

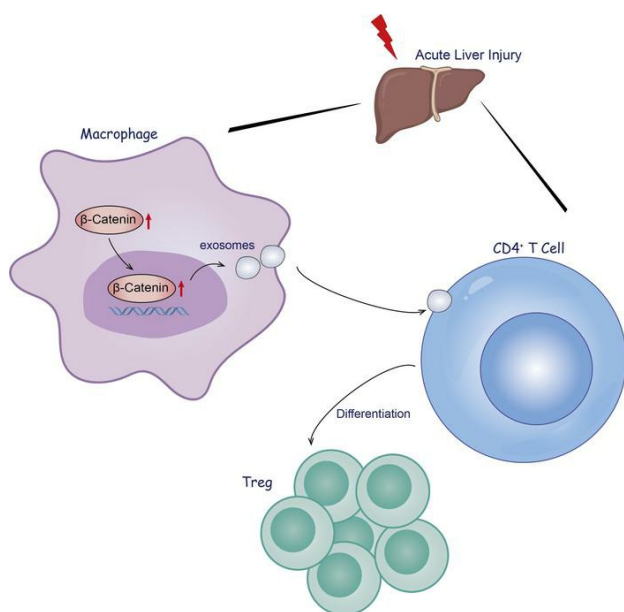
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Graphical abstract



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β -catenin disruption decreases macrophage exosomal α -SNAP and impedes Treg differentiation in acute liver injury

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Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; IL-10, interleukin-10; IL-2, interleukin-2; APAP, acetaminophen; LPS, lipopolysaccharide; D-GalN, D-galactosamine; Treg, regulatory T cells; BMDM, bone marrow-derived macrophages; α -SNAP, alpha soluble NSF attachment protein; *Napa*, NSF attachment protein alpha.

Summary

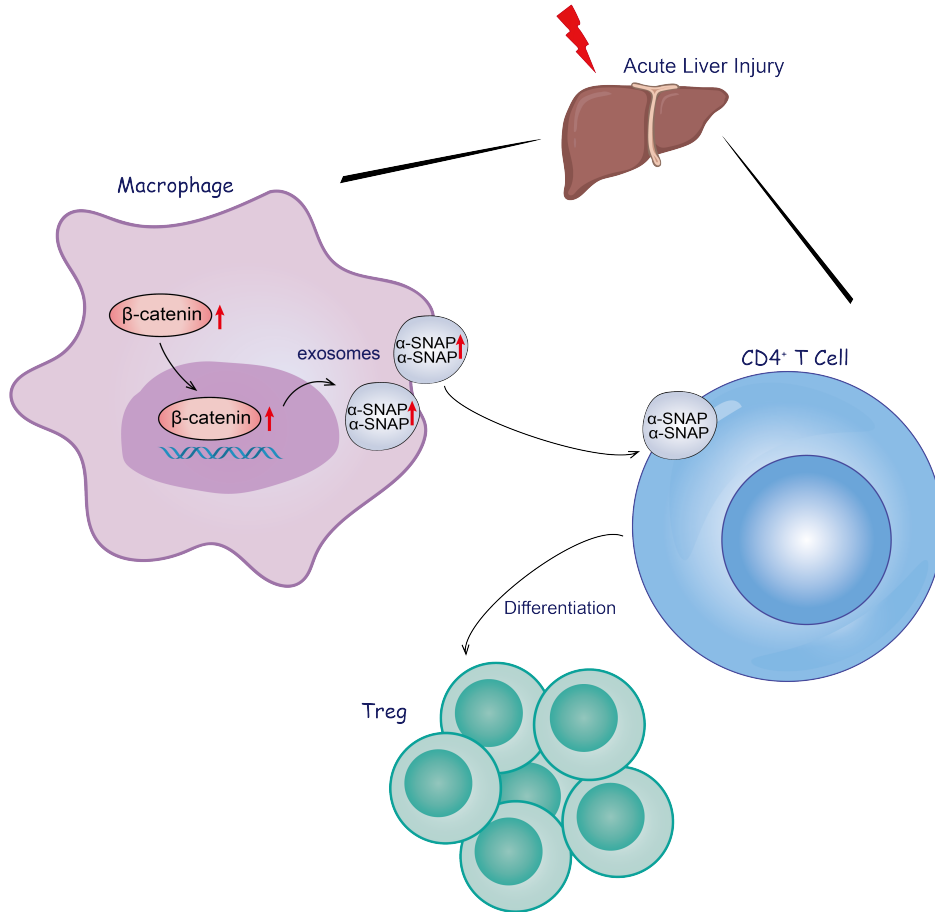
APAP or D-GalN/LPS induced β -catenin activation in infiltrating macrophages. Furthermore, β -catenin activation regulates the exosomal α -SNAP level of macrophages, thereby influencing Treg differentiation during acute liver injury.

Abstract

Hepatic macrophages and regulatory T cells (Tregs) play an important role in the maintenance of liver immune homeostasis, but the mechanism by which hepatic macrophages regulate Tregs in acute liver injury remains largely unknown. Here, we found that the hepatic Treg proportion and β -catenin expression in hepatic macrophages were associated with acetaminophen (APAP) and D-galactosamine (D-GalN)/lipopolysaccharide (LPS)-induced acute liver injury. Interestingly, β -catenin was markedly upregulated only in infiltrating macrophages, but not in resident Kupffer cells. Myeloid-specific β -catenin knockout mice showed an increased inflammatory cell infiltration and hepatocyte apoptosis. Moreover, myeloid β -catenin deficiency decreased the hepatic Treg proportion in the injured liver. Mechanistically, in vitro co-culture experiments revealed that macrophage β -catenin modulated its exosome composition, and influenced Treg differentiation. Using mass spectrometry-based proteomics, we identified that macrophage β -catenin activation increased the level of exosomal α -SNAP, which in turn promoted Treg differentiation. Overall, our findings demonstrated a molecular mechanism that macrophage β -catenin regulated the Treg proportion in the liver by enhancing the expression of exosomal α -SNAP, providing insights into the pathophysiology of acute liver injury.

Keywords: β -catenin; Treg; macrophage; exosome; acute liver injury

Graphical Abstract.



Schematic illustration of macrophage β -catenin in the regulation of hepatic Tregs in acute liver injury. APAP or D-GalN/LPS induced β -catenin activation in infiltrating macrophages. Furthermore, β -catenin activation regulates the exosomal α -SNAP level of macrophages, thereby influencing Treg differentiation during acute liver injury.

Introduction

Liver injury and acute liver failure, commonly caused by drug overdose such as acetaminophen (APAP) or by bacterial endotoxin/lipopolysaccharide (LPS), is a significant clinical problem in most countries (1, 2). During the past decades, it is well documented that liver macrophages play central roles in initiating, perpetuating and even restricting inflammation in the liver (3). Evidence from mouse models and early clinical studies support the notion that pathogenic macrophage subsets can be potentially translated into novel therapeutic strategies for patients with liver disease (3). However, developing macrophage-targeted therapies against liver diseases faces some challenges. Among them, the main challenge is macrophage heterogeneity and context-dependent functions. Liver macrophages can be divided into resident macrophages (called Kupffer cells) and infiltrating macrophages (called monocyte-derived macrophages), which have highly specialized functions in liver disease (4). Our recent studies also indicated that targeting macrophages could alleviate liver damage during acute liver injury (5-8). However, due to the high plasticity of macrophages in response to their microenvironment, the exact mechanism of macrophage function in various liver diseases is not fully understood. Therefore, further detailed understanding of the distinct pathogenic involvement of liver macrophages may open new perspectives for the treatment of liver diseases.

Regulatory T cells (Tregs) as a distinct lineage of CD4⁺ T lymphocytes, are also essential for the maintenance of immune homeostasis in the liver (9, 10). The primary function of Tregs is immune suppression, which by secreting IL-10, TGF- β , and other cytokines. Deficiency or disruption of Tregs was reported to result in autoimmune and inflammatory diseases in both humans and animals (11, 12). In addition, Tregs have been shown to be an important factor in the attenuation of liver injury (13). Indeed, Tregs depletion augmented pro-inflammatory cytokines, and exacerbated liver injury, while adoptive transfer of Tregs ameliorated APAP-induced acute liver injury (14).

Although previous reports have shown the regulatory role of liver macrophages on Tregs (15), the mechanism by which macrophages interact with Tregs in acute liver injury have not been fully understood. Exosomes are small, single-membrane, secreted organelles of ~30 to ~200 nm in diameter that have the same topology as the cell and are enriched in selected proteins, lipids, nucleic acids, and glycoconjugates (16). As extracellular carriers, exosomes are important mediators of intercellular communication and play an important role in many aspects of human health and disease, including development, immunity, tissue homeostasis, cancer, and neurodegenerative diseases (16-19). There is growing evidence that macrophage-derived exosomes participate in the regulation of various liver diseases (20, 21). Recently, exosomal long noncoding RNA (lncRNA) H19 has been shown to play a pivotal role in ConA-induced hepatitis through the HIF-1 α -p53 signaling pathway (20). In addition, previous evidence suggests that not only IL-6 could promote palmitic acid induction of miR-223 enriched exosomes in mouse and human macrophages, but also its levels correlate positively with miR-223 levels in the serum of NAFLD patients (21).

