

The novel combination of sirolimus and bortezomib prevents graft-versus-host disease but maintains the graft-versus-leukemia effect after allogeneic transplantation

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Citation: Caballero-Velázquez T, Sánchez-Abarca LI, Gutierrez-Cosío S, Blanco B, Calderon C, Herrero C, Carrancio S, Serrano C, del Cañizo C, San Miguel JF, and Pérez-Simón JA. The novel combination of sirolimus and bortezomib prevents graft-versus-host disease but maintains the graft-versus-leukemia effect after allogeneic transplantation. Haematologica 2012;97(9):1329-1337. doi:10.3324/haematol.2011.058677

Online Supplementary Design and Methods

Cell cultures

Peripheral blood mononuclear cells (PBMC) from volunteer donors' buffy coats were isolated by density gradient centrifugation using Ficoll-Paque solution and allowed to adhere to the tissue culture dish (BD, Franklin Lakes, NJ, USA). After 2 h at 37°C non-adherent cells were collected, washed and resuspended in a culture medium consisting of RPMI 1640 L-glutamine (2 mM), penicillin (100 UI/mL) and streptomycin (10 mg/mL) plus 10% human AB serum (Sigma). More than 90% of the monocytes / macrophages were eliminated during monitoring by flow cytometry (*data not shown*) and T cells were further purified using anti-CD3 magnetic beads (Miltenyi Biotec, Auburn, CA, USA) as required.

Activation analysis

We seeded 2×10^5 non-adherent lymphocytes per well in 96-well plates and cultured: a) in medium alone; b) stimulated with plate-bound anti-CD3 (5 µg/mL) plus soluble anti-CD28 (2.5 µg/mL); or c) stimulated in a mixed lymphocyte culture. To do this, 1×10^5 non-adherent lymphocytes are mixed and co-cultured with 1×10^5 irradiated lymphocytes from a second donor.

Different concentrations of sirolimus, bortezomib or a combination of the two (at doses of 0.5 nM and 5 nM of sirolimus and 100 nM of bortezomib for the combination studies) were added. Activation assays were performed on the second day of culture. Cells were stained using the following four-color combination of MoAbs: anti-CD25-FITC / anti-cytoplasmic IFN γ -PE / anti-CD4-PerCP-Cy5.5 / anti-CD40L-allophycocyanin, all purchased from BDB, except for anti-CD25-PE which was obtained from Immunotech. For intracellular cytokine staining, brefeldin A (10 µg/mL) was added during the last 4 h before acquisition. Simultaneous staining for intracytoplasmic IFN γ and CD40L and surface antigens was performed using the IntraStain Fixation and Permeabilization kit (Dako Cytomation, Denmark). Data were acquired on a FACSCalibur flow cytometer (BDB) using the CellQuest program (BDB) and analyzed using Infinicyt software (Cytognos, Salamanca, Spain).

Proliferation and apoptosis assessment

For the proliferation assays, 2×10^5 non-adherent lymphocytes stained

with the green fluorescent dye PKH-67 (Sigma) were seeded in 96-well plates and cultured in medium alone or stimulated with plate-bound anti-CD3 (5 µg/mL) plus soluble anti-CD28 (2.5 µg/mL) MoAbs. Bortezomib and/or sirolimus were added to the culture as previously specified. After four days, cells were collected, stained with CD25-PE, 7-amino-actinomycin (7AAD) and anti-CD3-APC MoAbs and analyzed by flow cytometry. The ModFit program was used to calculate the percentage of resting (PKH^{high}CD25⁻) and proliferating cells.

To detect apoptosis, cell cultures were managed as previously described and the Annexin V-PE / 7 AAD apoptosis detection kit from BD Pharmingen was used. Briefly, a minimum of 5×10^5 T lymphocytes were washed and resuspended in Binding Buffer (1:10 diluted in H₂O), maintaining a cell concentration of 1×10^6 / mL. Annexin V-PE and 7-AAD 5 µL were each added and incubated for 15 min. In order to identify activated T lymphocytes, anti-CD25-FITC and anti-CD3-APC were also added. For every condition, 50,000 events were collected and analyzed. The percentage of annexin V-PE plus 7-AAD negative lymphocytes was calculated using the Paint-A-Gate Pro program (BD).

Cytokine assays

To measure the release of cytokines IL-2, IL-4, IL-6, IL-10, tumor necrosis factor (TNF)- α and interferon (IFN- γ) by stimulated T cells, we used the BD Human Th1/Th2 Cytokine CBA kit (BDB). The assays were performed according to the manufacturer's instructions on supernatant collected four days after stimulation with anti-CD3 (5 µg/mL) and anti-CD28 (2.5 µg/mL). For the Th1/Th2 cytokine CBA kit, 50 µL of supernatant were stained with the mixture of human cytokine capture bead suspension and the phycoerythrin (PE) detection reagent. After 3 h of incubation, samples were washed and then analyzed in a FACSCalibur (BDB) flow cytometer using the BD CBA program. Human Th1/Th2 cytokine standards provided with the kit were appropriately diluted and used in parallel with samples to prepare the standard curves.

