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Consequences of the photodynamic treatment of resting and activated peripheral T lymphocytes

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Abstract

The impact of the immunomodulatory photosensitizer benzoporphyrin derivative monoacid ring A (BPD-MA, verteporfin) and visible light on the survival and surface receptor pattern of resting and activated murine T cells was evaluated. T cells treated for 48 h with immobilized anti-CD3 monoclonal antibody upregulated expression of the interleukin-2 receptor α-chain (CD25), transferrin receptor (CD71), the apoptosis-regulating Fas receptor (CD95), contained a greater level of the anti-apoptotic protein Bcl-2 and accumulated significantly more BPD-MA than their unactivated counterparts. Activated T cells displayed a modestly greater susceptibility to the photodynamic induction of DNA fragmentation than resting T cells. Resting T cells treated with sub-lethal levels of BPD-MA and light did not exhibit changes in surface levels of CD3, CD4, CD8, CD28, CD45 or T cell receptor (TCR) β-chain structures. However, levels of major histocompatibility complex (MHC) class I antigens were decreased while the density of Thy-1.2 (CD90) increased on these cells. Photodynamically treated T cells failed to express optimal CD25 levels when exposed to the mitogenic anti-CD3 antibody. Activated T cells treated with sub-lethal levels of BPD-MA and light exhibited lower CD25 levels, a temporary block in cell cycle transition, but unaltered expression of MHC Class I, CD3, CD4, CD8, CD45, CD54, CD71, CD122 (IL-2R β-chain) or TCR β-chain antigens 24 h afterward. Resting and activated T lymphocytes differ in susceptibility to PDT-mediated apoptosis but both types are sensitive to anti-proliferative effects the treatment exerts at sub-lethal photosensitizer levels. The marked sensitivity of activated T cells to photodynamic inactivation likely contributes to the immunomodulatory action of BPD-MA.

Keywords: Apoptosis; Interleukin-2 receptor; Lymphocyte activation; Photodynamic therapy; Photosensitizers; T lymphocytes

Abbreviations: BPD-MA, benzoporphyrin derivative monoacid ring A; FACS, fluorescence activated cell sorter; ICAM-1, intercellular adhesion molecule-1; FITC, fluorescein isothiocyanate; IL-2R, interleukin-2 receptor; LED, light emitting diode; MHC, major histocompatibility complex; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PDT, photodynamic therapy; PE, phycoerythrin; PI, propidium iodide; PWM-SCCM, pokeweed mitogen-stimulated spleen cell-conditioned medium; TCR, T cell receptor

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1. Introduction

Photodynamic therapy (PDT) in its conventional form is a cancer treatment that utilizes light-sensitize compounds, commonly porphyrin derivatives, to achieve clinical effects (Gomer et al., 1989; Henderson and Dougherty, 1992). In comparison to cancer indications, immunologic applications of this procedure have received comparatively little attention. The porphyrin photosensitizers hematoporphyrin derivative (HpD) (Elmets and Bowen, 1986), Photofrin® (Musser and Fiel, 1991) and benzoporphyrin derivative monoacid ring A (BPD-MA, verteporfin) (Simkin et al., 1997) in combination with visible light irradiation impaired the immunologically-mediated murine contact hypersensitivity response. BPD-MA has a light absorption spectrum which permits photodynamic activation at 690 nm, a wavelength of light which effectively penetrates living tissue (Levy, 1995). BPD-MA combined with whole body red light exposure inhibited the development of experimental murine T cell-mediated autoimmune conditions including adjuvant arthritis (Chowdhary et al., 1994; Ratkay et al., 1994) and an adoptively-transferred form of autoimmune encephalomyelitis (Leong et al., 1996) without altering parameters of central immune responsiveness (Chowdhary et al., 1994; Leong et al., 1996). It is apparent that PDT may be utilized to influence immune reactivity.

No overriding mechanism to account for the immunomodulatory action of PDT has been unveiled, although evidence for the selective depletion of activated T lymphocytes has been forwarded (Obochi et al., 1995). Mitogen-activated murine splenocytes were more sensitive to photodynamic killing with HpD than quiescent spleen cells (Canti et al., 1981). Activated murine spleen cells accumulated greater amounts of BPD-MA and were more susceptible to photodynamic killing with this compound than their resting counterparts (Obochi et al., 1995). As this earlier investigation employed unseparated spleen cells as the test population, it was important to determine how BPD-MA might affect the survival of purified T lymphocytes in order to minimize possible influences of other cell types. Since T lymphocytes play a pivotal role in many aspects of immunity, it is critical to define the impact of PDT upon this cell type.

In the present work, it was found that T cells activated by antibody cross-linking of the T cell receptor (TCR) complex in vitro were more susceptible to the induction of apoptosis with BPD-MA and light than their resting counterparts. Furthermore, treatment at sub-lethal levels of BPD-MA and light influenced the expression of specific surface receptors and growth characteristics of resting and activated T cells. The photodynamic impairment of activated T lymphocytes may contribute to the immunomodulatory action of PDT.

2. Materials and methods

2.1. Animals, T cell purification and activation

Male DBA/2 mice (Charles River Canada, St. Constance Quebec) of 8–12 weeks of age served as cell donors for all experiments. Animals were kept under 12 h light:12 h dark and supplied with standard rodent laboratory food (Ralston Purina) and acidified water ad libitum. Animal maintenance was in accordance with the Canadian Council of Animal Care guidelines.

RPMI 1640 medium containing 5% heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml), all from Gibco BRL (Burlington, Ontario) with 50 μM 2-mercaptoethanol was used for all cell work. T lymphocytes were isolated by passing erythrocyte-depleted spleen cell suspensions over Type 200 L nylon scrubbed wool (DuPont Canada, Mississauga, Ontario) or T cell immuno-affinity (R&D Systems, Minneapolis, MN) columns. Preparations routinely consisted of $>85\%$ T cells and $<10\%$ B cells as indicated by their labeling with the monoclonal antibodies 145-2C11 (anti-CD3, Leo et al., 1987) and RA3-6B2 (anti-CD45R-B220, Coffman, 1982), respectively. CD4+ and CD8+ T lymphocytes were present within these preparations in a ratio of approximately 2.5:1 as determined by flow cytometric analysis. Fewer than 2% of the isolated cells labeled with the anti-MHC Class II ($I\text{-}A^d$) antibody 39-10-8 (PharMingen, San Diego, CA).

