Photodynamic Therapy of Murine Mastocytoma Induces Specific Immune Responses against the Cancer/Testis Antigen P1A

Pawel Mroz, Fatma Vatansever, Angelika Muchowicz, and Michael R. Hamblin

Abstract
Photodynamic therapy (PDT) involves the intravenous administration of photosensitizers followed by illumination of the tumor with visible light, leading to local production of reactive oxygen species that cause vascular shutdown and tumor cell death. Antitumor immunity is stimulated after PDT because of the acute inflammatory response that involves activation of the innate immune system, leading to stimulation of adaptive immunity. We carried out PDT using benzoporphyrin derivative and 690-nm light after 15 minutes, in DBA/2 mice bearing either the mastocytoma, P815, which expresses the naturally occurring cancer/testis antigen P1A, or the corresponding tumor P1.204 that lacks P1A expression. Tumor cures, significantly higher survival, and rejection of tumor rechallenge were obtained with P815, which were not seen with P1.204 or seen with P815 growing in nude mice. Both CD4 and CD8 T cells had higher levels of intracellular cytokines when isolated from mice receiving PDT of P815 tumors than P1.204 tumors and CD8 T cells from P815-cured mice recognized the peptide epitope of the P1A antigen (LPYLGWLVF) using pentamer staining. Taken together, these findings show that PDT can induce a potent antigen- and epitope-specific immune response against a naturally occurring mouse tumor antigen.

Introduction
Photodynamic therapy (PDT) involves the intravenous administration of a photosensitizing drug followed by activation of the drug with nonthermal light of a specific wavelength (1). The anticancer effect of PDT is a consequence of a low-to-moderately selective degree of photosensitizer uptake by proliferating malignant cells, direct cytotoxicity of reactive oxygen species and severe vascular damage that impairs blood supply to the treated area. The biologic effects of PDT are considered to be limited to the particular tissue areas exposed to light. In addition, PDT leads to activation of tumor-directed, systemic immune responses (2). PDT as a treatment procedure has been accepted by the U. S. Food and Drug Administration for use in endobronchial and endoesophageal cancer and also as a treatment for premalignant and early malignant lesions of skin (actinic keratosis), bladder, breast, stomach, and oral cavity (3).

PDT is thought to be particularly effective at activating an immune response against a locally treated tumor by effectively engaging both innate and adaptive arms of the immune systems in the host responses to cancer (2). PDT stimulates the release or expression of a mixture of proinflammatory and acute phase response mediators from the treated site (4–6). The local trauma that threatens the integrity of tumor microenvironment is readily recognized by the body and proinflammatory mediators are subsequently released to preserve homeostasis. As a result, a powerful acute inflammatory response, involving the accumulation of neutrophils and other inflammatory cells in large numbers at the PDT-treated site, is triggered (7, 8). The activation of the complement system has in particular emerged as a powerful mediator of PDT anti-tumor effects (9–13). Complement not only acts as a direct mediator of inflammation but also stimulates cells to release secondary inflammatory mediators, including cytokines IL-1β (interleukin-1β), TNF-α, IL-6, IL-10, G-CSF, thromboxane, prostaglandins, leukotrienes, histamine, and coagulation factors (4).

To attack and eliminate malignant lesions, the immune system must use CTL that recognize tumor antigens. These are molecules that stimulate specific immunity and are presented by MHC class I molecules on the surface of target tumor cells (14). The immune response is triggered upon epitope recognition by specific T cells through T-cell receptors, after which the activated T cells exhibit cell proliferation (clonal expansion), functional differentiation, and develop effector function (15). Epitopes are groups of amino acids that are generally significantly smaller than the antigens they are derived from, and there is a possibility of a single antigen containing numerous different epitopes.
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(although not all of them may be available for immune response). Among various tumor antigens discovered to date, three broad classes have been defined (16, 17). One is encoded by cancer–testis genes that are expressed in various tumors, but not in normal tissues (18, 19); a second class is composed of differentiation antigens of the melanocytic lineage, which are present on most melanomas but also on normal melanocytes (20–22); and a third class comprises antigens that appeared because of mutations in genes commonly expressed in all tissues or genes introduced to the tumor cell genome by viruses (23–27). Immunotherapy uses various strategies to target those tumor antigens with significant degrees of success in both preclinical studies and early-phase clinical trials (28, 29).

