/antagonists were used that maintained in the co-cultures in an appropriate time as indicated. upon the completion of the incubation time, following of medium removed and cells washed 2x in ice-cold D-PBS to remove any excess proteins, the 20µl of ice-cold 2x sodium dodecyl sulphate (SDS) buffer 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 collected and transferred to 1.5 Eppendorf Tube then kept on ice for cool down. after that, by using an ultrasonic probe (Sonics Vibra cell) the lysates were sonicated for 10-second bursts until lysate resembled froth, and it was left for 30 minutes. Then using centrifuged at 12,000-14,000g, 4°C for 30 minutes, samples were centrifuged to segregated the insoluble material. (Mohammed and Georgopoulos, manuscript in preparation).

2.7.4. Protein Quantification

In order to perform western blot, the protein concentration in lysate samples has to be determined. To do so, by using of the most reliable protein estimation termed protein assay (a Coomassie protein reagent assay kit (Pierce cat# PN23236)), which involved of serial concentration of proteins standard. the protein lysates were diluted 2:23 in deionised water and then using a transparent 96-well flat bottomed plate, 10ul from diluted samples were aliquoted into a plate as well as a seven-point standard curve of 0-1mg/mL (0, 25, 125, 250, 500, 750 1000µg/mL) BSA (Pierce Cat# PN23208). after that 200ul from ambient temperature Coomassie reagent was added to each well and mixed gently by pipetting. based on a standard curve that is generated from known protein standards after the absorbance was then measured using a FLUOstar OPTIMA (BMG Labtech) plate reader at Abs 595nm against a dH2O control. thus the estimation of the protein concentration for each lysate was obtained by MARS analysis software 2.0 (BMG Labtech). (Mohammed and Georgopoulos, manuscript in preparation).

2.7.5. SDS-Polyacrylamide gel Electrophoresis (SDS-PAGE)

After protein lysates were quantified and diluted to 13ul. A total of 20ul protein samples were prepared 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 water bath was utilised to denature the samples for 10 minutes heating in a 70°C. Following to place the 10-well NuPAGETM Novex electrophoresis pre-cast gels (Invitrogen Cat# NP0321), the total of made up 800mL of 1x NuPAGETM MES SDS running buffer (Invitrogen Cat# NP0002) was flowed in both the outer and inner chambers. in order to prevent any Oxidation reactions and prior to loading of the samples, the 200µl of NuPAGETM antioxidant (Invitrogen Cat # NP0005) was added. Further, alongside of the samples loaded, a total of 500 µl mixture of 10 blue-stained recombinant proteins (10-250 kD) marker of protein size (Figure 2.7) were loaded and then the and the gel was run at 200V for 35 minutes (Mohammed and Georgopoulos, manuscript in preparation).

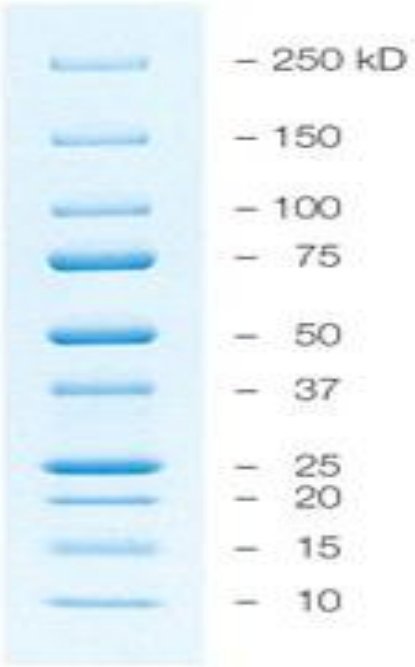


Figure 2.5: Precision plus Protein standard.

protein standards are seprated by using 10-well NuPAGETM Novex electrophoresis pre-cast gels. http://www.selectscience.net/products/precision-plus-protein-all-blue-standards-(161-0373)/?prodID=195607

2.7.6. Electrophoretic membrane transfer

When the electrophoretically-seperated proteins process finished. due to their high protein-binding affinity, the Immobilon-FLTM polyvinylidine difluoride membrane (PVDF; Millipore) was used, also using an Xcell IITM blot module (Invitrogen), to

transfer electrophoretically-seperated proteins from the gel. after that the membrane was immersed in methanol, washed with dH2O and finally was dipped in 0.5x “Towbin” transfer buffer with 20% (v/v) methanol along with the required number of blotting pads and WhatmanTM filter paper (Fisher). the gel membrane sandwich was then put together cathode to anode as follows; 2x blot pads, filter paper, gel, PVDF membrane, filter paper and 2x blot pads (as shown in figure 2.8 A and B). by utilising the Xcell SureLockTM Mini-Cell, the blot module was inserted in, followed filled with transfer buffer in inner side only, but the outer chamber was filled with ice-cold dH2O and the transfers were performed on ice at 25V for 2h (Mohammed and Georgopoulos, manuscript in preparation).

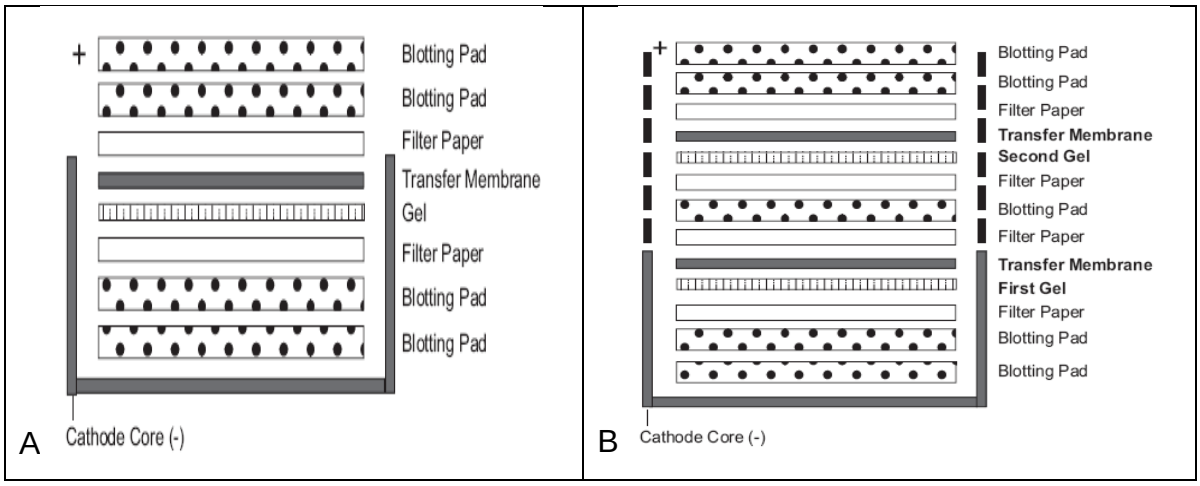


Figure 2.6: A and B. Single sandwich and double sandwich.

The diagram illustrates protein transfer during immunoblotting.

2.7.7. Membrane immunolabelling and visualisation using the Li-Cor Odyssey system

After the protein transfer process finished, and the membrane become ready for labelling. the 50:50 (v/v) Odyssey blocking buffer (Li-Cor Cat# 927-4000):10mM TBS pH 7.6 at ambient temperature was utilised to block the membrane in order to remove or minimise any non-specific binding, thus the membrane was incubated on a plate rocker for 1 hour. on a rocking platform overnight at 4°C, the membrane was incubated with 5-8mL primary antibody diluted in TBS+0.1% (v/v) Tween-20. Following the washing of membrane with TBS+0.1% (v/v) Tween-20 for 3-5 minutes, the 10mL appropriate infra-red secondary antibody (Table 2.2) was added and probed for 1 hour at ambient temperature on a rocker and prevented from direct light. then, prior to visualization using an OdysseyTM Infra-red Imaging system (Li-Cor) the membrane

was washed 1x 1x for 5 minutes with TBS. and then the densitometry was performed using Odyssey V3.1 software (Li-Cor) relative to Cytokeratin 8/18 as showed in an appendix (Dunnill et al., 2016).

2.8. Flow cytometry

2.8.1. Background

The machine of Flow cytometry is widely used in several field including biological and interestingly in medical field as well. by using parameter method which involves; Light scattering to facilitate analyse the chemical and physical characteristics of particles a technology, such as detection of fluorescence-negative cells, and gating of fluorescence measurements, and discrimination of cell types on the basis of shape and morphology.

2.8.2. Flow Cytometric Detection of CD40

The prostate cancer cell lines were cultured till 70-80% confluent cells in order to screen CD40 receptor on cell lines surface. after that cells were trypsinised and collected from a flask by using centrifuge, and cells were suspended with about 5mL of DR5% medium. using haemocytometer for cells counting which was about 1x106 cells were collected and re-suspended in 100µl of Facs buffer (1X PBS/1% FBS). by utilising Eppendorf tube, the 1x106 counted cells were divided to two for each conditions as following; two non-stain (NS) that used for assaying health cells, two for Con-PE (Phycoerythrin) which 2.5 µl was added to two tubes used as a negative control, and two for CD40 antibody which was 2.5 µl added to the Ab tubes to detect CD40 of the acquired cell population. Followed by incubation of all tubes prepared at 4ºC for 20-30 minutes, they were washed with 700µl of FACS buffer, then at 1500 RPM for 5 minutes' cells were centrifuged. Before starting determine and acquired by flow cytometry, cells were washed with 300µl of FACS buffer was added to each tube and all tubes mixed well. the generated data were analysed using a Guava EasyCyte 26 instrument and associated software (Millipore) (Mohammed and Georgopoulos, manuscript in preparation).

2.9. Detection of ROS

2.9.1. H_2DCFDA

Chloromethyl derivative of 6-carboxy-2', 7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) (Invitrogen 1795384) is a compound used as a ROS sensor that easily penetrate inside the cells passively, furthermore, it is lack of fluorescent, however, when acetate groups are removed by intracellular esterase and oxidation in the cell cytosol emits green fluorescence as an indication of ROS. this experiment was performed as shown by Dunnill et al (Dunnill et al., 2016).

2.9.2. ROS-Glo

ROS relates to a group of different types of reactive oxygen derivatives, superoxide is only one of them. Superoxide anion radical that is generated in cells and acts as a signalling molecule, which can lead to cell stress or death. in the most cases, the species of ROS are transformed to another type which has the longest half-life of all ROS in cultured cells termed H2O2 ROS determination, the ROS-Glo kit (Promega G8820) was used to measure H2O2 levels. upon of ROS generation (H2O2) that reacts with its substrate provided in the Kit. when detection reagent of ROS-Glo added which containing recombinant luciferase and D-Cysteine generates a luminescent signal (which is proportional to H2O2 level) that was detected by luminescence measurements as shown in Figure 2.3. The experiment was carried out, without MMC treated of fibroblast 3T3 CD40L and 3T3 Neo cells were cultured overnight in a white 96-well plate. Then, followed by harvested of target prostate cells, were loaded with H2O2 substrate at conc. 25µM for 30mins in suspension and incubated in incubator at 37°C and 5% CO2. after that the loaded cells were co-cultured with effector cells and incubated for 3 and 4h post receptor ligation. the H2O2 was used as a positive control at final concentration 2mM before the addition of the second reagent (ROS-Glo). then the preparation of ROS-Glo reagent was performed and added at 100µL/well and luminescence was then measured on a FLUOstar OPTIMA (BMG Labtech) plate reader. Data were analysed by using MARS software as shown by(Albarbar and

Georgopoulos, manuscript in preparation).

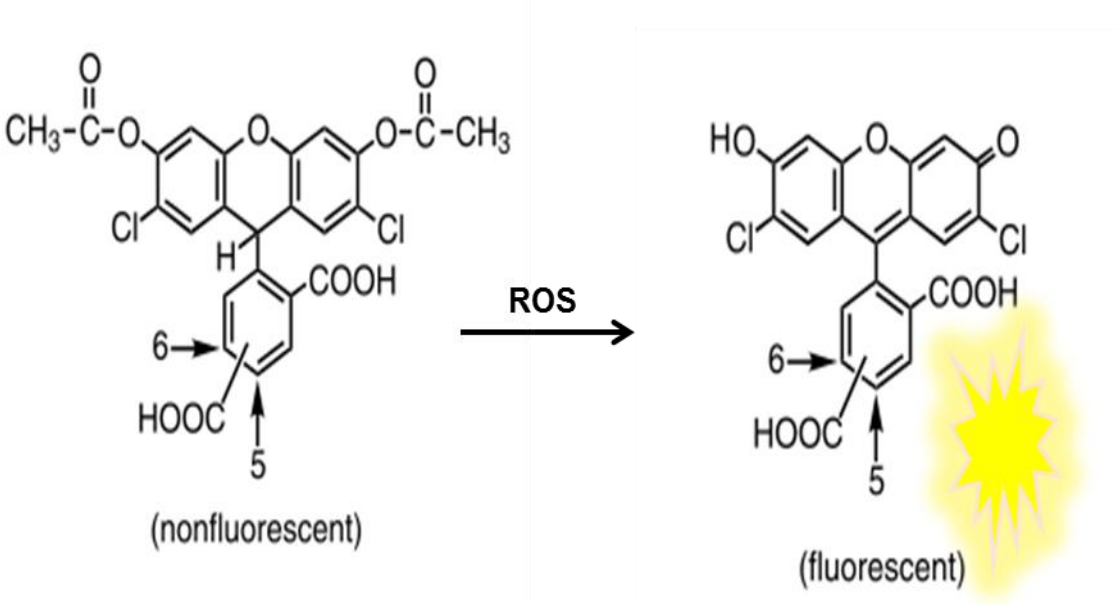


Figure 2.7: The principle of ROS detection using H2DCFDA.

The cell-permeant 2', 7'-dichlorodihydrofluorescein (H_2DCFDA), also termed as dichlorofluores *cin* diacetate that used as a useful indicator for ROS. It is chemically designed to penetrate inside the cells, as well as to be lack of any fluorescence until acetyl groups are removed by intracellular esterase(s) and ROS is induced within the cells. This converted non-fluorescent H_2DCFDA to the highly fluorescent 2',7'-dichlorofluorescein (DCF). The levels of fluorescence intensity emits are an indication of the intracellular concentration of ROS.

Adapted from https://www.thermofisher.com/order/catalog/product/D399.

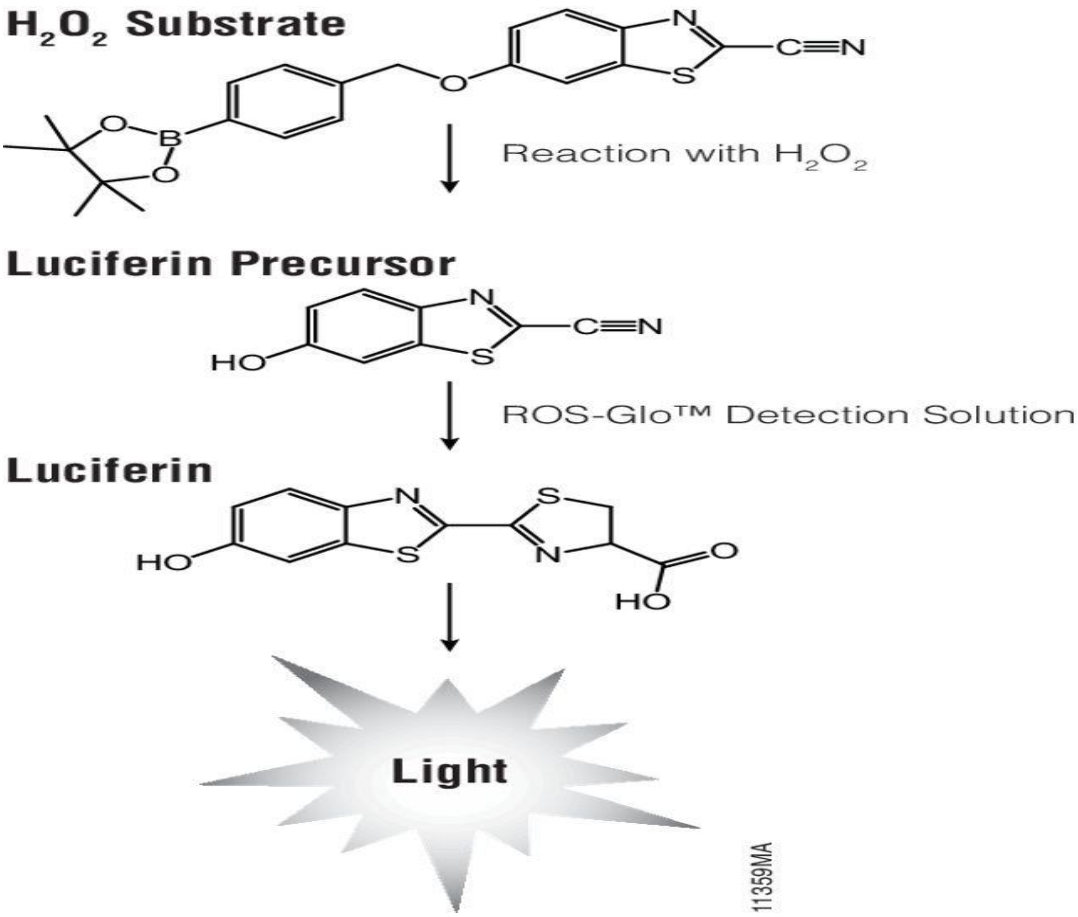


Figure 2.8: ROS-Glo assay principle.

The CM-H_2DCFDA compound utilised as a useful indicator for ROS detection, which passively penetrate inside the cell. It is a weak or non-fluorescent itself, however gives green fluorescence when acetate groups are removed by intracellular esterases and oxidation in the cells that occurs as a response to variety of stimuli which is eventually lead to ROS induction . Upon the addition of H_2O_2 substrate produces luciferin precursor as a result of the presence of H_2O_2. Then the addition of ROS-Glo™ detection reagent reacts with luciferin precursor generates a luminescent signal (LIGHT) that can be detected by the plate reader. The luminescent signal is proportional to H_2O_2 level which corresponds to ROS level. Figure was adapted from Promega's manual protocol for the ROS-Glo assay.

2.10. Detection of cytokine secretion by PCa cells upon CD40 ligation

2.10.1. Preparation of samples (supernatants)

Following treatement with MMC as explained in section 3.9, effector cells (3T3-Neo and 3T3-CD40L cell which express their CD40L surface) were cultured in 24 well plate at $6x10^4$ cells/well, (Two duplicate for each condition). Then plates were incubated at 37ºC and 5% CO2 for overnight. The medium was replaced by culturing target cells including DU145, LNaP and PC-3CD40 (500 µL; $6x10^4$ cells/well) and plates were incubated at 37ºC and 5% CO_2. Upon completion of the incubation time of post receptor ligation, supernatants were collected at specific times: 1.5,3, 6, 12, 24, 36 and 48hrs, centrifuged, aliquoted and stored in -80°C.

2.10.2. GM-CSF , interlukin-8 and interlukin-6

The secretion of cytokines including GM-CSF, IL-6 and IL-8 were estimated in co-culture supernatants, utilising ELISA kit (Catalogue number DGM00) and DuoSet ELISA/Human IL-6,8 kit respectively that were purchased from R&D system/Quantikine. For the detection of GM-CSF, GM-CSF plate was already coated by the company produced and ready for blocking step, but in the case of detection of for IL-6 and IL-8 plates were coated with 100µL/well capture antibody that prepared in PBS (without carrier protein). all plates were covered with adhesive strip and incubated overnight at room temperature. the 100µL of sample supernatants or diluted standards in reagent diluent was added to each well. then in the next day, followed by aspirated and three time washed with 400µL of wash buffer, the plates were then incubated 2h in room temperature. then plates were washed again as mentioned, and 100µL of working dilution of secondary antibody was added. in dark at room temperature, the Streptavidin-HRP was added to each well and incubated for 20mins, then followed by washed steps as previous, 100µL of substrate solution was added to each well and incubated for 20mins at room temperature. after that stope solution was gently used and added to each plates, then at 455nm wavelength, absorbance was measured and data were analysed and presented as concentration in pg/mL.

2.11. Statistical analysis

Following data collection and analysed utilising Microsoft Excel. and after mean of all replicates (5-6) values ±SEM calculated with error bars representing ± the standard error of mean and presented asvalues ±SEM . the Minitab 17 software was used and P-value was prduced by using two tailed paired student t-test.

Chapter 3: **Investigation of CD40-mediated apoptosis in PCa cells**

3.1. Overview

CD40 is a member of the TNFR family and belongs to the non-death domain containing receptors group, also referred to as the TIM domain-containing subgroup (Albarbar et al., 2015). Although it plays a central role in the functioning of the immune system, CD40 is expressed on several types of epithelial cells, endothelial, smooth muscle, fibroblasts and keratinocytes as well as a variety of carcinoma cells (Rokhlin et al., 1997; Schönbeck and Libby, 2001; van Kooten and Banchereau, 1997; van Kooten and Banchereau, 2000). By contrast, expression of its main ligand CD40L is tightly controlled and CD40L is expressed on activated T cells, it can be detected on dendritic cells (DCs), basophils, eosinophils, monocytes, macrophages, and neutral killer (NK) cells (Schönbeck and Libby, 2001).

Despite the activatory role it plays in the immune system and the lack of a death domain in its cytoplasmic tail, many studies have been demonstrated that soluble CD40 agonist can induce growth inhibition in several epithelial carcinoma cells of various region (Tong and Stone, 2003; Vonderheide et al., 2007). These agonists tend to be weakly pro-apoptotic and become highly effective only in combination with specific pharmacological inhibitors (e.g. cycloheximide) or chemotherapy agents (Afford et al., 2001; Bugajska et al., 2002; Georgopoulos et al., 2006; Georgopoulos et al., 2007; Hess and Engelmann, 1996). By contrast, previous work in our laboratory has demonstrated that receptor activation by membrane-presented CD40L (mCD40L) represents a highly pro-apoptotic signal (Georgopoulos et al., 2006; Georgopoulos et al., 2007; Hill et al., 2008; Shaw et al., 2005). mCD40L triggers extensive apoptosis in a variety of carcinoma cells whilst their normal epithelial counterparts are refractory (Bugajska et al., 2002; Dunnill et al., 2016; Shaw et al., 2005); by contrast, soluble agonists (soluble trimeric CD40L or agonistic anti-CD40 antibody) are weakly pro-apoptotic (Bugajska et al., 2002; Shaw et al., 2005).

3.2. CD40 receptor activation by mCD40L (CD40L) using a co-culture model

In order to accomplish CD40–CD40L interactions mediated by membrane CD40L (mCD40L), growth-arrested mouse NIH3T3 derivatives (effector) cells manipulated to express membrane CD40L were co-cultured with prostate carcinoma (target) cells. Previous work in our laboratory that involved optimisation of experimental techniques

in order to determine cell death mediated by CD40 using this co-culture system, has allowed accurate and reliable determination and quantification of mCD40L-mediated cell death utilising 96-well plate format assays (Dunnill et al., 2016; Georgopoulos et al., 2006). However, such optimisations were performed in other carcinoma cells types.

Therefore, in this study, further optimisation experiments were performed in order to establish and determine appropriate experimental conditions for determining mCD40L-mediated death in PCa cells (which included determination of optimal cell density and incubation times for PCa cells). The fibroblasts (NIH3T3 effector cells) that were transfected to express CD40L on their surface were termed “3T3CD40L” (and the signal “mCD40L”); homologous mock-transfected control NIH3T3 cells, served as negative (background) controls and were termed “3T3Neo” throughout this study. In addition, for the potential culture-related genetic drift, and to prevent loss of CD40L expression, 3T3CD40L cells were routinely cultured in the presence of G418 antibiotic (0.5mg/ml), with omission of the antibiotic during co-culture with target epithelial cells.

3.3. Objectives

The specific aims were:

- To determine whether PCa cell lines including (DU145, PC-3 and LNCaP), as well as prostate cancer cells express CD40.
- To determine the regulation of CD40 on carcinoma cells by pro-inflammatory cytokine (IFN-γ and TNF-α) treatment.
- To perform and optimise for the first time the co-culture system to activate CD40 on target PCa cell lines by using effector cells expressing mCD40L.
- To determine if CD40 and its signalling can mediate cell death
- To engineer de novo expression of CD40 in receptor negative PC-3 by using retrovirus transduction to express CD40 and determine whether mCD40L could cause apoptosis.
- Use soluble CD40L or agonistic anti-CD40 antibody (G28-5 mAb) to treat the target PCa cells in order to determine the difference between these soluble agonists and mCD40L.
- To perform assays for the detection of a) loss of membrane integrity, b) caspase activity and c) DNA fragmentation in order to determine the type of death triggered by CD40 in these cells.

3.4. Detection of CD40L and CD40 expression

In order to perform co-cultures, it was essential to ensure that effector and target cells PCa expressed CD40L and CD40, respectively. Therefore, the expression of CD40 on PCa cells lines was detected by both western blotting and flow cytometry assays. In addition, the expression of CD40L at cell membrane (3T3 fibroblasts) was confirmed by flow cytometry.

3.4.1. Flow cytometry

Flow cytometry is a technique used to detect specific proteins on the surface (mainly) or inside cells (following permeabilisation). Cells are immune-labelled with a fluorochrome-conjugated antibody to determine protein expression. The principle of cytometry depends on scanning each single cell, as they pass through, an excitation light source (laser) which is scattered and fluorescence is emitted as light from the excitation source strikes the moving cell. This fluorescence is measured for each individual cells and it is proportional to the level of protein expression. An example of flow cytometric analysis optimisation is provided in Figure 3.1, it show how to apply Gating strategies were used in flow cytometry to determine protein expression in DU145 cells.

The surface of CD40L and CD40 receptor were investigated for both effector cells (3T3 fibroblasts) and target cells (PCa) respectively, alongside with EJ cells (urothelial carcinoma cells) that served as positive controls based on previous studies by our group (Georgopoulos et al., 2006; Georgopoulos et al., 2007). The experiments were carried out as mentioned in the Materials and Methods (section 2.9.). 3T3-CD40L cells were positive for CD40L expression (expressed high amount of protein) when compared with control 3T3Neo which were negative, as illustrated in Figure 3.2. CD40 receptor was detectible on both of DU145 and LNCaP cells, although to a different extent, whereas PC3 was negative, compared with positive controls EJ cells as shown in Figure 3.3.

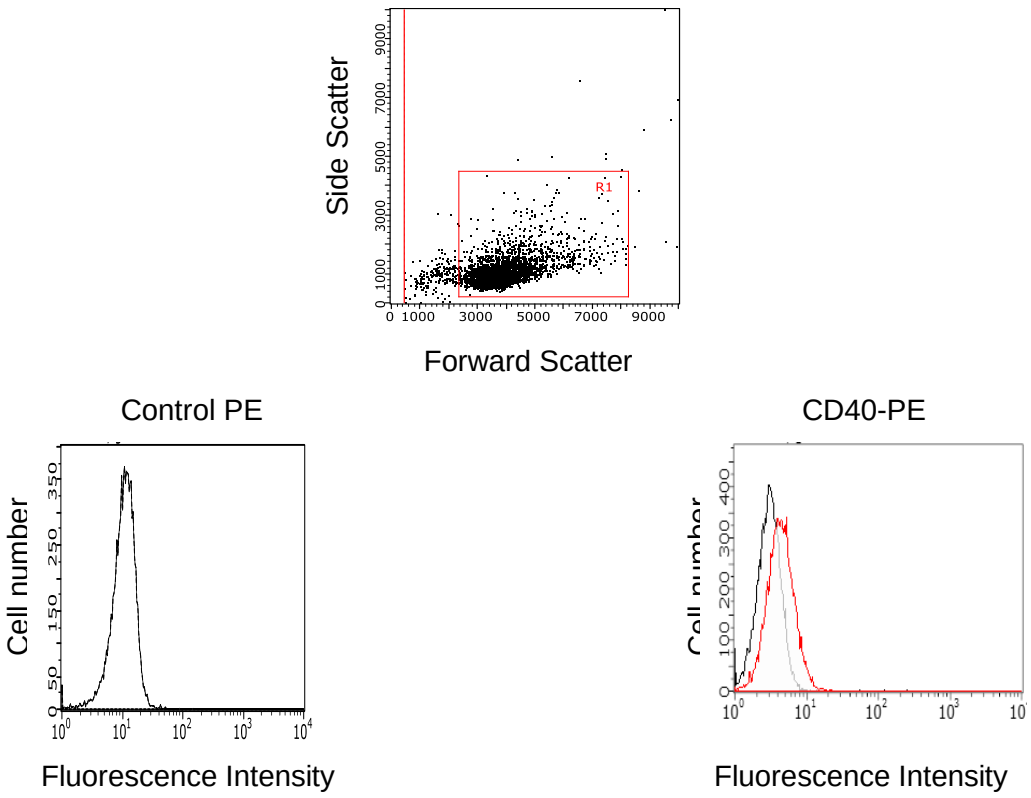


Figure 3.1:Example of flow cytometry optimisation.

Upper panel indicates Forward and Side scatter parameter detection for the determination of the viability of the cell population (the appropriate healthy cell population was gated using region R1). Cells analysed represent the DU145 cell line. The lower left histogram depicts control isotype antibody PE, lower right histogram depicts CD40 expression using CD40 PE conjugated antibody compared with control PE.

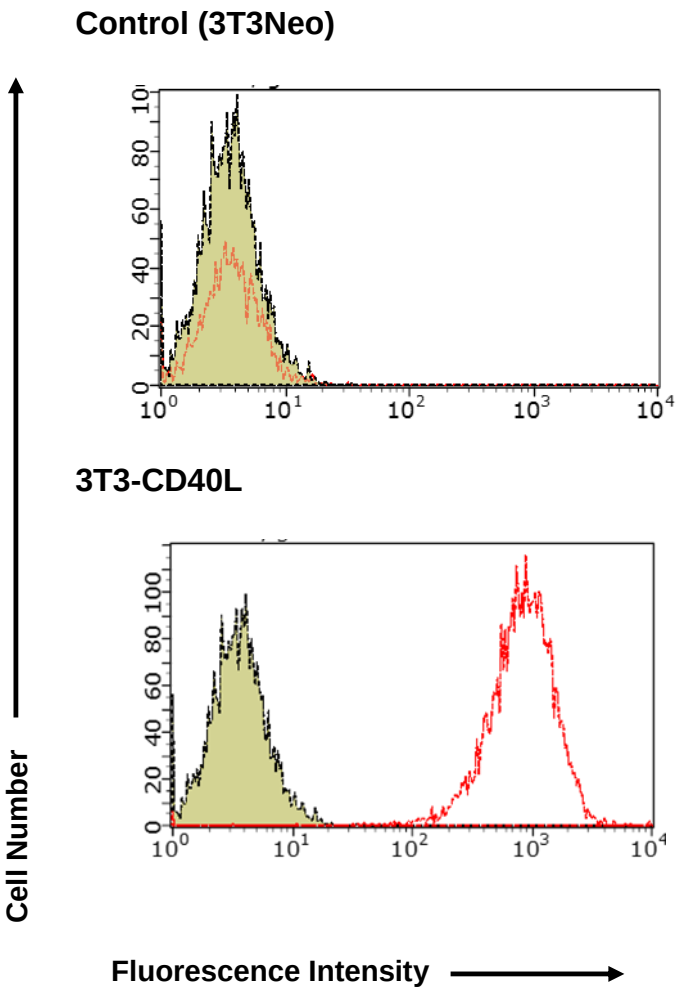


Figure 3.2: Flow cytometry analysis of CD40L expression.

Expression of CD40L on the cell surface of fibroblast 3T3CD40L and 3T3Neo were detected by flow cytometry. Cells were cultured until approximately 80% confluent and were harvested by trypsinisation. Cells were counted and adjusted at 0.25 x10^6 cells/100µl of FACS buffer. Cells were incubated with PE-conjugated mouse anti-human CD40L (empty red histogram), and a control PE-conjugated isotype-matched control Ab was also used (filled green histogram). Cells were acquired on a Guava EasyCyte flow cytometer and results analysed using Guava software.

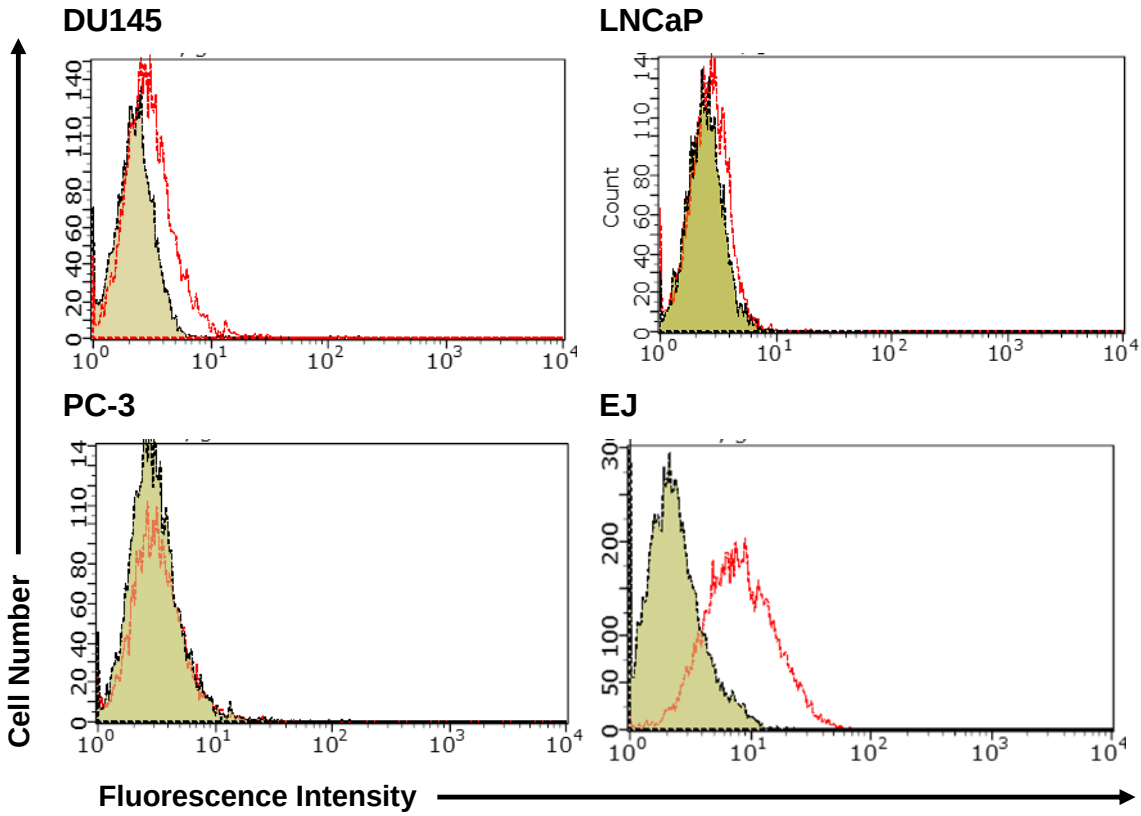


Figure 3.3: Flow cytometric analysis of CD40 expression on PCa.

Expression of CD40 receptor on the cell surface of PCa (DU145, LNCaP and PC-3) and EJ cells were detected by flow cytometer. Cells were cultured until approximately 80% confluent and were harvested by trypsinisation. Cells were counted and adjusted at 0.25 x10^6 cells/100µl of FACS buffer. Cells were incubated for 20-30 minutes with PE-conjugated mouse anti-human CD40 (2.5µl in 25 µl cell suspension FACS buffer) (empty red histogram), and a control PE-conjugated isotype-matched control Ab was also used (1/10 in FACS buffer) (filled green histogram). Results representative of a total two independent experiments for each experiment duplicates to be more performed(n=2). Cells were acquired on a Guava EasyCyte flow cytometer and results analysed using GuavaSoft software.

3.4.2. Western blotting

Thus, the expression of proteins CD40 were investigated in target cells (PCa). Experiments were performed as explained in material and methods (section 3.7.2). CD40 expression was detectable in DU145 and LNCaP cells, however, PC-3 cells were negative, as illustrated in figure 3.4 and in agreement with the Flow cytometry results.

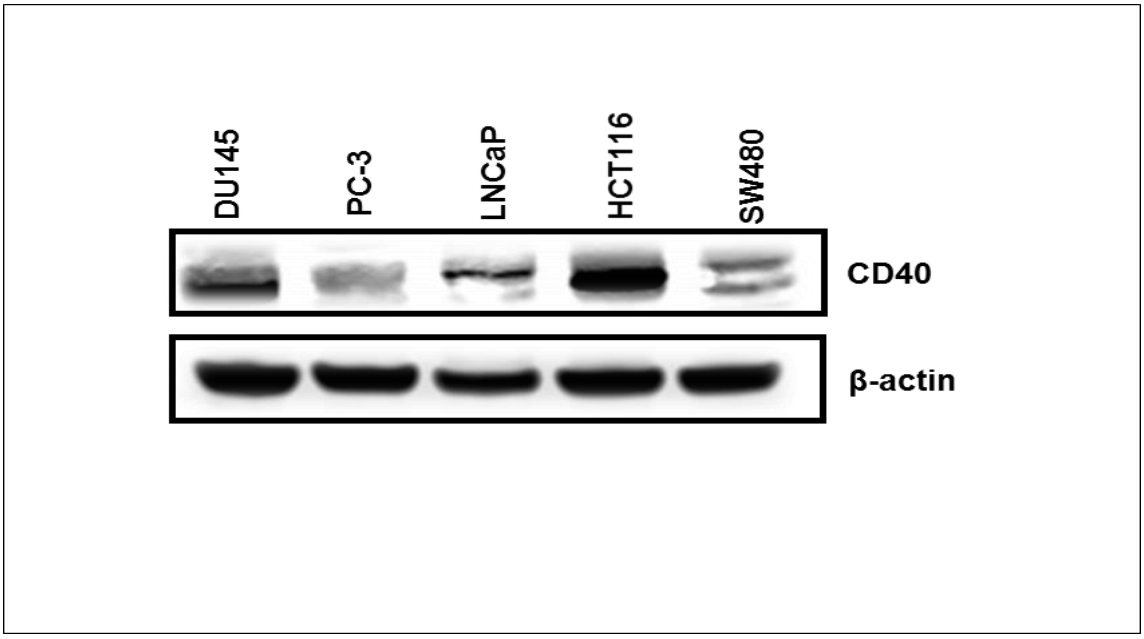


Figure 3.4: Expression of CD40 on PCa by Western blotting.

Western blot analysis for CD40 expression on PCa cells (PC-3, LNCaP and DU145 cells). Both of colorectal cancer cells HCT116 and SW480 were serving as a positive and negative control, respectively. Total amount of protein loading was 40µg/well. The membranes were incubated over night 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 for loading confirmation, the membrane was incubated with the primary antibody (diluted at 1:25000) and secondary antibody goat-anti mouse IgG Alexa 680 (diluted 1:10000). Membranes were scanned on Licor Odyssey Infra-Red Imaging System.

3.5. CD40 expression in prostate cancer stem cells (CSC)

There is evidence suggesting that prostate cancer development is dependent on the existence of prostatic cancer stem cells (CSC), also termed as tumour-initiating cells (TIC). The importance of such CSC cells in prostate carcinogenesis is in line with observation in other cancer types, including brain cancer, breast cancer, colon cancer, hepatic carcinoma, lung cancer, melanoma, ovarian cancer and pancreatic cancer. CSCs possess the ability to self-renew and to give rise to the heterogeneous lineages of cancer cells. For the prostate, these cells are characterized by the expression of specific markers CD44+/$\alpha_2\beta_1$/CD133+, after they had been identified and isolated (Jaworska et al., 2015; Maitland et al., 2011).

Prostate CSC were as kindly gift from Prof Norman Maitland and Dr Fiona Frame (Department of Biology, University of York) and were isolated from patients with different prostate cancer stages, and maintained (for limited passages) in complete KSFM medium, as described in the Methods (section 2.4.5). Microscopy images of prostate cancer stem cells cultured are provided in Figure 3.5.

This part of the work aimed to determine the expression of CD40 in prostate cancer stem cells alongside with prostate cancer cell (DU145), by using both western blotting and flow cytometry assays. Furthermore, the expression of CD40 was confirmed as shown in Figure 3.6, 3.7.

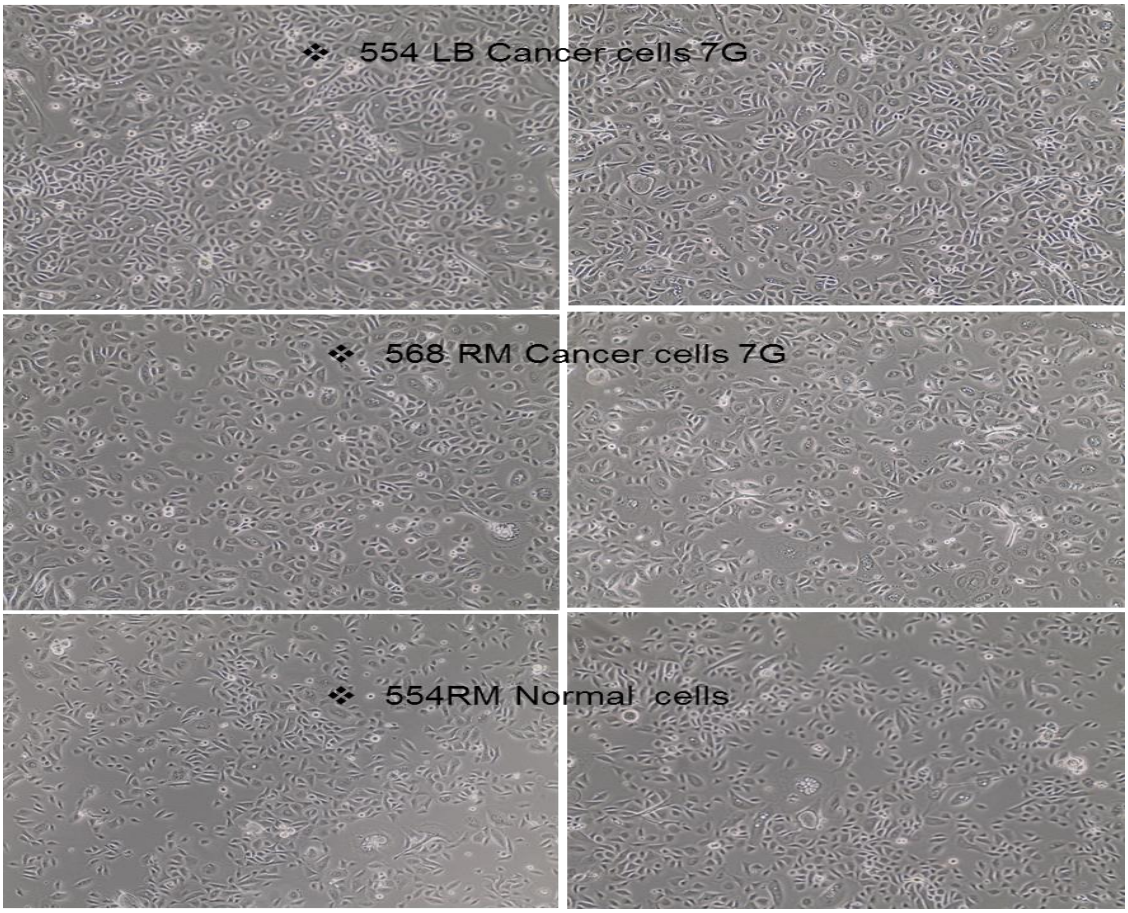


Figure 3.5: Microscopy images of prostate cancer stem cells.

Csc were cultured in T25 flask cell+vented cap, at passage 3 and 4 which were isolated from different patients as indicated.554 LB isolated from left base(Gleason7), 568 RM isolated from right middle (Gleason 7) and 554 isolated from right middle (normal).

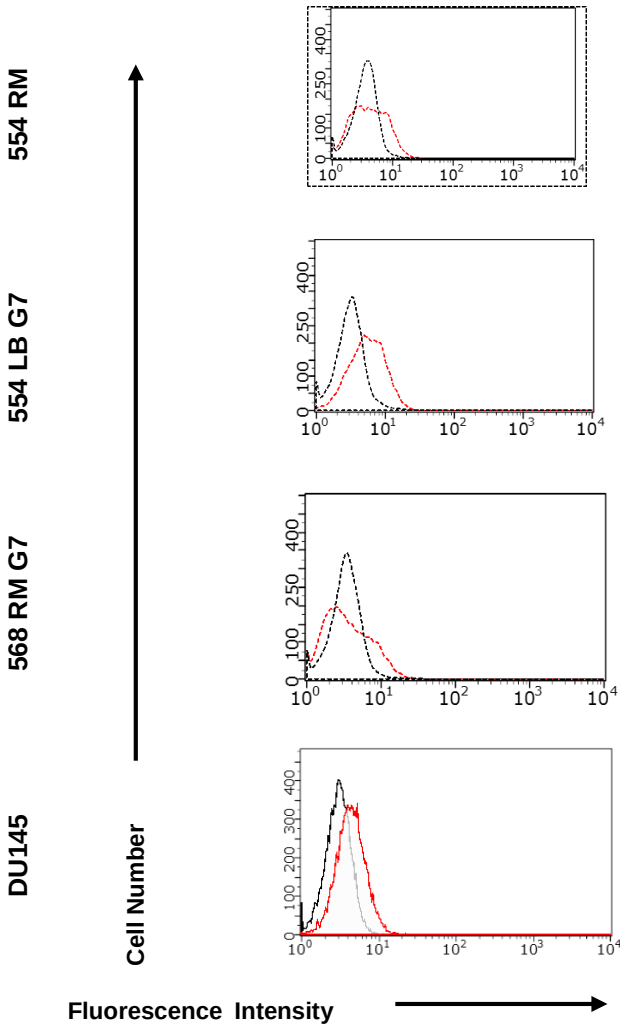


Figure 3.6: Flow cytometric analysis of CD40 expression on Csc.

Expression of CD40 receptor on the cell surface of Csc (554 LB isolated from left base (Gleason7), 568 RM isolated from right middle (Gleason 7) and 554 isolated from right middle (normal), alongside with DU145 cells were detected by flow cytometry. Csc cells were cultured in T25 flask cell+ vented cap until approximately 80% confluent and were harvested by trypsinisation. Cells were counted and adjusted at 0.25 x10^6 cells/100µl of FACS buffer. Cells were incubated for 20-30 minutes with PE-conjugated mouse anti-human CD40 (2.5µl in 25µl cell suspension FACS buffer) (empty red histogram), and a control PE-conjugated isotype-matched control Ab was also used (1/10 in FACS buffer) (empty black histogram). Cells were acquired on a Guava EasyCyte flow cytometer and results analysed using GuavaSoft software.

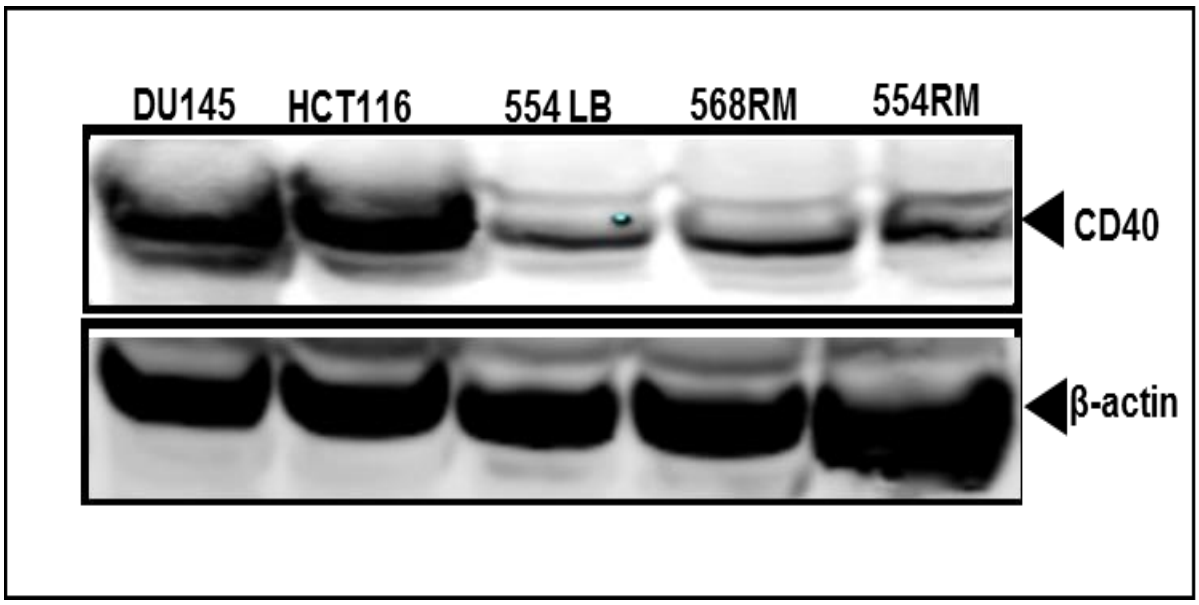


Figure 3.7: Expression of CD40 on Csc by Western blotting.

Western blot analysis for CD40 expression on Csc cells (554 LB isolated from left base (Gleason7), 568 RM isolated from right middle (Gleason 7) and 554 isolated from right middle (normal)), alongside with DU145 and (colorectal cancer) HCT116 cells. Both served as a positive control. Total amount of protein loading was 20µg/well. The membranes were incubated over night with primary antibody (CD40 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 for loading confirmation, the membrane was incubated with the antibody (diluted at 1:25000) and secondary antibody goat-anti mouse IgG Alexa 680 (diluted 1:10000). Membranes were scanned on Licor Odyssey Infra-Red Imaging System.
Key words: RM; right middle. LB; left base. (Based on biopsy sample location).

3.6. Regulation of CD40 expression by pro-inflammatory cytokines in PCa cells

The regulation of CD40 expression in epithelial cells by pro-inflammatory cytokines such as TNF-α and IFN-γ is well documented in various cell types (Bugajska et al., 2002; Schwabe et al., 2001; Wingett et al., 1998) and more specifically CD40 surface expression is upregulated following treatment with these cytokines in epithelial cells (Georgopoulos et al., 2007). Furthermore, in comparison, IFN-γ tends to be more effective than TNF-α in terms of CD40 upregulation in positive cells as shown by ourselves (Bugajska et al., 2002; Georgopoulos et al., 2007) and others (Rissoan et al., 1996). In contrast, treatment of receptor negative cells by both cytokines alone or in combination cannot induce CD40 expression (Georgopoulos et al., 2007).

Thus, the regulation of surface CD40 expression by pro-inflammatory cytokines was assessed. PCa cells were cultured and left overnight and the following day, cells were treated either with 1000U/mL TNF-α or IFN-γ for a period of 48 h and CD40 expression assessed by flow cytometry (the same gating principle followed for these experiments is provided in Figure 3.1), and as detailed in the Methods (section 2.9).

Representative results from such experiments are shown in Figure 3.8. Both TNF-α or IFN-γ caused marked upregulation of CD40 expression on DU145 cells, which was more pronounced by IFN-γ. The responses were different when compared to the other two cell lines, LNCaP and PC-3. IFN-γ induced significant upregulation of CD40 on LNCaP cells, however when treated with TNF-α cells underwent extensive cell death as shown in microscopy images Figure 3.9. By contrast, PC-3 treated with either TNF-α or IFN-γ, which was negative for CD40 expression, did not induce *de novo* CD40 expression with neither. Our data are anticipated and in agreement with previous studies (Chopra et al., 2004; Georgopoulos et al., 2006; Georgopoulos et al., 2007).
In order to observe any morphological changes in response to treatment with TNF-α or IFN-γ, microscopy images were taken, which showed that IFN-γ and TNF-α treatment appeared to promote cell growth or had no effects on the cell growth for 2 of the 3 PCa lines, with the exception of LNCaP cells treated with TNF-α. As shown in Figure 3.9, such treatment caused extensive cell death in this cell line.

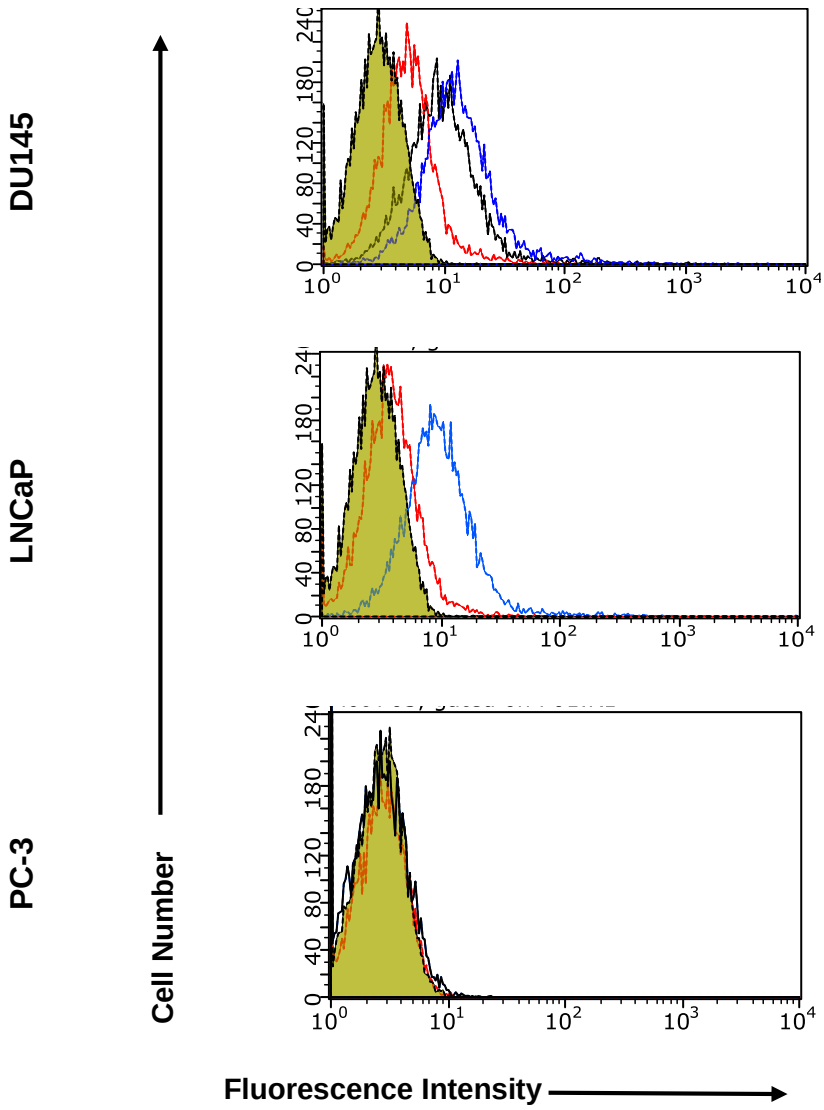


Figure 3.8: Regulation of CD40 expression by pro-inflammatory cytokines.

PCa cells were seeded in the presence or absence of either IFN-γ or TNF-α for 48h in 6-well plates. Cells were collected, counted and adjusted at 0.25 x10^6 cells/100µl of FACS buffer. Cells were incubated for 20-30 minutes with PE-conjugated mouse anti-human CD40 (empty red histogram), and a control PE-conjugated isotype-matched control Ab was also used (filled green histogram). Cells were treated with 1000U/mL of IFN-γ (unfilled blue histograms) or TNF-α (unfilled black histograms). Then analysed after 48h, Cells were acquired on a Guava EasyCyte flow cytometer and results analysed using GuavaSoft software.

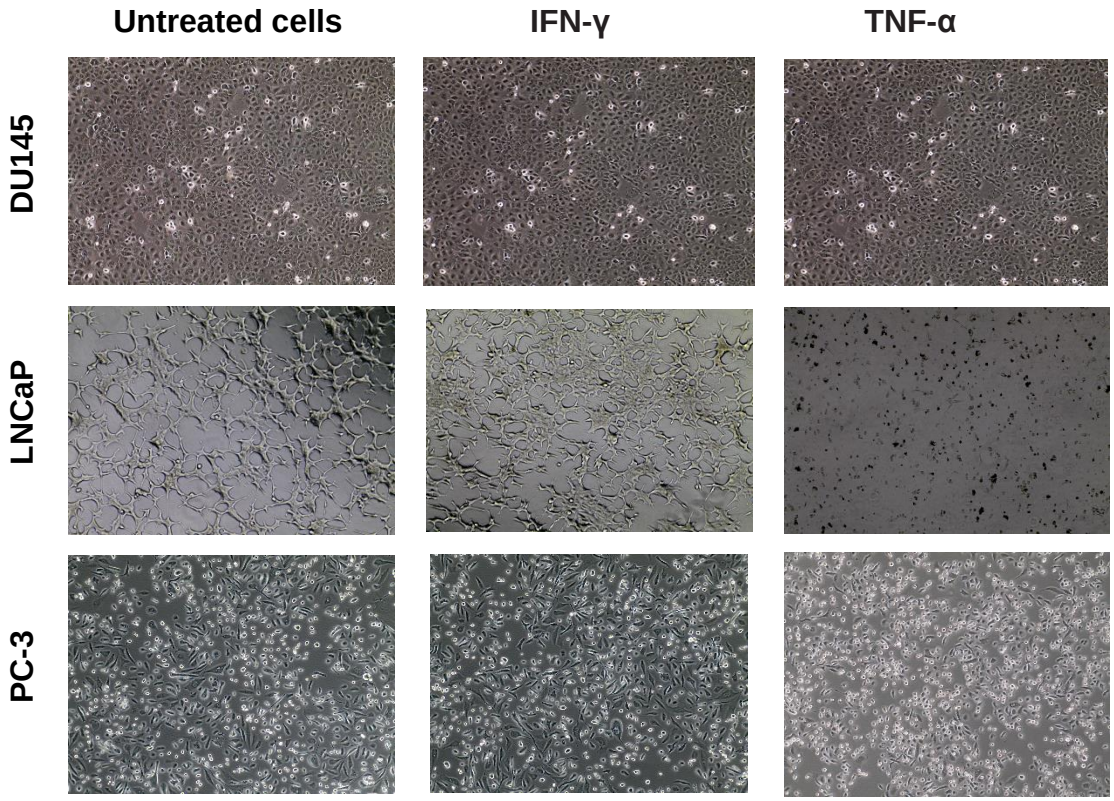


Figure 3.9: Microscopy images of untreated and treated PCa cells with IFN-γ and TNF-α.

PCa cells (DU145, LNCaP and PC-3) were cultured in 96 well plate overnight, on the following day; cells were treated with IFN-γ and TNF-α at concentrations 1000U/mL. And then incubated for 48h at 37°C. Phase contrast microscopy images were taken following 48 h incubation time at 100x magnification using an EVOSXL inverted microscope (PeqLab).

3.7. Retroviral transduction of PC-3 cells to express CD40

Retrovirus-mediated transduction can be utilised to insert a gene of interest stably within a population of rapidly dividing cells. This method is much more effective than many transfection protocols, resulting in less cellular stress and/or death. In fact, it has been demonstrated that the transduction methods used in our laboratory are often >90% efficient (Crallan et al., 2006).

In order to produce CD40-negative PC-3 cell line derivatives that expressed the cDNA encoding human CD40, retrovirus transduction was utilised as described elsewhere (Georgopoulos et al., 2006; Georgopoulos et al., 2007). The precise methodology followed for virus preparation, PC-3 cell transduction and initial selection in G418 antibiotic is detailed in the Methods (section 2.5.3.1).

Initial pre-titration experiments were carried out, with PC-3 target cells seeded in 24-well plates and treated with different concentrations of G418 antibiotic as indicated in Figure 3.10. Cells were routinely visualised using phase-contrast microscopy, which showed that G418 exhibited potent cytotoxic effects at concentrations >500µg/mL, thus this was selected as optimal concentration for selection purposes.

Moreover, during the transfection process, microscopy images were taken as shown in Figure 3.11. The first series of transduced PC-3 cells were observed 72 h following incubation in 500µg/mL G418 (images 1, 2 and 3) compared with control (Non transfected cells incubated in 500µg/mL G418 antibiotic) which had completely perished by 48h (image 6). Transduced cells continued growing after passage (split ratio 1:3) and were maintained in 500µg/mL G418 antibiotic (image 4 and 5).

Flow cytometry analysis results showed that PC-3 transfected with PLXSN-CD40 retrovirus was CD40-positive and expressed high levels of CD40 protein. Moreover, this expression was found to be stable through several passages in culture. No CD40 expression was detected in isogenic PC-3 cells transduced in parallel with control PLXSN (empty) virus, whilst EJ cells served as positive control (Figure 3.12). Furthermore, Western blotting experiments (Figure 3.13) confirmed CD40 expression in agreement with the flow cytometry data.

It should be noted that, as demonstrated in flow cytometry analyses (Figure 3.12), despite adequately lengthy selection of transduced PC-3 cells in antibiotic, there appeared to be two populations of transfected PC-3 CD40, and a negative population

was maintained alongside the positive one. Despite efforts to exclude the negative population by prolonged antibiotic treatment (as well as increasing the concentration of G418 from 0.4 to 0.8 and later on to 1mg/mL) in order to kill any non-resistance cells, the CD40-negative sub-population could not be eliminated.

To resolve this it was decided that the CD40-positive cells would be selected by fluorescence associated cell sorting (FACS) which was carried out at the Leeds Institute of Cancer and Pathology at the University of Leeds (in collaboration with Dr Liz Illett and Prof Alan Melcher) (for more details see material and methods section 2.6 and Appendix I). Sorted PC-3-CD40 cells were cultured and passaging was kept to a minimum and CD40 detected by flow cytometry. Despite FACS sorted, routine flow cytometry analysis shown there were still two populations of cells, however, it was noted that the results had shown slight improvement in terms of the proportion of positive cells (38.48%) comparing with negative cells (61.16%) as seen in Figure 3.14.

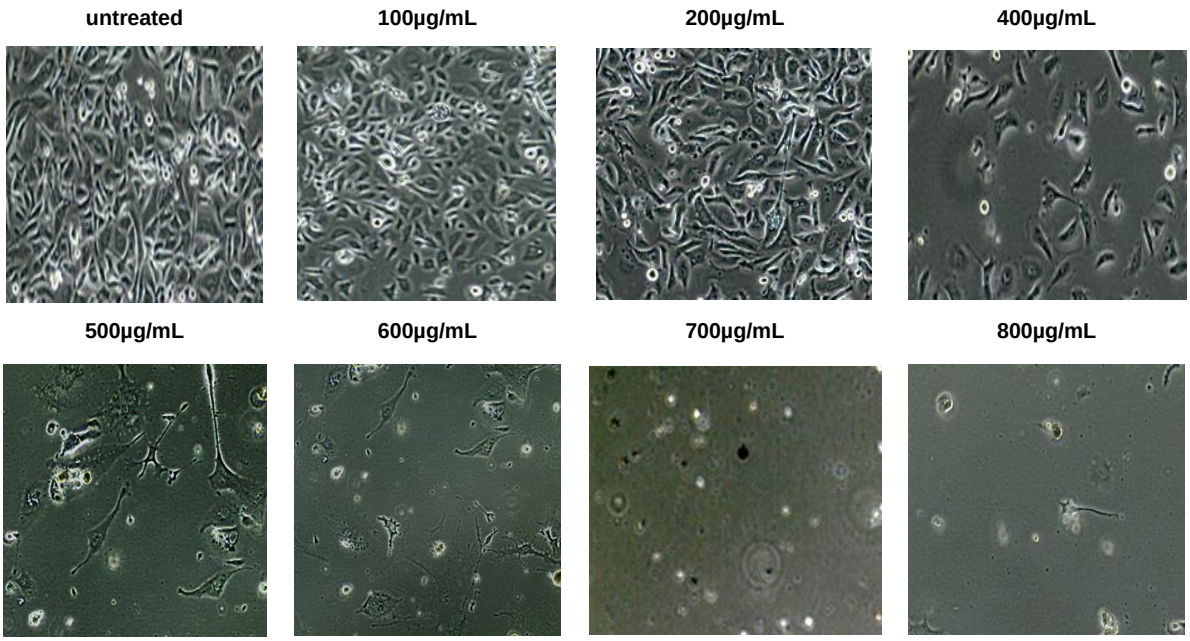


Figure 3.10: Titration for determination of optimal concentration of G418 for antibiotic-based selection.

$6x10^4$ PC-3 target cells were seeded in 24-well plates with DR5% supplemented 1% LG. At the same time, cells were treated with different concentration of G418 antibiotic as indicated. Cells were incubated at 37° C and 5% CO_2. Untreated cells served as a control. Cells were routinely visualised by phase contrast microscopy

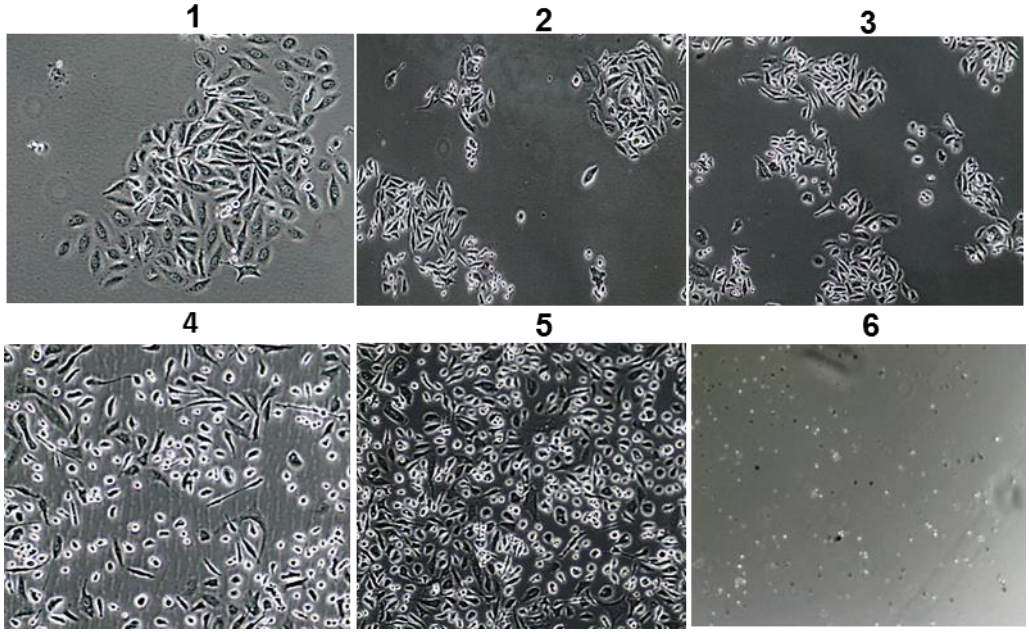


Figure 3.11: Microscopy images during retrovirus transduction process in PC-3 cells.

Images 1, 2 and 3 show example cultures with the first series of transduced PC-3 cells observed after 72h incubation time in 500µg/ml G418 (compared with control, non-transduced cells which were dead image 6). Transfected cells continued growing after passage (split ratio 1:3) and were maintained in 500µg/mL G418 antibiotic (images 4 and 5).