The β -catenin signaling is an evolutionary conserved pathway that is necessary for maintaining immune homeostasis (22, 23). As a core component of the cadherin protein complex, the stabilization of β -catenin is essential for the activation of β -catenin signaling (24). Additionally, β -catenin, acts as an intracellular signal transducer in the WNT signaling pathway, is expressed throughout the adult liver (25). Changes in the localization and signaling of β -catenin following liver injury (surgical resection, toxic insult, infection, metabolic insult, or tumor growth) suggest that the β -catenin signaling pathway is an attractive target for liver disease treatment (26, 27). Recently, β -catenin signaling pathway has been shown to be important in restoring tissue integrity following acute liver toxicity (28), but it remains largely unknown as to how macrophage β -catenin regulates Tregs during acute liver injury.

In this study, we identified a molecular mechanism of cell-cell communication between hepatic macrophages and Tregs during acute liver injury. We demonstrated that the hepatic Treg proportion and β -catenin expression in hepatic macrophages were

associated with APAP and D-GalN/LPS-induced acute liver injury. Importantly, using myeloid-specific β -catenin knockout (KO) mice and mass spectrometry-based proteomics, we documented that β -catenin modulated macrophage-derived exosomal α -SNAP level, which in turn influenced Treg differentiation during acute liver injury.

Results

β -catenin is upregulated in infiltrating macrophages, but not in resident Kupffer cells during acute liver injury

To investigate the involvement of β -catenin in acute liver injury, we detected the expression of β -catenin in the liver tissues following APAP and D-GalN/LPS-induced acute liver injury. As shown in Figure 1A, the mRNA levels of β -catenin in the liver tissues was markedly increased in injured livers compared to control livers. Meanwhile, we examined the protein levels of β -catenin in liver tissues by western blot. Obviously, β -catenin was phosphorylated at Ser552 in the liver after APAP or D-GalN/LPS injection, resulting in enhanced activation of hepatic β -catenin (Figure 1B). Using double immunofluorescence staining, we found that the increased expression of β -catenin was primarily localized in hepatic macrophages (Figure 1C). To explore the expression of β -catenin in different macrophage population during acute liver injury, we isolated non-parenchymal cells from healthy and injured mouse livers. Interestingly, flow cytometry analysis indicated that β -catenin was markedly upregulated only in infiltrating macrophages (marked by CD11b^{hi}F4/80^{int}), but not in resident Kupffer cells (marked by CD11b^{int}F4/80^{hi}) (Figure 1D, E). Furthermore, we found that the increased expression of active β -catenin (non-phospho Ser33/37/Thr41) was primarily localized in hepatic CD11b⁺ macrophages (Figure 1F). Consistent with these results, flow cytometry analysis showed that active β -catenin was markedly upregulated in infiltrating macrophages (marked by CD11b^{hi}F4/80^{int}) (Supplementary Figure 1). These observations suggested that β -catenin activation in hepatic infiltrating macrophages is

associated with APAP and D-GalN/LPS-induced liver damage. In addition, flow cytometry analysis revealed that the proportion of CD4⁺CD25⁺Foxp3⁺ Tregs in the liver tissue was markedly increased after APAP or D-GalN/LPS injection (Figure 1G).

Myeloid β -catenin deficiency aggravates liver injury and reduces hepatic Treg proportion

To further clarify whether β -catenin expression in hepatic macrophages is directly correlated with acute liver injury and the increased Treg proportion, we generated myeloid-specific β -catenin-deficient (β -catenin^{M-KO}) and β -catenin-proficient (β -catenin^{FL/FL}) mice and subjected them to APAP and D-GalN/LPS toxicity (Supplementary Figure 2). Compared to β -catenin^{FL/FL} mice, the livers in β -catenin^{M-KO} mice showed severe hemorrhage, swollen hepatocytes and larger necrotic area (Figure 2A), and higher serum ALT, AST levels (Figure 2B). As apoptosis/necrosis of hepatocytes is the major culprit underlying acute liver injury, we next analyzed the hepatocellular apoptosis by TUNEL staining. Livers in myeloid β -catenin deficiency mice revealed an increased proportion of apoptotic TUNEL⁺ cells compared with control livers (Figure 2C). These data were confirmed by increased activation of caspase-3 in β -catenin^{M-KO} compared with that in β -catenin^{FL/FL} mice (Figure 2D). Moreover, the β -catenin^{M-KO} mice liver was accompanied by augmented the infiltration of CD11b⁺ macrophages and MPO⁺ neutrophils (Figure 2E, F). Importantly, flow cytometry analysis showed that myeloid β -catenin disruption markedly reduced Treg proportion in acute liver injury (Figure 2G). These data demonstrated that myeloid β -catenin is essential for controlling the proportion of hepatic Tregs in APAP and D-GalN/LPS-induced liver injury.

Macrophage β -catenin disruption depresses Treg differentiation *in vitro*

Next, we further explored the association between macrophage β -catenin activation and Treg differentiation *in vitro*. BMDMs were isolated from β -catenin^{FL/FL} and β -catenin^{M-KO} mice and stimulated with LPS. Western blot analysis showed that LPS stimulation-induced β -catenin was phosphorylated at Ser552, resulting in augmented

β -catenin activation in BMDMs (Figure 3A). To test the hypothesis that macrophage β -catenin regulated Treg differentiation, we established the BMDM/naïve CD4⁺ T cell co-culture system *in vitro* (Figure 3B). We disrupted β -catenin in BMDMs using a β -catenin siRNA (Supplementary Figure 3A). Obviously, flow cytometry analysis showed that the Foxp3⁺ Treg population was reduced in response to inhibition of β -catenin in BMDMs (Figure 3C). Consistent with these results, the β -catenin^{M-KO}-BMDMs exhibited lower Foxp3⁺ Treg induction and IL-10 levels compared to β -catenin^{FL/FL}-BMDMs (Figure 3D, Supplementary Figure 4). Collectively, these data suggested that macrophage β -catenin plays a critical role in Treg differentiation.

Macrophage β -catenin regulates Treg differentiation via the exosome pathway

Given that the potent regulation of macrophage β -catenin on Treg differentiation, and that exosome plays a key role in cell-cell communication and maintenance of liver homeostasis (29, 30), we proposed that macrophage β -catenin may regulate Treg differentiation via the exosome pathway. GW4869 is a commonly used chemical inhibitor that blocks the release of exosomes. As shown in Figure 4A, GW4869 treatment in BMDMs decreased Treg proportion. Next, we isolated and characterized BMDMs-derived exosomes. The representative morphology of purified exosomes, a characteristic saucer-like shape, was observed by transmission electron microscopy (Figure 4B). In addition, nanoparticle tracking analysis (NTA) showed that the exosomes had an average diameter ranging from 50 to 150 nm (Figure 4C). Western blotting confirmed that exosomes expressed and enriched the known exosome markers TSG101, CD9 and CD63 (Figure 4D). To determine whether CD4⁺ T cells can take up BMDMs-secreted exosomes, we used red fluorescent dye PKH26 to label the BMDMs-derived exosomes and then added them into the culture medium of naïve CD4⁺ T cells. Confocal microscopy analysis showed that BMDMs-derived exosomes were taken up by naïve CD4⁺ T cells after 8 h of co-culture (Figure 4E, Supplementary Figure 5). Furthermore, to determine whether macrophage β -catenin influenced exosome-mediated Treg differentiation, we incubated naïve CD4⁺ T cells with exosomes isolated from β -catenin^{M-KO} and β -catenin^{FL/FL}-BMDMs. As expected, flow

cytometry analysis revealed that β -catenin^{M-KO}-BMDM exosomes exhibited lower induction of Foxp3⁺ Tregs compared to β -catenin^{FL/FL}-BMDM exosomes (Figure 4F). Taken together, these results suggested that macrophage β -catenin modulates its exosomes and influences Treg differentiation.

Macrophage β -catenin deletion decreases exosomal α -SNAP and impedes Treg differentiation

To explore the potential mechanism by which macrophage-derived exosomes regulate Treg differentiation, a proteomic analysis that assessed the protein composition of the exosomes isolated from BMDMs was performed. We conducted volcano plot and heatmap analysis to visualize the patterns of differential protein expression, which clearly delineated distinct expression profiles between the β -catenin^{FL/FL}-exosomes and β -catenin^{M-KO}-exosomes (Figure 5A, B). Among these differentially expressed proteins, α -SNAP was reported to be essential for Treg differentiation (31). Next, we validated the expression of *Napa* in the livers, which is coding for α -SNAP. qRT-PCR analysis revealed that the expression of *Napa* in the livers were greatly increased after APAP or D-GalN/LPS injection (Figure 5C). Importantly, myeloid β -catenin deficiency decreased *Napa* expression in APAP and D-GalN/LPS-injured livers (Figure 5D). Consistently, the expression of *Napa* was markedly reduced in β -catenin^{M-KO}-BMDMs compared to β -catenin^{FL/FL}-BMDMs (Figure 5E). Moreover, we evaluated the α -SNAP protein levels in BMDM-exosomes by western blot. Consistent with these changes, the α -SNAP expression was decreased in β -catenin^{M-KO}-BMDMs-derived exosomes compared with β -catenin^{FL/FL}-BMDMs-derived exosomes (Figure 5F).

