Examining plasminogen activator-inhibitor-1 and C-reactive protein regulation by glucocorticoids and pro-inflammatory cytokines in liver cell lines

by

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Declaration

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ABSTRACT

Current knowledge suggests that a continued elevated acute phase response (APR), which is often referred to as low-grade inflammation, occurs during insulin resistance. The levels of acute phase proteins (APPs), such as plasminogen activator-inhibitor-1 (PAI-1), C-reactive protein (CRP), and serum amyloid A (SAA) are significantly increased during this response, hence why they are commonly used as biological markers for type 2 diabetes (T2D). In the liver, APPs are regulated by the peripheral mediators of stress (i.e., endogenous glucocorticoids (GCs)) and inflammation (i.e., pro-inflammatory cytokines), with both implicated in the development of insulin resistance. Exogenous GCs are routinely prescribed in the management of inflammatory disorders. However, long-term GC treatment can exaggerate insulin resistance as it could possibly disrupt the interplay between endogenous GCs and cytokines, leading to a recurrent, chronic APR. Current knowledge suggests that when it comes to the APR, GCs, which are traditionally known for its anti-inflammatory properties, exert more pro-inflammatory behaviour, by further strengthening cytokine-induced APP expression. Whilst this cooperative regulation by GCs and pro-inflammatory cytokines have been extensively studied and demonstrated for SAA, fewer have studied this phenomenon in regards to PAI-1 and CRP, especially in the liver. In addition, previous studies investigating the possible co-regulation of PAI-1 and CRP by GCs and pro-inflammatory cytokines, included only interleukin-6 (IL-6) or tumour necrosis factor-alpha (TNF-α) as representative pro-inflammatory cytokine, respectively. Therefore, the current study examined the expression of both PAI-1 and CRP in response to GCs (synthetic and endogenous) in the presence of either pro-inflammatory cytokine, TNF-α or IL-6, in a murine (BWTG3)- and human (HepG2) liver cell line. The effects were investigated at both the mRNA- and intracellular protein level, using real-time semi-quantitative polymerase chain reaction (qPCR) and western blotting, respectively. In addition, the potential underlying molecular mechanism governing the action of these biological mediators to affect APP expression were investigated, using a luciferase promotor-reporter assay. PAI-1 and CRP levels generally remained elevated in response to individual and combinatorial treatments with GCs and pro-inflammatory cytokines, at intracellular protein- and promotor level. However, only under certain conditions, cooperativity was displayed between the test compounds, where PAI-1 and CRP expression were further potentiated, more than what was observed with either test compound alone. APPs are secreted proteins; therefore, the rate of protein synthesis and export presumably plays a role in determining intracellular APP expression and highlights the importance of measuring both

intra- and extracellular APP expression, the latter of which the current study did not determine. In addition, the increase in PAI-1 and CRP promoter activity does suggest that the molecular mechanism of action whereby GCs and pro-inflammatory cytokines increase PAI-1 and CRP levels is at the promoter level. Whether only the proximal promotor is involved remains to be elucidated. Taken together, the findings of the current study give credence to the current knowledge available on the subject of cooperative regulation of APPs by GCs and pro-inflammatory cytokines. It is clear that GCs favour an increase in APP expression, at times enhancing the cytokine-induced PAI-1 and CRP expression. However, GCs were also able to antagonise the cytokine-induced PAI-1 and CRP expression, which is in line with their anti-inflammatory role. Ultimately, our findings highlight the diversity and complexity to the often-contradictory nature of GCs and their crosstalk with inflammatory mediators.

Keywords: acute phase proteins, acute phase response, cooperative regulation, C-reactive protein, glucocorticoids, hepatic insulin resistance, inflammation, plasminogen activator-inhibitor-1, pro-inflammatory cytokines, stress

OPSOMMING

Bestaande kennis dui daarop dat ’n verhoogde akute fase reaksie (AFR), ook bekend as laegraadse inflammasie, tydens insulienweerstandigheid voorkom. Die vlakke van akute fase proteïene (AFPe), soos plasminogeen aktiveerder-inhibeerder-1 (PAI-1), C-reaktiewe proteïen (CRP) en serum amiloïed A (SAA) word aansienlik verhoog tydens hierdie reaksie, en word dus gereeld gebruik as biologiese merkers vir tipe 2 diabetes (T2D). In die lewer word AFPe deur die perifere molekules van stres (endogene glukokortikoïede (GKe)) en inflammasie (pro-inflammatoriese sitokiene) gereguleer, met beide wat ook in die ontwikkeling van insulienweerstandigheid geïmpliseer word. Eksogene GKe word gereeld voorgeskryf in die behandeling van inflammatoriese afwykings. Langtermyn GK-behandeling kan egter insulienweerstandigheid vererger, omdat dit dalk die wisselwerking tussen endogene GKe en sitokiene kan versteur, wat tot ’n herhaalde, chroniese AFR kan lei. Bestaande kennis dui daarop dat, met betrekking tot die AFR, GKe, wat tradisioneel bekend is vir hul anti-inflammatoriese eienskappe, geneig is om meer pro-inflammatories op te tree deur sitokien-geïnduseerde AFP-uitdrukking verder te versterk. Hoewel hierdie samewerkende regulering deur GKe en pro-inflammatoriese sitokiene vir SAA breedvoerig bestudeer en gedemonstreer is, is daar minder studies oor dié verskynsel ten opsigte van PAI-1 en CRP, veral in die lewer. Daarby het vorige studies ook slegs interleukin-6 (IL-6), in die geval van CRP, of tumor nekrose faktor-alfa (TNF-α), in die geval van PAI-1, gebruik as verteenwoordiger sitokien in kombinasie met die GK. Die huidige studie ondersoek dus die uitdrukking van beide PAI-1 en CRP in reaksie op GKe (sinteties en endogeen) in die teenwoordigheid van óf pro-inflammatoriese sitokien, TNF-α of IL-6, in ’n muriene (BWTG3) en menslike (HepG2) lewersellyn. Die uitwerkings is op beide mRNA- en intrasellulêre proteïenvlak ondersoek, met behulp van onderskeidelik intydse semi-kwantitatiewe polimerase kettingreaksie en western klad. Daarbenewens is die moontlike onderliggende molekulêre meganisme ondersoek, wat die aksie van hierdie biologiese molekules beheer om die uitdrukking van beide AKPe te beïnvloed, met behulp van ’n lusiferase promotor-verslaggewer toets. Die vlakke van PAI-1 en CRP was meestal verhoog in reaksie op individuele en gesamentlike behandelings met GKe en pro-inflammatoriese sitokiene, op proteïen- en promotorvlak. Maar samewerking tussen die toetsverbindings is slegs onder sekere omstandighede getoon, waar PAI-1- en CRP-uitdrukking verder versterk was, meer as wat met elke toetsverbinding op sy eie waargeneem was. AFPe is afgeskeide proteïene; daarom speel die tempo van proteïensintese en -uitvoer vermoedelik ’n rol om intrasellulêre AFP-uitdrukking te bepaal, en beklemtoon dit die belangrikheid om beide

intra- en ekstrasellulêre AFP-uitdrukking te bepaal, waarvan laasgenoemde nie in die huidige studie uitgevoer is nie. Verder dui die toename in PAI-1- en CRP promotor-aktiwiteit daarop dat die molekulêre aksiemeganisme waardeur GKe en pro-inflammatoriese sitokiene die vlakke van PAI-1- en CRP verhoog, op die promotorvlak is. Of slegs die proksimale promotor betrokke is, moet nog bepaal word. Gesamentlik gee die bevindinge van die hierdie studie geloofwaardigheid aan die beskikbare kennis oor die onderwerp van samewerkende regulering van AFPe deur GKe en pro-inflammatoriese sitokiene. Dit is duidelik dat GKe ’n toename in AFP-uitdrukking verkies, wat onder sekere omstandighede die sitokien-geïnduseerde PAI-1- en CRP-uitdrukking verbeter. GKe kan ook egter die sitokien-geïnduseerde PAI-1- en CRP-uitdrukking teenstaan, wat in lyn is met hul anti-inflammatoriese rol. Ten slotte, beklemtoon ons bevindings die diversiteit en kompleksiteit van die dikwels teenstrydige aard van GKe en hul kruiskommunikasie met inflammatoriese molekules.

Sleutelwoorde: akute fase-proteïene, akute fase reaksie, C-reaktiewe proteïen, glukokortikoïede, hepatiese insulienweerstandigheid, inflammasie, ko-regulering, plasminogeen aktiveerder-inhibeerder-1, pro-inflammatoriese sitokiene, stres

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ALPHABETICAL LIST OF ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
AF-1	Activation function-1
AmpR	Ampicillin resistance
ANOVA	Analysis of Variance
AP-1	Activator protein-1
APPs	Acute phase proteins
APR	Acute phase response
APS	Ammonium persulfate
BMI	Body mass index
Bp	Base pair
CAF	Central analytical facility
CBG	Corticosteroid binding globulin
cDNA	Complementary DNA
C/EBP-β	CCAAT-enhancer-binding protein beta
C/EBP-δ	CCAAT-enhancer-binding protein delta
ChIP	Chromatin immunoprecipitation
CHO	Chinese hamster ovary
CHX	Cycloheximide
Cort	Corticosterone
CRH	Corticotropin releasing hormone
CRP	C-Reactive protein
Ct	Cycle threshold
DBD	DNA-binding domain
Dex	Dexamethasone
DD	Death domain
DISC	Death-inducing signalling complex
DM	Diabetes Mellitus
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
EtOH	Ethanol

F	Cortisol
FADD	Fas associated *via* death domain
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Glucocorticoid
GLUT4	Glucose transporter 4
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp130	Glycoprotein 130
GRα/β	Glucocorticoid receptor α/β
GRB2	Growth factor receptor-bound protein 2
GRE	Glucocorticoid response element
h	Hour
H	Hinge region
HPA	Hypothalamic-pituitary-adrenal
HRP	Horseradish peroxidase
Hsp90	Heat shock protein 90
IFN	Interferon
IKK	IκB kinase
IL-6	Interleukin-6
IR	Insulin receptor
IRS-1/2	Insulin receptor substrate-1/2
JAK1	Janus kinase 1
JNK	c-Jun N-terminal kinase
kb	Kilobases
kDa	Kilodalton
LB	Luria-Bertani or Lysogeny broth or Luria Broth
LBD	Ligand binding domain
Luc	Luciferase
mAb	Monoclonal antibody
MPK-1	MAPK phosphatase-1
MAPK	Mitogen-activated protein kinase
mIL-6R	Membrane-bound interleukin-6 receptor
MKKs	Mitogen-activated protein kinase kinases
mRNA	Messenger RNA

mTNF-α	Membrane-bound tumour necrosis factor-alpha
mTOR	Mammalian target of rapamycin
Na_2EDTA	Disodium ethylene diamine tetra-acetic acid
NBRE	NGFI-B responsive element
NF-κB	Nuclear factor kappa B
NIDDM	Noninsulin-dependent diabetes mellitus
NR3C1	Nuclear receptor subfamily 3 group C member 1
NTD	N-terminal domain
Nur77	Nuclear receptor-77
Oligo	Oligonucleotide
O/N	Overnight
ori	Origin of replication
qPCR	Semi-quantitative real-time PCR
PAI-1	Plasminogen activator-inhibitor-1
PBS	Phosphate buffered saline
Pen/Strep	Penicillin/streptomycin
PEPCK	Phosphoenolpyruvate carboxykinase
PGE_2	Prostaglandin E_2
PI3K	Phosphoinositide 3-kinases
PKC	Protein kinase C
G9PC	glucose-6-phosphatase
RE	Restriction enzyme
RIP-1	Receptor-interacting protein-1
RLU	Relative light units
RNA	Ribonucleic acid
RO	Reverse osmosis
SAA	Serum amyloid A
SBE	Smad binding element
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHP2	Src homology region 2 (SH2)-containing protein tyrosine phosphatase 2
sIL-6R	Soluble interleukin-6 receptor
SOS	Son of sevenless
STAT3	Signal transducer and activator of transcription 3

sTNF-α	Soluble tumour necrosis factor-alpha
SOCS-3	Suppressor of cytokine signalling 3
T2D	Type 2 diabetes
TAE	Tris-acetate-EDTA
TBS	Tris buffered saline
TBST	Tris buffered saline containing Tween
TE	Tris-EDTA
TF	Transcription factor
TGF-β	Transforming growth factor-beta
TNF-α	Tumour necrosis factor-alpha
TNFR1/2	TNF receptor-1/2
TPE	Tris-phosphate-EDTA
TTP	Tristetraproline
TRADD	TNF receptor type 1-associated death domain
TRAF2	TNF receptor-associated factor-2
vs	*Versus*
WHO	World Health Organisation
WT	Wild-type

LIST OF TABLES

LIST OF FIGURES

TABLE OF CONTENTS

CHAPTER ONE: LITERATURE REVIEW

1.1. Introduction

Over the last few decades, the incidence of diabetes mellitus (DM) has been rapidly expanding, making it one of the leading public health challenges not only in South Africa but worldwide. The global prevalence of diabetes is projected to increase from 463 million in 2019 to 700 million by 2045, denoting a net increase of 51% [1]. In addition, it is among the top ten leading causes of death worldwide, with the World Health Organization (WHO) projecting DM to be the seventh leading cause of death in the world by 2030 [2]. Currently DM is classified as either: i) gestational DM, ii) type 1 DM (T1D) or iii) type 2 DM (T2D). The latter, previously called adult-onset diabetes or non-insulin-dependent, is the predominant form, comprising 90% of all DM cases. T2D is traditionally considered a metabolic disorder and a major non-communicable disease, which is mainly attributed to the initial development of insulin resistance [3]. The term 'insulin resistance' implies a reduced sensitivity of peripheral target tissues, which include adipose, muscle, and liver tissues, to normal circulating concentrations of insulin [4]. The liver is especially of interest, since it is not only the first organ reached by secreted insulin but also the first organ where insulin resistance is presumably initiated [5]. In the liver, insulin resistance is manifested by the inhibition of glycogen synthesis and impaired suppression of hepatic glucose output [6]. This causes chronic hyperglaecemia, which if persists, could lead to the body failing to launch an effective immune response against infection, ultimately leading to sepsis and death [7]. Although it is well established that insulin resistance is central to the pathogenesis of T2D, it remains unclear how this abnormality arises at a molecular level. Contrasting data exist on what the principal molecular defects are which lead to insulin resistance.

Although numerous factors contribute to the development of T2D, including obesity, a common theme throughout literature portrays inflammation and stress as key role players. A close link exists between chronic inflammation and insulin resistance [8], hence why T2D is currently regarded as a chronic, low grade inflammatory state [9]. Inflammation is regulated by several biochemical mediators, of which cytokines are the most important. Studies have implicated pro-inflammatory cytokines, including tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6), in the pathophysiology of insulin resistance and its complications [10][11]. Similarly, glucocorticoids (GCs), steroidal stress hormones, have been demonstrated to cause insulin resistance *in vivo* [12]. Stress *via* GC signalling can activate the acute phase response (APR), a part of the innate immune response.

It has been reported that an elevated APR occurs during insulin resistance [13]. Acute phase proteins (APPs) which significantly increase during the APR, are also associated with insulin resistance, and predict the development of T2D [14]–[20]. The levels of the APPs, plasminogen activator inhibitor-1 (PAI-1), C-reactive protein (CRP), and serum amyloid A (SAA) are upregulated during the diabetic state, hence why these compounds are commonly also used as biological markers for T2D [21]. The inducibility of these APPs by both the early- (pro-inflammatory cytokines) and late (GCs) -stage acute phase mediators suggest that they are synthesized and presumably play an essential role throughout the APR. The fact that certain APPs can be induced by both pro-inflammatory cytokines and GCs is interesting considering GCs are more known for their anti-inflammatory properties [22]. Traditionally GCs and the majority of pro-inflammatory cytokines are well known to antagonize each other's activity [23]. However, current knowledge suggest that GCs selectively regulate gene expression. When it comes to innate immune responses such as the APR, GCs exert more pro-inflammatory behaviour, converging their signal with that of pro-inflammatory cytokine signalling, to further increase the expression of certain APPs. Ultimately, by doing so, GCs reinforce the innate immune system and APR [24].

Taking the above-mentioned associations with insulin resistance into account, three smaller projects forming part of a larger study within our research group have been initiated **(Fig. 1.1)**. We speculate that APPs may be the correlative link between the physiological elements (stress and inflammation) and the development of insulin resistance. Speelman *et al*. investigated the effect of APPs (PAI-1, CRP, and SAA) on the hepatic insulin signalling pathway [25]. Here, the findings suggested that certain APPs possibly play a pivotal role in the development of hepatic insulin resistance, as it was found that PAI-1 and CRP attenuated key proteins in the insulin signalling pathway, leading to the downregulation of hepatic insulin signalling. In the current study these earlier observations are extended by investigating the possible co-regulation of the APPs, PAI-1 and CRP, by GCs and pro-inflammatory cytokines. In addition, the modulation of of either pro-inflammatory cytokine action by the GC and the modulation of the GC action by either pro-inflammatory cytokine will be analysed. It is important to examine regulation from both perspectives, as it has become abundantly clear that both the stress and immune system have bidirectional effects on each other [26]. The modulation of the stress system by the immune system has not been well studied, especially at the peripheral level. Whilst cooperative regulation of SAA by GCs and various pro-inflammatory cytokines have

been extensively studied [27], mostly reporting from the former perspective, fewer have studied this phenomenon in regards to PAI-1 and CRP.

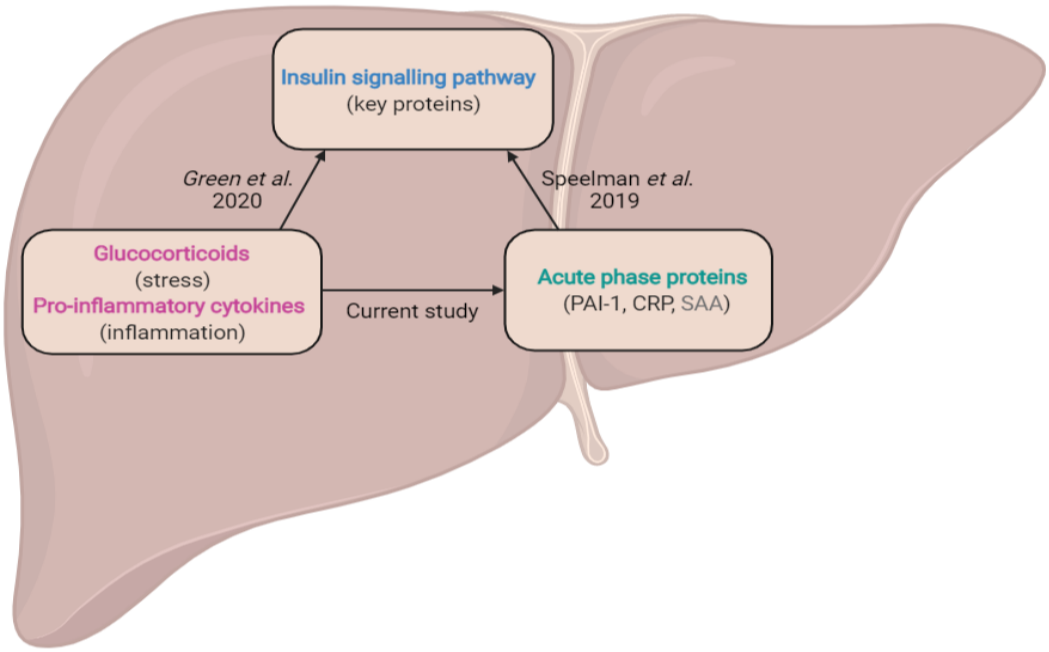


Figure. 1.1. The current study forms part of a larger project within the Verhoog research group. Acute phase proteins may be the correlative link between the physiological elements, stress (represented by glucocorticoids) and inflammation (represented by pro-inflammatory cytokines) and the development of hepatic insulin resistance. Three smaller projects were initiated, with the common goal to bring more evidence to this argument. Image created by author using BioRender.

In this literature review, the knowledge linking insulin resistance to each of the following components: inflammation, the stress system, the APR, and APPs, will be reviewed. In addition, a summary of each component will be provided. The last section in this chapter will provide an overview of the current knowledge available on cooperative regulation of APPs by the GCs and pro-inflammatory cytokines.

1.2. Inflammation and its role in insulin resistance

Inflammation is at the root of most chronic health conditions; this is no different in the pathophysiology of T2D. Several lines of evidence have reported a link between chronic inflammation and insulin resistance [21], however the precise mechanism by which chronic inflammation can evoke insulin resistance remains unclear. The argument has been made that the link between DM and inflammation can be explained by the presence of obesity [28]. In fact, adipose tissue is a source of low-grade inflammation, releasing both the pro-inflammatory cytokines, TNF-α and IL-6 [28]. Dandona and colleagues have proposed that chronic over-nutrition (obesity) can result in the over production of pro-inflammatory cytokines, which

might impair insulin action by suppressing insulin signal transduction [29]. It is however important to note that inflammation is a heterogenous concept, and the association between different inflammatory markers and the incidence of DM vary. Today, systemic low-grade inflammation is widely accepted to be associated with T2D [30][31].

Inflammation is regulated by numerous biochemical signals, which include three major categories of inflammatory mediators: 1) cytokines; 2) GCs, and 3) growth factors [32]. Cytokines are multi-functional molecules, which primarily function as stimulators of APP gene expression. They can be divided into three categories: i) pro-inflammatory cytokines (TNF-α, IL-1-α/β, IL-8), ii) anti-inflammatory cytokines (IL-4, IL-10, IL-13, TGF-β 3) and iii) IL-6-type cytokines (IL-6, IL-11) which have a dual function as both pro- and anti-inflammatory [33]. On the other hand, GCs (discussed in the next section) together with growth factors, such as insulin, function as modulators of cytokine action. In T2D, the balance between anti-inflammatory and pro-inflammatory cytokines are shifted towards pro-inflammation, potentially causing or amplifying the health complications found in this disease state [34].

Since pro-inflammatory cytokines have been associated with insulin resistance and T2D, the section below will review the role each cytokine plays in the development of insulin resistance. In addition, this section will provide an overview of the TNF-α and IL-6 general signalling pathways. However, it must be emphasized, that the cytokine signalling networks are very complex. Although we will evaluate the individual biological profiles of both TNF-α and IL-6, it must be kept in mind that cytokines interact in a complex fashion with each other as well as other factors to regulate gene expression.

> ***'Individual cytokines can be thought of as words which bear informational content. Although individual cytokines may, on occasion, communicate a complete message, more commonly the actual messages received by cells probably resemble sentences, in which it is the combination and sequence of words which convey information.'*** – Kushner, Irving (1993) [35] using a crude simile to describe the complexity of the cytokine signalling language

1.2.1. Tumour necrosis factor-α (TNF-α)

TNF-α is a pleiotropic cytokine, primarily secreted by immune cells, such as macrophages and a broad variety of non-immune cells, including adipocytes [36]. This signalling molecule has

a myriad of diverse functions, including energy metabolism, cell differentiation, proliferation, apoptosis, and the regulation of innate immunity. Importantly TNF-α is also involved in pathological processes such as systemic inflammation [36] where it has the important role in orchestrating the release of pro-inflammatory cytokines, including IL-6. In hepatocytes, TNF-α participates in promoting APP synthesis during the APR [37].

1.2.1.1. TNF-α signalling

Human TNF-α is produced as a homotrimer transmembrane protein which has two isoforms. It is synthesized as a membrane integrated 27 kDa protein (mTNF-α) that undergoes proteolytic cleavage, resulting in a bioactive 17 kDa soluble molecule (sTNF-α). The latter being the form in which TNF-α circulates throughout the body and executes its potent endocrine function, i.e., 'the ability to act at physiological sites, far away from the site of its synthesis' [38].

TNF-α signals through two transmembrane TNF-α receptors, TNFR1 (p55/60) and TNFR2 (p75/80). TNFR1 is constitutively expressed across all human tissues and when it comes to TNF-α signalling is presumed to be its main mediator. Whereas, TNFR2 expression is typically found in cells of the immune system, and plays a major role in the lymphoid system [38]. Since the majority of TNF-α's biological effects are mediated *via* TNFR1 activation, the following discussion will focus mainly on the effects of TNFR1 signalling and the pathways involved **(Fig. 1.2)**. It should also be stressed that these pathways are extraordinarily complex.

Whereas TNFR2 generally mediates the effects of the mTNF precursor, TNFR1 is the main receptor for sTNF. Thus sTNF-α binding to TNFR1 leads to the induction of diverse intricate signalling pathways: i) NF-κB, ii) p38/JNK and iii) caspase signalling **(Fig. 1.2)**. The NF-κB and p38/JNK pathways are activated to help maintain cell survival and promote inflammation by increasing gene transcription of cytokines, cytokine receptors, growth factors, among others. Whereas activation of the caspase pathway promotes apoptotic cell death [36].

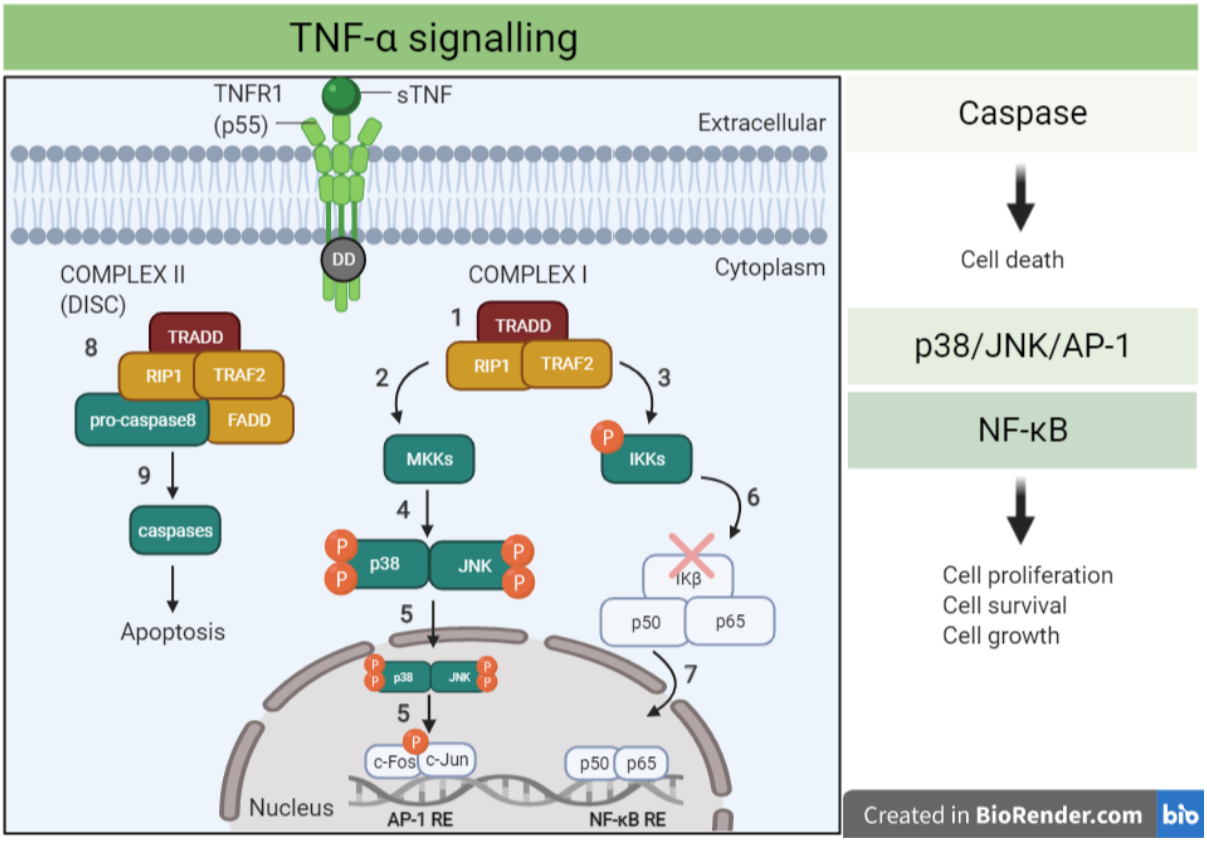


Figure 1.2. Schematic illustration of TNFR1-induced signalling *via* complex I and II. TNF-α (sTNF) binding to TNFR1 leads to the recruitment of TRADD, TRAF2 and RIP1, forming complex I (1). Complex I subsequently activate specific MKKs (2) as well as the IKK complex, consisting of three subunits (3). MKKs activate the MAPKs, p38 and JNK (4) *via* dual phosphorylation. This allows both MAPKs to translocate to the nucleus where they enhance the transcriptional activity of transcription factors, like AP-1 *via* phosphorylation (5) The IKKβ subunit of the IKK complex phosphorylates NF-κB-bound IκBα leading to its proteasomal degradation (6). This allows NF-κB, consisting of two heterodimers, p50/p65, to translocate to the nucleus (7), where it induces the transcription of many genes. Complex II is formed after FADD and procaspase-8 binds to complex I (8). The clustering of procaspase-8 results in its autoactivation, which enables it to activate several effector caspases (9), and signals toward apoptosis. Image was reproduced and adapted from Wullaert *et al.*, 2007 [39]. *Note: sTNF-α = soluble tumor necrosis factor-alpha, mTNF-α = membrane-bound tumor necrosis factor-alpha TNFR1/2 = Tumor necrosis factor receptor 1/2, TRADD = TNF receptor type 1-associated death domain, RIP1 = Receptor-interacting protein 1, TRAF2 = TNF receptor-associated factor 2, FADD = Fas Associated Via Death Domain, NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells, JNK = c-Jun N-terminal kinase, MKKs = mitogen-activated protein kinase kinases, IKKs = IκB kinases, AP-1 = Activator protein-1*

i. *NF-κB pathway*

TNF-α signal complex I is formed upon TNF-α binding to TNFR1, which leads to the recruitment of TNF receptor type 1-associated death domain (TRADD), TNF receptor-associated factor 2 (TRAF2), and receptor-interacting protein 1 (RIP1) as illustrated in **Fig. 1.2**. This complex by default phosphorylates and triggers the activation of IκB kinases (IKK)-αβγ complex. Next, the IKKβ subunit phosphorylates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-bound IκBα, leading to its proteasomal degradation. This allows the transcription factor, NF-κB, consisting of p65 (RelA)/p50 heterodimers, to translocate into the nucleus where it recognises and binds to specific DNA sequences in promoters of regulated

genes [39]. Subsequently, NF-κB regulates the expression of many different genes, including those associated with cell survival and the production of pro-inflammatory cytokines, such as IL-6 [40]. Moreover, NF-κB itself can transcriptionally induce *tnf*, as well as *traf2*, and thereby further amplify the TNF-α/TNFR1 signalling pathways. Despite TNF-α signalling not yet being fully understood, the pro-inflammatory effects of TNF-α are predominantly understood to be because of its ability to activate NF-κB [41].

ii. *p38/JNK/AP-1 pathway*

As shown in **Fig. 1.2**, TNF-α signal complex I also induces the activation of mitogen-activated protein kinases (MAPKs), such as c-Jun N-terminal kinase (JNK) and p38. Both are activated by dual phosphorylation on tyrosine and threonine residues *via* mitogen-activated protein kinase kinases (MKKs). Upon activation, they translocate into the nucleus, where they phosphorylate and enhance the transcriptional activity of transcription factors, including activator protein-1 (AP-1). The AP-1 protein, consisting of the c-Jun and c-Fos subunits, is an important promotor of inflammation by regulating the production of pro-inflammatory cytokines, proliferation as well as prevention of apoptosis [41].

iii. *Caspase pathway*

When the survival pathway is inhibited, TNFR1 can induce a cell death response, since it contains a death domain (DD). Binding of fas associated *via* the death domain (FADD) and procaspase-8 to complex I, results in the formation of complex II or otherwise known as the death-inducing signalling complex (DISC) **(Fig. 1.2)**. The clustering of procaspase-8 results in its autoactivation, which enables it to activate several effector caspases, which ultimately signals toward apoptosis and necrosis [39].

1.2.1.2. The role of TNF-α in insulin resistance

Amongst the broad-spectrum of pro-inflammatory cytokines, TNF-α, was the first to be implicated in the pathogenesis of T2D as it was found to interfere with insulin signalling [10]. Since then studies have emerged indicating serum levels of TNF-α to be positively correlated with the pathophysiology of insulin resistance [13][42][43], with the general consensus that elevated TNF-α can cause insulin resistance. TNF-α is also seen as a mediator of insulin resistance in obesity, as it is overexpressed in human and rodent adipose tissue, and can block the action of insulin *in vitro* and *in vivo* [10]. In support of this, studies have found animal models of obesity and insulin resistance to produce significantly higher TNF-α levels compared

to their lean counterparts, whilst also revealing a strong correlation between TNF-α levels and obesity (BMI = body mass index) [10][44]

In murine models of obesity and T2D where TNF-α was found to be markedly increased, pharmacological or genetic neutralization of TNF-α or its receptors improved insulin signalling, leading to reduced gluconeogenesis in fat and muscle [45][46]. In support of this finding, Hotamisligil *et al*. found that when TNF-α was neutralized in obese rats, the peripheral uptake of glucose in response to insulin was significantly increased [10]. In addition, a study by the same research group in TNF-α receptor knockout mice showed increased insulin sensitivity and an improved glucose tolerance [44].

Several potential mechanisms by which TNF-α directly impairs insulin signalling have been proposed. The majority of studies suggest that TNF-α contributes to the development of insulin resistance through induction of serine kinases that attenuate insulin receptor (IR) and insulin receptor substrate (IRS)-1/2 signalling pathways [47]–[49]. *In vitro* studies reported that exposure of cultured adipocytes, hepatoma and Chinese hamster ovary (CHO) cells to TNF-α caused insulin resistance by activating JNK and IKKβ signalling cascades. This resulted in an increase in the inhibitory serine phosphorylation of IRS-1, making IRS-1 a poor substrate for insulin receptor-activating kinases, ultimately impairing insulin action [44][50]–[53]. Additionally, *in vivo* studies in muscle and fat tissue of obese rats also showed TNF-α to promote insulin resistance by inducing serine phosphorylation of IRS-1, ultimately inhibiting the IR tyrosine kinase activity [44][54]. The abovementioned findings have shown that chronic exposure to TNF-α impairs insulin signalling in major insulin-sensitive tissues, such as the skeletal muscle and adipose tissue. The mechanism by which TNF-α impairs hepatic insulin signalling is not clearly defined.

1.2.2. Interleukin-6 (IL-6)

IL-6 is a pleiotropic cytokine, produced after TNF-α and IL-1 stimulation [55], by numerous different cell types, including those found in the immune system [56]. Outside of the immune system, hepatocytes among various other cells, are targets of IL-6. The biological effects of IL-6 differ according to target tissues, and ranges from immune regulation, proliferation, and differentiation of hematopoietic cells to inflammation at sites of tissue injury [36]. In the liver, IL-6 acts as a chief regulator of the APR following a triggering event [32][57], by stimulating

the hypothalamic pituitary adrenal (HPA)-axis to produce cortisol in humans, the feedback of which inhibits the production of IL-6 [55].

1.2.2.1. IL-6 signalling

Human IL-6 is produced as a 26 kDa four helix-bundle cytokine [58]. IL-6-mediated effects are exerted through two pathways known as classic and trans-signalling. In the former pathway, IL-6 binds to the membrane-bound IL-6R (mIL-6R, R refers to receptor), whereas in the latter pathway IL-6 binds to a soluble form (sIL-6R). It is believed that IL-6 classic signalling is important for the APR and promotes anti-inflammatory and regenerative effects, while IL-6 trans-signalling promotes pro-inflammatory responses [59]–[61].

Under normal conditions, circulating IL-6 prefers to bind to sIL-6R, but during some inflammatory conditions, where plasma IL-6 levels are elevated, IL-6 acts *via* trans-signalling [62]. Generally speaking, IL-6 binds to the α-chain of its cognate receptor, mIL-6R (CD126/gp80). Only a few cell types, including hepatocytes, express mIL-6R on their cell surface, and therefore can directly respond to IL-6. Subsequently, this ligand-receptor complex associates with two subunits of gp130, a membrane glycoprotein and signal transducing receptor, triggering its homodimerization **(Fig. 1.3)**. This forms a stimulatory complex (IL-6/IL-6R/gp130), which can now induce autophosphorylation and activation of Janus kinases (JAKs), (JAK1, JAK2) [63]. Activated JAKs can activate two major pathways: i) JAK1/STAT3 and ii) Ras/ERK/MAPK **(Fig. 1.3)**. As a result APP expression, adhesion molecules, chemokines, anti-apoptotic proteins and cytokines are induced [36][64]. Additionally, the complex can activate other signalling pathways, such as phosphoinositide 3-kinases (PI3K)/Akt, but this pathway will not be discussed as it is primarily involved in prostate cancer cells [65].

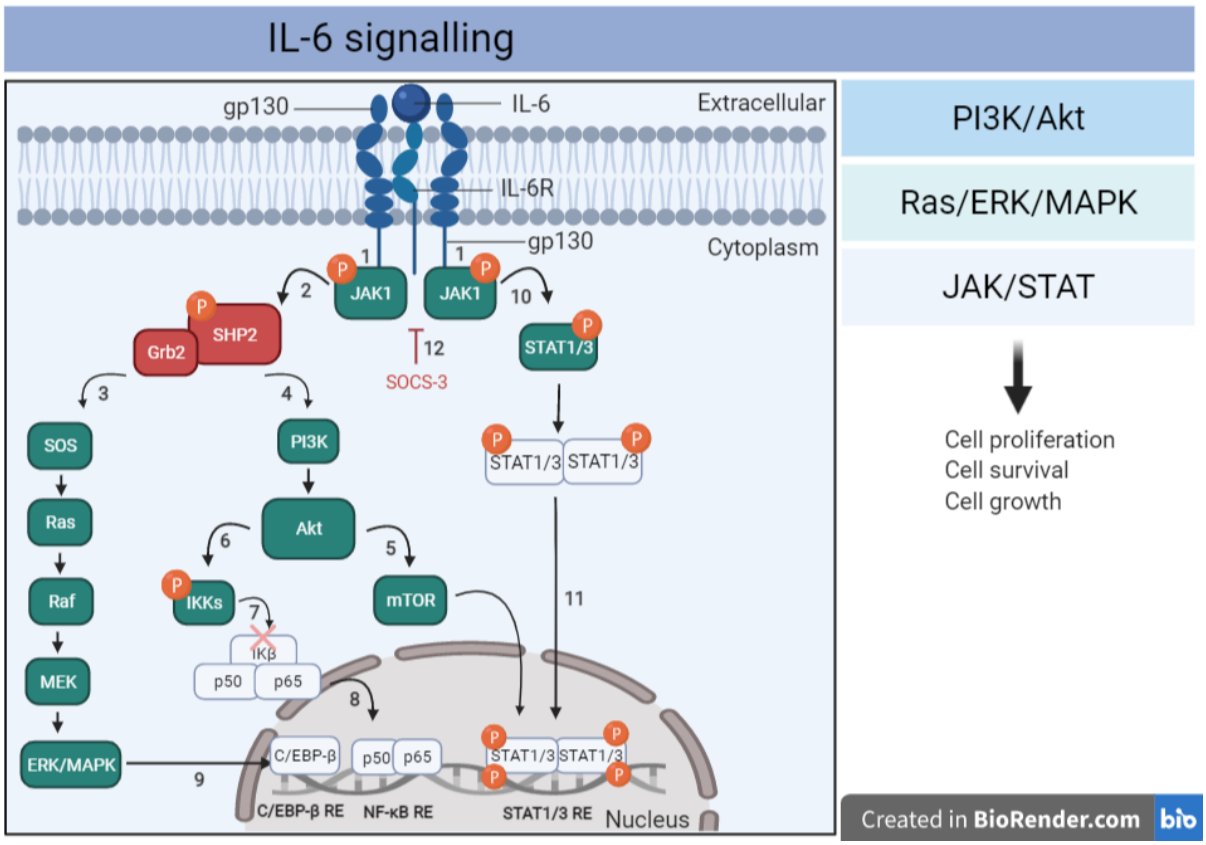


Figure 1.3. Schematic illustration of IL-6 signalling. The IL-6 receptor complex activates gp130-associated JAKs *via* autophosphorylation (1). Activated JAKs recruit SHP2 and Grb2 (2), which then stimulates activation of the Ras/ERK/MAPK (3) and PI3K/Akt (4) pathways, respectively. Activation of Akt can either lead to activation of mTOR (5) which upregulates SOCS-3 transcription or it can activate the IKK complex (6). IKKβ subunit of the IKK complex phosphorylates NF-κB-bound IκBα leading to its proteasomal degradation (7). This allows NF-κB, consisting of two heterodimers, p50/p65, to translocate to the nucleus (8), where it transcriptionally induces many genes. In addition, activation of the ERK/MAPK pathway can activate transcription factors, such as C/EBP-β. Furthermore, activated JAKs leads to the recruitment, phosphorylation and translocation of STAT3 to the nucleus (10). Here STAT3 dimers bind to DNA and induce APP and SOCS-3 expression (11). In addition, SOCS-3 inhibits JAKs (12), acting as negative regulator of IL-6 signalling. Image was reproduced and adapted from Taub, 2004 [66] & Akbari *et al*., 2018 [62]. *Note: APP = acute phase proteins, APR = acute phase response, C/EBP-β = CCAAT/Enhancer binding protein beta, gp130 = glycoprotein 130, Grb2 = Growth factor receptor-bound protein 2, IKKs = IκB kinases, IL-6 = interleukin-6, IL-6R = interleukin-6 receptor, JAK1 = Janus kinase 1, ERK/MAPK = extracellular signal-regulated kinases/mitogen-activated protein kinase, mTOR = mammalian target of rapamycin, NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells, PI3K = Phosphoinositide 3-kinases, SHP2 = Src homology region 2 (SH2)-containing protein tyrosine phosphatase 2, SOCS-3 = Suppressor of cytokine signaling 3, SOS = son of sevenless, STAT3 = Signal transducer and activator of transcription 3*

i. *JAK/STAT pathway*

Receptor activation by IL-6 initiates the activation of JAK1, which in turn leads to tyrosine phosphorylation and activation of the transcription factors, signal transducer and activator of transcription 3 (STAT3) and src homology region 2 (SH2)-containing protein tyrosine phosphatase 2 (SHP2), the latter triggering the MAPK pathway **(Fig. 1.3)**. Beyond the tyrosine phosphorylation of STAT3, which leads to its dimerization and translocation to the nucleus, serine phosphorylation of STAT3 is required for its maximal activation [67].

Several protein kinases, including mammalian target of rapamycin (mTOR), have been reported to phosphorylate STAT3 at its serine residue [67]. Fully activated STAT3 is now able to translocate to the nucleus as a dimer where it binds to specific response elements in the promotors of IL-6 responsive genes. One of these genes encodes the suppressor of cytokine signalling (SOCS) protein, which is rapidly induced by IL-6 *via* the JAK/STAT pathway [67]. Thereby, acting as a negative feedback inhibitor of this pathway in order to prevent excessive inflammation by terminating IL-6 signalling. SOCS proteins bind directly to tyrosine-phosphorylated JAK1 or activated cytokine receptors to suppress cytokine signalling [67]. This statement is supported by *in vivo* studies in the liver, reporting that deletions of SOCS-3 led to prolonged STAT3 activation following IL-6 stimulation [68]. Ultimately, the IL-6/JAK1/STAT3 pathway regulates the expression of several genes leading to an increase in cell growth, differentiation, and survival [69]. In the liver, this process promotes the APR, hepatoprotection and liver regeneration.

ii. ***Ras/ERK/MAPK pathway***

As shown in **Fig. 1.3**, SHP2 is another mediator of IL-6 signalling, following its recruitment to phosphorylated gp130, and subsequent tyrosine phosphorylation by JAK1. Phosphorylated SHP2 associates with the adaptor protein, growth factor receptor-bound protein 2 (Grb2), which subsequently recruits and binds to son of sevenless (SOS). This series of interactions between these proteins lead to the activation of Ras, a monomeric small GTPase switch protein responsible for the activation of downstream signalling proteins such as extracellular signal-regulated kinases 1 or 2 (ERK1/2) and MAPK **(Fig. 1.3)** [69]. In addition, it has been shown that activation of the ERK/MAPK pathway can activate the transcription factor, C/EBP-β [70], *via* phosphorylation, whereafter it moves across the nuclear membrane to interact with its own cognate response elements (CAAT box) in the mammalian promotor regions of IL-6-responsive genes [71]. Overall, the Ras/ERK/MAPK pathway mediates diverse effects including cell growth, stimulation of APPs and immunoglobulin synthesis [69].

1.2.2.2. The role of IL-6 in insulin resistance

Under normal physiological conditions, the expression of IL-6 is short-lived, contributing to tissue injury, and decreasing when tissue homeostasis is restored [36]. However, in the pathogenesis of diseases with an inflammatory background, including insulin resistance and T2D, the abnormal and chronic production of IL-6 plays a major role [49]. Several studies have reported circulating IL-6 concentrations to be positively correlated with obesity, insulin

resistance, and T2D in human subjects [11][13][72][73]. In particular studies have shown peripheral administration of IL-6 in rodents to induce insulin resistance [74][75]. The exact mechanism by which IL-6 induces insulin resistance appears to be complicated and versatile [76]–[82].

In brief, IL-6 induces insulin resistance in muscle and adipose tissue *via* activation of the JAK/STAT3 pathway, leading to the reduction of the glucose transporter, GLUT4, and IRS-1 expression [76]. Several studies in isolated hepatocytes and in the liver of experimental animals have linked the elevated levels of IL-6 to the inhibition of insulin signalling. *In vivo* studies in the liver, have shown that IL-6 blunted the ability of insulin to suppress gluconeogenesis and insulin-stimulated IRS-2 associated PI3K activity [77]. *In vitro* studies using both mouse hepatocytes and human hepatocarcinoma cells (HepG2) have demonstrated that IL-6 impairs insulin signalling by enhancing SOCS-3 expression, which impairs IRS-1 tyrosine phosphorylation [78][79]. In line with this, it has been proposed that mTOR plays an important role in IL-6-induced hepatic insulin resistance by regulating the activation of STAT3 [80]. Consequently, SOCS-3 expression increases leading to the impairment of insulin signalling [80]. In the liver tissue of obese mice, chronic exposure to IL-6 selectively induced insulin resistance, while systemic depletion of IL-6 improved hepatic insulin action [79][81]. Furthermore, evidence exists supporting the role of hepatic NF-κB activation in the development of insulin resistance. Mice models of obesity, with chronic activation of NF-κB, resulting in recurrent IKKβ activation, caused profound hepatic insulin resistance. However, injection of IL-6 neutralizing antibodies proved to be effective by improving hepatic insulin resistance in these mice [82]. Altogether these findings suggest a causative role for IL-6 in the development of insulin resistance, especially in the liver.