T cell activation was achieved with immobilized anti-CD3 antibody (Jenkins et al., 1990). Mono-

clonal antibody 145-3C11 (Leo et al., 1987) (Boehringer Mannheim, Dorval, Quebec) was coated onto sterile flat-bottomed polystyrene petri dishes (10 ml/plate) or 96-well microtiter plates (0.1 ml/well) at 10 μg/ml in sterile phosphate-buffered saline (PBS). Microtiter plates were employed for cytotoxicity assays while petri dishes were used to prepare activated T cells for surface antigen studies. After 3 h at 37°C, the coating solution was removed and containers were washed 3 times with PBS. T cells (5×10^5 cells/ml) were cultured in 0.2 ml (96-well plates) or 10 ml (petri dishes) volumes at 37°C under 5% CO_2. In select experiments, cultures were supplemented with recombinant human interleukin-2 (rIL-2, Amgen Biologicals, Thousand Oaks, CA). Cells were harvested by gentle swirling and removed by pipet.

2.2. Photodynamic treatment and assessment of cell viability

Liposomally-formulated BPD-MA (QLT PhotoTherapeutics, Vancouver, B.C., Canada) was reconstituted at 2.1 mg/ml with sterile, distilled water. Further dilution was with culture medium. All cell work with the photosensitizer was carried out under light-attenuated conditions. To test the impact of PDT upon T cell activation, freshly purified T lymphocytes were seeded into 96-well microtiter plates at 1×10^5 cells per well in 0.2 ml. Cells were incubated with BPD-MA at 37°C in the dark for 1 h, exposed to 690 nm light delivered from light emitting diodes (LED) (Hunt et al., 1995) and added to anti-CD3 antibody-coated plates. T cells activated for 48 h prior to PDT were treated with BPD-MA and light as described above for resting T cells except that these cells remained in the presence of the anti-CD3 antibody during all manipulations. Proliferative responses and cytotoxicity were assessed by the MTT (3-[4-,5-dimethylthiazol-2-yl]-2,4-diphenyl tetrazolium bromide, Sigma) colorimetric assay (Mosmann, 1983; Hunt et al., 1995). Replicates of four were performed at each BPD-MA concentration. Color development was terminated after 4 h at 37°C in the presence of MTT and read with an automated microtiter plate reader (Dynatech, Hamilton, VA) at a wavelength of 590 nm. Absorbance values for wells containing medium alone were subtracted from the result obtained for wells containing cells. Results are given as a percentage of the absorbance obtained for cells treated with light alone. The BPD-MA concentration required to produce a 50% reduction in cell viability (lethal dose 50%, LD_{50}) or a 50% inhibition (inhibitory concentration 50%, IC_{50}) of the response to the anti-CD3 antibody was interpolated from regression lines plotting BPD-MA concentration vs. % cell response.

Cell viability was also assessed by propidium iodide (PI) dye exclusion studies. PI is excluded from living cells. PI was added at 20 μg/ml to $\sim 1 \times 10^5$ cells and cell fluorescence was read immediately using the Elite software package with an Epics XL flow cytometer (Coulter Electronics, Hialeah, FL). The strong fluorescent signal of PI clearly distinguishes dead from live cells.

To evaluate the effect of PDT upon surface receptor expression of resting T cells, these cells were maintained for 24 h after treatment in the presence of 10% pokeweed mitogen spleen cell conditioned medium (PWM-SCCM, StemCell, Vancouver, Canada) as a source of cell maintenance factors. Cells were harvested and labeled with monoclonal antibodies as described below.

2.3. Fluorescence-activated cell sorter (FACS) analysis

To assess their purity, activation state or influence of PDT upon antigen expression, $\sim 5 \times 10^5$ cells in 0.2 ml of PBS containing 5% FCS and 0.05% NaN_3 were incubated with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies specific for MHC class I ($H\text{-}2D^d$), CD3 ε-chain (Leo et al., 1987), CD4, CD8, IL-2 receptor (IL-2R) α-chain (CD25) (Ortega et al., 1984), CD28 (Gross et al., 1992), leukocyte common antigen (CD45) (Ledbetter and Herzenberg, 1979), intercellular adhesion molecule-1 (ICAM-1, CD54) (Scheynius et al., 1993), Thy-1.2 (CD90) (Ledbetter and Herzenberg, 1979), Fas (CD95) (Ogasawara et al., 1993), IL-2R β-chain (CD122) (Tanaka et al., 1991) or the TCR β-chain (Kubo et al., 1989). A phycoerythrin (PE)-conjugated antibody against the transferrin receptor (CD71) (Kemp et al., 1987) was employed. The distribution of the structures recognized by these monoclonal antibodies is described in Table 1. PE-

Table 1
Distribution and function of the T lymphocyte surface antigens evaluated in this study

Antigen	Cellular distribution	Function
CD3 ε-chain	pan-T cell	TCR signal transduction
CD4	helper T cells	TCR co-receptor, MHC Class II recognition
CD8	cytotoxic T cells	TCR co-receptor, MHC Class I recognition
CD25 (IL-2R α-chain)	activated lymphocytes	IL-2 binding
CD28	most T cells	CD80/CD86 counter-receptor, signal transduction
CD45 (leukocyte common antigen)	pan-leukocyte	signal transduction
CD54 (ICAM-1)	activated leukocytes	cell adhesion
CD71 (transferrin receptor)	proliferating cells	iron uptake
CD90 (Thy-1.2)	pan-T cell	signal transduction?
CD95 (Fas)	activated T cells	apoptotic signaling
CD122 (IL-2R β-chain)	most T cells	IL-2 binding, signal transduction
TCR β-chain	most T cells	peptide antigen recognition
MHC Class I	all nucleated cells	peptide antigen presentation

and FITC-conjugated antigen-specific and isotype control monoclonal antibodies were from PharMingen. After 30 min on ice, cells were washed twice with buffer and fixed in 1% *p*-formaldehyde in PBS. Fluorescence signals (5000 cells analyzed) were analyzed with the flow cytometer. Dead cells and debris were gated out. Mean channel fluorescence intensity (MCFI) values (in arbitrary units) were obtained. To normalize data from different experiments, cell surface antigen levels were expressed as a percentage of the MCFI determined for cells treated with light alone. A PDT-induced change in the MCFI value of $\geq 20\%$ with a standard deviation (S.D.) less than this difference was considered significantly different from the control result. Exposure of light-protected, resting and anti-CD3 activated T cells to BPD-MA up to 200 ng/ml did not alter the labeling efficiency of the antibodies utilized in this study (data not shown).

