# Rapamycin is highly effective in murine models of immune-mediated bone marrow failure

**Xingmin Feng,[1] Zenghua Lin,[1,2] Wanling Sun,[1,3] Maile K. Hollinger,[1] Marie J. Desierto,[1] Keyvan Keyvanfar,[1] Daniela Malide,[4] Pawel Muranski,[1] Jichun Chen,[1] and Neal S. Young[1]**

[1]Hematology Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA; [2]Department of Hematology, Affiliated Hospital of Nantong University, Nantong, Jiangsu, China; [3]Department of Hematology, Xuanwu Hospital, Capital Medical University, Beijing, China and [4]Light Microscopy Core Facility, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA

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Correspondence: fengx2@nhlbi.nih.gov

# Supplemental Materials and Methods

## Blood counts, cell staining and flow cytometry

Blood was collected from the retro-orbital sinus into Eppendorf tubes containing EDTA. Complete blood count (CBC) was performed in a HemaVet 950 analyzer (Drew Scientific, Inc., Waterbury, CT). After euthanasia by $CO_2$, BM cells were extracted from tibiae and femurs, filtered through 95 µM nylon mesh, counted by a Vi-Cell counter (Beckman Coulter, Miami, FL), stained with antibody mixtures on ice for 30 minutes in FBS-supplemented RPMI 1640 (Life Technilogies), and fluorescent signal was acquired using BD FACSCanto II and BD LSRFortessa flow cytometers operated by FACSDiva software (Becton Dickson, San Diego, CA). In some experiments, stained BM and spleen cells were sorted using a FACSAria (Becton Dickson) in order to collect $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells for RNA extraction.

Monoclonal antibodies for murine CD3 (clone 145-2C11), CD4 (clone GK 1.5), CD8 (clone 53-6.72), CD11b (clone M1/70), CD25 (Clone 7D4), CD45R (clone RA3-6B2), CD45.1 (clone A20), CD45.2 (clone 104), CD48 (clone HM48-1), CD117 (c-Kit, clone 2B8), CD150 (SLAM, clone TC15-12F12.2), erythroid cells (clone Ter119), granulocytes (Gr1/Ly6-G, clone RB6-8C5), stem cell antigen 1 (Sca1, clone E13-161), CD11a, Granzyme B, and FoxP3 (Clone MF-14) were from Biolegend (San Diego, CA). Anti-mouse CD95 (Fas, clone Jo2), CD178 (FasL, clone MFL3), Ki67, CD44, and CD62L antibodies as well as annexin V apoptosis kits were purchased from BD Biosciences (San Diego, CA). Antibodies were conjugated to FITC, phycoerythrin (PE), PE-cyanin 5 (PE-Cy5), PE-cyanin 7 (PE-Cy7), allophycocyanin (APC) or brilliant violet 421 (BV421).

Regulatory T cells in the spleen were evaluated by flow cytometry. Briefly, the cells were first stained with surface markers, washed, resuspended in 1 mL cold Fix/Perm buffer (eBioscience), and incubated at 4°C for 1 hour. After washing with 2 mL of permeabilization buffer (eBioscience), cells were stained with FOXP3 antibodies at 4°C for 30 minutes in the dark. Finally, cells were washed with 2 mL permeabilization buffer and analyzed on BD LSRFortessa flow cytometers.

### Histology and confocal microscopy

Sternums from BMF CByB6F1 mice (5 Gy TBI + 5 $\times$ $10^6$ B6 LN cell infusion) were collected after animals were euthanized at day 14 post LN cell infusion. Immunofluorescence labeling and confocal imaging were performed using sternum whole mounts preparations, as previously described (19). Specimens were fixed with 4% paraformaldehyde at room temperature (RT) for 1 hr. After washing with PBS three times for 15 minutes, specimens were immediately stained for megakaryocytes with anti-mouse CD41-FITC (1:50, BD Biosciences) for 3 hours, followed by DAPI (1:1000 dilution; Invitrogen) for nuclei at RT for 40 minutes. All images were acquired by CLSM with a Zeiss LSM 510 confocal system (Confocal Zeiss MicroImaging).

### Cytokine measurement

Cytokines in plasma were measured using MILLIPLEX MAP Mouse Magnetic Bead Panel (EMD Millipore, Billerica, MA), according to the manufacturer's instructions.

### RNA isolation and gene expression analyses by PCR array

Total RNA was isolated from cultured T cells or sorted from BM with the RNeasy Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Then RNA was digested with RNase free DNase I (Qiagen) and assessed using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, Delaware). First strand cDNA was synthesized with 200 ng of total RNA using $RT^2$ First Strand Kit (Qiagen). Quantitative analyses of mRNA expression of T cell anergy and immune tolerance-related genes was performed using mouse PCR arrays (PAMM-074Z, Qiagen), according to the manufacturer's instructions.

