Inflammation, Vol. 42, No. 5, October 2019 (© 2019)
DOI: 10.1007/s10753-019-01032-y

ORIGINAL ARTICLE

Novel Piperazino-Enaminones Decrease Pro-inflammatory Cytokines Following Hemarthrosis in a Hemophilia Mouse Model

Chen Zhong,[1] Doreen Szollosi,[2,7] Junjiang Sun,[3,4,7] Baolai Hua,[5] Ola Ghoneim,[2] Ashley Bill,[2] Yingping Zhuang,[6] and Ivan Edafiogho[2]

Abstract— Hemarthrosis is the primary cause of hemophiliac arthropathy (HA). Pro-inflammatory cytokines are thought to play an important role in the pathogenesis of HA, and thus, anti-cytokine approaches may be used as an adjuvant therapy. A novel series of enaminone compounds (JODI), that contain the N-aryl piperazino motif, have been shown *in vitro* to reduce pro-inflammatory cytokines and thus may be efficacious *in vivo*. In this report, we will assess whether JODI can suppress multiple cytokines which might be potentially responsible for joint inflammation in a mouse model of hemarthrosis. The results showed that JODI significantly improved the survival after LPS treatment, and most pro-inflammatory cytokines/chemokines were decreased significantly after JODI administration. In the hemophilia mouse model, hemarthrosis resulted in local cytokine/chemokine changes, represented by elevated pro-inflammatory (IL-6, MCP-1, MIP-1α, MIP-1β) and pro-angiogenic (VEGF and IL-33) cytokines, and decreased anti-pro-inflammatory cytokines IL-4 and IL-10. The changes were reversed by administration of JODI, which can be used as a novel approach to manage hemophilia arthropathy.

KEY WORDS: hemophilia; hemarthrosis; cytokines; pro-inflammatory; piperazino-enaminones.

Chen Zhong, Doreen Szollosi, and Junjiang Sun contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10753-019-01032-y) contains supplementary material, which is available to authorized users.

[1] Shanghai Key Laboratory of New Drug Design, School of Pharmacy, East China University of Science and Technology, Shanghai, China
[2] Department of Pharmaceutical Sciences, University of Saint Joseph, School of Pharmacy and Physician Assistant Studies, Hartford, CT, USA
[3] Division of Molecular Pharmaceutics, Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA
[4] Gene Therapy Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA
[5] Department of Hematology, Clinical Medical College, Yangzhou University, Yangzhou, China
[6] State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, China
[7] To whom correspondence should be addressed to Doreen Szollosi at Department of Pharmaceutical Sciences, University of Saint Joseph, School of Pharmacy and Physician Assistant Studies, Hartford, CT, USA. E-mail: dszollosi@usj.edu; and Junjiang Sun at Gene Therapy Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA. E-mail: sunjj@med.unc.edu

0360-3997/19/0500-1719/0 © 2019 Springer Science+Business Media, LLC, part of Springer Nature

INTRODUCTION

Bleeding into the joints is a major complication of severe hemophilia. Bleeding can happen at any site; however, most bleeding occurs internally into the joints or muscles [1]. The combination of iron (derived from lysed red blood cells), inflammation, synovial hypertrophy, and neovascularization results in a vicious cycle for repeated bleeding. Recurrent bleeding into joints results in a crippling arthritis, called hemophilia arthropathy (HA). The associated chronic pain and joint impairment negatively impact the quality of life of hemophilia patients [2]. Once HA is established in a patient, it is essentially irreversible [3], especially in patients where bleeding has already developed in the target joint. Late prophylaxis on severe hemophilia with pre-existing arthropathy has been found to lead to decreased bleeding and pain, but no reduction in structural arthropathy progression [4]. Indeed, even the current prophylactic regimens do not fully prevent joint bleeding, and some patients may still develop joint disease [5, 6]. The management of HA and associated bone damage is still a major concern, especially in undeveloped countries [7, 8]. Even if the current prophylactic regimens do not fully prevent joint bleeding, some patients may still develop joint disease [5, 6].

The pathophysiology by which joint bleeds cause articular cartilage and subchondral bone destruction remains largely unknown [9]. Nonetheless, pro-inflammatory cytokines have been suggested to play an important role in the pathogenesis of HA [10, 11], whereas anti-cytokine approaches may offer protection against joint damage after hemarthrosis.

Enaminones represent a novel group of compounds possessing a variety of therapeutic properties including, but not limited to, anti-inflammatory, antitussive, and anti-epileptic effects [12]. Recent studies modifying the enaminone scaffold, by adding the N-aryl piperazine motif, have been extensively explored in *in vitro* studies to examine the effect on pro-inflammatory cytokines as well as chemokine signaling [13, 14]. In this report, we explored the safety and efficacy of such a novel anti-inflammatory agent, JODI-19, to reduce pro-inflammatory cytokine and chemokines in synovial lavage after hemarthrosis induction in a hemophilia mouse model.

MATERIALS AND METHODS

Materials

The chemical synthesis of all JODI compounds was reported previously [13, 14]. JODI-19 was selected from the series of compounds as one of the most promising compounds *in vitro* and was chosen for the *in vivo* testing. JODI-19 was first dissolved with dimethyl sulfoxide (DMSO); then, normal saline was slowly added by slowly mixing on vortex until fully dissolved. Lipopolysaccharide (LPS) (*Escherichia coli* serotype O111:B4) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and diluted in sterile normal saline. A single dose of 5 mg/kg was given by intraperitoneal injection.

Animals

FIX knockout C57BL/6J (FIX KO) mice were bred in-house [15]. Wild-type (WT) C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Given the majority of hemophilia patients are males, only male mice were used in hemophilia mouse study. Mice at the age of 8 to 12 weeks were used for the study. All investigations were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. Blood samples were collected from the retro-orbital plexus into 1:9 parts 3.2% citrated sodium, and the platelet-poor plasma was stored at − 80 °C before further measurement.

Safety Studies

Given the well-documented efficacy of JODI compound as inflammatory antagonist in *in vitro* study, we first tested the safety of JODI-19 *in vivo* in WT mice. Animals were weighed before recruitment into the study; thereafter, doses ranging from 6 mg/kg (“low dose”), 30 mg/kg (“middle dose”), to 120 mg/kg (“high dose”) were designed, normal saline containing 12.5% DMSO (a concentration of DMSO used in “high-dose” group) as the control group. JODI-19 was given daily by subcutaneous (s.c.) injection for a total of five doses. Three days after the last dose, whole blood samples were collected for hematological and liver/kidney tests.

