TUMOUR PHOTODYNAMIC THERAPY-INDUCED CHANGES OF COMPLEMENT GENE EXPRESSION

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Abstract

Photodynamic therapy (PDT), a clinically established modality for treatment of tumours and other lesions, involves the administration of a light-activated drug that is activated at the targeted site by exposure to light. Light energy transforms the drug into a chemically active state and, in the presence of oxygen, leads to the formation of toxic oxygen derivatives. This oxidative, PDT-inflicted damage elicits a response from the host that plays an important role in the outcome of therapy.

A major element of this host response is the activation of the complement system. Complement proteins are produced in the liver and it was presumed until recently that all components were synthesized there and released into the circulation. Recently, it has become clear that other cells besides hepatocytes are capable of synthesizing some or all of the cascade components. The purpose of this study was to examine the origin of complement production by the semi-quantitative RT-PCR analysis of key complement component genes C3, C5 and C9. Specifically we wanted to determine whether up-regulation of these genes occurred following PDT and if so, was it happening in the liver or locally at the tumour. Our hypothesis was: PDT results in an increase of the tumour-localized expression of key complement component genes.

An in vivo time course experiment using Lewis Lung Carcinoma (LLC) growing in C57BL/6J mice examined the local and hepatic expression of C3, C5 and C9 following PDT. The results indicated no hepatic up-regulation of these genes but local expression in the tumour significantly increased (3.5 fold for C3, 3.2 fold for C5 and 1.7 fold for C9) at 24 hours following PDT light administration. These levels declined but remained elevated until five days post-treatment. Because this peak time point coincides with
immune cell infiltration in vivo we wanted to examine the ability of macrophages in vitro to respond to PDT-treated LLC cells. This was done by co-incubating macrophages with PDT-treated LLC cells. Gene expression analysis revealed that macrophages significantly up-regulate the expression of complement genes C3, C5 and C9 by 8 hours following treatment and that this increase of gene expression results in a significant increase of complement protein levels in these cells by 16 hours co-incubation as compared to the control (no PDT) as determined by FACS. It was also discovered that malignant LLC cells themselves produce basal levels of complement proteins, but do not respond to PDT by increasing complement gene expression.

Further experiments revealed that PDT-induced complement gene up-regulation was less pronounced in tumours growing in Toll-like receptor 4 (TLR-4) knockout mice compared to tumours in WT hosts. Using a series of specific inhibitors or blocking antibodies in the in vitro system with macrophages co-incubated with PDT treated tumour cells, it was confirmed that the TLR signaling pathway leading to NF-κB activation has a major role in this phenomenon. The results of this thesis shed light on complement engagement during the local host response which carries implications for the effective treatment of tumours by this therapy. These components may be harnessed and targeted to improve therapy by means of better controlling the complement response to PDT and therefore controlling a large component of the critical immune response which is necessary for a positive outcome after therapy.
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**Abbreviations**

(ALA) 5-aminolevulinic Acid  
(APAAP) Apoptosis Protease Activating Factor  
(APC) Antigen Presenting Cell  
(APS) Ammonium persulfate  
(ATP) Adenosine Tri-Phosphate  
(BPD) Benzoporphyrin Derivative  
(CR) Complement Receptor  
(CRP) C-Reactive Protein  
(CTL) Cytotoxic T Lymphocyte  
(DAF) Decay Accelerating Factor  
(DEPC) Diethylpyrocarbonate  
(dNTP) deoxyNucleotide Tri Phosphate  
(ERK) Extracellular Signal Regulated Kinase  
(ETC) Electron Transport Chain  
(FDA) Food and Drug Administration  
(FITC) Fluorescein Isothiocyanate  
(GAPDH) Glyceraldehyde-3-Phosphate Dehydrogenase  
(HBSS) Hank’s Buffered Saline Solution  
(HpD) Hematoporphyrin Derivative  
(HSP) Heat Shock Protein  
(ICAM) Intercellular Adhesion Molecule  
(IFN) Interferon  
(Ig) Immunoglobulin  
(IL) Interleukin  
(IRAK) Interleukin-1 Receptor Associated Kinase  
(JNK) c-Jun N-terminal Kinase  
(KO) Knock Out  
(LDL) Low Density Lipoprotein  
(LLC) Lewis Lung Carcinoma  
(mAb) Monoclonal antibody  
(mCRP) Membrane-bound Complement Regulatory Protein  
(MAC) Membrane Attack Complex  
(MAPK) Mitogen Activated Protein Kinase  
(MASP) (MBL)-associated Serine Protease  
(MBL) Mannose Binding Lectin  
(MCP) Membrane Cofactor Protein  
(MHC) Major Histocompatibility Complex  
(MIP) Macrophage Inflammatory Protein  
(mTHPC) meta-tetrahydroxyphenylchlorin  
(MyD88) Myeloid Differentiation Protein 88  
(NaOaC) Sodium Acetate  
(NF-kB) Nuclear Factor Kappa B  
(PARP) Poly (ADP-Ribose) Polymerase  
(PCR) Polymerase Chain Reaction
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>(PDT)</td>
<td>Photodynamic Therapy</td>
</tr>
<tr>
<td>(PE)</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>(PMN)</td>
<td>Polymorphonuclear Leukocyte</td>
</tr>
<tr>
<td>(PRR)</td>
<td>Pattern Recognition Receptor</td>
</tr>
<tr>
<td>(PS)</td>
<td>Phosphatidyl Serine</td>
</tr>
<tr>
<td>(ROS)</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>(RT-PCR)</td>
<td>Reverse Transcriptase Polymerase Chain Reaction</td>
</tr>
<tr>
<td>(SAP)</td>
<td>Serum Amyloid P component</td>
</tr>
<tr>
<td>(TAM)</td>
<td>Tumour Associated Macrophage</td>
</tr>
<tr>
<td>(TIR)</td>
<td>Toll/IL-1R</td>
</tr>
<tr>
<td>(TIRAP)</td>
<td>TIR Domain Containing Adaptor Protein</td>
</tr>
<tr>
<td>(TLR)</td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td>(TEMED)</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>(TNF)</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>(Tollip)</td>
<td>Toll Interacting Protein</td>
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<tr>
<td>(TRAF)</td>
<td>Tumour Necrosis Factor Receptor Associated Factor</td>
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<tr>
<td>(WT)</td>
<td>Wild Type</td>
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Introduction and Literature Review

Section 1: The History and Current Clinical Uses of Photodynamic Therapy

I. History of Photodynamic Therapy

The healing properties of light have been recognised and applied to treat various skin diseases such as vitiligo, psoriasis, and skin tumours since antiquity (1, 2). However, it was not until more recently, that “phototherapy” began to be used more routinely in medicine (1). Light has been used in the modern treatment of rickets, tuberculosis, scurvy and smallpox (1, 2). Photo-chemotherapy, which combines the application of a photosensitizing agent followed by exposure to light has also been used by civilizations dating back over 3000 years (1). Psoralens have been used to treat psoriasis and vitiligo by the ancient Egyptians and North American Indians and are still used today in modern immunotherapy (1). It was not until 1900 however, that Oscar Raab showed that “phot chemotherapy” causes cell death. He observed that acridine in combination with light exposure induces death of paramecium in vitro (1, 2). In 1903 Jesionek and Tappeiner observed therapeutic effects of topically applied eosin and white light exposure on skin tumours. They termed this effect “photodynamic action” which is still used today to describe the primary and secondary reactions which ensue following “photodynamic therapy” (PDT) (1, 2, 3). Photodynamic therapy, then, is the use of photosensitive compounds in combination with light to treat disease where cell death is desired. The discovery by Jesionek et al. paved the way for further investigation into, and development of, this phenomenon.

In 1911 Haussmann characterized the photo-sensitivity changes, photo-toxicity and biological effects of hematoporphyrin (4). It wasn’t until the 1940s that the tumour localizing properties of hematoporphyrin were described and in the 1950s and 1960s
Lipson and Schwartz purified the mixture of porphyrins that comprise hematoporphyrin and developed Hematoporphyrin Derivative (1, 5, 6). Hematoporphyrin Derivative (HpD) could be used in much lower doses and was more than twice as phototoxic as its predecessor. It was subsequently found that the extent of the photoreaction was dependent upon the drug dose, the time between administration of the drug and the light exposure, as well as the light dose. The properties of HpD were exploited during the investigation of tumour therapy and tumour detection using this drug (1, 2).

Diamond et al. (1972) followed by Dougherty et al. (1975) were the first to utilize the tumour-localizing and phototoxic properties of HpD to treat cancerous lesions (7, 8). The first human studies followed suit and in 1978 Dougherty and colleagues reported the results of the first large-scale human study involving photodynamic therapy using HpD. In this study, 113 primary and secondary skin tumours (squamous cell carcinoma and basal cell carcinoma) were treated using HpD and red light. Ninety-eight tumours completely regressed, 13 partly responded and 2 showed no response (9). This pioneering work led to the development of porphimer sodium "Photofrin"® (FDA approval, 1993), a purified version of HpD and the most widely used photosensitizer today, both in the clinic and in the lab (14). Photofrin has been approved for clinical use for the palliative treatment of solid tumours where other treatments failed (10). It has also been approved for the treatment of early and late stage lung cancers, surface gastric tumours, oesophageal adenocarcinoma, bladder and cervical cancers (14). Clinical trials involving Photofrin are currently being carried out in order to better determine the value of Photofrin-based PDT for the treatment of prostate cancer, head and neck malignancies, pre-malignant conditions of the oesophagus, bladder and lung cancers in addition to many other conditions (11, 12, 14, 15).
II. Clinical Trials of Photofrin-based PDT

One advantage of porfimer sodium is that because of its widespread use, it has been better characterized than other, alternative drugs. In addition, Photofrin has consistently shown strong tumour ablation and low toxicity to normal cells in the absence of light (14). Clinical trials are ongoing in the evaluation of Photofrin based PDT for the treatment of solid tumours. Barrett’s esophagus, although already approved for PDT treatment in Canada, is under investigation using Photofrin and the largest clinical trial published to date was done by Overholt et al. (1999) which demonstrated that in all patients treated, 80% of treated Barrett’s cells returned to normal squamous cell epithelia (13). Brain tumours are also a target for Photofrin-based PDT. Light is administered using an optical fiber and PDT is done in concert with surgery and immediately following the resection of malignant glial tumours (12). Head and neck cancers as well as cancers of the oral cavity are also at the forefront of research in photomedicine involving porfimer sodium and have less morbidity and mortality associated with treatments than traditional surgeries or radiation (14). A clinical study was done using PDT to treat breast cancer that had progressed to the chest wall. Patients treated by photodynamic therapy showed tumour necrosis at the site of treatment and initial regression of metastatic disease. It was concluded that Photofrin-based PDT is a good treatment option for this disease and provides good clinical outcome (15).

III. Clinical Trials Using Second Generation Photosensitizers

Many trials have shifted from Photofrin to evaluation of other photosensitizing drugs, termed “second generation photosensitizers”, which show a better depth of tumour penetration, lower skin sensitivity following treatment and a smaller time period between administration of drug and light treatment (1, 11, 14). Second generation
photosensitizers are being developed with the aim of creating a pure chemical drug with more effective biological properties that act at a greater tissue depth. A number of clinical trials are being executed using second generation drugs, which have led to their increasing acceptance and characterization in the field of photomedicine (14). The only systemic second generation drug approved for cancer therapy is temoporhin and it is used primarily for the palliative therapy of advanced head and neck cancers. Verteporfin (Benzoporphyrin Derivative, BPD) is widely and effectively used for the treatment of an ophthalmic condition of the macula termed “macular degeneration”. Although not used for the treatment of cancer, this drug shows properties that could be harnessed for use in cancer therapy (14). Aminolevulinic acid (ALA) is the only topical drug approved (FDA 1999) for the treatment of skin cancer (both pre-malignant melanomas and melanomas in addition to actinic keratosis) (14, 16). ALA is a precursor of protoporphyrin IX which is the reactive component responsible for photodamage when activated by light at 595 nm (14, 17). ALA has also been used in clinical trials to test the concept of PDT for photorejuvination for areas of skin that have been damaged by the sun (16, 18). Chlorin e6 is another photosensitizer which is gaining clinical recognition as an effective method of treating melanoma. In a study by Sheleg et al. it was shown that it was possible to achieve full tumour regression with no recurrence after only one PDT treatment using chlorin e6 (19). Finally, a drug which has received much attention over the last few years in both experimental and clinical trials is meta-tetrahydroxyphenylchlorin (mTHPC). It has been used in the study of many different cancers but most recently in a study done by Hopper et al. (2004) it was used for the treatment of oral squamous cell carcinoma. This form of cancer is normally treated by conventional radiotherapy and surgery. However those modalities often result in impaired function of the treated area. Following this
study involving 114 patients, it was determined that mTHPC-mediated PDT for the treatment of early oral squamous cell carcinoma is an excellent alternative to traditional modalities and offers very good cosmetic and functional results and a very promising response (20).

Section 2: Why Photodynamic Therapy?

