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Highlights

- Compensatory erythropoiesis occurs during termination of emergency granulopoiesis.
- In $Fanc^c$ mice, this induces DNA damage and apoptosis in erythroid progenitors.
- Ruxolitinib protected erythroid progenitors and enhanced oxidant-stress handling.
- Ruxolitinib prolonged survival in Fancc-/- mice during emergency granulopoiesis.
- $Fanc^c$ hematopoietic stem cells were also protected, and cell stress decreased.
Ruxolitinib ameliorates progressive anemia and improves survival during episodes of emergency granulopoiesis in Fanconi C⁻/ mice.

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Running title: Ruxolitinib improves survival in Fanconi C⁻/ mice.

Key words: DNA-repair, innate immunity, DNA-damage response, erythropoiesis, granulopoiesis, inflammasome

Abstract

Fanconi Anemia (FA) is an inherited disorder of DNA repair with hematologic manifestations that range from anemia to bone marrow failure (BMF) to acute myeloid leukemia (AML). In a murine model of FA (Fanc⁻/ mice), we found BMF was accelerated by repeated attempts to induce emergency (stress) granulopoiesis; the process for granulocyte production during the innate immune response. Fanc⁻/ mice exhibited an impaired granulocytosis response and died with profound anemia during repeated challenge.
In the current study, we found erythropoiesis and serum erythropoietin decreased in \textit{Fancc}^{-/-} and wild type (Wt) mice as emergency granulopoiesis peaked. Serum erythropoietin returned to baseline during steady state resumption, and compensatory proliferation of erythroid progenitors was associated with DNA-damage and apoptosis in \textit{Fancc}^{-/-} mice, but not Wt mice. The erythropoietin receptor activates Janus kinase 2 (Jak2) and we found treatment of \textit{Fancc}^{-/-} mice with ruxolitinib (Jak1/2-inhibitor) decreased anemia, enhanced granulocytosis, delayed clonal progression and prolonged survival during repeated emergency granulopoiesis episodes. This was associated with a decrease in DNA damage and apoptosis in \textit{Fancc}^{-/-} erythroid progenitors during this process. Transcriptome analysis of these cells identified enhanced activity of pathways for metabolism of reactive oxygen species, and decreased apoptosis and autophagy related pathways, as major ruxolitinib-effects in \textit{Fancc}^{-/-} mice. In contrast, ruxolitinib primarily influenced pathways involved in proliferation and differentiation in Wt mice. Ruxolitinib is approved for treatment of myeloproliferative disorders and graft versus host disease, suggesting the possibility of translational use as a bone marrow protectant in FA.

**Introduction**

Fanconi anemia (FA) is an inherited disorder caused by mutation of one of the 21+ Fanconi DNA-repair genes (1). The FA phenotype may include skeletal anomalies and various carcinomas (2,3). Hematopoietic abnormalities include anemia with progression to BMF in childhood, or clonal progression to AML in adolescence (4). Allogenic stem cell transplantation is the main approach to severe FA hematologic manifestations (5).

Fanconi proteins A-C, E-G, L and M constitute a ligase that mono-ubiquitinates Fanconi D2 and I (6,7). The latter two associate with DNA during S phase and, upon ubiquitination, assemble a complex that repairs DNA-cross links and rescues collapsed or stalled replication forks (8,9). Mice with engineered
disruption of Fanconi genes do not develop BMF or AML at steady state. Since the laboratory environment does not expose mice to the stress of infectious challenge, as experienced by human FA patients, we tested the impact of such challenges (10,11).

Emergency granulopoiesis is the process for granulocyte production during the innate immune response (10,12). It is characterized by S phase shortening and accelerated differentiation; decreasing time for replication fork rescue and stressing the Fanconi DNA-repair pathway (13,14). In mice, emergency granulopoiesis is studied by injecting pathogens or an antigen/adjuvant combination (Alum) (10,15). These stimuli induce equivalent IL1β-dependent responses, but Alum injection is not complicated by chronic infection or death (16,17). Wild type (Wt) mice tolerate multiple episodes of induced emergency granulopoiesis at four-week intervals; with maximal granulocytosis at two weeks and steady state resumption by four weeks (10,11). However, such stimuli did not induce granulocytosis in Fance−/− mice and most developed fatal anemia after several challenges (10,11).

In Fance−/− mice, hematopoietic stem cell (HSC) function was impaired by even a single Alum-injection, with AML developing in mice not succumbing to anemia during repeated challenges (10). Emergency granulopoiesis attempts in Fance−/− mice was associated with apoptosis of HSCs and differentiating granulocytes (10). Treatment with an IL1R antagonist prevented these complications, consistent with the role of IL1β in inflammasome-dependent emergency granulopoiesis (10,13). Tp53 haplo-insufficiency rescued emergency granulopoiesis in Fance−/− mice, decreased anemia and improved survival, but hastened AML (11).