2.4. Cell cycle analysis

T cell cycle status was evaluated using PI to stain nuclear DNA (Fleming et al., 1993). Cells (5×10^5/tube) were washed twice with PBS and treated with 1 ml of PBS containing 1% Triton X-100, PI (10 μg/ml) and DNAse-free, RNAse A (100 μg/ml), all from Sigma, for 30 min at 4°C in the dark. Fluorescence (5000 nuclei analyzed) was measured by flow cytometry on a logarithmic scale at 620 nm. The resultant histogram was divided into nuclei with 2N DNA (G0/G1 stages), ≥2N DNA (S/G2/M stages) and total DNA.

2.5. Preparation of cell extracts and detection of Bcl-2

To prepare whole cell lysates, T lymphocytes were first washed twice with ice-cold PBS. Cell pellets were treated with 1 ml of lysis buffer (1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 20 mM Tris pH 8.0 supplemented with 1 mM phenylmethylsulfonyl fluoride, aprotinin (0.15 U/ml) and 1 mM sodium orthovanadate) for 20 min on ice and centrifuged for 10 min at $15{,}800 \times g$ at 4°C. Cell extract protein concentrations were determined with the BCA™ assay (Pierce, Rockford, IL) and separated (6 μg protein/lane) by sodium dodecyl sulfate polyacrylamide gel electrophoresis in 12% gels under reducing conditions (Laemmli, 1970). Proteins were transferred to nitrocellulose membrane at 100 V for 60 min. Membranes were blocked for 30 min at room temperature in PBS with 5% skim milk powder and 0.05% Tween 20 (PBS-T) and treated for 45 min at room temperature with a rabbit IgG anti-Bcl-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1 μg/ml. Membranes were washed with PBS-T and then probed with anti-rabbit IgG-horseradish peroxidase (Santa Cruz) (1:5000 in PBS-T with 1% skim milk powder) for 30 min at room temperature. Membranes were washed extensively

with PBS-T and proteins were revealed with the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL). Blots were viewed with an HP ScanJet 4c (Hewlett Packard, Palo Alto, CA) and band densities were measured using 1D Image Analysis Software (Eastman Kodak, Rochester, NY).

2.6. BPD-MA uptake

To assess the association of BPD-MA with resting and anti-CD3 activated T cells, 5×10^5 cells were incubated with the photosensitizer (0, 10 or 100 ng/ml) in 0.5 ml of medium containing 5% FCS at 37°C in the dark for 1 h. Cells were washed once and analyzed immediately with the flow cytometer using a laser excitation wavelength of 488 nm and an emission wavelength of 690 nm (Hunt et al., 1995). MCFI values were obtained for each sample.

2.7. Detection of DNA fragmentation

DNA degradation was measured by PI staining and flow cytometry (Nicoletti et al., 1991). Three hours following light irradiation, 5×10^5 cells were washed twice with ice-cold PBS and fixed in 80% ethanol at 4°C for 1 h. Cells were washed with PBS and stained with PI (50 μg/ml) in PBS with DNAse-free RNAse (5 U/ml). Fluorescence was measured by single parameter flow cytometry. The percentage of apoptotic hypodiploid (< 2N DNA) cells was calculated from the resultant histogram. PDT-induced DNA fragmentation was calculated from the equation:

$$\text{DNA fragmentation} = \frac{\text{PDT-treated cells (\% < 2N DNA)-light-treated cells (\% < 2N DNA)}}{\text{100-light-treated cells (\% < 2N DNA)}.} \times 100.$$

3. Results

3.1. T cell characterization and induction of DNA fragmentation by PDT

Freshly purified CD3 + splenic T cells expressed low CD54, CD71 and CD95 levels (Fig. 1A). CD25 was undetectable for these cells. T cells cultured with immobilized anti-CD3 monoclonal antibody for 48 h exhibited positive surface expression of CD25, CD54, CD71 and CD95. CD3 levels were lower on anti-CD3 activated T cells than resting T cells. Time course studies demonstrated that T cell CD25 levels increased from 0 to 72 h and were declining by 96 h after exposure to the anti-CD3 antibody (data not shown). Evaluation of cell cycle status indicated that the vast majority (~ 98%) of freshly isolated T cells contained 2N amounts of DNA (Fig. 1B). After 48 h in the presence of the anti-CD3 antibody, a sizeable proportion (~ 25%) of cell nuclei contained > 2N levels of DNA. Bcl-2 levels were over 5-fold higher within extracts prepared from activated T cells than resting T cells as determined by Western immunoblot analysis and gel band image densitometry (Fig. 1C). T cells maintained in medium for 48 h did not metabolize the MTT dye, while T cells cultured with anti-CD3 antibody for 48 h exhibited strong MTT dye reduction activity (data not shown). For resting and anti-CD3 activated T cells, PDT-mediated DNA fragmentation was detected at photosensitizer concentrations from 5 to 50 ng/ml at 3 h post-irradiation (Fig. 1D). At BPD-MA concentrations of 10 or 50 ng/ml, a significantly greater proportion of activated T cells exhibited photodynamically-induced DNA fragmentation than resting T cells. Fewer quiescent T cells treated with BPD-MA at 50 ng/ml exhibited DNA fragmentation than at certain lower concentrations (10 and 20 ng/ml) of the photosensitizer. Similar levels of DNA fragmentation were produced by 3 h for light-irradiated anti-CD3 activated T cells treated with BPD-MA at 10, 20 or 50 ng/ml. PDT-induced DNA fragmentation was not detectable at this sampling time for resting or activated T cells when BPD-MA concentrations of < 5 or at 100 ng/ml were employed.

