

# Extracorporeal photopheresis reverses experimental graft-versus-host disease through regulatory T cells

Erin Gatz,<sup>1</sup> Clare E. Rogers,<sup>1</sup> Shawn G. Clouthier,<sup>1</sup> Kathleen P. Lowler,<sup>1</sup> Isao Tawara,<sup>1</sup> Chen Liu,<sup>2</sup> Pavan Reddy,<sup>3</sup> and James L. M. Ferrara<sup>1</sup>

<sup>1</sup>Department of Pediatrics, University of Michigan Cancer Center, Ann Arbor; <sup>2</sup>Department of Pathology, University of Florida College of Medicine, Gainesville; and <sup>3</sup>Department of Internal Medicine, University of Michigan Cancer Center, Ann Arbor

**Extracorporeal photopheresis (ECP), a technique that exposes isolated white blood cells to photoactivatable 8-methoxypsoralen and ultraviolet A radiation, is used clinically to treat cutaneous T-cell lymphoma and immune-mediated diseases such as graft-versus-host disease (GVHD). ECP is thought to control these diseases in part through**

**direct induction of lymphocyte apoptosis, but its effects on the immune system beyond apoptosis remain poorly characterized. We have developed a novel method for incorporating ECP treatment into well-established and clinically relevant murine models of GVHD to examine its effects during an ongoing immune response. We demonstrate that**

**the transfer of cells treated with ECP reverses established GVHD by increasing donor regulatory T cells and indirectly reducing the number of donor effector lymphocytes that themselves had never been exposed to psoralen and ultraviolet A radiation. (Blood. 2008;112:1515-1521)**

## Introduction

Extracorporeal photopheresis (ECP) exposes isolated white blood cells to photoactivatable 8-methoxypsoralen (8-MOP) and ultraviolet A radiation (PUVA).<sup>1</sup> 8-MOP is normally biologically inert, but in the presence of UVA light it cross-links DNA by forming covalent bonds with pyrimidine bases and causes apoptosis.<sup>2</sup> ECP has been used to treat cutaneous T-cell lymphoma (CTCL) and, more recently, immune-mediated diseases such as graft-versus-host disease (GVHD), the major complication of allogeneic bone marrow transplantation (BMT).<sup>3,4</sup>

GVHD occurs when donor T cells respond to foreign histocompatibility antigens presented by host antigen-presenting cells<sup>5,6</sup> and results in significant morbidity and mortality. Prevention of GVHD has been limited either to depletion of donor T cells or the use of broadly immunosuppressive drugs that cause profound immunodeficiency as well as toxicities such as hypertension, diabetes, and aseptic bone necrosis.<sup>4,7</sup> ECP appears to be an effective therapy for both acute and chronic GVHD, even in some patients who are refractory to conventional immunosuppressive therapy. While randomized, controlled, multicenter studies are still needed, few side effects have been associated with ECP treatment and several reports of the efficacy of ECP for the treatment of both acute and chronic GVHD are impressive.<sup>3,4</sup>

PUVA treatment is known to induce the apoptosis of all exposed leukocytes, but it is unlikely that the direct induction of lymphocyte apoptosis fully accounts for its clinical efficacy, given that less than 10% of circulating leukocytes are exposed to PUVA during an ECP procedure. The effects of ECP on the immune system beyond the induction of apoptosis are still poorly understood, however, largely because of a lack of mechanistic animal models for the diseases historically treated with ECP. We have developed a novel method

for incorporating ECP treatment into 2 well-defined models of GVHD to examine the effects of ECP treatment on established disease. We demonstrate that infusing ECP-treated cells into allogeneic bone marrow (BM) transplant recipients significantly reduces established severe GVHD, suppresses allogeneic responses of donor effector T cells that have themselves never been exposed to PUVA, and increases the number of Foxp3<sup>+</sup> T regulatory cells (Tregs) derived from both the donor T-cell and bone marrow grafts. This increase in Tregs was observed early after the infusion of ECP-treated cells, remained stable for several weeks, and was required to reduce GVHD and mortality after allogeneic BMT.

## Methods

### Mice

Female C57Bl/6 (B6:H-2<sup>b</sup>, CD45.2<sup>+</sup>Thy1.2<sup>+</sup>), B6.Ly-5a (B6-Ly5.2:H-2<sup>b</sup>, CD45.1<sup>+</sup>Thy1.2<sup>+</sup>), and B6D2F1 (H-2<sup>bxd</sup>, CD45.2<sup>+</sup>Thy1.2<sup>+</sup>) were purchased from Charles River Laboratories (Wilmington, MA). Female B6.PL-Thy1a (B6-Thy1.1: H-2<sup>b</sup>, CD45.2<sup>+</sup>Thy1.1<sup>+</sup>) and C3H.SW (H-2<sup>b</sup>, CD45.2<sup>+</sup>Thy1.2<sup>+</sup>) were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were at least 8 weeks of age prior to use and were cared for according to the Guidelines for Laboratory Animal Medicine at the University of Michigan.

### Bone marrow transplantation

C3H.SW mice were conditioned with a single dose of 1000 to 1100 cGy total body irradiation (TBI; <sup>137</sup>Cs source), followed by intravenous infusion of 5.0 × 10<sup>6</sup> bone marrow (BM) cells plus 0.5 to 1.0 × 10<sup>6</sup> positively selected CD90<sup>+</sup> T cells from allogeneic (B6-Ly5.2 or B6-Thy1.1) or

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syngeneic (C3H.SW) donor mice on day 0. In some experiments, conditioned C3H.SW mice were infused with  $5.0 \times 10^6$  bone marrow and  $10^6$  T cells from B6 mice to allow identification of B6-Ly5.2→C3H.SW ECP-treated cells 3 days after injection. Conditioned B6 and C3H.SW recipient mice were infused with  $5.0 \times 10^6$  T cell-depleted B6-Ly5.2 BM cells plus  $1.0 \times 10^6$  purified B6-Thy1.1 T cells to distinguish origin from BM and T-cell grafts. B6D2F1 mice were conditioned with a single dose of 900 cGy, and then infused with  $5.0 \times 10^6$  bone marrow and  $2.0 \times 10^6$  T cells from B6-Ly5.2 or B6D2F1 donors. Mice were housed in sterile microisolator cages and given autoclaved hyperchlorinated (pH = 3.0) drinking water for 3 weeks following BMT.

