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MLKL deficiency inhibits DSS-induced colitis independent of intestinal microbiota

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ABSTRACT

The maintenance of intestinal tissue homeostasis is vital for the resistance against inflammatory bowel diseases (IBDs). Necroptosis is identified as an alternative mode of regulated cell death, which plays a pivotal role in tissue homeostasis. Thus, the roles of RIP3-mediated necroptosis in intestinal inflammation have been extensively studied. However, the biological implications of the mixed lineage kinase-like protein (MLKL), a molecule downstream of RIP3 in gut remain unclear. In this study, the role of MLKL in DSS-induced colitis was examined, and the contribution of gut microbiota was also determined. Compared with non-littermate WT mice, the survival rate, clinical score, intestinal damage and intestinal mucosal barrier integrity of non-littermate MLKL-deficient mice are significantly improved. MLKL deficiency prevents inflammatory cytokines production and MAPK signaling activation. Hence, MLKL deficiency inhibits DSS-induced colitis. Moreover, we proved that DSS susceptibility difference between two genotypes is not driven by intestinal microbiota based on the co-housing of two non-littermate genotypes and qPCR detection of fecal dominant bacterial taxa.

1. Introduction

Inflammatory bowel disease (IBD) refers to chronic, nonspecific inflammatory gastrointestinal disorder and is comprised of two main clinical forms of Crohn's disease and ulcerative colitis (Maloy and Powrie, 2011). The precise aetiology of IBDs remains unclear. A healthy gut has evolved diverse regulatory mechanisms that tightly control the immune reaction and cooperate to maintain intestinal immune homeostasis. Maintaining tissue homeostasis is vital during infection and inflammation. Similarly, normal intestinal homeostasis is not only essential for mounting protective immunity to pathogenic invasion, but also important for the pathogenesis of IBD. Necroptosis is identified as an alternative mode of mode of regulated cell death which plays a pivotal role in tissue homeostasis. Hence, the role of necroptosis in the pathogenesis of IBD has been studied. Indeed, the absence of RIP3 can reduce intestinal inflammation induced by FADD OR caspase-8 knockout, indicating that RIP3-dependent necroptosis promotes intestinal inflammation (Weinlich et al., 2013; Welz et al., 2011).

The mixed lineage kinase-like protein (MLKL), a member of the pseudokinase family, was originally identified as a critical necroptosis mediator that operates downstream of the receptor interacting protein RIP3 (Sun et al., 2012; Wu et al., 2013). Despite the RIP3-MLKL axis in cell death and inflammatory signaling is extensively investigated, the biological function of MLKL in the intestinal mucosal barrier remains elusive. Previously we found that MLKL promotes inflammasome activation in intestinal epithelial cells to inhibit pathogenic bacteria colonization (Yu et al., 2018). In this study, the DSS-induced mouse colitis model was used to investigate the role of MLKL gene in the pathogenesis of ulcerative colitis. The dynamic crosstalk between host immune and intestinal microbes represents one of the fundamental features of intestinal homeostasis. Moreover, the development of IBD is closely related to the disorder of intestinal microecology (Galvez et al., 2017; Zhang et al., 2017). Hence, the impact of intestinal microbiota on the colitis-regulated effect of MLKL was also investigated.

2. Materials and methods

2.1. Mice

MLKL$^{-/-}$ mice (gift from Dr. Jiahuai Han, Xiamen University, China) (Wu et al., 2013) were backcrossed to C57BL/6 background for eight generations. Two genotypes were housed in separated room. For

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Table 1
Primers used for Real-time PCR.

Gene	Sequence
Bacteroides	F:5'- GGTTCTGAGAGGAGGTCCC -3'
	R:5'- CTGCCTCCCGTAGGAGT -3'
Prevotellaceae	F:5'- CCAGCCAAGTAGCGTGCA -3'
	R:5'- TGGACCTTCCGTATTACC -3'
TM7	F:5'- GCAACTCTTTACGCCCAGT -3'
	R:5'- GAGAGGATGATCAGCCAG -3'
Lactobacillus	F:5'- AGCAGTAGGGAATCTTCCA -3'
	R:5'- CACCGCTACACATGGAG -3'
Staphylococcus	F:5'- TTTGGGCTACACACGTGCTACAATGGACAA -3'
	R:5'- AACAACTTTATGGGATTTGCWTGA -3'
Streptococcus spp.	F:5'- AGATGGACCTGCGTTGT -3'
	R:5'- GCTGCCTCCCGTAGGAGTCT -3'
Enterococcus spp.	F:5'- CCCTTATTGTTAGTTGCCATCATT -3'
	R:5'- ACTCGTTGTACTTCCCATTGT -3'
Bifidobacterium spp.	F:5'- ATTCTGGCTCAGGATGAACGC -3'
	R:5'- CTGATAGGACGCGACCCCAT -3'
A.muciniphila	F:5'- AGAGGTCTCAAGCGTTGTTCGGAA -3'
	R: 5'- TTTCGCTCCCCTGGCCTTCGTGC -3'
Firmicutes	F:5'- GGAGYATGTGGTTTAATTCGAAGC A -3'
	R:5'- AGCTGACGACAACCATGCAC -3'
Proteobacteria	F:5'- TCGTCAGCTCGTGTYGTGA -3'
	R:5'- CGTAAGGGCCATGATG -3'
Bacteroidetes	F:5'- GAGAGGAAGGTCCCCCAC -3'
	R: 5'- CGCTACTTGGCTGGTTCAG -3'
Clostridium cluster IV F	F:5'-GCACAAGCAGTGGAGT-3'
	R: 5'-CTTCCTCCGTTTTGTCAA-3'
MIB	F:5'- CCAGCAGCCGCGGTAATA-3'
	R: 5'- CGCATTCCGCATACTTCTC-3'
SFB	F:5'- AGGAGGAGTCTGCGGCACATTAGC-3'
	R: 5'- CGCATCCTTTACGCCCAGTTATTC-3'
P. gingivalis	F:5'- CTTGACTTCAGTGGCGGCAG-3'
	R: 5'- AGGGAAGACGGTTTTCACCA-3'
E. coli	F:5' CATGCCGCGTGTATGAAGAA–3'
	R: 5'- CGGGTAACGTCAATGAGCAAA-3'
Bacteroides fragllis	F:5'- TTTAAAGGGAGCGTAGGTGGATTG-3'
	R: 5'- CTCAGTGTCAGTTGCAGTCCAGT-3'
Eubacteria (Universal)	F:5'- ACTCCTACGGGAGGCAGCAGT -3'
	R: 5'- ATTACCGCGGCTGCTGGC -3'

co-housing studies, the SPF non-littermate WT and MLKL$^{-/-}$ mice (4-week-old) were co-housed at 1 : 1 ratio for 4 weeks. Adult male or female 8- to 10-week-old mice were used except in co-housing experiments. All animal studies were approved by the Animal Welfare and Research Ethics Committee at Jilin University.

