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Intestinal IL-33 promotes microbiota-derived trimethylamine *N*-oxide synthesis and drives metabolic dysfunction–associated steatotic liver disease progression by exerting dual regulation on HIF-1α

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Abstract

Background and Aims: Gut microbiota plays a prominent role in the pathogenesis of metabolic dysfunction–associated steatotic liver disease (MASLD). IL-33 is highly expressed at mucosal barrier sites and regulates intestinal homeostasis. Herein, we aimed to investigate the role and mechanism of intestinal IL-33 in MASLD.

Approach and Results: In both humans and mice with MASLD, hepatic expression of IL-33 and its receptor suppression of tumorigenicity 2 (ST2) showed no significant change compared to controls, while serum soluble ST2 levels in humans, as well as intestinal IL-33 and ST2 expression in mice were significantly increased in MASLD. Deletion of global or intestinal IL-33 in mice alleviated metabolic disorders, inflammation, and fibrosis associated with MASLD by reducing intestinal barrier permeability and rectifying gut microbiota dysbiosis. Transplantation of gut microbiota from IL-33 deficiency mice prevented MASLD progression in wild-type mice. Moreover, IL-33 deficiency resulted in a decrease in the abundance of trimethylamine *N*-oxide–producing bacteria. Inhibition of trimethylamine *N*-oxide synthesis by 3,3-dimethyl-1-butanol mitigated hepatic oxidative stress in mice with MASLD. Nuclear IL-33 bound to hypoxia-inducible factor-1α and suppressed its activation, directly damaging the integrity of the intestinal barrier. Extracellular IL-33 destroyed the balance of intestinal Th1/Th17 and

Abbreviations: DMB, 3,3-dimethyl-1-butanol; FMO3, flavin monooxygenase 3; FMT, fecal microbiota transplantation; HFD, high-fat diet; HIF-1α, hypoxia-inducible factor-1α; IECs, intestinal epithelial cells; MASLD, metabolic dysfunction–associated steatotic liver disease; PA, palmitic acid; ST2, suppression of tumorigenicity 2; TMA, trimethylamine; TMAO, trimethylamine *N*-oxide; Tregs, regulatory T cells; WT, wild type.

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facilitated Th1 differentiation through the ST2-*Hif1a*-*Tbx21* axis. Knockout of ST2 resulted in a diminished MASLD phenotype resembling that observed in IL-33 deficiency mice.

Conclusions: Intestinal IL-33 enhanced gut microbiota–derived trimethylamine *N*-oxide synthesis and aggravated MASLD progression through dual regulation on hypoxia-inducible factor-1α. Targeting IL-33 and its associated microbiota may provide a potential therapeutic strategy for managing MASLD.

INTRODUCTION

Metabolic dysfunction–associated steatotic liver disease (MASLD), formerly known as NASLD, is a metabolic disease characterized by hepatic lipid accumulation and accompanied by cardiometabolic risk factors, which has become the most common chronic liver disease worldwide.[1] Without effective intervention, MASLD would further progress to steatohepatitis, cirrhosis, and HCC, contributing to its increasing global burden.[2] In addition to intrahepatic factors, such as lipotoxicity, oxidative stress, autophagy, and innate immune response, intestinal homeostasis is closely associated with MASLD pathogenesis.[3] Metabolism, inflammation, and fibrosis are intricately intertwined in MASLD progression; therefore, targeting only one aspect cannot effectively reverse or halt its development, making successful treatment challenging.[4]

Studies have shown that in the early stage of a fatty liver, before obvious lipid deposition occurs in the liver, there is intestinal barrier leakage and chronic inflammation present in the intestine.[5] The gut-liver axis refers to the communication between the gut and the liver, and gut dysbiosis can exacerbate liver diseases through this axis. The gut microbiota and its metabolites, such as short-chain fatty acids, bile acids, and trimethylamine *N*-oxide (TMAO), are the key participants mediating liver diseases.[6] Gut bacterial choline trimethylamine (TMA)-lyase converts choline to TMA, which is circulated to the liver where flavin monooxygenase 3 (FMO3) oxidizes TMA to TMAO, subsequently releasing into the serum.[7] Numerous animal or preclinical experiments have provided evidence that strategies based on gut microbiota regulation, including fecal microbiota transplantation (FMT), probiotics, prebiotics, and synbiotics, can restore intestinal homeostasis and effectively reduce metabolic disorders and inflammation.[8]

IL-33, a member of the IL-1 family, is highly expressed in the mucosal barrier.[9] In intestinal diseases, IL-33 plays a key role in regulating intestinal epithelial cells (IECs) and a variety of immune cells.[10,11] Moreover, intestinal IL-33 exerts remote regulatory functions through modulation of gut microbiota, thereby effectively affecting systemic physiology.[12] Studies have reported on the involvement of IL-33 in diverse liver diseases, including chronic hepatitis B, liver cirrhosis, and HCC.[13] However, the precise contribution of IL-33 in MASLD is still not fully understood. Conflicting results have been observed in studies regarding the impact of exogenous administration or endogenous deficiency of IL-33 on steatohepatitis, primarily due to the predominant focus on liver-derived IL-33.[14,15] Considering its limited expression in the liver and potential restricted effects, it is crucial to explore the role of intestine-derived IL-33 in liver diseases, particularly since chronic liver disease often coincides with intestinal homeostasis disorder.

In this study, we present the novel role of intestinal IL-33 in MASLD. Our findings suggest that intestinal IL-33 exerts a dual regulatory effect on hypoxia-inducible factor-1α (HIF-1α) in both IECs and Th1 cells, thereby exacerbating disruption of the intestinal barrier. This dysregulation further promotes gut microbiota–derived TMAO synthesis, ultimately leading to MASLD progression. Targeting IL-33 and its associated gut microbiota holds promise for ameliorating metabolic imbalances, inflammation, and fibrosis associated with MASLD.

METHODS

Patient sample collection

We enrolled 46 patients with MASLD and 38 controls in this study. The clinical features are shown in Supplemental Table S1, http://links.lww.com/HEP/I528. Patients were diagnosed with hepatic steatosis by ultrasound and/or liver histology with the presence of at least 1 of 5 cardiometabolic risk factors according to the international guidelines of MASLD.[1] Patients with evidence of secondary causes of steatosis or alternative diagnoses were excluded, including the extent of alcohol intake, hepatic steatosis due to drug or genetic factors, chronic hepatitis (hepatitis B and hepatitis C), autoimmune liver disease, and severe extrahepatic disease. The serum samples were collected from a physical examination center, and liver samples were obtained from surgical patients undergoing partial hepatectomy. The research

was conducted in accordance with both the Declarations of Helsinki and Istanbul. All participants gave written informed consent. All procedures were approved by the Clinical Trial Ethics Committee of Huazhong University of Science and Technology (2024S091).

Animals

The 6–8-week-old male C57BL/6 mice were used for all experiments. *Il33*$^{fl/fl}$ mice and Villin-Cre transgenic mice were obtained from Youdu Biosciences. Intestinal epithelial-specific IL-33 knockout mice (*Il33*$^{\Delta IEC}$) were generated by crossing *Il33*$^{fl/fl}$ mice with Villin-Cre mice, and their *Il33*$^{fl/fl}$ littermates were used as controls. Global IL-33 knockout mice (*Il33*$^{-/-}$) were kindly provided by Professor Fang Zheng from the Huazhong University of Science and Technology. Global suppression of tumorigenicity 2 (ST2) knockout mice (*Il1rl1*$^{-/-}$) was obtained from Cyagen Biosciences. Their wild-type (WT) littermates were used as controls. All animals were maintained in a specific pathogen-free environment on a standard 12-hour light-dark cycle with ad libitum access to food and water at the Animal Experiment Center of Tongji Hospital. All experiments were approved by the Tongji Hospital Animal Ethics Committee (TJH-202009004) and followed the ARRIVE guidelines. Additional information on methods can be found in Supplemental Methods and Supplemental Tables S2, S3, http://links.lww.com/HEP/I528.

RESULTS

The level of IL-33 in the intestine, instead of the liver, was elevated in MASLD

To investigate the role of IL-33 in MASLD, we measured serum levels of IL-33 and its receptor ST2 in human samples. We observed a significant increase in soluble ST2 levels in patients with MASLD compared to the controls (Figure 1A), while the levels of IL-33 were undetectable in both groups (data not shown). The binding of released IL-33 to ST2 on immune cells exerts its extracellular functions, while soluble ST2 acts as a decoy receptor to limit the excessive biological effects of IL-33.[16] Meanwhile, no significant difference was observed in hepatic IL-33 and ST2 expression between patients with MASLD and controls (Figure 1B). Next, we established a murine MASLD model and found that compared to the chow group, both mRNA and protein levels of intestinal IL-33 and ST2 (encoded by *Il1rl1*) were significantly upregulated in the high-fat diet (HFD) with 60 kcal% fat group (Figures 1C, D), while no discernible difference was observed in liver. Furthermore, immunohistochemistry revealed widespread distribution of IL-33 in the intestinal epithelium and lamina propria (Figure 1E). These data suggest that intestinal IL-33 may exert an impact on MASLD.