Induction of Hemarthrosis and Synovial Lavage Collection

Hemarthrosis was induced by puncture of the joint capsule using a Hamilton syringe with a 30.5-G needle *via* a small (~ 0.5 mm) incision of the skin overlying the patella as described previously [16, 17].

Synovial lavage was collected by immersing the harvested joint tissues into normal saline. At each time point, immediately postmortem, the treated knee joints were collected by sectioning the femur and tibia/fibula 1 cm from the joint, exposing the joint space by cutting the ligament and immersing the tissues into an Eppendorf tube containing 0.5 ml normal saline. The tube was then put on a 2D rocker (Benchmark, Fisher Scientific) shaking in cool room for 4 h. Finally, the samples were centrifuged at 350*g* for 10 min at 4 °C, and the supernatants were harvested as the synovial lavage. The samples were stored at − 80 °C before measurement.

Efficacy of JODI-19 in Hemarthrosis Model

As outlined in Fig. 1, hemarthrosis was induced at D0 and D7 in FIX KO mice. Following two episodes of hemarthrosis, JODI-19 treatment was initiated with two doses at the second time joint injury and another dose at 24 h thereafter (4 h time point only one dose of JODI-19). The dose chosen is 30 mg/kg based on the survival weight as displayed in Fig. 4. At each defined time point, a pilot of animals ($n = 3$~8/time point) was sacrificed; plasma and synovial lavage ("SF") were collected for cytokine measurement.

Analysis of Cytokines and Chemokines

Cytokines were measured from samples of plasma and synovial lavage, including interleukin (IL)-1, IL-4, IL-6, IL-10, IL-17, IL-33, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, vascular endothelial growth factor (VEGF), and tumor necrosis factor alpha (TNF-α). Cytokines were measured on a Luminex MAGPIX system (Luminex Corporation, Austin, TX, USA), equipped with LUMINEX xPONENT® software using custom kits (BioRad Laboratories, Hercules, CA). Cytokine levels were expressed in picograms per milliliter (pg/ml). Levels below the detection limit of each cytokine were defined as 0 pg/ml.

Statistical Analyses

Data are shown as the mean ± standard error of the mean. Analysis by one-way analysis of variance for multiple comparisons was performed using GraphPad Prism v7 (GraphPad Software, Inc., La Jolla, CA) unless further defined. A p value of < 0.05 was considered statistically significant.

RESULTS

In Vivo Safety Profiles of JODI-19

First, to investigate the safety of this novel compound *in vivo*, WT mice received 5 consecutive doses of JODI-19, doses ranging from low dose of 6 mg/kg to the highest dose of 120 mg/kg. Compared with the control group that received the solvent to prepare JODI compound, the treatment group showed no change for all hematological parameters, including body weight (Fig. 2a) as well as kidney and liver functions (Fig. 3). No significant changes in body weight were seen in the mice from the start of the study to the last day 3 days after their fifth dose (Fig. 2b). The result showed that this novel compound has a relatively wide window of safety for use *in vivo*.

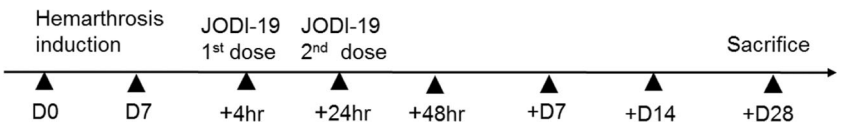

Fig. 1. Study design for *in vivo* efficacy of JODI on hemarthrosis. In FIX KO mice, hemarthrosis was induced at D0 and D7. Following two episodes of hemarthrosis, JODI treatment was initiated with two doses at the second time joint injury and another dose at 24 h thereafter (4 h time point only one dose of JODI). The dose chosen is 30 mg/kg. A pilot of animals ($n = 3$~8/time point) were sacrificed at each defined time point. Plasma and synovial lavage ("SF") were collected for cytokine measurement.

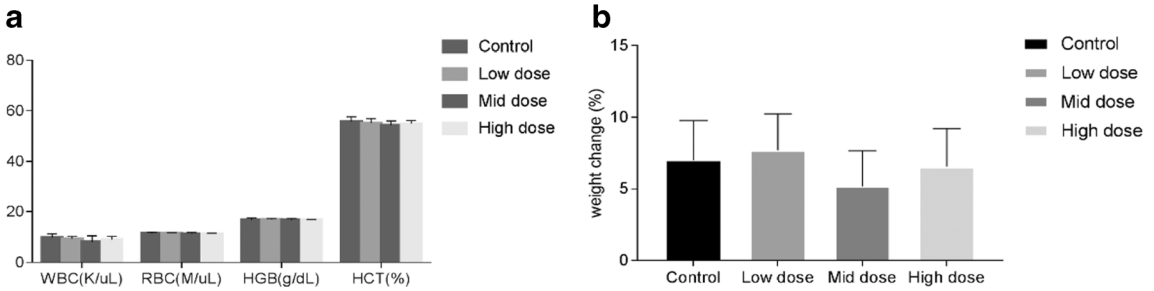

Fig. 2. *In vivo* safety profile of JODI compound: hematological tests and body weight. WT mice received JODI 19 subcutaneous (s.c.) injection daily for a total of 5 doses. **a** Three days after the last dose, whole blood was collected for hematological tests. **b** Mice were weighed before recruitment into the study and at the end of the observation for body weight change. "Control": normal saline containing 12.5% DMSO (concentration of DMSO used in "high-dose" group); "low dose": 6 mg/kg; "middle dose" = 30 mg/kg; "high dose" = 120 mg/kg. Each value represents the mean ± SD ($n = 5$).

JODI-19 Improved the Survival of LPS Treatment by Decreasing Pro-inflammatory Cytokines/Chemokines

Given the high safety profiles of JODI in WT mice, we sought to determine whether JODI can improve cytokine and chemokine profiles following LPS-induced endotoxemia. FIX KO mice were injected with LPS (5 mg/kg, i.p.) first, immediately followed by a single dose of subcutaneous injection. Then, JODI was injected at different doses. Four hours later, blood was collected for cytokine measurement. At 48 h, the experiment was terminated due to some treatment animals losing > 30% of their body weight. Results showed that administration of JODI significantly improved the survival in LPS-treatment hemophilia mice as shown in Fig. 4. The benefit of protection might be from blockage of pro-inflammatory cytokines or chemokines, as most cytokines at 4 h post-LPS treatment, including IL-1, IL-6, MCP-1, MIP-1α, MIP-1β, and TNF-α, decreased significantly after JODI administration (Fig. 5). IL-10, a putative anti-inflammatory cytokine, was not significantly impacted. Although we did not see statistical significance, there is a slight trend that a dose-response pattern exists between cytokine reduction and JODI dose (Fig. 4). At 48 h, cytokine levels were also detected in animals that survived, as shown in Fig. 6; most of the cytokines returned to baseline or relatively lower levels.