2.2. DSS colitis and clinical scoring

Colitis is induced by addition of 2% DSS (molecular mass 36,000–50,000 Da, MP Biomedicals) to drinking water. After 7 days, DSS was replaced with normal drinking water. Mice were weighed daily. Stool consistency, the presence of occult blood and disease activity index (DAI) were determined every 2 days by a previous scoring system (Alex et al., 2009).

2.3. Assessment of colon inflammation and immunohistochemistry

Tissue samples were harvested on the 8th day and fixed in buffered formalin solution (4%). The sections were stained with hematoxylin and eosin (H&E). Tissue pathology scores were determined as previous described (Alex et al., 2009). For immunohistochemistry, the sections were stained with F4/80 (BioLegend), mucin 2 (Santa Cruz), and claudin 3 (Abcam) antibodies. Cellular apoptosis was analyzed by TUNEL staining (KeyGEN Biotech).

2.4. Western blotting

The colon tissues were flushed free of feces with pre-cooled PBS, and then homogenized in lysis solution supplemented with complete protease inhibitor cocktail, and then centrifuged at 12,000 rpm for 20 min. The supernatants were collected and used for western blotting analysis. The membranes were incubated with primary antibodies against p-P38, total P38, p-ERK, total ERK, p-JNK, total JNK (Cell Signaling) and GAPDH (Proteintech).

2.5. Cytokine measurements

The colon tissues were homogenized mechanically in cold PBS (at a ratio of 6 mL per gram tissue) containing complete protease inhibitor cocktail and 1% Triton X-100. Tissue homogenates were then centrifuged at 12,000 rpm for 20 min. The supernatants were collected and used for inflammatory cytokines examination with ELISA kits following the R&D Systems instruction.

2.6. Real-time PCR

DNA was extracted from fecal samples using TIANamp DNA Stool DNA Kit (Qiagen, Beijing, China). Bacterial abundance was quantified by Real-time PCR using specific bacterial 16S rRNA gene primers in 200 ng DNA. The sequences of specific 16S rRNA gene target primer and universal eubacterial primer were listed in Table 1.

2.7. Statistical analysis

The data were expressed as mean ± SEM. All experiments were independently performed three times. Student's unpaired *t*-test was used to assess the difference when two groups were compared ($^{*}p < 0.05$ and $^{**}p < 0.01$). Statistical analysis was performed by GraphPad Software.

3. Results

3.1. MLKL deficiency facilitates host resistance to DSS-induced colitis

To investigate the function of MLKL in the intestine, we induced colitis in WT and MLKL$^{-/-}$ mice by feeding them with 2% DSS in drinking water for 7 days. We initially examined the mortality rate. We found that MLKL$^{-/-}$ mice displayed a significantly lower mortality compared with WT mice (Fig. 1A). Colitis susceptibility in MLKL$^{-/-}$ and WT mice was evaluated daily by monitoring body weight and clinical features including diarrhea and stool bleeding. Consistently, WT mice showed a marked reduction in body weight, and exhibited severe stool bleeding, diarrhea, and disease activity index (DAI) compared with MLKL$^{-/-}$ mice (Fig. 1B–E).

To further characterize the effect of MLKL in DSS-induced colitis, the mice were sacrificed on day 8. The colonic tissues of WT mice were significantly shorter than those of MLKL$^{-/-}$ mice (Fig. 2A-B), indicating that WT mice had a severe colonic damage. This observation was further supported by hematoxylin and eosin (H&E)-staining (Fig. 2C). Histopathology scores were more severe in WT mice than in MLKL$^{-/-}$ mice, represented by increased crypt destruction, ulceration, and submucosal edema (Fig. 2D). The maintenance of epithelial barrier