Deletion of intestinal IL-33 ameliorated MASLD through restoration of the impaired intestinal barrier

Considering the distinct change in intestinal IL-33 levels, we focused on the impact of IL-33 on the intestine. Upon *Il33* deletion in the intestine (Figure 2A), we observed a significant reduction in serum levels of orally administered 4 kDa fluorescein isothiocyanate-dextran and lipopolysaccharide in HFD-fed *Il33*$^{\Delta IEC}$ mice compared to HFD-fed *Il33*$^{fl/fl}$ mice (Figure 2B). Moreover, the mRNA levels of tight junction proteins, that is, *Tjp1* (encoding ZO-1) and *Ocln* (encoding occludin), were upregulated in *Il33*$^{\Delta IEC}$ mice (Figure 2C). The expression of other genes related to intestinal homeostasis, including *Muc2* and *Reg3g*, was also restored. These results indicated that deletion of intestinal IL-33 ameliorated HFD-induced intestinal barrier leakage, which was an important driver of intestinal homeostasis imbalance and MASLD progression.

We next evaluated the effect of intestinal IL-33 on MASLD-associated liver phenotypes and found that body weight, epididymal white adipose tissue weight, and inguinal white adipose tissue weight were significantly reduced in HFD-fed *Il33*$^{\Delta IEC}$ mice compared to *Il33*$^{fl/fl}$ controls (Figures 2D, E). Serum lipid levels, including triglyceride, cholesterol, and free fatty acids, were also downregulated (Figure 2F). To assess the insulin resistance condition, we performed a glucose tolerance test and insulin tolerance test, which revealed decreased blood glucose levels and AUC for insulin tolerance test in *Il33*$^{\Delta IEC}$ mice (Figure 2G). Consistently, compared with *Il33*$^{fl/fl}$ controls, HFD-fed *Il33*$^{\Delta IEC}$ mice exhibited reduced expression of genes associated with lipid synthesis (*Srebp2*) and absorption (*Cd36*), as well as increased expression of lipid consumption-related genes (*Ppara* and *Cpt1a*) (Figure 2H). Deletion of intestinal IL-33 also decreased the expression of genes associated with proinflammatory cytokines (*Tnf*, *Il1b*, and *Il6*) and fibrosis (*Acta2*, *Tgfb1*, and *Col1a1*) (Figure 2H). Furthermore, serum levels of ALT and AST were lower in HFD-fed *Il33*$^{\Delta IEC}$ mice (Figure 2I). The histological evaluation showed that knockout of intestinal IL-33 resulted in a reduction in both the size and number of lipid droplets, as well as a decrease in inflammatory cell infiltration and collagen fiber deposition in the liver (Figure 2J). In addition, we also constructed global *Il33* knockout (*Il33*$^{-/-}$) mice and found that knockout of IL-33 ameliorated MASLD progression, including decreased WAT weight, lipid levels, and hepatic enzymes (Supplemental Figures S1A–I, http://links.lww.com/HEP/I528). The data above demonstrated that intestine-derived IL-33 exacerbated hepatic metabolic disorders, inflammation, and fibrosis. Conversely, knockout of

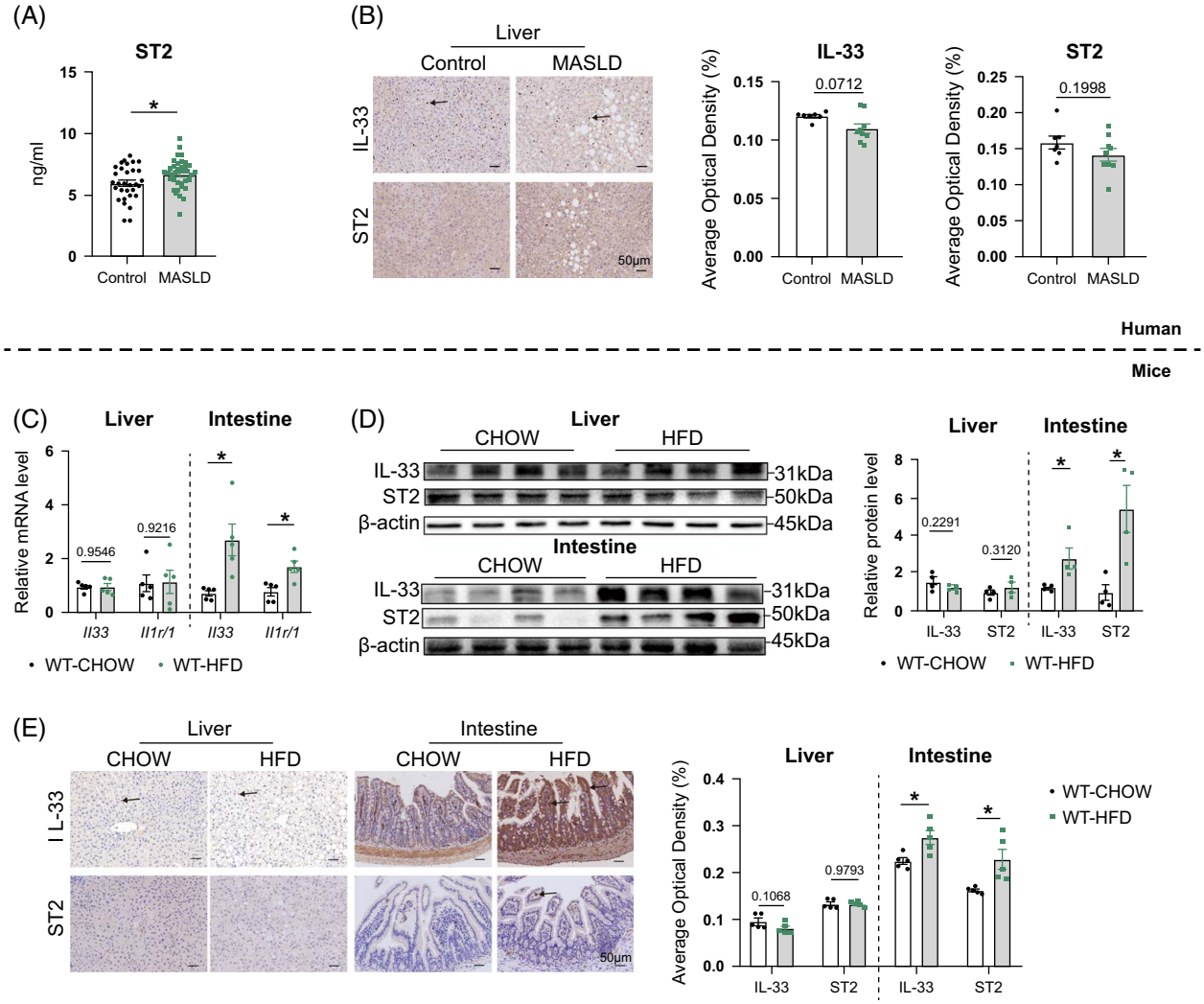

FIGURE 1 Intestinal IL-33 and ST2 were increased in MASLD. (A) Serum ST2 levels in patients with MASLD (n = 31) and controls (n = 37) detected by ELISA. (B) Hepatic IL-33 and ST2 expression in patients with MASLD (n = 7) and controls (n = 9) detected by IHC. Arrows indicate positive cells. Scale bars: 50 μm. WT mice were fed HFD or chow diet for 24 weeks. (C–E) IL-33 and ST2 (encoded by *Il1rl1*) expression in the liver and intestine detected by (C) qPCR (n = 5/group), (D) WB (n = 4/group), and (E) IHC (n = 5/group). Arrows indicate positive cells. Scale bars: 50 μm. Data are represented as mean ± SEM. Statistical differences were determined by unpaired 2-tailed *t* test or Mann-Whitney test. **p* < 0.05. Abbreviations: HFD, high-fat diet; IHC, immunohistochemistry; MASLD, metabolic dysfunction–associated steatotic liver disease; qPCR, realtime fluorescence quantitative polymerase chain reaction; ST2, suppression of tumorigenicity 2; WB, western blot; WT, wild type.

intestinal IL-33 ameliorated diet-induced MASLD phenotypes.

