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# Polyphenolic Extract (PE) from Olive Oil Exerts a Potent Immunomodulatory Effect and Prevents Graft-versus-Host Disease in a Mouse Model

Isabel Alvarez-Laderas[1,†], Teresa L. Ramos[1,†], Mayte Medrano[1], Rocío Caracuel-García[1], María Victoria Barbado[1], Marina Sánchez-Hidalgo[2], Rosario Zamora[3], Catalina Alarcón-de-la-Lastra[2], Francisco J. Hidalgo[3], José Ignacio Piruat[1], Teresa Caballero-Velázquez[1,‡], José Antonio Pérez-Simón[1,‡,*]

[1] *Department of Hematology, Instituto de Biomedicina de Sevilla (IBIS/CSIC/CIBERONC), Hospital Universitario Virgen del Rocío, Universidad de Sevilla, Seville, Spain*
[2] *Department of Pharmacology, Faculty of Pharmacy, Universidad de Sevilla, Seville, Spain*
[3] *Instituto de la Grasa, Consejo Superior de Investigaciones Científicas, Campus Universitario-Edificio 46, Seville, Spain*

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A B S T R A C T

Polyphenols are a group of chemical substances found in plants, with immunomodulatory, antiproliferative, and anti-inflammatory properties that might be useful in the prophylaxis and treatment of graft-versus-host disease (GVHD). Polyphenolic extract (PE) obtained from extra virgin olive oil (EVOO) decreased the activation and proliferation of activated T cells. In addition, a decreased production of proinflammatory cytokines was observed upon exposure to PE. Western blot assays showed a marked inhibition of Akt phosphorylation and nuclear translocation of NF-$\kappa$B in activated T cells. In a murine model of acute GVHD, we observed that mice that received a diet supplemented in PE (600 ppm) presented a higher survival rate and lower risk of developing GVHD when compared with the group that received a control diet. Histopathologic examination showed a significantly lower gut involvement in mice receiving PE, with a decrease in proinflammatory cytokines (IL-2, IL-17, and TNF-$\alpha$) in serum and the reestablishment of butyrate concentration in the gut. In conclusion, PE obtained from EVOO exerted a potent immunomodulatory effect, reducing the activation and proliferation of activated T cells and the production of proinflammatory cytokines. In a murine model of acute GVHD, a PE-supplemented diet reduced the incidence and severity of the disease and increased survival after transplantation.
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## INTRODUCTION

Graft-versus-host disease (GVHD) is one of the most common complications after allogeneic hematopoietic stem cell transplantation (allo-HSCT) [1]. It remains a significant cause of treatment failure, resulting in a mortality of >20% and a morbidity of >50% in transplant recipients [2]. Acute GVHD (aGVHD) is mediated by alloreactive immunocompetent donor T cells as well as the delivery of proinflammatory cytokines that induce damage in target organs such as skin, liver, or gastrointestinal tract [3,4].

Polyphenols are a group of chemical substances found in plants that are characterized by the presence of more than 1 phenol unit with 1 or more hydroxyl group [5]. The polyphenols can be obtained from grains, legumes, fruits, vegetables, extra virgin olive oil (EVOO), red wine, and tea [6]. These substances exercise their effect on T cells through different pathways related to cell proliferation, differentiation, and/or activation [7,8]. Specifically, polyphenols can favor regulatory T cell functions, decrease IFN-$\gamma$ production by stimulated T cells [9,10], and prevent autoimmunity and allergy [11,12]. Interestingly, a green tea polyphenol, epigallocatechin-3-gallate (EGCG), has been found to protect from allograft rejection and attenuates GVHD [13].

EVOO is a source of phenolic compounds such as glycoside oleuropein, hydroxytyrosol, and tyrosol. Olive oil polyphenolic extracts (PEs) have antioxidant, immunomodulatory, antiproliferative, and anti-inflammatory effects [14-16].

In the current study, we have evaluated the effect of PEs obtained from olive oil on T cell activation in vitro and the potential utility of a diet enriched in PEs in aGVHD prophylaxis in a murine model. We confirmed that PE reduces T cell activation, proliferation, and proinflammatory cytokine secretion in vitro. Finally, we also confirmed in a murine model of aGVHD

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*Correspondence and reprint requests: Jose A. Perez-Simon, MD, PhD, Department of Hematology, Instituto de Biomedicina de Sevilla (IBiS), Campus Hospital Universitario Virgen del Rocío, Avenida Manuel Siurot s/n, 41013 Seville, Spain.
*E-mail address:* josea.perez.simon.sspa@juntadeandalucia.es (J.A. Pérez-Simón).
† Equal contributors
‡ Share senior authorship

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that a PE-supplemented diet reduced the incidence and severity of the disease and increased survival after transplantation.

## METHODS

### *Human Samples*

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy volunteer donors. All samples were obtained from the regional center of blood donation of the University Hospital Virgen del Rocío (Seville, Spain). The local ethics committee provided institutional review board approval for this study, and informed consent was obtained from all donors, by the Declaration of Helsinki. PBMCs from buffy coats were isolated by density gradient centrifugation using a Ficoll Paque solution (Biosciences, Uppsala, Sweden).

### *Extraction PE and Characterization of Phenolic Compounds*

PE was obtained by the method described by Vázquez Roncero et al. [17] (Supplementary Methods). The methanolic extract was concentrated in vacuum under a stream of nitrogen at <35°C until it reached a syrup consistency. Finally, it was lyophilized and stored at −80°C.

### *Proliferation and Viability Assays*

For the proliferation assays, $2 \times 10^5$ PBMCs stained with the green fluorescent dye PKH-67 (Sigma-Aldrich, Saint Louis, MO, United States) were seeded in 96-well plates (Biofil, China) at a density of $2 \times 10^5$ cells/well in RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 $\mu$g/mL streptomycin (all reagents were purchased from Gibco-Invitrogen, Paisley, UK), and 10% human AB serum (Sigma-Aldrich) under different culture conditions: in the absence of stimulus or stimulated with plate-bound anti-CD3 (5 $\mu$g/mL) and soluble anti-CD28 (2.5 $\mu$g/mL) monoclonal antibodies (mAbs) (BD Biosciences, San Jose, CA). Different concentrations (0, 10, 45, and 60 $\mu$g/mL) of PE were added to each culture condition. After 18 hours, the cells were collected; stained with CD25-FITC, CD4-APC-H7, CD8-PE-Cy7, CD-64-PE, 7-amino-actinomycin D (7AAD), and anti-CD3-APC mAbs; and acquired in a FACSCanto II flow cytometer (BD Biosciences) using a FACS Diva software program (BD Biosciences). The ModFit program was used to calculate the percentage of resting ($PKH^{high}CD25^{-}$) and proliferating cells.

For cell viability, cell cultures were managed as previously described. After 18 hours, unstimulated or stimulated cells were collected and stained with anti-CD25-FITC, anti-CD3-APC mAbs, 7AAD, and Annexin V-PE using the PE Annexin V Apoptosis Detection Kit (BD Pharmingen, San Jose, CA) according to manufacturer`s instructions. For every condition, 50,000 events were acquired on a FACSCanto flow cytometer (BD Biosciences) using the CellQuest software program, and the percentage of Annexin V-PE cells was calculated using the Infinicyt software (Cytognos, Salamanca, Spain).

### *Cytokine Assays*

After 48 hours, with the same culture conditions previously described, supernatants were collected to calculate the concentration of different cytokines (IL-2, IL-4, IL-6, IL-10, TNF-$\alpha$, and IFN-$\gamma$) by flow cytometry. For that purpose, we used the BD Human Th1/Th2 Cytokine Cytometric Bead Array kit (BD Biosciences) according to manufacturer's instruction, and the samples were analyzed using the BD Cytometric Bead Array software (BD Biosciences).

