Journal of Innovative Optical Health Sciences
Vol. 6, No. 4 (2013) 1350039 (7 pages)

DOI: 10.1142/S1793545813500399

World Scientific
www.worldscientific.com

ANTI-TUMOR RESPONSES INDUCED BY LASER IRRADIATION AND IMMUNOLOGICAL STIMULATION USING A MOUSE MAMMARY TUMOR MODEL

FEIFAN ZHOU*, XIAOSONG LI†, SHENG SONG*,
JOSEPH T. ACQUAVIVA III‡, ROMAN F. WOLF§, ERIC W. HOWARD¶
and WEI R. CHEN‡,‖

*MOE Key Laboratory of Laser Life Science &
Institute of Laser Life Science
South China Normal University
Guangzhou, P. R. China

†Department of Oncology
The First Affiliated Hospital of Chinese PLA General Hospital
Beijing 100048, P. R. China

‡Department of Engineering and Physics
University of Central Oklahoma
Edmond, Oklahoma, USA

§Department of Comparative Medicine, College of Medicine
University of Oklahoma Health Sciences Center
940 S.L. Young Blvd., Oklahoma City
Oklahoma, USA

¶Department of Cell Biology
University of Oklahoma Health Sciences Center
940 S.L. Young Blvd., Oklahoma City
Oklahoma, USA
‖wchen@uco.edu

Received 4 May 2013
Accepted 19 August 2013
Published 1 October 2013

Anti-tumor immunological response induced by local intervention is ideal for treatment of metastatic tumors. Laser immunotherapy was developed to synergize photothermal interaction with immunological stimulation for cancer treatment. Using an infrared laser, indocyanine green (ICG, as a light absorbing agent), and glycated chitosan (GC, as an immunostimulant), laser immunotherapy has resulted in tumor suppression and anti-tumor responses in pre-clinical

as well as clinical studies. To further understand the mechanism of laser immunotherapy, the effects of laser and GC treatment without specific enhancement of laser absorption were studied. Passive adoptive immunity transfer was performed using splenocytes as immune cells. Spleen cells harvested from tumor-bearing mice treated by laser + GC provided 60% immunity in naive recipients. Furthermore, cytotoxicity and TNF-α secretion by splenocytes from treated mice also indicated that laser + GC induced immunity was tumor-specific. The high level of infiltrating T cells in tumors after laser + GC treatment further confirmed a specific anti-tumor immune response. Therefore, laser + GC could prove to be a promising selective local treatment modality that induces a systemic anti-tumor response, with appropriate laser parameters and GC doses.

Keywords: Laser immunotherapy; glycated chitosan; anti-tumor immunity; metastatic cancers.

1. Introduction

Laser immunotherapy (LIT) was developed to combine photothermal interaction with immunological stimulation to treat metastatic tumors.[1,2] Its selective photothermal effect serves as the first line of assault on the tumor, using a combination of a near-infrared laser irradiation and a light-absorbing dye.[3] An immunological stimulant is used concurrently to induce immunological responses. A new compound, glycated chitosan (GC), derived from chitosan by attaching galactose molecules to the chitosan molecules, was developed as a novel immunostimulant.[1,4] GC, as a water soluble compound, is more suitable for *in vitro* and *in vivo* biomedical applications.[5,6] LIT using dye-enhanced thermal interaction and GC has been proven to be highly effective in the treatment of metastatic tumors in animal studies and pre-clinical studies.[7–11]

The immune system can respond to cancer cells by reacting against tumor-specific antigens or tumor-associated antigens. Previously, antibodies binding to the plasma membrane of both living and preserved tumor cells were detected in sera from LIT-cured tumor bearing rats using histochemical analysis.[2] The induction of tumor-selective antibodies were also revealed by western blot analysis performed in sera from rats that were successfully treated by LIT.[2] Further investigation on how immune system responds to LIT is crucial in understanding the mechanism of LIT and in effectively applying this new therapy for clinical use. Specifically, the functions of different components in LIT need to be clarified to further improve the efficacy of LIT.

