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# Deletion of Alloantigen-Activated Cells by Aminolevulinic Acid-Based Photodynamic Therapy

**Eric A. Hryhorenko[1,2], Allan R. Oseroff[1], Janet Morgan[1] and Kate Rittenhouse-Diakun*[2,3,4]**

Departments of [1]Dermatology, [2]Microbiology and Immunology, Roswell Park Cancer Institute, Buffalo, NY, USA and [3]Departments of Clinical Laboratory Science and [4]Microbiology, State University of New York at Buffalo, Buffalo, NY, USA

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## ABSTRACT

**Protoporphyrin IX (PpIX), an endogenously synthesized photosensitizer, can transiently accumulate in activated lymphocytes following administration of the heme precursor 5-aminolevulinic acid (ALA). One possible mechanism of this in lymphocyte accumulation is that actively dividing cells use intracellular iron stores for cytochrome and DNA synthesis and thus do not inactivate PpIX, the photoactive precursor of heme, by iron incorporation. This selective accumulation in activated cells should allow targeting by photodynamic therapy (PDT). To determine the effect of this accumulation, we studied PDT effects on the *in vitro* correlate of transplantation rejection: the one-way mixed lymphocyte reaction (MLR). Selective phototoxicity was determined by photoirradiating ALA-treated, MLR-activated cells and measuring subsequent stimulation either in a secondary MLR or with phytohemagglutinin (PHA). We found that proliferation of MLR-activated lymphocytes incubated with ALA and treated with light was only 12–20% of controls (ALA+, no light) after rechallenge with the stimulator cells ($P < 0.05$), although their response to nonspecific PHA stimulation was similar to controls. Thus alloantigen-specific depletion was shown. The data suggest a role for ALA-PDT in the treatment of diseases that require the selective elimination of activated lymphocytes and possibly as an immunomodulator.**

## INTRODUCTION

Research is active in the area of clinically applicable immunosuppressive agents, because currently used drugs either have toxic side effects, are incomplete in their effectiveness or lack selectivity. Currently used immunosuppressive drugs (1) include those that inhibit T-cell activation and proliferation, such as cyclosporine A and FK 506 that inhibit interleukin (IL)†-2 production; rapamycin that inhibits IL-2-promoted $G_0$ to $G_1$ progression and leflunomide that inhibits proliferation of stimulated T-cells by inhibiting cytokine action. All of these therapies require continuous use of drug to effect graft tolerance or depression of autoimmune disease. Additional attempts to down-regulate T-cell activation have been made by binding antibody–toxin conjugates to T-cell surface molecules (2) including CD3 (3,4), CD2 (5), and CD25 (6). However, problems with these therapies include the development of human antimouse antibody and incomplete removal of effector cells (7).

Cyclosporine and related immunosuppressive therapies have allowed great strides in organ transplantation and have improved the clinical prognosis for patients with autoimmune disorders, but more tools are needed in both these areas to improve the clinical prognosis for a greater percentage of these patients. An ideal drug would be one that would cause clonal deletion of activated cells, so that it need only be used for short time periods to eliminate reactive cells. One such drug may be the endogenously synthesized photosensitizer, protoporphyrin IX (PpIX) (8–11).

The synthesis of PpIX occurs in the penultimate step of the heme synthesis pathway(12). It accumulates in cells following the addition of the heme precursor aminolevulinic acid (ALA) that bypasses the negative feedback of heme on the pathway (12). The PpIX is a photosensitive compound that, when exposed to the correct wavelength of light, induces a photochemical reaction that generates reactive oxygen species (13), which destroy cells by various mechanisms (14,15). Mechanisms include modifications of intracellular proteins and lipid peroxidation that alter cell function(s) resulting in cell membrane and organelle (such as mitochondria) destruction (16), which in turn can lead to death by apoptosis and necrosis(17–20).

Because PpIX can accumulate in actively dividing cells such as some cancer cells and activated lymphocytes (18,21), it may be possible to eliminate activated cells selectively while leaving naive cells relatively unscathed. This technique would be useful in the areas of transplantation rejection and control of autoimmune diseases in which activated immune cells are responsible for much of the damage.

We examined the feasibility of selectively destroying one-way mixed-lymphocyte reaction (MLR) immune cells using ALA-photodynamic therapy (PDT) (Fig. 1). Human periph-

*To whom the correspondence may be addressed at: Department of Clinical Laboratory Science, SUNY at Buffalo, NY 14214, USA.

