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

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Running title: Ruxolitinib with cyclosporine for immune bone marrow failure

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**Author 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.

**Conflict of interest:** Ruxolitinib was provided by Incyte Corporation, the manufacturers of ruxolitinib. Incyte did not have any input into the study design, data analysis, or presentation of results. NIH has a cooperative research and development with Novartis. No other authors have relevant COI.

**Data sharing statement:** RNA sequencing data are available under GEO series accession number GSE240867.
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. Immunosuppressive therapy (IST) with antithymocyte 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. 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. Among JAK inhibitors, ruxolitinib (RUX) is an orally-administrated selective ATP-competitive JAK1/2 kinase inhibitor, currently licensed to treat primary myelofibrosis (PMF) and graft versus host disease (GVHD). 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 Tregs. In our initial experiments, RUX was administered as either a food additive or via gavage at a standard dose equivalent to ~60 mg/Kg; 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. 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 CBByB6F1 mice for 4 and 12 weeks moderately decreased WBC, RBC, HGB, and lymphocytes (LYM), but did not affect NEU or PLT (Figures S1A&B, Figure 1A). Reduction in proportions of peripheral blood CD4+ and CD8+ T cells were also seen (Figure S1B, Figure 1A). In contrast to the high rates of thrombocytopenia in PMF and GVHD patients treated with RUX, 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. 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 platelet production in vivo in an irradiation mouse model. 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 (Figure S1B, Figure 1A). RUX reduced total BM cell numbers at 10 weeks (Figure S1C) and 16 weeks (Figure 1B) but did not affect Lin-Sca-1+CD117+ cells (KSL), myeloid progenitor cells (MP), or lymphoid progenitor cells (CLP). RUX did not affect the in vitro function of HSPCs evidenced by colony forming unit (CFU) assay (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. Most importantly, RUX-treated
donor BM cells showed normal ability to engraft lethally irradiated recipient mice at different dilutions in an irradiation protection assay (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 HSPCs 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 Gy total body irradiation (TBI) followed by injection of 5 ×10^6 lymph node (LN) cells from B6 donors to induce BMF (Figure 2A). Both CsA and RUX were administered at low doses: CsA at 25 mg/Kg (insufficient for the treatment of murine immune BMF) and RUX 15mg/kg BID (twice daily gavage, below typical therapeutic doses of 30-90 mg/Kg BID in mice) 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 two 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 (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 (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 of 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 (Figure S2C). Despite having lower total BM cells (Figure S2C) and a lower proportion and total number of myeloid progenitors (MP, Figure S2D), the mice who received RUX and CsA had a higher proportion and total number of KSL cells (Figure S2D) with normal CFU frequencies in the BM (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.

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 3 treatment groups separated from untreated BMF group in the 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.
References


Figures & legends

**Figure 1. Hematoxicity of ruxolitinib (RUX) 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 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 RUX-treated or normal control (CON) donor BM cells at 16 weeks at 1:128 dilution to transplant into lethally irradiated CBByB6F1 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.

**Figure 2. Therapeutic effects of low-dose ruxolitinib (RUX), cyclosporine A (CsA), and RUX+CsA combination on murine immune bone marrow failure (BMF).** A) To induce BMF, eight-week old female CBByB6F1 mice were pre-irradiated at 5 Gy total body irradiation (TBI) and infused with 5 ×10⁶ lymph node (LN) cells/mouse from B6 female donors. BMF mice were untreated (BMF), or were treated with low-dose RUX (RUX, 15 mg/Kg gavage twice daily, 5 days/week for 2-3 weeks, Incyte Corporation, Wilmington, DE) or low-dose CsA (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, 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 BM T cells from untreated and treated BMF mice. BM CD4$^+$ and CD8$^+$ T cells were sorted from mice (N=5, 8, 8, 10 for BMF, CsA, 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 vs 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 vs 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.
Fig. 1

A

- WBC
- RBC
- HGB
- PLT

Months:

- CON
- RUX

B

- BM#
- KSL#
- MP#
- CLP#

- CON
- RUX

C

- CFU

CFUs/20000 BM cells:

- CON
- RUX

D

Irradiation protection

% Survival:

- CON
- RUX

E

RNA sequencing using Lin\(^+\)Kit\(^+\) cells

Leading LogFC dim 2 (21%)

Leading LogFC dim 1 (24%)
Letter to Editor

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Supplemental figures
Supplemental Figure 1. Hematoxicity of ruxolitinib (RUX) in normal mice. A) Normal CB\(\text{ByB6F1}\) mice were fed with standard Purina 5002 Rodent chow without (CON) or the addition of 2000 mg/Kg of INCB01842 (RUX, Incyte Corporation, Wilmington, DE; composed by Research Diets, Inc, New Brunswick, NJ) for 4 weeks [N=8 for normal control mice (CON), N=10 for RUX-chow feeding mice (RUX)] or 12 weeks (N=15 for CON, N=15 for RUX) respectively in 2 separate experiments, and then switched back to standard chow until animals were bled and euthanized at 10 weeks or 16 weeks to evaluate RUX toxicity. BM cells from RUX-treated or CON mice at different dilutions were used for BM transplant into lethally irradiated (11 Gy) CB\(\text{ByB6F1}\) recipient mice, the survival was recorded at 30 days. B) Peripheral white blood cell (WBC), neutrophil (NEU), red blood cell (RBC), hemoglobin (HGB), platelet (PLT), lymphocyte (LYM) counts and CD4 and CD8 percentages during 10 weeks. C) Total BM cell numbers, Lin\(^{-}\) Sca-1\(^{+}\)CD117\(^{+}\) (KSL), myeloid progenitor (MP), and common lymphoid progenitor cell (CLP) numbers in the BM at 10 weeks. D) Colony forming unit (CFU) assay with BM cells at 10 weeks. E) Irradiation protection assay with RUX-treated or CON donor BM cells at 10 weeks at different dilutions to transplant into lethally irradiated CB\(\text{ByB6F1}\) recipient mice. Survival of recipients was monitored and recorded for 30 days. *, P<0.05; **, P<0.01; ***, P<0.001, ****, P<0.0001.
Supplemental Figure 2. Therapeutic effects of low-dose ruxolitinib (RUX), cyclosporine A (CsA), and RUX+CsA combination on murine immune bone marrow failure (BMF). A) Proportions of peripheral blood CD4+, CD8+, and CD45R+ lymphocytes, as well as Fas expression and apoptosis in blood non-T cells at day 14 after BMF initiation. B) PD-1, FasL, CD25, and CD38 on BM CD4+ and CD8+ T cells at day 14 after BMF initiation. C-D: Survival study. At the end of 12-week observation, survived RUX+CsA-treated mice (N=7) were bled and euthanized and were compared with normal mice (CON, N=5) to examine hematopoietic recovery: complete blood counts (WBC, NEU, RBC, and PLT) and total BM cells (C); Hematopoietic stem and progenitor cells including Lin−Sca-1−CD117+ (KSL) and myeloid progenitor (MP) cells (D); Colony-forming unit (CFU) assay with BM cells from survived RUX+CsA-treated BMF mice and normal CON mice (E). *, P<0.05; **, P<0.01; ***, P<0.001, ****, P<0.0001.