CHARACTERIZATION OF OPSONINS WITH POTENTIAL FOR ENGAGEMENT IN REMOVAL OF DYING TUMOUR CELLS FOLLOWING PHOTODYNAMIC THERAPY

by

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ABSTRACT

It has become evident that mice bearing tumours treated with photodynamic therapy (PDT) exhibit three hallmarks of acute phase response: neutrophilia, hypothalamic-pituitary-adrenal (HPA) axis activation and the release of acute phase proteins. The latter and a subset of the innate immune system are the subjects of this research for they have been shown to play critical roles in the clearance of dead and dying cells. Among these respective proteins are participants in a rapid and non-inflammatory dead cell removal process, which is believed to be important for the outcome of tumour PDT. Therefore, innate immune proteins including early complement components (C1q, mannose-binding lectin (MBL) and ficolins), acute phase proteins including pentraxins (serum amyloid P component (SAP) & pentraxin-3 (Ptx3)), and heat shock protein-70 (Hsp70) were the focus of our investigation. These proteins are implicated to be soluble sensors of molecular patterns expressed on dying cells. Identification of the critical candidates from this selection, and modulation of their actions could optimize the PDT-induced development of tumour specific immunity.

Hypothesis: Early complement components (C1q, MBL and ficolins) and pentraxins (SAP & Ptx3) are known to be involved in a rapid and non-immunogenic dead cell disposal method. Characterization of their activity and of Hsp70 when faced with the burden of clearing PDT killed solid tumour cancer cells would be beneficial in development of new therapeutic approaches.
The first objective of this project was to determine the genes most involved in the removal of apoptotic cells. \textit{In vivo} gene expression studies were performed using Lewis Lung Carcinomas (LLC) growing in C57Bl/6J mice. By performing real-time PCR on different tissue samples collected from naïve, untreated and PDT-treated mice, the expression of seven genes were evaluated: early complement components C1q, MBL-A, ficolins A & B; pentraxins SAP and Ptx3; and Hsp70. Among these candidates, Hsp70, SAP and ficolin B showed the most pronounced gene up-regulation \textit{in vivo} in response to PDT at both the local treated site (tumour) and at a systemic site (liver). These three proteins were therefore further investigated in this project. In order to pinpoint the sources responsible for the elevated expressions of Hsp70, SAP and ficolin B, \textit{in vitro} gene expression studies were performed using mouse peritoneal macrophages (IC-21), mouse hepatomas (Hepa 1-6) and LLC tumour cell line. The investigated genes were found to become highly up-regulated in PDT-treated LLC cells. Moreover, untreated macrophages and hepatoma cells up-regulated their SAP and Hsp70 genes respectively, when co-incubated with PDT-treated LLC cells.

Our second objective was to examine the mechanisms responsible for systemic up-regulation of Hsp70, SAP and ficolin B. Since tumour PDT activates the hypothalamic-pituitary-adrenal (HPA) axis in the host, we attempted to discover any links between the HPA activation and up-regulation of these genes in the liver. Experiments were done to test the effects of the glucocorticoid (dexamethasone), its receptor antagonist (mifepristone) and its synthesis inhibitor (metyrapone) on naïve mice and mice with PDT-treated tumours, demonstrated that the up-regulation of the
investigated three genes is at least partially mediated by the activation of the HPA axis and the release of glucocorticoids.

The final objective of this study was to determine whether Hsp70 would act as an acute phase protein, produced and released systemically by the liver in response to PDT induced trauma. Lower levels of Hsp70 were found in livers of mice with PDT treated tumours compared to mice with untreated tumours. This demonstrates that Hsp70 is being released from the liver in response to tumour-localized PDT-induced trauma. In addition, LLC cells treated in vitro with PDT, but not untreated LLC cells, were found to bind extracellularly added Hsp70 protein. These findings suggest that released Hsp70 is capable of binding to PDT-damaged and dying tumour cells, and indicate that this protein can have a critical role in the removal of dying cells.

The results of the experiments have demonstrated that among the investigated proteins Hsp70, SAP and ficolin B are highly transcribed at the local (tumour) and at a systemic (liver) site in response to tumour PDT treatment. Based on this evidence and previously published data, the three proteins studied appear to be the main candidates responsible for the effective removal of dead cancer cells. Their activity in clearing large loads of killed tumour cells could influence the development of the adaptive immune response towards cancer cells, destroying the primary tumour as well as any metastases or re-occurrences of the same type of cancer. Further studies aimed at elucidating the activity of these proteins in dead cell removal should identify therapeutic targets with potential for improved curative outcome.
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ABBREVIATIONS

'02: Singlet oxygen
5-ALA: 5-aminolevulinic acid
7-AAD: 7-amino-actinomycin D
AIDS: Acquired immunodeficiency syndrome
AP-1: Activator protein 1
APC: Antigen presenting cells
APR: Acute phase response
ARC: Animal Research centre
ATCH: Adrenocorticotropic hormone
BLYS: B lymphocyte stimulator
C3aR: C3a receptor
C4BP: C4b binding protein
C5aR: C5a receptor
C5L2: C5a receptor L2
CD40L: CD40 ligand
cDNA: Complementary strand DNA
CNS: Central nervous system
COX-2: Cyclooxygenase 2
CR: Complement receptor
CRH: Corticotrophin-releasing hormone
CRP: C-reactive protein
CT: Threshold cycle
CTL: Cytotoxic T lymphocyte
Cyclic AMP: Cyclic adenosine monophosphate
D5W: Dextrose 5% in Water
DC: Dendritic cell
ddH₂O: Deionized distilled water
DEPC: Diethylpyrocarbonate
dsDNA: Double-stranded DNA
E: Efficiency
EDTA: Ethylenediamine tetraacetic acid
ELAM-1: Endothelial cell-leukocyte adhesion molecule-1
ELISA: Enzyme-linked immunosorbent assay
ER: Endoplasmic reticulum
FBS: Fetal bovine serum
FDC: Follicular dendritic cell
FITC: Fluorescein isothiocyanate
FS: Forward scatter
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GC: Glucocorticoid
GM-CSF: Granulocyte-macrophage colony-stimulating factor
GR: Glucocorticoid receptor
HBSS: Hank’s balanced salt solution
HPA: Hypothalamic-pituitary-adrenal (axis)
HPD: Hematoporphyrin derivative
Hsp: Heat shock protein
Hsp70: Heat shock protein-70
I/R: Ischemia-reperfusion
iC3b: Inactivated C3b
ICAM-1: Intercellular adhesion molecule-1
iDC: Immature dendritic cell
IFN-γ: Interferon-gamma
IGF-I: Insulin-like growth factor-I
IgG: Immunoglobulin G
IL: Interleukin
iNOS: Inducible nitric oxide synthase
LLC: Lewis lung carcinoma
LOX-1: Low density lipoprotein receptor-1
LPS: Lipopolysaccharide
MAC: Membrane attack complex
MASP: MBL-associated proteases
MBL: Mannose-binding lectin
mCRP: Membrane complément regulatory protein
MGI: Mouse genome informatics
MHC: Major histocompatibility complex
Msrl: Macrophage scavenger receptor-1
m-THPC: Meta-tetra hydroxyphenyl chlorine
NCBI: National center for biotechnology information
NF-kB: Nuclear factor-kappa B
NK: Natural killer
NO: Nitric oxide
PARP: Poly ADP-ribose polymerase
PBS: Phosphate buffered saline solution
PC: Phosphatidylcholine
PCR: Polymerase chain reaction
PDT: Photodynamic therapy
PE: Phosphatidylethanolamine
PE: Phycoerythrin
PEG400: Polyethylene Glycol 400
PGE2: Prostaglandin E2
PLA2: Phospholipase A2
PS: Phosphatidylserine
Ptx3: Pentraxin-3
qRT-PCR: Quantitative reverse transcriptase polymerase chain reaction
R²: Coefficient of determination
ROS: Reactive oxygen species
SAA: Serum amyloid A
SAP: Serum amyloid P component
SCC: Squamous cell carcinoma
SE: Standard error
SLE: Systemic lupus erythematosus
snRNP: Small nuclear ribonucleoprotein
SS: Side scatter
TAE: Tris-Acetate-EDTA
TGF-β: Transforming growth factor β
TLR: Toll-like receptor
TMB: Tetramethylbenzidine
TNF-α: Tumour necrosis factor α
UDG: Uracil DNA glycosylase
β-ME: β-mercaptoethanol
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1) INTRODUCTION

1.1) Waste disposal hypothesis

The justification behind this project emanates from the “Waste-Disposal Hypothesis” which elegantly illustrates the most plausible pathological mechanism responsible for the development of the autoimmune disease, Systemic Lupus Erythematosus (SLE) [1]. This hypothesis proposes that massive apoptosis and failure of the appropriate clearance by macrophages, immature dendritic cells and complement, potentially leads to the onset of an autoimmune response [2]. This explains the importance of complement and pentraxin proteins for the anti-inflammatory clearance of apoptotic cells from tissues [3, 4]. It further demonstrates the complications caused by the failure of such innate immune components in ridding the body of dead and dying cells. Impaired removal, and therefore accumulation, of apoptotic cells due to the absence of the early complement components and pentraxins could evoke an autoimmune response. This is supported by the very high correlations (>90%) between C1q and serum amyloid P component (SAP) deficiencies and the predisposition to autoimmunity in both man and mouse [5, 6]. The fact that altered levels of complement and pentraxins could induce an adaptive immune response is very intriguing. This phenomenon can be utilized by manipulating the levels of key complement and pentraxin proteins involved in clearance of apoptotic tumour cells to elicit an adaptive immune response against tumours. Inducing such tumour specific immunity could very well eradicate the primary lesion as well as any secondary metastases.
Figure 1.1: Waste disposal hypothesis. Panel A: There is an array of ligands and receptors on apoptotic cells and macrophages that make efferocytosis an extremely efficient process. Binding of proteins such as C1q, C-reactive protein and IgM may initiate complement mediated opsonization of apoptotic cells. These cells are engulfed following the ligation of complement receptors on macrophages. Other bound proteins such as pentraxins (serum amyloid P component) mask auto-antigens and promote safe removal of apoptotic cells. After efferocytosis, macrophages release the anti-inflammatory cytokine TGF-β. Panel B: Excess of apoptotic cells accompanied by failure of receptor-ligand mediated uptake may lead to the initiation of adaptive immune response. In the presence of inflammatory cytokines including GM-CSF, TNF-α and IL-1, antigen presenting cells mature after efferocytosis. T cells are activated upon dendritic cell mediated presentation of auto-antigens in the presence of co-stimulatory molecules and cytokines. Panel C: Activated T cell expression of co-stimulatory molecules and cytokines are involved in maturation of B cells that have taken up auto-antigens from apoptotic cells. The auto-reactive B cells divide and mature into plasma cells that secrete auto-antibodies. Walport, M.J., Complement- Second of Two Parts. N Engl J Med, 2001. 344(15): p. 1140-1144. Copyright © [2001] Massachusetts Medical Society. All rights reserved.
Apoptosis, or programmed cell death, is recognized as an essential process during development and the maintenance of normal tissue homeostasis. Apoptosis is a tightly regulated mode of cell death that occurs without inflammation. However, when faced with an abnormally large load of apoptotic cells and/or defective removal capacity as a result of hampered opsonization by pentraxins and complement, non-ingested cells can proceed to secondary necrosis. Accompanied by swelling and eventual bursting, these necrotic cells release inflammatory and toxic contents, leading to severe tissue injury [7]. Apoptotic cells are also potential sources of intracellular auto-antigens. The autoimmune disease SLE is characterized by the presence of auto-antibodies to a wide range of cellular and nuclear antigens, including DNA, RNA, chromatin, ribonucleoproteins (Ro, La), poly (ADP-ribose) polymerase (PARP), nucleosomes and histones [8], which are present within apoptotic blebs [9]. It is remarkable that 17 of the known apoptotic protease substrates have been identified as auto-antigens or are constituents of larger complexes such as nucleosomes that contain a protein recognized by auto-antibodies [10]. It is believed that during programmed cell death caspase enzymes will cleave these well known auto-antigens, revealing potentially auto-reactive epitopes [11-13]. Due to harboring such dangerous materials, efficient elimination of apoptotic cells and thus prevention of unwanted immune reactions is essential. The clearance of apoptotic cells by phagocytes not only functions to remove them from the surrounding tissue but also serves to protect against local damage resulting from uncontrolled leakage of noxious contents.

Phagocytosis is the final common end stage of cells undergoing apoptosis. The characteristic changes in the plasma membrane occurring during the apoptotic process
and the binding of complement and pentraxin proteins enable the recognition and efficient removal of apoptotic cells by phagocytes including macrophages and dendritic cells (DCs) [7]. Among the opsonins for apoptotic cells, complement factors including C1q, MBL, and complement-activating members of the pentraxin family such as SAP, CRP and Ptx3 play an important role [14]. The binding of these proteins and also natural antibodies to apoptotic cells may promote the activation of complement, leading to the clearance of apoptotic cells by ligation of complement receptors. Once the macrophages have engulfed the apoptotic cells opsonized with classical short pentraxins such as CRP and complement, they secrete immunosuppressive cytokines, such as transforming growth factor β (TGF-β) and interleukin 10 (IL-10) that prevent DC maturation [1]. Similar to macrophages, DCs show suppressed cytokine production upon their exposure to apoptotic cells and even stop cytokine production when stimulated through their iC3b receptors [15, 16]. Furthermore, antigens delivered to DCs via apoptotic cells induce tolerance in vivo [17].

If the clearance of apoptotic cells is impaired, for example, when the availability of complement components or pentraxins is limited in combination with a large apoptotic load, cells may undergo post-apoptotic secondary necrosis, leading to the release of their toxic and pro-inflammatory contents. Uptake of post-apoptotic debris by phagocytes in the presence of inflammatory cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor α (TNF-α) and interleukin 1 (IL-1) [1], induce their subsequent activation/maturation and release of more inflammatory cytokines. Alternatively, post-apoptotic/secondary necrotic cells may be opsonized with
auto-antibodies, promoting their uptake through Fc receptors on phagocytes and leading to the propagation of an inflammatory response. During inflammation, DCs mature, express co-stimulatory signals and enhance their ability to cross-present antigens. After arrival at lymph nodes, they may stimulate T cells which in turn will maintain their viability [18]. Activated T cells that express co-stimulatory molecules and cytokines including an important member of the tumour necrosis family, B lymphocyte stimulator (BLyS), also referred to as zTNF-4, help the maturation process of B cells that have taken up antigens from apoptotic cells through their antibody receptors. The reactive B cells divide and mature into plasma cells that secrete antibodies.

The engagement of the adaptive immune system associated with SLE can not be explained just by the large numbers of apoptotic cells associated with this disease. Other diseases characterized by proportionally high numbers of apoptotic cells such as acquired immunodeficiency syndrome (AIDS), systemic vasculitis, and chemotherapy or irradiation-treated malignancies generally do not generate high titers of specific antibodies such as those present in SLE [10]. This clear distinction could be due to the fact that in the majority of SLE cases, deficiencies of complement and pentraxins can hamper the body’s ability to remove the apoptotic/soon to be necrotic cells. Therefore, an adaptive immune response will arise when large quantities of apoptotic cells are generated in the presence of functional defects associated with their clearance [19].
1.2) Proteins involved in dead cell clearance

1.2.1) Complement system

The complement system is part of the innate branch of the host defense system and has multiple biological effects, most of which contribute to the inflammatory reaction. It is made up of over 30 cell membrane and plasma proteins that react with one another to opsonize or kill invading organisms. Complement components activate leukocytes and endothelial cells and induce inflammatory responses that help fight and clear infections [20-22]. Aside from these well-known functions, complement has been shown to be actively involved in the equally important task of opsonizing and clearing immune complexes and altered host cells such as apoptotic cells. This system of opsonization also plays an important role in modulating the adaptive immune system and inducing tolerance [1, 2, 23, 24].

1.2.1.1) Complement pathways

There are three pathways of complement activation; the classical, the lectin and the alternative pathways. The classical pathway is triggered by natural or elicited antibodies or direct binding of the complement component C1q to activating structures such as pathogens and altered host cells [20, 22, 23]. The lectin pathway is initiated by the recognition and binding of lectin proteins such as mannose-binding lectin (MBL) and ficolins to carbohydrates such as N-acetyl glucosamine and mannose on microbes and altered host cells [25]. The alternative pathway is somewhat different from the first two modes in that it by undergoes a low-grade spontaneous activation [20, 21].
Introduction

Figure 1.2: Complement system. Complement activation can be triggered by three pathways, the classical pathway, the mannose-binding lectin (MBL) pathway and the alternative pathway. The classical pathway is initiated by the binding of C1 complex (consisting of C1q, two molecules of C1r and C1s) to antibody-antigen complexes, or to other structures such as pentraxins or apoptotic cells. The MBL pathway is initiated by binding of the complex of MBL and MBL-associated proteases 1 and 2, MASP1 and MASP2 respectively, to designated carbohydrates on cell surfaces. The alternative pathway is activated following low-grade spontaneous hydrolysis of C3 in plasma and subsequent covalent binding of C3b to hydroxyl groups on cell-surface carbohydrates and proteins. Each complement activating pathway generates a C3 convertase (C4b2a/C3bBbP), which mediates cleavage of C3, followed by activation of a common terminal complement pathway and formation of the membrane attack complex (MAC) (C5b-9). Walport, M.J., Complement- First of Two Parts. N Engl J Med, 2001. 344(14): p. 1058-1066. Copyright © [2001] Massachusetts Medical Society. All rights reserved.
1.2.1.2) Complement receptors

The most important action of complement is to facilitate the uptake and removal of targeted entities. This occurs through specific recognition of bound complement components by complement receptors (CRs) on phagocytes. Complement receptors bind complement opsonized cells. CR1 (CD35) binds C3b, C4b, and iC3b. CR3 (CD11b/CD18) binds iC3b. CR1 and CR3 are especially important in inducing phagocytosis of bacteria with complement components on their surfaces. CR2 (CD21) binds C3d, iC3b, C3dg. It is found mainly on B cells, acting as a part of the B cell co-receptor complex [22]. CR4 (CD11c/CD18) binds iC3b. CR3 and CR4 are integrins. CR3 is known to be important for leukocyte adhesion and migration, while CR4 is only known to function in phagocytic responses [22, 26, 27].

1.2.1.3) Complement and apoptotic cells

One of the key functions of the complement system is its participation in the removal of apoptotic cells. The importance of this removal is supported by the evidence gathered from cases in which deficiencies of early components of the classical complement pathway, such as C1q, are strongly associated with susceptibility to systemic lupus erythematosus (SLE). Dying cells contain both antigens and adjuvants sufficient to initiate an autoimmune response [28, 29]. Finding SLE-targeted auto-antigens within apoptotic cells has led to the development of the waste disposal hypothesis [2].
Immature dendritic cells (iDCs) have been recognized as one of the major players in the removal of apoptotic cells. Verbovetski and colleagues [23], showed that opsonization of apoptotic Jurkat cells by autologous iC3b increased the efficiency of their uptake by iDCs but inhibited DC maturation. It has been suggested that the uptake of apoptotic cells by iDCs is mediated by the receptors αvβ3 (the vitronectin receptor) [30] and αvβ5 [31] with participation of scavenger receptor CD36 [31]. iDCs ingest antigens by phagocytosis, macropinocytosis or receptor-mediated endocytosis. After ingestion of stimulatory entities such as pathogens, iDCs are triggered to undergo a developmental program called maturation [32]. However, after interaction with apoptotic cells, iDCs not only do not up-regulate, but rather down-regulate MHC class II and CD86 co-stimulatory molecules, showing the possible role of DCs in the induction of anergy rather than priming autoimmune T cells. This is further emphasized by anergy of iDCs exposed to iC3b opsonized apoptotic cells. These iDCs do not up-regulate MHC II and CD86, nor release IL-12 after stimulation by CD40L or LPS [23].

When committed to apoptosis, cells follow an orderly process of nuclear condensation, surface blebbing, cytoplasmic contraction, and packaging of cellular components within membranes before their budding from the cell as apoptotic bodies [9]. Also during this process, tissue transglutaminases are responsible for cross linking proteins that prevent the leakage of intracellular constituents from late apoptotic cells. Therefore normal conditions, apoptotic cells are sealed [33, 34].
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Well characterized surface changes during programmed cell death include loss of phospholipid asymmetry and exposure of such molecules as phosphatidylserine (PS) on the cell surface. Another important change is the alteration of membrane carbohydrates, increased expression of fucose and N-acetyl-glucosamine due to redistribution or clustering of glycoproteins. These changes suggest that lectins including mannose-binding lectin (MBL) that bind such altered carbohydrates might have an important role in apoptotic cell clearance [7]. C1q also may be involved in such a task because recent studies have demonstrated the high affinity of this lectin molecule for surface blebs of apoptotic cells, emphasizing its participation in apoptotic cell removal [35].