†*Abbreviations:* ALA, δ-aminolevulinic acid; BPD, benzoporphyrin derivative; HLA, human leukocyte antigen; MLR, one-way mixed-lymphocyte reaction; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PDT, photodynamic therapy; PHA, phytohemagglutinin; PpIX, protoporphyrin IX.

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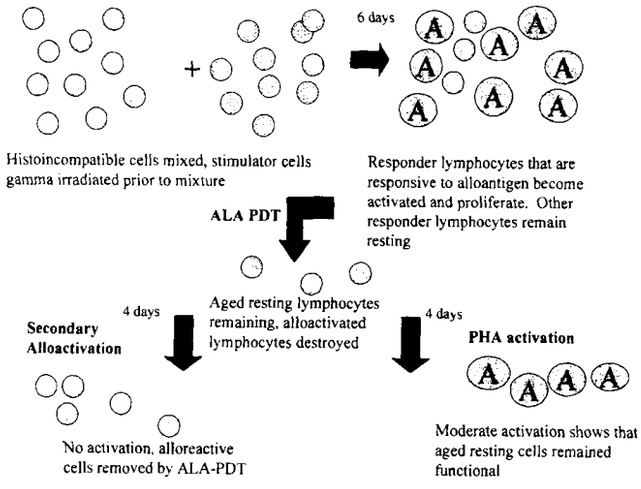


**Figure 1.** A schematic of the protocol utilized for the experiment shown in Fig. 3. This experiment was designed to determine if secondary alloactivation could be prevented by ALA-PDT, while leaving the reactivity to other stimuli, in this case PHA, unaffected.

eral blood mononuclear cells (PBMC) activated by allogeneic antigens from a histoincompatible donor were treated with ALA ± white light. Following treatment, the cells were treated in one of two ways. Either (1) additional stimulators were added to the treated cells from the original donor, creating a secondary or primed MLR to assess functional response of the alloantigen activated cells after treatment or (2) phytohemagglutinin (PHA) was added to the treated cells to assess the functional response of the resting cell population after treatment. These assays provided a method to examine if activated cells can be specifically eliminated from a population of cells with ALA-PDT by analyzing the responses to the original specific stimulus and also to the nonspecific mitogen, PHA.

## MATERIALS AND METHODS

*Cells and media.* Human PBMC from healthy donors were isolated using density centrifugation over a Ficoll-Paque gradient (Pharmacia LKB, Uppsala, Sweden). Following two washes of the mononuclear cell layer (250 g, 5 min) with sterile phosphate-buffered saline (PBS, pH 7.2, without $Ca^{2+}$ and $Mg^{2+}$), the PBMC were counted and resuspended in RPMI-1640 supplemented with L-glutamine (2.0 m*M*) and minimum essential medium nonessential amino acids (0.1 m*M*) (Life Technologies, Grand Island, NY) and containing 20% heat-inactivated pooled AB human serum (Pel-Freez, Brown Deer, WI). In some experiments during ALA incubation and light treatment, lower serum concentrations (1%) were used. Cells were incubated at 37°C with 5% $CO_2$ unless stated otherwise.

*Light source.* The light source used for irradiations was from two 500 W halogen lamps filtered to supply light at 18 mW/cm$^2$ between 400 and 720 nm.

*Preparation of stimulators and activation of cells.* The PBMC (stimulators) from healthy donors were irradiated with 4000 rads from a gamma source ($Ce^{137}$). Following irradiation, equal numbers of stimulator PBMC were combined with responder PBMC from a histoincompatible donor and incubated for 6 days to create the MLR. Control wells received responders only. In some experiments, cells were stimulated with the mitogen PHA (Sigma, St. Louis, MO) at 5 μg/mL.

*Dark toxicity.* Aliquots of 5 × 10$^6$ stimulator and responder PBMC from human leukocyte antigen (HLA)-disparate donors were prepared in an MLR as above. Cells were then collected and centrifuged. The conditioned medium from the MLR-activated culture was saved. The PBMC were washed once with PBS and resuspended to 1 × 10$^6$ cells/mL of medium containing 1% human serum. Aliquots of 100 μL of cells were plated per well of a 96 well round-bottom plate (Falcon, NJ). Freshly prepared ALA in 1% medium was added to the cells at final concentrations between 0 and 5 m*M* (six replicates per concentration). Following the ALA addition, all further work was done in the dark. Plates were incubated for 4 h to allow PpIX synthesis, centrifuged (1000 ***g***, 5 min) and the medium containing ALA was discarded. An aliquot of 100 μL of conditioned medium plus 100 μL fresh medium was added to each well. Plates were returned to the incubator and 30 h later tritiated thymidine was added to each well (1 μCi/well). Eighteen hours later, cells were harvested using a semiautomated cell harvester (Skantron, Sterling, VA) and cpm determined in Ecoscint scintillation fluid (National Diagnostics, GA) using a liquid scintillation counter (Packard, Downers Grove, IL).

