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Intestinal IL-25 prevents high-fat diet-induced obesity by modulating the cholesterol transporter NPC1L1 expression in the intestinal epithelial cells

Siranart Jeerawattanawart[1,2] & Pornpimon Angkasekwinai[1,3]✉

The intestine is essential for digestion and nutrient absorption, and its altered function contributes to metabolic dysregulation and obesity-induced intestinal inflammation. Intestinal immune responses have been associated with the regulation of metabolic dysfunction during obesity. Given that the epithelial cell-derived cytokine IL-25 has been demonstrated to regulate metabolic disorders, we sought to examine the role of intestinal IL-25 in modulating a high-fat diet (HFD)-induced obesity. We found that mice on a high-fat diet exhibited decreased IL-25 expression in the small intestine. Intestinal IL-25 mRNA levels displayed an inverse association with plasma triglycerides, total cholesterol, glucose levels, and the expression of the cholesterol transporter *Npc1l1* in the intestine. In HFD-induced obesity, transgenic mice overexpressing IL-25 in the intestinal epithelial cells demonstrated diminished mRNA expression of intestinal genes related to glucose, cholesterol, and fat absorption, along with chylomicron production, while also systemically decreasing plasma glucose, total cholesterol, and triglyceride levels, fat accumulation, and weight gain. In vitro, IL-25 treatment of human intestinal Caco-2 cells directly decreased cholesterol uptake and downregulated the expression of NPC1L1 and its transcriptional regulator, SREBP2. These findings highlight IL-25 as a potential modulator in the intestine that regulates intestinal cholesterol absorption and systemic metabolism in obesity.

Keywords IL-25, Intestine, NPC1L1, Cholesterol absorption, Obesity

Obesity, characterized by excessive accumulation of body fat within adipose tissue[1], remains a critical global public health challenge[2] and has become a major risk factor for non-communicable diseases (NCDs) and metabolic syndrome such as hypertension, hyperlipidemia, hyperglycemia, and cardiovascular disease[3]. Moreover, obesity is associated with low-grade chronic inflammation[4,5], as evidenced by elevated plasma levels of TNF-α and IL-6 in obese patients and diet-induced obese mice, which lead to the progression of insulin resistance-mediated diabetes[4,6]. In addition to the elevated inflammatory markers present in circulation during obesity, inflammation within adipose tissue is significantly linked to the development of obesity and insulin resistance. In both obese humans and mice, increased secretion of IL-6, TNF-α, and monocyte chemoattractant protein-1 (MCP-1) by adipocytes and adipose tissue macrophages directly contributes to the development of insulin resistance[4,5,7–11]. On the other hand, other organs such as the liver[12], kidney[13], muscle[14], brain[15], and intestine also play significant roles in obesity-associated inflammation and metabolic dysfunction.

Alteration in the intestinal morphology and function has been observed in obese humans and animal models, including increased intestinal length[16,17], a higher absorptive surface area[16,17], and enhanced intestinal permeability[18,19] and absorption[17,20]. Previous studies demonstrated that obesity significantly augments intestinal glucose absorption by upregulating the expression of glucose transporters, such as GLUT2[21] and SGLT1[22,23], which are associated with insulin resistance and glycemia. Moreover, an increase in intestinal NPC1L1, a cholesterol transporter that facilitates intestinal cholesterol absorption has been observed in type 2

[1]Department of Medical Technology, Faculty of Allied Health Sciences, Thammasat University, Pathum Thani 12120, Thailand. [2]Faculty of Medical Technology, Rangsit University, Pathum Thani 12000, Thailand. [3]Research Unit in Molecular Pathogenesis and Immunology of Infectious Diseases, Thammasat University, Pathum Thani 12120, Thailand. ✉email: upornpim@tu.ac.th; p.akswn@gmail.com

diabetes patients with higher BMI[24] and mice fed with HFD-induced hyperlipidemia[25]. Inhibition of NPC1L1 using the drug ezetimibe serves as an alternative treatment for hypercholesterolemia in obese individuals[26,27]. Related study using microarray analysis of the intestine following high-fat diet administration showed alteration in the gene involved in fat absorption including FATP4, CD36, MTP, and apoA-IV genes[28]. Moreover, high-fat diet enhanced inflammatory environment in the intestine that was associated with dysregulated glucose and lipid homeostasis during diet-induced obesity. The inflammatory cytokine TNF-α was found to be elevated in the small intestine of mice subjected to a high-fat diet, which corresponded with increased body weight gain, adiposity, and enhanced plasma insulin and glucose levels[29]. Moreover, this cytokine could impair intestinal cholesterol metabolism by downregulating the expression of cholesterol efflux-related gene ABCA1 and reducing HDL efflux in the Caco-2 cells[30].

IL-25, a cytokine derived from the intestinal epithelial cells, serves as a key modulator of type 2 immunity and plays role in maintaining intestinal barrier integrity[31–33] and promoting protective immunity against helminth infections[34–36]. IL-25 also has a role in modulating metabolic dysfunction, as evidenced by significantly attenuated body weight gain and improved glucose homeostasis in obese mice following IL-25 treatment[37]. Consistently, treatment of HFD fed mice with IL-25 enhanced ILC2 levels and M2 macrophages, thereby improving hepatic steatosis[38]. IL-25 has also been shown to modulate lipid metabolism in M2 macrophages by augmenting mitochondrial respiratory capacity and lipolysis[39]. Moreover, it plays a direct role in adipocytes by maintaining adiponectin levels under homeostatic conditions and mitigating inflammatory responses during low-grade inflammation[40]. A recent study revealed that IL-25 treatment inhibited short-term high-fructose diet-induced fatty acid synthesis, leading to a reduction of triglyceride accumulation in the liver[41]. Despite several studies illustrating the significant functions of IL-25 in the systemic regulation of metabolic diseases and obesity, its specific role in sustaining intestinal metabolic homeostasis, another characteristic of diet-induced obesity, remains unclear. We hypothesized that intestine-derived IL-25 is integral for the maintenance of intestinal metabolic homeostasis and may contribute to obesity prevention.

In the present study, we aimed to investigate the contribution of intestinal IL-25 expression in regulating the intestinal lipid absorption in a high-fat diet-induced obesity model. Intestinal cytokine IL-25 transcript levels were found to be negatively correlated with blood triglyceride, total cholesterol and glucose levels as well as with the expression of cholesterol transporter NPC1L1 in a high-fat diet-induced obesity model. The overexpression of IL-25 in the intestine resulted in diminished expression of genes associated with glucose, cholesterol and triglyceride absorption, as well as chylomicron formation in the intestines, and exerted a systemic effect by lowering plasma glucose, total cholesterol, triglyceride levels, fat accumulation, and weight gain. In vitro treatment of human intestinal Caco-2 cells with IL-25 directly reduced cholesterol uptake and the expression of NPC1L1 and its positive transcription factor, SREBP2. Our study revealed additional roles of IL-25 in high-fat diet-induced obesity through the regulation of cholesterol transport in the intestine.

Results

High-fat diet alters systemic metabolism and the expression of genes related to the absorption of glucose,cholesterol and triglycerides in the intestine of BALB/c mice.

Obesity is associated with chronic low-grade inflammation in several tissues[42,43]. The intestine has gained highlighting for its potential role in metabolic dysfunction and obesity-induced intestinal inflammation[16]. Within the small intestine, jejunum has been suggested to play a role in regulating glucose and lipid absorption under the influence of insulin[21,44] and serves as a hallmark contributor to obesity-related alteration in the intestine. We first investigated the effects of a high-fat diet on metabolic changes in BALB/c mice by feeding them either a normal chow diet (NCD) or high-fat diet (HFD) for 8 weeks. Our study successfully established the obesity model, as HFD-fed mice showed a significant increase in body weight starting from week 2 of the feeding period compared to NCD-fed mice (Fig. 1A). Consistent with these body weight gains, the food efficiency ratio (FER) indicated that HFD-fed mice were more efficient to convert food intake into body weight gain than NCD-fed mice even though their daily food intake was lower (Fig. 1B). Furthermore, eWAT (epididymal white adipose tissue) weight was markedly increased in HFD-fed mice (Fig. 1C), coupled with enhanced liver weight, which correlated with elevated liver triglyceride levels (Fig. 1D). Compared to NCD-fed mice, HFD-fed mice had higher plasma glucose, total cholesterol, and triglyceride levels (Fig. 1E).

To further investigate intestinal changes during HFD-induced obesity, we analyzed the expression of genes associated with glucose absorption, cholesterol transport, fatty acid transport and chylomicron formation in the jejunum of NCD-and HFD-fed mice using quantitative real-time PCR. The jejunum of HFD-fed mice exhibited increased expression of glucose absorption-related genes, such as *Sglt1* and *Glut2*, but not *Glp1*, compared to the NCD group (Fig. 2). Similarly, genes involved in cholesterol absorption (*Npc1l1*), fat absorption (*Fatp4* and *Cd36*), and chylomicron production (*Mogat2*, *Apobec1*, *Mttp*, *Dgat1*, *Acat2*) were upregulated in the jejunum of HFD group (Fig. 2). Thus, the jejunum of mice subjected to a high-fat diet exhibited substantial activation of genes linked to nutrient absorption and chylomicron synthesis, suggesting the involvement of the intestine in obesity-related metabolic dysfunction.

