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Immune imbalance in obesity-associated diseases

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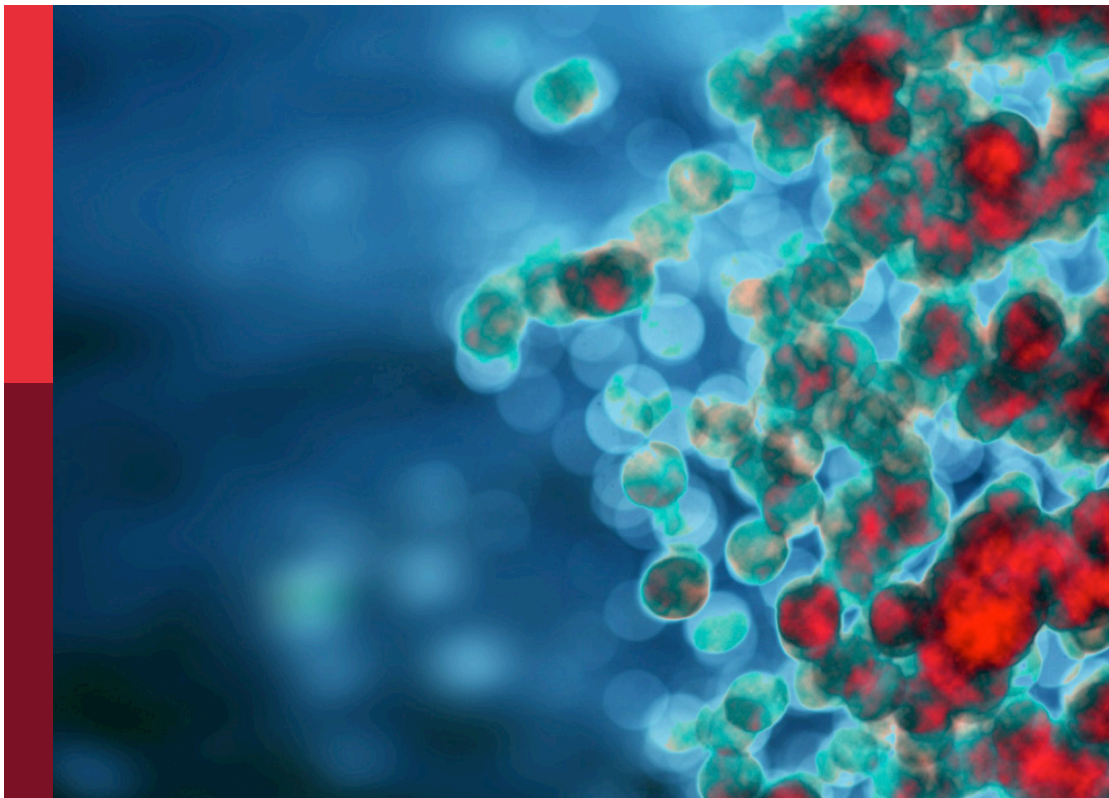

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Immune imbalance in obesity-associated diseases

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SYSTEMATIC REVIEW
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Effect of Exercise Training on Body Composition and Inflammatory Cytokine Levels in Overweight and Obese Individuals: A Systematic Review and Network Meta-Analysis

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Objective: This study aimed to compare and rank the effectiveness of aerobic exercise (AE), resistance training (RT), combined aerobic and resistance training (CT), and high-intensity interval training (HIIT) on body composition and inflammatory cytokine levels in overweight and obese individuals by using network meta-analysis (NMA).

Methods: We searched the PubMed, Cochrane, Embase, Web of Science, and EBSCO databases to identify randomized controlled trials investigating the effects of exercise training on inflammatory cytokines in overweight and obese patients. The retrieval period was from inception to November 2021. Two reviewers independently screened the retrieved articles, extracted the pertinent data, and assessed the risk of bias of the included studies; then, they used Stata 16.0 and Review Manager 5.3 to perform an NMA.

Results: A total of 38 studies involving 1317 patients were included in this study. The results of the NMA indicated that AE had the greatest effect on weight loss (SUCRA=78.3; SMD=−0.51, 95% CI: −0.70, −0.33); CT had the greatest effect on reducing body mass index (SUCRA=70.7; SMD=−0.46, 95% CI: −0.81, −0.10), waist circumference (SUCRA=93.4; SMD=−1.86, 95% CI: −2.80, −0.93), percentage body fat (SUCRA=79.6; SMD=−1.38, 95% CI: −2.29, −0.48), interleukin-6 level (SUCRA=86.4; SMD=−1.98, 95% CI: −3.87, −0.09), and tumor necrosis factor-α level (SUCRA=79.4; SMD=−2.08, 95% CI: −3.75, −0.42); AE (SMD=0.51, 95% CI: −1.68, 2.69), RT (SMD=0.15, 95% CI: −3.01, 3.32), CT (SMD=1.78, 95% CI: −1.35, 4.92), and HIIT (SMD=2.29, 95% CI: −1.27, 5.86) did not significantly increase the adiponectin level.

Conclusion: The current results suggest that CT is the best exercise modality for improving body composition and inflammatory status in overweight and obese individuals. More rigorous randomized control trials are needed for further validation.

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Keywords: exercise, body composition, inflammatory cytokine, overweight and obese individuals, network meta-analysis

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1 INTRODUCTION

Obesity prevalence worldwide has risen to the pandemic levels over the past 50 years (1). Approximately more than 2.1 billion adults are overweight or obese, of whom 1.5 billion are overweight and 640 million are obese (2). Based on the current trends, the global obesity rate will reach 18% in men and will exceed 21% in women by 2025 (3). Obesity has become one of the major health problems threatening the world today, and it is closely related to a range of diseases, such as cardiometabolic, digestive, respiratory, musculoskeletal, neurological, and infectious diseases (4, 5). The global economic cost of obesity and its complications is estimated to be US$2 trillion (2).

The adipose tissue is an organ specialized for long-term energy storage, and it grows through the increase in the number of adipocytes and in the size of each adipocyte when there is a surplus of nutrients (6). The adipose tissue plays an important role in systemic metabolic integration given its ability to produce and release a variety of inflammatory cytokines, such as leptin, adiponectin, tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) (7, 8). When there is excessive adipose tissue mass, the unbalanced expression of pro- and anti-inflammatory adipokines may result in a metabolic dysfunction (7). On the one hand, the adipose tissue of obese individuals is infiltrated by a large number of macrophages (7). On the other hand, free fatty acid exposure promotes the change in macrophage phenotype from the anti-inflammatory M2 type to the pro-inflammatory M1 type; M1 macrophages in turn promote the production of pro-inflammatory cytokines (9, 10). These cytokines could amplify inflammation locally and distally by stimulating the secretion of pro-inflammatory molecules from other tissues, leading to systemic low-grade chronic inflammation (11). Thus, obesity is often accompanied by chronic low-grade inflammation. It is worth noting that obesity-induced inflammation involves multiple organs, including adipose, heart, skeletal muscle, pancreas, liver, and brain (12). Moreover, it can lead to several diseases, such as cardiovascular disease, diabetes mellitus, nephropathy, nonalcoholic fatty liver disease, cancer, autoimmune, and neurodegenerative disorders, which severely burden global health (13).

Obesity interventions mainly include lifestyle changes, dietary restrictions, increased physical activity, use of drugs, and surgery, when necessary (14). Among the lifestyle interventions, increased physical activity is important for obesity management (14). The beneficial effects of exercise training on body composition have been studied in the existing network meta-analysis. They found aerobic exercise as well as combined aerobic and resistance training are better forms of exercise for improving anthropometric outcomes (15, 16). More importantly, regular exercise training plays an essential role in reducing the risk of chronic metabolic and cardiorespiratory diseases partly due to the anti-inflammatory effects of exercise (17). Many meta-analyses and systematic reviews have studied the effect of exercise training on inflammatory cytokines, focusing on people with type 2 diabetes mellitus, metabolic syndrome, middle-aged and older adults, cancer survivors, and others (18–21). They found that exercise training can improve the level of related inflammatory markers in these groups. Obesity is closely related to the above diseases. Therefore, it is of great significance to study the effect of exercise on inflammatory factors in overweight and obese individuals. Previous reviews have discussed the effect of exercise training on chronic inflammation and its underlying mechanisms, arguing that exercise training can reduce chronic systemic inflammation in obese individuals through a variety of mechanisms (17, 22). However, the anti-inflammatory effect of exercise training is inseparable from the exercise type and intensity. Currently, the main exercise modalities for overweight and obese people include aerobic exercise (AE), resistance training (RT), combined aerobic and resistance training (CT), and high-intensity interval training (HIIT). A meta-analysis has shown that AE decreases the levels of C-reactive protein (CRP), TNF-α, and IL-6 (20). Moreover, studies have shown that RT and CT can improve the inflammatory status of overweight and obese individuals (11, 23–25). Another study has shown that HIIT demonstrates anti-inflammatory effects similar to those of CT, and it is an effective treatment strategy for overweight and obese people who need to improve their inflammatory status but have insufficient time (26). Most of the current meta-analyses investigating the effect of exercise training on inflammatory status in overweight and obese people focuse on children (27, 28). And most of them are pairwise meta-analyses. However, a pairwise meta-analysis cannot rank the effects of different interventions. Therefore, as to which type of exercise is the most effective in improving the inflammatory status of overweight and obese patients remains unknown.

Network meta-analysis (NMA) is a technique used to evaluate multiple interventions in a single analysis by combining direct and indirect evidence (29). NMA allows for the comparison of the relative effectiveness between any pair of interventions, as well as ranks the effectiveness of different interventions (29). Therefore, this paper aimed to conduct an NMA of existing randomized controlled trials (RCTs) in order to compare different exercises and comprehensively evaluate and rank their intervention effects on body composition [body weight (BW), body mass index (BMI), waist circumference (WC), percentage body fat (%BF)] and on inflammatory cytokines (CRP, TNF-α, IL-6, IL-10, and adiponectin) in overweight and obese individuals.

2 METHODS

This systematic review and NMA are reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses for Network Meta-Analyses (PRISMA-NMA) (30). The study protocol was registered in the PROSPERO International Prospective Register of Systematic Reviews (Registration number: CRD42022303165).

2.1 Search Strategy

We searched for articles in five electronic databases (PubMed, Cochrane, Embase, Web of Science, and EBSCO), and the

retrieval period was from the date of their inception to November 2021. The literature search was performed according to the PICOS strategy, as follows: (P) Population: overweight or obese individuals; (I) Intervention: exercise; (C) Comparator: exercise intervention or no-exercise control; (O) Outcomes: body composition and inflammatory cytokine levels; and (S) Study type: RCTs. The main search terms were obesity, overweight, exercise, training, inflammation, C-reactive protein, interleukin, tumor necrosis factor, and randomized controlled trial. The reference lists of the selected articles were also searched to supplement the eligible studies. The detailed search strategy is shown in the online supplementary **Tables S1, 2**.

2.2 Study Selection

Guided by the inclusion and exclusion criteria, two researchers independently screened the studies using the EndNote software. Any disagreements in the process were resolved through a discussion or by consulting a third party, whenever necessary.

The inclusion criteria were as follows (1): The study must be an RCT. (2) The study subjects must be overweight or obese (BMI $\geq$ 25 kg/m^2). (3) The intervention group must have adopted an exercise intervention (e.g., AE, RT, CT, or HIIT) for at least 4 weeks, whereas the controls had a non-exercise routine and maintained their previous lifestyle. The classification of exercise training is shown in **Table S3**. (4) Outcome measures included at least one inflammatory cytokine (IL-6, IL-10, CRP, TNF-α, and adiponectin). (5) The study must be published in English.

The exclusion criteria were as follows: (1) The exercise intervention was combined with diet control or other lifestyle changes. (2) The subjects had other diseases, such as diabetes and cardiovascular disease. (3) The full text or other pertinent data cannot be obtained after contacting the author. (4) The material is a conference abstract, a dissertation, or a case report. (5) Duplicate publications.

2.3 Data Extraction

Two reviewers independently extracted the following information: (i) lead author; (ii) year of publication; (iii) country; (iv) sample size; (v) mean age; (vi) mean baseline; (vii) follow-up BW, BMI, %BF, WC, and IL-6, IL-10, CRP, TNF-α, and adiponectin levels; and (viii) details of the exercise intervention (type, frequency, duration, and intensity).

2.4 Risk of Bias of the Individual Studies

Two authors independently assessed the risk of bias (ROB) of the included studies using the Cochrane Risk of Bias Tool (31), which covers seven domains: (i) randomized sequence generation, (ii) treatment allocation concealment, (iii) blinding of participants and personnel, (iv) blinding of outcome assessment, (v) incomplete outcome data, (vi) selective reporting, and (vii) other sources of bias. For each source of bias, the studies were classified as having a low, high, or unclear risk (if reporting was insufficient to allow for the assessment of a particular domain). If there were any disagreements, a third party will be consulted for discussion and decision.

2.5 Statistical Analysis

We used Review Manager 5.3 for the pairwise meta-analysis. For the NMA, we used the "mvmeta" and "network" packages in Stata 16.0. In this study, the outcome indicators were the continuous variables; standardized mean difference (SMD) and 95% confidence interval (CI) were used as effect indicators. If a study involves more than one intervention groups adopting activities that fall within the same type of exercise training (e.g., stair exercise and downstairs exercise are classified as AE), the data for those intervention groups were pooled. The heterogeneity (I^2) and P values for the direct comparison of the exercise patterns for the intervention group with those of the control group were obtained through pairwise meta-analysis, and then NMA was carried out. The relationship between exercise interventions is presented using a network diagram. In the network geometry, the dot size represents the sample size, and the line connecting the dots indicates that direct comparison studies involving two exercise modes do exist (32). The greater the number of direct comparison studies between two interventions, the thicker the connecting line will be; otherwise, the thinner it becomes (32). If there is no connecting line between two motion modes, NMA was used for indirect comparison. First, the inconsistency factors (IF) and their 95% CI were calculated to evaluate the consistency of each closed loop; consistency is indicated by the lower limit of 95% CI being equal to 0 (33). Then, the inconsistency model is used to test for inconsistency; if P>0.05, the inconsistency is not significant, and thus the consistency model is used for analysis (32). At the same time, the node-splitting method is used to check the local inconsistency; if P > 0.05, the result is credible (34). The area under the cumulative ranking probability diagram (SUCRA) was used to rank and compare the effects of various exercise training interventions (35). SUCRA values range from 0 to 100%. The higher SUCRA values, and the closer to 100%, the higher the likelihood that a therapy is in the top rank or one of the top ranks (36). Thus, higher SUCRA values indicate better effects of an exercise intervention. Finally, the risk of publication bias was evaluated by using a correction comparison funnel. We also performed subgroup analyses and sensitivity analyses to explore the reasons for heterogeneity in pairwise meta-analyses.