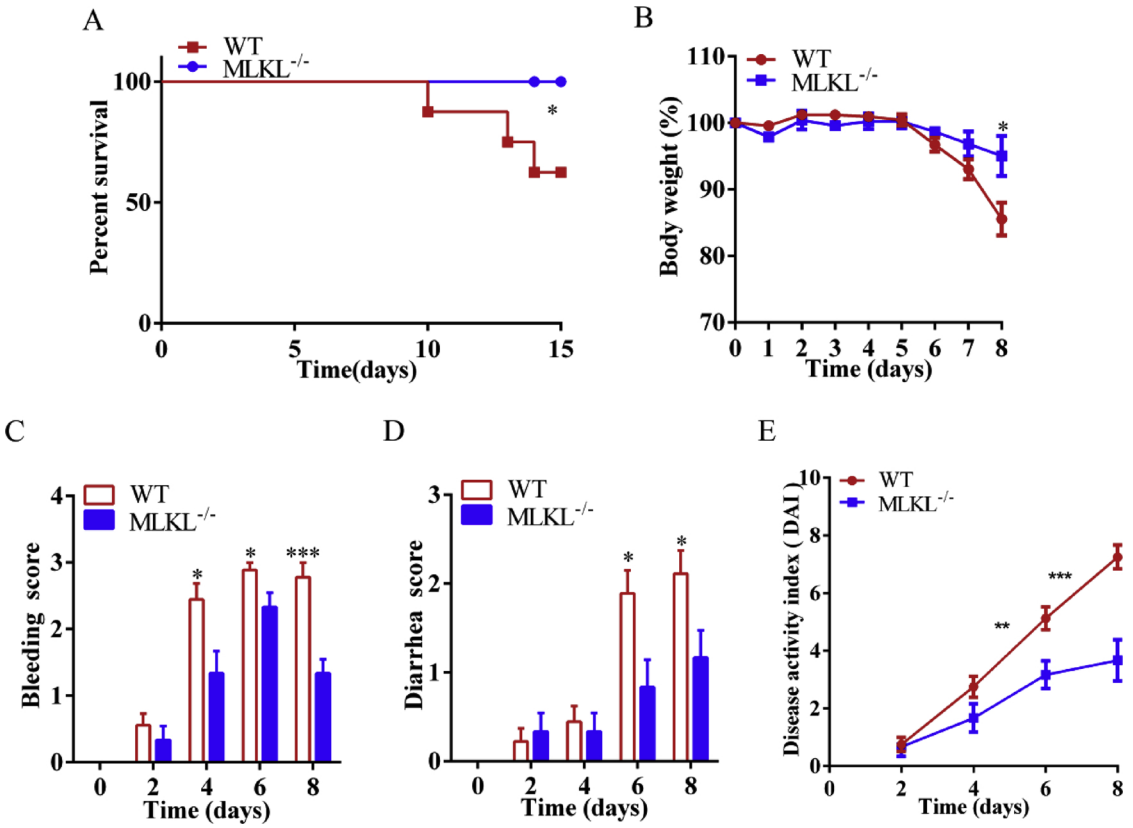

Fig. 1. MLKL promotes colitis development. The non-littermate WT and MLKL$^{-/-}$ mice (n = 10 each group) were fed with 2% DSS in drinking water for 7 days. (A) Survival and (B) body weight were detected daily; (C) Rectal bleeding, (D) stool consistency, and (E) DAI were scored every 2 days. Data represent mean ± SEM, *P < 0.05, ***P < 0.001. These experiments and analyses were performed on mice that were separately housed according to genotype.

integrity is essential for avoiding colitis. Therefore, we subsequently investigated whether MLKL affect the epithelial barrier integrity. We found that there was no difference in basal claudin 3 expression between two genotypes. However, the level of claudin 3 expression in WT mice was significantly lower than in MLKL$^{-/-}$ mice after DSS administration. Moreover, mucin expression has a similar pattern (Fig. 2E). Taken together, these data indicated that MLKL contributes DSS-induced colitis and which deficiency leads to lower colitis susceptibility in mice.

3.2. MLKL deficiency inhibits hyper-inflammation in DSS-induced colitis

Disturbed inflammatory cytokines production characterized by increased IL-6 and TNF-α production is one of the features of IBD. To further determine the pathologic changes, the amounts of cytokines and chemokines in colon tissue homogenates of MLKL$^{-/-}$ mice and WT mice were examined. On day 8, MLKL$^{-/-}$ mice had significantly decreased production of IL-1β, IL-6 and KC than WT mice (Figs. 3A, B and D), although TNF-α secretion was comparable between both genotypes (Fig. 3C). To further determine the inflammatory response, the recruitment of macrophage in colon tissue was examined. MLKL deficient mice exhibited markedly reduced recruitment of macrophages (Fig. 3E). Conclusively, these results suggested that MLKL deficiency prevents inflammatory cytokines production and inflammatory cells infiltration.

3.3. MLKL deficiency prevents proinflammatory MAPK activation

Mitogen-activated protein kinases (MAPK) chains are one of the important pathways in eukaryotic signaling networks and play a key role in regulating inflammatory response. Therefore, we subsequently investigated whether there is a reduced MAPK signaling activation contributing to the reduced severity to colitis in MLKL$^{-/-}$ mice. Consistent with the decreased inflammatory cytokines production in MLKL$^{-/-}$ mice, we found that MLKL deficiency resulted in reduced phosphorylation of p38, ERK, JNK in colon (Fig. 4A). No difference in total p38, ERK, JNK was detected in WT and MLKL$^{-/-}$ mice (Fig. 4A). MLKL is an executor of necroptosis. Overt epithelial cell death results in increased leakage of intracellular contents which induces inflammatory response and increases severity to colitis. Therefore, we examined the impact of MLKL on epithelial cell death. However, we did not observe any difference in epithelial cell death between two genotypes as measured by TUNEL staining (Fig. 4D). Collectively, these results suggested that MLKL deficiency prevents the activation of proinflammatory MAPK signaling pathway during DSS-induced colitis.

3.4. MLKL has little influence on intestinal microbial community

The intestinal mucosal barrier includes microbiota, epithelial cell layer, and a variety of immune cells (Zhu and Ma, 2018). A large number of intestinal microbes are distributed in the intestinal, compete