**Protein extraction and immunoblotting**

Proteins from T cells were extracted with extraction reagents (Thermo Scientific, Rockford, IL) in the presence of a complete cocktail of protease inhibitors (Roche, Madison, WI) and quantified using the BCA Protein Assay Kit (Thermo Scientific). Proteins (20 μg) were separated in Novex 4-12% Tris-Glycine Mini Gel and electro-transferred onto 0.2 μm pore size PVDF membranes (Life Technologies, Grand Island, NY). Non-specific sites on membranes were blocked with 3% non-fat dry milk in 0.1% Tween 20/PBS. Anti-mTOR, S6K1, S6, AKT and their phosphorylated antibodies, anti-NFAT1, and β-action antibodies were used to detect their protein levels (Cell Signaling Technology). Immunoblotting was performed using enhanced chemiluminescence reagents in order to visualize immunoreactive proteins. The images were analyzed by FUJI software to quantify the intensities of each band relative to actin.

### Cell culture

Mouse LN cells (2.0 x $10^6$) were cultured in 1.0 ml of RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 ug/ml streptomycin (Invitrogen) in a 24-well tissue culture plate. To stimulate naïve T cells, anti-mouse CD3 (hamster-anti-mouse 145-2C11) and CD28 antibodies were used at concentrations of 1 μg/mL and 2 μg/mL, respectively, with or without cyclosporine and rapamycin. Proteins were extracted at 5 hours and 12 hours for Western blotting. T cell activation was evaluated by flow cytometry at day 1 and day 3, respectively. Mouse LN cells were labeled with the carboxyfluorescein succinimidyl ester (CFSE, Invitrogen), and stimulated with anti-CD3 (1 μg/ml) and CD28 antibodies (2 μg/ml), in the presence or absence of cyclosporine or rapamycin and cultured for 3 days. In case of regulatory T cell function analysis, FACS-sorted regulatory T cells were added to CFSE-labeled effector T cells at 1:1 ratio. T cell proliferation based on CFSE dye dilution was evaluated by flow cytometry.

### Hematopoietic progenitor assays

Three × $10^4$ BM mononuclear cells were cultured in 1 mL semisolid methylcellulose (containing IL3, IL6, SCF, and EPO). Colonies were cultured at 37°C with 5% $CO_2$, with a water dish to maintain optimal humidity. Colonies were counted at day 7.

