

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

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ABSTRACT

Acquired aplastic anemia, the prototypical bone marrow failure disease, is characterized by pancytopenia and marrow hypoplasia. Most aplastic anemia patients respond to immunosuppressive therapy, usually with anti-thymocyte globulin and cyclosporine, but some relapse on cyclosporine withdrawal or require long-term administration of cyclosporine to maintain blood counts. In this study, we tested efficacy of rapamycin as a new or alternative treatment in mouse models of immune-mediated bone marrow failure. Rapamycin ameliorated pancytopenia, improved bone marrow cellularity, and extended animal survival in a manner comparable to the standard dose of cyclosporine. Rapamycin effectively reduced Th1 inflammatory cytokines interferon- γ and tumor necrosis factor- α , increased the Th2 cytokine interleukin-10, stimulated expansion of functional regulatory T cells, eliminated effector CD8⁺ T cells (notably T cells specific to target cells bearing minor histocompatibility antigen H60), and preserved hematopoietic stem and progenitor cells. Rapamycin, but not cyclosporine, reduced the proportion of memory and effector T cells and maintained a pool of naïve T cells. Cyclosporine increased cytoplasmic nuclear factor of activated T-cells-1 following T-cell receptor stimulation, whereas rapamycin suppressed phosphorylation of two key signaling molecules in the mammalian target of rapamycin pathway, S6 kinase and protein kinase B. In summary, rapamycin was an effective therapy in mouse models of immune-mediated bone marrow failure, acting through different mechanisms to cyclosporine. Its specific expansion of regulatory T cells and elimination of clonogenic CD8⁺ effectors support its potential clinical utility in the treatment of aplastic anemia.

Introduction

Aplastic anemia (AA) is a disease of bone marrow (BM) failure characterized by pancytopenia and marrow hypocellularity. In most patients, this is due to immune attack of hematopoietic stem and progenitor cells (HSPCs) by auto-reactive T cells.¹ Standard immunosuppressive therapy (IST) with horse anti-thymocyte globulin (ATG) and cyclosporine A (CsA) is effective in 60-70% of AA patients, resulting in hematologic recovery. However, patients who have responded to IST often relapse after CsA withdrawal or are dependent on continued CsA administration in order to maintain blood counts.² The overall and complete response rates to immunosuppressive therapy have increased to almost 100% with the addition of the thrombopoietin mimetic eltrombopag, but relapse on discontinuation of CsA may be especially problematic in these patients.³ ATG and CsA appear to partially eliminate and functionally suppress activation of expanded CD8⁺ effector T-cell clones.⁴ However, oligoclonal are often not eliminated, and relapse is likely due to their reactivation and renewed destruction of HSPCs and precursors. In the clinic, therapeutic strategies to achieve tolerance are highly desirable in order to avoid compli-

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cations of recurrent pancytopenia that may require re-initiation of transfusions, hospitalizations for neutropenic fever, and control of chronic toxicity due to repeated interventions.

Human AA has been modeled in mice by adaptation of historic “runt disease” in which infusion of lymph node (LN) cells into recipients mismatched at MHC or minor histocompatibility (minor-H) antigen loci produced BM failure with severe pancytopenia and marrow hypoplasia that mimics human AA.^{5,6} Like human AA, treatment of murine BM failure in these models with CsA and other immunosuppressive agents ameliorates disease. These models have been used to test the plausibility of immune mechanisms suggested by the study of patients and patient cells.

In the search for an alternative and/or supplementary treatment for AA and BM failure, we turned our attention to rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR) pathway that has been used in a variety of animal models of human diseases, such as murine experimental allergic encephalomyelitis, nephritis, lupus erythematosus, and inflammatory bowel disease.⁷⁻¹² In the clinic, rapamycin is used to treat autoimmune hepatitis and uveitis,^{13,14} and to prevent rejection in solid organ and hematopoietic stem cell transplantation.¹⁵⁻¹⁷

In this study, we employed murine models to test efficacy of rapamycin as a therapy for immune-mediated BM failure, based on its well-characterized immunosuppressive activity and its tolerogenic role in organ transplantation, and aimed at its application as prophylaxis or salvage treatment of AA patients at risk of relapse. We were especially interested in comparing the mechanisms of action between rapamycin and CsA.

Methods

Animals, induction of BM failure, and immunosuppressive therapies

Inbred C57BL/6 (B6) and FVB/N (FVB), congenic C.B10-H2b/LilMcd (C.B10) and B6-Cg-Tg(CAG-DsRed*MST)1Nagy/J (DsRed), and hybrid CByB6F1/J (CByB6F1) mice were all originally obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and were bred and maintained in National Institutes of Health animal facilities (Bethesda, MD, USA) under standard care and nutrition. All animal studies were approved by the Animal Care and Use Committee at the National Heart, Lung, and Blood Institute.

Induction of immune-mediated BM failure was performed as previously reported.^{5,6} In brief, LN cells from B6 or DsRed donors were homogenized, washed, filtered and intravenously injected into sex-matched CByB6F1 or C.B10 recipients pre-irradiated with 5 Gy of total body irradiation (TBI) 4-6 hours (h) earlier (or LN cells from FVB donors were infused into 6.5-Gy-pre-irradiated B6 recipients). Mice were used at 8-12 weeks of age in all experiments. In most experiments, animals were bled and euthanized 12-18 days (d) later to obtain tissue for histology and cytology. Some mice were maintained long-term to measure survival under normal animal care conditions.

Rapamycin was obtained from LC Laboratories (Woburn, MA, USA), dissolved in pure ethyl alcohol (The Warner-Graham Company, Cockeysville, MD, USA) at 50 mg/mL for storage at -30°C, diluted to 200 µg/mL with a 5% PEG-400 (polyethylene glycol MW 400, Sigma-Aldrich, St. Louis, MO, USA) and 5% Tween-80 (Sigma-Aldrich) solution before use,¹⁸ and administered through intraperitoneal injection (i.p.) once daily for 5-13 d at 2

mg/kg. CsA (NDC 0078-0109-01, Novartis Pharmaceutical Corporation, East Hanover, NJ, USA) was diluted in Iscove's Modified Dulbecco's Medium (IMDM) to 5 mg/mL and injected (i.p.) into animals at 50 mg/kg body weight once daily for 5-10 d. Both drugs were filtered through a 0.22 µm syringe-driven Millex-GS filter (Millipore Corporation, Billerica, MA, USA) before injection.

Methods regarding flow cytometry analyses, regulatory T-cell isolation and functional analysis, cytokine measurement, transcriptome assay, cell culture, immunoblotting, and hematopoietic progenitor assays are described in the *Online Supplementary Appendix*.

Statistical analysis

Data obtained from complete blood count, BM cell counting and flow cytometry were analyzed by unpaired t-test, variance analyses, and multiple comparisons using GraphPad Prism statistical software. Data are presented as means with standard errors. The differences in survival among different groups of animals were evaluated by log rank test. $P < 0.05$ was considered statistically significant.

Results

Rapamycin ameliorates pancytopenia and BM hypoplasia in AA mice

To evaluate the potential prophylactic effect of rapamycin in immune-mediated murine BM failure, we first infused LN cells from B6 donors into MHC-mismatched, pre-irradiated CByB6F1 recipients (Figure 1A) and induced severe BM failure in recipient animals with dramatically decreased white blood cells (WBCs), red blood cells (RBCs), platelets (PLT), and total BM cells on d13 after LN cell infusion (Figure 1B). Treatment with rapamycin at 2 mg/kg/day for 13 d (d0-12) preserved BM cellularity and ameliorated peripheral blood pancytopenia (Figure 1B). Relative to TBI and normal controls, BM failure mice had marked expansion of T cells in the BM, while rapamycin eliminated most BM-infiltrating T cells, especially CD8⁺ T cells (Figure 1C). Using DsRed mice on the B6 background as donors, we found that BM-infiltrating lymphocytes were essentially donor-derived (Figure 1D), consistent with previous observations.¹⁹ In TBI and normal control mice, BM had dense DAPI staining to show high cellularity with megakaryocytes scattered throughout the BM cavity; no DsRed LN cells were present (Figure 1D, upper panel). In untreated BM failure mice, the marrow cavity was infiltrated with DsRed LN cells with reduced BM cellularity and no visible megakaryocytes. In contrast, rapamycin-treated BM failure mice had very few DsRed LN cells in the BM, much higher BM cellularity, and abundant megakaryocytes (Figure 1D, lower panel).