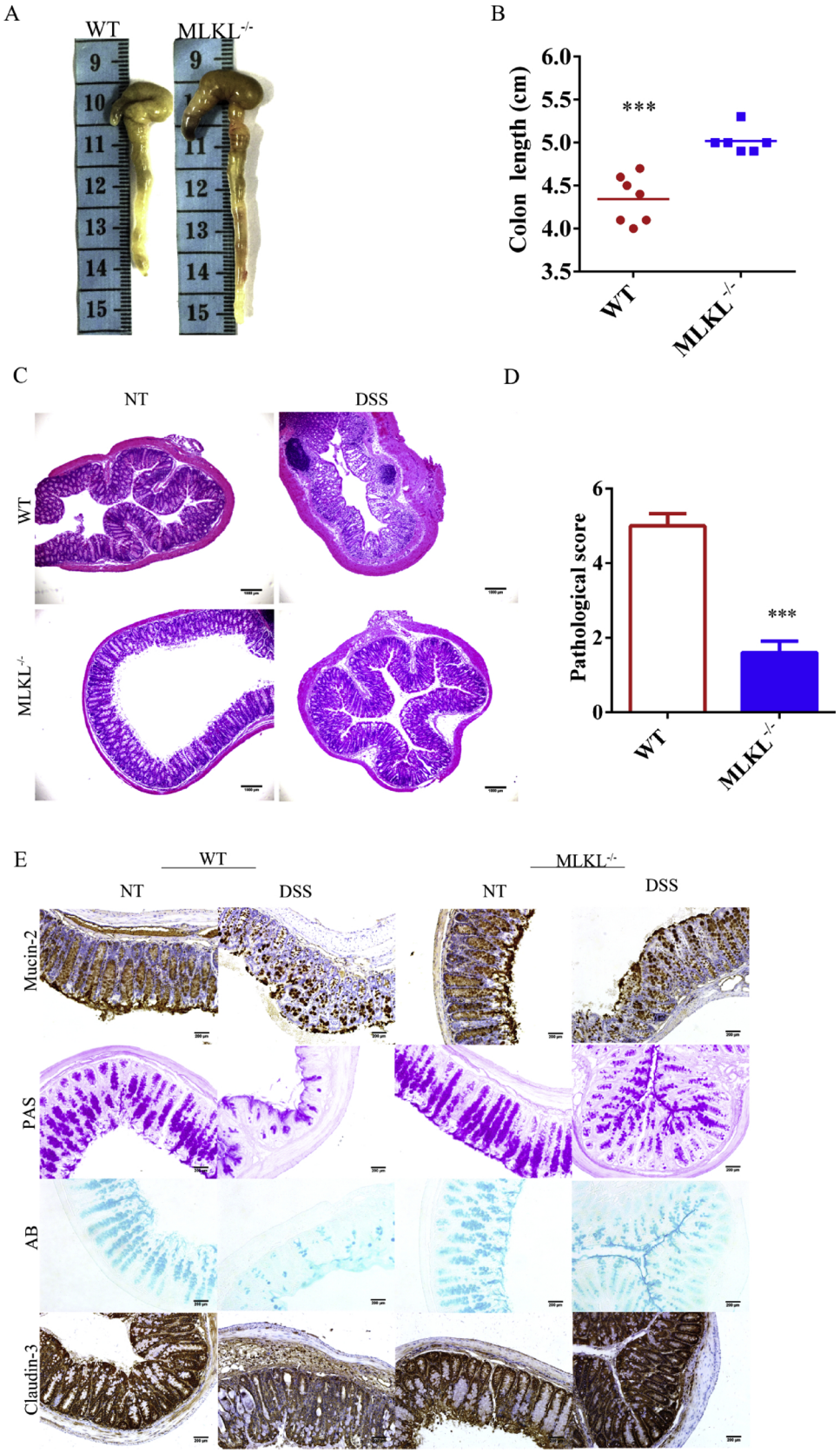

Fig. 2. MLKL deficiency reduces colonic damage and disruption of intestinal mucosal barrier integrity. The non-littermate MLKL$^{-/-}$ (n = 6) and WT (n = 7) mice were sacrificed on day 8. (A) Gross image of colon; (B) Colon length; (C) Colonic tissues were stained with H&E. Images were shown at magnifications of 40. (D) Pathological score of colonic tissues. ***P < 0.001. (E) Colonic tissues were stained with Mucin 2, AB, PAS and Claudin 3. Images were shown at magnifications of 40. These experiments and analyses were performed on mice that were separately housed according to genotype.

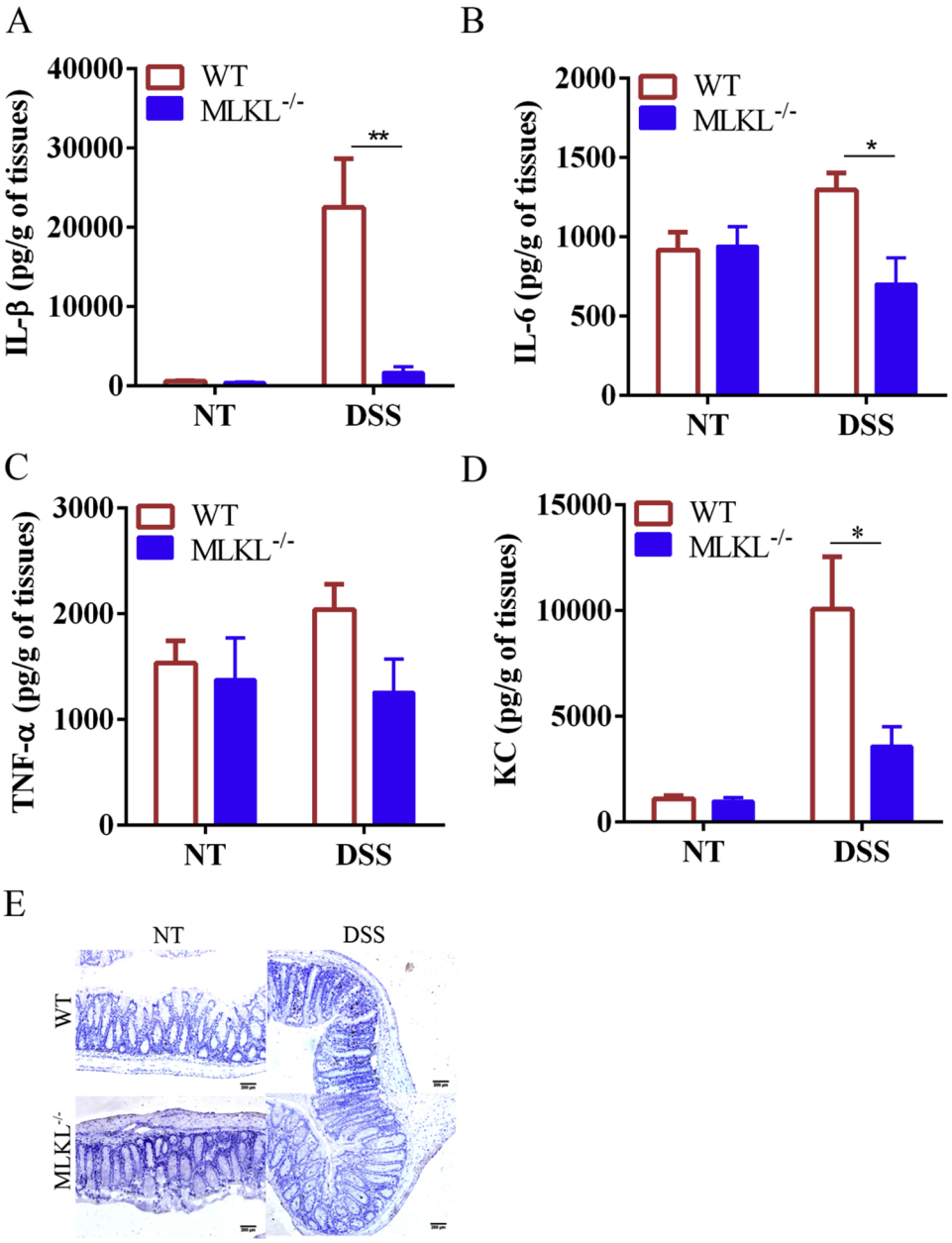

Fig. 3. MLKL promotes colonic inflammatory cytokines production and inflammatory cell chemotaxis. The non-littermate mice (n = 5 each group) were sacrificed on day 8, the amounts of cytokines in homogenized colon tissues were examined. (A) IL-1β; (B) IL-6; (C) TNFα; (D) KC; (E) The recruitment of macrophage in colon tissue was examined. *P < 0.05, **P < 0.01. These experiments and analyses were performed on mice that were separately housed according to genotype.

