Musculin Deficiency Aggravates Colonic Injury and Inflammation in Mice with Inflammatory Bowel Disease

Jing Yu, Yijia Liu, Wei Zhang, Xue Yang, Wanqi Tang, Huaping Liang, Suiyan Li, Wenda Gao, and Jun Yan

Abstract—Intestinal inflammatory reactions and resulting tissue injuries are two major aspects of inflammatory bowel disease (IBD). The regulatory factors involved in the pathogenesis of IBD remain unclear. Recent studies showed that musculin (MSC) as a transcription suppressor participates in the regulation of certain immune functions. The purpose of this study was to determine the impact of MSC deficiency on colonic injury and inflammatory reaction under IBD, where wild-type (WT, +/+) and MSC-knockout (MSCKO, MSC−/−) mice were induced for disease by dextran sulfate sodium (DSS) in drinking water. Immunohistochemistry hematoxylin-eosin (H&E) staining, enzyme-linked immunosorbent assay (ELISA), and quantitative real-time polymerase chain reaction (qRT-PCR) were used to analyze the matching samples from groups of different genotypes. The colonic epithelial injury in the MSC−/− IBD group was much severer than that in the +/+ IBD group, concurrent with higher IL-22 levels from the supernatant of ex vivo cultured colon tissues in the MSC−/− IBD group than those in the +/+ IBD group. The mRNA levels of IL-22 in mesenteric lymph nodes (MLN) also manifested similar tendency. MSC deficiency may enhance the inflammatory reactions in the gut via excessive secretion of IL-22, leading to aggravated colonic epithelial injury under IBD.

KEY WORDS: musculin (MSC); colonic injury; inflammation; inflammatory bowel disease (IBD).

INTRODUCTION

Inflammatory reactions unequivocally underlying the development of almost all trauma and diseases are the most common event for pathogenesis. Consequently, tissue injury triggered by inflammatory reaction becomes the inevitable outcome in such process. As a chronic, recurrent inflammatory disease of the gastrointestinal tract, inflammatory bowel disease (IBD) has an increasing incidence over the globe [1, 2]. Although much has been learned about its pathogenesis relating to gut injury and inflammation, regulatory factors involved in this process still remain unclear. The lack of this knowledge dampens our ability to...
improve the efficacy of various clinical intervention strategies for IBD.

Musculin (MSC), a basic helix-loop-helix (bHLH) transcript factor, is highly conserved in mammals [3, 4]. Previous studies showed that MSC not only plays a vital role in myogenesis of skeletal muscle, tissue repair, differentiation, and regeneration [5–7], but also directly participates in the regulation of inflammatory reaction and immune function [8–10]. However, it is still unknown whether MSC may regulate intestinal inflammation so as to influence the pathogenesis of colonic injury under IBD.

In this study, MSC-knockout (KO, −/−) mice were compared side-by-side with wild type (+/+) animals in a murine model of IBD induced by dextran sulfate sodium (DSS). The impact of MSC deficiency on colonic injury and inflammatory reaction was determined by in vivo and ex vivo experiments. Our results suggest that the MSC/IL-22 axis could be a key molecular duet, regulating the delicate balance between excessive intestinal inflammation and tissue repair.

MATERIALS AND METHODS

Mice

MSC−/− mice in C57BL/6 background were designed by us and made by BioCytogen, China. Wild-type (WT, +/-) C57BL/6 mice were purchased from the Experimental Animal Center, Research Institute of Surgery, Daping Hospital, Army Medical University (Third Military Medical University), China. All mice were housed under special pathogen free (SPF) condition. All animal procedures in the study were approved by the Experimental Animal Welfare and Ethics Committee of Army Medical University (Third Military Medical University).

Reagents and Kits

DSS was purchased from MP Biomedicals, France, and TRIzol was from Invitrogen, USA. The kits of enzyme-linked immunosorbent assay (ELISA) for mouse IL-10, IL-17A, IL-22, and TNF-α were from Boster, China. PrimeScript RT Kit with gDNA Eraser and SYBR Premix Ex Taq™ were products from Takara, Japan. The primers of quantitative real-time polymerase chain reaction (qRT-PCR) for IL-17A, IL-22, and MSC were synthesized by Generay, China (Table 1).