### *Western Blot Assays*

The PBMCs were cultured as previously described with different concentrations (0, 10, 45, 60 $\mu$g/mL) of PE. After 18 hours of culture, cells were lysed for protein preparation and were separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels and electroblotted onto PVDF membranes. Blots were immunoblotted with antibodies against phosphorylated Akt (-T308) (Cell Signaling Technology, Danvers, MA), NF-$\kappa$B p65 (Cell Signaling Technology), YY1 (Abcam, Cambridge, UK), and $\beta$-actin (Abcam). The membranes were then washed with Tween-Tris-buffered saline and incubated with either an horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG antibody (Jackson ImmunoResearch, Cambridge House, UK) or HRP-conjugated donkey anti-mouse IgG antibody (Jackson ImmunoResearch). Antibody detection was performed with an enhanced chemiluminescence reaction (Santa Cruz Biotechnology, Dallas, Texas USA).

For separation and extraction of cytoplasmic and nuclear soluble proteins, we used the Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Scientific, Massachusetts, US).

### *Graft-versus-Leukemia Assays*

T cells were purified from the spleen of BALB/C and C57BL/6 mice by immunomagnetic depletion of non-T cells using the Pan T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purity of isolated populations was routinely >95%.

Cocultures between isolated T cells with target cells, murine leukemic cells A20 (ATCC TIB-208), and C1498 (ATCC TIB-49) were performed at 3:1 and 5:1 ratio in a 96-well plate. The coculture was incubated at 37°C without shaking. The cell cultures were analyzed after 24 hours by flow cytometry to study the percentage of apoptosis using the following panel: Annexin V/7AAD, CD3-APC, CD4-V400, CD8-V421, and CD45-APC-H7 (BD Biosciences).

To study the effect of PE in lymphocyte cytotoxic activity, splenocytes from BALB/C and C57BL/6 mice were cultured ($1 \times 10^6$) with or without activation (CD3/CD28) in a 48-well plate with different concentrations of PE (0, 10, 45, and 60 $\mu$g). After 48 hours, the cells were recovered, washed, counted, and cocultured with C1498 and A20 (murine leukemic cells) (1:5) in a 96-well plate for 24 hours. Afterward, the cells were recovered and analyzed by flow cytometry for the percentage of viable cells (Annexin/7ADD BD kit).

### *Acute GVHD Murine Model*

BALB/c (H2d) and C57BL/6 (H2b) mice were purchased from Charles River Laboratory (St. Germain sur l'Arbresle, France). Animals between 8 and 12 weeks of age were housed in microisolator cages and maintained under specific pathogen-free conditions in the animal facility of the Institute of Biomedicine of Seville, Spain. All procedures were used following the Spanish and European Union guidelines and after approval of the local bioethics committee.

BALB/c mice received total body irradiation (850 cGy divided into 2 fractions, from a $Cs^{137}$ source) that was followed by an intravenous injection of $5 \times 10^6$ C57BL/6 bone marrow (BM) cells with $5 \times 10^6$ splenocytes. Mice were monitored and weighed twice a week.

Mice were randomized into 5 experimental groups during all experimental periods. The mice that underwent transplantation with BM and splenocytes (aGVHD) were divided into 2 groups: (A) received a standard diet (2014S Harlan Laboratories, Indianapolis, IN)—GVHD control diet and (B) received a standard diet (2014S Harlan Laboratories) supplemented with 600 ppm PE obtained from the EVOO GVHD PE diet. Control mice were also included in the study: the group that underwent transplantation with BM from C57BL/6 and received control diet and the group that underwent transplantation with BM from C57BL/6 and received the PE diet. The total body irradiation group was also included, with mice receiving irradiation without stem cell support.

The diet started from day −5 to day +100 post-transplantation. The severity of GVHD was assessed by the percentage of weight change, a parameter that has been found to be a reliable indicator of systemic GVHD in this and several other murine models.

A threshold of 10% weight loss was used to signify the presence of moderate GVHD. Also, the degree of systemic GVHD was assessed by a scoring system described by Cooke et al. [18] that incorporates 5 clinical parameters: weight loss, posture (hunching), activity, fur texture, and skin integrity.

After day +5 and +21 post-transplantation, aGVHD was assessed by histopathologic analysis of the small and large bowels and skin. Tissues were fixed in 4% neutral-buffered formalin, embedded in paraffin, cut into 5-$\mu$m-thick sections, and stained with hematoxylin and eosin (Merck KGaA, Darmstadt, Germany). For immunofluorescence experiments, the tissues were labeled with anti-CD3 (RD Systems), anti-IFN-$\gamma$ (ab9657; Abcam), and anti-Ki67 (ab15580; Abcam). Slides were mounted using Vectashield H-1000 medium (Vector Labs, Peterborough, United Kingdom). Images were visualized with an Olympus BX-61 light microscope (Olympus Optical, Tokyo, Japan). Captured images were handled using Adobe Photoshop CS6 (Adobe Systems, San Jose, CA).

Histopathologic analysis of small and large bowels and skin was also conducted to further evaluate aGVHD. The slides were examined by a pathologist in a blinded fashion using a severity scale [19,20]. The severity scale ranged from 0 to 4: 0, normal; 0.5, focal and rare; 1, focal and mild; 2, diffuse and mild; 3, diffuse and moderate; and 4, diffuse and severe. Changes in the colon (crypt regeneration, crypt epithelial cell apoptosis, crypt loss, surface colonocyte attenuation, lamina propria inflammatory cell infiltrate, mucosal ulceration, and thickness of the mucosa) and skin (epidermal/dermal lymphocytic infiltrate, dyskeratotic epidermal keratinocytes and epidermal thickening) were analysed and scored.

Intestinal GVHD was evaluated by crypt apoptosis bodies per 10 crypts.

Cytokine levels were analyzed from serum samples obtained from heart puncture after 5 days post-transplantation. To measure the cytokines granulocyte-macrophage colony stimulating factor, IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-17A, IL-23, TNF-$\alpha$, and IFN-$\gamma$, we used the mouse MACSPlex Cytokine 10 kit (Miltenyi Biotec) according to the manufacturer's instructions and then acquired them in a FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ). The analysis was performed with FCAP Array Analysis Software (BD Biosciences).

The quantity of butyrate in feces was determined in the mice on the day before they started the PE diet and 14 days after transplantation. Approximately 400 mg of thawed feces was homogenized with 1.8 mL milli-Q water. Then, 0.4 mL $H_2SO_4$ (50%) was added, and the mixture was vortexed for 2 minutes.

Then, 0.8 g NaCl, 1 mL diethyl ether, and 20 $\mu$L internal butyrate standard (100-mM solution of butyric acid-$d_8$ in methanol) were added, and the mixture was vortexed again. The phases were separated by orbital shaking for 1 hour and centrifugation at 3000 rpm for 5 minutes at room temperature. The superior phase was recovered and injected in a Shimadzu GCMS-QP2010 Ultra Standard Gas Chromatograph-Mass Spectrometer (Shimadzu, Kyoto, Japan).

The concentrations, expressed in nmol of butyrate per gram of faces, were calculated using the linear regression equations obtained for the corresponding standard curves, which were obtained using 10 different concentrations of butyrate.

