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MANF regulates splenic macrophage differentiation in mice

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ABSTRACT

Splenic immune cells, especially macrophages, play a key role in multiple pathological processes. With a proved anti-inflammatory and immunoregulatory function of mesencephalicastrocyte-derived neurotrophic factor (MANF) in inflammatory disorders, how MANF affects splenic immune cells in physiological and pathophysiological situations is still unknown. In this study, we constructed mono-macrophage-specific MANF knockout (Mø MANF$^{-/-}$) mice and found the increased splenic M1 macrophages, but no significant change of splenic morphology and size compared with wild type (WT) mice. Also, we established the pathophysiological situation of carbon tetrachloride (CCl_4)-induced hepatic fibrosis. Under the hepatic fibrosis, splenic M2 macrophages and CD138$^+$ plasma cells were significantly increased in Mø MANF$^{-/-}$ mice. Consistently, we found the increased TGF-β1 level in serum and spleen of Mø MANF$^{-/-}$ mice as well. Mono-macrophage-specific MANF knockout did not affect the number of splenic T and B cells under both the normal and hepatic fibrosis conditions. Our results suggest a distinct regulation of MANF on splenic immune cells and a specific regulation of MANF on the differentiation of splenic macrophages, which may exert a significant impact on physiological and pathophysiological processes of the spleen.

1. Introduction

As the largest lymphoid organ, the spleen contains a variety of specialized subsets of lymphocytes and myeloid cells locating in multiple functionally distinct regions: macrophages in marginal zone and red pulp, T cells in periarteriolar lymphoid sheath (PALS) and B cells in germinal center (GC) [1]. A number of pathological situations are able to trigger splenomegaly and hypersplenism, affect the splenic immune microenvironment and regulate pathological processes, such as sepsis and some chronic liver diseases [2,3]. Clinically, hepatic fibrosis, liver cirrhosis and other chronic liver diseases are often accompanied by splenic abnormalities [4,5].

The spleen is connected to the liver through the portal vein system [6,7]. During the onset of chronic liver diseases, portal hypertension is induced to further lead to splenomegaly and hypersplenism [3,8]. Splenomegaly is one of diagnostic criteria for many chronic liver diseases [9,10]. Reversely, spleen-derived immune cells and transforming growth factor-β1 (TGF-β1) migrate to the liver to affect chronic hepatic diseases [11,12]. In the process of hepatic fibrosis, splenic macrophages have been proven to play an important role in formation and degradation of extracellular matrix (ECM), further affecting hepatic fibrosis [13,14]. Previous studies have suggested the critical role of TGF-β1 as a pro-fibrogenic cytokine in hepatic fibrosis. Importantly, splenic macrophages have been proven to be a key source of TGF-β1 [15,16]. TGF-β1 secreted by splenic macrophages greatly promotes hepatic fibrosis. It has been reported that splenectomy facilitates to relieve hepatic fibrosis by decreasing spleen-derived TGF-β1 [17,18]. Therefore, relying on the portal vein system, the liver and spleen interact with each other immunologically, suggesting a close association between spleen dysfunction and chronic hepatic diseases.

Mesencephalic astrocyte-derived neurotrophic factor (MANF) belongs to the neurotrophic factor family and has the protective effect on neurons [19–23]. For non-neuronal cells, MANF is a secretary protein induced by endoplasmic reticulum (ER) stress to protect against ER stress-induced damages [19–21,24–26]. MANF is widely expressed in multiple tissues and organs, including spleen [27,28]. We have

Abbreviations: MANF, mesencephalic astrocyte-derived neurotrophic factor; TGF-β1, Transforming growth factor β; Mø, macrophage; ER, endoplasmic reticulum; TNF-α, Tumor Necrosis Factor-α; IL-1β, Interleukin-1β; iNOS, Inducible Nitric Oxide Synthase; Arg-1, arginase-1; UPR, unfolded protein response

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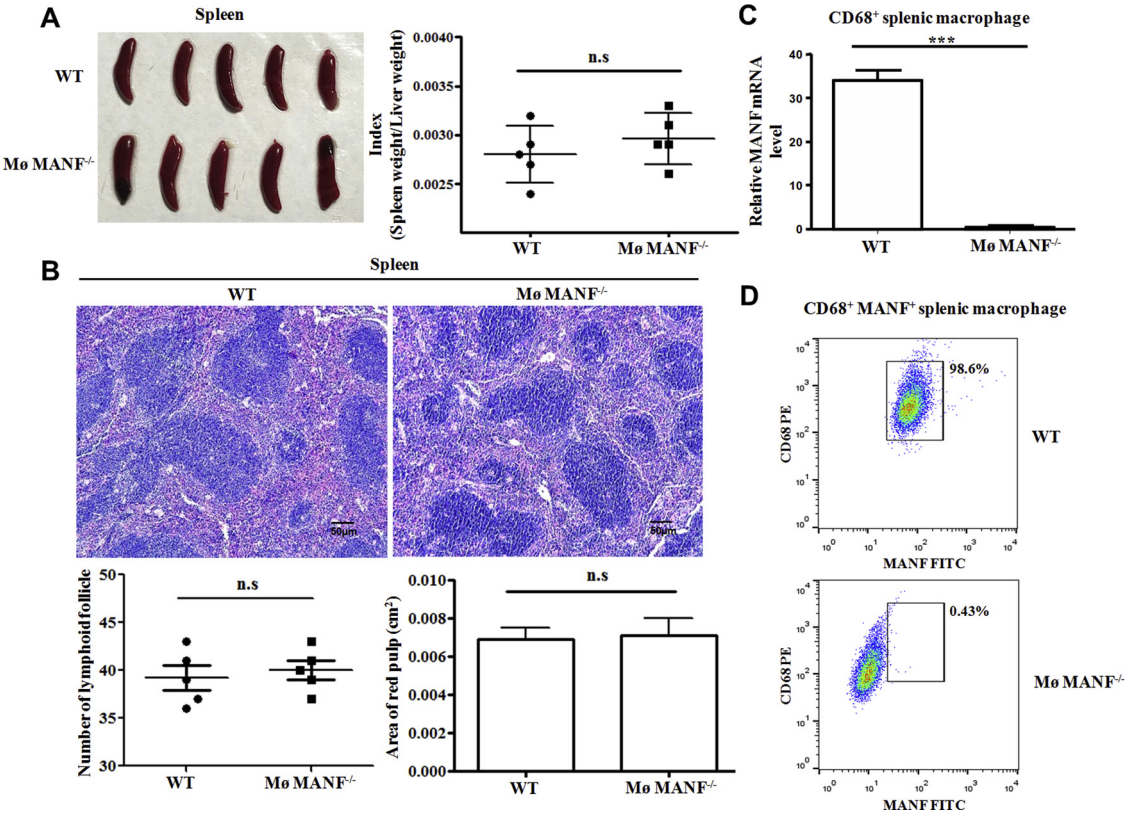