3.2. BPD-MA uptake by resting and activated T cells

BPD-MA uptake by 48 h anti-CD3 activated T cells was significantly greater than that of freshly isolated T cells as determined by flow cytometry (Fig. 2A). At 690 nm, the fluorescence strength of anti-CD3 activated T cells was approximately 2.5

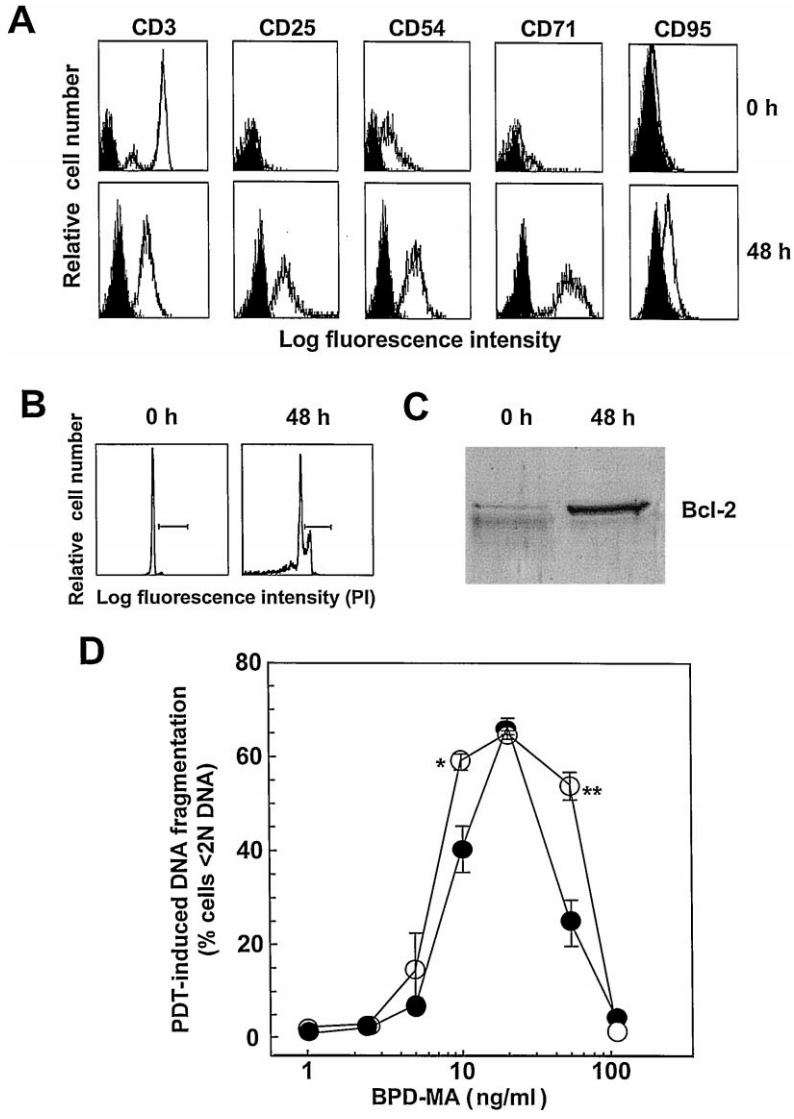

Fig. 1. Splenic T cells treated with immobilized anti-CD3 monoclonal antibody exhibit an activated phenotype. (A) Freshly isolated T cells or T cells exposed to anti-CD3 antibody for 48 h were labeled with fluorochrome-conjugated monoclonal antibodies against CD3, CD25 (IL-2R α-chain), CD54 (ICAM-1), CD71 (TrfR) or CD95 (Fas). These flow cytometric results are denoted by line tracings while those for isotype-matched control antibodies are represented by the filled areas. (B) T cell DNA status was appraised by PI staining and flow cytometry. Cell nuclei with $>2N$ amounts of DNA are denoted by horizontal bars. (C) Bcl-2 levels within lysates of freshly purified T cells (lane 1) and T cells maintained for 48 h in the presence of immobilized anti-CD3 antibody (lane 2) were determined by Western immunoblot analysis. Bands were visualized by a chemiluminescence. (D) DNA fragmentation (cells with $<2N$ DNA) was determined for quiescent (●) and anti-CD3 activated (○) T cells treated with PDT. Cells were incubated with BPD-MA for 1 h and treated with light (5 J/cm^2). DNA fragmentation levels were determined 3 h later by PI staining and flow cytometry. Mean ($\pm$S.D.) percentage of light-treated cells exhibiting $<2N$ DNA corresponded to $2.5 \pm 1.4\%$ (seven experiments) and $21.1 \pm 3.1\%$ (five experiments) for quiescent and anti-CD3 activated T cells, respectively. $* P < 0.01$; $** P < 0.001$ by unpaired Student's *t*-test.