PDT is a minimally invasive treatment which can be re-administered a number of times with no systemic toxicity such as caused by chemotherapy, radiotherapy and surgery. PDT is not toxic to normal or non-targeted cells in the absence of light and has very few serious side effects. PDT elicits an immune response against the tumour and can indeed lead to long term tumour cure and can eliminate metastases when used in conjunction with surgery. Finally, PDT is an enticing palliative treatment or alternative option to those patients who have not responded well to other mainstream modalities or who exhibit malignancy in places not accessible to surgery (10-12, 14, 20)

I. Mechanisms of Photodynamic Therapy

Photodynamic therapy requires the administration of a photosensitive drug, light of a specific wavelength and the presence of molecular oxygen. At a time following the selective uptake of drug into the tumour, it is activated by light of a specific wavelength depending on the properties of the photosensitizer. The drug, upon absorption of the light energy can undergo two types of reactions termed Type I and Type II (10). Type I reactions differ from Type II with respect to the initial steps where in Type I reactions, free-radical intermediates, in the presence of oxygen lead to toxic oxygen products. In Type II reactions, which dominate following PDT, the highly reactive singlet oxygen is produced directly by energy transfer from the triplet-state drug to molecular oxygen (10).
Singlet oxygen in addition to other toxic oxygen derivatives like hydrogen peroxide and the hydroxyl radical can be very destructive to the biological integrity of the cell. These potent species oxidize macromolecules in their vicinity such as lipids, proteins and nucleic acids which in turn can lead to the loss of an intact membrane and cause cell death by either apoptosis or necrosis. This immediate oxygen-mediated trauma can induce the expression of a variety of genes involved in the response to this damage including early response genes, cytokines, complement, matrix metalloproteinases, and the genes of the acute phase response (21, 22, 23). Singlet oxygen, which has been shown to be the primary cause of cellular damage following PDT, has a very short lifespan (<0.04μs) and radius of action (<0.02μm) (10, 21). The extent and efficiency of the cellular damage resulting from photooxidation therefore, is dependent upon the concentration of drug, the dose of light and oxygen supply (24). It follows then that the degree of photosensitizer uptake in tumour-associated cells in addition to the oxygen supply, is of crucial importance to the level of photochemical damage received at the site of illumination.

II. Photosensitizer localization and Cellular Uptake

The tumour localizing properties of these light sensitive drugs is what enables their use for the photodynamic treatment or detection of solid tumours. The chemical composition of the drugs along with the properties of solid tumours is what enables this preferential localization (10). Leaky vasculature, high metabolic rate, poor lymphatic drainage, an elevated number of low-density lipoprotein (LDL) receptors and an increased population of macrophages are all properties of solid tumours that lead to the selective increase of the localization of photosensitizers to the tumour (10). An important determinant of the extent of Photofrin accumulation is the composition of a tissue. This
is illustrated by comparing Photofrin accumulation in the spleen versus in a solid tumour. The spleen shows significantly higher accumulation of Photofrin than the tumour but it has a much higher number of cells per gram of tissue than a solid tumour (24). On a ‘per gram’ of tissue comparison the tumour far outweighs the spleen as to the level of drug accumulation in the same animal (25). The extent of photosensitizer accumulation is not only dependent on tissue composition but more specifically it is related to the cellular distribution within the heterogeneous environment of the tumour (24, 25). Further evidence for photosensitizer heterogeneity beyond its preferential accumulation in certain cells within the tumour is the variation of its access to the blood supply which has been shown to rely on the proximity to blood vessels (26). Host immune cell content in the tumour, especially resulting from infiltrating macrophages is related directly to the increased retention of Photofrin in the tumour (25). Korbelik et al. (27) demonstrated that there exists a large population of host macrophages subverted by the tumour for sustenance and the maintenance of local homeostasis. Among these tumour-associated macrophages (TAMs) there is a sub-population responsible for the elevated accumulation of Photofrin which at 24 hours post injection was shown to exceed levels in malignant cells by more than three fold (28). The retention in these macrophages accounts for most of the drug located within the solid tumour (27). This TAM sub-population is in an activated state characterized by increased size and granularity and a high expression of interleukin-2 receptors (25). Further studies by Korbelik have shown that TAMs contain sometimes more than 13X the levels of Photofrin retained in the malignant cell population and that the lowest concentrations of photosensitizer were found in the non-macrophage population of infiltrating immune cells (25, 27, 28). Relative selectivity of photosensitizer accumulation in TAMs is universal and does not depend on the species of
animal, the origin of the tumour (implanted or spontaneous), the immunogenicity of the tumour, or the cellular composition of the tumour (28). Photofrin uptake by macrophages in vitro is greater than the uptake by malignant tumour cells and is related to their phagocytic nature, as was demonstrated by the use of factors that modify macrophage activity (29).

IV. Drug Delivery

The localization of photodynamic compounds used in PDT can be a serious limitation to the efficacy of treatment and therefore there has been research into possible methods to improve delivery which include: oil dispersions, LDL, liposomes, polymeric particles or hydrophilic polymer-photosensitizer conjugates (10, 30, 31, 32). The most desirable system of drug delivery would be one which maximizes tumour uptake while minimizing the uptake by normal tissues (10, 30, 31, 32). It has been well documented that Photofrin binds to lipoproteins, plasma albumin and other serum proteins which affect its uptake into cells (31-35). LDL content of serum and the relative expression of LDL-receptors on the surface of tumour cells and normal cells has been examined in order to determine the effect of these components on photosensitizer retention, clearance and inhibition (31-33). Photofrin delivered via LDL can increase drug uptake into tumours due to the increase of LDL receptors on the surface of transformed cells (32) but the presence of high LDL in the serum can prove inhibitory to the uptake of Photofrin into cells (31). LDL is perhaps not the only determinant of the extent of Photofrin delivery and uptake by the tumour. Other serum proteins and macromolecules may also play a crucial role as demonstrated by the substantial differences in composition between mouse serum, human serum and fetal bovine serum which affect photosensitizer delivery (33). Hence delivery and retention of Photofrin and other photosensitizing drugs is
dependent not only on their intrinsic chemical composition but also on the way in which they interact with the components of the serum and therefore the design of these drugs or their carriers must account for all the factors involved (30).

V. Light Sources and Delivery

PDT is a light-dependent process and therefore requires light to be administered locally to the tumour. Tumours at various sites in the body can be reached by optical fibres coupled to the light source (36, 37). Light sources used for the application of PDT can vary but are typically either laser or lamp sources emitting in the red region of the light spectrum, 630-750 nm (36). The wavelength of light used is dependant on the photosensitizer and is also governed by the depth and location of the tumour (36, 37). For example, a deeper reaching tumour will require a photosensitizer that can be activated by light of a longer wavelength, one which can penetrate deeply enough to activate drug localized towards the base of the tumour. In addition, tumours located within the body, like Barrett’s esophagus will need to be accessed by a specially designed laser diode attachment.

Section 3: PDT-induced events leading to the host response

I. Photodynamic Action

Photodynamic action comprises the direct killing effect of activated drugs on the cells in the tumour and the indirect effect caused by a breakdown of the vasculature and the activation of the host response (2, 10). Primary oxidative stress is induced immediately during and following light administration and is responsible for the initial destruction of the tumour by (primarily) singlet oxygen (21-23). Secondary reactions ensue as a result of vascular collapse (loss of oxygen supply) and comprise both the
action of oxidative and nitrosative stress (toxic nitrogen derivatives) generated through the formation of the superoxide anion, toxic nitrogen species (NO) and even activated complement proteins resulting from ischemia reperfusion (2, 10, 38). Ischemia reperfusion injury results from the sudden reintroduction of oxygen to the tissue following a period of oxygen deprivation. The reintroduction of oxygen allows the conversion of xanthine dehydrogenase into the oxidant-producing xanthine oxidase which induces the formation of xanthine from hypoxanthine and the consequent release of a variety of potent reactive oxygen species (39, 40). Ischemia reperfusion-mediated injury to the vasculature is a potent initiator of an immune response through complement activation, neutrophil sequestration and the invasion of other inflammatory cells which can have a very serious impact on the outcome of therapy (38, 41, 42).

Due to the lack of accumulation of photosensitizer in nuclei, direct damage to DNA causing mutation at sublethal doses of PDT tends not to occur, although some strand breaks or chromosomal aberrations have been documented (43, 44). The plasma membrane, lysosomes and mitochondria are all major sites of the oxidative trauma caused by the primary and secondary reactions of PDT (45). The extent to which each of these sites is directly affected by PDT determines at least in part the form of cell injury and mode of cell death (45). The uptake of porphyrins like Photofrin through the plasma membrane is primarily by receptor mediated endocytosis with subsequent distribution to other compartments within the cell (46). Photofrin and ALA are known to localize primarily in the mitochondria and therefore inflict PDT damage to the integrity of the electron transport chain (ETC), leading to decreased levels of adenosine tri phosphate (ATP) and the release of cytochrome-C which binds to apoptosis protease activating factor (APAF-1), activating caspases that induce apoptosis (10, 47, 48, 49). Reports on
the damage to plasma membranes include observations of swelling, blebbing, and phospholipid scrambling (22, 51, 52). Many plasma membrane enzymes such as the Na+/K+ pump are inhibited leading to loss of membrane transport, an increase of Ca^{2+} concentration, and an increased permeability facilitating more photosensitizer uptake (52, 53, 54). In addition, lipid peroxidation and the regulation of surface antigen expression are affected and all of these events lead inevitably to cell death (55, 56, 10).

Section 4: HOST RESPONSE

To respond to tissue injury or invasion by a pathogen, the body is equipped with an immune system whose role is to contain and repair damage, destroy the pathogen or altered self cells and protect the body against further intrusion of non-self/ altered-self molecules. Immunity can be divided into two main branches: innate immunity and adaptive immunity. Innate immunity is a non-specific, first line of defense against injury or invasion. It consists of cellular components such as mast cells, neutrophils and macrophages that express a variety of pattern recognition receptors (PRRs) that bind conserved motifs on pathogens and endogenous host proteins outside the cell which act as "danger signals". In addition, non-cellular protein components, such as those of the complement cascade and those of the acute phase response, execute non-specific action against unprotected entities. Acute phase reactants promote the release of cytokines which serve to alert circulating host cells to migrate to the affected tissue. Complement proteins serve several roles. They bind to the surfaces of damaged cells or pathogens, flagging them for removal by phagocytic leukocytes (neutrophils and macrophages). They serve as acute phase proteins that amplify the host response, and they form a complex, the membrane attack complex (MAC), which lyses unwanted cells. All of these
components help to promote the activation of the adaptive immune response in order to achieve long-term antigen-specific immunity.

The adaptive arm of the immune response is specific because it is capable of recognising and targeting specific pathogens or altered self molecules that express unique peptides not expressed by the host. It is comprised primarily of T and B lymphocytes that interact with the antigen presenting cells like macrophages and dendritic cells which present antigen on MHC molecules expressed on their surface. This is a very specific interaction which facilitates the execution of their antigen-specific responses that lead to long term immunity.

PDT-inflicted damage to the tumour results in an abundance of dead or dying cells and the release of a variety of “danger signals”. The damage sustained by the body must be contained, damaged cells repaired or removed, dead cells and cell fragments eliminated in order to restore tissue and systemic homeostasis. This is done through the activation of immune proteins such as those of the acute phase response and the complement cascade, and by the recruitment leukocytes (neutrophils, monocytes, and dendritic cells) followed by lymphocytes (T-cells and B-cells).

I. Cell Death, Danger Signals, and Signalling Pathways

Severe PDT-induced trauma leads cells to initiate either a rescue response or to undergo cell death by necrosis (uncontrolled) or apoptosis (controlled) (10, 57, 58, 59). Photofrin is known to localize in mitochondrial membranes and primarily promotes cell death by apoptosis through the release of cytochrome C and the activation of caspase 3 (60, 61) unless initial oxidative damage to respiratory enzymes is too great, then cells undergo necrosis (59). Apoptosis is a regulated cellular process and as such requires intracellular signalling cascades and the involvement of many proteins. Heat shock
proteins (HSPs), molecular chaperones for damaged proteins, have been shown to be induced following PDT (65) and HSP70 for example, has been shown to be expressed on the surface of PDT-treated cells (66). HSPs have been shown to act as extracellular ligands that activate intracellular signalling pathways. A widely used mechanism involves the phosphorylation and dephosphorylation of tyrosine-kinases for the regulation of the stress-induced MAPK pathway. However, the details of how this pathway is activated in PDT have not been completely elucidated (62). Another signalling pathway involving protein kinases is one which activates the transcription factor, nuclear factor kappa B (NF-κB) which is involved in the activation of a number of immune genes and therefore has implications for the host response to PDT (63, 64). Moreover, NF-κB has been reported to be intimately involved with poly(adenosine diphosphate-ribose) polymerase, or PARP, which becomes up-regulated following genotoxic stress, ischemia and immune stimulants (67, 68). PARP is a family of nuclear enzymes involved in several different cellular processes involving signalling pathways following stress (68). PARP-1 is of special significance as it has been shown to be a crucial co-activator of the immune transcription factor NF-κB, which also plays a fundamental role in the activation of p53 and therefore is a key regulator not only of cell death by apoptosis or cell survival but is also responsible for the transcription of pro-inflammatory genes like interferon-gamma (IFN-γ), tumour necrosis factor-α (TNF-α), and adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1) and P-selectin (67, 68). This may be of relevance to PDT-induced cellular stress and the balance between necrotic and apoptotic cell death and the up-regulation of genes involved in inflammation and the acute phase response which play a pivotal role in the curative outcome of treatment (67).
Unlike the events induced by the process of apoptosis, cell death by necrosis results in the abundant release of endogenous "danger signals", which promote the activation of an acute host response through their recognition by immune receptors on surviving tumour-associated leukocytes and those infiltrating from the vasculature (67, 69, 70). These danger signals include: lipid fragments, heat shock proteins, complement proteins, and pieces of the extracellular matrix and plasma proteins, all of which do not normally exist outside the intact cell and are recognized by infiltrating immune effectors when exogenously present, thereby activating an immune response (67, 71, 72, 73).

The PDT-induced immune response resulting from the combination of events following oxidative trauma involves the production and activity of several different components which work in concert and jointly contribute greatly to the overall outcome of therapy (10, 69, 70, 72, 74).

II. ACUTE PHASE RESPONSE

PDT elicits a strong acute phase response resulting from the induced production of pro-inflammatory cytokines, primarily interleukin-6 (IL-6) but also TNF-α and IL-1. The expression of all of these cytokines is enhanced by the complement system activated by PDT (75). This is a non-specific host response following tissue trauma which orchestrates the infiltration of immune cells like neutrophils, monocytes/macrophages, mast cells and lymphoid cells in order to remove damaged tissue and to further destroy altered-self material such as remaining tumour cells (72, 75). This initial local response has far-reaching effects on many organs of the body, most prominently the liver which is the site of the production of major acute phase proteins including complement components, pentraxins, C-reactive protein (CRP) and serum amyloid P component (SAP) These acute phase proteins accumulate in PDT-treated tumours where they bind
to the damaged elements thereby promoting their removal by phagocytic cells (75, 76, 77-81).

