Efficacy of combined low-dose ruxolitinib and cyclosporine in murine immune bone marrow failure

Immune aplastic anemia (AA) is a bone marrow failure (BMF) syndrome characterized by pancytopenia and hypocellular bone marrow (BM) due to hematopoietic stem and progenitor cell (HSPC) destruction by activated T cells.[1] Immunosuppressive therapy (IST) with anti-thymocyte globulin (ATG) and cyclosporine A (CsA), with eltrombopag, a thrombopoietin agonist, is first line treatment for severe AA patients who are older or lack a fully matched sibling donor for stem cell transplant.[1] However, ATG requires hospitalization and is preferably administered at specialized centers, as it can cause significant infusion reactions and other toxicities. Therefore, development of a low-risk oral therapy is a key goal of BMF research.

Targeting the JAK pathways has proven efficacious in many diseases, in particular immune-mediated and inflammatory disorders.[2,3] Among JAK inhibitors, ruxolitinib (RUX) is an orally-administrated selective ATP-competitive JAK1/2 kinase inhibitor, currently licensed to treat primary myelofibrosis (PMF)[4] and graft-*versus*-host disease (GVHD).[2,3] We reported that RUX successfully treats BMF in mice by reversing cytopenias, resulting in prolonged survival and little toxicity, likely due to its suppression of T-cell activation and proliferation, reduction in inflammatory cytokines, and expansion of regulatory T cells (Treg).[5] In our initial experiments, RUX was administered as either a food additive or via gavage at a standard dose equivalent to ~60 mg/kg;[6] prior dosing in mice in other disease models has ranged from 30-90 mg/kg. However, RUX causes cytopenias in PMF and GVHD, potentially limiting its use in BMF patients. In our animal work, RUX did induce mild anemia in normal mice although neutrophils (NEU) and platelets (PLT) were unaffected in a 2-week short-term toxicity study.[5] Herein, we have further assessed long-term RUX hematoxicity in normal mice. All animal studies were approved by the Animal Care and Use Committee at the National Heart, Lung, and Blood Institute. RUX-chow was first administered to achieve a dose of ~60 mg/kg (full dose). Extended feeding in normal CByB6F1 mice for 4 and 12 weeks moderately decreased white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), and lymphocytes (LYM), but did not affect NEU or PLT (*Online Supplementary Figure S1A, B;* Figure 1A). Reduction in proportions of peripheral blood $CD4^+$ and $CD8^+$ T cells were also seen (*Online Supplementary Figure S1B;* Figure 1A). In contrast to the high rates of thrombocytopenia in PMF and GVHD patients treated with RUX,[2-4] we did not observe a reduction of PLT in normal mice after extended RUX treatment. Previously we had also observed that in BMF mice, PLT in animals receiving RUX recovered rapidly compared with untreated mice.[5] Our observations are in agreement with a report that RUX may stimulate CD41/CD42b expression and megakaryocyte differentiation in human K562 and Meg-01 cells *in vitro* and augment PLT production *in vivo* in an irradiation mouse model.[7] Of interest, WBC (both NEU and LYM) and HGB returned to normal levels after RUX withdrawal, indicating that RUX-associated hematopoietic side-effects are transient. RUX reduced $CD8^+$ more than $CD4^+$ T cells in blood (*Online Supplementary Figure S1B;* Figure 1A). RUX reduced total BM cell numbers at 10 weeks (*Online Supplementary Figure S1C*) and 16 weeks (Figure 1B) but did not affect $Lin^-Sca\text{-}1^+CD117^+$ cells (KSL), myeloid progenitor cells (MP), or lymphoid progenitor cells (CLP). RUX did not affect the *in vitro* function of HSPC evidenced by colony forming unit (CFU) assay (*Online Supplementary Figure S1D;* Figure 1C), similar to our earlier observations in the short-term study, indicating that RUX hematoxicity in mice is relatively mild and reversible.[5] Most importantly, RUX-treated donor BM cells showed normal ability to engraft lethally irradiated recipient mice at different dilutions in an irradiation protection assay (*Online Supplementary Figure S1E;* Figure 1D). In the long-term toxicity study, Lin^-CD117^+ cells from RUX-treated and normal donor BM cells had comparable molecular features, displaying similar transcriptome distribution in multidimensional scaling plot (Figure 1E), further confirming minimal impact on HSPC by RUX.

