Cardiac Injury in Lupus

Lauren Therese Bourke

Department of Medicine

University College London

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Declaration

I Lauren Bourke confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Date:

Abstract

Systemic lupus erythematosus (SLE) carries a significantly enhanced risk of developing cardiovascular disease (CVD) and remains a leading cause of death in these patients, accounting for ~25% of all causes of mortality. Although there is clear evidence linking accelerated atherosclerosis to SLE (and consequently an increase in cardiovascular events), another factor that may contribute to CVD related morbidity and mortality is reperfusion injury that occurs post-ischaemia. This is termed ischaemic / reperfusion (I/R) injury and is a known important contributor to the size of the eventual infarct in the heart, which in animal studies has been shown to account for up to 40-50% of the final infarct size.

Hydroxychloroquine (HCQ), originally an anti-malarial drug, is now used to treat autoimmune disorders, including SLE. HCQ has been shown to modulate inflammation in rheumatic diseases such as SLE and rheumatoid arthritis as well as have potential cardiovascular benefits in these patients. One of the keys aims of this thesis was to explore the potential use of HCQ in reducing cardiac I/R injury. HCQ was found to be cardioprotective in an *in vitro* neonatal cardiomyocytes simulated I/R injury model as well as in an *in vivo* cardiac I/R injury model. This was found to be through an ERK-dependent mechanism which was blocked in the presence of the ERK inhibitor U0126 both *in vitro* and *in vivo*.

Another relevant question addressed in this thesis was if I/R injury is enhanced in lupus. There is evidence from an autoimmune prone mouse model that lupus IgG are pathogenic in mesenteric I/R injury. However, no study as yet has investigated human lupus IgG in a heart model. IgG was purified from the serum of SLE patients (aPL +ve vs aPL –ve), antiphospholipid syndrome (APS) patients, juvenile onset SLE (JSLE) patients and healthy volunteers. The pre-treatment of neonatal rat cardiomyocytes with IgG from all 3 patient groups enhanced simulated I/R injury. However, the most pathogenic were those who were aPL positive. Interestingly, JSLE patients who were all aPL negative, enhanced I/R injury to similar levels as those who tested positive in the adult patient cohort. An enhanced p38 MAPK phosphorylation was observed in the presence of aPL positive IgG and this pathogenic effect was blocked in the presence of the p38 inhibitor SB23580.

The results obtained in this thesis have identified a potential role for HCQ in the cardiovascular field as a cardioprotective therapeutic in myocardial I/R injury. Additionally, IgG purified from patients with SLE, APS and JSLE have been shown to accelerate myocardial I/R injury.

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Publications

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Contents **Page Number**

List of Figures

Chapter I

Chapter III

Chapter IV

List of Tables

Chapter 1

Chapter II

Abbreviations

1D-PAGE	1 dimensional-polyacrylamide gel electrophoresis
3-MA	3-Methyladenine
β_2GPI	beta-2 glycoprotein I
aCL	anticardiolipin
ACR	American College of Rheumatology
ADCC	Antibody-dependent cell mediated cytotoxicity
AIF	Apoptosis-inducing factor
Akt	Protein kinase B
AMISTAD	Acute Myocardial Infarction Study of Adenosine
ANA	Antinuclear antibody
AP-1	Activator protein 1
Apaf1	Apoptotic protease activating factor 1
APC	Antigen presenting cell
aPL	Antiphospholipid antibodies
APS	Antiphospholipid syndrome
Atg	Autophagy-related gene
ATP	Adenosine triphosphate
AVN	Avascular bone necrosis
BAD	Bcl-2-associated death promoter
BAFF	B cell activating factor
BAK	Bcl-2 homologous antagonist killer
BAX	Bcl-2-like protein 4
Bcl-2	B cell lymphoma 2

BH	Bcl-2 homology
BID	BH3 interacting-domain death agonist
BILAG	British Isles Lupus Assessment Group
BIM	B cell lymphoma 2 interacting mediator of cell death
BlyS	B-lymphocyte stimulator
c-FLIP	cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein
CAD	Caspase activated DNase
CAPS	Catastrophic antiphospholipid syndrome
CARD	Caspase recruitment domain
CDC	Complement-dependent cytotoxicity
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CPK	Creatine phosphokinase
CREB	cAMP-response element-binding
CVD	Cardiovascular disease
DCs	Dendritic cells
DIL	Drug-induced lupus
DISC	Death-inducing signalling complex
DLE	Discoid lupus erythematosus
DNA	Deoxyribonucleic acid
dsDNA	double stranded DNA
EBV	Epstein Barr Virus
ECLAM	European Consensus Lupus Activity Measure
ELK1	ETS domain-containing protein
EMA	European Medicines Agency
ENAs	Extractable nuclear antigens
eNOS	endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
EULAR	The European League Against Rheumatism
FADD	Fas-Associated protein with Death Domain
FADH	Flavin adenine dinucleotide
FasL	Fas ligand

FasR	Fas receptor
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
GSLs	Glycosphingolipids
GSK-3β	Glycogen synthase kinase-3 beta
H202	Hydrogen peroxide
HCQ	Hydroxychloroquine
HDL	High-density lipoproteins
HLA	Human leukocyte antigen
HLA-DR	Human leukocyte antigen receptor
HPLC	High performance liquid chromatography
I/R	Ischaemia/reperfusion
IAP	Inhibitor of apoptosis protein
ICAD	Inhibitor of caspase activated DNase
IFN	Interferon
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1	Interleukin 1
IL-8	Interleukin 8
IP	Intraperitoneal
IPC	Ischaemic pre-conditioning
JNK	c-Jun N-terminal kinase
JSLE	Juvenile onset systemic lupus erythematosus
LA	Lupus anticoagulant
LC3	Microtubule-associated protein 1A/1B-light chain 3
LDL	Low-density lipoproteins
LN	Lupus nephritis
mAb	monoclonal antibody
MAPK	Mitogen-activated protein kinase
Mdm2	Mouse double minute 2 homolog
MEK1/2	Mitogen-activated protein kinase
MI	Myocardial Infarction
MPTP	Mitochondrial permeability transition pore

MRI	Magnetic resonance imaging
NADH	Nicotinamide adenine dinucleotide
NO	Nitric oxide
NO	Nitrogen oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
$ONOO.^-$	Peroxinitrite anion
p38	p38 mitogen-activated protein kinases
P90RSK	p90 ribosomal S6 kinase
PCTs	Placebo controlled trials
PDK1	Phosphoinositide-dependent kinase-1
PI3K	Phosphoinositide 3-kinase
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PKA	Protein kinase A
PM	Pregnancy morbidity
Puma	p53 up regulated modulator of apoptosis
RA	Rheumatoid Arthritis
RIP	Receptor interacting protein
RIPK3	Receptor-interacting protein kinase 3
RISK	Reperfusion injury salvage kinase
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT-PCR	Real time polymerase chain reaction
SLAM	Systemic Lupus Activity Measure
SLE	Systemic Lupus Erythematosus
SLEDAI	Systemic Lupus Erythematosus Disease Activity Index
Sm	Smith
Smac	Second mitochondria-derived activator of caspases
SNP	Single nucleotide polymorphism
snRNP	Small nuclear ribonucleoprotein
SOD	Superoxide dismutase
ssDNA	single stranded DNA
STAT1	Signal transducers and activators on transcription 1
Th1/2	T helper cell 1/2

TLRs	Toll like receptors
TNF-α	Tumour necrosis factor alpha
TNFR1	Tumour necrosis factor receptor
TRADD	Tumour necrosis factor receptor type 1-associated DEATH domain protein
TTC	Tetrazolium chloride
UV	Ultraviolet
VT	Venous and arterial thrombosis
WHO	World Health Organisation
XIAP	X-linked inhibitor of apoptosis protein

CHAPTER I

INTRODUCTION

Overview of Chapter I

This chapter aims to give an overview of old and current literature that encompasses the role of cardiac injury within lupus. It will be split into two clear sections; the first I/R injury and the second SLE. Each section will detail the pathogenesis, clinical features and current treatments available. It will then conclude by summarizing the role of I/R injury in SLE and potential overlaps in relation to pathogenesis for the two.

1.1 Ischaemia/reperfusion Injury

1.1.1 Myocardial Infarction

Cardiovascular disease (CVD) is a leading cause of morbidity and mortality in the world and in 2012 it was reported by the World Health Organisation (WHO) that an estimated 17.5 million people died as a result of CVD, primarily through myocardial infarction (MI) and stroke. The latest WHO estimates suggest that by 2030 around 23 million people will die annually due to CVD (World Health Organisation 2012).

Survivors of MI and indeed stroke often suffer post-infarct morbidity due to irreversible tissue damage, which causes major organ dysfunction and consequently significant functional compromise. Thus, the size of the resulting infarcted tissue is critical in defining the outcome. Patients who present with an acute MI require urgent myocardial reperfusion either via thrombolysis or primary cutaneous coronary angioplasty which should be administered as early as possible (Bassand J 2005). Despite widespread and timely use of these procedures, morbidity and mortality rates remain high. Therefore the development of new strategies for cardioprotection which are able to reduce infarct size and hence improve clinical outcomes is still an unmet need (Topol 2003).

1.1.2 Myocardial ischaemia/reperfusion (I/R) Injury

Occlusion of the coronary artery results in myocardial cell death primarily through necrosis during the ischeamia phase. Reperfusion of the coronary artery, whilst being an essential requirement in order to restore cardiac function, can paradoxically result in further myocardial injury and an increase in infarct size (Eefting F 2004). This phenomenon of reperfusion-induced damage was first described in 1960 and was termed ischaemia/reperfusion (I/R) injury (Jennings RB 1960). Reperfusion-induced injury is due to cell death of cardiac myocytes which were viable until blood flow was restored following ischaemia. Animal studies have suggested that I/R injury may account for 40-50% of the final infarct size post-MI (Yellon 2007; Simonis G 2012). Although medical reperfusion following ischaemia can be successful, cardiac failure after reperfusion stands at 25% and mortality due to I/R injury estimated at 10% (Keeley EC 2003). Understanding the molecular mechanisms that mediate I/R injury and identification of potential targets to modulate this has led to

a number of therapeutic candidates that are currently under development. The publication of the AMISTAD (Acute Myocardial Infarction Study of Adenosine) study showed for the first time, in a multi-center trial, that a drug targeting I/R injury could reduce infarct size in humans (Mahaffey KW, Bolli R et al. 1999).

I/R injury targets oxygen dependent cells within tissue and organs including the heart, liver and brain, which can be exposed to either periods of cold or warm ischaemia. Typically cold ischaemia occurs during transplantation after organ harvesting, when static cold preservation occurs (Salahudeen 2004). In contrast, warm ischaemia results following organ trauma such as stroke and MI. The cell populations that are affected will have varying degrees of resistance to the different types of ischaemia (Halazun K 2007). An example is cardiac endothelial cells which are relatively resistant to warm ischaemia (Pompilio G 1997), whereas hepatocytes in the liver are more sensitive (Berthiaume F 2009). Whilst organs can tolerate prolonged cold or warm ischemia, it is during reperfusion that significant cellular damage can occur through mechanisms such as apoptosis-induced cell death.

Sections 1.1.3 and 1.1.4 will outline the main mechanisms that are thought to regulate cell death in I/R injury and are summarised in figure 1.1.

1.1.3 Myocardial Ischaemia Injury

Under normal conditions all cells require energy supplied via mitochondrial oxidative phosphorylation and therefore cannot be independently supported by anaerobic glycolysis (Jassem 2004). Consequently, during ischaemia the absence of oxygen causes oxidative phosphorylation to be halted, resulting in mitochondrial membrane depolarization, ATP depletion and inhibition of mitochondrial contractile function. The absence of oxygen leads to anaerobic glycolysis being switched on, lactate accumulation, and a subsequent lowering of the intracellular pH to less than 7.0 (Hausenloy 2013). In turn, this leads to an increase in the number of protons causing the Na^+-H^+ ion exchanger to be activated, preventing protons from entering the cell in exchange for Na^+ being transferred in. Furthermore, a decrease in ATP during ischaemia inhibits Na^+-K^+ ATPase causing further intracellular Na^+ overload. In response to this Na^+ influx, the $2Na^+$-Ca^{2+} ion exchanger is activated resulting in intracellular Ca^{2+} overload as the cell attempts to remove this excess Na^+ (Avkiran and Marber 2002). ATP depletion can lead to morphological changes such as cytoskeleton depletion and formation of blebs at the cell surface. If ischaemia persists irreversible damage occurs

which is mainly associated with mitochondrial swelling, severe damage to plasma membrane and swelling of lysosomes (Buja 1998). A consequence of dysfunction of the energetic machinery of the cell (mitochondrial oxidative powerhouse and glycolytic pathway) is irreversible and therefore even if the cellular energetic machinery were to be restored, the irreparable damage to the cell membrane and genome leads to cell death usually via necrosis. However, when blood flow is restored to cells that have been made ischaemic but have yet to die, this can be beneficial but in contrast injury can actually be exacerbated. As a result, reperfusion can actually cause additional cell death and the mechanisms through which this can occur will be discussed in the following section.

1.1.4 Myocardial Reperfusion Injury

1.1.4.1 The Inflammatory Response: Neutrophils and The Complement Pathways

Cardiac I/R injury causes attraction, activation, adhesion and migration of neutrophils to the site of injury resulting in local as well as remote organ damage. This damage is via the release of inflammatory mediators such as cytokines and ROS as well as the local activation of the complement system (Dong, 1999).

There are three main pathways in the complement system, which can be activated through different mechanisms. The classical pathway is regulated by antigen-antibody interaction, which activates C1q, followed by C2- and C4-dependent cleavage of C3 (by C3 convertase) leading to cleavage of C5 (by C5 convertase). In the lectin pathway, serum mannose binding lectin (MBL), which is homologous to C1q, recognizes microbial surface mannose and triggers activation of MBL-associated proteases (MASP1-3). This interaction leads to the same activation of C3 and C5. The final pathway is the alternative pathway which is activated by presence of lipopolysaccharide (LPS) leading to C3 binding to factor B and forms a complex which is cleaved to form the alternative C3 convertase. Properdin amplifies this activation and stabilizes the complex allowing cleavage of C3b and formation of the alternative C5 convertase. All three pathways lead to C3 and C5 cleavage resulting in C5a and C5b-9 being produced. These main components of the complement system are believed to be responsible for complement pathway mediated cardiac I/R injury (Padua R 1998).

The classical complement pathway is the most extensively studied in I/R injury, and studies have suggested that pre-existing natural IgM may be responsible for activating this complement-mediated I/R injury (Zhang M 2004). These self-reactive natural antibodies may participate in the early stages

of I/R injury by being recruited to the site of injury when ischaemic antigens become exposed. Recruitment of natural IgM antibodies to these neoepitpes could be responsible for subsequent activation of complement (Zhang M 2006). The identification of natural IgM antibodies that cause I/R injury provides a crucial link between initiation and activation of complement after reperfusion of ischaemic tissue. This could subsequently lead to identification of neoantigens formed during I/R for IgM antibodies to bind to and therefore identify potential new therapeutic targets.

Although these neoepitopes that natural IgM antibodies bind to are still being identified, there have already been some experimental studies which have shown a significant reduction in MI. These studies have involved therapeutic strategies designed to inhibit the inflammatory process at the time of myocardial reperfusion. This includes using antibodies against cell-adhesion molecules (Ma X 1991; Hayward R 1999) and inhibiting activation of the complement pathway (Vakeva A 1998). However, studies in a clinical setting targeting inflammation pathways have had largely negative outcomes (Investigators 2007; Atar D 2009). Whilst inhibiting inflammatory pathways may not be able to reduce cardiac I/R injury alone, these mechanisms clearly contribute to injury in combination with other factors.

1.1.4.2 Reactive Oxygen Species

Fatty acids are the major fuel choice of cardiac tissue for the cardiac mitochondria supply of acetyl-coenzyme A (acetyl-CoA). Beta oxidation of fatty acids generates acetly-CoA as well as nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). This is then fed into the Krebs cycle and used to generate further NADH and $FADH_2$ (Jassem 2004). However, when the supply of oxygen is reduced the amount of NADH and $FADH_2$ produced is decreased leading to insufficient energy production for regular cardiac function. To try and counteract this, NADH and $FADH_2$ are transported to the mitochondria to undergo oxidative phosphorylation, therefore allowing generation of ATP. Unfortunately, as oxygen is the terminal electron acceptor in complex 4, NADH and $FADH_2$ cannot be metabolised due to the lack of oxygen, therefore preventing ATP production and so tissue injury begins (Giordano 2005).

During oxidative phosphorylation, electrons are usually and sequentially transferred to oxygen, which along with protons being pumped across the membrane, produce water molecules. Occasionally ‘electron leakage’ occurs, whereby oxygen molecules with odd numbers of electron in their outermost electron shell are formed, these are highly reactive and are known as reactive oxygen

species (ROS) (Giordano 2005). The oxygen molecules produced include $O_2^{\cdot}$ (a superoxide anion) which is formed due to the donation of a single electron, a process which is catalysed by xanthine oxidase (Li 2002). Additionally, the superoxide anion can accept a further electron to form a peroxide anion and can become protonated to form hydrogen peroxide H_2O_2 (Giordano 2005). This process would normally be catalysed by the anti-oxidant enzyme superoxide dismutase (SOD), leading to catalysis of H202 to water. There are also other free radical species which the heart produces which make up another group called reactive nitrogen species (RNS). This includes superoxide anion combining with nitrogen oxide (NO) to produce peroxinitrite $ONOO^{\cdot-}$ (Li 2002; Giordano 2005). In I/R injury ROS production becomes excessive and therefore the cellular physiological mechanisms for lowering ROS levels are overwhelmed, leading to cell injury. This can be severe and is known to lead to extensive cell death (Stowe 2009). There are three pathological responses that have been shown to occur as a consequence of the elevated ROS levels; lipid peroxidation of membranes, nuclear and mitochondrial DNA fragmentation and sulfhydryl-mediated protein cross-linkage. All three processes contribute to cell injury but the severity depends on the time and strength of the I/R injury insult (Venardos K 2007).

The generation of ROS during ischaemia can be inhibited using pharmacological inhibitors of mitochondrial electron transport, with studies suggesting complex III being the main site of ROS production in ischaemia (Chen Q 2003). However, interestingly it has been shown that these same inhibitors have little effect on ROS production during reperfusion suggesting they are produced via distinct mechanisms in the individual stages of I/R injury (Becker 2004).

1.1.4.3 Intracellular Calcium Overload

Reperfusion allows recovery of the mitochondria, therefore restoring the mitochondrial membrane potential that is responsible for calcium (Ca^{2+}) entry via the mitochondrial Ca^{2+} uniporter. This influx of Ca^{2+} subsequently induces the opening of the mitochondrial permeability transition pore (MPTP, see section 1.1.4.5). Studies have shown that pharmacologic antagonists of the sarcolemma Ca^{2+} channel (Herzog W 1997) or the mitochondrial Ca^{2+} uniporter (Miyamae M and Figueredo 1996), administered at the onset of myocardial reperfusion reduces MI size by up to 50%. However, not all experimental studies targeting Ca^{2+} influx as a therapeutic strategy have yielded positive results. Clinical studies of calcium channel blockers administered at the onset of myocardial reperfusion have shown no overall beneficial effects (Bär F 2006).

1.1.4.4 Restoration of Physiological pH

During ischaemia, intracellular pH decreases to below 7.0 due to anaerobic glycolysis producing lactic acid (Hausenloy 2013). However, during reperfusion, the washout of lactate and activation of the Na^+-H^+ exchanger restores physiological pH. This shift in pH causes MPTP opening, therefore contributing to cardiomyocyte death (Hausenloy 2013). Potential treatments to target this defect include utilizing an acidic buffer which has been shown to reduce the cardiac infarct size (Qian T 1997), as well as slowing the recovery of physiological pH using Na^+H^+ exchanger inhibitors (Avkiran and Marber 2002).

1.1.4.5 Mitochondrial permeability transition pore

The mitochondrial membrane potential is controlled by the MPTP, which is a large, non-specific pore, spanning the inner and outer mitochondrial membrane (Clerk A 2003). In ischaemia the MPTP remains closed due to the low pH within the cell but during the first few minutes of reperfusion the pores open as the pH is restored. This leads to an increase in proton levels and ROS production, as well as continued inhibition of ATP synthesis (Griffiths EJ 1995). The transition pores formed within the mitochondria mean that cytosolic ATP can gain access to mitochondrial ATPase, contributing to further ATP loss (Weiss J 2003). Sustained opening of the pore leads to the proton gradient and electrical potential across the inner membrane to collapse causing oxidative phosphorylation uncoupling. Additionally, under the force of osmotic pressure the matrix swells, and whilst the inner membrane is able to adapt through cristae remodeling the outer membrane is unable to and so eventually ruptures (Gustafsson AB 2008). This means that pro-apoptotic mediators such as cytochrome c, apoptosis-inducing factor (AIF) and endonuclease G are released into the cytosol, which further induces apoptosis pathways (Yang JC 1998). This injury process is enhanced by a decrease in DNA synthesis and repair leading to cell death (Eefting F 2004). Potential therapeutic strategies include preventing MPTP opening during reperfusion by administering MPTP inhibitors such as the immunosuppressant cyclosporine A (CsA). This is known to bind to and inhibit cyclophilins, which regulate protein folding *in vivo*. For immunosuppression CsA binds cyclophilin A preventing translocation of calcineurin, thereby inhibiting translocation of transcription factors and reducing interleukin-2 (IL-2) production. However, in the setting of the heart CsA been shown to bind to cyclophilin D, a regulator in MPTP opening. The first studies in isolated rat hearts with CsA showed inhibition of MPTP through outputs such as maintained calcium levels and membrane potential (Haunsenloy 2002). Further studies have shown that CsA can reduce MI size by 40-50% in

both small and large animal MI models (Hausenloy D 2003; Skyschally A 2010). However, due to the adverse side effects and non-specific selectivity of CsA for the MPTP a more specific and novel MPTP inhibitor needs to be developed.

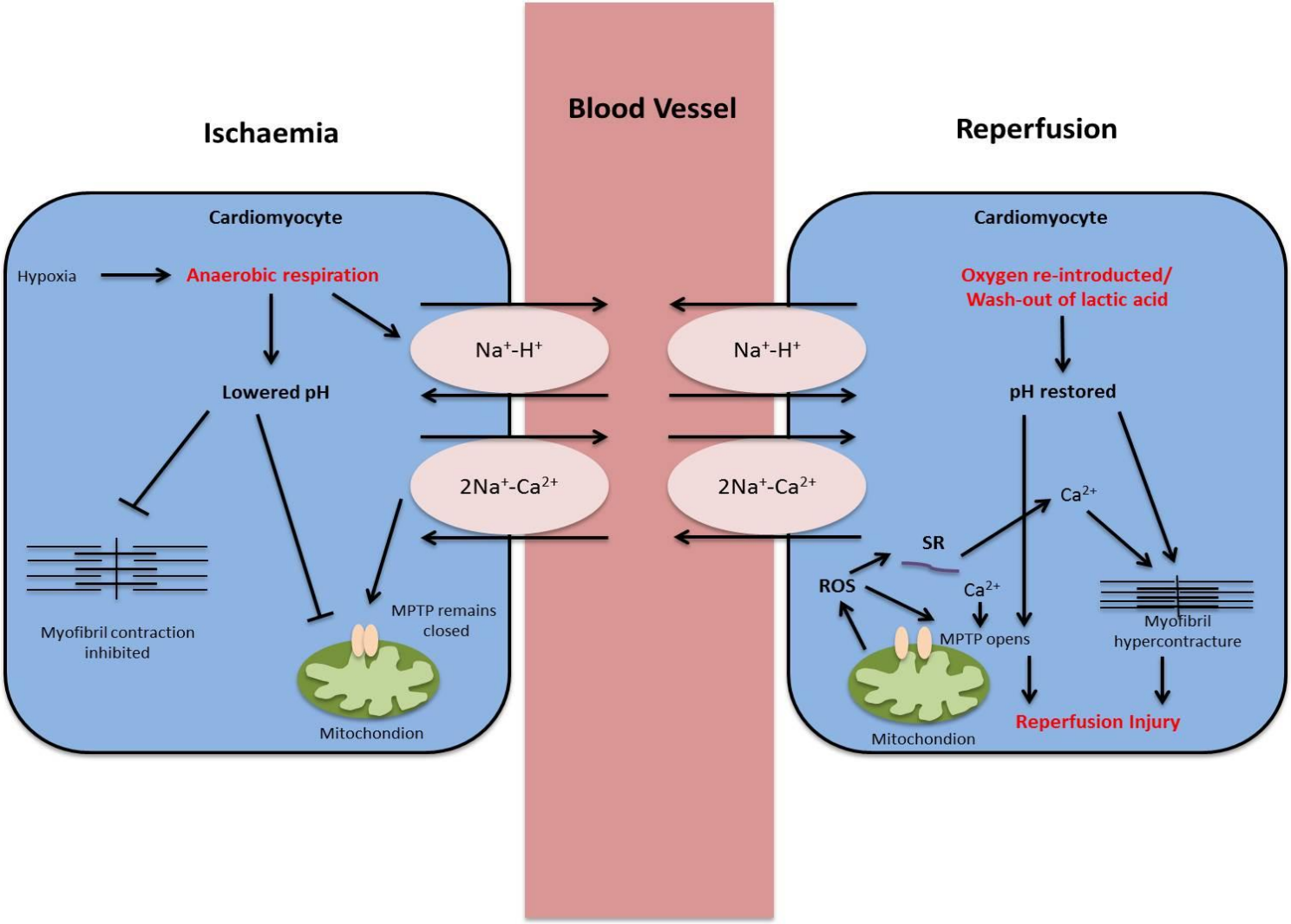


Figure 1.1 An overview of myocardial ischaemia/reperfusion injury. A schematic diagram showing the principle mechanisms that regulate I/R injury in the heart. Abbreviations: ROS, Reactive oxygen species; MPTP, Mitochondrial permeability transition pore; SR, sarcoplasmic reticulum; Na, Sodium; Ca, Calcium. Adapted from (Hausenloy 2013).

1.2 Cell Death in I/R injury

During I/R injury there are two major types of cell death in cardiomyocytes that are thought to occur to varying degrees; apoptosis and necrosis. During ischaemia necrosis plays a prominent role due to irreversible damage to cells and tissues. Apoptosis induction is also observed, however it is during reperfusion that apoptosis has been shown to play a prominent role. This has been shown in many *in vivo* animal models which have shown that prolonged myocardial ischaemia is predominantly due to an increase in necrosis, but reperfusion leads to an enhancement in apoptosis (Gottlieb, Burleson et al. 1994; Fliss 1996; Dumont E 2000; Hofstra L 2000; Eefting F 2004).

1.2.1 Necrosis

Necrosis is the premature death of cells in living tissue caused by external factors such as trauma, toxins and infection, which leads to unregulated digestion of cell components. It is morphologically characterised by a gain in cell volume, swelling of organelles, plasma membrane rupture and subsequent loss of intracellular contents (Krysko D 2008). An example of a trauma that can cause cells to undergo necrosis is hypoxia or anoxia resulting in ischaemic tissue. Interference with the blood supply of an organ results in tissue being deprived of access to substances necessary for metabolic nourishment. It has been thought for a long time that necrosis is merely an accidental and uncontrolled form of cell death, however there is now evidence suggesting that its execution may be finely regulated by signal transduction pathways and catabolic mechanisms (Kung G 2011). This has led to the identification of a new type of necrotic cell death, namely necroptosis (Degterev 2005). Originally this term was used for any form of active necrosis identified. However, more recently it has been suggested that it should refer only to necrotic cell death dependent on receptor-interacting protein kinase 3 (RIPK3) (Linkermann 2014).

1.2.2 Apoptosis: Extrinsic and Intrinsic pathways

Whereas the mechanisms through which necrosis occurs in a programmed fashion remain unclear, apoptosis (a form of controlled cellular suicide) is a highly regulated process. Kerr, Wyllie and Curie first described the term apoptosis in 1972 (Kerr J 1972) as the morphologically distinct form of cell death although other characteristics have since been identified. Apoptosis results in the formation of apoptotic bodies that contain cellular components, which are phagocytised by other cells (Taylor R 2008). Chromatin condensation and fragmentation as well as cell shrinkage and plasma membrane

budding to release apoptotic bodies are common features seen in apoptotic cells (Daniel R 1995). Today it is recognised as the distinctive mode of 'programmed' cell death, which eliminates cells that have been genetically determined for this fate. It is initiated and controlled through precise signalling mechanisms, which contain multiple checkpoints as well as anti-apoptotic programmes being induced at the same time. Apoptosis is a complex phenomenon, which requires co-ordinated and often energy dependent processes to be activated (Elmore 2007) as summarised in figure 1.2. The molecular process of apoptosis encompasses the use of dormant proteases (caspases) which cleave many cellular substrates leading the onset of cell death (Adams 2003). Cysteine-dependent, aspartyl specific proteases, proteases, or caspases, are proteins which exist in the cell as inactive zymogens until they are proteolytically cleaved at which time they become involved in the programmed death of a cell (Adams 2003). Caspases are evolutionarily conserved and are found in insects, nematodes, hydra and mammals (Hengartner 2000). They can be divided into two groups; those which initiate the cell death and those which execute it. The initiator caspases include caspase 2, 8, 9 and 10 whilst the executioner caspases consist of caspase 3, 6 and 7 (Boatright 2003).

Two main pathways of caspase activation have been identified. The first involves (intracellular) intrinsic damage or inadequate cytokine support and the second (extrinsic) is activated by 'death receptors' located on the cell surface (Elmore 2007).

1.2.2.1 Extrinsic pathway

The extrinsic pathway is activated due to ligands binding to death receptors such as FasL and tumour necrosis factor alpha (TNF-α) (Fulda 2000). Members of the TNF receptor family share a similar cysteine rich extracellular domain and have a cytoplasmic domain of approximately 80 amino acids called the 'death domain'. This death domain plays a critical role in transferring death signals from the cell surface to intracellular signalling pathways to induce apoptosis (Park H 2007). To date a number of ligands and corresponding death receptors have been identified, with the best characterised being FasL/FasR and TNF-α/TNFR1. These examples have been shown to have clusters of receptors, which bind with homologous trimeric ligands. When ligand binding occurs, cytoplasmic adaptor proteins are recruited such as the adaptor molecules Fas associated death domain (FADD) protein for FasL/FasR and tumour necrosis factor receptor type 1-associated death domain (TRADD) protein for TNF-α/TNFR1, with subsequent recruitment of FADD and Receptor-interacting serine/threonine-protein kinase 1 (RIPK1). The FADD adaptor protein is responsible for bridging the receptor and caspase-8 by associating with procaspase-8 via dimerization of the death

effector domain. At this stage the formation of the death-inducing signalling complex (DISC) occurs allowing the auto-catalytic activation of procaspase-8 to occur. This subsequently leads to caspase-8 activation, which is the beginning of the execution phase that allows apoptosis to be induced. This extrinsic or death receptor mediated apoptosis can be inhibited by the protein cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (c-FLIP) which can bind FADD and caspase-8, making them inactive (Tait 2010).

1.2.2.2 Intrinsic pathway

The second apoptotic signalling pathway is the intrinsic pathway which is induced due to a range of diverse, non-receptor-mediated stimuli that produce intracellular signals. These signals directly target cells and initiate events within the mitochondria (Tait 2010). The induction of the intrinsic pathway can produce intracellular signals which can activate pro-apoptotic or anti-apoptotic proteins, depending on the stimuli (Elmore 2007).

All stimuli that have been shown to play a role in the intrinsic pathway, cause inner mitochondrial membrane changes, which can lead to the opening of the MPTP (Tait 2010). This causes loss of mitochondrial trans-membrane potential and the release of two main groups of pro-apoptotic proteins from the intermembrane space to the cytosol. Cytochrome c, Smac/DIABLO and the serine protease HtrA2/Omi make up the first group and are responsible for activating caspase-dependent mitochondrial pathways (Eefting F 2004). Additionally, cytochrome c binds and activates Apaf-1 as well as procaspase-9 forming an 'apoptosome', causing caspase-9 activation. The other two proteins in this group are thought to promote apoptosis by inhibiting inhibitors of apoptosis proteins (IAP) however studies suggest that inhibiting Smac/DIABLO and HtrA2/Omi is not sufficient to prevent apoptosis being induced and therefore they should not be labelled as pro-apoptotic (Elmore 2007). The second group of pro-apoptotic proteins include AIF, endonuclease G and caspase activated DNase (CAD), which are released from the mitochondria once apoptosis has been induced, therefore they play a role much later within the apoptosis pathway. AIF translocates to the nucleus and causes DNA fragmentation as well as condensation of peripheral nuclear chromatin (Cande C 2007). Endonuclease G also translocates to the nucleus and cleaves nuclear chromatin to produce oligonucleosomal DNA fragments (Li L 2001).

1.2.2.3 Interaction and convergence of extrinsic and intrinsic pathways

The intrinsic and extrinsic pathways are not entirely independent of one another. For instance the pro-apoptotic Bcl-2 family member BH3 interacting-domain death agonist (Bid) can be cleaved by caspase-8 during death receptor apoptotic signalling, and also by other caspases, granzyme B, calpains and cathepsins. Although Bid is primarily cleaved by caspase-8 it can target other Bcl-2 family members in the intrinsic pathway and alter their activation as highlighted in *figure 1.2* (Billen L 2009). Both the extrinsic and intrinsic pathways lead into the execution phase which begins with activation of execution caspases-3,7 and 9. This causes cytoplasmic endonucleases to be activated for degradation of nuclear material as well as activating proteases to degrade nuclear and cytoskeletal proteins. Caspase-3 is considered the most important execution caspase and can be activated by any initiator caspase (caspase-8,9 or 10). Caspase-3 specifically activates CAD by cleaving ICAD to allow its release. CAD is responsible for degrading chromosomal DNA within the nuclei as well as chromatin condensation (Mcllroy D 1999). Fragmentation of DNA is a hallmark of apoptosis and therefore is defined as one of the main late stage events, making it a good marker of apoptosis. Another important role of caspase-3 is to reorganise the cytoskeleton and form apoptotic bodies (Daniel R 1995). A key substrate of caspase-3 is gelsolin, an actin binding protein, which acts as a nucleus for actin polymerization and can also bind phosphatidylinositol biphosphate, linking actin organisation and signal transduction (Elmore 2007).

The final stage of apoptosis is phagocytic uptake of apoptotic cells with phospholipid asymmetry and phosphatidyserine externalization to the surface of apoptotic cells being a hallmark of this phase. It is this appearance of apoptotic cells, which facilitates phagocytic recognition for uptake and disposal (Erwig L-P 2008). The figure below (figure 1.2) summarises the two main pathways involved in apoptosis.

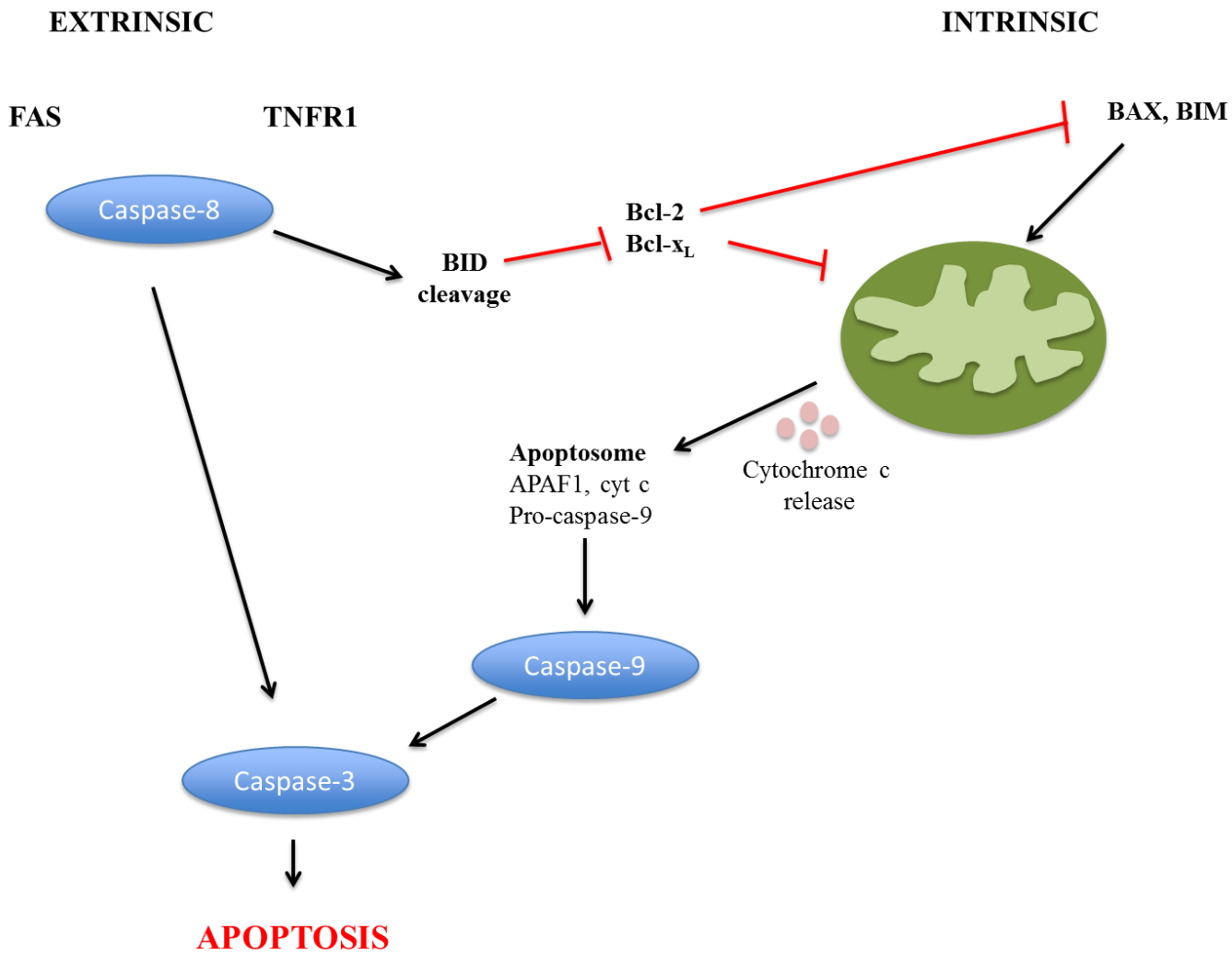


Figure 1.2 An overview of the intrinsic and extrinsic apoptotic pathways. A schematic illustrating the main mechanisms that regulate the intrinsic and extrinsic pathways in apoptosis. Abbreviations: APAF1, Apoptotic protease activating factor 1; BAX, Bcl-2-like protein 4; BID, BH3 interacting-domain death agonist; BIM, B cell lymphoma 2 interacting mediator of cell death; Bcl-2, B cell lymphoma 2; TNFR1, tumour necrosis factor receptor. Adapted from (Mak 2002)

1.2.3 Inhibition of apoptosis

Due to apoptosis playing a critical role in the pathogenesis of many diseases, targeting its inhibition constitutes a potential therapeutic target. Inhibitors of apoptosis (IAPs) are a family of proteins which contain a caspase recruitment domain (CARD) and a zinc finger binding domain allowing them to directly bind to and inactivate caspases (Wei Y 2008). Induction of the mitochondrial death signal, results in release of IAPs such as the proteins SMAC/DIABLO and Omi/HtrA2 from the mitochondria so that they can undergo cleavage to form active proteins (Wei Y 2008). Although IAPs role is to prevent apoptotic cell death, they themselves are regulated and therefore proteins such as Smac when proteolytically cleaved can block their action. XIAP is an example of an apoptosis inducing factor (IAP), which is a major constituent of native apoptosome where it binds to and inhibits caspase-3, caspase-7 and caspase-9 (Deveraux Q 1997). However, Smac can competitively bind to caspase-9 and remove the inhibitory effect of XIAP (Srinivasula S 2001).

Stimulation of other targets has also been shown to block apoptosis from occurring, such as pro-survival pathways PI3K/Akt and ERK. They elucidate their mechanisms by inhibiting pro-apoptotic proteins or up regulating of anti-apoptotic proteins which has been shown to reduce I/R injury and therefore is a reversible process. An example of this has been shown in studies where mice lacking Fas showed reduced cardiac I/R injury following an MI, as did transgenic mice that over express Bcl-2, therefore showing the same protective effect (Lee P 2003). It has also been shown in transgenic models that inhibiting the expression of Bax can prevent cytochrome c release from mitochondria therefore inhibiting the decrease in the mitochondrial membrane potential leading to protection from apoptosis induction (Elmore 2007).

1.2.4 Is apoptosis relevant in the pathogenesis of myocardial I/R Injury?

It is accepted that occlusion of the coronary artery results in ischaemic injury and myocyte cell death by necrosis. This underpins the focus of therapeutics on angioplasty and thrombolysis to limit the onset of ischaemic injury (Gottlieb, Burleson et al. 1994). However, whilst necrosis contributes largely to myocardial infarct size, apoptosis was also shown to play a role in ischaemia and be accelerated during reperfusion injury. *Gottlieb et al* showed using microscopic analysis and DNA laddering that apoptosis contributed to infarct area development in I/R injury (Gottlieb, Burleson et al. 1994). Studies have conflicting views on the degree to which apoptosis contributes to overall total cell death. Studies in ventricular myocytes of the hearts of patients who died of acute myocardial

infarction (AMI) show apoptosis plays a role, most prominent in the border zone of infarcts (Krijnen P 2002). *In vivo* animal models have shown apoptosis in the infarcted myocardium spreads to the border zone after 45 minutes of reperfusion injury. Additionally long-term *in vivo* cardiac I/R injury models showed enhanced apoptosis from 24 hours up to 12 weeks after ischaemia in the infarcted area and border zone (Palojoki E 2001). It has also been indicated that apoptosis alone can induce heart failure, highlighting its importance (Wencker D 2003). Additionally multiple studies, whereby apoptosis has been inhibited, have shown large reductions in infarct size. An example of this is inhibition of the pro-apoptotic protein Bcl-2, which led to a reduction in the myocardial infarct size of mice from 69.9% to 36.6% (± 7.3%) (Chen Z 2001). Whilst the majority of studies suggest that apoptosis plays a significant role in overall cell death there are others which have dismissed its importance. This is mainly due to unresolved issues with the contribution of apoptosis to the cell death within I/R injury (McCully J 2004). An example is the methodological issues surrounding detection of apoptosis which have been questioned by some. *Ohno et al* (Ohno M 1998) showed that using immunogold electron microscopy and *in situ* nick end labelling that coronary artery occlusion in the rabbit resulted in detection of necrosis and not apoptosis. Additionally *Taimor et al* (Taimor G 1999) could only identify induction of necrosis in isolated rat myocytes exposed to hypoxia-reoxygenation *in vitro*.

Inhibition of pro-apoptotic and promotion of anti-apoptotic pathways clearly have a role in I/R injury, however whether their contribution to total myocardial cell death is significant enough to be a valid therapeutic target remains unknown. Although many drugs have been shown to inhibit apoptosis and result in a significant amount of cardioprotection *in vitro* sand *in vivo*, results have been disappointing in clinic. This will be discussed further in section 1.8.

1.2.5 Alternative mechanisms of cell death in I/R Injury

As with most biological processes it has been shown that multiple pathways exist in cardiac I/R injury which lead to cell death, independent of apoptosis and necrosis. Studies have shown that cell death can occur in a caspase-independent mediated fashion via apoptosis through granzymes. Studies have shown that T cells and natural killer cells destroy virally infected cells by secreting perforin, a protein which enables granzymes to enter a cell and trigger apoptosis (Chowdhury 2008). Granzyme B has been shown to enter cells and instigate both the extrinsic pathway through caspase-8 cleavage as well as cleavage of Bid leading to MPTP opening in the intrinsic pathway (Bolvin W 2009). The

process of autophagy has also been implicated in apoptosis, however its role in cardiac I/R injury remains unclear as discussed in the next section.

1.3 Autophagy

The word autophagy is derived from the Greek word for 'self-eating' and defines the catabolic process through which cells turn over their own contents. Autophagy is a recycling pathway which refers to pathways which eliminate cytoplasmic components by delivering them to mammalian lysosomes or plant and yeast vacuoles. It doesn't refer to the proteasome and its role in cellular degradation (Boya, Reggiori et al. 2013). It has been shown to participate in a range of physiological processes including adaptation to starvation, innate and adaptive immunity, lifespan extension and cell death (Zhang S 2013). To date, three main types of autophagy have been described; macro autophagy, micro autophagy and chaperone-mediated autophagy (Mizushima 2007). Micro-autophagy involves recruitment of targeted components to the lysosomal membrane, which subsequently invaginates and pinches off. In contrast, chaperone-mediated autophagy involves proteins carrying the pentapeptide KFEQ-like sequence to be recognized by Hsc70 chaperone. This then associates with the integral lysosome membrane protein LAMP-2A and subsequently translocation of the bound protein into the lysosome interior (Boya P 2013). This section will focus on macroautophagy given that it is the best characterised type of autophagy and thus hereafter the term autophagy refers in fact to macroautophagy. A hallmark of autophagy is the formation of a double membrane vesicle, called the autophagosome. Upon induction of autophagy, there are five key stages, the first being formation of the phagosphore (also known as nucleation). This is followed by Atg proteins (specifically Atg5-Atg12) being recruited to the phagosphore. An example is beclin 1 which is the mammalian homologue of Atg6 and responsible for regulation of autophagy but also the crosstalk of apoptosis and autophagy due to its interactions with Bcl-2 family members (Kang R 2011). Once assembled they interact with other molecules and lipidation of microtubule-associated protein 1 light chain 3 (LC3) (mammalian homologue of Atg8) leads to its assembly on the extending phagosphore membrane and conversion of it from LC3-I to LC3-II. With the proteins successfully assembled, capture of random and/or selective targets for degradation occurs. The complete autophagosome subsequently fuses to the lysosome to expose their content to the hydrolases in the liposomal interior to allow them to be digested (Boya, Reggiori et al. 2013). The resulting metabolites are transported into the cytoplasm where they used either for synthesis of new macromolecules or used as a source of energy (Boya P 2013). This process is summarised in figure 1.3 at the end of this section.

In general it has been suggested that autophagy selects random targets for degradation as it appears to engulf cytosol indiscriminately. However, it is increasingly being suggested that LC3-II can act as a receptor at the phagosphore by interacting with 'adaptor' molecules on the target (e.g. protein aggregates) to promote selective uptake. An example of this is p62/SQSTM1 which promotes turnover of poly-ubiquitinated protein aggregates (Shaid S 2013).

Multiple studies have shown that there is an increase in the number of autophagosomes in the heart during I/R injury. Initially it was observed in fetal mouse hearts in organ cultures, which had been subjected to a combination of hypoxia and glucose deprivation, followed by reperfusion. Later studies showed that brief ischaemia (20 minutes) wasn't sufficient to induce autophagy in Langendorff-perfused rabbit hearts and in this cause autophagy was not induced until reperfusion, however during longer periods of ischaemia (i.e. 40 minutes) it can be (Gustafsson 2009). Multiple factors are thought to induce autophagy in I/R injury, such as generation of ROS, the opening of the MPTP and endoplasmic reticulum (ER) stress. The functional role of autophagy within I/R injury is debatable and intense investigations *in vivo* and *in vitro* by multiple groups have been done. Interestingly, up regulation of autophagy has been reported to both contribute and be the cause of cell death in the heart (Ma S). Experiments performed in the lab of my co-supervisor Dr Stephanou have suggested that autophagy plays a cardioprotective role in I/R injury, specifically by STAT1 negatively regulating autophagy. Therefore, when STAT1 deficient mice were exposed to cardiac I/R injury *in vivo* cell death was reduced and autophagy was up regulated (McCormick J 2012). *Matsui et al* also showed that autophagy was protective during ischaemia but that during reperfusion it was detrimental and caused cell death. Beclin1 (+/-) heterozygous mice showed reduced levels of autophagy in reperfusion compared to wild-type and this correlated with decreased apoptosis during reperfusion and reduced infarct size.

However, other groups have shown that enhanced autophagy contributed to cell death in cardiac I/R injury. Examples include a study demonstrating that when autophagy was inhibited by using 3-methyladenine (3-MA), cell death was reduced in the rat cardiomyocyte derived cell line H9c2 in simulated I/R injury (Aki T 2003). Additionally, in beclin 1 heterozygous knockout (beclin $1^{+/-}$) mice, a reduction in cardiac I/R injury and therefore myocyte death was observed (Matsui Y 2007). Another study has shown that both *in vitro* and *in vivo* that calpain, a protease activated in the presence of calcium, can cleave Atg5 causing it to translocate to the mitochondria and bind Bcl-2 to stimulate apoptosis (Yousefi, 2006). This evidence suggests that increases in calcium would activate

autophagy as well as convert some autophagy machinery such as Atg5 to play a role in activating apoptosis. Finally, activation of c-Jun NH2-terminal Kinases (JNK) a mitogen activated protein kinase (MAPK), which has been shown to induce apoptosis, leads to an increase in Beclin-1. It is therefore clear that cross-talk through calpain, calcium flux, Bcl-2 family members and signalling kinases allows regulation of autophagy to be linked to other death pathways active in I/R injury (Murphy 2008).

Autophagy currently remains a debatable topic in relation to its contribution to cardiac I/R injury. This section has presented studies providing evidence for autophagy antagonizing cardiac pathogenesis as well as it contributing to further damage. The key could be in suppressing excessive autophagy, whilst allowing basal autophagy to still occur. However, due to the underlying mechanism of autophagy remaining unclear, the debate continues.

Mechanisms to induce cell death such as apoptosis, necrosis and perhaps even autophagy have been identified as the key players in cardiac I/R injury. However, the regulation of these processes involves a vast range of inter-related signalling pathways and proteins, making it a very complex task to reduce cell death. The next chapter will discuss the mechanisms that are thought to regulate cardiac I/R injury.

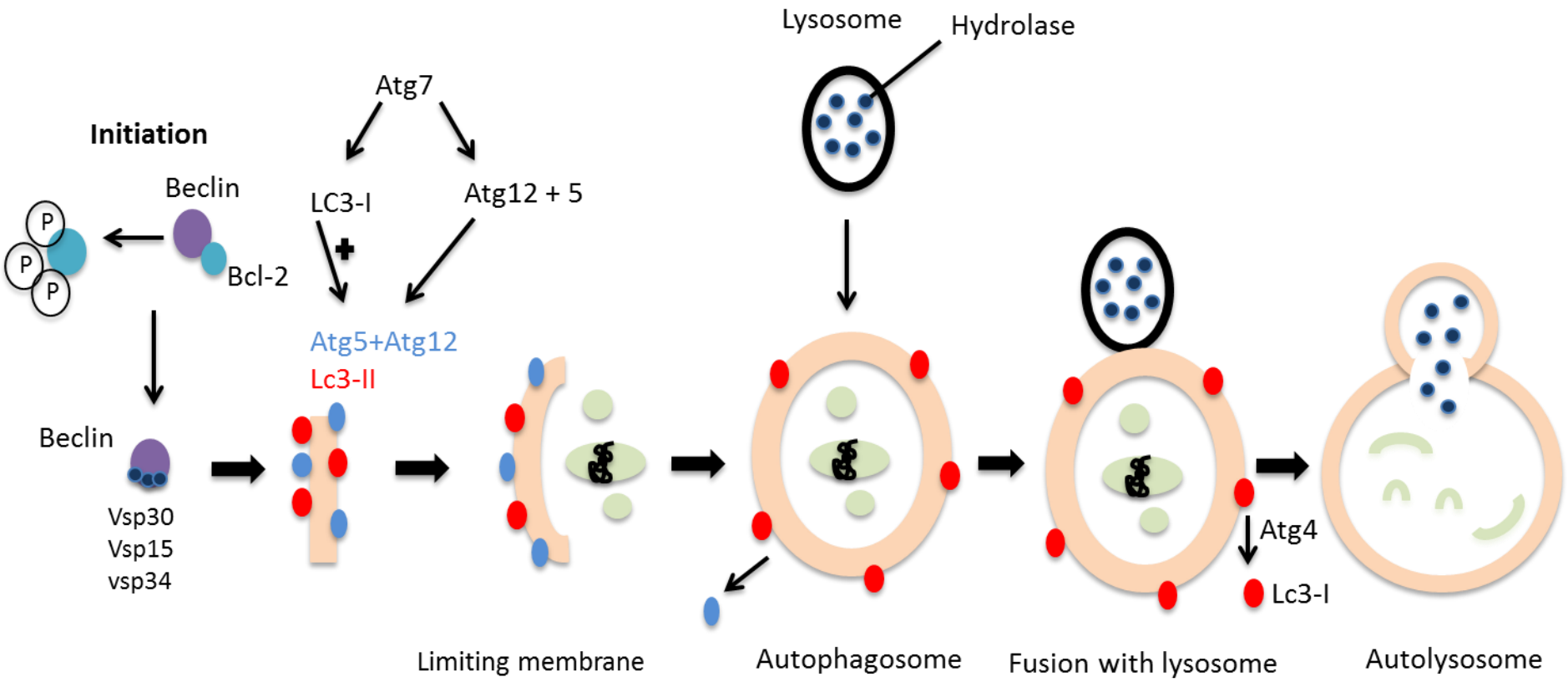


Figure 1.3 Schematic overview of autophagy. Autophagy begins with the isolation of double-membrane-bound structures inside an intact cell. These membrane structures elongate and mature, and LC3 is recruited to the membrane (conversion from LC3-I to LC3-II). The elongated double membranes form autophagosomes, which sequester cytoplasmic proteins and organelles such as mitochondria. The autophagosomes mature and fuse with the lysosome to become autolysosomes. Abbreviations: Atg, autophagy related gene; Lc3, microtubule-associated protein 1A/1B-light; APAF, apoptotic protease activating factor 1; BID, BH3 interacting-domain death agonist; BAX, bcl-2-like protein 4; BIM, Bcl-2-like protein 11; Bcl-2, B-cell lymphoma 2; Bcl-. Adapted from (Fleming A 2011)

1.4 The Regulation of Myocardial I/R Injury

1.4.1 The Mitogen Activated Protein Kinase Family

There are some processes, in particular those which require a degree of energy, that appear to only occur in the reperfusion stage (Borgers M 1987). An example of this is activation of various signalling pathways including the MAPK family. They are a family of serine-threonine kinases, which have been implicated in cell differentiation, survival and proliferation (Cobb 1999). MAPK have been shown to be heavily involved in I/R injury regulation in both a protective and detrimental way (Abe J 2000). Innate immune signals received through toll like receptors (TLRs) results in the activation of two main pathways, one being the MAPK pathway. They are activated in response to a large variety of signals including inflammatory cytokines, ischaemia, DNA damage and oxidative stress. Their function is to transduce signals from the extracellular environment to regulatory elements in the cell. Activation of MAPKs occurs through a cascade of upstream kinases including MAPKKK (MAPK Kinase Kinase), which phosphorylates a dual specificity protein kinase MAPKK (MAPK Kinase) which in turn phosphorylates the MAPK (Figure 1.4). This process allows signal amplification but also additional regulation to allow the kinetics, duration and level of activity to be precisely managed (Keshet 2010). Mammalian MAPK can be divided into four subgroups based on their structure and function; ERKs (Extracellular signal-regulated kinases), p38MAPKs, JNKs and ERK5 (Extracellular signal-Regulated Kinase-5) or BMK. Studies have suggested that MAPKs may be implicated in apoptosis but their precise role of regulation may be kinase specific. The activation of MAPKs in the heart, especially in the cardiomyocytes has been widely demonstrated *in vitro* and *in vivo* (Yue T 2000) .

ERK consists of two isoforms; ERK1 (p44) and ERK2 (p42) and is activated in response to primarily growth factors and mitogens. These stimuli act through G-protein coupled receptors, which activate the small G-protein Ras, which in turn activates Raf leading to phosphorylation of MAP2Ks MEK1 and MEK2 (Shaul 2007). Activation of ERK promotes many processes including; entry to the cell cycle, differentiation, cell migration and inhibition of apoptosis. ERK 1/2 has many substrates but p90 ribosomal S6 kinase (p90RSK) is a key player, mediating the majority of ERK signal transductions. Essential functions of p90RSK include regulation of gene expression via phosphorylation of various transcription factors such as cAMP-response element-binding (CREB) protein. Additionally it has more recently been shown to phosphorylate the serine 112 residue of the pro-apoptotic protein Bcl-2 associated death promoter (BAD), which leads to inhibition of BAD-mediated apoptosis. Therefore, this suggests that ERK promotes cell survival by inhibiting

components of the apoptotic pathway (e.g. BAD) but also increasing transcription of pro-survival genes (e.g. CREB).

Yue et al demonstrated that all MAPKs are activated during cardiac I/R injury, for both anti-apoptotic and pro-apoptotic functions. Inhibition of ERK 1/2 with the inhibitor PD98059 enhanced I/R induced apoptosis in cultured cardiomyocytes and perfused isolated rat hearts, therefore suggesting a pro-survival role (Yue T 2000). In contrast, studies have shown that inhibition of p38 MAPK with the inhibitor SB23580 results in a reduction in myocardial damage (Martin J 2001) and improved cardiac function (Nagarkatti 1998). Inhibition of p38 MAPK also leads to a reduction in inflammatory cytokines such TNF-α, interleukin-1 (IL-1) and interleukin-8 (IL-8), therefore reducing the pro-inflammatory response which is known to contribute to cardiac I/R injury (Zhang 2008). As a result of this it has been suggested that p38 MAPK inhibitors should be used as a therapeutic. However there have also been reports that inhibition leads to a poorer prognosis, due to involvement in developmental, differentiation and proliferation and so its use remains a controversial view (Kumphune S 2012).

The findings from these studies suggests that MAPK pathways provide potential therapeutic targets to abrogate cell death caused during I/R injury through up-regulation of the protective ERK 1/2 or inhibition of pro-death p38 MAPK and JNK.