To assess whether rapamycin has comparable efficacy to CsA, a standard treatment for AA patients and murine BM failure models (50 mg/kg, i.p. d0-9),^{5,6} we tested different regimens of rapamycin and found that all rapamycin treatments were effective, with the d0-12 treatment group produced optimal improvements in BM cellularity and peripheral blood counts relative to the standard CsA d0-9 treatment group (Figure 2A). In parallel survival experiments, untreated BM failure mice died within three weeks after LN cell infusion while short-term treatments with rapamycin d0-4 were ineffective and animals

died within one month (Figure 2B). In contrast, rapamycin treatment regimens of d0-9, d0-12 and d3-12 were all effective and maintained animal survival to more than 100 days, comparable to CsA d0-9 treatment ($P < 0.0001$ vs. untreated BMF) (Figure 2B). We measured complete blood counts (CBCs) in TBI, rapamycin d0-9 and d0-12 groups at 28, 42 and 100 d. RBC and PLT counts in rapamycin-treated mice recovered to similar levels as in TBI controls at all

three time points; recovery of WBC was slower until 42 days but reached TBI control levels at 100 d. BM cellularity in rapamycin-treated mice increased to similar levels as in TBI controls when the mice were euthanized and evaluated at 100 d (Figure 2C).

To mimic human disease treatment, we further delayed initiation of rapamycin injection until d5 (Rapa-D5) or d7 (Rapa-D7) post LN infusion and found that these delayed

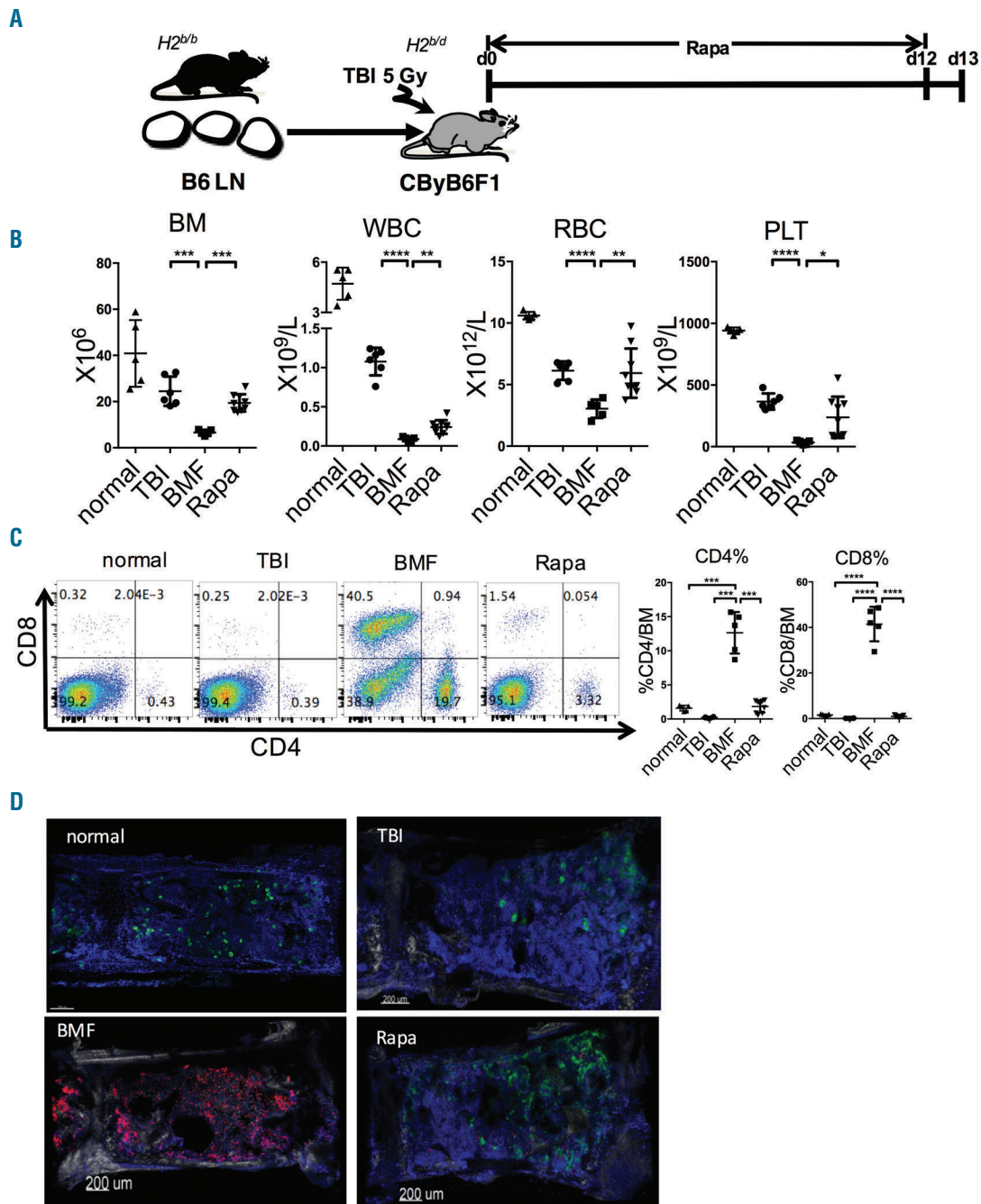


Figure 1. Rapamycin ameliorates bone marrow (BM) failure (BMF). (A) CByB6F1 mice received 5 Gy total body irradiation (TBI) and infusion of 5×10^6 B6 lymph node (LN) cells to induce BMF. Some BMF mice received daily rapamycin injections at 2 mg/kg/day for 13 days (d). Mice were bled and euthanized at d13 for analyses. (B) BM nucleated cell number, white blood cell (WBC), red blood cell (RBC) and platelets (PLT) counts in normal (n=5), TBI (n=6), BMF (n=5), and rapamycin-treated mice (n=9). (C) Flow cytometric analyses of CD8⁺ and CD4⁺ T cells infiltrating BM. (D) Confocal images of sternums. Specimens were fixed with 4% paraformaldehyde and subsequently stained for megakaryocytes with anti-mouse CD41-FITC (green) and for nuclei with DAPI (blue), respectively. DsRed LN cells are visible (red) without staining. Representative images from 3 mice/group are shown. Original magnification, x100. Rapa: rapamycin. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