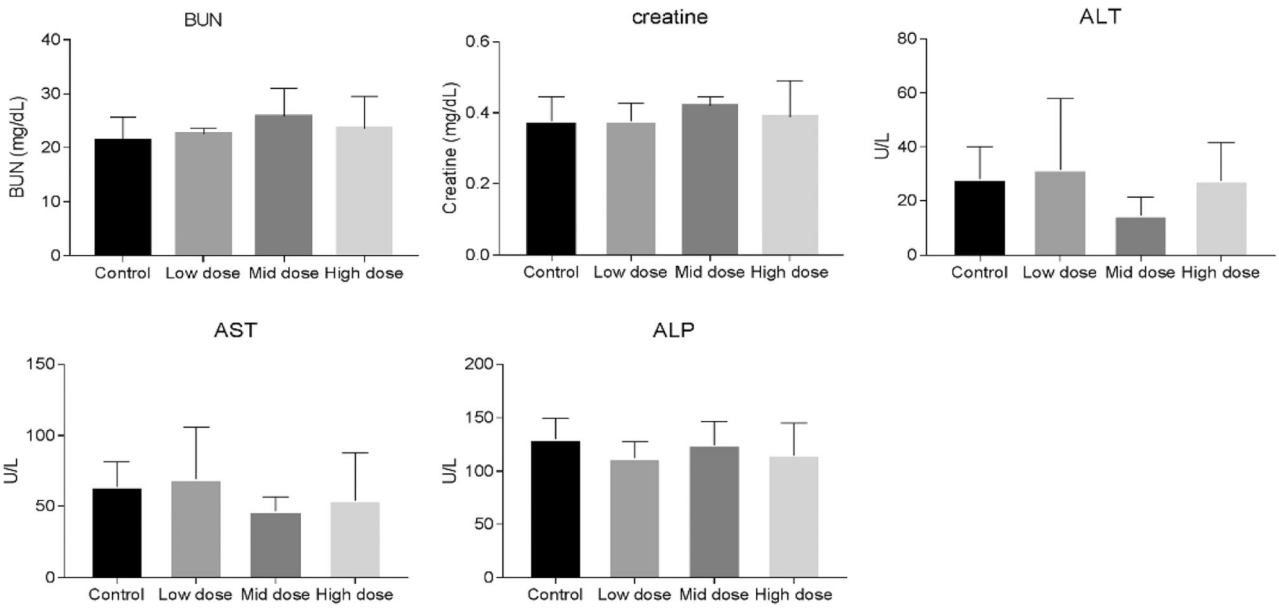

Fig. 3. *In vivo* safety profile of JODI compound: kidney function tests. WT mice received JODI 19 subcutaneous (s.c.) injection daily for a total of five doses. Three days after the last dose, whole blood was collected for liver/kidney test. Each value represents the mean ± SD ($n = 5$).

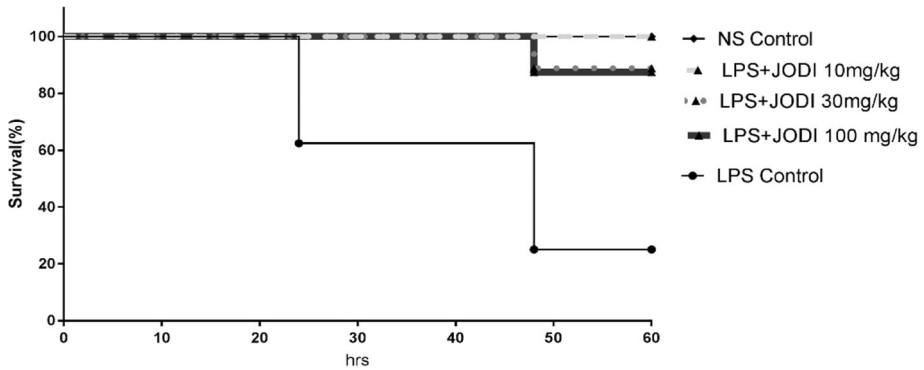

Fig. 4. JODI improved the survival of LPS treatment. FIX KO mice were injected with LPS (5 mg/kg, i.p.), immediately followed by a single dose of s.c. JODI at different doses. Because some treated animals lost > 30% weight, the experiment was terminated at 48 h. "NS control": normal saline i.p., followed by JODI dissolvent (NS with DMSO); "LPS control": LPS 5 mg/kg, i.p. injection, followed by JODI dissolvent (normal saline with DMSO); "LPS + LD": LPS 5 mg/kg, i.p., followed by low dose (LD) of JODI-19 at 10 mg/kg s.c.; "LPS + MD": LPS 5 mg/kg, i.p., followed by middle dose of JODI-19 at 30 mg/kg s.c.; "LPS + HD": LPS 5 mg/kg, i.p., followed by high dose of JODI-19 at 100 mg/kg s.c. Each value represents the mean ± SD ($n = 5$).

Efficacy of JODI-19 in Hemarthrosis Model

Pro-inflammatory cytokines, IL-1β, IL-6, keratinocyte-derived cytokines, and MCP-1 in synovial fluid have been reported to be involved in the development of hemophilic arthropathy [3, 4].We hypothesized that the novel JODI compound can prevent the excessive pro-inflammatory cytokine/chemokine product after joint bleeding in a hemophilia mouse model. Given that VEGF has been recognized as a strong pro-angiogenic cytokine to promote neo-angiogenesis, in this setting of cytokine measurement, VEGF was added.

As shown in Fig. 7, in the circulation (plasma), only IL-6 at the early time point (4 h) was found elevated. JODI treatment was found to decrease IL-6 levels significantly. The majority of the cytokines/chemokines were not affected systemically.