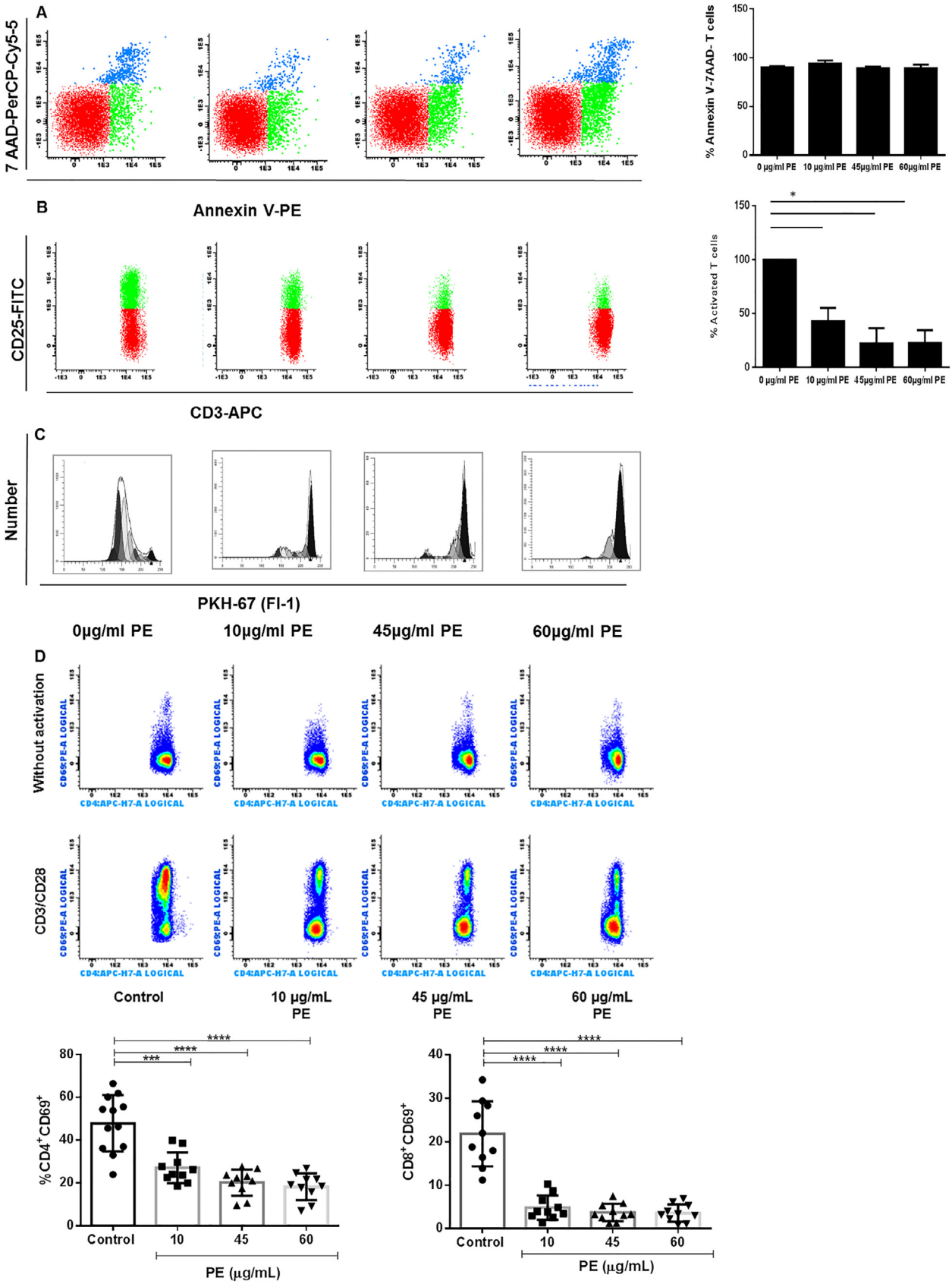


**Figure 1.** Effect of olive oil polyphenols on T cell viability, activation, and proliferation. Peripheral blood mononuclear cells were stimulated in the presence of different concentrations of PE. (A) Dot plot flow cytometry analysis of Annexin V/7AAD and percentage of viable cells. (B) Dot plot image and percentage of T cell activation ($CD3^+/CD25^+$) stimulated with anti-CD3 plus anti-CD28 and cultured in the presence of PE (10, 45, and 60 $\mu$g/mL). (C) T cell proliferation assessed by PKH-67 dilution of unstimulated or stimulated T cells cultured with PE (10, 45, and 60 $\mu$g/mL). (D) Percentage of CD64 cells among $CD4^+$ and $CD8^+$ T cells unstimulated or stimulated in the presence of different concentrations of PE. Data are represented by the mean ± SME of 4 to 6 independent experiments. $^*P < .05$. $^{***}P < .001$. $^{****}P < .0001$.

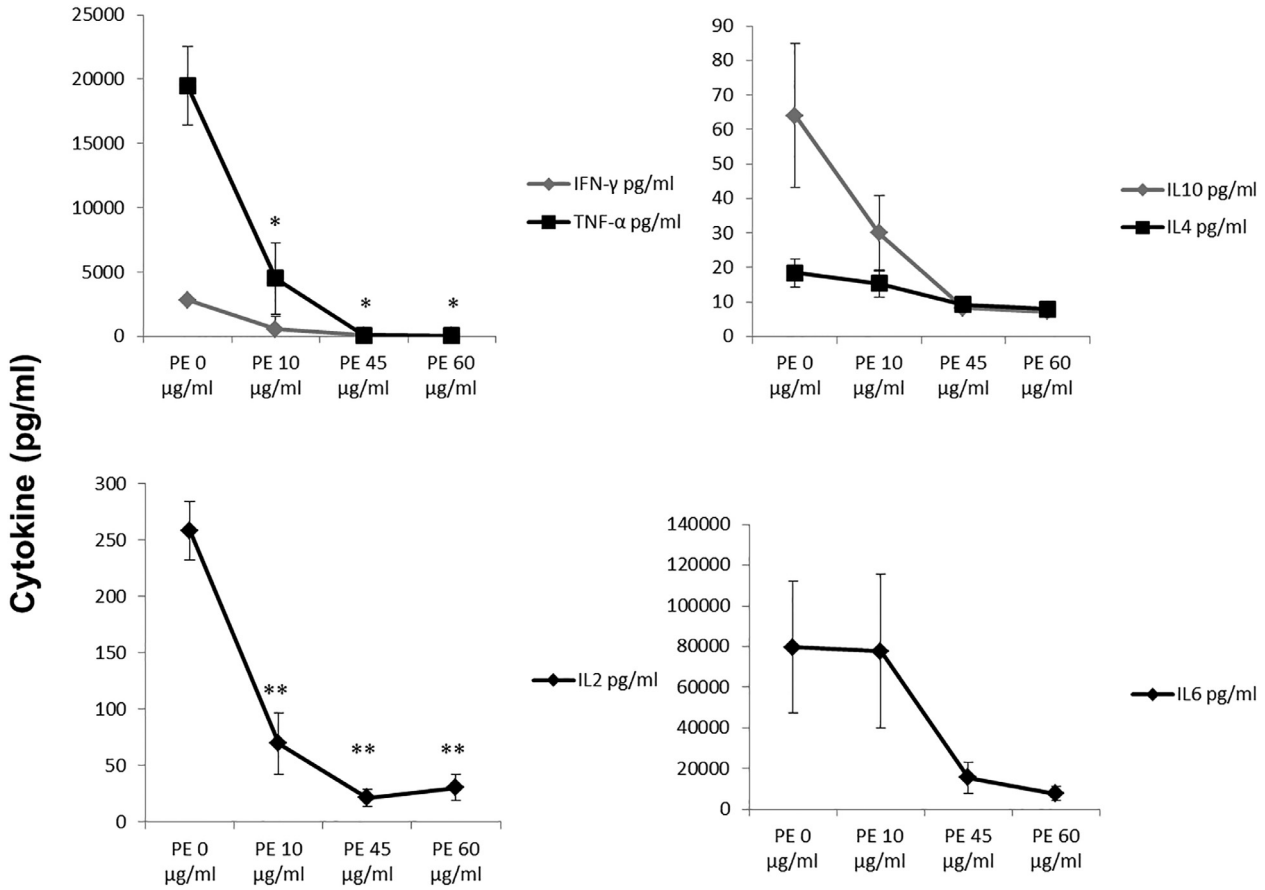


**Figure 2.** Effect of PE on T cell cytokine secretion. The concentration of IL-2, IFN-$\gamma$, TNF-$\alpha$, IL-4, IL-6, and IL-10 in the culture supernatant of PBMCs stimulated in the presence of different concentrations PE (10, 45, and 60 $\mu$g/mL) is shown. Data are represented by the mean ± SEM of 3 independent experiments. $^{*}P < .05$, $^{**}P < .01$ (with respect to the stimulated untreated sample).