III. Cellular Effectors of PDT-Induced Host Response

III a. Neutrophils

Of hematopoietic origin, neutrophils or polymorphonuclear leukocytes (PMNs) enter circulation, acting as sentinels in immune surveillance. They are characterized by the presence of cytoplasmic granules containing oxidative and cytotoxic agents, which facilitate their role in the degradation of dead or foreign materials (82). Following PDT, as a result of the acute phase response, there is a marked complement- and cytokine-dependent increase in the number of circulating neutrophils, that respond to these stimuli by efflux from the bone marrow and other non-circulating pools and then they are recruited to the damaged tumour (78). Neutrophils have been identified as an essential component for therapeutic benefit (83). They are the first immune cells to arrive at the tumour and to begin mediating the clearance and degradation of irreparably damaged tissue (78). The activation of complement and the release of IL-1, IL-8 and TNF-α contribute to the up-regulation of adhesion molecules like P and E selectins as well as ICAM-1 on the surface of endothelial cells lining the damaged site and thus facilitates the migration of neutrophils from the circulation into the tissues (84). The rapid invasion of neutrophils into the tumour in response to the presence of pro-inflammatory mediators is sustained by further production of pro-inflammatory cytokines by the neutrophils themselves. Their ability to rapidly produce and release cytokines, in addition to toxic derivatives, makes them essential not only to the destruction of abnormal or dead cells but also to the recruitment of monocytes/macrophages, mast cells and T-cells which further mediate the immune response (85, 86, 87).
Neutrophils represent a potent inflammatory weapon in tissue injury and thus an essential component in the response to PDT (78-80, 88). It is in a complement-dependent manner that neutrophils followed by monocytes/macrophages and lymphocytes migrate in high numbers to contain the damage, destroy the tumour and mediate inflammation. Moreover, a role in antigen-specific immune responses is indicated by the surface expression of MHC II molecules on neutrophils in PDT-treated tumours (78, 79). This indicates that neutrophils are recognizing non-self (tumour-specific) molecules and presenting them to T lymphocytes in the context of MHC peptides. This could have a significant impact on the activation of long-term tumour resistance by means of adaptive immunity.

**III b. Macrophages**

Macrophages are a specialized phagocytic leukocyte that, unlike neutrophils, are long-lived and found as residents in many tissues in the absence of inflammation where they localize upon differentiation from monocytes (87). Two types of macrophages exist where type-1 is IL-12, IL-23 producing and immune-promoting and type-2 is IL-10 producing and promotes immuno-tolerance (89). Many of their functions overlap with those of neutrophils. However, macrophages often play a housekeeping role throughout the body by digesting dead or dying cells and maintaining tissue homeostasis (87, 88). The uptake or phagocytosis of apoptotic cells by macrophages, which is generally anti-inflammatory and primarily complement dependent, is critical to the proper regulation of the immune system, particularly through their ability to present signals of tolerance to T-cells (90). Macrophages are equipped with a multitude of surface receptors which facilitate their vast and specialized role in immunity and tissue homeostasis. These include integrins, scavenger receptors, complement receptors (CRs), in addition to a
number of PRRs such as Toll-like receptors and CD14 which signal the activation of several immune genes through NF-κB (71, 87-91). Macrophages share a common origin with dendritic cells and are specialized for antigen presentation. Because of their interaction with other leukocytes and lymphocytes and their wide tissue distribution they are integral to the development of both local and systemic inflammatory responses (87).

Macrophages comprise a major cellular portion (~30%) of untreated solid tumours and accumulate a large proportion of tumour-localized Photofrin (26, 28). In addition they are the most numerous leukocyte to infiltrate the tumour following treatment by PDT and are thought to play a major role in the curative outcome of therapy (80, 85). Macrophages possess many cell surface receptors (ie PRRs) designed to detect foreign material such as bacteria and viruses. However these same receptors have been shown to bind materials that are not normally found outside the cell, such as heat shock proteins (HSPs), phosphatidyl serine (PS), lipid fragments and other intracellular proteins which are released by apoptotic and necrotic cells following PDT (55, 56, 71). These latter components are characterized as ‘danger signals’ which alert macrophages through their receptors and activate them (71, 92). Activated macrophages are capable of synthesizing all of the complement components (93) and can restore humoral immunity in complement deficient mice through bone marrow transplantation (94). They also secrete a number of pro-inflammatory as well as anti-inflammatory cytokines and can both amplify the immune response through positive feedback or repress it through receptor mediated intracellular signalling (87, 95). In PDT they play an important role in the removal of dead or critically damaged tumour cells, neutrophils and other macrophages. The activity of macrophages is important for continuing the immune response and links it to the activation of the adaptive immune system through antigen
presentation. Therefore, they contribute to long term tumour immunity following PDT (71, 80, 96).

III c. Lymphocytes

Adaptive immunity enables long-term, antigen-specific immunity executed by T and B lymphocytes. Naïve T-cells develop in the thymus as either CD4+ or CD8+ and move into the circulation once mature (97). CD8+ T-lymphocytes are known as cytotoxic T-cells and are MHC-I restricted and therefore associate with virally infected cells expressing viral antigen associated with MHC-I. In addition, CTLs that are tumour-specific play a role in immune surveillance and the elimination of tumour cells as they arise (97). In PDT, CTLs have been shown to become activated and are of interest in research concerning tumour vaccines (69). CD4+ T-lymphocytes are known as T-helper cells and can be of type T_H1 or T_H2. These cells are MHC-II restricted and associate with antigen presenting cells like dendritic cells, macrophages and B lymphocytes. CD4+ T_H1 cells activate macrophages through their production of IFN-γ, IL-2, and TNF-β whereas CD4+ T_H2 cells are responsible for stimulating B-cells to differentiate by secreting IL-4, IL-5, IL-6, IL-10 and IL-13 (97). In PDT, the depletion of CD8+ cells impaired therapeutic outcome more than did the depletion of CD4+ cells indicating that CTL’s contribute more to the therapeutic benefit of PDT than do CD4+ T-cells (80). Finally, natural killer T-cells (NK) make up less than 10% of circulating lymphocytes and were named for their tumouricidal abilities in the absence of MHC restriction (97).

B lymphocytes are generated in the bone marrow independent of antigen and migrate to secondary lymphoid organs where they encounter antigen (97). Those B-cells whose antigen receptors encounter an antigen to which they are specific will remain in the secondary lymphoid organ where the B-cells become activated either to secrete
antibody in association with antigen-specific T-cells (CD4+ T_{H2}) or to become memory B-cells. B-cells that bind a complement-immune complex have dramatically increased signalling leading to increased activity (97).

Tumour cure rates drop drastically in SCID mice deficient in lymphoid cell populations indicating that therapeutic efficacy is dependant on the proper functioning of adaptive immunity (98, 99).

IV. Complement

Complement proteins and the complement cascade are major components of the innate immune system and are also thought to be necessary for effective activation of the adaptive immune response (100). The complement system comprises more than 30 proteins including plasma proteins and receptors. It is involved in the recognition and elimination of pathogens and altered host cells through several mechanisms (100, 101). Activation of the system can occur through three separate pathways: the classical pathway, the lectin pathway, or the alternative pathway (Figure 1). Each of these pathways is initiated by different recognition molecules (100).
IV a. Complement Activation

The classical pathway of complement activation occurs with the binding of circulating C1q to the activating complex (ie altered-self, pathogen) and the binding of an IgM heavy chain to the bound C1q (100, 102). Binding of at least two C1q molecules to the immune complex causes autoactivation of the associated serine protease C1r that in turn cleaves and activates C1s. Activated C1s subsequently cleaves the next component C4 into C4a and C4b. C4a is an anaphylatoxin with chemotactic abilities and C4b opsonizes or targets the activator for phagocytosis. C4b is bound by the active component of cleaved C2, C2a. This complex C4b2a is a C3 convertase which is able to cleave the central component of the complement cascade, C3 (100, 102).

The lectin pathway of complement activation is activated by the presence of carbohydrate structures that become expressed/uncovered on apoptotic cells, and by high
mannose-containing polysaccharides common to many pathogens (100). The binding of mannose-binding lectin (MBL) to the activating surface, along with its binding to (MBL)-associated serine protease-1 (MASP-1), and MASP-2 proteins, leads to cleavage and activation of C4 and C2 and the formation of the C3 convertase (100).

The alternative pathway of complement activation occurs through the low-level spontaneous cleavage of C3 to C3a and C3b. C3a is a potent anaphylatoxin capable of augmenting the immune response and complement production whereas C3b bound to plasma protein factor B forms a C3 convertase (100). This pathway becomes truly activated or amplified when C3b binds an activator which lacks complement regulatory proteins. This is a major amplification step governed by a positive feedback loop where the more C3 cleaved, the more C3b bound to factor B is produced and the more C3 is cleaved (100). Activating complexes like pathogens and altered self molecules lack the complement regulatory proteins necessary to suppress this pathway and provoke full activation (100).

These three initial activation pathways are all routes to the formation of C3 convertase and the activation of C3 by its cleavage to C3a and C3b. Once C3 has been cleaved, the terminal pathway of the complement system can be activated through a series of sequential cleavages by proteases, leading to the formation of the membrane attack complex (MAC), assembled by the sequential recruitment of terminal proteins C5b, C6, C7, C8 and C9 (100, 103). All of these proteins must be present on the surface of the activation complex in order for MAC formation and lysis of the pathogen or altered host cell (100, 102, 103).
IV b. The multiple roles of complement proteins

IV b-1. Opsonization

Some complement proteins are involved in the opsonization or ‘flagging’ of altered host cells or pathogens for phagocytosis by macrophages and neutrophils which recognize the deposited complement proteins through specific complement receptors. C1q, and C3 fragments C3b and iC3b, all covalently bind to the surface of their targets which become recognized and phagocytosed by leukocytes expressing the CR1, CR3 and/or CR4 complement receptors (100, 102, 104). Macrophages bound to opsonized surfaces show up to a 10-fold increase in engulfing activity (105).

IV b-2. Anaphylatoxins

Active members of the complement system which play a major role in the amplification of the immune response and inflammation are the anaphylatoxins (99, 102). C3a, C4a and most especially C5a are potent instigators of myeloid cell sequestration through interaction with their receptors (C3aR, C4aR and C5aR) and initiate strong positive feedback mechanisms to induce further complement production and the synthesis of cytokines and chemokines (ie IL-8 and IL-1β) and are often the major culprits in complement-related autoimmunity (100-102, 106-107). C3a and C5a (and to a lesser degree, C4a) initiate intracellular responses in macrophages, mast cells and neutrophils through interaction with their receptors C3aR and C5aR respectively (100). These anaphylactic proteins can mediate the release of lysosomal enzymes from leukocytes, the release of histamine from mast cells and the recruitment of these cells from circulation (100, 108-110). The acute phase response is largely mediated by the activation of anaphylatoxins C3a and C5a which induce the synthesis of TNF-α, IL-1β, and IL-6, all of which have important roles in the regulation of expression of acute phase proteins such as
C-Reactive Protein (CRP) and Serum Amyloid P component (SAP) (111). Furthermore, C5a is largely responsible for the early recruitment of T-cells to the affected tissue and their sensitization to the activator complex. Along with their receptors, C3a and C5a also help regulate B-cell functions. This represents one area where complement is responsible for the bridging of innate immunity with acquired immunity (112-114).

IV b-3. The Membrane Attack Complex

MAC is the aggregation of complement components C5b to C9 to form the C5b9 complex on the surface of pathogenic or altered host cells. C5b is able to bind to C6 and form a metastable bimolecular complex. This complex is able to bind to C7 to form C5b67. C5b-7 is able to insert itself into the membrane lipid bilayer of cells. Each inserted C5b-7 complex is able to bind a C8 which stabilizes the insertion and causes small pores to form. This complex of C5b-8 becomes a C9 acceptor and can bind up to 16 C9s to complete the MAC. The assembly of these components leads to cell lysis because of the loss of membrane integrity caused by the formation of pores (100, 101). The receptor-independent binding of this complex also activates numerous signalling pathways such as those leading to activation of NF-κB (115, 116). A high level of MAC formation in tissues is representative of complement activation and is indicative of its procession to completion.

IV c. Complement Regulators

Due to the systemic presence of circulating complement under normal conditions and its potent pro-inflammatory and destructive abilities, tight control of the system is crucial to prevent normal host cells from succumbing to complement-mediated attack (100). The host has defences against complement deposition on normal cells mediated by complement regulators that can be either secreted plasma inhibitors or membrane-bound
inhibitors (117-118). These complement inhibitor proteins prevent the formation of the C3 convertases or of the MAC. Soluble inhibitors include: C1 inhibitor, C4b binding protein, factor I, factor H, and S protein, whereas membrane-bound regulatory proteins (mCRPs) include: membrane cofactor protein (MCP, CD46), decay accelerating factor (DAF) and protectin (CD59) (117). mCRPs are found in most tissues and on all circulating cells and have been shown to be up-regulated in tumour cells thereby allowing them to escape immune surveillance (118, 119). During complement activation, complement binds to cells not displaying these inhibitory molecules and will exceed the concentration of soluble inhibitors thereby allowing its sequential and significant activity in innate and adaptive immune activation (119).

IV d. Complement Synthesis

Plasma concentrations of complement proteins range from 2 μg/mL (factor D of the alternative pathway) to 2 mg/mL (C3) and are primarily synthesized in the liver by hepatocytes and released into the circulation (120). In the late 1960s it was found that plasma C proteins increase in concentration in response to inflammation and are implicated in the acute phase response which further demonstrates their liver-derivation (121). Later, it was determined that hepatocytes are not the only source of complement. Discovery that C1q, factor D and C7 were not found in the liver led to the search for the source of extrahepatic production of complement proteins (93, 122, 123). Sources of extra-hepatic complement production may contribute to plasma levels as well as be important to tissue homeostasis, as well as provide an immediate local defence against altered host cells or invading pathogens (93, 123, 124-126). Cells that have been shown to produce complement proteins include; endothelial cells, epithelial cells (GI and lung), fibroblasts, adipocytes, brain cells (astrocytes, microglia and neurons) and leukocytes.
macrophages, neutrophils) (122). Because of its variety of roles it is not surprising that local and hepatic complement synthesis are differentially regulated (93). Local complement production by macrophages and neutrophils may be of critical importance in inflammation. Macrophages have been shown to be capable of synthesizing all components of the complement system and are thought to be of major importance in local production of complement (122). Wild-type bone marrow transplanted into complement-deficient knock-out mice restored humoral immunity by local synthesis by macrophages thus revealing the extent and importance of local complement production by macrophages (94).

