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CD36 deficiency aggravates macrophage infiltration and hepatic inflammation by up-regulating MCP-1 expression of hepatocytes through HDAC2-dependant pathway

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ABSTRACT :

Aims: Cluster of differentiation 36 (CD36) is involved in the development of non-alcoholic steatohepatitis (NASH). Excess CD36 facilitates liver cells taking fatty acid and activates inflammatory signals to promote hepatic steatosis and inflammation. However, CD36-deficiency paradoxically promotes non-alcoholic fatty liver disease by unknown mechanisms. We explored the probable molecular mechanism of hepatic inflammation induced by CD36 deficiency. ***Results:*** CD36 deletion in mice ($CD36^{-/-}$ mice) specifically increased monocyte chemotactic protein -1 (MCP-1) in hepatocytes, promoted macrophage migration to liver and aggravated hepatic inflammatory response and fibrosis. The nuclear expression of histone deacetylase 2 (HDAC2) which highly expresses in wild-type hepatocytes and has an inhibitory effect on acetyl histone 3 (H3) was reduced in CD36 deficiency hepatocytes. Consequently, the level of acetyl H3 binding to MCP-1 promoters was increased in CD36 deficient hepatocytes, causing hepatic specific MCP-1 transcriptional activation. Reduction of nuclear HDAC2 in both $CD36^{-/-}$ mice liver and cultured hepatocytes was due to reduction of intracellular reactive oxygen species (ROS) level, while supplement of low concentration hydrogen peroxide (H_2O_2) overcame the suppression of HDAC2 caused by CD36 deficiency, decreasing MCP-1 gene transcription and microphage migration. ***Innovation:*** Our results provide first evidence that decreased ROS production by CD36 deletion was also harmful for livers. The fine balance of CD36 plays an important role in maintaining balances of hepatic ROS and nuclear HDAC2 which could be a potential new therapeutic strategy for the prevention of NASH development. ***Conclusion:*** CD36 deficiency promoted the development of NASH by facilitating the transcription of MCP-1 in hepatocytes, due to the reduction of ROS and nuclear HDAC2.

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INTRODUCTION

Cluster of differentiation 36, also known as fatty acid translocase (FAT), belongs to the scavenger receptor family and is an integral membrane protein found on the surface of many cell types in vertebrates (36). In its function as a facilitator of long-chain fatty acid transport and other biologically active lipids such as oxidized low density lipoprotein (ox-LDL), CD36 is believed to play an important role in the development of non-alcoholic fatty liver disease (NAFLD). Patients with NAFLD have elevated hepatic CD36 expression, which is positively correlated with liver fat content and insulin resistance (28). Experimental up-regulation of CD36 is sufficient to increase hepatic fatty acid uptake and triglyceride (TG) storage (6). Although the high expression of hepatic CD36 is closely related to NAFLD, CD36 deficiency paradoxically promotes the development of NAFLD. In the clinic, patients with genetic CD36 deficiency, which is relatively frequent in Asian and African populations, have been reported to exhibit hyperlipidemia, insulin resistance, and a propensity to develop symptoms of “metabolic syndrome” including fatty liver and atherosclerosis (18). When administered a high glucose or high fat diet, mice lacking CD36 exhibit increased plasma free fatty acid and TG levels and decreased hepatic insulin sensitivities (15). CD36 deletion exacerbates the steatosis by impairing hepatic triglyceride and ApoB secretion in homozygous ob/ob mice (31). These studies suggest that either CD36 over-expression or deletion causes hepatic steatosis and that the function of CD36 as a fatty acid transporter may not explain the conflicting results regarding hepatic steatosis.

CD36 has recently been identified as an important regulator of inflammatory response and function as a pattern recognition receptor (PRR) that conducts signals and activates inflammatory pathways such as Toll-like receptor (TLR), c-Jun N-terminal kinase(JNK) and Nuclear factor-κB (NF-κB) signals (17,22,36). Studies have shown that an increased expression of macrophage CD36 and other scavenger receptors contributes to hepatic macrophage infiltration and the development of non-alcoholic steatohepatitis (NASH) (5,6). However, in present study, we found that livers of mice

with global CD36 gene knockout exhibit an enigmatic increase of macrophage infiltration and inflammation, indicating a largely unknown role for CD36 in regulating macrophage migration.

Macrophage migration and infiltration are regulated by chemokines and their receptors, particularly monocyte chemotactic protein-1 (MCP-1). MCP-1, also called CCL2, is a key chemokine in the development of NASH, and its up-regulation promotes macrophage accumulation, inflammation, fibrosis and steatosis (2). The gene expression of cytokines including MCP-1 are usually regulated by transcription factors such as NF-κB and repressors on the gene promoter and enhancer regions (37). In addition to NF-κB, epigenetic modification, especially histone acetylation, is the most common and important mechanism regulating the gene transcription of chemokines. It has been demonstrated that the NF-κB signal is inhibited by CD36 deficiency (19), suggesting that increased macrophage migration and hepatic inflammation in CD36 deficiency are caused by NF-κB independent pathways.

Acetyl histone3 (H3), which is usually inhibited by histone deacetylases (HDACs), is a transcription factor that binds to MCP-1 promoters. Co-operation of histone acetyl transferases (HATs) and histone deacetylases (HDACs) keeps the balance of histone acetylation: activation of HATs or inhibition of HDACs promotes gene transcription by inducing hyper-acetylation of core histones. The activities of HDACs are regulated by intracellular reactive oxygen species (ROS) levels (4), and CD36 has been reported to participate in the production of intracellular ROS. Holloway, G. P. et al suggested that CD36 is positioned on the outer mitochondrial membrane, upstream of long-chain acyl-CoA synthetase, thereby contributing to the regulation of mitochondrial fatty-acid transport and beta-oxidation (38). CD36 is also involved in transduction of intracellular signals such as mitogen-activated protein kinase (MAPK) signals to regulate ROS formation (27). Deletion of CD36 decreases intracellular ROS levels (9).

In a “two-hit model” of NAFLD development, ROS-mediated inflammation has been considered to be the second hit, which has been proposed to cause the transition of hepatic steatosis to more severe NASH (10). The accumulation of lipids in hepatocytes impairs the oxidative capacity of mitochondria and increases the

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generation of ROS, which triggers cell death and the production of inflammatory cytokines, ultimately resulting in the development of NASH (35). However, accumulating evidence has now indicated that ROS at low/moderate levels, especially the relatively stable hydrogen peroxide (H_2O_2) molecule, can function as an intracellular second messenger (39). Many ROS-mediated responses protect cells against oxidative stress and maintain "redox homeostasis". In addition, physiological ROS levels are necessary for the prevention of hepatic steatosis in zebrafish larvae (32). The dual roles of ROS in development of NAFLD are similar to that of CD36, suggesting the development of NASH in CD36 knockout mice is probably related to the decreased ROS production induced by CD36 deficiency.

In this study, we explored whether CD36 deficiency inhibited hepatic HDACs by reducing ROS levels, and whether the decrease of HDACs increased acetyl histone3 (H3) binding to MCP-1 promoters, consequently enhancing MCP-1 expression and increasing hepatic macrophage infiltration as well as promoting NASH development in murine models and in vitro cellular experiments.

RESULTS

CD36 deletion promoted the development of NASH in mouse livers.

Age and weight matched wide-type (WT) mice and CD36 knockout ($CD36^{-/-}$) mice were fed with a normal chow diet (NCD) or high fat diet (HFD) for 14 weeks. Data of liver sections showed that there was substantially more ballooning degeneration, inflammatory infiltration (hematoxylin &eosin staining, HE), lipid deposits (Oil Red O staining, ORO) and fibrosis (Sirius Red staining, SR) in $CD36^{-/-}$ mouse livers than in WT mouse livers in both the NCD and HFD groups (figure 1A). The mRNA expression of cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin -6 (IL-6) (figure 1B), TG content (figure 1C), mRNA of fibrosis markers including collagen I (col I) and collagen IV (col IV) (figure 1D) in $CD36^{-/-}$ mouse livers was much higher than in WT mouse livers, in both the NCD and HFD groups. These data indicated that the deletion of CD36 in mice promoted liver inflammation and fibrosis, which contributed to the development of NASH, regardless of the diet.

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In contrast, results from cell culture experiments were markedly different from the in vivo data. The mRNA levels of cytokines (TNF-α, IL-1β, IL-6) in both the primary hepatocytes and liver macrophages were decreased in the $CD36^{-/-}$ group compared to WT group in the absence and presence of palmitate (PA) (figure 1E). HepG2 cells and THP-1 cells were transfected with negative control (NCi) or CD36 siRNA (CD36i) and treated with or without PA for 24 hours. PA treatment promoted the mRNA expression of cytokines (TNF-α, IL-1β, IL-6), which was attenuated by CD36 RNAi, both in HepG2 cells and THP-1 cells (figure 1F). These results demonstrated that a deficiency of CD36 in cultured hepatocytes or macrophages alleviated PA induced inflammation.

Increased macrophage infiltration is responsible for the elevated inflammation and fibrosis of $CD36^{-/-}$ mouse livers.