To further confirm macrophage-derived exosomal α -SNAP regulates Treg differentiation, we overexpressed α -SNAP in RAW264.7 macrophages through transfection with α -SNAP plasmid (Supplementary Figure 3B). We found that the Foxp3⁺ Treg population was markedly increased following α -SNAP overexpression (Figure 5G). To further define that macrophage β -catenin modulates Treg differentiation via exosomal α -SNAP, we overexpressed α -SNAP in β -catenin^{M-KO}-BMDMs (Supplementary Figure 3C). Then, the exosomes from β -catenin^{M-KO}-

BMDMs+pCMV and β -catenin^{M-KO}-BMDMs+pCMV- α -SNAP were collected to co-culture with naïve CD4⁺ T cells. We found that α -SNAP overexpression in BMDMs reversed the reduction in Treg differentiation caused by β -catenin deficiency (Figure 5H). Similarly, the overexpression of α -SNAP in naïve CD4⁺ T cell also markedly increased the proportion of Foxp3⁺ Tregs (Figure 5I).

Recent reports have shown that NF- κ B was inhibited by α -SNAP restriction, which can cause a severe block in Foxp3⁺ T cell development in mice (31). In addition, NF- κ B has been shown to be necessary for Treg development and function (32-34). To determine whether the expression of NF- κ B p65 was affected by α -SNAP overexpression in naïve CD4⁺ T cell, we extracted the protein from T cell. As expected, α -SNAP overexpression in naïve CD4⁺ T cell markedly enhanced NF- κ B p65 expression (Figure 5J). Collectively, these results indicated that macrophage β -catenin modulates Treg differentiation via exosomal α -SNAP.

Discussion

Macrophages and Tregs are key players in initiating and shaping liver immune responses, which play central roles in injury recognition and homeostasis maintenance in acute liver injury. However, the mechanisms by which hepatic macrophages regulate Tregs in acute liver injury have not been fully elucidated. In this study, we documented that macrophage β -catenin was markedly upregulated and hepatic Treg proportion was increased in acute liver injury. Importantly, β -catenin was upregulated only in infiltrating macrophages, but not in resident Kupffer cells. Furthermore, we showed that disruption of myeloid β -catenin promoted inflammatory cell infiltration and hepatocyte apoptosis, and decreased the Treg proportion during acute liver injury. Mechanistically, macrophage β -catenin regulated Treg differentiation by modulating the expression of exosomal α -SNAP. Collectively, these findings highlight the importance of macrophage β -catenin in hepatic Treg proportion and liver inflammation, and provide evidence and

rationale for therapies by targeting β -catenin in macrophages.

Accumulating evidence demonstrates that hepatic macrophages and Tregs play an important role in modulating inflammatory liver diseases (3, 35). As the first line of defense against pathogens such as bacteria and viruses, macrophages can be roughly divided into two populations because of the heterogeneity in the liver, comprising both liver resident Kupffer cells and infiltrating macrophages with considerable functional plasticity (3, 4). Moreover, macrophages are highly plastic and adapt their phenotype according to the signals derived from the hepatic microenvironment, which explains their manifold and even opposing functions during progression and regression of injury (3). Previous studies showed that depletion of either or both populations of liver macrophages could delay liver repair and histological recovery in acute liver injury (36). However, it has also been reported that infiltrating macrophages and resident Kupffer cells display different ontogeny and functions in acute liver injury (37). Indeed, Kupffer cells are reduced in APAP-induced liver injury, in contrast, infiltrating macrophages become the predominant macrophage subset at the necroinflammatory phase (24 h postchallenge) (37, 38). In keeping with these studies, our results showed that up-regulation of β -catenin expression was observed only in infiltrating macrophages, but not in resident Kupffer cells. These findings further support the notion that liver macrophages display a remarkable heterogeneity in response to microenvironmental signals during acute liver injury. Previous studies have demonstrated that the active macrophage was tightly connected to Treg homeostasis. Moreover, the proinflammatory macrophages have been identified to cause Th17/Treg imbalance (39). Meanwhile, the higher proportion of Tregs was found in patients with acute hepatitis A (40). Similarly, the present study indicated that hepatic Treg proportion was also increased in APAP and D-GalN/LPS-induced acute liver injury. The increased proportion of hepatic Tregs may be required for compensation of the liver to maintain essential physiological functions. Intriguingly, in this study, we demonstrated that the

increase of Treg proportion in the liver was regulated by macrophage β -catenin during acute liver injury. These findings provide a detailed emphasis on the relevance of macrophages or Tregs as an effective immunotherapeutic option for liver diseases.

Various signaling pathways have been implicated in the maintenance of liver immune homeostasis, including the β -catenin signaling. The β -catenin pathway is mostly inactive in the healthy liver, however it can be re-activated during cell renewal and regenerative processes, as well as in certain pathological conditions (41). Indeed, the expression of β -catenin in the liver is increased in acute liver injury, which is essential for liver injury repair (26, 28, 42, 43). In keeping with these results, our data revealed that the β -catenin expression in hepatic infiltrating macrophages was markedly upregulated. Additionally, the recently described role of β -catenin pathway in regulating immune cell infiltration renewed the interest, given its potential impact on responses to immunotherapy strategies (24). The pro-inflammatory macrophages could hinder hepatic Treg differentiation in the liver injury, but activation of β -catenin signals can facilitate M2 macrophage polarization (44, 45). Besides, previous studies showed that macrophage Wnt/ β -catenin signaling contributed to liver functional compensation during acute liver injury (46). Consistently, the present study demonstrated that the blockade of macrophage β -catenin decreased the hepatic Treg proportion and exacerbated APAP and D-GalN/LPS-induced liver damage, which highlighted the importance of macrophage β -catenin during acute liver injury. However, it remains unclear how APAP leads to activation of β -catenin in macrophages. It has been reported that in response to inflammatory injury, lung endothelial cells release the Wnt signaling modulator Rspodin3, which activates β -catenin signaling in lung interstitial macrophages during acute lung injury (47). Moreover, the Wnt signaling in liver endothelial cells was activated during acute liver injury, which could affect liver cell proliferation (46). These studies raise the possibility that macrophage β -catenin may be activated by the secretion of Wnt signal from liver endothelial cells during APAP-induced acute liver injury. Further work is needed to elucidate the precise mechanism of macrophage β -catenin activation during acute liver injury.

Although β -catenin was reported to play a role in macrophage migration (48), our results did show that increased CD11b⁺ macrophages and MPO⁺ neutrophils were detected in livers of β -catenin^{M-KO} mice in two independent acute liver injury models. A possible explanation is that signals derived from the hepatic microenvironment during liver injury are complex and these signals integrally regulate the recruitment of macrophages. Moreover, the previous study (49) and our current data demonstrated that myeloid specific β -catenin knockout aggravated liver inflammation and damage. In this case, the increased inflammatory cytokines and chemokines contributed to the infiltration of monocytes into the liver, which give rise to large numbers of inflammatory monocyte-derived macrophages. Additionally, macrophages, act as sentinel cells reside in tissues, initiate neutrophil recruitment by controlling and inducing various processes, such as an increase in permeability of local blood vessels and the release of chemokines (50, 51) during tissue inflammation. Moreover, our present data showed that the β -catenin^{M-KO} mice liver was accompanied by the augmented infiltration of CD11b⁺ macrophages after liver injury. Therefore, the increased MPO⁺ neutrophils in livers of β -catenin^{M-KO} mice might be mainly caused indirectly by the β -catenin disruption in macrophages.

Macrophage-derived exosomes are shown to play an important role in acute liver injury (52, 53). Our study also indicated that LPS-stimulated macrophage-derived exosomes could be taken up by naïve CD4⁺ T cells and regulated Treg differentiation. Meanwhile, GW4869 treatment decreased the proportion of Foxp3⁺ Tregs, further indicating that exosomes are the vital medium of communication between macrophages and T cells. Next, we further explore the potential mechanism of the macrophage-derived exosomes regulating the Treg differentiation by the exosomal mass spectrometry-based proteomics. Our results showed that the α -SNAP expression was markedly down-regulated after β -catenin was blocked. This finding is in agreement with a recent report demonstrated a crucial role of α -SNAP in Foxp3⁺ Treg differentiation in vivo as well as in vitro (31). Moreover, we validated the expression of α -SNAP in vivo and in vitro, and found that macrophage β -catenin specific knockout down-regulated the α -

SNAP level. NF- κ B pathway was identified as an important signaling for Foxp3⁺ T cell development regulated by α -SNAP (31). In agreement with previous findings, our results indicated that α -SNAP overexpression markedly enhanced the expression of NF- κ B p65. However, further mechanistic work is needed to fully understand whether the reduction of Tregs is the main reason for the exacerbation of acute liver injury in β -catenin^{M-KO} mice, and how macrophages β -catenin regulates the expression of exosomal α -SNAP. Previous studies from other groups and our own have demonstrated that β -catenin signaling facilitates M2 polarization both in vitro and in various in vivo models, and thus regulates the progression of various diseases including tissue inflammation, fibrosis and tumor (8, 45, 54). Meanwhile, macrophage β -catenin was shown to play an important role in regulating the NLRP3-driven inflammatory response through crosstalk with other signaling pathways (6, 49). In the current study, our findings reveal that macrophage β -catenin modulates its exosomal α -SNAP and influences Treg differentiation during acute liver injury.