The relationship between cytokine expression associated with intestinal inflammation and homeostasis and systemic glucose and lipid levels.

As intestinal immune responses have been implicated in the development of metabolic dysfunction linked to obesity[45], we investigated the expression of intestinal cytokines in a diet-induced obese condition. The mRNA expression levels of the proinflammatory cytokine (*Tnfa*) and the epithelial cell-derived cytokines associated with maintenance of intestinal homeostasis (*Il25*, *Tslp*, *Il33*) were analyzed in jejunum of NCD- and HFD-fed mice using quantitative real-time PCR. The HFD group exhibited increased *Tnfa* expression relative to the NCD group, alongside a significant decrease in *Il25* mRNA expression, whereas *Tslp* and *Il33* levels were unchanged

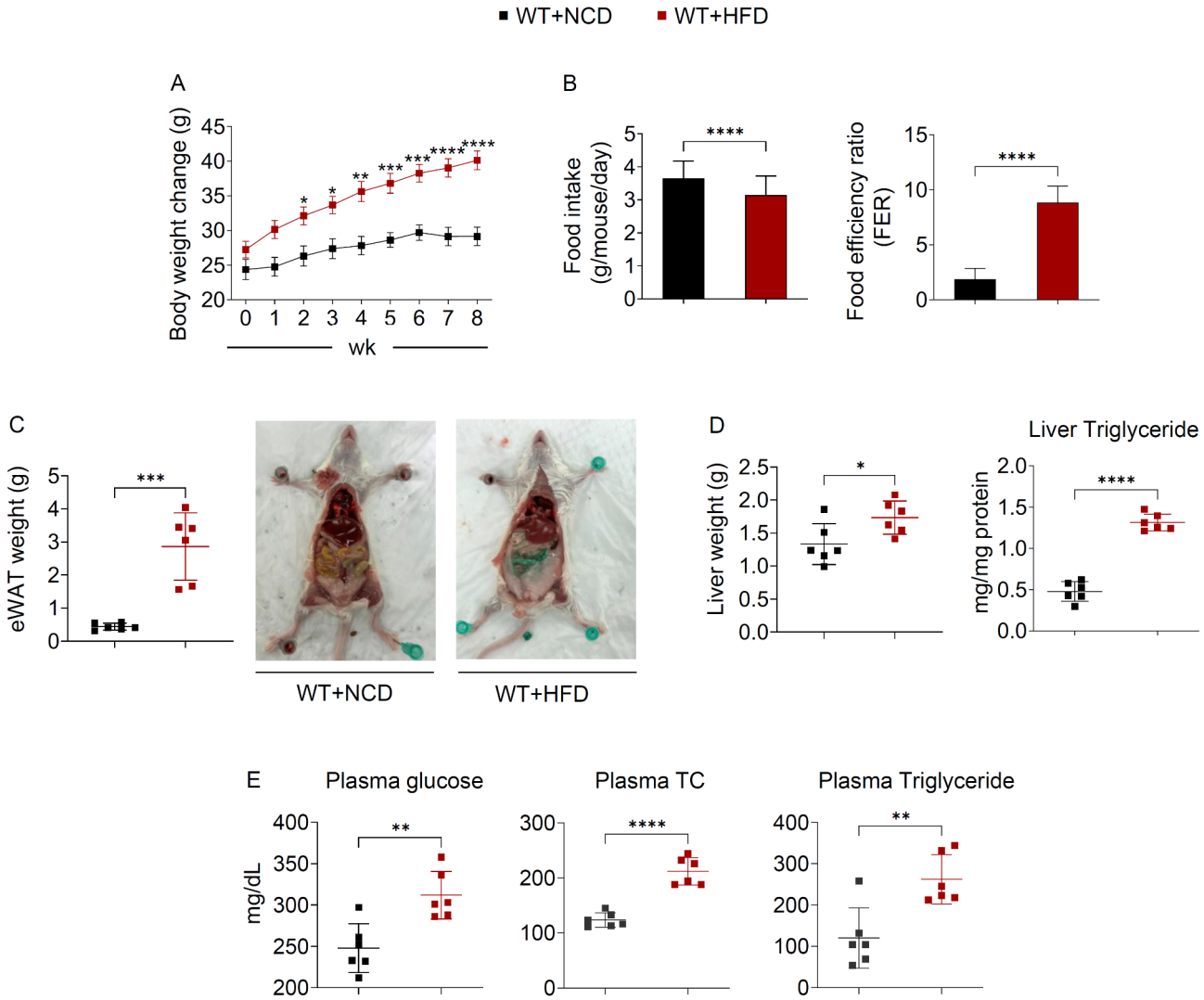

Fig. 1. **Systemic metabolic changes in high-fat diet-induced obesity of BALB/c mice.** BALB/c mice were fed with NCD or HFD for 8 weeks and metabolic changes were evaluated. (A) Body weight gain (g) was measured weekly between each group. (B) Food intake (g/mouse/day) and Food efficiency ratio (FER) were calculated as the ratio of body weight gain (g/day) to food intake (g/day), multiplied by 100, for each group. (C) eWAT weight (g) and representative images of abdominal fat in each group after 8 weeks of feeding. (D) Liver weight (g) and the amounts of liver triglycerides (mg/mg protein) in each group after 8 weeks of feeding. (E) Plasma concentration of glucose, total cholesterol (TC), and triglyceride (mg/dL) in each group after 8 weeks of feeding. Graphs depict mean ± SD of three independent experiments, with $n = 6$ mice per group. Significance was determined using two-way ANOVA with Dunnett's multiple comparisons test (Fig. 1A) and Student's t-test analysis (Fig. 1B-E) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

(Fig. 3A). We further assessed the association between the expression levels of these cytokines in the intestine and the levels of plasma glucose, total cholesterol, and triglycerides. Spearman's correlation analysis revealed a strong negative association between the expression of *Il25* and plasma glucose (r=-0.8571, $p = 0.0025$), plasma total cholesterol (r=-0.8085, $p = 0.0065$), and plasma triglyceride (r=-0.7781, $p = 0.0108$). In contrast, no significant association was found between these parameters and intestinal *Tnfa* gene expression (Fig. 3B). Moreover, Spearman's correlation analysis demonstrated a significant negative correlation between *Il25* expression and the cholesterol absorption-related gene *Npc1l1* (r=-0.7781, $p = 0.0107$) in the jejunum of HFD-fed mice (Fig. 3C), but not with glucose absorption-related gene *Sglt1* (r=-0.3040, $p = 0.3910$) or fat absorption-related genes *Fatp4* (r=-0.5228, $p = 0.1246$) and *Cd36* ($r = 0.07879$, $p = 0.8382$) (Fig. 3C). These findings suggested that the downregulation of intestinal cell-derived cytokines *Il25* in obesity may contribute to systemic lipid and glucose metabolism changes.

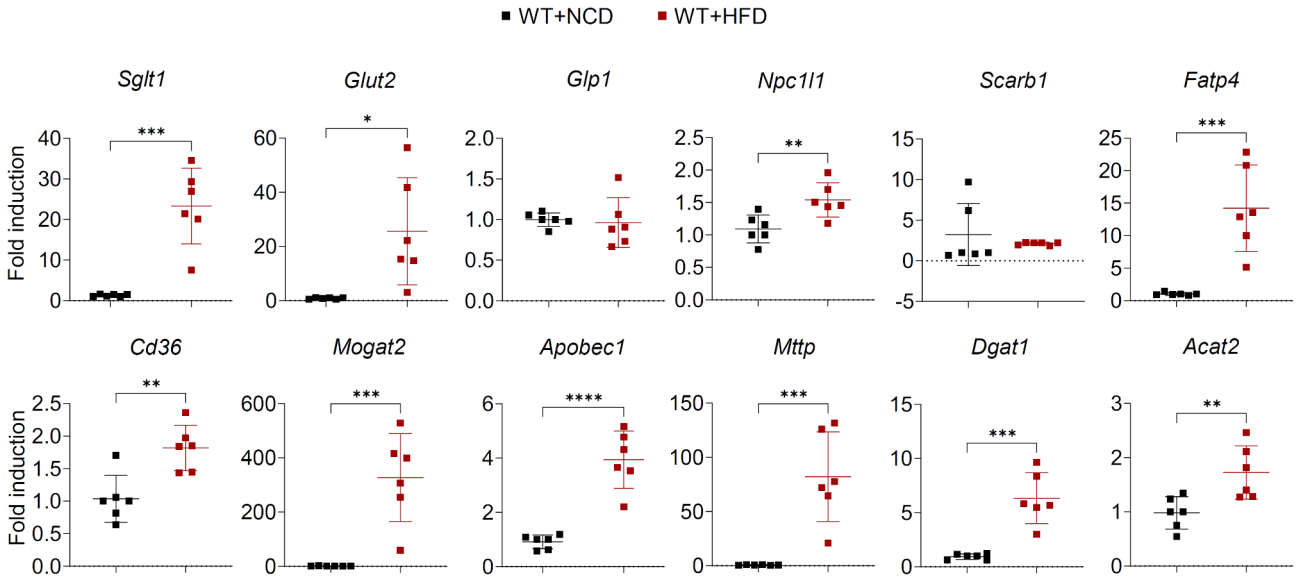

Fig. 2. Alterations in intestinal glucose and lipid absorption gene expression in BALB/c mice with high-fat diet-induced obesity. Quantitative real-time PCR analysis of glucose absorption (*Sglt1*, *Glut2*, *Glp1*), cholesterol transport (*Npc1l1*, *Scarb*), fatty acid transport (*Fatp4*, *Cd36*) and chylomicron production (*Mogat2*, *Apobec1*, *Mttp*, *Dgat1*, *Acat2*) in the jejunum of NCD-and HFD-fed mice after 8 weeks of feeding. The mRNA expression data are presented as fold induction over actin (*Actb*) expression, with the mRNA levels in NCD-fed mice set as 1. Graphs depict mean ± SD of three independent experiments, with $n = 6$ mice per group. Significance was determined using Student's t-test analysis (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