Defining molecular mechanisms for progressive anemia during the innate immune response may suggest approaches to bone marrow protection in FA. In the current work, we hypothesize that erythroid suppression during emergency granulopoiesis is followed by a compensatory erythropoietic burst during steady state resumption. Impaired DNA-repair in FA may induce apoptosis via the DNA-damage response, resulting in anemia during this proliferative stress. Jak2-activation by the erythropoietin (Epo)
receptor mediates erythroid proliferation and differentiation (18); suggesting Jak-inhibition might protect FA erythroid progenitors. However, IL1β stimulates granulocyte colony stimulating factor (G-CSF) production, enhancing Jak2-activation in granulocyte progenitors during emergency granulopoiesis (10,19). And, HSCs from Jak1+ or Jak2+ mice exhibit impaired cell cycle entry or self-renewal (20,21). Therefore, Jak1/2-inhibition may blunt any emergency granulopoiesis response in FA and enhance HSC quiescence.

Ruxolitinib is Jak1/2-inhibitor approved for use in myeloproliferative neoplasms and graft vs host disease (22,23). Availability of ruxolitinib for translation to human use encouraged us to investigate it as a bone marrow protectant for FA in pre-clinical studies.

Methods

Mice and emergency granulopoiesis assays. Fancc+/− mice were a gift from D.W. Clapp (Indiana University, Indianapolis, IN) (24-26). Mice were injected intraperitoneally (IP) every four weeks with Alum (aluminium chloride/ovalbumin) or heat-killed Candida albicans for emergency granulopoiesis, or saline as a steady state control. Alum was prepared and injected as described (10,15). C. albicans cultures were grown at 30°C, inactivated at 70°C for 1 hour, and injected (50 x 10⁶/10 gms body weight) (27). Mice were treated with ruxolitinib (45 mg/kg IP 5 days/week; MedChemExpress, Monmouth Junction, NJ) or vehicle control (28).

Blood was obtained by tail vein phlebotomy and analyzed by automated cell counter at experiment initiation and every two weeks. May-Grünwald-Giemsa-stained blood smears were hand-counted for myeloid blasts (300 cells/slide) (10). Images were digitally captured by light microscopy (x40). Plasma Epo and IL1β was determined using commercial kits (RayBiotech, Norcross, GA).

Cohorts included 10 mice. There was no blinding and no animals were excluded from analysis. Mice
were sacrificed for hemoglobin <6.0 g/dl or deteriorating body condition score. Variance between versus within groups were assessed to assure significance.

**Flow cytometry for population distribution, apoptosis, cell cycle and DNA damage:** Bone marrow erythroid progenitors were identified by labeling with anti-mouse PE-CD71 (BD Biosciences, Franklin Lakes, NJ) or APC-Ter119 (Invitrogen, Carlsbad, CA). Bone marrow granulocyte-monocyte progenitors (GMP) or hematopoietic stem cells (LSK) were identified by lineage negative (Lin−) selection (Lineage Depletion Kit, Miltenyi Biotech, Auburn, CA), followed by anti-mouse FITC-Sca1, Brilliant Violet 421-CD117, PE-Cyanine7-CD16/CD32, and PE-CD34 staining (eBioscience, San Diego, CA).

Apoptosis of CD71+ or Lin−ckit+ cells was determined by Annexin V staining (Annexin V-PE Apoptosis Detection Kit, BD Pharmingen) (10). Other cells were labeled with 5-ethynyl-2′-deoxyuridine (EdU; 10 μM) and analyzed for S phase (Click-iT Assay System, Life Technologies, Carlsbad, CA) (29) and/or stained with phospho-H2AX (S139) PE antibody (Invitrogen) (30).

**Western blotting and phospho-Stat3 assay:** CD71+ murine bone marrow cells were erythropoietin stimulated for two days in DME with 10% fetal calf serum, 1% pen-strep, SCF, holotransferrin (R&D Systems, Minneapolis, MN). Lysates (30 μg) of CD71+ cells were separated by 10% SDS-PAGE, transferred to nitrocellulose, and serially probed with Fanconi C and GAPDH antibodies (31). Three independent lysates were analyzed, and representative blots shown.

Stat3 and pY705-Stat3 were quantified by ELISA (Cell Signaling Technology, Danvers, MA). Three independent lysates were analyzed in duplicate.
Quantitative real-time PCR and transcriptome analysis: RNA was isolated using TRIZOL reagent (Invitrogen). Real-time PCR primers were designed with Applied Biosystems software (Grand Island, NY) and PCR performed with SYBR green by the “standard curve” method. Three independent samples were evaluated in triplicate and normalized to Actin.

Stranded total RNA-seq was conducted in the Northwestern University NUSeq Core. Total RNA samples were checked for quality on Agilent Bioanalyzer 2100 (Agilent Biotech, Santa Clara, CA) and quantity with Qubit fluorometer (Thermo Fisher Scientific, Waltham PA). The Illumina TruSeq Stranded Total RNA Library Preparation Kit was used to prepare sequencing libraries (Illumina Biotech, San Diego, CA). Libraries were sequenced with an Illumina HiSeq 4000 Sequencer (single-end 50 bp reads).

