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Photodynamic Therapy-mediated Immune Response against Subcutaneous Mouse Tumors[1]

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ABSTRACT

The curative ability of photodynamic therapy (PDT) is severely compromised if treated tumors are growing in immunodeficient hosts. Reconstitution of severe combined immunodeficient (scid) mice with splenocytes from naïve immunologically intact BALB/c mice did not improve the response to Photofrin-based PDT of EMT6 tumors growing in these animals. In contrast, adoptive transfer of BALB/c splenocytes containing EMT6 tumor-sensitized immune cells had a dramatic effect on tumor regrowth after PDT. For instance, full restoration of the curative effect of PDT was achieved with scid mice that received splenocytes from BALB/c donors that were cured of EMT6 tumors by PDT 5 weeks before adoptive transfer. Splenocytes obtained from donors cured of EMT6 tumors using X-rays were much less effective. Selective *in vitro* depletion of specific T-cell populations from engrafting splenocytes indicated that CTLs are the main immune effector cells responsible for conferring the curative outcome to PDT in this experimental model, whereas helper T lymphocytes play a supportive role. The immune specificity of these T-cell populations was demonstrated by the absence of cross-reactivity between the EMT6 and Meth-A tumor models (mismatch between tumors growing in splenocyte donors and recipients). The immunocompetent BALB/c mice that received adoptively transferred splenocytes containing PDT-generated, tumor-sensitized immune cells also benefited from the improved outcome of PDT of tumors they were bearing. This was demonstrated not only with the fairly immunogenic EMT6 tumor model but also with weakly immunogenic Line 1 carcinomas. The results of this study indicate that PDT is a highly effective means of generating tumor-sensitized immune cells that can be recovered from lymphoid sites distant to the treated tumor at protracted time intervals after PDT, which asserts their immune memory character. It is also shown that the treatment of tumors by PDT creates the conditions necessary for converting the inactive adoptively transferred pre-effector, tumor-sensitized immune cells into fully functional antitumor effector cells. An additional finding of this study is the evidence of NK cell activation in PDT-treated Meth-A sarcomas.

INTRODUCTION

The positive results obtained with PDT[3] in a clinical setting (1) have stimulated much interest in the mechanisms responsible for determining the efficacy of this treatment modality. In particular, important advances have recently been made in the understanding of PDT-elicited, antitumor immune responses and their relevance to the therapeutic benefit of this approach (1, 2). Briefly, at least three major factors appear to be involved in the induction of a strong immune response against PDT-treated cancers. PDT-mediated oxidative stress triggers a variety of cellular signal transduction pathways (1, 3) that lead to increased expression of stress proteins and the induction of downstream early response genes, the products of which are transcription factors regulating the expression of various genes. Of particular importance, PDT has been shown to activate nuclear factor-κB and AP-1, which in turn control the expression of various cytokines and other immunologically important genes (4, 5). Among the cytokines whose expression has been reported to be modulated by PDT are IL-6, IL-10, and tumor necrosis factor-α (6, 7), whereas several others including IL-1β, IL-2, and granulocyte-colony stimulating factor may also be affected (8, 9). PDT is also known to increase the expression of various genes involved in cell adhesion or antigen presentation, and these may further contribute to the development of the inflammatory/immune response elicited by this therapy (10).

Another important factor that contributes to the induction of PDT-mediated immune responses is the proinflammatory damage generated in cellular membranes and the vasculature of treated tumor and normal tissues (1, 2). These photooxidative lesions are responsible for the extensive release of various potent inflammatory mediators that provoke a prompt and strong inflammatory reaction at the PDT-treated site. A dominant event in such PDT-induced inflammation is a rapid and massive invasion of activated inflammatory cells, including neutrophils/granulocytes, mast cells, and monocytes/macrophages, from the circulation to the PDT-treated site (1). These cells appear to be the main contributors to the inflammation-primed immune development process associated with PDT (1, 2).

The nature, rate, and extent of tumor cell death induced by PDT may also play a crucial role in determining the generation of effective antitumor immune response. Large amounts of cellular debris are generated at a tumor site within a short time interval of PDT treatment. The particular nature of such material facilitates the uptake and presentation of putative tumor antigens by macrophages and dendritic cells recruited to the tumor site in response to PDT-induced inflammatory signals, ensuring the recognition of tumor-specific epitopes by T lymphocytes and their subsequent activation (1).

The initial photooxidative injury (inflicted during exposure of solid cancers to photodynamic light) triggers a variety of responses, some of which indirectly lead to tumor destruction. Hence, in addition to the direct killing of tumor cells, secondary events including ischemia (subsequent to vascular damage), ischemia-reperfusion injury, the antitumor activity of activated inflammatory cells, and tumor-specific T lymphocytes may contribute to the eradication of PDT-treated lesions (2). Although the immune reaction may be less important than the other antitumor effects in the stages of early tumor ablation after PDT, its role can be decisive in attaining long-term tumor control. We have demonstrated that lymphoid populations are essential for preventing the regrowth of PDT-treated mouse EMT6 sarcomas (11). The dose of Photofrin-based PDT that was fully curative for EMT6 tumors growing in immunocompetent BALB/c mice attained only initial ablation, but not permanent cures, with the same tumor model grown in immunodeficient scid or nude mice. The curative effect of PDT was restored in radiation chimeras in which immunodeficient host mice were reconstituted with BALB/c mice bone marrow (allowing these mice to acquire functionally active lymphocytes; Ref. 11). The continuation of this work, described in the present report, examines the generation of tumor-sensitized immune cells by PDT and the capacity of PDT to activate these cells when adoptively transferred to tumor-bearing hosts.