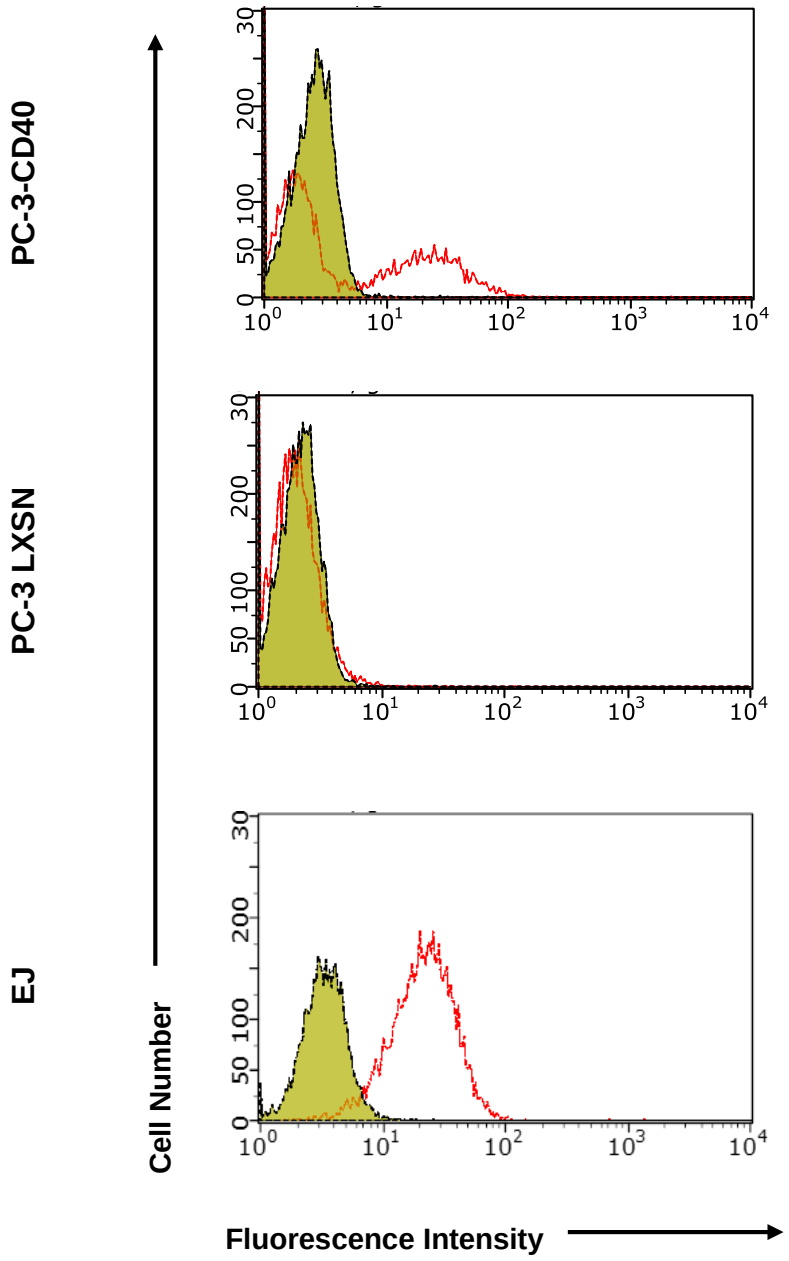


Figure 3.12: Flow cytometric analysis of CD40 expression on retrovirus transduced PC-3 cells (PC-3-CD40).

Expression of CD40 receptor on the cell surface of transduced PC-3 cells (PC-3CD40, PC-3LXN) detected by flow cytometry. Cells were maintained in 500µg/ml G418 and cultured till approximately 80% confluent and were harvested by trypsinisation. Cells were counted and adjusted at 0.25 x10^6 cells/100µl of FACS buffer. Cells were incubated for 20-30 minutes with PE-conjugated mouse anti-human CD40 2.5µl in a 25 µl cell suspension in FACS buffer (empty red histogram), and a control PE-conjugated isotype-matched control Ab was also used (1/10 in FACS buffer) (filled green histogram). EJ cells served as a positive control. Cells were acquired on a Guava EasyCyte flow cytometer and results analysed using GuavaSoft software.

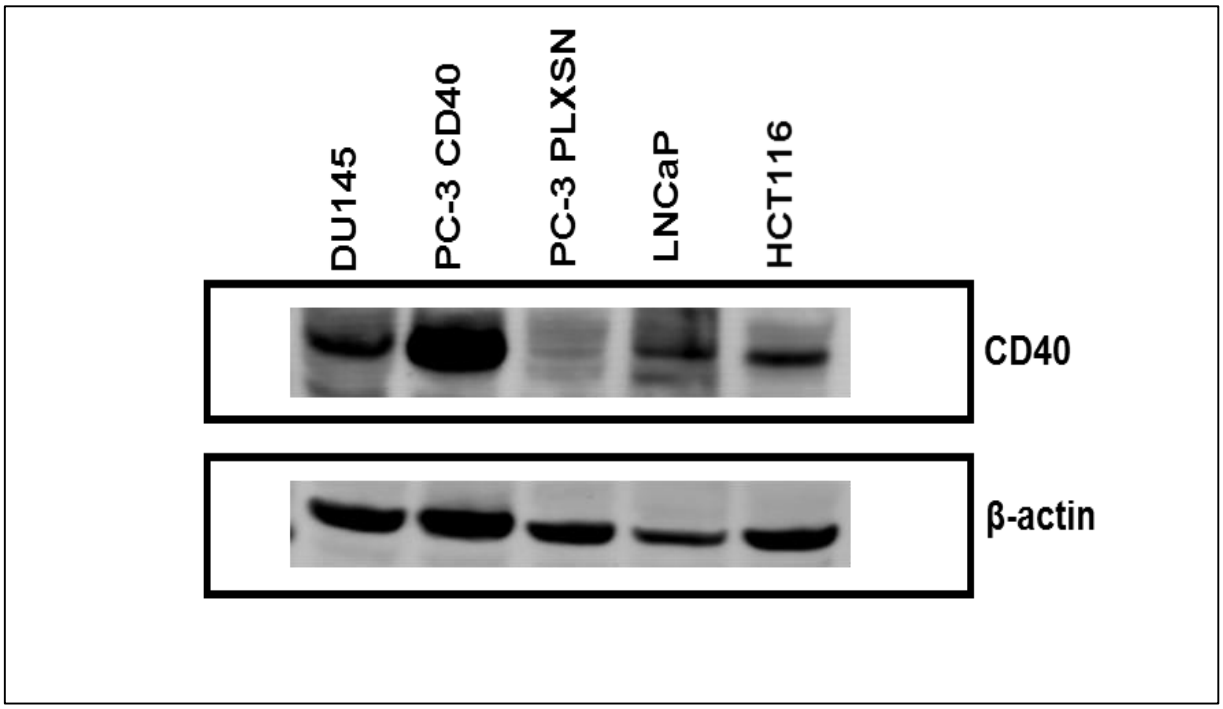


Figure 3.13: Expression of CD40 detected by Western blotting.

Western blot analysis for CD40 expression in PCa cells DU145, PC3-CD40, and PC-3PLXSN and LNCaP cells, as well as the CRC HCT116 cell line (positive control). Total amount of protein loading was 40µg/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 was used as specificity and loading control, the membrane was incubated with the antibody diluted at 1:25000 and secondary antibody goat-anti mouse IgG Alexa 680 diluted 1:10000. Membranes were scanned on a Licor Odyssey Infra-Red Imaging System.

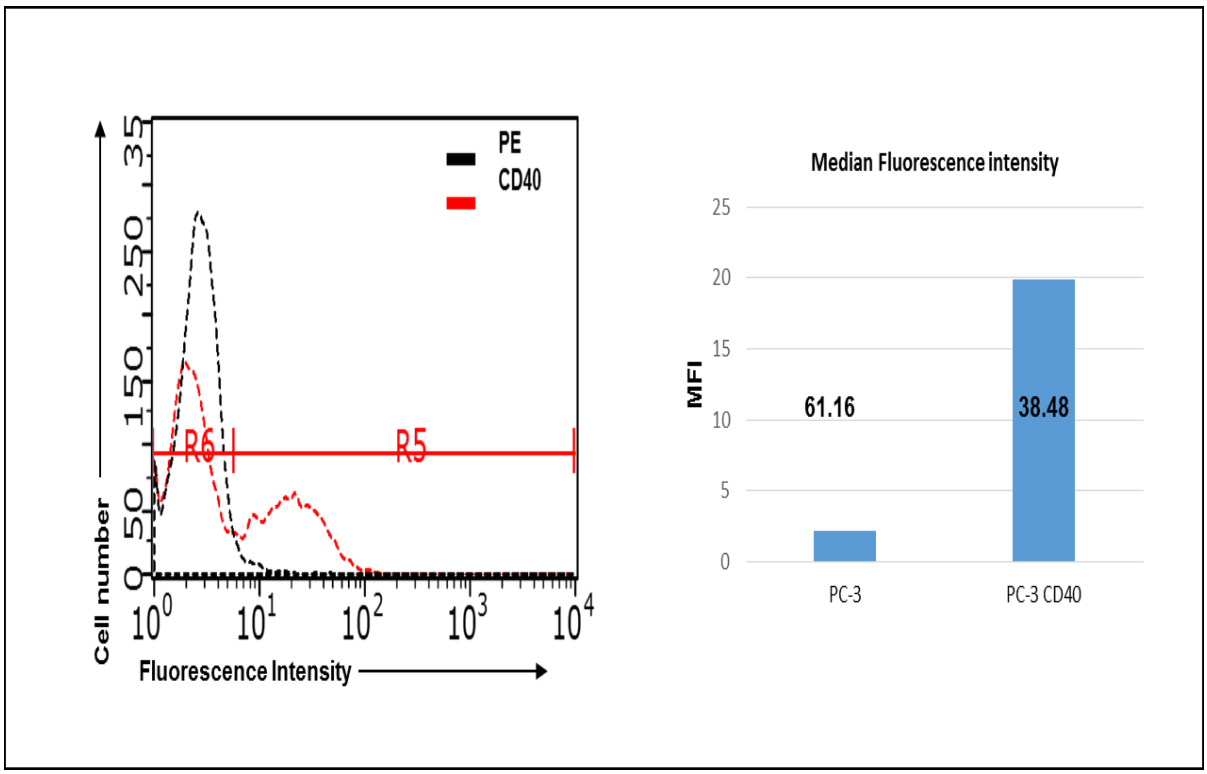


Figure 3.14: Expression of CD40 by flow cytometry on PC-3-CD40 cell populations following fluorescence activated cell sorting (FACS).

Expression of CD40 receptor on the cell surface of PC-3 transfected after cell sorting (performed at the University of Leeds and detailed in the text and the Methods section) was detected by flow cytometry. Cells were maintained in 0.8mg/ml G418 and cultured till approximately 80% confluent and were harvested by trypsinisation. Cells were counted and adjusted at 0.25 x10^6 cells/100µl of FACS buffer. Cells were incubated for 20-30 minutes with PE-conjugated mouse anti-human CD40 2.5µl in 25 µl cell suspension FACS, and a control PE-conjugated isotype-matched control Ab was also used (1/10 in FACS buffer). Cells were acquired on a Guava EasyCyte flow cytometer and results analysed using Guava software. Regions (gates) R5 and R6 represent CD40-positive and CD40-negative populations, respectively (left panel) and their MFI values have been plotted on the graph on the right.

3.8. Optimisation of culture conditions for the detection of mCD40L-mediated cell death

3.8.1. Cell viability assays

As part of the optimisation of the co-culture system, it was essential to determine optimal cell densities for the target PCa cells. This was carried out measuring cell viability using the MTS assay.

The principle of this assay (MTS) is based on measuring mitochondrial (metabolic) activities, by adding a single reagent (a tetrazolium compound 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS)). Only viable cells are able, during a defined incubation time (3-4h), to reduce MTS to a water-soluble coloured formazan product. By contrast non-proliferating or dying cells lose their ability to do so. Formazan levels correspond to cell biomass, which can be quantified spectrophotometrically at a wavelength of 492nm.

The panel of PCa cell lines DU145, PC-3-CD40 and LNCaP were cultured at three different cell densities ($8x10^3$, $1x10^4$ and $1.2x10^4$ per well in 96-well plates) as well as three incubation time points as indicated in Figure 3.15. After the incubation time, cell viability was measured using the MTS-based CellTiter 96® AQueous One solution assay as explained in the Methods.

It was observed that DU145 cells proliferate faster than PC-3-CD40 and LNCaP and they reached maximal absorbance more rapidly for all cell densities and time points tested. Overall, in most cases, cell viability in all lines at densities $1x10^4$ and $1.2x10^4$ cells/well exhibited nearly the same values at 48 and 72h. Based on these results, the cell density $1x10^4$ and incubation time 48 h appeared optimal.

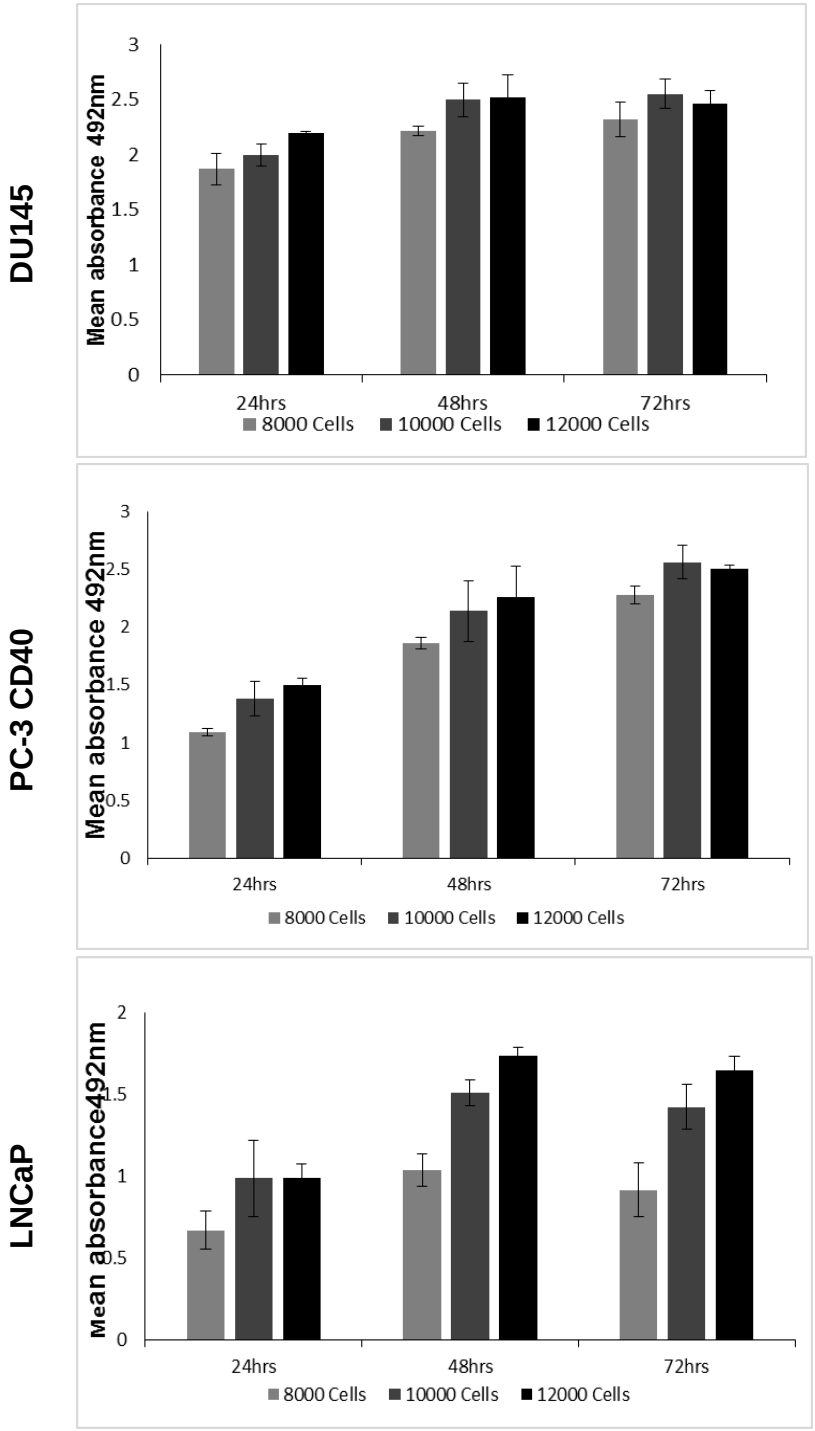


Figure 3.15: Cell viability assays for PCa cells.

Cells were seeded in 96 well transparent plates at the indicated cell densities for the three incubation times shown. After incubation, 20µl of CellTiter 96® AQueous One solution was add to wells and plates then were incubated at 37C in 5% CO_2 for approximately 4 h. Cell viability was measured using a FLUOstar OPTIMA plate reader at wavelength of 492nm. Bars are correspond to mean Abs values of 4-6 technical replicates ± SEM. Results are representative of two independent experiments.

3.8.2. Detection of cell death using the CytoTox-Glo assay

The cytoTox-Glo was tested for CD40-mediated cell death. In the co-culture system. Intitial experiments as described in materials and methods section 2.6. In addition, an important issue has been addressed by our group that was the CytoTox-Glo assay does not differentiate specifically between dead epithelial cells and effector (3T3CD40L and 3T3Neo) cells that are used in co-culture experimentIn order to allow subtraction of background RLU that related to 3T3CD40L and 3T3Neo cells and their luminescence measured following substrate addition which obtained raw data , the assay was included an effectors cells cultured alone.i.e [(3T3CD40L/DU145 – 3T3CD40L)] or [(3T3Neo/DU145 – 3T3Neo)]. then the data were analised as following i.e. [(3T3CD40L+DU145) /(DU145+ 3T3CD40L)] or [(3T3Neo+DU145) / (DU145+3T3Neo)] this stape resulted in obtain fold increase.

To perform co-cultures, previously optimised methodologies in our laboratory have defined at $1x10^4$ cells/well is the optimal cell density for effecter cells (3T3CD40L, 3T3Neo) (Dunnill et al., 2016; Georgopoulos et al., 2007). However, it terms of co-culture ratios of “effector: target” cells, different ratios may be required for different carcinoma target cells e.g. 1:0.8 and 1:1 for urothelial (UCC) and colorectal (CRC) cell lines, respectively. For effector: target cell optimisation in this study, a series of initial experiments were performed. Moreover, CD40-positive PCa cells (DU145, and LNCaP) were co-cultured in 96 well white plates at three different cell densities ($8x10^3$, $1x10^4$ and $1.2x10^4$ cell/ well), and incubation times (24, 48 and 72h) after effecter cells were growth arrested by treatment with MMC optimal concentration (10µg/mL), seeded into multi-wells and co-culture experiments performed as detailed in Section 2.8.

As shown in Figures 3.17 and 3.18, The results showed that co-culture of target cells with 3T3CD40L cells resulted in high levels of cell death compared with control (3T3Neo), in particular at $1x10^4$ cell density and 48h incubation time for both cell lines (DU145 and LNCaP). Furthermore, no cell death was detect in all cell density at 24h for both cell lines.

Consequently, by looking at both optimised results, cell viability assay and CytoTox-Glo assay, the cell density $1x10^4$ and 48h incubation time were selected as optimal cell density and incubation time for our future work.

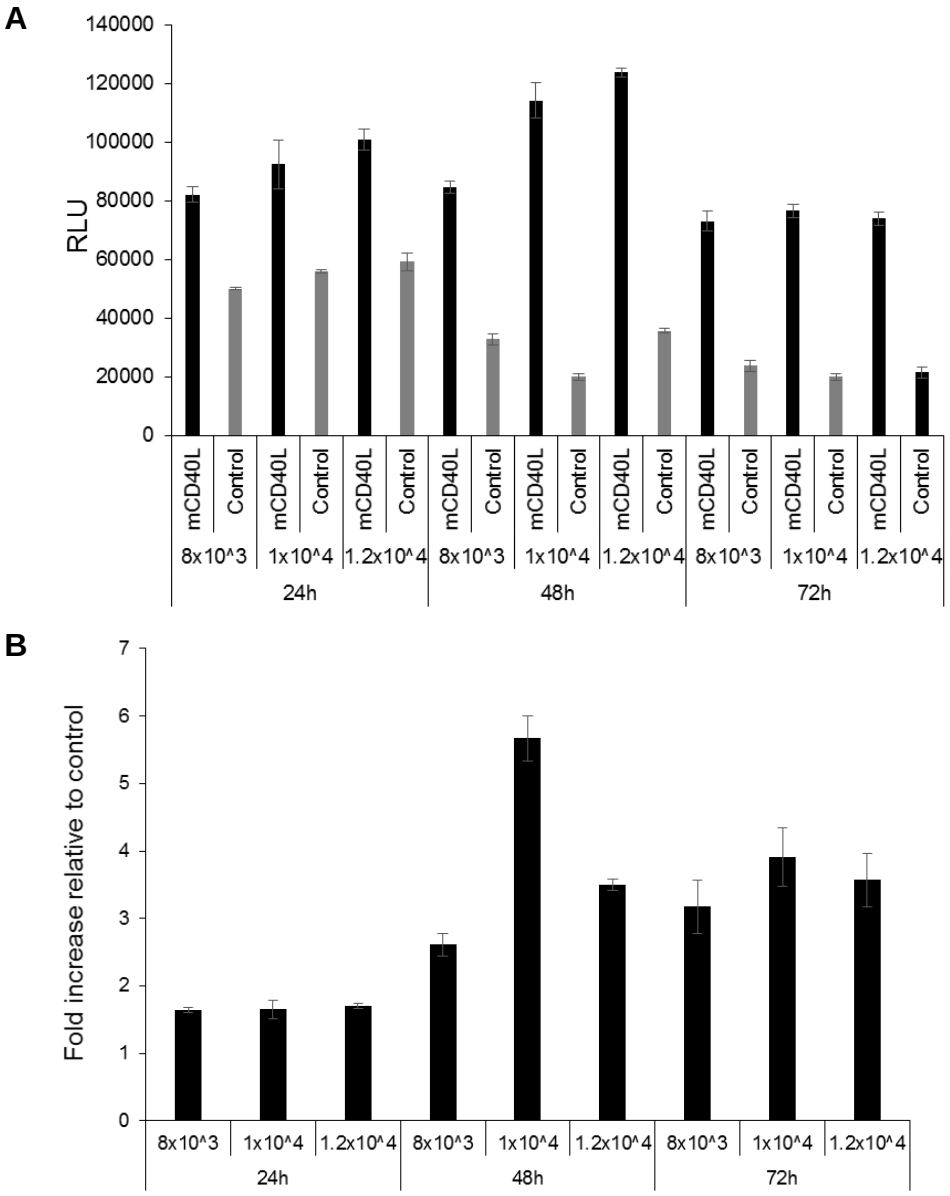


Figure 3.16: Optimization of the CytoTox-Glo assay for DU145 cells.

After treatment with 10µg/ml MMC, $1x10^4$ cells/well of 3T3Neo and 3T3CD40L were co-cultured in 96-well white plates with PCa cells (DU145) seeded at different desnities $8x10^3$, $1x10^4$ and $1.2x10^4$ in DR medium supplemented with 5% FCS and 1% L-glutamine. Plates were incubated for 24,48 and 72 h at 37ºC/5% CO_2. 50µl 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 luminescence values of 5 replicates ± SD. A) Background corrected RLU readings of CytoTox-Glo for DU145/3T3CD4L and DU145/3T3Neo. B) Fold change against control of background corrected RLU readings of CytoTox-Glo for DU145 cells.

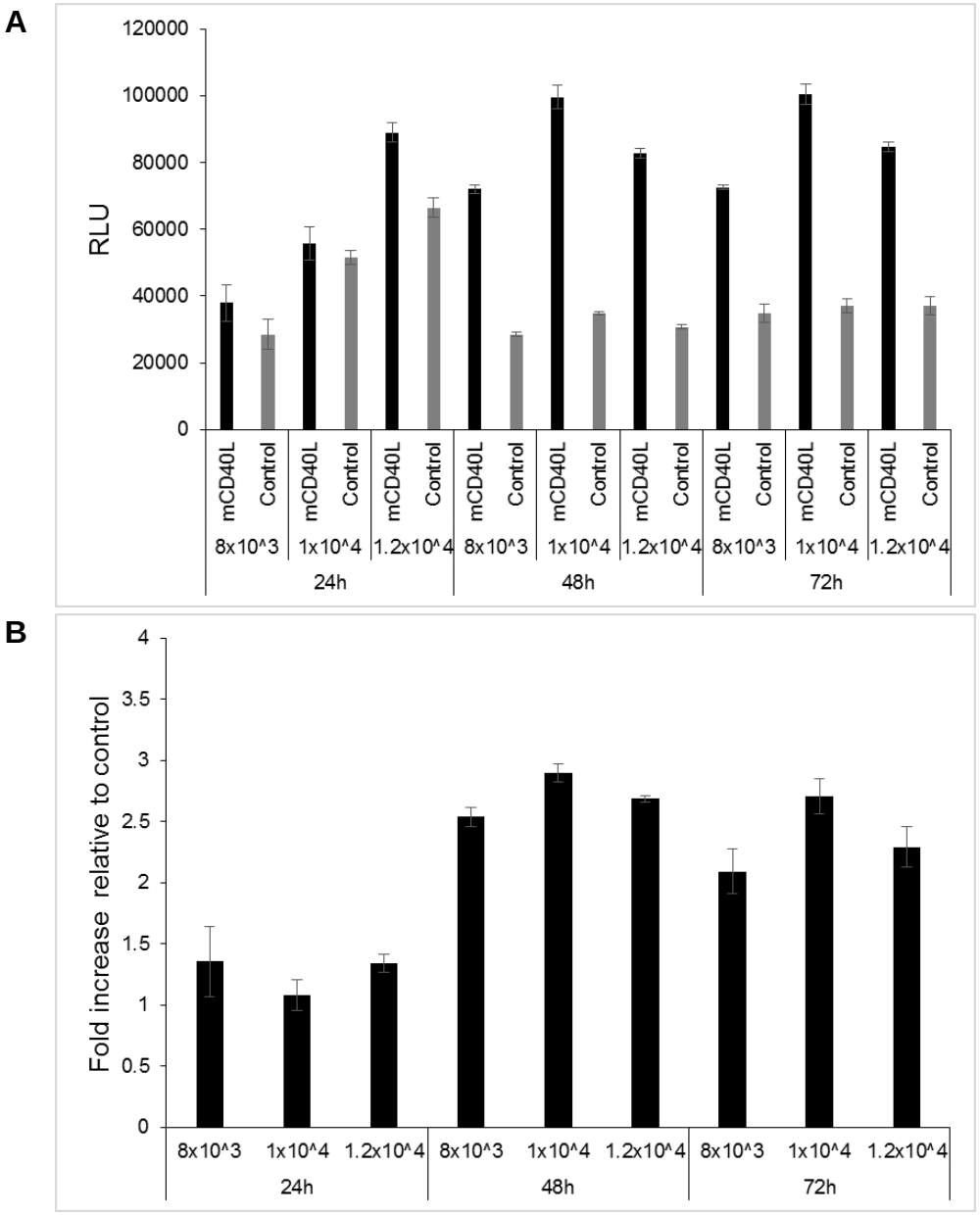


Figure 3.17: Optimization of the CytoTox-Glo assay for LNCaP cells.

After treatment with 10µg/ml MMC, $1x10^4$ cells/well of 3T3Neo and 3T3CD40L were co-cultured in 96-well white plates with PCa cells (LNCaP) seeded at different desnities $8x10^3$, $1x10^4$ and $1.2x10^4$ in DR medium supplemented with 5% FCS and 1% L-glutamine. Plates were incubated for 24,48 and 72 h at 37ºC/5% CO_2. 50µl 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 luminescence values of 5 replicates ± SD. A) Background corrected RLU readings of CytoTox-Glo for LNCaP/3T3CD4L and LNCaP/3T3Neo. B) Fold change against control of background corrected RLU readings of CytoTox-Glo for LNCaP cells.

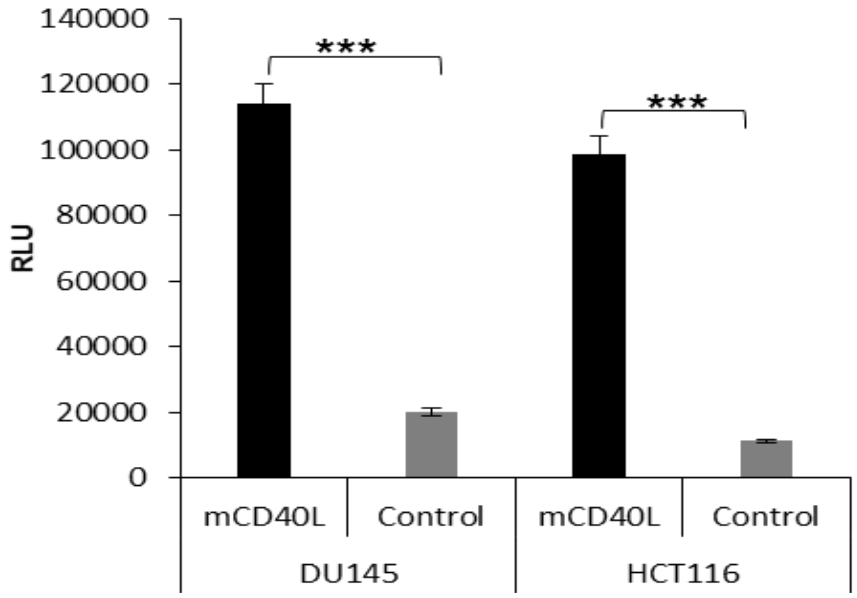
3.9. mCD40L causes extensive PCa cell death at 48 h detected by the CytoTox-Glo assay.

It has been previously demonstrated that CD40 ligation by CD40L membrane trigger apoptotic cell death in epithelial cells in particular colorectal cancer cells (Georgopoulos et al., 2007), and more recently in urothelial cancer cells (Dunnill et al., 2016). Following initial cell density-related optimisation experiments, co-cultures for the detection of mCD40L-mediated death in the PCa cell lines were performed alongside established controls, i.e. cell lines such as HCT116, the susceptibility of which to mCD40L-mediated cell death has been extensively characterised in our laboratory (Dunnill et al., 2016; Georgopoulos et al., 2007). Here it has been showen that mCD40L induced high levels of cell death particularly in DU145 cells, which exhibited ~6-fold increase relative to controls as demonstrated in (Figure 3.19 A and B). LNCaP cells were less yet clearly susceptible to mCD40L-mediated killing by showing approximately 3-fold increase (Figure 3.19 C and D).

The FACS analysis experiment results (Figure 3.8), which showed that cytokine, induced CD40 expression, so the hypothesis was that increased receptor expression might increase susceptibility to CD40-mediated death. Therefore, further experiments were performed in order to determine whether pro-inflammatory cytokines such as TNF-α and IFN-γ lead to cell death amplification. Thus, CytoTox-Glo experiments were performed, DU145 and LNCaP were treated with TNF-α and IFN-γ then cells were co-cultured with effecter cells (3T3Neo and 3T3CD40L). The results shown that no cell death increased compared with untreated cells, as illustrated in Figure (3.21).

Following establishment of PC-3 derivatives engineered to express CD40 (PC-3-CD40 cells), similar co-culture experiments were performed to determine whether re-expression of CD40 could render these cells susceptible to mCD40L-mediated cell death. It has been previously demonstrated that carcinoma cells that retained CD40 expression are highly susceptible to CD40-induced apoptosis (Bugajska et al., 2002; Georgopoulos et al., 2006). As shown in Figure 3.20, *de novo* CD40 expression in PC-3-CD40 conferred susceptibility to mCD40L-mediated death, and a 3-fold induction compared to control (PC-3LXSN).

A


140000
120000
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RLU
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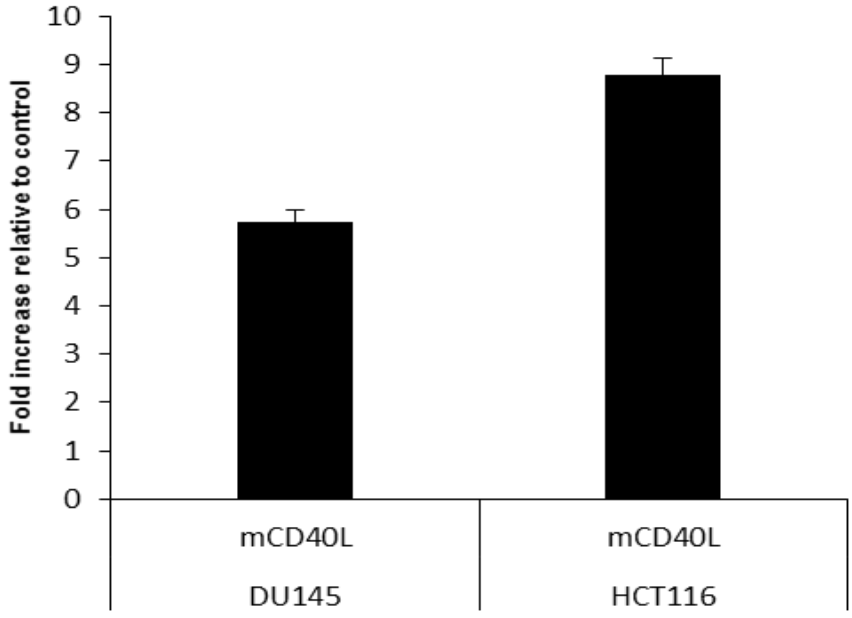
mCD40L
Control
mCD40L
Control
DU145
HCT116

B


10
9
8
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Fold increase relative to control
mCD40L
DU145
mCD40L
HCT116

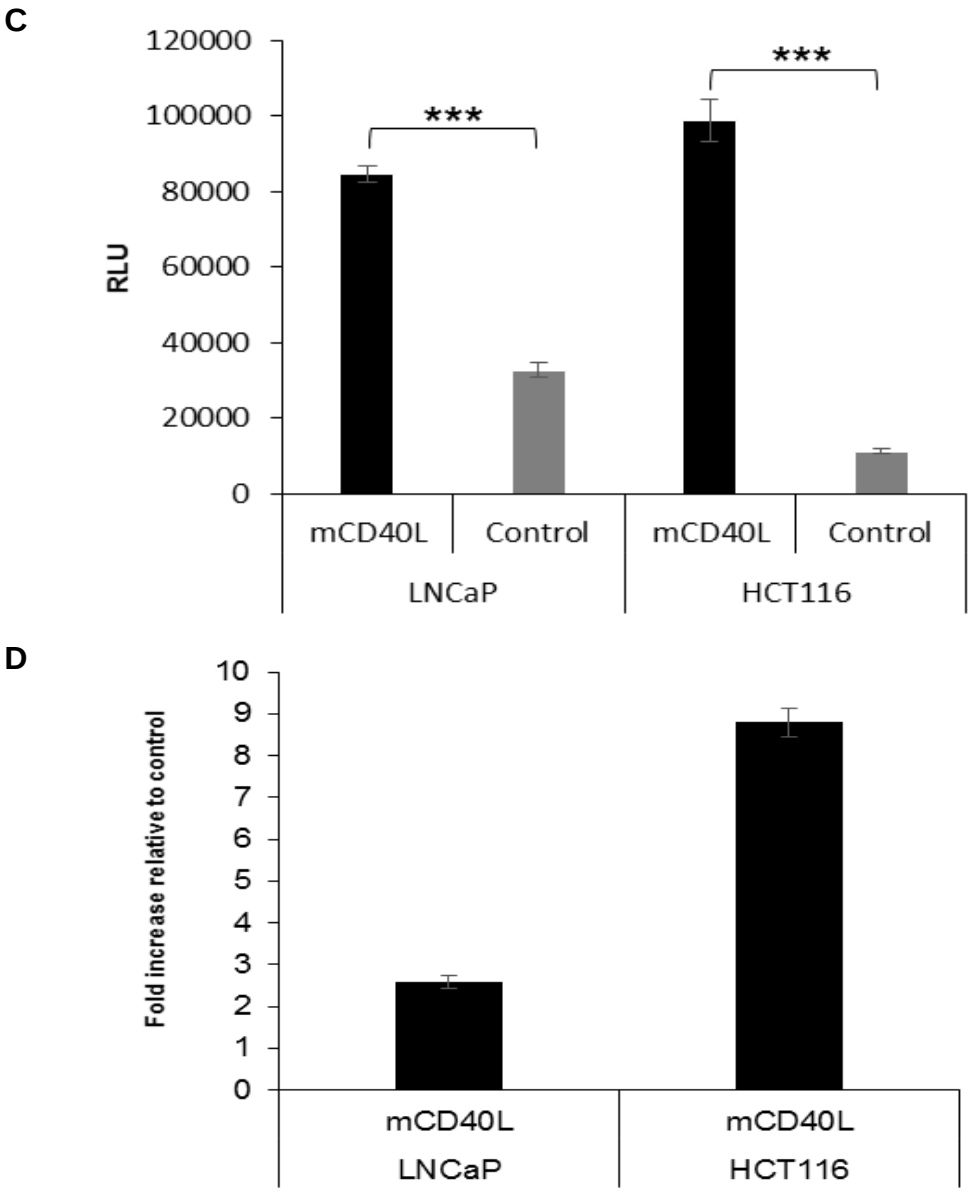


Figure 3.18: Detection of mCD40L-mediated death in CD40-positive PCa lines by Cyto-Tox Glo assay.

$1x10^4$ PCa cells (DU145 and LNCaP) alongside with colorectal cancer cells (HCT116 cells serving as control) were co-cultured with $1x10^4$ MMC-treated 3T3CD40L (mCD40L) or 3T3Neo (Control) in DR medium supplemented with 5% FCS and 1% L-glutamine. Plates were incubated for 24,48 and 72 h at 37ºC/5% CO_2. 50µl 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 luminescence values of 5 replicates ± SD. The figure shows background corrected RLU (A and C) and fold increase (B and D) of CytoTox-Glo for DU145 and LNCaP.Stats: ***p-value<0.001, paired t-test, target cells/control vs target cells/mCD40L, as indicated.

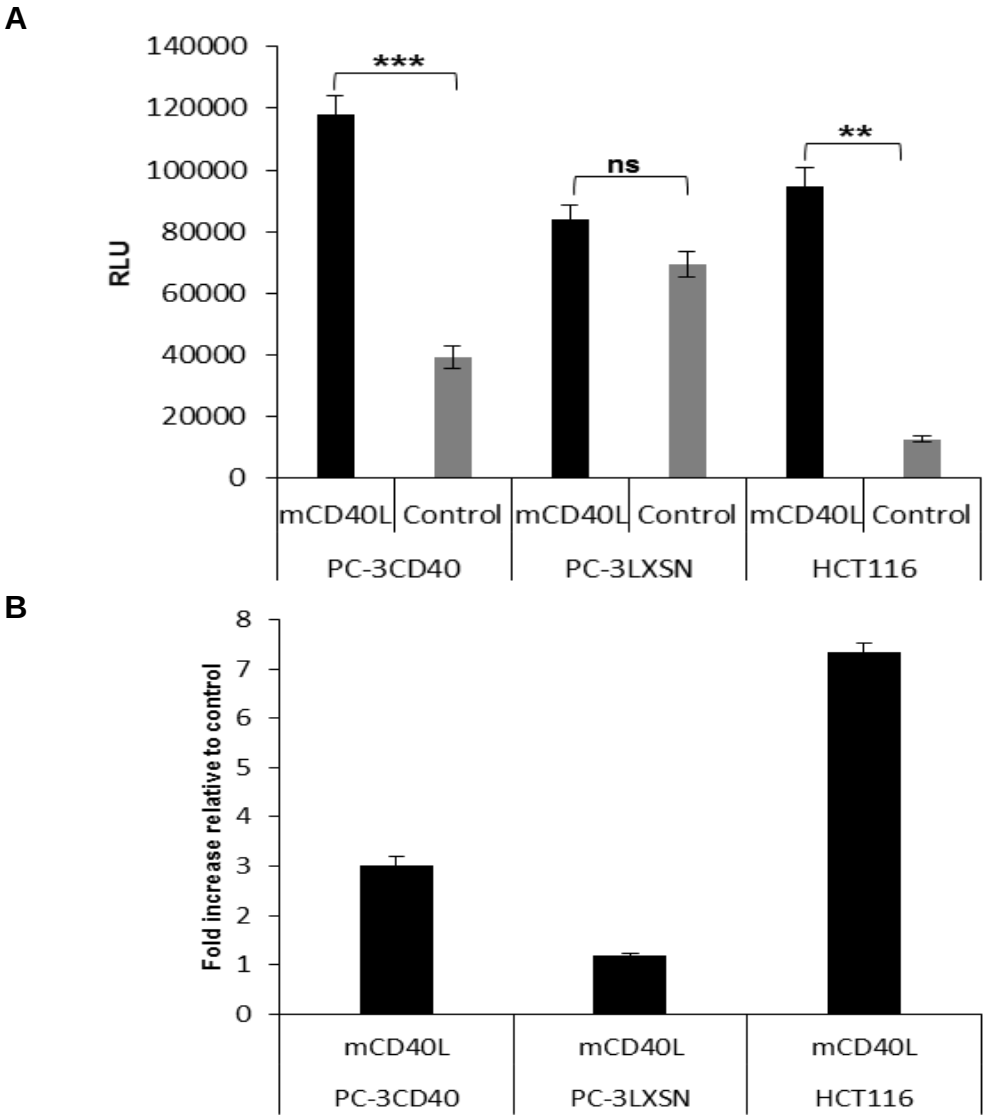


Figure 3.19: Detection of mCD40L-mediated death in transduced PC-3 cells by the Cyto-Tox Glo assay.

$1x10^4$ PCa cells (PC-3CD40 and PC-3LXSN) alongside with colorectal cancer (HCT116 cells serving as control) were co-cultured with $1x10^4$ MMC-treated 3T3CD40L (mCD40L) or 3T3Neo (Control) in DR medium supplemented with 5% FCS and 1% L-glutamine. Plates were incubated for 24,48 and 72 h at 37ºC/5% CO_2. 50µl 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 luminescence values of 5 replicates ± SD Bars correspond to mean values of 4-6 replicates ± SD.The figure shows background corrected RLU and fold increase A and B of CytoTox-Glo for PC-3CD40/mCD4L and PC-3CD40/Control. Stats: ns. non-significant; **, $p < 0.01$; ***, $p < 0.001$, paired student t-test, target cells/control vs target cells/mCD40L, as indicated.

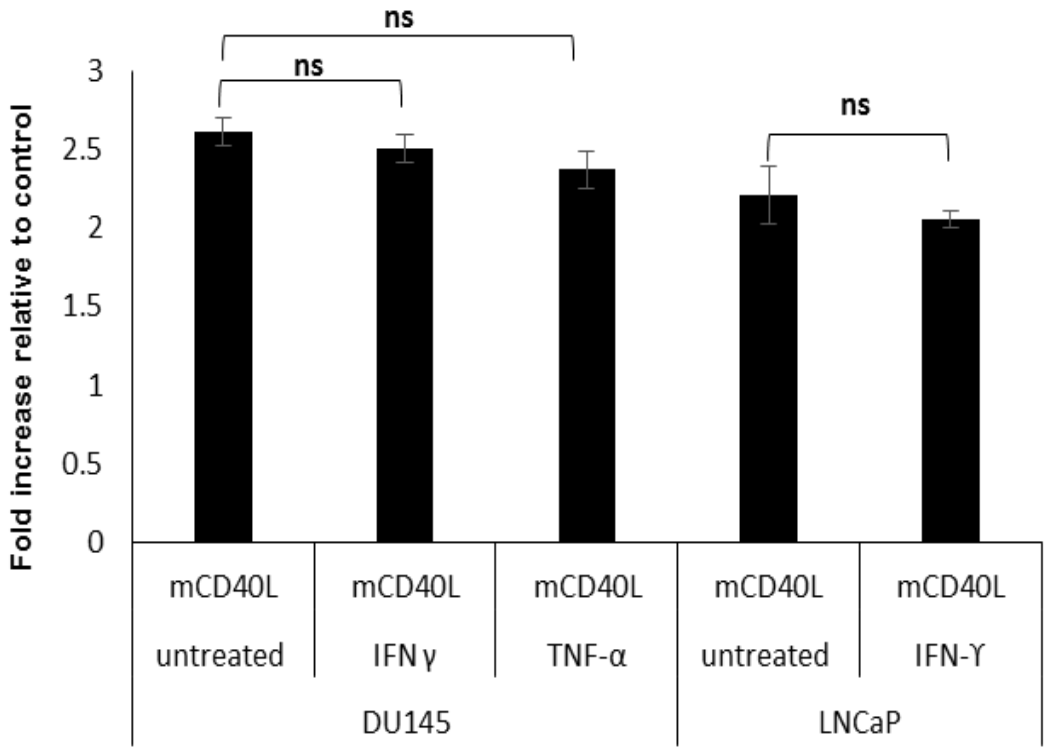


Figure 3.20: Assessment of the effect of pro-inflammatory cytokine TNF-α and IFN-γ treatment on mCD40L-mediated death by the Cyto-Tox Glo assay.

Following treatment with 1000U/mL either IFN-γ or TNF-α**,** 1x10^4 PCa cells (DU145 and LNCaP) were co-cultured with 1x10^4 MMC-treated mouse fibroblast cells 3T3CD40L (mCD40L) or 3T3Neo (Control) in DR medium supplemented with 5% FCS and 1% L-glutamine. Plates were incubated for 48 h at 37ºC/5% CO_2. 50µl 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 luminescence values of 5 replicates ± SD, results are representative of two experiments. The figure illustrates fold increase. Stats: ns. non-significant; paired student t-test, target cells/control vs target cells/mCD40L, as indicated.

3.10. Investigation of the effect of soluble CD40 agonists in PCa cell lines

Georgopoulos and colleagues have previously demonstrated mCD40L-mediated apoptosis in bladder carcinoma and colorectal cancer cells and also demonstrated no apoptosis is triggered by soluble agonistic CD40 (Georgopoulos et al., 2006; Georgopoulos et al., 2007). In addition, agonistic anti-CD40 mAb G28-5 did not induce apoptosis. But, there evidnece suggesting this antibody can inhibit cell growth in PCa cell lines (Rokhlin et al., 1997). Therefore, in this study, the ability of soluble CD40 agonistic to affect the proliferation rate and/or induce apoptosis was investigated by utilising both cell proliferation and the CytoTox-Glo assay.

Cells were treated with 10µg/mL agonistic anti- CD40 mAb G28-5, and cross-linked with affinity-purified human serum protein-adsorbed goat anti-mouse IgG at 5µg/mL. Then cells were incubated for 48h and experiments performed as described in Materials and Methods.

Our results demonstrate that cells did not exhibit any changes in proliferation, or any signs of cytotoxic response when treated with agonistic anti-CD40 mAb G28-5 as illustrated in Figure 3.22 and 3.23 respectively.

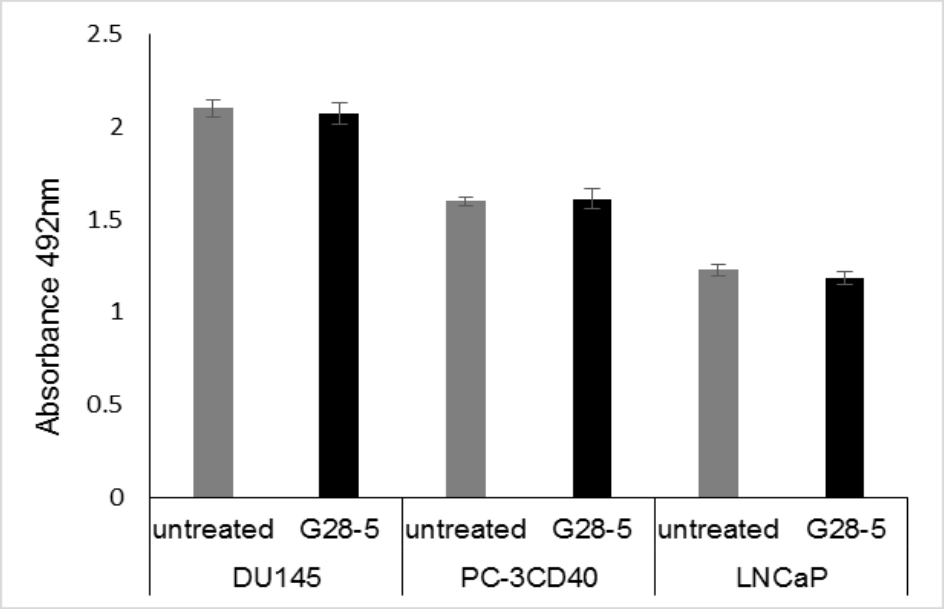
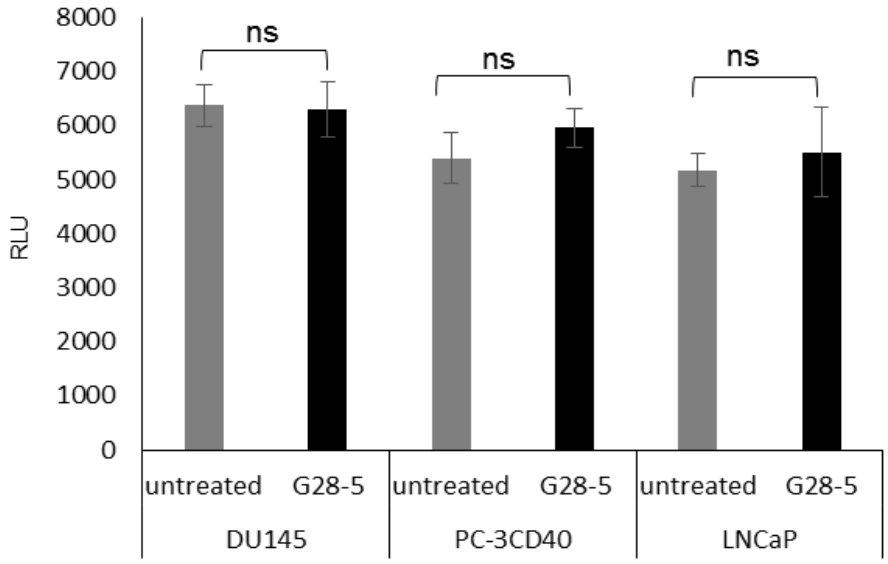


Figure 3.21:Effects of agonistic anti- CD40 mAb G28-5 treatment on PCa cells viability.

PCa cells DU145, PC-3-CD40 and LNCaP were seeded in transparent 96-well plates alone or treated with 10µg/mL agonistic anti-CD40 mAb G28-5, and cross-linked with affinity-purified human serum protein-adsorbed goat anti-mouse IgG at 5 µg/mL. Then, cells were incubated for 48h. 20µL of MTS solution was added to each well and incubated for approximately 4h. Cell viability was determined by a FLUOstar OPTIMA (BMG Labtech) plate reader at a wavelength of 492nm. Data are represented as mean values ±SEM. Results are representative of three experiments.

A



B

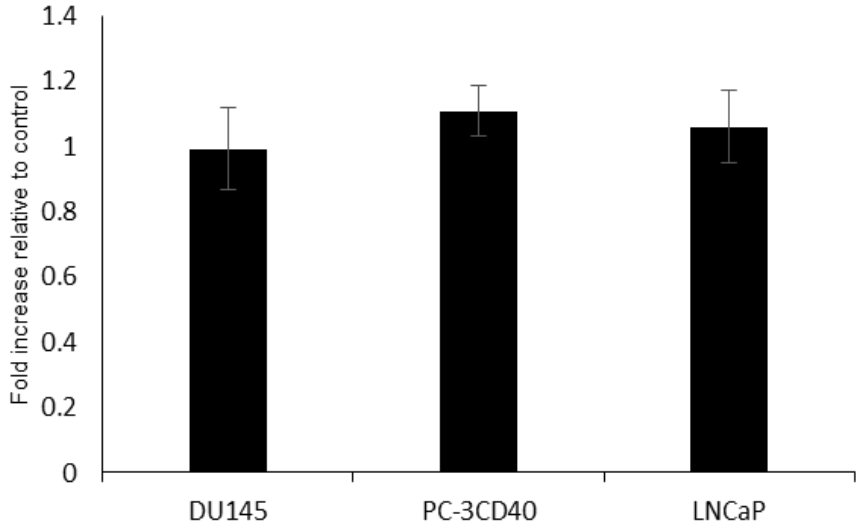


Figure 3.22: Detection of cell death following PCa cells treated with agonistic anti-CD40 mAb G28-5.

PCa cells DU145, PC-3-CD40 and LNCaP were seeded in white 96-well plates. Cells were treated with 10µg/mL agonistic anti- CD40 mAb G28-5, and cross-linked with affinity-purified human serum protein-adsorbed goat anti-mouse IgG at 5µg/m. Then, cells were incubated for 48h. CytoTox-Glo reagents were prepared and added to each well and then luminescence was measured by a FLUOstar OPTIMA (BMG Labtech) plate reader. Results are representative of two experiments .Data are presented as raw data (A) and fold increase (B. Data are represented as mean values ±SEM. Stats: ns. non-significant; paired student t-test for control cells vs treated cells, as indicated.

3.11. Detection of caspase activation by mCD40L using the SensoLyte caspase-3/7 assay

Recent study utilizing the SensoLyte Homogeneous Caspase 3/7 assay and showed that Caspase-3 becomes active within 48 h post CD40 ligation (Dunnill et al., 2016). In this study the caspase 3/7 activity was measured in PCa cells after co-culture for 24, 48, and 72h, which was based on previous work in our laboratory for the detection of mCD40L-mediated induction of Caspase 3/7 activity (Dunnill et al., 2016). In addition, for the analysis of results from these assays, the same principle, as in the CytoTox-Glo assays (Section 3.8), was employed, which involved a) appropriate calculations for background 3T3 cell-related readings.b). The optimisation of target cell (section 3.8.2). Furthermore,it was considered that that there is a specific time window within which caspase 3/7 activation takes place. therefore, the plates were measured frequently, (i.e., immediately, after 5, 10, 30 minutes and overnight).The results demonstrated that interaction between mCD40L (3T3-CD40L) and the target cells DU145 , LNCaP and PC-3CD40 versus 3T3-Neo , did not show significant caspase 3/7 activity at any incubation time as shown in Figure A and B 3.25 and A and B 3.26.

Consequently, further explorative functional experiments were performed to determine whether caspases are critical in mCD40L triggred cell death. Thus, PCa cells were treated with a general biochemical caspase inhibitor, the pan-caspase inhibitor z-VAD, CytoTox-Glo apoptosis assays were performed as mentioned before (in section 2.8). The results showed that the addition of pan-caspase z-VAD did not block the death, but even enhanced it, in particular in DU145, which where cell death doubled, following ligation with mCD40L/PCa compared with control PCa/Neo. Whereas z-VAD blocked death in mCD40L/HCT116 compared with controls HCT116/Neo. Not only this, in another independent experiment, alongside of aforementioned general caspase inhibitor z-VAD, another of general biochemical caspase inhibitor was used, a CAS-BIND^{TM} PRO, which is commercially available as a more effective inhibitor than zVAD, to confirm that CD40-mediated apoptosis is caspase independent. The results were similar to the previous observations with exceptional of non-death enhanced when cells were treated with a CAS-BIND^{TM} PRO. In addition,colorctal cancer cell HCT116 was used positive control which has been confirmed before by our group (Dunnill et al., 2016). Results were representative as fold increase as shown in Figure 3.27.

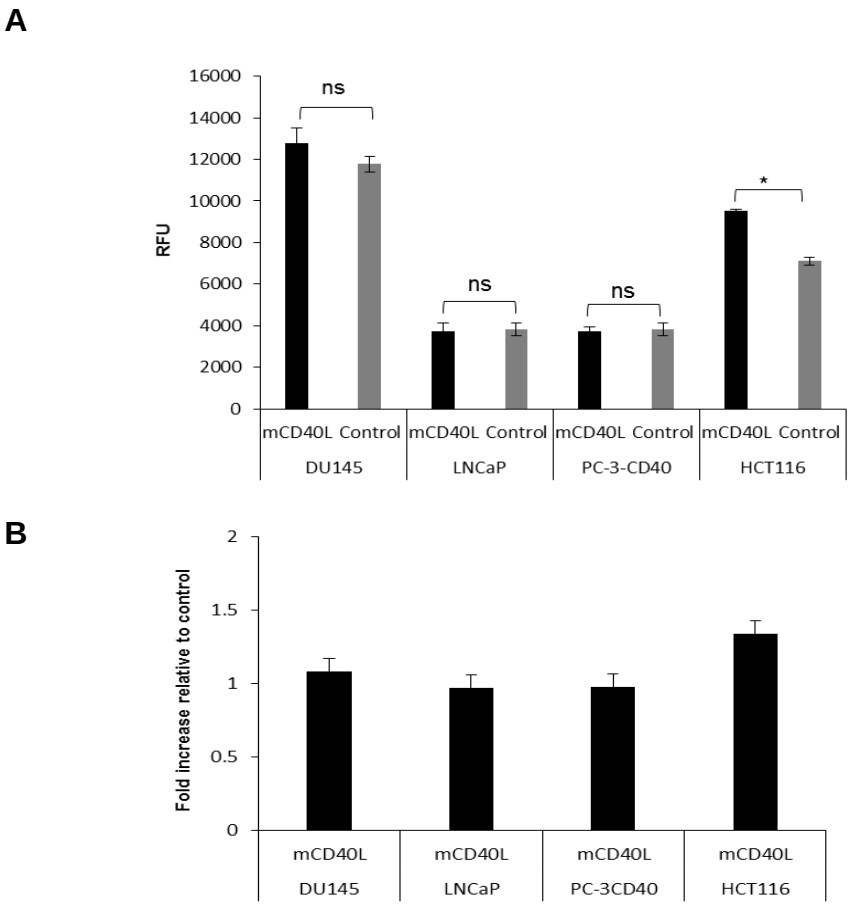


Figure 3.23: Detection of CD40-induced caspase 3/7 activation after 24h post-ligation.

MMC treated 1x10^{4}cells/well mouse fibroblasts (3T3CD40L and 3T3Neo) were seeded overnight then, co-cultured with 1x10^4 cells/well PCa cells (DU145,LNCaP and PC-3CD40) alongside with colorectal cancer cells HCT116 serving as a positive control in 96-well white plates in DR medium supplemented with 5% FCS and 1% L-glutamine. Plates were incubated for 24 h at 37ºC/5% CO_2. After 50µL of medium was added, 50µl substrate of the Anaspec assay reagent was added to each well and then plates were incubated 20 minutes in dark place and fluorescence was measured by a FLUOStar Optima plate reader and background-corrected RLU readings deduced as described in the Materials and methods and background-corrected RFU (Relative Fluorescence Units) calculated as described in the Materials and methods. Bars are proportional to mean values of 4-6 replicates ± SEM; results are representative of three experiments. The top figure (A) illustrates the background corrected. The bottom figure (B) illustrates fold increase relative to control. Stats: ns. non-significant; *, $p < 0.05$, student t-test, target cells/control vs target cells/mCD40L, as indicated.

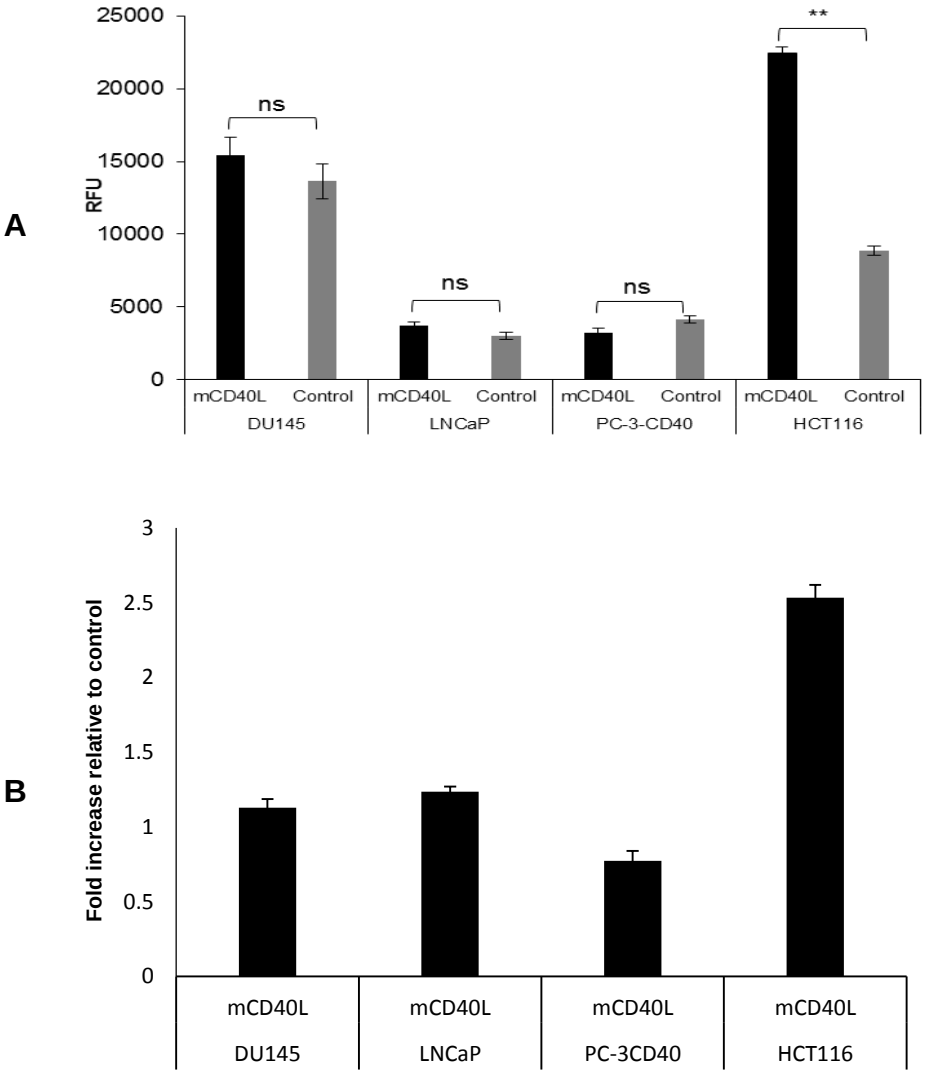


Figure 3.24: Detection of CD40-induced caspase 3/7 activation after 48h post-ligation.

MMC treated 1x10^{4}cells/well mouse fibroblasts (3T3CD40L and 3T3Neo) were seeded overnight then, co-cultured with 1x10^4 cells/well PCa cells (DU145,LNCaP and PC-3CD40) alongside with colorectal cancer cells HCT116 serving as a positive control in 96-well white plates in DR medium supplemented with 5% FCS and 1% L-glutamine. Plates were incubated for 48 h at 37ºC/5% CO_2. After 50µL of medium was added, 50µl substrate of the Anaspec assay was added to each well and then plates were incubated 20 minutes in dark place and fluorescence was measured and background-corrected RFU (Relative Fluorescence Units) calculated as described in the Materials and methods. Bars are proportional to mean values of 4-6 replicates ± SEM; results are representative of three experiments. The top figure (A) illustrates the background corrected. The bottom figure (B) illustrates fold increase relative to control. Stats: ns, non-significant, **, $p < 0.01$, paired student t-test, target cells/control vs target cells/mCD40L, as indicated.

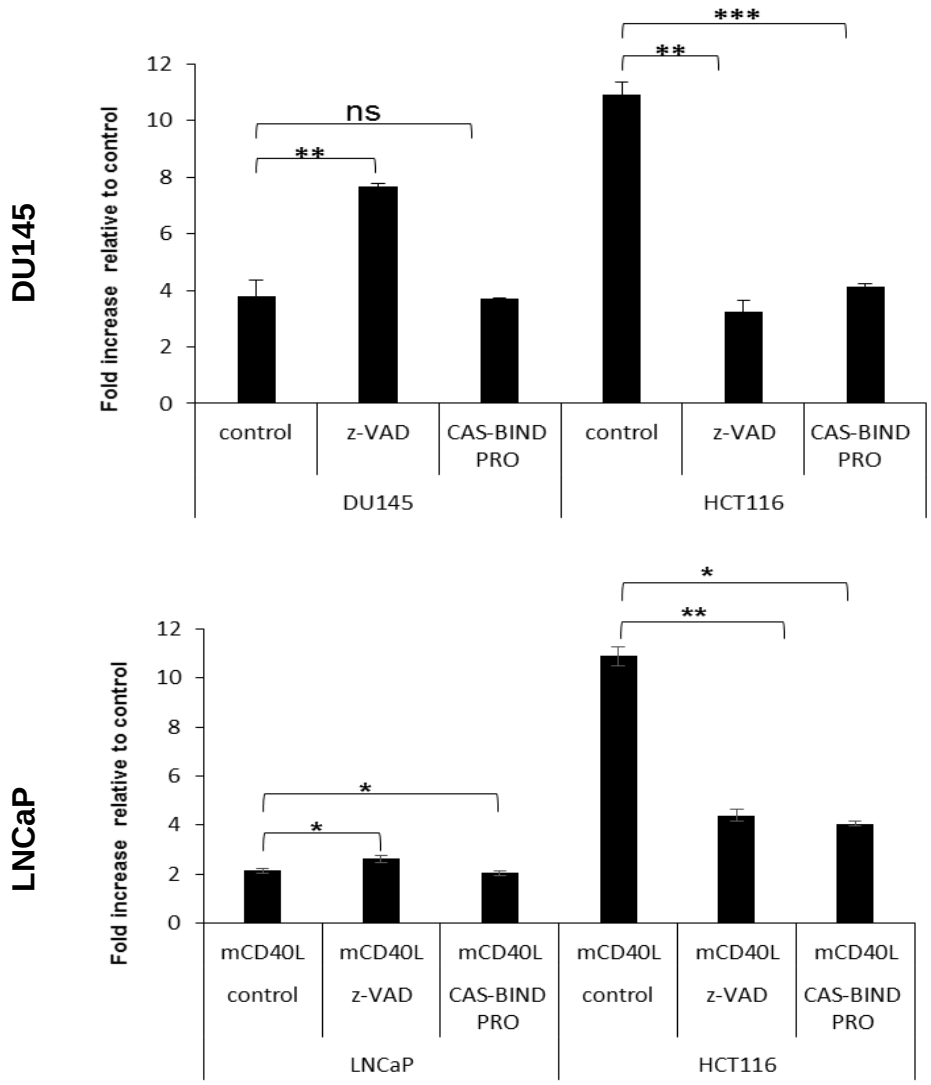


Figure 3.25: Effects of general caspase inhibitors (z-VAD and CAS-BINDTM PRO).

MMC treated 1x10^{4}cells/well mouse fibroblasts (3T3CD40L and 3T3Neo) were seeded overnight then, co-cultured with 1x10^4 cells/well PCa cells (DU145 and LNCaP) alongside with colorectal cancer cells HCT116 serving as a positive control in 96-well white plates in DR medium supplemented with 5% FCS and 1% L-glutamine. In addition, cells were treated either with general caspases inhibitor (z-VAD) (100µM) or with CAS-BIND^{TM} PRO (10µM). After 48h at 37ºC / 5% CO_2. Apoptosis was assessed by CytoTox-Glo assay. 50µl 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 luminescence values of 4-6 replicates ± SD, results are representative of two experiments. The top figure illustrates DU145 cells. The bottom figure illustrates LNCaP cells. Stats: ns; non-significant, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, paired student t-test, target cells/control vs target cells/mCD40L, as indicated.