The molecular identity of a number of both mouse and human tumor antigens has been well defined (16), the peptide sequence displayed in MHC-I molecules is known, and specific CTL lines that kill the tumor cells expressing these antigens have been produced. Furthermore, there have been efforts made to introduce defined tumor antigens into mouse tumor cell lines to have reproducible models to study. These model antigens are usually "foreign" proteins such as β-galactosidase from bacteria (30), ovalbumin from chicken eggs (31), and influenza hemagglutinin from a virus (32). Our laboratory was the first to recognize the role of tumor antigens in antitumor immune response after PDT (33). Recently, we also showed that PDT could induce a highly potent antigen-specific, systemic immune response capable of causing regression in distant established tumors expressing a model tumor antigen (34).

However successful this approach may be in laboratory, artificial model tumor antigens are not clinically relevant as they cannot truly reproduce the properties of naturally occurring tumor antigens. Therefore, in this study we examined antigen-specific PDT–induced antitumor immune response in a more clinically relevant model by using a naturally occurring cancer antigen, namely, the mouse homolog of human melanoma-associated antigen (MAGE)-type antigen termed P1A. P1A is the best-described nonmutated mouse cancer–testis tumor antigen and has been used for many immunotherapy studies (35–37). We decided to use this cancer–testis antigen, as it is well studied and established in the literature, and is expressed in testis as well as in cancers, but not at all or at very low levels in other tissues (18, 19, 38, 39). The novelty and significance of this study is that, in addition to using a tumor model that expresses a naturally occurring tumor antigen, thus making the study clinically relevant, we used nude mice and pentamer staining in in vivo studies to show the epitope-specific nature of the immune response.

Materials and Methods

Cell lines

P815 wild-type (parental) and P1.204 (P815 derived) cell lines were a gift from Dr. Thomas Gajewski from the Department of Pathology, University of Chicago (Chicago, IL). Cells were cultured in RPMI medium with 1-glutamine and NaHCO₃ supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL; all from Sigma) at 37°C in 5% CO₂ in 75 cm² flasks (Falcon, Invitrogen).

Reverse transcription PCR for P1A

Total RNA from P815 and P1.204 cells was prepared using the RNeasy Mini Kit (Qiagen Inc.). Reverse transcription was done using a Reverse Transcription System at the suggested conditions (Invitrogen). One microgram of total RNA was used in each reaction. PCR was done using the following primers and thermal cycle conditions: 5V-CCCTTCTATGACCTCACT- CATGG-3V [glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward], 5V-CTGCTTCCACACCTTTTGATGTC-3V (GAPDH reverse), 5V CCGAATTCTGTCGAATGCCTCATTAA- CAAAGAA-3V (P1A forward), and 5V-CTGCTAGATGGCAA- CTGCATTCAAGGTTGAG-3V (P1A reverse). 94°C × 5 minutes (94°C × 1 minute, 58°C × 1 minute, and 72°C × 1 minute) × 30 cycles, and 72°C × 7 minutes. PCR products were separated on 1% agarose gels and visualized by ethidium bromide techniques.

Flow cytometry analysis of MHC class I molecule levels

P815 and P1.204 cells were fixed and incubated at room temperature (22°C–25°C) for 1 hour with PE-conjugated anti-H2-Dd antibody (BD Pharmingen). PE isotype antibody and unstained cells were used as controls. Cells were washed twice in 1 mL of PBS and analyzed on FACSCalibur (BD).

Photosensitizer and light source

Liposomal benzoporphyrin derivative mono acid ring A (verteporfin for injection, BPD, QLT Inc.) was prepared by diluting the powder to a concentration of 0.3 mg/mL in sterile 5% dextrose. A 1-W 690-nm diode laser (B&W Tek Inc.) was coupled into a 0.8-mm diameter fiber and a lens was used to obtain a uniform spot.

In vitro PDT

Of note, 5,000 P815 and P1.204 cells were plated per well in 96-well plates and incubated for 1 hour with 200 nmol/L BPD. After incubation the medium was replaced with 200 µL of fresh medium and PDT was conducted. Of note, 690 nm laser light dose was varied and fluences of zero (dark toxicity) to 2 J/cm² were delivered at an irradiance of 50 mW/cm² to each well separately (4 wells represented a group). Controls entailed cells with no treatment and cells with light alone at the highest fluence or with photosensitizer alone. At the completion of the illumination, the plates were returned to the incubator for 24 hours before initiating further studies. A 4-hour MTT colorimetric assay was used that measures mitochondrial reductase activity. This assay correlates well with colony-forming assays as a measure of cell viability, as has been described previously. The absorbance for MTT assay was read at 560 nm.