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[3] The abbreviations used are: PDT, photodynamic therapy; IL, interleukin; NK, natural killer; scid, severe combined immunodeficient; mAb, monoclonal antibody.

MATERIALS AND METHODS

Tumor Models and Mice. The tumors used were grown in syngeneic BALB/cJ mice. The EMT6 mammary sarcoma (12) and Meth-A fibrosarcoma (13), which are fairly immunogenic tumor models, were maintained by biweekly passage using i.m. tumor brei inoculation. The experimental tumors were initiated by implanting 1×10^6 tumor cells s.c. into a lower dorsal site. The Line 1 carcinoma (14), a weakly immunogenic tumor model obtained from Dr. E. M. Lord (University of Rochester Medical Center, Rochester, NY), was maintained *in vitro*, and 2×10^5 cells were used for s.c. tumor implantation. The EMT6 and Meth-A tumors were also implanted into immunodeficient scid mice (BALB/cJ-scid.TO). Female mice 7–9 weeks of age were used in the experiments.

PDT. Six days after tumor inoculation, the mice received Photofrin (10 mg/kg i.v.), and the tumors they were bearing were illuminated 24 h later. During the light treatment, the mice were restrained unanesthetized in lead holders exposing their backs. The fluence rate was 120–130 mW/cm^2. The tumor size at the time of treatment was 5–7 mm (largest diameter), with thickness not exceeding 3.5 mm. The 630 ± 10 nm monodirectional beam was delivered from a tunable light source (model A5000 with a 1-kW xenon bulb, manufactured by Photon Technology International, Inc.) through a 5-mm core diameter liquid light guide 2000A (Luminex, Munich, Germany).

The individual treatment groups consisted of 8–10 mice. After treatment, the mice were inspected three times per week for signs of tumor regrowth. No sign of tumor recurrence at 90 days post-PDT qualified as a cure. Statistical analysis of the results was based on the log-rank test.

Adoptive Transfer of Splenocytes. Spleens excised from donor mice were carefully teased apart to release cells into suspension without enzymatic digestion. Erythrocytes were immediately removed by lysis in ice-cold ammonium chloride buffer, the leukocyte suspension was filtered through a layer of 50-μm pore size polyester mesh and promptly transferred into recipient mice ($1–2 \times 10^7$ cells/mouse) via tail-vein injection. In most cases, the adoptive transfer was performed 2 days before tumor inoculation and 9 days before PDT treatment ("schedule one").

Splenocyte donors were either naïve or tumor cured BALB/c mice. In the latter case, the tumors (implanted 7 days earlier) were treated by either X-rays or Photofrin-based PDT. This was done 5 weeks before the hosts were sacrificed and their spleens used for the adoptive transfer. For the X-ray treatment (35 Gy at 3.33 Gy/min), the mice were immobilized in the same lead holders as used for PDT, which shielded their body (importantly, spleen and other organs) while fully exposing the tumor to the radiation beam. The mice were turned 180° midway through irradiation to optimize the dose uniformity throughout the tumor volume. The source of irradiation was a Philips RT250 (250 kVp, 0.5 mm Cu).

Depletion of NK Cells *in Vivo*. Rabbit anti-mouse/rat asialo-GM1 polyclonal antibody (Cedarlane Laboratories, Ltd., Hornby, Ontario, Canada), injected i.v. two times in a 5-day interval (20 μl/mouse), was used to deplete NK lymphocytes in BALB/c and scid mice. The antibody titer of this preparation was approximately 1:1000, as determined using an agglutination assay.

Complement-mediated Depletion. After lysis of erythrocytes, spleen cells were incubated 40 min (on ice) in tissue culture supernatants (1:1) of hybridomas producing either anti-mouse CD4 (clone GK1.5) or anti-mouse CD8 (clone 3.155) monoclonal antibodies. Cells were then washed by centrifugation and resuspended in HBSS containing 2% fetal bovine serum and warmed to 37°C. Low-tox guinea pig complement (Cedarlane) was added at a final dilution 1:10, and the samples were incubated for 45 min at 37°C. Dead cells where then removed using a Ficoll-metrizoic acid gradient [formed by mixing 36 ml of 14% w/v Ficoll 400 and 15 ml of 33% metrizoic acid (Sigma M-4762)]. The live cells (collected from the top of the gradient) were washed, counted, and transferred to the recipient mice by i.v. injection. Aliquots were examined by flow cytometry to verify depletion of target populations.

Flow Cytometry. Spleen or blood samples were (after lysis of erythrocytes) stained with mAbs directed against mouse leukocyte membrane antigens CD4, CD8, CD44, CD45R, and Ly-6Gy to determine the proportion of major leukocyte populations and/or their activation status. The mAbs, purchased from PharMingen (San Diego, CA), were directly conjugated with fluorescent markers (FITC, phycoerythrin, or CyChrome). Flow cytometry analysis was performed on a Coulter Epics Elite ESP (11).