Although early complement components appear to be important for the rapid clearance of apoptotic cells, cells must be protected from the assembly of later components in order not to provoke an inflammatory response triggered by the complement system. Formation of anaphylatoxins (mainly C5a and C3a) and opsonins (mainly C3b) promote inflammatory responses. Therefore, the presence of complement inhibitors such as C4b binding protein (C4BP), a major inhibitor of the classical pathway, may be very important for the inhibition of inflammation close to apoptotic cells [24]. During the initial stage of apoptosis, cells expose negatively charged phospholipids including phosphatidylserine on their surfaces. The vitamin K-dependent protein S has a high affinity for this type of phospholipid. In human plasma, 60%-70% of protein S circulates in complex with C4b binding protein (C4BP). Therefore protein S, due to its high affinity for negatively charged phospholipids, localizes C4BP to areas where such phospholipids are exposed such as in apoptotic cells. C4BP is able to interact with the
complement protein C4b and regulate the complement system by preventing further assembly of down-stream molecules on the surface of the apoptotic cells [24].

1.2.1.4) **Complement as a bridge between the innate and the adaptive immune systems**

The complement system is also capable of linking the innate and the adaptive immune systems. The evidence for this comes from experimental results showing that a transient reduction of the amount of circulating complement C3 led to disruption of the antibody response. It is becoming increasingly clear that complement enhances B cell immunity, principally via the complement receptors CD21 and CD35 [25, 36]. Uptake of C3d-coated antigens by cognate B cells via the B cell antigen receptor and co-engagement of the CD21-CD19-CD81 co-receptors, lowers the threshold of B cell activation and provides an important survival signal [25, 37]. Follicular Dendritic Cells (FDCs) also have relatively high expressions of CD21 and CD35, and this provides an effective mechanism for the retention of C3-coated immune complexes within the lymphoid compartments [25, 38].

Control of activated CD4\(^+\) T cells in the periphery is mediated by their specific elimination, anergy and transformation into regulatory T cells. The complement system seems to participate in the development of human regulatory T cells via co-stimulation of CD3 and CD46. Cross-linking of CD3 and CD46 on human CD4\(^+\) T cells led to induction of a regulatory T phenotype and release of IL-10 [39]. The induced regulatory T cells...
proliferate in culture, block activation of bystander T cells and differentiate into memory cells [39].

C3a and C5a peptides released during the activation of the complement cascade are also involved in bridging the innate and the adaptive immune systems. They serve as potent ligands for G protein-coupled chemo-attractant receptors referred to as C3aR and C5aR respectively [26, 40, 41]. These receptors are expressed on a wide range of inflammatory cells, such as neutrophils, mast cells, eosinophils, basophils and lymphocytes. Aside from acting as chemo-attractants, C3a and C5a have an influence on the fate of naïve CD4<sup>+</sup> (Th0) T cells. It has been shown that activation of C5aR and signalling through CD88 induces IL-12 production by antigen presenting cells (APCs) which drives the Th1 response, whereas signalling through C5L2, another receptor for C5a, inhibits the Th1 response and/or promotes the Th2 response [42-44]. Conversely, C3b and iC3b can suppress IL-12 production by APCs and favour the development of a Th2 response. C3a may induce Th2 cytokine release from basophils/mast cells and promote Th2 polarization during sensitization on APCs. It may also interact with activated T cells to maintain a Th2 response [26].
1.2.2) Pentraxins

Pentraxins are pattern recognition proteins of the acute-phase response. They are characterized by a cyclic pentameric structure and show strong inter-species homology [45]. The classical short pentraxins, C-reactive protein (CRP), and serum amyloid P component (SAP) are produced in the liver in response to inflammatory mediators [46]. Pentraxin-3 (Ptx3) is a prototypic long pentraxin and can be produced by a variety of cells in response to inflammatory cytokines [47, 48]. Pentraxins play a major role in the innate immune response against microbes and in the regulation of scavenging of cellular debris [45]. Binding of these pattern recognition molecules to apoptotic cells has also been demonstrated by recent studies [49-51]. Opsonization of apoptotic cells with pentraxins can lead to the binding of C1q and subsequent activation of complement [50] followed by complement receptor (CR3 & CR4) mediated phagocytosis [52, 53]. However, pentraxin opsonization may also directly promote uptake of apoptotic cells by phagocytes [7, 54].

Human SAP and CRP, classical short pentraxins share 51% of amino acid identity and 59% nucleotide sequence identity. Their genes are closely linked, being located in band q2.1 of chromosome 1. In humans, CRP acts as an acute phase protein with its circulating levels increasing to up to 1000-fold during an acute phase response. However, in some other animal species such as laboratory mice, SAP rather than CRP reacts as the acute phase protein [46, 55].
1.2.2.1) Serum amyloid P component (SAP)

Serum amyloid P component (SAP) is a calcium-dependent pentameric glycoprotein. It has been suggested that this molecule has crucial roles in vivo because mice with targeted depletion of the SAP gene developed antinuclear antibodies, reminiscent of the human autoimmune disease SLE [9]. These observations were interpreted as evidence of a role for SAP in controlling the degradation of chromatin. In the presence of calcium ions, SAP has been shown to bind to several ligands among which DNA [56], chromatin [57, 58], histones [59, 60] and phosphoethanolamine containing compounds such as phosphatidylethanolamine [61, 62] are of great importance. In normal cells, phospholipids are distributed asymmetrically between the inner and outer leaflet of the cell membrane, with most amino-phospholipids such as phosphatidylserine and to a lesser extent phosphatidylethanolamine located in the inner leaflet of the plasma membrane [63-66]. During apoptosis, one of the earliest events is the loss of this asymmetry, leading to the exposure of phosphatidylserine (PS) phosphatidylethanolamine (PE) and phosphatidylcholine (PC) in the outer leaflet [14, 67]. In a study by Familian and colleagues it was demonstrated that in fact SAP binds to PE exposed after membrane phospholipids flip-flop on the surface of apoptotic cells [5, 49].

1.2.2.2) C-reactive protein (CRP)

CRP, another member of the classical short pentraxins acts as an acute phase protein in humans and its serum levels rise dramatically in response to the released pro-
inflammatory cytokines such as IL-1 and TNF-α. Various studies have demonstrated that CRP specifically binds to phosphorylcholine in membrane bilayers [68] as well as to H1-containing chromatin [69] and small nuclear ribonucleoproteins (snRNP) [70]. However, it is becoming clear that the main target of CRP on apoptotic cells are phosphorylcholine moieties that become accessible as a result of oxidation of phosphatidylcholine molecules [71]. Despite amplification of the classical pathway, CRP paradoxically attenuates the formation of the membrane attack complex on the surfaces of apoptotic cells, thereby protecting the cells from lysis. This effect was achieved by the recruitment of factor H, a complement regulatory protein that accelerates the decay of the C3 and C5 convertases [72]. Elevated serum CRP significantly increased phagocytosis of apoptotic cells by macrophages and sustained the production of TGF-β by these phagocytes [50]. It should be emphasized however that the presence of C1q is necessary for the CRP anti-inflammatory clearance of apoptotic cells [50].

1.2.2.3) Pentraxin-3 (Ptx3)

Pentraxin-3 (Ptx3) is also a member of the pentraxin family of acute-phase proteins which is capable of binding to apoptotic cells [49, 51]. This protein is structurally related to, but distinct from, classical cyclic pentameric short pentraxins. The C-terminal half of Ptx3 aligns with the full-length sequence of CRP and SAP, whereas the N-terminal region does not show any homology with these other proteins [73]. Ptx3 expression is induced by inflammatory cytokines such as IL-1β and TNF-α in a variety of cell types, including endothelial cells and monocytes. The expression of Ptx3 increases early during inflammation, preceding the rise of expression of SAP and CRP [74].
Therefore it is a perfect candidate for interaction with cells dying at inflammatory sites. C1q also interacts with immobilized Ptx3 via its globular head region and in turn activates the classical complement pathway [75]. Specifically, Ptx3 enhances C1q binding and complement activation on apoptotic cells. A unique signature of Ptx3 is that upon binding to apoptotic cells, it inhibits their phagocytosis by dendritic cells (DC), indicating a regulatory role for this prototypic long pentraxin in the clearance of apoptotic cells by professional antigen-presenting cells (APC). Based on its biological role in clearance of apoptotic cells during inflammation, it may be very well involved in the protection against the onset of an adaptive immune response [51].

Therefore, the conclusion arrived from the above evidence is that pentraxins and the classical complement pathway appear to work in concert to promote the clearance of apoptotic cells in an anti-inflammatory context.
1.2.3) Heat shock proteins

Heat shock proteins (Hsp) are one of the most abundant and highly conserved soluble intracellular proteins found in all prokaryotic and eukaryotic organisms [76-78]. These proteins were initially identified by their expression after exposure of cells to elevated temperatures. In addition, Hsps are also up-regulated by a large array of stressful stimuli including environmental (UV radiation, heat shock, heavy metals, oxidative stress and amino acid deprivation), pathological (viral, bacterial or parasitic infections, fever, inflammation, malignancy or autoimmunity) or physiological stimuli (growth factors, cell differentiation, hormonal stimulation, or tissue development) [79]. Hsps are a family of polypeptides distributed across different sub-cellular compartments such as the cytosol, nucleus, endoplasmic reticulum (ER), and mitochondria. They are classified according to their molecular weight, for instance 70kDa Hsps belong to the Hsp70 family [80]. Located in the cytosol and the nucleus, Hsp70s are the most conserved and the best studied among their class. They include the constitutively expressed Hsp73 (Hsc70) and the stress-inducible Hsp70 (Hsp72) [81].

1.2.3.1) The many roles of heat shock proteins

Hsps are involved in multiple vital cellular processes during normal and stress conditions [82]. Their best known role is that of molecular chaperones, involved in the folding of nascent proteins, degradation of aberrantly folded or mutated polypeptides, translocation of polypeptides across membranes, assembly of macromolecule structures and maintenance of membrane receptors [79, 80, 83-85], all of which enables them to confer cyto-protection following stressful insults. Aside from being chaperons, they are
established as the first adjuvants of endogenous/mammalian origin [86]. Due to having peptide-binding sites analogous to that of class I histocompatibility proteins, members of the Hsp70 family are capable of shuttling antigens to major histocompatibility complex (MHC) molecules [84]. This adjuvant characteristic of Hsps was identified in the field of cancer immunology, in studies where the investigators were looking for cancer-specific antigens, by their ability to elicit protective immunity to cancer challenges. Purification of the fractionated cancer homogenates which elicited protection, revealed Hsps of the Hsp70 or Hsp90 family, including Hsp70, gp96 and Hsp90 to be the active components. Hsps purified from a given cancer were observed to elicit protective immunity specific to that particular cancer, whereas normal tissue Hsps did not confer such immunity [87-89]. Blachere and colleagues further demonstrated via in vitro studies that a combination of non-immunogenic peptides chaperoned by Hsps elicited antigen-specific CD8$^+$ cytotoxic T lymphocytes (CTL) [86]. Vaccination with non-covalent mycobacterial Hsp70-peptide complex has also been shown to establish CD4$^+$ T-cell anti-peptide immunity [90], which also would promote the induction of an antibody response. In another study, immunization with cancer-derived Hsp-peptide complexes were found to elicit, in addition to the CD8$^+$ and CD4$^+$ T cell response, an NK response that may be crucial for adaptive immunity [91].

1.2.3.2) Heat shock protein receptors

The observation that extremely small quantities of Hsp-peptide complexes were effective in inducing specific immunity led to the suggestion that professional APCs possess Hsp-specific receptors that take up Hsp-peptide complexes with specificity [92].
Indeed, it has been demonstrated recently that these complexes can be taken up into non-acidic compartments via the scavenger receptor CD91 (α2-macroglobulin receptor) [93] and the low density lipoprotein receptor (LOX-1) [94]. These peptides are then processed and presented by MHC class I molecules of the APC, initiating the activation of cytotoxic T lymphocytes (CTL) [93]. Other proteins implicated of having Hsp receptor activity on plasma membranes of macrophages and APCs include CD40 [95-97], macrophage scavenger receptor (Msr1) [98], Toll-like receptor-2 (TLR-2) and TLR-4 [99].

1.2.3.3) Heat shock proteins as danger signals

Apart from acting as potent adjuvants, heat shock proteins, specifically Hsp70, seem to possess a cytokine activity [99]. Dying cells, especially necrotic, have been shown to release gp96, Hsp90 and Hsp70. Upon interacting with their receptors on antigen-presenting cells (APC), these Hsps serve as danger signals [92, 100] and stimulate macrophages to secrete cytokines and induce expression of antigen-presenting and co-stimulatory molecules on the DCs. Hsp70 induction of pro-inflammatory cytokines may proceed by its binding to TLR-2 and TLR-4 receptors and the subsequent activation of MyD88/IRAK/NF-κB signal transduction pathway [97]. TLR-2 and TLR-4 may also synergize to greatly augment Hsp70 induced cytokine production [97]. Ligation of these receptors results in phosphorylation of the intracellular inhibitory subunit I-κBα and subsequent activation of nuclear transcription factor NF-κB. This leads to up-regulation of genes encoding pro-inflammatory and immune mediatory proteins such as tumour necrosis factor α (TNF-α), interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 12 (IL-12), co-stimulatory molecules from the B7 family and adhesion molecules.
including endothelial cell-leukocyte adhesion molecules (ELAM-1, E-selectin) [95, 97, 101].

In a recent study, Arispe and colleagues also demonstrated that Hsp70 and Hsc70 are capable of binding selectively and with high affinity to phosphatidylserine moieties uncovered on plasma membranes. This would also implicate Hsps of having an immunoregulatory role in the removal of dying host cells [82, 95].
1.3) **Acute phase response**

Acute phase response (APR) refers to a regulated series of distant systemic changes that occur in response to a localized tissue injury [102]. It is regarded as a stress response at the level of the organism initiated with the purpose of creating an overall protective systemic environment required for coping with the inflicted insult and containing the disrupted homeostasis. In humans, stimuli that commonly give rise to the APR include bacterial and, to a lesser extent, viral infections; surgical or other trauma; neoplasm; burn injury; tissue infarction; various immunologically mediated inflammatory states; strenuous exercise [103]; heatstroke; and childbirth. Psychological stress [104] and chronic fatigue syndrome [105] are also associated with acute phase changes. In a given individual, APR represents the integrated sum of multiple, separately regulated changes induced primarily by inflammation-associated cytokines and their modulators including endocrine hormones and other circulating molecules. The organ systems participating in APR include the liver, brain, adrenal gland, bone, muscle and adipose tissue.

1.3.1) **Liver and acute phase proteins**

The liver is responsible for modifying the concentration of a large number of plasma proteins called the acute phase proteins. These changes largely reflect reprogramming of plasma protein gene expressions in hepatocytes. The primary mediator inducing APR and triggering acute phase protein release is IL-6 but a number of other cytokines, alone or in concert, may also be involved [106]. Plasma proteins that show 25% change of their levels, either an increase or a decrease, are known as positive and negative acute phase proteins, respectively. Important positive acute phase proteins
include CRP (or SAP in mice), serum amyloid A (SAA), plasminogen, protein S, and complement system proteins such as MBL, C3, C4, factor B, Cl inhibitor, and C4b-binding protein [107-110]. Recently recognized negative human acute phase proteins include transthyretin, α-fetoprotein, T4-binding globulin, and insulin-like growth factor-I (IGF-I) [111-113]. The plasma protein response to inflammatory stimuli seen in hepatocytes can serve as a paradigm for the APR in other organs and therefore the term APR is frequently employed in a narrow sense to refer only to changes in the concentration of acute phase proteins.

1.3.2) APR and the hypothalamic-pituitary-adrenal (HPA) axis

Involvement of the brain may result in the activation of the hypothalamic-pituitary-adrenal (HPA) axis resulting in an increased synthesis of hormones including corticotrophin-releasing hormone (CRH), adrenocorticotropic hormone (ATCH), and glucocorticoids such as cortisol [114]. Fever is also another manifestation of the brain’s involvement in the APR. The mentioned neuroendocrine hormones have stimulatory effects on other participants of the APR including the adrenal gland. ATCH induces the production and release of cortisol (glucocorticoid) and aldosterone (mineralocorticoid) from the adrenal cortex. Signals from the sympathetic nervous system also act on the adrenal gland and bring about the release of adrenal catecholamines (epinephrine) from the adrenal medulla [114].
1.3.3) APR and metabolic changes

APR involvement of the bones/hematopoietic system includes the suppression of erythropoiesis, induction of leukocytosis and thrombocytosis, and the overall loss of bone mass. Other metabolic changes will also take place in the muscle and adipose tissue [115]. In the muscle, these include proteolysis and decreased protein synthesis which will result in the loss of skeletal muscle. Alterations of lipid metabolism, specifically the loss of adipose tissue results, at least in part, from inhibition of lipoprotein lipase production by cytokines [115]. Increase in serum triglycerides, very-low-density lipoproteins, and low-density lipoproteins could also be seen [116].
1.4) Photodynamic therapy (PDT)

1.4.1) History of photodynamic therapy

The therapeutic properties of light have been recognized and utilized for the treatment of various diseases such as vitiligo, psoriasis and skin cancer since the ancient civilizations of Egypt, India and China [117, 118]. However, it was not until the 20th century that the full extent and applications of light in treating disease “phototherapy” was realized and exploited [118]. After discovering that red and ultraviolet light could treat smallpox pustules and cutaneous tuberculosis respectively, Niels Finsen began the modern era of light therapy for which was awarded a Nobel Prize in 1903 [117, 118]. Photo-chemotherapy was first discovered by Oscar Raab in 1900 who reported that the compound Acridine in combination with certain light wavelengths induced death of Paramecium [118, 119]. In 1903 Herman Von Tappeiner and A. Jesionek observed therapeutic effects of topically applied Eosin and white light exposure on skin tumors and termed this effect “photodynamic action”. This term is still used today and refers to the reactions following “photodynamic therapy” (PDT) [117-119]. In 1911 W. Hausmann characterized the photo-toxicity and biological effects of hematoporphyrin, a member of the family of photosensitive molecules called the porphyrins, on the skin of mice [119]. Taking this one step further, Friedrich Meyer-Betz tested the effects of hematoporphyrin on his own hand and in 1913 was the first to treat humans with porphyrin based PDT [119]. Samuel Schwartz developed a hematoporphyrin derivative (HPD) in 1955 that was found to be twice as phototoxic and could be used at much smaller doses than unmodified hematoporphyrin [120]. Few years later Lipson and Baldes ushered in a new era of PDT at the Mayo Clinic using HPD [121, 122]. Diamond et al. (1972) was the first to utilize
the tumour-localizing and phototoxic properties of HPD to treat malignancies in the lab including rat implanted gliomas [123]. A significant breakthrough occurred in 1975 when Thomas Dougherty and colleague reported that activation of HPD using red light completely destroyed mammary tumour growth in mice [124]. These studies were followed by the first human trials with HPD for the treatment of cancers such as bladder (Kelly, 1975) [125] and skin (Dougherty, 1975-8)[126].

1.4.2) Benefits of photodynamic therapy

PDT is a minimally invasive treatment which can be re-administered a number of times. Unlike chemotherapy, radiotherapy and surgery it causes very few acute side effects and no systemic toxicity. Photosensitizers are non-toxic in the absence of light. The very short half-life and high reactivity of PDT-induced reactive oxygen species (ROS) (<0.04μs), enforces a very small radius of action (<0.02μm) [127]. Due to limited migration potential of ROS, sites of photodamage in PDT treated tumour closely match the localization of photosensitizer molecules at the time of illumination. This leads to a very controlled and precisely treated site which limits damage to the immediate surrounding healthy tissue. Contrary to ionizing radiation, PDT induces rapid and overwhelming apoptotic and necrotic cell death in a dose dependent manner [128, 129]. Furthermore, PDT elicits an immune response against the tumour and may induce tumour specific immunity [130, 131] which could eradicate the primary as well as any secondary metastases. Therefore, PDT is an excellent alternative to traditional treatment modalities because it offers very good cosmetic and functional results. For example in treating oral squamous-cell carcinomas where radiotherapy and surgery result in impaired function of
the treated area, PDT can be used successfully [132]. Finally, this modality is an enticing palliative treatment or alternative option to those patients who have not responded well to other mainstream therapies or who exhibit malignancy in places not accessible to surgery [132-136].

1.4.3) Photofrin-based PDT in clinical use

Further work on HPD led to the development of porphimer sodium “Photofrin”®, a partially purified form of HPD which is a mixture of mono-, di-, and oligomers that all contain the porphyrin moiety. In 1993, PDT was first approved in Canada using photofrin for the treatment of bladder cancer. This drug is also the most widely used photosensitizer today, both in the clinic and in research [136]. Photofrin-based PDT has been approved for the treatment of cancers such as cervical and gastric cancers in Japan; endobroncheal and esophageal cancers in North America, Europe and Japan; and papillary bladder cancer in Canada [119]. It also has been approved for clinical use for the palliative treatment of solid tumours where other treatments have failed [133]. As the most popular drug in the field of PDT, Photofrin possesses consistent and strong tumour ablating properties, low toxicity to normal cells in the absence of light and better defined characteristics compared to the alternative PDT drugs [136].