with exogenous microbes to space, and nutrients, and then withstand the invasion of pathogenic microbes (Liu et al., 2018). In addition, the intestinal microbiota interacts with the host to regulate intestinal barrier function and maintain intestinal homeostasis (Scott et al., 2018; Sun and Jia, 2018). Therefore, we further analyzed whether the DSS susceptibility difference between WT and MLKL$^{-/-}$ mice is due to a difference in the intestinal microbiota composition. Bacterial specific 16S rRNA genes were quantitatively measured by real-time PCR analysis of the fecal bacterial DNA. Firstly, we detected the intestinal microbiota at phylum level, including Firmicutes, Bacteroidetes, Proteobacteria, and TM7. There were no significant differences at phylum level between the two genotypes (Figs. 5A). Subsequently, the bacterial population in WT and MLKL$^{-/-}$ mice were determined at genus and species levels. We found that the abundances of some known mouse gut commensal and colitogenic bacteria including *Clostridium* cluster IV, *A. muciniphila*, *Lactobacillus*, *Staphylococcus*, *Streptococcus.* spp., *Enterococcus. spp.*, *Bacteroides fragilis*, MIB, *P. gingivalis*, SFB and *E. coli* were comparable in two genotypes (Fig. 5B-C). Although MLKL$^{-/-}$ mice showed a higher abundance of *Prevotellaceae* and a lower abundance of *Bifidobacterium.*spp., but the differences were rather subtle

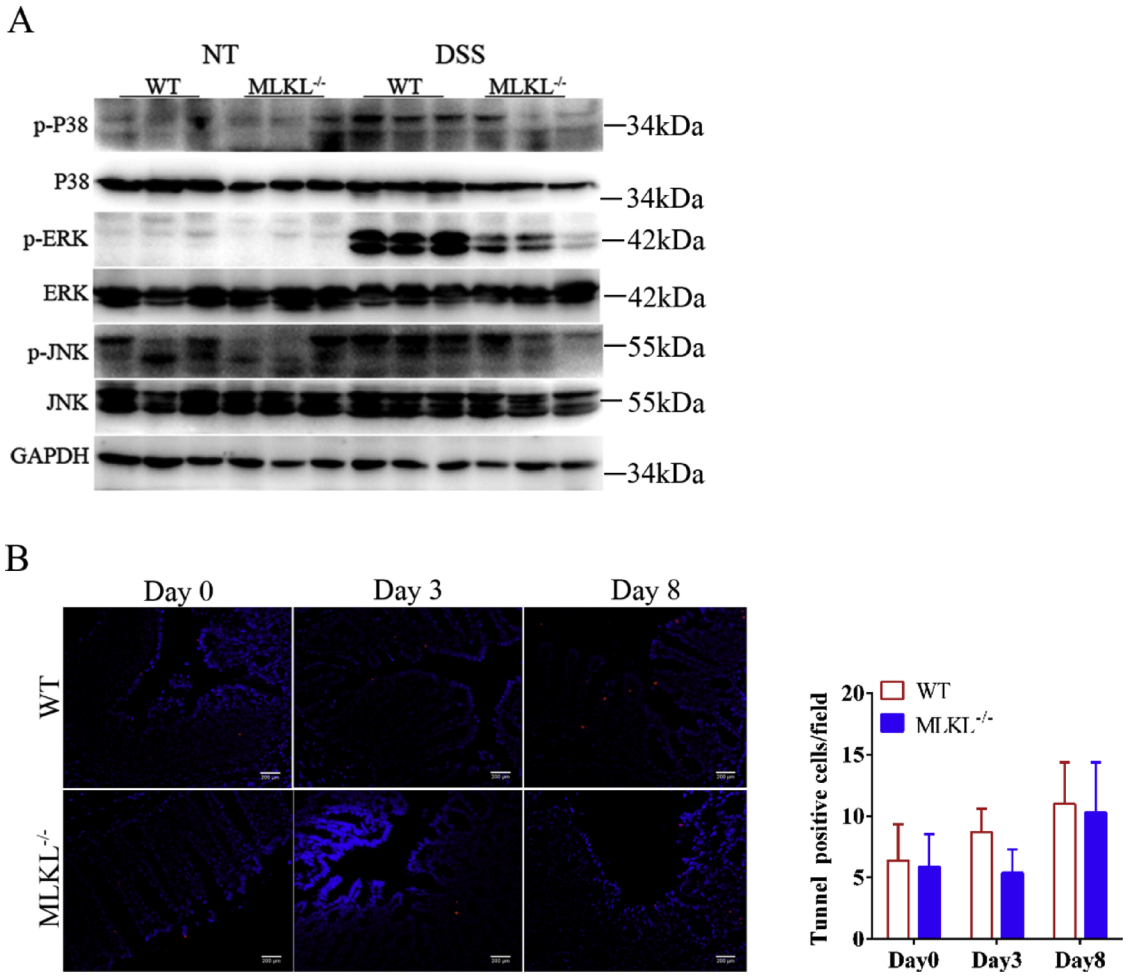

Fig. 4. MLKL deficiency inhibits MAPK signaling activation rather than cell death. The non-littermate WT (n = 7) and MLKL$^{-/-}$ (n = 6) mice were sacrificed on day 8 following DSS administration. (A) The colon sections were analyzed for p-P38, total-P38, p-ERK, total ERK, and p-JNK, total JNK by Western blotting. Data are representative of at least two independent experiments. (B) The colon sections collected after DSS administration were analyzed for cell death by TUNEL staining. These experiments and analyses were performed on mice that were separately housed according to genotype.

(less than one order of magnitude) (Fig. 5B). Taken together, MLKL has little effect on intestinal microbiota composition based on qPCR detection of fecal dominant bacterial taxa.

