



# Biology of Blood and Marrow Transplantation

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## Biology

### The Ex Vivo Treatment of Donor T Cells with Cosalane, an HIV Therapeutic and Small-Molecule Antagonist of CC-Chemokine Receptor 7, Separates Acute Graft-versus-Host Disease from Graft-versus-Leukemia Responses in Murine Hematopoietic Stem Cell Transplantation Models



Kenneth A. Fowler<sup>1</sup>, Kelin Li<sup>2</sup>, Christopher B. Whitehurst<sup>1,3</sup>, Danny W. Bruce<sup>1</sup>, Nathaniel J. Moorman<sup>1,3</sup>, Jeffrey Aubé<sup>2</sup>, James M. Coghill<sup>1,4,\*</sup>

<sup>1</sup> Lineberger Comprehensive Cancer Center, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

<sup>2</sup> Eshelman School of Pharmacy, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

<sup>3</sup> Department of Microbiology and Immunology, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

<sup>4</sup> Department of Internal Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

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#### A B S T R A C T

Despite recent advances in therapy, allogeneic hematopoietic stem cell transplantation (HSCT) remains the only curative option for a range of high-risk hematologic malignancies. However, acute graft-versus-host disease (aGVHD) continues to limit the long-term success of HSCT, and new therapies are still needed. We previously demonstrated that aGVHD depends on the ability of donor conventional T cells ( $T_{\text{cons}}$ ) to express the lymph node trafficking receptor, CC-Chemokine Receptor 7 (CCR7). Consequently, we examined the ability of cosalane, a recently identified CCR7 small-molecule antagonist, to attenuate aGVHD in mouse HSCT model systems. Here we show that the systemic administration of cosalane to transplant recipients after allogeneic HSCT did not prevent aGVHD. However, we were able to significantly reduce aGVHD by briefly incubating donor  $T_{\text{cons}}$  with cosalane ex vivo before transplantation. Cosalane did not result in  $T_{\text{con}}$  toxicity and did not affect their activation or expansion. Instead, cosalane prevented donor  $T_{\text{con}}$  trafficking into host secondary lymphoid tissues very early after transplantation and limited their subsequent accumulation within the liver and colon. Cosalane did not appear to impair the intrinsic ability of donor  $T_{\text{cons}}$  to produce inflammatory cytokines. Furthermore, cosalane-treated  $T_{\text{cons}}$  retained their graft-versus-leukemia (GVL) potential and rejected a murine P815 inoculum after transplantation. Collectively, our data indicate that a brief application of cosalane to donor  $T_{\text{cons}}$  before HSCT significantly reduces aGVHD in relevant preclinical models while generally sparing beneficial GVL effects, and that cosalane might represent a viable new approach for aGVHD prophylaxis.

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## INTRODUCTION

Despite recent advances in therapy, allogeneic hematopoietic stem cell transplantation (HSCT) remains the sole curative option for a range of high-risk hematologic malignancies. The ultimate success of HSCT continues to be limited by acute graft-versus-host disease (aGVHD), however, and new therapy options are still needed. Our research group previously demonstrated that aGVHD depends on the ability of donor “conventional” nonregulatory T cells ( $T_{\text{cons}}$ ) to express CC-Chemokine Receptor 7 (CCR7), a G protein-coupled receptor critical for the normal trafficking of lymphocytes and dendritic cells into lymph nodes and the splenic white pulp [1,2]. Donor T cells knocked

out at the CCR7 locus ( $CCR7^{-/-}$ ) generated greatly attenuated aGVHD responses in multiple mouse HSCT model systems. Nevertheless,  $CCR7^{-/-}$   $T_{\text{cons}}$  retained their ability to mount beneficial graft-versus-leukemia (GVL) antitumor immune responses, and  $CCR7^{-/-}$  regulatory T cells were able to diminish aGVHD. In addition, at least 2 studies involving human patients have linked the proportion of CCR7-expressing T cells in the donor stem cell product to the subsequent development of aGVHD [3,4].

Based on these data, we deemed CCR7 to be an attractive therapy target and undertook a high-throughput screening effort to identify small-molecule antagonists of the receptor. This work revealed for the first time that cosalane, a compound originally developed as a human immunodeficiency virus (HIV) therapeutic, has an intrinsic ability to block human and murine CCR7 function in vitro in response to both of its natural ligands, CCL19 and CCL21 [5]. As a result, we explored the use of

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\* Correspondence and reprint requests: James M. Coghill, MD, 125 Mason Farm Road, 5230D Marsico Hall, CB 7599, Chapel Hill, NC 27599

E-mail address: [jcoghill@email.unc.edu](mailto:jcoghill@email.unc.edu) (J.M. Coghill).

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cosalane as a new approach to prevent aGVHD in mouse HSCT model systems and present our findings here.

Initial attempts to attenuate aGVHD through the systemic administration of cosalane to mice post-transplantation were unsuccessful, likely due to the compound's hydrophobicity and high albumin binding. Nonetheless, we were able to significantly reduce aGVHD by briefly incubating donor  $T_{\text{cons}}$  with cosalane *ex vivo* before transplantation. Cosalane's effects were observed in multiple strain combinations, including a xenogeneic aGVHD model, were dose-dependent, and did not appear to result in  $T_{\text{con}}$  toxicity. Furthermore, cosalane did not impair early stem cell engraftment when bone marrow (BM) cells were also exposed to the compound before transplantation and appeared to spare GVL effects against murine P815 mastocytoma cells. Collectively, these data indicate that cosalane may represent a viable new approach for specific aGVHD prophylaxis following HSCT.

## METHODS

### Mice

C57BL/6 ("B6"; H-2<sup>b</sup>), B6xDBA/2 F1 ("B6D2"; H-2<sup>bxd</sup>), BALB/c (H-2<sup>d</sup>), and NSG mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Enhanced green fluorescent protein (eGFP)-expressing B6 mice were generated as described previously [6]. CCR7<sup>-/-</sup> mice that had been backcrossed 4 times onto a C57BL/6 background (B6.129P2-CCR7<sup>tm1Dgen</sup>) were obtained from The Jackson Laboratory. These mice were further backcrossed in our laboratory to 8 generations. All animal experiments were performed in accordance with protocols approved by The University of North Carolina's Institutional Animal Care and Use Committee.

### Transplantation Procedures

Recipient mice were irradiated on transplantation day -1. Recipients were then administered T cell-depleted (TCD) BM cells with or without CD25<sup>-</sup> column-purified conventional T cells ( $T_{\text{cons}}$ ) to induce aGVHD as described previously [7,8]. In brief, whole BM cells were collected from the femurs and tibias of donor mice, lysed of RBCs using an ammonium chloride/potassium carbonate buffer, and depleted of T cells by a negative selection column purification process using anti-CD90.2 ferromagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). For  $T_{\text{con}}$  preparations, whole donor splenocytes were lysed of RBCs and then applied to a total T cell isolation column (Cedarlane Laboratories, Burlington, ON, Canada). The flow-through was then purified via a second negative selection approach. For this, the cells were incubated with PE-conjugated antibodies against B220 (to remove any residual contaminating B cells) and CD25 (to deplete regulatory T cells and activated effector T cells), followed by anti-PE ferromagnetic beads. The cells were then applied to a magnetic column (Miltenyi Biotec) and the final flow-through collected. Following this procedure, the final  $T_{\text{con}}$  inoculum consisted of both CD25<sup>-</sup>CD4<sup>+</sup> and CD8<sup>+</sup> T cells at an approximate 1:1 ratio.

For *ex vivo* cosalane incubations, cell populations were suspended in serum/protein-free injectable saline. They were then incubated with cosalane at varying concentrations or with an equal volume of DMSO vehicle for 1 hour at 37 °C before transplantation. The cells were then administered immediately to recipients via tail vein injection. For xenograft experiments, human peripheral blood mononuclear cells (PBMCs) were obtained from leukocytes obtained from the Gulf Coast Regional Blood Center (Houston, TX) using a Ficoll gradient.

### Cosalane

Cosalane was obtained from Southern Research (Birmingham, AL) or prepared in-house according to published protocols [9,10]. The cosalane derivatives phenylalanine cosalane (p-cosalane) and tert-butyl cosalane (tb-cosalane) were kind gifts from the Mark Cushman laboratory.

### $T_{\text{con}}$ Proliferation Assay

Purified CD25<sup>-</sup> B6  $T_{\text{cons}}$  were incubated with cosalane at the indicated concentrations or an equal volume of DMSO vehicle for 1 hour at 37 °C. The cells were then washed and cultured on 24-well plates that had been previously incubated overnight with anti-CD3 and anti-CD28 antibody at 10 µg/mL. The cells were cultured for 72 hours in complete medium supplemented with murine IL-2 at 100 IU/mL and then counted with a hemocytometer.

### Organ eGFP Quantification

Recipients were euthanized, and their organs were removed. Individual organs were immediately homogenized in PBS plus protease inhibitor without any pooling of tissues, and absolute eGFP levels then determined with an

enzyme-linked immunosorbent assay (ELISA) kit (Cell Biolabs, San Diego, CA) as described previously [1].

### Organ Cytokine Quantification

Recipient mice were euthanized, and their organs were removed and homogenized. Total organ IFN- $\gamma$  and tumor necrosis factor levels were then determined by ELISA (eBioscience, San Diego, CA).

### P815 GVL Model and *In Vivo* Imaging

Luciferase-transfected P815 murine mastocytoma cells (H-2<sup>d</sup>) were a kind gift from the Jonathan Serody laboratory. P815 cells were cultured and then mixed with TCD BM cells before transplantation. A tumor dose of  $2.5 \times 10^4$  P815 cells was used for all experiments. Recipients were then serially imaged using an IVIS Kinetic Optical real-time imaging system (Caliper Life Sciences, Hopkinton, MA) to monitor for *in vivo* tumor growth twice weekly. For imaging, recipients were dosed with 3 mg of D-Luciferin (PerkinElmer, Waltham, MA) by i.p. injection at 10 minutes before the procedure. Mice were then anesthetized with isoflurane during image acquisition. Unless indicated otherwise, an exposure time of 4 seconds was used for all images.

### Statistical Analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA). Survival curves were constructed using the Kaplan-Meier method, and median survival times were compared using the log-rank test. Unless indicated otherwise, continuous variables were compared using the nonparametric Mann-Whitney *U* test. *P* values <.05 were considered significant. Error bars represent SEM.