3 RESULTS

3.1 Literature Selection

A total of 4903 studies were identified in the abovementioned databases and in other sources, and 3760 articles were left after the duplicates were removed. After the titles and abstracts were screened, 3588 articles were excluded. Finally, after the full texts were read, 38 RCTs were included in the NMA. The detailed process for the study search and selection is presented in **Figure 1**.

3.2 Characteristics of the Included Studies

The basic characteristics of the included studies (n=38) are shown in **Table S4** (11, 23–26, 37–61). A total of 1317 subjects

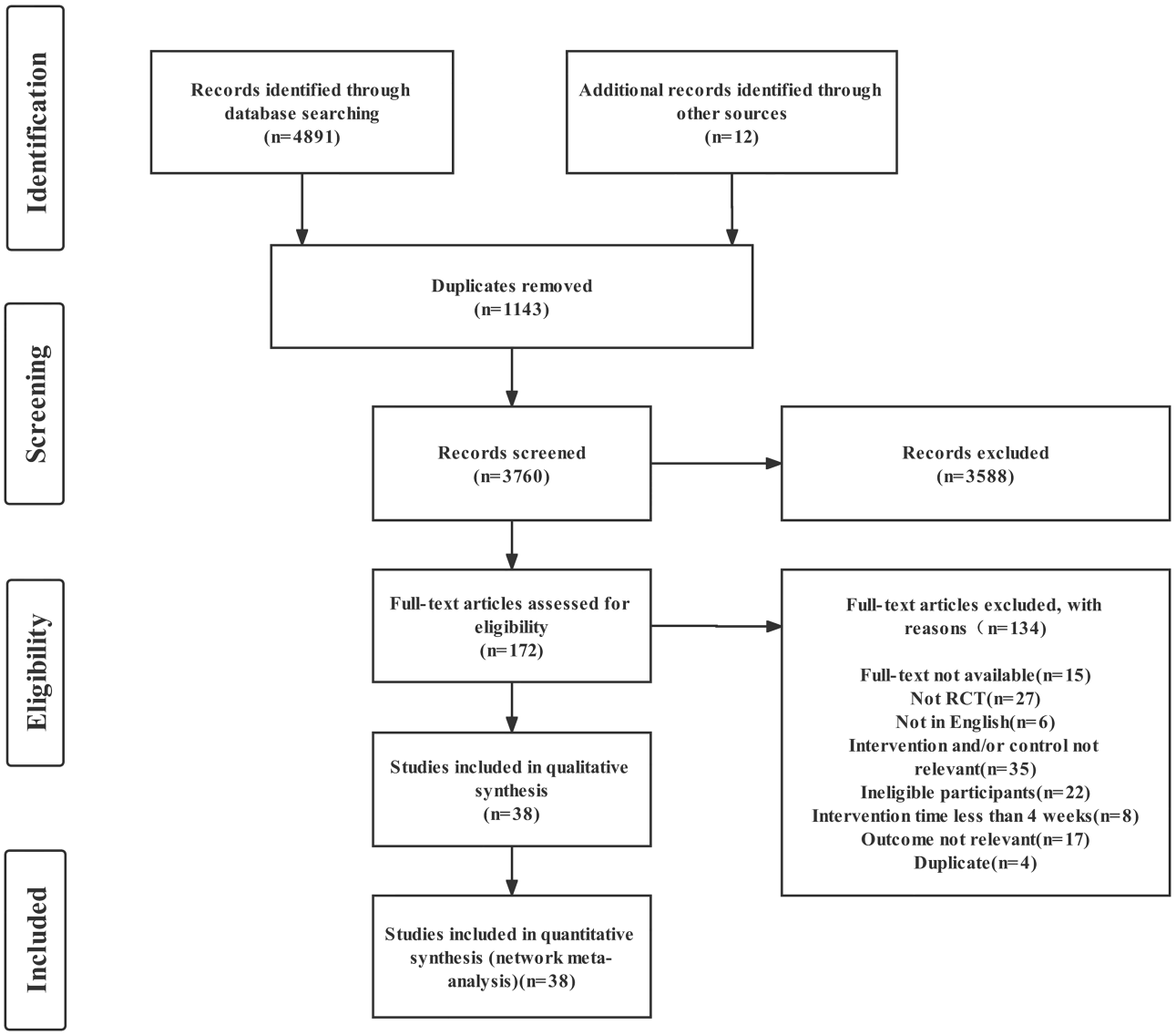

FIGURE 1 | PRISMA flow diagram.

were included in this study, that is, 868 in the experimental group and 449 in the control group. There were 31 control groups and 58 exercise intervention groups. In the exercise intervention groups, the distribution of the adopted interventions was as follows: AE (n=27), RT (n=12), CT (n=11), and HIIT (n=8). Among the control groups, one group did not engage in exercises outside the school physical education class; another group did not engage in exercises but did engage in stretching, knitting, and health lectures; the other groups had no exercise. The duration of the interventions ranged from 4 weeks to 48 weeks; the majority of the interventions lasted for 12 weeks (n=19). As regards exercise frequency, three times a week was prescribed in most studies (n=29). **Figures 2A–G** shows the available direct comparisons studies for BW (30 studies), BMI (24 studies), WC (12 studies), %BF (30 studies), TNF-α (17 studies), IL-6 (23 studies), and adiponectin (14 studies).

3.3 ROB

Of the 38 RCTs, 7 reported the generation of random sequences, and the rest only mentioned random assignment. Due to the nature of the interventions, none of the included studies met the criteria for double blinding of the subjects and implementers. Nevertheless, all studies met the criteria for the blinding of outcome indicators, and they showed good data integrity as well as avoided selective reporting. In four studies, attrition was high, which may affect the integrity of data. Three studies possibly have other biases, and the rest did not have other biases. Details about the ROB are shown in **Figure S1**.

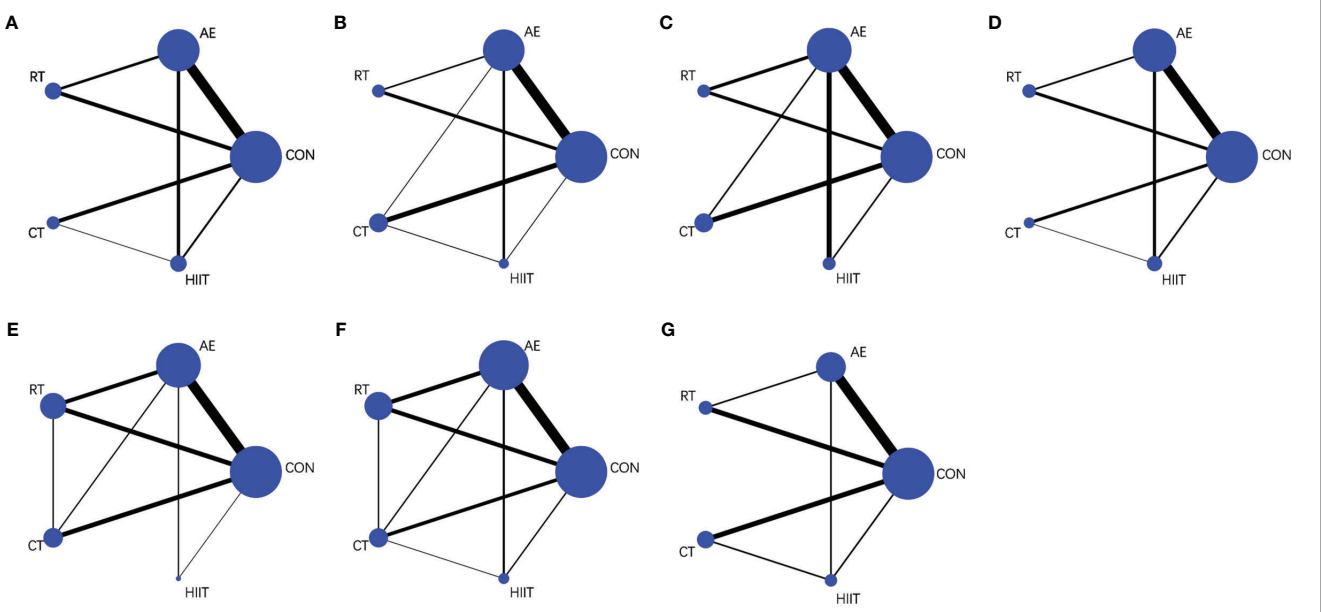

FIGURE 2 | Net graphs for **(A)** body weight, **(B)** body mass index, **(C)** waist circumference, **(D)** percentage body fat, **(E)** tumor necrosis factor-alpha, **(F)** interleukin-6, and **(G)** adiponectin. AE, aerobic exercise; RT, resistance training; CT, combined aerobic and resistance training; HIIT, high-intensity interval training; CON, control group. The size of the nodes represents the number of participants in an intervention, and the thickness of lines between interventions represents the number of studies that compare them.

3.4 Pairwise Meta-Analysis and NMA

3.4.1 BW

The pairwise meta-analysis results demonstrated that exercise effectively reduced the BW in the intervention groups relative to that in the control group (SMD=−0.48; 95% CI: −0.69, −0.27; P<0.0001; I^2 = 53%; studies: n=25; **Table S5**). The results of the consistency analysis based on the NMA indicated that, compared with the control group, the intervention groups that adopted AE (SMD=−0.51; 95% CI: −0.70, −0.33; P<0.05), CT (SMD=−0.46, 95% CI: −0.76, −0.17; P<0.05), and HIIT (SMD=−0.49; 95% CI: −0.79, −0.20; P<0.05) showed a significantly reduced BW. No significant difference in BW was observed between the control group and the RT group (SMD=−0.26; 95% CI: −0.54, 0.03; P>0.05) (**Figure 3**, **Figure S2**). The SUCRA probability sorting result showed that AE (SUCRA=78.3) had the highest probability of being the best exercise intervention for weight loss, whereas RT (SUCRA=31.9) is most likely the least effective exercise intervention (**Table S6**, **Figure S3**).

3.4.2 BMI

The pairwise meta-analysis results indicated that exercise intervention effectively reduced the BMI in the intervention group relative to that in the control group (SMD=−0.41; 95% CI: −0.68, −0.14; P=0.003; I^2 = 65%; studies: n=20; **Table S5**). The results of the consistency analysis based on the NMA showed that, compared with the control group, the intervention groups that adopted AE (SMD=−0.42; 95% CI: −0.68, −0.17; P<0.05) and CT (SMD=−0.46; 95% CI: −0.81, −0.10; P<0.05) showed a significantly reduced BMI. No significant difference in BMI was observed among the HIIT (SMD=−0.43; 95% CI: −0.95, 0.09; P>0.05), RT (SMD=−0.29; 95% CI: −0.70, 0.13; P>0.05), and control groups (**Figure 3**, **Figure S4**). The SUCRA probability sorting result showed that CT (SUCRA=70.7) is most likely the best exercise intervention for lowering BMI (**Table S6**, **Figure S5**).

3.4.3 WC

Data from the pairwise meta-analysis showed that exercise intervention effectively reduced the WC in the intervention group relative to that in the control group (SMD=−1.22; 95% CI: −1.74, −0.70; P<0.00001; I^2 = 81%; studies: n=9; **Table S5**). The results of the consistency analysis based on the NMA demonstrated that, compared with the control group, the groups that adopted AE (SMD=−1.22; 95% CI: −1.85, −0.59; P<0.05) and CT (SMD=−1.86; 95% CI: −2.80, −0.93; P<0.05) showed a significantly a reduced WC. No significant difference in WC was observed among the RT (SMD=−1.01; 95% CI: −2.09, 0.07; P>0.05), HIIT (SMD=−0.71; 95% CI: −1.69, 0.27; P>0.05), and control groups (**Figure 3**, **Figure S6**). The SUCRA probability sorting result showed that CT (SUCRA=93.4) is most likely the best exercise intervention for WC reduction (**Table S6**, **Figure S7**).

3.4.4 %BF

The pairwise meta-analysis results revealed that the exercise interventions effectively reduced the %BF in the intervention group relative to that in the control group (SMD=−1.07; 95% CI: −1.47, −0.68; P<0.00001; I^2 = 85%; studies: n=25; **Table S5**).

A

Body weight	Body mass index				
	AE	0.14 (−0.30, 0.57)	−0.04 (−0.44, 0.37)	−0.01 (−0.52, 0.50)	0.42 (0.17, 0.68)
	−0.26 (−0.55, 0.04)	**RT**	−0.17 (−0.71, 0.37)	−0.15 (−0.79, 0.50)	0.29 (−0.13, 0.70)
	−0.05 (−0.39, 0.29)	0.21 (−0.20, 0.61)	**CT**	0.03 (−0.53, 0.59)	0.46 (0.10, 0.81)
	−0.02 (−0.31, 0.27)	0.23 (−0.16, 0.62)	0.03 (−0.36, 0.42)	**HIIT**	0.43 (−0.09, 0.95)
	−0.51 (−0.70, −0.33)	−0.26 (−0.54, 0.03)	−0.46 (−0.76, −0.17)	−0.49 (−0.79, −0.20)	**CON**

B

Waist circumference	Percentage body fat				
	AE	0.33 (−0.56, 1.21)	−0.35 (−1.37, 0.66)	−0.27 (−1.09, 0.55)	1.03 (0.51, 1.55)
	−0.20 (−1.28, 0.87)	**RT**	−0.68 (−1.91, 0.55)	−0.59 (−1.74, 0.55)	0.70 (−0.14, 1.55)
	0.65 (−0.40, 1.70)	0.85 (−0.55, 2.25)	**CT**	0.09 (−1.04, 1.21)	1.38 (0.48, 2.29)
	−0.51 (−1.41, 0.39)	−0.30 (−1.66, 1.05)	−1.16 (−2.47, 0.15)	**HIIT**	1.30 (0.46, 2.14)
	−1.22(−1.85, −0.59)	−1.01 (−2.09, 0.07)	−1.86 (−2.80, −0.93)	−0.71 (−1.69, 0.27)	**CON**

C

Interleukin-6	Tumor necrosis factor-α				
	AE	−0.03 (−1.60, 1.54)	−0.78 (−2.66, 1.09)	−0.24 (−3.21, 2.73)	1.30 (0.17, 2.42)
	−0.15 (−1.80, 1.51)	**RT**	−0.76 (−2.82, 1.31)	−0.21 (−3.51, 3.09)	1.33 (−0.19, 2.85)
	0.80 (−1.23, 2.83)	0.94 (−1.33, 3.21)	**CT**	0.55 (−2.88, 3.98)	2.08 (0.42, 3.75)
	−0.88 (−3.20, 1.45)	−0.73 (−3.45, 1.99)	−1.67 (−4.34, 0.99)	**HIIT**	1.54 (−1.52, 4.60)
	−1.18 (−2.42, 0.06)	−1.04 (−2.74, 0.67)	−1.98 (−3.87, −0.09)	−0.30 (−2.66, 2.05)	**CON**

D

Adiponectin					
	AE				
	0.35 (−3.23, 3.94)	**RT**			
	−1.28 (−4.98, 2.42)	−1.63 (−6.06, 2.80)	**CT**		
	−1.79 (−5.53, 1.95)	−2.14 (−6.83, 2.55)	−0.51 (−4.49, 3.47)	**HIIT**	
	0.51 (−1.68, 2.69)	0.15 (−3.01, 3.32)	1.78 (−1.35, 4.92)	2.29 (−1.27, 5.86)	**CON**

FIGURE 3 | Matrix of the network meta-analysis results of **(A)** body weight and body mass index, **(B)** waist circumference and percentage body fat, **(C)** interleukin-6 and tumor necrosis factor-alpha, and **(D)** adiponectin. AE, aerobic exercise; RT, resistance training; CT, combined aerobic and resistance training; HIIT, high-intensity interval training; CON, control group. Each cell shows the SMD, along with the 95% CI.