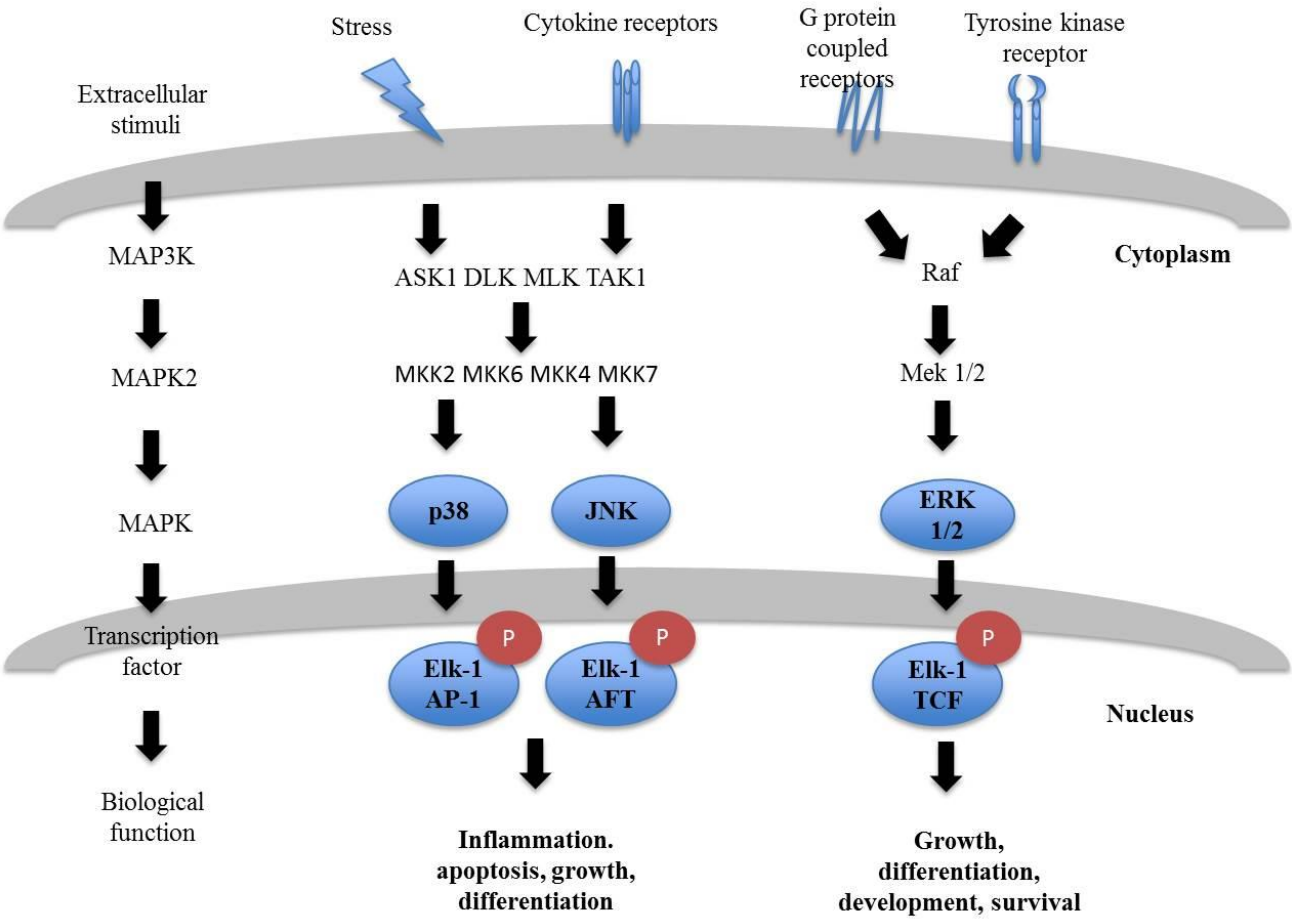


Figure 1.4 The MAPK signalling cascade. MAPK signalling is controlled by a cascade of MAP3Ks>MAP2K>MAPK. Stimuli such as cytokines, tyrosine kinases and growth factors all activate MAP3Ks causing activation of the main MAPKs; p38, JNK and Akt. Abbreviations: MAPK; mitogen-activated protein kinase, p-38; p38 mitogen-activated protein kinases, JNK; c-Jun N-terminal kinase, ERK1/2; extracellular signal-regulated kinase, Elk-1; ETS domain containing protein, AP-1; Activator protein 1, MKK; Map kinase kinase, Raf; proto-oncogene serine/threonine-protein kinase. Figure adapted from Cellsignalling.com.

1.4.2 PI3K/Akt pathway

PI3K is a lipid and protein kinase which catalyses the phosphorylation of the inositol ring of phosphoinositides at the D3 position as well as phosphorylating non-muscle tropomyosin, which is involved in ß-adrenergic receptor endocytosis. Reports have identified PI3K as playing a role in cytoprotection and apoptosis reduction (Koyasu 2003). The role of PI3K in cardioprotection is due to generation of phosphatidylinositol 3,4,5-triphosphate (PIP3) which facilitates PDK1 phosphorylation of substrates such as p70S6K, Akt and Protein Kinase A (PKA). An increase in Akt phosphorylation has been observed in pre-conditioning, with this activation inhibited using PI3K inhibitors suggesting it is responsible for its activation. Akt is believed to induce cardioprotection by phosphorylating target proteins such as GSK-3β, endothelial nitric oxide synthetase (eNOS) and pro-apoptotic Bcl-2 family member BAD (Tong H 2002). Phosphorylation of BAD targets it to 14-3-3 protein where it is sequestered and therefore its pro-apoptotic actions are blocked. Studies have shown that overexpression of Akt leads to blocking of hypoxia-mediated activation of caspase-3 and caspase-9. Akt also phosphorylates and activates the ubiquitin ligase mdm2 which is known to reduce I/R injury-induced cell death (Davidson S 2006).

It has been published that Akt can phosphorylate and activate eNOS to produce NO, which has been shown to have cardioprotective effects in I/R injury (Roviezzo F 2007). Also, during ischaemia low pH and low levels of oxygen allow intracellular nitrite can also be converted into NO by deoxymyoglobin or xanthine oxidoreductase. The effects of NO are thought to be on mitochondrial K^+/ATP by production of guanylyl cyclase, which via protein kinase G, can result in the opening of the mitochondrial potassium ATP channel leading to an influx of postassium ions and disruptions to membrane potential. Conversely, excess NO can lead to post-translational modifications to proteins including S-nitrosylation, which is a reversible modification that has the ability to protect thiol groups from further oxidation and thus may have a protective role (Dos Santos P 2002).

1.4.3 The Reperfusion Injury Salvage Kinase Pathway

Activation of pro-survival kinases during the first stages of ischaemia has been hypothesised to attenuate reperfusion-induced cell death. Activation of these pro-survival kinases such as ERK 1/2 and Akt at reperfusion appears to be sufficient to elucidate protection and therefore these cascade pathways have been given the term reperfusion injury salvage kinase (RISK) pathway (figure 1.5) (Davidson S 2006). Due to their cardioprotective roles it has been suggested that the ability to

manipulate and up-regulate these pathways in reperfusion may provide a potential approach to limit reperfusion-induced cell death (Hausenloy 2004).

A number of growth factors and other agents have been shown to be cardioprotective when administered during the early stages of reperfusion, with their mechanism of action linked to activation of this RISK pathway. An example of this is insulin, which has been shown to activate the PI3K-Akt kinase pathway. *Jonassen et al* (Jonassen AK 2000) showed that administration of glucose-insulin-potassium (GIK) to rats at reperfusion reduced heart infarct size. This cardioprotective effect shown with insulin has been linked to phosphorylation of Akt and BAD, with increased phosphorylation being seen in comparison to control hearts not treated with insulin (Jonassen AK 2000). Another agent which has been identified as having cardioprotective effects is the peptide urocortin, which is released by myocytes in response to stress e.g. ischaemia (Brara B 1999). It has been shown that prolonged hypoxia causes an enhanced presence of urocortin during reoxygenation which leads to reduced cell death via increased phosphorylation of ERK 1/2 (Brara B 1999). Finally, fibroblast growth factor (FGF) has been shown to protect at reperfusion via recruitment of the ERK 1/2 signalling cascade (Padua RR and Source 1998). FGF is a polypeptide growth factor, which is implicated in cell proliferation, survival and apoptosis. It has been shown that when administered during the reperfusion stage in an *in vivo* rat model of MI that it is cardioprotective (Cuevas P 1999) and this is modulated by reduction of apoptotic cell death (Cuevas P 1997). The activation of these pro-survival pathways at the time of reperfusion appears to regulate downstream effectors such as BAD, BAX, p70S6K and eNOS which are thought to be responsible for the protection associated with activation of the RISK pathway. One way in which this may cause protection is inhibition of the MPTP opening. It has previously been shown that pharmacological inhibitors of MPTP opening leads to cardioprotection (Hausenloy DJ 2002). Inhibition of the pro-apoptotic proteins BAD, BAX and p53, which are thought to regulate the MPTP opening, may therefore be a mechanism through which the RISK pathway elucidates its protectiveness (Hausenloy 2004).

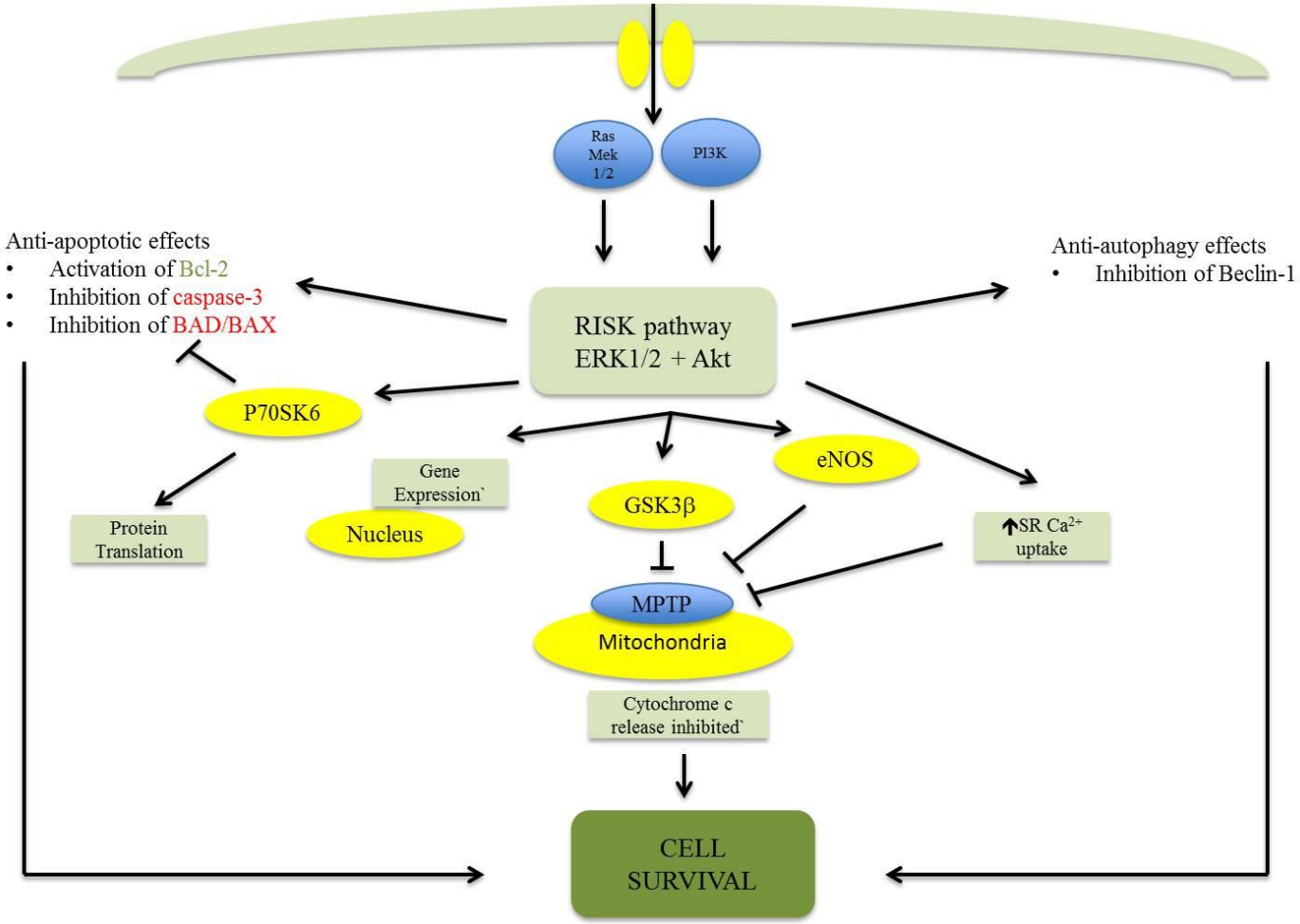


Figure 1.5 The RISK signalling pathway. RISK signalling involves two main kinases; Akt and ERK which when activated have both and-apoptotic effects and anti-autophagic effects, which promote cell survival. Abbreviations: MPTP; mitrochondrial permeability transition pore, GSK3β; glycogen synthase kinase 3 beta, eNOS; endothelial nitric oxide synthase, P70SK6; Ribosomal protein S6 kinase beta-1, ERK1/2; extracellular signal-regulated kinase, MEK; mitogen extracellular-regulated kinase, Bcl-2; B cell lymphoma-2, BAX; Bcl-2-like protein 4, BAD; Bcl-2-associated death promoter. Figure adapted from (Hausenloy 2004).

1.4.4 The Bcl-2 family

The Bcl-2 family of proteins have been found to be key players in the apoptotic death programme, particularly in mitochondrial regulation and caspase activation. This family of proteins contain both pro-apoptotic (Bax and Bak) and pro-survival members (Bcl-XL and Bcl-w) and therefore act as critical life-death decision point within the common pathway of apoptosis (Tsujimoto 1998). The pro-apoptotic Bcl-2 members can be characterised into either 'BH (Bcl-2 homology)-multi' proteins with three conserved domains; BH1, BH2 and BH3 and include Bax and Bak. The second group contain only the BH3 domain and are therefore known as 'BH3-only proteins' and include Bid, Bad and Puma (Burlacu 2003).

Bcl-xl and Bcl-2 are localised in the mitochondrial membrane as well as the endoplasmic reticulum and nuclear envelope and prevent the release of factors that contribute to apoptosis being initiated. This includes cytochrome c and AIF from the mitochondrial inter-membrane space to the cytoplasm. Once here they can activate caspases and induce apoptosis. AIF release is regulated by the opening of the MPTP which in turn is regulated by Bcl-2 and Bcl-XL (Tsujimoto 1998).

The pro-apoptotic proteins such as Bax reside in the cytosol prior to apoptosis, but following a pro-apoptotic signal undergoes a conformational change allowing it to translocate to the mitochondria where it participates in disrupting the mitochondrial membrane (Crow M 2004). Bax has been shown to integrate into the mitochondrial membrane and form pores to allow cytochrome c release (Cory S 2003).

Another pro-apoptotic member is BAD which in its inactive state is phosphorylated by survival signals and binds to 14-3-3 scaffold proteins in the cytoplasm (Yang E 1995). There are two main residues thought to be phosphorylated in the inactive form of BAD; serine 112 and serine 136. They have been shown to be regulated by distinct survival kinases, namely serine 112 by ERK and serine 136 by Akt (Xianjun F 1999). Upon abrogation of survival signals BAD is dephosphorylated, dissociates from 14-3-3 and then localises to the mitochondria. The free BAD molecules are then able to interact with either Bcl-2 or Bcl-Xl and block their anti-apoptotic functions. Studies have shown that the BH3 domain of BAD specifically interacts with Bcl-2 and Bcl-Xl to inhibit them (Adachi 2002).

This introduction thus far has detailed the mechanisms which define and mediate myocardial I/R injury. Given that a key aim of this thesis is to explore these mechanisms in relation to the autoimmune disorder systemic lupus erythematosus (SLE), the subsequent sections will therefore outline the clinical features, pathogenesis and current treatments for SLE.

1.5 Autoimmune disease

In society we are continually exposed to organisms that are inhaled, swallowed or inhabit our skin and mucous membranes (Janeway 2002). The ability of these organisms to cause disease relies on the pathogenicity of an organism but also, and perhaps more importantly, how our defense mechanisms respond to eliminate the pathogen. The immune system comprises of a complex network of cells, humoral factors, cytokines and lymphoid organs. The immune system defense is divided into two parts, namely the innate and adaptive immune response, although in practice they overlap and have much interaction (Delves 2000).

The innate immune system is the first line of defense against infection and is described as a broad response mainly involving phagocytic cells such as neutrophils, monocytes and macrophages, which can release pro-inflammatory mediators such as mast cells and natural killer cells. Additionally, there is a molecular component of the innate response comprising of complement and cytokines such as interferons (IFN) (Medzhitov 2000; Janeway 2002). The second stage of the immune response, the adaptive, does not become activated until sometime after exposure to an antigen. However, its delayed response allows it to react with high antigenic specificity as well as have the ability to generate memory allowing for a more rapid response of subsequent antigenic exposures. Antigen-specific B and T cells surface receptors bind to antigens and specialised antigen presenting cells (APC) display the antigen to the lymphocytes allow them to combine to provide a response to the antigen. Ultimately, the adaptive and innate immune responses work collaboratively to eliminate pathogens (Delves 2000). This is particularly important in cancer, where studies have suggested that the immune system plays a role in surveillance for tumours. The use of both mouse models of cancer and humans with cancer have shown strong evidence to suggest that certain immune cell types, effector molecules and pathways can function as extrinsic tumour suppressor mechanisms (Swann J 2007).

The complex nature of the immune system is perhaps best illustrated when it goes wrong. An example of this is autoimmunity, whereby failure of an organism to recognise its own constituents as

self, resulting in an immune response which is what drives the pathogenesis of an autoimmune disorder. Human autoimmune disorders can be divided into organ specific and systemic diseases. Examples of organ specific diseases include Hashimoto's thyroiditis and insulin-dependent diabetes mellitus. These diseases involve an immune response directed primarily against a single organ or gland. In contrast, a systemic disease is directed against a broad range of tissues and present manifestations in a variety of organs. This pathogenesis results from cell mediated responses and cellular damage caused by autoantibodies or immune complexes. Examples include systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and the antiphospholipid syndrome (APS) (Goldsby R 2003).

1.5.1 Systemic Lupus Erythematosus

SLE is a chronic, multisystem autoimmune disorder, which can be characterised by inflammation of many organ systems including skin, blood elements, kidneys, and heart (Rahman 2008). The reported prevalence of SLE in the general population is 20-150 cases per 100,000 (Lawrence RC 1998; Chakravarty EF 2007; Pons-Estel GJ 2010) and in women this rate has been shown to vary from 164 (white) to 406 (African American) per 100,000 (Lawrence RC 1998). Improvements in detection of the disease, particularly in mild cases has led to incidence rates tripling in the last 40 years of the 20th century (Uramoto KM 1999). However, it remains that prevalence is notably higher in individuals of Afro-Caribbean extraction and in females of childbearing age (Tsokos 2011).

The bi-modal pattern of mortality in SLE was first described by Urowitz who suggested that early death results from active lupus and infection followed by death in later life being primarily due to cardiovascular disease (Urowitz MB 1976). The life expectancy of lupus patients has improved from a 50% 4-year survival rate in the 1950s to a 15 year survival rate of 80% today. However, a patient who is diagnosed at 20 years still has a 1 in 6 chance of dying by 35 years of age (Rahman 2008). A multi-national study of 9,547 patients with SLE showed 1,255 deaths and approximately 25% (313) were as a result of cardiovascular disease (Bernatsky S 2005). Due to the complex nature of SLE, a significant amount of research has been carried out to define the causes and identify potential targets for treatment. Hence, the pathogenesis of SLE will be detailed in the next section of this thesis.

1.5.2 Pathogenesis of SLE

1.5.2.1 Autoantibodies

The main disturbance to the function of the immune system is production of pathogenic autoantibodies. There a growing number of autoantibodies identified in association with SLE. Many are thought to be pathogenic but their individual roles remain largely unclear (Ehrenstein 1999).

These autoantibodies are mostly directed against several self-molecules found in the nucleus, but also to antigens found in the cytoplasm and on the cell surface. Antibodies to nuclear cellular components are the most frequently observed and those common ones detected included antinuclear antibodies (ANA), anti-double stranded DNA (ds-DNA), anti-nucleosome antibodies and antibodies to extractable nuclear antigens such as anti-Ro (SS-a), anti-La (SS-B), anti-ribonucleoprotein (RNP), anti-Sm and anti-histone (Morrow W 1999; Franceschini 2005). ANA antibodies are present in more than 95% of patients and therefore remains the most sensitive test for SLE (Mok 2003) but are not specific as many other autoimmune conditions are also associated with ANA positivity and up to 10% of the healthy population harbor these antibodies. Conversely, anti-dsDNA antibodies are less sensitive, but more specific. Anti-dsDNA antibodies are detected in approximately 70% of patients with SLE, however they are specific to SLE and rarely found in other diseases or healthy individuals (Isenberg 1987). Hence the tests ANA (due to its high sensitivity) and anti-dsDNA (due to its high specificity) are both included as two of the 11 criteria for the classification of SLE (Hochberg 1997), (see table 1.1). There is a strong association of these antibodies with lupus nephritis and they can be found enriched in the kidney, as well as patients with active nephritis often having elevated anti-dsDNA levels (Bootsma H 1995).

More recently it has been proposed the nucleosome is the principal antigen which contributes to the pathogenesis of SLE. The nucleosome (chromatin) is the term given to the native complex of DNA and histones and has the ability to become immunogenic under certain conditions. Impaired clearance of apoptotic cell debris is a hallmark of SLE and predisposes to the disease through accumulation of chromatin breakdown and DNA fragmentation from dying cells (Herrmann M 1998), exposing nuclear antigenic material normally not exposed to the immune system and thus lowering the threshold for loss of tolerance in susceptible individuals. Auto-antibodies directed against nucleosomes were originally described in lupus mouse models and correlation between anti-

nucleosome antibodies and renal disease has been shown in SLE patients (Cervera R 2003; Duzgun N 2007).

Approximately 30-40% of patients with lupus test positive for another group of auto-antibodies, namely the antiphospholipid antibody (aPL) and therefore additionally have the antiphospholipid syndrome (APS), which is discussed in section 1.5.10 of this thesis. The aPL are a family of autoantibodies that exhibit a broad range of target specificities and affinities, recognising a combination of phospholipids and proteins that bind to phospholipids. Initially it was believed that aPL bound to phospholipid directly, however it is now known that the most pathogenic aPL actually bind not directly to phospholipids but via proteins complexed to anionic phospholipids (Galli M 1990). In 1990, three groups independently discovered that anticardiolipin (aCL) antibodies did not bind cardiolipin directly but instead via a protein 'co-factor', the phospholipid-binding plasma protein β_2GPI (Galli M 1990; Matsuura E 1990; McNeil H 1990). However, binding is also thought to occur via other co-factors such as prothrombin, annexin V, protein S and C (M Sorice 2000), although their relevance remains uncertain. Not all aPL are pathogenic and they can be found in healthy adults and patients with infectious, malignant or drug-related disorders who do not go on to develop APS (Keeling D 2012). This led to the distinction of two populations of aPL; non-pathogenic aPL, which bind neutral or anionic phospholipid and do not require serum cofactors and pathogenic aPL, which occur in the APS and bind negative phospholipid, requiring the presence of serum cofactors such as β_2GPI to do so (Keeling D 2012). There are a number of proposed mechanisms that have been suggested to contribute to autoantibody production, however, the exact mechanism for the generation of aPL remains unknown. The current theories as to the causes of SLE are described in *section 1.5.4* of this thesis.

1.5.2.2 B and T cells

Functional immune system alterations to B cell, T cell and monocytic lineage cells have been characterised in SLE. These alterations result in polyclonal activation of B cells, increased numbers of antibody producing cells and subsequent autoantibody production and immune complex formation (Mok 2003). B and T cell activation requires stimulation by specific antigens, however it has been shown in mice that bacterial DNA, viral antigens and chemicals such as pristine can induce production of anti-DNA antibodies (Hahn 1998). Additionally, self-antigens such as DNA-protein and RNA-protein complexes have also been shown to induce autoantibody production (James J 1995). Both these environmental antigens and self-antigens can be taken up by APC or bind to antibodies on the surface of B cells. These antigens are then processed and presented to T cells via

their human leukocyte antigen (HLA) molecules, which in turn leads to B cell production of autoantibodies (Mok 2003).

B cell activation is abnormal in SLE, with increased numbers of activated cells which have increased intra-cytoplasmic calcium responses as well as being more sensitive to cytokines such as interleukin 6 (IL-6) (Linker-Israeli M 1991). Epitope spreading has also been shown in murine and human SLE whereby B cells are more prone to polyclonal activation by antigens and cytokines (Monneaux 2002). The B lymphocyte stimulator (BLyS), also known as BAFF, is a soluble ligand of the TNF cytokine family and a prominent regulator of B cell differentiation, homeostasis and selection (Moore PA 1999). BLyS levels alters survival signals and, therefore, selective apoptosis of autoantibody-producing B cells. High levels of BLyS cause exacerbation of the SLE disease state, due to relaxation of B cell selection and, therefore, enhancement of autoantibody production (Cancro 2009). This important feature of the BLyS ligand has made it an important therapeutic target, which will be expanded on in section 1.5.8.6.

T cell function abnormalities include lower peripheral blood levels as well as a skew of their function towards B cell activation, hence increase antibody production (Linker-Israeli M 1990). Early events in T cell activation have been shown to be defective, therefore causing a reduced capacity to proliferate in response to mitogenic stimulation, thereby reducing interleukin-2 (IL-2) production (Alcocer-Varela 1982). It previously has been suggested that altered lipid metabolism contributes to SLE pathogenesis with their presence altering early steps during T cell receptor (TCR) stimulation (Jury E 2007). The identification of a role for lipid raft-associated glycosphingolipids (GSLs) identifies a potential new therapeutic target (Leong 1994). Recent work has focused on changes due to alterations in the metabolic processing of lipid biosynthesis. CD4+ T cells from SLE patients have been shown to display an altered profile GSLs compared with that of healthy controls (McDonald G 2014).

1.5.2.3 Cytokines

Several cytokines are thought to play a role in the pathogenesis of SLE, such as previously mentioned IL-2. More recently, interleukin 10 (IL-10) has been identified as being elevated in the serum of SLE patients (Houssiau FA 1995). IL-10 is a T helper 2 (Th2) cell cytokine which acts a stimulator of B cell proliferation and differentiation and, therefore, a potential mediator of polyclonal B cell activation in SLE (Mok 2003). Another cytokine which has been shown to contribute to SLE

pathogenesis is IL-6 which is known to induce terminal differentiation of B lymphocytes into antibody-forming cells as well as contributing to differentiation of T cells into effector cells (Tackey E 2004). Finally, IFN-α is thought to be a key cytokines linked to SLE pathogenesis and promote activation of dendritic cells (DCs), B and T cells, endothelial cells, neuronal cells, renal resistant cells and other tissues (Bertsias G 2012). Activation of the IFN pathway has previously been linked to the presence of autoantibodies specific for RNA-associated proteins. RNA-mediated activation of TLR is an important mechanism contributing to production of the key cytokine IFN-α as well as other pro-inflammatory cytokines and hence TLRs are described in more detail in the following subsection (Visentini M 2009).

1.5.2.4 Toll like receptors

Patients with lupus are unique in their ability to activate endosomal sensors such as TLRs by nucleic acids (DNA and RNA) (Rahman 2006). Studies have shown that mice carrying deletions of *Tlr7* and *Tlr9* have reduced disease activity. When mice have double deletion of both *Tlr7* and *Tlr9* the reduction in disease activity is even greater than when independent, therefore suggesting that they both contribute to disease pathogenesis (Santiago-Raber ML 2010). In a healthy individual there are barriers which prevent this kind of sensor activation, however in SLE, molecules called 'alarmins' are released due to stress and have the ability to facilitate transfer (Wu 2012). Examples include neutrophil extracellular traps (NETs) and immune complexes. The activation of TLRs forms part of the enhanced innate immune response in SLE. They are receptors found on cell membranes most commonly but also in the cytosol and endosomal compartments (Rahman 2006). The TLRs are responsible for engaging with antigens expressed on autoreactive B or T lymphocytes leading to their activation. They are also responsible for the activation of APCs such as DCs, enhancing their ability to present their auto-antigens. It has been shown that chromatin containing immune complexes are 100-fold more efficient in stimulating lupus B cells due to the presence of nucleic acids as well as the combined B cell receptor activation and TLR stimulation (Bertsias G 2012). There are two main types of DCs; plasmacytoid dendritic cells (pDCs) and myeloid dendritic cells (mDCs). pDCs have been likened to IFN alpha factories due to the large quantities of the type 1 interferon upon activation by antigens or auto antigens recognised by the innate immune receptors (Niewold T 2010). In contrast, mDCs are involved in antigen presentation with immature conventional mDCs promoting tolerance and mature auto reactivity.

1.5.2.5 Immune complexes

Immune complexes and complement activation pathways are responsible for mediating effector function and tissue injury. In healthy individuals, immune complexes are cleared by Fc and complement receptors. Failure to clear these complexes results in tissue deposition and tissue injury at sites (Morrow W 1999). The clearance of immune complexes by phagocytic cells is defective in patients with SLE (Salmon J 1996). This is partly due to a reduction in CR1 receptors but also to allelic polymorphisms of the IgG receptor, therefore reducing clearance of IgG2 and IgG3 containing complexes (Kiss E 1996; Morrow W 1999). Tissue damage is also responsible for recruitment of inflammatory cells, reactive oxygen intermediates, and production of inflammatory cytokines and modulation of the coagulation cascade (Davies, Peters et al. 1992).

As well as defective immune complex clearance it has also been shown that apoptotic cell clearance is also impaired in patients with SLE (Herrmann M 1998). This persistent circulation of apoptotic waste can serve as an immunogen for autoreactive lymphocyte activation as well as an antigen for immune complex formation (M 2001).

1.5.2.6 Apoptosis

Apoptosis acts as a source of auto antigens and molecules with the ability to induce cytokine activity (e.g. IFN-α) (Salmon 1999). The increased spontaneous apoptosis as well as UV induced apoptosis in skin cells that SLE patients are susceptible to as well as the impaired clearance of apoptotic peripheral blood cells all provide cell blebs which are rich in lupus auto antigens (Casciola-Rosen LA 1994; Casciola-Rosen L 1996). Under normal circumstances these apoptotic cells would be cleared by macrophages, therefore not producing an immune response, however this is impaired in patients with SLE (Herrmann M 1998). The reason for this defect is not clear, however it is thought that quantitative or qualitative defects in the early part of complement activation, such as C2, C4 or C1q may play a role (Korb 1997).

1.5.2.7 Complement

Activation of the complement pathways is commonly observed in patients with SLE and has been shown to lead to deposition of complement components at sites of tissue injury such as the renal glomeruli. This finding strongly suggests that complement activation contributes to tissue damage in SLE. Additionally, it has been shown that complement is important in generating an autoimmune response (e.g. in SLE) but that deficiencies in the classical complement pathway can infact cause SLE. An example is a lack of C4, which causes a decreased elimination of self-reactive B cells. This

lack of C4 has been shown in mouse models to reduce ability for negative selection (Tsokos 2011). Additionally, decreases in C1q are known to reduce the body's ability to clear necrotic waste, which is a common characteristic of SLE (Manderson AP 2004) and anti-C1q antibodies are associated with SLE.

Following on from defining the pathogenesis of SLE, this chapter will now discuss the clinical features which result of the disease progression.

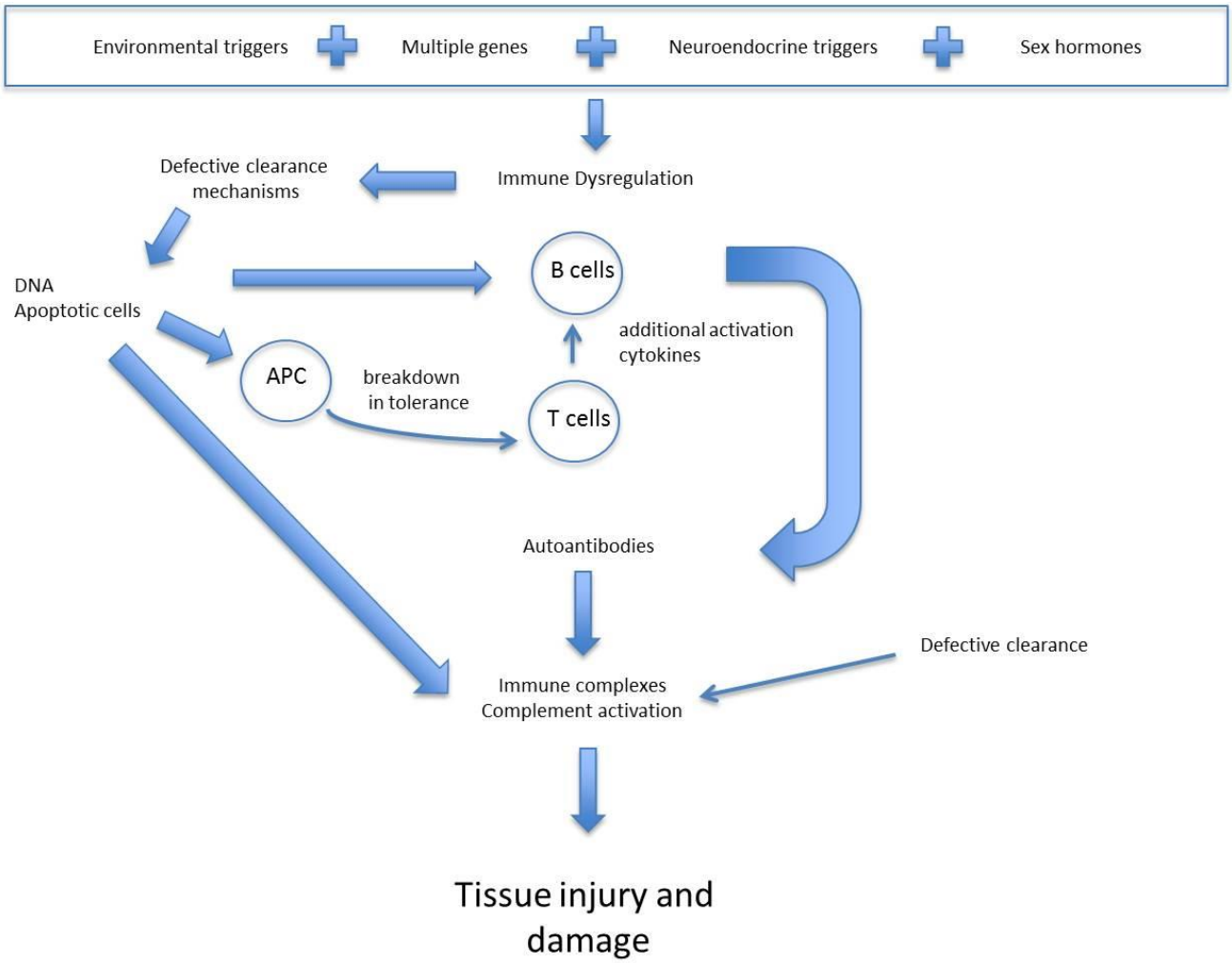


Figure 1.6 The Pathogenesis of Systemic Lupus Erythematosus
An overview of the main mechanisms that have been identified as contributing to the pathogenesis of the SLE disease.

1.5.3 Clinical Features of SLE

SLE disease progression is characterised by periods of remission and relapse. Non-specific symptoms are common in SLE and include fatigue, fever and anorexia. A severe and for many the most disabling symptom of SLE is fatigue (Morrow W 1999). The most frequent symptom of the musculoskeletal system is arthralgia which affects approximately 90% of patients with SLE (Morrow W 1999). Additionally myalgia, muscle weakness and tenderness have been reported in up to 60% of patients (Isenberg DA 1981). Other common features include the classic butterfly rash and pleuritic chest pain. Table 1.1 summarises the revised classification criteria for SLE (Tan EM 1982; Hochberg 1997) listing the clinical features commonly associated with SLE.

MANIFESTATION	DEFINITION/CHARACTERISTICS
Malar rash	Erythematosus rash over malar area, sparing nasolabial folds
Discoid rash	
Photosensitivity	
Oral Ulcers	Usually painless and observed by physician
Arthritis	Non-erosive, involving two or more joints
Serositis	Pleuritis and/or Pericarditis
Renal disorder	Persistent proteinuria (>0.5g/24 hours or >3+ on dipstick if quantification unavailable)
Neurological disorder	Seizures or psychosis (unprovoked)
Haemolytic disorder	Haemolytic anaemia or leucopoenia or lymphopenia or thrombocytopenia
Anti-nuclear antibodies	Raised titre at any point
Immunological disorder	Raised anti-native DNA antibody or Presence of anti-Sm antibody or Positive aPL (based on abnormal serum level of IgG or IgM aCL or positive LA or false positive test result for Syphilis sustained for at least six months

Table 1.1 Classification criteria for the diagnosis of SLE. Adapted from *Tan et al* (Tan EM 1982) and *Hochberg et al* (Hochberg 1997). Out of the 11 criteria four are required, either serially or simultaneously, to make a diagnosis of SLE. Abbreviations: Sm, Smith; aPL, antiphospholipid; LA, lupus anticoagulant, IgG; immunoglobulin G, IgM; immunoglobulin M.

1.5.3.1 Mucocutaneous Features

It is acknowledged that mucocutaneous involvement in SLE affects the majority of patients. The classic lupus 'butterfly' rash is elevated, often painful and precipitated by exposure to sunlight. This kind of rash can last for a number of days but may also persist for weeks and is commonly associated with other inflammatory manifestations of the disease. Other rashes such as discoid lupus erythematosus (DLE) develop in as many as 25% of SLE patients. This type of lesion are small and discrete, however infiltrate dilated hair follicles (follicular plugging) (Bertsias G 2012).

Alopecia occurs in the majority of SLE patients and therefore the term 'lupus hair' has been characterised by thin hair that easily fractures. The hair loss normally corresponds to increased disease activity and therefore normally grows back as disease activity subsides. Photosensitivity occurs in 60-100% of SLE patients who are defined as developing a rash after minimum exposure to ultraviolet (UV) lights.

1.5.3.2 Musculoskeletal features

In approximately 50-95% of patients with SLE there are signs of the musculoskeletal system being affected (Bertsias G 2012). Most commonly arthritis is observed with join involvement classically being non-erosive, non-deforming arthralgias in a distribution similar to that seen in patients with RA. It most commonly affects small joints in the hands, wrists and knees and may be the primary symptom observed or accompany other lupus manifestations during a flare. Another less common feature is myositis where general myalgia and muscle tenderness with proximal muscle weakness is observed during a flare of the disease. Finally avascular bone necrosis (AVN) is seen in typically 5-15% of patients (Mok 2003; Ghaleb R 2011)and is a form of acute joint pain presented in the latter course of SLE, which causes mortality and disability. Factors, which are known to induce AVN, include ischaemia and necrosis to the bone via Raynaud's phenomenon, vasculitis and the APS.

1.5.3.3 Renal Complications

Renal involvement in SLE is common, with 40-70% of all patients being affected as well as it being a major cause of mortality and hospital admissions. Immune complexes form and deposit themselves in the kidney causing intra-glomerular inflammation. This inflammation leads to recruitment of leucocytes and activation and proliferation of renal cells. This disruption in renal functions leads to lupus nephritis (LN) due to proteinuria of various levels being present in the urine. Lupus nephritis presents with proteinuria and may lead to nephrotic syndrome (proteinuria, hypoalbuminaemia and peripheral oedema), hypertension and deteriorating renal function if not identified early and treated.

1.5.3.4 Nervous System

The central nervous system (CNS) involvement in SLE is thought to be one that is most difficult to diagnose and though less common than renal, skin or musculoskeletal involvement, it remains a major cause of morbidity and mortality in patients with SLE. Clinical manifestations observed include both central and peripheral nervous systems and they must be segregated from infections, metabolic complications and drug-induced toxicity. The diagnosis of CNS involvement is a major challenge in the clinical setting with milder symptoms such as migraine, cognitive dysfunction (20-30% patients) and minor psychiatric disorders being the most difficult to link to SLE. However, it has been reported that migraines are the most prevalent manifestation in SLE patients but linking this directly to disease activity is very difficult therefore the inclusion of migraine among SLE disease activity features remains questionable (Sciascia S 2013).

1.5.3.5 Pleura and Lungs

Most commonly pleuritis is observed in SLE with 45-60% of patients affected and frequently presents with pleuritic chest pain, which can occur in the presence or absence of a pleural effusion. The effusions are linked to higher glucose and lower lactate dehydrogenase levels, compared with pleural effusions seen in patients with RA (Bertsias G 2012).

1.5.3.6 Haematologic features

Often haematologic abnormalities are the presenting symptom in SLE, with manifestations such as anaemia, leucopenia and autoimmune thrombocytopaenia. Anaemia is common in SLE and has been shown to correlate with disease activity (Giannouli S 2005). A common cause is suppressed erythropoiesis due to chronic inflammation or autoimmune haemolytic anaemia. Leucopenia is also common with white blood cell counts of below 4500/mm^3 (4-11x10^9 is normal) reported in about 30-40% of cases, particularly when disease activity is high (Bertsias G 2012).

1.5.3.7 Cardiovascular features

Patients with lupus have increased and accelerated atherosclerosis with a greater prevalence of cardiovascular and cardiac valvular disease. Studies have also shown an increased risk of MI and stroke; however this increased risk cannot be explained by traditional CVD risk factors. Valvular heart disease has been likened to the presence of aPL, with the most common abnormality being diffuse thickening of the mitral and aortic valves (Gabrielli F 1995).

The relative risk of suffering a MI in patients with SLE is high, with a 5-10 fold increase in the possibility of developing cardiovascular disease when compared to the background age and gender matched population (Elliott JR 2007). The relative risk of developing cardiovascular disease is particularly high in pre-menopausal women, who have a 50-fold increase of atherosclerotic disease for women aged 35-44, in comparison to age and gender matched controls (Manzi, Selzer et al. 1999). This higher relative risk of early cardiovascular disease in patients with SLE underlines the importance for identifying therapeutic targets, that when modulated may improve morbidity and mortality outcomes in these patients. Whilst it is important to investigate treatments that are able to prevent an MI from occurring, naturally a significant number of patients with SLE due to their increased risk, will ultimately suffer from an event. It is, therefore, also important to explore whether patients with lupus are more susceptible to I/R cardiac injury, should an MI occur. Addressing this question is important, because if I/R cardiac injury is also worse in SLE and pathways are identified in lupus that may promote this enhanced I/R injury, then targeted intervention could have translational benefits. Previous studies have shown that the SLE-like B6.MRL/*lpr* mice have accelerated and enhanced intestinal I/R injury. Additionally I/R-induced injury resistant *Rag-1*$^{-/-}$ mice infused with whole IgG from old B6.MRL/*lpr* mice showed restored intestinal I/R injury (Fleming S 2004). To date there has been little focus on the heart in terms of investigating I/R injury and SLE.

Whilst there is a good understanding of the pathogenesis and the clinical manifestations of SLE, the causes are less well defined. The next section will discuss some of the current theories as to why individuals are more susceptible to this disease.

1.5.4 Causes of SLE

The aetiology of SLE includes both genetic and environmental components with the female sex strongly influencing pathogenesis. All these factors are believed to lead to loss of tolerance manifested by immune responses against endogenous nuclear antigens. However, it is important to remember that SLE is highly multifactorial disease and it is likely that many causes of disease are yet to be discovered.

1.5.4.1 Genetic Factors

Studies have shown that SLE patient's siblings are 30 times more likely to develop SLE when compared to those with no affected sibling (Bertsias G 2012). Genome-wide association studies

using hundreds of thousands of single nucleotide morphism (SNP) markers has allowed the discovery of SLE related genes to be identified. Additionally, it has allowed identification of specific genes related to immune response and inflammation (e.g. HLA-DR), DNA repairs (e.g. TREX1), adherence of inflammatory cells to the endothelium (ITGAM) and tissue response to injury (KLK1) (Hom G 2008). Nevertheless, monozygotic twin concordance is 25%, which though higher than background prevalence in the population, implies that other non-genetic factors play a role in conferring risk (Grennan DM 1997).

1.5.4.2 Epigenetic Factors

More recently the role of epigenetic modifications such as DNA methylation and post-translational modifications of histones have been explored in SLE (Ballestar E 2006). This sort of modification can be inherited or can be due to environmental influence. Epigenetics refers to changes in gene expression, which are caused by mechanisms independent of DNA base sequence alterations. Research has suggested that differences in DNA methylation status of certain genes could explain, at least in part, differences observed in some identical twins that are discordant for SLE (Jeffries 2011). DNA accessibility to transcription factors and, therefore, gene expression is regulated by histone modifications e.g. acetylation as well as DNA methylation. An example is the recruitment of histone deacetylase 1 to the IL2 promoter suppressing its expression (Mishra N 2003). Many believe that epigenetics may be the missing factor, which links genetic and environmental risk factors (Jaenisch 2003).

1.5.4.3 Environmental Factors

Environmental factors such as medication, smoking and exposure to UV light are thought to be candidate triggers of SLE (Tsokos 2011). Epstein Barr virus (EBV) has also been identified as a possible cause of SLE as it is thought to reside and interact with B cells and promote IFN-α production by pDCs. Therefore, it is suggested that at least in part lupus could be triggered in response to viral infection driven by elevated IFN-α levels, a central cytokine in SLE (Obermoser 2010). Certain drugs have been linked to induction of autoantibodies in a significant number of patients, most of who do not develop signs of autoantibody-associated diseases. However, over 100 drugs have been associated with causing drug-induced lupus (DIL). Although the cause of DIL is not well understood, it is believed that a genetic predisposition may play a role. For example, when patients with slow acetylator enzymes are given drugs that are metabolized by acetylation they are more likely to develop disease (Bertsias G 2012). Also, it has been suggested these drugs may alter

gene expression in CD4+ T cells by inhibiting DNA methylation and inducing over-expression of LFA-1 antigen, therefore promoting auto reactivity (Zhou 2008).

1.5.4.4 Hormonal Factors

It has been shown in murine models that oestrogen or prolactin can lead to an autoimmune phenotype with an increase in mature autoreactive B cells (Bertsias G 2012). The Nurses' Health Study showed that increased risk of developing SLE was associated with oral contraceptive (relative risk 1.9 compared to never users). These findings suggest that hormones can contribute to autoimmune development in murine models, however interestingly the use of oral contraceptive does not increase disease flares in women who have stable disease (Sanchez-Gierrero 2005). However, other studies have shown that as long as disease state is stable then there is no increased risk of disease flare, with only 7 out of 91 patients taking oral contraception having a severe disease flare (Petri M 2005). Another important factor is pregnancy, however it has been shown that although in some cases disease flares are induced by pregnancy, levels of oestradiol and progesterone are actually lowered in the second and third trimester when compared to healthy matched controls (Doria A 2008) and this may account for the variation in flare rate seen during pregnancy in patients with SLE.

1.5.5 Diagnosis

1.5.5.1 Serological Tests

A number of serological tests, whereby serum is tested for autoantibodies, have been developed to diagnose rheumatic diseases. An example is the ANA antibody assay, which is known to be extremely sensitive (95% when using human cultured cells as the substrate) and relatively easy to carry out (Bertsias G 2012). A limitation of the ANA assay is its specificity for SLE. ANAs are present in many other rheumatic diseases as well as observed in infections, as well as some 10% of healthy individuals testing positive. Due to this issue other assays have also been develop, namely an assay for antibodies to extractable nuclear antigens (ENAs) and antibodies to Sm (Smith) and Ro are those most frequently seen in lupus. The first identified lupus antigen was the nucleosome, a complex of DNA and histones (Rumore 1990). Autoantibodies to single stranded DNA (ssDNA) and individual histones are common in SLE and antibodies to dsDNA are found in up to 70% of SLE patients. Antibodies for dsDNA are 95% specific for the disease and, therefore, are very valuable disease marker but clearly less sensitive than ANA. Other antibodies, which have been correlated with SLE antibodies to some ENAs, namely anti-Sm (Smith) antibodies and these are found in 10-

20% of SLE patients (Bertsias G 2012). Additionally, anti-ribosomal antibodies are found in 12-16% of patients with SLE, but are less sensitive than anti-dsDNA or anti-Sm antibodies (Nagai T 2005).

1.5.5.2 Prognostic markers

The analysis of large cohorts has identified clusters of autoantibodies associated with distinct SLE features as well as serum anti-dsDNA titers being correlated with LN. The aPL antibodies are associated with features of APS, CNS involvement, LN and death. The ENA autoantibodies anti-Ro and anti-La have been correlated to neonatal lupus and congenital heart block in neonates of seropositive mothers (Hausenloy 2013).

1.5.6 Classification criteria and activity indices

A criterion for the SLE was first developed in 1971 but has since been revised several times, with the latest in 2007. The criterion allows patients to be distinguished from those without the disease. The American College of Rheumatology ACR classification was developed for clinical studies of lupus to ensure that cases reported in the literature do in fact have the disease (Bertsias G 2012). However, due to the dynamic nature of the disease, diagnosis can often be challenging. Whilst this criteria is recognised as being used in standard practice, several caveats have been identified. Most importantly, this set of criteria was developed to identify patients with an established disease profile and therefore may exclude those who are early in the disease progression or have limited organ involvement. As well as disease diagnosis, it is important to scale disease activity. This is because lupus is such a complex and varying disease from patient to patient and such scale allows appropriate treatment plans to be formed. The activity of the disease needs to be distinguished from permanent damage as this has an effect on long-term prognosis as well as identifying the appropriate treatment. There are a number of validated global as well as organ-specific activity indices used to measure the activity of a patients disease. An example is the British Isles Lupus Assessment Group Scale (BILAG) which provides individual organ/system assessment (Hay E 1993). This was updated in 2004 (BILAG 2004) due to concerns over the division of organs and systems for the purpose of activity assessment (Isenberg D). Other global score systems exist which provide an overall measure of activity. Examples include the European Consensus Lupus Activity Measurements, Systemic Lupus Activity Measure (SLAM), and Systemic Lupus Erythematosus Disease Activity Index (SLEDAI). All the different assessment scales have been validated against one another and therefore all can be recommended for monitoring a patients disease progression (Romero-Diaz J 2011). Whilst the majority of SLE patients are diagnosed during their adulthood, up to 20% of cases are diagnosed

before the age of 16 and therefore termed childhood-onset or juvenile-onset SLE (JSLE) (Amaral B 2014).

1.5.7 Juvenile Systemic Lupus Erythematosus

Although the manifestations of JSLE are broadly similar to that of in adult-onset, differences do exist. It is accepted that the age at which the disease begins can have a significant impact on the severity and outcome of the disease (Descloux E 2009). It has been suggested that JSLE has a more aggressive nature, with renal organ involvement being more frequent greater and long-term treatment (e.g. immunosuppressant) being required (Amaral B 2014). This leads to poorer prognosis, resulting in earlier morbidity and increased mortality rates due to factors such as a higher risk of cumulative damage. Studies have shown that a notable percentage of adolescent-onset patients develop cardiovascular events and cancer, and whilst this was not higher than in adult-onset, these diseases are uncommon in younger patients suggesting an increased susceptibility (Frostegard 2005).

Correct disease management of both adult and childhood onset SLE is crucial, and as part of this it is important to have correct treatment plans. The following section outlines current treatments available to patients.

1.5.8 Treatments

1.5.8.1 Non-pharmacologic management

There is little advice for non-pharmacologic treatment of SLE; however EULAR and ACR guidelines make some limited recommendations. They offer guidelines on safe sun protection, as one-third of patients are known to be photosensitive. This includes avoiding direct exposure and using suitable sun protection (e.g. clothing and sunblock to block UVA and UVB rays) (Ioannou 2002). It is also advised that smoking be prevented as it has been shown smokers have a significantly higher SLEDAI score than previous smokers or those that have never smoked (Ghuassey, 2003). Diet is also advised on, with vitamin D and calcium intake particularly important, especially in patients receiving long-term glucocorticoid therapy.

1.5.8.2 Pharmacological treatments

Guidelines were developed by the ACR in 1999 and the EULAR taskforce in 2008 and they are the most commonly used guidelines but they do have some limitations, particularly the ACR guidelines

because of the outdated proposal. This means that newer medications aren't included, simply because they were not available. Additionally, the treatment of SLE patients is highly personal and therefore each case has to be assessed individually (Gladman 1999 and Bertsais 2008). Mild symptoms such as arthralgias and cutaneous manifestations are generally treated by antimalarials and non-steroidal anti-inflammatory drugs (NSAIDs), whereas oral corticosteroids and cytotoxic agents are used for more severe disease. Depending on organ involvement and severity, other drugs such as cyclophosphamide, immunosuppressive agents and tacrolimus may also be used.

Biologics are increasingly used in lupus. The most recent agent on the market is belimumab, which is a human monoclonal antibody that inhibits B cell activating factor (BAFF) and is now licensed for patients with SLE with no renal nor CNS manifestations for moderate to severe disease.

1.5.8.3 NSAIDs

NSAIDs are prescribed in the majority of patients with SLE due to 90% developing arthralgias or polyarthritis (Cojocaru M 2011). However, NSAIDs can have significant risks associated with long-term use (Bertsais 2008) such as having an adverse effect on renal function in the setting of lupus nephritis. Their mechanism targets inhibition of cyclooxygenase-2 (COX-2), which in turn inhibits the production of prostaglandins that protect the lining of the gastrointestinal tract. This makes gastrointestinal bleeding a possible side effect and therefore patient blood count and serum creatinine are monitored as well as educating patients on possible symptoms of bleeding (e.g. bloody stools and urine) (Gladman 1999). For this reason they are often co-prescribed with gastroprotective agents such as proton pump inhibitors. Additionally, there is also evidence to suggest that NSAIDS may enhance the risk of developing CVD by up to 35% (Capone ML 2010).

1.5.8.4 Glucocorticoids

Glucocorticoids are a group of immunosuppressive agents used in the majority of patients with some cohort studies suggesting up to 80% of patients use the therapy as a 'maintenance treatment' (Mosca 2011). However, high doses are normally reserved for treatment of patients with active major organ involvement (Parker 2007). The concern is that long-term complications at high doses are common and often severe and can include hypertension, diabetes, weight gain, osteoporosis and infections through immunosuppression, therefore those treated for long periods of time are monitored closely. Also long-term steroid use is linked with higher levels of damage incidences (Ruiz-Arruza I 2014).

1.5.8.5 Cytotoxic/immunosuppressive agents

The main treatment of active lupus with major organ involvement is broad spectrum immunosuppression, whilst at the same time aiming to prevent organ damage (Ioannou 2002). Patients who fail to respond to less toxic drugs such as antimalarials are next considered for immunosuppressive agents including cyclophosphamide, mycophenolate mofetil and azathioprine. Cyclophosphamide is normally administered intravenously to decrease the risk of bladder damage and often results in lower overall cumulative dose. Mycophenolate mofetil targets an enzyme in the body called inosine monophosphate dehydrogenase that is important for the formation of DNA in cells. By interfering with DNA, the medication impairs function of immune system cells that become overactive in autoimmune diseases such as lupus (Karim M 2002).

1.5.8.6 Belimumab (Benylsta)

In March 2011 The Food and Drug Administration (FDA) and European Medicines Agency (EMA) approved belimumab for treatment of adults with active, autoantibody-positive SLE. This drug has a unique mechanism in that it targets B-cell dysfunction by inhibiting BLyS (Stohl 2013). It blocks the binding of soluble BLyS, a B cell survival factor, to receptors on B cells. Although belimumab does not directly bind to B cells it inhibits their survival due to its interactions with BLyS, this includes targeting autoreactive B cells. Additionally, it reduces the differentiation of B cells into antibody producing plasma cells (Furie R 2008).

1.5.8.7 Rituximab

Rituximab is a chimeric monoclonal antibody (mAb) that was originally developed for the treatment of B cell lymphomas (Grillo-López AJ 1999). It is directed against the cell surface molecule CD20 which is present on B cells when in the immature stage and remains until they differentiate into plasma cells (Eisenberg 2005). Rituximab has been shown to trigger cell death through complement-dependent cytoxocity (CDC), antibody-dependent cell mediated cytotoxicity (ADCC) and direct induction of apoptosis. Although initial studies were positive, the LUNAR and EXPLOER studies are two double-blind phase II/III randomized, placebo-controlled trials (RCTs) with rituximab in SLE or lupus nephritis (n = 257 and 144, respectively) which failed to meet their primary or secondary end points (Merrill J 2010; Rovin B 2012). There are a number of issues with these trials, such as the excessively high use of steroids and choice of outcome measures that may have contributed to these negative results. Multiple, large open labelled studies have confirmed clear benefit, with greatest indication for efficacy in early disease (Ezeonyeji 2012) and, hence, this has now become an established treatment in SLE.

1.5.9 Antimalarials

Antimalarials are used in around 80-90% of patients with SLE (Gladman 1999). Hydroxychloroquine (HCQ) is the most commonly prescribed antimalarial agent and this is due to its decreased likelihood of causing ocular and gastrointestinal adverse conditions as compared to chloroquine. It has been used for over 60 years in the treatment of inflammatory disorders and despite its numerous clinical applications the molecular mechanisms by which it elucidates it effects are poorly understood. It is very similar to chloroquine except for the addition of a hydroxyl group to the side chain and beta hydroxylation of the N-ethyl substituent. HCQ is orally administered and is absorbed into the gastrointestinal tract rapidly leading to a large volume of distribution. A variety of cytochrome P450 enzymes convert it to an active metabolite called desethyl HCQ (Lee S 2011). Post-commencement of therapy, it can take between 3-6 months before maximal clinical efficacy is achieved. It is recommended that a dose of 200-400 mg is administered daily, resulting in physiological concentrations of 1-2 µg/mL in patient blood being achieved (Costedoat-Chalumeau N 2006).

As previously mentioned, little is known about the mechanisms of action through which HCQ is able to reduce symptoms and improve patient conditions. However, there are some mechanisms which have been identified to date. An example is its ability to reduce inflammation by blocking pro-inflammatory pathways (Willis R 2012). This includes decreasing production of cytokines such as interleukins and IFN-α, which are known to be up-regulated in SLE (Crow 2010). HCQ is believed to be able to interfere with physiological functions of subcellular compartments that rely on an acidic environment due to their weak base nature. It is able to enter lysosomes as well as other acidic compartments where it disrupts functions which depend on an acidic milieu such as inflammatory mediator secretion, autoantibody production and receptor recycling (Fox 1993). Another example of the disruption it can induce is in respect to TLRs, which play a pivotal role in the innate immune response. It has been shown that HCQ can inhibit TLR-9 pathways as well as TLR-7 and TLR-3. It was originally thought that HCQ inhibited endosomal compartment acidification due to its weak base nature, therefore inhibiting activation. However, more recent studies suggest it can interact with nucleic acids which act as ligands for TLR-9 and TLR-7, therefore preventing ligand binding (Kuznik A and Jerala 2011). This may be the reason why pro-inflammatory cytokines such as interferon's and interleukins are reduced, as these TLRs are known to stimulate cytokine production.

