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Characteristics of complement activation in mice bearing Lewis lung carcinomas treated by photodynamic therapy

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Abstract

Following treatment of Lewis lung carcinomas (LLC) by Photofrin-mediated photodynamic therapy (PDT), tumor tissues and sera of host mice were collected for the analysis of complement activity. Elevated tumor C3 levels were detected between 1 and 24 h after PDT, while serum C3 levels increased significantly at 24 h post therapy. Increased alternative complement pathway activity in the serum was evident between 1 and 3 days post PDT. Blocking C3a- or C5a-receptors in the host mice decreased the efficacy of PDT in producing LLC tumor cures, supporting the importance of complement action in PDT-mediated tumor destruction.

Keywords: Photodynamic therapy; Complement system; C3; Mouse LLC tumor model

1. Introduction

Photodynamic therapy (PDT) is a treatment approved by health agencies throughout the world for oncological and non-oncological applications [1,2]. It combines the administration of a light-sensitive drug (photosensitizer) and lesion-directed activation of the photosensitizer with light of appropriate wavelength [1]. Energy transfer from the activated photosensitizer to molecular oxygen leads to the production of reactive oxygen species, dominated by singlet oxygen, which then binds to various cell substrates causing cytotoxicity [3,4]. Cancer tissue damage inflicted by PDT provokes the induction of an inflammatory and immune responses that both have an important role in the therapy outcome [3]. The elicited tissue-destructive inflammatory action is particularly important as a pivotal contributor to the PDT-mediated damage of tumor vasculature. Recently, the complement system was identified as a major instigator and propagator of this PDT-induced inflammatory response [5,6].

Complement was first identified as a heat-labile substance in serum that 'complemented' antibodies in the killing of bacteria, and its nomenclature simply followed the historical order of discovery of each protein. More than 30 proteins make up this cascade of unidirectional enzymatic reactions which lead to

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a number of biological effects establishing its role as a major effector system of inflammation, innate and adaptive immunity [7–9]. The primary source of plasma complement proteins is the liver, but various types of cells have been implicated in local complement production [10].

The central complement component C3 may be activated by three different pathways: the antibody-dependent classical, and the antibody-independent alternative and lectin pathways. All three pathways lead to the terminal step of the cascade with the assembly of the complement proteins C5b through C9, forming the membrane attack complex, a lytic pore inserted in targeted cell membranes [7,8]. Activation of the complement cascade also results in the generation of anaphylatoxins that promote white blood cell chemotaxis, in particular by neutrophils, to migrate toward the site of injury. In tumors the result can be a sequestration and aggregation of these cells to promote cell plug formation leading to blood flow stasis, with deprivation of oxygen and nutrients to tumor cells. The complement system is activated by a variety of disease-associated stimuli including antibodies or immune (antibody–protein) complexes, via the classical pathway; or the alternative pathway by injured or ischemic tissue, altered cell surfaces or infections [7].

Complement activity is a highly regulated system that mediates inflammatory responses, be it to infection, or wounded tissue. What is usually considered to be a beneficial response of the host to maintain homeostasis and to promote healing, can also lead to severe damage of normal, healthy tissue. These complications have been noted in transplant rejection, glomerular and myocardial ischemia-reperfusion injury, and burn injury [11–13]. Activated complement reactions can induce damage to target cells and this could provide an advantage to the host during rejection of tumors treated by PDT [6].

In the early 1980s, a few reports indicated complement activation following photodynamic therapy [14–16]. However, these studies were conducted on normal cutaneous tissue. Our recent studies have shown that activated complement is a major mediator of PDT-induced neutrophilia [5,17] and provided evidence of PDT-induced deposition of the membrane-attack complex of complement on mouse tumors [5]. We have also reported that the effects of PDT at the cellular level, including the induced surface expression of heat shock protein 70 [18] and apoptosis, directly instigate the activation of the complement system [19].

This communication describes the kinetics of systemic and treated tumor-localized activation of the complement system following PDT and demonstrates that the activity of C3a and C5a anaphylatoxins contributes to the cure rate of PDT-treated tumors.

2. Materials and methods

Lewis lung carcinomas (LLC) [20] were grown in syngeneic C57BL/6 mice. The cell line was maintained in vivo by biweekly intramuscular tumor brei inoculation, as described previously [21]. For experiments, two million cells suspended in serum-free media were inoculated subcutaneously on the depilated backs of mice using a 26-gauge needle. All animal protocols were approved by the Animal Ethics Committee of the University of British Columbia, Vancouver, Canada.