*Light dosimetry.* Responder and stimulator PBMC were combined and incubated for 6 days as described above. The MLR-activated PBMC were then washed once with PBS and resuspended to 1 × 10$^6$ cells/mL of medium containing 1% serum. A 100 μL aliquot of MLR-activated cells was plated per well in 96 well plates. The ALA (4.0 m*M* final concentration) or medium was added to six replicate wells and plates were incubated for 4 h. Following incubation, plates were irradiated with 0, 10, 20, 30 or 45 J/cm$^2$ of light. Following light treatment each plate was centrifuged at 1000 ***g*** and the medium containing ALA was discarded. Conditioned medium (100 μL) was added to each well along with 100 μL of fresh medium and plates were incubated for an additional 48 h, 8 days total, with addition of tritiated thymidine to each well (1 μCi/well) 18 h before the completion of the incubation (48 h total). Cells were harvested and cpm determined.

*Secondary MLR.* Responder and stimulator PBMC were combined and incubated for 6 days as described above. Activated PBMC were collected and washed once with PBS and resuspended to 1 × 10$^6$ cells/mL of medium containing 1% serum. The MLR-activated cells (100 μL) were plated per well of two 96 well plates. Six replicate wells received ALA (4 m*M* final concentration) or medium. As a control, some wells received 100 μL 6 day responders (nonMLR-activated cells, 1 × 10$^6$ cells/mL) with ALA or medium. Cells were incubated for 4 h and were then either irradiated (20 J/cm$^2$)(plate 1) or kept in the dark (plate 2). During the 4 h incubation period, fresh stimulators were obtained from the original donor, gamma irradiated and resuspended to 1 × 10$^6$ cells/mL 10% medium. After irradiation plates were centrifuged at 1000 ***g*** and the medium containing ALA discarded. The MLR conditioned medium (100 μL) was added to the wells containing MLR-activated cells and 100 μL of responder alone conditioned medium was added to the control wells. Then, 100 μL (1 × 10$^6$ cells/mL) of stimulators in fresh medium was added to half the wells to create the secondary MLR and 100 μL of PHA in medium (5 μg/mL final concentration) was added to the remaining half of the wells to provide activation of the resting, nonalloantigen-specific T cells. Additional control wells included fresh stimulators plus PHA, MLR-activated cells plus medium (no additional stimulus) and medium alone (no cells for background control) (Fig. 1). Both plates were returned to the incubator for 3 days, tritiated thymidine was added (1 μCi/well) and 18 h later cells were harvested and cpm determined (see above). This entailed a total incubation time of 10 days.

*Statistics.* Experiments were carried out three to five times in six replicates per treatment group, using different healthy donors. Students' *t*-test was used to determine significance of these gaussianly distributed results. Light dosimetry and dark toxicity experiments were carried out three times, with different responders and stimulators in each run. *P* values less than 0.05 were considered significant.

The experiment depicted in Fig. 3 was performed five times with two different donors and two different recipients. The experiment depicted in Fig. 5 was performed three times, using three different donors and three different recipients. Representative experiments are shown and similar patterns were obtained in each experiment. Experiments varied according to individual donor responsiveness.

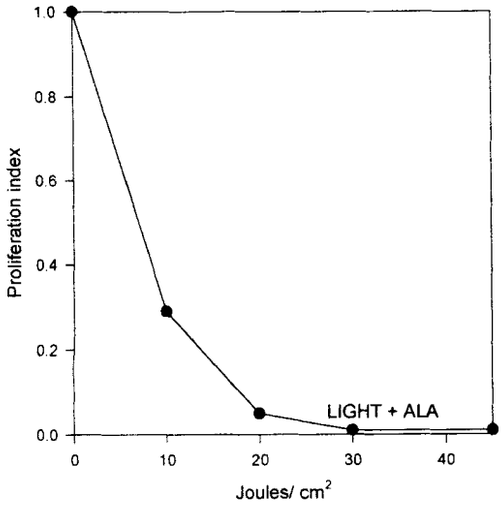


**Figure 2.** Light dosimetry of PBMC treated with ALA. The PBMC were incubated for 4 h with and without 4 m*M* ALA. Proliferation index was determined by dividing all sample cpm by control cpm that did not receive light. The light source was a halogen lamp with a dichroic and infrared filter (400–700 nm).