## Supplemental Figures

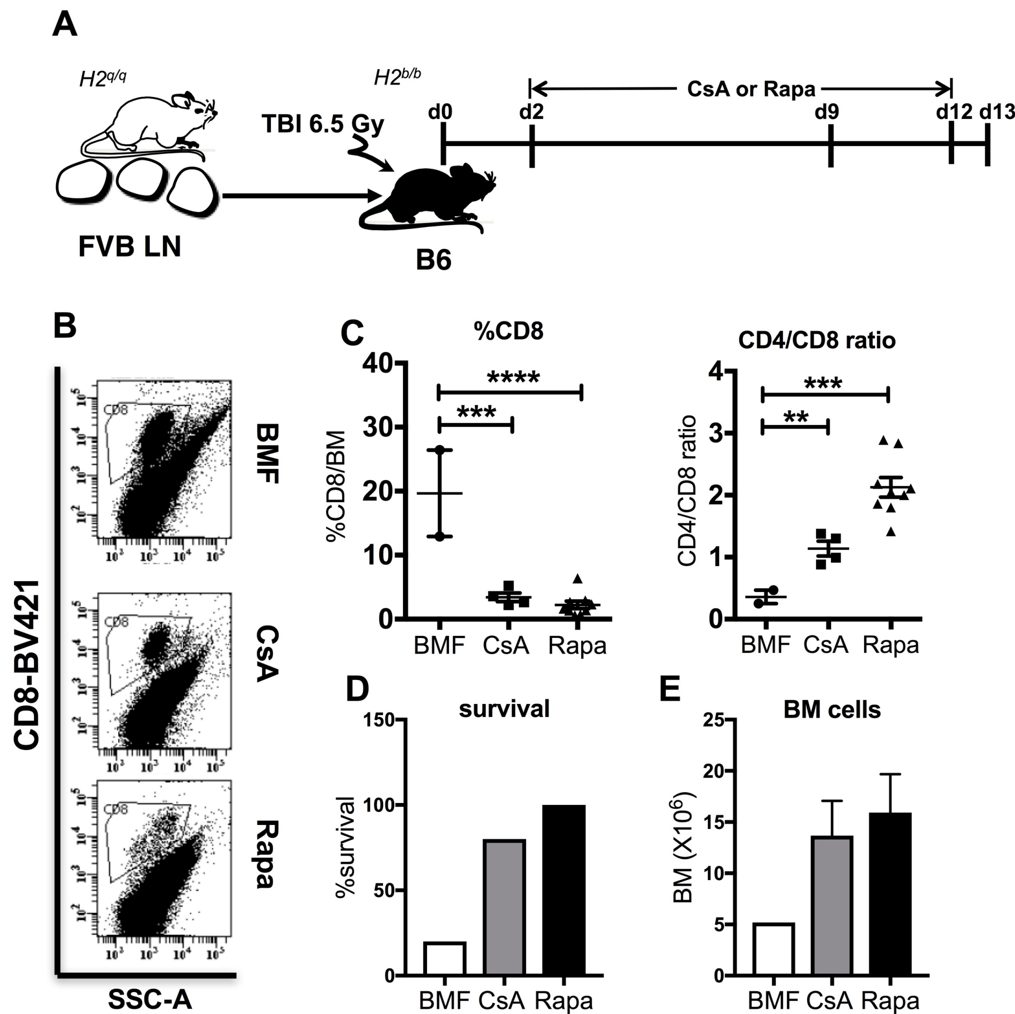


**Figure S1. Rapamycin provides effective treatment to B6 mice with immune-mediated BM failure.** (**A**) B6 mice irradiated with 6.5 Gys TBI were infused with $5 \times 10^6$ FVB LN cells to induce BM failure (BMF, n=8), some BMF mice received 11 days (d2-12) of CsA (50 mg/kg i.p, n=5) or Rapa (2 mg/kg, i.p., n=11). (**B**) BM CD8 T cell percentage was high in BMF mice, intermediate in CsA-treated BMF mice, and low in Rapa-treated BMF mice. (**C**) BMF mice with Rapa treatment had the lowest CD8 T cell percentage and highest CD4/CD8 T cell ratio in the BM relative to BMF and CsA-treated BMF mice. (**D**) Two of 8 BMF mice survived beyond day 10 for sample analyses, 4 of 5 CsA-treated BMF mice survived to day 13 while all 11 Rapa-treated BMF mice survived to day 13 for measurements. (**E**) Total BM cell recovery was higher in CsA-treated and Rapa-treated mice relative to the two survived BMF mice without treatment. **, p<0.01; ***, P<0.001; ****, p<0.0001. Rapa, rapamycin; CsA, cyclosporine; TBI, total body irradiation.

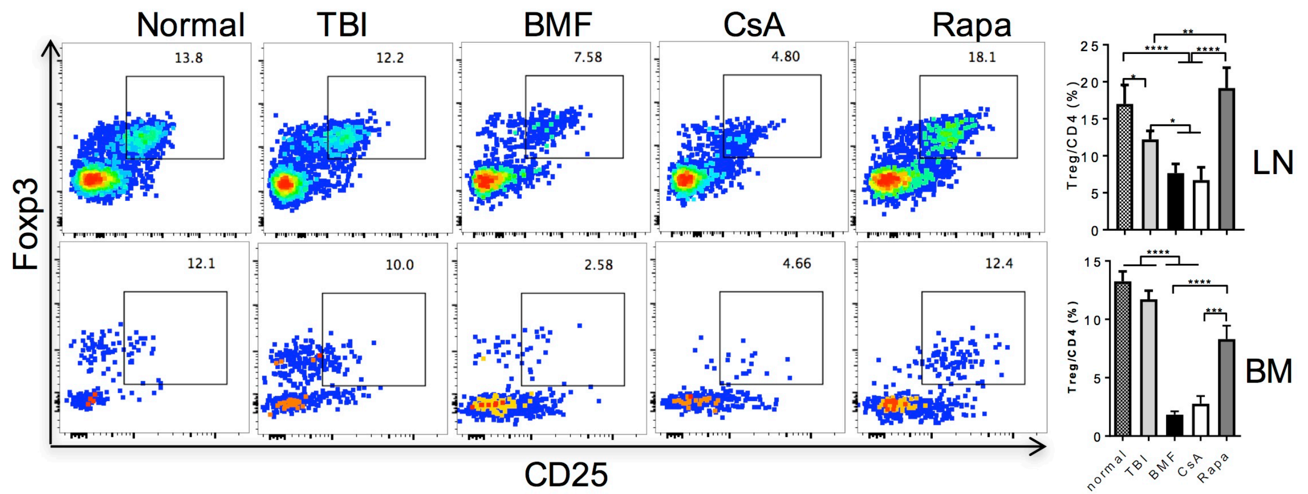


**Figure S2. Rapamycin expands regulatory T cells in lymph nodes and bone marrow.** CByB6F1 mice irradiated with 5 Gys TBI were infused with $5 \times 10^6$ B6 LN cells to induce BM failure (BMF, n=5), some BMF mice received 10 days of CsA (50 mg/kg i.p, n=5) or 12 days of Rapa (2 mg/kg, i.p., n=5). TBI (n = 5) and normal (n = 5) mice were used as controls. Regulatory T cells ($CD4^+CD25^+FoxP3^+$) in LN and BM were evaluated at day 13 post LN injection. **, p<0.01; ***, p<0.001; ****, p<0.0001. Rapa, rapamycin; CsA, cyclosporine; TBI, total body irradiation.