RESULTS

Adoptive Transfer of Naïve or Tumor-sensitized Immune Cells. s.c. EMT6 tumors growing in syngeneic BALB/c mice can be effectively cured by PDT. Exposure of these tumors to a light dose of 110 J/cm^2 24 h after the host mice received 10 mg/kg of Photofrin administered i.v. (the PDT dose that will be called "standard" in this report) resulted in a rapid ablation of these lesions (Fig. 1). No sign of tumor recurrence was observed up to 90 days posttreatment, which qualifies as tumor cure. In contrast, the same PDT treatment of EMT6 tumors growing in scid mice was not curative. Despite a comparable initial response (lesions not palpable 1 day after PDT), all of the tumors treated in scid mice regrew within 3 weeks. As shown in our earlier work (11), this result can be attributed to the absence of functionally active lymphocytes in scid mice. The engraftment of splenocytes from naïve BALB/c mice was not effective in restoring the curative effect of PDT in EMT6 tumor-bearing scid recipients (the difference in tumor response between this treatment group and no transfer group is not statistically significant, $P < 0.15$; Fig. 1). A similar result was obtained using T cells purified from the spleens of naïve BALB/c mice (11). The adoptive transfer in these experiments was performed according to "schedule one," in which the cells are injected into scid recipients 2 days before they are implanted with EMT6 tumors that are allowed to grow for an additional 7 days before PDT treatment. This treatment schedule was chosen based on our experience with adoptive transfer of spleen-derived T cells using the same experimental model (11). It is important to emphasize that EMT6 tumors grow at a similar rate in both BALB/c and scid mice and that adoptive transfer of lymphocytes alone had no detectable effect on tumor growth. The same was the case with the growth of tumors in scid mice engrafted with splenocytes by adoptive transfer protocols described elsewhere in this work.

A marked improvement in the response of EMT6 tumors to PDT was observed with the scid hosts engrafted with splenocytes from BALB/c donors that had been cured of EMT6 tumors. Cures after the "standard" PDT dose were obtained in over one-third of the scid recipients after

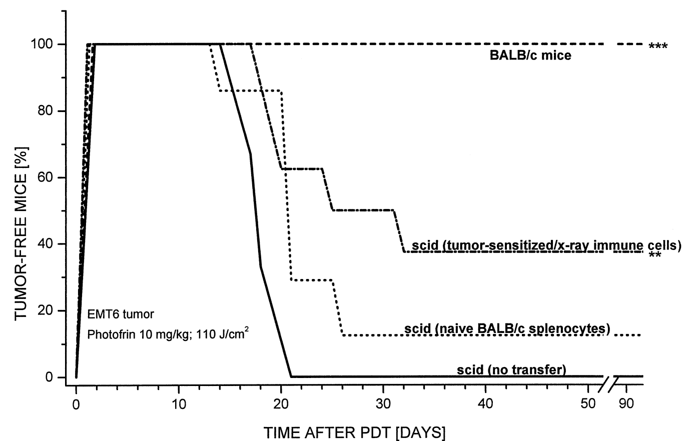

Fig. 1. The effect of adoptively transferred splenocytes from BALB/c mice on the PDT response of s.c. EMT6 tumors growing in scid mice recipients. scid mice received spleen cells ($1–2 \times 10^6$/mouse). Two days later, these mice were implanted with EMT6 tumors, which were allowed to grow for 7 days before being treated by PDT. The splenocyte donors were either naïve BALB/c mice or BALB/c mice that 5 weeks before the adoptive transfer were cured from EMT6 tumors using X-rays (35 Gy) and thus had spleen-residing "tumor-sensitized/X-ray immune cells." The PDT treatment of EMT6 tumors growing in scid mice that were either splenocyte recipients or not involved in spleen cell transfer consisted of Photofrin administration (10 mg/kg, i.v.), followed 24 h later by tumor-localized light exposure to 110 J/cm^2. The mice were observed afterward for signs of tumor growth. Tumor-free mice at 90 days post-PDT were considered cured. The response of EMT6 tumors growing in BALB/c mice (not involved in spleen cell transfer) to the same PDT treatment is also included. Statistical significance of responses compared to "scid (no transfer)" group: ***, $P < 0.0001$; **, $P < 0.01$.

adoptive transfer of splenocytes obtained from BALB/c mice in which X-ray treatment (35 Gy) was used to eradicate EMT6 tumors 5 weeks before they served as splenocyte donors (Fig. 1). This result suggests that the transferred spleen cell populations contained immune cells sensitized to the EMT6 tumor (as could be expected with this relatively immunogenic tumor model), which became activated in the recipient scid mice once the tumors they were bearing were PDT treated.

PDT-generated, Tumor-sensitized Immune Cells. Using PDT ("standard" dose) to treat EMT6 tumors in BALB/c mice 5 weeks before transferring their splenocytes to scid mice fully restored the therapeutic effect of PDT in the recipients (Fig. 2). This was manifested as a 100% cure of EMT6 tumors with the adoptive transfer performed according to the "schedule one" and just slightly lower cure rate when the adoptive transfer was delayed to 1 day before the PDT treatment of tumors growing in the recipients. In both cases, the tumors were treated with the "standard" PDT dose. In contrast, the engraftment of spleen cells containing lymphocyte populations sensitized (by PDT) against a different tumor had no therapeutic benefit. This was demonstrated using BALB/c donors implanted previously with Meth-A sarcomas. The tumors were eradicated by PDT (Photofrin 10 mg/kg; 150 J/cm^2) 5 weeks before the spleens of these mice provided cells that were adoptively transferred to scid mice (according to "schedule one"), which were subsequently implanted with EMT6 tumors and PDT treated (Fig. 2).