Fig. 1. Mono-macrophage-specific MANF knockout does not affect the morphology and size of spleen. (A) MANF knockout in mono-macrophages does not affect the size and weight of spleen. The spleens were collected from WT (n = 5) and Mø MANF$^{-/-}$ (n = 5) mice. The index was calculated as a ratio of spleen weight/liver weight. (B) MANF knockout in mono-macrophages does not affect the morphology of spleen. Splenic tissues were stained by hematoxylin-eosin (HE). The number of lymphoid follicle and the area of red pulp were calculated by Image J. Splenic macrophages were purified by CD68^{+} fluorescence-activated cell sorting. qRT-PCR (C) and flow cytometric analysis (D) were performed to confirm the mono-macrophage-specific MANF knockout in spleen. Data are expressed as mean ± SD. n = 5, *** $p < 0.001$, compared with WT. n.s, no significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

previously demonstrated that MANF is expressed in red pulp and marginal zone of spleen, especially in plasma cells and macrophages, but not in T and B cells [29]. Macrophages mainly have two subsets, including the pro-inflammatory M1 macrophage and anti-inflammatory M2 macrophage [30,31]. In addition, MANF has been proven to have the immunoregulatory effect on the M1/M2 macrophage differentiation to promote damage repair and neuroprotective effect [32]. Accordingly, It is possible that MANF is a key regulatory factor to determine the splenic macrophage differentiation.

In this study, we investigated the effects of MANF on splenic macrophages differentiation through constructing mono-macrophage-specific MANF knockout mice. We also observed the impact of the splenic immune microenvironment on the splenic macrophage-derived MANF by using CCl_4-induced hepatic fibrosis mice. Our study demonstrates that MANF plays the different roles in regulating the splenic macrophage differentiation in the healthy and hepatic fibrosis conditions, respectively.

2. Materials and methods

2.1. Antibodies

Anti-MANF (Abcam, Cambridge, UK, ab67271), anti-CD206 (Abcam, ab64693), anti-B220 (Abcam, ab10558), anti-CD138 (Abcam, ab34164), anti-CD68 (Abcam, ab955), anti-TGF-β1 (Abcam, ab31913), anti-Fizz-1 (Abcam, ab39626), anti-YM-1 (Abcam, ab192029), anti-GAPDH (Proteintech, Wuhan, China, 60004-1-Ig).

2.2. Mice

MANF fl/fl LYZ2-cre-T mice with mono-macrophage-specific MANF knockout, which were named as Mø MANF$^{-/-}$ mice, were constructed by mating between MANF conditional knockout mice and LYZ2-cre mice. MANF conditional knockout mice were obtained from Professor Jia Luo of the University of Kentucky as a gift. All mice were raised in SPF-class animal laboratory. All mice studies were conducted according to protocols approved by the Animal Ethics Committee of Anhui Medical University.

2.3. Hepatic fibrosis model

All mice were divided into two groups: Mø MANF$^{-/-}$ and WT C57BL/6J mice. All 6-weeks-old male C57BL/6J mice were injected intraperitoneally with 2 μl/g CCl_4 three times a week for 8 weeks according to the previous report [33]. The weight of mice was monitored weekly. After 8 weeks, all mice were sacrificed. Spleens and livers were removed and weighed.

2.4. Western blot

The expressions of GAPDH and MANF were examined by Western blot. Peritoneal macrophages from WT and Mø MANF$^{-/-}$ mice were collected and lysed. For each sample, 5 μg of total protein was separated by the reduced SDS-PAGE, then transferred to PVDF membrane and incubated with antibodies, including anti-MANF (Abcam, ab67271) and anti-GAPDH (Proteintech, 60004-1-Ig).