Intestinal IL-25 overexpression prevented metabolic alterations in a HFD-induced obesity model

Given that intestinal IL-25 expression has been linked to alteration in systemic lipid and glucose metabolism changes, we further investigated whether intestinal IL-25 overexpression influenced systemic glucose and lipid metabolisms. The iFABP IL-25 transgenic mice, which IL-25 specifically overexpressed in the epithelial cells of the mucosal layer of the small intestine[46], and their littermate controls were subjected to a high-fat diet for approximately 8 weeks to evaluate systemic and intestinal metabolic changes. These transgenic mice demonstrated a significant elevation in IL-25 levels exclusively in the jejunum, with no alterations in other organs, such as the liver and eWAT, nor in systemic blood levels, as observed by the unchanged serum IL-25 levels relative to wild-type littermate controls (Supplementary Fig. S1). Moreover, iIL-25 transgenic mice on a NCD exhibited no significant differences in body weight changes, food intake, food efficiency ratio, the weight of eWAT and liver, liver triglyceride levels, or plasma levels of glucose, total cholesterol and triglycerides when compared to NCD-fed wild-type littermate controls, suggesting normal metabolic homeostasis was maintained (Supplementary Fig. S2). Compared with littermate controls, iIL-25tg mice fed with HFD exhibited a significant reduction in body weight changes from week 5 of the feeding period (Fig. 4A) with comparable daily food intake (Fig. 4B) and a marked reduction in food efficiency ratio (Fig. 4B). Additionally, the weight of eWAT and liver as well as liver triglyceride levels in iIL-25tg mice were significantly reduced compared with control mice (Fig. 4C-D). Interestingly, compared to control mice given HFD, iIL-25tg mice greatly diminished plasma glucose, total cholesterol and triglyceride levels (Fig. 4E). Altogether, our data indicated that intestinal IL-25 expression influences systemic lipid and glucose metabolic alteration.

Overexpression of intestinal IL-25 modulates the expression of intestinal genes involved in nutrient absorption and chylomicron formation during HFD-induced obesity

To better understand the link between IL-25 expression and the absorption of glucose and cholesterol in the intestine, we analyzed the expression of genes involved in nutrient absorption and formation of chylomicrons in the jejunum of iIL-25tg mice and littermate control, both fed with HFD. Notably, compared with HFD-fed littermate control group, the jejunum from HFD-fed iIL-25tg showed a reduced expression of genes involved in glucose absorption (*Sglt1*, *Glut2*), cholesterol absorption (*Npc1l1*), fatty acid absorption (*Fatp4*, *Cd36*), and formation of chylomicrons (*Mogat2*, *Apobec1*, *Mttp*, *Dgat1*) (Fig. 5A). As we observed a significant negative correlation between *Il25* expression and the cholesterol absorption-related gene *Npc1l1* in the jejunum of HFD-fed mice, we further performed western blot analysis to compare the NPC1L1 protein expression in jejunal tissue lysates between the HFD-fed iIL-25tg group and the HFD-fed littermate control group. Consistent with mRNA expression, we observed a markedly diminished level of NPC1L1 protein expression in the jejunum of HFD-fed iIL-25tg mice compared to the HFD-fed littermate control group (Fig. 5B). Thus, overexpression of IL-25 in the intestine decreased systemic lipid and glucose metabolic levels, and the expression of intestinal genes involved in nutrient absorption and formation of chylomicrons during HFD-induced obesity. Together with the above

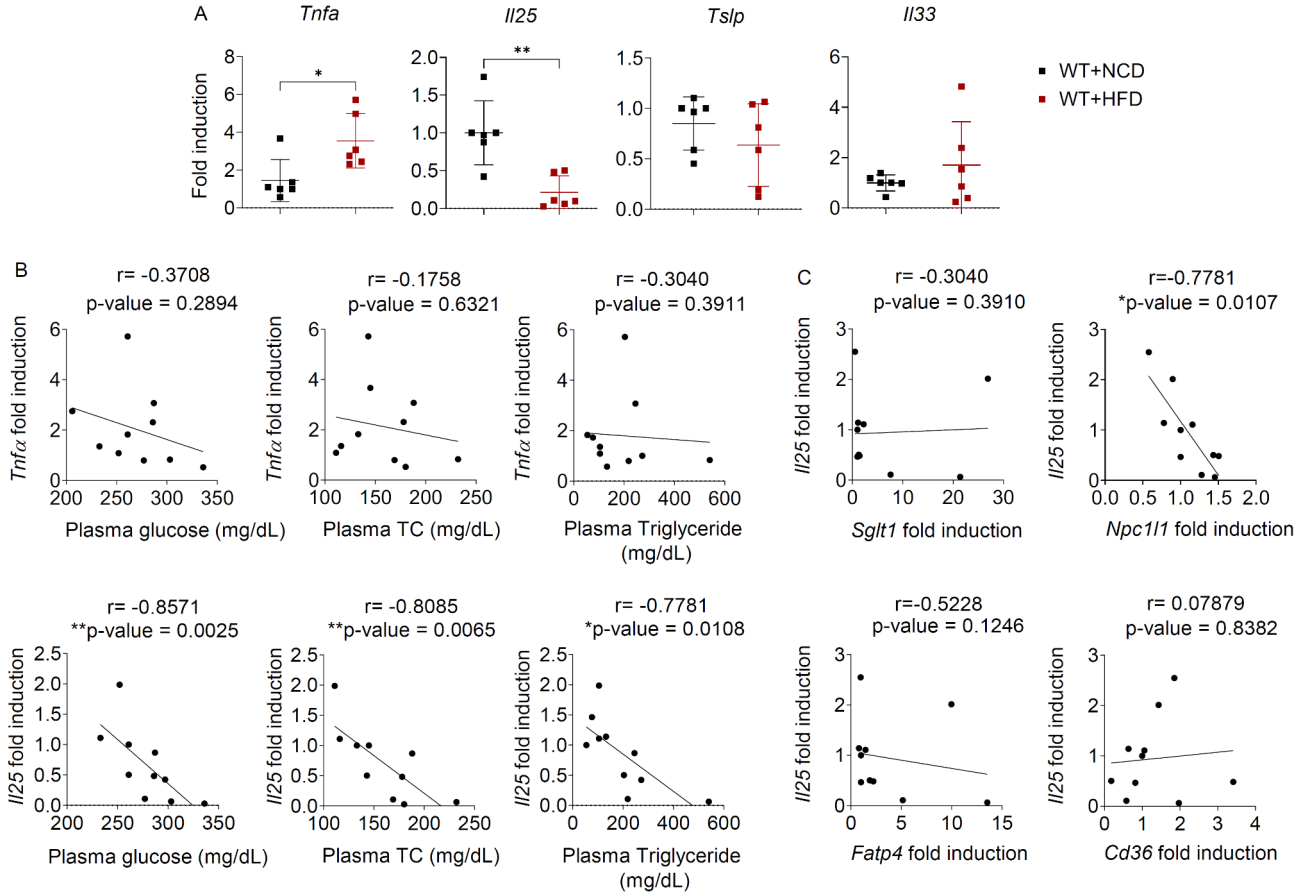

Fig. 3. The association between intestinal IL-25 expression and the systemic and intestinal metabolic changes in high-fat diet-induced obesity. BALB/c mice were fed with NCD or HFD for 8 weeks. The alteration of intestinal cytokine gene expression was determined and associated with various metabolic parameters. (A) Quantitative real-time PCR analysis of proinflammatory cytokine (*Tnfa*) and epithelial cell-derived cytokines (*Il25*, *Tslp*, *Il33*) in the jejunum of NCD-and HFD-fed mice. The mRNA expression data are presented as fold induction over actin (*Actb*) expression, with the mRNA levels in NCD-fed mice set as 1. Graphs depict mean ± SD of three independent experiments, with $n = 6$ mice per group. Significance was determined using Student's t-test analysis. (B) The correlation between plasma glucose, total cholesterol (TC), and triglycerides (mg/dL) and the mRNA expression levels of intestinal cytokines *Tnfa* (upper row) and *Il25* (bottom row), with $n = 10$ mice from HFD group was analyzed using Spearman's rank test. (C) The association between the expression level of *Il25* gene and genes related to glucose and lipid absorption in the jejunum, including *Sglt1*, *Npc1l1*, *Fatp4*, *Cd36*, with $n = 10$ mice from HFD group was analyzed using Spearman's rank test. Correlation coefficients (r) and p values are provided. ($^{*}p < 0.05$, $^{**}p < 0.01$)

findings, reduced levels of IL-25 in the intestines during HFD-induced obesity may influence systemic lipid and glucose metabolisms by altering the expression of nutrient absorption transporter and lipid metabolism in the small intestine.