### Systemic and histopathologic analysis of GVHD

Survival after BMT was monitored daily and clinical GVHD was assessed weekly using a previously described scoring system<sup>8</sup> that generates a composite GVHD score composed of individual scores for weight loss, posture, mobility, skin integrity, and fur texture. Histopathological changes of GVHD were quantified in liver, skin, and intestine, as described,<sup>8</sup> by a single pathologist using coded slides.

### ECP treatment

Splenocytes from separate cohorts of B6→C3H.SW (to treat B6-Ly5.2 or B6-Thy1.1→C3H.SW BM transplant recipients), B6-Ly5.2→C3H.SW (to treat B6→C3H.SW BM transplant recipients), or B6→B6D2F1 (to treat B6-Ly5.2→B6D2F1 BM transplant recipients) mice were harvested 14 days after BMT (date of maximal donor cell expansion) and processed into single-cell suspension in Leibovitz media (L-15; Gibco, Grand Island, NY). Red blood cells (RBCs) were lysed using Sigma RBC Lysing Buffer (Sigma-Aldrich, St Louis, MO) per the manufacturer's protocol followed by 2 washes in L-15 at room temperature (RT). The splenocytes were counted using trypan blue exclusion and suspended to  $7.0 \times 10^6$ /mL in RT L-15 containing 8-MOP (UVADEX; Therakos, Exton, PA;  $C_f = 200$  ng/mL). The cells were incubated in the dark at RT for 30 minutes with intermittent mixing, then transferred into sterile polystyrene vented-top flasks for exposure to 2 J/cm<sup>2</sup> UVA light from a UVAR light set (provided by Therakos). The light set was calibrated periodically using a UVX meter fitted with a UVA detector (UVP, Upland, CA) to maintain a 2 J/cm<sup>2</sup> dose for all studies. After UVA exposure, the cells were washed twice and suspended in L-15 at  $30 \times 10^6$  cells/250  $\mu$ L for intravenous injection. Untreated splenocytes were handled identically but not exposed to 8-MOP and UVA.

### In vivo CD25 depletion

Anti-CD25 mAb-producing hybridoma PC61.5 was purchased from ATCC (Manassas, VA) and was cultivated with CD-hybridoma media (Gibco) in the CELLLine classic 1000 flask (Wilson Wolf Manufacturing, New Brighton, MN). PC-61 mAb was concentrated from culture supernatant using ammonium sulfate and was dialyzed against PBS. C3H.SW transplant recipients were injected intraperitoneally with 0.5 mg anti-mouse CD25 PC61 or isotype control mAb (Rat IgG1) 1 day after BMT, and 0.1 mg 1 day after each infusion of ECP-treated cells. Depletion of CD25<sup>+</sup> cells was verified by flow cytometry using a non-cross-reactive anti-CD25 mAb, 7D4, before the onset of ECP and 1 week following the last infusion.

### In vitro MLR cultures

B6-Ly5.2 CD90<sup>+</sup> T cells (responders,  $2.0 \times 10^6$ /mL) were cultured with B6D2F1 bone marrow-derived dendritic cells (stimulators) at a 10:1 ratio, respectively, in T-25 vented-top flasks or flat-bottom 96-well plates. ECP-treated or control splenocytes (B6→B6D2F1, day + 14) were added at a 1:1 ratio with responder cells. Cultures were incubated at 37°C for 60 hours. Ninety-six-well cultures were pulsed for an additional 12 hours with 1  $\mu$ Ci (0.037 MBq) <sup>3</sup>H-thymidine per well to determine proliferation in triplicate samples. Bulk cultures in flasks were harvested, counted, and added at a 1:1 ratio to responder cells in fresh cultures containing 10:1 B6 responders to B6D2F1 stimulators, or stained for intracellular Foxp3 or IFN- $\gamma$ , as described in "Flow cytometry."

