Colchicine reduces inflammation in a humanized transgenic murine model of sickle cell disease

Sickle cell diseases (SCD) are caused by a mutation in the β-globin gene that causes deoxygenated hemoglobin to polymerize, resulting in rigid, fragile, sickle-shaped red blood cells.[1,2] Prominent clinical features are hemolytic anemia; hyperalgesia; chronic and acute pain; wide-spread organ damage; and premature death (USA median age ~50 years). SCD pathobiology involves oxidative stress, ischemia/reperfusion injury, and activation of the innate immune system with sterile inflammation and "cytokine storm". Inflammation in SCD is chronic with acute exacerbations, and complex with many cellular and molecular components. Among these, mast cell activation and interleukin (IL)-6 and C-reactive protein (CRP) are chronically and acutely increased in SCD.[3,4] CRP contributes to inflammation through the activation of the complement system, a component of innate immunity. CRP has been used to monitor both disease activity and response to therapy in many inflammatory conditions involving both innate and acquired immunity.[5]

Colchicine is a drug used for the treatment or prevention of symptoms of gout and other non-infectious inflammatory conditions. Recently, it has been shown to improve outcomes in atherosclerotic cardiovascular disease (ASCVD) in which, like SCD, inflammation is a secondary pathological process.[6] Colchicine effects in ASCVD and other conditions include reductions in IL-6 and CRP. Among five selected agents or agent classes of anti-inflammatory drugs - colchicine, non-steroidal anti-inflammatory drugs, corticosteroids, canakinumab, and imatinib - only colchicine has been shown to inhibit each of four selected inflammatory mechanisms - inflammatory cytokines, inflammasomes, mast cell (MC) activation, and neutrophil adhesion and superoxide release. Among the five anti-inflammatory agents or agent classes, only colchicine is a tubulin inhibitor (TI). Among TI, colchicine has unique properties.[7] Tubulin is a dynamic structural protein, and TI are classified as stabilizers (sTI) or destabilizers (dTI). dTI are associated with five binding sites named by an agent or agent class: colchicine, taxanes, laulimalide, epothilones, and vinca alkaloids. Among TI, only colchicine is used as an anti-inflammatory agent, with all others targeted to various types of cancer.

We hypothesized that colchicine might reduce inflammation and improve the clinical course of SCD. We sought evidence for this by examining the effect of colchicine on inflammation in the HbSS-BERK humanized transgenic mouse model of SCD. These sickle mice are knockout for murine α and β globins and express human α and β^S (>99% of total β) globins on a mixed genetic background. They have severe disease with hyperalgesia, inflammation, extensive organ damage, and pathology consistent with SCD; in contrast, HbAA-BERK mice that express normal human α and β globins do not exhibit hyperalgesia or organ pathology. Only female mice were studied, as male mice are fragile with less tolerance to experimental manipulations, rendering them unsuitable for this sort of study.

Female ~4-month-old mice were treated with colchicine 100 μg/kg body weight (Tocris Bioscience, Bristol, United Kingdom) or vehicle (phosphate-buffered saline) intraperitoneally once daily for 14 days. Hyperalgesia testing was performed at 1 hour (h) and 24 h after the first treatment and on day 7 and day 14.[8] Then mice were humanely euthanized; blood was collected by cardiac puncture; and 4 mm diameter dorsal skin punch biopsies were collected for mast cell analysis[9] or incubated in culture media for 24 h to analyze secreted inflammatory cytokines. All experiments were performed following protocols approved by the Institutional Animal Care and Use Committee. Data were analyzed using GraphPad Prism software version 9.3.1 (GraphPad Prism Inc., San Diego, CA).

Serum amyloid protein (SAP) is a murine acute-phase reactant with extensive (60-70%) sequence homology with human CRP; it is commonly used as a surrogate for CRP in mouse studies.[10] Consistent with our hypothesis, plasma IL-6 levels were ~67% lower (~130 *vs*. ~48 μg/g protein; $P<0.01$), and plasma SAP levels were ~45% lower (~33 *vs*. ~18 μg/g protein; $P<0.01$) in colchicine-treated mice compared to vehicle (Figure 1A).

