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High-Protein Diet Intervention Based on Whey Protein Supplementation Improves Weight Cycling by Reducing Metaflammation

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ABSTRACT: Background

Weight cycling, characterized by repeated weight regain after weight loss in individuals with obesity, exacerbates long-term metabolic burden and increases the risk of chronic diseases. Exploring intervention strategies based on food components is therefore of great significance.

Objective

This study investigates the effects of a high-protein diet (HPD) supplemented with whey protein on weight change and metaflammation, exploring its underlying molecular mechanisms.

Methods

Ninety-four subjects with obesity and weight cycling were recruited for a three-month HPD intervention supplemented with whey protein. Body composition, basal metabolism, and biochemical parameters were assessed pre- and post-intervention. A weight-cycling mouse model was used to compare the effects of HPD and calorie-restricted diet (CRD) interventions on metaflammation and weight cycling. Bioinformatics and machine learning were utilized to analyze adipose tissue metaflammation and immune microenvironment changes focusing on the regulation of biochemical pathways.

Results

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The HPD led to significant reductions in body weight, body fat percentage, waist-to-hip ratio, and visceral fat index, alongside improvements in muscle mass, blood glucose, lipid levels, and blood pressure ($P < 0.05$). The HPD significantly reduced systemic and adipose tissue metaflammation via the TLR4-NLRP3 pathway. Protein levels of NLRP3 and TLR4 in white adipose tissue were lower in the HPD and CRD groups compared to the weight cycling group ($P < 0.05$). Machine learning identified key genes predicting weight regain—IFNGR1 and SCRN2. Single-cell analysis revealed enriched macrophages and IFNGR1-high NK cells, suggesting their roles in metaflammation inhibition.

Conclusion

Supplementation of a high-protein diet with whey protein effectively reduces metaflammation, decreases body weight and fat, and improves metabolic health in individuals with obesity and weight cycling. These benefits are associated with downregulation of the TLR4-NLRP3 pathway and activation of the Wnt pathway. Metaflammation driven by M1 macrophages and NK cells is linked to weight cycling, with IFNGR1 and SCRN2 serving as potential markers for long-term weight control.

Keywords Whey protein supplementation, High protein diet, Weight cycling, Obesity， Metaflammation

1. Introduction

The health risk of obesity is great and reducing obesity and overweight has become a major public health challenge. Most individuals with obesity who achieve weight loss subsequently experience weight regain, with approximately 60% returning to their original weight within five years[1]. This repeated pattern of weight loss followed by weight regain is commonly referred to as weight cycling[2]. Weight cycling can lead to additional weight gain and unfavorable changes in body composition[3-5]. Furthermore, it may impair insulin secretion, with fluctuations in weight being associated with a heightened risk of microvascular complications in individuals with type 2 diabetes[6, 7]. Obesity and weight cycling differ in their characteristics. Obesity is a condition characterized by body weight significantly exceeding the recommended standards, serving as a static indicator of an individual's current weight status[8]. Intervention measures aimed at obesity typically prioritize weight loss as the primary goal. In contrast, weight cycling refers to a dynamic and longitudinal process that involves the repeated loss and regain of body weight over extended periods. This can be viewed as the "trajectory" of an individual's weight history, encompassing not only weight loss but also subsequent fluctuations in weight. Intervention strategies targeting weight cycling emphasize both achieving effective weight reduction and implementing measures to prevent weight regain, thereby promoting long-term weight stability and maintenance[9].

Weight cycling is closely associated with the concept of "obesity memory" within the immune cells of adipose tissue[10]. During obesity and subsequent weight loss, immune cells within adipose tissue undergo changes in their quantity, proportion, and phenotype. These alterations often persist after weight loss, contributing to a phenomenon referred to as "obesity memory"[11]. This phenomenon heightens the risk of weight regain, as weight cycling may be linked to sustained low-grade inflammation driven by the "obesity memory" of immune cells[12]. Metaflammation is characterized by chronic, subacute-level inflammation involving the infiltration of immune cells into adipose tissues. In animal models that undergo weight cycling, weight reduction has been shown to intensify inflammation in adipose tissue, promote weight regain, and worsen glucose intolerance, with inflammation failing to return to baseline levels [6]. This persistent

inflammatory state contributes to the progression of chronic metabolic diseases by continuously activating multiple inflammatory pathways, including NLRP3/caspase-1/IL-1β, NF-kB, p38 MAPK, IL-6/STAT3, and PI3K/AKT. These pathways influence a complex multi-organ crosstalk network involving adipose tissue, muscle, and liver [13]. Therefore, efforts towards effectively reducing metaflammation is a critical strategy for mitigating weight regain and preventing weight cycling [10, 14].–

However, despite being a highly safe and effective approach, research on nutritional interventions for weight cycling remains limited. Emerging studies suggest that supplementing with resistant starch may improve the gut microbiome, increasing the prevalence of microbial communities with anti-inflammatory properties, thereby helping to prevent weight cycling[15]. Following dieting and weight loss in mice, flavonoid intervention has been shown to effectively mitigate weight rebound. This effect is associated with a significant increase in the expression of the primary thermogenic factor, uncoupling protein 1 (UCP1), in brown adipose tissue[16]. A high-protein diet is often linked to lower dietary inflammatory factors, and a higher protein-to-carbohydrate ratio appears to be more advantageous for weight loss. Previous studies have shown that feeding a high-protein diet to mice after weight loss can prevent weight rebound. The underlying mechanism includes reducing intestinal lipid absorption and inhibiting the growth of tissue lactobacilli, thereby preventing fat accumulation[17]. To date, no studies have specifically focused on nutritional interventions for individuals with weight cycling obesity. Additionally, the impact of high-protein diets on the immune environment surrounding adipose tissue in weight cycling animals, as well as the deeper changes in metaflammation, remains unexplored.

This study aims to investigate the impact of high-protein diets on weight cycling in both humans and mice, with a particular focus on metaflammation. It will also explore the effects on immune cells associated with adipose tissue in animal models. Additionally, the study seeks to uncover potential molecular mechanisms, providing both theoretical and practical insights for interventions in weight cycling and the prevention of related metabolic disease risks.

2. Method

2.1 Study Population and Data Collection.

2.1.1. Study population

The study utilized a cohort from the Weight Cycling Population at Peking Union Medical College Hospital (WC-PUMCH), a prospective, single-center study including 180 adults with weight cycling and obesity. Recruitment took place from April 2022 to January 2023, and the study is registered as NCT05311462 on ClinicalTrials.gov. Approved by the PUMCH Ethics Committee (ZS3067, K3171) and adhering to Helsinki principles, this study involved 94 individuals without baseline metabolic syndrome. Detailed inclusion and exclusion criteria were provided in Supplement Text 1. The participant flow diagram is shown in Supplement F1. The intervention diet plan included a high-protein, calorie-restricted regimen (20-25 kcal/kg of body weight per day), with protein intake set at 1.5-2 g/kg of body weight per day. Whey protein powder (Nutrasumma, Arizona, USA) was used to supplement the diet and meet the desired protein intake,

contributing 20%-30% of total energy. The exercise plan involved 40 minutes of aerobic exercise and 20 minutes of resistance exercise daily.The program required strict adherence for three months, during which participants were asked to record their weight daily. Throughout the intervention, the researchers encouraged patients to record their daily diets and calculate nutrient intake using a WeChat mini-program (Yingkang, Shanghai, China). Additionally, weekly dietary guidance and follow-up sessions were provided to ensure participants remained compliant with the program.

2.1.2. Information Collection & Sample Testing

A questionnaire was used to capture demographics, habits, and medical histories. Baseline and 3-month measures included body weight (BW), height, hip and waist circumferences, blood pressure (BP), body composition (via bioimpedance,SECA, China), and resting metabolic rate (measured with Delta Trac II, SensorMedics, Anaheim, CA). After a 10-12 hour fast, blood samples were collected and centrifuged (15 minutes at 3000 r/min). Routine tests for fasting blood glucose (FBG), total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and renal/liver function were performed onsite. Serum samples were stored at -80℃ for later analysis via ELISA.

2.2 Animal Experiment

This study involving C57BL/6 mice was approved by the Animal Ethics Committee of Fujian Medical University (No. IACUC FJMU 2023-0234) and adhered to National Institutes of Health guidelines. Fifty 8-week-old male mice were divided into five groups based on different feeding protocols: the Normal Control (NC) group, the Obesity (OB) group, the Weight Cycling (WC) group, the Weight Cycling plus High-Protein Diet (WC+HPD) group, and the Weight Cycling plus Caloric Restriction Diet (WC+CRD) group. Detailed group assignments and feeding protocols are provided in Figure 4A and Supplemental Text 2. Mice were housed under standard conditions ((23±2)℃, 12-hour light-dark cycle) with ad libitum access to water. Weekly monitoring included body weight (BW) and food intake. At the study's end, an oral glucose tolerance test (OGTT) was performed before CO2-induced euthanasia. Body composition, including BW, lean mass, fat percentage, and fat-free mass, was assessed using a small animal body composition analysis system (EM-SCAN, SA-3000 Multi-Detector, Springfield, USA). Serum, adipose tissue, gastrocnemius, and liver samples were collected; serum was stored at -80℃, and tissues were either snap-frozen or fixed in 4% paraformaldehyde for subsequent analyses (see Supplement F2).

2.3 Bioinformatic analysis

2.3.1 Data source

Data acquisition for bioinformatics analysis is provided in Supplement F3.The transcriptome dataset GSE24432 was downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/), with details provided in Supplement Text 3. Differentially expressed genes (DEGs) were identified using the 'limma' package, with significance determined at $P < 0.05$ and $|\log2FC| > 0.585$ [18].