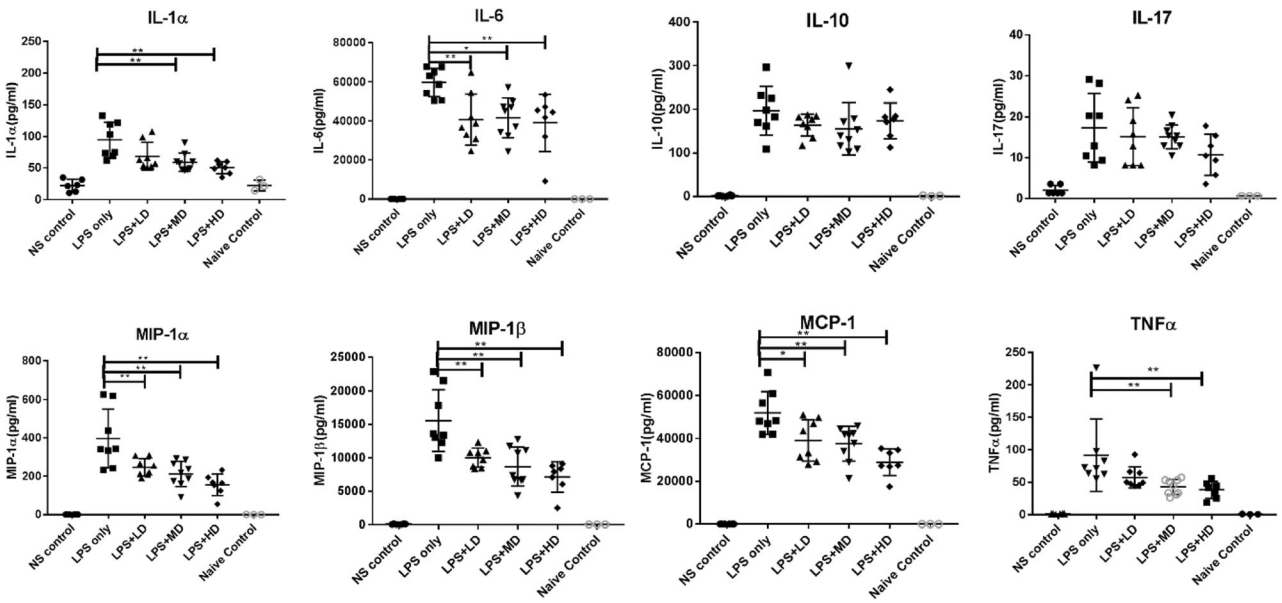

Fig. 5. JODI decreased pro-inflammatory cytokines/chemokines after LPS treatment. Blood was collected 4 h later from FIX KO mice with LPS treatment. Multiple cytokine measurements were performed. "Naïve control": plasma from untreated FIX KO mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Each value represents the mean ± SD ($n = 5$).

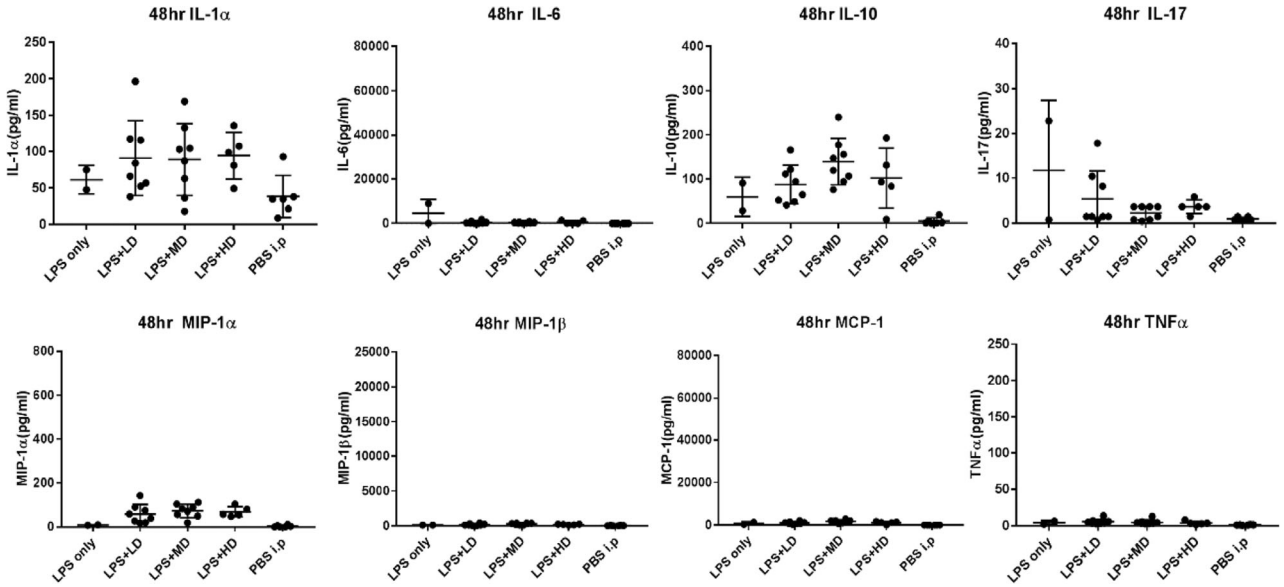

Fig. 6. Pro-inflammatory cytokines/chemokines at 48 h after LPS treatment. Blood was collected 48 h later from FIX KO mice in Fig. 3. Multiple cytokine measurements were performed. $*p < 0.05$. Each value represents the mean ± SD ($n = 5$).