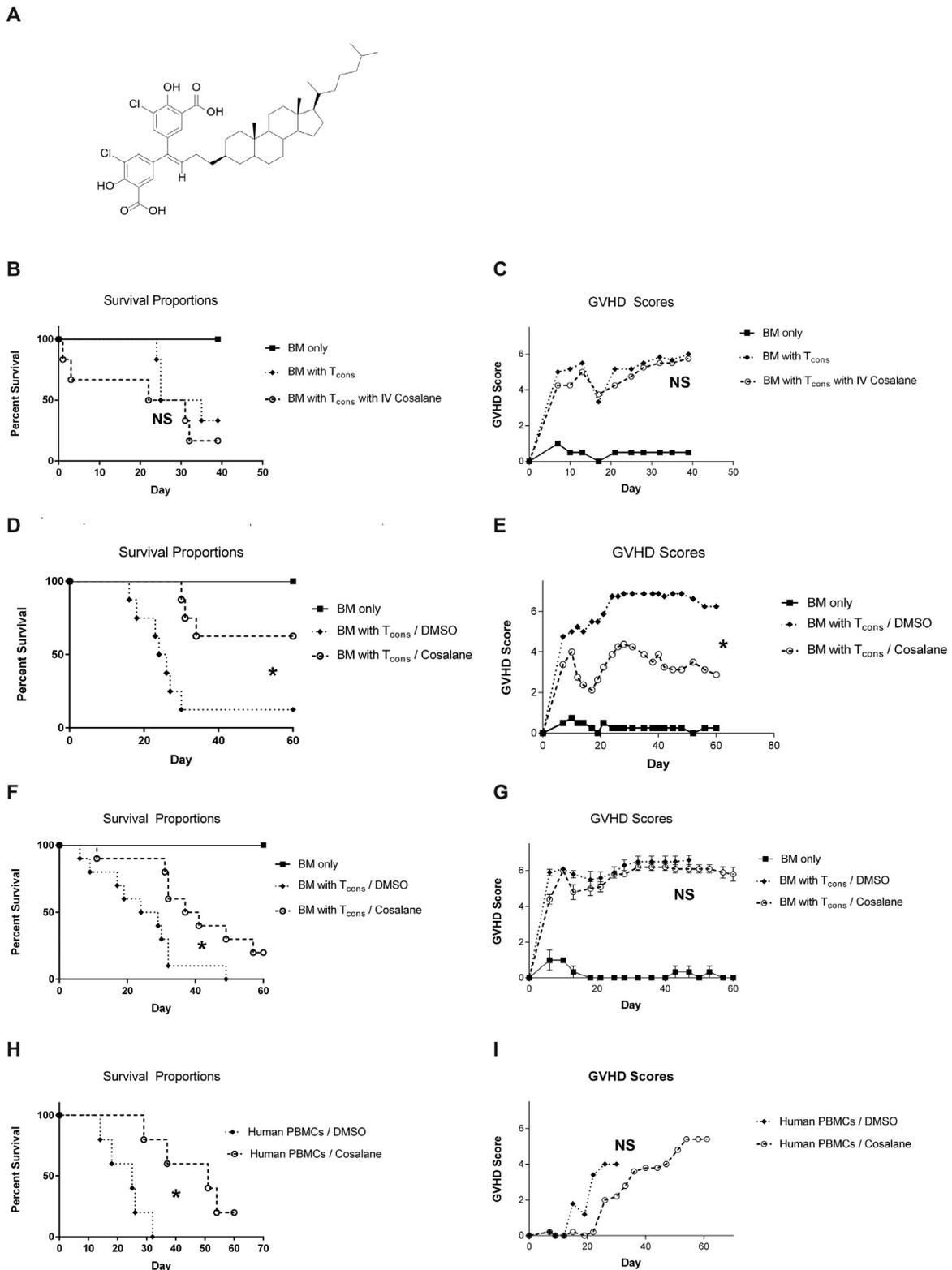
## RESULTS

### Cosalane Does Not Prevent aGVHD When Dosed Systemically to Mice after Transplantation but Is Active *ex Vivo*

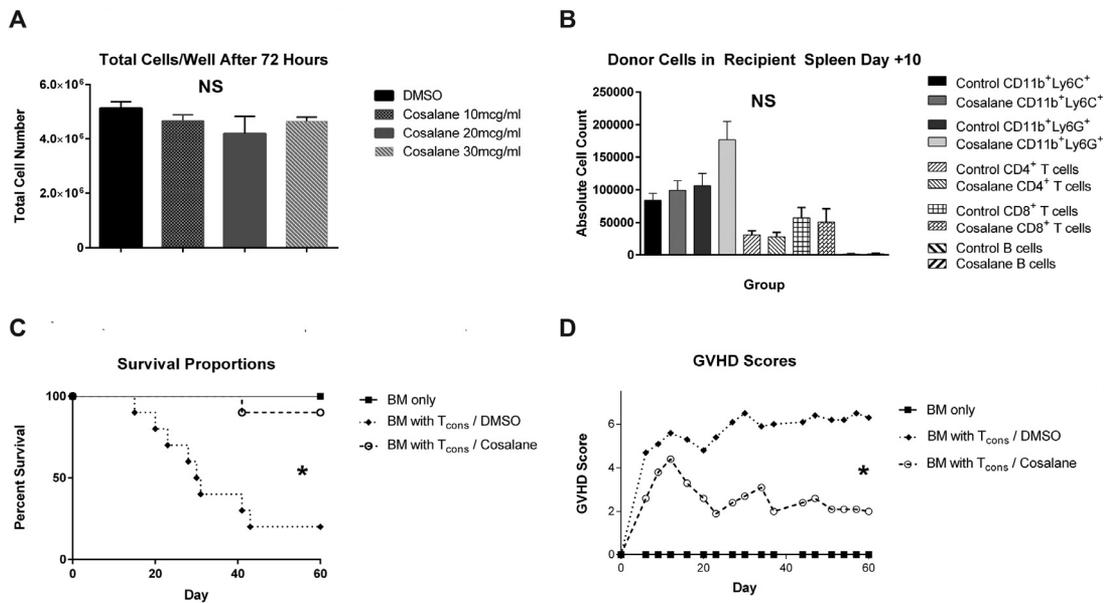
Initial work focused on whether cosalane (structure depicted in Figure 1A) could limit aGVHD when dosed to murine recipients after HSCT. For these experiments, we used a standard C57BL/6 ("B6" H-2<sup>b</sup>) into C57BL/6  $\times$  DBA2 ("B6D2" H-2<sup>bxd</sup>) parent in an F1 model system. Based on the limited existing literature describing cosalane administration to rodents [11], we dosed the compound at 10 mg/kg via i.v. injection on the day of transplantation and then again every 3 days for a total of 3 doses. Control mice were administered an equivalent volume of DMSO vehicle. Mice were then followed for overall survival and scored for aGVHD twice weekly using a validated scoring system [12]. As shown in Figure 1B and C, cosalane did not attenuate aGVHD and may have actually worsened clinical outcomes.

Subsequent work has revealed that 10 mg/kg was an excessively high dose to administer to irradiated recipients, and that dosages of 2 to 5 mg/kg are much better tolerated. Nevertheless, given the technical challenges of repeated i.v. drug administration and cosalane supply considerations, we explored the use of an *ex vivo* dosing strategy as an alternative means to apply the compound. For these experiments, purified  $T_{\text{cons}}$  were incubated with cosalane at 10 µg/mL (13 µM) or DMSO vehicle diluted in saline at 37 °C for 1 hour immediately before transplantation. They were then mixed with untreated TCD BM cells and administered to recipients by tail vein injection. Recipients received no additional cosalane after transplantation. As depicted in Figure 1D and E, control mice given  $T_{\text{cons}}$  incubated with DMSO in saline developed aggressive aGVHD and demonstrated poor survival. In contrast, those receiving  $T_{\text{cons}}$  incubated with cosalane in saline had lower aGVHD scores and improved median survival times.

We next ensured that cosalane's ability to attenuate aGVHD was not strain-dependent. For this work, we used a completely MHC-mismatched B6 into BALB/c (H-2<sup>d</sup>) allograft model (Figure 1F and G) and a human xenogeneic aGVHD system (Figure 1H and I). Although cosalane's effects were not as complete in these highly aggressive models, in both instances, mice receiving cosalane-treated cells demonstrated prolonged



**Figure 1.** Donor  $T_{\text{cons}}$  exposed to cosalane ex vivo generate attenuated aGVHD responses. (A) The chemical structure of cosalane. (B and C) B6D2 mice were irradiated to 950 rads on transplantation day -1. On day 0, recipients were administered  $3 \times 10^6$  TCD B6 BM cells with or without  $4 \times 10^6$  whole splenic B6  $T_{\text{cons}}$  (CD25<sup>-</sup>CD4<sup>+</sup> and CD25<sup>-</sup>CD8<sup>+</sup> T cells in a 1:1 ratio). Those mice receiving  $T_{\text{cons}}$  were dosed with cosalane at 10 mg/kg or with an equal volume of DMSO vehicle via i.v. injection on day 0 immediately before transplantation and then again on post-transplantation days +3 and +6. BM only,  $n = 2$ ; all other treatment groups,  $n = 6$ . (B) Mice were followed for survival.  $P > .05$  for survival curve comparison between BM/ $T_{\text{cons}}$  and BM/ $T_{\text{cons}}$  with cosalane groups by the log-rank test. (C) Mice were scored for aGVHD twice weekly using a validated scoring system. Recipients were assigned a score of 0 to 2 for 5 separate clinical parameters: weight loss, activity, kyphosis, fur ruffling, and skin breakdown. Individual scores were then summed for a total score ranging from 0 to 10.  $P > .05$  for aGVHD score comparison between BM/ $T_{\text{cons}}$  and BM/ $T_{\text{cons}}$  with cosalane groups on day +40 by the Mann-Whitney test. (D and E) B6D2 mice were lethally irradiated on day -1. On day 0, recipients were administered  $3 \times 10^6$  TCD B6 BM cells with or without  $4 \times 10^6$  whole B6  $T_{\text{cons}}$ . Donor  $T_{\text{cons}}$  were incubated ex vivo with cosalane diluted in normal saline at 10  $\mu\text{g}/\text{mL}$  or an equal volume of



