Format for ANSWERING REVIEWERS

December 20, 2013

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 6434-review.doc).

Title: Immune response after photodynamic therapy increases anti-cancer and anti-bacterial effects

Author: Eleonora Reginato, Peter Wolf, Michael R Hamblin

Name of Journal: *World Journal of Immunology*

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The manuscript has been improved according to the suggestions of reviewers:

Referee #1

This paper reviewed innate and adaptive immune responses induced by photodynamic therapy for cancer and infection. The manuscript covers broad aspects of immunological effects of PDT and is well written although the content is similar to that of Mroz et al. (ref. 100). There are several points to be improved. First of all, page number should be added to each page.

Authors stated that this review focus on immune responses induced by PDT against pathogens as well as tumors (page 2, lines 3-5). However, concerning immune responses by PDT against pathogens, authors focused only on the innate immune response against bacterial arthritis (Tanaka et al.; ref. 9). Authors mentioned that PDT is capable of killing a large variety of pathogens such as bacteria, parasitic protozoa, fungi, yeasts and viruses (page 9, lines 7, 8). Refer the papers describing the PDT effects on parasitic protozoa, fungi, yeast and viruses, not only on bacteria.

→ Response: A couple of sentences with references have been added as follows:
"There are literature reports of PDT on its effects on certain species of fungus, including both filamentous fungi (*Tricophyton* (92) and *Aspergillus* (93)) and yeasts (*Saccharomyces* (94) and *Candida albicans* (95, 96)). Also several types of virus have been tested for the affection by PDT, including herpes viruses HSV-1 (97) (PDT by methylene blue and light), enveloped RNA viruses from two different families, Semliki Forest Virus (SFV, *Togaviridae*) and vescicular stomatitis virus (VSV, *Rhabdoviridae*) (PDT by buckminsterfullerene and light) (98) and others (99)."

In addition, the description of the difference of PDT effects on cancer and bacterial infection at page 10, lines 13-22 is not clear. Is there no evidence that PDT induces B cell-mediated adaptive immune response against bacterial infection?

→ Response: To the best of our knowledge, hitherto nothing is known about humoral responses induced by PDT against bacterial infection. In order to make a clearer description of the difference of PDT effects on cancer and bacterial infection, we modified the respective paragraph slightly and added the following sentence:

"However, to the best of our knowledge, nothing is known yet about humoral responses induced by PDT against bacterial infection."

There are several grammatical and nongrammatical errors to be revised through the manuscript. Authors should check and correct them. They are listed below.
Page 2, line 2 (Core Tip section). "procedure" reads "procedures".
Page 2, line 14 (Abstract section). "is capable to affect" reads "is capable of affecting". The usage of "is capable to" is grammatically wrong.
Page 2, line 18. Check the usage of "able to".
Page 2, line 2 from the bottom (Key Words). "Photodynamic therapy" reads "photodynamic therapy.
Page 3, lines 19, 23. Authors use "Balb/c" here, but use "BALB/c" in page 6, line 10 from the bottom. Use one style through the manuscript.
Page 4, lines 3-6. The sentence "DAMPs are…" is too long.
Page 4, line 2 from the bottom. "the massive infiltration to the tumor by the immune cells" should be "massive infiltration of the immune cells to the tumor".
Page 5, line 18. Check the usage of "capable of".
Page 5, line 21. "?" should be a period ".".
Page 5, line 7 from the bottom. "CTL" should be spelled out first.
Page 5 line 6 from the bottom. "DC" reads "DCs".
Page 5 line 1 from the bottom. "CD8+ cells" reads "CD8+ T cells".
Page 6 line 1. "in absence" reads "in the absence".
Page 6 lines 8, 9. Authors used (i), (ii), (iii) here, but used (1), (2), (3) at lines 17, 20, 21 of the same page. Use one style.
Page 6, line 11 from the bottom. "wild type" reads "wild-type".
Page 6, line 5 from the bottom. Check the usage of "able to".
Page 7, line 23. "recognizes" reads "recognize".
Page 7, line 4. "tat" reads "that".
Page 8, line 7. "evidences" reads "evidence".
Page 8, line 24. "MHCI" should be spelled out when it appears for the first time (page 5, line 10 from the bottom).
Page 9, line 2. Refer the paper or review that described "PDT in the field of microbiology over 100 years ago".
Page 9, line 7. "is capable to kill" reads "is capable of killing".
Page 10, line 4. "such effect" reads "such an effect".
Page 10, line 15. "is capable to stimulate" reads "is capable of stimulating".

Page 10, line 24. "is capable to pronouncedly activate" reads "is capable of pronouncedly activating".

