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Selective Depletion of a Thymocyte Subset *in Vitro* with an Immunomodulatory Photosensitizer

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Conventional photodynamic therapy (PDT) utilizes light-absorbing compounds that have anti-cancer activity upon visible light irradiation. PDT has also been utilized for the treatment of certain immune conditions. To further understand the action of PDT upon immune cells, DBA/2 mouse thymocytes were treated with the photosensitizer benzoporphyrin derivative monoacid ring A (BPD-MA, verteporfin) and/or an apoptosis-inducing anti-Fas (APO-1, CD95) monoclonal antibody. Nanomolar levels of BPD-MA in combination with nonthermal visible light irradiation rapidly induced apoptosis as gauged by DNA fragmentation assays. Thymocytes were modestly more sensitive to PDT-induced apoptosis than mature splenic T cells. BPD-MA and light or the anti-Fas antibody decreased $CD4^+CD8^+$ cell numbers while relatively sparing $CD4^-CD8^-$, $CD4^+CD8^-$, and $CD4^-CD8^+$ thymocytes. In combination, anti-Fas antibody and PDT augmented activity levels of the apoptosis-related protease caspase-3, cleavage of the caspase-3 substrate poly-(ADP) polymerase, and the proportion of cells exhibiting DNA fragmentation and further impacted $CD4^+CD8^+$ thymocyte survival. Although $CD4^+CD8^+$ thymocytes had the greatest sensitivity to photodynamic depletion, BPD-MA was taken up by the other major thymocyte subsets with equal or greater avidity. Since $CD4^+CD8^+$ thymocytes are selectively impacted by PDT and anti-Fas antibody can act in concert with PDT to further cytotoxicity, thymocytes may be useful for the identification of factors that govern immune cell susceptibility to this form of phototherapy.

Key Words: **apoptosis; benzoporphyrin derivative; Fas; photodynamic therapy; thymocytes.**

INTRODUCTION

Photodynamic therapy (PDT) engages the photophysical properties of certain light-absorbing compounds to generate cytotoxic effects within target tissues by catalyzing the formation of reactive oxygen intermediates, particularly singlet oxygen (1). In combination with visible light, porphyrin- and phthalocyanine-based photosensitizers induce apoptotic cell death in different tumor cell lines (2–4). Importantly, these photosensitizers are nontoxic in the absence of light. PDT can also be used in nononcologic indications (5). In mice, PDT inhibits responses to topically applied haptens (6, 7) and limits pathogenic changes associated with autoimmune conditions including adjuvant arthritis (8) and experimental autoimmune encephalomyelitis (9). Further, PDT has been used for the treatment of psoriasis, a human condition with an immune basis (5). PDT may affect these immunologically mediated events by influencing the function and/or survival of antigen-presenting cells and effector T lymphocytes (6–12). Mitogen- and anti-CD3-activated murine splenic T cells accumulate higher amounts of photosensitizer and are more susceptible to PDT than their unactivated counterparts (10, 11). These data suggest that depletion of activated T cells may contribute to the immunomodulatory activity of PDT.

Bone-marrow-derived T lymphocyte precursors undergo selection and maturation processes in the thymus to yield cells with rearranged T cell receptor (TCR) genes that define the antigen recognition specificity of these cells (13). Thymocyte maturation proceeds on the basis of selection events that ultimately yield mature T lymphocytes that lack reactivity to host antigens but have the capacity to interact with self major histocompatibility complex molecules. Thymocyte maturation is traceable by the expression of specific surface receptors as well as the general topographical position of these cells (14). Within the thymic subcapsular region, immature $CD3^-$ T cells do not express either CD4 or CD8 (13, 14). Thymocytes subsequently coexpress both molecules (double-positive thymocytes) at the time of TCR gene rearrangement within the cortical zone (13, 14). Further maturation steps yield $CD4^+CD8^-$ (helper) or $CD4^-CD8^+$ (cytotoxic) single-positive T cells that move into the medulla region prior to their release into the

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circulation (13, 14). Deletion of self-reactive thymocytes occurs at the $CD4^+CD8^+$ stage (15, 16). These cells are removed by an apoptotic process (17).

Apoptosis is a tightly regulated form of cell death, characterized by distinct morphologic and biochemical changes including membrane blebbing, cell shrinkage, and DNA fragmentation (18, 19). Many apoptosis-related events are attributable to the action of a group of intracellular cysteine proteases, with cleavage specificity for proteins at aspartic acid residues, that are activated during the death process (18, 19). Members of this family of enzymes have been termed caspases (*c*ysteinyl *asp*artate-specific protein*ases*) (19). Various exogenous agents including anti-TCR complex antibodies, glucocorticoids, and γ-irradiation induce apoptosis in normal mouse thymocytes (18, 20). Apoptosis may be triggered by the binding of the Fas receptor (APO-1, CD95) with its natural ligand (FasL) (21) or agonistic anti-Fas antibodies (22). Fas is expressed by many different cell types and its presence signifies that these cells may be receptive to apoptosis-inducing signals from FasL-bearing cells (21). In the periphery, Fas–FasL interactions serve to limit the proliferation of activated T cells, promote the lysis of virally infected cells by cytotoxic T cells, and contribute to the maintenance of a state of immune privilege in different tissues by imperiling the survival of activated inflammatory cells (18, 19). Although most thymocytes express Fas, and $CD4^+CD8^+$ thymocytes lose viability when exposed to specific anti-Fas monoclonal antibodies, $CD4^+CD8^-$, $CD4^-CD8^+$, and $CD4^-CD8^-$ thymocytes generally tolerate this treatment (23, 24). It is generally recognized that agonistic anti-Fas antibodies may mimic the action of FasL for the induction of apoptosis (18).

In contrast to its impact upon mature T cell populations (10, 11), no information is available regarding the influence of PDT upon immature T cell populations. The present study examined whether thymocyte subsets might differ in their sensitivity to PDT. The possibility that Fas ligation might act in concert with the photodynamic effect upon these cells was also appraised.

MATERIALS AND METHODS

Animal maintenance and cell isolation. Male DBA/2 mice (Charles River Canada, St. Constance, Quebec) of 8–10 weeks of age were held under 12-h light:12-h dark and supplied with standard rodent laboratory food and acidified water *ad libitum*. Animals were maintained in direct accordance with the guidelines set by the Canadian Council of Animal Care. 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), and 50 μM 2-mercaptoethanol was used for all cell work. Thymocyte suspensions were made by pressing the tissue through a stainless-steel mesh with a 3-ml syringe plunger.