For PDT, Photofrin® (porfimer sodium, kindly provided by Axcan Pharma Inc., Mont-Saint-Hilaire, Quebec, Canada), was reconstituted in 5% dextrose in double distilled water and used at a concentration of 10 mg/kg. A volume of 0.2 ml/20 g mouse was administered intravenously (i.v.) 24 h prior to light treatment. Light was delivered superficially to the tumors through an 8 mm diameter liquid light guide, model 77638 (Oriel Instruments, Stratford, CT). The light guide was attached to a high throughput fiber illuminator (Sciencetech Inc., London Ontario, Canada) equipped with a 150 W QTH lamp with integrated ellipsoidal reflector and a 630 ± 10 nm interference filter. Fluence rate delivered was 100 mW/cm^2 at total light dose of 150 or 180 J/cm^2, as indicated. During light treatment individual animals were restrained un-anesthetized in lead holders designed to expose only the sacral region of their backs to light. The light spot encompassed the tumor plus $\sim$1 mm of surrounding skin. This treatment was not painful; mice experienced only a minor stress due to the immobilization as reported earlier [17].

At various time points after PDT, mice were sacrificed and LLC tumors were excised. Immediately

following excision, the tumors were weighed and placed in 1 ml of PBS containing 10 μl of a protease inhibitor cocktail (Sigma Chemical Company, St Louis, MO). Each tumor was homogenized for 10 s with a Polytron PT 3100 homogenizer (Kinematica AG, Switzerland), placed on ice, and centrifuged at 20,000 × *g* in a Micromax bench-top ultracentrifuge (International Equipment Company, Needham Heights, MA, USA) to obtain supernatant. The supernatant was stored at −80 °C.

Serum was harvested from whole blood collected by cardiac puncture. After thirty minutes at room temperature, the serum was separated from clotted blood by centrifugation.

The content of C3 in sera and tumor homogenates of mice following PDT was assessed by a sandwich enzyme-linked immunosorbent assay (ELISA). Goat anti-mouse C3 F(ab′)2 fragments (1:1000; Cappel Laboratories, Durham, NC, USA) in PBS was added to coat the wells of a NUNC Maxisorp 96-well plate followed by overnight incubation at 4 °C. The wells were blocked with 5% heat-inactivated fetal bovine serum in PBS, 0.05% Tween-20 and 0.001% dextran sulfate solution. After 1.5 h of incubation at 37 °C the blocking solution was decanted, then standard and test samples (diluted in blocking solution) were added to the plate and incubated for 1 h at 37 °C. All test sera were diluted $2.5–5 \times 10^4$ times prior to being added to their designated wells in the test plate. After washing, HRP-conjugated goat anti-mouse C3 (1:5000; Cappel) in PBS/0.05% Tween-20/1% normal goat serum was added and incubated at 37 °C for an additional hour. The plate was washed and 0.4 mg/ml *o*-phenylenediamine dihydrochloride (OPD) in phosphate–citrate buffer pH 5.0 was added to each well for 20 min at 37 °C. The reaction was stopped by the addition of 150 μl 1 M H_2SO_4 per well and absorbance determined at 490 nm in a Dynex MRX microplate reader (Dynex Technologies Inc., Chantilly, VA, USA). The mouse C3 standard, a β-glycoprotein of M.W. 210,000 consisting of two non-identical, disulfide-linked polypeptide chains, was isolated and purified from the plasma of male DAB/2J (C5-deficient) mice (The Jackson Laboratory, Bar Harbor, ME, USA) using the protocol based on SP/QAE-Sephadex chromatography [22]. Lowry protein assay kit purchased from Pierce Biotechnology Inc. (Rockford, IL) was used for determining tumor protein contents in order to express the measured C3 in micrograms per milligrams of total tumor protein.

A semi-quantitative ELISA based on rat anti-mouse monoclonal antibody to C3/C3b/iC3b/C3c produced by the 3/26 hybridoma [23] was used as an alternate means for monitoring PDT-induced changes in the levels of tumor C3 and its fragments. Undiluted tumor homogenate supernatants were placed in the wells of a 96-well NUNC Maxisorp plate in 100 μl aliquots. The plates were incubated for 1.5 h at 37 °C, then washed with PBS/0.05% Tween-20 and blocking solution (as described above) was added to each well with additional 1.5 h incubation at 37 °C. The plates were washed and 3/26 hybridoma supernatant was added to each well. Following 1-h incubation at 37 °C the wells were washed and a 1:100 dilution of HRP-goat anti-rat IgG (Oncogene Research products, Boston, MA) was added to each well for 30 min at 37 °C. Following three more washes, OPD solution was added to each well as described for the C3 sandwich ELISA and the reaction stopped with 1 M H_2SO_4. Absorbance was read at 490 nm on a Dynex MRX micro-plate reader. The results were expressed as relative absorbance compared to the level in untreated tumors.