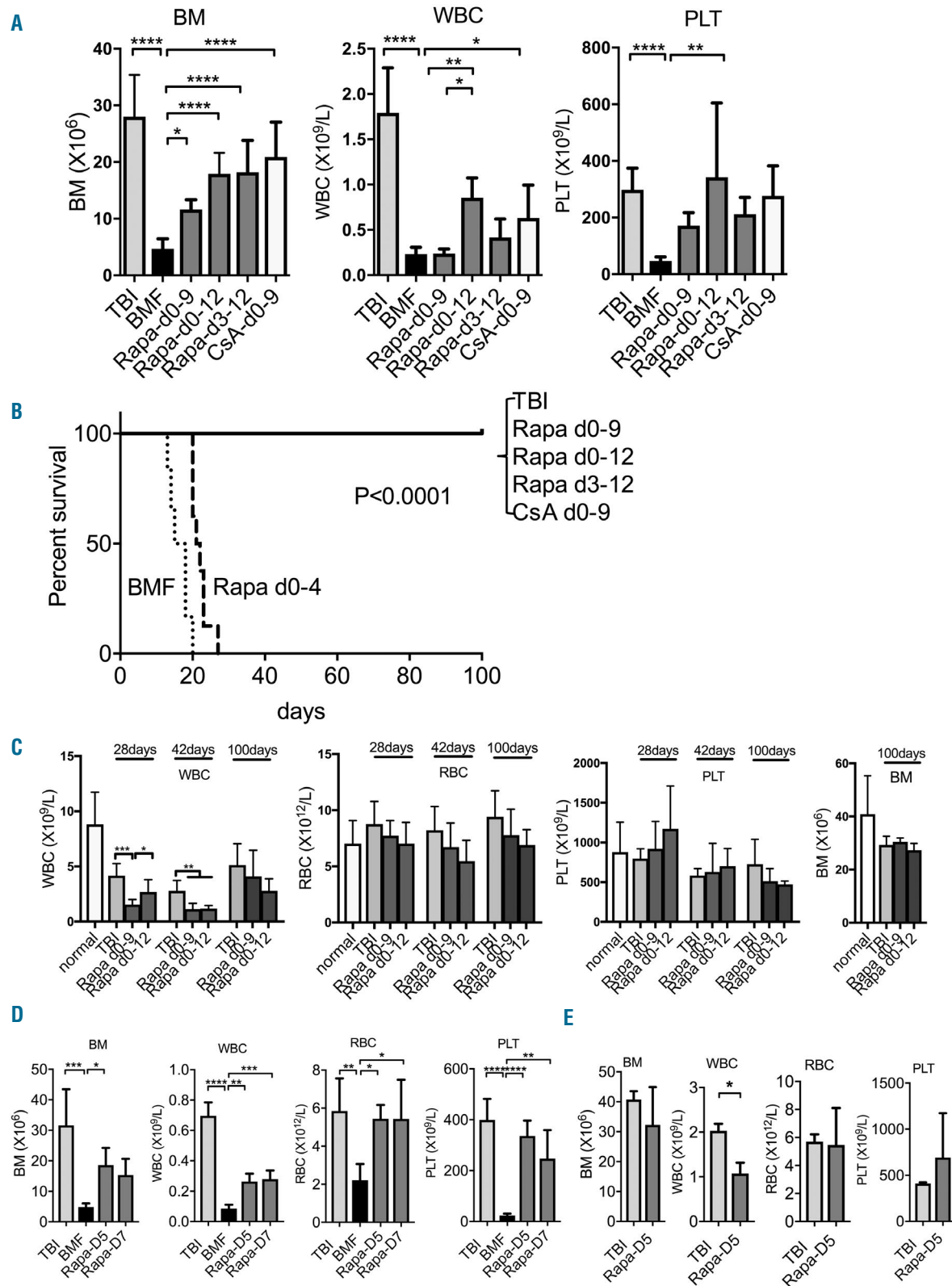


Figure 2. Efficacy of rapamycin in treatment of bone marrow (BM) failure. (A) Comparison of BM cell number, white blood cell (WBC) and platelet (PLT) count in different regimens of rapamycin at day (d)13. CByB6F1 BM failure (BMF) mice were treated with cyclosporine (CsA, 50 mg/kg/day) at the standard duration of d0-9, or with rapamycin (2 mg/kg/day) d0-9, d0-12, and d3-12, respectively. n=10 for each group, except n=5 for total body irradiation (TBI). (B) Survival curves using different treatment regimens. TBI, Rapa d0-9, Rapa d3-12, CsA d0-9 versus BMF or Rapa d0-4; P<0.0001. n=8 for Rapa d0-9 and d0-12, respectively; n=4 for TBI control. (C) Recovery of complete blood counts in rapamycin-treated BMF mice at d28, d42 and d100 (n=8 for Rapa d0-9 and d0-12, respectively; n=4 for TBI control). Normal mice were used as a reference (n=3). BM cellularity was evaluated at 100 days. (D) Delayed rapamycin treatment to d5 (Rapa-D5, n=5) and d7 (Rapa-D7, n=5) following BMF induction (n=4) also preserved BM cells and alleviated pancytopenia when animals were evaluated at d18. n=5 for TBI. (E) Delayed rapamycin treatment to d5 (Rapa-D5, n=5) led to 100% animal survival with blood and BM cellularity comparable to that of TBI control (n=3) when animals were analyzed at ten weeks. Rapa: rapamycin. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

rapamycin treatments also ameliorated pancytopenia and BM failure, with a better response in the Rapa-D5 than the Rapa-D7 group (Figure 2D). Furthermore, the efficacy of the delayed treatment (Rapa-D5) was sustainable, with BM cellularity and CBCs reaching similar levels to TBI controls at ten weeks post-BM failure, although the recovery of WBC was slower than that of TBI controls (Figure 2E). We also tested the efficacy of rapamycin in another BM failure model by infusing LN cells from FVB donors into sublethally-irradiated B6 recipients²⁰ (Online Supplementary Figure S1A). Rapamycin eliminated CD8⁺

T cells in BM, increased the CD4/CD8 T-cell ratio, and improved animal survival, comparable to the efficacy of CsA (Online Supplementary Figure S1B-E), indicating that rapamycin is effective in ameliorating BM failure independent of strains.

Rapamycin expands regulatory T cells and modulates T-cell function

Based on the optimal efficacy and survival of rapamycin d0-12 mice, we chose this regimen as standard treatment relative to the previously-established CsA d0-9 treatment