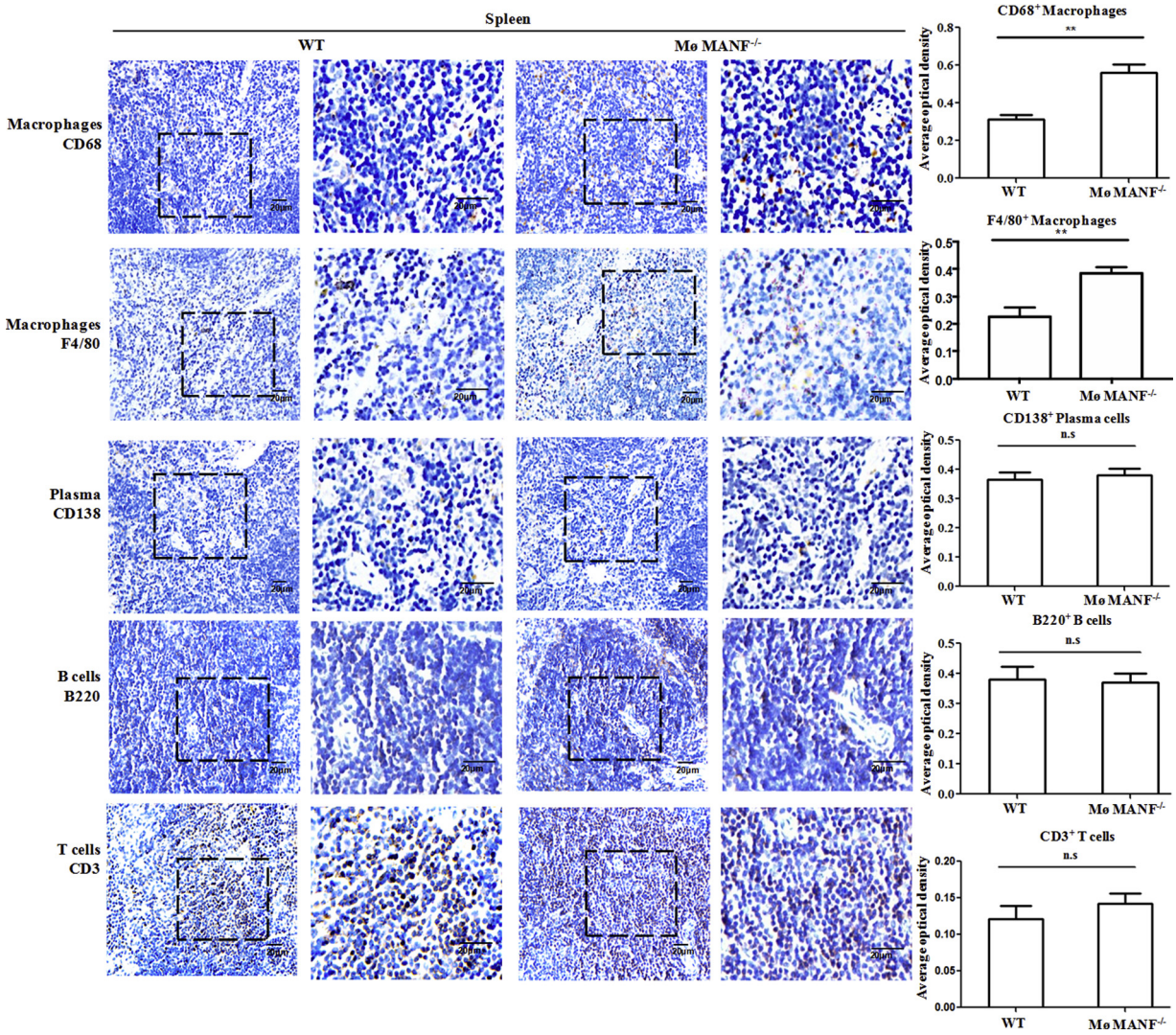

Fig. 2. Mono-macrophage-specific MANF knockout differentially regulates splenic immune cells. The splenic macrophages, plasma, B and T cells were detected by immunohistochemical assay with the specific antibodies of anti-CD68, anti-F4/80, anti-CD138, anti-B220 and anti-CD3, respectively. The number of the immunoreactive positive cells was reflected by the average optical density, which was calculated by Image J. Data are expressed as mean ± SD. n = 5, ** $p < 0.01$, compared with WT. n.s, no significance.

2.5. Immunohistochemistry

Splenic tissues were fixed by 10% formaldehyde for 24 h, and then paraffin embedding was performed. The immunohistochemical method was carried out as described previously [32]. Paraffin sections were deparaffinized in dimethylbenzene for 2 h and rehydrated through 100%, 90%, 80%, 70% ethanol. For hematoxylin-eosin staining (HE), sections were stained with hematoxylin for 6 min. After rinsing with water for 15 min, sections were stained with eosin for 3 s, following with rinse for 5 min. Sections were placed in dimethylbenzene for 40 min. For antibody staining, sections were incubated with the goat serum for 0.5 h and antibodies for 2 h in 37℃. After that, sections were stained with 3, 3′-diaminobenzidinetetrahydrochloride (DAB) and hematoxylin was used for counterstain. Images were obtained by Olympus Microscope BX53 and cellSens Standard software.

2.6. Immunofluorescent staining

The immunofluorescent staining was carried out as described previously [32]. Paraffin sections were deparaffinized and rehydrated, then incubated with 5% BSA for 0.5 h and antibodies for 2 h in 37℃. Anti-CD68 and anti-MANF were used. Nuclei were stained with DAPI for 10 min. Images were obtained by Olympus Microscope BX53/IX71 and cellSens Standard software.

2.7. Real time-quantitative PCR

Total RNA was extracted from spleen or purified $CD68^+$ splenic macrophages by using Trizol reagent according to manufacturer's protocol. Reverse transcription was performed by using PrimeScript RT reagent Kit (TaKaRa Bio, Dalian, China). Primers for real time-qPCR were: iNOS, foward 5′-GCAGAGATTGGAGGCCTTGTG-3′ and reverse 5′-GGGTTGTTGCTGAACTT

CCAGTC-3′; TNF-α, forward 5′-CAGGAGGGAGAACAGAAACT CCA-3′ and reverse 5′−CCTGGTTGGCTGCTTGCTT-3′; IL-1β, forward 5′-TCCAGGATGAGGA

CATGAGCAC-3′ and reverse 5′-GAACGTCACACACCAGCAGG TTA-3′; IL-6, forward 5′−CCACTTCACAAGTCGGAGGCTTA-3′ and reverse 5′-GCAAGTGCAT

CATCGTTGTTCATAC-3′; Arg-1, forward 5′-AGACAGCAGAGGAGG TGAAGAG

-3′ and reverse5′-CGAAGCAAGCCAAGGTTAAAGC-3′; Fizz-1, forward 5′-GGTCCCAGTGCATATGGATGAGACCATAG-3′ and reverse 5′-CACCTCTTC