**IV e. Complement in PDT**

PDT-treated cells are the targets of complement proteins C3b, iC3b and C5b which act as opsonins, flagging them for recognition by innate immune recognition receptors on the surfaces of immune cells (71, 96, 126). Opsonization of PDT-damaged cells can attract circulating and resident macrophages, neutrophils, dendritic cells and lymphoid cells. Complement receptors on the surfaces of these cells trigger intracellular signalling pathways which result in the up-regulation of expression of pro-inflammatory cytokines (71). In addition, it has been shown that complement plays a significant role during the induction of PDT-induced neutrophilia (41, 96). Complement components C3a and C5a have also been shown to be of importance in the curative outcome of PDT since the blockage of their receptors resulted in decreased cure rates of Lewis Lung Carcinoma tumours (126). Complement activation at the treated tumour site results in an increase of MAC formation as detected by immuno-histochemistry to C5b-9 and further supports the role of complement in the host response to PDT (41). In addition, nearby immune cells displaying complement receptors or other innate immune receptors such
TLR-2 and TLR-4 are able to detect breakdown products of the extracellular matrix, fibronectin, lipid fragments and exogenous proteins like HSP70 among other danger signals massively released by the PDT-treated tumour (71). TLRs are innate PRRs that have the ability to recognize danger signals and initiate intracellular signalling mechanisms in coordination with other receptors (like complement receptors) and adaptor molecules like myeloid differentiation factor 88 (MyD88) and TIR domain containing adaptor protein (TIRAP), that lead to the up-regulation of various pro-inflammatory cytokines and chemokines through the activation of NF-κB (71). In the case of PDT, TLRs on the surface of resident or infiltrating immune cells such as macrophages may play an important role during the host response to PDT-induced damage (126, 66). Oxidative damage to PDT treated tumour cells either directly by the action of singlet oxygen or indirectly by PDT-induced ischemia reperfusion injury causes activation of the complement system (128, 129). Following its initial activation at the tumour site an increase of C3 has been demonstrated both in the tumour and in the blood, following treatment by PDT.

V. TLR signalling pathways

TLRs are intimately involved in innate immunity as PRRs that are capable of detecting a wide range of danger signals and signalling the activation of transcription factors that control the expression of more than 2000 genes whose products include inflammatory proteins (Figure 2) (130). There are at least 13 of these receptors (TLR1-TLR13), found primarily on the surfaces of immune cells (71, 128). TLRs are type I transmembrane receptors characterized by extracellular leucine-rich repeats and an intracellular domain called Toll/IL-1R (TIR) domain which shares homology with the interleukin-1 receptor (IL-1R) (130). In most cases, intracellular adaptor proteins like
MyD88 are required for signalling the NF-κB pathways. MyD88 interacts with TLRs through its TIR domain and also contains a death domain which enables its interaction with IL-1 receptor-associated kinase (IRAK). Another adaptor protein, Tollip (Toll-interacting protein) helps to recruit IRAK to TLR (130). TIRAP facilitates a MyD88-independent TLR signalling for NF-κB activation and may be responsible for providing downstream signalling specificity for TLRs (131).

Activation of TLRs by exogenous ligands recruits the cytoplasmic adaptor proteins that mediate the activation of IRAK which in turn interacts with tumour necrosis factor receptor associated factor 6 (TRAF6) that leads to the activation of NF-κB, or JNK (c-jun-N-terminal kinase), ERK (extracellular signal regulated kinase), and p38 which facilitate the formation of the gene transcription complex AP-1 (activating protein 1) through the MAPK cascade (71).

PDT results in the massive release of danger signals from PDT-treated tumours which could engage the activity of TLRs expressed in myeloid and lymphoid cells that have the ability to activate the transcription of genes associated with and necessary for the host response to this damage (66).
Section 5: Rationale for the thesis project

The complement cascade is a critical component of innate immunity and important for bridging innate and adaptive immune responses which have been shown to play crucial roles in the host response to PDT. Complement is up-regulated following PDT and is known to be an important factor in the therapeutic outcome of this type of tumour treatment. However, many questions as to its role the host-response to this treatment remain and discoveries are needed to answer remaining questions regarding its functions and may be valuable in devising ways to improve responses to PDT.
**Hypothesis:**

PDT causes an increase in the tumour-localized expression of key complement component genes.

**Specific Aims of This Project:**

1. To determine the source of increased complement gene expression following PDT

2. To determine the cells responsible for the increase of local complement gene expression using *in vitro* models with tumour cells and macrophages

3. To investigate cellular signaling pathways underlying the PDT-induced up-regulation of complement genes
Materials and Methods

The mice used in the experiments were 8-12 week old C57Bl/6J, B6.129S4-C3\textsuperscript{tmleer} (C3 KO), or B6.KB2-Cln\textsuperscript{mmd}/MsrJ (TLR-4 KO) males/females, and were kept in the Joint Animal Facility at the B.C. Cancer Research Centre where they were provided with food and water ad libitum. The Animal Ethics Committee of the University of British Columbia approved all protocols.

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Lewis Lung Carcinoma (LLC) tumours (132) were grown in syngenic, immunocompetent C57BL/6J mice and were maintained in vivo by tumour brei inoculation. Mice were sacrificed using CO\textsubscript{2} gas and tumours were extracted aseptically from the hind legs using forceps and a #22 scalpel blade. Tumours were minced by chopping and cutting using two #22 scalpel blades and, for brei, were passed successively through a 20 and 22 gauge needle. The brei was then suspended in phosphate buffered saline solution (PBS) at 5X the volume of tumour. 0.2 mL of tumour brei was injected into the hind legs of anaesthetised mice using a 22-gauge needle.

For experiments, minced tumour tissue was placed in an enzyme cocktail consisting of 0.3 mL each of collagenase Type IV (Sigma-Aldrich Co., St. Louis MO) (0.24 mg/mL), Dispase (Boehringer, Mannheim, Germany) (0.18 mg/mL), and DNAase Type I (Sigma), (0.6 mg/mL), diluted to a final volume of 5 mL with cold PBS. The suspension was rotated and incubated at 37°C for 30 minutes at which time the suspension was immediately forced through a 100 micron filter using a 6 cc syringe and pelleted by centrifugation at 800 rpm for 10 minutes. The supernatant was discarded and the tumour cells counted using a hemocytometer and re-suspended in PBS to yield a concentration of
2-3 $\times 10^6$ tumour cells/0.04 mL for subcutaneous injection into the sacral dorsal region of each recipient.

All subcutaneous tumours were treated with PDT at approximately 8-9 days post-inoculation when the tumours reached 8-10 mm in diameter, slightly larger than the optimal PDT-size.

b. *In vitro* culture

LLC cells were cultured at 37°C, 5% CO$_2$ and 95% humidity, in alpha-minimal essential medium (Sigma) supplemented with heat inactivated 10% fetal bovine serum (Hyclone Laboratories Inc., Logan, Utah, USA), 100$\mu$g/mL streptomycin and 100 Units/mL penicillin (Sigma) where they adhered to the bottom of T75cm$^2$ tissue culture flasks. Cells were allowed to grow until near confluent and then were treated for 3-5 minutes with Trypsin-EDTA (Sigma) and washed with complete medium, collected by centrifugation at 800 rpm and resuspended in PBS at a concentration of 3 $\times 10^6$ cells/0.04 mL for subcutaneous injection into the sacral dorsal region of recipient mice.

In the case of *in vitro* studies, a predetermined number of cells was plated into 3 cm diameter Petri dishes so that there would be approximately 1 $\times 10^6$ cells at the time of treatment. Just prior to PDT-treatment the cells were washed twice with PBS and resuspended in 0.5 mL of protein-free and serum-free medium (S8284, Sigma).

II. Macrophages

a. Tumour-associated Macrophages

TAMs were obtained from subcutaneous LLC tumours by preparing a tumour cell suspension as described above. 3 $\times 10^6$ cells were plated into 3 cm diameter Petri dishes in 2 mL serum-free medium and incubated for 20 minutes at 37°C to allow the macrophages to preferentially adhere to the Petri dish. Non-adherent cells (cancer and
stromal cells) were aspirated off and the attached macrophages were washed once with PBS and left in 2 mL of complete medium. Before being co-incubated with treated/untreated LLC cells they were washed once with PBS and overlaid with 1mL of protein-free and serum-free medium. This differential attachment procedure is routinely used in our laboratory (27).

**b. Splenic Macrophages**

Splenic macrophages were obtained from the spleens of mature female mice by scraping the spleens with forceps and the dull side of a scalpel blade in PBS followed by centrifugation at 800 rpm. The cells were then suspended in a hypotonic lysis buffer solution (10 mM KHCO₃, 150 mM NH₄Cl, 0.1 mM EDTA (pH 8)) to remove red blood cells, incubated on ice for 20 minutes, centrifuged and resuspended in complete medium (26). 4 x 10⁶ cells were plated into 3 cm diameter Petri dishes in 2 mL of complete medium overnight until needed. Just prior to treatment the cells were washed once with PBS and resuspended in 1 mL of protein-free and serum-free medium.

**III. Photodynamic Therapy**

Photofrin® obtained from Axcan Pharma Inc. (Mont-Saint-Hilaire, Quebec, Canada), was reconstituted in 5% dextrose in H₂O and used in the photodynamic therapy of all *in vivo* and *in vitro* tumours. For *in vivo* experiments, Photofrin® was injected intravenously through the tail vein at a concentration of 10 mg/kg (0.2 mL/20 g mouse). For *in vitro* experiments it was added to the 25 mm diameter culture inserts with a 0.02 μm anapore membrane base (Nalge Nunc International, Naperville, IL., USA) at a concentration of 20 μg/mL where 16 μl of a 2.5 μg/mL stock was added per tissue culture insert (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 thorough-put fiber illuminator (Sciencetech Inc., London, Ontario, Canada), was delivered through an 8 mm core diameter liquid light guide (model 77638, Oriel instruments, Stratford, CT, USA). In vivo tumours received a light dose of 150 J/cm² and in vitro cells received a dose of 1 J/cm².

In selected experiments, the effects of PDT based on photosensitizer benzoporphyrin derivative (BPD), provided by QLT Phototherapeutics Inc. was also tested. In this case, BPD (2.5 mg/kg i.v.) was administered 3 hours before tumour illumination (100 J/cm²).

Animals given light treatment were restrained unanesthetized in lead holders exposing only the dorsal sacral region where the tumours were located.

IV. Sample Collection for RNA Isolation

a. Tumour Extraction

At the appropriate time following light treatment (or no treatment) mice were sacrificed using CO₂ gas. Tumours were excised using surgical scissors and forceps and immediately placed in 1 mL of cold TRI® Reagent (Sigma). The sample was then homogenised for approximately 1 minute (until the tissue was sufficiently broken up) and incubated at room temperature for 5 minutes before being put on ice. All samples were frozen in TRI reagent (Sigma) at −80°C before RNA isolation. Livers were harvested and homogenized in the same way as the tumours.
b. Cell Collection

Macrophages or tumour cells were collected by adding 1 mL of Trizol (Invitrogen Co., Carlsbad, California, USA) to the Petri dish and scraping the cells gently with a rubber policeman. The cells were collected with a pipette and transferred to a 1.5 mL centrifuge tube and frozen at -80°C for later RNA isolation.

c. RNA Isolation and Purification

Workspace and pipettes were cleaned using RNase-Zap (Sigma) and all pipetting was done with aerosol-free, plugged ART pipette tips (Sigma).

Samples in TRI reagent were removed from the -80°C freezer, thawed at 37°C in a water bath and then placed on ice. They were then transferred from 5 mL tubes to smaller 1.5 mL centrifuge tubes (in vivo samples) and spun at 12,000 rpm for 10 minutes at 4°C. Samples were placed on ice and the clear aqueous supernatants collected and transferred to another tube containing 200 µl of chloroform containing isoamyl alcohol. The samples were vortexed for 15 seconds and left at room temperature for 10 minutes, again spun at 12,000 rpm for 10 minutes at 4°C, and the aqueous phase then was transferred to another tube. Using acid phenol (pH 5) and chloroform, the samples were “cleaned” and excess protein and TRI reagent/Trizol was removed. An equal volume of acid phenol was added to the aqueous phase (containing RNA) and 1/5 the total volume of chloroform was added. Samples were vortexed for 15 seconds, centrifuged at 12,000 rpm at 4°C and the aqueous supernatant transferred to a new tube for the process to be repeated. After phenol cleansing, samples were precipitated with 10% 3M NaOAc (pH 5.2) and 100% ethanol (2.5X volume) and incubated for 1 hour at -20°C. Following this incubation the samples were centrifuged at 12,000 rpm for 20 minutes at 4°C. The supernatant was discarded and only the RNA pellet remained at the
bottom of the tube. One mL of 75% ethanol was added to each tube with gentle shaking and the RNA was again pelleted by centrifugation at 12,000 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet was dried at room temperature (approximately 10 minutes). Finally the pellet was resuspended and dissolved in DEPC (diethylypyrocarbonate) treated water and kept at −80°C until use.