Response of scid Mice Cured by PDT to EMT6 Tumor Rechallenge. scid mice that were successfully cured of EMT6 tumors by a combination of PDT and adoptive transfer of PDT-generated tumor-sensitized immune cells (Fig. 2) were rechallenged with 1×10^6 EMT6 tumor cells 90 days after the initial therapy. In some of these mice, tumor appearance following rechallenge was considerably delayed and was followed by complete regression after a period of very slow growth, whereas in the others the tumors grew much slower than in naïve scid or BALB/c mice (data not shown). These results demonstrate the long-term persistence of the antitumor immune response induced in scid mice by adoptive lymphocyte transfer and PDT.

Meth-A Response after Adoptive Transfer or NK Cell Depletion. Comparable experiments carried out using the Meth-A tumor model yielded similar results. The "schedule one" adoptive transfer of Meth-A-sensitized splenocytes (generated in BALB/c mice using PDT, as described above) completely restored the curative effect of PDT in the scid recipients bearing Meth-A tumors (Fig. 3).

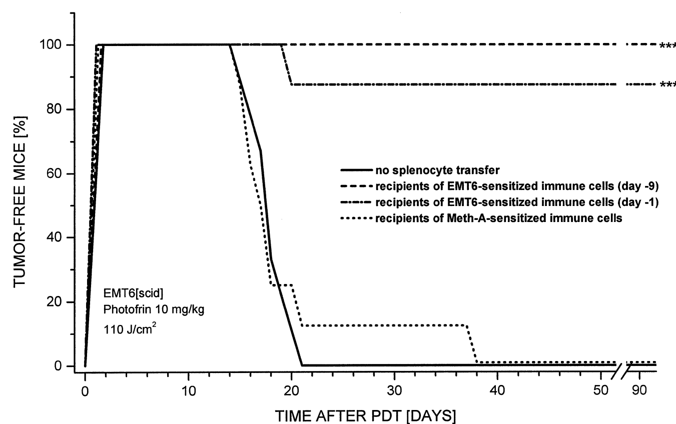

Fig. 2. The effect of adoptively transferred EMT6 or Meth-A tumor-sensitized immune cells on the PDT response of s.c. EMT6 tumors growing in recipient scid mice. scid mice received splenocytes containing either EMT6 or Meth-A tumor-sensitized immune cells, which were generated by PDT treatment (Photofrin, 10 mg/kg, plus 110 J/cm^2 for EMT6 or 150 J/cm^2 for Meth-A) of tumors growing in donor BALB/c mice 5 weeks before the adoptive transfer. The adoptive transfer was performed either at 9 days before the PDT treatment of EMT6 tumors growing in recipient scid mice (same as described in Fig. 1) or at 1 day before PDT. The PDT exposure of tumors in scid mice and other experimental details were as described in Fig. 1. Statistical significance of responses compared to "no splenocyte transfer" group: ***, $P < 0.0001$.

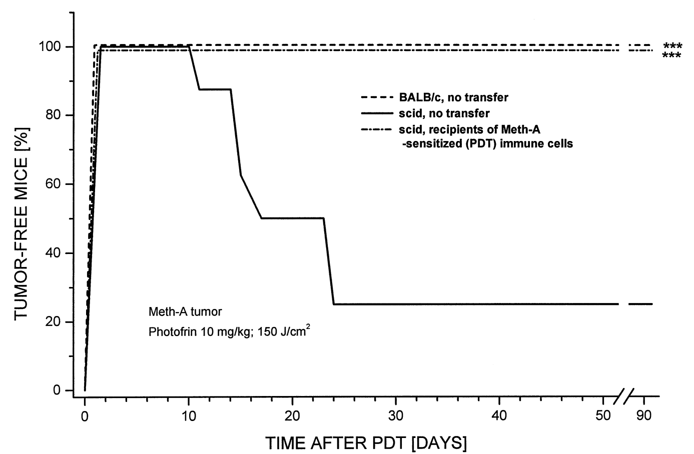

Fig. 3. The effect of adoptively transferred Meth-A tumor-sensitized immune cells on PDT response of s.c. Meth-A tumors growing in recipient scid mice. The scid mice received splenocytes containing Meth-A tumor-sensitized immune cells, which were generated by PDT treatment of these tumors in donor BALB/c mice, as described in Fig. 2. The PDT treatment of Meth-A tumors growing in scid mice that were either splenocyte recipients or not involved in spleen cell transfer consisted of Photofrin administration (10 mg/kg, i.v.), followed 24 h later by tumor-localized exposure to 150 J/cm^2. The timing of adoptive transfer and other details were as described in Fig. 1. The response of Meth-A tumors growing in BALB/c mice (not involved in spleen cell transfer) to the same PDT treatment is also included in the graph. Statistical significance of responses compared to "scid, no transfer" group: ***, $P < 0.001$.