Animal tumor model

All experiments were carried out according to a protocol (number 2004N000001) approved by the Subcommittee on Research Animal Care (Institutional Animal Care and Use Committee) at Massachusetts General Hospital (Boston, MA) and were in accord with NIH guidelines. DBA/2 and BALB/c Nu/Nu mice (6–8 weeks old) were purchased from Charles River Laboratories. Mice were inoculated with 350,000 cells.
subcutaneously into the depilated right thigh. Two orthogonal dimensions (a and b) of the tumor were measured two to three times a week with vernier calipers. Tumor volumes were calculated as follows: volume = \(4\pi/3 \times (a+b)/4^3\). When tumors reached a diameter of 5 to 7 mm (9 days after inoculation), PDT was conducted.

**PDT and tumor response**

Tumor-bearing mice were anesthetized with intraperitoneal injection of 87.5 mg/kg of ketamine and 12.5 mg/kg xylazine and BPD (1 mg/kg in 5% dextrose solution) was administered intravenously via the supraoculat plexus. Control mice received 5% dextrose only. Fifteen minutes after BPD injection illumination was conducted. A total fluence of 120 J/cm\(^2\) was delivered at a fluence rate of 100 mW/cm\(^2\) with a spot diameter of 1 cm covering the tumor and a border of 2 to 3 mm normal tissue. Mice were allowed to recover in an animal warmer after PDT and showed no adverse effects of the treatment. The mice were sacrificed when any of the tumor diameters exceeded 1.5 cm.

**Rechallenge**

Mice surviving 90 days after PDT were subsequently rechallenged with 350,000 cells of P815 or P1.204 in the contralateral thigh and monitored for another 60 days. Naïve control mice were inoculated with the same sample of cells to confirm tumorigenicity.

**Lymphocyte preparation**

Inguinal lymph nodes and spleens from mice in all experimental groups were harvested 5 days after PDT or on day 14 of experiment in control mice, homogenized and passed through a 70-μm mesh nylon cell strainer (BD Falcon) to make single-cell suspensions that were used for further experiments. For each study group we used \(n = 5\) mice to extract the cells that were pooled.

**Adoptive transfer**

A total of \(2 \times 10^6\) of isolated lymph node cells from control and PDT-treated DBA/2 P815 tumor–bearing mice were intravenously injected into BALB/c Nu/Nu mice (6–8 weeks old) immediately after inoculation with P815 tumors. Mice were monitored for tumor growth and survival as described above.

**Intracellular cytokine production in splenocytes**

We used the Mouse Intracellular Cytokine Kit (BD Biosciences) to measure the levels of cytokines (TNF-α and IL-2) in the isolated splenocytes. The assay was conducted according to the manufacturer’s instructions. In brief, single-cell suspensions of splenocytes were subsequently used in the primary activation culture to stimulate production of cytokines for 4 hours using Leukocyte Activation Cocktail. The splenocytes obtained from the stimulation culture were subsequently suspended in BD Pharmingen Stain Buffer and the PE-conjugated anti-IL-2 or anti-TNF-α antibodies were added for overnight incubation at 4°C. In addition, FITC-conjugated anti-CD4 or anti-CD8 antibody was added. The controls included PE and FITC isotype antibodies, unstained control as well as blocking antibody. Manufacturer provided MiCK-1 cytokine positive control cells were used as positive controls.

**Pentamer staining**

The R-PE–labeled Pro5 MHC pentamer for P1A epitope (H-2L\(^d\), LPYLGLWLF) was a generous gift from ProImmune. The assay was conducted according to the manufacturer’s instructions (www.proimmune.com). Briefly, \(2 \times 10^6\) of isolated lymphocytes from P815 control and PDT-treated mice were washed in a wash buffer and suspended in 50 μL aliquots. Next 10 μL of labeled pentamer was added and incubated in room temperature (22°C–25°C) for 10 minutes. The samples were washed in 2 mL of wash buffer and costained with anti-CD8 FITC antibody for 20 minutes. Washed samples were subsequently used for a fluorescence-activated cell sorting (FACS) analysis (FACScalibur, BD Biosciences). FACS scattergrams were analyzed by first gating for size and CD8 expression on FL1 versus FSC dot-plot and next by replotting CD8-positive cells on FL1 versus FL2 dot-plot to assess percentage of CD8 pentamer–double positive cells. Two separate assays were conducted.