T cells were isolated by the passage of erythrocyte-depleted spleen cell suspensions over T cell immunoaffinity (R&D Systems, Minneapolis, MN) columns. Preparations routinely consisted of $>$90% T cells and $<$5% B cells as indicated by their labeling with monoclonal antibodies against CD3 (25) and CD45R-B220 (26), respectively. Viable cell numbers were determined by trypan blue dye exclusion.

Photodynamic treatment and assessment of cell viability. Liposomally formulated benzoporphyrin derivative monoacid ring A (BPD-MA, verteporfin) (27) (QLT PhotoTherapeutics, Inc., Vancouver, B.C., Canada) was reconstituted in sterile, distilled water. Further dilution was with culture medium. Cells were handled in the presence of the photosensitizer under low light conditions. To assess their sensitivity to PDT, 2×10^5 thymocytes were seeded into quadruplicate wells of 96-well microtiter plates in 0.2 ml of culture medium. Cells were incubated with BPD-MA for 1 h at 37°C in the dark and then exposed to 690 nm light (5 J/cm^2) delivered from light-emitting diode panels. Plates were returned to the incubator overnight and cell survival was assessed by the MTT 3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide (Sigma Chemical Co., St. Louis, MO) colorimetric assay (28). Color development was terminated after 4 h in the presence of MTT at 37°C. Color intensity was measured with a 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 with the test wells.

Influence of anti-Fas antibody on thymocyte and T cell viability. To assess their sensitivity to a Fas-directed stimulus, thymocytes or T cells were incubated with hamster IgG monoclonal antibodies (Pharmingen, San Diego, CA) against mouse Fas (22) or the unrelated antigen trinitrophenol. To evaluate their sensitivity to the anti-Fas antibody in combination with PDT, thymocytes were incubated with BPD-MA for 1 h and either light-protected or exposed to light. Cells were then maintained for 24 h at 37°C without or in the presence of hamster IgG or the anti-Fas monoclonal antibody. Cell viability was assessed by the MTT assay.

DNA fragmentation assay. The state of cellular DNA was assessed with a propidium iodide (PI, Sigma) fluorescence technique (4, 29). Cells (5×10^5) were washed twice with ice-cold PBS and fixed in 80% ethanol at 4°C for 1 h (4). Cells were washed with PBS and stained with PI (50 μg/ml) in PBS containing DNAse-free RNAse (5 U/ml, Sigma). Cell-associated PI fluorescence was measured using the Elite software package and an Epics XL flow cytometer (Coulter Electronics,

Inc., Hialeah, FL). The percentage of apoptotic hypodiploid (<2N DNA) cells was calculated from the resultant histogram.

Fluorescence-activated cell sorter (FACS) analysis. To define their subset, thymocytes (5×10^5) in 0.2 ml of phosphate-buffered saline, pH 7.3, containing 5% FCS, 0.05% NaN_3, were incubated with phycoerythrin (PE)-conjugated anti-CD4 and fluorescein isothiocyanate (FITC)-conjugated anti-CD8 rat monoclonal antibodies (Pharmingen). Fas expression was assessed with FITC-conjugated monoclonal antibody Jo2 (22). Isotype control monoclonal antibodies were from Pharmingen. Cells were incubated with antibody for 30 min on ice, washed twice with buffer, and fixed in PBS containing 1% *p*-formaldehyde. Cell fluorescence was analyzed by flow cytometry. Dead cells and debris were gated out and this procedure was verified by PI dye uptake studies. For CD4/CD8 studies, cell fluorescence was evaluated with two-color flow cytometric analysis using appropriate signal compensation techniques. The percentage of cells within each thymocyte subset (10,000 cells analyzed) was determined. Mean channel fluorescence intensity (MCFI) values (in arbitrary units) for thymocyte Fas expression levels (5000 cells analyzed) were recorded.

BPD-MA uptake. To gauge the association of BPD-MA with the major thymocyte subsets, freshly prepared thymocytes were incubated without or with BPD-MA (140 nM) for 1 h at 37°C in 0.2 ml of medium in 96-well microtiter plates at 2×10^5 cells per well. Cells were washed twice with FACS buffer and treated with fluorochrome-conjugated anti-CD4 and anti-CD8 monoclonal antibodies as described above. Thymocytes (10^5 events per sample) were read immediately on the flow cytometer using three-color analysis and appropriate color compensation procedures. BPD-MA fluorescence was read at 690 nm, an emission wavelength distinct from those of FITC and PE. MCFI values for cell-associated BPD-MA were obtained for each thymocyte subset and compared to the result obtained for cells not incubated with BPD-MA, but labeled with the anti-CD4 and anti-CD8 antibodies.

Preparation of cell lysates and determination of caspase-3 activity. For the preparation of cell extracts, thymocytes were treated within 6-well culture plates at 1.8×10^6 cells/ml (10 ml/well). Lysates were prepared by first washing the cells twice with ice-cold PBS. The cell pellet was treated with 0.4 ml of a buffer containing 1% Nonidet P-40, 10% glycerol, 20 mM Tris, pH 8.0, 137 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, aprotinin (0.15 U/ml), and 1 mM sodium orthovanadate for 20 min on ice (4). All these reagents were from Sigma. Extracts were centrifuged for 10 min at 15,800*g* at 4°C.

Protease assays were performed by the addition of 50 μl of thymocyte lysate to 100 μl of cell lysis buffer containing 40 μM of the caspase-3 substrate acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin (Ac-DEVD-AMC, Calbiochem, Cambridge, MA) (4). Following 4 h at 37°C, fluorescence was measured with a CytoFluor 2350 (PerSeptive Biosystems, Burlington, ON) set at excitation and emission wavelengths of 380 and 460 nm, respectively.

Western immunoblot analysis. The status of poly-(ADP-ribose) polymerase (PARP) was evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Thymocyte lysates (1×10^7 cell equivalents per lane) were separated within 10% gels and transferred to nitrocellulose (4). Membranes were blocked with 5% skim milk powder in PBS–Tween (0.05%) for 30 min. Rabbit polyclonal anti-mouse PARP antibody (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA) was added at 1 μg/ml for 45 min at room temperature. Membranes were again washed and treated for 30 min with a goat anti-rabbit IgG horseradish peroxidase conjugate (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:5000 in blocking buffer. Proteins were detected using the enhanced chemiluminescence assay system (Amersham Canada Limited, Oakville, ON) and bands visualized by autoradiographic techniques. The PARP p85 fragment was viewed with a HP ScanJet 4c (Hewlett Packard, Palo Alto, CA) and band densities were measured using 1D image analysis software (Eastman Kodak Company, Rochester, NY).

Statistical analysis. Treatment effects were compared using two-way analysis of variance and the Bonferroni multiple comparison test (NCSS Statistical Software, Kaysville, UT). The paired Student *t* test was employed to compare group means where appropriate. A probability (*P*) value of <0.05 was used to demarcate statistical significance. Means with standard deviations (SD) are given in figures.