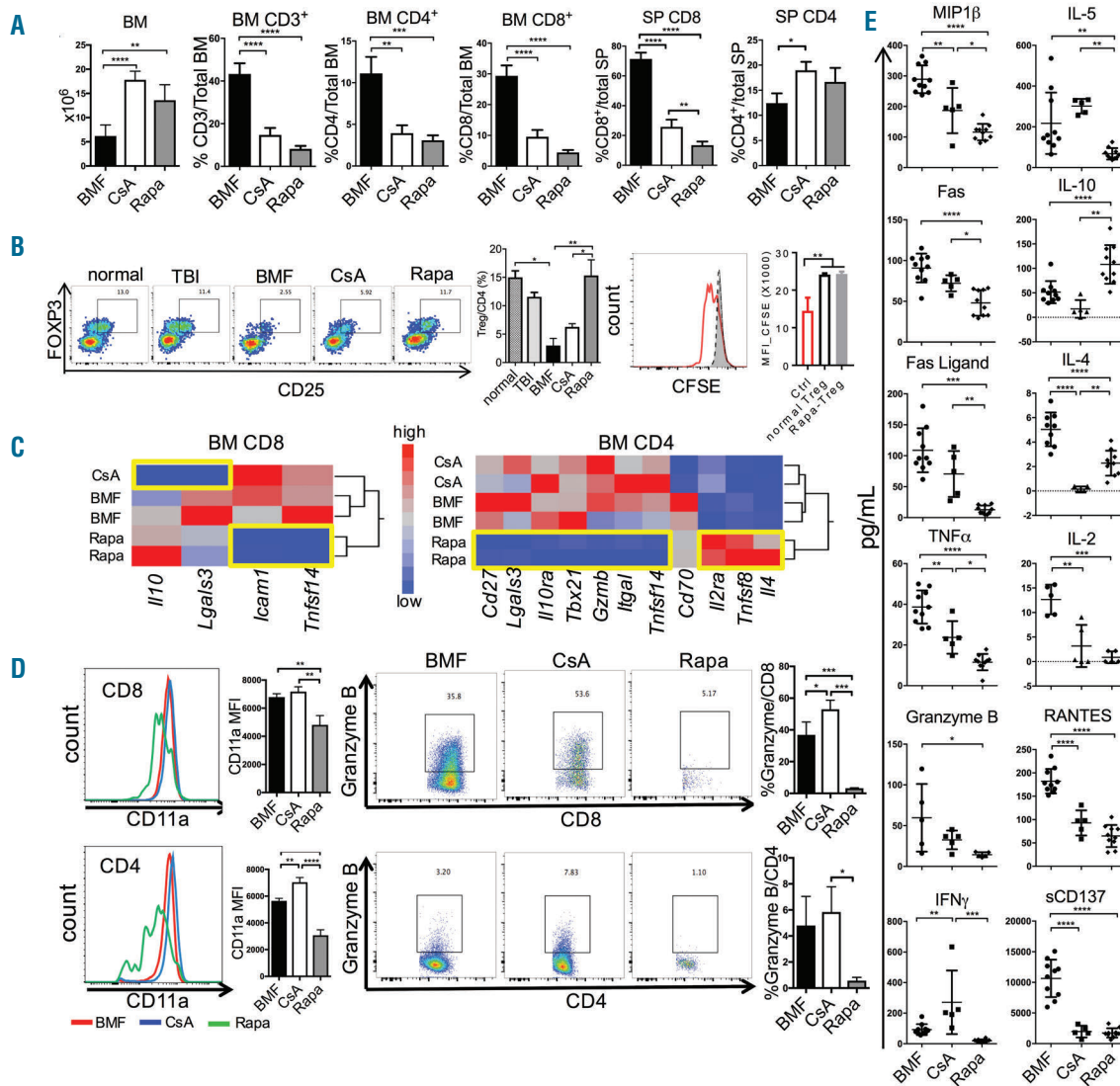


Figure 3. Rapamycin expands regulatory T cells and suppresses effector T-cell function. (A) Both cyclosporine (CsA) and rapamycin were efficient in improving total bone marrow (BM) cell number and in decreasing frequencies of CD3⁺, CD8⁺, and CD4⁺ T cells in total BM cells (n=10 for each group, except n=5 for cyclosporine). (B) Rapamycin increased regulatory T cells in the spleen of BM failure (BMF) mice compared with untreated and CsA-treated mice, but comparable with total body irradiation (TBI, n=3) and normal (n=3) mice. The regulatory T cells (CD4⁺CD25⁺) in rapamycin-treated mice were FACS-sorted, and were tested for functionality in suppressing proliferation of CFSE-labeled effector T cells upon T-cell receptor (TCR) stimulation on day (d)5. Red graph represents effector T cells alone; dashed open graph with regulatory T cells from normal mice; gray closed graph with regulatory T cells from rapamycin-treated mice. (C) Transcriptome analyses of BM-infiltrating CD8⁺ and CD4⁺ T cells using a PCR-based array. Each row represents one pooled population of CD8⁺ or CD4⁺ T cells from the same groups; each column represents one gene with more than 2-fold differences between any two groups. Blue: low expression level; red: high expression level. (D) Validation of CD11a (Itga1) and granzyme B expression in BM-infiltrating T cells in rapamycin-treated or CsA-treated mice by flow cytometry (n=3 for each group). (E) Plasma was collected from peripheral blood of BMF mice (n=10) and rapamycin-treated (n=10) or CsA-treated (n=5) mice at d13 post lymph node (LN) injection and analyzed for T-cell-related cytokines by Luminex. Rapa: rapamycin. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

in follow-up studies. In a new experiment of B6=>CByB6F1 LN cell infusion-induced AA, both CsA and rapamycin mitigated BM failure by suppressing CD8⁺ and CD4⁺ T-cell infiltration in the BM. In the spleen, rapamycin exerted greater suppression of CD8⁺ T cells than did CsA, though both drugs preserved CD4⁺ T-cell numbers (Figure 3A). Strikingly, the spleens of rapamycin-treated mice had a much higher proportion of CD4⁺CD25⁺FoxP3⁺ regulatory T (Treg) cells than in CsA-treated or untreated BM failure animals (Figure 3B), which was also observed in the BM and LN (Online Supplementary Figure S2). Isolated Tregs from the spleens of rapamycin-treated mice were capable of suppressing the proliferation of effector cells reflected by decreased CFSE dilution after T-cell receptor (TCR) stimulation, similar to Tregs derived from normal mice (Figure 3B), suggesting that the expanded Tregs by rapamycin are functionally competent.

We sorted BM CD4⁺ and CD8⁺ T cells from BM failure mice with or without rapamycin or CsA treatment and performed transcriptome analyses focusing on genes related to T-cell function using a PCR-based array. Genes showing more than 2-fold changes in expression between any two groups (BM failure, CsA, and rapamycin) were

plotted in a heat map. In CD8⁺ T cells, rapamycin suppressed expression of *Icam1* and *Tnfrsf14*, whereas CsA suppressed expression of *Lgals3* (Figure 3C, left). In CD4⁺ T cells, rapamycin suppressed expression of *Cd27*, *Lgals3*, *Il10ra*, *Itgal*, *Tbx21*, *Gzmb*, *Tnfrsf14*, and *Cd70* but increased expression of *Il-2ra*, *Tnfrsf8*, and *Il-4*, while CsA down-regulated the expression of *Cd70* only without affecting expression of other genes (Figure 3C, right). Thus, rapamycin and CsA appeared to affect different molecular pathways while modulating immune activity. Consistent with our transcriptome analyses, we found that rapamycin, but not CsA, reduced CD11a, a protein encoded by the *Itgal* gene, on the cell surface of both BM CD4⁺ and CD8⁺ T cells (Figure 3D, left). More strikingly, rapamycin abrogated intracellular granzyme B expression in residual CD4⁺ and CD8⁺ T cells in BM. In contrast, CsA increased the frequency of granzyme B-secreting T cells (Figure 3D, right).

Plasma cytokine levels were measured by Luminex assays to further evaluate differences in the T-cell secretory profiles of rapamycin-treated and CsA-treated mice (Figure 3E). Plasma MIP1 β , Fas, Fas ligand, and TNF- α were reduced by both CsA and rapamycin, but the reduc-

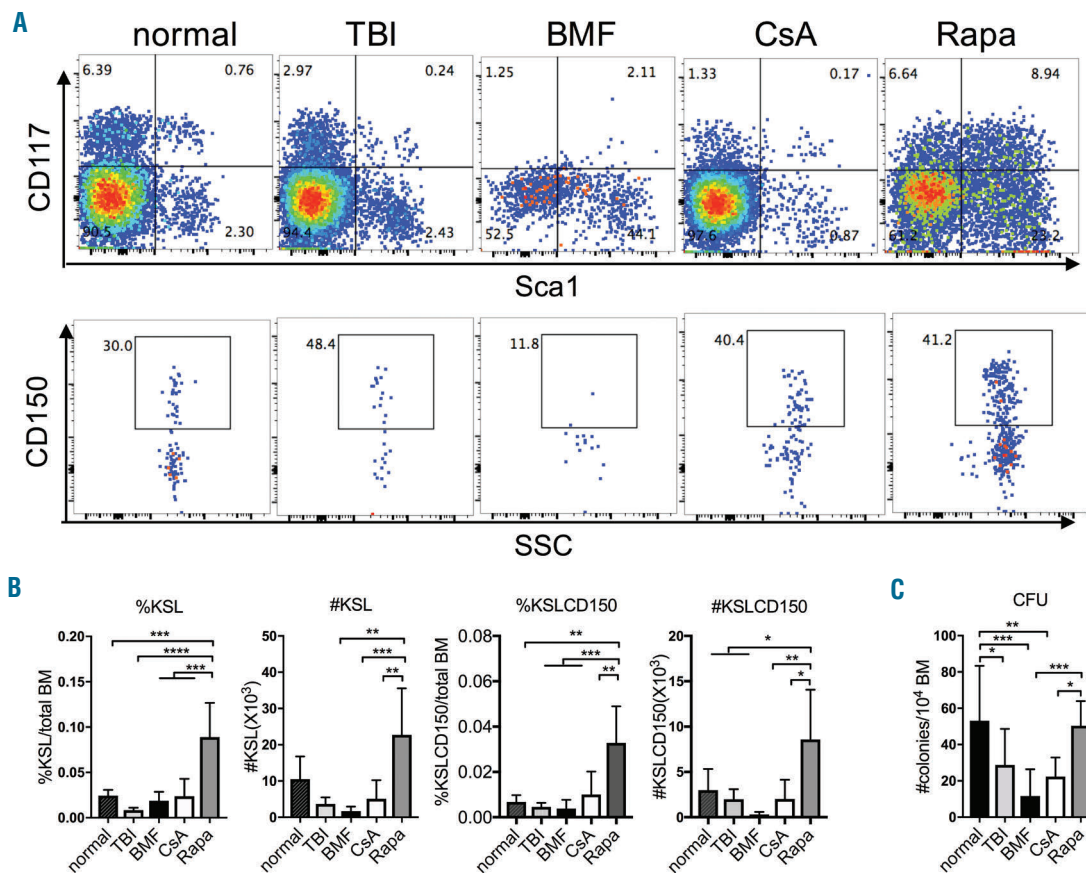


Figure 4. Rapamycin preserves hematopoietic stem and progenitor cells in bone marrow (BM) failure mice. (A) Representative flow cytometry analyses of BM KSL (upper panel, c-kit⁺Sca1⁺ in Lin⁻) and KSLCD150 (lower panel, CD150⁺ in KSL cells) in normal mice, total body irradiation (TBI), untreated, cyclosporine (CsA)-treated, and rapamycin-treated BM failure (BMF) mice on day (d)13. (B) Frequencies and absolute numbers of total BM KSL and KSLCD150⁺ cells in normal mice, TBI, untreated, CsA-treated, and rapamycin-treated BMF mice. (C) Colony-forming unit (CFU) assay of BM cells from CsA-treated and rapamycin-treated BMF mice. 3×10^4 total BM cells were seeded into 1 mL methylcellulose culture medium; total colonies were counted after ten days. $n=5$ for each group. Rapa: rapamycin. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

tion of these cytokines was greater with rapamycin than with CsA. Granzyme B levels were significantly reduced only by rapamycin, consistent with transcriptome and flow cytometry data (Figure 3C and D). Also, consistent with transcriptome data, CsA induced higher levels of