**Figure 2.** Cosalane does not affect T<sub>con</sub> proliferation in vitro and does not impair early donor lymphoid or myeloid engraftment after HSCT. (A) B6 T<sub>cons</sub> were purified and incubated with increasing concentrations of cosalane or equal volumes of DMSO vehicle in normal saline for 1 hour at 37° C. The cells were then washed and cultured on 24-well plates precoated with anti-CD3 and anti-CD28 antibody in the presence of supplemental IL-2 for 72 hours. The cells per well were then quantified.  $P > .05$  for all cell number comparisons by the Mann-Whitney test. (B) Irradiated B6D2 recipients underwent transplantation with  $3 \times 10^6$  eGFP<sup>+</sup> B6 TCD BM cells plus  $4 \times 10^6$  eGFP<sup>+</sup> B6 T<sub>cons</sub>. The entire donor stem cell product (TCD BM cells plus T<sub>cons</sub>) was incubated with either cosalane (15  $\mu$ g/mL) or DMSO vehicle before transplantation. On day +10, total numbers of donor (eGFP<sup>+</sup>) cells in the spleen were determined by flow cytometry.  $n = 5$  mice per treatment group.  $P > .05$  for all pairwise cell number comparisons between DMSO and cosalane cell recipients by the Mann-Whitney test. (C and D) B6D2 recipients underwent transplantation as in (B) but were followed for survival and scored for aGVHD. BM only,  $n = 4$ ; all other treatment groups,  $n = 10$ . In (C),  $*P = .0006$  for survival curve comparison between BM with T<sub>cons</sub>/DMSO and BM with T<sub>cons</sub>/cosalane groups. In (D),  $*P = .0007$  for aGVHD score comparison between BM with T<sub>cons</sub>/DMSO and BM with T<sub>cons</sub>/cosalane groups on day +60.

survival times. Collectively, these data indicate that a brief incubation of donor immune cells with cosalane can reduce aGVHD, and that this effect does not appear to be strain- or species-specific.

### Cosalane Exposure Does Not Result in Cytotoxicity or Impair BM Engraftment

Previous reports have indicated that cosalane is nontoxic to cells in vitro, with a therapeutic index  $>100^9$ . Nevertheless, we set out to ensure that cosalane's ability to prevent aGVHD is not the result of the drug killing the donor T<sub>con</sub> inoculum or limiting these cells' intrinsic ability to undergo activation and expansion. Purified B6 T<sub>cons</sub> were isolated as before and incubated with cosalane at increasing concentrations ranging from 10  $\mu$ g/mL (13  $\mu$ M, the concentration used for the B6 into B6D2 survival experiment described in Figure 1D and E) to 30  $\mu$ g/mL (39  $\mu$ M) or an equal volume of DMSO diluted in normal saline. The cells were then washed, placed on 24-well plates precoated with anti-CD3 and anti-CD28 immunoglobulin, and cultured for 72 hours in medium containing supplemental IL-2. Following incubation, all the T<sub>con</sub> groups exhibited robust growth in vitro, and no differences in cell numbers were noted after the culture period (Figure 2A).

Following up on these data, we determined whether cosalane exposure could potentially cause injury to hematopoietic stem cells if BM were also exposed to cosalane ex vivo. Notably, this situation would be potentially relevant to human transplantation, in which the donor stem cell product is typically not separated into separate cellular components before administration. Purified T<sub>cons</sub> and TCD BM cells were obtained from B6 donors transgenic for eGFP, mixed together, and then incubated with cosalane or DMSO. The cells were subsequently administered to irradiated B6D2 recipients, and early BM and T<sub>con</sub> engraftment then determined by flow cytometry within the host spleen on post-transplantation day +10. Donor-derived cells were distinguished from residual host cells by virtue of their eGFP positivity. As shown in Figure 2B, cosalane exposure did not produce any differences in donor monocyte, neutrophil, T cell, or B cell numbers in the spleen at this time point.

We next determined whether cosalane treatment of the entire stem cell product (BM plus T<sub>cons</sub>) would limit the compound's ability to reduce aGVHD or potentially result in delayed mortality due to eventual BM failure. As depicted in Figure 2C and D, cosalane treatment of the whole stem product appeared to attenuate aGVHD at least as well as what was observed previously with the isolated treatment of T<sub>cons</sub> alone. Furthermore, no clinical signs of delayed BM failure were

DMSO in normal saline for 1 hour at 37° C. The cells were then mixed with untreated TCD B6 BM cells and administered immediately to recipient mice by tail vein injection. Mice were then followed for survival and scored for aGVHD. BM only,  $n = 4$ ; BM with T<sub>cons</sub>/DMSO,  $n = 8$ ; BM with T<sub>cons</sub>/cosalane,  $n = 8$ . In (D),  $*P = .044$  for survival curve comparison between BM with T<sub>cons</sub>/DMSO and BM with T<sub>cons</sub>/cosalane groups by the log-rank test. In (E),  $*P = .026$  for aGVHD comparison between BM with T<sub>cons</sub>/DMSO and BM with T<sub>cons</sub>/cosalane on day +60. (F and G) BALB/c mice were irradiated to 800 rads on day -1. On day 0, recipients were administered  $5 \times 10^6$  TCD B6 BM cells with or without  $5 \times 10^5$  whole B6 T<sub>cons</sub> incubated ex vivo with cosalane at 15  $\mu$ g/mL in normal saline alone or an equal volume of DMSO vehicle in saline. Mice were then followed for survival and scored for aGVHD. BM only,  $n = 3$ ; all other treatment groups,  $n = 10$ . In (F),  $*P = .01$  for survival curve comparison between BM with T<sub>cons</sub>/DMSO and BM with T<sub>cons</sub>/cosalane groups. In (G),  $P > .05$  for aGVHD score comparison between BM with T<sub>cons</sub>/DMSO and BM with T<sub>cons</sub>/cosalane groups on day +47. (I and J) NSG mice were irradiated to 200 rads on day -1. On day 0, recipients were given  $7 \times 10^6$  human PBMCs incubated ex vivo with DMSO or cosalane at 15  $\mu$ g/mL. Mice were then followed for survival and scored for aGVHD.  $n = 5$  in both treatment groups. In (I),  $*P = .0064$  for survival curve comparison. In (J),  $P > .05$  for aGVHD score comparison on day +30.

observed, with 90% of the cosalane group surviving to the end of the study observation period. Collectively, these data indicate that cosalane does not induce any apparent cytotoxicity in vitro at doses up to 39  $\mu\text{M}$ , and that a whole marrow product can be safely exposed to compound without any loss of compound efficacy or apparent impairment in myeloid or lymphoid engraftment.

### ***Cosalane Affects Donor $T_{\text{con}}$ Function in a Dose-Dependent Fashion and Is More Potent than 2 Similar Structural Derivatives***

We next set out to formally determine whether cosalane's effects are dose-dependent. For this work, we incubated purified B6  $T_{\text{cons}}$  with DMSO or increasing concentrations of cosalane *ex vivo*: 10  $\mu\text{g}/\text{mL}$  (13  $\mu\text{M}$ ), 20  $\mu\text{g}/\text{mL}$  (26  $\mu\text{M}$ ), and 30  $\mu\text{g}/\text{mL}$  (39  $\mu\text{M}$ ). The cells were then mixed with untreated TCD B6 BM cells and administered to irradiated B6D2 recipients. Recipient mice were followed for survival and scored for aGVHD twice weekly. As depicted in Figure 3A and B, we observed improved transplantation outcomes with increasing dosages of cosalane during the preincubation step.

Building on these data, we went on to repeat transplants using a B6 into BALB/c completely MHC mismatched allograft model (Figure 3C and D) and a human PBMC into NSG xenogeneic system (Figure 3E and F) with higher compound doses. Compared with the outcomes observed previously (Figure 1F–I), cosalane's effects were more complete and more durable, with most recipients surviving to the end of the study period. Thus, at higher dosages, cosalane allowed for nearly complete aGVHD protection in 3 separate model systems.

Our laboratory subsequently evaluated the activity of 2 cosalane derivatives, phenylalanine cosalane (p-cosalane) and tert-butyl cosalane (tb-cosalane), in which the amino acid phenylalanine or a tert-butyl group respectively is conjugated to each of cosalane's two carboxylic acid groups. For this work, we used a B6 into B6D2 model system and incubated donor  $T_{\text{cons}}$  *ex vivo* with identical doses of cosalane, p-cosalane, tb-cosalane, or DMSO vehicle before transplantation. Recipients were then followed for survival and scored for aGVHD. As depicted in Figure 3G and H, neither derivative appeared to be as active as the parent compound. Given the dose-dependent nature of cosalane's aGVHD protective effects, however, we repeated this experiment with higher concentrations of each derivative. As shown in Figure 3I and J, both structures were able to improve median survival times at higher doses, with recipients of p-cosalane-treated  $T_{\text{cons}}$  showing the lowest aGVHD scores. Collectively, these data indicate a class effect for this family of compounds. Nevertheless, neither of the derivatives was as potent as the parent structure, suggesting that the identity of the compound's hydrophilic head group is particularly relevant to the drug's ability to ameliorate aGVHD after HSCT.