RESULTS

Impact of BPD-MA and Light on Thymocyte DNA Fragmentation

Approximately 10% of thymocytes treated with medium, light, or BPD-MA alone 3 h before contained a subdiploid amount of DNA (Fig. 1A). However, almost twice as many cells incubated with BPD-MA and exposed to light 3 h previously had subdiploid DNA levels. After 24 h of culture, about one-third of cells treated with medium, light, or BPD-MA alone exhibited DNA fragmentation. Although mouse thymocytes display a propensity to undergo apoptosis *in vitro* (30), treatment with PDT led to a twofold increase in the

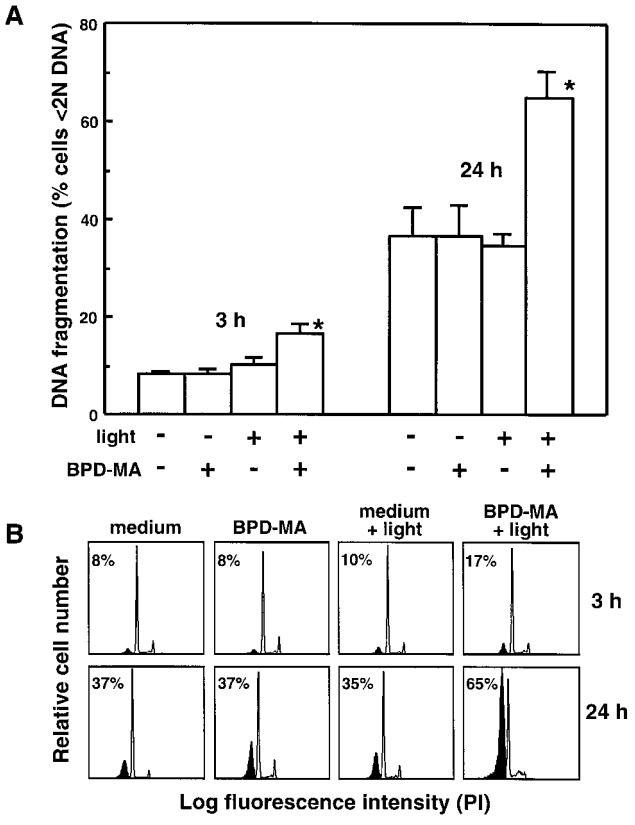

FIG. 1. PDT induces thymocyte DNA fragmentation. Thymocytes were incubated in the presence or absence of BPD-MA (3.5 nM) for 1 h at 37°C then either light-protected or exposed to 690 nm light (5 J/cm^2). DNA fragmentation was assessed 3 or 24 h later by PI staining and flow cytometry. (A) The percentage of cells containing subdiploid amounts of DNA was determined for thymocytes prepared from three individual mice. (B) Representative flow cytometric profiles for thymocyte DNA at 3 and 24 h posttreatment are shown. Events within the filled peak on the left side of each histogram represent cells containing subdiploid amounts of DNA. The percentage of apoptotic cells is given within each histogram. * $P < 0.05$ in comparison to the result for all other treatment groups at this sampling time.

number of cells with subdiploid DNA levels. Flow cytometric profiles of thymocyte DNA staining patterns are presented in Fig. 1B.

Sensitivity of Thymocytes and T Cells to PDT and Fas-Induced Apoptosis

Thymocyte CD3 expression was bimodal with most cells bearing low levels of this antigen while a small proportion of these cells had relatively high CD3 levels (Fig. 2A). Purified splenic T cells expressed uniformly high CD3 levels. Most thymocytes expressed Fas antigen while about one-half of the T cells expressed this marker and at a relatively low level. Light-irradiated thymocytes exhibited DNA fragmentation at lower concentrations of BPD-MA than splenic T cells (Fig. 2B). With higher BPD-MA concentrations, low DNA fragmentation levels were present. At a 140 nM concentration of BPD-MA, little or no PDT-induced DNA fragmentation was detected for either cell type. Nevertheless, at photosensitizer levels of ≥15 nM, overall thymocyte and T cell viability was totally compromised, as determined by MTT assays performed 24 h post-PDT (data not shown).

Combined Effect of PDT and Anti-Fas Antibody

Thymocyte viability was reduced in a drug dose-dependent fashion by approximately 60% 24 h follow-

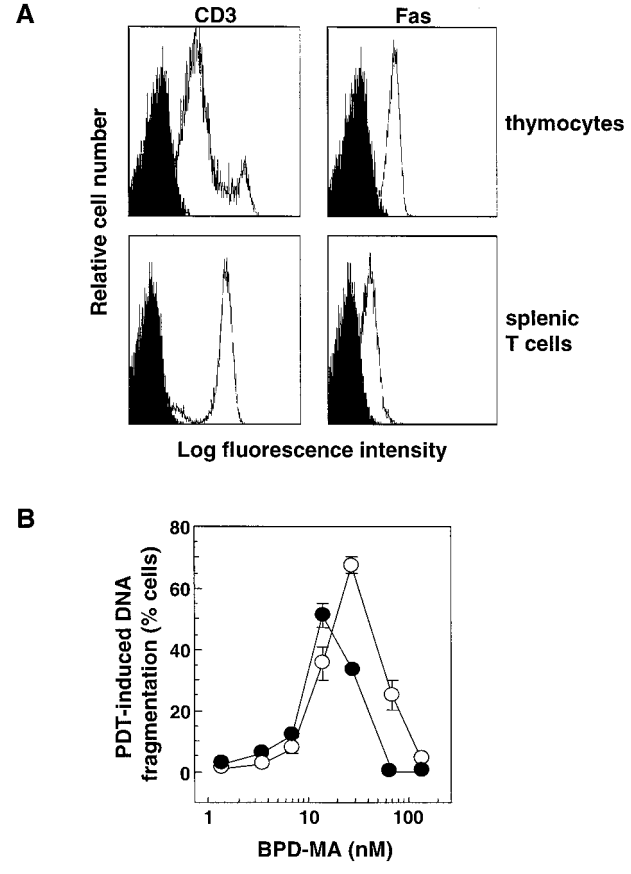

FIG. 2. Thymocytes display modestly greater sensitivity to PDT-induced DNA fragmentation than splenic T cells. (A) Freshly prepared thymocytes and purified splenic T cells were characterized with hamster IgG monoclonal antibodies against CD3 and Fas (line tracings). The flow cytometric result obtained with the hamster IgG control antibody is represented by the filled areas. (B) To assess their sensitivity to PDT-induced apoptosis, cells were incubated with different amounts of BPD-MA for 1 h at 37°C and then irradiated with light. DNA status was evaluated 3 h later by PI staining and flow cytometric analysis. To determine the percentage of cells exhibiting PDT-induced DNA fragmentation, the value obtained for thymocytes (12.1 ± 2.8%, $n = 12$) and T cells (2.8 ± 2.1%, $n = 12$) treated with light alone was subtracted from each experimental value.