Gut microbiota–derived from IL-33 knockout mice attenuated MASLD progression

To identify the effect of IL-33 on intestinal homeostasis, we collected fecal samples and performed 16s rRNA sequencing to assess the change in gut microbiota (PRJNA1086241, http://www.ncbi.nlm.nih.gov/bioproject/1086241). We found that HFD induced alterations in the abundance (chao1) and the distribution (PCA) of gut microbiota in WT mice, whereas these changes were not evident between HFD-fed *Il33*$^{-/-}$ mice and WT mice (Supplemental Figure S2A, http://links.lww.com/HEP/I528). Upon further analysis, compared with WT mice, HFD-fed *Il33*$^{-/-}$ mice exhibited lower levels of *Bacteroides_ovatus*,[17] *Blautia*, *Ileibacterium*, *Alistipes*, and *Tyzzerella*,[18] which had been reported to be associated with metabolic diseases, including MASLD, atherosclerosis, and obesity (Figure 3A). *Il33*$^{-/-}$ mice exhibited an elevated relative abundance of beneficial bacteria like *Muribaculum* and *Acetatifactor*[19] while displaying reduced levels of proinflammatory bacteria including *Marvinbryantia*, *Desulfovibrio*,[20] *Allobaculum*, *Providencia*,[21] *Dorea*, *Gordonibacter*, and *Turicibacter*.[22] Therefore, the gut microbiota profiling suggested that IL-33 knockout alleviated gut microbiota dysbiosis in MASLD.

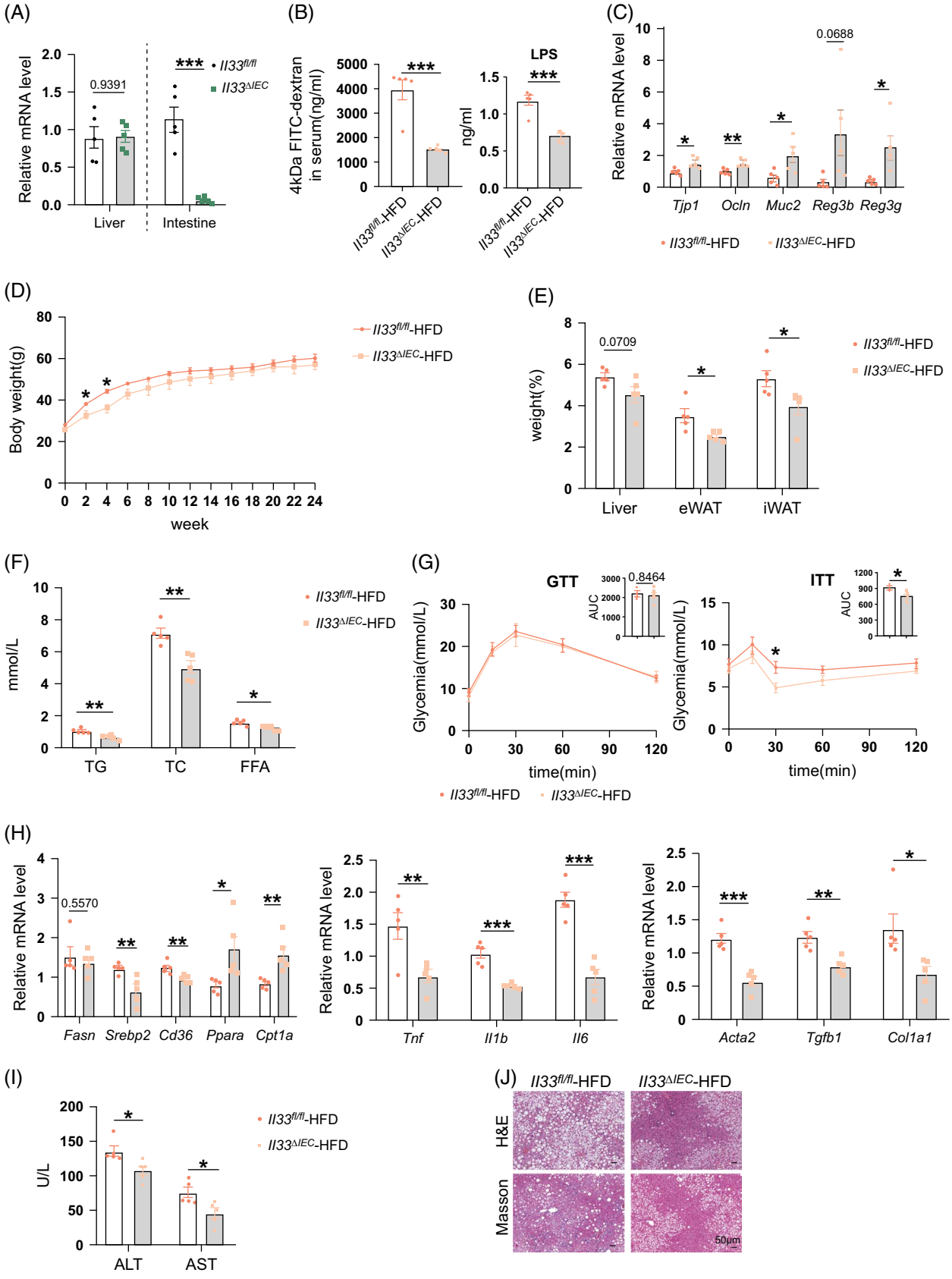

FIGURE 2 Intestinal IL-33 deficiency alleviated MASLD by preserving intestinal barrier integrity. *Il33*$^{fl/fl}$ and *Il33*$^{\Delta IEC}$ mice were fed HFD for 24 weeks. (A) Hepatic and intestinal mRNA levels of *Il33* detected by qPCR. (B) Serum 4 kDa FITC-dextran levels and LPS levels. (C) Intestinal mRNA levels of *Tjp1*, *Ocln*, *Muc2*, *Reg3b*, and *Reg3g* detected by qPCR. (D) Body weight over time. (E) Liver, eWAT, or iWAT-to-body weight.

(F) Serum TG, TC, and FFA content levels. (G) GTT and ITT with corresponding AUCs. (H) Hepatic mRNA levels of genes involved in lipogenesis (*Fasn* and *Srebp2*), fatty acid transport (*Cd36*) and fatty acid oxidation (*Ppara* and *Cpt1a*), inflammation (*Tnf*, *Il1b*, and *Il6*), fibrosis (*Acta2*, *Tgfb1*, and *Col1a1*) detected by qPCR. (I) Serum ALT and AST levels. (J) Representative images displaying hepatic H&E staining and Masson staining in liver sections. Scale bars: 50 μm. Data are represented as mean ± SEM (n = 5/group). Statistical differences were determined by unpaired 2-tailed *t* test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Abbreviations: eWAT, epididymal white adipose tissue; FFA, free fatty acids; FITC, fluorescein isothiocyanate; GTT, glucose tolerance test; H&E, hematoxylin & eosin; HFD, high-fat diet; ITT, insulin tolerance test; iWAT, inguinal white adipose tissue; LPS, lipopolysaccharide; MASLD, metabolic dysfunction–associated steatotic liver disease; qPCR, realtime fluorescence quantitative polymerase chain reaction; TC, cholesterol; TG, triglyceride; WT, wild type.

To investigate the role of IL-33-regulated gut microbiota in MASLD, we performed FMT. First, we collected fecal microbiota from HFD-fed *Il33*$^{-/-}$ mice and transferred them into HFD-fed WT mice as the allogeneic group (WT→WT). Autologous FMT was set as the control group (*Il33*$^{-/-}$→WT). After 7 weeks of FMT, we examined MASLD-related phenotypes (Figure 3B). Our results showed that transplantation of gut microbiota from *Il33*$^{-/-}$ mice alleviated the severity of MASLD, evidenced by the decreased liver and WAT weight, lower serum lipid content, improved insulin resistance, liver functions, and fibrosis (Figures 3C–H). Reverse validation was also performed. Recipients of gut microbiota from HFD-fed WT mice (WT→*Il33*$^{-/-}$) exhibited more severe MASLD features compared with the control group (*Il33*$^{-/-}$→*Il33*$^{-/-}$), characterized by the increased liver and inguinal white adipose tissue weight, lipid content, insulin resistance, liver enzymes, and fibrosis (Supplemental Figures S2B–G, http://links.lww.com/HEP/I528). We also performed FMT in intestinal epithelial IL-33 deficiency mice and obtained consistent results (Figures 3C–H, Supplemental Figures S2B–G). Taken together, the above results suggested that the influence of intestinal IL-33 on MASLD progression was mediated by gut microbiota.