Consistent with our observations of reduced systemic inflammation with colchicine treatment, we observed that skin-conditioned media from colchicine-treated mice showed significantly reduced levels of three pro-inflammatory cytokines compared to skin-conditioned media from vehicle-treated mice: granulocyte-macrophage colony-stimulating factor (GM-CSF), 40% reduction ($P<0.05$); IL-3, 40% reduction ($P<0.01$); and interferon γ (IFNγ), 20% reduction ($P<0.05$) (Figure 1B). Of note, GM-CSF can antagonize the therapeutic increase in fetal hemoglobin expression caused by hydroxyurea (HU), the most effective SCD disease-modifying drug (DMD).[11] Thus, colchicine-induced reduction in GM-CSF might enhance HU effectiveness. Conditioned media levels of the anti-inflammatory cytokine IL-10 were reduced by 20% ($P<0.05$). We speculate that this reflects downregulation of the inflammatory loop that stimulates IL-10 production. Conditioned media cytokine levels not affected by colchicine were IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-12, IL-17, monocyte chemo-at-

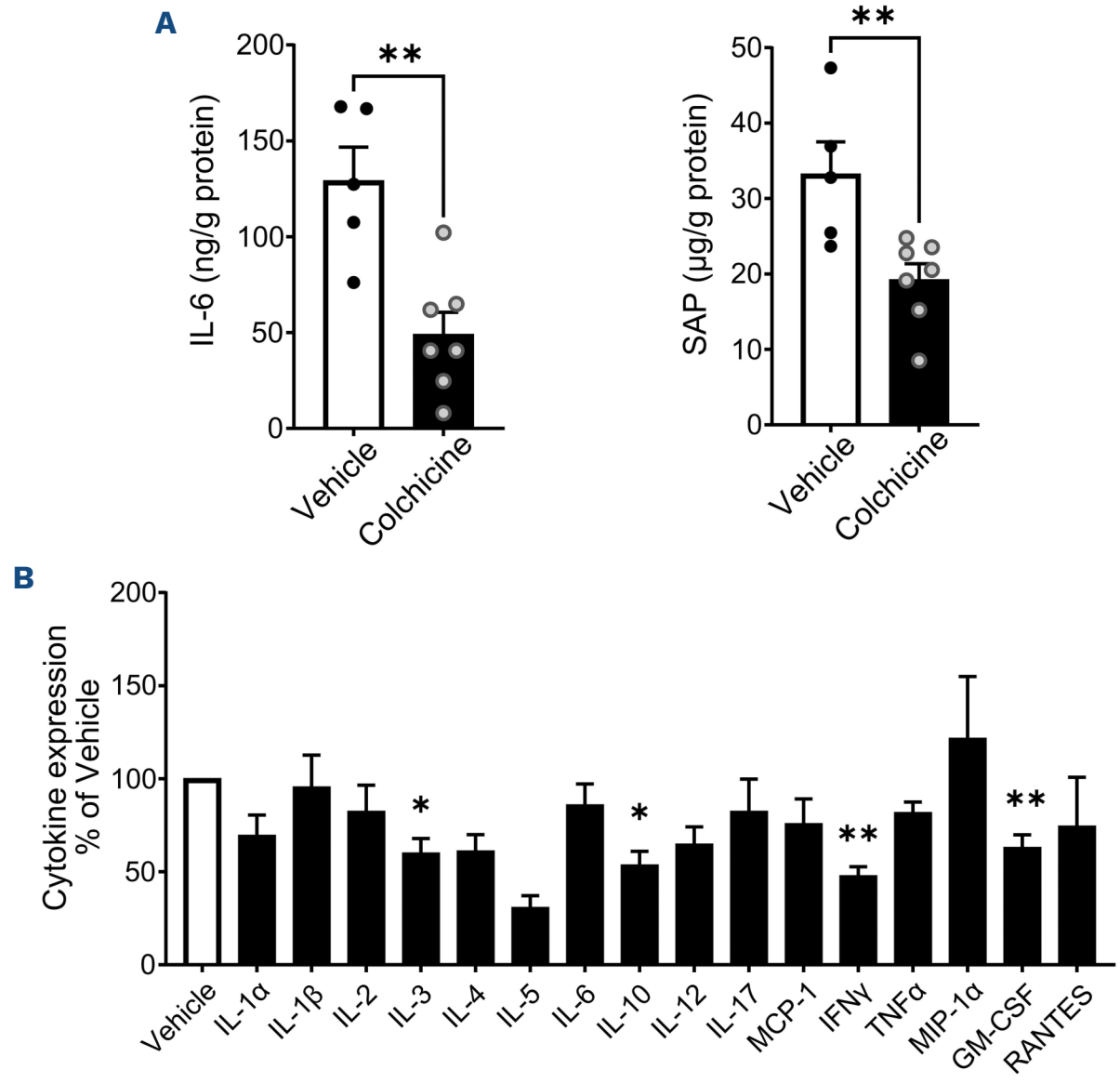

Figure 1. Colchicine attenuates inflammation in sickle mice. Female HbSS-BERK sickle mice were treated daily with 100 µg/kg/day colchicine or vehicle (phosphate-buffered saline). After 14 days of treatment, mice were euthanized, blood collected, and punch biopsies of dorsal skin were incubated in culture media for 24 hours. Data are expressed as mean ± standard error of the mean. (A) Plasma interleukin (IL)-6 and serum amyloid protein in vehicle- and colchicine-treated mice. (B) Concentrations in skin-conditioned media of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17, monocyte chemo-attractant protein 1, tumor necrosis factor α, macrophage inflammatory protein-1α, and regulated on activation, normal T-cell expressed and secreted protein as percentage of vehicle-treated control. Cytokines were analyzed using a microplate-based array (Quansys Biosciences, Logan, UT, USA), a sandwich enzyme-linked immunosorbent assay in microscale. Data expressed as colchicine-treated as percent of control. SAP: serum amyloid protein; GM-CSF: granulocyte-macrophage colony-stimulating factor.