1.4.4) Second generation photosensitizers

The “second generation photosensitizers” were developed to absorb light at longer wavelengths to facilitate better light penetration and therefore drug activation deeper in the tumour. Some of these compounds also cause less skin photosensitivity and
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considerably cut down the 4-6 week sunlight ban required for patients treated with Photofrin-based PDT [118, 134, 136]. A systemic second generation drug that has received much attention and approval for treatment of head and neck cancer and possible use for the treatment of prostate and pancreatic cancers is meta-tetra hydroxyphenyl chlorine (m-THPC) “Foscan”® or Temoporfin. This drug is also administered for palliative treatment of advanced head and neck cancer that is unsuitable for radiotherapy, surgery or chemotherapy, or that has not responded to these treatments [137, 138]. Topical 5-aminolevulinic acid (5-ALA) “Levulan”® and its methylesther “Mevix”® are approved and widely used second generation photosensitizers for the treatment of actinic keratosis and basal-cell carcinoma of the skin [119].

1.4.5) Photodynamic therapy mechanisms of action

Photodynamic therapy inflicts its tumouricidal properties via three mechanisms of action. (A) The initial injurious impact is due to direct oxidative damage caused by reactive oxygen species (ROS), mediating injury to cellular proteins and lipids. These may in turn give rise to lethal photo-oxidative lesions with both apoptotic and necrotic cell death [133, 139, 140]. (B) PDT damages the normal and tumour vasculature and disrupts blood flow, causing severe ischemia leading to tumour infarction [133]. The ischemia developed during the treatment of tumours by PDT is followed by a restoration of blood flow and induction of ischemia-reperfusion (I/R) injury in PDT- treated tumours [141]. (C) Activation of the inflammatory/immune response is another method of PDT induced cytotoxic action against the tumour. Inflicting phototoxic lesions and I/R injury brings about various inflammation-specific events following the treatment of tumours.
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with PDT, including: (i) release of inflammatory cytokines and chemokines, arachidonic
acid metabolites and various other inflammatory mediators; (ii) pro-inflammatory
changes in vascular endothelium; (iii) complement activation and engagement of other
plasma cascade systems (kinin-generation, coagulation, and fibrinolysis); (iv) invasion of
inflammatory cells; (v) activation of NF-κB [139, 142-144].

Direct cytotoxicity of photodynamic therapy requires the administration of a
photosensitive drug "photosensitizer", light of a specific wavelength and the presence of
molecular oxygen. When exposed to its optimally absorbing wavelength of light, the
photosensitizer becomes activated from a ground to an excited state. It can then undergo
two kinds of reactions termed type I and type II [119, 133]. In a type I reaction, the
activated photosensitizer reacts directly with cellular substrates such as membranes or
other molecules, transferring a hydrogen atom to form intermediate free radicals. These
free radicals in turn will interact with oxygen to produce toxic reactive oxygen species
(ROS) such as singlet oxygen, hydrogen peroxide and hydroxyl radicals. These highly
potent reactive species oxidize various cellular substrates including lipids and proteins
and in turn can lead to cellular toxicity and cell death [145]. In type II reactions, an
activated sensitizer can transfer its energy directly to molecular oxygen to form ROS.
Type I and II reactions occur simultaneously, and the ratio between them depends on the
type of sensitizer used, the concentrations of substrates and oxygen, as well as the
binding affinity of the sensitzers for the substrates [145]. The extent of photodamage
caused by PDT is dependent on many independent factors which include: the type of
photosensitizer used; tumor localization and location of the sensitizer - extracellular
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versus intracellular; the total drug dose administered; the total light exposure dose; the light fluence rate; oxygen availability; the time between the administration of the drug and light exposure [119].

Figure 1.3: Photodynamic therapy mechanisms of action. Following the activation of a photosensitizer via specific wavelengths of light, two types of reactions take place. First, activated sensitizers can react with substrates creating radicals. These radicals in turn may further react with oxygen and produce singlet oxygen ($^1$O$_2$), a highly reactive oxygen species (type I). Alternatively, the activated photosensitizer can interact directly with oxygen to form $^1$O$_2$ and subsequently induce $^1$O$_2$ dependent oxidative damage (type II). Reprinted with permission from Macmillan Publishers Ltd: [Nature Reviews Cancer] (Dolmans, D.E.J.G.J., D. Fukumura, and R.K. Jain, PHOTODYNAMIC THERAPY FOR CANCER. Nature Reviews Cancer, 2003. 3(5): p. 380-387.), copyright © (2003)

The insults inflicted on tumours by PDT are potent inflammatory and immune stimulating trauma. PDT-induced pro-inflammatory events include: (i) primary oxidative damage; (ii) direct complement activation; (iii) damage to the extracellular matrix; (iv) direct damage of the vascular wall; (v) engagement of cellular immunity.
1.4.6) Cascade of events after PDT-induced trauma

Primary oxidative damage is the initiator of all the subsequent events taking place in the PDT-treated tumour. The modifications/damage inflicted to cellular lipids and proteins by the reactive oxygen species (ROS) are responsible for the induction of various types of stress proteins. Shortly after PDT, phospholipase activation and the elevation of intracellular calcium occur due to photo-oxidative damage to cellular constituents such as membrane lipids [146]. These are examples of typical yet crucial events eliciting early protein-kinase-mediated signal transduction pathways. Tyrosine phosphorylation, catalyzed by membrane-associated src family kinases, was observed as early as 20s after initiation of PDT [147]. PDT activated phospholipases release arachidonic acid metabolites from the membrane phospholipids [148, 149]. These liberated lipids may be participating in cell signaling pathways involved in the expression of stress proteins [150], including heat shock proteins (Hsp). Gomer and colleagues demonstrated increased levels of Hsp70 mRNA and protein after PDT [151]. Expression of other early stress proteins such as c-fos and c-jun are also induced by PDT-mediated oxidative damage [152]. Some of these early stress-induced products function as transcription factors for inflammatory genes. For instance NF-κB and AP-1 (c-fos and c-jun heterodimeric complex) are among the transcription factors activated after PDT [142, 153]. The consequence of such transcription factor activity is the expression of various immunologically important molecules including cytokines, chemokines, adhesion proteins, growth factors, enzymes involved in arachidonic acid metabolism (COX-2) and immune co-stimulation molecules [133].
1.4.7) PDT and the complement system

PDT treatment directly activates the complement system because this arm of innate immunity recognizes injured autologous cells [154, 155]. The complement activation by PDT can be attributed to different mechanisms of action. It has been demonstrated that various forms of oxidative damage can directly activate the complement system [156]. Therefore the induced oxidative damage could be responsible for PDT-mediated complement engagement. PDT instigated basement membrane exposure of the tumour vasculature also prompts the complement activation [157, 158]. Other events brought about by PDT including apoptosis [159], ischemia [160], and cytokine release [161] may down-regulate the membrane complement regulatory proteins (mCRP) and therefore reduce the inherent complement resistance of the altered autologous cells. The activated complement system is capable of stimulating a range of inflammatory responses that promote the engagement of the cellular arm of the immune system (neutrophils and other leukocytes) [155].

Endothelial cells sustaining PDT damage lesions will contract and therefore expose the basement membrane in the vessel walls [162, 163]. The exposed sub-endothelial matrix promotes the rapid binding and activation of complement [158], and attachment of neutrophils and platelets [164], all of which bring about the release of pro-inflammatory agents [165].
1.4.8) PDT and cellular immunity

The engagement of cellular immunity is manifest as the sequestration of neutrophils, mast cells and macrophages to the PDT-treated tumours. Their activity contributes to the eradication of the tumour and also to the induction of tumour specific immunity. Inflammatory mediators produced after PDT including complement proteins as well as cytokines, chemokines, coagulation cascade components, histamine and arachidonic acid metabolites are responsible for activation and accumulation of inflammatory cells in PDT treated tumours [143]. Expression of endothelial adhesion molecules such as ICAM-1 and E-selectin in tumour vasculature after PDT also plays an important role in the invasion of such inflammatory cells [166]. Neutrophils are the first and the most abundant immune cells infiltrating the PDT-treated tumour within 1 minute of treatment onset [167]. They produce a wide range of toxic agents such as reactive oxygen species (ROS) and nitric oxide (NO), which they will release into phagocytic vacuoles and/or the extracellular environment [168] destroying cancer cells [169-173]. Under appropriate conditions, these cells can also produce cytokines and chemokines [174], express MHC class II molecules and act as antigen-presenting cells [175-178]. Dying neutrophils will discharge stimulators that trigger another wave of inflammatory cell invasion, including more neutrophils as well as mast cells and monocytes.

Similar to neutrophils, mast cells show significant increase in their numbers within 5 minutes of the light treatment initiation [167]. These cells are powerful mediators of inflammation. PDT-induced complement activation may be responsible for
mast cell degranulation [179] which contributes to vascular permeability therefore increasing phototoxic tissue damage and neutrophil infiltration [180].

Macrophages are the most numerous population of stromal leukocytes in many tumours [181-183]. PDT-destroyed tumour macrophages are quickly repopulated by new waves of monocytes/macrophages, summoned by PDT-released stimuli [167, 184, 185]. These newly activated arrivals, unlike their tumour resident predecessors, serve in the eradication of tumour cells [182]. In a mouse SCCVII tumour model, macrophages repopulating the post PDT treated tumours showed a five-fold greater tumouricidal effect than the resident macrophages [167]. Macrophage activity in PDT-treated tumours is also regulated by a variety of induced inflammatory stimuli such as cytokines and chemokines [186]. Similar to neutrophils, macrophages infiltrating PDT-treated tumours may become stimulated to produce toxic levels of NO [187]. These two types of leukocytes will also up-regulate complement receptors, CD11b and CD11c, upon infiltration and may be highly important in removal of complement opsonized cells following PDT [187].

1.4.9) PDT and adaptive immunity

PDT-generated tumour-specific immunity was reported upon tumour re-challenge experiments by Canti and colleagues [188]. Specific in vivo depletion studies demonstrated that both helper and cytotoxic T cells are responsible for this PDT-induced adaptive immunity [189]. In further studies, Korbelik and colleagues demonstrated that PDT is highly effective in generating tumour-specific sensitized immune memory cells that can be recovered from lymphoid sites distant from the treated tumour at the
appropriate time. These cells, when transferred into immunodeficient scid mice were able to restore PDT-based tumour curability [130]. Therefore based on these results, it is apparent that the conditions created by tumour PDT treatment are favorable not just for the initiation of innate but also adaptive immunity. Expression of various molecules including cytokines, chemokines, adhesion and co-stimulatory proteins engage the innate immunity, but also promote cancer specific antigen presentation and development of tumour specific adaptive immunity.
2) HYPOTHESIS

Early complement components (C1q, MBL and ficolins) and pentraxins (SAP & Ptx3) are known to be involved in a rapid and non-immunogenic dead cell disposal process. Characterization of their activity and of Hsp70 when faced with the burden of clearing PDT-killed solid tumour cancer cells would be beneficial in development of new therapeutic approaches.

3) SPECIFIC AIMS

1. Determine the most significant genes involved in the removal of apoptotic cells and identify the sources responsible for their elevated expressions.

2. Examine the mechanisms responsible for the up-regulation of genes encoding the significant proteins.

3. Determine whether Hsp70 acts as an acute phase protein, produced and released by the liver, and binds to PDT-damaged cells.
4) MATERIALS AND METHODS

4.1) Animal model

The Animal Ethics Committee of the University of British Columbia had approved all protocols. The mice used in the experiments were 8-12 week old C57Bl/6J females, and kept in the Animal Research Centre (ARC) at the BC Cancer Research Centre. They were housed on Allentown ventilated racks (http://www.allentowninc.com/PDFs/MicroEnviroSystems.pdf), 4 mice per cage. After injection with Photofrin®, the mice were quarantined on smaller altD racks (http://www.altdesign.com/asp/flex_pi_24M.asp) that were kept in the dark. The food used was the lab diet food – High fat 5058 25lb bag and Low fat 5053 25lb bag. http://www.labdiet.com/indexlabdiethome.htm.

4.2) Tumor model

4.2.1) In vivo

Mouse Lewis Lung Carcinoma (LLC) (ATCC Number: CRL-1642) tumours were grown in syngeneic, immunocompetent C57Bl/6J mice and maintained in vivo by tumour brei inoculation. Tumour bearing (maintenance) mice were sacrificed using CO₂ gas, the tumours were extracted aseptically from the hind legs using forceps and surgical scissors and minced by chopping using a number 22 scalpel. For the brei, the minced tumors were passed 10 times through 18 and then 20 gauge needles respectively so the homogenate that was suspended in phosphate buffered saline solution (PBS) would flow smoothly. After washing the recipients’ legs with ethanol, 0.1 mL of tumour brei was injected into
each thigh using a 20 gauge needle. If the implanting brei was frozen in liquid nitrogen, 0.4 mL was injected into each thigh. Maintenance was started from LLC cells grown in \textit{vitro} and required 0.2 mL injection of $2 \times 10^6$ LLCs into each thigh.

For experiments, minced tumour tissue was suspended in PBS at $5 \times$ the tissue volume. An enzyme cocktail consisting of 0.3 mL of collagenase Type IV [4 mg/mL] (Sigma-Aldrich Co., St. Louis MO), 0.3 mL Dispase [3 mg/mL] (Boehringer, Mannheim, Germany), and 0.3 mL DNAase Type I [10 mg/mL] (Sigma), was added for every 5 mL tumour-PBS suspension. This final suspension was vigorously shaken to mix the contents, and then incubated at 37° Celsius for 30 minutes. After 15 minutes, the mixture was re-suspended by brief shaking. Following the incubation, the suspension was forced through a 100 micron filter using a 5 cc syringe and pelleted by centrifugation at 1000 rpm for 10 minutes at 20°C. The supernatant was discarded and the cells were washed with the same aliquot of PBS. Tumour cells were then counted using a hemocytometer and re-suspended in an appropriate volume of PBS. Each mouse received a 0.05 mL subcutaneous injection of $2-3 \times 10^6$ tumour cells in the sacral dorsal region.

All subcutaneous tumours were treated with photodynamic therapy (PDT) at approximately 14 days post-inoculation when they had reached 8-10 mm in diameter.
Materials and Methods

Figure 4.1: Hemocytometer slide and coverslip.
Coverslip is applied to slide and cell suspension is added to the counting chamber. Each counting chamber has a $3 \times 3$ mm grid. Cells in the four corner squares (1, 2, 3 and 4) and the central square (5) are counted using a hand-held counter. An average count per square is determined. (Cells touching the middle line of the triple lines bordering the top and left hand sides of each square are counted and those from the bottom and right hand sides of the squares are excluded. Cell number per mL is calculated using the following formula:

$$\text{Cells/mL} = (\text{Average count per square}) \times (\text{Dilution factor}) \times (10^4)$$

Total cells = (Cells/mL) $\times$ (Total original volume of cell suspension)

$10^4$ is the volume correction factor for the hemacytometer: each square is $1 \times 1$ mm and the depth is 0.1 mm. Figure reprinted by permission from Dr. William H. Heidcamp, ©1995
4.2.2) In vitro culture

LLC cells were cultured at 37°C, 5% CO₂ and 95% humidity, in alpha-minimal essential medium (GIBCO Invitrogen Cell Culture, Carlsbad, California, USA) supplemented with heat inactivated fetal bovine serum (10%) (Hyclone Laboratories Inc., Logan, Utah, USA), [100 µg/mL] streptomycin and [100 Units/mL] penicillin (Sigma), and adhered to the bottom of T175 cm² plastic cell culture flasks (Corning Incorporated Life Sciences, Lowell, MA, USA) (Cat.No. 431080). The cells were allowed to grow until near confluency and treated for 10 minutes with Trypsin-EDTA (GIBCO), collected with complete medium, pelleted by centrifugation at 1000 rpm for 10 minutes at 20°C, and re-suspended in complete medium at appropriate concentrations.

In the case of in vitro studies, a predetermined number of cells were plated into 3.5 cm diameter Petri dishes, so there would be approximately 0.7-0.8 x 10⁶ cells at the time of treatment (two days post-plating). Photofrin® was added 24 hrs before light treatment. Just prior to light treatment, the cells were washed with PBS and 1 mL protein-free/serum-free medium (Sigma, Cat.No. S8284) was added to each Petri dish.

In vitro cell images were taken using the Axiovert 40 CFL inverted microscope, a AxioCam MRm scientific 1.4 mega pixel digital camera and AxioVision 4.2 digital imaging software (Carl Zeiss Canada Ltd., Toronto, Ontario).
4.3) Photodynamic therapy

Photofrin® (Axcan Pharma Inc., Mont-Saint-Hilaire, Quebec, Canada) was used as the photosensitizing drug in all the experiments. This drug was reconstituted in 5% dextrose in H$_2$O to achieve a stock concentration of 2.5 mg/mL. For in vivo experiments every 1 mL of prepared stock, Photofrin® [2.5 mg/mL] was diluted in 1.5 mL of 1 x sterile PBS solution to achieve a working concentration of 1 mg/mL. This solution was then injected intravenously through the tail vein at a concentration of 10 mg/kg (0.2 mL per 20 g mouse). For in vitro experiments, the final Photofrin® concentration of 20 μg/mL was achieved by adding 16 μL of a [2.5 mg/mL] stock Photofrin® to 3.5 cm diameter Petri dishes or 3.0 cm diameter cell culture inserts yielding a final total volume of 2.0 mL. The drug was administered 24 hours before light treatment for both the in vivo and in vitro experiments. Light of 630 ± 10 nm (the absorption peak for Photofrin®), generated from a 150W QTH lamp equipped high throughoutput fibre illuminator (Sciencetech Inc., London, Ontario), was delivered through an 8mm core diameter liquid light guide (model 77638, Oriel instruments, Stratford, CT, USA). In vivo tumours received a light dose of 150 J/cm$^2$ and in vitro cells received a dose of 1 J/cm$^2$. The exposure time, producing the appropriate dose was calculated using the following equation: 

$$\text{Exposure Time} = \left( \frac{(r)^2 \pi \text{(Light dose)}}{(\text{Power output})} \right) \times \left( \frac{1 \text{ minute}}{60 \text{ seconds}} \right)$$

Where $r$ is the radius of the area being illuminated, reported in centimetres. Light dose is expressed in J/cm$^2$. Power output is in Watts or J/s. The equation provides the required exposure time in minutes. The decimals values are multiplied by 60 seconds to obtain the appropriate time in minutes and seconds.
Animals about to receive light treatment were restrained un-anaesthetized in lead holders, exposing only the dorsal sacral region where the tumours were located.

4.4) In vitro co-incubation experiment

Cell types implicated in PDT-induced up-regulation of the genes of interest were investigated using the in vitro cultured mouse Lewis Lung Carcinoma (LLC) cell line, the SV40 transformed mouse peritoneal macrophage (IC-21) cell line and the mouse hepatoma (Hepa 1-6) cell line. In these experiments, a predetermined number of LLC cells were plated into 3.0 cm diameter Millicell-®PCF cell culture inserts to collect approximately 0.7-0.8×10^6 cells at the time of treatment (two days post plating). Millicell-®PCF cell culture inserts (Millipore Corporation, Billerica, MA, USA) (Cat.No. PIHP 03050) contained Isopore™ membranes (polycarbonate) and were especially designed for transport/permeability applications. These PCF inserts had a pore size of 0.4 μm and a pore density of 1×10^8. Photofrin® was added 24 hrs before light treatment to the appropriate cell culture inserts. IC-21 and Hepa 1-6 cells were also plated into 6-well plates at approximately 0.7-0.8×10^6 cells per well. Twelve wells of IC-21s were divided equally into 4 groups: 4 hr incubation with untreated LLCs; 8 hr incubation with untreated LLCs; 4 hr incubation with PDT treated LLCs; and 8 hr incubation with PDT treated LLCs. Twelve cell culture inserts containing LLCs were also divided equally into 4 groups: 4 hrs untreated; 8 hrs untreated; 4 hrs after PDT; and 8 hrs after PDT. Just prior to PDT and co-incubation, all the cells were washed with 1×PBS, and protein-free/serum-free medium was added to each well and cell culture insert. PDT was performed on the LLC groups designated 4 and 8 hrs after PDT and all the inserts (treated
and untreated) were immediately transferred to their appropriate IC-21 groups (untreated LLC inserts were transferred to the IC-21 group incubating with untreated LLCs; treated inserts were transferred to the IC-21 group incubating with PDT treated LLC). Four hours into the co-incubation, the 4 hr untreated groups and the 4 hr treated groups (IC-21 + LLC co-incubating sets) were removed from the incubator, and the inserts were taken out of the 6-well plates. Cells were collected and processed separately using the Qiagen RNeasy® Plus Mini Kit, isolating and purifying their respective total RNA. The same procedure was done at eight hours into co-incubation and the 8 hr untreated and treated groups were removed and processed. Two-step real-time RT-PCR was performed on total RNA to analyze the gene expressions of Hsp70, SAP and ficolin B in the untreated and treated LLCs and their respective co-incubated IC-21s. The same co-incubation experiment was repeated using Hepa 1-6 cells instead of IC-21s.