It should be noted that the EMT6 and Meth-A tumor models exhibit certain differences in their response to PDT. In BALB/c mice, Meth-A tumors were somewhat more PDT resistant than EMT6 (it takes 150 J/cm^2 compared with 110 J/cm^2 to reach 100% cures). In contrast, Meth-A tumors growing in scid mice were more sensitive to PDT than EMT6 tumors. The PDT treatment of Meth-A tumors that is fully curative in BALB/c mice cured ~25% of these tumors growing in scid hosts. Cures of EMT6 tumors growing in scid mice were not achieved, even with a PDT dose that is double the 100% curative dose in BALB/c hosts (11). The different responsiveness of these two tumor models to PDT when growing in scid mice could possibly reflect their different sensitivity to NK cells, which are functionally active in scid mice despite the immunocompromised status (absence of T and B lymphocyte activity) of these animals. To test whether NK cell activity contributes to PDT-mediated Meth-A cures in scid mice, these cells were depleted in tumor-bearing animals after PDT treatment. This was achieved using the polyclonal antibody asialo-GM1, which is an established agent for *in vivo* depletion of NK cells (15). i.v. injection of 10–25 μl of this reagent into mice results in >90% reduction in the NK cell activity. The effect of NK depletion was tested with BALB/c or scid mice bearing Meth-A fibrosarcomas. Mice received asialo-GM1 immediately after PDT and again 5 days later. As shown in the inset to Fig. 4, depletion of NK cells in immunocompetent BALB/c mice had no significant effect on PDT-mediated tumor cures. However, the depletion of NK cells in scid mice significantly reduced the response of Meth-A tumors to PDT (Fig. 4).

Depletion of CD4$^+$ and CD8$^+$ T Cells from Splenocytes Used for the Adoptive Transfer. To further characterize the immune cell types present in adoptively transferred splenocytes that confer the curative outcome of PDT treatment in scid hosts, specific populations were selectively eliminated from spleen cell suspensions before they were injected into recipients. This was achieved using standard complement-mediated *in vitro* lysis of either CD4$^+$ or CD8$^+$ T lymphocytes present in spleen cell suspensions prepared from BALB/c mice cured of EMT6 tumors by PDT treatment, as described above. The

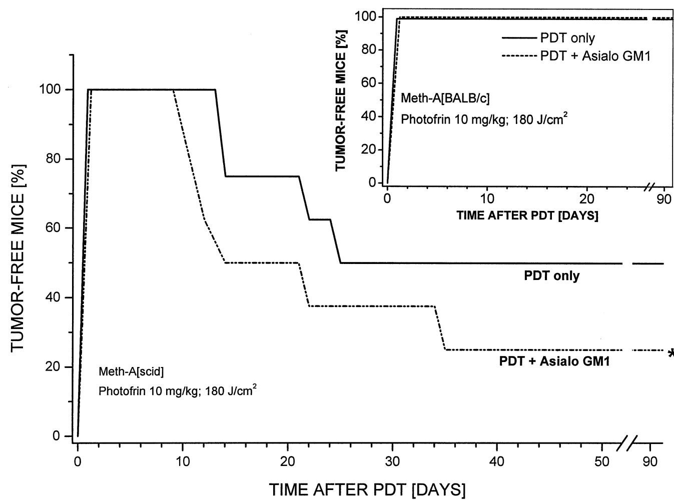

Fig. 4. The effect of NK cell depletion on the response of Meth-A tumors to PDT. Tumor-bearing scid mice were given Photofrin (10 mg/kg), and 24 h later, the tumors were exposed to the light dose of 180 J/cm^2. The asialo-GM1 polyclonal antibody (20 μl/mouse, i.v.) was administered twice, immediately after the termination of light treatment and 5 days later. Other details were as described in Fig. 1. *Inset,* results of the same treatments involving BALB/c instead of scid mice as tumor-bearing hosts. Statistical significance of responses compared to "PDT only" group: $*$, $P < 0.05$.

depletion of CD8$^+$ T lymphocytes completely abrogated the curative benefit conferred by the transfer of nonselected splenocyte populations, because the outcome of therapy did not differ from that seen with naïve splenocytes (Fig. 5). In contrast, the adoptive transfer of splenocytes from which CD4$^+$ T cells were eliminated only partially decreased the curative benefit obtained with the engraftment of nonselected splenocyte populations.

Analysis of Spleen and Blood T Lymphocytes in Adoptive Transfer Donors and Recipients. The results of flow cytometry analysis examining the CD4$^+$ and CD8$^+$ T cell content and the expression of the CD44 antigen (a cell adhesion receptor associated with activation of these cells) are shown in Table 1. There was no detectable difference between the content of helper and cytotoxic T cells or the expression of CD44 in the spleens of naïve BALB/c mice and BALB/c mice cured from EMT6 tumor by PDT 5 weeks earlier. However, the presence of increased numbers of CD45RB^{low}CD44^{high} cells (memory cells) in the latter group was reported recently by Gollnick *et al.* (16), who were working with the same experimental model. Immune memory cells are notoriously difficult to identify by flow cytometry.