The work presented here is an *in vivo* study designed to understand the immunological mechanisms of LIT in the treatment of murine mammary tumors, using a combination of a 980 nm laser irradiation and GC stimulation. Splenocytes activation and T cells infiltration were analyzed to understand the immunological response induced by laser + GC treatment.

2. Materials and Methods

2.1. *Animal tumor model*

EMT6 cells (1×10^6) in a 100-μl solution were injected into the flank region of female Balb/c mice, age 6–8 weeks. Animals were used in experiments 7 to 10 days after tumor cell inoculation, when the tumors reached a size of approximately 300 mm^3.

2.2. *Laser + GC treatment of animal tumors*

Tumor-bearing mice were divided into four different treatment groups (10 mice/group). A solution of 100 μl containing 5 mg/ml (25 mg/kg) GC was directly injected into the center of each tumor, 2 h before irradiation with a 980-nm laser. The light was delivered to the tumor noninvasively using a fiber optic delivery system. The power density at the treatment area, which encompassed the tumor and 0.5 to 1 cm of the surrounding skin, was 0.75 W/cm^2 for a treatment duration of 10 min. During laser irradiation, mice were anesthetized with an intraperitoneal injection of pentobarbital sodium and were restrained in a specially designed holder. After treatment, the mice were observed daily and the tumors were measured every other day for a period of 100 days.

2.3. *Determination of spleen cell cytotoxicity after laser + GC treatment*

For cell death statistic analysis *in vivo*, the tumors were harvested 3 h after treatment and physically dissociated. Single cells in suspension were stained with Annexin-V-FITC (Becton Dickinson, Mountain View, CA, USA), and analyzed by FACScanto II flow cytometry (Becton Dickinson) with excitation at 488 nm. Fluorescent emission of FITC was measured at 530 nm.

Seven days after treatment of the EMT6 tumors in mice, mouse spleens were harvested and co-cultured with mitomycin-C-treated EMT6 cells stained with calcein acetoxymethyl. Stimulated effector cells were tested for cytolytic activity against EMT6 cells five days later by fluorescence detection of calcein acetoxymethyl in the tumor cells, which were excited with 488-nm light and detected with emission at 530 nm using a microplate reader (INFINITE M200). The cytolytic effect of splenocytes was expressed as % specific lysis, defined as (Emcon-Emtre)/Emcon $\times$ 100% (where Emcon was the emission intensity at 530 nm of control cells and Emtre was the emission intensity at 530 nm of treated cells).

2.4. *Adoptive immunization*

Seven days after treatment, the treated mice were terminated and their spleens were dissected free of fat. Single cell suspensions were prepared by mechanical disruption of mouse spleens into medium with 10% FCS. Spleen cells and viable tumor cells were counted on a hemocytometer before admixing. The admixture had a 500:1 spleen cell to tumor cell ratio. Naive mice were inoculated with a 0.2-ml admixture containing 5×10^7 spleen cells and 10^5 tumor cells.

2.5. *Infiltration of T cells to tumor sites after laser + GC treatment*

Seven days after treatment, the treated mouse tumors were excised, dissociated into single cell suspensions and labeled with FITC-conjugated anti-CD4 or phycoerythrin-conjugated anti-CD8 mAb (eBioscience) and analyzed by FACS with excitation at 488 nm. Fluorescent emission of FITC was measured at 530 nm and that of phycoerythrin complexes at 575 nm.

2.6. *Detection of TNF-α secreted by spleen cells due to laser + GC treatment*

To detect TNF-α secretion by splenocytes from treated mice, harvested splenocytes were incubated with mitomycin C-treated tumor cells in 24-well tissue culture plates. After incubation of 48 h, the supernatants were collected for ELISA detection.

