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Research paper

Mesenteric lymph node CD4$^+$ T lymphocytes migrate to liver and contribute to non-alcoholic fatty liver disease

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is characterized by altered intestinal microbiota and intestinal immune disorder. Here we investigated the role of mesenteric lymph node (MLN) CD4$^+$ T lymphocytes in NAFLD. In high fat diet (HFD)-fed mice, the percentage ratios of Th1 to Th2 cells and Th17 to Treg cells were imbalanced in MLNs. Co-culture assays showed MLN CD4$^+$ T lymphocytes from HFD-fed mice tended to migrate to the liver and promoted hepatic inflammation. Adoptive transfer of MLN CD4$^+$ T lymphocytes from NAFLD mice to HFD-fed mice resulted in higher transaminase, worse hepatic inflammation and lipid accumulation. Antibiotics and probiotics were administrated to regulate intestinal microbiota, and the restoration of MLN Th1/Th2 and Th17/Treg cells in alleviated NAFLD were found. In summary, MLNs CD4$^+$ T subtype cells may involve in NAFLD, and the restoration of MLN CD4$^+$ T subtype cells ratio by regulating intestinal bacteria could be the new strategies.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) has become one of the most common chronic liver disorders worldwide [1]. The pathogenesis of NAFLD has been studied extensively, but its mechanism is still far from clear. Gut-derived factors are believed to play important roles in NAFLD. The intestinal barriers in NAFLD is weakened by reduced mucus and antimicrobial peptides secretion, and impaired tight junctions [2,3]. Then the intestinal permeability is increased, which facilitates the enteric pathogenic antigen crossing the barrier and entering into the liver, and eventually promotes NAFLD in turn [4,5].

Intestinal barriers are consist of several parts, in which the immune barrier plays important roles [6]. As a component of gut-associated lymphoid tissue (GALT), mesenteric lymph nodes (MLNs) act as a secondary lymphoid tissue. Antigen presenting cells contact antigens at the mucous layer and present them to secondary lymphoid tissues, such as Peyer's patches (PP) and MLNs, where T cells immune response happens and the effector T cells are induced home into the mucosa and exhibit immune effects [7]. The protective roles of MLNs in confining bacterial infection and limiting systemic disease have been demonstrated using several infection models [8,9]. Based on these data, MLNs can be considered as a central processing unit, that receive signals from intestinal immunological monitoring stations and response [10]. In high fat diet (HFD)-fed mice, the MLN immune dysfunction was observed [11], indicating damage to the intestinal barrier plays important roles in NAFLD. Moreover, our previous study has revealed increased lymphocytes in livers of NAFLD mice than control mice, and lymphocytes in the MLNs other than the lymphocyte from the thyroid、spleen、and bone marrow of NALFD mice had a propensity to migrate to the liver, which contributed to the progression of NAFLD [12], thus, we hypothesize that the MLN immune response, especially by CD4$^+$ T cells, may be involved in NAFLD.

Healthy intestinal bacteria contribute to maintain human health. Healthy Microbiota can inhibit the translocation of bacteria and keep intestinal barrier in a steady state [13]. Increasing evidences link NAFLD with the disturbance of intestinal microbiota [14,15]. Antibiotics and probiotics have been used to treat the dysbacteriosis and obesity [16,17]. Gut bacteria have effects on the differentiation and

Abbreviations: NAFLD, non-alcoholic fatty liver disease; MLN, mesenteric lymph node; HFD, high fat diet; ND, normal diet; GALT, gut-associated lymphoid tissue; PP, Peyer's patches; CFU, colony forming unit; ALT, Alanine aminotransferase; AST, Aspartate Aminotransferase

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proliferation of T cells in the intestine and MLNs [18,19]. Whether changes of gut microbiota are related to the changes of T cells in the MLNs in NAFLD has not been fully investigated.

In this study, we investigated the changes of $CD4^+$ T subtypes in MLNs and liver in HFD-fed mice, and explored the role of MLN $CD4^+$ T lymphocytes in the progression of NAFLD. We found that HFD induced remarkable increase in Th1 and Th17 cell proportions, and reduction in Th2 and Treg cell proportions in MLNs. In addition, transfer of MLN $CD4^+$ T cells, isolated from HFD-fed mice, aggravated NAFLD. The proportion balances between Th1 and Th2 cells, and Th17 and Treg cells were partly restored after administration of antibiotics or probiotics, and improvement of NAFLD was also observed. Based on these data, we proposed that the balance of $CD4^+$ T cells subtypes in MLN may involved in the progression of NAFLD.

2. Materials and methods

2.1. Animal model

Male 6–8-week C57BL/6J mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China), and raised in the experimental animal center of Peking University People's Hospital. All mice were fed with a normal diet (ND) or a HFD (45%) for 12 weeks, and antibiotics (216 mg/kg neomycin and 92 mg/kg polymyxin B) [20] or 10^8 colony forming unit (CFU) *Lactobacillus* [21] were gavaged once a day for the last four weeks to induce the change of intestinal microbiota respectively. Liver, MLNs, and serum were collected after mice were sacrificed. All of the procedures were validated by the ethical committee of Peking University People's Hospital.

2.2. Histology

Liver tissue was paraffinized and sectioned (5 μm), and stained with Hematoxylin and Eosin (H&E) following a standard procedure.

2.3. Biochemical measurement

Serum levels of Alanine aminotransferase (ALT) and Aspartate Aminotransferase (AST) were measured using an automatic biochemical detector (Labospect 008, HIACHI Ltd, Japan).

2.4. Isolation of lymphocytes and flow cytometry

Lymphocytes from blood in the liver were eliminated using PBS perfusion via the portal vein. Single-cell suspensions were prepared by mechanically homogenizing liver tissue through a 100 μm filter. Gradient centrifugation with 40% and 80% Percoll (GE healthcare, Uppsala, Sweden) was used to isolate the intrahepatic lymphocytes. Single-cell suspensions of MLN were prepared by mechanical disruption of MLN and through a 100 μm filter. After 5 h of Cell Stimulation Cocktail (eBioscience) incubation, surface markers (CD4-FITC, CD25-APC) of lymphocytes were stained. Intracellular markers (IFN-γ-PerCP/Cy5.5, IL-4-PE, IL-17-APC) of lymphocytes were stained after the permeabilized with the Intracellular Fixation & Permeabilization Buffer Set (eBioscience), and intranuclear marker (Foxp3-PE) was stained according to the manufacturer instruction of Foxp3 Staining buffer Set (eBioscience). All samples were detected using the FACS Calibur flow cytometer (BD immunocytometry systems, NJ, USA). Data were analyzed using Flowjo 7.6 (Flowjo, Oregon, USA).