1.3. Glucocorticoids: the hormone and its signalling pathway

GCs are essential steroid hormones with potent anti-inflammatory capabilities that primarily function to modulate and suppress inflammation [83]. Naturally produced GCs such as cortisol and corticosterone are principally secreted by the human and rodent adrenal cortex, respectively. The effective anti-inflammatory activity of endogenous GCs are exploited by the pharmaceutical industry through the synthesis of synthetic GCs, such as dexamethasone (Dex) and prednisone [22]. These derivative steroid drugs are routinely prescribed in the management of many inflammatory disorders [84]. Endogenous GCs are mainly secreted in response to a stress which is defined as: ‘a state of threatened homeostasis, provoked by a stressor - either an

internal or external stimulus' [85]. The following section will review the current status of knowledge on the molecular sequence of events that unfolds when GCs reach its target organs and binds to its receptor. In addition, as GCs have been associated with insulin resistance and T2D, the section below will review the role it plays in the development thereof.

1.3.1. Glucocorticoid signalling

The stress response system, which also include the sympathetic nervous system (SNS), is essential for human survival and involves the activation of the HPA-axis, a multi-step hormonal process [86][87]. Upon exposure to stress (environmental or physiological) the hypothalamus is activated to release corticotropin-releasing hormone (CRH). In turn, CRH targets the anterior pituitary gland to secrete adrenocorticotropic hormone (ACTH). Next, ACTH acts on the cortex of the adrenal glands situated above each kidney to synthesize and release endogenous GCs such as cortisol into systemic circulation **(Fig. 1.4)**. Once within the blood stream, cortisol will move to target organs in the periphery, such as the liver, where they will exert their tissue-specific effects [87]. In the liver specifically, GCs stimulate gluconeogenesis, which provide systemic glucose needed to fuel the stress response [88]. In return, GCs control the regulation of basal HPA-axis activity, establishing a regulatory feedback loop [86]. It is important to note, that although a stress-induced increase in GCs is adaptive in the short term, anomalous GC secretion may result in health complications [83]. In addition, prolonged exposure to synthetic GCs, have shown to lead to side effects, including weight gain or obesity [89].

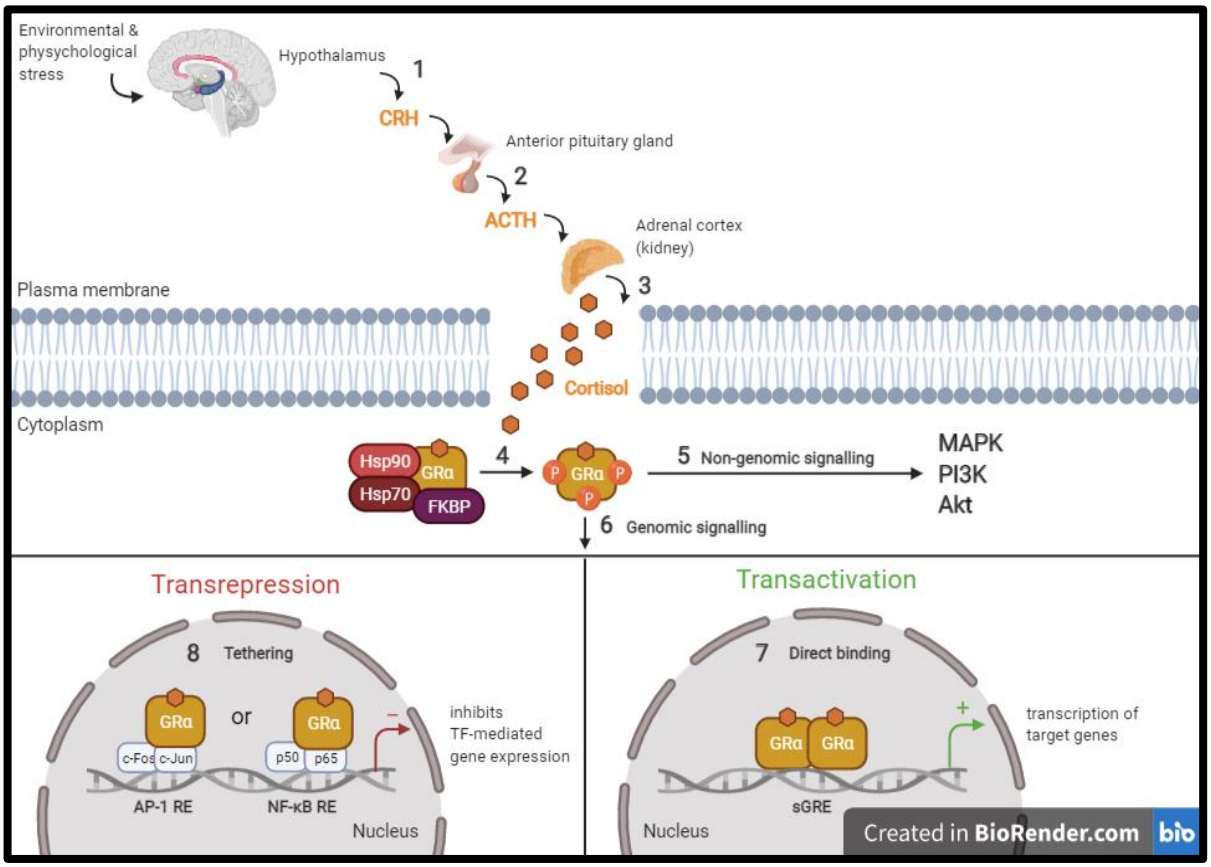


Figure 1.4. Schematic illustration of the HPA-axis and glucocorticoid signalling pathways. Upon exposure to environmental and physiological stress the hypothalamus is stimulated to secrete CRH (1), which stimulates the release of ACTH by the anterior pituitary gland (2). In turn, ACTH targets the adrenal glands of the kidney to synthesize and secrete cortisol into the bloodstream (3). Cortisol can readily enter the cell, where this hormone binds to the GR in the cytoplasm (4). This induces a conformational change in the receptor, where it becomes hyperphosphorylated and dissociates from its chaperone molecules, such as heat shock proteins (HSPs) (4). The GR can signal in a non-genomic manner (5) or translocate into the nucleus where it exerts its actions through genomic mechanisms (6). In the nucleus, the GR can either dimerise (7) to transactivate anti-inflammatory genes or tether to pro-inflammatory transcription factors (TFs) as a monomer (8) to inhibit cytokine production, known as transrepression. Image was reproduced and adapted from Cruz-Topete & Cidlowski, 2015 [87]. *Note: CRH = Corticotropin-releasing hormone, ACTH = Adrenocorticotropic hormone, AP-1 = Activator protein-1, Hsp90/70 = Heat shock protein 90/70, FKBP = Immunophilins, MAPK = Mitogen-activated protein kinase, PI3K = Phosphoinositide 3-kinases, NF-κB = Nuclear factor kappa-light-chain-enhancer of activated B cells, GR = glucocorticoid receptor, sGRE = simple glucocorticoid response element*

GCs are lipophilic molecules which exert their physiological and pharmacological effects by diffusing across the cell membrane to bind to their cognate intracellular receptor, the glucocorticoid receptor (GR) [90]. Whilst synthetic GCs, such as Dex almost exclusively activate the GR, endogenous GCs can also activate the mineralocorticoid receptor (MR), which alongside the GR belongs to the nuclear receptor superfamily [24]. The GR is expressed in virtually all cell types, tissues and organs [87]. The ligand-bound GR functions as a transcription factor which can modulate the transcription of a large number of genes *via* well-described genomic and less well understood non-genomic signalling mechanisms [91]. Human GR is transcribed from the *nr3c1* gene, and due to alternative splicing of this gene has two

main protein isoforms, i.e., GRα and GRβ, which only differ in their C-terminal regions [92]. GRα is the classic receptor, which elicits most of the biological actions of GCs. Whereas, GRβ does not bind active GC agonists [87] and its biological relevance remains unclear. The expression of GRα is higher than GRβ in tissues [87].

The GR consists of three major functional domains, each fulfilling a specific function (**Fig. 1.5**) [87]. Firstly, the N-terminal domain (NTD) contains a transcriptional activation function-1 (AF-1) domain, which consists of residues that undergo several post-translational modifications. As a result, the NTD is important for the recruitment of basal transcriptional machinery [87]. Secondly, the central DNA-binding domain (DBD) is involved in GR dimerization, nuclear localization and binding of the GR to glucocorticoid response elements (GREs), within the promoter regions of GC-responsive genes [87]. Lastly, the C-terminal ligand-binding domain (LBD) contains an AF-2 domain which interacts with the chaperone protein, heat shock protein 90 (Hsp90), in a ligand-dependent manner and allows the GR to bind to natural or synthetic GCs [87]. Furthermore, the hinge region links the DBD and LBD.

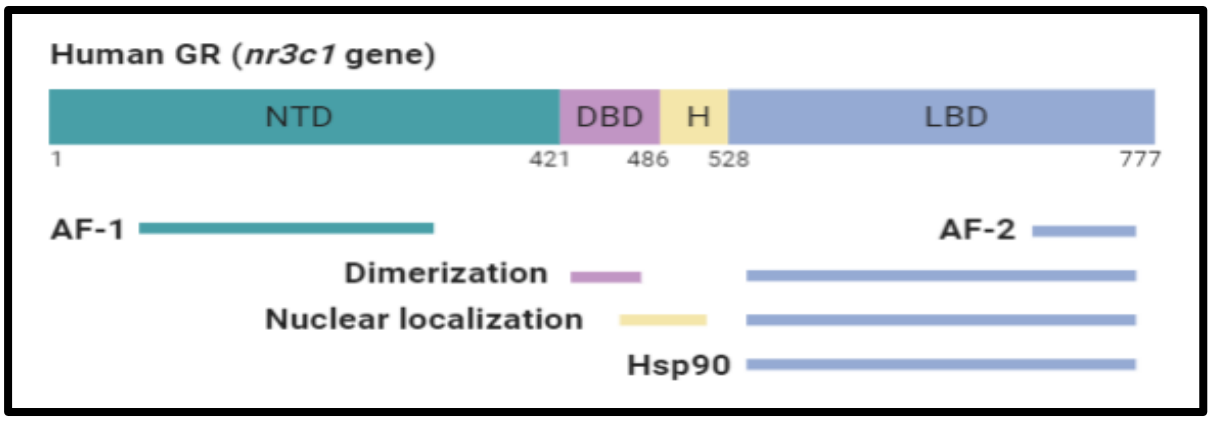


Figure 1.5. Schematic illustration of the human GR structure. Linear representation of the 777 amino acid GR showing the principal domains and regions involved in transactivation (AF-1 and AF-2), dimerization, nuclear localization and Hsp90 binding. Image was inspired by Cruz-Topete & Cidlowski, 2015 [87]. *Note: NTD = N-terminal domain, DBD = DNA binding domain, H = hinge region LBD = ligand binding domain, AF = activation function*

> ***''GC signalling is not merely a simplified signal transduction pathway but a complex homeostatic system that functions co-ordinately with other systems to help individuals cope with stressful stimuli''*** *– Nicolaides et al., 2015* [91]

Studies are surfacing, adding new layers of complexity to GR signalling [90][91]. To simplify this complex signalling system, only the general steps as illustrated in **Fig. 1.4** will be discussed. Within the target cell, the unliganded GRα (hereafter referred to as GR) is

predominantly retained in the cytoplasm complexed with Hsp90/70 chaperones, as well as other accessory proteins, such as the immunophilins (FKBP) [24][93]. This forms an inactive multi-protein complex, where the GR is considered to be functionless. A GC signalling cascade is triggered upon lipophilic GCs entering the cell and binding to the cytoplasmic GR *via* its LBD. This leads to conformational changes in the receptor which results in dissociation of the multi-protein complex and hyperphosphorylation of the GR [24]. Subsequently, allowing the receptor to either translocate along microtubules into the nucleus by virtue of a sequence in the DBD, or interfere with signal transduction components in the cytoplasm. Once within the nucleus, monomeric GR can assume different conformations and exert its actions *via* various genomic mechanisms as displayed in **Fig. 1.6**.

The homodimeric GR can interact directly with GREs in the promoter (regulatory) regions of GC target genes, ultimately, influencing their transcription rate in a positive manner (referred to as transactivation). In addition, as a monomer, the ligand-activated GR can bind to negative GREs (nGREs) whereby transcription of the targeted genes are inhibited (referred to as transrepression) [24]. In addition, activated GR bound to GREs can interact with neighbouring DNA-bound transcription factors, commonly known as composite regulation. Alternatively, the activated GR can influence gene expression independently of GREs through protein-protein interactions with other DNA-bound transcription factors, known as tethering [24].

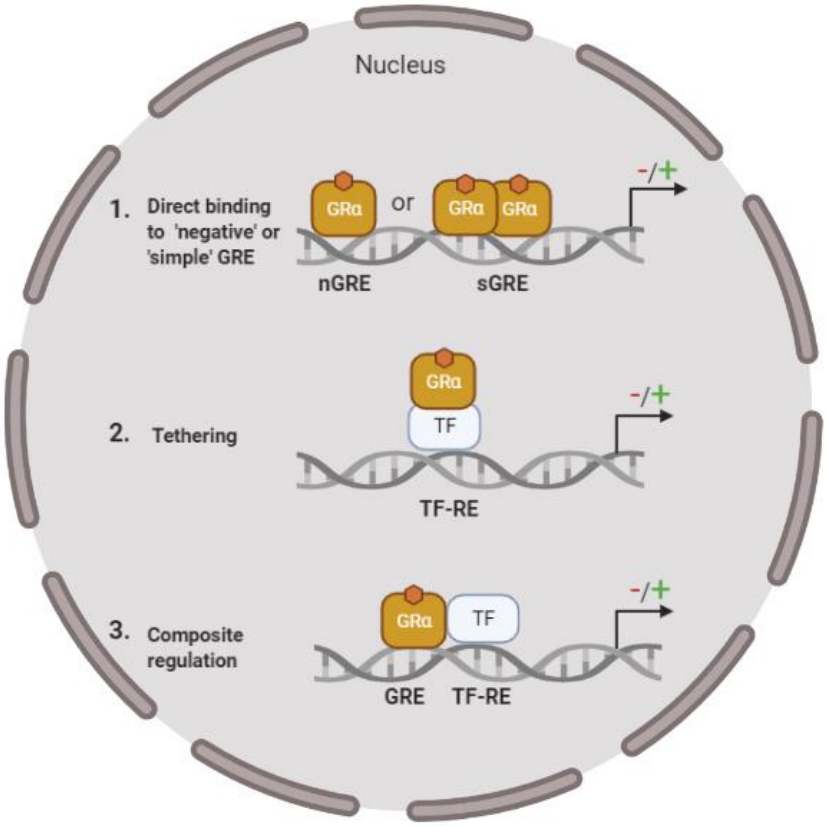


Figure 1.6. Schematic illustration of the different GR genomic signalling mechanisms. The GR can repress or enhance the transcription of target genes by (1) directly binding to negative (nGRE) or simple (sGRE) glucocorticoid-response elements, respectively, (2) by tethering itself to other transcription factors (TFs), or (3) by directly binding to GREs and interacting with neighbouring TFs (known as composite regulation). Image was adapted from Necela & Cidlowski, 2004 [94] using BioRender.

Traditionally, transactivation is generally mediated by a GR homodimer directly binding to 'simple' GRE sites (sGRE), thereby activating the gene transcription of multiple anti-inflammatory genes, such as tristetraproline (TTP) **(Fig. 1.4)** [87]. On the other hand, transrepression is generally mediated *via* a tethering mechanism whereby the monomeric GR interacts with other transcription factors, thereby inhibiting their gene targets **(Fig. 1.4)** [94].

During inflammation a signalling cascade is triggered, leading to the activation of pro-inflammatory transcription factors, NF-κB and AP-1. As mentioned earlier, the former is composed of the p50 and p65 (RelA) subunits, whereas the latter is composed of the c-Jun and c-Fos subunits. However, the GR can inhibit the transcriptional activity of both NF-κB and AP-1 by binding to a GRE and simultaneously interacting with the p65 and c-Jun subunits, respectively (also known as composite regulation or negative tethering) [87]. Ultimately, leading to the repression of their capacity to induce pro-inflammatory gene transcription [91]. However, positive tethering by the GR *via* C/EBP [95][96], NF-κB [96] and STAT3 [94] has also been shown.

In addition to the GR's genomic signalling, this steroid receptor can signal in a non-genomic manner, which is poorly understood. What is known to researchers thus far is that the non-genomic effects of the GR involves its ability to directly modulate signal transduction pathways, including MAPK, P13K, and Akt pathways **(Fig. 1.4)** [87][97]. However, the detailed mechanism and biological implications have not been fully elucidated. Current knowledge suggests that GCs have inhibitory effects on the pro-inflammatory MAPK signalling pathway by inducing the expression of proteins, such as MAPK phosphatase-1 (MPK-1), which inhibits p38. Thus preventing the induction of multiple pro-inflammatory genes encoding cytokines, which ultimately resolves the inflammatory process and restores homeostasis [87][97]. Whether the ligand activated GR directly interacts with any components of these signal transduction pathways remain unknown and requires further investigation.

1.3.2. The role of glucocorticoids in insulin resistance

As mentioned earlier, exogenous GCs, such as Dex, are routinely prescribed in the management of inflammatory disorders. However, long-term GC treatment can reportedly exaggerate insulin resistance [98]. In fact, the ligand-activated GR is responsible for the increase in the production of the gluconeogenic enzymes, glucose-6-phosphatase (G6PC) and phosphoenolpyruvate carboxykinase (PEPCK) [99][100], which upregulates gluconeogenesis, thereby increasing hepatic glucose production. However, studies have also demonstrated that the synthetic GC, Dex can interfere with insulin signalling more directly by decreasing IRS-1 [101] and AKT protein expression [102] in mouse adipocytes, and decreasing tyrosine phosphorylation of the IR and IRS-1 in muscle and liver cells [103]. Altogether these findings suggest a causative role for exogenous GCs in the development of insulin resistance in all the major insulin target tissues.

1.4. The acute phase response: current status of knowledge

> ***'The maintenance of physiologic homeostasis during the ups and downs of daily living is assured by a number of physiological mechanisms. From time to time, however, perturbing events occur which represent a threat to the integrity of the organism'*** – Kushner, Irving (1982) [104]

Under normal circumstances, homeostatic maintenance is ensured by a number of physiological mechanisms. However, deviations from this stable internal state may occur,

imposing a serious threat to an individuals' health. These homeostatic deviations can include any process leading to tissue damage including infection, inflammation, and stress, among others [104][105]. As a result, the body responds to these disturbances in a predetermined and well-coordinated manner, with a local inflammatory response accompanied by a large number of systemic responses [105]. These complex physiological events are collectively referred to as the APR, which involves the cardiovascular and central nervous system (CNS) systems as well as major organs like the liver [35].

The APR is the cornerstone of the innate immune response [106], which is the body's first line of defence against inflammatory stimuli. The activation of the APR is proportional to the severity of this inflammatory stimulus [107]. The APR occurs at the very beginning of the inflammatory process, showing a rapid response that peaks within the first 48 hours, but can continue up to three to five days [108][109]. Two main responses are generated by the APR, namely the local and systemic responses **(Fig. 1.7)**. The local response is manifested as acute inflammation, with a prominent aspect being the dilation and leakage of blood vessels, resulting in tissue edema (swelling) and apparent redness [32]. Whereas the systemic response constitutes an array of changes, which are primarily expressed as fever, increased release of several hormones, leucocytosis and the drastic rearrangement of plasma protein synthesis [110]. Below a brief overview of the APR and the sequence of events it entails, is given.

During the early part of the APR, at the local site of insult, cells of the innate immune system are activated. These cells primarily include white blood cells (leukocytes), such as blood monocytes and tissue macrophages, which are responsible for initiating the APR cascade [32]. Additionally, other events such as mast cell degranulation and aggregation-induced platelet activation can lead to the release of chemotactic mediators, such as transforming growth factor-beta (TGF-β) [32]. TGF-β stimulates the migration and infiltration of more macrophages and monocytes to the site of insult [32]. Together, activated monocytes and macrophages secrete a number of pro-inflammatory cytokines, known as 'alarm' cytokines, into the bloodstream, most notable of which are TNF-α and IL-1 [32][111]. These cytokines stimulate the synthesis and release of a range of secondary cytokines from local stromal cells (fibroblasts), including, IL-6. IL-6 promotes the systemic effects of the APR in diverse tissues, ranging from the liver to the bone marrow [112].

Thus, as the APR is initiated, pro-inflammatory cytokines are first produced and act locally in an autocrine manner. Additionally, these cytokines can act in a paracrine manner, by recruiting adjacent stromal cells, including fibroblasts and endothelial cells [113]. As a result, a second wave of pro-inflammatory cytokines are produced and released in larger quantities, further potentiating the cytokine-induced response. Ultimately, these early events of the APR, ensure that all cells of the body have the potential to be involved in the initiation and propagation of this important homeostatic response.

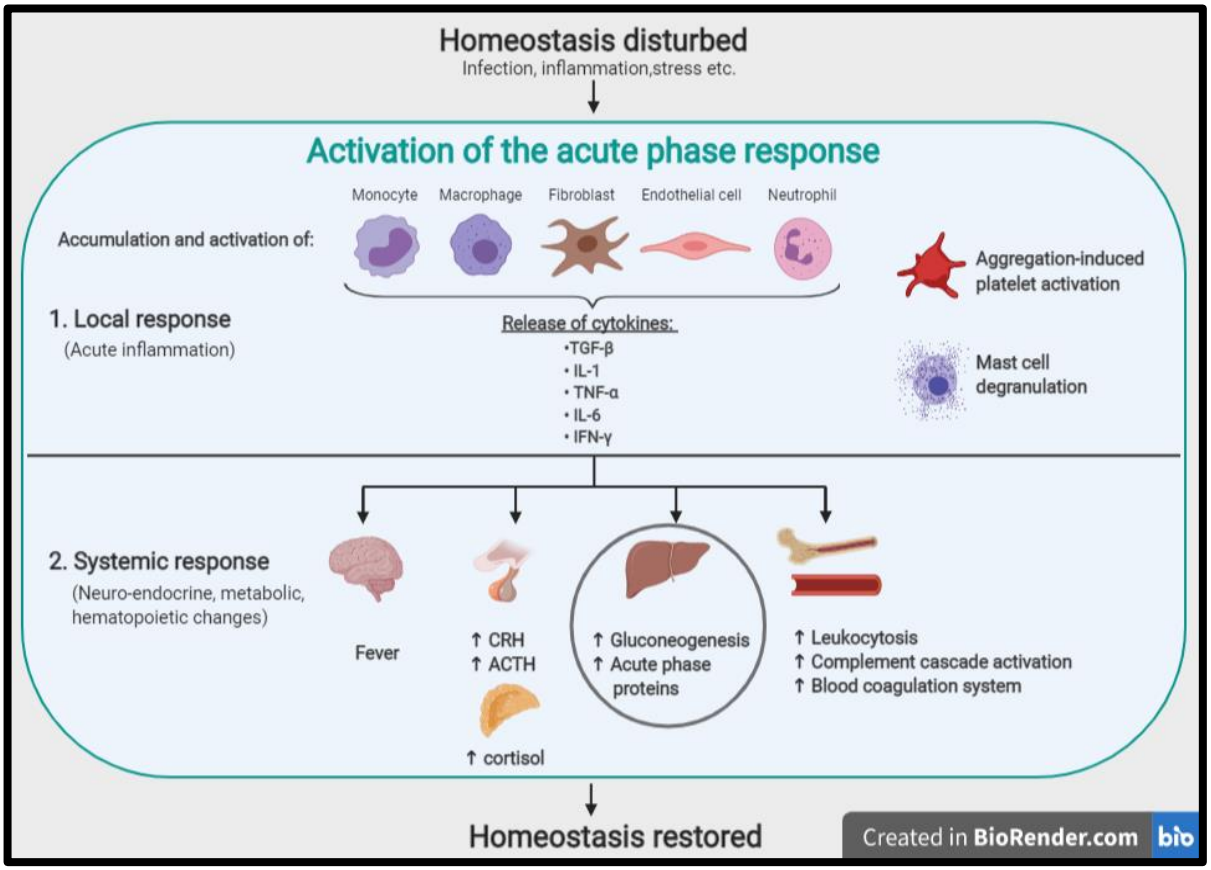


Figure 1.7. Schematic illustration of the different phases that occur during the acute phase response. In response to various disturbances (infection, inflammation, stress), the acute phase response (APR) is activated and generates two responses: the local (1) and systemic (2) responses. The local response is known as acute inflammation, which results in the release of pro-inflammatory cytokines from sites of insult. Whereas the systemic response results in a number of physiological processes which, most importantly, includes increased production of acute phase proteins in the liver (pointed out with a grey circle). Ultimately, the APR functions to restore homeostasis. Image was reproduced and adapted from Heinrich et al. 1990 [112], Paltrinieri, 2008 [108] and Anglin *et al.*, 2010 [114]. *Note: IL = interleukin, TNF = tumour necrosis factor, IFN = interferon, TGF = tumour growth factor, CRH = corticotropin-releasing hormone, ACTH = adrenocorticotropic hormone*

As the APR progresses, pro-inflammatory cytokines leave the local site of insult and enter the bloodstream, where they act distally in a paracrine manner [113]. Ultimately, these signalling molecules are responsible for the induction of a wide range of systemic inflammatory effects, which include a host of neuro-endocrine, metabolic, and hematopoietic changes [114]. The

primary characteristic features of the systemic APR, including (i) generation of fever, (ii) increase in the release of several hormones, (iii) leucocytosis, and (iv) changes in the concentration of serum APPs, will be discussed below. Other prominent features of the APR include, an increase in the activation of the complement cascade and blood coagulation system [109] as well as increased gluconeogenesis [115][116], which will not be discussed in detail. The latter metabolic process is promoted by both IL-1 and TNF-α, in order to fuel obligated glucose-consuming cells during the course of the APR [33].

The induction of fever is one of the earliest recognised features of the APR in the body. This change in thermoregulation results from exogenous pyrogens, such as lipopolysaccharides (LPS), which activate monocytes and macrophages to release endogenous pyrogens, such as pro-inflammatory cytokines: IL-1, TNF-α, IL-6, and interferon-gamma (IFN-γ) [114]. As a result, these pro-inflammatory cytokines act on the CNS and anterior hypothalamus to locally produce intermediate molecules, such as prostaglandin E_2 (PGE_2) [32][109]. PGE_2 stimulates the sympathetic nerve to activate the hypothalamic centres responsible for raising the temperature set point, ultimately increasing body temperature [114]. Additionally, pro-inflammatory cytokines can stimulate adipocytes to release the leptin hormone, which can directly stimulate the thermoregulatory centres in the hypothalamus [114]. Fever can be seen as a beneficial outcome of the APR, since it can increase the rate of enzyme reactions, thus speeding up the body's metabolism [116]. However, a body temperature too high, may cause serious damage, including organ damage and failure.

In addition to causing fever, pro-inflammatory cytokines, including IL-1, TNF-α, and IL-6, act on the HPA-axis to generate CRH and ACTH, which subsequently induces the production and release of the endogenous GC, cortisol [104][112]. Cortisol is regarded to locally play an anti-inflammatory role by inhibiting cytokine-gene expression. Thus, providing a negative feedback loop, which may be one of the most important mechanisms in the termination of the APR. However, at a systemic level, cortisol can be involved in determining leucocytosis [108]. Increased cortisol levels have been found to be responsible for the rapid increase in circulating leukocytes such as neutrophils, by causing mature neutrophils to detach from the endothelium and enter the blood [108]. Additionally, the synthesis rate of a number of other hormones are increased, including insulin, glucagon, etc. [104]

Another important hallmark of the APR is increased leucocytosis [110]. This process is broadly defined as an increase in the white blood cell (leukocyte) concentration [116]. Briefly, IL-1 and TNF-α, released in the local inflammatory response, stimulate the bone marrow competent cells to produce cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF). This cytokine is necessary for the growth and development of neutrophils [108], which are released from bone marrow storage pools, to further contribute to more pro-inflammatory cytokines being released into circulation.

An integral part of the APR is the modification of more than 200 APPs in the liver [117]. APPs are defined as plasma proteins whose expression levels significantly changes during inflammation. Changes in the levels of APPs largely reflect altered production by hepatocytes, the parenchymal cells of the liver [32]. The hepatocytes respond to cytokines produced during the APR *via* their cell-surface receptors, as discussed in section 1.1. Although IL-1 and TNF-α can promote the synthesis of certain APPs in the liver, IL-6 is the major inducer of most APPs, as shown in studies involving hepatocytes [112]. IL-6 induces the synthesis of the full spectrum of APPs [33], which can be grouped as either positive, if they increase by at least 25% during inflammatory states, or negative, if they decrease [104]. Other metabolic changes as a result of the APR, include muscle (protein) catabolism, which leads to increased transfer of amino acids from the muscle to the liver [115]. Additionally, the decrease in negative APPs, such as albumin and transferrin, are suggested to allow for unused amino acids to instead be used to produce positive APPs [117]. Furthermore, the decrease of the negative APP, corticosteroid binding globulin (CBG), results in an increase in the bioavailability of GCs [118]. GCs, like pro-inflammatory cytokines, have been found to upregulate APP expression (discussed in the next section).

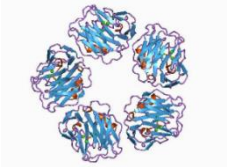
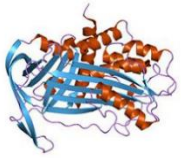
Taken together, the APR is regulated in a highly complex manner, and it is this complexity that accounts for the diverse changes that occur during this response. The entire cycle of the APR is designed to re-establish homeostasis [115] by counteracting the underlying challenge and contributing to the resolution and activation of the tissue repair process [106][113]. Hence, why the APR represents a phenomenon of enormous biological importance. However, as with all inflammation-associated phenomena, the APR is not uniformly beneficial. Although short term-activated APR is generally understood to serve a homeostatic function, the systemic response of the APR can be prolonged by persistent stimulation or disruption of normal control mechanisms, converting to a recurrent chronic state of inflammation [113]. Chronic APR can

have several disease implications, including metabolic syndrome X, atherosclerosis, cardiovascular disease, and T2D [26]. Interestingly, T2D has been suggested to be an 'acute phase disease' [119]. The development of T2D as a result of chronic APR is of special interest to this study. Therefore, an overview of APPs with a specific focus on, PAI-1 and CRP, will be discussed in the following section.

1.5. Introduction to the acute phase proteins (APPs)

As discussed in the previous section, hepatic protein synthesis is reprioritized with APPs being either downregulated (referred to as 'negative APPs') or upregulated (referred to as 'positive APPs') during the APR. Negative APPs include important metal-binding proteins (transferrin) or carrier proteins (albumin, CBG). Whereas positive APPs (SAA, CRP, PAI-1) constitute an important aspect of the systemic defence mechanism and APR. Positive APPs are further classified as minor, moderate or major, depending on the magnitude of their response. Traditionally, major proteins, including SAA and CRP, represent those that increase 10- to 1000-fold. These proteins are normally present in plasma in only trace amounts but increase dramatically within the first 24to 48 hours after a triggering event. Moderate proteins, including PAI-1, represent those that increase 2- to 10-fold, while minor proteins represent those with only a modest increase [35]. This section will introduce the APPs of interest in our study, namely PAI-1 and CRP, by giving a broad overview of each **(Table 1.1)**. Both are especially of interest, as increased PAI-1 [120]–[123] and CRP [11][122][124][125] levels are a common feature encountered among individuals with T2D. In addition, many studies exist which support the role of PAI-1 [14]–[17] and CRP [18]–[20] in the development of insulin resistance. As a result, both proteins are routinely used as biological markers for this disease.

Table 1.1. Overview of the positive acute phase proteins, CRP and PAI-1.

	C-reactive protein (CRP)	**Plasminogen activator inhibitor-1 (PAI-1)**
***Quaternary structure**	Cyclic homo-pentameric; each subunit consists of 2 β-sheets and 1 long α-helix 	Single chain glycoprotein consisting of 3 β-sheets and 8 to 9 α-helices 
Levels during APR	Major (increase 10 to 1000-fold)	Moderate (increase up to 50%)
Physiological role	Innate immune system: activation of complement, promotes apoptosis, induces phagocytosis and NO release.	Inhibits the activation of plasminogen (inhibits plasma fibrinolytic activity) leading to blood clotting

**Images: courtesy of the European Bioinformatics Institute*
Table created by the author of this study.

1.5.1. Plasminogen activator inhibitor-1 (PAI-1)

Plasminogen activator inhibitor-1 (PAI-1), also named Serpin E1, belongs to a superfamily of serine-protease inhibitors (SERPINs). It is produced and released into circulation by primarily endothelial cells but also by a variety of other cell types, including hepatocytes and adipocytes [126]. The latter explains why PAI-1 is a well-known adipocytokine, as its levels are markedly increased along with the accumulation of fat [17]. This possibly explains the correlation between elevated PAI-1 levels and obesity, a risk factor for T2D.

PAI-1 is produced and secreted as a single chain glycoprotein consisting of three β-sheets and eight to nine α-helices **(Table 1.1)**. It is composed of 379 amino acids with a molecular weight of 45 kDa. The main physiological role of PAI-1 is as key negative regulator of fibrinolysis through its role as the principle inhibitor of both urokinase- (*u-PA*) and tissue-plasminogen activator (*t-PA*) [127]. Under normal conditions, u-PA and t-PA are able to convert the inactive enzyme plasminogen to its active form, plasmin. As a result, plasmin can degrade many blood plasma proteins, including fibrin clots **(Fig. 1.8)**. The process of fibrin degradation is known as fibrinolysis. PAI-1 is therefore capable of inhibiting intravascular fibrinolysis, which leads to blood clotting or coagulation (haemostasis). Therefore, if an individual is injured, the action of PAI-1 leads to blood clotting, which prevents further blood loss *via* sealing off damaged

blood vessels [126]. In addition to PAI-1's role in haemostasis (normal blood coagulation), it is thought to be involved in migration and remodelling of body tissues [126].

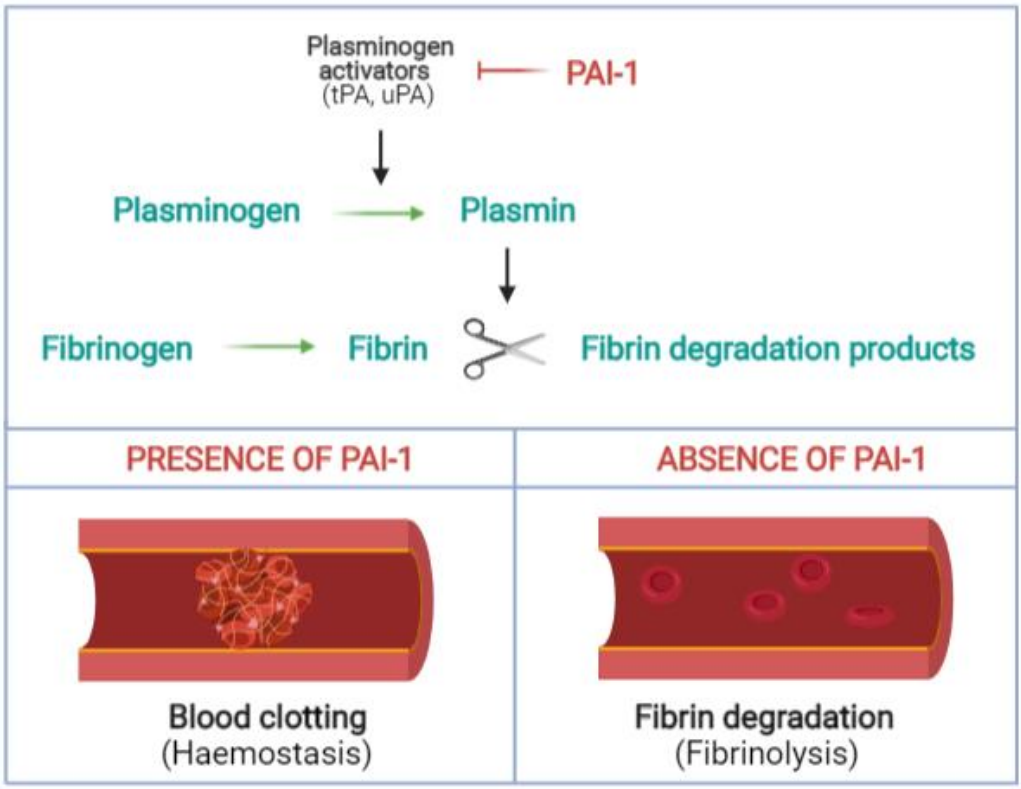


Figure 1.8. A schematic of the role PAI-1 plays in the fibrinolytic system. During the process of fibrinolysis, u-PA and t-PA, convert the inactive enzyme, plasminogen, to its active form, plasmin. Subsequently, plasmin degrades fibrin clots (blood plasma proteins) into fibrin degradation products. PAI-1 is the main inhibitor of both t-PA and u-PA, therefore inhibiting fibrinolysis, leading to blood clotting (haemostasis). Image was created by the author of this study, using BioRender. Note: t-PA = tissue-plasminogen activator, u-PA = urokinase-plasminogen activator, PAI-1 = plasminogen activator inhibitor-1

Circulating PAI-1 levels vary more than any other component of the fibrinolytic system. This could be due to PAI-1 production being stimulated by a wide variety of signalling molecules, including IL-1, TNF-α, and TGF-β [128]. In addition, PAI-1 has been indicated as a major stress-induced gene [129]. As previously explained, activation of the HPA-axis by stressors, lead to an increase in the secretion of GCs, which are able to induce PAI-1 expression *in vivo* [55][130]. In healthy individuals, normal active PAI-1 plasma concentration ranges from 5 to 20 ng/ml [127]. This concentration range is suggested to be sufficient to control fibrinolysis [127]. However, under pathological conditions, several tissues produce substantial amounts of PAI-1 in response to inflammatory cytokines. As a result, elevated PAI-1 concentrations have been observed consistently in blood from patients suffering with T2D. Cho *et al*. reported basal plasma PAI-1 antigen concentration to be significantly higher in diabetic subjects (36 ng/ml) compared to in the control group (18 ng/ml) [121]. In line with this, Festa *et al*. reported that

diabetic individuals had higher baseline levels of PAI-1 (15 to 37.5 ng/ml) compared to the control group (9 to 27 ng/ml) [122]. In addition, obese individuals, many of whom exhibit insulin resistance, were found to exhibit a three-fold elevation of PAI-1 in blood, compared with values in healthy lean individuals [131].

1.5.2. C-Reactive Protein (CRP)

In 1930, Tillet and Francis were the first to discover C-reactive protein (CRP), while investigating the sera of patients infected with pneumococcal pneumonia. They reported that CRP reacted with the capsular (C)-polysaccharide of *Streptococcus pneumoniae*. Today much more is known of this molecule since its initial discovery. CRP, also named Pentraxin 1 (PTX1), is a member of the highly conserved pentraxin family of proteins which include other structurally related molecules such as SAA. It is primarily synthesized in liver hepatocytes but also moderately by endothelial cells, macrophages, lymphocytes, smooth muscle cells, and adipocytes [132]. The CRP molecule is produced as a cyclic/ring-shaped homo-pentameric protein, which involves five identical non-covalently bound 23 kDa subunits arranged symmetrically around a central pole **(Table 1.1)** [133]. Each subunit is composed of 206 amino acids folded into two anti-parallel β-sheets with a flattened jelly-roll topology. In addition, a long α-helix folds against one of the β-sheets and the remainder of the secondary structure constitutes of random coils [133].

The main physiological role of CRP lies with the innate immune system, where it acts as an early defence system against foreign infectious pathogens. CRP exhibits anti-inflammatory activities including: (i) activation of the classical complement pathway [134], through binding to the C1q molecules, resulting in pathogens being opsonized and as a result cleared [133], (ii) promoting apoptosis or phagocytosis of damaged cells and lastly (iii) displaying an anti-inflammatory effect by inhibiting neutrophil (leukocytes) action [132]. CRP participates in the systemic response to inflammation, increasing up to 1000-fold. Its levels start to rise after six to eight hours and peaks by 48 hours, after an inflammatory event [135]. Serum concentration of CRP increases dramatically during acute and chronic inflammation, in response to a variety of inflammatory cytokines, including TNF-α and IL-6 and in some non-inflammatory conditions such as stress [136]. Hence why the measurement of CRP levels is widely used to monitor various inflammatory states [136]. Highly variable plasma levels, ranging from 0.8 to 3 μg/ml, are found in healthy individuals [132]. Factors such as polymorphisms in the CRP gene, could contribute to these variations [132]. However, CRP concentrations between 2 and

10 µg/ml are considered to indicate metabolic inflammation, which could lead to the development of insulin resistance [137]. This is supported by Festa and colleagues, which found a significant relation of CRP to the development of T2D, with diabetic individuals having higher baseline levels of CRP (1.3 to 5.9 µg/ml) compared to the control group (0.8 to 3.4 µg/ml) [122].

The regulation of each APP is uniquely complex, with pro-inflammatory cytokines, GCs, and growth factors being some of its main mediators. Both *in vitro* and *in vivo* studies have reported the regulation of PAI-1 and CRP expression to be closely influenced by the pro-inflammatory cytokines, TNF-α and IL-6, and hormones such as GCs [126]. Identifying the possible regulatory binding sites *via* which these signalling molecules act to trigger a change in PAI-1 and CRP levels, could lead to a better understanding of the molecular mechanism behind their action. A large variety of signalling pathways and transcriptional machinery exist which impact PAI-1 and CRP expression, and will be discussed below.

1.5.3. The regulation of PAI-1 by glucocorticoids or pro-inflammatory cytokines

Experimental *in vivo* studies performed in animal models, which include baboons [138][139], rodents [140][141], and human subjects [142], have shown that IL-6 and TNF-α are major contributors to the increase of PAI-1 **(Addendum A: Table A1)**. In addition, experimental *in vitro* studies performed in endothelial cells [140][143]–[148], epithelial cells [149], vascular smooth muscle cells [150], adipocytes [151]–[154], and hepatocytes [155][156] have shown TNF-α to significantly upregulate PAI-1 expression **(Addendum A: Table A1)**. This suggests that the regulation of PAI-1 by TNF-α is not dependent on cell type. Samad *et al.* showed that neutralisation of TNF-α or deletion of both TNFRs (p55 and p75) resulted in significantly reduced PAI-1 expression in adipose tissue of mice [157]. The mechanism by which TNF-α induces PAI-1 expression in endothelial cells was delineated by Gruber *et al.*; they identified the transcription factor, nuclear receptor-77 (Nur77) to be part of the TNF-α-induced PAI-1 expression [146]. In addition, they reported that Nur77 drives the PAI-1 transcription *via* direct binding to a NGFI-B responsive element (NBRE), and that the human PAI-1 proximal promotor contains this functional binding motif from -261 to -254 bp. In addition, Hou *et al.* proposed that NF-κB was involved in TNF-α-induced PAI-1 expression in bovine aortic endothelial cells [147]. However, according to de Martin *et al.* the PAI-1 promotor lacks a consensus sequence for NF-κB [158]. Furthermore, another study identified protein kinase C

(PKC) as a central mediator of TNF-α-mediated increase of PAI-1 levels in adipocytes, since the inhibition of the PKC pathway almost completely inhibited this response [154]. However, the mechanism of action whereby TNF-α increases PAI-1 levels in liver tissue is yet to be elucidated.

In addition to TNF-α, experimental *in vitro* studies performed in a human hepatoma cell line (HepG2), murine and human primary hepatocytes [159][160], and human primary adipocytes [161] have shown IL-6 to significantly upregulate PAI-1 **(Addendum A: Table A1)**. Dong *et al.*, found that in HepG2 cells, IL-6-mediated PAI-1 promotor activity was mediated by the transcription factor, C/EBP-δ positioned between -226 to -213 bp in the human PAI-1 (*SERPIN1*) promoter [160]. In addition, JAK/STAT signalling was also reported to be responsible for the IL-6-induced PAI-1 expression in HepG2 cells. However, whether IL-6 induces C/EBP-δ *via* STAT3 is not clear. It should be mentioned, that studies do exist which have found IL-6 to have little to no effect on PAI-1 expression in primary human and murine hepatocytes [140][139][156] and HepG2 cells [155] [159][162]

Like the pro-inflammatory cytokines, GCs have also been shown to influence PAI-1 levels **(Addendum A: Table A1)**. GC-induced PAI-1 expression appears to not be cell type specific as a number of *in vitro* studies, using Dex as a representative GC, have reported it to occur in adipocytes [163]–[165], hepatocytes [130][166], fibrosarcoma cells [167]–[169], monocytes [170], trophoblasts [171], and epithelial cells [149]. PAI-1 induction by Dex is mediated by the GC-activated GR and its association with the GRE in the minimal promotor of the human PAI-1 gene [166]–[168][172]. This has been proven by studies showing that Dex-induced PAI-1 expression is completely dependent upon the GR pathway, by using RU-486, an inhibitor of the GR [149][169]. Riccio *et al.*, found that the human PAI-1 gene contains a GRE motif in its promotor region at position -299 [168]. While van Zonneveld *et al.* identified the location of one-half of a putative GRE within -305 and +75 of the human PAI-1 gene, *via* which Dex induced PAI-1 promotor activity [173].

Collectively, the mechanism underlying IL-6 or Dex-induced PAI-1 regulation have been well defined, with the following transcription factors, respectively, being implicated: C/EBP-δ and the GR. In addition, studies have also mapped the binding site of each transcription factor in the minimal promotor of the human PAI-1 gene. However, contrasting findings exist on whether IL-6 influences PAI-1 expression in liver cells. Furthermore, although TNF-α has been

shown to be a major inducer of PAI-1 expression in a range of cell types, exactly how it controls PAI-1 expression in liver cells remain enigmatic.