The results of the consistency analysis based on the NMA showed that, compared with the control group, the groups that adopted AE (SMD=−1.03; 95% CI: −1.55, −0.51; P<0.05), CT (SMD=−1.38; 95% CI: −2.29, −0.48; P<0.05), and HIIT (SMD=−1.30; 95% CI: −2.14, −0.46; P<0.05) showed a significantly reduced %BF. No significant difference in %BF was observed between the RT (SMD=−0.70; 95% CI: −1.55, 0.14; P>0.05) and control groups (**Figure 3**, **Figure S8**). The SUCRA probability sorting result showed that CT (SUCRA=79.6) is most likely the best exercise intervention for reducing %BF, whereas the least effective exercise is most likely RT (SUCRA=36.7; **Table S6**, **Figure S9**).

3.4.5 CRP

The pairwise meta-analysis results demonstrated that exercise intervention effectively reduced the CRP level in the intervention group relative to that in the control group (SMD=−0.76; 95% CI: −1.11, −0.41; P<0.0001; I^2 = 78%; studies: n=20). In the 20 included studies, there were 12 items for AE, 5 items of RT, 5 items of CT, and 1 item of HIIT. A subgroup analysis involving three exercise modes was carried out, and the results indicated that AE (SMD=−0.43; 95% CI: −0.78, −0.09; P=0.01; I^2 = 63%), RT (SMD=−0.77; 95% CI: −1.27, −0.27; P=0.003; I^2 = 48%), and CT (SMD=−1.89; 95% CI: −3.33, −0.48; P=0.009; I^2 = 92%) significantly reduced the CRP level in the intervention groups (**Table S5**).

3.4.6 TNF-α

The pairwise meta-analysis showed that exercise intervention effectively reduced the TNF-α level in the intervention group relative to that in the control group (SMD=−1.36; 95% CI: −1.90, −0.82; P<0.00001; I^2 = 91%; studies: n=22; **Table S5**). The results of the consistency analysis based on the NMA revealed that, compared with the control group, the groups that adopted CT (SMD=−2.08; 95% CI: −3.75, −0.42; P<0.05)

and AE (SMD=−1.30; 95% CI: −2.42, −0.17; P<0.05) showed significantly reduced the TNF-α level. No significant difference was observed among the RT (SMD=−1.33; 95% CI: −2.85, 0.19; P>0.05), HIIT (SMD=−1.54; 95% CI: −4.60, 1.52; P>0.05), and control groups (**Figure 3**, **Figure S10**). The SUCRA probability sorting result showed that the different exercise methods reduced the TNF-α level in the following order: CT (SUCRA=79.4), HIIT (SUCRA=58.0), RT (SUCRA=53.9), AE (SUCRA=52.9), and CON (SUCRA=5.8) (**Table S6**, **Figure S11**).

3.4.7 IL-6

The pairwise meta-analysis revealed that exercise intervention effectively reduced the IL-6 level in the intervention groups relative to that in the control group (SMD=−0.85; 95% CI: −1.42, −0.27; P=0.004; I^2 = 91%; studies: n=19; **Table S5**). The results of the consistency analysis based on the NMA showed that, compared with the control group, the groups that adopted CT (SMD=−1.98; 95% CI: −3.87, −0.09; P<0.05) showed significantly reduced IL-6 level. No significant difference in IL-6 level was observed among the AE (SMD=−1.18; 95% CI: −2.42, 0.06; P>0.05), RT (SMD=−1.04; 95% CI: −2.74, 0.67; P>0.05), HIIT (SMD=−0.30; 95% CI: −2.66, 2.05; P>0.05), and control groups (**Figure 3**, **Figure S12**). The SUCRA probability sorting result showed that CT (SUCRA=86.4) is most likely the best exercise intervention for reducing IL-6 (**Table S6**, **Figure S13**).

3.4.8 IL-10

The 8 included studies consisted of 8 control groups, 6 AE groups, 2 RT groups, and 2 CT groups. The pairwise meta-analysis presented evidence that exercise intervention effectively improved the IL-10 level in the intervention groups relative to that in the control (SMD=2.96; 95% CI: 1.39, 4.53; P=0.0002; I^2 = 96%; **Table S5**).

3.4.9 Adiponectin

The pairwise meta-analysis indicated that the adiponectin level in the exercise intervention group did not significantly differ from that in the control group (SMD=0.52; 95% CI: −0.11, 1.15; P=0.11; I^2 = 88%; studies: n=12; **Table S5**). The results of the consistency analysis based on the NMA showed that the adiponectin level did not significant differ among the AE (SMD=0.51; 95% CI: −1.68, 2.69; P>0.05), RT (SMD=0.15; 95% CI: −3.01, 3.32; P>0.05), CT (SMD=1.78; 95% CI: −1.35, 4.92; P>0.05), HIIT (SMD=2.29; 95% CI: −1.27, 5.86; P>0.05), and control groups (**Figure 3**, **Figure S14**).

3.5 Inconsistency

The 95% CI for the inconsistency factor value for the closed loop involved in each index contains 0, indicating a good consistency for each closed loop; thus, the direct comparison evidence and indirect comparison evidence are quite consistent, and there is little difference in the impact of the results on the entire NMA (**Figures S15-21**). All of the inconsistency models showed that the P values were >0.05, indicating that there was no inconsistency. Therefore, the consistency model was used for analysis. Finally, the node splitting method shows that there is no inconsistency between the direct and indirect evidence.

3.6 Publication Bias or Small Sample Effect Test

The indexes involved in the study were tested for publication bias (**Figures S22-28**). The indexes for WC and TNF-α were asymmetric in the funnel plots, suggesting that there was a certain publication bias or small sample effect, which may have had a certain impact on the results of the corresponding indexes. The funnel plots for the other indicators were basically symmetrical, suggesting that there is a low possibility of publication bias or a small sample effect in the current study.

3.7 Sensitivity Analyses of Pairwise Meta-Analysis

In order to test whether the results of the paired meta-analysis are stable and reliable, we performed sensitivity analyses for BW, BMI, WC, %BF, CRP, TNF-α, IL-6, IL-10, and adiponectin. Sensitivity analysis showed that the overall results were solid and stable after the removal of each study (**Figures S29-37**).

4 DISCUSSION

This systematic review and NMA compared the effects of different exercise interventions on body composition and inflammatory cytokine levels in overweight and obese individuals. This study included 38 RCTs that adopted four exercise interventions and with a total sample size of 1353. The results confirmed the beneficial effects of exercise interventions on body composition and inflammatory status of overweight and obese individuals. Furthermore, CT is most likely the best exercise intervention for improving body composition (BMI, WC, and %BF) and inflammatory cytokine levels (IL-6 and TNF-α) for this population.

4.1 Effect of Exercise Training on Anthropometric Outcomes in Overweight and Obese Individuals

The results showed that exercise can effectively reduce the BW, BMI, WC, and %BF of obese patients, consistent with the results of a previous pairwise meta-analysis (62). Using NMA, we observed that AE had a better effect on weight loss, whereas CT demonstrated greater effectiveness in reducing BMI, WC, and %BF.

The AHA/ACC/TOS Guidelines for the management of overweight and obesity in adults argue that a sustained weight loss of 3%–5% is likely to reduce obesity-related complications and offers greater benefits resulting from greater weight losses (63). Our finding showed that AE, CT, and HIIT could effectively reduce the weight of overweight and obese patients, and AE is possibly the most effective exercise intervention. A previous NMA showed that long-term adherence to a regular moderate-

to-vigorous AE can significantly reduce BW compared with having a no-exercise lifestyle (15). Another meta-analysis revealed that 12 weeks to 12 months of AE can moderately reduce weight and can lower the risk of cardiovascular diseases (64). Consistent with previous findings, we also found that resistance training alone is not ideal for weight loss (16). The possible main reason is that RT alone is more helpful in maintaining or even increasing lean body mass. Therefore, RT is not useless for overweight and obese people. A study has shown that adding RT to caloric restriction can almost completely prevent the loss of lean body mass caused by caloric restriction, which is especially important in overweight and obese older adults (65).

Apart from weight, we also explored BMI, BF%, and WC. BMI is a powerful predictor of overall mortality, but it has limitations in reflecting changes in adipose tissue and lean muscle (5, 66). Meanwhile, %BF demonstrates higher specificity when considering the contribution of other tissue types to weight and body composition (67). Compared with BF %, WC reflects the status of abdominal obesity, and it is closely associated with the risk of cardiovascular diseases (5). In general, BMI and BF% reflect the degree of overall adiposity, whereas WC reflects the degree of central adiposity. Our study showed that CT will most likely exert the best effect in reducing BMI, %BF, and WC in overweight and obese individuals. An NMA investigating the effect of exercise intervention in obese patients has found that the combined exercise intervention involving AE and RT is the most promising intervention to reduce WC and %BF (16). A prospective cohort study also suggests that CT is more effective in preventing obesity (68). AE is beneficial for increasing energy and lipid utilization (69). The possible reason as to why RT can induce positive changes in body composition is that it increases skeletal muscle mass, further improving the basal metabolic rate and energy expenditure (70). Furthermore, lipolytic activation is delayed in obese individuals, and RT may play a role by upregulating adipose tissue lipolysis and by increasing energy expenditure (71, 72).

Our results demonstrated the important role of exercise intervention in obesity management and further confirmed the superiority of AE and CT over other forms of exercise in improving body composition. However, it is worth noting that dietary control cannot be ignored in obesity management. The current guidelines for medical care of obese patients point out that a structured lifestyle intervention program designed for weight loss should include healthy dietary plans, physical activities, and behavioral interventions (73). An NMA on the impact of long-term lifestyle programs on weight loss and cardiovascular risk factors in overweight/obese participants also suggests that diet combined with exercise intervention can be highly recommended for long-term obesity management, and dietary intervention has advantages over exercise intervention in anthropometric results (74). In a word, we recommend that overweight and obese people should adopt AE combined with RT as their primary form of exercise, while paying attention to caloric restriction.

4.2 Effect of Exercise Training on Pro-Inflammatory Cytokine Levels in Overweight and Obese Individuals

IL-6, TNF-α, and CRP are important pro-inflammatory factors, and their levels are elevated in people with obesity (7, 17). Studies have shown that exercise training can reduce obesity-related chronic inflammation by affecting the inflammatory mediators from various sources, including adipose tissue, muscle tissue, endothelial cells, and circulating immune cells (22). The pairwise meta-analysis showed that exercise intervention could significantly reduce the levels of IL-6, TNF-α, and CRP in overweight and obese people. Since the relevant literature on CRP indicators does not meet the requirements for an NMA, we conducted an NMA on IL-6 and TNF-α, and the results showed that CT had the highest probability of being the best exercise intervention for reducing IL-6 and TNF-α levels.

The circulating levels of IL-6 and TNF-α are directly associated with adiposity and insulin resistance (8). A previous review discussing the effects of physical activity on inflammatory mediators suggested that the combination of AE and RT is the best form of exercise to improve one's inflammatory state, consistent with our findings (75). CT may reduce the level of inflammatory cytokines in overweight and obese individuals through the following mechanisms. Firstly, CT reduces the release of inflammatory cytokines by reducing body fat, especially visceral fat. Adipose tissue is a rich source of inflammatory cytokines, and the current results and previous findings have shown that CT is likely to be the best exercise intervention to reduce %BF and abdominal fat (76, 77). Secondly, CT is superior to AE and RT in improving muscle protein synthesis and myocellular quality (78). Moreover, exercise promotes the production of skeletal muscles and the release of muscle-derived cytokines (such as IL-6), which play significant anti-inflammatory and metabolic functions (79). Thirdly, Inflammatory monocytes (CD14+CD16+) are highly "proinflammatory", and are potent producers of inflammatory proteins (80). Previous research indicated that CT can reduce the percentage of inflammatory monocytes in circulation (80). And another study has shown that CT can reduce CD14 + cell surface expression of toll-like receptor 4 (TLR4) and lower lipopolysaccharide-(LPS) stimulated IL-6 production (81). More high-quality studies are needed to further explore the mechanism by which CT can more effectively improve the degree of inflammation.

CRP is a chronic systemic inflammatory marker capable of predicting cardiovascular events (82). A large-scale cross-sectional study has shown that CRP is positively correlated with BMI (83). Consistent with our results, the findings of a meta-analysis indicated that exercise training reduces CRP levels and that exercise results in a greater reduction in CRP when accompanied by a reduction in BMI or %BF, further confirming the importance of improving body composition to reduce the levels of anti-inflammatory factors (84). A previous review also suggested that the unfavorable inflammatory profile related to increased adiposity can be improved during a period of weight loss (85). The results of our subgroup analysis according to

exercise mode showed that AE, RT, and CT could effectively reduce CRP levels. Although the effect of exercise interventions cannot be ranked, based on the effectiveness of CT in reducing BMI and %BF, we can speculate that CT may be more effective in reducing CRP levels. Interestingly, a meta-analysis also compared the effects of exercise training and caloric restriction on inflammatory markers (86). The above study found that exercise training combined with caloric restriction could effectively improve the circulating concentrations of inflammatory factors, and caloric restriction was more effective than exercise training in reducing CRP levels (86).

We also attempted to analyze other pro-inflammatory cytokines such as IL-1β, Monocyte chemoattractant protein-1 (MCP-1), leptin, and IL-18. Their production is upregulated in the obese state, leading to the development of a chronic inflammatory state (7). However, there are few studies on these inflammatory factors, and the number cannot meet the minimum literature amount of meta-analysis.