tractant protein 1, tumor necrosis factor α, macrophage inflammatory protein-1 α, and regulated on activation, normal T-cell expressed and secreted protein. All of these are elevated in human SCD plasma,[12,13] but we have no human SCD plasma samples from patients exposed to colchicine for comparison.

Previous studies in sickle mice indicate that disease-associated MC activation results in the release of substance P, tryptase, and multiple inflammatory cytokines from skin and dorsal root ganglia that leads to neurogenic inflammation and nociceptor activation.[8] Colchicine reduced skin MC degranulation by ~75% (P<0.0001), and we attribute reductions in GM-CSF, IL-3, and IFNγ to this effect (Figure 2).

We did not observe changes in circulating blood cell parameters (see the *Online Supplementary Figure S1*). Although colchicine can cause myelosuppression, it does not cause significant myelosuppression in SCD or other conditions at currently recommended clinical doses.[6] We also did not observe changes in hyperalgesia (*Online Supplementary Figure S2*). Given the chronic nature of inflammation and tissue damage in SCD, we speculate that starting colchicine treatment in younger mice and continuing dosing for a longer interval might attenuate the development of inflammation and consequent organ damage.

The current SCD drug armamentarium is inadequate, and introduction of a drug that targets SCD inflammation might improve outcomes. Although the four DMD approved in the USA for the treatment of SCD - hydroxyurea, voxelotor, L-glutamine, and crizanlizumab - have some secondary anti-inflammatory effects, significant inflammation persists in persons with SCD despite taking one or more of these drugs.[14] Non-steroidal anti-inflammatory agents are commonly used to treat pain in SCD, but they have not been shown to have disease-modifying effects in SCD. For unclear reasons, corticosteroids have mixed effects, and in general are avoided. In a small trial, the anti-