2.3.2 Gene function enrichment analysis based on DEGs

We used the “ggplot2” “pathview” “circlize” package to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses on DEGs, respectively. Protein-protein interaction analysis (PPI) was performed based on Metascape, and the functional modules of highly correlated proteins were identified. Gene set enrichment analysis (GSEA) was performed on the gene expression matrix through the “clusterProfiler” package. And "c2. Cp. Kegg. V7.0. Symbols. The GMT" was selected as the reference gene set.

2.3.3 Screening of diagnostic markers based on machine learning

This study employed three machine learning methods—least absolute shrinkage and selection operator (LASSO) logistic regression, random forest (RF), and support vector machine-recursive feature elimination (SVM-RFE)—to identify diagnostic markers that distinguish between the two groups of individuals. Detailed analysis methods are provided in Supplement Text 4.

2.3.4 Single-cell RNA sequencing data analysis

We downloaded the GSE182233 dataset from the GEO database, which provided single-cell gene expression profiles of immune cells isolated from mouse epididymal adipose tissue using anti-CD45 magnetic beads[11]. Single-cell RNA sequencing data of two weight-maintaining mouse samples and two weight-cycling mouse samples included in this dataset were selected and analyzed using the Seurat V4.1.0 package. For detailed analysis method, see Supplement Test 5.

2.4 Metabolomics and Lipidomics

Metabolomics and lipidomic analysis were performed on serum samples, with specific detection details provided in Supplement Test 6. First-order molecular weight matching was conducted using the Human Metabolome Database (HMDB) and LipidMaps to identify metabolites. Differential metabolites were then analyzed for pathways using MetaboAnalyst (https://www.metaboanalyst.ca), followed by visualization.

2.5 Total RNA isolation and qRT-PCR

Total RNA was extracted from tissues using the standard TRIZOL method (Quanshijin, China, H10318). First-strand cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Tiangen Biotech Beijing Co., Ltd., China, KR118-02). All qRT-PCR reactions were performed on ABI 7500 Real-Time PCR System (Applied Biosystems, USA). The level of target gene expression was normalized against the GADPH gene. Primer sequences are presented in the Supplementary Table 1. RNA relative expression was measured using the 2 ΔΔCT method (cycle threshold).

Table 1 Changes in clinical parameters of individuals with weight cycling and obesity to the Three-Month High-Protein diet (n=94)

	0-Month	3-Month	P-value[a]
Age (years)	32.62 ± 6.79	-	-
Gender (Female%)	70 (74.47%)	-	- BMI
（kg/m^2）	30.56 ± 2.15	27.19 ± 2.56	< 0.05
Weight（kg）	84.20 ± 11.02	74.98 ± 10.90	< 0.05
Fat mass（%）	40.92 ± 5.02	38.02 ± 5.91	< 0.05
SMM（%）	27.38±3.46	28.67±4.00	< 0.05

WHR	0.88 ± 0.06	0.85 ± 0.06	< 0.05
ALT (U/L)	23.30 ± 12.68	17.99 ± 8.00	< 0.05
AST (U/L)	19.59 ± 6.09	18.81 ± 5.53	0.246
ALB (g/L)	46.00 ± 1.97	46.71 ± 2.35	< 0.05
Cr (μmol/L)	66.10 ± 11.85	66.70 ± 10.57	0.378
Urea (mmol/L)	4.37 ± 1.13	4.78 ± 1.10	< 0.05
GLU (mmol/L)	5.28 ± 0.40	4.88 ± 0.37	< 0.05
UA (μmol/L)	362.44 ± 85.09	354.44 ± 87.27	0.221
TC (mmol/L)	4.99 ± 0.81	4.59 ± 0.92	< 0.05
TG (mmol/L)	1.18 ± 0.54	0.81 ± 0.36	< 0.05
HDL-c (mmol/L)	1.24 ± 0.23	1.24 ± 0.24	0.845
LDL-c (mmol/L)	3.20 ± 0.76	2.98 ± 0.83	< 0.05
Systolic blood pressure (mmHg)	119.76 ± 12.05	118.80 ± 10.02	< 0.05
Diastolic blood pressure (mmHg)	78.37 ± 8.47	74.79 ± 7.69	< 0.05
HR (bpm)	74.34 ± 9.70	74.34 ± 9.71	0.634
BMR (%)	94.98 ± 10.16	94.24 ± 10.64	0.56
Handgrip strength (kg)	32.55 ± 10.32	32.51 ± 9.33	0.954
REE (kcal)	1592.85 ± 268.13	1491.45 ± 278.75	< 0.05
RQ	0.81 ± 0.07	0.79 ± 0.08	0.07
Phase angle (°)	5.18 ± 0.51	4.98 ± 0.47	< 0.05
Visceral-fat index	3.17 ± 1.12	2.45 ± 0.86	< 0.05

2.6 Western Blotting

Mouse adipose and liver tissue lysates in RIPA buffer were quantified using the BCA assay. Proteins were electrophoresed, transferred to PVDF membranes, and blocked with 5% milk for 1 hour. Membranes were incubated with primary antibodies (see Supplement Table 2) overnight at 4℃, followed by 2-hour incubation with secondary antibodies. After applying ECL solution for 1 minute, β-actin was used as an internal control. Band intensities were analyzed using ImageJ.

2.7 ELISA

The human and mouse serum levels of cytokines, adipokines, muscle factors, and gastrointestinal hormones were measured using ELISA kits according to the manufacturer's instructions. The specifications of the kits used are provided in Supplement Table 3.

2.8 Histological analysis

Adipose tissue and liver samples were fixed in 4% paraformaldehyde at 4℃ overnight, then processed for paraffin embedding and cut into 4-μm thick sections. These slides were stained with hematoxylin and eosin (H&E) for general pathological inspection. For lipid deposition analysis, cryosections of liver tissues were stained with Oil Red O. All sections were examined using a Nikon Eclipse TE 2000-U microscope (Nikon, Tokyo, Japan).

2.9 Immunofluorescence Staining and Immunohistochemistry

Immunofluorescence staining of paraffin-embedded tissues involved dewaxing, rehydration, antigen retrieval, BSA blocking, and overnight incubation with primary antibodies. Immunohistochemistry on mouse liver sections used primary antibodies per standard protocols, HRP & DAKO kits, followed by hematoxylin counterstaining. Images were analyzed using a Nikon Eclipse TE 2000-U microscope (details in Supplement Table 4).

2.10 Statistical Analyses

Statistical analysis was performed using SPSS 25.0 and GraphPad Prism 9. Data are presented as mean ± SEM (for animals) or SD (for populations). Statistical tests included paired t-test for pre-post comparisons, one-way ANOVA for multiple groups, Mann-Whitney U test for pairwise comparisons, and repeated-measures ANOVA for serial data. A P value < 0.05 was considered statistically significant.

3. Results

3.1 Weight loss and metabolic improvement in people with weight cycling after HPD intervention

A total of 94 drug-naive and non-metabolic syndrome subjects with a history of weight cycling and obesity completed a 3-month high-protein diet program. After HPD intervention, body weight, BMR, and Fat mass% were significantly reduced, SMM% was significantly increased (Figure 1 A-D), and blood glucose and lipid profiles were significantly improved (Table 1). Due to physiological differences between men and women, analyses were conducted separately for each gender. The observed changes were generally consistent with those in the overall subject population (Figure 1 A-D; Supplement Table 5-6). After HPD intervention, serum levels of inflammatory markers IL-1β and IL-18 decreased significantly (P < 0.05), while IL-6 levels showed no significant change. Insulin levels also decreased significantly, and insulin sensitivity improved (P < 0.05). The adipose factor leptin decreased significantly (P < 0.05), whereas the muscle factor irisin increased significantly and GDF-8 (Myostatin) levels decreased. Additionally, gastrointestinal hormones PYY, GLP-1, and motilin increased significantly (P < 0.05) (Figure 1 E-N). This indicates that the 3-month HPD intervention significantly promotes weight loss in individuals with non-metabolic syndrome, weight cycling, and obesity. It also leads to improvements in body composition, blood sugar and its regulatory factors, cardiovascular disease risk factors, circulating inflammatory markers, adipose factors, muscle factors, and gastrointestinal hormones.