Murine IBD Model

A total of 44 10-week-old male mice with 20 g (g) average body weight, including 22 +/- and 22 MSC−/− mice, were randomly divided into 4 groups (n = 11/group) as follows: +/- control, MSC−/− control, +/- IBD, and MSC−/− IBD. IBD was induced by providing 4% DSS in drinking water on an ad libitum basis for continuous 7 days (d). The control groups were provided with drinking water without DSS. The dynamic changes in body weight, bloody stool, animal behavior, and survival status of each group were recorded every day.

Ex Vivo Culture of Colon Tissues

Mice were sacrificed 7 days after DSS induction of IBD, and the length of the colon was measured for each mouse. Colon tissues were then excised and rinsed with phosphate buffer solution (PBS) for 3–5 times to remove contents in the enteric cavity. The colon tissues were cut into 1 square centimeter (cm²) pieces and seeded in a 24-well plate (1 piece in each well) in 1.0 mL RPMI-1640 medium and cultured under 5% CO2 at 37 °C. Twenty-four hours later, the supernatants from the ex vivo culture of colon tissues from different mice of each group were harvested, and the cytokine levels for IL-10, IL-17A, IL-22, and TNF-α were measured by ELISA according to the kit manufacturers’ instructions.

qRT-PCR

Total RNA from mouse mesenteric lymph nodes (MLN) was extracted with 1 mL TRIzol, and 1 microgram (µg) RNA template was reverse transcribed into cDNA with the PrimeScript RT kit. qRT-PCR was performed on a Bio-Rad CFX Manager Optics Real-Time PCR System, using the primers listed in Table 1. The qRT-PCR conditions were as follows: 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C. The expression levels of IL-17A, IL-22, and MSC relative to GAPDH were determined in triplicates on the basis of the comparative 2−ΔΔCT method.

Histological Analysis

Colon tissues were fixed by 4% paraformaldehyde and embedded in paraffin before being processed for staining with hematoxylsin and eosin (H&E). Colitis pathology and infiltration were blindly reviewed by two independent pathologists. Histology scoring was performed in different groups.
Statistical Analysis

ELISA and qRT-PCR data were presented as mean value ± standard deviation (SD) and analyzed with the multiple comparison Sidak method of one-factor variance (ANOVA) in GraphPad 6.02 software. Statistical significance was reached if P value is less than 0.05 (P < 0.05).

RESULTS

DSS-Induced IBD in +/- and MSC−/− Mice

Without DSS treatment, mice in the +/- control group and MSC−/− control group showed normal growth and behavior, i.e., active movement, glossy hair, stable body weight, and normal stool. In contrast, 4 days after DSS treatment, mice in the +/- IBD group and MSC−/− IBD group started to show several symptoms, such as wiggling and shivering, loss of body weight, messy hair, and diarrhea. Moreover, blood streak around the anus could be found in several mice. Seven days after DSS treatment, 1 mouse each from the +/- IBD group and MSC−/− IBD group died, respectively. Furthermore, bloody stools were seen in other mice in the IBD groups. Compared with the control groups without DSS treatment, the IBD groups had darker intestine color and local adhesions in some of the colon regions (Fig. 1a).

MSC Deficiency Aggravates Colonic Injury Under IBD

On the 7th day after DSS induction of IBD, colons of mice in the IBD groups were significantly shorter than those in the non-treated control groups (+/+ IBD vs. +/- control: P < 0.01; MSC−/− IBD vs. MSC−/− control: P < 0.0001). However, the difference of colon lengths between +/- and MSC−/− in the control groups and IBD groups was not obvious (P > 0.05) (Fig. 1b, c).

Although MSC deficiency does not seem to affect colon length, detailed histochemistry analysis revealed that in the absence of MSC, colon injury during DSS-induced IBD did get aggravated. At steady state without DSS treatment, the structures of colonic tissue in both control groups were intact with clear borders and well-aligned epithelial cells. There was no significant morphological difference between the two control groups. DSS treatment clearly triggered colon injury in both the +/- IBD group and MSC−/− IBD group. The results of H&E staining showed the damage of colonic epithelium, infiltration of inflammatory cells, and disarranged and swelling of epithelial cells in both IBD groups. Notably, combined with the results of histology score, the degree of injury in the MSC−/− IBD group was much severer than that in the +/- IBD group (Fig. 2).