V. Semi-Quantitative RT-PCR

a. cDNA Synthesis

Complementary strand DNA (cDNA) was synthesised from 1 μg of total RNA using products from Invitrogen. One μg of RNA was added to a PCR tube along with 1 μL dNTP mix (10 mM each of dATP, dTTP, dGTP, dCTP) and 1 μL of oligo (dT)12-18 (500 μg/mL) primers and topped to a total of 12 μL with DEPC water. This was incubated at 65°C for 5 minutes in the thermocycler (MJ Research, Waltham, MA, USA) and immediately chilled on ice. Eight μL of master mix (4 μl Superscript II buffer (5X), 2 μL DTT (0.1M), 0.1 μl RNase inhibitor-cloned (10 U/μL), 0.9 μl DEPC water, and 1 μL superscript II reverse transcriptase (200 U/μL per 20 μL reaction) was added to each tube. This was spun down using a PCR mini centrifuge and left at room temperature for 10 minutes followed by 50 minutes at 42°C and 70°C for 10 minutes in the thermocycler.

b. Oligo Nucleotide Primers

The complete cDNA sequences for the four genes of interest (mouse complement components C3, C5, C9 and the housekeeping gene glyceraldehydes-3-phosphate dehydrogenase, GAPDH) were found on NCBI’s Genbank (www.ncbi.nlm.nih.gov). Primers designed to be specific to the 3’ end of the mRNA sequence of the genes of interest were constructed to be 18-22 nucleotides in length and were ordered from Qiagen.
Table 1: Description of oligo-nucleotide primer pairs used in PCR reactions.

<table>
<thead>
<tr>
<th>gene</th>
<th>primer sequence (5'-3')</th>
<th>alignment</th>
<th>Tm °C</th>
<th>PCR product size</th>
</tr>
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<tr>
<td></td>
<td>GGCCCTCCTGTATTATGG</td>
<td>right</td>
<td>62.7</td>
<td></td>
</tr>
<tr>
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<td>GAAAAGCCCAACACCAGC</td>
<td>left</td>
<td>56.3</td>
<td>151bp</td>
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<tr>
<td></td>
<td>GGACAACCATAAACACCATAG</td>
<td>right</td>
<td>57.2</td>
<td></td>
</tr>
<tr>
<td>C5</td>
<td>CCTCTGGCTTGGAAACCTA</td>
<td>left</td>
<td>59.2</td>
<td>157bp</td>
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<td></td>
<td>ACCAACACCCCTGACCTGCTA</td>
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<td></td>
<td>CACTGCCCCATCCAGAGAAT</td>
<td>right</td>
<td>57.2</td>
<td></td>
</tr>
</tbody>
</table>

c. Polymerase Chain Reaction (PCR)

All PCR reactions were performed in a PTC-100 Thermal Cycler (MJ Research). PCR was done using reagents from Invitrogen and according to their outlined procedure. For a 20 μL reaction, 2 μL of magnesium-free 10X PCR buffer [200mM Tris-HCl, 500mM KCl], 0.6 μL of Mg2+ (50mM), 0.4 μL dNTP mix (10 mM each), 1 μL of each oligo-DNA primer (10 mM) (Qiagen), 1 μL of cDNA, 0.2 μL Taq DNA polymerase (units) and 13.8 μL of sterile DEPC-treated ddH2O. A master mix was made for all primer sets and 19 μL added to 1 μL of cDNA in 0.15 mL PCR tubes (VWR). 30 cycles with the following parameters were done: Step 1-95°C for 2 minutes, Step 2-95°C for 30 seconds, step 3-54°C (C3, C9), 55°C (C5), 58°C (GAPDH), step 4-72°C 1 minute, step 5-72°C for 7 minutes, step 6-4°C for 10 minutes. The results of the PCR under these parameters on both a positive control (mouse DNA) and a negative control (water) are shown in figure 3.
parameters on both a positive control (mouse DNA) and a negative control (water) are shown in figure 3.

![Image of a gel electrophoresis with markers and bands](image)

**Figure 3**: Positive and negative controls to demonstrate the proper functioning of the Polymerase Chain Reaction and its parameters for the primer pairs designed to amplify GAPDH, and the complement component genes C3, C5 and C9. GAPDH, C3, C5 and C9 were amplified from mouse DNA using the primer sets described in Table 1 under the conditions discussed above. These are designated + as they represent the positive control for this reaction. The negative control is designated -, where the PCR template is water.

d. Poly-acrylamide Gel Electrophoresis

A 50% acrylamide/bis-acrylamide stock (73.05 g acrylamide + 1.95 g bis-acrylamide in 150 mL) was used to make a 12% acrylamide gel. For each gel 12 mL of 50% acrylamide stock was added to 10 mL of 5X TBE (108 g TRIS buffer + 55 g Boric acid + 40 mL of 0.5M EDTA in 2L of ddH$_2$O) topped with ddH$_2$O to a volume of 50 mL. To polymerize the acrylamide 100 µL of tetramethylethylenediamine (TEMED) and 50 µL of 20% APS (ammonium persulfate) was added and the solution mixed by inverting 3 times. It was poured into the plates and the plates were left for about 1 hour or until the gel was polymerised.
Two μL of 10X loading buffer (dye) was added to the 20 μL PCR products and 10 μL was loaded into the gel. The gel was run at 160 volts for approximately 1 hour and 45 minutes at which time the gels were stained with SYBR-green nucleic acid stain (Invitrogen). Forty μL of SYBR green (10,000X) were diluted in 400mL of ddH₂O and the gels were stained for 40 minutes before being scanned.

e. Gel Imaging

Gels were scanned on the STORM (Amersham) Imager. They were placed on the scanner bed over plastic wrap and removed from the glass plate gently using water and adherence to the plastic wrap. Air bubbles were removed gently by pushing them to the sides then scanner properties were selected. The area of scan was entered and the phospho-blue selection was picked. The scan took between 7 and 10 minutes depending on the area selected. The images generated were used in the quantification of gene expression.

f. Image Quantification

Gene expression was quantified using ImageQuant software (Amersham Biosciences, Piscataway, NJ, USA). Individual band intensity was measured in terms of pixels per unit area compared to the background intensity. For each sample the intensity of the gene of interest (C3, C5 and C9) was normalized against the intensity of GAPDH for that same sample thus giving a new “normalized” value for gene intensity which can be used for the analysis of gene expression.
VI. Antibody Staining and Flow Cytometry

Flow cytometry was done using the Coulter Epics Elite ESP apparatus from Coulter Electronics (Hialeah, FL). A 488 nm laser was used to excite the fluorescent dye fluorescein isothiocyanate (FITC) and phycoerythrin (PE) whose emissions were recorded through 530 +/- 15 and 580 +/- 10 nm bandpass filters respectively. 20,000 cells were analyzed per sample and dead cells were eliminated on the basis of side and forward light scatter.

a. C3 expression

Flow cytometry was used to detect the levels of C3 protein in TAMs coincubated for 16 hours with untreated LLC cells or with PDT-treated LLC cells. TAMs were collected in 500 μL of cold PBS, centrifuged at 800 rpm and resuspended in 500 μL Hanks Buffer. The cells were fixed and permeabilized using Cytofix/Cytoperm solution (PharMingen, BD Biosciences, Mississauga, Ontario, Canada). The cells were then suspended in 200 μL of wash solution and split into two aliquots. The samples were centrifuged, the supernatants discarded and each antibody diluted in Perm/Wash buffer provided in the kit added. Aliquot A received 150 μL of FITC-conjugated-goat-anti-mouse-C3 (Cappel, ICN Pharmaceuticals Inc., Aurora, OH, USA) and Aliquot B received 150 μL of FITC-chromePure goat IgG (Jackson Immune Research Laboratories, West Grove, PA, USA) for background staining used as a control. Samples were incubated on ice, away from light, for 30 minutes before being spun down and re-suspended in 400 μL of HBSS prior to sorting.

b. Percentage of macrophages present after their isolation from tumour or spleen.
Flow cytometry was used to measure the percentage of macrophages remaining after their isolation by adherence, from tumours or spleens. Suspended in 500 µL of HBSS and following the brief procedure outlined above, cells were stained with a mAb to the macrophage marker F4/80 conjugated to PE (Serotec Inc, Oxford, UK) prior to sorting.

VII. Experiments:

a. Hepatic versus tumour-localized complement gene expression following photodynamic therapy.

To analyze the expression of complement genes C3, C5 and C9 in the livers and tumours of mice treated with PDT the following experiment was done. The livers and tumours of 24 mature wild-type C57Bl/6J mice in 6 groups of 4, bearing 8-10 mm LLC tumours growing subcutaneously were harvested from control (untreated) and PDT (Photofrin)-treated mice at 3 hrs, 6 hrs, 8 hrs, 24 hrs and 5 days following treatment. The livers and tumours were homogenized in TRI reagent (Sigma) and total RNA isolated for semi-quantitative RT-PCR analysis of hepatic and tumour-localized expression of the key complement component genes C3, C5 and C9.

b. Tumour-localized complement gene expression in wild type mice versus TLR-4 KO mice, and C3KO mice.

Two groups each of 3 mature wild-type C57Bl/6J, B6.KB2-cln8mnd/msrJ (TLR-4 knock-out), and B6.12954-C3<sup>tm1err</sup> (C3 knock-out) mice were inoculated subcutaneously with LLC cells from <i>in vitro</i> culture. All tumours were grown to 8-10 mm as the largest diameter and 1 group from each mouse strain was treated by PDT. The other group was left untreated and used as control. At 24 hours following light treatment the tumours were harvested and homogenized in TRI reagent (Sigma) for total RNA isolation. Semi-
quantitative RT-PCR was performed in order to determine the expression patterns of complement components C3, C5 and C9 in each mouse type before and after PDT treatment.

c. Complement gene expression in TAMs co-incubated with untreated LLC cells and PDT-treated LLC cells.

There were six treatment groups, each plated in triplicate: a) untreated TAMs, b) untreated LLC cells, c) PDT-treated TAMs, d) PDT-treated LLC cells, e) untreated TAMs with untreated LLC cells in insert, f) untreated TAMs with PDT-treated LLC cells in inserts. Following 8 hrs incubation in protein and serum-free medium at 37°C, cells were collected in TRI reagent (Sigma) and semi-quantitative RT-PCR was performed on the total RNA to examine the expression of complement genes C3, C5 and C9.

d. The analysis of complement gene expression in WT or TLR-4 KO splenic macrophages co-incubated with PDT-treated LLC.

To demonstrate that complement activation in response to co-incubation with PDT-treated LLC cells was not unique to TAMs, splenic macrophages harvested from either WT or TLR-4 KO mice were co-incubated with PDT-treated or untreated LLC cells. Macrophages from spleens incubated alone or co-incubated with untreated LLC cells served as controls and then were compared to macrophages co-incubated with PDT-treated LLC cells (20 μg/mL Photofrin, 1 J/cm²). At 8 hrs following PDT-treatment and co-incubation the macrophages were collected in TRI-reagent (Sigma) and semi-quantitative RT-PCR was performed to determine the effect of exposure to PDT-treated LLC on the expression of complement genes C3, C5 and C9. An additional group with wild type macrophages incubated for 8 hours with lipopolysacharride (LPS) derived from E. coli 0111:B4 (Sigma) at 0.1 μg/mL was also included.
e. Analysis of complement gene expression by LLC cells in vitro.

Twelve groups of $1 \times 10^6$ LLC cells were plated in triplicate. One group was used as a control and left untreated. Another control group was co-incubated with untreated LLC cells. Of the 10 remaining groups, 5 were treated with PDT and collected for RT-PCR analysis at 3 hrs, 6 hrs, 9 hrs, 12 hrs and 16 hrs. The last 5 groups were left untreated but were co-incubated with LLC treated with PDT and collected at 3 hrs, 6 hrs, 9 hrs, 12 hrs and 16 hrs post-treatment. Semi-quantitative RT-PCR was used to determine the ability of PDT-treated LLC cells to produce complement C3, C5 and C9.

f. The effect of various inhibitors on the expression of C3, C5 and C9 in TAM co-incubated with PDT-treated LLC in vitro.

Ten groups of WT TAMs were plated in triplicate. All TAMs were left untreated but were co-incubated with untreated LLC (control), and PDT-treated cells alone or PDT-treated LLC cells with the addition of the following inhibitors of: HSP70 (polyclonal K-20 blocking antibody) (20 μg/mL) from Santa Cruz Biotechnology Inc., San Diego, CA, USA), selective NF-kB inhibitor SN50 (Calbiochem 481480) (10 μg/mL), blocking peptide of TIRAP (co-adaptor molecule necessary for TLR-4 signalling) (150 μg/mL) (Calbiochem 613570), TLR-4 blocking antibody MTS 510 (20 μg/mL) (Santa Cruz Biotechnology). Controls included the antibody control isotype anti-rat IgG (20 μg/mL), antibody isotype anti-chicken IgY (20 μg/mL) and 10 μl DMSO. Cells were co-incubated for 8hrs following PDT treatment (20 μg/mL Photofrin, 1 J/cm²), collected in TRI reagent (Sigma) and RT-PCR was done on total RNA for analysis of the expression of complement genes C3, C5 and C9 in addition to GAPDH (control). The same experiment was done using spleen-derived macrophages and the effects of an antibody to
TLR-2 (clone T 2.5 from HyCult Biotechnology bv, Hornby, ON) on complement gene expression at 8 hours following PDT treatment was tested (Santa Cruz Biotechnology).

g. FACS analysis to determine whether TAMs co-incubated with PDT treated LLC increase C3 protein production.

One experiment consisting of two groups of wild-type TAMs were plated in quadruplicate and co-incubated with either untreated or PDT-treated LLC cells. Twelve hours before the end of the incubation, GolgiPlug protein transporter (an inhibitor based on brefeldin A; BD Pharmingen, San Diego, Ca, USA) was added to each sample at a concentration of 1 μg/mL in order to prevent protein from being secreted from the cells. After 16 hrs of incubation, TAMs were collected in 1 mL of PBS and prepared for FACS analysis as described previously. Intracellular staining for C3 was done with paraformaldehyde-fixed and saponin-permeabilized cells by flow cytometry where intracellular C3 protein was detected using FITC-conjugated anti-C3 antibody.

h. FACS analysis to determine the purity of macrophage populations (splenic or TAM) following their isolation and culture.