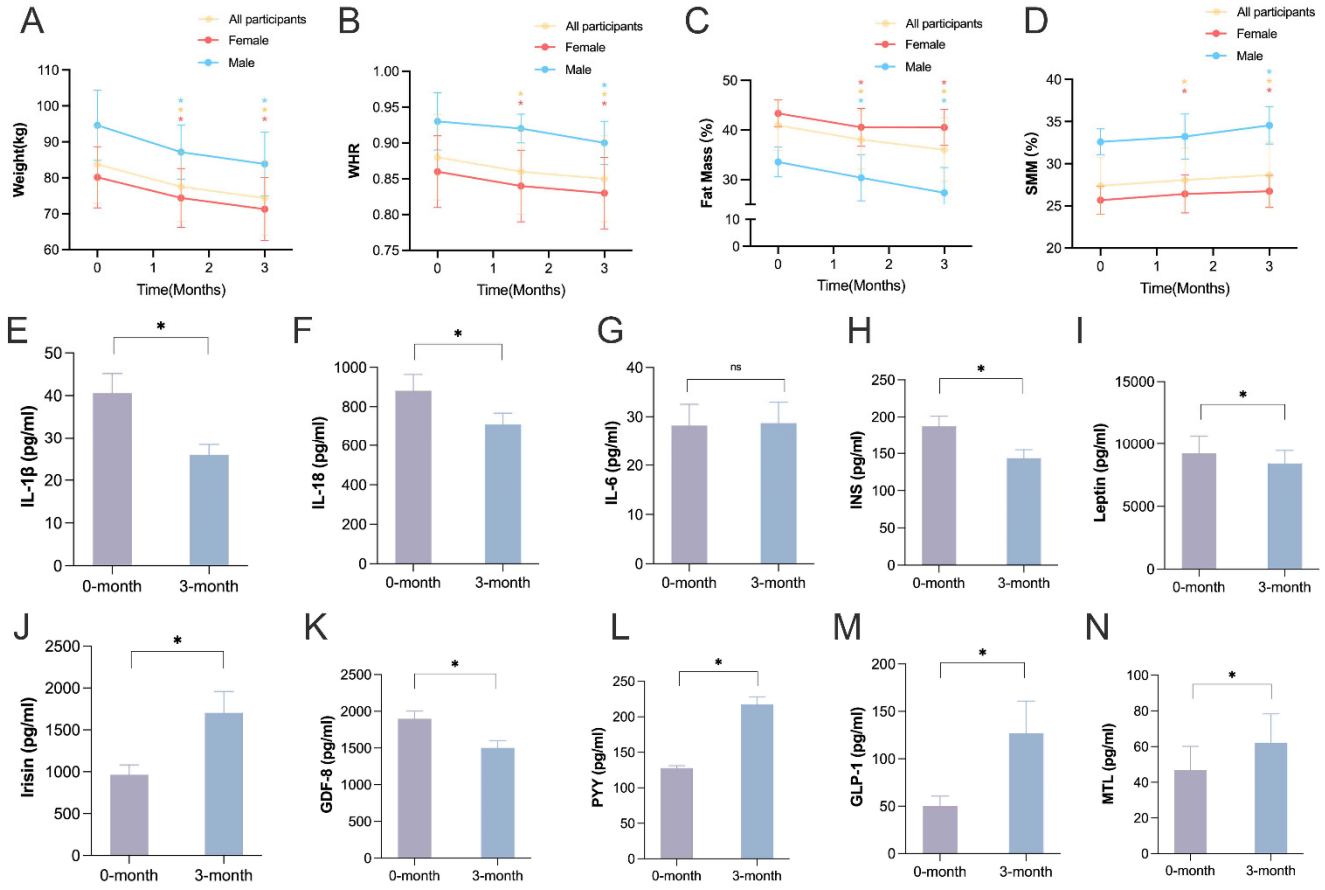


Figure 1 Comparisons between two time points were conducted using paired t-tests. Repeated measures data were analyzed using repeated measures analysis of variance. Bonferroni correction was applied for pairwise comparisons. *It indicates significant differences compared to the baseline, $P < 0.05$. In the line graph, red represents females, blue represents males, and yellow represents the overall population. In the bar graph, gray represents the overall population's biomarker levels at 0 months, and light blue represents the overall population at 3 months. (A) Weight. (B) WHR. (C) FAT Mass.(D) SMM. Serum levels of inflammatory factors/muscle factors and adipokines in subjects: (E) IL-1β. (F) IL-18. (G) IL-6. (H) Insulin (INS). (I) Leptin. (J) Irisin. (K) Growth Differentiation Factor-8, GDF-8(GDF-8) . (L) Peptide YY(PYY). (M) Glucagon-like peptide-1 (GLP-1). (N) Motilin (MTL).

3.2 Local metaflammation in subcutaneous adipose tissue is significant in people with poor weight loss

Based on prior clinical trial outcomes, we hypothesized that local adipose tissue alterations impact body composition, circulating markers, and clinical indicators. Thus, to investigate weight cycling rebound and intervention targets, we analyzed a baseline fat transcriptome dataset from weight rebound participants. Classifying subjects by weight rebound (< 10% good weight loss vs. > 50% poor weight loss), we identified 192 differential genes (DRGs), with 95 upregulated and 97 downregulated (Figure 2A). Clustering DRGs yielded five modules, four linked to weight gain and metaflammation (Figure 2B). Key modules encompassed adipogenesis (Module 1), inflammation biomarkers (Module 2), adipocyte extracellular matrix remodeling (Module 3), and the Notch pathway linked to fat metabolism, inflammation, and fibrosis (Module 4). GO enrichment highlighted metaflammtion and adipogenesis processes (Figure 2C-E). KEGG and GSEA analyses highlighted immune-inflammatory pathways, supporting the GO enrichment findings and revealing pathways involved in appetite and weight regulation, such as the Relaxin signaling pathway. These results suggest potential targets for further investigation (Figure 2F-G).

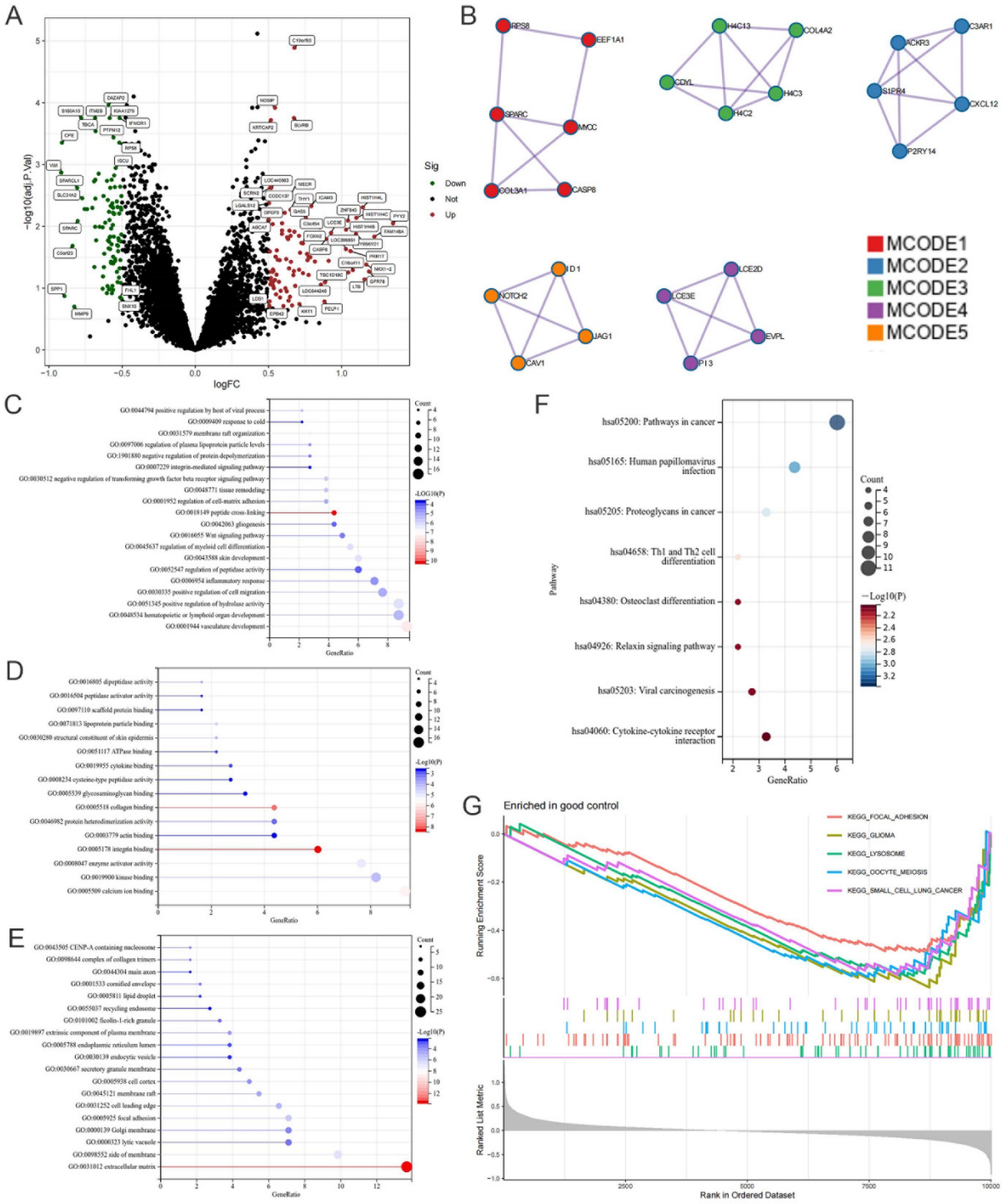


Figure 2 DRGs analysis of GEO adipose tissue dataset: (A) DRGs volcano map, (B) DRGS GO analysis of five module GEO adipose tissue data sets obtained by cluster analysis: biological processes obtained by (C)-(E) GO enrichment analysis, KEGG and GSEA enrichment analysis of DRGs for GEO adipose tissue dataset: (F) KEGG enrichment analysis, (G) GSEA enrichment analysis.

3.3 Identify markers in adipose tissue that predict weight rebound

To predict weight loss outcomes, we screened predictive biomarkers using three machine-learning methods on DRG differential expression. IFNGR1 and SCRN2 emerged as key genes (Figure 3A-D). A predictive nomogram based on these genes distinguished weight loss outcomes with AUC values of 0.925 (IFNGR1) and 0.895 (SCRN2), indicating robust classification (Figure 3E). The poor control group showed

decreased IFNGR1 and increased SCRN2 expression (Figure 3F). DCA analysis revealed the nomogram's superiority over chance in predicting outcomes within a high-risk threshold (Figure 3G). The clinical impact curve confirmed the model's predictive ability (Figure 3H). These markers, linked to proteolysis and metaflammation, suggest avenues for inflammatory cytokine exploration and HPD intervention efficacy.