3.5. The colitis resistance of MLKL-deficient mice is not mediated by microbiota

The interplay between gut microbiota and host genetics plays a key role in the pathogenesis of IBD. Co-housing different genotypes in the same cage after weaning results in a similar microbial communities (Thiemann et al., 2017). To further examine if the microbiota contribute to the effect of MLKL on DSS-induced colitis, non-littermate WT and MLKL$^{-/-}$ mice were co-housed for 4 weeks to balance the abundance of the fecal microbiota. The subtle fecal abundance difference of several species between two genotypes totally disappeared after co-housing by qPCR (Fig. 6A-B). To allow for independent maturation of microbiota that experienced the same environmental exposures early in life, we repeated the single-housed experiment with WT and MLKL$^{-/-}$ {Lemire, 2017 #1159}{Lemire, 2017 #1159} mice. Subsequently, the single-housed and co-housed mice were challenged with DSS. We found that the co-housing experiments did not alter the difference in colitis severity between WT and MLKL$^{-/-}$ mice. Just like the discrepancy between two single-housed genotypes, co-housed MLKL$^{-/-}$ mice were still less sensitive to colitis compared to co-housed WT mice, determined by body weight changes, rectal bleeding, stool consistency and DAI (Fig. 6C–F). In agreement with this finding, H&E staining of histologic sections taken from the colons after DSS treatment showed a reduced epithelium damage in co-housed MLKL$^{-/-}$ mice compared to co-housed WT mice (Fig. 7A–D). These results revealed that co-housed WT and MLKL$^{-/-}$ mice had a similar disease trend during DSS-induced colitis progression compared with single-housed mice. Hence, the colitis resistance of MLKL-deficient mice may not correlate with intestinal microbiota.

4. Discussion

Programmed cell death has a vital role in maintaining tissue homeostasis. Necroptosis, a regulated, protease-independent cell death, requires specific mediator such as receptor-interacting protein kinase 3 (RIP3). MLKL, an interacting target of RIP3 was also discovered as a key player that mediates necroptosis (Sun et al., 2012). Due to the release of

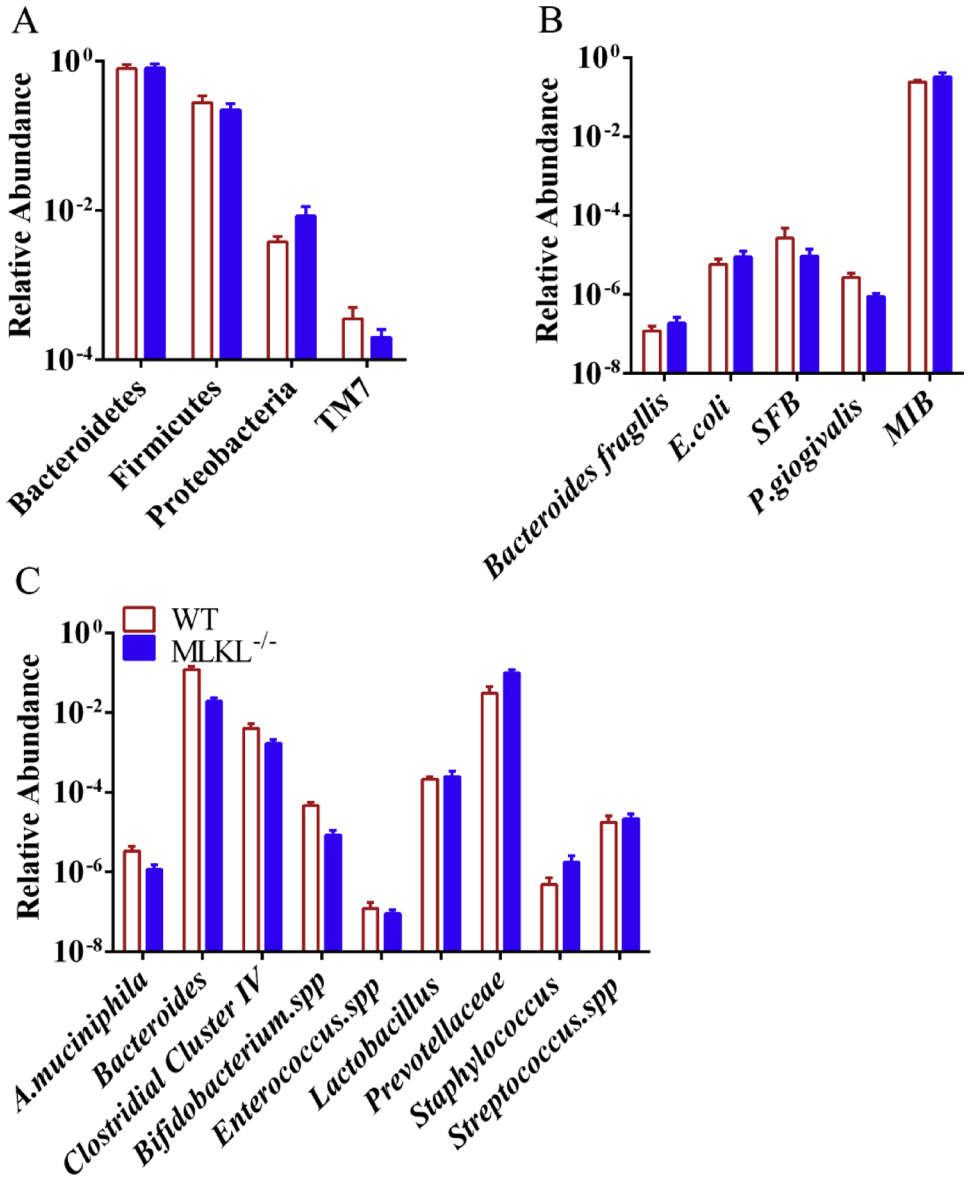

Fig. 5. Relative abundance of specific bacterial taxa is subtly altered by MLKL genotype. (A–C) DNA was isolated from feces of non-littermate WT and MLKL$^{-/-}$ mice (n = 6 each group). Bacterial 16S rRNA were quantitatively measured by real-time PCR. Data represent mean ± SEM, *P < 0.05, **P < 0.01. These experiments and analyses were performed on mice that were separately housed according to genotype.

immunostimulatory intracellular contents include damage-associated molecular patterns (DAMPs) following cell-membrane rupture, necroptosis is considered to be a proinflammatory response (Weinlich et al., 2017). Necroptosis has been shown to be involved in the development of inflammation and autoimmune diseases (Galluzzi et al., 2017; Newton and Manning, 2016).