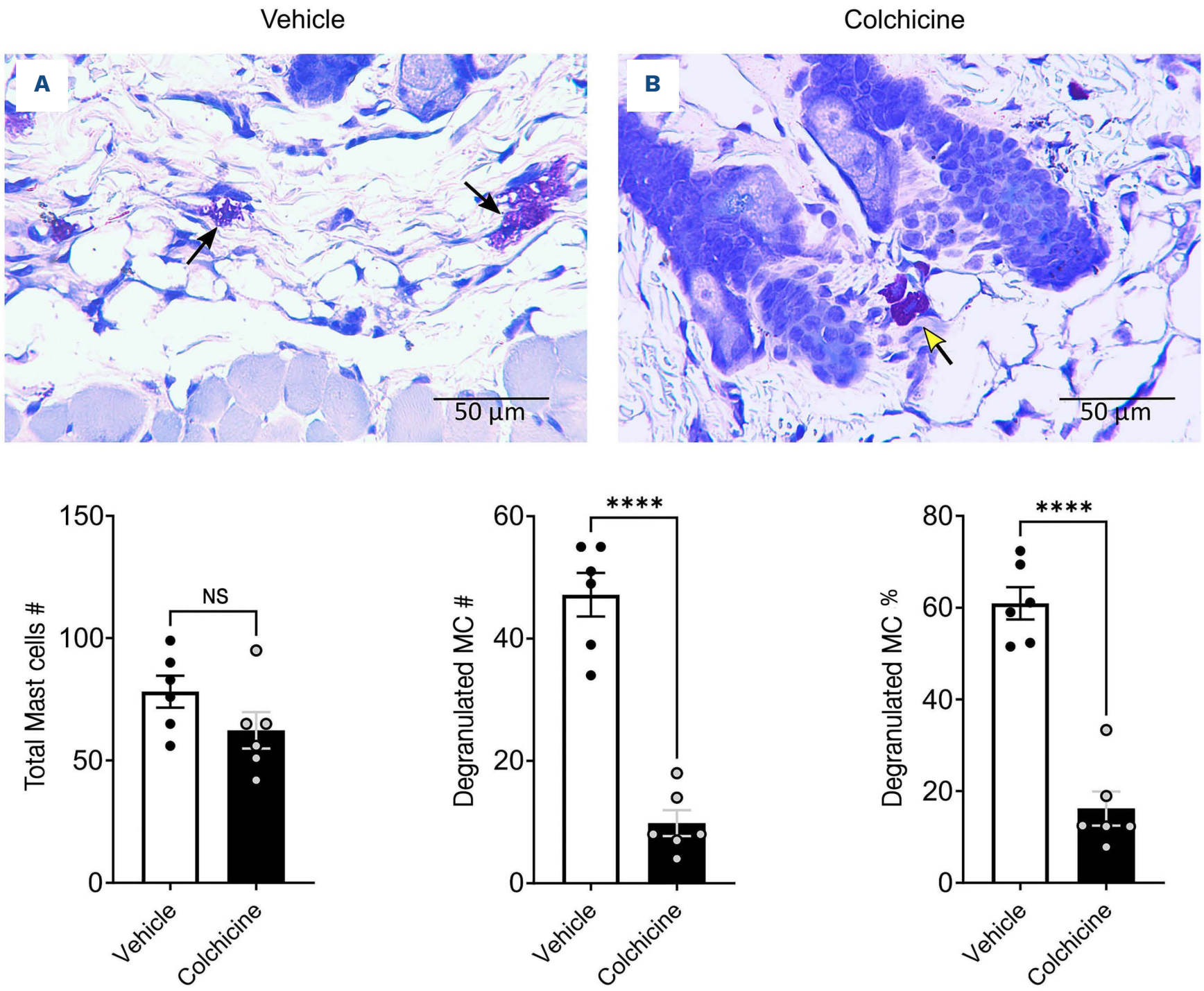

Figure 2. Colchicine inhibits mast cell degranulation in the skin of transgenic humanized sickle mice. (A) Black arrow: degranulated mast cell (MC). (B) Yellow arrow: intact MC. Toluidine blue stain was prepared by dissolving 0.25 g toluidine blue (Sigma-Aldrich) in 35 mL distilled water, 15 mL ethanol 100%, and 1 mL HCL. After deparaffinization, the skin sections were incubated in toluidine blue for 1 minute at room temperature, washed with distilled water, and air-dried. The stained specimens were observed under an Olympus Microscope BH-2. MC were recognized by red-purple metachromatic staining color on a blue background. MC were counted in 20 fields, 4 fields per slide, and expressed as total MC number, number of degranulated MC, and percentage of degranulated cells. Degranulated MC were defined as cells associated with ≥8 granules outside the cell membrane. Analyzed with unpaired *t* test, two-tailed in GraphPad Prism software; software ($P<0.0001$); one outlier in the colchicine-treated cohort was detected by Grubb's test. NS: not significant

IL-1β antibody, canakinumab, showed a nominal reduction in CRP and trends toward improvement in multiple clinical endpoints, but none of these were statistically significant, and the indication has not been pursued.

We are aware of only one other study of colchicine in SCD, in which colchicine was shown to attenuate inflammation and cardiac damage in a transgenic mouse model.[15]

Reduced inflammation due to colchicine treatment might result in reduced pain, preservation of end-organ function, and prolonged survival in SCD. As colchicine has a unique mechanism of action and minimal side-effects at currently recommended clinical doses, patients might benefit from combination of colchicine with one or more of the established DMD. Further research is warranted.

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Contributions

RTF conducted experiments, mast cell staining and analysis, analyzed data, prepared the data for publication, prepared figures and the visual abstract. HMC conducted hyperalgesia experiments. SBK bred, phenotyped and prepared sickle mice. NRG conducted the ELISA assays. DAA participated in experimental design and cytokine analysis. GJV analyzed histopathology. KG participated in experimental design, supervision of the study, data analysis and interpretation, and manuscript editing. JDR proposed the study, participated in the experimental design and interpretation of results, and wrote the manuscript.

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Data-sharing statement

Original data can be made available on reasonable request to a corresponding author.

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