Read quality was evaluated using FastQC. Adapters were trimmed and reads of poor quality or aligning to rRNA filtered by Trim Galore (www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Cleaned reads were aligned to the mouse genome (mm10) using STAR (36). Read counts for each gene were calculated using HTSeq-Counts in conjunction with a gene annotation file for mm10 from Ensembl (useast.ensembl.org/index.html) (37). A comprehensive QC report was generated using MultiQC (38). Differential expression was determined using DESeq2 (39). The cutoff for significant differences was an FDR-adjusted p-value <0.05. Metascape was used for pathway analysis (40).

Reporter assays. Murine Stat3 cDNA (Addgene, Cambridge, MA) was subcloned into the pcDNAamp vector (32). Stat3 MISSION shRNA in the pLKO plasmid was obtained from Sigma-Aldrich (St. Louis, MO). Knockdown was ≥70%. Scrambled control shRNAs were generated using the Promega website and subcloned into pLKO (33,34). Reporter constructs with pGL3-basic vector (Promega) and 1.0 kb of FANCC 5' flank were generated as described (10).

HEL cells were obtained from Dr. L.C. Platanias (Northwestern University, Chicago), maintained
under standard conditions and authenticated annually (35). Cells (32 × 10^6/ml) were transfected by electroporation with Stat3-expression or control vector, or shRNA Stat3 or control vector (30 μg), a firefly luciferase reporter construct with 1 kb FANCC 5' flank or empty pGL3 vector (20 μg), and a CMV/renilla luciferase reporter (0.5 μg, for transfection efficiency). Some transfectants were treated with 30 ng/ml of IL1β or erythropoietin for 24 hours (31). Six independent experiments were performed in duplicate.

**Statistical analysis:** Statistical significance was determined by one way ANOVA for comparisons that vary one factor, and by two way ANOVA for comparisons that varied two factors. SigmaPlot/Stat software was used for this analysis (SPSS Inc, Chicago, IL). P values ≤0.05 were considered statistically significant. Error bars represent ± SEM.

**Study approval and method sharing:** Animal studies were approved by the Animal Care and Use Committees of Northwestern University and Jesse Brown VA Medical Center. Original data is available from e-eklund@northwestern.edu.

**Results**

*Ruxolitinib increased survival of Fance−/− mice during emergency granulopoiesis.* To mimic repeated infectious challenges, we injected Fance−/− or Wt mice every four weeks with Alum to induce emergency granulopoiesis, or saline as a steady state control (10,11). Mice were treated daily with ruxolitinib (Jak1/2-inhibitor) or vehicle control (28). Although 50% of Fance−/− mice died after the second Alum injection, 80% of ruxolitinib treated mice survived three episodes (p<0.001, n=10 for Alum injected
*Fanc*−/− mice with versus without ruxolitinib) (Figure 1A) (10). Wt mice survived more than five such injections without morbidity, with or without ruxolitinib.

We evaluated circulating granulocytes in these mice during multiple episodes of injection with Alum or saline (as a steady state control) (Figure 1B). In Wt mice, the number of peripheral blood granulocytes two weeks post Alum injection was consistent over three injection episodes (one way ANOVA, p=0.2, n=10). Granulocyte counts two weeks post Alum injection in ruxolitinib treated Wt mice were also not significantly different over three injections (one way ANOVA, p=0.63, n=10), but were significantly less compared to Alum injection without ruxolitinib treatment (two way ANOVA, p=0.0001, n=10). In *Fanc*−/− mice, the number of peripheral granulocytes two week after Alum injection decreased over three injection episodes (one way ANOVA, p=0.004, n=10 at the start of the experiment). However, in ruxolitinib treated *Fanc*−/− mice, granulocyte counts at the two week time point post Alum injection were consistent during three injection cycles (one way ANOVA, p=0.32, n=10). Therefore, ruxolitinib treated *Fanc*−/− mice had increased circulating granulocytes two weeks after Alum injection, over three injections, compared to Alum injection without ruxolitinib treatment (two way ANOVA, p=0.003, n=10 at the start of the experiment).
Peripheral blood Hemoglobin (Hgb) decreased progressively over three Alum injection episodes in Fancc−/− mice (one way ANOVA, p<0.001, n=10 at the start of the experiment) (10,11). Hgb also decreased in ruxolitinib treated Fancc−/− mice during three Alum injection episodes (one way ANOVA, p<0.001, n=10 at the start), but this decrease was significantly less than in Alum injected Fancc−/− mice.
without ruxolitinib treatment (two way ANOVA, p=0.005, n=10 at start) (Figure 1C). Protection from progressive anemia correlated with survival.

Similarly, circulating platelets decreased during multiple Alum injections in Fancc−/− mice (one way ANOVA, p<0.001, n=10 at the start of the experiment) or in ruxolitinib treated, Alum injected Fancc−/− mice (one way ANOVA, p<0.001, n=10 at the start). However, ruxolitinib treatment significantly lessened this effect (two way ANOVA, p=0.02, n=10 at start) (Figure 1D). In Wt mice, neither Hgb concentration (two way ANOVA, p=0.44, n=10) nor platelet counts (two way ANOVA, p=0.52, n=10) were different over three episodes of injection of Alum versus saline. The addition of ruxolitinib treatment did not alter Hgb or platelet counts during multiple episodes of Alum injection in Wt mice (two way ANOVA, p>0.3, n=10 with versus without ruxolitinib for Hgb or platelet counts over the course of the experiment).