IBD is a kind of chronic, nonspecific inflammatory gastrointestinal disorder whose pathogenesis is unclear. Hence, it is necessary to unveil the role of necroptosis in the pathogenesis of IBD. RIP3 is a molecule upstream of MLKL in the necroptotic signaling pathway. Genetic deficiency in RIP3 can reduce intestinal inflammation induced by tissue-specific ablation of FADD OR caspase-8, demonstrating that RIP3-dependent necroptosis induces intestinal inflammation (Weinlich et al., 2013; Welz et al., 2011). However, there are inconsistent reports as to the role of RIP3 in DSS-induced colitis. Moriwaka et al. showed that RIP3$^{-/-}$ mice were more susceptible to colitis, while Newton et al. showed no difference in colitis phenotype exists between RIP3$^{-/-}$ and WT mice (Moriwaki et al., 2014; Newton et al., 2016). The discrepancy of the two studies might result from the duration of DSS treatment. Comparing to the extensive studies of RIP3 function in intestinal inflammation, the biological role of MLKL in the intestinal mucosal barrier remains elusive. In a previous work we showed that MLKL promotes inflammasome activation in intestinal epithelial cells to inhibit pathogenic bacteria colonization (Yu et al., 2018). In this study, we found that MLKL deficient mice were more resistant to DSS-induced colitis, as reflected by decreased mortality, lower clinical score (body

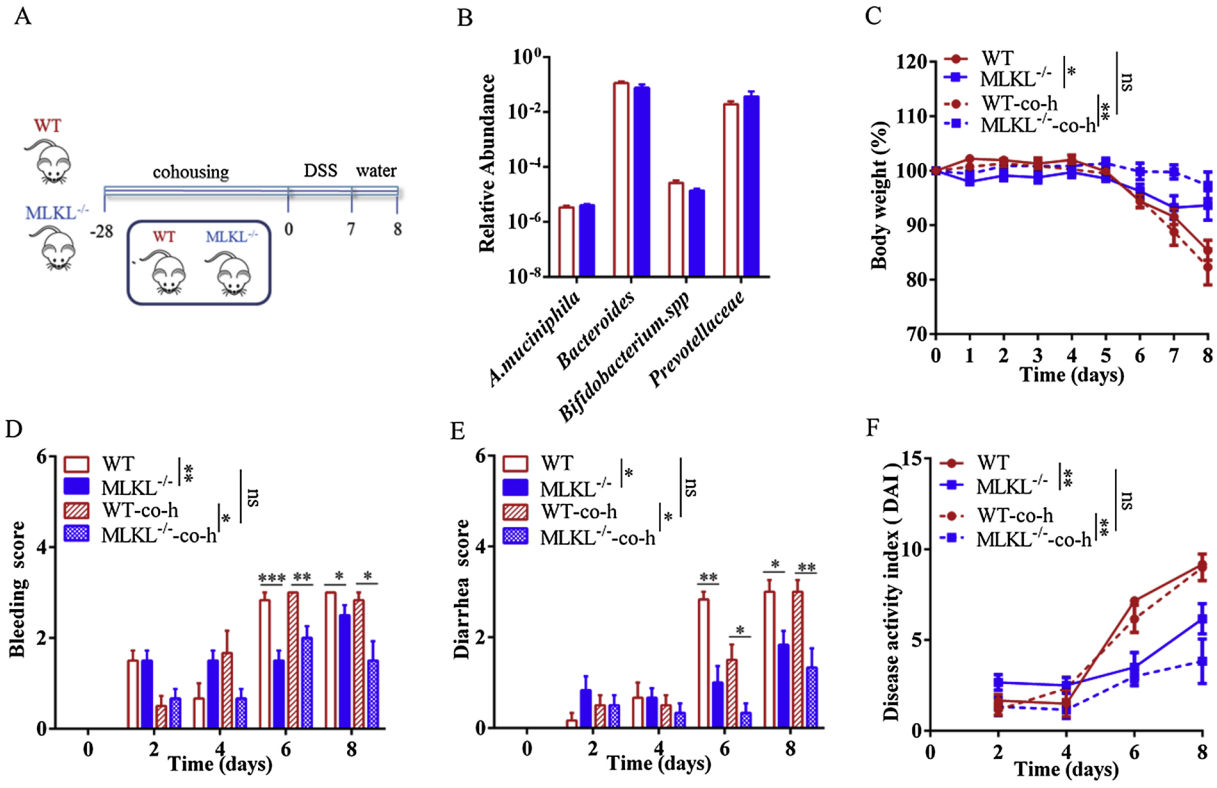

Fig. 6. The colitis development is not improved in WT mice after co-housing. The non-littermate WT and MLKL$^{-/-}$ mice were co-housed for 4 weeks, and then were fed with 2% DSS in drinking water. (A) WT and MLKL$^{-/-}$ mice were co-housed for 4 weeks and induced with DSS; (B) Bacterial 16S rRNA were quantitatively measured by real-time PCR after co-housing; (C) Body weight, (D) rectal bleeding, (E) stool consistency and (F) DAI of single-housed (WT and MLKL$^{-/-}$) and co-housed (WT-co-h and MLKL$^{-/-}$-co-h) mice were shown. *P < 0.05, **P < 0.01.

weight loss, stool consistency, and rectal bleeding), mild colonic shortening, and lower histological score (inflammatory cell infiltration, hyperplasia and epithelial damage). Strikingly, this finding is consistent with the observation that MLKL is highly expressed in inflamed intestinal mucosa of children with inflammatory bowel diseases (Pierdomenico et al., 2014).

As a critical executor of necroptosis, MLKL mediates pore forming on cell membrane, which results in leakage of intracellular contents and inflammatory response. Interestingly, we found that the resistance of MLKL deficiency against DSS-induced colitis cannot be explained by epithelial cell death. Although MLKL deletion significantly reduced inflammatory cytokines production, there was no difference in epithelial cell death between WT and MLKL$^{-/-}$ mice following DSS administration. To further examine the effect of MLKL on intestinal inflammation, the activation of inflammatory signaling was examined. A reduce in the phosphorylated levels of p38, ERK and JNK was observed in colon of MLKL$^{-/-}$ mice compared to that of WT mice. Previous studies also found that MLKL is required for p38 and JNK activation (Wang et al., 2016; Zhao et al., 2012). Hence, it implicates that MLKL promotes intestinal inflammation development via enhancing MAPK signaling activation.