### ***Cosalane Limits aGVHD by Blocking $T_{\text{con}}$ Accumulation in the Colon and Liver, with Minimal Action on Donor BM Cells***

Most of our allogeneic transplantation experiments up to this point had involved the treatment of purified donor  $T_{\text{cons}}$  composed of a mixture of  $\text{CD}25^{-}\text{CD}4^{+}$  and  $\text{CD}25^{-}\text{CD}8^{+}$  cells (Figures 1D–G and 3A–D). In treating this particular population, we were able to consistently reduce aGVHD and improve survival, thereby establishing donor  $T_{\text{cons}}$  as a critical cosalane drug target. However, previous studies have shown that donor BM-derived antigen-presenting cells, particularly  $\text{CCR}7^{+}$  dendritic cells, play a role in augmenting the aGVHD process [13,14]. Furthermore, in our own experiments in which donor BM cells and donor  $T_{\text{cons}}$  were exposed to cosalane

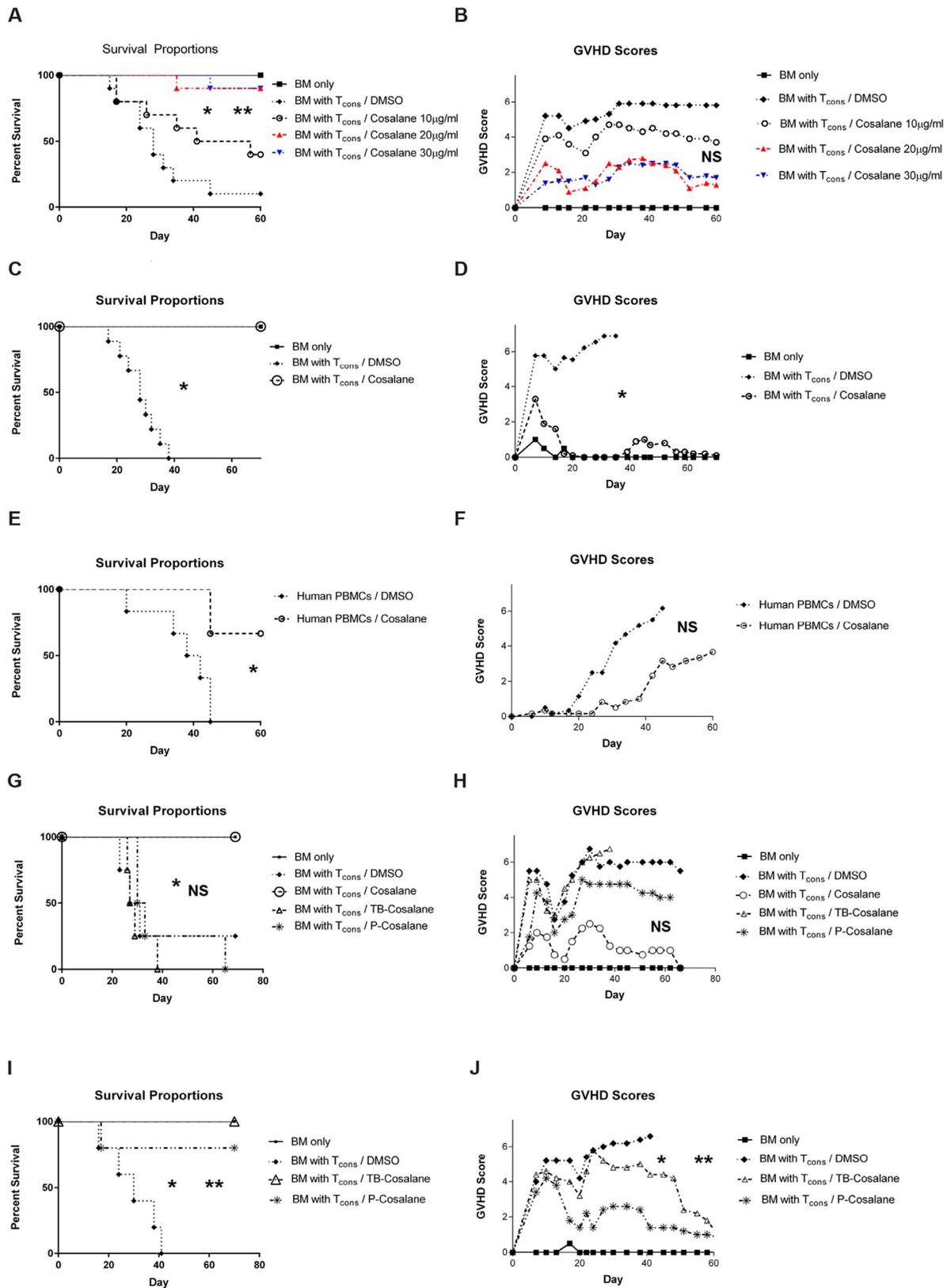
pretransplantation (Figure 2C and D), we achieved excellent long-term aGVHD control. As a result, we questioned whether recipient mice might derive a benefit from the isolated treatment of donor BM cells alone or a synergistic benefit from the combined treatment of donor  $T_{\text{cons}}$  and BM.

To formally evaluate this possibility, we performed a B6 into B6D2 haplotype-matched transplantation in which we pretreated donor BM cells, donor  $T_{\text{cons}}$ , both populations, or neither population with cosalane before HSCT. Given our finding that cosalane's effects are dose-dependent, each cell population was incubated in an identical concentration of drug. Recipients were then followed for survival and scored for aGVHD twice weekly. As shown in Figure 4A and B, the isolated treatment of purified donor BM cells alone did not significantly improve outcomes. Conversely, the isolated treatment of donor  $T_{\text{cons}}$  once again resulted in substantial attenuation of aGVHD, with 100% survival in this instance. Notably, the combined treatment of BM cells and  $T_{\text{cons}}$  did not appear to further reduce aGVHD scores. Nevertheless, the combination group appeared to do equally well, with 100% of recipients surviving to the end of the study period. Collectively, these data confirm our previous findings that cosalane can be safely administered to a whole donor stem cell product (BM cells plus T cells), but also indicate that cosalane's protective effects occur primarily via action on mature  $T_{\text{cons}}$  contained in the graft.

Given that cosalane appears to act primarily on  $T_{\text{cons}}$ , we elected to focus on this population in the remainder of our studies. Because cosalane is known to block  $\text{CCR}7$  in vitro [5], we hypothesized that the compound would limit donor  $T_{\text{con}}$  trafficking to host lymph nodes after transplantation [1,2]. To evaluate this, eGFP<sup>+</sup> B6  $T_{\text{cons}}$  were incubated with cosalane or DMSO vehicle and then transplanted with untreated eGFP<sup>-</sup> B6 TCD BM cells into irradiated eGFP<sup>-</sup> B6D2 recipients. On day +7, recipients were killed and their secondary lymphoid tissues (SLTs; mesenteric lymph nodes [MLNs], inguinal lymph nodes [ILNs], and spleen) were removed along with 3 important aGVHD target organs: colon, lung, and liver. These tissues were then homogenized, and donor  $T_{\text{con}}$  accumulation in each site was compared between recipients of cosalane and recipients of vehicle-treated cells using an anti-eGFP ELISA approach. As depicted in Figure 4C, no differences in donor  $T_{\text{con}}$  accumulation were noted in either host SLTs or aGVHD target organs at this time point.

Subsequent to this, we performed an identical transplantation but harvested recipient organs on day +14, a later time point at which clinical differences between the 2 treatment groups are typically more prominent (Figure 4D). As before, we found no differences in donor  $T_{\text{con}}$  accumulation within host lymphoid sites; however, by the second post-transplantation week, we detected a significant reduction in donor  $T_{\text{cons}}$  within the colon and liver. Taken together, these experiments did not suggest any generalized deficit in  $T_{\text{con}}$  expansion, as the eGFP signal was similar in a majority of sites in both treatment groups. Instead, cosalane was specifically limiting donor  $T_{\text{con}}$  accumulation in 2 critical aGVHD target organs while having surprisingly little impact on their expansion within the SLTs.

Following up on these data, we evaluated cosalane's effects on inflammatory cytokine production after HSCT. No differences in total TNF or  $\text{IFN-}\gamma$  levels were noted in the spleen, MLN, ILNs, or lung on either post-transplantation day +7 (Figure 4E) or day +14 (Figure 4F). Conversely, consistent with our  $T_{\text{con}}$  in vivo trafficking data, both TNF and  $\text{IFN-}\gamma$  levels were significantly reduced in the host colon on day +14 in the cosalane treatment group (Figure 4F). Collectively, these data do not support any overall deficiency in inflammatory cytokine production following cosalane exposure; rather, they indicate



**Figure 3.** Cosalane functions in a dose-dependent fashion and is more potent than 2 structurally similar derivatives. (A and B) B6D2 mice were lethally irradiated on day -1. On day 0, recipients were administered  $3 \times 10^6$  TCD B6 BM cells with or without  $4 \times 10^6$  B6  $T_{cons}$ . Donor  $T_{cons}$  were incubated ex vivo with DMSO vehicle or cosalane diluted in normal saline at increasing concentrations ranging from 10  $\mu\text{g}/\text{mL}$  to 30  $\mu\text{g}/\text{mL}$ . The cells were then mixed with untreated TCD B6 BM cells and administered immediately to recipient mice by tail vein injection. Mice were then followed for survival and scored for aGVHD. BM only,  $n = 4$ ; all other treatment groups,  $n = 10$ . In (A),  $*P = .020$  for survival curve comparison between BM with  $T_{cons}/\text{cosalane}$  10  $\mu\text{g}/\text{mL}$  and BM with  $T_{cons}/\text{cosalane}$  30  $\mu\text{g}/\text{mL}$ ;  $**P = .0165$  for survival curve comparison between BM with  $T_{cons}/\text{cosalane}$  10  $\mu\text{g}/\text{mL}$  and BM with  $T_{cons}/\text{cosalane}$  20  $\mu\text{g}/\text{mL}$ ;  $**P = .0165$  for survival curve comparison between BM with  $T_{cons}/\text{cosalane}$  10  $\mu\text{g}/\text{mL}$  and BM with  $T_{cons}/\text{cosalane}$  30  $\mu\text{g}/\text{mL}$ . In (B),  $P > .05$  for aGVHD score comparison between BM with

that, with the exception of the liver, organ cytokine level differences between the cosalane and control groups generally parallel differences in the number of infiltrating donor  $T_{\text{cons}}$ .