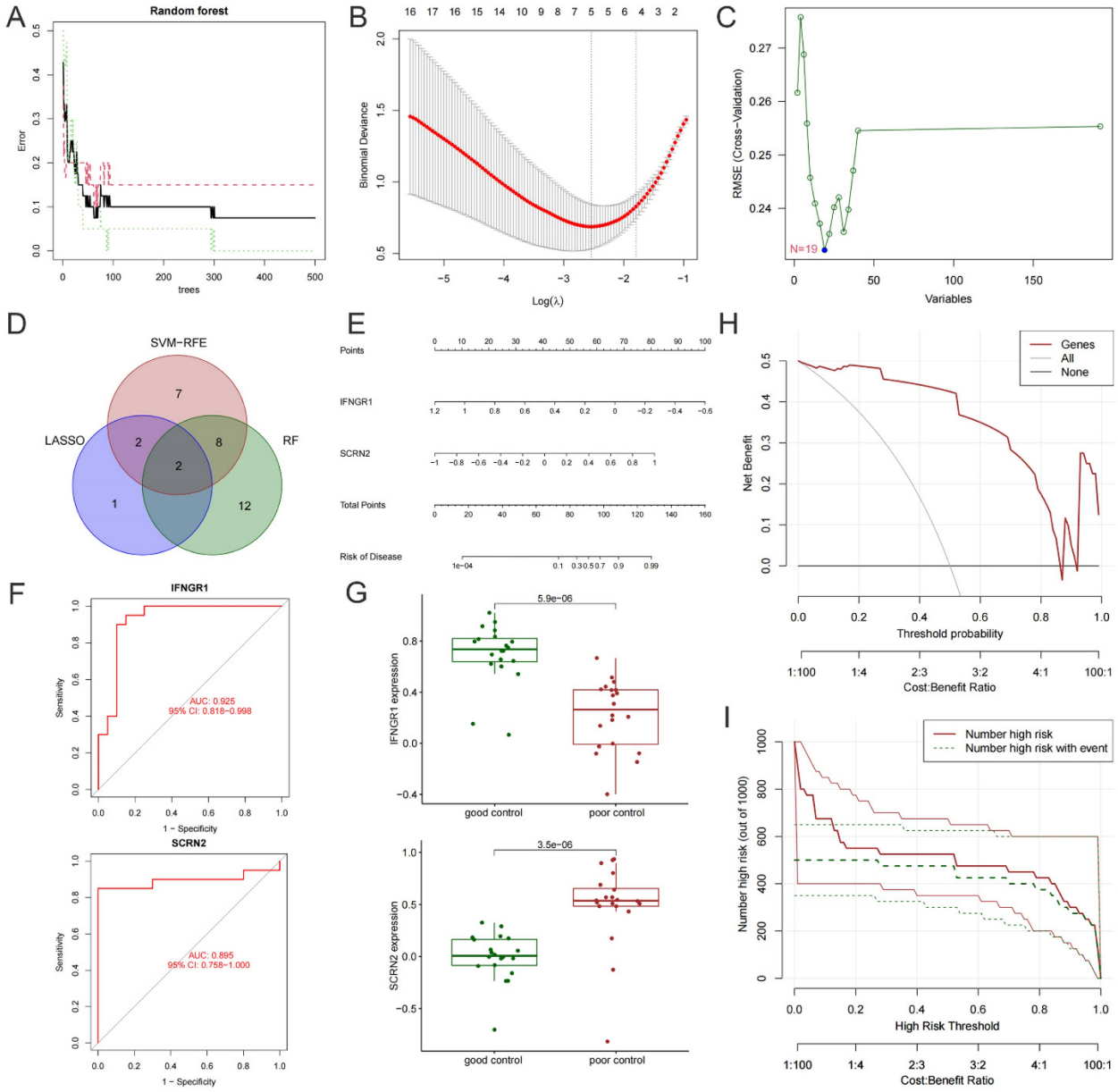
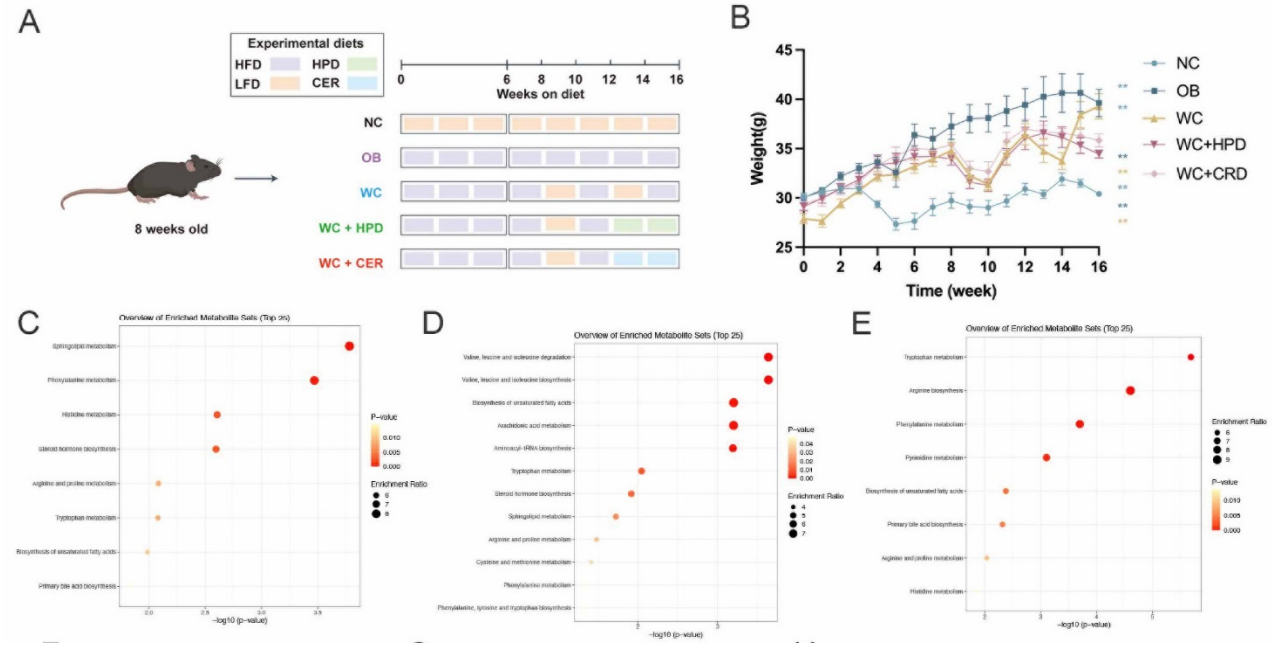


Figure 3 Identification of markers predicting weight regain from the GEO adipose tissue dataset: (A) RF screening results, (B) LASSO regression screening results, (C) SVM-RFE screening results, (D) Wayne diagram of the screening results of three models. Identification of key genes of weight regain IFNGR1 and SCRN2: (E) Predictive nomogram of IFNGR1 and SCRN2, (F) AUC curves of IFNGR1 and SCRN2, (G) Expression levels of IFNGR1 and SCRN2 in adipose tissue of the two groups, DCA and clinical impact curves of IFNGR1 and SCRN2 linear table models, markers of weight regain prediction: DCA curve of (H) linear table model, (I) Clinical impact curve of line table model.

3.4 The animal model of weight cycling confirmed that high-protein diet effectively reduced fat droplet accumulation in muscle and liver

We investigated the effects of high-protein diet (HPD) intervention on weight-cycling mice, contrasting it with 66.7% energy restriction (Figure 4A). Mice gained weight rapidly upon high-fat diet exposure, with WC and OB groups exhibiting significant weight gain over NC ($P < 0.01$). Following 16 weeks, WC+HPD

mice exhibited marked weight loss compared to OB and WC, suggesting HPD's short-term efficacy (Figure 4B). Non-targeted metabolomics revealed differences in unsaturated fatty acids, amino acids, and sphingolipids, with NC-WC and NC-OB different pathways sharing unsaturated fatty acid and bile acid pathways, while OB-WC different pathways implicated chronic inflammation (Figure 4C-E). Subcutaneous (sWAT) and epididymal (eWAT) fat indices were elevated in WC and OB, mitigated by HPD but not calorie restriction (CRD). Notably, HPD significantly reduced sWAT in WC+HPD vs. OB/WC and eWAT in WC+HPD vs. WC, highlighting its effectiveness in visceral and subcutaneous fat reduction. Muscle content was lower and fat content higher in OB and WC compared to NC ($P < 0.001$), with HPD reversing these trends but CRD showing no significant change (Figure 4F-G). ITT results indicated insulin resistance in WC mice, alleviated by HPD (Figure 4H). Histological analysis (HE for WAT, BAT; Oil Red O for liver, gastrocnemius) revealed enlarged adipocytes in OB and WC, less pronounced in WC, and significant improvement in WC+HPD/CRD, with WC+HPD showing slight advantage. Liver and muscle trends mirrored these findings (Figure 4I). In summary, HPD effectively reduced weight, improved insulin sensitivity, and modulated adipose tissue and metabolic profiles in weight-cycling mice, suggesting its potential as a therapeutic strategy for obesity-related metabolic disorders.