2.5. $CD4^+$ T lymphocytes isolation and cultivation

$CD4^+$ T lymphocytes from MLN were sorted by using a MACS CD4 positive selection kit (Mitenyi Biotec, Gladbach, Germany). T lymphocytes were cultured at 37℃ with 5% CO_2 using the RPMI 1640 medium (Gibco, USA), supplemented with 10% fetal bovine serum (FBS)(Gibco, USA) and 1% 100 × penicillin/streptomycin.

2.6. Isolation of primary hepatocyte and co-culture assay

Primary hepatocytes were isolated with two-step perfusion method, and purified using Percoll gradients as previously described [22]. Hepatocytes (4×10^5) were plated in lower chamber of rat tail collagen pre-coated 12-well transwell plates and cultured using 1.5 ml DMEM (glucose 4500 mg/L, L-glutamine, 110 mg/L sodium pyruvate, Gibco) containing 10% FBS and 1% 100× penicillin/streptomycin. Hepatocytes were pre-incubated at 37℃ for 3 h and allowed to adhere to the plate. After three PBS washes, 2×10^6 $CD4^+$ T lymphocytes or $CD4^+$ T lymphocytes supernatants were added to the upper chamber and both cell types were co-cultured for 24 h.

2.7. Liver homogenate and transwell assay

100 mg liver tissue was weighed and homogenized using a laboratory blender in 5 ml RPMI 1640 medium. Samples were centrifuged for 10 min at 20000 × *g* to eliminate cell debris. In the 24-well transwell plate, 0.2 ml 20% liver homogenates and 0.4 ml RPMI 1640 medium were added to the lower chamber, and 10^6 $CD4^+$ T cells in 0.2 ml RPMI 1640 medium were added to the upper chamber. The number of T cells migrating to the lower chamber was counted and the chemotactic index was calculated after 6 h of culture.

2.8. Adoptive $CD4^+$ T lymphocytes transfer

$CD4^+$ T lymphocytes were isolated from MLNs of mice fed with ND or HFD for 12 weeks as described above. 1×10^6 cells/200 μl PBS were injected into mice fed with ND and HFD for 11 weeks via the caudal vein, and mice were sacrificed one week later.

2.9. RNA extraction and real-time polymerase chain reaction (PCR)

All tissues and cells were homogenized in TRIzol (Life Technologies, USA) and total RNA was extracted, followed by the synthesis of cDNA with Revert Aid First Strand cDNA Synthesis Kit (Thermo, Vilnius, Lithuania). Real-time PCR reactions were performed using the StepOne Plus Real-Time PCR System (Applied Biosystems, Waltham, MA, USA).

Table 1
SYBR Green PCR Primer sequences of target genes in mice.

Primers	Forward 5′ → 3′	Reverse 5′ → 3′
GAPDH	TCAACAGCAACTCCCACTCTTCCA	ACCCTGTTGCTGTAGCCGTATTCA
TNF-a	CAGCCGATGGGTTGTACCTT	GGCAGCCTTGTCCCTTGA
IL-6	AAGTCGGAGGCTTAATTACACATGT	CCATTGCACAACTCTTTTCTCATTC
IL-10	TGGAGCAGGTGAAGAGTGATTTT	TCAAATTCATTCATGGCCTTGT
FAS	GCCATGCCCAGAGGGTGGTT	TTCTTGCGATACACTCTGGTGG
ACC-1	CCCTCAGACACCCACATCTT	CAGAGCTCAGAAAGGGGTTG
PPAR-α	GAGAAGTTGCAGGAGGGGATTGTG	AAGACTACCTGCTACCGAAATGGG
FGF-21	TCCAAATCCTGGGTGTCAAA	CAGCAGCAGTTCTCTGAAGG