IL-33 deficiency reduced gut microbiota–mediated synthesis of TMA and subsequent induction of hepatic oxidative stress

In addition to the aforementioned alterations in gut microbiota, IL-33 deficiency also induced a significant reduction of specific gut bacteria in MASLD mice, including *Lachnoclostridium*, *Providencia*, *Desulfovibrio*, *Blautia*, and *Prevotella* (Figure 3A). Notably, these bacteria exhibited a high abundance of choline TMA-lyase (*Cut C/D*),[23,24] an enzyme involved in choline metabolism leading to TMA, a precursor of TMAO. Moreover, it has been reported that the abundance of the bacteria, including *Prevotella*, *Ruminococcus*, *Alistipes*, *Desulfovibrio*, and *Providencia,* in HFD-fed WT mice was positively related to TMAO levels.[25]

Further, we employed untargeted metabolomics to investigate serum metabolites associated with gut microbiota. By applying variable important in projection > 1 and $p < 0.05$, we identified 60 differential metabolites between HFD-fed and chow diet–fed WT mice, as well as 10 differential metabolites between HFD-fed *Il33*$^{-/-}$ and WT mice (Supplemental Tables S4, S5, Supplemental Figures S3A and 4A, http://links.lww.com/HEP/I528 and Figure 4A). Compared with the chow group, the choline metabolism pathway was upregulated in HFD-fed WT mice (Supplemental Figure S3B, http://links.lww.com/HEP/I528). Importantly, we found a downregulation of LysoPC(22:4(7Z,10Z,13Z,16Z)), a metabolite involved in choline metabolism, in *Il33*$^{-/-}$ HFD-fed mice (Figures 4A, B). LysoPC (22:4(7Z,10Z,13Z,16Z)) also showed a positive correlation with *A2*, *Allobaculumn*, *Barnesiella*, *Clostridia_UCG-014*, *Prevotellaceae_NK3B31_group*, *Prevotellaceae_UCG-001*, *Ruminococcus*, and *ruminantium*, which had high abundance in HFD-fed WT mice (Supplemental Figure S3C, http://links.lww.com/HEP/I528). These results led us to focus on TMA, the key metabolite of gut microbiota involved in choline metabolism. We detected the levels of TMAO and its precursor TMA in serum, both of which were upregulated in HFD-fed WT mice compared to chow diet–fed mice (Figure 4C). IL-33 deletion decreased the levels of TMAO and TMA in HFD-fed mice. The raw materials of TMA, including choline, L-carnitine, and betaine, showed no difference between these 2 MASLD groups (Supplemental Figure S3D, http://links.lww.com/HEP/I528). The oxidation of TMA by hepatic FMO3 is the final step in TMA production, as well as the rate-limiting step.[7] However, there was no difference in mRNA and protein levels of FMO3 among the 4 groups (Supplemental Figures S3E, F, http://links.lww.com/HEP/I528), suggesting that changes in TMAO were associated with intestinal IL-33 rather than hepatic FMO3.

Studies have reported that TMAO aggravates oxidative stress, apoptosis, and mitochondrial dysfunction in cardiovascular diseases,[26] and oxidative stress has been identified as the primary factor contributing to MASLD.[27] We observed that intestinal IL-33 deletion suppressed hepatic oxidative stress, as evidenced by reduced malondialdehyde levels, and increased total antioxidant capacity, glutathione peroxidase, and superoxide dismutase levels (Figure 4D, Supplemental Figure S4A, http://links.lww.com/HEP/I528). 3,3-dimethyl-1-butanol (DMB), a naturally occurring plant extract and structural analog of choline, effectively inhibits the utilization of choline by gut microbiota for TMA synthesis.[26] We treated HFD-fed mice with DMB to inhibit TMAO synthesis, which decreased the extent of hepatic oxidative stress (Figure 4E). Compared

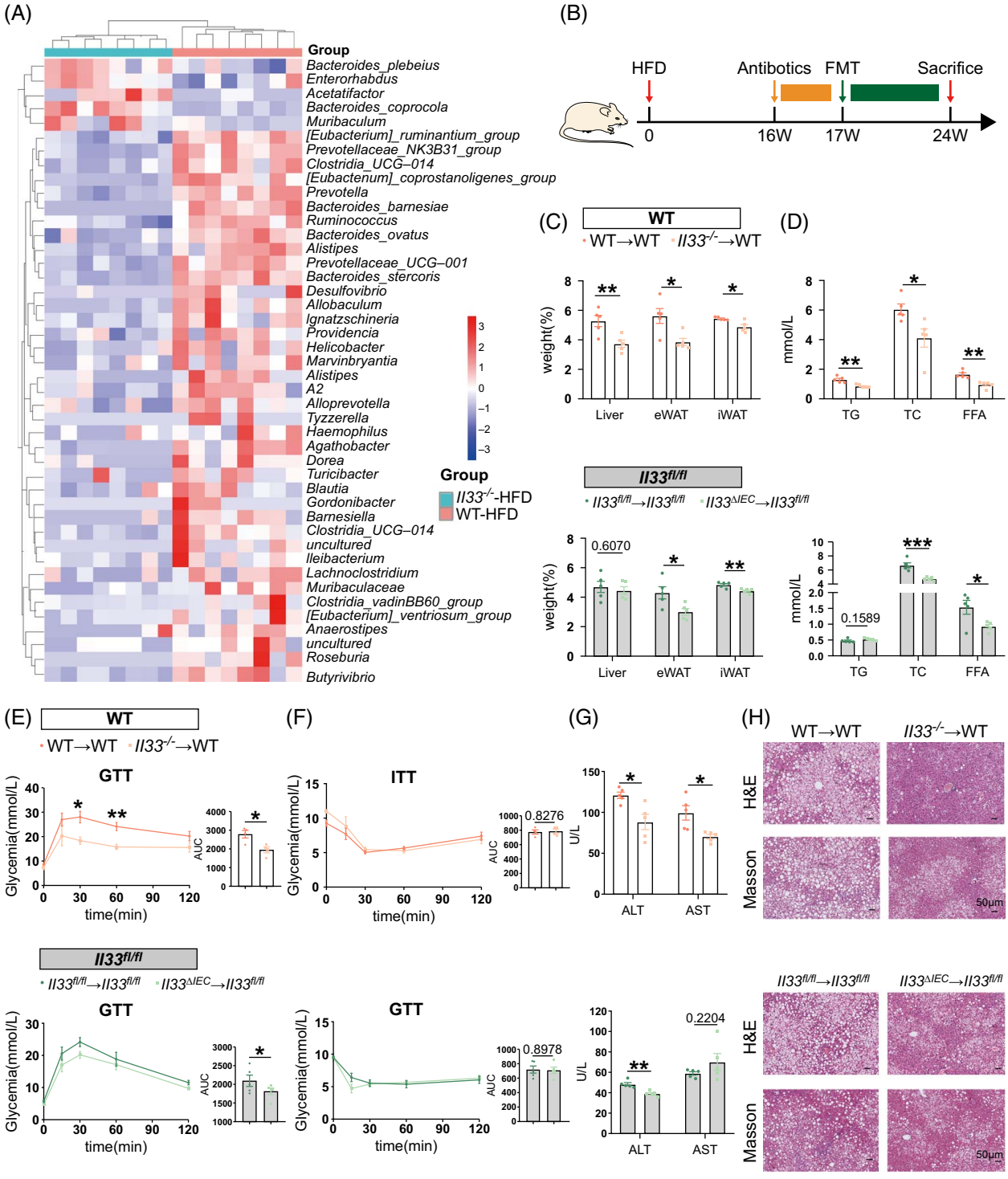

FIGURE 3 IL-33 deficiency improved dysbiosis of gut microbiota in MASLD. (A) Heatmap of differential species for gut microbiota in HFD-fed *Il33*$^{-/-}$ mice versus HFD-fed WT mice (n = 8/group). (B) Experimental design of FMT study. HFD-fed WT mice received fecal microbiota from WT mice (WT → WT) or *Il33*$^{-/-}$ *mice* (*Il33*$^{-/-}$ → WT). HFD-fed *Il33*$^{fl/fl}$ mice received fecal microbiota from *Il33*$^{fl/fl}$ mice (*Il33*$^{fl/fl}$→*Il33*$^{fl/fl}$) or *Il33*$^{\Delta IEC}$ mice (*Il33*$^{\Delta IEC}$ →*Il33*$^{fl/fl}$). (C) Liver, eWAT, and iWAT-to-body weight. (D) Serum TG, TC, and FFA content levels. (E) GTT with corresponding AUCs. (F) ITT with corresponding AUCs. (G) Serum ALT and AST levels. (H) Representative images displaying hepatic H&E staining and Masson staining in liver sections. Scale bars: 50 μm. Data are represented as mean ± SEM (n = 5/group unless specially noted). Statistical differences were determined by unpaired 2-tailed *t* test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Abbreviations: eWAT, epididymal white adipose tissue; FFA, free fatty acids; FMT, fecal microbiota transplant; GTT, glucose tolerance test; H&E, hematoxylin & eosin; HFD, high-fat diet; ITT, insulin tolerance test; iWAT, inguinal white adipose tissue; MASLD, metabolic dysfunction–associated steatotic liver disease; TC, cholesterol; TG, triglyceride; WT, wild type.