In an attempt to reduce the dose and therefore toxicity of RUX while also retaining efficacy, we treated BMF mice with a combination of CsA and low-dose RUX. To allow for dose reduction of RUX, gavage rather than chow was used. CByB6F1 mice were pre-irradiated with 5 Gys total body irradiation (TBI) followed by injection of 5×10^6 lymph node (LN) cells from C57BL/6 donors to induce BMF[8] (Figure 2A). Both CsA and RUX were administered at low doses: CsA at 25 mg/kg (insufficient for the treatment of murine immune BMF[9]) and RUX 15 mg/kg twice daily gavage (BID, below typical therapeutic doses of 30-90 mg/kg BID in mice[10,11]) to keep stable drug concentration because RUX has a short terminal half-life of approximately 3 hours. Using low-dose RUX or CsA monotherapy as controls, we found that RUX and CsA combined therapy significantly improved WBC, NEU, RBC, and PLT 2 weeks after BMF initiation (Figure 2B). combined-therapy but neither monotherapy reduced blood $CD4^+$ and $CD45R^+$ cells; all treatment groups had decreased blood $CD8^+$ T cells relative to BMF mice, with RUX and CsA combined therapy group having the lowest frequencies of $CD8^+$ T cells (Figure S2A). Combined therapy also reduced Fas expression and apoptosis of blood non-T cells (*Online Supplementary Figure S2A*). Combined therapy increased residual BM cells (RBM, excluding T cells), suppressed $CD4^+$

and CD8⁺ T cells, reduced RBM apoptosis, and increased RBM viability (Figures 2C), when compared to BMF mice with or without monotherapy with RUX or CsA. Furthermore, combined therapy reduced expression of PD-1, FasL, CD25, and CD38 in BM CD4⁺ and CD8⁺ T cell, while RUX or CsA monotherapy reduced these activation and functional markers only in CD4⁺ but not in CD8⁺ T cells (*Online Supplementary Figure S2B*). Thus, low-dose RUX and CsA combined therapy effectively suppressed T-cell activation and alleviated immune-mediated BM destruction.

In a long-term survival study, we monitored animals for 12 weeks: all untreated BMF mice died within 3 weeks, and 90% of BMF mice in the low-dose RUX or CsA monotherapy groups were dead by 12 weeks. In contrast, 70% (7/10) of mice in the low-dose RUX and CsA combination therapy group survived to the end of the 12-week study (Figure 2D). The surviving mice had similar NEU, RBC, and PLT counts to normal control CByB6F1 mice (*Online Supplementary Figure S2C*). Despite having lower total BM cells (*Online Supplementary Figure S2C*) and a lower proportion and total number of myeloid progenitors (MP; *Online Supplementary Figure S2D*), the mice who received RUX and CsA had a higher proportion and total number of KSL cells (*Online Supplementary Figure S2D*) with normal CFU frequencies in the BM (*Online Supplementary Figure S2E*). Thus, low-dose RUX and CsA combined therapy augmented hematopoietic recovery and significantly increased animal survival with restored HSPC functionality. Findings from our study are compatible with reports showing that combined therapy of RUX and other agents may enhance therapeutic efficacy.[12-14]