Previous studies reported that monocyte-sourced exosomes from blood samples of patients activated macrophages and increased pro-inflammatory cytokines in acute lung injury (55). Similarly, Pei et al. observed a significant increase in the levels of exosomal SCIMP in bronchoalveolar lavage fluid and serum of pneumonia patients. They demonstrated that exosomal SCIMP regulated communication between macrophages and neutrophils in pneumonia (56). In addition, macrophage-derived exosomal aminopeptidase N from the plasma of patients aggravated sepsis-induced acute lung injury by regulating necroptosis of lung epithelial cells (57). While we did not directly validate the signaling pathway of macrophage-sourced exosomal α -SNAP in patients with acute liver injury, our data in vivo and in vitro demonstrate the communication between hepatic macrophages and Tregs via the exosome pathway. The aforementioned findings suggest that circulating exosomes might exert a key role in regulating cell-to-cell communication by releasing proteins, mRNAs, microRNAs (miRNAs), and long noncoding RNAs (lncRNAs).

In summary, we investigated the association between macrophage β -catenin and hepatic Treg proportion in APAP and D-GalN/LPS-induced acute liver injury, and identified a regulatory mechanism of cell-cell communication between hepatic macrophages and Tregs during acute liver injury. We demonstrated that macrophages β -catenin influenced Treg proportion by regulating the exosomal α -SNAP level. Although we cannot rule out that macrophage β -catenin activation may regulate Tregs production through other pathways, our findings add a non-negligible mechanism for the crosstalk between macrophages and CD4⁺ T cells, providing insights into the pathophysiology of acute liver injury.

Materials and Methods

Sex was not considered as a biological variable.

Animals

The floxed β -catenin (β -catenin^{FL/FL}) mice (Jackson Laboratory, Bar Harbor, ME) and the mice expressing Cre recombinase under the control of the Lysozyme 2 (Lyz2) promoter (LysM-Cre; Jackson Laboratory) were used to generate myeloid-specific β -catenin knockout (β -catenin^{M-KO}) mice. In brief, two steps were used to generate β -catenin^{M-KO} mice. First, a homozygous loxP-flanked β -catenin mouse was mated with a homozygous Lyz2-Cre mouse to generate the F1 mice that were heterozygous for a loxP-flanked β -catenin allele and heterozygous for the Lyz2-Cre. Next, these F1 mice were backcrossed to the homozygous loxP-flanked β -catenin mice, resulting in the generation of β -catenin^{M-KO} (25% of the offspring), which were homozygous for the loxP-flanked β -catenin allele and heterozygous for the Lyz2-Cre allele (Supplementary Figure. 2). Mouse genotyping was performed by using a standard protocol with primers described in the JAX genotyping protocols database. Animals at 6–8 weeks of age were used in all experiments. The mice were bred in a standard environment with a 12h light/dark cycle.

Mouse Acute Liver Injury Model

APAP-induced acute liver injury: APAP solution was freshly prepared for each experiment by dissolving APAP in PBS at a concentration of 10 mg/ml and warmed to 40 °C. Mice were fasted for 14 h and then either given PBS (i.p.) or APAP (300 mg/kg, i.p.). Mice were sacrificed for collecting serum and liver tissues at 24 h after APAP injection.

D-GalN/LPS-induced acute liver injury: The mice were fasted for 14 h and then either injected intraperitoneally with a dose of 30 µg/kg LPS plus 600 mg/kg D-GalN. Mice were sacrificed for collecting serum and liver tissues at 5 h after D-GalN/LPS injection.

Histology, Immunohistochemistry, and Immunofluorescence Staining

Fresh liver tissues were fixed in 4% paraformaldehyde and embedded in paraffin, and sectioned into 5 µm sections. Liver sections were stained with hematoxylin and eosin (H&E). Liver monocyte and neutrophil infiltration were detected by immunohistochemistry staining, using primary rabbit anti-mouse CD11b Ab (ab133357, Abcam, MA, USA) and primary rabbit anti-mouse MPO Ab (14569S, Cell Signaling Technology, MA, USA), respectively. Cleaved caspase-3 was detected using primary rabbit anti-mouse cleaved caspase-3 Ab (9662S, Cell Signaling Technology). The secondary biotinylated goat Anti-Rabbit IgG (5220-0336, SeraCare, Shanghai, China) was used for immunohistochemistry staining. The average number of positive cells was quantified by analyzing at least 10 random high-power fields per animal, with Image-Pro Plus software.

CD68 and β-catenin double-positive macrophages were identified by primary rabbit antimouse CD68 (ab125212, Abcam) and rabbit monoclonal antibody β-catenin (8480, Cell Signaling Technology), respectively. CD11b and active β-catenin double-positive macrophages were identified respectively by primary rabbit antimouse CD11b (AF6396, Beyotime, Shanghai, China) and rabbit monoclonal antibody active β-catenin (non-phospho Ser33/37/Thr41) (8814, Cell Signaling Technology). Images were captured using an inverted Olympus fluorescence microscope.

Hepatocellular Function Assay

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, which are an indicator of hepatocellular injury, were measured by an automated chemical analyzer (Olympus Automated Chemistry Analyzer AU5400, Tokyo, Japan).

TUNEL Assay

The apoptosis of hepatocytes was identified with an apoptosis detection kit (S7110, EMD Millipore Co, Merck KGaA, Darmstadt, Germany). Cells with nuclear-positive staining by fluorescent antibodies to detect the DNA fragmentation characteristic of apoptosis in formalin-fixed paraffin-embedded liver sections. The TUNEL-positive cells were visually identified by fluorescence microscopy.

Flow Cytometry Analysis

We initially anesthetized mice with 0.1 mL 1% pentobarbital to prepare non-parenchymal liver cells. Then 20 mL pre-infusion solution (D-Hanks solution containing 0.5% phosphatase inhibitor) and 20 mL post-infusion solution (Hanks solution containing 0.5% phosphatase inhibitor, 0.002% DNase I, 0.05% collagenase IV, 1% BSA) were perfused from the inferior vena cava to liver, respectively. The flow rate was 5 mL/min. Perfused livers were dissected and teased through 200-mesh filter screen. The hepatocytes were removed by centrifuging at $50 \times g$ for 5 min. The collected supernatant was centrifuged at $400 \times g$ for 5 min and red blood cell lysis buffer was used to lysed red blood cells, then, non-parenchymal cells were obtained by centrifugation at $400 \times g$ for 5 min again.

For analysis of the mice regulatory T-cell population in the liver, non-parenchymal cells were incubated with fluorescein isothiocyanate (FITC)/anti-CD3 (11-0032-82, eBioscience, Chicago, USA), allophycocyanin (APC)/anti-CD4 (17-0041-82, eBioscience) and phycoerythrin-cyanine7 (PE-Cy7)/anti-CD25 (25-0251082, eBioscience) antibodies for 30 min at 4°C in the dark. After extensive washing, cells were fixed and permeabilized with Foxp3 Fixation/Permeabilization working solution according to the manufacturer's instruction (00-5523-00, eBioscience) and incubated

with phycoerythrin (PE)/anti-Foxp3 antibody (12-4771-82, eBioscience) for 30 min at RT in the dark.

To detect the expression of β -catenin/active β -catenin (non-phospho Ser33/37/Thr41) in hepatic macrophages, non-parenchymal cells were incubated with fluorescein isothiocyanate (FITC)/anti-CD11b (557396, BD Biosciences, New Jersey, USA) and Phycoerythrin-Cyanine7 (PE-Cy7)/anti-F4/80 (123114, Biolegend, California, USA) antibodies for 30 min at 4°C in the dark. After extensive washing, cells were fixed, permeabilized for 30 min at RT, then the cells were blocked for 30 min at RT with PBS containing 5% BSA. Then the cells were incubated with rabbit monoclonal antibody β -catenin (8480, Cell Signaling Technology)/active β -catenin (non-phospho Ser33/37/Thr41) (8814, Cell Signaling Technology), antibody for 1 h and Goat Anti-Rabbit IgG/APC antibody (bs-0295G-APC, Bioss, Beijing, China) for 30 min at RT.

To analyze the Tregs (Foxp3⁺) proportion of naïve CD4⁺ T cells in the co-culture experiments *in vitro*, cells were stained with APC/anti-CD4 antibody for 30 min at 4°C in the dark. After extensive washing, cells were fixed and permeabilized with Foxp3 Fixation/Permeabilization working solution according to the manufacturer's instruction and incubated with PE/anti-Foxp3 antibody for 30 min at RT in the dark. Stained cells were measured at BD FACS Aria™ III flow cytometer (BD Biosciences).

Western Blot Analysis

Protein was extracted from liver tissue or cells with RIPA lysis buffer contains 1% PMSF, 1% proteinase and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, USA), then protein concentrations were determined by BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL). Equal amounts of proteins (25–50 μ g) were separated by 12% SDS-polyacrylamide gel electrophoresis and electro-transferred onto a PVDF membrane (Bio-Rad, CA, USA). The membrane was blocked with 5% dry milk and 0.1% Tween-20 TBS buffer (TBST, pH 7.4) for 1 h and incubated overnight at 4°C in primary antibodies, followed by the corresponding secondary antibodies. The used primary antibodies included β -catenin (A19657, A11932, Abclonal, Wuhan, China), β -tubulin

(10068-1-AP, Proteintech, Chicago, USA), GAPDH (AC001, ABclonal), p-β-catenin (5651, Cell Signaling Technology), β-Actin (AC038, ABclonal), TSG101 (ab125011, Abcam), CD9 (A19027, ABclonal), CD63 (ab217345, Abcam), α-SNAP (MA5-36194, Invitrogen, California, USA), NF-κB p65 (8242, Cell Signaling Technology). The membrane was imaged by ECL Senicapture gel image acquisition software (Peiqing, Shanghai, China).