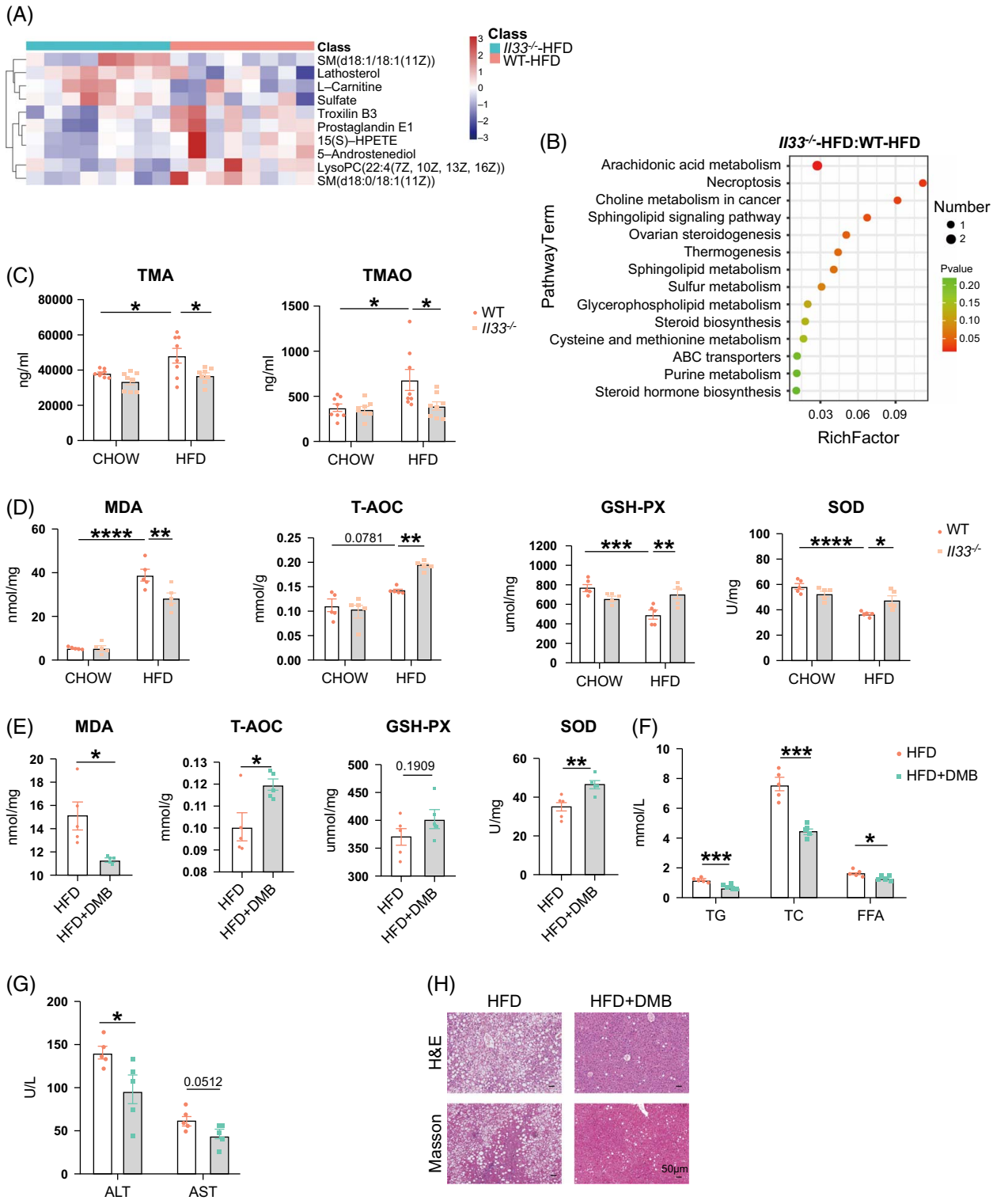

FIGURE 4 Deletion of IL-33 alleviated hepatic oxidative stress by reducing the synthesis of gut microbiota–derived TMAO. (A) Heatmap of differential metabolites in the serum of HFD-fed *Il33*$^{-/-}$ mice versus HFD-fed WT mice (n = 8/group). (B) KEGG pathway enrichment analysis of the differential metabolites in serum (n = 8/group). (C) Serum TMAO and TMA levels in WT mice and *Il33*$^{-/-}$ mice fed HFD or chow diet (n = 8/group). (D) Hepatic MDA, T-AOC, GSH-PX, and SOD levels. HFD-fed WT mice were treated with DMB for 8 weeks. (E) Hepatic MDA, T-AOC, GSH-PX, and SOD levels. (F) Serum TG, TC, and FFA content levels. (G) Serum ALT and AST levels. (H) Representative images displaying hepatic H&E staining and Masson staining in liver sections. Scale bars: 50 μm. Data are represented as mean ± SEM (n = 5/group unless specially noted). Statistical differences were determined by unpaired 2-tailed *t* test or one-way ANOVA with the Tukey post-test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. Abbreviations: DMB, 3,3-dimethyl-1-butanol; FFA, free fatty acids; GSH-PX, glutathione peroxidase; H&E, hematoxylin & eosin; HFD, high-fat diet; KEGG, Kyoto Encyclopedia of Genes and Genomes; MDA, malondialdehyde; SOD, superoxide dismutase; T-AOC, total antioxidant capacity; TC, cholesterol; TG, triglyceride; TMA, trimethylamine; TMAO, trimethylamine *N*-oxide; WT, wild type.

with no drug intervention, DMB treatment alleviated metabolic disorders, inflammation, and fibrosis in MASLD (Figures 4F–H, Supplemental Figures S4B–G). Collectively, the data above suggested that IL-33 deficiency reduced gut microbiota–mediated synthesis of TMAO and subsequent induction of hepatic oxidative stress, thereby preventing the progression of MASLD.

Nuclear IL-33 inhibited the activation of protective HIF-1α in IECs and enhanced intestinal permeability

IL-33 is a multifunctional protein with distinct intracellular and extracellular roles.[28] Intracellularly, IL-33 mostly localizes in the nucleus of parenchymal cells and acts as a nuclear factor to regulate gene transcription. We observed that IL-33 was mainly located within the intestinal epithelium (Figure 1E). We isolated IECs from the intestine and observed higher levels of nuclear IL-33 in the MASLD group (Figure 5A). In addition, compared with chow diet–fed WT mice, downstream genes regulated by HIF-1α, including *Tff3*, *Cdh1*, and *Adora2b*, were inhibited in HFD-fed mice (Figure 5B). Unlike other tissues, the intestinal mucosa experiences physiological hypoxia, which promotes HIF-1α translocation into the nucleus for transcriptional regulation rather than degradation.[29] Among them, ITF encoded by *Tff3* protects the intestinal barrier by repairing damaged mucosa.[30] E-cadherin encoded by *Cdh1* is involved in the intestinal barrier construction,[31] and *Adora2b* coordinates the resolution of intestinal inflammation.[32] Knockout of IL-33 resulted in increased levels of HIF-1α in the nucleus, as well as increased expression of *Tff3*, *Cdh1*, and *Adora2b* (Figures 5A–C).

Next, we used Caco-2, a human IEC line, to study the link between IL-33 and HIF-1α. We found that palmitic acid (PA) directly induced damage to IECs, leading to decreased tight junction proteins and increased levels of IL-33 (Supplemental Figures S5A–D, http://links.lww.com/HEP/I528). Concurrently, PA also inhibited the activation of HIF-1α (Supplemental Figure S5E, http://links.lww.com/HEP/I528). We also examined the effects of LPS, mainly produced by dysregulated gut microbiota, on Caco-2 and observed no difference in IL-33 (Supplemental Figures S5A, B, http://links.lww.com/HEP/I528), which suggested that PA rather than LPS serves as the primary cause for intestinal IL-33 changes in MASLD. In addition, we found that stimulation with IL-33 alone did not affect intestinal epithelial function (Supplemental Figures S5F, G, http://links.lww.com/HEP/I528), implying that nuclear IL-33 may directly damage IECs rather than in an autocrine form. After that, we used siRNA to disturb IL-33 expression (Supplemental Figures S5H, I, http://links.lww.com/HEP/I528). The constrained IL-33 restored tight junction protein expression under PA stimulation (Figures 5D–F). Notably, the expression of HIF-1α and its downstream genes were increased under IL-33 siRNA disturbance (Figure 5E). We also used HIF-1α siRNA and found that it inhibited the downstream genes and aggravated damage to the intestinal barrier integrity (Supplemental Figures S5H–J, http://links.lww.com/HEP/I528). Next, we confirmed the interaction between IL-33 and HIF-1α in the nucleus under PA stimulation by immunofluorescence and coimmunoprecipitation analysis (Figures 5G, H). The results above suggested that nuclear-localized IL-33 directly suppressed activation of the protective HIF-1α downstream pathway in IECs; thus, the inhibition of IL-33 expression alleviated intestinal epithelial injury.