Figure 4.2: In vitro co-incubation experiment. First row: Hepa 1-6, IC-21 and LLC are the cell lines used. Second row: LLCs grown in Millicell-®PCF cell culture inserts are PDT treated using the light source and co-incubated with Hepa 1-6 and IC-21 cells.
4.5) Sample collection and RNA isolation

4.5.1) Tissue collection for RNA isolation

At appropriate times following light treatment (or no treatment) mice were sacrificed using CO₂ gas. Tissues were excised using surgical scissors and forceps and immediately placed in 1.5 mL of cold TRIzol® Reagent (Invitrogen) and kept on ice. The samples were then homogenised for approximately 1 minute (three 20 second intervals). Following homogenisation, the tissues were stored at −80°C until RNA isolation.

4.5.2) RNA isolation from tissue homogenates

Workspace and pipettes were cleaned with RNase-AWAY™ (Invitrogen) and all pipetting was done using aerosol-free, ART Self-Sealing Barrier pipette tips (VWR International, Mississauga, Ontario). The samples were removed from the −80°C freezer and thawed at room temperature. They were then centrifuged at 13000 rpm for 10 minutes at 4°C (using a mini-centrifuge) to remove cell debris. The clear supernatants were transferred to new 2.0 mL microcentrifuge tubes. 200 μL of chloroform for every 1 mL of Trizol used was added. Samples were shaken vigorously for 20 seconds and incubated at room temperature for about 5 minutes. Then they were centrifuged at 13000 rpm for 15 minutes at 4°C. Two phases of liquid were present. The colourless upper aqueous phase containing the RNA prep was carefully transferred to a fresh 2.0 mL microcentrifuge tube for the phenol:chloroform RNA purification procedure.
4.5.3) RNA purification from tissue homogenates

4.5.3.1) Phenol:chloroform RNA purification and ethanol precipitation

Equal volume of phenol solution (Sigma, Cat.No. P4682) as the aqueous was added to each tube and then shaken vigorously for 20 seconds. After 5 minutes of incubation at room temperature, chloroform-isoamyl alcohol mixture (Sigma, Cat.No. P25668), at about 0.4 times the volume of phenol, was added and the tubes were shaken vigorously for another 20 seconds. After 5 minutes of incubation at room temperature, the samples were centrifuged at 13000 rpm for 15 minutes at 4°C. The two liquid phases were separated with the upper aqueous layer containing the RNA. Subsequently, the layer containing the RNA was transferred to a new 2.0 mL microcentrifuge tube and a second wash, identical to the first, using phenol was done to remove any impurities. At this stage, the aqueous layer was very clean and the purification was continued by adding an equal amount of chloroform as the aqueous layer to each sample. The tubes were shaken vigorously for 20 seconds and incubated at room temperature for 5 minutes. They were then centrifuged at 13000 rpm for 10 minutes at 4°C. Again two liquid phases were present. The colourless upper aqueous phase containing the RNA was transferred to a new tube and mixed with 3M sodium acetate (3 M NaOAc, pH: ~ 5.2) at about 0.1 times the volume of the aqueous phase. Isopropanol (Sigma, Cat.No. 195162) was added at 2 times the volume of the samples (RNA prep + NaOAc) for RNA precipitation. The liquids were mixed thoroughly by vigorous shaking and incubated at -20°C overnight (a minimum of 1 hour incubation is needed). Centrifugation of samples at 13000 rpm for 10 minutes at 4°C was performed to pellet the precipitated RNA. The supernatant was removed and the RNA pellet was washed with 1 mL of 75% ethanol. Following
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centrifugation at 13000 rpm for 10 minutes at 4°C, the supernatant was discarded and the pellet was air dried for approximately 30-45 minutes. Next, the precipitated RNA was dissolved in 50 µL of UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Cat.No. 10977-015).

4.5.3.2) RNA concentration reading and DNase digestion

The concentration of the RNA samples was determined using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Bancroft Building, Wilmington, DE, USA). Sufficient amounts of RNA samples were then carried over for DNase digestion, removing genomic DNA contaminations. Appropriate amounts of RQ1 RNase-Free DNase, RQ1 RNase-Free DNase 10× Reaction Buffer (Promega Corporation, Madison, WI, USA) (Cat.No. M610A), and UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Cat.No. 10977-015) were added to each RNA sample. Following incubation at 37°C for 30 minutes, the DNase treatment was terminated by adding an appropriate volume of DNase Stop Solution (Promega, Cat.No. M610A) and incubation at 65°C for 10 minutes.

4.5.3.3) Phenol:chloroform RNA purification and ethanol precipitation

At this point, the phenol:chloroform RNA purification and ethanol precipitation was repeated to remove any protein and ion contaminations left behind from the DNase treatment step. The precipitated RNA was dissolved in 50 µL of UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Cat.No. 10977-015) and the concentration was determined using the NanoDrop® ND-1000 UV-Vis
Spectrophotometer (NanoDrop Technologies). The samples were then stored at -80°C until use.

4.5.4) Cell culture collection for RNA isolation

Mouse Lewis Lung Carcinoma (LLC) cell line (ATCC Number: CRL-1642), SV40 transformed mouse peritoneal macrophage (IC-21) cell line (ATCC Number: TIB-186) and mouse hepatoma (Hepa 1-6) cell line (ATCC Number: CRL-1830) were collected and processed using Qiagen RNeasy® Plus Mini Kit (Qiagen Inc., Valencia, CA, USA) (Cat.No. 74134). Appropriate volumes of β-mercaptoethanol (β-ME) (Sigma, Cat.No. 63689) were added to the Qiagen kit supplied RLT Plus buffer to prepare the working lysis buffer. Cells grown in monolayer (in 3.5 cm diameter Petri dishes/3.0 cm diameter cell culture inserts) were collected by adding the recommended volume of RLT Plus buffer + β-ME, and scraping using a rubber policeman. Following homogenization, the cell lysates were transferred to 2.0 mL microcentrifuge tubes and frozen at -80°C for future RNA isolation.
4.5.5) RNA isolation and purification from cell culture extracts

Cell culture samples were collected and processed using Qiagen RNeasy® Plus Mini Kit (Cat.No. 74134). The detailed procedures described here were in accordance with the Qiagen RNeasy® Plus Mini Handbook.

Figure 4.3: Schematics of RNA isolation using Qiagen RNeasy® Plus Mini Kit. After thawing the homogenized lysates at room temperature, they were transferred to the gDNA Eliminator spin columns placed in 2.0 mL collection tubes. Following centrifugation for 30 seconds at ≥8000×g (≥10,000 rpm), the flow-through was mixed with one volume of 70% ethanol. Up to 700 μL of the samples at a time were transferred to the RNeasy spin column placed in 2.0 mL collection tubes. Samples were
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centrifuged for 15 seconds at $\geq 8000 \times g \ (\geq 10,000 \ rpm)$ and the flow-throughs were discarded. (Sample volumes exceeding 700 $\mu$L were centrifuged in successive aliquots in the same RNeasy spin column). Added to each RNeasy spin column was 700 $\mu$L of the Qiagen kit-supplied RW1 buffer and they were spun for 15 seconds at $\geq 8000 \times g \ (\geq 10,000 \ rpm)$ to wash the spin column membranes. The flow-through was discarded. Next, 500 $\mu$L of the Qiagen kit-supplied RPE buffer was added to each RNeasy spin column and centrifuged for 15 seconds at $\geq 8000 \times g \ (\geq 10,000 \ rpm)$ to wash the spin column membrane. After discarding the flow-through, the spin column membrane wash was repeated using a second 500 $\mu$L aliquot of the RPE buffer. Centrifugation for 2 minutes at $\geq 8000 \times g \ (\geq 10,000 \ rpm)$ ensured that no ethanol was carried over during RNA elution. RNeasy spin columns were then placed in new 2.0 mL collection tubes and centrifuged at full speed for 1 minute to eliminate any possible carryover of RPE buffer or residual flow-through. Spin columns were then placed in new 1.5 mL collection tubes, 50 $\mu$L of RNase-free water was directly added to the column membranes and centrifuged for 1 min at $\geq 8000 \times g \ (\geq 10,000 \ rpm)$ to elute the RNA. Concentrations of RNA samples were determined using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies).
4.6) qRT-PCR: Quantitative reverse transcriptase (RT) – polymerase chain reaction (PCR)

Many cellular functions are regulated by changes in gene expressions. Quantitation of transcription levels would help to understand gene function and overall cellular status. This information can be obtained by analysis of messenger RNA (mRNA). Real-time quantitative reverse transcriptase PCR is the latest innovation in the filed of PCR technology that provides a sensitive, reproducible and accurate method for determining mRNA levels in cells. The method is based on the detection of a fluorescent signal produced and monitored during the amplification process, without the need for post-PCR processing. SYBR® Green I based real-time PCR works by utilizing the double-stranded (ds) DNA minor groove binding ability of this fluorescent dye. In qPCR, as dsDNA accumulates, SYBR® Green I generates signals that are proportional to the DNA concentration and that can be detected using the real-time PCR instrument. Unlike conventional (end-point) PCR, real-time PCR allows the accumulation of amplified product to be detected and measured as the reaction progresses, enabling the determination of the starting template copy numbers with accuracy and high sensitivity over a wide dynamic range.

The two-Step Real-Time Quantitative RT-PCRs were done using Invitrogen’s SuperScript™ III Platinum® Two-Step qRT-PCR Kit with SYBR® Green (Cat.No. 11735-040).
4.6.1) First-strand cDNA synthesis

Complementary strand DNA or cDNA samples were synthesised from 0.132-1 μg of total RNA using Invitrogen's SuperScript™ III Platinum® Two-Step qRT-PCR Kit with SYBR® Green (Cat.No. 11735-040). Up to 1 μg of total RNA was added to PCR tubes along with 10 μL of 2× reverse transcriptase (RT) Reaction Mix (containing oligo(dT)$_{20}$, random hexamers, MgCl$_2$, and dNTPs in a buffer formulation optimized for use in qPCR), 2 μL RT Enzyme Mix (containing SuperScript™ III RT and RNaseOUT™ Recombinant Ribonuclease Inhibitor), and DEPC-treated water to achieve a final volume of 20 μL. The contents were mixed gently and the tubes were spun down for few seconds and kept on ice. A 96-Well GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) was used for cDNA synthesis by providing the following parameters:

- 25°C for 10 minutes
- 42°C for 50 minutes
- 85°C for 5 minutes

At this point, the samples were spun down and chilled on ice. One micro-litre (2 U) of *E.coli* RNase H and 1 μL of DEPC-treated water were added to each sample and incubated at 37°C for 20 minutes. *E.coli* RNase H was used to remove the RNA template from the cDNA:RNA hybrid molecule after first-strand synthesis to increase the sensitivity of downstream reactions. When finished, the reaction mixtures were stored at -20°C until use.
4.6.2) Real-time quantitative PCR (qPCR) primer and amplicon designs

The complete genomic and cDNA sequences for the 7 genes of interest (mouse complement components: Clq, MBL-A, ficolin A, ficolin B; mouse pentraxins: serum amyloid P component (SAP), Ptx3; mouse heat shock protein 70 (Hsp70)), and housekeeping gene (mouse glyceraldehydes-3-phosphatde dehydrogenase, GAPDH) were found on the Entrez, The Life Sciences Search Engine [http://www.ncbi.nlm.nih.gov/gquery/gquery.fcgi]. Based on these sequences, we designed the primers as 17-22 nucleotides and checked them with NetPrimer software [http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html] from PREMIER Biosoft International, a web-based program rating primer suitability. Most primers used were given a rating of 100%, meaning they did not form hairpins, dimers and did not contain palindromes or repeats. Most were designed to be exon/exon spanning primers, alerting of any genomic DNA contaminations. Primers were designed to produce 75-200bp long amplicons. Templates with long (>4) single base repeats were avoided, and all amplicons were blasted using Mouse Genome Informatics (MGI) MouseBLAST (WU-BLAST 2.0.) [http://mouseblast.informatics.jax.org/] to ensure that only the sequence of interest was being amplified. The primers were ordered in “desalted” format from Invitrogen’s online Custom Primers ordering website. [https://catalog.invitrogen.com/index.cfm?fuseaction=orderAssemble.simpleAddPrimerToCart&primerTypeCode=D&lid=DNABasic]. The concentrations of MgCl₂ producing the lowest C_T values were selected as the optimal MgCl₂ concentrations. C_T or threshold cycle is the cycle number at which fluorescent signal from the accumulated amplified product surpasses the background fluorescence levels and therefore is detectable.
## Materials and Methods

Table 4.1: Description of oligo-nucleotide primer pairs used in qPCR reactions.

Table 4.1 presents the genes of interest and their NCBI accession numbers used in this study; their respective primer pair sequences and alignments; their melting temperatures (Tm) in degrees Celsius; respective ratings based on NetPrimer software shown in percentage; optimal PCR MgCl₂ concentrations in mM; and their resulting amplicon size.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Alignment</th>
<th>Tm</th>
<th>Rating</th>
<th>MgCl₂</th>
<th>Amp. size</th>
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<tr>
<td>Hsp70</td>
<td>ATCACCATCACCAACGACAAG</td>
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<td>4.0 mM</td>
<td>236bp</td>
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<td>NM 010478</td>
<td>GAGATGACCTCCTGGCACTT</td>
<td>Anti-sense</td>
<td>56.16</td>
<td>100%</td>
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<td></td>
</tr>
<tr>
<td>SAP (Apes)</td>
<td>TTTTAGAGCCTTTTGGTCAGA</td>
<td>Sense</td>
<td>55.39</td>
<td>100%</td>
<td>3.5 mM</td>
<td>146bp</td>
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<tr>
<td>NM 011318</td>
<td>AAGGTCAAAGTAGGTTGGGA</td>
<td>Anti-sense</td>
<td>54.36</td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ptx3</td>
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<td>NM 008987</td>
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<td>100%</td>
<td>4.0 mM</td>
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<td>Anti-sense</td>
<td>57.93</td>
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<tr>
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<td>100%</td>
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<td></td>
</tr>
</tbody>
</table>
4.6.3) Relative real-time quantitative polymerase chain reaction (qPCR)

4.6.3.1) Real-time PCR system and reagents

All relative qPCRs were performed using the Applied Biosystems 7500 Real-Time PCR System and Invitrogen's SuperScript™ III Platinum® Two-Step qRT-PCR Kit with SYBR® Green (Cat.No. 11735-040).

4.6.3.2) Reaction components

Reaction samples were prepared in accordance with instructions supplied by Invitrogen. Component volumes were scaled down to achieve 25 μL reaction mixtures.

Each reaction mixture was prepared by adding 12.5 μL of Platinum® SYBR® Green qPCR SuperMix-UDG, 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 0.5 μL ROX reference dye (10× diluted), 2.75 μL cDNA from the first-strand synthesis reaction, 0.25 μL [50 mM] MgCl₂ if needed, and DEPC-treated water to reach the final reaction volume to 25 μL.

<table>
<thead>
<tr>
<th>Components</th>
<th>Single rxn @ 3.5 mM [MgCl₂]</th>
<th>Single rxn @ 4.0 mM [MgCl₂]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platinum® SYBR® Green qPCR SuperMix-UDG</td>
<td>12.5 μL</td>
<td>12.5 μL</td>
</tr>
<tr>
<td>Forward primer (10 μM)</td>
<td>0.5 μL</td>
<td>0.5 μL</td>
</tr>
<tr>
<td>Reverse primer (10 μM)</td>
<td>0.5 μL</td>
<td>0.5 μL</td>
</tr>
<tr>
<td>ROX Reference Dye</td>
<td>0.5 μL</td>
<td>0.5 μL</td>
</tr>
<tr>
<td>MgCl₂ (50 mM)</td>
<td>-</td>
<td>0.25 μL</td>
</tr>
<tr>
<td>DEPC-treated water</td>
<td>8.25 μL</td>
<td>8.0 μL</td>
</tr>
<tr>
<td>cDNA from the first-strand synthesis reaction</td>
<td>2.75 μL</td>
<td>2.75 μL</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>25.0 μL</strong></td>
<td><strong>25.0 μL</strong></td>
</tr>
</tbody>
</table>

*Table 4.2: Components of 25 μL qPCR reactions and their contributing volumes.*
Platinum® SYBR® Green qPCR SuperMix-UDG contained Platinum® Taq DNA polymerase, SYBR® Green I dye, Tris-HCl, KCl, 6 mM MgCl₂, 400 μM dGTP, 400 μM dATP, 400 μM dCTP, 800 μM dUTP, uracil DNA glycosylase (UDG), and stabilizers. Platinum® Taq DNA polymerase is a recombinant Taq DNA polymerase complexed with a proprietary antibody that blocks polymerase activity at ambient temperatures. Activity was restored after the denaturation step of PCR cycling, providing an automatic hot start in PCR for increased sensitivity, specificity and yield. UDG and dUTP in the qPCR SuperMix prevented the reamplification of carryover PCR products between reactions. dUTP ensured that any amplified DNA contains uracil, while UDG removed uracil residues from single or double stranded DNA. A UDG incubation step before PCR cycling destroyed any contaminating dU-containing product from previous reactions. UDG was then inactivated by the high temperatures during normal PCR cycling, allowing the amplification of genuine target sequences. SYBR® Green I included in the qPCR SuperMix, is a fluorescent dye that binds directly to double-stranded DNA (dsDNA). In qPCR, as dsDNA accumulates, the dye generates a signal that is proportional to the DNA concentration and one that can be detected using real-time PCR instruments. For multiple reactions, a master mix for each gene was prepared by adding the appropriate volume of Platinum® SYBR® Green qPCR SuperMix-UDG, gene specific forward and reverse primer pairs, ROX reference dye (10× diluted), and DEPC-treated water. The master mixes were added to clear, elevated half-skirt 96-well plates (Axygen Scientific, Inc., Union City, CA, USA) (Cat.No. PCR-96-AB-C), and then the unique reaction components, first-strand cDNAs, representing different samples were added.
4.6.3.3) qPCR cycling parameters

The following cycling parameters were used for all the PCR runs:

- 50°C for 2 minutes (UDG incubation)
- 95°C for 2 minutes
- 50 cycles of: 95°C for 15 seconds 60°C for 60 seconds

Dissociation analyses were performed after each run, and the specificity of each qPCR reaction was confirmed by analyzing the melting curve signatures of the reaction product.

4.6.3.4) Optimized qPCR reactions

Linear standard curves (high coefficient of determination, $R^2 > 0.980$), high amplification efficiencies (90-105%), and consistency across replicate reactions were achieved which illustrated optimized qPCR reactions. Standard curves were generated by amplifying 10-fold serial dilutions of cDNA samples. They were constructed by plotting the log of the sample dilution against the $C_T$ values obtained from amplification of each dilution. The equation of the linear regression line, along with the coefficient of determination ($R^2$) were used to evaluate whether the qPCR assay was optimized. The $R^2$ value of a standard curve represents how well the experimental data fit the regression line, that is, how linear the data is. Linearity, in turn, gives a measure of the variability across assay replicates and whether the amplification efficiency is the same for different starting template copy numbers. (A significant difference in observed $C_T$ values between
replicates will lower the $R^2$ value). $R^2$ values $> 0.980$ are indications of optimized qPCR assays. The amplification efficiency, $E$, was calculated from the slope of the standard curve using the following formula:

$$E = 10^{1/slope}$$

$$\% \text{ Efficiency} = (E-1) \times 100\%$$

$$\% \text{ Efficiency} = (10^{1/slope} - 1) \times 100\%$$

Efficiency close to 100% is the best indicator of a robust, reproducible assay. For optimal qPCR reactions amplification efficiencies of 90-105% are desired.