We obtained similar results in mice injected with heat killed Candida as an alternative method to activate emergency granulopoiesis (Supplemental Figure 1).

To investigate erythropoiesis during emergency granulopoiesis, we determined the effect of Alum-injection on plasma erythropoietin (Epo). In Wt and Fancc−/− mice, we found comparable plasma levels of Epo and IL1β prior to injection (p>0.5, n=6 for comparison between genotypes) (Supplemental Figure 2). Serum IL1β peaked two weeks after Alum injection in both genotypes but returned to pre-treatment levels by four weeks (one way ANOVA for three time points for each genotype p<0.01, n=6). IL1β levels were not different in Wt versus Fancc−/− mice over these three time points (two way ANOVA, p>0.4, n=6). Serum Epo fell two weeks after Alum injection in both genotypes (one way ANOVA for three time points for either genotype, p<0.002, n=6). Epo levels were not significantly different in the two genotypes at these three time points (two way ANOVA, p>0.3, n=6). Ruxolitinib did not influence these cytokines in either genotype (two way ANOVA p>0.5, n=6 with versus without ruxolitinib for Alum injected, Wt or Fancc−/− mice).
Ruxolitinib protected proliferating Fancc-/- erythroid progenitors and hematopoietic stem cells from DNA-damage and apoptosis during emergency granulopoiesis. In FA, we hypothesize that erythroid progenitor proliferation during steady state resumption induces apoptosis via the DNA-damage response. To determine the impact of emergency granulopoiesis on erythroid progenitor viability, we quantified CD71+AnnexinV- bone marrow cells (viable erythroid progenitors). We compared Alum-injected Wt or Fancc-/- mice to steady state controls two or four weeks after injection; peak emergency granulopoiesis versus steady state resumption. For these studies, flow cytometry results were used to calculate absolute numbers of cells in each category, based on the number of total bone marrow mononuclear cells that were harvested from femurs of each mouse.

In Wt mice, CD71+AnnexinV- cells decreased two weeks after Alum-injection, but returned to initial baseline value by four weeks (Figure 2A), indicating reversible suppression (one way ANOVA, p=0.003, n=6 for three time points). At steady state, there were significantly fewer viable CD71+ bone marrow cells in Fancc-/- mice compared to Wt (comparing two genotypes at three time points with saline injection, two way ANOVA, p=0.002, n=6) and ruxolitinib did not alter this (two way ANOVA, p=0.001, n=6) (Figure 2A). Alum induced a greater decrease in viable CD71+ cells in Fancc-/- mice versus Wt (comparing two genotypes at three time points, two way ANOVA, p<0.001, n=6). Four weeks after Alum injection, viable Fancc-/-CD71+ cells had not returned to pre-injection levels with or without ruxolitinib (one way ANOVA, p<0.001, n=6 for either comparison). However, the decrease in viable cells over the four weeks was less in ruxolitinib treated Fancc-/- mice compared to Alum-injection alone (two way ANOVA, p=0.015, n=6 for Alum with versus without ruxolitinib).
We next investigated proliferation (EdU+) and DNA damage (phospho-H2AX+) in Wt or Fancc-/- bone marrow erythroid progenitors during steady state resumption (Alum versus saline injection at 2.5 weeks). For these studies, flow cytometry results were expressed as the percent of the total CD71+
population that was EdU$^+$ or the percent of the CD71$^+$phospho-H2AX$^+$ population that was EdU$^+$. We found the percent of total CD71$^+$ cells that were EdU$^+$ was increased in Wt mice 2.5 weeks after injection of Alum compared to saline steady state control, with or without ruxolitinib (Figure 2B). These cells did not exhibit DNA damage (Figure 2B). Conversely, the percent of total CD71$^+$ bone marrow cells that were EdU$^+$ was less in Fance$^{+/-}$ mice 2.5 weeks after Alum injection compared to saline injected steady state control (Figure 2B), and the percent of CD71$^+$pH2AX$^+$ cells that were EdU$^+$ was increased with Alum versus saline control. At this time point, proliferating CD71$^+$ bone marrow cells were significantly more abundant in ruxolitinib treated, Alum-injected Fance$^{+/-}$ mice compared to mice injected with Alum alone, and the percent of proliferating CD71$^+$ with DNA damage was less (Figure 2B). This suggested ruxolitinib protected Fance$^{+/-}$CD71$^+$ cells from proliferation-related DNA damage and apoptosis.

We also studied these events in bone marrow Lin$^-\text{ckit}^+$ cells from Wt or Fance$^{+/-}$ mice two weeks post injection with Alum or saline. For these studies, the lineage negative population was selected by antibody depletion chromatography followed by flow cytometry to identify the ckit$^+$ subpopulation. In saline injected steady state controls, we found significantly fewer viable Lin$^-\text{ckit}^+$ cells in Fance$^{+/-}$ mice compared to Wt at two weeks after injection, with or without ruxolitinib treatment (Figure 2C). Although viability of Lin$^-\text{ckit}^+$ cells from Alum versus saline injected Wt mice was not different at this time point (one way ANOVA, p=0.6, n=6), apoptosis of Fance$^{+/-}$ Lin$^-\text{ckit}^+$ cells was enhanced by Alum versus saline injection (one way ANOVA, p=0.002, n=6). However, apoptosis of Fance$^{+/-}$ Lin$^-\text{ckit}^+$ cells was not different in ruxolitinib treated, Alum injected mice versus saline injected steady state controls (one way ANOVA, p=0.1, n=6) (Supplemental Figure 3).