### Flow cytometry

Cells in single-cell suspension were isolated from mice after BMT or from in vitro cultures. Cells were added to 96-well V-bottom plates ( $2.0 \times 10^5$ – $5.0 \times 10^5$  per well) and Fc receptors were blocked for 10 minutes on ice in PBS containing 2% fetal bovine serum (FACS wash) containing 1:400 Fc Block (purified CD16/32, clone 2.4G2; BD Biosciences, San Jose, CA) followed by cell surface staining with fluorochrome-labeled anti-mouse mAbs or isotype controls as listed at the end of this paragraph (1:200 dilutions in FACS wash) for 20 minutes on ice. After washing, the cells were either suspended in FACS wash and transferred into 5-mL polystyrene round-bottom tubes (BD Falcon) for immediate analysis or fixed using BD Fix and Lyse (BD Biosciences) and stored overnight at 4°C before analysis. For Foxp3 staining,  $2.0$  to  $3.0 \times 10^6$  cells per well were stained for cell surface markers, suspended in BD Fix and Lyse, and stored in V-bottom wells at 4°C overnight. The cells were then washed with 1 $\times$  permeabilization (Perm) buffer (eBioscience, San Diego, CA) and then incubated for 15 minutes on ice in BD Fc block (BD Biosciences) diluted 1:400 in 1 $\times$  Perm buffer. After centrifugation, the cells were suspended in antimouse Foxp3 or isotype control mAb diluted 1:100 in 1 $\times$  Perm buffer for 30 minutes on ice. The cells were washed twice with 1 $\times$  Perm buffer and once with FACS wash before being suspended in 200 to 300  $\mu$ L FACS wash and transferred to 5-mL tubes for immediate analysis. IFN- $\gamma$  production by responder CD8<sup>+</sup> T cells was assessed following in vitro restimulation with 2  $\mu$ g/mL ionomycin and 50 ng/mL PMA for 5 hours with or without brefeldin A (BD GolgiPlug; BD Biosciences). Cells not stimulated with PMA/ionomycin were also included as a negative control. Cells were stained for cell surface markers, fixed, permeabilized, and stained intracellularly with anti-mouse IFN- $\gamma$  as described in the BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit manual<sup>9</sup> (BD Biosciences). A BD FACS Aria and FACS Diva software (BD Biosciences) were used for analysis of in vivo (6-color) Treg samples; all other samples were analyzed using a BD FACS Calibur and CellQuest software (BD Biosciences). The following antibodies and their isotype controls were used: anti-mouse CD4 (PerCP-Cy5.5 and Pacific Blue, clone RM4-5, rat IgG2a), CD8a (PE, APC, and APC-Cy7, clone 53-6.7, rat IgG2a), CD19 (APC, clone 1D3, rat IgG2a), CD25 (APC-Cy7 and PerCP-Cy5.5, clone PC61, rat IgG2a), CD25 (FITC, clone 7D4, rat IgM), CD45.1 (FITC, clone A20, Ms IgG2a), CD45.2 (PE and PerCP-Cy5.5, clone 104, Ms IgG2a), CD90.1 (FITC, clone HIS51, rat IgG2a), IFN- $\gamma$  (PE, clone XMG1.2, rat IgG1) (all from BD Biosciences), anti-mouse CD45.1 (APC, clone A20, Ms IgG2a; Southern Biotech, Birmingham, AL), Foxp3 (PE, clone FJK-16, rat IgG2a; eBioscience).

### Statistical analysis

Survival curves were plotted using Kaplan-Meier estimates. The Mann-Whitney *U* test was used for the statistical analysis of ex vivo data and clinical scores and the Mantel-Cox log-rank test was used to analyze survival data. *P* less than .05 was considered statistically significant.

## Results

### Infusion of cells treated with ECP reduces GVHD following allogeneic BMT

Because only approximately 10% of circulating leukocytes are exposed to PUVA during an ECP procedure, we hypothesized that cells treated with ECP would modulate the function of lymphocytes that had not been exposed to ECP. We tested this hypothesis in a well-defined allogeneic BMT model of GVHD where donor and recipient are identical at the major histocompatibility complex (MHC) but mismatched at multiple minor histocompatibility antigens (miHAs) (B6→C3H.SW) and GVHD is mediated by donor CD8<sup>+</sup> T cells.<sup>5</sup> C3H.SW mice received 11 Gy total body irradiation and received a transplant of  $5.0 \times 10^6$  bone marrow and  $10^6$  T cells from either syngeneic (C3H.SW) or allogeneic (B6-Ly5.2) donors. Thirty million splenocytes from a second

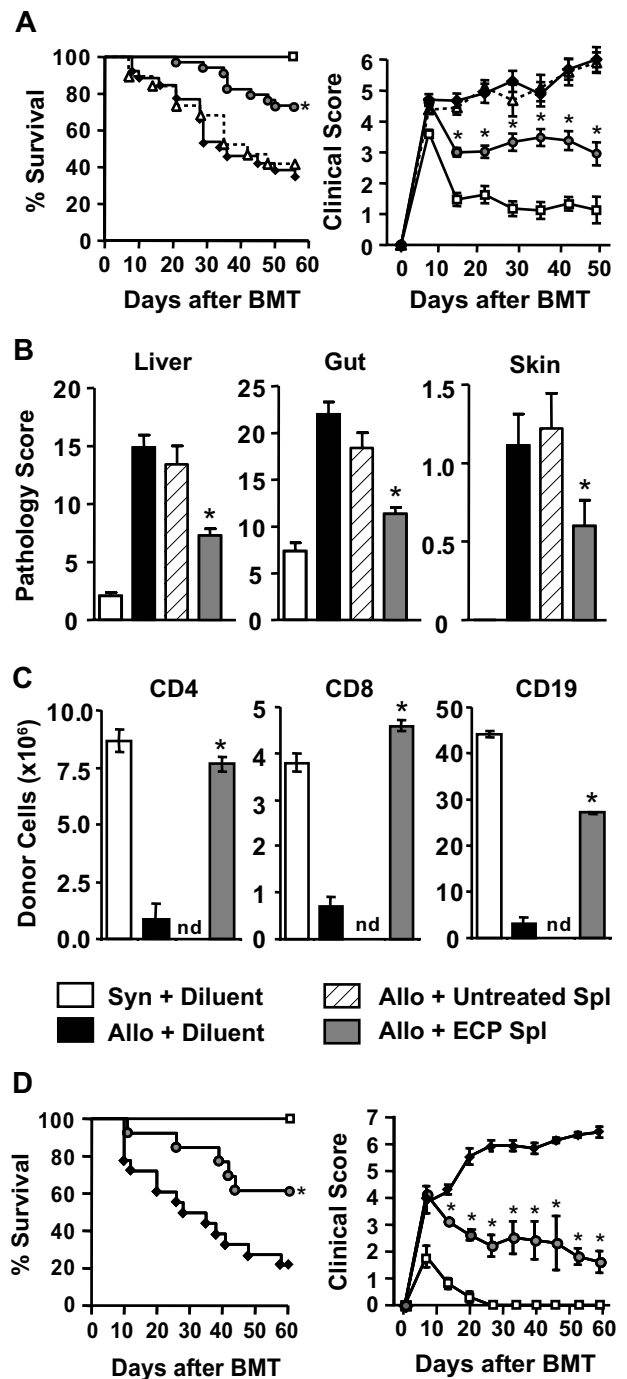
cohort of B6→C3H.SW BM transplant recipients were isolated, exposed to doses of PUVA used clinically *ex vivo*, and infused intravenously into the first cohort of BM transplant recipients 7 days after BMT (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article), when donor T cells had already differentiated and trafficked to GVHD target organs, causing clinical disease. We assumed that the spleen would be representative of the peripheral blood and therefore chose  $30 \times 10^6$  splenocytes to treat and reinfuse because that quantity represents 10% of the splenocytes present on day +14 of this GVHD model. Splenocytes from the second BMT cohort that were exposed to PUVA were all (> 98%) annexin-V<sup>+</sup> within 24 hours of exposure and were undetectable in the spleen, thymus, and lymph nodes 3 days after infusion (data not shown). As shown in Figure 1A, 1 injection of ECP-treated cells suppressed ongoing clinical GVHD and 4 weekly injections improved both survival and GVHD clinical scores compared with controls injected with either untreated splenocytes or diluent. Mice receiving ECP-treated cells also showed significantly less histopathological damage in all 3 GVHD target organs (Figure 1B) and dramatically improved immune reconstitution 56 days after BMT (Figure 1C). All mice demonstrated complete (> 99.8%) donor engraftment and showed no evidence of any PUVA-treated splenocytes in their lymphoid organs (data not shown). We confirmed these results in a second, haploidentical model (B6-Ly5.2→B6D2F1) where GVHD is driven by CD4<sup>+</sup> T cells.<sup>10</sup> Injection of ECP-treated cells from parallel cohorts of mice with GVHD significantly increased survival at day 60 compared with controls, and significantly decreased GVHD clinical scores (Figure 1D). Surprisingly, the infusion of untreated splenocytes did not measurably worsen GVHD (Figure 1A and data not shown).