3. Results

3.1. *In vivo tumor killing effects of laser + GC*

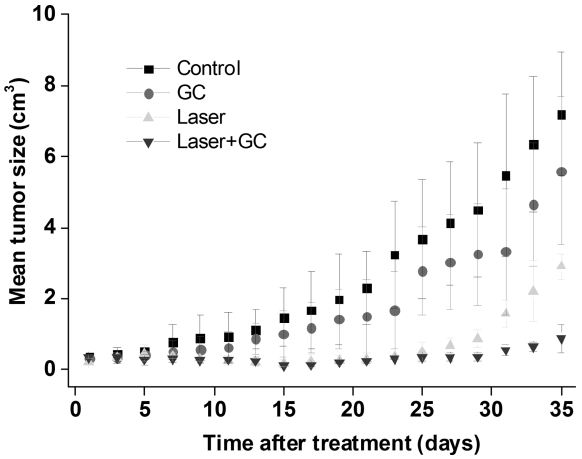
After the tumor size reached approximately 300 mm^3, the animals were divided into four different groups (10 mice per group) and treated by laser, GC or laser + GC. After treatment, the mice were observed daily and the tumor volume was measured using a caliper every other day. The mice treated by laser or laser + GC had an average tumor burden noticeably smaller than that of the control mice [see Fig. 1(a)]. To determine tumor destruction by the treatment, single cells dissociated from treated tumors were analyzed by FACS. Laser or laser + GC treatment induced about 50% cell death rate [see Fig. 1(b)].

3.2. *Anti-tumor immune effect of laser + GC treatment*

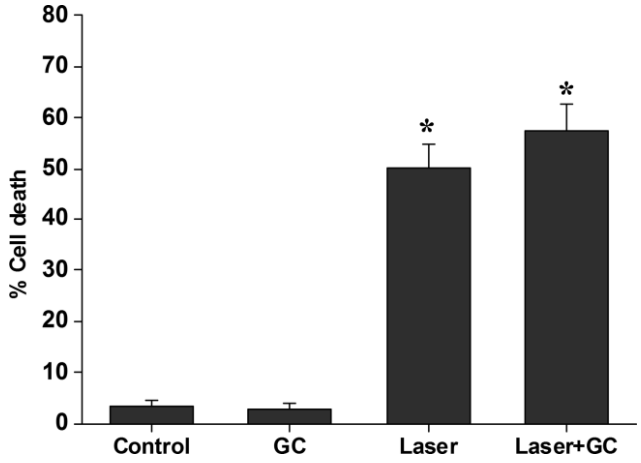
Splenocytes from treated mice were harvested as immune cells, and were admixed with viable tumor cells at a ratio of 500:1. Naive mice were inoculated by 10^5 viable tumor cells with 5×10^7 splenocytes harvested from mice of different treatment groups. Figure 2 shows the survival rates of mice inoculated with the mixture of splenocytes and tumor cells. The splenocytes from laser + GC treated mice protected 60% of the recipients, while the splenocytes from laser treated mice protected only 20% of the recipients.

3.3. *Induction of anti-tumor immunity by laser + GC treatment*

Seven days after treatment, mice were sacrificed and splenocytes were harvested. Levels of TNF-α secretion from the splenocytes were detected using



(a)



(b)

Fig. 1. *In vivo* cytotoxicity assays. EMT6 cells were subcutaneously injected in the flanks of Balb/c female mice, and treatment took place when tumors reached a size of approximately 300 mm^3. Tumors were treated with intratumoral injections of different components, followed by laser irradiation (0.75 W/cm^2 for 10 min): (i) Control, (ii) GC (25 mg/kg), (iii) Laser only, (iv) laser + GC (25 mg/kg). (a) Volumetric changes in tumor sizes of different treatment groups. (b) Analysis of cell death. Tumors were excised 3 h after different treatments, dissociated into single cell suspensions and labeled with Annexin V-FITC, analyzed by FACS. Bars, means ± SD ($n = 4$), *$p < 0.05$.