Retrospective studies have suggested that HCQ acts as a cardioprotective agent in patients as well as research studies, identifying improvements in factors which typically cause cardiovascular disease. This includes reduced platelet aggregation (thrombosis) and improved lipid profiles. This data is summarised in table 1.2

Reference	Study design	Population	Findings
*Studies included are evidence-based level A or B studies with various study designs.			
Abbreviations: aPL, antiphospholipid antibody; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.			
Thrombosis*			
Ho *et al.* (2005)[58]	Observational cohort (LUMINA)	442 patients with SLE and known aPL status	Hydroxychloroquine use protective against thrombosis in univariate analysis (OR 0.536)
Ruiz-Irastroza *et al.* (2006)[53]	Observational prospective cohort	232 patients with SLE	Antimalarial use protective against thrombosis (HR 0.28)
Sisó *et al.* (2008)[59]	Cohort	206 patients with lupus nephritis, 56 of whom previously used antimalarial agents	Previous hydroxychloroquine use protective against thrombosis (5% in hydroxychloroquine users vs 17% in non-users)
Kaiser *et al.* (2009)[60]	Cohort	1,930 patients with SLE	Hydroxychloroquine use thromboprotective after propensity analysis (OR 0.62)
Tektonidou *et al.* (2009)[61]	Longitudinal case–control	144 aPL positive patients with SLE matched with 144 aPL-negative patients with SLE	Hydroxychloroquine use protective against thrombosis in both groups (aPL positive patients: HR per month 0.99; aPL negative patients: HR per month 0.98)
Jung *et al.* (2010)[62]	Nested case–control	162 patients with SLE, 54 of whom had a history of thrombotic event	Ever-use of antimalarial agents thromboprotective (OR 0.32) Overall 68% reduction in thrombotic events in users of antimalarial drugs
Lipid profile*			
Wallace *et al.* (1990)[33]	Retrospective	155 patients with SLE or RA subdivided according to exposure to hydroxychloroquine and/or steroids	Addition of hydroxychloroquine to steroids reduced levels of LDL cholesterol and triglyceride by 15% compared with steroids alone
Petri *et al.* (1994)[63]	Longitudinal cohort (Hopkins)	264 patients with SLE	Hydroxychloroquine use associated with an 8.94 mg% reduction in serum total cholesterol level
Kavanaugh *et al.* (1997)[64]	Double blind, prospective	17 patients with SLE	Hydroxychloroquine associated with a mean decrease of 11.6 mg/dl in serum total cholesterol level
Borba *et al.* (2001)[65]	Case–control	60 patients with SLE (subdivided according to exposure to hydroxychloroquine and steroids) and 30 healthy controls	Chloroquine use associated with elevated level of HDL cholesterol as compared with no therapy, and with lower level of VLDL cholesterol in the group taking chloroquine plus steroids

Table 1.2 Summary of published research suggesting HCQ is cardioprotective. Adapted from Wallace et al (Wallace D 2012).

Patients with lupus typically show increased ‘bad cholesterol’ such as low-density lipoproteins (LDLs) and reduced ‘good cholesterol’ such as high-density lipoproteins (HDLs). These lipids have the ability to build up in plaque, increasing the risk of a cardiovascular event. A study of a cohort of 264 patients with SLE showed that HCQ mediated lower serum cholesterol independently of steroid administration. Patients on HCQ showed lower levels of atherogenic lipids such as cholesterol, triglycerides and LDLs, with the effect on HDLs remaining conflicted (Morris S 2011). This improved lipid profile is an important mechanism of action for HCQ in patients with lupus and helps reduce their risk of complications associated with the disease.

Antimalarials have been shown to significantly reduce the risk of thrombovascular events in lupus patients. One study observed 54 SLE patients with thrombovascular events that were matched to controls for the calendar year of their first visit, duration of follow up and highest disease activity during a 2 year observation period. It was shown that the risk of all thrombovascular events was reduced by 68% for patients on HCQ (Hyejung J 2010).

Annexin A5 is a potent anticoagulant protein, which works by crystallizing over phospholipid bilayers therefore preventing them from being available for coagulation reactions. Patients with SLE are commonly found to be positive for aPL antibodies which have been shown to disrupt annexin A5 binding. This prevents annexin A5 exerting its anti-coagulant properties leading to increased risk of thrombosis. It has been observed that HCQ reverses aPL antibody-mediated disruption of annexin A5 binding on phospholipid bilayers (Rand J 2010). This mechanism of action further supports the suggestion that HCQ is able to exert anti-thrombotic effects.

The adverse effects of antimalarials are mild and infrequent. However, rare but serious adverse effects include cardiomyopathy, arrhythmias and neuromyopathy. Retinal toxicity is also a concern from HCQ treatment, and although incidence is low thousands of individuals take the drug and therefore this must be monitored. It is therefore suggested that patients eye sight is checked prior to taking HCQ to act as a baseline and then checked annually from 5 years after treatment commencement (Marmor M 2011). Therefore, based on current evidence of multiple beneficial effects of HCQ in SLE, which includes improved disease activity control, reduced regularity and severity of flares, reduced risk of cardiovascular events and reduced overall mortality and favourable safety profile, it is recommended by many clinicians that patients with SLE be treated with HCQ indefinitely (Wallace D 2012).

Within SLE 40-50% of patients can also develop a subsequent autoimmune disorder, the APS, this is termed secondary APS. However, it has now been established that it can develop independently from any other underlying disease and this is therefore called primary APS (Grossman 2004). Its relevance to this thesis is that its main pathogenic effect is mediation of thrombosis and therefore it will be expanded upon in the next section of this chapter.

1.5.10 The Antiphospholipid Syndrome

APS is a pro-thrombotic disorder which associates aPL with hypercoagulability (Levine J 2002). There are two distinct hallmarks of APS; VT (venous and/or arterial thrombosis) and PM (pregnancy morbidity). It most commonly targets the deeps veins of the lower limbs and cerebral arterial circulation, however any organ can be targeted (Giannakopoulos 2013). APS is caused by the persistence of pathogenic aPL, detectable by the anti-aCL and/or anti-β_2GPI and/or lupus anticoagulant (LA) assays (Miyakis S 2006). β_2GPI is a protein of approximately 53 kDa, composed of 5 homologous polypeptide subunits, which are termed domains I through to V, with domain V being responsible for binding anionic phospholipid.

A small subgroup of patients develop catastrophic antiphospholipid syndrome (CAPS) and this is characterised by multiple thrombi in small vascular beds resulting in multi-organ failure and high mortality (Sciascia S 2012). CAPS represents less than 1% of all APS patients, but is severe with a 30-50% mortality rate even in the setting of optimal treatment (Asherson RA 1998; Asherson RA 2001).

1.5.10.1 Classification criteria of the APS

The criteria outlined that definite APS is considered to be present in a patient with at least one clinical manifestation (as outlined in table 1.3) and a positive test for circulating aPL (including LA, aCL and anti-β_2GPI) detected twice, at least 12 weeks apart (Miyakis S 2006).

Clinical Criteria	
Vascular Thrombosis	One or more clinical episodes of arterial, venous or small vessel thrombosis, in any tissue or organ. Thrombosis must be confirmed by objective validated criteria
Pregnancy morbidity	One or more unexplained deaths of a morphologically normal foetus at or beyond the 10^{th} week of gestation, with healthy foetal morphology documented by ultrasound or examination of foetus. One or more premature births of a morphologically normal neonate before the 34^{th} week of gestation because of eclampsia or severe pre-eclampsia defined according to standard definitions or recognised features of placental failure. Three or more unexplained consecutive spontaneous abortions before the 10^{th} week of gestation, with maternal anatomical or hormonal abnormalities and paternal and maternal chromosomal causes excluded.
Laboratory Criteria	
The presence of LA in plasma on two or more occasions which are at least 12 weeks apart, detected according to the guidelines of the International Society on Thrombosis and Haemostasis The presence of aCL of IgG and/or IgM isotype in the serum or plasma of the patient, present in medium or high titres (ie. above 40 G/MPLU or greater than the 99^{th} percentile) The presence of the anti-β_2GPI antibody of IgG and/or IgM isotype in the serum or plasma of a patient on two or more occasions at least 12 weeks apart, measured by a standardised ELISA.	

Table 1.3 Clinical and laboratory criteria for the APS
This table outlines the clinical and laboratory classification criteria of the APS as defined in the 'International consensus statement on an update of the classification criteria for definite APS' (Miyakis S 2006).

1.5.10.2 aPL pathogenicity

Both clinical data and experimental evidence have shown a direct link between aPL antibodies and the manifestations of recurrent venous and arterial thromboembolic events. A number of clinical studies have shown significant association between increased aPL levels and venous (Schulman S 1998) and arterial (Brey R 2001) thrombosis. In a prospective study *Vaarala et al* compared 133 patients who developed a cardiac end point, defined as death or non-fatal MI, to 133 control subjects. Patients who developed a cardiac end point had high levels of aCL compared to control patients (Vaarala O 1995). Additionally a study by *Meroni et al* showed a significant association between anti-β_2-GPI antibodies and MI occurrence when comparing 172 women with a history of MI and 172 with no history of thrombosis (Meroni P 2007).

A 'two hit hypothesis' has been suggested to explain the clinical observations occur only occasionally even when there is a persistent presence of aPL. This theory suggests that the antibodies act as the primary hit and induce a thrombophilic state, however clotting only takes place in the presence of another thrombophilic state (the second hit e.g. MI) (Shoenfeld Y 2006).

The pathogenic effects of aPL have been widely explored, particularly in endothelial cells due to the enhanced risk of suffering from thrombosis. Human monoclonal and polyclonal aPL have been demonstrated to induce a signalling cascade similar to that observed with LPS. There is evidence to suggest TLR2 and TLR4 could be implicated in aPL activation in endothelial cells. *Pierangeli et al* (Pierangeli SS 2007) demonstrated that TLR4 is involved in the pathogenesis of aPL-mediated thrombosis and aPL activation of endothelial cells. Two strains of mice (LPS responsive vs unresponsive) were injected with the following IgG; IgG purified from 2 patients with primary APS (1 with a history of thrombosis and 1 with no history of APS and PM), 1 healthy control IgG and 1 polyclonal IgG from an aPL-negative patient with SLE. The mice were then subjected to mechanical pinch induced thrombi to their right femoral vein. The IgG purified from APS patients produced significantly larger thrombi in comparison to the non-APS samples in LPS responsive mice. LPS unresponsive mice treated with APS IgG showed a significant reduction in thrombus size suggesting that TLR4 is implicated in the pathogenic effects of APS IgG in this animal model. *Vega-Ostertg et al* (Vega-Ostertag ME 2007) have shown that activation of p38 MAPK (a downstream target of TLR4) leads to aPL mediated thrombosis as well as aPL mediated activation of endothelial cells. They demonstrated that IgG from 6 aPL positive patients increased phosphorylation of p38 MAPK in endothelial cells compared with IgG from 2 healthy controls. Activation of p38 is known to

promote an inflammatory response via the production of cytokines and chemokines, therefore its activation is thought to contribute to the pathogenesis of SLE (Iwata Y 2003).

The pathogenic role of SLE derived autoantibodies in cardiac I/R injury has been relatively unexplored, although some work that has been published on the role of natural IgM which was discussed in *section 1.5.2.2* of this chapter. However, there has been some interesting work published in relation to pathogenic IgG and its contribution to enhanced mesenteric I/R injury. Previously it has been shown that in mesenteric I/R injury an autoimmune prone mouse model is more susceptible to I/R injury as compared to a sham control. Furthermore, when IgG from a lupus mouse model is injected into a Rag-1 deficient mouse, which has no B or T lymphocytes, cell injury is accelerated in comparison to control (Fleming S 2004). *Fleming et al* (Fleming S 2004) next went on to show that aPL antibodies restore mesenteric I/R Injury in Complement Receptor 2/Complement Receptor 1-Deficient Mice, therefore suggesting a role for them in mediating I/R injury. They identified β_2GPI as a binding site for aPL antibodies, however proposed that multiple neoantigens are expressed in response to I/R injury and therefore these auto-antibodies could be also binding other sites.

To date no one has extensively explored the role of myocardial I/R injury in lupus or indeed APS. It would be expected that due to the enhanced risk of cardiovascular complications, that lupus contributes to enhanced I/R injury in the heart. The next section will therefore discuss the possible mechanisms through which this could occur by speculating on common pathogenic processes which are present in both SLE and cardiac I/R injury

1.6 I/R injury in SLE

Accelerated atherosclerosis is widely accepted as a major cause of cardiovascular related morbidity and mortality in SLE, and this is currently the research focus of a number of groups around the world. However, what remains relatively unexplored is the role that I/R injury may be play in cardiac related morbidity and mortality in lupus. Many of the molecular mechanisms that mediate I/R, such as oxidative stress and enhanced apoptosis, are mechanisms that may be amplified in lupus and therefore one may speculate that I/R may be enhanced in SLE and contribute to patient morbidity and mortality. Understanding the relationship between lupus and I/R injury in greater depth, and identifying dominant common pathways that may be targeted may provide a novel target for ultimately improving outcomes in patients with SLE. This section will briefly describe the main pathogenic mechanisms which are both involved in SLE and I/R injury.

The presence of apoptotic bodies and enhanced apoptosis induction in SLE may cause accelerated apoptosis and, therefore, enhanced myocardial death in I/R injury due to stress pathways already being activated (Salmon 1999). Although it is known that apoptosis is a defined factor that influences lupus development, how these abnormalities trigger the disease are not well understood. A better understanding of the link would give a greater insight in to how this may affect the severity of I/R injury cell death via apoptosis induction.

MAPK have also been shown to play a role in SLE by their activation contributing to disease activity. For example ERK and JNK have both been shown to have increased activation correlating with increased disease activity (Richardson 2010). Specifically the MAPK pathway has been shown to have a role in regulation, selection, differentiation and maturation of T cells. Multiple abnormalities in T cell pathways have been linked to autoimmunity suggesting there might be a common denominator in pathogenesis of autoimmune diseases. Studies in SLE-prone mice with active SLE have been shown to have increased JNK activation and that treatment with complementary-determining regions peptide hCDR1 led to a decrease in JNK, lympohocyte apoptosis and caused amelioration of the SLE manifestations (M Rapoport 2005). In comparison, as previously reported for I/R injury, the role of MAPK can be kinase specific. When ERK activation is diminished, SLE induction was observed meaning a balance between pro-survival and pro-apoptotic MAPK is essential. Additionally, it is possible that local activation of MAPK family occurs; therefore an observation made in one target organ could be different to that seen in another

Multiple studies have suggested that the innate immune system and TLRs are relevant in both I/R injury and SLE, however it remains unclear to what extent the precise mechanisms of the innate immune system activation which may overlap between the two conditions. Interestingly, it has been shown that in a subset of SLE patients (20-40%) aPL target TLR2 and TLR4 activation and therefore it is possible that this subset of patients may be more prone to I/R injury (Satta N 2011)

ROS evidently play an important role in both cell death in I/R injury and pathogenicity in SLE. Given that ROS are known to promote pro-apoptotic signalling pathways and necrosis in I/R injury, one may hypothesize that I/R injury is enhanced in patients with SLE due to the increased baseline levels of ROS observed in this disease.

The role of complement in I/R injury and SLE is conflicting in that activation during I/R injury appears to contribute to pathogenicity through induction of various inflammatory pathways. In

contrast in SLE it has been suggested there is complement deficiency which is thought to be responsible in part for defective clearance of apoptotic cells. It would, therefore, suggest that those with SLE should have reduced I/R injury due to complement being relatively deficient, though this may be offset through an enhancement of apoptosis and increased baseline ROS levels. Studies have shown a role of antibodies in both I/R injury and SLE however patients with SLE typically have low complement levels and if complement is a dominant mechanism in I/R injury then it may be conceivable that the low complement effect seen in lupus may have a protective effect. Nevertheless, it is possible that due to the auto reactive nature of antibodies from patients with SLE that they may have a lower threshold for pathogenic IgM and therefore be more susceptible to I/R injury. Another feature of the immune response which is important is the enhancement of neutrophils in SLE patients. This may be a significant contributing factor to accelerated I/R injury due to enhanced neutrophil infiltration causing a larger inflammatory response and impaired degradation of neutrophil NETS as a source of ROS and nuclear antigenic material. The activation of complement may enhance I/R injury despite the relative deficiency of complement observed in SLE.

All the factors mentioned in this section could be important contributors to accelerated I/R injury in SLE. In order to explore the role of SLE on severity of I/R injury it is important to have robust model systems and, therefore, the next section will summarise the main animal models currently available to study myocardial I/R injury and the mechanisms, which contribute to severity.

1.7 Animal models in I/R injury

Myocardial ischaemia has been extensively studied in cardiovascular research; however its true meaning is still being debated (Hearse 1994). The majority of investigators define ischaemia as abnormalities between oxygen and substrates supplied to the heart, therefore preventing normal function. This is due to the heart depending on oxygen for oxidative phosphorylation, an essential metabolic process which has the ability to provide sufficient energy for normal myocardial contraction. Myocardial ischaemia is therefore an in balance between oxygen supply and demand, which leads to anaerobic respiration and reduced contractile function (Pieter D 1998).

The most common experimental modes in cardiac research are the isolated whole heart and cultured cardiac cells. The use of cell cultures is a common approach due to the versatility, economy and convenience compared with whole animal experiments (Chlopcíková S 2001). The neonatal rat cardiomyocytes model enables researches to study morphological, biochemical and

electrophysiological characteristics of the heart. Most importantly, *in vitro* studies allow multiple comparative experiments at once, unlike *in vivo* where specific conditions have to be kept, therefore limiting the number of questions that can be answered. Apoptosis is a pathological phenomenon that has been widely studied in neonatal rat cardiomyocytes, with the ability to study cell morphology, metabolism and contractile activities easily. Additionally the expression of proteins, regulation of their expression and the effect of drugs on their expression has been widely studied in this model (Pieter D 1998).

In contrast *in vivo* models of myocardial I/R injury provide more robust and transnationally relevant data. They have the ability to facilitate studies to verify the effectiveness of a therapy as well as various routes of time and application. Functional characterisation using echocardiographic investigations or magnetic resonance imaging (MRI) during ischaemia and reperfusion provide invaluable data. Additionally, histological and molecular biological experiments can be performed to objectify success of therapy and to identify specific mechanisms and targets.

Both *in vitro* and *in vivo* systems provide individual and combined valuable data. Neonatal rat cardiomyocytes are more accessible and allow the testing of multiple components. However, once the main targets have been identified the use of *in vivo* models is much more appropriate. Models of cardiac I/R injury are valuable, however given the number of therapeutics which show promise in the lab but fail to translate to the clinic, some have questioned the value of experimental I/R injury. There are a number of suggestions as to why these models are not as robust as they should be in relation to species, age, sex and most importantly duration of ischaemia and reperfusion (Hausenloy D 2010). The following section will expand on this further in relation to past and current treatment strategies to target I/R injury.

1.8 Therapeutic Strategies for I/R Injury

For the majority of patients myocardial ischaemia will have already taken place and therefore the opportunity to intervene is significantly reduced. However, although the restoration of blood flow (i.e. reperfusion) remains the most effective treatment, it also causes additional cell death therefore identifying a new therapeutic target. Nevertheless, there remains no effective treatment for preventing lethal myocardial reperfusion injury. This is despite the substantial progress in understanding the mechanisms of I/R injury, as well as the associated attempt to translate this to patient care. Some believe that this could be due to alternative mechanisms, which are yet to be

explored, whilst others believe it is due to the naive approach that a single intervention could be successful in treating such a complex process. Despite the lack of positive outcomes so far, investigations continue and the following sections will discuss the pharmacological and mechanical interventions currently being explored.

1.8.1 Therapeutic intervention for reperfusion injury

Targeting individual components of lethal myocardial injury such as oxidative stress, calcium overload and pH correction (as discussed in *section 1.1.4* previously) remains unsuccessful (Hausenloy 2013). The main problem with this targeted approach is that either adverse side effects are observed or that although protection is seen in animal models it does not translate to the clinic. An example of this is the recently published IMMEDIATE clinical trial, which investigated the effect of metabolic modulation using intravenous glucose insulin potassium (GIK) therapy. This was administered in the ambulance to patients suspected of suffering acute myocardial ischaemia with potential acute coronary syndrome. However, using a primary end point of progression to acute MI no difference was observed, although patients administered GIK therapy experienced less cardiac arrest and in-hospital mortality compared with those administered placebo (Selker H 2012). Also, as previously mentioned CsA was shown to reduce MI size by 40-50% in both small and large animal MI models (Hausenloy D 2003; Skyschally A 2010). This pharmacological agent has been perhaps one of the most promising with a small study suggestion that inhibition of MPTP may be of benefit. This pilot trial of 58 patients showed that administration of CsA at the time of reperfusion was associated with a smaller infarct then with placebo (Piot C 2008). This data is now being expanded in a larger clinical trial.

However, there are a number of therapeutic strategies which have shown promise and clinical trials are currently in progress to define the clinical outcomes of patients. This includes mechanical interventions such as therapeutic hypothermia (Duncker D 1996) and pharmacological therapies such as sodium nitrite. The latter has recently been shown in two human models to improve functionality of ischaemic myocardium as well as protection against vascular I/R injury when administered prior to ischaemia (Ingram T 2013). Additionally the concept of pre- and post-conditioning has been vastly explored as a type of intervention and will be discussed in the next section.

1.8.2 Pre-conditioning and Post-conditioning

Ischaemic pre-conditioning (IPC) was originally described by *Murray et al* in 1986 and consisted of four cycles of 5 minutes ischaemia and 5 minutes of reperfusion prior to sustained ischaemia. Since then others have used variations of this protocol with similar results. Studies have shown that pre-conditioning can reduce infarct size, lactate generation and rate of fall of ATP (Murry C 1986). It has also been shown to reduce arrhythmias and reduce contractile dysfunction. It has been reported that pre-conditioning leads to activation of signalling pathways, which has consequential effects during prolonged ischaemia leading to protection. It has also been shown that pre-conditioned hearts have less anaerobic glycolysis during ischaemia and, therefore, have lower lactate levels. Protection offered by pre-conditioning is time dependent and it has been suggested this is due to the fact that signalling pathways activation decays with time. These pathways are activated due to the release of agonists such as adenosine and opioids which bind to G protein coupled receptors subsequently activating cardioprotective signalling pathways (Downey J 2007). The RISK pathway has particularly been shown to mediate IPC-induced cardioprotection (Tong H 2000; Fryer R 2001). An example is the PI3K pathway which, when activated, results in up regulation of targets such as p70SK6 and Akt. Also, signalling through endosomal signalling and B-arresting results in activation of ERK and other kinases and phosphatases. Studies have shown that inhibition of PI3K and ERK 1/2 abrogated the IPC-induced infarct limiting effect and also abolished the IPC-induced phosphorylation of Akt and ERK 1/2 (Hausenloy 2004). The activation of these targets leads to subsequent activation of further downstream signalling pathways. There are drugs such as sildenafil which have been shown to mimic the action of pre-conditioning. Mechanistic studies have shown that it exerts protection through NO generation (from eNOS/iNOS), activation of ERK 1/2 signalling and opening of ATP-sensitive potassium channels (Kukreja R 2005).

In 2003, *Zhao et al* showed that intermittent reperfusion following ischaemia (post-conditioning) can reduce infarct size, this was termed post-conditioning (Zhao Z 2003). The duration and number of reperfusion intervals required has been reported to largely depend on the species. This protection is thought to involve signalling pathways similar to IPC, which includes molecules such as Akt, and ERK.

There are currently a number of clinical trials underway in the UK to assess the potential of pre-conditioning as a valid therapeutic to reduce I/R injury. This includes the ERICCA trial, a 16 centre 1610 patient randomised controlled clinical trial, sponsored by the NIHR/MRC/BHF (£1.5 million

research grant), to investigate whether remote ischaemic preconditioning can improve clinical outcomes in patients undergoing coronary artery bypass graft surgery (Hausenloy D 2012).

Ultimately reperfusion injury remains a valuable therapeutic target and, therefore, research is required to identify therapies to reduce its severity. Although, thus far, the reduction of reperfusion injury has been relatively unsuccessful, further research is required to continue to search for a molecule that can fulfil the requirements of this drug-targeted therapy. Additionally, there is potential for success using drugs that can mimic or enhance pre-conditioning, such as that previously shown with sildenafil.

1.9 Introduction Summary

This introduction has aimed to give an insight into the complex nature of I/R injury by describing the various types of cell death and the mechanisms by which cell death in I/R injury may occur and be regulated. Furthermore, due to the role of cardiac injury in SLE being some prominent in relation to mortality and morbidity this introduction then went on to highlight features of this disease. This includes the pathogenesis, clinical features and causes of SLE as well as outlining the current therapeutic treatments. This thesis will now describe the data collected in this project to address the aims which follow on from this introduction.

1.10 Aims of PhD

- To establish an *in vitro* model of simulated I/R injury model in the cardiac cell line H9c2 and neonatal rat cardiomyocytes

- To characterise the effect of pre-treating neonatal rat cardiomyocytes in this *in vitro* model with

    - HCQ – a therapeutic currently used to treat SLE patients to investigate if it has cardiovascular benefits in simulated I/R injury
    - purified IgG from patients with SLE, JSLE and APS to assess their contribution to I/R injury

- To explore the mechanisms through which HCQ and purified IgG exert their effects in simulated I/R injury

- To use an *in vivo* cardiac I/R injury model to replicate data produced in the *in vitro* simulated I/R injury model

CHAPTER II

MATERIALS, METHODOLGICAL PRINCIPLES AND DETAILS OF PROCEDURES

Overview of Chapter II

The first part of this chapter, after listing the general materials used, describes the three models of simulated I/R injury that were used in this thesis. This includes two *in vitro* models; a rat cardiomyoblast cell line H9c2 and primary neonatal rat cardiomyocytes, as well as a third *in vivo* rat cardiac I/R injury model.

The second part of this chapter describes the methodological principles for purifying IgG from the whole blood of patients with APS, SLE ± APS and healthy volunteers. This also includes purification, concentration and endotoxin removal from serum samples. This is followed by a description of the antibody detections assays for cardiolipin and β_2GPI.

The final part focuses on describing the techniques for detecting alterations in levels of proteins in signalling pathways which are being targeted. This is followed by a description of the alternative cell death assays that were used to study simulated I/R injury.

2 Materials, Methodological Principles and Details of Procedures

2.1 Chemicals, General Materials and Equipment

2.1.1 Chemicals

All solid chemicals were dissolved in ddH_2O and adjusted to the correct pH with 0.1 M HCl or 0.1 M NaOH. They were then autoclaved or filter sterilised with 0.22 µm syringe driven filter units (Millipore, Bedford, MA, USA).

2.1.2 General materials and equipment

15 mL and 50 mL sterile tubes	91015T, Helena Biosciences, Sunderland, UK
1.5 mL microfuge tubes	Eppendorf AC, Hamburg, Germany
Centrifuges	Heraeus Fresco 17 (Thermo Scientific)
	Megafuge 40R (Thermo Scientific)
Nanodrop	ND-1000 Spectrophotometer (LabTech International)

2.1.3 Cell culture reagents

Dulbecco's modified Eagles medium (DMEM) (D5546, Sigma) supplemented with 1% penicillin/streptomycin (10 000 U/ mL) (P43333, Sigma) and varying volumes of foetal calf serum (FCS) (F6765, Sigma) as described below:

H9c2 rat cardiomyoblast cell line supplemented with 10% FCS
Neonatal rat cardiomyocytes supplemented with:
Plating medium – 15% FCS
Maintenance medium – 1% FCS

2.1.4 General buffers and solutions

Phosphate buffered saline (PBS) pH 7.4. One PBS tablet (Invitrogen, Paisley, UK) was added per 500 mL sterile water. To make PBS/0.1% Tween, 1 mL "Tween 20" detergent was added to 1 L PBS.

2.1.5 Specific buffers and solutions

Neonatal rat cardiomyocyte preparation

A stock solution of 500 mLs isolation buffer was prepared by dissolving the following components in 500 mL of distilled water and the pH adjusted to pH 7.35

3.4 g 116 mM NaCl
2.38g 20 mM HEPES
0.06g 0.77 mM NaH_2PO
0.5g 5.5 mM glucose
0.2g 5.4 mM KCl
0.05g 0.4 mM $MgSO_4$

For each preparation the following enzymes were dissolved in 100 mLs of Isolation buffer:

250 U/mg collagenase type II CLS2 (Worthington, New Jersey, USA)
25 mg pancreatin from porcine pancreas (P-3292, Sigma)

Simulated I/R Injury

A stock solution of Esumi buffer was made by dissolving the following components in 1 L of distilled water and the pH adjusted to 7.4

137 mM NaCl
3.8 mM KCl
0.9 mM $CaCl_2$
4 mM HEPES
10 mM Glucose

A stock solution of ischaemic buffer was made by dissolving the following components in 1L of distilled water and the pH adjusted to 6.2:

137 mM NaCl
12 mM KCl
0.49 mM $MgCl_2$
0.9 mM $CaCl_2$
4 mM HEPES
20 mM sodium lactate

10 mM deoxyglucose

Aliquots of 50 mL were stored at -20ºC and only thawed once.

Protein Extraction

Cell lysates were extracted using 100 µL of lysis buffer composed of the following:

50 nM Tris-HCl pH 7.4
150 mM NaCl
5 mM EDTA
1 mM EGTA
1% NP-40
0.1% SDS
0.5% Na-Deoxycholate
1 mM Na_3VO_4
A complete mini protease inhibitor cocktail tablet (Roche) was added and aliquots of 1 mL were stored at -20ºC and thawed only once.

Western Blot reagents

A stock solution of 10x running buffer was made by dissolving the following components in 1 L of distilled water:

30 g Tris base
144 g glycine
10 g sodium dodecyl sulphate (SDS)
The antibodies used and their dilution factors are described in table 2.1.

Primary Antibody	**Dilution with 4% (w/v) non-fat milk**	**Company**
Rabbit anti-phospho-p42/p44 MAPK (ERK 1/2)	1:1000	Cell Signalling
Rabbit anti-Erk 1 (C-16)	1:1000	Santa Cruz Biotechnology
Rabbit anti-phospho p38MAPK (Thr^{180}/Tyr^{182})	1:1000	Cell Signalling
Rabbit anti-p38MAPK	1:1000	Cell Signalling
Rabbit anti-phospho-SAPK/JNK (Thr^{183}/Tyr^{185})	1:1000	Cell Signalling
Rabbit anti-SAPK/JNK	1:1000	Cell Signalling
Rabbit anti-phospho Akt (Ser^{473})	1:1000	Cell Signalling
Rabbit anti-Akt	1:1000	Cell Signalling
Rabbit anti-cleaved caspase-3 (Asp^{175})(5A1E))	1:1000	Cell Signalling
Rabbit anti-cleaved caspase-8 (Asp^{391})	1:1000	Cell Signalling
Rabbit anti-cleaved caspase-9 (Asp^{315})	1:1000	Cell Signalling
Rabbit anti-BAD	1:1000	Cell Signalling
Rabbit anti-BAD (Ser^{112})	1:1000	Cell Signalling
Rabbit anti-BAD (Ser^{136})	1:1000	Cell Signalling
Rabbit anti-LC3B	1:1000	Abcam
Mouse anti-SQSTM1/p62	1:1000	Abcam
Mouse anti-GAPDH	1:500	Abcam

Table 2.1 Antibodies used for Western blot and the dilution factors used.

2.2 Cell Culture

2.2.1 H9c2 rat cardiomyoblast cell line

H9C2 rat cardiomyoblast cells (H9c2 (2-1) (ATCC CRL-1446)) were cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin (10 000 U/ml, 10 000 µg/ml)(Gibco). Sub-confluent cells (70-80%) were sub-cultured 1:4. The cells were trypsinized and plated in cell culture plates and grown for 24 hours before treatments.

2.2.2 Animals

This study was performed in accordance with the United Kingdom. Home Office Animals (Scientific Procedures) Act 1986. *In vivo* studies were performed by a core facility at The Centre for Biomedical Imaging (CABI), performed using their project license.

2.2.2.1 Neonatal rat cardiomyocyte isolation

Neonatal rat cardiomyocytes were isolated according to an established protocol (Stephanou A 2000). Cardiac myocytes were dissociated from the hearts of 1-2 day old rats using 250 U/mg collagenase type II CLS2 (Worthington) and 25 mg of pancreatin from porcine pancreas (Sigma #P-3292) in 100 mL of oxygenated digestion buffer (116 mM NaCl, 20 mM HEPES, 0.77 mM NaH_2PO_4, 5.5 mM glucose, 5.4 mM KCl, 0.4 mM $MgSO_4$, pH 7.35). Pups were culled and hearts isolated into a Petri dish of isolation buffer and finely cut into small pieces. The hearts were pipetted into a 25 cm^3 TC flask and buffer removed. The hearts were rinsed once with 7 mLs of digestion buffer, allowed to settle and the buffer discarded. Fresh digestion buffer was added and incubated at 37 ^oC for 15 minutes. The flask was gently shaken, the hearts allowed to settle and the buffer discarded. This digestion process was repeated 8 times but the digestion buffer removed into 2 mL of FCS and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and re-suspended in 4 mLs FCS and stored at 37 ^oC whilst the subsequent digestions were carried out. The cells were then collected in a 50 mL falcon and centrifuged for 5 minutes at 1000 rpm. The supernatant was discarded and the cells were re-suspended in 15% plating media (DMEM supplemented with 15% FCS, 1% p/s) and pre-plated in a 175 cm^3 flask for 1 hour to allow fibroblasts to stick down. The media was then removed (containing the myocytes) and cells were plated out at $2x10^6$ cells/well in a cell culture plate pre-coated with 1% gelatin. Cells were allowed to attach and the next day media

was replaced with 1% maintenance media (DMEM supplemented with 1% FCS, 1% P/S). Cells were confluent and beating synchronously 24-48 hours after isolation.

2.2.2.2 Determination of cell number

Cell numbers were determined using a haemocytometer with an aliquot of 10ul cell suspension. The following formula was used to estimate cell number

Number of cells in all quadrants X 10^4 = number of cells / ml

2.2.2.3 Simulated *in vitro* I/R injury model

Control cells were kept in a cell culture incubator (5% CO_2 in air) and media was replaced with Esumi buffer (137 mM NaCl, 3.8 mM KCl, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2$, 4mM HEPES and 10 mM Glucose). Cell culture media was replaced with ischaemic buffer (137 mM NaCl, 12 mM KCl, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2$·H2O, 4 mM HEPES, 20 mM sodium lactate, and 10 mM deoxyglucose (pH 6.2)) and cells transferred to an hypoxic chamber pre-warmed to 37°C. Simulated ischaemia was achieved by addition of 5% CO_2 in balanced argon to displace oxygen from the chamber. Cells were subjected to simulated ischaemic injury for a period of between 4 and 6 hours, after which ischaemic buffer was replaced with DMEM containing 1% (v/v) FBS and cultured in 5% CO_2 in air (reoxygenation to simulate reperfusion) for the indicated times.

2.2.2.4 *In vivo* rat cardiac I/R injury model

I/R injury in the rat was performed as previously described (Carr C 2011). Male Sprague Dawley rats were given a week to acclimatize before commencing the experiment. The surgery was performed as a core service by UCL based in the Centre for Advanced Biomedical Imaging (CABI). Rats were dosed with HCQ (Sigma) 200 mg/kg daily for 3 days via gavage (or water only as a control) and pre-treated with 1mg of the ERK 1/2 inhibitor U0126 (Promega) via intraperitoneal (IP) injection (or water only as a control) 30 minutes prior to surgery. The technicians administering the reagents, undertaking the injury and performing histological analyses were blind to the identity of the regents that were given. Briefly, the rat is anaesthetised, intubated and ventilated. The heart is exposed through a thoracotomy and blood flow to the left anterior descending (LAD) artery occluded by placing a suture around the LAD. After 1 hour the suture is released, the incision closed and the animal recovered. Following 24 hours reperfusion the animal is sacrificed using pentobarbitone, the heart removed and flushed with 2ml saline solution through the left ventricle. The LAD is re-

occluded before injecting with Evans Blue dye (1 ml/4%) through the left ventricle. The heart is then stored for 24 hr at -20 °C and the following day cut into slices 1mm thick. Staining using tetrazolium chloride (TTC) at 37 °C is then performed followed by fixation using formaldehyde. The heart slices are then scanned and analysed using Image J (NIH). The dual staining allows measurement of the area of infarction and area at risk to be calculated.

2.3 Whole blood hydroxychloroquine detection assay

HCQ concentrations in rat whole blood were assayed by using high performance liquid chromatography (HPLC) coupled with fluorescence detection. This assay was performed by Dr Benoit Blanchet, Laboratory of Pharmacology, Hôpitaux de Paris. Chromatographic separation was achieved on Xterra® Phenyl (250 mm x 4.6 mm, 5 µm; Waters, Milford, USA) associated with a guard column packed with the same bonded phase. The mobile phase consisted of a mixture of glycine buffer (pH 9.6, 100 mM,) and methanol (46:54; v/v), and was delivered at a flow rate of 1.2 mL/min throughout the 16-minute run. Chromatography was performed at 50°C. The excitation and emission wavelengths are 320 and 380 nm, respectively.

Regarding sample preparation, 20 µl of quinine (internal standard) at 50 µg/mL was added to 200 µl of whole blood (calibration standard, internal quality control or rat sample). After mixing, 400 µl of cold methanol and 50 µl of cupric sulphate (3 nM) were added before a 2-minute vortex step with a VX-2500 Multi-Tube Vortexer® (VWR, Fontenay Sous Bois, France). Then, the tubes were centrifuged 15 minutes (13000 rpm, room temperature). The supernatant was transferred into a plastic vial for chromatography, and then 20 µl of each sample was injected into the chromatographic system. Calibration curves were linear from 25 to 1560 ng/mL. The intra- and inter-assay coefficients of analytical variabilities were both less than 10%. The lower quantification limit was 25 ng/mL.

2.4 Organ lysate Hydroxychloroquine detection assay

Regarding tissue lysates, the extraction protocol was similar to that for whole blood sample preparation as described previously (section 2.3). However, 200 µl whole blood sample were substituted to 100 µl tissue lysate sample + 100 µl of RIPA 1X for all tissues except liver (RIPA 0.2X). Finally, tissue lysate instead of whole blood was used to build the standard calibration curve. This assay was performed by Dr Benoit Blanchet, Laboratory of Pharmacology, Hôpitaux de Paris.

2.5 Purification of IgG from human serum

2.5.1 Patient samples

Serum samples from 3 individuals (Table 2.2) for the H9c2 preliminary study and 34 individuals for the neonatal rat cardiomyocyte study (Table 2.3) were obtained for this study from patients under the care of University College London Hospital (UCLH), London, UK. Serum samples were selected on the basis that they fulfilled the American College of Rheumatology Classification Criteria (Tan EM 1982; Hochberg 1997). All subjects donated blood after informed consent and the study was approved by the local ethics committee (REC 11/LO/0330).

Patient number	Cardiovascular Event	Date of Event	Date of Sample
113	Ischaemic stroke	Sept 2011	June 2011
345	Myocardial infarction	March 2009	July 2009
522	Ischaemic stroke	May 2010	Nov 2010

Table 2.2 SLE patients whose serum samples were used for H9c2 rat cardiac cell line preliminary studies. Samples used with taken either prior (113) or after (345 and 522) the described event.

	APS (n=6)	SLE/ aPL +ve (n=5)	SLE/ aPL –ve (n=5)	JSLE (n=8)	Healthy controls (n=10)
Age (Mean ± SEM)	55 (±4.5)	45.2 (±5.1)	42.8 (±6.3)	16.5 (±0.5)	35.2 (±4.7)
Sex	3 F/3 M	5 F	5 F	7 F/1 M	8 F/2 M
Serum aCL (Mean GPLU ± SEM)	5.84 (±1.7)	91.6 (±19.7)	3.58 (±0.3)	49.62 (±16.1)	5.4 (±0.7)
Serum anti-β2GPI (Mean SU ± SEM)	5.45 (±1.15)	50.0 (±20.65)	3.62 (±0.34)	43.26 (±15.83)	4.12 (±0.58)

Table 2.3 Clinical and laboratory features of patients and healthy volunteer controls used in the neonatal rat cardiomyocyte study. Abbreviations: aCL, anti-cardiolipin antibodies; anti-β_2GPI, anti β_2-glycoprotein-1 antibodies; SEM, standard error of the mean; SLE, systemic lupus erythematosus; JSLE, juvenile onset systemic lupus erythematosus; SU, Standard units; GPLU, IgG phospholipid units

2.5.2 Purification and concentration of IgG

IgG was purified from serum by protein G sepharose affinity chromatography (Thermo Scientific) as previously described (Lambrianides A 2010). In summary, the column was washed with 5 mL of binding buffer (0.1 M sodium phosphate pH 7.2). Patient or control serum was diluted 1:2 with binding buffer; filter sterilised and placed in the column, so that serum IgG binds to the protein G within the matrix of the column. The column was then washed with 10 mL of binding buffer and the IgG eluted into 3 mL elution buffer (0.1 M glycine pH 2-3). Glycine in the elution buffer lowers the pH to detach the IgG from the column. The eluted fraction was then neutralised by adding 300 μL 1 M Tris pH 8-9. Finally, the columns were washed with 5 mL of elution buffer and 5 mL of PBS and then stored in 5 mL of storage solution (0.02% sodium azide in PBS).

IgG from the collected fraction was concentrated using an Amicon ultra centrifugal filter (Millipore) with a molecular weight cut off of 30 KDa. The eluted fraction from IgG purification was placed in the filter, which was spun at 7,500 g for 20 minutes. This process retains IgG (of molecular weight 150 KDa), which was then washed twice with 2 mL PBS and centrifuged at 7,500 g for 20 minutes, before being transferred to clean microcentrifuge tubes and made up to a total volume of 1 mL with PBS. The concentration of purified and concentrated IgG was determined using the Nanodrop ND-1000 Spectrophotometer (LabTech International).

2.5.3 Endotoxin removal

Patient and control IgG samples were passed through Detoxi-Gel^{TM} Endotoxin removing columns (Thermo Scientific) to remove endotoxin. The column contains immobilised polymixin B that binds the lipid A portion of bacterial lipopolysaccharide (LPS) and thus removes it from contaminated samples. Endotoxin removal was performed under sterile conditions as follows: each column was sequentially washed with 5 mL sodium deoxycholate, followed by 5 mL water and then 5 mL PBS. The purified IgG sample was added to the column and the flow through discarded. Following which the IgG was eluted from the column by the addition of 1 mL of PBS and stored at -20°C. The column was then washed with 1% sodium deoxycholate, then water and finally 25% ethanol in which it was stored at 4°C.

The IgG samples were confirmed to be endotoxin free (<0.25 endotoxin units (EU)/ml) by the *Limulus* Amoebocyte Lysate assay. Endotoxin standard (Sigma) was reconstituted in endotoxin free water to obtain a stock concentration of 4000 EU/mL. This stock was then diluted to make a range of standard concentrations, from 400 EU/mL to 0.015 EU/mL in sterile capped polystyrene tubes. Next,

E-Toxate (Sigma) was reconstituted in 5 mL endotoxin free water. The E-Toxate will react with endotoxin present in a sample, forming a gel. 100 µL of purified IgG at 500 µg/mL, as well as 100 µL PBS, endotoxin free water and the endotoxin standard serial dilutions (400 EU/mL to 0.015 EU/mL) were placed in non-siliconised glass tubes. Next, 100 µL of E-Toxate was added to each tube and the tubes were incubated at 37°C for one hour. After incubation the tubes were inverted once to determine whether a gel was formed. In the presence of a gel a sample was considered as endotoxin positive to a threshold determined by the endotoxin standard, whilst absence of a gel confirmed that the IgG sample was free of endotoxin. The assay was able to detect levels of endotoxin greater or equal to 0.25 EU/ml, as determined by the endotoxin standard. Endotoxin removal was repeated on those samples where a gel had formed until they were endotoxin negative.

2.5.4 Detection of aCL antibodies

The aCL activity of purified IgG and also the aCL activity of patient and control serum before IgG purification was confirmed by enzyme-linked immunosorbent assay (ELISA). A 96 well Polysorb plate (Nunc, Thermo Scientific) was marked vertically to divide it in to a test half and a control half. Wells in the test half were coated with 50 µL of 50 µg / L CL 9Sigma) (diluted in ethanol) and wells in the control half of the plate were coated with 50 µL of ethanol alone. Plates were left uncovered and incubated overnight at 4 ºC. The following day plates were washed twice with PBS and then blocked with 100 µL of 10% FCS/PBS at room temperature for 1 hour to block non-specific binding of IgG to the plastic. Plates were then washed twice with PBS. Next, either purified IgG diluted to 500 µg/mL in 10% FBS/PBS was added to row A and E on both test and control sides of the plate and 50 µL of PBS was added to all other wells. Purified IgG was serially diluted (1:2) down 4 wells of the plate by transferring 50 µL and mixing. In the case of patient and control serum, serum was diluted 1:50 with 10% FBS/PBS and 50 µL of each sample was added in triplicate on both the test half and control half of the plate. 50 uµL of a standard containing polyclonal IgG of known CL binding activity defined as IgG phospholipid units (GPLU) (Louisville APL Diagnositics, Inc. Texas, USA) was added to each plate, on both the test (column 1-row A-H) and control half (column 7-row A-H). Plates were incubated for 90 minutes at room temperature before being washed three times with PBS. Goat anti-human IgG alkaline phosphatase conjugate (Sigma) was diluted 1:1000 in 10% FBS/PBS and 50 µL was added to each well of the plate. Plates were incubated for 1 hour at room temperature before being washed three times with PBS. Finally 50 µL of diethanolamine (DEA) buffer, diluted as follows: 1 mL buffer in 4 mL water plus 1 p-nitropenylphosphate (p-NPP) tablet (KPL), was added to each well of the plate. Plates were incubated for 30 minutes at room

temperature before being read on a TECAN GENios Microplate reader at 405 nm. Background binding to control wells lacking CL was subtracted from binding to CL in corresponding test wells and then expressed relative to the standard as GPLU.

2.5.5 Detection of anti-β_2GPI antibodies

The anti-β_2GPI activity of purified IgG and the anti-β_2GPI activity of patient and control serum before IgG purification was also confirmed by ELISA. A maxisorb plate (Nunc, Thermo Scientific) was marked vertically to divide it in two halves: the test half and the control half. Wells in the test half were coated with 50 µL of 4 µg/mL β_2GPI (Louisvilled APL Diagnostics, Inc. Texas, USA) diluted in PBS and wells in the control half of the plate were coated with 50 µL of PBS alone. Plates were incubated overnight at 4ºC before being washed twice with PBS/0.1% Tween. Plates were blocked with 150 µL of 0.5% gelatin in PBS and incubated for 1 hour at 37ºC. Following this step plates were washed three times with PBS/0.1% tween. Next either purified IgG or patient or control serum was added to the plate. Keeping column 1 and 7 empty for the standard, in the case of purified IgG 100 µL of purified IgG diluted to 500 µg/mL in 1% bovine serum albumin (BSA) was added to row A and E on both the test and control side of the plate and 50 µL PBS was added to all other wells. Purified IgG was serially diluted (1:2) down 4 wells of the plate by transferring 50 µL and mixing. In the case of patient and control serum, serum was diluted 1:50 with 1% BSA/PBS and 50 µL of each sample was added in triplicate on both the test half and control half of the plate. 50 µL of an in house standard diluted at 1:400 was also used and serially diluted (1:2) down 6 wells on both the test half (column 1) and control half (column 7) of the plate. The in house standard is a patient with positive aPL but no APS. 100 standard units (SU) of the in house standard is equivalent to 125 ng/mL HCAL (IgG Sapporo standard- Centre for Diease Control, USA). Plates were incubated for 1 hour at room temperature and then washed three times with PBS/0.1% Tween. Goat anti-human IgG alkaline phosphatase conjugate (Sigma) was diluted 1:1000 in 1% BSA/PBS and 50 µL was added to each well of the plate. Plates were incubated for 1 hour at room temperatre before being washed three times with PBS/0.1% Tween. Finally 50 µL of DEA buffer, diluted as for aCL ELISA was added to each well of the plate and incubated for 30 minutes at room temperature before being read on a TECAN GENios Microplate reader at 405 nm. Background binding to control wells lacking β_2GPI was subtracted from binding to β_2GPI in corresponding test wells and then expressed relative to the in-house standard as SU.

2.6 RNA extraction, quantification, detection and characterisation

2.6.1 RNA extraction

Cells were washed with 1 mL diethylpyrocarbonate (DEPC) treated PBS before 1 mL TRIzol (Invitrogen) was added to each well and incubated for 5 minutes at room temperature (TRIzol is a monophasic solution of phenol and guanidine isothiocyanate, which maintains the integrity of RNA whilst disrupting cells and dissolving cell components). Next 200 µl chloroform was added, mixed and then incubated for 3 minutes at room temperature before centrifugation at 14,000g for 15 minutes at 4 °C to separate RNA. The clear upper aqueous phase containing the RNA was removed and mixed with 500 µL isopropanol per sample, and left for 5 minutes at room temperature. Samples were then centrifuged at 14,000 g for 10 minutes at 4 °C following which the supernatant was removed and 1 mL 80% ethanol was added per sample. Samples were mixed before being centrifuged at 5,000 g for 5 minutes at 4 °C and supernatant was removed and the pellet air-dried. 30 µl of DEPC treated water and 1 µl RNase (Promega) was added per sample. RNA concentration was measured on the Nanodrop ND-1000 Spectrophotometer and samples were then diluted to 1 µg/µl using DEPC treated water.

2.6.2 cDNA synthesis

1 µl random primers (Promega, 1 µg RNA and 1 µl dNTP (Promega)) were added to sterile microcentrifuge tube and made up to a total volume of 12 µL with DEPC treated water. This mixture was heated to 65 °C for 5 minutes and then chilled on ice. 4 µL of 5x first strand buffer (250 mM Tris-HCL pH 8.3, 375 mM KCL, 15mM $MgCl_2$) and 2 µl 0.1 M Dithiothreitol (DTT) was added to the sample and incubated at room temperature for 2 minutes. Finally 1 µl of SuperScript II Reverse Transcriptase (Invitrogen) was added to the sample, which was incubated at 42°C for 50 minutes. The reaction was then stopped by heating the sample to 70°C for 15 minutes.

2.6.3 Real Time Polymerase Chain Reaction (RT-PCR)

RT-PCR of cDNA products was performed to amplify and semi-quantify Hsp70 and Hif1α to establish if simulated I/R injury was successful. cDNA was first diluted 1:5 with DEPC treated water. The following mixture was added to a PCR tube: 10 µL SYBR Green master mix (Invitrogen), 5 µL DEPC treated water, 2 µL of a 5 µM Forward (Fw) and Reverse (Rv) Primer working solution (sequences below) and 1 µL cDNA.

Hsp70 Fw: 5' CGAAGAGAGCGGCTGAAGGCTC 3'

Hsp70 Rv: 5' GGGACGGTTACGGTCGAGAGG 3'

Hif1α Fw: 5' TCAAGAGCTCCTGAGCGTTTCCTAATCTCATTC 3'

Hif1α Rv: 5' AGCTACGCGTCCTGGTCCACAGAAGATGTTT 3'

GAPDH Fw: 5' GAG TCA ACG GAT TTG GTC GT 3'

GAPDH Rv: 5' TTG ATT TTG GAG GGA TCT CG 5'

Samples were run on a DNA Engine Opticon continuous fluorescence detector (MJ Research) under the following conditions: Initial denaturation: 95°C for 3 minutes, followed by 45 cycles of: 95°C for 30 seconds, 64°C for 30 seconds and 72°C for 30 seconds. A melt curve was performed from 65°C to 95°C, read every 0.3°C and held for 1 second between reads, in order to ensure only one primer product was made. Following RT-PCR the presence of a PCR product was confirmed by gel electrophoresis through a 1.5% agarose gel.

The cycle threshold (C_t) of the Hsp70 and Hif1α primer products were normalised to the C_t of the housekeeping gene GAPDH primer products (ΔC_t), following this $\Delta\Delta C_t$ was calculated. These $\Delta\Delta C_t$ values were used to calculate the fold change ($\Delta\Delta C_t^2$) between Hsp70 or Hif1α and the housekeeping gene GAPDH.

2.7 Protein quantification, detection and characterisation

2.7.1 Protein Extraction

Cell lysate extracts were prepared as follows: the 6 well TC plate was placed on ice and each well was washed with 1 mL ice cold PBS. Next 100 µL of lysis buffer (50 nM, Tris-HCL pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1% NP-40, 0.1% SDS, 0.5% Na-Deoxycholate, 1mM Na_3VO_4 and complete mini protease inhibitor cocktail tablets (Roche)) was added to each well and incubated on ice for 10 minutes. Cells were then scrapped from the bottom of the wells and transferred to ice cold microcentrifuge tubes. Then cells were lysed by passage through a 22 gauge needle five times and placed on ice for a further 10 minutes. Next lysates were spun at 16,000g at 4°C for 5 minutes to pellet cell debris. Following centrifugation lysate supernatants were transferred to microcentrifuge tubes and stored at -20 °C for future analysis.

Organ lysates were prepared as follows: organs were removed, washed in PBS, snap-frozen in liquid nitrogen and stored at -80°C until use. Organs were crushed in the presence of liquid nitrogen using a mortar and pestle. Organ lysate was then lysed in 1 mL of lysis buffer (50 nM, Tris-HCL pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1% NP-40, 0.1% SDS, 0.5% Na-Deoxycholate, 1mM Na3VO4 and complete mini protease inhibitor cocktail tablets (Roche)) on ice for 10 minutes. The cells were then lysed by a dounce homogenizer and spun at 16, 000g at 4°C for 5 minutes to pellet cell debris. Following centrifugation lysate supernatants were transferred to microcentrifuge tubes and stored at -20 °C for future analysis.

2.7.2 Determination of protein concentration

Protein concentration was determined using the BCA protein Assay (Thermo Scientific). First protein standards were made by diluting a 2 mg/mL bovine serum albumin standard to eight concentrations ranging from 25 µg/ml to 2000 µg/ml. Working reagent was then prepared by mixing 50 parts of BCA reagent A with 1 part BCA reagent B. 5 µl of either the albumin standard or cell lysate were pipetted into wells of a maxisorp plate. Then 195 µl of working reagent was added to each well and the plate was incubated at 37 °C for 30 minutes. The maxisorp plate was read on a TECAN GENios Microplate reader at 560 nm. Protein concentrations of the cell lysates were determined from a standard curve produced by the albumin standards.

2.7.3 One dimensional PAGE (1D-PAGE)

2.7.3.1 Resolving gel

Proteins were separated according to molecular weight devised by Lamelli (1970) using mini-protean vertical slab gel apparatus (Bio-Rad, UK). N, N, N', N' – tetramethylethylenediamine (TEMED) and ammonium persulphate (APS) were added to the polyacrylamide solution (30% acrylamide mix) with stirring prior to casting of gels. Gels were over-laid with water-saturated butan-2-ol and left to polymerise for 1 hour at room temperature

2.7.3.2 Stacking gel

A stacking gel (5 %) was used to concentrate the proteins into tight bands before entering the resolving gel. Before casting of the stacking gel the butan-2-ol was removed and the resolving surface washed with water to remove excess butan-2-ol. After pouring the stacking gel on top of the resolving gel a 10 well comb was placed in the stacking gel to allow polymerisation for 1 hour. The composition of the resolving and stacking gels is described in table 2.4.

	Volumes (ml)			
	8% gel	10% gel	12% gel	Stacking Gel (5%)
30% (w/v) Acrylamide mix[1]	2.7	3.3	4.0	1.7
1.5M Tris HCL (pH 8.8)	2.5	2.5	2.5	
1M Tris HCL (pH 6.8)				1.25
10% (w/v) SDS	0.1	0.1	0.1	0.1
10% (w/v) APS[2]	0.1	0.1	0.1	0.1
TEMED[3]	0.004	0.004	0.004	0.01
dH_2O	4.6	4.0	3.3	6.8

Table 2.4 The composition of resolving and stacking gels for SDS-PAGE. [1]Acrylamide bis = N,N'-Methylene-bis-acrylamide. [2]APS = ammonium persulfate. [3]TEMED = NNNN-tetramethylethalinediamine.

2.7.4 Western Blotting

Sample buffer (125mM Tris-HCl pH 6.8, 5 % (w/v) SDS, 0.25 % (w/v) bromophenol blue, 25 % (w/v) glycerol, 10 mM DTT) was added to bring the protein concentration to 20 µg and boiled for three minutes before loading. Samples were loaded on a 1D-PAGE gel and electrophoresed at 150 volts in protein running buffer (191.8 mM glycine, 25 mM Tris, 1 % (w/v) SDS). Gel was run until the dye reached the bottom of the plate.

2.7.5 Transfer of protein to nitrocellulose

Proteins which had been separated on SDS-PAGE gels were transferred to Hybond C membranes (Amersham, Bucks, UK) using a wet-transfer method described by Towbin, 1979 (Towbin H 1979). The stacking gel was removed and the resolving gel placed onto a sponge and two pieces of filter paper, which had been pre-soaked in transfer buffer (protein running buffer containing 25 % (w/v) ethanol). A piece of Hybond-C membrane was cut to the same size as the gel and pre-soaked in transfer buffer. The membrane was places on the surface of the gel and two pieces of pre-soaked filter paper and a second sponge were placed on top. Proteins were transferred to the membrane at 100 volts for 1 hour.

2.7.6 Immunodetection of proteins on western blots

The membrane was incubated in 4 % (w/v) non-fat milk for 1 hour at room temperature to block non-specific protein binding. Antibodies were dissolved in 4 % (w/v) non-fat milk; the membrane added and probed over-night at 4^{o}C. Membranes were washed in phosphate buffered saline containing 0.1 % Tween 20 (Sigma-Aldrich, UK) three times for 5 minutes each to removed unbound primary antibody. The membranes were then added to 4 % (w/v) non-fat milk containing horse radish peroxidase (HRP) conjugated secondary antibody (1:1000 dilution, Dako, UK), incubated for 1 hour at room temperature and washed three times for 5 minutes each to remove unbound secondary antibody.