Alum-injection decreased the percent of the total Fance$^{+/-}$ Lin$^-\text{ckit}^+$ cell population that was EdU$^+$ at 2.5 weeks compared to saline control, but increased percent of Lin$^-\text{ckit}^+$pH2AX$^+$ cells that were EdU$^+$ (one way ANOVA, p<0.02, n=6 for either comparison with Alum versus saline) (Figure 2D). Ruxolitinib reversed these Alum-effects in Fance$^{+/-}$ bone marrow at this time point; increasing the percent of total Lin$^-$
ckit⁺ cells that were EdU⁺ and decreasing the percent of proliferating cells with DNA damage (one way ANOVA, p=0.003, n=6 for comparison of Alum injected mice with versus without ruxolitinib) (Supplemental Figure 3).

In Wt bone marrow, Alum increased the percent of Lin⁻ckit⁺ cells that were EdU⁺ at 2.5 weeks compared to saline injected controls (Figure 2D). However, there were significantly more EdU⁺ cells with DNA-damage in Wt mice injected with Alum versus Alum plus ruxolitinib treatment at this time point (one way ANOVA, p=0.002, n=6). Therefore, ruxolitinib selectively protected proliferating Fancc⁻/⁻ or Wt Lin⁻ckit⁺ from DNA-damage during emergency granulopoiesis.
Ruxolitinib stabilized Fancc<sup>-/-</sup> erythroid progenitors and hematopoietic stem cells during emergency granulopoiesis. We next determined if ruxolitinib increased abundance of bone marrow erythroid progenitors during emergency granulopoiesis in Fancc<sup>-/-</sup> mice. As described above, data from flow cytometry was used to calculate absolute numbers of cells in various populations, based on numbers of total bone marrow mononuclear cells in murine femurs. In Wt mice, there were significantly fewer differentiating erythroid progenitors (Ter119<sup>+</sup> cells) two weeks after injection of Alum compared to saline injection (Figure 3A). At four weeks, numbers of these cells were equivalent in Alum and saline injected mice (one way ANOVA, p=0.5, n=6). Wt Ter119<sup>+</sup> cells were more abundant two weeks post Alum
injection with versus without ruxolitinib treatment (one way ANOVA, p=0.02, n=6). Abundance of Ter119⁺ bone marrow cells was not significantly different in saline injected Fance⁻/⁻ mice versus Wt (two way ANOVA, p=0.15, n=6 at the two time points). However, there were significantly fewer Fance⁻/⁻ Ter119⁺ cells compared to Wt two weeks after Alum injection (Figure 3A). Abundance of Fance⁻/⁻ Ter119⁺ bone marrow cells was not different at two versus four weeks post Alum injection (one way ANOVA, p=0.22, n=6), but was less than cells from saline injected Fance⁻/⁻ mice at these two time points (two way ANOVA, p=0.004, n=6). Fance⁻/⁻ Ter119⁺ bone marrow cells were less abundant two or four weeks post Alum injection compared to cells from ruxolitinib treated, Alum injected mice at these time
points (two way ANOVA, p=0.01, n=6).

We also considered the effect of ruxolitinib on LSK cells. In Wt mice, there were fewer LSK cells two weeks after injection with Alum versus saline control (Figure 3B). There were also fewer LSK cells in ruxolitinib treated, Alum injected Wt mice versus saline injected mice at this time point (one way ANOVA, p=0.02, n=6). However, at four weeks, Wt LSK cell abundance was not different after Alum versus saline injection (one way ANOVA, p=0.04, n=6). Bone marrow LSK cells were less abundant two weeks after Alum injection in Fance⁻/⁻ mice compared to Wt mice (Figure 3B). At two and four weeks, Alum injected, ruxolitinib treated Fance⁻/⁻ mice had more LSK cells than Alum alone (two way ANOVA, p<0.001, n=6 with versus without ruxolitinib).

We also investigated the impact of ruxolitinib on bone marrow granulocyte/monocyte progenitor cells (GMP) and maturing granulocytes. Consistent with peripheral blood counts, numbers of either GMP or Gr1⁺ cells two weeks after Alum-injection in Wt mice versus saline injection were less with Jak1/2-inhibition (Figure 3C and 3D). GMPs or granulocytes were more abundant two weeks after Alum-injection in ruxolitinib treated Fance⁻/⁻ mice compared to mice injected with Alum alone (Figure 3C and 3D); an effect consistent with improved granulocytosis.