Distinguishing between the pathways of complement activation was based on using $MgCl_2$/EGTA buffer (that inhibits the classical pathway) and zymosan as the activating surface in the assay for the alternative pathway, while employing antibody-opsonized cells in the assay for the antibody-dependent classical pathway. For assessment of alternative complement pathway activation, zymosan A particles, derived from the yeast cell wall of *Saccharomyces cerevisiae* (Sigma) were suspended at a concentration of 1×10^9/ml in endotoxin-free Dulbecco's PBS (DPBS) and activated by double-boiling for 60 min, then washing twice in DPBS before being added to reaction tubes. The reaction tubes contained: 10 μl of zymosan particle suspension, 10 μl ethylene glycol-bis(2-aminoethyl)-*N,N,N′,N′*,-tetraacetic acid (EGTA) and $MgCl_2$ to their respective final concentrations 10 and 5 mM, 10 μl of test serum or tumor homogenate sample and 70 μl of DPBS to make a total of 100 μl volume per tube. The reaction tubes were incubated at 37 °C for 30 min to allow C3 to bind to the zymosan particles,

indicative of alternative pathway activation. The reactions were stopped with the addition of 20 mM ethylenediaminetetraacetic acid (EDTA) and the tubes set immediately on ice. The particles were centrifuged and washed in Dulbecco's PBS (DPBS)/1% FBS, then incubated on ice for 30 min with FITC-conjugated goat anti-mouse C3 antibodies (Cappel). The particles were washed and re-suspended in 0.5 ml DPBS/1% FBS prior to analysis by flow cytometry. FITC-conjugated whole molecule goat IgG served as the isotype control. This experimental design for measuring residual alternative pathway activity was adapted from an earlier described protocol [24,25].

Experimental design for classical pathway activation was loosely based on a protocol described by Molina et al. [26] and Quigg et al. [27]. Mouse carcinoma SCCVII cells grown in culture were harvested using 3 mg/ml dispase in Hanks' Mg^{2+}/Ca^{2+}-free buffer and suspended in alpha-MEM containing 2 mM $MgCl_2$ and 0.15 mM $CaCl_2$ (alpha-MEM/Mg/Ca) at a concentration of 2×10^5 per 100 μl. To each 100 μl vial of cells was added 5 μg of mouse anti-cytokeratin five and eight monoclonal antibody (Chemicon International, Temecula, CA, USA) diluted in 80 μl of alpha-MEM/Mg/Ca and 20 μl of the serum sample to be tested. The reaction was allowed to proceed at 37 °C for 60 min (with no evidence of cell lysis). The reaction was stopped by the addition of 100 μl of 100 mM EDTA to all samples and the reaction tubes set on ice immediately. All samples were washed in DPBS/1% FBS and stained for the presence of mouse C3 using FITC-conjugated goat anti-mouse C3 antibody. All flow cytometry was undertaken using the Coulter Epics Elite ESP apparatus from Coulter Electronics (Hialeah, FL).

Three groups of eight LLC tumor-bearing mice were treated with either PDT only, or PDT followed immediately by a bolus i.v. injection of either 1 mg/kg C5a receptor antagonist, a synthetic cyclic hexapeptide AcF-[OPdChaWR] [28], or 50 μg/mouse goat antibody to mouse C3a receptor (sc-20138, Santa Cruz Biotechnology, Santa Cruz, California). The isotype control for anti-C3aR was ChromePure goat IgG obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Following initial tumor ablation, the mice were monitored for tumor re-growth following treatment. Mice surviving tumor-free at 90 days following PDT were considered to be cured.

Statistical analysis. In all serum assays the data are presented as mean ± standard error of the mean (SEM). Analysis of variance using one-way ANOVA and a post hoc comparison using the LSD test were used to test for significant differences between control and treated samples. $P<0.05$ was considered a statistically significant difference. For tumor cure rate statistical significance was determined by the log-rank test. Statistical analyses were performed using the software program Statistica 6.1 (StatSoft, Inc.).