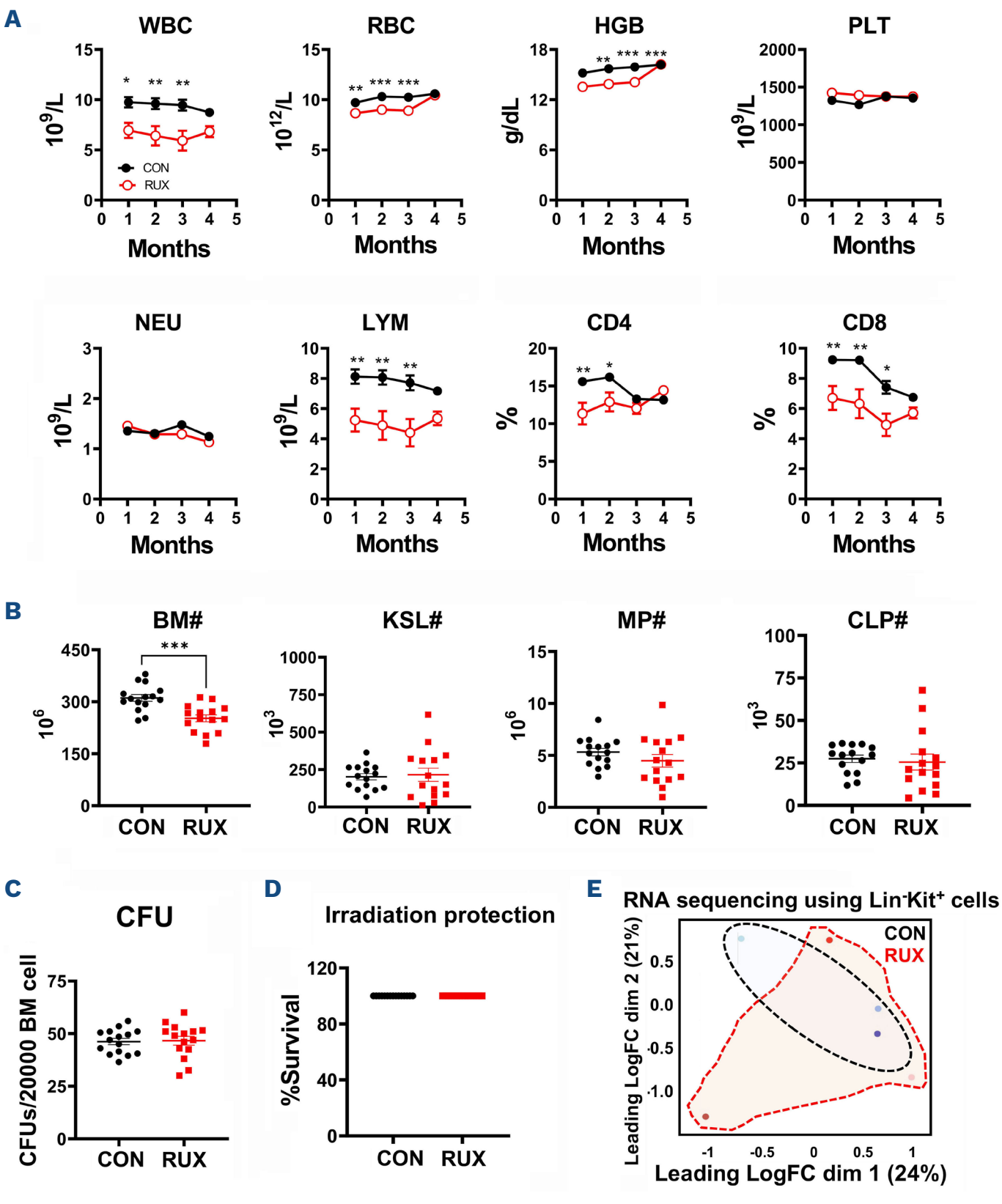


Figure 1. Hematoxicity of ruxolitinib in normal mice. (A) Peripheral white blood cell (WBC), neutrophil (NEU), red blood cell (RBC), hemoglobin (HGB), platelet (PLT), lymphocyte (LYM) counts and CD4 and CD8 percentages during 16 weeks. (B) Total bone marrow (BM) cell numbers, Lin⁻Sca-1⁺CD117⁺ (KSL), myeloid progenitor (MP), and common lymphoid progenitor cell (CLP) numbers in the BM at 16 weeks. (C) Colony forming unit (CFU) assay with BM cells at 16 weeks. (D) Irradiation protection assay with ruxolitinib (RUX)-treated or normal control (CON) donor BM cells at 16 weeks at 1:128 dilution to transplant into lethally irradiated CByB6F1 recipient mice. Survival of recipients was monitored and recorded for 30 days. (E) RNA sequencing: multidimensional scaling plot of Lin⁻CD117⁺ cells from RUX-treated mice at 16 weeks and from CON mice. Data are available under GEO series accession number GSE240867. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. LogFC: log fold change.