The contrary results from liver and cultured cells indicated that the likely reason for elevated inflammation in CD36 deficient livers was due to the interaction between hepatocytes and macrophages. The immunohistochemistry staining showed that there were many more F4/80 (marker of macrophages) positive cells in $CD36^{-/-}$ mouse livers (figure 2A) than the WT mouse livers, in the absence and presence of HFD. The "crown-like" structure (indicated by the arrow in figure 2A), which was considered a common histological feature for steatohepatitis, was increased in the $CD36^{-/-}$ mouse livers compared to the WT mouse livers. The mRNA expression of F4/80 was also increased in livers of $CD36^{-/-}$ mice (figure 2A).

We then administered gadolinium chloride ($GdCl_3$), which inhibits macrophage migration to the liver, to mice that were fed with HFD for 10 weeks. $GdCl_3$ is an earth metal salt that is widely used as an in vivo macrophage selective inhibitor (1) and has been shown to depress macrophage numbers and phagocytic activity in the livers of experimental animals (17,30). Interestingly, $GdCl_3$ administration decreased macrophage infiltration (F4/80 staining and F4/80 mRNA levels), cytokines expression (mRNA of TNF-α, IL-1β and IL-6), and fibrosis (SR staining and mRNA levels of col I, col IV) in $CD36^{-/-}$ mouse livers compared to WT mouse livers, whereas the lipid deposit (ORO staining and TG level) was still higher in $CD36^{-/-}$ mouse livers than that in WT mouse livers (figure 2B). These results indicated that the inhibition of hepatic

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macrophage migration could alleviate the liver inflammation and fibrosis that was induced by CD36 deletion in vivo but it was not able to improve the steatosis in CD36$^{-/-}$ mouse livers.

In an in vitro co-culture system, increasing macrophage migration induced by CD36 deletion in hepatocytes accounts for an increase in cytokine expression.

Next, we set up a co-culture system of HepG2 and PMA differentiated THP-1 cells in transwell chambers to study two cell interactions without (-PA) or with palmitate treatment (+PA). Treatment of PA increased THP-1 migration (figure 3A: I vs V, II vs VI, III vs VII, IV vs VIII), which may be due to increased release of various monocytes/ macrophages chemoattracting agents (e.g. MCP-1, MIP-1, MIP-2 and nucleotides) in palmitate-treated cells (14,34). Interestingly, in the absence and presence of PA, CD36 RNAi in THP-1 cells decreased THP-1 migration in the co-culture system (figure 3A III vs I, VII vs V), whereas CD36 RNAi in HepG2 increased THP-1 migration in the co-culture system (figure 3A II vs I, VI vs V). CD36 RNAi in HepG2 overrode the suppression caused by CD36 RNAi in THP-1 (figure 3A IV vs I, VIII vs V) suggesting that the overall biological effect of CD36 knock-down in both hepatocytes and THP-1 was to increase macrophage migration. The same conclusion could be obtained from co-culture experiments of primary hepatocytes and liver macrophages isolated from WT and CD36$^{-/-}$ mice livers (figure S1).

We then cultured HepG2 cells with different amounts of THP-1 cells, and both were transfected with CD36 siRNA to mimic the situation of increasing macrophage infiltration in CD36-deficient livers. The concentrations of cytokines in the supernatant of the co-culture system increased as the amounts of THP-1 cells in the upper chamber increased (figure 3B). This suggested that the overall effect of CD36 deficiency is an inflammatory response due to large numbers of macrophage migration. Even the inflammatory responses of each cell (HepG2 and THP-1 cells) were diminished in CD36 deficiency conditions, as shown in figure 1F.

Elevated MCP-1 expression in CD36-deficient hepatocytes should be responsible for the increased macrophage infiltration induced by CD36 deletion.

We tested chemokine expression in different models. We found that the levels of

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chemokines (including MCP-1, MIP-1 and MIP-2) were higher in CD36$^{-/-}$ mouse livers than in WT mouse livers, regardless of the diets administered to the mice (figure 4A). After suppression of macrophage migration by $GdCl_3$, MIP-1 and MIP-2 mRNA in the liver decreased, whereas the MCP-1 mRNA was still higher in CD36$^{-/-}$ mouse livers than that in WT mouse livers (figure 4B). In HepG2 cells (figure 4C), MCP-1 mRNA was increased in the CD36i group compared with the NCi group in the absence or presence of PA, whereas MIP-2 mRNA was not different between the NCi and CD36i groups. In THP-1 cells (figure 4D), both the mRNA of MCP-1 and MIP-2 was decreased in the CD36i group compared to the NCi group. These in vitro results indicated that the elevated MCP-1 was specifically derived from CD36-deficient hepatocytes.

We then designed a Chromatin Immunoprecipitation (ChIP) assay to test whether the expression of CD36 affected the level of acetyl histone3 (H3) binding to MCP-1 promoters (figure 4E). In HepG2 cells, the level of acetyl H3 binding to MCP-1 promoters was increased in the CD36i group compared to the NCi group. In contrast, in THP-1 cells, the level of acetyl H3 binding to MCP-1 promoters was decreased in the CD36i group. This corresponded to the changes of MCP-1 mRNA expression, indicating that CD36 tissue deletion specifically regulated MCP-1 expression through histone acetylation.

CD36 deletion inhibited nuclear HDAC2 expression which regulated MCP-1 expression in hepatocytes but not in macrophages.

Next, we screened the expression of HDACs in mouse livers and cultured hepatocytes. HDAC2 mRNA expression decreased both in CD36$^{-/-}$ mouse livers and CD36 RNAi HepG2 cells (figure S2), indicating that increased MCP-1 expression was probably regulated by HDAC2.

We then determined the expression and distribution of HDAC2 in mouse livers and cultured cells by IHC staining. We found that in WT mouse livers, HDAC2 positive signals were located in hepatocytes, especially in hepatocellular nuclei (as indicated by the solid line arrow in figure 5A), with no signals in non-parenchymal cells (as indicated by the dashed arrow in figure 5A). The positive HDAC2 staining was decreased in the

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hepatocellular nuclei of CD36$^{-/-}$ mouse livers, indicating that CD36 deficiency attenuated the active expression of HDAC2 in hepatocytes (figure 5A). In primary cultures of mouse hepatocytes and HepG2 cells, an HDAC2 positive stain was observed in both the nuclei and cytoplasm of WT hepatocytes (figure 5B I) and in NCi HepG2 cells (figure 5C I) but was decreased in the nuclei of CD36$^{-/-}$ hepatocytes (figure 5B II) and CD36i HepG2 cells (figure 5C II). However, in mouse liver macrophages and THP-1 cells, the HDAC2 staining was very weak in the nuclei of all groups, including WT and CD36$^{-/-}$ liver macrophages (figure 5B III, IV), NCi and CD36i THP-1 cells (figure 5C III, IV), indicating that the levels of HDAC2 in macrophages were too low to be altered by CD36 deficiency. These observations were also confirmed by the results of Western blotting for nuclear HDAC2 in HepG2 and THP1 cells (figure 5D and figure S3).

Next, we utilized the HDAC inhibitor TSA and HDAC2 RNAi (HDAC2i) to down-regulate HDAC2 activities in HepG2 and THP-1 cells. In HepG2 cells, MCP-1 mRNA was increased in the TSA or HDAC2i groups compared to the corresponding controls (figure 5E). In THP-1 cells, MCP-1 mRNA was not changed in the TSA or HDAC2i groups, compared to the control or NCi (figure 5F). Collectively, these results indicated that suppressed nuclear HDAC2, especially in hepatocytes, promoted MCP-1 expression.

The disturbance of ROS production in CD36-deficient hepatocytes is responsible for the suppressed nuclear HDAC2 and increased expression of MCP-1.

Because HDAC activity was reported to be closely related to intracellular ROS levels, we examined H_2O_2 levels in the liver tissue and the ROS content of cultured HepG2 cells. The H_2O_2 level in mouse livers in the HFD group was much higher than that in the NCD group. However, the H_2O_2 level in CD36$^{-/-}$ mouse livers was much lower than that in WT mouse livers in both the NCD and HFD groups, indicating that CD36 deletion attenuated H_2O_2 production in the liver (figure 6A). In HepG2 cells, the ROS content was elevated in cells that were treated with PA and was decreased in CD36i HepG2 compared with NCi HepG2 (figure 6B).

We then treated HepG2 cells with the supplement of a low concentration (50μmol/L) of H_2O_2. Under the treatment of H_2O_2, nuclear HDAC2 staining appeared again in CD36i HepG2 cells (figure 6CII). Moreover, with the supplement of H_2O_2, MCP-1 mRNA expression was no longer increased in CD36i HepG2 cells (figure 6D). ChIP assays showed that level of acetyl H3 binding to MCP-1 promoters was clearly decreased, which was consistent with increased level of HDAC2 binding to MCP-1 promoters, after treatment with 50μM H_2O_2 in CD36 RNAi HepG2 cells (figure 6E). Furthermore, CD36RNAi in HepG2 cells no longer promoted differentiated THP1 cells migration with the supplement of 50μM H_2O_2 (figure 6F). All these results suggested that CD36 deficiency promotes MCP-1 expression by inhibiting ROS production and HDAC2 and that maintaining a balance of hepatic ROS could prevent macrophage migration induced by CD36-deficiency.