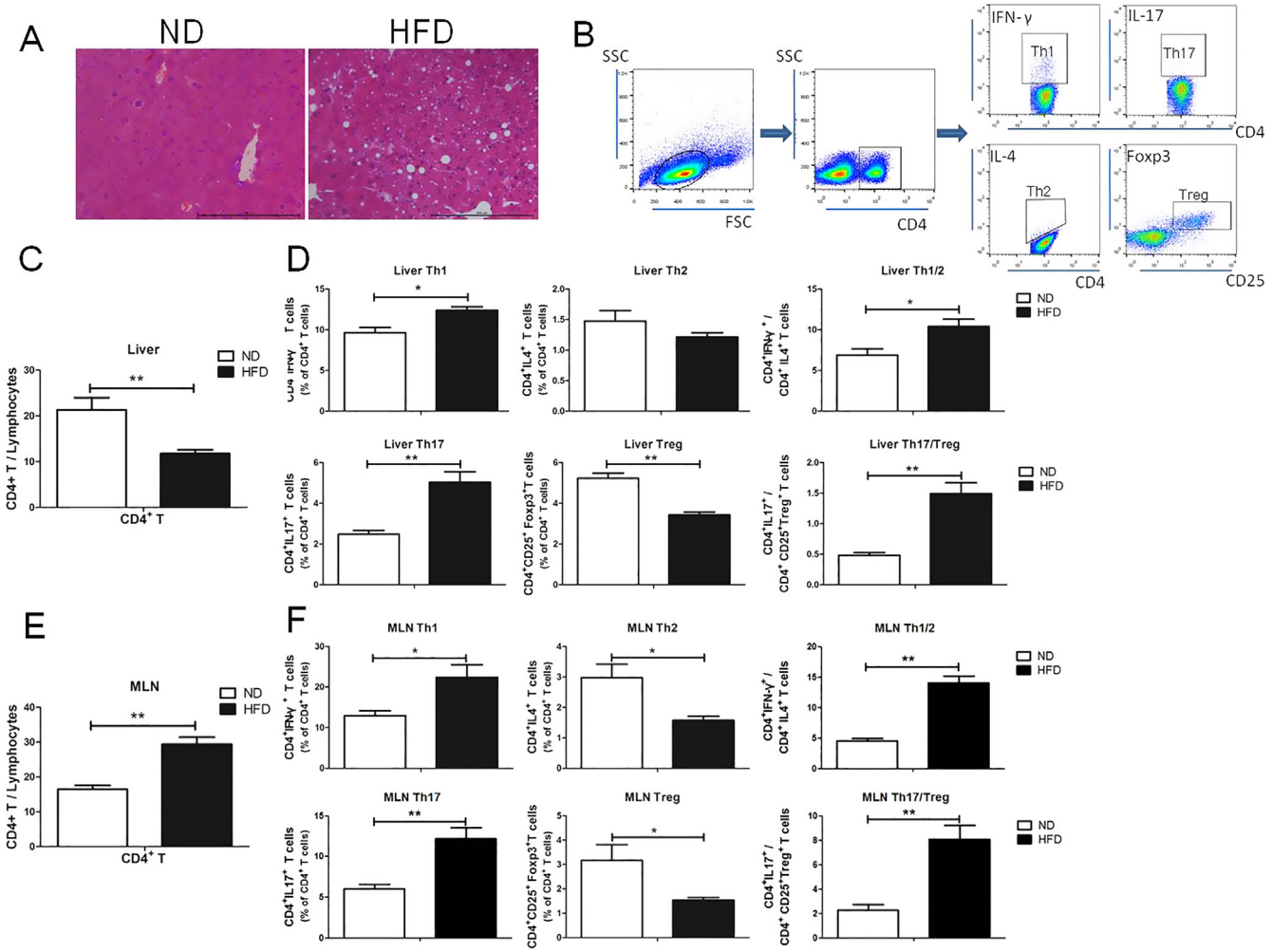

Fig. 1. Alteration of $CD4^+$ T cell subtypes in the liver and MLN of HFD-fed mice. Representative hematoxylin and eosin (H&E, ×200) staining of mouse liver. (B) The gating strategy of flow cytometry for liver and mesenteric lymph node (MLN). The proportion of $CD4^+$ T lymphocytes (C), $CD4^+IFN\text{-}\gamma^+$ Th1 cells, $CD4^+IL\text{-}4^+$ Th2 cells, $CD4^+IL\text{-}17^+$ Th17 cells, $CD4^+CD25^+Foxp3^+$ Treg cells, and the percentage ratios of Th1 to Th2 cells, Th17 to Treg cells (D) in liver of HFD- and normal diet (ND)-fed mice. The proportion of $CD4^+$ T lymphocytes (E), $CD4^+IFN\text{-}\gamma^+$ Th1 cells, $CD4^+IL\text{-}4^+$ Th2 cells, $CD4^+IL\text{-}17^+$ Th17 cells, $CD4^+CD25^+Foxp3^+$ Treg cells, and the percentage ratios of Th1 to Th2 cells, Th17 to Treg cells (F) in MLNs of HFD- and normal diet (ND)-fed mice. N = 6, values represent means ± SEM, $^*P < 0.05$, $^{**}P < 0.01$.

Data were analyzed using the $2^{-\triangle\triangle Ct}$ method. Primer sequences were given in Table 1.

2.10. Statistical analysis

Results are presented as the mean ± SEM. Data were analyzed using SPSS 17.0 (IBM, NY, USA), and statistical significance ($p < 0.05$) was determined using a Student's *t* test or one-way ANOVA. Graphs were prepared using GraphPad Prism (GraphPad Software Inc., La Jolla, USA).

3. Results

3.1. Alteration of $CD4^+$ T cell subtypes in the liver and MLN of HFD-fed mice

To investigate the role of hepatic and intestinal immune system in NAFLD, we analyzed the proportion of $CD4^+$ Th subsets in the liver and MLNs of mice fed with ND or HFD for 12 weeks. The NAFLD model was verified by the liver pathology (Fig. 1A). The gating strategy of flow cytometry for liver and MLNs is shown in Fig. 1B. $CD4^+$ T lymphocytes decreased in liver of HFD-fed mice ($p < 0.01$) (Fig. 1C). The percentages of $CD4^+IFN\text{-}\gamma^+$ Th1 cells and $CD4^+IL\text{-}17^+$ Th17 cells were increased remarkably ($p < 0.05$) and $CD4^+CD25^+Foxp3^+$ Treg cells was decreased significantly ($p < 0.05$), which lead to the increased percentage ratios of Th1/Th2 ($p < 0.05$), and Th17/Treg cells ($p < 0.01$) in the livers of HFD-fed mice compared with ND-fed mice (Fig. 1D). $CD4^+$ T lymphocytes increased in the MLNs of HFD-fed mice ($p < 0.01$) (Fig. 1E). Similar with results of liver, increased percentages of Th1 ($p < 0.05$) and Th17 ($p < 0.01$) cells, decreased Th2 ($p < 0.05$) and Treg ($p < 0.05$) cells, and increased percentage ratios of Th1/Th2 ($p < 0.01$), Th17/Treg cells ($p < 0.01$) in MLNs were observed in HFD group (Fig. 1F). These results showed that changes of CD4+ T cell subtype is consistant in liver and MLN in HFD group, which suggest there maybe some relationship between the intestinal immune and liver immune in NAFLD.

3.2. MLN $CD4^+$ T lymphocytes tended to migrate to the liver and induce hepatocyte injury

To evaluate whether MLN $CD4^+$ T cells could migrate to the liver and affect hepatocytes, we performed co-culture experiments *in vitro*. A higher chemotactic index of MLN $CD4^+$ T cells isolated from HFD-fed mice to liver homogenates than ND-fed mice were observed in the