Flow cytometry was used to measure the percentage of macrophages remaining after their isolation from tumours or spleens by sorting cells positive for F4/80. Following the isolation procedure, mentioned previously, cells were scraped from the Petri dish using a rubber policeman and suspended in 500 μl HBSS. Samples were centrifuged for 10 minutes at 1000 rpm and then were stained with the appropriate mAb (PE-conjugated anti-F4/80 antibody + FITC-conjugated antibody to GR-1 or the immunoglobulin isotype control) (PharMingen) by incubating for 30 minutes on ice in the dark before being re-suspended in 400 μl of HBSS prior to sorting for F4/80 + GR-1 positive cells.
VIII. Statistical Analysis

All data represented graphically are shown as the mean ± standard deviation. A non-paired student’s t-test was done to compare the difference between two means. Differences were considered significant when p<0.05.
Results

Section 1

I. Photofrin-based PDT-induced local increase in the expression of complement genes C3, C5 and C9

Twenty-four mice bearing subcutaneous LLC tumours were divided into 6 groups. One group was not treated with PDT and was used as a control. The other 5 groups were treated with PDT and the tumours and livers were harvested at 3 hrs, 6 hrs, 8 hrs, 24 hrs and 5-days post-treatment. Total RNA was isolated and all samples were analyzed for the expression of complement genes C3, C5 and C9 as well as the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for comparison using semi-quantitative RT-PCR. The data were derived from gels as shown in Figure 4 which visually demonstrates the intensity of expression of the PCR products resulting from the amplification of GAPDH, C3, C5 and C9 transcripts by RT-PCR performed on the total RNA isolated from untreated and PDT-treated tumours at 24 hours following therapy.
Figure 4: Photofrin-based PDT induces a tumour localized up-regulation of complement genes at 24 hours following treatment. LLC tumours growing subcutaneously in C57BL/6J mice were treated with Photofrin-based PDT (Photofrin 10 mg/kg i.v. followed 24 hours later by 150 J/cm²), and excised 24 hours after PDT treatment. Total RNA isolated from this and control tumours was used for RT-PCR based analysis of the expression of selected genes. The 10% polyacrylamide gel stained with SYBR green nucleic acid stain shows the expression of complement genes before and at 24 hours after treatment by PDT. Depicted above is the expression profile of one representative profile from each group. GAPDH (G) is the housekeeping gene expressed constitutively in all cells, while C3, C5 and C9 are key complement genes whose expression is compared to that of GAPDH and M is the 100 bp ladder.

The results shown in Figure 5 depict the average normalized, (compared to GAPDH), expression of C3, C5 and C9 in untreated tumours, and PDT-treated tumours at 3 hrs, 6 hrs, 8 hrs, 24 hrs and 5-days post-treatment. The results indicate that expression of the complement genes C3, C5 and C9 does not increase in untreated tumours but shows an increasing trend from 3 hrs, 6 hrs to 8 hrs following treatment. However a significant increase was evident of C3, C5 and C9 expression in tumours at 24 hrs following treatment by PDT with levels of expression remaining higher than normal at 5-days post treatment.
The average fold-increase in gene expression for C3, C5 and C9 at 24 hrs post-treatment is shown in Figure 6. C3 expression increased to as high as 5.7 times greater than in untreated tumours and both C5 and C9 increased significantly with levels as high as 4.8 and 1.9 times levels seen in untreated tumours respectively.

![Figure 5: Tumour-localized expression of key complement genes C3, C5 and C9 after Photofrin-based PDT. Subcutaneous LLC tumours were either untreated or PDT-treated as described for Figure 2. The tumours were harvested and homogenized in TRI reagent (Sigma) at 3 hrs, 6 hrs, 8 hrs, 24 hrs and 5 days following treatment. Semi-quantitative RT-PCR was done to determine the expression pattern of complement genes C3, C5 and C9. Data is presented as the mean intensity of expression normalized against the housekeeping gene GAPDH + standard deviation, *p< 0.05 compared to untreated control.](image-url)
II. Photofrin-based PDT does not cause an increase of the expression of hepatocyte-derived complement genes C3, C5 and C9

The livers from the same mice whose LLC tumours were analyzed were also harvested at 3 hrs, 6 hrs, 8 hrs, 24 hrs, and 5-days following treatment using PDT. Four mice comprised each group and 4 mice were used as controls where no treatment was applied. The results show no significant difference of hepatic expression of C3, C5 and C9 at any time tested following PDT treatment as compared to the untreated control (Figure 7).

Figure 6: Average fold increase of tumour-localized expression of key complement genes C3, C5 and C9 at 24 hours following Photofrin-based photodynamic therapy. The data for 24 hours post PDT from Figure 3 are presented as the average fold increase + standard deviation for C3, C5 and C9 compared to the untreated control.
Figure 7: Hepatic complement gene expression (C3, C5, C9) at various time points following Photofrin-based PDT. C57Bl/6J mice bearing subcutaneous LLC tumours were treated by tumour-localized PDT as described for Figure 2. Livers collected from control and PDT-treated mice were homogenized in TRI reagent (Sigma). Data is represented as expression of C3, C5 and C9 normalized to GAPDH. Bars represent standard deviation.

III. There is no increase of either hepatic or tumour-localized expression of complement genes following BPD-based PDT.

Two groups of 4 mice were used in this experiment where 1 group acted as a control and the other group was treated with PDT. Livers and tumours were harvested from untreated control mice and from those treated with BPD-based PDT 24 hours post-treatment. Results shown in Figure 8 demonstrate that there was no up-regulation of complement component genes C3, C5 and C9 at 24 hours after PDT treatment either in the liver or in the tumour. This is in contrast to the results obtained with Photofrin-PDT where there was a significant up-regulation of all 3 genes in the tumour at 24 hours following treatment.
Figure 8: The expression of complement genes C3, C5 and C9 in tumours and livers after treatment with BPD or Photofrin-based PDT. Subcutaneous LLC tumours were treated with either BPD-based PDT (BPD 2.5 mg/kg, 100 J/cm²) or Photofrin-based PDT as described for Figure 2. Tumours and livers were excised and homogenized in TRI reagent, total RNA was isolated and semi-quantitative RT-PCR analysis was performed. Results are shown as the normalized intensity of expression compared to GAPDH. Bars are standard deviation. *p<0.05 compared to untreated control.

Section 2

I. Procedures used to isolate macrophages (TAMs and splenic) used in in vitro studies resulted in > 90% of cells identified as macrophages as determined by FACS

Macrophages for use in our in vitro studies were isolated from either tumours or spleens. To confirm that our procedure did indeed result in the isolation of macrophages and not other cells, a simple FACS experiment was done. The FACS analysis demonstrates that 97.6% of cells isolated are macrophages (TAMs) from tumour cell
suspension as indicated by positive PE staining with the mature myeloid macrophage cell surface marker F4/80 plus FITC staining for myeloid cell surface marker GR-1 as shown in Figure 9A. Figure 9B shows cells isolated by our procedure from spleens resulted in > 90% of cells identified as macrophages using PE conjugated antibody to F4/80. Figure 9C is a plot of cell number versus fluorescence intensity.
Figure 9: The procedure used for the isolation of macrophages resulted in 97.6% positive PE + FITC staining for tumour and > 90% PE staining from spleen as determined by FACS. A) Cells from a LLC tumour cell suspension were plated in serum-free medium on a 3 mm Petri dish and incubated for 20 minutes at 37°C to allow adherence of macrophages to the Petri dish. Subsequently, the dish was washed once with cold PBS and the remaining cells were incubated in complete media before being used in the experiment. For flow cytometry analysis, the cells were detached using a rubber policeman and stained for the macrophage marker F4/80 using a PE conjugated antibody and for GR-1 using a FITC conjugated antibody. Shown is a representative dot plot obtained by flow cytometry. B) The spleen macrophages were detached using a rubber policeman and stained with PE-conjugated antibody raised against mouse macrophage marker F4/80. Shown is a representative dot plot graph obtained by flow cytometry analysis of these cells. C) A representative graph of cell count versus PE log fluorescence for isotype control (anti-mouse IgG in blue) and for cells positive for F4/80 in green.

II. PDT induces a significant increase of C3 by spleen-derived macrophages at 8 hours following treatment

To identify the optimal time point for complement gene up-regulation in vitro using spleen-derived macrophages, a time course experiment analyzing C3 expression was
performed. Macrophages were isolated from the spleens of C57Bl/6J mice and plated in triplicate. They were then co-incubated with non-treated (8 hrs) or PDT-treated LLC cells for 3 hours, 5 hours, 8 hours and 16 hours at which times macrophages were harvested, total RNA extracted, and RT-PCR analysis was done. Figure 10 shows a peak time point of 8 hours where there was a significant up-regulation of C3 by spleen-derived macrophages co-incubated with PDT-treated LLC cells in vitro.

Figure 10: PDT induces a significant increase of C3 at 8 hours following light treatment. C3 expression by spleen-derived macrophages co-incubated with non-PDT-treated LLC cells and with PDT-treated (20 μgL/mL Photofrin, 150 J/cm² light) LLC cells at 3 hrs, 5 hrs, 8 hrs and 16 hrs after light treatment. RT-PCR was done on total RNA. Data are presented as the C3 band intensity relative to control (NT). * p<0.05 compared to non-treated group (NT).
III. PDT induces a significant increase of the expression of key complement genes by macrophages co-incubated with PDT-treated LLC cells.

To determine whether tumour-associated macrophages (TAMs) are stimulated to up-regulate complement gene expression when in the presence of PDT-treated tumour cells, the following experiment was performed. TAMs were isolated from a cell suspension made from subcutaneous LLC tumours growing on the backs of C57Bl/6J mice, and plated in triplicate. They were then co-incubated with PDT-treated or untreated LLC cells. As controls, triplicates of untreated TAMs and LLC cells were plated alone. In addition, for comparison, triplicates of TAMs alone and LLC cells alone were treated with PDT. All cells were harvested at 8 hrs post-treatment in TRI reagent (Sigma) and RT-PCR was performed on total RNA to analyze the expression of complement genes C3, C5 and C9.

As shown in Figure 11, LLC cells alone (untreated and treated), TAMs alone (untreated and treated) and TAMs co-incubated with untreated LLC cells displayed almost identical levels of basal C3, C5 and C9 expression. Therefore PDT had no effect on complement gene expression in TAMs or LLC cells alone. Results indicate however, that TAMs co-incubated for 8 hrs with PDT-treated LLC show a significantly higher expression of all three complement component genes.
**IV. PDT induced up-regulation of complement gene C3 results in an increase in C3 protein expression in TAMs co-incubated with PDT-treated LLC.**

Results shown in Figure 11 indicate that TAMs co-incubated with PDT-treated LLC cells are stimulated to significantly increase the expression of key complement genes C3, C5 and C9. We determined whether this increase of gene expression translated into an increase of protein production levels. Two groups of TAMs were plated in triplicate.
One group was co-incubated for 16 hrs with PDT-treated LLC cells and the other group with untreated LLC cells. After the first 6 hours of incubation, GolgiPlug (PharMingen) was added to prevent protein from exiting the cells. At 16 hrs post-treatment the TAMs were collected and stained intracellularly with either FITC-conjugated antibody to C3 or to IgG (control) in preparation for flow cytometry. The results demonstrate an increase of fluorescence intensity for the TAMs incubated with treated LLC cells compared to the control, indicating a significant increase of C3 protein expression in TAMs co-incubated with PDT-treated LLC cells (Figure 12). Therefore the increase of C3 gene expression in TAMs co-incubated with PDT-treated LLC cells is associated with an increase of C3 protein levels.
Figure 12: C3 protein levels increase in TAMs co-incubated with PDT-treated LLC cells relative to the control. TAMs derived from LLC tumours were plated in quadruplicate and one group co-incubated with untreated LLC cells and the other with PDT-treated LLC cells (Photofrin 20 μg/mL and 1 J/cm²). TAMs were collected after 16 hrs and stained with a control antibody or with an antibody to C3. Samples were analyzed by flow cytometry. A) C3 fluorescence relative to the control is shown indicating a 1.6 fold increase of protein expression intensity (*p<0.05 compared to untreated control). Bar is standard deviation. B) C3 fluorescence in untreated samples (dotted line) versus treated samples (solid line).

Section 3

1. The expression of C3, C5 or C9 does not increase over time in PDT-treated LLC cells alone or in untreated LLC cells co-incubated with PDT-treated LLC cells.

After results indicated that LLC cells in vitro were capable of producing C3, C5 and C9 it was necessary to determine the extent of that production in order to better understand the localized up-regulation of complement genes in PDT-treated tumours in vivo. 12 groups of LLC cells were plated in triplicate and treated with 20 μg/mL Photofrin and 1J/cm² of light except for 2 control groups left untreated. Another 6 groups of triplicate LLC cells plates were left untreated and co-incubated with control LLC cells and with PDT-treated LLC cells. At 3 hrs, 6 hrs, 9 hrs, 12 hrs and 16 hrs following
treatment the cells were collected in TRI-reagent (Sigma), and RT-PCR was performed on total RNA to determine the expression pattern of C3, C5 and C9. Figure 13a, Figure 13b and Figure 13c demonstrate that in both the LLC cells alone treated with PDT and untreated LLC co-incubated with treated LLC there is no significant increase of complement gene C3, C5 or C9 expression. Therefore PDT has no effect on the expression of complement genes C3, C5 and C9 in LLC cells \textit{in vitro}.

![Graph showing normalized band intensity for C3 over treatment time](image)
Figure 13a-c: Complement gene expression does not increase in LLC cells treated by PDT or in those co-incubated with PDT-treated LLC. 18 groups of LLC cells were plated in triplicate. 8 groups were left untreated and 10 were treated with 20 ug/mL Photofrin and 1 J/cm² of light. 5 groups of untreated LLC were co-incubated with 5 groups of treated LLC, 5 groups of PDT-treated LLC were left alone, 1 group of untreated LLC was left alone and the other one was co-incubated with untreated LLC. Cells were collected at 3 hrs, 6 hrs, 9 hrs, 12 hrs and 16 hrs post-PDT treatment. Data are represented as mean (normalized to GAPDH) intensity of C3, C5 or C9 expression + standard deviation. *p<0.05 compared to PDT-treated LLC cells alone.
Section 4

I. There is a significant increase of the local expression of complement gene C3 in tumours 24 hrs post-PDT treatment in both wild-type and TLR-4 knock out mice but not in C3 knock out mice.