IL-25 treatment reduced cholesterol uptake in human intestinal Caco-2 cell model

The negative association between the expression of IL-25 and cholesterol absorption-related gene *Npc1l1*, coupled with reduced intestinal *Npc1l1* expression and plasma cholesterol in iIL-25tg mice, prompted us to investigate whether IL-25 may regulate cholesterol absorption in the intestinal epithelial cells. To further investigate the function of IL-25 in controlling intestinal cholesterol metabolism, the intestinal epithelial Caco-2 cells were treated with human recombinant IL-25 for 24 h. These cells were then incubated with fluorescent cholesterol analog 25-NBD-cholesterol for 30 min, followed by measuring cholesterol uptake using flow cytometry analysis. Compared to the untreated control, we found that IL-25 treatment inhibited the cholesterol uptake in Caco-2 cells, as evidenced by decreased mean fluorescence intensity and the percentage of 25-NBD-cholesterol in the IL-25 treatment group (Fig. 6A). We also assessed the mRNA expression of genes related to the cholesterol uptake (*Npc1l1*, *Scarb1*), fatty acid transporter (*Cd36*, *Fatp4*), and cholesterol efflux (*Abca1*, *Abcb1*, *Abcg5*, *Abcg8*) in Caco-2 cells using quantitative real-time PCR. As shown in Fig. 6B, treatment with IL-25 decreased the mRNA expression of *Npc1l1* but not that of other genes. Our findings revealed that IL-25 expression may reduce cholesterol absorption in the intestine by downregulating NPC1L1 expression.

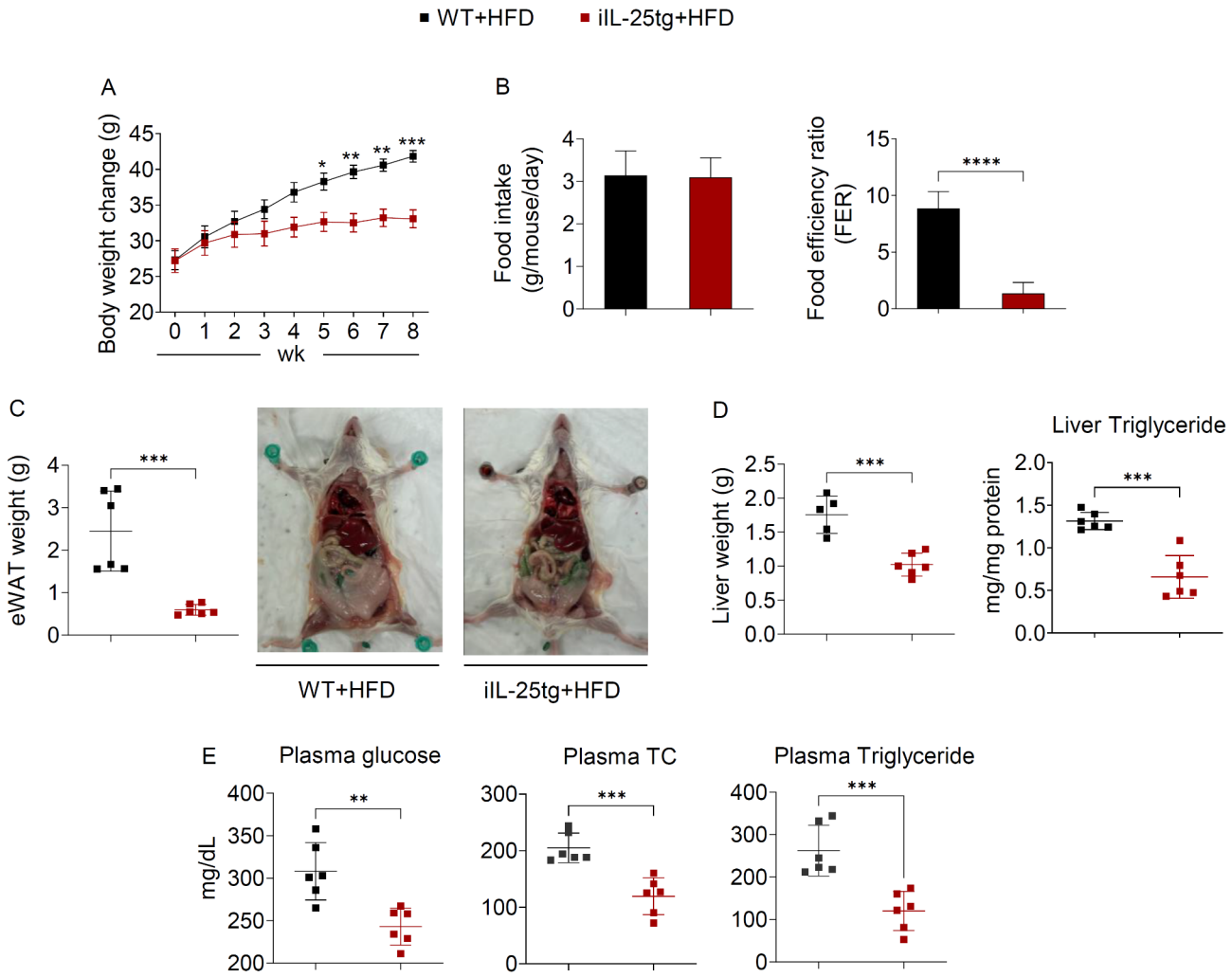

Fig. 4. The effect of high-fat diet feeding on systemic metabolic changes in iIL-25 transgenic mice. iIL-25tg or littermate control BALB/c mice were fed with HFD for 8 weeks and metabolic changes were evaluated. (A) Body weight gain (g) was measured weekly between each group. (B) Food intake (g/mouse/day) and Food efficiency ratio (FER) were calculated for iIL-25tg or littermate control BALB/c mice fed with HFD by dividing body weight gain (g/day) by daily food intake (g/day), multiplied by 100. (C) eWAT weight (g) and representative picture of abdominal fat in each group after 8 weeks of feeding. (D) Liver weight (g) and the amounts of liver triglycerides (mg/mg protein) in each group after 8 weeks of feeding. (E) Plasma concentration of glucose, total cholesterol (TC), and triglyceride (mg/dL) in each group after 8 weeks of feeding. Graphs depict mean ± SD of three independent experiments, with $n = 6$ mice per group. Significance was determined using two-way ANOVA with Dunnett's multiple comparisons test (Fig. 4A) and Student's t-test analysis (Fig. 4B-E) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

IL-25 regulates NPC1L1 expression by downregulating the transcription factor SREBP2 in Caco-2 cells

Considering that IL-25 was found to decrease cholesterol absorption in Caco-2 cells, we elucidated the mechanisms by which IL-25 modulates the expression of cholesterol transporter *Npc1l1* that essential for promoting cholesterol absorption[47]. We conducted western blot analysis to validate the mRNA expression analysis of *Npc1l1* in Caco-2 cells. Corresponding to the mRNA results, we observed a reduction in NPC1L1 protein expression in these cells treated with IL-25 (Fig. 7A). Previous studies have shown that transcription factors, such as *Hnf4a* and *Srebp2*, are involved in regulating the expression of *Npc1l1* in Caco-2 cells[48]. In addition, the transcription factor *Hnf1a* has been found as a regulator of *Npc1l1* expression in human liver HuH7 cells[49]. To explore potential regulatory mechanisms, we examined the mRNA expression of *Hnf1a*, *Hnf4a* and *Srebp2* in Caco-2 cells, both with and without IL-25 treatment. Although the mRNA levels of *Hnf1a* and *Hnf4a* expression remained unchanged, IL-25 treatment resulted in a significant reduction in *Srebp2* mRNA expression (Fig. 7B). The protein expression of SREBP2 was further assessed in Caco-2 cells from each condition group. IL-25 treatment significantly reduced the protein expression of mature form of SREBP2 (mSREBP2), whereas the expression of the precursor form of SREBP2 (pSREBP2) remained unchanged (Fig. 7C). Additionally, we