***Statistical Analysis***

Most statistical analyses were performed using IBM SPSS Statistics 19 software (SPSS, Inc., Chicago, IL). Statistical significance was assessed by the Student *t* test or 1-way analysis of variance (ANOVA) test. Pairwise comparisons were performed using the Mann-Whitney test with Bonferroni correction.

A 2-way measurement of repeated multiple ANOVA (2-way ANOVA) was performed to analyze the signs of GVHD at different times, and survival curves were plotted using Kaplan-Meier estimates, and a log-rank test was used to compare survival rates. Statistical significance in all tests was defined as $P < .05$. All graphics were done using the PRISM software package (GraphPad Software, La Jolla, CA).

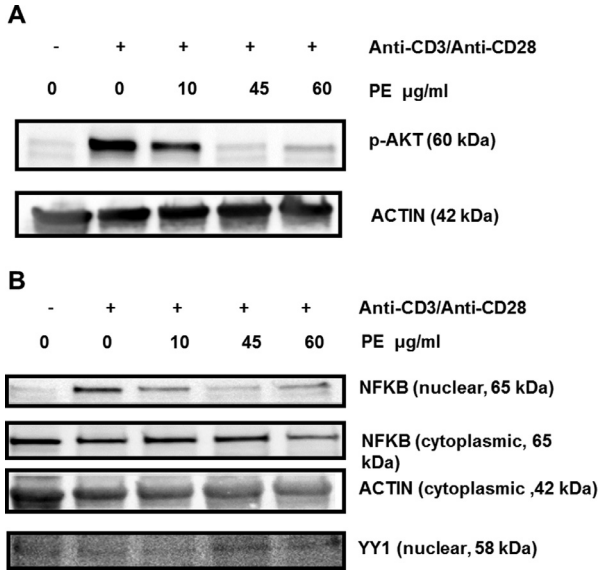


**Figure 3.** Western blot analysis of p-Akt protein phosphorylation (A) and NF-$\kappa$B expression (C) in stimulated peripheral blood mononuclear cells for 18 hours with anti-CD3 and anti-CD28 mABs, in the presence of different concentrations of PE (10, 45, and 60 $\mu$g/mL). Representative image of 3 independent experiments.

## RESULTS

### *Chemical Composition of PE from EVOO*

Phenolic content of PE determined by $^{1}$H NMR (proton nuclear magnetic resonance) spectroscopy was performed. The 2 most common phenolic compounds were hydroxytyrosol (36.49 $\mu$mol/100 g) and tyrosol (30.32 $\mu$mol/100 g).

### *Effect of Olive Oil PE on T Cell Activation, Proliferation, and Apoptosis Induction*

First, the effect of PE obtained from EVOO on the viability and activation in stimulated T cells was assessed. We observed no effect on T cell viability (Figure 1A) when we added PE to the cultures. By contrast, PE decreased the activation of stimulated T-lymphocytes, as shown in Figure 1B, and reduced cell proliferation (Figure 1C). The expression of CD64, a classical early marker of lymphocyte activation, was also evaluated in the different $CD4^{+}$ and $CD8^{+}$ T cells unstimulated or stimulated with CD3/CD28. The effect of the PE on these subpopulations was similar to what observed with CD25 (Figure 1D).