**Statistics**

All values are expressed as the mean ± SD and all experiments were repeated at least twice with comparable results. Differences between means were tested for significance by one-way ANOVA. The Fisher exact test was used for proportions of animals surviving between two groups. Survival analysis was conducted using the Kaplan–Meier method and a log-rank test. \(P\) values of less than 0.05 were considered significant.

**Results**

**In vitro experiments**

We used a pair of previously described tumors that includes P1A antigen–positive mouse mastocytoma P815 and a cell line derived from P815, P1A antigen–negative P1.204. We measured the expression of the P1A gene in both cell lines \(in vitro\) as well as \(ex vivo\) in tumors (Fig. 1A and B) as well as the levels of MHC class I molecules (Fig. 1C). In addition, we compared the \(in vitro\) susceptibility with PDT (Fig. 1D). The BPD-mediated PDT was somewhat more effective against P1.204 cells, most probably due to the fact that P1.204 cells grow as a suspension, making them more susceptible to PDT treatment. Nevertheless, the result of this \(in vitro\) experiment suggests that the much better response seen \(in vivo\) to PDT of P815 tumors than the response seen with P1.204 tumors is not due to a possible inherent higher sensitivity of P815 cells to PDT. Rather, the reverse is true and P1.204 cells are inherently more sensitive to PDT.
The occurrence of cancer metastases from occurring both in control and in PDT-treated immunocompetent mice the immune response prevents the capability of metastasis; however, it is strongly likely that in the absence of the immune system, we carried out cross-challenge studies. In this set of experiments, mice cured of P1A antigen–positive tumors P815 that survived tumor free for 90 days after PDT were inoculated with P1A antigen–negative P1.204 cells and vice versa. One of 4 mice rechallenged with the P1.204 tumors rejected the rechallenge and survived more than 60 days, whereas all tumors progressed when mice received P815 tumors.

**Adaptive immune system is necessary for PDT antitumor effects**

To corroborate our results indicating that PDT antitumor effects are indeed mediated by the activation of the immune system, we repeated the experiments with P1A antigen–positive P815 tumors in immunocompromised BALB/c Nu/Nu mice. PDT treatment produced a local response similar to that observed in immunocompetent DBA/2 mice; however, there was no survival advantage over nontreated controls and no permanent cures were observed (Fig. 3A). The difference between PDT of P815 tumors in DBA2 mice and PDT in nude mice was highly significant ($P < 0.0001$ and, interestingly, there was also a significant difference between survival of no treatment controls in nude and DBA2 mice ($P = 0.012$). This observation shows that there is an immune response against P815 in untreated DBA2 mice that is insufficient to save any mice from death but does give a slower growth rate than in nude mice.

To provide additional evidence for the involvement of the immune system in antitumor PDT effects, we also carried out adoptive transfer experiments (Fig. 3B). In this set of experiments, we initially treated P815 tumors growing in immunocompetent DBA/2 mice with PDT, and 5 days later we harvested the lymph node cells and transferred them intravenously into the immunocompromised BALB/c Nu/Nu. The control mice received lymph node cells from control, nontreated P815–bearing DBA/2 mice. Before lymph node cell transfer, the immunocompromised BALB/c Nu/Nu in all experimental groups were inoculated with P815 tumors. The adoptive transfer results of these experiments are shown in Figure 3C. In the adoptive transfer experiments, both the tumors in immunocompetent and immunocompromised mice were inoculated with P815 tumors. The adoptive transfer results of these experiments are shown in Figure 3C. In the adoptive transfer experiments, both the tumors in immunocompetent and immunocompromised mice were inoculated with P815 tumors.
transfer significantly delayed the development and progress of P815 tumors and led to significant survival advantage.

These results provide strong evidence that the curative effects observed in the case of P1A antigen–positive P815 tumors were mediated by PDT activated, antigen-specific, and transferable immune response, and that the lack of functional adaptive immune system abrogates this effect.