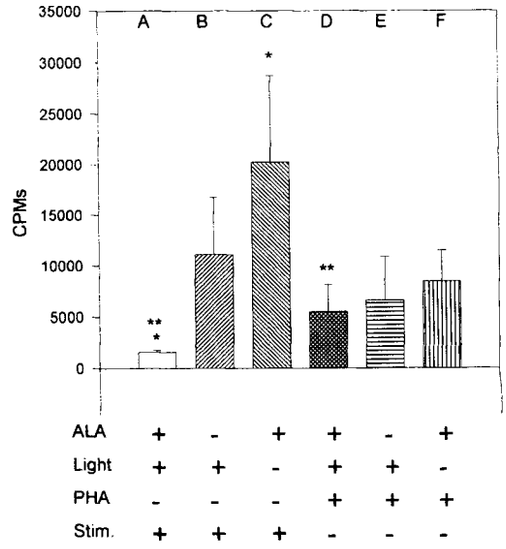


**Figure 3.** Selective elimination of MLR-activated PBMC by ALA-PDT. The MLR-activated PBMC were incubated with (+) or without (−) ALA for 4 h. Following incubation, gamma-irradiated stimulators (Stim.) or PHA were added to the wells. Proliferation was measured by a tritiated thymidine incorporation assay. The data on the graph for each sample are the mean of six sample wells; the error bars represent 1 SD. *, ** $P < 0.05$; Students' t-test, unpaired.

## RESULTS

### Dark toxicity and light dosimetry

Prior to light irradiation of the mixed lymphocyte-activated PBMC, ALA concentrations and light doses were examined for inhibition of cell proliferation. A concentration of 4 mM ALA showed no dark toxicity and was used for the remainder of the study (data not shown). Figure 2 illustrates the proliferation index of MLR-activated cells incubated for 4 h with 4 m*M* ALA and then exposed to various light doses from a halogen lamp. Tritiated thymidine uptake of the MLR-activated cells decreased with increasing light dose. A light dose of 20 J/cm$^2$ (95% inhibition) was chosen for the remaining experiments.

### Selective elimination of MLR-activated human PBMC by ALA-PDT

We determined if MLR-activated PBMC were selectively killed by PDT by treating MLR-activated cells with ALA + light and then restimulating the same activated population specifically with a secondary MLR (original antigenic stimulus) or by stimulating the resting and activated cells non-specifically with PHA. A lack of proliferation following addition of fresh stimulators to the ALA + light-treated cells would indicate removal of activated lymphocytes responding to the MLR stimulus by the ALA light treatment. Successful activation of this treated population with PHA would indicate that PBMC that were not reactive with the alloantigens, and thus not activated prior to the ALA and light treatment, were not killed and could still function. Taken together, these data would indicate that selective killing of MLR-activated clones had occurred (Fig. 1). Figure 3 shows the results from one representative experiment (n = 5). There is significant killing of MLR-activated cells treated with ALA and light compared to ALA-treated, MLR-activated cells left in the dark (*) (A *vs* C). There was no significant difference between MLR-activated cells exposed to light alone compared to ALA without light (B *vs* C). Thus, both ALA and light were required for killing. These results indicate that cells, which responded in the MLR, were selectively eliminated by the PDT treatment as hypothesized and thus were not available for secondary alloantigen-induced proliferation.

To show that lymphocytes that were unresponsive to the MLR stimulus were unaffected by PDT, we used a PHA stimulus after ALA-PDT (D). Following light treatment and ALA incubation for 10 days in culture (D) there are still cells that can be activated by PHA. However, the control of MLR + ALA + PHA + dark (F) showed greater stimulation by PHA than the same sample in the presence of light (D). This is not surprising because the activated alloantigen-reactive cells can also be stimulated by PHA, and these were removed. Comparing MLR-activated cells treated with ALA + light and restimulated with the original donor (A) with MLR-activated cells treated with ALA + light and restimulated with PHA (**) (D), we see that lymphocytes remained that were responsive to PHA but not to secondary alloantigen stimulation. The difference in these (A *vs* D)(**) values was statistically significant in some of the repetitions of this experiment but not in all. To view the differential effects of the ALA treatment on resting and activated cells in a manner that would incur greater differences, we increased the population of resting cells by adding a fresh population of unprimed responder cells to all the wells prior to the addition of ALA and light (Fig. 4). Figure 5 illustrates a representative of the results of the experiments (n = 3). A pattern similar to Fig. 3 was observed, showing a statistically significant difference ($P < 0.05$) in the alloantigen response of ALA + light-treated cells compared to cells treated with light alone (G *vs* H) and a statistically significant difference between the response of the ALA-PDT-treated cells response