3. Results

The activity of the complement system is best evaluated by monitoring the C3 component, the key protein in the complement cascade. The kinetics of C3 level changes in the circulation of mice bearing PDT-treated LLC tumors is shown in Fig. 1(a). Compared to baselines measured on naïve (tumor-free) mice, the serum level of C3 was already significantly ($P<0.05$) elevated in mice bearing untreated subcutaneous LLC tumors. One hour after PDT treatment, C3 levels decreased significantly compared to pre-treatment levels. Levels of C3 in the circulation returned to pre-treatment levels within 3 h after PDT, followed by a significant rise at the 24 h time point. After remaining elevated close to this peak value for around 1 day, C3 levels declined but still remained significantly higher than in untreated tumor-bearing mice at 72 h after treatment. The quantity of C3 in the sera of PDT-treated mice returned to pre-treatment levels within 1 week following treatment.

Changes in serum C3 content are not necessarily reflecting the activation status of complement system. Hence, aliquots of serum samples utilized for C3 ELISA were used for the assessment of alternative and classical complement pathways of activation (Fig. 1(b)). Alternative pathway activity was determined by exposing serum samples to activated zymosan particles at physiological conditions required for alternative pathway activation to proceed including buffer containing $MgCl_2$ and the classical

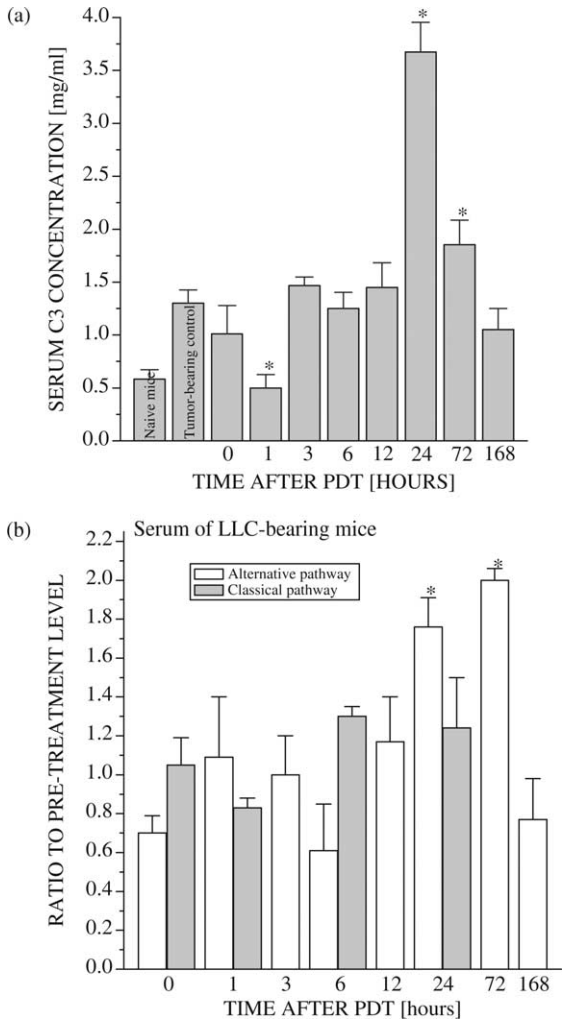

Fig. 1. C3 levels and complement activity in the sera of mice bearing PDT-treated LLC tumors. LLC tumors were treated by PDT (Photofrin 10 mg/kg plus 150 J/cm^2) and serum samples collected at various time points following treatment. The samples were used for the determination of (a) concentration of C3 content determined by ELISA, and (b) extent of engagement of alternative and classical complement activation pathways. Columns represent mean ± SEM, *$P < 0.05$ compared to tumor-bearing controls; $n = 4$.

pathway blocker calcium chelator EGTA. The potential for complement activation via this pathway remained unchanged until a significant increase at 24 h post PDT, which persisted at 72 h time point and returned to the pre-treatment level at 7 days post PDT. In contrast, complement activation potential via classical pathway, assessed by C3 deposition on antibody-opsonized tumor cells, showed no significant changes during the post-PDT treatment observation period.