4.3 Effect of Exercise Training on Anti-Inflammatory Cytokine Levels in Overweight and Obese People

IL-10 and adiponectin are significant anti-inflammatory factors. It has been reported that the circulating levels of IL-10 and adiponectin are lower in obese individuals than in normal-weight people (7, 17). Some studies have shown that exercise training can improve the levels of IL-10 and adiponectin, whereas other studies have not observed significant changes in both factors after exercise (11, 23, 59). We conducted only pairwise meta-analyses of IL-10 due to the small number of articles focusing on IL-10. The results showed that IL-10 levels significantly increased in overweight and obese individuals who adopted exercise regimens relative to that in the control group. IL-10 can promote the switch of macrophage phenotype from M1 to M2; M2 macrophages can upregulate IL-10 production, significantly enhancing the ability of IL-10 to exert anti-inflammatory effects and consequently improve insulin resistance and obesity-related complications (87). A study suggested that exercise training can increase circulating numbers of regulatory T cells, which mainly release anti-inflammatory cytokines such as IL-10 (22). Another study has shown that exercise training increases the level of muscle-derived IL-6 (88). IL-6 creates an anti-inflammatory environment by inducing anti-inflammatory cytokines such as IL-10 and IL-1Ra and inhibiting TNF-α production in adipose tissue and infiltrated macrophages (89). Furthermore, a review has shown that exercise reduces adipose tissue mass and adipocyte size, reduces macrophage infiltration, and promotes the macrophage phenotype changes from the pro-inflammatory M1 type to the anti-inflammatory M2 type, which may help increase the release of anti-inflammatory cytokines (e.g., IL-10 and adiponectin) from the adipose tissue (17). In addition, exercise training may reduce endothelial cell inflammation by increasing the number of endothelial progenitor cells, blood flow, laminar shear stress, and reducing the release of adhesion molecules, which can promote macrophages to switch from pro-inflammatory M1-type to anti-inflammatory M2-type (22). However, the results of both pairwise meta-analyses and NMA showed that adiponectin level increased after the exercise intervention, but the difference was not statistically significant.

Adiponectin is an adipose tissue-secreted factor that is negatively correlated with obesity, and its circulating levels can be used as a key marker of adipose tissue health (90). The decrease in adiponectin expression may be related to obesity or obesity-related metabolic disorders, such as insulin resistance, hyperlipidemia, and atherosclerosis (27). A meta-analysis has shown that exercise increases the level of adiponectin in overweight and obese people compared with the no-exercise regimen and the control, inconsistent with our results (91). However, the aforementioned study argues that the results for adiponectin are unreliable because they included small trials reporting extreme effects, as well as studies with high heterogeneity (91). Moreover, the authors were more inclined to speculate that exercise may have little to do with significant changes in adiponectin level (91). A review on the response and adaptation of adiponectin to acute and chronic exercise suggests that in some cases, adiponectin levels are not affected after exercise despite the reduction in body fat or BMI on the one hand; on the other hand, it seems that a training that aims to improve health and reduce weight and body fat will increase adiponectin levels at rest (92). The reasons for this discrepancy may be manifold. The most likely reason is the difference in the duration of intervention. A 24-week intervention study has reported that a moderate-to-high-intensity combined exercise increased the serum concentrations of adiponectin in middle-aged obese men (11). Another study has shown that 1 year of regular moderate-intensity RT significantly increased the level of adiponectin in overweight women (48). However, most of the studies included in the current study had a short intervention time, mostly about 12 weeks. Furthermore, the possible reasons include the initial degree of inflammation of the subjects, blood collection time, menstrual cycle, and intensity of exercise intervention, among others.

Besides IL-10 and adiponectin, we also focused on anti-inflammatory factors such as IL-4, IL-13, IL-1ra, and transforming growth factor β (TGF-β). Unfortunately, we found so little literature on these anti-inflammatory factors that a meta-analysis was impossible.

4.4 Strengths and Limitations

This study has several strengths. First, this paper is the first to use NMA to analyze the impact of different training modalities on inflammatory cytokines in overweight and obese individuals. Furthermore, the indicators included in this paper are relatively comprehensive and can effectively reflect the changes in body composition and inflammatory status. However, our study has some limitations. First, although the superiority of CT was demonstrated here, we did not take into account the sequence of performing AE and RT. And due to their limited number, the studies were not classified according to exercise intensity. Second, there is a high risk of heterogeneity in the pooled results of paired meta-analyses due to the differences in exercise intensity, exercise form, exercise time, exercise

frequency, exercise equipment, settings, sample size, and article quality among studies. Heterogeneity was not fully resolved by sensitivity and subgroup analyses, and the results should be interpreted with caution. Third, the number of studies on different exercise interventions varied greatly; for example, 27 studies involved AE, while only 8 studies involved HIIT. Furthermore, during the literature selection process, not all existing literature could be included because the original text for some studies could not be found, and some studies used geometric means. Finally, since the included studies were all human trials, it was difficult to observe double blinding.

5 CONCLUSION

Our study demonstrated that exercise intervention could effectively improve body composition and chronic inflammatory status in overweight and obese individuals. More importantly, the results of this NMA suggested that CT is most likely the best exercise intervention for reducing BMI, WC, %BF, IL-6, and TNF-α in overweight/obese individuals. It is recommended that exercise prescriptions for overweight and obese people will involve a combination of AE and RT.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.921085/full#supplementary-material

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REVIEW
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Obesity, Inflammation, and Immune System in Osteoarthritis

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Obesity remains the most important risk factor for the incidence and progression of osteoarthritis (OA). The leading cause of OA was believed to be overloading the joints due to excess weight which in turn leads to the destruction of articular cartilage. However, recent studies have proved otherwise, various other factors like adipose deposition, insulin resistance, and especially the improper coordination of innate and adaptive immune responses may lead to the initiation and progression of obesity-associated OA. It is becoming increasingly evident that multiple inflammatory cells are recruited into the synovial joint that serves an important role in pathological changes in the synovial joint. Polarization of macrophages and macrophage-produced mediators are extensively studied and linked to the inflammatory and destructive responses in the OA synovium and cartilage. However, the role of other major innate immune cells such as neutrophils, eosinophils, and dendritic cells in the pathogenesis of OA has not been fully evaluated. Although cells of the adaptive immune system contribute to the pathogenesis of obesity-induced OA is still under exploration, a quantity of literature indicates OA synovium has an enriched population of T cells and B cells compared with healthy control. The interplay between a variety of immune cells and other cells that reside in the articular joints may constitute a vicious cycle, leading to pathological changes of the articular joint in obese individuals. This review addresses obesity and the role of all the immune cells that are involved in OA and summarised animal studies and human trials and knowledge gaps between the studies have been highlighted. The review also touches base on the interventions currently in clinical trials, different stages of the testing, and their shortcomings are also discussed to understand the future direction which could help in understanding the multifactorial aspects of OA where inflammation has a significant function.

Keywords: osteoarthritis, obesity, innate immunity, adaptive immunity, synovium & osteoarthritis, biomechanics, T cells

1 INTRODUCTION

Obesity and OA are two major health problems prevalent in our society today. According to the Australian Institute of Health and Welfare, 2 in 3 (67%) of the adult population were overweight or obese in 2018 (1). It is reported that 1 in 3 (6.9 million) people suffer from musculoskeletal conditions, including arthritis which constitutes the top disease burden (2). Obesity increases the risk of OA in both weight-bearing (knee) and non-weight-bearing (hand) joints, and obesity doubles the lifetime risk of symptomatic OA compared to individuals with a BMI below 25 (3). The dynamic environment of joints is constantly subjected to mild damage through motions, and in some joints, weight-bearing (knee and hip) leads to compression, resulting in a state of persistent wound healing and repair processes. As a result, the articular cartilage and neighboring bone must continually rebuild where synthesis and degradation are a constant process (4). These activities necessitate the activation of anabolic and catabolic enzymes in bones and cartilage. Traditionally, the pathogenesis of OA was considered non-inflammatory in origin, with mechanical stress leading to cartilage destruction (5). However, recent studies suggest that OA has an inflammatory component with inflammatory cell infiltration of the synovial membrane (6–8). Recent research highlights that obesity and increased periprosthetic infections are strongly linked according to Australian Orthopaedic Association registry data, and the risk increases for the morbidly obese (9). This data highlights that the obese environment is highly complex at both the systematic and local levels. Adipose tissue (AT) plays a crucial role in the regulation of metabolic activities storing excess energy as triglycerides and converting them into fatty acids and glycerol, in required places. They also perform an additional role in secreting adipokines. Adipokines are a key player that regulates the homeostasis in inflammation, immunity, reproduction, angiogenesis, fibrinolysis, regulating appetite, coagulation, and insulin sensitivity (10). It is becoming evident with piles of novel studies that correlate obesity-induced adipokine production which leads to the onset of OA (11, 12). A large genome-wide associated study was run to identify the susceptibility risk loci. The study found that over 140 genes were associated with OA and majority of the variants were localized in the non-coding region which made it inconspicuous. Hence, it is essential to establish the functional link between genomic and disease-relevant alteration on multimolecular levels (13–15).

1.1 Comparative View on the Healthy Synovial Microenvironment and OA Synovium Microenvironment

Synovium is a soft tissue found in the diarthrodial joints, tendon sheaths and bursae. They have consecutive layers of cells, where inner layer is called the intima and the outer layer is called the subintima. The intimal layer houses the macrophages and fibroblasts while the subintima has blood and lymphatic vessels, fibroblast and infiltration of cells in a collagenous extracellular matrix (16). The intimal layer is 20- 40mm thick in cross-section and the subintima can be up to 5mm in thickness (16, 17). The synovium is responsible for maintaining the functional activity of articular cartilage and the well-being of chondrocytes by producing lubricin and hyaluronic acid (i.e. synovial fluid) (16). Numerous factors contribute to synovial joint homeostasis, including regular expression of protective lubricin, fibroblast-like synoviocytes (FLS) secretion of matrix metalloproteinase (MMPs), immune centralized role is played by resident macrophages and FLS, regulated entry and exit of leukocytes involved in immune surveillance, and local regulation by cytokines and growth factors (4).

Synovitis is increasingly recognized as a prevalent symptom of OA, both in early and late stages, and as such, it provides a potential target for treatment, both for symptom relief and structural alteration. OA synovial tissue (ST) has a lower overall inflammatory profile than Rheumatoid arthritis (RA), although it is greater than healthy controls. From the synovial membranes of OA patients, a range of immune cells from both the innate and adaptive immune families have been discovered. Lindblad et al. found that inflammation in the synovium adjacent to cartilage elicited a stronger inflammatory response, with T cells surrounded by B cells and plasma cells (18). Revell et al. observed lymphoid follicle growth in OA synovial membrane throughout the same period, emphasizing the relevance of B lymphocytes and granulocytes in the pathogenesis of OA (19)

2 ROLE OF INNATE IMMUNITY IN OBESITY AND OA

The innate immune system is a primitive mechanism that humans inherit from invertebrates that use germline-encoded proteins to recognize pathogens. Upon exposure to these pathogens, the innate immune cells either kill the pathogens directly or recruit the adaptive immune system through a series of events. Cells of the innate immune system consist of macrophages, dendritic cells (DCs), and natural killer cells (NK) which recognize Pathogen-Associated Molecular Patterns (PAMPs) or Damage-Associated Molecular Patterns (DAMPs) that originate from highly conserved parts of microbes and use a diverse set of patterns recognition receptors (PRR) molecules like

Abbreviations: OA, Osteoarthritis; AT, Adipose Tissue; ST, Synovial Tissue; DCs, Dendritic cells; NK, Natural killer cells; RA, Rheumatoid Arthritis; ER- α, Estrogen receptor- α; FLS, Fibroblast-Like Synoviocyte; MMPs, Matrix Metalloproteinases; IPFP, Infrapatellar Fat Pad; SCAT, Subcutaneous AT; VAT, Visceral AT; SWAT, Subcutaneous White AT; PAMPs, Pathogen-Associated Molecular Patterns; DAMPs, Damage-Associated Molecular Patterns; PRR, Pattern Recognition Receptors; LPS, Lipopolysaccharide; IFN-γ, Interferon gamma; IL-#, Interleukins-#; iNOS, Inducible Nitric Oxide Synthase; WAT, White AT; TNF-α, Tumor necrosis factor-alpha; ATM, AT Macrophages; MMe, Metabolic Activation; TLR4, Toll-like receptor 4; SNPs, Single Nucleotide Polymorphisms; NKG2D, Natural Killer Group 2D; H_2O_2, Hydrogen peroxide; HIF-1a, Hypoxia-inducible factor 1-alpha; RORγt, Retinoic-acid receptor-related orphan γt; ACC1, Acetyl CoA Carboxylase 1; T2DM, Type 2 Diabetes Mellitus; GC, Germinal Centre; RvD1, Resolvin D1; MAPKs, Mitogen-Activated Protein Kinases.

lipopolysaccharide (LPS) (20). One of the potential mechanisms of LPS leakage into the systemic circulation and sensitization of the immune system is through dysbiosis gut-associated with obesity. The leakage has been implicated in the development of low-grade inflammation both systematically through the release of LPS and locally in the small intestine and ST (21, 22).

Classically, the innate immune system is activated by host responses to PAMPs generated by interactions with invariable pattern recognition receptors (PRRs) on synovial joint immune cells such as neutrophils, macrophages, monocytes, and DC. PRRs are a group of cell surface, endosomal, and cytosolic receptors that include Toll-like and NOD-like receptors (23). When PRRs are activated within tissues such as the joint, they initiate rapid-onset inflammatory responses, which are then followed by the commencement of adaptive immune responses and, lastly, healing responses in the case of tissue damage. The **Figure 1** shows the innate immune cells and their upregulation/downregulation in physiological inflammation and pathological inflammation.

2.1 Monocytes and Macrophages in Obesity and OA

In AT, macrophages are the critical mediators of inflammation and the most abundant infiltrated cells. There is four times increase in macrophage density in the obese mice model compared to lean mice models (25). In obese mouse models, macrophages are localized in the crown-like structure around the larger adipocytes (Tab.1) (26). Macrophages are heterogeneous as characterized by cytokine secretions and surface marker expression functions. They have broadly divided into M1 or classically activated macrophages and M2 or activated macrophages (27). M1, which is considered as pro-inflammatory, is induced by pro-inflammatory mediators like LPS and interferon-gamma (IFN-γ) and thereby secretes cytokines like interleukins (IL-6 and IL-1β), inducible nitric oxide synthase (iNOS), and tumor necrosis factor-alpha (TNF-α); whereas M2 is induced by IL-4 and IL-13 and secretes anti-inflammatory IL-10, IL-1 decoy receptor, and arginase which further blocks the IL-1β and iNOS activity. White AT (WAT) in obese tissue has been observed to increase the number of macrophages and mast cells compared to lean tissue leading to increased gene expression of TNF- α and a remarkable increase in IL-6 and iNOS levels (25, 28).