#### Cells treated with ECP modulate allogeneic T-cell responses in vivo and in vitro

Although no ECP-treated cells were detectable in the spleen 72 hours after infusion, at 96 hours they had caused a 3-fold decrease in CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> donor effector splenocytes that had never been directly exposed to PUVA (Figure 2). ECP-treated cells also suppressed allogeneic T-cell responses in vitro. B6D2F1 bone marrow-derived dendritic cells (stimulators) and B6-Ly5.2 responder T cells were cocultured as described in "In vitro MLR cultures" at a 1:10 ratio, respectively. We added splenocytes from B6→B6D2F1 mice that were either untreated or treated with ECP *ex vivo* to B6-Ly5.2 responders at a 1:1 ratio. Sixty hours later, cultures containing ECP-treated splenocytes contained half the number of Ly5.2<sup>+</sup>CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> responders, and T-cell proliferation in response to allogeneic stimuli was reduced by 50% (Figure 3A).

#### Splenocytes treated with ECP increase Foxp3<sup>+</sup> T regulatory cells in MLR cultures and recipients of allogeneic BM transplant

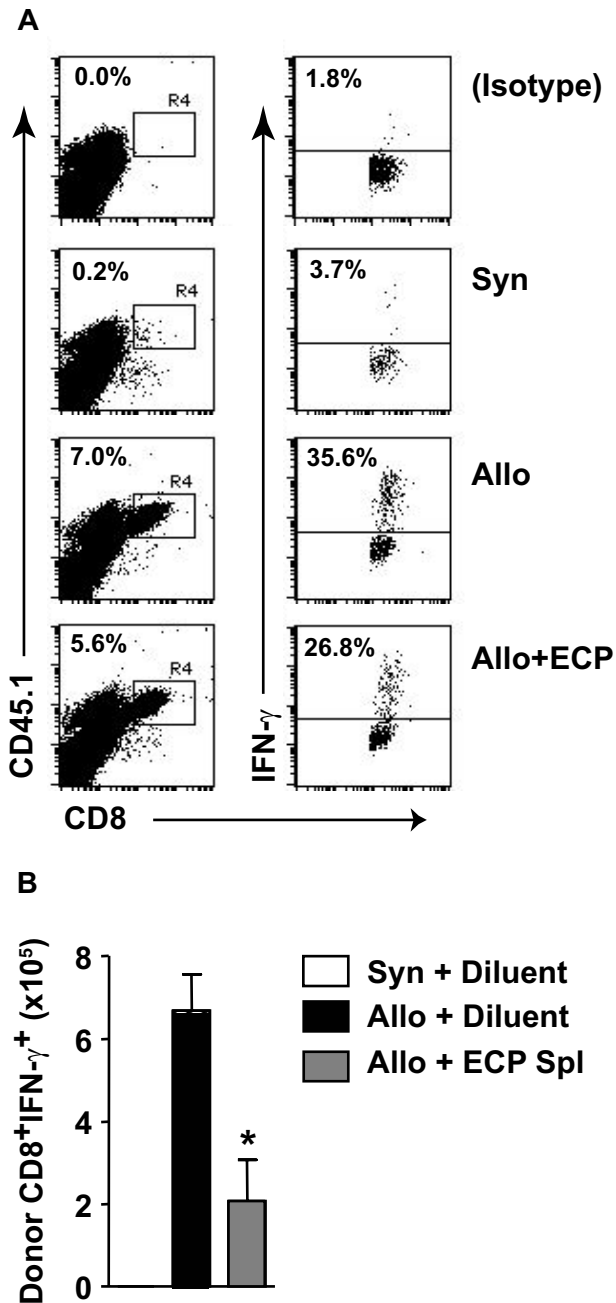
Within 72 hours, MLR cultures containing ECP-treated splenocytes contained increased percentages of donor CD4<sup>+</sup>Foxp3<sup>+</sup> cells (Figure 3B), resulting in an average 1.8-fold increase in the number of donor CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs per well compared with MLR cultures incubated with media or control splenocytes (data not shown). In addition, cells from MLR cultures containing ECP-treated splenocytes did not proliferate in response to secondary stimulation with fresh B6D2F1 stimulator dendritic cells (DCs) (data not shown), and their bulk transfer from the first MLR to an identical second set of MLR cultures suppressed allogeneic proliferation of fresh responders more than 200-fold (Figure 3C). We