Quantitative RT-PCR Analysis

Liver tissues or cell cultures were lysed with TRIzol (Invitrogen) and then RNA was purified according to the manufacturer's instructions. RNA (0.5 µg) was reverse transcribed to cDNA by the PrimeScript RT reagent kit with gDNA Eraser (Takara Biotechnology Co. Ltd, Osaka, Japan). cDNA was quantified by real-time PCR using the Hieff™ qPCR SYBR® Green Master Mix (YEASEN, Shanghai, China) and CFX 96 Detection System (Bio-Rad, Hercules, CA). Primer sequences used for the amplification of *Ctnnb1*, *Napa* and *Gapdh* are shown in Supplementary Table 1. Target gene expressions were calculated by their ratios to the housekeeping gene *Gapdh*.

Isolation of BMDMs and naïve CD4⁺ T Cells

Murine primary BMDMs were isolated, as described (5). In brief, bone marrow cells were harvested from the femurs and tibias of β-catenin^{FL/FL} and β-catenin^{M-KO} mice and cultured in DMEM supplemented with 10% FBS and 20% L929-conditioned medium.

Using EasySep™ Mouse naïve CD4⁺ T cell Isolation Kits (Stem Cell Technologies, Vancouver, Canada) to purify naïve CD4⁺ T cells from the mouse spleen (purity > 90%).

Cell Culture and Co-culture System

In the Treg differentiation assay, 3 × 10⁵ naïve CD4⁺ T cells were cocultured with 3 × 10⁵ BMDMs in RPMI 1640 complete medium containing soluble anti-CD3 (1 µg/ml) (100340, Biolegend), soluble anti-CD28 (1 µg/ml) (102116, Biolegend), and recombinant murine IL-2 (5 µg/ml) (212-12, Peprotech). Cells were harvested for Foxp3 analysis after 3 days.

To verify the role of exosomes, BMDMs were pretreated with 5 μ M GW4869 (D1692, Sigma-Aldrich, St. Louis, USA) or DMSO for 6 h. Then the pretreated BMDMs were cocultured with naïve CD4⁺ T cells for 3 days, during which GW4869 treatment was continuously performed.

For the coculture of exosomes and naïve CD4⁺ T cells, 3 \times 10⁵ naïve CD4⁺ T cells were prestimulated with soluble anti-CD3 (1 μ g/ml), soluble anti-CD28 (1 μ g/ml), and recombinant murine IL-2 (5 ng/ml) for 3 days. The BMDM-exosomes (30 μ g/ml) were added to the system, and co-culture was continued for 3 days.

Plasmid and siRNA

The pCMV- α -SNAP plasmid and empty vector were purchased from MIAOLING Biology (Wuhan, China). The oligoes were purchased from GenePharma (Shanghai, China) and the sequences of β -catenin siRNA were as follows: 5'-CCAGGUGGUAGUUAUAATT-3' (sense si β -catenin), 5'-UUUAUUAACUACCACC-UGGTT-3' (antisense si β -catenin).

***In Vitro* Transfection**

BMDMs (1 \times 10⁶/well) were transfected with the pCMV- α -SNAP plasmid or β -catenin siRNA by Lipo8000 transfection reagent (C0533, Beyotime, Shanghai, China) according to the manufacturer's instructions. After 48 h, cells were supplemented with 500 ng/mL LPS for an additional 6 h.

Exosome-labelling for Cellular Uptake

To examine the uptake of BMDM exosomes by naïve CD4⁺ T cells *in vitro*, BMDM exosomes were labeled using a PKH27 red fluorescent labeling kit (MINI26, Sigma Aldrich). The labeled BMDM exosomes were then incubated with the naïve CD4⁺ T cell at 37 °C for 8 h. The exosome-treated naïve CD4⁺ T cells were then fixed and imaged by confocal microscopy (Leica Microsystems, Wetzlar, Germany).

Isolation and Characterization of BMDM-exosomes

The concentrated medium was performed by differential centrifugation to isolate the exosomes. All centrifugations and procedures were carried out at 4°C to minimize protein degradation. Cells present in the concentrated medium were removed by low-speed centrifugation at 300 × g for 10 min. The cleared supernatant was then sequentially centrifuged at 2000 × g for 10 min and 10,000 × g for 30 min to remove any remaining cell debris/microvesicles. The solution was subsequently ultracentrifuged at 100,000 × g for 70 min to pellet exosomes. The exosomes were resuspended in PBS and stored at -80°C for further use.

The approximately 5 µL of the exosome suspension sample was applied to carbon coated copper grids for 10 min at room temperature (24°C) and negatively stained with 2% phosphotungstic acid solution (pH=6.5). The morphology of exosomes was observed by HT7700 transmission electron microscope (TEM) (Hitachi, Tokyo, Japan). Particle size distribution and concentrations were measured by nanoparticle tracking analysis (NTA) (Particle Metrix, Germany). Positive biomarkers (TSG101, CD9 and CD63) were detected by western blot.

Liquid Chromatography-Mass Spectrometry (LC/MS) Analysis

The protein extraction and LC/MS Analysis was done on protein from BMDM-exosomes by DLMBiotech (Wuhan, China). In brief, the extracted peptides in each sample were analyzed using label-free quantification on an LC-MS online with a Q Exactive Plus mass spectrometer (Thermo Scientific, USA) coupled to an EASY-nLC 1200 system (Thermo Scientific, USA). The analysis was operated in a data-dependent acquisition (DDA) mode. The full mass spectrometry survey scan resolution was set to 70000 with an automated gain control (AGC) target of 3×10^6 for a scan range of 340-1,800 m/z and a maximum injection time of 20 ms. The higher-energy collision dissociation fragmentation was performed at a normalized collision energy of 28%. The MS2 AGC target was set to 2×10^5 with a maximum injection time of 50 ms, and the dynamic exclusion was set to 35 s.

The MS/MS data was searched against the concatenated target/decoy Mouse

Uniprot (58) database as of Jan 01, 2023, with only reviewed and canonical sequences used. MaxQuant (V1.6.6) was then used to infer protein identifications. Label-free quantification and subsequent median normalization of each protein were performed using weighted spectral counting (59).

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (60) partner repository with the dataset identifier PXD051425.

Enzyme-Linked Immunosorbent Assay (ELISA)

The concentrations of IL-10 were measured with ELISA kits (RK00016, Abclonal) according to the manufacturer's instructions.

Study approval

All animal protocols were approved by the Animal Care and Use Committee of Wuhan University in China (Permit No: WP20220117). The animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Statistical Analysis

Statistical analysis was done using two-tailed Student's *t* test or one-way analysis of variance (ANOVA). Results were shown as mean \pm standard deviation; *P* values < 0.05 were considered statistically significant. All analyses were performed by Prism 7.0 (GraphPad Software).

Data Availability Statement: All nonsequencing data are contained within the Supporting Data Values XLS file. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (60) partner repository with the dataset identifier PXD051425.

Author contributions statement: RZ and YL performed *in vitro* and *in vivo* experiments, and data analysis; MZ, BL and WZ performed *in vitro* experiments; CL and HH contributed to the study concept and research design; RZ and CL wrote the manuscript. All authors contributed to the article and approved the submitted version.