3.12. Detection of apoptotic cell death using DNA fragmentation assay

Cell death can exhibit distinct morphological changes and DNA fragmentation is often a hallmark used to define the event of apoptosis. It can thus be a powerful and robust assay to detect apoptosis. DNA fragmentation assay has been demonstrated by our group as a reliable tool to assess apoptotic cell death (Bugajska et al., 2002; Georgopoulos et al., 2006; Georgopoulos et al., 2007), as well as by others (Abreu-Martin et al., 1995; Gavrieli et al., 1992; Jänicke et al., 1998).

Based on the principle of the DNA fragmentation detection assay (the principle of the assay is schematically illustrated in Figure 2.5), unlike the CytoTox-Glo assay, only target cells are required to be labelled by 5-bromo-2'-deoxyuridine (BrdU) specific antibodies for detection, which means there is no need to include “effector cell-alone” controls for background subtraction. Accordingly,it provided an additional benefit by excluding any probability of interference of the effector cells in the experimental readings obtained. Greater amounts of fragmented DNA labelled with BrdU only represent a greater number of target cells that have undergone apoptosis.

Therefore, the level of DNA fragmentation was measured on a panel of PCa cells, and independent experiments were performed in the presence or absence of general biochemical caspase inhibitor CAS-BIND^{TM} PRO. Thus, PCa cells were labelled with (BrdU) specific antibody for 2 h at a concentration of 10µM according to the manufacturer’s instructions. Cells were then co-cultured as described in section 2.8. After supernatants were collected, the experiment was completed in an ELISA plate as described in material and methods section 2.7.5. Data was collected and presented as the % of apoptotic/dead epithelial cells which was calculated relative to doxorubicin which was used as a positive control at a concentration of 5µM (the optimal concentration determined in our laboratory) (as detailed in section 2.7.5). Results obtained from these experiments showed that mCD40L caused in both DU145 and LNCaP relatively high level of cell death based on DNA fragmentation in both cases (both with and without treatment of a general biochemical caspase inhibitor CAS-BIND^{TM} PRO) in particular DU145 was about 80%, 90% before and after treatment respectively. Whereas for LNCaP cells the result was ~50% and 60% before and after treatment respectively, as illustrated in Figures 3.28.and 3.29.

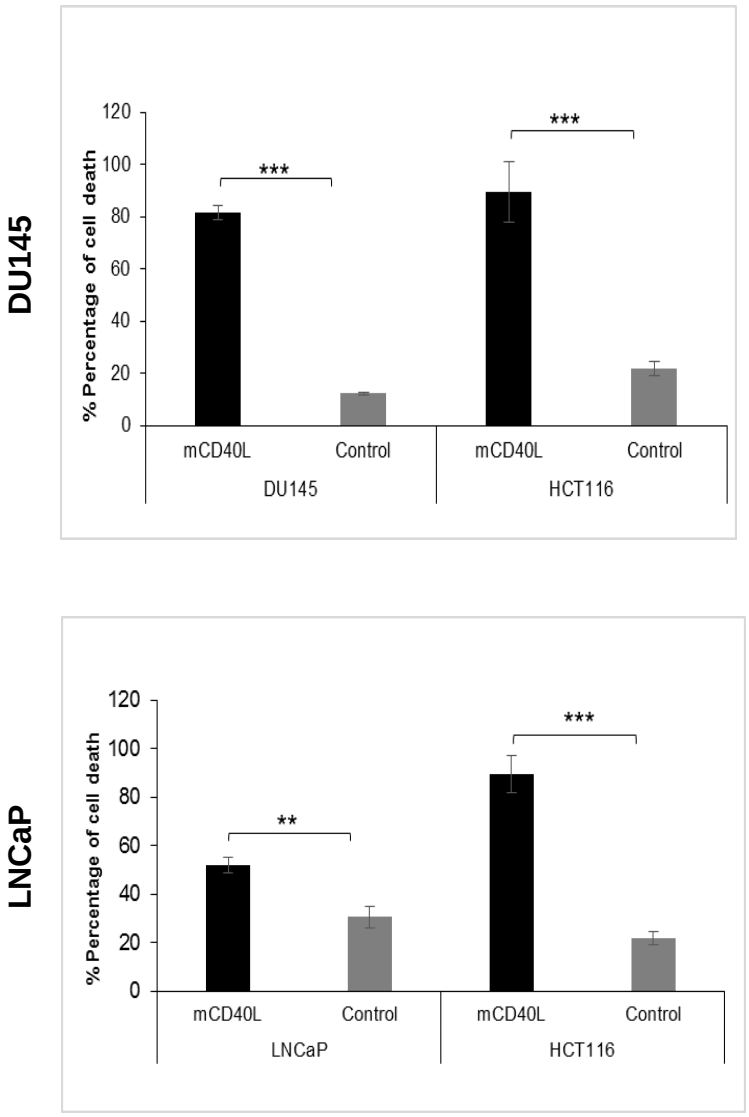


Figure 3.26: Detection of DNA fragmentation mediated by mCD40L.

$1x10^4$ of BrdU labelled PCa cells (DU145 and LNCaP) were co-cultured with fibroblast 3T3 $1x10^4$ cells/well,(Neo or mCD40L) in DR medium supplemented with 5% FCS and 1% L-glutamine in white 96-well plates. And then incubated for 48h then supernatents were collected. Colorectal cancer HCT116 cell was serving as a positive control. ELISA assay was performed and a FLUOstar OPTIMA plate reader measured absorbance and the percentage of apoptotic cells was calculated (as described in 2.6.5). The top figure illustrates DU145 cells. The bottom figure illustrates LNCaP cells. Bars correspond to mean values ±SEM. Results are representative of two experiments. Stats: **, $p < 0.01$, ***, $p < 0.001$ paired student t-test for co-cultured DU145, LNCaP and HCT116 /control (Neo) cells vs mCD40L/DU145, LNCaP and HCT116, as indicated.

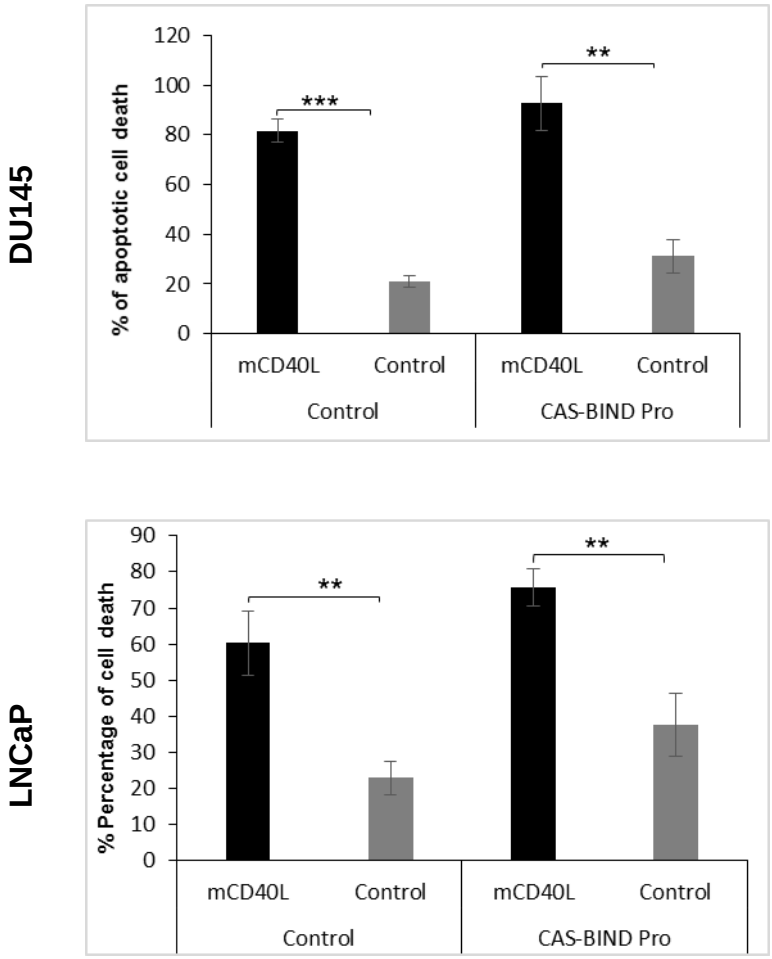


Figure 3.27: Detection of DNA fragmentation mediated by mCD40L after treatment with caspase inhibitor CAS-BINDTM PRO.

After treated with general biochemical caspase inhibitor CAS-BIND™ PRO (10µM), $1x10^4$ of BrdU labelled PCa cells (DU145 and LNCaP) were co-cultured with fibroblast 3T3 $1x10^4$ cells/well,(Neo or mCD40L) in DR medium supplemented with 5% FCS and 1% L-glutamine in white 96-well plates. And then incubated for 48h then supernatents were collected. Colorectal cancer HCT116 cell was serving as a positive control. ELISA assay was performed. A FLUOstar OPTIMA plate reader measured absorbance and the percentage of apoptotic cells was calculated (as described in 2.6.5). The top figure illustrates DU145 cells. The bottom figure illustrates LNCaP cells. Bars are correspond to mean values ±SEM. results are representative of two experiments .Stats: **, $p < 0.01$, ***, $p < 0.001$ paired student t-test for co-cultured DU145, LNCaP and HCT116 /control (Neo) cells vs mCD40L/DU145, LNCaP and HCT116, as indicated.

Summary

The main aim of this chapter was to assess that the validity of co-culture system for stimulation of CD40 ligation my mCD40L in PCa cells *in vitro* and to use a variety of methodologies to investigate CD40-mediated cell death.

- Viability assays demonstrated that target cells (PCa) were optimally proliferating only at 48h at $1x10^4$ cell density, which was in agreement with optimla results observed using the CytoTox-Glo assay following optimisation (incubation time and cell density). Therefore, these were selected as optimal incubation times and cell density values throughout this study.
- The results have confirmed that PCa (target) cells as well as prostate cancer stem cells (CSC) expres CD40 as shown by flow cytometry; this was also confirmed by using western blotting. Furthermore, CD40 can be regulated by pro-inflammatory cytokines such as TNF-α and IFN-γ, however PCa cells shown different response after treatment with these cytokines. Although treatment up-regulated CD40 expression this did not translate into cell death signal increase.
- Due to lack of CD40 expression the PC3 cell line was engineered to de novo express CD40 by retrovirus transduction. The flow cytometry and western blotting results showed that with PC-3-CD40 was receptor positive and expressed high levels of CD40 protein. Moreover, this expression was found to be stable in several passages in culture.
- Three 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 apoptosic. The results shown that mCD40L triggered extensive cell death in DU145, whilst LNCaP cells showed moderate level of cell death. Furthermore, The CytoTox-Glo assay results showed that *de novo* CD40 protein on PC-3CD40 transfected was able to trigger cell death. By contrast, when cells were treated with agonistic anti- CD40 mAb G28-5 they not show any growth arrest or cell death. This finding confirms the importance of the quality of CD40 signal in determining functional outcome (death or no death).
- The Sensolyte Homogenous caspase-3/7 assay was used to detect effect caspase 3/7 activity in apoptotic cells. The results demonstrated that mCD40L did not trigger any significant (detectable) activity of caspase-3/7 in all cell lines; this indicates that CD40 may induce caspase-independent cell death. This is

supported by experiments involving pan-caspase inhibitors, the pan-caspase inhibitors z-VAD and CAS-BIND™ PRO.

- The DNA fragmentation test was also used to detect cell death and provide an additional confirmation that CD40 trigger apoptotic death that was independent of caspases, as mCD40L induced DNA fragmentation that was not blocked following treatment with pan-caspase inhibitor CAS-BIND™ PRO.

Chapter 4: **Investigation of intracellular signalling pathways activated by mCD40L in PCa cells**

4.1. Introduction

CD40 is a member of the non-classical death receptor subfamily of the TNFR superfamily, also termed as TRAF interaction motif (TIM) containing receptors (Russo et al., 2010). Due to lack of direct kinase activity of CD40's cytoplasmic domain, initiation of signal cascades occurs through the TIM domain and is mediated via TRAF adaptor protein recruitment following ligand-receptor binding and activation (Albarbar et al., 2015; Bishop et al., 2007). These events can result in activation of signalling pathways such as MAPKs ERK1/2, JNK and p38 (section 1.8.). Up-regulation of death receptor ligands, specifically FasL and TRAIL (and thus signalling 'cross-talk' with other TNFRs), was originally hypothesised as the mechanism of CD40-mediated apoptosis in carcinoma cells (Eliopoulos et al., 2000). Moreover, another an important intracellular molecular, Reactive Oxygen Species (ROS) can be involved in downstream signalling by activation of apoptosis signalling kinase 1 (ASK1) (Davis, 2000; Dhanasekaran and Reddy, 2008; Soga et al., 2012). The balance of these signalling cascades can ultimately control cell fate, with responses ranging from cell survival to cell death.

Work in the previous chapter demonstrated for the first time the ability of membrane-presented (mCD40L), but not soluble CD40 agonist, to induce apoptosis in PCa cells. However, the precise regulation and/or roles of the TRAF adaptor proteins and MAPK pathways in CD40 signalling in PCa cells are to-date unknown, the specific aims of the work described in this chapter were to:

- To optimise western blot techniques for the detection of intracellular proteins involved with CD40 signalling.

- To detect TRAF1, 2, 3, 5 and 6 expression activation following CD40 signalling triggered by mCD40L using immunoblotting and by utilising protein-specific antibodies for each type of TRAF.

- Utilise western blot techniques to detect CD40 receptor-mediated ERK1/2, JNK/AP-1 and p38 expression/activation and use specific pharmacological inhibitors for ERK1/2 (U0126), JNK (SP600125) and p38 (SB202190) to investigate the functional role of these MAPKs as well as pro-apoptotic proteins involved in CD40 triggered death by mCD40L using CytoTox-Glo assays.

- Determine ROS levels upon CD40 activation by mCD40L using commercially available assays, including ROS-Glo and CM-H_2DCFDA assays. To determine whether ROS production may have a functional involvement in CD40 mediated apoptosis, functional inhibition experiments using the two well-characterised antioxidants, N-acetyl-cysteine (NAC) and propyl gallate (PG), were performed.

- Perform immunoblotting experiments to investigate whether death ligands (FasL, TRAIL) were induced during cell death mediated by CD40 triggered by mCD40L, thus investigate whether receptor cross-talk was a possible mechanism of CD40 killing.

- Carry out inhibition experiments to determine whether Ca^{2+} ions release from the Endoplasmic Reticulum (ER) and thus an ER-stress type of cell death may represent the mechanism of apoptosis upon CD40 activation by mCD40L.

4.2. Optimisation of immunoblotting techniques for detection of protein expression in epithelial and non-epithelial cultured cells

Co-culture experiments (effector fibroblasts and target PCa cells) were employed in this study not only for cell death detection assays but also for immunoblotting purposes, to determining epithelial cell protein expression following receptor ligation. This poses experimental constraints, one such challenge being in that cell lysates obtained from co-cultures contain both effector cell (fibroblast) and target cell (epithelial) proteins (see section 2.8.). Previous studies documented that classical loading controls, such as housekeeping proteins β-actin, which are naturally abundantly present and detectable in most mammalian cell types. (Hofmann, 2009; Hofmann and De Lanerolle, 2006). On the contrary, epithelial cells exclusively express cytokeratin proteins (e.g. CK8 and CK18) (Moll et al., 1982). Thus, CKs represent common markers for epithelial cells, and have been used as a loading control for immunoblotting experiments for co-cultures in our laboratory (Bugajska et al., 2002; Georgopoulos et al., 2006), and more recently (Dunnill et al., 2016).

The first experiments for the optimisation of such metholdologies for PCa cells involved investigating whether CKs were detectable in cultured epithelial (target cells) and fibroblast (effector cells) cells alone. For CKs detection, western blot experiments were performed as described in material and methods (section 2.8) using CK-specific antibodies. As illustrated in Figure 4.1, expression of CK8 and 18 was detectable in epithelial cells including DU145, LNCaP and PC-3CD40 versus fibroblasts (effector cells) (3T3Neo and3T3CD40L) that, as expected, lack CKs. Furthermore, EJ (urothelial) and HCT116 (colorectal) cancer cells were examined alongside, and will be used when it is applicable throughout of this study as positive controls (Figure 4.1).

The second stage of this optimisation was to ensure correct and sensitive detection of epithelial proteins in lysates following co-culture experiments. Having described above how immunoblotting could allow us to distinguish between epithelial cells from fibroblast cells by epithelial cell-specific markers (CK18), instead of detection of β-actin, CK18 was used as loading control for immunoblotting and densitometry-based normalisation (this would important if adjustment of loading were necessary). The reason for this is that when lysates are loaded for electrophoresis, followed by protein transfer, the equivalent protein content of target cells from co-cultures of both target/control (Neo) and target/mCD40L cells were correctly calculated, as previous

studies have optimised previously (Bugajska et al., 2002; Georgopoulos et al., 2006) and more recently in our laboratory (Dunnill et al., 2016). Thus, for all of co-culture experiments, band intensity for CK8, 18 for DU145, LNCaP and PC-3CD40 as well as HCT116 and EJ cells were quantified by the Li-Cor Odyssey analysis software. Then correction based on CK18 band intensity (normalisation) was performed. Following band intensity-based normalisation of lysates as illustrated in Figure 4.2A), subsequent immunoblotting experiments were performed with equal protein from epithelial cells ensured, as illustrated in Figure 4.2B.

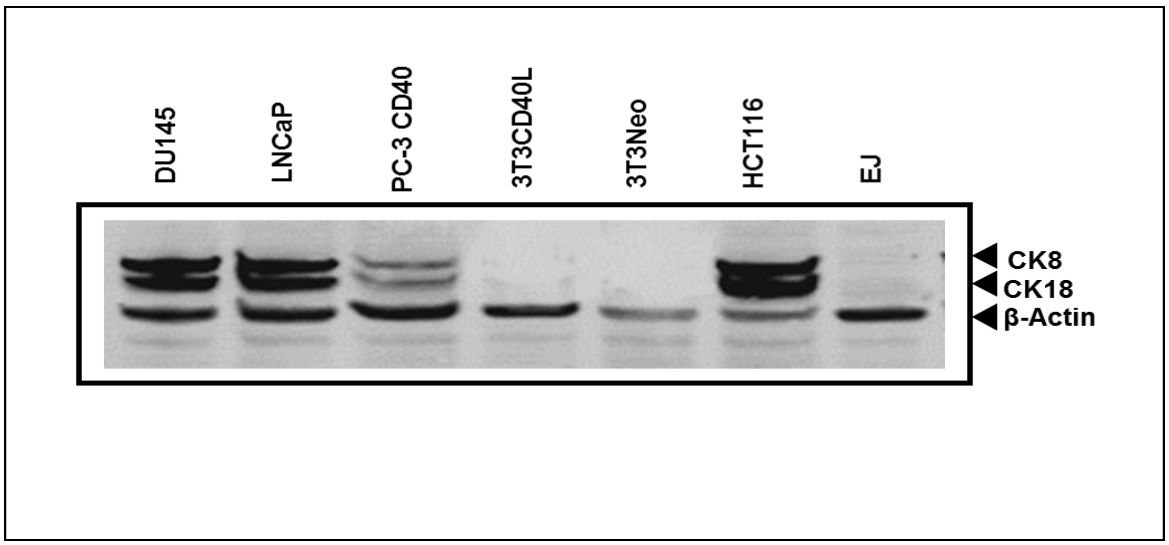


Figure 4.1: Investigation of CK (CK8 and CK18) expression in PCa cell lines and in effector cells (fibroblasts).

Cell lysates were prepared from target cells including (DU145, LNCaP and PC-3CD40) as well as effector cells (3T3CD40Land 3T3Neo), and HCT116 and EJ. 20µg of cell lysates as indicated were loaded per well. Lysates were resolved by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and protein transfer onto PVDF membrane followed. The membrane was labelled overnight with monoclonal anti-CK8-18 (Zym5.2) in TBS/Tween 0.1% (1:1000 dilution). After membrane was washed with TBS/Tween 0.1%, it was incubated with monoclonal antibody for β-actin (1:25,000 dilution) in TBS/Tween 0.1%. .The membrane was labelled with goat anti-mouse IgG Alexa 680 antibody (1:10,000) in TBS/Tween 0.1% for 1h before being scanned at channel 700nm using an Odyssey™ Infra-red Imaging system (Li-Cor). The expected molecular weights of CK8, 18 and β-actin were 52.5, 50 and 42kDa, respectively.

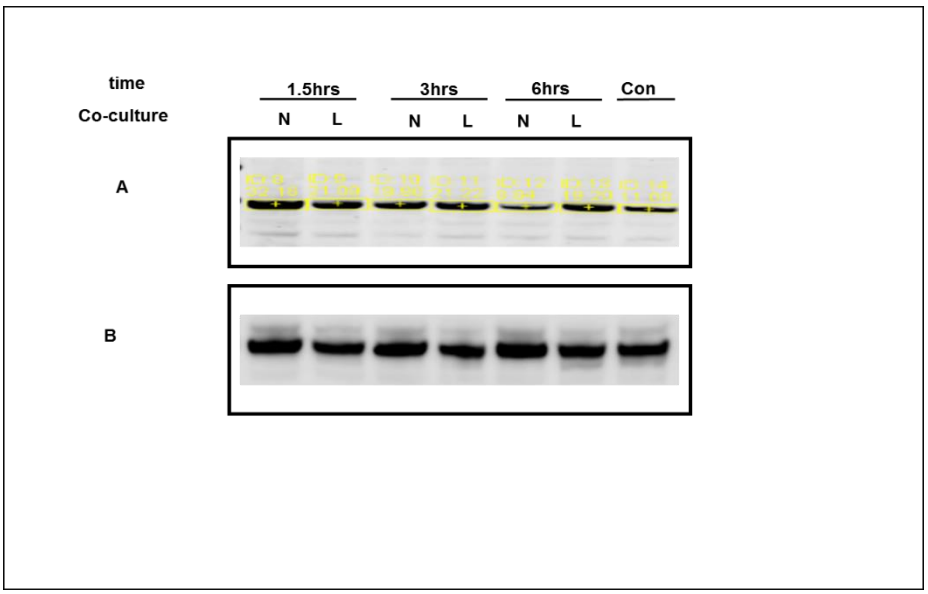


Figure 4.2: Densitometry analysis for detection of CK18 protein band intensities values (for normalisation purposes).

Cell lysates were prepared from target cell DU145 and colorectal cancer cells HCT116 serving as a control, as well as effector cells (3T3CD40L and 3T3Neo). 20µg of cell lysates as indicated were loaded per well. Lysates were resolved by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and protein transfer onto PVDF membrane followed. The membrane was labelled overnight with monoclonal anti-CK18 (Zym5.2) in TBS/Tween 0.1% (1:1000 dilution). The membrane was labelled overnight with monoclonal anti-CK18 in TBS/Tween 0.1% (1:1000 dilution). After membrane was washed with TBS/Tween 0.1%, it was incubated with goat anti-mouse IgG Alexa 680 antibody (1:10,000) in TBS/Tween 0.1%, for 1h before being scanned at channel 700nm using an Odyssey^{TM} Infra-red Imaging system (Li-Cor). The expected molecular weights of CK18 was 50kDa. The top panel A representative of results from “first run” immunoblotting experiment with quantification of band intensities for CK18 expression (before loading correction). Whereas as the bottom panel B, “second run” immunoblotting experiment following densitometry analysis (after loading correction). Key word: N: co-culture 3T3-Neo cells with DU145, and L: co-culture 3T3-CD40L with DU145.

4.3. Regulation of TRAF expression following CD40 ligation by mCD40L

Unlike classical death receptors, CD40 can induce extensive apoptosis in many types of carcinoma cells, due to its ability to recruit adaptor proteins TRAFs, which play a major role in transduction of downstream signalling, leading to the amplification of kinase signal cascades (Albarbar et al., 2015; Wajant et al., 2001).

The first cytoplasmic signalling protein showing association with CD40 was TRAF3 (Bishop et al., 2007). Previous studies demonstrated that TRAF regulation upon CD40 activation in carcinoma cells is very different, with TRAF2 being down regulated, whereas TRAF 1, 3 were upregulated (Georgopoulos et al., 2006). TRAF2 can activate survival signals mediated by activation of NF-kB following TNFR2 and CD40 activation (Rothe et al., 1995a). Via specific sites on receptor C-terminal cytoplasmic domains, TRAF3 can interact with TRAF1, 2 and 5, and mediates Epstein–Barr virus(EBV) and induced B cell proliferation and activation of NF-κB (Izumi et al., 1999). In addition, TRAF 5 and 6 were discovered as adaptor proteins that can also bind to CD40 (Ishida et al., 1996b; Nakano et al., 1996). Other studies reported that both TRAFs were implicated in NF-κB activation (Aizawa et al., 1997; Akiba et al., 1998), moreover overexpressed TRAF5 and 6 resulted in activation of NF-κB, JNK and p38 (Song et al., 1997). Furthermore, in terms of cell death, previous studies demonstrated that recruitment of TRAF3 may play a major role for transmission of cell death signalling triggered by CD40 and LTβR (Eliopoulos et al., 1996; Georgopoulos et al., 2006; Machlis et al., 1997; Rooney et al., 2000; VanArsdale et al., 1997).

Georgopoulos and colleagues have previously demonstrated differential regulation of TRAF proteins by CD40 in normal and malignant cells, and suggested that TRAF3 for the induction of death by CD40 (Georgopoulos et al., 2006). More recently work , involving knockdown experiments in different cancer types including bladder and colorectal cancer carried out in our laboratory has extensively characterised which of the TRAFs may be regulated by CD40 and play a functional role in apoptosis (Dunnill et al., 2016). However, how mCD40L triggers CD40 TRAF signalling in PCa cells, remain unexplored. Therefore, as soluble agonists (e.g. agonistic antibody G28-5) are incapable of triggering any cell death, this project focused on ligation of CD40 by membrane ligand (mCD40L) to identifyIntracellular proteins signalling involved.

To do so, PCa cell lines were co-cultured with control and mCD40L-expressing effector cells and TRAF1, 2, 3, 5 and 6 proteins were examined by immunoblotting following CD40 ligation using human specific antibodies. Immunoblotting showed the induction of TRAF1, which was markedly and rapidly increased as early as 1.5 hour with further increases at 3 and 6h. By contrast, TRAF2 dramatically decreased as early as 1.5, and at 3h, but remained stable at 6h in the DU145 cell line as illustrated in Figure 4.3. Results shown in Figure 4.4 illustrate that in DU145, LNCaP and in the PC-3-CD40 transduced cell line up-regulation of TRAF3 was observed. This occurred within 1.5h and continued at 6h post-ligation, although in PC-3-CD40 cells less TRAF3 was observed at 6h, compared with co-culture with the control cells. Of note, when such experiments were performed for TRAF5 and 6, no or little detectable expression was shown in Figure 4.5.

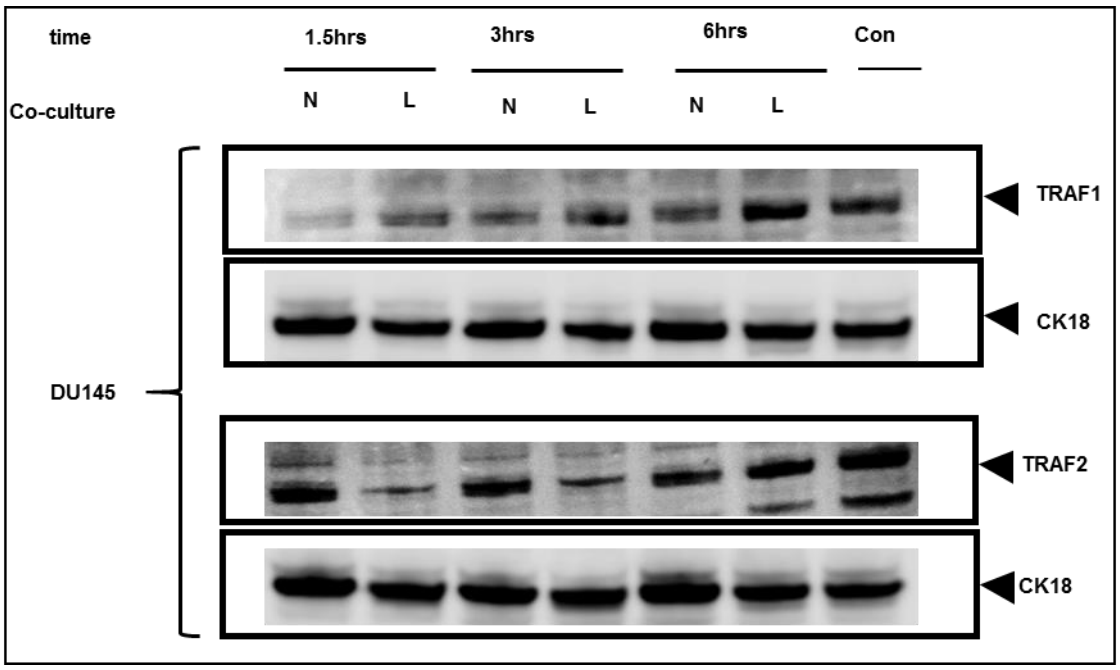


Figure 4.3: TRAF1 and 2 regulation following CD40 ligation by mCD40L in DU145 PCa cells.

Cell lysates were prepared from target cell DU145 and colorectal cancer cells HCT116 serving as a control, as well as effector cells (3T3CD40L and 3T3Neo). 20µg of cell lysates as indicated were loaded per well. Lysates were resolved by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and protein transfer onto PVDF membrane followed. The membranes were labelled overnight at 4ºC with primary polyclonal antibody (anti-TRAF1 or 2) in TBS/Tween 0.1% (1:250 dilution). After membranes were washed with TBS/Tween 0.1%, they were incubated overnight with monoclonal anti-CK18 in TBS/Tween 0.1% (1:1000 dilution). Then the membranes were labelled for 1h with secondary antibody goat anti-rabbit IgG IRDye 800 (1:10000 dilution) for TRAF1 or 2 detection and with goat anti-mouse antibody IgG IRDye 680 (1:10000 dilution) for CK18 before being scanned at channel 700nm using an Odyssey™ Infra-red Imaging system (Li-Cor). The expected molecular weight of TRAF1 and 2 were52 and 50kDa respectively. Key word: N: co-culture 3T3-Neo cells with DU145, and L: co-culture 3T3-CD40L with DU145.

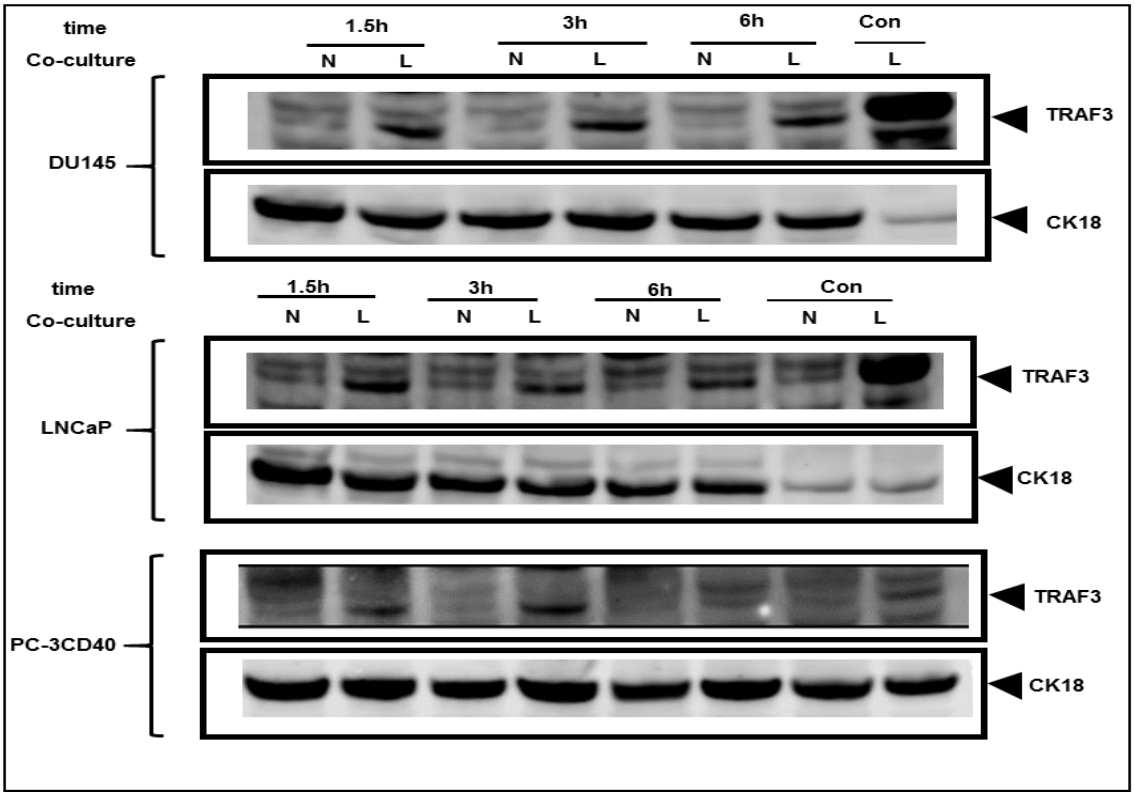


Figure 4.4: TRAF3 regulation following CD40 activation in PCa cells.

Cell lysates were prepared from target cells including (DU145, LNCaP and PC-3CD40) as well as effector cells (3T3CD40Land 3T3Neo), and EJ only in the case of DU145 and LNCaPcell. Whereas, HCT116 in the case of PC-3CD40 (both cells are serving as a positive control). 20µg of cell lysates as indicated were loaded per well. Lysates were resolved by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and protein transfer onto PVDF membrane followed. The membranes were labelled overnight at 4ºC with primary polyclonal antibody (anti-TRAF3) in TBS/Tween 0.1% (1:250 dilution), then incubated overnight with anti-CK18 antibody 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 TRAF3 detection and with goat anti-mouse antibody IgG IRDye 680 (1:10000 dilution) for CK18 before being scanned at channel 700nm using an Odyssey™ Infra-red Imaging system (Li-Cor).). The expected molecular weights of TRAF3 was 50kDa. Key word: N: co-culture 3T3-Neo cells with either DU145, LNCaP or PC-3CD40. L: co-culture 3T3-CD40L with either DU145, LNCaP or PC-3CD40.

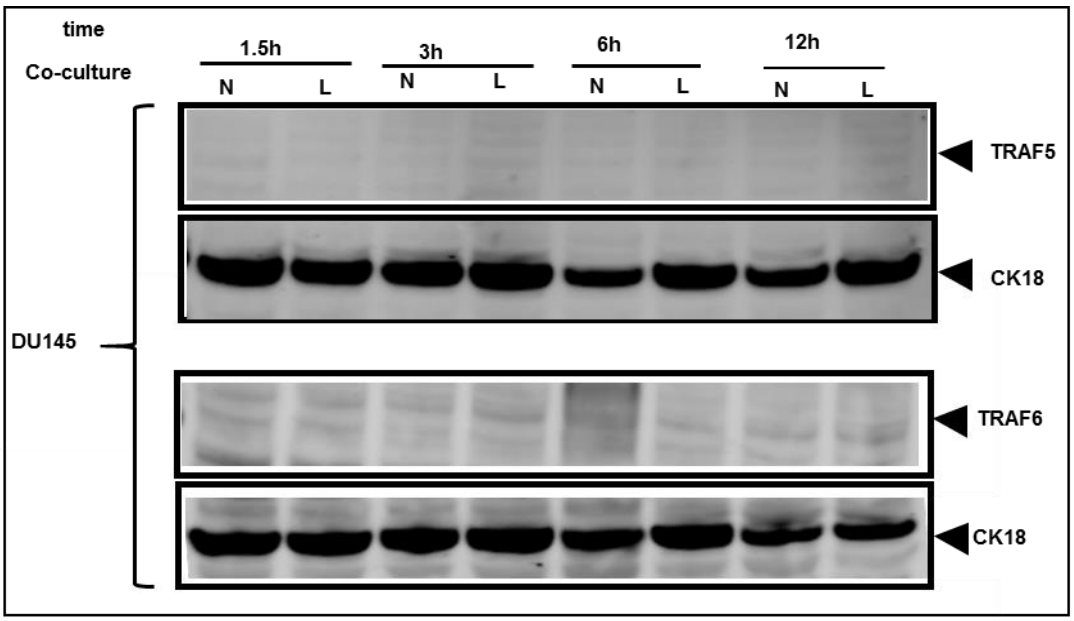


Figure 4.5: Involvement of TRAF5 and 6 following CD40 ligation by mCD40L in DU145 PCa cells.

Cell lysates were prepared from target cell DU145, as well as effector cells (3T3CD40L and 3T3Neo). 20µg of cell lysates as indicated were loaded per well. Lysates were resolved by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and protein transfer onto PVDF membrane followed. The membranes were labelled overnight at 4ºC with primary polyclonal antibody anti-TRAF5 or with primary monoclonal antibody anti-TRAF6 in TBS/Tween 0.1% (1:250 dilution). After membranes were washed with TBS/Tween 0.1%, they were incubated overnight with monoclonal anti-CK18 in TBS/Tween 0.1% (1:1000 dilution). Then the membranes were labelled for 1h with secondary antibody goat anti-rabbit IgG IRDye 800 (1:10000 dilution) for TRAF5 or with goat anti-mouse antibody IgG IRDye 680 (1:10000 dilution) for detection of TRAF6 and CK18, before being scanned at channel 700nm using an Odyssey™ Infra-red Imaging system (Li-Cor). The expected molecular weight of TRAF5 and 6 were 55 and 50kDa respectively. Key word: N: co-culture 3T3-Neo cells with DU145, and L: co-culture 3T3-CD40L with DU145.

4.4. Expression of MKK4 and MKK7 during CD40-mediated apoptosis induced by mCD40L in PCa cells

Mitogen-Activated Protein kinases (MAPKs) JNK and p38 play a crucial role in cell death and inflammation, and they are regulated by upstream MAPKKs, in particularly MKK4 and MKK7 (Wagner and Nebreda, 2009). MAPKs are a part of the protein kinase superfamily (Boulton and Cobb, 1991; Goitre et al., 2014; Rossomando et al., 1989) and are important key mediators involved in signal transduction from the plasma membrane to the nucleus. MAPKs are activated through an amplification process that involves phosphorylation cascades (Zhang et al., 2014). These cascades consist of a set of three evolutionarily conserved, sequentially acting kinases: (1) a MAPK, (2) a MAPK kinase (MAP2K), and (3) a MAPK kinase kinase (MAP3K).

In general, MEKKKs (MAP kinase kinase kinase) are Ser/Thr kinases that are activated via phosphorylation or through interaction with small GTP proteins of the Ras/Rho family in response to a wide range of extracellular stimuli that include mitogens, cytokines, UV ionizing radiation, osmotic stress, oxidative stress (Goitre et al., 2014). This leads to phosphorylation and activation of downstream MEKK proteins including MKK-3, -4, 6, and -7 as well as MEK1/2 which in turn phosphorylate a MAPK (JNK and p38). MKK4 and MKK7 in particular activate JNK in response to external stimuli (Kültz and Avila, 2001). MAP kinases cascades ultimately lead to MAPK-mediated activation of transcription factors which allows the regulation of expression of a wide variety of genes (Bermudez et al., 2010; Châtel, 2009; Sabio and Davis, 2014).

Previous studies have shown that that MKK4 is mainly activated by environmental stress and MKK7 by cytokines; MKK4 and MKK7 activate JNK in response to external stimuli, also activation of JNK in CD40-mediated apoptosis has been reported by our laboratory and others (Davis, 2000; Dunnill et al., 2016; Elmetwali et al., 2010a; Georgopoulos et al., 2006).

Consequently, the aim of this work for the first time to examine whether MKK4 and/or MKK7 are activated during CD40 signalling induced by mCD40L in PCa cell lines. Immunoblotting experiments were performed using human phospho-specific antibodies demonstrated that MKK7 but not MKK4 was activated in response to mCD40L as shown by its phosphorylation in Figure 4.7. Phosphorylation of MKK7 was detected as early as 1.5h post-ligation, and levels continued to rise until 12h.

4.5. ASK1 regulation during CD40 mediated apoptosis

Structurally, ASK1 is a 1374 amino acid protein in humans consisting of a kinase domain (residues 670-938) bordered by N- and C-terminal regulatory domains. The interaction between MAP3K (ASK -1)/MAP2K (MKK4/7 or MKK3/6)/MAPK1 (JNK, p38) plays a major role in signal transduction pathways. In particular ASK-1 which functions as a MAP3K that activates the MAP2Ks MKK4/7 or MKK3/6, results in the activation of the MAPKs JNK and p38, respectively, which ultimately led to a variety of cellular responses such as apoptosis (Kim et al., 2002; Shiizaki et al., 2013). ASK1 is activated in response to oxidative stress, endoplasmic reticulum stress, calcium stress, and inflammatory signals (Dasari et al., 2012; Kadowaki et al., 2005; Szegezdi et al., 2006). Furthermore, previous studies reported that interactions of TNFα with the TNF Receptor (TNFR), resulted in activation of TRADD, which in turn promotes binding of TNF receptor associated factors (TRAFs), with interactions between TRAFs and ASK-1 leading to activation of downstream kinases and the initiation of apoptosis (Ashkenazi and Dixit, 1998; Matsuzawa and Ichijo, 2001). Moreover, recent work in our laboratory has shown that there appears to be a link between MKK4 and JNK activation, and MAP3K/ASK-1 was identified as the link connecting the signalling axes CD40/TRAF3 and MKK4/JNK/AP-1 (Dunnill et al., 2016). In addition, ASK-1 activation can lead to direct phosphorylation of MKK4 via a specific recognition sequence (Dunnill et al., 2016; Ichijo et al., 1997).

Consequently, using immunoblotting and a phospho-ASK1 antibody it was investigated for the first time whether CD40 ligation by mCD40L in PCa cell can regulate ASK-1 activation. The results showed ASK1 activation as evidenced by rapid phosphorylation observed within 3 h post-ligation which then subsided at later time points, as illustrated in Figure 4.6.

4.6. The regulation of MAPKs during CD40 signalling in PCa cells

MAPKs in mammalian cells can be divided into 5 groups: the group of the Extracellular activated kinases (ERK1 / 2), the group of c-Jun N-terminal Kinases (JNK), which are also termed as stress-activated protein kinases (SAPK). The p38 MAP kinases, as well as ERK3/4 and ERK5 (Roux and Blenis, 2004), as discussed in detail in section 1.6.13.

ERK1/2 largely control cell proliferation pathways (Chang and Karin, 2001; Kyriakis and Avruch, 2012). However, other studies reported that ERK1/2 might implicated in cell death responses via loss of proliferation, it has been demonstrated that ERK1/2 are activated when HeLa cells are treated with cisplatin (Wang et al., 2000). Furthermore, inhibition of ERK1/2 led to stimulation of survival pathway in renal cell lines and primary cultures of renal proximal tubular cells (Kim et al., 2005b; Nowak et al., 2004). In many cases, JNK is very a strong pro-apoptotic mediator. For instance, it has been demonstrated that in response to chemotherapy drugs JNK can be activated (Hayakawa et al., 2004; Potapova et al., 2001); moreover, other studies have demonstrated that CD40 activation in carcinoma cells and RANK ligation in osteoclast precursor cells contributes to cell death with activation of JNK (Boyle et al., 2003; Eliopoulos et al., 2000; Elmetwali et al., 2010a; Georgopoulos et al., 2006).

The p38-MAP kinase is associated with response to extracellular stimuli such as UV radiation, osmotic stress, pro-inflammatory cytokines and hypoxia (Dhanasekaran and Reddy, 2008), via tyrosine kinase receptors and G protein-coupled receptors (Chen et al., 2001). The MKKs responsible for the activation of the p38-MAPK are MKK3 and MKK6. Upstream of the MKKs, are a large number of MAP3Ks that lead to the activation of p38 (Zarubin and Jiahuai, 2005). The activation of p38 has a plethora of biological effects. p38 often has a pro-apoptotic effect and is involved in the regulation of the cell cycle (Chi et al., 2006; Herlaar and Brown, 1999; Zhang and Liu, 2002).

As mentioned above, CD40 ligation by mCD40L has been shown to activate JNK in UCC (urothelial) and CRC (colorectal) carcinoma cells (Crallan et al., 2006; Dunnill et al., 2016; 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 (Johnson and Lapadat, 2002; Sabapathy and Wagner, 2004). Moreover, CD40 ligation in both B cells and carcinoma cells led to TRAF recruitment and eventually activation of the NF-kB and AP-1 pathways (Georgopoulos et al., 2006; Grammer and Lipsky, 2000). Activation of these transcription factors can induce cell survival and proliferation or cell death in a signal- and cell type-specific manner (Shaulian and Karin, 2002).

Therefore, this study aimed to investigate whether JNK and/or p38 were activated during CD40 mediated apoptosis in PCa cells. Moreover, the possible functional role of these kinases as well as their downstream transcription factors was tested using

specific pharmacological inhibitors of JNK, MEK/ERK and p38, as well as generic inhibitors of the NF-kB and AP-1 transcription factors. The effects of these inhibitors on mCD40L-mediated cell death were assessed using CytoTox-Glo death detection assays. Initial pre-titration experiments were carried out in order to determine the optimal concentration for these components using cell viability assay (see Appendix ii for MAPK inhibitors titrations).

Immunoblotting analysis demonstrated that JNK phosphorylation occurs within1.5 h post CD40 ligation for DU145, this expression was sustained until 6h as shown in Figure 4.8. Moreover, results from functional inhibition experiments showed that mCD40L-mediated death in DU145 and LNCaP cells was significantly and/or completely abrogated in a dose-dependent fashion by AP-1 inhibitor (NDGA) and JNK inhibitor (SP600125) in both DU145 and LNCaP cell lines. By contrast, treatment with NF-κB inhibitor (PDTC), MEK/ERK inhibitor (U0126) had no effect on CD40 mediated killing as illustrated in Figures 4-10,11, 13, 14), these observations are supported by previous findings in UCC cells (Bugajska et al., 2002; Georgopoulos et al., 2006). Interestingly, although p38 expression of activated p38 MAPK was not detectable over a number of experiments using different phospho-p38 specific antibody by immunoblotting assays (Figure 4.9), functional inhibition results (using SB202190) showed that p38 blockade attenuated cell death, an observation similar to that for JNK. As shown in Figures 4-12

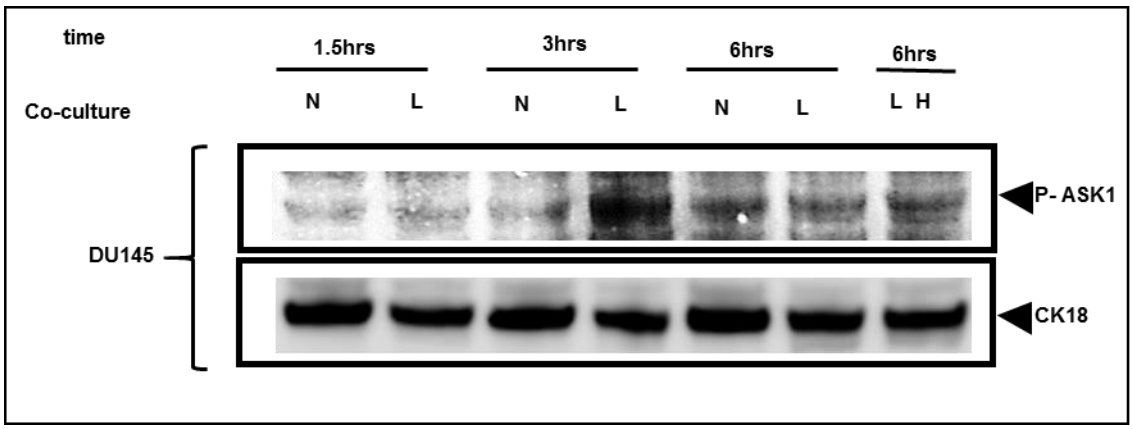


Figure 4.6: Activation of ASK1 in response to mCD40L.

Cell lysates were prepared from target cell DU145 and colorectal cancer cells HCT116, as well as effector cells (3T3CD40L and 3T3Neo). 20µg of cell lysates as indicated were loaded per well. Lysates were resolved by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and protein transfer onto PVDF membrane followed. The membrane was labelled overnight at 4ºC with an anti-phospho-ASK1 (Thr845) antibody in TBS/Tween 0.1% (1:1000 dilution), and then with an anti-CK18 antibody (1:2000 dilution). The membrane was incubated for one hour with goat anti-mouse in TBS/Tween 0.1% IgG IRDye 800 (1:10,000 dilution) for the detection of phospho-ASK1 and with goat anti-mouse IgG Alexa 680 (1:10,000 dilution) for the detection of CK18, before being scanned at channel 700 and 800nm using an Odyssey™ Infra-red Imaging system (Li-Cor). The expected molecular weight of phospho-ASK1 was 50kDa. Key word: N: co-culture 3T3-Neo cells with DU145, and L: co-culture 3T3-CD40L with DU145 or HCT116 cells.

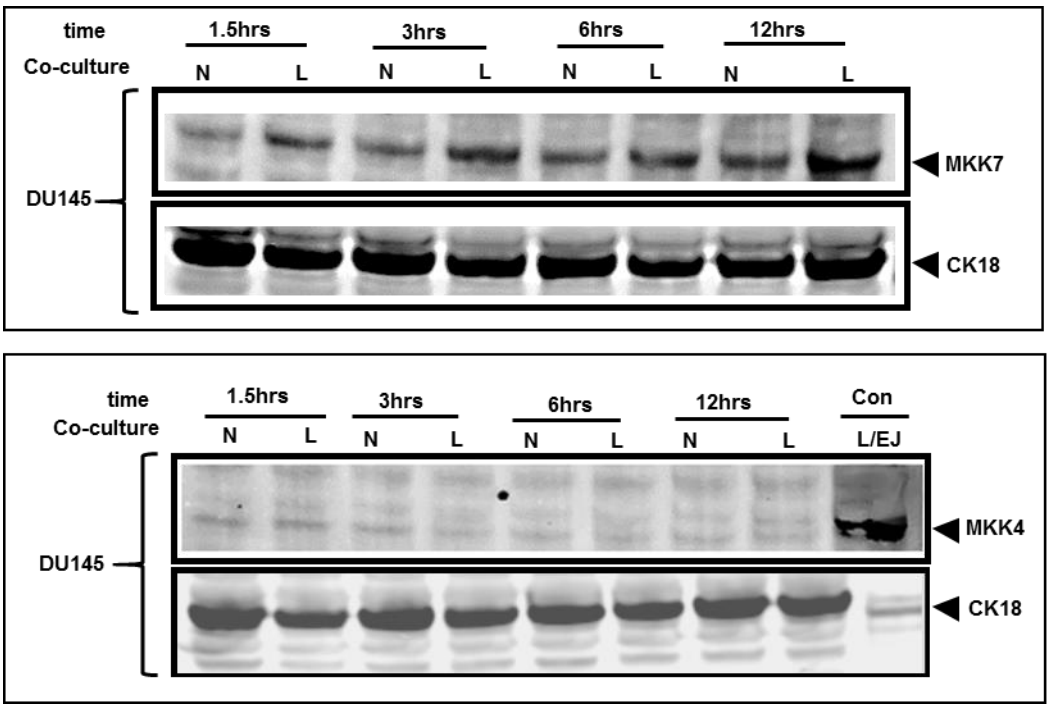


Figure 4.7: The activation of MKK4 and MKK7 by mCD40L.

Cell lysates were prepared from target cell DU145 and bladder cancer cell EJ only in the case of MKK4 detection, as well as effector cells (3T3CD40L and 3T3Neo). 20µg of cell lysates as indicated were loaded per well. Lysates were resolved by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and protein transfer onto PVDF membrane followed. The membrane was labelled overnight at 4ºC with primary polyclonal antibody (anti-p-MKK7) or (anti-p-MKK4) in TBS/Tween 0.1% (1:500 dilution) and then with an anti-CK18 antibody in TBS/Tween 0.1% (1:2000 dilution). The membrane was incubated for one hour with goat anti-rabbit in TBS/Tween 0.1% IgG IRDye 800 (1:10,000 dilution) for the detection of phospho-MKK7 and MKK4, or with goat anti-mouse IgG Alexa 680 (1:10,000 dilution) for the detection of CK18, before being scanned at channel 700 and 800nm using an Odyssey™ Infra-red Imaging system (Li-Cor). The expected molecular weight of phospho-MKK7 and MKK4 was 48, 44kDa respectively. Key word: N: co-culture 3T3-Neo cells with DU145, and L: co-culture 3T3-CD40L with DU145 or HCT116 cells.

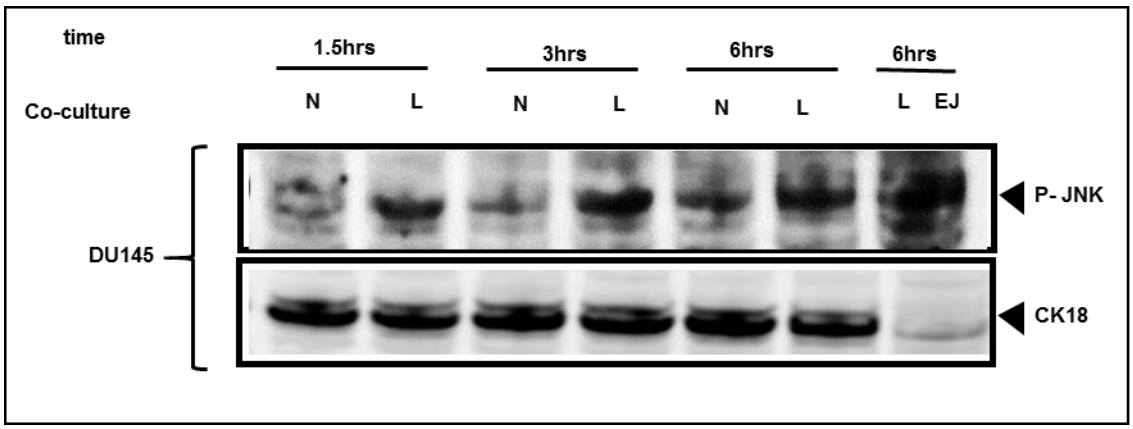


Figure 4.8: The activation of JNK upon CD40 ligation induced by mCD40L.

Cell lysates were prepared from target cell DU145, alongside with UUC bladder cancer cell line EJ serving as a positive control, as well as effector cells (3T3CD40L and 3T3Neo). 20µg of cell lysates as indicated were loaded per well. Lysates were resolved by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and protein transfer onto PVDF membrane followed. The membranes were labelled overnight at 4ºC with primary monoclonal antibody (anti-p-JNK) in TBS/Tween 0.1% (1:500 dilution), and then with anti-CK18 antibody in TBS/Tween 0.1% (1:2000 dilution). The membrane was then labelled for one hour with secondary antibody goat anti-mouse IgG IRDye 680 (1:10,000 dilution) for p-JNK detection and with goat anti-mouse antibody IgG IRDye 680 (1:10000 dilution) for CK18, before being scanned at channel 700,800nm using an Odyssey™ Infra-red Imaging system (Li-Cor). The expected molecular weight of p-JNK was 46kDa. Key word: N: co-culture 3T3-Neo cells with DU145, and L: co-culture 3T3-CD40L with DU145 or EJ.

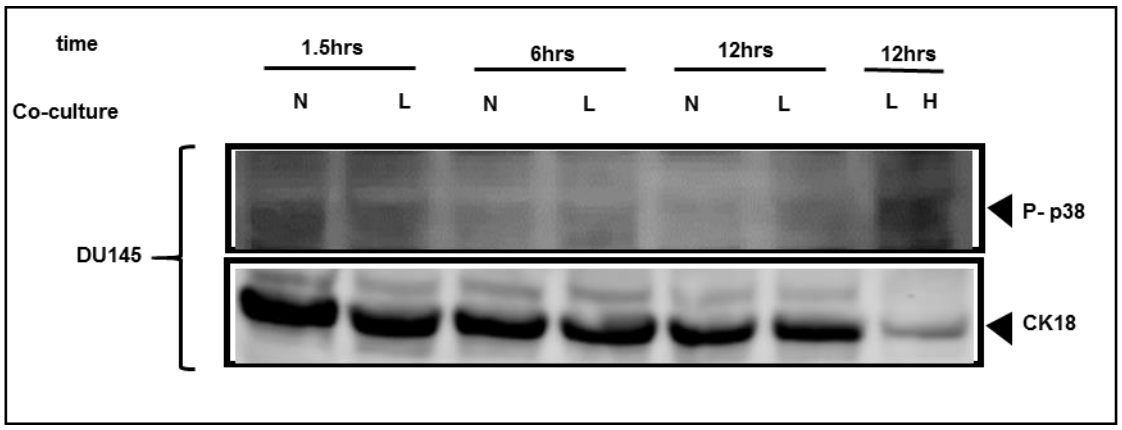


Figure 4.9: The activation of p38 following CD40 ligation.

Cell lysates were prepared from target cell DU145, alongside with colorectal cancer cell line HCT116 serving as a positive control, as well as effector cells (3T3CD40L and 3T3Neo). 20µg of cell lysates as indicated were loaded per well. Lysates were resolved by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and protein transfer onto PVDF membrane followed. The membrane was labelled overnight at 4ºC with primary polyclonal antibody (anti-p-p38) in TBS/Tween 0.1% (1:500 dilution). Then with anti-CK18 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 (1:10,000 dilution)] for p-p38 detection and with goat anti-mouse antibody IgG IRDye 680 (1:10000 dilution) for CK18, before being scanned at channel 700,800nm using an Odyssey™ Infra-red Imaging system (Li-Cor). The expected molecular weight of p-p38 was 43kDa. Key word: N: co-culture 3T3-Neo cells with DU145, and L: co-culture 3T3-CD40L with DU145 or HCT116.

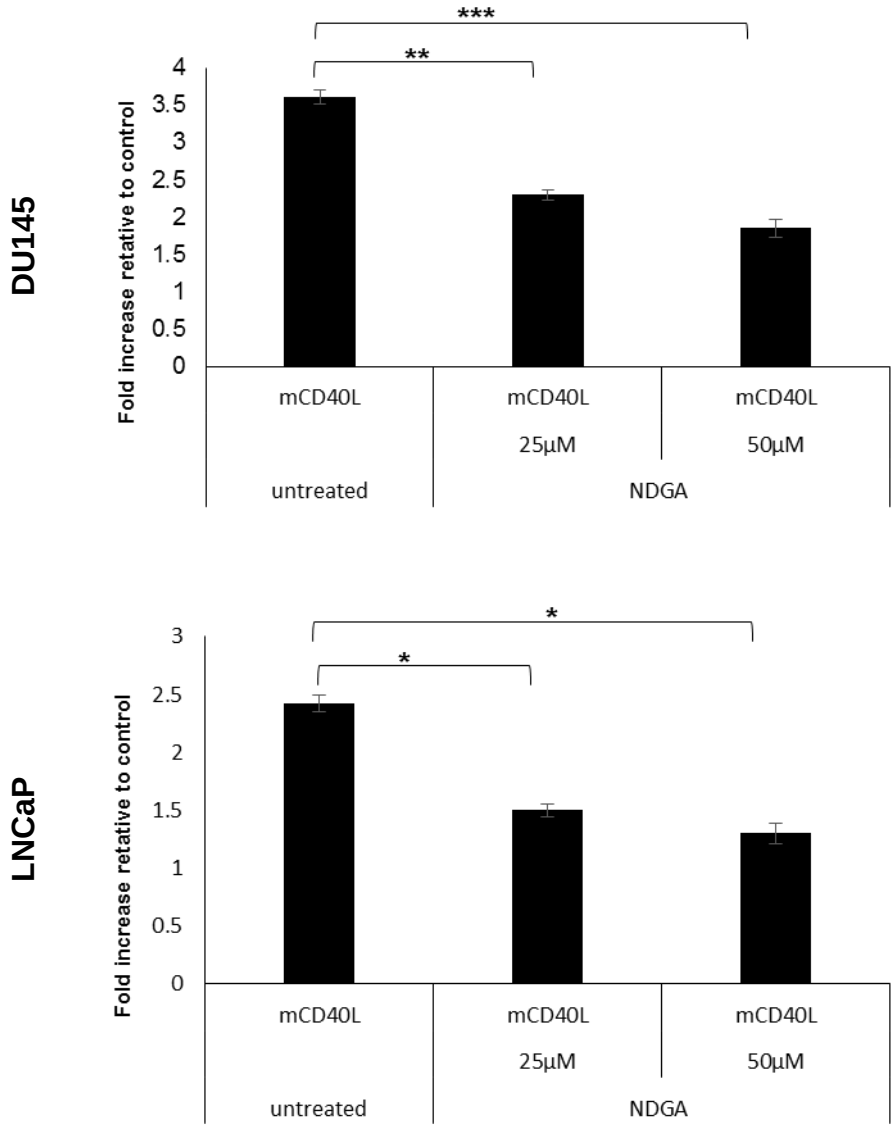


Figure 4.10 :Effects of AP-1 inhibition on mCD40L-mediated cell death.

MMC treated 1x10^{4}cells/well mouse fibroblasts (3T3CD40L and 3T3Neo) were seeded overnight then, co-cultured with 1x10^4 cells/well PCa cells (DU145 and LNCaP) in 96-well white plates in the absence or presence of AP-1 inhibitor (NDGA) at concentration 50µM and 25µM, in DR medium supplemented with 5% FCS and 1% L-glutamine. Cells were incubated for 48h at 37ºC and 5% CO_2. 50µl 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 luminescence values of 5 replicates ± SD. Stats: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, paired student t-test for co-cultured DU145 or LNCaP cells with mCD40L cells vs co-cultured cells with Neo plus NDGA, as indicated.

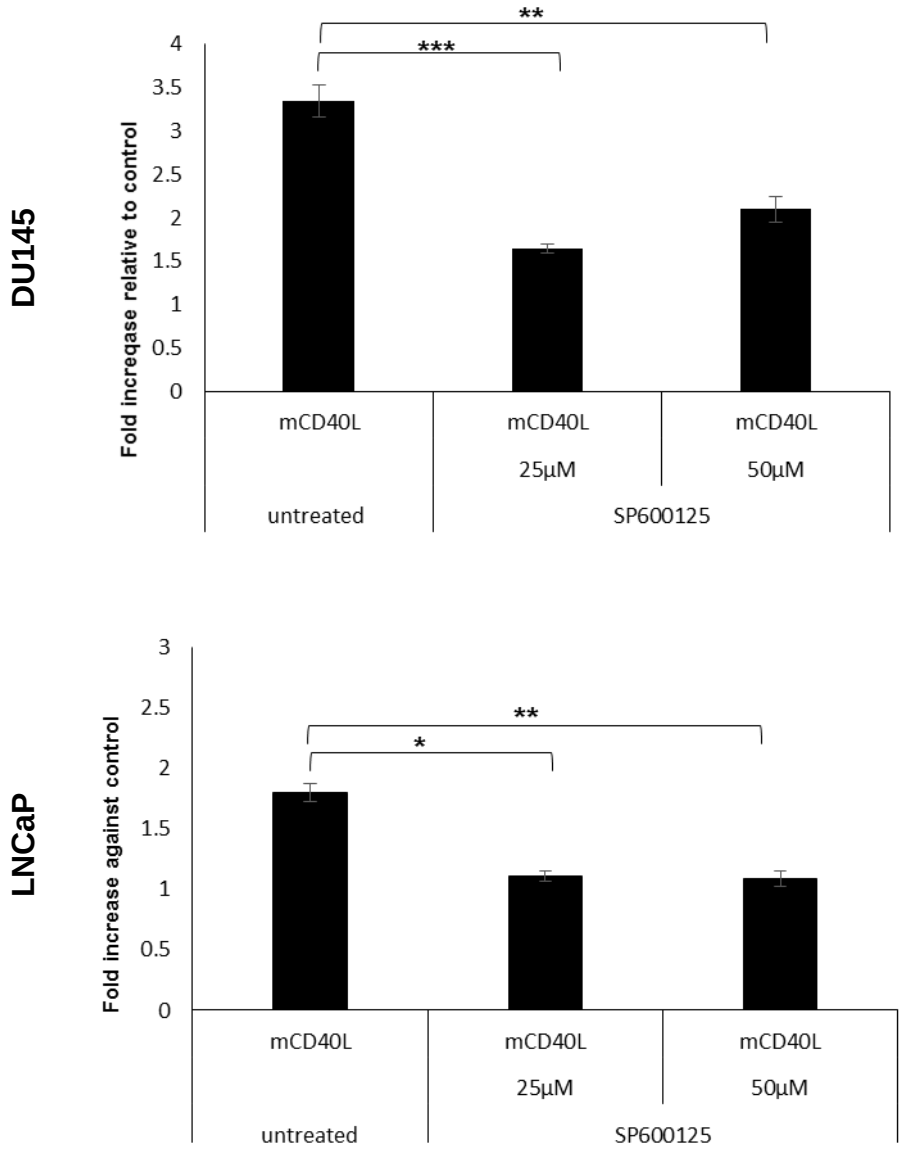


Figure 4.11: Effects of JNK inhibition on mCD40L-mediated cell death.

MMC treated 1x10^{4}cells/well mouse fibroblasts (3T3CD40L and 3T3Neo) were seeded overnight then, co-cultured with 1x10^4 cells/well PCa cells (DU145 and LNCaP) in 96-well white plates in the absence or presence of JNK inhibitor (SP600125), at concentration 50µM and 25µM in DR medium supplemented with 5% FCS and 1% L-glutamine. Cells were incubated for 48h at 37ºC and 5% CO_2. 50µl 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 luminescence values of 5 replicates ± SD. Stats; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, paired student t-test for co-cultured DU145 or LNCaP cells with mCD40L cells vs co-cultured cells with Neo plus SP600125, as indicated.