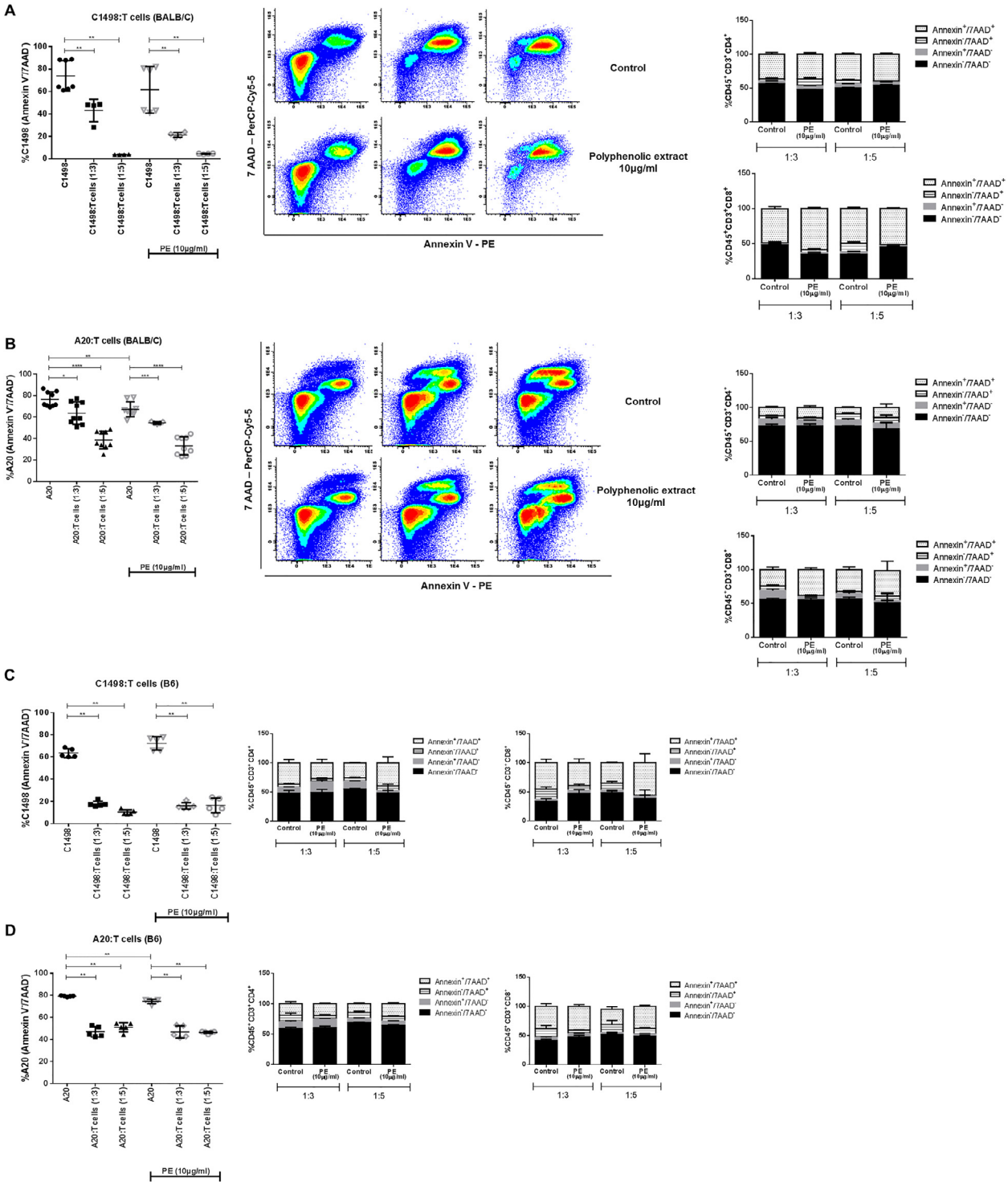


**Figure 4.** Cytotoxic effect of T cells against murine leukemic cells treated with PE. (A, B) Flow cytometry of isolated T cells from the spleen of BALB/C mice cocultured with A20 (n = 4) and C1498 (n = 4) at a ratio of 1:3 and 1:5, with or without PE (10 $\mu$g/mL). Right panels show the percentage of Annexin V and 7AAD+ cells among $CD4^+$ and $CD8^+$ T cells. (C, D) Flow cytometry of isolated T cells from the spleen of C57BL/6 (B6) mice cocultured with A20 (n = 4) and C1498 (n = 4) at a ratio of 1:3 and 1:5, with or without PE (10 $\mu$g/mL), and percentage of Annexin V and $7AAD^+$ cells among $CD4^+$ and $CD8^+$ T cells. Data are represented by the mean ± SEM. $^*P < .05$, $^{**}P < .01$ (with respect to A20 and C1498).

### *Effect of PE on T Cell Cytokine Secretion*

PE induced a dose-dependent decrease in Th1 (IFN-$\gamma$, IL-2, and TNF-$\alpha$)/Th2 (IL-4, IL-6, and IL-10) cytokine secretion in vitro. At a concentration of 10 $\mu$g/mL, PE induced a significant decrease in TNF-$\alpha$ and IL-2 secretion (Figure 2).

### *PE Reduces the Expression of Proteins Related to Proliferation and Survival*

Western blot assays were performed to identify pathways involved in T cell survival and proliferation affected by exposure to PE. PE reduced AKT phosphorylation and nuclear

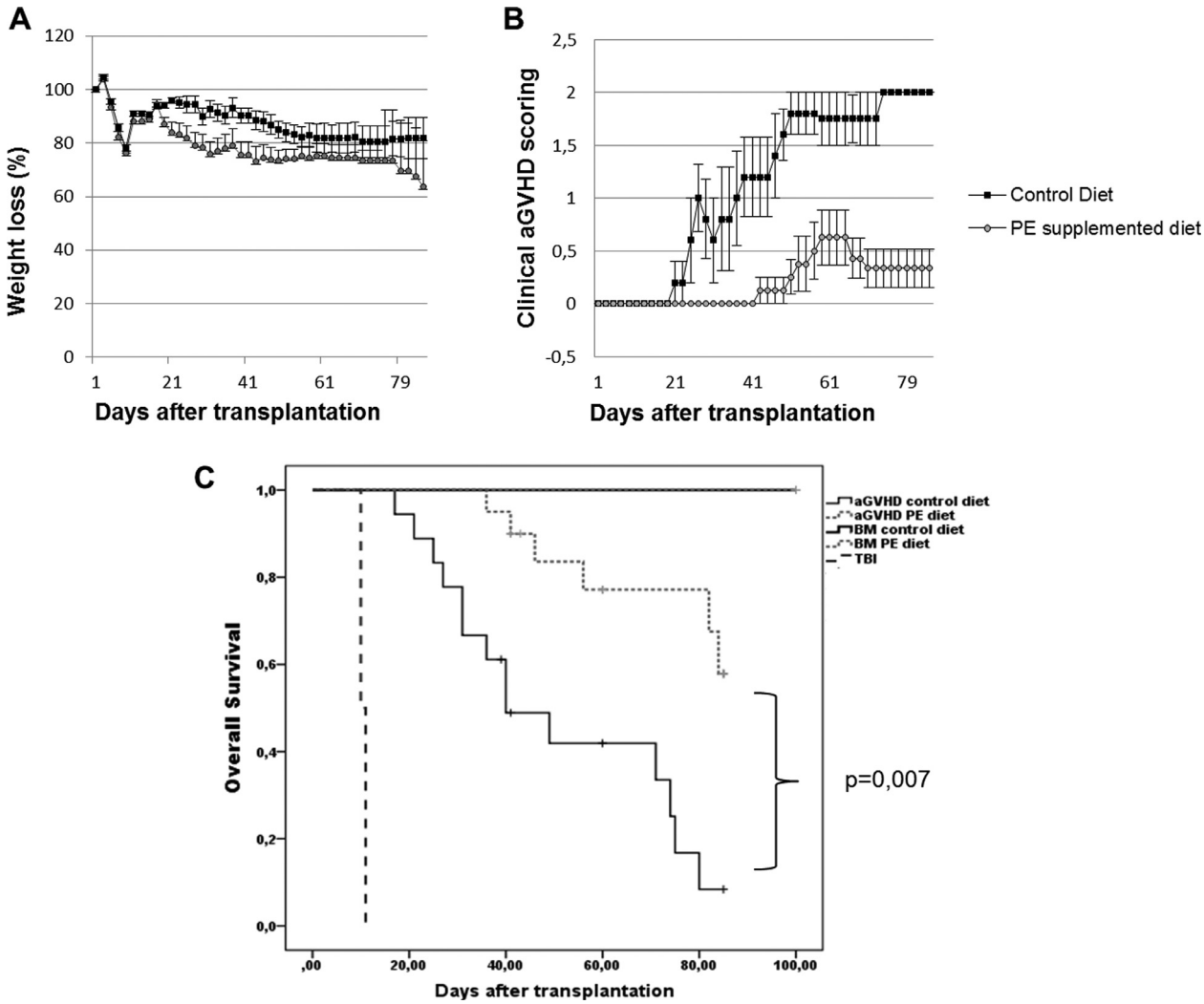


**Figure 5.** Acute GVHD murine models. (A) Percentage of body weight loss ($P \leq .05$ between days +21 and +45 after transplantation) of mice transplanted with $5 \times 10^6$ bone marrow cells and $5 \times 10^6$ splenocytes receiving a standard diet or a standard diet supplemented with 600 ppm PE obtained from EVOO. (B) Median aGVHD score of transplanted mice that received a control diet or a PE diet ($P \leq .05$ for day 37 post-transplantation). (C) Kaplan-Meier curve representing overall survival of different experimental groups: total body irradiation (TBI; n = 2), BM-transplanted group that received the standard diet (n = 2), BM-transplanted group that received the standard diet supplemented with 600 ppm PE obtained from EVOO (n = 2), GVHD group that received the standard diet (n = 18), and GVHD group that received the standard diet supplemented with 600 ppm PE obtained from EVOO (n = 20). Combined data from 2 to 3 (n = 4 to 5 mice for each experiment group) experiments are shown.

translocation of NF-$\kappa$B of T cells at all doses, including a diminution with a concentration superior to 45 $\mu$g/mL (Figure 3A,B).

### *Effect of T Cells against Murine Leukemic Cells*

Once we confirmed that PE was able to decrease T cell activation and proinflammatory cytokine secretion, we next studied the effect of T cells exposed to PE on tumor viability by flow cytometry using 7AAD and Annexin V staining. In the presence of T cells from BALB/C and C57BL/6 mice, the viability of A20 and C1498 leukemic cells significantly decreased (Figure 4). Remarkably, this effect did not change in the presence of PE (10 $\mu$g/mL).