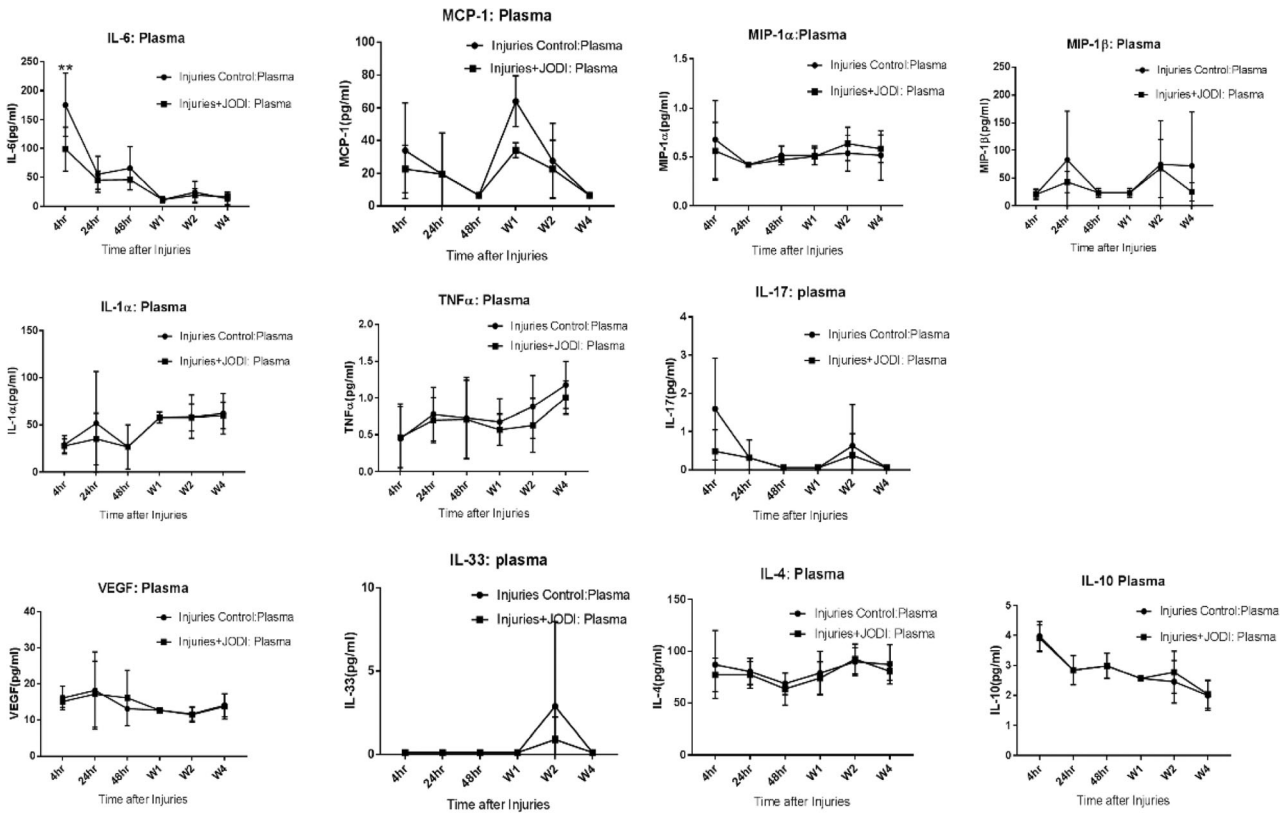

Fig. 7. Efficacy of JODI-19 in hemarthrosis model: cytokine/chemokine changes in circulation. Blood was collected at each defined time point in Fig. 1. Multiple cytokine measurements were performed. $**p < 0.01$. Each value represents the mean ± SD ($n = 5$).

In contrast to what was found in the circulation, cytokines and chemokines in synovial lavage showed mixed patterns as shown in Fig. 8. Cytokines/chemokines were increased at early time points and gradually dropped, which represent the acute phase reaction after the induction of hemarthrosis. These include IL-6, MCP-1, MIP-1α, and MIP-1β. Elevated cytokines/chemokines persisted relatively with fluctuation at certain time points (W1 or W2), which were represented by IL-1, TNF-α, and IL-17. Strikingly different to other cytokines/chemokines, there was a steady increase with time as seen for VEGF and IL-33. In any situation, in which cytokines/chemokines increased following hemarthrosis, JODI administration has suppressed the increase. On the contrary to cytokine/chemokine elevation following hemarthrosis, it is also interesting to see a very different pattern in which levels of IL-4 and IL-10 dropped sharply at week 2, while the drop was reversed by JODI administration. As the hemophilic mice developed markedly increased joint diameter after first hemarthrosis induction and a further increase after second hemarthrosis, JODI administration significantly prevented the joint swollen as shown in Fig. S1.

The result clearly showed that in the hemophilia mouse model, hemarthrosis resulted in local cytokine/chemokine changes, represented by elevated pro-inflammatory (IL-6, MCP-1, MIP-1α, MIP-1β) and pro-angiogenic (VEGF and IL-33) cytokines, and decreased anti-inflammatory cytokines IL-4 and IL-10. Both changes were reversed by JODI administration.

DISCUSSION

In this report, the safety of JODI was first validated in WT mice that received five consecutive doses of JODI-19, ranging from a lower dose of 6 mg/kg to the high dose of 120 mg/kg. In FIX KO mice, JODI significantly improved survival after LPS-induced endotoxemia possibly by decreasing pro-inflammatory cytokines including IL-1, IL-6, and IL-17. Furthermore, FIX KO mice were subjected to two episodes of hemarthrosis (at D0 and D7), and JODI treatment