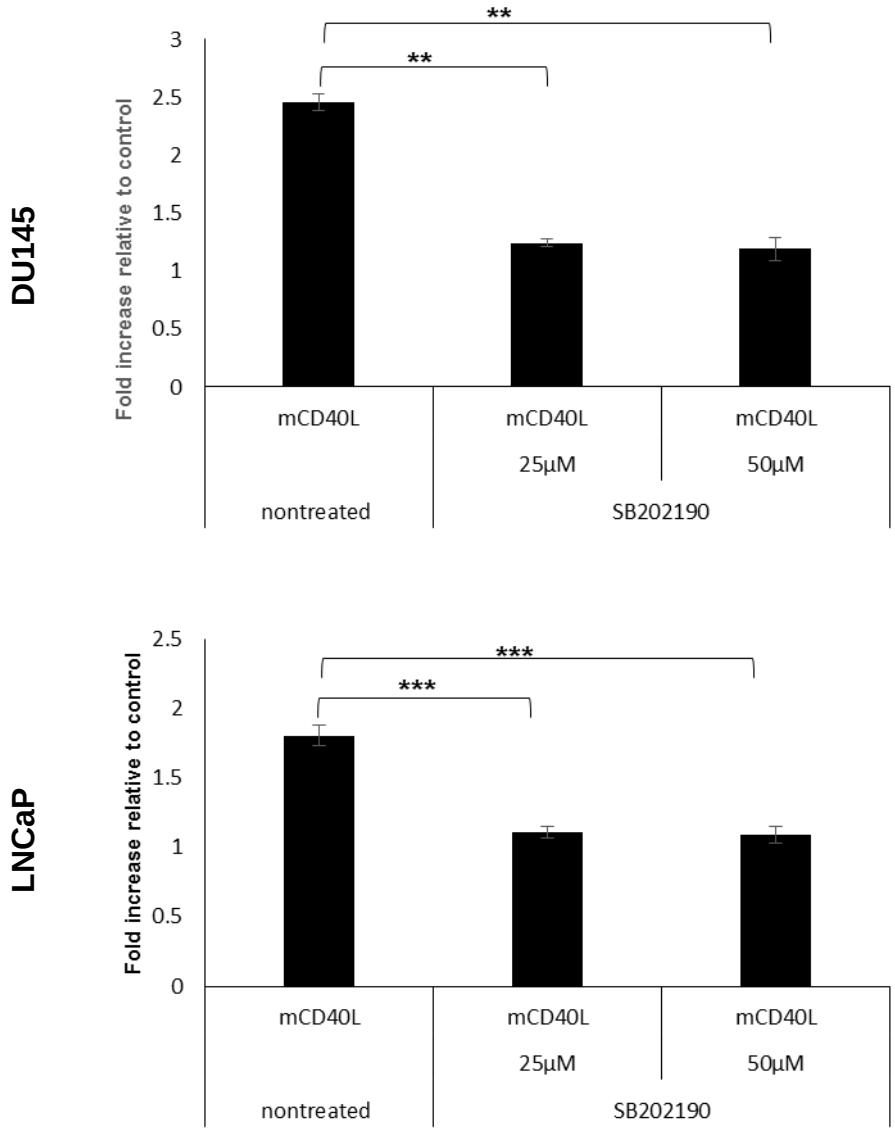


Figure 4.12: Effects of p38 inhibition on mCD40L-mediated cell death.

MMC treated 1x10^{4}cells/well mouse fibroblasts (3T3CD40L and 3T3Neo) were seeded overnight then, co-cultured with 1x10^4 cells/well PCa cells (DU145 and LNCaP) in 96-well white plates in the absence or presence of p38 inhibitor (SB202190), at concentration 50µM and 25µM in DR medium supplemented with 5% FCS and 1% L-glutamine. Cells were incubated for 48h at 37ºC and 5% CO_2. 50µl 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 luminescence values of 5 replicates ± SD. Stats: **, $p < 0.01$; ***, $p < 0.001$, paired student t-test for co-cultured DU145 or LNCaP cells with mCD40L cells vs co-cultured cells with Neo plus SB202190, as indicated.

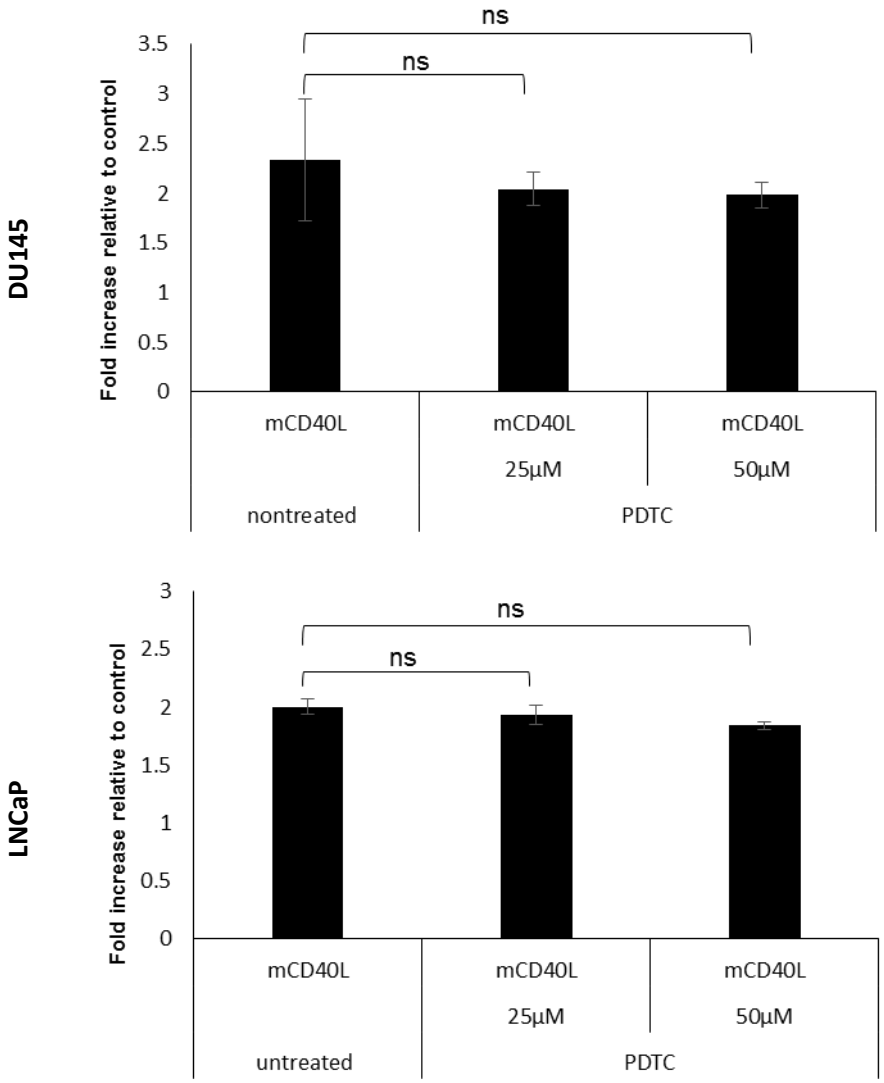


Figure 4.13: Effects of NF-kB inhibition on mCD40L-mediated cell death.

MMC treated 1x10^{4}cells/well mouse fibroblasts (3T3CD40L and 3T3Neo) were seeded overnight then, co-cultured with 1x10^4 cells/well PCa cells (DU145 and LNCaP) in 96-well white plates in the absence or presence of NF-kB inhibitor (PDTC), at concentration 50μM and 25μM, in DR medium supplemented with 5% FCS and 1% L-glutamine. Cells were incubated for 48h at 37ºC and 5% CO_2. 50μl 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 luminescence values of 5 replicates ± SD. Stats: ns. non-significant, paired student t-test for co-cultured DU145 or LNCaP cells with mCD40L cells vs co-cultured cells with Neo plus PDTC, as indicated.

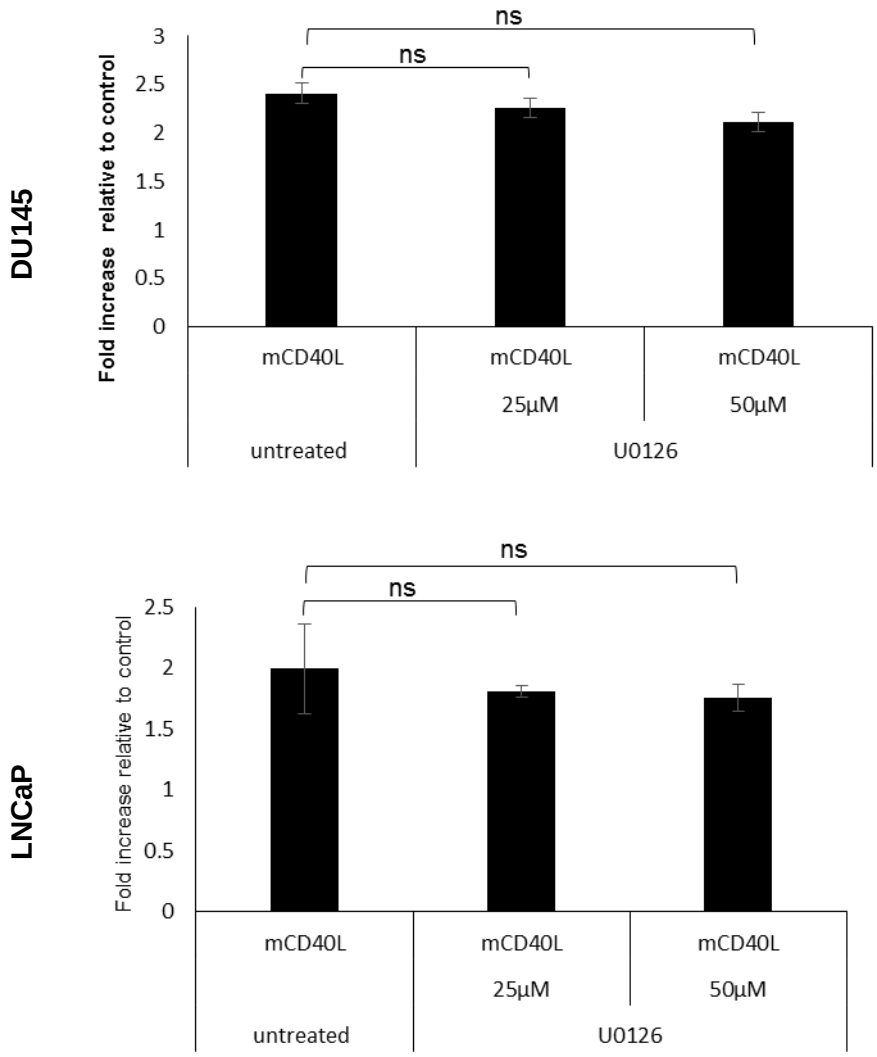


Figure 4.14: Effects of MEK/ERK inhibition on mCD40L-mediated cell death.

MMC treated 1x10^{4}cells/well mouse fibroblasts (3T3CD40L and 3T3Neo) were seeded overnight then, co-cultured with 1x10^4 cells/well PCa cells (DU145 and LNCaP) in 96-well white plates in the absence or presence of MEK/ERK inhibitor (U0126), at concentration 50µM and 25µM , in DR medium supplemented with 5% FCS and 1% L-glutamine. Cells were incubated for 48h at 37ºC and 5% CO_2. 50µl 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 luminescence values of 5 replicates ± SD. Stats: ns. non-significant; paired student t-test for co-cultured DU145 or LNCaP cells with mCD40L cells vs co-cultured cells with Neo plus U0126, as indicated.

4.7. Regulation of pro-apoptotic proteins in mCD40L signalling

It has been shown that CD40 engagement by mCD40L in epithelial carcinoma cells caused down-regulation of anti-apoptotic Bcl-2 protein whilst inducing expression of the pro-apoptotic molecules Bak and Bax (Bugajska et al., 2002). Moreover, pro-apoptotic proteins Bax and Bak are essential in CD40 mediated apoptosis by inducing MOMP in both UCC and CRC cells (Dunnill et al., 2016) and release of cytochrome c in parallel with translocation of Bax to the mitochondria. These observations indicate that Bax plays an important role during CD40-mediated apoptosis and thus may be used as downstream marker of apoptosis mediated by TRAF3 and JNK (Mohamed and Georgopoulos, unpublished observations). Also these findings are supported by another study, which demonstrated that retrotranslocation of Bax is essential in order to protect cells from commitment to programed cell death (Schellenberg et al., 2013; Todt et al., 2013; Todt et al., 2014). In addition, previous studies reported that Bax and Bak played a major role in Endoplasmic reticulum (ER) stress-induced apoptosis by being directly and indirectly linked with downstream effectors of pro-apoptotic IRE1α which caused cytochrome c release from the mitochondria (Hetz et al., 2006; Klee et al., 2009; Scull and Tabas, 2011).

To investigate whether Bax and Bak were induced by mCD40L signalling in PCa cells, the expression of Bax and Bak was investigated following CD40 ligation at 1.5, 3 and 6h using immunoblotting techniques and by utilising human protein-specific antibodies for Bak and Bax detection. As shown in Figure 4.15, the expression of pro-apoptotic protein Bax was detected within 3 and 6h post ligation (note correct band on the blot indicated by arrow), whereas Bak was detected at 6h.

Further investigation to determine whether ROS implicated in CD40/CD40Lsignalling may be linked to ASK1 activation and therefore mediated cell downstream signalling,is the subject of the work carried out in the following section.

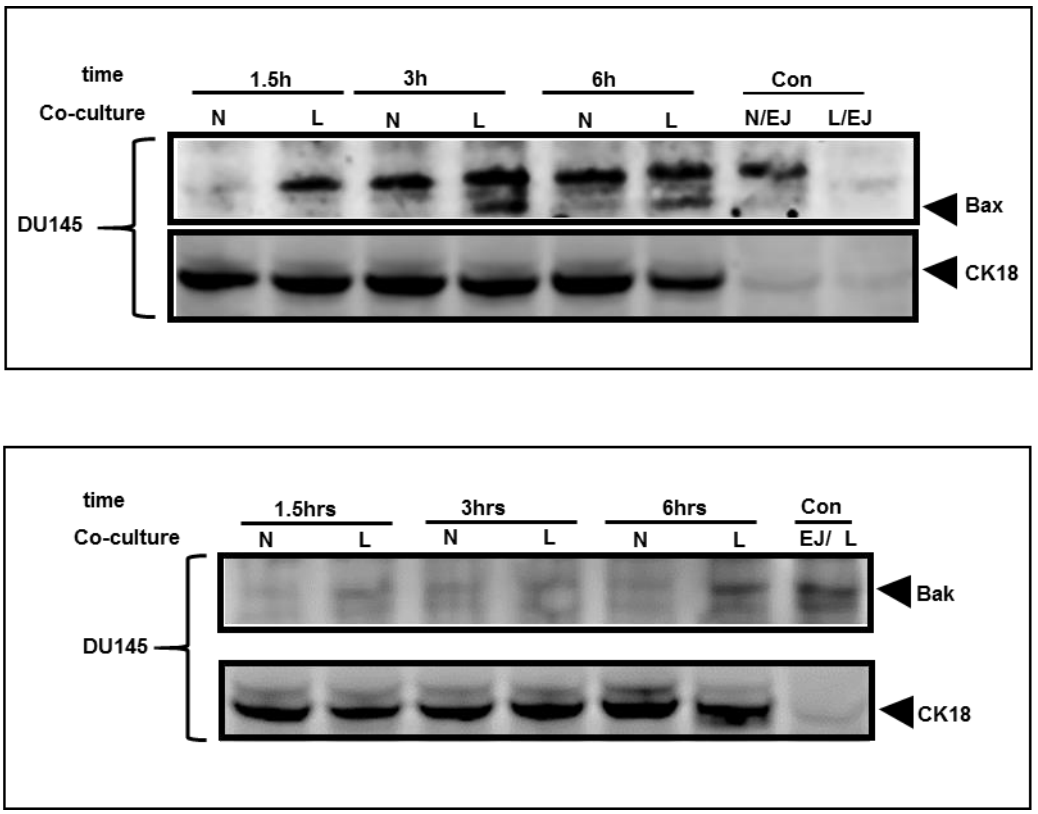


Figure 4.15: Bax and Bak expression following CD40 activation in PCa cells.

Cell lysates were prepared from target cell DU145, as well as effector cells (3T3CD40L and 3T3Neo). 20µg of cell lysates as indicated were loaded per well. Lysates were resolved by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and protein transfer onto PVDF membrane followed. The membranes were labelled overnight at 4ºC with primary monoclonal and polyclonal antibody (anti-Bax and Bak) in TBS/Tween 0.1% (1:500 dilution) and then with an anti-CK18 and anti-CK18 antibody in TBS/Tween 0.1% (1:2000 dilution). The membranes were then labelled for one hour with secondary antibody goat anti-mouse 680 (1:10,000 dilution) for Bak detection or goat anti-rabbit IgG IRDye 800 (1:10,000 dilution), before being scanned at channel 700,800nm using an Odyssey™ Infra-red Imaging system (Li-Cor). The expected molecular weight of Bax and Bak were 21 and 28kDa respectively. Key word: N: co-culture 3T3-Neo cells with DU145, and L: co-culture 3T3-CD40L with DU145. Control represent UUC bladder cancer cell line EJ serving as a positive control, L/EJ co-culture 3T3-CD40L with EJ only in the top panel, N/EJ and L/EJ in the bottom panel.

4.8. Investigation of the role of Reactive oxygen species (ROS) in CD40-mediated apoptosis

Several forms of reactive oxygen species (ROS) are produced in mammalian cells, including the radical superoxide anion ($.O_2$ -), nitric oxide (NO.) and hydroxyl radical (.OH). The hydroxyl radical is a highly reactive species that can change purine and pyrimidine bases of DNA and lead to DNA damage (Matés et al., 2010; Matés et al., 2012). ROS also include non-radical components such as hydrogen peroxide (H_2O_2). There are two of sources of cellular ROS under physiological conditions: a) the mitochondrial electron transport chain and b) the NADPH oxidase (NOX) complex and peroxidases (by several cellular components such as cell membrane and endoplasmic reticulum(ER)) (Dickinson and Chang, 2011; Lennicke et al., 2015).

Oxidative stress describes a state of the cell in which the oxidative processes predominate due to an imbalance in the formation and elimination of ROS. Mainly via their direct toxic effects (DNA damage, lipid peroxidation, protein denaturation), ROS play an important role in the pathogenesis of many diseases. These include cardiovascular diseases, diabetes, cancer, neurodegenerative diseases and aging processes (Jomova and Valko, 2011; Pala and Gürkan, 2008; Valko et al., 2007). Yet, ROS can also act as signalling molecules and activate signal transduction cascades (D'Autréaux and Toledano, 2007). The antioxidant system, which includes endogenous antioxidant enzymes (such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione (GSH)) play a major role in regulating and balancing cellular ROS levels. Recently accumulating evidence shows that Trx and GSH systems are function in parallel way and have many overlapping functions (Lu and Holmgren, 2014). The antioxidant system is regulated through multiple genes such as Ref-1, Thioredoxin (Trx), as well as the master regulator Nrf-2 that belongs to the family of Cap'n'Collar (CNC) transcription factors, which are characterized by basic leucine zipper elements (bZIP). It can bind to the ARE elements (antioxidant response element) and promoters of different genes and activate their transcription. Scavenging of ROS through the action of the antioxidant system results in the prevention of cellular oxidative damage (Fruehauf and Meyskens, 2007; Poljsak et al., 2013). Imbalance in ROS level has various effects in cell fate with elevated ROS resulting in cell stress (e.g. following chemotherapy or UV exposure), cytotoxicity, apoptosis and necrosis (Ambrosone, 2000; Dunnill et al., 2016; Halliwell, 2007; Hoeijmakers, 2009; Sweeney

et al., 2000). By contrast, low or moderate ROS levels most likely promote cell growth, differentiation and survival (Dunnill et al., 2015; Hamanaka and Chandel, 2010).

Some members of TNFR utilise ROS, which is induced as a consequence of the interaction of TRAF with NADPH oxidases (Matés et al., 2012). For example, production of ROS in CD40 activation through 5-lipoxygenase pathway and a TRAF3-NADPH oxidase association (Aggarwal, 2004; Dickinson and Chang, 2011). Furthermore, previous studies reported that ROS triggers NF-κB, P38 and JNK activation as a consequence of CD40 ligation in Raji human B cells (Aggarwal, 2004; Dickinson and Chang, 2011). In addition, CD154-mediated apoptosis of hepatocytes involves ROS generation that is amplified during hypoxia-reoxygenation (Bhogal et al., 2012).

It has been demonstrated that the MAP3K signalling pathway is activated in response to oxidative stress (ROS), with one of these MAP3K, mainly ASK1 being the main "sensor" of oxidative stress. Normally ASK1 is inactive, but ROS elevation mediates release and auto-phosphorylation of Trx (redox-sensing protein and an inhibitor for ASK1) (Gotoh and Cooper, 1998; Liu and Min, 2002; Saitoh et al., 1998). Furthermore, in some of the TNFRs (LTβR and TNF-α) the signal transduction downstream cascades were triggered by production of ROS which in turn led to activation of ASK1 (Chen et al., 2003; Gotoh and Cooper, 1998), and more recently this was for the first time demonstrated in CD40-mediated apoptosis in carcinoma cells (Dunnill et al., 2016).

To determine the possible role of ROS, in this study, two assays were used to measure ROS levels following CD40 activation: ROS-Glo and CM-H_2DCFDA (as described in section 2.10.2). The ROS-Glo™ H_2O_2 assay is a homogeneous, rapid and sensitive luminescent assay that was designed to measure hydrogen peroxide (H_2O_2) levels only. as most cellular ROS are converted to H_2O_2 and have the longest half-life of all ROS in cell culture.

The PCa cell line DU145 cells was selected as representative cell line to measure ROS levels due to its naturally high CD40 expression. Tests involved co-cultures of DU145 cells with control (Neo) and mCD40L-expressing cells, whilst the PC-3 cell line served as negative control. In the ROS-Glo assay, unlike the CytoTox-Glo and caspase detection assays, no "cells alone" background control subtractions were necessary as only target cells were treated (loaded) with the H_2O_2 detection substrate (ROS-Glo, as

detailed in section 2.10.2). The results in this assay showed that significant ROS level was observed in DU145 cells compared with control, no induction was seen, in PC-3 which was expected as it CD40 negative data was presented as Relative Luminescence Unit (RLU) as shown in Figure 4.16. However, it must be noted that the luminesce readings obtained in this assay were relatively very low, so it is not clear how biologically significant the obtained data is. To confirm the validity of the findings, an independent ROS detection assay was employed, which was the CM-H_2DCFDA reagent and detection of ROS was carried out by flow cytometry.

Therefore, experiments were preformed involving co-cultures of DU145 cells with control (Neo) and mCD40Lcells, alongside with well characterised EJ (UCC cell lines) which served as the positive control for this assay (Dunnill et al., 2016). The effector cells could be excluded from the analysis as only target cells (DU145and EJ) were labelled with a redox-sensitive fluorescence H2DCFDA at concentration 10µM, for 30 minutes, then cells were co-cultured with fibroblast effector cells (control Neo and 3T3CD40L cells) for 1.5,3, and 6hrs post receptor ligation. Fluorescence was then measured by flow cytometry (for more details, CM-H_2DCFDA experiment is detailed in section 2.10.1). Pre titration experiments were preformed to determine the optimal reagent concentration, thus DU145 cells were labelled with different H2DCFDA concentrations (5, 10 and 20µM) and cells treated with H_2O_2 served as a positive control. 10 µM was the optimal concentration (data not shown). The observations of this assay showed that no ROS activity was observed DU145 cells (red histogram mCD40L/DU145) in 1.5, 3 and (6h not shown) compared with negative control (black histogram Neo/DU145). Furthermore, the expected ROS elevation in UCC cells (EJ) was observed, in accordance with previous findings in our laboratory (Dunnill et al., 2016) (Figure 4.17).

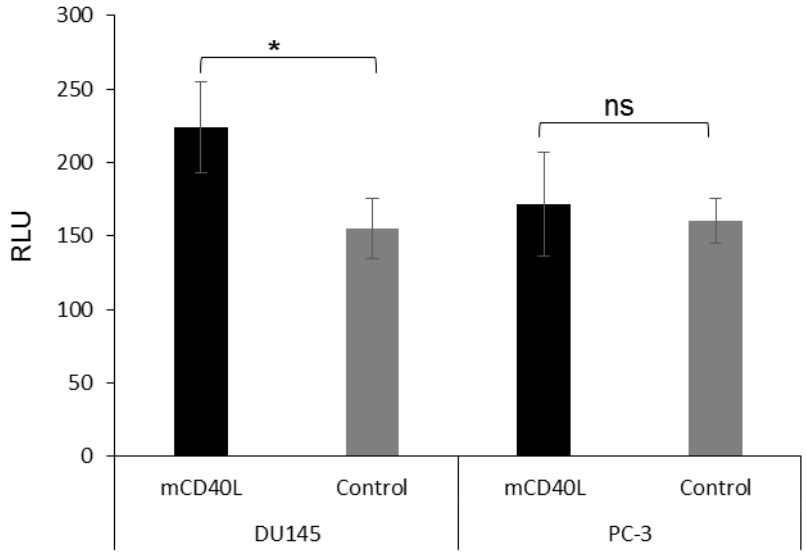


Figure 4.16: Detection of ROS induction in PCa cells following CD40 ligation.

Following pretreatment with H_2O_2 substrate for 30mins, PCa cells (DU145 and PC-3, latter are negative for CD40 expression and serving as negative control) were co-cultured with $1x10^4$ cells/well $1x10^4$cells/well mouse fibroblasts (3T3CD40L and 3T3Neo). Then Cells were incubated for 3hrs at 37ºC and 5% CO_2. 100µL ROS-Glo Detection Solution was added to the wells and luminescence was determined by a FLUOstar OPTIMA (BMG Labtech). Data were collected, analysed and presented as RLU. Data are represented as mean values ±SEM. Stats: ns. non-significant; *, $p < 0.05$; paired student t-test for co-cultured DU145 or PC-3 cells with control cells vs co-cultured cells with mCD40Lcells, as indicated.

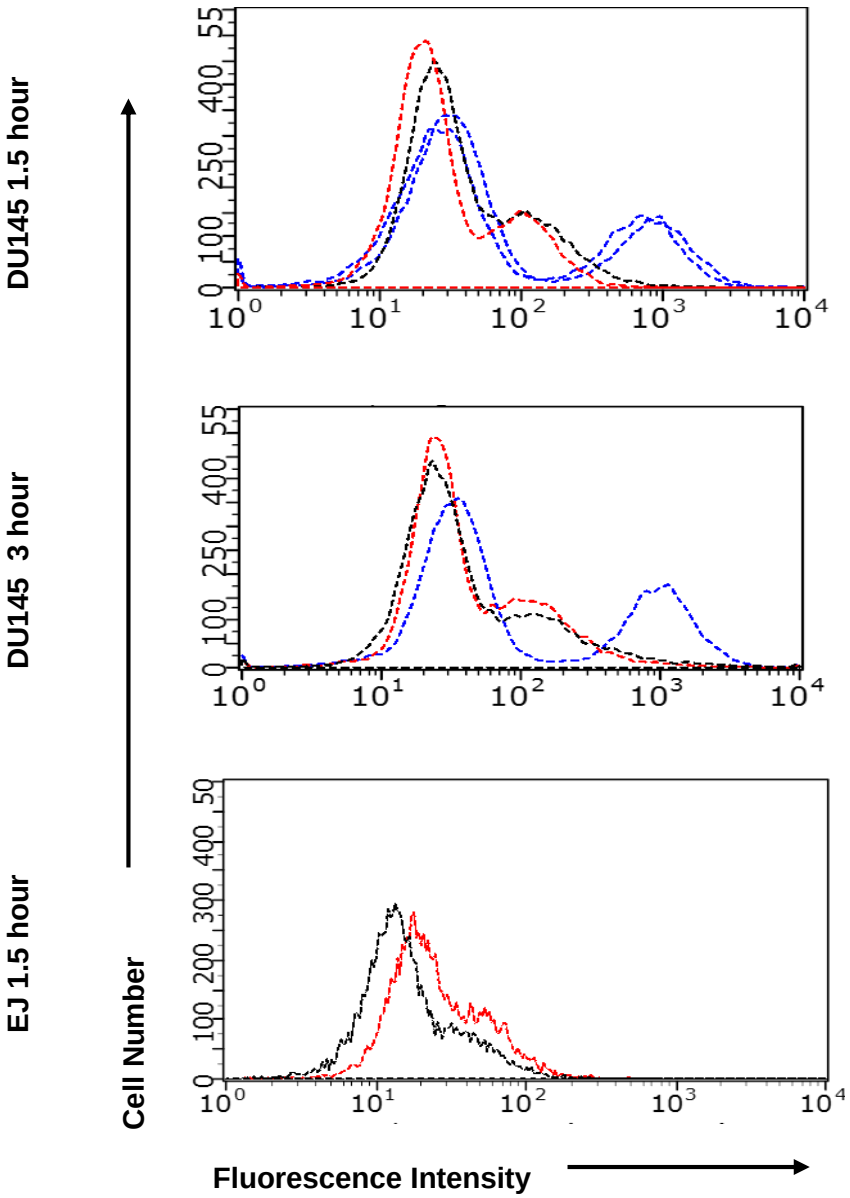


Figure 4.17: Detection of ROS induction in PCa cells following CD40 ligation by flow cytometry.

Following loading with a redox-sensitive fluorescence H2DCFDA at concentration 10µM, for 30 minutes in PBS in suspension, $3x10^5$ PCa (DU145) alongside with EJ (served as a positive control) were co-cultured with $3x10^5$ fibroblasts (3T3Neo and 3T3CD40L) cells in 6 well plates. Following CD40 ligation for 1.5 and 3h, cells were washed with PBS and harvested. Fluorescence was then measured by flow cytometry on a Guava EasyCyte green channel.

Key: DU145 or EJ co-cultured with 3T3CD40L: red dotted histograms; DU145 or EJ co-cultured with 3T3Neo: black dotted histograms; cells treated with H_2O_2 (hydrogen peroxide) as a positive control: blue dotted histograms.

4.9. Effects of the antioxidants NAC and PG on CD40-mediated apoptosis by mCD40L

Although little (if any) ROS elevation was observed above, in order to ensure that ROS definitely had no effect in the induction of CD40-mediated cell death, functional inhibition experiments using two well-characterised antioxidants were performed. More specifically, the antioxidant/ROS scavenger N-acetyl L-cysteine (NAC) and propyl gallate (PG) were employed (Dunnill et al., 2016). Pre-titration experiments using theCellTiter 96® AQ_{ueous} One Solution Cell Proliferation assay were used to determine an effective dose for all cell lines, including effector cells (3T3Neo and 3T3CD40L) and PCa cell (DU145 and LNCaP) (for more detail see section 2.7.2, and assay data shown in Appendix III). These experiments showed that the optimal concentrations were NAC at 5mM and PG at 0.1µM. Cells were pre-treated either with NAC for 3h or PG for 2h and then cells were co-cultured with control (Neo/DU145) or (mCD40L/DU145) alongside with UCC EJ cells serving as a positive control for 48h and the CytoTox-Glo assay was performed. Results showed neither NAC nor PG during co-culture utilising the optimal antioxidant concentration had any effects on mCD40L induced cell death as determined by CytoTox-Glo on PCa (DU145) cells. By contrast, in UCC cells (EJ) showed a marked reduction of cell death as illustrated in Figure 4.18 and 4.19 respectively.

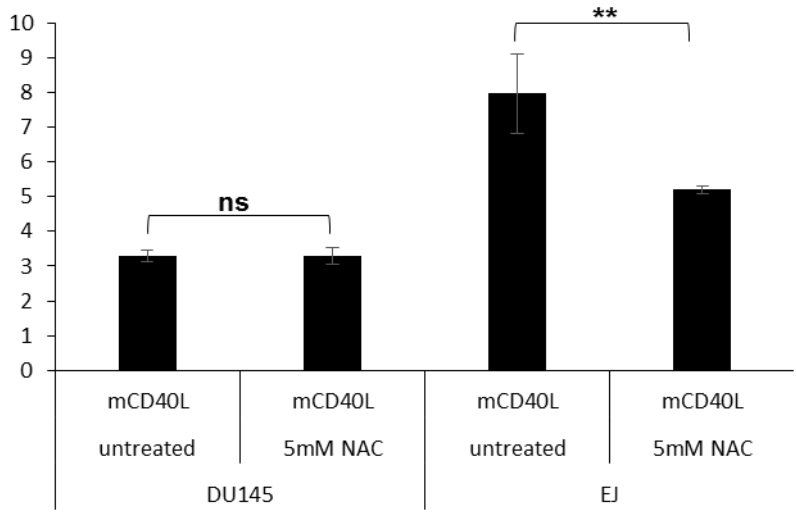


Figure 4.18: Effects of antioxidant NAC on CD40-mediated apoptosis triggered by mCD40L.

MMC treated 1x10^{4}cells/well mouse fibroblasts (3T3CD40L and 3T3Neo) were seeded overnight then. Then they were co-cultured with 1x10^4 cells/well PCa cells (DU145) and UCC EJ cells pre-treated with NAC for 3hrs (in a T25 flask at concentration 5mM) in 96-well white plates. In the absence or presence of NAC, cells were incubated for 48hrs at 37ºC and 5% CO_2, in DR medium supplemented with 5% FCS and 1% L-glutamine. Prepared 50µl 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 luminescence values of 5 replicates ± SD. Stats; ns. non-significant; **, $p < 0.01$; paired student t-test for co-cultured DU145 or EJ cells with mCD40L cells vs co-cultured cells with Neo plus NAC, as indicated.

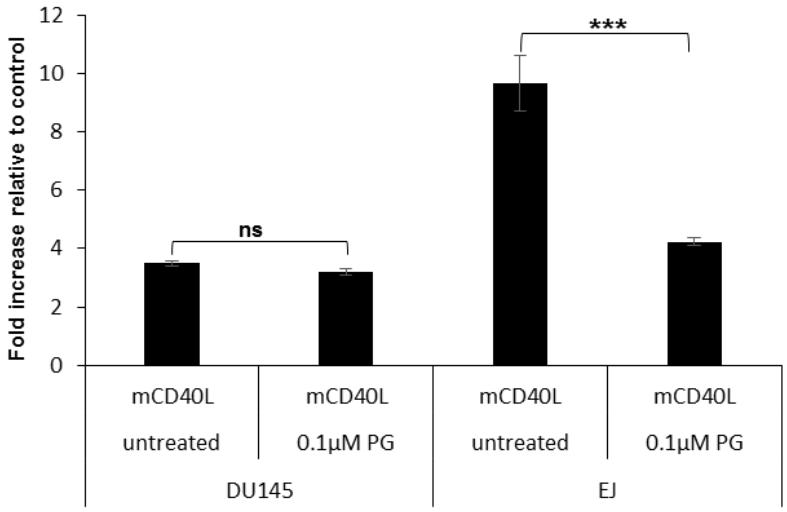


Figure 4.19: Effects of the antioxidant PG on CD40-mediated apoptosis.

MMC treated 1x10^{4}cells/well mouse fibroblasts (3T3CD40L and 3T3Neo) were seeded overnight. Then they were co-cultured with 1x10^4 cells/well PCa cells (DU145) and UCC EJ cells were pre-treated with PG for 2h (in a T25 flask at concentration 0.1µM) in 96-well white plates. In the absence or presence of PG, cells were incubated for 48hrs at 37ºC and 5% CO_2, in DR medium supplemented with 5% FCS and 1% L-glutamine. Prepared 50µl 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 luminescence values of 5 replicates ± SD. Stats; ns. non-significant; ***, $p < 0.001$; paired student t-test for co-cultured DU145 or EJ cells with mCD40L cells vs co-cultured cells with Neo plus PG, as indicated.

4.10. Investigation of the possibility of receptor cross -talk in the induction of CD40-mediated apoptosis (detection of TNFR death ligands)

Previous studies have revealed that upon CD40-mediated apoptosis in carcinoma cells expression of TNFR death ligands are induced and they may be implicated in cell death, in particularly FasL, TRAIL and TNFa (Afford et al., 1999; Afford et al., 2001; Eliopoulos et al., 2000). However, findings in our laboratory have shown that in bladder (UCC) cancer cells CD40 killing does not involve receptor cross-talk but a direct intrinsic pathway of apoptosis (Dunnill et al., 2016; Georgopoulos et al., 2006). Interestingly however, more recent findings in our laboratory have demonstrated that CD40 ligation induces TRAIL (but not FasL) within 1.5 h in colorectal (CRC) cell lines and this is sustained until 12h. Furthermore, CD40-induced TRAIL causes cytotoxicity in CRC cells via an autocrine, but not paracrine/juxtacrine mechanism, as functional blockade of TRAIL with mAb RIK2 had no effect on CD40-mediated apoptosis (Mohamed and Georgopoulos, manuscript in preparation).

Consequently, the possibility of such cross-talk in CD40-mediated killing in PCa cells was investigated. Immunoblotting experiments showed that upon CD40 ligation no up-regulation of TRAIL or FasL was observed between 1.5 to 12h post-ligation, as illustrated in Figures (4.20, 21) and (4.22, 23) for TRAIL and FasL respectively. The experiments included CRC cell line HCT116 that served as a positive control for TRAIL detection. Collectively, our data suggested that mCD40L-induced cell death occurs via a direct mechanism, and with agreement with previous study on CD40-mediated apoptosis on UCC cells (Georgopoulos et al., 2006).

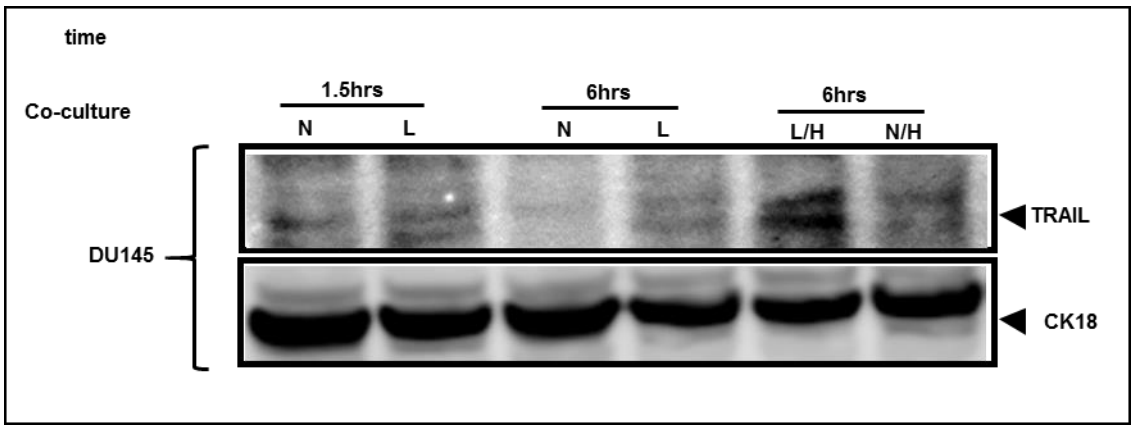


Figure 4.20: Detection of TRAIL expression following CD40 ligation.

Cell lysates were prepared from target cell DU145, alongside with colorectal cancer cell line HCT116 serving as a positive control, as well as effector cells (3T3CD40L and 3T3Neo). 20µg of cell lysates as indicated were loaded per well. Lysates were resolved by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and protein transfer onto PVDF membrane followed. The membrane was labelled overnight at 4ºC with primary polyclonal antibody (anti-TRAIL) in TBS/Tween 0.1% (1:500 dilution) and then with an anti-CK18 in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one an 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 CK18, before being scanned at channel 700,800nm using an Odyssey™ Infra-red Imaging system (Li-Cor). The expected molecular weight of TRAIL was 28kDa. Key word: N: co-culture 3T3-Neo cells with DU145 orHCT116, and L: co-culture 3T3-CD40L with DU145 or HCT116.

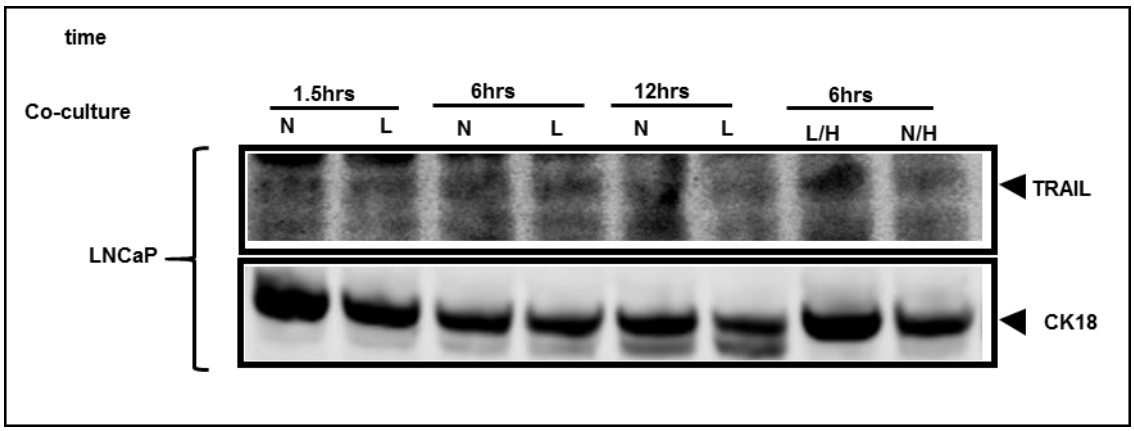


Figure 4.21: Detection of TRAIL expression following CD40 ligation.

Cell lysates were prepared from target cell LNCaP, alongside with colorectal cancer cell line HCT116 serving as a positive control, as well as effector cells (3T3CD40L and 3T3Neo). 20µg of cell lysates as indicated were loaded per well. Lysates were resolved by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and protein transfer onto PVDF membrane followed. The membrane was labelled overnight at 4ºC with primary polyclonal antibody (anti-TRAIL) in TBS/Tween 0.1% (1:500 dilution) and then with an anti-CK18 in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one an 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 CK18, before being scanned at channel 700,800nm using an Odyssey™ Infra-red Imaging system (Li-Cor). The expected molecular weight of TRAIL was 28kDa. Key word: N: co-culture 3T3-Neo cells with LNCaP or HCT116, and L: co-culture 3T3-CD40L with LNCaP or HCT116.

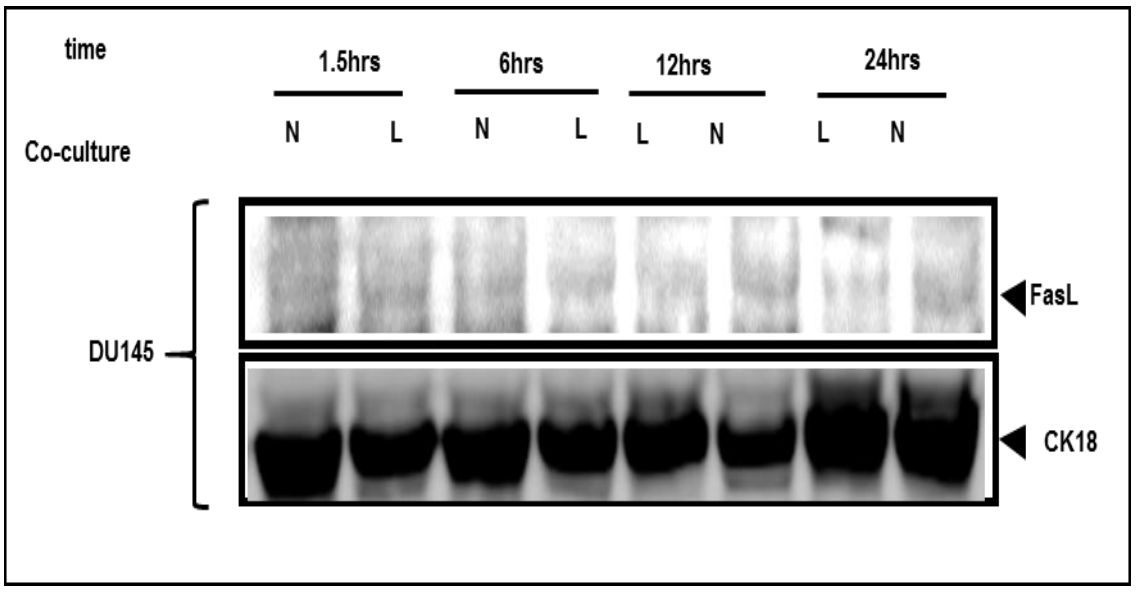


Figure 4.22: Detection of FasL expression following CD40 ligation.

Cell lysates were prepared from target cell DU145, as well as effector cells (3T3CD40L and 3T3Neo). 20µg of cell lysates as indicated were loaded per well. Lysates were resolved by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and protein transfer onto PVDF membrane followed. The membrane was labelled overnight at 4ºC with primary polyclonal antibody (anti-FasL) in TBS/Tween 0.1% (1:500 dilution) and then with an anti-CK18 antibody in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one an 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 CK18, before the membrane being scanned at 700nm and 800nm using an OdysseyTM Infra-red Imaging system (CK18 was serving as loading controls). The expected molecular weight of FasL was 40kDa. Key word: N: co-culture 3T3-Neo cells with DU145, and L: co-culture 3T3-CD40L with DU145.

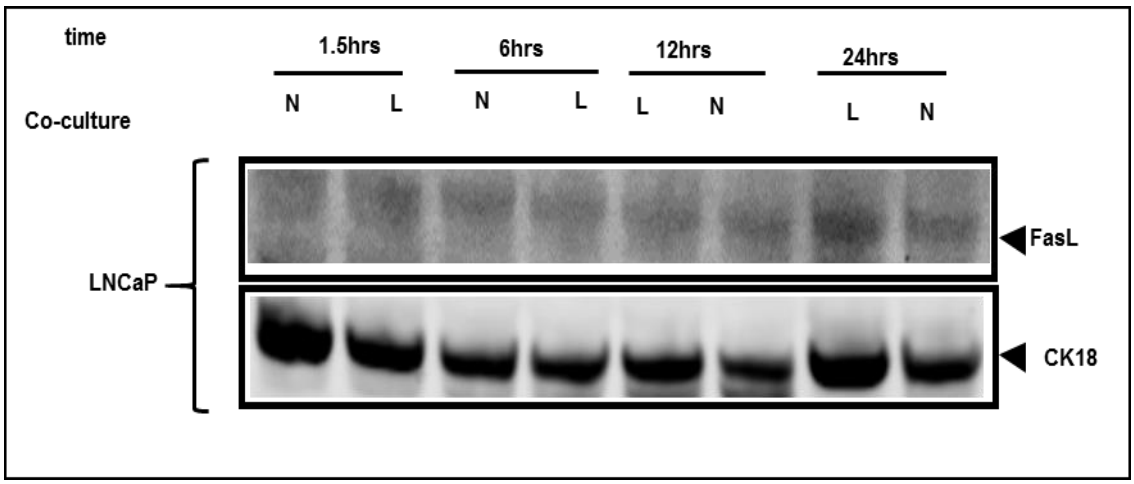


Figure 4.23: Detection of FasL expression following CD40 ligation.

Cell lysates were prepared from target cell LNCaP, as well as effector cells (3T3CD40L and 3T3Neo). 20µg of cell lysates as indicated were loaded per well. Lysates were resolved by SDS-PAGE using 4-12% (w/v) Bis-Tris gels and protein transfer onto PVDF membrane followed. The membrane was labelled overnight at 4ºC with primary polyclonal antibody (anti-FasL) in TBS/Tween 0.1% (1:500 dilution) and then with an anti-CK18 antibody in TBS/Tween 0.1% (1:2000 dilution). The membrane was then incubated for one an 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 CK18, before the membrane being scanned at 700nm and 800nm using an OdysseyTM Infra-red Imaging system (CK18 was serving as loading controls). The expected molecular weight of FasL was 40kDa. Key word: N: co-culture 3T3-Neo cells with LNCaP, and L: co-culture 3T3-CD40L with LNCaP.

4.11. Preliminary investigations on the role of Endoplasmic Reticulum (ER) Stress in CD40 apoptosis

Eukaryotic cells have the ability to respond to endoplasmic reticulum (ER) stress. Furthermore, this can occur through a process termed folding and modification in the ER (Di Sano et al., 2006), in order to maintain properly folded secretory proteins (Scull and Tabas, 2011). However, when these processes are obstructed and the cells have failed to repair the ER stress and the damage is comprehensive. As a consequence of this, an unfolded protein response UPR proteins are activated and initiate diverse signalling responses, resulting in mobilisation of ER calcium (Ca^{2+}) stores and sensitises mitochondria to direct pro apoptotic stimuli, and triggers apoptosis (Di Sano et al., 2006; Scull and Tabas, 2011). In addition, it has been reported that UPR sensor proteins play a crucial role in cell death by apoptosis (Hetz, 2007) in particularly when the UPR pathways of adaptation and cell survival are insufficient to repair the unfolded protein load (Hetz and Glimcher, 2008; Hetz, 2007). Furthermore, Ca^{2+} ions have been shown to be released leading to mitochondrial permeability transition and functional collapse; under these conditions Grp78/BiP and GADD153/CHOP proteins are mainly upregulated (Orrenius et al., 2015). Moreover, previous studies demonstrated that upon ER stress induced by TN using murine embryonic cells cytochrome c is released from mitochondria independently of apoptosome formation (Di Sano et al., 2006). It has also been shown that ER-stress-induced apoptosis depends on Ca^{2+} release (Di Sano et al., 2006), and these observation were confirmed by usage of two inhibitors of Ca^{2+} namely dantrolene, which is a strong inhibitor of Ca^{2+} release from ER and BAPTA, which is a Ca^{2+} chelator within the cytosol (Di Sano et al., 2006; Orrenius et al., 2015).

The findings presented in this (chapter 3), in particular the results from the caspase detection, DNA fragmentation assay and CytoTox-Glo assays, combined with functional inhibition experiments with pan-caspase inhibitors, collectively suggest that cells death occurs in a caspase independent fashion, thus indicating a possible involvement of ER/Ca^{2+} ion-release in cell death by CD40 ligation. Consequently, the involvement of Ca^{2+} release was investigated using the two inhibitors of Ca^{2+} Dantrolene and BAPTA mentioned above.

Initial pre-titration experiments for both inhibitors (Dantrolene, BAPTA) were carried out, in order to select the optimal concentration. It was observed that the optimal concentration for BAPTA was 8µM. Whereas, Dantrolene not toxic was observed for the range of Dantrolene concentration tested (See Appendix IV). Before co-culture of mCD40L-expressing effector cells (3T3CD40L or with 3T3Neo) with DU145 cells, the target cells (DU145) were pre-treated for one h in suspension either with Dantrolene at concentration 4µM, 6µM or with BAPTA at 4µM, 8µM. Results from these preliminary experiments demonstrated that neither Dantrolene nor BAPTA caused any detectable reduction in the level of cell death as illustrated in Figure 4.24.

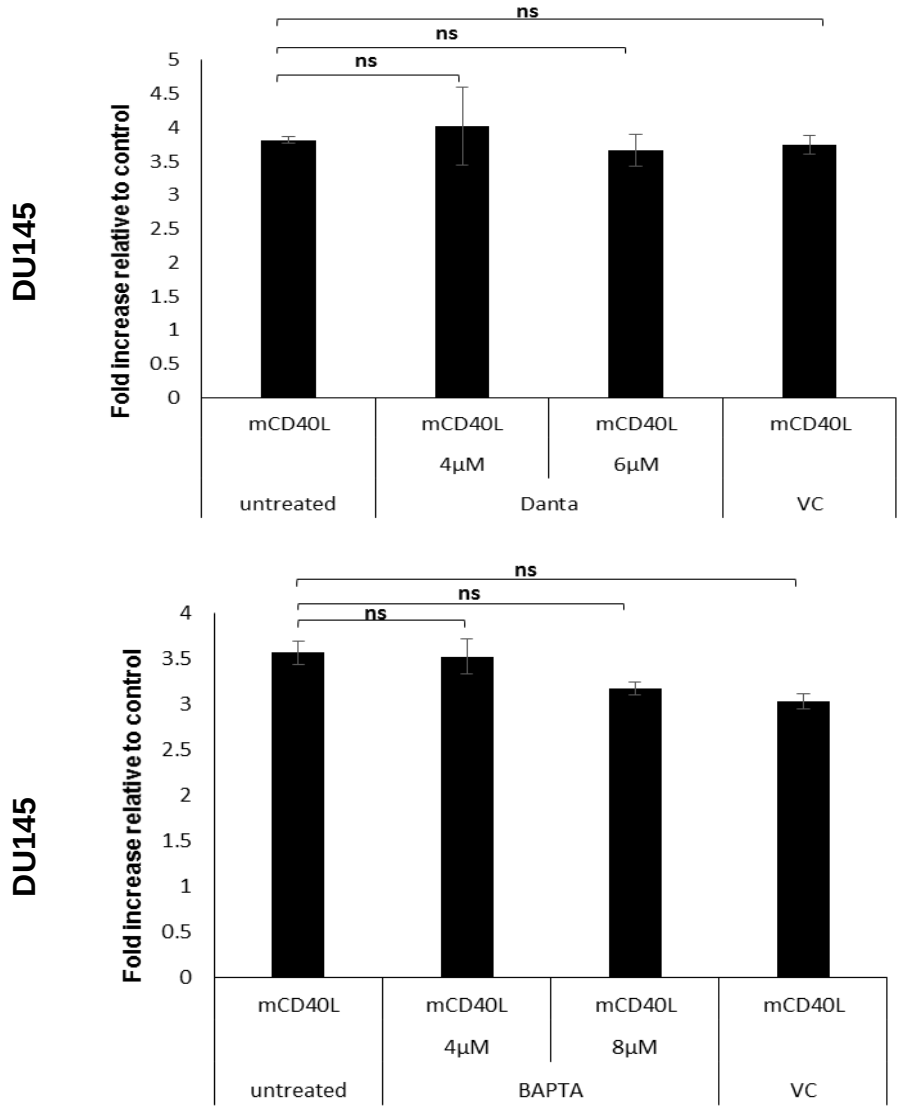


Figure 4.24: Effect of calcium ion release inhibitors on CD40 killing in PCa cells.

MMC treated 1x10^{4}cells/well mouse fibroblasts (3T3CD40L and 3T3Neo) were seeded overnight. then co-cultured with 1x10^4 cells/well PCa cells DU145 after pre-treated with either Dantrolene (Danta) or BAPTA for one an hour in suspension, as indicated in 96-well white plates in DR medium supplemented with 5% FCS and 1% L-glutamine. Plates were incubated for 48h at 37ºC/5% CO_2. Then prepared of 50µl CytoTox-Glo reagents were added to wells and luminescence was measured by a FLUOstar OPTIMA (BMG Labtech) plate reader and background-corrected RLU readings deduced as described in the Materials and methods. Bars are proportional to mean values of 4-6 replicates ± SD. Results are representative of three experiments. The top Figure illustrates DU145 treated with Danta The bottom Figure illustrates DU145 treated with BAPTA. Stats: ns. non-significant; paired student t-test for co-cultured DU145 cells with mCD40L cells vs co-cultured cells with 3T3Neo plus Danta or, BAPTA as indicated.

Summary

- In this chapter optimisation of immunoblotting techniques for detection of protein expression in epithelial and non-epithelial cultured cells was performed. Epithelial cells exclusively expressed cytokeratins (CK8 and CK18) so that the intracellular proteins involved in cell death can be detectable by utilising the co-culture system.
- Following CD40 activation, rapid TRAF3 induction was observed both in cells naturally expressing CD40 (DU145) and in cells de novo expressing the receptor (PC-3-CD40 cells). Induction of TRAF3 was accompanied by concurrent upregulation of TRAF1 and down-regulation of TRAF2. Other TRAF proteins such as TRAF6 were not detectable.
- Investigations in MAPK expression levels following CD40 activation by mCD40L showed that MAPK3 ASK1 was activated (as evident by induction of ASK1 phosphorylation), which was accompanied by the phosphorylation of MAPK2 MKK7 but not activation of MKK4. Activation of MKK7 was then followed by the detection of phosphorylation of the MAPK JNK. No activation of p38 was detectable following CD40 ligation.
- Functional inhibition experiments were then performed using specific pharmacological inhibitors to identify functional roles for these MAPKs in CD40-mediated apoptosis. It was shown that: a) Inhibition of JNK and p38 significantly abrogated CD40-mediated death in DU145 and LNCaP cells, despite p38 not being detected by immunoblot; b) no effects were observed using inhibitors for NF-kB and MEK/ERK; c) inhibition of JNK and downstream transcription factor AP-1 attenuated CD40-mediated death.
- Results presented are representative of a number of different some of cell lysates (n=4). For exact quantitative and publication purposes densitometry should be performed.
- Pro-apoptotic mitochondrial death (intrinsic apoptosis pathway) related proteins Bax and Bak were both detectable by western blot after 3 and 6h.

- Reactive oxygen species (ROS) release was investigated using independent assays. Although the ROS-Glo assays demonstrated statistically significant results, the luminescence values were quite low and the findings were not corroborated by the independent assay based on use of H2DCFDA and flow cytometry. Moreover, pharmacological inhibition using ROS scavenger/antioxidant NAC and propyl gallate showed that neither NAC nor PG had an effect on mCD40L-induced cell death. The results imply that ROS are involved in CD40-mediated apoptosis in PCa cell lines.

- CD40-mediated apoptosis in PCa cells does not appear to involve cross-talk with other receptors, as there was no detectable induction of death ligands such as TRAIL and FasL; this suggests that mCD40L-induced cell death in PCa cells occurs via a direct mechanism.

- As ROS were not involved in cell death induction, the possible role of Ca^{2+} in the cell death was investigated; yet, inhibitors dantrolene and BAPTA did not attenuate apoptosis. However, these results were limited and preliminary and more extensive optimisation would be necessary, together with the inclusion of appropriate positive controls.

Chapter 5: **Investigation of pro-inflammatory cytokine secretion by mCD40L *versus* soluble agonist in PCa cell lines**

5.1. Introduction

Cytokines are extracellular signalling molecules whose molecular weight is typically less than 80 kDa and are produced by many immuno-inflammatory mainly lymphoid cells, as well as other cell types including cancer cells. The main cytokines were originally discovered in the 1950s, however the identification of their structure and function took many years to be fully characterised (Akira et al., 2006).

Cytokines exert their cellular effects either locally by auto- or paracrine mechanisms or sometimes at a distance (in endocrine fashion) (Banchereau and Steinman, 1998). They represent a set of fundamental cellular communication molecules that mainly drive the activation and differentiation of a repertoire of immune cells (Banchereau and Steinman, 1998). The cytokine-dependent recruitment of infiltrating effector immune cells (monocytes, dendritic, T cells and NK cells) to the sites of infection is crucial for the eradication of pathogenic invaders and infected cells (Elgueta et al., 2009). Immune responses during neoplastic disease can detected via secretion of chemotactic factors produced by the carcinoma cells. The secretion of these cytokines is an essential characteristic of immune cells which play a fundamental role in the functional activation of these cells and the orientation of the immune response (Kourilsky and Truffa-Bachi, 2001; Xavier and Podolsky, 2007). Cytokines represent a large variety of mediators and are divided into ten different subgroups (Fresno et al., 1997). As the present study focused on a defined set of cytokines, below the pro-inflammatory cytokines IL-6, IL-8 and GM-CSF are discussed in detail.

5.1.1. Interleukin-6 (IL-6)

This is a cytokine originally identified as a T cell-derived factor acting on B cells (Kishimoto, 1989). IL-6 is produced predominantly by monocytes, macrophage and epithelial cells, but can also be induced in many cell types such as endothelial cells, fibroblasts, T and B lymphocytes, mast cells and neutrophils (Van Snick, 1990). It thus regulates the physiological functions (proliferation, survival, differentiation and activation) of numerous cell types. The multiple biological activities of IL-6 include the ability to stimulate the differentiation of myeloid cells to promote the production of Ig by B cells, and proteins of the acute phase of inflammation in the liver and as the elevation of body temperature. It is therefore involved in immune regulation, hematopoiesis, inflammation as well as oncogenesis (Kishimoto, 1989; Van Snick, 1990).

5.1.2. Interleukin-8 (IL-8)

IL-8 is the first chemokine demonstrated by different groups in 1988 (Baggiolini et al., 1995). This pro-inflammatory chemokine is primarily involved in the attraction of neutrophils. It is expressed by different immune cells, such as monocytes / macrophage and DC, and non-immune, such as epithelial, endothelial cells and fibroblasts (Najakshin et al., 1999). IL-8 performs its biological functions through its interaction with 2 receptors (CXCR1 and CXCR2) present on the surface of different cell types such as neutrophils, endothelial cells and even some cancer cells and tumours associated macrophage (TAM). Increased expression of IL-8 is found in some tumours where it promotes angiogenesis; IL-8 may mimic the function of Vascular Endothelial Growth Factor (VEGF) and stimulates endothelial cell survival and proliferation. Its expression can be induced by inflammatory cytokines (e.g. TNFα, IL-6) and Toll-like Receptor (TLR) agonists (e.g. LPS) (Baggiolini et al., 1995; Najakshin et al., 1999).

5.1.3. Granulocyte-macrophage colony-stimulating factor (GM-CSF)

GM-CSF induces the proliferation and activation of erythroid and myeloid cells, including granulocytes, macrophages, eosinophils (Metcalf, 1993; Ogawa, 1993). In synergism with Interferon-gamma (IFN-GM), GM-CSF effects the differentiation of mononuclear cells into tumoricidal macrophages. The lifetime of such macrophages and of granulocytes with tumoricidal potential is extended by GM-CSF (Metcalf, 2008). In addition, the combination of GM-CSF and IL-4 stimulates the generation of professional antigen-presenting cells from monocytes. In contrast to the MHC class I, II and other costimulatory molecules, GM-CSF induces the expression of CD40 on DC (Metcalf, 2008).

5.1.4. Cytokine secretion by the TNFR family

Besides to their fundamental roles in controlling cell fate and regulate many biological events such as, proliferation, differentiation and cell death in various cell types, previous study also showed that TNFR members are capable to induce cytokine secretion in epithelial cells. in particular, member of TNF-α and Fas are exhibited lL-8 secretion on colorectal cancer cell (Abreu-Martin et al., 1995), in addition, in another member of TNFR LTβR which highly related TNFR to CD40, indicated that IL-8 is induced upon receptor activation via agonistic anti-LTβR monoclonal antibody in A375 cells (malignant melanoma) (Degli-Esposti et al., 1997).

CD40 / CD40L interactions in B cells is important to humeral immune response. in the activated T cells which mainly expressed CD40L 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 plasma cells, antibody-producing cells for specific antigens that provide immunisation to the second infection (Hassan et al., 2014).

More recently, it has been demonstrated that membrane presented CD40L (mCD40L) induced secretion of cytokines IL-6, IL-8 and GM-CSF in some carcinoma cell lines of CRC and UCC origins (Georgopoulos et al., 2007). Remarkably, IL-8 secretion was induced when cells were treated with soluble CD40 agonist (and to a lesser extent IL-6 secretion was observed); however, GM-CSF was only induced following CD40 ligation by mCD40L (Georgopoulos et al., 2007). In line with these observations, in normal and malignant epithelial cells and also in human colonic fibroblasts, IL-6 and IL-8 secretion was reported and this secretion was dependent on NF-κB activation (Cagnoni et al., 2004; Gallagher et al., 2002; Gelbmann et al., 2003; Schwabe et al., 2001). The ability of CD40 to mediate secretion of pro-inflammatory cytokines in PCa cells has not yet been investigated. Therefore, having demonstrated for the first time that CD40 ligation by membrane-presented (mCD40L), but not soluble (G28-5 mAb) agonist, induces cell death in PCa lines, to further explore the biological consequences of mCD40L-mediated death, this study examined whether a) CD40 ligation induces cytokine secretion, and b) whether the mode of receptor ligation affects not only cell fate (death versus no death) but also the repertoire of secreted cytokines. More specifically, the aims of this chapter were:

- Induce receptor ligation by mCD40L versus soluble agonist (G28-5) and collect culture supernatants to measure secretion of cytokines IL-6, IL-8 and GM-CSF.

- Detect cytokine secretion in the PCa panel of DU145, LNCaP and PC-3-CD40 cells; these were either co-cultured with 3T3-CD40L and 3T3Neo cells, or treated with soluble CD40 agonist G28-5 mAb for a series of incubation periods post receptor ligation

- Detection of cytokines secretion was quantified by ELISA assays and data were analysed as described in the methods (section 2.9).

- Detect cytokine secretion at 1.5, 3 6, 12, 36 and 48h post receptor ligation.

5.2. Determine of pro-inflammatory cytokine secretion following CD40 activation by mCD40L in PCa cells

When ligation of CD40 was carried out by mCD40L in DU145 cells, rapid and sustained induction of IL-6 and IL-8 secretion was observed. Interestingly, marked induction of GM-CSF was also detected, albeit at later time points (Figure 5.1). By contrast, soluble agonist in the form of agonistic mAb G28-5 caused no induction of any of the cytokines tested, even though the antibody was highly cross-linked (Figure 5.2).

Similar experiments in LNCaP cells showed dramatic induction of both IL-6 and IL-8 secretion by mCD40L but not soluble agonist (Figures 5.3 and 5.4), which was more marked than that observed in DU145 cells. However, in contrast to DU145, mCD40L did not induce GM-CSF secretion in the LNCaP cell line (Figure 5.3).

Finally, the ability of CD40 to induce cytokine secretion was tested in the transduced PC-3 cells. As *de novo* expression of CD40 in these cells (PC-3-CD40) could induce cell death, these investigations aimed at testing whether death was accompanied by pro-inflammatory cytokine secretion. As seen in Figures 5.5 and 5.6, respectively, mCD40L, but not soluble agonist, induced rapid secretion of IL-6, IL-8 and notably also GM-CSF (though to a lesser extent) in PC-3-CD40 cells. Interestingly however, this secretion was rapid and by 12 and particularly 24 h, the secretion of these cytokines had subsided (Figure 5.5).

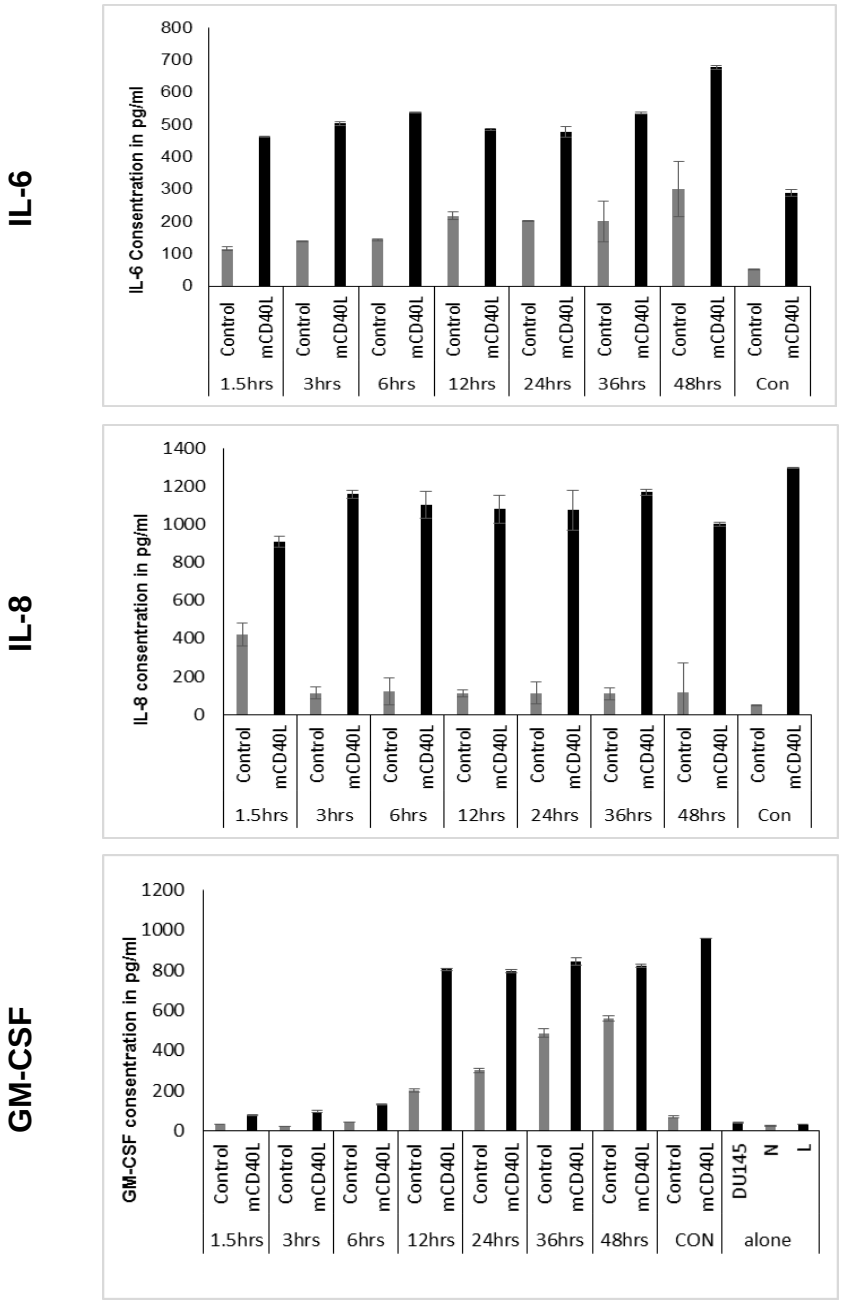


Figure 5.1: mCD40L-mediated cytokine secretion in DU145 cells.