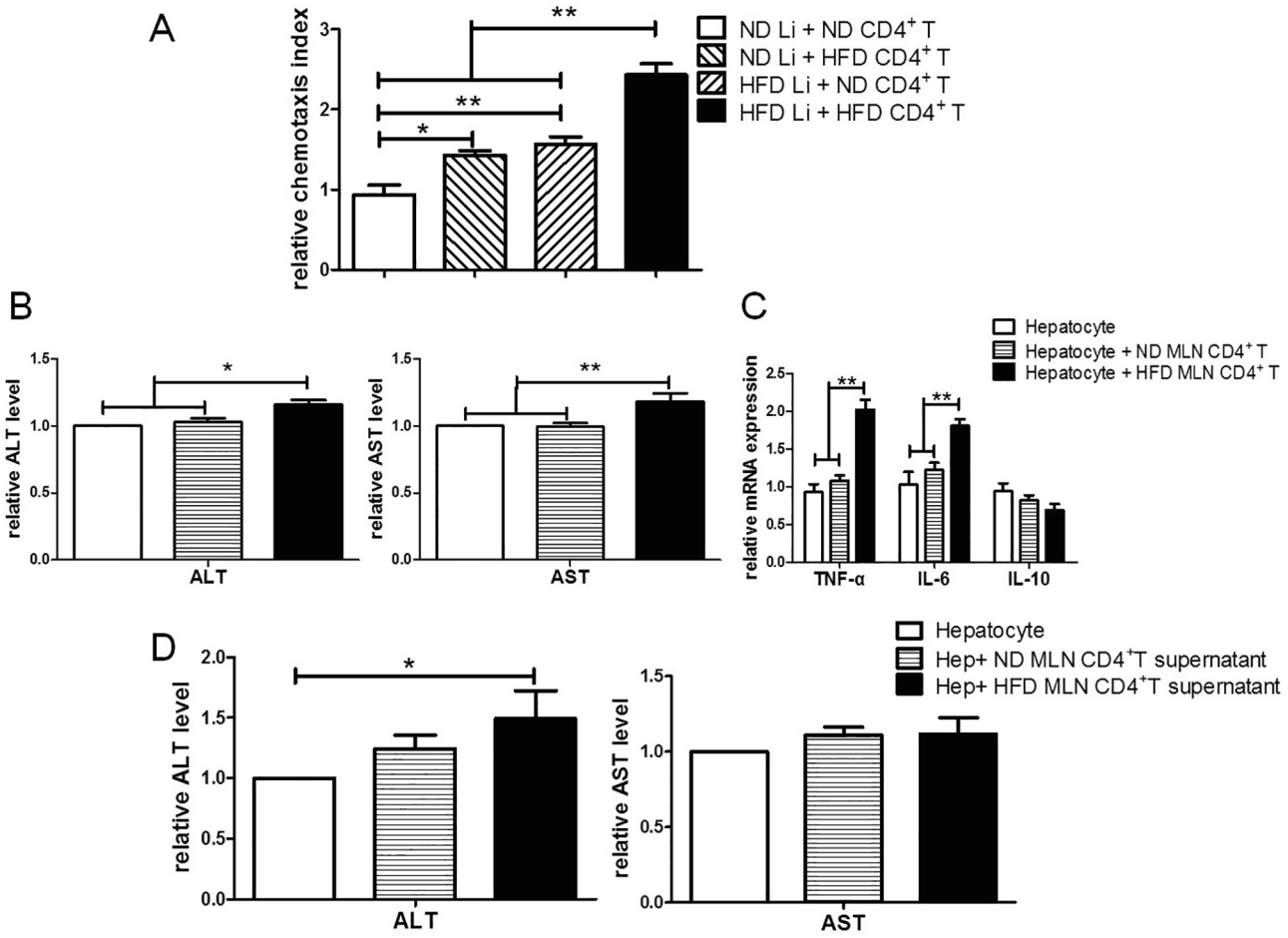

Fig. 2. MLN CD4$^+$ T lymphocytes tended to migrate to the liver and induce hepatocyte injury. (A) Relative chemotaxis index of mesenteric lymph node (MLN) CD4$^+$ T lymphocytes to liver homogenate. (B) Aminotransferase of the culture supernatant in the co-culture system of primary hepatocytes and MLN CD4$^+$ T lymphocytes. (C) Relative mRNA expression levels of genes responsible for inflammation, specifically, *tnf-α*, *interlukin (il)-6* and *il-10* of hepatocytes. (D) Aminotransferase of the culture supernatant in the co-culture system of primary hepatocytes and MLN CD4$^+$ T lymphocytes cultural supernatant. N = 3(repeated twice), values represent means ± SEM, $^*P < 0.05$, $^{**}P < 0.01$. Abbreviations: ND Li, liver homogenate from normal diet (ND)-fed mice; HFD Li, liver homogenate from high fat diet (HFD)-fed mice; ND CD4$^+$ T, MLN CD4$^+$ T lymphocytes from ND-fed mice; HFD CD4$^+$ T, MLN CD4$^+$ T lymphocytes from HFD-fed mice.

transwell system, irrespective of whether liver homogenates were made from ND- or HFD-fed mice (Fig. 2A). In the co-culture experiment of primary hepatocytes and CD4$^+$ T cells, MLN CD4$^+$ T cells from HFD-fed mice caused elevation of ALT ($p < 0.05$) and AST ($p < 0.01$) levels in supernatants, and promoted the TNF-αand IL-6 mRNA expression of hepatocytes, while MLN CD4$^+$ T cells from ND-fed mice failed to do so (Fig. 2B,C). Similar with these results, MLN CD4$^+$ T cells supernatant from HFD-fed mice promoted the level of AST ($p < 0.05$), but ND MLN CD4$^+$ T cells did not (Fig. 2D). These data showed that migration of MLN CD4$^+$ T cells to the liver was increased in NAFLD and might damage the hepatocyte both in vivo and in vitro.

3.3. Adoptive transfer of MLN CD4$^+$ T cells aggravated NAFLD

To see whether the supplement of MLN CD4$^+$ T cells might aggravate the inflammation and lipid metabolism of NAFLD, we used an adoptive T cell transfer method in HFD-fed mice. Histological assessment also showed that mice receiving HFD MLN CD4$^+$ T cells showed maximum lipid accumulation and inflammatory infiltration focus (Fig. 3A). Transfer of MLN CD4$^+$ T cells isolated from HFD mice resulted in significantly higher ALT ($p < 0.05$), AST ($p < 0.05$) and glucose ($p < 0.05$) levels in serum than PBS group and mice receiving ND MLN CD4$^+$ T cells group (Fig. 3B and C). Compared with PBS group, increased mRNA expression of IL-6 ($p < 0.05$) was observed in HFD-fed mice receiving ND MLN CD4$^+$ T cells (Fig. 3D), as well as higher levels of TNF-α ($p < 0.01$), IL-6 ($p < 0.01$), FAS ($p < 0.01$) and lower level of IL-10 ($p < 0.01$) mRNA expression in HFD-fed mice transferred with HFD MLN CD4$^+$ T cells (Fig. 3D and E). As compared with ND MLN CD4$^+$ T cells group, HFD MLN CD4$^+$ T cells transfer induced higher mRNA expression of TNF-α ($p < 0.05$) (Fig. 3D) and FAS ($p < 0.05$) (Fig. 3E). These results indicated that MLN CD4$^+$ T cells from HFD-fed mice could promote hepatic inflammation and lipid accumulation.

3.4. Adoptive transfer of MLN CD4$^+$ T cells had no effects on liver of ND-fed mice

To see whether the supplement of MLN CD4$^+$ T cells had an effect on the normal liver, we used an adoptive T cell transfer method in ND-fed mice. The histological assessment, ALT, AST or glucose levels did not show any variation among PBS, ND and HFD MLN CD4$^+$ T cells (Fig. 4A–C). Transfer of MLN CD4$^+$ T cells, either from ND- or HFD-fed mice, nearly had no effects on the mRNA expression of inflammatory cytokines or lipid metabolism in the liver, except for IL-10 ($p < 0.05$) (Fig. 4D and E). These data suggested that MLN CD4$^+$ T cells did not impair the normal liver.