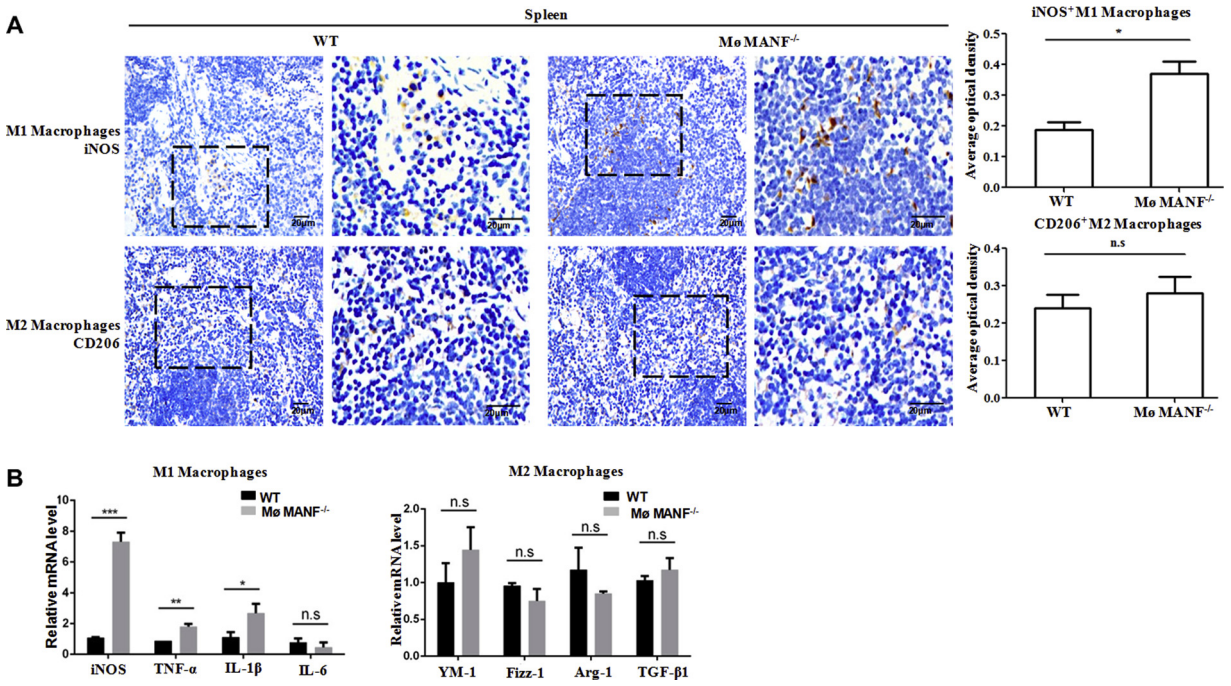

Fig. 3. Mono-macrophage-specific MANF knockout specifically increases the number of splenic M1 macrophages. (A) The markers of M1 and M2 macrophages in splenic tissues of WT and Mø MANF$^{-/-}$ mice were detected by immunohistochemical staining with the specific antibodies of anti-iNOS and anti-CD206, respectively. The number of the immunoreactive positive cells was reflected by the average optical density, which was calculated by Image *J*. (B) The mRNA levels of cytokines and markers derived from M1 and M2 macrophages were determined by real time quantitative PCR. Data are expressed as mean ± SD. n = 5, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with WT. n.s, no significance.

ACTCGAGGGACAGTTGGCAGC-3′; YM-1, forward 5′-CATTCAGTC AGTTATCA

GATTCC-3′ and reverse 5′-AGTGAGTAGCAGCCTTGG-3′; TGF-β1, forward 5′-GGATACCAACTATTGCTTCAGCTCC-3′ and reverse 5′-AGG CTCCAAATATA

GGGGCAGGGTC-3′; MANF, forward 5′-TCACATTCTCACCAGCC ACT-3′ and reverse 5′-ATCTGGCTGTCCTTCTTCTTAA-3′.

2.8. Sirius red staining

Paraffin sections were deparaffinized in dimethylbenzene for 2 h and rehydrated through 100%, 90%, 80%, 70% ethanol. For Sirius red staining, sections were stained with Sirius red staining solution for 1 h, followed by washing with water for 30 min. Images were obtained by Olympus Microscope BX53 and cellSens Standard software.

2.9. Peritoneal macrophages isolation and culture

Mice were sacrificed and sterilized with alcohol for 5 min. Then, mice were intraperitoneally injected with 5 ml Dulbecco's Modified Eagle Medium (DMEM). The intraperitoneal DMEM was collected, following with centrifugation for 4 min. After removing the supernatant, the remaining macrophages were re-suspended and cultured in DMEM for 24 h.

2.10. Splenic macrophages purification and flow cytometric analysis

The splenic cell suspension from WT and Mø MANF$^{-/-}$ mice was produced according to the previous report [34]. Splenic macrophages were purified by CD68^{+} fluorescence-activated cell sorting. Briefly, the splenic cell suspension was used for staining with PE-labeled CD68 antibody (BD Biosciences, CA, USA, 566387). After washing with PBS, CD68^{+} fluorescence-activated cell sorting was performed. Then, the purified CD68^{+} splenic macrophages were used for intracellular MANF detection. The cell permeabilization was performed by BD Cytofix/Cytoperm™ Plus Fixation/Permeabilization Kit (BD Biosciences, 554715) according to the kit manual. After that, intracellular MANF was labeled by anti-MANF (Abcam, ab67271) and FITC-anti-Rabbit IgG (Jackson, PA, USA, 111-095-003), followed by flow cytometric analysis for MANF expression in CD68^{+} splenic macrophages. Beckman Coulter MoFlo XDP High-Speed Cell Sorter System and CytoFLEX Flow Cytometer were used for macrophage sorting and flow cytometric analysis respectively.

2.11. Enzyme-linked immuno sorbent assay (ELISA)

The serum was collected from WT and Mø MANF$^{-/-}$ mice with hepatic fibrosis. TGF-β1 level in serum was examined by the Mouse TGF-β1 ELISA Kit (R&D Systems, Minneapolis, USA, MB100B) according to the manufacturer's instruction.

2.12. Statistics

The average optical density, lymphoid follicle number and red pulp area were calculated by Image J. The statistical comparison was conducted by the two-way ANOVA. p value < 0.05 indicates a significant difference. An asterisk (*), two asterisks (**) and three asterisks (***) mean $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively. n.s means no significance. Error bars indicate mean ± SEM. Data are representative of three independent experiments.

3. Results

3.1. MANF knockout in mono-macrophages has no effect on splenic morphology and size

We first examined the effect of mono-macrophage-derived MANF on spleen in the healthy condition. In WT and Mø MANF$^{-/-}$ mice, the ratio of spleen to liver weight was analysed, showing no significant difference (Fig. 1A). Then, we examined whether the spleen structure