DU145 cells were co-cultured with MMC-treated 3T3CD40L (mCD40L) or 3T3Neo (Control) cells for the indicated time points. EJ/3T3 co-cultures at 24 h served as a +ve control (denoted as Con/CON). After 1.5, 3, 6, 12, 24, 36 and 48h, culture supernatants were collected and secretion of IL-6, -8 and GM-CSF was quantified using cytokine specific ELISA on a FLUOstar OPTIMA plate reader. Supernatants were also collected from cultures of DU145 as well as 3T3CD40L and 3T3Neo cells (denoted DU145, N and L alone, respectively) at 24 h. Data are represented as mean values ±SEM of cytokine concentration (pg/mL) for 2 replicates.

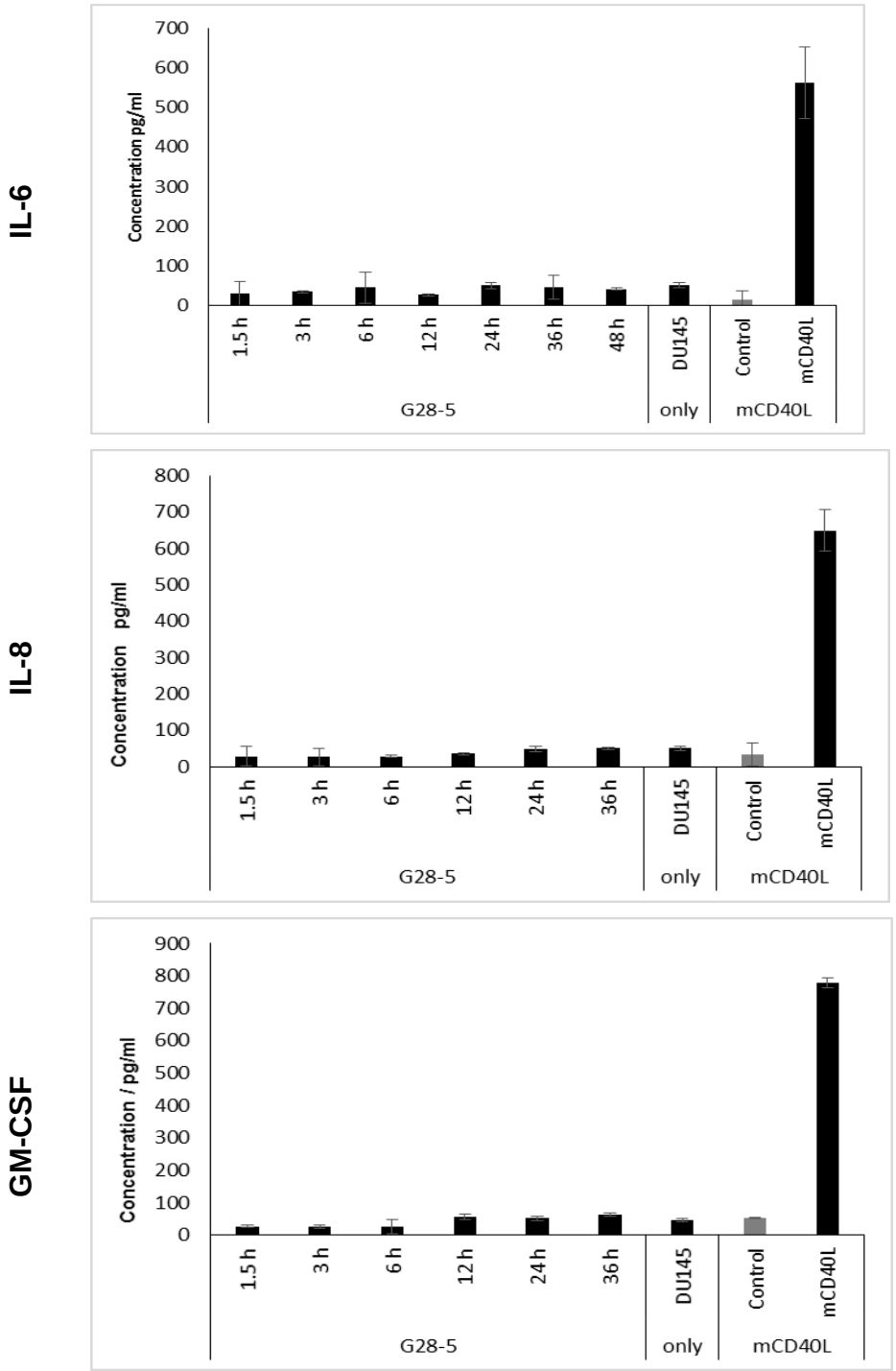


Figure 5.2: G28-5 mAb-mediated cytokine secretion in DU145 cells.

DU145 cells were treated with agonistic anti CD40 mAb G28-5 at 10µg/mL cross-linked with anti-mouse IgG at 5µg/mL for the indicated time points. EJ/3T3 co-cultures at 24 h served as a +ve control (CON). After 1.5, 3, 6, 12, 24 and 36h, culture supernatants were collected and secretion of IL-6, -8 and GM-CSF was quantified using cytokine specific ELISA on a FLUOstar OPTIMA plate reader. Supernatants were also collected from cultures of PCa cells (DU145) alone ('DU145 only'). Data are represented as mean values ±SEM of cytokine concentration (pg/mL) for 2 replicates.

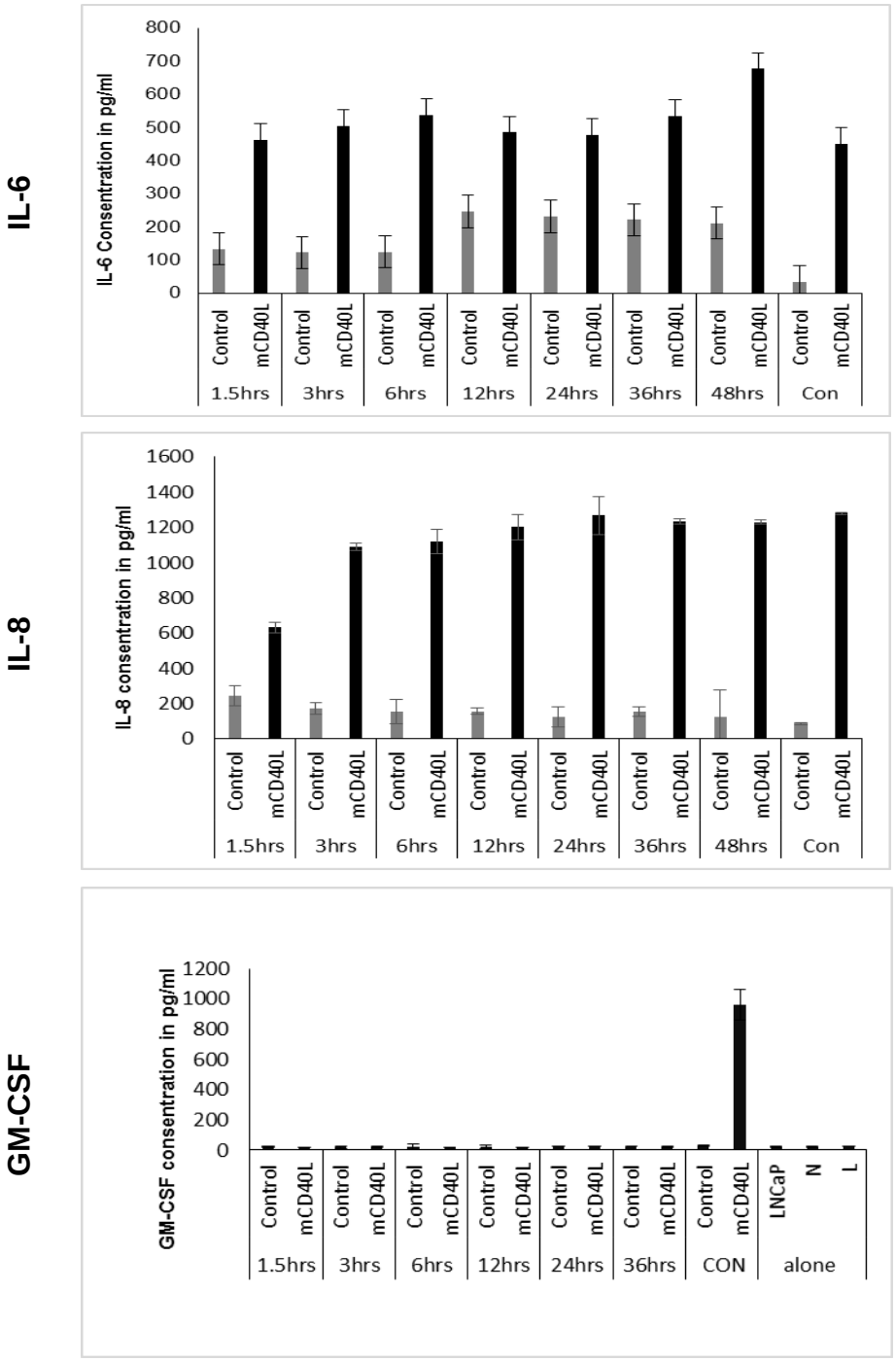


Figure 5.3: mCD40L-mediated cytokine secretion in LNCaP cells.

LNCaP cells were co-cultured with MMC-treated 3T3CD40L (mCD40L) or 3T3Neo (Control) cells for the indicated time points. EJ/3T3 co-cultures at 24 h served as a +ve control (denoted as Con/CON). After 1.5, 3, 6, 12, 24 and 36h, culture supernatants were collected and secretion of IL-6, -8 and GM-CSF was quantified using cytokine specific ELISA on a FLUOstar OPTIMA plate reader. Supernatants were also collected from cultures of LNCaP as well as 3T3CD40L and 3T3Neo cells (denoted DU145, N and L alone, respectively) at 24 h. Data are represented as mean values ±SEM of cytokine concentration (pg/mL) for 2 replicates.

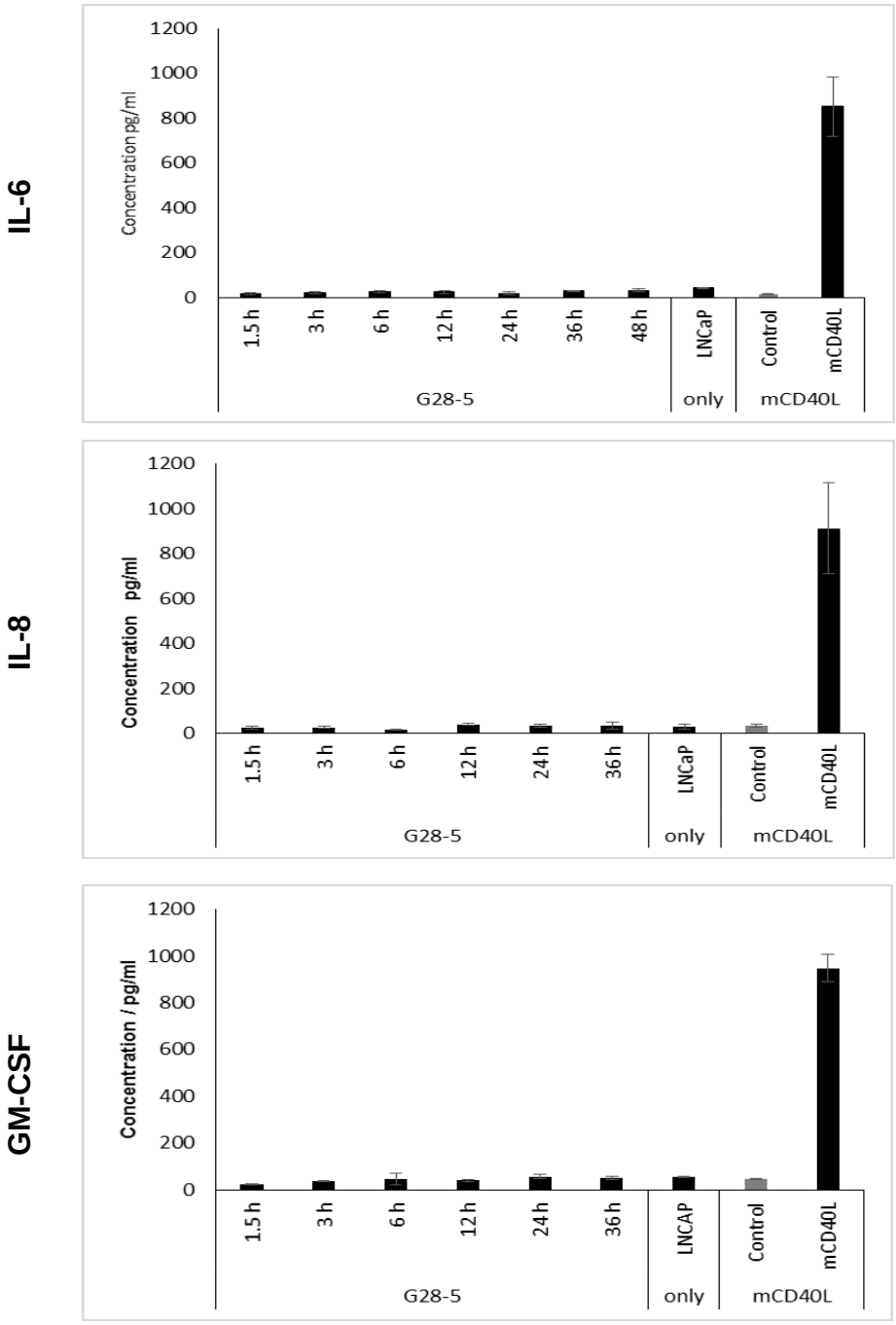


Figure 5.4: G28-5 mAb-mediated cytokine secretion in LNCaP cells.

LNCaP cells were treated with agonistic anti CD40 mAb G28-5 at 10µg/mL cross-linked with anti-mouse IgG at 5µg/mL for the indicated time points. EJ/3T3 co-cultures at 24 h served as a +ve control (CON). After 1.5, 3, 6, 12, 24 and 36h, culture supernatants were collected and secretion of IL-6, -8 and GM-CSF was quantified using cytokine specific ELISA on a FLUOstar OPTIMA plate reader. Supernatants were also collected from cultures of PCa cells (LNCaP) alone ('LNCaP only'). Data are represented as mean values ±SEM of cytokine concentration (pg/mL) for 2 replicates.

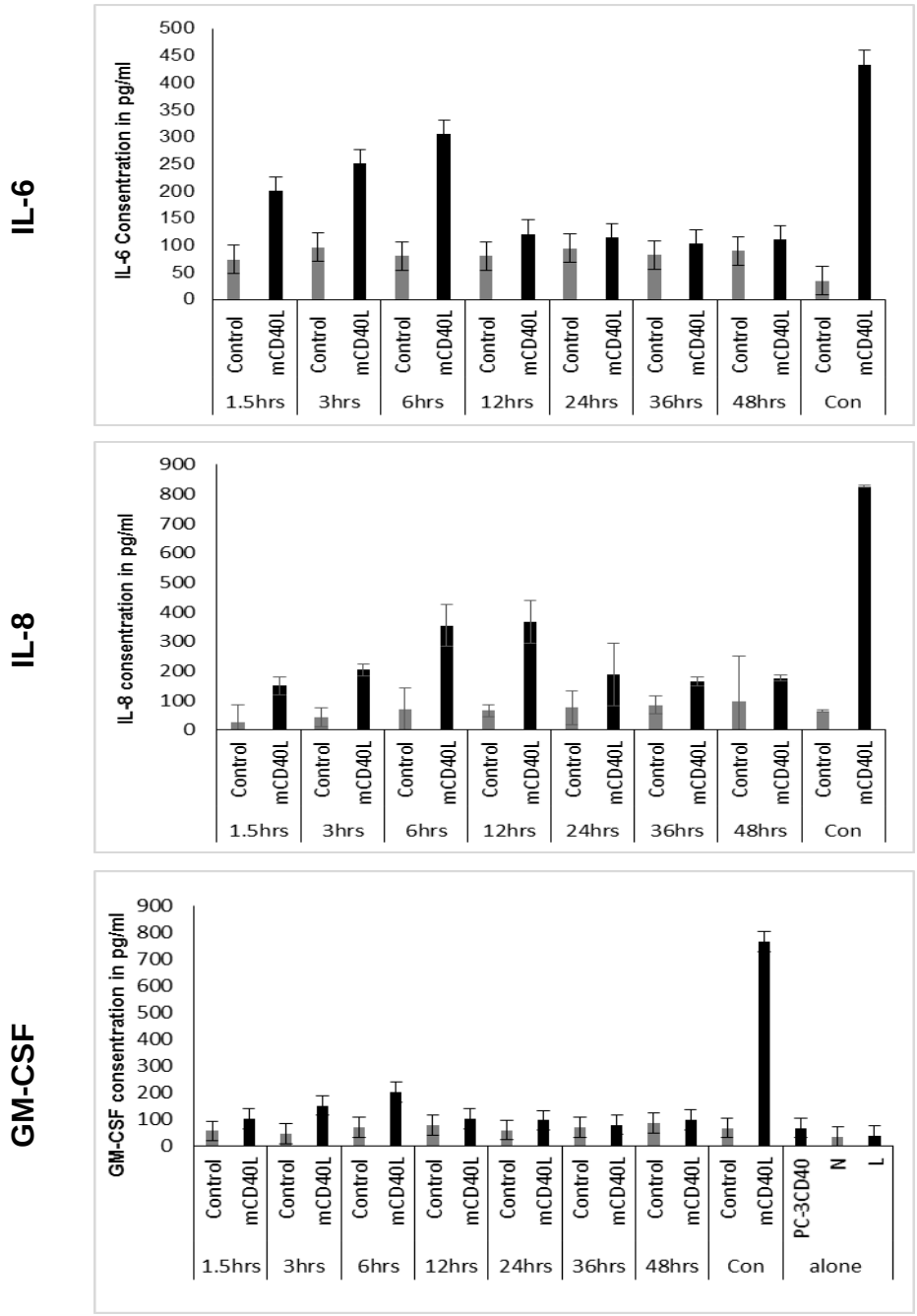


Figure 5.5: mCD40L-mediated cytokine secretion in PC-3-CD40 cells.

PC-3CD40 cells were co-cultured with MMC-treated 3T3CD40L (mCD40L) or 3T3Neo (Control) cells for the indicated time points. EJ/3T3 co-cultures at 24 h served as a +ve control (denoted as Con/CON). After 1.5, 3, 6, 12, 24, 36 and 48h, culture supernatants were collected and secretion of IL-6, -8 and GM-CSF was quantified using cytokine specific ELISA on a FLUOstar OPTIMA plate reader. Supernatants were also collected from cultures of LNCaP as well as 3T3CD40L and 3T3Neo cells (denoted PC-3CD40, N and L alone, respectively) at 24 h. Data are represented as mean values ±SEM of cytokine concentration (pg/mL) for 2 replicates.

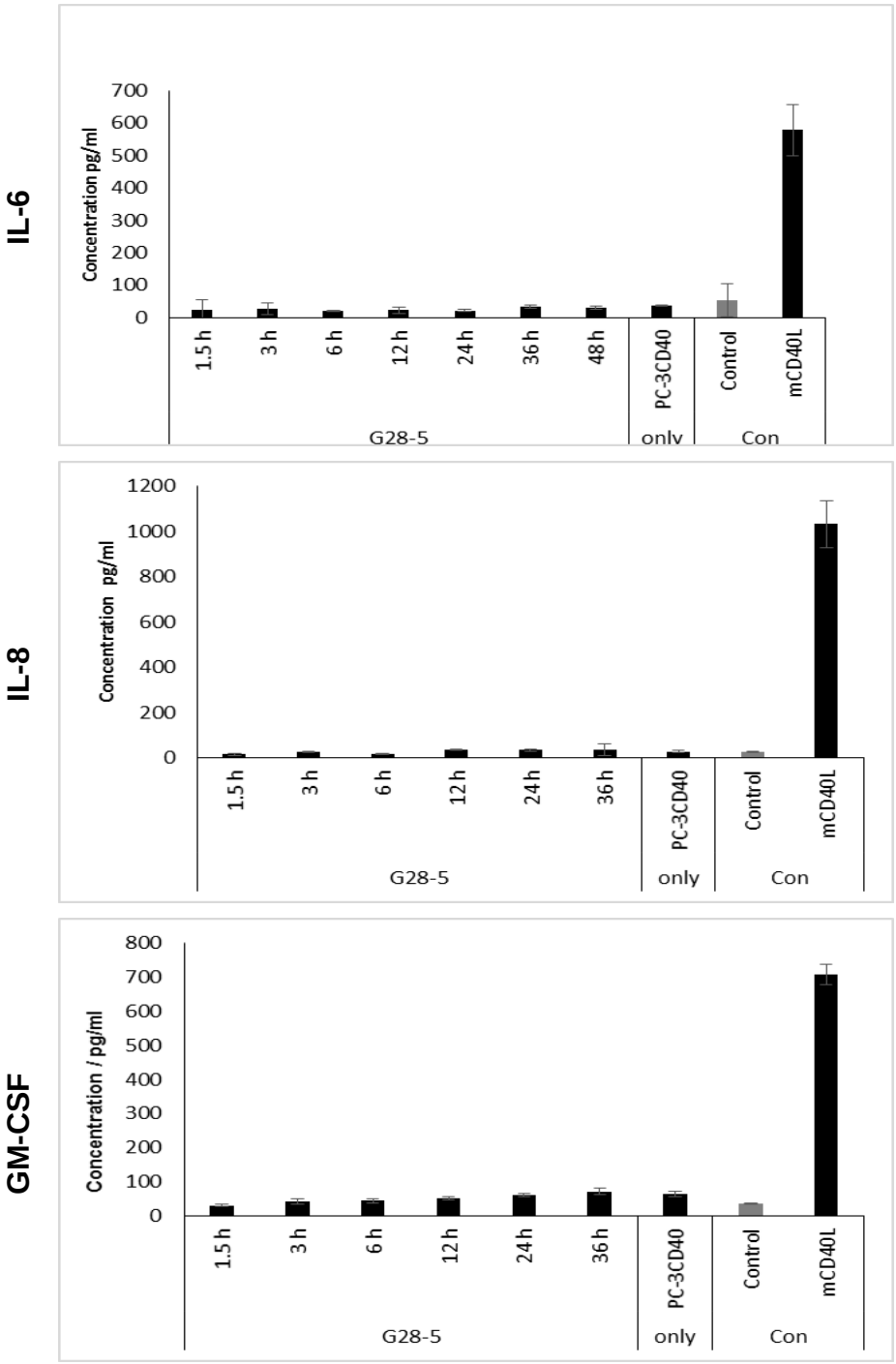


Figure 5.6 :G28-5 mAb-mediated cytokine secretion in PC-3CD40 cells.

PC-3CD40 cells were treated with agonistic anti CD40 mAb G28-5 at 10µg/mL cross-linked with anti-mouse IgG at 5µg/mL for the indicated time points. EJ/3T3 co-cultures at 24 h served as a +ve control (CON). After 1.5, 3, 6, 12, 24 and 36h, culture supernatants were collected and secretion of IL-6, -8 and GM-CSF was quantified using cytokine specific ELISA on a FLUOstar OPTIMA plate reader. Supernatants were also collected from cultures of PCa cells (PC-3CD40) alone ('PC-3CD40 only'). Data are represented as mean values ±SEM of cytokine concentration (pg/mL) for 2 replicates.

Summary

- When ligation of CD40 was carried out by mCD40L in DU145 cells, rapid and sustained induction of IL-6 and IL-8 secretion was observed. Interestingly, marked induction of GM-CSF was also detected, albeit at later time points. By contrast, soluble agonist in the form of agonistic mAb G28-5 caused no induction of any of the cytokines tested, even though the antibody was highly cross-linked.
- Similar experiments in LNCaP cells showed dramatic induction of both IL-6 and IL-8 secretion by mCD40L but not soluble agonist which was more marked than that observed in DU145 cells. However, in contrast to DU145, mCD40L did not induce GM-CSF secretion in the LNCaP cell line.
- Finally, the ability of CD40 to induce cytokine secretion was tested in the transduced PC-3 cells. As de novo expression of CD40 in these cells (PC-3-CD40) could induce cell death, these investigations aimed at testing whether death was accompanied by pro-inflammatory cytokine secretion. mCD40L, but not soluble agonist, induced rapid secretion of IL-6, IL-8 and notably also GM-CSF (though to a lesser extent) in PC-3-CD40 cells. Interestingly however, this secretion was rapid and by 12 and particularly 24 h, the secretion of these cytokines had subsided these observations were in line with the results with the UCC line EJ used alongside as a positive control, which exhibited a high degree of pro-inflammatory cytokine secretion including IL-6, IL-8 and GM-CSF.
- Agonistic anti-CD40 mAb (G28-5), unlike mCD40L, did not cause any pro-inflammatory cytokine secretion.
- These observations not only provide evidence for the biological effects of CD40 ligation in PCa cells but also constitute further confirmation for the importance of the quality of CD40 signal in determining functional outcome in carcinoma cells.

Chapter 6: **General Discussion**

6.1. Thesis background

Based on previous studies the quality of the CD40 signal is essential in the functional outcome of CD40 ligation. Several studies in our laboratory demonstrated that CD40 ligation by cell-surface presented agonists and in particular membrane CD40L (mCD40L) caused extensive apoptosis specifically in carcinoma cells (Bugajska et al., 2002; Georgopoulos et al., 2006; Georgopoulos et al., 2007; Hill et al., 2008). By contrast, a number of studies have shown that soluble CD40 agonists are at best weakly pro-apoptotic and only become significantly pro-apoptotic by pharmacological intervention (Afford et al., 2001; Bugajska et al., 2002; Choudhury et al., 2003; Eliopoulos et al., 2000; Hess and Engelmann, 1996). In addition, more recent work from our laboratory has shed light into the tumour specificity of CD40 as well as providing a better understanding of the differences in soluble versus membrane-presented agonists in terms of their pro-apoptotic capacity. Moreover, this work showed that although soluble agonist alone could not induce death, combinatorial treatment incorporating soluble CD40 agonist and pharmacological inhibition of Trx-1 was functionally equivalent to the signal triggered by mCD40L (Dunnill et al., 2016).

Although mCD40L, but not soluble agonist, can trigger a strong pro-apoptotic signal in several carcinoma cell types, including urothelial and colorectal (Dunnill et al., 2016; Georgopoulos et al., 2006; Georgopoulos et al., 2007; Hill et al., 2008), the role of CD40 on prosate cancer cells remain unknown. Consequently, this work represents the first systematic study that aimed to address the hypothesis that if CD40 is present in PCa cells, it is capable of inducing a potent pro-apoptotic signal. In order to achieve CD40 ligation by membrane ligand (mCD40L), a co-culture *in vitro* model was used, whereby target PCa cells were co-cultured with third-party (murine fibroblasts 3T3CD40L (engineered to express mCD40L) and their control counterparts 3T3Neo.

6.2. Establishment of the co-culture system of PCa and effector cells

6.2.1. CD40L expression on murine fibroblast cells

The main cognate CD40 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, activated dendritic cells, natural killer (NK) cells, activated platelets, mast cells, endothelial cells, smooth muscle cells and in some very rare cases on epithelial cells (van Kooten and Banchereau, 2000).

In order to perform co-cultures as described in the Methods (section 2.8), it was essential to verify the CD40L protein expression on murine fibroblast effector (3T3CD40L and 3T3Neo) cells. In order to deliver the signal NIH3T3 fibroblasts were used that had been previously stably transfected with expression plasmids bearing the sequences coding for CD40L and neomycin resistance gene (3T3-CD40L cells); controls transfected with empty plasmid coding for neomycin resistance alone (3T3-Neo cells) were also employed throughout this study. Cells were initially screened by flow cytometry to confirm CD40L expression on cell surface (Figure 3.2), as reported elsewhere (Bugajska et al., 2002).

6.2.2. Confirmation of CD40 expression, its regulation by pro-inflammatory cytokines on target PCa cells and the effect of CD40 ligation

CD40 is known to be expressed widely on immune system cells, such as B cells, dendritic cells, basophils, eosinophils and monocytes. Furthermore, CD40 can be detected on endothelial cells, keratinocytes, smooth muscle cells, epithelial cells and fibroblasts (van Kooten and Banchereau, 2000). In addition, it has been demonstrated in a variety of cells of non-haematopoietic origin, including bladder cancer cells, where the CD40 was originally identified, as well as in ovarian, breast, nasopharynx, liver, cervical, colorectal (Dunnill et al., 2016; Georgopoulos et al., 2007) as well as in renal and also prostate carcinoma cells (Palmer et al., 2004; Rokhlin et al., 1997).

This study examined the expression of CD40 in prostate cell lines in metastasis stages, in particular DU145, LNCaP and PC-3 cells, and provided evidence that all the tested cells expressed CD40 with the exception of PC-3 cells. Our observations are in agreement with previous studies (Palmer et al., 2004; Rokhlin et al., 1997) which reported that DU145 cell lines expressed high levels of CD40, but no CD40 was detected on PC-3 cells, thus these observations were identical with our results.

These observations appear to be in contrast with a previous study, which demonstrated that CD40 was not detected on PCa cell lines (Moghaddami et al., 2001). Interestingly this mainly immunohistochemistry based study demonstrated that the pattern of CD40 expression was continuous on basal epithelial cells of normal and hyperplastic prostate glands, and also expression of CD40 was detectable in glands that featured prostatic intraepithelial neoplasia, however this expression was discontinuous. In contrast, glandular epithelial cells in prostate adenocarcinoma did not express CD40. The present study is partly in agreement with the above report, in that some expression of the CD40 was reported in neoplastic tissue. Of note, Moghaddami *et al* did not assess any metastatic tissue CD40 expression, and CD40 expression in our study was demonstrated in the metastatic cell lines tested. Therefore, at later stages of malignant transformation, the expression of CD40 may increase in PCa tissues.

This study demonstrated for the first time that ligation of CD40 on CD40-postive PCa lines caused apoptosis in these cells, as seen in both DU145 cells and to a lesser extent in LNCaP cells. By contrast, CD40-ve cells PC-3 were refractory to CD40 ligation. Equally importantly, only receptor ligation by mCD40L, and not soluble agonist, could cause cell death, as soluble agonist (cross-linked G28-5 mAb) was not pro-apoptotic. This will be discussed in detail in subsequent sections.

As demonstrated in Chapter 3, the confirmation of CD40 expression on PCa cells allowed investigations on the effects of pro-inflammatory cytokines (IFN-γ and TNF-α) on the regulation of CD40 expression. The rationale for this part of the work was that it was of interest to determine whether a pro-inflammatory cytokine milieu could regulate CD40 receptor expression then eventually enhancing the cell death when the receptor is activated.

The pro-inflammatory cytokine IFN-γ is classified as a growth inhibitor as well as a growth factor for many types of cells. On one hand, IFN-γ can induce activation of signal transducer activator of transcription 1 (Stat1) and interferon regulatory factor-1 (IRF-1). Stat1 represents a transcription factor, which plays an essential role in cell differentiation, cell cycle and cell death (Asao and Fu, 2000). There is evidence that interaction of IRF-1 with an interferon stimulated response element (ISRE) which Acquired IRF-1 plays crucial roles in regulating of target gene expression (Asao and Fu, 2000). Furthermore, it has been demonstrated that upon the activation of both

Stat1 and IRF-1 expression resulted in cell proliferation inhibition which indicated that IFN-γ acts as a growth inhibitor (Chin et al., 1997; Sato et al., 1998). On the other hand, when cells are lost Stat1 expression, IFN-γ may regulate cell survival pathway (Bromberg et al., 1996). Bugajska *et al* demonstrated that cells are treated with IFN-γ, they became capable to regulate a variety of co-stimulator proteins expression such as CD40 both on immune cells and normal as well as malignant epithelial cells (Bugajska et al., 2002). As shown in Chapter 3 (Figure 3.8), treatment with IFN-γ overall led to an increase in CD40 expression in CD40-positive PCa cells compared with controls cells, however this did not cause de novo CD40 expression in CD40-ve cells, and this is supported by previous studies by our group (Bugajska et al., 2002; Georgopoulos et al., 2007).

The second pro-inflammatory cytokine used in such experiments was TNF-α to determine any effects on CD40 expression. TNF-α is a member of the TNFSF and it can bind two receptors: death receptor (TNFRI) and non-death receptor (TNFRII) (Cabal-Hierro and Lazo, 2012). Furthermore, it has various biological effects on different cell signalling types, whether these effects are cell survival or cell death (Giroir et al., 1992; Hernandez-Caselles and Stutman, 1993). *In vitro*, not only killing human tumour cells specificelly, TNF-α has growth either inhibitory or cytotoxic effects at the same time in normal cell types and human tumour cells. furthermore, TNF-α can medaited cell death in the persence of protein synthesis such as CHX. (Meager, 1991; Porter, 1990; Ruggiero et al., 1987). Thus necrotic and apoptotic cell death are triggered resulted in TNF-α can induction on mouse fibroblasts *in vitro* and *in vivo* (Kamata et al., 2005). Moreover, it can cause a characteristic type of death known as necroptosis under particular circumstances (Vandenabeele et al., 2010). Interestingly also, TNF-α Induces a range of biological responses associated with apoptosis/survival in both normal epithelial and prostate cancer cells (Chopra et al., 2004). Consistent with this and as shown in Chapter 3 (Figure 3.8), TNF-α showed less effect on CD40 positive PCa cells (DU145) compared with IFN-γ and TNF-α treatment promoted cell growth or had no effects on CD40 negative PC-3 cells, however, LNCaP cells underwent rapid apoptosis as reported previously (Chopra et al., 2004).

Of note, cytokine pre-treatment of PCa cells, although in most cases increased receptor expression, particularly in DU145 cells, overall it is did not statistically significantly increase the level of cells induced by CD40 ligation. Therefore, it appears

that the level of receptor expression already present on PCa cells is adequate to achieve a clear and significant functional outcome.

6.3. CSCs expression of CD40 protein

Several studies have previously demonstrated that tumours in vivo originate from tumour-initiating cells referred to as cancer stem cells (CSCs) and such cells can be identified and characterised based on expression of specific markers (Agliano et al., 2017; Bekaii-Saab and El-Rayes, 2017; Dragu et al., 2015). CSCs are central in the tumour organisation, they are critical in driving tumour development and are able to migrate from the primary tumour through bloodstream. Particularly, in prostate cancer only quite a small population from these cells (rare cancer stem cells) are able to initiate a secondary tumour, by invade surrounding tissues and cause metastatic disease (Agliano et al., 2017). Furthermore, CSCs are considered as one of the most likely reasons for the inadequacy or ineffectiveness (leading to recurrence and metastasis) of conventional therapies, including chemotherapy or radiation therapy and may be responsible for deterioration after treatment due to increasing DNA repair capacity and overexpression of drug efflux pumps. Thus, all these challenges have made the targeting and elimination of cancer diseases are complicated task. However, the ability to identify and isolate CSCs may facilitate the study of these cells which could have profound therapeutic implications for all of the various types of cancer including prostate (Agliano et al., 2017; Bekaii-Saab and El-Rayes, 2017; Dragu et al., 2015). Prostatic CSCs demonstrate the characteristic expression of the CD133 marker, which allows their isolation and characterisation, as demonstrated by the work of Dr Anne Collins and colleagues at the University of York (Maitland et al., 2011).

Therefore, one part of the present study aimed to provide preliminary data on the expression of CD40 on prostate CSCs as well as assessing whether these cells may be susceptible to mCD40L-mediated cell death. As targeting CSCs is a rational approach to preventing tumor regrowth and the development of resistance, this part of this study would test the possibility of CD40-based targeting of such CSCs and thus may have significant implications for cancer therapy.

Preliminary experiments on independent tumour derived cells lines provided for the first time evidence that similarly to other PCa cell lines, CSC cells were CD40+ve. These results were demonstrated for three independent specimens, including normal cells as well as cells from a metastasis stage, as shown by western blot analysis and flow cytometry (Figure 3.6, 3.7.). However, difficulties were encountered in expanding

these cell cultures further and in assessing CSC cell's susceptibility to CD40 ligation; this would involve co-culture experiments with 3T3 effector cell derivatives and posed several challenges. On one hand, CSCs are primary and undifferentiated cells cultured in specific serum-free medium however, effector 3T3CD40L and 3T3Neo cells require serum-containing medium. Whilst trying to address these issues experimentally and optimise conditions for these experiments, CSC cells did not withstand adequate passages in culture due to their relative low numbers and eventually demonstrated features of stress resulting ultimately to loss of replicative capacity and demonstrated signs of replicative senescence. Therefore, within the time constraints of this project, it was not possible to assess the possibility that CD40 ligation may provide the potential of CSC-targeted CD40-mediated cell death.

6.4. Engineered CD40 expression converts non-susceptible carcinoma cells (PC-3) to susceptible to CD40-induced apoptosis

It is possible to utilise the retrovirus infection (transduction) method to incorporate a gene of interest stably within a population of rapidly dividing cells. It has previously been demonstrated that the transgene can be retained in UCC cells (Bugajska et al., 2002) and in our laboratory; it has been shown that de novo CD40 expression renders malignant cells susceptible to death (Georgopoulos et al., 2006). Therefore, this project examined whether restoration of CD40 expression could restore apoptotic susceptibility, and we engineered receptor expression using a retroviral vector. Transduced PC-3 (PC-3-CD40) cells expressed high levels of CD40 and were susceptible to CD40-mediated apoptosis, whereas control (PC-3-neo) cells remained refractory.

6.5. Insights into the activation of CD40 utilising various agonist formats

The outcome of death receptors and non-classical death receptors (TNFR members) in the regulation of cell fate including of tumour growth proliferation/differentiation or induce cell death (apoptosis) has been under extensive research for decades, in addition, the quality of signal and on tumour types well as the cellular context, are play a crucial role in these member's outcome as investigated by the nature of the agonist used as reviewed recently (Albarbar et al., 2015). Moreover, in one of non-classical death receptors, CD40, previous studies demonstrated that CD40 interaction with

CD40 agonists may regulate tumour cell growth (Eliopoulos and Young, 2004; Tong and Stone, 2003), nevertheless, Georgopoulos and others demonstrated that the quality' of the CD40 signal is a key for functional outcome of CD40L/CD40 in carcinoma cells (Dunnill et al., 2016; Georgopoulos et al., 2006).

6.5.1. Soluble CD40 agonists are not growth inhibitory or pro-apoptotic in PCa cells

A number of studies have demonstrated that soluble CD40 agonists (soluble form of recombinant CD40L or agonistic anti-CD40 antibodies) might exhibit growth inhibition effects on cancer cells, yet often this is little or very modest. In particular, this has been reported for carcinoma cells of various origins including ovarian, breast, bladder (urothelial) and colorectal tumour cells *in vitro* (Bugajska et al., 2002; Georgopoulos et al., 2006; Jiang et al., 2007; Vonderheide et al., 2007). Moreover, only one previous report has examined the role of soluble CD40 agonist in in prostate cancer cells (Rokhlin et al., 1997). These agonists only have the ability to inhibit cell growth in most cases and could not induce apoptosis alone, however they only become capable to induce apoptosis by pharmacological intervention (Afford et al., 2001; Bugajska et al., 2002; Hess and Engelmann, 1996). A more recent study from our laboratory provided a novel approach, demonstrating that combinatorial treatment using soluble CD40 agonist and Trx-1 inhibitor provides a death signal that is functionally equivalent to the signal triggered by mCD40L (Dunnill et al., 2016).

It was, therefore, important to determine if similar effects could be triggered via soluble CD40 agonist on the panel of PCa cells, as shown in Chapter 3 (Figure 3.23) The findings from this study revealed that soluble CD40 agonist is incapable to trigger CD40-mediated PCa cell killing as shown by assays for measuring cell death (CytoTox-Glo). Furthermore, this study demonstrated that treatment with soluble CD40 agonist did result in any growth inhibition, and this is in contrast with previous studies demonstrated that soluble CD40 agonist (agonistic anti-CD40 antibody) can exhibits growth inhibition affect in particular on PCa cells (Rokhlin et al., 1997). Despite systematic attempts to do so, such as using different cell densities, this study could not reproduce the above previously published data, suggesting that the signals of soluble CD40 agonists are weak, as they may not trigger adequate receptor cross-linking and thus cell death.

6.5.2. mCD40L is a potent pro-apoptotic signal and is associated with pro-inflammatory cytokine secretion in PCa cells

The findings discussed above strongly indicated that CD40 soluble agonists are unable either to induce cell death or even to inhibit growth. It has been suggested that the signals of CD40 soluble agonists are weak, as they may not induce efficient receptor cross-linking , in comparison to a membrane presented signal in the form of membrane-bound mCD40L (or by 'forcing' agonistic antibody presentation to the cell surface) to trigger activation of CD40 receptor (Bugajska et al., 2002; Georgopoulos et al., 2006; Vonderheide et al., 2007). This is not idiosyncratic to CD40, as it appears that one fundamental property of the TNFR family signalling in general is related to "signal quality" (i.e. the degree of receptor cross-linking), which determines the outcome of receptor ligation, as reviewed recently (Albarbar et al., 2015).

In this context, this study demonstrated for the first time that the membrane signal in the form of membrane-bound mCD40L can trigger efficient and pro-apoptotic activation of CD40 in malignant prostate cancer cell lines. Previously, several studies have demonstrated that CD40 ligation by mCD40L caused extensive cell death in urothelial (UCC), colorectal (CRC) and ovarian carcinoma cells whilst normal cells were refractory (Bugajska et al., 2002; Dunnill et al., 2016; Elmetwali et al., 2016; Elmetwali et al., 2010b; Georgopoulos et al., 2006; Georgopoulos et al., 2007; Hill et al., 2008). This study showed for the first time that mCD40L-CD40 interactions induced extensive apoptotic cell death on malignant (DU145 and LNCaP) cells. Furethermore, *de novo* CD40 expression (PC-3-CD40 cells) rendered malignant cells susceptible to death. Overall, these findings are in agrrement with previous studies demonstrating that mCD40L-CD40 interactions induced death in cells of different origins (Dunnill et al., 2016; Elmetwali et al., 2016; Georgopoulos et al., 2006; Georgopoulos et al., 2007). Of note, because of the lack of pro-apoptotic potential by soluble agonist, this study focused on mCD40L in order to identify intracellular components involved in the death signalling pathway and did not examine any effects by soluble agonist.

Despite the inability of soluble agonist to induce cell death. it was of interest to compare mCD40L and soluble G28-5 mAb), to provide further evidence as to whether CD40 ligation can induce cytokine secretion in PCa cells and compare the effects of apoptotic and non apoptotic ligation; this would further address whether the 'quality' of the CD40 signal is important in the functional outcome of CD40 ligation. It has been reported that CD40-mediated growth-inhibition or death in carcinoma cells can be accompanied

by induction of IL-6 and/or IL-8 (Alexandroff et al., 2000; Cao et al., 2005; Eliopoulos et al., 1996; Gallagher et al., 2002) and previous work ocmparing mCD40L and soluble agonists has provided evidence for differential induction of cytokine secretion in both UCC and CRC cell lines, in particular IL-6 and GM-CSF (Georgopoulos et al., 2007). In addition, recent study in our laboratory on another member of the non classical death, Lymphotoxin receptors LTβR and HVEM (with the former being related structurally and functionally with CD40) have demonstrated that membrane-presented LIGHT-mediated cell killing was accompanied by induction of pro-inflammatory cytokines such as IL-6, IL-8 and GM-CSF in CRC and UCC cells (Albarbar and Georgopoulos, manuscript in preparation).

Partially in line with these previous observations, this study showed for the first time that mCD40L, but not soluble agonist (G28-5), induced rapid cytokine secretion. In particular, induction of pro-inflammatory cytokines IL-6, IL-8 and GM-CSF was observed, as shown in chapter 5 (Figures 5.1-6). Our observations demonstrate that CD40/mCD40L can directly modulate cytokine production in PCa cells, as it induced secretion of IL-8, a potent neutrophil chemoattractant that sustains neutrophil recruitment and activation (Martinez et al., 2004) as well as lymphocytes in a variety of inflammatory diseases (Belperio et al., 2000). However, agonistic anti-CD40 antibody (G28-5) appeared not to be efficient at mediating cytokine release, and results showed that none of these cytokines (IL-6, IL-8 and GM-CSF) was secreted in response. More importantly, these findings strengthened the evidence supporting the notion that the 'nature' of the CD40 signal determines the level of the cytokines secreted by PCa cells, as mCD40L specifically also mediates GM-CSF secretion. GM-CSF is of particular interest as it is a pleiotropic cytokine, which is crucial in the generation of successful immune responses. In addition, critically, previous studies have demonstrated that tumour cell-released GM-CSF *in vivo* facilitates recruitment of mononuclear cells and macrophages as well as dendritic cells, and enhances contact-dependent macrophage-mediated to engulfment of cells (tumour cells) (Kielian et al., 1999; Shinohara et al., 2000). The study also observed IL-6 cytokine secretion following CD40 activation; IL-6 is also a pleiotropic cytokine that plays critical roles in the acute phase reaction, inflammation, haematopoiesis and bone metabolism (Mansell and Jenkins, 2013). Overall, these observations are in agreement with previous work demonstrating that the activation CD40 by mCD40L caused IL-8 and GM-CSF secretion in some carcinoma cell lines of CRC and UCC cells (Georgopoulos et al.,

2007). However, it is noted that in such previous studies, even soluble agonist was capable of inducing some, albeit far more modest, IL-8 cytokine secretion. Therefore, it is possible that the lack of any effect by the G28-5 agonist will indicate a specific requirement for mCD40L to induce adequate cytokine secretion in PCa cells.

In overall, data discussed above showed that PCa cell lines are undergoes highly apoptotic cell death mediated by CD40 ligation (CD40L/CD40) , and this accompanied by induction of proinflammatory cytokines secretion. Therefore, the presented data may lead to target approach for PCa treatment.

6.6. CD40-mediated apoptosis occurs in a caspase-independent fashion and does not involve induction of death ligands

A defined number of major signaling pathways, but mainly the extrinsic and intrinsic signalling axes, in mammalian cells can lead to apoptosis which in most cases involves caspase activation (Elmore, 2007). However in some cases these (and particularly the intrinsic pathway) may also operate via caspase-independent mechanisms (Orrenius et al., 2015), as described above (see Introduction chapter). Georgopoulos et al (2006) have 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); moreover recent work in our laboratory has provided evidence that upon the activation of CD40 cross-talk may occur between extrinsic and intrinsic apoptotic pathways by observing CD40-mediated induction of TRAIL-mediated, caspase-10 in colorectal cancer cells (manuscript in preparation). Recently more support that CD40 mediated apoptosis in carcinoma cells is caspase dependent has also been published (Dunnill et al., 2016) which utilised fluorimetric assays.

This study has provided for the first time evidence that the mechanism of CD40-mediated death in PCa does not require caspase activity unlike the mechanism of CD40-mediated apoptosis in malignant urothelial (UCC) (Dunnill et al., 2016) and colorectal (CRC) cells (Mohamed et al, manuscript in preparation), as well as very recently in renal cancer cells (Ibraheem and Georgopoulos, unpublished observations). This study has shown that no caspase activitiy was observed upon mCD40L-mediated CD40 ligation triggered cell death. Although sensitive caspase-specific assays were used for detection of caspase activity as previously (Dunnill et al., 2016), no significant results were observed (as. shown in chapter 3 Figures 3.25 and

26). These observations were enhanced by the findings that CD40-mediated death was not abrogated by the pan-caspase inhibitor z-VAD. Not only this, similar result observed in another independent experiment with a different and 10x more efficient general biochemical caspase inhibitor (CAS-BIND™ PRO). In summary, CD40 triggers a cell death type that is via caspase-independent mechanisms in PCa cells (Figure 3.27).

Nevertheless, in this study it is not understood, how the nature of caspases independent cell death mechanistic occurs or identifying the molecules are responsible for the DNA fragmentation in absence of caspases activity as showed above. It was on this hypothesis that the release of different mitochondrial apoptogenic factors may occur and facilitate to large scale of DNA fragmentation as demonstrated in previous studies (Kroemer and Martin, 2005; Norberg et al., 2010; Wang et al., 2012). Furthermore, it was demonstrated that the caspases independent factors such as apoptosis inducer factor (AIF) and endonuclease G (Endo G), and when released to cytosol, they are capable to translocate into nucleus and played a major role in execution phase, subsequently, caused DNA fragmentation, similar to caspases 3/7 function, in case of apoptosis dependent caspases (Norberg et al., 2010; Wang et al., 2012). Furthermore, to achieve this experimentally and carry out an in depth investigation in future, it is suggested that cell fractionation technic would utilised in order to isolate unwanted fractions of cell contains from various cellular compartments, more specifically, to compare the existence of both AIF and Endo G in cytosol and mitochondrial fragments, then the isolated fractionation approach will be followed by lysis and by using specific phosphorylated antibody for AIF and Endo G the western blot technique will be involved. For more confirmation, functional experiments assay required, by using specific inhibitor for these factors followed by DNA fragmentation assay to determine their effects during CD40-mediated apoptosis.

Having shown that CD40 induced death and this was via a caspase-independent mechanism, in DU145, LNCaP and PC-3-CD40 cells. This study also found that CD40 activation caused significant levels in DNA fragmentation in these cells (Figure 3.28); this indicated that death was apoptotic, as DNA fragmentation is one of the hallmarks of apoptotic cell death (although relatively less DNA fragmentation was detected in LNCaP cells compared with DU145 and controls). Interestingly, however, DNA fragmentation was induced in the presence of caspase inhibitor CAS-BIND™ PRO

(Figure 3.29). These findings clearly provided further evidence that CD40 mediated death displays apoptotic features yet does not involve caspase activity.

In this study, the possible involvement of the classical death TNFSF members TRAIL and Fas were investigated follwing CD40 activation in PCa cells. It has been demonstrated that CD40-mediated apoptosis in hepatocytes requires induction of the Fas pathway (Afford et al., 1999; Afford et al., 2001). Furthermore, it was previously suggested that CD40 killing may involve up-regulation of death receptors and ligands, specifically FasL and TRAIL (Eliopoulos et al., 2000). TRAIL and FasL belong to the classical death TNF members and induce apoptosis via Fas (CD95), and DR4 (TRAIL-R1) and/or DR5 (TRAIL-R2), respectively (Steele et al., 2006). Furthermore, recent studies in our laboratory demonstrated that mCD40L-mediated CD40 ligation triggered rapid TRAIL induction (but not FasL upregulation) in CRC cells (Mohamed et al, manuscript in preparation). However, this was not the case in CD40-mediated apoptosis in UCC cells and cell death was triggered directly without cross-talk with death receptor pathways (Georgopoulos et al., 2006). In support of these last findings above, results in the present study showed that mCD40L-induced cell death may also occur via a direct mechanism. Immunoblotting data showed that FasL and TRAIL were not up regulated following CD40 activation (chapter 4). This therefore argues against autotropic/paratropic killing by induction of death receptors/ ligands. In other words, CD40 in PCa cells triggers death that does not involve cross-talk with the extrinsic apoptotic pathway, and are more in line with observations demonstrating that mCD40L-induced cell death in UCC cells occurs via a direct mechanism and did not involve death receptors/ligands (Georgopoulos et al., 2006). Yet unlike these reports, CD40-mediated killing in PCa cells is not caspase-dependent, thus suggesting that CD40-mediated cell death in PCa is unique and adds to the incredible variety exhibited by the CD40 receptor in the precise mechanisms via which it mediated cell death in carcinoma cells of different origins.

6.7. Differential Regulation role of TRAF adaptor proteins upon CD40 activation

The present work has identified for the first time that CD40-mediated signalling in PCa cells engages TNFR-associated factors (TRAFs), and this subsequently led to a downstream signalling cascade through mitochondrial intrinsic pathways (this will be discussed in the next section).

Despite the lack of instrinsic catalytic activity, CD40's cytoplasmic tail transduces signals into cells initiated by the binding of TRAF family of adaptor proteins (TRAF-1, -2, -3, -4, -5, -6) (Aggarwal, 2000), as described above (Introduction). Previous studies have demonstrated rapid recruitment of TRAFs from the cytoplasm to the receptor's cytoplasmic domain following CD40L-CD40 interactions. Some of the TRAFs, such as members TRAF1, TRAF2, TRAF5, and TRAF6 tend to act as positive regulators of CD40L-CD40 signalling, while TRAF3 has been implicated as a negative regulator (Hauer et al., 2005). However, almost all such studies have involved investigations on CD40-mediated effects in lymphoid cells (Bishop, 2004; Bishop et al., 2002; Bishop et al., 2007). Nevertheless, studies that have been carried out in our laboratory have specifically demonstrated TRAF regulation by CD40 in normal and malignant epithelial cells (UCC and CRC cells) (Dunnill et al., 2016; Georgopoulos et al., 2006) (Mohamed et al, manuscript in preparation).

As shown in Chapter 4 (Figures 4.3, 4.4), this study found for the first time that TRAF1, 2, and 3 were induced by mCD40L-CD40 interactions in PCa cells. Our findings demonstrated that the regulation of TRAF1 and TRAF3 by mCD40L was rapid as TRAF induction was observed as early as 1.5 h post receptor ligation. In addition, it appeared that TRAF2 expression was downregulated early (1.5 and 3h) and returned back to normal levels later on.

TRAF3 is a CD40-associated adaptor protein that allows the receptor to transmit signals for MAPKs induction, and often acts as an inhibitor of NF-κB activation pathway; such signals may result in multifaceted cell responses varying from epithelial cell death to cell growth and survival (Häcker et al., 2011). It has been reported that TRAF3 activation is associated with cell death in HT29 cells and in human embryonic kidney (HEK293T) cells (Force et al., 1997) when the lymphotoxin-β receptor was studied, a receptor that is closly related to CD40 (Sanjo et al., 2010; VanArsdale et al., 1997). Our studies demonstrated a novel pattern of rapid TRAF3 up-regulation in PCa cell lines; furthermore, it was shown that mCD40L-CD40 interactions caused TRAF3 induction in PCa cell lines that naturally express CD40 as well as in cells engineered to express the receptor. Although no functional blocking experiments were performed (i.e. no expression of dominant negative TRAF3 mutant, or RNAi-mediated TRAF3 knock-down), these findings are suggestive of a role for TRAF3 in CD40-mediated apoptosis. These observations are supported by previous studies demonstrating that

TRAF3 activation has an essential role in CD40-mediated CRC and UCC cells death as shown by RNAi-mediated stable TRAF3 knockdown experiments (Dunnill et al., 2016)(Mohamed et al, manuscript in preparation). Soluble CD40 agonists do not stabilise TRAF3 as effectively as does membrane agonist, further supporting the thought that CD40 cross-linking is a key factor for high TRAF3 expression (Elmetwali et al., 2010a; Georgopoulos et al., 2006). Although the molecular weight of TRAF3 is 65 kDa full length, interestingly it was detected at approximate MW 50 kDa, however, previous studies have been reported that TRAF3 protein has several splice variantes that can produce different isoforms (Gamper et al., 2001; Van Eyndhoven et al., 1999). Overall, the observations on TRAF3 regulation are in agreement with studies in CRC cells (Mohamed et al, manuscript in preparation), as well as recent studies on the lymphotoxin receptors (Albarbar and Georgopoulos, unpublished observations) and in UCC cells (Dunnill et al., 2016). This is not only a novel observation in the context of PCa cells, but it also adds to the similarities of CD40 signalling with the lymphotoxin receptor (LT-βR and HVEM) receptor system.

Data in this study demonstrated that alongside with TRAF3, TRAF1 was expressed which may responsible for down regulation of TRAF2 as previously demonstrated(Henkler et al., 2003; Leo et al., 2001), these an observation in agreement with previous study demonstrated that TRAF1 was up regulated while, TRAF2 which was degraded, which also suggested that as a result of downstream signalling triggered that caused an extensive cell death, TRAF3 and TRAF2 may be competing and resulted in TRAF2 degraded whereas,TRAF3 is upregulated (Georgopoulos et al., 2006). It has been previously described that TRAF2 is associated with survival pathway which occurs through proliferation and NF-kB activation, while TRAF1 is related with pro-apoptosis pathway (Henkler et al., 2003). furthermore, consistent with this observations, it was showed that TRAF2 was degraded upon CD40 activation on B cell lines .(Bishop et al., 2002). In this study also, it appeared that TRAF5 and 6 were not detectable and may not be as important as TRAF1 and 3 in cell death (chapter 4 Figure 4.5).

6.8. The roles of MAPKs in pro-apoptotic CD40 signalling in PCa cells

The ‘stress’-associated intracellular signalling molecules MAPKs can modulate various biological activities including cell differentiation, cell survival and cell death, and are classified into subgroups: ERK1/2, JNK and p38 (as detailed in the Introduction and in

chapter 4) (Georgopoulos et al., 2006; Mebratu and Tesfaigzi, 2009). The first identification of the downstream signalling events triggered by mCD40L-CD40 interactions in carcinoma cells were carried out by Georgopoulos *et at* (Georgopoulos et al., 2006). TRAF3 and JNK/AP-1 were induced and appeared to play a role in the regulation of cell death mediated by mCD40L; these observations were later confirmed by Young and colleagues (Elmetwali et al., 2010a). More recently, it was shown that rapid (1.5h) induction of TRAF3, by relocalisation and stabilisation on CD40, initiates a signalling cascade that resulted in activation of 'stress' MAPK signaling pathways (Dunnill et al., 2016) (Mohamed et al, manuscript in preparation). Further, the MAPK signaling pathways, in particular those driven by p38 and JNK, can play crucial role in the control of cell death. C-Jun N-terminal kinase/ stress kinase (JNK) can play an important role in activating of apoptosis (He et al., 2006). Previous studies have demostrated that JNK activation is often regulated by MKK4 and/or MKK7 following activation by MAP3K ASK1 (Cargnello and Roux, 2011; Wagner and Nebreda, 2009). Knockdown experiments revealed that MKK4 was induced following CD40 ligation and it directly regulated the phosphorylation of JNK and apoptosis overall. By contrast, MKK7 did not appear to be induced by CD40 ligation (Dunnill et al., 2016).

Consistent with the role of JNK in cell death, and as part of our investigation of proximal and more cytosolic signalling components of the apoptotic pathway triggered by CD40, this study demonstrated that JNK/AP-1 was rapidly up-regulated (1.5h) in parallel with TRAF3 as demonstrated in chapter 4 (Figure 4.8). These observations suggest that TRAF3 may directly (through MKKK and MKK) influence the phosphorylation status and activation of JNK, which in turn induces AP-1 and subsequently triggers cell death. This functional significance of the activation of phospho-JNK in CD40 mediated cell death was evident by the fact that both JNK and AP-1 inhibitors blocked cell death (Figure 4.10 and 4.11). In addition, this study has found that unlike the observations in UCC cells, MKK7 but not MKK4, was gradually upregulated following CD40 ligation (Figure 4.7), suggesting that MKK7 may be responsible for JNK activation, although further experiments would be necessary to clarify its exact functional roles. These data are in agreement with the knowledge that JNK activation is often regulated by MKK4 and/or MKK7 (Cargnello and Roux, 2011; Wagner and Nebreda, 2009).

The activation of the stress kinase p38 plays a critical role in cell death (Adams et al., 2000; Cuadrado and Nebreda, 2010). Studies in our laboratory demonstrated a role for activated p38 in CD40-mediated apoptosis in CRC cells, as p38 phosphorylation

was observed and an inhibitor of p38 completely attenuated death in CRC cells (Mohammed et al, manuscript in preparation). In this study, the stress kinase p38 could not be detected, yet, an inhibitor of p38 attenuated death (Figure 4.9 and 12). Thus both MAPKs p38 and JNK appear important in apoptosis in agreement with the reported importance of both of these proteins during the induction of apoptosis (Cuadrado and Nebreda, 2010; Deacon and Blank, 1999; Dhanasekaran and Reddy, 2008{Browning, 1999 #636). Moreover, also it has been identified that MKK 7/4 and MKK 6/3, are the two MAPK kinases specific activators for JNK and p38, respectively (Deacon and Blank, 1999). This raises the possibility that MKK3 or MKK6 might be involved in CD40-signalling and future studies could address this hypothesis. Phospho-MKK7 was readily detectable and increased within 1.5 h post CD40 ligation, an observation suggesting this could be the driver of JNK activation (Wagner and Nebreda, 2009).

ASK1 often plays an essential role in apoptosis triggered by TNFR members as has been reported (Albarbar et al., 2015). In particular, in the context of the highly-related member to CD40 (structurally and functionally) LTβR has been shown that TRAF3 can interact with ASK1, MKK4/7 and JNK (Chung et al., 2002). Furthermore, in CD40 signalling in UCC cells, ASK1 has been identified as the missing link in the CD40/TRAF3 – MKK4/JNK signalling axis (Dunnill et al., 2016). Immunoblotting data in the present study has shown for the first time in PCa cells that CD40/ mCD40L ligation activated ASK1 as evidenced by specific, activatory phosphorylation at Thr845, which was observed ~3 h post ligation. (Figure 4.6). Collectively these and the above observations suggest that activation of ASK1 may lead to phosphorylation of MKK7, leading to JNK activation, and thus drive mCD40L-induced apoptosis. However, further experiments (ASK1 knockdown) are required in order to provide unequivocal evidence for exact functional roles. Should such a functional role be demonstrated, these observations would be in agreement with previous studies demonstrateing that ASK1 activation causes phosphorylation of MKK4/7 and leads to JNK activation (Dunnill et al., 2016; Ichijo et al., 1997).

6.9. ROS is not implicated in CD40-mediated cell death in PCa cells

ROS were initially thought to be just natural by-products of mitochondrial oxidative metabolism and molecules that merely caused cell damage. Yet, it is now widely recognised that they are secondary messengers able to homeostatically balance cell

proliferation/survival as well as death (Ray et al., 2012; Terada, 2006). Furthermore, previous studies reported that ROS are implicated in various hallmarks of cancer (Hanahan and Weinberg, 2011), due to their different effects on cellular molecules and their ability to regulate angiogenesis, invasion and proliferation (Paletta-Silva et al., 2013). Many members of the TNFRSF exploit ROS to control various biological activities, such as cell survival and apoptosis by activating oxidative stress-responsive MAPK signalling pathways (Shen and Pervaiz, 2006). In the context of CD40, until recently only one study had demonstrated that CD40 generates ROS via TRAF3 and NADPH oxidase (Nox) in B-cells (Ha and Lee, 2004).

The activation of ASK1 is delicately linked with the intracellular redox status (Ichijo et al., 1997), and recent work in our laboratory showed that in response to CD40 driven oxidative stress ASK1 is activated as active downregulation of Trx is observed (Dunnill et al., 2016)(Mohammed et al, manuscript in preparation). More recently, in our laboratory, it has been demonstrated that activation of LTβR generates ROS in CRC and UCC cells (Albarbar and Georgopoulos, unpublished observations). In addition, our group has demonstrated that CD40-mediated apoptosis in UCC and CRC cells involves rapid, TRAF3-dependent induction of ROS in a NOX-dependent fashion (Mohammed et al, manuscript in preparation)(Dunnill et al., 2016).

Therefore, having reported ASK1 induction in PCa cells, this study investigated the involvement of ROS in CD40 mediated death. Figures 4.16-17 showed that ROS production could not be detected upon CD40 activation, using two independent ROS detection assays. In support of this, functional ROS inhibition experiments performed showed that unlike CRC and UCC cells, it appeared that ROS is not critical for CD40 mediated death in PCa cells as both antioxidants NAC and PG (propyl gallate) did not inhibit CD40-mediated death (Figures 4.18-19). Although these observations are interesting, it is clearly paradoxical how ASK1 is phosphorylated in response to mCD40L-CD40 ligation in the absence of ROS induction. Because phospho-ASK1 may be linked directly with TRAF3 following mCD40L-CD40 interaction, as it has been previously linked to TNF and lymphotoxin ligand-associated cell responses (Chen et al., 2003; Kim et al., 2005a; Matsuura et al., 2002; Nishitoh et al., 1998). It would be useful if future studies investigated whether ROS inhibition could block ASK1 phosphorylation to determine the possibility of ROS-independent ASK1 activation, which is possible as recently reported in non-epithelial cells (Derstine et al., 2016).

6.10. Activation of the intrinsic mitochondrial pathway and pro-apoptotic protein regulation by mCD40L signalling in PCa cells

The pro apoptotic Bcl-2 proteins Bax and Bak are predominantly found in the cytosol of non-apoptotic cells and are rapidly recruited to mitochondria during apoptosis induction (Schellenberg et al., 2013). Bax and Bak are thought to translocate to mitochondria following an apoptotic stimulus and are associated with induction of MOMP to trigger cell death (Nechushtan et al., 2001; Schellenberg et al., 2013).

In this study, we have shown for the first time that the pro-apoptotic Bcl-2 proteins Bax and Bak are up-regulated following CD40 ligation on PCa cells, observations showing similarity to those from studies reporting a role for Bak and Bax and the mitochondria in CD40-induced cell death in other model systems. Early studies on CD40 showed induction of Bak and Bax by mCD40L in UCC cells (Bugajska et al., 2002). Mitochondrial outer membrane permeabilisation (MOMP) was evident by the release of cytochrome c into the cytoplasm in CRC cells (Mohammed et al, manuscript in preparation). In addition, up-regulation of Bax and Bak was was also demonstrated as functionally essential and JNK/AP-1-mediated during mCD40L-induced apoptosis (Dunnill et al., 2016), as Bak and particularly Bax were essential in cell death and their induction fully dependent on JNK/AP-1 activation. Moreover, previous studies demonstrated that that LTβR/HVEM activation by LIGHT/IFN-γ in MDA-MB-231 and HT29 cells led to the upregulation of Bak, Bax, observation supported by recent studies in our laboratory (Albarbar and Georgopoulos, unpublished findings).