Extracellular IL-33 disrupted intestinal Th1/Th17 balance by promoting Th1 differentiation through ST2-*Hif1a*-*Tbx21* axis

Long-term exposure to HFD stimulation induced a state of low-grade inflammation in the intestine and enhanced the release of IL-33 by IECs (Supplemental Figure S5D, http://links.lww.com/HEP/I528). Because of the widespread expression of its receptor ST2 on immune cells, released IL-33 was thought to be a pleiotropic cytokine acting in innate and adaptive immunity.[33] We collected immune cells from the intestinal lamina propria and analyzed different CD4+ T-cell subsets by flow cytometry. The percentage of Th1 was upregulated, and the proportion of Th17 was downregulated in HFD-fed mice compared to chow diet–fed WT mice (Figure 6A). No change was observed in Th2 and regulatory T cells (Tregs) between these 2 groups. Knockout of global IL-33 resulted in a decrease in the proportion of Th1 and an increase in Th17 in HFD-fed mice; this phenomenon was also observed in HFD-fed *Il33*$^{\Delta IEC}$ mice (Figure 6B). It is reported that excessive Th1 responses by secreting IFN-γ weaken the intestinal barrier and lead to systemic chronic inflammation.[34] In obesity, intestinal Th17 plays a beneficial role by protecting the intestinal barrier and inhibiting intestinal lipid absorption.[35] We also examined the expression levels of transcription factors and cytokines that are associated with T-cell differentiation and function (Supplemental Figures S6A, B, http://links.lww.com/HEP/I528). Among them, T-bet (encoded by *Tbx21*) is regarded as a key regulator of Th1 differentiation and IFN-γ production.[36] RORγt (encoded by *Rorc*) is an important transcription factor for Th17 cells, promoting the secretion of IL17A.[37] The knockout of IL-33 reduced Th1-related *Tbx21* and *Ifng* expression, increased *Rorc* and *Il17a* expression, and had no influence on the expression of *Gata3* and *Foxp3*, which were associated with Th2 or Tregs differentiation.[38] To investigate the

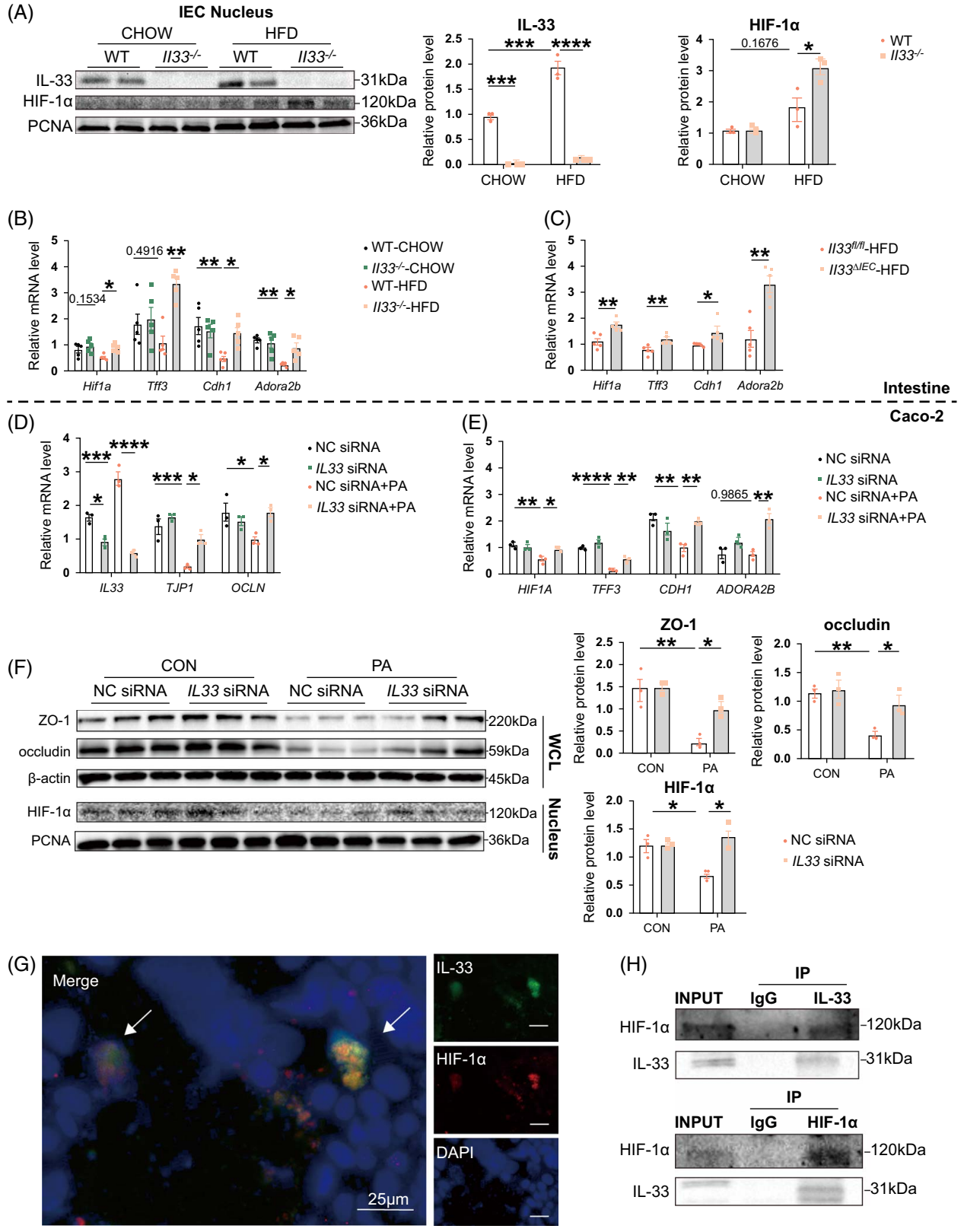

FIGURE 5 Nuclear IL-33 increased intestinal permeability by inhibiting the activation of HIF-1α in IECs. (A) Nuclear IL-33 and HIF-1α levels in IECs of WT mice and *Il33*$^{-/-}$ mice fed HFD or chow diet detected by WB. (B, C) Intestinal mRNA levels of *Hif1a*, *Tff3*, *Cdh1*, and *Adora2b* detected by qPCR (n = 5/group). Caco-2 cells under *IL33* siRNA or NC siRNA transfection were stimulated with PA. (D) mRNA levels of *IL33*, *TJP1*, and *OCLN* detected by qPCR. (E) mRNA levels of *HIF1A*, *TFF3*, *CDH1*, and *ADORA2B* detected by qPCR. (F) Protein levels of ZO-1 and occludin in WCL and HIF-1α in nucleus detected by WB. (G) Colocalization of IL-33 (green) and HIF-1α (red) detected by immunofluorescence (arrowheads). Scale bars: 25 μm. (H)

Coimmunoprecipitation of the interaction between IL-33 and HIF-1α in the nucleus. Data are represented as mean ± SEM (n = 3/group unless specially noted). Statistical differences were determined by unpaired 2-tailed *t* test or one-way ANOVA with the Tukey post-test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. Abbreviations: HFD, high-fat diet; HIF-1α, hypoxia-inducible factor-1α; IECs, intestinal epithelial cells; NC, negative control; PA, palmitic acid; qPCR, realtime fluorescence quantitative polymerase chain reaction; WCL, whole-cell lysates; WB, western blot; WT, wild type.

regulatory effect of IL-33 on intestinal immunity, we further constructed *Il1rl1*$^{-/-}$ mice and observed that ST2 deficiency decreased the percentage of Th1 cells and the expression of intestinal *Tbx21* and *Ifng*, and increased Th17 cells, as well as of *Rorc* and *Il17a* expression (Figure 6C, Supplemental Figure S6C). The amelioration of intestinal inflammation was concomitant with an improvement in the integrity of the intestinal barrier, as well as a reduction in metabolic disorders, inflammation, and fibrosis of the liver in *Il1rl1*$^{-/-}$ HFD-fed mice (Supplemental Figures S6D–J, http://links.lww.com/HEP/I528). These results indicated that extracellular IL-33 might be involved in T-cell differentiation and function through ST2.