DISCUSSION

Liver lobules are formed by parenchymal cells, such as hepatocytes, and non-parenchymal cells, including Kupffer cells, sinusoidal endothelial cells, and stellate cells. In NASH, the defining pathological element is hepatocellular injury, as evidenced by ballooning, Mallory bodies and apoptosis. Hepatocytes are considered to be a major source of the inflammatory response in NASH-affected liver (13). In addition to hepatocytes, activated Kupffer cells can launch a biochemical attack and initiate interactions with hepatocytes and other liver cells by releasing a variety of biologically active mediators including cytokines, chemokines, eicosanoids, proteolytic enzymes, ROS, and nitric oxide (3). An enlarged Kupffer cell pool, which is usually expanded by the migrated monocytes/macrophages from circulation, is believed to contribute to the onset of NASH by interacting with hepatocytes (24). However, the role of hepatocytes on macrophage migration remains unclear.

In this study, we demonstrated that CD36 deletion attenuated the expression of inflammatory cytokines both in hepatocytes and macrophages when they were cultured alone. However, when hepatocytes and macrophages were together in liver tissue or in an in vitro co-culture system, CD36 deletion could not attenuate but instead

promoted cytokine secretion as a consequence of increased macrophage infiltration. CD36 deletion in hepatocytes alone or both in hepatocytes and macrophages increased macrophage migration and inflammatory cytokine secretion in a co-culture system of hepatocytes and macrophages. Targeted inhibition of macrophage infiltration into the liver by $GdCl_3$ administration effectively relieved the increased hepatic inflammation and fibrosis induced by CD36 deletion. These results suggest that CD36 deletion could induce an increased macrophage pool in the liver and increase the interaction between hepatocytes and macrophages. This could completely compensate for the decrease in cytokine secretion by CD36 deletion in hepatocytes and macrophages. In addition, $CD36^{-/-}$ decreased fatty acid up-take, but $CD36^{-/-}$ did not reduce lipid accumulation in mouse livers, probably because of a deficiency in very low-density lipoprotein (VLDL) excretion by hepatocytes (31). The overall effect of CD36 deficiency promotes hepatic inflammatory response, lipid accumulation and liver fibrosis.

The role of CD36 in the regulation of macrophage migration has been studied extensively in models of arthrosclerosis; however, the results are controversial (20). A study from Harb (16) and Kuchibhotla (25) has suggested that CD36 expression promotes macrophage migration, whereas the results from Park (33) suggest that the engagement of CD36 by ox-LDL inhibits macrophage migration. One potential explanation for these apparently conflicting results is that different ligands binding to CD36 may promote different intracellular signaling pathways related to cellular migration and inflammation. Other than the recognition of different ligands, CD36 is also expressed in different cells and tissues. In this study, we first demonstrated that CD36 deletion in hepatocytes promoted macrophage migration, whereas CD36 deletion in hepatic macrophages inhibited macrophage migration, indicating that the CD36 of parenchymal cells or non-parenchymal cells might play different roles in hepatic inflammation. The different regulation of macrophage migration by CD36 depends on the differential expression of MCP-1 in different cells.

MCP-1 expression can usually be stimulated by cytokines such as TNF-α (24)，but in this study, MCP-1 was elevated, whereas cytokines and other chemokines such as

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MIPs were decreased in CD36$^{-/-}$ mouse livers after the inhibition of liver macrophages. This indicates that MCP-1 in CD36$^{-/-}$ mouse livers was elevated prior to other cytokines and was derived from hepatocytes. The result was confirmed by in vitro experiments showing that MCP-1 was elevated whereas cytokines and MIPs were decreased in CD36 RNAi HepG2 cells. These changes were consistent with the results of a macrophage migration assay. Thus, this is the first demonstration that MCP-1 derived from hepatocytes plays a key role in hepatic macrophage infiltration and hepatic inflammation in CD36 deficiency models.

Furthermore, we explored the mechanism by which CD36 tissue deletion specifically induced the expression of MCP-1 in hepatocytes but not in macrophages. We demonstrated that CD36 deletion regulated MCP-1 expression at the transcriptional level by changing the acetylation of histones binding to the MCP-1 promoters. A balance of HATs and HDACs controls the histone acetylation. Approximately 18 different HDACs have been identified and grouped into 2 families and 4 classes in eukaryotic cells: Zn-dependent HDACs including HDAC1 through 11 and NAD-dependent Sirtuins (8). Many studies have shown that MCP-1 expression is regulated by HDACs. In hepatic stellate cells, HDAC1 is recruited to specific regulator regions of MCP-1 and suppress MCP-1 expression (12); HDAC3 has been reported to mediate allergic skin inflammation by regulating MCP-1 expression(23). Many HDACs are involved in the development of NASH, such as HDAC3 and SIRT1 (26). However, the regulation of MCP-1 by HDACs and CD36 deficiency remains unclear. We found that HDAC2 mRNA was decreased after screening for the expression of 11 classic HDACs in CD36$^{-/-}$ mouse livers and CD36-deficient hepatocytes. HDAC2 belongs to class I HDAC (comprisingHDAC1, HDAC2, HDAC3 and HDAC8), which is ubiquitously expressed in all tissue types (7). HDAC2, with its highly related sister protein HDAC1, is present in the mammalian nucleus as part of stable multi-protein complexes, such as Sin3A, NuRD and CoREST complexes (21). Simultaneous deletion of HDAC1 and HDAC2 in T-cells and embryonic stem cells causes a 50% decrease in total HDAC activity therefore; they are recognized as the predominant HDACs in the mammalian nucleus (11). We demonstrated that in the mouse liver, HDAC2 is primarily expressed

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in hepatocytes and only rarely in non-parenchymal cells. CD36 deletion clearly inhibited nuclear expression of HDAC2 in hepatocytes but had no impact on the expression of HDAC2 in macrophages. MCP-1 mRNA expression was promoted by the treatment of the HDAC inhibitor TSA or HDAC2 RNAi in hepatocytes rather than in macrophages. Furthermore, CD36 deletion in hepatocytes promoted MCP-1 expression by suppressing the nuclear expression of HDAC2, which does not apply to macrophages because of the extremely low expression of HDAC2.

Numerous studies have shown that HDACs are redox-sensitive. The relationship between ROS, HDAC activity, and acetylation status may depend on the intensity of ROS, endogenous HDAC activity, and the experimental cell types used (29). ROS has a bi-directional impact on the activity of HDACs: strong oxidative stress induces hypoacetylation, while weak oxidative stress induces hyperacetylation, even in the same cell line (4). A decreased oxygen environment inhibits the activities of HDACs (40).

CD36 is important to maintain intracellular ROS homeostasis, as it could modulate ROS production through mediating lipid uptake, facilitating fatty acid into mitochondria for oxidation, and activating redox signaling such as MAPK (27). In this study, we found that CD36 deletion decreased the hepatic ROS levels in vivo and in vitro. Supplementation with ROS (H_2O_2) improved the nuclear expression of HDAC2, decreased acetyl H3 binding to MCP-1 promoters, and eliminated the elevation of MCP-1 expression in CD36 deficient hepatocytes. This suggests that the decreased ROS level by CD36 deletion contributes to the suppressed nuclear HDAC2 and elevated MCP-1 in CD36 deficient hepatocytes, which promoted hepatic macrophage infiltration and the development of NASH in $CD36^{-/-}$ mice. The over-production of ROS has been regarded to be a deleterious process that induces an inflammatory response and pathological conditions. Our data suggest that decreased ROS production by CD36 deletion was also harmful for mouse livers, making them susceptible to developing NASH by suppressing the expression of HDAC2 and promoting the expression of MCP-1 in hepatocytes.

In conclusion, excess CD36 facilitates the transport by liver cells of fatty acids and

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activates inflammatory signals, thereby promoting hepatic steatosis and inflammation; whereas, deletion of CD36 induces hepatic macrophage infiltration and inflammation by increasing the expression of MCP-1 in hepatocytes because of reducing ROS production and suppressing nuclear HDAC2 (figure 7). CD36 deficiency cannot alleviate the development of NASH in mouse models. The physiological levels of CD36 in hepatocytes are important to keep a balance of ROS, macrophage migration, and the inflammatory response. Maintaining a balance of hepatic ROS and nuclear HDAC2 could be a potential new therapeutic strategy for the prevention of NASH development in CD36-deficient individuals.

INNOVATION:

It is generally acceptable that over-production of ROS has been regarded to be a deleterious process that induces an inflammatory response, causing the second hit for NASH development in the “two-hit model”. However, we demonstrated that decreased ROS production by CD36 deletion was also harmful for livers. The fine balance of CD36 plays an important role in maintaining balances of hepatic ROS and nuclear HDAC2 which could be a potential new therapeutic strategy for the prevention of NASH development.