IFN- γ , and IL-5, but lower levels of IL-10 and IL-4 than did rapamycin (Figure 3C). Plasma IL-2, RANTES, and sCD137 levels were similar in both CsA-treated and rapamycin-treated mice. Thus, rapamycin significantly down-regulated cytokines related to Th1 immune

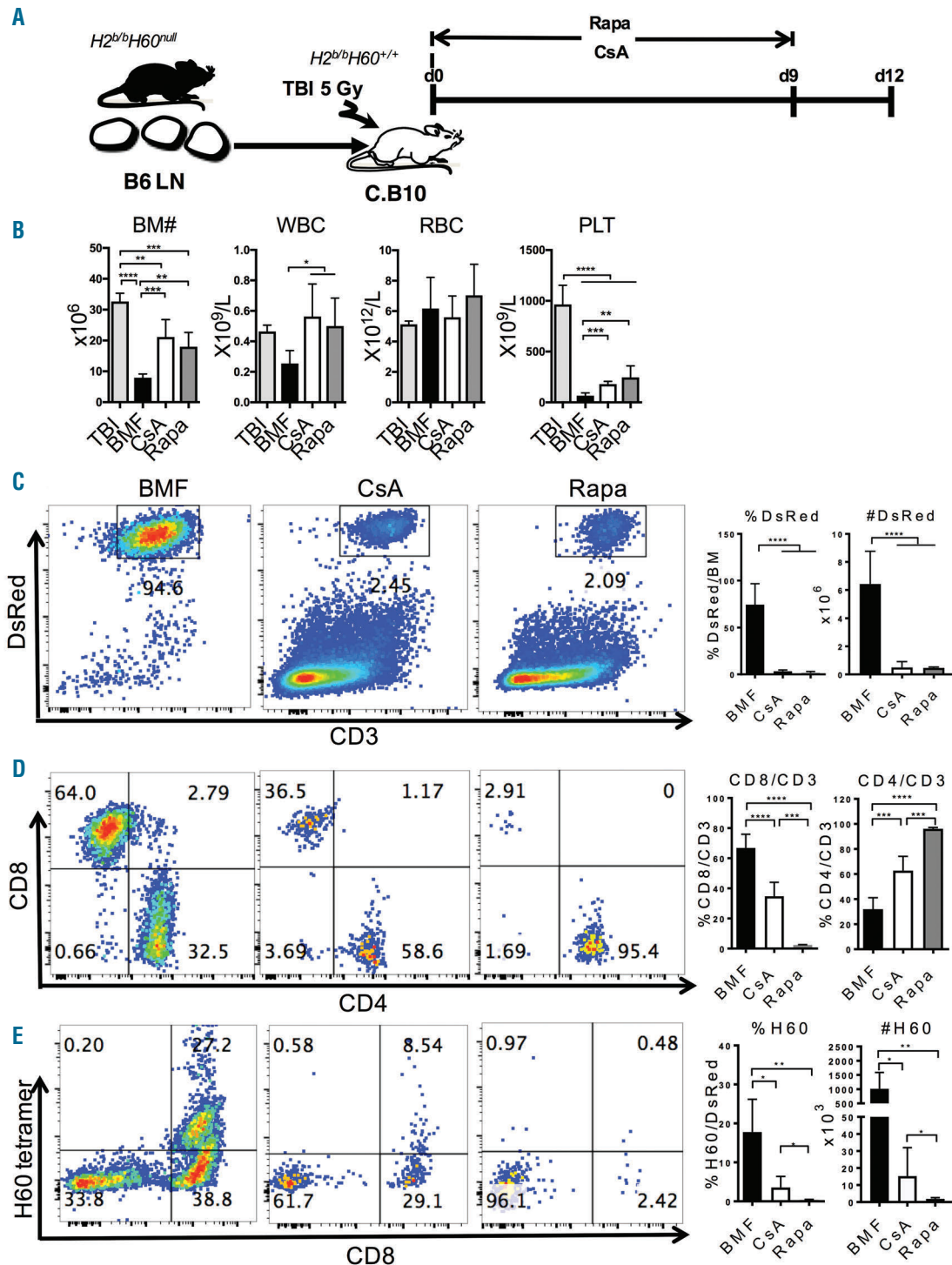


Figure 5. Eradication of antigen-specific T cells by rapamycin in a minor histocompatibility antigen mismatched bone marrow (BM) failure (BMF) model. (A) C.B10 mice received 5 Gy total body irradiation (TBI) and infusion of 5×10^6 DsRed B6 lymph node (LN) cells to induce BM failure (BMF). Some BMF mice received daily rapamycin injections at 2 mg/kg/day or cyclosporine (CsA) at 50 mg/kg/day for ten days. Mice were bled and euthanized at day (d)12 for analyses. (B) BM cell number, white blood cell (WBC), red blood cell (RBC), and platelet (PLT) counts in TBI control (n=4), BMF (n=7), CsA-treated (n=7) and rapamycin-treated (n=9) mice. (C) Frequency and absolute number of DsRed CD3⁺ T cells in total BM. (D) Proportion of CD8⁺ and CD4⁺ T cells in DsRed T cells. (E) Frequency and absolute number of H60-specific CD8⁺ T cells in DsRed CD3⁺ T cells. Representative flow cytometry analyses are shown. Rapa: rapamycin. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

responses, such as IFN- γ , and up-regulated cytokines related to Th2 immune responses, such as IL-10. CsA produced a different profile, more similar to that of Th1-like cytokine than rapamycin.

Rapamycin preserves HSPCs

We examined the efficacy of rapamycin and CsA in preserving HSPCs as defined by KSL (c-Kit⁺Sca1⁻Lin⁻) and KSL-SLAM (c-Kit⁺Sca1⁻Lin⁻CD150⁺) markers. Rapamycin