Although mature T lymphocytes were virtually nonexistent in samples from naïve scid mice, significant numbers of these cells were found in the spleen and blood of scid mice engrafted with splenocytes from BALB/c donors. The most striking difference between the recipients of splenocytes from naïve donors and recipients from donors cured previously from EMT6 tumor by PDT was highly elevated levels of circulating and spleen-residing CD8$^+$ T cells in the latter group. In both groups, the incidence of CD4$^+$ T cells was generally low, whereas the CD44 antigen was highly expressed in spleen-residing CD4$^+$ and even more so in spleen CD8$^+$ T cells.

PDT and Adoptive Transfer with BALB/c Recipients. The outcome of PDT treatment of EMT6 tumors growing in immunocompetent BALB/c mice engrafted with splenocytes from BALB/c donors containing PDT-generated EMT6 tumor-sensitized immune cells is shown in Fig. 6*A*. The PDT dose used for treating the tumors growing in these recipients was decreased (by lowering the light dose to 50 J/cm^2) to have limited cure rates in the PDT-only reference group. The results show that adoptive transfer improved the effect of therapy in these recipients, although the outcome was not fully curative.

The same type of experiment was performed with another tumor model, Line 1 carcinoma (also syngeneic to BALB/c mice), which, unlike EMT6, is a weakly immunogenic tumor (14). Future BALB/c donors were implanted with Line 1 carcinoma and treated by PDT (Photofrin 10 mg/kg; 180 J/cm^2). The mice showing no signs of tumor regrowth 5 weeks later were sacrificed, and their splenocytes were transferred to naïve BALB/c mice that were subsequently implanted with Line 1 tumor and PDT treated ("schedule one" protocol). The results (Fig. 6*B*) show that the splenocyte transfer improved the curative effect of PDT in BALB/c host mice in a manner comparable with that observed with the EMT6 tumor model.

DISCUSSION

It is well established that immunogenic tumors, such as EMT6, induce the generation of tumor-sensitized T lymphocytes in host mice. If the host animals are cured of tumor (*e.g.,* by surgical excision or X-ray treatment), these immune cells will maintain long-term resistance to rechallenge with the same tumor. In experiments combining PDT and adoptive transfer, the presence of tumor-sensitized T cells among the splenocytes of BALB/c mice cured previously from EMT6 tumors, and their absence from naïve BALB/c spleen cell populations, made a critical difference to therapy outcome. Significant levels of cures of PDT-treated EMT6 tumors growing in engrafted scid mice were achieved only in the former case.

Severe deficiency in the activity of lymphoid populations in scid mice (17) is responsible for the absence of cures of PDT-treated EMT6 tumors growing in these animals (11). It appears that the adoptive transfer of naïve BALB/c splenocytes was inadequate to reconstitute the T-cell activity in scid recipients to the level functioning in the immunocompetent BALB/c mice. This is likely due to the abnormalities in lymphoid tissues (17), which may hinder restoration of orderly immune cell activity in engrafted scid mice. Upon stimulation provided by PDT treatment of EMT6 tumors in scid recipients, the engrafted EMT6 tumor-sensitized T-cell populations are apparently much easier to activate than naïve splenocytes. Selective trafficking of tumor-sensitized lymphocytes to the tumor could be one of the factors responsible for that difference. In particular, considerably higher levels of circulating and spleen-residing CTLs were found 1 week after PDT in scid mice that received tumor-sensitized immune

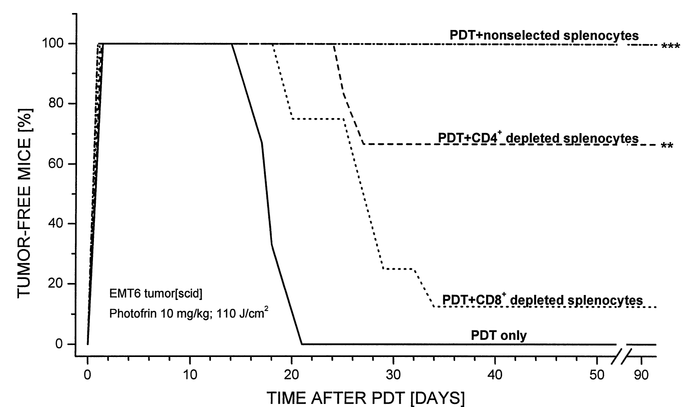

Fig. 5. The effect of adoptively transferred EMT6 tumor-sensitized immune cell populations depleted of CD4$^+$ or CD8$^+$ T cells on the PDT response of s.c. EMT6 tumors growing in scid mice recipients. The splenocytes containing PDT-generated EMT6 tumor-sensitized immune cells (same as described in Fig. 2) were subjected to the complement-mediated *in vitro* depletion of either CD4$^+$ or CD8$^+$ T cells before being used for the adoptive transfer that was performed as described in Fig. 1. The PDT treatment of EMT6 tumors growing in scid mice that were recipients of either nonselected, CD4$^+$-depleted, or CD8$^+$-depleted splenocytes, or not involved in spleen cell transfer, as well as other experimental details were also as described in Fig. 1. Statistical significance of responses compared with "PDT only" group: $***$, $P < 0.0001$; $**$, $P < 0.001$.