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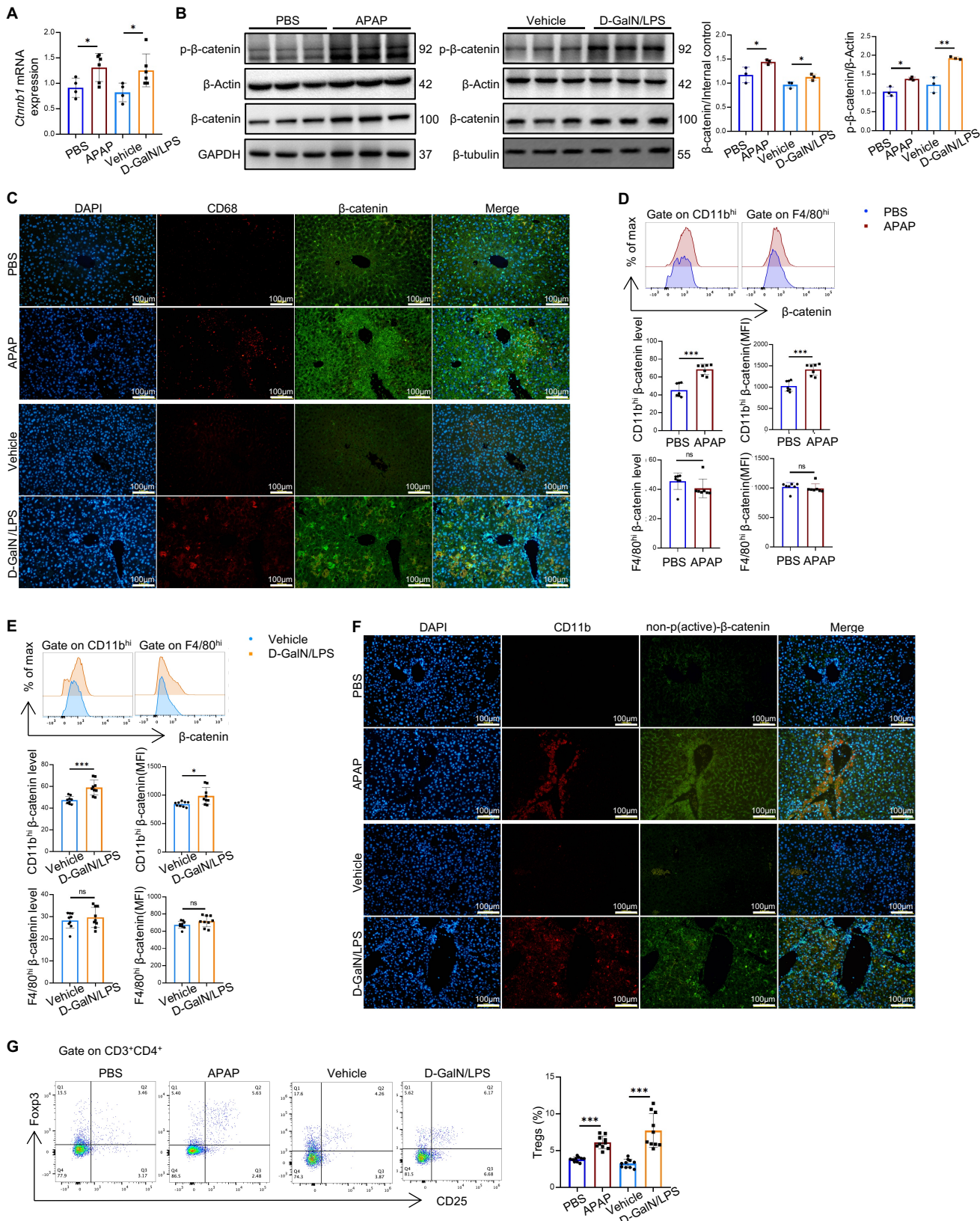


Figure 1. β -catenin is upregulated in infiltrating macrophages, but not in resident Kupffer cells

For APAP overdose-induced acute liver injury, male mice were i.p. injected with APAP (300 mg/kg BW) or same volume of PBS after overnight fasting and the mice were sacrificed 24 h after injection. For D-GalN/LPS-induced acute liver injury, male mice were intraperitoneally (i.p.) injected with 600 mg/kg BW (body weight) of D-GalN and 30 μ g/kg BW of LPS, or same volume of vehicle, and the mice were sacrificed 5 h after injection. (A) Western blot analysis total β -catenin/ p- β -catenin (Ser552) expression in the liver tissues. (B) qRT-PCR assisted detection of β -catenin in the liver tissues (n = 4-6 samples/group). (C) Immunofluorescence images of staining with antibodies against CD68 (red) and β -catenin (green). Nuclei were labeled with DAPI (blue). Scale bar, 100 μ m. (D-E) The expression of β -catenin in hepatic macrophages was examined by flow cytometry (n = 7-9). (F) Immunofluorescence images of staining with antibodies against CD11b (red) and active β -catenin (non-phospho Ser33/37/Thr41) (green). Nuclei were labeled with DAPI (blue). Scale bar, 100 μ m. (G) The proportion of hepatic CD25⁺Foxp3⁺ Tregs gated on CD3⁺CD4⁺ T cells was analyzed by flow cytometry (n = 10 samples/group). Scale bar, 100 μ m. Data are presented as individual values and represent the mean \pm SD. * P < 0.05, *** P < 0.001 versus nondiabetic controls by one-way ANOVA in A, B, G and two-tailed Student's t test in D, E.

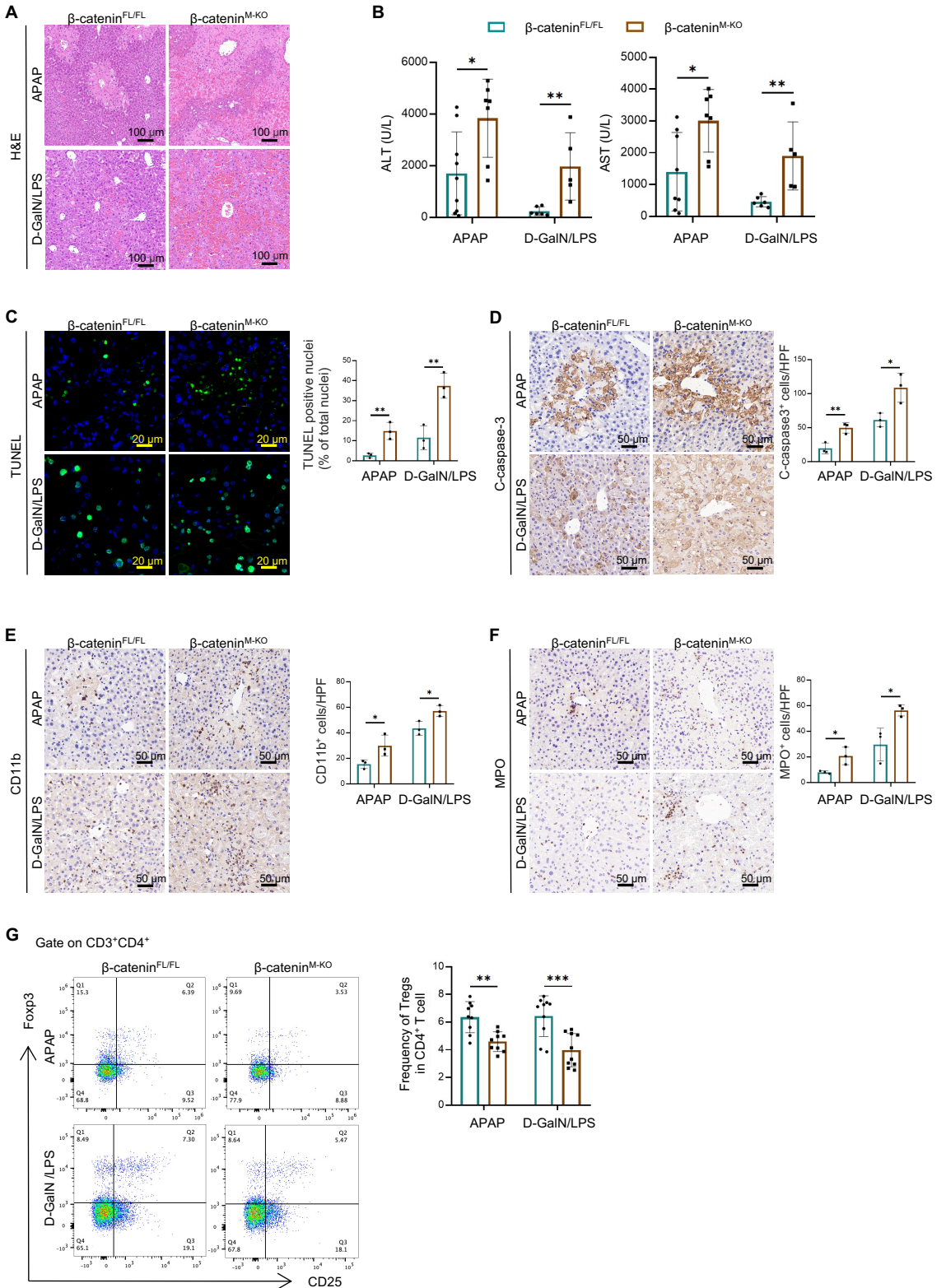


Figure 2. Myeloid β -catenin deficiency aggravates liver injury and reduces hepatic Treg proportion

(A) Representative pictures of H&E staining of liver sections. (B) Hepatocellular function, as assessed by serum ALT/AST levels (IU/L) ($n \geq 5$ samples/group). (C) Representative images of TUNEL-staining liver sections and quantification of TUNEL-positive nuclei per high-power field for at least 6 fields per sample. (D-F) Immunohistochemistry staining and quantification of cleaved caspase-3⁺, CD11b⁺ macrophages and MPO⁺ neutrophils in the livers. (G) The proportion of hepatic CD25⁺Foxp3⁺ Tregs gated on CD3⁺CD4⁺ T cells was analyzed by flow cytometry ($n = 9-10$ samples/group). Scale bars, 50/100 μm . Data are presented as individual values and represent the mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus nondiabetic controls by one-way ANOVA.