Next, we evaluated ex vivo lymphocyte cytotoxic activity in the presence of PE. After 48 hours in the presence of different concentrations of PE, the splenocytes from BALB/C and C57BL/6 mice (with and without the presence of stimulation) were cocultured with 2 different murine leukemic cells for 24 hours. As shown in the Supplementary Figure S1, the splenocytes were able to maintain a significant cytotoxic activity against leukemic cells. Accordingly, a decrease of C1498 and A20 cell viability was observed after coculture with T cells that were previously treated with different concentrations of PE.

### *Treatment of Mice with PE Improves Survival and Clinical Stage of the aGVHD Murine Model*

To examine the efficacy of a diet rich in PE from EVOO to improve survival and decrease the risk of aGVHD in vivo, we used a GVHD murine model.

As shown in Figure 5, mice receiving a supplemented diet had a significantly lower score ($P \leq .05$ after day 37 post-transplantation) and a significantly lower weight loss between days +21 and +45 after transplantation ($P \leq .05$) (Figure 5A,B), as well as a significantly increased survival as compared with the mice that received a standard diet (Figure 5C).

We next quantified 10 different cytokines (granulocyte-macrophage colony stimulating factor, IFN-$\gamma$, IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-17A, IL-23, and TNF-$\alpha$) from the plasma of mice that received a standard or supplemented diet on day 5

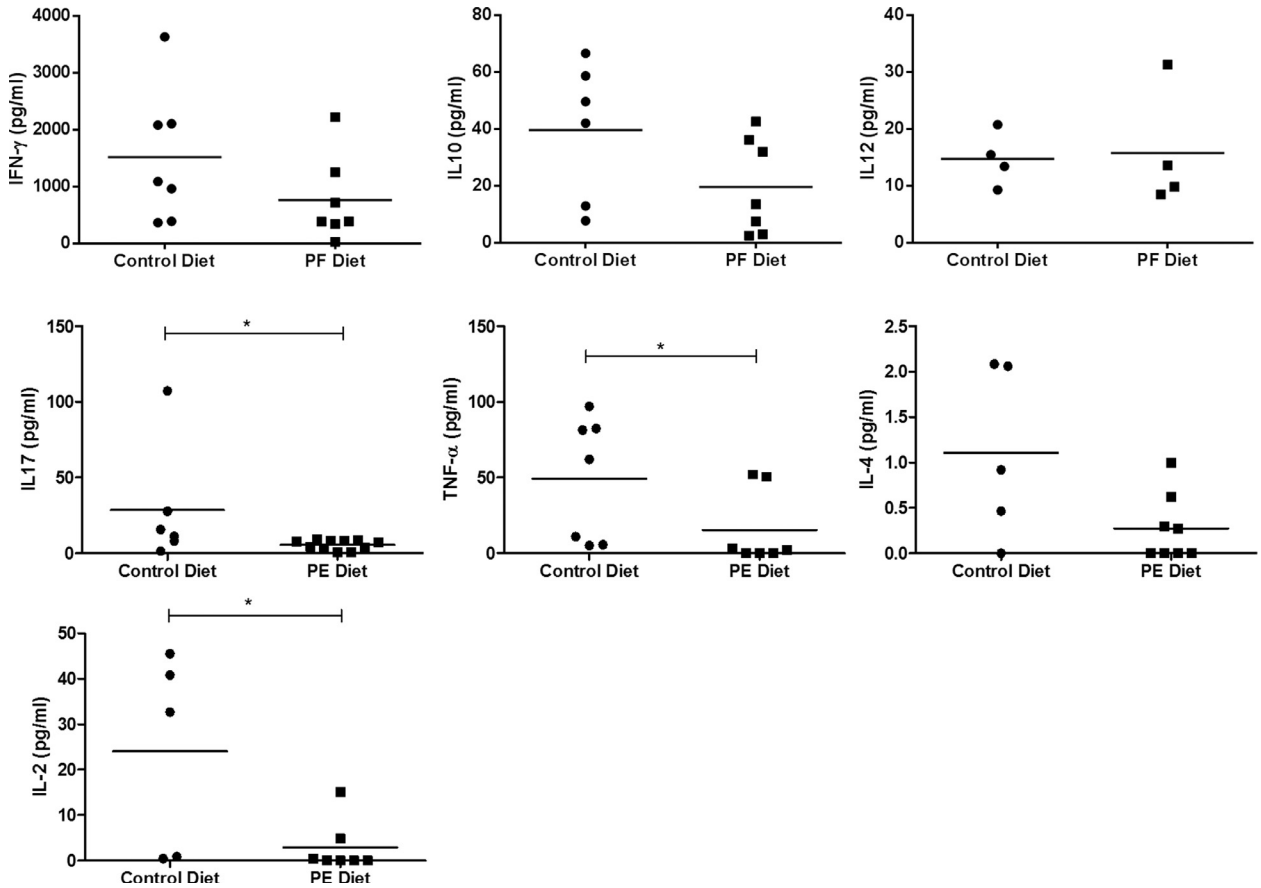


**Figure 6.** Determination of cytokine concentrations (IFN-$\gamma$, IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-17, and TNF-$\alpha$) in the plasma of mice that underwent transplantation with $5 \times 10^6$ bone marrow cells and $5 \times 10^6$ splenocytes receiving a standard diet or a standard diet supplemented with 600 ppm PE obtained from EVOO 5 days after transplantation. Data are represented as the median of 4 to 7 serum mice/group from 2 independent experiments. *$P < .05$ (Mann-Whitney test).

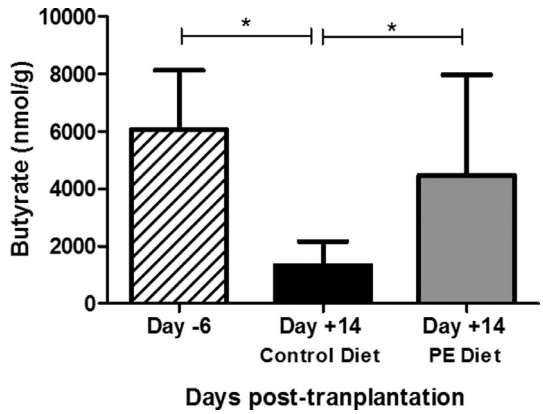


**Figure 7.** Butyrate feces analysis. The concentration of butyrate in the feces of animals before transplant (day −6; n = 6) and 14 days post-transplantation among mice receiving a standard diet (n = 5) or a standard diet supplemented with 600 ppm PE obtained from EVOO (n = 9). Data are represented by the mean ± SEM. *$P < .05$.

post-transplantation. We observed a significantly decreased concentration of proinflammatory cytokines, including IL-2 ($P = .0253$), IL-17 ($P = .0365$), and TNF-$\alpha$ ($P = .0209$), among the mice receiving the supplemented diet (Figure 6).

We determined the concentration of butyrate in the feces of the different mice groups on day −6 before transplant and 14 days post-transplantation. We observed that the group that received the PE-enriched diet recovered the concentration of butyrate in the feces (Figure 7).

To evaluate aGVHD target organs, histologic analysis of skin and intestine was performed on days 5 and 21 after transplantation.

The skin of both subgroups displayed pathologic findings of aGVHD, including hyperkeratosis, focal hypergranulosis, focal vacuolization, and presence of apoptotic bodies in the basal layer of the epidermis as well as a focal inflammatory infiltrate in the dermis (Figure 8). Despite no significant differences between subgroups in the skin, the control diet group displayed a significantly worse GVHD score between days 5 and 21 post-transplantation.