Table 1 *Flow cytometry analysis of T lymphocytes before and after adoptive transfer of spleen cells from BALB/c to scid mice*

Sample[a]	$CD4^+$ T cells		$CD8^+$ T cells	
	%	CD44 expression	%	CD44 expression
Control BALB/c				
Spleen	20–30	+	15–25	++
EMT6(PDT) cured BALB/c[b]				
Spleen	20–30	+	15–25	++
scid, naïve splenocyte recipients[c]				
Spleen	3–5	+++	25–35	++++
Blood	1–3	+	10–15	++
scid, recipients of EMT6 tumor-sensitized immune spleen cells[b]				
Spleen	3–5	++	40–70	++++
Blood	4–6	+	40–70	++

[a] Samples from at least four mice were independently analyzed for each treatment group.
[b] Samples taken 5 weeks after curative PDT (suspensions used for adoptive transfer).
[c] Samples taken 16 days after the adoptive transfer, *i.e.*, 7 days after PDT treatment (details described in Figs. 1 and 2).

cells than in recipients of naïve splenocytes (Table 1). A factor of critical importance for the restoration of the curative effect of PDT in scid mice appears to be the incidence of tumor-sensitized T cells in splenocyte populations adoptively transferred to these hosts. The levels present in the spleens of donors cured of tumor by X-rays were evidently too low to secure the fully curative effect of PDT (Fig. 1) but were sufficiently high in the spleens of donors cured from the tumor by PDT (Fig. 2). This suggests that PDT is a highly effective means of generating tumor-sensitized immune cells *in vivo*.

The difference in the results with X-rays and PDT suggests that both the nature and extent of tumor cell death impact upon the magnitude of the elicited antitumor immune responses. Thus, although lethally irradiated B16 tumor cells, which die via a slow postmitotic process, are poorly immunogenic, equivalent tumor cells transfected with herpes simplex virus-thymidine kinase and killed *in situ* by gancyclovir, elicit strong antitumor immunity (18). Similar results have been obtained using tumor cells transfected with cytosine deaminase that were killed rapidly by administration of 5-fluorocytosine (19). A possible explanation is that rapid and massive release of tumor cell debris may enhance the uptake and presentation of tumor antigens by tumor-associated antigen-presenting cells. Immunological processes have little direct impact on the responses to treatment with ionizing radiation, which induces mainly slow postmitotic or apoptotic death. In contrast, necrotic cell death that generates a vigorous inflammatory response is characteristic for PDT response.

Recent advances in adoptive immunotherapy have established that the tumor-sensitized T lymphocytes generated in tumor-bearing hosts are arrested in a "pre-effector" stage and require further activating signals to mature into fully functional antitumor effector cells (20). These signals, provided by tumor antigen-specific activation through the T cell receptor/CD3 complex along with costimulatory cytokines (such as IL-2) and other accessory signals, are shut off by immunosuppressive signals in hosts with progressively growing tumors (20). Tumor-sensitized T cells transferred into scid mice remained in the "pre-effector" stage (hence not affecting tumor growth) until the treatment of tumor by PDT provided the necessary conditions to convert them into fully active immune effector cells. These conditions are obviously met by the dramatic changes induced by PDT in the tumor microenvironment. The destruction of tumor tissue eliminates its immunosuppressive dominance, whereas the release of various cytokines and other inflammatory/immune mediators that activate diverse types of host cells (1, 2) seems to create the necessary stimulus for the activation of adoptively transferred pre-effector cells.

An important characteristic of PDT-induced immune reaction appears to be the dominance of the cellular arm of the immune system carried by various types of activated myeloid and lymphoid effector cells, including neutrophils, mast cells, monocytes/macrophages, helper T cells, cytotoxic T cells, and NK cells (1, 2). With respect to NK cells, their contribution to the cures of PDT-treated Meth-A sarcomas growing in scid mice was revealed in this work upon selective depletion of these cells from the hosts initiated immediately after PDT (Fig. 4). On the other hand, the depletion of NK cells had no influence on the curative effect of PDT against Meth-A tumors growing in BALB/c hosts. These findings may reflect the capability of