Ruxolitinib treatment of Fance⁻/⁻ mice during emergency granulopoiesis increased anti-oxidant pathways in erythroid progenitors and decreased activity of apoptotic pathways in HSCs. To investigate mechanisms for effects of ruxolitinib on Fance⁻/⁻ murine bone marrow, we performed transcriptome analysis of CD71⁺Ter119⁺ or Lin⁻ckit⁺ cells. We performed RNA-Seq on cells isolated from Wt or Fance⁻/⁻ mice three weeks after Alum-injection (during steady state resumption), with or without ruxolitinib. Gene ontology analysis was applied to identify differences in pathway activity between groups.
Ruxolitinib treatment of Alum-injected mice significantly altered erythroid progenitor transcriptomes, but effects were different in the two genotypes. In Fanc^c^- CD71^+
Ter119^+ cells, ruxolitinib treatment of Alum-injected mice increased activity of pathways involved in catabolism of reactive oxygen species, erythrocyte development, negative regulation of apoptotic signaling and autophagy (Figure 4A). Conversely, activity of pathways involved in carbohydrate and protein catabolism was decreased in Fanc^c^- mice by ruxolitinib during emergency granulopoiesis (Figure 4A). In contrast, the major effect of ruxolitinib CD71^+
Ter119^+ cells from Alum-injected Wt mice was decreased activity of cell cycle, cell division and cell growth pathways (Figure 4B).
We confirmed RNA-Seq results for genes involved in metabolism of reactive oxygen species (Cat and Gpx1) in independent RNA quantification experiments with CD71\textsuperscript{+}Ter119\textsuperscript{+} bone marrow cells isolated 3 weeks after injection of Alum or saline; with versus without ruxolitinib (Figure 4C). Ruxolitinib effects on transcriptomes in Wt erythroid progenitors were consistent with inhibition of Jak2 signaling pathways involved in proliferation. In contrast, transcriptomes in F ance\textsuperscript{--} erythroid progenitors indicated protection from oxidant stress.

Ruxolitinib also differentially altered Lin\textsuperscript{+}ckit\textsuperscript{+} transcriptomes in Alum-injected F ance\textsuperscript{--} mice compared to Alum-injected Wt. In F ance\textsuperscript{--}Lin\textsuperscript{+}ckit\textsuperscript{+} cells from Alum-injected mice, ruxolitinib decreased activity of pathways involved in mitosis, apoptosis, respiratory electron transport and endoplasmic reticulum stress, but increased activity in pathways mediating myeloid leukocyte differentiation and

![Figure 5: Jak1/2-inhibition during emergency granulopoiesis normalized transcriptomes in F ance\textsuperscript{--} hematopoietic stem cells.](image)

Wt or F ance\textsuperscript{--} mice were injected with Alum to induce emergency granulopoiesis, and treated daily with a Jak1/2 inhibitor (ruxolitinib) or vehicle control. Mice were sacrificed at 3 weeks and Lin\textsuperscript{+}ckit\textsuperscript{+} bone marrow cells analyzed by RNA-Seq. Gene ontology identified pathway activities altered by ruxolitinib in Alum-injected; A. F ance\textsuperscript{--} mice versus B. Wt mice. Differences indicated as \text{-log10}(P). C. Expression differences were confirmed for key genes in independent experiments. One way ANOVA was used to determine differences in expression of individual genes for various conditions, as indicated by a symbol and connecting bracket. Comparisons are not made between genes. For Aldh18a1 or Slfn2, comparison of Alum treatment in Wt versus FA indicated by \text{* (p<0.02 for either comparison); and FA mice treated with Alum versus Alum plus ruxolitinib by \text{**}(p<0.03 for either). For Tcirg4 or Ptpn4, comparison of FA
mRNA translation (Figure 5A). In Wt Lin’ckit’ cells from Alum-injected mice, ruxolitinib decreased activity of pathways mediating MAPK and intracellular signaling, and leukocyte differentiation (Figure 5B). This indicated enhanced granulocytosis in ruxolitinib treated Fancec mice during emergency granulopoiesis, but inhibition in Wt, consistent with peripheral blood and bone marrow analysis.

We confirmed some transcriptome differences in independent studies with Lin’ckit’ cells from Alum-injected mice (Figure 5C). This included differential expression of Aldh genes (known to protect FA cells from DNA damage; 41), and genes involved in growth/differentiation during immune processes (Slfn2 and Ptn14).
Ruxolitinib delayed AML progression in Fancc<sup>−/−</sup> mice during repeated episodes of emergency granulopoiesis. We found that AML had developed in all Fancc<sup>−/−</sup> mice surviving three or more Alum injections (Figure 6A) (10,11). In contrast, ruxolitinib treatment of Fancc<sup>−/−</sup> mice resulted in survival of 80% of mice through three Alum injections, with AML developing in only ~20% of survivors. This was reflected in a greater abundance of circulating myeloid blasts in Alum injected Fancc<sup>−/−</sup> mice in the absence of ruxolitinib treatment at eight and twelve weeks post Alum injection (two way ANOVA, p<0.001 comparing two treatment conditions at two time points) (Figure 6B). In these studies, AML was defined as >10% myeloid blasts in the bone marrow or circulation, based on histology (Figure 6C). Consistent with this, bone marrow flow cytometry at eight weeks identified increased absolute numbers
of LSK cells and differentiation block, as indicated by a decrease in Gr1+ cells, in Alum-injected Fancc−/− mice that were not treated with ruxolitinib (Figure 6D). We also found a statistically significant increase in spleen size in mice with versus without AML; with or without ruxolitinib.