In order to determine molecular changes in T cells post therapies, BM CD8⁺ and CD4⁺ T cells from untreated BMF mice and those treated for 2 weeks were subjected to RNA sequencing (Figure 3). Transcriptomes of BM CD8⁺ T cells of combination therapy mice had a distinct distribution from low-dose RUX or CsA monotherapy groups; both monotherapy groups overlapped while all three treatment groups separated from untreated BMF group in the

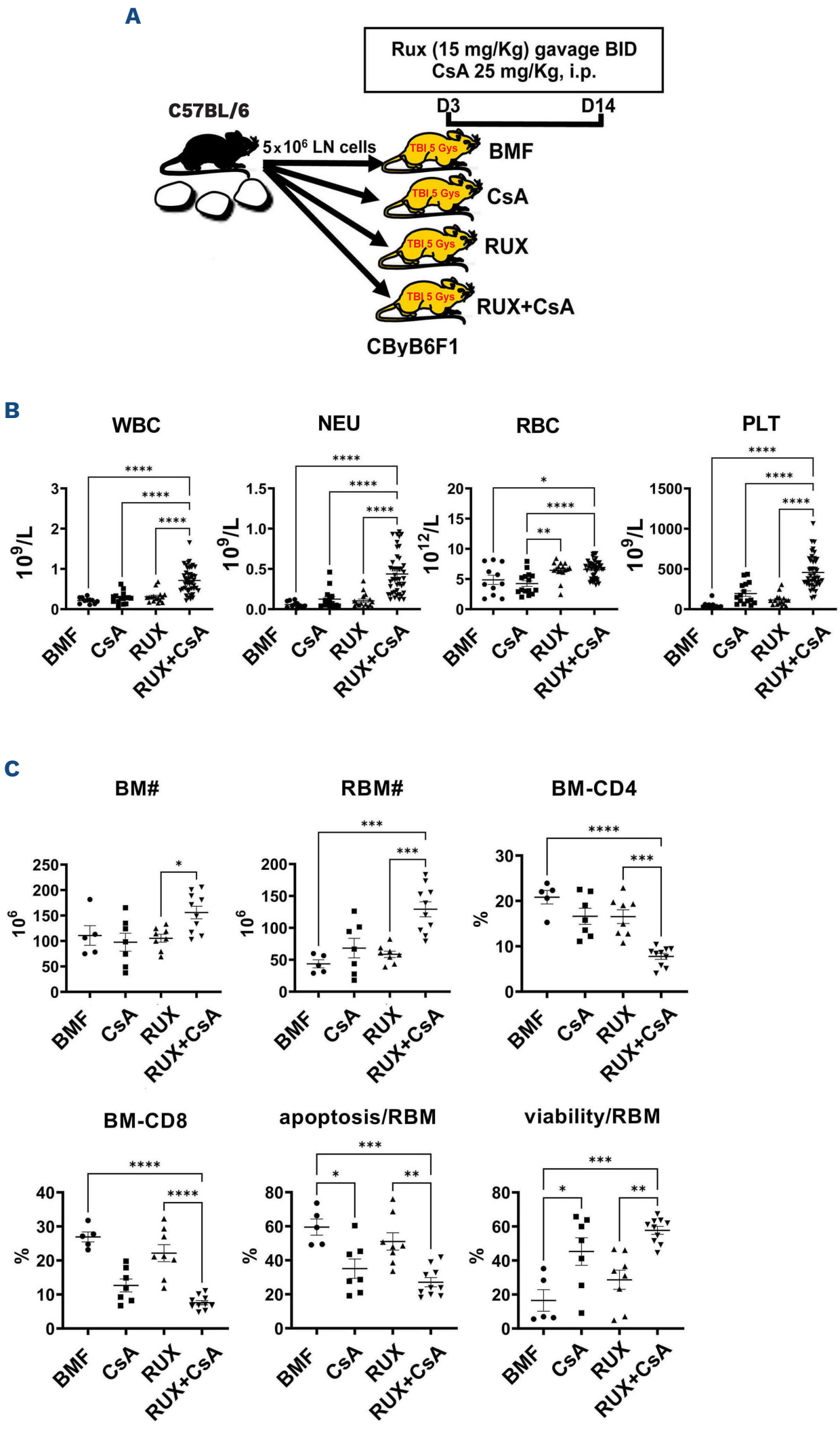
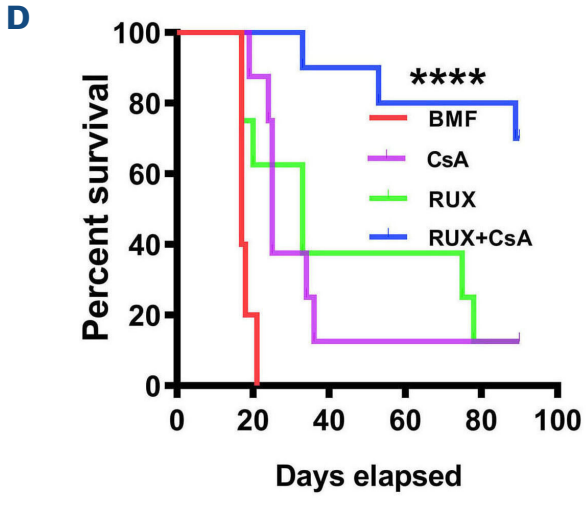