To further investigate the mechanisms underlying local production of complement components in tumours following PDT wild-type C57Bl/6J (WT) and knock out (KO) mutant mice of the same genetic background were used (4 mice per treatment group). C3 KO mice were used in order to confirm that C3 upregulation in WT mice is due to host cells and that PDT does not upregulate C3 in these KOs. TLR-4 KO mice were used in order to investigate one of the possible pathways through which PDT-induced complement gene expression occurs and whether this pathway is implicated in the regulation of any or all of C3, C5 and C9. This KO was used because TLR-4 is known to be implicated in the control of a number of genes involved in the host response through NF-κB signalling. All mice were bearing LLC tumours on their backs and 1 group of each genotype were treated with 10 mg/kg Photofrin and 150 J/cm² of light and the other group was left untreated and used as a control. Tumours were extracted at 24 hours following PDT light treatment, immediately homogenized and total RNA was isolated for RT-PCR analysis of the expression of C3, C5 and C9. Figure 14 shows the normalized expression (relative to GAPDH) of C3 in WT, C3 KO, and TLR-4 KO mice. Results indicate that in WT mice there is a significant increase although to a lesser degree of the local expression of C3 which is in accordance with the data shown in Figure 5. They further show evidence for the expression of C3 in the C3 KO mice in both the PDT treated tumours and the untreated tumours. There was no significant difference in C3 expression in these two groups. Since the C3 gene is knocked out in these hosts, the detected C3 expression obviously originated from the LLC cells. Finally, the results
indicating a significant increase of the tumour localized expression of C3 in PDT-treated compared to untreated TLR-4 KO mice.

Figure 14: Complement gene C3 expression of PDT treated versus untreated tumours growing in wild type, C3 KO and TLR-4 KO mice. Two groups each of wild-type, C3 knock-out and TLR-4 knock-out mice bore LLC tumours on their backs. One group of each was treated with 10 mg/kg Photofrin and 150 J/cm² of light and the other was left untreated. Semi-quantitative RT-PCR was done on the total RNA isolated from the tumours excised at 24 hours post PDT. The data are represented as the mean C3 expression normalized to GAPDH + standard deviation. * p<0.05 compared to untreated control.

Figure 15 shows the normalized expression (relative to GAPDH) of C5 in untreated and PDT-treated LLC tumours growing in wild-type, C3 KO, and TLR-4 KO mice. Results indicate that in WT mice there is a marked increase of the local expression of C5 which is in accordance with the data shown in Figure 5. They further indicate that there was an increase of localized expression of C5 in C3 knock-out mice while no increase of the expression of C5 was detected in the TLR-4 knock-out mice.
Figure 15: Complement gene C5 expression of PDT treated versus untreated tumours growing in wild type, C3 KO and TLR-4 KO mice. The expression of C5 was determined employing semi-quantitative RT-PCR on the total RNA from the same tumours as used for the C3 expression analysis described for Figure 14. The data are represented as the mean C5 expression normalized to GAPDH + standard deviation. * p<0.05

Following the same procedure as listed previously for analysis of C3 and C5 expression, the expression of C9 was also analyzed. The data represented in Figure 16 demonstrated a local increase of the expression of C9 in PDT-treated compared to untreated LLC tumours growing in WT mice which is in accordance with the data shown in Figure 5. However, there was no increase of tumour-localized expression of C9 in C3 KO or TLR-4 KO mice.
Figure 16: Complement gene C9 expression of PDT treated versus untreated tumours growing in wild type, C3 KO and TLR-4 KO mice. As in the procedure described in Figures 14 and 15 the results obtained by semi-quantitative RT-PCR for C9 are presented as the mean normalized expression ± standard deviation. *p<0.05 compared to untreated control.

Section 5

I. PDT induces a larger increase of complement gene expression by WT spleen-derived macrophages co-incubated with PDT-treated LLC cells than by TLR-4 KO cells

Macrophages isolated from the spleens of WT or TLR-4 KO mice were plated in triplicate and co-incubated with untreated LLC cells (control) or with PDT-treated LLC cells for 8 hours to determine the expression of complement component genes C3, C5 and C9 in response to signals expressed by PDT-treated tumour cells. Following co-incubation, the macrophages were collected in TRI reagent and semi-quantitative RT-PCR was done on the total RNA. Figure 17 shows the expression of C3, C5 and C9 by spleen-derived macrophages (WT (A) and TLR-4 KO (B)) co-incubated with untreated and PDT-treated LLC cells. There was an increase of the expression of these key
complement genes by wild-type spleen macrophages exposed to PDT-treated LLC cells after 8 hrs co-incubation confirming previous results. It is also evident that there was an increase of the expression of these genes by TLR-4 KO macrophages co-incubated with PDT-treated LLC cells. However, this increase was not as pronounced as that by WT macrophages. For example, C3 increased by almost 40% in WT macrophages after exposure to PDT-treated LLC cells but only by 20% in TLR-4 KO macrophages.
**Figure 17**: Expression of C3, C5 and C9 genes by WT and TLR-4 KO spleen macrophages co-incubated with PDT-treated LLC cells. The spleen-derived macrophages were co-incubated with untreated LLC cells or with PDT-treated LLC cells. Samples were collected at 8 hrs post treatment in TRI reagent and RT-PCR was done on the total RNA. The above expression levels are for C3, C5 and C9 by both WT (A) and TLR-4 KO (B) macrophages alone, or co-incubated with either untreated LLC cells or PDT-treated LLC cells and the data are presented compared to the control (untreated macrophages alone). * p<0.05 when compared to non-treated macrophages alone.

**Section 6**

I. The marked PDT-induced increase of complement gene expression by WT TAMs co-incubated with PDT treated LLC cells is attenuated by the addition of inhibitors of HSP70, TIRAP, NF-κB and TLR-4.

Our previous results have demonstrated that PDT induces a significant increase of the expression of complement genes C3, C5 and C9 *in vivo* and *in vitro* by macrophages co-incubated with PDT-treated LLC cells. To investigate the cellular signalling pathways involved in the local up-regulation of these genes following treatment by PDT, TAMs were co-incubated with PDT-treated LLC cells with or without the addition of various...
inhibitors or blocking antibodies. Some of the proteins involved in TLR signalling were targeted due to the possible PDT-induced up-regulation of complement genes through NF-κB. If an inhibitor/blocker of one of these proteins stops the PDT-induced increase of complement expression then it is likely that this protein is, at least partially, involved in the signalling for this increase. Inhibitors of the transcription factor NF-κB, the Toll receptor adaptor protein TIRAP, antibodies blocking HSP70 or TLR-4, or their associated controls (DMSO used as a solvent for the TIRAP blocking peptide, and antibody isotype controls anti-chicken IgY and anti-rat IgG) were added to the Petri dishes containing TAM and PDT-treated LLC cells in the inserts. The results in Figure 18 show, as expected, a large increase of the expression of C3, C5 and C9 in TAMs coinubated with PDT treated LLC cells alone. An increase of expression was also seen in the control samples containing DMSO or the isotype antibody controls. This increase of gene expression was attenuated by the use of the tested inhibitors/blockers. Anti-HSP70 and the NF-κB inhibitor diminished the increase of the induced C3 and C5 expression and completely blocked the up-regulation of C9. The TIRAP blocking peptide and anti-TLR-4 also inhibited the induced up-regulation of C3 and prevented any significant induction of C5 and C9 up-regulation.
Figure 18: C3, C5 and C9 expression by TAM co-incubated with PDT treated LLC cells in the presence or absence of various inhibitors/blockers. Ten groups of wild-type TAMs were plated in triplicate. One group was incubated alone and another with untreated LLC cells to serve as controls. The remaining groups were co-incubated with PDT-treated LLC cells with the addition of inhibitors/blockers to NF-kB, HSP70, TLR-4 and TIRAP, or appropriate control agents (DMSO (TIRAP), chicken IgY (HSP70) or rat IgG (TLR-4)). Macrophages were collected in TRI reagent after 8 hrs and semi-quantitative RT-PCR was done on total RNA. The results are presented as the normalized intensity of expression relative to the control (TAMs alone). *p<0.05 for PDT induced effect relative to TAM alone. ^p<0.05 for inhibitor/blocker induced effect compared to its isotype control.

II. The addition of an antibody to TLR-2 reduces the PDT induced increase of C3 by spleen-derived macrophages

Finally, the effect of an antibody to TLR-2 on PDT-induced complement gene expression was tested. Spleen-derived macrophages were co-incubated with non-treated and PDT-treated LLC cells for 8 hours. C3 expression increased significantly by 8 hours.
in the absence of antibody and with the addition of antibody isotype control (mouse IgG).

The addition of the antibody to TLR-2 prevented this significant increase of C3 showing expression levels not significantly different from the non-treated sample (Figure 19).

Figure 19: The addition of an antibody to TLR-2 decreases the PDT-induced increase of C3 by spleen-derived macrophages. Macrophages were isolated from spleen cells, plated in triplicate and co-incubated for 8 hours with non-treated or PDT-treated LLC cells with and without the addition of antibody isotype control to mouse IgG or to TLR-2. Total RNA was extracted and semi-quantitative RT-PCR analysis was done to determine the expression of C3. The data are represented as band intensity relative to control. *p<0.05 compared to control (NT). ^p<0.05 compared to isotype control.
Discussion

The destruction of solid tumours caused by the biological and chemical reactions of PDT induces a rapid and powerful host response which includes activation of the acute phase response, leading to inflammation with the activation of circulating leukocytes, lymphocytes, and the amplification of the innate immune response resulting, optimally in adaptive immunity and long term tumour control (69, 71, 80). Of significant importance and with various roles in the immune response, is the complement cascade. Proteins of the complement system have been shown to increase in concentration following PDT in both the blood and the tumour (126). A rise in C3 protein levels occurs following PDT (71, 126). Also, assembly of the terminal MAC was detected both in vivo and in vitro on the surfaces of PDT treated cells (41, 127). Results obtained from the depletion of complement using cobra venom factor cause a reduction of tumour cure rates. The opposite effect is seen with activators of complement (133). The role of complement in PDT has not been completely elucidated. However, it is obvious that it plays a significant and positive role when stimulated following treatment. Its potential effects in PDT include the marking of apoptotic cells and cell debris for phagocytosis by infiltrating neutrophils and macrophages, the recruitment of these immune cells by chemotaxis and by the stimulation of cytokine release, the direct lysis of target tumour cells and the activation of T-cells through enhanced antigen presentation by antigen presenting cells (APCs) (71, 96, 126). Because of its wide range of action, complement is of great importance to the host response which is activated in PDT.
I. Hepatic versus tumour localized complement expression after PDT

Complement is synthesized primarily in the liver, but it can be made by other cells in a local environment (93, 122). The results from our experiments demonstrate that there is a high level of constitutive expression but no increase of the expression of key complement component genes C3, C5 and C9 in the livers of tumour-bearing mice following treatment by PDT. However, there is localized expression of C3, C5 and C9 in the untreated tumour and following Photofrin-based PDT treatment there is a significant increase of the expression of all three complement genes tested 24 hours post-treatment with levels remaining high at 5 days post treatment. Expression levels increased by more than 3-fold for both C3 and C5 at 24 hrs post-treatment and 2-fold for C9. Furthermore, when the level of expression was compared to that in the liver at the same time point, tumour-localized expression reached almost double that of hepatic expression at 24 hrs post-treatment. Complement gene expression possibly becomes increased in the tumour at 24 hours following light treatment because of the time it takes for circulating neutrophils and monocytes to be recruited from the blood and tissue storage pools to the tumour. It has been shown previously in our lab (78, 79) that neutrophil levels in the blood and in the tumour increase as early as 1 hour following PDT and infiltrate the tumour in high numbers until 24 hours following treatment. Because most cells in the tumour are destroyed immediately by direct PDT inflicted injury, there are no local cells able to produce complement until they have been recruited from other areas.

In contrast, PDT using BPD did not result in any increase of complement gene expression locally in the tumour or by hepatocytes in the liver. This finding may be explained by the location of action of these photosensitizers which have different properties. The effectiveness of BPD depends on its high concentration in the circulation
and therefore it exerts its damage on the tumour vasculature. Photofrin, however, localizes more to the tumour parenchyma and exerts its effects there in addition to the vasculature (133). It could be that there is a greater stimulation of local complement gene expression when damage is inflicted to the tumour parenchyma rather than to the vasculature.

It is apparent from these results that there is significant stimulation of complement gene expression locally at the PDT-treated tumour when Photofrin is used as the photosensitizer. This local response may be of critical importance to the host response to PDT and may contribute more to the effectiveness of PDT than does systemic complement. Because of the drastic increase in the levels of complement gene expression locally at the tumour, some protein product maybe be entering the circulation and contributing to the rise of humoral C3 content that has been documented (126, 127).

The liver is comprised of three distinct cell types: hepatocytes which are generally involved in metabolism; Kupffer cells which play a role in immunomodulation by synthesizing immunostimulatory and inhibitory factors; and endothelial cells which generally contribute to pro-inflammatory signals by producing IL-1 and IL-6 (134). Hepatocytes have receptors for a variety of secreted factors including TNF, IL-1, and IL-6, that when bound, can increase the expression of complement proteins and acute phase reactants (134, 135). These cells however, are strongly regulated by cross-talk signals from Kupffer cells that can limit the systemic response (134). For example, in response to sepsis, a condition of excess bacteria in the blood, the liver is stimulated to produce a large amount of TNF and to up-regulate IL-1 and IL-6 gene expression. This increases the production of acute phase reactants, including some complement proteins. However, Kupffer cells can downregulate this response and are involved in the uptake and
clearance of these factors (134). This highly modulated response indicates that there is a control mechanism to prevent an excessive host response which can potentially have negative consequences to the system. Evidence for an increase of C3 gene expression has been documented in both hepatocytes and macrophages in response to LPS or IL-1 indicating that regulation is pre-translational (135, 136). This same effect was not seen for other complement factors tested; C2 and factor B (136), indicating that differential regulation of these proteins occurs and that the responses seen may be specific not only to the individual protein but also to the activating source.