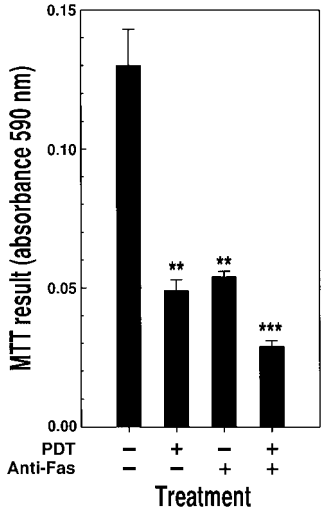

FIG. 3. Anti-Fas antibody adds to the cytotoxic effect of PDT upon thymocyte viability. Thymocytes were treated with BPD-MA (3.5 nM) and 690 nm light (5 J/cm^2) and then maintained with or without the anti-Fas monoclonal antibody Jo2 at 5 μg/ml. Cell survival was assessed by the MTT colorimetric assay 24 h posttreatment. Results from one of two identically performed experiments yielding highly similar findings are shown. ** $P < 0.01$ in comparison to the result for thymocytes maintained in medium. *** $P < 0.01$ in comparison to the result obtained with all other treatments.

ing treatment with BPD-MA and light as determined by MTT dye reduction (Fig. 3). Addition of the anti-Fas antibody reduced thymocyte viability by a similar amount. The hamster IgG control antibody (5 μg/ml) did not affect thymocyte viability (data not shown). Addition of anti-Fas antibody to cultures of thymocytes treated with BPD-MA and light reduced thymocyte MTT dye reduction activity by a further 20%, a statistically significant ($P < 0.01$) effect. Splenic T cell viability was not significantly affected by a 24-h culture with either the control or the anti-Fas antibody (data not shown).

For light-irradiated thymocytes treated with BPD-MA (3.5 or 7 nM) and the anti-Fas antibody, DNA fragmentation levels attained within 3 h were greater than the sum of these treatments when applied independently (Fig. 4). In these experiments, the number of thymocytes containing subdiploid DNA levels was increased further by approximately 16 and 32% when these cells were treated with BPD-MA at 3.5 or 7 nM, respectively, and in combination with anti-Fas antibody (Fig. 4). In contrast, the level of DNA fragmentation induced in thymocytes treated with the hamster IgG control monoclonal antibody combined with BPD-MA and light was the same as that produced by BPD-MA and light alone. Relative thymocyte DNA fragmentation levels among these treatment groups were maintained whether PDT was applied before or after the addition of anti-Fas antibody and if DNA fragmentation was evaluated 3 or 5 h posttreatment (data not shown).

Flow cytometric analysis confirmed that the $CD4^+CD8^+$ cells were the predominant (>75%) thymocyte subset (Fig. 5). Treatment with PDT or anti-Fas antibody significantly decreased the percentage of $CD4^+CD8^+$ thymocytes and increased the relative representation of $CD4^+CD8^-$, $CD4^-CD8^+$, and $CD4^-CD8^-$ cells within these preparations (Fig. 5, Table 1). In combination, anti-Fas antibody and PDT further reduced $CD4^+CD8^+$ cell numbers and led to an increase in the proportion of $CD4^-CD8^-$, $CD4^+CD8^-$, and $CD4^-CD8^+$ thymocytes within these preparations. PDT and anti-Fas antibody reduced the proportion of $CD4^+CD8^+$ thymocytes by 21 and 15%, respectively. Together, PDT and the anti-Fas antibody reduced the relative representation of $CD4^+CD8^+$ thymocytes by approximately 57%, a decrease greater than expected if these agents had acted in an additive fashion. Light alone or BPD-MA in the absence of light did not affect thymocyte survival or the impact of the anti-Fas antibody. Furthermore, the hamster IgG control antibody did not modify the impact of PDT upon the thymocyte subpopulations. Modestly lowered CD4 and CD8 levels were evident for $CD4^+CD8^+$ thymocytes treated with PDT and/or the anti-Fas antibody.

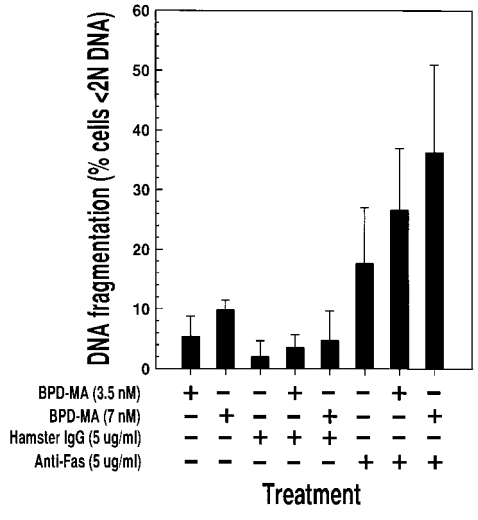

FIG. 4. Thymocytes treated with PDT and anti-Fas antibody exhibit higher DNA fragmentation levels than when these treatments are applied separately. Cells treated with BPD-MA and light (5 J/cm^2) were placed in medium alone or with hamster IgG or the anti-Fas monoclonal antibody. DNA status was determined 3 h later by PI staining and flow cytometric analysis. Thymocytes obtained from nine different mice were tested separately in three independent experiments. The extent of DNA fragmentation for cells maintained in medium alone was subtracted from the result obtained for treated cells. For these experiments, subdiploid DNA levels were present within 13.1 ± 2.4% of control thymocytes.