The intestinal microbiota dysbiosis leads to a variety of diseases, such as chronic liver disease, obesity, and diabetes, so the intestinal microbial communities are of great significance to our healthy life (Jackson et al., 2015; Porras et al., 2017). In order to further investigate whether the reduced sensitive to colitis in MLKL$^{-/-}$ mice was due to the imbalance of intestinal microbes. We compared relative abundance of many bacterial taxa in stool and no significant difference was found. Some early studies also showed that NOD1, NOD2, NLRP6 and several TLRs do not influence intestinal microbial community structure (Lemire et al., 2017; Robertson et al., 2016, 2013; Shanahan et al., 2014; Ubeda et al., 2012). We further co-housed WT and MLKL$^{-/-}$ mice to balance the intestinal microbiota composition and found that the body weight changes, the clinical pathological scores and histopathological changes were comparable to single-housed WT and MLKL$^{-/-}$ mice respectively. Thus, based on the co-housing of two genotypes and qPCR detection of fecal dominant bacterial taxa, we speculate that colitis resistance of MLKL-deficient mice may not correlate with intestinal microbiota. However, the difference of microbial composition exist between different gut compartments and sites such as stool, lumen, mucosa, cecum, and colon. The bacterial diversity of intestinal microbiota is under-represented in feces. Hence, further studies using germ-free mice and 16S rRNA sequencing of intestinal microbiota are needed to clarify the crosstalk between MLKL and intestinal microbiota.

5. Conclusion

In summary, our results show that MLKL promotes DSS-induced colitis. We also observed MLKL enhances proinflammatory MAPK signaling. While understanding the exact mechanism of MLKL regulating colitis awaits further investigation, this study underscores the importance of MLKL inhibition may be a potential strategy for the prevention and treatment of inflammatory bowel disease.

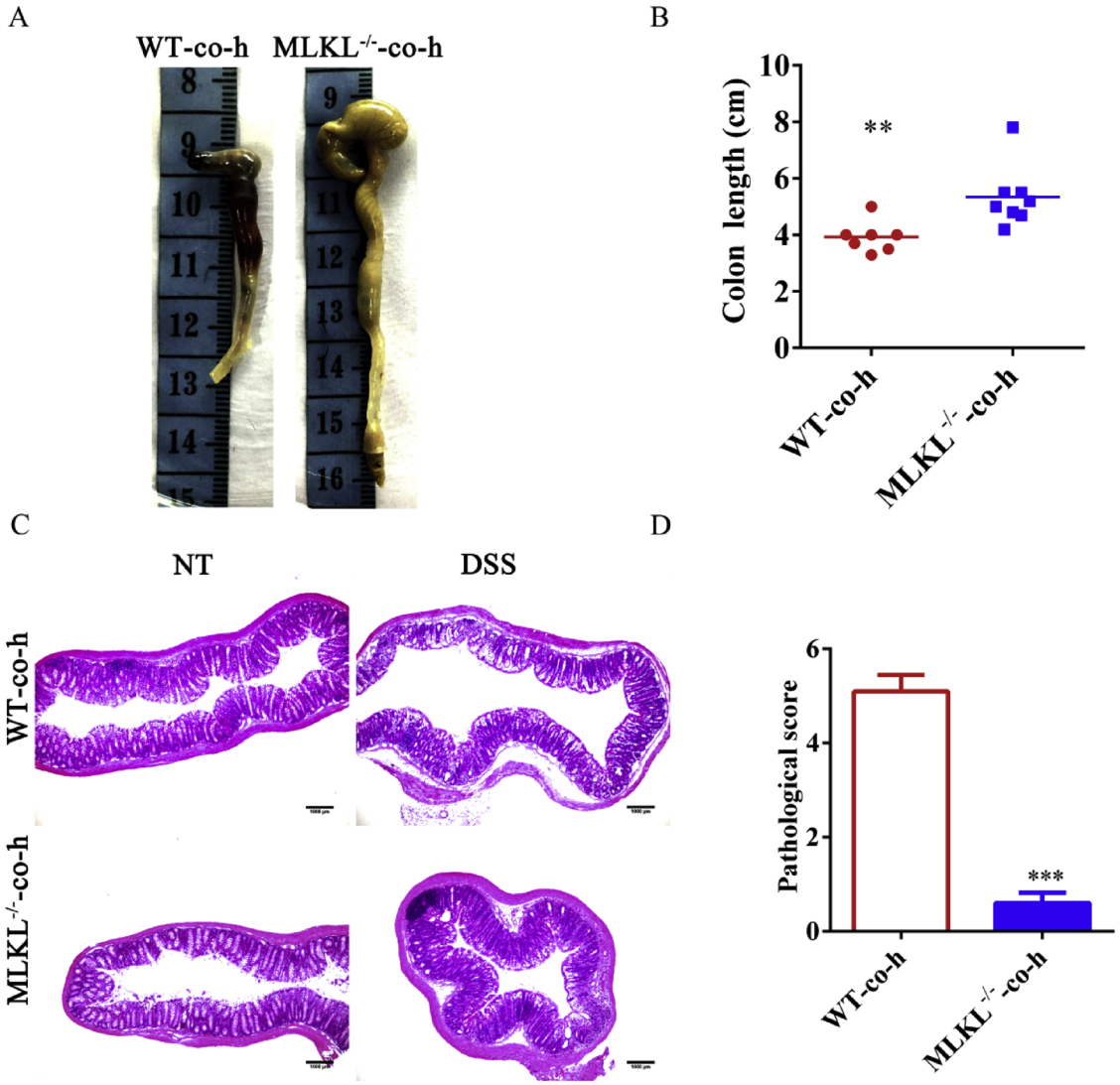

Fig. 7. The colonic damage is not improved in WT mice after co-housing. The non-littermate WT and MLKL$^{-/-}$ mice were co-housed for 4 weeks, then were fed with 2% DSS in drinking water for 7 days. (A) Gross image of colon; (B) Colon length; (C) Colon tissues were stained with H&E, images were shown at magnifications of 40; (D) Pathological score. ***P $<$ 0.001.

Author contributions

ZJ, QD, YYJ and CW designed experiments. ZJ and QD performed the experiments. ZJ and YYJ wrote the manuscript. HGQ, QXX, DCT read the manuscript.

Conflict of interest

The authors have declared that no competing interests exist.

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