The cytokine expression of macrophages differs in its polarisation. Even though macrophage polarisation is not binary, it is expressed in a continuum. M1 macrophages have been shown to express IFN-γ and LPS driven macrophage phenotypes, whereas M2 refers to macrophage phenotypes triggered by IL-4 or IL-13. Furthermore, this representation reveals the mixed signals that lead to cytotoxic function of M1, anti-inflammatory, tissue remodelling, and repair from M2 macrophage polarisation (29). Regulation of macrophage polarization and functions are tightly controlled through the activation of several interconnected pathways. Among the few factors, STAT1 and STAT3/STAT6 transcription factors are crucial in the balance of macrophage activation. STAT1 activation promotes M1 polarisation, and in contrast, STAT3/6 activation by IL-4, IL-13, and IL-10 leads to increased M2 polarisation (30). In addition, IL-4 has been shown to induce c-Myc that activates the interferon regulatory factors4 (IRF4) axis, resulting in M2 promotion by inhibiting IRF5 mediated M1

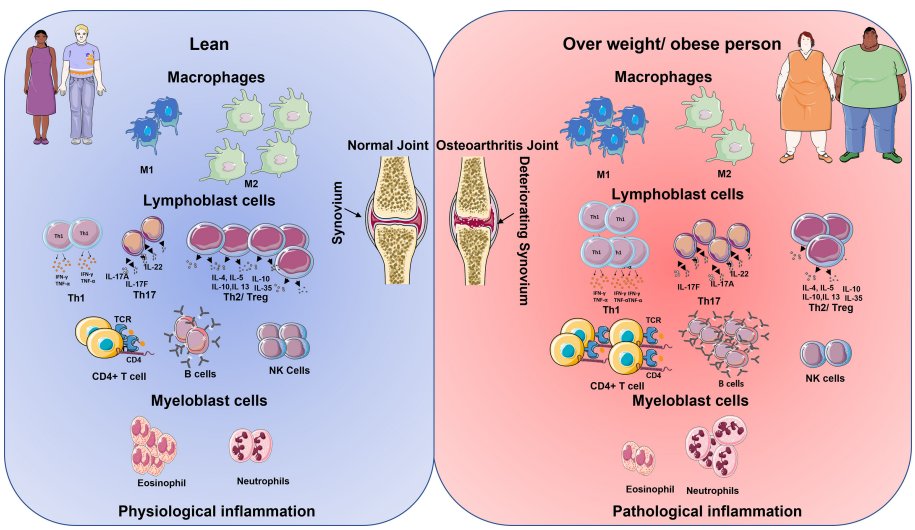

FIGURE 1 | Schematic diagram shows the immune cell being up and downregulated in lean and overweight/obese individuals. The cells on the left represent the lean physiological aspects of the immune system and on the right, the obese immune system (24). In lean population during inflamed condition the macrophages M1 cells are lower in comparison to M2 cells. However, the reverse is seen in the obese inflammation. Lymphoblastic cells like Th1, Th17, T helper, and B cells are lower in comparison with Treg cells and NK cells in lean inflammation population. However, in obese population, there is an increase in the production of Th1, Th17, T helper cells, B cells but reduction in the number of T reg cells and NK becomes low. Granulocytes like the eosinophiles are higher in comparison with neutrophiles in lean inflammation condition, however, the reverse is observed with obese population in their inflammatory condition.

polarization. IRF4 plays a role in developing a subset of myeloid cDC2 specializing in Th2 responses in mice models (31).

The characteristic differentiating surface markers of M1 differing from M2 is CD11c expression in inflammation and insulin resistance in human obesity (32). Lumeng et al. demonstrated that lean mice have predominant M2 phenotype and obese mice expressed M1 phenotype (26). However, in human obesity, multiple *in vivo* studies revealed that AT macrophages (ATM) adopt mixed M1/M2 phenotypes (30, 33). In obesity, ATM adopts a prominent metabolically activated state with increased lysosomal activity (34). This state of metabolic activation (MMe) is induced by diverse stimuli like free fatty acids, high insulin, high glucose levels (35). A "phenotypic switch" occurs in diet-induced obese mice where a shift in polarization towards M1 activation takes place over days to weeks from the M2 macrophages, which are dominant in AT of lean mice (26). ATM in an MMe are a significant source of inflammatory cytokines, and their production can be modulated by NADPH oxidase 2 activity during the progression of obesity (34). In obese condition, due to adipocyte hypertrophy, the secretion of chemo-attractants like MCP-1/CCL2 lead to macrophage recruitment and production of TNF-α, IL-6, and IL-1β, which acts as pro-inflammatory signals (35). Morris et al. demonstrated that ATM in obese models had increased expression of MHC II and T cell co-stimulatory molecules, thus processing antigens and inducing antigen specific $CD4^+$ T cell population (36). ATM serves as a crucial link between innate and adaptive immunity in obesity. However, an M2 phenotypic switch can be induced through activation of PPAR-γ, thereby protecting against M1 activation and insulin resistance (37). This can potentially promote adipocyte lipid storage and prevent lipotoxicity and adipocyte death (38). De Jong et al. (39) has shown that BMI-related features of immune cell profile in subcutaneous AT (SCAT) and visceral AT (VAT) could not be detected in the infrapatellar fat pad of OA patients. Animal and clinical studies have primarily revealed that obesity does not increase the number of crown-like structures in the infrapatellar fat pad (39). Macrophages are the most widely distributed cell types in the OA synovium, mostly present along with the lining layer (40). Wood et al. reclassified OA into classical OA (cOA), which is predominantly associated with cartilage remodelling features, and inflammatory OA (iOA) subset characterized by a proliferation signature (41). Insulin-like growth factor-binding protein 5 (IGFBP-5) is overexpressed in cOA, which is associated with the negative regulation of inflammatory mediators. High-temperature requirement 1 and EGF-containing fibulin-like extracellular matrix protein 1 overexpression is observed in cOA, which modulate the synovial fibroblasts to produce cartilage catabolic MMPs and negatively regulate chondrogenesis, respectively. Macrophages in iOA were most closely aligned to macrophages associated with inflammatory arthritis, which is strongly linked to cell cycle processes. iOA macrophages have increased expression of MK167, which demonstrates the increased proliferation and chymotrypsin-like protease that reflects a pro-inflammatory environment, thus developing a positive feedback loop (41). Various DAMPs have been identified, leading to the activation of macrophages, and inducing inflammation (20).

Through etarfolatide imaging, activated macrophages are present in patients with knee OA rather than resting macrophages (42). TGF-β1 levels (2) expressed by ST macrophages in synovial fluid are found to be a strong predictor for knee OA progression (43). Recently, Wood et al. (41) described the heterogeneity of synovial macrophages in OA, and the classical description of either pro-inflammatory (M1) or anti-inflammatory (M2) paradigm is not aligning with the functional status of tissue macrophages seen in OA. RNA-seq data from synovial OA shows a mixture of both M1- and M2-related genes can be expressed in synovial macrophages across OA (41). So, the traditional M1/M2 classification is no longer an accurate description for different types of synovial macrophages in OA and other inflammatory arthritis. Animal studies indicate that complex and multiple factors could be involved in the development of OA-like synovial insulin resistance (44), gut microbiota (45), and dietary fatty acid composition (46, 47). In a study to assess the synovial inflammation between early and late OA, the intensity of macrophage infiltration increased with increasing histological grade of OA (48). Multiple studies have implicated macrophage-mediated inflammatory response in the pathogenesis of OA (28, 49). However, Harasymowicz et al. (50) reported that the synovium and fat pad of obese patients had shown increased macrophage infiltration and higher toll-like receptor 4 (TLR4) gene expression. TLR4 recognizes LPS along with its extracellular components such as MD-2 and CD14 (51). Furthermore, there was an increase of CD14+ CD206+ M2- type macrophage in infrapatellar fat pad (IPFP) and ST. However, peroxisome proliferator-activated receptor γ and adiponectin were expressed in lower levels in IPFP and ST of obese patients compared to lean patients (50). These demonstrated that the M1/M2 paradigm does not fit with the obesity-induced macrophage activation. In aged mice, genetic deletion of TLR4 prevented the development of OA from high-fat diet-induced obesity (52). Multiple TLR single nucleotide polymorphisms (SNPs) have been implicated as genetic associations with knee OA like T-1486C SNP in TLR9 (53, 54), TLR3 SNPs (55), TLR7, and TLR 8 SNPs (56). These potential mechanisms need to be further explored to better understand the role of macrophages in obesity-related OA.

2.2 Neutrophils in Obesity and OA

In morbid obese individuals, it was demonstrated that the blood neutrophils have a low bactericidal capacity and adherence capacity (57). It is also demonstrated that neutrophil stimulation with LPS results in increased hydrogen peroxide (H_2O_2) production for pathogen clearance (58). Neutrophils are known to use high rates of aerobic glycolysis for ATP generation through hypoxia-inducible factor-1alpha (HIF-1a) (59). These activations of the HIF signaling pathway increase their survival, glycolytic metabolism, production of antimicrobial peptides, thereby effectively fighting against infection (58). Recent evidence demonstrates that in obese and asthma patients, obesity leads to shifting the Th2/eosinophilic response towards

Th17/neutrophilic profile, contributing to the worsening of asthma (60). Xu et al. demonstrated the systemic effects of obesity in the human population by identifying the increased neutrophil percentage and increased expression of genes involved in neutrophil activation like neutrophil elastase and myeloperoxidase (57, 61).

Another study has shown that the release of basal superoxides, formyl- methionyl- leucyl- phenylalanine-stimulated superoxide, and opsonized zymosan-stimulated superoxides were elevated compared to that of lean controls (62). However, phagocytosis, CD11b surface expression, and adherence of neutrophils did not have any significant changes compared to the lean model. The study was drawn to a conclusion by observing the high level of superoxide production, chemotactic activity, normal phagocytotic activity, and adherence are significant signs of subjects with obesity carrying neutrophils being primed and having the capacity to fight infection. Since the neutrophils are in the prime state, they may participate in the pathogenesis of obesity-related diseases, including OA (62).

The role of neutrophils in OA pathogenesis is relatively unknown, whereas RA is well characterized. According to de Lange-Brokaar et al. (63), there are varying levels of neutrophils present in the synovium of OA. Hsueh et al. demonstrated the presence of neutrophils in synovial fluid through increased expression of elastase (43). Levels of elastase in synovial fluid are strong predictors of knee OA progression (43). Hsueh et al. found synovial fluid elastase and TGFβ are vital players in knee OA, reflecting the synchrony of neutrophils and macrophages population in the pathogenesis and worsening of OA (43). These studies do not have a consistent outcome on their results that could help in understanding the role of neutrophils in OA, further studies must be done to establish the independent role and their signalling cascade in obesity-linked OA.

2.3 Eosinophils in Obesity and OA

Eosinophils have a protective role in obese mice. Two prominent studies showed that an increase in the eosinophil numbers had reduced the fat in mice (64, 65). The increase in weight gain and glucose intolerance, this was associated with the reduced eosinophils (65). Contradictorily, in another study, the eosinophils population was increased in AT of high-fat diet-induced mice by chronic helminth infection and soluble helminth egg antigen. In treated mice, it was observed that the weight gain was less and less of fat mass gain, adipocyte size was smaller, glucose uptake improved, and AT insulin sensitivity also increased (66, 67).

Contradictory results were seen between mice and human studies with regards to the eosinophil activity in obesity. The positive coexistence of eosinophils with obesity was observed in an epidemiological study between body mass index or metabolic syndrome and blood eosinophil counts (68, 69). Similarly, Moussa et al. studies that the increase in the number of eosinophils in the SCAT was directly associated with metabolic syndrome (70). The study exhibited that circulating and SCAT eosinophils were two times higher in metabolic syndrome patients and correlated with each other. The findings were novel and seminal, where the eosinophils increased in SCAT in metabolic syndrome patients which is directly associated with the pro-inflammatory status. Therefore, it was concluded that dysregulation of SCAT biology contributes to metabolic syndrome in humans (70). A specific population of eosinophils was identified in patients with RA however a proper understanding of eosinophils and their functions in OA is still under investigation (71). A detailed study is needed to define how this innate immune cell type reacts when the joint microenvironment homeostasis is affected by this multifactorial disease. The role of eosinophils in obesity and OA has been elucidated in **Table 1**.

2.4 DCs in Obesity and OA

DCS are specialized antigen-presenting cells that link adaptive and innate immune responses. DCs are a heterogeneous collection of cells distinguished by differential expression of essential transcription factors such as IRF8 and IRF4. This system recognizes two types of conventional/myeloid DCs (cDCs) and the plasmacytoid DCs (pDCs) (102). Myeloid cDC1 promotes the T helper type1(Th1) and NK responses through IL-12 and activates CD8+ T cells through MHC class I (103). Myeloid Cdc2 in human blood responds well to LPS, flagellin, Poly IC and R 848 as they are well equipped with a wide range of lectins, TLRs, NOD-like receptors, and RIG-I like receptors (104). Human Cdc2 is stimulated and produces huge quantities of IL-12 compared to Cdc1. Cdc2 has been shown as potent activators of Th1, Th2, Th17, and CD8+ T cells. The third type of DCs called the inflammatory dendritic cells, defined as monocyte derived DCs, are seen in the inflammatory state. In humans, these cells are demonstrated in various settings like synovitis, psoriasis, inflammatory bowel disease (103). The heterogeneity of these cell surface markers makes it difficult to develop a clear consensus on other immune cells like monocyte, macrophage, or DCs to describe these cells. Segura et al. demonstrated that inflammatory DCs induce Th17 cell differentiation through stimulation of memory CD4+ T cells to produce IL-17 (105). They further proposed that these cells are derived from monocytes and are involved in the induction and maintenance of Th17 cell responses (105). Activated DCs were demonstrated to be increased in numbers in AT of obese non-diabetic humans compared to lean subjects (75). These activated DCs regulated the AT inflammation by regulating the switch towards Th17 cell is insulin resistance associated with obesity (75).

TLR family plays a fundamental role in the activation of DCs in OA, especially TLR4, promotes obesity-induced OA in the mouse model (Tab.1) (52). In the experimental OA model, increased DCs were found to significantly upregulate TLR 1-8 mRNA levels (76). TLR3 was significantly elevated in DCs from these experimental OA models in mice. These data suggested that the inflammatory activity of DCs in OA occurs through activation of membrane TLRs.

TABLE 1 | Details cellular functions of the innate and adaptive immune cells on the review are mentioned in detail along with the references.