### **Cosalane Prevents aGVHD in a CCR7-Associated Manner**

As noted previously, our initial premise for using cosalane to attenuate aGVHD was based on its demonstrable antagonistic effects on CCR7, a chemokine receptor critical for T cell trafficking into SLTs [2]. However, our *in vivo* trafficking data indicated a minimal impact on the ability of donor  $T_{\text{cons}}$  to accumulate within the host spleen or lymph nodes on post-transplantation days +7 and +14 and suggested that cosalane's anti-aGVHD properties actually might be CCR7-independent. To more definitively ascertain the extent to which cosalane's protective effects are linked to CCR7, we induced lethal aGVHD with CCR7<sup>-/-</sup>  $T_{\text{cons}}$ , reasoning that if CCR7 were indeed critical for its action, then the drug should be less active in this setting. Notably, aGVHD is not entirely prevented by the genetic absence of CCR7 on donor  $T_{\text{cons}}$ ; rather, aGVHD is greatly attenuated compared with that induced by an equivalent dose of wild-type (WT) donor  $T_{\text{cons}}$ . We previously found that the degree of aGVHD attenuation observed in the absence of CCR7 is somewhat model-dependent, with outcome differences less pronounced with higher degrees of MHC mismatch between the donor and recipient strains [1]. As a result, for this work we chose an aggressive, completely MHC-mismatched B6 into BALB/c system. In addition, we performed dose-finding work with CCR7<sup>-/-</sup> B6 donor mice to arrive at an appropriate donor  $T_{\text{con}}$  dose that would consistently induce lethal aGVHD at an intensity/severity approximating what is typically observed with  $5 \times 10^5$  WT cells. After evaluating a range of different doses, we identified a donor  $T_{\text{con}}$  inoculum of  $3 \times 10^6$  CCR7<sup>-/-</sup> B6  $T_{\text{cons}}$  per recipient as optimal. CCR7<sup>-/-</sup>  $T_{\text{cons}}$  were pretreated with DMSO or cosalane *ex vivo* at 15  $\mu\text{g}/\text{mL}$ , the same dose that we used for the survival studies depicted in Figure 1F and G using an identical B6 into BALB/c strain combination. As shown in Figure 5A and B, and in contrast to what we observed with WT B6 donor  $T_{\text{cons}}$ , cosalane appeared to be completely inactive at this dose.

We previously found cosalane to afford nearly complete aGVHD protection in a B6 into BALB/c system at a higher incubation concentration of 30  $\mu\text{g}/\text{mL}$  (Figure 3C and D). As a result, we repeated our CCR7<sup>-/-</sup> B6 into BALB/c transplantation, but preincubated the donor  $T_{\text{cons}}$  with an identical cosalane concentration of 30  $\mu\text{g}/\text{mL}$ . As shown in Figure 5C and D, again cosalane's effects were much less complete in the absence of donor  $T_{\text{con}}$  CCR7, even at a higher drug dose. Nevertheless,

cosalane's protective effects were not entirely abrogated in this case, with recipients of cosalane-treated CCR7<sup>-/-</sup> B6  $T_{\text{cons}}$  demonstrating longer survival times and a trend toward lower aGVHD scores compared with control mice. Collectively, these data indicate that cosalane does appear to exert its protective effects in a CCR7-associated manner; however, its modest residual efficacy even in the absence of the receptor suggests activity against additional target(s) yet to be determined.

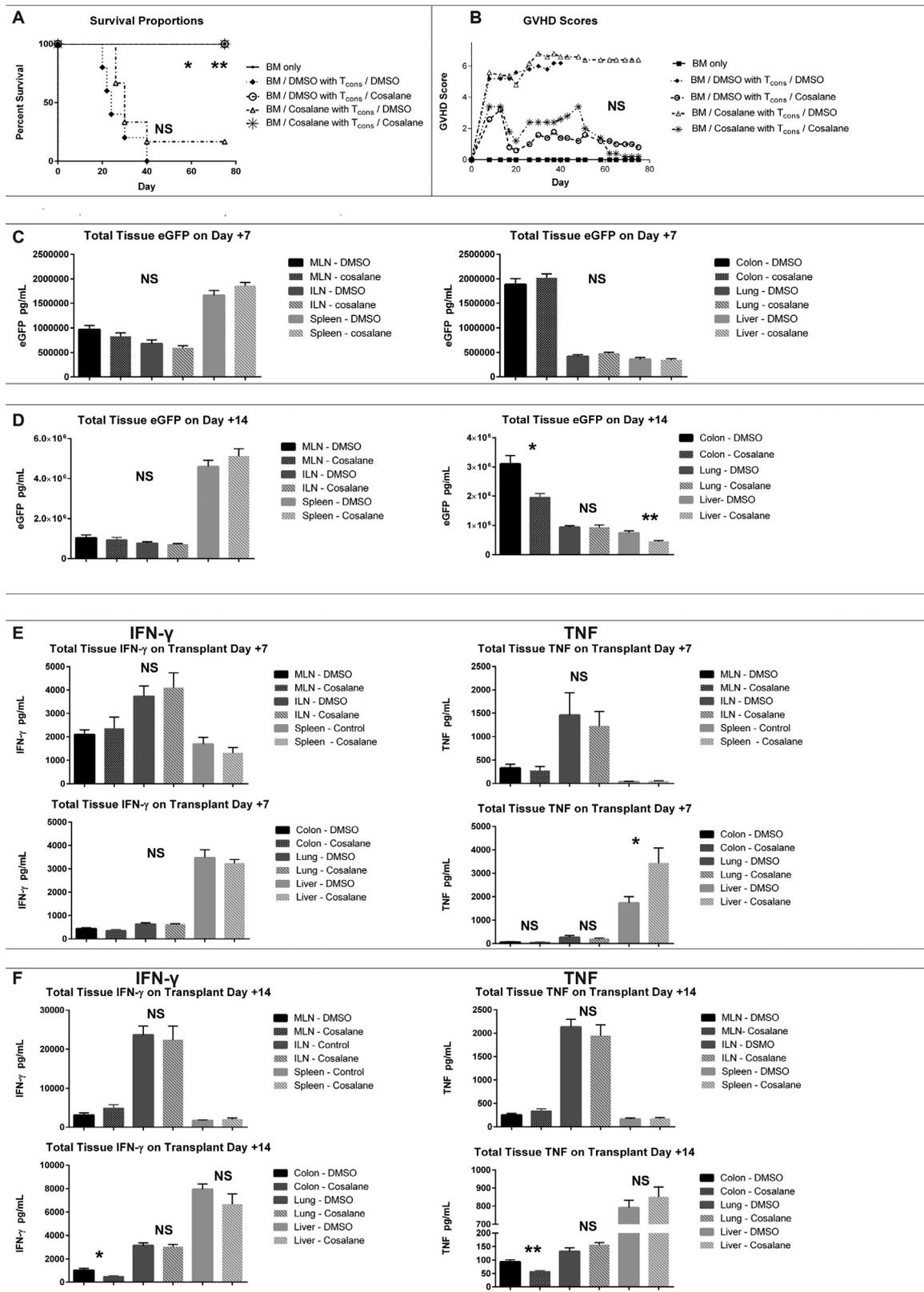
In light of these survival data linking cosalane's ability to attenuate aGVHD to CCR7, we revisited our initial hypothesis that cosalane might impair donor  $T_{\text{con}}$  trafficking into host lymphoid sites. Our previous *in vivo* trafficking measurements performed on post-HSCT days +7 and +14 (Figure 4C and D) failed to show any differences in lymphoid accumulation between cosalane and vehicle-treated  $T_{\text{cons}}$ . It should be noted, however, that significant  $T_{\text{con}}$  activation and expansion would have already occurred by these time points, and that donor immune cells could have recirculated back into the lymph nodes via the afferent lymphatics. Furthermore, previous reports have indicated that a partial deficiency in CCR7 signaling can result in delayed but paradoxically increased T cell expansion within lymph nodes over time owing to altered negative feedback mechanisms [15]. As a result, subtle effects of cosalane on early lymphocyte trafficking out of the bloodstream might have been overlooked.

To more definitively ascertain whether cosalane could impair donor  $T_{\text{con}}$  homing into host SLTs, we transplanted cosalane- or DMSO-treated eGFP<sup>+</sup>  $T_{\text{cons}}$  into irradiated B6D2 recipients and then harvested their organs for analysis by anti-eGFP ELISA after only 36 hours, a time at which  $T_{\text{con}}$  expansion and/or recirculation would be minimal. Of note, an ELISA approach is extremely sensitive and allows for direct eGFP quantitation within postirradiated atrophied lymphoid sites without the need for pooling of tissues. As shown in Figure 5E, at this time point, we detected significant reductions in donor  $T_{\text{cons}}$  within the spleen, ILNs, and MLNs in those mice given cosalane-treated cells. Thus, cosalane does indeed limit the trafficking of donor  $T_{\text{cons}}$  into host lymphoid sites early after transplantation. The observed differences in the number of donor  $T_{\text{cons}}$  within SLTs become less prominent over time, however, and are no longer detectable by the end of the first week post-transplantation.

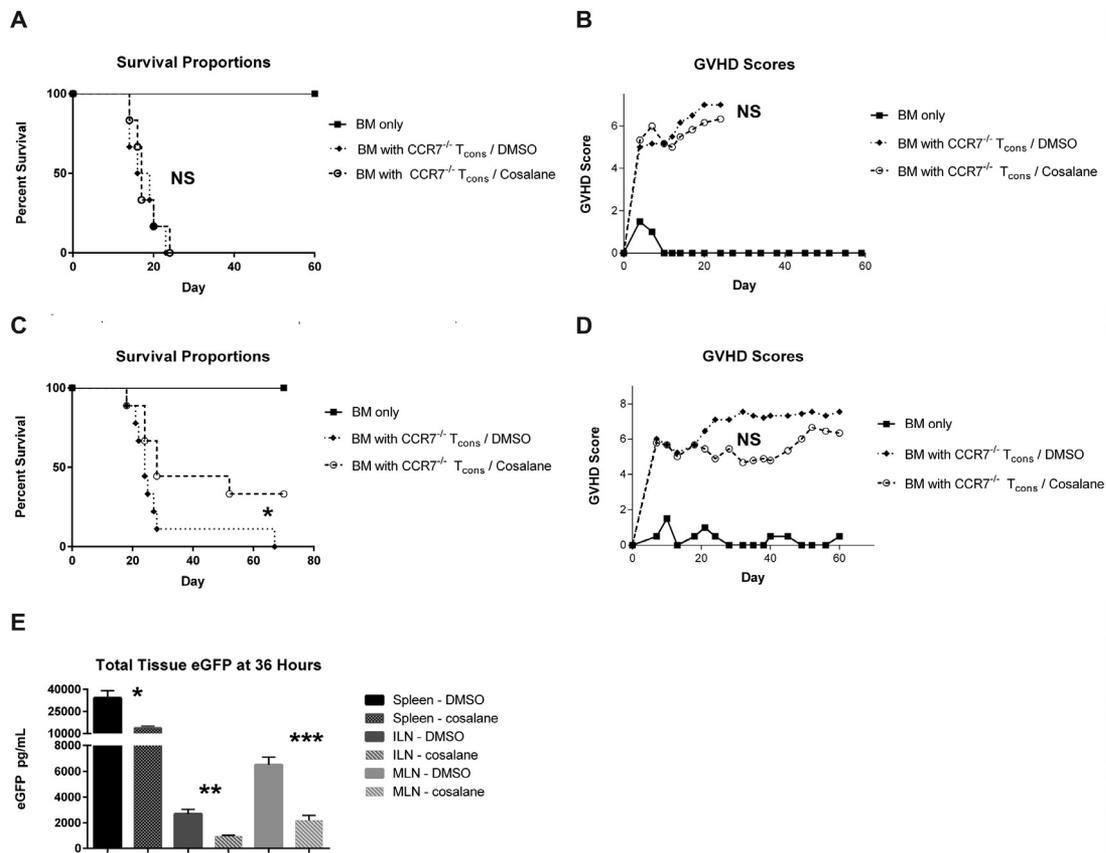
### **Cosalane Treatment of Donor $T_{\text{cons}}$ before HSCT Limits aGVHD but Spares GVL Effects**