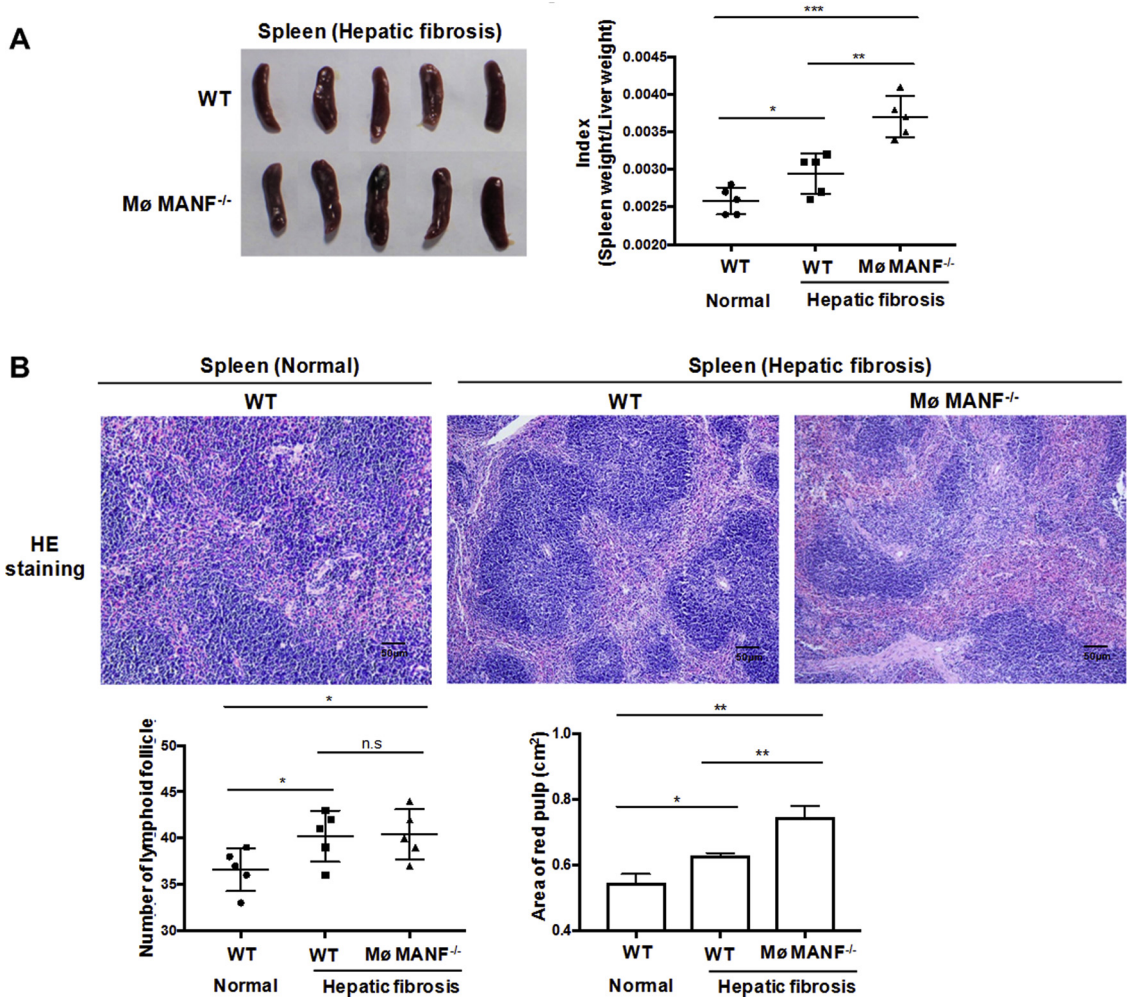

Fig. 4. Mono-macrophage-specific MANF knockout exacerbates splenomegaly in hepatic fibrosis mice. (A) MANF knockout in mono-macrophages increases the size and weight of spleen in hepatic fibrosis. The spleens in health or hepatic fibrosis were collected from WT (n = 5) and Mø MANF$^{-/-}$ (n = 5) mice. The index was calculated as a ratio of spleen weight/liver weight. (B) MANF knockout in mono-macrophages increases red pulps area of spleen in hepatic fibrosis. Splenic tissues were stained by hematoxylin-eosin (HE). The number of lymphoid follicle and the area of red pulp were calculated by Image *J*. Data are expressed as mean ± SD. n = 5, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with WT. n.s, no significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was changed in Mø MANF$^{-/-}$ mice. Results in Fig. 1B showed both splenic lymphoid follicle and red pulp area had no change between WT and Mø MANF$^{-/-}$ mice. The mono-macrophage-specific MANF knockout in Mø MANF$^{-/-}$ mice was confirmed (Supplemental Fig. 1). Also, we used CD68^{+} fluorescence-activated cell sorting to purify CD68^{+} splenic macrophages from WT and Mø MANF$^{-/-}$ mice. qRT-PCR and flow cytometric results in Fig. 1C and D showed the absence of MANF in splenic macrophages of Mø MANF$^{-/-}$ mice. Therefore, no significant splenomegaly was found in Mø MANF$^{-/-}$ mice.

3.2. MANF knockout in mono-macrophages specifically up-regulates splenic M1 macrophages

To investigate the effect of mono-macrophage-derived MANF on splenic immune cells, we examined the splenic macrophages, plasma, T and B cells in WT and Mø MANF$^{-/-}$ mice by immunohistochemistry. We found that splenic CD68^{+} or F4/80^{+} macrophages in Mø MANF$^{-/-}$ mice were more than that in WT mice, whereas there was no significant difference in splenic plasma, T and B cells (Fig. 2). Also, the splenic inducible nitric oxide synthase (iNOS)$^{+}$ M1 macrophages in Mø MANF$^{-/-}$ mice were significantly increased, compared with WT mice (Fig. 3A, upper panel). Consistently, iNOS, Tumor Necrosis Factor-α (TNF-α) and Interleukin-1β (IL-1β) mRNA were also increased in Mø MANF$^{-/-}$ mice (Fig. 3B). However, there was no difference in splenic CD206^{+} M2 macrophages between the two groups (Fig. 3A, lower panel). The expressions of M2 macrophage-related genes YM-1, Fizz-1, arginase-1 (Arg-1) and TGF-β1 in spleen remained unchanged (Fig. 3B). Therefore, the mono-macrophage-specific MANF knockout only affects splenic M1 macrophages, rather than M2 macrophages, plasma, T and B cells in the healthy condition.

3.3. MANF knockout in mono-macrophages exacerbates splenomegaly in hepatic fibrosis mice

To examine whether mono-macrophage-derived MANF affects splenic morphology and function under the abnormal condition, we used CCl_4 to induce hepatic fibrosis in WT and Mø MANF$^{-/-}$ mice. The hepatic fibrosis model was successfully established. Results from HE and Sirius red staining showed the significantly increased peripheral inflammatory cells infiltrating into the hepatic fibrosis tissue and collagen fiber respectively after mono-macrophage-specific MANF knockout, indicating that MANF knockout in mono-macrophages promoted hepatic fibrosis (Supplemental Fig. 2). We found the hepatic fibrosis-induced splenomegaly became more severe in Mø MANF$^{-/-}$