Consistent with these reports, this study revealed that CD40 ligation by mCD40L caused rapid induction of pro-apoptotic proteins Bax and to a lesser extent Bak in PCa cells (as early as 3 and 6h post-ligation respectively, as shown in chapter 4 Figure 4.15). Although this would require appropriate further studies, it is tempting to speculate that the possible mechanism via which pro-apoptotic proteins Bax and Bak were activated is JNK/AP-1 phosphorylation or p38 activation (or both). These hypotheses are supported by a) previous studies which demonstrated that JNK inhibitor reduced the activation and mitochondrial translocation of Bax and Bak, as well as the cytosolic release of cytochrome c induced by luteolin (Lee et al., 2005); b) reports that JNK activation is often linked to pro-apoptotic Bcl-2 protein expression and subsequently activation of intrinsic mitochondrial pathway (Jin et al., 2006); and c) studies in our laboratory (Mohammed et al, manuscript in preparation)(Dunnill et al.,

2016) demonstrating dependency of Bax/Bak induction on JNK/AP-1. Schematic diagram showed that cell signalling pathway mediated by mCD40.

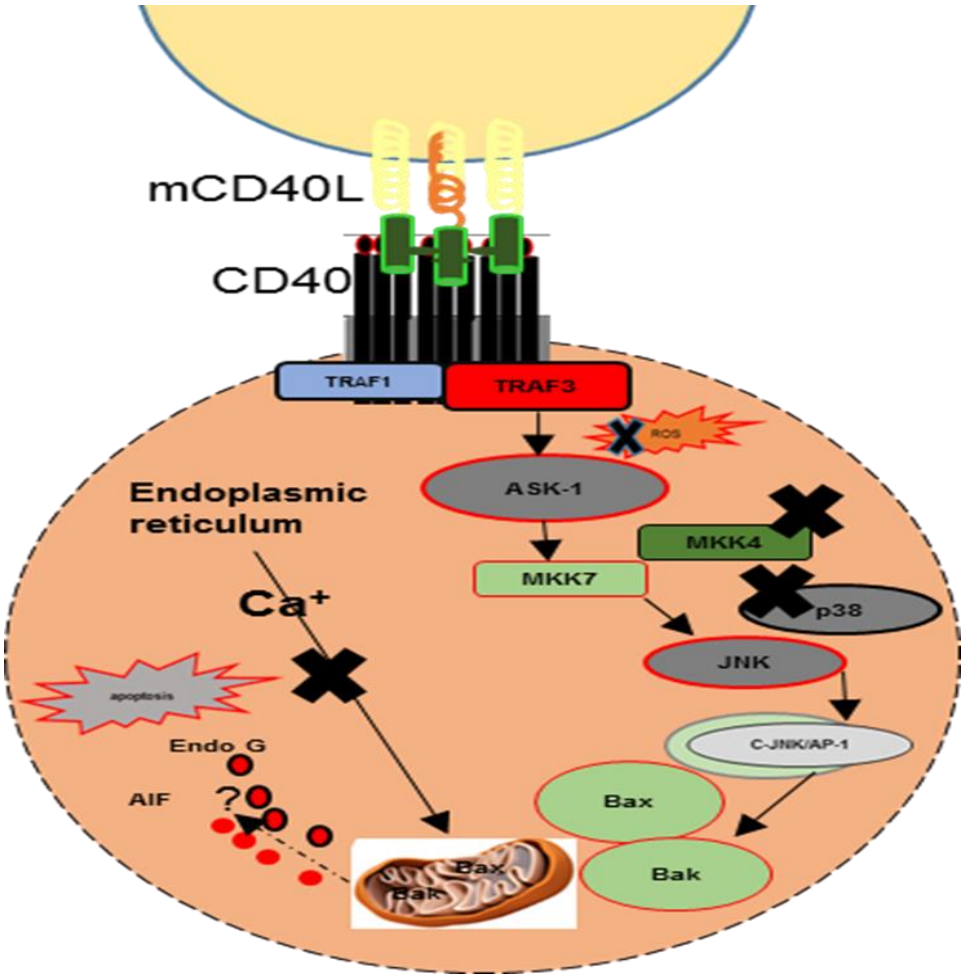


Figure 6.1 Apoptosis regulated by CD40 in PCa.

Schematic diagram illustrated that cell signalling pathway mediated by mCD40 which interacted with the receptor, as a result of receptor activation, TRAF3 recruited and stabilised and thus, cell signalling initiated. Unlike other region of cancer (bladder and colorectal), ASK-1 activated may in directed way with ROS generation, and subsequently phosphorylation of MAPKK MKK7 but not MKK4 and followed by JNK to activate the JNK/AP-1 which resulted in pro-apoptotic protein expressed such as Bax and Bak. As the caspases do not involve in this pathway, AIF or Endo G may involve and responsible for large scale of DNA fragmentation.

Interestingly, the results of this study indicate that despite a number of similarities to other cell models, CD40 mediated death in PCa cells is apoptotic yet occurs in a caspase-independent fashion, a highly novel observation. Moreover, as ROS were not functionally responsible for death, yet MOMP features were observed, other possible mechanisms were investigated. It has been reported that the ER-stress mediated apoptotic cell death through mitochondrial pathway by release of Ca2+ ions which causing change in an integrity of mitochondrial outer membrane permeabilisation (Derstine et al., 2016). Therefore, in this study the possibility of involvement of ER-stress mediated apoptotic cell death was investigated,using the inhibitors such as dantrolene and BAPTA, showed that such functional interference did not attenuate apoptosis. However, these results were quite limited, dose-response curves would need to be refined and more extensive optimisation would be necessary, together with the inclusion of appropriate positive controls. This would provide more appropriate evidence, as well as potentially explain the mechanism of ROS independent activation of ASK1 for instance.

6.11. Conclusion and future directions

By optimising and utilising an appropriate and robust PCa cell model, this study has confirmed expression of CD40 in PCa cells and studies on human PCa cell lines with either natural or engineered CD40 expression demonstrated for the first time that these cells are highly susceptible to CD40-mediated killing. Apoptosis is only achieved by mCD40L, as soluble receptor agonist did not cause cell death or growth inhibition, and CD40 ligation by mCD40L but not G28-5 mAb was accompanied by pro-inflammatory cytokine secretion. Despite a number of similarities to the signalling pathways observed in other models (UCC and CRC) characterised in our laboratory. Including TRAF adaptor protein engagement, DNA fragmentation, activation of stress kinases (JNK and p38), MOMP and upregulation of Bcl-2 proteins, it appears that in PCa cells death by CD40 occurs via a caspase-independent apoptotic pathway, MKK7 but not MKK4 is activated, Ask-1 is activated independently of redox status and ROS are not essential in the induction of apoptosis.

These findings have not only generated highly novel observations in terms of the ability of CD40 to induce PCa cell death, but have also added to our knowledge of the intriguingly multifaceted effects of CD40 in carcinoma cells, which appear to be not only context and quality of signal-dependent, but clearly are also highly cell type-

specific. This is a highly fascinating observation as it implies that the same receptor, whilst engaging signalling pathways with some common intracellular mediators, the precise signalling pathways can differ both in their exact nature and in their components/composition. Moreover, in addition to providing interesting biological evidence for the mechanisms of CD40 apoptosis, these observations may also represent a promising, targeted approach for PCa treatment. The ability to lead to extensive apoptosis in PCa cells has a potential therapeutic promise. However, induction of apoptosis was exclusively by membrane presented ligand, which poses potential therapeutic obstacles. However, work in our laboratory (Dunnill et al, 2016) has shown that understanding the precise mechanisms of CD40-mediated apoptosis can allow the discovery of novel appropaches to render a soluble CD40 agonist functionally equivalent to mCD40L. Therefore, it is essential that more studies delve further into the complexities of CD40 system signalling.

Although the present study has provided a number of lines of evidence on the ability of CD40 to kill PCa cells and the mechanisms via which this takes place, more studies would be necessary to provide better and stronger functional links for the role of specific intracellular mediators in apoptosis. Additional work should focus on understanding the precise roles of specific components involved (e.g. TRAF3, ASK1) as well as Bak/Bax and other mitochondrial death-related mediators' proteins, as well as for instance whether release of AIF or Endo G occurs during apoptosis and their contribution to DNA fragmentation. We believe that our work has for the first time provided evidence that TRAF3 may be central and be linked with/activate a MKK7/JNK mCD40L signalling axis. However, future work should focus on knocking down these intracellular proteins to identify their roles in mediating downstream signalling. Finally, and equally importantly, it would be of outmost importance to investigate the ability of CD40 to induce apoptosis in tumour-initiating, CD133-ve prostate CSCs, as stem cell-specific CD40-mediated death would represent an extremely promising and novel for therapeutic intervention in prostate cancer. The ability to kill efficiently and cause rapid pro-inflammatory cytokine secretion, whilst at the same time targeting what is potentially the cellular driver of carcinogenesis (CSCs), renders CD40-mediated killing an ideal potential therapeutic tool for PCa therapy in the near future.

Chapter 7: **Reference**

Reference

Abou-Saleh, H., Yacoub, D., Théorêt, J.-F., Gillis, M.-A., Neagoe, P.-E., Labarthe, B., Théroux, P., Sirois, M. G., Tabrizian, M., Thorin, E., 2009. Endothelial progenitor cells bind and inhibit platelet function and thrombus formation. Circulation 120, 2230-2239.

Abreu-Martin, M. T., Vidrich, A., Lynch, D. H., Targan, S. R., 1995. Divergent induction of apoptosis and IL-8 secretion in HT-29 cells in response to TNF-alpha and ligation of Fas antigen. The Journal of Immunology 155, 4147-4154.

Acehan, D., Jiang, X., Morgan, D. G., Heuser, J. E., Wang, X., Akey, C. W., 2002. Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. Molecular cell 9, 423-432.

Adams, J. M., 2003. Ways of dying: multiple pathways to apoptosis. Genes & development 17, 2481-2495.

Adams, R. H., Porras, A., Alonso, G., Jones, M., Vintersten, K., Panelli, S., Valladares, A., Perez, L., Klein, R., Nebreda, A. R., 2000. Essential role of p38α MAP kinase in placental but not embryonic cardiovascular development. Molecular cell 6, 109-116.

Adrain, C., Creagh, E. M., Martin, S. J., 2001. Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2. The EMBO journal 20, 6627-6636.

Afford, S. C., Randhawa, S., Eliopoulos, A. G., Hubscher, S. G., Young, L. S., Adams, D. H., 1999. CD40 activation induces apoptosis in cultured human hepatocytes via induction of cell surface fas ligand expression and amplifies fas-mediated hepatocyte death during allograft rejection. Journal of Experimental Medicine 189, 441-446.

Afford, S. C., Ahmed-choudhury, J., Randhawa, S., Russell, C., Youster, J., Crosby, H. A., Eliopoulos, A., Hubscher, S. G., Young, L. S., Adams, D. H., 2001. CD40 activation-induced, Fas-dependent apoptosis and NF-κB/AP-1 signaling in human intrahepatic biliary epithelial cells. The FASEB Journal 15, 2345-2354.

Aggarwal, B. B., 2000. Tumour necrosis factors receptor associated signalling molecules and their role in activation of apoptosis, JNK and NF-κB. Annals of the rheumatic diseases 59, i6-i16.

Aggarwal, B. B., 2004. Nuclear factor-κB: the enemy within. Cancer cell 6, 203-208.

Agliano, A., Calvo, A., Box, C., 2017. The challenge of targeting cancer stem cells to halt metastasis. Seminars in Cancer Biology. Elsevier.

Ahonen, C. L., Manning, E. M., Erickson, L. D., O'Connor, B. P., Lind, E. F., Pullen, S. S., Kehry, M. R., Noelle, R. J., 2002. The CD40-TRAF6 axis controls affinity maturation and the generation of long-lived plasma cells. Nature immunology 3, 451-456.

Aizawa, S., Nakano, H., Ishida, T., Horie, R., Nagai, M., Ito, K., Yagita, H., Okumura, K., Inoue, J., Watanabe, T., 1997. Tumor necrosis factor receptor-associated factor (TRAF) 5 and TRAF2 are involved in CD30-mediated NFκB activation. Journal of Biological Chemistry 272, 2042-2045.

Akiba, H., Nakano, H., Nishinaka, S., Shindo, M., Kobata, T., Atsuta, M., Morimoto, C., Ware, C. F., Malinin, N. L., Wallach, D., 1998. CD27, a member of the tumor necrosis factor receptor superfamily, activates NF-κB and stress-activated protein kinase/c-Jun N-terminal kinase via TRAF2, TRAF5, and NF-κB-inducing kinase. Journal of Biological Chemistry 273, 13353-13358.

Akira, S., Uematsu, S., Takeuchi, O., 2006. Pathogen recognition and innate immunity. Cell 124, 783-801.

Albarbar, B., Dunnill, C., Georgopoulos, N. T., 2015. Regulation of cell fate by lymphotoxin (LT) receptor signalling: Functional differences and similarities of the LT system to other TNF superfamily (TNFSF) members. Cytokine & growth factor reviews 26, 659-671.

Alderson, M. R., Armitage, R. J., Tough, T. W., Strockbine, L., Fanslow, W. C., Spriggs, M. K., 1993. CD40 expression by human monocytes: regulation by cytokines and activation of monocytes by the ligand for CD40. The Journal of experimental medicine 178, 669-674.

Alexandroff, A. B., Jackson, A. M., Paterson, T., Haley, J. L., Ross, J. A., Longo, D., Murphy, W., James, K., Taub, D. D., 2000. Role for CD40–CD40 ligand interactions in the immune response to solid tumours. Molecular immunology 37, 515-526.

Allen, R. C., Armitage, R. J., Conley, M. E., Rosenblatt, H., Jenkins, N. A., Copeland, N. G., Bedell, M. A., Edelhoff, S., Disteche, C. M., Simoneaux, D. K., 1993. CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. SCIENCE-NEW YORK THEN WASHINGTON- 259, 990-990.

Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W., Yuan, J., 1996. Human ICE/CED-3 protease nomenclature. Cell 87, 171.

Alturaihi, H., S Hassan, G., Al-Zoobi, L., Salti, S., Darif, Y., Yacoub, D., Akoum, S. E., Oudghiri, M., Merhi, Y., Mourad, W., 2015. Interaction of CD154 with different receptors and its role in bidirectional signals. European journal of immunology 45, 592-602.

Amarante-Mendes, G., Green, D., 1999. The regulation of apoptotic cell death. Brazilian journal of medical and biological research 32, 1053-1061.

Ambrosone, C. B., 2000. Oxidants and antioxidants in breast cancer. Antioxidants & redox signaling 2, 903-917.

Anand, S. X., Viles-Gonzalez, J. F., Badimon, J. J., Cavusoglu, E., Marmur, J. D., 2003. Membrane-associated CD40L and sCD40L in atherothrombotic disease. THROMBOSIS AND HAEMOSTASIS-STUTTGART- 90, 377-384.

André, P., Nannizzi-Alaimo, L., Prasad, S. K., Phillips, D. R., 2002a. Platelet-derived CD40L the switch-hitting player of cardiovascular disease. Circulation 106, 896-899.

André, P., Prasad, K. S., Denis, C. V., He, M., Papalia, J. M., Hynes, R. O., Phillips, D. R., Wagner, D. D., 2002b. CD40L stabilizes arterial thrombi by a β3 integrin–dependent mechanism. Nature medicine 8, 247-252.

Antonsson, B., Martinou, J.-C., 2000. The Bcl-2 protein family. Experimental cell research 256, 50-57.

Armitage, R. J., Macduff, B., Spriggs, M., Fanslow, W., 1993. Human B cell proliferation and Ig secretion induced by recombinant CD40 ligand are modulated by soluble cytokines. The Journal of Immunology 150, 3671-3680.

Armitage, R. J., Fanslow, W. C., Strockbine, L., Sato, T. A., Clifford, K. N., Macduff, B. M., Anderson, D. M., Gimpel, S. D., Davis-Smith, T., Maliszewski, C. R., 1992. Molecular and biological characterization of a murine ligand for CD40. Nature 357, 80-82.

Arron, J. R., Pewzner-Jung, Y., Walsh, M. C., Kobayashi, T., Choi, Y., 2002. Regulation of the subcellular localization of tumor necrosis factor receptor–associated factor (TRAF) 2 by TRAF1 reveals mechanisms of TRAF2 signaling. The Journal of experimental medicine 196, 923-934.

Aruffo, A., Farrington, M., Hollenbaugh, D., Li, X., Milatovich, A., Nonoyama, S., Bajorath, J., Grosmaire, L. S., Stenkamp, R., Neubauer, M., 1993. The CD40 ligand, gp39, is defective in activated T cells from patients with X-linked hyper-IgM syndrome. Cell 72, 291-300.

Asao, H., Fu, X.-Y., 2000. Interferon-γ has dual potentials in inhibiting or promoting cell proliferation. Journal of Biological Chemistry 275, 867-874.

Ashkenazi, A., Dixit, V. M., 1998. Death receptors: signaling and modulation. Science 281, 1305.

Avruch, J., 2007. MAP kinase pathways: the first twenty years. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research 1773, 1150-1160.

Baggiolini, M., Loetscher, P., Moser, B., 1995. Interleukin-8 and the chemokine family. International journal of immunopharmacology 17, 103-108.

Bajorath, J., 1998. Detailed comparison of two molecular models of the human CD40 ligand with an x-ray structure and critical assessment of model-based mutagenesis and residue mapping studies. Journal of Biological Chemistry 273, 24603-24609.

Bajorath, J., Aruffo, A., 1997. Construction and analysis of a detailed three-dimensional model of the ligand binding domain of the human B cell receptor CD40. Proteins: Structure, Function, and Bioinformatics 27, 59-70.

Bajorath, J., Marken, J. S., Chalupny, N. J., Spoon, T. L., Siadak, A. W., Gordon, M., Noelle, R. J., Hollenbaugh, D., Aruffo, A., 1995. Analysis of gp39/CD40 interactions using molecular models and site-directed mutagenesis. Biochemistry 34, 9884-9892.

Banchereau, J., Steinman, R. M., 1998. Dendritic cells and the control of immunity. Nature 392, 245-252.

Banchereau, J., Bazan, F., Blanchard, D., Briè, F., Galizzi, J., Van Kooten, C., Liu, Y., Rousset, F., Saeland, S., 1994. The CD40 antigen and its ligand. Annual review of immunology 12, 881-926.

Barrett, T. B., Shu, G., Clark, E. A., 1991. CD40 signaling activates CD11a/CD18 (LFA-1)-mediated adhesion in B cells. The Journal of immunology 146, 1722-1729.

Bavendiek, U., Libby, P., Kilbride, M., Reynolds, R., Mackman, N., Schönbeck, U., 2002. Induction of tissue factor expression in human endothelial cells by CD40 ligand is mediated via activator protein 1, nuclear factor κB, and Egr-1. Journal of Biological Chemistry 277, 25032-25039.

Beatty, G. L., Torigian, D. A., Chiorean, E. G., Saboury, B., Brothers, A., Alavi, A., Troxel, A. B., Sun, W., Teitelbaum, U. R., Vonderheide, R. H., 2013. A phase I study of an agonist CD40 monoclonal antibody (CP-870,893) in combination with gemcitabine in patients with advanced pancreatic ductal adenocarcinoma. Clinical Cancer Research 19, 6286-6295.

Bekaii-Saab, T., El-Rayes, B., 2017. Identifying and targeting cancer stem cells in the treatment of gastric cancer. Cancer.

Belperio, J. A., Keane, M. P., Arenberg, D. A., Addison, C. L., Ehlert, J. E., Burdick, M. D., Strieter, R. M., 2000. CXC chemokines in angiogenesis. Journal of leukocyte biology 68, 1-8.

Benson, R. J., Hostager, B. S., Bishop, G. A., 2006. Rapid CD40-mediated rescue from CD95-induced apoptosis requires TNFR-associated factor-6 and PI3K. European journal of immunology 36, 2535-2543.

Bergtold, A., Desai, D. D., Gavhane, A., Clynes, R., 2005. Cell surface recycling of internalized antigen permits dendritic cell priming of B cells. Immunity 23, 503-514.

Bermudez, O., Pagès, G., Gimond, C., 2010. The dual-specificity MAP kinase phosphatases: critical roles in development and cancer. American Journal of Physiology-Cell Physiology 299, C189-C202.

Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T. B., Oukka, M., Weiner, H. L., Kuchroo, V. K., 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 441, 235-238.

Bhogal, R. H., Weston, C. J., Curbishley, S. M., Adams, D. H., Afford, S. C., 2012. Activation of CD40 with platelet derived CD154 promotes reactive oxygen species dependent death of human hepatocytes during hypoxia and reoxygenation. PloS one 7, e30867.

Bishop, G. A., 2004. The multifaceted roles of TRAFs in the regulation of B-cell function. Nature Reviews Immunology 4, 775-786.

Bishop, G. A., Hostager, B. S., 2001. Signaling by CD40 and its mimics in B cell activation. Immunologic research 24, 97-109.

Bishop, G. A., Hostager, B. S., Brown, K. D., 2002. Mechanisms of TNF receptor-associated factor (TRAF) regulation in B lymphocytes. Journal of leukocyte biology 72, 19-23.

Bishop, G. A., Moore, C. R., Xie, P., Stunz, L. L., Kraus, Z. J., 2007. TRAF proteins in CD40 signaling. TNF Receptor Associated Factors (TRAFs). Springer, pp. 131-151.

Boldin, M. P., Goncharov, T. M., Goltseve, Y. V., Wallach, D., 1996. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1-and TNF receptor–induced cell death. Cell 85, 803-815.

Bollinger, C. R., Teichgräber, V., Gulbins, E., 2005. Ceramide-enriched membrane domains. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research 1746, 284-294.

Bossy-Wetzel, E., Newmeyer, D. D., Green, D. R., 1998. Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and

independently of mitochondrial transmembrane depolarization. The EMBO journal 17, 37-49.

Boulton, T. G., Cobb, M. H., 1991. Identification of multiple extracellular signal-regulated kinases (ERKs) with antipeptide antibodies. Cell regulation 2, 357-371.

Bourgeois, C., Rocha, B., Tanchot, C., 2002. A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory. Science 297, 2060-2063.

Boussiotis, V. A., Nadler, L. M., Strominger, J. L., Goldfeld, A. E., 1994. Tumor necrosis factor alpha is an autocrine growth factor for normal human B cells. Proceedings of the National Academy of Sciences 91, 7007-7011.

Boyle, W. J., Simonet, W. S., Lacey, D. L., 2003. Osteoclast differentiation and activation. Nature 423, 337-342.

Braesch-Andersen, S., Paulie, S., Koho, H., Nika, H., Aspenström, P., Perlmann, P., 1989. Biochemical characteristics and partial amino acid sequence of the receptor-like human B cell and carcinoma antigen CDw40. The Journal of Immunology 142, 562-567.

Brancho, D., Tanaka, N., Jaeschke, A., Ventura, J.-J., Kelkar, N., Tanaka, Y., Kyuuma, M., Takeshita, T., Flavell, R. A., Davis, R. J., 2003. Mechanism of p38 MAP kinase activation in vivo. Genes & development 17, 1969-1978.

Briscoe, D. M., Alexander, S. I., Lichtman, A. H., 1998. Interactions between T lymphocytes and endothelial cells in allograft rejection. Current opinion in immunology 10, 525-531.

Bromberg, J. F., Horvath, C. M., Wen, Z., Schreiber, R. D., Darnell, J. E., 1996. Transcriptionally active Stat1 is required for the antiproliferative effects of both interferon alpha and interferon gamma. Proceedings of the national academy of sciences 93, 7673-7678.

Brown, K. D., Hostager, B. S., Bishop, G. A., 2001. Differential Signaling and Tumor Necrosis Factor Receptor–associated Factor (TRAF) Degradation Mediated by CD40 and the Epstein-Barr Virus Oncoprotein Latent Membrane Protein 1 (LMP1). Journal of Experimental Medicine 193, 943-954.

Brown, K. D., Hostager, B. S., Bishop, G. A., 2002. Regulation of TRAF2 signaling by self-induced degradation. Journal of Biological Chemistry 277, 19433-19438.

Bruey, J.-M., Ducasse, C., Bonniaud, P., Ravagnan, L., Susin, S. A., Diaz-Latoud, C., Gurbuxani, S., Arrigo, A.-P., Kroemer, G., Solary, E., 2000. Hsp27 negatively regulates cell death by interacting with cytochrome c. Nature cell biology 2, 645-652.

Brunner, T., Wasem, C., Torgler, R., Cima, I., Jakob, S., Corazza, N., 2003. Fas (CD95/Apo-1) ligand regulation in T cell homeostasis, cell-mediated cytotoxicity and immune pathology. Seminars in immunology, Vol. 15. Elsevier, pp. 167-176.

Bugajska, U., Georgopoulos, N. T., Southgate, J., Johnson, P. W., Graber, P., Gordon, J., Selby, P. J., Trejdosiewicz, L. K., 2002. The effects of malignant transformation on susceptibility of human urothelial cells to CD40-mediated apoptosis. Journal of the National Cancer Institute 94, 1381-1395.

Burlacu, A., 2003. Regulation of apoptosis by Bcl-2 family proteins. Journal of cellular and molecular medicine 7, 249-257.

Bush, T. J. V., Bishop, G. A., 2008. TLR7 and CD40 cooperate in IL-6 production via enhanced JNK and AP-1 activation. European journal of immunology 38, 400-409.

Cabal-Hierro, L., Lazo, P. S., 2012. Signal transduction by tumor necrosis factor receptors. Cellular signalling 24, 1297-1305.

Cagnoni, F., Oddera, S., Giron-Michel, J., Riccio, A. M., Olsson, S., Dellacasa, P., Melioli, G., Canonica, G. W., Azzarone, B., 2004. CD40 on adult human airway epithelial cells: expression and proinflammatory effects. The Journal of Immunology 172, 3205-3214.

Cain, K., Bratton, S. B., Cohen, G. M., 2002. The Apaf-1 apoptosome: a large caspase-activating complex. Biochimie 84, 203-214.

Cain, K., Langlais, C., Sun, X.-M., Brown, D. G., Cohen, G. M., 2001. Physiological concentrations of K+ inhibit cytochrome c-dependent formation of the apoptosome. Journal of Biological Chemistry 276, 41985-41990.

Candé, C., Cecconi, F., Dessen, P., Kroemer, G., 2002. Apoptosis-inducing factor (AIF): key to the conserved caspase-independent pathways of cell death? Journal of cell science 115, 4727-4734.

Cao, W., Cavacini, L. A., Tillman, K. C., Posner, M. R., 2005. CD40 function in squamous cell cancer of the head and neck. Oral oncology 41, 462-469.

Cargnello, M., Roux, P. P., 2011. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiology and Molecular Biology Reviews 75, 50-83.

Caux, C., Massacrier, C., Vanbervliet, B., Dubois, B., Van Kooten, C., Durand, I., Banchereau, J., 1994. Activation of human dendritic cells through CD40 cross-linking. The Journal of experimental medicine 180, 1263-1272.

Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P., Lanzavecchia, A., Alber, G., 1996. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: TT help via APC activation. The Journal of experimental medicine 184, 747-752.

Chai, J., Du, C., Wu, J.-W., Kyin, S., Wang, X., Shi, Y., 2000. Structural and biochemical basis of apoptotic activation by Smac/DIABLO. Nature 406, 855-862.

Chakrabarti, S., Blair, P., Freedman, J. E., 2007. CD40-40L signaling in vascular inflammation. Journal of Biological Chemistry 282, 18307-18317.

Chakrabarti, S., Varghese, S., Vitseva, O., Tanriverdi, K., Freedman, J. E., 2005. CD40 ligand influences platelet release of reactive oxygen intermediates. Arteriosclerosis, thrombosis, and vascular biology 25, 2428-2434.

Chan, F. K.-M., Chun, H. J., Zheng, L., Siegel, R. M., Bui, K. L., Lenardo, M. J., 2000. A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. Science 288, 2351-2354.

Chan, H., Reed, J. C., 2005. TRAF-dependent association of protein kinase Tpl2/COT1 (MAP3K8) with CD40. Biochemical and biophysical research communications 328, 198-205.

Chang, B. S., Minn, A. J., Muchmore, S. W., Fesik, S. W., Thompson, C. B., 1997. Identification of a novel regulatory domain in Bcl-xL and Bcl-2. The EMBO journal 16, 968-977.

Chang, L., Karin, M., 2001. Mammalian MAP kinase signalling cascades. Nature 410, 37-40.

Châtel, A., 2009. Voies de signalisation des MAP kinases et apoptose chez l'éponge Suberites domuncula et la moule Mytilus galloprovincialis. Université de Bretagne occidentale-Brest.

Chen, M.-C., Hwang, M.-J., Chou, Y.-C., Chen, W.-H., Cheng, G., Nakano, H., Luh, T.-Y., Mai, S.-C., Hsieh, S.-L., 2003. The role of apoptosis signal-regulating kinase 1 in lymphotoxin-β receptor-mediated cell death. Journal of Biological Chemistry 278, 16073-16081.

Chen, W., Liu, L., Luo, Y., Odaka, Y., Awate, S., Zhou, H., Shen, T., Zheng, S., Lu, Y., Huang, S., 2012. Cryptotanshinone activates p38/JNK and inhibits Erk1/2 leading to caspase-independent cell death in tumor cells. Cancer prevention research 5, 778-787.

Chen, Z., Gibson, T. B., Robinson, F., Silvestro, L., Pearson, G., Xu, B.-e., Wright, A., Vanderbilt, C., Cobb, M. H., 2001. MAP kinases. Chemical reviews 101, 2449-2476.

Cheung, E. C., Melanson-Drapeau, L., Cregan, S. P., Vanderluit, J. L., Ferguson, K. L., McIntosh, W. C., Park, D. S., Bennett, S. A., Slack, R. S., 2005. Apoptosis-inducing factor is a key factor in neuronal cell death propagated by BAX-dependent and BAX-independent mechanisms. Journal of Neuroscience 25, 1324-1334.

Chi, H., Barry, S. P., Roth, R. J., Wu, J. J., Jones, E. A., Bennett, A. M., Flavell, R. A., 2006. Dynamic regulation of pro-and anti-inflammatory cytokines by MAPK phosphatase 1 (MKP-1) in innate immune responses. Proceedings of the National Academy of Sciences of the United States of America 103, 2274-2279.

Chin, Y. E., Kitagawa, M., Kuida, K., Flavell, R. A., Fu, X.-Y., 1997. Activation of the STAT signaling pathway can cause expression of caspase 1 and apoptosis. Molecular and cellular biology 17, 5328-5337.

Chipuk, J. E., Moldoveanu, T., Llambi, F., Parsons, M. J., Green, D. R., 2010. The BCL-2 family reunion. Molecular cell 37, 299-310.

Choi, K.-S., Song, E.-K., Yim, C.-Y., 2008. Cytokines secreted by IL-2-activated lymphocytes induce endogenous nitric oxide synthesis and apoptosis in macrophages. Journal of leukocyte biology 83, 1440-1450.

Chopra, D. P., Menard, R. E., Januszewski, J., Mattingly, R. R., 2004. TNF-α-mediated apoptosis in normal human prostate epithelial cells and tumor cell lines. Cancer letters 203, 145-154.

Choudhury, J. A., Russell, C. L., Randhawa, S., Young, L. S., Adams, D. H., Afford, S. C., 2003. Differential induction of nuclear factor-κB and activator protein-1 activity after CD40 ligation is associated with primary human hepatocyte apoptosis or intrahepatic endothelial cell proliferation. Molecular biology of the cell 14, 1334-1345.

Chung, J. Y., Park, Y. C., Ye, H., Wu, H., 2002. All TRAFs are not created equal: common and distinct molecular mechanisms of TRAF-mediated signal transduction. Journal of cell science 115, 679-688.

Clark, E. A., Ledbetter, J. A., 1986. Activation of human B cells mediated through two distinct cell surface differentiation antigens, Bp35 and Bp50. Proceedings of the National Academy of Sciences 83, 4494-4498.

Clodi, K., Asgary, Z., Zhao, S., Kliche, K. O., Cabanillas, F., Andreeff, M., Younes, A., 1998. Coexpression of CD40 and CD40 ligand in B-cell lymphoma cells. British journal of haematology 103, 270-275.

Crallan, R., Georgopoulos, N. T., Southgate, J., 2006. Experimental models of human bladder carcinogenesis. Carcinogenesis 27, 374-381.

Craxton, A., Magaletti, D., Ryan, E. J., Clark, E. A., 2003. Macrophage-and dendritic cell—dependent regulation of human B-cell proliferation requires the TNF family ligand BAFF. Blood 101, 4464-4471.

Crompton, M., 1999. The mitochondrial permeability transition pore and its role in cell death. Biochemical Journal 341, 233-249.

Cuadrado, A., Nebreda, A. R., 2010. Mechanisms and functions of p38 MAPK signalling. Biochemical Journal 429, 403-417.

Cuschieri, J., Maier, R. V., 2005. Mitogen-activated protein kinase (MAPK). Critical care medicine 33, S417-S419.

D'Autréaux, B., Toledano, M. B., 2007. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. Nature reviews Molecular cell biology 8, 813-824.

Danese, S., de la Motte, C., Reyes, B. M. R., Sans, M., Levine, A. D., Fiocchi, C., 2004. Cutting edge: T cells trigger CD40-dependent platelet activation and granular RANTES release: a novel pathway for immune response amplification. The Journal of Immunology 172, 2011-2015.

Dasari, S., Chambers, M. C., Martinez, M. A., Carpenter, K. L., Ham, A.-J. L., Vega-Montoto, L. J., Tabb, D. L., 2012. Pepitome: evaluating improved spectral library search for identification complementarity and quality assessment. Journal of proteome research 11, 1686-1695.

Davies, C. C., Mak, T. W., Young, L. S., Eliopoulos, A. G., 2005. TRAF6 is required for TRAF2-dependent CD40 signal transduction in nonhemopoietic cells. Molecular and cellular biology 25, 9806-9819.

Davis, R. J., 2000. Signal transduction by the JNK group of MAP kinases. Cell 103, 239-252.

de Almagro, M. C., Vucic, D., 2015. Necroptosis: Pathway diversity and characteristics. Seminars in cell & developmental biology, Vol. 39. Elsevier, pp. 56-62.

Deacon, K., Blank, J. L., 1999. MEK kinase 3 directly activates MKK6 and MKK7, specific activators of the p38 and c-Jun NH2-terminal kinases. Journal of Biological Chemistry 274, 16604-16610.

Declercq, W., Denecker, G., Fiers, W., Vandenabeele, P., 1998. Cooperation of both TNF receptors in inducing apoptosis: involvement of the TNF receptor-associated factor binding domain of the TNF receptor 75. The Journal of Immunology 161, 390-399.

Degli-Esposti, M. A., Davis-Smith, T., Din, W. S., Smolak, P. J., Goodwin, R. G., Smith, C. A., 1997. Activation of the lymphotoxin beta receptor by cross-linking induces chemokine production and growth arrest in A375 melanoma cells. The Journal of Immunology 158, 1756-1762.

Degterev, A., Boyce, M., Yuan, J., 2003. A decade of caspases. Oncogene 22, 8543-8567.

Dejardin, E., 2006. The alternative NF-κB pathway from biochemistry to biology: pitfalls and promises for future drug development. Biochemical pharmacology 72, 1161-1179.

Dejean, L. M., Martinez-Caballero, S., Guo, L., Hughes, C., Teijido, O., Ducret, T., Ichas, F., Korsmeyer, S. J., Antonsson, B., Jonas, E. A., 2005. Oligomeric Bax is a component of the putative cytochrome c release channel MAC, mitochondrial apoptosis-induced channel. Molecular biology of the cell 16, 2424-2432.

DeKruyff, R. H., Rizzo, L. V., Umetsu, D. T., 1993. Induction of immunoglobulin synthesis by CD4+ T cell clones. Seminars in immunology, Vol. 5. Elsevier, pp. 421-430.

Deng, G.-M., Zheng, L., Chan, F. K.-M., Lenardo, M., 2005. Amelioration of inflammatory arthritis by targeting the pre-ligand assembly domain of tumor necrosis factor receptors. Nature medicine 11, 1066-1072.

Derstine, R. J., Naik, M. U., Naik, U. P., 2016. The MAP3K Apoptosis Signal-Regulating Kinase 1 (ASK1) Functions As a Calcium-Dependent Regulator of Platelet Activation Downstream of the G (α) q-PLC Signaling Axis. Am Soc Hematology.

Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B., Martinou, J.-C., 1999. Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. The Journal of cell biology 144, 891-901.

Deveraux, Q. L., Leo, E., Stennicke, H. R., Welsh, K., Salvesen, G. S., Reed, J. C., 1999. Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. The EMBO journal 18, 5242-5251.

Dhanasekaran, D. N., Reddy, E. P., 2008. JNK signaling in apoptosis. Oncogene 27, 6245-6251.

Di Sano, F., Ferraro, E., Tufi, R., Achsel, T., Piacentini, M., Cecconi, F., 2006. Endoplasmic reticulum stress induces apoptosis by an apoptosome-dependent but caspase 12-independent mechanism. Journal of Biological Chemistry 281, 2693-2700.

Dickinson, B. C., Chang, C. J., 2011. Chemistry and biology of reactive oxygen species in signaling or stress responses. Nature chemical biology 7, 504-511.

Dragu, D. L., Necula, L. G., Bleotu, C., Diaconu, C. C., Chivu-Economescu, M., 2015. Therapies targeting cancer stem cells: Current trends and future challenges. World journal of stem cells 7, 1185.

Du, C., Fang, M., Li, Y., Li, L., Wang, X., 2000. Smac, a mitochondrial protein that promotes cytochrome c–dependent caspase activation by eliminating IAP inhibition. Cell 102, 33-42.

Dunnill, C., Ibraheem, K., Mohamed, A., Southgate, J., Georgopoulos, N., 2016. A redox state-dictated signalling pathway deciphers the malignant cell specificity of CD40-mediated apoptosis. Oncogene.

Dunnill, C., Patton, T., Brennan, J., Barrett, J., Dryden, M., Cooke, J., Leaper, D., Georgopoulos, N. T., 2015. Reactive oxygen species (ROS) and wound healing: the functional role of ROS and emerging ROS-modulating technologies for augmentation of the healing process. International wound journal.

Eferl, R., Wagner, E. F., 2003. AP-1: a double-edged sword in tumorigenesis. Nature Reviews Cancer 3, 859-868.

Ekert, P. G., Silke, J., Hawkins, C. J., Verhagen, A. M., Vaux, D. L., 2001. DIABLO promotes apoptosis by removing MIHA/XIAP from processed caspase 9. The Journal of cell biology 152, 483-490.

Elgueta, R., Benson, M. J., De Vries, V. C., Wasiuk, A., Guo, Y., Noelle, R. J., 2009. Molecular mechanism and function of CD40/CD40L engagement in the immune system. Immunological reviews 229, 152-172.

Eliopoulos, A. G., Young, L. S., 2004. The role of the CD40 pathway in the pathogenesis and treatment of cancer. Current opinion in pharmacology 4, 360-367.

Eliopoulos, A. G., Davies, C., Knox, P. G., Gallagher, N. J., Afford, S. C., Adams, D. H., Young, L. S., 2000. CD40 induces apoptosis in carcinoma cells through activation of cytotoxic ligands of the tumor necrosis factor superfamily. Molecular and cellular biology 20, 5503-5515.

Eliopoulos, A. G., Dawson, C. W., Mosialos, G., Floettmann, J. E., Rowe, M., Armitage, R. J., Dawson, J., Zapata, J., Kerr, D. J., Wakelam, M., 1996. CD40-induced growth inhibition in epithelial cells is mimicked by Epstein-Barr Virus-encoded LMP1: involvement of TRAF3 as a common mediator. Oncogene 13, 2243-2254.

Ellmark, P., Borrebaeck, C. A., 2003. Pre-assembly of the extracellular domains of CD40 is not necessary for rescue of mouse B cells from anti-immunoglobulin M-induced apoptosis. Immunology 108, 452-457.

Elmetwali, T., Young, L. S., Palmer, D. H., 2010a. CD40 ligand-induced carcinoma cell death: a balance between activation of TNFR-associated factor (TRAF) 3-dependent death signals and suppression of TRAF6-dependent survival signals. The journal of immunology 184, 1111-1120.

Elmetwali, T., Salman, A., Palmer, D., 2016. NORE1A induction by membrane-bound CD40L (mCD40L) contributes to CD40L-induced cell death and G1 growth arrest in p21-mediated mechanism. Cell death & disease 7, e2146.

Elmetwali, T., Searle, P. F., McNeish, I., Young, L. S., Palmer, D. H., 2010b. CD40 ligand induced cytotoxicity in carcinoma cells is enhanced by inhibition of metalloproteinase cleavage and delivery via a conditionally-replicating adenovirus. Mol Cancer 9, 52.

Elmore, S., 2007. Apoptosis: a review of programmed cell death. Toxicologic pathology 35, 495-516.

Emery, J. G., McDonnell, P., Burke, M. B., Deen, K. C., Lyn, S., Silverman, C., Dul, E., Appelbaum, E. R., Eichman, C., DiPrinzio, R., 1998. Osteoprotegerin is a

receptor for the cytotoxic ligand TRAIL. Journal of Biological Chemistry 273, 14363-14367.

Eskes, R., Desagher, S., Antonsson, B., Martinou, J.-C., 2000. Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. Molecular and cellular biology 20, 929-935.

Evan, G., Littlewood, T., 1998. A matter of life and cell death. Science 281, 1317-1322.

Ferri, K. F., Kroemer, G., 2001. Organelle-specific initiation of cell death pathways. Nature cell biology 3, E255-E263.

Fesik, S. W., Shi, Y., 2001. Controlling the caspases. Science 294, 1477-1478.

Festjens, N., Vanden Berghe, T., Vandenabeele, P., 2006. Necrosis, a well-orchestrated form of cell demise: signalling cascades, important mediators and concomitant immune response. Biochimica et Biophysica Acta (BBA)-Bioenergetics 1757, 1371-1387.

Fischer, U., Jänicke, R., Schulze-Osthoff, K., 2003. Many cuts to ruin: a comprehensive update of caspase substrates. Cell Death & Differentiation 10, 76-100.

Fisher, W. R., Taniuchi, H., Anfinsen, C. B., 1973. On the role of heme in the formation of the structure of cytochrome c. Journal of Biological Chemistry 248, 3188-3195.

Force, W. R., Cheung, T. C., Ware, C. F., 1997. Dominant negative mutants of TRAF3 reveal an important role for the coiled coil domains in cell death signaling by the lymphotoxin-β receptor. Journal of Biological Chemistry 272, 30835-30840.

Forte, M., Bernardi, P., 2006. The permeability transition and BCL-2 family proteins in apoptosis: co-conspirators or independent agents? Cell Death & Differentiation 13, 1287-1290.

Fotin-Mleczek, M., Henkler, F., Hausser, A., Glauner, H., Samel, D., Graness, A., Scheurich, P., Mauri, D., Wajant, H., 2004. Tumor necrosis factor receptor-associated factor (TRAF) 1 regulates CD40-induced TRAF2-mediated NF-κB activation. Journal of Biological Chemistry 279, 677-685.

Foy, T. M., Laman, J. D., Ledbetter, J. A., Aruffo, A., Claassen, E., Noelle, R. J., 1994. gp39-CD40 interactions are essential for germinal center formation and the development of B cell memory. The Journal of experimental medicine 180, 157-163.

Fresno, M., Kopf, M., Rivas, L., 1997. Cytokines and infectious diseases. Immunology today 18, 56-58.

Fruehauf, J. P., Meyskens, F. L., 2007. Reactive oxygen species: a breath of life or death? Clinical Cancer Research 13, 789-794.

Fu, W.-N., Kelsey, S. M., Newland, A. C., Jia, L., 2001. Apaf-1XL is an inactive isoform compared with Apaf-1L. Biochemical and biophysical research communications 282, 268-272.

Gallagher, N., Eliopoulos, A., Agathangelo, A., Oates, J., Crocker, J., Young, L., 2002. CD40 activation in epithelial ovarian carcinoma cells modulates growth, apoptosis, and cytokine secretion. Molecular Pathology 55, 110.

Gamper, C., Omene, C. O., van Eyndhoven, W. G., Glassman, G. D., Lederman, S., 2001. Expression and function of TRAF-3 splice-variant isoforms in human lymphoma cell lines. Human immunology 62, 1167-1177.

Gao, J., Bernatchez, C., Sharma, P., Radvanyi, L. G., Hwu, P., 2013. Advances in the development of cancer immunotherapies. Trends in immunology 34, 90-98.

Gaur, U., Aggarwal, B. B., 2003. Regulation of proliferation, survival and apoptosis by members of the TNF superfamily. Biochemical pharmacology 66, 1403-1408.

Gavrieli, Y., Sherman, Y., Ben-Sasson, S. A., 1992. Identification of programmed cell death in situ via specific labelling of nuclear DNA fragmentation. Journal of cell Biology 119, 493-493.

Gelbmann, C., Leeb, S., Vogl, D., Maendel, M., Herfarth, H., Schölmerich, J., Falk, W., Rogler, G., 2003. Inducible CD40 expression mediates NFκB activation and cytokine secretion in human colonic fibroblasts. Gut 52, 1448-1456.

Georgopoulos, N. T., Steele, L. P., Thomson, M., Selby, P. J., Southgate, J., Trejdosiewicz, L. K., 2006. A novel mechanism of CD40-induced apoptosis of carcinoma cells involving TRAF3 and JNK/AP-1 activation. Cell Death & Differentiation 13, 1789-1801.

Georgopoulos, N. T., Merrick, A., Scott, N., Selby, P. J., Melcher, A., Trejdosiewicz, L. K., 2007. CD40-mediated death and cytokine secretion in colorectal cancer: A potential target for inflammatory tumour cell killing. International journal of cancer 121, 1373-1381.

Giroir, B. P., Brown, T., Beutler, B., 1992. Constitutive synthesis of tumor necrosis factor in the thymus. Proceedings of the National Academy of Sciences 89, 4864-4868.

Goitre, L., Trapani, E., Trabalzini, L., Retta, S. F., 2014. The Ras superfamily of small GTPases: the unlocked secrets. Ras Signaling: Methods and Protocols, 1-18.

Goldstein, J. C., Waterhouse, N. J., Juin, P., Evan, G. I., Green, D. R., 2000. The coordinate release of cytochrome c during apoptosis is rapid, complete and kinetically invariant. Nature cell biology 2, 156-162.

Golstein, P., Kroemer, G., 2007. Cell death by necrosis: towards a molecular definition. Trends in biochemical sciences 32, 37-43.

Gormand, F., Briere, F., Peyrol, S., Raccurt, M., Durand, I., Ait-Yahia, S., Lebecque, S., Banchereau, J., Pacheco, Y., 1999. CD40 expression by human bronchial epithelial cells. Scandinavian journal of immunology 49, 355-361.

Gotoh, Y., Cooper, J. A., 1998. Reactive oxygen species-and dimerization-induced activation of apoptosis signal-regulating kinase 1 in tumor necrosis factor-α signal transduction. Journal of Biological Chemistry 273, 17477-17482.

Grammer, A. C., Lipsky, P. E., 2000. CD40-mediated regulation of immune responses by TRAF-dependent and TRAF-independent signaling mechanisms. Advances in immunology 76, 61-178.

Grammer, A. C., Bergman, M. C., Miura, Y., Fujita, K., Davis, L. S., Lipsky, P. E., 1995. The CD40 ligand expressed by human B cells costimulates B cell responses. The Journal of Immunology 154, 4996-5010.

Grassmé, H., Jendrossek, V., Bock, J., Riehle, A., Gulbins, E., 2002. Ceramide-rich membrane rafts mediate CD40 clustering. The Journal of Immunology 168, 298-307.

Grassmé, H., Jekle, A., Riehle, A., Schwarz, H., Berger, J., Sandhoff, K., Kolesnick, R., Gulbins, E., 2001. CD95 signaling via ceramide-rich membrane rafts. Journal of Biological Chemistry 276, 20589-20596.

Gray, D., Bergthorsdottir, S., van Essen, D., Wykes, M., Poudrier, J., Siepmann, K., 1997. Observations on memory B-cell development. Seminars in immunology, Vol. 9. Elsevier, pp. 249-254.

Grech, A., Quinn, R., Srinivasan, D., Badoux, X., Brink, R., 2000. Complete structural characterisation of the mammalian and Drosophila TRAF genes: implications for TRAF evolution and the role of RING finger splice variants. Molecular immunology 37, 721-734.

Grewal, I. S., Xu, J., Flavell, R. A., 1995. Impairment of antigen-specific T-cell priming in mice lacking CD40 ligand. Nature 378, 617-620.

Grewal, I. S., Borrow, P., Pamer, E. G., Oldstone, M. B., Flavell, R. A., 1997. The CD40–CD154 system in anti-infective host defense. Current opinion in immunology 9, 491-497.

Grimaldi, J. C., Torres, R., Kozak, C. A., Chang, R., Clark, E. A., Howard, M., Cockayne, D. A., 1992. Genomic structure and chromosomal mapping of the murine CD40 gene. The Journal of Immunology 149, 3921-3926.

Gross, A., McDonnell, J. M., Korsmeyer, S. J., 1999. BCL-2 family members and the mitochondria in apoptosis. Genes & development 13, 1899-1911.

Gulbins, E., Grassmé, H., 2002. Ceramide and cell death receptor clustering. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids 1585, 139-145.

Guo, L., Pietkiewicz, D., Pavlov, E. V., Grigoriev, S. M., Kasianowicz, J. J., Dejean, L. M., Korsmeyer, S. J., Antonsson, B., Kinnally, K. W., 2004. Effects of cytochrome c on the mitochondrial apoptosis-induced channel MAC. American Journal of Physiology-Cell Physiology 286, C1109-C1117.

Gupta, S., 2003. Molecular signaling in death receptor and mitochondrial pathways of apoptosis (Review). International journal of oncology 22, 15-20.

Ha, Y. J., Lee, J. R., 2004. Role of TNF receptor-associated factor 3 in the CD40 signaling by production of reactive oxygen species through association with p40phox, a cytosolic subunit of nicotinamide adenine dinucleotide phosphate oxidase. The Journal of Immunology 172, 231-239.

Häcker, H., Tseng, P.-H., Karin, M., 2011. Expanding TRAF function: TRAF3 as a tri-faced immune regulator. Nature Reviews Immunology 11, 457-468.

Hager, A. C. M., Ellmark, P., Borrebaeck, C., Furebring, C., 2003. Affinity and Epitope Profiling of Mouse Anti-CD40 Monoclonal Antibodies. Scandinavian journal of immunology 57, 517-524.

Halliwell, B., 2007. Oxidative stress and cancer: have we moved forward? Biochemical Journal 401, 1-11.

Hamanaka, R. B., Chandel, N. S., 2010. Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes. Trends in biochemical sciences 35, 505-513.

Hanahan, D., Weinberg, R. A., 2011. Hallmarks of cancer: the next generation. Cell 144, 646-674.

Harada, H., Becknell, B., Wilm, M., Mann, M., Huang, L. J.-s., Taylor, S. S., Scott, J. D., Korsmeyer, S. J., 1999. Phosphorylation and inactivation of BAD by mitochondria-anchored protein kinase A. Molecular cell 3, 413-422.

Hassan, G. S., Merhi, Y., Mourad, W. M., 2009. CD154 and its receptors in inflammatory vascular pathologies. Trends in immunology 30, 165-172.

Hassan, S. B., Sørensen, J. F., Olsen, B. N., Pedersen, A. E., 2014. Anti-CD40-mediated cancer immunotherapy: an update of recent and ongoing clinical trials. Immunopharmacology and immunotoxicology 36, 96-104.

Hauer, J., Püschner, S., Ramakrishnan, P., Simon, U., Bongers, M., Federle, C., Engelmann, H., 2005. TNF receptor (TNFR)-associated factor (TRAF) 3 serves as an inhibitor of TRAF2/5-mediated activation of the noncanonical NF-κB pathway by TRAF-binding TNFRs. Proceedings of the National Academy of Sciences of the United States of America 102, 2874-2879.

Haxhinasto, S. A., Bishop, G. A., 2004. Synergistic B Cell Activation by CD40 and the B Cell Antigen Receptor ROLE OF B LYMPHOCYTE ANTIGEN RECEPTOR-MEDIATED KINASE ACTIVATION AND TUMOR NECROSIS FACTOR RECEPTOR-ASSOCIATED FACTOR REGULATION. Journal of Biological Chemistry 279, 2575-2582.

Hayakawa, J., Mittal, S., Wang, Y., Korkmaz, K. S., Adamson, E., English, C., Omichi, M., McClelland, M., Mercola, D., 2004. Identification of promoters bound by c-Jun/ATF2 during rapid large-scale gene activation following genotoxic stress. Molecular cell 16, 521-535.

Hayashi, M., Shimada, Y., Inomata, M., Ohno-Iwashita, Y., 2006. Detection of cholesterol-rich microdomains in the inner leaflet of the plasma membrane. Biochemical and biophysical research communications 351, 713-718.

He, L., Wu, X., Siegel, R., Lipsky, P. E., 2006. TRAF6 regulates cell fate decisions by inducing caspase 8-dependent apoptosis and the activation of NF-κB. Journal of Biological Chemistry 281, 11235-11249.

Hegde, R., Srinivasula, S. M., Zhang, Z., Wassell, R., Mukattash, R., Cilenti, L., DuBois, G., Lazebnik, Y., Zervos, A. S., Fernandes-Alnemri, T., 2002. Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. Journal of Biological Chemistry 277, 432-438.

Heidenreich, A., Bastian, P. J., Bellmunt, J., Bolla, M., Joniau, S., van der Kwast, T., Mason, M., Matveev, V., Wiegel, T., Zattoni, F., 2014. EAU guidelines on prostate cancer. Part 1: screening, diagnosis, and local treatment with curative intent—update 2013. European urology 65, 124-137.

Heiskanen, K. M., Bhat, M. B., Wang, H.-W., Ma, J., Nieminen, A.-L., 1999. Mitochondrial Depolarization Accompanies Cytochrome cRelease During Apoptosis in PC6 Cells. Journal of Biological Chemistry 274, 5654-5658.

Hellman, P., Eriksson, H., 2007. Early activation markers of human peripheral dendritic cells. Human immunology 68, 324-333.

Hengartner, M. O., 2000. The biochemistry of apoptosis. Nature 407, 770-776.

Henkler, F., Baumann, B., Fotin-Mleczek, M., Weingärtner, M., Schwenzer, R., Peters, N., Graness, A., Wirth, T., Scheurich, P., Schmid, J. A., 2003. Caspase-mediated cleavage converts the tumor necrosis factor (TNF) receptor-associated factor (TRAF)-1 from a selective modulator of TNF receptor signaling to a general inhibitor of NF-κB activation. Journal of Biological Chemistry 278, 29216-29230.

Henn, V., Slupsky, J. R., Gräfe, M., Anagnostopoulos, I., Förster, R., Müller-Berghaus, G., Kroczek, R. A., 1998. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. Nature 391, 591-594.

Herlaar, E., Brown, Z., 1999. p38 MAPK signalling cascades in inflammatory disease. Molecular medicine today 5, 439-447.

Hermann, A., Schrör, K., Weber, A.-A., 2002. CD40 ligand (CD40L) does not stimulate proliferation of vascular smooth muscle cells. European journal of cell biology 81, 213-221.

Hernandez-Caselles, T., Stutman, O., 1993. Immune functions of tumor necrosis factor. I. Tumor necrosis factor induces apoptosis of mouse thymocytes and can also stimulate or inhibit IL-6-induced proliferation depending on the concentration of mitogenic costimulation. The Journal of Immunology 151, 3999-4012.

Hess, S., Engelmann, H., 1996. A novel function of CD40: induction of cell death in transformed cells. Journal of Experimental Medicine 183, 159-167.

Hetz, C., Glimcher, L., 2008. The daily job of night killers: alternative roles of the BCL-2 family in organelle physiology. Trends in cell biology 18, 38-44.

Hetz, C., Bernasconi, P., Fisher, J., Lee, A.-H., Bassik, M. C., Antonsson, B., Brandt, G. S., Iwakoshi, N. N., Schinzel, A., Glimcher, L. H., 2006. Proapoptotic BAX and BAK modulate the unfolded protein response by a direct interaction with IRE1α. Science 312, 572-576.

Hetz, C. A., 2007. ER stress signaling and the BCL-2 family of proteins: from adaptation to irreversible cellular damage. Antioxidants & redox signaling 9, 2345-2356.

Hill, A., Chapel, H., 1993. X-linked immunodeficiency. The fruits of cooperation. Nature 361, 494-494.

Hill, K. S., Errington, F., Steele, L. P., Merrick, A., Morgan, R., Selby, P. J., Georgopoulos, N. T., O'Donnell, D. M., Melcher, A. A., 2008. OK432-activated human dendritic cells kill tumor cells via CD40/CD40 ligand interactions. The Journal of Immunology 181, 3108-3115.

Hoeijmakers, J. H., 2009. DNA damage, aging, and cancer. New England Journal of Medicine 361, 1475-1485.

Hofmann, W. A., 2009. Cell and molecular biology of nuclear actin. International review of cell and molecular biology 273, 219-263.

Hofmann, W. A., De Lanerolle, P., 2006. Nuclear actin: to polymerize or not to polymerize. J Cell Biol 172, 495-496.

Hollenbaugh, D., Grosmaire, L. S., Kullas, C. D., Chalupny, N. J., Braesch-Andersen, S., Noelle, R. J., Stamenkovic, I., Ledbetter, J., Aruffo, A., 1992. The human T cell antigen gp39, a member of the TNF gene family, is a ligand for the CD40 receptor: expression of a soluble form of gp39 with B cell co-stimulatory activity. The EMBO journal 11, 4313.

Horton, D. B., Libby, P., Schönbeck, U., 2001. Ligation of CD40 on vascular smooth muscle cells mediates loss of interstitial collagen via matrix metalloproteinase activity. Annals of the New York Academy of Sciences 947, 329-336.

Hostager, B. S., Bishop, G. A., 1999. Cutting edge: contrasting roles of TNF receptor-associated factor 2 (TRAF2) and TRAF3 in CD40-activated B lymphocyte differentiation. The Journal of Immunology 162, 6307-6311.

Hostager, B. S., Catlett, I. M., Bishop, G. A., 2000. Recruitment of CD40 and tumor necrosis factor receptor-associated factors 2 and 3 to membrane microdomains during CD40 signaling. Journal of Biological Chemistry 275, 15392-15398.

Hostager, B. S., Haxhinasto, S. A., Rowland, S. L., Bishop, G. A., 2003. Tumor necrosis factor receptor-associated factor 2 (TRAF2)-deficient B lymphocytes reveal novel roles for TRAF2 in CD40 signaling. Journal of Biological Chemistry 278, 45382-45390.

Hsing, Y., Hostager, B. S., Bishop, G. A., 1997. Characterization of CD40 signaling determinants regulating nuclear factor-kappa B activation in B lymphocytes. The Journal of Immunology 159, 4898-4906.

Hsu, H., Xiong, J., Goeddel, D. V., 1995. The TNF receptor 1-associated protein TRADD signals cell death and NF-κB activation. Cell 81, 495-504.

Hsu, H., Shu, H.-B., Pan, M.-G., Goeddel, D. V., 1996. TRADD–TRAF2 and TRADD–FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell 84, 299-308.

Hu, Y., Ding, L., Spencer, D. M., Núñez, G., 1998. WD-40 repeat region regulates Apaf-1 self-association and procaspase-9 activation. Journal of Biological Chemistry 273, 33489-33494.

Huang, J., Jochems, C., Talaie, T., Anderson, A., Jales, A., Tsang, K. Y., Madan, R. A., Gulley, J. L., Schlom, J., 2012. Elevated serum soluble CD40 ligand in cancer patients may play an immunosuppressive role. Blood 120, 3030-3038.

Hussein, M., Berenson, J. R., Niesvizky, R., Munshi, N., Matous, J., Sobecks, R., Harrop, K., Drachman, J. G., Whiting, N., 2010. A phase I multidose study of dacetuzumab (SGN-40; humanized anti-CD40 monoclonal antibody) in patients with multiple myeloma. Haematologica 95, 845-848.

Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K., Gotoh, Y., 1997. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science 275, 90-94.

Iezzi, G., Sonderegger, I., Ampenberger, F., Schmitz, N., Marsland, B. J., Kopf, M., 2009. CD40–CD40L cross-talk integrates strong antigenic signals and microbial stimuli to induce development of IL-17-producing CD4+ T cells. Proceedings of the National Academy of Sciences 106, 876-881.

Igney, F. H., Krammer, P. H., 2002. Death and anti-death: tumour resistance to apoptosis. Nature Reviews Cancer 2, 277-288.

Ishida, T., ToJo, T., Aoki, T., Kobayashi, N., Ohishi, T., Watanabe, T., Yamamoto, T., Inoue, J.-I., 1996a. TRAF5, a novel tumor necrosis factor receptor-associated factor family protein, mediates CD40 signaling. Proceedings of the National Academy of Sciences 93, 9437-9442.

Ishida, T., Mizushima, S.-i., Azuma, S., Kobayashi, N., Tojo, T., Suzuki, K., Aizawa, S., Watanabe, T., Mosialos, G., Kieff, E., 1996b. Identification of TRAF6, a novel tumor necrosis factor receptor-associated factor protein that mediates signaling from an amino-terminal domain of the CD40 cytoplasmic region. Journal of Biological Chemistry 271, 28745-28748.

Izumi, K. M., McFarland, E. C., Ting, A. T., Riley, E. A., Seed, B., Kieff, E. D., 1999. The Epstein-Barr virus oncoprotein latent membrane protein 1 engages the tumor necrosis factor receptor-associated proteins TRADD and receptor-interacting protein (RIP) but does not induce apoptosis or require RIP for NF-κB activation. Molecular and cellular biology 19, 5759-5767.

Jabara, H. H., Laouini, D., Tsitsikov, E., Mizoguchi, E., Bhan, A. K., Castigli, E., Dedeoglu, F., Pivniouk, V., Brodeur, S. R., Geha, R. S., 2002. The binding site for TRAF2 and TRAF3 but not for TRAF6 is essential for CD40-mediated immunoglobulin class switching. Immunity 17, 265-276.

Janeway Jr, C. A., Travers, P., Walport, M., Shlomchik, M. J., 2001. Macrophage activation by armed CD4 TH1 cells.

Jänicke, R. U., Sprengart, M. L., Wati, M. R., Porter, A. G., 1998. Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. Journal of Biological Chemistry 273, 9357-9360.

Jaworska, D., Król, W., Szliszka, E., 2015. Prostate cancer stem cells: research advances. International journal of molecular sciences 16, 27433-27449.

Jiang, E., He, X., Chen, X., Sun, G., Wu, H., Wei, Y., Zhao, X., 2007. Expression of CD40 in ovarian cancer and adenovirus-mediated CD40 ligand therapy on ovarian cancer in vitro. Tumori 94, 356-361.

Jiang, X., Wang, X., 2000. Cytochrome c promotes caspase-9 activation by inducing nucleotide binding to Apaf-1. Journal of Biological Chemistry 275, 31199-31203.

Jin, H. O., Park, I. C., An, S., Lee, H. C., Woo, S. H., Hong, Y. J., Lee, S. J., Park, M. J., Yoo, D. H., Rhee, C. H., 2006. Up-regulation of Bak and Bim via JNK downstream pathway in the response to nitric oxide in human glioblastoma cells. Journal of cellular physiology 206, 477-486.

Johnson, D., Lanahan, A., Buck, C. R., Sehgal, A., Morgan, C., Mercer, E., Bothwell, M., Chao, M., 1986. Expression and structure of the human NGF receptor. Cell 47, 545-554.

Johnson, G. L., Lapadat, R., 2002. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 298, 1911-1912.

Jomova, K., Valko, M., 2011. Advances in metal-induced oxidative stress and human disease. Toxicology 283, 65-87.

Jürgensmeier, J. M., Xie, Z., Deveraux, Q., Ellerby, L., Bredesen, D., Reed, J. C., 1998. Bax directly induces release of cytochrome c from isolated mitochondria. Proceedings of the National Academy of Sciences 95, 4997-5002.

Kadowaki, H., Nishitoh, H., Urano, F., Sadamitsu, C., Matsuzawa, A., Takeda, K., Masutani, H., Yodoi, J., Urano, Y., Nagano, T., 2005. Amyloid β induces neuronal cell death through ROS-mediated ASK1 activation. Cell Death & Differentiation 12, 19-24.

Kaestner, L., 2016. Prostate cancer update: men's health-prostate cancer. The Specialist Forum, Vol. 16. New Media Publishing, pp. 10-12.

Kamata, H., Honda, S.-i., Maeda, S., Chang, L., Hirata, H., Karin, M., 2005. Reactive oxygen species promote TNFα-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. Cell 120, 649-661.

Kang, J.-O., Sucov, H. M., 2005. Convergent proliferative response and divergent morphogenic pathways induced by epicardial and endocardial signaling in fetal heart development. Mechanisms of development 122, 57-65.

Karin, M., Lin, A., 2002. NF-κB at the crossroads of life and death. Nature immunology 3, 221-227.

Karin, M., Gallagher, E., 2005. From JNK to pay dirt: jun kinases, their biochemistry, physiology and clinical importance. IUBMB life 57, 283-295.

Karmann, K., Hughes, C., Schechner, J., Fanslow, W. C., Pober, J. S., 1995. CD40 on human endothelial cells: inducibility by cytokines and functional regulation of adhesion molecule expression. Proceedings of the National Academy of Sciences 92, 4342-4346.

Karpusas, M., Hsu, Y.-M., Wang, J.-h., Thompson, J., Lederman, S., Chess, L., Thomas, D., 1995. 2 Å crystal structure of an extracellular fragment of human CD40 ligand. Structure 3, 1031-1039.

Kataoka, T., Budd, R., Holler, N., Thome, M., Martinon, F., Irmler, M., Burns, K., Hahne, M., Kennedy, N., Kovacsovics, M., 2000. The caspase-8 inhibitor FLIP promotes activation of NF-κB and Erk signaling pathways. Current Biology 10, 640-648.

Kaufmann, S. H., Mesner Jr, P. W., Samejima, K., Toné, S., Earnshaw, W. C., 1999. Detection of DNA cleavage in apoptotic cells. Methods in enzymology 322, 3-15.

Khanna, R., Cooper, L., Kienzle, N., Moss, D. J., Burrows, S. R., Khanna, K. K., 1997. Engagement of CD40 antigen with soluble CD40 ligand up-regulates peptide transporter expression and restores endogenous processing function in Burkitt's lymphoma cells. The Journal of Immunology 159, 5782-5785.

Khong, A., Brown, M. D., Vivian, J. B., Robinson, B. W., Currie, A. J., 2013. Agonistic anti-CD40 antibody therapy is effective against postoperative cancer recurrence and metastasis in a murine tumor model. Journal of Immunotherapy 36, 365-372.

Kielian, T., Nagai, E., Ikubo, A., Rasmussen, C. A., Suzuki, T., 1999. Granulocyte/macrophage-colony-stimulating factor released by adenovirally transduced CT26 cells leads to the local expression of macrophage inflammatory protein 1α and accumulation of dendritic cells at vaccination sites in vivo. Cancer Immunology, Immunotherapy 48, 123-131.