In addition to these PDT-induced changes in systemic C3 levels and complement activity in host mice, C3 levels markedly increased in PDT-treated LLC tumors (Fig. 2). The results of C3 ELISA measurements in tumor homogenates reveal a significant rise evident already at 1 h after PDT that peaked 2 h later with a greater than fourfold increase in C3 tumor content compared to pre-treatment values. Very high tumor C3 levels persisted at 6 and 12 h after PDT. The values at 24 h post PDT were lower but still elevated. The tumors completely regressed after this time point and further samples could not be collected. A similar kinetics of increase in C3 protein levels in PDT-treated LLC tumors was obtained by ELISA measurement based on a different antibody, rat monoclonal raised against mouse C3 fragments that binds also intact C3 (see Section 2). The peak increase

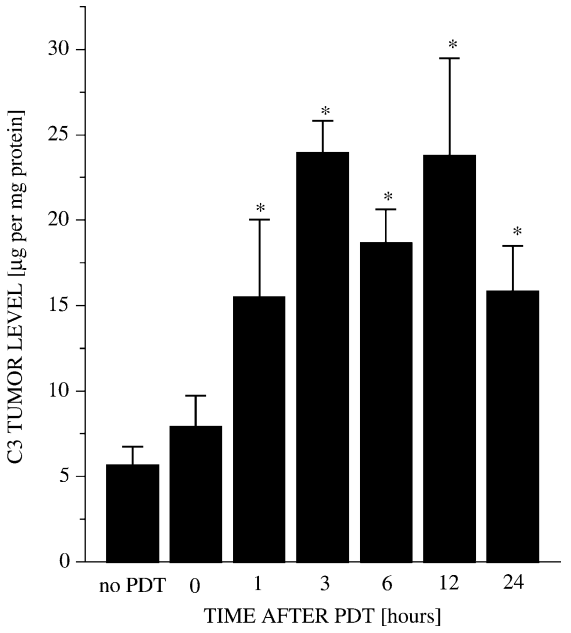

Fig. 2. C3 levels in LLC tumors following PDT. LLC tumors were treated as in Fig. 1, then excised at 0, 1, 3, 6, 12, and 24 h following treatment ($n = 4$ per time point). Immediately following excision the tumors were homogenized and the supernatants analyzed by C3 ELISA. *$P < 0.05$ compared to untreated controls. Each time point represents mean ± SEM.

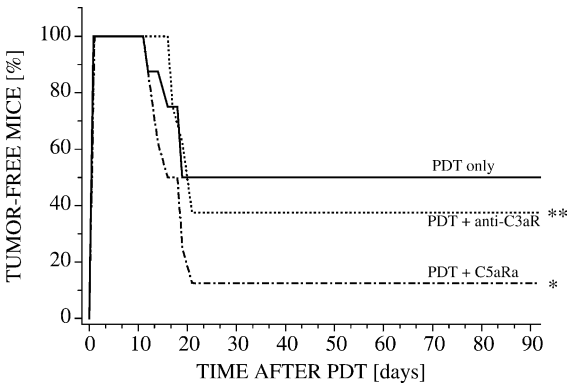

Fig. 3. The role of C3a and C5a receptors on PDT-mediated LLC tumor cures. The PDT treatment of LLC tumors was as described in Fig. 1 with the exception of light dose increased to 180 J/cm^2. Goat anti-mouse C3a receptor antibody (50 μg/mouse) and C5a receptor antagonist AcF-[OPdChaWR] (1 mg/kg) were injected i.v. immediately post PDT light delivery. Treatment groups consisted of eight mice. Following treatment, the mice were monitored for signs of tumor regrowth. No sign of tumor at 90 days post PDT qualified as a cure. *$P<0.05$ compared to PDT only; **$P<0.05$ compared to PDT only based on pooled data from two identical experiments.

in post-PDT tumor C3 levels was also about fourfold with this antibody (data not shown).

To examine directly the significance of complement activity on PDT cures, LLC tumors were treated with either PDT alone or PDT followed immediately by treatment blocking the receptors of the anaphylatoxins C3a and C5a. While PDT alone resulted in approximately 50% tumor cures, significantly decreased tumor cure rates were obtained with adjuvant treatments using either antibodies to C3aR or C5aR antagonist (Fig. 3). The use of these blockers without PDT treatment produced no significant effect on tumor growth, while post PDT treatment with nonspecific goat immunoglobulin (isotype control for anti-C3aR) produced no detectable impact on PDT response (data not shown).

4. Discussion

This report confirms and characterizes in more detail our earlier findings [5,17] that PDT treatment of solid tumors induces the activation of host's complement system. This is manifested by the increased levels of C3 protein both systemically (in the serum) and in targeted lesions, as well as in the deposition of the terminal attack complex of complement in the vascular endothelium and parenchyma of PDT-treated tumors [5].