Any therapy that reduces aGVHD also has the potential to impair beneficial GVL effects. Consequently, we evaluated cosalane's effects on GVL immunity using a well-described

$T_{\text{cons}}/\text{cosalane}$  10  $\mu\text{g}/\text{mL}$  and BM with  $T_{\text{cons}}/\text{cosalane}$  20  $\mu\text{g}/\text{mL}$  or BM with  $T_{\text{cons}}/\text{cosalane}$  30  $\mu\text{g}/\text{mL}$  on day +60. (C and D) BALB/c mice were irradiated to 800 rads on day -1. On day 0, recipients were administered  $5 \times 10^6$  TCD B6 BM cells with or without  $5 \times 10^5$  B6  $T_{\text{cons}}$ . Donor  $T_{\text{cons}}$  were incubated *ex vivo* with DMSO vehicle or cosalane diluted in normal saline at 30  $\mu\text{g}/\text{mL}$ . The cells were then mixed with untreated TCD B6 BM cells and administered immediately to recipient mice by tail vein injection. Mice were then followed for survival and scored for aGVHD. BM only, n = 4; BM with  $T_{\text{cons}}/\text{DMSO}$ , n = 9; BM with  $T_{\text{cons}}/\text{cosalane}$ , n = 10. In (C), \* $P < .0001$  for survival curve comparison between BM with  $T_{\text{cons}}/\text{DMSO}$  and BM with  $T_{\text{cons}}/\text{cosalane}$  groups. In (D), \* $P < .0001$  for aGVHD score comparison between BM with  $T_{\text{cons}}/\text{DMSO}$  and BM with  $T_{\text{cons}}/\text{cosalane}$  groups on day +35. (E and F) NSG mice were irradiated to 200 rads on day -1. On day 0, recipients were administered  $7 \times 10^6$  human PBMCs that had been incubated *ex vivo* with DMSO or cosalane at 35  $\mu\text{g}/\text{mL}$ . Mice were then followed for survival and scored for aGVHD. n = 6 mice per treatment group. In (E), \* $P = .0049$  for survival curve comparison. In (F),  $P > .05$  for aGVHD score comparison between the human PBMCs/DMSO group and the human PBMCs/cosalane group on day +45. (G and H) B6D2 mice were lethally irradiated on day -1. On day 0, recipients were administered  $3 \times 10^6$  TCD B6 BM cells with or without  $4 \times 10^6$  B6  $T_{\text{cons}}$ . Donor  $T_{\text{cons}}$  were incubated *ex vivo* with DMSO vehicle or cosalane, p-cosalane, or tb-cosalane diluted in normal saline at 20  $\mu\text{g}/\text{mL}$ . The cells were then mixed with untreated TCD B6 BM cells and administered immediately to recipient mice by tail vein injection. Mice were then followed for survival and scored for aGVHD. BM only, n = 2; all other treatment groups, n = 4. In (G), \* $P = .040$  for survival curve comparison between BM with  $T_{\text{cons}}/\text{DMSO}$  and BM with  $T_{\text{cons}}/\text{cosalane}$ ;  $P > .05$  for survival curve comparison between BM with  $T_{\text{cons}}/\text{DMSO}$  and BM with  $T_{\text{cons}}/\text{p-cosalane}$  or BM with  $T_{\text{cons}}/\text{tb-cosalane}$ . In (H),  $P > .05$  for aGVHD score comparison between BM with  $T_{\text{cons}}/\text{DMSO}$  and BM with  $T_{\text{cons}}/\text{p-cosalane}$  on day +62 and between BM with  $T_{\text{cons}}/\text{DMSO}$  and BM with  $T_{\text{cons}}/\text{tb-cosalane}$  on day +38. (I and J) B6D2 mice were lethally irradiated on day -1. On day 0, recipients were administered  $3 \times 10^6$  TCD B6 BM cells with or without  $4 \times 10^6$  B6  $T_{\text{cons}}$ . Donor  $T_{\text{cons}}$  were incubated *ex vivo* with DMSO vehicle or p-cosalane or tb-cosalane diluted in normal saline at 30  $\mu\text{g}/\text{mL}$ . The cells were then mixed with untreated TCD B6 BM cells and administered immediately to recipient mice by tail vein injection. Mice were then followed for survival and scored for aGVHD. BM only, n = 2; all other treatment groups, n = 5. In (I), \* $P = .0269$  for survival curve comparison between BM with  $T_{\text{cons}}/\text{DMSO}$  and BM with  $T_{\text{cons}}/\text{p-cosalane}$ ; \*\* $P = .0039$  for survival curve comparison between BM with  $T_{\text{cons}}/\text{DMSO}$  and BM with  $T_{\text{cons}}/\text{tb-cosalane}$ . In (J), \* $P = .0079$  for aGVHD score comparison between BM with  $T_{\text{cons}}/\text{DMSO}$  and BM with  $T_{\text{cons}}/\text{p-cosalane}$  on day +41; \*\* $P = .0238$  for aGVHD score comparison between BM with  $T_{\text{cons}}/\text{DMSO}$  and BM with  $T_{\text{cons}}/\text{tb-cosalane}$  on day +41.



**Figure 4.** Cosalane limits donor  $T_{con}$  accumulation within the liver and colon. (A and B) B6D2 mice were lethally irradiated on day -1. On day 0, recipients were administered  $3 \times 10^6$  TCD B6 BM cells with or without  $4 \times 10^6$  B6  $T_{cons}$ . Donor TCD BM cells, donor  $T_{cons}$ , both cell populations, or neither were incubated ex vivo with cosalane at  $15 \mu\text{g}/\text{mL}$  for 1 hour before transplantation. Cells not treated with cosalane were incubated with an equal volume of DMSO vehicle. Mice were then followed for survival and scored for aGVHD. BM only,  $n=2$ ; all other treatment groups,  $n=5$ . In (A),  $P > .05$  for survival curve comparison between BM/DMSO with  $T_{cons}/\text{DMSO}$  and BM/cosalane with  $T_{cons}/\text{DMSO}$  groups;  $P = .0018$  for survival curve comparison between BM/DMSO with  $T_{cons}/\text{DMSO}$  and BM/DMSO with  $T_{cons}/\text{cosalane}$  groups;  $**P = .0018$  or survival curve comparison between BM/DMSO with  $T_{cons}/\text{DMSO}$  and BM/cosalane with  $T_{cons}/\text{cosalane}$  groups. In (B),  $P > .05$  for aGVHD score comparison between BM/DMSO with  $T_{cons}/\text{DMSO}$  and BM/cosalane with  $T_{cons}/\text{DMSO}$  groups on day +40. (C and D) B6D2 mice were lethally irradiated on day