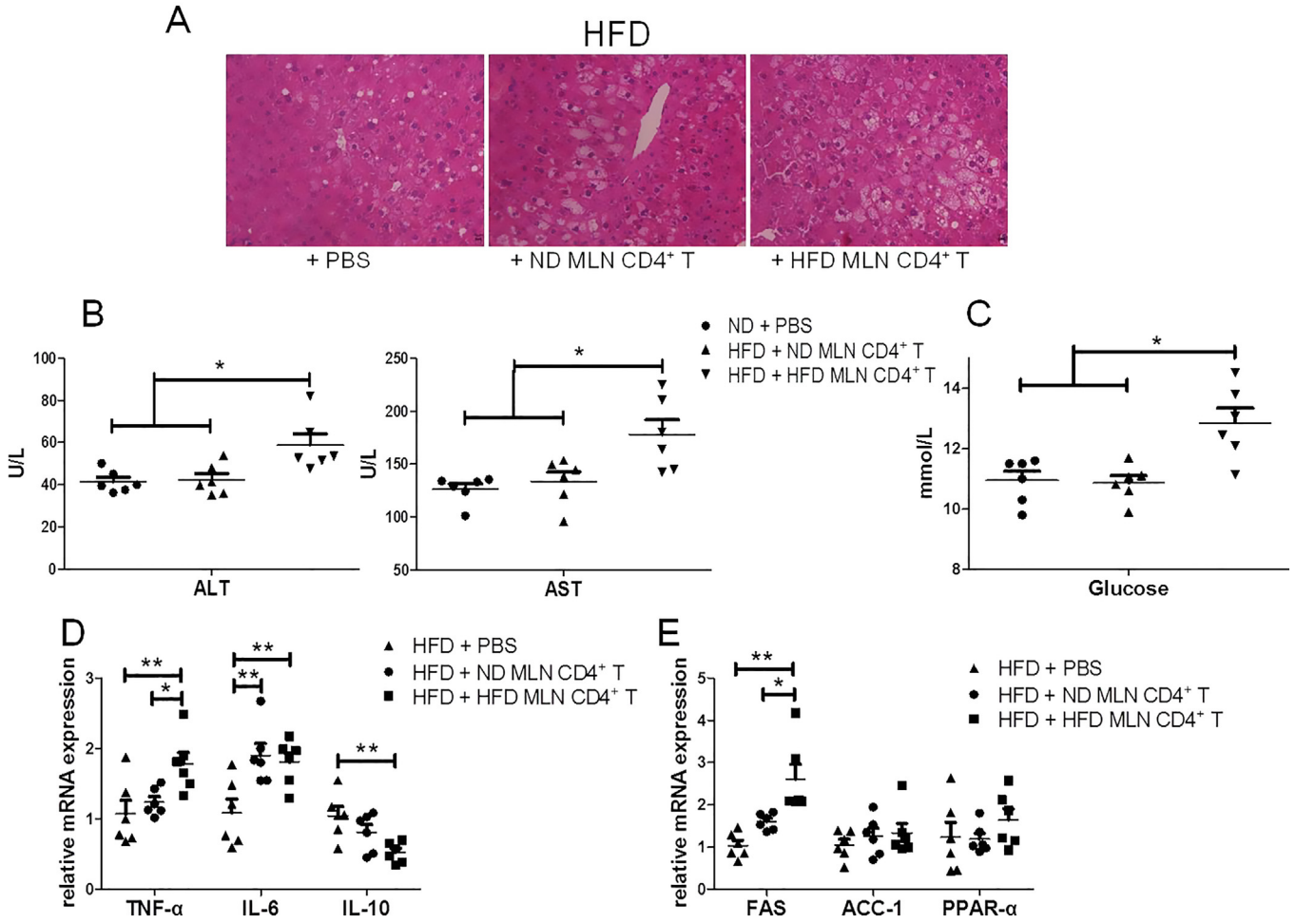

Fig. 3. Adoptive transfer of MLN CD4$^+$ T cells aggravated NAFLD. High fat diet (HFD)-fed mice were given injections of PBS and MLN CD4$^+$ T cells from normal diet (ND) or HFD-fed mice. (A) Representative hematoxylin and eosin (H&E, ×200) staining of mouse liver. Aminotransferase (B) and glucose (C) levels in serum. Relative mRNA expression levels of genes responsible for inflammation and lipid metabolism of mouse liver (D and E), specifically, *tnf-α*, *interlukin (il)-6*, *il-10*, *fatty acid synthase complex (fas)*, *acetyl-CoA carboxylase-1(acc-1)* and *peroxisome proliferator-activated receptor-α (ppar-α)*. N = 6, values represent means ± SEM, $^*P < 0.05$, $^{**}P < 0.01$.

3.5. Administration of antibiotics or Lactobacilli alleviated non-alcoholic fatty liver disease

To verify the effects of intestinal bacteria on NAFLD, antibiotics and lactobacillus were administrated to HFD mice. Among PBS, Antibiotics and Lactobacilli groups, there was no significant difference in body weight (Fig. 5A). Pathological assessment of livers showed that Antibiotics and Lactobacilli administration resulted in less inflammatory infiltration foci and lipid accumulation than that seen in the PBS group (Fig. 5B). We can see the decreased ALT ($p < 0.01$) and AST ($p < 0.01$) levels in the Antibiotics and Lactobacillus groups, which suggested regulation of microbiota could alleviate the liver injury in NAFLD (Fig. 5C). The mRNA expression of IL-6 ($p < 0.01$) and TNF-α ($p < 0.01$) was decreased and FGF-21 ($p < 0.05$) expression was increased remarkably in livers of Antibiotics and Lactobacillus groups compared with PBS group (Fig. 5D). These results indicated that regulation of bacteria could improve the inflammatory state of NAFLD.

3.6. Administration of antibiotics and Lactobacillus altered the balance of CD4$^+$ T lymphocyte subsets in mesenteric lymph node

CD4$^+$ T lymphocytes in MLNs play important roles in defense against intestinal bacteria, and CD4$^+$ T cells in the liver are considered to be involved in NAFLD. Therefore, we examined whether Antibiotics and Lactobacillus administration induces alterations of CD4$^+$ T lymphocytes in MLNs and in the liver. Descended CD4$^+$ T lymphocytes proportion in MLNs of HFD-fed mice were observed in Antibiotics and Lactobacillus groups ($p < 0.01$) (Fig. 6A). As compared with PBS group, the proportion of Th2 cells ($p < 0.01$) and Treg cells ($p < 0.01$), which play an anti-inflammatory character, was increased in the MLNs of Antibiotics and Lactobacillus groups (Fig. 6B and C); the proportion of Th1 cells ($p < 0.01$) and Th17 cells ($p < 0.01$), which play a pro-inflammatory characters, decreased significantly in the MLNs of Antibiotics and Lactobacillus groups, as well as percentage ratios of Th1/Th2 and Th17/Treg cells (Fig. 6B and C). Ascended CD4$^+$ T lymphocytes proportion in liver of HFD-fed mice were observed in the Antibiotics and Lactobacillus groups ($p < 0.01$) (Fig. 6D). The proportion of Treg ($p < 0.01$), not Th2 cell in the liver showed an increasement similar to the changes of CD4$^+$ T cell subtypes seen in MLNs (Fig. 6E and F); Th1 ($p < 0.01$), Th17 ($p < 0.01$) cells proportions and percentage ratios of Th1/Th2, Th17/Treg cells decreased in the Antibiotics and Lactobacillus groups (Fig. 6E and F). These data indicated that the alleviation of NAFLD might be related to the changes in CD4$^+$ T lymphocyte subtypes proportion.