**Figure 4.4: SAP standard curve for the assessment of reaction optimization.**

Figure 4.4 represents the standard curve generated using 10-fold dilutions of a template amplified on the Applied Biosystems 7500 Real-Time PCR System to evaluate the assay's efficiency. The calculated amplification efficiency is 99.25%.
4.6.4) Real-time qPCR data analysis: Relative quantification

All the qPCR reactions performed were relative quantifications, normalized to the reference gene GAPDH. Reference genes are characterised by possessing constant expression levels across all test samples and whose expressions are not affected by the experimental treatment under study. The $\Delta C_T$ method using a reference gene was used for analysing the relative gene expressions. The relative expression levels of the target genes in different samples were determined following these steps:

1. $C_T$ of the target genes were normalized to that of the reference (ref) gene GAPDH, in all the test and the calibrator samples (naïve/untreated samples)

   \[ \Delta C_T^{(test)} = C_T^{(ref, test)} - C_T^{(target, test)} \]

   \[ \Delta C_T^{(calibrator)} = C_T^{(ref, calibrator)} - C_T^{(target, calibrator)} \]

2. Expression ratios were calculated for each target and calibrator samples

   \[ \text{Ratio (target/ref)} = 2^\Delta C_T \]

3. To calculate relative expression, each sample ratio was normalized to that of the calibrator

   \[ \text{Calibrator normalized expression} = \frac{2^\Delta C_T^{(calibrator)}}{2^\Delta C_T^{(calibrator)}} \]

   \[ \text{Test normalized expression} = \frac{2^\Delta C_T^{(test)}}{2^\Delta C_T^{(calibrator)}} \]

The results obtained were fold increase/decrease of the target genes in the test samples relative to the calibrator samples (naïve/untreated samples) and were normalized to the expression of the reference gene GAPDH.
4.7) **Agarose gel electrophoresis**

4.7.1) **Gel procedure**

The gels were run to verify the products amplified by the real-time PCR. One litre of 50× TAE buffer stock solution was prepared by mixing 242 g Tris base, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA (pH 8.0), appropriate volume of deionized distilled water (ddH₂O) and the pH adjusted to 8.0. The working 1× TAE buffer was prepared by 50× dilution of the stock in ddH₂O. Two percent w/v gels were prepared by adding 1g agarose powder (Sigma, Cat.No. A9539-50G) to a 100 mL volumetric flask containing 50 mL of 1× TAE buffer. The mixture was swirled to disperse the agarose, covered with aluminium foil and heated using a microwave on high power for 30 seconds. The flask was removed briefly and shaken gently, put back and heated for 15 more seconds. Following heating, the flask was swirled for a few minutes and allowed to cool down to about 55°C. SYBR® Green I Nucleic Acid Gel Stain (2.5 µL) (Invitrogen, Cat.No. S7563) was added (diluting the SYBR® Green 20000×) and the flask swirled to disperse the dye. The Owl EasyCast™ model B1 horizontal mini gel electrophoresis system (Owl Separation Systems Inc., Portsmouth, NH, USA), was used in conjunction with a VWR AccuPower electrophoresis power supply model 300 120 V 60 Hz (VWR International, Mississauga, Ontario). The UVT gel tray and the comb (B1-14; teeth thickness: 1.5 mm; teeth width: 4.4 mm) were placed in the buffer chamber in the casting position and the mixture was poured into the gel tray. Once solidified, the tray was turned 90 degrees, the comb was removed, an appropriate volume of 1× TAE buffer was added and then the samples were loaded. The GeneRuler™ DNA Ladder, Low Range, ready-to-use (Fermentas Life Sciences, Burlington, Ontario) (Cat.No. SM1203), and
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O’GeneRuler™ 100bp DNA Ladder, ready-to-use (Fermentas Life Sciences, Cat.No. SM1143) were diluted 3× using UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Cat.No. 10977-015) and 2 µL of the mixtures were loaded into the appropriate wells. qPCR products were first diluted 2× using UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, Cat.No. 10977-015). Subsequently, 5 volumes of diluted samples were mixed with 1 volume of 6× Orange Loading Dye Solution (Fermentas, Cat.No. R0631) and 10 µL of the mixtures were loaded into the appropriate wells. The gels were run at 60 volts : 30 mAmps for 3 hours.

4.7.2) Gel imaging

The DNA bands were visualized using a Herolab UVT-28M, standard – mid-range (312 nm) UV Transilluminator (Herolab GmbH Laborgeräte, Wiesloch, Germany), and documented with a Kodak Digital Science DC50 Electrophoresis Documentation and Analysis System 120 (Eastman Kodak Company, Rochester, New York, USA).
Figure 4.5: Quantitative RT-PCR amplicons.
As shown in the above photograph, all the bands fall within their appropriate size ranges. Ficolin B (FB) amplicon is 130 bps. GAPDH (GAP) amplicon is 167 bps. Hsp70 amplicon is 236 bps. SAP amplicon is 146 bps. Ptx3 amplicon is 93 bps. MBL-A (MBL) amplicon is 175 bps. Ficolin A (FA) amplicon is 152 bps. C1q amplicon is 221 bps.

4.8) Enzyme-linked immunosorbent assay (ELISA)

4.8.1) ELISA procedure

ELISA was used for the detection and quantification of Hsp70 in tissue extracts and serum samples. Stressgen’s Hsp70 StressXpress ELISA kit was used (Assay Designs, Inc., Ann Arbor, Michigan, USA) (Cat.No. EKS-700A) for detection. Before starting, the appropriate reagents were brought to room temperature. Recombinant Hsp70 standard (10 μg/mL stock solution of inducible Hsp70 protein) and samples (liver and serum) were prepared by diluting in Sample Diluent 1 (buffer to dilute serum samples and accompanying standards) or Sample Diluent 2 (buffer to dilute cell lysates and tissue extracts and accompanying standards). Prepared standards, blanks (Sample Diluents 1 or 2) and samples, all in 100 μL volumes were added to duplicate wells of an anti-Hsp70
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Immunoassay Plate (12 × 8 removable strips pre-coated plate with mouse monoclonal antibody specific for inducible Hsp70). The immunoassay plate was then covered and incubated at room temperature for 2 hours. Following the incubation, the wells were emptied and washed 4× with 200 µL aliquots of 1× wash buffer (buffer solution and surfactant). Hundred micro-liters (100 µL) of anti-Hsp70 antibody (rabbit polyclonal antibody specific for inducible Hsp70) were added to each well and the plate covered and incubated at room temperature for 1 hour. Following 4× wash with 1× wash buffer, 100 µL of Hsp70 conjugate (horseradish peroxidase conjugated anti-rabbit IgG) were added to each well. After incubation at room temperature for 1 hour, the wells were washed 4× with 1× wash buffer and 100 µL of TMB substrate (stabilized tetramethylbenzidine substrate) were then added to each well. The plate was incubated at room temperature for 30 minutes. Subsequently, the reactions were stopped by adding 100 µL of Stop Solution 2 (an acid solution to stop the colour reaction) to each well. Absorbance was measured at 450nm wavelength using the DYNEX MRX® Revelation™ ELISA microplate reader (DYNEX Technologies, Chantilly, VA, USA). For each step in the procedure, total dispensing time for the addition of the reagents and samples to the assay plate did not exceed 20 minutes.

4.8.2) Sample preparation

4.8.2.1) Liver samples

Mouse liver sections ranging from 0.17 g to 0.92 g were homogenized in 1 mL PBS. An appropriate amount of protease inhibitor cocktail (50 µL/g) (Sigma, Cat.No. P8340) was added. The mixtures were spun at 13000 rpm for 10 minutes to remove cell
debris and 1 mL of this supernatant was then mixed with 1 mL of 1 x Extraction Reagent (buffer for preparation of cell and tissue extracts). This final supernatant was diluted 10 x with Sample Diluent 2 to remove matrix interference and prepare the samples for ELISA.

4.8.2.2) Serum samples

Blood was collected by heart puncture and allowed to clot at room temperature for 30 minutes. The samples were centrifuged at 2700 x g for 10 minutes, and the serum was carefully collected and transferred to polypropylene tubes. Required dilutions of the serum samples were prepared using Sample Diluent 1.

4.8.3) Hsp70 standard curves

4.8.3.1) Liver standard curve

The Recombinant Hsp70 Standard, diluted with Sample Diluent 2, was used to generate a standard curve with 7 points ranging from 0.78-50 ng/mL for liver samples. Seven polypropylene tubes were labelled with the appropriate concentrations of 50 ng/mL, 25 ng/mL, 12.5 ng/mL, 6.25 ng/mL, 3.125 ng/mL, 1.56 ng/mL, and 0.78 ng/mL. Nine hundred and ninety five micro-litres (995 μL) of Sample Diluent 2 were added to the tube labelled 50 ng/mL (tube #1). Five hundred micro-litres (500 μL) of Sample Diluent 2 were added to the rest of the tubes (tubes #2, 3, 4, 5, 6 and 7). Five micro-litres (5 μL) of the Hsp70 Standard stock solution (10 μg/mL) were added to tube #1 (50 ng/mL) and mixed thoroughly. Nine hundred micro-litres (900 μL) from tube #1 (50 ng/mL) were then transferred to tube #2 (25 ng/mL) and mixed thoroughly. Similarly, 2-fold serial dilutions were carried out to generate the remaining standards (500 μL from tube #2 to
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tube #3, mixed thoroughly, etc.) up to and including tube #7. Five hundred micro-litres (500 μL) of Sample Diluent 2 were added to another polypropylene tube (tube #8) which served as the assay blank (0 ng/mL).

Figure 4.6: Liver serial dilution schematics.

4.8.3.2) Serum standard curve

The Recombinant Hsp70 Standard, diluted with Sample Diluent 1, was used to generate a standard curve with 10 points ranging from 0.049-25 ng/mL for serum samples. Eleven polypropylene tubes were labelled with the appropriate concentrations of 50 ng/mL, 25 ng/mL, 12.5 ng/mL, 6.25 ng/mL, 3.125 ng/mL, 1.56 ng/mL, 0.780 ng/mL, 0.390 ng/mL, 0.195 ng/mL, 0.098 ng/mL, and 0.049 ng/mL (tubes #1-#11 respectively). Sample Diluent 1, 995 μL, was added to tube #1 (50 ng/mL). Five micro-litres (5 μL) of the Hsp70 Standard stock solution (10 μg/mL) were added to tube #1 (50 ng/mL) and mixed thoroughly. Five hundred micro-litres (500 μL) of Sample Diluent 1 were added to the rest of the tubes (tubes #2-11). Five hundred micro-litres (500 μL) from tube #1 (50 ng/mL) were then transferred to tube #2 (25 ng/mL) and mixed thoroughly. Similarly, the
serial dilutions were carried out to generate the remaining standards (500 μL from tube #2 to tube #3, mixed thoroughly, etc.) up to and including tube #11. Five hundred micro-litres (500 μL) of Sample Diluent 1 were added to another polypropylene tube (tube #12) which served as the assay blank (0 ng/mL). Samples from tubes #2-11 (0.049-25 ng/mL) were used to generate the serum Hsp70 standard curve. The Recombinant Hsp70 Standard was aliquoted appropriately to avoid more than two freeze/thaw cycles.

**Figure 4.7: Serum serial dilution schematics.**

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**4.8.4) ELISA data analysis**

The average of the duplicate absorbance measurements for each standard, sample, and blank was calculated. The average absorbance value obtained for the blank (0 ng/mL Hsp70) was subtracted from the values obtained for standards and samples. To generate the standard curves, the Recombinant Hsp70 Standard concentrations and their corresponding absorbance were plotted on a log to log scale graph. The best fit lines were determined for the data points. Trendline equations were used to calculate the log of the concentration of the experimental samples. Upon taking the anti-logs of the values, and subsequently multiplying them by their respective dilution factors, total sample Hsp70
concentrations were determined. In order to assess the Hsp70 concentrations per unit weight for the liver samples, the total Hsp70 concentrations were then divided by the total weight of their respective starting samples. In these ELISA assays, values were considered statistically significant if they were greater than $2 \times$ the standard deviation of the zero (0 ng/mL), anything less was considered assay noise.

**Figure 4.8: Hsp70 standard curves for liver and serum samples.** The standard curves were produced by graphing the log of absorbance vs. log of concentration of 2-fold serially diluted Recombinant Hsp70 protein provided. For liver standard curve, Recombinant Hsp70 protein was diluted in Diluent 2 and for serum standard curve, Recombinant Hsp70 protein was diluted in Diluent 1.
4.8.5) ELISA performance characteristics

Liver samples were diluted 10× with the Sample Diluent 2 to remove any matrix interference during the assay. An ELISA was performed using $\sqrt{10}$ -fold serial dilutions of the liver homogenates with Sample Diluent 2, which clearly validated the appropriateness of 10× dilution of the samples. A liver sample homogenate with 50.175 ng/mL Hsp70 concentration was used for the serial dilutions. Four polypropylene tubes were labelled #1-4. Two hundred and twenty five micro-litres (225 µL) of Sample Diluent 2 were added to tubes #1-4. Hundred and four micro-litres (104 µL) of liver sample were then transferred to tube #1 and mixed thoroughly. Similarly, $\sqrt{10}$ -fold serial dilutions were carried out to generate the remaining solutions (104 µL from tube #2 to tube #3, mixed thoroughly, etc.). Two hundred and twenty five micro-litres (225 µL) of Sample Diluent 2 were added to another polypropylene tube (tube #5) which served as the assay blank (0 ng/mL). Linearity of the graph confirmed by a high coefficient of determination ($R^2$) value, showed no matrix effect and assay interference when using the dilutions performed in the experiment.
ELISA Performance for Liver Sample Serial Dilutions

![ELISA Performance for Liver Sample Serial Dilutions](image)

Log of Absorbance

<table>
<thead>
<tr>
<th>Log of Dilution</th>
<th>y = 0.8602x + 0.5043</th>
</tr>
</thead>
<tbody>
<tr>
<td>-2.5</td>
<td></td>
</tr>
<tr>
<td>-2</td>
<td></td>
</tr>
<tr>
<td>-1.5</td>
<td></td>
</tr>
<tr>
<td>-1</td>
<td></td>
</tr>
<tr>
<td>-0.5</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>-0.20</td>
<td></td>
</tr>
<tr>
<td>-0.40</td>
<td></td>
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<tr>
<td>-0.60</td>
<td></td>
</tr>
<tr>
<td>-0.80</td>
<td></td>
</tr>
<tr>
<td>-1.00</td>
<td></td>
</tr>
<tr>
<td>-1.20</td>
<td></td>
</tr>
<tr>
<td>-1.40</td>
<td></td>
</tr>
<tr>
<td>-1.5</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4.9: ELISA performance curve.**
ELISA performance was checked using serial sample dilutions. The high coefficient of determination, $R^2 > 0.980$, illustrated that no matrix interference was present at the tested dilutions, $\left(\frac{1}{\sqrt{10}}\right)^n$, $n = \text{positive integers}$.

### 4.9) Flow cytometry and antibody staining

#### 4.9.1) Flow cytometry parameters

Flow cytometry was performed using a dual laser apparatus Coulter Epics Elite ESP (Coulter Electronics, Hialeah, FL, USA). The staining with monoclonal antibodies were visualised by the fluorescent dyes fluorescein isothiocyanate (FITC) and phycoerythrin (PE), which were excited via a 488 nm laser. Emissions were split by a dichroic mirror and recorded after passing through 530 nm ± 15 and 580 nm ± 10 nm bandpass filters for the detection of FITC and PE respectively. Twenty thousand (20,000) cells were analyzed per sample. Also, forward and side light scatter (FS and SS) were recorded for each cell. The dead cells in samples were easily distinguished by their
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decreased FS and SS values. Additionally, the time of flight parameter was used for gating out cell doublets.

4.9.2) Staining procedure

*In vitro* cultured LLC cells were treated by Photofrin-PDT (Photofrin 20 μg/mL for 24 hours in complete growth medium followed by light dose of 1 J/cm²) and then left in culture for 3 hours. The cells were then collected and transferred to 5.0 mL test tubes, kept on ice and shielded from light. Following centrifugation at 1000 rpm for 10 minutes at 5°C, the supernatants were removed. Samples were resuspended in Hank’s balanced salt solution (HBSS) plus 2% heat inactivated fetal bovine serum (FBS) (GIBCO Invitrogen Cell Culture, Cat.No. 10082-147) and 0.09% sodium azide (Sigma, Cat.No. S8032), at [1.0×10⁶/mL]. Subsequently, 200 μL of the samples were spun at 1000 rpm for 10 minutes at 5°C, supernatants were removed and 100 μL of Mouse BD Fc Block™ monoclonal antibody (BD Pharmingen™, Franklin Lakes, NJ, USA) (Cat.No. 553141) were added to each tube in order to block Fc receptor mediated non-specific binding of immunoglobulins. Samples were vortexed and incubated on ice in the dark. After 30 minutes, 400 μL of HBSS were added to each sample. Cells were centrifuged at 1000 rpm for 10 mins at 5°C, the supernatants were removed and 100 μL of mouse anti-Hsp70 FITC conjugated monoclonal antibody (Assay Designs, Inc., Ann Arbor, Michigan, USA) (Cat.No. SPA-810FI) were added to each tube, vortexed and incubated at 4°C in the dark for 30 minutes. HBSS 400 μL, were added to each sample and supernatants were removed after centrifuging at 1000 rpm for 10 minutes at 5°C. Following a second wash with 400 μL of HBSS, the cells were resuspended in 400 μL of HBSS (flow cytometry...
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buffer). All the samples were kept on ice in the dark prior to analysis. The same procedure was performed using FITC conjugated annexin V (BD PharMingen™, Cat.No. 556419) to detect the amount of annexin V bound to PDT-damaged LLC cells in the absence or presence of exogenously added Hsp70 protein. Annexin V is a phospholipid-binding protein that has a high affinity for phosphatidylserine, which makes it a sensitive probe for identifying apoptotic cells. Annexin V-FITC was added at (5 µL/test) following the manufacturer instructions (including the use of a special binding buffer with defined calcium and salt concentrations: 10 mM Hepes/NaOH, pH 7.4 with 140 mM NaCl, 2.5 mM CaCl₂).

*In vitro* cultured LLC cells were treated by Photofrin-PDT (Photofrin 20 µg/mL for 24 hours in complete growth medium followed by light dose of 1 J/cm²) and then left in culture for 4, 8, or 24 hours. The cells were then collected and resuspended in phosphate buffered saline (PBS) at \[1.0 \times 10^6 /\text{mL}\]. Two hundred micro-liters (200 µL) of this suspension were incubated with (0.25 µg/sample) of 7-amino-actinomycin D (7-AAD) obtained from BD PharMingen™, (BD Biosciences, Mississauga, Ontario) (Cat.No. 559925) and (5 µL/sample) of 488 Caspase-3 substrate (a component of NucView caspase-3 assay kit produced by Biotium Inc., Hayward, CA, USA) (Cat.No. 30029-T); for 30 minutes at room temperature. The latter rapidly enters cells and is cleaved by caspase-3 to form a bright green dye that binds to DNA of apoptotic cells. On the other hand, 7-AAD is a nucleic acid dye that can enter only necrotic cells, and formerly apoptotic cells undergoing secondary necrosis.
In an alternative staining procedure, FITC-conjugated annexin V (BD PharMingen) (Cat.No. 556419) was used instead of the 488 Caspase-3 substrate. Annexin V-FITC was added at (5 μL/test) following the manufacturer’s instructions (including the use of a special binding buffer with defined calcium and salt concentrations: 10 mM Hepes/NaOH, pH 7.4 with 140 mM NaCl, 2.5 mM CaCl₂).

4.10) PDT cell death analysis

Flow cytometry was used in this section to analyse the distribution of cell death between apoptosis and necrosis after in vitro PDT treatment of LLC cells. An example of the obtained fluorescence data is shown in Figure 4.10. The results summarized in Table 4.10 serve as information illustrating the distribution of cell death patterns after the PDT treatment used in this work. The results with two types of staining (caspase-3 substrate or annexin V) were in a good agreement. The data indicate that at least a portion of initially apoptotic cells has undergone later secondary necrosis. Complicating factors for cell death analysis at later time intervals are the disintegration of dead cells and proliferation of survivors.

<table>
<thead>
<tr>
<th>Time after PDT</th>
<th>% Apoptotic cells</th>
<th>% Necrotic cells</th>
<th>% Viable cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 hours</td>
<td>10 (±1)</td>
<td>1 (±0.2)</td>
<td>89 (±1.2)</td>
</tr>
<tr>
<td>8 hours</td>
<td>20 (±1.5)</td>
<td>42 (±1)</td>
<td>38 (±2.5)</td>
</tr>
<tr>
<td>24 hours</td>
<td>0</td>
<td>35 (±5)</td>
<td>65 (±5)</td>
</tr>
</tbody>
</table>

Table 4.10: Distribution of PDT-induced cell death in vitro. This table reveals the percentage of cells dying via apoptosis or necrosis at different time intervals after PDT treatment of in vitro LLC cells. Numbers in parentheses are standard errors. % viable cells are calculated by subtracting the % apoptotic and necrotic cells from 100. SE for viable cells is calculated by adding the apoptotic and necrotic SE respectively.
Figure 4.10: Cell death flow cytometry dot plot. The graph shows a dot plot (dots representing individual cells) presenting a representative example of the results obtained with cells collected at 4 hours after PDT treatment. The extent of fluorescence associated with caspase-3 activity (ordinate) and 7-AAD staining (abscissa) is depicted in arbitrary units per cell.
4.11) Pharmaceutics

The three drugs in this thesis were used to examine the role of glucocorticoids (GC) in regulation of the Hsp70, SAP and ficolin B genes systemically. They included dexamethasone, mifepristone and metyrapone. Dexamethasone is a synthetic glucocorticoid, mifepristone is a GC receptor antagonist and metyrapone is an inhibitor of GC synthesis.

Approved by the FDA in 1958, dexamethasone is a synthetic glucocorticoid used as an anti-inflammatory or immunosuppressive agent. It is roughly 20-30 times more potent than hydrocortisone and 5-7 times more potent than prednisone. Dexamethasone is usually selected for the management of cerebral edema because of its superior ability to penetrate the CNS. Similar to other glucocorticoids, unbound dexamethasone readily crosses cell membranes and binds to its cytoplasmic receptor. Modifying transcription and ultimately protein synthesis, dexamethasone inhibits leukocyte infiltration at the site of inflammation, interferes with mediators of the inflammatory response and suppresses humoral immunity. The anti-inflammatory actions of this drug are thought to involve phospholipase A\textsubscript{2} inhibitory proteins, lipocortins. Dexamethasone is available as oral, parenteral, topical (spray) and ophthalmic dosage forms [190]. Purchased from Sigma (Cat.No. D1756) in powder form, this drug was dissolved in methanol and further diluted with D5W to achieve a working concentration of 10 µg/mL and 40 µg/kg was injected to mice intra-peritoneally [191, 192].