**Figure 1. ECP-treated cells reduce GVHD and mortality after allogeneic BMT.** (A) Survival and clinical GVHD scores after BMT. C3H.SW mice received syngeneic (C3H.SW; □, n = 15) or allogeneic (B6-Ly5.2) BM transplant followed by 4 weekly infusions of diluent (◆, n = 26) or of B6→C3H.SW splenocytes that were untreated (△, n = 19) or treated with ECP (●, n = 34); ● versus △ or ◆, \**P* < .004. (B) Histopathology scores 56 days after BMT (n = 8–20 per group); ▨ versus ▩, \**P* < .03. (C) Spleen reconstitution with donor lymphocytes (B6-Ly5.2) 56 days after BMT (n = 6–8 per group); ▨ versus ▩, \**P* < .02. (D) Survival and clinical GVHD scores after haploidentical BMT. B6D2F1 mice received syngeneic (B6D2F1; □, n = 6) or allogeneic (B6-Ly5.2) BMT followed by 4 weekly infusions of diluent (◆, n = 18) or of B6→B6D2F1 splenocytes that were treated with ECP (●, n = 13); ● versus ◆, \**P* < .02. Data are means (± SD) pooled from at least 2 independent experiments.

hypothesized that these Tregs mediated the reversal of GVHD in vivo. Indeed, one infusion of ECP-treated cells caused the number of donor CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> splenocytes in mice that underwent transplantation to nearly double within 4 days (Figure 4B left





**Figure 2. ECP-treated splenocytes reduce CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> effector T cells in vivo.** C3H.SW recipient mice received  $5.0 \times 10^6$  bone marrow and  $10^6$  T cells from syngeneic (C3H.SW) or allogeneic (B6-Ly5.2) donors on day 0 followed by infusion of B6 $\rightarrow$ C3H.SW ECP-treated cells on day 7. IFN- $\gamma$  production by donor CD8<sup>+</sup> effector cells was measured 96 hours later. (A) Representative flow cytometry data of unseparated splenocytes gated on whole-cell scatter. Percentage of donor (CD45.1<sup>+</sup>) CD8<sup>+</sup> T cells in R4 (left) or producing IFN- $\gamma$  (right) indicated. (B) Numbers of donor CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells per spleen.  $\square$  versus  $\blacksquare$ , \* $P < .05$ . Data are means ( $\pm$  SD) pooled from 3 independent experiments ( $n = 9-20$  per group).

panel). The number of Tregs remained significantly elevated for more than a month in the spleen, mesenteric lymph nodes (MLNs), and the thymus (Figure 4B middle and right panels). As shown in Table S1, the increase in Tregs 11 days after transplantation was due to a significant increase in the percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells, whereas the increases at 35 and 49 days were due to increased donor cell reconstitution at these sites. Identical increases were observed at all sites when Treg cells were

defined as CD4<sup>+</sup>Foxp3<sup>+</sup> cells (data not shown) and all CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> and CD4<sup>+</sup>Foxp3<sup>+</sup> cells were additionally GITR<sup>+</sup> ( $99\% \pm 1\%$ ). As shown in Figure 4C, 4 days after the infusion of ECP-treated cells (day 11), all Tregs originated from mature T cells in the BMT inoculum. However, 1 week after the fourth infusion of ECP-treated cells (day 35), increased numbers of Tregs had also derived from the bone marrow graft and differentiated in the thymus (Figure 4D).

#### Regulatory T cells are required for reversal of GVHD by ECP treatment

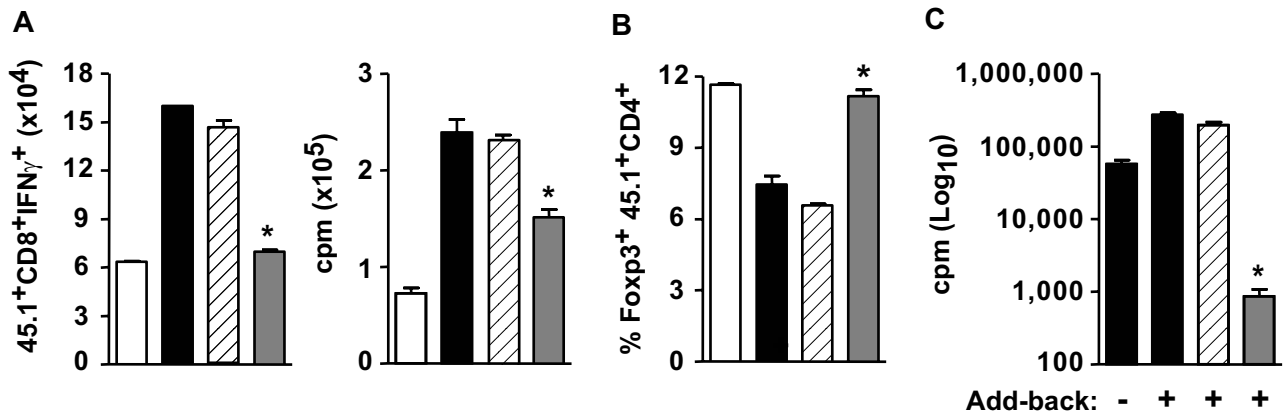
We tested whether Tregs were required to reverse GVHD in this system by incapacitating them in vivo. We injected BM transplant recipients with anti-CD25 (PC61) or isotype control mAb before and after the infusion of ECP-treated splenocytes, as described in "In vivo CD25 depletion." PC61 renders Foxp3<sup>+</sup> Tregs nonfunctional in vivo,<sup>11</sup> and completely prevented the increases in Foxp3<sup>+</sup> cells (Figure 5A) and lymphocyte reconstitution (data not shown) previously observed after infusion of ECP-treated cells. Anti-CD25 also abolished the ability of cells treated with ECP to reverse GVHD, which was now equally severe and lethal in both groups (Figure 5B,C), confirming the requirement of Tregs for disease reversal. Importantly, depletion of CD25 did not augment GVHD clinical scores or the rate of GVHD-associated mortality compared with syngeneic and allogeneic controls (data not shown), and the infusion of ECP-treated cells remained effective following injections of isotype control mAb, significantly enhancing day-56 survival from 44% to 78% ( $P = .02$ ) and reducing GVHD clinical scores ( $5.6 \pm 0.2$  vs  $3.4 \pm 0.6$ ;  $P = .006$ ).