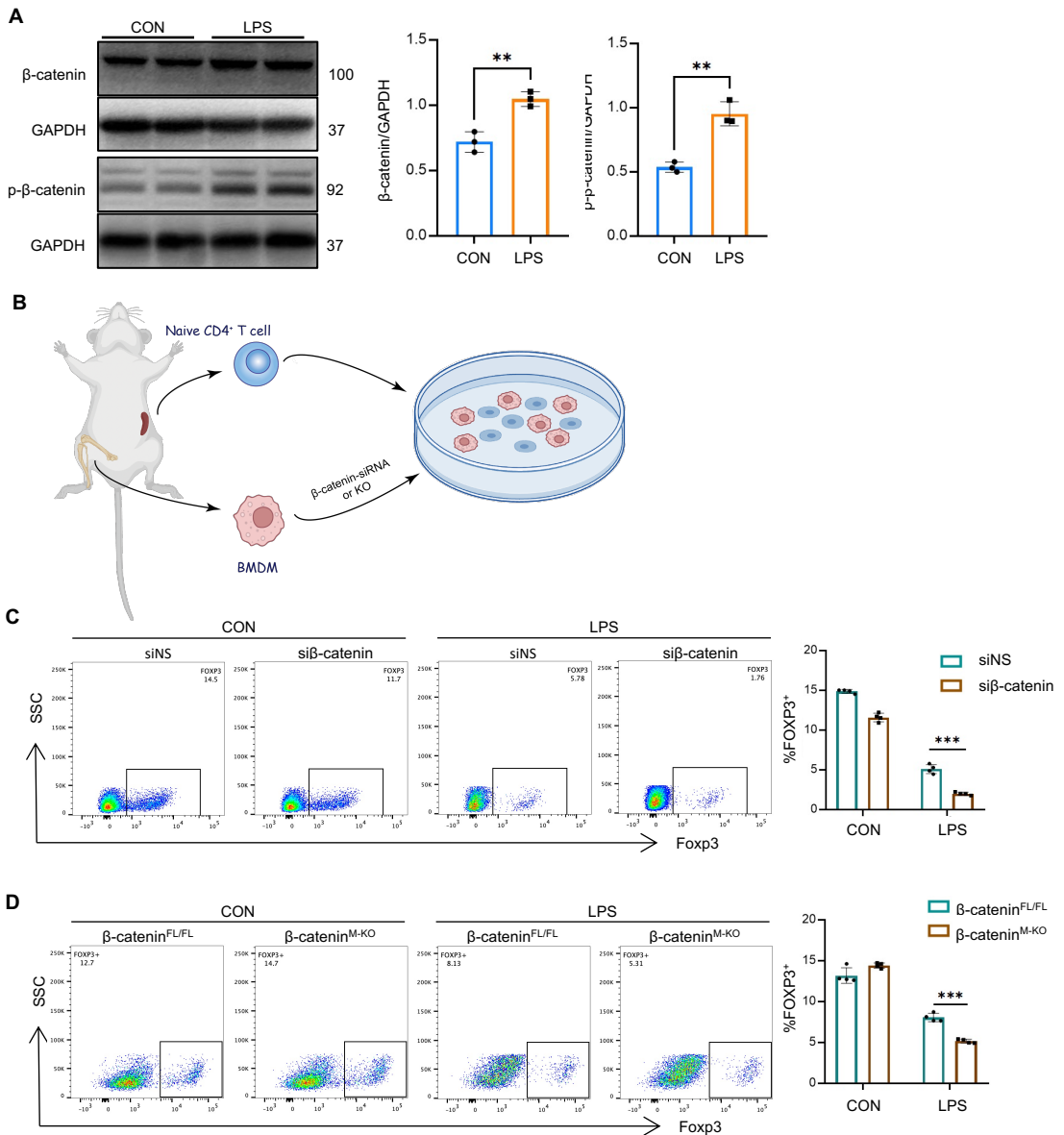


Figure 3. Macropage β -catenin disruption depresses Treg differentiation *in vitro*

(A) Western blot analysis and quantification of the expression of total β -catenin/ p- β -catenin (Ser552) in BMDMs after LPS (500 ng/mL) treatment for 6 h. (B) Co-culture diagram. (C) The si β -catenin or siNS-transfected BMDMs were stimulated with LPS (500 ng/mL) or PBS for 6 h, respectively, and then cocultured with naïve CD4⁺ T cells for 3 days, the induction of Foxp3⁺ Tregs was analyzed by flow cytometry (n = 4). (D) BMDMs from β -catenin^{FL/FL} and β -catenin^{M-KO} mice were stimulated with LPS (500 ng/mL) or PBS for 6 h, respectively, and then cocultured with naïve CD4⁺ T cells for 3 days, the induction of Foxp3⁺ Tregs was analyzed by flow cytometry (n = 4). Data are presented as individual values and represent the mean \pm SD. ** $P < 0.01$, *** $P < 0.001$ versus nondiabetic controls by two-tailed Student's *t* test in A and one-way ANOVA in C, D.

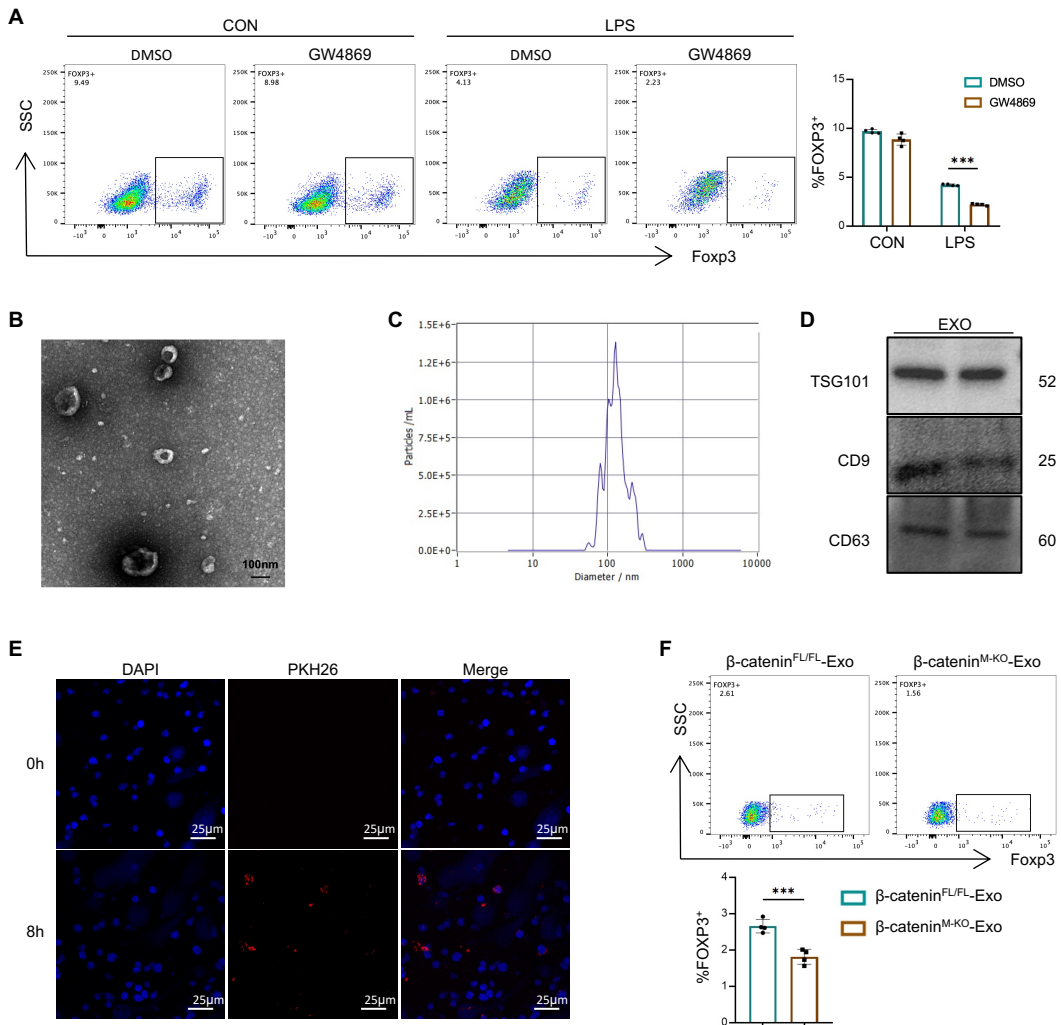
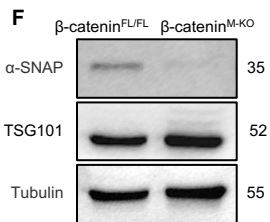
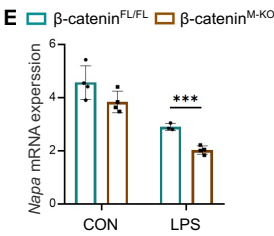
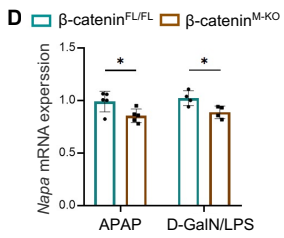
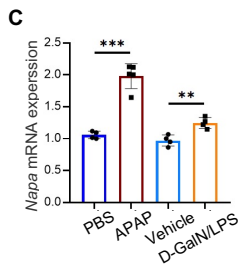
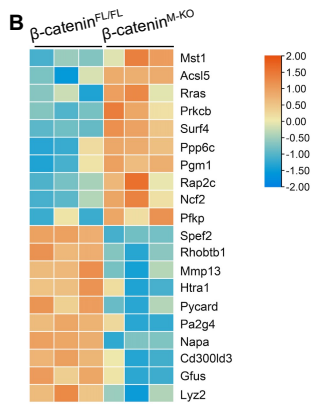
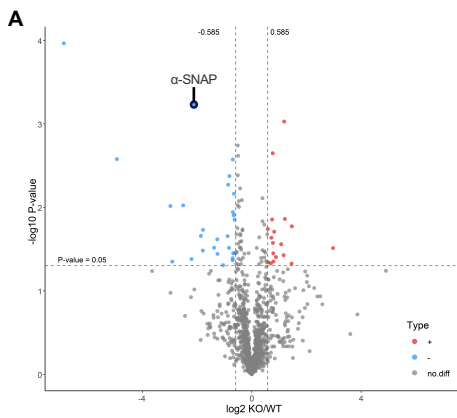
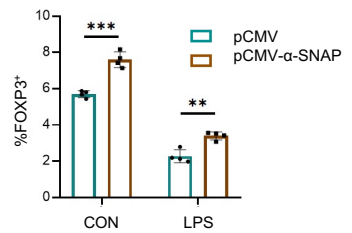
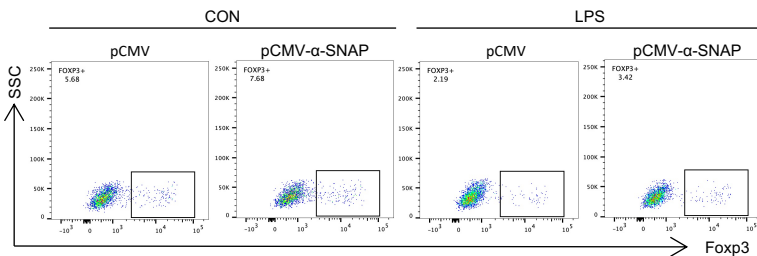