1.5.4. The regulation of CRP by glucocorticoids or pro-inflammatory cytokines

Current knowledge of the regulation of CRP synthesis is mainly gained from studies in human hepatoma cells. In addition, there has been increasing evidence of a well-established relationship between CRP and IL-6, which is intimately involved in the hepatic regulation of the APR [174]. Therefore, CRP is well-known to be primarily synthesized by IL-6-dependent hepatic biosynthesis [11]. This has further been proven by Wendling *et al.* [175] and Bataille *et al.* [176] whom both demonstrated *in vivo*, that production of CRP in humans was blocked by anti-IL-6 treatment. IL-6 has been shown to significantly upregulate CRP levels in experimental *in vitro* studies performed in human hepatoma cells [177]–[186], primary hepatocytes [174][186][187], mouse hepatoma cells [88], adipocytes [188], and coronary smooth muscle cells [189], as summarised in **Addendum A: Table A2**, suggesting that the regulation of CRP by IL-6 is not dependent on cell type. In Hep3B cells, a human hepatoma cell line, IL-6 have been reported to induce CRP expression by activating transcription factors STAT3 and C/EBP-β. C/EBP-β binds to positions -52 and -219 on the CRP promoter [182], with STAT3 binding between these two positions, to a site at -108 on the promoter [183]. STAT3 has also been shown to act on the CRP promoter in a mouse hepatic cell line, confirming its role in driving CRP expression [190].

Whilst IL-6-induced CRP regulation has been extensively studied, few studies have investigated the effect of TNF-α on CRP regulation **(Addendum A: Table A2)** Some studies have observed TNF-α to have no detectable effect on CRP protein synthesis [174][180], while others have observed a significant increase in CRP protein release and mRNA expression in response to TNF-α [189], depending on the *in vitro* cell system used. The molecular mechanism regarding which signalling pathway and transcriptions factors are involved in TNF-α-induced CRP regulation, remains to be elucidated. Similarly, although a few studies have demonstrated Dex-induced CRP expression as summarised in **Addendum A: Table A2** [88][180], the molecular mechanism of action thereof is unclear. It is unknown whether functional GREs are present in the CRP promoter, which would facilitate direct DNA binding of the ligand-activated GR. The GR is also known to regulate gene transcription *via* tethering to other transcription

factors such as STAT3 [94], C/EBP [95][96], and NF-κB [87][96], which could possibly be the mechanism in which GCs increase CRP expression.

Taken together, the mode of action governing IL-6-induced CRP expression has been well established, with studies finding C/EBP-β, STAT3, and NF-κB (p50/p65) to be involved. In addition, the binding sites for each transcription factor within the proximal promotor of the human CRP gene have also been identified. We are left with two unanswered questions; namely i) what is the underlying mechanism of Dex-induced CRP expression, especially in liver cells? and ii) does TNF-α have an effect on CRP expression in liver cells and if so, which transcriptional regulators are involved?

1.6. Co-regulation of APPs by GCs and pro-inflammatory cytokines

Despite the traditional knowledge that many pro-inflammatory cytokine induced-genes are profoundly repressed by GCs, studies have shown no repression, partial repression or even enhancement by GCs of the expression of such genes [191]. In recent years it has been suggested that GCs selectively regulate gene expression. For example, it has now become clear that GC effects on innate immunity are not universally anti-inflammatory [192]. The APR, where the synthesis of several APPs is increased, is the cornerstone of the innate immune response (discussed in section 1.4). Interestingly, a small number of APPs have been identified to be co-regulated by the combination of GCs and pro-inflammatory cytokines. This demonstrates how these seemingly opposing signalling molecules can work cooperatively with one another to upregulate the expression of genes involved in the APR. This concept of APP co-regulation by GCs and pro-inflammatory cytokines, was first reported in the late 1980s when Baumann *et al*. reported the stimulating effect of GCs on IL-6-induced APP mRNA expression of haptoglobin and α1-antichymotrypsin (α1-ACT) [193]. Since this initial discovery, a considerable body of knowledge has accumulated over the years concerning the possible crosstalk between GCs and pro-inflammatory cytokines to induce APP expression, which will be discussed in the following paragraphs **(Addendum A: Table A3)**.

A study by Liu *et al*. reported that Dex (400 ng/ml) and IL-6 (10 ng/ml) induced the protein synthesis of the murine APP, SIP24/24p3, in mouse embryonic fibroblasts (Balb/c 3T3) and mouse liver (BNL) cells [194] to an extent much greater than the sum of the effects of each test

compound alone. They suggested that the signalling molecules exerted their effects through different pathways independently leading to the possible synergism observed. Furthermore, an additive effect on SIP24/24p3 protein synthesis was observed when Dex was combined with TNF-α (1000 ng/ml) in the BNL cells. This additive effect was speculated to be due to the respective pathways of the signalling molecules overlapping. Another study by Diitrich *et al.* reported that Dex (100 nM) further enhanced IL-6 (20 ng/ml)-induced γ-fibrinogen (FGG) and α1-ACT mRNA expression, in both primary murine hepatocytes and human hepatoma (HepG2) cells [195]. This group demonstrated how Dex interferes with IL-6-induced expression of the feedback inhibitor, SOCS3, thereby leading to enhanced FGG and α1-ACT expression. They suggested that the GC exerted a dual activity by further inducing IL-6-induced APPs expression (pro-inflammatory) and by dampening IL-6-induced SOCS3 expression (anti-inflammatory). Furthermore, a study by Lannan *et al.* reported that the mRNA and protein expression of SerpinA3 (also known as α-AACT) were significantly upregulated in lung (A549) cells by means of co-administration with TNF-α (1000 ng/ml) and either Dex (10 nM) or cortisol (100 nM). These effects were beyond that expected from treatment of either compound alone [27]. The authors also proposed that the synergistic co-regulation of SerpinA3 expression was due to the GC- and TNF-α signalling pathways interacting in novel way(s). The study proceeded with an *in vivo* experiment in lung and liver tissue of adrenalectomized mice, where they observed an additive increase of SerpinA3 mRNA expression in response to co-treatment with Dex (1 mg/kg) and TNF-α (60 μg/kg). In addition, chromatin immunoprecipitation (ChIP) assays indicated that GR binding at the SerpinA3 transcription starting site was more robust when cells were co-treated with Dex and TNF-α.

The possible co-regulation by GCs and pro-inflammatory cytokines of the APPs, SAA, PAI-1, and CRP, have been investigated by a number of research groups [27][88][149]. [180][181][195]–[201]. Although, it seems that most studies have focused on the co-regulation of SAA by GCs and pro-inflammatory cytokines, based on the amount of literature available on this subject.

SAA expression by various pro-inflammatory cytokines in the presence of a GC is enhanced *in vitro* as well as *in vivo*, which appears not to be dependent on cell type or concentrations of either pro-inflammatory cytokine or GC. For example, Castell *et al.* reported that Dex (100 nM) and IL-6 (10 ng/ml) synergistically induced SAA protein synthesis and secretion, in human primary hepatocytes [196]. In other words, Dex enhanced the action of IL-6-induced

SAA protein synthesis, and *vice versa*. In addition, a study by Ganapathi *et al.* reported that induction of SAA protein synthesis by the combination of IL-1α (40 ng/ml) plus IL-6 (10 ng/ml) was potentiated by Dex (1 µM), in Hep3B cells [180]. Another group, Meek *et al.* reported that SAA1, SAA2, and SAA4 mRNA expression was effectively induced by IL-1β or IL-6 (10 ng/ml), but only in the presence of Dex (1 µM), in human adult aortic smooth muscle cells [197]. In addition, Ito and colleagues, reported that only the combination of Dex (1 mg/kg) plus IL-6 (2 µg/mouse) increased SAA protein production in mice, compared to each treatment on its own. Interestingly, the SAA protein production was markedly elevated when the combination treatment with Dex and IL-6 was followed by an additional treatment with IL-1β (2 µg/mouse) [198]. Furthermore, a study by Thorn *et al.* [199] reported that Dex (50 nM) enhanced TNF-α or IL-6 (10 ng/ml) driven induction of the SAA1 promotor, in HepG2 cells. A similar study by Lannan *et al.* [27], reported that Dex (10 nM) cooperatively upregulated SAA1/2 mRNA expression in concert with TNF-α (1000 ng/ml), in A549 lung cells. Dittrich *et al*. [195] have also reported that Dex (4 mg/kg) increased IL-6 (100 µg/kg)-induced hepatic SAA mRNA expression and serum protein of treated mice. The authors showed that this effect elicited by Dex was dependent on SOCS3 expression and the SOCS3 recruiting motif within the gp130 receptor. Visser *et al.* reported that the combination of Dex (10 nM) plus IL-6 (10 ng/ml) significantly increased SAA mRNA expression, compared to IL-6 but not Dex alone, in BWTG3 cells, a mouse hepatocyte cell line [88]. Although a significant increase in both intra- and extracellular SAA protein levels were seen after co-treatment with both agents, it was not more than what was observed with either agent alone. The most recent study by Su & Weindl, reported that Dex (100 nM) further enhanced TNF-α (10 ng/ml)-induced SAA1/2 mRNA expression and secretion in keratinocytes [200]. In addition, co-immunoprecipitation (co-IP) assays indicated that the transcription factors, GR, STAT3, and NF-κB, mediated this TNF-α and Dex-induced SAA1 mRNA expression.

Whilst cooperative regulation of SAA by GCs and various pro-inflammatory cytokines have been extensively studied, fewer have studied this phenomenon in regard to PAI-1 and CRP. In addition, the array of pro-inflammatory cytokines used is limited with most either reporting on TNF-α or IL-6. To the best of our knowledge, only two research groups have investigated the possible co-regulation of PAI-1, with both studies focussing on co-regulation by Dex and TNF-α [149][201]. Both studies reported an augmented increase in PAI-1 expression in the presence of Dex and TNF-α compared to individual treatments, albeit not in liver cells. Yamamoto *et al*. showed that combined treatment with Dex (1 µM) plus TNF-α (10 ng/ml) for

48 hours had an additive effect on PAI-1 mRNA expression in vascular endothelial cells (HUVECs) [201]. Similarly, Kimura *et al.* showed that Dex (100 nM) enhanced TNF-α (10 ng/ml)-stimulated PAI-1 mRNA expression but not protein production in human proximal renal tubular cells (HPTECs), by an additonal 4.8-fold, after 24 hours [149]. Unfortunately it is still unknown how PAI-1 expression is affected by GCs and other pro-inflammatory cytokines, such as IL-6 and IL-1β. In addition, as PAI-1 is primarily produced in the liver- and adipose cells [55], it remains undetermined whether cooperative regulation by GCs and pro-inflammatory cytokines occur in these cell types. This is especially important and requires to be investigated considering that PAI-1 levels are chronically high in a diabetic state [121][122] and has also been implicated in insulin resistance [131].

To the best of our knowledge, only three research groups have investigated the possible co-regulation of CRP expression by GCs and pro-inflammatory cytokines. Like PAI-1, the primary source of CRP, which is also elevated in a diabetic state [124][202], is the liver. Presumably this is the reason why the few studies investigating co-regulation of CRP by GCs and a pro-inflammatory cytokine were all conducted in liver cell models. Unfortunately, only IL-6 was used as a representative pro-inflammatory cytokine in all three studies [88][181] [196]. Similar to the above-mentioned studies whereby SAA and PAI-1 expression are co-regulated by GCs and pro-inflammatory cytokines, IL-6-induced CRP expression was either augmented [181] or remained unchanged [88][196] in the presence of Dex. Specifically, Castell *et al*. discovered that human primary hepatocytes co-stimulated with Dex (100 nM) plus IL-6 (10 ng/ml) for 20 hours, strongly induced extracellular CRP protein synthesis (23-fold), more than what was observed with Dex (1.7-fold) but not with IL-6 (22-fold) alone [196]. The lack of an amplified response observed by the authors in response to co-treatment with Dex and IL-6 when compared to IL-6 treatment alone, could possibly be attributed to maximal CRP protein synthesis being reached with 10 ng/ml IL-6 in primary hepatocytes. It would be interesting to examine how Dex affects IL6-mediated increase in CRP expression at a lower concentration of IL-6. Nonetheless, Depraetere *et al*. reported that HepG2 cells co-stimulated for 72 hours with IL-6 (10 ng/ml) and a higher Dex concentration (i.e., 1 µM) than used by Castell and colleagues, cooperatively increased (14-fold) the magnitude of extracellular CRP protein release significantly above what was seen with Dex (1.5-fold)- and IL-6 (9-fold) alone [181]. Whilst this does suggest that an augmented increase in CRP expression might be dependent on the concentration of test compounds used, the length of exposure to both signalling molecules could also attribute to the cooperativity observed as Depraetere *et al*. did

treat significantly longer with the test compounds (20- *versus* 72 hours). Highlighting that cooperativity between GCs and pro-inflammatory cytokines might be dependent on both dose as well as duration of exposure. For example, Visser *et al*. found that extracellular CRP protein levels remained unchanged when BWTG3 cells, were co-stimulated with Dex (10 nM) for 24 hours plus IL-6 (10 ng/ml) for 3 hours, compared to either agent alone [88]. Interestingly, the authors did observe Dex to significantly lower IL-6-induced intracellular CRP protein levels. While, both agents alone or combined did not significantly modulate each other effects at mRNA level.

Taken together, it is clear, that in regard to APPs, GCs favour an increase in APP expression, sometimes under the right conditions enhancing cytokine-induced APP expression, as mentioned above for a number of APPs including ones associated with insulin resistance such as SAA, CRP, and PAI-1. Whether this cooperativity between GCs and pro-inflammatory cytokines is unique to APP regulation is unclear. Traditional dogma suggests that GCs and pro-inflammatory cytokines primarily antagonise each other's activity [23]. However, as discussed in section 1.3, GCs are not simply anti-inflammatory, since in recent years it has become abundantly clear that when GCs act in a pro-inflammatory manner by cooperatively enhancing cytokine-mediated APP expression, this reinforces the innate immune system and APR [203]. On the other hand, it is important to remember that neither hormonal nor cytokine networks function in isolation [34]. Thus, many more future contributions to our understanding of APP regulation by GCs and pro-inflammatory cytokines will be made by examining the complicated crosstalk between these signalling molecules.

1.7. Concluding remarks

Although it is well established that insulin resistance is central to the pathogenesis of T2D, how this abnormality arises at a molecular level remains to be elucidated. Inflammation, stress, and APPs, such as PAI-1 and CRP, have all been individually linked to the development of insulin resistance [14]–[20].

This study forms part of a larger project within the Verhoog research group. Ultimately, we wish to provide insight into whether stress (represented by GCs) and inflammation (represented by pro-inflammatory cytokines) affect hepatic insulin signalling *via* the production of APPs. We thus ask the question of whether GC- and pro-inflammatory cytokine-induced insulin resistance develops as a result of an increase in these APPs ('indirect effect')? To answer this

question, our research group have previously shown the APPs, PAI-1 and CRP, disrupt hepatic insulin signalling at key nodes of the insulin signalling pathway (unpublished data) [25].

In the current study these earlier observations are extended, by investigating the possible co-regulation of the APPs, PAI-1, and CRP, by GCs and pro-inflammatory cytokines. Presently the extent of cooperative regulation by GCs and pro-inflammatory cytokines on PAI-1 and CRP expression remain largely unexplored in the literature. The 'why' behind the occurrence of co-regulation has become clear, but a knowledge gap still remains regarding the 'how' i.e., the molecular mechanism of action. No studies have yet been conducted, to the best of our knowledge, examining i) the possible cooperative regulation of PAI-1 expression by GCs and pro-inflammatory cytokines in liver cells, ii) the possible cooperative regulation of PAI-1 expression by Dex and IL-6, iii) the possible cooperative regulation of CRP expression by Dex and TNF-α or importantly iv) the possible cooperative regulation of PAI-1 and CRP by GCs and pro-inflammatory cytokines in the same model system in addition to comparing different pro-inflammatory cytokines. Ultimately, the results obtained could provide further proof that GCs and pro-inflammatory cytokines crosstalk with one another to increase PAI-1 and CRP expression, as well as contribute to the existing literature available on this subject of cooperative regulation.

1.8. Research question and Hypothesis

Do GCs (representing stress) and pro-inflammatory cytokines (representing inflammation) co-regulate APPs (PAI-1 and CRP) in hepatoma cells? Based on previous literature I hypothesise that in the liver, GCs and pro-inflammatory cytokines will cooperatively regulate PAI-1 and CRP expression, at both the mRNA and protein level.

1.9. Research aims

Aim 1: Determine whether the glucocorticoid, Dex, and/or pro-inflammatory cytokines, TNF-α and IL-6, affect PAI-1 and CRP mRNA expression in hepatoma cells?

Aim 2: Determine if glucocorticoids (Dex, corticosterone, and cortisol) and/or pro-inflammatory cytokines, TNF-α and IL-6, affect PAI-1 and CRP intracellular protein expression in hepatoma cells?

Aim 3: Investigate whether the molecular mechanism underlying the regulation of PAI-1 and CRP expression by the glucocorticoid, Dex, and/or pro-inflammatory cytokines, TNF-α and IL-6, are at the promotor level?

CHAPTER TWO: METHODS AND MATERIALS

2.1. Test compounds

The test compounds used in this study, included a potent synthetic GRα agonist, Dexamethasone (Dex) [(11β,16α)-9-Fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione]**,** and the endogenous GCs, corticosterone (Cort) [11β,21-Dihydroxy-4-pregnene-3,20-dione] and cortisol (F) [(11β)-17 α,21-Trihydroxypregn-4-ene-3,20-dione], which were purchased from Sigma-Aldrich. In addition, two pro-inflammatory cytokines were included in this study, namely tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), which were both obtained from Thermo-Fisher. All glucocorticoids (GCs) were dissolved in 100% absolute ethanol (EtOH) (stock solution: 10^{-2} M), since this was recommended as suitable solvent for the hydrophobic GCs. Subsequently, serial dilution (10^{-3} M to 10^{-6} M) were prepared from the stock solution, and stored at -20 °C. For all experiments, GCs were diluted 1000 times in unsupplemented low glucose (1000 mg/L)-Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich). Final GC concentrations of 10 nM (10^{-8} M), 100 nM (10^{-7} M) and 1 µM (10^{-6} M) were used for treatment of cells. Lyophilised recombinant human TNF-α and IL-6 powder were reconstituted in autoclaved distilled water and 100 mM acetic acid, respectively, to a stock concentration of 1 mg/ml. A working solution of 20 µg/ml each was prepared using low glucose-DMEM supplemented with 10% (v/v) FBS (Thermo-Fisher), and stored at -20 °C. For all experiments, TNF-α and IL-6 were diluted 1000 times to a final concentration of 20 ng/ml using unsupplemented low glucose-DMEM.

2.2. Mammalian cell culture

2.2.1 Cell Growth and Maintenance

Murine hepatoma (BWGT3) cells were obtained from the University of Gent, Belgium, and human hepatoma (HepG2) cells were purchased from Cellonex **(refer to Table 2.1 & Fig. 2.1 for more information)**. Both cell lines were cultured and maintained in 75 cm^2 culture flasks (Bio-Smart Scientific) containing low glucose (1000 mg/L)-DMEM containing 1 mM sodium pyruvate and 17.9 mM sodium bicarbonate purchased from Sigma-Aldrich and supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin (Pen/Strep) (10 000 U/ml penicillin and 10 mg/mL streptomycin) (Sigma-Aldrich), hereafter referred to as complete DMEM. Cells were kept in an incubator set at 37 ºC in an atmosphere of 97% relative humidity and 5% CO_2. Both cell cultures were passaged twice weekly with 1x trypsin-EDTA solution (Sigma-Aldrich). To neutralize the activity of the trypsin, complete DMEM was added to the cells. The

cell lines employed were routinely monitored for *Mycoplasma* contamination using Hoechst staining, to ensure that only *Mycoplasma*-negative cells were used in experiments **(Addendum B: Fig. B1)**.

For mRNA experiments, BWGT3 cells were seeded in 6-well plates (Bio-Smart Scientific) at a density of 5 x 10^5 cells per well, as a better total RNA yield was observed compared to seeding the cells in 12-well plates. Whereas, for protein experiments BWGT3 and HepG2 cells were seeded in 12-well plates (Bio-Smart Scientific) at a density of 2 x 10^5 cells per well. For transfection experiments, BWTG3 cells were seeded in 10 cm^2 dishes at a density of 5 x 10^5 cells per dish and re-seeded in 96-well plates (Bio-Smart Scientific) at a density of 45 000 cells per well.

Table 2.1. Information on immortalized cell lines used in this study.

Cell line	Organism	Cell type	Morphology	Characteristics
BWTG3	*Mus musculus*, mouse	Hepatoma (Liver cancer) cells	Epithelial adherent	Elongated extensions
HepG2	*Homo sapiens*, human			small aggregates (clumps) of irregular shaped cells, colonies

Figure 2.1. Morphology of immortalised cell lines used in this study. Since the liver is the main source of acute phase protein (APP) production, such as C-reactive protein (CRP) and plasminogen activator inhibitor-1 (PAI-1), mouse (BWTG3) (left) and human (HepG2) (right) hepatoma cell lines were used as *in vitro* models. Images are not according to scale and shown for visual purposes only.

2.2.2 Treatment conditions

For time course experiments, to establish the time point at which the test compounds mentioned in section 2.1 maximally induces PAI-1 and CRP protein expression, BWGT3 and HepG2 cells were seeded in 12-well plates (Bio-Smart Scientific) at a density of 2 x 10^5 (BWTG3) and 4 x 10^5 (HepG2) cells per well. Once the cells reached 70-80% confluency, complete medium was replaced with unsupplemented low glucose-DMEM for 24 hours. Subsequently, cells were treated with vehicle controls (0.1% EtOH or complete DMEM) and the test compounds. The final concentration of the test compounds Dex, Cort, and F were 10^{-8} M, 10^{-7} M, and 10^{-6} M, whilst for TNF-α and IL-6 it was 20 ng/ml. Cells were exposed to the test compounds for 24, 48 and 72 hours.

For PAI-1 and CRP and protein analysis, BWGT3 and HepG2 cells treated with increasing concentrations (10^{-8} M, 10^{-7} M, and 10^{-6} M) of Dex or Cort (BWTG3) or F (HepG2) with or without 20 ng/ml TNF-α or IL-6 for 48 hours. Both PAI-1 and CRP mRNA analysis and promoter-reporter experiments, cells were incubated for 24 hours with increasing concentrations of Dex (10^{-8} M, 10^{-7} M, 10^{-6} M) and/or TNF-α or IL-6 (20 ng/ml), for 24 hours. To allow for the same vehicle control (0.1% EtOH and 0.1% complete medium combined) to be used across all treatments including single treatments, it was ensured that EtOH was added to the wells containing cytokines to a final concentration of 0.1% and complete DMEM was added to the wells containing GCs to a final concentration of 0.1%.

2.3. Total RNA Extraction

For mRNA analysis, BWTG3 cells were treated as described in section 2.2.2. Total RNA from cultured cells was extracted using Tri-reagent as per the manufacturer's protocol (Sigma-Aldrich). Briefly, treatment medium was removed from each well and the cells lysed by the addition of 400 µl Tri-reagent per well, and placed at -20 °C overnight to promote cell lysis. The cell lysates were transferred into 1.5 ml microcentrifuge tubes after thawing, followed by the addition of 80 µl chloroform to extract total RNA (ribosomal, messenger, and transfer RNA). The cells were vortexed for 1 minute, followed by centrifugation at 14000 rpm for 20 minutes at 4 °C to separate into three layers. Since RNA is confined in the clear aqueous phase, this phase was collected (100 µl) and transferred into a clean 1.5 ml microcentrifuge tube, followed by the addition of 100 µl isopropanol. Next, samples were vortexed for 1 minute and RNA precipitation was facilitated by placing the samples at -20 °C for an extended period of

time (preferably more than two weeks) to enhance RNA precipitation. To pellet the RNA, samples were centrifuged at 14 000 rpm for 30 minutes at 4 °C. The supernatant was discarded, and the pellet was washed with 500 µl 70% DEPC-treated EtOH (EtOH was diluted in diethyl pyrocarbonate (DEPC) treated water) and vortexed for 1 minute followed by centrifugation at 14000 rpm for 5 minutes at 4 °C. This wash step was repeated three times to ensure the removal of any contaminants. With the final wash step, the supernatant was removed, and the pellet air-dried for 20 minutes to remove remaining traces of EtOH. The pellet was dissolved in 20 µl DEPC-treated water and incubated at 55 °C for 5 minutes on a heating block. RNA samples were stored at -80 °C. The RNA concentration and purity of the samples were assessed using a NanoDrop spectrophotometer (Central analytical facility (CAF), Stellenbosch). An $A_{260/280}$ ratio close to 2.0 was expected, since this indicates pure RNA. To confirm RNA integrity, the presence of intact 18S and 28S ribosomal RNA was determined. Total RNA (1 µg) was loaded on a 1% (w/v) agarose gel, containing biocide [204], which degrades any traces of RNases, and stained with Nancy-520 (Sigma-Aldrich). Subsequently after gel electrophoresis at 60V for 1 hour, the agarose gel was immediately visualized under ultraviolet light using the Gel Doc™ XR+ Imaging System (Bio-Rad) with Image Lab™ Software **(Addendum C1: Fig. C1)**.

2.4. Reverse-Transcription (cDNA synthesis)

Total RNA was reverse transcribed to complementary DNA (cDNA) using the ImProm-II Reverse Transcription System cDNA synthesis kit (Promega) as per manufacturer's instructions. RNA is very easily degraded by RNases; therefore, it is more convenient to use the more stable cDNA for analysis of mRNA. Briefly, 2 µg RNA was added into a 0.5 ml thin-walled PCR tube followed by the addition of 50 µg/ml oligoDT primers, which targets the 3' end poly(A) tail of mRNA. Nuclease-free water was subsequently added to a final volume of 5 µl. The reaction mixture was incubated at 70 °C for 5 minutes and immediately chilled on ice for 10 minutes. Thereafter, 15 µl of the second reaction mixture (master mix) was added to each sample. A master mix for a single sample, contains the following reagents: 4 µl ImProm-II 5X reaction buffer, 0.9 µl $MgCl_2$ (co-factor), 1 µl dNTP mix (extends the cDNA strand), 20 U (0.5 µl) Recombinant RNasin® ribonuclease inhibitor and 1 µl ImProm-II reverse transcriptase (transcribes cDNA from mRNA), with the volume adjusted to 15 µl with nuclease-free water. Next, samples were incubated at 25 °C for 5 minutes to allow annealing of the oligoDT primers, followed by an incubation step at 45 °C for 60 minutes allowing the extension of the cDNA strand. The final step involved placing the samples at 70 °C for 15

minutes to inactivate the reverse transcriptase. Finally, the cDNA samples were chilled on ice for 5 minutes and stored at -20 °C until analysis.

2.5. Semi-quantitative real-time polymerase chain reaction (q) RT-PCR

All qPCR reactions were carried out on a LightCycler 96 Real-Time PCR System (Roche Applied Science). For each cDNA sample, a master mix was prepared, containing the following reagents: 6.25 µl SYBR green (Kapa SYBR Fast qPCR master mix, containing the DNA polymerase enzyme and necessary reagents for the PCR reaction, Roche Applied Science), 0.5 µl forward primer, 0.5 µl reverse primer (both 10 µM final concentration), and 1.75 µl DEPC-treated water to a final volume of 9 µl. SYBR Green is a fluorescent dye that binds to the minor groove of double stranded DNA (dsDNA), emitting fluorescence when bound. This allows the levels of amplified product to be monitored. LightCycler multiwell plates were used (Celtic Molecular Diagnostics) for the qPCR reaction in which 9 µl of the master mix was added to each well and 1 µl of the cDNA sample (template) or nuclease-free H_2O (non-template control). Each reaction was performed in duplicate. Predeveloped validated mouse PAI-1, CRP, and 18S primer sets were purchased from Integrated DNA Technologies (Whitehead Scientific). The details regarding these specific primer sets are shown in **Table 2.2**. The efficiency of each primer set was calculated by making a dilution curve from cDNA (untreated sample) **(Addendum C2: Fig. C2)**. The efficiency is a measure of how much PCR product is amplified per cycle, 1 indicating no product and 2 indicating that every PCR product is amplified. LightCycler® 96 software (Roche Applied Science) was used to set the PCR thermal cycling conditions, which were as follows: 95 ºC for 3 seconds (pre-incubation or activation), followed by 45 cycles of amplification: 95 ºC for 3 seconds (denaturation), 55 ºC (mCRP) or 60 ºC (mPAI-1 & m18S) for 10 seconds (annealing), and 72 ºC for 3 seconds (elongation or extension). This was followed with a melting step at 95 °C for 10 seconds. Since the expression of 18S was not influenced by the treatment conditions (low variations in Ct values between different samples), it was used as an internal control against which samples were normalised. The melting curve analysis was performed to confirm the amplification of a single product in each sample **(Addendum C3: Fig. C3)**. Relative quantification of the target genes was performed by using comparative $2^{-\Delta\Delta CT}$ method, with the vehicles arbitrarily set to 1. This was then normalized relative to the respective reference gene transcript levels. PCR products were separated by 2% (w/v) agarose gel electrophoresis at 100 V for 1 hour, to confirm the

correct amplicon size of the amplified PAI-1, CRP, and 18S products and visualized under ultraviolet light using the Gel Doc™ XR+ Imaging System (Bio-Rad) with Image Lab™ Software. For determination of the correct amplicon size, samples were compared to the 100 bp DNA ladder RTU (Celtic Molecular Diagnostic).

Table 2.2. Details of primers used for semi-quantitative real-time PCR amplification.

Species	Gene	Strand	Sequence (5' → 3')	Amplicon length (bp)	Annealing temperature (°C)	Primer efficiency
Mouse	CRP	Forward	CCCTATGGGAGAATGGTTGG	96	55	2.46
		Reverse	TGGTCCTCTCCCAGGTTACA			
	PAI-1	Forward	TTCAGCCCTTGCTTGCCTC	115	60	2.51
		Reverse	ACACTTTACTCCGAAGTCGGT			
	18S	Forward	GTAACCCGTTGAACCCCATT	151	60	2.33
		Reverse	CCATCCAATCGGTAGTAGCG			

2.5.1. Primer design

The National Centre for Biotechnology Information (NCBI) provides a primer design tool, Primer-BLAST. Nucleotide mRNA sequences of PAI-1, CRP and 18S were found in the NCBI database. All the primers were designed to have the following:

(i) **GC-content less than 60%:** *high GC content will reduce PCR efficiency; GC bonds are stronger (require more energy to break) and less specific, because of the triple H-bond. Therefore, GC-rich regions are more prone to mispairing with other GC-rich regions.*

(ii) **Yielding a product of less than 200 bp:** *this maximise the chance of amplifying with high PCR efficiency; the bigger the amplicon the faster the fluorescence level will be reached in earlier Ct cycles, thus masking the actual effect of the gene.*

(iii) **Exon junction (intron spanning):** *exclusively targets cDNA and not genomic DNA which contains introns between exon-exon junctions.*

2.5.2. Primer preparation

Primers were delivered as lyophilized powders in vials, and these were picofuged for a few seconds to collect the primers at the bottom of the vial. The primers were resuspended in TE buffer **(Addendum D)** to yield a primer stock solution of 100 µM, from which a working

solution (10 µM) of both forward and reverse primers were prepared. Primers were stored at -20 °C.

2.6. Western Blot Analysis

2.6.1. Preparation of protein lysates

For western blot analysis, BWTG3 and HepG2 cells were treated as described in section 2.2.2. Whole cell lysates were prepared: briefly, cells were washed with 1x PBS and lysed with 150 µl passive lysis buffer containing freshy added protease inhibitor tablet (Roche Applied Science) as well as phenylmethylsulphonyl fluoride (PMSF). The combination of protease inhibitors, which inhibits serine and cysteine proteases, are required for complete protection of proteins in cell lysates. In addition, cell lysis was promoted with an overnight freeze-thaw cycle and thereafter detached from plates using cell scrapers and transferred to clean microcentrifuge tubes to which 5x SDS-reducing buffer **(Addendum D)** was added. Proteins in the lysates were denatured by boiling for 15 minutes at 95 °C , and stored at -20 °C until further analysis.

2.6.2. SDS-PAGE and Western Blotting

For gel electrophoresis, protein lysates were separated on a 10% polyacrylamide gel at a constant voltage of 75 V for 15 minutes, and 150 V for 1 hour, in 1x running buffer **(Addendum D)**. A colour prestained protein standard broad range (245-25 kDa) protein ladder was used as a marker for protein size (Inqaba Biotechnology). Following electrophoresis, the proteins were transferred onto a Hybond™ ECL™ nitrocellulose membrane (AEC Amersham) for 2 hours at a constant current of 0.18 A in cold 1x transfer buffer **(Addendum D)**. The membranes were subsequently blocked in 5% (w/v) fat-free milk powder prepared in 1x Tris buffered saline (TBS) containing Tween-20 (1x TBS-T) **(Addendum D)** for 90 minutes at room temperature. Membranes were subsequently rinsed with 1x TBS-T and incubated overnight (18-20 hours) at 4 ºC while agitating, in the appropriate antibody diluted in TBS-T **(Table 2.3)**. Hsp90 was used as loading control to normalise for equal loading of protein across all wells, which was found not to be influenced by any of the test compounds **(Addendum E1&E2)**. Membranes were washed with TBS-T for 10 minutes, followed by two 5-minute washes. Following the washing steps, the membranes were incubated for 90 minutes at room temperature with the appropriate secondary antibody diluted in 5% (w/v) fat-free milk powder in TBS-T **(Table 2.3)**. Membranes were subsequently washed as previously described. Proteins were visualised using Clarity™ Western ECL substrate (Bio-Rad) for enhanced

chemiluminescence and visualized using the MyECL or iBright Imager (Thermo-Fisher). ImageJ software was used to quantify all western blot images.

Table 2.3. A list of specific antibodies and dilutions used for Western blot analysis.

Primary (1°) Antibody	Catalogue Number	Dilution	Secondary (2°) Antibody	Catalogue Number	Dilution
[1]**PAI-1** (D9C4) rabbit mAb	11907	1:1000	[4]Goat anti-rabbit IgG-HRP	A9169	1:1000
[2]**Anti-C reactive Protein antibody** (26D7) mouse mAb	ab50861	1:1000	[2]Rabbit pAb to mouse IgG (HRP)	ab97046	1:7500
[3]**HSP 90α/β** (F-18) mouse mAb	sc-13119	1:5000			

[1]Cell Signalling Company (CST)
[2]Abcam
[3]Santa Cruz Biotechnology
[4]Sigma-Aldrich

2.7. Plasmid DNA preparation

2.7.1. Engineering of plasmid constructs

The pGL4.10/PAI-1 (PAI-1-Luc) promoter-reporter plasmid was kindly provided by Dr Y. Kawarada (Nagoya City University, Japan). This plasmid was generated by ligating the human PAI-1 promoter region (−800/+71) with the luciferase reporter vector, pGL4.10, as described in Kawarada *et al.*, 2016 [205]. The wild-type (WT) CRP reporter-promoter construct, 5'Δ10CAT3M or pCRP-luc (WT CRP -300), was kindly provided by Dr A. Agrawal (East Tennessee University, USA). WT CRP -300 was generated by ligating the human promoter region (-300/-1) with the luciferase reporter vector, pGL2 basic, as described in Kleemann *et al.*, 2003 [206]. See **Addendum F2, Fig. F1** for a schematic representation of the luciferase reporter vector maps used for each construct.

2.7.2. Transformation of bacterial cells

E. coli DH5α competent cells were transformed by heat shock with the plasmids, PAI-1-Luc & WT CRP -300, respectively. Briefly, plasmid DNA (5 µl) was added to competent cells (100 µl) and the resulting mixture was incubated on ice for 30 minutes. This was followed by heat shock at 42 ºC for 30 seconds, followed by cooling the plasmid DNA-bacterial mix on ice for 5 minutes. Subsequently, room temperature Super Optimal Broth with Catabolite repression (SOC) medium (950 µl) was added to the mixture and vigorously shaken at 37 ºC for 1 hour. Finally, several dilutions of the bacterial suspension (undiluted, 20x, 50x, 100x) were prepared and spread onto LB (Lysogeny broth)-agar plates **(Addendum D)** containing 50 µg/ml ampicillin antibiotic **(Addendum D)**. Plates were incubated at 37 ºC overnight. Ampicillin was chosen as suitable antibiotic, since both plasmid constructs contain an ampicillin resistant gene. Thus, if the bacterial cells are successfully transformed with the respective plasmid DNA, the cells will be able to grow in the presence of this antibiotic. In addition, a negative control was included, where no growth was expected.

2.7.3. Plasmid DNA extraction

Following transformation, a starter culture was prepared, where single *E. coli* colonies were picked from agar plates and inoculated into LB-medium (5 ml). This culture was vigorously shaken at 37 ºC for 6 hours. Subsequently, 1 ml of the starter culture was added to 250 ml LB-medium containing ampicillin. This culture was vigorously shaken at 37 ºC overnight. The following day, the OD_{600} of the overnight culture was measured, to ensure that the culture was still in the exponential growth phase. Bacterial cells were centrifuged at 6000 x g for 10 minutes at 4 °C to form a pellet. Plasmid DNA purifications were performed using the NucleoBond Xtra Maxi plasmid kit (Macherey-Nagel) according to the manufacturer's instructions. Briefly, the supernatant was decanted, and the remaining pellet resuspended in a buffer containing RNase, to remove any excess RNA. The kit relies on the lysis of bacterial cells using an alkaline solution containing the detergent SDS. Proteins tagged with SDS, as well as chromosomal DNA, precipitate and aggregate upon neutralisation of the solution. Plasmid DNA, however, remains soluble and was subsequently exposed to a wash and elution step. Thereafter, the eluate containing the plasmid DNA was precipitated with isopropanol and underwent centrifugation (15000 x g, 30 minutes, 4 °C) to form a pellet. The supernatant was discarded, and the pellet retained and washed with 70% EtOH and centrifuged at 15000 x g for 5 minutes at room temperature. Once again, the supernatant was removed, and the pellet air-

dried for 15 minutes to remove remaining traces of EtOH. DNA pellets were resuspended in Tris-EDTA (TE) buffer (1-1.5 ml) depending on the size of the pellet, and stored at -20 °C.

2.7.4. Measurement of plasmid DNA concentration and A260/280 ratio

The concentration and purity of the isolated plasmid DNA was determined by measuring the absorbance at 260 nm and 280 nm **(Addendum F3, Table F2)**. The concentration can be determined using the Beer-Lambert law, which predicts a linear change in absorbance with concentration. Quantification measurements were performed using a NanoDrop ND-1000 UV-vis spectrophotometer (CAF, Stellenbosch). As a blank, TE buffer was used, since the plasmid DNA was resuspended in this solution. An $A_{260/280}$ ratio of around 1.8 is expected [207], which is indicative of pure DNA free from contamination of organic solvents, salts and/or proteins.

2.7.5. Restriction enzyme digestion

A further plasmid DNA quality assurance was performed by restriction enzyme (RE) digestion. Single digests were performed in a 1.5 ml microcentrifuge tube, using 1 µg of plasmid DNA and 2000 U of the RE, SalI-HF (New England Biolabs). 2.5 µl 10x NE Buffer was added to this mixture, followed by the addition of Milli-Q water, to a final volume of 25 µl. In the case of undigested samples, no restriction enzyme was added. The digested and undigested plasmid samples were incubated at 37 ºC for 15 minutes. Digestion was terminated by adding 5 µl $MgCl_2$.

2.7.6. Agarose gel electrophoresis

Following RE digestion, linearized (digested) and non-linearized (undigested) plasmid were separated on a 0.5% (w/v) agarose gel to confirm the quality and integrity of the plasmids i.e., if the isolated plasmid DNA is supercoiled and therefore suitable for transient transfection. Firstly, 6x gel loading dye (5 µl) was added to each sample. The dye serves three main functions: 1) provides colour to the samples to facilitate loading, 2) contains high glycerol content, which allows settling of the samples to the bottom of the well and 3) migrates independently from samples, allowing the user to estimate the migration of DNA. Subsequently, agarose in 1x TAE buffer **(Addendum D)** was prepared at a concentration of 0.5% for plasmid DNA with a predicted size of >5000 bp. Agarose gels stained with Nancy-520 (Sigma-Aldrich) were allowed to set at room temperature. Subsequently, after loading 20 µl of each mixture, electrophoresis was performed at 100 V for approximately 1 hour. The gel

was visualized under UV light using the MyECL Imager (Pierce Thermo Scientific) **(Addendum F4: Fig. F2)**. For determination of the plasmid DNA size, samples were compared to the O'GeneRuler 1 kB Plus DNA Ladder (Thermo-Fisher).

2.8. Transient transfections

Cells were plated into a 10 cm^2 dish, to allow for equal transfection efficiency across the dish. When cells reached approximately 60 to 70% confluency, they were transiently transfected using the lipid-mediated transfection reagent, X-tremeGENE-HP DNA (Roche Applied Science), according to the manufacturer's instructions. Briefly, the transfection reagent forms a lipid vesicle around the negatively charged exogenous plasmid DNA, allowing it to pass through the phospholipid membrane, into the cell cytoplasm. The cells were transfected with 0.5 µg/µl of PAI-1-Luc (3:1) or 5 µg/µl WT CRP -300 (1:1). The transfection reagent and plasmid DNA mix were added to the cells in a drop wise manner. Following this, cells were incubated for 24 hours and subsequently re-plated into 96-well plates (Bio-Smart Scientific) at a density of 45 000 cells per well.

2.9. Luciferase Promoter Reporter Assay

For promoter reporter experiments, BWTG3 cells were transiently transfected and treated as described in sections 2.8 and 2.2.2, respectively. After treatment for 24 hours, cells were lysed with 35 µl passive lysis buffer **(Addendum D)**. The cells were agitated for 10 minutes at room temperature to ensure the coverage of cells with buffer, and subsequently stored at -20 ºC overnight, to aid with cell lysis. Luciferase activity was determined using the luciferase assay kit (Promega) according to the manufacturer's instructions. Briefly, once cells were thawed, 10 µl of each lysate was transferred to a white 96-well clinical plate (Whitehead Scientific) and 25 µl luciferase substrate was added to each well. The luciferase assay system generates light through a chemical reaction involving luciferin, which forms the product molecule oxyluciferin. As a result, light is produced which is measured using a Veritas™ microplate luminometer (Turner BioSystems). Therefore, promotor activity, expressed as relative light units (RLU), is quantitively measured as an end point experiment based on the amount of luciferase protein translated (light emitted). Luciferase promotor activity was normalized to total protein using the bicinchoninic acid assay (BCA) protein determination method (Thermo Scientific) described in section 2.10. Luciferase promotor activity was also compared relative to the vehicle controls.

2.10. Protein determination

The bicinchoninic acid assay (BCA) protein determination method (Thermo-Fisher) was used to measure the total protein content of the cell lysates prepared for the promoter reporter assay described in section 2.9. The BCA assay is a colorimetric assay, based on the reduction of Cu^{2+} to Cu^{1+}. The latter ion is chelated by two BCA molecules to form a tetradentate purple complex. Firstly, albumin (BSA) standards were prepared by performing eight serial dilutions of 1 ml albumin concentrate stock (2.0 mg/ml dissolved in a 0.9% aqueous NaCl solution containing sodium azide) in passive lysis buffer to concentrations ranging from 25-2000 µg/ml. Passive lysis buffer was used since the cell lysates used for the luciferase promoter reporter assay were lysed with this buffer. Next, the BCA working reagent was prepared as described by the manufacturer: for each cell lysate, 1 part of reagent B (containing 4% cupric sulphate) and 50 parts of reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) were mixed (1:50, reagent B: A), referred to as the working reagent. For the reaction in the transparent 96-well microplate, 10 µl of cell lysate or the prepared standards in triplicate was added to each well. Hereafter, 200 µl of working reagent was added to each well. To protect the microplate from light, it was wrapped in foil and incubated at 37 °C for 30 minutes. The reaction forms a purple colour product and the BCA/Cu^{1+} complex exhibits a strong absorbance at 562 nm, hence why the absorbance was measured at 562 nm, using the Multiskan Sky UV/Vis plate reader (Thermo-Fisher Scientific). A standard curve was plotted using GraphPad Prism® version 5 software. The protein concentration of each cell lysate was measured by interpolating the absorbance readings of each cell lysate into the standard curve and using the equation of the linear region, taking the Beer-lambert law into account.

2.11. Data and Statistical analysis

Graphical representation and statistical analysis were conducted using the GraphPad Prism® version 5.0 software. Data is expressed as the mean ± standard deviation (SD) if two independent experiments (n=2) and mean ± standard error of mean (SEM) if three or more independent experiments (n=3<) were performed. One-way ANOVA with Dunnett's Multiple Comparison's post-test was used when comparing each treatment to the vehicle control or each co-treatment to either pro-inflammatory cytokine alone treatment. Two-way ANOVA with Bonferroni post-test was used to compare each co-treatment to the respective concentration of GC on its own. When comparing each treatment to the vehicle, an asterisk

symbol (*) was used. When comparing each co-treatment with its respective GC- or cytokine only treatment, a hashtag- (#) and ampersand symbol (&) were used, respectively (***/###/&&&: $p<0.001$, **/##/&&: $p<0.01$, */#/&: $p<0.05$). No statistical differences ($p>0.05$) are not shown in the figures. In addition, statistical analysis was not performed for treatments consisting of only two independent experiments (n=2).

CHAPTER THREE: RESULTS

It has been reported that an elevated APR occurs during insulin resistance [13]. The levels of APPs, such as PAI-1 and CRP, are significantly increased during this response, hence why they are commonly used as biological markers for T2D [21]. In addition, APPs are regulated by GCs and pro-inflammatory cytokines, which have also been implicated in the development of insulin resistance [10][11]. It thus begs the question whether GC- and pro-inflammatory cytokine-induced insulin resistance develops as a result of an increase in these APPs? In an attempt to answer this question, our research group previously showed that the APPs, PAI-1 and CRP, were able to disrupt insulin signalling at key nodes of the insulin signalling pathway (unpublished data) [25]. This finding highlighted the role of APPs as possible causative agents in the development of insulin resistance. In the current study, we extend these earlier observations, by investigating the possible co-regulation of the expression of the APPs, PAI-1, and CRP, by GCs and pro-inflammatory cytokines. Although studies do exist investigating this possible co-regulation, our study sets out to further contribute to the knowlegde on this topic currently available in the literature.