#### Discussion

A primary goal of these studies was to model the clinical ECP treatment of GVHD. We implemented 2 well-defined murine models of GVHD, one with donor and host differing only at minor histocompatibility antigens (B6 $\rightarrow$ C3H.SW) where CD8<sup>+</sup> T cells mediate disease and the other a parent into F1 (B6 $\rightarrow$ B6D2F1) model where GVHD is mediated primarily by CD4<sup>+</sup> T cells. Our system used splenocytes obtained from cohorts of mice with ongoing GVHD to model the cells from a patient undergoing apheresis. Importantly, doses of UVADEX (methoxsalen) and UVA light were equivalent to those used clinically. Approximately 10% of circulating leukocytes are exposed to PUVA during 2 consecutive days of clinical ECP treatment. We chose a dose of 30 million cells, approximately 10% of the viable donor leukocytes present in the spleens of ECP spleen donor cohorts 14 days after transplantation, assuming that the mouse spleen is representative of peripheral blood as a cell source early after transplantation.

These experiments demonstrate an important mechanism whereby ECP reverses GVHD by reducing allogeneic responses of donor effector T cells and generating Foxp3<sup>+</sup> Tregs from donor cells that had not been directly exposed to PUVA, ruling out a mechanistic role for direct apoptosis of effector cells. This increase in Tregs was observed early after the infusion of ECP-treated cells, remained stable for several weeks, and was required to reduce GVHD and mortality after allogeneic BMT.

Mice receiving ECP-treated cells demonstrated improved immune reconstitution, which is consistent with reduced GVHD and with the relative immunocompetence of patients who receive ECP therapy compared with immunosuppressive therapy.<sup>12,13</sup> Recent



**Figure 3. ECP-treated splenocytes reduce allogeneic T-cell responses and increase Foxp3 expression in vitro.** (A,B) B6-Ly5.2 responder T cells were incubated at a 10:1 ratio with syngeneic B6 (□) or allogeneic B6D2F1 (■) stimulator DCs for 60 hours in the presence of untreated (▨) or ECP-treated (▩) B6→B6D2F1 splenocytes at a 1:1 ratio with responders. (A) Numbers of responder CD8<sup>+</sup>IFN-γ<sup>+</sup> cells per well (left) and proliferation of bulk responder cells (right); ▩ versus ■ or ▨, \**P* < .05. (B) Percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> cells; ▩ versus ■ or ▨, \**P* < .05. (C) Proliferation of fresh MLR cultures containing 10 B6-Ly5.2 responder T cells per B6D2F1 stimulator DC after cells harvested from panels A-B were added at a 1:1 ratio with responders. – indicates nothing added; +, cells added from primary cultures as colored in panels A,B; ▩ versus ■ or ▨, \**P* < .05. Syngeneic control groups had low background proliferation (< 2.0 × 10<sup>3</sup> cpm) that was unaffected by addition of cells from panel A,B (data not shown). Data are means (± SD) pooled from at least 2 independent experiments (n = 6-9 wells per group).

research has demonstrated that apoptotic cells can exert profound modulatory effects on both the innate and adaptive immune systems.<sup>14,15</sup> Other models of ECP treatment have altered immune responses by transferring apoptotic cells during immunologic homeostasis in the absence of relevant antigen (eg, to prevent priming in a delayed-type hypersensitivity model<sup>16</sup> or rejection of skin allografts<sup>17,18</sup>). Indeed, interventions that prevent GVHD, such as the transfer of  $\gamma$ -irradiated apoptotic cells, must occur simultaneously with the injection of donor effector cells to be successful.<sup>19</sup> Even so, GVHD mortality in those studies was delayed but not prevented and all animals eventually succumbed to disease. By contrast, our experiments used ECP-treated cells to treat established GVHD. ECP-treated cells not only reduced mortality, but suppressed donor effector T cells a full week after they had encountered host antigen-presenting cells, at a time when they had already clonally expanded and trafficked to GVHD target organs.<sup>20</sup>