IMMUNE CELL AND ITS CHARACTERISTICS	OBESITY	OA
INNATE IMMUNE SYSTEM		
Increased ATM population and pro-inflammatory state	(26)	
Role of LPS and TLR 4 in macrophage activation and pro-inflammatory state		(50, 52)
Role of PPAR y in macrophage activation	(37, 38)	(50)
NK cell dysregulation	(72, 73)	(74)
Dendritic cells activation	(75)	(76)
Heterogeneity/Phenotypic switch M1/M2	(26, 30, 33)	(41, 50)
ADAPTIVE IMMUNE SYSTEM		
Th9 cells and IL-9		(77)
TH2/Eosinophil to Th17/Neutrophil transition	(60)	
Increased Th1 and IFN -y production	(78, 79)	
Role of PPAR-Y in T cells	(80)	
Biphasic expression of Th17 in obesity	(81, 82)	
Role of IL-17/Th17 in arthritis		(83–86)
Negative regulation of adipogenesis by IL-17		
Inhibitory and anti-inflammatory role of Treg in obesity and metabolic syndrome	(79, 87)	
Lack of Tfh in OA		(88)
Regulation of Th17/Treg in obesity/hypoxia	(89–91)	
Treg influence of M1 infiltration in AT	(92)	
Cytotoxic T cells	(93–95)	(96)
Role of CD8+ T cells in macrophage recruitment in obesity	(97)	
B cell – autoantibodies production	(98)	(99)
B cells infiltration	(100)	(99, 101)

3 ADAPTIVE IMMUNE CELLS IN OBESITY AND OA

Lymphocytes constituted 10% of non-adipocyte cells, including T cells, B cells, NK cells, NK T cells, and ILC2. In mice models, deficient mature lymphocytes revealed greater weight gain than WT mice fed a high-fat diet (HFD). DIO mice lacking ab T and B cells led to worsening the VAT and skeletal muscle inflammation compared to WT DIO mice (106). During the development of obesity, there is a relative increase of $CD8^+$ T cells and a decrease in Treg cells. Both $CD4^+$ and $CD8^+$T cells play a crucial role in the recruitment of ATM and polarisation (97). The accumulation of $CD8^+$ T cells into AT precedes macrophage infiltration (79). Duffaut et al. demonstrated that human AT lymphocytes and murine AT lymphocytes differ in the relative proportion of T lymphocyte subsets (107). Compared to murine AT infiltration, there is no adipocyte production of CXCL12 in human subcutaneous white AT (SWAT) or VAT (107). However, in Class II/III obese subjects instead of CXCL12, there is increased expression of CCL20, CCL20 receptor (CCR6) in VAT (108). Various adaptive cells roles are discussed elaborately to understand how they are differentially expressed from the normal to in obesity and OA microenvironment. The **Figure 1** shows the adaptive immune cells and their upregulation/downregulation in physiological inflammation and pathological inflammation.

3.1 NK Cells in Obesity and OA

Several studies have shown inconsistent outcomes in terms of increasing or decreasing NK cell numbers. These discrepancies could be correlated with strain/species-dependent of the rodent models and their metabolic attributes or differences in the development, degradation, or migration of the NK cells (109–111). Compartmental NK cell distribution has also been studied along with the discrepancies mentioned above. The study found an increased number of NK cells in the blood and spleen but a decreased amount in the liver tissue of obese rats compared to lean littermates (43). A human study on the high BMI(>40kg/m2) obesity group demonstrated that both NK cell levels and functions were significantly compromised compared to lean subjects (72). The NK cells in obesity are characterized by decreased production of IFN-γ, granzymes, perforin, and reduced numbers (73). However, these characteristics are reversed by weight loss surgery and exercises (73).

Huss et al. demonstrated that NK cells infiltrate the synovium, but they are characterized by a quiescent phenotype which is consistent with post-activation exhaustion (74). These cells are found to have lost the capability to produce IFN- γ on cytokine stimulation (74). Recently, Jaime et al. has demonstrated the characteristics mentioned above of limited cytotoxicity of NK cells in the synovium of OA compared to peripheral blood (112). These NK cells in synovium expressed a lower amount of granzyme B and perforin (112). ATM express the NK group 2D (NKG2D) ligand Rae-1, making them a target for NK cell lysis (73). Babic et al. demonstrated that NKG2D promotes increased Th1 and Th17 pro-inflammatory production and causes antigen-induced arthritis (113).

NK cells secrete antimicrobial peptides like LL-37 (114). These are critical antimicrobial agents expressed on the surface of epithelial cells, which act as a barrier against bacterial invasion. The functional characteristics of these antimicrobial peptides produced by NK cells need detailed study with regards to obesity and OA.

3.2 T Cells in Obesity and OA

Obesity is promoted by T cells by recruiting macrophages into AT (97). In one of the studies where they stimulated T cells from

an obese individual, it was found the T cells had limited insulin binding in comparison with lean individuals. Furthermore, the T cells obtained from obese individuals with type 2 diabetes mellitus (T2DM), expressed 40% fewer insulin receptors in comparison to obese individuals without T2DM. It was inferred that T2DM exacerbates defects due to obesity (115, 116).

Infiltration of T cells in the joints is the hallmark of OA. At any given time, healthy joints have few tissue-resident T cells within the synovium or in synovial fluid (117). Furthermore, the joint homeostasis is maintained by these tissue-specific T cells, infiltration of the pathogenic T cells occurs only when there is an inflammatory event. This activation of these cells could be in both antigen-dependent and independent cascading. The presence of both mono and oligoclonal T cells points towards antigen-specific proliferation (7). Moreover, the proliferation of T cells in response to the chondrocytes and synoviocytes membrane antigens also was existing in the circulation of some OA patients (118). Certain amino acid sequence from aggrecans is recognized in a few OA patient T cell population, these aggrecans constitutes a major part of the normal cartilage, but also be prone to autoantigen production within the joints (119). All the above data suggest OA is driven by the joint-derived antigen that is followed up by the production of the aberrant systemic and local T cell population. Haynes et al. in 2002 demonstrated that the presence of large cellular aggregates in OA synovial membrane in expressed T cell markers associated with immune activation and antigen presentation (120). T cells are predominantly found in the sub-lining layer of the synovium and to some extent in the deep layer (19, 40). The MNC infiltrates found in OA consist of T cells expressing early, intermediate, and late activation antigens (121). However, the decreased expression of CD3 zeta protein in OA suggests chronic T cell stimulation (122). It is found that CD80, which is an inducible co-stimulatory ligand involved in T cell stimulation, is expressed in synovial aggregates from OA (120). A relative abundance of $CD4^+$ T cells is found in OA ST with a $CD4^+$/$CD8^+$ T cell ratio of 5:1 compared to 2:1 in normal synovium (8). This shows that the T helper cells are potentially involved in the pathogenesis of OA. Different types of T cells and their role in obesity and OA are summarized on **Table 1**.

3.2.1 Th1/Th2 Cells in Obesity and OA

IL-12 stimulates the naïve T cells to differentiate into Th1 cells. In VAT and SAT obese and non- T2DM human subjects have a 10-20 fold greater frequency of Th1 than Th2 (123). Increased frequency of Th1 in VAT and SAT is correlated with IL-6 and CRP levels (27). Under hypoxic conditions, Th1 cells lose the capability to produce IFN-γ through a HIF - dependent manner (124). The low oxygen tension state will activate HIF-1, which leads to phosphorylation of STAT3, a transcription factor for IL-17 and differentiation to Th17, and inhibition of Th1 (124, 125). Khan et al. demonstrated that T cell deficiency is associated with reduced IFN-γ levels, reducing AT inflammation and metabolic dysfunction (106). Higher levels of IFN-γ promote Th1 polarisation, this is associated with waist circumference (126).

In OA, there is no variation in cell count numbers of circulating Th1 cells in the peripheral blood compared to healthy controls. However, there is an increase in Th1 cells in OA patients in the synovial fluid and synovial membrane. These cells are found in the sub-lining layer of synovium and IFN-γ^+ in nature compared to IL-4^+ cells (127). The origin of IFN-γ needs to be explored further in OA to identify the cell of origin. On the contrary, in OA condition, current studies reveal that Th2 cells play a limited role in pathogenesis (127). This is demonstrated through multiple studies showing minimal alterations in Th2 cells in peripheral blood, synovial fluid, and synovial membrane. There is also evidence of low concentrations of IL-4, IL-10 levels in synovial fluid, and there is an absence of IL-4 and IL-5 in the synovial membrane of OA patients (121). A clear subpopulation study will help in establishing whether Th1/2 cells could aid in managing OA.

3.2.2 Th17 in Obesity and OA

Th17 is a subset of $CD4^+$ T cells characterized by the secretion of pro-inflammatory cytokines like IL-17, IL-22, and IL-21 and plays an essential role in various autoimmune diseases (128). Th17 cell differentiation is induced by TGF- β, IL-6, IL-21 and maintained by IL-23 (81, 128). Ivanov et al. (129) demonstrated retinoic-acid receptor-related orphan γt (RORγt), an orphan nuclear receptor as the key transcription factor in the differentiation of Th17 cell lineage. RORγt induces genes encoding IL-17 and thus the manifest response to IL-6 and TGF-β. Bettelli et al. (Tab.1) (89) delineated that IL-6 can completely inhibit the generation of T reg cells induced by TGF- β and IL-23 is independent of the above cytokines in the polarisation of naïve $CD4^+$ T cells (89). However, Yang et al. demonstrated that STAT3 activated by both IL-6 and IL-23 played a critical role in the development of Th17 cells (125). Moreover, STAT3 regulated the expression of RORγt, and deficiency of STAT3 resulted in impaired RORγt expression, which led to elevated expression of T-box (Th1) and Forkhead box P3 (T reg cells) (125). Thus TGF-β has a dual role in inducing the anti-inflammatory T regs or the pro-inflammatory Th17 cells depending on the IL-6 state (89, 129).

Winer et al. demonstrated the increased presence of Th17 cells in diet-induced obesity and then on numerous studies confirmed the increased Th17 bias (128) (82). Interestingly, IL-17 shows a biphasic response in obesity and T2 DM development where at later stages IL-17 levels decrease, thus predisposing to increased adipogenesis (128). Transcriptional profiling of Th17 cells revealed increased acetyl CoA carboxylase 1 (ACC1) is an essential regulator of Th17 differentiation *in vitro* and pathogenicity *in vivo* through modulating RORγt (83). Thus, ACC1 forms the link between fatty acid synthesis and Th17 obesity-related pathology regulation. IL-17 functions as a negative regulator of adipogenesis and glucose metabolism and helps in delaying the development of obesity (90).

However, in hypoxia conditions, increased expression of HIF-1a promotes Th17 cells production and reduces T reg cell expression (Tab.1) (91). When T cells are activated through

antigen stimulation, there is a metabolic switch to glycolysis mediated through HIF-1 in Th17 cells and not in T reg cells (91). Furthermore, Th1 and Th2 cell differentiation were largely independent of HIF1a, but HIF1a deficiency significantly impaired Th17 differentiation and IL-17 production (91).

Garidou et al. demonstrated that IL-17/RORγt deficient CD 4^+T cells could induce T2DM and obesity (130). A HFD induces ileum dysbiosis and reduces antigen-presenting ability to induce Th17 cell differentiation. However, HFD feeding can stimulate Th17 cell development in the spleen, thus accelerating the onset of some autoimmune diseases like collagen-induced arthritis (83, 84).

In OA, it is widely accepted that Th17 cells are present in synovial fluid and ST (127). However, there is a discrepancy in the circulating Th17 cells in OA vs healthy group. These discrepancies require further investigation and potentially could explain the biphasic role of IL-17 in obesity. IL-17 can induce the production of IL-6 and IL-8 (ligand for CXCR2) through its effects on synoviocytes or normal skin fibroblasts, leading to recruiting cells of granulocytic lineage like neutrophils and protecting against bacterial infection (131, 132).

There are two contradicting studies regarding the expression of Th17 cells, in OA microenvironment, Zhang et al. demonstrated no difference between circulating Th17 cells or IL-17 plasma levels between OA and healthy controls (133). However, recently Qi et al. demonstrated in a study of 25 OA patients that the number of circulating Th17 cells and IL-17 levels are significantly elevated in the OA group compared to healthy controls (85). A study comparing RA and OA ST expression of Th17 cells found that the frequency of Th17 cells is elevated in OA but lower than RA (Tab.1) (86). More studies are required to have a better understanding of the role of Th17 and IL-17 in OA.

3.2.3 Cytotoxic T Cells in Obesity and OA

$CD8^+$ T cells are less prominent in VAT in humans compared to $CD4^+$ T cells, but $CD8^+$ T cell numbers in VAT are positively correlated with the BMI of subjects. In obese mice models, The increased expression of HIF-1a is linked to an increase in CD8+ T cell influx and 2a and GLUT1 due to the hypoxic environment present in AT (93). mTORC1- HIF1 pathway controls the fate of $CD8^+$ cytolytic T cells through glucose metabolism and glycolysis (94). This is a PI3K-Akt independent mechanism, and GLUT1 expression is linked to promoting the survival of macrophages by facilitating glycolysis in a hypoxic environment (93, 94). However, in mice models, $CD8^+$ T cells exhibit perforin-dependent killing of dendritic cells and other T cells to limit abnormal T cell activation in a physiological situation (95, 134). This shows the biphasic characteristics of $CD8^+$ T cells depending upon the environment.

In OA, even though helper T cells are abundant in ST, cytotoxic T cells occur sparsely in various layers of ST. In synovial lymphoid, aggregates found in OA $CD8^+$ T cells are found in the periphery (120).In anterior cruciate ligament transection OA models, increased activation of $CD8^+$ T cells are manifested, and these cells show increased expression of tissue inhibitor of MP1 (Tab.1) (96). Saejung et al. demonstrated that perforin production is significantly lower in the blood of OA patients compared to healthy subjects (135). Recently, follicular helper cell expressing CD8 instead of CD4 has been found in germinal centres of SLO (136), so a more in-depth analysis of $CD8^+$ T cells needs to be done to differentiate the type of cells activated in OA.

3.2.4 Treg Cells in Obesity and OA

T reg cells are found highly enriched in visceral AT of lean mice, but it is markedly reduced in obesity and insulin resistance (Tab.1) (87). iNKT cells in VAT regulate Treg cell homeostasis, and innate lymphoid cell group 2 (group 2 innate lymphoid cells ILC2s are a recently discovered subpopulation of innate lymphocytes with critical immunological and homeostatic roles in many organ locations, particularly the lung. These cells are found in the lung and other peripheral organs, and they grow locally after birth and during postnatal lung development) controls Treg cells through a direct interaction of co-stimulatory molecules such as (ICOS) and ICOS ligand (137, 138). Under the influence of TGF-β, naïve T cells differentiate into Treg cells (138, 139). Treg produces IL-10 and thereby suppresses the inflammation and maintains insulin sensitivity. IL-10 limits the M1 macrophage infiltration of WAT by suppressing monocyte chemotactic protein -1 (MCP-1) (Tab.1) (92).

In non-obese OA, Treg cells were found in increased numbers in peripheral blood, but on the other side, lower secretions of IL-10 were noticed from Treg cells (140). This drop-in IL-10 is linked to lower levels of T cell immunoglobulin and mucin domain-containing protein 3, a checkpoint receptor similar to PD-1 (140). Moradi et al. demonstrated increased T reg cells in both OA and RA ST and activated effector memory phenotype (141).