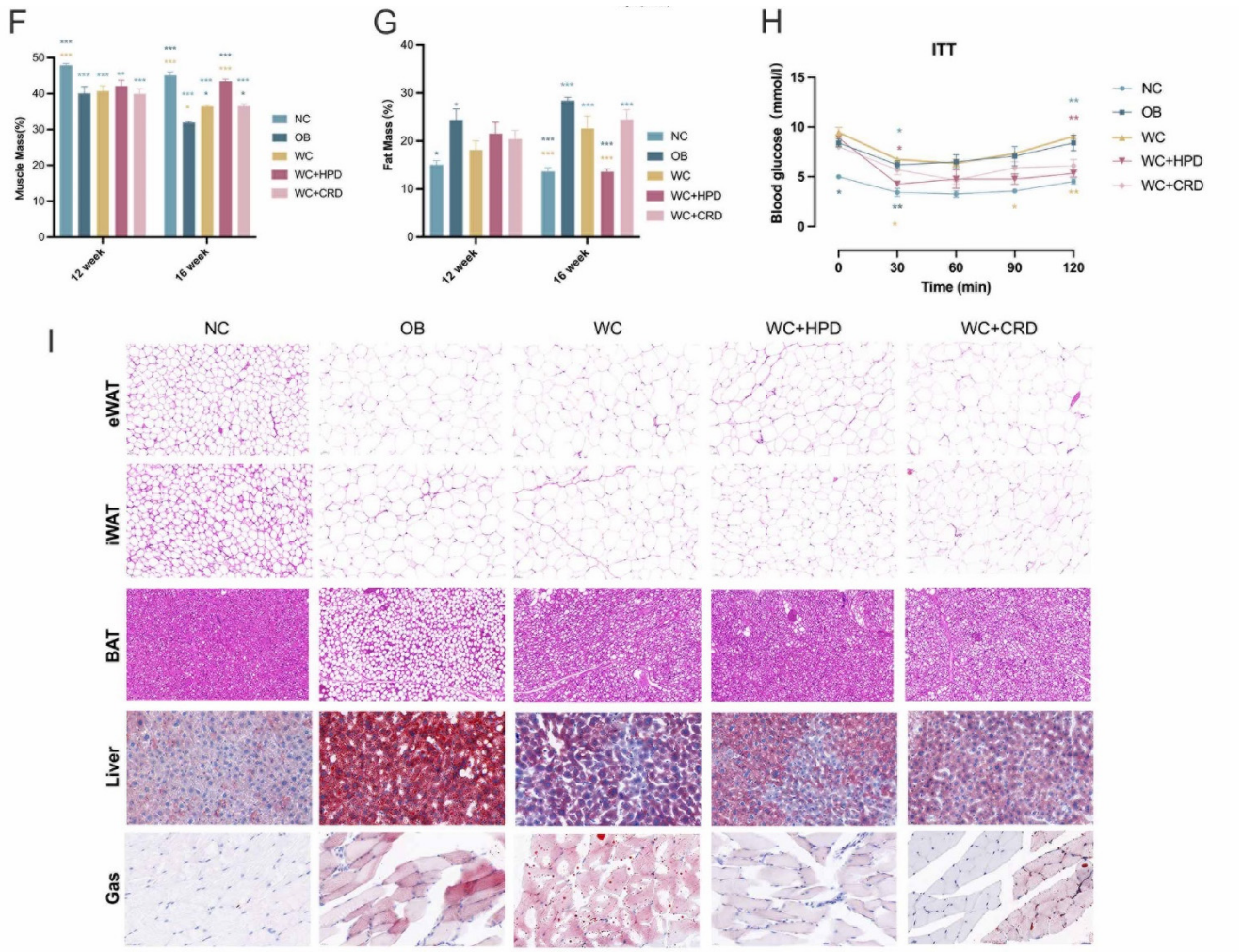


Figure 4 (A) Mouse experimental protocol (B) Weight change of mice in each group of experimental mice, modeling and intervention mode (0-16 weeks of the experiment) (C)-(E) KEGG enrichment analysis of differential metabolites in different groups of mice: all are enrichment pathways. Body composition levels of mice in each group after modeling (after 12 weeks) and intervention (after 16 weeks) : (F) muscle content, (G) fat content, (H) ITT results of mice in each group, (I) eWAT, iWAT, BAT, liver and Gastrocnemius (Gas) pathology of mice in each group: eWAT, eWAT, iWAT and BAT were HE staining, 200× 50μmol/L; Liver and Gas, oil red O staining, 400×, scale 20μmol/L* $P < 0.05$, ** $P < 0.01$.

3.5 High-protein diet effectively reduces metaflammation in adipose tissue and liver

Obese mice exhibited altered TNF-α, IL-6, IL-1β levels, with weight cycling mice showing reduced levels but persisting low-grade inflammation. High-protein diet (HPD) significantly reduced systemic inflammation more than calorie restriction (CRD) ($P < 0.05$, Figure 5A-C). Short-term weight loss in weight-cycling (WC) mice reduced NLRP3, Caspase1, IL-18 transcripts in epicardial (eWAT) and subcutaneous adipose tissue (sWAT) ($P < 0.05$), with HPD showing greater reductions ($P < 0.05$) than CRD, except for TLR4 ($P > 0.05$) (Figure 5D-K). In brown adipose tissue (BAT), only HPD reduced NLRP3, Caspase1, IL-18 transcripts ($P < 0.05$) (Supplement F4). Similar protein level changes were observed (Figure5 L-Q). Both interventions reduced Wnt signaling pathway activation but did not affect STAT1 phosphorylation (Supplement F4). These findings underscore the efficacy of HPD in mitigating metaflammation.

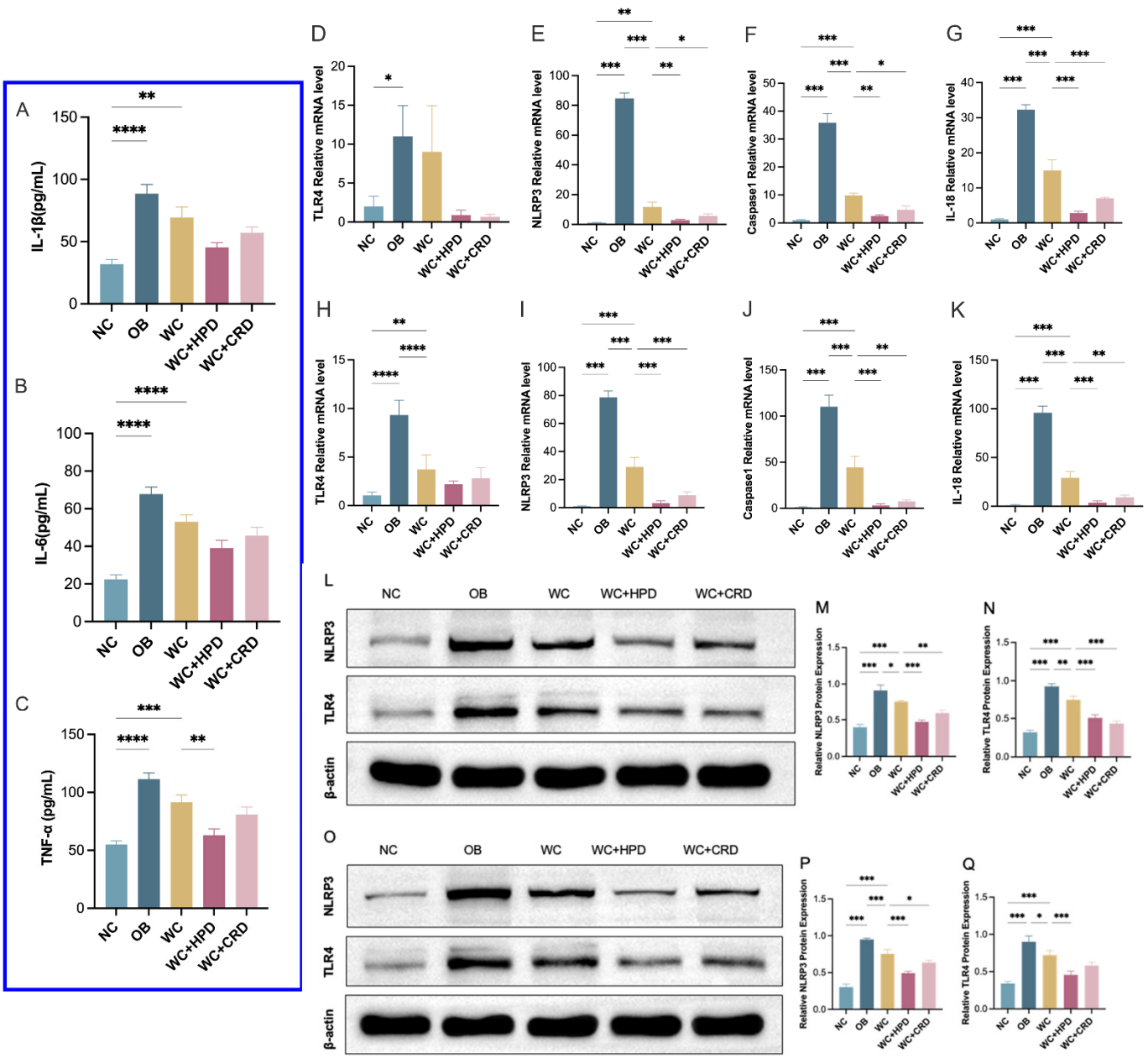
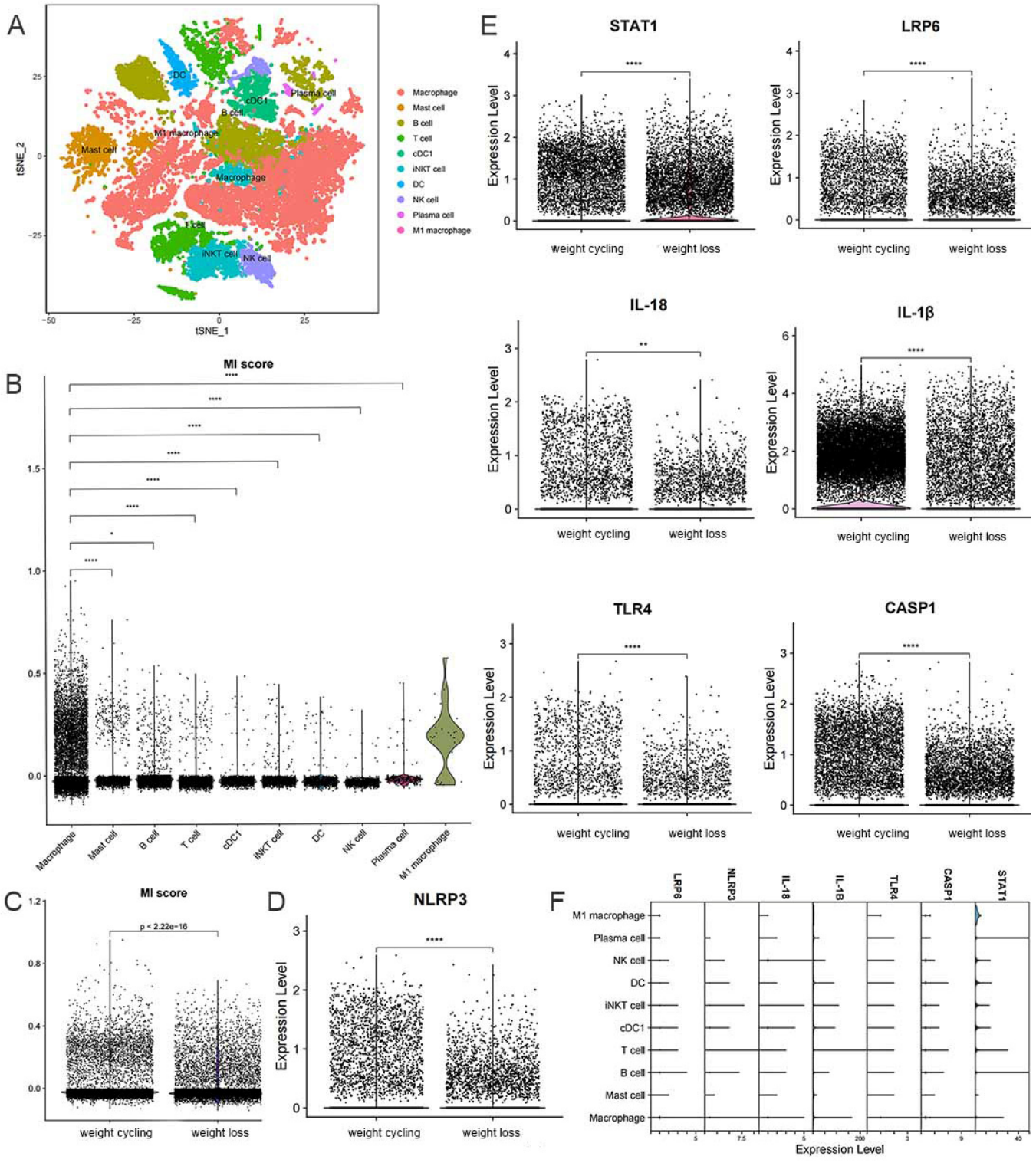