ELISA. After incubating with mitomycin C-treated EMT6 cells, the splenocytes harvested from laser + GC treated mice significantly increased TNF-α secretion, as shown in Fig. 3(a). These results indicated a tumor-specific immune response when the mouse tumors were treated with laser + GC. In addition, laser + GC treatment increased the cytotoxicity of splenocytes [see Fig. 3(b)], indicating

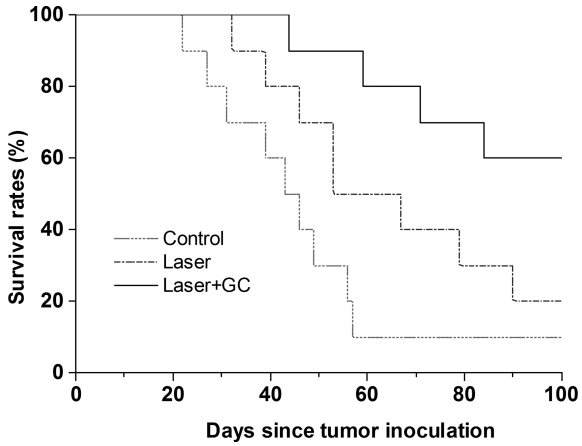


Fig. 2. Anti-tumor immune effect of laser + GC treatment. Animal survival mice in the adoptive immunity experiments using splenocytes from treated mice as immune cells. Viable tumor cells were admixed with splenocytes from different mice, then injected into naive mice. The splenocytes to tumor cell ratio was 50,000,000:100,000 per mouse. The splenocytes from mice treated by laser + GC protected 60% of the recipients and splenocytes from mice treated by laser only protected 20% of the recipients.

the induction of tumor-specific cytotoxic T lymphocytes (CTLs), in the presence of target tumor cells. Next, we assessed the recruitment of T cells into the tumors 7 days after different treatments. There was a dramatic increase in the percentage of both CD4+ and CD8+ cells within the tumor after laser + GC treatment (see Fig. 4). These results indicate that laser + GC treatment induces specific anti-tumor immune responses, mediated by T cell response.

4. Discussion

The ideal treatment modality for cancer, particularly metastatic cancer, should achieve a systemic, tumor-specific immunological response through a minimally invasive, local intervention. Such an approach could potentially suppress local tumors and at the same time eradicate metastases at distant sites, while providing anti-tumor immunity to the host with minimal adverse side effects. Photothermal interaction using laser is an ideal local intervention due to its precise energy delivery to target tissue and the sensitivity of tumor tissue to temperature increase.[12,13]

Anti-tumor immune response can be significantly enhanced by introducing immunological stimulants to the tumors, particularly when combined with