Western blot analysis

We seeded 5×10^6 Jurkat T cells in 6-well plates (Corning, NY, USA) and these were stimulated with plate-bound anti-CD3 (5 µg/mL) and soluble anti-CD28 (2.5 µg/mL) mAbs (BD Biosciences). Either medium, sirolimus 5 nM, bortezomib 100 nM, or the combination sirolimus 5 nM + bortezomib 100 nM was added at the beginning of the culture. After

48 h cells were washed with PBS (Gibco) and lysed in ice-cold lysis buffer (140 mM NaCl, 10 mM EDTA, 10% glycerol, 1% Nonidet P-40, 20 mM Tris pH 7.0, 1 μ M pepstatin, 1 μ g/mL aprotinin, 1 μ g/mL leupeptin, 1 mM sodium orthovanadate). Samples were centrifuged at 13,000 rpm at 4°C for 10 min and supernatants were collected. Cell extracts were subjected to 10% SDS/PAGE and blotted onto PVDF membrane (Millipore). Membranes were incubated with rabbit anti-pAkt (1:500) (Cell Signaling), mouse anti-pErk (1:500) (Santa Cruz Biotechnology Inc.) and anti- α tubulin (Santa Cruz Biotechnology Inc.). Membrane-bound first-step antibodies were reacted with horseradish peroxidase-conjugated anti-rabbit (1:5000) or anti-mouse (1:1000) (Amersham™) and bands were visualized with an ECL Western Blotting Detection Kit (Amersham™).

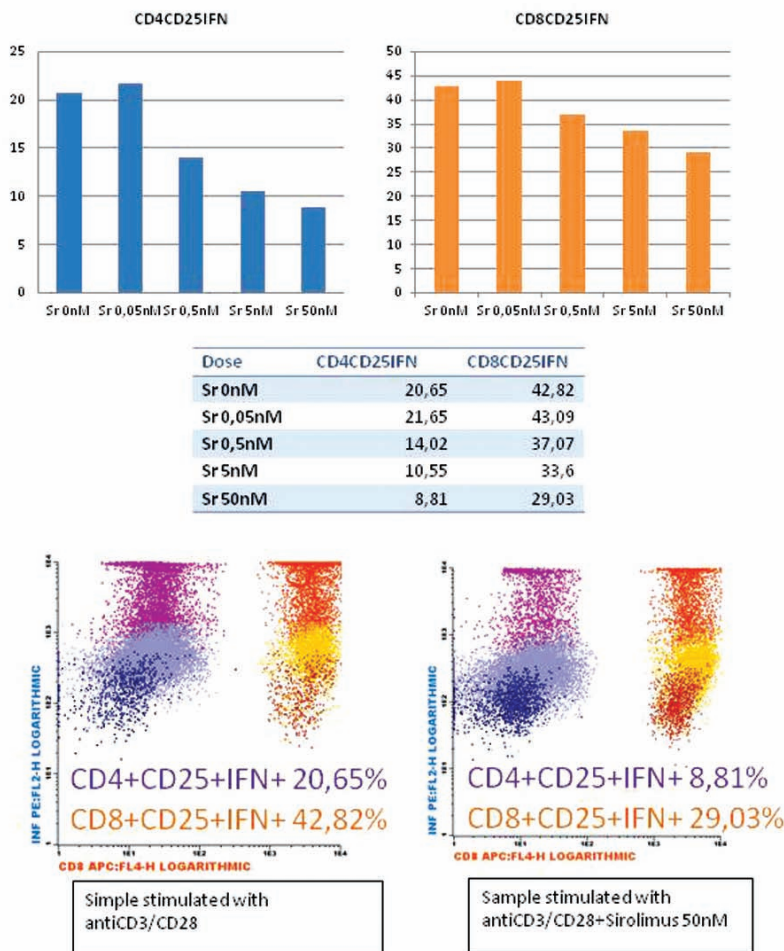
In Western blot assays, tubulin served as a loading control. pErk and pAkt band intensities were quantified using the Scion image program, normalized relative to the quantity of their respective tubulin bands, and expressed as fold change with respect to the untreated sample.