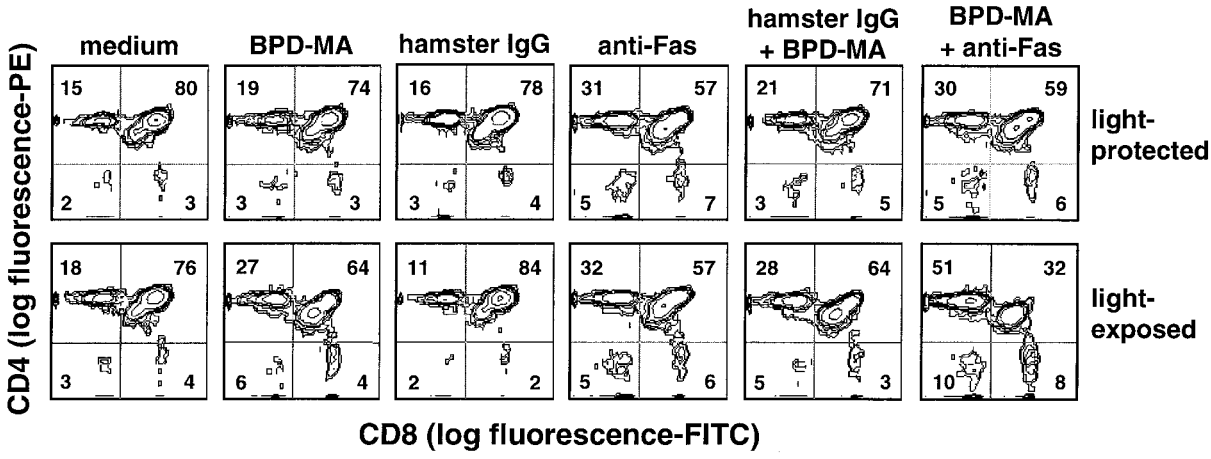

FIG. 5. Anti-Fas antibody and PDT compromise $CD4^+CD8^+$ thymocyte survival. Thymocytes were treated with BPD-MA (0 or 3.5 nM) and 690 nm light (0 or 5 J/cm^2). Cells were then maintained for 24 h in the absence or presence of hamster IgG or anti-Fas antibody at 5 μg/ml. Following staining with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 monoclonal antibodies, cell fluorescence was analyzed by dual-color flow cytometry. The percentage of cells within each thymocyte subset is given in each quadrant of the contour plots. Reduced CD4 and CD8 levels are discernable for $CD4^+CD8^+$ thymocytes treated with anti-Fas antibody and/or BPD-MA and light. Flow cytometric profiles from one of three consecutive, identically performed experiments are shown. Highly similar results were obtained in each experiment and are summarized in Table 1.

Accumulation of BPD-MA by Thymocyte Subsets

For thymocytes incubated with BPD-MA, the anti-CD4 and anti-CD8 monoclonal antibody labeling pattern was the same as that for thymocytes maintained in medium alone (Fig. 6A). Three-color flow cytometric analyses revealed that $CD4^-CD8^-$ thymocytes exhibited the highest relative degree of BPD-MA uptake followed, in descending order, by $CD4^-CD8^+$, $CD4^+CD8^-$, and $CD4^+CD8^+$ cells (Fig. 6B). When a 10-fold lower concentration of BPD-MA was tested, the same relative degree of BPD-MA uptake among the

TABLE 1

PDT Combined with Anti-Fas Antibody[a] Causes Extensive Depletion of the $CD4^+CD8^+$ Thymocyte Subset

Treatment	Thymocyte subset (% cells)			
	$CD4^+CD8^+$	$CD4^+CD8^-$	$CD4^-CD8^+$	$CD4^-CD8^-$
Light-protected				
Medium	75.5 ± 3.8	17.5 ± 3.5	3.2 ± 0.5	3.8 ± 1.5
BPD-MA	73.8 ± 2.6	19.2 ± 1.8	3.9 ± 0.8	3.1 ± 0.2
Hamster IgG	74.2 ± 4.2	18.6 ± 4.3	3.7 ± 0.1	3.5 ± 1.0
Anti-Fas	59.1 ± 2.3*	29.2 ± 2.6	5.7 ± 1.5	6.0 ± 1.6
BPD-MA + hamster IgG	72.9 ± 2.3	19.4 ± 2.6	3.8 ± 0.9	3.9 ± 0.7
Anti-Fas + BPD-MA	60.0 ± 1.4*	29.2 ± 3.2	5.3 ± 1.4	5.4 ± 1.4
Light-exposed				
Medium	74.9 ± 2.0	18.2 ± 0.6	3.3 ± 0.8	3.7 ± 1.3
BPD-MA	63.8 ± 4.0*	27.5 ± 3.7	3.7 ± 0.2	5.1 ± 0.7
Hamster IgG	78.0 ± 5.6	15.9 ± 4.2	2.7 ± 0.8	3.4 ± 1.1
Anti-Fas	59.3 ± 2.4*	29.7 ± 2.7	5.6 ± 1.2	5.4 ± 1.0
BPD-MA + hamster IgG	63.7 ± 0.6*	27.3 ± 2.3	5.0 ± 1.5	4.0 ± 1.8
Anti-Fas + BPD-MA	31.8 ± 10.4**	51.6 ± 13.3**	9.1 ± 1.3**	7.5 ± 2.9*

Note. The major thymocyte subsets were delineated by flow cytometric analyses performed 24 h posttreatment. Results (mean ± SD) are from three consecutive independent experiments.

[a] BPD-MA was applied at 3.5 nM. The 690-nm light dose was 5 J/cm^2. Hamster IgG and the anti-Fas monoclonal antibody Jo2 were used at 5 μg/ml.

* $P < 0.05$ compared to light-protected or irradiated thymocytes maintained in medium.

** $P < 0.05$ compared to the result obtained for all other treatment groups.

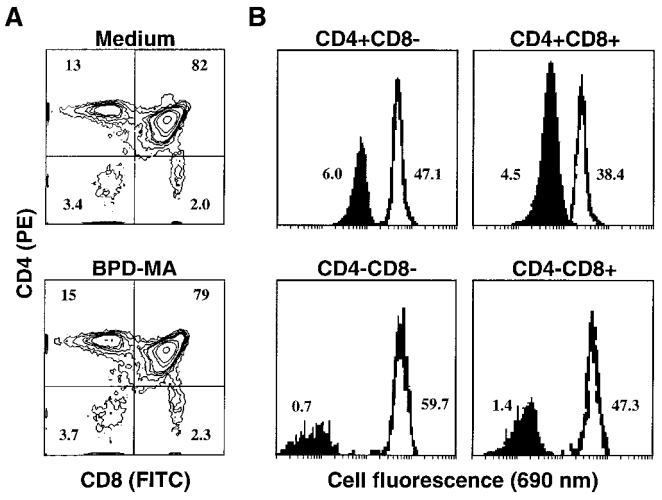

FIG. 6. Murine thymocyte subsets differ in their uptake of BPD-MA. (A) Freshly prepared thymocytes were incubated in the absence or presence of BPD-MA (140 nM) for 1 h at 37°C and then labeled with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 monoclonal antibodies. Contour plots show flow cytometric analyses of CD4 and CD8 expression by thymocytes incubated with or without BPD-MA. The percentage of cells within each thymocyte subset is given. (B) The association of BPD-MA with each thymocyte subset (fluorescence intensity at 690 nm) was assessed by three-color flow cytometric analysis. Fluorescence profiles obtained for cells incubated without (filled areas) or in the presence of BPD-MA (bold lines) within each subset (minimum of 5000 cells analyzed) are shown. MCFI values determined for cells incubated in the presence of medium alone or with BPD-MA are given. A second experiment yielded highly similar results.

four major thymocyte subsets existed, although the signal strength for BPD-MA was proportionally lower (data not shown). When this experiment was performed using anti-CD4 and anti-CD8 monoclonal antibodies conjugated to FITC and PE, respectively, a highly similar pattern for thymocyte subset BPD-MA uptake was observed (data not shown).