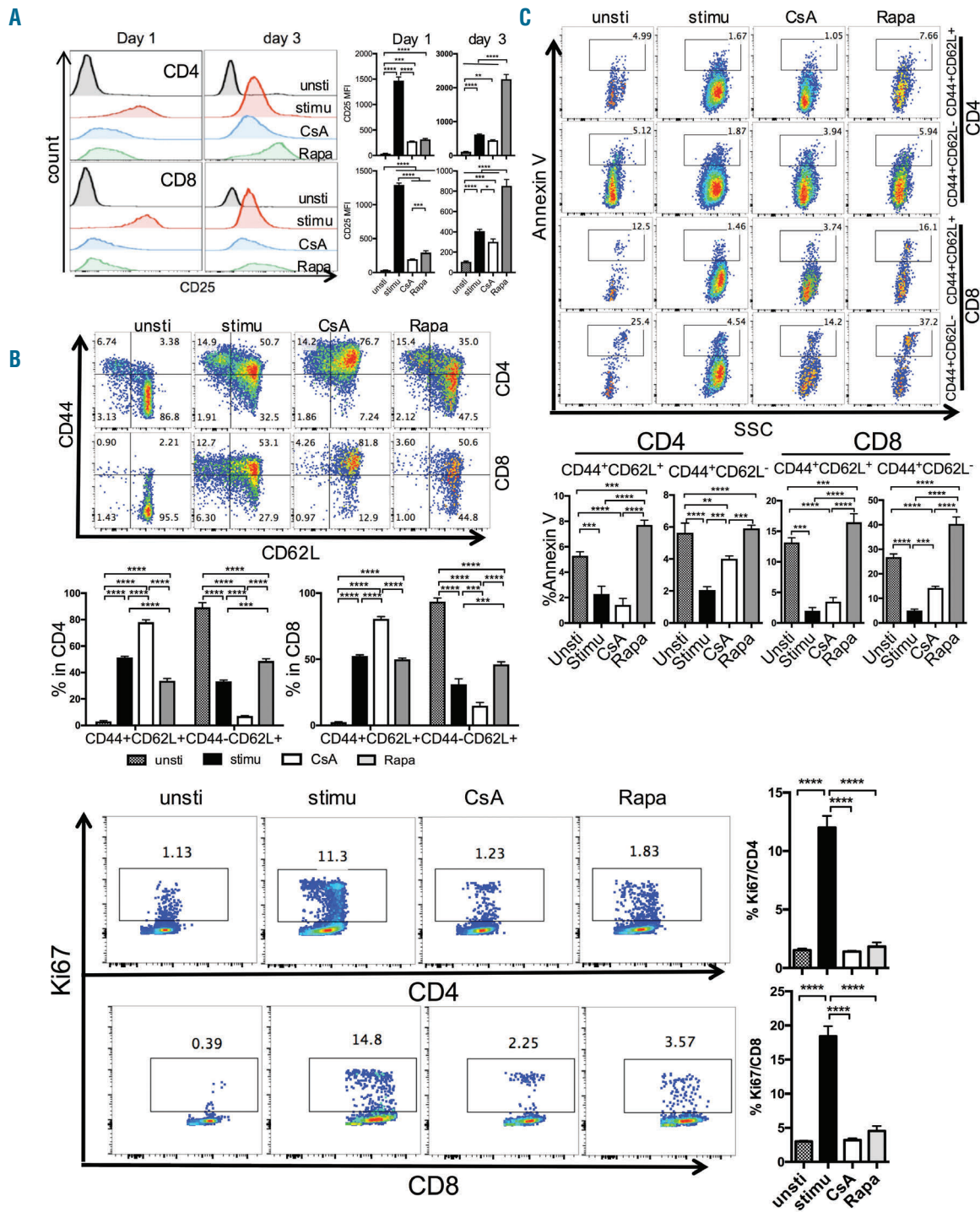


Figure 6. Effects of rapamycin (Rapa) and cyclosporine on T-cell activation, proliferation and T-cell phenotype post-T-cell receptor (TCR) stimulation *in vitro*. (A) Suppression of CD25 expression by cyclosporine (CsA) and rapamycin upon TCR stimulation on day (d)1, and increase of CD25 expression by rapamycin on d3. (B) Representative flow cytometric figures and statistics of different memory phenotypes based on CD44 and CD62L expression in CsA-treated and rapamycin-treated lymph node (LN) T cells on d3. (C) Apoptosis in different T-cell phenotypes in CsA and rapamycin-treated LN T cells on d2. n=3 for each group. (D) Effect of rapamycin and CsA on T-cell proliferation in total CD4⁺ and CD8⁺ T cells. Rapa: rapamycin; unsti: unstimulated; stimu: stimulated. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

increased the frequency of the c-Kit+Sca-1⁺ population in Lin⁻ cells compared to untreated or CsA-treated BM failure mice (Figure 4A, upper panel). Frequencies of CD150⁺ cells in KSL cells were similar in each group (30-50%) but lower in the BM failure group (Figure 4A, lower panel). Calculating total BM cells, rapamycin increased frequencies and absolute numbers of both KSL and KSLCD150⁺ cells as compared with other groups (Figure 4B). When BM cells were cultured *in vitro* in semisolid medium, BM cells from rapamycin-treated BM failure mice formed significantly more total colonies than did cells from CsA-treated or untreated BM failure mice (Figure 4C). Thus, rapamycin effectively preserved HSPCs in animals with immune-mediated BM failure, with an efficacy comparable to that of standard CsA treatment.

Rapamycin eradicates antigen-specific effector T cells

The effectiveness of rapamycin was further evaluated in the B6-DsRed=>C.B10 LN cell infusion-induced BM failure model, in which C.B10 mice express the dominant minor-H antigen H60 and B6 mice carry a null allele at H60 locus, allowing us to detect antigen-specific T cells⁵ (Figure 5A). We treated BM failure C.B10 mice with CsA or rapamycin for ten days and evaluated their effects on H60-specific T cells. At d12, untreated BM failure mice had decreased BM cellularity and peripheral blood cell counts. Both CsA and rapamycin ameliorated pancytopenia and improved BM cellularity (Figure 5B). Flow cytometry analyses show that BMs of untreated BM failure mice were infiltrated with large numbers of DsRed donor T cells, but both rapamycin and CsA markedly reduced the proportion of these cells in BM (Figure 5C). Most infiltrating DsRed cells in untreated BM failure mice were CD8⁺ T cells; rapamycin treatment eliminated almost all of the CD8⁺ T cells within DsRed population and more than 90% of the remaining DsRed cells were CD4⁺ T cells. While suppressing donor DsRed cell infiltration to a similar extent as that achieved by rapamycin, CsA maintained a substantial proportion of CD8⁺ T cells in the residual DsRed cells (Figure 5D). As expected, a large clone of H60-specific CD8⁺ T cells had infiltrated the BM of untreated AA mice. In rapamycin-treated mice, very few H60-specific CD8⁺ T cells in DsRed cells were present in recipient animals, and after CsA treatment, H60-specific CD8⁺ T cells were reduced in number but could be observed in host marrows (Figure 5E).

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Rapamycin eliminates memory-like and effector T cells but retains naïve T cells *in vitro*

To understand the mechanisms related to the different effects of CsA and rapamycin on T cells, we investigated T-cell activation, proliferation, and phenotypic differentiation. Stimulating mouse LN cells *in vitro* with anti-mouse CD3 and CD28 antibodies induced T-cell activation. Both CsA and rapamycin inhibited T-cell activation on d1, with reduced CD25 expression following TCR stimulation. On

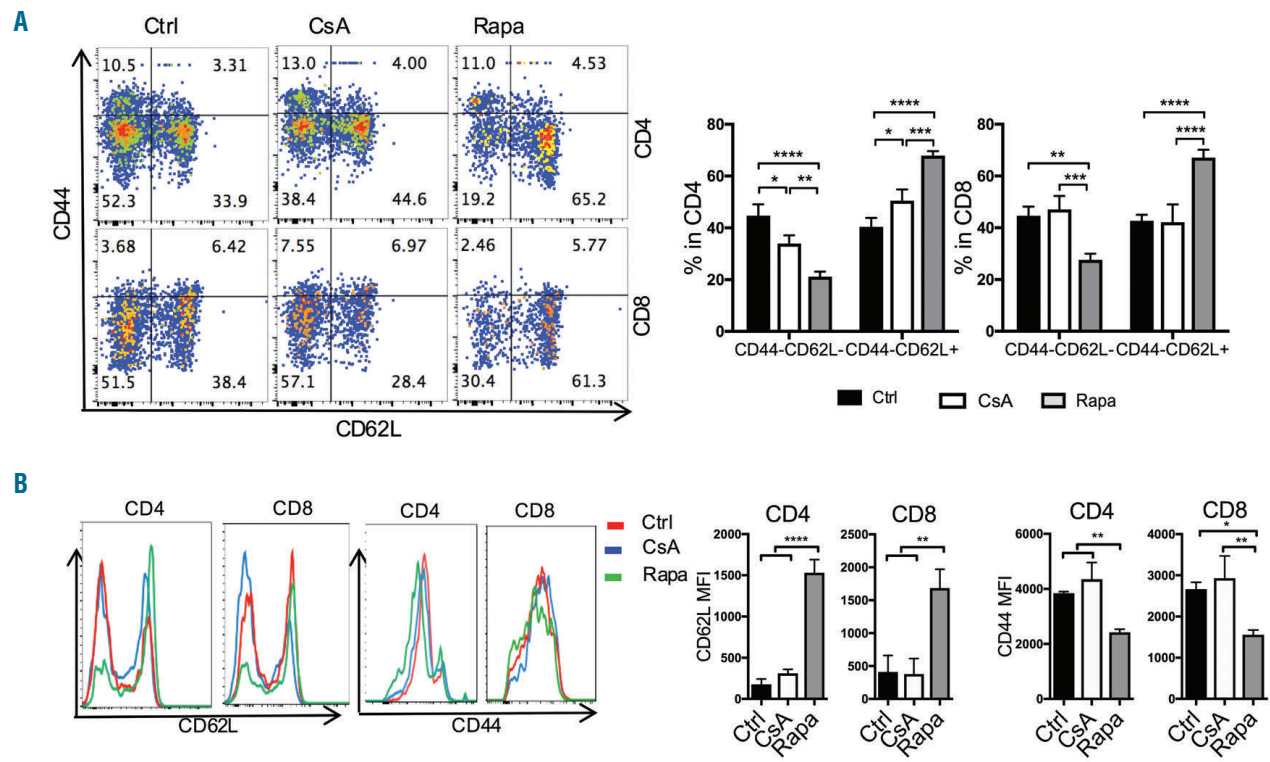


Figure 7. Preservation of naïve T cells and elimination of active effector T cells by rapamycin after *in vivo* T-cell activation. (A) Representative flow cytometry figures and statistics of different cell phenotypes of spleen CD4⁺ and CD8⁺ T cells from CByB6F1 mice that received B6 lymph node (LN) injection (10x10⁶) and subsequent treatment with rapamycin (Rapa, 2 mg/kg) or cyclosporine (CsA, 50 mg/kg) *via i.p.* for three days. Statistics of two major populations CD44⁻CD62L⁻ (active effector) and CD44⁺CD62L⁺ (naïve) are shown. (B) CD62L and CD44 expression in spleen CD4⁺ and CD8⁺ T cells in CsA-treated and rapamycin-treated CByB6F1 mice after LN injection. n=5 for each group. Ctrl: LN injection without treatment. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