4. Discussion

Either “two hits” or “multiple hits” hypothesis emphasize the role of gut-derived factors, especially gut microbiota and cytokines, in NAFLD [23,24], while the intestinal immune system is relatively neglected. The

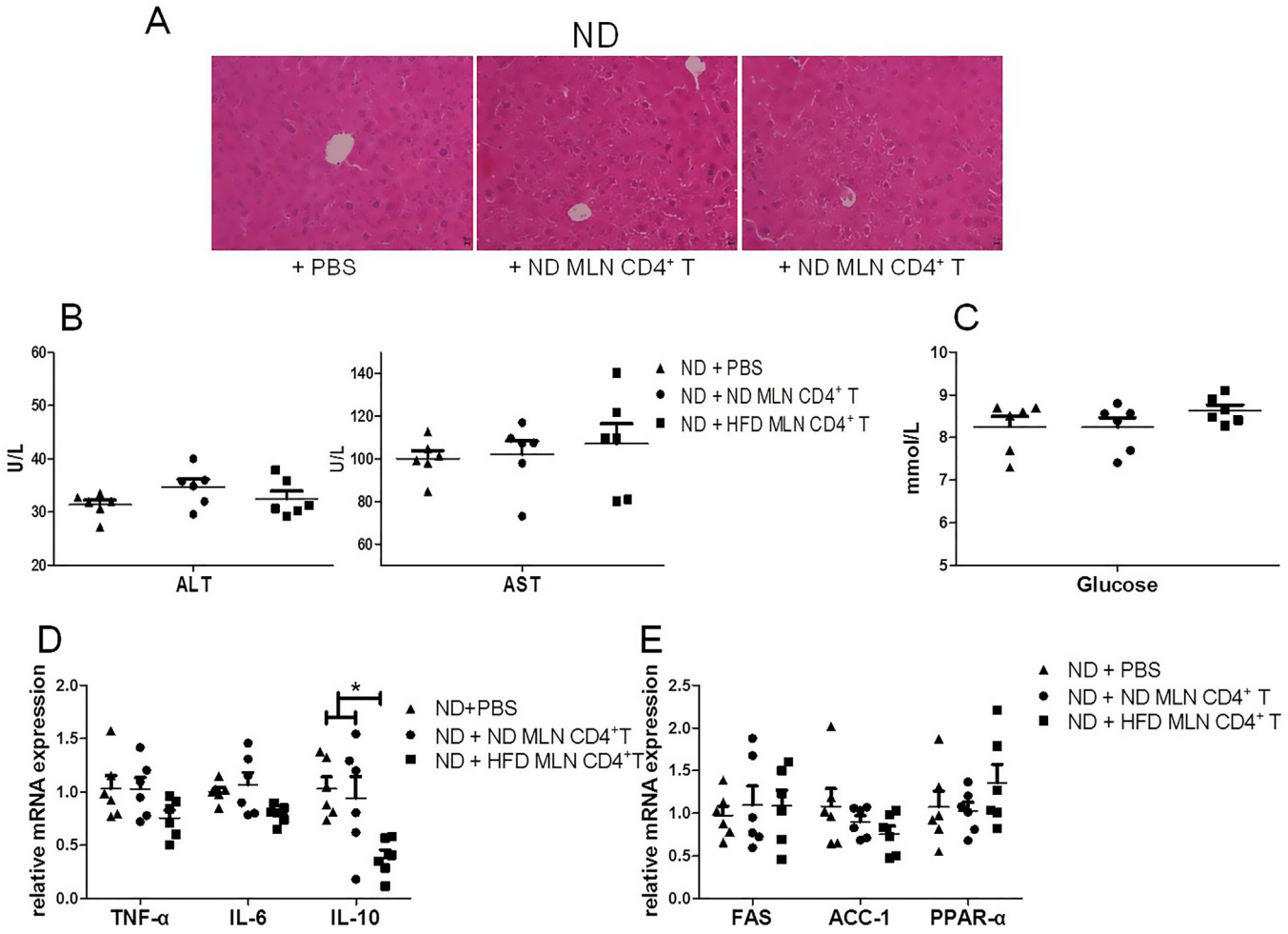

Fig. 4. Adoptive transfer of MLN CD4$^+$ T cells had no effects on liver of ND-fed mice. Normal diet (ND)-fed mice were given injections of PBS and MLN CD4$^+$ T cells from ND or high fat diet (HFD)-fed mice. (A) Representative hematoxylin and eosin (H&E, ×200) staining of mouse liver. Aminotransferase (B) and glucose (C) levels in serum. Relative mRNA expression levels of genes responsible for inflammation and lipid metabolism of mouse liver (D,E), specifically, *tnf-α*, *interlukin (il)-6*, *il-10*, *fatty acid synthase complex (fas)*, *acetyl-CoA carboxylase-1(acc-1)* and *peroxisome proliferator-activated receptor-α (ppar-α)*. N = 6, values represent means ± SEM, $^*P < 0.05$, $^{**}P < 0.01$.

present study identified alteration in the proportions of MLN CD4$^+$ T lymphocytes, i.e. increased percentage ratios of Th1/Th2 and Th17/Treg cells in HFD-fed NAFLD mice. Transfer of MLN CD4$^+$ T lymphocytes isolated from NAFLD mice to recipient mice resulted in higher ALT, AST and hepatic inflammation. Benefits to the liver due to administration of antibiotics or probiotics were also related to the restoration of CD4$^+$ T subsets balance in MLN.

The percentage ratios of Th1/Th2 and Th17/Treg are all increased in MLN of HFD group. Gut-associated lymphoid tissue (GALT) consists of immune cells in the intestinal wall and lymphoid tissue related to the gut, such as peyer's patches, lymphoid follicle and MLNs. GALT contributes to the defense of the host against gut microbiota examining blood through the lymphatic pathway [25,26]. Among which, MLN is one of the main units of immunoreaction, and provides immune effector cells to the lamina propria, which is the major region of defense of GALT against gut microbiota. Gautreaux MD *et al* found elevated loads of bacteria in the parenteral organ and blood, as well as in MLN when CD4$^+$ T cells were eliminated, which could be reversed by adoptive transfer of splenetic CD4$^+$ T cells. Moreover, the transferred CD4$^+$ T cells mainly migrated to spleen and MLN [27,28]. There is also a correlation between MLN CD4$^+$ T cell subtype and serum LPS [29]. These results indicated the indispensable role of MLN CD4$^+$ T cells in the intestinal barrier. Notably, different CD4$^+$ T cell subtypes playing different functions. Th1 and Th17 cells are considered to be involved in immune response against microbial, i.e. pro-inflammatory reactions, and Th2 and Treg cells play antagonistic roles [10,30,31]. Increased Th1/Th2 and Th17/Treg ratios in MLN show that the delicate balance of pro-inflammation and anti-inflammation responses are broken in intestines of NAFLD mice. Similar to our study, Mao JW *et al* also observed decreased Treg cells in MLN of NAFLD mice [32].