Figure 2. Therapeutic effects of low-dose ruxolitinib, ruxolitinib, and ruxolitinib plus ruxolitinib combination on murine immune bone marrow failure. (A) In order to induce bone marrow failure (BMF), 8-week old female CByB6F1 mice were pre-irradiated at 5 Gys total body irradiation (TBI) and infused with 5×10^6 lymph node (LN) cells/mouse from C57BL/6 female donors. BMF mice were untreated (BMF), or were treated with low-dose ruxolitinib (RUX, 15 mg/kg gavage twice daily, 5 days/week for 2-3 weeks, Incyte Corporation, Wilmington, DE) or low-dose cyclosporine A (CsA, 25 mg/kg i.p. once daily, 5 days/week for 2 weeks, Perrigo, Minneapolis, MN) monotherapy, or RUX and CsA combination therapy (RUX+CsA). All treatments started at day 3 following LN infusion. Animals were bled and euthanized at 2 weeks for cellular analyses, or were kept for 12 weeks to monitor survival. (B) Mice were bled at day 14 after LN cell infusion to analyze white blood cells (WBC), neutrophils (NEU), red blood cells (RBC), and platelets (PLT). (C) In one study (N=5, 8, 8, 10 for BMF, CsA, RUX, and RUX+CsA groups), mice were euthanized at day 14 and BM cells were extracted from bilateral tibiae and femurs to analyze BM cell counts, residual BM cell counts (RBM, BM cells excluding T cells), and proportions of CD4⁺ T cells, CD8⁺ T cells, apoptosis and viability of RBM. (D) In another study (N=5, 7, 8, and 10 for BMF, CsA, RUX, and RUX+CsA groups), mice were monitored for 12 weeks after 3-week treatment to record animal survival. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.