By contrast, when we analyzed the intestine, we observed significant differences between the 2 groups regarding the number of apoptotic bodies per 10 crypts on days 5 and 21 after transplantation (Figure 9). The intestine of the control group showed a median of 10.25 apoptotic bodies for every 10 crypts as compared with 4.25 apoptotic bodies for every 10 crypts in animals that received the supplemented diet 5 days post-transplantation (Figure 9A). After 21 days, significant differences in the number of apoptotic bodies again were observed upon comparing the group receiving the PE-supplemented diet versus the control group diet (Figure 9B). Moreover, the previous group had a moderate and the latter a mild focal inflammatory infiltrate. When we compared the histologic score in the intestine, significant differences between the control diet and PE diet on day 5 after transplantation were observed (Figure 9C).

In addition, we analyzed the expression of CD3, Ki67 (proliferation marker), and IFN-$\gamma$ in mice colon and small

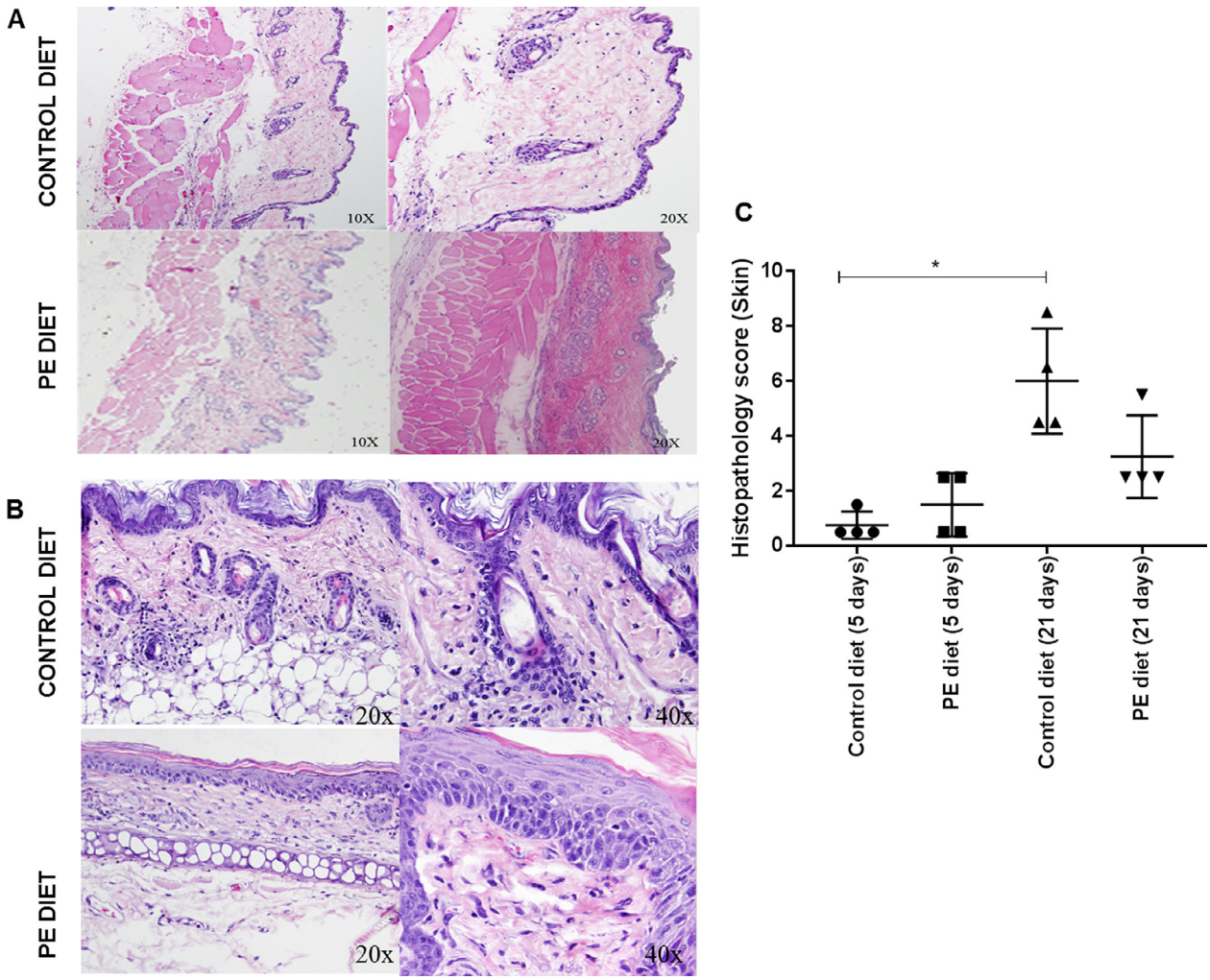


**Figure 8.** Histopathologic skin analysis of aGVHD mouse model. Photomicrographs in hematoxylin and eosin–stained sections of skin samples collected on day 5 (A) and 21 (B) after transplantation. Magnification is indicated in each photomicrograph. (C) Data of histologic GVHD score (single blind evaluation, n = 4/ group, data pooled from 1 experiment). Data are represented by the mean ± SEM. $^{*}P < .05$.

intestine 5 days post-transplantation. As can be observed in the Supplementary Figure S2 (small intestine) and Supplementary Figure S3 (large intestine), proliferating CD3 T cells ($CD3^{+}Ki67^{+}$) were significantly increased in the control group. The presence of IFN-$\gamma^{+}$ T cells was also increased in the samples from the small and large intestine from the control group. This is in contrast to the data observed in bone marrow, lymph nodes, and spleen where no significant differences were observed in terms of T cell activation or proliferation between both groups (data not shown).

## DISCUSSION

After allogeneic transplantation [1], GVHD is the most devastating complication hampering the success of the procedure. It is mainly due to the activation of alloreactive T cells, which induce cytotoxic damage in normal host tissues [3]. Prevention strategies for the reduction of GVHD are based on the combination of calcineurin [21] inhibitors plus methotrexate or mycophenolate mofetil, among other options; however, with these standard approaches, the risk of acute and chronic GVHD ranges between 25% and 70% among patients receiving transplantation from a sibling donor.

In the current study, we have evaluated the immunomodulatory effect of the EVOO PE in the prophylaxis of aGVHD. We have demonstrated that PE present in EVOO reduces T cell activation and proliferation in vitro. These results are in accordance with previous studies showing that PE decreased IFN-$\gamma$ production by stimulated T cells. More specifically, Zou et al. [22] described that resveratrol inhibits T cell activation through the Sirt1 protein. The inhibitory effect of apigenin on T cell activation has also been described in response to concanavalin A [23].

In addition, we have found that PE decreases pAKT in stimulated T-lymphocytes. This is in agreement with previous studies showing that EGCG, a compound found in green tea, inhibits the PI3K/AKT pathway by decreasing the levels of the p85 and p110 subunits of PI3K and the levels of P-AKT [24]. Furthermore, curcumin treatment inhibits AKT phosphorylation in different cancer cell types [25].