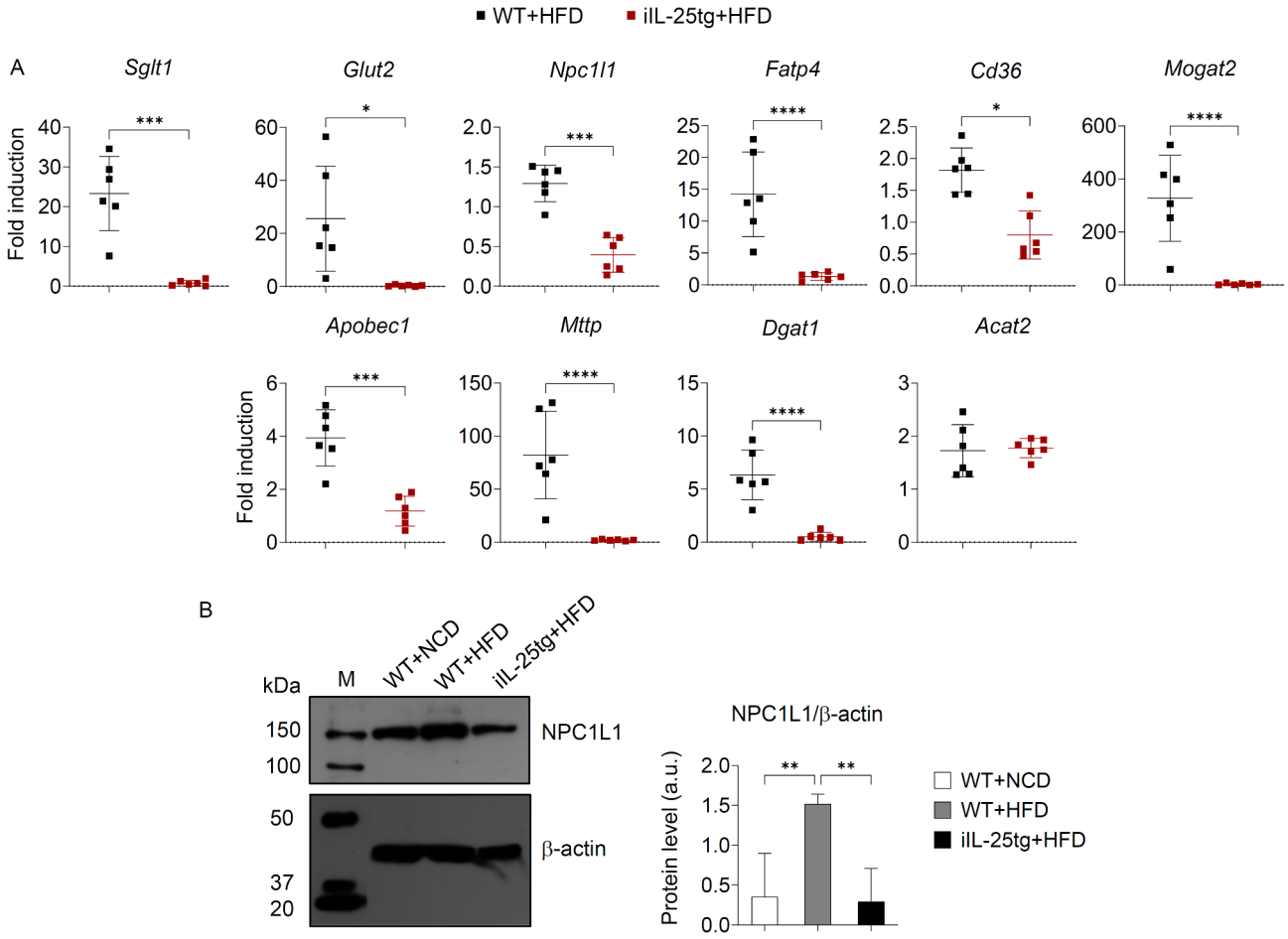

Fig. 5. Overexpression of intestinal IL-25 promoted the expression of gene related to the lipid and glucose absorption in the intestine during high-fat diet-induced obesity. iIL-25tg or littermate control BALB/c mice were fed with HFD for 8 weeks and the expression of gene involved in nutrient absorption and chylomicron production was evaluated in the jejunum. (A) Quantitative real-time PCR analysis of glucose absorption (*Sglt1*, *Glut2*), cholesterol transport (*Npc1l1*), fatty acid transport (*Fatp4*, *Cd36*) and chylomicron production (*Mogat2*, *Apobec1*, *Mttp*, *Dgat1*, *Acat2*) in the jejunum of iIL-25tg or littermate control HFD-fed mice. The mRNA expression data are presented as fold induction over actin (*Actb*) expression, with the mRNA levels in littermate control NCD-fed mice set as 1. Graphs depict mean ± SD of three independent experiments, with $n = 6$ mice per group. (B) Western blot analysis of NPC1L1 and β-actin in the jejunal lysate of HFD-fed iIL-25tg or littermate control mice compared with NCD-fed littermate control mice. Lane M represents molecular weight protein marker (kDa). The molecular weights of NPC1L1 and β-actin are 145 kDa and 42 kDa, respectively. Bar graph represents the protein level measured by the intensity of protein bands in arbitrary units. The intensity of β-actin expression was normalized with that of NPC1L1 expression. The full-length blots are presented in Supplementary Fig. S4. Graphs depict mean ± SD of three independent experiments. Significance was determined using Student's t-test and one-way ANOVA followed by Turkey post hoc analysis (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

assessed the influence of IL-25 treatment on the expression of SREBP2 target genes, such as *Hmgcr* and *Ldlr*, in Caco-2 cells. Following IL-25 treatment, we observed a decrease in the mRNA expression of *Hmgcr*, but *Ldlr* expression remained unaffected (Fig. 7D). Our findings suggest the potential involvement of IL-25 in regulating transcription factor for NPC1L1 (SREBP2) and its target gene *Hmgcr*. Altogether, these data indicated that IL-25 may modulate NPC1L1 expression by inhibiting mature SREBP2 levels in the intestinal epithelial Caco-2 cells.

Discussion

Recent studies have demonstrated the role of intestinal immunity in the regulation of metabolic disorders, including obesity[16,50,51]; however, the contribution of intestinal cytokines to systemic lipid metabolism in the context of diet-induced obesity remains unclear. In our study, a high-fat diet altered not only systemic lipid and glucose metabolic levels but also the expression of intestinal cytokines. The reduced expression of intestinal IL-25 was negatively associated with elevated systemic lipid and glucose metabolic levels in HFD-induced obesity. The transgenic overexpression of IL-25 in intestinal epithelial cells altered whole-body metabolism and influenced the expression of genes associated with nutrient absorption and cholesterol transport. IL-25 treatment in human