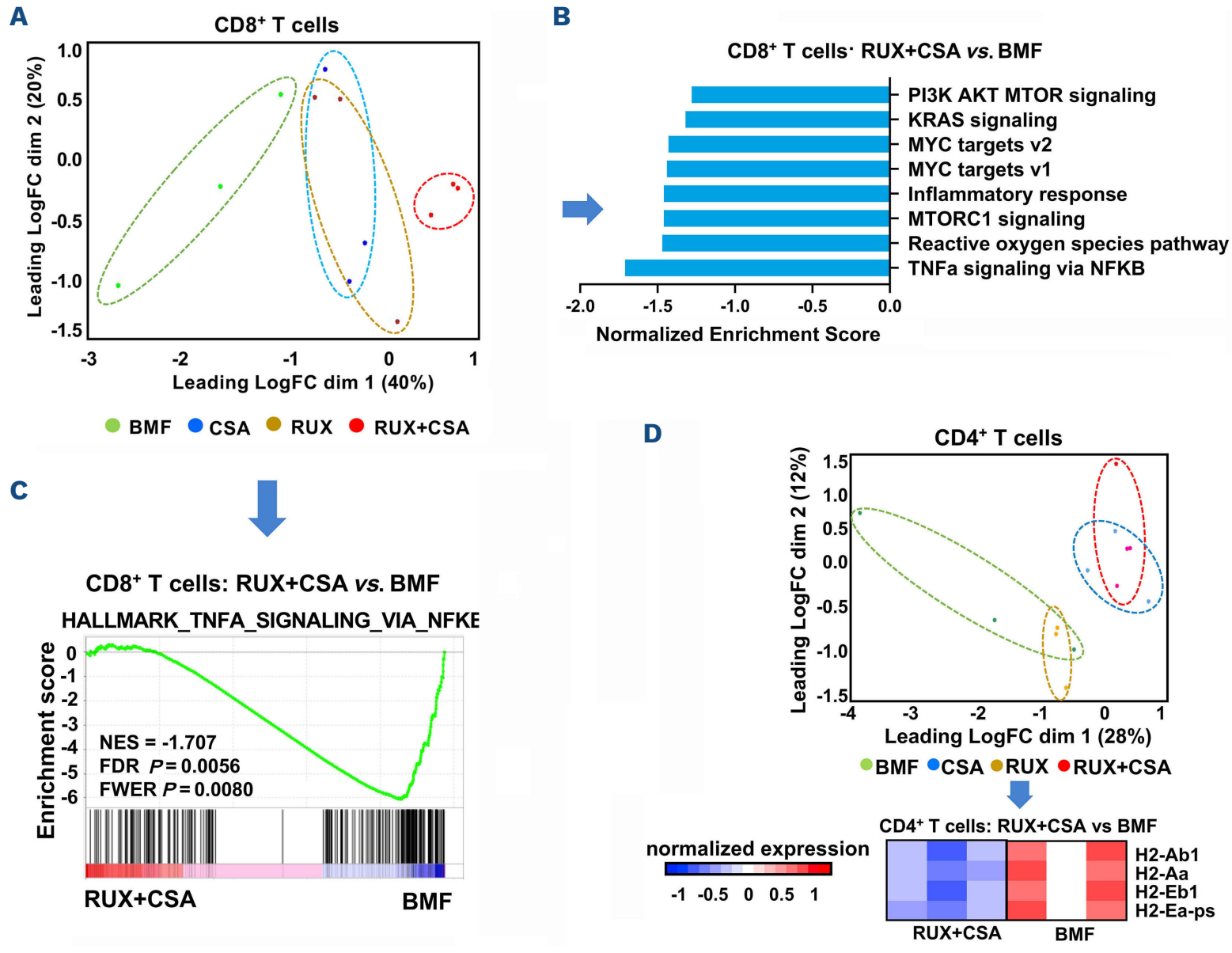


Figure 3. RNA sequencing of bone marrow T cells from untreated and treated bone marrow failure mice. Bone marrow (BM) CD4⁺ and CD8⁺ T cells were sorted from mice (N=5, 8, 8, 10 for bone marrow BM failure [BMF], cyclosporine A [CsA], ruxolitinib [RUX], and RUX+CsA groups) at 2 weeks, and pooled into 3 samples/group. RNA was extracted from pooled samples and applied to RNA sequencing. (A) CD8⁺ transcriptome distribution of untreated BMF, RUX, CsA, and RUX+CsA-treated BMF mice in multidimensional scale plot. (B) Top gene sets identified by Genomatrix Generanker to be downregulated in BM infiltrated CD8⁺ T cells from RUX+CsA-treated mice, compared to those from untreated BMF control mice. (C) Gene set enrichment analysis of CD8⁺ T cells in RUX+CsA *versus* untreated BMF. NES: normalized enrichment score. (D) CD4⁺ transcriptome distribution of untreated BMF, RUX, CsA, and RUX+CsA-treated BMF mice in multidimensional scale plot, and heat map of MHC-II genes downregulated in BM infiltrated CD4⁺ T cells from RUX+CsA-treated *versus* untreated BMF control mice. A red-blue color scale depicts gene expression levels (red indicates high, blue low). Data are available under GEO series accession number GSE240867.