MATERIALS AND METHODS

Animals and diets

CD36 knockout mice created on a C57BL/6J background were kindly provided by Dr. Maria Febbraio (Lerner Research Institute, U.S.). Mice were randomly fed a normal chow diet (NCD) composed of 10% kcal% fat (Research Diets Inc., NJ, U.S.) or a high fat diet with 1.25% cholesterol and 0.5% cholic acid (HFD) containing 40% kcal% fat (Research Diets Inc., NJ, U.S.) for 14 weeks before sacrifice. To inhibit the function of liver macrophages, WT and $CD36^{-/-}$ mice were administered 1% gadolinium chloride ($GdCl_3$, Sigma) solution (10 mg/kg) twice a week through intra-peritoneal injection and fed with HFD for 10 weeks before sacrifice. All animals received humane care

according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985).

Cell culture and treatments

HepG2 cells were cultured in DMEM with 10% fetal bovine serum (FBS, Equetech Bio Inc, TX, U.S.), and THP-1 cells were cultured in RPMI 1640 medium containing 10% FBS, in an incubator kept at 37˚C with5% CO_2. Before experiments, THP-1 cells were differentiated into macrophages by incubation with 200 nmol/L phorbol 12-myristate 13-acetate (PMA, Sigma) for 48 h. The preparation of primary hepatocytes and liver macrophages from mouse livers followed the protocol in the supplementary material. Before harvesting, the cells were pre-incubated for at least 12 hours in serum-free medium and then incubated for 24 hours in serum-free medium (control) or serum-free medium containing 0.16 mmol/L sodium palmitate (PA, Sigma), or 100μmol/L trichostatin A (TSA, Millipore), or 50μmol/L H_2O_2 solution (Sigma). siRNA was transfected using X-tremeGENE siRNA Transfection Reagent (Roche) according to the manufacturer's instructions.

Co-culture system and migration assay

HepG2 cells and differentiated THP-1 cells were co-cultured using 6-well transwell plate inserts with a 0.4μm porous membrane (Corning) to separate the lower chamber of the serum-starved HepG2 from the upper chamber of serum-starved THP-1. For the in vitro migration assay, transwell inserts with an 8μm porous membrane (Corning) were used. After allowing migration for 16 hours, the migratory cells on the lower side of the membrane were stained with 4,6-diamidino-2-phenylindole. The average number of migratory cells in each well was counted from four random fields under the microscope. The cytokine content in the supernatants of the co-culture system was determined by Milliplex Analyst (Millipore).

Preparation of primary hepatocytes and macrophages from mouse livers

We anesthetized 8 week old WT or $CD36^{-/-}$mice and opened the abdominal cavity. We cannulated the portal vein and immediately perfused it with modified Hanks' buffered saline. The liver was infused with a collagenase solution (100 mg collagenase in 150

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ml Hank's buffer) by a peristaltic pump at a rate of 1.5 ml/min for 20 minutes. The liver was removed and sliced into small pieces which were placed in collagenase solution and filtered with a 100 mm cell filter. The cell suspension was centrifuged at 800rpm for 5 min, and the deposits were primary hepatocytes. The supernatant was placed into another tube and centrifuged twice at 2000rpm for 5 min. The deposits were macrophages. The hepatocytes and macrophages were cultured in DMEM containing 20% FCS and maintained in an incubator at 37°C,5% CO_2.

Liver histology

Paraffin-embed liver sections(4μm) were stained with hematoxylin and eosin (HE). For immunohistochemistry, the sections were incubated in 3% hydrogen peroxide followed by 1% BSA in PBS, and then overnight (4˚C) with anti-CD36 (1:100, Santa Cruz) or anti-F4/80 (1:50, Biolegend) or anti-HDAC2(1:100, Millipore). Acommercial kit (Zsbio, China) was used to perform the histochemical reaction and was counterstained with hematoxylin. For Oil Red O staining (ORO), frozen sections were stained for 30 minutes then counterstained with hematoxylin. For Picro-Sirius Red staining (SR), sections were incubated with 0.5% Sirius Red F3B (Sigma) in saturated aqueous picric acid for at least 1 hour, then washed with 0.1% acetic acid solution 3 times. Results were examined by light microscopy.

Real time PCR

Total RNA was extracted from the liver of mice or cultured cells by RNAiso Plus reagent (Takara). By using a cDNA synthesis kit (Takara), 1.0μg of total RNA was converted to first strand complementary DNA in a 20μl reaction system. Real-time-PCR was performed in a real-time PCR machine (Bio-Rad) using SYBR Green dye. The thermal cycling program was 5 min at 95˚C for enzyme activation and 40 cycles of denaturation for 15 s at 95˚C, 15 s annealing at 55˚C and 15 s extension at 72˚C. Beta-actin was used as an internal control gene. All the primers were designed by Primer Express Software V2.0 (Applied Biosystems, UK) (See also Table S1 and S2).

Chromatin immunoprecipitation (ChIP) assay

The procedure was performed according to the manufacturer's instructions with a

Magna ChIP G kit (Millipore). The MCP-1 promoter-specific primers are shown in Supplementary Material (See Table S1).

TG content, ROS and H_2O_2 assays

These assays were performed according to the manufacturer's instructions with a TG Assay kit (Millipore) and hydrogen peroxide assay kit (Beyotime). For ROS assays, cells were incubated with DCFH for 40min and relative fluorescence unit (RFU) of DCF were measured at indicated time using an ROS assay kit (Beyotime).

Western blotting

Nuclear protein was extracted using a nuclear extraction kit (Abcam). Equal amounts of nuclear protein was resolved on SDS-PAGE and transferred to PVDF membrane (Millipore). The blots were incubated with the primary antibodies anti-HDAC2 (Millipore), anti-Lamin (Proteintech) and secondary antibodies HRP-conjugated goat anti-rabbit IgG (Zsbio). Blotted proteins were detected with the Odyssey Imaging System (LI-COR Biosciences). Quantification was performed with Image J software.

Statistical analysis

All the data were analyzed by Graph Pad Prism 5.0. A t-test was used to compare the statistical relevance of the two groups. For groups of three or more, analysis of variance (one-way ANOVA) with post test using Tukey was performed. Two-way ANOVA was used to determine the interactions between two factors. Data are expressed as the mean ± standard error of the mean (SEM). $P < 0.05$ was considered to be significant. All the data were from at least three separate experiments.

Acknowledgements

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Aurthor Disclosure Statement:

No competing financial interests exit.

Abbreviations Used:

1. CD36 = cluster of differentiation 36;
2. NASH = non-alcoholic steatohepatitis;
3. MCP-1 = monocyte chemotactic protein-1;
4. H3 = histone3;
5. HDAC = histone deacetylase;
6. ROS = reactive oxygen species;
7. NAFLD = non-alcoholic fatty liver disease;
8. TG = triglyceride;
9. ApoB = apolipoprotein B;
10. PRR = pattern recognition receptor;
11. TLR = toll-like receptor;
12. JNK = c-Jun N-terminal kinase;
13. NF-κB = nuclear factor-κB;
14. HAT = histone acetyl transferases ;
15. MAPK = mitogen-activated protein kinase;
16. FAT = fatty acid translocase;
17. WT = wide type ;
18. NCD = normal chow diet；
19. HFD = high fat diet；
20. $GdCl_3$ = gadolinium chloride；
21. FCS = fetal calf serum;
22. PMA = phorbol 12-myristate 13-acetate;
23. PA = palmitate;
24. TSA = Trichostatin A；
25. ChIP = chromatin immunoprecipitation;
26. H_2O_2 = hydrogen peroxide;

27. MIP-1 = macrophage inflammatory protein-1;
28. MIP-2 = macrophage inflammatory protein-2;
29. NC = negative control;
30. VLDL = very low-density lipoprotein

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Figure legends:

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Figure-1:

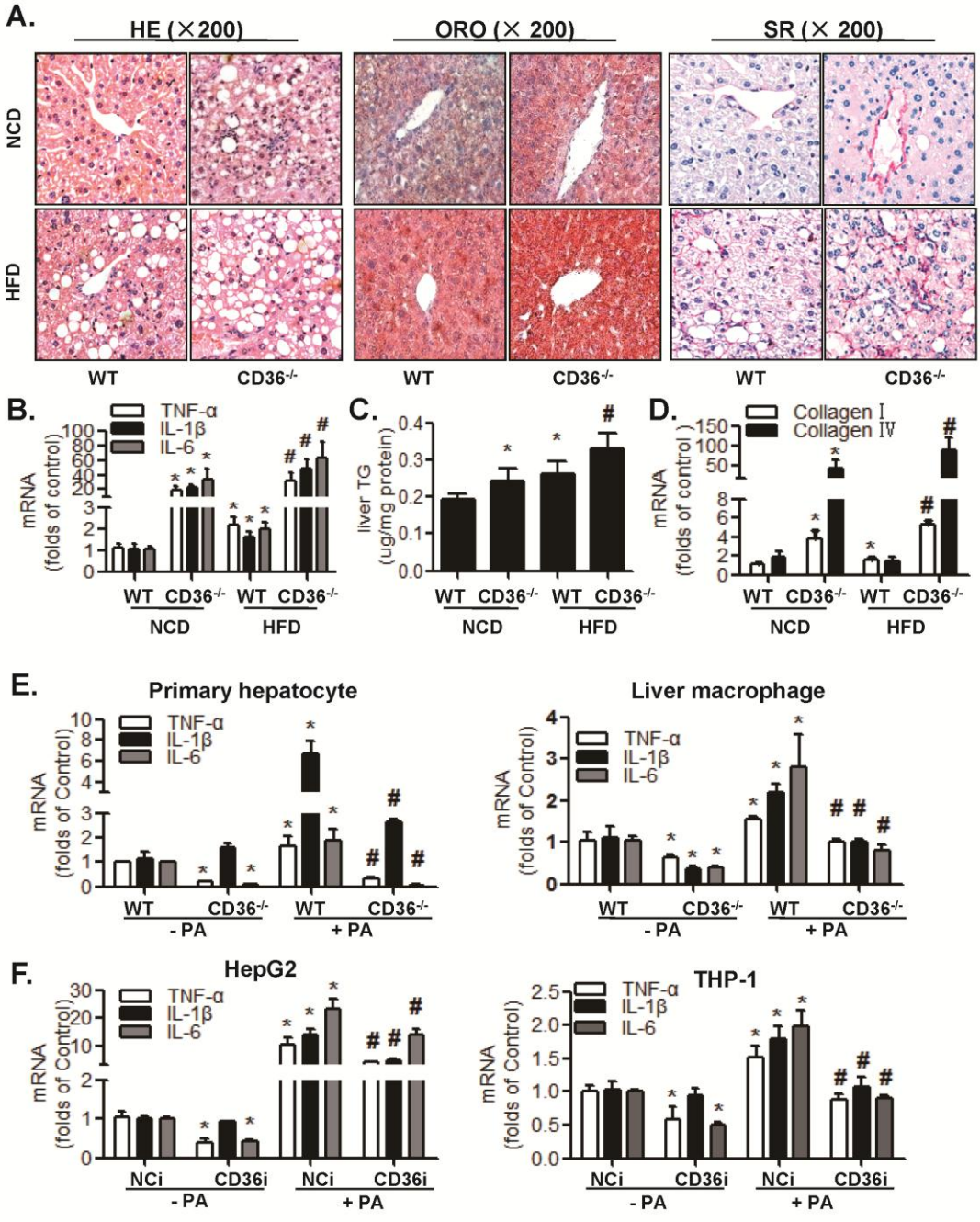

Figure1. CD36 deletion promoted the development of NASH in mouse livers.

(A) The histological changes in liver tissue were examined by hematoxylin-eosin (HE) staining. The lipid accumulation in the livers was verified by oil red O (ORO) staining. Liver fibrosis was examined by Sirius Red (SR) staining. (B) The mRNA expression of cytokines (TNF-α, IL-1β, IL-6) in the liver tissues of WT and CD36-/- mice was examined by real time PCR, n≥5, *P<0.05 versus WT in NCD, #P<0.05 versus WT in HFD. (C) Triglyceride (TG) content was determined by an ELISA kit, n≥5, *P<0.05 versus WT in NCD, #P<0.05 versus WT in HFD. (D) The mRNA expression of fibrosis markers (collagen I, collagen IV) was examined by real time PCR, n≥5, *P<0.05 versus WT in NCD, #P<0.05 versus WT in HFD. (E) The mRNA expression of cytokines in primary hepatocytes and liver macrophages from WT and CD36-/- mice treated with or without 0.16 mmol/L palmitate(PA) were examined by real time PCR, n>5, *P<0.05 versus WT -PA, #P<0.05 versus WT +PA. (F) The mRNA expression of cytokines in HepG2 and THP-1 cells transfected with NC siRNA (NCi) or CD36 siRNA (CD36i) were examined by real time PCR, n>5, *P<0.05 versus NCi -PA, #P<0.05 versus NCi +PA. To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars.

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Figure-2:

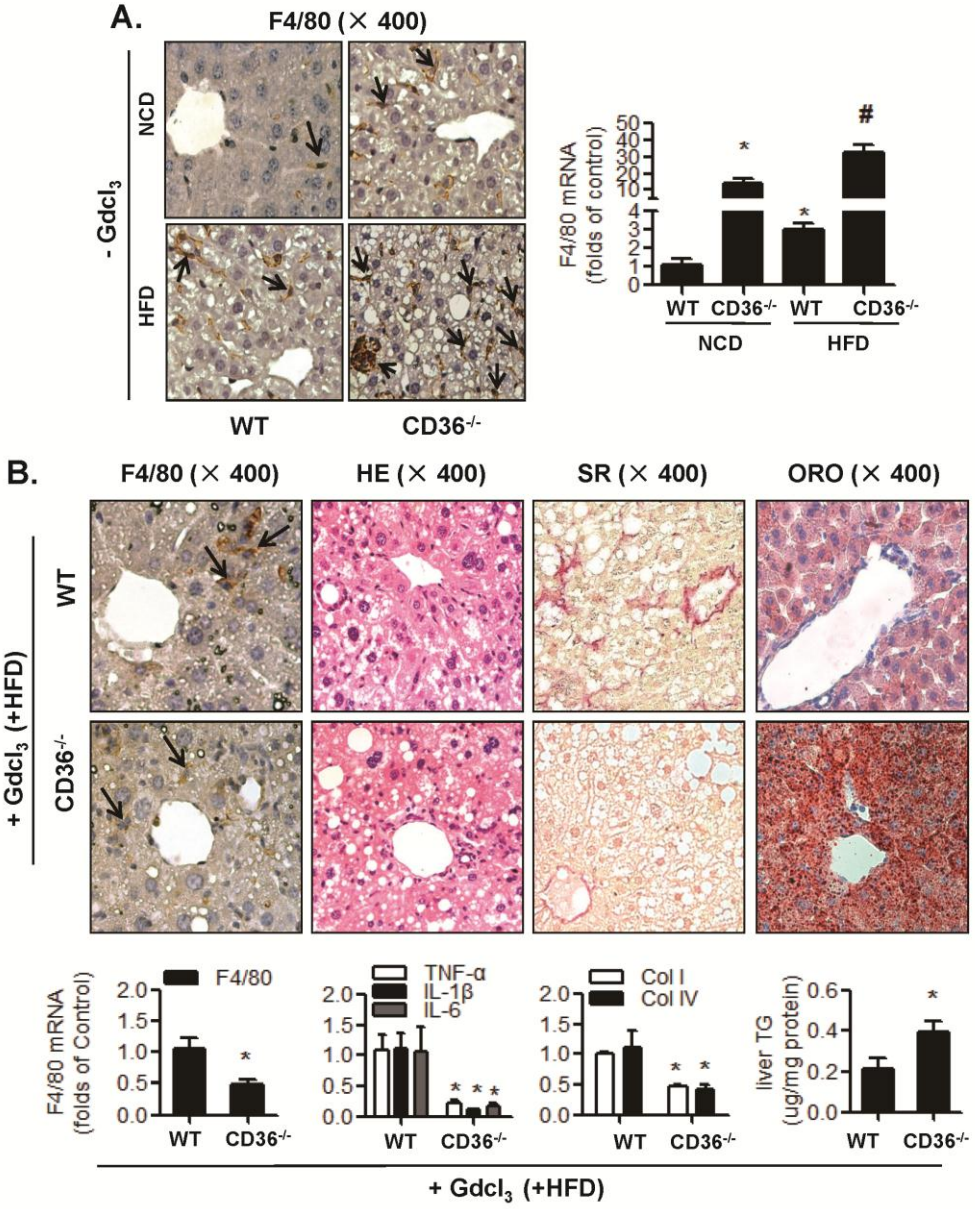

Figure2. Increased macrophage infiltration is responsible for the elevated

inflammation and fibrosis in CD36$^{-/-}$ mice livers.

(A) Immunohistochemistry staining of macrophage marker F4/80 in liver sections of WT and CD36$^{-/-}$ mice fed with NCD or HFD. And mRNA of F4/80 of these groups was tested by RT-PCR, n≥3, *P<0.05 versus WT and NCD, $^{\#}$P<0.05 versus WT and HFD. (B) Liver sections from WT and CD36$^{-/-}$ mice administered by gadolinium chloride ($GdCl_3$) and fed with HFD were stained by IHC of F4/80, HE, SR, and ORO. The mRNA expression of F4/80, cytokines and collagens was examined by real time PCR, and TG content was determined by ELISA kit, n≥5, *P<0.05 versus control (WT). To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars.