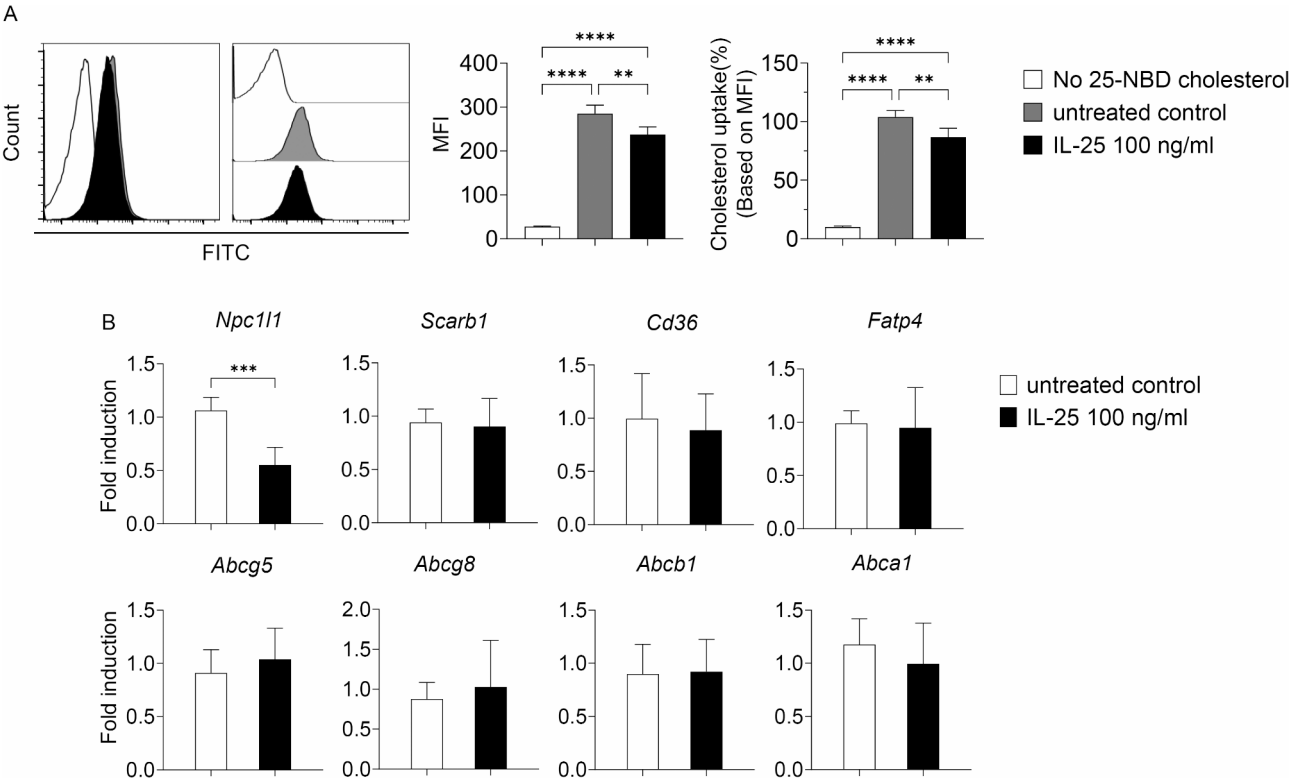

Fig. 6. The effect of IL-25 treatment on cholesterol uptake in Caco-2 cells. Caco-2 cells were cultured in 24-well plate for 21 days. Cells were treated with or without 100 ng/mL human recombinant IL-25 for 24 h before analysis of cholesterol uptake using 25-NBD cholesterol by flow cytometric analysis and gene expression by quantitative real time PCR. (A) Representative histogram and graph show mean fluorescence level (MFI) and percentage (%) of cholesterol uptake in Caco-2 cells. (B) Quantitative real time PCR analysis showed the mRNA expression level of genes related to cholesterol uptake transport (*Npc1l1*, *Scarb1*), fatty acid transporter (*Cd36*, *Fatp4*), and cholesterol efflux (*Abcg5*, *Abcg8*, *Abca1*, *Abcb1*). Data are presented as fold induction over actin (*Actb*) expression, with the mRNA levels in the control untreated cell set as 1. Graphs depict mean ± SD of three independent experiments. Significance was determined using one-way ANOVA followed by Turkey post hoc analysis and Student's t-test (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

intestinal Caco-2 cells resulted in a reduction in cholesterol uptake and decreased expression of the cholesterol absorption NPC1L1, along with related transcription factor SREBP2.

In our study, BALB/c mice subjected to a high-fat diet exhibited increased body weight gain, lipid accumulation in both eWAT and liver, elevated plasma lipid profile and glucose levels, and significant alterations in the intestine, including the expression of cytokines and genes involved with glucose, cholesterol and fat absorption, as well as chylomicron formation. Previous research has suggested that obesity is linked to the intestinal inflammation[16,19,29]. A prior study revealed a strong positive correlation between ileal *Tnfa* mRNA expression and plasma glucose levels in C57BL/6 mice subjected to a HFD for 16 weeks[29]. While we observed an increase in the mRNA expression of the proinflammatory cytokine *Tnfa* in the jejunum of HFD-fed mice, we did not detect any correlation between *Tnfa* expression and the levels of plasma lipid and glucose. The discrepancy results may be attributed to the difference in the mouse strain used in our investigation, BALB/c mice, a strain recognized for its Th2-type immune response[52]. C57BL/6 mice exhibit greater susceptibility to diet-induced obesity and glucose intolerance than other strains[53,54]. Moreover, the duration of HFD feeding may also account for the observed discrepancies. Besides the expression of *Tnfa* in the jejunum, we also evaluated additional cytokines related to the regulation of intestinal immune response and revealed a decrease in intestinal IL-25 expression in HFD-induced obesity. Previous reports indicated a reduction in IL-25 and TSLP mRNA expression in the intestinal tuft cells following HFD feeding, suggesting the involvement of intestinal tuft cells in metabolic regulation[55]. Correlation analysis revealed an inverse relationship between intestinal *Il25* mRNA levels and plasma glucose, total cholesterol, and triglyceride levels. More importantly, we found a significant negative correlation between intestinal *Il25* and *Npc1l1* mRNA levels, suggesting that alterations within the intestine, particularly IL-25 expression, may influence systemic metabolism during obesity by regulating the expression of genes associated with nutrient absorption and cholesterol transport in the intestine.

Transgenic mice that overexpressed IL-25 in intestinal epithelial cells and were subjected to a high-fat diet exhibited diminished body weight gain, decreased lipid accumulation, and reduced plasma levels of glucose, total cholesterol, and triglyceride. This systemic effect was accompanied by intestinal changes, including reduced expression of gene involved intestinal glucose absorption, cholesterol absorption, fatty acid absorption, and

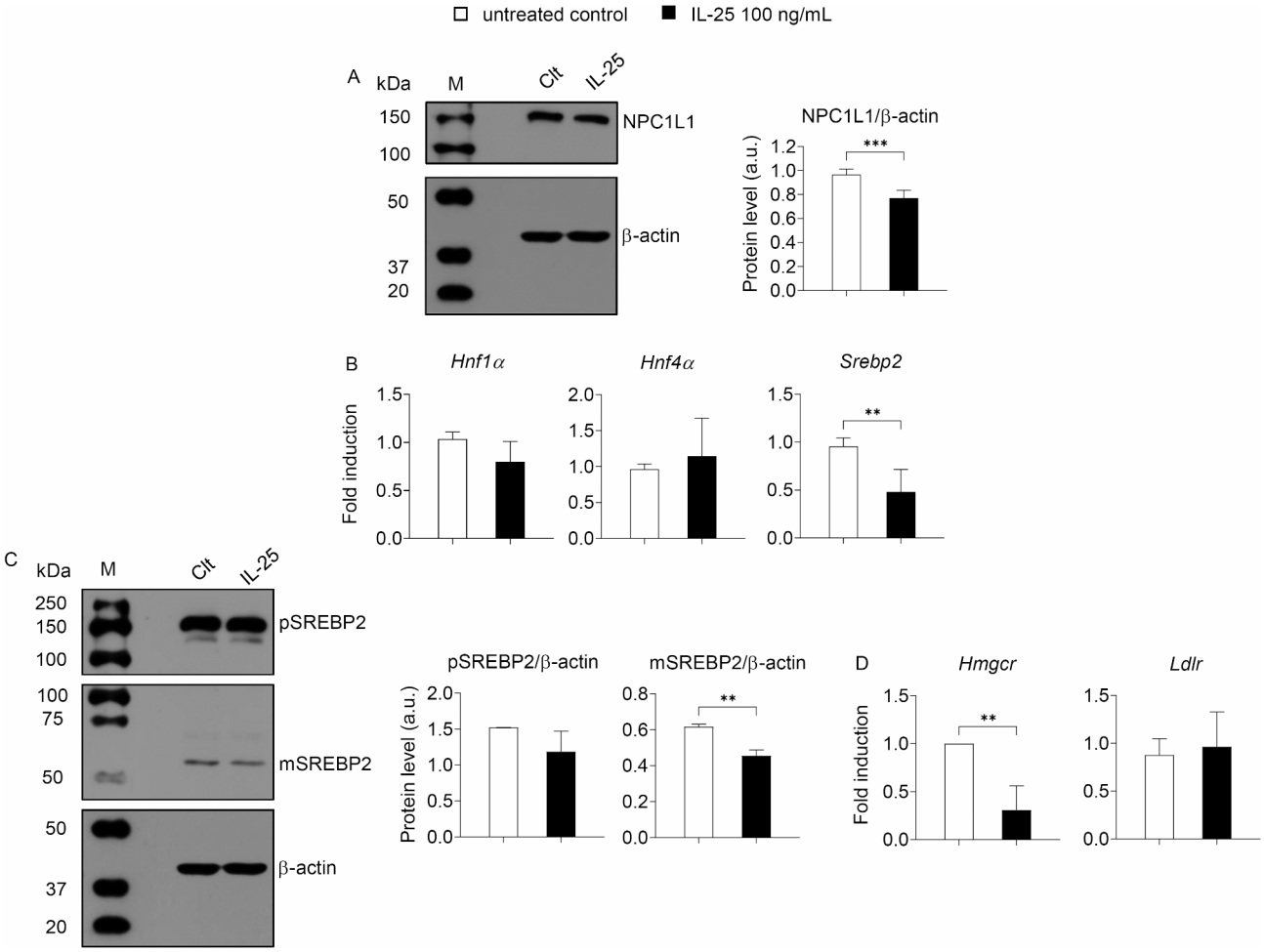

Fig. 7. The effect of IL-25 on the expression of NPC1L1 and their positive transcription regulator in Caco-2 cells. Caco-2 cells were cultured in 24-well plate for 21 days. Cells were treated with or without 100 ng/mL human recombinant IL-25 for 24 h. (A) Western blot analysis of NPC1L1 and β-actin. Lane M represents molecular weight protein marker (kDa). The molecular weights of NPC1L1 and β-actin are 145 kDa and 42 kDa, respectively. Bar graph represents the protein level by measuring intensity of protein bands in arbitrary units. The intensity of β-actin was normalized with the intensity of NPC1L1. The full-length blots are presented in Supplementary Fig. S5. (B) Quantitative real time PCR analysis of *Hnf1a*, *Hnf4a*, and *Srebp2* mRNA expression. Data are presented as fold induction over actin (*Actb*) expression, with the mRNA levels in the control untreated cell set as 1. (C) Western blot analysis of pSREBP-2, mSREBP-2 and β-actin. Lane M represents molecular weight protein marker (kDa). The molecular weights of pSREBP-2, mSREBP-2, and β-actin are 126 kDa, 55 kDa, and 42 kDa, respectively. Bar graph represents the protein level by measuring intensity of protein bands in arbitrary units. The intensity of β-actin was normalized with the intensity of pSREBP-2 and mSREBP-2. The full-length blots are presented in Supplementary Fig. S5. (D) Quantitative real time PCR analysis of *Hmgcr* and *Ldlr* mRNA expression. Data are presented as fold induction over actin (*Actb*) expression, with the mRNA levels in the control untreated cell set as 1. Graphs depict mean ± SD of three independent experiments. Significance was determined using Student's t-test (**$p < 0.01$, ***$p < 0.001$).