MSC Deficiency Showed Opposite Effects on the Levels of Pro- and Anti-inflammatory Cytokines in Ex Vivo Culture of Colon Tissues Under IBD

We consistently observed enhanced IL-22 production in MSC−/− mice, regardless of being at steady state or under IBD (Fig. 3). This suggests that MSC could be a negative regulator of IL-22 synthesis. As IL-22 is a feature cytokine released by Th17 cells, we also looked at the two pro-inflammatory cytokines associated with this T cell subset, IL-17A and TNF-α. Interestingly, MSC deficiency caused a consistent reduction of IL-17A (P < 0.0001) and TNF-α (P < 0.05) under IBD while it showed the same tendency (IL-17A P < 0.05, TNF-α ns) at steady state (Fig. 3). This suggests that MSC may be necessary for a full-fledged Th17 response. IL-10 and IL-22 are both from the IL-10 cytokine family but with anti- and pro-inflammatory functions. In line with this, compared with steady state, IL-10 as an inhibitory cytokine was downregulated (P < 0.01) under IBD, regardless of being in +/- or MSC−/− mice. MSC deficiency further reduced IL-10
production (Fig. 3), suggesting that MSC may reciprocally regulate pro- and anti-inflammatory cytokines, esp. IL-22 and IL-10.

MSC Deficiency Reciprocally Regulate the mRNA Levels of IL-22 and IL-17A in MLN Under IBD

To confirm the above cytokine ELISA data, we measured the mRNA levels of IL-22 and IL-17A in the MLN of mice from different groups. Indeed, MSC deficiency dramatically increased IL-22 mRNA levels during IBD ($P < 0.0001$). In contrast, IL-17A mRNA levels were decreased significantly in MSC$^{-/-}$ MLN under IBD ($P < 0.0001$) (Fig. 4). MSC has been reported to be induced in lymphocytes under inflammatory conditions. We confirmed that MSC is highly expressed only in the $+/+$ mice under IBD ($P < 0.05$), and no MSC messages can be detected in MLN at steady state or in MSC$^{-/-}$ mice. Thus, our results strongly suggest that MSC may be involved in regulating the development of inflammation through modulating cytokine expression, esp. IL-22 and IL-17A.

DISCUSSION

IBD is a chronic recurrent inflammatory disease in the gastrointestinal tract that consists of ulcerative colitis (UC) and Crohn’s disease (CD) and has a typical feature of worldwide epidemic incidence [11]. Studies showed that continuous inflammation in the colonic mucous membrane is an important pathologic process of UC [12], while the affected site in CD also locates in the colon. Thus, colonic
Fig. 2. Histochemistry analysis of colon tissues from representative mice of different groups. a +/+ control. b MSC−/− control. c +/+ IBD. d MSC−/− IBD. e histology score. Scale bar indicates 50 micrometers (μm).
injury is the common ultimate stage for both UC and CD [13]. However, what factors regulate the above process are not fully known.

The imbalance between colonic inflammation and epithelium repair directly influences the development and outcome of IBD. It is necessary to maintain an active colonic epithelium repair process under the inflammatory circumstance in the gut. Excessive inflammatory reaction will aggravate colonic epithelium damage. As a member of the IL-10 family, IL-22 plays a vital role of a double-edged sword. In particular, a proper level of IL-22 may protect the integrity of colonic epithelium, inhibit cellular apoptosis, and promote tissue repair, so as to maintain immune homeostasis in the gut [14–16]. On the contrary, over-expression of IL-22 will lead to excessive inflammatory reaction, which may trigger the pathological process of IBD and even induce autoimmune disorders [17, 18]. Thus, following IBD induction, the level of IL-22 in the colonic microenvironment directly regulates the pathological process versus epithelium repair, and hence the disease outcome. Previous studies have shown that IL-22 levels are regulated by IL-22-secreting cells, which mainly reside in the intestinal mucosa, especially in MLN [19]. A consensus in this field is that the response of the intestinal mucosal system to the inflammatory reactions under disease status is dysregulated [20–24]. Determining what factors contribute to this (dys)regulation has been highly sought.