Both the synthetic GC, Dex, and natural occurring GCs, corticosterone and cortisol, in rodents and humans, respectively, were included in this study. Exogenous GC treatment is commonly prescribed to treat and combat chronic inflammation, which is associated with insulin resistance and diabetes [208]. It is for this reason; Dex was specifically included as a representative synthetic GC. Under normal conditions, the endogenous GC, cortisol, plays a fundamental role in the termination of the APR, as it functions as negative feedback system by downregulating pro-inflammatory cytokine production in humans [83]. The GCs were used at three physiologically relevant concentrations (10 nM, 100 nM, and 1 µM), based on the knowledge that under normal conditions, cortisol is secreted in a circadian manner with its levels peaking in the morning (138 to 635 nM) and decreasing in the afternoon (83 to 359 nM) [209].

In addition, two well-studied, early-release pro-inflammatory cytokines, TNF-α and IL-6, were included, as both play an important role in the APR. The former indirectly promotes APP expression *via* orchestrating the production of cytokines, whereas the latter is understood to have a more direct effect on APP expression [112]. To the best of our knowledge, information regarding circulating cytokine levels in the body remain scarce and rather contradictory. Both healthy individuals and diabetic patients display inconsistent cytokine levels, which are far less (<0.2 ng/ml) [210] compared to the concentrations used in literature (between 1 to 100 ng/ml)

(Addendum A: Tables A1&A2). As a result, a single concentration of 20 ng/ml was chosen for each pro-inflammatory cytokine, as based on the literature this concentration is commonly used to stimulate cytokine signalling *in vitro* [182][147]. Because the liver is the main source of APPs, both the murine (BWTG3) and human (HepG2) hepatoma (liver carcinoma) cell lines were chosen as suitable *in vitro* models for this investigation, allowing for the determination of any cell line specific effects. The cells were cultured in low glucose-DMEM (containing 5.55 mM glucose) supplemented with FBS, which approximates normal blood sugar levels *in vivo* [211]. In addition, cells were serum starved with unsupplemented low glucose-DMEM for 24 hours prior to treatment. Taking these steps limited the intereference of factors, other than our test compounds, on PAI-1 and CRP expression.

The current study tested the hypothesis whether hepatoma cells co-treated with GCs and pro-inflammatory cytokines, would cooperatively regulate PAI-1 and CRP expression, at both mRNA and protein level. For measuring mRNA and intracellular protein expression, real-time semi-quantitative (q)-PCR and western blotting were utilised, respectively. Firstly, the transcriptional regulation of PAI-1 and CRP in response to the synthetic GC, Dex, and/or either pro-inflammatory cytokine, TNF-α or IL-6 was determined. Next, the optimal time required to affect intracellular PAI-1 and CRP protein expression in response to either the endogenous- (cortisol or corticosterone) or synthetic (Dex) GC, or the pro-inflammatory cytokines (TNF-α or IL-6) in each hepatoma cell line was established. Subsequently, the results obtained from the time course experiments were used in choosing a suitable time point to investigate what effect GCs co-treated with either TNF-α or IL-6 would have on intracellular PAI-1- and CRP protein expression i.e., at translational level. Lastly, to elucidate the potential molecular mechanisms governing the action of these signalling molecules to affect the expression of both APPs were investigated. The following question was formulated: Is the predicted increase in APP expression as a result of an increase in promotor activity of the APPs? To answer this question, cells were transfected with luciferase reporter constructs containing either the human PAI-1 or CRP minimal promotor. The mRNA and promoter experiments were only conducted in the mouse hepatoma cell line, due to reasons which will be addressed in each respective section.

3.1 The effects of GC and/or pro-inflammatory cytokines on PAI-1 and CRP mRNA expression

APP levels primarily increase during the APR in response to stress and inflammation aiding in restoring homeostasis. However, prolonged exposure to stress and inflammatory mediators such as GCs and pro-inflammatory cytokines can result in a prolonged APR and sustained APP production [113]. Chronic exposure to some APPs, like PAI-1 and CRP are associated with various disease states including T2D as previously mentioned [122]. PAI-1 and CRP mRNA expression in BWTG3 cells in response to Dex alone or in combination with the pro-inflammatory cytokines, TNF-α and IL-6 was firstly determined (**Figs 3.1&3.2)**. Due to difficulty experienced with the human PAI-1 and CRP primers mRNA expression was not investigated in the HepG2 cell line.

3.1.1 TNF-α and IL-6 differentially regulate PAI-1 mRNA expression, whilst Dex did not affect either PAI-1 or CRP mRNA expression in the BWTG3 cell line

Although stress and inflammatory mediators such as GCs and pro-inflammatory cytokines are associated with increased APP expression, they appear to influence PAI-1 and CRP mRNA expression differently. Dex at all three concentrations tested had no significant effect ($p>0.05$) on PAI-1- **(Fig. 3.1)** and CRP **(Fig. 3.2)** mRNA expression, relative to the vehicle control. Although both cytokines are categorised as pro-inflammatory mediators, it was observed that TNF-α and IL-6 differentially affected the mRNA levels of the two APPs. TNF-α had no significant effect ($p>0.05$) on PAI-1 mRNA expression compared to the vehicle control **(Fig. 3.1A)**, whereas IL-6 significantly ($p<0.01$) increased PAI-1 mRNA expression **(Fig. 3.1B)**. In contrast, both TNF-α **(Fig. 3.2A)** and IL-6 **(Fig. 3.2B)** had no significant ($p>0.05$) effect compared to the vehicle control on CRP mRNA expression. However, caution should be exercised when interpreting the findings with the pro-inflammatory cytokines because of the high variability between the independent biological repeats as indicated by the error bars.

In summary, the exogenous GC, Dex did not affect PAI-1 and CRP mRNA expression, whilst the two pro-inflammatory cytokines differentially regulated the mRNA expression of PAI-1.

3.1.2 PAI-1 and CRP mRNA expression are affected similarly in response to Dex co-treated with TNF-α, but not IL-6 in the BWTG3 cell line

Whilst single treatments with either 20 ng/ml TNF-α or Dex, at all three concentrations tested, had no significant effect on both PAI-1 and CRP mRNA expression, co-treatment with 10^{-8} M Dex, the lowest concentration used, plus TNF-α significantly ($p<0.01$) increased the mRNA levels of both APPs when compared to basal expression. Similarly, although not as pronounced as with 10^{-8} M Dex, TNF-α co-treated with the highest concentration of Dex used (10^{-6} M) significantly ($p<0.05$) increased PAI-1 and CRP mRNA expression compared to the vehicle control. Thus, an increase in PAI-1 and CRP mRNA expression was only observed when the BWTG3 cells were co-treated with TNF-α and either 10^{-8} M- or 10^{-6} M Dex. Furthermore, 10^{-8} M Dex co-treated with TNF-α also lead to a significant ($p<0.01$) increase in mRNA expression of both APPs compared to 10^{-8} M Dex alone **(Figs 3.1A & 3.2A)**, of which the latter was unable to induce PAI-1 and CRP mRNA expression.

In contrast, PAI-1 mRNA levels remained unaffected when compared to vehicle control in response to Dex at all concentrations used when co-treated with IL-6 **(Fig. 3.1B)**. Moreover, Dex appears to antagonise IL-6-induced PAI-1 mRNA expression by decreasing PAI-1 mRNA back to basal levels. However, significance was not established, possibly due to the variability observed between independent biological repeats for the IL-6 single treatment **(Fig. 3.1B)**. A significant ($p<0.05$) increase in CRP mRNA levels compared to vehicle control was observed when BWTG3 cells were co-treated with IL-6 and the highest concentration (10^{-6} M) of Dex used **(Fig. 3.2B)**. Surprisingly, CRP mRNA levels significantly ($p<0.01$) decreased when the cells were co-treated with IL-6 and a lower concentration (10^{-7} M) of Dex. Whereas CRP mRNA levels remained unaffected by the lowest concentration (10^{-8} M) of Dex used co-treated with IL-6 **(Fig. 3.2B)**. In contrast, CRP mRNA levels were mostly upregulated when the BWTG3 cells were co-treated with TNF-α and various concentrations of Dex **(Fig. 3.2A)**.

In summary, both PAI-1- and CRP mRNA expression in BWTG3 cells were similarly affected by Dex when co-treated with TNF-α, but not IL-6. A significant ($p<0.05$) increase in both PAI-1- and CRP mRNA expression was observed in response to co-treatment with TNF-α and the highest and lowest concentrations of Dex used in the study **(Figs 3.1A & 3.2A)**. In contrast, the effect co-treatment of IL-6 and Dex has on CRP mRNA levels, compared to the vehicle control, appear to be dependent on the concentration of Dex used. CRP mRNA levels

significantly ($p<0.05$) increased in response to IL-6 co-treated with Dex at the highest concentration used, significantly ($p<0.01$) decreased in response to IL-6 co-treated with a lower concentration of Dex used and remained unaffected in response to IL-6 and the lowest concentration of Dex used **(Fig. 3.2B)**. Furthermore, the IL-6-mediated increase in PAI-1 mRNA was inhibited by Dex at all concentrations tested.

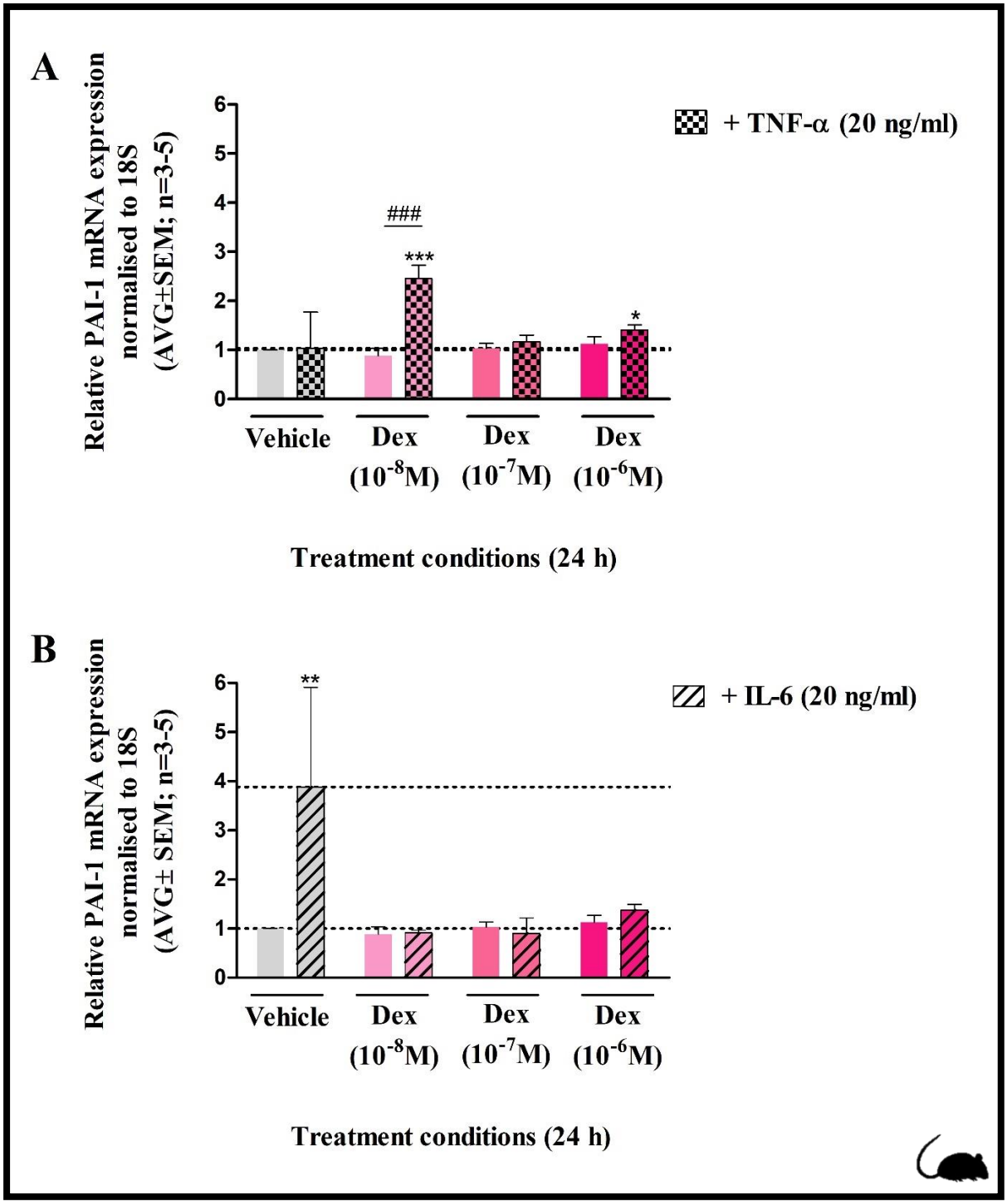


Figure 3.1. PAI-1 mRNA expression in mouse hepatoma cells in response to co-treatment with Dex and either TNF-α or IL-6. BWTG3 cells were serum starved for 24 hours (h) followed by co-treatment with various concentrations (10^{-8} M, 10^{-7} M, 10^{-6} M) of the synthetic GC, dexamethasone (Dex) combined with either (A) TNF-α or (B) IL-6 for 24 h. Hereafter, total RNA was isolated, cDNA synthesized, and analysed using qPCR with primers specific to PAI-1 and the internal control, 18S. The relative PAI-1 mRNA expression was normalised to the relative 18S mRNA expression. The data were plotted as fold change relative to the vehicle mRNA (combination of ethanol and supplemented DMEM), which was set to 1. Results displayed are representative of three to five independent biological repeats (± SEM), where each condition was performed in duplicate (two technical repeats). One-way ANOVA with Dunnett's Multiple Comparison's post-test was used when comparing each treatment to the vehicle control, whereas two-way ANOVA with Bonferroni post-test was used to compare each co-treatment to the respective concentration of Dex on its own When comparing each co-treatment with its respective Dex- or cytokine only treatment, a hashtag- (#) and ampersand symbol (&) were used, respectively (*: $p<0.05$, **: $p<0.01$, ***/###: $p<0.001$).

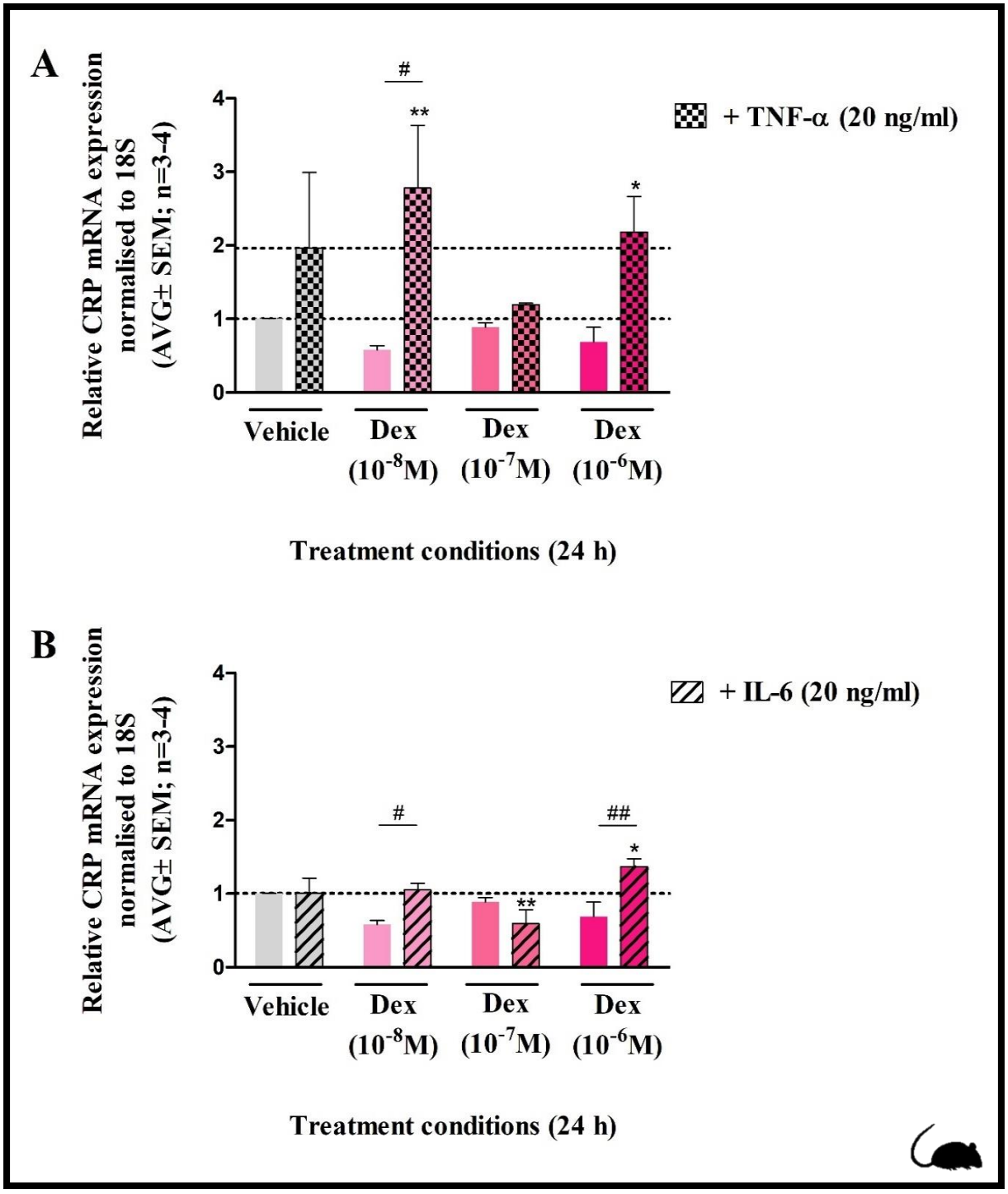


Figure 3.2. CRP mRNA expression in mouse hepatoma cells in response to co-treatment with Dex and either TNF-α or IL-6. BWTG3 cells were serum starved for 24 hours (h) followed by co-treatment with various concentrations (10^{-8} M, 10^{-7} M, 10^{-6} M) of the synthetic GC, dexamethasone (Dex) combined with either (A) TNF-α or (B) IL-6 for 24 h. Hereafter, total RNA was isolated, cDNA synthesized, and analysed using qPCR with primers specific to PAI-1 and the internal control, 18S. The relative CRP mRNA expression was normalised to the relative 18S mRNA expression. The data were plotted as fold change relative to the vehicle mRNA (combination of ethanol and supplemented DMEM), which was set to 1. Results displayed are representative of three to five independent biological repeats (± SEM), where each condition was performed in duplicate (two technical repeats). One-way ANOVA with Dunnett's Multiple Comparison's post-test was used when comparing each treatment to the vehicle control, whereas two-way ANOVA with Bonferroni post-test was used to compare each co-treatment to the respective concentration of Dex on its own When comparing each co-treatment with its respective Dex- or cytokine only treatment, a hashtag- (#) and ampersand symbol (&) were used, respectively (*/#: $p<0.05$, **/##: $p<0.01$).

3.2 Time-dependent effects of GCs or pro-inflammatory cytokines on PAI-1 and CRP protein expression

Establishing whether the effects of GCs and the pro-inflammatory cytokines on PAI-1 and CRP mRNA level are also reflected at the protein level was next established. Because some of the test compounds failed to affect PAI-1 and CRP expression at the transcriptional level at 24 hours **(Fig. 3.1)**, we first set out to investigate whether the length of exposure to the test compounds could affect intracellular PAI-1- and CRP protein expression. The normal APR in the liver is known to peak at 48 hours after a triggering event, but can proceed up to days [32]. In light of this, the time course study included three time points (24-, 48- and 72 hours) in order to determine the optimal treatment time with the selected test compounds. For the purpose of the time course study a single GC concentration of a 100 nM (10^{-7} M) was chosen, as the majority of studies in the literature which have investigated the regulation of APP expression by GCs, included this specific concentration **(Addendum A: Tables A1&A2**). The relative PAI-1- and CRP protein expression at each time point were normalised to time 0 (vehicle control), which was set to 1. For consideration of equal protein loading, Hsp90 was used as a loading control as it was shown to be not significantly regulated by the test compounds **(Addendum E1&E2)**.

Both the synthetic GC, Dex and the endogenous GC, corticosterone (Cort), lead to an increase in PAI-1 protein expression in the murine cell line **(Fig. 3.3A&B).**In the BWTG3 cells, a significant ($p<0.05$) increase in PAI-1 protein levels was observed in response to 24- and 48 hours Dex treatment. In contrast, PAI-1 protein levels were unaffected when BWTG3 cells were treated with Dex for 72 hours (**Fig. 3.3A)**. Furthermore, in the HepG2 cells, PAI-1 protein expression appeared to be unaffected by Dex although statistical analysis could not be done as only two biological independent repeats were performed **(Fig. 3.4A)**. Like Dex, a significant increase in PAI-1 protein expression was found in the BWTG3 cells in response to the endogenous GC, Cort **(Fig. 3.3B)**. However in contrast to Dex, a longer exposure to the endogenous GC, Cort was required before a significant ($p<0.05$) increase in PAI-1 protein expression was observed in the BWTG3 cells **(Figs 3.3A&B)**. PAI-1 protein expression was only significantly ($p<0.05$) increased in response to 48 hours Cort treatment **(Fig. 3.3B)**. Additionally, although statistical analysis could not be performed as only two biological independent repeats were done, in the HepG2 cells it does appear that a slight increase in PAI-1 protein expression was observed in response to 48 hours cortisol (F) treatment **(Fig. 3.4B)**.

Like with the GCs, the effect of the pro-inflammatory cytokines on PAI-1 protein expression was also time dependent and varied between cell lines. No significant ($p>0.05$) effect on PAI-1 protein expression was observed in response to TNF-α- and IL-6 exposure at all three timepoints in the BWTG3 cells. However, although not statistically significant ($p>0.05$), exposure to TNF-α for 48- and 72 hours and IL-6 for 48 hours appeared to increase PAI-1 protein expression **(Fig 3.3C&D)**. In the HepG2 cells however, TNF-α treatment for both 24- and 48 hours lead to a significant ($p<0.01$) increase in PAI-1 protein expression, which returned to basal levels when treated with TNF-α for 72 hours **(Fig 3.4C)**. In contrast, HepG2 cells treated with IL-6 had no significant effect on PAI-1 protein levels although a slight decrease was observed at 24 hours followed by a slight increase at 48 hours **(Fig. 3.4D)**.

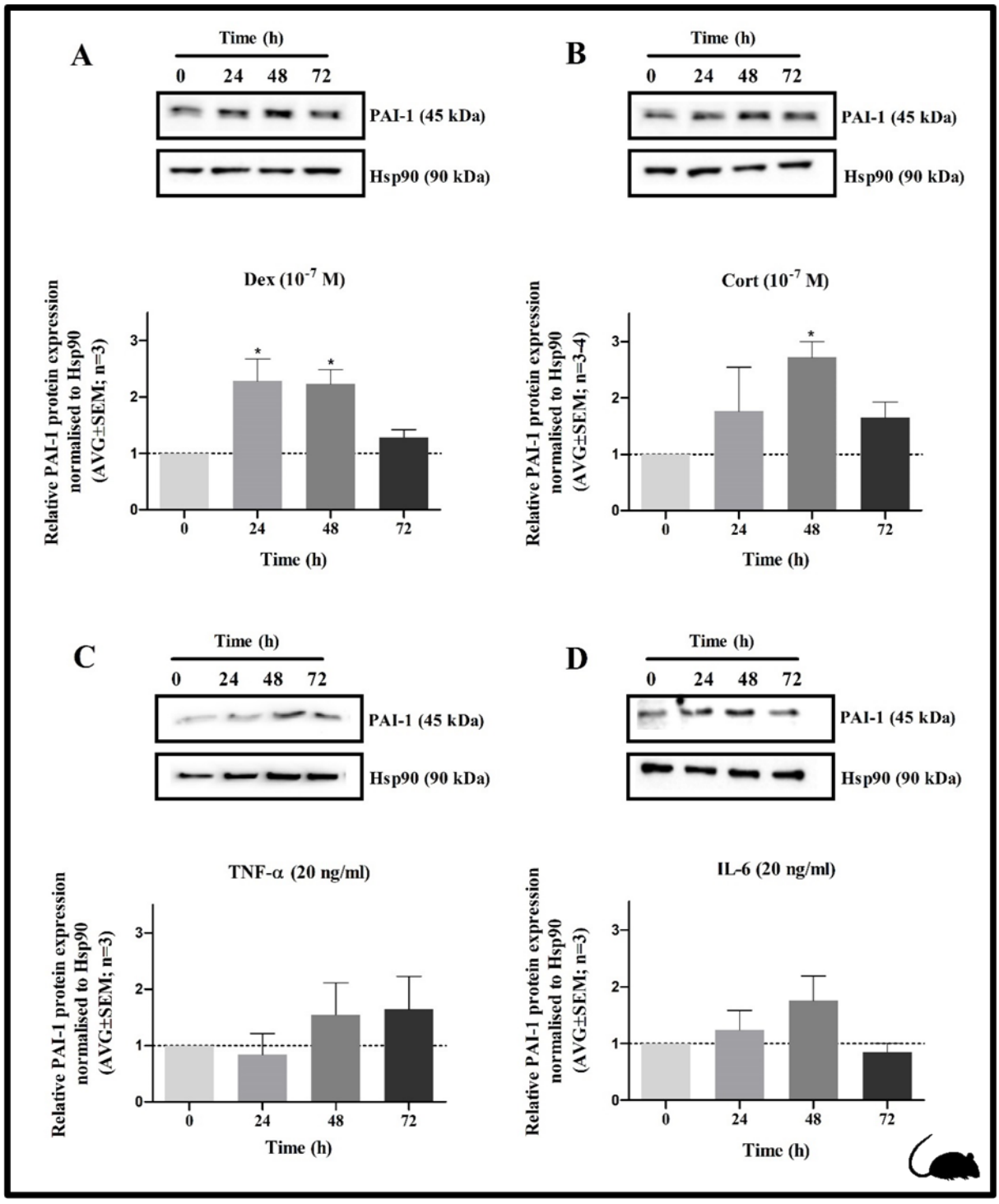


Figure 3.3. Intracellular PAI-1 protein levels in a mouse hepatoma cell line over time in response to GCs, TNF-α, or IL-6. Total protein was extracted from BWTG3 cells treated with 10^{-7} M of (A) the synthetic GC, dexamethasone (Dex) or (B) endogenous GC, corticosterone (Cort), and 20 ng/ml of the pro-inflammatory cytokines, (C) TNF-α or (D) IL-6, for indicated time periods (0, 24, 48, 72 hours (h)). PAI-1 protein was identified as a single 45 kDa band using a monoclonal anti-rabbit PAI-1 antibody. Hsp90 was used as loading control. Densities of the western blot bands were measured by ImageJ software and illustrated as bar graphs. The relative PAI-1 protein expression levels at each time point were normalised to the loading control and compared with that at time 0, which was set to 1 (represented by the black dotted line). Results displayed are representative of three to four independent biological repeats (± SEM). Statistical analysis was performed using one-way ANOVA with Dunnett's Multiple Comparison's post-test (*: $p<0.05$).

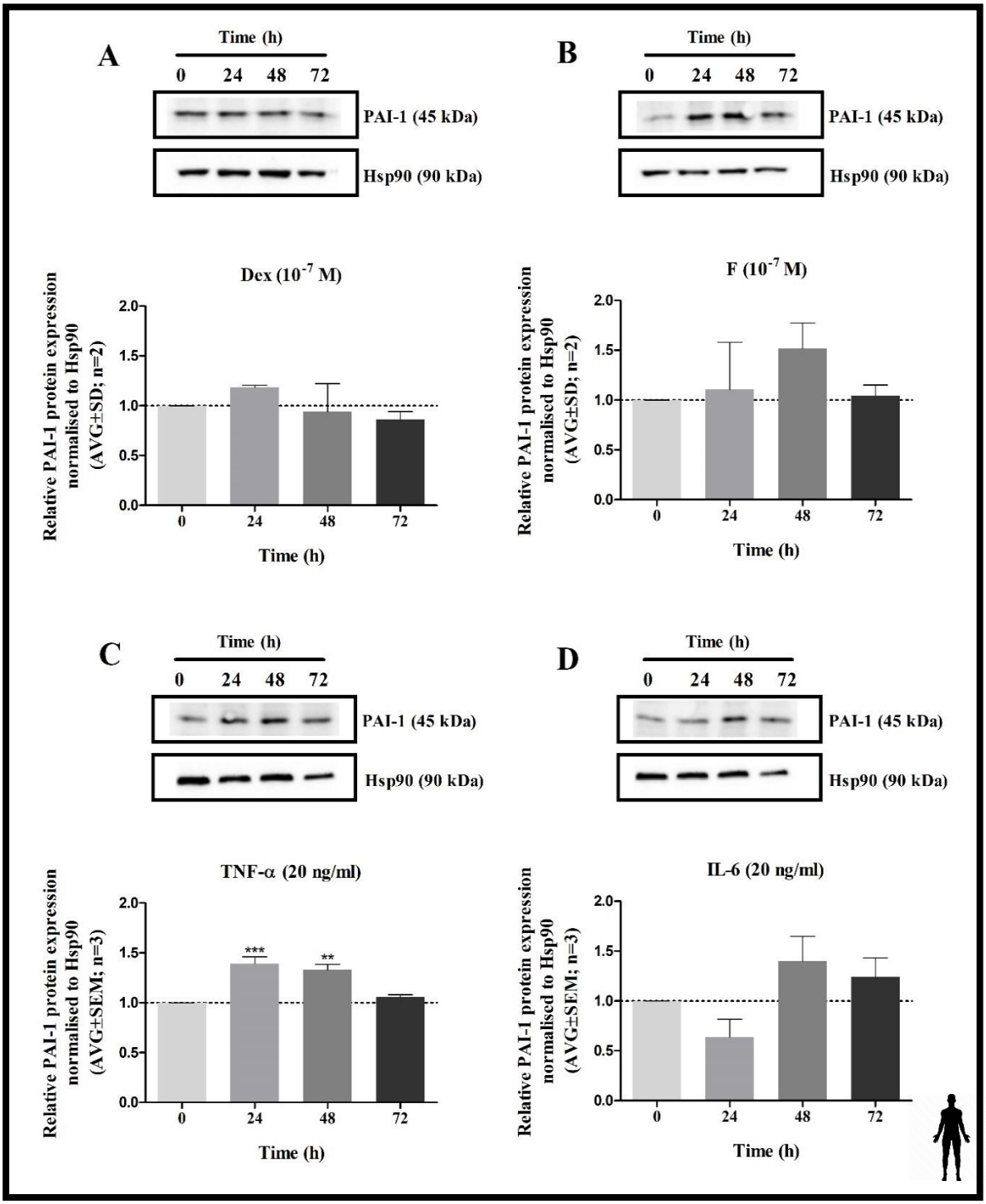


Figure 3.4. Intracellular PAI-1 protein levels in a human hepatoma cell line over time in response to GCs, TNF-α, or IL-6. Total protein was extracted from HepG2 cells treated with 10^{-7} M of (A) the synthetic GC, dexamethasone (Dex) or (B) endogenous GC, cortisol (F), and 20 ng/ml of the pro-inflammatory cytokines, (C) TNF-α or (D) IL-6, for indicated time periods (0, 24, 48, 72 hours (h)). PAI-1 protein was identified as a single 45 kDa band using a monoclonal anti-rabbit PAI-1 antibody. Hsp90 was used as loading control. Densities of the western blot bands were measured by ImageJ software and illustrated as bar graphs. The relative PAI-1 protein expression levels at each time point were normalised to the loading control and compared with that at time 0, which was set to 1 (represented by the black dotted line). Results displayed are representative of two (± SD) to three independent biological repeats (± SEM). Statistical analysis was performed using one-way ANOVA with Dunnett's Multiple Comparison's post-test (**p<0.01, ***p<0.001). In addition, statistical analysis was not performed for treatments consisting of only two independent experiments (n=2), as in the case of graphs A and B.

Both the synthetic GC, Dex and the endogenous GCs lead to an increase in CRP protein expression in both the murine and human hepatoma cell lines, albeit to different extents **(Fig. 3.5A *vs* Fig. 3.6A)**. Although an increase in CRP protein expression in response to Dex treatment was observed from 24 hours, significance was only established after 72 hours treatment in the BWTG3 cells ($p<0.05$) **(Fig 3.5A)**. Similar to the BWTG3 cells, in HepG2 cells Dex treatment resulted in an increase in CRP protein levels, with a significant increase ($p<0.01$) obtained at 24 and 48 hours **(Fig 3.6A)**. Like Dex, the endogenous GCs also resulted in an increase in CRP protein levels albeit to different degrees. CRP protein levels only started to increase at 48 hours exposure to the endogenous murine GC, Cort, **(Fig. 3.5B)**, whereas in the HepG2 cells, F the endogenous GC in humans at already 24 hours increased CRP protein levels **(Fig. 3.6B)**. The highest fold increase in CRP protein expression for both endogenous GCs was shown at 48 hours **(Figs 3.5B & 3.6B)**.

The effect of each pro-inflammatory cytokine on CRP protein expression seems to differ between cell lines (possible species-specific differences). Although, a slight increase in CRP protein levels was detected after 48- and 72-hour exposure to TNF-α in BWTG3 cells, significance could not be established for either time points ($p>0.05$) **(Fig. 3.5C)**. In contrast, a significant increase in CRP protein levels was already observed in HepG2 cells treated with TNF-α for 24 hours, which remained significantly ($p<0.05$) elevated up to 72 hours **(Fig. 3.6C)**. Like TNF-α treatment, a slight but not significant ($p>0.05$) increase in CRP protein levels in response to IL-6 treatment for 48- and 72 hours was found in BWTG3 cells **(Fig. 3.5D)**. CRP protein expression also appeared to increase in response to IL-6 at all the time points investigated in the HepG2 cells although significance could not be established due to large error between independent experiments **(Fig. 3.6D)**.

Taken together, both PAI-1- and CRP protein expression in both the BWTG3 and HepG2 cells were increased by the test compounds albeit to different extents. An increase in both PAI-1- and CRP protein levels were generally observed in response to the test compounds at 48 hours, which was chosen as the time point for subsequent combinatorial treatments. Treating the cells for a duration of 48 hours would allow us to examine how a combination of GCs plus pro-inflammatory cytokines would affect APP expression at intracellular protein level.

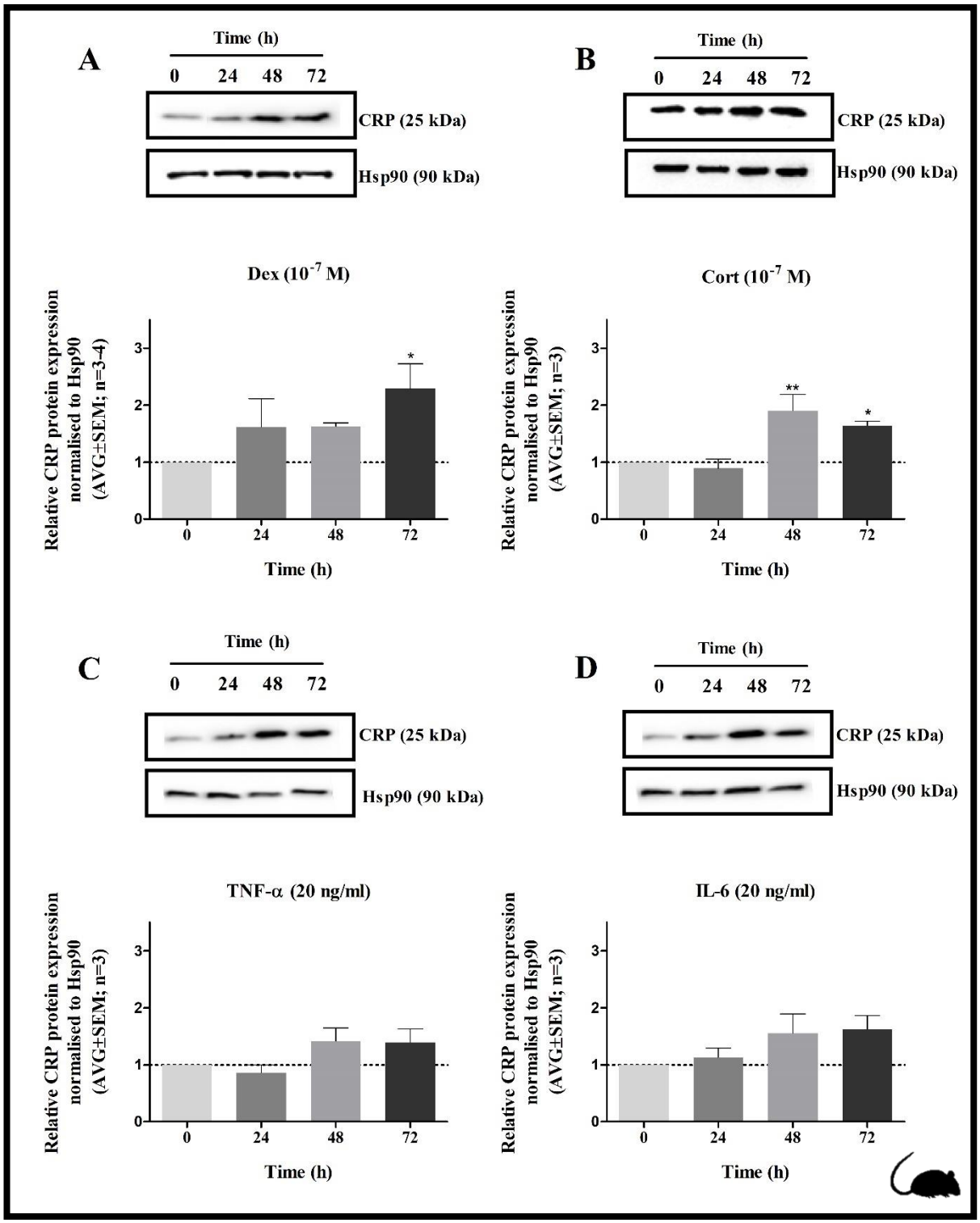


Figure 3.5. Intracellular CRP protein levels in a mouse hepatoma cell line over time in response to GCs, TNF-α, or IL-6. Total protein was extracted from BWTG3 cells treated with 10^{-7} M of (A) the synthetic GC, dexamethasone (Dex) or (B) endogenous GC, corticosterone (Cort), and 20 ng/ml of the pro-inflammatory cytokines, (C) TNF-α or (D) IL-6, for indicated time periods (0, 24, 48, 72 hours (h)). CRP protein was identified as a single 26 kDa band using a monoclonal anti-mouse CRP antibody. Hsp90 was used as loading control. Densities of the western blot bands were measured by ImageJ software and illustrated as bar graphs. The relative CRP protein expression levels at each time point were normalised to the loading control and compared with that at time 0, which was set to 1 (represented by the black dotted line). Results displayed are representative of three to four independent biological repeats (± SEM). Statistical analysis was performed using one-way ANOVA with Dunnett's Multiple Comparison's post-test (*: $p<0.05$, **: $p<0.01$).

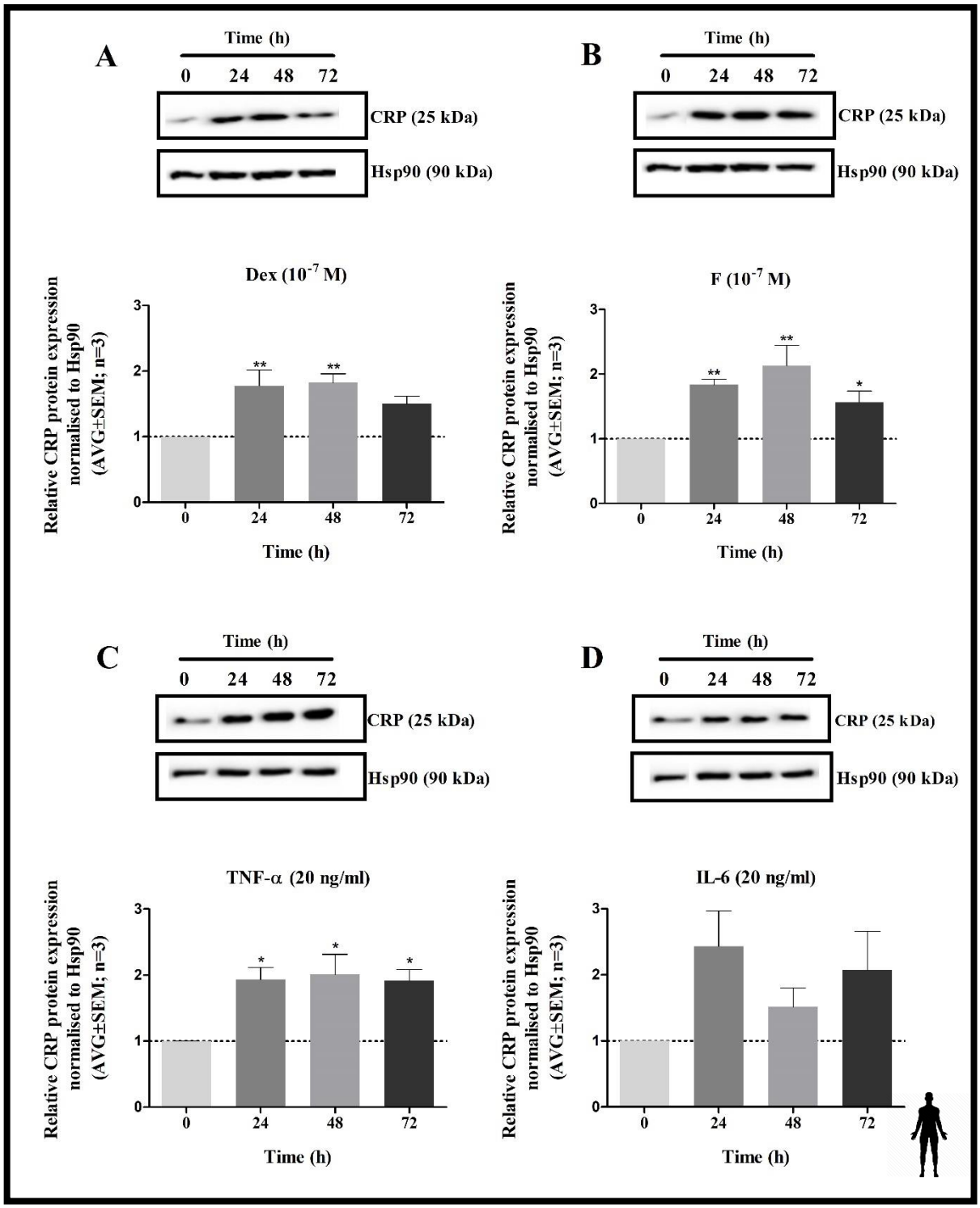


Figure 3.6. Intracellular CRP protein levels in a human hepatoma cell line over time in response to GCs, TNF-α, or IL-6. Total protein was extracted from HepG2 cells treated with 10^{-7} M of (A) the synthetic GC, dexamethasone (Dex) or (B) endogenous GC, cortisol (F), and 20 ng/ml of the pro-inflammatory cytokines, (C) TNF-α or (D) IL-6, for indicated time periods (0, 24, 48, 72 hours (h)). CRP protein was identified as a single 26 kDa band using a monoclonal anti-mouse CRP antibody. Hsp90 was used as loading control. Densities of the western blot bands were measured by ImageJ software and illustrated as bar graphs. The relative CRP protein expression levels at each time point were normalised to the loading control and compared with that at time 0, which was set to 1 (represented by the black dotted line). Results displayed are representative of three independent biological repeats (± SEM). Statistical analysis was performed using one-way ANOVA with Dunnett's Multiple Comparison's post-test (*: $p<0.05$, **: $p<0.01$).

3.3 The effects of GCs and/or pro-inflammatory cytokines on PAI-1- and CRP expression at protein level

Having determined the optimal treatment time with the selected test compound to induce intracellular PAI-1 and CRP protein expression, BWTG3 and HepG2 cells were treated for 48 hours with increasing concentrations (10^{-8} M, 10^{-7} M, and 10^{-6} M) of Dex or endogenous GC in the absence and presence of 20 ng/ml of either, TNF-α or IL-6 (**Figs 3.7-3.14**). As previously mentioned, what occurs at the mRNA level of a protein does not necessarily reflect at its protein level. This could be due to complex and varied post-transcriptional modifications (microRNA regulation) which could occur between the transcript and protein product [212][213], or the export of proteins, which could affect the amount of protein present.

Both pro-inflammatory cytokines in the absence of GCs resulted in a significant ($p<0.01$) increase in PAI-1- and CRP protein levels regardless of the cell line used **(Figs 3.7-3.14)**, which was not observed for Dex.

3.3.1. PAI-1 protein expression in response to GCs in the absence or presence of pro-inflammatory cytokines

Differential regulation of PAI protein expression in response to Dex in the absence and presence of the pro-inflammatory cytokines was observed in the mouse and human hepatoma cell lines **(Fig. 3.7 *vs* Fig. 3.8)**. Firstly, in the BWTG3 cells, Dex at all concentrations tested significantly ($p<0.05$) induced an increase in PAI-1 protein expression **(Fig. 3.7)**. The addition of either TNF-α or IL-6, had no significant effect ($p>0.05$) on PAI-1 protein expression when compared to either the GC at the relevant concentration or to the appropriate pro-inflammatory cytokine alone **(Fig. 3.7)**. In contrast to the results obtained in the BWTG3 cells, in the HepG2 cells, Dex at all concentrations tested was unable to significantly affect PAI-1 protein levels **(Fig. 3.8)**. Moreover, both TNF-α- and IL-6-induced increases in PAI-1 protein levels were significantly ($p<0.05$) antagonised by Dex, which was not observed in the murine hepatoma cell line **(Fig. 3.8 *vs* Fig. 3.7)**. In addition, this antagonising effect by Dex appears to be dose-dependent in the HepG2 cells.

Similar to Dex, Cort the endogenous GC in rodents, significantly ($p<0.05$) increased PAI-1 protein expression in BWTG3 cells, although 10^{-8} M Cort had no significant effect ($p>0.05$) on PAI-1 protein expression when compared to vehicle control **(Fig. 3.9)**. In regard to the

combinatorial treatments, Cort had no significant ($p>0.05$) effect on the increase in PAI-1 protein levels mediated by either TNF-α or IL-6 in the BWTG3 cells **(Fig. 3.9)**. On the other hand, 10^{-8} M Cort co-treated with IL-6 significantly ($p<0.01$) increased PAI-1 protein expression when compared to 10^{-8} M Cort only treatment **(Fig. 3.9B)**.