Proteins were visualised using enhanced chemiluminescence (ECL, Amersham Biosciences, UK). Excess PBS-T was removed by placing the membrane on tissue; the ECL reagent was pipetted on to the membrane and incubated at room temperature for 1 minute. Excess ECL reagent was removed and the membranes exposed to photosensitive film to visualise the proteins of interest. The intensity of protein bands was quantified by densitometric analysis (QuantityOne software, Biorad, USA). Results were expressed as a ratio of relative expression of phosphorylated protein to total protein.

Phosphorylated and total protein for the same signalling protein was analysed on the same membrane. After incubation and detection of the phosphorylated protein membranes were washed twice for 5 minutes with 0.2 M sodium hydroxide to remove bound antibodies. Membranes were then re-blocked for 1 hour in 4 % (w/v) non-fat milk and re-probed with their respective total antibody.

2.8 Immunofluorescence

Cells were seeded on UV irradiated coverslips at 1 $x10^5$ cells/well in a 24 well plate. Cells were washed with cold PBS and fixed with 4 % paraformaldehyde (w/v) for 15 minutes at room temperature. PBS containing 0.1% (w/v) Triton-X-100 was used to permeabilise for 5 minutes and blocked for 1 hour in 5% bovine serum albumin in PBS. Cells were then incubated in primary mouse anti-desmin antibody (Santa Cruz) diluted in PBS containing 2.5% (w/v) BSA at 1/1000 dilution for 1 hour at room temperature. Cells were washed three times with PBS and then incubated in Alexa-Fluor secondary antibody (emission wavelengths Alexa488, Alexa456, Molecular Probes, USA) diluted in 2.5% BSA for 1 hour at room temperature in the dark. Cell nuclei were stained with DAPI. Coverslips were mounted onto glass slides using fluorescence mounting media (DAKO, UK) and images acquired using a Zeiss Axioscope inverted fluorescent microscope (Zeiss, JP).

2.9 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

DNA nick end labelling was performed to demonstrate DNA fragmentation. Cells were seeded at $1x10^5$ cells/well in a 24 well plate on UV irradiated glass coverslips. Cells were treated as per the experiment and then washed with cold PBS and fixed with 4 % paraformaldehyde (w/v) at room temperature for 15 minutes. *In situ* cell death was detected in cultured cardiomyocytes by using TUNEL assay kit (Roche Diagnostics, Meylan, France) according to the manufactures instructions. Briefly, cells were incubated in permeabilisation solution (0.1 % triton X-100, 0.1 % sodium citrate in PBS) for 2 minutes on ice and then washed three times with PBS. Positive control cells were incubated with DNase I (1:10 dilution of RQ1 RNase free DNase, Promega in 10x Reaction Buffer RQ1 DNase, Promega) for 15 minutes to induce DNA breaks and then washed three times with PBS. Cells were then incubated with 50 µl / slide TUNEL reaction mix for 60 minutes in a 37°C humidified chamber. Cells were then washed and stained to identify nuclei with DAPI (NucBlue® Fixed Cell ReadyProbes™ Reagent, Invitrogen) at room temperature for 5 minutes. Coverslips were mounted onto glass slides using fluorescence mounting media (DAKO, UK) and images acquired using a Zeiss Axioscope inverted fluorescent microscope (Zeiss, JP). The percentage of TUNEL positive cardiomyocytes (with green fluorescent nuclei) was measured at 25X magnification in three

randomly chosen fields. The proportion of TUNEL positive cardiomyocytes was expressed as a percentage of the total cells counted (DAPI positive).

2.10 3-(4,5-Dimethyl-thiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium assay

To estimate cell viability, neonatal rat cardiomyocytes were seeded into 24 well plates in 500 µL of 1% FBS/DMEM media and incubated at 37°C, 5% CO_2. Cells were exposed to 4 hours simulated ischaemia and various time points of reoxygenation. Following simulated I/R injury 100 µL of the CellTiter 96 Aqueous One Solution (Promega) was added to each well and cells were incubated for 1 hour in a 37°C, 5% CO_2 incubator. 100 µL aliquots of the supernatant were transferred to a 96-well plate and absorbance was read on a TECAN GENios Microplate reader at 492 nm.

2.11 Statistical analysis

For each outcome the experiments were performed at least three times independently and data are expressed as mean ± SEM of these independent experiments. Data was tested for normality using the Kolmogorov-Smirnov test (when >5 data points) or by plotting data on a normality plot. All data was identified as being normally distributed therefore statistical analysis was undertaken using one-way analysis of variance (ANOVA) and assessed for overall statistical significance at level ($p<0.05$). A post-hoc Tukey analysis was performed to compare each individual mean with all other means. The unpaired t test was used to make direct comparisons of two data sets which were age and gender matched (i.e. cells treated with aPL +ve IgG vs aPL –ve IgG). Data analysis was performed using the GraphPad Prism software program (GraphPad Software, San Diego, CA).

CHAPTER III

HCQ is cardioprotective and IgG purified from patients with SLE, APS and JSLE are pathogenic in an *in vitro* simulated cardiac I/R injury model

Overview of Chapter III

This chapter focuses on the use of an *in vitro* simulated cardiac I/R injury model to observe changes in the level of cell death in the presence of different potential modulators. This chapter can therefore be broadly divided into three parts:

The first part is the optimisation of this I/R injury model system in the rat cardiac cell line H9c2 and in neonatal rat cardiomyocytes.

The second part looks at the effect of the drug HCQ on cell death in these two *in vitro* simulated cardiac I/R injury models using various output measures of apoptosis and total cell death.

The final section explores the effect of IgG purified from patients with SLE, APS and JSLE on cell death in these two *in vitro* simulated I/R injury models. This includes optimisation of the dose of IgG to be used as well as the sub-division of patients depending on their aPL activity levels.

3.1 Simulated I/R injury in H9c2 rat cardiac cell line

3.1.1 Introduction

In the cardiovascular field it is recognized that inducing 30% apoptotic cell death is optimal for *in vitro* models of cardiac injury (Shen M 2012). To achieve this, the duration of hypoxia (simulated ischaemia) and reoxygenation (simulated reperfusion) must be optimised. Preliminary experiments were carried out in cardiac myoblast cell line H9c2 obtained from within our research group (original source H9C2 (2-1) (ATCC® CRL-1446™)). This cell line was used to establish optimal cell culture conditions to select the most appropriate time points for simulated I/R injury. This *in vitro* simulated cardiac I/R injury model is induced in two stages; firstly cells are placed in a hypoxic chamber in the presence of an ischaemic buffer, containing 2-deoxyglucose to inhibit glycolysis. A mix of argon/CO_2 is pumped into the chamber to create a hypoxic environment due to the ability of the argon to layer over the CO_2, displacing molecular oxygen. Reoxygenation is induced by removing the cells from the chamber and replacing the ischaemic buffer with 1% FCS, DMEM. The cells are returned to a cell incubator (5% CO_2 in air, 37°C) for the reoxygenation phase to allow oxygen to be reintroduced.

3.1.2 Hsp70 and Hif1α are up-regulated in simulated I/R injury in H9c2 rat cardiac cell line

Firstly, an experiment was carried out to ensure that hypoxia was being induced. The mRNA levels of two proteins; Hsp70 and Hif1α levels were measured after a period of hypoxia and then subsequent reoxygenation. Hsp70 is a heat shock protein and therefore is induced in stress, whereas Hif1α is a hypoxic induced protein.

Hif1α (a) and Hsp70 (b) mRNA levels increased during hypoxia induction as shown in *figure 3.1*. However, in reoxygenation Hsp70 continued to have high mRNA levels due to cells still being in a stressed environment. In contrast Hif1α mRNA levels decreased due to cells no longer being in a hypoxic setting. This experiment confirmed that a hypoxic environment is created when cells are placed in the chamber and upon reoxygenation, oxygen is being reintroduced into the system, therefore simulated I/R injury was successfully being induced.

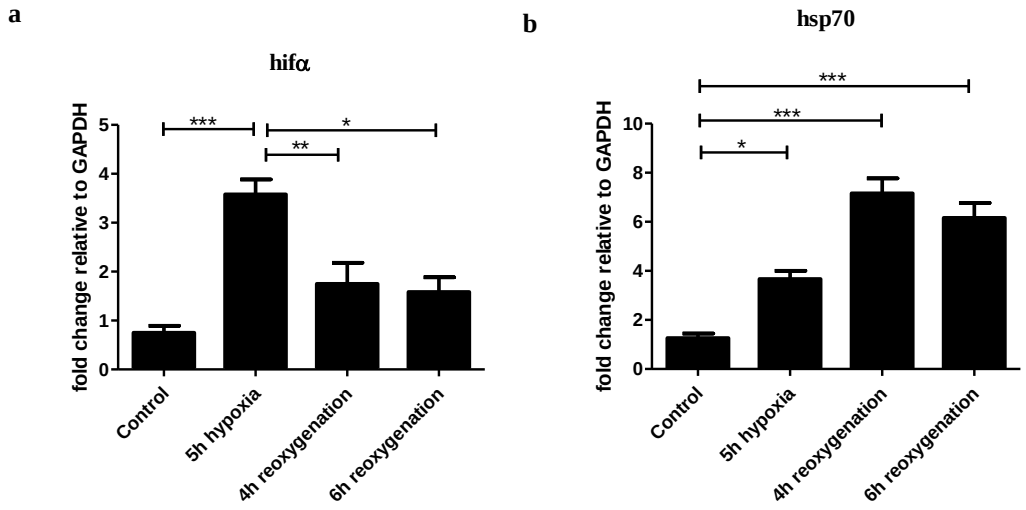


Figure 3.1 Confirmation of simulated I/R injury in H9c2 cell line. Cells were placed in a hypoxic chamber in ischaemic buffer for 5 hours followed by reoxygenation for 4 or 6 hours in 1% FCS/DMEM. mRNA levels of hif1α (a) and hsp70 (b) were then investigated using RT-PCR and levels expressed relative to the housekeeper GAPDH. Graph shows mean ±SEM of quantitative analysis from 3 independent experiments. Statistical analysis determined by one way ANOVA using post-hoc Tukey to compare all columns (* $p<0.05$, ** $p<0.005$, *** $p<0.0005$).

3.2 Optimisation of TUNEL to detect apoptotic cells

3.2.1 Introduction

To optimise our *in vitro* model to achieve 30% apoptosis a method to detect apoptosis had to be selected and it was decided that TUNEL would be used. This kit identifies late stage apoptosis by the enzyme TdT catalysing the addition of fluorescently labelled nucleotides onto the free 3'-hydroxl terminal of double and single stranded breaks. These breaks are induced during DNA fragmentation, a hallmark of terminal apoptosis.

3.2.2 Results of TUNEL optimisation in H9c2 cells

A preliminary experiment was carried out to optimise the use of a positive and negative control for the TUNEL assay. The positive control was treated with DNase I, to induce DNA fragmentation in all cells. The negative control was treated with the reagent minus the TdT enzyme, to eliminate potential fluorescent background.

When cells were pre-treated with DNase I, DNA fragmentation was induced in all cells therefore TUNEL positivity was 100% (*figure 3.2.a*). The negative control was treated with the TUNEL stain containing no enzyme therefore TUNEL positivity was zero (*figure 3.2.b*).

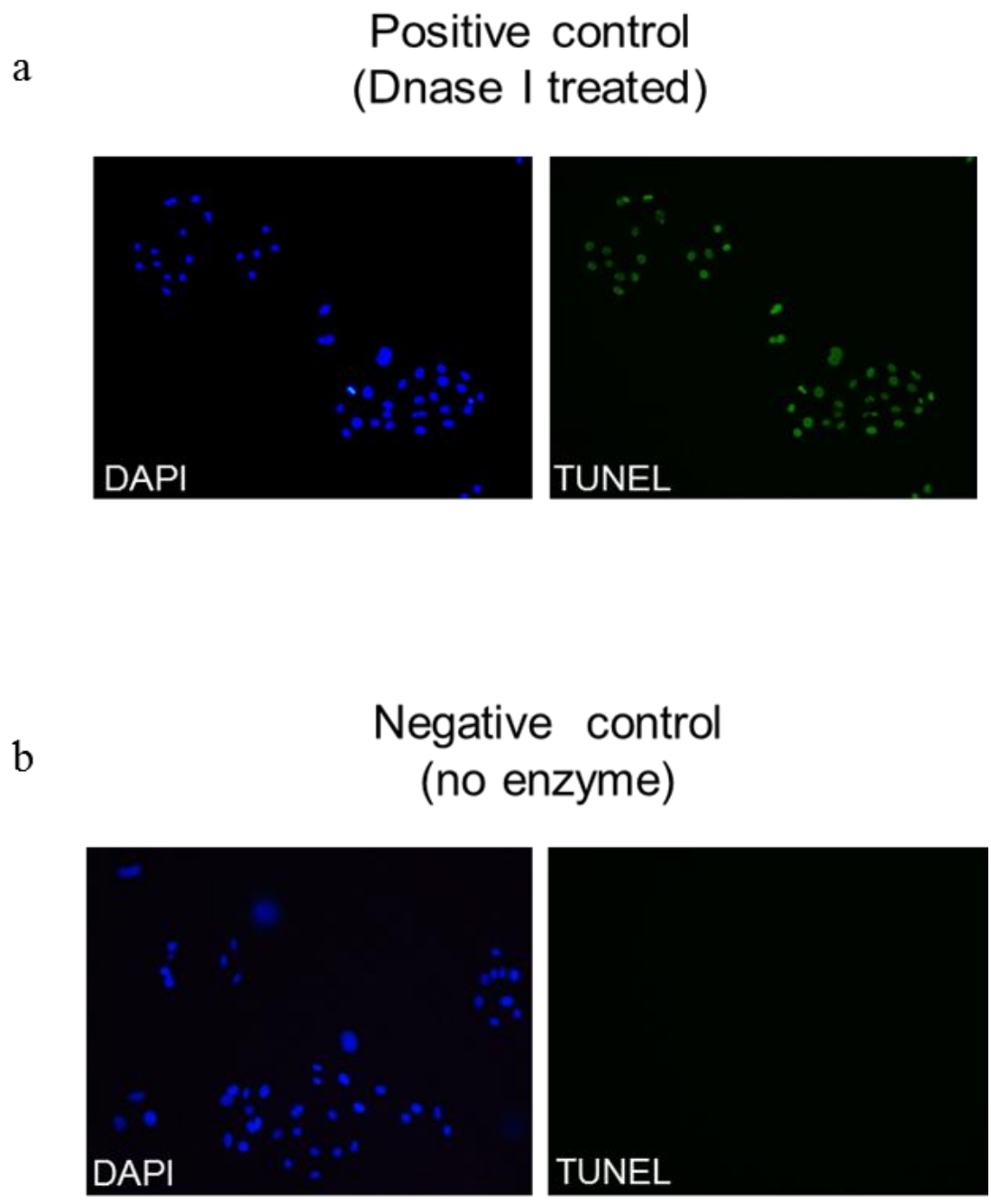


Figure 3.2 Optimisation of controls for TUNEL assay in cardiac H9c2 cell line. For the positive control cells were treated for 15 minutes with DNase I followed by DAPI and TUNEL staining (a) and for the negative control cells were treated with DAPI and TUNEL (minus TdT enzyme) (b).

3.3 Optimisation of simulated I/R injury in H9c2 rat cardiac cell line

3.3.1 Introduction

Once optimisation of the TUNEL technique was complete an experiment was performed to optimise simulated I/R injury in the H9c2 cardiac cell line. Cells were seeded at 1 $x10^5$ cells per well in a 24 well plate on glass coverslips and the following day subjected to 5 or 6 hours of hypoxia in ischaemic buffer. This was followed by a range of reoxygenation timepoints carried out in normal media (1% FCS in DMEM) in a cell incubator (5 % CO_2 in air, 37°C). This was done to assess the duration required to induce approximately 30 % apoptosis/TUNEL positivity. The percentage of apoptosis for each condition was determined by counting the number of TUNEL positive cells and calculating its percentage relative to total number of cells (DAPI positive cells). Images were taken for DAPI positive and TUNEL positive as shown in *figure 3.3.a.*

3.3.2 Results of simulated I/R injury

Optimisation experiments showed that either 5 hours hypoxia followed by 16 hours reoxygenation (*figure 3.3.b*) or 6h hypoxia followed by 4-6 hours reoxygenation (*figure 3.3.c*) achieved approximately 30% TUNEL positivity. This is the desired amount of apoptosis to be induced, as suggested by experiments previously performed in my lab (Scarabelli T 2001; Barry S 2009).

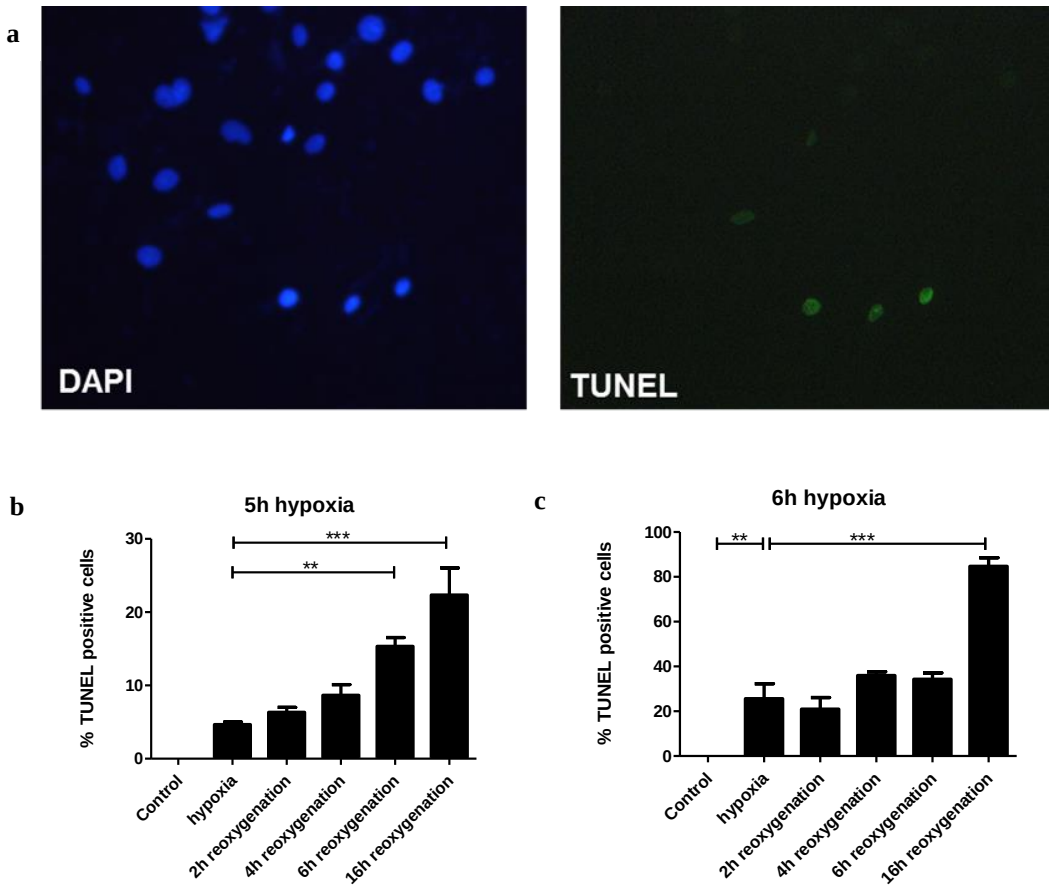


Figure 3.3 Optimisation of simulated I/R injury in H9c2 cardiac cell line for TUNEL assay. Cells were seeded at 1 x10^5 cells/well in a 24 well plate on glass coverslips and the next day were exposed to 5 hours (b) or 6 hours (c) hypoxia in ischaemic buffer followed by a range of reoxygenation time points in 1% FCS in DMEM. DNA nick end labelling was then performed to demonstrate DNA fragmentation and identify TUNEL positive cells (a). Graph shows mean ±SEM of quantitative analysis from 3 independent fields. Statistical analysis determined by 1 way ANOVA using post-hoc Tukey to compare all columns (** $p<0.005$, *** $p<0.0005$).

3.4 The effect of pre-treatment with IgG from patients with SLE in simulated I/R injury in H9c2 rat cardiac cell line

3.4.1 Introduction

Preliminary experiments were next carried out to explore the effects of IgG purified from patients with SLE on apoptosis in I/R injury. Patients selected had suffered a thrombotic event and the serum used was taken as close to the event date as possible. IgG from a healthy control was also tested to ensure the effects observed were specific to SLE patient IgG. Cells were incubated with 500 µg/mL IgG overnight (for 16 hours) at 37ºC and then exposed to simulated I/R injury. IgG was present in culture at 500 µg/mL throughout the experiment.

3.4.2 Results of pre-treating H9c2 cardiac cell line with IgG from SLE patients in simulated I/R injury

IgG from patients 113 and 522 increased apoptosis in simulated I/R injury suggesting they play a pathogenic role whereas healthy and patient 345 IgG have no effect on I/R injury (*figure 3.4*). This preliminary data suggests that IgG from patients 113 and 522 have the ability to be pathogenic in both hypoxia and reperfusion, but with the greatest increase in apoptosis being during reoxygenation.

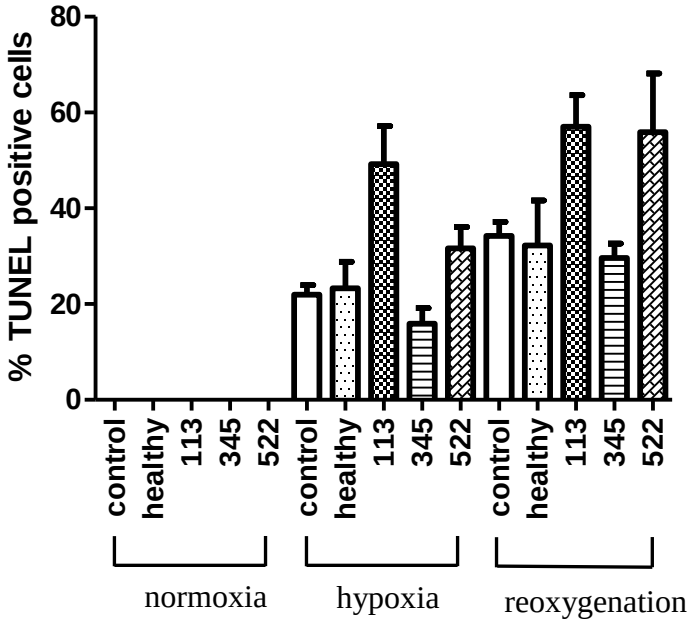


Figure 3.4 IgG purified from patients with SLE has differential effects on the number of TUNEL positive cells in H9c2 cardiac cell line in simulated I/R injury Cells were incubated with 500 µg/mL purified IgG from a healthy control and 3 patients overnight prior to simulated I/R injury (5h hypoxia + 16h reoxygenation). Cells were fixed in 4% PFA and the percentage of TUNEL positive cells was assessed. Graph shows mean ±SEM of quantitative analysis from 3 independent fields.

3.5 The effect of pre-treatment with Hydroxychloroquine (HCQ) in simulated I/R injury in H9c2 rat cardiac cell line

3.5.1 Introduction

Additionally as part of this project a preliminary experiment was also carried out to explore the effects of HCQ on apoptosis in simulated I/R injury. Cells were pre-treated with 1 µg/mL HCQ and the following day cells were exposed to simulated I/R injury, with HCQ present throughout. A dose of 1 µg/mL was used due to this correlating with blood concentrations achieved in patients currently treated with HCQ.

3.5.2 Results of pre-treatment with HCQ in simulated I/R injury in H9c2 rat cardiac cell line

Incubation of cells with HCQ reduces apoptosis in simulated I/R injury after 16h reoxygenation from 50% (±SD 8) to 35% (±SD 4). It also appeared to reduce apoptosis after hypoxia alone from 27% (±SD 2) to 18% (±SD 4), however to a lesser extent than observed after reoxygenation (*figure 3.5*). Therefore this preliminary data suggests that HCQ may be cardioprotective.

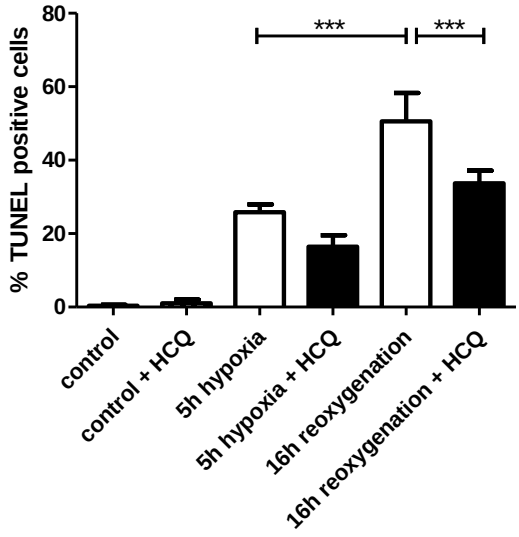


Figure 3.5 Pre-treatment with HCQ reduces TUNEL positive cells in H9c2 cardiac cell line in simulated I/R injury. Cells were pre-incubated with 1 μg/mL HCQ and the following day exposed to simulated I/R injury. Cells were fixed in 4% PFA the percentage of TUNEL positive cells were assessed. Graph shows mean ±SEM of quantitative analysis from 3 independent fields. Statistical analysis determined by 1 way ANOVA using post-hoc Tukey to compare all columns (*** $p<0.0005$).

3.6 Isolation of neonatal rat cardiomyocytes

3.6.1 Introduction

Neonatal rat cardiomyocytes are the most translationally relevant *in vitro* model available in the cardiovascular field to mimic *in vivo* I/R injury. It was decided that upon the completion of preliminary experiments in cardiac myoblast H9c2 cell line that subsequent experiments would be carried out in these primary cells. Cardiomyocytes are isolated from 1-2 day old pups by extracting their hearts and digesting in collagenase type 2 and pancreatin. Isolated cells are pre-plated for 1 hour to allow contaminating fibroblasts to attach. The enriched myocyte prep is then plated on 1% gelatin coated cell culture plates. The following day the cells should have attached and can be seen beating under the microscope.

3.6.2 Isolated neonatal rat cardiomyocytes stain positive for desmin

Due to the nature of the prep it is important to identify that myocytes are successfully being isolated and therefore immunofluorescence was performed to stain for Desmin, a common cardiac muscle marker. Immunofluorescences showed isolated cells were positive for desmin, which is a subunit of intermediate filaments in skeletal muscle tissue, smooth muscle tissue, and cardiac muscle tissue and therefore myocytes are positively stained. Contaminating fibroblasts do not stain positive for desmin therefore allowing their contamination to be assessed. It was identified that approximately 90% of cell preparations are myocyte positive; therefore fibroblast contamination after pre-plating is negligible (*figure 3.6*).

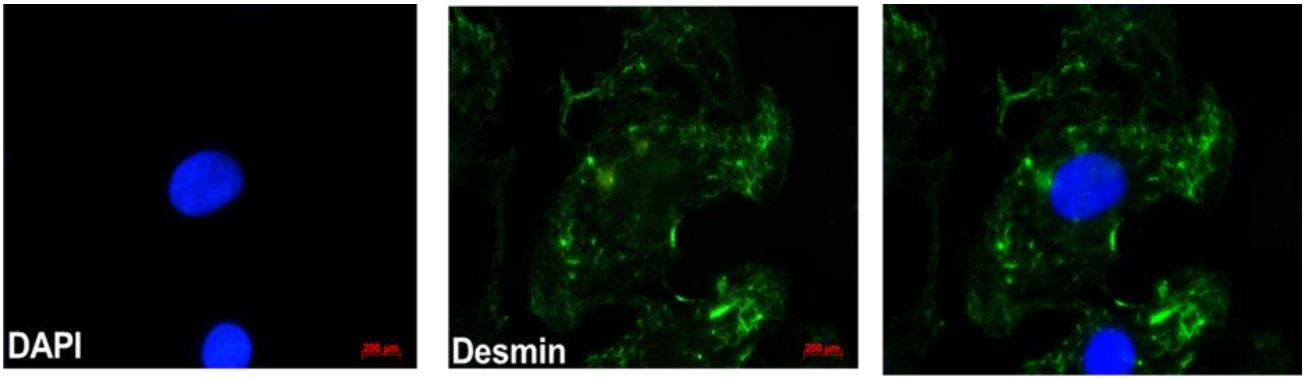


Figure 3.6 Immunofluorescence to confirm isolation of neonatal rat cardiomyocytes Cells were seeded on UV irradiated glass coverslips at 1 x10^5 cells/well in a 24 well plate. Cells were fixed in 4% PFA and immunofluorescence for desmin (green) was performed and nuclei were stained with DAPI (blue).

3.7 Optimisation of simulated I/R injury in neonatal rat cardiomyocytes

3.7.1 Introduction

Primary cells are more susceptible to stress induction such as simulated I/R injury in comparison to cells lines and therefore optimisation experiments to determine conditions in the neonatal cardiomyocytes were carried out. Previous experiments carried out in our lab using neonatal cardiomyocytes suggested 4h hypoxia followed by 16h reoxygenation was optimal and this was therefore confirmed.

3.7.2 Results of optimisation of simulated I/R injury in neonatal rat cardiomyocytes

It was confirmed that 4 hours hypoxia followed by 16 hours reoxygenation was suitable to induce 30% apoptosis (*figure 3.7*), therefore for subsequent TUNEL experiments in the neonatal rat cardiomyocytes these conditions were used.

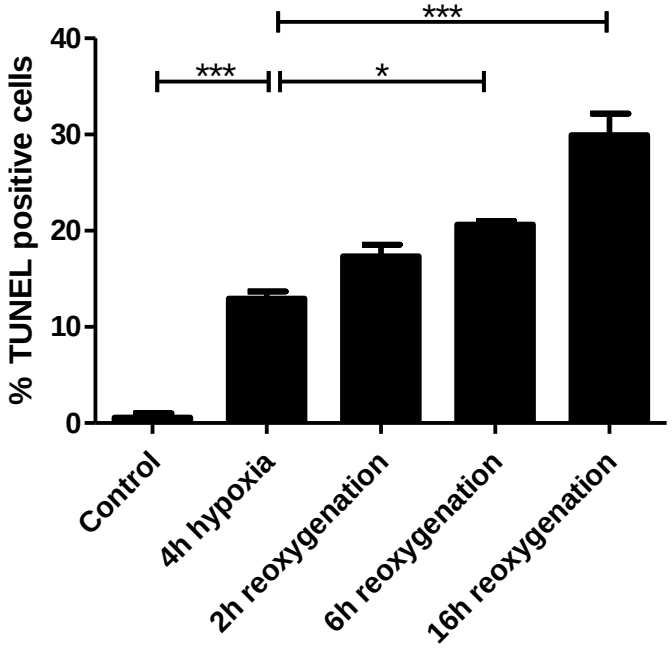


Figure 3.7 Optimisation of simulated I/R injury in neonatal rat cardiomyocytes Simulated I/R injury was induced (4h hypoxia + indicated times of reoxygenation) and cells were fixed in 4% PFA for TUNEL analysis. Graph shows mean ±SEM of quantitative analysis from three independent fields. Statistical analysis determined by 1 way ANOVA using post-hoc Tukey to compare all columns (* $p<0.05$, *** $p<0.0005$).

3.8 The effect of pre-treatment with HCQ in simulated I/R injury on cell death in neonatal rat cardiomyocytes

3.8.1 Introduction

The main focuses of this project has been to identify the role of HCQ in I/R injury. This is due to preliminary data collected from the H9c2 cardiac rat cell line suggesting a considerable protective effect being observed through the reduction of apoptosis. This has the potential to identify a new mechanism of action for HCQ, providing further evidence to suggest its importance as a cardioprotective agent in patients with lupus and in the wider cardiovascular field.

3.8.2 Pre-treatment of neonatal rat cardiomyocytes with HCQ in simulated I/R injury reduces apoptotic positive cells in TUNEL

Cells were seeded on UV irradiated glass coverslips at $1x10^5$ cells/well in a 1% gelatin coated 24 well plate. Once cells had attached and were beating they were pre-treated with 2 µg/mL HCQ and the following day simulated I/R injury was carried out. HCQ was present throughout the experiment and in all subsequent experiments.

The pre-treatment of neonatal rat cardiomyocytes with HCQ reduced apoptosis when simulated I/R injury is induced (*figure 3.8*). When the cells are only exposed to hypoxia and then stained for TUNEL positivity, 20.65% (±SD 7.38), stain positive and when exposed to re-oxygenation for 16 hours TUNEL positivity is enhanced to 30.13% (±SD 7.047)($p<0.005$). However, if the cells are pre-incubated with HCQ, then this enhancement of TUNEL positivity during the re-oxygenation stage is completely abrogated back down to that observed at hypoxia alone (16.93% (±SD 3.002)($p<0.0005$). Low levels of apoptosis are detected in control cells in both the absence and presence of HCQ due to natural cell turnover. This demonstrates that HCQ is cardioprotective in this *in vitro* simulated I/R injury model.

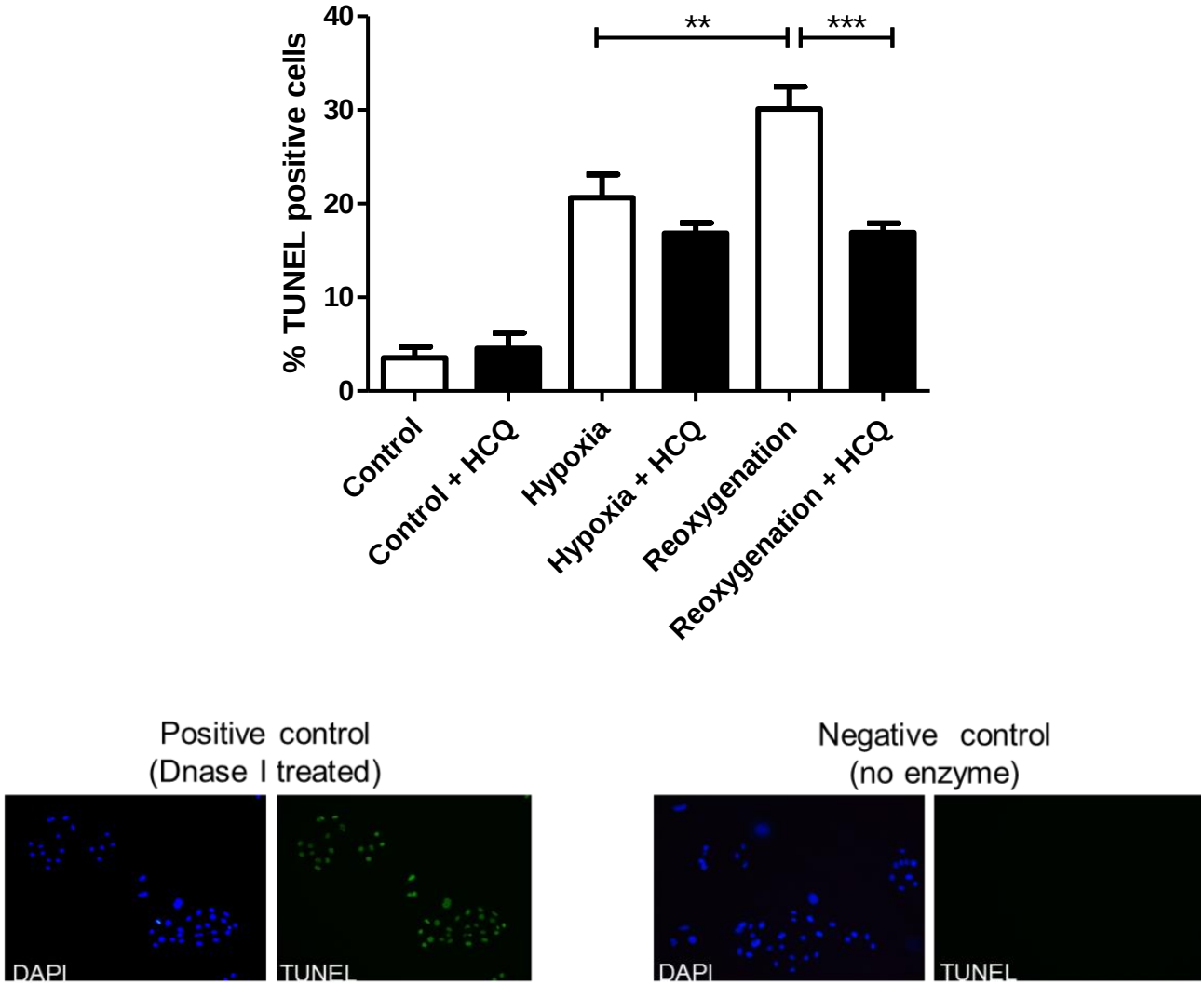


Figure 3.8 HCQ reduces TUNEL positive cells in neonatal rat cardiomyocytes in simulated I/R injury Cells were pre-treated with 2 μg/mL HCQ. The following day cells were exposed to simulated I/R injury (4h hypoxia and 16h reoxygenation), fixed in 4% PFA and the percentage of TUNEL positive cells was assessed. Graph shows ±SEM of quantitative analysis from 9 independent experiments. Statistical analysis determined by 1 way ANOVA using post-hoc Tukey to compare all columns (** $p<0.005$, *** $p<0.0005$).

3.8.3 Pre-treatment of neonatal rat cardiomyocytes with HCQ in simulated I/R injury reduces caspase-3 cleavage

This finding was very interesting and therefore it was decided that it should be confirmed using another method that allows detection of apoptosis. Apoptosis can be induced via a variety of mechanisms which all converge to induce cleavage of the execution caspase, caspase 3. Thus cleaved caspase-3 is a prominent marker of apoptosis and so this HCQ experiment was repeated to detect cleaved caspase-3.

Cells were plated at $1.5x10^6$ cells/well in a 6 well plate, they were pre-treated with 2 µg/mL HCQ and the following day simulated I/R injury was carried out. A dose of 2 µg/mL was used due to this correlating with the blood concentration of HCQ achieved in patients of between 1-2 µg/mL. Cells were placed in the hypoxic chamber (argon/CO_2) for 4 hours in the presence of ischaemic buffer and subsequently media was replenished and cells were reoxygenated in a cell incubator (5% CO_2 in air, 37°C) for 2 and 4 hours. Shorter periods of reoxygenation were used due to caspase-3 cleavage occurring at an early stage of apoptosis in comparison to DNA fragmentation, which was previously quantified using TUNEL. Cell lysates were collected and separated using Western Blotting to allow detection of cleaved caspase-3.

The pre-treatment of neonatal rat cardiomyocytes with HCQ reduces caspase-3 cleavage, therefore suggesting a cardioprotective role in this *in vitro* model of simulated I/R injury (*figure 3.9*). Cleaved caspase-3 is increased during reoxygenation (0.24 relative to GAPDH (±SD 0.09)($p<0.0005$) when compared to cells in optimal conditions (0.03 relative to GAPDH (±SD 0.03)). In the presence of HCQ this increase in cleaved caspase-3 is significantly reduced by 54.16% (0.11 relative to GAPDH (±SD 0.05)($p<0.05$). This further clarifies that HCQ is cardioprotective in simulated I/R injury.

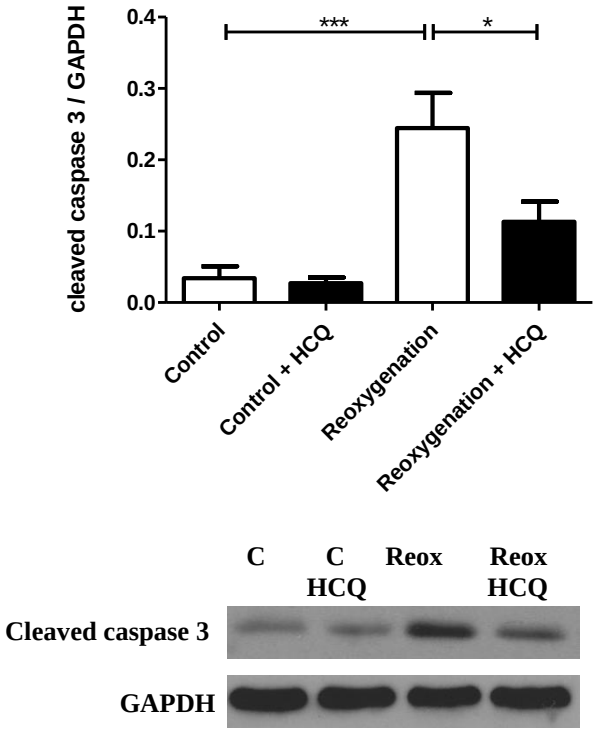


Figure 3.9 HCQ reduces cleaved caspase-3 in neonatal rat cardiomyocytes in simulated I/R injury Cells were pre-treated with 2 µg/mL HCQ. The following day cells were exposed to simulated I/R injury (4h hypoxia + 4h reoxygenation) and the level of cleaved caspase-3 was assessed by western blot. Graph shows ±SEM of quantitative analysis from 4 independent experiments. Statistical analysis determined by 1 way ANOVA using post-hoc Tukey to compare all columns (* $p<0.05$, *** $p<0.0005$).

3.8.4 Pre-treatment of neonatal rat cardiomyocytes with HCQ reduces total cell death in simulated I/R injury

Data produced thus far has focused on apoptotic cell death within I/R injury; however it is important to assess the relevance of HCQ and its protective effect when looking at total cell death. This allows assessment of it protection in simulated I/R injury to be quantified to determine is significance and translational relevance. A cell viability assay was therefore used to assess reduction in overall cell viability in simulated I/R injury and the effects of HCQ on this viability. A colorimetric cell proliferation assay confirmed that HCQ causes a reduction in total cell death in cells exposed to simulated I/R injury of 57.89% (±SD 7.14, p=0.0213) when compared to cells not incubated with pathways activated by stress signals inducing apoptosis (*figure 3.10*).

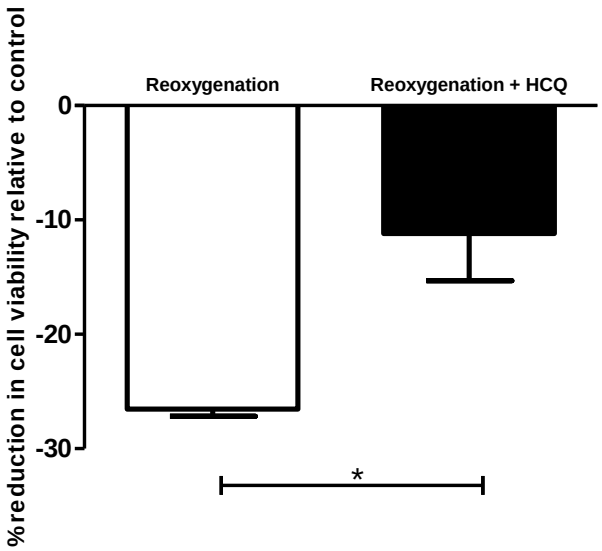


Figure 3.10 HCQ reduces total cell death in neonatal rat cardiomyocytes in simulated I/R injury Cells were pre-treated with 2 µg/mL HCQ. The following day cells were exposed to simulated I/R injury (4h hypoxia + 4h reoxygenation) and a cell viability assay was performed. Graph shows ±SEM of quantitative analysis from 4 independent experiments. Statistical analysis determined by unpaired t-test (p=0.0213).

3.9 Control experiments to confirm IgG had a direct effect on apoptosis in neonatal rat cardiomyocytes

3.9.1 Introduction

Preliminary studies in the H9c2 rat cardiac cell line suggested that IgG purified from the serum of patients with SLE is pathogenic in simulated I/R injury. This experiment was therefore carried out in our primary cell system using neonatal rat cardiomyocytes. More samples were tested and categorised depending on their antiphospholipid antibody status as well as testing samples from JSLE and APS patients.

3.9.2 Patient and healthy control IgG selection and characteristics

Purified IgG from 34 patients were selected for use in experiments in this chapter as outlined in section 2.7.2 of Material and Methods. Six samples were selected from patients with primary APS, five were selected for SLE/aPL +ve, five were selected for SLE/aPL –ve, eight for JSLE and the remaining ten were healthy controls.

The ability of purified and endotoxin free IgG to bind CL and β_2GPI at the experimental concentration (500 μg/mL) was then determined (*figure 3.11*).

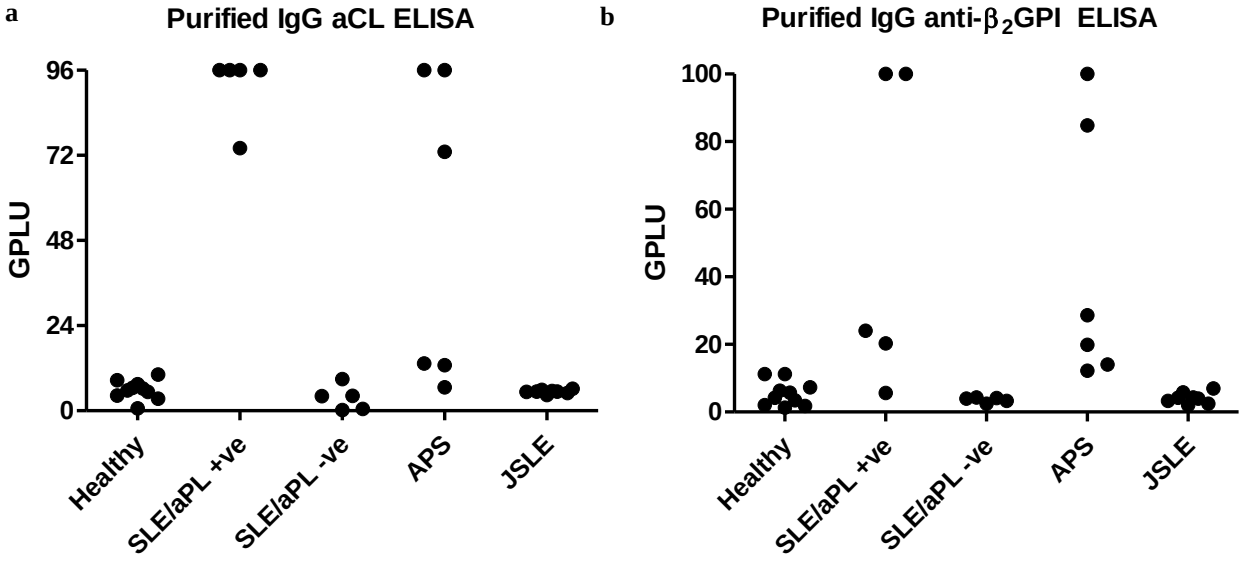


Figure 3.11 IgG aCL and anti-β_2GPI levels
All purified IgG was tested at 500 µg/mL for binding to aCL (a) and anti-β_2GPI (b). IgG samples that have activity above the cut-off of these assays are plotted as the upper limit of the assay (96 GPLU or 100 SU). Positivity is defined as above 17 GPLU (aCL) and 9 GPLU (anti-β_2GPI) based on the testing of 200 healthy controls (±3 SD).

3.9.3 Pre-treatment of neonatal rat cardiomyocytes with LPS has no effect on cleaved caspase-3 in simulated I/R injury

Prior to testing patient samples some control experiments were performed. One of the challenges when purifying IgG from serum of patients is contamination with endotoxins, which could potentially activate signalling pathways in target cells. Therefore cells were incubated with varying concentrations (1 ng/ml to 10 µg/ml) of LPS to determine if neonatal rat cardiomyocytes are sensitive to endotoxin and if so determine the threshold concentration of endotoxin required to cause an effect. It was found that there was no significant difference in levels of cleaved caspase-3 when cells were treated with increasing concentrations of LPS overnight and the following day exposed to simulated I/R injury (*figure 3.12a*). However, as it is routine in our lab to run purified samples through endotoxin removal columns and this was still carried out to ensure there was minimal endotoxin contamination (<0.25 endotoxin units (EU/mL).

3.9.4 Pre-treatment of neonatal rat cardiomyocytes with BSA has no effect on cleaved caspase-3 in simulated I/R injury

It was also important to show that any differences observed with IgG from patients was due to the effects of the IgG and not due to another factor such as protein overloading. When cells are overwhelmed with high concentrations of protein (e.g. BSA), stress pathways may be activated and therefore could contribute to changes in levels of cleaved caspase-3. Varying concentrations (100 µg/ml to 500 µg/ml) were incubated with cells overnight prior to simulated I/R injury. No difference was observed when compared with control cells (*figure 3.12b*).

3.9.5 Pre-treatment of neonatal rat cardiomyocytes with IgG purified from the serum of healthy controls has no effect on cleaved caspase-3 in simulated I/R injury

It was essential to assess whether IgG purified from healthy serum was pathogenic in our simulated I/R injury model. A pilot experiment was therefore carried out in which cells were pre-treated overnight with varying concentrations of healthy IgG (100 µg/ml to 500 µg/ml) followed by simulated I/R injury. No effect was seen in the presence of healthy IgG on cleaved caspase-3 following simulated I/R cardiac injury suggesting healthy IgG is not pathogenic up to a concentration of 500 µg/ml (*figure 3.12c*). It was thus decided that in subsequent experiments IgG would be used at a concentration of 500 µg/ml to maximise the potential signal observed with IgG purified from patients.

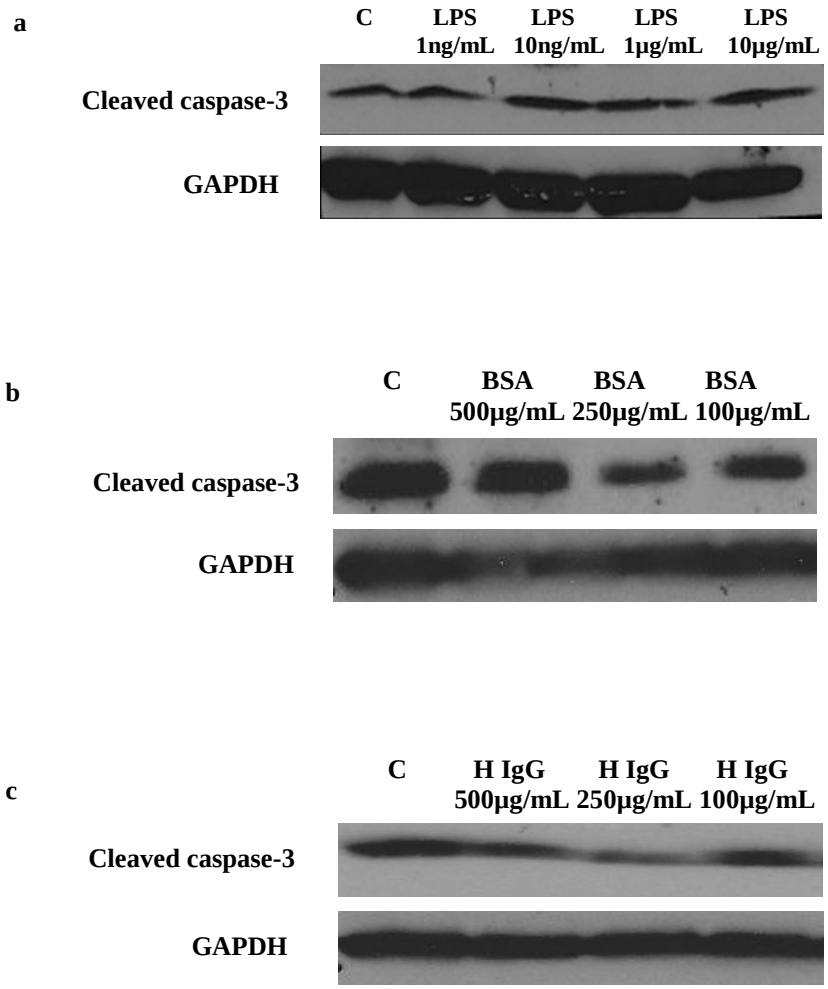


Figure 3.12 Neonatal rat cardiomyocytes are not sensitive to LPS, BSA and healthy IgG. Neonatal rat cardiomyocytes were treated with a range of LPS concentrations (1ng/mL to 10 µg/mL) or a range of BSA concentrations (100 µg/mL to 500 µg/mL) or a range of healthy patient IgG (100 µg/mL to 500 µg/mL) overnight and the following day exposed to simulated I/R injury before cell lysates were collected and probed for cleaved caspase-3 using western blot (n=1).

3.10 IgG from patients with SLE, JSLE and APS increase apoptosis in neonatal rat cardiomyocytes in simulated I/R injury

3.10.1 Introduction

SLE carries a significantly enhanced risk of developing cardiovascular disease (CVD) and remains a leading cause of death in these patients. There is clear evidence linking accelerated atherosclerosis to SLE, and as a consequence, an increase in cardiovascular events. Another factor which could contribute to increased morbidity and mortality related to CVD is enhanced reperfusion injury post MI. The factors that could contribute to this are yet to be explored, however there is some evidence using a lupus mouse model that lupus IgG antibodies are pathogenic within a mesenteric I/R injury model. No study as yet has investigated human lupus IgG in a heart I/R injury model.

3.10.2 Pre-treatment of neonatal rat cardiomyocytes with IgG purified from patients with SLE increases cleaved caspase-3 in a dose dependent manner in simulated I/R injury

An initial experiment was carried out to confirm at what concentration IgG from SLE patients was pathogenic. Patient 113 from preliminary studies was used due to previously identifying it as being pathogenic in H9c2 rat myoblast cell line. The most significant increase in cleaved caspase-3 was at a concentration of 500 µg/mL (*figure 3.13*). As previous experiments demonstrated no toxicity with healthy IgG at this concentration, this dose was chosen for all subsequent experiments.

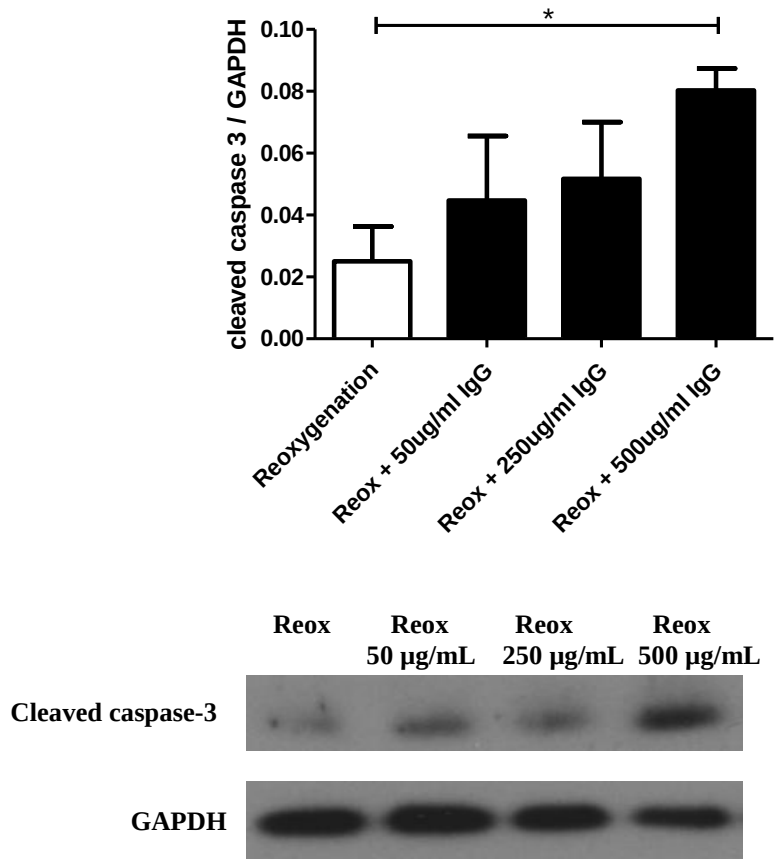


Figure 3.13 IgG from a patient with SLE increases cleaved caspase-3 in a dose dependent manner in neonatal rat cardiomyocytes exposed to simulated I/R injury. Neonatal rat cardiomyocytes were pre-treated with a range of IgG concentrations (50 µg/mL to 500 µg/mL) purified from patient 113 (SLE/aPL +ve). The following day cells were exposed to simulated I/R injury and the cell lysates collected and probed for cleaved caspase-3 using western blot. Graph shows ±SEM of quantitative analysis from 4 independent experiments. Statistical analysis determined by 1 way ANOVA using post-hoc Tukey to compare all columns (* $p<0.05$).

3.10.3 Pre-treatment of neonatal rat cardiomyocytes with IgG purified from patients with SLE enhanced cleavage of caspase-3 in simulated I/R injury

Cells were incubated with IgG purified from healthy controls and SLE patients overnight and the following day exposed to simulated I/R injury. IgG purified from the blood of patients with SLE significantly increased cleaved caspase-3 when compared to healthy age and gender matched controls in simulated I/R injury. Healthy IgG had no significant effect on cleaved caspase-3 levels, however SLE IgG increased levels by a mean of 49.7% (±SD 16.31) when compared with control cells (*figure 3.14*).

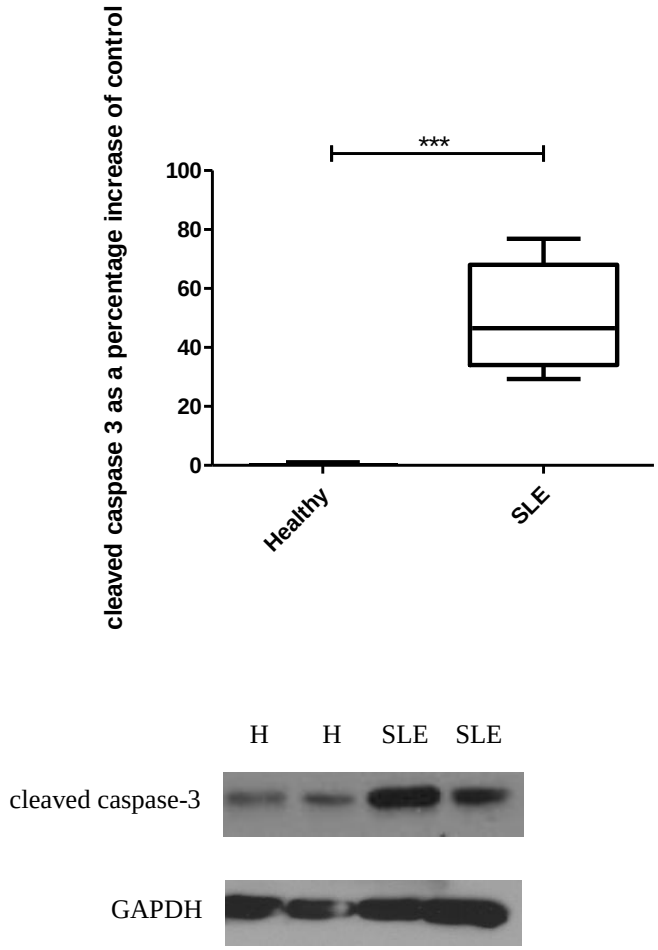


Figure 3.14 IgG from patients with SLE enhances cleaved caspase-3 in neonatal rat cardiomyocytes in simulated I/R injury. Cells were pre-treated with healthy or SLE IgG. The following day cells were exposed to simulated I/R injury (4h hypoxia + 4h reoxygenation) and the level of cleaved caspase-3 was assessed by western blot. Graph shows ±SEM of quantitative analysis from 10 SLE patients and 10 healthy controls. Statistical analysis determined by unpaired t-test (*** $p<0.0005$).