Figure-3:

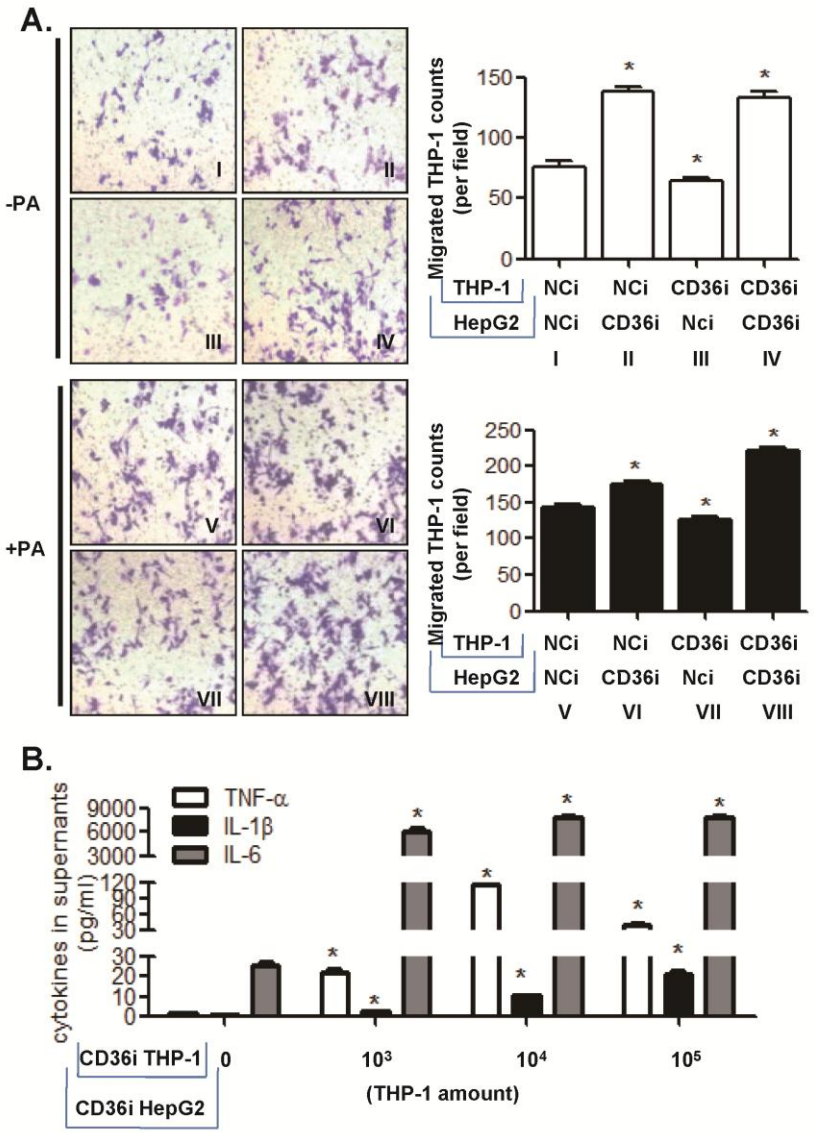

Figure3. In vitro co-culture system, increasing macrophage migration induced

by CD36 deletion in hepatocytes accounts for the increasing cytokine expression.

(A) Migrated THP-1 cells co-cultured with HepG2 cells after NCi or CD36i were stained by 4,6-diamidino-2-phenylindole and counted, *P<0.05 versus NCi HepG2 with NCi THP-1. (B) The cytokine contents in the supernatant of the co-culture system in whichCD36i HepG2 cells co-cultured with increasing amounts of CD36i THP-1 cells were determined by Milliplex Analyst, n=5, *P<0.05 versus THP-1. To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars.

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Figure-4:

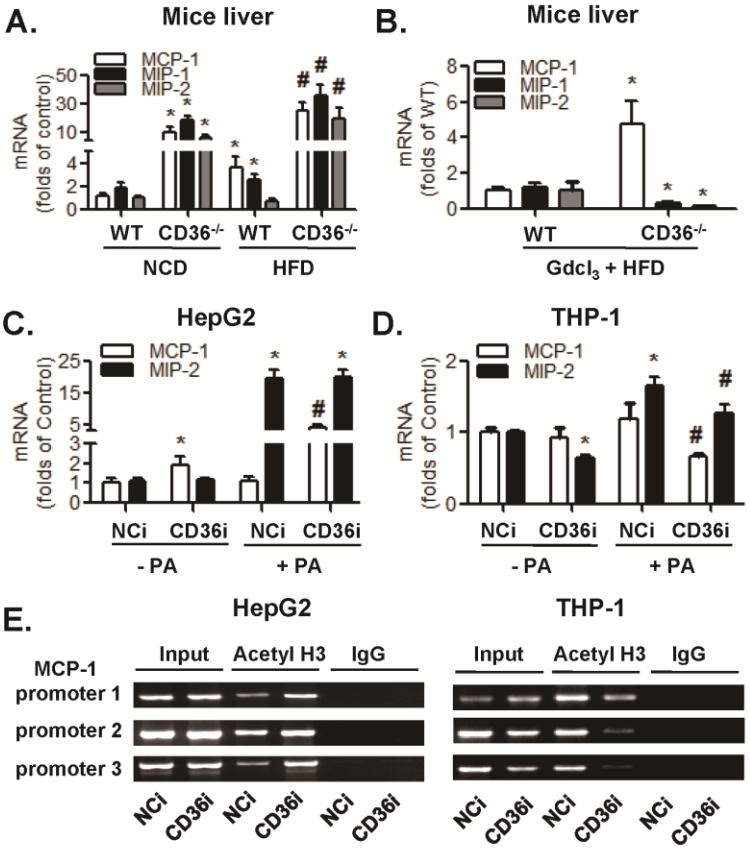

Figure4. Elevated MCP-1 expression in CD36-deficient hepatocytes could be

responsible for the increased macrophage infiltration induced by CD36 deletion.

The mRNA expression of chemokines (MCP-1, MIP-1,MIP-2) in mouse liver without Gdcl3 administration(A), mouse liver with Gdcl3 administration(B), CD36 RNAi HepG2 cells(C) and CD36 RNAi THP-1 cells(D) were measured by real time PCR, n≥5, *P<0.05 versus corresponding control(WT in NCD or NCi -PA), $^{\#}$P<0.05 versus WT in HFD or NCi +PA. (E) Chromatin immunoprecipitation (ChIP) was used to examine the levels of acetyl histones binding to MCP-1 promoters in NCi or CD36i HepG2 cells and THP-1 cells.

Figure-5:

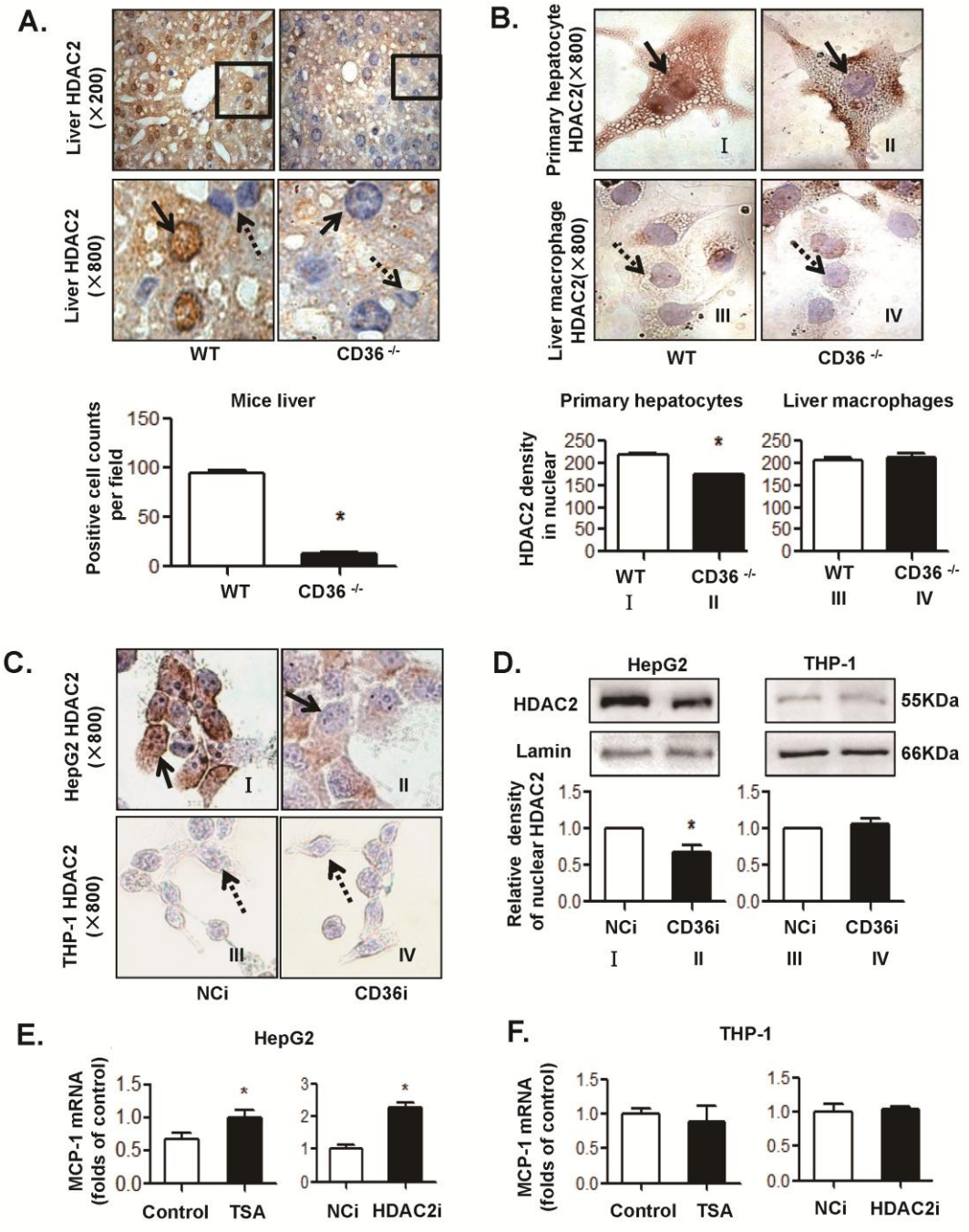

Figure5. CD36 deletion inhibited nuclear HDAC2 expression, which regulated

MCP-1 mRNA expression in hepatocytes, but not in macrophages.