In the HepG2 cells the endogenous GC, F only at 10^{-8} M and 10^{-6} M was able to increase PAI-1 protein expression **(Fig. 3.10)**, unlike Dex the synthetic GC which was unable to increase the protein levels of this APP at any of the concentrations used in the HepG2 cells **(Fig. 3.8)**. Furthermore, whilst F generally had no significant effect on TNF-α-mediated increase in PAI-1 protein expression, co-treatment of 10^{-7} M F with TNF-α resulted in a significant ($p<0.001$) increase in PAI-1 protein levels when compared to the 10^{-7} M F treatment alone **(Fig. 3.10A)**. Moreover, whilst both 10^{-6} M F- and IL-6 only treatments resulted in a significant ($p<0.05$) increase in PAI-1 protein levels, surprisingly co-treatment of these two compounds resulted in PAI-1 levels similar to that observed with basal expression, suggesting that at this concentration of F the two compounds are antagonising each other **(Fig. 3.10B)**.

Thus, to summarise, whilst PAI-1 protein expression was upregulated by both TNF-α- and IL-6 only treatments in both the murine and human liver cells, cell line specific effects in response to the GCs were observed. PAI-1 protein expression in the BWTG3 cells appeared to be more sensitive to GC treatment as both Dex (at all concentrations tested) and the endogenous GC, Cort (at the two highest concentrations tested) increased PAI-1 protein levels **(Figs 3.7 & 3.9)**. Whilst in the HepG2 cells Dex (at all concentrations tested) had no effect on PAI-1 protein expression compared to vehicle control **(Fig. 3.8)**. Despite this, F at concentrations of 10^{-8} M and 10^{-6} M was able to induce a significant increase in PAI-1 protein expression **(Fig. 3.10)**. However, although the PAI-1 protein regulation was less sensitive to GCs in HepG2 cells, TNF-α- and IL-6-mediated increase in PAI-1 protein expression was more affected by the GCs in the human hepatoma cell line. For example, both TNF-α- and IL-6-mediated increase in PAI-1 protein expression was antagonised by Dex **(Fig. 3.8)**, and 10^{-6} M F antagonised IL-6-induced PAI-1 protein expression **(Fig. 3.10B)** in the HepG2 cells. Furthermore, also in the HepG2 cells, 10^{-7} M F potentiated TNF-α-induced PAI-1 protein levels **(Fig. 3.10A)**. These results suggest that PAI-1 regulation by GCs in the presence and absence of pro-inflammatory cytokines are cell line specific.

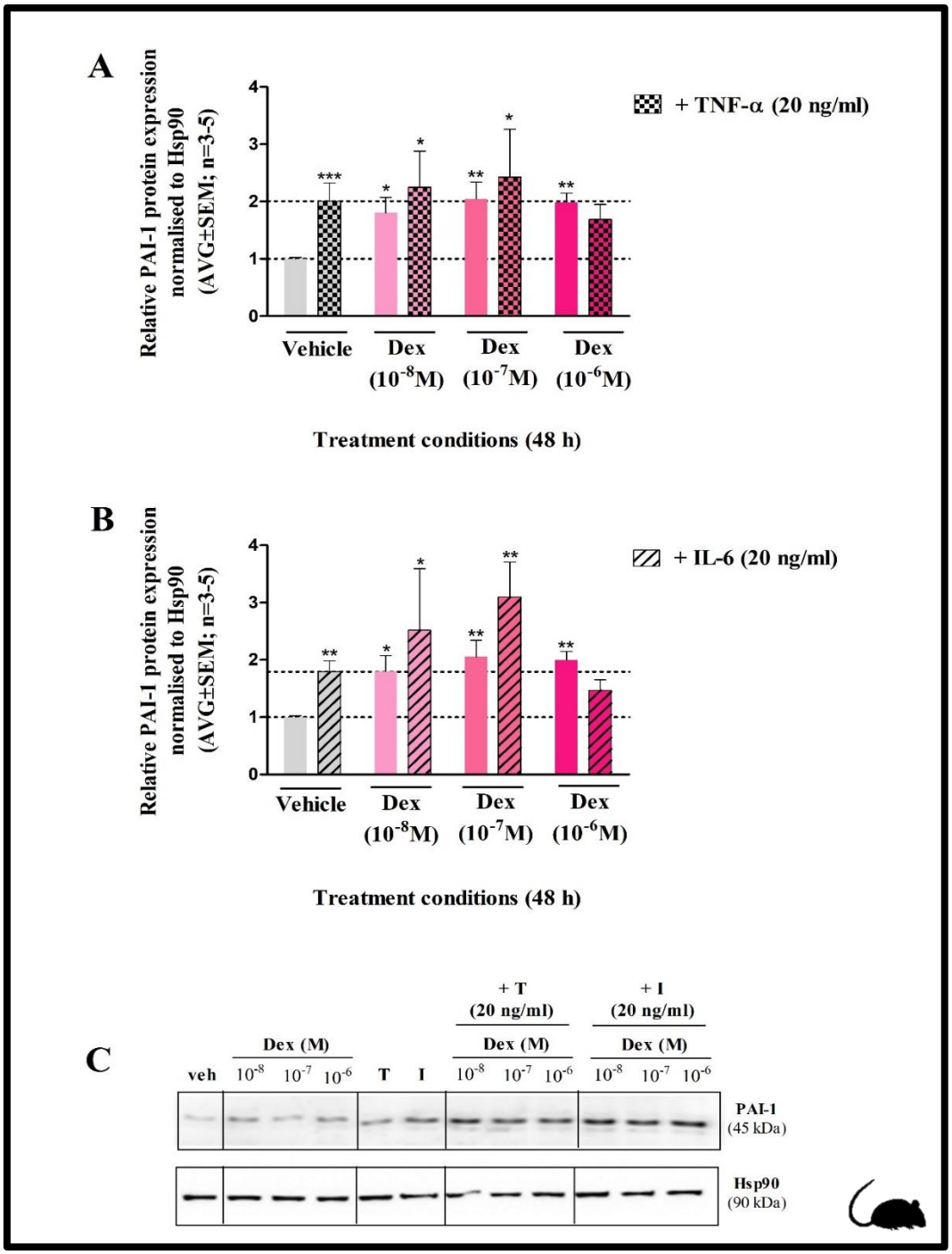


Figure 3.7. Intracellular PAI-1 protein levels in a mouse hepatoma cell line response to single- and co-treatment with Dex and either TNF-α or IL-6. Total protein was extracted from BWTG3 cells after 48 hours (h) treatment with various concentrations of (A) the synthetic GC, dexamethasone (Dex), or 20 ng/ml of TNF-α (T) or IL-6 (I), in addition to the GC co-treated with either (B) TNF-α or (C) IL-6. A representative blot is shown in (D). Densities of the western blot bands were measured by ImageJ software and illustrated as bar graphs. The relative PAI-1 protein expression was normalised to the loading control (Hsp90) and compared with the vehicle control, which was set to 1 (represented by the black dotted line). Results displayed are representative of three to five independent biological repeats (± SEM). One-way ANOVA with Dunnett's Multiple Comparison's post-test was used when comparing each treatment to the vehicle control or each co-treatment to either pro-inflammatory cytokine alone treatment. Two-way ANOVA with Bonferroni post-test was used to compare each co-treatment to the respective concentration of Dex on its own. When comparing each treatment to the vehicle, an asterisk symbol (*) was used. When comparing each co-treatment with its respective Dex- or cytokine only treatment, a hashtag- (#) and ampersand symbol (&) were used, respectively (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

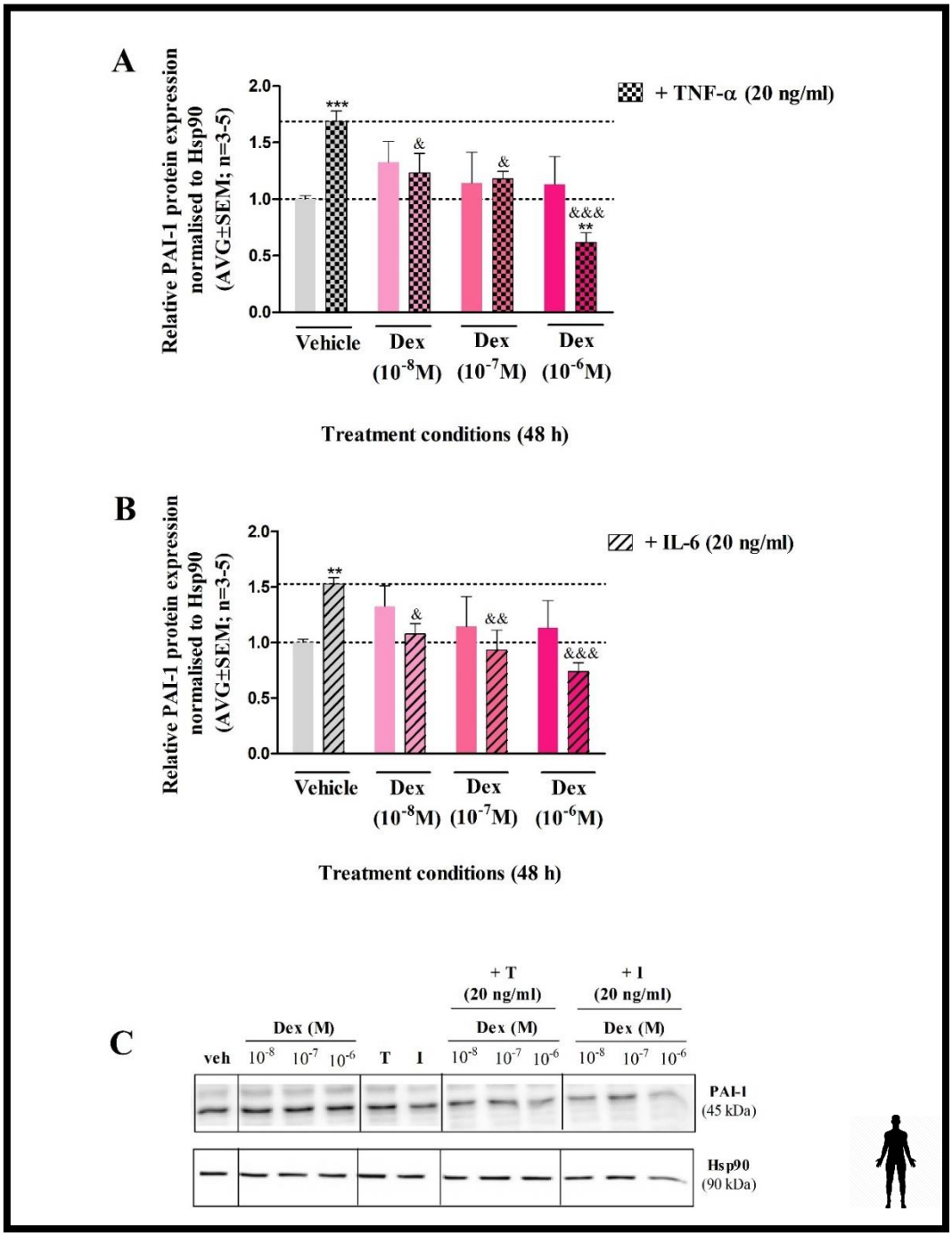


Figure 3.8. Intracellular PAI-1 protein levels in a human hepatoma cell line response to single- and co-treatment with Dex and either TNF-α or IL-6. Total protein was extracted from HepG2 cells after 48 hours (h) treatment with various concentrations of (A) the synthetic GC, dexamethasone (Dex), or 20 ng/ml of TNF-α (T) or IL-6 (I), in addition to the GC co-treated with either (B) TNF-α or (C) IL-6. A representative blot is shown in (D). Densities of the western blot bands were measured by ImageJ software and illustrated as bar graphs. The relative PAI-1 protein expression was normalised to the loading control (Hsp90) and compared with the vehicle control, which was set to 1. Results displayed are representative of three to five independent biological repeats (± SEM). One-way ANOVA with Dunnett's Multiple Comparison's post-test was used when comparing each treatment to the vehicle control or each co-treatment to either pro-inflammatory cytokine alone treatment. Two-way ANOVA with Bonferroni post-test was used to compare each co-treatment to the respective concentration of Dex on its own. When comparing each treatment to the vehicle, an asterisk symbol (*) was used. When comparing each co-treatment with its respective Dex- or cytokine only treatment, a hashtag- (#) and ampersand symbol (&) were used, respectively (*/&: $p<0.05$, **/&&: $p<0.01$, ***/&&&: $p<0.001$).

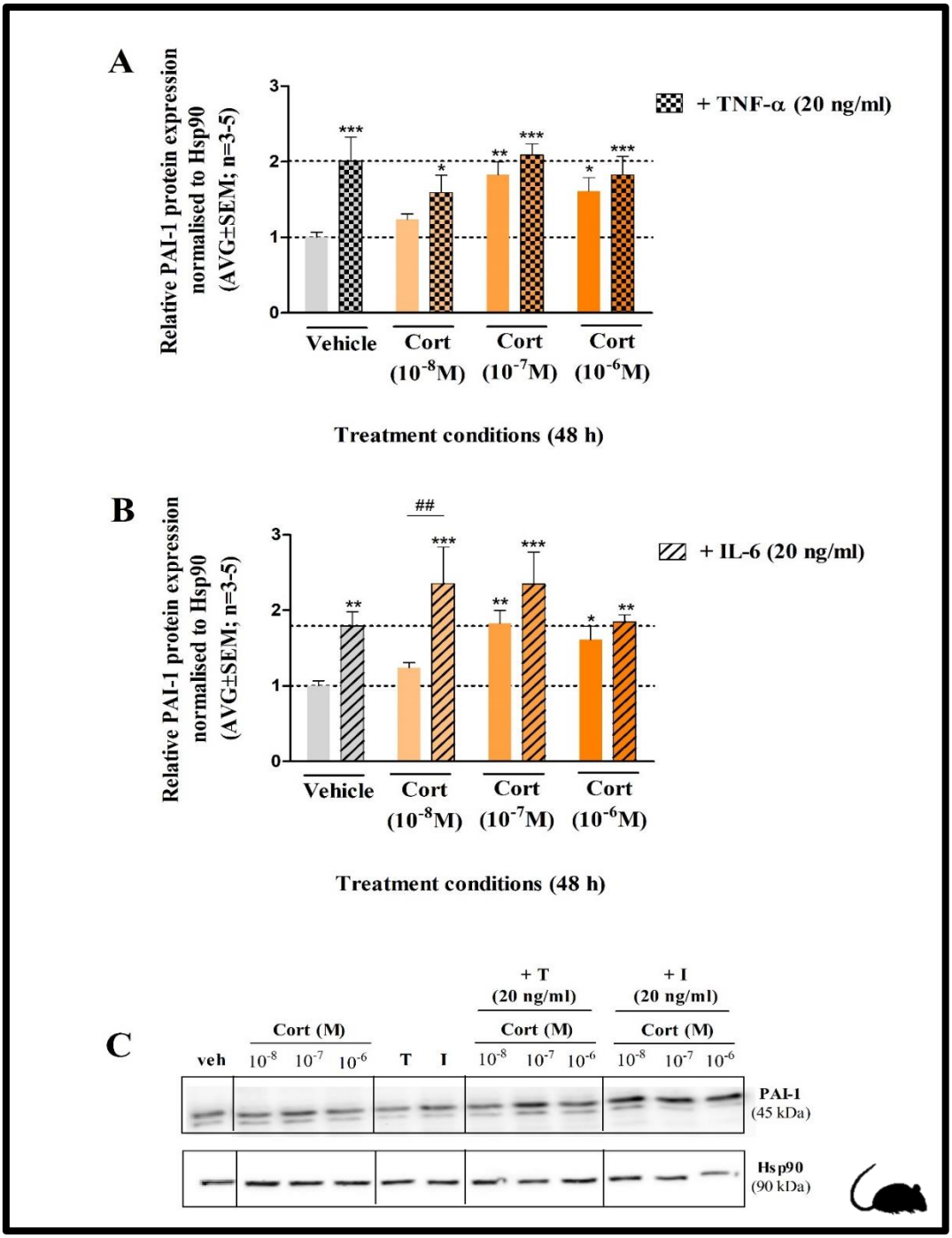


Figure 3.9. Intracellular PAI-1 protein levels in a mouse hepatoma cell line response to single- and co-treatment with Cort and either TNF-α or IL-6. Total protein was extracted from BWTG3 cells after 48 hours (h) treatment with various concentrations of (A) the endogenous GC, corticosterone (Cort), or 20 ng/ml of TNF-α (T) or IL-6 (I), in addition to the GC co-treated with either (B) TNF-α or (C) IL-6. A representative blot is shown in (D). Densities of the western blot bands were measured by ImageJ software and illustrated as bar graphs. The relative PAI-1 protein expression was normalised to the loading control (Hsp90) and compared with the vehicle control, which was set to 1. Results displayed are representative of three to five independent biological repeats (± SEM). One-way ANOVA with Dunnett's Multiple Comparison's post-test was used when comparing each treatment to the vehicle control or each co-treatment to either pro-inflammatory cytokine alone treatment. Two-way ANOVA with Bonferroni post-test was used to compare each co-treatment to the respective concentration of Cort on its own. When comparing each treatment to the vehicle, an asterisk symbol (*) was used. When comparing each co-treatment with its respective Cort- or cytokine only treatment, a hashtag- (#) and ampersand symbol (&) were used, respectively (*: $p<0.05$, **/##: $p<0.01$, ***: $p<0.001$).

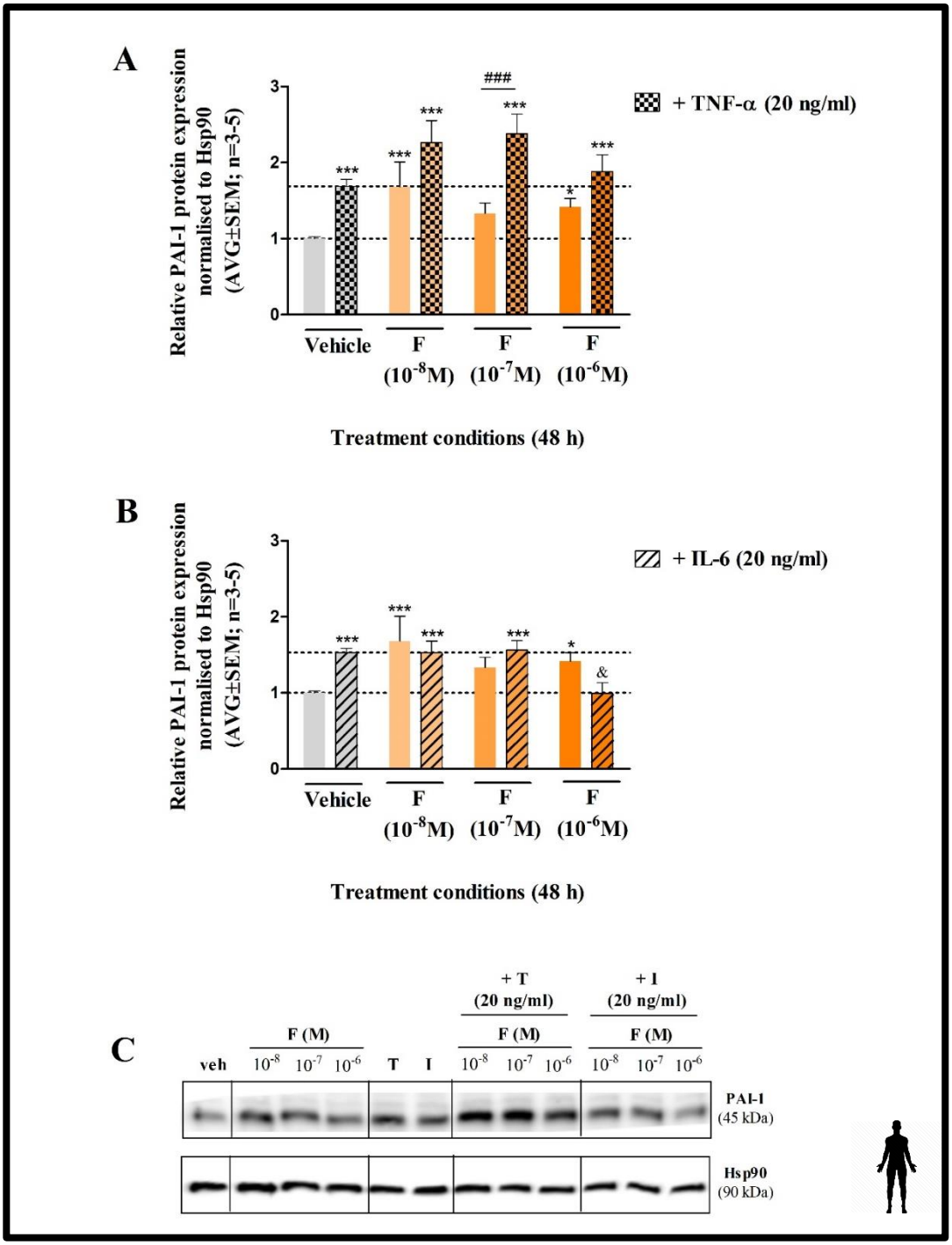


Figure 3.10. Intracellular PAI-1 protein levels in a human hepatoma cell line response to single- and co-treatment with F and either TNF-α or IL-6. Total protein was extracted from HepG2 cells after 48 hours (h) treatment with various concentrations of (A) the endogenous GC, cortisol (F), or 20 ng/ml of TNF-α (T) or IL-6 (I), in addition to the GC co-treated with either (B) TNF-α or (C) IL-6. A representative blot is shown in (D). Densities of the western blot bands were measured by ImageJ software and illustrated as bar graphs. The relative PAI-1 protein expression was normalised to the loading control (Hsp90) and compared with the vehicle control, which was set to 1. Results displayed are representative of three to five independent biological repeats (± SEM). One-way ANOVA with Dunnett's Multiple Comparison's post-test was used when comparing each treatment to the vehicle control or each co-treatment to either pro-inflammatory cytokine alone treatment. Two-way ANOVA with Bonferroni post-test was used to compare each co-treatment to the respective concentration of F on its own. When comparing each treatment to the vehicle, an asterisk symbol (*) was used. When comparing each co-treatment with its respective F- or cytokine only treatment, a hashtag- (#) and ampersand symbol (&) were used, respectively (*/&: $p<0.05$, ***/###: $p<0.001$).

3.3.2. CRP protein expression in response to GCs in the absence or presence of pro-inflammatory cytokines

CRP protein expression had a similar response to Dex in the absence and presence of the pro-inflammatory cytokines in the murine and human hepatoma cell lines **(Fig. 3.11 *vs* Fig. 3.12)**. Firstly, in the BWTG3 cells, Dex at all concentrations tested significantly ($p<0.05$) induced an increase in CRP protein expression compared to the vehicle control **(Fig. 3.11)**. The addition of either TNF-α **(Fig. 3.11A)** or IL-6 **(Fig. 3.11B)**, had no significant effect ($p>0.05$) on CRP protein expression when compared to either the GC at the relevant concentration or the appropriate pro-inflammatory cytokine.

Similar to the results obtained in the BWTG3 cells, in the HepG2 cells, Dex at all concentrations tested significantly ($p<0.01$) increased CRP protein expression compared to the vehicle control **(Fig. 3.12)**. Like observed in the BWTG3 cells, CRP protein expression remained significantly ($p<0.01$) elevated when HepG2 cells were co-treated with Dex in the presence of either pro-inflammatory cytokine. Overall, the addition of either TNF-α **(Fig. 3.12A)** or IL-6 **(Fig. 3.12B)**, had no significant effect ($p>0.05$) on CRP protein expression when compared to either the GC at the relevant concentration or the appropriate pro-inflammatory cytokine. The exception to this was 10^{-7} M Dex co-treated with IL-6, which potentiated ($p<0.05$) both Dex- and IL-6-induced CRP protein expression, more than what was observed for either test compound alone **(Fig. 3.12B)**.

Similar to Dex treatment in the BWTG3 cells, Cort significantly ($p<0.01$) increased CRP protein expression compared to the vehicle control **(Fig. 3.11 *vs* Fig. 3.13)**. In regard to the combinatorial treatments, CRP protein levels remained significantly ($p<0.05$) elevated although not significantly ($p>0.05$) more than what was observed for either test compound alone **(Fig. 3.13)**. Similar to Dex treatment in the HepG2 cells, F significantly ($p<0.01$) increased CRP protein expression relative to the vehicle control **(Fig. 3.12 *vs* Fig. 3.14)**. In regard to the combinatorial treatments, CRP protein levels appear to remain elevated although not significantly ($p>0.05$) more than what was observed for either test compound alone **(Fig. 3.14)**. However, co-treatment with the lowest concentration of F used (10^{-8} M) and either TNF-α **(Fig. 3.14A)** or IL-6 **(Fig. 3.14B)** did appear to slightly decrease CRP protein levels, which is not statistically different to basal levels ($p>0.05$). This suggests that at a concentration of 10^{-8} M, F and either cytokine appear to antagonise each other.

Thus, to summarise, both the murine and human liver cells were equally responsive to single treatment with GCs or either pro-inflammatory cytokine to increase CRP protein expression. In addition, when Dex **(Figs 3.11 & 3.12)** or Cort **(Fig. 3.13)** were in the presence of either pro-inflammatory cytokine, CRP protein levels remained significantly ($p<0.05$) elevated, but not more than what was observed for each test compound on its own. This occurred with the exception of 10^{-7} M Dex co-treated with IL-6, which did in fact exhibit cooperativity, as CRP protein levels were more pronounced compared to treatment with either test compound alone **(Fig. 3.12B)**. In contrast to the above-mentioned results, the lowest concentration of F used combined with either TNF-α **(Fig. 3.14A)** or IL-6 **(Fig. 3.14B)** appear to repress CRP protein expression back to basal expression. However, at higher concentrations of the GC this apparent repression appears to be lifted **(Fig. 14)**.

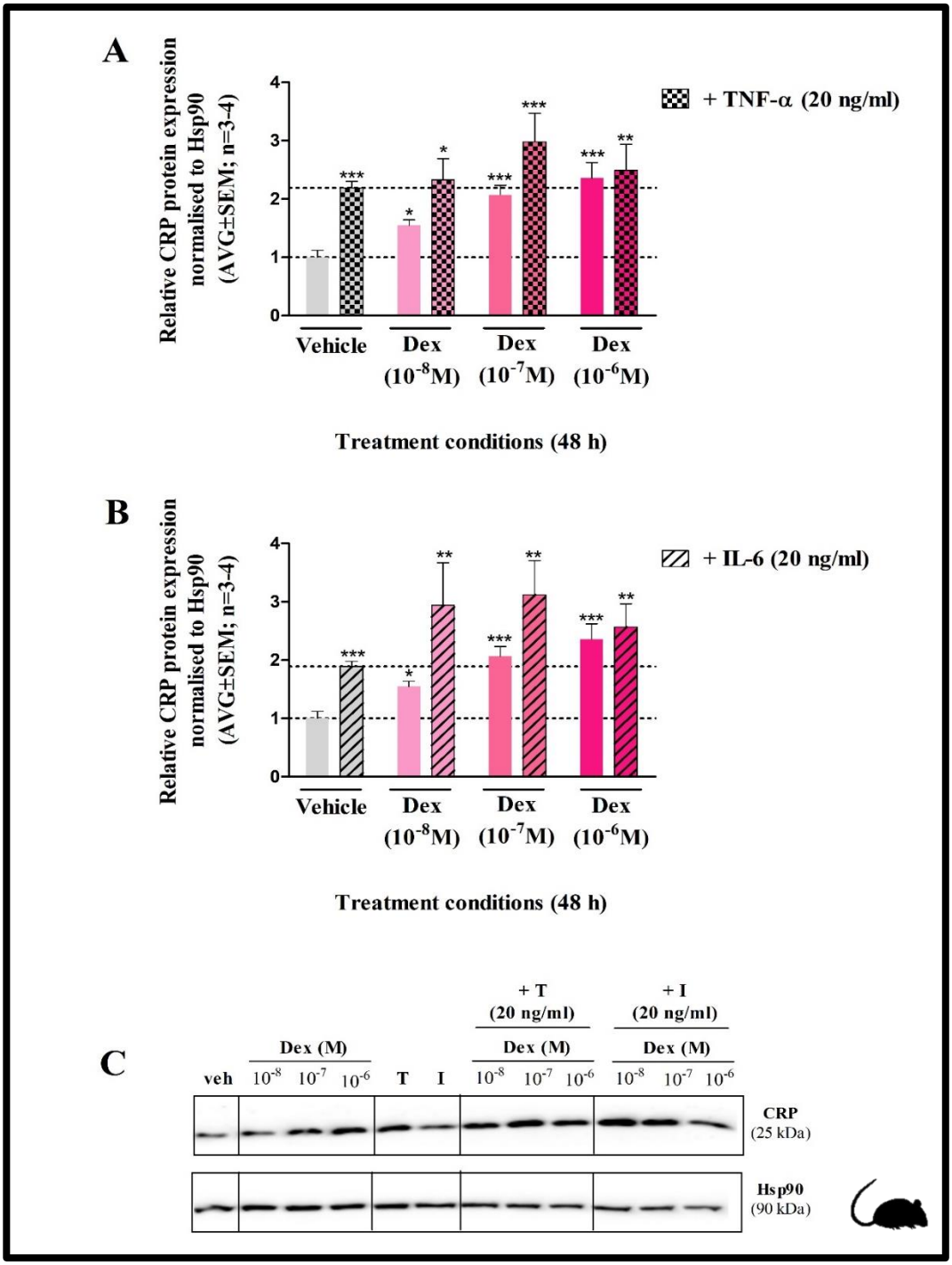


Figure 3.11. Intracellular CRP protein levels in a mouse hepatoma cell line response to single- and co-treatment with Dex and either TNF-α or IL-6. Total protein was extracted from BWTG3 cells after 48 hours (h) treatment with various concentrations of (A) the synthetic GC, dexamethasone (Dex), or 20 ng/ml of TNF-α (T) or IL-6 (I), in addition to the GC co-treated with either (B) TNF-α or (C) IL-6. A representative blot is shown in (D). Densities of the western blot bands were measured by ImageJ software and illustrated as bar graphs. The relative CRP protein expression was normalised to the loading control (Hsp90) and compared with the vehicle control, which was set to 1. Results displayed are representative of three to four independent biological repeats (± SEM). One-way ANOVA with Dunnett's Multiple Comparison's post-test was used when comparing each treatment to the vehicle control or each co-treatment to either pro-inflammatory cytokine alone treatment. Two-way ANOVA with Bonferroni post-test was used to compare each co-treatment to the respective concentration of Dex on its own. When comparing each treatment to the vehicle, an asterisk symbol (*) was used. When comparing each co-treatment with its respective Dex- or cytokine only treatment, a hashtag- (#) and ampersand symbol (&) were used, respectively (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

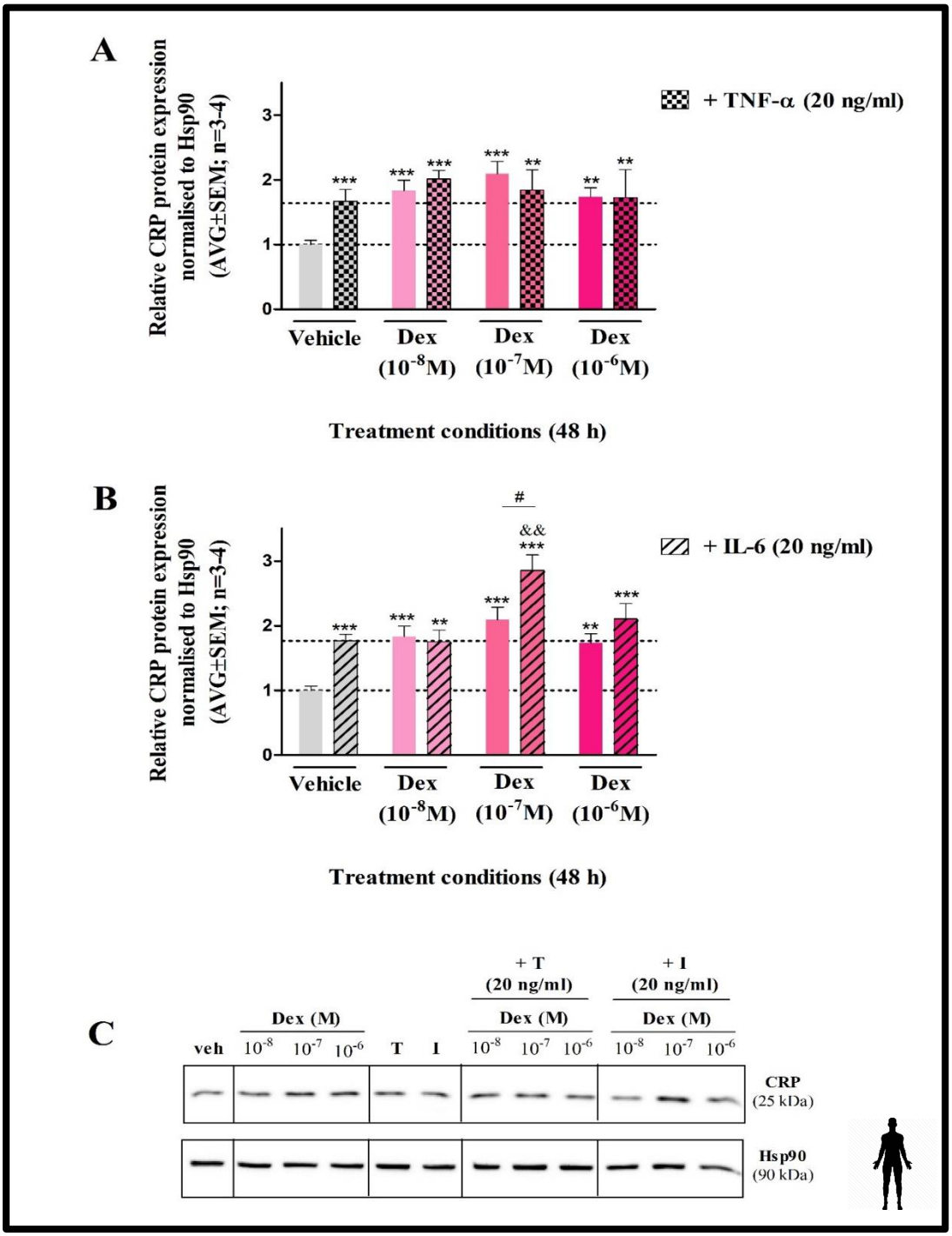


Figure 3.12. Intracellular CRP protein levels in a human hepatoma cell line response to single- and co-treatment with Dex and either TNF-α or IL-6. Total protein was extracted from HepG2 cells after 48 hours (h) treatment with various concentrations of (A) the synthetic GC, dexamethasone (Dex), or 20 ng/ml of TNF-α (T) or IL-6 (I), in addition to the GC co-treated with either (B) TNF-α or (C) IL-6. A representative blot is shown in (D). Densities of the western blot bands were measured by ImageJ software and illustrated as bar graphs. The relative CRP protein expression was normalised to the loading control (Hsp90) and compared with the vehicle control, which was set to 1. Results displayed are representative of three to four independent biological repeats (± SEM). One-way ANOVA with Dunnett's Multiple Comparison's post-test was used when comparing each treatment to the vehicle control or each co-treatment to either pro-inflammatory cytokine alone treatment. Two-way ANOVA with Bonferroni post-test was used to compare each co-treatment to the respective concentration of Dex on its own. When comparing each treatment to the vehicle, an asterisk symbol (*) was used. When comparing each co-treatment with its respective Dex- or cytokine only treatment, a hashtag- (#) and ampersand symbol (&) were used, respectively (#: $p<0.05$, **/&&: $p<0.01$, ***: $p<0.001$).

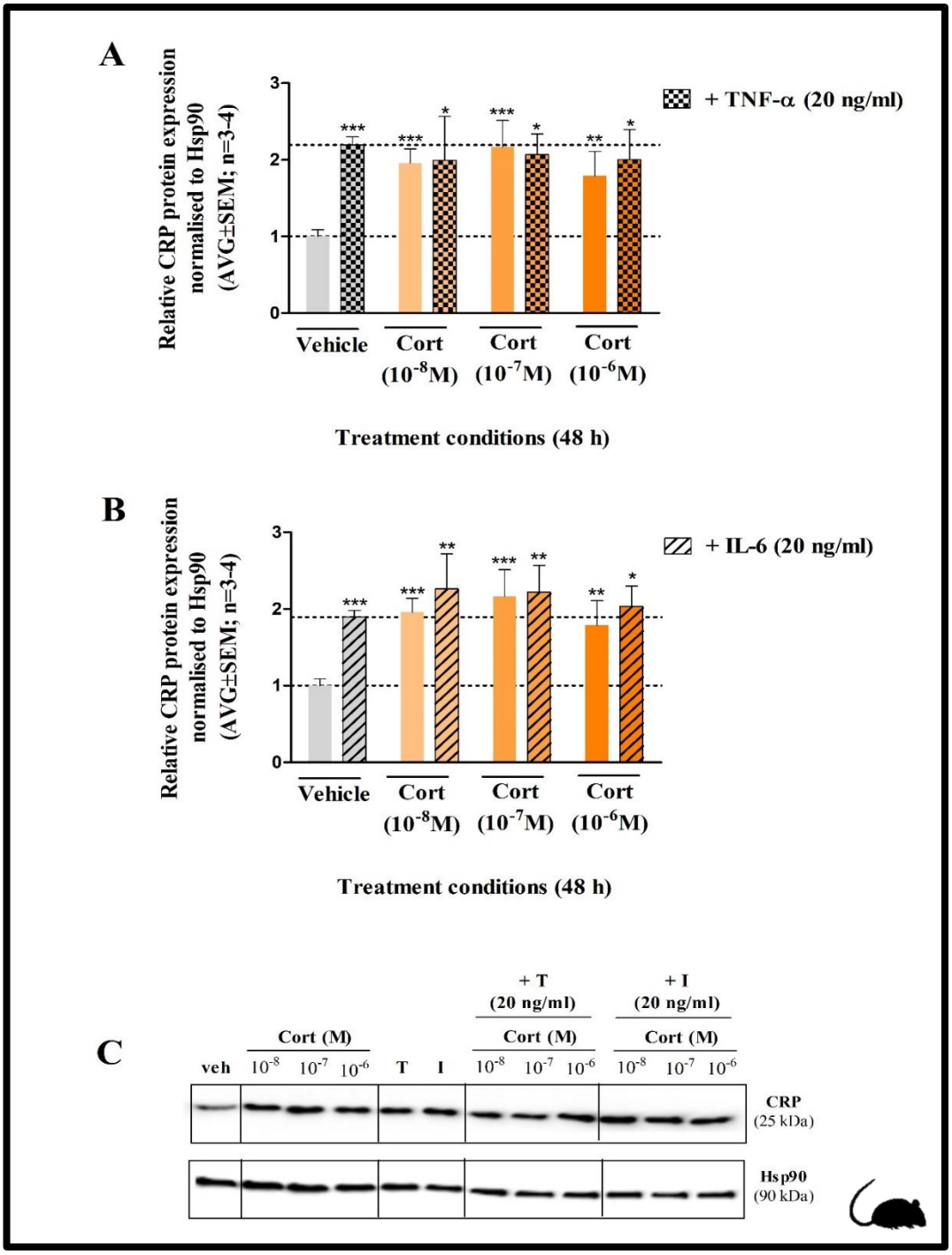


Figure 3.13. Intracellular CRP protein levels in a mouse hepatoma cell line response to single- and co-treatment with Cort and either TNF-α or IL-6. Total protein was extracted from BWTG3 cells after 48 hours (h) treatment with various concentrations of (A) the endogenous GC, corticosterone (Cort), or 20 ng/ml of TNF-α (T) or IL-6 (I), in addition to the GC co-treated with either (B) TNF-α or (C) IL-6. A representative blot is shown in (D). Densities of the western blot bands were measured by ImageJ software and illustrated as bar graphs. The relative CRP protein expression was normalised to the loading control (Hsp90) and compared with the vehicle control, which was set to 1. Results displayed are representative of three to four independent biological repeats (± SEM). One-way ANOVA with Dunnett's Multiple Comparison's post-test was used when comparing each treatment to the vehicle control or each co-treatment to either pro-inflammatory cytokine alone treatment. Two-way ANOVA with Bonferroni post-test was used to compare each co-treatment to the respective concentration of Cort on its own. When comparing each treatment to the vehicle, an asterisk symbol (*) was used. When comparing each co-treatment with its respective Cort- or cytokine only treatment, a hashtag- (#) and ampersand symbol (&) were used, respectively (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

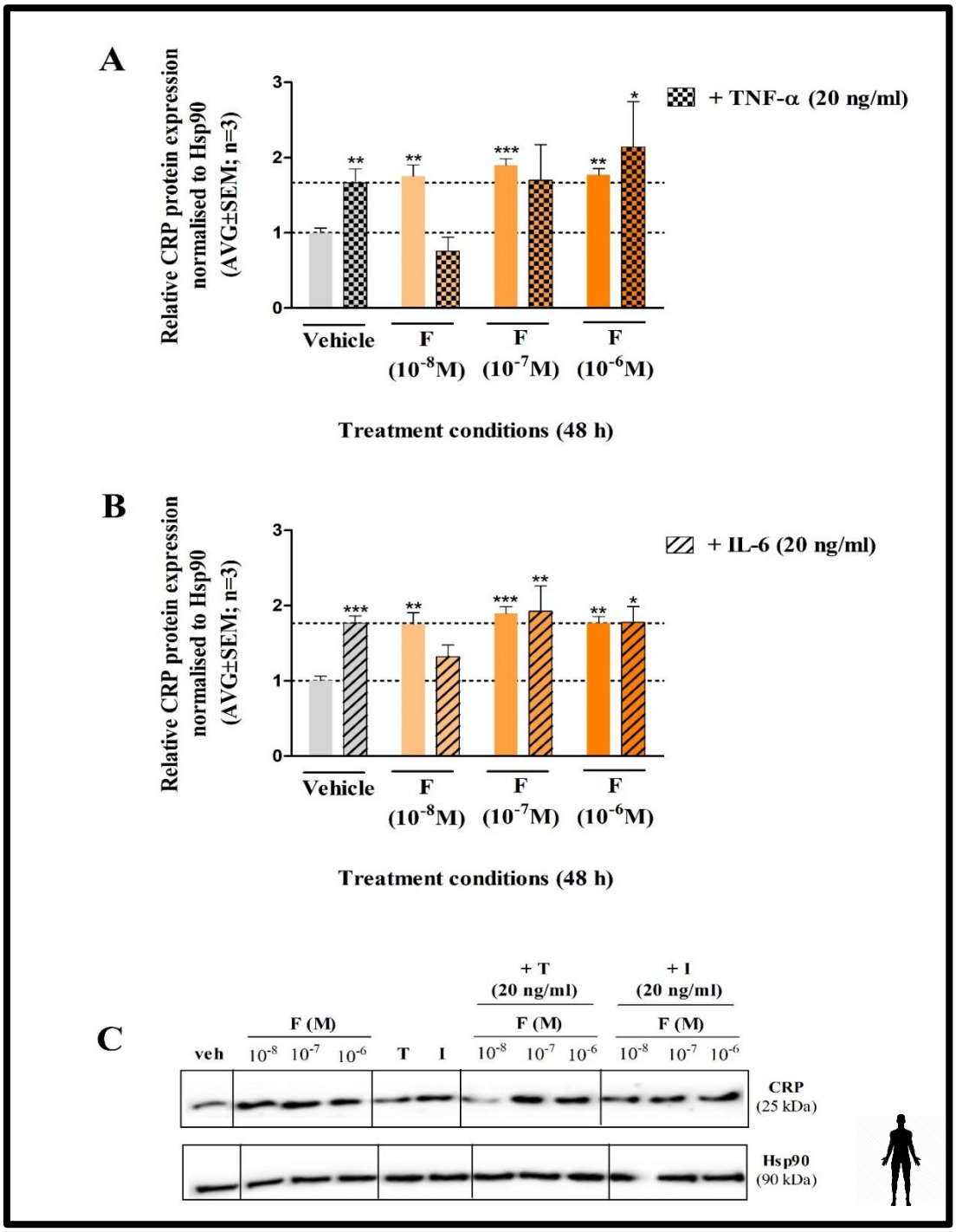


Figure 3.14. Intracellular CRP protein levels in a human hepatoma cell line response to single- and co-treatment with F and either TNF-α or IL-6. Total protein was extracted from HepG2 cells after 48 hours (h) treatment with various concentrations of (A) the endogenous GC, cortisol (F), or 20 ng/ml of TNF-α (T) or IL-6 (I), in addition to the GC co-treated with either (B) TNF-α or (C) IL-6. A representative blot is shown in (D). Densities of the western blot bands were measured by ImageJ software and illustrated as bar graphs. The relative CRP protein expression was normalised to the loading control (Hsp90) and compared with the vehicle control, which was set to 1. Results displayed are representative of three independent biological repeats (± SEM). One-way ANOVA with Dunnett's Multiple Comparison's post-test was used when comparing each treatment to the vehicle control or each co-treatment to either pro-inflammatory cytokine alone treatment. Two-way ANOVA with Bonferroni post-test was used to compare each co-treatment to the respective concentration of F on its own. When comparing each treatment to the vehicle, an asterisk symbol (*) was used. When comparing each co-treatment with its respective F- or cytokine only treatment, a hashtag- (#) and ampersand symbol (&) were used, respectively (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

3.4 The effects of GCs and/or pro-inflammatory cytokines on PAI-1- and CRP promotor activity

Next, to better understand at the molecular level how GCs and the pro-inflammatory cytokines, TNF-α and IL-6, affect PAI-1 and CRP levels, the promoter activities of each of the APP was measured. BWTG3 cells were transiently transfected with plasmid DNA containing the endogenous human PAI-1 or CRP minimal promoter, each linked to a luciferase (Luc)-reporter. As a result, the Luc gene was placed under control of either the PAI-1 or CRP minimal promotors. Promotor activity, expressed as relative light units (RLU), was quantitively measured based on the amount of luciferase protein translated (light emitted), as described in detail in Chapter 2 (Materials and Methods). Results were normalized to total protein in each well, determined by the BCA protein determination assay. To ensure the successful transfection of cells with either PAI-1 Luc or WT CRP -300 plasmids, wells containing untransfected cells were included as a negative control. Little to no light was emitted from these wells, which confirmed successful transfection. Furthermore, the experimental design for this investigation, mimicked the treatment regime used for mRNA experiments, which allowed for direct comparisons between what was observed at mRNA level *versus* promotor level. Due to difficulty experienced transfecting the HepG2 cells with our plasmid constructs, promotor activity for both APPs were not investigated in the HepG2 cell line.