d3, however, expression of CD25 was increased in CD4⁺ T cells following rapamycin exposure (Figure 6A), probably due to the Treg-stimulatory effect of rapamycin.

Activation generally results in T-cell differentiation into different phenotypes. We determined T-cell phenotypes after exposure to rapamycin or CsA in combination with anti-mouse CD3 and CD28 mAb *in vitro*. CD4⁺ and CD8⁺ T cells without stimulation retained a naïve phenotype (CD44⁺CD62L⁺), but effector memory (CD44⁺CD62L⁻) and central memory (CD44⁺CD62L⁺) phenotypes were induced by stimulation with CD3 and CD28 antibodies. TCR-stimulated T cells exposed to CsA or rapamycin showed phenotypes similar to stimulated T cells on d1, but a substantial proportion of CD4⁺ and CD8⁺ T cells treated with rapamycin displayed markers of a naïve phenotype on d3 after stimulation. In the presence of CsA, however, T cells retained their central memory phenotype

(Figure 6B). Rapamycin induced more apoptosis in central memory and effector memory CD4⁺ and CD8⁺ T cells than did TCR stimulation and CsA treatment, especially in CD8⁺ T cells (Figure 6C).

To investigate the effects of each drug on T-cell proliferation, we measured Ki67 expression in TCR-stimulated T cells on d2 by flow cytometry. Both CsA and rapamycin suppressed Ki67 frequencies in CD4⁺ and CD8⁺ T cells with respect to TCR-stimulation controls (Figure 6D).

Rapamycin eliminates effector T cells and retains naïve T cells *in vivo*

To test if rapamycin and CsA have the same effect on T-cell differentiation *in vivo* as they do *in vitro*, we injected B6 LN cells into unirradiated CByB6F1 mice to activate the host immune system, with or without administration of rapamycin (2 mg/kg, *i.p.*) or CsA (50 mg/kg, *i.p.*) for three

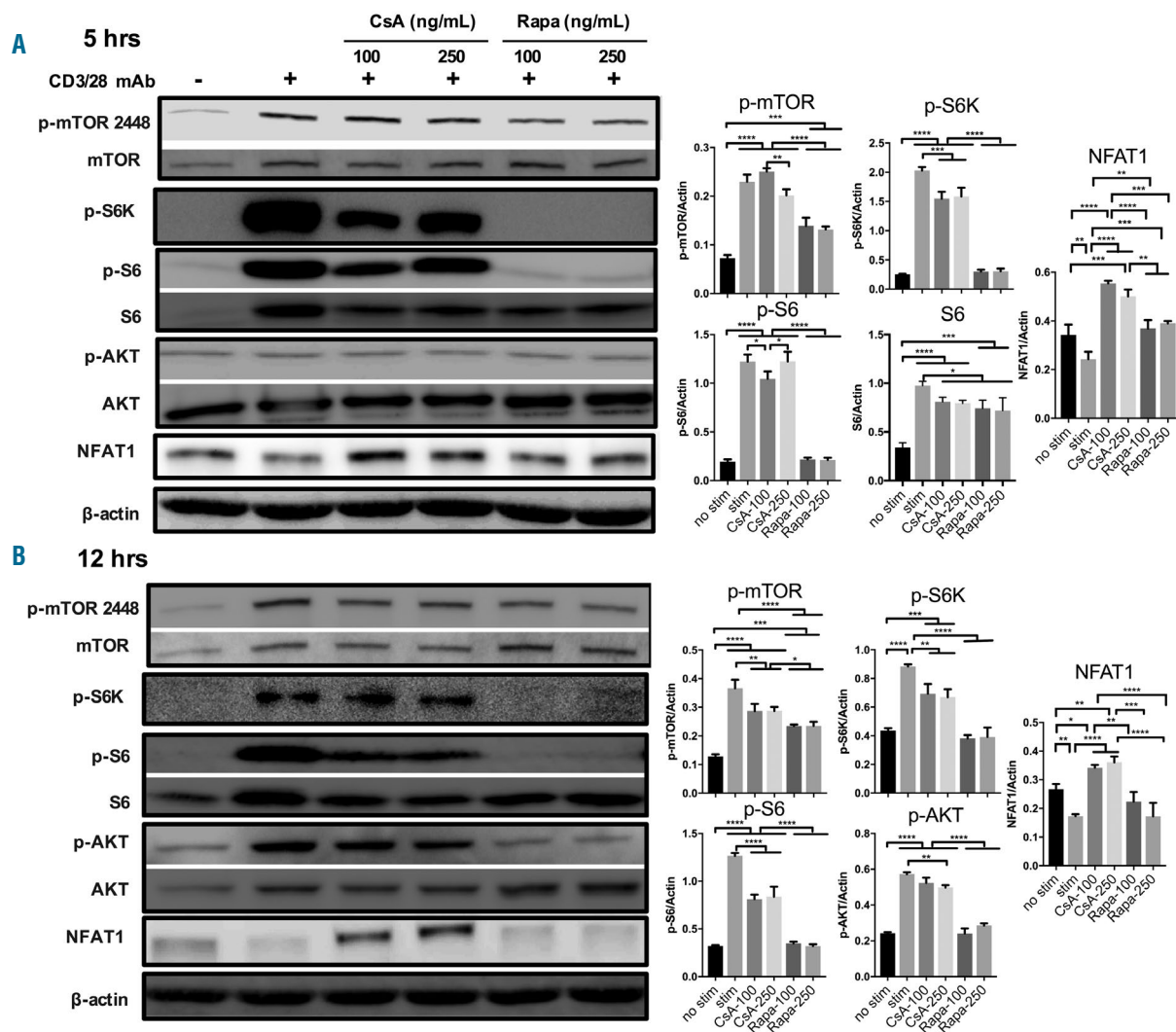


Figure 8. Effects of rapamycin and cyclosporine on phosphorylation of mTOR pathway components after T-cell receptor (TCR) stimulation. B6 lymph node (LN) cells were stimulated with CD3/CD28 mAb in the presence or absence of different concentrations of cyclosporine (CsA) or rapamycin for 5 hours (h) (A) and 12 h (B), respectively, and proteins were extracted, immunoblotted to detect mTOR, p-mTOR, p-S6K, p-S6, S6, p-AKT, AKT, and NFAT1, with β-actin expression used as a loading control. The bands were quantified based on β-actin levels, the proteins differentially expressed between rapamycin and CsA are shown in bar graphs. Representative figures from 3 separate experiments are shown. Bands of p-S6K at 12 h were visible when exposure time was extended. Rapa: rapamycin; no stim: no stimulation; stim: stimulation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

days. On d4, spleen cells were collected and T-cell phenotypes were evaluated by flow cytometry. Active effector (CD44⁺CD62L⁻) T cells and naïve (CD44⁺CD62L⁺) T cells differed among the three groups: LN cell injection without drug treatment induced expansion of CD44⁺CD62L⁻ active effector T cells in both CD4⁺ and CD8⁺ T-cell fractions, while rapamycin reduced the frequencies of this population in both CD4⁺ and CD8⁺ T cells, and CsA reduced the effector population in CD4⁺ but not in CD8⁺ T cells. Rapamycin induced a marked increase in CD44⁺CD62L⁺ naïve CD4⁺ and CD8⁺ T cells; CsA only slightly increased naïve CD4⁺ T cells (Figure 7A), confirming our observations *in vitro* (Figure 6B). When we measured median fluorescence intensity of CD44 or CD62L in CD4⁺ and CD8⁺ T cells, rapamycin decreased CD44 but increased CD62L fluorescence intensity compared to CsA-treated or untreated mice (Figure 7B), suggesting suppression of effector T cells and retention of naïve T cells by rapamycin.