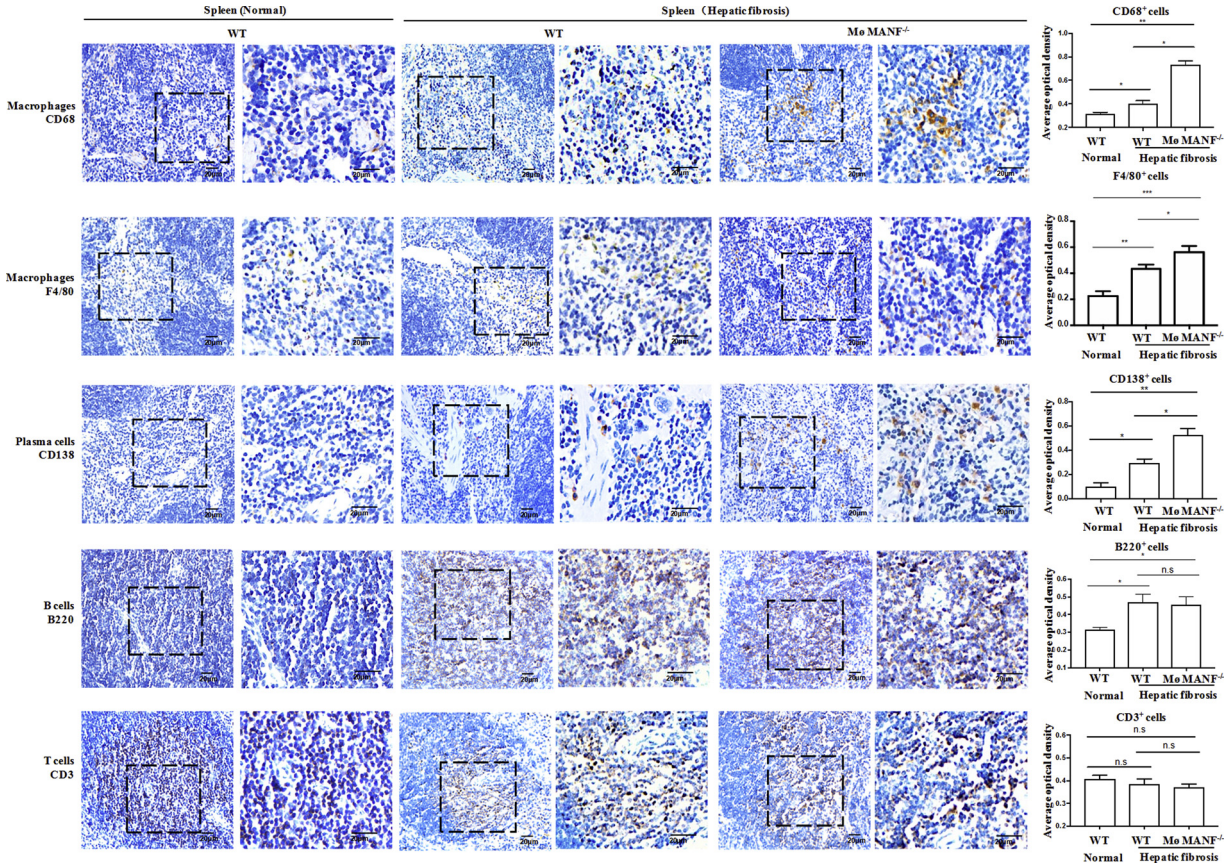

Fig. 5. Mono-macrophage-specific MANF knockout differentially regulates splenic immune cells in hepatic fibrosis mice. The splenic macrophages, plasma, B and T cells in the healthy or hepatic fibrosis mice were detected by immunohistochemical assay with the specific antibodies of anti-CD68, anti-F4/80, anti-CD138, anti-B220 and anti-CD3, respectively. The number of the immunoreactive positive cells was reflected by the average optical density, which was calculated by Image J. Data are expressed as mean ± SD. n = 5, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s, no significance.

mice than that in WT mice (Fig. 4A). In hepatic fibrosis mice, HE staining showed no significant difference of lymphoid follicle, but a significant increase of red pulps area in Mø MANF$^{-/-}$ mice (Fig. 4B), compared with WT mice. Therefore, under the pathophysiological condition of hepatic fibrosis, the splenomegaly in Mø MANF$^{-/-}$ mice may be attributed to the increase of red pulp.

3.4. MANF knockout in mono-macrophages differentially regulates splenic immune cells in hepatic fibrosis mice

We have mentioned above that under the healthy condition, MANF knockout in mono-macrophages only increased macrophages, but did not affect plasma, T and B cells in spleen. We wondered whether MANF loss in mono-macrophages affected splenic immune cells under the hepatic fibrosis condition. We used CCl_4-induced hepatic fibrosis model to detect splenic macrophages, plasma, T, and B cells in both WT and Mø MANF$^{-/-}$ mice. We found more splenic macrophages and plasma cells in Mø MANF$^{-/-}$ mice compared with WT mice. However, splenic T and B cells did not show any significant difference (Fig. 5), indicating MANF could affect splenic plasma cells and macrophages in hepatic fibrosis.

3.5. MANF knockout in mono-macrophages specifically up-regulates splenic M2 macrophages in hepatic fibrosis mice

To further investigate whether mono-macrophage-derived MANF affects splenic macrophage differentiation in hepatic fibrosis, we tested M1 and M2 macrophages in spleen. We found that splenic CD206$^+$, Fizz-1$^+$ and YM-1$^+$ M2 macrophages in Mø MANF$^{-/-}$ mice were significantly increased, compared with WT mice. However, splenic iNOS$^+$ M1 macrophages had no difference (Fig. 6A). Similarly, in the spleen of Mø MANF$^{-/-}$ mice, the expressions of M2 macrophage-related genes, including Fizz-1, YM-1 and TGF-β1 were up-regulated, while the expressions of M1 macrophage-related genes, including iNOS, TNF-α, IL-1β, and IL-6 remained unchanged (Fig. 6B). These results suggest that MANF knockout in mono-macrophages specifically promotes M2 polarization of splenic macrophages under the hepatic fibrosis condition, which is distinct from that in the healthy condition.

3.6. MANF knockout in mono-macrophages increases the serum and splenic TGF-β1 in hepatic fibrosis mice

As a key M2 macrophage-derived cytokine, the splenic TGF-β1 has been reported to greatly aggravate hepatic fibrosis [3,12]. To investigate whether the TGF-β1 level was elevated along with the increase of M2 macrophages in Mø MANF$^{-/-}$ mice under the status of hepatic fibrosis, immunohistochemistry and ELISA assay were performed to detect TGF-β1 in the spleen tissue and serum, respectively. Results showed the increased TGF-β1 in both the spleen tissue and serum of Mø MANF$^{-/-}$ mice under the hepatic fibrosis condition, compared with WT mice (Fig. 7A and B), which was consistent with the enhanced M2 macrophage polarization triggered by MANF knockout in mono-macrophages.