Mifepristone (RU-486) is a synthetic steroid with potent antiprogesterone and
antigliucocorticoid activities, antagonizing progesterone and glucocorticoid receptors. Initially used as an antiprogestagen that can terminate early pregnancy, mifepriston, at higher doses will also inhibit the glucocorticoid receptor (GR). Negatively affecting GR, this drug blocks feedback regulation of the HPA axis and subsequently increases endogenous ACTH and cortisol levels. Because of its ability to inhibit glucocorticoid action, mifepristone also has been studied as a potential therapeutic agent in patients with hypercorticalism, currently being prescribed for inoperable patients with ectopic ACTH secretion or adrenal carcinoma who have failed to respond to other therapeutic manipulations [193]. Purchased from Sigma (Cat.No. M8046) in powder form, this drug was dissolved in PEG400 with 2% ethanol to achieve a working concentration of 10 mg/mL and 40 mg/kg was injected to mice subcutaneously [194].

Metyrapone is an oral agent commonly used in tests of adrenal function. It may also be used for pituitary-function tests. This compound is a relatively selective inhibitor of steroid synthesis. It inhibits 11-hydroxylation, interfering with cortisol and corticosterone synthesis. Normally after administration of metyrapone there is a compensatory increase of pituitary ACTH release and adrenal 11-deoxycortisol secretion. Two-fold or greater increase of the urinary 17-hydroxycorticoid excretion is also observed [195]. Purchased from Sigma (Cat.No. 856525) in powder form, this drug was dissolved in PBS to achieve a working concentration of 25 mg/mL and 100 mg/kg was injected to mice intra-peritoneally 15 minutes before PDT [194].
4.12) **Statistical analysis**

All data represented in the graphs are shown as the means plus standard errors. Non-parametric Wilcoxon-Mann-Whitney U tests were performed to compare the differences between two means and Kruskal-Wallis analysis was done to compare the differences between the means of multiple sample groups. Differences were considered statistically significant at level $P<0.05$. Microsoft Excel, and GraphPad Prism® Version 4.0 software (GraphPad Software, Inc., San Diego, CA, USA) were used for statistical analysis and graphing purposes.
5) RESULTS

5.1) PDT-induced changes in the expression of genes for Hsp70, pentraxin and complement proteins at the local (tumour) and systemic (liver and spleen) sites

In order to identify which among the proteins known to be engaged in the removal of cell corpses play an important role in the removal of dead cells in PDT-treated tumours, gene expression profiles of the possible candidates were analyzed locally (in tumour) and systemically (in liver and spleen).

Twenty-four mature female C57Bl/6J mice bearing 8-10 mm subcutaneous LLC tumours were separated into 4 groups. One group was not treated and was used as the control. The remaining 3 groups were treated with PDT (Photofrin [10 mg/kg] injected intravenously via the tail vein followed 24 hours later by tumour-localized light treatment, delivering a dose of 150 J/cm²). Subsequently, tissues such as tumours, livers, spleens and blood were collected at 4 hrs, 8 hrs, and 24 hrs post light treatment. Samples were also collected from 6 healthy mice to serve as the designated naïve control group. Tumours, livers and spleens were homogenized in TRIzol® Reagent (Invitrogen). The total RNA was isolated from each tissue and relative quantitative two-step real-time RT-PCR was performed and the expression levels of the candidate genes were determined. The genes under investigation included early complement components C1q, MBL-A, and ficolins A and B; the pentraxins serum amyloid P component (SAP) and pentraxin 3 (Ptx3); and heat shock protein 70 (Hsp70). In all the gene expression studies, the
Results

housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels were also determined and used to normalize the PCR data. An additional control group was used to assess the impact of Photofrin injection without light treatment on the expression levels of the investigated genes.

5.1.1) Tumour

The results in Figure 5.1 show the GAPDH normalized gene expression values of early complement components Clq, MBL-A, ficolins A and B; pentraxins SAP and Ptx3; and Hsp70 in untreated and PDT treated tumours collected at 4, 8 and 24 hrs post light treatment. The results indicate that among the 7 genes studied, Hsp70, SAP, ficolin B and Ptx3 gene expression levels are maximally and significantly (P<0.05) up-regulated at 4 hrs, 24 hrs, 8 hrs and 4 hrs post treatment respectively. The relative tumour gene expression profiles for Hsp70, SAP, ficolin B and Ptx3 are shown in Figure 5.2. All illustrated relative gene expressions in PDT treated tumours are compared to the levels in untreated tumours. Hsp70 gene expression increased to as high as ~170-fold at 4 hrs post treatment relative to the expression level in untreated tumours. Thereafter, its up-regulation gradually subsided and was only around 9-fold at 24 hrs after PDT. Relative SAP gene expression gradually increased to over 4-fold at 24 hrs. Ptx3 gene exhibited around 18-fold increase at 4 hrs post treatment relative to untreated tumours. Its levels subsequently subsided to ~3.6-fold at 8 hrs, and then to ~1.6-fold at 24 hrs after PDT. Ficolin B relative gene expression increased gradually reaching over 18-fold up-regulation at 8 hrs post PDT and then subsided to less than 2-fold at 24 hrs after PDT.
Figure 5.1: The effect of tumour PDT on tumour-localized expression of genes encoding Hsp70, SAP, ficolin A & B, Ptx3, C1q and MBL-A. LLC tumours growing subcutaneously in C57Bl/6J mice were treated with Photofrin-based PDT (Photofrin [10 mg/kg] injected intravenously; followed 24 hours later by tumour-localized light treatment, delivering a dose of 150 J/cm²). Tumours were excised at 4, 8 and 24 hours after PDT, total RNA was isolated using Trizol and relative quantitative two-step real-time RT-PCR was performed to analyze the expression levels of selected genes. All the samples were normalized to GAPDH expression levels. Bars are standard errors.
Figure 5.2: Relative gene expression profiles of Hsp70, SAP, ficolin B and Ptx3 in PDT-treated tumours. Mice bearing LLC tumours were PDT treated and gene expression analysis of tumour tissue was performed as described for Figure 5.1. Present are the values of GAPDH normalized gene expression from Figure 5.1 relative to that in the untreated tumour samples. The inserted graph presents the same relative expression profiles for all the genes tested. * = time points at which these genes were expressed at statistically significantly different levels relative to the untreated tumour samples (P<0.05). Bars are standard errors.
5.1.2) Liver

Figure 5.3 shows the GAPDH normalized gene expressions of early complement components C1q, MBL-A, ficolins A and B; pentraxins SAP and Ptx3; and Hsp70 in livers of naïve (tumour-free) and tumour-bearing mice, collected at 4, 8 and 24 hrs post light treatment. The results indicate that among the 7 genes studied Hsp70, SAP and ficolin B gene expression levels were highly up-regulated after treatment. The relative liver expression profiles for these 3 genes are shown in Figure 5.4. All illustrated relative gene expressions in livers of PDT treated mice are compared to the levels in livers of mice bearing untreated tumours. Hsp70 gene expression levels were lower in livers of naïve mice. The expression levels reached a 17.6-fold increase at 4 hrs after PDT. Thereafter, Hsp70 gene up-regulation subsided to ~ 7-fold at 8 hrs and then to ~ 5-fold at 24 hrs after PDT. SAP gene expression was at 0.5-fold in naïve livers. The expression levels for this gene gradually increased reaching 1.5-fold at 4 hrs, ~ 7-fold at 8 hrs and 10-fold at 24 hrs after PDT. Ficolin B relative gene expression was negligible in naïve livers, where following PDT treatment, relative expression levels rose to 3-fold after 4 hrs, 4-fold after 8 hrs and finally to ~ 13-fold after 24 hrs.
Figure 5.3: The effect of PDT on the liver-localized expression of genes encoding Hsp70, SAP, ficolin A & B, Ptx3, C1q and MBL-A following the treatment. LLC tumours growing subcutaneously in C57Bl/6J mice were treated with Photofrin-based PDT as described for Figure 5.1. Mice were sacrificed 4, 8 and 24 hours after PDT. Total RNA was isolated from excised liver tissues using Trizol and relative quantitative two-step real-time RT-PCR was performed to analyze the expression levels of selected genes. All the values were normalized with GAPDH. Bars are standard errors.
Figure 5.4: PDT-induced changes in relative gene expression profiles for Hsp70, SAP and ficolin B in livers. Columns represent the values of GAPDH normalized gene expressions from Figure 5.3 relative to that in the untreated samples. The inserted graph presents the same relative expression profiles for all the genes tested. * = time points at which these genes express statistically significant difference relative to the values from the livers of untreated mice (P<0.05). Bars are standard errors.
5.1.3) Spleen

Figure 5.5 shows the GAPDH normalized gene expression values for the early complement components Clq and ficolin B as well as for Hsp70 in spleens of untreated, and PDT treated mice. Spleens of mice bearing PDT-treated tumours were collected at 4 and 24 hrs post light treatment. The results indicate that among the 3 genes studied, ficolin B and Hsp70 became up-regulated post treatment. The relative spleen gene expression profiles for Hsp70 and ficolin B are shown in Figure 5.6. All illustrated relative gene expressions in spleens of PDT treated mice are compared to the levels in spleens of untreated tumour-bearing mice. Hsp70 gene expression was at 0.5-fold in spleens of naïve mice. At 4 hrs after PDT, it elevated slightly to 1.3-fold, but the levels dropped to 0.6-fold at 24 hrs after PDT. Ficolin B gene expression levels were found to increase 3-fold at 4 hrs after PDT, and subsequently eased to 2-fold at 24 hrs after PDT.
Figure 5.5: Expression levels of Hsp70, ficolin B and C1q genes in the spleens of naïve, untreated and PDT-treated tumour bearing mice. LLC tumours growing subcutaneously in C57Bl/6J mice were treated with Photofrin-based PDT as described for Figure 5.1. Mice were sacrificed at 4 and 24 hours after PDT. Total RNA was isolated from excised spleen tissues using Trizol and relative quantitative two-step real-time RT-PCR was performed to analyze the expression levels of selected genes. All the values were normalized to GAPDH. Bars are standard errors.
Figure 5.6: PDT-induced changes in relative gene expression profiles for Hsp70 and ficolin B in spleens. Columns represent the ratios of GAPDH normalized gene expression shown in Figure 5.5 compared to that in the spleens of untreated tumour-bearing mice. The inserted graph presents the same relative expression profiles for Hsp70, ficolin B and C1q genes tested. * = points at which genes are expressed at statistically significantly different levels compared to the values from spleens taken from untreated mice ($P<0.05$). Bars are standard errors.
In summary (table 5.1), the results demonstrate the up-regulation of 3 out of 7 investigated genes locally and systemically. Hsp70, SAP and ficolin B show both local (tumour) and systemic (liver and spleen) up-regulation in response to PDT.

Although the Ptx3 gene was found to be highly expressed in PDT treated tumours, its systemic expression was maximal in the livers of untreated mice. Therefore, it is highly unlikely that Ptx3 is up-regulated systemically in response to PDT and consequently was not studied further.

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<td>MBL-A</td>
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Table 5.1: Summary of PDT-induced changes in the expression of investigated genes. This table is based on the results obtained with mice bearing LLC tumours treated by Photofrin-PDT. All gene expression data are first normalized by GAPDH and represent fold-changes relative to tumour-bearing untreated controls. a = time after PDT; b = tumor-free control.
Results

The results obtained with control groups consisting of mice treated with Photofrin alone demonstrated that in the absence of light exposure the photosensitizing drug could not induce significant up-regulation of these genes (Figure 5.7). The data even suggest a trend for Photofrin-induced down-regulation for liver Hsp70, tumour SAP and tumour ficolin B genes, but none of the changes in the gene expression levels were statistically significant.

![Relative Gene Expression after "Photofrin only" Treatment](image)

**Figure 5.7: The effect of Photofrin treatment on the relative gene expression profiles of Hsp70, SAP and ficolin B in the liver and tumour.** Mice bearing LLC tumours were given an intravenous injection of Photofrin [10 mg/kg]. The mice were sacrificed 24 hours later and tissues were excised for gene expression analysis as described for Figure 5.1. Columns represent the values of GAPDH normalized gene expressions relative to that in Photofrin-untreated tumour bearing mice. Liver and tumour samples were from the same cohort of mice. Bars are standard errors. No changes in gene expression levels are statistically significant.
5.2) Hsp70, SAP and ficolin B genes are highly up-regulated in a PDT-treated Lewis Lung Carcinoma (LLC) cell line

5.2.1) PDT induces a pronounced Hsp70, SAP, and ficolin B gene expression up-regulations in LLC treated cells

Values of GAPDH normalized gene expressions of Hsp70, SAP and ficolin B in LLC cells collected 4 and 8 hrs after PDT relative to that in the untreated samples are shown in Figure 5.8. Hsp70 gene expression is increased over 40-fold in PDT treated LLC cells after 4 hrs. This increase was even more dramatic (close to 280-fold) at 8 hrs after PDT. Expression of the SAP gene increased almost 12-fold in PDT treated LLC cells after 4 hrs and over 90-fold in PDT treated LLC cells after 8 hrs. The ficolin B gene also showed increased expression in response to PDT treatment. Its expression levels increased close to 5-fold in PDT treated LLC cells after 4 hrs and then over 80-fold in PDT treated LLC cells after 8 hrs.

A control experiment was performed to check the effects of the photosensitizing drug alone on the Hsp70, SAP and ficolin B gene expressions in LLC cells grown in vitro. Following 24 hrs of incubation with Photofrin at a final concentration of 20 μg/mL, LLC cells were collected and total RNA was isolated. Two-step real-time RT-PCR revealed that Hsp70 and SAP gene expressions were not significantly influenced and ficolin B levels were even down-regulated in response to Photofrin.
Results

Relative Gene Expression in *In Vitro* LLC Cells

Figure 5.8: Relative gene expression profiles for Hsp70, SAP and ficolin B in PDT treated LLC cells. *In vitro* cultured LLC cells were exposed to Photofrin [20 μg/mL] for 24 hours and then treated with 630±10nm light (1 J/cm²). Cells were collected at 4 or 8 hours after light treatment for determining the expression of investigated genes by two-step real-time RT-PCR. Columns depict the values of GAPDH normalized gene expressions relative to that in the untreated samples. * = points at which genes are expressed at statistically significantly different relative to their respective controls ($P<0.05$). Bars are standard errors.
5.2.2) Up-regulation of SAP, and ficolin B genes in IC-21 cells co-incubated with PDT-treated LLC cells

Figure 5.9 shows the values of GAPDH normalized gene expressions of Hsp70, SAP and ficolin B in IC-21 cells collected at 4 and 8 hrs after co-incubation with PDT-treated LLC cells relative to that in the IC-21 cells co-incubated with untreated LLC cells. While the Hsp70 relative gene expression exhibited no significant change after 4 and 8 hr co-incubation, the SAP relative gene expression increased almost 6-fold in IC-21 cells co-incubated 4 hrs with PDT treated LLC cells. That level subsided almost to pre-treatment levels during the next 4 hours. The ficolin B gene expression in IC-21 cells co-incubated for 4 hrs with PDT-treated LLCs showed no significant change but after 8 hours of co-incubation it exhibited more then 2-fold up-regulation.
Figure 5.9: Relative gene expression profiles for Hsp70, SAP and ficolin B in IC-21 cells co-incubated with PDT treated LLC cells. LLC cells growing in culture inserts were treated by PDT as described for Figure 5.8, and the inserts were then transferred to dishes with IC-21 macrophages for 4 or 8 hr co-incubations. This was followed by harvesting IC-21 cells for determining the expression of investigated genes by two-step real-time RT-PCR. Columns present the values of GAPDH normalized gene expressions relative to that in the IC-21s incubated with untreated LLCs. * = time points at which these genes are expressed at statistically significantly different relative to their respective controls ($P<0.05$). Bars are standard errors.
5.2.3) Presence of PDT-treated LLC cells induce up-regulation of Hsp70 gene in Hepa 1-6 cells

The experiment involving co-incubation with PDT-treated LLC cells was also performed with Hepa 1-6 cells instead of IC-21. Untreated and PDT treated LLCs were incubated for 4 and 8 hrs with Hepa 1-6 cells in the same manner as before. At the end of the 4 and 8 hr co-incubations, the Hepa 1-6 cells were collected and two-step real-time RT-PCR was performed on total RNA to analyze the gene expressions of Hsp70, SAP and ficolin B. The results shown in Figure 5.10 illustrate the values of GAPDH normalized gene expressions of Hsp70, SAP and ficolin B in Hepa 1-6 cells collected at 4 and 8 hrs after co-incubation relative to that in Hepa 1-6 cells incubated with untreated LLC cells. Hsp70 relative gene expression in Hepa 1-6 cells increased 4-fold and 4.6-fold after 4 and 8 hr co-incubations, respectively. SAP gene expression remained close to pre-treatment levels after 4 hr co-incubation and was remained at 0.7-fold relative to the control after 8 hr co-incubation, but this decrease was not statistically significant. Ficolin B gene expression did not change significantly after 4 and 8 hr co-incubations, remaining at ~1-fold relative to the level in control Hepa 1-6 cells.
Results

Figure 5.10: Relative gene expression profiles for Hsp70, SAP and ficolin B in Hepa 1-6 cells co-incubated with PDT treated LLCs. Following the procedure for co-incubation with PDT-treated LLC cells described for Figure 5.9, Hepa 1-6 cells were harvested for two-step real-time RT-PCR. Columns present the ratio of GAPDH normalized gene expressions relative to that in Hepa 1-6 cells incubated with untreated LLCs. * = point at which gene expression has statistically significant difference compared to the levels in respective controls (P<0.05). Bars are standard errors.
Results

5.3) Systemic Hsp70, SAP and ficolin B gene expression up-regulations are mediated partially by glucocorticoids (GCs)

Since the observed changes of the expression of the investigated genes are typical of an acute phase response which, in turn, is influenced by corticoid hormones, we examined whether glucocorticoids have a direct impact on the expression of these genes in the liver tissue.

5.3.1) The up-regulation of liver Hsp70 and SAP genes in mice treated by dexamethasone

Twenty-four mature female tumour-free C57Bl/6J mice were separated into 4 groups receiving treatments with either mifepristone, dexamethasone, dexamethasone + mifepristone (Dexa.+Mifep.) or vehicle (solvent) alone. Mifepristone, a competitive antagonist of the glucocorticoid receptor, was injected subcutaneously at a 40 mg/kg dose known to be effective in rodent models. Dexamethasone, a potent synthetic glucocorticoid, was injected intra-peritoneally at 40 μg/kg dose. For the combined treatment group, dexamethasone injection was followed immediately by an injection of mifepristone. The appropriate amounts of dexamethasone and mifepristone were delivered in 100 μL injection volumes per mouse. The purpose of the vehicle control group was to determine the effects of dexamethasone and mifepristone vehicles (solvents) on the expressions of our genes of interest. For the vehicle group, 100 μL of PEG400 with 2% ethanol was injected subcutaneously followed by 100 μL of D5W diluted methanol injected intra-peritoneally per mouse. The mice were sacrificed and
their livers excised at 4 hrs after the injections and homogenized in TRIzol® Reagent (Invitrogen). Total RNA was isolated, relative quantitative two-step real-time RT-PCR was performed and the expression levels of the Hsp70, SAP and ficolin B were determined. The housekeeping gene GAPDH expression levels were also determined and used to normalize the PCR data. The results shown in Figure 5.11 show the GAPDH normalized gene expression values and relative fold changes of Hsp70, SAP and ficolin B obtained in this experiment. The results indicate that liver Hsp70 and SAP genes were up-regulated by dexamethasone treatment and that this effect was inhibited by the glucocorticoid receptor antagonist, mifepristone. Ficolin B gene expression was not significantly affected by these treatments. The presented relative gene expressions are shown compared to the levels in the vehicle control group. Hsp70 gene expression increased 2-fold in the livers of mifepristone injected mice. Dexamethasone induced a strong up-regulation of Hsp70 gene reaching almost 5-fold increase in livers of injected mice. Following the treatment with the combination of dexamethasone and its receptor antagonist, liver Hsp70 expression levels increased only 1.4-fold compared to that of the vehicle group.

SAP gene expression decreased 0.5-fold level in the livers of mifeprisitne injected mice relative to those of vehicle group. Injection of dexamethasone induced ~ 4-fold up-regulation of this gene. The injections of both dexamethasone and mifepristone resulted in the down-regulation of liver SAP gene expression to 0.5-fold levels compared to that of the vehicle group. Ficolin B relative gene expression in the livers of treated mice appeared unaffected by the injection of mifepristone, dexamethasone, or the
combination of both. Statistical analysis confirmed that the gene expressions of Hsp70 and SAP in the dexamethasone group were significantly higher ($P<0.05$) compared to that in the mifepristone, their combination, and the vehicle control groups.