In the current study, cytokine secretion assays confirmed that PE reduced Th1 and Th2 cytokine secretion by stimulated T cells. Activation and proliferation of T-lymphocytes result in an increased transcription of cytokine genes and their receptors, thus producing a massive release of proinflammatory mediators (TNF-$\alpha$, IL-1, IFN-$\gamma$) that cause tissue damage. Accordingly, the number of T-lymphocytes producing IL-2 has been related to the severity of GVHD. In a murine model of aGVHD, we have verified that those animals receiving a

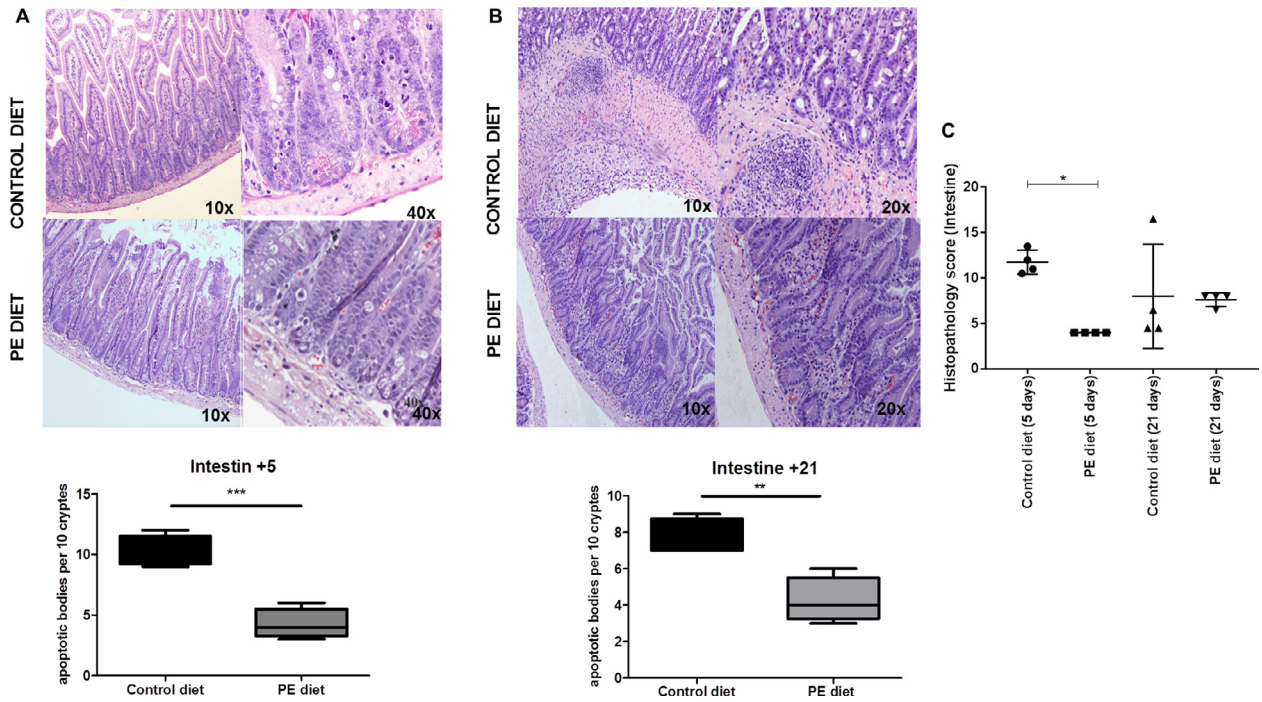


**Figure 9.** Histopathologic gut analysis of aGVHD mouse model. Photomicrographs in hematoxylin and eosin–stained sections of intestine samples collected on day 5 (A) and 21 (B) after transplantation. Magnification is indicated in each photomicrograph. The quantification of apoptotic bodies per 10 crypts in an aGVHD mouse model. Intestine samples, from mice receiving standard diet and standard diet supplemented with PE, collected on days 5 and 21 after transplantation. (C) Data of histologic GVHD score (single blind evaluation, n = 4/ group, data pooled from 1 experiment). Data are represented by the mean ± SEM. $^{*}P < .05$. $^{**}P < .01$. $^{***}P < .001$.

supplemented diet with PE had lower levels of proinflammatory cytokines, with significant differences for IL-2, IL-17, and TNF-$\gamma$ at 5 days post-transplant, compared with animals receiving a control diet. In this regard, Aparicio-Soto et al. [26] demonstrated that an EVOO-rich diet significantly reduced the production of TNF-$\alpha$, IL-6, IL-10, and IL-17 in lipopolysaccharide-stimulated splenocytes from lupus-induced mice as compared with those animals that were fed with a standard diet. Interestingly, it has been described that oleuropein glucoside decreases the production of IL-1$\beta$ [27]. Moreover, a clinical trial in patients with coronary heart disease showed that the consumption of EVOO enriched in PE was associated with a decreased production of IL-6 and lower levels of C-reactive protein [28]. All these studies support the idea that PEs modulate the immune response and have potent anti-inflammatory activity.

Finally, we have demonstrated that the use of a diet enriched in PE from EVOO decreases the occurrence of aGVHD in a murine model. The use of a diet supplemented with PE significantly improved mice survival and ameliorated aGVHD clinical signs. It has been described that some chemical derivatives of plants can reduce the severity of GVHD and graft rejections [29]. This was the case for PG27 extracted from *Tripterygium wilfordii*, which reduced GVHD in a murine model of allogeneic bone marrow transplantation [30]. Kanamune et al. [31] showed that EGCG inhibited the activation of T-lymphocytes in vitro, and in a murine model, splenocytes treated with this polyphenol were less potent in inducing GVHD. Subsequently, Choi et al. [32] evaluated the use of EGCG by intraperitoneal administration in different GVHD animal models without obtaining improvement in survival or weight reduction. However, a recent study showed that EGCG ameliorated aGVHD and reduced GVHD-related target organ damage by increasing regulatory T cell number and reducing oxidative stress [33]. In the current study, histopathologic analysis showed that animals that received the supplemented diet had fewer apoptotic bodies in the crypts as compared with those that did not receive PE. However, no significant differences were observed in skin, which suggests a local effect in gastrointestinal mucosa. Moreover, we did not observe significant differences in T cell activation or proliferation or in Treg numbers in the bone marrow, blood, and spleen of mice receiving or not the EVOO-supplemented diet. Accordingly, our data suggest that this diet has a local effect in the gut without modifying the immune response systemically. Considering the main role of the gut in the pathophysiology of GVHD, mainly through cytokine secretion, this local effect could finally lead to a lower systemic GVHD score while maintaining a graft-versus-leukemia effect. Although further in vivo studies will be required to confirm this aspect, in vitro assays do confirm that EVOO does not hamper the cytotoxic effect of T cells on leukemic cells.

Different studies have shown that patients with GVHD present an altered intestinal microbiota that correlates with GVHD severity [34]. These changes in the microbiota result in alterations in the levels of microbial metabolites such as short-chain fatty acids. A recent report showed that butyrate, a short-chain fatty acid derived from microbiota, which is a preferred energy source for intestinal epithelial cells (IECs), significantly decreases after allo-HSCT [35]. Mathewson et al. [35] showed that a decreased amount of butyrate in CD326$^{+}$IECs results in decreased histone acetylation, which is restored upon administration of exogenous butyrate. Butyrate restoration also improved IEC junctional integrity, decreased apoptosis, and mitigated GVHD. In our study, we demonstrated a significant decrease in the concentration of butyrate in feces of mice that developed aGVHD and a restoration of butyrate levels in mice receiving a diet supplemented in PE from EVOO. Interestingly, different studies have shown that the

supplementation with PE might select butyrate-producing bacteria (such as Clostridiaceae) and improve gut bacterial abundance [36]. Our data do not directly address the changes in the amount of butyrate-producing bacteria and the effects of GVHD protection. However, a growing body of evidence indicates that we can change the GVHD pathophysiology in not only suppressing the donor immune system but also manipulating the host response to injury. Considering the critical role of the gastrointestinal tract on the pathophysiology of GVHD, these data represent a significant finding in the current study.

In summary, PEs obtained from EVOO are immunomodulatory agents, being able to decrease T cell activation and proliferation in cell culture and inhibiting the production of proinflammatory cytokines. In addition, its use as a dietary supplement in a murine model of aGVHD increases the survival of mice and decreases the severity of the disease, mainly in the digestive tract.

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## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.bbmt.2019.11.019.

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