Expression and distribution of HDAC2 in liver tissue (A), primary hepatocytes and liver macrophages (B), HepG2 and THP1 cells (C) were determined by IHC. Nuclear HADC2 positive cells were counted, and relative density of nuclear HDAC2 were measured by Image J, n≥5, *P<0.05 versus the corresponding controls (WT or NCi). (D) The protein expression of nuclear HDAC2 in HepG2 and THP1 cells were determined by western blotting. The mRNA expression of chemokines (MCP-1) in HepG2 cells (E) and THP-1 cells (F) treated with TSA or HDAC2 RNAi was determined by real time PCR. n≥3, *P<0.05 versus the corresponding controls (TSA=0 or NCi). To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars.

Figure-6:

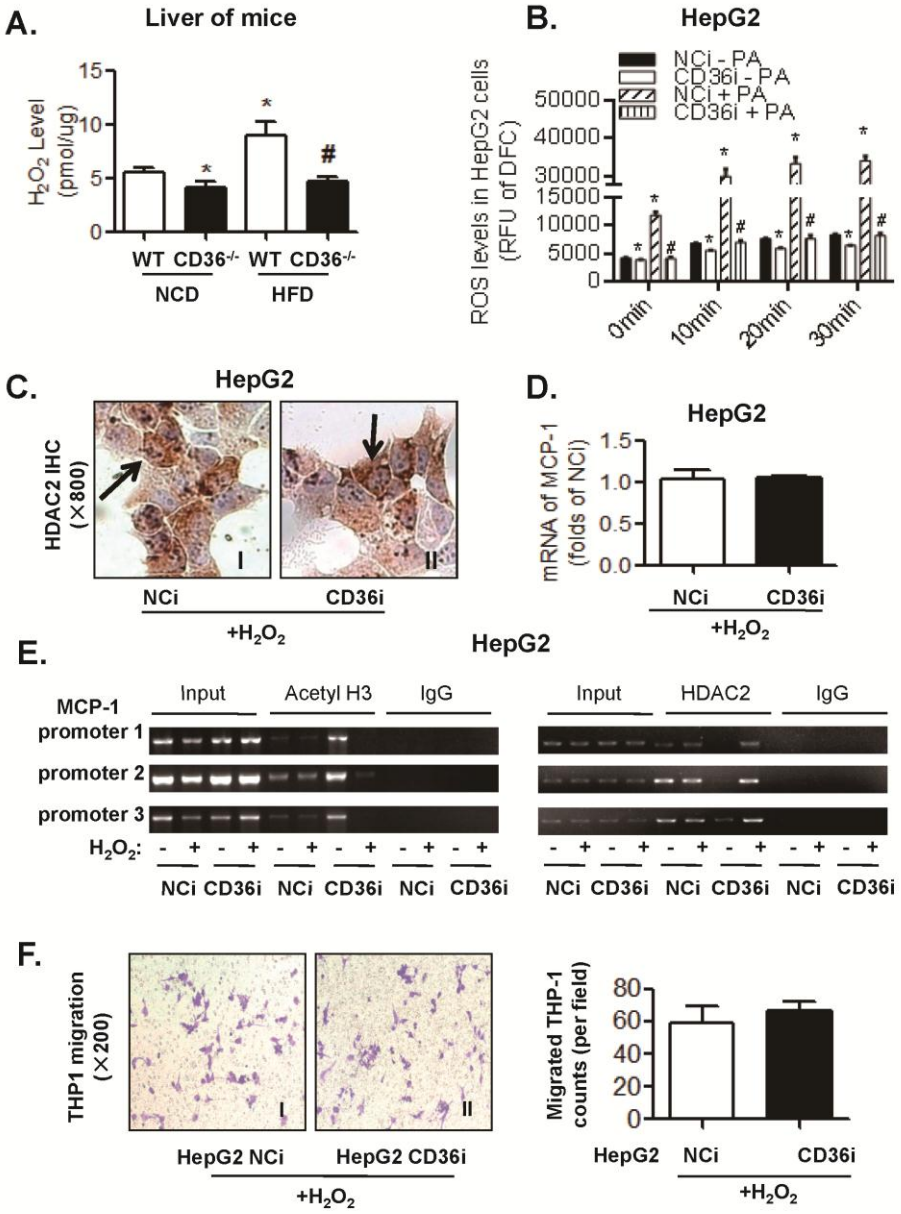

Figure6. The disturbance of ROS production in CD36-deficient hepatocytes is

responsible for the suppression of nuclear HDAC2 and the increased expression of MCP-1 mRNA.

(A) H_2O_2 content in liver tissue was verified by spectrophotometer, n=6, *P<0.05 versus the control (WT in NCD), #P<0.05 versus WT in HFD. (B) ROS in HepG2 cells was labeled by fluorescent probes and tested by spectrophotometer at indicated time, n=6, *P<0.05 versus the control (NCi,-PA), #P<0.05 versus NCi +PA. (C) HepG2 cells transfected by NC or CD36 siRNA were treated with serum-free medium containing 50μmol/L H_2O_2 for 24 hours. IHC staining of HDAC2 was performed to test the nuclear expression of HDAC2. (D) The mRNA expression of MCP-1 in HepG2 cells treated with 50μmol/L H_2O_2 were determined by real time PCR, n≥3. (E) ChIP assay of acetyl H3 and HDAC2 binding to MCP-1 promoters in HepG2 cells treated with or without H_2O_2 (50μmol/L). (F) With the supplement of H_2O_2, migrated THP-1 cells co-cultured with NCi or CD36i HepG2 cells were stained by 4,6-diamidino-2-phenylindole and counted, *P<0.05 versus NCi HepG2 with NCi THP-1. To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars.

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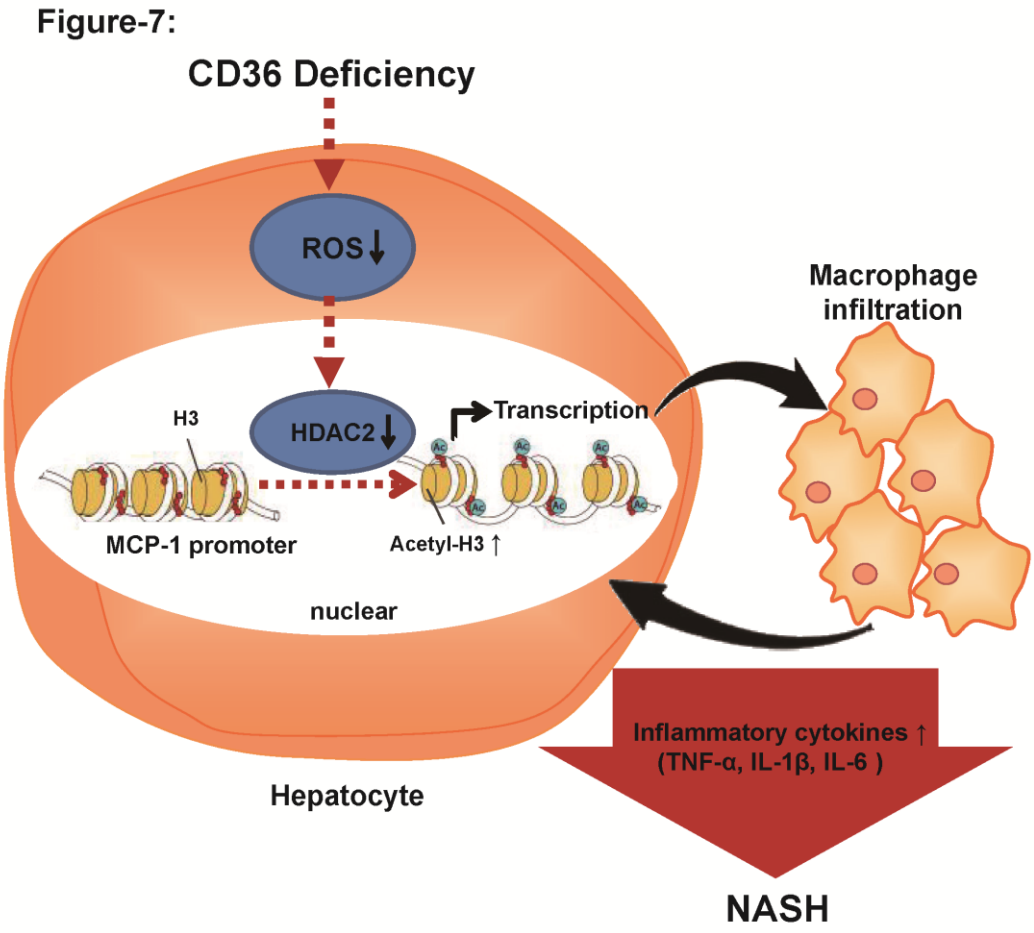

Figure7: The mechanism of CD36 deficiency promoting the development of

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NASH.