Page 10, line 6 from the bottom. ", that" reads ", which". The sentence is too long.

Table 1. "Treg" should be "Treg (T regulatory cells)".

Figure 1. The fonts of alpha in TNF-alpha and beta in IL-1beta are too large.

"hv" should be "h nu (Greek letter)".

"PMNS" should be "PMN" or "PMNs".

Figure 2. "hv" should be "h nu (Greek letter)".

References. Check the abbreviated form of journal names. For example, the journal name of your paper (ref. 72) should be "Br J Cancer" instead of "BJC".

→ Response: All errors have been corrected in the text, Figures and Tables.

Referee #2

General comments

1. **Side effects of PDT (photodynamic therapy) appeared to be underestimated.**

Response 1: In the present manuscript we reviewed the immunostimulatory properties of PDT, focusing on anti-cancer and anti-bacterial applications. However, in the "Conclusions" section we specified that PDT has been described also as having immunosuppressive (potentially side) effects, as follows:

"Moreover, PDT has been linked also to immunosuppressive effects. Such immunosuppressive effects have been established in model of suppression of induction of contact hypersensitivity (CHS) (i.e. afferent immune response), which involves the application of a hapten to the skin, followed by re-challenge (102), and suppression of delayed-type hypersensitivity (Mantoux) reactions (i.e. efferent immune response) for instance in healthy Mantoux-positive volunteers (103, 104). In particular, such immunosuppressive responses seem to be dependent on the rate of light delivery (105) and anatomic site of PDT (106)."

2. **Erroneous sentences and irrelevant information are found throughout the text.**

Response 2: We believe that all information included in the manuscript is relevant for the topic of the review and properly described.

Major comments

1. **The manuscript should have focused more on 'cellular and molecular mechanisms' on the induction of damage-associated molecular patterns (DAMPs) and cell death-associated molecular patterns (CDAMPs) by PDT.**

Response 1: We included in the manuscript an entire section and dedicated a table for the description of DAMPs. We also added in the text more details about induction of DAMPs by PDT as follows:

"Korbelik *at el*. (22) found that SCCVII cancer cells treated by *in vitro* photofrin-PDT expose on the surface heat shock proteins (HSPs) such as HSP60, HSP70 and GRP94 (GRP - glucose-regulated protein) and release HSP70 to the extracellular space. Interestingly, when PDT was applied in *in vivo* settings,

they found a different spectrum of DAMPs exposed on the surface of treated SCCVII cells. While HSP70 was still exposed, HSP60 and GRP94 were no longer detected and replaced by GRP78 on the surface of PDT-treated SCCVII cancer cells. This study indicated for the first time that the DAMPs associated with PDT can differ in the same cancer cells between *in vitro* and *in vitro* settings (22).
It is worth mentioning also that the spectra of DAMPs exposed and/or released after PDT correlate with the sub-cellular localization patterns of the PS, where the ROS-based stress is originated. For instance, PSs targeting the endoplasmatic reticulum (ER) (e.g. hypericin) are known to cause surface exposure of calreticulin (CRT); conversely, Photofrin (whose localization is mostly associated with lipid membranes)-PDT, has been linked primarily to surface exposure of HSP70 (23, 24).
Further investigations on cellular and molecular mechanisms are certainly required to establish in more detail the correlations between DAMPs and PDT."

2. **PDT can cause the cell death not only on tumor cells, but also on normal cells including immune cells that could have caused the cancerous or immune-compromised conditions. Furthermore, the inhibition of Treg by PDT has (have) potential to induce autoimmune disease. Authors should describe this (these) impact (pros and cons) with proper references.**

Response 2: One of the main advantages of PDT is its double selectivity, due first by the higher degree localization of the photosensitizer within more rapidly proliferating cancer cells as compared to normal cells and second by the precise delivery of light to the tumor mass, that makes the treatment spatially selective and protects the surrounding healthy cells from the insult (Firczuk M, Nowis D, Golab J: PDT-induced inflammatory and host responses. *Photochem Photobiol Sci*, 2010; 10: 653-663). Our recent preliminary clinical data revealed that PDT of esophageal squamous cell carcinoma does not have any significant effect on the level of Treg (Reginato E *et al.*, Unpublished data). Neither the systemic level of Treg cells circulating in the blood, nor the local level of Treg infiltrated in the tumor mass was significantly affected at a short or long periods after PDT. In view of these reports, it is most likely that the Treg cells death caused by PDT does not have any relevant impact on the mechanisms for the maintenance of the immune homeostasis in patients.