3.4.1. PAI-1 promotor activity is increased in response to Dex in the absence or presence of either TNF-α or IL-6

PAI-1 promoter activity was significantly ($p<0.05$) increased by Dex at all the concentrations tested by ~2-fold, relative to the vehicle control **(Fig. 3.15)**. Similarly, both pro-inflammatory cytokines, TNF-α **(Fig. 3.15A)** and IL-6 **(Fig. 3.15B)**, significantly ($p<0.001$) increased PAI-1 promoter activity, also by ~2-fold. Generally, the ability of Dex to induce PAI-1 promotor activity was not significantly ($p>0.05$) influenced by the presence of either TNF-α **(Fig. 3.15A)** or IL-6 **(Fig. 3.15B)**. Additionally, a slight but not significant decrease ($p>0.05$) in PAI-1 promoter activity was observed in response to 10^{-6} M Dex co-treated with TNF-α **(Fig 3.15A)** and 10^{-8} M Dex co-treated with IL-6 **(Fig 3.15B)**.

3.4.2. Dex in the absence and presence of either TNF-α or IL-6 induces CRP promotor activity

Similar to what was observed with the PAI-1 promoter activity, all concentrations of Dex tested was able to significantly ($p<0.001$) increase CRP promoter activity, relative to the vehicle control **(Fig. 3.16)**. Like Dex, TNF-α **(Fig. 3.16A)** and IL-6 **(Fig. 3.16B)** each also significantly ($p<0.001$) increased CRP promoter activity. On the whole, Dex-mediated increase in CRP promoter activity was not significantly ($p>0.05$) affected by co-treating with either TNF-α **(Fig. 3.16A)** or IL-6 **(Fig. 3.16B)**. However, 10^{-8} M Dex co-treated with TNF-α potentiated both Dex- and TNF-α only induced CRP promoter activity ($p<0.001$) **(Fig. 3.16A)**, and 10^{-6} M Dex co-treated with IL-6 augmented both Dex- and IL-6 only induced CRP promoter activity ($p<0.01$) **(Fig. 3.16B)**.

Taken together, Dex as well as the pro-inflammatory cytokines, TNF-α and IL-6, increased PAI-1- and CRP promotor activity, which remained unaffected when Dex was co-treated with either of the pro-inflammatory cytokines **(Fig. 3.15 & 3.16)**. This was with the exception of 10^{-8} M Dex co-treated with TNF-α and 10^{-6} M Dex co-treated with IL-6, which further increased CRP promoter activity, more than what was observed with either test compound on its own.

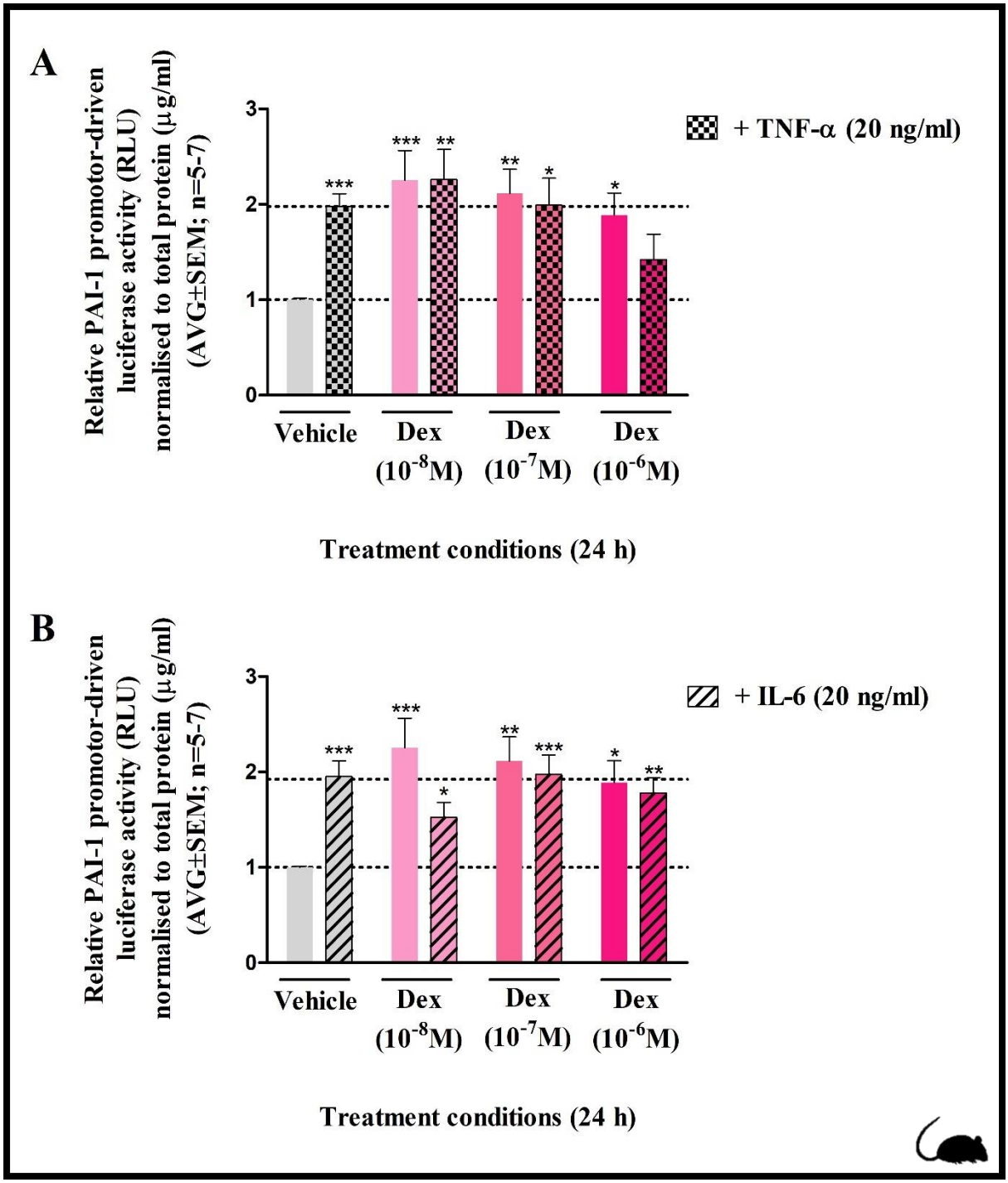


Figure 3.15. PAI-1 promotor activity in mouse hepatoma cells in response to co-treatment with Dex and either TNF-α or IL-6. BWTG3 cells were transiently transfected with PAI-1-Luc promoter-reporter construct. Cells were co-treated with various concentrations (10^{-8} M, 10^{-7} M, 10^{-6} M) of dexamethasone (Dex) in the absence and presence of 20 ng/ml of either (A) TNF-α or (B) IL-6 for 24 hours (h). Luciferase activity was measured in relative light units (RLU) and normalised to protein concentration. The response of each treatment was plotted relative to the vehicle, which was set to 1. Results displayed are the averages (± SEM) of five to seven independent biological repeats with each condition performed in triplicate (three technical repeats). One-way ANOVA with Dunnett's Multiple Comparison's post-test was used when comparing each co-treatment to either pro-inflammatory cytokine alone treatment. Two-way ANOVA with Bonferroni post-test was used to compare each co-treatment to the respective concentration of Dex on its own. When comparing each co-treatment with its respective Dex- or cytokine only treatment, a hashtag- (#) and ampersand symbol (&) were used, respectively (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

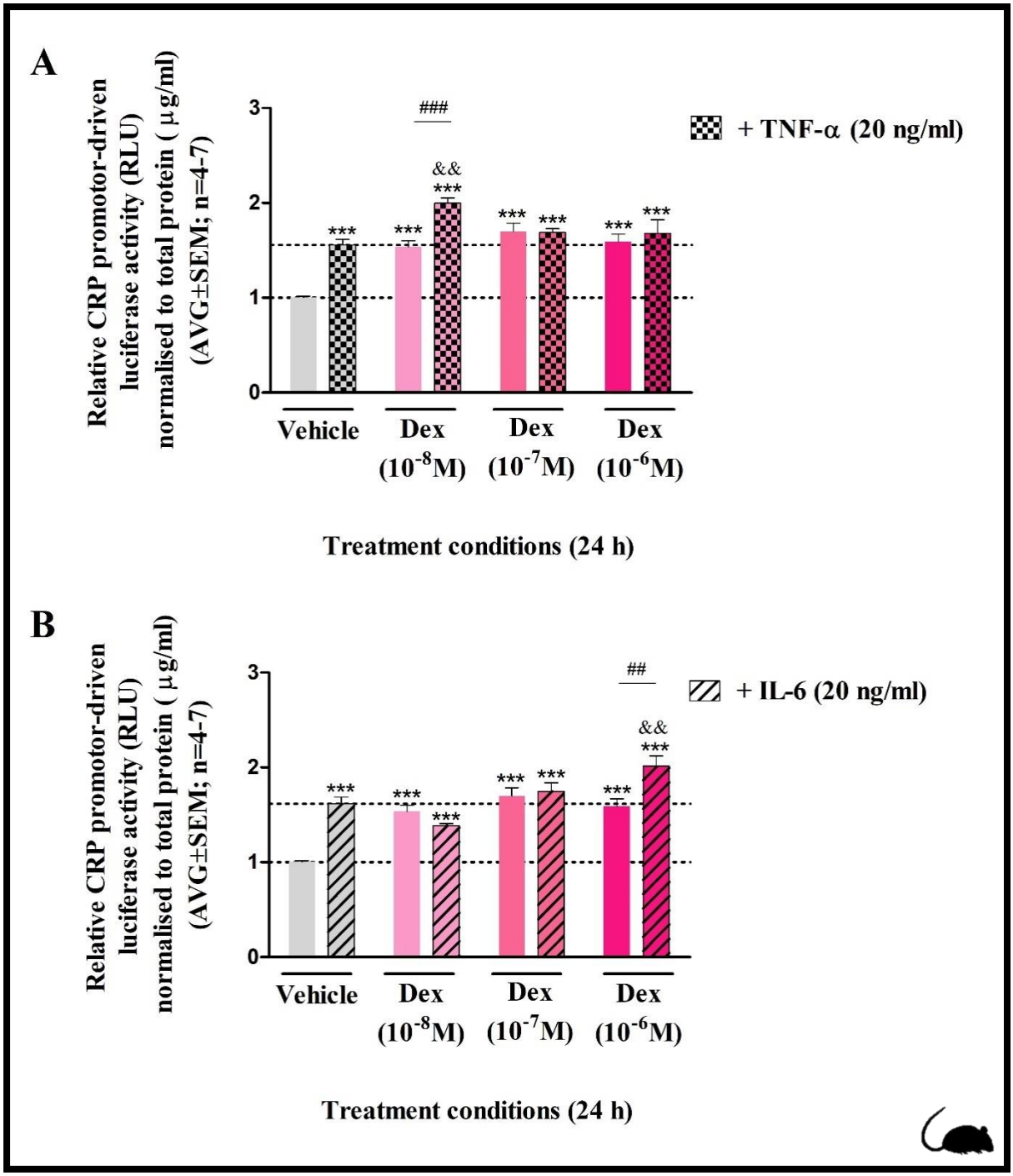


Figure 3.16. CRP promotor activity in mouse hepatoma cells in response to co-treatment with Dex and either TNF-α or IL-6. BWTG3 cells were transiently transfected with WT CRP -300 promoter-reporter construct. Cells were co-treated with various concentrations (10^{-8} M, 10^{-7} M, 10^{-6} M) of dexamethasone (Dex) in the absence and presence of 20 ng/ml of either (A) TNF-α or (B) IL-6 for 24 hours (h). Luciferase activity was measured in relative light units (RLU) and normalised to protein concentration. The response of each treatment was plotted relative to the vehicle, which was set to 1. Results displayed are the averages (± SEM) of four to seven independent biological repeats with each condition performed in triplicate (three technical repeats). One-way ANOVA with Dunnett's Multiple Comparison's post-test was used when comparing each co-treatment to either pro-inflammatory cytokine alone treatment. Two-way ANOVA with Bonferroni post-test was used to compare each co-treatment to the respective concentration of Dex on its own. When comparing each co-treatment with its respective Dex- or cytokine only treatment, a hashtag- (#) and ampersand symbol (&) were used, respectively (##/&&: $p<0.01$, ***/###: $p<0.001$).

CHAPTER FOUR: DISCUSSION, CONCLUSION AND FUTURE DIRECTIONS

4.1 Introduction

T2D, the most common form of DM, is an escalating public health crisis not only in South Africa but worldwide. Although it is well established that insulin resistance is the main risk factor for T2D, it remains unsolved what the principal molecular defects are which lead to this pathogenesis. A common theme throughout literature portrays inflammation (represented by pro-inflammatory cytokines) and stress (represented by GCs) as key role players in the development of insulin resistance [21][98]. Generally, pro-inflammatory cytokines, such as TNF-α and IL-6, are understood to play a causative role in the development of insulin resistance, by interfering with insulin signalling **(refer to Chapter 1, sections 1.2.1.2 & 1.2.2.2)** Similarly, GCs have also been demonstrated to directly interfere with insulin signalling *in vitro* and *in vivo* [103][214]–[216]. Furthermore, it has been reported that an elevated APR occurs during insulin resistance [119].

APPs, such as PAI-1, CRP, and SAA, are upregulated during the diabetic state, hence why they are commonly used as biological markers for T2D [21]. The APR is regulated in a highly complex manner by many factors of which pro-inflammatory cytokines and the endogenous GCs are most important. Although short term-activated APR is understood to serve a homeostatic function, the systemic response of the APR can be prolonged by persistent stimulation or disruption of normal control mechanisms, converting to a recurrent chronic state of inflammation [113]. Traditional knowledge would suggest that under normal circumstances, GCs function as negative feedback system of the APR by downregulating pro-inflammatory cytokine production, thereby bringing the inflammatory response to a halt. Interestingly, several studies have also shown the synthetic GC, Dex, to enhance pro-inflammatory cytokine-induced APP expression, thereby reinforcing the innate immune system and APR [149][180][193][195][199][200].

In the current study we extended on these earlier observations, by investigating the possible co-regulation of the APPs, PAI-1, and CRP, by GCs and pro-inflammatory cytokines, both at the mRNA and protein level. Whilst cooperative regulation of SAA by GCs and various pro-inflammatory cytokines have been extensively studied [27], fewer have studied this phenomenon in regards to PAI-1 and CRP. In this study, the effect the presence of either pro-inflammatory cytokine had on GC-mediated APP regulation and *vice versa*, were analysed. It

is important to examine regulation from both perspectives, as it has become abundantly clear that both the stress and immune system have bidirectional effects on each other [26].

Therefore, based on the literature, it was hypothesized that GCs and pro-inflammatory cytokines would cooperatively regulate PAI-1 and CRP expression, at both mRNA and protein level. To test this hypothesis, the effect of GCs (synthetic and endogenous) in the presence of either pro-inflammatory cytokine (TNF-α or IL-6) on the expression of APPs (PAI-1 and CRP) in hepatoma cells (BWTG3 and HepG2) was investigated. Liver cells were used as it is a major source of APPs, such as PAI-1 and CRP. Both cell lines proved to be generally responsive to single treatment with either of the selected signalling molecules, and therefore allowed us to examine how a combination thereof would affect the expression levels of the APPs investigated.

In this chapter the findings of this thesis will be discussed in context with the literature. The chapter has been divided into two sections: Firstly, the effects of GCs and/or pro-inflammatory cytokines on PAI-1- and CRP expression at both transcriptional (mRNA) and translational (intracellular protein) level will be discussed, in sub-sections 4.2.1 and 4.2.2, respectively. Subsequently, to elucidate the molecular mechanism underlying the regulation of PAI-1 and CRP expression by GCs and/or pro-inflammatory cytokines, the affect at the promoter level is reviewed in sub-sections 4.3.1 and 4.3.2, respectively. Finally, to conclude the findings of this MSc thesis, the latter, will be placed in a broader context with the literature, followed by future prospectives.

4.2 Effects of glucocorticoids and pro-inflammatory mediators on APPs expression

4.2.1. PAI-1 regulation by stress and inflammatory mediators

4.2.1.1. PAI-1 regulation in response to individual treatments

PAI-1 protein levels were increased by both GCs and pro-inflammatory cytokines in the liver cell lines used except for Dex, which only increased PAI-1 protein expression in the BWTG3 cells. The increase in PAI-1 protein levels was not necessarily reflected at mRNA level **(Addendum G: Table G1)** in the BWTG3 cells, with neither Dex nor TNF-α influencing PAI-1 mRNA expression.

Conflicting results exist regarding Dex-mediated regulation of PAI-1 mRNA expression in the literature. Some studies have reported Dex to have no effect on mRNA expression [156][159] in liver cells, whilst others have observed Dex-induced increase in PAI-1 mRNA expression in various cell types including those derived from the liver [149][163][165]–[167][169]. Thus, the lack of Dex-mediated PAI-1 mRNA regulation does somewhat agree with the literature, although an increase in PAI-1 protein expression was observed, suggesting that the lack of mRNA regulation observed in response to Dex could be attributed to length of treatment. From the protein time course data **(Fig. 3.3A)**, a significant increase in PAI-1 protein levels were already observed at 24 hours Dex exposure, suggesting that Dex-induced PAI-1 mRNA expression could possibly have been observed if treated with Dex for less than 24 hours, as mRNA precedes protein synthesis.

Several studies have shown enhanced intra- and extracellular PAI-1 protein expression in response to Dex in various cell types, including rat hepatoma cells [166], human and mouse adipocytes [164][165], human adipose tissue [163], human fibrosarcoma cells [167][169], human trophoblast cells [171], human epithelial cells [163], and human monocytes [170]. To the best of our knowledge, no studies have investigated the effect of Dex or the endogenous GCs, corticosterone and cortisol, on PAI-1 protein expression in human or murine liver cells. Therefore, the current study is the first to report GC-induced increase in intracellular PAI-1 protein expression in the murine and human hepatoma cell lines. Dex-mediated PAI-1 protein expression was not observed in the HepG2 cells, whereas already at 24 hours significant increase in PAI-1 protein expression was observed in the BWTG3 cells. In contrast, an increase in PAI-1 protein expression in response to both corticosterone and cortisol was observed at 48 hours treatment in BWTG3 and HepG2 cells, respectively. This would suggest that the rate of PAI-1 protein synthesis in response to the GCs differ, with the potent GR agonist Dex possibly inducing PAI-1 synthesis at a faster rate in murine liver cells and might explain why no PAI-1 protein expression was observed in the HepG2 cells in response to Dex. However definitive conclusions regarding the rate of PAI-1 protein synthesis comparing Dex *versus* the endogenous GCs cannot be made without determining the $t_{1/2}$ for PAI-1 protein synthesis, which should include treatment times earlier than 24 hours. In addition, the differences in PAI-1 protein expression in response to the GCs in HepG2 cells could also be compounded by the rate of PAI-1 export, which might differ between the two cell lines. Unfortunately, there are no data available on the rate of PAI-1 export in either the BWTG3 or HepG2 cells to confirm this posit. Nonetheless, in support of this theory, Andreassen *et al*. using human

fibrosarcoma cells reported that Dex (1 µM) increased intracellular PAI-1 protein levels within 4 hours, which levelled off after 20 hours, whilst extracellular PAI-1 levels were significantly increased after 16 hours and continued to increase during the 48-hour incubation. Therefore, it would have been interesting to have also measured the extracellular PAI-1 protein levels in the current study and is something that should be considered in future studies **(refer to section 5.5)**.

Similar to Dex, TNF-α had no effect on PAI-1 mRNA as previously mentioned, which is in stark contrast to several lines of evidence from the literature, which found concentrations of TNF-α ranging between 5 to 50 ng/ml, to be potent enough to induce PAI-1 mRNA expression [141][144]–[146][148]–[150][153][154][165]. However, these studies were not performed in liver cells, and exposure to TNF-α was much shorter compared to the current study. To the best of our knowledge, only Seki & Gelehrter used liver cells in their study [156]. They found that 1 hour exposure to TNF-α (50 ng/ml) increased PAI-1 mRNA by 4-fold, returning to basal levels after 4 hours in murine primary hepatocytes. This could explain the lack of TNF-α-induced PAI-1 mRNA expression in the current study as both the concentration of TNF-α used as well as exposure time differs to that of Seki & Gelehrter. In addition, although primary hepatocytes and hepatoma cells are both of hepatic origin, they appear to show considerable differences in growth behaviour and expression of acute phase genes [217].

Whilst, 24 hours exposure to TNF-α had no effect on PAI-1 mRNA expression in the BWTG3 cells, increase in protein expression was observed in both the BWTG3- and HepG2 cells in response to 48 hours TNF-α treatment **(Addendum G: Table G1)**. Others using a higher concentration of TNF-α (50 ng/ml) have also shown it to be a potent inducer of PAI-1 protein synthesis in HepG2 cells [155] and human and rat hepatocytes [140], after 24 hours. Similarly, lower concentrations of TNF-α ranging from 5 to 10 ng/ml were reported to markedly induce PAI-1 protein accumulation in human and mouse adipocytes after 24 hours [141][153]. TNF-α-mediated increase in PAI-1 protein appears not to be cell type dependent, albeit dependent on the TNF-α concentration. Several research groups have shown PAI-1 protein synthesis to be induced by TNF-α in a number of different cell types, including human smooth muscle cells [150], human and bovine endothelial cells [143][145], and human epithelial cells [145].
While neither Dex nor TNF-α induced PAI-1 mRNA expression, BWTG3 cells treated with 20 ng/ml IL-6 for 24 hours resulted in an approximately 3.9-fold increase in PAI-1 mRNA expression. This finding is consistent with results from previous studies performed in human

hepatoma (HepG2) cells, murine, and human primary hepatocytes, and human adipocytes [159]–[161]. Dong *et al*. reported that IL-6 at a lower concentration of 1 ng/ml significantly increased PAI-1 mRNA expression by 2-fold in both HepG2 cells and mouse primary hepatocytes after 4 hours treatment [160]. Similarly, Healy & Gelehrter reported that IL-6 at a much higher concentration of 40 ng/ml had a significant effect on PAI-1 mRNA expression, increasing its levels by up to 2.5-fold, in HepG2 cells after 4 hours [159]. IL-6-induced PAI-1 mRNA expression is not limited to the liver, as Rega *et al*. reported that IL-6 at an even higher concentration of 100 ng/ml significantly increased PAI-1 mRNA expression by 10-fold, in human adipocytes after 24 hours [161].

Surprisingly, although IL-6-induced PAI-1 mRNA expression have been reported in HepG2 cells, other studies using the same cell line [155][159][162] as well as human and murine hepatocytes [139] have found IL-6 to have no detectable effect on extracellular PAI-1 protein synthesis after 24 hours. Our time course data support these findings, as IL-6 had no effect on intracellular PAI-1 protein expression in both murine and human hepatoma cell lines after 24 hours treatment **(Figs 3.3D & 3.4D)**. However, IL-6 was capable of increasing intracellular PAI-1 protein levels after 48 hours, suggesting that the rate of protein synthesis and export here too plays a role and highlights the importance of measuring both intra- and extracellular APP expression as well as at different time points.

4.2.1.2. PAI-1 regulation in response to combinatorial treatments

Although both Dex, at all concentrations tested, and TNF-α only treatment had no effect on PAI-1 mRNA expression, co-treatment of 10^{-8} M- and 10^{-6} M Dex with TNF-α led to a significant increase of PAI-1 mRNA expression in the BWTG3 cells **(Fig. 3.1A)**. Interestingly, while no studies exist, to the best of our knowledge, investigating possible co-regulation of PAI-1 mRNA expression by Dex and TNF-α in liver cells, studies performed in human endothelial [201] and epithelial cells [149], have reported their cooperative effect on PAI-1 mRNA expression, beyond that observed from either signalling molecule alone. These studies are in agreement with the results obtained in the current study in regard to combinatorial treatment with Dex and TNF-α.

This cooperative effect observed suggests cross talk between GC- and TNF-α signalling, which has been reported for other APP such as SerpinA3 [163]. Lannan *et al*. in fact demonstrated using ChIP analysis that binding of the ligand-activated GR at the SerpinA3 transcriptional

start site was more robust when cells were exposed to Dex (10^{-8} M) plus TNF-α [27]. The authors of this particular study also suggested that the GR is allowed to bind and mediate the effects of its ligand, Dex, because of TNF-α possibly promoting local unwinding of chromatin, although this still needs to be confirmed experimentally.

Interestingly, this cooperative effect of 10^{-8} M Dex and TNF-α was not reflected at protein level in the BWTG3 cells. The increase in PAI-1 protein levels in response to either Dex or TNF-α remained unaffected when the murine cells were co-treated with Dex and TNF-α, although slight but not significant ($p>0.05$) increases were observed when TNF-α was co-treated with 10^{-8}- and 10^{-7} M Dex. Similarly, TNF-α co-treated with corticosterone had little effect on the already increased PAI-1 protein expression of TNF-α- and corticosterone (10^{-7}- & 10^{-6} M) only treatment in the BWTG3 cells **(Fig. 3.9A)**. In addition, GCs co-treated with IL-6 also lead to a slight, but not significant ($p>0.05$) increase compared to either treatment alone, which could suggest some co-operativity between the GCs and IL-6 in the BWTG3 cells. However due to variability between independent biological repeats causing large error this needs to be confirmed for co-treatments of GCs with the pro-inflammatory cytokines.

Meanwhile, whilst the presence of Dex had no effect on either cytokine-induced PAI-1 protein levels in the murine liver cell line, a different regulatory pattern emerged in the human liver cell line. In the HepG2 cells, the presence of various concentrations of Dex significantly ($p<0.05$) antagonised both TNF-α- and IL-6-induced intracellular PAI-1 protein expression **(Fig. 3.8)**. This antagonism by Dex of either pro-inflammatory cytokine-induced PAI-1 protein expression was most noticeable with the highest concentration (10^{-6} M) of Dex used. These cell type specific effects could be attributed to different expression levels of key proteins in GC- and cytokine signalling pathways such as different levels of the GR, NF-κB, or STAT3 proteins and requires further investigation. Also, to confirm antagonism in the HepG2 cells, measuring extracellular PAI-1 protein levels should be considered in future studies as the decrease in cytokine-induced intracellular PAI-1 in response to Dex treatment could be attributed to a faster rate of export from the cells as mentioned earlier. This is however merely speculative especially since PAI-1 mRNA levels were not examined in the HepG2 cells.

Co-regulation of PAI 1 protein levels by Dex and TNF-α have been reported in cell types other than the liver. Kimura *et al*. using human renal epithelial cells showed that the presence of Dex

(10^{-7} M) significantly modulated TNF-α (10 ng/ml)-induced extracellular PAI 1 protein production by an additional 2.3-fold, after 24 hours [13]. Thus, emphasising the need to also investigate extracellular PAI 1 protein expression and to consider the rate of protein synthesis as well as export.

Unlike Dex, co-treatment of cortisol with the pro-inflammatory cytokines had little effect on either TNF-α or IL-6-induced PAI-1 protein expression in the HepG2 cells. However, 10^{-6} M cortisol did antagonise IL-6-mediated increase in PAI-1 expression. This different effect between cortisol and the synthetic GC could be attributed to the fact that Dex is a more potent GR agonist due to its higher binding affinity for the GR [218], suggested by cortisol only able to antagonise IL-6-induced PAI-1 protein expression at the highest concentration tested i.e., 10^{-6} M. It should also be noted that cortisol and corticosterone can bind to other receptors such as the mineralocorticoid receptor (MR) [83], which could also explain these differences observed between the synthetic- and endogenous GC.

Taken together, cooperative regulation of PAI-1 by the GCs and the pro-inflammatory cytokines was only really observed at the mRNA level and only with the lowest concentration of Dex tested in the presence of TNF-α. Very little cooperative effects by the GCs and the pro-inflammatory cytokines were observed at the protein level, mainly due to huge error between independent biological repeats. In fact, various concentrations of Dex were able to significantly ($p<0.05$) antagonise both TNF-α- and IL-6-induced intracellular PAI-1 protein expression in the human liver cell line, most notably with the highest concentration of Dex used. Nonetheless, co-treatments mainly resulted in little to no changes to the individual treatments on their own in the murine liver cell line. This emphasises again that GCs cannot always only be considered anti-inflammatory.

4.2.2. CRP regulation by stress and inflammatory mediators

4.2.2.1. CRP regulation in response to individual treatments

CRP protein levels were increased by both GCs and pro-inflammatory cytokines in the liver cell lines. The increase in CRP protein levels was not reflected at the mRNA level **(Addendum G: Table G1)**. in the BWTG3 cells, with none of the cell signalling mediators able to significantly influence CRP mRNA expression.

As previously mentioned, Dex at concentrations of 10^{-8}M to 10^{-6} M had no effect on CRP mRNA, which is in agreement with results from a previous study also performed in BWTG3 cells. Visser *et al*. reported that Dex at a concentration of 10^{-8} M had no effect on CRP mRNA expression after 24 hours [88]. The lack of mRNA regulation observed in response to Dex could be attributed to length of treatment. From the protein time course data **(Fig. 3.5A)**, an increase in CRP protein levels were already observed at 24- and 48 hours Dex exposure, suggesting that Dex-induced CRP mRNA expression could possibly have been observed if treated with Dex for less than 24 hours, as mRNA precedes protein synthesis. To the best of our knowledge, no studies have investigated the effect of the endogenous GCs, on CRP protein expression in human or murine liver cells. Therefore, this is the first study to report corticosterone- and cortisol-induced increase in intracellular CRP protein expression in murine and human hepatoma cell lines.

Although endogenous- and synthetic GCs produce different metabolites, both elicited a similar biological response in regards to intracellular CRP protein expression. Conflicting data exists regarding the effects of Dex on intra- and extracellular CRP protein expression, as both an increase [88][180] as well as no effect [181] in response to Dex have been reported. In agreement with the current study, Visser *et al*. reported that Dex, at a concentration of 10^{-8} M, significantly increased intra- and extracellular CRP protein expression in BWTG3 cells after 24 hours [88]. In addition, Ganapathi *et al*. reported that Dex (10^{-7} M) increased extracellular CRP protein synthesis by 2.2-fold in human hepatoma (Hep3B) cells after 24 hours [180]. The findings of both groups are consistent with the time course results from the current study, where an increase in intracellular CRP protein levels in response to Dex were observed in the BWTG3- **(Fig. 3.5A)** and HepG2 cells **(Fig. 3.6A)**. In contrast, Depraetere *et al*. found that Dex (10^{-6} M) had no effect on extracellular CRP protein secretion in HepG2 cells after 72 to 96 hours [181]. It is possible that they lost the effect of Dex on CRP protein expression due to stimulating the cells for too long a period, as in the current study and previous groups observed a response after 24- or 48 hours.

Similar to Dex, IL-6 had no effect on CRP mRNA expression as earlier mentioned, which is consistent with results from a previous study also performed in the BWTG3 liver cell line [88]. In contrast, studies performed in human hepatoma (PLC/PTF/5) cells [179] and human- and mouse primary hepatocytes [174][187], have shown treatments between 9- to 24 hours with IL-6 at concentrations ranging from 10- to 100 ng/ml to induce CRP mRNA expression to up

to 9-fold. Thus, the lack of IL-6-mediated CRP mRNA regulation observed in the current study could be attributed to length of treatment, and IL-6-induced CRP mRNA expression could possibly have been observed if treated with IL-6 for less than 24 hours.

Whilst, 24 hours exposure to IL-6 elicited no effect on CRP mRNA expression in the BWTG3 cells in the current study, an increase in protein expression was observed in both the BWTG3- and HepG2 cells in response to 48 hours IL-6 treatment **(Addendum G: Table G1)**. Others using a two times lower concentration of IL-6 (10 ng/ml) compared to the current study, have also observed an increase in both intra- and extracellular CRP protein expression in BWTG3 cells [181] and extracellular CRP protein secretion in Hep3B cells [177]. The latter study also found IL-6 at a lower concentration of 5 ng/ml to induce extracellular CRP protein synthesis in NPLC/PRF/5 cells [180]. Interestingly, IL-6 (10 ng/ml) have also been shown to increase extracellular CRP protein synthesis by 22.5-fold in human primary hepatocytes [174]. As previously mentioned, heterogeneity does exist between hepatic primary and established cell lines [217]. Thus, possibly explaining the considerable difference in fold-induction observed for CRP protein expression in the primary cell culture as opposed to that in carcinoma cells. Furthermore, studies have shown IL-6 to induce extracellular CRP protein production in other cell types, including human primary adipocytes [188] and human coronary smooth muscle cells [189].

TNF-α had no significant effect on CRP mRNA expression as earlier mentioned. However, due to variability between independent biological repeats causing large error, the exact effect 20 ng/ml TNF-α has on CRP mRNA expression in the BWTG3 cells remain inconclusive. To the best of our knowledge, only one study has investigated the effect of TNF-α on CRP mRNA expression. Calabro *et al.* found TNF-α (50 ng/ml) to significantly induce CRP mRNA expression by 2.5-fold, after 48 hours [189]. However, this study was performed in human coronary artery smooth muscle cells, and the authors used a higher concentration of TNF-α, and also exposed the cells to TNF-α for much longer compared to the current study.

Whilst, 24 hours exposure to TNF-α had no effect on CRP mRNA expression in the BWTG3 cells, a significant increase in protein expression was observed in both the BWTG3- and HepG2 cells in response to 48-hour TNF-α treatment **(Addendum G: Table G1)**. This finding is in disagreement with results from previous studies, where TNF-α concentrations ranging from 0.1 to 200 ng/ml, had no effect on extracellular CRP protein synthesis in human primary

hepatocytes [174] and Hep3B cells [180], after 24 hours. However, from the time course data **(Fig. 3.6C)**, a significant increase in intracellular CRP protein levels were already observed at 24 hours TNF-α exposure in the HepG2 cells. This would suggest that the rate at which intracellular CRP protein is exported from the human liver cell into the extracellular environment could be much slower, and might explain why the above-mentioned studies detected an insignificant amount of extracellular CRP protein in response to TNF-α after 24 hours. Unfortunately, there are no data available on the rate of CRP export in liver cells to confirm this argument.

4.2.2.2. CRP regulation in response to combinatorial treatments

Although both Dex, at all concentrations tested, and either pro-inflammatory cytokine only treatment had no effect on CRP mRNA expression, co-treatment of 10^{-8} M and 10^{-6} M Dex with TNF-α **(Fig. 3.2A)**, and co-treatment of 10^{-6} M Dex and IL-6 **(Fig. 3.2B)** led to a significant ($p<0.05$) increase of CRP mRNA expression in the BWTG3 cells after 24 hours. The increase in CRP mRNA does appear to be dependent on the concentration of Dex. To the best of our knowledge, only one group has investigated the possible co-regulation of CRP mRNA expression by GCs and pro-inflammatory cytokines [88]. Visser *et al.* found that Dex (10^{-8} M) and IL-6 (10 ng/ml) alone and combined had no effect on CRP mRNA expression in BWTG3 cells. Their findings do, however, somewhat differ from that found in the current study, as Dex (10^{-8} M) appeared to decrease CRP mRNA expression in the BWTG3 cells, and with the addition of IL-6 (20 ng/ml) this effect was significantly attenuated **(Fig. 3.2B)**. It is likely that these disparate findings are due to different treatment regimens used between the Visser *et al*. *versus* the current study. They sequentially treated cells with Dex for 24 hours followed by treatment with a two times lower concentration of IL-6 for 3 hours. Whereas, in the current study Dex and IL-6 were introduced to the cells concurrently. In a recent review by Desmet *et al.*, it was suggested that pro-inflammatory genes are synergistically increased when GCs and pro-inflammatory cytokines are administrated simultaneously [203], which is supported by our findings. Therefore, it would be interesting to examine how treatment with GCs prior to addition of the pro-inflammatory cytokine (known as GC priming), as applied by Visser *et al.*, would affect the regulation CRP.

In the current study, intracellular CRP protein levels remained significantly ($p<0.05$) increased after BWTG3- and HepG2 cells were co-treated with either pro-inflammatory cytokine and 10^{-8} - and 10^{-6} M of Dex or corticosterone, although generally not significantly ($p>0.05$) more

than what was observed with either GC or cytokine on its own **(Figs. 3.11-3.13)**. Interestingly, only cortisol at the lowest concentration used, deviated from this pattern of regulation, as in combination with either cytokine, CRP protein levels were no different to that of basal levels **(Fig. 3.14)**. To the best of our knowledge, only three research groups have investigated the possible co-regulation of CRP protein expression by GCs and pro-inflammatory cytokines, in liver cells (human primary hepatocytes [196], HepG2 cells [181] & BWTG3 cells [88]). Unfortunately, in all three studies only IL-6 was used as a representative pro-inflammatory cytokine, at a concentration of 10 ng/ml. Firstly, Depraetere *et al*. found that HepG2 cells co-treated with Dex (10^{-6} M) and IL-6 for 48 hours cooperatively increased extracellular CRP protein expression more than what was observed with either test compound alone [181]. This is analogous to the finding in the current study, where co-treatment with Dex, although at a lower concentration of 10^{-7} M, and IL-6 significantly induced intracellular CRP protein expression in the HepG2 cells, more than what was observed for either test compound alone **(Fig. 3.12B)**. Furthermore, Castell *et al*. found that human primary hepatocytes co-treated with Dex (10^{-7} M) and IL-6 for 20 hours, potentiated extracellular CRP protein expression more than what was observed with Dex, but not IL-6 alone [196]. Lastly, Visser *et al*. found that BWTG3 cells co-treated with Dex (10^{-8} M), and IL-6 increased extracellular CRP protein expression, although not more than what was observed with either test compound alone. In addition, they found that the presence of Dex was able to significantly decrease the IL-6-induced intracellular CRP protein expression back to basal expression [88]. This in stark contrast to what was observed in the current study, as it was found that in the same murine cell line, the presence of Dex (10^{-8} M) appeared to strengthen IL-6-induced intracellular CRP protein expression **(Fig. 3.11B)**. However, Visser *et al*. mentioned that optimisation for their study was only performed at the level of GR protein expression, unlike the current study, which optimised CRP protein expression in both liver cell lines in response to each test compound *via* performing a time course.

Taken together, similar to PAI-1, cooperative regulation of CRP by the GCs and the pro-inflammatory cytokines was only really observed at the mRNA level and only with the lowest concentration of Dex tested in the presence of TNF-α and the highest concentration of Dex tested in the presence of IL-6. Very little cooperative effects by the GCs and the pro-inflammatory cytokines were observed at the protein level, mainly due to huge error between independent biological repeats. Overall CRP protein levels remained significantly elevated in response to co-treatments, however there was little to no changes compared to individual

treatments on their own. This nonetheless emphasises again that GC-mediated effects are not simply anti-inflammatory and at least in regard to APPs, co-regulation between pro-inflammatory cytokines and GCs occurs, which might be related to the initial response to stress [203]. In addition, as observed for PAI-1, there appears to be a poor correlation between what was observed for all treatments (single and combined) on CRP mRNA *versus* protein level. This discrepancy could be attributed to (i) different treatment times (24- *versus* 48 hours) used in each experiment and, (ii) the involvement of other levels of regulation (post-transcriptional modifications) between the transcript and protein product.

4.3 Involvement of the proximal promoter in PAI-1 and CRP gene regulation

The regulation of PAI-1 and CRP by the GCs in the absence and presence of TNF-α and IL-6 shown at mRNA and protein level could be attributed to gene transcription activation. Studies have identified many *cis*-acting sequences and trans-activating transcription factors involved in the transcriptional induction of PAI-1 and CRP by pro-inflammatory cytokines, such as TNF-α and IL-6, or the synthetic GC, Dex. The proximal promoter region of both PAI-1 and CRP contain recognition sites for transcription factors involved in GC-, TNF-α- and IL-6 signalling as shown in **Fig. 4.1**. The current study shows that both Dex and the pro-inflammatory cytokines induce PAI-1- and CRP promoter activity, which remains generally unaffected with combinatorial treatments.

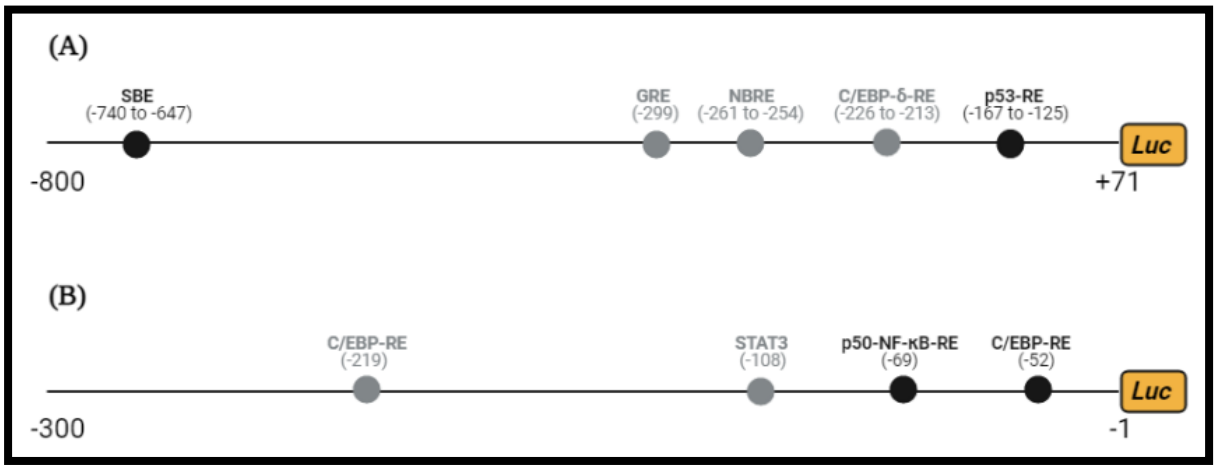


Figure 4.1. Schematic representation of the proximal promotor region for the (A) PAI-1 and (B) CRP gene used in the current study. The relative positions of transcription factor recognition sites identified in each promoter are shown. The known response elements (RE) contained in each promoter are indicated in either black (obtained from the research paper of the respective authors which kindly gifted each plasmid) or grey (obtained from the literature). Diagrams were created by the author of this study. *Note: GRE = glucocorticoid responsive element; p50-NF-κB-RE = p50 n*uclear factor kappa B responsive element*; C/EBP-RE = CCAAT-enhancer-binding protein response element; NBRE = NGFI-B response element; SBE = smad binding element; STAT3 = signal transducer and activator of transcription 3; p53-RE = p53 responsive element*

4.3.1. PAI-1 promotor regulation by stress and inflammatory mediators

There is good evidence that GC-induced PAI-1 expression is mediated by the GC-activated GR and its association with the GRE in the proximal promotor of the human PAI-1 gene [166]–[168][172][173]. Riccio *et al.* not only found Dex to induce PAI-1 mRNA expression in human fibrosarcoma cells, but also identified that the most conserved hexanucleotide (TGT(T/C)CT) of the GRE was present at -299 in the anti-sense orientation, in the regulatory region of the human PAI-1 gene [168]. In addition, Dex-induced PAI-1 promotor activity was shown by Van Zonneveld *et al.* to be mediated *via* the Dex-bound GR directly interacting with one-half of a presumable GRE with enhancer-like properties located within the region between nucleotides -305 and +75 of the human PAI-1 promoter, transfected into rat hepatoma (FTO-2B) cells [173]. The PAI-1-Luc construct used in the current study should encompass at least one GRE, as the construct includes this specific regulatory region explaining the Dex-induced PAI-1 promotor activity shown **(Fig. 3.15)**. The presence of GREs within the promoter region of the PAI-1 promoter also suggest the molecular mechanism of action whereby PAI-1 levels are increased in response to GCs.

TNF-α increased PAI-1 promotor activity in BWTG3 cells, by 2-fold **(Fig. 3.15A)**. However, the mechanism of action whereby TNF-α increases PAI-1 expression in liver cells remain unclear. However, some studies do provide a possible mechanism of action whereby TNF-α induces PAI-1 expression in endothelial cells. For example, Hou *et al*. identified a distal TNF-α responsive enhancer of the PAI-1 gene, which contained a NF-κB-binding site (5´-TGGAATTTCT-3´) at –14889/-14880, able to bind NF-κB (p50 and p65), as well as mediate the response to TNF-α [147]. However, according to de Martin *et al*. the human PAI-1 promotor lacks a consensus sequence for NF-κB [158]. More recently, Gruber *et al.* delineated the mechanism by which TNF-α induces PAI-1 mRNA expression in endothelial cells (HUVECs); they identified that the transcription factor, Nur77 to be part of TNF-α-induced PAI-1 expression [146]. In addition, they reported that Nur77 drives the PAI-1 transcription *via* direct binding to a NBRE. The human PAI-1 proximal promotor in fact contains a functional binding motif for Nur77 from -261 to -254 bp. Therefore, it is possible that this Nur77-NRBE complex contributes to the TNF-α-induced PAI-1 promotor activity observed in the current study, provided the presence of this transcription factor in the BWTG3 cells can be confirmed.

IL-6-mediated PAI-1 promoter activity was shown by Dong *et al*. to be mediated by a C/EBP-δ motif positioned between -226 to -213 bp in the human PAI-1 promotor [160]. They reported that IL-6 (1 ng/ml) increased PAI-1 promotor activity in HepG2 cells transfected with the human PAI-1 promoter (-829 to +36 bp), by 2.4-fold. Similarly, the current study observed a 2-fold induction in PAI-1 promotor activity in response to IL-6 **(Fig. 3.15B**), which could possibly also be mediated *via* C/EBP-δ.

Co-treatment of Dex with either pro-inflammatory cytokine did not influence the increase in PAI-1 promoter activity observed with either test compounds on their own. Overall, the results on a synthetic promotor-reporter differ from what was observed for the endogenous PAI-1 gene, which suggest that the molecular mechanism of action whereby GCs and the pro-inflammatory cytokines, TNF-α and IL-6, increase PAI-1 levels is at the promoter level. Whether only the proximal promotor is involved remains to be elucidated. It is possible that promotor-reporters may not reflect the regulation on full-length endogenous regulatory region of the gene as it would be in the native chromatin structure.