Influence of PDT on Thymocyte Fas Expression Levels

Thymocytes subjected to BPD-MA and light 3 h before displayed surface Fas levels similar to those of thymocytes treated with medium, BPD-MA, or light alone (data not shown). In comparison to control cells, thymocytes treated with PDT 24 h before had approximately 20% lower Fas expression (MCFI) levels (data not shown).

Influence of PDT and Anti-Fas Antibody on Caspase-3 Activity and PARP Processing

Caspase-3-like activity was detectable for thymocytes treated with anti-Fas antibody as determined by a fluorogenic peptide assay (Fig. 7A). Little or no induced activity was detectable for light-irradiated thymocytes treated with BPD-MA, hamster IgG, or BPD-MA plus hamster IgG. However, PDT combined with anti-Fas antibody increased caspase-3-like activity by 57% compared to the effect of antibody alone. Western immunoblot analysis revealed the presence of low levels of the p85 cleavage fragment of PARP within lysates prepared from irradiated thymocytes treated with medium, BPD-MA, hamster IgG, or hamster IgG plus BPD-MA (Fig. 7B). Treatment with anti-Fas antibody increased thymocyte lysate PARP p85 levels. A further 20% increase in thymocyte PARP p85 levels was achieved with anti-Fas antibody in combination with PDT, as determined by densitometric analysis.

DISCUSSION

Apoptosis may ensue in tumor cells (2–4, 31) as well as immune cells (11, 12) treated with cytotoxic levels of different photosensitizers and visible light. Human myeloid leukemia HL-60 cells treated with BPD-MA and light contained activated caspase-3, a member of a family of cell death-associated proteases, and exhibited evidence of the degradation of specific intracellular proteins and DNA fragmentation (4, 31). Caspase-3

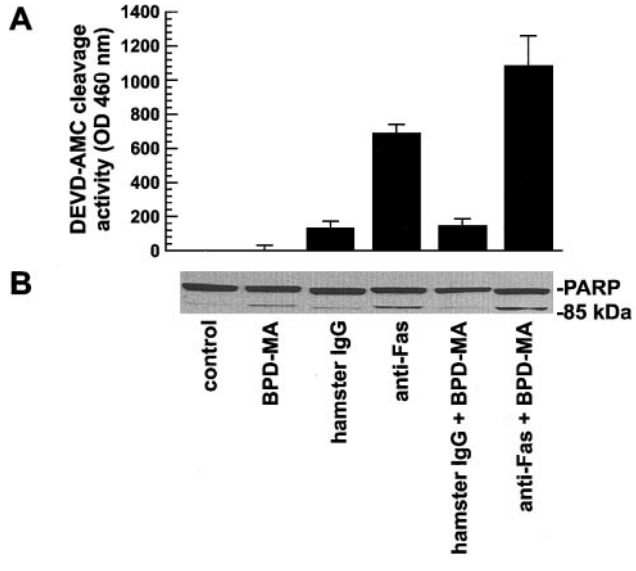

FIG. 7. PDT in combination with anti-Fas antibody produce elevated levels of thymocyte caspase-3-like activity and PARP processing. (A) Cell lysates, prepared 3 h post-light irradiation, were incubated with the fluorogenic caspase-3 substrate Ac-DEVD-AMC for 4 h. The mean protease activity level for lysates prepared from treated thymocytes treated with light alone corresponded to 838 ± 21 (arbitrary) units. This value was subtracted from each experimental result to yield an estimate of induced caspase-3 activity. (B) The status of the DNA repair enzyme PARP was evaluated by separating cell lysates prepared 3 h post-light irradiation by SDS–PAGE followed by immunoblot analysis with anti-mouse PARP polyclonal antibody. The position of PARP and its 85-kDa cleavage fragment are indicated.

also partcipates in the apoptotic death of murine thymocytes treated with various cytotoxic agents (30). Ligation of the Fas receptor and its subsequent trimerization mobilizes caspase-8 (FLICE/MACH/Mch5) which in turn processes caspase-3, leading to the full execution of the protease cascade (19, 32–34). Caspase-3 activity is linked DNA fragmentation (35, 36). Since PDT with BPD-MA (4, 31) as well as Fas receptor ligation (37) cause caspase-3 activation and DNA fragmentation, certain apoptosis-related biochemical events triggered by these agents may be similar.

$CD4^+CD8^+$ thymocyte numbers were reduced after the treatment of thymocytes with PDT as well as anti-Fas antibody. In combination, PDT and anti-Fas antibody further increased the number of cells exhibiting DNA fragmentation and decreased $CD4^+CD8^+$ thymocyte numbers. Fas^+ $CD4^+CD8^+$, but not Fas^+ $CD4^-CD8^-$, $CD4^+CD8^-$, or $CD4^-CD8^+$ thymocytes, are susceptible to Fas-mediated killing (22, 23). $CD4^+CD8^+$ thymocytes are also susceptible to killing with glucocorticoids (38) and anti-TCR ligands (39). Thus, $CD4^+CD8^+$ thymocytes are not only sensitive to Fas receptor ligation but to other proapoptotic stimuli including PDT.

$CD4^+CD8^+$ thymocytes down-regulate surface CD4 and CD8 expression during apoptosis (40, 41). Modestly reduced surface CD4 and CD8 levels were evident for $CD4^+CD8^+$ thymocytes treated with PDT and/or Fas ligation. However, the extent of these decreases was insufficient to account for the observed reduction in the representation of the $CD4^+CD8^+$ subset produced by the treatments. Differential BPD-MA uptake might contribute to the impact of PDT upon the $CD4^+CD8^+$ thymocyte subset. Activated mouse T lymphocytes (10, 11) and human leukemia cell types (42) accumulate greater amounts of BPD-MA than their quiescent or normal counterparts, a trait associated with heightened susceptibility to photodynamic killing. Flow cytometric studies indicated that $CD4^-CD8^-$ thymocytes accumulated approximately 10 times more BPD-MA than $CD4^+CD8^-$ and $CD4^+CD8^+$ cells and twice as much of the photosensitizer as $CD4^-CD8^+$ thymocytes. Relative to other subsets, $CD4^-CD8^-$ thymocytes are in a proliferative state (13), an attribute that might promote BPD-MA uptake by these cells (10, 11, 42). Thus, the selective impact of PDT upon the $CD4^+CD8^+$ thymocyte subset appears related to the low resistance of this cell type to proapoptotic stimuli.