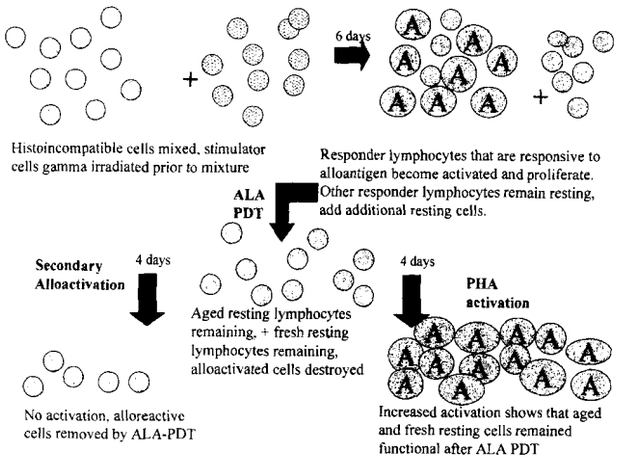


**Figure 4.** A schematic of the protocol utilized for the experiment shown in Fig. 5. As in Fig. 3, this experiment was designed to determine if secondary alloactivation could be prevented by ALA-PDT, while leaving the reactivity to other stimuli, in this case PHA, unaffected. This protocol includes the addition of fresh resting PBMC, so that the differential effects of the ALA and light treatment can be better evaluated.

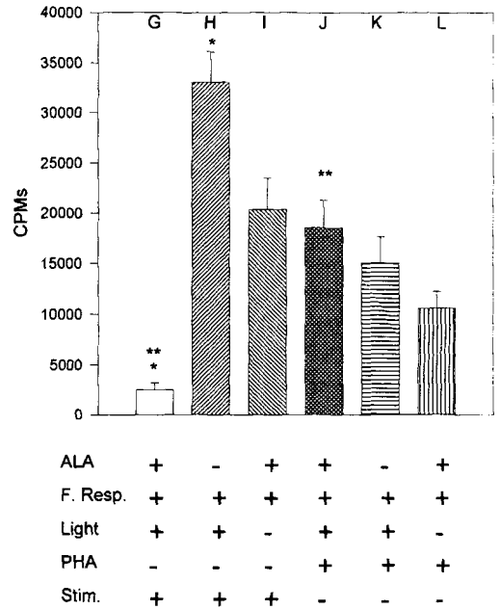


**Figure 5.** Addition of fresh responders to the MLR selective elimination experiment. Fresh responders (F. Resp.) were added to MLR-activated PBMC and incubated for 4 h with (+) and without (−) ALA. Following incubation, gamma-irradiated stimulators (Stim.) or PHA were added to the wells. Proliferation was measured by a tritiated thymidine incorporation assay. The data on the graph are the mean of six wells for each sample, the error bars are 1 SD. *,**$P < 0.05$, Students' t-test, unpaired.

to alloantigen (G) compared to the response to PHA (J). The increased amount of resting cells in this modified experiment allowed an increased responsiveness to PHA, and the (**) difference between (G) and (J) was statistically significant in each trial of this experiment. This held true for all the donors tested again, demonstrating selective elimination of the alloantigen response, while allowing resting cells to remain unscathed.

There was a possibility that the fresh responders could affect the secondary MLR proliferation even though we measured it on day 4, while primary MLR activation of these fresh cells is maximum on day 6. In addition, stimulated MLR culture fluid may cause early activation of the fresh responders in a bystander effect. To determine the extent of these effects, we compared the MLR-activated cells + fresh responders + stimulators + MLR conditioned medium on day 4 with a control lacking the previously activated MLR cells. Both light and dark treatment were measured. The results (Fig. 6) show that the contribution of these factors to the secondary MLR reaction was less than 16% and thus were not a substantial contributor to the response. Therefore we concluded that PDT with ALA could selectively remove alloactivated cells leaving resting lymphocytes. The resting lymphocytes were still responsive to a different antigenic challenge and could confer a protective response to infectious disease.