CD36 deficiency decreased ROS production in hepatocytes, which removed the inhibition of HDAC2 on the level of acetyl-H3 binding to the MCP-1 promoter, initiating the transcription of the MCP-1 gene. Sequentially the increased expression of MCP-1 from hepatocytes enhanced the infiltration of macrophages in the liver, which elevated cytokine excretion and triggered the development of NASH in CD36 deficient individuals. To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars.

Figure legends for supplementary figures:

Figure S1. Migration assay of liver macrophages co-cultured with primary hepatocytes.

Primary liver macrophages isolated from WT or CD36$^{-/-}$ mice livers were co-cultured with primary hepatocytes isolated from WT or CD36$^{-/-}$ mice livers, with or without PA treatment. The migrated macrophages were stained by 4,6-diamidino-2-phenylindole and counted at least 5 fields, *P<0.05 versus WT macrophages with WT hepatocytes.

Figure S2. mRNA levels of HDACs in mice liver and cultured cells.

mRNA levels of HDACs (HDAC1-11) in mice livers (A) and HepG2 cells (B) were examined by real time PCR, n≥5, *P<0.05 versus WT in NCD or NCi.

Figure S3. The full unedited blot of nuclear HDAC2 in HepG2 and THP1 cells.

Table S1: PCR Primers of human gene.

Gene-specific primers of human	Sequences(5′ to 3′)
Human β-actin	Forward: 5′-CCTGGCACCCAGCACAAT-3′
	Reverse: 5′-GCCGATCCACACGGAGTA-3′
Human TNF-α	Forward: 5′-AGGACCAGCTAAGAGGGAGA-3′
	Reverse: 5′-CCCGGATCATGCTTTCAGTG-3′
Human IL-1β	Forward: 5′-GGAGAATGACCTGAGCACCT-3′

	Reverse: 5′-GGAGGTGGAGAGCTTTCAGT-3′
Human IL-6	Forward: 5′-AGTCCTGATCCAGTTCCTGC-3′
	Reverse: 5′-AAGCTGCGCAGAATGAGATG-3′
Human Collagen Ⅰ	Forward: 5′-GGAAACGTAAGCGTGGTGAAA-3′
	Reverse: 5′- TGTCACACAGTTGGCAAGGAA-3′
Human Collagen Ⅳ	Forward: 5′-CTTGCCTTCCCGTATTTAGCA-3′
	Reverse: 5′-GATCTGTCGTTTCTCTGGGCATA-3′
Human MCP-1	Forward: 5′-CAAGCAGAAGTGGGTTCAGGAT-3′
	Reverse: 5′-TCTTCGGAGTTTGGGTTTGC-3′
Human MIP-2α	Forward: 5′- AAGATGCTGAAAAATGGCAAATC-3′
	Reverse: 5′- GAACAGCCACCAATAAGCTTCCT-3′
Human MCP-1 promoter 1	Forward: 5′- ACCCTCATTTTCCCCATA-3′
	Reverse: 5′- AGGCCATCTCACCTCATC-3′
Human MCP-1 promoter 2	Forward: 5′- AATGCGGTCCACCAAGTT-3′
	Reverse: 5′- AATGGCTGGGCTGAGTTT-3′
Human MCP-1 promoter 3	Forward: 5′- CTAGCAACAGCCTCCTAA-3′
	Reverse: 5′- CTGGGTTAGTCTCAGCCT-3′
Human HDAC1	Forward: 5′- CGCCAAGTGTGTGGAATTTG-3′
	Reverse: 5′- GCCTCCCAGCATCAGCATA-3′
Human HDAC2	Forward: 5′-ACATGAGCAATGCGGAGAAAT -3′
	Reverse: 5′-TCTGCCATCTTGTGGTACAGTGA -3′
Human HDAC3	Forward: 5′-CCTTTTCCAGCCGGTTATCA-3′
	Reverse: 5′- ACAATGCACGTGGGTTGGT-3′
Human HDAC4	Forward: 5′- TCAGATCGCCAACACATTCG-3′
	Reverse: 5′- ACGGGAGCGGTTCTGTTAGA-3′
Human HDAC5	Forward: 5'-TTGCCTGGGCCCTACGA-3'
	Reverse: 5'-GGGTTCAGAGGCTGTTTTGC-3'
Human HDAC6	Forward: 5′- TCGCTGCGTGTCCTTTCAG-3′
	Reverse: 5′- -GCTGTGAACCAACATCAGCTCTT -3′
Human HDAC7	Forward: 5′- CAAGAGCAAGCGAAGTGCTGTA-3′
	Reverse: 5′- TTCAGAATCACCTCCGCTAGCT-3′
Human HDAC8	Forward: 5'-CGCTGGTCCCGGTTTATATC-3'
	Reverse: 5'-TGGCCAGGGAGTCACACAT-3'
Human HDAC9	Forward: 5'-GCCCACCACACATCATTGG-3'

	Reverse: 5'-AATGTGTACTTGTAGGATGGAGATGTTC-3'
Human HDAC10	Forward: 5'-TGACCCCAGCGTCCTTTACT-3'
	Reverse: 5'-CCAGAAGCGCCCATGCT-3'
Human HDAC11	Forward: 5'-ACAACCCAGCTGTACCAGCAT-3'
	Reverse: 5'-CGCGGCGAGTACACGATT-3'

Table S2: PCR Primers of mouse gene.

Gene-specific primers of human	Sequences(5′ to 3′)
Mouse β-actin	Forward: 5′-CGATGCCCTGAGGCTCTTT-3′
	Reverse: 5′-TGGATGCCACAGGATTCCAT-3′
Mouse TNF-α	Forward: 5′-CAGCCGATGGGTTGTACCTT-3′
	Reverse: 5′-GGCAGCCTTGTCCCTTGA-3′
Mouse IL-1β	Forward: 5′-ACTCATTGTGGCTGTGGAGA-3′
	Reverse: 5′-TTGTTCATCTCGGAGCCTGT-3′
Mouse IL-6	Forward: 5′- CCACGGCCTTCCCTACTTC-3′
	Reverse: 5′- TTGGGAGTGGTATCCTCTGTGA-3′
Mouse Collagen Ⅰ	Forward: 5′-CAACCTGGACGCCATCAAG-3′
	Reverse: 5′- CAGACGGCTGAGTAGGGAACA-3′
Mouse Collagen Ⅳ	Forward: 5′- CCGAGCCAGTCCATTTATAGAATG-3′
	Reverse: 5′- CAGCGAAGCCAGCCAGAA -3′
Mouse MCP-1	Forward: 5′- GTCTGTGCTGACCCCAAGAAG-3′
	Reverse: 5′- TGGTTCCGATCCAGGTTTTTA-3′
Mouse MIP-1α	Forward: 5′-CCCGAGCAACACCATGAAG-3′
	Reverse: 5′- CCACGAGCAAGAGGAGAGAGA-3′
Mouse MIP-2	Forward: 5′- TGGGCTGCTGTCCCTCAA -3′
	Reverse: 5′- CCCGGGTGCTGTTTGTTTT-3′
Mouse HDAC1	Forward: 5′- GTGGCTACACCATCCGGAAT-3′
	Reverse: 5′-GGCCACCGCTGTTTCGTA-3′
Mouse HDAC2	Forward: 5′- CGGTGTTTGATGGACTCTTTGA-3′
	Reverse: 5′- CACAGCCCCAGCAACTGAA-3′
Mouse HDAC3	Forward: 5'-CCCCGATGTGGGCAACT-3'
	Reverse: 5'-AGGCGATGAGGTTTCATTGG-3'
Mouse HDAC4	Forward: 5′- CACTGACGCTGCTAGCAATGA-3′
	Reverse: 5′- TCACACGGGCAGGATTCA-3′
Mouse HDAC5	Forward: 5'-CGCCTCCCTCCTACAAATTG-3'
	Reverse: 5'-GGAAAGTCATCACGGCTGTCA-3'
Mouse HDAC6	Forward: 5'-GACAGCGAAAGAGTAGGCACAA-3'
	Reverse: 5'-AGGTGGCGCTGGATTCC-3'
Mouse HDAC7	Forward: 5'-GCCTACCCTGACGGCTATCC-3'
	Reverse: 5'-CCTGGTCCCCTACCCAGATC-3'

Mouse HDAC8	Forward: 5'-CGGCCAGACCGCAATG-3'
	Reverse: 5'-TCCCTTTGATGTAGTTGAGGATTTG-3'
Mouse HDAC9	Forward: 5'-CAAATGAAGCTGACGCAAATG-3'
	Reverse: 5'-CATCGCAGCATCTGATTGGA-3'
Mouse HDAC10	Forward: 5'-GGCTGCTCCTCCACCATAATAA-3'
	Reverse: 5'-GCCCACCTTGACCGATTTC-3'
Mouse HDAC11	Forward: 5'-TGGGCATGAGCGAGACTTC-3'
	Reverse: 5'-GCGGTTGTAAACATCCATGATG-3'