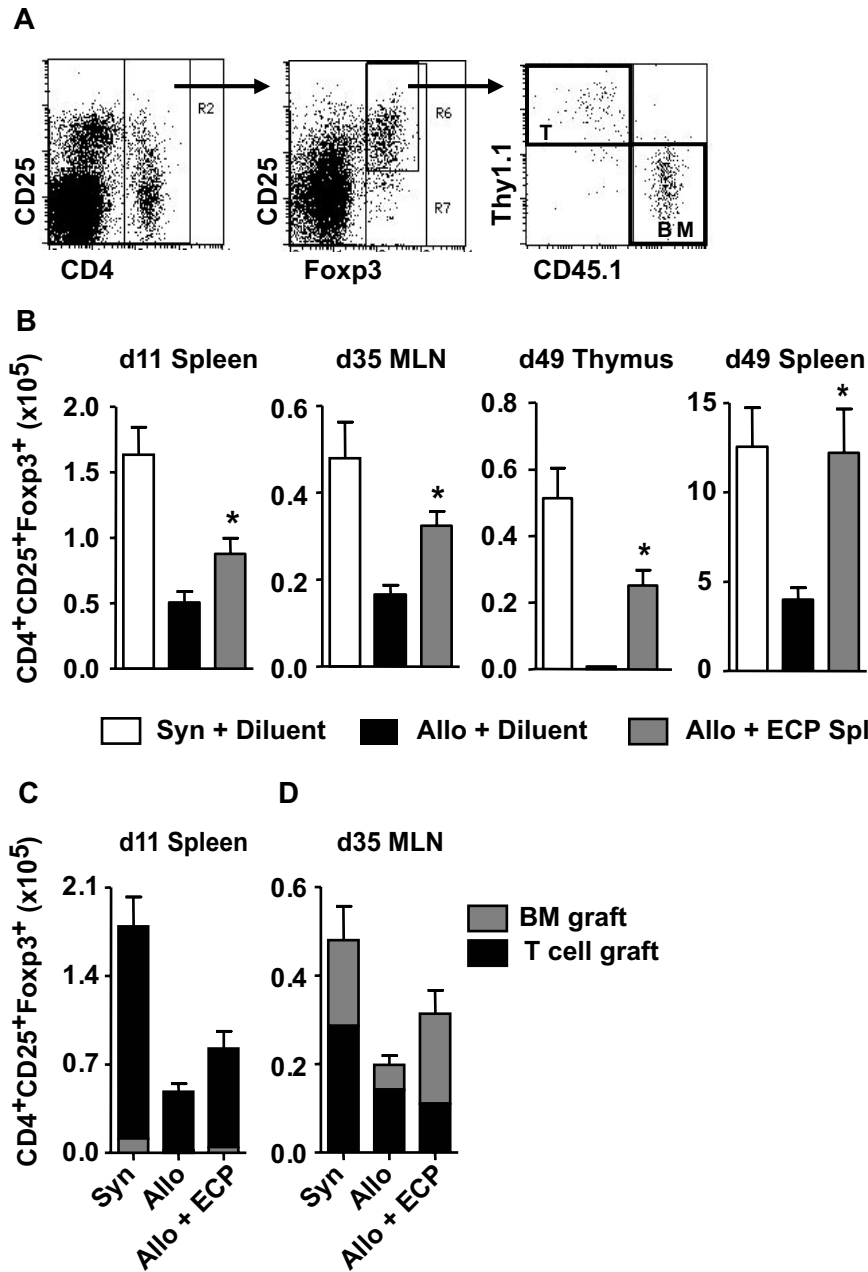
Other groups have reported that the infusion of apoptotic cells delivered during immune homeostasis increases CD4<sup>+</sup>CD25<sup>+</sup> T cells that can partially prevent subsequent priming of DTH responses<sup>16</sup> or GVHD.<sup>19</sup> Our study extends these results and demonstrates the ability of ECP to increase CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells and functionally change the response of effector T cells during an established immune reaction.

Several studies suggest that GVHD can be considered an imbalance of effector T cells and Tregs.<sup>21,22</sup> Edinger et al demonstrated that the simultaneous injection of purified Tregs and donor effector cells suppressed the early expansion of alloreactive donor T cells, and thereby prevented GVHD in a murine model.<sup>23</sup> Consistent with these observations, one recent clinical study has shown that increased numbers of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells in the graft are associated with a reduced risk of GVHD and that the ratio of Treg to effector T cells (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>) is significantly reduced in patients with GVHD.<sup>22</sup> Another small study has also recently observed increases in Tregs in patients with GVHD who responded to ECP treatment.<sup>24</sup> Our data support these observations and demonstrate that ECP induces profound increases in Tregs early after transplantation, and generates Tregs de novo from bone marrow progenitors while reducing effector T-cell responses. Importantly, the ability to generate Tregs in vivo circumvents the need for ex vivo isolation and adoptive transfer of Treg cells for disease treatment or prevention. Our data suggest that the early use

of ECP treatment during the clinical course of a disease may warrant investigation not only for GVHD, but for other diseases with deficits in Tregs, such as autoimmune diseases and solid organ rejection.<sup>4</sup>

Our studies do not address the role of cytokines such as IL-10 or TGF- $\beta$ , or determine whether a distinct subpopulation of Tregs is responsible for reducing GVHD following infusions of ECP-treated splenocytes. Levels of serum IL-10 were unchanged 4 days following the first infusion, and its production by CD4<sup>+</sup> T cells following 60-hour coculture with both allogeneic stimulators and apoptotic splenocytes was undetectable (data not shown). Others have reported that only the CD62L<sup>+</sup> subpopulation of CD4<sup>+</sup>CD25<sup>+</sup> cells protects against murine GVHD when administered with the bone marrow inoculum on the day of BMT<sup>25</sup> and that increased numbers of these cells are observed in the peripheral blood following clinical ECP treatment.<sup>24</sup> In our model, CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>+</sup> Tregs are undetectable in the spleens 14 days after allogeneic BMT and comprise less than 2% of Tregs by day 35, compared with nearly 20% in syngeneic recipients (data not shown). These cells were expanded nearly 5-fold after 4 infusions of ECP-treated splenocytes, but we also observed increased numbers of Tregs that express CD103<sup>+</sup>, another T-cell homing molecule, which represented a much more abundant population (data not shown). Future studies will address both of these important questions more thoroughly as well as to determine the optimal cell dose and number of infusions to achieve a clinical benefit.

