CD47 Deficiency Triggers Hemolytic Anemia and Alters the Bone Marrow Niche During Fracture Repair

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INTRODUCTION: We have previously demonstrated that the global deletion of CD47 impairs long bone fracture healing with decreased mineralization and chondrogenesis [1]. This is accompanied by dysregulated stromal cell proliferation and decreased stemness. CD47 is a ubiquitously expressed cell surface receptor known for its role as a 'don't eat me' signal by binding to SIRPα, allowing healthy cells to avoid immune clearance. This is a two-step process where cells lacking CD47 also express an 'eat me signal' (i.e. foreign antigens or mutagens) and are cleared. Thus, a CD47 deletion is not lethal because most healthy cells don't express 'eat me' signals. However, mature red blood cells (RBCs), which lack nuclei may be perceived as abnormal and cleared in the absence of CD47. We hypothesize that an absence of CD47 results in RBC clearance, which promotes erythropoiesis in the bone marrow, disrupts long bone fracture healing, and limits nutrient and oxygen delivery, leading to stromal stress and dysfunction.

METHODS: All studies were conducted under IACUC approval. 18-month-old C57 Bl/6 (WT) mice (n=8M) and CD47-null mice (n=4M, 3F) underwent stabilized right femoral fractures using a 23-gauge needle for stabilization followed by a mid-shaft fracture. Extended-release buprenorphine (sc 1mg/kg) was given pre-operatively. Body weights (pre-fracture and at harvest) and spleen weights (at harvest) were recorded. Bone marrow was flushed from injured and contralateral femurs 3 days post fracture (dpf) (n=4M WT, n =3M, 1F CD47-null). Marrow was stained with erythropoietic markers Ter119-APC and CD71-FITC. CD11b-PE, B220-PE, and Gr1-PE were used to exclude myeloid and lymphatic lineages. Samples were fixed with 4% PFA and run using the Miltenyi MacsQuant Analyzer; analysis was performed on FlowJo v10. Pro-erythroblasts (CD71+/Ter119lo) and erythroblasts (CD71+/Ter119hi) were quantified. Complete blood counts and reticulocyte analysis were completed pre and post fracture on the Heska HT5 Element and MacsQuant Analyzer, respectively. For scRNAseq, 20-week-old WT and CD47-null mice underwent tibia fractures (samples consisted of cells pooled from 2M+2F) and were harvested at 7dpf. Stromal cells were isolated and GSEA hallmark analysis was performed using the log2FC. The FDR cutoff was set to <0.25. Sex was not considered a main factor in this study as we have previously demonstrated minimal impact of sex in the CD47-null fracture model [1].

RESULTS: While CD47-null mice weighed less than WT pre- and post-fracture, their spleen weight was found to be ~1.5 times greater in size (**Fig 1A**). We found a decrease in total RBC count, hemoglobin, and hematocrit in CD47-null mice both pre- and post-fracture, consistent with anemia (**Fig 1B**). Importantly, we found that this decrease is exacerbated with fracture in both WT and CD47-null mice suggesting reduced systemic RBC availability. To interrogate the anemic phenotype, we assessed reticulocyte percentages and found this to be increased in the CD47-null mice and even more so with fracture (**Fig 1C**). Next, we assessed the relative abundance of pro-erythroblasts (ProE) and mature erythroblasts (EryB) in injured bone marrow and found there to be more of these intermediate immature erythropoietic populations in the CD47-null mice compared to the WT (**Fig 1D-E**). We observed decreases in proE and EryB in injured compared to contralateral limbs, demonstrating a fracture-specific influence on the marrow composition. The absence of CD47 leads to increased erythropoiesis as evidenced by the increase in reticulocytes, proE, and EryB. In addition to resulting in decreased circulating mature RBCs, changes in the marrow niche can place stress on the stromal cells that contribute to the fracture callus. We have previously demonstrated that the CD47-null fracture callus undergoes delayed healing with reduced proliferation, osteogenesis and chondrogenesis. To better understand the changes in stromal cell behavior, we performed GSEA hallmark analysis on the stromal population using the unfiltered log2FC and observed increased stress response signals including complement, oxidative phosphorylation, p53 pathway, and ROS pathway activation compared to the WT callus, which has hallmark pathways associated with chondrogenesis (**Fig 1F**). Two-sided t-test was used to compare spleen weights. Two-way ANOVA was utilized for multiple comparison analyses. *P<0.05, **P<0.01, ***P<0.001. We did not observe sex-specific effects in our study and chose to combine data from both sexes when applicable to reduce the use of animals.