Figure 5 The levels of serum inflammatory cytokines in each group were as follows: (A) IL-1β,(B)IL-6, (C)TNF-α; mRNA levels of TLR4-NLRP3 pathway in epididymal fat of mice in each group were as follows: (D)TLR4,(E)NLRP3,(F) Caspase1,(G)IL-18; mRNA levels of TLR4-NLRP3 pathway in subcutaneous fat of mice in each group: (H)TLR4, (I) NLRP3, (J) Caspase1, (K) IL-18; The protein levels of epididymal fat protein TLR4-NLRP3 pathway of mice in each group: (L) the levels of epididymal fat protein TLR4 and NLRP3, (M) the relative expression level of NLRP3, (N) the relative expression level of TLR4; (O) the levels of subcutaneous fat protein TLR4 and NLRP3; (P) the relative expression level of NLRP3; (Q) the relative expression level of TLR4. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

3.6 Macrophages mediate metaflammation in adipose tissue of weight-cycling groups

In adipose tissue of weight-cycling mice, immune cells including macrophages, M1 macrophages, iNKT cells, NK cells, and plasma cells were abundant (Figure 6A). Macrophages, with higher abundance than M1 macrophages and elevated MI scores, emerged as key inducers of metaflammtion (Figure 6B). Genes implicated in metaflammtion (NLRP3, STAT1, LRP6, IL-18, IL-1β, TLR4, CASP1) showed higher expression in weight-cycling mice compared to weight-loss mice, with macrophages exhibiting more pronounced expression than M1 macrophages (Figure 6C-F). Validation of immune cells associated with

metaflammtion revealed significant infiltration of adipose tissue macrophages M1 in OB and WC groups, with WC+HPD showing more pronounced anti-inflammatory effects and WC+CRD also improving in subcutaneous and epididymal fat, brown fat, and liver (Figure 6G-H, Supplement F5). NK cell infiltration and activation were also evident in OB and WC groups, with WC+HPD and WC+CRD demonstrating improvements, but no significant difference between them; no significant differences were observed in brown fat and liver (Fig. 6I-J, Supplement F5). These findings suggest interrelated metaflammtion across adipose tissues and the liver, with white adipose tissue changes in NK cell accumulation and activation being more pronounced.



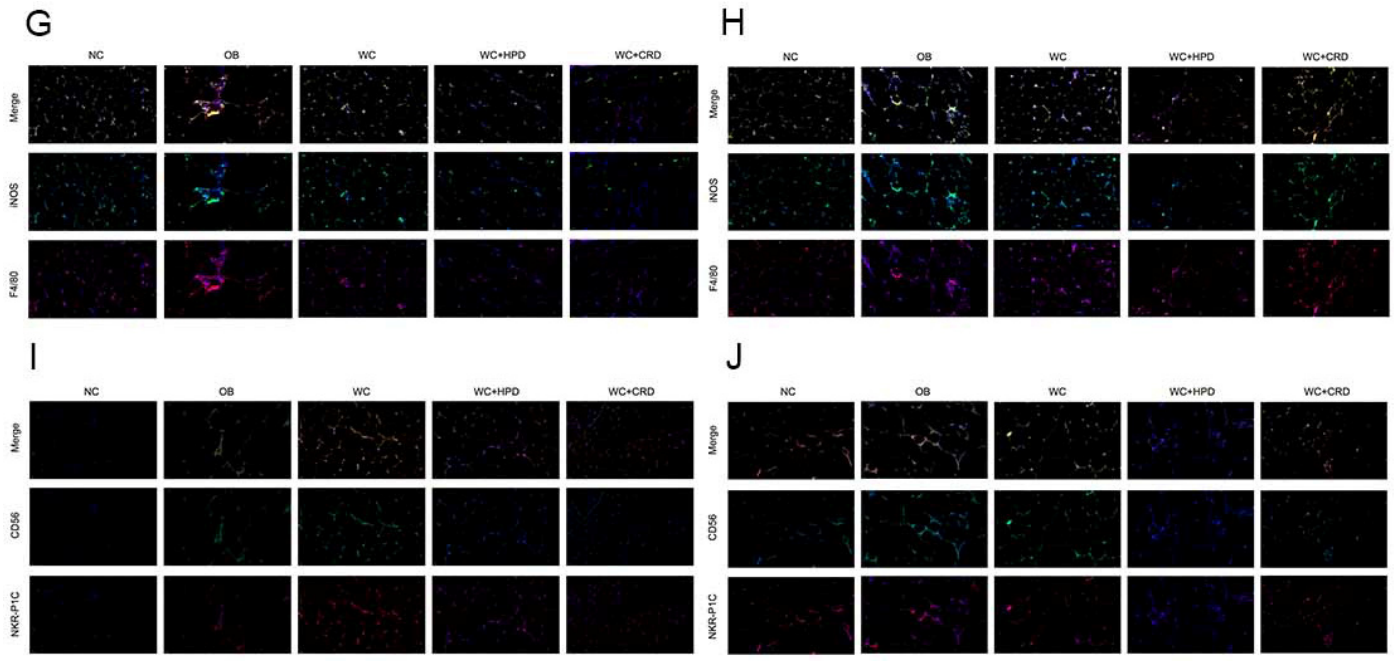


Figure 6 Metaflammation levels in poor control and good control groups. (A) tSNE is used for dimensionality reduction, cell classification, and definition. (B) Comparison of MI scores of various immune cells in the poor control group. (C) Comparison of MI scores between the poor control group and the good control group. (D-E) Comparison of the expression of metabolic inflammation-related genes between the poor control group and the good control group. (F) Expression levels of metabolic inflammation-related genes in various immune cells. Confocal laser scanning microscopy image: 400×, scale bar, 50 μm. G) Immunostaining for iNOS and F4/80 in sWAT, H) Immunostaining for iNOS and F4/80 in eWAT, J) Immunostaining for CD56 and NKR-P1C in sWAT, I) Immunostaining for CD56 and NKR-P1C in eWAT.* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.001$.