multidimensional scaling plot (Figure 3A). Pathway analysis revealed immune activation and proliferation pathways related to the pathogenesis of BMF to be suppressed in CD8⁺ T cells of mice receiving combination therapy, compared with untreated BMF mice, such as PI3K, AKT, mTOR signaling, KRAS signaling, MYC targets, inflammatory response, and TNFα signaling pathways (Figure 3B). Gene set enrichment analysis also demonstrated that TNFα signaling in CD8⁺ T cells in combination therapy was suppressed (Figure 3C). In the multidimensional scaling plot of CD4⁺ T cells, transcriptome distribution of combination therapy overlapped with CSA monotherapy. Although no enriched pathways were found in BM CD4⁺ T cells, MHC-II gene expression that was previously found to be elevated in BMF[15] was suppressed by combination therapy (Figure 3D), suggesting inhibition of T-cell activation, consistent with flow cytometry results. In summary, RUX hematoxicity is mild and reversible in normal mice, mainly affecting red blood cells. Low-dose RUX and CsA combination therapy in BMF mice prolonged survival and resulted in sustained improvements in peripheral blood counts when compared to low-dose RUX or CsA monotherapy. Findings from this pre-clinical study support an approach to combine lower doses of RUX with CsA in patients to minimize hematologic toxicity.

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Disclosures

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Contributions

XF and JC designed research, performed experiments, analyzed data and wrote the paper. ALM, ZW, JD, NA, and HM performed experiments and analyzed results. HL, ZW, and SG analyzed RNAseq data. NSY and EMG designed research, analyzed data and edited the paper.

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Data-sharing statement

RNA sequencing data are available under GEO series accession number GSE240867.

References

1. Young NS. Aplastic Anemia. N Engl J Med. 2018;379(17):1643-1656.
2. Zeiser R, von Bubnoff N, Butler J, et al. Ruxolitinib for glucocorticoid-refractory acute graft-versus-host disease. N Engl J Med. 2020;382(19):1800-1810.
3. Zeiser R, Polverelli N, Ram R, et al. Ruxolitinib for glucocorticoid-refractory chronic graft-versus-host disease. N Engl J Med. 2021;385(3):228-238.
4. Harrison C, Kiladjian JJ, Al-Ali HK, et al. JAK inhibition with ruxolitinib versus best available therapy for myelofibrosis. N Engl J Med. 2012;366(9):787-798.
5. Groarke EM, Feng X, Aggarwal N, et al. Efficacy of JAK1/2 inhibition in murine immune bone marrow failure. Blood. 2023;141(1):72-89.
6. Huarte E, Peel MT, Verbist K, et al. Ruxolitinib, a JAK1/2 inhibitor, ameliorates cytokine storm in experimental models of hyperinflammation syndrome. Front Pharmacol. 2021;12:650295.
7. Yang S, Tang X, Wang L, et al. Targeting TLR2/Rac1/cdc42/JNK pathway to reveal that ruxolitinib promotes thrombocytopoiesis. Int J Mol Sci. 2022;23(24):16137.
8. Chen J, Lipovsky K, Ellison FM, Calado RT, Young NS. Bystander destruction of hematopoietic progenitor and stem cells in a mouse model of infusion-induced bone marrow failure. Blood. 2004;104(6):1671-1678.
9. Knospe WH, Steinberg D, Gratwohl A, Speck B. Experimental immunologically mediated aplastic anemia (AA) in mice: cyclosporin A fails to protect against AA. Int J Cell Cloning. 1984;2(4):263-271.
10. Das R, Guan P, Sprague L, et al. Janus kinase inhibition lessens inflammation and ameliorates disease in murine models of hemophagocytic lymphohistiocytosis. Blood. 2016;127(13):1666-1675.
11. Spoerl S, Mathew NR, Bscheider M, et al. Activity of therapeutic JAK 1/2 blockade in graft-versus-host disease. Blood. 2014;123(24):3832-3842.
12. Gagliardi TA, Milner J, Cairo MS, Steinberg A. Concomitant ruxolitinib and ibrutinib for graft-versus-host disease (GVHD): the first reported use in pediatric patients. Cureus. 2022;14(9):e29195.
13. Joly JA, Vallee A, Bourdin B, et al. Combined IFN-gamma and JAK inhibition to treat hemophagocytic lymphohistiocytosis in mice. J Allergy Clin Immunol. 2023;151(1):247-259.
14. Rai S, Grockowiak E, Hansen N, et al. Inhibition of interleukin-1beta reduces myelofibrosis and osteosclerosis in mice with JAK2-V617F driven myeloproliferative neoplasm. Nat Commun. 2022;13(1):5346.
15. Erie AJ, Samsel L, Takaku T, et al. MHC class II upregulation and colocalization with Fas in experimental models of immune-mediated bone marrow failure. Exp Hematol. 2011;39(8):837-849.