3.10.4 Pre-treatment of neonatal rat cardiomyocytes with IgG purified from patients with SLE enhances TUNEL positivity in simulated I/R injury

TUNEL was used as an additional method to assess if IgG from patients with SLE is pathogenic. IgG purified from patients with SLE or healthy age and gender matched controls was incubated with cells overnight and the following day cells were exposed to simulated I/R injury. Data showed that IgG from SLE patients caused a significant increase in TUNEL positivity of a mean of 54.17% (±SD 8.13), when compared to control cells (*figure 3.15*) whereas healthy IgG had no significant affect.

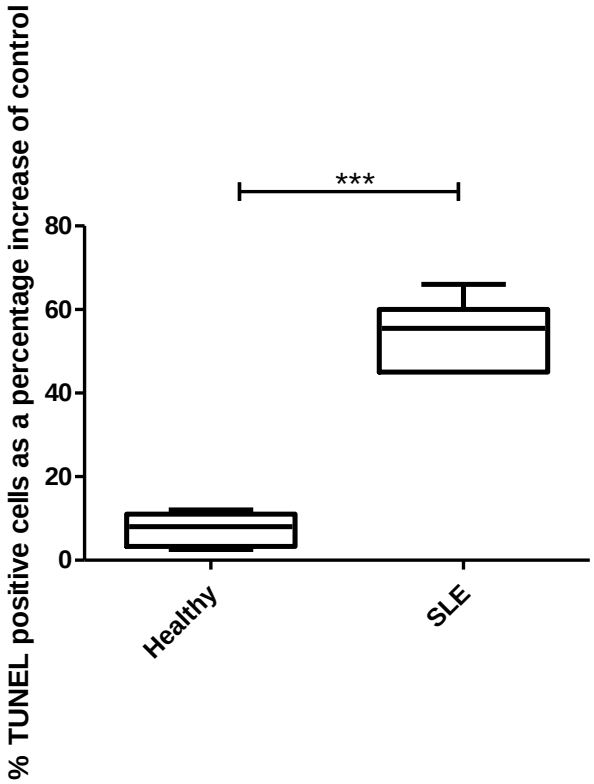


Figure 3.15 IgG from patients with SLE enhances the number of TUNEL positive cells in neonatal rat cardiomyocytes exposed to simulated I/R injury. Neonatal rat cardiomyocytes were pre-treated with 500 µg/mL IgG from either healthy or SLE patients and the following day cells were exposed to simulated I/R injury (4h hypoxia + 16h reoxygenation). Cells were fixed in 4% PFA and the percentage of TUNEL positive cells was assessed. Graph shows mean ± SEM of quantitative analysis from 6 SLE patients and 5 healthy controls. Statistical analysis determined by unpaired t test ($p<0.0001$)

3.10.5 Pre-treatment of neonatal rat cardiomyocytes with IgG purified from patients with JSLE and primary/secondary APS enhances cleaved caspase-3 in simulated I/R injury

The SLE group was next split into those who tested antiphospholipid antibody (aPL) positive and those who were negative. Those who are positive are diagnosed as having 'secondary' APS, additionally a group titled 'primary' APS, which occurs in patients who have APS alone and no other autoimmune disease was also included. It was observed that within the SLE group, the aPL positive patients had a mean of 68.15% (±SD 13.53) increase in caspase-3 cleavage compared to control cells (*figure 3.16*). This was significantly higher than that seen in the SLE patients who were aPL negative and had a mean of 40.66% (±SD 8.7). Additionally, IgG from patients with primary APS caused a significant increase in cleaved caspase-3 with a mean of 67.94 (±SD 12.41), similar to that seen in patients with secondary APS (*figure 3.16*). All aPL-IgG samples harboured anti-ß$_2$GPI (ß$_2$GPI) antibodies (*figure 3.16*). This finding suggests a potential aPL dependent mechanism through which IgG from patients is pathogenic in this simulated I/R injury model.

Due to the availability of samples from an adolescent rheumatology clinic samples were also tested from patients with juvenile onset SLE (JSLE) to observe if their IgG also resulted in accelerated I/R injury. It was observed that JSLE IgG caused a mean increase of 63.98% (±SD 17.99) in cleaved caspase-3 compared with control cells (*figure 3.16*). Interestingly, these JSLE patients are all aPL negative, however appear to follow the levels of pathogenicity observed in adult onset SLE patients who are aPL positive.

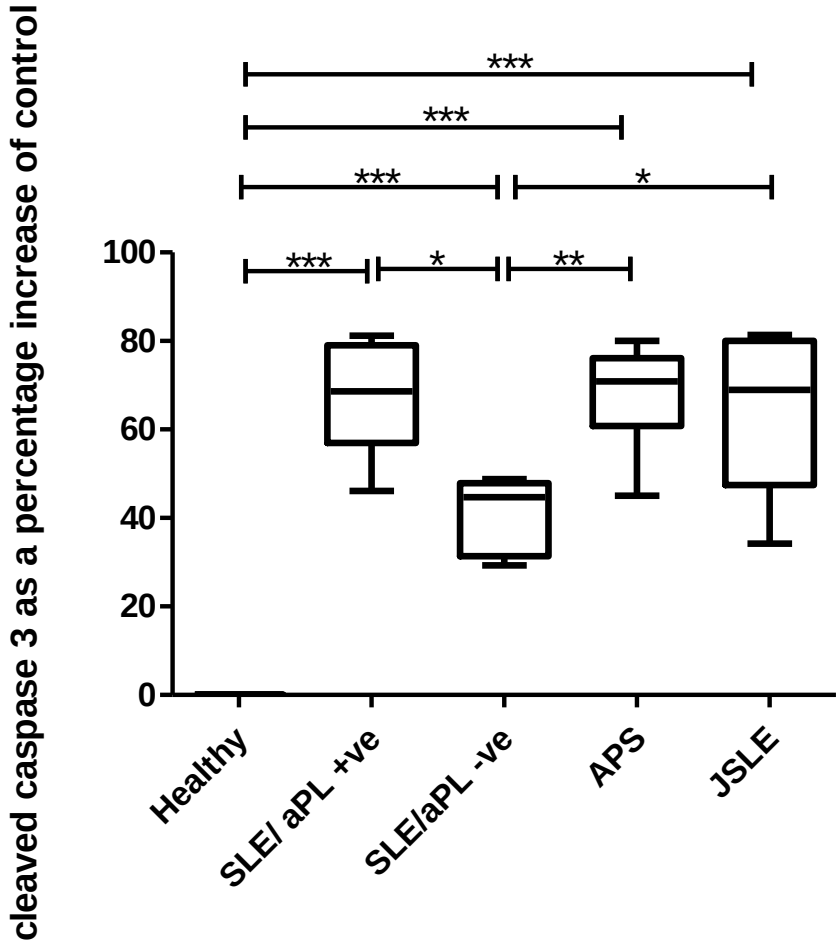


Figure 3.16 IgG from patients with JSLE, SLE/aPL +ve, SLE/aPL –ve and APS enhance cleaved caspase-3 in neonatal rat cardiomyocytes in simulated I/R injury . Cells were pre-treated with healthy, JSLE, SLE/aPL –ve, SLE/aPL +ve or APS IgG. The following day cells were exposed to simulated I/R injury (4h hypoxia + 4h reoxygenation) and the level of cleaved caspase-3 was assessed by western blot. Graph shows ± SEM of quantitative analysis from 8 JSLE, 5 SLE/aPL +ve patients, 5 SLE/aPL –ve patients, 6 APS patients and 10 healthy controls. Statistical analysis determined by 1 way ANOVA using post-hoc Tukey to compare all columns (* $p<0.5$,*** $p<0.0005$).

3.11 Discussion

The data in this chapter indicates that the drug HCQ is cardioprotective in I/R injury. Additionaly IgG purified from the serum of SLE, APS and JSLE patients were found to be pathogenic in simulated I/R injury.

The experiments described in this chapter begin by establishing the optimal conditions for simulated I/R injury in cardiac derived cells with the aim to establish approximately 30% enahncement of apoptosis during reoxygeneation post hypoxia. This apoptosis induction is the accepted percentage of apoptosis for *in vitro* simulated I/R injury, that mimcs what is observed *in vivo* (Fujio Y 2000; Shen M 2012). In the *in vitro* model of simulated I/R injury the terminology used to describe the two stages of I/R injury is different. The ischaemic phase induced during a MI refers to occlussion of blood flow and therefore restriction of oxygen supply to tissue. In this *in vitro* model a hypoxic environment is created, which is the state in which tissue in an *in vivo* setting is maintained. This is achieved by a mix of CO_2/argon gas being pumped into a chamber where the argon forms a layer over the CO_2 causing displacement of molecular oxygen. The reperfusion phase which occurs *in vivo* refers to resotration of blood flow and therefore in the *in vitro* setting this is instead referred to as reoxygenation, due to oxygen being re-introduced into the system. To confirm that successful simulated I/R injury was being , the mRNA levels of two proteins; Hif1α and Hsp70 were assessed. Hif1α is a hypoxic induced protein and therefore as expected levels of mRNA were increased only in the hypoxic stage. Hsp70 is a heat shock protein induced in the presence of stress and therefore mRNA levels increased for both hypoxia and reoxygenation (*chapter 3, figure 3.1*). This experiment confirmed that hypoxia was being introduced and that upon reoxygenation, cells were no longer in a hypoxic environment, but were still in a stressed state, consistent with the setting of I/R injury.

Opimisation experiments were completed using TUNEL, which stains cells positive that have DNA fragmentation, a late-stage marker of apoptosis. In the H9c2 cardiacmyoblast cell line 5 hours hypoxia followed by 16 hours reoxygenation or 6 hours hypoxia followed by 6 hours reoxygenation was optimal (*chapter 3, figure 3.3*). In the neonatal rat cardiomyocytes 4h hypoxia followed by 16 hours reoxygenation satisified the criteria for *in vitro* simulated I/R injury (*chapter 3, figure 3.7*).

A novel aspect of this study is the investigation of the role of HCQ in myocardial I/R injury. These experiments were peformed due to patients with SLE, prescribed HCQ, have less cardiovascular events (Venuturupalli S 2012) due to the drugs lipid lowering effects and ability to reduce platelet aggregation (Hyejung J 2010; Rand J 2010; Morris S 2011). No investigation into the effects of HCQ

on cardiac I/R injury has been performed and given its effects on the lysosome and autphagy the hypothesis was that it may affect I/R injury also. Recent studies in the heart by *Zhou et al* (Zhou 2013) have shown that HCQ can significantly increase phosphorylation of the pro-survival kinase Akt, without significantly impacting expression of phosphorylated p53 protein in the peri-infarct myocardium. Consequently, HCQ can inhibit cardiomyocyte apoptosis in the peri-infarct myocardium. Another recently published study has shown that HCQ can protect in an *in vivo* model of renal I/R injury (Todorovic Z 2014). Interestingly, a study looking at the role of HCQ in liver I/R injury showed protection in early stage injury, however, HCQ was shown to actually cause damage in late stage I/R injury (Fang H 2013). Exploring the effects of HCQ in cardiac I/R injury will provide further evidence for its use in patients with SLE, due to their increased risk of cardiovascular disease. Additionaly, it may provide evidence for the use of HCQ in the wider cardiovascular field i.e for patients who are at risk of developing CVD.

It was observed that the percentage of TUNEL positive cells in cells pre-treated with HCQ in simulated I/R injury was enhanced upon induction of hypoxia and further increased during reoxygenation. Interestingly, upon reoxygenation the percentage of TUNEL positive cells was signifcantly decreased in the presence of HCQ. This was observed in both a cardiac H9c2 rat myoblast cell line (*chapter 3, figure 3.5*) and primary neonatal rat cardiomyocytes (*chapter, figure 3.8*). In neonatal rat cardiomyocytes, HCQ abborgated levels of TUNEL positivty down to levels observed in hypoxia alone. This observation was confirmed using another method to detect apoptosis, caspase-3 cleavage via western blot. This was done to strengthen the finding that HCQ reduced apoptosis. Apoptosis occurs via two main pathways; the extrinsic and intrinsic, however these pathways converge to induce cleavage of the execution caspase, caspase-3 (Taylor R 2008). This therefore makes it a good indicator of total apoptosis occuring within a cell population. This set of experiments showed significantly increased levels of caspase-3 cleavage after 2 hours of reoxygenation compared with control cells. Furthermore, after 2 hours of reoxygenation there was a statistically significant decrease in cleaved caspase-3 in the presence of HCQ, which was also observed at 4 hours (*chapter 3, figure 3.9*). This experiment required earlier timepoints of simulated I/R injury to be explored due to caspase-3 cleavage being an earlier marker of apoptosis in comparison to TUNEL. These findings further validated our hypothesis that HCQ is cardioprotective in simulated I/R injury.

I/R injury leads to extensive cell injury and death through apoptosis but also through another mechanism of cell death, necrosis. The effects of HCQ on total cell viability were therefore explored

in the neonatal rat cardiomyocytes. It was observed that the same protective mechanism could be demonstrated when looking at total cell death (*chapter 3, figure 3.10*). Data was presented as a percentage reduction in cell viability relative to control cells. It was observed that there was a significant decrease in cell viability following reoxygenation. However, in the presence of HCQ there was a significant increase in cell viability. The role of apoptosis in I/R injury is subject to conflicting evidence in the cardiovascular field (Eefting F 2004; McCully J 2004), however my data suggests that it plays a prominent role, due to the observation that total cell death is reduced significantly. However, it should also be noted that HCQ's protective effect may not just be reducing apoptosis and therefore its role in inhibiting necrosis should not be ruled out. Whilst it is well established that necrosis induction is mainly due to irreversible damage to cells there is now the suggestion that necrosis may also be able to occur through a program-dependent manner (Elmore 2007). Therefore, exploring the effects of HCQ in relation to necrosis in simulated I/R injury may be an important area to explore in the future. The observation that HCQ has a protective effect on total cell death increases evidence to suggest this drugs translational relevance. Nevertheless, it should be acknowledged that this is preliminary *in vitro* data, however these initial findings underpinned subsequent experiments to investigate the protective effects of HCQ *in vivo* in a rat cardiac I/R injury model and the results are presented in Chapter 5 of this thesis. It is also of interest to explore how HCQ is exerting its protective mechanism. It has previously been shown to inhibit autophagy by altering the pH of the lysosome therefore causing accumulation of autophagosomes. The role of autophagy in cardiac I/R injury is controversial; some believe its activation enhances cell death (Noh HS 2010), whilst others believe that its activation leads to protection (Hamacher-Brady A 2006; McCormick J 2012). There are other potential targets of HCQ including the pro-survival kinases ERK 1/2 and Akt which have been shown to have increased phosphorylation in I/R injury to promote cell survival (Haunsenloy 2007). Other drugs such as sildenafil have previously been shown to enhance phosphorylation of ERK 1/2 to induce protection in cardiac I/R injury (Das A 2009). Subsequent experiments presented in chapter 4 of this thesis aim to dissect out the mechanism through which HCQ is cardioprotective.

The second aim of this project was to explore the pathogenicity of IgG purified from patients with lupus in this simulated I/R injury model. Preliminary experiments in the H9c2 cardiomyoblast cell line showed promising results. Previously published data has suggested that IgG is pathogenic in a murine mesenteric I/R injury model due to accelerated I/R injury in the presence of IgG from a lupus prone mouse (Fleming S 2004). However, there is no literature to suggest the effect of IgG in the cardiac I/R injury setting has been explored. Three patients were selected for an initial experiment,

and it should be noted that they had all had a form of cardiovascular event as detailed in *chapter 2, table 2.2*. TUNEL data suggested that increased apoptosis was observed in cells that were pre-treated with two out of the three patient samples (113 and 552) in both hypoxia and reoxygenation (*chapter 3, figure 3.4*). Interestingly these patients are positive for aPL antibodies, whereas patient 345 is not. aPL have been heavily implicated in an increased risk of thrombosis and enhanced inflammatory response due to binding to and altering endothelial cell responses and other cells in thrombosis and inflammation such as monocytes (de Laat, Derksen et al. 2004; Giannakopoulos B 2007; Rand J 2010), however their role in I/R injury to date has not been explored. This preliminary experiment therefore suggests that IgG from patients with SLE may be pathogenic in simulated I/R injury, and this is further enhanced in patients who are positive for aPL. However, this is a preliminary experiment in a cell line. Following on from this observation this experiment was repeated in neonatal rat cardiomyocytes and the cohort of patients was expanded, with the effect confirmed.

Control experiments were primarily carried out to establish the sensitivity of these neonatal rat cardiomycoyte cells to human IgG. Firstly, a range of doses (1 ng/mL to 10 µg/mL) of LPS were pre-incubated with cells overnight before exposure to simulated I/R injury. This was done to observe whether LPS altered levels of cleaved caspase-3 in cells. IgG purified from serum of patients (and healthy controls) is contaminated with endotoxin. Our lab regularly purifies samples to remove endotoxin to below <0.25 endotoxin units (EU)/ml, however it is important to establish how sensitive cells are to endoxtoin to ensure effects observed are in fact due to a direct effect of the IgG and not due to LPS contamination. It was found that none of the doses of LPS administered to cells altered levels of cleaved caspase 3 (*chapter 3, figure 3.12a*). Additionally, cells were pre-treated with a range of concentrations of BSA (100 µg/mL to 500 µg/mL) to ensure that high levels of protein did not alter cell function and induce cell death. Again, at a range of concentrations there was no effect on levels of cleaved caspase-3 (*chapter 3, figure 3.12b*). Finally, cells were pre-treated with a range of healthy IgG concentrations to see at what dose healthy IgG enhanced pathogenicity in simulated I/R injury. In this model, up to a concentration of 500 µg/mL, there was no change in cleaved caspase-3 observed (*chapter 3, figure 3.12c*). This experiment was repeated with IgG purified from a patient with SLE to see at what doses cleaved caspase-3 was enhanced. From as little as 50 µg/mL IgG an increase in cleaved caspase-3 was observed, however maximal increase was observed with 500 µg/mL (*chapter 3, figure 3.13*). Due to healthy IgG having no effect on cells at this concentration it was decided that subsequent experiments would use a dose of 500 µg/mL.

IgG was subsequently purified from 10 SLE patients and 10 healthy age and gender matched controls. Neonatal rat cardiomyocytes were pre-treated with 500 µg/mL IgG from SLE patients and

the following day exposed to simulated I/R injury. In the presence of IgG from patients with SLE cleaved caspase 3 was significantly increased (*chapter 3, figure 3.14*). This was further confirmed using the TUNEL assay, where a significant increase in TUNEL positive cells was also observed in the presence of IgG from patients with SLE in comparison to healthy age and gender matched controls (*chapter 3, figure 3.15*). Due to the initial observation that only aPL positive patient IgG was pathogenic, the patient cohort was split into those who were aPL positive (5 patient samples) vs aPL negative (5 patient samples) and an additional group of patients who had APS (i.e.aPL positive but didn't have SLE) (6 patient samples) were included. IgG purified from the blood of patients with SLE with aPL positivity and IgG from patients with the APS but without SLE significantly enhanced apoptosis in cardiomyocytes post-reoxygenation as compared to IgG from patients with SLE without aPL (*chapter 3, figure 3.16*). All groups enhanced caspase-3 cleavage to a greater extent than that observed with IgG from healthy volunteers. It should be noted that all aPL-IgG samples harboured ß$_2$GPI and aCL antibodies (*chapter 3, figure 3.11*). These results were of great interest as previously published work has shown that autoimmune prone mice with high titers of autoantibodies have accelerated mesenteric I/R injury (Fleming S 2004). Further studies by this group showed that aPL antibodies were specifically responsible for accelerated mesenteric I/R injury, in complement receptor 2/complement receptor 1 deficient mice (Fleming S 2004). Previous work by my lab and others has shown that aPL positive IgG activates p38 MAPK leading to enhanced thrombosis (Vega-Ostertag ME 2007; Lambrianides A 2010). This signalling pathway is therefore of interest and data regarding this work is presented in chapter 4 of this thesis.

Lastly, a fourth group of patients were included in this set of experiments, those with JSLE (8 patient samples). Patients with childhood onset SLE compared to those with adult-onset disease have greater prevalence of renal disease, more haematological disease and generally have a worse prognosis with significantly greater standardised mortality ratios as compared to adult onset SLE (Amaral B 2014). Hence, it was felt relevant to test this group separately. All JSLE patients were aPL negative, however interestingly they caused a significant increase in cleaved caspase 3, to the same extent as observed with adult aPL positive patients (*chapter 3, figure 3.17*). IgG purification yields a polyclonal population and therefore it is possible that an unidentified subset of IgG is present in both aPL positive adult onset SLE and JSLE patients, that is not present in the aPL negative group. However, it is also possible that another IgG population, yet to be identified in the purified JSLE IgG, targets the same antigen as aPL antibodies. Further experiments are required to explore these possibilities.

Limitations of these experiments include that this simulated I/R injury model gives a restricted view into the kinetics of the protective effect of HCQ and the pathogenic role if IgG from patients. It would be of interest to look at longer time points to see if the effects observed and preserved long term. Additionally, we were unable to access healthy controls which were age matched to our JSLE cohort.

In summary, an *in vitro* simulated I/R injury model has been optimized in H9c2 rat cardiomyoblast cell line and neonatal rat cardiomyocytes. Pre-treatment with the drug HCQ has been shown to offer cardioprotective in these *in vitro* cardiac I/R injury models. Subsequently, experiments have explored the effects of IgG purified from serum of patients with SLE (aPL positive and negative), APS and JSLE. It was observed that all IgG enhanced cleaved caspase-3 when compared to healthy age and gender matched controls. However, IgG from adults who were aPL positive and JSLE patients (who are aPL negative) caused a significantly greater increase in cleaved caspase-3 compared to adults who were aPL negative. The next chapter of this thesis aims to explore potential mechanisms of action for the observations presented in this chapter.

CHAPTER IV

The mechanisms through which HCQ is cardioprotective and IgG purified from SLE, APS and JSLE patients are pathogenic in an *in vitro* simulated cardiac I/R injury model

Overview of Chapter IV

This chapter aims to expand on observations observed when both the rat cardiac H9c2 cell line and neonatal rat cardiomyocytes are exposed to simulated *in vitro* I/R injury after pre-treatment with various the drug HCQ and IgG purified from patients with SLE, APS and JSLE. It focuses on potential mechanisms of action that cause these modulators to have an effect.

The first part will focus on HCQ and how it is cardioprotective in these models of cardiac I/R injury. Specifically, the role of signalling pathways and how inhibition of specific pathways alters the ability of HCQ to be protective.

The second part of this chapter will define specific signalling pathways, which appear to be targeted by pathogenic IgG from SLE, APS and JSLE patients. Inhibitors of target molecules show that the pathogenic effect of these antibodies can be blocked.

4.1 The regulation of kinases in neonatal rat cardiomyocytes when pre-treated with HCQ in simulated I/R injury

4.1.1 Introduction

Confirmation of a cardioprotective role for HCQ via two methods led to subsequent experiments to identify how this effect was being produced. I/R injury consists of multiple pathways and mechanisms, which account for the myocardial injury and cell death observed. It was decided that western blotting would be used to assess the levels of kinases, which have been previously implicated in I/R injury. This included the MAPK family, which consists of a series of serine-threonine kinases that are involved in cell proliferation, differentiation and survival. These kinases have been shown to be heavily implicated in I/R injury in both a pro and anti-apoptotic fashion. Furthermore PI3K-Akt pathway, also implicated in I/R injury in a pro-survival role, was also explored.

4.1.2 ERK phosphorylation is up regulated in neonatal rat cardiomyocytes in simulated I/R injury when pre-treated with HCQ

The ERK 1/2 signalling pathway is a part of the MAPK family and has been shown to play a cardioprotective role in I/R injury by contributing to the RISK pathway, which promotes survival mechanisms to protect against cell death. The levels of this protein were detected using western blotting to evaluate whether HCQ mediates its protective effects through enhancing phosphorylation of the pro-survival kinase ERK 1/2.

ERK 1/2 phosphorylation occurs at low levels in control cells, however is significantly up regulated in reoxygenation due to pro-survival mechanisms being activated. This observed phosphorylation is significantly increased in the presence of HCQ during reoxygenation (*figure 4.1*). In reoxygenation cells p-p44 ERK has a mean of 0.55 (±SD0.12 (n=6)) versus an increase in HCQ treated cells to 0.8 (±SD0.04 (n=6)) relative to total p44 ERK. For p-p42 ERK reoxygenation cells had a level of 0.37 (±SD0.23 (n=6)) versus 0.93 (±SD0.12 (n=6)) relative to total p42 ERK. This suggests that HCQ could be mediating its protective effect via ERK phosphorylation.

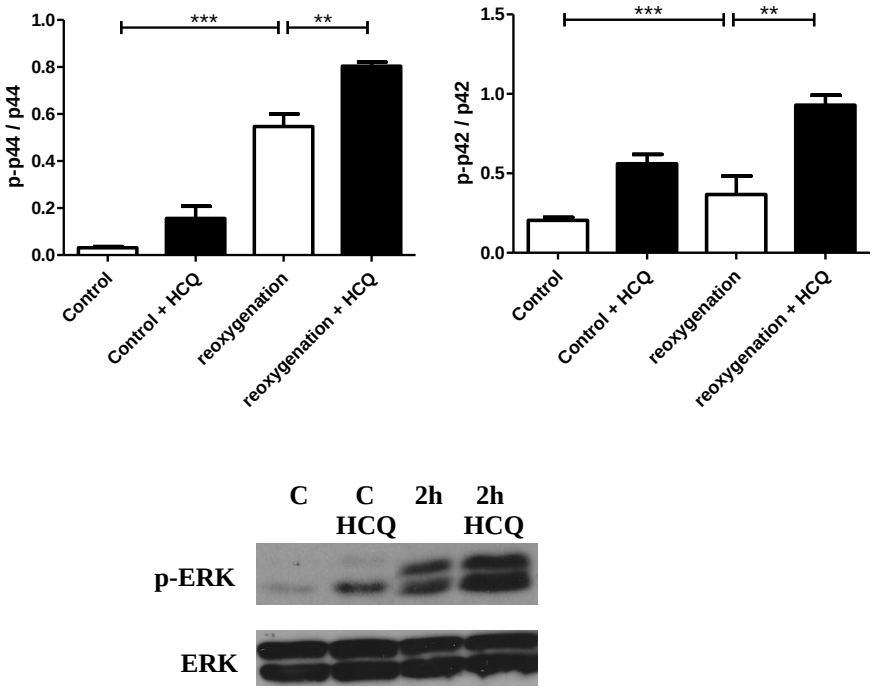


Figure 4.1 HCQ increases the pro-survival kinase ERK 1/2 phosphorylation in neonatal rat cardiomyocytes in simulated I/R injury Cells were pre-treated with 2 µg/mL HCQ and the following day cells were exposed to simulated I/R injury (4h hypoxia + 4h reoxygenation) and the levels of p42/p44 ERK phosphorylation assessed using western blot. Graph shows ±SEM of quantitative analysis from 5 independent experiments. Statistical analysis determined by 1 way ANOVA using post-hoc Tukey to compare all columns (** $p<0.005$, *** $p<0.0005$).

4.1.3 Increasing HCQ concentration increases ERK phosphorylation in neonatal rat cardiomyocytes in simulated I/R injury

The effect of increasing HCQ concentration was also explored to see the effects on ERK 1/2 phosphorylation. Cells were pre-treated with HCQ concentrations ranging from 250 ng/mL to 2 µg/mL prior to simulated I/R injury to identify whether ERK phosphorylation can be increased in a dose-dependent manner.

Western blot analysis showed that with increasing concentration of HCQ there was an increase in ERK 1/2 phosphorylation, further supporting the evidence to suggest HCQ enhances activation (*figure 4.2*).

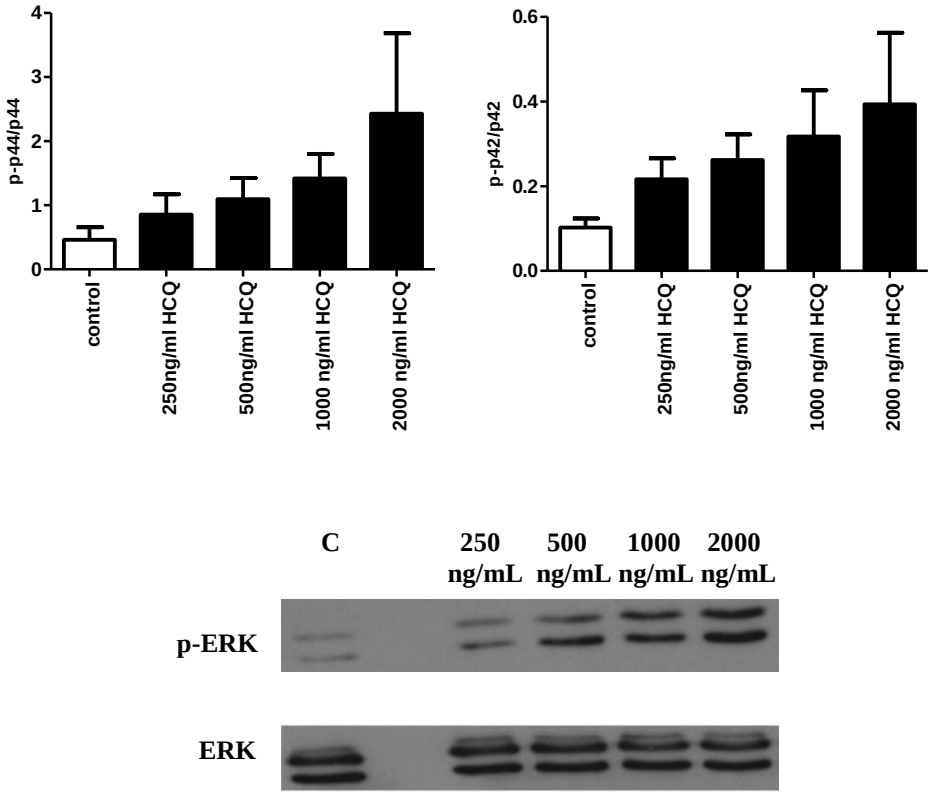


Figure 4.2 HCQ increases the pro-survival kinase ERK 1/2 phosphorylation in a dose-dependent manner in neonatal rat cardiomyocytes in simulated I/R injury Cells were pre-treated with varying concentrations (250 ng/ mL to 2 µg/ mL) HCQ . The following day cells were exposed to simulated I/R injury (4h hypoxia + 4h reoxygenation) and the levels of ERK phosphorylation assessed using western blot. Graph shows ±SEM of quantitative analysis from 3 independent experiments.

4.1.4 Pre-treatment of neonatal rat cardiomyocytes with HCQ does not affect the regulation of Akt, p38 MAPK nor JNK in simulated I/R injury

Other MAPK family members, as well as Akt, a member of the PI3K signaling cascade were assessed by western blot to see if HCQ mediated an effect on their activation also. The MAP kinases p38 MAPK and JNK have been implicated in promoting apoptosis in I/R injury whereas Akt is a pro-survival kinase and therefore prevents it. It was observed that HCQ had no significant effect on levels of other kinases detected by western blotting in simulated I/R injury (*figure 4.3*).

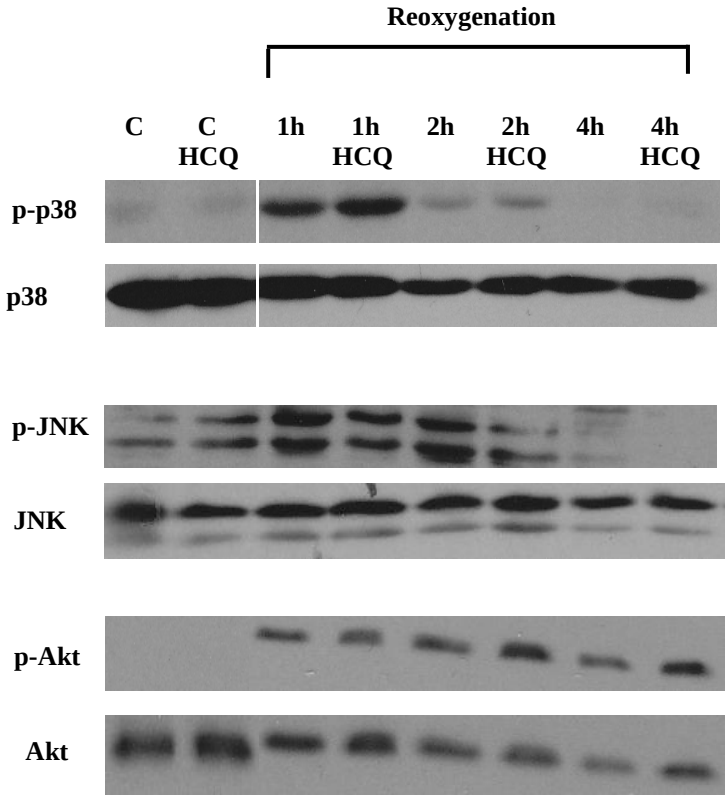


Figure 4.3 HCQ does not significantly alter the phosphorylation levels of other kinases in neonatal rat cardiomyocytes exposed to simulated I/R injury Cells were pre-treated with 2 µg/mL HCQ. The following day cells were exposed to simulated I/R injury (4h hypoxia + 1, 2 or 4h reperfusion) and the levels p-38, JNK and Akt phosphorylation were assessed using western blot (n=1).

4.2 Inhibiting ERK phosphorylation blocks HCQ cardioprotection in neonatal rat cardiomyocytes in simulated I/R injury

4.2.1 Introduction

Due to ERK 1/2 phosphorylation being identified as a potential mechanism through which HCQ could be reducing apoptosis it was decided that subsequent experiments would be performed to inhibit this phosphorylation. This was to see if this protective effect could be reversed and was explored using a commercially available inhibitor (U0126 (Cell Signalling)) which inhibits MEK 1/2, directly upstream of ERK 1/2. An optimisation experiment was done to confirm successful inhibition using two concentrations of the inhibitor; 10 µM and 1 µM. Cells were treated with U0126 for 2 hours prior to simulated I/R injury and the inhibitor was present in media and buffer throughout the experiment.

4.2.2 U0126 inhibits ERK 1/2 phosphorylation in neonatal rat cardiomyocytes

The ERK 1/2 inhibitor U0126 successfully inhibited ERK phosphorylation in simulated I/R injury at a concentration of 10 µM (*figure 4.4*). The inhibitor also enhanced caspase-3 cleavage due to pro-survival mechanisms being blocked, causing further apoptosis to occur. Immunoblots were also probed for other kinases to confirm specificity. The phosphorylation levels of JNK and Akt were unchanged in the presence of the inhibitor.

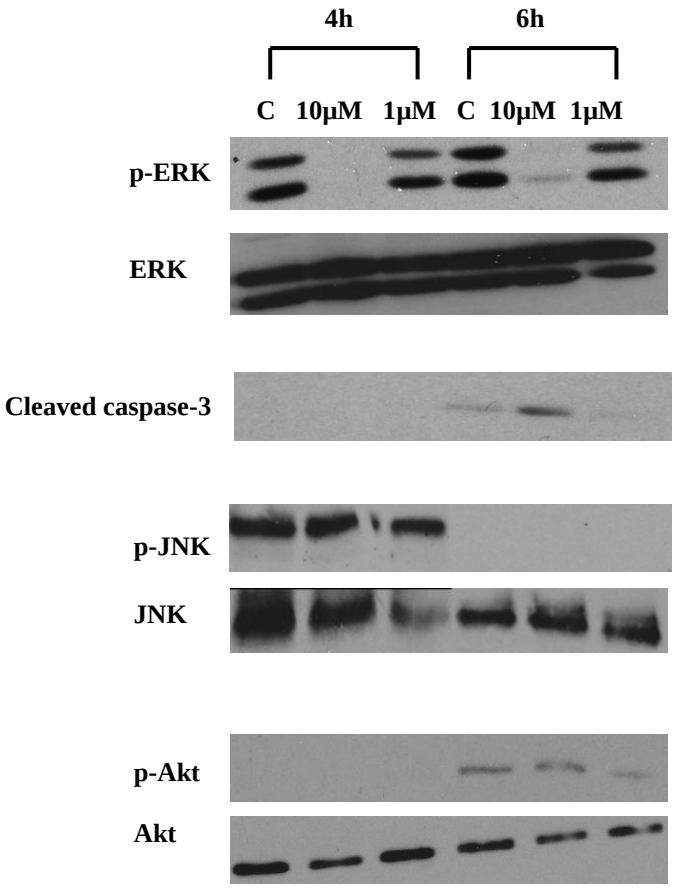
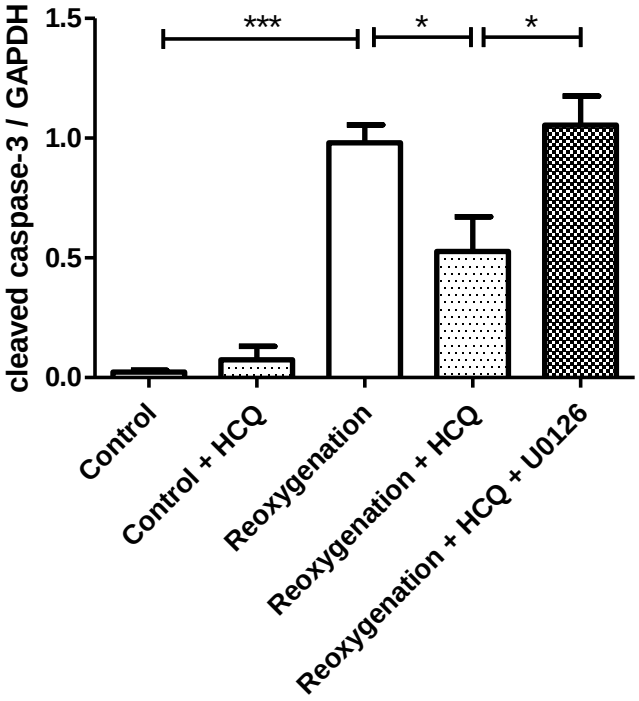


Figure 4.4 U0126 selectively inhibits ERK phosphorylation in neonatal rat cardiomyocytes. Cells were pre-treated for 2h with 1uM or 10uM of the ERK inhibitor U0126 and then exposed to 4hr of hypoxia followed by 2h or 4h reoxygenation in simulated I/R injury. Levels of ERK, JNK and Akt phosphorylation as well as cleaved caspase-3 were assessed using western blot.

4.2.3 Inhibiting ERK phosphorylation reverses the protective effect of HCQ in neonatal cardiomyocytes in simulated I/R injury

Following optimisation an experiment was carried out to see if the presence of U0126 prevented HCQ from having a cardioprotective effect in simulated I/R injury.

Inhibition of ERK 1/2 phosphorylation reverses the cardioprotective effect observed in cells pre-treated with HCQ (*figure 4.5*). The reduction in caspase-3 cleavage observed in the presence of HCQ during reoxygenation (0.53 (±SD0.25 (n=3))) is increased to 1.06 (±SD0.21 (n=3)) when U0126 and HCQ are present. This increase in cleaved caspase-3 restores levels to that seen in reoxygenation alone (0.98 (±SD0.21 (n=3)). This finding further validates the hypothesis that HCQ is cardioprotective in simulated I/R injury and that this mechanism is mediated through upregulation of the pro-survival kinase ERK.



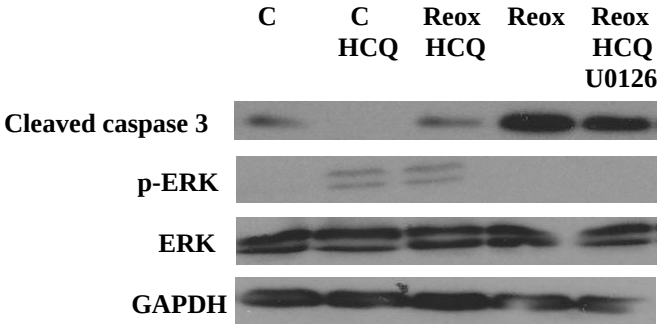


Figure 4.5 The ERK inhibitor U0126 blocks the cardioprotective effect of HCQ in neonatal rat cardiomyocytes in simulated I/R injury Cells were pre-treated with 2 µg/ mL HCQ and the following day incubated with 10 µM U0126 for 2h followed by simulated I/R injury (4h hypoxia + 4h reoxygenation). Graph shows ±SEM of quantitative analysis from 3 independent experiments. Statistical analysis determined by 1 way ANOVA using post-hoc Tukey to compare all columns (* $p<0.05$, *** $p<0.0005$)

4.2.4 The ERK inhibitor U0126 does not inhibit phosphorylation of ERK5

Studies have suggested that the ERK inhibitor U0126 can also inhibit phosphorylation of another kinase, ERK5 which has also been shown to have protective effects in I/R injury (Nishimoto 2006). Levels of ERK 5 phosphorylation were therefore also explored to rule out its involvement in the protective effects mediated by HCQ. Results showed no difference in phosphorylation of ERK5 in the presence of the U0126 inhibitor and HCQ suggesting that it plays no role in the protective effects observed (*figure 4.6*).

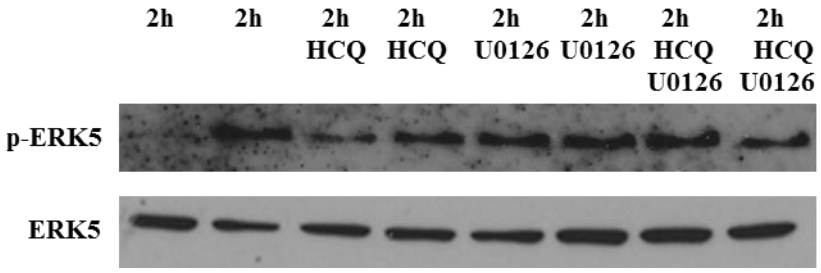


Figure 4.6 ERK5 phosphorylation is not altered in neonatal rat cardiomyocytes in simulated I/R injury Cells were pre-treated with 2 μg/ mL HCQ and the following day incubated with 10 μM U0126 for 2h followed by simulated I/R injury (4h hypoxia + 4h reoxygenation) (n=2).

4.3 Pre-treatment of neonatal rat cardiomyocytes with HCQ targets intrinsic pathways in simulated I/R injury

4.3.1 Introduction

Upon identifying that HCQ protects in this *in vitro* model of cardiac I/R simulated injury through inhibiting cleavage of caspase-3 and identifying a role or ERK phosphorylation, potential molecular pathways through which this may occur were explored. There are two main pathways that lead to cleavage of caspase-3, the extrinsic and intrinsic. Key proteins in these pathways were therefore identified and differences were explored to identify a specific signaling pathway through which protection is occurring.

4.3.2 Pre-treatment of neonatal rat cardiomyocytes with HCQ inhibits cleaved caspase-9 in simulated I/R injury

The two main pathways of apoptosis are regulated by different execution caspases, the extrinsic by caspase-8 cleavage and the intrinsic by caspase-9 cleavage. It would be expected that if HCQ is targeting an ERK 1/2 dependent mechanism that this would result in reductions in the intrinsic pathway (i.e. caspase-9) due to the RISK pathway targeting Bcl-2 family members (Hausenloy 2004). Cells pre-treated overnight with HCQ and then exposed to simulated I/R injury showed reduced cleavage of caspase-9 when compared with control cells (*figure 4.7a*). Levels of caspase-9 in cells exposed to reoxygenation had a mean of 0.79 (±SD0.23) (n=3)), which was reduced to 0.44 (±SD0.27 (n=3)) in HCQ treated cells. However, there was no difference in levels of cleaved caspase-8 (*figure 4.7b*), suggesting that protection is via an intrinsic signaling pathway.

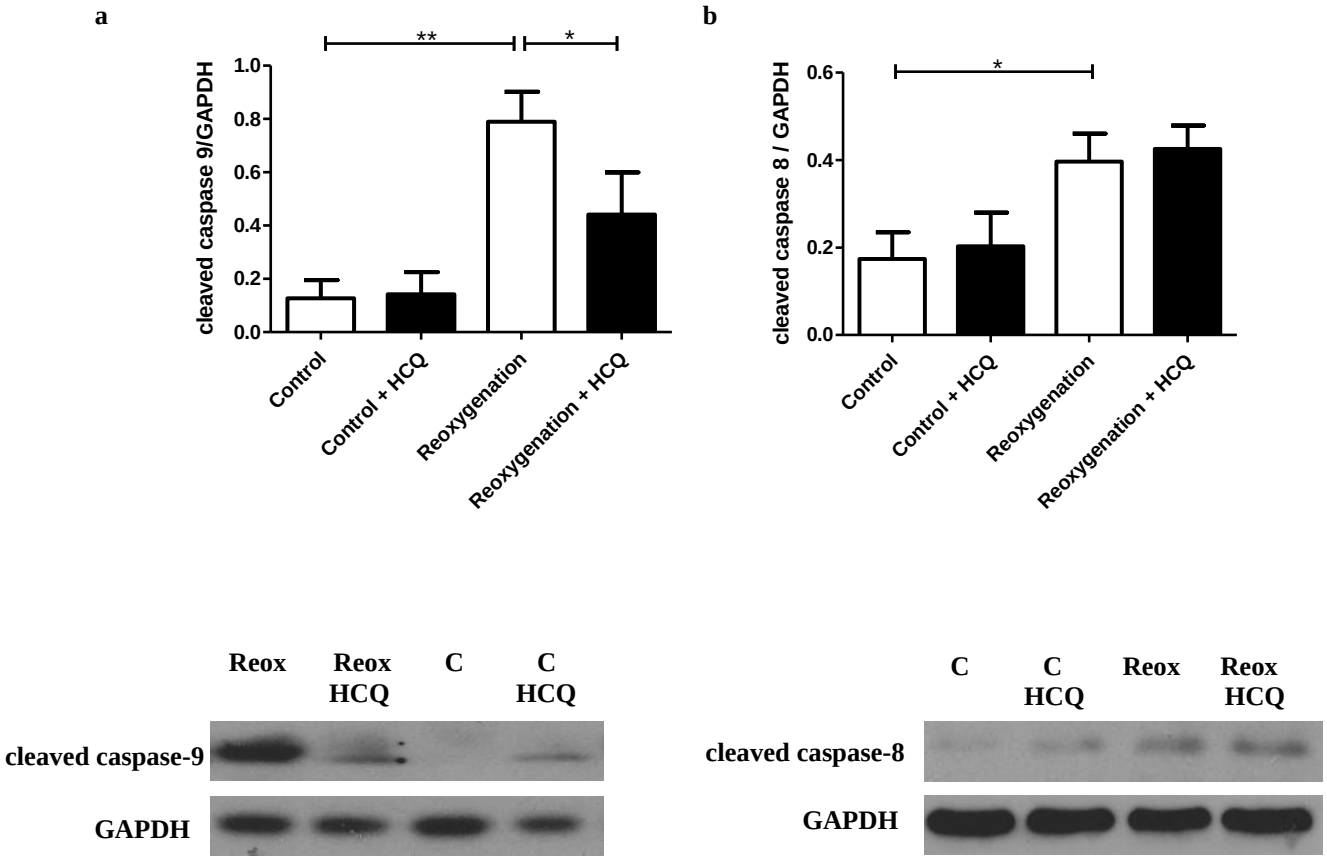


Figure 4.7 HCQ reduces cleaved caspase 9 in neonatal rat cardiomyocytes in simulated I/R injury Cells were pre-treated with 2 µg/ mL HCQ and the following day exposed simulated I/R injury (4h hypoxia + 4h reoxygenation). Cleaved caspase-9 (a) and cleaved caspase-8 (b) were assessed by western blot. Graph shows ±SEM of quantitative analysis from 3 independent experiments. Statistical analysis determined by 1 way ANOVA using post-hoc Tukey to compare all columns (* $p<0.05$, ** $p<0.005$)

4.3.3 Pre-treatment of neonatal rat cardiomyocytes with HCQ blocks dephosphorylation of BAD at serine 112

Upon identifying changes in caspase-9 cleavage proteins which could connect enhancement of ERK phosphorylation to inhibition of caspase-9 cleavage were explored. The pro-apoptotic protein BAD, which is a key regulator of apoptotic proteins such as the Bcl family, was identified. BAD is activated upon dephosphorylation at two main serine residues; 112 and 136. They are regulated independently of each other, by ERK and Akt respectively. It was therefore hypothesized that if HCQ truly acted through an ERK dependent manner then serine 112 de-phosphorylation would be blocked in the presence of HCQ. In cells pre-treated with HCQ de-phosphorylation of BAD was prevented at serine-112 but not serine-136 (*figure 4.8*). This suggests that HCQ is cardioprotective through increased ERK phosphorylation, which in turn blocks dephosphorylation of the pro-apoptotic protein BAD at serine-112 making it inactive. This subsequently prevents BAD from interacting with and inactivating pro-survival proteins such as Bcl-2/Bcl-$2_{XL.}$ This allows these survival proteins to prevent Bax/Bak triggered apoptosis, therefore reducing cleaved caspase-9 (Gross A 1999).

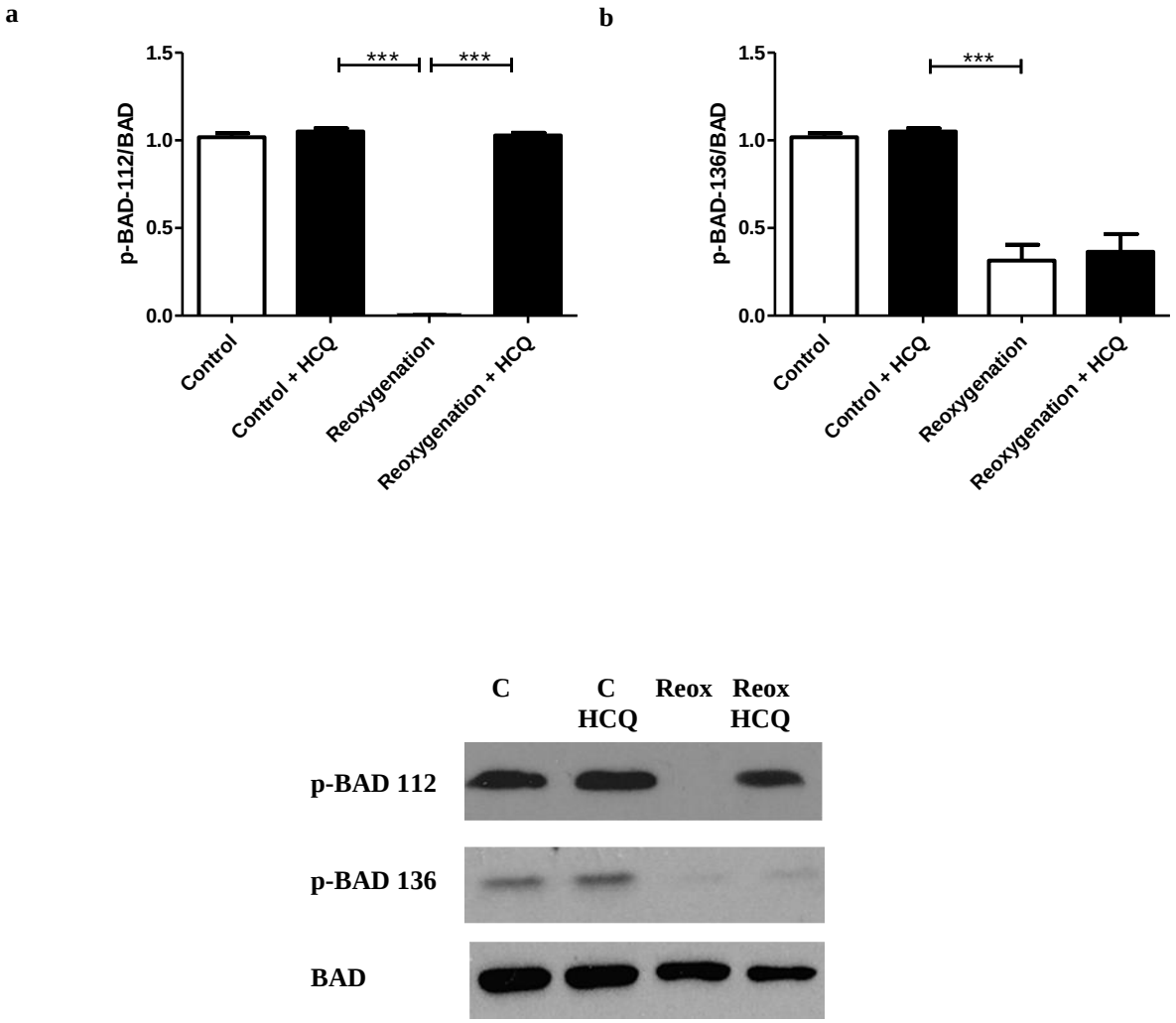


Figure 4.8 HCQ blocks de-phosphorylation of serine 112 but not serine 136 of BAD in neonatal rat cardiomyocytes in simulated I/R injury Cells were pre-treated with 2 µg/ mL HCQ and the following day exposed to simulated I/R injury (4h hypoxia + 4h reoxygenation). Levels of phosphorylation at serine 112 (a) and serine 136 (b) were assessed by western blot. Graph shows ±SEM of quantitative analysis from 3 independent experiments. Statistical analysis determined by 1 way ANOVA using post-hoc Tukey to compare all columns (*** $p<0.0005$)

4.4 HCQ is most protective when cells are pre-treated and incubated with HCQ throughout simulated I/R injury

4.4.1 Introduction

It is of translational importance to explore whether it is necessary for HCQ to be present throughout the experiment in order to be protective or whether administering it during the simulated ischaemic stage or during simulated reperfusion is just as beneficial.

4.4.2 HCQ abrogates cleavage of caspase-3 in neonatal rat cardiomyocytes only when cells are pre-treated with the drug and it is present throughout simulated I/R injury

Cells were incubated with HCQ at different stages of the experimental protocol and exposed to simulated I/R injury as shown in *figure 4.9a.* Maximal inhibition of cleaved caspase-3 with HCQ was observed when HCQ was present throughout the experiment.

4.4.3 Maximal enhancement of ERK phosphorylation in neonatal rat cardiomyocytes occurs when cells are pre-treated with HCQ and it is present throughout simulated I/R injury

Additionally when cells were pre-treated with HCQ at different stages and exposed to simulated I/R injury, maximal enhancement of ERK phosphorylation was observed when the drug was present throughout the experiment (*figure 4.9b*).

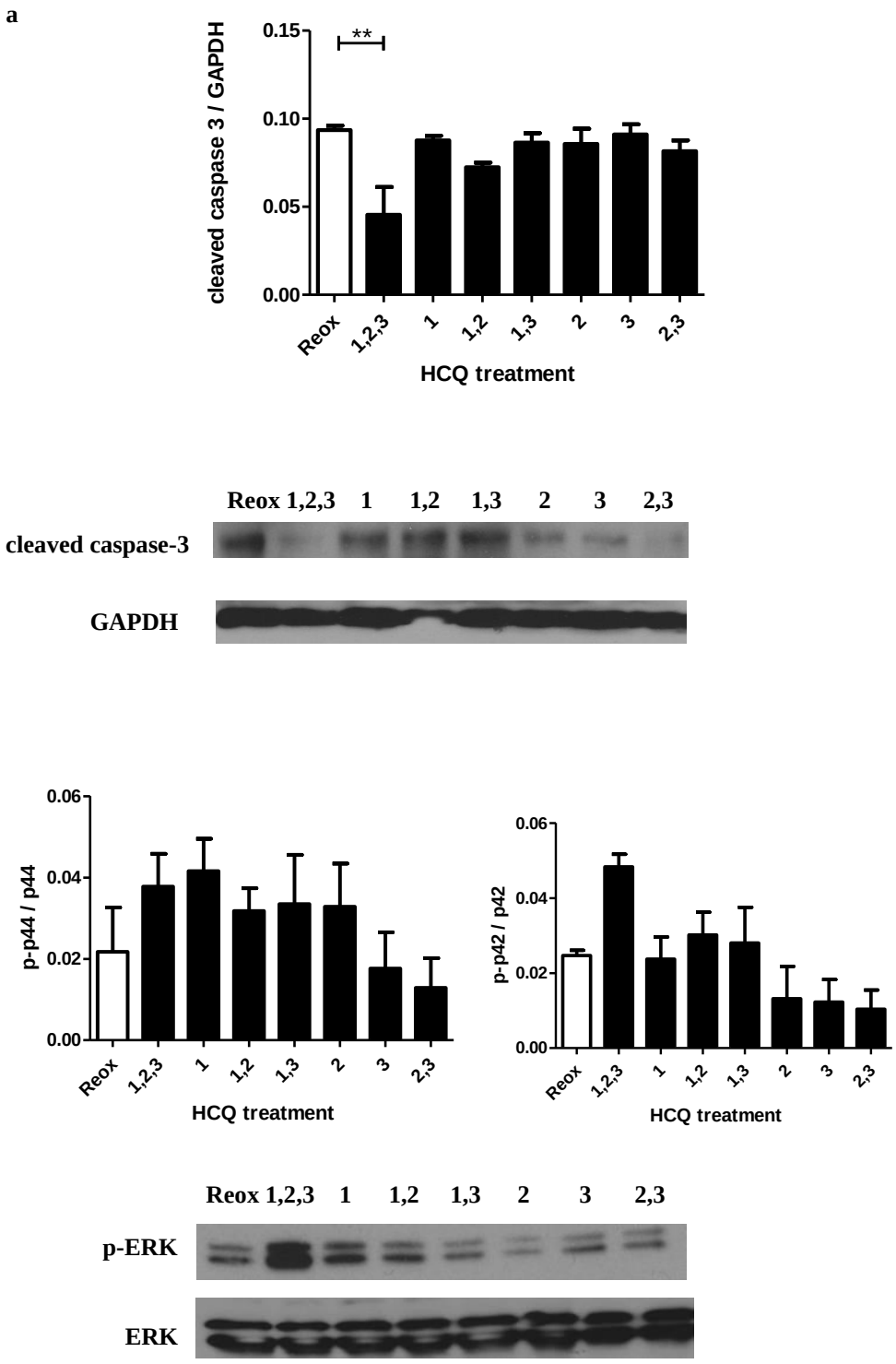


Figure 4.9 HCQ inhibits cleavage of caspase-3 and increases ERK phosphorylation in neonatal rat cardiomyocytes most effectively when cells are pre-treated with the drug and it is present throughout I/R injury Cells exposed to simulated I/R injury (4h hypoxia + 4h reoxygenation) were treated with 2 µg/mL HCQ at various times; 1-pre-treatment overnight, 2-treatment during simulated ischaemia, 3-treatment during simulated reperfusion. Western blotting was used to assess levels of cleaved caspase-3 and ERK phosphorylation. Graph shows ±SEM of quantitative analysis from 4 independent experiments. Statistical analysis determined by 1 way ANOVA using post-hoc Tukey to compare all columns (** $p<0.005$).