3.7 IFN-γR1 expression mediates the heterogeneity of macrophages and NK cells in the metaflammation process

The intricate interplay between immune cells, particularly macrophages and NK cells, governs the orchestration of metaflammtion. Cellchat analysis underscored the robust communication intensity of NK cells despite their lower metabolic inflammatory activity (Figure 7A). Distinct immune responses emerged, with macrophages exhibiting upregulated inflammatory and adipogenic pathways, while NK cells showed downregulation of TNF-α/NF-κB signaling, indicative of metaflammtion inhibition. NK cells additionally exhibited upregulation of cell stress-related pathways and robust responses to IFN-γ and IFN-α (Figure 7B). This concurs with prior findings that adipocyte-NK cell spatial interactions induce IFN-γ production, activating macrophages' pro-inflammatory phenotype. Given IFN-γR1's crucial role in IFN-γ signaling and its downregulation in adipose tissue of individuals with poor weight control, we explored its expression dynamics in macrophage and NK cell development. IFN-γR1 expression in NK cells did not align with MI scores, whereas in macrophages, IFN-γR1high and IFN-γR1low populations coexisted (Figure 7D-E). This implies IFN-γR1high NK cells may curb metaflammtion and weight regain. High IFN-γR1 in NK cells reduced cell stress and inflammation, whereas in macrophages, it augmented IFN responses and metaflammtion-related pathways (Supplement F6). Our findings underscore IFN-γ/IFN-γR's centrality in lipid metabolism and inflammation, revealing immune cell heterogeneity.

Further, we delved into the intercellular communication mechanisms between NK cells and macrophages. Close communication was observed, with NK cells preferentially signaling to macrophages through MIF-mediated pathways, while macrophages utilized CCL6-CCR2 (Figure 8A-C). This autocrine/paracrine network extends to T cells, cDC1 in the MIF network, and mast cells in the CCL network (Figure 8D), elucidating the complex interplay in adipose tissue during weight loss. Moreover, the histochemical analysis of IFN-γR1 marker in liver samples mirrored similar patterns, with reduced staining in OB and WC groups and increased staining in WC+HPD and WC+CRD groups, with WC+HPD showing the most pronounced effect (Figure 8E). Collectively, these findings provide insights into the intricate communication networks between immune cells, particularly macrophages and NK cells, in modulating metaflammation and lipid metabolism.

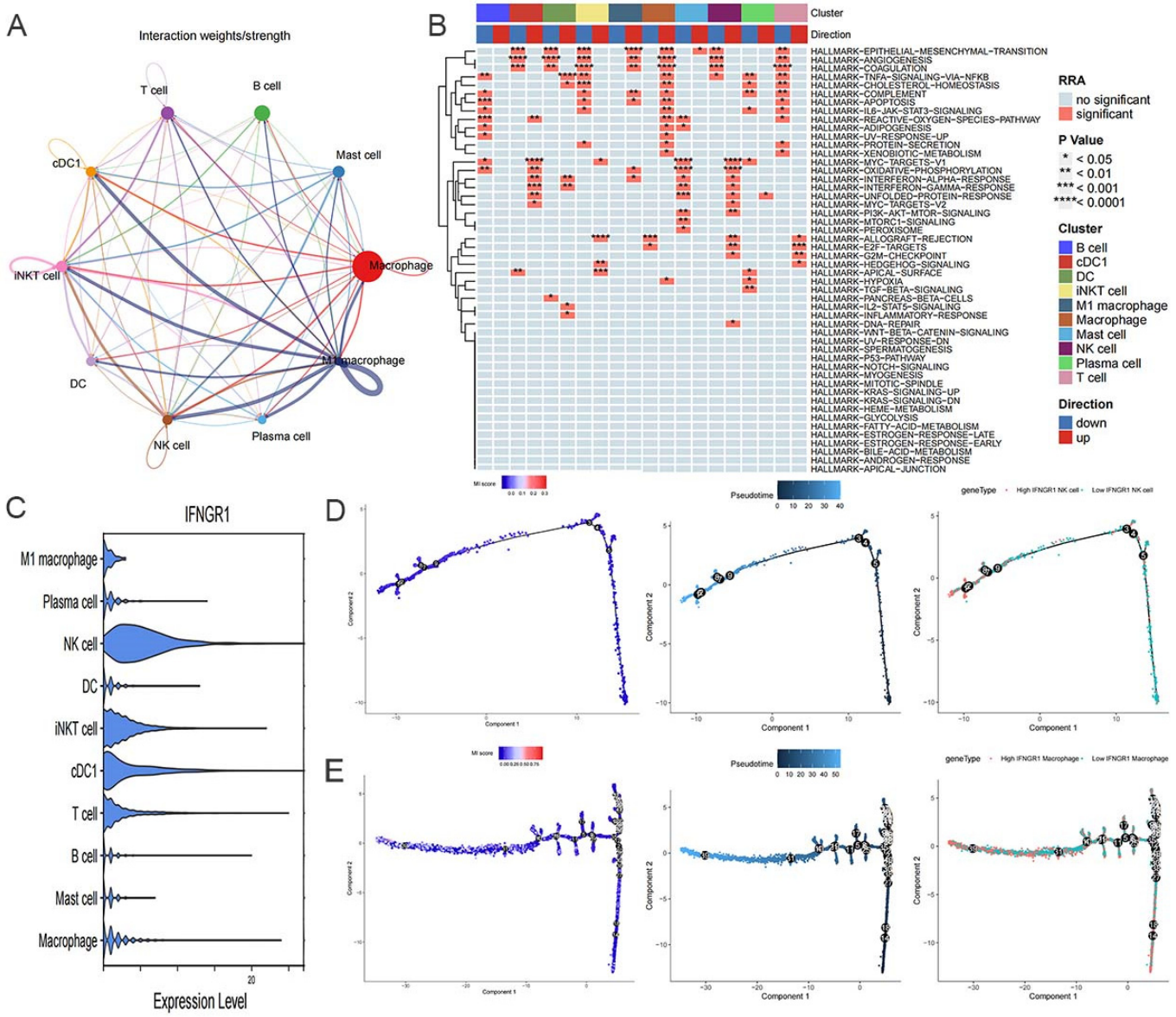


Figure 7 Multiple single-cell RNA-Seq analysis methods reveal the biological characteristics of NK cells and macrophages. (A) The intensity of interactions between various immune cells. (B) Activation of signaling pathways in various cells. (C) Expression of IFN-γR1 in various cells. (D) Pseudotime trajectory analysis of MI score in NK cells. Cells are colored based on MI score (left), pseudotime (middle) and IFN-γR1 expression (right). (E) Pseudotime trajectory analysis of MI score in macrophages. Cells are colored based on MI score (left), pseudotime (middle) and IFN-γR1 expression (right).