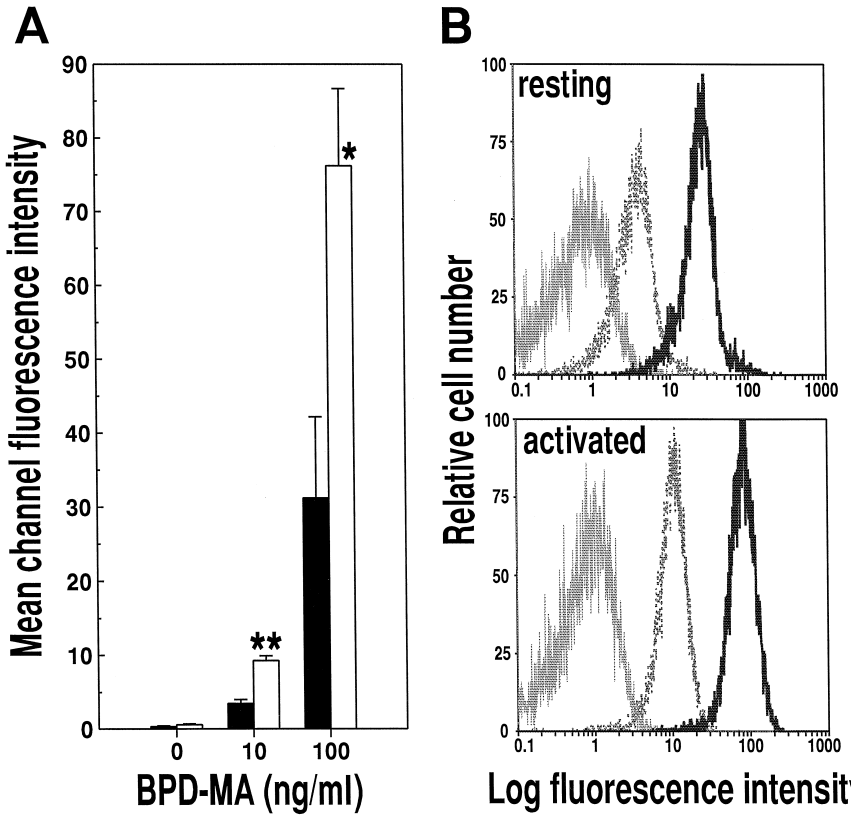

Fig. 2. (A) T cells activated for 48 h with anti-CD3 antibody accumulate greater amounts of BPD-MA than freshly isolated T cells. Cells were incubated with BPD-MA for 1 h at 37°C, washed with medium and cell-associated fluorescence determined immediately by flow cytometry at an absorbance wavelength of 690 nm. The fluorescence of cells not exposed to BPD-MA corresponds to cell autofluorescence. MCFI values were determined from three to four independent experiments. (B) Profiles of cell-associated BPD-MA fluorescence for resting and 48 h anti-CD3 activated T lymphocytes incubated with BPD-MA at 0 (light lines), 10 (hatched lines) or 100 (bold lines) ng/ml are presented. $* P < 0.005$; $** P < 0.001$ by paired Student's *t*-test.

times greater than of resting cells whether these cells were incubated with BPD-MA at 10 or 100 ng/ml. The autofluorescence of 48 h anti-CD3 activated T cells (MCFI = 0.57 ± 0.14) was modestly greater than that of freshly isolated T cells (MCFI = 0.33 ± 0.10). Representative flow cytometric profiles for BPD-MA-associated fluorescence of resting and activated T cells are presented in Fig. 2B.

3.3. Influence of PDT upon the surface receptor pattern of resting T cells

Surface TCR β-chain, CD3, CD4, CD8, CD28 and CD45 levels for unactivated T cells treated with BPD-MA and light and then maintained at 37°C for 24 h were no different than those treated with light alone (Fig. 3A). CD90 levels exhibited a dose-related increase of up to 60% at this sampling time. MHC Class I levels were approximately 20% lower after treatment at the highest concentration of BPD-MA. There was no significant difference in the level of interaction of isotype control antibodies with light-treated or PDT-treated cells. In the absence of light, BPD-MA did not affect the surface receptor expression levels of inactivated T cells (data not shown). Flow cytometric profiles of the labeling patterns obtained for CD45, CD90 and MHC Class I are shown in Fig. 3B.

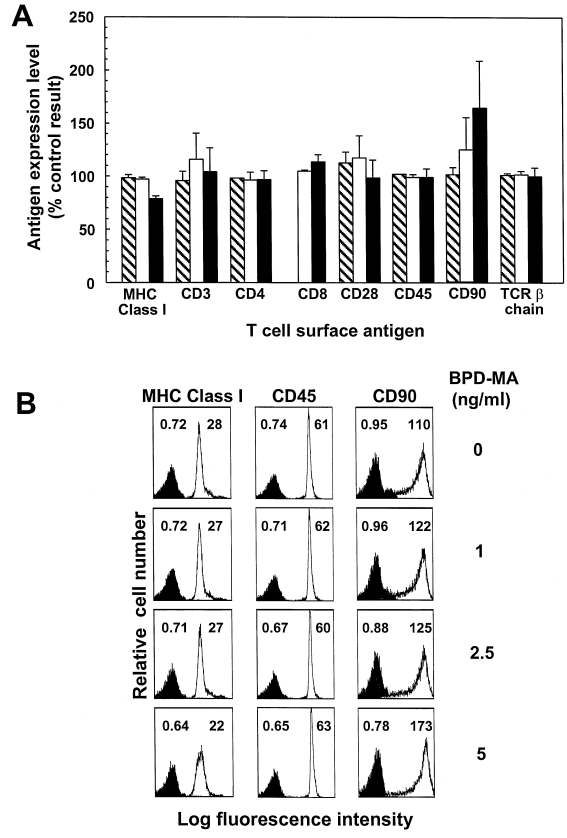

Fig. 3. Treatment of resting T cells with PDT increases surface CD90 (Thy-1.2) expression and lowers levels of MHC Class I antigens. (A) Freshly purified T cells were incubated with BPD-MA at 1 (cross-hatched), 2.5 (□) or 5 (■) ng/ml for 1 h at 37°C, exposed to 690 nm light (5 J/cm^2) and maintained for 24 h in medium containing 10% PWM-SCCM. Cells were labeled with monoclonal antibodies against different surface antigens and then analyzed by flow cytometry. Results, given as a percentage of the MCFI value obtained for cells treated with light alone, were obtained in three to four independent experiments. CD8 expression was not evaluated for cells treated at the lowest BPD-MA concentration. (B) Flow cytometric profiles for MHC Class I, CD45 and CD90 (line tracings) and the isotype control antibodies (filled areas) for T cells analyzed 24 h following PDT are shown. Results for CD45 exhibit an example of antigen expression unaltered after PDT. MCFI values obtained with the control and antigen-specific antibodies are given within each panel.

3.4. T cells treated with BPD-MA and light exhibit a reduced proliferative potential and depressed CD25 levels

For light-irradiated resting T cells treated over a limited concentration range of BPD-MA, DNA fragmentation levels produced by 3 h post-irradiation were inversely related to the capacity of these cells to proliferate in response to immobilized anti-CD3 antibody as determined by MTT dye reduction 48 h later (Fig. 4A). Maximum levels of DNA fragmentation were produced with BPD-MA at 20 ng/ml. At higher concentrations (50 and 100 ng/ml) of BPD-MA, little or no PDT-induced DNA fragmentation was detectable although these cells did not proliferate in the presence of the anti-CD3 antibody. For T cells treated with PDT prior to exposure to anti-CD3 antibody, the IC_{50} for BPD-MA corresponded to 3.5 ± 0.3 ng/ml (three experiments), as determined by the MTT method. Cell viability was preserved at higher concentrations of BPD-MA ($IC_{50} = 6.2 \pm 0.3$ ng/ml, three experiments) as assessed by PI dye exclusion studies. In comparison to T cells treated with light alone prior to their exposure to anti-CD3 antibody, CD25 levels of PDT-treated cells were lower in a drug dose-related manner, resulting in approximately 75% lower CD25 levels at the highest concentration (5 ng/ml) of BPD-MA (Fig. 4B). Representative flow cytometric profiles for these studies are shown (Fig. 4C). For light-irradiated T cells treated with BPD-MA at 1 and 2.5 ng/ml, but not 5 ng/ml, supplementation with rIL-2 was associated with CD25 expression levels equivalent to those of light-treated control cells after 48 h in the presence of anti-CD3 antibody.