Thymocytes were marginally more sensitive to the induction of PDT-mediated apoptosis than splenic T cells. At high BPD-MA levels, little or PDT-induced DNA fragmentation was detectable for light-irradiated thymocytes or T cells. At these higher photosensitizer doses the apoptotic pathway may be circumvented and cells undergo death through necrosis.

Chemotherapeutic agents may sensitize tumor cells to Fas-directed cytotoxicity by elevating Fas expression (43). Treatment of thymocytes with PDT had no such effect. At 3 h postirradiation, thymocytes treated with PDT had unchanged Fas levels, and by 24 h, the density of Fas on PDT-treated thymocytes was less than that of control cell preparations. This change indicates that PDT may negatively influence thymocyte Fas expression and/or that the cells surviving the photodynamic treatment are those bearing lower Fas levels. Treatment of thymocytes with PDT could act in concert with the anti-Fas antibody by destabilizing cellular organelles such as mitochondria (1), a primary site for apoptosis regulation (19, 44–46). Human HeLa cells treated with cytotoxic levels of BPD-MA and light exhibited the rapid appearance of cytochrome *c* in the cytosol, a primary event associated with apoptosis induced by many agents, leading to the activation of several different caspase family members (46). PDT and anti-Fas antibody instigated a level of caspase-3-like activity in thymocytes greater than that produced by either stimulus independently as determined by protease assays. Furthermore, more extensive processing of the DNA repair enzyme PARP, a caspase-3 substrate (35), was observed for thymocytes treated with PDT and anti-Fas antibody. Treatment of murine YAC-1 lymphoma cells with the photosensitizer Photosan 3 and light combined with tumor necrosis factor-α (TNF-α), had an additive impact upon cell survival (47). TNF-α induces apoptosis in some cell types upon binding to specific surface receptors, and paralleling the effect of Fas ligation, triggers caspase-8 mobilization (16, 19). PDT in combination with signaling through apoptosis-regulating surface cell receptors such as Fas or TNF-α, may lower the threshold required for the induction of T lymphocyte death.

$CD4^+CD8^+$ thymocytes are highly susceptible to the effects PDT, a result that parallels the sensitivity of these cells to various immunomodulatory agents. Of potentially greater significance, Fas cross-linking combined with PDT had an additive impact against the survival of $CD4^+CD8^+$ thymocytes. The combined effect of PDT and anti-Fas antibody upon these cells was definable by a convergence of intracellular caspase activity induced by the two different treatments. Delineating the influence of PDT on the sensitivity of peripheral T cells to Fas-mediated signaling may lead to a fuller understanding of the immunomodulatory action of PDT.

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REFERENCES

1. Gomer, C. J., Rucker, N., Ferrario, A., and Wong S., Properties and applications of photodynamic therapy. *Radiat. Res.* **120,** 1–18, 1989.
2. Agarwal, M. L., Clay, M. E., Harvey, E. J., Evans, H. H., Antunez, A. R., and Oleinick, N. L., Photodynamic therapy induces rapid cell death by apoptosis in L5178Y mouse lymphoma cells. *Cancer Res.* **51,** 5993–5996, 1991.
3. Luo, Y., Chang, C. K., and Kessel, D., Rapid initiation of apoptosis by photodynamic therapy. *Photochem. Photobiol.* **63,** 528–534, 1996.
4. Granville, D. J., Levy, J. G., and Hunt, D. W. C., Photodynamic therapy induces caspase-3 activation in HL-60 cells. *Cell Death Differ.* **4,** 623–628, 1997.
5. Levy, J. G., Photodynamic therapy. *Trends Biotechnol.* **13,** 14–17, 1995.
6. Elmets, C. A., and Bowen, K. D., Immunological suppression in mice treated with hematoporphyrin derivative photoradiation. *Cancer Res.* **46,** 1608–1611, 1986.
7. Simkin, G. O., King, D. E., Levy, J. G., Chan, A. H., and Hunt, D. W. C., Inhibition of contact hypersensitivity with different analogs of benzoporphyrin derivative. *Immunopharmacology* **37,** 221–230, 1997.
8. Chowdhary, R. K., Ratkay, L. G., Neyndorff, H. C. Richter, A., Obochi, M., Waterfield, J. D., and Levy, J. G., The use of transcutaneous photodynamic therapy in the prevention of adjuvant-enhanced arthritis in MRL/lpr mice. *Clin. Immunol. Immunopathol.* **72,** 255–263, 1994.
9. Leong, S., Chan, A. H., Levy, J. G., and Hunt, D. W. C., Transcutaneous photodynamic therapy alters the development of an adoptively transferred form of murine experimental autoimmune encephalomyelitis. *Photochem. Photobiol.* **64,** 751–757, 1996.
10. Obochi, M. O. K., Canaan, A. J., Jain, A. K., Richter, A. M., and Levy, J. G., Targeting activated lymphocytes with photodynamic therapy: Susceptibility of mitogen-stimulated splenic lymphocytes to benzoporphyrin derivative (BPD) photosensitization. *Photochem. Photobiol.* **62,** 169–175, 1995.
11. Hunt, D. W. C., Jiang, H., Granville, D. J., Chan, A. H., Leong, S., and Levy, J. G., Consequences of the photodynamic treatment of resting and activated peripheral T lymphocytes. *Immunopharmacology* **41,** 31–44, 1999.
12. King, D. E., Jiang, H., Simkin, G. O., Obochi, M. O. K., Levy, J. G., and Hunt, D. W. C., Photodynamic alteration of the surface receptor expression pattern of murine splenic dendritic cells. *Scand. J. Immunol* **49,** 184–192, 1999.
13. Rothenberg, E. V., The development of functionally responsive T cells. *Adv. Immunol.* **51,** 85–214, 1992.
14. Robey, E., and Fowlkes, B. J., Selective events in T cell development. *Annu. Rev. Immunol.* **12,** 675–705, 1994.
15. Kisielow, P., Bluthmann, H., Staerz, U. D., Steimetz, M., and von Boehmer, H., Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature $CD4^+CD8^+$ thymocytes. *Nature* **333,** 742–746, 1988.
16. Fowlkes, B. J., Schwartz, R. H., and Pardoll, D. M., Deletion of self-reactive thymocytes occurs at a $CD4^+CD8^+$ precursor stage. *Nature* **334,** 620–623, 1988.
17. Surh, C. D., and Sprent, J., T-cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature* **372,** 100–103, 1994.
18. Nagata, S., Apoptosis by death factor. *Cell* **88,** 355–365, 1997.
19. Granville, D. J., Carthy, C. M., Hunt, D. W., and McManus B. B., Apoptosis: Molecular aspects of cell death and disease. *Lab. Invest.* **78,** 893–913, 1998.
20. Chao, D. T., Linette, G. P., Boise, L. H., White, L. S., Thompson, C. B., and Korsmeyer, S. J., Bcl-X_L and Bcl-2 repress a common pathway of cell death. *J. Exp. Med.* **82,** 821–828, 1995.
21. Suda, T., Takahashi, T., Goldstein, P., and Nagata, S., Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell* **75,** 1169–178, 1993.
22. Ogasawara, J., Watanabe-Fukunaga, R., Adachi, M., Matsuzama, A,. Kusugai, T., Kitamura, Y., Itoh, N., Suda, T. and, Nagata, S., Lethal effects of the anti-Fas antibody in mice. *Nature* **364,** 806–809, 1993.
23. Nishimura, Y., Ishii, A., Koayashi, Y., Yamasaki, Y., and Yonehara, S., Expression and function of mouse Fas antigen on immature and mature T cells. *J. Immunol.* **154,** 4395–4403, 1995.
24. Ogasawara, J., Suda, T., and Nagata, S., Selective apoptosis of $CD4^+CD8^+$ thymocytes by the anti-Fas antibody. *J. Exp. Med.* **81,** 485–491, 1995.
25. Leo, O., Foo, M., Sachs, D. H., and Samelson, L. E., and Bluestone, J., Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA* **84,** 1374–1378, 1987.
26. Coffman, B., Surface antigen expression and immunoglobulin rearrangement during mouse pre-B cell development. *Immunol. Rev.* **69,** 5–23, 1982.
27. Richter, A. M., Kelly, B., Chow, J. Liu, D. J., Towers, G. H. N., Dolphin, D., and Levy, J. G., Preliminary studies on a more effective phototoxic agent than hematoporphyrin. *J. Natl. Cancer Inst.* **79,** 1327–1332, 1987.
28. Mosmann, T., Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65,** 55–63, 1983.
29. Telford, W. G., King, L. E., and Fraker, P. J., Rapid quantitation of apoptosis in pure and heterogeneous cells populations using flow cytometry. *J. Immunol. Methods* **172,** 1–16, 1994.
30. Alam, A., Braun, M. Y., Hartgers, F., Lesage, S., Cohen, L., Hugo, P., Denis, F., and Sekaly, R.-K., Specific activation of the cysteine protease CPP32 during the negative selection of T cells in the thymus. *J. Exp. Med.* **186,** 1503–1512, 1997.
31. Granville, D. J., Jiang, H., An, M. T., Levy, J. G., McManus, B. M., and Hunt, D. W. C., Overexpression of Bcl-XL prevents caspase-3-mediated activation of DNA fragmentation (DFF) produced by treatment with the photochemotherapeutic agent BPD-MA. *FEBS Lett.* **422,** 151–154, 1998.
32. Boldin, M. P., Goncharov, T. M., Goltsev, Y. V., and Wallach, D., Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell* **85,** 803–815, 1996.
33. Muzio, M., Chinnaiyan, A. M., Kischkel F. C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J. D., Zhang, M., Gentz, R., Mann, M., Krammer, P. H., Peter, M. E., and Dixit, V. M., FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell* **85,** 817–827, 1996.
34. Hirata, H., Takahashi, A., Kobayashi, S., Yonehara, S., Sawai, H., Okazaki, T., Yamamoto, K., and Sasada, M., Caspases are activated in a branched protease cascade and control downstream processes in Fas-induced apoptosis. *J. Exp. Med.* **187,** 587–600, 1998.
35. Casciola-Rosen, L., Nicholson, D. W., Chong, T., Rowan, K. R., Thornberry, N. A., Miller, D. K.,and Rosen, A., Apopain/CPP32 cleaves proteins that are essential for cellular repair: A funda-