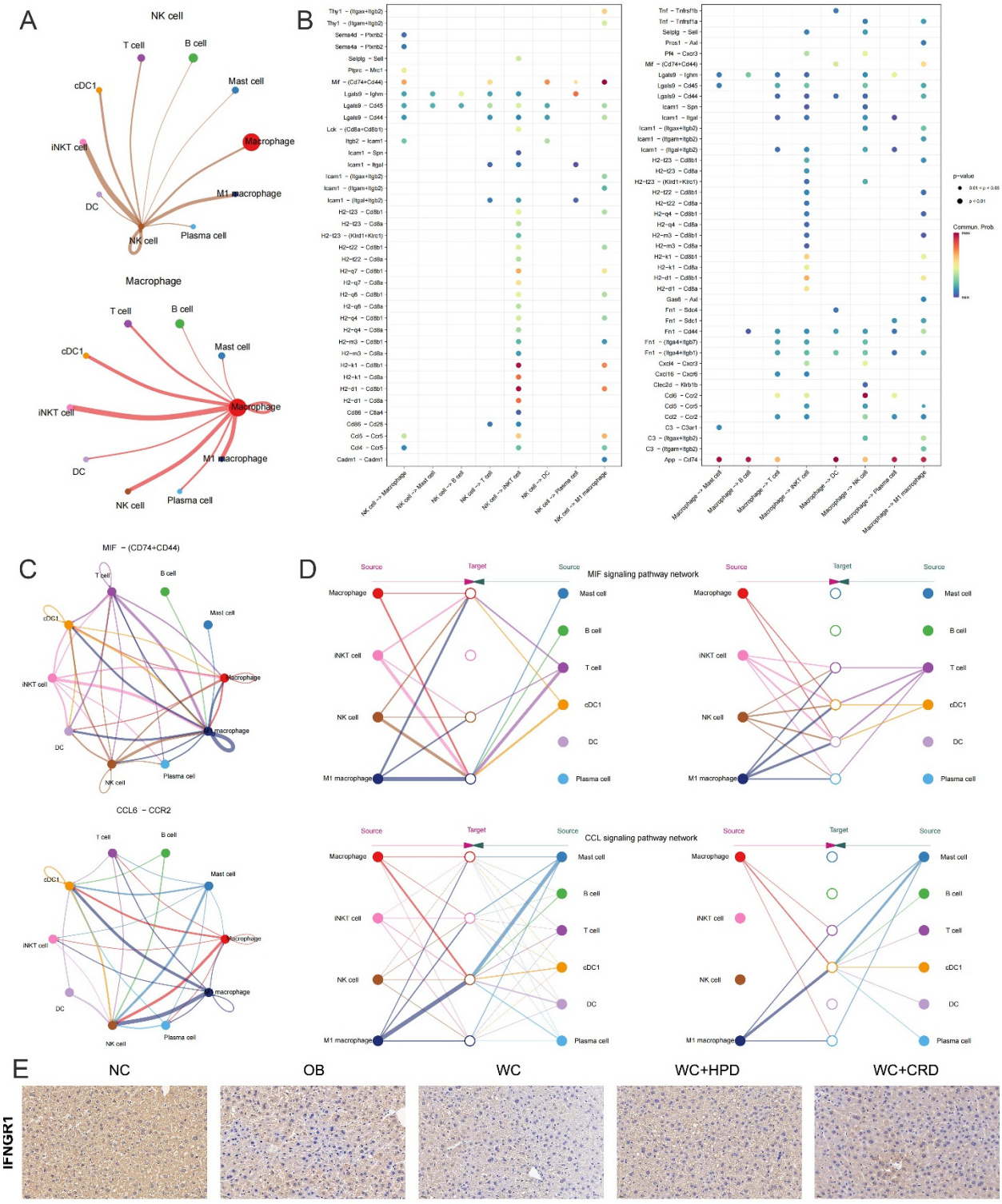


Figure 8 Analysis of communication pathways between NK cells and macrophages. (A) Chord diagram showing the strength of specific cell-to-cell interactions. (B) Communication pathways and their strengths between specific cells. (C) The weights of the MIF signaling pathway and the CCL signaling pathway in the communication between various types of cells. (D) Hierarchical diagram showing the cell-to-cell communication network based on the MIF signaling pathway and the CCL signaling pathway. (E) Immunohistochemistry of IFN-γR1 in liver; 200 x, the scale is 20 μm.

4. Discussion

The impact of weight cycling, characterized by repeated cycles of weight loss and regain, on health, remains controversial[19, 20]. While some studies suggest that weight cycling may increase the risk of heart disease, diabetes, and subsequent obesity[6, 21, 22] , other evidence indicates that the so-called yo-yo effect

might not adversely affect weight, body composition, or metabolic rate. Our research found no correlation between the number of weight cycles and future weight loss outcomes. However, individuals with obesity who experience weight cycling often lose confidence in their ability to achieve sustainable weight loss due to repeated weight rebounds and slow progress [9].

We investigated the role of a high-protein diet (HPD) while considering its potential negative effects. We selected healthy individuals without chronic kidney disease (CKD), metabolic syndrome, or a history of weight cycling among the obese to complete a three-month intervention. In the animal model, we compared a short-term 4-week HPD with a 30% energy restriction program [23]. For the first time, we observed that HPD effectively reduces weight in individuals with a history of weight cycling and obesity, improves insulin sensitivity, blood lipids, blood pressure, and other high-risk factors for metabolic and cardiovascular diseases, and lowers levels of inflammatory factors and adipokines. It also enhances appetite-regulating hormones and muscle factors. Notably, obesity and energy restriction are typically associated with muscle mass loss; however, in this study, HPD significantly increased skeletal muscle mass percentage (SMM%). These results suggest that HPD can effectively reduce body weight (particularly fat) and protect against muscle loss, thus preventing sarcopenic obesity. A systematic review indicates that a 1% increase in protein intake can improve obesity remission probability by 6%, with HPD potentially increasing the success rate of weight loss by 50%. Higher protein intake (> 60 g/day or 90 g/day) may further benefit weight loss[24]. Thus, appropriately increasing protein intake can help prevent sarcopenic obesity[25]. Our study examined a high-protein dietary regimen based on caloric restriction, aimed at promoting weight loss in patients with weight cycling obesity by increasing the proportion of protein in the diet. In this study, the high-protein diet intervention included not only calorie control but also an exercise program. This design is based on the clinical consensus that a combination of energy restriction and moderate physical activity is a fundamental lifestyle intervention for weight loss[26-28]. The fundamental cause of obesity arises from an imbalance between energy intake and expenditure, typically resulting from overeating, low energy output, and sedentary behavior. Therefore, controlling calorie intake is essential[29]. Physical exercise plays an important role in maintaining weight, especially when combined with other weight loss therapies such as medication or nutritional interventions[30, 31]. Specifically, resistance exercise not only helps maintain weight by improving body composition and increasing muscle mass, but also enhances the effectiveness of weight loss medications in preventing weight cycling[32]. Physical exercise effectively boosts the levels of appetite-regulating hormones such as glucagon-like peptide-1 (GLP-1) and peptide tyrosine tyrosine (PYY), thereby increasing satiety and complementing the effects of high-protein diets[33]. Additionally, exercise is essential for maintaining existing muscle mass and promoting the formation of new muscle tissue, further enhancing the benefits of a high-protein diet[34, 35]. In the future, we will design more precise randomized controlled clinical trials to continue to explore the underlyding mechanisms and effects.

This study established a weight-cycling mouse model and observed that after HPD intervention, serum levels of IL-1β, IL-6, and TNF-α were significantly reduced compared to the WC group. Additionally, the

transcription and protein expression of NLRP3 inflammasome-related genes in various adipose tissues were significantly decreased, while the transcription and protein expression of Wnt pathway-related genes were significantly increased. We also noted a significant increase in IFN-γR1 marker expression and a reduction in M1 macrophage infiltration and NK cell activation levels in adipose tissue. Research by Guo et al. indicates that HPD can effectively prevent weight regain in mice by inhibiting fat accumulation and preventing the growth of lactic acid bacteria, which may also affect adipose tissue metaflammation due to changes in the intestinal flora [17]. The effects of HPD on metaflammtion are consistent across both animal and human studies[36]. In individuals with weight cycling, IL-1β and IL-18 levels in peripheral blood were significantly reduced after three months of HPD intervention, although IL-6 levels did not change significantly. Among inflammation-related adipose and muscle factors, irisin levels were significantly increased, leptin levels significantly decreased, and insulin levels were reduced. This indicates that HPD intervention significantly impacts NLRP3 inflammasome and leptin-related metaflammtion and improves insulin sensitivity. Additionally, HPD has been shown in some studies to reduce chronic inflammation, with whey protein hydrolyzate demonstrating anti-inflammatory activity through the regulation of intestinal flora in animal experiments[6].

Analysis of human adipose tissue transcriptome data sets also found similar mechanisms. Differential genes were significantly enriched in adipogenesis and development, inflammation-related biomarkers and fat metabolism, inflammation and fibrosis, and NLRP3, STAT1, LRP6, IL-18, IL-1β, TLR4, and CASP1, important genes for metaflammtion, were more significantly expressed in the epididymal adipose tissue of weight cycling mice. In our previous research, we found that ferroptosis and the metaflammtion it mediates play an important role in long-term weight control[37]. Some studies have found that in a weight-cycling model, the metabolic levels of fat macrophages Elevated, secreting higher levels of inflammatory factors[11, 14]; in addition, T cell depletion and enhanced macrophage lipid processing were also seen in weight-cycling mice. These data suggest that post-obesity weight loss can prime adipose macrophages to enhance inflammation when weight is restored. At the same time, we also identified two markers, IFNGR1 and SCRN2. Some studies have found that white adipocyte differentiation and IFNγ mediators of obesity- or insulin-induced macrophage-cholesterol network dysregulation and increased macrophage cholesterol accumulation, and the IFNγ-macrophage pathway is a mediator of obesity/insulin resistance and accelerated atherosclerosis[38]. SCRN2 is related to proteolysis and is involved in human growth and development[39]. This suggests that this gene is involved in proteolysis, digestion, and utilization and also influences the response to the HPD method, which can impact the outcome of weight loss.

We have discovered the association between weight cycling and metaflammtion in the analysis of general transcriptomes, so we further used single-cell transcriptional data to explore the changes in weight cycling and obesity-related immune cells. We found that weight-cycling mice contain macrophages, M1 macrophages, iNKT cells, and NK cells. Macrophages have the highest MI score and are significantly correlated with the expression of NLRP3 series and STAT1 series metaflammtion genes, while NK cells have

the highest MI score. lowest score. However, in cell communication analysis, both macrophages and NK cells are highly active. Trajectory analysis combined with the IFN-γR1 identified by our screening revealed that IFN-γR1high NK cells have an inhibitory effect on metaflammation and weight regain and that macrophages mediate metaflammation in adipose tissue.

Future studies should include follow-up periods of 1-2 years or longer to assess the long-term impact of HPD on preventing weight cycling. Additionally, there is no standardized method for establishing a mouse body weight cycling model. While most studies use alternating high-protein diets (HPD) and normal diets (ND) as the primary approach, there is no consensus on the frequency of cycle alternation and feeding regimens[6, 9].

5. Conclusion

Our results suggest that the underlying cause of weight cycling and obesity may be the long-term inflammatory infiltration of M1 macrophages and NK cells. HPD intervention effectively reduces body weight and fat, increases muscle mass to support sustained weight maintenance, and improves blood lipids, blood pressure, and glucose tolerance while reducing metaflammation. These effects may be achieved by decreasing NLRP3-related metaflammation and mitigating the impact of IFNGR1-associated M1 macrophage infiltration and NK cell activation.

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Ethical approval

This study was approved by the Human Ethics Committee of PUMC Hospital (No.ZS3067, K3171) and the Animal Ethics Committee of Fujian Medical University (No. IACUC FJMU 2023-0234), Beijing, China.

Data availability

Data will be made available on request.

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Declarations of interests

The authors declared no conflict of interest.

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