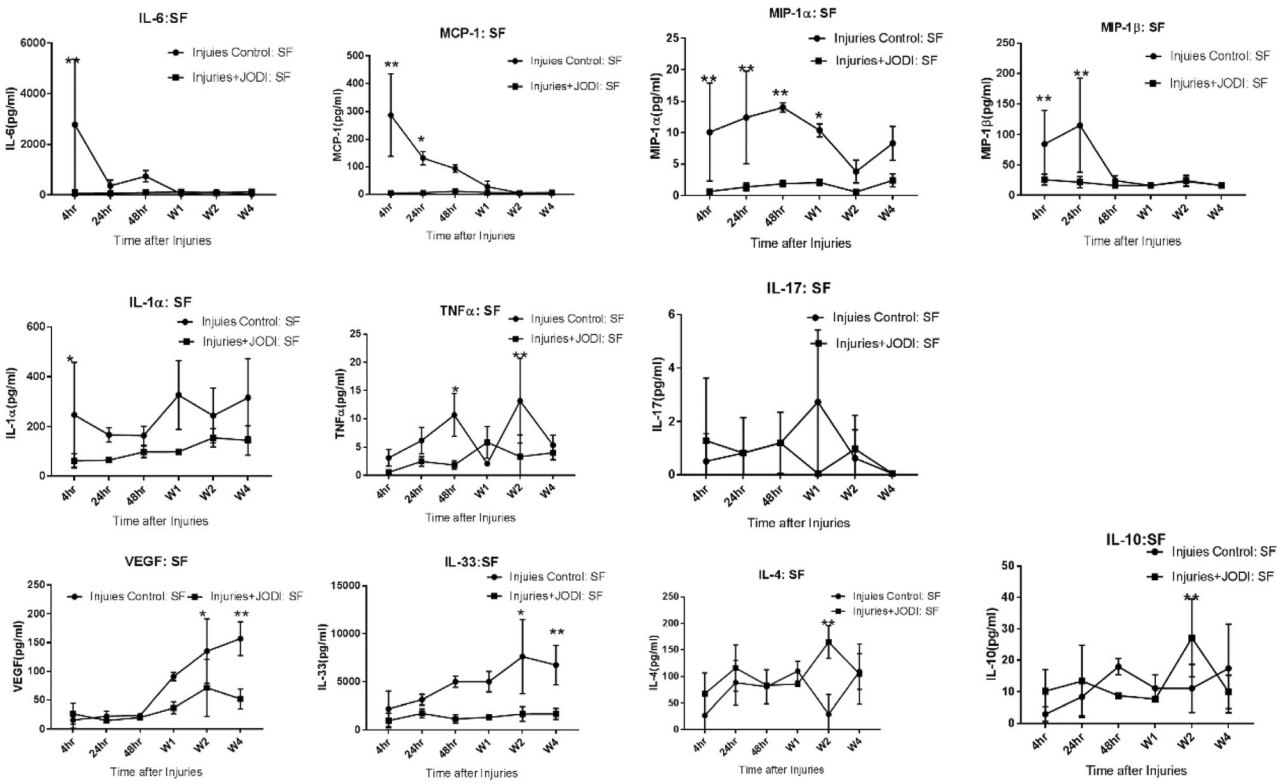

Fig. 8. Efficacy of JODI-19 in hemarthrosis model: cytokine/chemokine changes in synovial lavage. Synovial lavage was collected at each defined time point shown in Fig. 1. Multiple cytokine measurements were performed. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Each value represents the mean ± SD ($n = 5$).

started after the second joint injury. At different time points from 4 h to 4 weeks after hemarthrosis, plasma and synovial lavage were collected. Even though the majority of the cytokines/chemokines (except for IL-6) were not impacted in the circulation, cytokines/chemokines in synovial lavage showed a strikingly different and mixed pattern. In synovial lavage, pro-inflammatory (IL-6, MCP-1, MIP-1α, MIP-1β) and pro-angiogenic (VEGF and IL-33) cytokines were increased following hemarthrosis, while JODI administration suppressed this increase. On the contrary, anti-pro-inflammatory cytokines IL-4 and IL-10 dropped sharply at week 2 following hemarthrosis; the drop was reversed by JODI administration. Given our *in vivo* findings, JODI-19 may be a novel approach to manage hemophilia arthropathy and its associated bone damage.

Development of HA is characterized traditionally by two major processes, i.e., synovial inflammation and cartilage degeneration [18]. In this respect, HA shares the same features with inflammatory joint diseases such as rheumatoid arthritis (RA). Synovial joints are susceptible to spontaneous bleeds due to the high vascularization of the synovium. The unique characteristics of local hemostasis in a joint make the joint a vulnerable tissue for spontaneous bleeding, specifically low coagulation cascade and increased fibrinolysis following hemarthrosis [19, 20]. For either pathway, the synovial-derived pro-inflammatory cytokines, plasmin, and matrix metalloproteinases (MMPs) play important roles in the degenerative processes. Bone remodeling, particularly following injury, can be rapidly modulated by a number of factors including pro-inflammatory mediators present due to injury and the final molecular effector receptor activator of nuclear factor κB (RANK), RANK ligand (RANKL), and osteoprotegerin (OPG), the natural decoy receptor for RANKL [21]. The OPG/RANKL ratio therefore signifies the sum of bone remodeling influences at a given time, where increases in the OPG/RANKL ratio indicate a pro-bone formation state. Any influence that uncouples this process can result in an overall change in bone density [22]. Pro-inflammatory cytokines released by local inflammatory cells in response to injury, like IL-6, negatively regulate osteoblast differentiation and bone resorption through osteoblastic production of downstream effectors such as RANKL that activate osteoclasts [23, 24].

Over-expression of cytokines/chemokines perpetuates the inflammatory response by recruiting additional innate immune cells, such as monocytes and neutrophils, inducing T cell differentiation. In an *in vitro* study, inhibiting IL-1β by recombinant human IL-1β monoclonal antibody or IL-1 receptor antagonist had a protective effect on blood-induced cartilage damage in a dose- and time-dependent manner [25]. Blocking IL-1 has been a successful treatment for RA and gout as well as other auto-inflammatory syndromes [26, 27]. Previous studies from our lab have found that administration of an IL-6 receptor antagonist in addition to clotting factor replacement after joint bleeding led to protection against bleed-induced joint damage in the hemophilia A mouse model [28], represented by a significant decline in synovial hyperplasia, hemosiderin deposits, and infiltration of macrophages; such protection can also be achieved in the hemophilia B mouse using an anti-TNF-α approach [29]. Interestingly, the efficacy of anti-TNF-α to decrease the synovitis and hemarthrosis has been reported in hemophilia arthropathy patients [30].

Data to characterize novel anti-inflammatory agent enaminone E121 have shown a marked decrease in the release of pro-inflammatory cytokines including TNF-α in macrophages stimulated with LPS [14]. Literature has described that it is able to increase the CCR2 antagonistic activity when an N-alkylated piperazine motif is added to the terminal end of the lead compound. It was assumed that incorporating the N-alkylated piperazine motif into the existing E121 enaminone pharmacophore could lead to a ligand with dual functionality as a cytokine inhibitor and chemokine receptor antagonist [14].

It is interesting to see VEGF and IL-33 in synovial lavage steadily increase with time while JODI administration prevented the increase. VEGF is a strong pro-angiogenetic cytokine that mediates neo-angiogenesis by sprouting of capillaries from existing blood vessels into the wound bed. Neo-angiogenesis is vital for healing and repair; however, in hemophilia, it has been well characterized that perivascular tissue factor expression is downregulated, and the newly formed vessels are tissue factor deficient ("fragile vessels") during wound healing. This renders hemophiliacs vulnerable to hemorrhage and creates a vicious circle of bleeding and rebleeding during healing [31]. In hemophilia patients with arthropathy, the number of proangiogenic macrophage/monocyte cells in the synovium was significantly elevated [32]. Indeed, an

open clinical trial by Intra-articular administration of anti-VEGF antibody for the Recurrent Hemarthroses caused Target Joints is ongoing (ClinicalTrials.gov identifier: NCT02060305). IL-33 is a newly discovered member of the IL-1 family and plays an important role in matrix synthesis and neovascularization [33]. In osteoarthritis and chronic inflammatory joint diseases, IL-33 and other endogenous molecules have deleterious effects on joints by recruiting immune cells to inflamed synovia and perpetuating the disease progression [34]. In the hemophilia mouse model following hemarthrosis, a temporary increase in M1 monocytes and more sustained increase in M2 monocytes have been noted in the joint lavage [35]. We hypothesized this can contribute to the continuous IL-33 production given the rebleeding chances can be greatly increased by which disruption of vessels facilitated IL-33 release and attendant inflammation.