We investigated mechanisms of leukemogenesis by analyzing transcriptomes of Lin−ckit+ bone marrow cells from Fancc−/− mice two weeks after the third Alum injection. Ruxolitinib treated, Alum-injected Fancc−/− mice had not developed AML at this point, but AML was found in surviving Alum-injected Fancc−/− mice not receiving ruxolitinib (Figure 6B).

Ruxolitinib treatment of Alum-injected Fancc−/− mice decreased activity of pathways involved in

![Figure 7: Transcriptome changes in Fancc−/− hematopoietic stem cells occurred during emergency granulopoiesis-induced clonal progression. Wt or Fancc−/− mice were injected with Alum to induce emergency granulopoiesis every four weeks, and treated daily with a Jak1/2-inhibitor (ruxolitinib) or vehicle control. Mice were sacrificed at 3 weeks in the 3rd cycle and Lin−ckit+ bone marrow cells analyzed by RNA-Seq. A. Gene ontology identified pathway activity altered by ruxolitinib in Alum-injected Fancc−/− mice. Differences are indicated as -log10(P). B. Gene expression differences were confirmed for key genes in independent experiments. For individual genes, one way ANOVA was used to determine significant differences in expression of individual genes under various conditions, as indicated by a symbol and connection by a bracket. Comparisons are not made between different genes. For Aldh18a1, Npm1 or Prkcd, comparison of Alum treatment in Wt versus FA mice is indicated by * (p<0.01 for each comparison); and FA mice treated with Alum versus Alum plus ruxolitinib by ** (p<0.02 for each comparison). For Tcirg1, comparison of Wt mice treated with saline versus Alum is indicated by * (p=0.01); Alum treatment in Wt versus FA by ** (p<0.02); and FA mice treated with Alum versus Alum plus ruxolitinib by # (p=0.02).]
cytokine production, protein kinase B or cAMP signaling (Figure 7A). Conversely, ruxolitinib treatment increased pathways involved in differentiation/activation of myeloid leukocytes and pluripotency of stem cells; suggesting protection from differentiation block. Ruxolitinib increased apoptotic pathway activity, associating Alum-induced leukemogenesis with an impaired DNA-damage response. In independent experiments, we confirmed expression results including decreased Aldh and Npm1 with ruxolitinib, but increased expression of differentiation related genes that may be decreased in AML (Tcirg1, Prkcd) (Figure 7B).

_Erythropoietin activated the FANCC promoter in erythroid progenitors._ During emergency granulopoiesis, IL1β increases Fanconi C (FancC) expression in murine granulocyte/monocyte progenitors (10,11). In the current studies, we found Epo increased FancC mRNA (by one way ANOVA, p<0.02, n=3 for with versus without Epo) (Figure 8A) and protein (Figure 8B, total protein normalized to 1) in CD71+ murine bone marrow cells.
In granulocyte/monocyte progenitors, Stat3 activated the FANCC promoter via a proximal promoter cis element (10,31). To study this during erythropoiesis, we transfected an erythroblast cell line (HEL) with a 1.0 kb FANCC promoter-reporter construct. Stimulation with Epo, but not IL1β, increased FANCC promoter activity in HEL cells (by one way ANOVA, p<0.001, n=7 for untreated versus Epo treated transfectants, but p=0.8, n=7 for untreated versus IL1β treated transfectants, n=7) (Figure 8C). FANCC promoter activity was increased by Stat3 overexpression (by one way ANOVA, p<0.001, n=7 for Stat3 expression vector versus vector control), but decreased by Stat3 shRNA knockdown (by one way ANOVA, p<0.001, n=7 for Stat3 shRNA knockdown versus scrambled control).
Epo or IL1β enhanced *FANCC* promoter activation by overexpressed Stat3 (by one way ANOVA, p<0.001 for reporter activity in Stat3 transfected cells with versus without Epo; p=0.002 for reporter activity in Stat3 transfected cells with versus without IL1β). Expression of endogenous total and phospho-Stat3 protein in HEL cells was also increased by these cytokines (by one way ANOVA, p<0.05, n=3 for comparison of total Stat3 in untreated cells versus cells treated with Epo or IL1β; p<0.05 for comparison of phospho-Stat3 in untreated versus with Epo or IL1β treatment) (Figure 8D). Therefore, expression of phospho-Stat3 was comparable in IL1β or Epo treated HEL cells, but only Epo enhanced *FANCC* promoter activity in these cells.

**Discussion**

Mice with Fanconi gene disruption do not develop overt BMF or AML at steady state, but attempts to stimulate emergency granulopoiesis induced both in *Fance<sup>-/-</sup>* mice (10). Emergency granulopoiesis failure was due to apoptosis of HSCs and differentiating progenitors, but progressive anemia was the cause of death (10). Mechanisms of anemia in *Fance<sup>-/-</sup>* mice during attempted emergency granulopoiesis were unclear. In the current studies, we found plasma Epo decreased as emergency granulopoiesis peaked in Wt and *Fance<sup>-/-</sup>* mice; perhaps due to IL1β-effects on renal Epo-producing cells (42). Plasma IL1β fell and Epo rose during steady state resumption in both genotypes. During this process, a proliferative burst expanded Wt erythroid progenitors, but induced DNA-damage and apoptosis in *Fance<sup>-/-</sup>* erythroid progenitors. Epo stimulation increased Fanconi C in these cells, suggesting the Fanconi DNA-repair pathway protects erythroid progenitors from genotoxic stress during the inflammatory response.