**Figure 5.11: The effect of dexamethasone, mifepristone and their combination on liver gene expression profiles for Hsp70, SAP and ficolin B.** Mature C57Bl/6 mice were injected with either dexamethasone [40 μg/kg, i.p.], mifepristone [40 mg/kg, s.c.] or their combination. Four hours later, the mice were sacrificed, the livers excised and processed for real-time RT-PCR based analysis of genes encoding Hsp70, SAP, and ficolin B. Columns represent the ratio of GAPDH normalized gene expressions compared to that in the control group (injected with solvent/vehicle only). The inserted graph represents absolute GAPDH-normalized gene expression values for all the tested groups. * = statistically significant difference compared to respective vehicle controls ($P<0.05$). Bars are standard errors.
5.3.2) Metyrapone prevents PDT-induced elevation of expression levels of the liver Hsp70, SAP and ficolin B genes

Metyrapone is an inhibitor of adrenal corticosteroid synthesis that is frequently used to block the production of glucocorticoids in vivo. In our study, this drug was dissolved in PBS and injected intra-peritoneally at 100 mg/kg dose delivered in 100 µL injections per mouse. The PDT group was treated with Photofrin-based PDT (Photofrin [10 mg/kg] injected intravenously; followed 24 hours later by tumour-localized light treatment, delivering a dose of 150 J/cm²). For the metyrapone plus PDT group, metyrapone was injected 15 minutes before the PDT light treatment. The treated mice were sacrificed and their livers were collected at 4 hrs post PDT and homogenized in TRIzol® Reagent. The total RNA was isolated, relative quantitative two-step real-time RT-PCR was performed and the expression levels of the Hsp70, SAP and ficolin B genes were determined. Figure 5.12 illustrates the GAPDH normalized gene expression values and relative fold changes of Hsp70, SAP and ficolin B in this experiment. The results indicate that PDT-induced elevation of Hsp70, SAP and ficolin B gene expression levels were at least partially inhibited by the metyrapone treatment. All the relative gene expressions are compared to the levels in the livers of the control group that consist of mice bearing untreated tumours. The results show that the treatment of tumours by PDT provoked an over 15-fold up-regulation of the expression of the Hsp70 gene in the livers of host mice. Metyrapone injection before PDT reduced this increase to 7-fold. Metyrapone injection alone caused no statistically significant effect on liver Hsp70 gene expression except for a trend suggesting a possibility of a small increase (presumably a reflection of non-specific stress). Liver SAP gene expression was also not significantly
affected by metyrapone alone. The expression of this gene increased almost 4-fold after PDT, but it should be kept in mind that a 4-hour interval is not optimal for SAP. A metyrapone injection before PDT reduced the expression level increase to 2-fold. For both the Hsp70 and SAP genes, metyrapone treatment blocked the PDT-induced increase in expression levels by almost 50%. The ficolin B gene expression remained unchanged at about 0.8-fold in the livers of metyrapone treated mice relative to untreated mice. PDT increased the ficolin B expression levels to almost 13-fold in the livers of treated mice. This increase was almost completely abrogated by metyrapone injection before PDT. The results suggest that metyrapone may have a more potent effect on ficolin B regulation than on Hsp70 or SAP genes.
Results

Figure 5.12: Relative gene expression profiles for Hsp70, SAP and ficolin B in livers of mice 4 hrs after PDT treatment and injection with metyrapone. Mice bearing subcutaneous LLC tumours were treated by PDT (as described for Figure 5.1), metyrapone [100 mg/kg, i.p.] or their combination (metyrapone injected 15 minutes before the onset of PDT light treatment). The mice were sacrificed at 4 hours after PDT and their livers were taken for real-time RT-PCR based analysis of the expression of genes encoding Hsp70, SAP and ficolin B. Columns represent the ratio of GAPDH normalized gene expressions compared to that in the untreated control group. The inserted graph presents the absolute GAPDH-normalized gene expression values for all the tested groups. * = statistically significant difference compared to untreated control group (P<0.05). ** = Statistically significant difference compared to PDT group (P<0.05). Bars are standard errors.
5.4) **Hsp70 proteins are produced and released from livers of PDT-treated mice, and travel and bind at the site of trauma**

To determine the impact of induced Hsp70 gene expression up-regulation in the livers of PDT treated mice, the levels of the protein product of this gene were measured in the sera and liver samples taken from mice bearing PDT-treated tumours.

5.4.1) **PDT-induced changes of serum Hsp70 levels**

Following standard PDT treatment of LLC tumours performed as in the previous experiments, blood was collected from the host mice at 4, 8, or 24 hours post therapy. For controls, blood was taken from naïve tumour-free mice and mice bearing untreated tumours implanted into the cohort used for PDT treatment. Hsp70 protein concentration measurement in sera derived from these blood samples was performed using a commercially available ELISA kit. The results reveal that mean serum levels of Hsp70, which were very low in naïve mice ~ [0.8 ng/mL] and may become slightly elevated (37% on average) in mice carrying untreated LLC tumours, show a rising trend at 4 hours after PDT followed by a downturn at 8 hours after PDT and further sharp drop at 24 hours after PDT (Figure 5.13). Serum Hsp70 levels at this last time-point reached statistically significant difference compared to the levels measured at earlier time-points and with untreated tumour controls. The ELISA data suggests that Hsp70 protein is released in the blood and is subsequently cleared by 24 hrs post PDT treatment, possibly by being sequestered at the site of trauma.
Results

Hsp70 Protein Levels in Serum

Figure 5.13: Hsp70 protein level profile in sera of naïve, untreated and PDT treated mice. Mice bearing subcutaneous LLC tumours were treated by PDT (as described for Figure 5.1), and their blood was collected at 4, 8 and 24 hours after PDT. Blood was also collected from tumour-free control mice and controls with untreated tumours. The serum samples were used for an ELISA-based Hsp70 protein measurement. * = statistically significant difference relative to that in the 24hrs after PDT group ($P<0.05$). Bars are standard errors.
Results

An additional control experiment was performed to check the effects of Photofrin alone on the Hsp70 protein levels in the sera. Eight mature female C57Bl/6J mice bearing 8-10 mm subcutaneous LLC tumours were separated into 2 groups. One group was not treated and used as the untreated control. The remaining group was injected with Photofrin [10 mg/kg]. At 24 hours following the intravenous Photofrin injection, blood samples were collected. The results of Hsp70 ELISA with sera from the collected blood indicate that in the absence of tumour light treatment Photofrin injection had no detectable impact on Hsp70 protein levels in the serum.

5.4.2) Liver Hsp70 protein levels decline after PDT

Mice bearing PDT-treated LLC tumours were sacrificed at 3, 5, or 9 hours after therapy and their livers excised. The concentration of Hsp70 protein in liver tissue homogenates was determined using ELISA. The results (Figure 5.14) show that liver Hsp70 levels sharply declined, with an over 30% drop evident at 3 hours post PDT and remained depressed even further at 9 hours post PDT.
**Results**

**Hsp70 Protein Levels in Livers *In Vivo***

*Figure 5.14: The effect of PDT on liver Hsp70 protein levels.* Mice bearing subcutaneous LLC tumours were treated by PDT as described for Figure 5.1. They were sacrificed at 3, 5 or 9 hours after therapy and their livers were taken for Hsp70 protein measurement based on ELISA. * = statistically significant difference compared to pre-treatment levels (\(P<0.05\)). Bars are standard errors.
5.4.3) *Ex-vivo* incubation reveals that PDT induces Hsp70 protein production in host livers

Halves of the liver tissues collected from naïve, tumour untreated, 3 and 5 hr post PDT treated mice for the previous experiment were used in the *ex-vivo* experiment, performed to test the possibility that Hsp70 proteins produced in the liver are in fact being released into the body. Both control liver samples were incubated *ex-vivo* for 9 hrs at 37°C. The liver samples collected 3 and 5 hrs post PDT were incubated *ex-vivo* for 6 and 4 hrs respectively in order to keep the total post-therapy time interval to 9 hours. Following incubation, liver samples and tissue supernatants were collected for ELISA, the results of which are illustrated in Figure 5.15. In order to display the results as total Hsp70 protein, the liver tissues and their supernatants were processed together. The Hsp70 protein levels determined in samples taken from naïve, tumour untreated and 3 hrs post PDT groups were very similar (around 65 ng per gram of liver). However livers collected from 5 hrs post PDT sacrificed mice and further incubated 4 hrs *ex-vivo*, produced significantly higher levels of Hsp70 protein.
Figure 5.15: Hsp70 protein levels in livers of naïve, tumour untreated and PDT treated mice incubated ex-vivo. Mice bearing subcutaneous LLC tumours were treated by PDT as described for Figure 5.1 and sacrificed at either 3 or 5 hours later. Their livers were excised and incubated ex-vivo for the duration needed to reach the total post-PDT time interval of 9 hours. Control livers from tumour-free mice and mice with untreated tumours were also included and incubated ex-vivo for 9 hours. Thereafter, liver samples and their supernatants were collected for ELISA-based Hsp70 measurement; the results are presented as total Hsp70 protein (liver plus supernatant). * = statistically significant difference compared to untreated group ($P<0.05$). Bars are standard errors.
5.4.4) Exogenous Hsp70 protein is capable of binding to PDT-treated LLC cells

The results of the previous experiments had led us to hypothesize that Hsp70 was released into the circulation. To determine whether it can be attracted to damaged tumour tissue, we designed an in vitro experiment to test whether Hsp70 protein is capable of binding to PDT-damaged LLC cells. In this experiment, Petri dishes with growing LLC cells were separated into 4 groups: Untreated LLC cells incubated without exogenous Hsp70 protein added; Untreated LLC cells incubated with exogenous Hsp70 protein added; PDT treated LLC cells without exogenous Hsp70 protein added; and PDT treated LLC cells with exogenous Hsp70 protein added. For PDT treatment, the cells were incubated with Photofrin [20 µg/mL] for 24 hours and then exposed to light [1 J/cm²]. Recombinant Hsp70 [10 µg/sample] was added immediately after PDT and cells were left in culture at 37°C for 3 hours. Cells were then collected, stained with FITC-conjugated antibody to Hsp70, and the levels of Hsp70 protein bound to the surface of LLC cells were detected using flow cytometry. As illustrated in Figure 5.16, both untreated groups with and without the added exogenous Hsp70 proteins presented a background fluorescence signal of about 1.2 a.u./cell. The PDT group not receiving the exogenous Hsp70 proteins expressed a 3-fold increase in Hsp70-associated fluorescence. This is in accordance with earlier findings in our laboratory that a fraction of intracellular Hsp70 moves to the cell surface after PDT treatment [196]. However, the PDT group that received the exogenous Hsp70 protein expressed significantly higher fluorescence at 4.7 a.u./cell compared to that obtained with PDT-treated LLC cells without exogenous Hsp70 protein added. This demonstrates that Hsp70 proteins can bind to PDT damaged cells.
**Exogenous Hsp70 Binding to PDT Treated LLC Cells**

**Figure 5.16:** Hsp70 protein added to culture medium binds to PDT-treated LLC cells. LLC cells growing *in vitro* were treated by PDT as described for Figure 5.8. Immediately after PDT, recombinant mouse Hsp70 was added to the cell medium [10 μg/sample] and the cultures were further incubated at 37°C. Other experimental groups included PDT-untreated LLC kept with or without added Hsp70 and PDT-treated cells left without the addition of Hsp70. After 3 hours of incubation, cells were harvested, stained with FITC-conjugated anti-mouse Hsp70 antibody and surface expression of Hsp70 was analyzed by flow cytometry. The results show the extent of Hsp70-associated fluorescence in arbitrary units per cell. * = value with statistically significant difference compared to that with PDT-treated cells not incubated with Hsp70. Bars are standard errors.
5.4.5) Hsp70 binding to PDT-treated LLC cells includes linking to phosphatidylserine expressed on the cells

Since Hsp70 was reported to bind phosphatidylserine [82], this experiment was performed to investigate the possible role of cell surface exposed phosphatidylserine in mediating the Hsp70 protein attachment to the PDT treated LLCs. Samples collected in the previous experiment were also stained with fluorochrome bound annexin V to determine the levels of annexin V binding to the PDT treated LLCs incubated for 3 hrs with and without exogenous Hsp70 protein added. As illustrated in Figure 5.17, the extent of annexin V binding to PDT treated LLCs incubated with exogenous Hsp70 protein was significantly lower than with PDT treated LLCs incubated without exogenous Hsp70 protein. This result suggests that Hsp70 proteins can bind to cells via the exposed phosphatidylserines on the cell membranes and therefore compete with annexin V for the available exposed phosphatidylserines. This in turn explains the decrease in the annexin V associated fluorescence observed in the PDT treated LLCs incubated for 3 hrs with exogenous Hsp70 protein added versus the PDT treated LLCs incubated for 3 hrs without exogenous Hsp70 protein added.
Figure 5.17: Annexin V binding to PDT-treated LLC cells incubated with or without added Hsp70. PDT-treated cells were incubated with or without added Hsp70 as described for Figure 5.16. The cells were then collected, stained with phycoerythrin-conjugated annexin V and analyzed by flow cytometry. The results show the extent of annexin V associated fluorescence in arbitrary units per cell. * = value statistically significantly different than the value obtained with other treatment group. Bars are standard errors.
6) DISCUSSION

6.1) Thesis rationale

When an organism is faced with a large number of dead cells, impaired clearance of this burden due to the absence of early complement components and pentraxins could result in an adaptive immune response. This possibility is supported by the very high correlations between C1q and serum amyloid P component (SAP) deficiencies and a predisposition to autoimmunity in both man and mouse, respectively [5, 6]. Therefore by creating large volumes of dead tumour cells and manipulating the key proteins involved in their removal, we can promote an adaptive immune response against tumours. As well modulating the early components of the complement system and/or pentraxins could also elicit the desired tumour specific immunity.

The proposed underlying mechanism for this phenomenon is as follows: Apoptotic cells are highly efficient at regulating the series of events that would ensure their disposal in a timely manner and without any immune complications in vivo. In vitro however, unphagocytosed apoptotic bodies as well as remaining cell fragments ultimately proceed to the secondary necrosis stage during which they swell and lyse. This scenario could also take place in vivo when the body is faced with abnormally large loads of apoptotic cells and a defective removal capacity. Non-ingested PDT-induced apoptotic cancer cells can proceed to secondary necrosis when an influx of water and extracellular ions mediate the swelling and eventual rupture of the cells and their organelles. Released cytoplasmic contents inflict tissue damage and result in an intense inflammatory response [197-200]. Uptake of post-apoptotic debris by phagocytes in the presence of
inflammatory cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor α (TNF-α) and interleukin 1 (IL-1) [1], induce subsequent phagocyte activation/maturation and the release of more inflammatory cytokines. Such events create the perfect condition for the engagement of the adaptive immune system. During inflammation, DCs mature, express co-stimulatory signals and enhance their ability to cross-present antigens. After arriving at the lymph nodes, they may stimulate and activate T cells [18]. Activated T cells that express co-stimulatory molecules and cytokines help mature B cells that have taken up antigens from PDT-induced apoptotic cancer cells. The reactive B cells could then divide and mature into plasma cells and secrete cancer specific antibodies [1].

Among the opsonins for apoptotic cells, complement factors including C1q, MBL, ficolins and complement-activating members of the pentraxin family such as SAP, CRP and Ptx3 seem to play important roles [14]. Therefore in this study mouse C1q, MBL-A and their homologues ficolin A & B; pentraxins SAP and Ptx3; and Hsp70 were evaluated for their potential involvement in the opsonization and removal of PDT-induced apoptotic cancer cells.

6.2) Opsonins with potential for removal of dying tumour cells

The first objective of this project was to determine the genes most involved in the removal of dead cells. In vivo gene expression studies were performed using Lewis Lung Carcinoma tumours (LLC) grown in C57Bl/6J mice. By performing real-time PCR on different tissue samples collected from naïve, untreated and PDT treated mice, expression
levels of the mentioned seven genes were evaluated. Among these candidates, Hsp70, SAP and ficolin B showed statistically significant gene up-regulations at both the local PDT treated site (tumour) and at a distant systemic site (liver). Our findings in combination with other studies will demonstrate the significance of these genes in the opsonization and removal of apoptotic cells.

Evaluating the Hsp70, SAP and ficolin B gene expressions in tumour, liver and spleen (figure 6.1) has helped us uncover other valuable information regarding some of the principal organs responsible for their synthesis. Figure 6.1A presents the Hsp70 expression values in tumours and livers of naïve, untreated and PDT-treated mice. Interestingly, tumours and livers of untreated mice expressed Ffsp70 at similar levels. As expected, PDT-treated tumours became the main source of Hsp70 expression after treatment. Despite this fact, tumours possessed the fastest rate of Hsp70 down-regulation between 8 and 24 hrs post PDT (56-fold faster than that of the liver). As shown in the graph, the tumour and liver Hsp70 curves are on a collision course after 24 hrs. Assuming the expression rates would hold beyond the 24 hr time point, one can speculate that very soon the liver becomes the major contributor to Hsp70 expression. This would support our proposed hypothesis that there is a new role for Hsp70 as an acute phase protein.

Comparing PDT-induced up-regulation of SAP in the tumour and the liver reveals a 20000-fold greater expression level of this gene in the liver (figure 6.1B). These results are in agreement with pervious studies reporting SAP as a major mouse acute phase protein of liver origin [201-203]. PDT treatment of the tumour greatly stimulated SAP
transcription in the liver which was still on the rise 24hrs after termination of the light
treatment.

Comparison of ficolin B expression in the tumour, liver and spleen clearly
identified the spleen as the major organ that transcribes this gene in naïve, untreated and
PDT-treated mice (figure 6.1C). Previous reports had implicated spleen myeloid cell
lineages in ficolin B expression [204], which is also supported by our gene expression
results. High levels of ficolin B expression were also detected in tumours after PDT
which might originate from tumour-infiltrating immune cells. To the best of our
knowledge, this is the first time that up-regulation of ficolin B has been reported in a
post-natal liver in response to PDT-induced tumour trauma. Even more interesting is the
fact that 24 hrs after treatment, the liver ficolin B expression is still on the rise while the
spleen’s and the tumour’s have plateaued or subsided. At 24 hrs the levels of ficolin B in
the liver had even reached the levels in the tumour. Observing such up-regulation at
systemic sites including the spleen and liver fuel speculations that ficolin B also may be
an acute phase protein.
Discussion

6.1A: Hsp70 Gene Expression Values

6.1B: SAP Gene Expression Values
Figure 6.1: Photofrin-based PDT induces up-regulation of Hsp70, SAP and ficolin B genes following treatment. LLC tumours growing subcutaneously in C57Bl/6J mice were treated with Photofrin-based PDT as described in figure 5.1. At 4, 8 and 24 hours post treatment, tumours, livers and spleens were excised. Total RNA was isolated and analyzed for the expression levels of selected genes. Panel A: Hsp70 expression levels in tumours and livers. Panel B: SAP expression levels in tumours and livers. Panel C: Ficolin B expression levels in tumours, livers and spleens. All the samples are normalized with GAPDH. Bars are standard errors.

In order to identify the sources responsible for the elevated expressions of Hsp70, SAP and ficolin B, in vitro gene expression studies were performed using mouse peritoneal macrophages (IC-21), mouse hepatomas (Hepa 1-6) and the LLC tumour cell line. The three investigated genes were highly up-regulated in PDT-treated LLC cells, supporting their in vivo tumour up-regulation. Moreover, untreated macrophages up-regulated SAP when co-incubated with PDT treated LLC cells, illustrating their ability to
transcribe this gene remotely in response to trauma. Ficolin B also showed an increase (although modest) in IC-21 cells, which would support the reports attributing ficolin B expression to myeloid cell lineages [204]. Since the liver is comprised of three distinct cell types, one of which is macrophage-like Kupffer cells [205], one can speculate that the observed liver ficolin B up-regulation may be due to transcription in these cells. Upon co-incubation with PDT-treated LLCs, Hepa 1-6 cells up-regulated their Hsp70 gene expression further suggesting that the in vivo liver Hsp70 expressions is due to transcription in the hepatocytes.

Using our in vitro co-incubation techniques, we have identified the possible sources responsible for the up-regulation of Hsp70, SAP and ficolin B in vivo. A further significant finding of this in vitro study is the ability of Hsp70 and SAP to be up-regulated remotely in untreated macrophages and hepatomas, further inferring the possibility of these proteins to act as acute phase reactants.