4.5 HCQ increases Lc3b-II accumulation in neonatal rat cardiomyocytes in simulated I/R injury

4.5.1 Introduction

There is little evidence in the literature as to the mechanism of action of HCQ, however it is known to inhibit autophagy through alteration of the lysosome pH, thus inhibiting lysozomal function (Fox 1993). This causes a build-up of autophagosomes with scaffold proteins such as Lc3b-II remaining attached to their membrane. Recent studies have shown that Lc3b-II accumulation is linked to enhanced ERK 1/2 phosphorylation (Martinez-Lopez N. Athonvarangkul D 2013).

4.5.2 HCQ increases Lc3b-II in neonatal rat cardiomyocytes in simulated I/R injury

When cells are pre-treated with HCQ overnight and subsequently exposed to simulated I/R injury levels of Lc3b-II accumulation are significantly increased (*figure 4.10*). This suggests that HCQ could be inducing cardioprotection via its ability to inhibit lysozomal uptake of atuophagosomes leading to their accumulation (as demonstrated through Lc3b-II accumulation in the presence of HCQ) thus providing a scaffold for ERK phosphorylation.

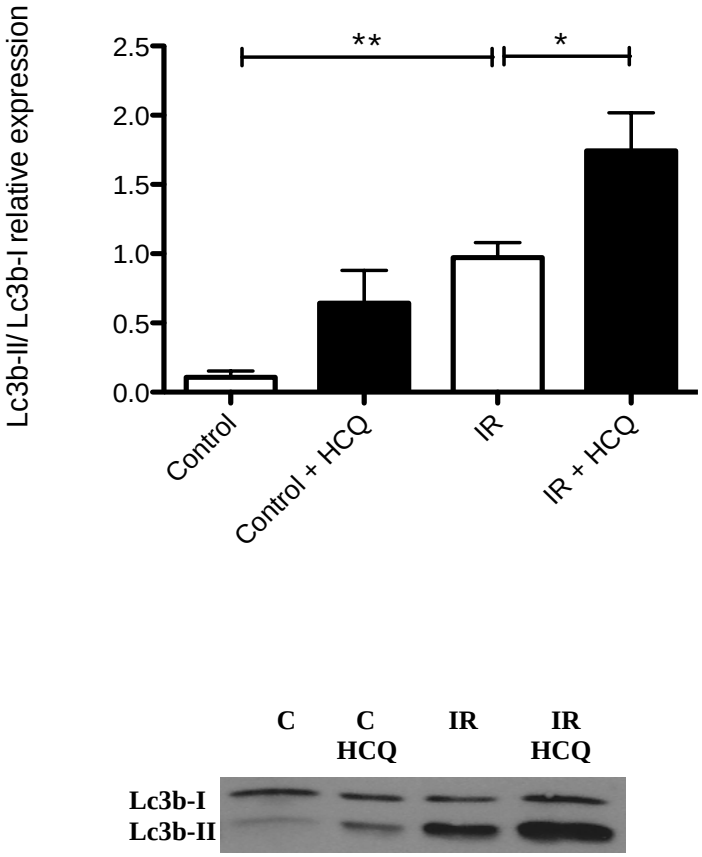


Figure 4.10 HCQ increases Lc3b-II in neonatal rat cardiomyocytes in simulated I/R injury Cells were pre-treated with 2 µg/ mL HCQ and the following day exposed to simulated I/R injury (4h hypoxia + 4h reoxygenation). Graph shows ±SEM of quantitative analysis from 6 independent experiments. Statistical analysis determined by 1 way ANOVA using post-hoc Tukey to compare all columns (* $p<0.05$, ** $p<0.0005$)

4.6. IgG purified from patients who are aPL positive increases p38 MAPK phosphorylation in neonatal rat cardiomyocytes exposed to simulated I/R injury

4.6.1 Introduction

Confirmation of a pathogenic role for IgG purified from SLE patients via two methods led to subsequent experiments to identify how this effect was being mediated. I/R injury consists of multiple pathways and mechanisms, which account for the myocardial injury and cell death observed. It was decided that western blotting would be used to assess the levels of kinases previously implicated in I/R injury, such as the MAPK family. This family is made of a series of serine-threonine kinases, which are involved in cell proliferation, differentiation and survival. These kinases have been shown to be heavily implicated in I/R injury in both a pro and anti-apoptotic fashion. Furthermore the PI3K-Akt pathway, also has a pro-survival role in I/R injury and therefore was also explored.

4.6.2 Pre-treatment of neonatal rat cardiomyocytes with IgG purified from patients who are aPL positive causes increased p38 MAPK phosphorylation compared with aPL negative patient samples in simulated I/R injury

When cells are pre-treated with IgG from SLE patients, different responses to levels in p38 MAPK phosphorylation are observed depending on the aPL state of the patient. IgG from patients with aPL caused enhanced phosphorylation of p38 MAPK mitogen activated protein kinases (p38 MAPK-MAPK), whereas IgG without aPL had no such effect (*figure 4.11*). Levels of p-p38 MAPK in aPL positive treated cells had a mean of 0.15 (±SD0.03 (n=3)), which is decreased to 0.005 (±SD0.002 (n=3)) in cells treated with IgG from aPL negative patients.

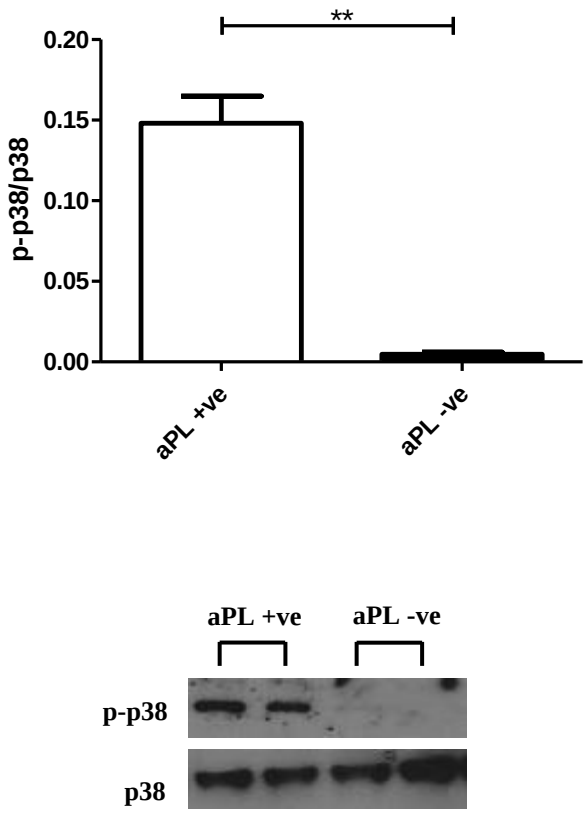


Figure 4.11 IgG purified from aPL positive patients increases the pro-apoptotic kinase p-38 MAPK phosphorylation in neonatal rat cardiomyocytes in simulated I/R injury Cells were pre-treated with 2 µg/mL HCQ. The following day cells were exposed to simulated I/R injury (4h hypoxia + 4h reoxygenation) and the levels of p38 MAPK phosphorylation assessed using western blot. Graph shows ± SEM of quantitative analysis from 4 aPL +ve and 4 aPL -ve patients. Statistical analysis determined by unpaired t-test (** p=0.0010).

4.6.3 Pre-treatment of neonatal rat cardiomyocytes with IgG purified from patients that are aPL positive show no difference in ERK or Akt phosphorylation compared with aPL negative patients when exposed to simulated I/R injury

No differences were seen with other pathways known to be involved in the regulation of I/R injury such as ERK 1/2 and Akt (*figure 4.12*).

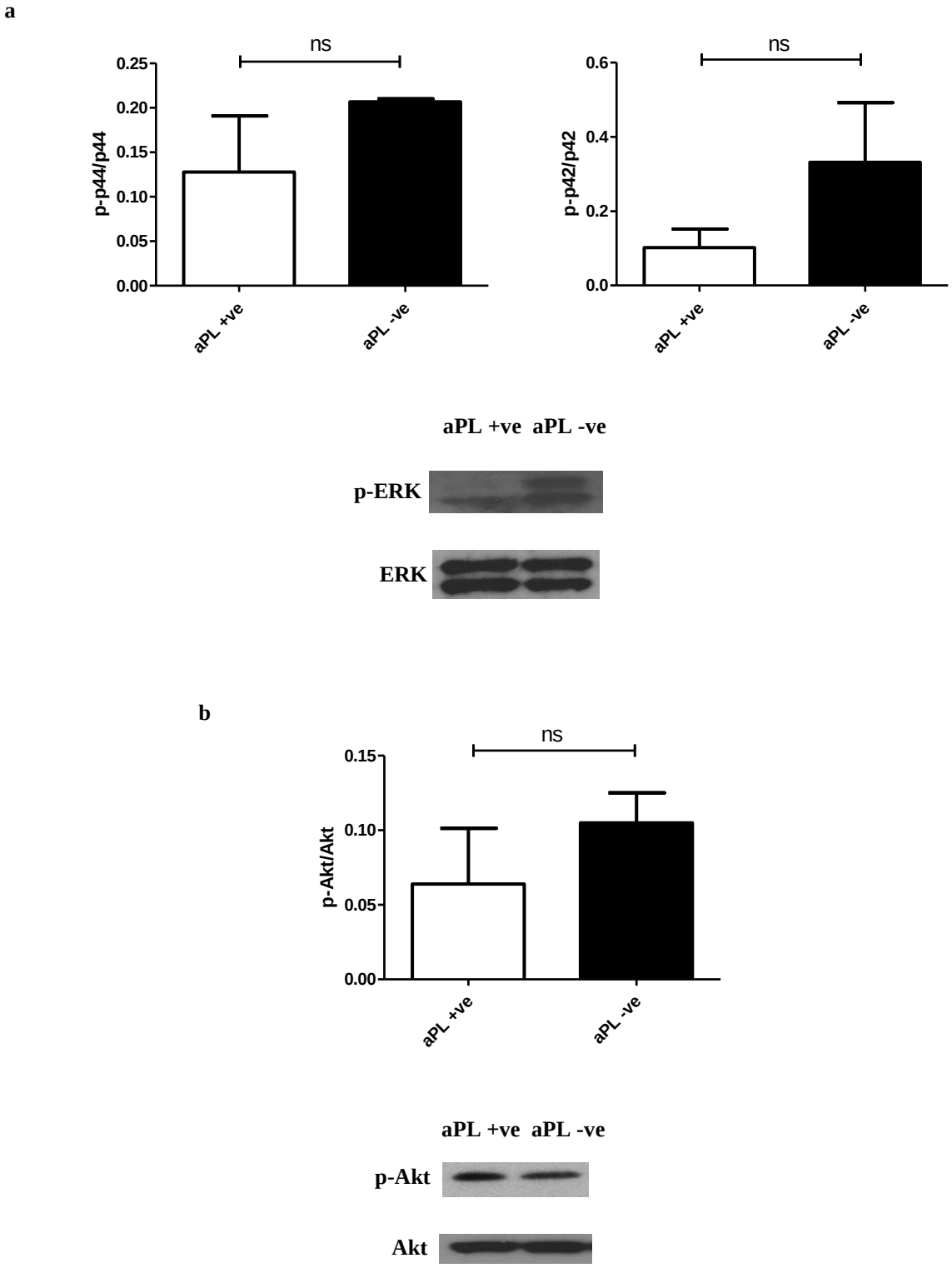


Figure 4.12 IgG purified from aPL positive patients doesn't alter other kinase phosphorylation levels in neonatal rat cardiomyocytes in simulated I/R injury Cells were pre-treated with 2 µg/mL HCQ. The following day cells were exposed to simulated I/R injury (4h hypoxia + 4h reoxygenation) and the levels of ERK 1/2 (a) and Akt (b) phosphorylation assessed using western blot. Graph shows ± SEM of quantitative analysis from 4 aPL +ve and 4 aPL negative patients. Statistical analysis determined by unpaired t-test.

4.7 Inhibiting p38 MAPK phosphorylation abrogates cleavage of caspase-3 in neonatal rat cardiomyocytes pre-treated incubated with aPL positive patient samples and exposed to simulated I/R injury

4.7.1 Introduction

Due to p38 MAPK phosphorylation being identified as a potential mechanism through which IgG from aPL positive patients could be enhancing apoptosis it was decided that subsequent experiments would be performed to inhibit this phosphorylation. This was to see if the pathogenic effect of aPL positive IgG could be reversed and was explored using a commercially available inhibitor (SB203580 (Cell Signalling)) which specifically inhibits p38 MAPK phosphorylation. An optimisation experiment was performed to confirm successful inhibition using 10 μM of the inhibitor. Cells were treated with SB208350 for 2 hours prior to simulated I/R injury and the inhibitor was present in media and buffer throughout the experiment.

4.7.2 p38 MAPK inhibitor optimisation

The p38 MAPK inhibitor SB203580 successfully inhibited p38 MAPK phosphorylation in simulated I/R injury at a concentration of 10 μM (*figure 4.13*). Successful inhibition is detected through a decrease in phosphorylation of MAPKAP-2, downstream of p-38 activation. This is due to the inhibitor blocking p38 activation by binding the ATP-binding pocket of p38 MAPK, but it does not block other kinases from phosphorylating p38. The inhibitor decreased caspase-3 cleavage due to pro-death pathways being blocked. Immunoblots were also probed for other kinases to confirm specificity. The phosphorylation levels of ERK and Akt were unchanged in the presence of the inhibitor.

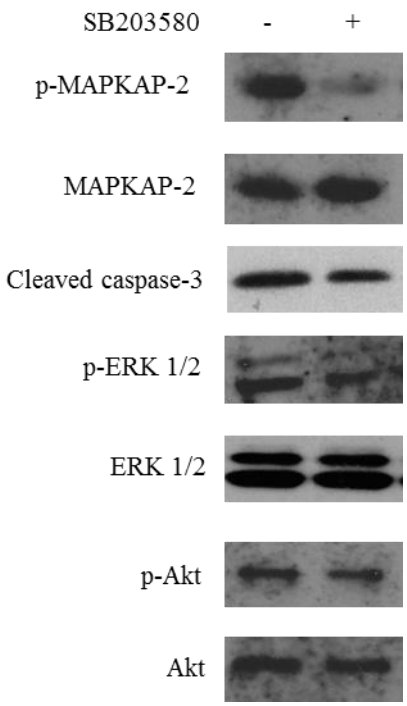


Figure 4.13 Optimisation of the p38 MAPK inhibitor SB203580 Cells were pre-treated with 10 μM SB203580 and the following day exposed to simulated I/R injury (4h hypoxia + 2h reoxygenation) and the levels of MAPKAP, ERK and Akt phosphorylation as well as cleaved caspase-3 were assessed using western blot (n=1).

4.7.3 Pre-treatment of neonatal rat cardiomyocytes with IgG from aPL positive patients in the presence of the p38 MAPK inhibitor SB203580 inhibits cleavage of caspase-3 when exposed to simulated I/R injury

Following optimization, an experiment was carried out to see if the presence of SB203580 prevented aPL positive IgG from having a pathogenic effect in simulated I/R injury.

Inhibition of p38 MAPK phosphorylation blocked the pathogenic effect observed in cells pre-treated with aPL +ve IgG (*figure 4.14*). The enhancement in caspase-3 cleavage observed in the presence of aPL positive IgG during reoxygenation was decreased by 52.81% (±SD14.46 (n=4)) when SB208350 and aPL positive IgG were present. In the presence of IgG from aPL negative patients the decrease in caspase-3 cleavage observed was significantly lower at 5.68% (±SD11.36 (n=4))(p=0.0022). This finding further validates the hypothesis that aPL poistive IgG is more pathogenic in simulated I/R injury that aPL negative IgG. Furthermore, enhanced pathogenicity in aPL poistive IgG is mediated through upregulation of the pro-apoptotic kinase p38 MAPK.

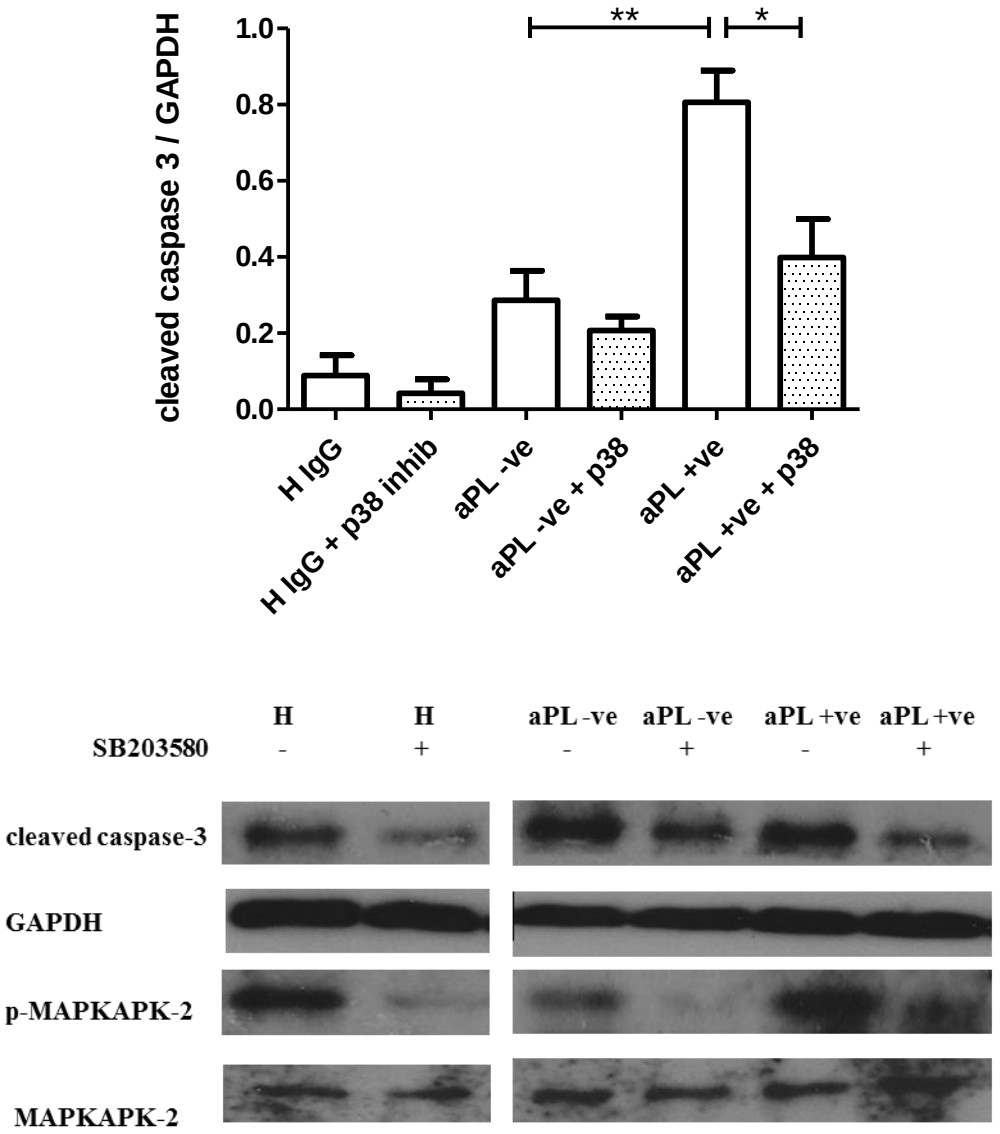


Figure 4.14 In the presence of the p38 MAPK inhibitor SB203580 IgG purified from aPL positive SLE patients cannot enhance cleaved caspase-3 in neonatal rat cardiomyocytes in simulated I/R injury Cells were pre-treated with 10 μM SB203580 and 2h later 500 μg/mL IgG. The following day cells were exposed to simulated I/R injury (4h hypoxia + 2h reoxygenation) and the levels of cleaved caspase-3 were assessed using western blot. Graph shows ±SEM of quantitative analysis from 3 healthy volunteers, 4 aPL +ve and 4 aPL –ve patients. Statistical analysis determined by unpaired t-test (* p=0.0256, ** p=0.0038).

4.8 IgG purified from patients with JSLE enhances p38 MAPK phosphorylation in neonatal rat cardiomyocytes exposed to simulated I/R injury

4.8.1 Introduction

Confirmation of a pathogenic role for IgG purified from JSLE patients, due to an increase in cleaved caspase-3, led to subsequent experiments to identify how this effect was being mediated. I/R injury consists of multiple pathways and mechanisms, which account for the myocardial injury and cell death observed. It was decided that western blotting would be used to assess the levels of the MAPK family, which have been previously implicated in I/R injury.

4.8.2 Pre-treatment of neonatal rat cardiomyocytes with IgG purified from patients with JSLE (aPL negative) have increased p38 MAPK and decreased Akt phosphorylation compared to adult aPL negative patients when exposed to simulated I/R injury

When cells are pre-treated with IgG from JSLE patients, there is enhanced p38 MAPK phosphorylation and decreased Akt phosphorylation. IgG from JSLE patients had a similar response to that of adult onset SLE who are aPL positive for phosphorylation of p38 MAPK (*figure 4.15*). However, JSLE IgG also appeared to decrease phosphorylation of Akt, a pro-survival kinase, albeit not as significantly as the alteration in p38 MAPK phosphorylation.

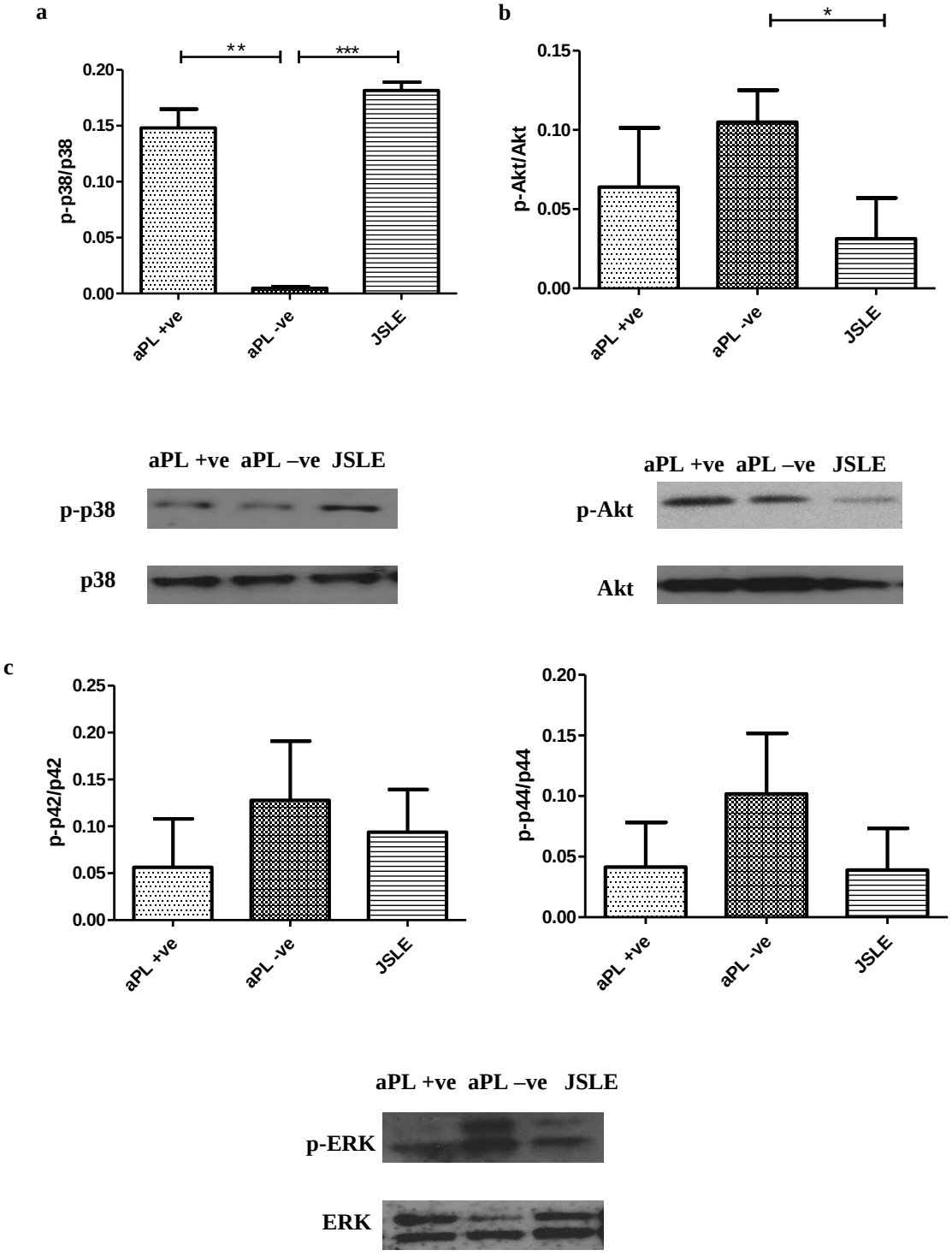


Figure 4.15 IgG purified from JSLE patients increases p38 MAPK phosphorylation and decreases Akt kinase phosphorylation in neonatal rat cardiomyocytes in simulated I/R injury Cells were pre-treated with 500 µg/mL IgG. The following day cells were exposed to simulated I/R injury (4h hypoxia + 4h reoxygenation) and the levels of p38 MAPK (a), Akt (b) and ERK 1/2 (c) phosphorylation assessed using western blot. Graph shows ±SEM of quantitative analysis from 4 aPL +ve, 4 aPL –ve and 3 JSLE patients. Statistical analysis determined by 1 way ANOVA using post-hoc Tukey to compare all columns (* $p<0.05$, *** $p<0.0005$)

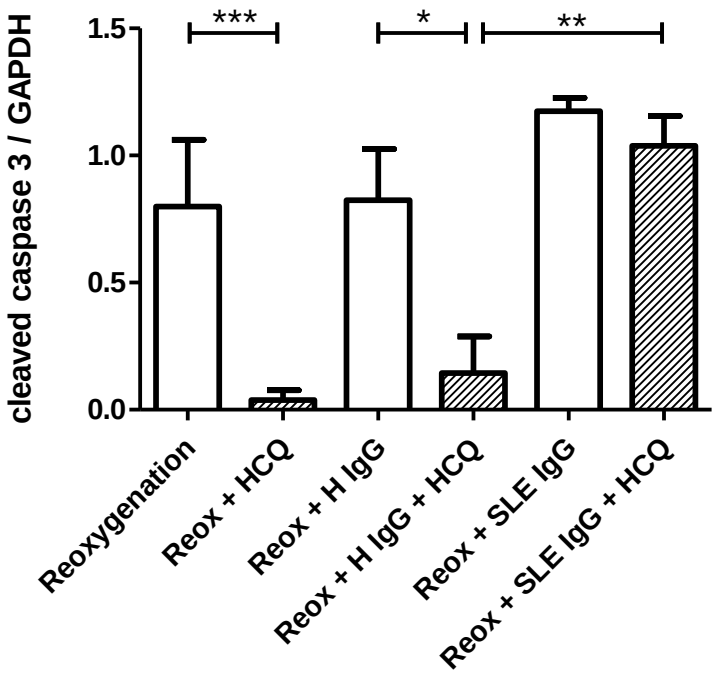
4.9 Pathogenic IgG prevents HCQ from being cardioprotective in neonatal rat cardiomyocytes in simulated I/R injury

4.9.1 Introduction

Whilst SLE patients treated with HCQ have been shown to be less susceptible to suffering a MI, many still do and mortality and morbidity remains high in these patients. Experiments were therefore performed to explore whether the protection shown by HCQ is altered in the presence of IgG purified from SLE patients.

4.9.2 Pre-treatment of neonatal rat cardiomyocytes with IgG from SLE patients blocks HCQ from reducing cleaved caspase-3 in simulated I/R injury

The five most pathogenic IgG (from aPL positive) SLE patients from previous experiments (i.e. increased cleaved caspase-3 the most) were selected and pooled. Neonatal rat cardiomyocytes were pre-treated overnight with HCQ and IgG and the following day exposed to simulated I/R injury. Five healthy control samples were also pooled and incubated with and without HCQ. In the presence of IgG purified from SLE patients HCQ was unable to inhibit cleaved caspase-3, however in the presence of IgG from healthy age and gender matched controls it was shown to still be cardioprotective (*figure 4.16*).



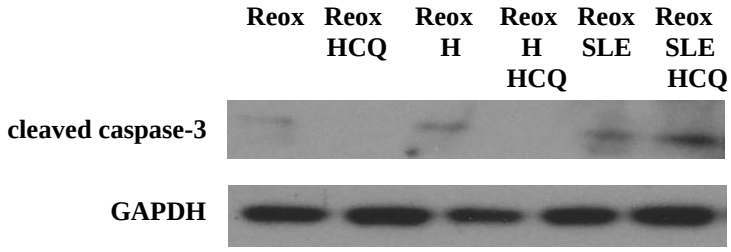


Figure 4.16 IgG purified from aPL positive SLE patients increases HCQ protective effects in neonatal rat cardiomyocytes in simulated I/R injury Cells were pre-treated with 2 μg/mL HCQ and 500 μg/mL IgG pooled from 5 healthy or aPL positive SLE patients. The following day cells were exposed to simulated I/R injury (4h hypoxia + 4h reoxygenation) and the levels of cleaved caspase-3 were assessed using western blot. Graph shows ±SEM of quantitative analysis from 3 independent experiments. Statistical analysis determined by 1 way ANOVA using post-hoc Tukey to compare all columns (* $p<0.05$, ** $p<0.005$, *** $p<0.0005$)

4.10 Discussion

Due to evidence suggesting that HCQ has a protective effect in simulated I/R injury, the next step was to identify potential mechanisms through which this may be occurring. It was decided that different signalling pathways would be explored that are known to play a prominent role in I/R injury. The MAPK family are a group of serine-threonine kinases involved in cell proliferation, survival and differentiation (Cobb 1999). Within I/R injury it is thought that they play both a pro- and anti-apoptotic role in a kinase specific manner. For example, p38 MAPK and JNK have been implicated in enhancing apoptosis-induced cell death in I/R injury. In contrast ERK 1/2, another MAPK, has been shown to induce pro-survival mechanisms when activated in I/R injury (Yue T 2000). ERK 1/2 has also be shown to play a prominent role in the RISK pathway, which consists of pro-survival mechanisms that are activated in the early stages of reperfusion to try and counteract the damaging effects of I/R injury (Davidson S 2006). Another member of this RISK pathway is the PI3K-Akt pathway which has been shown to have protective effects by phosphorylating target proteins such as GSK-3β, endothelial nitric oxide and pro-apoptotic Bcl-2 family member BAD (Tong H 2002). Western blotting was used to determine whether HCQ elucidated its cardioprotection through altered regulation of these various kinases. It was identified that ERK 1/2 phosphorylation was significantly enhanced in the presence of HCQ in simulated I/R injury (*chapter 4, figure 4.1*). This was interesting due to it previously being published that ERK 1/2 is activated in response to ischaemia in the early stages of reperfusion to counteract apoptotic pathways which have been induced (Yue T 2000). This finding was confirmed when levels of ERK 1/2 phosphorylation were investigated in the presence of increasing concentrations of HCQ. It was detected that as the concentration of HCQ was increased, ERK 1/2 phosphorylation was up regulated to a maximum concentration of 2 µg/ml of HCQ (*chapter 4, figure 4.2*). Phosphorylation levels of other kinases were detected, but no significant differences were observed in the presence of HCQ in simulated I/R injury (*chapter 4, figure 4.3*).

Following the finding that HCQ enhanced ERK 1/2 phosphorylation a commercially available ERK 1/2 inhibitor (U0126) was purchased. Optimisation experiments were carried out to ensure successful inhibition of ERK 1/2 could be achieved. As shown by previous studies, 10 µM U0126 induced complete inhibition of ERK 1/2 (Frias MA 2007) when cells were incubated 1 hour prior to simulated I/R injury (*chapter 4, figure 4.4*).

Optimisation experiments with the U0126 inhibitor showed that enhanced caspase-3 cleavage was seen in the presence of the inhibitor alone suggesting that ERK 1/2 activation occurs in simulated

reperfusion to act as a regulator of apoptosis. Protein lysates from this experiment were also used to confirm that the inhibitor was specific to ERK 1/2 inhibition. Other MAPK family members including JNK and p38 MAPK as well as the pro-survival kinase Akt detected no changes in phosphorylated levels in the presence of HCQ (*chapter 4, figure 4.4*).

Following optimisation, an experiment was performed to identify if in the presence of an ERK 1/2 inhibitor (U0126) HCQ's cardioprotective effects were reversed. It was observed that in simulated I/R injury caspase-3 cleavage was induced and as shown previously in *chapter 4, figure 4.1* this was reduced in cells pre-treated with HCQ. However, when cells were incubated with both HCQ and the ERK inhibitor the inhibition of caspase-3 cleavage with HCQ was no longer observed (*chapter 4, figure 4.5*). This experiment therefore suggests that HCQ is mediating its cardioprotective effect at least in part through up-regulation of the anti-apoptotic kinase ERK 1/2.

An additional control experiment was performed due to a recent publication suggesting that HCQ enhances another kinase, ERK5 and this has been shown to have a vasoprotective effect in endothelial cells (Le N 2014). Additionally, previous studies have suggested that U0126 may also inhibit the MEK5/ERK5 pathway (Nishimoto 2006) and therefore it was possible that HCQ could be exerting its protective effect instead via ERK5. It was found that in simulated I/R injury there was no difference in levels of ERK5 phosphorylation in the presence of HCQ and furthermore no alteration in ERK5 phosphorylation in the presence of the inhibitor U0126 (*chapter 4, figure 4.6*). This therefore excluded the possibility of an MEK5/ERK5 mediated protective mechanism, although it should be noted that this experiment is n=2 and further validation should be done.

ERK 1/2 protects against apoptosis through a number of target molecules downstream, including the pro-apoptotic Bcl-2 family member BAD. The pro-apoptotic activity of BAD is regulated through its phosphorylation at serine-112 and serine-136. Studies have shown that ERK phosphorylation specifically regulates serine-112 and Akt is responsible for phosphorylation of serine-136 (Xianjun F 1999). Results showed that in untreated cells, simulated I/R injury causes dephosphorylation of BAD at both serine-112 and serine-136, in order to activate BAD. However, in the presence of HCQ, dephosphorylation of serine-112 is blocked (*chapter 4, figure 4.7*) due to the increased ERK phosphorylation previously shown. No difference was observed in the de-phosphorylation of serine 136 in the presence of HCQ, therefore further supporting the concept that HCQ protection is mediated through ERK.

Following on from this observation one would expect that HCQ is protective through regulation of cleaved caspase-9. As previously described, there are two main pathways through which apoptosis

occurs; the extrinsic and intrinsic. The extrinsic pathway leads to cleavage of caspase-8 and the intrinsic cleavage of caspase-9, with subsequent merger into combined cleavage of caspase-3. BAD dephosphorylation has been shown to directly activate the opening of the mitochondrial permeability transition pore (MPTP), a hallmark of the intrinsic pathway (Kinnally K 2011). Therefore, one would hypothesis that inactive BAD would lead to a reduction in MPTP opening and subsequently reduced cleavage of capsase-9. Experiments showed that indeed in the presence of HCQ there was a reduction in cleaved caspase-9, but not caspase-8 when cells were exposed to simulated I/R injury (*chapter 4, figure 4.8*).

Further *in vitro* data suggests that HCQ treatment is required prior to simulated I/R injury in order for it to have a maximal protective effect as seen by a reduction in cleaved caspase-3 (*chapter 4, figure 4.9a*) and maximal enhancement of ERK phosphorylation (*chapter 4, figure 4.9b*). Protection with HCQ was only observed in cells treated with HCQ prior to simulated ischaemia and continued incubation throughout simulated ischaemia and reperfusion. In relation to translational relevance, a critical issue is that in humans HCQ takes a number of weeks to achieve steady state concentrations. However administering the standard dose (200 mg to 400 mg daily) as well as a loading dose, has been shown to speed this process up (Furst DE 1999) as well as HCQ being used in higher doses in cancer trials (1000 mg daily) (Goldberg S 2012).

The cytoplasmic ubiqituin-like protein Atg8 (LC3) targets autophagosomes through binding to the autophagosome membrane and integrating into the lipid bilayer through undergoing lipidation to LC3-II via ATG7, ATG5-ATG12, and ATG16. LC3-II in turn recruits cargo adaptor proteins (also known as autophagy receptors), such as p62, Nbr1, or NIX which recruit cargo such as damaged organelles or ubiquinated aggregates from the cytoplasm and are delivered to the lysosome, broken down and then recycled. HCQ inhibits autophagy through inhibition of lysosomal function and this has recently been exploited as a target for cancer treatment (Yang Z 2011). Inhibition of lysosomal function therefore results in an accumulation of autophagosomes in the cytoplasm and hence LC3-II. Interestingly, recent evidence points towards LC3-II acting as a scaffold for the phosphorylation of ERK 1/2 (Martinez-Lopez N 2013). It could therefore be hypothesised that HCQ results in the accumulation of autophagosomes and LC3-II, which in turn serves to enhance phosphorylation of ERK 1/2 as observed. This hypothesis is supported by our observation that LC3-II is increased in cardiomyocytes incubated with HCQ (*chapter 4, figure 4.10),* however further experiments are required, as discussed in the future work section of this thesis.

In parallel, experiments were performed to identify potential mechanisms through which IgG from patients with aPL caused pathogenicity in simulated I/R injury. As previously mentioned, the MAPK family are a group of serine-threonine kinases involved in cell proliferation, survival and differentiation (Cobb 1999). Within I/R injury, it is thought that they play both a pro- and anti-apoptotic role, in a kinase specific manner. For example, ERK 1/2 has been shown to induce pro-survival mechanisms when activated in I/R injury but in contrast p38 MAPK and JNK have been implicated in enhancing apoptosis-induced cell death in I/R injury (Yue T 2000). It was observed that cells treated with IgG that was aPL positive, enhanced phosphorylation of p38 MAPK, whereas IgG without aPL had no such effect (*chapter 4, figure 4.11*). No differences were seen with other kinases such as ERK 1/2 and Akt (*chapter 4, figure 4.12*). This data complemented previous work in our lab and others, who have shown that aPL positive IgG leads to p38 MAPK activation in monocytes, which is believed to play a major role in inducing thrombotic events in APS patients (Vega-Ostertag ME 2007; Lambrianides A 2010). Activation of p38 MAPK is known to be mediated through TLR4, however preliminary experiments in this thesis suggested neonatal rat cardiomyocytes are not sensitive to LPS stimulation and therefore TLR4 activation. Previous studies have suggested that aPL mediated p38 MAPK activation in a variety of cell types could be through TLR4 activation (Poulton K 2011). However, within cardiac I/R injury it has been shown that p38 MAPK activation can also occur in response to other signals such as oxidative stress (Su H 2013). Furthermore, studies have shown that in autoimmune prone mice, pathogenic IgM natural antibodies target specific neoepitopes to exert their pathogenic effects and activate signalling pathways during I/R injury (Zhang M 2004). Therefore, it can be hypothesized that aPL positive antibodies bind , unidentified neoepitopes which are exposed during cardiac I/R injury, leading to activation of p38 MAPK. Further experiments are required to explore this theory and are discussed in the future work section of this thesis.

Following on from the finding that aPL positive IgG enhanced p38 MAPK phosphorylation, a commercially available p38 MAPK inhibitor (SB203580) was purchased. Optimisation experiments were carried out to ensure successful inhibition of p38 MAPK could be achieved. As shown by previous studies, 10 μM SB203580 induced complete inhibition of p38 MAPK, when cells were incubated 1 hour prior to simulated I/R injury (*chapter 4, figure 4.13*). Protein lysates from this experiment were also used to confirm that the inhibitor was specific to p38 MAPK inhibition. Other MAPK family members including ERK 1/2 as well as the pro-survival kinase Akt detected no changes in phosphorylated levels in the presence of HCQ (*chapter 4, figure 4.13*).

Subsequent experiments were performed to identify if in the presence of the p38 MAPK inhibitor (SB23580) the pathogenic effects of aPL positive IgG were reversed. It was observed that in simulated I/R injury caspase-3 cleavage was induced and as shown previously in *chapter 3, figure 3.16* this was enhanced most significantly in cells pre-treated with aPL positive IgG. However, when cells were incubated with both IgG and the p38 MAPK inhibitor this increased caspase-3 cleavage was no longer observed (*chapter 4, figure 4.14)*. Interestingly, levels of cleaved caspase-3 were reduced to that seen in aPL negative IgG treated cells. However, cleaved caspase-3 was still greater than that observed in the presence of healthy IgG suggesting that other mechanisms contributing to pathogenicity most likely exist. This experiment therefore suggests that IgG that is aPL positive is mediating its pathogenic effect, at least in part, through up-regulation of the pro-apoptotic kinase p38 MAPK. However, further experiments are required to identify additional mechanisms through which aPL positive and negative IgG accelerates injury in this *in vitro* cardiac I/R injury model.

Another observation of chapter 3 was that IgG from patients with JSLE enhanced simulated I/R injury compared with healthy controls. However, it should be noted that healthy controls could not be age matched. Interestingly, these samples were all aPL negative but followed the trend of adult onset SLE/aPL +ve and APS samples. Their effect on p38 MAPK phosphorylation was explored and it was shown that JSLE IgG treated cells had the same enhanced phosphorylation as that observed in aPL positive IgG treated cells (*chapter 4, figure 4.15*). As previously mentioned in chapter 3, IgG purification yields a polyclonal population and therefore it is possible that an unidentified subset of IgG is present in both aPL positive adult onset SLE and JSLE patients, that is not present in the aPL negative group. However, it is also possible that another IgG population, yet to be identified in the purified JSLE IgG, targets the same antigen as aPL antibodies. The finding that JSLE IgG increases p38 MAPK phosphorylation suggests that JSLE IgG is targeting the same unidentified neoepitope as aPL positive IgG patients. However, the discovery that JSLE IgG also decreases the pro-survival kinase Akt suggests that JSLE IgG may regulate additional signalling pathways. It should be noted however that only 3 JSLE patient samples have been tested to date and therefore further validation is required.

Finally, this chapter explored the effect of treating cells with both HCQ and IgG from SLE patients. Patient IgG was pooled from 5 SLE patients who had previously shown the most pathogenicity in this simulated I/R injury model (all aPL positive), with IgG also being pooled from 5 healthy age and gender matched controls. Results showed that in the presence of IgG from healthy controls, HCQ was able to still have a cardioprotective effect. However, interestingly, cells pre-treated with IgG from SLE patients prevented HCQ from being cardioprotective in this simulated I/R injury model. It

would be interesting to compare aPL positive and aPL negative patients and investigate what effects there is on ERK 1/2 and p38 MAPK phosphorylation. There is evidence that cardiovascular events are less frequent in SLE patients treated with HCQ, however no evidence to suggest less deaths. Nevertheless, it is possible to predict that in patients without SLE, that HCQ could be protective, and therefore this should be explored further as discussed in the future work section of this thesis.

CHAPTER V

Hydroxychloroquine is cardioprotective in an *in vivo* cardiac I/R injury model- an effect mediated through ERK1/2

Overview of Chapter V

This final chapter aims to validate the *in vitro* data that has so far shown HCQ is cardioprotective in simulated I/R injury within an *in vivo* model of cardiac I/R injury. The first part is dedicated to optimisation of the correct dose of HCQ to rats in order to achieve a blood concentration of 1-2 μg/mL. Additionally weight was monitored throughout dosing to ensure there was no toxicity with administration of HCQ.

The second part of this chapter goes on to look at the effects of HCQ in this model of cardiac I/R injury, with infarct size quantified via histological analysis. Following on from this, further experiments were performed to look at ERK1/2 phosphorylation and the effect of the ERK 1/2 inhibitor U0126 in the presence of HCQ within this model.

5.1 Optimisation for treatment of rats with HCQ in an *in vivo* cardiac I/R injury model

5.1.1 Introduction

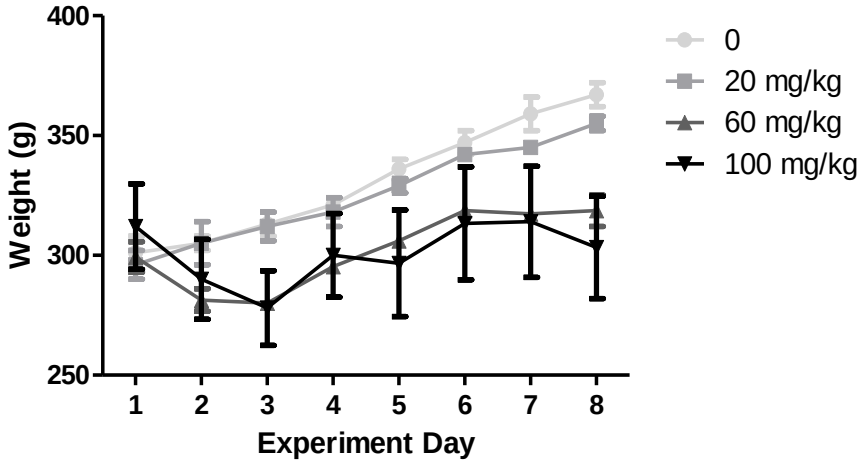
Previous chapters have discussed the finding that in an *in vitro* simulated I/R injury model the drug HCQ inhibits apoptosis and overall cell death. Additionally, protection was shown to be through an ERK-dependent mechanism. The next step was to demonstrate that this protection *in vitro* translated to protection *in vivo*.

An *in vivo* cardiac rat I/R injury model that has been established within UCL (The Centre for Biomedical Imaging (CABI)) was used. Notably, optimisation experiments were carried out for the administration method and dose of HCQ that should be given to the rats, with particular important emphasised on achieving a transnationally relevant concentration of HCQ in the rat blood (1-2 µg/mL). To achieve this in a short term model would take high doses of HCQ, which had to be non-toxic, therefore the weight of the rats and their drinking water consumption were monitored throughout optimisation studies.

5.1.2 Optimisation 1. Rats dosed with HCQ via drinking water have both reduced water consumption and weight accumulation

Rats were dosed with 20, 60 or 100 mg/kg HCQ for one week, via their drinking water. A reduction in their water intake and subsequent weight accumulation (*figure 5.1a*) was observed suggesting that the rats disliked the taste. Due to this reduced water intake, bottles containing drug were substituted for normal drinking water at various points to prevent dehydration. Dextrose was added to the water to try and mask the taste of the drug, however this did not alter the water intake and subsequent rat weights. Additionally, rat blood was taken to assess the concentration of HCQ using HPLC. Due to inconsistent intake of HCQ, blood concentrations were varied and the level of 1-2 µg/mL desired wasn't achieved (*figure 5.1b*).

a



b

Rat No.	Dose (mg/kg)	MDOH (ng/ml)	desethyl-hydroxychloroquine (ng/ml)	hydroxychloroquine (ng/ml)
1	0	<50	<25	<50
2	0	<50	<25	<50
3	20	420	104	171
4	20	442	93	184
5	60	717	131	299
6	60	185	28	85
7	60	770	133	239
8	100	487	56	104
9	100	940	169	382
10	100	<50	<25	<50

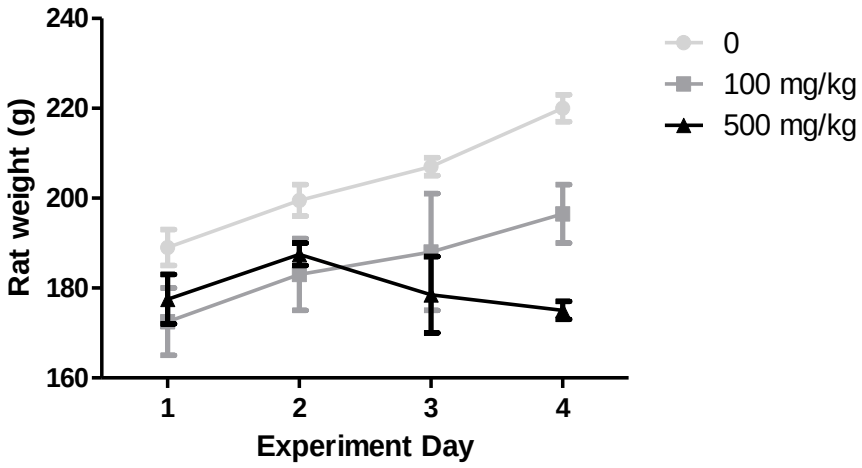
Figure 5.1 Optimisation 1. The weight of rats and their HCQ blood concentrations. Rats were dosed with varying concentrations of HCQ (0, 20, 60 and 100 mg/kg) in their drinking water for seven days and their weight (a) was recorded daily. HPLC was used to detect levels of the two metabolites of HCQ (MDOH and desethly-hydroxychloroquine) a well as total HCQ (b).

5.1.3 Optimisation 2. Rats dosed with HCQ for three days via gavage accumulate weight normally

Due to administering HCQ via drinking water being unsuccessful, dosing via gavage was explored. Rats were dosed with 100 mg/kg and 500 mg/kg for three days and their weight was monitored to ensure toxicity did not occur. Weight gain occurred in the rats in a normal manner for the 100 mg/kg dose, however 500 mg/kg appeared to reduce the amount of weight put on suggesting potential drug toxicity (*figure 5.2a*).

After three days of treatment with HCQ (or water alone), rats were scarified and blood samples taken. Blood concentrations of HCQ were measured using HPLC to detect two metabolites MDOH (methylenedioxyhydroxyamphetamine) and desethyl-HCQ, as well as total HCQ. MDOH represents the active metabolite. This optimisation showed much more consistency in levels of total HCQ between rats within the same group. Rats dosed with 100 mg/kg HCQ achieved approximately 600-700 ng/mL HCQ (*figure 5.2b*). We observed toxicity at 500 mg/kg and therefore excluded this as a potential dose, which was further confirmed by detection of HCQ over the desired threshold previously stated as 1-2 µg/mL (*figure 5.2b*). We therefore decided for subsequent experiments to use a dose of 200 mg/kg to achieve a desired HCQ blood concentration, which also does not prove toxic.

a



b

Rat No.	Dose (mg/kg)	MDOH (ng/ml)	desethyl-hydroxychloroquine (ng/ml)	hydroxychloroquine (ng/ml)
1	0	<25	<25	<25
2	0	<25	<25	<25
3	100	1266	223	623
4	100	1389	273	741
5	500	1810	375	6165
6	500	2450	525	5410

Figure 5.2 Optimisation 2: The weight of rats and their HCQ blood concentrations. Rats were dosed with varying concentrations of HCQ (0, 100 and 500 mg/kg) for three days, their weight was recorded daily (a) and when sacrificed, blood was collected. HPLC was used to detect levels of the two metabolites of HCQ (MDOH and desethly-hydroxychloroquine) as well as total HCQ (b).

5.1.4 Optimisation 2. Rats dosed with HCQ for three days via gavage have detectable levels of HCQ in their organs

Additionally in this optimisation experiment organ lysates were harvested and processed to detect HCQ levels. We felt it important to ensure accumulation of HCQ occurs in organs, specifically the target organ, the heart. *Figure 5.3* confirms detection of HCQ in the heart and liver, with low levels in the brain.

a

Heart

Rat No.	Dose (mg/kg)	MDOH (ng/ml)	desethyl-hydroxychloroquine (ng/ml)	hydroxychloroquine (ng/ml)
1	0	<25	<25	<25
2	0	<25	<25	<25
3	100	41.9	23.5	82.3
4	100	75.8	47.3	156.0
5	500	358.5	323.0	3190.0
6	500	273.8	246.3	2366.6

b

Liver

Rat No.	Dose (mg/kg)	MDOH (ng/ml)	desethyl-hydroxychloroquine (ng/ml)	hydroxychloroquine (ng/ml)
1	0	<25	<25	<25
2	0	<25	<25	<25
3	100	537.9	180.9	404.0
4	100	453.1	132.9	291.9
5	500	1287.5	842.9	9407.7
6	500	1700.0	1116.0	10366.0

c

Brain

Rat No.	Dose (mg/kg)	MDOH (ng/ml)	desethyl-hydroxychloroquine (ng/ml)	hydroxychloroquine (ng/ml)
1	0	<25	<25	<25
2	0	<25	<25	<25
3	100	<25	<25	10.1
4	100	<25	<25	20.9
5	500	<25	<25	84.2
6	500	<25	<25	94.5

Figure 5.3 Optimisation 2: The concentration of HCQ in rat organ lysates. Rats were dosed with varying concentrations of HCQ (0, 100 and 500 mg/kg) for three days and when sacrificed the heart (a), liver (b) and brain (c) were collected. HPLC was used to detect levels of the two metabolites of HCQ (MDOH and desethly-hydroxychloroquine) as well as total HCQ.

5.2 HCQ significantly reduces the infarct size of rats in an *in vivo* cardiac I/R injury model

5.2.1 Introduction

Following optimisation experiments a protocol was established whereby HCQ was administered at a dose of 200 mg/kg daily for 3 days via gavage. The technicians administering the reagents, undertaking the injury and performing histological analysis were blind to the identity of the reagents that were given.

5.2.2 HCQ was detected in the blood of rats dosed with HCQ

Rats were treated with HCQ for three days via gavage in an *in vivo* cardiac I/R injury model and using HPLC levels of the two metabolites of HCQ (MDOH and desethly-hydroxychloroquine) as well as total HCQ were detected. A mean level of 1895 ng/mL (±SD436.9) (*figure 5.4*), therefore the desired level of HCQ in the blood of rats was achieved.

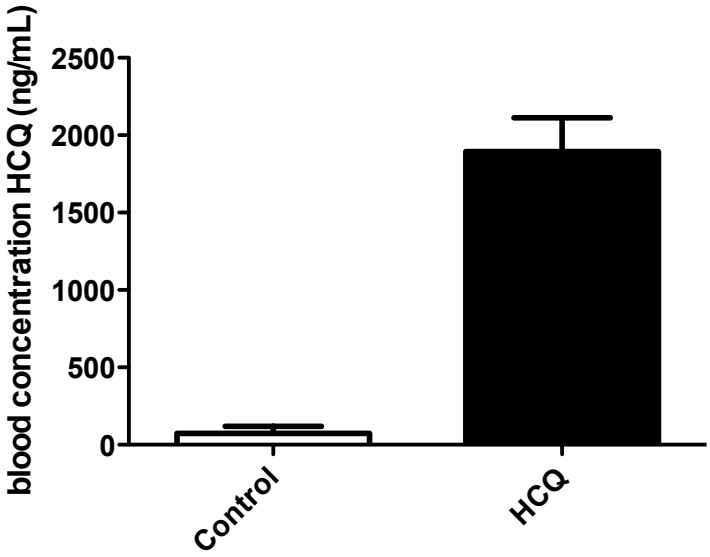
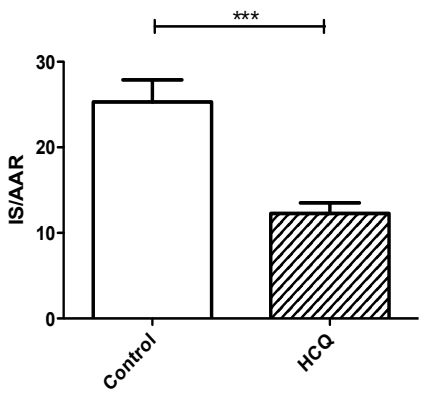


Figure 5.4 Detection of HCQ in the blood of rats dosed with HCQ Rats were dosed by gavage with 200mg/ml HCQ for three days (3 control and 3 HCQ treated). When sacrificed blood was taken and assessed using HPLC to detect levels of the two metabolites of HCQ (MDOH and desethly-hydroxychloroquine) and total HCQ (as shown in this figure).

5.2.3 HCQ reduced infarct size in an *in vivo* rat cardiac I/R injury model

In the group treated with HCQ the infarct size (IS) over the area at risk (AAR) (IS/AAR) were significantly reduced as compared to the water-only control group (*figure 5.5a*). The mean IS/AAR for control rats was 25.32% (± SD8.1 (n=10) which was reduced to a mean of 12.28% (± SD5.8 (n=11)) in HCQ treated rats (*figure 5.5a*). This represents a significant reduction of 51.5% (p=0.0002) in IS/AAR. This is also seen when looking at infarct size alone where the mean IS for control rats was 13.15 (± SD5.65 (n=10)) which was reduced to a mean of 6.7% (± SD2.1 (n=6)) (*figure 5.5b*). Finally, *figure 5.5c* shows the size of AAR for each rat, which is a measure of occlusion consistency. There was no difference in AAR between rats in all groups, therefore highlighting the reproducibility of the results.

a



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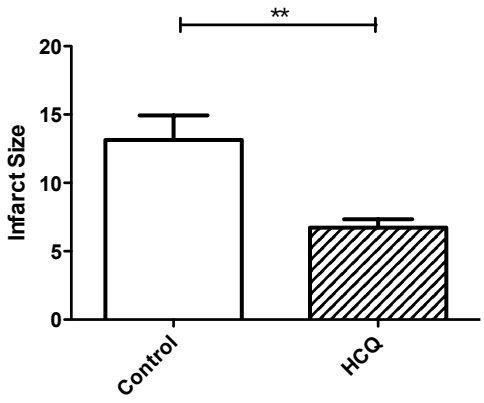

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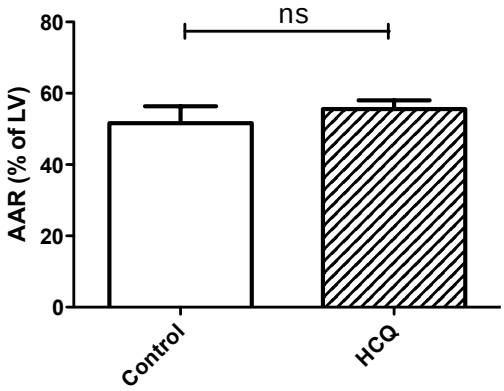

Figure 5.5 Infarct size relative to area at risk (IS/AAR) is reduced in rats pre-treated with HCQ and then subjected to cardiac I/R injury *in vivo*. Rats were dosed by gavage with 200mg/ml HCQ for three days prior to surgery (1 hour occlusion of LAD artery + 24 reperfusion) and when sacrificed hearts were stained with Evans blue and TTC to assess area of infarct size (IS) (b) and area at risk (AAR) (c). The data is presented as IS/AAR as shown in figure a. All graphs show mean ±SEM of quantitative analysis from 10 control and 11 HCQ treated rats. Statistical analysis determined by unpaired t-test (*** p =0.0002)(a) and (** p=0.0022)(b).