Figure 4. Macrophage β -catenin regulates Treg differentiation via the exosome pathway

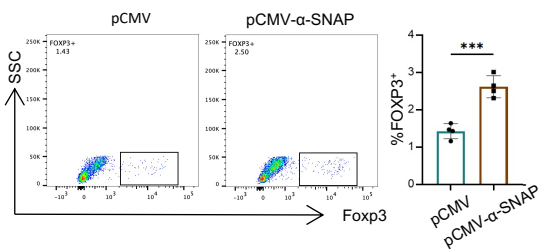
(A) Naïve CD4⁺ T cells were cocultured with BMDMs treated with GW4869 (5 μ M) for 3 days, and the induction of Foxp3⁺ Tregs was detected by flow cytometry (n = 4). (B) Exosomes isolated from BMDM supernatants (BMDM-exosomes) were analyzed by transmission electron microscopy (scale bar, 100 nm). (C) Size distribution of exosomes. (D) Western blot assay indicated the expression of TSG101, CD9 and CD63 proteins in exosomes. (E) BMDM-exosomes were labeled with PKH26 (red) and then co-cultured with naïve CD4⁺ T cells for 8 h. The T cells were collected for fluorescence confocal microscopy to detect exosomes uptake and the nuclear location was determined by DAPI (blue) staining (scale bar, 25 μ m). (F) BMDMs from β -catenin^{FL/FL} and β -catenin^{M-KO} mice were stimulated with LPS (500 ng/mL) or PBS for 6 h. Then the naïve CD4⁺ T cells were co-cultured with BMDM-exosomes for 3 days, the induction of Foxp3⁺ Tregs was analyzed by flow cytometry (n=4). Data are presented as individual values and represent the mean \pm SD. ****P* < 0.001 versus nondiabetic controls by one-way ANOVA in A and two-tailed Student's *t* test in F. Representative of three experiments.



G $\alpha\text{-SNAP}$ overexpression in RAW264.7



H $\alpha\text{-SNAP}$ overexpression in $\beta\text{-catenin}^{\text{M-KO}}$ -BMDMs



I $\alpha\text{-SNAP}$ overexpression in naïve CD4+ T cell

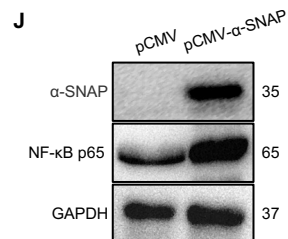
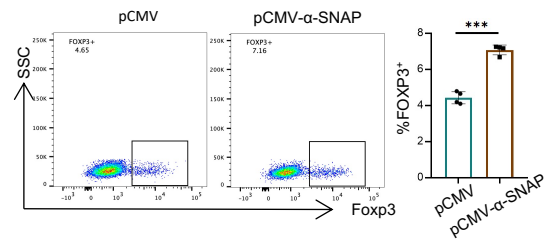
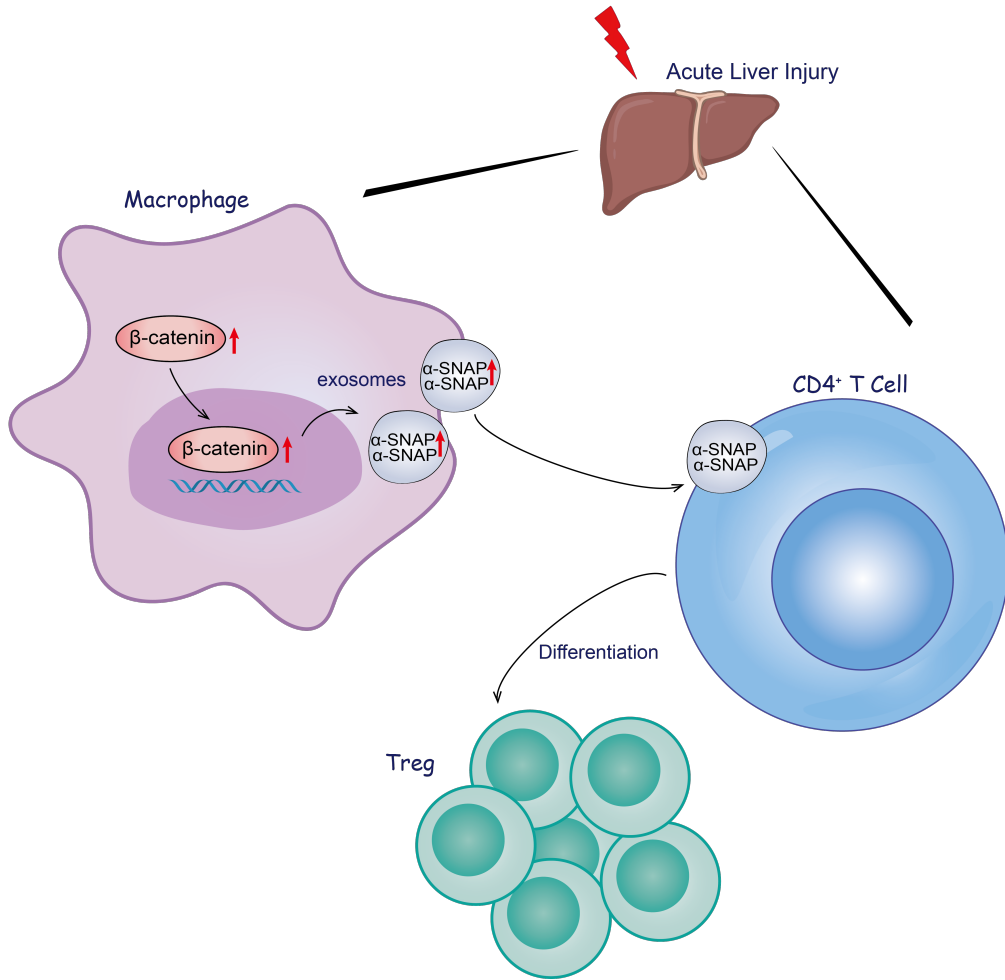


Figure 5. Macrophage β -catenin deletion decreases exosomal α -SNAP and impedes Treg differentiation

BMDMs from β -catenin^{FL/FL} and β -catenin^{M-KO} mice were stimulated with LPS (500 ng/mL) for 6 h. The protein expression profiles in BMDM-Exosomes were analyzed by LC/MS analysis (n = 3). (A) Volcano plot presented differentially expressed proteins in two groups. (B) Heatmap of proteins that differed significantly ($p < 0.05$) in two groups. (C-E) *Napa* mRNA expression in the mice liver/BMDM. (F) Western-assisted validate the expression of α -SNAP in BMDM-exosomes. (G) The pCMV- α -SNAP or pCMV transfected RAW264.7 cells were stimulated with LPS (500 ng/mL) or PBS for 6 h, respectively, and then co-cultured with naïve CD4⁺ T cells for 3 days, the induction of Foxp3⁺ Tregs was analyzed by flow cytometry (n = 4). (H) The pCMV- α -SNAP or pCMV-transfected β -catenin^{M-KO}-BMDMs were stimulated with LPS (500 ng/mL) for 6 h, then the exosomes from β -catenin^{M-KO}-BMDMs+pCMV and β -catenin^{M-KO}-BMDMs+pCMV- α -SNAP were collected to co-culture with naïve CD4⁺ T cells for 3 days. The induction of Foxp3⁺ Tregs was analyzed by flow cytometry (n = 4). (I) The induction of Foxp3⁺ Tregs was detected by flow cytometry after naïve CD4⁺ T cells transfected with pCMV- α -SNAP or pCMV (n = 4). (J) Western-assisted validate the expression of NF- κ B p65 in naïve CD4⁺ T cells transfected with with pCMV- α -SNAP or pCMV. Data are presented as individual values and represent the mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus nondiabetic controls by one-way ANOVA in C, D, E, G and two-tailed Student's *t* test in H, I. Representative of three experiments.



Graphical Abstract. Schematic illustration of macrophage β -catenin in the regulation of hepatic Tregs in acute liver injury

APAP or D-GalN/LPS induced β -catenin activation in infiltrating macrophages. Furthermore, β -catenin activation regulates the exosomal α -SNAP level of macrophages, thereby influencing Treg differentiation during acute liver injury.