Afterward, we investigated the effect and mechanism of IL-33 on T cells ex vivo. We isolated spleen naïve CD4+ T cells from WT and *Il1rl1*$^{-/-}$ mice and cultured them under Th1 differentiation conditions with or without supplementation of IL-33. IL-33 administration increased ST2 expression and promoted naïve T-cell differentiation into Th1 cells (Figure 6D). However, ST2 deletion significantly decreased Th1 differentiation and *Ifng* expression. Our findings revealed that IL-33 stimulation upregulated the expression of both *Tbx21* and *Hif1a* in Th1 cells, while knockout of ST2 abolished these effects (Figures 6E, F). Studies have shown that IL-33 increased *Hif1a* expression through ST2 signaling.[39] Furthermore, HIF-1α could facilitate *Tbx21* transcription.[40] These results suggested that the IL-33/ST2 axis promoted Th1 cell differentiation through the HIF-1α signaling pathway. In addition, ST2 deficiency increased the proportion of Th17 cells under Th17 differentiation conditions (Supplemental Figures S6K–L, http://links.lww.com/HEP/I528). HIF-1α has been reported to promote Th17 cell differentiation.[41] However, we did not observe differences in *Hif1a* expression between WT and *Il1rl1*$^{-/-}$ Th17 cells (Supplemental Figure S6L, http://links.lww.com/HEP/I528), indicating that the inhibitory effect exerted by IL-33 on Th17 cell development was not mediated through modulation of HIF-1α activity. Collectively, extracellular IL-33 induced intestinal Th1/Th17 imbalance and promoted Th1 cell differentiation through the ST2-*Hif1a*-*Tbx21* axis, and knockout of IL-33/ST2 could alleviate intestinal inflammation.

DISCUSSION

IL-33 exhibits divergent roles, either protective or pathogenic, in different diseases, depending on the function and predominance of the target cells.[33] Recombinant IL-33 protects against *Trichuris muri* infection by inducing IL-4, IL-9, and IL-13.[42] However, IL-33 increases intestinal permeability and inhibits Th17 immunity through ST2 in colitis.[10] Understanding the role and mechanism of IL-33 in MASLD may provide potential strategies for the prevention and treatment of MASLD. Previous studies have presented conflicting results regarding the impact of IL-33 on steatohepatitis, and the mechanism has not been clarified. Exogenous administration of IL-33 aggravated liver inflammation and fibrosis in mice following 20 weeks of HFD feeding,[15] whereas endogenous knockout of IL-33 showed no significant effect on liver fibrosis progression in mice fed an HFD for 12 weeks.[14] The divergences might be caused by different feeding periods since the mice typically showed only hepatic steatosis and mild inflammation without evident fibrosis after 12 weeks of HFD feeding. In our study, we established MASLD models by feeding mice with HFD for 24 weeks to induce inflammation and fibrosis and identified the pathogenic role of IL-33 in MASLD progression through its exacerbation of metabolic disorders, inflammation, and fibrosis. Enhanced Th2 response and M2 macrophage activation in the liver were suggested to be involved in IL-33-mediated steatohepatitis progression,[15] but given its limited expression in the liver, IL-33 likely exerts its effects through additional mechanisms in MASLD.

IL-33 is highly expressed at mucosal barrier sites and affects the development of intestinal disease.[9,33] Intestinal barrier plays a pivotal role in the pathogenesis of metabolic diseases by facilitating the translocation of gut microbiota and their metabolites from the gut to the liver, thereby inducing metabolic disorders and inflammation.[5] In our study, we observed quite low expression levels of IL-33 in the liver of both humans and mice, with no significant difference in hepatic IL-33 expression between the MASLD group and the control. Interestingly, we found a significant increase in IL-33 levels in the intestine of MASLD mice. More importantly, IL-33 aggravated intestinal barrier leakage and homeostasis disruption, drawing our attention to the function of intestinal IL-33 in MASLD. Notably, specific deletion of intestinal epithelial IL-33 indeed attenuated intestinal injury and ameliorated MASLD-related liver phenotypes, providing additional evidence for the influence of intestine-derived IL-33 in promoting MASLD progression. However, further investigation is required to elucidate the key mediators contributing the IL-33-driven alteration of the gut-liver axis in MASLD.

To analyze IL-33-induced alterations in gut microbiota, we performed 16S rRNA sequencing and found that knockout of IL-33 reduced the abundance of microbiota associated with metabolic disorders and

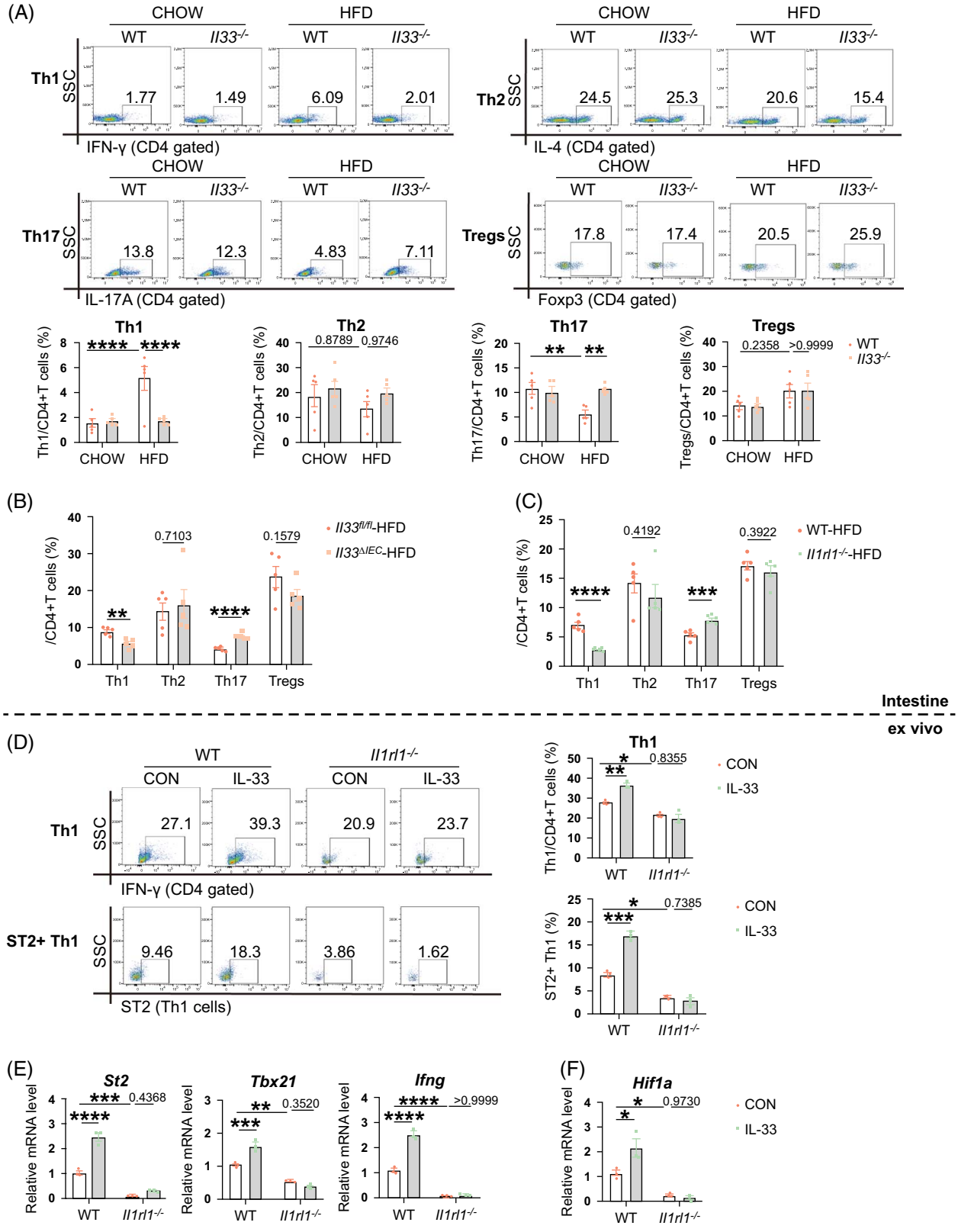

FIGURE 6 Released IL-33 disrupted Th1/Th17 balance in the intestine by promoting Th1 differentiation through the ST2-*Hif1a*-*Tbx21* axis. (A–C) Representative flow cytometry plots and proportions of Th1 (IFN-γ + CD4+ T cells), Th2 (IL-4 + CD4+ T cells), Th17 (IL17A + CD4+ T cells), and Tregs (Fopx3 + CD4+ T cells) in intestinal lamina propria. Spleen naïve CD4+ T cells from WT mice and *Il1rl1*$^{-/-}$ mice were cultured under Th1 differentiation conditions, with or without treatment of IL-33. (D) Representative flow cytometry plots and proportion of Th1 and ST2+ Th1 cells (n = 3/group). (E) mRNA levels of *Il1rl1*, *Tbx21*, and *Ifng* detected by qPCR (n = 3/group). (F) mRNA levels of *Hif1a* detected by qPCR (n = 3/group). Data are represented as mean ± SEM (n = 5/group unless specially noted). Statistical differences were determined by unpaired 2-tailed *t* test or one-way ANOVA with the Tukey post-test. **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001. Abbreviations: qPCR, realtime fluorescence quantitative polymerase chain reaction; Tregs, regulatory T cells; WT, wild type.