mental principle of apoptotic death. *J. Exp. Med.* **183,** 1957–1964, 1996.

36. Janicke, R. U., Spengart, M. L., Wati, M. R., and Porter, A. G., Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. *J. Biol. Chem.* **273,** 9357–9360, 1998.
37. Enari, M., Talanian, R. V., Wong, W. W., and Nagata, S., Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. *Nature* **380,** 723–726, 1996.
38. Cohen, J. J., Programmed cell death in the immune system. *Adv. Immunol.* **50,** 55–85, 1991.
39. Finkel, T. H., Cambier, J. C., Kubo, R. T., Born, W. K., Marrack, P., and Kappler, J. W., The thymus has two functionally distinct populations of immature $\alpha\beta+$ T cells: One population is deleted by ligation of $\alpha\beta$TCR. *Cell* **58,** 1047–1054, 1989.
40. Swat, W., Ignatowicz, L., von Boehmer, H., and Kiselow, P., Clonal deletion of immature $CD4^+8^+$ thymocytes in suspension culture by extrathymic antigen-presenting cells, *Nature* **351,** 150–153, 1991.
41. Kishimoto, H., Surh, C. D., and Sprent, J., Upregulation of surface makers on dying thymocytes. *J. Exp. Med.* **181,** 649–655, 1995.
42. Jamieson, C. H. M., McDonald, W. N., and Levy, J. G., Preferential uptake of benzoporphyrin derivative by leukemic versus normal cells. *Leukocyte Res.* **14,** 209–219, 1990.
43. Micheau, O., Solary, E., Hammann, A., Martin, F., and Dimanche-Boitrel, M.-T., Sensitization of cancer cells treated with cytotoxic drugs to Fas-mediated cytotoxicity. *J. Natl. Cancer Inst.* **89,** 783–789, 1997.
44. Zamzami, N., Susin, S. A., Marchetti, P., Hirsch, T., Gomez-Monterry, I., Castedo, M., and Kroemer, G., Mitochondrial control of nuclear apoptosis. *J. Exp. Med.* **183,** 1533–1544, 1996.
45. Kroemer, G., The proto-oncogene Bcl-2 and its role in regulating apoptosis. *Nature Med.* **3,** 614–620, 1997.
46. Granville, D. J., Carthy, C. M., Jiang, H., Shore, G. C., McManus, B. M., and Hunt, D. W. C., Rapid cytochrome *c* release, activation of caspases 3, 6, 7 and 8 followed by Bap31 cleavage in HeLa cells treated with photodynamic therapy. *FEBS Lett.* **437,** 5–10, 1998.
47. Reiter, I., Schwamberger, G., and Krammer, B., Effect of photodynamic pretreatment on the susceptibility of murine tumor cells to macrophage antitumor mechanisms. *Photochem. Photobiol.* **66,** 384–388, 1997.

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