It is also noteworthy to see that in JODI-treated animals, IL-4 and IL-10 levels were significantly increased instead of decrease for the rest of cytokines, especially at the week 2 time point. IL-4 and IL-10 have been recognized with the anti-inflammatory/regulatory characteristics that can play a role to "rebalance" the microenvironment in the joint space toward a "calm" joint. IL-4 and IL-10 can prevent cartilage damage upon blood exposure *in vitro* [36, 37], and intra-articular injection with the combination of IL-4 and IL-10 after induction of hemarthrosis is able to alleviate cartilage damage [38]. As we saw the elevated cytokines/chemokines persisted relatively with the fluctuation of IL-1, TNF-α, and IL-17 especially at W1 or W2, we hypothesized that re-bleeding may occur from the inflamed and hyper-vascularized synovium, which has been attributed to the pathogenicity of hemophilia arthropathy.

CONCLUSION

In the present study, a novel enaminone compound JODI-19 suppressing multiple cytokines responsible for joint inflammation was explored in a mouse model of hemarthrosis. Hemarthrosis resulted in local cytokine/chemokine changes, represented by elevated pro-inflammatory (IL-6, MCP-1, MIP-1α, MIP-1β) and pro-angiogenic (VEGF and IL-33) cytokines, and decreased anti-pro-inflammatory cytokines IL-4 and IL-10. Both changes can be reversed by JODI administration to demonstrate that JODI-19 may be a promising and effective therapeutic avenue to manage hemophilia arthropathy.

ACKNOWLEDGMENTS

The authors acknowledge the Animal Histopathology and Lab Medicine Core at UNC-CH for multiple cytokine measurements and hematological and liver/kidney tests.

AUTHORS' CONTRIBUTION

All authors reviewed the article and approved its content. JS and DS designed the study. CZ and JS performed the *in vivo* study and wrote the manuscript. DS, OG, AB, and IE prepared the compound and revised the manuscript. BH and YZ revised the manuscript.

FUNDING INFORMATION

This work was partly supported by a research grant from Asklepios BioPharmaceutical (to J.S.). It was also supported by "the Fundamental Research Funds for the Central Universities."

COMPLIANCE WITH ETHICAL STANDARDS

All investigations were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

Conflict of Interest. The authors declare that they have no conflicts of interest.

REFERENCES

1. Srivastava, A., A.K. Brewer, E.P. Mauser-Bunschoten, et al. 2012. Guidelines for the management of hemophilia. *Hemophilia* 19: e1–e47.
2. Soucie, J.M., S.D. Grosse, A.E. Siddiqi, et al. 2017. The effects of joint disease, inhibitors and other complications on health-related quality of life among males with severe hemophilia A in the United States. *Hemophilia* 23: e287–e293.