formation of chylomicrons. These data suggested that the intestinal IL-25 may play a role in controlling nutrient absorption and lipid metabolism within the small intestine, thereby influencing systemic lipid and glucose metabolic alteration. Previous study demonstrated that the increased expression of IL-25 in small intestinal tuft cells was associated with improving insulin sensitivity and glucose tolerance, as well as reductions in liver weight, eWAT mass, body weight, fat to lean mass ratio, and GABA receptor expression in obese mice[55]. By analysis in lamina propria and eWAT, these alterations were shown to be unrelated to the involvement with type 2 immune cells[55]. It is likely that the intestinal IL-25 may exert their effects by modulating intestinal epithelial cell function and type 2 immune response. In addition to the association between obesity and changes in intestinal immune composition, intestinal dysbiosis, characterized by an imbalance in gut microbiota composition has been reported to promotes the pathogenesis of obesity in both murine models and humans[57–60]. Further studies are required to investigate whether intestinal IL-25 expression may alter microbiota composition, resulting in the regulation of intestinal metabolisms that mitigate systemic obesity-induced chronic low-grade inflammation. Besides IL-25 cytokine expression, the role of its binding receptors, IL-17RB and IL-17RA, in the intestine

during HFD-induced obesity remains unclear, necessitating further investigation to elucidate their involvement in intestinal metabolic homeostasis.

Although we observed no association between *Il25* and *Sglt1* mRNA expression in the intestines of HFD-fed mice, related study by Smith et al.[56] demonstrated that IL-25 treatment in HFD-fed C57BL/6 mice inhibited glucose absorption and *Sglt1* activity in the intestines, without altering intestinal *Sglt1* mRNA expression[56]. Similar to this finding, HFD-fed iIL-25tg mice exhibited lower plasma glucose levels and reduced mRNA expression of *Sglt1* and *Glut2* in the jejunum. Additionally, glucose uptake using the fluorescent glucose analog 6-NBDG and the mRNA expression of genes related to glucose transport and metabolism was reduced in Caco-2 cells that were treated with IL-25 (Supplementary Fig. S3), suggesting the importance of IL-25 in regulating intestinal glucose absorption and improving glucose homeostasis. Moreover, the effect of IL-25 in regulating cholesterol uptake in human intestinal epithelial Caco-2 cells was revealed. IL-25 treatment decreased cholesterol uptake and the expression of cholesterol transporter NPC1L1, along with their positive transcription regulator, the mature form of SREBP-2. This form of SREBP-2 has been shown to transcriptionally activate the NPC1L1 expression and involve with NPC1L-1-mediated cholesterol absorption in the Caco-2 cells[61]. It is likely that IL-25 influences the regulation of intestinal cholesterol absorption by downregulating NPC1L1 expression through SREBP2 activation, which contributes to decreased plasma cholesterol levels. In support of our findings, a previous study indicated that the overexpression of intestinal SREBP2 correlated with elevated serum cholesterol levels, suggesting that the intestine significantly contributes to the regulation of cholesterol homeostasis in the body[62]. Alternatively, the inhibition of NPC1L1 was also found to suppress fat absorption through downregulating intestinal FATP4 and improve glucose tolerance[63]. In this context, IL-25 may reduce intestinal expression of NPC1L1, thereby contributing to the maintenance of systemic glucose or lipid homeostasis. Intestinal IL-25 expression may regulate chylomicron formation; however, more investigations, including the measurement of plasma ApoB48 (Apolipoprotein B-48) levels, are necessary to elucidate the underlying mechanisms.

In conclusion (Fig. 8), this study demonstrated the role of intestinal IL-25 in modulating systemic lipid and glucose metabolism, nutrient absorption, and cholesterol transport, suggesting its potential as a therapeutic target for HFD-induced metabolic disorders. IL-25 expression in the intestine was associated with reduced body weight gain and lipid accumulation in the liver and eWAT. IL-25 significantly influenced intestinal function by reducing cholesterol absorption, potentially impacting systemic lipid metabolism. Further studies will explore how intestinal IL-25 signaling influences synthesis and secretion of chylomicrons by the intestine and the contribution of microbiota composition. These factors may collectively shape intestinal and systemic metabolism, providing protection against diet-induced obesity.

Materials and methods

Animals

Sex-matched male and female BALB/c mice, aged 6 to 8 weeks -old were purchased from Nomura Siam International Co., Ltd., Thailand. All genetically modified iFABP-IL-25 transgenic mice, sex-matched male and female mice, aged 6 to 8 weeks (kindly provided by Dr. Yui-Hsi Wang, University of Cincinnati, Cincinnati Children's Hospital Medical Center)[46] were bred and maintained under specific pathogen-free conditions within the animal facility of Thammasat University. The genotyping of these mice was validated using primers specific to iIL-25 transgenic mice, which included the forward primer (5′-CAGGACCGAATCTCTGCTTT-3′) and the reverse primer (5′-TCAAGTCCCTGTCCAACTCA-3′) as previously described[46,64]. All animal experiments were conducted in accordance with the relevant ARRIVE guidelines (https://arriveguidelines.org) and received approval from the Thammasat University Animal Care and Use Committee (019/2023).

In vivo mouse models of diet-induced obesity

The diet-induced obesity model was performed as previously described[65]. Mice ($n = 6$–10 per group) were fed either a normal chow diet with 10% energy from fat (Testdiet®, irradiated) or a high-fat diet with 60% energy from fat (Testdiet®, irradiated) for 8 weeks. Body weight and food intake were monitored weekly throughout the experiment. The food efficiency ratio (FER, %) was calculated by dividing body weight gain (g/day) by food intake (g/day) and multiplying by 100[66]. At the end of the feeding period, mice were sacrificed by CO_2 inhalation to assess metabolic changes by capturing a photo of fat accumulation in the abdominal cavity, collecting blood samples for measurements of glucose, triglyceride, and total cholesterol levels in plasma and weighing the eWAT and liver. Liver and jejunum tissues were excised, snap-frozen in liquid nitrogen, and kept at -80 °C for subsequent analysis.

Quantitation of liver triglyceride content

Triglyceride content in liver was measured according to previously described with some modifications[67]. Approximately 250 mg of liver tissue was washed with cold sterile PBS, homogenized with 1 mL of 5% IGEPAL (Sigma-Aldrich) on ice, and then gently heated at 95 °C for 30 min before being allowed to cool to room temperature. Homogenate tissue was centrifuged at 10,000 x g at 4 °C for 10 min. The supernatant was collected and diluted at 1:10 in sterile Ultrapure water (Type I) to determine protein concentration using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Triglyceride concentration was then determined with the Stanbio Triglyceride LiquiColor™ (Stanbio Laboratory) by measuring absorbance at 490 nm and calculating it to a standard curve (ranging from 1.5625 to 200 mg/mL). Liver triglyceride content was normalized to the total protein concentration of each sample.