3. **Type of light and wavelength, intensity are important factors for PDT, and therefore Table(s) to compare would help the readers of WJI.**

Response 3: There is an enormous variety of PDT settings in terms of light wavelenght, fluence rate, light dose and PSs among the treatments for different diseases. It would be difficult to compare so many and different approaches of PDT in a reasonable number of tables that could be added to the paper.

4. **Future prospecting and implication including clinical trials should be covered.**

Response 4: In the "Conclusions" section we summarized our view about the future perspectives of PDT and its present and potential utilization in clinical practice and follows:
"Further studies using a better targeted and dose-controlled PDT treatment would help to expand the knowledge on the activation/suppression of the immune system and the possibilities to improve it in clinical practice.
The proven ability of PDT to trigger inflammation and improve the anti-tumor immune response could be successfully employed in tandem with other treatment modalities, to combat cancer and to achieve

long-term tumor control. Nevertheless, up to now PDT remains clinically underutilized. We must realize that with all probability it will take several years of further investigations and clinical trials before the use of PDT becomes a clinically accepted standard practice in cancer patients.
The innate immune responses seem to be of crucial importance also in the relatively new field of PDT as anti-microbial treatment. The activation of neutrophils after PDT, their mobilization from the bone marrow and their attraction to the site of inflammation appear to be important mechanisms, significantly potentiating the antibacterial effects, e.g. in bacterial arthritis mouse models. However, it still remains to be elucidated whether the activation of the host neutrophils is applicable also to other infection models, with other classes of pathogens and/or using different PS. Many years of intense research will be required providing answers to these intriguing questions."

5. **The section 'PDT for infections' and 'Immune responses in anti-bacterial PDT' are not informative and should be re-written.**

Response 5: Parts of the two paragraphs have been edited.

Minor comments

1. **Non-cytokine reactants, such as serum amyloid P components (SAP), mannose-binding lectin A (MBL-A) and C-reactive protein, should be explained in-depth.**

Response 1: More details and proper references have been added as follows:
"SAP and CRP belong to the pentaxin family proteins and are involved in acute immunological responses (35). They are specialized in facilitating the phagocytosis and removal of dying cells such as those killed in PDT-treated tumors. SAP production and release is a hallmark acute phase reactant response in mice, but in humans CRP is a more important acute phase reactant than SAP and PDT dose-dependent up-regulation of CRP has been demonstrated in human lung tumor A549 cells (35). MBL-A is another important acute phase reactant with functional attributes similar to SAP (36)."

2. **It would be better briefly explained a photo sensitizer (PS).**

Response 2: A brief explanation about PSs has been added as follows:
"Most of the PSs used in PDT are based on a tetrapyrrole structure, similar to that of the protoporphyrin contained in hemoglobin (4). They have an absorption peak between 600 and 800 nanometers (nm) (red to deep red), since light at lower wavelengths would not penetrate efficiently through the tissue and light at longer wavelengths than 800 nm would not have sufficient energy to initiate a photochemical reaction and generate a substantial yield of ROS (4)."
Other desirable properties of PS agents and oncologic PSs that have come to clinical trial have already been published elsewhere (Allison RR, Sibata CH (2010) Oncologic photodynamic therapy photosensitizers: a clinical review. *Photodiagnosis Photodyn Ther*, 7(2):61-75).

3. **Common features of Treg should be summarized into Table or Figure format would be helpful.**

Response 3: A table (Table 3) summarizing the common features of Treg cells described also in the text has been added.

4. Compare and contrast the procedures and types of PDT in Table format would be helpful.

Response 4: Please see above response 3 from "Major Comments".

5. Detailed explanation on photo sensitizer (PS) should be well described.

Response 5: Please see above response 2 from "Minor Comments".

6. There are many references mentioned (are) missing.

Response 6: All the references cited in the text are listed in the Bibliography.

7. Following references should be included;

- **Kalluru P et al. 2013. Photosensitization of Singlet Oxygen and In vivo Photodynamic Therapeutic Effects Mediated by PEGylated W_{18} O_{49} Nanowires. Angew Chem Int Ed Engl. 2013 Oct 18**
- **Kamkaew A and Burgess K. 2013. Double-Targeting Using a TrkC Ligand Conjugated to Dipyrrometheneboron Difluoride (BODIPY) Based Photodynamic Therapy (PDT) Agent. J Med Chem. 2013 Oct 10;56(19):7608-14.**

Response 7: We are very thankful to the referee for the interesting references suggested. In particular, the first example of "nanomaterial-mediated PDT" published by Kalluro *et al*. is a new emerging hint in the field of PDT. However, we believe that the contents of these two publications do not match the main topic of the present review.

Thank you again for publishing our manuscript in the *World Journal of Immunology*.

Sincerely yours,

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