3.2.5 Th9 in OA

IL-9 producing Th cell subsets have been identified recently, and they are closely associated with autoimmune responses in RA, EAE, and systemic lupus erythematosus (142). These cells and a higher level of IL-9 have been detected in the synovial fluid and peripheral blood of RA and psoriatic arthritis (PsA) patients (77). In the same study, IL-9 levels are increased in peripheral blood and synovial fluid in patients with OA but not as high as RA or PsA patients (77). Roy et al. recently demonstrated that Th9 cells are highly glycolytic compared to other Th cells. Th9 cells differentiation is further enhanced under hypoxic conditions (143). Also, Th9 cells generate a significantly large amount of ATP through the glycolytic pathway, and this increase in glycolysis is brought in by the mTOR-HIF-1a signaling pathway (143). There are no studies conducted in obese tissue samples for Th9 cell and IL-9 expression. Investigation into the tissue specificity of Th9 cell lines in obese conditions could unravel essential answers in establishing a bridge between obesity and OA.

3.2.6 T Follicular Helper Cells (TFH) in OA

TFH cells are found in lymphoid tissue follicles, where they stimulate B cells to generate immunoglobulins. CXCR5, PD-1,

ICOS, CD40L, Bcl-6, and IL-21 are among the genes expressed by these cells (130). Shan et al. recently discovered a greater number of TFH cells in the peripheral blood of OA patients compared to healthy people (Tab.1) (84). They discovered that in OA, greater levels of serum IL-21 and expression of IL-21$^+$TFH cells were linked to disease activity (84). Zhu et al. found an increased number of TFH cells in OA, which were positively linked with CD3$^+$CD4$^+$CXCR5-PD-1$^+$ T cells and Th17 cells in a comparable investigation utilizing peripheral blood (131). However, in a study of ST by Chu et al., there are no signs for TFH cells in both OA and normal tissue ST samples compared to RA (Tab.1) (88).

In the hypoxic condition, TFH cells are regulated by mTOR complexes 1 and 2 through HIF. Cho et al. demonstrated the role of HIF in the interaction between CD4+ T cells and germinal centre (GC) B cells (144). In TFH cells, HiF2a induces the expression of CD154, which is essential for the stimulation of CD40 on GC B cells (144). In obesity models, there is a differential expression of TFH in secondary lymphoid organs and other tissue regions (60). A detailed study in the tissues obtained from overweight/obese OA patients would throw light on uncovering the signalling cascading involved in the development of OA in obese patients.

3.3 B Cells in Obesity and OA

Different B-cell subpopulations in humans can be identified in peripheral blood and other organs by differential expression of various surface markers. These various subgroups represent various levels of development, activation, and differentiation. The B cell regulation of T cells occurs through the secretion of pro-inflammatory cytokines and pathogenic autoantibodies (IgG class) (145). In mice models, B1 cells are shown to produce IgM (natural antibody), which are anti-inflammatory, whereas B2 cells seen in VAT produce IgG that are pathogenic cells associated with obesity-associated inflammation (100). B1 cell-associated IgM production in AT is inversely correlated with circulating monocyte chemoattractant protein1 such that it blunts M1-like macrophage-mediated inflammation in DIO mice models (100). Furthermore, B1 derived IgM antibodies exhibit both direct and indirect anti-inflammatory activities and are considered to protect against diet-induced chronic inflammation in some cases (146–148). As B1 cells are very rich in omental AT, it is an essential regulator for VAT function (149). However, unlike B-2 cell–derived IgG, which exacerbates inflammation, B-1 cell–derived natural IgM inhibits inflammation (150–153). B-1 cells have been shown to reduce VAT inflammation, glucose intolerance, and IR in diet-induced obese mice (100, 154). A detailed study into the role of B cells subpopulation is required to understand their role in obesity which ultimately will aid in understanding their role in OA.

In classical OA, B cells are found in low numbers in ST compared to RA (63). However, it is reported that OA ST has relatively more B cells inflammatory infiltrates. These infiltrated B cells in ST were oligoclonal, suggesting an antigen-driven expansion (155). Furthermore, sequencing of complementarity determining regions of B cells indicated that these cells had been clonally expanded (101). In a high-fat obese mouse model, Schott et al. demonstrated early synovial B cell infiltration and activation of numerous inflammatory pathways. These B cells are potentially considered novel mediators of early obesity-associated OA (Tab.1) (99). The activated B cells will become plasma cells, leading to increased antibody production (101). Multiple studies revealed auto-antibodies against cartilage-derived proteins in OA like osteopontin, cartilage intermediate layer protein (CLIP), YKL-39, fibulin, and collagen (156). Antibody production plays a major role in any disease, epically OA as inflammation is a huge influencing factor. This statement comes as contradictory evidence to the role of regulatory B cells (Breg) which are proven to suppress inflammation in various diseases, including RA (157). Th study found that in SF, the B cells producing IL10 were directly present in the ex vivo and increased upon stimulation, this proves that one of the main sources of IL10 is B cells and affects OA patients. Furthermore, the study also analysed the functional analysis of blood to investigate IgM$^+$ CD27$^+$ B cells in OA patients. These IgM$^+$CD27$^+$ B cells were observed to secrete increased levels of IL10 but decline in the levels of CD80 and CD86 in comparison with non- IgM$^+$CD27$^+$ B cells. The blood IgM$^+$CD27$^+$ B cells were found to suppress the production of IFN γ which is an autologous expression of T cells, this could be a reaction to an over production of IL10. Moreover, they found that OA patients had lower levels of IL10$^+$ B cells in the synovial fluid. When the study was concluded that IgM$^+$CD27$^+$ B cells subset in OA patients denoted as the major source of IL10 secreting B cells type in the SF and had the capacity to regulate functions in OA (157). So, a very close study on signalling cues will establish a clear understanding the role of B cells in obesity and OA, which could also eventually lead to developing intervention in the early stages of development in OA.

4 OTHER INFLUENCING FACTORS

4.1 Mechanical Loading and Inflammation

Obesity induces a number of pathological changes to the whole knee joint structure, including abnormal loading on the joint, joint malalignment and muscle weakness (158). Obesity has long been associated with unequal distribution of the mechanical loading in the knee joint apart from the disruption in the physiological condition caused due to inflammation (158). Although "inflammation is a helpful process meant to confine and destroy threats to the host organism," prolonged inflammation-induced changes in joint homeostasis in obesity-induced OA may decrease inflammation resolution and contribute to tissue regeneration failure (159). The articular cartilage is one such essential structure in the joints that lacks regenerative capacity when under abnormal acute or long-term mechanical loading. Under these abnormal conditions, they are prone to lesions that lead to OA (160). Various studies have shown that mechanical loading has led to the activation of inflammatory pathways and their channels like IL-1β, TNF-α, NF-κB, Wnt, microRNA, and oxidative stress pathways (160, 161). These are the few of the main pathways that lead to

regulating joint inflammation, activating key degradation enzymes in articular cartilages such as MMPs and aggrecanases, including chondrocyte apoptosis, extracellular matrix (ECM) degradation, subchondral bone dysfunction, and synovial inflammation which ultimately lead to OA (161). Chondrocytes maintain the catabolic and anabolic process homeostasis, slowly turning over the cartilage extracellular matrix. Progressive cartilage degradation indicates the chondrocyte imbalance, which ultimately favors the catabolic processes. The activities of chondrocytes are influenced by soluble mediators, like growth factor and cytokines, local matrix composition, and biophysical factors, which also includes mechanicals (sensed by mechanoreceptors) or osmotic stress (162). Wang et al. compared the preserved with damaged cartilage, they identified the levels of estrogen receptor- α (ER-α) was significantly low in the damaged cartilage in comparison with preserved cartilage in both human and mice samples (163). Furthermore, they used a 3-dimensional culture model, when the induced mechanical loading suppressed the level of ER- α in the chondrocytes that lead concomitant upregulation of OA phenotype. The study demonstrated the independent role of ER- α with respect to mechanical loading and its effect on the chondrocyte phenotype (163). Clinical and animal studies revealed that increased joint loading, whether acute or cumulative contact stress, might affect the composition, structure, metabolism, and mechanical characteristics of articular cartilage, subchondral bone, and other joint tissues, ultimately leading to OA (78, 162, 163). Moreover, there was a clinical study conducted using 3D giant analysis, where the 32 young obese individuals and 16 normal weight age-matched individuals were tested based on the mechanics of knee and ankle joints. The analysis was based on kinematic and kinetic data which revealed that knee flexion was less, greater knee ab-adduction angle during the gait cycle test, and knee flex-extension moment abnormalities were observed. Reduction in the range of motions together with a lower peak of ankle plantarflex or moment and power during terminal stances at the ankle joints were noted (164).

4.2 Role of AT Metabolism in Obesity and OA

The organization of white AT is found in various depots in the body, this includes under the skin (subcutaneous), within the abdominal cavity (visceral) and in small depots within the most organs. Statistics show that up to 10- 20% of the adipose in men are depots in the visceral and 5-8% in women (165). Moreover, the inclination of developing type 2 diabetes and metabolic syndrome is strongly associated with the accumulation of visceral fat (166–168). In obesity-associated metabolic syndrome, the AT lipid storage dysfunction leads to rising in the level of circulatory free fatty acids which gets reposited in visceral fat, liver, muscle, and pancreatic β- cells which leads to the deposition of ectopic lipids that causes lipotoxicity (169). The highlighted feature of lipotoxicity is an accumulation of reactive lipid aldehydes. This includes the 4- hydroxynonenal and malondialdehyde and subsequent protein carbonylation (170). In addition to lipotoxicity, changes in antioxidant enzymes activity, expression, and particular gene variations, as well as oxidative alterations of mitochondrial DNA (mtDNA), have been examined as possible indicators of metabolic disorders. Reduced activity of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), as well as a substantial drop in the GSH/GSSG ratio, were detected in obese individual peripheral blood mononuclear samples (171).

Previous studies have shown that more inflammatory cytokines are secreted into the circulation with excess fat secretion, with higher levels of adipokines and inflammatory protein in obese individuals. Furthermore, the cellular interaction between macrophages and adipocytes resulted in adipose-associated inflammatory responses. The characteristic M1 phenotype for obesity has been known to increase the production of adipokines, and in turn adiponectin which regulates the obesity-induced inflammation in OA and modulates immune responses (12, 172, 173). The concentration of adiponectin was reported to be higher in concentration in OA patients in comparison with controls. Further studies have reinforced the concentration of adiponectin and leptin was closely linked to female gender, body mass index, and synovial inflammation, these findings pointed out the prospective role of adiponectin in the serious inflammatory components of OA (174). A study by Kroon et al. showed that levels of leptin in the serum has close relation with OA and is associated with the partial adiposity. However, adiponectin levels are not associated with OA condition (175). These findings have thrown a light on how AT has a major influence on the OA.

4.3 Role of Insulin Resistance in Obesity and OA

Obesity link to various diseases, particularly insulin resistance and type 2 diabetes mellitus (T2DM). Evidence based reporting suggests AT is very versatile in terms of their metabolic flexibility due to the energy demands and being able to cope with large and rapidly evolving balance between fasting and feeding through the day and also fine tune to long term changes in energy balance with tissue expansion and reduction (176). This versatility of the AT especially the feature of expansion and reduction is detrimental of the AT health and systemic metabolic homeostasis, and in changes to the responses are likely to a contributing factor to the heterogeneity in the metabolic health observed in people with obesity (177–179). The ground-breaking discovery in mice AT produces proinflammatory cytokines that causes insulin resistance and discovery of accumulation of AT macrophages in obese population, has led to hypothesis that adipose inflammation is a major driver of insulin resistance in obese population (25, 180). Even though, there are significant increase in inflammatory macrophages and gene expression of the proinflammatory proteins in subcutaneous abdominal AT in individuals with metabolically unhealthy obesity in comparison with metabolically healthy obesity, it is very hard to deter whether it is the cause or effect of insulin resistance (181). The

free fatty acid concentration associated with obesity and T2DM could have an adverse effect on pancreatic β cells. 30% of insulin secretion is due to circulating free fatty acids in the basal condition in people with or without diabetes (182). Furthermore, there is a strong link that associates obesity and increase in the rate of free fatty acid in the bloodstream and delivery of it to the body tissues (183). Although, there are strong studies exhibiting the increase in the level of plasma free fatty acids concentration, these are important cause of liver and muscle insulin resistance, various evidence become questionable when conflicting data emerge with the real world scenarios in contrast to the experimental set ups (184). Several studies prove that lipolysis of AT triglycerides is very sensitive to insulin, the postprandial suppression of lipolysis and plasma FFA concentrations is often the same in lean and obese human subjects because the greater postprandial increase in plasma insulin in obese subjects can possibly make up for their increased fat mass (185–187). Insulin resistance and relation to obesity is still a conundrum which needs a clear pathway mapping relation between T2DM and increase in the inflammatory macrophages in subcutaneous layer AT.

The role of diabetes in OA has various contradicting view obtained from clinical data. A group from Puerto Rico et al. did a cross sectional study on 202 subjects, they found that patients with OA had diabetes mellitus were 49% however, only 26.5% of patients with diabetes mellitus had OA. The study also examined and proved that number of females with diabetes mellitus were more to have knee or hand OA were more than males. Various other aspects were also considered like the age, gender, education level, obesity, exercise, and osteoporosis, even with which the patients with diabetes mellitus had 2.18 times the risk of hand or knee OA in comparison with nondiabetic patients (188). A 3-years follow-up study was conducted to understand the gender effect of diabetes mellitus on OA. Out 559 patients who were over the age 50 or over the male patients with T2DM developed a narrower joint space than female (189).

On the contrary various other studies supported no link between OA and diabetes. A systemic review that consisted of 31 independent study were the sample space was 295,100 supported the notion of OA and diabetes are two independent factor and not only diabetes is a sole contributor to the development of OA, moreover, the study also suggested that the higher body mass is the main associate from inducing OA (190, 191).

The molecular signalling cascades between diabetes and OA has not been well explored. A study on extracellular glucose level on chondrocytes showed, low concentration of extracellular glucose concentration (5- 10mM) there was increase in the glucose transporter 1 (GLUT1) expression in healthy chondrocytes, when the concentration was increased (25-75mM) the level of GLUT1 decreased significantly (192). Rosa et al. exhibited that chondrocytes isolated from health individuals were able to adjust to the fluctuation of high glucose levels. However, chondrocytes from OA patients were not able to regulate GLUT1 expression (193). Autophagy was reduced in diabetic group in both human chondrocytes and mouse studies which paves a new direction in understanding the cartilage degradation in diabetes condition with regards to defective autophagy. Rapamycin, a pharmacological activator of autophagy, reduced cartilage breakdown, proteoglycan loss, inflammation, and lowered MMP13 expression in experimentally produced db/db OA animals (194, 195). Both animal and human studies have yielded inconsistent data to arrive at conclusion whether diabetes is independent of OA or there could be unexplored pathways that should be examined for establishing a detailed pathways between diabetes and OA.