Since Jak2 is activated by the Epo-receptor, we tested the ability of pharmacologic Jak1/2 inhibition (with ruxolitinib) to protect erythroid progenitors from proliferative stress. We found ruxolitinib treatment abrogated anemia and prolonged survival in *Fance<sup>-/-</sup>* mice during multiple emergency granulopoiesis attempts. This was associated with decreased DNA-damage and apoptosis in *Fance<sup>-/-</sup>* erythroid
progenitors. However, the mechanism was slightly different than anticipated.

To investigate ruxolitinib-related molecular mechanisms, we performed transcriptome analysis of erythroid progenitors from Alum-injected \textit{Fancc}^{+/+} or Wt mice. We found different effects of ruxolitinib on transcriptomes in the two genotypes. In cells from Alum-injected \textit{Fancc}^{+/+} mice, ruxolitinib enhanced activity of pathways involved in erythroid differentiation; consistent with improved anemia. However, the major ruxolitinib effect was to increase capacity for detoxification of reactive oxygen species by \textit{Fancc}^{-/-} erythroid progenitors. This was associated with decreased activity of apoptosis-associated pathways, suggesting resolution of oxidative stress enhanced cell survival during the innate immune response. A role for Jak2 in responding to, and modulating, oxidant stress has also been suggested in epithelial cells (43,44). In Wt erythroid progenitors from Alum-injected mice, ruxolitinib primarily modulated proliferation-associated pathways; consistent with Epo-induced Jak2 signaling in these cells.

Jak1/2 enhance HSC cell cycle entry/progression, suggesting ruxolitinib might protect these cells during proliferative stress (20,21,45). Encouragingly, ruxolitinib decreased DNA-damage and apoptosis of Lin^−ckit^+ cells in \textit{Fancc}^{−/−} mice during emergency granulopoiesis attempts, and stabilized cell numbers. We found the decrease in DNA damage was specific to proliferating cells, consistent with our hypothesis. In these studies, we used pH2AX as an indicator of DNA damage (also referred to as γH2AX) (46). This mark is an early indicator of the cellular response to dsDNA damage and is frequently tracked in Fanconi anemia studies. Other indicators of DNA damage could also be employed to further investigate molecular mechanisms for cell death under these conditions and the role of Jak1/2. These studies are of interest for the future. Since Jak1 is activated by IL2, IL6, IL10 and interferons, modulating the response to these cytokines may contribute to observed effects (47). Studies to separate consequences of inhibiting Jak1 versus Jak2 are being pursued in the laboratory.

We also found differences in ruxolitinib effects on Lin^−ckit^+ cell transcriptomes from Alum-injected \textit{Fancc}^{−/−} versus Wt mice. In the former, ruxolitinib decreased \textit{Aldh} expression, perhaps indicating a
decrease in toxic aldehydes due to decreased bone marrow stress (41). Ruxolitinib also enhanced pathways involved in myeloid leukocyte differentiation and mRNA translation; consistent with improved granulocytosis in these mice. Conversely, ruxolitinib decreased activity of pathways associated with leukocyte commitment and inhibited MAPK signaling in Wt Lin^+ kit^+ cells from Alum-injected mice; consistent with impaired emergency granulopoiesis. Impaired activation of Jak2 by the G-CSF-receptor may also contribute to these effects (48). Ruxolitinib did not normalize emergency granulopoiesis in Fancc^−/− mice, but decreased bone marrow oxidant stress plus decreased proliferative stress may have improved both granulocytosis and erythrocytosis during such challenges.

Rescue of emergency granulopoiesis by ruxolitinib in Fancc^−/− mice did not accelerated AML, as we found with Tp53 haplo-insufficiency. In contrast, clonal progression during multiple emergency granulopoiesis attempts was delayed in these mice. This was associated with decreased expression of AML associated-genes in Lin^− kit^+ cells including Npm1, but increased expression of differentiation associated genes, including Tcirg1 and Prkcd (49-51). These genes are of interest for future studies.

Therapeutics to mitigate the impact of infections challenge on FA bone marrow might include IL1-receptor antagonists or prophylactic antibiotics. However, clinically relevant IL1-R antagonists require injection and chronic antibiotic use may be complicated by pathogen resistance. Ruxolitinib is an oral agent approved for myeloproliferative disorders or graft versus host disease. Toxicities, including cytopenias in 10-20% of patients, are not generally prohibitive (23). Although neutropenia may occur in ruxolitinib-treated children, enhanced emergency granulopoiesis in Fancc^−/− mice suggest this complication may be relatively less problematic in FA patients. These studies suggest a translational potential for this approach, although dosing for optimal effect and targeting use to high-risk clinical situations is indicated.

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References