## DISCUSSION

The MLR classically is used to show alloreactivity between individuals in transplantation studies(24–26). In our studies we used an MLR to show selective elimination of activated human lymphocytes from a population of cells with ALA-PDT while leaving resting cells relatively unharmed. Responder lymphocytes activated by alloantigens and treated with ALA-PDT were prevented from responding to the original stimulus but not to the mitogen PHA (Figs. 3 and 5). The PHA responsiveness was similar for cells receiving ALA + light (Fig. 3, column D) or either of the components alone (Fig. 3, column E or F). The specificity of the elimination was shown again in our modified experiment (Figs. 4 and 5). In this expansion of the previous protocol, the addition of the fresh resting cells showed the specificity of the elimination in a more obvious manner, by increasing the number of responsive resting cells that remained after activated cell removal.

The response of resting cells to PHA following ALA-PDT differs from that of Barrett *et al.* who found that lymphocytes treated with exogenous PpIX followed by longwave

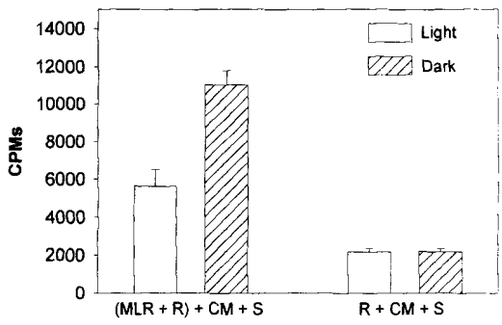


**Figure 6.** The effects of MLR-conditioned medium on fresh responders. Responders (R) plated with MLR-conditioned medium (CM) and stimulators (S) were compared to MLR-activated cells plated with fresh responders plus conditioned medium plus stimulators. Light and dark controls were done. Proliferation was measured with a tritiated thymidine incorporation assay. The data on the graph are the mean of six wells for each sample, the error bars are 1 SD.

UV light had a decreased proliferation to PHA. The difference may be due to the use of exogenous PpIX in Barrett *et al.* compared to ALA-induced endogenous PpIX in our work. Endogenous PpIX is synthesized in mitochondria while exogenous PpIX partitions into plasma membranes and other lipophilic sites (27). Activated lymphocytes synthesize more PpIX from ALA than resting lymphocytes, providing selectivity. In addition to selectivity, use of exogenous ALA instead of exogenous PpIX can affect the efficacy of the PDT treatment. Tabata and colleagues have shown that ALA-induced PpIX was photodynamically more efficient than exogenously added PpIX because of localization at sensitive sites and production of more monomeric, photodynamically active PpIX (27).

The results discussed indicate a potential utility of ALA-PDT in eliminating specifically activated lymphocytes. These data significantly extend our prior work on mitogen-activated cells (21), to demonstrate that the ALA-PDT can inhibit a highly specific alloantigen response, without compromising the proliferative capability to a different stimulus. The feasibility of using PDT to selectively destroy activated cells previously has been shown using different photosensitizers (28). North and colleagues used benzoporphyrin derivative (BPD) to selectively eliminate activated lymphocytes, $CD25^+$ and $HLA\text{-}DR^+$, from a population of cells (28).

The capability of selectively eliminating a subpopulation of cells from an immune response could expand the clinical utility of PDT. A new research area includes studies with various photosensitizers to treat autoimmune diseases and transplantation rejection. Ratkay, Chowdhary and coworkers used BPD-PDT to treat adjuvant-enhanced arthritis in MLR-lpr mice. Leong and colleagues delayed the development of adoptively transferred murine experimental autoimmune encephalomyelitis with BPD-PDT (31). Obochi *et al.* found prolonged murine skin allograft survival with low-dose BPD-based PDT was attributed to a decrease of both the major histocompatibility complex and expression of costimulatory molecules (B7) on Langerhans cells rather than cell death (32). The rationale for adding ALA-PDT immunotherapy to this list is based on the selectivity of ALA-PDT for activated cells under iron stress.

The selectivity of ALA-PDT for activated lymphocytes when treating tissues after transplantion is an advantage over immunosuppressive chemicals such as corticosteroids, cyclophosphamide and azathioprine that tend to be nonspecific (33). More specific treatments using polyclonal and monoclonal antibodies to selectively target cells involved in graft rejection have other drawbacks (34) that may be absent or decreased with ALA-PDT. In the meantime PpIX-PDT from exogenous ALA is a potential treatment strategy that, because of its selective action on activated lymphocytes, may be useful in the treatment of transplantation rejection and autoimmune diseases.

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