5.2.4 Rats dosed with HCQ for three days via gavage have significantly increased ERK phosphorylation level in an *in vivo* cardiac I/R injury model

The hearts were collected from the sacrificed rats and processed to be used in western blotting. The level of ERK phosphorylation was detected, and as seen *in vitro*, HCQ increased phosphorylation of ERK, suggesting a potential mechanism of protection (*figure 5.6*).

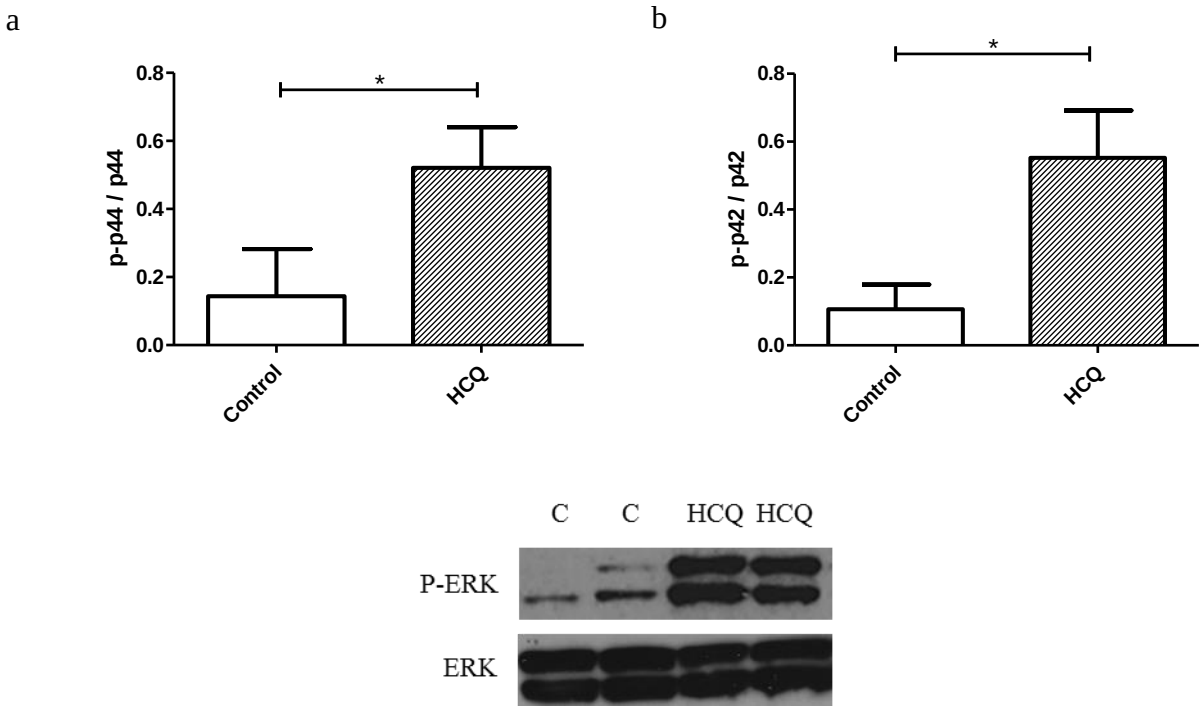

Figure 5.6 ERK phosphorylation is increased in HCQ treated rats subjected to cardiac I/R injury. Rats were dosed by gavage with 200 mg/ml HCQ for three days prior to surgery (1 hour occlusion of LAD artery + 24 reperfusion). All graphs show mean ±SEM of quantitative analysis from 6 control and 8 HCQ treated rats. Statistical analysis determined by Mann Whitney U test (p42 (p=0.0293) and p44 (p=0.02)).

5.3 The ERK inhibitor U0126 blocks protection by HCQ in an *in vivo* rat cardiac I/R injury model

5.3.1 Introduction

Due to previously discussed *in vitro* data showing that when ERK phosphorylation is prevented that HCQ no longer reduces apoptosis, it was decided that this inhibitor would be used *in vivo* to see if it had the same effect. As shown in *chapter 5, figure 5.6,* in HCQ treated rats ERK phosphorylation was enhanced which parallels the *in vitro* data presented in chapter 4.

5.3.2 Optimisation of the ERK inhibitor U0126

It was important to establish the correct dose of the inhibitor in order to inhibit ERK phosphorylation in the absence or presence of HCQ. Due to HCQ appearing to enhance ERK phosphorylation as shown in *figure 5.6,* inhibitor optimization experiments were performed in rats previously dosed with 200 mg/kg for three days. Literature suggested that a single intraperitoneal (IP) injection of the inhibitor at a concentration of 1 mg 30 minutes prior to surgery would be sufficient to inhibit ERK phosphorylation and yield no toxicity (Alderliesten M 2007). Our experimental protocol allowed for three days of dosing following by IP injection of U0126 on day 4. The rats were then left for 4 hours or 24 hours as they would have if the surgery had been carried out before being sacrificed. *Figure 5.7* shows complete inhibition of ERK phosphorylation in rats treated with U0126.

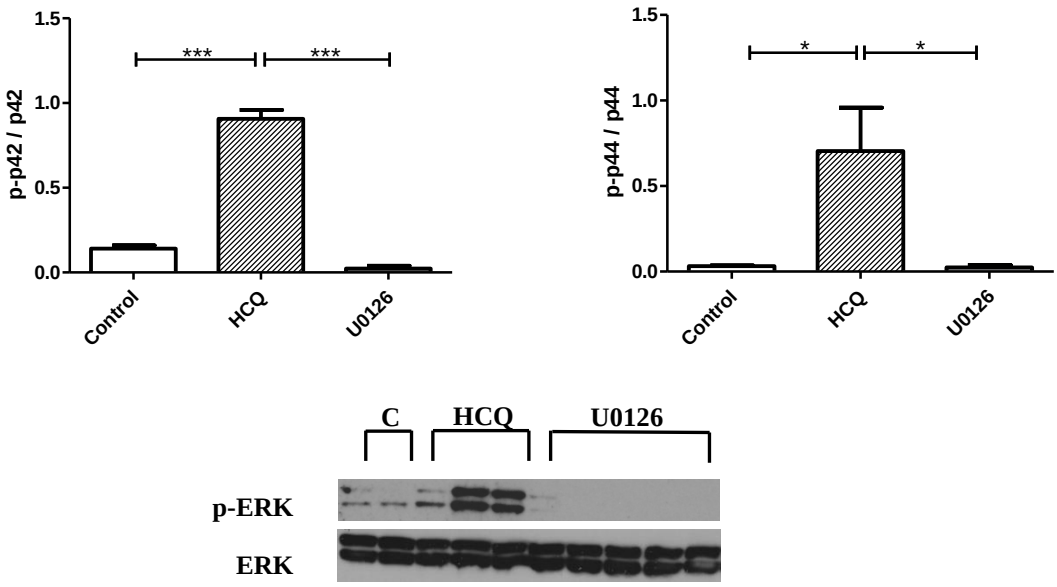

Figure 5.7 ERK phosphorylation is inhibited by U0126. Rats were dosed by gavage with 200 mg/ml HCQ for three days and then administered 1 mg U0126 via IP 30 minutes before surgery. Rats were sacrificed 24 hours later and hearts were collected for western blotting. All graphs show mean ±SEM of quantitative analysis from 3 control, 3 HCQ treated and 4 U0126 treated rats. Statistical analysis determined by one way ANOVA (* $p<0.05$, *** $p<0.0005$)

5.3.3 The ERK 1/2 inhibitor U0126 increases Akt phosphorylation in rats exposed to cardiac I/R injury

To ensure that U0126 was specifically inhibiting ERK 1/2 activation other kinases were looked at in the heart lysates. A significant increase in Akt phosphorylation was observed in the heart lysates of rats treated with U0126 (*figure 5.8a*), however no difference in p38 MAPK phosphorylation was observed (*figure 5.8b*).

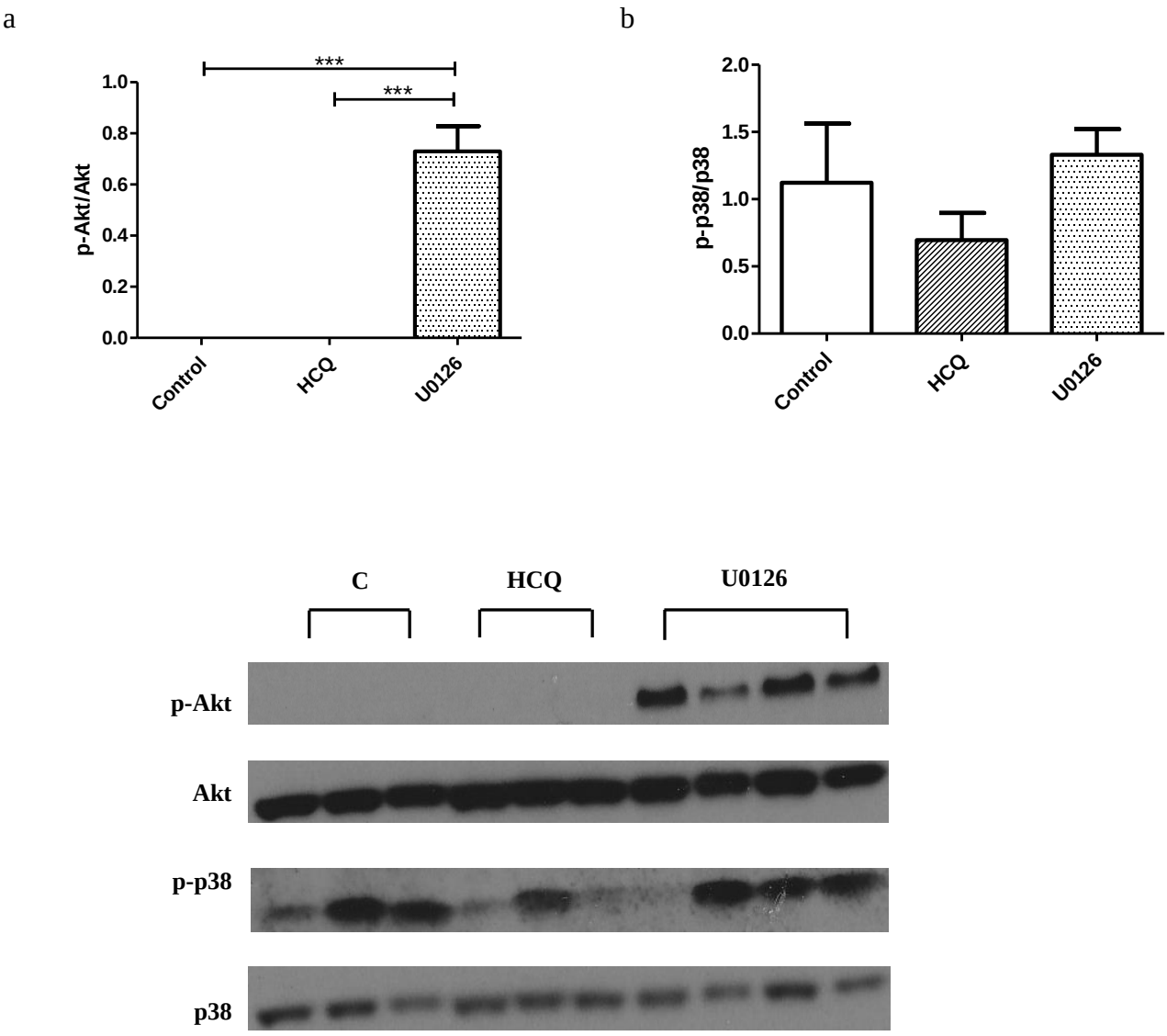

Figure 5.8 The ERK inhibitor U0126 enhances Akt phosphorylation in rats exposed to cardiac I/R injury. Rats were dosed by gavage with 200 mg/ml HCQ for three days and then administered U0126 via IP 30 minutes before surgery. Rats were sacrificed 24 hours later and hearts were collected for western blotting. All graphs show mean ±SEM of quantitative analysis from 3 control, 3 HCQ treated and 4 U0126 treated rats. Statistical analysis determined by one way ANOVA (*** $p<0.0005$)

5.3.4 Infarct size is reduced in rats dosed with HCQ and then subjected to cardiac injury *in vivo* through an ERK 1/2 dependent manner

Rats treated with HCQ cause a significant decrease in infarct size relative to area at risk (IS/AA) as shown in *figure 5.9a*. However, in the presence of HCQ and U0126, infarct size is no longer reduced and is restored back to that seen in control rats (*figure 5.9a*). Control rats have a mean IS/AAR of 25.32% (± SD8.1 (n=10) which was reduced to a mean of 12.28 % (± SD5.8 (n=11)) in HCQ treated rats. In the presence of the ERK inhibitor U0126 alone there is no significant difference in infarct size compared with control rats (mean IS/AAR of 22.31% (± SD12.3 (n=6))). However, in the presence of HCQ and U0126 the mean infarct size was 23.86% (± SD11.23 (n=5)), therefore blocking the protective mechanisms seen in rats treated with HCQ alone.

Additionally when looking at infarct size alone, there is a significant difference between HCQ and HCQ with U0126 treated rats (*figure 5.9b*). The area at risk remains constant, again highlighting the consistency of the surgical technique (*figure 5.9c*).

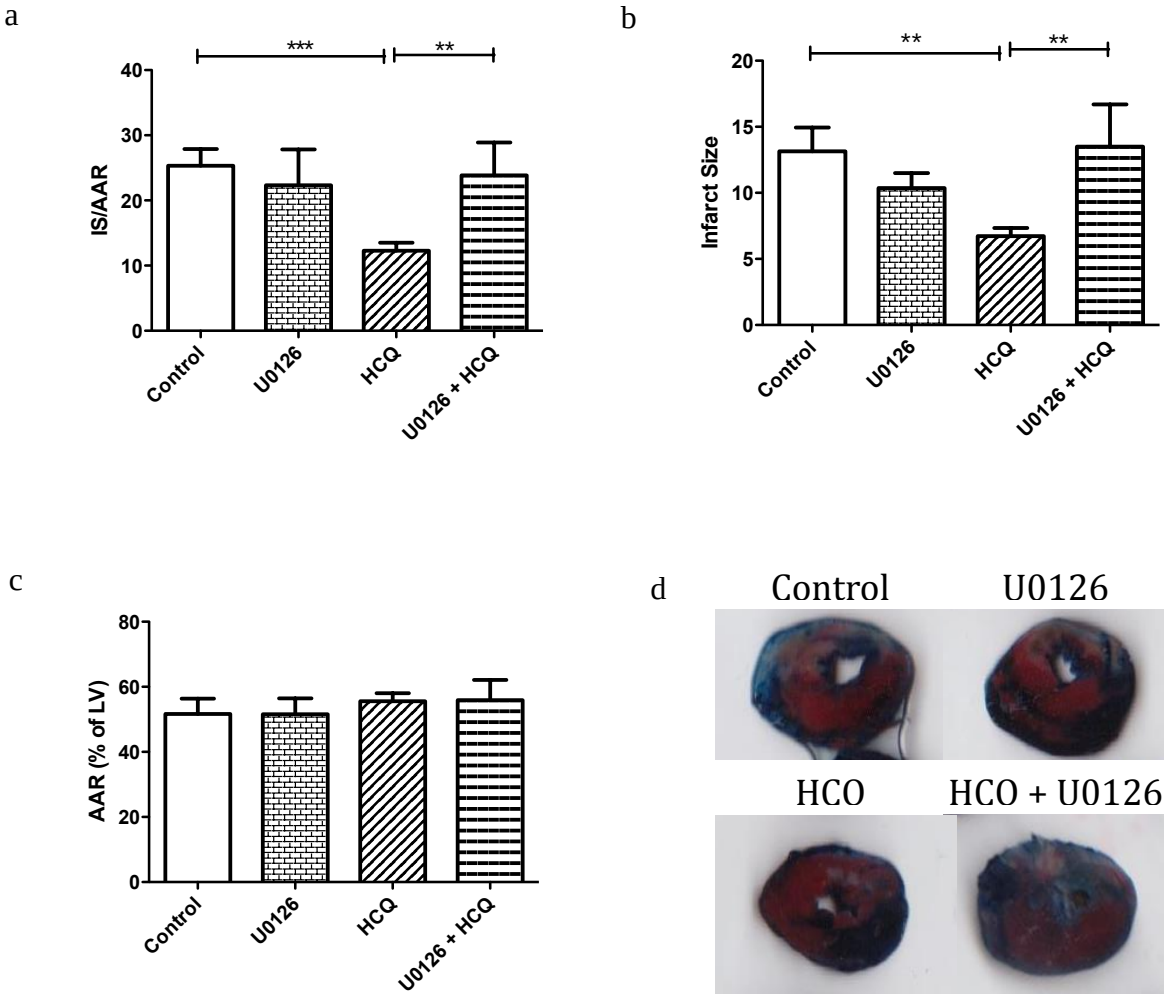

Figure 5.9 Protection by HCQ is blocked by the ERK inhibitor U0126 in a rat *in vivo* cardiac I/R injury model. Rats were dosed by gavage with 200mg/ml HCQ for three days and administered U0126 via IP injection 30 minutes prior to surgery (1 hour occlusion of LAD artery + 24 reperfusion). The data is presented as IS/AAR as shown in figure a. When sacrificed hearts were stained with Evans blue and TTC to assess area of infarct size (IS) (b) and area at risk (AAR) (c). Figure d represents tissue staining for all groups. All graphs show mean ±SEM of quantitative analysis from 10 control, 6 U0126 treated, 11 HCQ treated and 5 U0126 + HCQ treated rats. Statistical analysis determined by unpaired t-test (** p =0.0002, *** p=0.0077)(a) and (** p=0.0022, ** p=0.0097)(b).

5.4 Discussion

The purpose of this chapter was to demonstrate that the cardioprotection by HCQ in the *in vitro* model translated to protection in an *in vivo* cardiac I/R injury model. To test this, a collaboration with a core-service, who have an *in vivo* I/R injury rat model, was set up at UCL and performed in line with standard protocols published previously (Stuckey D 2012). The administration of HCQ was first optimized with the goal of achieving 1-2 µg/mL blood concentration, which translates to levels achieved in patient blood. The first optimisation consisted of 3 doses of HCQ; 20, 60 and 100 mg/kg administered via drinking water over a period of 7 days. It was observed from the beginning that the rats disliked the taste of the drug, particularly at 60 and 100 mg/kg and therefore had a reduced intake of water. This led to a reduction in weight gain (*chapter 5, figure 5.1a*) and low and inconsistent levels of HCQ detected in the rat blood (*chapter 5, figure 5.1b*). Due to dosing rats via their drinking water yielding low and inconsistent levels of HCQ in their blood it was decided a new approach had to be taken. Optimisation 2 consisted of administering HCQ via gavage for three days at a concentration of 100 and 500 mg/kg, as suggested by previously published data (Pareek A 2009). This time rats dosed with 100 mg/kg HCQ accumulated weight at a normal rate, with 500 mg/kg proving to be slightly toxic causing weight gain to plateau (*chapter 5, figure 5.2a*). Furthermore, blood levels of HCQ were much more consistent between rats in the same group, with 100 mg/kg achieving slightly under the desired concentration and 500 mg/kg yielding 5 times above (*chapter 5, figure 5.2b*). Additionally, concentrations of HCQ were measured in organ lysates to ensure that HCQ was accumulating in target organs i.e. heart (*chapter 5, figure 3*). From this optimisation it was concluded that administering HCQ via gavage was much more consistent, and a dose of 200 mg/kg was decided upon which yielded a blood concentration of 1895 ng/mL ±SD436.9 (*chapter 5, figure 5.4*).

Rats were dosed with HCQ 200 mg/kg daily for 3 days via gavage (or water only as a control) and then exposed to I/R cardiac injury and sacrificed 24 hours post reperfusion. The technicians administering the reagents, undertaking the injury and performing histological analysis were blind to the identity of the regents that were given. In the group treated with HCQ the infarct size relative to the area at risk (AAR) was significantly reduced as compared to water only control group (*chapter 5, figure 5.5a*). Infarct size alone also showed a significant decrease (*chapter 5, figure 5.5b*), whereas the AAR size remained dependable, highlighting consistency in the protocol (*chapter 5, figure 5.5c*). Heart lysates were collected and when probed for ERK, the group of rats that received HCQ had significantly enhanced phosphorylation of ERK as compared to rats treated with water alone (*chapter 5, figure 5.6*). Hence the protection observed *in vitro* with HCQ in a simulated model of I/R injury is also observed with I/R cardiac injury *in vivo* in rats and HCQ and also enhances phosphorylation of the pro-survival kinase ERK *in vivo* in hearts.

In keeping with the *in vitro* data previously reported in this thesis the next step was to use the ERK inhibitor U0126 to see if this protective effect could be blocked. An optimisation experiment was performed whereby rats were dosed with HCQ for three days and then injected (intraperitoneal (IP)) with 1 mg of U0126 (or water only as a control) 30 minutes prior to surgery. This treatment with U0126 yielded complete abrogation of ERK phosphorylation as shown in *figure 5.6*. Other kinases were also probed for to ensure U0126 specifically inhibited ERK 1/2 inhibition. There was no significant difference in p38 MAPK phosphorylation (*chapter 5, figure 5.7a*), however Akt phosphorylation was significantly increased in rats dosed with U0126 (*chapter 5, figure 5.8b*). This may be due to mechanisms being activated to try and counteract the reduction in ERK phosphorylation, as previously reported (Hausenloy D 2004). It is thought that this feedback is either via p70s6k or BAD which are both known to interact with Akt and ERK. It would be expected that if a vital protective mechanism such as ERK phosphorylation was inhibited then infarct size would infact be enhanced in the presence of U0126. This increase in Akt phosphorylation therefore presents an explanation as to why there is no enhancement in infarct size relative to AAR when ERK phosphorylation is inhibited.

Results showed that when rats are dosed with HCQ and then treated with U0126 infarct size over AAR is restored to that observed in control rats (*chapter 5, figure 5.8*). Infarct size alone is also significantly reduced in the presence of HCQ but restored in the presence of HCQ and U0126. As previously shown AAR remains consistent highlighting the reproducibility of the data. This data suggests that HCQ is exerting its protective effect through increased phosphorylation of ERK as previously reported *in vitro*.

Limitations of this experiment include that the model used is short term and therefore the long term effects of HCQ on myocyte viability, cardiac function and cardiac remodelling haven't been explored. Additionally due to evans blue and TTC staining being required to assess infarct size no post immunofluorescence could be performed or markers of apoptosis such as cleaved caspase-3 be measured. In future it would be ideal to use imaging techniques such as magnetic resonance imaging (MRI) to assess infarct size so that heart tissue can be used for alternative analysis.

In summary, this chapter has confirmed previous *in vitro* data in a cardiac *in vivo* I/R injury model. Firstly, when HCQ is administered to rats to achieve a dose of 1-2 µg/mL in rat blood there is a significant decrease in infarct size relative to AAR. Additionally when the ERK 1/2 inhibitor U0126 is administered to rats this protection is blocked suggesting that protection is via enhanced ERK phosphorylation.

This chapter concludes the data presented in this thesis, however chapter 6 will discuss the overall findings of this project as well as identify further work to be carried out in order to expand on this data further.

CHAPTER VI

Overall Findings and Future Experiments

Overview of Chapter VI

This chapter gives an overview of the main findings of this thesis. Firstly it will discuss the principal findings regarding the drug HCQ and its protective effects in cardiac I/R injury. The second part will discuss the role of pathogenic IgG autoantibodies from patients with SLE, JSLE and APS in this *in vitro* model of cardiac I/R injury. This chapter will then discuss the potential for future projects to take this work forward as well as identify potential new hypotheses.

6.1 Overall findings

MI carries a significant burden of both mortality and morbidity post-infarct. Prompt diagnosis and treatment through thrombolysis or angioplasty remains the mainstay of treatment and long-term outcomes are determined by both the site and extent of the infarct. The infarct size is not just dependent on damage ensued during ischaemia. An important contributor to the size of the eventual infarct is injury that occurs to the myocardium during the reperfusion phase, which in animal studies has to been shown to account for up to 40-50% of the final infarct size (Yellon D 2007). The dominant mechanisms through which cardiomyocytes die during the ischaemic phase is necrosis. However, during the re-perfusion phase a complex interplay of multiple pathways and mechanisms are activated which ultimately culminate in cell death primarily through apoptosis (Eefting F 2004). These mechanisms have been studied extensively and revolve around dysregulation of mitochondrial function and eventual rupture of mitochondrial membrane releasing multiple pro-apoptotic mediators into the cytoplasm, generation of ROS, initiation of inflammatory response pathways further aggravating injury, complement, autophagy and TLR activation to name but a few (Verma S 2002).

HCQ was originally an anti-malarial drug, however it is now used to treat autoimmune disorders such as SLE. It has been shown to reduce inflammation as well as have potential cardiovascular benefits in these patients (Wallace D 2012). This includes lowering atherogenic lipids such as cholesterol, triglycerides and LDL levels (Morris S 2011). Additionally, it has been shown to have anti-thrombotic effects, with the risk of suffering from an event reducing by 68% in patients with SLE (Jung H 2010). Despite cardiac morbidity and mortality being enhanced in patients with lupus (Esdaile JM 2001), the effect of HCQ on cardiomyocyte survival in relation to I/R injury prior to this thesis had yet to be explored.

Work carried out in H9c2 rat cell line and neonatal rat cardiomyocytes identified a cardioprotective effect of HCQ in simulated I/R injury. Previous work has suggested a protective role for HCQ in renal and liver I/R injury (Fang H 2013; Todorovic Z 2014), however this was the first suggestion for a protective role in the heart. To validate the findings observed *in vitro*, an *in vivo* rat cardiac I/R injury model was utilised to see if the same protective effect with HCQ could be seen. After optimization of the model to ensure equivalent HCQ concentrations *in vivo*, a reduction of 51.5% in infarct size relative to area at risk was observed in HCQ treated rats compared with controls gavaged with water alone. Subsequent experiments explored specific mechanisms through which HCQ may be mediating its protective effect. Pro-survival kinases such as ERK 1/2 and Akt have been identified as being modulators of cardioprotection and constitute the RISK pathway (Haunsenloy

2007). The RISK pathway is up-regulated prior to I/R injury, therefore allowing maximal protection to be induced during the reperfusion stage (Davidson S 2006). Therefore, enhancing components of the protective RISK pathway through pre-conditioning offers an appealing therapeutic target to ameliorate I/R injury (Yang X 2010). Pharmacological pre-conditioning is an area of great therapeutic interest, and drugs such as sildenafil have been shown to be cardioprotective through enhancing ERK phosphorylation prior to simulated I/R injury (Das A 2009). Results in this thesis showed a significant increase in ERK 1/2 phosphorylation in HCQ treated cells in the *in vitro* neonatal rat cardiomyocyte I/R injury model as well as the *in vivo* cardiac I/R injury model. Furthermore, when the ERK 1/2 inhibitor U0126 was incubated with cells or administered to rats, protection was blocked in the presence of HCQ. The work from this thesis has the potential to underpin the rationale for future clinical studies, investigating a re-purposed role for HCQ as a protective agent for I/R injury as described below in section 6.2.

The other focus of this project was the role of IgG purified from whole blood of patients with SLE in cardiac I/R injury. The possibility of cardiac I/R injury being enhanced in lupus is relevant, given that there is increased cardiovascular morbidity and mortality rates observed in lupus, yet possible explanations for this are yet to be explored. There is some evidence from a lupus mouse model within a mesenteric I/R injury model that lupus IgG are pathogenic (Fleming S 2004) but no study as yet has investigated human lupus IgG in a heart model. In humans, there is no large-scale study to our knowledge that has compared cardiac infarct sizes between patients with lupus and age and gender matched controls. Therefore the aim of this section of the project was to explore the effect of IgG in the *in vitro* neonatal rat cardiomyocytes model of simulated I/R injury.

It was found that the pre-treatment of neonatal rat cardiomyocytes with IgG purified from patients with SLE enhanced caspase-3 cleavage and TUNEL positivity in cells exposed to simulated I/R injury. When this group of patients was sub-divided into those positive for aPL antibodies versus those that were negative, it was found that there was a significantly greater increase in cleaved caspase-3 for aPL positive patients compared to aPL negative. This suggested that aPL positive patients have a mechanism of action which differs to that in aPL negative patients for causing pathogenicity in this simulated I/R injury model. Furthermore primary APS patients who are by definition aPL positive (but do not have SLE) showed similar levels of increase caspase-3 cleavage as SLE/aPL positive. Another group of patients was included that consisted of patients with JSLE that were all aPL negative. Interestingly, they followed the trend of adult onset aPL positive patients, in that their caspase-3 cleavage was more significant than adult onset aPL negative. It should be noted that the purified IgG is a polyclonal population. Therefore, an explanation for this finding may

be that they share a common pathogenic subset of autoantibodies as aPL positive patients that is yet to be discovered.

Due to the observation that aPL positive patients had enhanced pathogenicity, mechanisms of action were explored. Work previously published by members of the lab of my supervisor Dr. Ioannou and his collaborators suggested that IgG derived from aPL positive patients causes enhanced p38 MAPK phosphorylation in monocytes (Lambrianides A 2010) whereas this was not observed with IgG purified from patients with SLE without aPL nor healthy volunteer controls. p38 MAPK is known to play a pro-apoptotic role in cardiac I/R injury (Kumphune S 2012) and therefore one would hypothesise that aPL positive IgG could be exerting its pathogenic effect through up regulation of this kinase. A commercially available p38 MAPK inhibitor (SB23580) was used to show that the enhanced pathogenicity of aPL positive patients is p38 MAPK dependent. Results showed that when cells were treated with IgG from aPL positive patients in the presence of SB23580, there was a significant decrease in cleaved caspase-3, to levels similar to that observed in the presence of aPL negative patient IgG. To conclude, this data suggests an aPL positive, p38 MAPK dependent mechanism, for aPL mediated pathogenicity in cardiac I/R injury as observed in this simulated *in vitro* model; however an increase in patient numbers is required to confirm this.

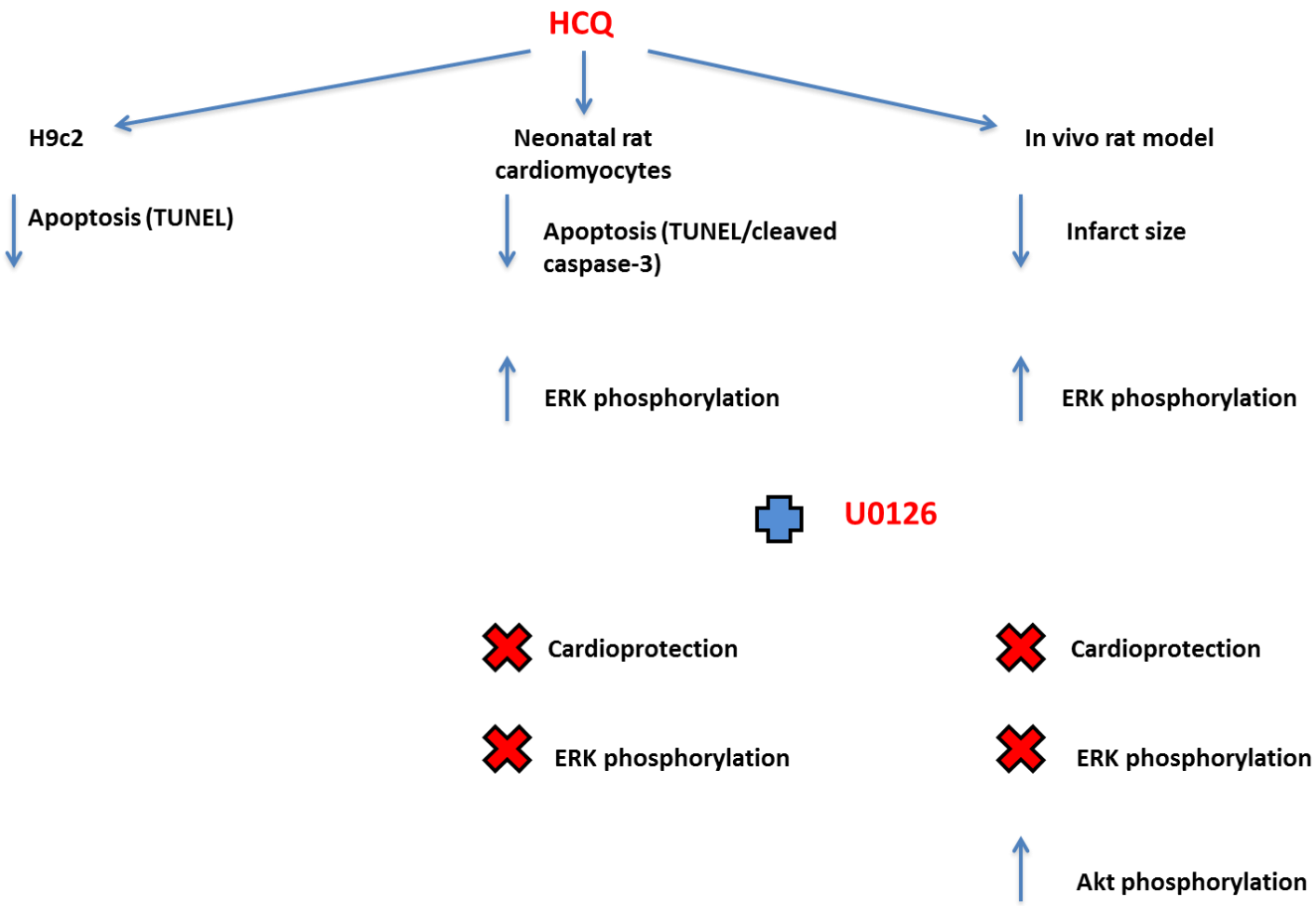

Figure 6.1. A summary of the main findings for HCQ. The drug HCQ causes a reduction in apoptosis in H9c2 and neonatal rat cardiomyocytes. This cardioprotection is also observed in an *in vivo* rat model of cardiac I/R injury. A proposed mechanism of action is enhanced phosphorylation of ERK 1/2 and therefore the addition of the ERK inhibitor U0126 blocked protection both *in vitro* and *in vivo*.

6.2 Future work

To take the findings of this thesis forward there a number of experiments that could be suggested. Due to the two distinct pathways of this work, future experimental suggestions will be split into two sections.

This thesis has made the observation that HCQ is protective in cardiac injury and therefore future experiments could aim to explore the role in protection against cardiac injury in detail. This should include dissecting out potential mechanisms related to autophagy and assessing whether reducing infarct size translates to improved cardiac function in the longer term, in terms of the effects on cardiac remodelling. At present, the success rate of lead candidates targeting pathways relevant to I/R injury progressing through to market has been poor. The advantage of HCQ is that it has a well-defined and favorable safety profile, having been in routine use within rheumatology (e.g. for SLE) for the last 50 years. This would greatly facilitate its development within clinical studies as a lead candidate for this indication as compared to a new chemical entity, which requires far greater regulatory hurdles to overcome. Additionally, studies such as exploring the effects of HCQ in long term models of cardiac I/R injury are essential. This would allow its effects on cardiac remodeling to be assessed. The use of MRI studies would allow the tracking of infarct size and function over time without having to sacrifice animal or stain tissue, allowing the assessment of effects on apoptosis and signalling pathways. If these extensive *in vivo* experiments support HCQ as a protective agent in cardiac I/R injury, with beneficial effects on cardiac function and remodeling over time, then this will provide the rationale for developing HCQ in human studies for ameliorating outcomes post MI. It should be noted that most likely HCQ would need to be given prophylactically. This is firstly due to data in this thesis suggesting that HCQ is most protective when cells are pre-treated *chapter 4, figure 4.16*. It has also been shown in SLE patients that HCQ can take a number of weeks to reach therapeutic levels (1-2 µg/mL) therefore complementing the *in vitro* data in the suggestion that HCQ would need to be administered prior to an MI, as is currently suggested for other cardioprotective drugs such as statins (Davignon 2004). However, it should be noted that it is possible to administer a loading dose of HCQ to achieve therapeutic blood levels quicker with no toxic effect, as shown in RA patients (Furst D 1999).

As well as defining the relevance of HCQ as a protective agent it is also important to further dissect the mechanisms through which it is exerting a cardioprotective effect. This thesis has identified a central role of ERK 1/2 phosphorylation, however as yet has not identified how this enhanced ERK 1/2 phosphorylation is mediated. It is therefore proposed that subsequent experiments would be

performed to look at the role of autophagy. HCQ is known to inhibit autophagy through inhibition of the lysosome, resulting in accumulation of autophagosomes. These autophagosomes have scaffold proteins such as LC3 attached to their membrane, which integrate into the lipid bilayer by undergoing lipidation to LC3-II via ATG7, ATG5-ATG12, and ATG16. LC3-II in turn recruits cargo adaptor proteins (also known as autophagy receptors), such as p62, Nbr1, or NIX which recruit cargo such as damaged organelles or ubiquinated aggregates from the cytoplasm and are delivered to the lysosome, broken down and then recycled (Rabinowitz 2010). The ability of HCQ to inhibit autophagy through inhibition of lysosomal function has been exploited as a target for cancer treatment recently (yang Z 2011). Inhibition of lysosomal function hence results in an accumulation of autophagosomes in the cytoplasm and hence LC3-II. Interestingly, recent evidence points towards LC3-II acting as a scaffold for the phosphorylation of ERK (Martinez-Lopez 2013). Hence, our hypothesis is that HCQ results in the accumulation of LC3-II on the accumulated autophagosomes, which in turn serves to enhance phosphorylation of ERK as observed. This hypothesis is supported by our observation that LC3-II is increased in cardiomyocytes incubated with HCQ. LC3-I lipidation to LC3-II is dependent on ATG5 and ATG7 and recycling of LC3 II to LC3-I via de-lipidation is dependent upon ATG4 (Satoo K 2009). Hence, depletion of ATG5 or ATG7 results in reduced LC3-II and conversely depletion of ATG-4 hence results in an accumulation of LC3-II which in turn leads to enhanced ERK phosphorylation (Martinez-Lopez 2013). Thus if this hypothesis is true, in the presence of HCQ, silenced ATG5 or ATG7 should inhibit the resultant accumulation of LC3-II which in turn should inhibit LC3-II mediated increase in ERK phosphorylation during simulated I/R injury and remove the protective effect of HCQ. Conversely, ATG4 silencing should result in LC3-II accumulation, enhanced ERK phosphorylation and mirror the protective effects seen with HCQ.

For the work produced concerning the pathogenicity of IgG derived from patients who are SLE/aPL positive, there are many avenues through which this work could be progressed. In the first instance, it is important to acknowledge that number of patient samples tested in the presence of the p38 inhibitor SB23580 should be expanded to make the data more robust. Anti-ß$_2$GPI antibodies mediate their pro-thrombotic pathogenicity through targeting domain I (DI) of ß$_2$GPI (Ioannou Y 2007; Ioannou Y 2010). The laboratory of my supervisor Dr Ioannou together with his collaborators have previously shown the IgG-APS mediated exacerbation of thrombosis in a validated *in vivo* model of APS may be inhibited by using recombinant DI (rDI) as a decoy peptide (Ioannou Y 2009). The lab is now developing this as a novel therapeutic for APS. Hence, it would be of interest to observe whether the pathogenic effects of IgG-APS in this simulated model of I/R injury is also dependent on binding DI of ß$_2$GPI.

Human rDI is expressed and purified within an E.coli expression system, as described previously. This system is now optimised to produce around 35-40 mg/L culture of purified, untagged, conformationally correct rDI. Purified IgG could be pre-incubated with rDI for 1 hour at room temperature prior to adding to the cardiomyocytes. This set of experiments will determine if the pathogenic effects of IgG-APS that are anti-ß$_2$GPI positive are dependent upon binding DI of ß$_2$GPI. Once this pathogenic population of aPL is confirmed *in vitro*, these can be affinity purified and used within a passive transfer animal model to confirm pathogenicity *in vivo*. This can be confirmed in the *in vivo* rat cardiac I/R injury model previously descried in this thesis.

The data produced in relation to the pathogenicity of JSLE IgG should be explored further. It would be of interest to identify the subset of pathogenic antibodies from this polyclonal population and/or the antigens that these antibodies are targeting to cause accelerated injury. Experiments whereby cells are incubated with IgG and exposed to simulated I/R injury should be performed. Cell lysates can then be used for pull-down experiments using dynabeads, to isolate specific IgG-protein interactions. This will allow identification of novel neoepitopes, which are being exposed during I/R injury, to be identified by mass spectrometry.

Finally, a preliminary study in this thesis showed that co-incubating cardiomyocytes with HCQ and IgG from an aPL positive patient blocked protection by HCQ. Firstly this finding should be validated further by increasing patient samples numbers, however it would also be of interest to see the effect of HCQ in the presence of IgG from SLE/aPL negative, APS and JSLE patients. This would determine if the enhanced phosphorylation of the pro-survival kinase ERK seen in the presence of HCQ ‘overrides’ the pro-death effects of p38-MAPK phosphorylation observed with IgG-APS or not. This will give insight into whether HCQ can protect in the presence of antibodies from patients with APS and SLE and, if so, to what extent and in which groups.

The outcome of this research may identify a new mechanism through which antibodies from patients with APS and lupus may cause cardiac damage following an MI. This research would then underpin future clinical studies to determine if patients with APS or lupus suffer larger heart attacks or strokes as compare to age and gender matched people without APS and lupus. Additionally it would also support animal *in vivo* studies of I/R injury to confirm and validate the findings. Ultimately, this may lead to identification of novel pathogenic roles for antibodies from patients with lupus / APS and investigation of potential new targets and therapeutics which hold the promise of improving outcomes for patients with these conditions and CVD.

Bibliography

Abe J, B. C. a. B. B. (2000). "Role of Mitogen-Activated Protein Kinases in Ischemia and Reperfusion Injury : The Good and the Bad." Circulation Research **86**: 607-609.

Adachi, M. a. I., K (2002). "The proapoptotic BH3-only protein BAD transduces cell death signals independently of its interaction with Bcl-2." Cell death and differentiation **9**(11): 1240-1247.

Adams, J. (2003). "Ways of dying: multiple pathways to apoptosis." Genes & Development **17**(20): 2481-2495.

Aki T, Y. K., Fujimiya T and Mizukami Y (2003). "Phosphoinositide 3-kinase accelerates autophagic cell death during glucose deprivation in the rat cardiomyocyte-derived cell line H9c2." Oncogene **22**(52): 8529-8535.

Alcocer-Varela, J. a. A.-S., D (1982). "Decreased production of and response to interleukin-2 by cultured lymphocytes from patients with systemic lupus erythematosus." J Clin Invest **69**(6): 1388-1392.

Alderliesten M, d. G. M., Oldenampsen, Judit J, Qin Y, Pont C, van Buren L, van de Water B. (2007). "Extracellular Signal-Regulated Kinase Activation during Renal Ischemia/Reperfusion Mediates Focal Adhesion Dissolution and Renal Injury." The American Journal of Pathology **171**(2): 452-462.

Amaral B, M. G., Ioannou Y, Isenberg D (2014). "A comparison of the outcome of adolescent and adult-onset systemic lupus erythematosus." Rheumatology.

Asherson RA, C. R., Piette JC, Font J, Lie JT and Burcoglu A (1998). "Catastrophic antiphospholipid syndrome. Clinical and laboratory features of 50 patients. ." Medicine (Baltimore) **77**(3): 195-207.

Asherson RA, C. R., Piette JC, Shoenfeld Y, Espinosa G and Petri MA (2001). "Catastrophic antiphospholipid syndrome: clues to the pathogenesis from a series of 80 patients." Medicine (Baltimore) **80**(6): 355-377.

Atar D, P. P., Schwitter J, Huber K, Rensing B, Kasprzak J, Butter C, Grip L, Hansen P, Süselbeck, T, Clemmensen P, Marin-Galiano M, Geudelin B and Buser P (2009). "Effect of Intravenous FX06 as an Adjunct to Primary Percutaneous Coronary Intervention for Acute ST-Segment Elevation Myocardial Infarction: Results of the F.I.R.E. (Efficacy of FX06 in the Prevention of Myocardial Reperfusion Injury) Trial." Journal of the American College of Cardiology **53**(8): 720-729.

Avkiran, M. and M. S. Marber (2002). "Na+/h+ exchange inhibitors for cardioprotective therapy: progress, problems and prospects." Journal of the American College of Cardiology **39**(5): 747-753.

Ballestar E, E. M. a. R. B. (2006). "The Epigenetic Face of Systemic Lupus Erythematosus." The Journal of Immunology **176**(12): 7143-7147.

Bär F, T. D., Dirksen M, Fernández-Ortiz A, Heyndrickx G, Brachmann J, Reiber J, Avasthy N, Tatsuno J, Davies M, Hibberd M and Krucoff M (2006). "Results of the first clinical study of adjunctive CAldaret (MCC-135) in patients undergoing primary percutaneous coronary intervention for ST-Elevation Myocardial Infarction: the randomized multicentre CASTEMI study." European Heart Journal **27**(21): 2516-2523.

Barry S, T. P., McCormick J, Knight R, Scarabelli T, Latchman D and Stephanou A (2009). "STAT3 deletion sensitizes cells to oxidative stress." Biochem Biophys Res Commun **385**(3): 324-329.

Bassand J, D. N., Filippatos G, Gitt A, Hamm C, Silber S, Tubaro M and Weidinger F (2005). "Implementation of reperfusion therapy in acute myocardial infarction. A policy statement from the European Society of Cardiology." European Heart Journal **26**(24): 2733-2741.

Becker, L. (2004). New concepts in reactive oxygen species and cardiovascular reperfusion physiology.

Bernatsky S, B. J., Joseph L, Rajan R, Zoma A, Manzi S, Ginzler S, Urowitz M, Gladman D, Fortin P, Petri M, Edworthy S, Barr S, Gordon C, Bae S, Sibley J, Isenberg D, Rahman A, Aranow C, Dooley M, Steinsson K, Nived O, Sturfelt G, Alarcón G, Senécal J, Zummer M, Hanly J, Ensworth S, Pope J, El-Gabalawy H, McCarthy T, St. Pierre Y, Ramsey-Goldman R, Clarke A, (2005). "An international cohort study of cancer in systemic lupus erythematosus." Athritis and Rheumatism **52**(5): 1481-1490.

Berthiaume F, B. L., Mokuno Y, MacDonald A, Jindal J, Yarmush M (2009). "Steatosis Reversibly Increases Hepatocyte Sensitivity to Hypoxia-Reoxygenation Injury." Journal of Surgical Research **152**(1): 54-60.

Bertsias G, C. R., Boumpas DT (2012). "Systemic lupus erythematosus: Pathogenesis and clinical features." EULAR textbook on rheumatic diseases: 476-505.

Billen L, S.-D. A., Andrews D (2009). "Bid: a Bax-like BH3 protein." Oncogene **27**: 93-104.

Boatright, K. a. S., G (2003). "Mechanisms of caspase activation." Current Opinion in Cell Biology **15**(6): 725-731.

Bolvin W, C. D., Hiebert P, Granville J (2009). "Intracellular versus extracellular granzyme B in immunity and disease:challenging the dogma." Laboratory Investigations **89**: 1195-1220.

Bootsma H, S. P., Derksen R, de Boer G, Wolters-Dicke H, Hermans J, Limburg P, Gmelig-Meyling F, Kater L, Kallenberg C. (1995). "Prevention of relapses in systemic lupus erythematosus." Lancet **345**(8965): 1595-1599.

Borgers M, S. L., Xhonneux R, Thoné F, and Overloop P (1987). "Changes in ultrastructure and Ca2+ distribution in the isolated working rabbit heart after ischemia. A time-related study." The American Journal of Pathology **126**(1): 92-102.

Boya P, P. F. a. C. P. (2013). "Emerging regulation and functions of autophagy." Nature Cell Biology **15**: 713-720.

Boya, P., F. Reggiori, et al. (2013). "Emerging regulation and functions of autophagy." Nat Cell Biol **15**(7): 713-720.

Brara B , S. A., Okosib A, Lawrence K , Knight R, Marberc M and Latchman D (1999). "CRH-like peptides protect cardiac myocytes from lethal ischaemic injury." Molecular and Cellular Endocrinology **158**(1-2): 55-63.

Brey R, A. R., Curb J, Sharp D, Ross W, Stallworth C and Kittner S (2001). "β2-Glycoprotein 1–Dependent Anticardiolipin Antibodies and Risk of Ischemic Stroke and Myocardial Infarction: The Honolulu Heart Program." Stroke **32**(8): 1701-1706.

Buja, M. E., M (1998). "Modes of Myocardial Cell Injury and Cell Death in Ischemic Heart Disease." Circulation **98**: 1355-1357.

Burlacu, A. (2003). "Regulation of apoptosis by Bcl-2 family proteins." Journal of Cellular and Molecular Medicine **7**(3): 249-257.

Cancro, M. D. C., D and Khamashta, M (2009). "The role of B lymphocyte stimulator (BLyS) in systemic lupus erythematosus." J Clin Invest **119**(5): 1066-1073.

Cande C, V. N., Garrido C and Kroemer G (2007). "Apoptosis-inducing factor (AIF): caspase-independent after all." Annual Review Immunology **25**: 561-586.

Capone ML, T. S., Rodriguez LG, Patrignani P. (2010). "NSAIDs and cardiovascular disease: transducing human pharmacology results into clinical read-outs in the general population." Pharmacol Rep **62**(3): 530-535.

Carr C, S. D., Tan J, Tan S, Gomes R, Camelliti P, Messina E, Giacomello A, Ellison G, Clarke K (2011). "Cardiosphere-Derived Cells Improve Function in the Infarcted Rat Heart for at Least 16 weeks - an MRI Study." PLoS One **6**(10).

Casciola-Rosen L, R. A., Petri M, and Schlissel M (1996). "Surface blebs on apoptotic cells are sites of enhanced procoagulant activity: implications for coagulation events and antigenic spread in systemic lupus erythematosus." PNAS **93**(4): 1624-1629.

Casciola-Rosen LA, A. G., Rosen A (1994). "Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes." J Exp Med **179**(4): 1317-1330.

Cervera R, V. O., Ramos-Casals M, Font J, García-Carrasco M, Sisó A, Ramírez F, Machuca Y, Vives J, Ingelmo M and Burlingame R W (2003). "Anti-chromatin antibodies in systemic lupus erythematosus: a useful marker for lupus nephropathy." Annals of the Rheumatic Diseases **62**(5): 431-434.

Chakravarty EF, B. T., Manzi S, Clarke AE, Ward MM (2007). "Prevalence of adult systemic lupus erythematosus in California and Pennsylvania in 2000: estimates obtained using hospitalization data." Arthritis & Rheumatism **56**(6): 2092.

Chen Q, V. E., Moghaddas S, Hoppel C and Lesnefsky E (2003). "Production of Reactive Oxygen Species by Mitochondria: CENTRAL ROLE OF COMPLEX III." Journal of Biological Chemistry **278**(38): 36027-36031.

Chen Z, C. C., Ho Y, Hamdy R and Chua B (2001). "Overexpression of Bcl-2 attenuates apoptosis and protects against myocardial I/R injury in transgenic mice." **280**(5): 2313-2320.

Chlopcíková S, P. J., Miketová P. (2001). "Neonatal rat cardiomyocytes--a model for the study of morphological, biochemical and electrophysiological characteristics of the heart." Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub **145**(2): 49-55.

Chowdhury, D. a. L., J (2008). "Death by a Thousand Cuts: Granzyme Pathways of Programmed Cell Death." Annual Review of Immunology **26**(1): 389-420.

Clerk A, C. S., Cullingford TE, Harrison JG, Jormakka M and Valks DM. (2003). "Regulation of cardiac myocyte cell death." Pharmacol Ther **97**: 223-261.

Cobb, M. (1999). "MAP kinase pathways." Progress in Biophysics and Molecular Biology **71**(3-4): 479-500.

Cojocaru M, C. I., Silosi I, Vrabie C (2011). "Manifestations of systemic lupus erythematosus " Maedica a journal of clinical medicine **6**(4): 330-336.

Cory S, H. D. a. A. J. (2003). "The Bcl-2 family: roles in survival and oncogenesis." Oncogene **22**: 8590-8607.

Costedoat-Chalumeau N, A. Z., Hulot J, Hammoud H, Aymard G, Cacoub P, Francès C, Wechsler B, Huong D, Ghillani P, Musset L, Lechat P and Piette J. (2006). "Low blood concentration of hydroxychloroquine is a marker for and predictor of disease exacerbations in patients with systemic lupus erythematosus." Arthritis & Rheumatism **54**(10): 3284-3290.

Crow, M. (2010). "Interferon-alpha: A Therapeutic Target in Systemic Lupus Erythematosus." Rheumatic Disease Clinics of North America **36**(1): 173-186.

Crow M, M. K., Nam Y and Kitsis R. (2004). "The Mitochondrial Death Pathway and Cardiac Myocyte Apoptosis." Circulation Research **95**(10): 957-970.

Cuevas P, C. F., Cuevas B, Gimenez-Gallego G, Martinez-Coso Vf (1997). "A non-mitogenic form of acidic fibroblast growth factor reduces neutro-
phil infiltration in rat ischemic reperfused heartmia/reperfusion. ." Eur J Med Reso
40: 155-161.

Cuevas P, C. F., Martinez-Coso V, Cuevas B, Fernandez-Ayerdi A, Reimers D, Asin-Cardiel E, Giménez-Gallego G (1999). "Cardioprotection from ischemia by fibroblast growth factor: role of inducible nitric oxide synthase." European Journal of Medical Research **4**(12): 517-524.

Daniel R, C. B. a. S. S. (1995). "Formation of Apoptotic Bodies Is Associated with Internucleosomal DNA Fragmentation during Drug-Induced Apoptosis." Experimental Cell Research **216**(1): 169-177.

Das A, S. F., Xi L, Rao Y, Kukreja R (2009). "ERK phosphorylation mediates sildenafil-induced myocardial protection against ischemia-reperfusion injury in mice." American Journal of Physiology - Heart and Circulatory Physiology **296**(5): H1236-H1243.

Davidson S, H. D., Duchen and Yellon D (2006). "Signalling via the reperfusion injury signalling kinase (RISK) pathway links closure of the mitochondrial permeability pore to cardioprotection." The International Journal of Biochemistry and Cell Biology **38**(3): 414-419.

Davies, K. A., A. M. Peters, et al. (1992). "Immune complex processing in patients with systemic lupus erythematosus. In vivo imaging and clearance studies." The Journal of Clinical Investigation **90**(5): 2075-2083.

Davignon, J. (2004). "The cardioprotective effects of statins." Curr Atheroscler Rep **6**(1): 27-35.

de Laat, H. B., R. H. W. M. Derksen, et al. (2004). "β2-glycoprotein I–dependent lupus anticoagulant highly correlates with thrombosis in the antiphospholipid syndrome." Blood **104**(12): 3598-3602.

Degterev, A. H., Z, Boyce, M. Li, Y. Jagtap, P. Mizushima, N. Cuny, G. Mitchison, T. Moskowitz, M and Yuan, J. (2005). "Chemical inhibitor of nonapoptotic cell death with therapeutic potenital for ischemic brain injury." Nature chemicals **1**: 112-119.

Delves, P. a. R., I (2000). "The Immune System." New England Journal of Medicine **343**(1): 37-49.

Descloux E, D. I., Cochat P, Vital-Durand D, Ninet J, Fabien N and Cimaz R (2009). "Influence of age at disease onset in the outcome of paediatric systemic lupus erythematosus." Rheumatology **48**(7): 779-784.

Deveraux Q, T. R., Salvesen G and Reed J. (1997). "X-linked IAP is a direct inhibitor of cell-death proteases." Nature **388**(300-304).

Doria A, T. A. a. L. M. (2008). "Challenges of lupus pregnancies." Rheumatology **47**(suppl 3): iii9-iii12.

Dos Santos P, K. A., Laclau M, Seetharaman S, Paucek P, Boudina S, Thambo J, Tariosse L, Garlid K (2002). Mechanisms by which opening the mitochondrial ATP- sensitive K+ channel protects the ischemic heart.

Downey J, D. A., Cohen M (2007). "Signaling pathways in ischemic preconditioning." Heart failure reviews **12**(3-4): 181-188.

Dumont E, H. L., van Heerde W, van den Eijnde S, Doevendans P, DeMuinck E, Daemen M, Smits Jos, Frederik P, Wellens H, Daemen Mat, Reutelingsperger C (2000). "Cardiomyocyte Death Induced by Myocardial Ischemia and Reperfusion: Measurement With Recombinant Human Annexin-V in a Mouse Model." Circulation **102**(13): 1564-1568.

Duncker D, K. C., Ishibashi Y, Herrlinger S, Pavek T, Bache R (1996). Effect of temperature on myocardial infarction in swine.

Duzgun N, Ş. M., GenÇ Y, Tutkak H (2007). "Antinucleosome Antibodies and Systemic Lupus Erythematosus." Annals of the New York Academy of Sciences **1109**(1): 421-428.

Eefting F, R. B., Wigman J, Pannekoek W, Liu W, Cramer M, Lips D, Doevendans P (2004). "Role of apoptosis in reperfusion injury." Cardiovascular Research **61**(3): 414-426.