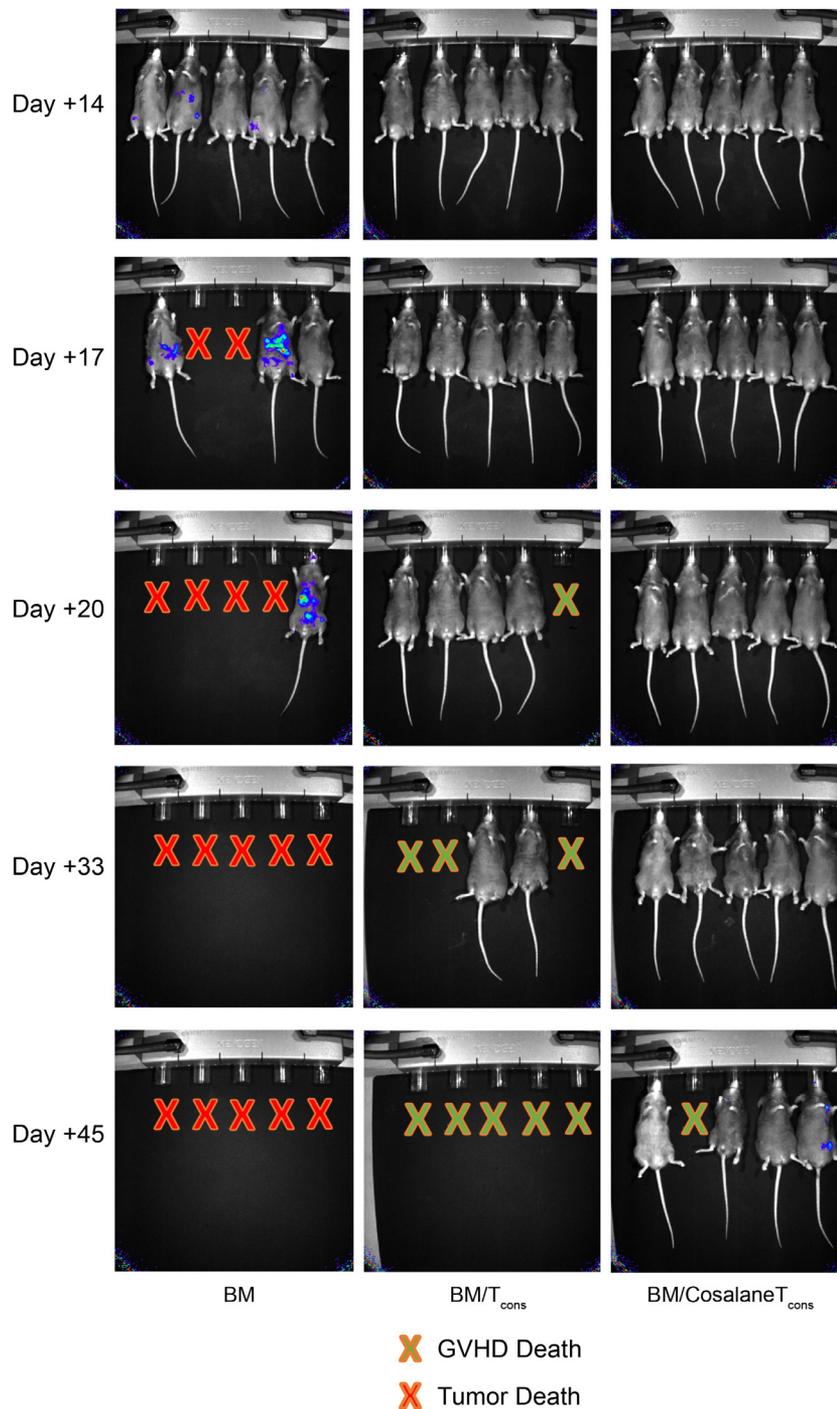
**Figure 5.** Cosalane functions in a CCR7-associated manner and alters donor T<sub>cons</sub> trafficking into SLT early after transplantation. (A and B) BALB/c mice were lethally irradiated on day -1. On day 0, recipients were administered  $5 \times 10^6$  TCD B6 BM cells with or without  $3 \times 10^6$  CCR7<sup>-/-</sup> B6 T<sub>cons</sub>. Donor T<sub>cons</sub> were incubated ex vivo with DMSO vehicle or cosalane diluted in normal saline at 15  $\mu$ g/mL. The cells were then mixed with untreated TCD B6 BM cells and administered immediately to recipient mice by tail vein injection. Mice were then followed for survival and scored for aGVHD. BM only, n = 2; all other treatment groups, n = 6. In (A),  $P > .05$  for survival curve comparison between BM with CCR7<sup>-/-</sup> T<sub>cons</sub>/DMSO and BM with CCR7<sup>-/-</sup> T<sub>cons</sub>/cosalane groups. In (B),  $P > .05$  for aGVHD score comparison between BM with CCR7<sup>-/-</sup> T<sub>cons</sub>/DMSO and BM with CCR7<sup>-/-</sup> T<sub>cons</sub>/cosalane groups on day +24. (C and D) BALB/c mice underwent transplantation as in (A) and (B); however, donor CCR7<sup>-/-</sup> B6 T<sub>cons</sub> were incubated ex vivo with DMSO vehicle or cosalane diluted in normal saline at 30  $\mu$ g/mL. BM only, n = 2; all other treatment groups, n = 9. In (C),  $*P = .0481$  for survival curve comparison between BM with CCR7<sup>-/-</sup> T<sub>cons</sub>/DMSO and BM with CCR7<sup>-/-</sup> T<sub>cons</sub>/cosalane groups. In (D),  $P > .05$  for aGVHD score comparison between BM with CCR7<sup>-/-</sup> T<sub>cons</sub>/DMSO and BM with CCR7<sup>-/-</sup> T<sub>cons</sub>/cosalane groups on day +67. (E) B6D2 mice were lethally irradiated on day -1. On day 0, recipients were administered  $5 \times 10^6$  eGFP<sup>+</sup> B6 T<sub>cons</sub>. Donor T<sub>cons</sub> were incubated ex vivo with DMSO vehicle or cosalane diluted in normal saline at 15  $\mu$ g/mL. Recipient mice were killed after 36 hours, and lymphoid organs were subsequently harvested and homogenized. Total eGFP levels were then determined by ELISA. n = 6 per treatment group.  $*P = .0043$  for spleen comparison between DMSO and cosalane groups by Mann-Whitney test;  $**P = .0022$  for ILN comparison;  $***P = .0022$  for MLN comparison.

P815 murine mastocytoma (H-2<sup>d</sup>) leukemia model. Here B6D2 mice were lethally irradiated and then given  $3 \times 10^6$  TCD B6 BM cells containing 25,000 luciferase-transfected P815 cells with or without  $4 \times 10^6$  B6 T<sub>cons</sub> to drive a GVL response. One-half of those mice receiving BM/tumor plus T<sub>cons</sub> received T<sub>cons</sub> incubated with DMSO, and the other half received cosalane-treated T<sub>cons</sub>. Recipients were subsequently evaluated by serial in vivo imaging to monitor tumor growth in each treatment group. In most instances, recipient mortality could be reasonably attributed to tumor versus aGVHD based on the tumor burden by imaging and clinical aGVHD scores at the time of death. As depicted in Figure 6, all the mice given BM cells plus tumor without T<sub>cons</sub> demonstrated aggressive tumor growth,

with 100% malignancy-related mortality. All the mice given BM/tumor plus DMSO-treated T<sub>cons</sub> (BM/T<sub>cons</sub>) rejected the P815 inoculum but developed aggressive aGVHD. In contrast, the mice given BM/tumor plus cosalane-treated T<sub>cons</sub> (BM/cosalane T<sub>cons</sub>) demonstrated substantial suppression of tumor growth and the longest overall survival times.

In GVL transplantation experiments, the aGVHD control group (BM/tumor/DMSO-treated T<sub>cons</sub> in this instance) frequently succumbs to lethal aGVHD before any tumor growth can occur. As a result, it is often difficult to compare longer-term GVL effects between these mice and mice given BM/T<sub>cons</sub> plus an anti-aGVHD therapy (BM/tumor/cosalane-treated T<sub>cons</sub>). To address this, we performed a similar experiment but

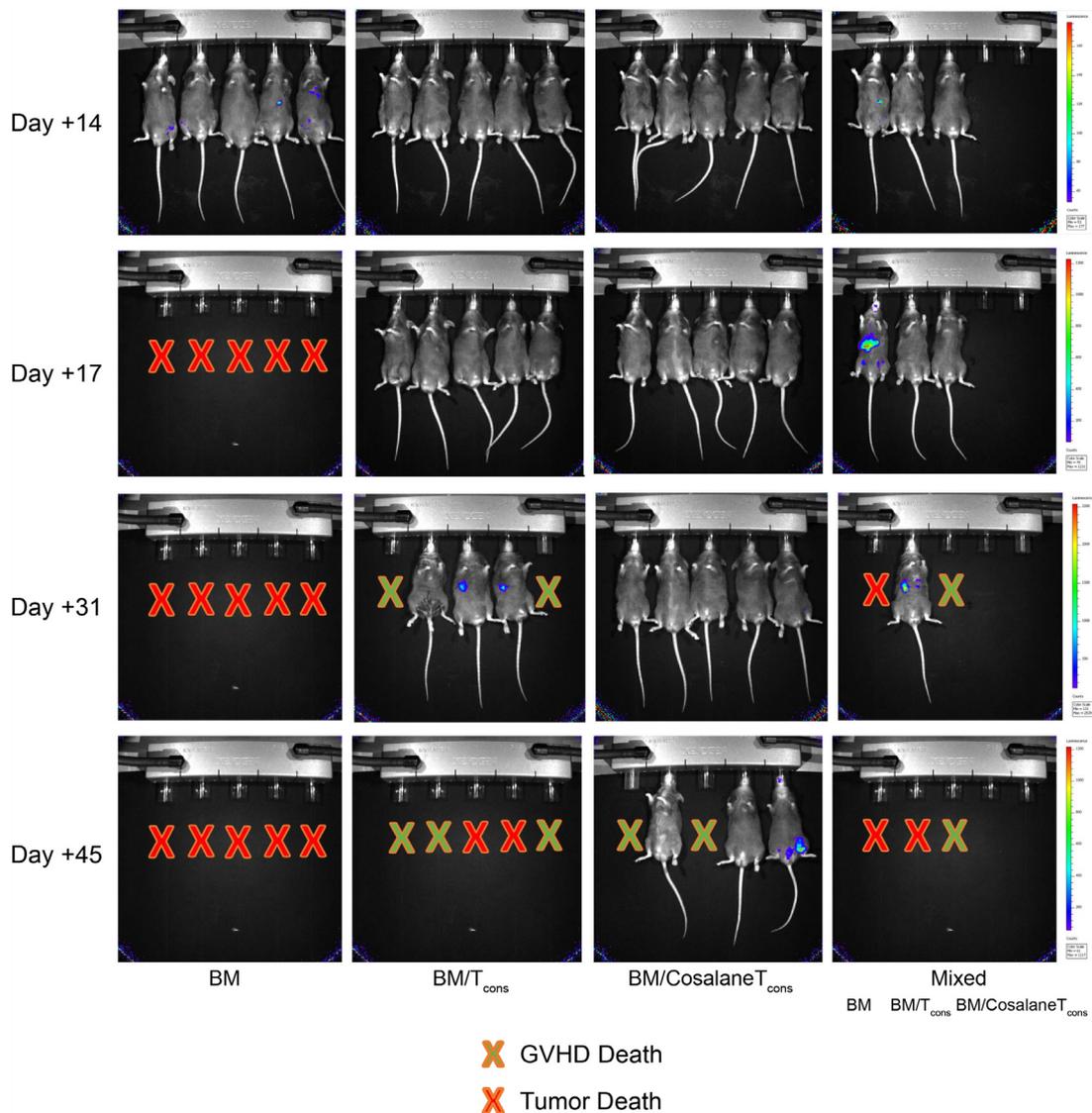
-1. On day 0, recipients were administered  $3 \times 10^6$  TCD eGFP<sup>-</sup> B6 BM cells plus  $4 \times 10^6$  eGFP<sup>+</sup> B6 T<sub>cons</sub>. Donor T<sub>cons</sub> were incubated ex vivo with DMSO vehicle or cosalane diluted in normal saline at 15  $\mu$ g/mL. The cells were then mixed with untreated TCD B6 BM cells and administered immediately to recipient mice by tail vein injection. Recipient mice were subsequently killed on day +7 (C) or day +14 (D). Lymphoid organs (left panels) and aGVHD target organs (right panels) were subsequently harvested and homogenized. Total eGFP levels were then determined using an anti-eGFP ELISA. n = 7 per treatment group. In (C),  $P > .05$  for all pairwise comparisons on day +7. In (D),  $*P = .0003$  for colon comparison between DMSO and cosalane groups by the Mann-Whitney test;  $**P = .0006$  for liver comparison. (E and F) B6D2 mice underwent transplantation as in (C) and (D), and their organs were removed and homogenized on day +7 (E) or day +14 (F). Total IFN- $\gamma$  (left panels) and TNF (right panels) was then determined by ELISA. n = 7 per treatment group. In (E),  $*P = .038$  for total TNF comparison in the liver on day +7 by the Mann-Whitney test. In (F),  $*P = .0015$  for total IFN- $\gamma$  comparison in the colon on day +14;  $**P = .0002$  for total TNF comparison in the colon on day +14.