4. Discussion

As a neurotrophic factor, the neural protection function of MANF has been well demonstrated in recent years [19,21]. Besides protection

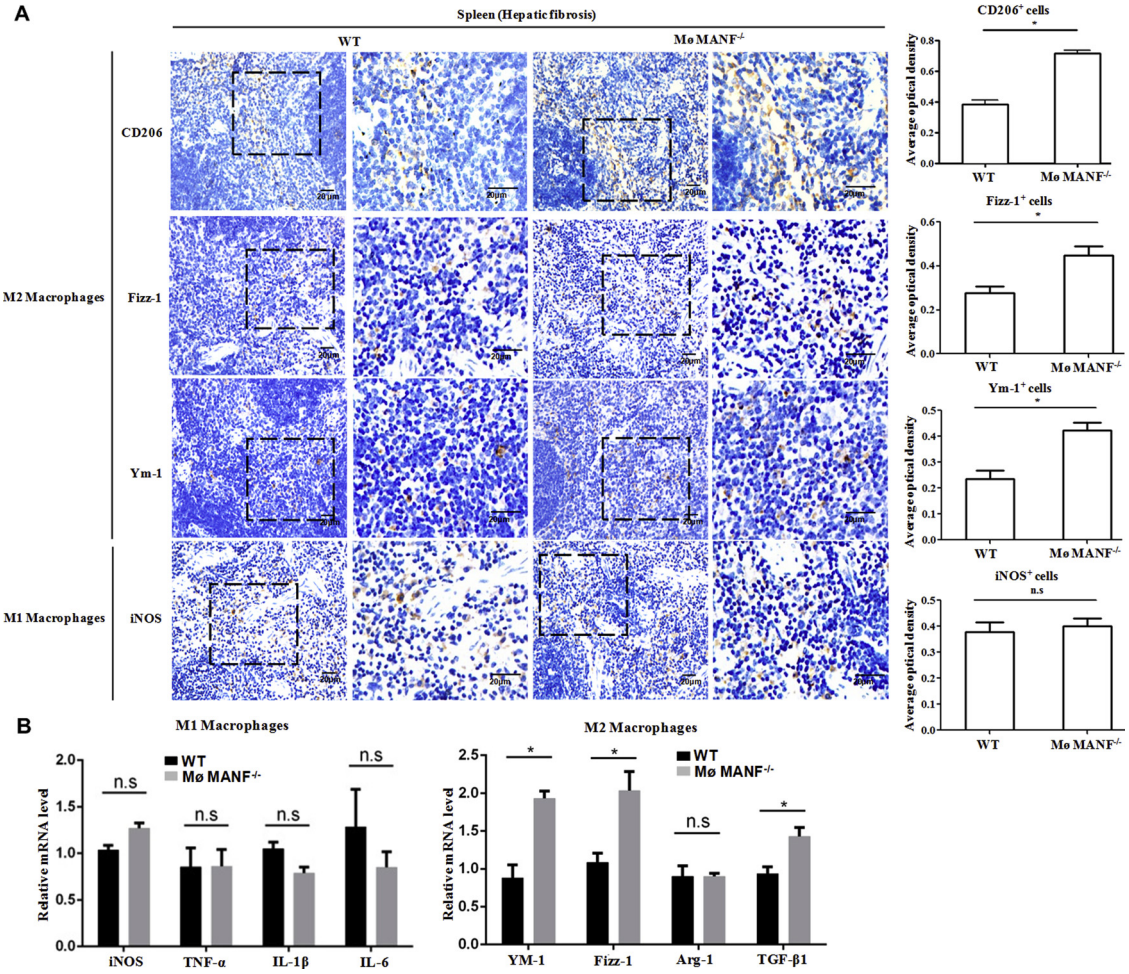

Fig. 6. Mono-macrophage-specific MANF knockout specifically increases the number of splenic M2 macrophages in hepatic fibrosis mice. (A) The markers of macrophages were detected by immunohistochemical staining with the specific antibodies of anti-iNOS for M1 macrophages, anti-CD206, anti-Fizz-1and anti-YM-1 for M2 macrophages, respectively. The number of the immunoreactive positive cells was reflected by the average optical density, which was calculated by Image J. (B) The mRNA levels of cytokines and markers derived from M1 and M2 macrophages were determined by real time quantitative PCR. Data are expressed as mean ± SD. n = 5, * $p < 0.05$, n.s, no significance.

against neuron apoptosis, MANF is greatly beneficial for animal behavioral and brain functional recovery, as well as differentiation and migration of neural progenitor cells to play a role of neural regeneration [22,23]. Some researches on *Drosophila* brain have shown that MANF silencing in glial cells prolongs growth and development of *Drosophila* and induces a new cell type of MANF immunoreactive Cells (MiCs) [35,36]. Moreover, there are a number of researches demonstrating the role of MANF in inflammatory reaction of multiple organs. It has been reported that exogenous MANF is able to provide protection against inflammation-induced human pancreatic beta cell death [37]. Also, our previous work has shown that MANF negatively affects the progress of autoimmune diseases [38]. MANF could be considered as a potential anti-inflammatory factor. Accordingly, we explore the influence of mono-macrophage-derived MANF on inflammation-induced hepatic fibrosis, focusing on the splenic immune change induced by hepatic inflammation and fibrosis in this study. In the healthy condition, mono-macrophage-specific MANF knockout triggers an increase of splenic M1 macrophages without the significant splenomegaly. Differently, in the hepatic fibrosis condition, mono-macrophage-specific MANF knockout causes both more severe splenomegaly and more splenic M2 macrophages compared with WT. There is a close association between the spleen and liver in the pathological condition of hepatic fibrosis. First, hepatic fibrosis is promoted by splenic macrophages and TGF-β1. Second, splenomegaly and hyperthyroidism are triggered by hepatic chemokines and High Mobility Group Box1 (HMGB1) [39–41]. Previous studies have demonstrated that splenectomy is able to relieve hepatic fibrosis by decreasing the serum TGF-β1 [12,42]. Also, hepatic monocytes and macrophages are able to be regulated by spleen [18]. Our results suggest that mono-macrophage-specific MANF knockout greatly promotes hepatic fibrosis and hepatic fibrosis-induced splenomegaly, implying the potential role of MANF in affecting splenic inflammatory response and hepatic fibrosis.