MSC, also known as activated B cell factor-1 (ABF-1) or represor of myogenesis (MyoR), has high homology in protein sequence and spatial structure from lower to higher mammals [25]. As a transcription factor and member of the basic helix-loop-helix (bHLH) family, MSC has two alternatively spliced isoforms, MSC1a and MSC1b, which are 201 and 180 amino acids respectively [26]. Studies showed that MSC not only participates in the processes of mammalian skeletal myogenesis, tissue development, differentiation, and regeneration [5–7], but also modulates inflammatory reaction via regulating immune cell differentiation and function under trauma and disease [8, 10, 27, 28]. However, it is not clear yet whether MSC

Fig. 3. The levels of secreted cytokines by ELISA in the ex vivo culture of colon tissues from different groups. a IL-22. b IL-17A. c TNF-α. d IL-10 (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).
Fig. 4. qRT-PCR comparison of mRNA levels in MLN of mice from different groups. a IL-22. b IL-17A. c MSC (*$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$).
may regulate the secretion of IL-22 in MLN in the colonic microenvironment.

To address these questions, herein we studied the effect of MSC deficiency on colonic injury and inflammatory reaction under IBD. Using the murine model of DSS-induced IBD, we found that the degree of colonic injury and inflammatory reaction in the MSC−/− IBD group was much severer than that in the +/+ IBD group. Moreover, our ex vivo measurement of cytokines released by colon tissues harvested from IBD mice indicates that MSC may be involved in the reciprocal regulation of IL-22 and IL-17A, in that compared with the +/+ IBD group, IL-22 production was increased while IL-17A, TNF-α, and IL-10 production was decreased in the MSC−/− IBD group. The same tendency is confirmed with qRT-PCR measurement of IL-22 and IL-17A mRNA levels in the MLN during IBD. Given the pro-inflammatory effect of IL-22 in many atopic and autoimmune disease settings, our results suggested that the MSC/IL-22 axis may play a vital role in colonic inflammatory reaction under IBD. The pro-inflammatory effect of IL-22 could override its protective function on epithelium repair and barrier integrity. Therefore, the tipped imbalance of colonic inflammatory reaction over epithelium repair by exuberant production of IL-22 in the absence of MSC could eventually lead to severer disease outcome in IBD. Any factors that cause the diminished expression of MSC in the gut may likewise trigger a similar inflammatory path towards IBD.

Although in our study we focused on the expression of MSC and IL-22 in MLN and colon tissues, our data do not provide a definitive answer as to what is the cellular identity of IL-22-secreting and MSC-expressing immune cells. Given the importance of maintaining barrier integrity and containing inflammatory reaction of the intestinal mucosal system, one of the initiating factors in many trauma conditions and systemic diseases, it is necessary for us to further identify the cellular components and signaling pathways involved in the MSC/IL-22 axis in future studies. This will in turn clarify the underlying mechanism of MSC/IL-22 for the pathogenesis of IBD and bring about better intervention strategy for this disease.

In summary, our study has shown that MSC, a previously underappreciated transcription factor, plays an important regulatory role in colonic injury and inflammatory reaction. MSC deficiency may enhance intestinal inflammatory reaction via excessive secretion of IL-22 and lead to aggravated colonic epithelial injury under IBD. Our findings suggest that MSC may act as a novel regulatory factor for maintaining the balance between excessive inflammatory reaction and tissue repair in the gut.

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AUTHOR CONTRIBUTION

J Yan designed the study, performed data management, and drafted the paper. J Yu and YJ L participated in the study design and performed the experiments and data analysis. W Z, X Y, and WQ T performed the experiments. HP L and SY L participated in the study. W G designed the MSC−/− mouse, provided several important suggestions, and improved English writing of the paper. All authors read and approved the final manuscript.

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COMPLIANCE WITH ETHICAL STANDARDS

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

Ethics Approval. All animal procedures were conducted according to the guidelines and with the approval of the Army Medical University (Third Military Medical University) Laboratory Animal Management Committee and approved by the Ethics Committee.

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