Kiener, P. A., Moran-Davis, P., Rankin, B. M., Wahl, A. F., Aruffo, A., Hollenbaugh, D., 1995. Stimulation of CD40 with purified soluble gp39 induces proinflammatory responses in human monocytes. The Journal of Immunology 155, 4917-4925.

Kim, D.-S., Kim, J.-H., Lee, G.-H., Kim, H.-T., Lim, J. M., Chae, S.-W., Chae, H.-J., Kim, H.-R., 2010. p38 Mitogen-activated protein kinase is involved in endoplasmic reticulum stress-induced cell death and autophagy in human gingival fibroblasts. Biological and Pharmaceutical Bulletin 33, 545-549.

Kim, D. H., Feinbaum, R., Alloing, G., Emerson, F. E., Garsin, D. A., Inoue, H., Tanaka-Hino, M., Hisamoto, N., Matsumoto, K., Tan, M.-W., 2002. A conserved p38 MAP kinase pathway in Caenorhabditis elegans innate immunity. Science 297, 623-626.

Kim, H. S., Cho, J. W., Hidaka, K., Morisaki, T., 2007. Activation of MEK–ERK by heregulin-β1 promotes the development of cardiomyocytes derived from ES cells. Biochemical and biophysical research communications 361, 732-738.

Kim, S. D., Moon, C. K., Eun, S.-Y., Ryu, P. D., Jo, S. A., 2005a. Identification of ASK1, MKK4, JNK, c-Jun, and caspase-3 as a signaling cascade involved in cadmium-induced neuronal cell apoptosis. Biochemical and biophysical research communications 328, 326-334.

Kim, Y. K., Kim, H. J., Kwon, C. H., Kim, J. H., Woo, J. S., Jung, J. S., Kim, J. M., 2005b. Role of ERK activation in cisplatin-induced apoptosis in OK renal epithelial cells. Journal of Applied Toxicology 25, 374-382.

Kinnally, K. W., Antonsson, B., 2007. A tale of two mitochondrial channels, MAC and PTP, in apoptosis. Apoptosis 12, 857-868.

Kishimoto, T., 1989. The biology of interleukin-6. Blood 74, 1-10.

Klaus, S. J., Berberich, I., Shu, G., Clark, E. A., 1994. CD40 and its ligand in the regulation of humoral immunity. Seminars in immunology, Vol. 6. Elsevier, pp. 279-286.

Klee, M., Pallauf, K., Alcalá, S., Fleischer, A., Pimentel-Muiños, F. X., 2009. Mitochondrial apoptosis induced by BH3-only molecules in the exclusive presence of endoplasmic reticular Bak. The EMBO journal 28, 1757-1768.

Kluck, R. M., Bossy-Wetzel, E., Green, D. R., Newmeyer, D. D., 1997. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 275, 1132-1136.

Knox, P. G., Davies, C. C., Ioannou, M., Eliopoulos, A. G., 2011. The death domain kinase RIP1 links the immunoregulatory CD40 receptor to apoptotic signaling in carcinomas. The Journal of cell biology 192, 391-399.

Koho, H., Paulie, S., Ben-Aissa, H., Jónsdóttir, I., Hansson, Y., Lundblad, M.-L., Perlmann, P., 1984. Monoclonal antibodies to antigens associated with transitional cell carcinoma of the human urinary bladder. Cancer Immunology, Immunotherapy 17, 165-172.

Kontoyiannis, D., Kotlyarov, A., Carballo, E., Alexopoulou, L., Blackshear, P. J., Gaestel, M., Davis, R., Flavell, R., Kollias, G., 2001. Interleukin-10 targets p38 MAPK to modulate ARE-dependent TNF mRNA translation and limit intestinal pathology. The EMBO journal 20, 3760-3770.

Koppold, B., Sauer, G., Buening, H., Hallek, M., Kreienberg, R., Deissler, H., Kurzeder, C., 2006. Chemotherapeutic agents enhance AAV2-mediated gene transfer into breast cancer cells promoting CD40 ligand-based immunotherapy. Journal of cancer research and clinical oncology 132, 787-794.

Korniluk, A., Kemona, H., Dymicka-Piekarska, V., 2014. Multifunctional CD40L: pro- and anti-neoplastic activity. Tumor Biology, 1-11.

Korthäuer, U., Graf, D., Mages, H. W., Brière, F., Padayachee, M., Malcolm, S., Ugazio, A. G., Notarangelo, L. D., Levinsky, R. J., Kroczek, R. A., 1993. Defective expression of T-cell CD40 ligand causes X-linked immunodeficiency with hyper-IgM.

Kothakota, S., Azuma, T., Reinhard, C., Klippel, A., Tang, J., Chu, K., McGarry, T. J., Kirschner, M. W., Koths, K., Kwiatkowski, D. J., 1997. Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. Science 278, 294-298.

Koul, H. K., Pal, M., Koul, S., 2013. Role of p38 MAP kinase signal transduction in solid tumors. Genes & cancer 4, 342-359.

Kourilsky, P., Truffa-Bachi, P., 2001. Cytokine fields and the polarization of the immune response. TRENDS in Immunology 22, 502-509.

Krajewski, S., Tanaka, S., Takayama, S., Schibler, M. J., Fenton, W., Reed, J. C., 1993. Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. Cancer research 53, 4701-4714.

Kroemer, G., Martin, S. J., 2005. Caspase-independent cell death. Nature medicine 11, 725-730.

Kroemer, G., Zamzami, N., Susin, S. A., 1997. Mitochondrial control of apoptosis. Immunology today 18, 44-51.

Kroemer, G., Galluzzi, L., Brenner, C., 2007. Mitochondrial membrane permeabilization in cell death. Physiological reviews 87, 99-163.

Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E. S., Baehrecke, E., Blagosklonny, M., El-Deiry, W., Golstein, P., Green, D., 2008. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death & Differentiation 16, 3-11.

Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E., Baehrecke, E., Blagosklonny, M., El-Deiry, W., Golstein, P., Green, D., 2009. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell death & differentiation 16, 3-11.

Krueger, A., Baumann, S., Krammer, P. H., Kirchhoff, S., 2001. FLICE-inhibitory proteins: regulators of death receptor-mediated apoptosis. Molecular and cellular biology 21, 8247-8254.

Kültz, D., Avila, K., 2001. Mitogen-activated protein kinases are in vivo transducers of osmosensory signals in fish gill cells. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 129, 821-829.

Kyriakis, J. M., Avruch, J., 1990. pp54 microtubule-associated protein 2 kinase. A novel serine/threonine protein kinase regulated by phosphorylation and stimulated by poly-L-lysine. Journal of Biological Chemistry 265, 17355-17363.

Kyriakis, J. M., Avruch, J., 2001. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiological reviews 81, 807-869.

Kyriakis, J. M., Avruch, J., 2012. Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update. Physiological reviews 92, 689-737.

Lee, H.-J., Wang, C.-J., Kuo, H.-C., Chou, F.-P., Jean, L.-F., Tseng, T.-H., 2005. Induction apoptosis of luteolin in human hepatoma HepG2 cells involving mitochondria translocation of Bax/Bak and activation of JNK. Toxicology and applied pharmacology 203, 124-131.

Lennicke, C., Rahn, J., Lichtenfels, R., Wessjohann, L. A., Seliger, B., 2015. Hydrogen peroxide–production, fate and role in redox signaling of tumor cells. Cell Communication and Signaling 13, 39.

Leo, E., Welsh, K., Matsuzawa, S.-i., Zapata, J. M., Kitada, S., Mitchell, R. S., Ely, K. R., Reed, J. C., 1999. Differential requirements for tumor necrosis factor receptor-associated factor family proteins in CD40-mediated induction of NF-κB and Jun N-terminal kinase activation. Journal of Biological Chemistry 274, 22414-22422.

Leo, E., Deveraux, Q. L., Buchholtz, C., Welsh, K., Matsuzawa, S.-i., Stennicke, H. R., Salvesen, G. S., Reed, J. C., 2001. TRAF1 is a substrate of caspases activated during tumor necrosis factor receptor-α-induced apoptosis. Journal of Biological Chemistry 276, 8087-8093.

Léveillé, C., Bouillon, M., Guo, W., Bolduc, J., Sharif-Askari, E., El-Fakhry, Y., Reyes-Moreno, C., Lapointe, R., Merhi, Y., Wilkins, J. A., 2007. CD40 ligand binds to α5β1 integrin and triggers cell signaling. Journal of Biological Chemistry 282, 5143-5151.

Levine, B., Deretic, V., 2007. Unveiling the roles of autophagy in innate and adaptive immunity. Nature Reviews Immunology 7, 767-777.

Levine, B., Sinha, S. C., Kroemer, G., 2008. Bcl-2 family members: dual regulators of apoptosis and autophagy. Autophagy 4, 600-606.

Lewis, T. S., McCormick, R. S., Emmerton, K., Lau, J. T., Yu, S.-F., McEarchern, J. A., Grewal, I. S., Law, C.-L., 2011. Distinct apoptotic signaling characteristics of the anti-CD40 monoclonal antibody dacetuzumab and rituximab produce enhanced antitumor activity in non-Hodgkin lymphoma. Clinical Cancer Research 17, 4672-4681.

Li, G., Sanders, J. M., Bevard, M. H., Sun, Z., Chumley, J. W., Galkina, E. V., Ley, K., Sarembock, I. J., 2008. CD40 ligand promotes Mac-1 expression, leukocyte recruitment, and neointima formation after vascular injury. The American journal of pathology 172, 1141-1152.

Li, H., Zhu, H., Xu, C.-j., Yuan, J., 1998. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 94, 491-501.

Li, J., Yuan, J., 2008. Caspases in apoptosis and beyond. Oncogene 27, 6194-6206.

Li, K., Li, Y., Shelton, J. M., Richardson, J. A., Spencer, E., Chen, Z. J., Wang, X., Williams, R. S., 2000. Cytochrome c deficiency causes embryonic lethality and attenuates stress-induced apoptosis. Cell 101, 389-399.

Li, L. Y., Luo, X., Wang, X., 2001. Endonuclease G is an apoptotic DNase when released from mitochondria. Nature 412, 95-99.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., Wang, X., 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91, 479-489.

Liang, H., Russell, R. S., Yonkers, N. L., McDonald, D., Rodriguez, B., Harding, C. V., Anthony, D. D., 2009. Differential effects of hepatitis C virus JFH1 on human myeloid and plasmacytoid dendritic cells. Journal of virology 83, 5693-5707.

Lievens, D., Eijgelaar, W. J., Biessen, E., Daemen, M., Lutgens, E., 2009. The multi-functionality of CD40L and its receptor CD40 in atherosclerosis. Thromb Haemost 102, 206-14.

Linette, G. P., Li, Y., RoTH, K., Korsmeyer, S. J., 1996. Cross talk between cell death and cell cycle progression: BCL-2 regulates NFAT-mediated activation. Proceedings of the National Academy of Sciences 93, 9545-9552.

Lingwood, D., Simons, K., 2010. Lipid rafts as a membrane-organizing principle. science 327, 46-50.

Lippens, S., VandenBroecke, C., Van Damme, E., Tschachler, E., Vandenabeele, P., Declercq, W., 2003. Caspase-14 is expressed in the epidermis, the choroid plexus, the retinal pigment epithelium and thymic Hassall's bodies. Cell Death & Differentiation 10.

Liston, P., Fong, W. G., Kelly, N. L., Toji, S., Miyazaki, T., Conte, D., Tamai, K., Craig, C. G., McBurney, M. W., Korneluk, R. G., 2001. Identification of XAF1 as an antagonist of XIAP anti-Caspase activity. Nature cell biology 3, 128-133.

Liu, Y., Min, W., 2002. Thioredoxin promotes ASK1 ubiquitination and degradation to inhibit ASK1-mediated apoptosis in a redox activity-independent manner. Circulation research 90, 1259-1266.

Locksley, R. M., Killeen, N., Lenardo, M. J., 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell 104, 487-501.

Longthorne, V. L., Williams, G. T., 1997. Caspase activity is required for commitment to Fas-mediated apoptosis. The EMBO journal 16, 3805-3812.

Lorenzo, H. K., Susin, S. A., Penninger, J., Kroemer, G., 1999. Apoptosis inducing factor (AIF): a phylogenetically old, caspase-independent effector of cell death. Cell death and differentiation 6, 516-524.

Loskog, A. S., Eliopoulos, A. G., 2009. The Janus faces of CD40 in cancer. Seminars in immunology, Vol. 21. Elsevier, pp. 301-307.

Lu, J., Holmgren, A., 2014. The thioredoxin antioxidant system. Free Radical Biology and Medicine 66, 75-87.

Luo, X., Budihardjo, I., Zou, H., Slaughter, C., Wang, X., 1998. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell 94, 481-490.

Ma, D. Y., Clark, E. A., 2009. The role of CD40 and CD154/CD40L in dendritic cells. Seminars in immunology, Vol. 21. Elsevier, pp. 265-272.

MacFarlane, M., Williams, A. C., 2004. Apoptosis and disease: a life or death decision. EMBO reports 5, 674-678.

Mach, F., Schönbeck, U., Bonnefoy, J.-Y., Pober, J. S., Libby, P., 1997. Activation of monocyte/macrophage functions related to acute atheroma complication by

ligation of CD40 induction of collagenase, stromelysin, and tissue factor. Circulation 96, 396-399.

Mach, F., Schönbeck, U., Fabunmi, R. P., Murphy, C., Atkinson, E., Bonnefoy, J.-Y., Graber, P., Libby, P., 1999. T lymphocytes induce endothelial cell matrix metalloproteinase expression by a CD40L-dependent mechanism: implications for tubule formation. The American journal of pathology 154, 229-238.

Machlis, G. E., Force, J. E., Burch Jr, W. R., 1997. The human ecosystem part I: the human ecosystem as an organizing concept in ecosystem management. Society & Natural Resources 10, 347-367.

Mackey, M. F., Wang, Z., Eichelberg, K., Germain, R. N., 2003. Distinct contributions of different CD40 TRAF binding sites to CD154-induced dendritic cell maturation and IL-12 secretion. European journal of immunology 33, 779-789.

Maitland, N. J., Frame, F. M., Polson, E. S., Lewis, J. L., Collins, A. T., 2011. Prostate cancer stem cells: do they have a basal or luminal phenotype? Hormones and Cancer 2, 47-61.

Malapati, S., Pierce, S. K., 2001. The influence of CD40 on the association of the B cell antigen receptor with lipid rafts in mature and immature cells. European journal of immunology 31, 3789-3797.

Malik, N., Greenfield, B. W., Wahl, A. F., Kiener, P. A., 1996. Activation of human monocytes through CD40 induces matrix metalloproteinases. The Journal of Immunology 156, 3952-3960.

Mansell, A., Jenkins, B. J., 2013. Dangerous liaisons between interleukin-6 cytokine and toll-like receptor families: a potent combination in inflammation and cancer. Cytokine & growth factor reviews 24, 249-256.

Mariani, S. M., Matiba, B., Armandola, E. A., Krammer, P. H., 1997. Interleukin 1β-converting enzyme related proteases/caspases are involved in TRAIL-induced apoptosis of myeloma and leukemia cells. The Journal of cell biology 137, 221-229.

Martinez, F. O., Sironi, M., Vecchi, A., Colotta, F., Mantovani, A., Locati, M., 2004. IL-8 induces a specific transcriptional profile in human neutrophils: synergism with LPS for IL-1 production. European journal of immunology 34, 2286-2292.

Martinou, J.-C., Youle, R. J., 2011. Mitochondria in apoptosis: Bcl-2 family members and mitochondrial dynamics. Developmental cell 21, 92-101.

Martinou, J.-C., Desagher, S., Antonsson, B., 2000. Cytochrome c release from mitochondria: all or nothing. Nature cell biology 2, E41-E43.

Martins, L. M., Iaccarino, I., Tenev, T., Gschmeissner, S., Totty, N. F., Lemoine, N. R., Savopoulos, J., Gray, C. W., Creasy, C. L., Dingwall, C., 2002. The serine protease Omi/HtrA2 regulates apoptosis by binding XIAP through a reaper-like motif. Journal of Biological Chemistry 277, 439-444.

Marzo, I., Brenner, C., Zamzami, N., Jürgensmeier, J. M., Susin, S. A., Vieira, H. L., Prévost, M.-C., Xie, Z., Matsuyama, S., Reed, J. C., 1998. Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. Science 281, 2027-2031.

Matés, J. M., Segura, J. A., Alonso, F. J., Márquez, J., 2010. Roles of dioxins and heavy metals in cancer and neurological diseases using ROS-mediated mechanisms. Free Radical Biology and Medicine 49, 1328-1341.

Matés, J. M., Segura, J. A., Alonso, F. J., Márquez, J., 2012. Oxidative stress in apoptosis and cancer: an update. Archives of toxicology 86, 1649-1665.

Matsuura, H., Nishitoh, H., Takeda, K., Matsuzawa, A., Amagasa, T., Ito, M., Yoshioka, K., Ichijo, H., 2002. Phosphorylation-dependent Scaffolding Role of JSAP1/JIP3 in the ASK1-JNK Signaling Pathway A NEW MODE OF REGULATION OF THE MAP KINASE CASCADE. Journal of Biological Chemistry 277, 40703-40709.

Matsuzawa, A., Ichijo, H., 2001. Molecular mechanisms of the decision between life and death: regulation of apoptosis by apoptosis signal-regulating kinase. The Journal of Biochemistry 130, 1-8.

Mazel, S., Burtrum, D., Petrie, H. T., 1996. Regulation of cell division cycle progression by bcl-2 expression: a potential mechanism for inhibition of programmed cell death. The Journal of experimental medicine 183, 2219-2226.

Meager, A., 1991. A cytotoxicity assay for tumour necrosis using a human rhabdomyosarcoma cell line. Journal of immunological methods 144, 141-143.

Mebratu, Y., Tesfaigzi, Y., 2009. How ERK1/2 activation controls cell proliferation and cell death: Is subcellular localization the answer? Cell cycle 8, 1168-1175.

Melter, M., Reinders, M. E., Sho, M., Pal, S., Geehan, C., Denton, M. D., Mukhopadhyay, D., Briscoe, D. M., 2000. Ligation of CD40 induces the expression of vascular endothelial growth factor by endothelial cells and monocytes and promotes angiogenesis in vivo. Blood 96, 3801-3808.

Metcalf, D., 1993. Hematopoietic regulators: redundancy or subtlety? BLOOD-NEW YORK- 82, 3515-3515.

Metcalf, D., 2008. Hematopoietic cytokines. Blood 111, 485-491.

Mihov, B., 2008. Biochemistry of apoptosis. Meditsinski Pregled/Medical Review 44, 14-19.

Milhas, D., Cuvillier, O., Therville, N., Clavé, P., Thomsen, M., Levade, T., Benoist, H., Ségui, B., 2005. Caspase-10 triggers Bid cleavage and caspase cascade activation in FasL-induced apoptosis. Journal of Biological Chemistry 280, 19836-19842.

Miura, M., Zhu, H., Rotello, R., Hartwieg, E. A., Yuan, J., 1993. Induction of apoptosis in fibroblasts by IL-1β-converting enzyme, a mammalian homolog of the C. elegans cell death gene ced-3. Cell 75, 653-660.

Moghaddami, M., Cohen, P., Stapleton, A. M., Brown, M. P., 2001. CD40 is not detected on human prostate cancer cells by immunohistologic techniques. Urology 57, 573-578.

Moll, R., Franke, W. W., Schiller, D. L., Geiger, B., Krepler, R., 1982. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell 31, 11-24.

Moore, C. R., Bishop, G. A., 2005. Differential regulation of CD40-mediated TNF receptor-associated factor degradation in B lymphocytes. The Journal of Immunology 175, 3780-3789.

Moran, A. E., Kovacsovics-Bankowski, M., Weinberg, A. D., 2013. The TNFRs OX40, 4-1BB, and CD40 as targets for cancer immunotherapy. Current opinion in immunology 25, 230-237.

Mottet, N., Bellmunt, J., Bolla, M., Briers, E., Cumberbatch, M. G., De Santis, M., Fossati, N., Gross, T., Henry, A. M., Joniau, S., 2017. EAU-ESTRO-SIOG guidelines on prostate cancer. Part 1: screening, diagnosis, and local treatment with curative intent. European urology 71, 618-629.

Mukundan, L., Milhorn, D. M., Matta, B., Suttles, J., 2004. CD40-mediated activation of vascular smooth muscle cell chemokine production through a Src-initiated, MAPK-dependent pathway. Cellular signalling 16, 375-384.

Mukundan, L., Bishop, G. A., Head, K. Z., Zhang, L., Wahl, L. M., Suttles, J., 2005. TNF receptor-associated factor 6 is an essential mediator of CD40-activated proinflammatory pathways in monocytes and macrophages. The Journal of Immunology 174, 1081-1090.

Muppidi, J. R., Siegel, R. M., 2004. Ligand-independent redistribution of Fas (CD95) into lipid rafts mediates clonotypic T cell death. Nature immunology 5, 182-189.

Naismith, J. H., Sprang, S. R., 1998. Modularity in the TNF-receptor family. Trends in biochemical sciences 23, 74-79.

Najakshin, A. M., Mechetina, L. V., Alabyev, B. Y., Taranin, A. V., 1999. Identification of an IL-8 homolog in lamprey(Lampetra fluviatilis): early evolutionary divergence of chemokines. European journal of immunology 29, 375-382.

Nakano, H., Oshima, H., Chung, W., Williams-Abbott, L., Ware, C. F., Yagita, H., Okumura, K., 1996. TRAF5, an activator of NF-κB and putative signal transducer for the lymphotoxin-β receptor. Journal of Biological Chemistry 271, 14661-14664.

Nakano, H., Sakon, S., Koseki, H., Takemori, T., Tada, K., Matsumoto, M., Munechika, E., Sakai, T., Shirasawa, T., Akiba, H., 1999. Targeted disruption of Traf5 gene causes defects in CD40-and CD27-mediated lymphocyte activation. Proceedings of the National Academy of Sciences 96, 9803-9808.

Nechushtan, A., Smith, C. L., Lamensdorf, I., Yoon, S.-H., Youle, R. J., 2001. Bax and Bak coalesce into novel mitochondria-associated clusters during apoptosis. The Journal of cell biology 153, 1265-1276.

Newby, A. C., 2007. Metalloproteinases and vulnerable atherosclerotic plaques. Trends in cardiovascular medicine 17, 253-258.

Niles, A. L., Moravec, R. A., Hesselberth, P. E., Scurria, M. A., Daily, W. J., Riss, T. L., 2007. A homogeneous assay to measure live and dead cells in the same sample by detecting different protease markers. Analytical biochemistry 366, 197-206.

Nishina, H., Bachmann, M., Oliveira-dos-Santos, A. J., Kozieradzki, I., Fischer, K. D., Odermatt, B., Wakeham, A., Shahinian, A., Takimoto, H., Bernstein, A., 1997. Impaired CD28-mediated interleukin 2 production and proliferation in stress kinase SAPK/ERK1 kinase (SEK1)/mitogen-activated protein kinase kinase 4 (MKK4)-deficient T lymphocytes. The Journal of experimental medicine 186, 941-953.

Nishitoh, H., Saitoh, M., Mochida, Y., Takeda, K., Nakano, H., Rothe, M., Miyazono, K., Ichijo, H., 1998. ASK1 is essential for JNK/SAPK activation by TRAF2. Molecular cell 2, 389-395.

Norberg, E., Orrenius, S., Zhivotovsky, B., 2010. Mitochondrial regulation of cell death: processing of apoptosis-inducing factor (AIF). Biochemical and biophysical research communications 396, 95-100.

Nowak, G., Bakajsova, D., Clifton, G. L., 2004. Protein kinase C-ϵ modulates mitochondrial function and active Na+ transport after oxidant injury in renal cells. American Journal of Physiology-Renal Physiology 286, F307-F316.

O'Grady, J., Stewart, S., Lowrey, J., Howie, S., Krajewski, A., 1994. CD40 expression in Hodgkin's disease. The American journal of pathology 144, 21.

Ogawa, M., 1993. Differentiation and proliferation of hematopoietic stem cells. Blood 81, 2844-2853.

Ola, M. S., Nawaz, M., Ahsan, H., 2011. Role of Bcl-2 family proteins and caspases in the regulation of apoptosis. Molecular and cellular biochemistry 351, 41-58.

Orrenius, S., Gogvadze, V., Zhivotovsky, B., 2015. Calcium and mitochondria in the regulation of cell death. Biochemical and biophysical research communications 460, 72-81.

Ottaiano, A., Pisano, C., De Chiara, A., Ascierto, P. A., Botti, G., Barletta, E., Apice, G., Gridelli, C., LAFFAIOLI, V. R., 2002. CD40 activation as potential tool in malignant neoplasms. Tumori 88, 361-366.

Oyadomari, S., Araki, E., Mori, M., 2002. Endoplasmic reticulum stress-mediated apoptosis in pancreatic β-cells. Apoptosis 7, 335-345.

Pala, F. S., Gürkan, H., 2008. The role of free radicals in ethiopathogenesis of diseases. Adv Mol Biol 1, 1-9.

Paletta-Silva, R., Rocco-Machado, N., Meyer-Fernandes, J. R., 2013. NADPH oxidase biology and the regulation of tyrosine kinase receptor signaling and cancer drug cytotoxicity. International journal of molecular sciences 14, 3683-3704.

Palmer, D., Hussain, S., Ganesan, R., Cooke, P., Wallace, D., Young, L., James, N., 2004. CD40 expression in prostate cancer: a potential diagnostic and therapeutic molecule. Oncology reports 12, 679-682.

Pandey, P., Saleh, A., Nakazawa, A., Kumar, S., Srinivasula, S. M., Kumar, V., Weichselbaum, R., Nalin, C., Alnemri, E. S., Kufe, D., 2000. Negative regulation of cytochrome c-mediated oligomerization of Apaf-1 and activation of procaspase-9 by heat shock protein 90. The EMBO journal 19, 4310-4322.

Papoff, G., Hausler, P., Eramo, A., Pagano, M. G., Di Leve, G., Signore, A., Ruberti, G., 1999. Identification and characterization of a ligand-independent oligomerization domain in the extracellular region of the CD95 death receptor. Journal of Biological Chemistry 274, 38241-38250.

Pardo, J., Wallich, R., Martin, P., Urban, C., Rongvaux, A., Flavell, R. A., Müllbacher, A., Borner, C., Simon, M. M., 2008. Granzyme B-induced cell death exerted by ex vivo CTL: discriminating requirements for cell death and some of its signs. Cell Death & Differentiation 15, 567-579.

Parrish, A. B., Freel, C. D., Kornbluth, S., 2013. Cellular mechanisms controlling caspase activation and function. Cold Spring Harbor perspectives in biology 5, a008672.

Paulie, S., Koho, H., Ben-Aissa, H., Hansson, Y., Lundblad, M.-L., Perlmann, P., 1984. Monoclonal antibodies to antigens associated with transitional cell carcinoma of the human urinary bladder. Cancer Immunology, Immunotherapy 17, 173-179.

Paulie, S., Ehlin-Henriksson, B., Mellstedt, H., Koho, H., Ben-Aissa, H., Perlmann, P., 1985. A p50 surface antigen restricted to human urinary bladder carcinomas and B lymphocytes. Cancer Immunology, Immunotherapy 20, 23-28.

Pellat-Deceunynck, C., Bataille, R., Robillard, N., Harousseau, J., Rapp, M., Juge-Morineau, N., Wijdenes, J., Amiot, M., 1994. Expression of CD28 and CD40 in human myeloma cells: a comparative study with normal plasma cells. Blood 84, 2597-2603.

Perkins, C. L., Fang, G., Kim, C. N., Bhalla, K. N., 2000. The role of Apaf-1, caspase-9, and bid proteins in etoposide-or paclitaxel-induced mitochondrial events during apoptosis. Cancer research 60, 1645-1653.

Petros, A. M., Olejniczak, E. T., Fesik, S. W., 2004. Structural biology of the Bcl-2 family of proteins. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research 1644, 83-94.

Pham, L. V., Tamayo, A. T., Yoshimura, L. C., Lo, P., Terry, N., Reid, P. S., Ford, R. J., 2002. A CD40 signalosome anchored in lipid rafts leads to constitutive activation of NF-κB and autonomous cell growth in B cell lymphomas. Immunity 16, 37-50.

Pinchuk, L. M., Klaus, S. J., Magaletti, D. M., Pinchuk, G. V., Norsen, J. P., Clark, E. A., 1996. Functional CD40 ligand expressed by human blood dendritic cells is up-regulated by CD40 ligation. The Journal of Immunology 157, 4363-4370.

Pitti, R. M., Marsters, S. A., Ruppert, S., Donahue, C. J., Moore, A., Ashkenazi, A., 1996. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. Journal of Biological Chemistry 271, 12687-12690.

Poljsak, B., Šuput, D., Milisav, I., 2013. Achieving the balance between ROS and antioxidants: when to use the synthetic antioxidants. Oxidative medicine and cellular longevity 2013.

Porter, A. G., 1990. Human tumour necrosis factors-α and-β: Differences in their structure, expression and biological properties. FEMS microbiology immunology 2, 193-199.

Potapova, O., Basu, S., Mercola, D., Holbrook, N. J., 2001. Protective role for c-Jun in the cellular response to DNA damage. Journal of biological chemistry 276, 28546-28553.

Pound, J. D., Gordon, J., 1997. Maintenance of human germinal center B cells in vitro. Blood 89, 919-928.

Prasad, K. S., Andre, P., He, M., Bao, M., Manganello, J., Phillips, D. R., 2003. Soluble CD40 ligand induces β3 integrin tyrosine phosphorylation and triggers platelet activation by outside-in signaling. Proceedings of the National Academy of Sciences 100, 12367-12371.

Pullen, S. S., Miller, H. G., Everdeen, D. S., Dang, T. T., Crute, J. J., Kehry, M. R., 1998. CD40-tumor necrosis factor receptor-associated factor (TRAF) interactions: regulation of CD40 signaling through multiple TRAF binding sites and TRAF hetero-oligomerization. Biochemistry 37, 11836-11845.

Pulverer, B. J., Kyriakis, J. M., Avruch, J., Nikolakaki, E., Woodgett, J. R., 1991. Phosphorylation of c-jun mediated by MAP kinases. Nature 353, 670-674.

Puthalakath, H., Huang, D. C., O'Reilly, L. A., King, S. M., Strasser, A., 1999. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. Molecular cell 3, 287-296.

Radeke, M. J., Misko, T. P., Hsu, C., Herzenberg, L. A., Shooter, E. M., 1987. Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature 325, 593-597.

Raman, M., Chen, W., Cobb, M., 2007. Differential regulation and properties of MAPKs. Oncogene 26, 3100-3112.

Ravagnan, L., Roumier, T., Kroemer, G., 2002. Mitochondria, the killer organelles and their weapons. Journal of cellular physiology 192, 131-137.

Ravagnan, L., Gurbuxani, S., Susin, S. A., Maisse, C., Daugas, E., Zamzami, N., Mak, T., Jäättelä, M., Penninger, J. M., Garrido, C., 2001. Heat-shock protein 70 antagonizes apoptosis-inducing factor. Nature cell biology 3, 839-843.

Ray, P. D., Huang, B.-W., Tsuji, Y., 2012. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cellular signalling 24, 981-990.

Reed, J. C., 2000. Mechanisms of apoptosis. The American journal of pathology 157, 1415-1430.

Régnier, C. H., Tomasetto, C., Moog-Lutz, C., Chenard, M.-P., Wendling, C., Basset, P., Rio, M.-C., 1995. Presence of a new conserved domain in CART1, a novel member of the tumor necrosis factor receptor-associated protein family, which is expressed in breast carcinoma. Journal of Biological Chemistry 270, 25715-25721.

Rissoan, M., Van Kooten, C., Chomarat, P., Galibert, L., Durand, I., THIVOLET-BEJUI, F., Miossec, P., Banchereau, J., 1996. The functional CD40 antigen of fibroblasts may contribute to the proliferation of rheumatoid synovium. Clinical & Experimental Immunology 106, 481-490.

Rizvi, M., Pathak, D., Freedman, J. E., Chakrabarti, S., 2008. CD40–CD40 ligand interactions in oxidative stress, inflammation and vascular disease. Trends in molecular medicine 14, 530-538.

Rodriguez, J., Lazebnik, Y., 1999. Caspase-9 and APAF-1 form an active holoenzyme. Genes & development 13, 3179-3184.

Rokhlin, O. W., Bishop, G. A., Hostager, B. S., Waldschmidt, T. J., Sidorenko, S. P., Pavloff, N., Kiefer, M. C., Umansky, S. R., Glover, R. A., Cohen, M. B., 1997. Fas-mediated apoptosis in human prostatic carcinoma cell lines. Cancer research 57, 1758-1768.

Rooney, I. A., Butrovich, K. D., Glass, A. A., Borboroglu, S., Benedict, C. A., Whitbeck, J. C., Cohen, G. H., Eisenberg, R. J., Ware, C. F., 2000. The lymphotoxin-β receptor is necessary and sufficient for LIGHT-mediated apoptosis of tumor cells. Journal of Biological Chemistry 275, 14307-14315.

Roskoski, R., 2012. ERK1/2 MAP kinases: structure, function, and regulation. Pharmacological research 66, 105-143.

Rossé, T., Olivier, R., Monney, L., Rager, M., Conus, S., Fellay, I., Jansen, B., Borner, C., 1998. Bcl-2 prolongs cell survival after Bax-induced release of cytochrome c. Nature 391, 496-499.

Rossomando, A. J., Payne, D. M., Weber, M. J., Sturgill, T. W., 1989. Evidence that pp42, a major tyrosine kinase target protein, is a mitogen-activated serine/threonine protein kinase. Proceedings of the National Academy of Sciences 86, 6940-6943.

Rothe, M., Sarma, V., Dixit, V. M., Goeddel, D. V., 1995a. TRAF2-mediated activation of NF-kappaB by TNF receptor 2 and CD40. Science 269, 1424.

Rothe, M., Pan, M.-G., Henzel, W. J., Ayres, T. M., Goeddel, D. V., 1995b. The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. cell 83, 1243-1252.

Rousset, F., Garcia, E., Banchereau, J., 1991. Cytokine-induced proliferation and immunoglobulin production of human B lymphocytes triggered through their CD40 antigen. The Journal of experimental medicine 173, 705-710.

Roux, P. P., Blenis, J., 2004. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiology and molecular biology reviews 68, 320-344.

Rubinstein, A. D., Kimchi, A., 2012. Life in the balance–a mechanistic view of the crosstalk between autophagy and apoptosis. J Cell Sci 125, 5259-5268.

Rudel, T., Bokoch, G. M., 1997. Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2. Science 276, 1571-1574.

Ruggiero, V., Latham, K., Baglioni, C., 1987. Cytostatic and cytotoxic activity of tumor necrosis factor on human cancer cells. The Journal of Immunology 138, 2711-2717.

Russell, P. J., Kingsley, E. A., 2003. Human prostate cancer cell lines. Prostate Cancer Methods and Protocols, 21-39.

Russo, M., Mupo, A., Spagnuolo, C., Russo, G. L., 2010. Exploring death receptor pathways as selective targets in cancer therapy. Biochemical pharmacology 80, 674-682.

Sabapathy, K., Wagner, E. F., 2004. JNK2: a negative regulator of cellular proliferation. Cell Cycle 3, 1520-1523.

Sabio, G., Davis, R. J., 2014. TNF and MAP kinase signalling pathways. Seminars in immunology, Vol. 26. Elsevier, pp. 237-245.

Sacks, D., Noben-Trauth, N., 2002. The immunology of susceptibility and resistance to Leishmania major in mice. Nature Reviews Immunology 2, 845-858.

Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., Ichijo, H., 1998. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. The EMBO journal 17, 2596-2606.

Saleh, A., Srinivasula, S. M., Balkir, L., Robbins, P. D., Alnemri, E. S., 2000. Negative regulation of the Apaf-1 apoptosome by Hsp70. Nature cell biology 2, 476-483.

Samejima, K., Tone, S., Earnshaw, W. C., 2001. CAD/DFF40 nuclease is dispensable for high molecular weight DNA cleavage and stage I chromatin condensation in apoptosis. Journal of Biological Chemistry 276, 45427-45432.

Sanjo, H., Zajonc, D. M., Braden, R., Norris, P. S., Ware, C. F., 2010. Allosteric regulation of the ubiquitin: NIK and ubiquitin: TRAF3 E3 ligases by the lymphotoxin-β receptor. Journal of Biological Chemistry 285, 17148-17155.

Sano, R., Reed, J. C., 2013. ER stress-induced cell death mechanisms. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research 1833, 3460-3470.

Saraste, A., 1999. Morphologic criteria and detection of apoptosis. Herz 24, 189-195.

Sato, M., Hata, N., Asagiri, M., Nakaya, T., Taniguchi, T., Tanaka, N., 1998. Positive feedback regulation of type I IFN genes by the IFN-inducible transcription factor IRF-7. FEBS letters 441, 106-110.

Sato, S., Sanjo, H., Takeda, K., Ninomiya-Tsuji, J., Yamamoto, M., Kawai, T., Matsumoto, K., Takeuchi, O., Akira, S., 2005. Essential function for the kinase TAK1 in innate and adaptive immune responses. Nature immunology 6, 1087-1095.

Scarlatti, F., Granata, R., Meijer, A., Codogno, P., 2009. Does autophagy have a license to kill mammalian cells? Cell Death & Differentiation 16, 12-20.

Schellenberg, B., Wang, P., Keeble, J. A., Rodriguez-Enriquez, R., Walker, S., Owens, T. W., Foster, F., Tanianis-Hughes, J., Brennan, K., Streuli, C. H., 2013. Bax exists in a dynamic equilibrium between the cytosol and mitochondria to control apoptotic priming. Molecular cell 49, 959-971.

Schendel, S. L., Montal, M., Reed, J. C., 1998. Bcl-2 family proteins as ion-channels. Cell Death & Differentiation 5.

Schlesinger, P. H., Gross, A., Yin, X.-M., Yamamoto, K., Saito, M., Waksman, G., Korsmeyer, S. J., 1997. Comparison of the ion channel characteristics of proapoptotic BAX and antiapoptotic BCL-2. Proceedings of the National Academy of Sciences 94, 11357-11362.

Schlom, J., Jochems, C., Gulley, J. L., Huang, J., 2013. The role of soluble CD40L in immunosuppression. Oncoimmunology 2, e22546.

Schönbeck, U., Libby, P., 2001. The CD40/CD154 receptor/ligand dyadRID="†" ID="†" Review. Cellular and Molecular Life Sciences CMLS 58, 4-43.

Schönbeck, U., Sukhova, G. K., Graber, P., Coulter, S., Libby, P., 1999. Augmented expression of cyclooxygenase-2 in human atherosclerotic lesions. The American journal of pathology 155, 1281-1291.

Schwabe, R. F., Schnabl, B., Kweon, Y. O., Brenner, D. A., 2001. CD40 activates NF-κB and c-Jun N-terminal kinase and enhances chemokine secretion on activated human hepatic stellate cells. The Journal of Immunology 166, 6812-6819.

Scull, C. M., Tabas, I., 2011. Mechanisms of ER stress-induced apoptosis in atherosclerosis. Arteriosclerosis, thrombosis, and vascular biology 31, 2792-2797.

Shamas-Din, A., Kale, J., Leber, B., Andrews, D. W., 2013. Mechanisms of action of Bcl-2 family proteins. Cold Spring Harbor perspectives in biology 5, a008714.

Shaulian, E., Karin, M., 2002. AP-1 as a regulator of cell life and death. Nature cell biology 4, E131-E136.

Shaw, N. J., Georgopoulos, N. T., Southgate, J., Trejdosiewicz, L. K., 2005. Effects of loss of p53 and p16 function on life span and survival of human urothelial cells. International journal of cancer 116, 634-639.

Shen, H.-M., Pervaiz, S., 2006. TNF receptor superfamily-induced cell death: redox-dependent execution. The FASEB Journal 20, 1589-1598.

Shepherd, D. M., Kerkvliet, N. I., 1999. Disruption of CD154: CD40 blocks generation of allograft immunity without affecting APC activation. The Journal of Immunology 163, 2470-2477.

Shibasaki, F., Kondo, E., Akagi, T., McKeon, F., 1997. Suppression of signalling through transcription factor NF-AT by interactions between calcineurin and Bcl-2.

Shiizaki, S., Naguro, I., Ichijo, H., 2013. Activation mechanisms of ASK1 in response to various stresses and its significance in intracellular signaling. Advances in biological regulation 53, 135-144.

Shimizu, S., Eguchi, Y., Kamiike, W., Funahashi, Y., Mignon, A., Lacronique, V., Matsuda, H., Tsujimoto, Y., 1998. Bcl-2 prevents apoptotic mitochondrial dysfunction by regulating proton flux. Proceedings of the National Academy of Sciences 95, 1455-1459.

Shinohara, H., Yano, S., Bucana, C. D., Fidler, I. J., 2000. Induction of chemokine secretion and enhancement of contact-dependent macrophage cytotoxicity by engineered expression of granulocyte-macrophage colony-stimulating factor in human colon cancer cells. The Journal of Immunology 164, 2728-2737.

Shoshan-Barmatz, V., Gincel, D., 2003. The voltage-dependent anion channel. Cell biochemistry and biophysics 39, 279-292.

Siegel, R. M., Frederiksen, J. K., Zacharias, D. A., Chan, F. K.-M., Johnson, M., Lynch, D., Tsien, R. Y., Lenardo, M. J., 2000. Fas preassociation required for apoptosis signaling and dominant inhibition by pathogenic mutations. Science 288, 2354-2357.

Singh, J., Garber, E., Vlijmen, H. V., Karpusas, M., Hsu, Y. M., Zheng, Z., Thomas, D., Naismith, J. H., 1998. The role of polar interactions in the molecular recognition of CD40L with its receptor CD40. Protein science 7, 1124-1135.

Soga, M., Matsuzawa, A., Ichijo, H., 2012. Oxidative stress-induced diseases via the ASK1 signaling pathway. International journal of cell biology 2012.

Son, Y., Cheong, Y.-K., Kim, N.-H., Chung, H.-T., Kang, D. G., Pae, H.-O., 2011. Mitogen-activated protein kinases and reactive oxygen species: how can ROS activate MAPK pathways? Journal of signal transduction 2011.

Song, H. Y., Régnier, C. H., Kirschning, C. J., Goeddel, D. V., Rothe, M., 1997. Tumor necrosis factor (TNF)-mediated kinase cascades: bifurcation of nuclear factor-κB and c-jun N-terminal kinase (JNK/SAPK) pathways at TNF receptor-associated factor 2. Proceedings of the National Academy of Sciences 94, 9792-9796.

Spriggs, M., Armitage, R., Strockbine, L., Clifford, K., Macduff, B., Sato, T., Maliszewski, C., Fanslow, W., 1992. Recombinant human CD40 ligand

stimulates B cell proliferation and immunoglobulin E secretion. The Journal of experimental medicine 176, 1543-1550.

Srinivasula, S. M., Hegde, R., Saleh, A., Datta, P., Shiozaki, E., Chai, J., Lee, R.-A., Robbins, P. D., Fernandes-Alnemri, T., Shi, Y., 2001. A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. Nature 410, 112-116.

Steele, L. P., Georgopoulos, N. T., Southgate, J., Selby, P. J., Trejdosiewicz, L. K., 2006. Differential susceptibility to TRAIL of normal versus malignant human urothelial cells. Cell Death & Differentiation 13, 1564-1576.

Su, Z., Yang, Z., Xu, Y., Chen, Y., Yu, Q., 2015. Apoptosis, autophagy, necroptosis, and cancer metastasis. Molecular cancer 14, 48.

Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., 1999. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397, 441-446.

Susin, S. A., Daugas, E., Ravagnan, L., Samejima, K., Zamzami, N., Loeffler, M., Costantini, P., Ferri, K. F., Irinopoulou, T., Prévost, M.-C., 2000. Two distinct pathways leading to nuclear apoptosis. The Journal of experimental medicine 192, 571-580.

Suttles, J., Stout, R. D., 2009. Macrophage CD40 signaling: a pivotal regulator of disease protection and pathogenesis. Seminars in immunology, Vol. 21. Elsevier, pp. 257-264.

Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K., Takahashi, R., 2001. A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. Molecular cell 8, 613-621.

Sweeney, C., McClure, G. Y., Fares, M. Y., Stone, A., Coles, B. F., Thompson, P. A., Korourian, S., Hutchins, L. F., Kadlubar, F. F., Ambrosone, C. B., 2000. Association between survival after treatment for breast cancer and glutathione S-transferase P1 Ile105Val polymorphism. Cancer Research 60, 5621-5624.

Szabó, I., Báthori, G., Wolff, D., Starc, T., Cola, C., Zoratti, M., 1995. The high-conductance channel of porin-less yeast mitochondria. Biochimica et Biophysica Acta (BBA)-Biomembranes 1235, 115-125.

Szegezdi, E., Logue, S. E., Gorman, A. M., Samali, A., 2006. Mediators of endoplasmic reticulum stress-induced apoptosis. EMBO reports 7, 880-885.

Tait, S. W., Green, D. R., 2010. Mitochondria and cell death: outer membrane permeabilization and beyond. Nature reviews. Molecular cell biology 11, 621.

Talanian, R. V., Quinlan, C., Trautz, S., Hackett, M. C., Mankovich, J. A., Banach, D., Ghayur, T., Brady, K. D., Wong, W. W., 1997. Substrate specificities of caspase family proteases. Journal of Biological Chemistry 272, 9677-9682.

Taniuchi, H., Basile, G., Taniuchi, M., Veloso, D., 1983. Evidence for formation of two thioether bonds to link heme to apocytochrome c by partially purified cytochrome c synthetase. Journal of Biological Chemistry 258, 10963-10966.

Terada, L. S., 2006. Specificity in reactive oxidant signaling: think globally, act locally. The Journal of cell biology 174, 615-623.

Thompson, C. B., 1995. Apoptosis in the pathogenesis and treatment of disease. Science 267, 1456.

Thornberry, N. A., 1998. Caspases: key mediators of apoptosis. Chemistry & biology 5, R97-R103.

Thornberry, N. A., Lazebnik, Y., 1998. Caspases: enemies within. Science 281, 1312-1316.

Timmer, J., Salvesen, G., 2007. Caspase substrates. Cell Death & Differentiation 14, 66-72.

Todt, F., Cakir, Z., Reichenbach, F., Youle, R., Edlich, F., 2013. The C-terminal helix of Bcl-xL mediates Bax retrotranslocation from the mitochondria. Cell Death & Differentiation 20, 333-342.

Todt, F., Cakir, Z., Reichenbach, F., Emschermann, F., Lauterwasser, J., Kaiser, A., Ichim, G., Tait, S. W., Frank, S., Langer, H. F., 2014. Differential retrotranslocation of mitochondrial Bax and Bak. The EMBO journal, e201488806.

Tong, A. W., Stone, M. J., 2003. Prospects for CD40-directed experimental therapy of human cancer. Cancer gene therapy 10, 1-13.

Tran, E. H., Azuma, Y.-T., Chen, M., Weston, C., Davis, R. J., Flavell, R. A., 2006. Inactivation of JNK1 enhances innate IL-10 production and dampens autoimmune inflammation in the brain. Proceedings of the National Academy of Sciences 103, 13451-13456.

Trapani, J. A., Smyth, M. J., 2002. Functional significance of the perforin/granzyme cell death pathway. Nature Reviews Immunology 2, 735-747.

Tsujimoto, Y., 2003. Cell death regulation by the Bcl-2 protein family in the mitochondria. Journal of cellular physiology 195, 158-167.

Uhlmann, E. J., D'Sa-Eipper, C., Subramanian, T., Wagner, A. J., Hay, N., Chinnadurai, G., 1996. Deletion of a nonconserved region of Bcl-2 confers a novel gain of function: suppression of apoptosis with concomitant cell proliferation. Cancer research 56, 2506-2509.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. The international journal of biochemistry & cell biology 39, 44-84.

Valladares, A., Álvarez, A. M., Ventura, J. J., Roncero, C., Benito, M., Porras, A., 2000. p38 Mitogen-activated protein kinase mediates tumor necrosis factor-α-induced apoptosis in rat fetal brown adipocytes. Endocrinology 141, 4383-4395.

Van den Eertwegh, A., Noelle, R. J., Roy, M., Shepherd, D. M., Aruffo, A., Ledbetter, J., Boersma, W., Claassen, E., 1993. In vivo CD40-gp39 interactions are essential for thymus-dependent humoral immunity. I. In vivo expression of CD40 ligand, cytokines, and antibody production delineates sites of cognate TB cell interactions. The Journal of experimental medicine 178, 1555-1565.

van Essen, D., Kikutani, H., Gray, D., 1995. CD40 ligand-transduced co-stimulation of T cells in the development of helper function. Nature 378, 620-623.

Van Eyndhoven, W., Gamper, C., Cho, E., Mackus, W., Lederman, S., 1999. TRAF-3 mRNA splice-deletion variants encode isoforms that induce NF-κB activation. Molecular immunology 36, 647-658.

van Kooten, C., Banchereau, J., 1997. Functions of CD40 on B cells, dendritic cells and other cells. Current opinion in immunology 9, 330-337.

van Kooten, C., Banchereau, J., 2000. CD40-CD40 ligand. Journal of leukocyte biology 67, 2-17.

van Loo, G., Schotte, P., Van Gurp, M., Demol, H., Hoorelbeke, B., Gevaert, K., Rodriguez, I., Ruiz-Carrillo, A., Vandekerckhove, J., Declercq, W., 2001. Endonuclease G: a mitochondrial protein released in apoptosis and involved in caspase-independent DNA degradation. Cell death and differentiation 8, 1136-1142.

Van Snick, J., 1990. Interleukin-6: an overview. Annual review of immunology 8, 253-278.

VanArsdale, T. L., VanArsdale, S. L., Force, W. R., Walter, B. N., Mosialos, G., Kieff, E., Reed, J. C., Ware, C. F., 1997. Lymphotoxin-β receptor signaling complex: role of tumor necrosis factor receptor-associated factor 3 recruitment in cell death and activation of nuclear factor κB. Proceedings of the National Academy of Sciences 94, 2460-2465.

Vandenabeele, P., Galluzzi, L., Berghe, T. V., Kroemer, G., 2010. Molecular mechanisms of necroptosis: an ordered cellular explosion. Nature reviews Molecular cell biology 11, 700-714.

Vander Heiden, M. G., Thompson, C. B., 1999. Bcl-2 proteins: regulators of apoptosis or of mitochondrial homeostasis? Nature cell biology 1, E209-E216.

Vander Heiden, M. G., Chandel, N. S., Schumacker, P. T., Thompson, C. B., 1999. Bcl-x L prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange. Molecular cell 3, 159-167.

Vanichakarn, P., Blair, P., Wu, C., Freedman, J. E., Chakrabarti, S., 2008. Neutrophil CD40 enhances platelet-mediated inflammation. Thrombosis research 122, 346-358.

Vardouli, L., Lindqvist, C., Vlahou, K., Loskog, A., Eliopoulos, A., 2009. Adenovirus delivery of human CD40 ligand gene confers direct therapeutic effects on carcinomas. Cancer gene therapy 16, 848-860.

Veldhoen, M., Hocking, R. J., Flavell, R. A., Stockinger, B., 2006. Signals mediated by transforming growth factor-β initiate autoimmune encephalomyelitis, but chronic inflammation is needed to sustain disease. Nature immunology 7, 1151-1156.

Vempati, U. D., Diaz, F., Barrientos, A., Narisawa, S., Mian, A. M., Millán, J. L., Boise, L. H., Moraes, C. T., 2007. Role of cytochrome C in apoptosis: increased sensitivity to tumor necrosis factor alpha is associated with respiratory defects but not with lack of cytochrome C release. Molecular and cellular biology 27, 1771-1783.

Verhagen, A. M., Coulson, E. J., Vaux, D. L., 2001. Inhibitor of apoptosis proteins and their relatives: IAPs and other BIRPs. Genome biology 2, 1.

Verhagen, A. M., Ekert, P. G., Pakusch, M., Silke, J., Connolly, L. M., Reid, G. E., Moritz, R. L., Simpson, R. J., Vaux, D. L., 2000. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. cell 102, 43-53.

Vidalain, P. O., Azocar, O., Servet-Delprat, C., Rabourdin-Combe, C., Gerlier, D., Manié, S., 2000. CD40 signaling in human dendritic cells is initiated within membrane rafts. The EMBO Journal 19, 3304-3313.

Vonderheide, R. H., Glennie, M. J., 2013. Agonistic CD40 antibodies and cancer therapy. Clinical Cancer Research 19, 1035-1043.

Vonderheide, R. H., Flaherty, K. T., Khalil, M., Stumacher, M. S., Bajor, D. L., Hutnick, N. A., Sullivan, P., Mahany, J. J., Gallagher, M., Kramer, A., 2007. Clinical activity and immune modulation in cancer patients treated with CP-870,893, a novel CD40 agonist monoclonal antibody. Journal of Clinical Oncology 25, 876-883.

Wagner, D. H., Stout, R. D., Suttles, J., 1994. Role of the CD40-CD40 ligand interaction in CD4+ T cell contact-dependent activation of monocyte interleukin-1 synthesis. European journal of immunology 24, 3148-3154.

Wagner, E. F., Nebreda, Á. R., 2009. Signal integration by JNK and p38 MAPK pathways in cancer development. Nature Reviews Cancer 9, 537-549.

Wajant, H., 2002. The Fas signaling pathway: more than a paradigm. Science 296, 1635-1636.

Wajant, H., 2015. Principles of antibody-mediated TNF receptor activation. Cell death and differentiation 22, 1727.

Wajant, H., Henkler, F., Scheurich, P., 2001. The TNF-receptor-associated factor family: scaffold molecules for cytokine receptors, kinases and their regulators. Cellular signalling 13, 389-400.

Walczak, H., Krammer, P. H., 2000. The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. Experimental cell research 256, 58-66.

Walke, D. W., Morgan, J. I., 2000. A comparison of the expression and properties of Apaf-1 and Apaf-1L. Brain research 886, 73-81.

Wang, D., Freeman, G. J., Levine, H., Ritz, J., Robertson, M. J., 1997. Role of the CD40 and CD95 (APO-1/Fas) antigens in the apoptosis of human B-cell malignancies. British journal of haematology 97, 409-417.

Wang, K., Yin, X.-M., Chao, D. T., Milliman, C. L., Korsmeyer, S. J., 1996. BID: a novel BH3 domain-only death agonist. Genes & development 10, 2859-2869.

Wang, L., Liu, L., Shi, Y., Cao, H., Chaturvedi, R., Calcutt, M. W., Hu, T., Ren, X., Wilson, K. T., Polk, D. B., 2012. Berberine induces caspase-independent cell death in colon tumor cells through activation of apoptosis-inducing factor. PloS one 7, e36418.

Wang, X., Martindale, J. L., Holbrook, N. J., 2000. Requirement for ERK activation in cisplatin-induced apoptosis. Journal of Biological Chemistry 275, 39435-39443.

Wang, Y., Zhang, P., Liu, Y., Cheng, G., 2010. TRAF-mediated regulation of immune and inflammatory responses. Science China Life Sciences 53, 159-168.

Wei, Y., Fan, T., Yu, M., 2008. Inhibitor of apoptosis proteins and apoptosis. Acta biochimica et biophysica Sinica 40, 278-288.

Weston, C. R., Davis, R. J., 2007. The JNK signal transduction pathway. Current opinion in cell biology 19, 142-149.

Widlak, P., Garrard, W. T., 2005. Discovery, regulation, and action of the major apoptotic nucleases DFF40/CAD and endonuclease G. Journal of cellular biochemistry 94, 1078-1087.

Widlak, P., Garrard, W., 2009. Roles of the major apoptotic nuclease-DNA fragmentation factor-in biology and disease. Cellular and Molecular Life Sciences 66, 263-274.

Widlak, P., Li, L. Y., Wang, X., Garrard, W. T., 2001. Action of Recombinant Human Apoptotic Endonuclease G on Naked DNA and Chromatin Substrates COOPERATION WITH EXONUCLEASE AND DNase I. Journal of Biological Chemistry 276, 48404-48409.

Wilson, K. P., Black, J.-A. F., Thomson, J. A., Kim, E. E., Griffith, J. P., Navia, M. A., Murcko, M. A., Chambers, S. P., Aldape, R. A., Raybuck, S. A., 1994. Structure and mechanism of interleukin-lβ converting enzyme.

Wingett, D. G., Vestal, R. E., Forcier, K., Hadjokas, N., Nielson, C. P., 1998. CD40 is functionally expressed on human breast carcinomas: variable inducibility by cytokines and enhancement of Fas-mediated apoptosis. Breast cancer research and treatment 50, 27-36.

Wykes, M., MacPherson, G., 2000. Dendritic cell–B-cell interaction: dendritic cells provide B cells with CD40-independent proliferation signals and CD40-dependent survival signals. Immunology 100, 1-3.

Xavier, R., Podolsky, D., 2007. Unravelling the pathogenesis of inflammatory bowel disease. Nature 448, 427-434.

Xia, M., Li, G., Ma, J., Ling, W., 2010. Phosphoinositide 3-kinase mediates CD40 ligand-induced oxidative stress and endothelial dysfunction via Rac1 and NADPH oxidase 2. Journal of Thrombosis and Haemostasis 8, 397-406.

Xie, P., Hostager, B. S., Munroe, M. E., Moore, C. R., Bishop, G. A., 2006. Cooperation between TNF receptor-associated factors 1 and 2 in CD40 signaling. The Journal of Immunology 176, 5388-5400.

Xu, Y., Cheng, G., Baltimore, D., 1996. Targeted disruption of TRAF3 leads to postnatal lethality and defective T-dependent immune responses. Immunity 5, 407-415.

Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T.-I., Jones, D. P., Wang, X., 1997. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 275, 1129-1132.

Yang, Y., Xue, S., Lu, Z., Xiao, F., Yin, Y., Zhang, Q., Wu, C., Wang, H., Wang, L., 2014. Antitumor effects of oncolytic adenovirus armed with PSA-IZ-CD40L fusion gene against prostate cancer. Gene therapy 21, 723.

Ye, H., Cande, C., Stephanou, N. C., Jiang, S., Gurbuxani, S., Larochette, N., Daugas, E., Garrido, C., Kroemer, G., Wu, H., 2002. DNA binding is required for the apoptogenic action of apoptosis inducing factor. Nature Structural & Molecular Biology 9, 680-684.

Yeh, W.-C., Shahinian, A., Speiser, D., Kraunus, J., Billia, F., Wakeham, A., de la Pompa, J. L., Ferrick, D., Hum, B., Iscove, N., 1997. Early lethality, functional NF-κB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. Immunity 7, 715-725.

Yellin, M. J., Winikoff, S., Fortune, S. M., Baum, D., Crow, M. K., Lederman, S., Chess, L., 1995. Ligation of CD40 on fibroblasts induces CD54 (ICAM-1) and CD106 (VCAM-1) up-regulation and IL-6 production and proliferation. Journal of leukocyte biology 58, 209-216.

Youle, R. J., Strasser, A., 2008. The BCL-2 protein family: opposing activities that mediate cell death. Nature reviews. Molecular cell biology 9, 47.

Young, L., Dawson, C., Brown, K., Rickinson, A., 1989. Identification of a human epithelial cell surface protein sharing an epitope with the C3d/epstein-barr virus receptor molecule of B lymphocytes. International Journal of Cancer 43, 786-794.

Zarubin, T., Jiahuai, H., 2005. Activation and signaling of the p38 MAP kinase pathway. Cell research 15, 11-18.

Zha, J., Harada, H., Yang, E., Jockel, J., Korsmeyer, S. J., 1996. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X L. Cell 87, 619-628.

Zhang, H., Xu, Q., Krajewski, S., Krajewska, M., Xie, Z., Fuess, S., Kitada, S., Pawłowski, K., Godzik, A., Reed, J. C., 2000. BAR: An apoptosis regulator at the intersection of caspases and Bcl-2 family proteins. Proceedings of the National Academy of Sciences 97, 2597-2602.

Zhang, W., Liu, H. T., 2002. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. Cell research 12, 9-18.

Zhang, Y., Li, R., Meng, Y., Li, S., Donelan, W., Zhao, Y., Qi, L., Zhang, M., Wang, X., Cui, T., 2014. Irisin stimulates browning of white adipocytes through mitogen-activated protein kinase p38 MAP kinase and ERK MAP kinase signaling. Diabetes 63, 514-525.

Zheng, L., Schickling, O., Peter, M. E., Lenardo, M. J., 2001. The death effector domain-associated factor plays distinct regulatory roles in the nucleus and cytoplasm. Journal of Biological Chemistry 276, 31945-31952.

Zimmermann, R., Hennig, B., Neupert, W., 1981. Different transport pathways of individual precursor proteins in mitochondria. European Journal of Biochemistry 116, 455-460.

Zirlik, A., Maier, C., Gerdes, N., MacFarlane, L., Soosairajah, J., Bavendiek, U., Ahrens, I., Ernst, S., Bassler, N., Missiou, A., 2007. CD40 ligand mediates inflammation independently of CD40 by interaction with Mac-1. Circulation 115, 1571-1580.

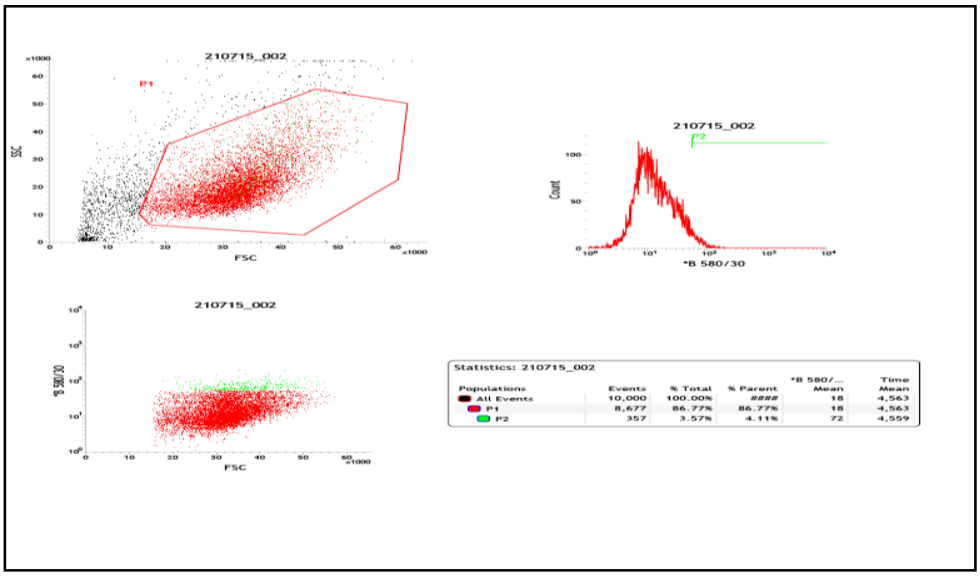
Zitvogel, L., Kepp, O., Senovilla, L., Menger, L., Chaput, N., Kroemer, G., 2010. Immunogenic tumor cell death for optimal anticancer therapy: the calreticulin exposure pathway. Clinical Cancer Research 16, 3100-3104.