We have no evidence to indicate that the liver is contributing (at the level of transcriptional up-regulation) to an increase of circulating complement proteins or to complement active at the tumour site which increases following PDT (126). It could be that signals released in response to localized PDT-induced damage do not elicit pre-translational control of complement in the liver. Translational control could be exerted there by increasing protein synthesis and release without an increase of mRNA synthesis. It is possible that regulatory mechanisms increase the speed of complement mRNA degradation in order to prevent the negative consequences of over activation. In addition, it may be that hepatocytes contain intracellular storage pools for complement proteins which can be quickly released for an immediate systemic response. It has been documented that neutrophils contain these storage pools for complement receptor proteins to aid in augmenting phagocytic efficiency when they are stimulated by activating structures (137). By the same logic it might be that hepatocytes also have cytoplasmic or membrane-bound complement storage pools. These could all be reasons for why we did not see any up-regulation of complement genes in the liver following PDT, where it would have been expected due to the nature of the signals released at the
damaged tumour and the documented increases of C3 levels in the blood (71, 126). Further studies using hepatic cells *in vitro* co-incubated with PDT-treated tumour cells might help to clarify what we have documented.

**II. Macrophages are the source of PDT-induced increase by local complement expression**

Tumour-localized up-regulation of C3, C5 and C9 did occur to a significant degree by 24 hours following treatment by PDT. We wanted to determine which cells in the tumour were responsible for this up-regulation of complement genes after PDT. An experiment using WT and C3 KO mice was done *in vivo* where the data obtained show that compared to wild-type mice, which showed a significant increase of tumour-localized C3, C5 and C9 gene expression at 24 hrs post-treatment, C3 KO mice, as expected showed no increase of C3 expression and only a slight increase of both C5 and C9 revealing that a host-cell type responds to PDT by up-regulating complement. We see small levels of complement gene expression in C3 KO mice compared to WT mice. There was a significant increase of C5 gene expression but no significant up-regulation of C9 mRNA in C3 KO tumours. Although the increase in C5 expression was significant, it was slight and it is likely that these genes are not activated to the same extent following PDT in C3 KO mice because their products can not be cleaved and activated without activated C3 products. Therefore it is possible that the activation of these key terminal complement genes is positively regulated in part by C3. To further determine which cells were responsible for this increased expression in the PDT-treated tumours and to identify an optimum time point *in vitro* at which an increase of C3 occurs, a time course experiment was done using spleen-derived macrophages co-incubated with LLC cells. This experiment revealed that a significant increase of C3 expression by spleen-derived macrophages occurs after 8 hours of co-incubation with PDT-treated LLC cells. Further
in vitro experiments using tumour-associated macrophages (TAMs) and LLC cells were done using a time point of 8 hours. These in vitro studies revealed that untreated TAMs exposed to PDT treated LLC cells significantly up-regulate the expression of C3, C5 and C9 at 8 hours post-treatment and that this correlates to an increase of local C3 protein levels at 16 hours post-treatment. This same effect was not seen in TAMs co-incubated with non-treated LLC cells thereby indicating that the PDT-treated tumour contains or releases signals that stimulate complement gene expression in macrophages (66, 71, 96). This indicates an important role for TAMs, either remaining resident TAMs not killed by PDT (less likely) or newly infiltrating TAMs (24 hours post-treatment), in the local production of complement proteins in the PDT-treated tumour. Neutrophils have been shown to infiltrate the tumour early following light treatment with increasing numbers toward 10 hours and macrophages (TAMs) follow usually within 6 hours (78, 79). If we assume that macrophages are significantly infiltrating the tumour at about 16 hours following PDT treatment and we have shown that complement expression significantly increases at 24 hours post-treatment in vivo, then it followsthat these TAMs in vitro produce complement after 8 hours of exposure to PDT-induced danger signals. This local production by macrophages infiltrating the tumour, as demonstrated by the significant increase in vivo at 24 hrs post PDT treatment, and in vitro after 8 hours co-incubation with PDT-treated LLC cells, may be of critical importance to the inflammatory process leading to tumour immunity and effective therapy. This increase of gene expression by TAMs in vitro was most prominent for C3 where levels rose to more than double the basal level seen in the controls.

Demonstrating that this ability of tumour associated macrophages to respond to PDT-treated tumour cells by increasing the expression of complement genes C3, C5 and
C9 was not unique to TAMs, a similar experiment was performed using wild-type macrophages isolated from spleens. This experiment showed that wild-type splenic macrophages also respond to signals released from PDT-treated LLC cells by up-regulating the expression of complement genes C3, C5 and C9. This further supports our notion that it is the exposure of newly recruited macrophages to PDT-induced signals that up-regulates complement transcription.

Notably, in vitro experiments also demonstrated the ability of LLC cells to synthesize all of these complement components. When examined further it was determined that the extent of complement transcription by LLC cells is minimal and does not increase following PDT. LLC cells themselves do not respond to signals released from PDT-damaged cells, possibly because they do not possess the necessary receptors for the PDT-induced danger signals (66, 71, 96). This is in accordance with other reports noting that malignant cells, more specifically epithelial cells in the lung, are capable of synthesizing complement proteins (138).

III. TLR signalling pathway is involved in macrophage upregulation of local complement after PDT

The experiments presented in this thesis thus far have shown that there is a tumour-localized increase of the expression of key complement genes C3, C5 and C9 following PDT. It was demonstrated that macrophages respond to signals from PDT-treated cells and are responsible for the increase of expression of complement genes. The questions that arise are what signals are they responding to and by which pathway are they signalled to up-regulate complement genes?

Macrophages express a large number of different cell-surface receptors including complement receptors and TLRs, both PRRs, which are capable of innate immune recognition of conserved bacterial sequences and more importantly in PDT, endogenous
host danger signals (66, 71, 127). Signals that may lead to the activation of the host response to PDT include HSPs (HSP70, HSP60 and GRP78), complement-opsonised materials, membrane lipid fragments caused by the activation of phospholipases, and products of membrane degradation (fibronectin, laminin, and collagen) (71). HSP70 is released from PDT-damaged cells and is up-regulated in cells under stress (66, 71, 139, 140). HSP70 normally resides inside the cell where it acts as a protein chaperone. When cells undergo necrosis or are under severe stress such as that resulting from PDT-induced trauma, HSP70 may escape, or be released from the cell (66, 141). HSP70 existing outside the cell is a potent danger signal which can be recognized by immune receptors like TLR-4 and TLR-2 on the surfaces of macrophages (140, 141). TLR-4 is an innate immune recognition receptor largely responsible, in coordination with other receptors like CD14, for intracellular signalling resulting in the up-regulation of inflammatory genes (142-144). HSP70/TLR-4 binding has been implicated in the signalling through the inflammatory transcription factor NF-κB which is responsible for activating a plethora of immune genes including IFN-γ, TNF-α, and interleukins (141, 145) (Figure 19). In fact, recent work in our laboratory has shown that upon co-incubation with PDT-treated tumour cells, macrophages elevate their production of TNF-α as a consequence of the TLR-NF-κB signalling triggered by the released HSP70 (66). The TLR signalling pathways could therefore play an important role in the outcome of PDT (71, 144). Could this pathway be responsible for locally up-regulating complement genes C3, C5 and C9 in macrophages following PDT treatment?

To determine whether TLR-4 might be involved in the signalling leading to the up-regulation of C3, C5 and C9 in vivo, an experiment was done involving WT mice, and TLR-4 KO mice. In TLR-4 KO mice, there was a significant increase of C3 expression,
a slight increase of C9 expression but interestingly there was no increase of C5 expression. These results indicate that TLR-4 may have a role in the stimulation of some local complement synthesis, namely, C5, however from this data it may not be essential to the signalling leading to the expression of C3. To further investigate the response seen in vivo it was necessary to perform an in vitro study of a similar nature. Results obtained from splenic macrophages isolated from TLR-4 KO mice co-incubated with PDT-treated LLC cells indicate a very similar pattern to what was seen in vivo, further supporting our claim that TLR-4 plays a role in the outcome of PDT and illustrating its involvement in complement gene regulation. From these data it appears as though C5 up-regulation is more dependent on TLR-4 signalling than are C3 and C9. Not much is known about the transcriptional control of complement genes and whether they have NF-κB binding sites or NF-κB controlled genes and therefore it is difficult to draw conclusions that can be related to the literature. These questions are currently being addressed (146).

Finally, to determine whether or not HSP70 signalling via TLR-4 and NF-κB is responsible for the increase of complement production by macrophages following PDT, an in vitro experiment was done involving WT TAMs co-incubated with PDT treated LLC cells in the presence or absence of inhibitors/blockers of HSP70, NF-κB, TIRAP and TLR-4. Results from this experiment demonstrate a significant increase of the expression of complement genes C3, C5 and a marked rise of C9 in wild-type TAMs following 8 hours of co-incubation with PDT-treated LLC cells. This increase of gene expression was significantly reduced by inhibitors/blockers of, NF-κB and HSP-70, and TIRAP but not by anti-TLR-4 which significantly inhibited only C5 expression. These results are similar to those obtained in vivo and to those obtained using KO macrophages in vitro where PDT-induced C3 expression significantly increased in TLR-4 KO
macrophages/mice in contrast to C5 which did not increase as it did in WT mice/macrophages/TAM. These results indicate that indeed the increase of tumour-localized complement gene expression (C3, C5 and C9) in macrophages is a product of the TLR-4/NF-κB pathway. It occurs possibly, at least in part, through the binding of TLR-4 on the surface of tumour associated macrophages to exogenous HSP70 released from PDT-damaged cells. This signalling activates the transcription factor NF-κB which localizes to the nucleus where it activates the transcription of key complement genes C3, C5 and C9. The fact that C3 and in some cases C5 and C9 up-regulation was not completely blocked by these inhibitors/blockers could be explained either by the incomplete action of these interfering agents, or by the existence of additional contributory signalling independent of the TLR-4-NF-κB pathway. Despite the lack of significant complement gene up-regulation in the presence of these inhibitors/blockers, there was still a lower level of gene expression comparable to that seen in untreated tumours. Some of this may be due to incomplete blockage by each of the inhibitors/blockers thereby allowing a small amount of transcription to occur. It could also be the product of the action of another transcription factor (not NF-κB) or perhaps due to alternative signalling receptors. Possible receptors contributing to the regulation of complement gene expression include other TLRs, complement receptors, in addition to other immune receptors on the surfaces of macrophages capable of intracellular signalling like IL, IFN and TNF receptors. To examine the role of TLR-2 in the PDT induced up-regulation of complement one final experiment was done using spleen-derived macrophages co-incubated with PDT-treated LLC cells. The results obtained for C3 using RT-PCR demonstrate that the PDT-induced increase of C3 by macrophages is
prevented by the addition of an antibody to TLR-2, indicating a shared role with TLR-4 in the activation of complement synthesis in macrophages following PDT.

**IV. Summary**

The results presented in this thesis help to explain the role of macrophages in the host response to PDT-inflicted injury on solid tumours. It has been documented that complement proteins are a necessary factor in innate immunity and inflammation which in turn has a role in the proper activation of T-cells and B-cells thus bridging innate immunity to adaptive immunity (105-121). In PDT, for effective cures and good therapeutic outcome it is necessary that the host response be intact and that neutrophils are sequestered to the tumour along with other immune cells like macrophages and lymphocytes. The sequestration of neutrophils to the tumour is complement dependent and when complement is depleted, the therapeutic benefit of PDT decreases and lower tumour cure rates are achieved (80, 96). This could be due to the lack of complement directly or it could be due to the lack of complement mediated effects. For this reason it is important to reveal the origin of complement that is produced following PDT so that it might be harnessed during therapy to improve tumour cures and to better understand the mechanism by which PDT destroys the solid tumour (147). The results presented in this paper unveil the origin of complement following PDT, revealing that a significant local production of C3, C5 and C9 in the tumour does occur. We further demonstrated that this production occurs in macrophages and is at least in part mediated by NF-κB activation occurring through TLR-4/TLR-2 signalling engaged by the binding of HSP70 released from PDT-damaged cells. Figure 19 shows a summary diagram of the results presented in this thesis.
Figure 20: Summary of the pathway by which complement genes C3, C5 and C9 are locally up-regulated in the tumour following treatment by PDT.

Conclusions

The application of PDT to solid tumours inflicts large physical and chemical damage at the tumour site. The body responds to this injury by mounting a host response to contain and repair the damaged tissue. In this process, tumour cells are recognized and destroyed by various components of the innate and adaptive immune systems resulting in long term tumour control. Without an intact and powerful host response the benefit of therapy becomes greatly reduced. The results presented in this thesis demonstrate that there is a tumour-localized up-regulation of the key complement genes C3, C5 and C9 by TAMs. They implicate the role of HSP70 released by PDT damaged cells through its interaction with TLR-4 receptors on the surfaces of macrophages which can signal intracellularly via NF-κB to increase complement gene expression. The results of this
study further clarify the involvement of the complement cascade in the host response to PDT-inflicted injury. This represents another stepping stone in the path leading to the understanding of the PDT-elicited responses that are essential to its success as a cancer therapy.

**Future Directions**

Future studies stemming from this research could include the further characterization of the pathways involved in the up-regulation of complement genes following PDT so that these processes may be harnessed for increasing the efficacy of therapy. In addition, the characterization of other immune components, such as those involved in the acute phase response (pentraxins) activated following PDT would benefit the overall understanding of the multitude of processes that ensue following the PDT inflicted injury to the tumour. Furthermore it would benefit our understanding of the complement system in general if more of its components were studied, both at the level of gene expression and at the level of protein expression both locally and in the liver. For example, it would be pertinent to study the expression levels of complement receptors and inhibitors in the liver and in the tumour before and after PDT treatment. In addition the expression profiles for TLRs should be studied to better understand the whole process leading to complement up-regulation in tumours following PDT treatment.
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