Our previous study showed the increased lymphocytes infiltration in the liver of NAFLD mice, and the migration of MLN lymphocytes other than the lymphocyte from the thyroid、spleen、and bone marrow of NALFD mice to the liver *in vivo*, which are more apparent in NAFLD mice [12]. Selective CD4$^+$ T lymphocyte loss was evidenced in NAFLD [33], thus, we hypothesized that CD4$^+$ T cells in MLN might involve in the pathogenesis of NAFLD, although their roles are not verified. This study not only verified the chemotaxis of MLN CD4$^+$ T cells to liver *in vitro*, but also showed their impairment to hepatocytes by the co-culture and adoptive transfer experiments in vivo. As far as the mechanism of hepatocyte impairment by the CD4+ T lymphocyte, several studies have reported that lymphocytes can affect hepatocytes via secreting cytokines and so on [34,35]. Lymphocytes in other GALT organs also have effects on NAFLD. Hong CP *et al.* showed that HFD reduced Th17 cells in the small intestine, and adoptive transfer of gut-tropic Th17 cells alleviated hepatic steatosis, whereas Treg cells failed to do so [36]. The gut-liver immune dialogue cannot be ignored in liver diseases, although lymphocytes in different GALT organs seem to paly various

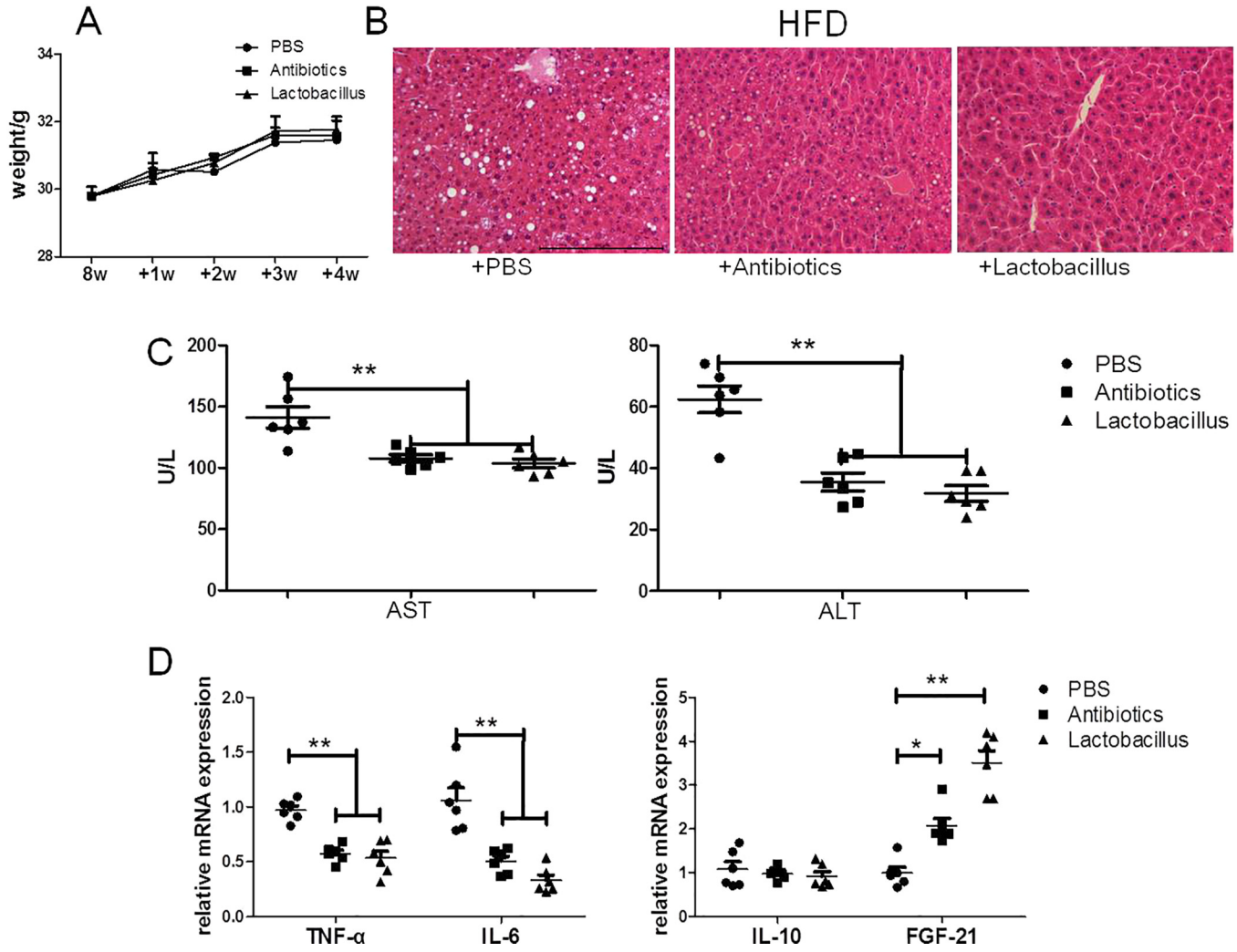

Fig. 5. Administration of antibiotics and Lactobacilli alleviated non-alcoholic fatty liver disease. High fat diet (HFD)-fed mice were given PBS, antibiotics and lactobacillus by gavage. Body weight (A) and representative hematoxylin and eosin (H&E, ×200) staining (B) of mouse liver. (C) Aminotransferase levels in serum. (D) Relative mRNA expression levels of genes responsible for inflammation, specifically, *tnf-α*, *interlukin (il)-6*, *il-10*, and *fibroblast growth factor-21* (*fgf-21)* of mouse liver. N = 6, values represent means ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$.

roles, and targeting the gut immune system in NAFLD is a promising strategy [37]. The chemotaxis of $CD4^+$ T subsets requires further investigation, because Th1, Th17 cells and Th2, Treg cells play opposite roles in NAFLD [38]. It is interesting that $CD4^+$ T cells, isolated from MLN of NAFLD mice, injured hepatocytes of HFD-fed mice in vitro and in vivo, but failed to do so to ND-fed mice. It seems that the simple steatosis make hepatocytes fragile for the impact of MLN $CD4^+$ T cells, which may play a role in the pathogenesis of NAFLD.