PDT treatment leads to increase in intracellular production of cytokines in splenocytes from mice bearing antigen–positive tumors

We compared to what extent the local PDT treatment of P1A antigen–positive and -negative tumors leads to activation of the immune system by measuring cytokines secreted in populations of CD4 T cells. These experiments revealed that PDT...
treatment of P1A antigen–positive P815 (but not P1A antigen–negative P1.204) tumors led to a 4-fold increase in TNF-α and more than 3-fold in IL-2 levels (Fig. 4), suggesting the active involvement of the T-helper cell type 1 (Th1) arm of adaptive immune response. To further evaluate the PDT ability to activate T cells, we measured and compared the levels of secreted cytokines by CD8+ T cells in control and PDT-treated splenocytes from mice bearing P1A antigen–positive P815 tumors. As can be seen in Fig. 5A, PDT led to a more than 3-fold increase in the levels of both TNF-α and IL2. These results strongly suggest that PDT of P1A antigen–positive tumors leads to strong activation of the Th1 arm of the adaptive immune system as well as to activation of cytotoxic CD8+ T cells.

Pentamer staining reveals development of P1A epitope–specific CD8+ T cells after PDT

To establish that PDT can indeed lead to recognition of a specific peptide epitope derived from the tumor antigen P1A, we used pentamer staining. The lymph node cells isolated from control or PDT-treated mice bearing P1A antigen–positive P815 tumors were incubated with P1A epitope (LPYLGWLVF)–specific pentamer and costained for CD8 marker (Fig. 5B). There was a significant difference (P < 0.01) between binding of P1A pentamer by CD8-positive T cells isolated from control or PDT-treated mice. These results strongly suggest that PDT does indeed induce recognition of MHC class I–bound epitope derived from the P1A antigen and provide additional evidence to support the notion that the expression of tumor antigen makes a significant difference in the final outcome of PDT of tumors.

Discussion

The P815 tumor is perhaps the best-studied mouse tumor in the immunology and immunotherapy fields and the antigens expressed by P815 were the first mouse tumor antigens to be identified. However, there have been no studies reported with
P815 tumors and PDT looking at the antigen-specific immune response. We designed a study that used the mastocytoma P815 tumor cells syngeneic to DBA/2 mice that are expressing P1A (a MAGE-type mouse antigen) in parallel with P1.204 tumor cells (a P815 variant, negative for P1A) to investigate the effectiveness of PDT in this very clinically relevant setting. These tumor cells have been widely used in murine tumor immunologic response studies (40–42) and at least four distinct antigens (P815: AB, C, D, and E) characterized by five epitopes (P1A, P1B, P1C, P1D, and P1E), capable of inducing CTL response, have been identified, with the main component of the CTL response against the P815 tumor being targeted against P815AB and P815E (43). The P815 antigen shares many characteristics found in human TAA genes (particularly those belonging to the MAGE family in melanomas and other tumors; ref. 44), and these antigens are not expressed in most adult tissues, with the exception of testis and placenta (37). The P1.204 is a cell line derived from an immune system escape variant P815 (42) that has lost the P815AB antigen but retains the P815E antigen. The response to PDT (of both tumors) is consistent with these factors.

We found that the two tumors had differences that were manifest in how the cells grew in vitro. P815 cells grew as adherent monolayers, whereas P1.204 cells grew as suspensions. This difference in the in vitro growth pattern probably accounted for differences that we found in the susceptibility of the two lines to PDT with liposomal BPD as well. As it happens, the suspension P1.204 cells were more susceptible to PDT than the adherent P815 cells, and this meant that the greater response to PDT we observed of P815 tumors in mice could not be attributed to an inherently greater susceptibility of the tumor cells (rather the reverse). The initial reaction of both tumor types to PDT was similar with a black eschar developing at the site of the tumor and accompanied by marked tumor shrinkage. The subsequent course of the mice, however, was very different. The majority of the mice with PDT-treated P815 tumors showed a regression and continued absence of the tumor that lasted up to 90 days of observation. The remaining 4 of 22 mice showed a local recurrence of the tumor, leading to sacrifice due to progressive growth between days 44 and 57. On the other hand, the 21 mice with PDT-treated P1.204 tumors did not fare as well. The first mouse needed to be sacrificed because of metastatic disease at day 31 and this continued to be necessary at intervals until only 5 of 21 mice remained alive and healthy at day 66. The difference in the response between the two tumor types was hypothesized to be due to immune response, especially as P1.204 cells were more susceptible to PDT.