DISCUSSION: Our findings demonstrate a dysfunction in CD47-null RBCs that suggests hemolytic anemia in which the mature RBCs are cleared in the spleen, with excessive compensatory erythropoiesis occurring in the bone marrow. These changes may have consequential effects on local iron concentration, oxygen tension at the fracture site, and nutrient delivery leading to stress in local cell populations. Decreased stromal cell proliferation, stem cell marker expression, and osteo/chondrogenic differentiation in the absence of CD47 could be due to changes in the marrow microenvironment secondary to these changes in RBC metabolism, and not necessarily a cell-autonomous effect. Continued work will interrogate RBC lifespan in circulation, oxygen tension and iron in the local callus environment and RBC-stromal cell crosstalk.

SIGNIFICANCE/CLINICAL RELEVANCE: CD47 has been therapeutically targeted in cancer treatment through the use of anti-CD47 antibodies to clear cancer cells that overexpress these 'eat me' signals while largely sparing healthy tissues. Here, we demonstrate that RBC are not protected from this clearance. While it is colloquially known that anemia can cause complications in healing, there is limited understanding about the effect of anemia on stromal cells and fracture repair. Our CD47-null fracture model represents a novel system to study the impact of hemolytic anemia in fracture repair. This approach provides valuable mechanistic insight to inform patient care and potentially develop novel therapeutic targets to improve fracture outcomes.

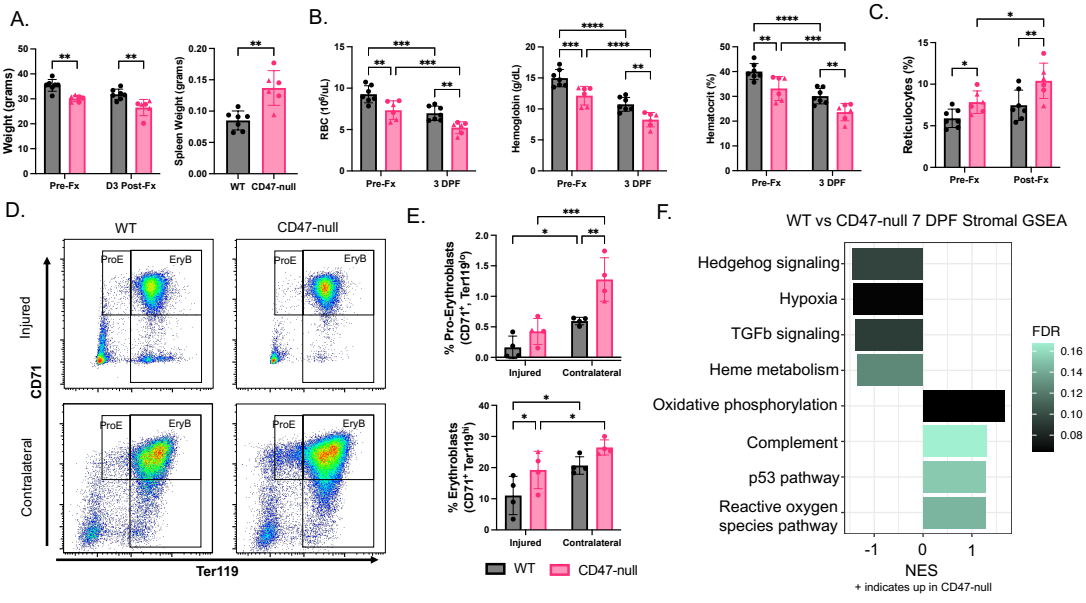

Figure 1: CD47-null mice have dysregulated RBC clearance and erythropoiesis: (A) Mouse weights (pre & post femur fracture) spleen weights post fracture. **(B)** RBC, Hemoglobin, and Hematocrit counts pre and post fracture. **(C)** Reticulocytes pre and post fracture. **(D)** Representative flow analysis for pro-erythroblasts (ProE) and erythroblasts (EryB). **(E)** Flow analysis of ProE and EryB in the fractured and contralateral limb, **(F)** GSEA analysis of hallmark pathways in the stromal cells 7 dpf. FDR <0.25. Circles represent male samples, triangles represent female. No statistical differences were observed between sexes. *P<0.05, **P<0.01, ***P<0.001, two-sided t test or two-way ANOVA for multiple comparisons.

1. Zondervan, R. L. & Capobianco, C.A. *Bone Research* (2025).