Gut microbiota are emerging as critical factors that shape the intestinal immune system, which includes the differentiation of $CD4^+$ T cell subsets [39–41]. As compared with that of the SPF mice, the intestinal immune response can be considered naïve in the absence of commensal microbiota in germ-free mice, which showed fewer and smaller Peyer's patches, lymphoid follicles and MLN, as well as a thinner lamina propria. Except for the morphologic changes, $CD4^+$ T cell numbers in the lamina propria and MLN decreased, including Th17 and Treg cells [39]. Meanwhile, HFD can induce alteration of gut bacteria both in NAFLD patients and HFD-fed mice [42]. Hence, the change in the intestinal immune response may be caused by the gut bacteria. To verify this hypothesis, antibiotics and probiotics was used to improve the dysbiosis in NAFLD mice, and the percentage balance of Th1/Th2 and Th17/Treg cells were restored. Benefits of strategies targeting gut bacteria may be related to the intestinal immune response in NAFLD. Besides, the MLN is embedded in the mesentery, and mesenteric stromal cells contribute to the activation of T cells [43]. The effects of antibiotics and probiotics on the mesentery should be taken into consideration for further studies.

In conclusion, our results indicated that the imbalance between Th1 and Th2 cells, and Th17 and Treg cells in MLN were caused by the disordered gut bacteria, and might involve in the pathological mechanism of NAFLD. Strategies targeting intestinal bacteria for alleviating NAFLD may be related to the recovery of $CD4^+$ T subsets balance.

Author contributions

Lin Su & Yulan Liu designed the study; Lin Su, Zhe Wu, Yujing Chi, Yang Song, Jun Xu and Jiang Tan performed the experiments; Lin Su, Zhe Wu, Xu Cong and Yulan Liu analyzed the results; Lin Su, Zhe Wu and Yulan Liu wrote the paper. All authors reviewed the manuscript.

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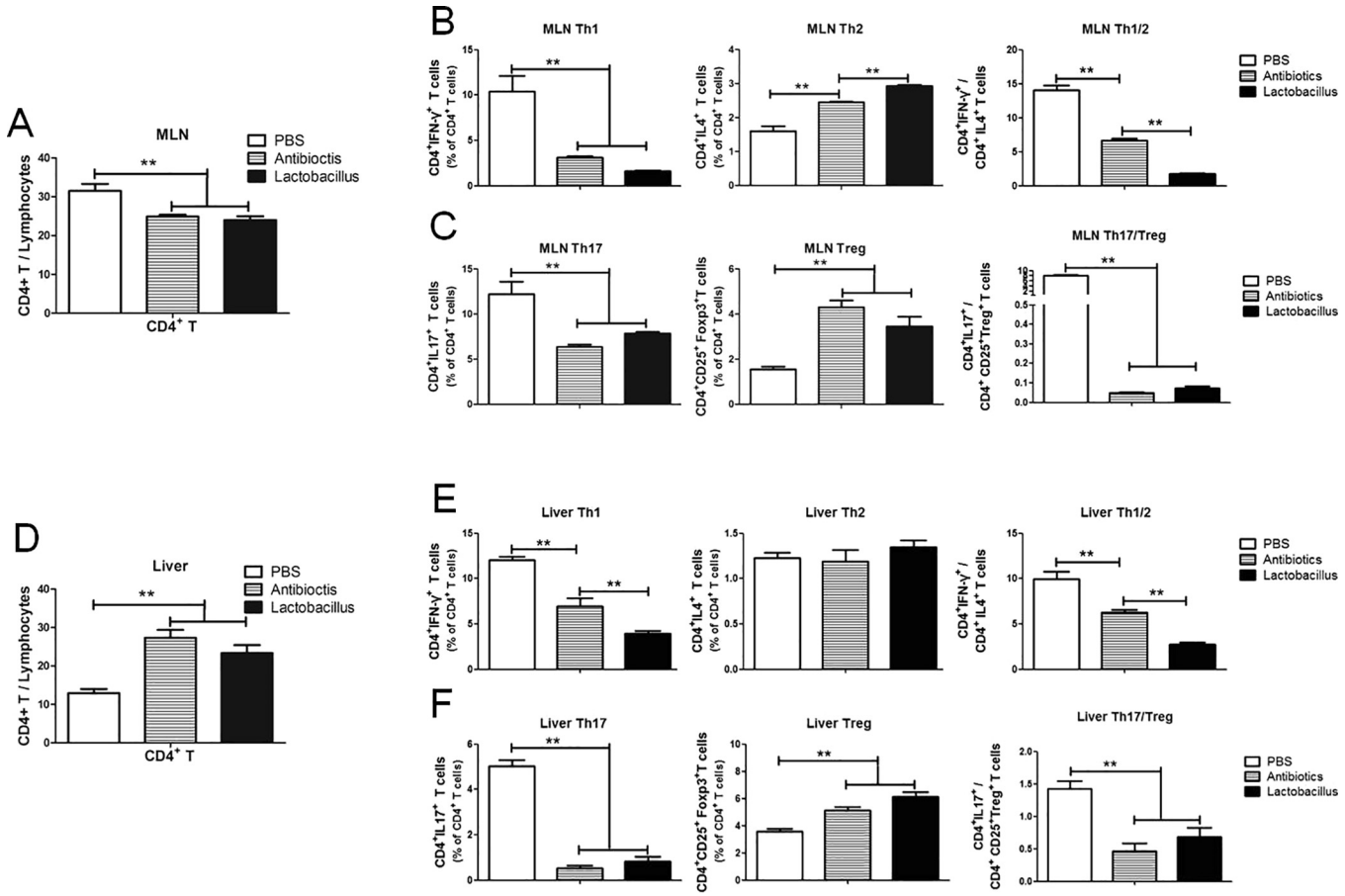

Fig. 6. Administration of antibiotics and Lactobacillus altered the balance of $CD4^+$ T lymphocyte subsets in mesenteric lymph node. High fat diet (HFD)-fed mice were given PBS, antibiotics and lactobacillus by gavage. The proportion of $CD4^+$ T lymphocytes (A), $CD4^+IFN\text{-}\gamma^+$ Th1 cells, $CD4^+IL\text{-}4^+$ Th2 cells, and the percentage ratio of Th1 to Th2 cells (B) and $CD4^+IL\text{-}17^+$ Th17 cells, $CD4^+CD25^+Foxp3^+$ Treg cells and the percentage ratio of Th17 to Treg cells (C) in mesenteric lymph node (MLN) of mouse. The proportion of $CD4^+$ T lymphocytes (D), $CD4^+IFN\text{-}\gamma^+$ Th1 cells, $CD4^+IL\text{-}4^+$ Th2 cells, and the percentage ratio of Th1 to Th2 cells (E) and $CD4^+IL\text{-}17^+$ Th17 cells, $CD4^+CD25^+Foxp3^+$ Treg cells and the percentage ratio of Th17 to Treg cells (F) in liver of mouse. N = 6, values represent means ± SEM, $^*P < 0.05$, $^{**}P < 0.01$.

Conflict of interest

The authors declare no conflict of interest.

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