3.5. Influence of PDT on surface antigen expression and cell cycle status of anti-CD3 activated T cells

For 48 h anti-CD3 activated T cells, the LD_{50} for BPD-MA corresponded to 3.3 ± 0.9 ng/ml (five independent experiments) as determined by MTT assays performed 24 h after light irradiation. For these experiments, assay absorbance (590 nm) values for light-treated anti-CD3 activated T cells averaged 0.656 ± 0.119 above background levels (~ 0.100). TCR β-chain, CD3, CD4, CD8, CD45, CD71, IL-2R β-chain (CD122) and MHC Class I expression levels of anti-CD3 activated T cells treated with BPD-MA and light 24 h previously were no different than those of light-irradiated T cells (Fig. 5A). CD95 expression was modestly higher on PDT-treated cells while CD25 levels were lower in a drug dose-related

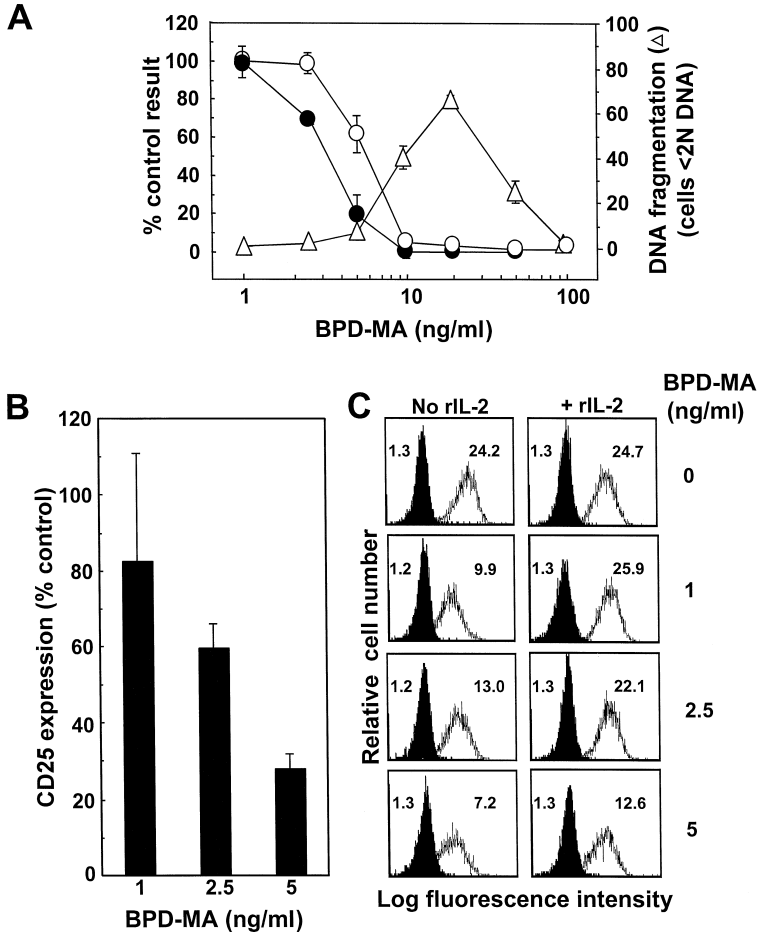

Fig. 4. Resting T cells treated with BPD-MA and light exhibit reduced proliferation and depressed CD25 levels. (A) Freshly-purified T cells were treated with BPD-MA and light (5 J/cm^2) and seeded into microtiter plate wells pre-coated with anti-CD3 antibody. DNA fragmentation (△) was assessed 3 h post-irradiation (seven experiments). Cell responses were assessed 48 h post-irradiation by MTT dye reduction (●) and PI dye exclusion (○) assays performed in parallel. For cells pretreated with light alone (100% response), PI studies indicated that $62.5 \pm 2.6\%$ were viable while absorbance (590 nm) values for these T cells averaged 0.781 ± 0.203 above background (~ 0.100) by MTT assay (three experiments). (B) CD25 expression by T cells pretreated with PDT and cultured with anti-CD3 antibody for 48 h was evaluated by flow cytometric analysis. Results are given as the percentage of the result obtained with cells treated with light alone (MCFI $= 26.9 \pm 8.0$ units, four experiments). (C) CD25 expression for T cells treated with light alone or BPD-MA and light 48 h before followed by exposure to immobilized anti-CD3 antibody without or with rIL-2 (200 U/ml) was assessed. Cells were stained with anti-CD25 (line tracings) or the isotype control antibody (filled areas). MCFI values obtained with these reagents are given in each panel. Results from one of four experiments are shown.

manner 24 h after PDT, resulting in approximately 40% lower CD25 levels at the highest concentration of BPD-MA. CD25 expression levels of anti-CD3 activated T cells approximated control MCFI values by 48 h after the photodynamic treatment (Fig. 5B). There was no difference in the degree of interaction of the control antibodies with light or PDT-treated anti-CD3 activated T cells. In the absence of light, BPD-MA did not affect the surface receptor expression levels of anti-CD3 activated T cells (data not shown). The extremely bright expression of CD90 by these cells prevented reliable assessment of the influ-