3. Nilsson, I.M., E. Berntorp, T. Lofqvist, et al. 1992. Twenty-five years' experience of prophylactic treatment in severe hemophilia A and B. *Journal of Internal Medicine* 232: 25–32.
4. Manco-Johnson, M.J., B. Lundin, S. Funk, C. Peterfy, D. Raunig, M. Werk, C.L. Kempton, M.T. Reding, S. Goranov, L. Gercheva, L. Rusen, V. Uscatescu, M. Pierdominici, S. Engelen, J. Pocoski, D. Walker, and W. Hong. 2017. Effect of late prophylaxis in hemophilia on joint status: a randomized trial. *Journal of Thrombosis and Haemostasis* 15: 2115–2124.
5. Manco-Johnson, Marilyn J., Thomas C. Abshire, Amy D. Shapiro, Brenda Riske, Michele R. Hacker, Ray Kilcoyne, J. David Ingram, Michael L. Manco-Johnson, Sharon Funk, Linda Jacobson, Leonard A. Valentino, W. Keith Hoots, George R. Buchanan, Donna DiMichele, Michael Recht, Deborah Brown, Cindy Leissinger, Shirley Bleak, Alan Cohen, Prasad Mathew, Alison Matsunaga, Desiree Medeiros, Diane Nugent, Gregory A. Thomas, Alexis A. Thompson, Kevin McRedmond, J. Michael Soucie, Harlan Austin, and Bruce L. Evatt. 2007. Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. *The New England Journal of Medicine* 357: 535–544.
6. Fischer, K., K. Steen Carlsson, P. Petrini, et al. 2013. Intermediate-dose versus high-dose prophylaxis for severe hemophilia: comparing outcome and costs since the 1970s. *Blood* 122: 1129–1136.
7. Simpson, M.L., and L.A. Valentino. 2012. Management of joint bleeding in hemophilia. *Expert Review of Hematology* 5: 459–468.
8. Arruda, V.R., B.S. Doshi, and B.J. Samelson-Jones. 2017. Novel approaches to hemophilia therapy: successes and challenges. *Blood* 130: 2251–2256.
9. Melchiorre, D., M. Manetti, and M. Matucci-Cerinic. 2017. Pathophysiology of hemophilic arthropathy. *Journal of Clinical Medicine* 6: 63.
10. Ovlisen, K., A.T. Kristensen, A.L. Jensen, et al. 2009. IL-1 beta, IL-6, KC and MCP-1 are elevated in synovial fluid from hemophilic mice with experimentally induced haemarthrosis. *Hemophilia* 15: 802–810.
11. Sen, D., A. Chapla, N. Walter, V. Daniel, A. Srivastava, and G.R. Jayandharan. 2013. Nuclear factor (NF)-kappaB and its associated pathways are major molecular regulators of blood-induced joint damage in a murine model of hemophilia. *Journal of Thrombosis and Haemostasis* 11: 293–306.
12. El-Hashim, A., S. Yousefi, I. Edafiogho, et al. 2010. Anti-inflammatory and immunosuppressive effects of the enaminone E121. *European Journal of Pharmacology* 632: 73–78.
13. Ghoneim, O.M., A. Bill, J. Dhuguru, D.E. Szollosi, and I.O. Edafiogho. 2018. Design, synthesis and biological evaluation of piperazino-enaminones as novel suppressants of pro-inflammatory cytokines. *Bioorganic & Medicinal Chemistry* 26: 3890–3898.
14. Szollosi, D.E., O.A. Ghoneim, M.K. Manzoor, et al. 2016. Novel piperazino-enaminones suppress pro-inflammatory cytokines and inhibit chemokine receptor CCR2. *Inflammation* 39: 2053–2061.
15. Lin, H.F., N. Maeda, O. Smithies, D.L. Straight, and D.W. Stafford. 1997. A coagulation factor IX-deficient mouse model for human hemophilia B. *Blood* 90: 3962–3966.
16. Sun, J., N. Hakobyan, L.A. Valentino, B.L. Feldman, R.J. Samulski, and P.E. Monahan. 2008. Intraarticular factor IX protein or gene replacement protects against development of hemophilic synovitis in the absence of circulating factor IX. *Blood* 112: 4532–4541.
17. Sun, J., B. Hua, E.W. Livingston, S. Taves, P.B. Johansen, M. Hoffman, M. Ezban, D.M. Monroe, T.A. Bateman, and P.E. Monahan. 2017. Abnormal joint and bone wound healing in hemophilia mice is improved by extending factor IX activity after hemarthrosis. *Blood* 129: 2161–2171.
18. Valentino, L.A., N. Hakobyan, C. Enockson, et al. 2012. Exploring the biological basis of hemophilic joint disease: experimental studies. *Hemophilia* 18: 310–318.
19. Drake, T.A., J.H. Morrissey, and T.S. Edgington. 1989. Selective cellular expression of tissue factor in human tissues, implications for disorders of hemostasis and thrombosis. *The American Journal of Pathology* 134: 1087–1097.
20. Storti, E., U. Magrini, and E. Ascari. 1971. Synovial fibrinolysis and hemophilic hemarthrosis. *British Medical Journal* 4: 812.
21. Deschaseaux, F., L. Sensebe, and D. Heymann. 2009. Mechanisms of bone repair and regeneration. *Trends in Molecular Medicine* 15: 417–429.
22. Baud'huin, M., F. Lamoureux, L. Duplomb, et al. 2007. RANKL, RANK, osteoprotegerin: key partners of osteoimmunology and vascular diseases. *Cellular and Molecular Life Sciences* 64: 2334–2350.
23. Kaneshiro, S., K. Ebina, K. Shi, C. Higuchi, M. Hirao, M. Okamoto, K. Koizumi, T. Morimoto, H. Yoshikawa, and J. Hashimoto. 2014. IL-6 negatively regulates osteoblast differentiation through the SHP2/MEK2 and SHP2/Akt2 pathways in vitro. *Journal of Bone and Mineral Metabolism* 32: 378–392.
24. Sims, N.A., B.J. Jenkins, J.M. Quinn, et al. 2004. Glycoprotein 130 regulates bone turnover and bone size by distinct downstream signaling pathways. *The Journal of Clinical Investigation* 113: 379–389.
25. van Vulpen, L.F., R.E. Schutgens, K. Coeleveld, et al. 2015. IL-1beta, in contrast to TNF alpha, is pivotal in blood-induced cartilage damage and is a potential target for therapy. *Blood* 126: 2239–2246.
26. Cavalli, G., and C.A. Dinarello. 2015. Treating rheumatological diseases and co-morbidities with interleukin-1 blocking therapies. *Rheumatology* 54: 2134–2144.
27. Latourte, A., A. Frazier, C. Briere, et al. 2013. Interleukin-1 receptor antagonist in refractory haemochromatosis-related arthritis of the hands. *Annals of the Rheumatic Diseases* 72: 783–784.
28. Narkbunnam, N., J. Sun, G. Hu, F.C. Lin, T.A. Bateman, M. Mihara, and P.E. Monahan. 2013. IL-6 receptor antagonist as adjunctive therapy with clotting factor replacement to protect against bleeding-induced arthropathy in hemophilia. *Journal of Thrombosis and Haemostasis* 11: 881–893.
29. Sun, Junjiang, Genlin Hu, and Paul E. Monahan. 2009. TNF-alpha antagonists augment factor replacement to prevent arthropathy in hemophilic mice. *Journal of Thrombosis and Haemostasis* 7 (Suppl 2): 225.
30. Melchiorre, D., M. Morfini, S. Linari, A.L. Zignego, M. Innocenti, and M. Matucci Cerinic. 2014. Anti-TNF-alpha therapy prevents the recurrence of joint bleeding in hemophilia and arthritis. *Rheumatology* 53: 576–578.
31. McDonald, A.G., K. Yang, H.R. Roberts, D.M. Monroe, and M. Hoffman. 2008. Perivascular tissue factor is down-regulated following cutaneous wounding: implications for bleeding in hemophilia. *Blood* 111: 2046–2048.
32. Acharya, S.S., R.N. Kaplan, D. Macdonald, O.T. Fabiyi, D. DiMichele, and D. Lyden. 2011. Neoangiogenesis contributes to the development of hemophilic synovitis. *Blood* 117: 2484–2493.
33. He, R., H. Yin, B. Yuan, T. Liu, L. Luo, P. Huang, L. Dai, and K. Zeng. 2017. IL-33 improves wound healing through enhanced M2 macrophage polarization in diabetic mice. *Molecular Immunology* 90: 42–49.

34. Nefla, M., D. Holzinger, F. Berenbaum, and C. Jacques. 2016. The danger from within: alarmins in arthritis. *Nature Reviews Rheumatology* 12: 669–683.
35. Nieuwenhuizen, L., R.E. Schutgens, K. Coeleveld, et al. 2014. Hemarthrosis in hemophilic mice results in alterations in M1-M2 monocyte/macrophage polarization. *Thrombosis Research* 133: 390–395.
36. van Meegeren, M.E., G. Roosendaal, N.W. Jansen, et al. 2012. IL-4 alone and in combination with IL-10 protects against blood-induced cartilage damage. *Osteoarthritis and Cartilage* 20: 764–772.
37. van Meegeren, M.E., G. Roosendaal, K. van Veghel, et al. 2013. A short time window to profit from protection of blood-induced cartilage damage by IL-4 plus IL-10. *Rheumatology* 52: 1563–1571.
38. van Meegeren, M.E., G. Roosendaal, K. Coeleveld, et al. 2013. A single intra-articular injection with IL-4 plus IL-10 ameliorates blood-induced cartilage degeneration in hemophilic mice. *British J Hematology* 160: 515–520.

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.