Due to its pivotal role in the complement system, C3 is a highly versatile and multi-functional molecule whose cleavage products serve as mediators of leukocyte functions and are essential elements for further progression of the complement cascade [7]. In mice bearing PDT-treated tumors, the levels of serum C3 first show a temporal decline at 1 h after therapy (suggesting its consumption above the rate of production and release into circulation) followed later by a pronounced rise persisting between 24 and 72 h after PDT (caused presumably by the release of C3 from the liver). The latter is a classical manifestation of acute phase reaction in mice [29], with C3 involved as acute phase reactant in the regulation of systemic host response triggered by tumor-localized PDT treatment [6]. As already indicated by our preliminary findings [17] and the in vitro studies [19], PDT-induced activation of the complement cascade proceeds primarily through the alternative pathway while the classical activation pathway appears not to be significantly involved (Fig. 1(b)). This is in concordance with the known ability of the alternative pathway to become activated by C3 deposition on tissue surfaces altered by injury, which in the case of PDT treatment is caused by photooxidative damage inflicted at the targeted site.

Our analysis of C3 content in PDT-treated tumors revealed a marked increase in the levels of this protein peaking at 3 h after therapy and remaining highly elevated until 24 h post PDT. After this time period there was no viable tumor tissue available for analysis, as tumors become ablated and eventually around 30% of them are cured by the PDT dose chosen in our experiments.

A specific circumstance in immunodetection of C3 is that the same epitopes are present in intact native molecule and its active fragments (C3b lacks only a small C3a fragment of C3). Hence, polyclonal goat anti-mouse C3 antibody used in our ELISA measurements could bind to C3 fragments in addition to native C3. However, it is unlikely that this distorted the results of our experiments focused on the measurement of fluid phase C3 in serum samples and tumor homogenates since C3 fragments localize on solid

surfaces. Nonetheless, basic findings obtained in our experiments were independently confirmed with a different antibody (rat monoclonal) that reacts preferentially with the cleaved C3 fragments [23].

Since there was no significant rise in serum C3 levels until 24 h post PDT, the source of increased C3 levels in the treated tumors at earlier time points could be tumor-localized production of this protein. This is supported by our findings of up-regulated expression of key complement genes (C3, C5 and C9) in PDT-treated LLC tumors [30], which we suggest to take place mainly in immune cells (particularly monocytes/macrophages) invading the tumor after PDT. Tumor-localized production of complement proteins triggered by PDT treatment, which may have an important role in the mechanism of destruction of cancerous lesions treated by this therapy, is in focus of our ongoing investigation.

Relevance of the induced engagement of the complement system is illustrated by the reduction in LLC tumor cure rates achieved through blocking anaphylatoxin C3a or C5a receptors immediately post PDT light delivery (Fig. 3). Functionally blocking the C3a receptor could involve preventing down-stream signaling that leads to mast cell degranulation and coincidental histamine release. Histamine is a potent vasodilator released by mast cells that accumulate in PDT-treated tumors [31] which also acts to activate neutrophils, a cell type required for efficient PDT [31–33]. Blocking C3a-C3aR and C5a-C5aR interactions and consequently signal transduction pathways mediated by these receptors with down-stream activation of MAP kinases responsible for the production of various cytokines [34] could inhibit neutrophil chemotaxis and cytotoxic activity by decreased neutrophil aggregation, degranulation, and superoxide production [35]. This action of complement anaphylatoxins on sequestration and activation of neutrophils and other myeloid effectors in PDT-treated tumors is supported by the results of our investigation of mediators involved in PDT-elicited neutrophilia [5].

In addition, complement activity is a major element of innate immune reaction involved in controlling the development of tumor-specific adaptive immune response that was shown to contribute to PDT-mediated tumor cures [3]. Fragments of C3 bound to tumor-derived proteins are known to greatly facilitate tumor antigen presentation to T and B cells [9], while various complement proteins also exert co-stimulatory action on T and B cells [7,36,37]. Approaches based on exploiting the extensive involvement of complement system in tumor response to PDT by its further amplification employing adjuvant treatments with complement activating agents seem to offer promising advances for improving the therapeutic effect of PDT [38].

In conclusion, activation of the complement system by Photofrin-based PDT in the LLC tumor model appears to reflect a natural response of innate immunity engaged to maintain homeostasis following acute tumor injury inflicted by PDT. The results of our study suggest that this response is of an overall benefit toward long-term tumor control by PDT applied to the treatment of solid tumors.

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