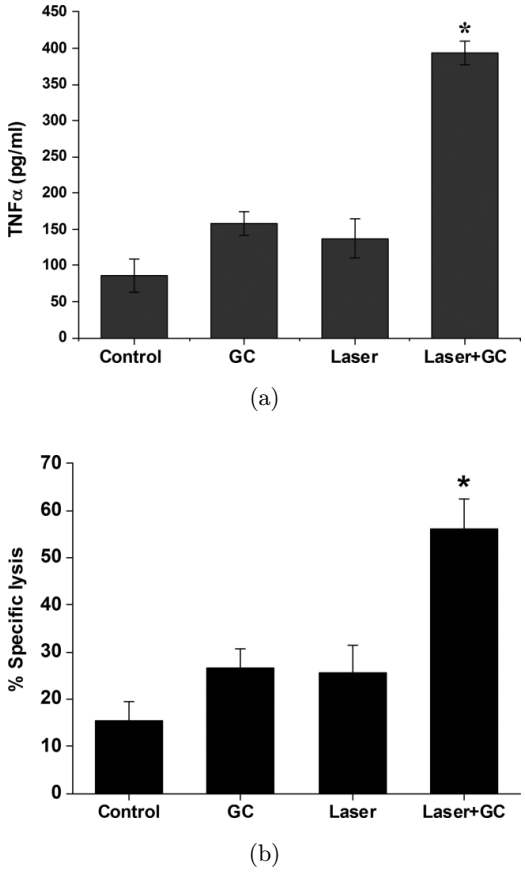


Fig. 3. Induction of anti-tumor immunity by different treatments. (a) TNF-α secretion by mouse splenocytes. Seven days after different treatments as indicated, splenocytes from treated mice were harvested and restimulated with mitomycin C-treated EMT6 cells for 2 days and their TNF-α secretion was detected by ELISA. The levels of TNF-α secretion by splenocytes from mice treated by laser + GC were noticeably higher than that from mice of the other treatment groups. Bars, means $\pm$ SD ($n = 5$), $^*p < 0.05$. (b) Tumor cytotoxicity of splenocytes from treated mice. Harvested splenocytes were restimulated with mitomycin C-treated EMT6 cells, stained by calcium acetoxymethyl, for 5 days. Cytolytic activity against EMT6 cells was tested by fluorescence assay. Bars, means $\pm$ SD ($n = 4$), $^*p < 0.05$.

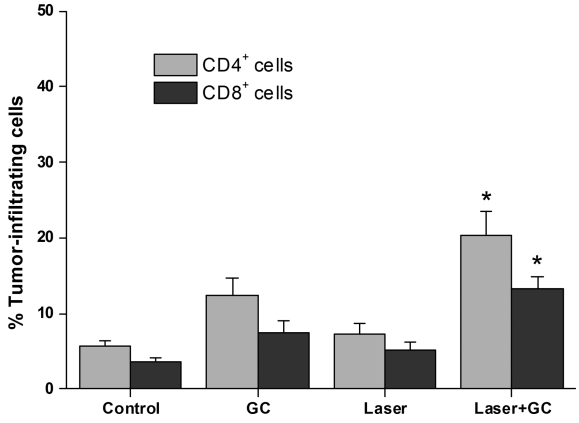


Fig. 4. Analysis of tumor-infiltrating CD4+ or CD8+ cells 7 days after different treatments of mouse tumors. Treated tumors were excised, dissociated into single cell suspensions, labeled with FITC-conjugated anti-CD4 or phycoerythrin-conjugated anti-CD8 mAb, then analyzed by FACS. Bars, means $\pm$ SD ($n = 4$), $^*p < 0.05$.

other interventions. When used appropriately, such immunostimulants can significantly improve the efficacy of cancer treatment by stimulating the host immune system, such as when *Corynebacterium parvum*, bacille Calmette-Guérin, or other immunoadjuvants were intratumorally administered in conjunction with photodynamic therapy treatment.[14–17] Some chemotherapy agents, such as gemcitabine, serve as effective boosters of immune response through induction of tumor-specific antigen overexpression when disrupting apoptotic tumor cells.[18] Other strategies, such as GM-CSF or interleukin-2, contribute to the improved availability of immune cells in the tumor vicinity, improving both antigen presentation and T-cell activation and proliferation.[19,20]

Our experiments show that the induced immunity could be passively transferred using splenocytes. After treatment, the splenocytes from mice treated by laser + GC protected 60% of normal recipient mice when the animals were injected with a mixture of the spleen cells and tumor cells, as shown in Fig. 2. In comparison, splenocytes from mice treated by laser only provided only a low level of protection to the recipient mice (see Fig. 2). These results indicate that laser + GC could induce long-term memory in immune cells, again, attributed to the effect of GC. It should be noted that previous studies using indocyanine green (ICG) as a light absorbing enhancer yielded much better outcomes in adoptive immunity transfer.[7] Therefore, appropriate thermal effect is needed to achieve optimal effects.

In vitro results also show immunological effects of laser + GC, particularly in inducing tumor-specific immune responses. The splenocytes from laser + GC treated mice showed noticeable increase in TNF-α secretion [see Fig. 3(a)]. The increased cytotoxicity of splenocytes from treated mice also demonstrated the effectiveness of laser + GC in inducing tumor-specific responses [see Fig. 3(b)]. The high level of T cells infiltrating tumors after laser + GC treatment (see Fig. 4) further confirmed