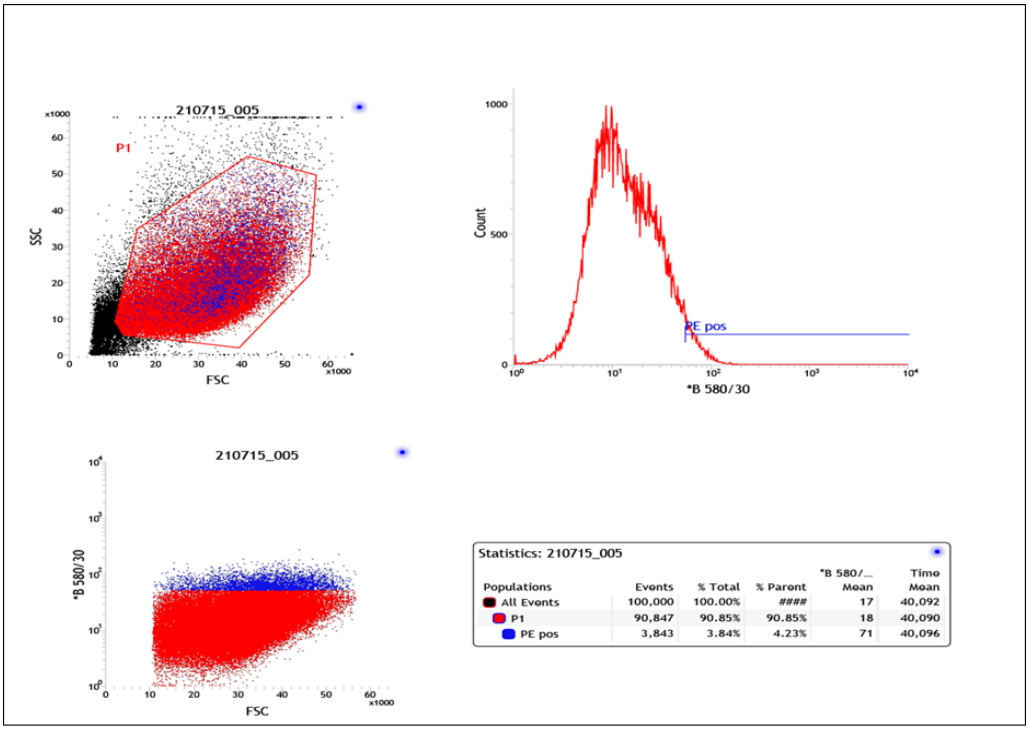
Chapter 8: **Appendix**

Appendix I:

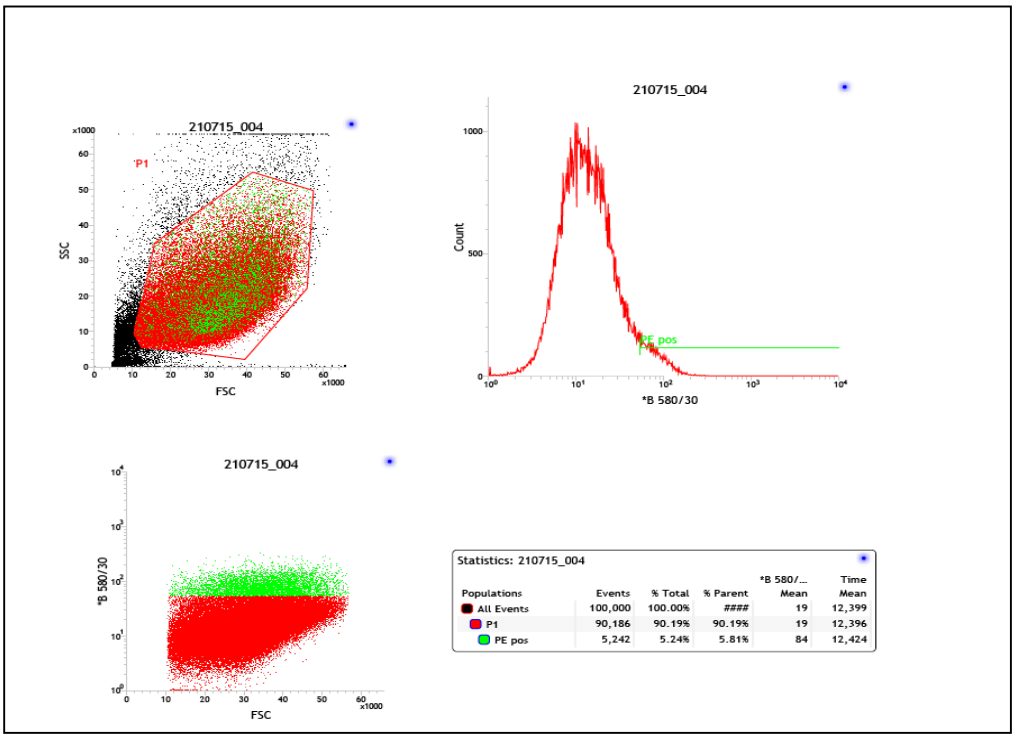
2. Cell Sorting by Flow Cytometry

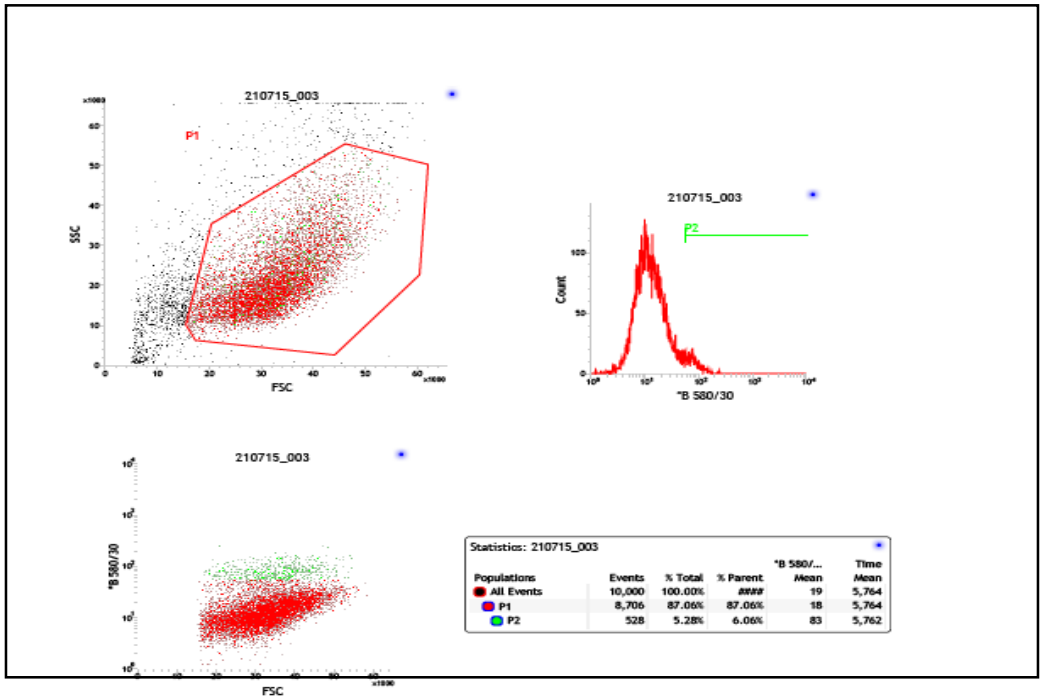
A





B





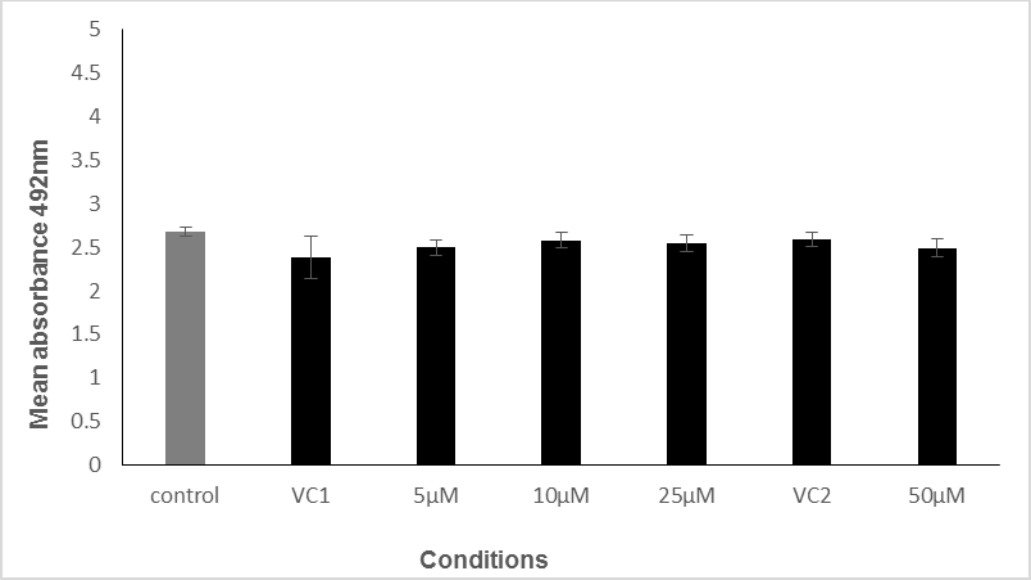
The sophisticated device of flow cytometry was used, it is able to use several parametric analysis to identify and sorting highly specific populations based on a specific antibody. Two samples of cells (PC-3CD40) were sorted A and B.

8.1. Appendix II:

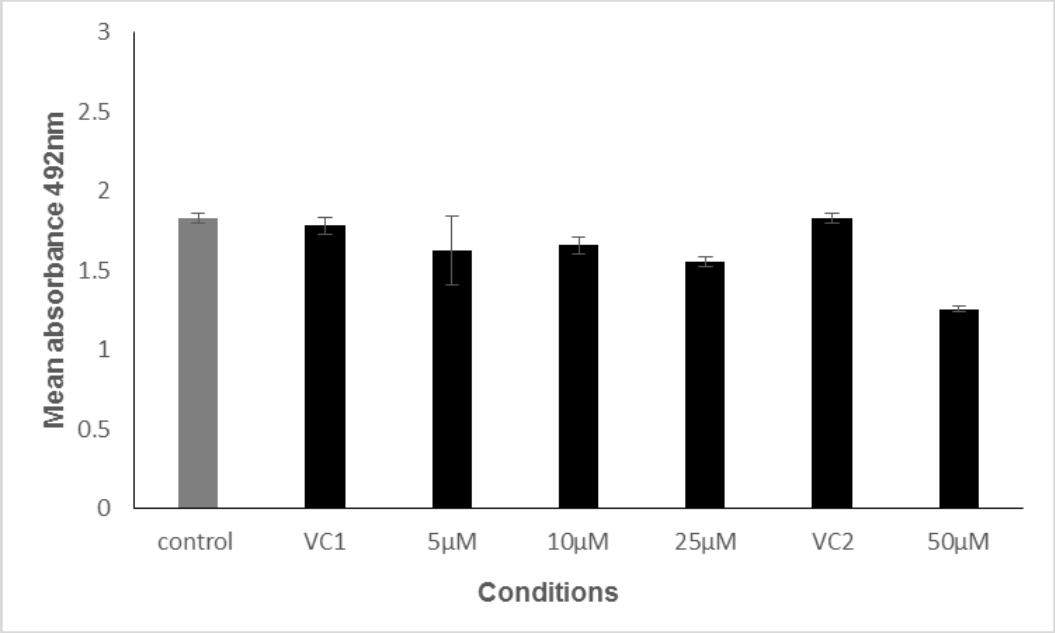
Titrations of MAPK inhibitors

1. MEK/ERK inhibitor (U0126)

A. DU145 cells

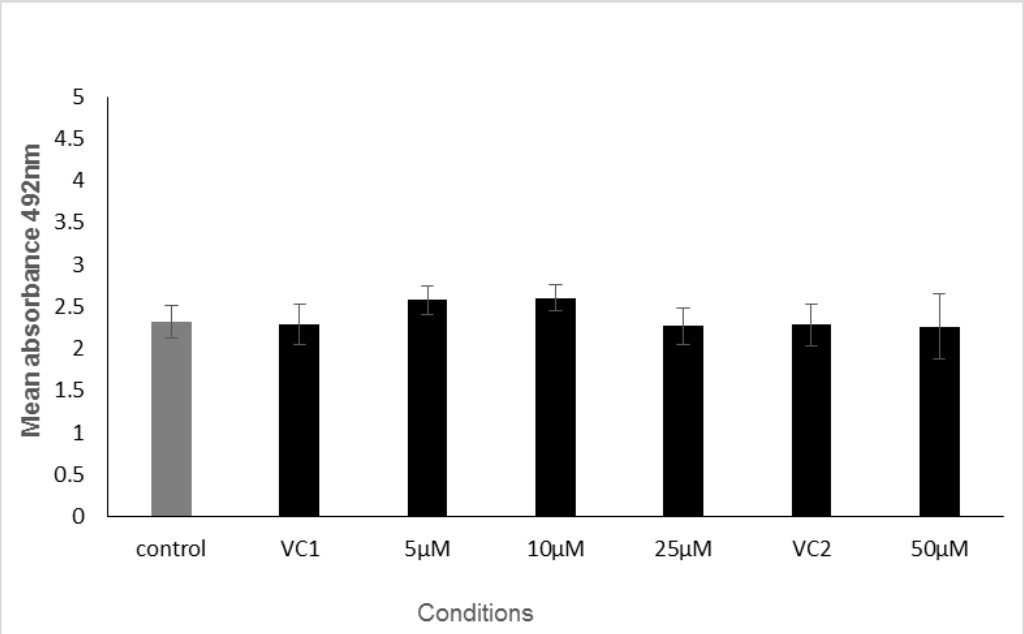


B. LNCaP cells

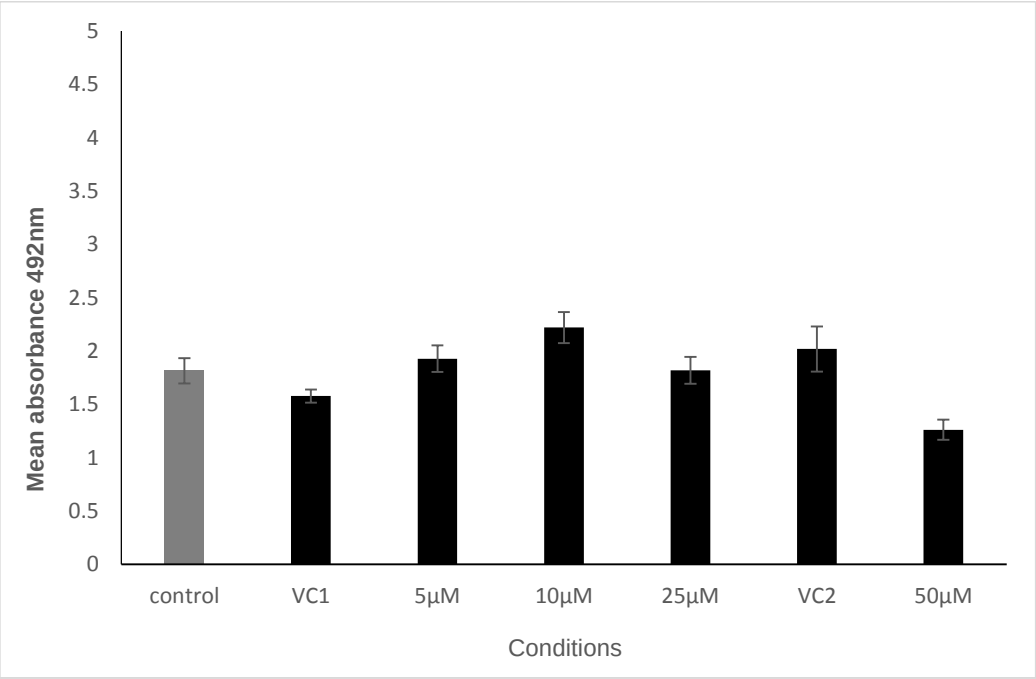


2. JNK inhibitor (SP600125)

A. DU145 cells

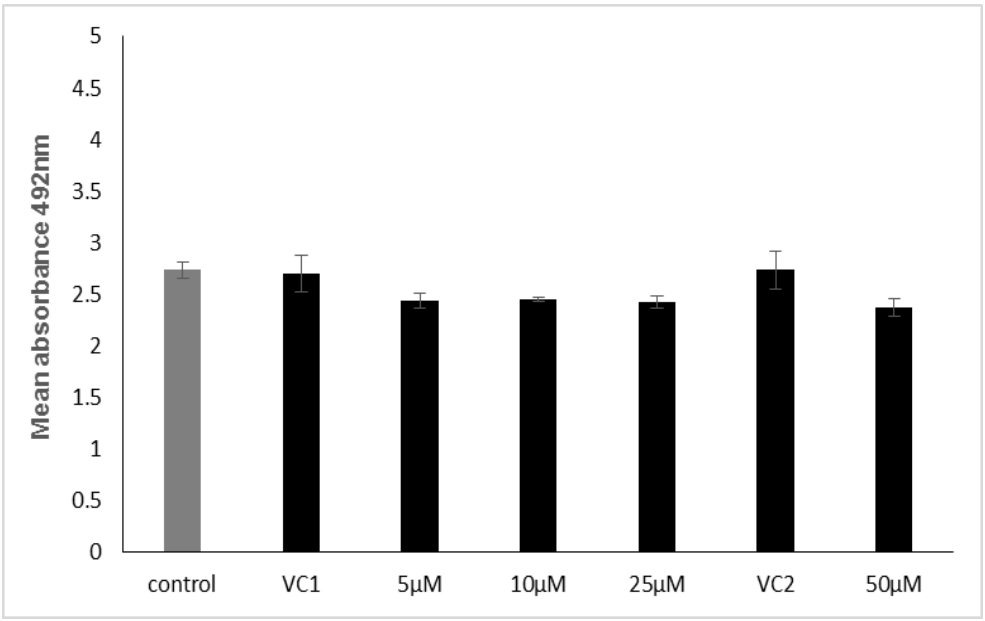


B. LNCaP cells

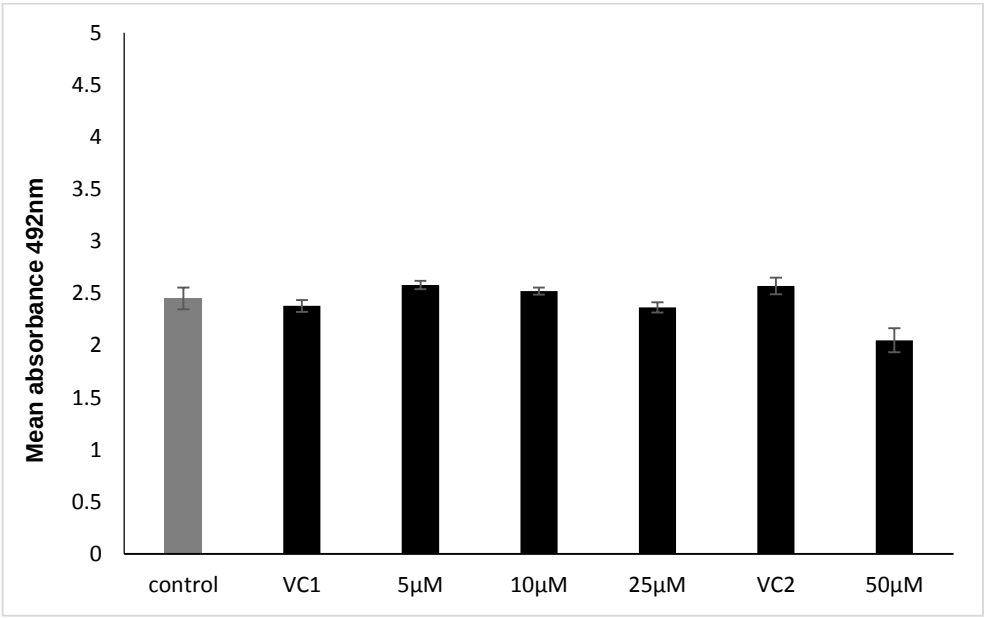


3. p38 inhibitor (SB202190)

A. DU145

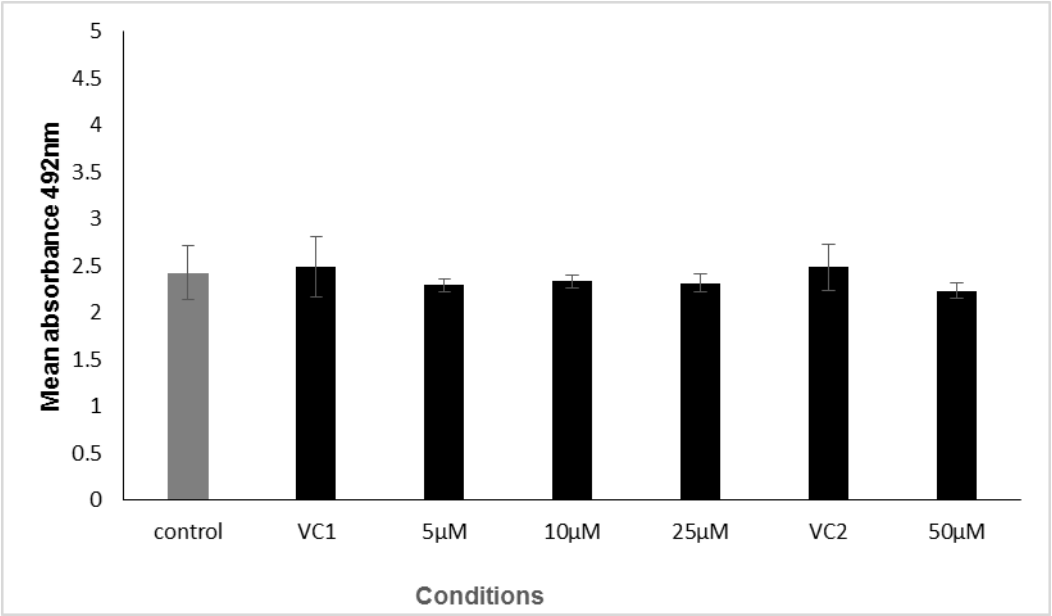


B. LNCaP

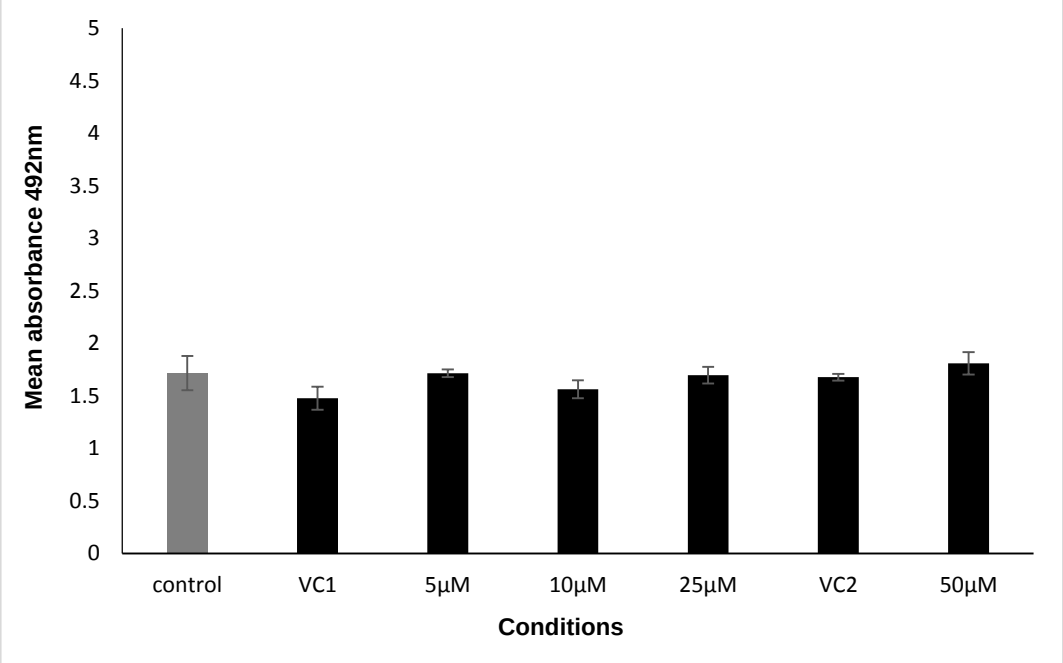


4. (NDGA) AP-1 inhibitor

A. DU145

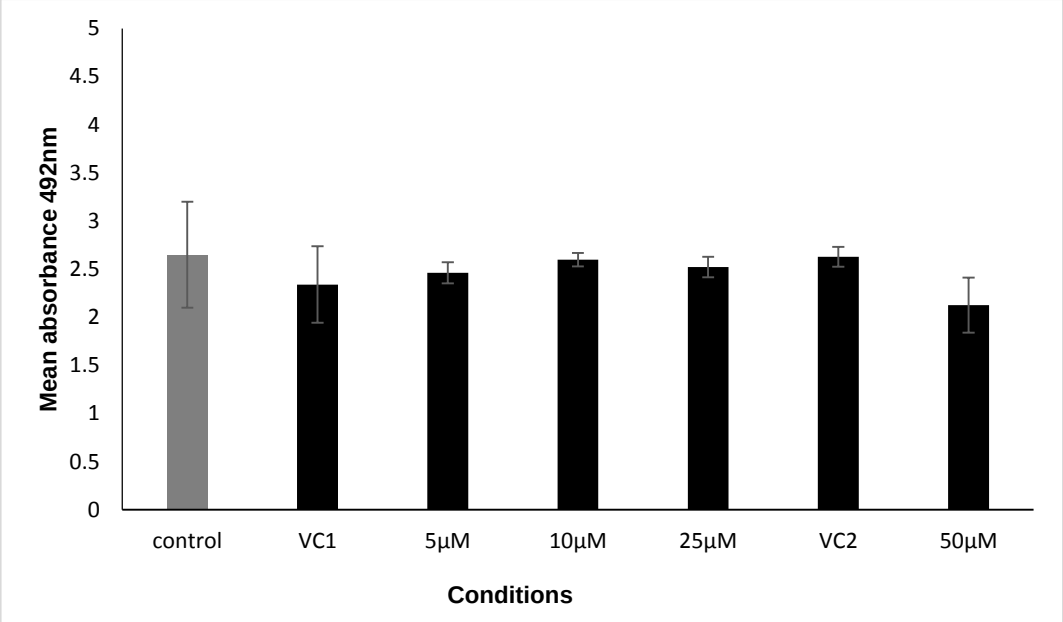


B. LNCaP

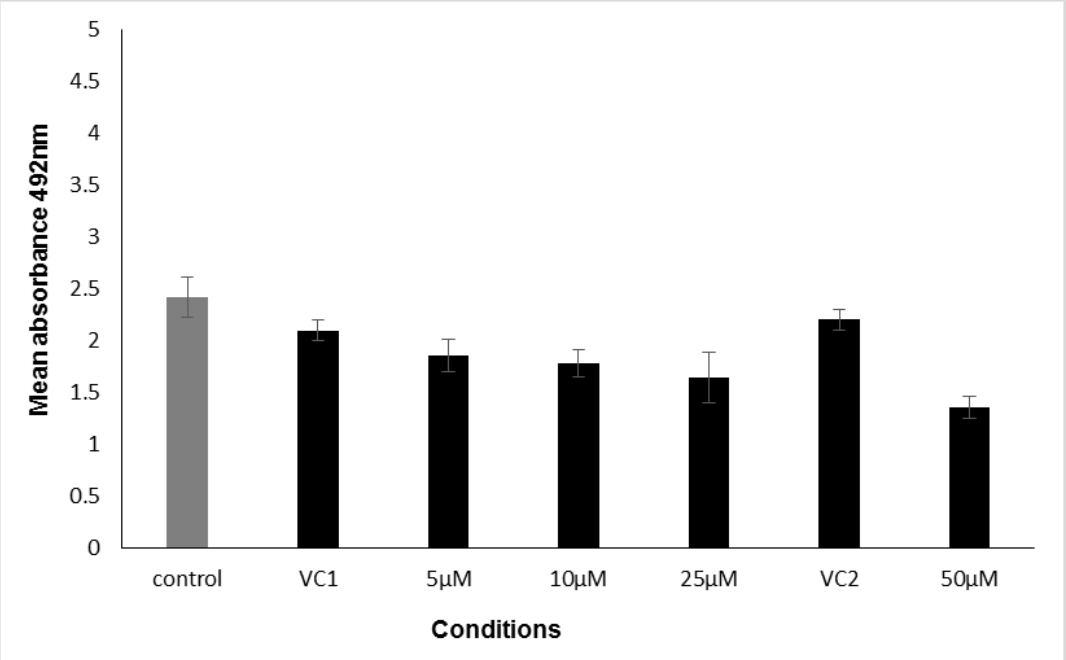


5. NF-κB inhibitor

A. DU145



B. LNCaP



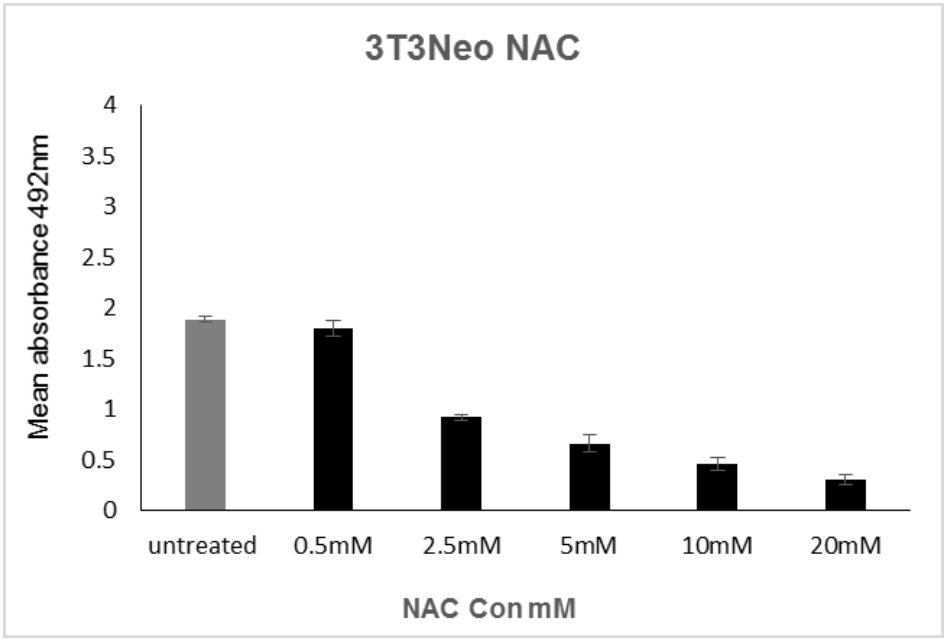
Effects of the MAPK inhibitors on carcinoma cell viability

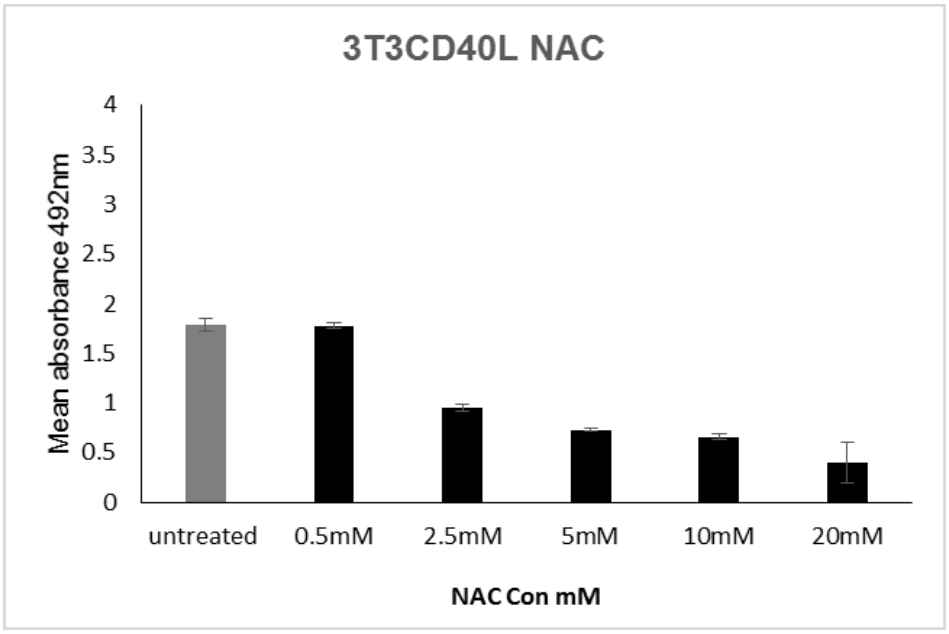
PCa cells (DU145 and LNCaP) were in 96-well plates. Cells were then treated with MAPKs inhibitors: MEK/ERK inhibitor (U0126), JNK inhibitor (SP600125), p38 inhibitor (SB202190), (NDGA) AP-1 inhibitor and NF-κB inhibitor as indicated, and vehicle control (VC) was also included (DMOS). 20μl of MTS solution was added to each well and incubated for 4hrs. Cell viability was determined by a FLUOstar OPTIMA (BMG Labtech) plate reader at absorbance 492nm. Data are represented as mean values ±SEM.

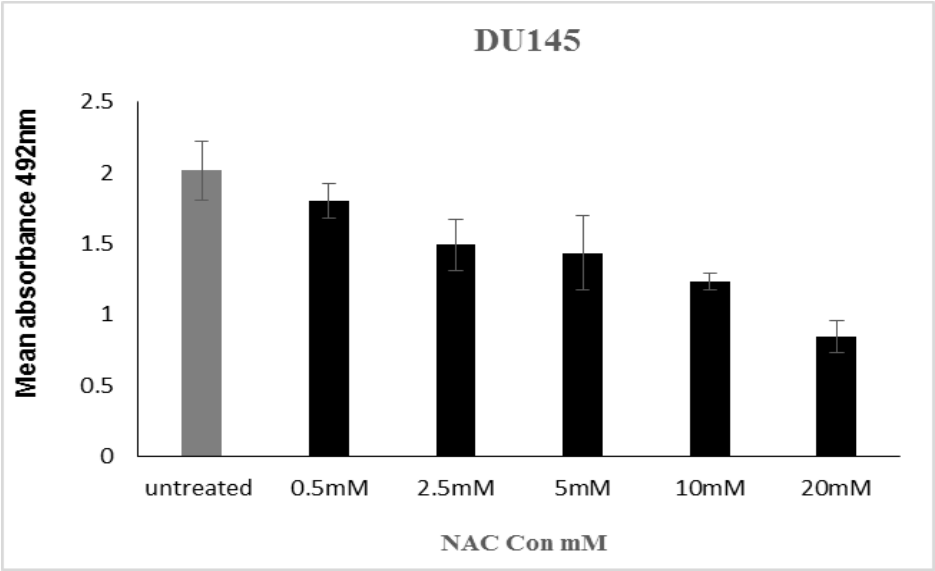
8.2. Appendix III:

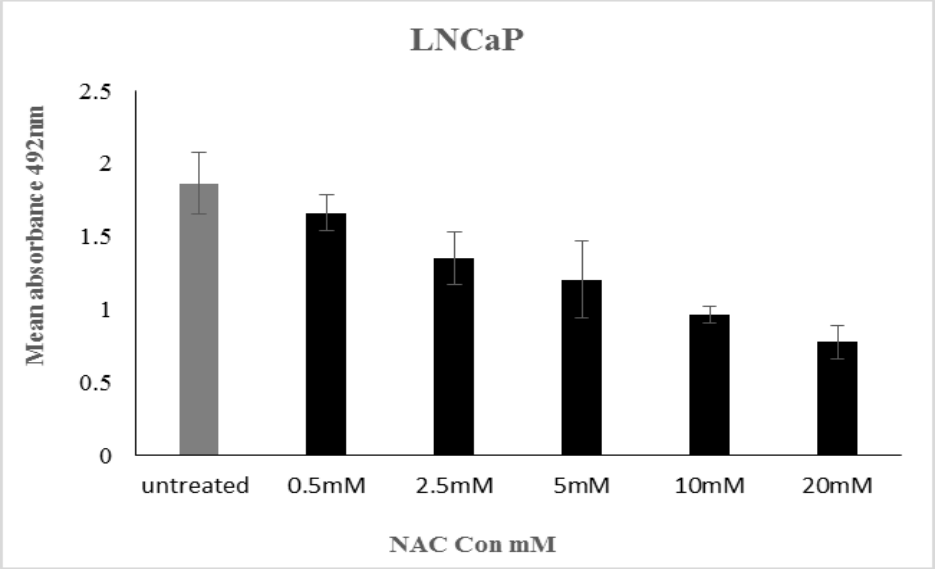
The antioxidant titration

A. NAC





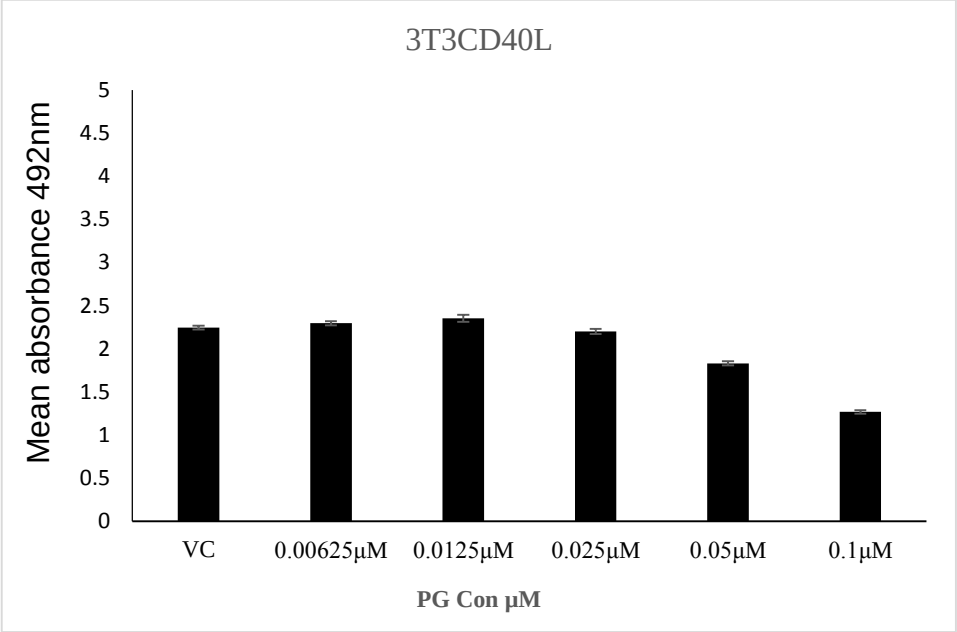


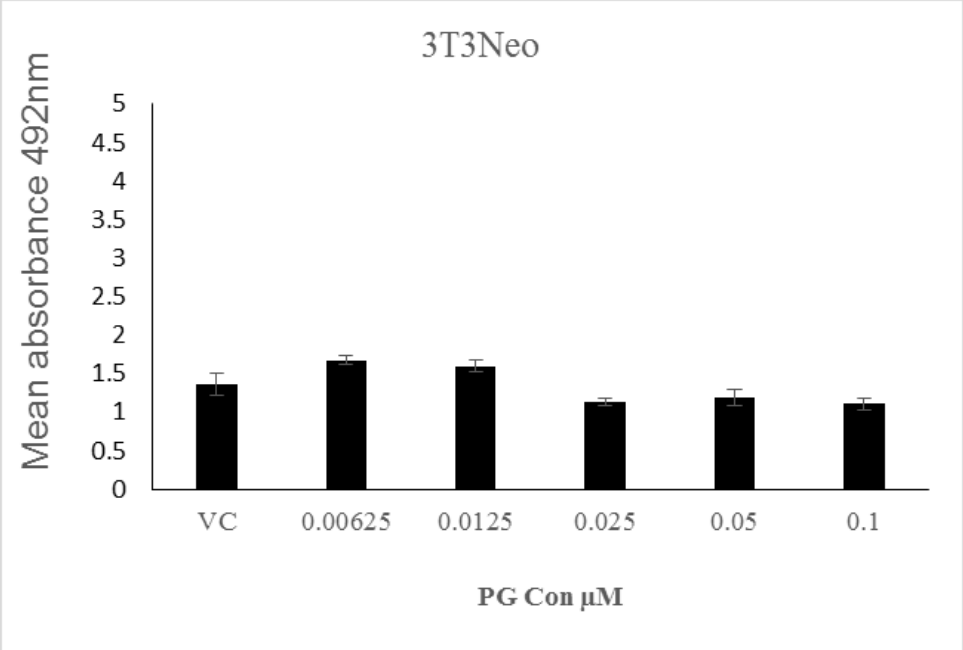


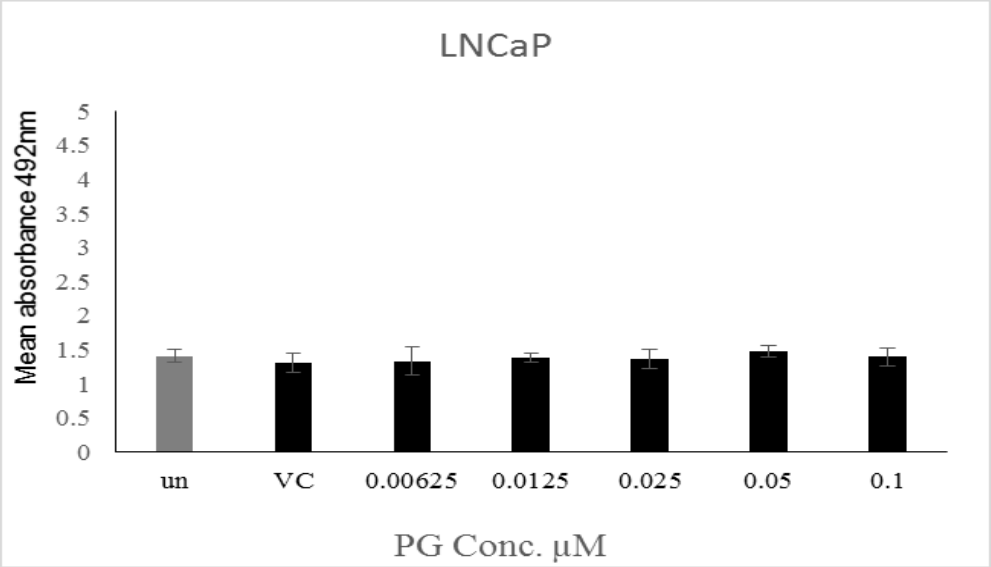
Effects of the antioxidant NAC on PCa cell viability

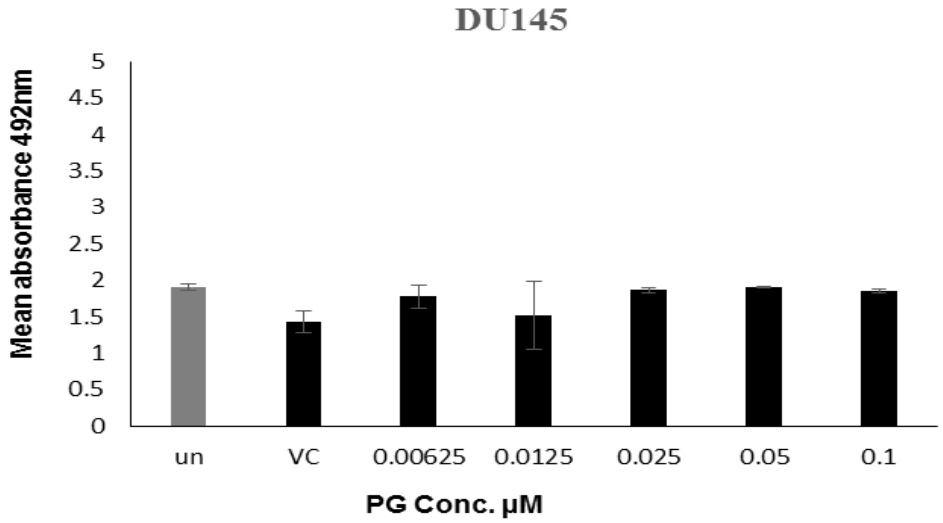
Affecter cells (3T3CD40L and 3T3Neo), and target cells (DU145 and LNCaP) were seeded in 96-well plates. Cells were then treated with NAC as indicated. 20µl of MTS solution was added to each well and incubated for 4hrs. Cell viability was determined by a FLUOstar OPTIMA (BMG Labtech) plate reader at absorbance 492nm.

B. propyl gallate (PG)







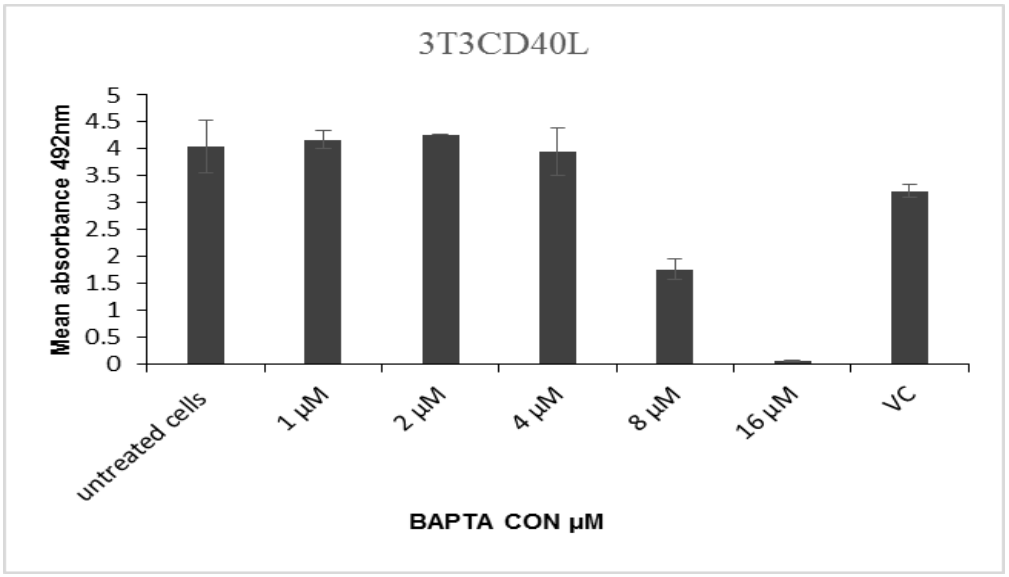


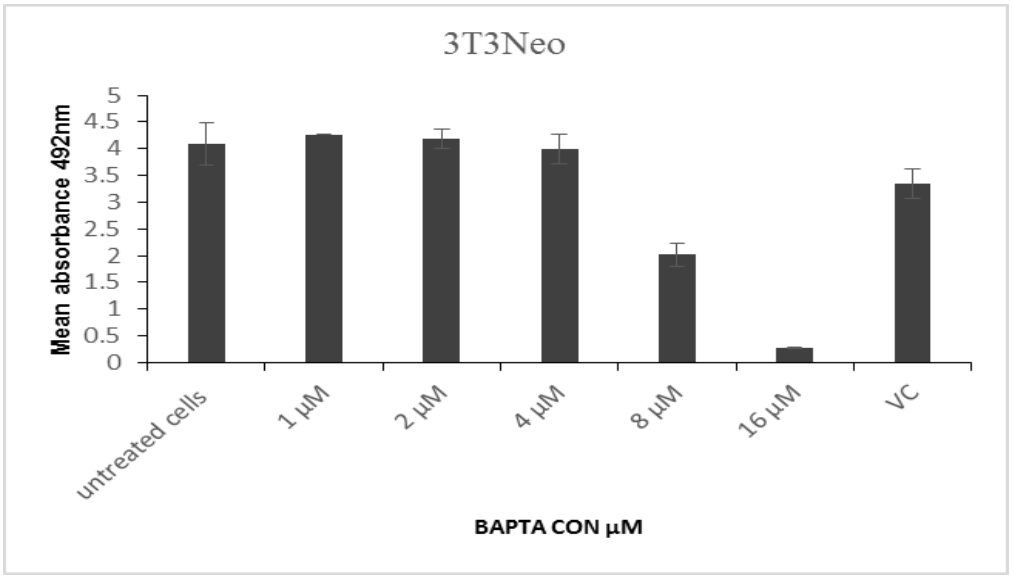
Effects of the antioxidant PG on fibroblast and PCa cell viability

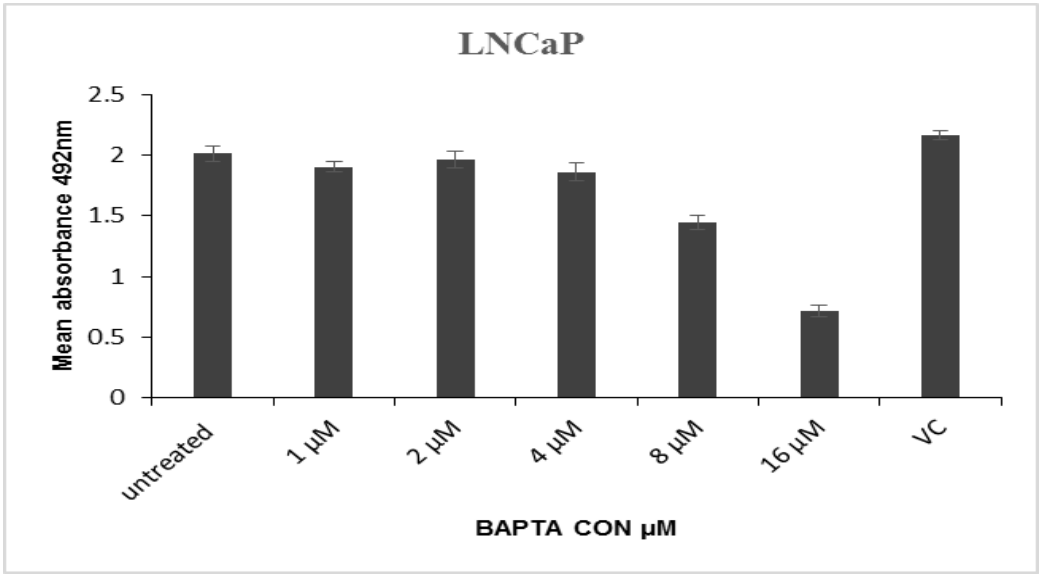
Affecter cells (3T3CD40L and 3T3Neo), and target cells (DU145 and LNCaP) were seeded in 96-well plates. Cells were then treated with PG as indicated. 20µl of MTS solution was added to each well and incubated for 4hrs. Cell viability was determined by a FLUOstar OPTIMA (BMG Labtech) plate reader at absorbance 492nm.

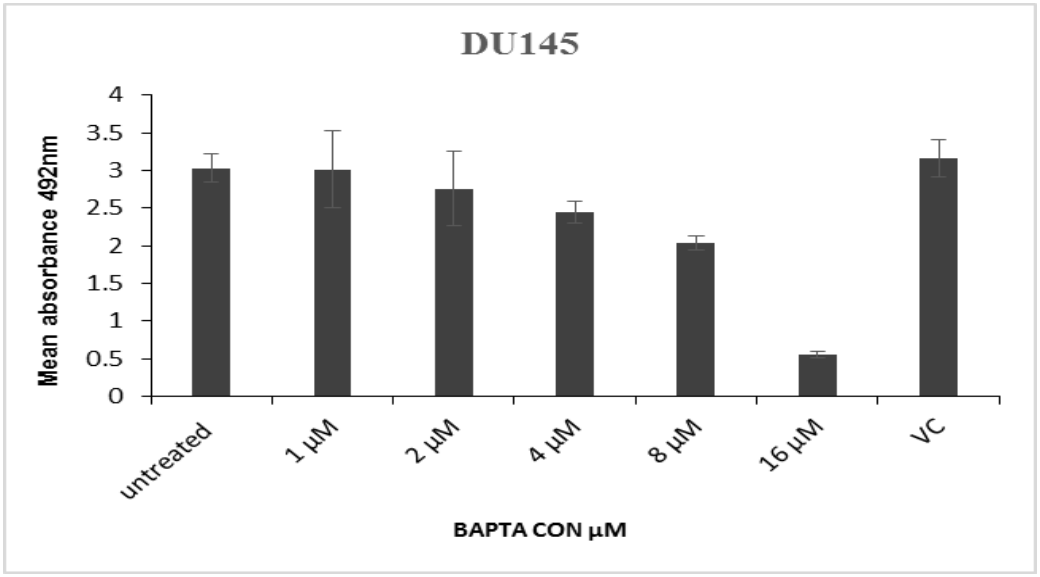
8.3. Appendix IV:

A. Titration of Ca^{+} inhibitor (BAPTA)







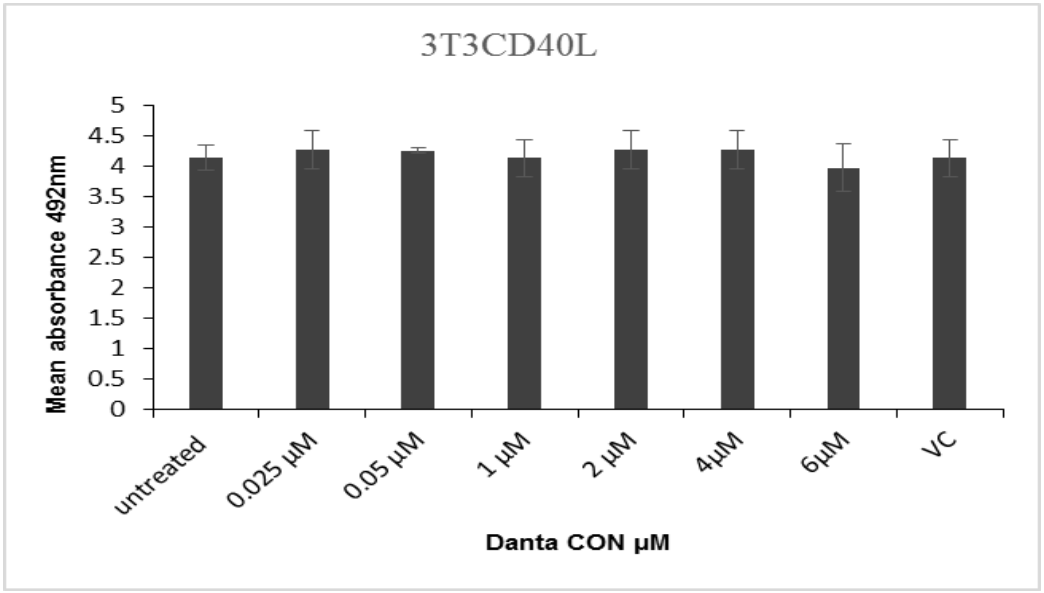


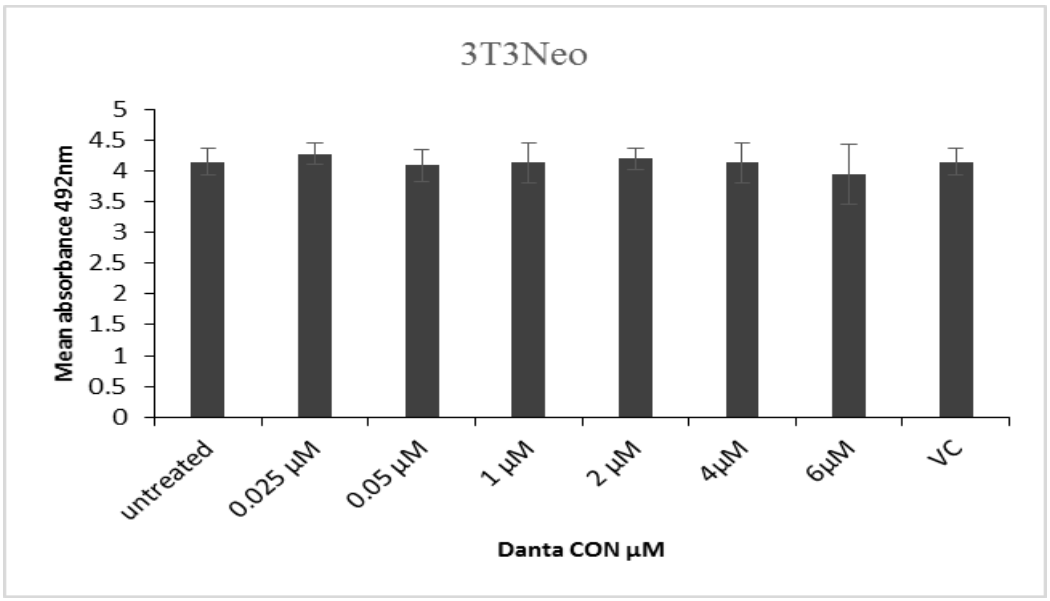
Effects of the Ca+ inhibitor (BAPTA) on fibroblast and PCa cell viability

Affecter cells (3T3CD40L and 3T3Neo), and target cells (DU145 and LNCaP) were seeded in 96-well plates. Cells were then treated with BAPTA as indicated. 20µl of MTS solution was added to each well and incubated for 4hrs. Cell viability was determined by a FLUOstar OPTIMA (BMG Labtech) plate reader at absorbance 492nm.

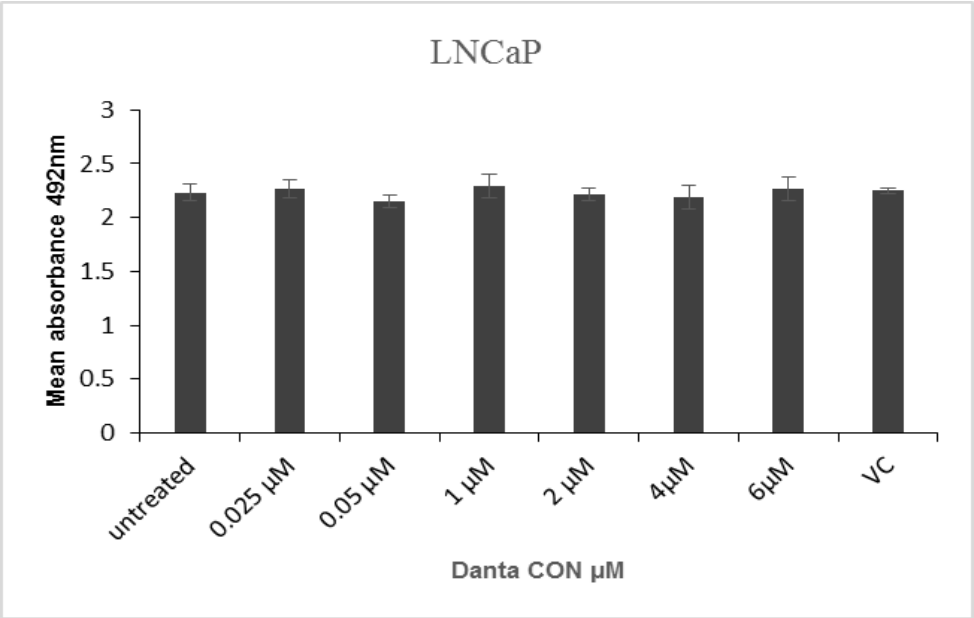
B. Titration of Ca^{+} inhibitor (Danta)

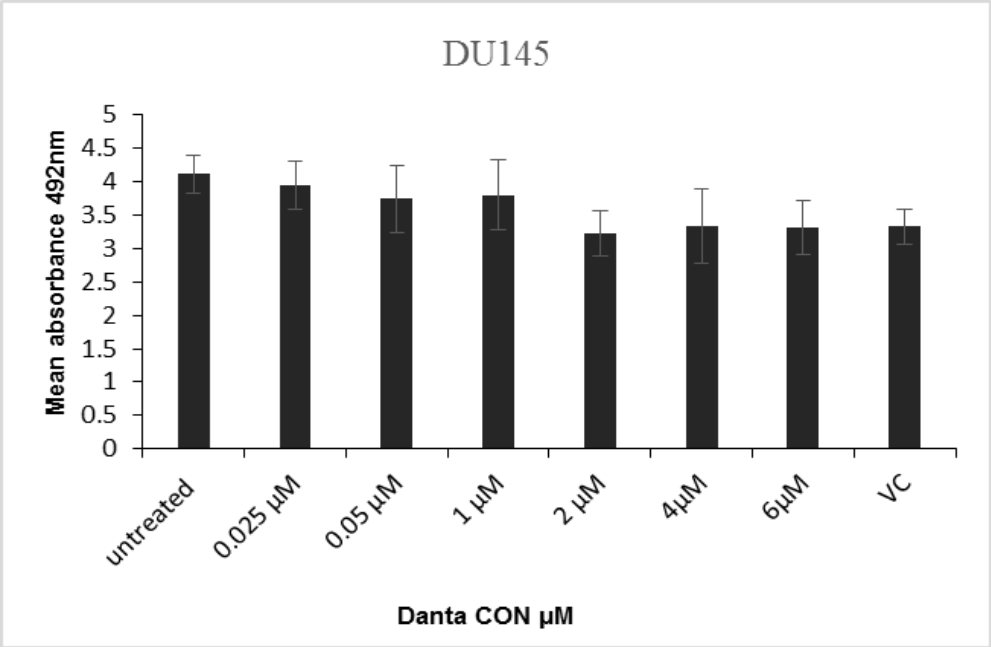
A. Affecter cells





B. Target cells

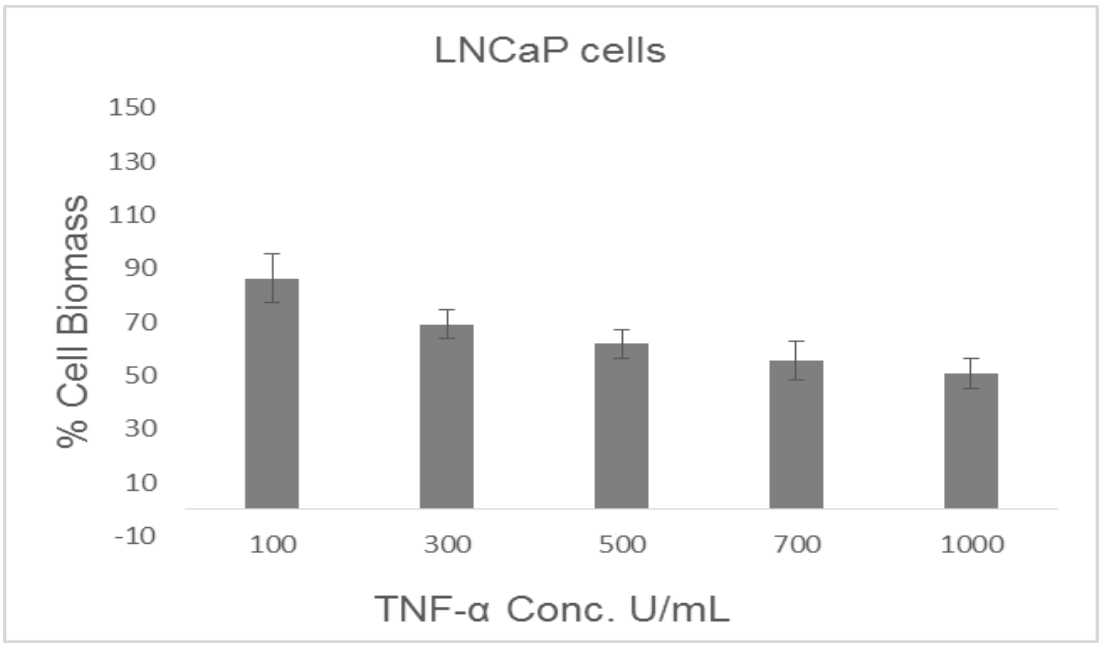




Effects of the Ca+ inhibitor (Danta) on fibroblast and PCa cell viability

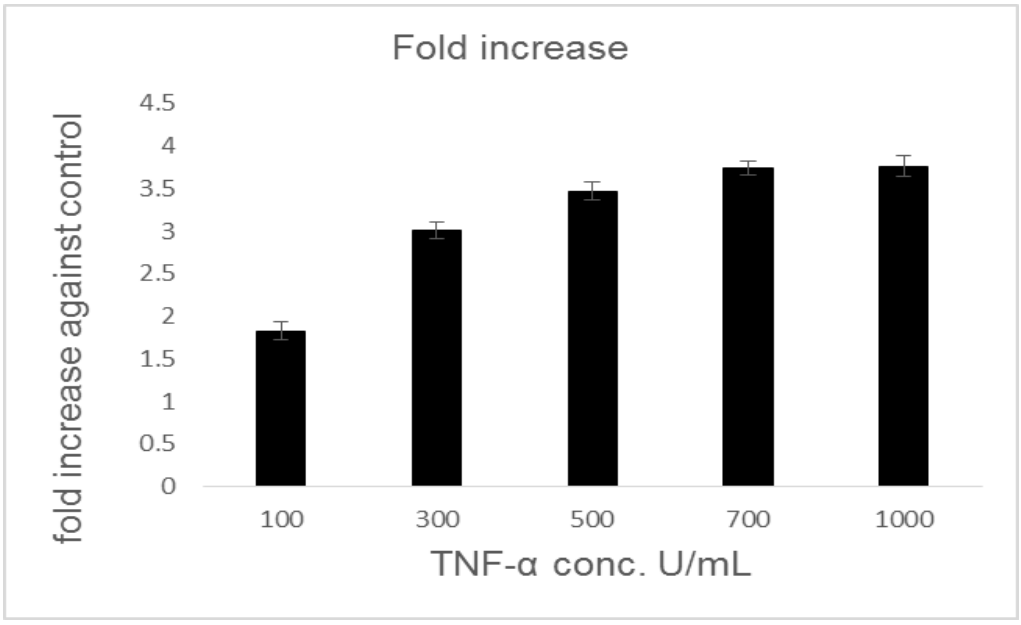
Affecter cells (3T3CD40L and 3T3Neo), and target cells (DU145 and LNCaP) were seeded in 96-well plates. Cells were then treated with Danta as indicated. 20µl of MTS solution was added to each well and incubated for 4hrs. Cell viability was determined by a FLUOstar OPTIMA (BMG Labtech) plate reader at absorbance 492nm.

LNCaP cells treated with TNF-α



Effects of the TNF-α on LNCaP cell viability

LNCaP cell was seeded in 96-well plates. Cells were then treated with TNF-α as indicated. 20µl of MTS solution was added to each well and incubated for 4hrs. Cell viability was determined by a FLUOstar OPTIMA (BMG Labtech) plate reader at absorbance 492nm.



Effects of the TNF-α on LNCaP CytoTox-Glo

8.4. Appendix V:

Protein Quantification and Western blot

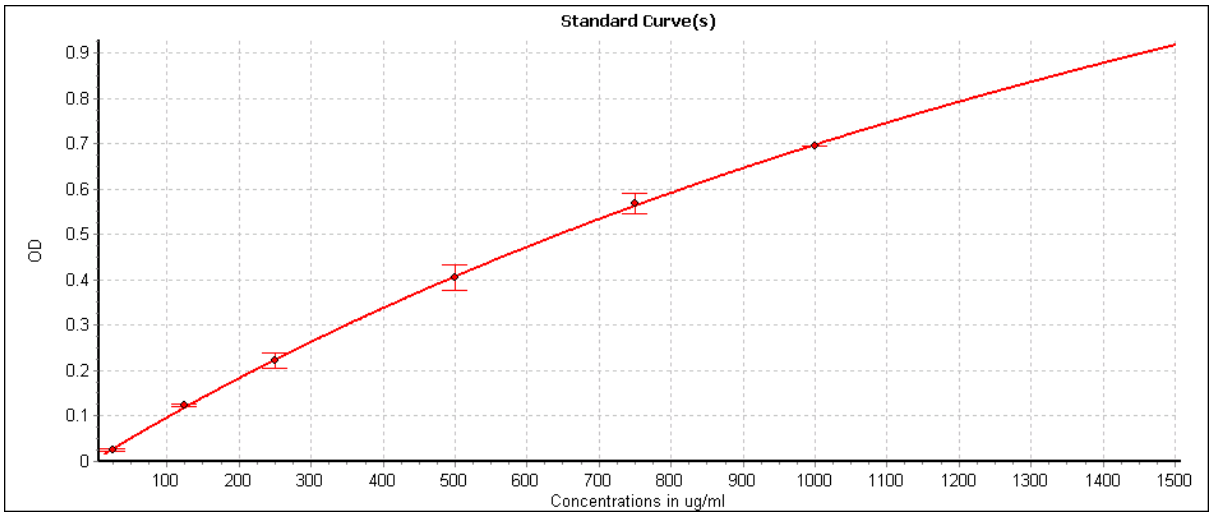


Figure shows Standard curve (4-parameter-fit) to measure protein concentration in DU145 cultured cell lines.

A

	1/2hrs	1/2hrs	1hrs	1hrs	1.5hrs	1.5hrs	6hrs	12hrs
	D+N	D+L	D+N	D+L	D+N	D+L	H+L	H+L
Average	6496.198	7302.158	5352.994	7535.65	6399.412	9700.794	6451.992	9952.995
ug/ul	6.496198	7.302158	5.352994	7.53565	6.399412	9.700794	6.451992	9.952995
V.O.S	**6.16**	**5.48**	**7.47**	**5.31**	**6.25**	**4.12**	**6.20**	**4.02**
H2O	6.84	7.52	5.53	7.69	6.75	8.88	6.80	8.98
RA	2	2	2	2	2	2	2	2
LDS	5	5	5	5	5	5	5	5
TOTAL	20	20	20	20	20	20	20	20

Table (A) shows protein concentration of DU145 co-cultured with effector cells lysate.

Samples of cell lysate were diluted with dH2O in small Eppendorf tube (dilution factor 12.5) and mix well to ensure the protein concentration readout by plate reader. 10µl of diluted lysate and protein standards were added in Microplate (96 well) in duplication. 200µl then of Coomassie blue reagent was added and incubated between 5-10 mins at room temperature. The absorbance of protein concentration was measured by plate reader.

B

	1hrs	1hrs	3hrs	3hrs	6hrs	6hrs	12hrs	12hrs
	D+N	D+L	D+N	D+L	D+N	D+L	H+L	H+L
Previous protein loading	6.157448	5.477833	7.472454	5.308103	6.250574	4.123374	6.199636	4.018891
Average intensity	2292.11	2082.51	2309.63	2094.65	2316.96	1806.54	2152.24	1528.89
Highest value of protein	2316.96	2316.96	2316.96	2316.96	2316.96	2316.96	2316.96	2316.96
Ave/Highest	0.989275	0.898811	0.996836	0.904051	1	0.779703	0.928907	0.659869
1-value	0.010725	0.101189	0.003164	0.095949	0	0.220297	0.071093	0.340131
1+value	1.010725	1.101189	1.003164	1.095949	1	1.220297	1.071093	1.340131
Next loading in 40ug/ml	**6.22**	**6.03**	**7.50**	**5.82**	**6.25**	**5.03**	**6.64**	**5.39**
H2O	6.78	6.97	5.50	7.18	6.75	7.97	6.36	7.61
RA	2	2	2	2	2	2	2	2
LDS	5	5	5	5	5	5	5	5
Total	20	20	20	20	20	20	20	20

Table (B) shows protein concentration of DU145 co-cultured with effector cells lysate after normalisation.