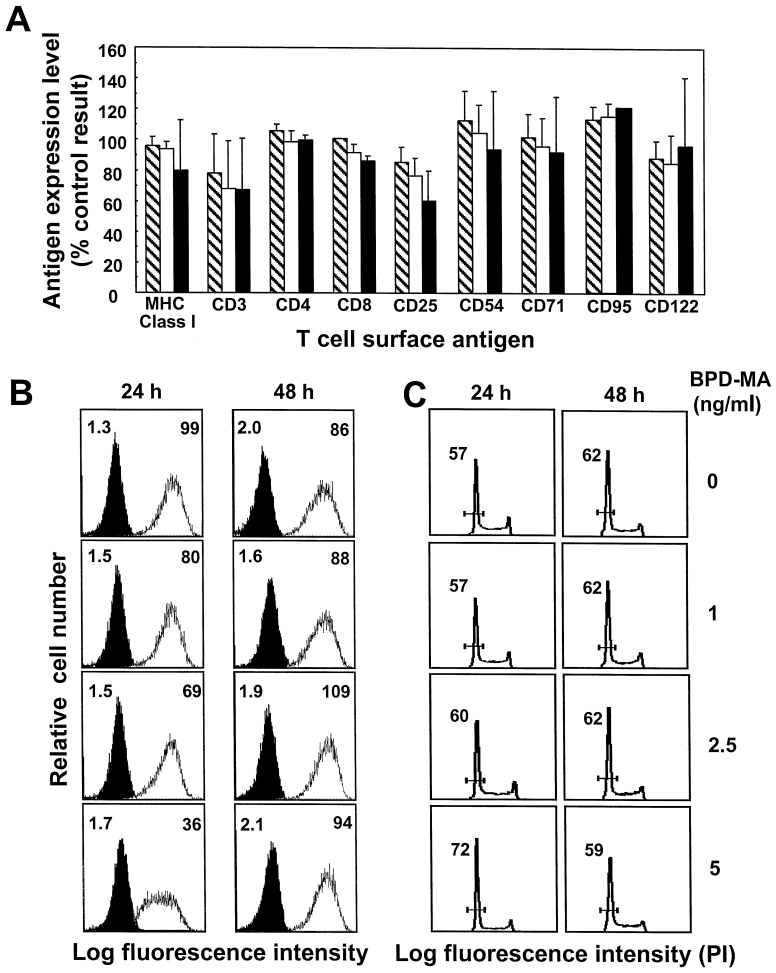

Fig. 5. The photodynamic treatment of anti-CD3 activated T lymphocytes does not alter CD3, CD4, CD8, CD28, CD45, CD54, CD71, CD122 or MHC Class I expression but results in depressed CD25 levels, a modest elevation in CD95 expression and a transient block in cell cycle progression. (A) Activated T cells treated with BPD-MA at 1 (cross-hatched), 2.5 (□) or 5 (■) ng/ml and light (5 J/cm^2) were labeled 24 h later with monoclonal antibodies against mouse T cell surface structures and analyzed by flow cytometry. Relative antigen expression levels (means with S.D.) are given as a percentage of the MCFI value of cells treated with light alone (three to ten independent experiments). For anti-CD3 activated T cells treated with light, MCFI values for CD25 expression levels averaged 69.3 ± 26.6 units (10 experiments). (B) Flow cytometric results for 48 h anti-CD3 activated T cells treated with BPD-MA and light 24 or 48 h before and labeled with the isotype control antibody (shaded areas) or anti-CD25 (line tracings) and their respective MCFI values are presented. (C) Cell cycle analyses were performed on T cells treated with BPD-MA and light 24 or 48 h previously using PI staining and flow cytometric analysis. The percentage of nuclei residing in the G0/G1 phases of the cell cycle (2N DNA) is given within each panel and this region is denoted by horizontal bars.

ence of PDT upon levels of this marker. In comparison to light-irradiated anti-CD3 activated T cells, a greater percentage of T cells treated with BPD-MA and light were in the G0/G1 phases of the cell cycle upon evaluation 24 h post-PDT (Fig. 5C). At 48 h post-PDT, the DNA profile of photodynamically-treated cells was indistinguishable from that of cells treated with light alone, with all groups having comparable percentages of cells with 2N amounts of DNA.

4. Discussion

T cells treated with anti-CD3 antibody, but not freshly purified T cells, expressed abundant CD25, CD54, CD71 and CD95 levels, representative of an activated T cell phenotype (Kemp, 1993; Scheynius et al., 1993; Nagata, 1997). In comparison to freshly isolated T cells, a much greater proportion of anti-CD3 activated T cell nuclei contained $>2N$ amount of DNA, indicative of a proliferating cell population. In agreement with a study in which splenocytes were activated with concanavalin A (Obochi et al., 1995), anti-CD3 activated T cells took up significantly more BPD-MA and were more sensitive to the induction of apoptosis upon light irradiation than their resting counterparts. BPD-MA associates with plasma low density lipoproteins (LDL) (Allison et al., 1994) and a greater density of LDL receptors on proliferating cells (Ho et al., 1979) may augment cellular delivery of the photosensitizer and increase the potential for photodynamic cell killing.

It has been determined that mitogen-activated human T cells accumulate higher amounts of the endogenous photosensitizer protoporphyrin IX (PPIX) than their resting counterparts following incubation with the heme precursor δ-aminolevulinic acid (Hryhorenko et al., 1998). This feature contributed to the depletion of the activated T cell type upon light irradiation (Hryhorenko et al., 1998). The differential impact of PDT against resting and activated T cells might possibly be related to an undiscovered capacity of BPD-MA to chelate intracellular iron thereby altering the fluorescence and photodynamic properties of the porphyrin. The fluorescent and photodynamic features of PPIX, a molecule with structural similarity to BPD-MA, are abolished following the insertion of iron into the parent structure (Hryhorenko et al., 1998). Upregulation of surface CD71 expression on the activated T cell reflects increased iron demand by these cells (Kemp, 1993). Lower amounts of available iron within activated T cells may serve to maintain the fluorescent and photodynamic properties of BPD-MA while higher levels of chelatable iron within resting T cells could have an attenuating effect upon these parameters.

Mitochondrial damage is widely documented for PDT-treated cells and many photosensitizers localize to this organelle (Gomer et al., 1989; Henderson and Dougherty, 1992). It has been suggested that mitochondrial peripheral benzodiazepine receptor (PBR) density directly correlates with cell sensitivity to photodynamic killing with porphyrin photosensitizers (Verma et al., 1998). Porphyrins, as well as other classes of molecules, avidly bind the PBR associated with the outer membrane of mitochondria (Verma et al., 1987), an organelle closely involved in the regulation of apoptotic cell death (see below). Although mitochondrial PBR density closely parallels that of Bcl-2 during normal T cell development (Carayon et al., 1996), it is undetermined whether PBR levels are modulated during T cell activation. The link between the mitochondrial PBR and the cytotoxic effect of porphyrin photosensitizers requires full elucidation.