inflammation, including *Bacteroides_ovatus*, *Tyzzerella*, *Desulfovibrio*, *Providencia,* *Turicibacter*, and so on.[17,18,20–22] Consequently, we conducted FMT experiments in mouse models. Transplantation of fecal microbiota from IL-33 deficient mice into WT mice ameliorated MASLD development. Conversely, fecal microbiota transplanted into IL-33 deficient mice from WT mice deteriorated the condition in knockout mice. These results confirmed the significance of IL-33-induced alteration in bacteria composition. It is noteworthy that IL-33 deletion reduced specific gut bacteria in MASLD, including *Lachnoclostridium*, *Providencia*, *Desulfovibrio*, *Blautia*, and *Prevotella*. These modified bacteria are found to possess choline TMA-lyase (*cut C/D*) and are able to degrade dietary choline to TMA, the precursor to TMAO.[7] Moreover, the knockout of IL-33 also reduced serum levels of TMAO in MASLD mice.

Several clinical studies have indicated that TMAO, a metabolite from gut microbiota, could serve as a promising biomarker associated with human health conditions, particularly in cardiovascular diseases.[26] Elevated levels of circulating TMAO are also associated with MASLD, mainly in patients with type 2 diabetes.[43] However, the underlying mechanism by which TMAO contributes to MASLD remains elusive. As reported, the pathogenic effect of TMAO in cardiovascular diseases is associated with oxidative stress, and inhibition of microbiota-dependent TMAO production by DMB reduces the development of hypertension and atherosclerosis.[26,44] Oxidative stress, resulting from an imbalance between reactive oxygen species production and the antioxidant defense system, is widely recognized as a pivotal contributor to hepatic injury and MASLD.[27] In this study, we observed a reduction in hepatic malondialdehyde levels and an increase in total antioxidant capacity, glutathione peroxidase, and superoxide dismutase levels in intestinal IL-33 deletion mice, indicating that intestinal IL-33 may contribute to the induction of hepatic oxidative stress. In addition, the administration of DMB also alleviated hepatic oxidative stress injury in MASLD, concomitant with improvements in metabolic disorders, inflammation, and fibrosis. These findings imply that TMAO plays a vital role in intestinal IL-33-induced MASLD progression by aggravating hepatic oxidative stress injury. After that, we explored the specific molecular mechanism by which intestinal IL-33 regulated TMAO-related intestinal homeostasis alterations.

IL-33 is predominantly expressed in parenchymal cells rather than immune cells and exerts multiple effects by functioning as a nuclear factor with transcriptional regulatory properties or as a cytokine mediating inflammatory immune responses.[10,28] For example, nuclear IL-33 inhibited Smad6 expression by blocking RUNX2 and promoted cancer development in chronic inflammation of the skin and pancreas.[45] Intriguingly, hyperoxia has been shown to augment IL-33-dependent group 2 innate lymphoid cell responses and induce asthmatic features in neonates.[46] The intestine is in a unique hypoxic state under physiological conditions, maintaining a large number of anaerobic bacteria colonization.[32] However, HFD disrupts the intestinal hypoxic environment, leading to higher oxygen concentration in the intestinal lumen.[47] Our data showed that HFD enhanced the synthesis of IL-33 in IECs, thereby promoting its nuclear translocation and extracellular release. Inspired by these findings, we then investigated the distinct nuclear and extracellular functions exerted by IL-33 in MASLD.

First, we explored the role of IL-33 in the nucleus of IECs. It is well established that HIF-1α is activated under hypoxic conditions and acts as a key regulator contributing to intestinal barrier integrity.[29,32] Here, we observed repression of HIF-1α in IECs in MASLD, while IL-33 deficiency enhanced the expression of HIF-1α and its downstream genes, including *Tff3*, *Cdh1*, and *Adora2b,* which play crucial roles in intestinal barrier construction and repair. In addition, in vitro experiments demonstrated that PA stimulation inhibited nuclear HIF-1α expression and induced severe damage to IECs, whereas interference with IL-33 siRNA restored HIF-1α activation, accompanied by the recovery of IECs. In contrast, recombinant IL-33 had no influence on the expression of nuclear HIF-1α in IECs. Furthermore, we investigated the molecular mechanism through which intestinal IL-33 regulated HIF-1α and revealed that nuclear IL-33 directly binds to HIF-1α, thereby inhibiting its activation and downstream signaling pathways. Notably, knockout of intestinal IL-33 restored HIF-1α activity and attenuated intestinal barrier damage.

Subsequently, we explored the role of extracellular IL-33 released by IECs. We examined the impact of IL-33 on intestinal CD4+ T cells, which were in the central part in maintaining homeostasis and orchestrating immune responses within the gastrointestinal tract.[48] Our study showed that HFD altered the distribution of intestinal CD4 + T cells in mice, characterized by an increase in the proportion of Th1 and a decrease in Th17, which is consistent with findings.[35] Knockout of IL-33 restored the intestinal Th1/Th17 balance by attenuating proinflammatory Th1 cell activity while enhancing protective Th17 cell responses. Similar to the impact of IL-33 deficiency, ablation of ST2, the receptor of IL-33, also abolished the detrimental impact exerted by IL-33 on intestinal immunity.

Further study revealed that in MASLD mice, the IL-33/ST2 signaling pathway upregulated the expression of the transcription factor *Tbx21* and the cytokine *Ifng*, which facilitate the differentiation and proinflammatory functions of Th1 cells. We next employed stimulation with recombinant IL-33 on primary naïve CD4+T cells from mice, and observed a significant augmentation in the differentiation of these cells toward the Th1 phenotype. Unexpectedly, exogenous IL-33 induced an enhancement of *Hif1a* expression in Th1 cells, contrasting with the inhibitory effect of nuclear IL-33 on HIF-1α activation in IECs, suggesting a divergent influence of IL-33 on HIF-

1α in T cells and IECs. These effects were abrogated upon ST2 deficiency, as evidenced by a decreased proportion of Th1 cells and downregulation of *Tbx21* and *Ifng* expression. In light of the above findings, as well as reports indicating HIF-1α enhances *Tbx21* expression,[40,49] we propose that IL-33 promoted Th1 differentiation and proinflammatory function through ST2-*Hif1a*-*Tbx21* axis and ultimately led to intestinal injury. Here, we provided a distinctive elucidation of how IL-33 governs intestinal homeostasis in MASLD, namely, by inducing disruption of the intestinal barrier and perturbation of intestinal homeostasis through dual regulation of HIF-1α function in IECs and Th1 cells.

CONCLUSIONS

In conclusion, our study demonstrates that IL-33 derived from IECs induced gut microbiota dysbiosis and TMAO synthesis, leading to hepatic oxidative stress injury and ultimately exacerbated metabolic disorders, inflammation, and fibrosis in MASLD. During this process, IL-33 exerts a dual regulatory effect on HIF-1α function in both IECs and Th1 cells. On the one hand, IL-33 binds to HIF-1α in the nucleus of IECs and inhibits its activation, thereby directly damaging the intestinal barrier. On the other hand, released IL-33 promotes Th1 differentiation and IFN-γ secretion through the ST2-*Hif1a*-*Tbx21* axis, aggravating the disruption of intestinal homeostasis. These findings suggest that targeting IL-33, along with its associated gut microbiota and metabolites, may represent an effective therapeutic strategy for MASLD.

AUTHOR CONTRIBUTIONS

Suping Hai performed the experiments, acquired and analyzed the data, and wrote the manuscript. Xitang Li and Erliang Xie acquired and analyzed the data. Wenhui Wu, Qiang Gao, Binghui Yu, Junjian Hu, Feiyang Xu, and Xizhe Zheng provided technical assistance and acquired part of the data. Bin-hao Zhang, Di Wu, and Weiming Yan provided and collected samples, participated in the data discussion, and provided suggestions. Xiaojing Wang and Qin Ning designed and supervised the project and revised the manuscript.

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CONFLICTS OF INTEREST

The authors have no conflicts to report.

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