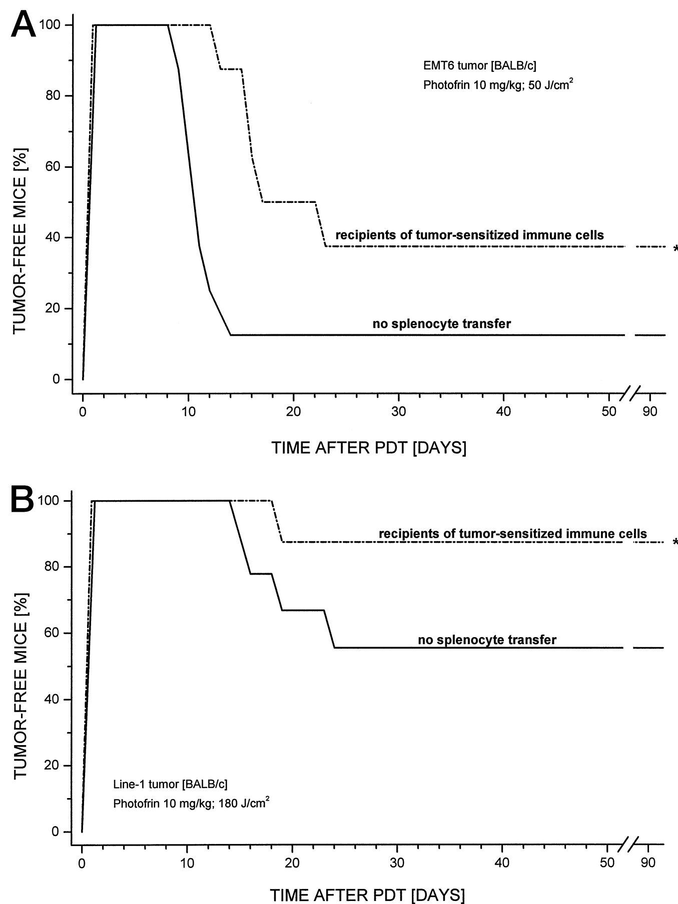

Fig. 6. The effect of adoptively transferred, PDT-generated, tumor-sensitized immune cells on the response of s.c. EMT6 or Line 1 tumors growing in BALB/c mice. s.c. EMT6 or Line 1 tumors growing in BALB/c mice were treated by PDT (Photofrin, 10 mg/kg, plus 110 J/cm^2 for EMT6 or 180 J/cm^2 for Line 1 tumors). Five weeks later, these mice served as donors of splenocytes that were transferred to naïve BALB/c mice. The recipient mice were implanted with the same tumor as the donors 2 days later, and the tumors were allowed to grow for 7 days before being treated by PDT using Photofrin (10 mg/kg) and 50 J/cm^2 for EMT6 tumors (*A*) or 180 J/cm^2 for Line 1 tumors (*B*). Other experimental details were as described in Fig. 1. Statistical significance of responses compared to "no splenocyte transfer" group: *, $P < 0.05$.

PDT-activated T lymphocyte populations in immunocompetent hosts to maintain tumor control, even in the absence of a contribution from activated NK cells. However, this may not be the case with tumors that are more susceptible to NK cells.

We showed that *in vivo* depletion of $CD8^+$ T cells from BALB/c mice immediately after the treatment of EMT6 tumors with Photofrin-based PDT markedly reduced the tumor cure rate (21). *In vivo* depletion of $CD4^+$ T lymphocytes or blocking the IL-2 receptor (using anti-CD25 mAbs) performed under the same experimental circumstances also reduced the curative rate of PDT-treated EMT6 tumors, but to a lesser degree. In agreement with these results are the findings from selective *in vitro* depletion experiments with engrafting splenocytes (Fig. 5). They show that tumor-sensitized CTLs are the main immune effector cell population responsible for conferring the curative outcome to PDT treatment of EMT6 tumors growing in engrafted scid mice. Tumor-sensitized helper T lymphocytes are also involved, but the curative effect is not completely abolished in their absence, which suggests that these cells have a supportive role. The immune specificity of these T lymphocyte populations is evidenced by the absence of cross-reactivity between the responsiveness of EMT6 and Meth-A tumors (Figs. 2 and 3). The fact that these cells can be recovered from distant lymphoid tissues (spleen) at protracted time intervals (5 weeks after the donor's tumor was eradicated) attests to their immune memory character.

The therapeutic potential of adoptively transferred PDT-generated tumor-sensitized immune cells was evident not only in immunodeficient mice (scids) but also in immunologically intact BALB/c recipients (Fig. 6). The latter case represents a classical adoptive immunotherapy that was combined with PDT in an effort to improve the cure rate of treated s.c. tumors. The presence of tumor-induced immunosuppressor T cells is known to limit the success of adoptive immunotherapy (20). The activity of these cells may have restricted the therapeutic benefit obtained in these experiments with BALB/c mice, in contrast to the experiments involving T cell-deficient scid mice. Nevertheless, the results in Fig. 6 demonstrate that the combination of PDT and adoptive immunotherapy produced a therapeutic benefit, even with a weakly immunogenic tumor model (Line 1 carcinoma), which indicates that the induction of PDT-mediated immune reaction is not restricted to strongly immunogenic tumors. This has important ramifications for clinical PDT, because most human tumors are poorly immunogenic.

Further improvements to the adoptive therapy protocols used in this study could be expected to produce additional enhancements in tumor cure rate in immunocompetent hosts. These include: (*a*) removal of L-selectin- positive immunosuppressor cells from the populations used for adoptive transfer (22); (*b*) augmenting the recruitment of antigen-presenting cells to tumor site by localized treatment with cytokines such as granulocyte/macrophage-colony stimulating factor and IL-3 (23, 24); and/or (*c*) *ex vivo* expansion and activation of tumor-sensitized lymphocytes (*e.g.,* using anti-CD3/IL-2 combination; 20). The use of PDT may address some critical issues in adoptive therapy. For instance, improved homing of adoptively transferred cells could be achieved due to the release of chemotactic factors triggered by PDT. Moreover, the PDT-induced release of IL-2 and other cytokines may permit the adjuvant systemic administration of IL-2 (frequently causing severe side effects in adoptive immunotherapy treatments) to be reduced or omitted. With respect to the latter, it should be noted that adoptive therapy combined with PDT was beneficial in this study, despite the fact that systemic IL-2 treatment (required in standard protocols using this therapy for treatment of solid tumors) was omitted.

Very encouraging initial results were obtained in our ongoing studies aimed at advancing the therapy of solid cancers, in which PDT is combined with the adoptive transfer of lymphocytes from tumor-draining lymph nodes and the above-mentioned strategies for improved adoptive immunotherapy protocols are applied (25).

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