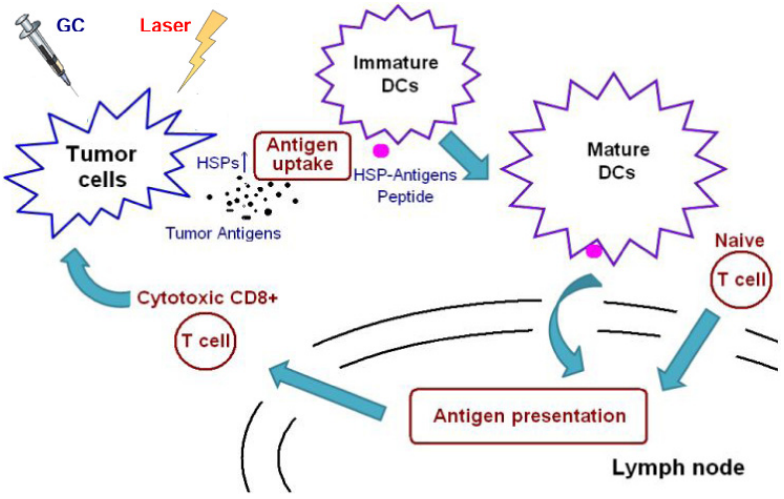


Fig. 5. The operation procedure and the working mechanism of laser–GC treatment of cancers. The laser irradiation causes the release of tumor antigens and heat shock proteins. GC injected to the treated tumor site recruits and activates antigen-presenting cells, such as dendritic cells (DCs). The activated DCs take up the tumor antigens and present them to the T cells. The activated T cells infiltrate tumors to eradicate residual tumor cells at the treatment sites and at the metastasizing sites.

the anti-tumor immune response, mediated by T cells.

The outcomes of the cancer treatment using laser irradiation and immunological stimulation depend on several parameters, including the intensity of the irradiation laser, the duration of the irradiation and the dose of the immunostimulant. The optimization of the treatment protocol is currently underway. The criteria for the optimization needs extensive studies since the relationships between thermal interaction and host immune response and between immunological stimulation and tumor-specific response are complex. Overall, the criterion should be the maximum destruction of viable tumor cells, reducing the tumor burden and releasing viable tumor antigens. Our future studies will certainly shed light on this challenging issue.

Although further investigation is needed, it can be hypothesized that the mechanism of laser + GC in tumor therapy relies on the synergistic interaction between the photothermal reaction and the immunological stimulation. The photothermal reaction reduces the tumor burden and at the same time exposes the tumor antigens; the immunoadjuvant *in situ* first stimulates the host immune system (as indicated by the results in Fig. 3(a)) and then directs the immune system against the specific tumor cells [as indicated by the results in Figs. 3(b) and 4], inducing T-cell immune response. The working mechanism of laser–GC treatment is depicted in Fig. 5. This method, therefore, could provide a systemic immunotherapy for each individual host without the usually required immune cross-reactivity.

Acknowledgment

This research is supported in part by grants from the US National Institutes of Health (R21 EB0155091), the US Fulbright Scholar Program and Immunophotonics, Inc. This work was also supported in part by grants from National Natural Science Foundation of China (No. 81000994) and Beijing Municipal Science and Technology Commission (No. Z121107001012080).

References

1. W. R. Chen, R. L. Adams, R. Carubelli, R. E. Nordquist, "Laser-photosensitizer assisted immunotherapy: A novel modality in cancer treatment," *Cancer Lett.* **115**, 25–30 (1997).
2. W. R. Chen, W. G. Zhu, J. R. Dynlacht, H. Liu, R. E. Nordquist, "Long-term tumor resistance induced by laser photo-immunotherapy," *Int. J. Cancer* **81**, 808–812 (1999).
3. W. R. Chen, R. L. Adams, S. Heaton, D. T. Dickey, K. E. Bartels, R. E. Nordquist, "Chromophore-enhanced laser-tumor tissue photothermal interaction using 808 nm diode laser," *Cancer Lett.* **88**, 15–19 (1995).
4. W. R. Chen, R. Carubelli, H. Liu, R. E. Nordquist, "Laser immunotherapy: A novel treatment modality