Statistical analysis

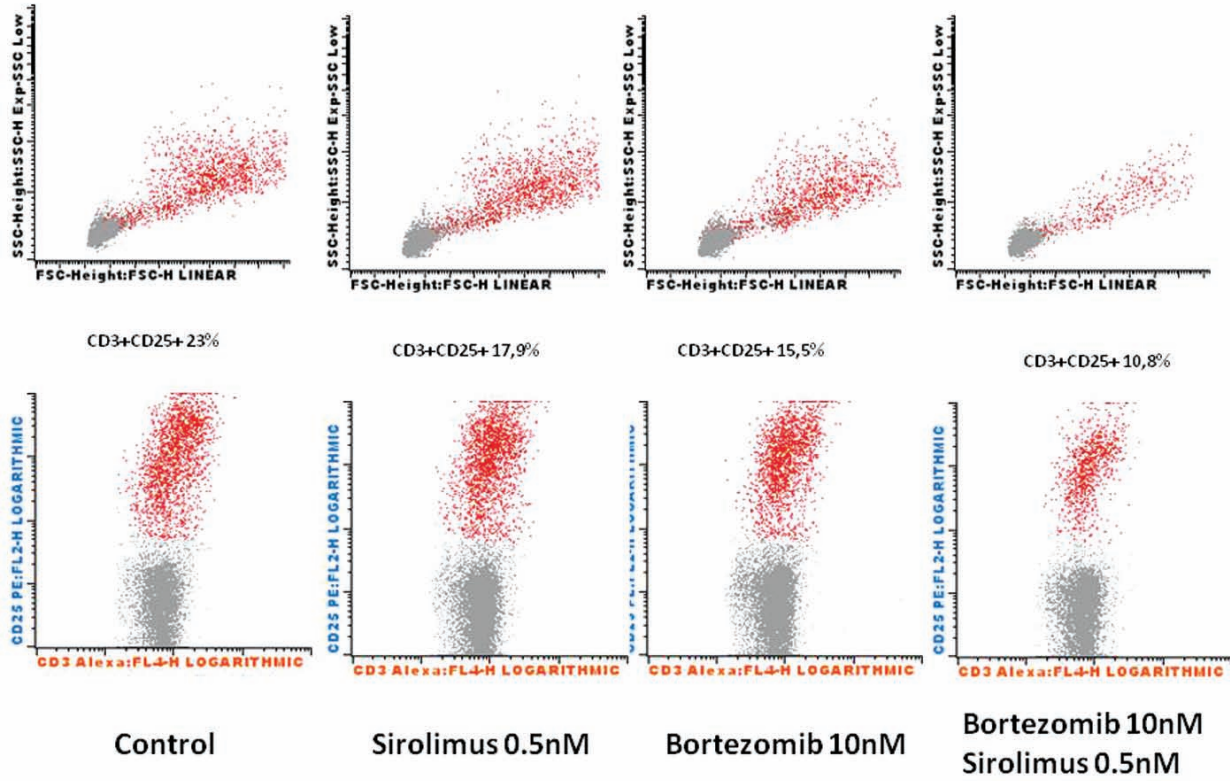
Mean values and their standard deviations, as well as the range and median, were calculated for each variable in SPSS (SPSS 11.0, Chicago, IL, USA). Groups were compared by analysis of variance with post-hoc Scheffé and Tukey tests performed to confirm differences between groups. A two-way measurement of repeated multiple analysis of variance (two-way MR-MANOVA) was made to compare the effect of the different drug doses within the different types of culture and to evaluate the effect of the drug on the signs of GVHD at different times. Kaplan-Meier product-limit estimates were used to evaluate the effect of the drug on survival. $P < 0.05$ was considered significant. Synergy between both drugs was evaluated with the Calcsyn software (Biosoft, Ferguson, MO, USA) based on the Chou–Talalay method.¹ This equation calculates a combination index (CI) with the following interpretation: CI > 1: antagonistic effect; CI = 1: additive effect; and CI < 1 synergistic effect.

References

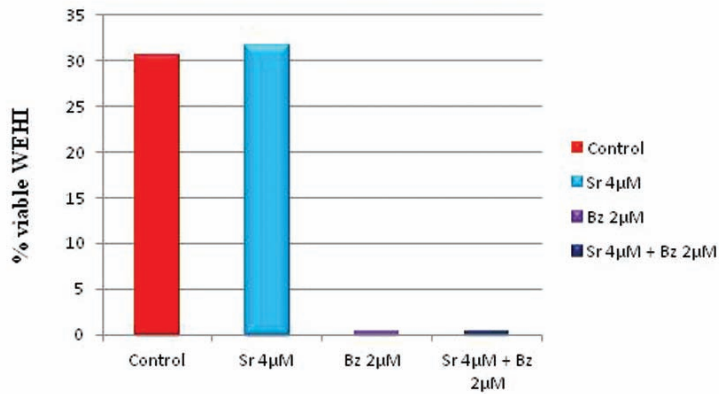
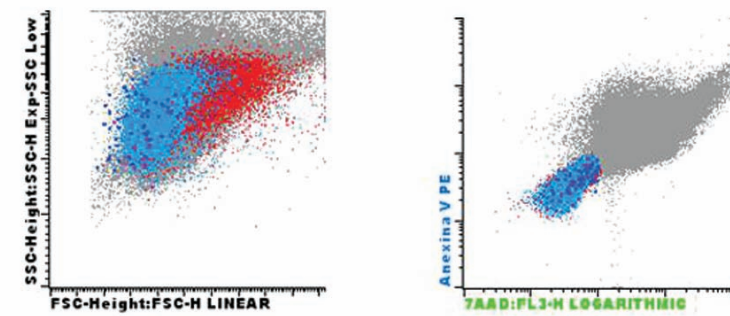
1. Chou TC, Talalay P Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme. Inhibitors. Adv Enzyme Regul. 1984;22:27–55.