Apoptosis ensues following the treatment of cells with diverse cytotoxic stimuli (Nagata, 1997; Hannun, 1997) including PDT (Agrarwal et al., 1991; Granville et al., 1997). Cellular evidence for this tightly controlled process is commonly provided by the presence of a regular DNA fragmentation pattern in agarose gels, corresponding to sub-diploid DNA levels as determined by flow cytometric analysis (Nicoletti et al., 1991). Human myeloid leukemia HL-60 cells treated with lethal levels of BPD-MA and light rapidly mobilized caspase-3, a member of a pro-apoptotic family of cysteine proteases, leading to the degradation of key intracellular proteins and DNA fragmentation (Granville et al., 1997). While it is uncertain whether all photosensitizers can induce apoptotic cell death (Luo et al., 1996), treatment of mouse T cells at defined cytotoxic levels of BPD-MA and light, as described in the present work, likely triggers the caspase pathway, since DNA fragmentation during apoptosis ensues as a direct consequence of caspase activation (Casciola-Rosen et al., 1996).

Anti-CD3 activated T cells contained a greater level of the anti-apoptotic protein Bcl-2 than resting T cells, consistent with the increased expression of Bcl-2 described for human lymphocytes (Reed et al., 1987) and mouse splenic T cells (Broome et al., 1995) exposed to activating stimuli. The position of Bcl-2 within the outer mitochondrial membrane influences cell survival by governing the intracellular distribution of pro-apoptotic factors (Kroemer, 1997). Enforced over-expression of Bcl-2 protects different cell types from apoptosis induced by various cytotoxic agents (Sentman et al., 1991). The modestly

greater sensitivity to DNA fragmentation of activated T cells, despite elevated Bcl-2 expression, suggests that additional regulatory factors influence the survival of photodynamically-treated T cells. Relative levels of other Bcl-2 family members of apoptosis-regulating factors (Reed, 1997) may be important in this regard. At relatively high cytotoxic concentrations of BPD-MA, fewer T cells exhibited DNA fragmentation after photoirradiation than at lower yet still cytotoxic concentrations of the photosensitizer. Outer membrane integrity may be rapidly compromised after the light treatment of T cells exposed to higher amounts of BPD-MA and the caspase pathway may not be invoked and necrotic death ensues. In comparison to quiescent T cells, a greater proportion of anti-CD3 activated T cells exhibited DNA fragmentation at a higher concentration of BPD-MA after light treatment. It is uncertain whether this dose effect is related to differences in membrane stability or the intracellular distribution of BPD-MA between these T cell forms.

Resting T cell levels of CD3, CD4, CD8, CD28, CD45 and TCR β-chain were unchanged 24 h after PDT at sub-lethal levels of BPD-MA and light. MHC Class I density was modestly diminished while CD90 expression levels were increased after this photodynamic treatment. All of the above markers, with the exception of CD90, are transmembrane proteins. The carboxy-terminus of CD90 is anchored within the cell membrane by a glycosylphosphatidylinositol moiety and molecules of this type exhibit greater lateral mobility within the membrane than transmembrane proteins (Robinson, 1991). Membrane perturbations produced by PDT (Girotti, 1990) could conceivably affect the distribution and density of CD90 on the cell surface.

Resting T cells treated with sub-lethal levels of BPD-MA and light exhibited deficient proliferative responses and low CD25 expression following exposure to the mitogenic anti-CD3 antibody. For these cells, high levels of DNA fragmentation detected at 3 h post-treatment corresponded to a complete loss of viability and an absence of proliferation as determined 48 h later. Importantly, at lower levels of BPD-MA, photodynamically-treated T cells remained viable after a treatment which lead to little or no proliferation. Oxidative stress provided by hydrogen peroxide or tumor-associated macrophages reduced CD3 ζ-chain, but not CD3 ε-chain, levels of mouse splenic T cells and responses of these cells to a specific antigen (Otsuji et al., 1996). Although CD3 ζ-chain levels were not evaluated, the sub-optimal responses produced by photodynamically-treated T cells was not associated with altered CD3 ε-chain levels, a change which might have affected the capacity of these cells to respond to the anti-CD3 antibody. Addition of rIL-2 to cultures of T cells previously treated with PDT was partially effective at restoring CD25 levels indicating that PDT-treated T cells may have a reduced capacity to elaborate IL-2. Pre-treatment of human blood MNC with nanomolar levels of PPIX and ultraviolet A light suppressed mitogen and rIL-2-stimulated proliferation by preventing up-regulation of the IL-2R complex (Barrett et al., 1994). PPIX and BPD-MA have similar molecular configurations and may produce comparable cellular effects as a result of the oxidative stress the photoactivation of these compounds places upon immune cells.

The lower CD25 expression levels of anti-CD3 activated T cells treated with BPD-MA and light was a notable effect since no alteration in surface density of MHC Class I, CD3, CD4, CD8, CD54, CD71, CD122 or TCR β-chain was observed. This dose-related effect upon these cells was transient and CD25 expression levels as well as the cell cycle status returned to control values by 48 h post-PDT. Binding of IL-2 to its multimeric αβγ high affinity receptor profoundly influences T lymphocyte biology by promoting the induction of nuclear proto-oncogenes, cell proliferation and differentiation (Minami et al., 1993). A depression in T cell CD25 expression produced by PDT would presumably lower IL-2R signaling activity, slow cell cycle transit and limit T cell differentiation. Treatment of whole blood from patients infected with the human immunodeficiency virus with BPD-MA and light reduced the proportion of MNC which expressed CD25 suggesting a selective depletion of activated lymphocytes from these samples (North et al., 1993). These findings could also be partially explained by a dampening of CD25 expression produced by the photodynamic treatment. CD95 expression levels of activated T cells were modestly increased 24 h following photodynamic treatment. Upregulated CD95 expression may lower the cellular threshold to apoptosis upon interaction

with cells expressing its counter receptor, Fas-ligand (Nagata, 1997). Relationships between PDT and CD95 signaling in the induction of T lymphocyte apoptosis are under study within the laboratory (Jiang et al., submitted manuscript).

PDT with BPD-MA may produce beneficial outcomes in autoimmune conditions through an accentuated accumulation in activated autoreactive T lymphocytes and the induction of cell death at low nanomolar drug levels upon photoirradiation. Furthermore, at sub-lethal photosensitizer and light levels, the proliferation and differentiation of this T cell form may be restricted. The present work indicates that activated T cells may be selectively targeted for photodynamic inactivation even in the setting of upregulated expression of the anti-apoptotic factor Bcl-2. This attribute may contribute to the effectiveness of PDT for the treatment of human autoimmune disease.

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