4.3.2. CRP promotor regulation by stress and inflammatory mediators

Although in the current study Dex at the various concentrations tested, increased CRP promotor activity **(Fig. 3.16)**, functional GREs have yet to be identified. Thereby the exact molecular mechanism whereby GCs induce CRP promoter activity remains to be elucidated. It is traditionally understood, that GCs exert their effects *via* binding to the GR, which following translocation to the nucleus, binds to GRE(s) in the promotors of GC-responsive genes. The lack of GR binding sites in the CRP promoter suggests GC-activated GR positively regulate the CRP levels *via* possible positive tethering to other transcription factors **(Chapter 1, Fig. 1.6)**. Positive gene regulation of ligand-activated GR *via* interacting with other transcription factors have been described [87][94]. It has been reported that the GR can tether to C/EBP [96][219], STAT3 [94], or NF-κB [87][96], to promote positive regulation of genes. Therefore, a tethering mechanism could possibly be involved in which GCs, such as Dex increase CRP expression.

Similarly, regulatory elements within the CRP promoter activated by TNF-α have not been identified, thus the increase in CRP promoter activity in response to TNF-α **(Fig. 3.16A)** remains to be elucidated. In contrast, the increase in IL-6-induced increase in CRP promoter

activity **(Fig. 3.16B)** could be attributed to the presence of both C/EBP and STAT3 as these transcription factor recognition sequences have been shown to be present [185][190]. The WT CRP -300 construct has two sites to which C/EBP can bind, namely -52 and -219 [182], with STAT3 binding between these two positions to a site at -108 on the promotor [183]. IL-6-induced CRP promoter activity shown in the current study is in agreement with other studies performed in human liver cell lines [178][182][185].

When various concentrations of Dex were combined with either TNF-α or IL-6, CRP promotor activity remained significantly ($p<0.001$) elevated. In addition, cooperative regulation of CRP promotor activity, more than what was observed with either signalling molecule alone, was observed for: (i) the lowest concentration of Dex (10^{-8} M) plus TNF-α **(Fig. 3.16A)** and (ii) the highest concentration of Dex (10^{-6} M) plus IL-6 **(Fig. 3.16B)**. This cooperativity displayed was also observed at mRNA level, and could be due to a positive tethering mechanism, where crosstalk occurs between the transcription factors involved in Dex-, TNF-α-, and IL-6 signalling, and the promotor of the CRP gene. For example, the GR along with other transcription factors (C/EBP, STAT3, NF-κB) could be co-recruited, to simultaneously occupy the promotor of the CRP gene to induce its expression. However, as mentioned earlier, the luciferase promotor-reporter assay used is an artificial system, in which the plasmids involved contain only the minimal promotor and not the fully endogenous gene of our proteins of interest.

Taken together, an increase in both PAI-1 and CRP levels could be attributed to the activation of the gene promoters of PAI-1 and CRP, respectively.

4.4 Conclusions

The hypothesis of the current study is partially accepted, as cooperative regulation of PAI-1 and CRP by GCs in the presence of either pro-inflammatory cytokine, were observed under some but not all experimental conditions. Generally, PAI-1 and CRP levels remained significantly elevated in response to combinatorial treatments, which occurred predominantly at the promotor- and protein level. However, the GCs were also able to antagonise the cytokine-induced PAI-1 and CRP expression under certain conditions, which is in line with their well-known anti-inflammatory role. Nonetheless, it has become abundantly clear, that GCs-mediated effects go beyond just that of being anti-inflammatory, by surprisingly promoting pro-inflammatory pathways. In the current study this was demonstrated as in combination with

either pro-inflammatory cytokine, under certain conditions, it cooperatively induced an increase in PAI-1 and CRP expression, more than what was observed with either test compound alone.

Conclusively, the findings of the current study in itself highlight the layered complexity of APP regulation by GCs and pro-inflammatory cytokines. It is difficult to identify a uniform pattern of regulation across all the components forming part of this study, as the crosstalk between GCs and pro-inflammatory cytokines appear to be rather complex. In addition, the current study only reflected a snapshot of parameters, and not the complete cytokine nor hormonal networks. Despite this limitation, our findings do highlight the importance of investigating the regulation of various APPs by GCs and pro-inflammatory cytokines in the same system, as the majority of studies tend to centralise their investigation around the regulation of a single APP. Ultimately, more research is required to disentangle the complexity of the findings of the current study, which will briefly be addressed in the next and final section.

4.5 Future directions

While undertaking this study, several ideas for future research worth investigating surfaced. These future prospects include, optimising HepG2 cells as a human cell model to study APP regulation, further investigating the underlying mechanism of co-regulation by GCs and/or cytokines on APP expression, determining if the GCs are metabolised in our liver cell lines, investigating extracellular PAI-1 protein expression and using an alternative treatment regime and/or cell model system.

4.5.1. Complete outstanding experimental work in HepG2 cells

Due to time constraints, owing to the COVID-19 pandemic, some experimental work could not be performed in the human hepatoma (HepG2) cell line. This was due to difficulty experienced with the human PAI-1 and CRP primers, which prevented investigating PAI-1 and CRP mRNA expression in the HepG2 cell line. In addition, efforts to transiently transfect the HepG2 cells with our plasmid constructs, in order to perform a luciferase promotor-reporter assay, proved unsuccessful, as these cells tend to grow in clumps or clusters, stacking on top of each other, when culturing **(Chapter 2, Fig. 2.1)**. As a result, minimal membrane surface is exposed, which could possibly compromise the uptake of the exogenous plasmid DNA. Future studies should consider attempting to overcome the above-mentioned challenges by perhaps i) designing and using primer sets with better specificity and from a more reliable manufacturer

and, ii) optimising transfection conditions to allow for greater transfection efficiency in the HepG2 cell line, such as cell density, length of exposure to transfection reagent or the amount of plasmid DNA.

4.5.2. Are certain transcription factors, such as the GR, co-recruited to the promotor of the PAI-1 or CRP genes?

In order to improve our understanding of PAI-1 and CRP co-regulation by GCs and pro-inflammatory cytokines, the current study can be extended to investigating the possible mechanism of action behind this phenomenon. Firstly, future studies could therefore ascertain whether the GR is required *via* a knock down study, using silencing RNA (siRNA) targeting the *nr3c1* gene. This gene specifically codes for the GR. Secondly, a chromatin immunoprecipitation assay (ChIP) could be performed to investigate the interaction between possible transcription factors involved and the promotor of the PAI-1 and CRP genes, i.e., to investigate if the GR binding site co-localises with sites for inflammatory transcription factors, such as NF-κB, C/EBP, or STATs. If these transcription factors are co-recruited to the PAI-1 and CRP gene promotors, this would confirm the theory that a positive tethering mechanism whereby the GR interacts with other transcription factors is responsible for PAI-1 and CRP gene regulation.

4.5.3. Are the GCs metabolized in the liver cell lines?

To the best of our knowledge, the BWTG3 cells have not been fully characterized in regards to its liver enzyme expression profile. However, liver cell lines, such as HepG2, express both phase I and II metabolising enzymes [220], which can metabolise steroidal compounds. According to Gentile *et al.,* Dex contains a 9α-fluorine group in its chemical structure, which is believed to disrupt the activity of the human liver enzyme, 11-β-hydrogenase type II, from metabolising Dex [221]. Taking this into consideration, it is understood that Dex would perhaps undergo less metabolic processing *in vitro* compared to the endogenous GCs, cortisol and corticosterone. In addition, there is evidence that liver enzymes transcript levels are much lower in carcinoma cell lines opposed to its levels in primary hepatocytes [25]. Nonetheless, mass spectrometry could be utilised to determine if the steroid substrates were metabolised to its respective metabolites in both liver cell lines. This would confirm if the GC ability of the steroids or its metabolites are responsible for the observed responses in the current study. If this proves that steroid metabolism did in fact occur, future studies could use liver enzyme inhibitors to block the GCs from being metabolised.

4.5.4. Extracellular protein expression

The lack of Dex-mediated effect on PAI-1 protein levels and the possible antagonistic behaviour displayed by Dex on both cytokine-induced PAI-1 protein expression, exclusively in the human liver cell line, is peculiar considering PAI-1 promoter activity was observed using a human PAI-1 promoter reporter construct. Nonetheless, this was not entirely unexpected, as GCs are well known to possess anti-inflammatory properties [23]. However, it cannot be dismissed that either the rate of Dex-induced PAI-1 protein synthesis or PAI-1 export might occur at a faster rate in HepG2 cells. Hence, future studies could explore this issue further by measuring extracellular PAI-1 protein levels (i.e., PAI-1 protein secreted into the media) in response to the test compounds *via* performing an enzyme-linked immunosorbent assay (ELISA) over time.

4.5.5. Alternative treatment regime

It is important to keep in mind that neither hormonal nor cytokine networks function in isolation. Their ratios in the body might be different, but these signalling molecules are still present in the system simultaneously. Therefore, examining the complicated interaction between GCs and pro-inflammatory cytokines are very relevant. Future studies should assess a combination (cocktail) of GCs and pro-inflammatory cytokines – for example treating cells with Dex plus TNF-α plus IL-6. Few studies have used this treatment-approach, where in most cases they observed an even bigger response in APP protein production [180][194][198].

Furthermore, one could also include another early release pro-inflammatory cytokine, namely IL-1-β, which shares similar biological properties to TNF-α [222]. It would be a much closer physiological reflection of what occurs in the human body, if a combination of all three cytokines (TNF-α plus IL-1-β plus IL-6) were introduced to the cells. Multiple studies have reported how the presence of IL-1-β synergistically enhanced the effect of IL-6-induced APP expression [178][183]–[185][194][198][199]. It has become apparent to the author of this study that changes in the expression of many APPs are mediated not by IL-6 alone but instead by a combination of pro-inflammatory cytokines.

In addition, most studies investigating APP expression in response to a combination of pro-inflammatory cytokines, administered the cytokines in simultaneously. However, the sequential appearance of IL-1-β followed by IL-6 and thereafter TNF-α have been demonstrated *in vivo*. Hence, the treatment regime used in the current study did not mimic

in vivo inflammatory environments. If future studies were to consider treating with a combination of cytokines, this order of cytokine release during the APR, should be kept in mind.

The current study did have its limitations, as it showed the importance of optimizing conditions for both APPs at the different levels of regulation. As the only optimisation in regards with APP expression was in the form of a time course experiment at protein level, the mRNA (and by default promotor) results could possibly reflect differently had conditions for each individual parameter been optimised in terms of length of exposure to the test compounds. However, taking into account that the transcription process occurs before mRNA is translated into a functional protein, as described by the central dogma of molecular biology, a shorter treatment time than used for the protein expression experiments was chosen. Despite this, future studies should consider (i) performing a time course study for PAI-1 and CRP mRNA expression in response to single treatment with Dex and either TNF-α or IL-6.

4.5.6. Alternative cell model system

Although the human and murine hepatoma cell lines, HepG2 and BWTG3, are good models for studies investigating APP regulation, they may not mimic the functional response of primary cells. This is due to whole body conditions and physiological factors not being properly reflected by carcinoma cell lines. However, the current study was a pilot study, thus employing the above-mentioned cell lines were the best approach, since it provided a more controlled system for the research to be conducted within. Future studies could alternatively use primary hepatocytes, which resemble a truer representation of the *in vivo* environment. The current study could also be replicated *in vivo* using an animal (mouse or rat) model. Factors may be present in animal models but not in cell culture that counteract the upregulation of APP expression by pro-inflammatory cytokines. Furthermore, the effect of co-treatment with GCs and/pro-inflammatory cytokines on PAI-1 and CRP expression in other insulin-sensitive tissues, such as adipocytes or muscle cells, can be investigated.

In summary, it is difficult to arrive at any concrete conclusions with regard to the complete comprehensive picture of PAI-1 and CRP regulation by both GCs and pro-inflammatory cytokines, without first addressing the short-comings of this study. The results from the additional future work mentioned above, would undoubtedly build upon and strengthen our understanding of PAI-1 and CRP regulation by both signalling mediators but also provide

answers to questions which surfaced during the course of this study. Nonetheless, the current study confirmed that combinatorial treatments with GCs and inflammatory mediators are generally capable of inducing PAI-1 and CRP expression in liver cells, even displaying cooperativity under certain experimental conditions. The promoter reporter results of the current study also suggest that regulation of these APPs occur at the promoter level, although the transcription factors involved remain to be elucidated.

To conclude the potentiation, rather than suppression of cytokine-induced APP expression by GCs, which was generally observed, highlight the need for a paradigm shift in how GC- and cytokine signalling and crosstalk between them is considered, as it is much more nuanced in its behaviour.

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ADDENDA

ADDENDUM A: Summaries of the literature pertaining to the regulation of the APPs

Table A1. Effects of glucocorticoids and pro-inflammatory cytokines on the regulation of PAI-1.

Model system	[Signalling molecule]	Effect	Treatment time	Technique	Reference
mRNA					
Human fibrosarcoma cells (HT-1080)	**Dex** (1 µM)	Increase (10-fold)	48 h	**Quantitative dot blot** (mRNA)	[167]
Human fibrosarcoma cells (HT-1080)	**Dex** (100 nM)	Time course: 24 h: increase of 11.09-fold (3.4 kb) and 7.8-fold (2.4 kb) 48 h: decreased with 2-fold (3.4 kb) and increase to 6-fold (2.4 kb)	24, 48 h	**Northern blot** (mRNA: 3.4 kb and 2.4 kb)	[169]
Human umbilical artery and vein endothelial cells (HUAEC)	**TNF-α** (50 ng/ml)	Increase (10- to 20-fold)	24 h	**Northern blot** (mRNA)	[140]
Rat hepatoma cells (HTC)	**Dex** **(100 nM)** (Maximal responses occur at approximately **50 nM)**	time- and concentration-dependent; increase of 4- to 5-fold (maximal increase after 8-10 h)	1 - 10 h	**Northern blot** (mRNA: 3.2 kb)	[166]
Bovine aortic endothelial cells (BAE)	**TNF-α** (25 ng/ml)	Increase (30-fold)	6 - 18 h	**Northern blot** (mRNA: 3 + 1.6 kb)	[144]
Human hepatoma cells (HepG2)	**IL-6** (40 ng/ml)	Increases of 3.5-fold (3.2 kb) and 2.5-fold (2.2 kb)	2 - 4 h	**Northern blot** (mRNA: 3.5 kb and 2.2 kb)	[159]
	Dex (Results not shown)	no effect			
Human vascular smooth muscle cells from mammary artery (HMASMC)	**TNF-α** (1-100 nM – significant induction) (10 nM – maximal effect)	Increases of 5-fold (3.4 kb) and 3-fold (2.4 kb)	12 h	**Northern blot** (mRNA: 3.4 kb and 2.4 kb)	[150]
Adipocytes (3T3-L1)	**TNF-α** (5 ng/ml)	Increase of 8-fold within 3 h; levels return to basal by 24 h	3 h	**qPCR** (mRNA)	[141]

Murine hepatocytes (AML 12)	**TNFα-** (50 ng/ml)	Increase of 4-fold within 1 h, levels return to basal by 4 h	1 h	**Northern blot** (mRNA)	[156]
	IL-6 (50 ng/ml)	no effect			
	Dex (100 nM)				
Human (visceral) adipose tissue	**Dex** (0.1–1 µM)	Increase (2-fold)	4 - 8 h	**Northern blot** (mRNA)	[163]
Human adipocytes	**TNF-α** (5 nM)	Increase (1.7-fold)	24 h	**qPCR** (mRNA)	[153]
Human endothelial cells (EA. hy 926)	**TNF-α** (50 ng/mL)	Increase (1.8-fold)	4 h	**qPCR** (mRNA)	[145]
Human umbilical vein endothelial cells (HUVEC)	**TNF-α** (10 ng/ml)	Increase (10-fold)	4 h	**qPCR** (mRNA)	[146]
Adipocytes (3T3-L1)	**TNF-α** (8 ng/mL)	Increase of 5- to 6-fold (3 h), 2-fold (24 h)	3, 24 h	**qPCR** (mRNA)	[154]
Human adipocytes of subcutaneous (SC) **and visceral** (V) **origin**	**IL-6** (100 ng/mL)	SC: Increase (9.5-fold) V: Increase (10-fold)	SC – 8 h V – 24 h	**qPCR** (mRNA)	[161]
Human hepatoma cells (HepG2) **Primary mouse hepatocytes**	**IL-6** (1 ng/mL)	Increase (2-fold)	4 h	**qPCR** (mRNA)	[160]
Bovine aortic endothelial cells (BAE)	**TNF-α** (1-30g/ml) 10 ng/ml (maximal)	dose response (6 h): dose-dependent increase time course (10 ng/ml): time-dependent increase, maximal after 6 h	Up to 24 h (Maximal at 6 h)	**Northern blot** (mRNA)	[148]

Human proximal renal tubular epithelial cells (HPTEC)	**Dex** (100 nM / 1 µM / 5 µM)	dose-response: Increase 3.2-fold (100 nM) 3.4-fold (1 µM) 2.2-fold (5 µM) time-course: Increase 5.4-fold (6 h) 3.2-fold (24 h) 2.3-fold (48 h)	6 h (Dex) 24 h (TNF-α)	**TaqMan quantitative PCR** (mRNA)	[149]
	TNF-α (10 ng/ml)	Increase of 2.5-fold (24 h)			
Human primary bone marrow adipocytes	**Dex** (100 nM / 1 µM)	Dose-response (24 h): Increase (100 nM), maximal at 1 µM (3.7-fold) time-course (1 µM): 9-fold (24 h), maximal at 12 h	12 h	**qPCR** (mRNA)	[165]
Protein					
Human fibrosarcoma cells (HT-1080)	**Dex** (1 µM)	intracellular protein: increase within 4 h and levelled off after 10 - 20 h extracellular protein: Increase after 16 h and continued to increase during the 48 h of incubation (5- to 10-fold)	4 – 10 h (intracellular) 16 - 48 h (extracellular)	**ELISA** (Intra- and extracellular)	[167]
Human endothelial cells (HEC)	**TNF-α** (0.01-1 ng/ml) (0.1-0.3 ng/ml – maximal)	dose-dependent increase (4-fold); time-dependent - required more than 6 h (maximal at 24 h)	6 – 24 h	**Fibrin enzymography** (production)	[143]
Human fibrosarcoma cells (HT-1080)	**Dex** (100 nM)	Increase (9-fold)	48 h	**Radioimmunoassay** (secretion)	[169]
Human and rat hepatocytes	**TNF-α** (50 ng/ml - maximal)	Increase	24 h	**Reverse fibrin autography** (Production of activity)	[140]
	IL-6 (0.05 – 50 ng/ml)	no effect			
Rat hepatoma cells (HTC)	**Dex** (50 nM)	Increase (5- to 10-fold), intracellular activity: increase as early as 1 h, maximal (6 - 8 h) extracellular activity (data not shown) increases after 2 - 4 h, maximal (10 h after)	6 - 8 h (intracellular) 10 h (extracellular)	**Esterolytic assay** (Intra- and extracellular activity)	[166]
Human hepatoma cells (HepG2)	**IL-6** (1000 ng/ml)	no effect	48 h	**Radio-immunoassay** (synthesis)	[155]
	TNF-α (50 ng/ml)	Increase (2- to 3-fold)			

Human hepatoma cells (HepG2)	**IL-6** (100 ng/ml)	no effect	24 h	**ELISA** (antigen)	[162]
Human monocytes	**Dex** (100 nM)	Increase (6-fold)	18 h	**ELISA** (antigen)	[170]
Human hepatoma cells (HepG2)	**IL-6** (40 ng/ml)	no effect (data not shown)	up to 24 h	**ELISA** (Extracellular)	[159]
Human vascular smooth muscle cells from mammary artery (HMASMC)	**TNF-α** (10 nM)	dose-dependent increase (4.2-fold) for 24 h, time-dependent increase (3.1-fold) after 31 h	24 - 31 h	**ELISA** (antigen/synthesis)	[150]
Adipocytes (3T3-L1)	**TNF-α** (5 ng/ml)	Increase (5-fold)	3 h	**ELISA** (antigen)	[141]
Human (visceral) adipose tissue	**Dex** (0.1–1 µM)	Time course (50 nM): time-dependent increase (1.7-fold) Dose response (24 h): Increase (1–10 nM), maximal (0.1–1 µM)	12 - 24 h (maximal)	**ELISA** (secretion/ production)	[163]
Adipocytes (3T3-L1)	**TNF-α** (10 ng/mL)	Increase (2.5-fold)	24 h	**Western blot** (Intracellular accumulation)	[151]
Human adipocytes	**TNF-α** (5 nM)	Increase (2.7-fold)	24 h	**ELISA** (Extracellular)	[153]
Adipocytes (3T3-L1)	**Dex** (0.25 µM)	Increase (2- to 2.5-fold)	40 h	**ELISA** (production/antigen)	[164]
Human Trophoblast cells (HTR-8/SVneo)	**Dex** (100 nM)	Increase (2-fold)	96 h	**ELISA** (antigen)	[171]
Human endothelial cells (EA. hy 926)	**TNF-α** (50 ng/mL)	Increase (2-fold)	20 h	**ELISA** (antigen)	[145]
Adipocytes (3T3-L1)	**TNF-α** (8 ng/mL)	Increase (5-fold)	24 h	**ELISA** (antigen)	[154]

Human preadipocytes and adipocytes of subcutaneous (SC) **and visceral** (V) **origin**	**IL-6** (100 ng/mL)	SC: Increase (3.5-fold) V: Increase (7-fold)	48 h	**ELISA** (antigen/production)	[161]
Bovine aortic endothelial cells (BAE)	**TNF-α** (10 ng/ml)	Dose response (6 h): increased in concentration-dependent manner Time course (10 ng/ml): increased in time-dependent manner, maximal increase after 6 h	6 h	**Western Blot** (Intracellular)	[148]
Human proximal renal tubular epithelial cells (HPTEC)	**Dex** (100 nM)	Dose response (24 h): not dose-dependent; maximal at 100 nM (2.6-fold) Time course (100 nM): 2.5-fold (6 h) 3.7-fold (24 h) 4.0-fold (48 h)	48 h	**Immunoassay** (Extracellular)	[149]
	Hydrocortisone (F) (1 µM)	Increase of 2.5-fold (24 h)			
	TNF-α (10 ng/ml)	Increase of 2.5-fold (24 h)			
Human primary bone marrow adipocytes	**Dex** (100 nM / 1 µM)	Dose response (24 h): Increase at 100 nM, maximal with 1 µM (2.4-fold)	24 h	**ELISA** (secretion/production)	[165]
Promotor activity					
Human endothelial cells (EA. hy 926)	**TNF-α** (50 ng/mL)	Increase (14.5-fold)	12 h	**Luciferase Assay** (Cells transfected with plasmid **p800 LUC** containing a PAI-1 promoter fragment **(+71 to -800)**	[145]
Bovine aortic endothelial cells (BAE)	**TNF-α** (20 ng/ml)	Increase (3-fold)	18 - 24 h	**Luciferase Assay** (Cells transfected with **0.2 µg human and mouse PAI-1 proximal promotor: pU18.8-11.4** for 36 – 48 h)	[147]
Human hepatoma cells (HepG2)	**IL-6** (1 ng/mL)	Increase (2.4-fold)	24 h	**Luciferase Assay** (Cells transfected with **1.5 µg human PAI-1 promoter -829 to +36 bp** for 24 h)	[160]
	IL-6 + IL-1β (1 ng/mL)	Increase (7.1-fold)			

Table A2. Effects of glucocorticoids and pro-inflammatory cytokines on the regulation of CRP.

Model system	[Signalling molecule]	Effect	Treatment time	Technique	Reference
mRNA					
Human hepatoma cells (PLC/PTF/5)	**IL-6** (10 ng/ml)	Increase (9-fold)	24 h	**Northern Blot** (mRNA)	[179]
Human primary hepatocytes	**IL-6** (10 - 100 ng/ml)	Increase (7-fold)	20 h	**Cytoblot** and **mRNA Hybridization**	[174]
	TNF-α (0.1 - 100 ng/ml)	no effect			
Human and mouse primary hepatocytes	**IL-6** (50 ng/ml)	'Highly induced'	9 h	**Northern Blot** (mRNA)	[187]
Human coronary artery smooth muscle cells (HCASMC)	**TNF-α** (50 ng/ml)	Increase (2.5-fold)	48 h	**qPCR** (mRNA)	[189]
Mouse hepatoma cells (BWTG3)	**Dex** (10 nM)	no effect	24 h (Dex) 3 h (IL-6)	**qPCR** (mRNA)	[88]
	IL-6 (10 ng/ml)				
Protein					
Human hepatoma cells (Hep3B) (NPLC/PRF/5)	**IL-6** (200 ng/ml - Hep3B) (5 ng/ml – NPLC/PRF/5)	Hep3B cells: no effect NPLC/PRF/5 cells: Increase	18 – 24 h	Immunoprecipitation and **SDS PAGE** (Extracellular protein synthesis and secretion)	[177]
Human primary hepatocytes	**IL-6** (10 - 100 ng/ml)	Increase (22.5-fold)	20 h	Immunoprecipitation and **SDS PAGE** (Extracellular protein synthesis and secretion)	[174]
	TNF-α (0.1 - 100 ng/ml)	no effect			
Human hepatoma cells (Hep3B)	**Dex** (20 nM, 100 nM, 1 µM)	<u>Dose response:</u> Increase 20 nM: mean 1.9-fold 100 nM: mean 2.2-fold	18 – 24 h	Immunoprecipitation and **SDS PAGE** (Extracellular protein synthesis and secretion)	[180]
	IL-6 (10 ng/ml)	Increase			

	TNF-α (10 ng/ml)	no effect			
Human hepatoma cells (HepG2)	**Dex** (1 µM)	no effect	72 – 96 h	**Radio-immunoassay** (secretion/production/release/accumulation)	[181]
	IL-6 (10 ng/ml)	Increase			
Human coronary artery smooth muscle cells (HCASMC)	**IL-6** (10 ng/mL)	Increase	48 h	**ELISA** (production/release)	[189]
	TNF-α (50 ng/ml)	Increase			
Primary cultures of human adipocytes from adipose tissue	**IL-6** (10 ng/mL)	Increase (2-fold)	48 h	**ELISA** (production)	[188]
Mouse hepatoma cells (BWTG3)	**Dex** (10 nM)	Increase	24 h (Dex) 3 h (IL-6)	**ELISA** (Intra- and extracellular)	[88]
	IL-6 (10 ng/ml)				
Promotor activity					
Human hepatoma cells (Hep3B)	**IL-6** (10, 20, 40, 100 ng/ml)	Increase, more active than IL-1β	24 h	**CAT assay** (Cells transfected with **plasmid 5'Δ-355** (CRP-CAT fusion) for 12 h)	[178]
	IL-6 (50 ng/ml) + **IL-1β** (100 ng/ml)	Cooperatively increased			
Human hepatoma cells (PLC/PRF/5)	**IL-6** (20 ng/mL)	Increase (2-fold)	16 - 24 h	**CAT assay** (Cells transfected with plasmids (**M40-3 CAT3M, M40-3-18 CAT3M)** containing site-specific mutations in one or two IL-6 responsive elements for 16 h)	[182]
Human hepatoma cells (Hep3B)	**IL-6** (10 ng/mL)	Increase of 2–3-fold (cells overexpressing STAT3); can also activate CRP promotor	24 h	**CAT assay** (Cells transfected with CRP promoter (-157 to +3)	[183]

	IL-6 + IL-1β (10 ng/mL)	synergistical increase		(Overexpression of STAT3: stably transfected with pRc/CMV-STAT3)	
Human hepatoma cells (Hep3B)	**IL-6** (10 ng/mL)	In the presence of overexpressed p50: Increase (4-fold)	24 h	**Luciferase reporter assay** (Transiently co-transfected with **–157 CRP-LUC** construct and expression vectors for **p50/p65** for 24 h)	[184]
	IL-6 + IL-1β (20 ng/mL)	In the presence of overexpressed p50: Increase (12-fold)			
Human hepatoma cells (Hep3B)	**IL-6** (10 ng/mL)	Luc-300 WT: Increase (5-fold) Luc-157 WT: Increase (2.5-fold)	24 h	**Luciferase reporter assay** (Cells transfected with **0.1 µg/well** in 6-well **Luc-300 WT and Luc-157 WT** for 16 h)	[185]
	IL-6 + IL-1β (1 ng/ml)	Luc-300 WT: IL-1β enhanced IL-6-induced effect by 5.1-fold Luc-157 WT: IL-1β enhanced IL-6-induced effect by 4.4-fold			

Table A3. Studies investigating the effect of co-treatment with GCs and pro-inflammatory cytokines on APPs.

APP	Model system	[Signalling molecule]	Effect	Treatment time	Technique	Reference
SAA **CRP**	**Human primary hepatocytes**	**Dex** (100 nM)	SAA: Increase (4-fold) CRP: Increase (1.7-fold)	20 h	**^{35}S metabolic labeling** (Protein synthesis)	[196]
		IL-6 (10 ng/ml)	SAA: Increase (5-fold) CRP: Increase (22-fold)			
		Dex + IL-6	SAA: Increase (27-fold) CRP: Increase (23-fold)			
Haptoglobin α1-ACT	**Human hepatoma cells** (HepG2)	**Dex** (1 µM)	No effect alone but **synergistically enhanced** the cytokine response of both APPs	6 - 18 h	**Northern Blot** (mRNA)	[193]
		IL-6 (25 ng/ml)	Increase both			
		IL-1α (50 ng/ml)	Increase both			
CRP	**Human hepatoma cells** (HepG2)	**Dex** (1 µM)	no effect 0.8-fold (48 h) 1.5-fold (72 h)	48, 72 h	**Radio-immunoassay** (secretion/production/ release/accumulation)	[181]
		IL-6 (10 ng/ml)	Increase 2.7-fold (48 h) 9-fold (72 h)			
		Dex + IL-6	**Cooperatively increased** 5.2-fold (48 h) 14-fold (72 h)			
SAA	**Human hepatoma cells** (Hep3B)	**Dex** (20 nM, 100 nM)	synthesis: no effect 20 nM: Increase of 1.6- to 5.2-fold (mean 3.0-fold) 100 nM: Increase of 2.1- to 7.8-fold (mean 4.4-fold)	18 - 24 h	**^{35}S metabolic labeling** (Protein synthesis) **Northern Blot** (mRNA)	[180]
		IL-6 (5 ng/ml)	mRNA: no effect			
		Dex (1 µM) **+ IL-6 + TNF-α**	in various combinations are all capable of influencing synthesis			

SAA1/2/4	**Human adult aortic smooth muscle cells**	**Dex** (1 µM) **+ IL-1β** (10 ng/mL)	Dex had no effect; only combined with either cytokine increased mRNA expression (20- and 50-fold)	20 h	**Hybridized with a ^{32}P-labeled human SAA1 cDNA** (mRNA)	[197]
		Dex (1 µM) **+ IL-6** (50 ng/mL)				
SAA3	**Female ICR mice** (C57BL/6)	**Dex** (1 mg/kg) **+ IL-6** (2µg/mouse) **+ IL-1β** (2µg/mouse)	only the combination of Dex + IL-6 increased the production of SAA, and the level were markedly elevated after stimulation with IL-1β.	11 h	**ELISA** (Intracellular protein)	[198]
SIP24/24p	**Mouse embryonic fibroblasts** (Balb/c 3T3) **Mouse liver cells** (BNL)	**Dex** (400 ng/ml)	Balb/c 3T3 cells: increase (2.3-fold) BNL cells: Increase (14. 6-fold)	24 h	**^{35}S metabolic labeling** (synthesis/production)	[194]
		TNF-α (1000 ng/ml)	Balb/c 3T3 cells: increase (2.5-fold) BNL cells: Increase (8.6-fold)			
		Dex + TNF-α	Balb/c 3T3 cells: **synergistic increase** (3-fold) BNL cells: no synergistic effect (5-fold)			
		IL-6 (10 ng/ml)	Balb/c 3T3 cells: increase (1.9-fold) BNL cells: no effect (1.2-fold)			
		Dex + IL-6	Balb/c 3T3 cells: **synergistic increase** (7-fold) BNL cells: obvious but weak synergistic effect (3-fold)			
		Dex + TNF-α + IL-6	Balb/c 3T3 cells**: synergistic increase** (10.8-fold) BNL cells: **additive effect** (23.7-fold)			
SAA1	**Human hepatoma cells** (HepG2)	**IL-6** (10 ng/ml)	Maximum increase at 3 h, decreased over time	up to 24 h	**Luciferase Assay** (Transfected with human A-SAA promoter luciferase reporter constructs: pGL3-SAA1pt for 16 - 20 h)	[199]
		TNF-α (10 ng/ml)	Increased over time			
		Dex (50 nM) + **TNF-α / IL-6**	Previously reported: Dex increased cytokine driven induction of SAA1 promoter at all time-points (2- to 3-fold)			

PAI-1	**Human umbilical vein endothelial cells** (HUVEC)	**Dex** (1 µM)	Increase (4.2-fold)	48 h	**RT-PCR** (mRNA)	[201]
		TNF-α (10 ng/ml)	Increase (1.8-fold)			
		Dex + TNF-α	Additive effect			
PAI-1	**Human proximal renal tubular epithelial cells** (HPTEC)	**Dex** (100 nM)	Time course mRNA increase 5.4-fold (6 h) 3.2-fold (24 h) 2.3-fold (48 h) Protein increase 2.5-fold (6 h) 3.7-fold (24 h) 4.0-fold (48 h)	6, 24, 48 h	**TaqMan quantitative PCR** (mRNA) **Immunoassay** (Extracellular protein)	[149]
		TNF-α (10 ng/ml)	mRNA + protein: Increase of 2-5-fold (24 h)			
		Dex + TNF-α	mRNA: Increase (4.8-fold) Protein: Increase (2.3-fold) (24 h)			
FGG **ACT** **SAA**	**Primary murine hepatocytes** **Human hepatoma cells** (HepG2) **WT mice** (Whole liver extracts)	**Dex** *In vitro*: 100 nM *In vivo:* 4 mg/kg	mRNA (FGG) no effect *in vitro*	4 h	**qPCR** (mRNA) **ELISA** (Intracellular protein)	[195]
		IL-6 *In vitro:* 20 ng/ml *In vivo*: 100 µg/kg	FGG Primary murine hepatocytes: Increase in mRNA (5-fold) HePG2: Increase in mRNA (2-fold) SAA (mice) Increase in mRNA (10-fold) and protein (3.5-fold)			
		Dex + IL-6	FGG Primary murine hepatocytes: Increase in mRNA (7.5-fold) HepG2: Increase in mRNA (3.5-fold) SAA (mice) Increase in mRNA (20-fold) and protein (4.7-fold)			
		Dex *In vitro:* 10 nM *In vivo:* 1 mg/kg	*In vitro*: mRNA: Increase of 10-fold (SerpinA3), slight increase (SAA1), no effect (SAA2) Protein: Increase (SerpinA3) *In vivo:* mRNA: increase in both lung (2.2-fold) and liver (3-fold) tissues			

SerpinA 3, SAA1/2	**Human lung cells** (A549 cells) **Adrenalectomized male mice** (C57BL/6)	**Cortisol** (100 nM)	mRNA: Increase (5-fold)	6 h	**qPCR** (mRNA) **Western Blot** (Intracellular protein)	[27]
		TNF-α *In vitro:* 10 ug/ml *In vivo*: 60 µg/kg	*In vitro*: mRNA: Increase of 25-fold (SerpinA3, SAA1), increase of 75-fold (SAA2) Protein: Increase (SerpinA3) *In vivo:* mRNA: increase in both lung and liver tissues (1.5-fold)			
		Dex + TNF-α	*In vitro*: mRNA: **synergistic increase** of 85-fold (SerpinA3), 200-fold (SAA1), 400-fold (SAA2) Protein: **synergistic increase** (SerpinA3) *In vivo:* mRNA: **additive increase** in both lung (3-fold) and liver (6-fold) tissues			
		Cortisol + TNF-α	mRNA: **synergistic increase** (42-fold)			
SAA, CRP	**Mouse hepatoma cells** (BWTG3)	**Dex** (10 nM) or **IL-6** (10 ng/mL)	CRP / SAA Intra-and extracellular protein: Increase mRNA: no effect	24 h (Dex) 3 h (IL-6)	**ELISA** (Intra- and extracellular protein) **qPCR** (mRNA)	[88]
		Dex + IL-6	CRP Extracellular protein: **'convergence of signal'** Intracellular protein: Dex decreased IL-6-induced CRP expression, but had no effect at extracellular protein or mRNA levels. IL-6 had no effect on the action of Dex. SAA Intra-and extracellular protein: increase, but not more than what was observed with either test compound alone mRNA: 'in presence of Dex, IL-6-induced SAA expression is higher than in the absence of Dex'			
		Dex (100 nM)	mRNA: Increase SAA1 – 100-fold (24 h) SAA2 – 25-fold (24 h) Secretion: Increase SAA1 - 15-fold			

SAA1/2	**Primary human keratinocytes** **Immortalised human keratinocytes** (HaCaT)	**TNF-α** (10 ng/ml)	mRNA: Increase in both SAA1 and SAA2, but not as much as with Dex alone Secretion: (SAA1) Keratinocytes: Slight increase HaCaT cells: Increase (2.5-fold)	2 h (secretion) 24 h (mRNA)	**ELISA** (secretion/production) **qPCR** (mRNA)	[200]
		Dex + TNF-α	mRNA: Increase SAA1 – 300-fold SAA2 – 85-fold Secretion: Increase (SAA1) Keratinocytes: 40-fold HaCaT cells: 10-fold			

ADDENDUM B: Mycoplasma-negative images

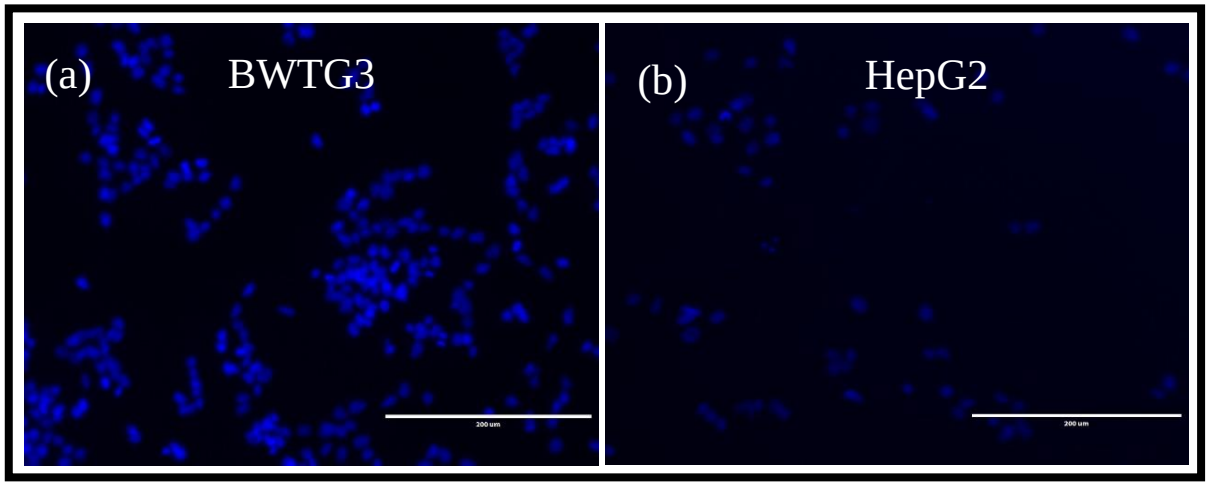


Figure B1. Mycoplasma negative (a) BWTG3 and (b) HepG2 cells. Cells were fixed with Carnoy's fixative containing glacial acetic acid and methanol and in a 1:3 ratio. Subsequently, cells were stained with the Hoechst 33258 dye (Sigma-Aldrich). The Hoechst-dye emits blue fluorescence when bound to dsDNA. Fluorescent images were obtained using the Olympus XI81 inverted fluorescent microscope. Both images show fluorescent nuclei of cells against a dark background with no traces of extra-nuclear fluorescence of mycoplasma DNA. *Scale: 200 µM*

ADDENDUM C: RNA isolation and semi-quantitative real-time PCR

C1: RNA isolated from BWTG3 cells were intact

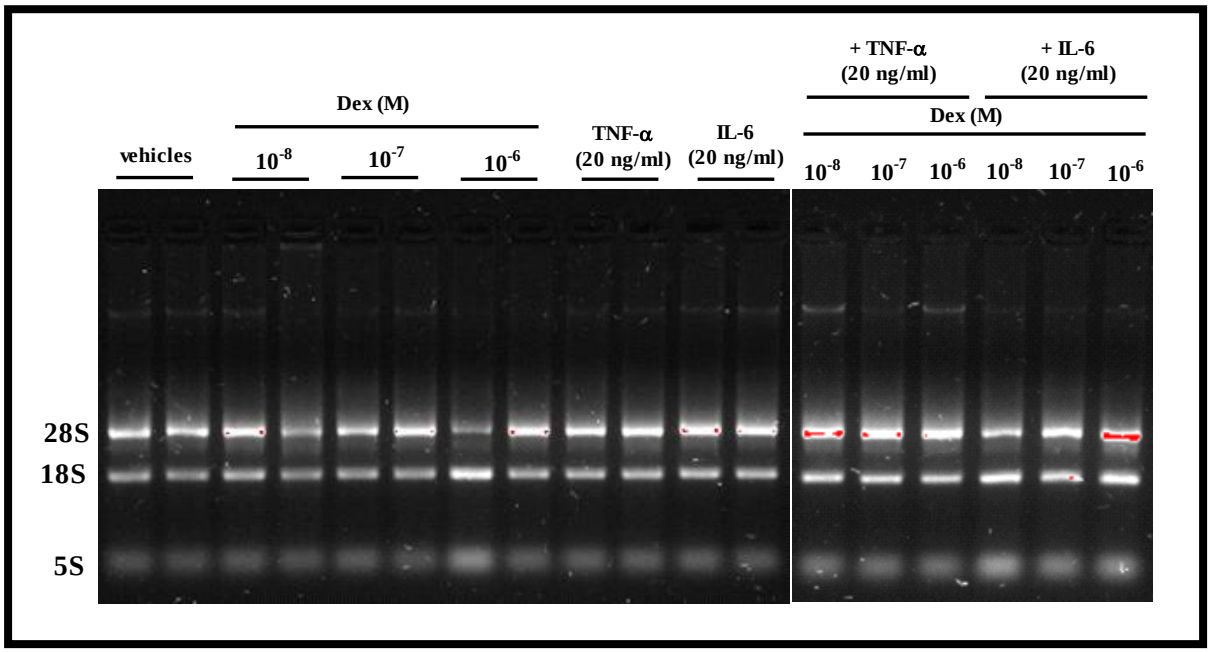
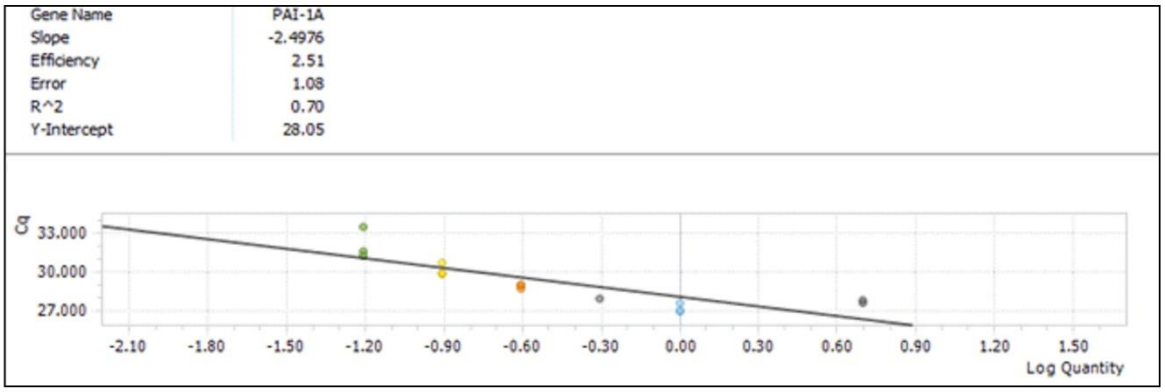
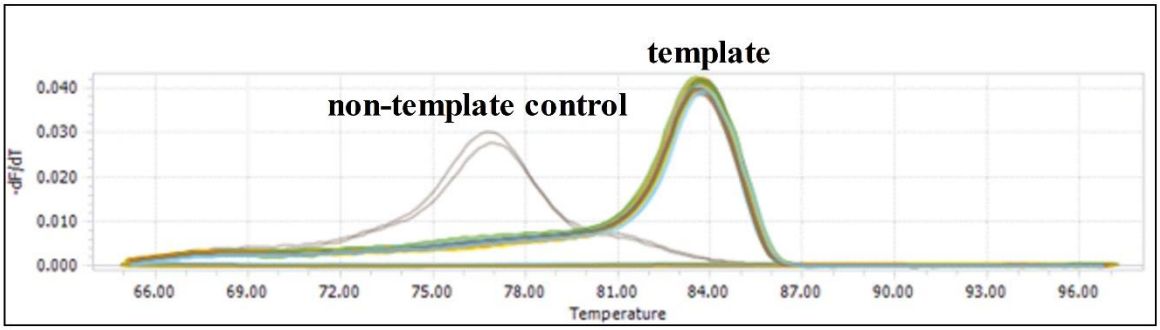


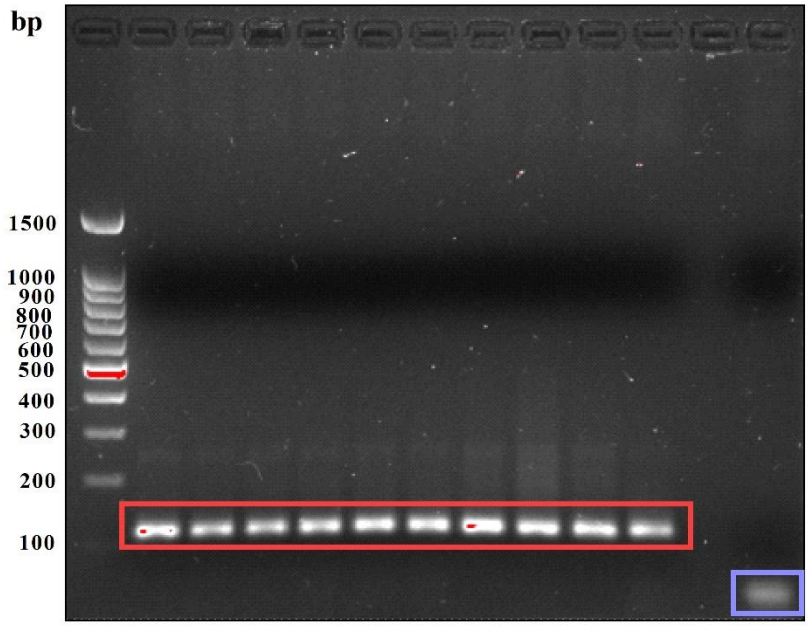
Figure C1. A representative 1% denaturing agarose gel indicating intact RNA isolated from BWTG3 cells. To confirm RNA integrity, the presence of intact 28S and 18S ribosomal RNA was determined. Cells were treated with 0.01% (v/v) ethanol combined with supplemented DMEM (vehicles), various concentrations of dexamethasone (Dex), 20 ng/ml TNF-α or IL-6, and various concentrations of Dex in the presence of TNF-α or IL-6. Total RNA was extracted from BWTG3 cells using Tri-reagent as described in Chapter 2, section 2.8. A total of 1 µg RNA of each sample was loaded onto a 1% (w/v) denaturing agarose gel, containing biocide, and stained with Nancy-520.