Rapamycin and CsA act on different signaling pathways

We stimulated T cells with CD3/CD28 mAb in the presence or absence of rapamycin or CsA to investigate molecular changes in mTOR and nuclear factor of activated T-cell (NFAT) signaling pathways. By immunoblot, TCR stimulation increased total mTOR and ribosomal S6 levels compared to unstimulated T cells. CsA or rapamycin exposure post-TCR stimulation did not alter the increased levels of mTOR and ribosomal S6. In contrast, rapamycin inhibited mTOR phosphorylation and almost eliminated phosphorylation of S6 kinase (S6K) and its downstream target S6, effects not seen with CsA. At 5 h, similar levels of total and phosphorylated AKT were detected in all conditions. Phosphorylated AKT was greatly increased in stimulated T cells at 12 h; rapamycin, but not CsA, almost completely suppressed phosphorylated AKT (Figure 8). Baseline NFAT1 was observed in unstimulated T cells at both 5 and 12 h. TCR stimulation reduced cytoplasmic NFAT1 levels at 5 h, which was further decreased at 12 h. In contrast, CsA exposure post-TCR stimulation increased cytoplasmic NFAT1 levels at 5 h, with higher NFAT1 levels becoming even more pronounced at 12 h. Rapamycin exposure appeared to increase NFAT1 levels at 5 h, but returned to similar levels as TCR stimulation alone at 12 h, indicating that rapamycin might affect NFAT1 transiently (Figure 8). Thus, rapamycin appeared to exert its immunosuppressive function by modulating mTOR activity, while CsA suppresses immune activity by interfering with the NFAT signaling pathway.

Discussion

In this study, we demonstrate that rapamycin effectively and reproducibly attenuated immune-mediated BM failure in AA mouse models, with efficacy similar to that of the standard dose of CsA. Rapamycin showed high efficacy in suppressing Th1 immune responses, eradicating pathogenic CD8⁺ T cells, stimulating immunosuppressive Treg cells, and protecting HSPCs. Our data indicate that modulation of mTOR activity and its downstream signaling molecules is key to the therapeutic efficacy of rapamycin, which differs from CsA immunosuppressive function through modulation of NFAT pathway.

Rapamycin, but not CsA, expanded Treg cells in BM

failure mice. This result is consistent with previous observations that activation of mTOR suppresses FoxP3 expression and that complete inhibition of mTOR activity in CD4⁺ T cells stimulates the generation of Treg cells even under activating conditions.²¹⁻²⁵ There are two distinct mTOR complexes, mTOR complex 1 (mTORC1) and mTORC2, and both contribute to suppression of FOXP3 expression.²⁶ In our *in vitro* experiments, rapamycin increased CD25 expression on CD4⁺ T cells at d3 post TCR stimulation. Increased plasma IL-10 concentration by rapamycin may also indicate enhanced immune regulatory function of Tregs, as IL-10 is a potent suppressor of effector T-cell proliferation.²⁷

Tregs are central to the maintenance of self-tolerance and tissue homeostasis: Treg impairment had been reported in human autoimmune and immune-mediated conditions.²⁸⁻³⁰ In patients with AA, both the frequency and absolute number of Treg cells are reduced.³¹ Furthermore, the superiority of horse over rabbit ATG in AA treatment correlates to preservation of Treg cells,³² specific human Treg subpopulations defined using mass cytometry may be useful as predictive biomarkers for response to IST in AA,³³ addition of rapamycin to anti-CD3/CD28 beads, high-dose IL-2, and all-trans retinoic acid led to more than 30-fold expansion of Treg from AA patients.³³ Thus, modulation of Treg number and function becomes an important determinant of therapeutic efficacy in AA.

Rapamycin selectively eradicated effector and memory T cells. TCR stimulation *in vitro* or MHC-mismatched LN injection *in vivo* augmented differentiation of T cells from naïve to central memory, effector memory, and active effector phenotypes. CsA maintained T cells with effector and memory phenotypes, which may provide a logical explanation for the frequent relapse of AA patients following withdrawal of CsA. In contrast, rapamycin augmented memory T-cell apoptosis, decreased effector and memory T cells, and increased naïve T cells. The efficacy of rapamycin on effector T cells was best demonstrated in the C.B10 BM failure mouse model in which rapamycin eradicated almost all H60-specific T cells. Our observations reflect an early report in a heart transplantation model in which rapamycin alone or in combination with co-stimulation blockade eradicated effector and memory T cells and induced immune tolerance.³⁴

Alteration of mTOR activity by rapamycin affects T-cell differentiation, since deficiency of an important mTORC1 activator RHEB (Ras homolog enriched in brain) in CD4⁺ T cells leads to normal Th2 but impaired Th1 and Th17 effector lineages through reduced activation of STAT4 and STAT3.²² Loss of STAT4 and STAT3 is also correlated with diminished expression of T-bet, a Th1 master transcription factor, and ROR γ t, a Th17 master transcription factor.³⁵ Our observation of reduced Tbet and Granzyme B in BM CD4⁺ T cells from rapamycin-treated mice is consistent with suppression of Th1 immune function. Thus, rapamycin not only induced Tregs and eradicated memory and effector T cells, but also skewed T-cell differentiation away from pro-inflammatory Th1 and Th17 subsets.

In T cells, mTOR bridges immune signals and metabolic cues to regulate T-cell responses. Active effector T cells preferentially utilize aerobic glycolysis to meet energy demands associated with expansion of cell numbers, cytolytic activity, and homing.^{36,37} Within the two mTOR complexes, the mTORC1-S6K1 axis is a crucial determinant of T-cell activation.³⁸ Although AKT is not down-

stream of the mTORC1 complex, we observed AKT phosphorylation to be abrogated by rapamycin but not by CsA. This effect was observed at 12 h but not at 5 h post-TCR stimulation, secondary to S6 phosphorylation, suggesting that mTORC2 inhibition requires prolonged treatment with rapamycin despite the fact that rapamycin primarily affects mTORC1.^{35,39} The PI3K-Akt-mTORC1-S6K1/2 axis was reported to control Th17 cell differentiation *via* downregulation of Gfi1, a negative regulator of Th17 differentiation.⁴⁰ There is an increased ratio of Th17 to regulatory T cells in AA,⁴¹ and the inhibitory effect of rapamycin on Th17 differentiation may be of benefit to AA patients treated with rapamycin.

The effectiveness of rapamycin in BM failure mouse models is relevant to the establishment of a clinical trial for AA patients. However, results from our previous randomized clinical trial suggested that treatment of AA patients with horse-ATG/CsA/rapamycin produced no improved beneficial effect than the standard horse-ATG/CsA treatment.⁴² CsA has been reported to prevent the induction of the cytosolic branched chain aminotransferase (BCATc). Blockade of BCATc increases phosphorylation of mTORC1 downstream targets, S6 and 4EBP-1,⁴³ which counteracts the inhibitory effect of rapamycin on

mTORC1, possibly explaining the ineffectiveness of concurrent CsA/rapamycin treatment in AA. Data from our current animal study showed the importance of treatment duration, as short treatments with either CsA or rapamycin were not effective. We selected our CsA treatment duration based on its effectiveness from our previous experience, since longer-term CsA has produced adverse effects in mice, including hunching and scruffy appearance, weight loss, and death, probably due to nephrotoxicity and hepatotoxicity.^{44,45} In contrast, mice treated with rapamycin appeared healthy and active.

In conclusion, rapamycin treated immune-mediated murine BM failure as compared to standard dose CsA. Our observations support the potential utility of rapamycin in the clinic for the treatment of AA. A phase II clinical trial application is under development at our institution (*clinicaltrials.gov* identifier: 02979873) to test rapamycin as prophylaxis in severe AA patients who are at high risk of relapse on withdrawal of long-term CsA.

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