6.3) Consequences of PDT-induced trauma: Initiation of APR and activation of the HPA axis

PDT-inflicted trauma initiates the acute phase response (APR) which is a highly conserved sequence of physiologic events that occur whenever a tissue injury is of sufficient consequence to require a systemic response [206]. As described in more detail in the Introduction, APR is characterized by metabolic and hormonal changes including immunological, neuroendocrinological and neurological modifications [207].
One of the immunological responses initiated by trauma is the production and release of cytokines. These intercellular signaling proteins are regulators of the host response to infection, inflammation, and trauma [208] and are the chief stimulators of the APR [209]. Besides having local effects mediating the inflammatory response to tissue injury, cytokines also initiate systemic changes. The primary cytokines engaged after trauma are interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α). They are released from activated tissue macrophages and circulating monocytes in the damaged tissue. IL-1 and TNF-α subsequently stimulate the production and release of more cytokines, in particular interleukin-6 (IL-6), the main instigator of APR [210]. Consequently the liver is stimulated to begin de novo synthesis of a wide variety of proteins that are important for the protection of the organism and restoration of homeostasis [192]. The principal constituents of these liver proteins are the acute phase reactants including C-reactive protein (CRP), serum amyloid P component (SAP), and coagulation factors such as fibrinogen, α2-macroglobulin and many more. These molecules participate in inflammatory mediation, scavenging and tissue repair [210]. Aside from triggering the production of more cytokines, IL-1, TNF-α and IFN-γ will induce the expression of pro-inflammatory mediators which include chemokines and enzymes such as type II phospholipase (PLA₂), cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS). They also up-regulate endothelial adhesion molecules which are essential for the adhesion of leukocytes prior to their emigration into tissues [208]. Consequences of these events are elevated levels of prostaglandins (PGE₂), leukotrienes, nitric oxide (NO) and the migration of activated neutrophils. These events collectively result in inflammation, tissue destruction and loss of function [208]. It is noteworthy to mention that PDT seems
to trigger the inflammatory and the acute phase response regardless of the targeted tissue. However the intensity of these two responses is far less pronounced when a normal rather than a tumourous tissue is treated. PDT-induced inflammation of tumour tissue is greater because of a more prominent exhibition of danger signal molecules in cancerous tissue. An example is the abundance of alkyl-lipid derivatives (highly potent inflammatory/immune activators) among degradation products of membrane lipids that are hardly detectable in the membranes of normal cells [211]. APR activation characterized by serum SAP elevation was also reported following PDT treatment of normal peritoneal tissue of mice, but increase was around 10 times lower than previously documented after tumour PDT treatment [212].

The neuroendocrinological response is initiated by complex interplay between neural and circulatory events [192]. Afferent neural impulses and cytokines generated at the site of injury can activate the hypothalamic-pituitary-adrenal (HPA) axis. The neural inputs travel along sensory nerve roots through the dorsal root of the spinal cord, up the spinal cord to the medulla to activate the hypothalamus [207]. Cytokines also act on the CNS to activate the HPA axis via a corticotrophin-releasing hormone (CRH) dependent/independent stimulation of adrenocorticotrophic hormone (ATCH) [114, 213, 214]. When the hypothalamus is appropriately activated, CRH is released into the pituitary portal veins and descends into the anterior pituitary. After binding to plasma membrane receptors, this 41 amino acid peptide stimulates the release and synthesis of ACTH via calcium and cyclic AMP as second messengers [215]. ACTH is a 39 amino
Discussion

acid peptide that increases the synthesis and immediate release of glucocorticoids (cortisol), other hormones such as aldosterone, adrenal androgens and their precursors.

**Figure 6.2: The hypothalamic-pituitary-adrenal (HPA) axis.** The HPA axis refers to the communication network between the hypothalamus, pituitary and the adrenal glands. It is linked with the immune response via a feedback loop in which specific cytokines signal the brain and activate the HPA axis. Following activation, there is an increase in cortisol secretion which in turn feeds back and suppresses the immune reaction. Copyright © CNSforum by The Lundbeck Institute.

Glucocorticoids (GC) act by binding to intra-cytoplasmic glucocorticoid receptors (GR). GCs enter target cells via facilitated diffusion and bind to their receptor at the carboxyl-terminus which contains an AF$_2$ domain and is responsible for hormone binding [216, 217]. The mid-portion of the GR contains a DNA binding structure consisting of 2 zinc fingers. After binding, the GC-GR complex undergoes a conformational change and
becomes activated during which it displaces a blocking protein from the receptor. Hsp90
is the blocking protein bound to the GR and it masks the nuclear localization signal
necessary for the subsequent migration of the activated GR to the nucleus. Upon removal
of Hsp90, the activated GR-GC complex translocates to the cell nucleus where it binds as
a dimer to hormone regulatory elements on target DNA molecules and induces or
represses their transcription [215, 218]. There are about 100 genes thought to be directly
regulated by glucocorticoids. There is evidence that the GC-GR complex also can
indirectly regulate genes through induction of anti-inflammatory proteins and
transrepression mechanisms [219].

The presence of GC response elements was found in the promoter regions of
some of the hepatic acute-phase proteins [220]. Also, there is substantial experimental
evidence to support the observation that GCs enhance cytokine, especially IL-6, effects
on hepatic cell protein synthesis during the acute phase response [221]. For example,
incubation of human hepatic cells with 50-100 nM concentrations of dexamethasone,
markedly increased cytokine-induced transcriptional activation of the serum amyloid A
gene [222]. Additionally, a single intraperitoneal injection of dexamethasone to normal
rats increased 5-fold the hepatic IL-6 receptor mRNA in harvested hepatocytes within 12
hrs [223]. An indirect contribution of GCs to the synthesis of acute-phase proteins is the
ability of these hormones to stimulate protein catabolism which frees up amino acids that
may be used in the liver to form new polypeptides, particularly acute-phase proteins
[207].
Discussion

6.4) Glucocorticoid effects on Hsp70, SAP and ficolin B genes

Since tumour PDT activates the hypothalamic-pituitary-adrenal (HPA) axis in the host [224], we attempted to discover any links between HPA activation and the up-regulation of Hsp70, SAP and ficolin B seen in the liver. As mentioned before, GCs have considerable influence on liver metabolism, especially on gene regulation [220, 221]. Consequently we hypothesized that release of glucocorticoids from the adrenal gland upon stimulation, may be responsible for the hepatic up-regulation of these three genes. Authenticating our postulate, naïve mice were injected with a synthetic glucocorticoid, dexamethasone, and its receptor antagonist, mifepristone, in our first experiment. Liver samples were collected 4 hrs post injections and Hsp70, SAP and ficolin B gene expressions were measured. Administration of dexamethasone indeed significantly increased the transcription of Hsp70, and SAP. This effect is dexamethasone specific because injection of mifepristone was able to inhibit the respective gene expressions.

In a complementary experiment, a glucocorticoid synthesis inhibitor, metyrapone, was administered to mice whose tumours were subsequently treated with PDT. In support of our hypothesis, metyrapone down-regulated the expressions of Hsp70 and SAP 4 hrs after light treatment. We concluded that Hsp70 and SAP gene expressions are at least partially regulated by glucocorticoids.

These two experiments, however, suggest that ficolin B may be regulated differently from Hsp70 and SAP. Interestingly, ficolin B seemed to be unaffected to stimulation with dexamethasone, mifepristone or the combination of both. Two
Discussion

possibilities may explain this. First, the concentrations of the drugs used may not have been appropriate to stimulate a ficolin B response. Second, the hepatic up-regulation of this gene may be dependent on more factors than just glucocorticoids. This latter scenario is supported by our second experiment, demonstrating the down-regulatory effects of metyrapone on the hepatic gene expression levels of ficolin B in mice carrying PDT treated tumours. A plausible explanation could be that the PDT treatment of the tumour creates other necessary factors, complementing the glucocorticoid's stimulatory effects on hepatic ficolin B expression. Therefore exclusive injection of dexamethasone may not be enough, however blocking the synthesis of glucocorticoids may eliminate a crucial ingredient, resulting in the inhibitory effects of metyrapone.

6.5) The many roles of heat shock protein 70 (Hsp70)

Heat shock proteins (Hsp) are highly conserved polypeptides found in all prokaryotic and eukaryotic organisms. Among them, the Hsp70 family constitutes the most conserved and best studied group, consisting of the stress-inducible Hsp70 (Hsp72: 72kDa), constitutively expressed Hsc70 (Hsp73: 73kDa), the mitochondrial Hsp75 (75kDa), and the endoplasmic reticulum (ER) Grp78 (78kDa) [81, 225]. In this study we have focused on the stress-inducible Hsp70.

Cellular levels of Hsp70 are very low in un-stimulated cells, however, transcription and translation of this protein is rapidly induced upon exposure to noxious stimuli [226]. The functions of intracellular Hsp70 include chaperoning proteins, limiting protein aggregation and facilitating protein refolding [227]. These functions enable
Hsp70 to protect and improve cell survival against a wide variety of stressors [227]. Aside from its intracellular molecular chaperone modality, the evidence implicates Hsp70 in other roles depending on the context of its localization and interaction. The immunological properties of Hsps were proposed and demonstrated in the last two decades of the 20th century. Specifically, Udono and Srivastava indicated that tumour-derived Hsp70-peptide complexes are capable of eliciting cancer specific immunity [87, 88]. Also it has been illustrated that this molecule can be expressed on outer cellular membranes [228-233] and even released from damaged cells [93, 234-236], including PDT-treated tumor cells [196]. Hsp70 cell surface localization has been recently verified on the cells of primary human tumours (head and neck cancers) [228]. PDT, chemotherapy, radiotherapy and hyperthermia also increase surface expression of this molecule on treated cancer cells [196, 228, 233]. Many studies have proposed different functions for surface Hsp70, including the stabilization and preservation of lipid membrane integrity in the face of deleterious circumstances [237]. These proteins have also been attributed to plasma membrane cation channel formation [238-240]. Yet another group has suggested the transport of Hsp70 as a part of a larger molecular complex involved in regulation of certain surface receptors [230]. Release of Hsp70 has been reported by different groups [93, 235, 236, 241-244].

Other results indicate that released/extracellular Hsp70 fits the criteria of an endogenous "danger signal". Postulated by Matzinger, [245, 246] the danger theory proposes that immune activation involves danger and non danger molecular schemas. It has been hypothesized that when the integrity of the host is threatened by noxious
stimuli, cells will release endogenous stress and/or damaged self-proteins (danger signals) which would alert and activate the immune system [245, 246]. When incubated with antigen presenting cells (APC), extracellular Hsp70 molecules act as potent cytokines. Binding with high affinity to APCs, exogenous Hsp70 elicit a rapid intracellular Ca\(^{2+}\) flux; activate the NF-κB and its subsequent nuclear translocation; augment the expression and release of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 [95, 243, 247-249]; induce the release of NO [250]; up-regulate co-stimulatory molecule expressions such as CD80 and CD86 [97, 99]; and bring about dendritic cell (DC) stimulation [95, 97, 251, 252]. Different receptors have been identified as participants in Hsp70-mediated signaling and uptake. Endocytotic receptors include CD91, LOX-1 [94, 96, 253], whereas certain other receptors are specifically involved in signaling (CD40, TLR-2/TLR-4) [96, 97, 101].

Upon further investigation, Hsp70 seems to be involved in more biological process than previously believed. For example, this protein has recently been implicated in binding selectively and with high affinity to phosphatidylserine moieties uncovered on damaged plasma membranes [82]. Moreover, Guzhova and colleagues discovered the presence of antibodies against Hsp70 in patients with autoimmune disease [236]. This evidence, together with studies demonstrating the PDT-mediated induction of Hsp70 [151, 254], has induced us to pursue this protein as a possible candidate involved in the removal of dead cells after PDT. Observing Hsp70’s immune modulating roles, its ability to bind altered plasma membranes, its documented synthesis in rodent liver in response to
exercise [255, 256] and its reported release into the circulation even in the absence of tissue injury [243], prompted us to hypothesize that Hsp70 may be an acute phase protein, released by the liver into the circulation in response to stress.

### 6.6) Hsp70: Possible acute phase reactant and dead cell opsonin

In order to confirm our hypothesis, subsequent experiments were performed. Mice carrying LLC tumours were treated with Photofrin-based PDT and their livers were collected at 3, 5 and 9 hrs post light treatment. ELISA was performed on the prepared liver homogenates. The results from these experiments illustrate an interesting trend. Liver Hsp70 protein levels were at their maximum concentrations in naïve and untreated tumor-bearing mice. Three hours after termination of the light treatment, Hsp70 concentrations were significantly lower than in the livers of naïve and untreated mice. The levels recovered slightly at 5 hrs post PDT which corresponds to the *in vivo* liver Hsp70 gene up-regulations observed previously in this study. Thereafter, Hsp70 dropped to a new significantly lower concentration compared to that in naïve livers. A plausible conclusion from these kinetics is the release of Hsp70 by the liver into the circulation.

A parallel study was performed to capture any Hsp70 released from the liver. Appropriate liver sections from the mice used in the previous experiment were further incubated *ex-vivo* upon excision. Hepatic fractions from naïve and untreated mice were incubated *ex-vivo* for 9 hrs. Those from mice sacrificed 3 and 5 hrs post light treatment were incubated for 6 and 4 hrs respectively. We hypothesized that by incubating these
liver sections *ex-vivo*, we would capture the released protein within the closed confinements of the culture dish and accurately measure, using ELISA, the amount of Hsp70 that would have been released *in vivo*. Interestingly, there was a significant increase of the liver Hsp70 levels collected 5 hrs post PDT which corresponds to the *in vivo* liver gene up-regulation seen 4 hrs post treatment. However to our surprise, there was about a 62% drop in the level of Hsp70 in the livers of naïve and untreated *ex-vivo* incubated samples compared to those observed in the *in vivo* experiment. This unexpected finding has complicated the interpretation of the *ex-vivo* data. Further experiments are required to evaluate the rate of loss of Hsp70 during the incubation which would clarify our *ex-vivo* results.

The next step taken to better illustrate the release of Hsp70 by the liver into the circulation was an experiment in which serum samples from naïve, untreated and PDT treated mice were analyzed using ELISA. The tabulated serum Hsp70 graph resembles a bell-shaped curve which is inversely correlated to *in vivo* liver Hsp70 concentrations. Derived from the previous experiment, the *in vivo* hepatic Hsp70 levels were at their highest in livers of naïve mice. However, serum Hsp70 levels in these mice were relatively low. This is logical because in healthy mice there is no stress or trigger what would necessitate the release of this stress-inducible protein. Livers of untreated mice with tumors had the second highest concentration of Hsp70. Moreover, serum samples taken from them had more Hsp70 than naïve animals. This might be due to the fact that the body senses the presence of the tumour as a stressor, therefore signaling the release of this protein from the liver. At the 4 hr time point, hepatic Hsp70 levels were lower than
the untreated. However serum collected 4 hrs after treatment contained the highest concentrations of Hsp70. Since PDT is a modality inflicting trauma, treating the tumour would serve as a powerful inducer of stress that may signal the release of Hsp70 from the liver, explaining the relatively lower hepatic and higher serum levels of Hsp70 protein. The same principle can be utilized to explain the liver and serum Hsp70 levels 8 hrs post PDT. However, there is a noticeable and important difference between the 4 and the 8 hr time points. At 8 hrs, the hepatic Hsp70 levels declined compared to 4 hrs. Serum concentrations of this protein were also lower in samples collected 8 hrs post PDT. This suggests that Hsp70 was depleted from the blood. The serum collected 24 hrs post PDT also continues this trend. In fact the 24 hr serum samples contained significantly lower Hsp70 than that collected from naïve, untreated, 4 and 8 hr post PDT treated mice. Observing this interesting drop in concentration, and knowing that Hsp70 is capable of binding to apoptotic cells [82], we hypothesized that blood Hsp70 is being attracted by the PDT-damaged apoptotic cells. In order to address this hypothesis, an in vitro experiment was performed in which LLC cells where treated with PDT and incubated in the presence or absence of exogenous Hsp70. After 3 hrs, samples were collected and Hsp70 bound to cell surfaces was measured using flow cytometry. The results indicated that indeed the levels of membrane-bound Hsp70 are significantly higher in the treated group incubated with exogenous Hsp70. To further explore this phenomenon and confirm the previously reported importance of phosphatidylserine moieties in this interaction [82], flow cytometry was extended by analyzing cells stained with fluorophore-conjugated annexin V. The significant decrease found in the annexin V-associated fluorescence in
the "exogenous Hsp70 added" group confirms that this protein competes with annexin V for binding to phosphatidylserine on the cell membranes.

In summary, our studies have demonstrated that liver originated Hsp70 may be released into the circulation upon infliction of PDT-induced trauma, and PDT-treated tumours appear to be the site absorbing this circulating Hsp70. This interaction is achieved at least in part by the presentation of phosphatidylserine on PDT damaged tumour cells and the affinity of Hsp70 for such moieties presented on compromised cells.
7) CONCLUSION

Photodynamic therapy is capable of inflicting overwhelming trauma to the targeted solid cancer tumours. Faced with such tremendous injury, the body mounts array of responses to engage and contain the damaged tissue. Tissue repair and reconstitution of homeostasis, which are dependent on the proper disposal of dead and damaged cells, soon follow. A subset of the innate immune system and acute phase proteins are among the crucial participants in the removal of dead cells. Absence and/or failure of these players, including the early complement components and pentraxins, when faced with large loads of apoptotic cells could result in the generation of an adaptive immune response.

This phenomenon could be utilized to elicit cancer specific immunity by manipulating the levels of key proteins involved in the clearance of PDT-induced apoptotic tumour cells. In order to achieve this, we examined the gene expressions of the early complement components (Clq, MBL-A and ficolins A & B), pentraxins (SAP & Ptx3) and Hsp70 to determine the genes most involved in the removal of apoptotic cells, and to establish an understanding of the mechanisms responsible for the transcription of these genes.

Among the genes studied, Hsp70, SAP and ficolin B show gene expression up-regulation at the local (tumour) and at the systemic (liver) sites and therefore appear to be the main candidates for the removal of PDT killed cancer cells. It is interesting to note that PDT-damaged LLC cells highly up-regulate the expression of Hsp70, SAP and
Conclusion

ficolin B genes, possibly to increase the efficiency of their removal. The systemic up-regulation of these 3 genes are mediated at least in part by the activation of the hypothalamic-pituitary-adrenal (HPA) axis and the release of glucocorticoids which demonstrate the importance of the systems and organs indirectly affected. Another significance of this study is the demonstration of a novel attribute of the Hsp70 protein. Based on the results described here, Hsp70 may act as an acute phase protein involved in the opsonization of dead cells.
8) FUTURE DIRECTIONS

We have only begun to explore the possibility of eliciting an anti-tumour adaptive immune response by manipulation of the innate immune system. There is strong evidence implicating the early complement components and pentraxins in the development of an adaptive immunity. Exploring this phenomenon, one would be tempted to try and utilize it as an arsenal in cancer immunotherapy.

In this thesis we have performed the pioneering work, studying the gene expressions of the early complement proteins (C1q, MBL-A, ficolins A & B), pentraxins (SAP, Ptx3) and Hsp70. By doing so, we discovered that Hsp70, SAP and ficolin B show significant gene up-regulation at the local PDT-treated (tumour) and at the systemic (liver) sites. Our results combined with those from previous studies suggest that Hsp70, SAP and ficolin B appear to be the main candidates for the removal of PDT-killed cancer cells.

Despite these findings, much more remains to be elucidated. Further studies supplementing the results of this project could be aimed at verifying the major candidates involved in opsonization and removal of dead cells. Although we looked at gene expressions of the suspected players, other proteins may be stored in protein deposits and discharged upon demand without prior up-regulation of their respective genes. Therefore proteomic studies exploring such potentials are valuable.
Future Directions

The chief unanswered question is whether the genomic/proteomic manipulation of the key candidates would have the desired immunological effects. In order to understand and interpret the results of such studies, more insight into the mechanism of action of these proteins are needed. One such area of research is the study of the receptors involved and the consequences of their engagement. As demonstrated for Hsp70, six different receptors may contribute to a wide variety of outcomes. The presence of different combination of receptors on distinct cells, at specific times, could spatially and temporally regulate the effects of Hsp70. Therefore cataloguing the complement, pentraxin and Hsp70 receptors would help us to better understand and to ultimately achieve the desired immunological effects by manipulating these receptors and/or their expressing cells.

The ex-vivo Hsp70 ELISA was performed to support the trends seen in vivo and also to validate our hypothesis that this stress-inducible protein is being released from the liver into the circulation. However, due to complications, the results of the ex-vivo study is very hard to interpret. Further experiments would be helpful to calculate the rate of loss of Hsp70 ex-vivo. Tabulating this unknown would allow us to quantify the amount of Hsp70 accumulated as the result of synthesis. Other in vivo experiments using Colchicine-like drugs that would stop the export and/or release of proteins from the liver could reveal the amount of Hsp70 protein released into the circulation as a result of PDT-induced tumour trauma.
Future Directions

Issues to be taken into consideration when interpreting the results from this thesis are the animal and tumour models used. Although the murine immune system and murine tumours closely resemble those of man, only clinical studies would demonstrate this with any certainty. Therefore it is the wish of this author to be able to perform preliminary investigations on patients' samples in the near future.
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# APPENDIX

## Ethics certificate

*THE UNIVERSITY OF BRITISH COLUMBIA*

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<th>Application Number:</th>
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<tr>
<td>Investigator or Course Director:</td>
<td>Mladen Korhelik</td>
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<td>Department:</td>
<td>Pathology &amp; Laboratory Medicine</td>
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<td>Animals:</td>
<td>Mice C3H/HeN, C57BL/6J, B612984C3 660</td>
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<td>Start Date:</td>
<td>June 16, 2005</td>
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<td>Approval Date:</td>
<td>June 2, 2006</td>
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<td>Funding Sources:</td>
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<tr>
<td>Grant Agency:</td>
<td>National Cancer Institute of Canada</td>
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<tr>
<td>Grant Title:</td>
<td>Relevance of complement activation in photodynamic therapy-mediated eradication of solid tumors</td>
</tr>
<tr>
<td>Unfunded title:</td>
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The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.