To confirm the involvement of the mouse immune system in the long-term response to PDT in these models, we rechallenged the cured mice with the identical tumor from which they were originally cured. Only mice cured from P1A antigen–positive P815 tumors reliably rejected the rechallenge, whereas 100% of the naive mice injected with either cell line grew tumors. This observation implies that in the case of P1A antigen–positive P815 tumors, a strong immunity developed after PDT sufficient to prevent tumor growth when a tumorigenic dose of cells was reinjected.

The P815 cell line (apart from P1A) expresses additional known antigens such as P1B and P1E and, presumably, the T cells that arise after PDT could recognize either P815AB or P815E epitopes. If this were the case, then mice cured of the P815 tumor could also be capable of rejecting a challenge with P1.204 tumors. Our hypothesis proved to be correct only in a limited number of mice, as seen in the cross-challenge study. The phenomenon of P815 tumors escaping a successful initial immune response is well documented (42, 45–47). For the P1A peptide antigen, LPYLGWLVF expressed in P185 progression was reported to occur due to antigenic loss (48).
PDT Induces Immune Response to Natural Tumor Antigen

In the ex vivo set of studies, we asked the question to what extent does PDT of P815 mastocytoma induce host antitumor immune response and whether the P1A antigen is recognized by T cells before and after PDT. Brichard and colleagues showed (49) that T cells isolated from DBA/2 mice bearing-growing P815 tumors primarily recognized either antigens AB or CDE, but not both. We measured, therefore, the levels of cytokines secreted by both CD4+ and CD8+ T cells to compare the effects of PDT treatment and we showed that PDT of P1A antigen–positive tumors led to marked increase in levels of TNF-α and IL-2. In addition, we were able to identify a population of CD8+ T cells able to effectively recognize an LPYLGLWLVF epitope from the P1A antigen. Finally, we were able to show that the lack of an adaptive immune system in nude mice could completely abrogate the antitumor effectiveness of PDT in mice bearing P1A antigen–positive P815 tumors. Moreover, survival of nude mice with P815 tumors could be significantly prolonged by adoptive transfer of activated lymph node cells isolated from immunocompetent mice with PDT-treated P815 tumors.

Our findings presented here are in accordance with a study published by Kablingu and colleagues (50) as well as our article (34), where we showed for the first time that PDT can lead to the development of systemic, antigen-specific immune response toward tumors expressing the model tumor antigen β-galactosidase. The results derived from this study, however, may have direct utility of the PDT application in clinical cancer therapy. First, if a tumor antigen is constitutively expressed in tumor tissues, PDT may be ultimately more successful in the patient population with tumors positive for that particular antigen. Second, although PDT of cells in vitro has been shown to generate anticancer vaccines that are superior to other cytotoxic strategies (1), the role of tumor antigens has never been addressed in this setting. Third, even though many solid tumors show heterogeneous expression of tumor antigens, recent data suggest that de novo induction of tumor antigens in solid tumors may represent a novel means to break tumor escape mechanisms (51). Fourth, the combination of PDT with various strategies that enhance the expression of tumor antigens and their presentation by MHC-I molecules may extend the benefits of this therapy to those cancers that are otherwise untreatable.

Collectively, the data presented in this study using tumors expressing clinically relevant, naturally occurring tumor antigen P1A, a murine homolog of cancer testis antigen, provide additional, strong evidence that the antigen expression in PDT-treated tumors leads to better treatment outcomes and should be considered as a positive factor in patient prognosis and clinical trials.

Disclosure of Potential Conflicts of Interest
P. Mroz is a consultant/advisory board member of Dermira. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: P. Mroz, M.R. Hamblin
Development of methodology: P. Mroz, M.R. Hamblin
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Mroz, M. Muchowicz
Analysis and interpretation of data (e.g., statistical analysis, biosatistics, computational analysis): P. Mroz, A. Muchowicz, M.R. Hamblin
Writing, review, and/or revision of the manuscript: P. Mroz, F. Vatansever, M.R. Hamblin
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Mroz
Study supervision: M.R. Hamblin

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