C2: Primer efficiency, melting curve and PCR product gel of each primer set

(a) PAI-1



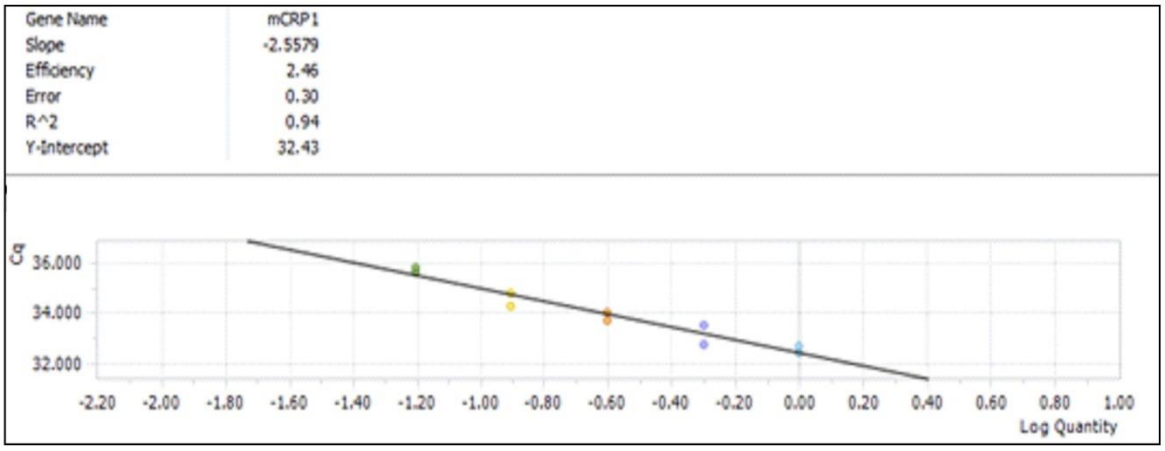


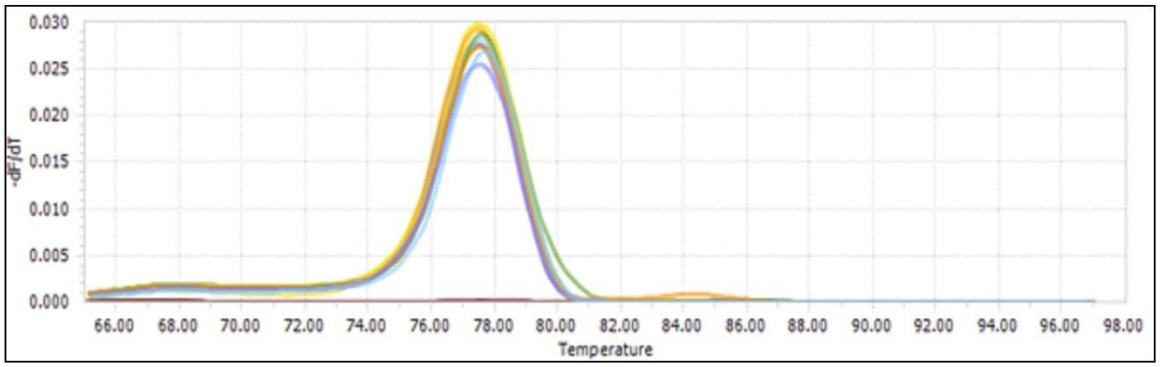


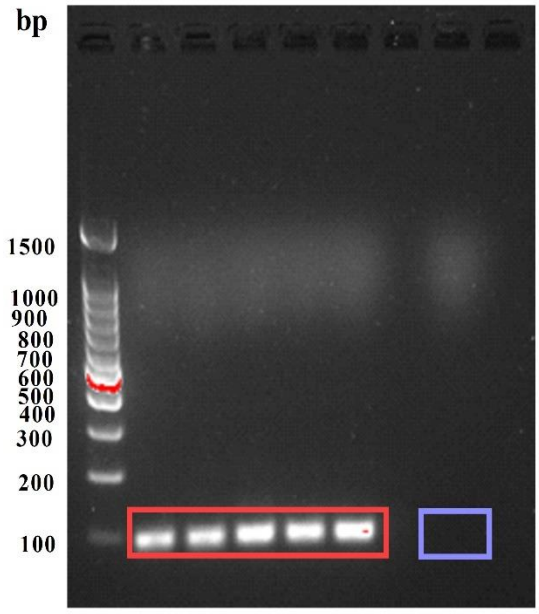
Expected product size: 115 bp

(Figure legend on page 147)

(b) CRP





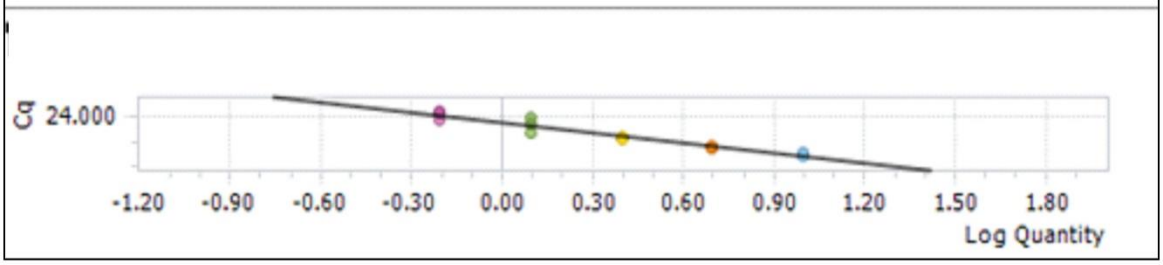


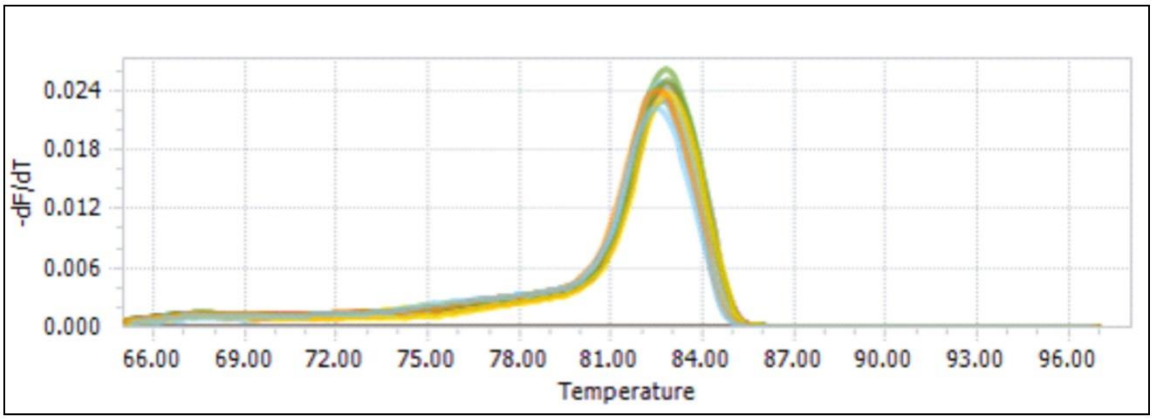
Expected product size: 96 bp

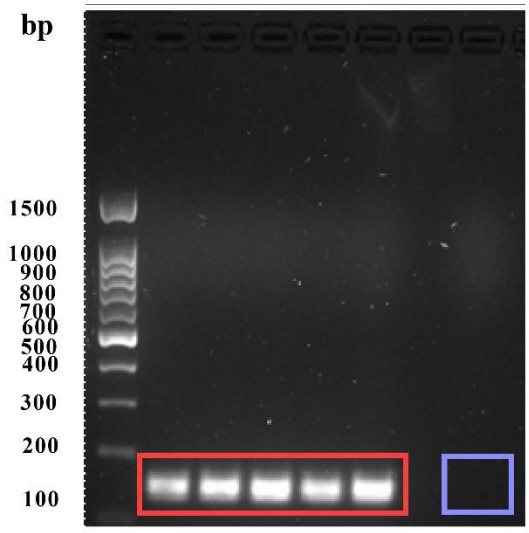
(Figure legend on page 147)

(c) 18S

Gene Name	m18S (Gr...
Slope	-2.7207
Efficiency	2.33
Error	0.34
R^2	0.93
Y-Intercept	23.50







Expected product size: 151 bp

(Figure legend on next page)

Figure C2. Primer efficiency melting curve for each primer set (a) mouse PAI-1, (b) mouse CRP and (c) mouse 18S used in this study; and corresponding 2% denaturing agarose gel indicating qPCR products. To confirm the amplification of a single product, melting curve analysis was performed. The efficiency of each primer set was calculated by making a dilution curve from cDNA (vehicle control); To confirm the correct amplicon size of the amplified gene product, agarose gel electrophoresis was performed. PCR products were separated by 2% (w/v) agarose gel electrophoresis, stained with Nancy-520. 100 bp DNA ladder (ThermoFisher Scientific); Blue block: PCR product (vehicle control); Red block: non template control.

ADDENDUM D: Buffers and solutions

10X TPE (Tris-phosphate-EDTA) (1 L)
• 108 g 10.8 % (w/v) Tris • 15.5 ml 1.6% (v/v) 85%-Phosphoric acid (1.679 g/ml) • 40 ml 4% (v/v) 0.5 M Na_2EDTA (pH 8.0) Final volume to 1 L using deionised water Store at room temperature
Function: common buffer solution for gel electrophoresis; solubilizes RNA or DNA while protecting it from degradation.

Passive Lysis Buffer (250 ml)
• 25 ml 10% (v/v) glycerol • 7 ml 2.8% (v/v) 10X TPE • 0.5 ml 0.2% (v/v) Triton X-100 Final volume to 250 ml using deionised water Store at 4 ºC
Function: concentrated cell lysis buffer, which promotes rapid lysis of cultured mammalian cells without the requirement of freeze-thaw cycles; it elicits only minimal coenterazine auto-luminescence, making it the lytic reagent of choice when processing cells for quantification of *Renilla* luciferase activities.

5X SDS-Reducing Lysis Buffer (10 ml)

- 5 ml 10% (w/v) SDS – break up membrane structures; important for high-quality protein separation; confers negative charge to polypeptide.
- 2 ml 20% (v/v) glycerol - increases sample density in order to facilitate gel loading and to ensure that each sample remain at the bottom of the well, preventing it from overflowing.
- 1 ml 10% (v/v) 1 M Tris-HCl buffer pH 6.8 – regulates the pH and osmolarity of lysate.
- 0.01 g Bromophenol Blue – small anionic dye; provides migration front to monitor separation process.

  Final volume to 10 ml using deionised water

  Store at -20 ºC (0.5 ml aliquots) and add 25 µl Beta-Mercapto-Ethanol (reduces disulphide bridges) per aliquot

Function: ionic denaturing detergent

0.5 M Na_2EDTA (pH 8.0)
(Disodium ethylene diamine tetra-acetic acid) (100 ml)

- 18.612 g 18.6% (w/v) Na_2EDTA (MW = 372.24 g/mol)

  Final volume to 100 ml using deionised water

  Adjust pH to 8.0 with NaOH

  Sterilize by autoclaving and store at room temperature

Function: prevents degradation of RNA or DNA and inactivates nucleases that require metal ions.

50X TAE (Tris-acetate-EDTA) (1 L)

- 242 g 24.2% (w/v) Tris
- 100 ml 10% (v/v) 0.5 M Na_2EDTA (pH 8.0)
- 57.1 ml 5.7% (v/v) glacial acetic acid

Final volume to 1 L using deionised water

Store at room temperature

Function: electrophoresis buffer for nucleic acids in agarose gels.

TE (Tris-EDTA) (500 ml)

- 10 ml 2% (v/v) 0.5 M Tris-HCl (pH 8.0)
- 100 µl Na_2EDTA (pH 8.0)

Final volume to 500 mL using DEPC-treated water.

Store at room temperature

Function: to solubilise DNA or RNA, while protecting it from degradation by DNase or RNases.

10% APS (Ammonium persulfate) (10 ml)

- 1 g 10% (v/v) APS

Final volume to 10 ml using deionised water

Store at -20 ºC

Function: used in the preparation of polyacrylamide gels; oxidising agent used in combination with TEMED, to catalyse the polymerization of acrylamide and bisacrylamide (setting agents).

10% (w/v) SDS (pH 7.2) (1 L)

- Dissolve 100 g 10% (w/v) SDS at 68 ˚C and adjust pH to 7.2 with HCl
  Final volume to 1 L using reverse osmosis water
  Store at room temperature

Function: Disrupts the tertiary structure of proteins allowing them to become linear; coats proteins to produce a uniform negative charge, in order for the charge to be proportional to the weight of the proteins.

1 M Tris-HCl Buffer (pH 6.8) (1 L)

- 121.4 g 12% (w/v) Tris
  Adjust pH to 6.8 with HCl
  Final volume to 1 L using reverse osmosis water
  Sterilize by autoclaving and store at room temperature

Function: organic compound used in buffer solutions for electrophoresis gels; to maintain a stable pH to hinder shifts that may influence cellular factors.

1.5 M Tris-HCl Buffer (pH 8.8) (1 L)

- 182.1 g 18.2% (w/v) Tris
  Adjust pH to 8.8 with HCl
  Final volume to 1 L using reverse osmosis water
  Sterilize by autoclaving and store at room temperature

Function: organic compound used in buffer solutions for electrophoresis gels; to maintain a stable pH to hinder shifts that may influence cellular factors.

10X Running buffer (pH 8.3) (2 L)

- 60.6 g 3% (w/v) Tris
- 288.2 g 14.4% (w/v) glycine

Final volume to 2 L using reverse osmosis water

Store at room temperature

Function: provides ions that carry a current and helps maintain the pH at a relatively constant value.

1X Transfer buffer (2 L)

- 200 ml 10% (v/v) 10X Running buffer
- 200 ml 10% (v/v) Methanol – assists with the dissociation of SDS from the protein and enhances the absorption of protein onto the membrane

Final volume to 2 L using reverse osmosis water

Store at 4 ˚C

10X TBS (Tris Buffered Saline) (2 L)

- 175.2 g 8.8% (w/v) NaCl
- 48.456 g 2.4% (w/v) Tris (50 mM)

Final volume to 2 L using reverse osmosis water

Adjust pH to 7.5 with HCl

Store at 4 ˚C (stable up to three months)

Function: stock solution

1X TBS (Tris Buffered Saline) (1 L)
• 100 ml 10% (v/v) 10X TBS Final volume to 1 L using reverse osmosis water Store at 4 ºC
Function: wash buffer; removes residual protein and prevents the formation of non-specific bands.

1X TBST (Tris Buffered Saline - 0.1% Tween) (1 L)
• 100 ml 10% (v/v) 10X TBS • 1 ml 0.1% (v/v) Tween 20 Final volume to 1 L using reverse osmosis water Store at 4 ºC
Function: wash buffer; the presence of Tween 20, a soap/detergent, helps to reduce non-specific protein-protein interaction without affecting antibody-antigen binding (blocking agent).

LB (Lysogeny Broth) medium (1 L)
• 20 g 2% (w/v) LB-broth Final volume to 1 L using deionised water Sterilize by autoclaving and store at room temperature
Function: Used for the growth of bacteria, since it is nutritionally rich medium.

Ampicillin stock (50 mg/ml) (15 ml)
• 0.75 g 5% (w/v) ampicillin Final volume to 15 ml using deionised water Filter sterilize using a 0.22-micron filter Store at -20 °C
Function: antibiotic

LB Agar Plates (250 ml)
• 11.25 g 4.5% (w/v) LB-agar Final volume to 250 ml using deionised water Sterilize by autoclaving Add ampicillin to a final concentration of 50 µg/ml
Function: creates a gel for the plated bacterial cultures to grow upon.

ADDENDUM E: Validation of the Hsp90 loading control expression consistency

When conducting Western blot analysis on target protein(s), a control is required to normalise to when quantifying the protein levels within a sample. This would allow for a more accurate representation of the levels of the protein(s) of interest and decrease the influence of human- or pipetting error (i.e., ensure equal loading of protein across all wells of the gel). In addition, it would compensate for if perhaps more protein was present in the one sample compared to the other. In theory, loading controls such as Hsp90 should not be regulated by different experimental conditions, as it is ubiquitously expressed in all eukaryotic cell types [223]. In the current study, we found that Hsp90 protein expression was minimally affected when exposed to the selected test compounds alone over various time points **(Fig. E1)** as well as stably expressed across all individual and combinatorial treatments for 48 hours **(Fig. E2)**. Therefore, we can confirm that using Hsp90 as loading control in the current study, proved to be a reliable method of standardizing our proteins of interest.

E1: The loading control was minimally affected in the time course study for each test compound alone

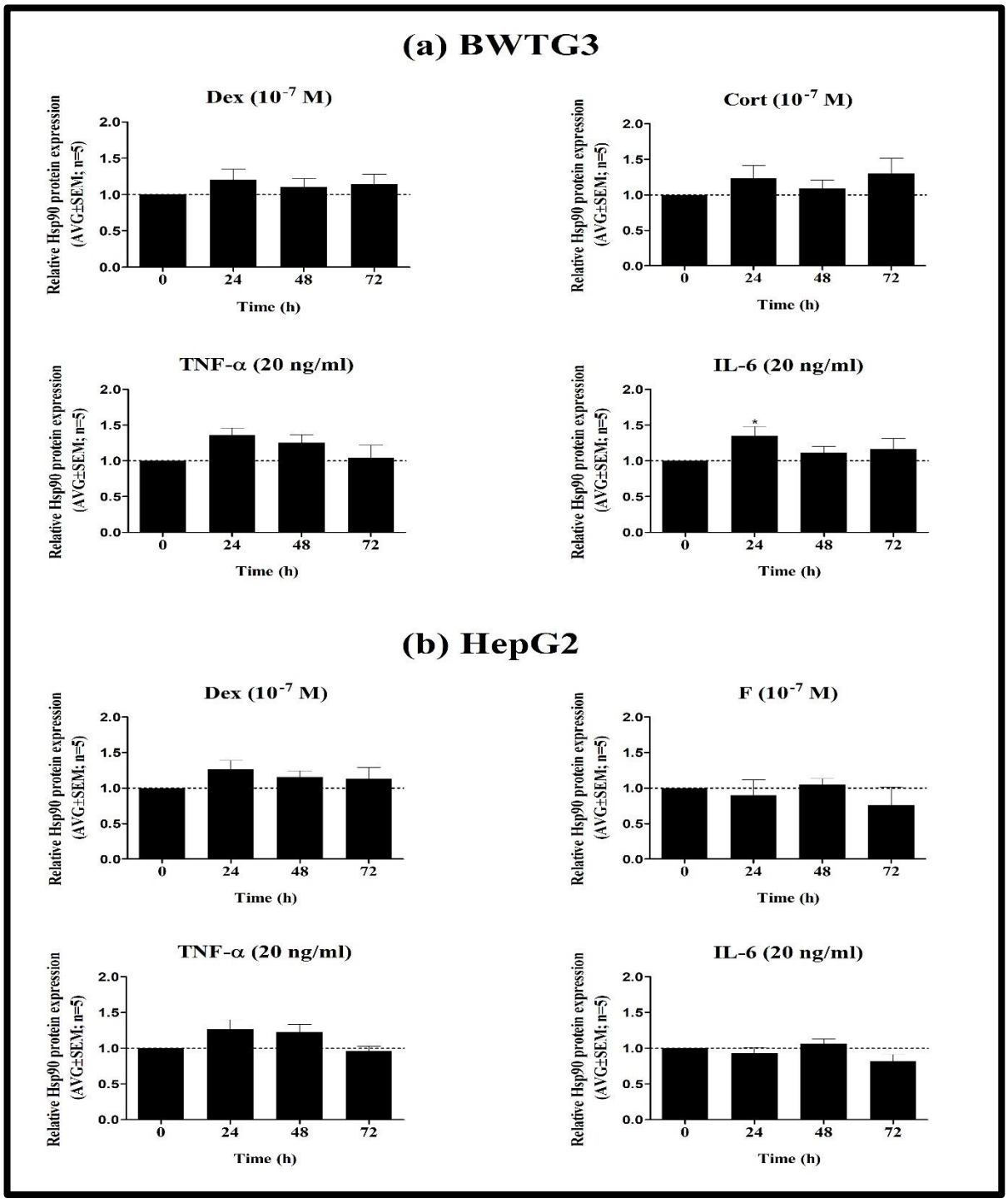


Figure E1. The loading control, Hsp90, was minimally affected across all time points by the selected test compounds in the (a) BWTG3 and (b) HepG2 cells. To verify the expression stability of the loading control, Hsp90, in response to individual treatment with dexamethasone (Dex), corticosterone (Cort), cortisol (F), TNF-α or IL-6 over a period of 24-, 48- and 72 hours (h), the data were plotted as the densitometry value for each treated sample relative to the densitometry value for the untreated sample (vehicle), which was set to 1 (represented by the black dotted line). Results displayed are representative of five independent experiments (± SEM). Statistical analysis was performed using one-way ANOVA with Dunnett's Multiple Comparison's post-test (*: $p<0.05$). No statistical differences ($p>0.05$) are not shown in the figures.

E2: The loading control was stably expressed across all individual and combinatorial treatments for 48 hours

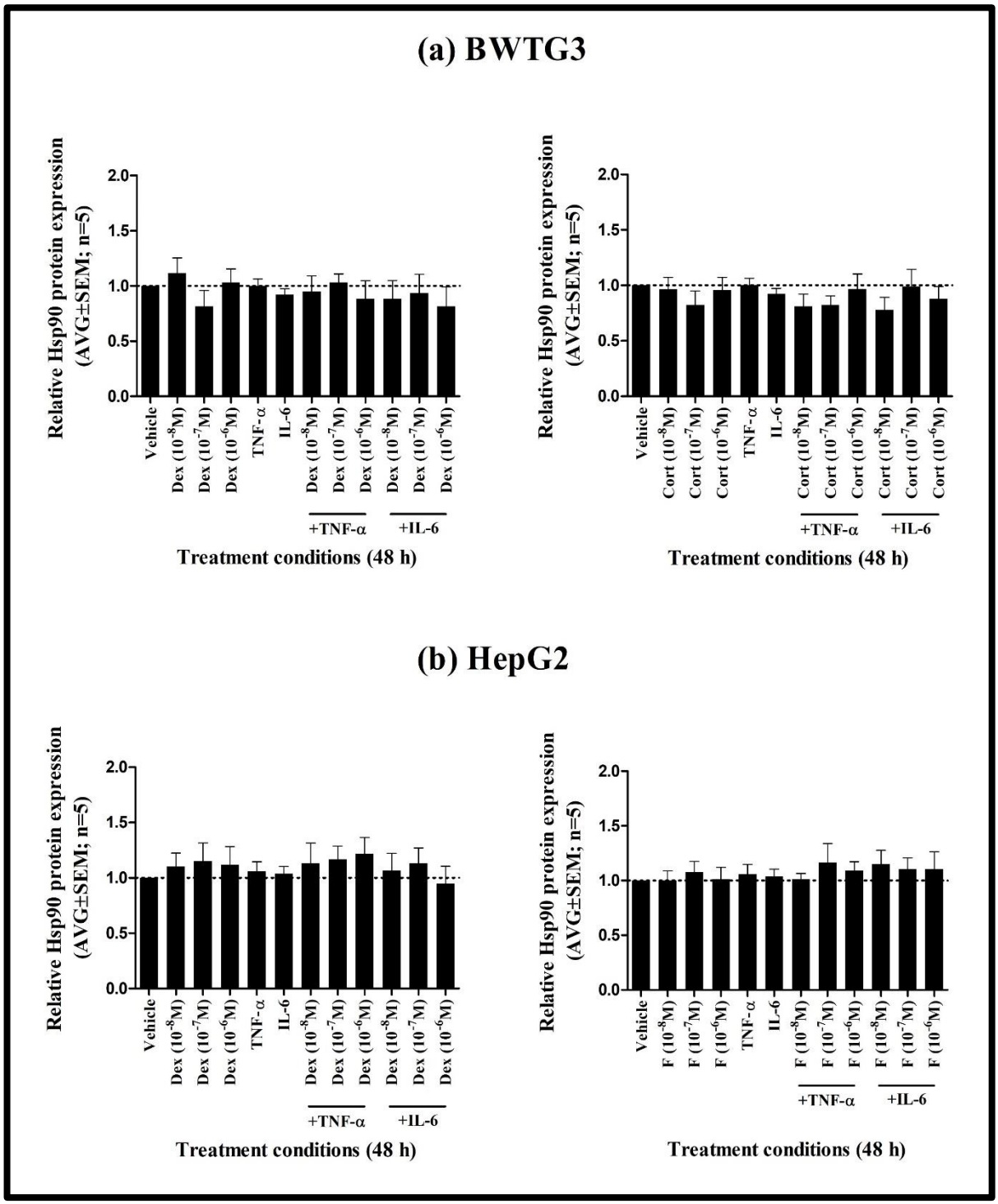


Figure E2. The loading control, Hsp90, was stably expressed across all treatments in the (a) BWTG3 and (b) HepG2 cells. To verify the expression stability of the loading control, Hsp90, in response to treatment with a panel of test compounds alone and in combination for 48 hours (h), the data were plotted as the densitometry value for each treated sample relative to the densitometry value for the untreated sample (vehicle), which was set to 1 (represented by the black dotted line). Results displayed are representative of five independent experiments (± SEM). Statistical analysis was performed using one-way ANOVA with Dunnett's Multiple Comparison's post-test. No statistical differences ($p>0.05$) are not shown in the figures.

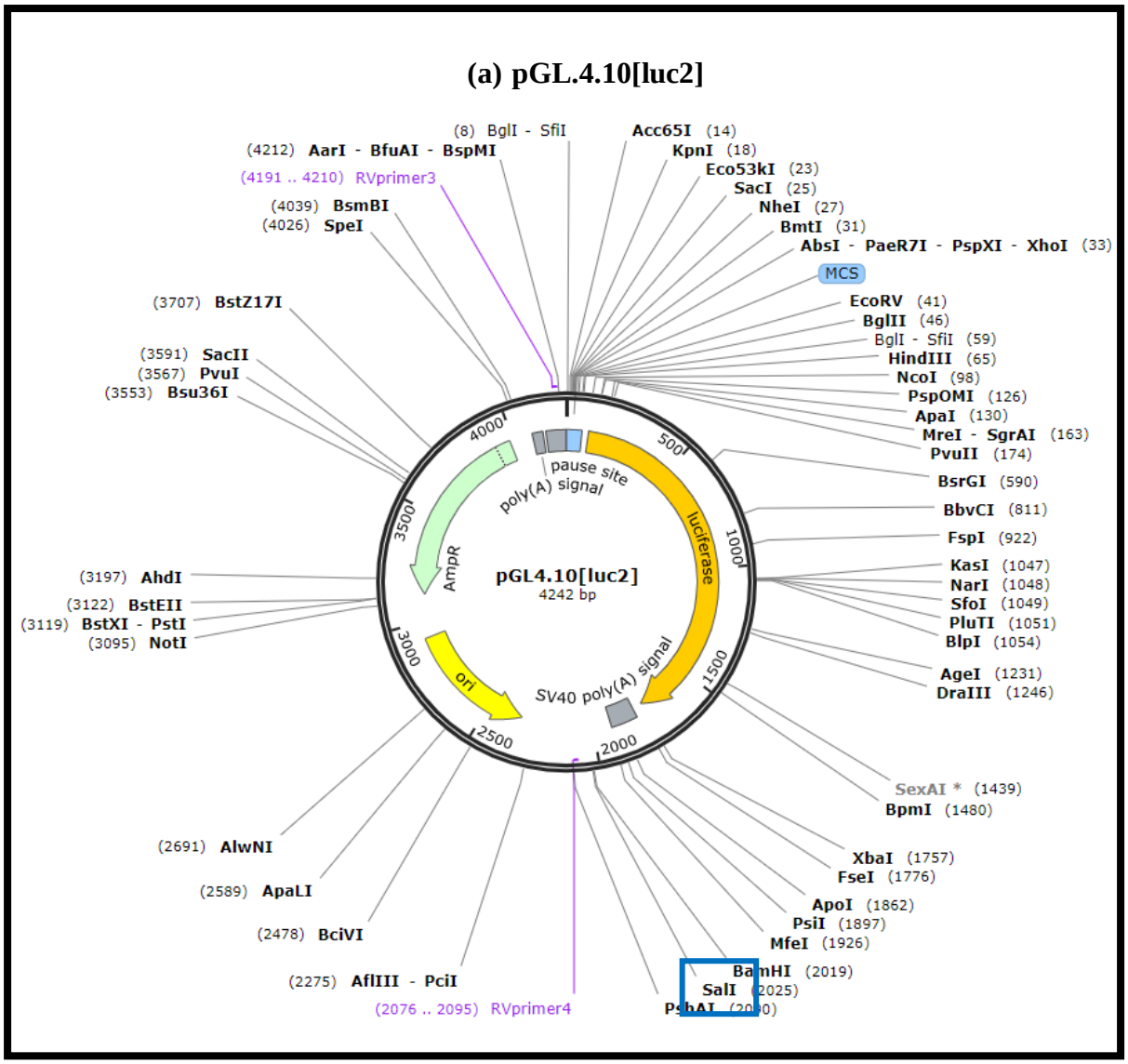
ADDENDUM F: Plasmid isolation and restriction enzyme digest

F1: List of plasmids used in the current study

Table F1. Promoter-reporter constructs (plasmids) used in this study.

Plasmid name	Vector	Vector + Insert Size (bp)	Source	Reference
PAI-1-Luc	**pGL.4.10[luc2]**	**4242 + 871 = 5113**	**Dr Yuki Kawarada** (Nagoya City University, Department of Cell Signalling, Graduate School of Pharmaceutical Sciences, Japan)	[205]
WT CRP -300	**pGL2-Basic Vector**	**5598 + 299 = 5897**	**Dr Alok Agrawal** (East Tennessee State University, Department of Biomedical Sciences, United State of America)	[185]

F2: Luciferase reporter vector maps



(Figure legend on next page)

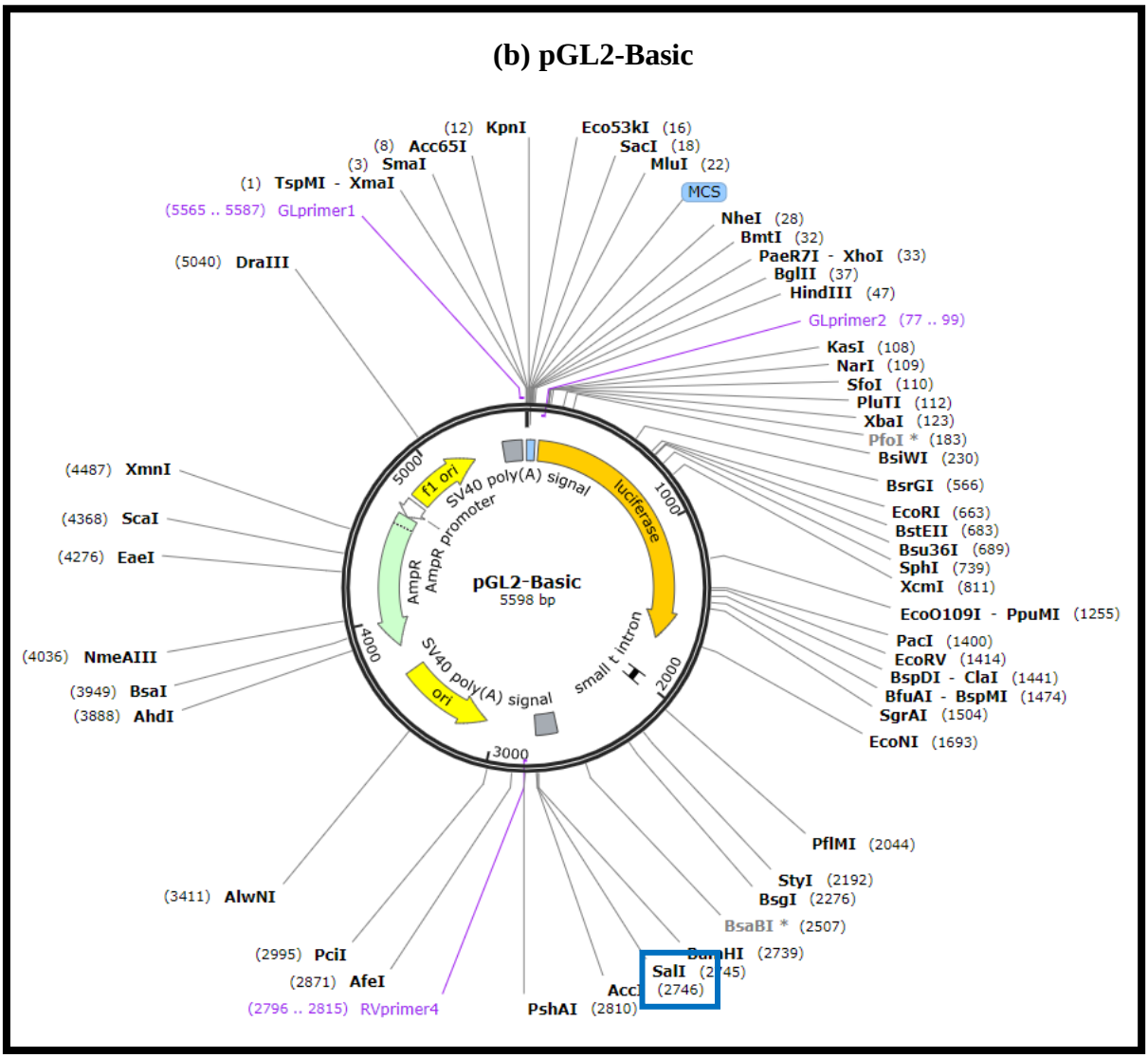


Figure F1. Luciferase reporter vector maps. The PAI-1-Luc and WT CRP -300 plasmids were generated by ligating a part of the human PAI-1 or CRP promotor region with the (a) pGL.4.10[luc2] or (b) pLG2-Basic luciferase reporter vectors, respectively. Various restriction enzyme binding sites are displayed in each image. The luciferase vector map (back bone) for each plasmid used in the current study was created using SnapGene®. The restriction enzyme site where the SalI restriction enzyme would linearize each plasmid is boxed in blue. *Note: AmpR = ampicillin resistant, bp = base pairs, ori = origin of replication.*

F3: Plasmid Isolation

To investigate which regulatory regions in the promotor of the APPs, PAI-1 and CRP might play a role in GC and/or pro-inflammatory cytokine-induced regulation, plasmid DNA encoding the minimal PAI-1-Luc and proximal WT CRP -300 promotor were isolated and purified.

Table F2. The concentration, A_{260}/A_{280} ratio and yield of isolated plasmid constructs.

	Concentration (ng/µl)	$A_{260/280}$	Yield (µg)
PAI-1-Luc	812	1.85	541
CRP WT -300	985	1.83	492

$A_{260}/_{280} \geq 1.8$: Pure DNA

$A_{260}/_{280} \geq 2$: Contamination with RNA

$A_{260}/_{280} \leq 1.8$: Contamination with protein and aromatic substances

The A_{260}/A_{280} ratio indicate that pure, good-quality DNA was obtained. The plasmid isolation NucleoBond Xtra Maxiprep System used has an expected yield of up to 1000 µg of plasmid DNA per preparation. In each case a loss of yield (half of expected yield) was observed when isolating both PAI-1-Luc and WT CRP -300 plasmids, as displayed in **Table F2**. It is possible that the other half of the expected yield was lost due to vigorous pipetting.

The success of further applications, such as transfection of cells followed by a promoter-reporter assay, are dependent on the yield and purity of the isolated plasmid DNA. To assess if any DNA contamination occurred during the process of plasmid isolation, the absorbance readings of each plasmid were measured at 260- and 280 nm, using a BioDrop spectrophotometer. Both DNA and RNA absorb UV light at 260 nm, while proteins containing aromatic amino acids absorb UV light at 280 nm. Therefore, if either or both contaminants (RNA or proteins) were present in the plasmid DNA samples, it would contribute to their total absorbance. Pure DNA has an expected A_{260}/A_{280} ratio of ≥1.8. An A_{260}/A_{280} ratio of 1.92 and 1.89 was obtained for PAI-1-Luc and WT CRP -300, respectively. These values indicate that pure, good-quality DNA was obtained.

F4: Restriction Enzyme Digest of the DNA plasmid constructs used in the current study

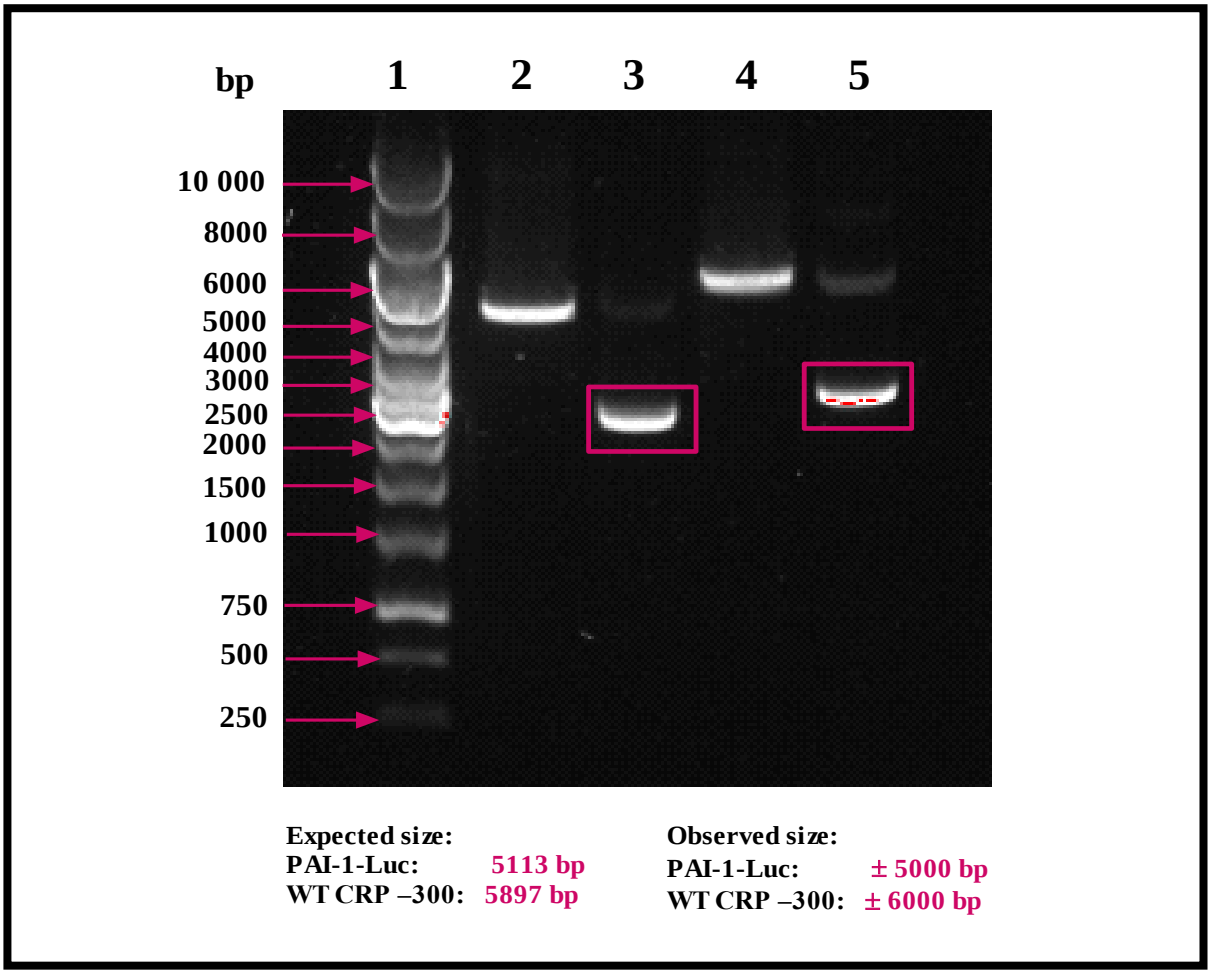


Figure F2. 0.5% Agarose gel containing Nancy-520 stain. The quality and integrity of the plasmid DNA for all constructs was assessed using restriction enzyme digestion and visualised on 1% agarose gel. Lane 1: O'GeneRuler 1 kb Plus DNA Ladder, Lane 2: PAI-1-Luc (single digest), Lane 3: PAI-1-Luc (undigested), Lane 4: WT CRP -300 (single digest), Lane 5: WT CRP -300 (undigested). Pink boxes represent the presence of supercoiled plasmid DNA.

Since pure plasmid DNA was obtained, next the quality and integrity (i.e., supercoiled, linear or nicked) of the plasmid DNA was determined. For this reason, a restriction enzyme (RE) digest was performed using the restriction enzyme, SalI-HF. Subsequently, agarose gel electrophoresis, using a 0.5% agarose gel, was performed to analyze the digested and undigested plasmid DNA **(Fig. F2)**. The plasmid DNA would migrate differently in the agarose gel, depending on the size of the DNA fragment and its integity. Together with the PAI-1-Luc or WT CRP -300 inserts, each plasmid vector should have a size of 5113- and 5897 bp, respectively.

Both plasmid sequences have a single recognition site for SalI, hence why this specific RE was chosen. Theoretically a single band was expected after both plasmids were subjected to a single digestion with the RE. Bands observed in lanes 2 and 4 are of correct size. In order for the BWTG3 cells to efficiently take up DNA, the isolated plasmid DNA should be supercoiled. Supercoiled DNA would migrate faster through the pores of the agarose gel as it is small and compact. Therefore, with the undigested plasmids we expected a single band, further down the agarose gel compared to the digested plasmids, to indicate their supercoiled integrity. A single band in lanes 3 and 5 **(boxed in pink in Fig. F2)** was observed, which appeared to represent mostly supercoiled DNA, although linear DNA is also present. Therefore, it is possible that each plasmid DNA was sheared due to pipetting too harshly.

ADDENDUM G: Summary of results

Table G1. Summary of the overall effects of single and co-treatment with GCs and/or pro-inflammatory cytokines on PAI-1 and CRP, at mRNA, promotor, and intracellular protein level, in BWTG3- and HepG2 cells relative to the vehicle. Data obtained from Figures 3.1-3.16.

	PAI-1				CRP			
	24 hours		48 hours		24 hours		48 hours	
	mRNA (BWTG3)	Promotor activity (BWTG3)	Protein (BWTG3)	Protein (HepG2)	mRNA (BWTG3)	Promotor activity (BWTG3)	Protein (BWTG3)	Protein (HepG2)
Dex (10^{-8} M)	-	↑ ***	↑ *	-	-	↑ ***	↑ *	↑ ***
Dex (10^{-7} M)	-	↑ **	↑ **	-	-	↑ ***	↑ ***	↑ ***
Dex (10^{-6} M)	-	↑ *	↑ **	-	-	↑ ***	↑ ***	↑ **
TNF-α (20 ng/ml)		↑ ***	↑ ***	↑ ***		↑ ***	↑ ***	↑ ***
IL-6 (20 ng/ml)	↑ **	↑ ***	↑ **	↑ **	-	↑ ***	↑ ***	↑ ***
Dex (10^{-8} M) + TNF-α	↑ ***	↑ **	↑ *	-	↑ **	↑ ***	↑ *	↑ ***
Dex (10^{-7} M) + TNF-α	-	↑ *	↑ *	-	-	↑ ***	↑ ***	↑ **
Dex (10^{-6} M) + TNF-α	↑ *	↑ ns	↑ ns	↓ **	↑ *	↑ ***	↑ **	↑ **
Dex (10^{-8} M) + IL-6	-	↑ *	↑ *	-	-	↑ ***	↑ **	↑ **
Dex (10^{-7} M) + IL-6	-	↑ ***	↑ **	-	↓ **	↑ ***	↑ **	↑ ***
Dex (10^{-6} M) + IL-6	-	↑ **	↑ ns	↓ ns	↑ *	↑ ***	↑ **	↑ ***
Cort/F (10^{-8} M)	ND	ND	-	↑ ***	ND	ND	↑ ***	↑ **
Cort/F (10^{-7} M)	ND	ND	↑ **	-	ND	ND	↑ ***	↑ ***
Cort/F (10^{-6} M)	ND	ND	↑ *	↑ *	ND	ND	↑ **	↑ **
TNF-α (20 ng/ml)	ND	ND	↑ ***	↑ ***	ND	ND	↑ ***	↑ **
IL-6 (20 ng/ml)	ND	ND	↑ **	↑ ***	ND	ND	↑ ***	↑ ***
Cort/F (10^{-8} M) + TNF-α	ND	ND	↑ *	↑ ***	ND	ND	↑ *	-
Cort/F (10^{-7} M) + TNF-α	ND	ND	↑ ***	↑ ***	ND	ND	↑ *	↑ ns
Cort/F (10^{-6} M) + TNF-α	ND	ND	↑ ***	↑ ***	ND	ND	↑ *	↑ *
Cort/F (10^{-8} M) + IL-6	ND	ND	↑ ***	↑ ***	ND	ND	↑ **	-
Cort/F (10^{-7} M) + IL-6	ND	ND	↑ ***	↑ ***	ND	ND	↑ **	↑ **
Cort/F (10^{-6} M) + IL-6	ND	ND	↑ **	-	ND	ND	↑ *	↑ *

↑ Increase ↓ Decrease - No Effect Inconclusive ND Not Determined

One-way ANOVA with Dunnett's Multiple Comparison's post-test was used when comparing each treatment to the vehicle control (***: $p<0.001$, **: $p<0.01$, *: $p<0.05$, ns: $p>0.05$).