Ehrenstein, M. (1999). "Antinuclear antibodies and lupus: causes and consequences." Rheumatology **38**(8): 691-693.

Eisenberg, R. (2005). "Update on rituximab." Annals of the Rheumatic Diseases **64**(suppl 4): iv55-iv57.

Elliott JR, M. S., Edmundowicz D (2007). "The role of preventative cardiology in systemic lupus erythematosus." Current Rheumatology Reports **9**(2): 125-130.

Elmore, S. (2007). "Apoptosis: A Review of Programmed Cell Death." Toxicologic Pathology **35**(4): 495-516.

Erwig L-P, H. P. M. (2008). "Clearance of apoptotic cells by pahgocytes." Cell death and differentiation **15**: 243-250.

Esdaile JM, A. M., Grodzicky T, Li Y, Panaritis C, du Berger R, Côte R, Grover SA, Fortin PR, Clarke AE, Senécal JL (2001). "Traditional Framingham risk factors fail to fully account for

accelerated atherosclerosis in systemic lupus erythematosus." Arthritis & Rheumatism **44**: 2331-2337.

Ezeonyeji, A. a. I., D (2012). "Early treatment with rituximab in newly diagnosed systemic lupus erythematosus patients: a steroid-sparing regimen." Rheumatology **51**(3): 476-481.

Fang H, L. A., Dahmen U and Dirsch O (2013). "Dual role of chloroquine in liver ischemia reperfusion injury: reduction of liver damage in early phase, but aggravation in late phase." Cell death and differentiation **4**.

Fang H, L. A., Dahmen U, Dirsch O. (2013). "Dual role of chloroquine in liver ischemia reperfusion injury: reduction of liver damage in early phase, but aggravation in late phase." Cell Death Dis **4**: e694.

Fleming A, N. T., Yoshimori T, Rubinsztein D (2011). "Chemical modulators of autophagy as biological probes and potential therapeutics." Nat Chem Biol **7**(1): 9-17.

Fleming S, E. R., Chai C, Girardi G, Holers V, Salmon J, Monestier M and Tsokos G (2004). "Anti-phospholipid antibodies restore mesenteric ischemia/reperfusion-induced injury in complement receptor 2/complement receptor 1-deficient mice." The Journal of Immunology **173**: 7055-7061.

Fleming S, E. R., Chai C, Girardi G, Holers V, Salmon J, Monestier M, Tsokos G (2004). "Anti-Phospholipid Antibodies Restore Mesenteric Ischemia/Reperfusion-Induced Injury in Complement Receptor 2/Complement Receptor 1-Deficient Mice." The Journal of Immunology **173**(11): 7055-7061.

Fleming S, M. M., Tsokos G (2004). "Accelerated Ischemia/Reperfusion-Induced Injury in Autoimmunity-Prone Mice." The Journal of Immunology **173**(6): 4230-4235.

Fliss, H. a. G., D (1996). "Apoptosis in Ischemic and Reperfused Rat Myocardium." Circulation Research **79**(5): 949-956.

Fox, R. (1993). "Mechanism of action of hydroxychloroquine as an antirheumatic drug." Semin Arthritis Rheumatology **23**(2): 82-91.

Franceschini, F. a. C., I. (2005). "Anti-Ro/SSA and La/SSB antibodies." Autoimmunity **38**(1): 55-63.

Frias MA, R. M., Gerber-Wicht C, Lang U. (2007). "Prostaglandin E2 activates Stat3 in neonatal rat ventricular cardiomyocytes: A role in cardiac hypertrophy." Cardiovasc Res **73**(1): 57-65.

Frostegard, J. (2005). "SLE, atherosclerosis and cardiovascular disease." Journal of Internal Medicine **257**(6): 485-495.

Fryer R, P. P., Hsu A, Gross G (2001). "Differential Activation of Extracellular Signal Regulated Kinase Isoforms in Preconditioning and Opioid-Induced Cardioprotection." Journal of Pharmacology and Experimental Therapeutics **296**(2): 642-649.

Fujio Y, N. T., Wencker D, Kitsis R, Walsh K (2000). "Akt Promotes Survival of Cardiomyocytes In Vitro and Protects Against Ischemia-Reperfusion Injury in Mouse Heart." Circulation **101**(6): 660-670.

Fulda, S. a. D., K (2000). "Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy." Oncogene **25**(34): 4798-4811.

Furie R, S. W., Ginzler EM, Becker M, Mishra N, Chatham W, Merrill JT, Weinstein A, McCune WJ, Zhong J, Cai W, Freimuth W (2008). "Biologic activity and safety of belimumab, a neutralizing anti-B-lymphocyte stimulator (BLyS) monoclonal antibody: a phase I trial in patients with systemic lupus erythematosus." Arthritis & Rheumatism **10**(5): 109.

Furst D, L. H., Baethge Bruce, Botstein G, Caldwell J, Dietz F, Ettlinger R, Golden H, McLaughlin G, Moreland L, Roberts N, Rooney T, Rothschild B, Sack M, Sebba A, Weisman M, Welch K, Yocum D (1999). "Dose-loading with hydroxychloroquine improves the rate of response in early, active rheumatoid arthritis: A randomized, double-blind six-week trial with eighteen-week extension." Arthritis & Rheumatism **42**(2): 357-365.

Furst DE, L. H., Baethge B, Botstein GR, Caldwell J, Dietz F, Ettlinger R, Golden HE, McLaughlin GE, Moreland LW, Roberts WN, Rooney TW, Rothschild B, Sack M, Sebba AI, Weisman

M, Welch KE, Yocum D. (1999). "Dose-loading with hydroxychloroquine improves the rate of response in early, active rheumatoid arthritis: a randomized, double-blind six-week trial with eighteen-week extension." Arthritis & Rheumatism **42**(2): 357-365.

Gabrielli F, A. E., Di Prima MA, Mazzacurati G, Masala C. (1995). "Cardiac valve involvement in systemic lupus erythematosus and primary antiphospholipid syndrome: lack of correlation with antiphospholipid antibodies." International Journal of Cardiology **51**(2): 117-126.

Galli M, B. T., Comfurius P, Maassen C, Hemker H C, Zwaal R, Bevers E, de Baets M, van Breda-Vriesman P (1990). "Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein cofactor." The Lancet **335**(8705): 1544-1547.

Galli M, C. P., Maassen C, Hemker HC, de Baets MH, van Breda-Vriesman PJ, Barbui T, Zwaal RF, Bevers EM. (1990). "Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein cofactor." Lancet **335**(8705): 1544-1547.

Ghaleb R, O. G., Ibrahim M (2011). "Avascular necrosis of bone in systemic lupus erythematosus." The Egyptian Rheumatologist **33**(1): 27-33.

Giannakopoulos B, P. F., Rahgozar S, Krilis S (2007). "Current concepts on the pathogenesis of the antiphospholipid syndrome." Blood **109**(2): 422-430.

Giannakopoulos, B. a. K., S (2013). "The Pathogenesis of the Antiphospholipid Syndrome." New England Journal of Medicine **368**(11): 1033-1044.

Giannouli S, V. M., Ziakas P and Tzioufas A (2005). "Anemia in Systemic lupus erythematosus: from pathophysiology to clinical assessment." Annals of the Rheumatic Diseases **65**(2): 144-148.

Giordano, F. (2005). "Oxygen, oxidative stress, hypoxia and heart failure." J Clin Invest **115**(3): 500-508.

Goldberg S, S. J., Neal J, Muzikansky A, Digumarthy S, Fidias P, Temel J, Heist R, Shaw A, McCarthy P, Lynch T, Sharma S, Settleman J, Sequist L (2012). "A Phase I Study of Erlotinib and Hydroxychloroquine in Advanced Non–Small-Cell Lung Cancer." Journal of thoracic oncology **7**(10): 1602-1608.

Goldsby R, K. T., Osborne BA, Kuby J (2003). "Immunology." W.H. Freeman and Company **Fifth edition**: 551.

Gottlieb, R. A., K. O. Burleson, et al. (1994). "Reperfusion injury induces apoptosis in rabbit cardiomyocytes." The Journal of Clinical Investigation **94**(4): 1621-1628.

Grennan DM, P. A., Manolios N, Huang Q, Hyland V, Dunckley H, Doran T, Gatenby P, Badcock C. (1997). "Family and twin studies in systemic lupus erythematosus." Dis Markers **13**(2): 93-98.

Griffiths EJ, H. A. (1995). "Mitochondrial non-specific pores remain closed during cardiac ischaemia, but open upon reperfusion." Biochem J **307**: 93-98.

Grillo-López AJ, W. C., Varns C, Shen D, Wei A, McClure A, Dallaire BK. (1999). "Overview of the clinical development of rituximab: first monoclonal antibody approved for the treatment of lymphoma." Semin Oncol **26**(5): 66-73.

Gross A, M. J., Korsmeyer S (1999). "BCL-2 family members and the mitochondria in apoptosis." Genes & Development **13**(15): 1899-1911.

Grossman, J. (2004). "Primary versus secondary antiphospholipid syndrome: Is this lupus or not?" Current Rheumatology Reports **6**(6): 445-450.

Gustafsson, Å. a. G., R (2009). "Autophagy in Ischemic Heart Disease." Circulation Research **104**(2): 150-158.

Gustafsson AB, G. R. (2008). "Recycle or die: the role of autophagy in cardioprotection." J Mol Cell Cardiol **44**: 654-661.

Hahn, B. (1998). "Antibodies to DNA." New England Journal of Medicine **338**(19): 1359-1368.

Halazun K, M. A., Aldouri A, Willis S, Ahmad N (2007). "Warm Ischemia in Transplantation: Search for a Consensus Definition." Transplantation Proceedings **39**(5): 1329-1331.

Hamacher-Brady A, B. N. a. G. R. (2006). "Enhancing Macroautophagy Protects against Ischemia/Reperfusion Injury in Cardiac Myocytes." The Journal of Biological Chemistry **281**: 29776-29787.

Haunsenloy, D., Maddock H, Baxter G and Yellon, D (2002). "Inhibiting mitochondrial permeability transition pore opening: a new paradigm for myocardial preconditioning." Cardiovascular Research **55**(3): 534-543.

Haunsenloy, D. a. Y., D (2007). "Reperfusion injury salvage kinase signalling: taking a RISK for cardioprotection." Heart Failure Reviews **12**(3-4): 217-234.

Hausenloy D, B. G., Bell R, Bøtker H, Davidson S, Downey J, Heusch G, Kitakaze M, Lecour S, Mentzer R, Mocanu M, ihaelaM, Ovize M, Schulz R, Shannon R, Walker M, Walkinshaw G, Yellon D (2010). "Translating novel strategies for cardioprotection: the Hatter Workshop Recommendations." Basic Research in Cardiology **105**(6): 677-686.

Hausenloy D, C. L., Laing, C, Kunst G, Pepper J, Kolvekar S, Evans R, Robertson S, Knight R, Ariti C, Clayton T, Yellon D (2012). "Effect of remote ischemic preconditioning on clinical outcomes in patients undergoing coronary artery bypass graft surgery (ERICCA): rationale and study design of a multi-centre randomized double-blinded controlled clinical trial." Clinical Research in Cardiology **101**(5): 339-348.

Hausenloy D, D. M., Yellon D (2003). "Inhibiting mitochondrial permeability transition pore opening at reperfusion protects against ischaemia–reperfusion injury." Cardiovascular Research **60**(3): 617-625.

Hausenloy D, M. M., Yellon D (2004). "Cross-talk between the survival kinases during early reperfusion: its contribution to ischemic preconditioning." Cardiovascular Research **63**(2): 305-312.

Hausenloy, D. a. Y., D (2004). "New directions for protecting the heart against ischaemic reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway." Cardiovascular Research **61**(3): 448-460.

Hausenloy, D. a. Y., D (2013). "Myocardial ischemia-reperfusion injury: a neglected therapeutic target." The Journal of Clinical Investigation **123**(1): 92-100.

Hausenloy DJ, M. H., Baxter GF, and Yellon DM (2002). " Inhibiting mitochondrial permeability transition pore opening: a new paradigm for myocardial preconditioning? ." Cardiovascular Research **55**: 534-543.

Hay E, B. P., Gordon P, Isenberg D, Maddison P, Snaith P, Symmons D, Viner D and Zoma A (1993). "The BILAG index: a reliable and valid instrument for measuring clinical disease activity in systemic lupus erythematosus." QJM: An International Journal of Medicine **86**(7): 447-458.

Hayward R, C. B., Shin Y, Scalia Rosario, Lefer A (1999). "Recombinant soluble P-selectin glycoprotein ligand-1 protects against myocardial ischemic reperfusion injury in cats." Cardiovascular Research **41**(1): 65-76.

Hearse, D. (1994). "Myocardial ischaemia: can we agree on a definition for the 21st century?" Cardiovas Res **28**(12): 1737-1744.

Hengartner, M. (2000). "The biochemistry of apoptosis." Nature **407**(6805): 770-776.

Herrmann M, V. R., Zoller OM, Hagenhofer M, Ponner BB, Kalden JR. (1998). "Impaired phagocytosis of apoptotic cell material by monocyte-derived macrophages from patients with systemic lupus erythematosus." Arthritis & Rheumatism **41**(7): 1241-1250.

Herrmann M, V. R., Zoller OM, Hagenhofer M, Ponner BB, Kalden JR. (1998). "Impaired phagocytosis of apoptotic cell material by monocyte-derived macrophages from patients with systemic lupus erythematosus." Arthritis & Rheumatism **41**(7): 1241-1250.

Herzog W, V. R., Schlossberg M, Edenbaum L, Scott H, Serebruany V (1997). "Short-term low dose intracoronary diltiazem administered at the onset of reperfusion reduces myocardial infarct size." International Journal of Cardiology **59**(1): 21-27.

Hochberg, M. (1997). "Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus." Arthritis & Rheumatism **40**(9): 1725.

Hofstra L, L. I., Dumont E, Boersma H, van Heerde W, Doevendans P, DeMuinck E, Wellens H, Kemerink G, Reutelingsperger C, Heidendal G (2000). "Visualisation of cell death in vivo in patients with acute myocardial infarction." The Lancet **356**(9225): 209-212.

Hom G, G. R., Modrek B, Taylor K, Ortmann W, Garnier S, Lee A, Chung S, Ferreira R, Pant P, Ballinger D, Kosoy R, Demirci F, Kamboh M, Amy H, Tian C, Gunnarsson I, Bengtsson A, Rantapää-Dahlqvist S, Petri M, Manzi S, Seldin M, Rönnblom L, Syvänen A, Gregersen P, Behrens T (2008). "Association of Systemic Lupus Erythematosus with C8orf13–BLK and ITGAM–ITGAX." New England Journal of Medicine **358**(9): 900-909.

Houssiau FA, L. C., Vanden Berghe M, Lambert M, Devogelaer JP, Renauld JC. (1995). "Serum interleukin 10 titers in systemic lupus erythematosus reflect disease activity." Lupus **4**(5): 393-395.

Hyejung J, R. B., Jiandong S, Zhaleh S, Dafna G, Murray U, Wendy Lou, and Paul F (2010). "The Protective Effect of Antimalarial Drugs on Thrombovascular Events in Systemic Lupus Erythematosus." American College of Rheumatology **62**(3): 863-868.

Ingram T, F. A., Bleasdale R, Ellins E, Margulescu A, Halcox J, James P (2013). "Low-Dose Sodium Nitrite Attenuates Myocardial Ischemia and Vascular Ischemia-Reperfusion Injury in Human Models." Journal of the American College of Cardiology **61**(25): 2534-2541.

Investigators, A. A. M. I. (2007). "Pexelizumab for acute st-elevation myocardial infarction in patients undergoing primary percutaneous coronary intervention: A randomized controlled trial." JAMA **297**(1): 43-51.

Ioannou Y, P. C., Giles I, Latchman DS, Isenberg DA and Rahman A (2007). "Binding of antiphospholipid antibodies to discontinuous epitopes on domain I of human beta(2)-glycoprotein I: mutation studies including residues R39 to R43." Arthritis & Rheumatism **56**(1): 280-290.

Ioannou Y, R.-P. Z., Pericleous C, Giles I, Papalardo E, Vargas G, Shilagard T, Latchman DS, Isenberg DA, Rahman A, Pierangeli S. (2009). "In vivo inhibition of antiphospholipid antibody-induced pathogenicity utilizing the antigenic target peptide domain I of beta2-glycoprotein I: proof of concept." J Thromb Haemost **7**(5): 833-842.

Ioannou Y, R. A. (2010). "Domain I of beta2-glycoprotein I: its role as an epitope and the potential to be developed as a specific target for the treatment of the antiphospholipid syndrome." Lupus **19**(4): 400-405.

Ioannou, Y. a. I., D (2002). "Current concepts for the management of systemic lupus erythematosus in adults: a therapeutic challenge." Postgraduate Medical Journal **78**(924): 599-606.

Isenberg D, R. A., Allen E, Farewell V, Akil M, Bruce I, D'Cruz D, Griffiths B, Khamashta M, Maddison P, McHugh N, Snaith M, Teh L, Yee C, Zoma A, and Gordon C "BILAG 2004. Development and initial validation of an updated version of the British Isles Lupus Assessment Group's disease activity index for patients with systemic lupus erythematosus." Rheumatology **44**(7): 902-906.

Isenberg DA, S. M. (1981). "Muscle Disease in systemic lupus erythematosus: a study of its nature, frequency and cause." J Rheumatol **8**(6): 917-924.

Isenberg, D. a. M., P (1987). "Detection of antibodies to double stranded DNA and extractable nuclear antigen." J Clin Pathol **40**(11): 1374-1381.

Iwata Y, W. T., Furuichi K, Sakai N, Matsushima K, Yokoyama H, Kobayashi K (2003). "p38 Mitogen-Activated Protein Kinase Contributes to Autoimmune Renal Injury in MRL-Faslpr Mice." Journal of the American Society of Nephrology **14**(1): 57-67.

Jaenisch, R. a. B., A (2003). "Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals." Nature Genetics **33**: 245-254.

James J, G. T., Scofield R, Harley J (1995). "Immunoglobulin Epitope Spreading and Autoimmune Disease After Peptide Immunization: Sm B/B'-derived PPPGMRPP and PPPGIRGP Induce Spliceosome Autoimmunity." J Exp Med **181**(2): 453-461.

Janeway, C. a. M., R (2002). "Innate immune recognition." Ann Rev Immunology **20**: 197-216.

Jassem, W. a. H., N (2004). "The role of mitochondria in ischemia//reperfusion injury in organ transplantation." Kidney Int **66**(2): 514-517.

Jeffries, M. a. S., A. (2011). "Epigenetics in systemic lupus erythematosus: leading the way for specific therapeutic agents." International Journal of Clinical Rheumatology **6**(4): 423-438.

Jennings RB, S. H., Smyth GA, Flack HA, Linn H (1960). "Myocardial necrosis induced by temporary occlusion of a coronary artery in the dog." Arch Pathol **70**: 68-78.

Jonassen AK, A. E., Riemersma RA, Mjøs OD, Larsen TS. (2000). "Glucose-insulin-potassium reduces infarct size when administered during reperfusion." Cardiovas Drugs Ther **12**(6): 615-623.

Jung H, B. R., Shariati-Sarabi Z, Gladmann D, Urowitz M, Lou W, Fortin P (2010). "The protective effect of antimalrial drugs on thrombovascular events in systemic lupus erythematosus." Arthritis and Rheumatism **62**(3): 863-868.

Jury E, F.-B. F. a. K. P. (2007). "Lipid rafts in T cell signalling and disease." Seminars in Cell & Developmental Biology **18**(5): 608-615.

Kang R, Z. H., Lotze M, Tang D. (2011). "The Beclin 1 network regulates autophagy and apoptosis." Cell death and differentiation **18**: 571-580.

Karim M, A. P., Cuadrado M, Abbs I, D'Cruz D, Khamashta M, Hughes G (2002). "Mycophenolate mofetil for systemic lupus erythematosus refractory to other immunosuppressive agents." Rheumatology **41**(8): 876-882.

Keeley EC, B. J., Grines CL (2003). "Primary angioplasty versus intravenous thrombolytic therapy for acute myocardial infarction: a quantitative review of 23 randomised trials." Lancet **361**: 13-20.

Keeling D, M. I., Moore G, Greer I, Greaves M (2012). "Guidelines on the investigation and management of antiphospholipid syndrome." British Journal of Haematology **157**(1): 47-58.

Kerr J, W. A., and Currie R (1972). "Apoptosis: A Basic Biological Phenomenon with Wide-ranging Implications in Tissue Kinetics." Br J Cancer **26**(4): 239-257.

Keshet, Y. a. S., R (2010). The MAP Kinase Signaling Cascades: A System of Hundreds of Components Regulates a Diverse Array of Physiological Functions. MAP Kinase Signaling Protocols. R. Seger, Humana Press. **661:** 3-38.

Kinnally K, P. P., Ryu S, Dejean L (2011). "Is mPTP the gatekeeper for necrosis, apoptosis, or both?" Biochimica et Biophysica Acta (BBA) - Molecular Cell Research **1813**(4): 616-622.

Kiss E, C. I., Cohen JH, Reveil B, Kávai M, Szegedi G. (1996). "CR1 density polymorphism and expression on erythrocytes of patients with systemic lupus erythematosus." Autoimmunity **25**(1): 53-58.

Korb, L. a. A., J (1997). "C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited." The Journal of Immunology **158**(10): 4525-4528.

Koyasu, S. (2003). "The role of PI3K in immune cells." Nat Immunol **4**(4): 313-319.

Krijnen P, N. R., Meijer C, Visser C, Hack C, Niessen H (2002). "Apoptosis in myocardial ischaemia and infarction." Journal of Clinical Pathology **55**(11): 801-811.

Krysko D, V. B. T., D'Herde K, Vandenabeele P (2008). "Apoptosis and necrosis: Detection, discrimination and phagocytosis." Methods **44**(3): 205-221.

Kukreja R, S. F., Das A, Ockaili R, Yin C, Bremer Y, Fisher P, Wittkamp M, Hawkins J, Chou E, Kukreja A, Wang X, Marwaha V, Xi L (2005). "Pharmacological preconditioning with sildenafil: Basic mechanisms and clinical implications." Vascular Pharmacology **42**(5–6): 219-232.

Kumphune S, C. S., Chattipakorn N (2012). "Role of p38 inhibition in cardiac ischemia/reperfusion injury." Eur J Clin Pharmacol **68**: 513-524.

Kung G, K. K., Kitsis R (2011). "Programmed Necrosis, Not Apoptosis, in the Heart." Circulation Research **108**(8): 1017-1036.

Kuznik A, B. M., Svajger U, Jeras M, Rozman B and R. Jerala (2011). "Mechanism of Endosomal TLR Inhibition by Antimalarial Drugs and Imidazoquinolines." The Journal of Immunology **186**(8): 4794-4804.

Lambrianides A, C. C., Pierangeli S, Pericleous C, Branch W, Rice J, Latchman DS, Townsend P, Isenberg DA, Rahman A, Giles IP (2010). "Effects of polyclonal IgG derived from patients with different clinical types of the antiphospholipid syndrome on monocyte signaling pathways." J Immunol **184**(12): 6622-6628.

Lambrianides A, C. C., Pierangeli S, Pericleous Charis, Branch W, Rice J, Latchman D, Townsend P, Isenberg D, Rahman A, Giles I (2010). "Effects of Polyclonal IgG Derived from Patients with Different Clinical Types of the Antiphospholipid Syndrome on Monocyte Signaling Pathways." The Journal of Immunology **184**(12): 6622-6628.

Lawrence RC, H. C., Arnett FC, Deyo RA, Felson DT, Giannini EH, Heyse SP, Hirsch R, Hochberg MC, Hunder GG, Liang MH, Pillemer SR, Steen VD, Wolfe F (1998). "Estimates of the prevalence of arthritis and selected musculoskeletal disorders in the United States." Arthritis & Rheumatism **41**(5): 778.

Le N, T. Y., Izawa-Ishizawa Y, Heo K, Lee H, Smrcka A, Miller B, Ko K, Ture S, Morrell C, Fujiwara K, Akaike M, Abe J (2014). "Identification of Activators of ERK5 Transcriptional Activity by High-Throughput Screening and the Role of Endothelial ERK5 in Vasoprotective Effects Induced by Statins and Antimalarial Agents." The Journal of Immunology **193**(7): 3803-3815.

Lee P, S. M., Lefer DJ, Factor SM, Walsh K, Kitsis RN (2003). " Fas pathway is a critical mediator of cardiac myocyte death and MI during ischemia-reperfusion in vivo." Am J Physiol Heart Circ Physiol **284**: 456-463.

Lee S, S. E., Bargman J (2011). "The role of antimalarial agents in the treatment of SLE and lupus nephritis." Nat Rev Nephrol **7**: 718-729.

Leong, K. K., ET. Feng, PH and Boey, ML. (1994). "Lipid profiles in patients with systemic lupus erythematosus." J Rheumatol **21**(7): 1264-1267.

Levine J, B. W., Rauch J (2002). "The Antiphospholipid Syndrome." New England Journal of Medicine **346**(10): 752-763.

Li, C. a. J., R (2002). Reactive species mechanisms of cellular hypoxia-reoxygenation injury.

Li L, L. X., Wang X (2001). "Endonuclease G is an apoptotic DNase when released from mitochondria." Nature **412**: 95-99.

Linker-Israeli M, D. R. J., Wallace D J, Prehn J, Ozeri-Chen T, Klinenberg J R (1991). "Elevated levels of endogenous IL-6 in systemic lupus erythematosus. A putative role in pathogenesis." The Journal of Immunology **147**(1): 117-123.

Linker-Israeli M, Q. F. J., Horwitz DA (1990). "CD8+ lymphocytes from patients with systemic lupus erythematosus sustain, rather than suppress, spontaneous polyclonal IgG production and synergize with CD4+ cells to support autoantibody synthesis." Arthritis & Rheumatism **33**(8): 1216-1225.

Linkermann, A. a. G., D (2014). "Necroptosis." New England Journal of Medicine **370**(5): 455-465.

M Rapoport, A. S., D Aharoni, O Bloch, H Zinger, M Dayan and E Mozes (2005). "Amelioration of SLE-like manifestatiions in (NZBxNZW)F1 mice following treatment with a peptide based on the complementarity determining region 1 of an autoantibody is associated with a down-regulation of apoptosis and of the pro-apoptotic factor JNK kinase." Clinical Immunology **117**(3): 262-270.

M Sorice, V. P., T Griggi, A Losardo, O Leri, M S Magno, R Misasi, and G Valesini (2000). "Specificity of anti-phospholipid antibodies in infectious mononucleosis: a role for anti-cofactor protein antibodies." Clinical and Experimental Immunology **120**(2): 301-306.

M, W. (2001). "Complement." New England Journal of Medicine **344**(15): 1140-1144.

Ma S, W. Y., Chen Y, Cao F "The role of the autophagy in myocardial ischemia/reperfusion injury." Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease(0).

Ma X, T. P., Lefer A (1991). "Antibody to CD-18 exerts endothelial and cardiac protective effects in myocardial ischemia and reperfusion." The Journal of Clinical Investigation **88**(4): 1237-1243.

Mahaffey KW, P. J., Barbagelata NA, DiCarli MF, Leesar MA, Browne KF, Eisenberg PR, , C. A. Bolli R, Molina-Viamonte V, Orlandi C, Blevins R, Gibbons RJ, Califf RM, Granger , et al. (1999). "Adenosine as an adjunct to thrombolytic therapy for acute myocardial infarction: results of a

multicenter, randomized, placebo-controlled trial: the Acute Myocardial Infarction STudy of

ADenosine (AMISTAD) trial." J.Am.Coll.Cardiol **34**: 1711-1720.

Mak, T. a. Y., W (2002). "Signaling for survival and apoptosis in the immune system." Arthritis Res **4**(Suppl 3): S243 - S252.

Manderson AP, B. M., Walport MJ (2004). "The role of complement in the development of systemic lupus erythematosus

" Annual Rev Immunol **22**: 431-456.

Manzi, S., F. Selzer, et al. (1999). "Prevalence and risk factors of carotid plaque in women with systemic lupus erythematosus." Arthritis & Rheumatism **42**(1): 51-60.

Marmor M, K. U., Lai T, Lyons J, Mieler W (2011). "Revised Recommendations on Screening for Chloroquine and Hydroxychloroquine Retinopathy." Ophthalmology **118**(2): 415-422.

Martin J, A. M., Quinlan R, Cohen P, Marber M (2001). "Ant-ischemic Effects of SB203580 Are Mediated Through the Inhibition of p38α Mitogen-Activated Protein Kinase: Evidence From Ectopic Expression of an Inhibition-Resistant Kinase." Circulation Research **89**(9): 750-752.

Martinez-Lopez N, A. D., Mishall P, Sahu S, Singh R (2013). "Autophagy proteins regulate ERK phosphorylation." Nat Commun **4**.

Martinez-Lopez N. Athonvarangkul D, M. P., Sahu S, Singh R (2013). "Autophagy proteins regulate ERK phosphorylation." Nat Commun **4**.

Martinez-Lopez, N. A., D. Mishall, P. Sahu, S. Singh, R (2013). "Autophagy proteins regulate ERK phosphorylation." Nature Communications **4**.

Matsui Y, T. H., Qu X, Abdellatif M, Sakoda H, Asano T, Levine B and Sadoshima J (2007). "Distinct roles of autophagy in the heart during ischemia and reperfusion." Circulation Research **100**: 914-922.

Matsuura E, I. Y., Fujimoto M, Ichikawa K, Koike T. (1990). "Anticardiolipin cofactor(s) and differential diagnosis of autoimmune disease." Lancet **336**(8708): 177-178.

McCormick J, S. N., Scarabelli T, Knight R, Latchman D, Stephanou A. (2012). "STAT1 deficiency in the heart protects against myocardial infarction by enhancing autophagy." Journal of Cellular and Molecular Medicine **16**(2): 386-393.

McCully J, w. H., Hsieh Y, Jones M and Levitsky S (2004). "Differntial contibution of necrosis and apoptosis in myocardial ischemia-reperfusion injury." Heart and Circulatoy Physiology **286**(5): 1923-1935.

McCully J, W. H., Hsieh Y, Jones M, Levitsky S (2004). "Differential contribution of necrosis and apoptosis in myocardial ischemia-reperfusion injury." American Journal of Physiology - Heart and Circulatory Physiology **286**(5): H1923-H1935.

McDonald G, D. S., Miguel L, Hall C, Isenberg D, Magee A, Butters T and Jury E (2014). "Normalizing glycosphingolipids restores function in CD4+ T cells from lupus patients." J Clin Invest **124**(2): 712-724.

Mcllroy D, S. H., Talanian R, Nagata S (1999). "Involvement of caspase 3-activated DNase in internucleosomal DNA cleavage induced by diverse apoptotic stimuli." Oncogene **18**(31): 4401-4408.

McNeil H, S. R., Chesterman C, and Krilis S (1990). "Anti-phospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: beta 2-glycoprotein I (apolipoprotein H)." PNAS **87**(11): 4120-4124.

Medzhitov, R. a. J., C (2000). "Innate Immunity." New England Journal of Medicine **343**(5): 338-344.

Meroni P, P. F., Foco L, Bernardinelli L, Fetiveau R, Mannucci P, Tincani A (2007). "Anti-beta 2 glycoprotein I antibodies and the risk of myocardial infarction in young premenopausal women." Journal of Thrombosis and Haemostasis **5**(12): 2421-2428.

Merrill J, N. M., Wallace D, Shanahan J, Latinis K, Oates J, Utset T, Gordon C, Isenberg D, Hsieh H, Zhang D, Brunetta P (2010). "Efficacy and safety of rituximab in moderately-to-severely active systemic lupus erythematosus: The randomized, double-blind, phase ii/iii systemic lupus erythematosus evaluation of rituximab trial." Arthritis & Rheumatism **62**(1): 222-233.

Mishra N, R. C., Brown DR, Ruiz P, Gilkeson GS (2003). "Histone deacetylase inhibitors modulate renal disease in the MRL-lpr/lpr mouse." J Clin Invest **111**: 539-552.

Miyakis S, L. M., Atsumi T, Branch D, Brey R, Cervera R, Derksen R, De Groot P, Koike T, Meroni P, Reber G, Shoenfeld Y, Tincani A, Vlachoyiannopoulos P, Krilis S (2006). "International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS)." Journal of Thrombosis and Haemostasis **4**(2): 295-306.

Miyamae M, C. S., Schiner M and V. M. Figueredo (1996). Attenuation of postischemic reperfusion injury is related to prevention of [Ca2+]m overload in rat hearts.

Mizushima, N. (2007). "Autophagy: process and function." Genes & Development **21**(22): 2861-2873.

Mok, C. a. L., C (2003). "Pathogenesis of systemic lupus erythematosus." J Clin Pathol **56**(7): 481-490.

Monneaux, F. a. M., S (2002). "Epitope spreading in systemic lupus erythematosus: Identification of triggering peptide sequences." Arthritis & Rheumatism **46**(6): 1430-1438.

Moore PA, B. O., Orr A, Pieri K, LaFleur DW, Feng P, Soppet D, Charters M, Gentz R, Parmelee D, Li Y, Galperina O, Giri J, Roschke V, Nardelli B, Carrell J, Sosnovtseva S, Greenfield W, Ruben SM, Olsen HS, Fikes J, Hilbert DM. (1999). "BLyS: member of the tumor necrosis factor family and B lymphocyte stimulator." Science **285**(5425): 260-263.

Morris S, W. M., Anthoe J, Sartorius J, Kirchner H, Dancea S, Bili A (2011). "Hydroxychloroquine use associated with improvement in lipid profiles in rheumatoid arthritis patients." Arthritis care and research **63**(4): 530-534.

Morrow W, N. J., Watts R, Isenberg D (1999). Autoimmune & Rheumatic disease, Oxford, Oxford University Press.

Murphy, E. a. S., C (2008). Mechanisms Underlying Acute Protection From Cardiac Ischemia-Reperfusion Injury.

Murry C, J. R., Reimer K (1986). "Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium." Circulation **74**(5): 1124-1136.

Nagai T, A. Y., Yanagida T, Yamamoto K, Hirohata S. (2005). "Anti-ribosomal P protein antibody in human systemic lupus erythematosus up-regulates the expression of proinflammatory cytokines by human peripheral blood monocytes." Arthritis & Rheumatism **52**(3): 847-855.

Nagarkatti, D. a. S., R (1998). "Role of p38 MAP Kinase in Myocardial Stress." Journal of Molecular and Cellular Cardiology **30**(8): 1651-1664.

Niewold T, C. D., Salloum R, Poole B (2010). "Interferon Alpha in Systemic Lupus Erythematosus." Journal of Biomedicine and Biotechnology **2010**: 8.

Nishimoto, S. a. N., E (2006). MAPK signalling: ERK5 versus ERK1/2.

Nishimoto, S. a. N., E (2006). "MAPK signalling: ERK5 versus ERK1/2." EMBO reports **7**(8): 782-786.

Noh HS, S. I., Ha JH, Hah YS, Baek SM and Kim DR (2010). "Propofol protects the autophagic cell death induced by the ischemia/reperfusion injury in rats." Mol Cells **30**(5): 455-460.

Obermoser, G. a. P., V (2010). "The interferon-α signature of systemic lupus erythematosus." Lupus **19**(9): 1012-1019.

Ohno M, T. G., Ohno A, Misao J, Hayakawa Y, Minatoguchi S, Fujiwara T, Fujiwara H (1998). ""“Apoptotic” Myocytes in Infarct Area in Rabbit Hearts May Be Oncotic Myocytes With DNA Fragmentation: Analysis by Immunogold Electron Microscopy Combined With In Situ Nick End-Labeling." Circulation **98**(14): 1422-1430.

Padua R, M. P., Doble B, Yu C, Zahradka P, Pierce G, Panagia V, Kardami E (1998). "FGF-2-induced Negative Inotropism and Cardioprotection are Inhibited by Chelerythrine: Involvement of Sarcolemmal Calcium-independent Protein Kinase C." Journal of Molecular and Cellular Cardiology **30**(12): 2695-2709.

Padua RR, M. P., Doble BW, Yu CH, Zahradka P, Pierce GN, Panagia V, Kardami E. and Source (1998). "FGF-2-induced negative inotropism and cardioprotection are inhibited by chelerythrine: involvement of sarcolemmal calcium-independent protein kinase C." J Mol Cell Cardiol **30**(12): 2695-2709.

Palojoki E, S. A., Eriksson A, Pulkki K, Kallajoki M, Voipio-Pulkki L, Tikkanen I (2001). Cardiomyocyte apoptosis and ventricular remodeling after myocardial infarction in rats.

Pareek A, Y. P., Tenpe C, Chandurkar N and Payghan R. (2009). "Effect of atorvastatin and hydroxychloroquine combination on blood glucose in alloxan-induced diabetic rats." Indian Journal of Pharmacology **41**(3): 125-128.

Park H, L. Y., Lin S, Wang L, Yang J, Wu H (2007). "The Death Domain Superfamily in Intracellular Signaling of Apoptosis and Inflammation." Annual Review of Immunology **25**(1): 561-586.

Parker, B. a. B., IN (2007). "High dose methylprednisolone therapy for the treatment of severe systemic lupus erythematosus." Lupus **16**(6): 387-393.

Petri M, K. M., Kalunian K, Grossman J, Hahn B, Sammaritano L, Lockshin M, Merrill J, Belmont M, Askanase A, McCune J, Hearth-Holmes M, Dooley M, Von Feldt J, Friedman A, Tan M, Davis J, Cronin M, Diamond B, Mackay M, Sigler L, Fillius M, Rupel A, Licciardi F, Buyon Jill P. (2005). "Combined Oral Contraceptives in Women with Systemic Lupus Erythematosus." New England Journal of Medicine **353**(24): 2550-2558.

Pierangeli SS, V.-O. M., Raschi E, Liu X, Romay-Penabad Z, De Micheli V, Galli M, Moia M, Tincani A, Borghi MO, Nguyen-Oghalai T, Meroni PL. (2007). "Toll-like receptor and antiphospholipid mediated thrombosis: in vivo studies." Ann Rheum Dis **66**(10): 1327-1333.

Pieter D, V., Mirella A. van den Doel, Sandra de Zeeuw, Dirk J. Duncker (1998). "Animal models in the study of myocardial ischaemia and ischaemic syndromes." Cardiovascular Research **39**: 121-135.

Piot C, C. P., Staat P, Thibault H, Rioufol G, Mewton N, Elbelghiti R, Cung T, Bonnefoy E, Angoulvant D, Macia C, Raczka F, Sportouch C, Gahide G, Finet G, André-Fouët X, Revel D, Kirkorian G, Monassier J, Derumeaux G, Ovize M (2008). "Effect of Cyclosporine on Reperfusion Injury in Acute Myocardial Infarction." New England Journal of Medicine **359**(5): 473-481.

Pompilio G, P. G., Rossoni G, Porqueddu M, Berti F, Barajon I, Petruccioli M, Guarino A, Aguggini G, Biglioli P, Sala A (1997). "Effects of Warm Ischemia on Valve Endothelium." Ann Thorac Surg **63**: 656-662.

Pons-Estel GJ, A. G., Scofield L, Reinlib L, Cooper GS (2010). "Understanding the epidemiology and progression of systemic lupus erythematosus." Semin Arthritis Rheumatology **39**(4): 257.

Poulton K, R. A. a. G. I. (2011). "Examining how antiphospholipid antibodies activate intracellular signaling pathways: a systematic review." Semin Arthritis Rheumatology **41**(5): 720-736.

Qian T, N. A., Herman B, Lemasters J (1997). Mitochondrial permeability transition in pH-dependent reperfusion injury to rat hepatocytes.

Rabinowitz, J. W., E (2010). "Autophagy and Metabolism." Scienc **330**: 1344-1348.

Rahman, A. a. E., R (2006). "The role of toll-like receptors in systemic lupus erythematosus." Springer seminars in immunopathology **28**(2): 131-143.

Rahman, A. a. I., D (2008). "Systemic Lupus Erythematosus." New England Journal of Medicine **358**(9): 929-939.

Rand J, W. X., Quinn A, Ashton A, Chen, Pojen P, Hathcock J, Andree H, Taatjes D (2010). "Hydroxychloroquine protects the annexin A5 anticoagulant shield from disruption by antiphospholipid antibodies: evidence for a novel effect for an old antimalarial drug." Blood **115**(11): 2292-2299.

Richardson, G. G. a. B. (2010). "Key role of ERK pathway signaling in lupus." Autoimmunity **43**(1): 17.

Romero-Diaz J, I. D., Ramsey-Goldman R (2011). "Measures of adult systemic lupus erythematosus: Updated Version of British Isles Lupus Assessment Group (BILAG 2004), European Consensus Lupus Activity Measurements (ECLAM), Systemic Lupus Activity Measure, Revised (SLAM-R), Systemic Lupus Activity Questionnaire for Population Studies (SLAQ), Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K), and Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index (SDI)." Arthritis Care & Research **63**(S11): S37-S46.

Roviezzo F, C. S., Di Lorenzo A, Brancaleone V, Mazzon E, Di Paola R, Bucci M, Cirino G (2007). "Protective role of PI3-kinase-Akt-eNOS signalling pathway in intestinal injury associated with splanchnic artery occlusion shock." British Journal of Pharmacology **151**(3): 377-383.

Rovin B, F. R., Latinis K, Looney R, Fervenza F, Sanchez-Guerrero J, Maciuca R, Zhang D, Garg J, Brunetta P, Appel G (2012). "Efficacy and safety of rituximab in patients with active proliferative lupus nephritis: The lupus nephritis assessment with rituximab study." Arthritis & Rheumatism **64**(4): 1215-1226.

Ruiz-Arruza I, U. A., Cabezas-Rodriguez I, Medina J, Moran M, Ruiz-Irastorza G (2014). "Glucocorticoids and irreversible damage in patients with systemic lupus erythematosus." Rheumatology **53**(8): 1470-1476.

Rumore, P. a. S., CR (1990). "Endogenous circulating DNA in systemic lupus erythematosus. Occurrence as multimeric complexes bound to histone." J Clin Invest **86**(1): 69-74.

Salahudeen, A. (2004). Cold ischemic injury of transplanted kidneys: new insights from experimental studies.

Salmon J, M. S., Schachter L, Arnett F, Ginzler E, Gourley M, Ramsey-Goldman R, Peterson M, Kimberly R (1996). "Fc gamma RIIA alleles are heritable risk factors for lupus nephritis in African Americans." The Journal of Clinical Investigation **97**(5): 1348-1354.

Salmon, M. a. G., C. (1999). "The role of apoptosis in systemic lupus erythematosus." Rheumatology **38**(12): 1177-1183.

Santiago-Raber ML, D.-S. I., Wu T, Li QZ, Uematsu S, Akira S, Reith W, Mohan C, Kotzin BL, Izui S (2010). "Critical role of TLR7 in the acceleration of systemic lupus erythematosus in TLR9-deficient mice." J Autoimmunity **34**(4): 339-348.

Satoo K, N. N., Kumeta H, Fujioka Y, Mizushima N, Ohsumi Y, Inagaki F (2009). "The structure of Atg4B-LC3 complex reveals the mechanism of LC3 processing and delipidation during autophagy." The EMBO Journal **28**: 1341-1350.

Satta N, K. E., Fickentscher C, Dunoyer-Geindre S, Boehlen F, Reber G, Burger D, de Moerloose P (2011). "Toll-like receptor 2 mediates the activation of human monocytes and endothelial cells by antiphospholipid antibodies." Blood **117**(20): 5523-5531.

Scarabelli T, S. A., Rayment N, Pasini E, Comini L, Curello S, Ferrari R, Knight R and Latchman D (2001). "Apoptosis of Endothelial cells precedes myocyte cell apoptosis in Ischemia/Reperfusion Injury." Circulation **104**: 253-256.

Schulman S, S. E., Granqvist S (1998). "Anticardiolipin Antibodies Predict Early Recurrence of Thromboembolism and Death Among Patients with Venous Thromboembolism Following Anticoagulant Therapy." The American Journal of Medicine **104**(4): 332-338.

Sciascia S, B. M., Baldovino S, Roccatello D, Khamashta M, Sannam G (2013). "Central nervous system involvement in systemic lupus erythematosus: Overview on classification criteria." Autoimmunity Reviews **12**(3): 426-429.

Sciascia S, L.-P. C., Roccatello D, Cuadrado M (2012). "Catastrophic antiphospholipid syndrome (CAPS)." Best Practice & Research Clinical Rheumatology **26**(4): 535-541.

Selker H, B. J., Sheehan P (2012). "Out-of-hospital administration of intravenous glucose-insulin-potassium in patients with suspected acute coronary syndromes: The immediate randomized controlled trial." JAMA **307**(18): 1925-1933.

Shaid S, B. C., Serve H, Dikic I (2013). "Ubiquitination and selective autophagy." Cell Death Differ **20**(1): 21-30.

Shaul, Y. a. S., R (2007). "The MEK/ERK cascade: From signaling specificity to diverse functions." Biochimica et Biophysica Acta (BBA) - Molecular Cell Research **1773**(8): 1213-1226.

Shen M, W. R., Zhao L, Gup H, Fan R, Cui Y, Wang Y, Yue S, Pei J (2012). "Resveratrol Attenuates Ischemia/Reperfusion Injury in Neonatal Cardiomyocytes and Its Underlying Mechanism." PLoS One **7**(12): 512223.

Shen M, W. R., Zhao L, Li J, Guo H, Fan R, Cui Y, Wang Y, Yue S, Pei J (2012). "Resveratrol Attenuates Ischemia/Reperfusion Injury in Neonatal Cardiomyocytes and Its Underlying Mechanism." PLoS ONE **7**(12): e51223.

Shoenfeld Y, B. M., Cervera R, Font J, Raschi E, Meroni P-L (2006). "Infectious origin of the antiphospholipid syndrome." Annals of the Rheumatic Diseases **65**(1): 2-6.

Simonis G, S. R. a. E. B. (2012). "Reperfusion injury in acute myocardial infarction." Critical Care **16**(2).

Skyschally A, S. R., Heusch G (2010). "Cyclosporine A at Reperfusion Reduces Infarct Size in Pigs." Cardiovascular Drugs and Therapy **24**(1): 85-87.

Srinivasula S, H. R., Saleh A, Datta P, Shiozaki E, Chai, J, Lee R, Robbins P, Fernandes-Alnemri T, Shi Y, Alnemri E (2001). "A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis." Nature **410**(6824): 112-116.

Stephanou A, B. B., Scarabelli T, Jonassen A, Yellon D, Marber M, Knight R, Latchman D (2000). "Ischemia-induced STAT-1 Expression and Activation Play a Critical Role in Cardiomyocyte Apoptosis." Journal of Biological Chemistry **275**(14): 10002-10008.

Stohl, W. (2013). "Future prospects in biologic therapy for systemic lupus erythematosus." Nat Rev Rheumatol **9**(12): 705-720.

Stowe, D. a. C., A (2009). "Mitochondrial Reactive Oxygen Species Production in Excitable Cells: Modulators of Mitochondrial and Cell Function." Antioxidant Redox Signal **11**(6): 1373-1414.

Stuckey D, C. C., Camellii P, Tyler D, Davies K, Clarke K. (2012). "In vivo MRI Characterization of Progressive Cardiac Dysfunction in the mdx Mouse Model of Muscular Dystrophy." plos one.

Su H, J. L., Xing W, Zhang W, Zhou H, Qian X, Wang X, Gao F, Sun X and Zhang H (2013). "Acute hyperglycaemia enhances oxidative stress and aggravates myocardial ishaemia/reperfusion injury: role of thioredoxin-interacting protein." J Cell Mol Med **17**(1): 181-191.

Swann J, S. M. (2007). "Immune surveillance of tumors." J Clin Invest **117**(5): 1137-1146.

Tackey E, L. P. a. I. G. (2004). "Rationale for interleukin-6 blockade in systemic lupus erythematosus." Lupus **13**(5): 339-343.

Taimor G, L. H., Hofstaetter B, Schlüter K.-D, Piper H.M (1999). Induction of necrosis but not apoptosis after anoxia and reoxygenation in isolated adult cardiomyocytes of rat.

Tait, S. a. G., D (2010). "Mitochondria and cell death: outer membrane permeabilization and beyond." Nat Rev Mol Cell Biol **11**(9): 621-632.

Tan EM, C. A., Fries JF, Masi AT, McShane DJ, Rothfield NF, et al (1982). "The 1982 Revised Criteria for Classification of Systemic Lupus Erythematosus." Arthritis & Rheumatism **25**: 1271-1277.

Taylor R, C. S., Martin S (2008). "Apoptosis: controlled demolition at the cellular level." Nat Rev Mol Cell Biol **9**: 231-241.

Todorovic Z, M. B., Basta-Jovanovic G, Radojevic Skodric S, Stojanovic R, Rovcanin B, Prostran M (2014). "Acute Pretreatment with Chloroquine Attenuates Renal I/R Injury in Rats." PLoS ONE **9**(3): e92673.

Todorovic Z, M. B., Basta-Jovanovic G, Skodric S, Stojanovic R, Rovcanin B and Prostran M (2014). "Acute pretreatment with Chloroquine Attenuates Renal I/R Injury in Rats." PloS one **9**(3).

Tong H, C. W., Steenbergen C, Murphy E (2000). "Ischemic Preconditioning Activates Phosphatidylinositol-3-Kinase Upstream of Protein Kinase C." Circulation Research **87**(4): 309-315.

Tong H, I. K., Steenbergen C, Murphy E (2002). "Phosphorylation of Glycogen Synthase Kinase-3Œ≤ During Preconditioning Through a Phosphatidylinositol-3-Kinase‚ÄìDependent Pathway Is Cardioprotective." Circulation Research **90**(4): 377-379.

Topol, E. (2003). "Current Status and Future Prospects for Acute Myocardial Infarction Therapy." Circulation **108**(16 suppl 1): III-6-III-13.

Towbin H, S. T., Gordon J (1979). "Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications." Proc Natl Acad Sci U S A **76**(9): 4350-4354.

Tsokos, G. (2011). "Systemic Lupus Erythematosus." New England Journal of Medicine **365**(22): 2110-2121.

Tsujimoto, Y. (1998). "Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria?" Genes to Cells **3**(11): 697-707.

Uramoto KM, M. C. J., Thumboo J, Sunku J, O'Fallon WM, Gabriel SE (1999). "Trends in the incidence and mortality of systemic lupus erythematosus, 1950-1992." Arthritis & Rheumatism **42**(1): 46.

Urowitz MB, B. A., Koehler BE, Gordon DA, Smythe HA, Ogryzlo MA. (1976). "The bimodal mortality pattern of systemic lupus erythematosus." Am J Med **60**(2): 221-225.

Vaarala O, M. M., Manninen V, Tenkanen L, Puurunen M, Aho K, Palosuo T (1995). "Anti-Cardiolipin Antibodies and Risk of Myocardial Infarction in a Prospective Cohort of Middle-Aged Men." Circulation **91**(1): 23-27.

Vakeva A, A. A., Rollins S, Matis L, Li L, Stahl G (1998). "Myocardial Infarction and Apoptosis After Myocardial Ischemia and Reperfusion: Role of the Terminal Complement Components and Inhibition by Anti-C5 Therapy." Circulation **97**(22): 2259-2267.

Vega-Ostertag ME, F. D., Romay-Penabad Z, Liu X, Taylor WR, Colden-Stanfield M and Pierangeli SS (2007). "Role of p38 mitogen-activated protein kinase in antiphospholipid antibody-mediated thrombosis and endothelial cell activation." J Thromb Haemost **5**(9): 1828-1834.

Vega-Ostertag ME, F. D., Romay-Penabad Z, Liu X, Taylor WR, Colden-Stanfield M, Pierangeli SS. (2007). "Role of p38 mitogen-activated protein kinase in antiphospholipid antibody-mediated thrombosis and endothelial cell activation." J Thromb Haemost **5**(9): 1828-1834.

Venardos K, P. A., Headrick J, Kaye D (2007). "Myocardial ischemia-reperfusion injury, antioxidant enzyme systems, and selenium: a review." Curr Med Chem **14**(14): 1539-1549.

Venuturupalli S, G. V. a. W. D. (2012). "Reconsidering antimalarials in systemic lupus erythematosus: development of translational clinical interest." J Rheumatol **39**(9): 1769-1771.

Verma S, F. P., Weisel R, Butany J, Rao V, Maitland A, Li R, Dhillon B, and Yau T (2002). "Fundamentals of Reperfusion Injury for the Clinical Cardiologist." Circulation **105**: 2332-2336.

Visentini M, C. V., Cagliuso M, Tinti F, Siciliano G, Trombetta A, Mitterhofer A, Quinti I (2009). "Regression of systemic lupus erythematosus after development of an acquired Toll-like receptor signaling defect and antibody deficiency." Arthritis & Rheumatism **60**(9): 2767-2771.

Wallace D, G. V., Weisman M, Venturupalli S (2012). "New insights into mechanisms of therapeutic effects of antimalarial agents in SLE." Nature Review Rheumatology **8**: 522-533.

Wallace D, G. V., Weisman M, Venuturupalli S (2012). "New insights into mechanisms of therapeutic effects of antimalrial agents in SLE." Nature Reviews Rheumatology **8**: 522-533.

Wei Y, F. T., Yu Miaomiao (2008). "Inhibitor of apoptosis proteins and apoptosis." Acta Biochimica et Biophysica Sinica **40**(4): 278-288.

Weiss J, K. P., Honda H, Ping P (2003). "Role of the Mitochondrial Permeability Transition in Myocardial Disease." Circulation Research **93**(4): 292-301.

Wencker D, C. M., Nguyen K, Miao W, Garantziotis S, Factor S, Shirani J, Armstrong R, Kitsis R. (2003). "A mechanistic role for cardiac myocyte apoptosis in heart failure." The Journal of Clinical Investigation **111**(10): 1497-1504.

Willis R, S. A., McGwin G, Martinez-Martinez LA, Gonzlez EB, Dang N, Papalardo E, Liu J, Vil LM, Reveille JD, Alarcn GS, Pierangeli SS (2012). "Effect of hydroxychloroquine treatment on pro-inflammatory cytokines and disease activity in SLE patients: data from LUMINA (LXXV), a multiethnic US cohort." Lupus **21**(8): 830-835.

World Health Organisation, W. (2012). "The 10 Leading Causes of Death in the World." World Health Organisation.

Wu, T. a. M., C (2012). "Lupus nephritis - alarmins may sound the alarm?" Arthritis Research & Therapy **14**(6): 129.

Xianjun F, S. Y., Astrid E, Muling M, Robert B, Douglas B, Gordon M (1999). "Regulation of BAD phosphorylation at serine 112 by the Ras-mitogen-activated protein kinase pathway." Oncogene **18**(48).

Yang E, Z. J., Jockel J, Boise L, Thompson C, Korsmeyer S (1995). "Bad, a heterodimeric partner for Bcl-xL and Bcl-2, displaces bax and promotes cell death." Cell **80**(2): 285-291.

Yang JC, C. G. (1998). "Induction of the mitochondrial permeability transition causes release of the apoptogenic factor cytochrome c." Free Radic Biol Med(24): 621-631.

Yang X, C. M., Downey J. (2010). "Mechanism of cardioprotection by early ischemic preconditioning." Cardiovascular Drugs Therapy **24**(3): 225-234.

yang Z, C. C., Huang S, Sinicrope F (2011). "The role of autophagy in cancer: Therapeutic Implications." Molecular Cancer Therapy **10**: 1533-1541.

Yang Z, C. C., Huang S, Sinicrope F (2011). "The Role of Autophagy in Cancer: Therapeutic Implications." Molecular Cancer Therapeutics **10**(9): 1533-1541.

Yellon D, H. D. (2007). "Myocardial Reperfusion Injury." New England Journal of Medicine **357**: 1121-1135.

Yellon, D. a. H., D (2007). "Myocardial Reperfusion Injury." New England Journal of Medicine **357**(11): 1121-1135.

Yue T, W. C., Gu J, Ma X, Kumar S, Lee J, Feuerstein G, Thomas H, Maleeff B, Ohlstein E (2000). "Inhibition of Extracellular Signal‚ÄìRegulated Kinase Enhances

Ischemia/Reoxygenation‚ÄìInduced Apoptosis in Cultured Cardiac Myocytes and Exaggerates Reperfusion Injury in Isolated Perfused Heart." Circulation Research **86**(6): 692-699.

Zhang M, A. W., Chiu I, Alicot E, Hung R, Ma M, Verna N, Xu M, Hechtman H, Moore F and Carroll M (2004). "Identification of a specific self-reactive IgM antibody that initiates intestinal ischemia/reperfusion injury." Biological Sciences-Immunology **101**(11): 3886-3891.

Zhang M, A. W., Chiu I, Alicot E, Hung R, Ma M, Verna N, Xu M, Hechtman H, Moore F and Carroll M. (2004). "Identification of a specific self-reactive IgM antibody that initiates intestinal ischemia/reperfusion injury." PNAS **101**(11): 3886-3891.

Zhang M, T. K., Alicot E, Vorup-Jensen T, Kessler B, Thiel S, Jensenius A, Ezekowitz A, Moore F and Carroll M. (2006). "Activation of the lectin pathway by natural IgM in a model of ischemia/reperfusion injury." J Immunol **177**: 4727-4734.

Zhang, M. a. C., L (2008). "Status of cytokines in ischemia reperfusion induced heart injury." Cardiovascular Hematol Disord Drug Targets **8**(3): 161-172.

Zhang S, W. J., Du Y, Shang J, Wang L, Wang J, Wang K, Bai K, Lv T, Li X and Liu H (2013). "Cell Autophagy and Myocardial Ischemia/Reperfusion Injury,." Ischemic Heart Disease.

Zhao Z, C. J., Halkos M, Kerendi F, Wang N, Guyton R, Vinten-Johansen J (2003). "Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning." American Journal of Physiology - Heart and Circulatory Physiology **285**(2): H579-H588.

Zhou, J., Li G, Wang Z, Wang L and Dong P (2013). "Effects of low-dose hydroxychloroquine on expression of phosphorylated Akt and p53 proteins and cardiomyocyte apoptosis in peri-infarct myocardium in rats." Experimental Cardiology **18**(2): 95-98.

Zhou, Y. a. L., Q (2008). "DNA methylation in T cells from idiopathic lupus and drug-induced lupus patients." Autoimmunity Reviews **7**(5): 376-383.