**Figure 6.** Cosalane-treated T<sub>cons</sub> remain capable of GVL effects. B6D2 mice were lethally irradiated on day -1. On day 0, recipients were administered  $3 \times 10^6$  TCD B6 BM cells and  $2.5 \times 10^4$  luciferase<sup>+</sup> P815 murine mastocytoma cells (H-2<sup>d</sup>) with or without  $4 \times 10^6$  B6 T<sub>cons</sub>. Donor T<sub>cons</sub> were incubated ex vivo with DMSO vehicle or cosalane diluted in normal saline at 15  $\mu$ g/mL. The cells were then mixed with untreated BM/tumor cells and administered immediately to recipient mice by tail vein injection. Mice were then serially imaged twice weekly following i.p. injection of luciferin. Selected imaging points are depicted. Recipient mortality was attributed to tumor versus aGVHD based on the tumor burden by imaging and clinical aGVHD scores at the time of death. Exposure time, 4 seconds for all panels. n = 5 per treatment group.

reduced the dose of T<sub>cons</sub> in the DMSO control group by 50%. Specifically, mice received  $3 \times 10^6$  TCD BM cells plus 25,000 P815 cells, BM/tumor plus  $2 \times 10^6$  DMSO-treated T<sub>cons</sub>, or BM/tumor plus  $4 \times 10^6$  cosalane-treated T<sub>cons</sub>. As shown in Figure 7, all mice given BM alone once again died from malignancy. Three of the 6 mice given  $2 \times 10^6$  DMSO T<sub>cons</sub> died from malignancy, and 3 died from aGVHD. Three of the 6 mice given

$4 \times 10^6$  cosalane-treated T<sub>cons</sub> died from aGVHD, and none succumbed to malignancy. Thus, in this experiment, cosalane-treated T<sub>cons</sub> generated an aGVHD response roughly equivalent to one-half as many untreated T<sub>cons</sub> while simultaneously producing a stronger GVL effect. These data indicate that cosalane was able to separate aGVHD effects from GVL effects in this model system.



**Figure 7.** Cosalane treatment of donor T<sub>cons</sub> separates aGVHD from GVL effects in a P815 tumor model. B6D2 mice were lethally irradiated on day -1. On day 0, recipients were administered  $3 \times 10^6$  TCD B6 BM cells and  $2.5 \times 10^4$  luciferase<sup>+</sup> P815 murine mastocytoma cells. Some recipients also received  $2 \times 10^6$  DMSO-treated B6 T<sub>cons</sub> or  $4 \times 10^6$  cosalane-treated B6 T<sub>cons</sub> (15  $\mu$ g/mL). Mice were then serially imaged twice weekly following i.p. injection of luciferin. Selected imaging points are depicted. Exposure time, 4 seconds for all panels. The “mixed” cage on the far right contained one mouse each of the BM/P815 only, BM/P815 plus DMSO T<sub>cons</sub>, and BM/P815 plus cosalane T<sub>cons</sub> groups; n = 6 per treatment group.

## DISCUSSION

Cosalane was originally developed as an HIV therapeutic. The drug initially appeared to be quite promising, with activity against multiple strains of resistant HIV and a range of enveloped viruses relevant to the HSCT setting, including cytomegalovirus and herpes simplex virus [16]. Cosalane has never been used clinically, however, likely owing to several important characteristics of the molecule. Cosalane exhibits very poor oral bioavailability [11], which generally precludes its use as a viable anti-HIV therapy. Furthermore, although cosalane can be successfully administered i.v., it exhibits high albumin avidity and a propensity to accumulate within the liver [11,17]. Despite these drawbacks, however, the HSCT setting represents a unique clinical scenario in which cosalane could be effectively used. Specifically, HSCT allows for the ex vivo manipulation of donor cells before administration. Thus, the bioavailability and pharmacokinetic concerns that thus far have limited cosalane's clinical translation would be much less of an issue.

At this time, we envision that cosalane could be applicable to the stem cell transplantation field in one of several ways. First, cosalane could be used as aGVHD prophylaxis at the time of HSCT. Based on our data, it would appear that an entire hematopoietic stem cell product could be safely treated with cosalane without any detrimental effects on early engraftment or long-term BM persistence. Second, because cosalane diminishes aGVHD by acting primarily on donor T<sub>cons</sub>, it could be used to limit the aGVHD potential of donor lymphocyte infusion (DLI) administered to boost donor chimerism and/or stave off early malignancy recurrence. Our own data suggest that cosalane selectively limits the ability of donor T<sub>cons</sub> to mediate aGVHD effects while appearing to spare their GVL potential. Although a precise mechanism for this separation of immune effects is not entirely clear, we suspect that the difference is primarily spatial in nature. Specifically, cosalane ultimately limits the accumulation of alloreactive T<sub>cons</sub> within aGVHD target organs without affecting their intrinsic activation,

expansion, or ability to mediate inflammatory cytokine production. Early after transplantation, cosalane does reduce donor  $T_{con}$  accumulation within host lymphoid tissues, important potential sites for leukemia growth. This effect is transient, however, and presumably of insufficient duration to result in significant tumor escape in our P815 GVL model.

Cosalane came to our group's attention during a search for small-molecule antagonists of CCR7, a chemokine receptor critical for aGVHD pathogenesis [5]. Nevertheless, cosalane's mechanism of action in attenuating aGVHD is incompletely understood. Previously, we demonstrated that  $CCR7^{-/-}$   $T_{cons}$  have an impaired ability to traffic to and expand within host SLTs. This in turn results in reduced  $T_{con}$  accumulation within the colon and liver by the second post-transplantation week. In the present study, cosalane-treated donor  $T_{cons}$  similarly demonstrated an impaired ability to traffic into host SLTs early after transplantation and accumulated to a lesser degree within gastrointestinal and hepatic tissues by post-transplantation day +14. Furthermore, studies using  $CCR7^{-/-}$   $T_{cons}$  showed that the compound is considerably less effective in limiting aGVHD when donor  $T_{cons}$  lack this particular receptor. Collectively, these data suggest that cosalane functions in a CCR7-associated manner. Nevertheless, several of our findings imply activity against other receptors beyond CCR7. Cosalane's effects on  $T_{con}$  trafficking into SLTs were rather modest, with treated and untreated cells accumulating to similar degrees within host ILNs, MLNs, and spleen by post-transplantation day +7. Furthermore, cosalane demonstrated some residual efficacy in limiting aGVHD even when donor  $T_{cons}$  were knocked out at CCR7.

As noted above, cosalane was originally developed as an antiviral agent; however, it was also shown to block CCR1-dependent chemotaxis in response to CCL5 (RANTES) [18]. This is potentially quite relevant to our own findings, because CCR1 was previously shown to be critical for gastrointestinal aGVHD in mouse transplantation models [19]. Cosalane's effects on other chemokine receptors are not well described and will be the focus of ongoing mechanistic studies. Nevertheless, based on currently available evidence, we suspect that cosalane limits aGVHD through its effects on CCR7 and 1 or more inflammatory chemokine receptors, which may include CCR1.

Cosalane is a poor membrane penetrator and appears to imbed in the outer leaflet of artificial phospholipid bilayers by way of its cholestane moiety [20]. Thus, given its negatively charged carboxylic head groups, it is possible that cosalane could exert its effects at the cell surface via completely nonspecific charge alterations, resulting in impaired cell adhesion, changes in cell shape, or generally impaired receptor signaling. Alternatively, the cholestane portion of the molecule conceivably could modify membrane fluidity and thus affect cell function in another nonspecific manner. Multiple findings would seem to argue against either possibility, however. In previous work, we examined the ability of a range of different cholesterol derivatives to block CCR7-dependent chemotaxis. All were found to be inactive, arguing against a nonspecific sterol effect on the membrane. Furthermore, we demonstrated that cosalane itself was approximately 10-fold more active against CCL19 compared with CCL21, indicating that the nature of the particular receptor ligand is important for the compound's action [5]. Similarly, in previous work describing cosalane's ability to block CCR1, the identity of the chemokine agonist critically influenced the compound's efficacy. Specifically, cosalane blocked CCR1-dependent chemotaxis in response to CCL5 but was inactive against the receptor's other 2 ligands, CCL3 and CCL4<sup>18</sup>. Moreover, in the present study, cosalane's ability

to prevent aGVHD after HSCT appeared to be linked to CCR7. Collectively, these findings indicate a complex range of activities that likely extend beyond a single target but appear to be confined to a limited number of particular receptor/ligand pairs.

We believe that our present study has several strengths. First, to our knowledge, it is the first study to demonstrate efficacy of cosalane in a relevant preclinical animal model against any disease process. Second, it is the first to demonstrate that a CCR7 small-molecule antagonist can limit aGVHD. Third, we used two separate murine allogeneic HSCT models, as well as a xenogeneic system, to evaluate cosalane's anti-aGVHD effects. This allowed us to realistically conclude that the compound's actions are not strain-specific, and that its benefits are not limited to murine immune cells.

Nonetheless, this study has several limitations. Cosalane and its derivatives are not commercially available, and thus drug supply was a frequent issue. As a result, our n values are somewhat small for some of the transplantation work. Furthermore, as described above, we are only able to describe a partial mechanism for cosalane's activity in the HSCT setting at the present time.

In summary, the existing antiviral drug cosalane demonstrates an intriguing ability to limit aGVHD while appearing to generally spare GVL effects when applied to donor  $T_{cons}$  before transplantation. Although we are cautiously optimistic about cosalane's therapeutic potential, additional work is needed to develop a better understanding of the compound's mechanism (s) of action and its off-target effects. These studies will be critical before any attempt is made to advance the drug to early-phase clinical trials.

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