Generally, the activated macrophages are designated to two main groups of pro-inflammatory M1 and anti-inflammatory M2 macrophages, and both of them have the distinct role in the pathogenic process. However, the unique role of M1 and M2 macrophages in different diseases is still undefined [43–45]. We have demonstrated that MANF protects against ER stress-induced injury [38,46]. Moreover, MANF has been proven to have the immunomodulatory effect on M1/M2 macrophage polarization in retina repair [32]. Therefore, MANF may play a crucial role in the regulation of splenic macrophage differentiation. Our previous study has demonstrated that MANF is mainly expressed in plasma cells and macrophages in human spleen [29]. In this study, we further found that MANF knockout in mono-macrophages resulted in an increase of splenic M1 macrophages, suggesting that the loss of MANF in macrophages may induce inflammation. More interestingly, in CCl_4-induced hepatic fibrosis, MANF knockout in mono-macrophages specifically increased splenic M2 macrophages. The

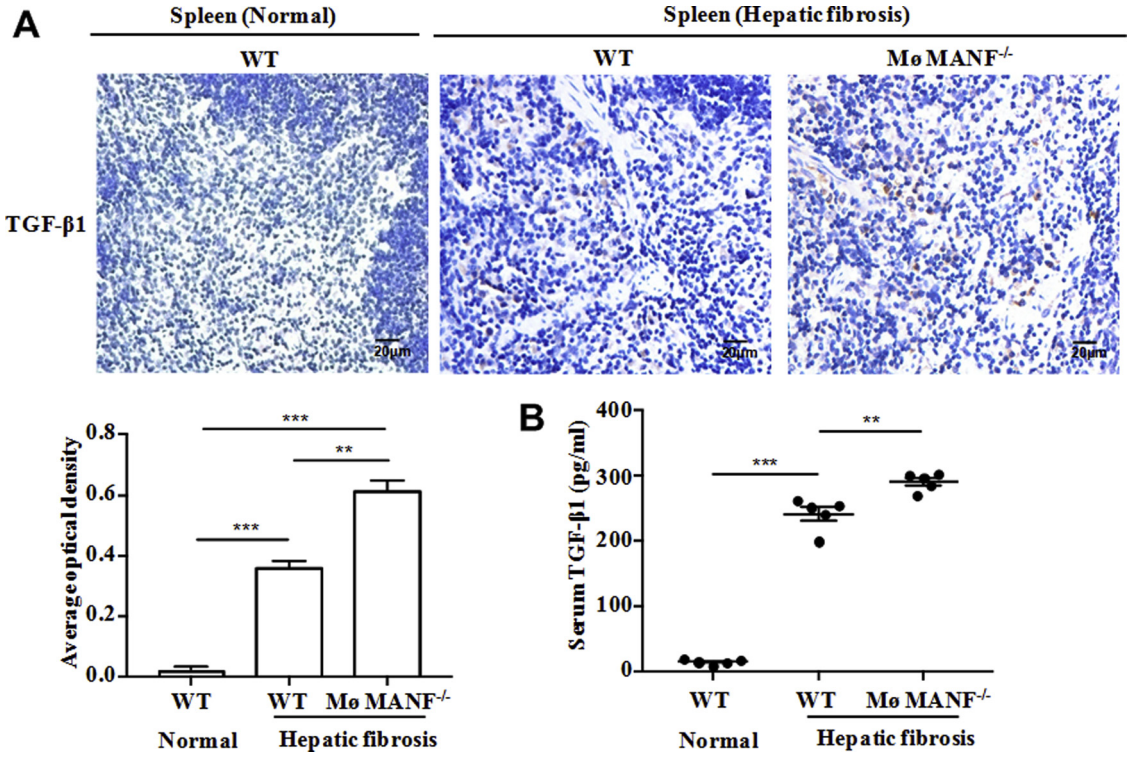

Fig. 7. Mono-macrophage-specific MANF knockout increases the serum and splenic TGF-β1 in hepatic fibrosis mice. (A) The splenic TGF-β1 was detected by immunohistochemical staining with the specific antibody of anti-TGF-β1. (B) The serum TGF-β1 level was determined by ELISA. Data are expressed as mean ± SD. n = 5, ** $p < 0.01$.

distinct role of MANF in splenic macrophage polarization in the healthy and hepatic fibrosis conditions may reflect the functional diversity of MANF.

TGF-β1, an important cytokine that promotes hepatic fibrosis, is mainly derived from M2 macrophages in spleen [15,16]. In hepatic fibrosis mice, we found the splenomegaly and hepatic fibrosis became more severe in Mø MANF$^{-/-}$ mice, indicating a stronger inflammatory response after mono-macrophage-specific MANF knockout. Considering the portal vein system between the liver and spleen, this result may be attributed to the increased splenic M2 macrophage-derived TGF-β1 secretion. In the healthy condition, although the splenic M1 macrophages and inflammatory cytokines, such as TNF-α and IL-1β, were increased in Mø MANF$^{-/-}$ mice, splenomegaly and chronic liver diseases were not induced. This phenomenon may be due to the fact that the pathogenic splenomegaly is associated with chronic liver diseases, but not MANF knockout in mono-macrophages. Therefore, the hepatic fibrosis-induced splenomegaly and splenic inflammatory response are closely associated with M2 macrophage-derived TGF-β1. MANF could affect the progression of chronic liver diseases by regulating the splenic macrophage M1/M2 differentiation.

In this study, we also found that MANF knockout in mono-macrophages did not affect splenic plasma cells in the healthy condition. However, in the hepatic fibrosis condition, splenic plasma cells in Mø MANF$^{-/-}$ mice were significantly increased compared with WT mice. The reason may be related to the fact that hepatic fibrosis-induced inflammation triggers ER stress and activates the unfolded protein response (UPR) [47,48]. UPR has been proven to be crucial for plasma cell differentiation [49–51]. Therefore, in hepatic fibrosis, the increased splenic plasma cells in Mø MANF$^{-/-}$ mice may be attributed to the enhanced inflammatory response, which further supports the previous finding that MANF inhibits inflammatory response and regulates immune function [19,31,45].

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For work assignment, Y. X. S. proposed the original idea and final model; Y. X. S. and D.W. designed the experiments; C. H. and X. L. performed the critical experiments; J. L., C. S. W., Y. F. H., R. J. and L. J. F. contributed to the experiments and discussion; C. H., Y. X. S., D.W. and L. J. F. analyzed the data and wrote the manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imlet.2019.06.007.

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