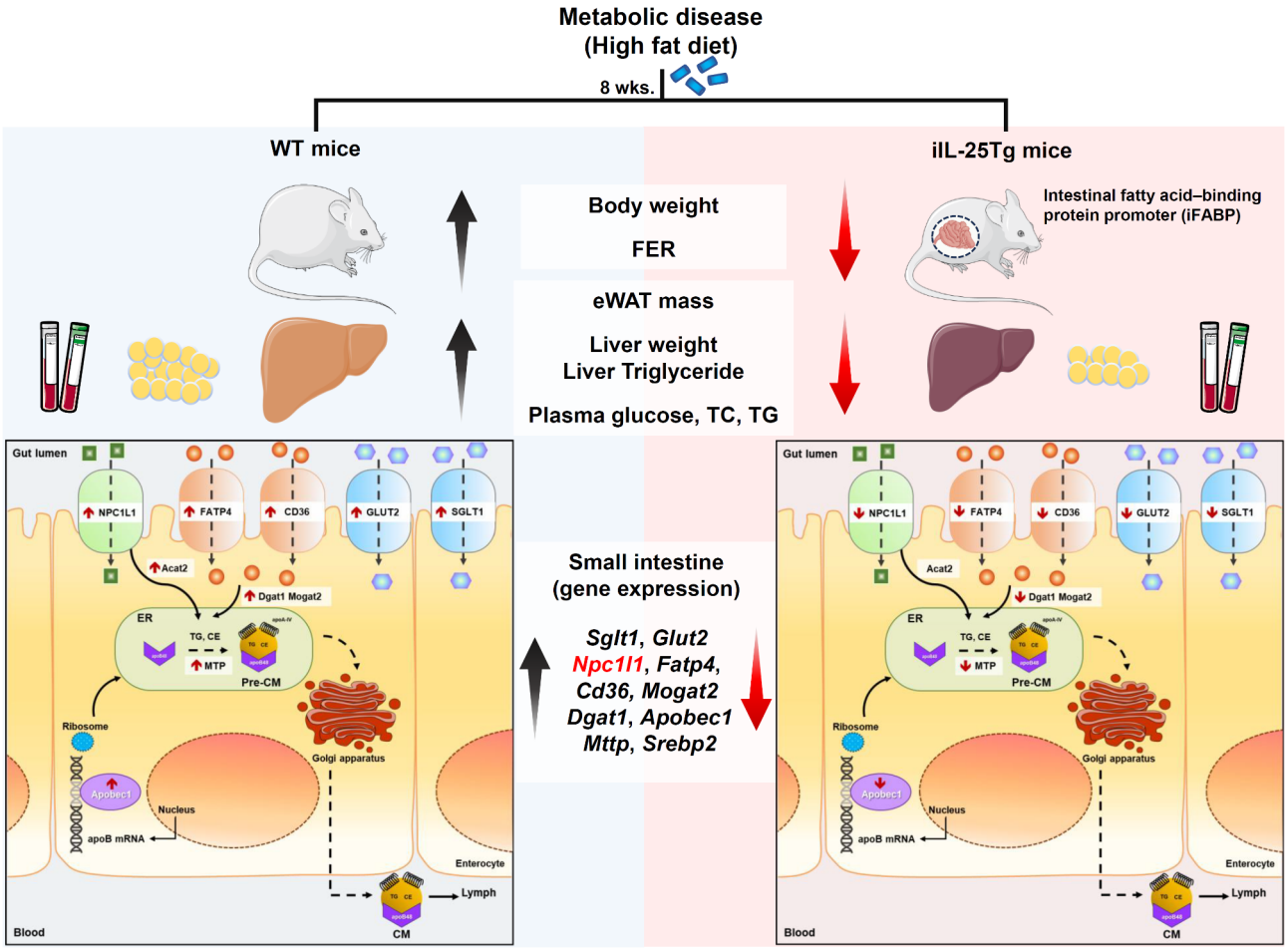

Fig. 8. A proposed role of IL-25 on systemic and intestinal metabolic changes in high-fat diet-induced obesity BALB/c mice. Intestinal IL-25 plays a crucial role in modulating systemic lipid and glucose metabolism. Moreover, IL-25 regulates intestinal function by decreasing cholesterol absorption, which may lead to systemic lipid metabolic changes. These findings suggest that targeting IL-25 could offer a strategy for modulating metabolic dysfunction associated with obesity.

Measurement of blood chemistry

Blood samples from mice in each condition were centrifuged at 3,000 rpm at 25 °C for 10 min to collect plasma samples. Plasma glucose levels were measured using an enzymatic method, while total cholesterol and triglyceride levels were determined via enzymatic colorimetric method on a Mindray BS620M analyzer, according to the manufacturer's instructions.

RNA extraction and quantitative RT-PCR

Jejunum tissue or Caco-2 cell samples were homogenized in the TRIzol® reagent (Invitrogen) to isolate total RNA, following the manufacturer's instructions. Total RNA (1 ng to 5 μg) was used to synthesize cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific™) which includes oligo dT primer, 10 mM dNTP mix, 5X Reaction buffer, RiboLock RNase Inhibitor and RevertAid M-MuLV Reverse Transcriptase. To assess the levels of target mRNA, cDNA samples were subjected to amplification using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories) with primers listed in Supplementary Table S1 on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). Endogenous actin (*Actb*) mRNA served as an internal control to normalize the expression levels of target.

In vitro effect of IL-25 on cholesterol uptake in Caco-2 cells

Human epithelial cell lines (Caco-2 cells, ATCC HTB-37) were grown in high-glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco™), supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S). Cells were maintained at 37 °C in a humidified chamber with 5% CO_2 as previously described[64]. Confluent cells (85–90%) were utilized for subsequent experimental analysis. The effect of IL-25 on cholesterol uptake in Caco-2 cells was evaluated as previously described with some modifications[68]. Briefly, Caco-2 cells were seeded at a density of 2×10^5 cells/well in 24-well plate, with the medium replaced every 2 days, and cultured for 21 days until reaching full confluence. After 21 days of incubation, cells were

incubated in serum-free medium for 2 h and treated with recombinant human IL-25 (IL-17E) as prepared in complete DMEM (100 ng/mL, R&D system) for 24 h. The concentration of IL-25 at 100 ng/mL was selected based on a previous study investigating the effect of IL-25 on primary intestinal epithelial cells[64]. Endotoxin levels in recombinant human IL-25 were validated by the manufacturer to be less than 0.10 EU/μg of protein, as determined using the Limulus Amebocyte Lysate (LAL) assay. To assess cholesterol uptake, Caco-2 cells were washed twice with sterile DPBS, resuspended in serum-free medium containing 10 μM 25-NBD cholesterol (Avanti® Polar Lipid, Inc.) and incubated at 37 °C for 30 min. The cells were then rinsed twice with cold sterile DPBS and harvested using with 0.25% trypsin-EDTA. After harvesting, cells were stained with propidium iodide (PI, Biolegend) and performed flow cytometry analysis using a BD FACSLyric cytometer (BD Biosciences). The collected data were analyzed with FlowJo™ Software (Tree Star, Inc.). Cholesterol uptake was expressed as the mean fluorescence intensity (MFI) and calculated by dividing the MFI value of each sample by the MFI value of untreated control samples, then multiplying with 100.

Western blot analysis

For protein expression analysis, Caco-2 cells from different treatment groups were washed twice with sterile DPBS and homogenized in cold modified RIPA buffer (50 mM Tris–HCl pH 7.4, 1% Triton X-100, 0.2% sodium deoxycholate, 0.2% sodium dodecylsulfate (SDS), 1 mM EDTA) containing 1 mM PMSF (Phenylmethanesulfonyl fluoride, Sigma-Aldrich) and protease inhibitor cocktail (Roche). Cell lysates were collected by centrifugation 12,000 rpm at 4 °C for 20 min. For western blot analysis, 30 μg protein lysates were separated by 8% SDS-PAGE (BioRad Laboratories) and transferred electrophoretically to a PVDF (Polyvinylidene fluoride) membrane (Millipore). The membrane was then blocked with 5% nonfat milk (Bio Basic) and incubated with the primary antibodies specific for NPC1L1 (1:1,000, Santa Cruz Biotechnology), SREBP2 (1:1,000, Abcam) at 4 °C with shaking at 80 rpm. After overnight incubation, the membrane was washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies, either goat anti-mouse IgG (heavy and light chain) (1:3,000, Bio-Rad Laboratories) or goat anti-rabbit IgG (heavy and light chain) (1:2000, Cell Signaling Technology). Immunoblots were visualized using ECL substrate (Bio-Rad Laboratories) and exposed to autoradiography Films (Kodak X-Ray Film, ABM Good). For loading control determination, the membrane was incubated in stripping buffer (1 M Tris pH 6.8, 20%SDS, β-mercaptoethanol) at 56 °C for 15 min. After washing with 1XTBST, the membrane was then blocked with 5% nonfat milk (Bio Basic) and incubated with the HRP-conjugated β-actin mouse monoclonal antibody (1:10,000, #AC-15, Sigma-Aldrich) at 4 °C with shaking at 80 rpm for 1 h. After washing with TBST, chemiluminescent detection is used to visualize the protein bands. The intensity of the specified band was measured using ChemiDoc Imaging System (Bio-Rad Laboratories). Protein quantification was performed using Image Lab software, with normalization to β-actin as the internal control.

Statistical analysis

Each experiment was performed at least three times. All data are presented as the mean value ± SD. Data were analyzed using the unpaired t test (two-tailed), the one-way ANOVA with Turkey's post hoc analysis, and two-way ANOVA followed by Dunnett's multiple comparisons test. The correlation study was conducted using Spearman's rank correlation coefficient. Statistical analysis was conducted using GraphPad Prism 10 Software, and a p-value of < 0.05 was considered statistically significant.

Data availability

All data are included in the manuscript and the supplementary information and can be obtained from the corresponding author upon request.

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Author contributions

S.J. contributed to data curation, formal analysis, investigation, methodology, validation, visualization, writing-original draft and writing-reviewing & editing. P.A. contributed to conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, supervision, validation, visualization, writing-original draft and writing-reviewing & editing.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to P.A.