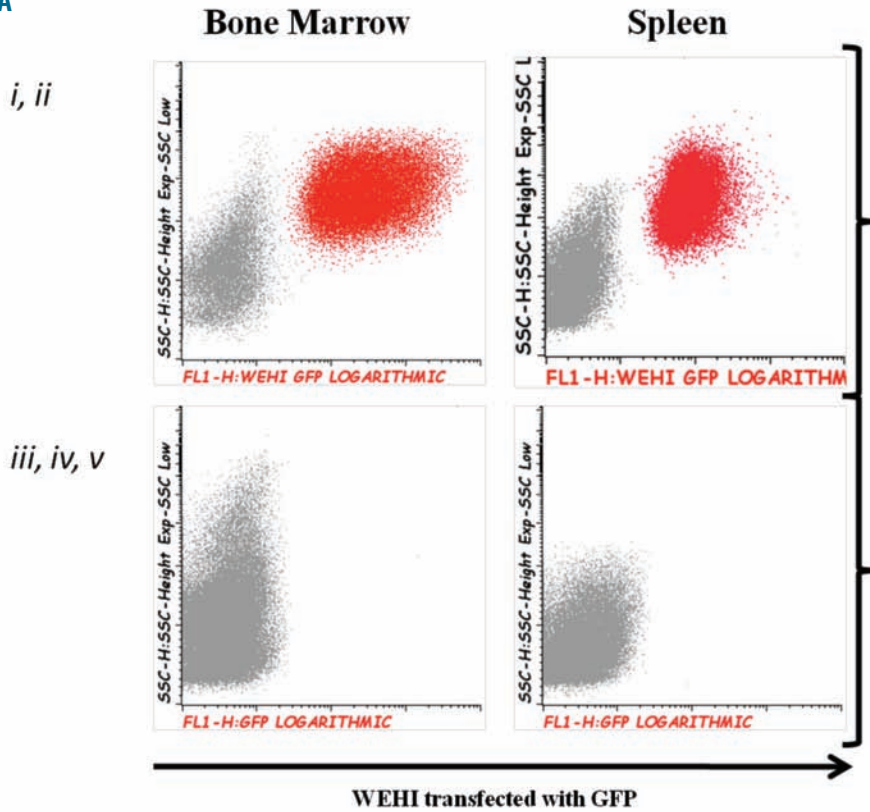
MLR C57BL6:BALBc 3:1



Online Supplementary Figure S2. Effect of Bz, Sr or both on activation of T cells after mixed culture of lymphocytes obtained from C57BL6 and BALB/C mice.



Online Supplementary Figure S3. Viability of WEHI cells after exposure to Bz, Sr or both.

A

Online Supplementary Figure S4. (A) Dot-plots showing bone marrow and spleen infiltration with WEHI cells transfected with GFP from BALB/c mice receiving 5×10^6 C57BL/6 BM cells after total body irradiation plus 5×10^4 WEHI cells either (i) on Day 0 without GVHD prophylaxis or (ii) with sirolimus from Day 0 to 12 post-transplant plus bortezomib on Days 0, 1 and 2 as GVHD prophylaxis plus WEHI cells on Day + 21. The same figures from bone marrow and spleen showing no infiltration from BALB/c mice receiving 5×10^6 C57BL/6 BM cells together with (iii) 5×10^4 WEHI on Day 0 plus 5×10^6 C57BL/6 splenocytes and GVHD prophylaxis with sirolimus plus bortezomib or (iv) 5×10^4 WEHI on Day 0 plus sirolimus and bortezomib without splenocytes or (v) 5×10^4 WEHI on Day 21 plus 5×10^6 C57BL/6 splenocytes and sirolimus plus bortezomib. (B) Kaplan-Meier curves representing overall survival in these groups. Samples were obtained four weeks after transplantation.

B