These studies also do not address the intriguing question of how ECP leads to clinical responses in GVHD on the one hand, where it appears to induce tolerogenic effects, and in CTCL on the other, where it generates tumor-specific cytotoxic CD8<sup>+</sup> T-cell responses.<sup>26-28</sup> We speculate that antigen-presenting cells may play a key role in mitigating these differential outcomes. Berger et al have demonstrated the rapid production of dendritic cells from peripheral blood monocytes after ECP treatment in CTCL patients<sup>26</sup> and the generation of antigen-loaded activated DC from monocytes that are capable of stimulating a vaccine-like response to CTCL in the presence of apoptotic Sézary cells.<sup>29</sup> But Spisek et al have recently demonstrated that DCs from cGVHD patients treated with ECP are immature in phenotype and that their activation/maturation was not induced by the uptake of ECP-treated apoptotic lymphocytes.<sup>30</sup> The

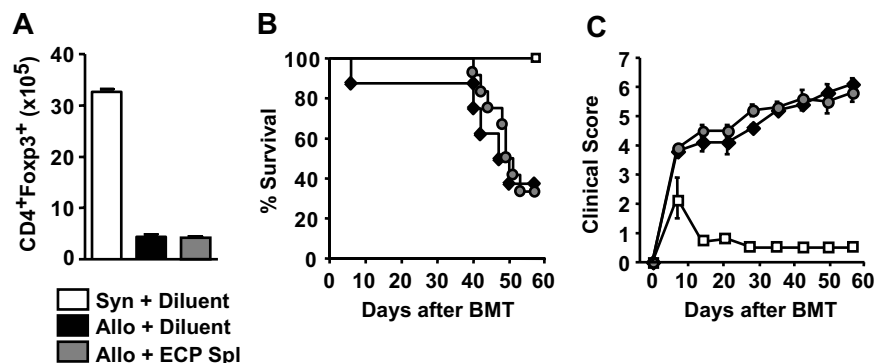


**Figure 4. Infusion of ECP-treated splenocytes increases donor Treg after allogeneic BMT.** B6 and C3H.SW (CD45.2<sup>+</sup>Thy1.2<sup>+</sup>) recipient mice received  $5.0 \times 10^6$  T cell–depleted B6-Ly5.2 (CD45.1<sup>+</sup>Thy1.2<sup>+</sup>) bone marrow and  $10^6$  B6-Thy1.1 (CD45.2<sup>+</sup>Thy1.1<sup>+</sup>) T cells followed by infusion of ECP-treated B6→C3H.SW splenocytes. (A) Gating strategy for Tregs using representative day-35 MLN data. CD4<sup>+</sup>Foxp3<sup>+</sup> or CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells were gated on whole cell scatter followed by segregation into CD45.1<sup>+</sup>Thy1.1<sup>-</sup> BM or CD45.1<sup>-</sup>Thy1.1<sup>+</sup> T-cell inoculum-derived cells. (B) Total numbers of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells after infusion of ECP-treated cells from second BMT cohort;  $\square$  versus  $\blacksquare$ ,  $P < .02$ . At day 11, mice had received 1 infusion of ECP-treated cells, and at day 35 they had received 4 infusions. (C,D) Numbers of donor CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells from the BM and T-cell grafts in the spleen and MLN 11 and 35 days after BMT, respectively. (C) Allo plus diluent versus allo plus ECP,  $\blacksquare$ ,  $P < .03$ ; (D) allo plus diluent versus allo plus ECP,  $\square$ ,  $P = .04$ . Bars represent means ( $\pm$  SD). Data pooled from 3 to 4 independent experiments (n = 12-20/group).

presentation of antigen by these immature DCs may lead to the inhibition of the T cells whose receptors they engage to promote the production of regulatory T cells. Indeed, 2 groups have shown a

role for APCs in ECP-induced prevention of immunity mediated by Tregs in murine models, implicating CD11c<sup>+</sup> DCs<sup>16</sup> and bone marrow–derived macrophages<sup>19</sup> in the prevention of DTH priming

**Figure 5. Tregs are required for reversal of GVHD by ECP.** (A-C) C3H.SW recipient mice received syngeneic (C3H.SW;  $\square$ , n = 3) or allogeneic (B6-Ly5.2) BM transplant and 4 weekly infusions of ECP-treated B6→C3H.SW splenocytes (gray circle, n = 12) or diluent ( $\blacklozenge$ , n = 8), as described in "Bone marrow transplantation" and "ECP treatment." All BM transplant recipients were injected intraperitoneally with 0.5 mg CD25-depleting mAb (PC61) on day 1 and 0.1 mg on days 8, 15, 22, and 29 after BMT. (A) Number of CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs 56 days after BMT (n = 3-4 per group). Bars represent means ( $\pm$  SD). (B) Survival. (C) Clinical GVHD scores.



and GVHD prevention, respectively. Thus, the immune response invoked by ECP treatment may be governed by the state of the antigen-presenting cells and the maturation signals present in the patient at the time of therapy. Further studies will be required to address this important issue.

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## Authorship

Contribution: E.G. designed, conducted, and analyzed all experiments; C.E.R. designed and performed in vivo Treg flow cytometry experiments; S.G.C. and K.P.L. provided technical support for bone marrow transplantations and infusion of apoptotic cells; I.T. purified and provided PC-61 mAb; C.L. performed histopathological analysis of GVHD; P.R. contributed intellectually throughout the project; J.L.M.F. helped with experimental design; E.G. and J.L.M.F. conceived the project and wrote the paper.

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Correspondence: Erin Gatza, 1500 E Medical Center Dr, 6410 CGC, Box 0942, Ann Arbor, MI 48109-0942; e-mail: egatza@umich.edu.