for metastatic tumors," *Mol. Biotechnol.* **25**, 37–43 (2003).
5. S. Song, F. Zhou, R. E. Nordquist, R. Carubelli, H. Liu, W. R. Chen, "Glycated chitosan as a new non-toxic immunological stimulant," *Immunopharmacol. Immunotoxicol.* **31**, 202–208 (2009).
6. F. Zhou, S. Song, W. R. Chen, D. Xing, "Immunostimulatory properties of glycated chitosan," *J. X-Ray Sci. Tech.* **19**, 285–292 (2011).
7. W. R. Chen, A. K. Singhal, H. Liu, R. E. Nordquist, "Laser immunotherapy induced antitumor immunity and its adoptive transfer," *Cancer Res.* **61**, 459–461 (2001).
8. W. R. Chen, H. Liu, J. W. Ritchey, K. E. Bartels, M. D. Lucroy, R. E. Nordquist, "Effect of different components of laser immunotherapy in treatment of metastatic tumors in rats," *Cancer Res.* **62**, 4295–4299 (2002).
9. M. F. Naylor, W. R. Chen, T. K. Teague, L. Perry, R. E. Nordquist, "*In situ* photo immunotherapy: A tumor-directed treatment modality for melanoma," *Br. J. Dermatol.* **155**, 1287–1292 (2006).
10. X. Li, M. F. Naylor, H. Le *et al.*, "Clinical effects of in situ photoimmunotherapy on late-stage melanoma patients: A preliminary study," *Cancer Biol Ther.* **10**(11), 1081–1087 (2010).
11. X. Li, G. L. Ferrel, M. C. Guerra *et al.*, "Preliminary safety and efficacy results of laser immunotherapy for the treatment of metastatic breast cancer patients," *Photochem. Photobiol. Sci.* **10**, 817–821 (2011).
12. L. J. Anghileri, J. Robert, *Hyperthermia in Cancer Treatment*, CRC Press, Boca Raton, FL (1986).
13. F. Zhou, D. Xing, Z. Ou, B. Wu, D. E. Resasco, W. R. Chen, "Cancer photothermal therapy in the near-infrared region by using single-walled carbon nanotubes," *J. Biomed. Opt.* **14**, 021009 (2009).
14. R. C. Myers, B. H. Lau, D. Y. Kunihira, R. R. Torrey, J. L. Woolley, J. Tosk, "Modulation of hematoporphyrin derivative-sensitized phototherapy with Corynebacterium parvum in murine transitional cell carcinoma," *Urology* **33**, 230–235 (1989).
15. Y. H. Cho, R. C. Straight, J. A. Smith Jr, "Effects of photodynamic therapy in combination with intravesical drugs in a murine bladder tumor model," *J. Urol.* **147**, 743–746 (1992).
16. G. Krosl, M. Korbelik, "Potentiation of photodynamic therapy by immunotherapy: The effect of schizophyllan (SPG)," *Cancer Lett.* **84**, 43–50 (1994).
17. M. Korbelik, V. R. Naraparaju, N. Yamamoto, "Macrophage-directed immunotherapy as adjuvant to photodynamic therapy of cancer," *Br. J. Cancer* **75**, 202–207 (1997).
18. P. Correale, M. G. Cusi, K. Y. Tsang *et al.*, "Chemoimmunotherapy of metastatic colorectal carcinoma with gemcitabine plus FOLFOX 4 followed by subcutaneous granulocyte-macrophage colony-stimulating factor and interleukin-2 induces strong immunologic and antitumor activity in metastatic colon cancer patients," *J. Clin. Oncol.* **23**, 8950–8958 (2005).
19. J. O. Armitage, "Emerging application of recombinant human granulocyte-macrophage colony-stimulating factor," *Blood* **92**, 4491–4508 (1998).
20. L. Cruz-Merino, E. Grande-Pulido, A. Albero-Tamarit, M. E. C. M. Viliena, "Cancer and immune response: Old and new evidence for future challenges," *Oncologist* **12**, 1246–1254 (2008).