5 ANTI-INFLAMMATORY THERAPEUTICS FOR OA

In this review there are extensive discussion on obesity and OA and the inflammatory immune profiling, however the influence of the drugs that aids in altering the immune profiling of OA is still under investigation. However, none of these interventions has been shown to significantly alter disease progression or successfully prevent eventual joint replacement in the advanced disease stages. Numerous drugs which influence the inflammatory pathways are being extensively studies, out of which few of their biologics and their alteration in OA is discussed below.

5.1 IL-1 Inhibitors

The expression of IL-1 has been extensively seen in cartilage, synovium, and SF in OA patients (196). The drugs that target IL family like the Anakinra and human IL-1 receptors type 1 monoclonal antibody AMG 108 are produced by genetic recombination (197, 198). A randomized trial with double blind, placebo control of AMG 108 was administered subcutaneously and intravenously every 4 weeks for 12 weeks. The patients showed insignificant improvement in OA symptoms but greater improvement in the pain control compared to placebo (197).

5.2 TNF-α Inhibitors

The proinflammatory cytokine, TNF-α produced by the synoviocytes and chondrocytes in OA, play a critical role in modulating the pain and structural damage in OA. Additionally, TFN-α is a key player in enhancing the production of proinflammatory cytokines like IL-6 and IL-8, also triggers the synthesis of MMP and cyclooxygenase and increases the level of NO production (199). Etanercept is one such drug that is a recombinant human necrosis factor type II antibody fusion protein. The study showed that the IA injection of etanercept compared with hydraulic acid, relived pain effectively in OA patients (200). However, A random double-blind placebo-controlled trail with subcutaneous injection of etanercept for 24 weeks did not show any pain reliving effects in patients with hand OA compared with placebo (201). Whereas etanercept treated for 52 weeks for joints showed radio graphical remodelling and less MRI bone marrow lesions (201). In this

study the application of etanercept was observed to decrease the level of MMP3, an important mediator of joint destruction (202). Conclusion to the study was given by the author to use this drug for etanercept for short term as a treatment with TNF-α inhibitor during OA flare ups (203). There are other drugs that are considered for TNF-α inhibition, like infliximab and adalimumab. While infliximab does not have any clinical trials supporting possible symptom and disease alleviating effects (203). Adalimumab did not have any significant effect on the erosive hand OA, did not affect synovitis or the bone marrow lesions in hand OA with MRI detected synovitis (204, 205).

5.3 Resolvin

Resolvin D1 (RvD1) is derived from omega3 fatty acid which is known to be a specialized proresolving mediator which has been proven to have anti- inflammatory and antiapoptotic effects in OA (206). Furthermore, the study showed that the drug inhibited the production of OA- FLS by promoting the yes-associated protein phosphorylation and protects the chondrocytes *via* inhibition of IL1β and MMP13 production which provides a potential experimental treatment of OA (98). However, there are no *in vivo* studies that are currently available to prove their potential anti- inflammatory properties and apoptotic properties.

5.4 Mitogen-Activated Protein Kinases (MAPKs) Interference in Pro-Inflammatory Pathways

MAPKs has shown some significant results with regards to murine models and clinical trials. In a murine destabilising OA model, local administration of a strong p38 MAPK inhibitor (PH-797804) decreased joint degradation and inflammation (207). In a clinical investigation involving knee OA patients, the efficacy of PH-797804 was compared to naproxen, however the findings have not yet been released (NCT01102660). FX-005, another therapeutic p38 MAPK inhibitor with sustained-release kinetics, was tested in a phase I/II knee OA study and shown to be superior to placebo in terms of pain reduction after 4 weeks (NCT01291914) (208). Direct TLR targeting might give even more upstream interference with OA immune activation; for example, a miR-21 inhibitor targeting TLR7 was able to cause long-lasting analgesia in an OA rat model.

5.5 Macrophage Immunomodulation

Hyaluronic therapy was found to recruit more anti-fibrotic macrophages which also helps in decreasing the pain (209). In a similar manner, a recent study found that administering alpha defensin-1 makes macrophages anti-inflammatory to an extend and reduces OA in a surgical model (210). These findings show that targeted anti-inflammatory therapy soon following knee injury may represent a viable future therapeutic strategy, justifying further experimental and, eventually, clinical research in OA.

Macrophage immunomodulation of M1 to M2 has become more prominent anti-inflammatory therapy target for researchers. The study by Li et al. showed extracellular vesical derived from human umbilical cord could be a potential target for promoting the M2 macrophages and secretion of anti-inflammatory cytokine IL10 (24). Their animal studies showed that the miRNA mediated M2 polarization was taking place which indicated the immunomodulation potential (24). All these results indicate that immunomodulation of macrophages has high potential for translation into humans.

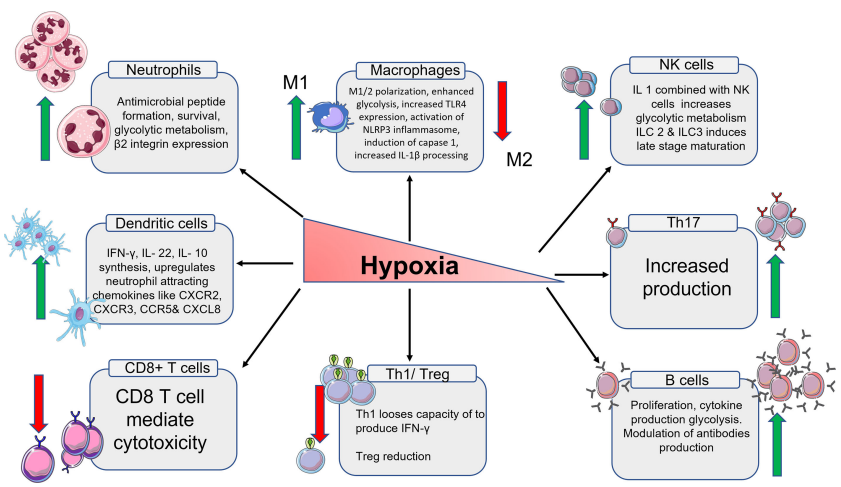

FIGURE 2 | Schematic representation of the immune cells and their products in hypoxia condition. From left, the expression of neutrophiles cells multiplies. The expression of M1 macrophages increases and M2 macrophages decreases. The total number of NK cells goes up which in turn increases the glycolytic metabolism. The number of Th17 cells goes up. B cells also increases along with high regulation of antibodies. Th1 cells and T reg cells production decreases due to low oxygen. The number T cytotoxic cells also decrease because of reduced oxygen. The number of DCs increases which intern increases the production of chemokines associated with DCs.

6 SUMMARY

Obesity is a key risk factor for the development of OA, which disrupts immune homeostasis and causes joint inflammation. OA is a multifactorial disease where various factors like mechanical loading, inflammation, and repairing of the ongoing injury are orchestrated in synchrony. Multiple studies have shown that inflammation plays a critical role in the development and progression of OA. PAMPs or DAMPs may have a role in the immune system's dysregulation in obesity, which can lead to OA or a higher risk of infection.

The many phases of macrophage development and activation in terms of gene expression and chemokine secretion are discussed, as well as how these expressions differ from obese individuals in various animal models. There is a detailed look at the numerous signaling pathways that lead to M1 and M2 macrophage polarisation inactivation and insubordinate behaviour as a result of chronic inflammation brought on by obesity. There is little information about osteoarthritic pathology for myeloid origin cells such as neutrophils and eosinophils, there are few evidence-based research connecting to animal and human obesity. Especially the role of eosinophils is still unclear with OA pathology. There are no relevant studies that centres on the role of eosinophile in the ST during the development of OA. DCs are antigen-presenting cells that have been widely investigated for their role in the immune system as well as how they behave in obese mouse models. The subpopulation of DCs and their role in various cellular activation cues are well explained. It is also taken into consideration that these subpopulations might follow a similar path in OA as well. The role of all the innate and immune cells in hypoxia condition has been summarized on **Figure 2**.

All lymphoid cells that play a role in active immunity have detailed descriptions of their functions, activation, and signaling. Various studies on the involvement of NK cells in obesity physiology have produced conflicting results. Their significance in ST in the context of OA has a well-established signaling cascade. However, chemokines released by NK cells are said to have antimicrobial qualities, but there is not enough evidence to back up their claims or their role in OA physiology. Evidence for T cell subtypes is given in a complete and detailed study. Although the data for cellular signaling is widely given, it is insufficient to comprehend the multiple connecting signals that link obesity and OA, eventually leading to synovial inflammation.

According to the research presented in this review, B cells release IgG antibodies as well as cytokines to regulate T cells in obese models. Obesity-related chronic inflammation is exacerbated by these antigen-secreting cells. Nonetheless, a detailed literature review is necessary to fully comprehend their role in OA and obesity.

To comprehend the disease pathophysiology and determine the similar immunological pathways implicated in OA and obesity, more research on ST and the influence of obesity on OA in terms of immune cells and their activities is needed. In obesity, various pathways contribute to immunological homeostasis loss, such as intestinal dysbiosis or hypoxia, that need to be investigated OA. Furthermore, many other cytokines associated with OA have also become therapeutic targets. Most of the therapies target the pro-inflammatory pathways like IL-1β, TNF-α, resolving, MAPKs and macrophage immunomodulation therapies which could effectively reduce the inflammation in OA without side effects.

AUTHOR CONTRIBUTIONS

UN drafted, critically revised, and designed the figures for the manuscript. IV designed the outline and drafted the manuscript and the table. AS structured the abstract, reviewed the concepts and language usage. XW, IP, and RC all were involved in editing the document. All the authors contributed to the article and approved the submitted version.

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Adipose tissue macrophage in obesity-associated metabolic diseases

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Adipose tissue macrophage (ATM) has been appreciated for its critical contribution to obesity-associated metabolic diseases in recent years. Here, we discuss the regulation of ATM on both metabolic homeostatsis and dysfunction. In particular, the macrophage polarization and recruitment as well as the crosstalk between ATM and adipocyte in thermogenesis, obesity, insulin resistance and adipose tissue fibrosis have been reviewed. A better understanding of how ATM regulates adipose tissue remodeling may provide novel therapeutic strategies against obesity and associated metabolic diseases.

KEYWORDS

adipose tissue macrophage, obesity, adaptive thermogenesis, insulin resistance, fibrosis, adipokines

Introduction

Obesity is an accumulation of adipose tissue resulting from an energy imbalance, which has been linked to numerous comorbid conditions including type 2 diabetes mellitus, nonalcoholic fatty liver disease (NAFLD), atherosclerosis, cancers as well as COVID-19 (1, 2). Adipose tissues, which include brown adipose tissue (BAT) and white adipose tissue (WAT), play critical roles in the maintenance of energy homeostasis. WAT store energy when nutrition is abundant, while BAT dissipate energy for heat production through a mitochondrial uncoupled respiration.

Besides adipocytes, many types of immune cells reside in both BAT and WAT to control adipose tissue homeostasis (3). Among these immune cells, macrophage is the most abundant population, constituting 5%-10% cell numbers of the adipose tissue in the lean state and increasing to 50% or more in the condition of extreme obesity both in humans and in mice (4). Macrophage is derived from embryo or adult bone marrow-derived circulating monocytes, which are essential in the maintenance of tissue homeostasis and play a vital role in different pathologies. Macrophage is a heterogeneous population of immune cells, such as Kupffer cell in liver, alveolar macrophage in lung, microglia in brain among many others. They play tissue-specific

functions in homeostatic and immune-related responses shaped by different local microenvironment (5, 6).

Adipose tissue is an energy reservoir which contains lots of lipids and acts as an important endocrine organ by secreting numerous factors. These lipids and factors generate a specific microenvironment that distinguishes adipose tissue from others and distinguishes adipose tissue macrophage (ATM) from macrophage in other tissues. There are two types of activated macrophage in adipose tissue, named M1 macrophage and M2 macrophage. In the adipose tissue of lean mice, most macrophages are M2 activated, which produce anti-inflammatory cytokines including interleukin-10 (IL-10) and TGF-β, contributing to resolution of inflammation and tissue homeostasis. But in obese mice, the adipose tissue recruits many M1 macrophages, which generate proinflammatory cytokines, causing adipose tissue inflammation and metabolic dysfunction (7, 8).

Here, we summarize the latest progresses of the metabolic implications of ATMs. We describe the polarization and recruitment of adipose tissue macrophages, and discuss their functions both in health and metabolic diseases, including thermogenesis, obesity, insulin resistance as well as adipose tissue fibrosis.

Macrophage polarization

M1/M2 polarization of macrophage is a process by which macrophages produce distinct functional phenotypes driven by microenvironmental stimuli in specific conditions. There are two types of macrophage polarization in adipose tissue, M1 and M2 macrophages. M1 macrophages are generated when stimulated with lipopolysaccharide (LPS) or Th1 proinflammatory cytokines such as IFN-γ. Meanwhile, M2 macrophages are induced by Th2 cytokines such as IL-4 and IL-13. M1 macrophages are usually characterized by enhanced phagocytic activity and increased secretion of proinflammatory cytokines (9). Phenotypically, M1 macrophages show enhanced expression of main histocompatibility complex class II (MHC-II), CD68, CD80 and CD86 both in mice and humans (10). These characteristics are mainly promoted by IFN-γ-mediated Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling or directly by pathogen associated molecular patterns (PAMPs) such as LPS. Thus, M1 macrophages, along with other innate immune cells, provide the first line of defense to fight against infectious pathogens and promotes Th1 immune response. Several pathways have been discoveried to regulate M1 activation. Transcription factor interferon regulatory factor 5 (IRF5) has been reported as a key player in the polarization of both human and mouse macrophages towards a proinflammatory M1-like phenotype by controlling expression of M1 markers, as well as Th1 and Th17 responses (11). STAT1, which is activated by LPS/TLR4 pathway, plays a critical role in M1 polarization (12, 13). Suppressor of cytokine signaling 3 (SOCS3) activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway to produce NO, which promotes expression of M1 markers and inhibits IL-10 expression (14). M2 macrophages have been initially identified during helminth infection, which promotes a Th2-polarized response. They are usually characterized by the expression of M2 markers including arginase 1 (ARG1), chitinase 3-like 3 (also known as YM1), FIZZ1 and CD206. Depending on the contexts and the expression of phenotypic markers, M2 macrophages can be subtyped into M2a, M2b, M2c and M2d ones (15, 16). M2a macrophages play a role in the Th2 response during parasite infections. They are typically induced by stimulation of IL-4 and IL-13, which are produced by eosinophils. M2a macrophages are characterized by high surface expression of CD206, ARG1, YM1, FIZZ1 and TGF-β, and they can promote fibrosis and wound healing. M2b macrophages show immune-regulated and anti-inflammatory effects which induced by IL-1 and TLR agonists such as LPS, expressing high levels of TNF superfamily, C-C motif chemokine ligand 1 (CCL1) and IL-10.M2c macrophages are induced in the presence of IL-10, TGF-β and glucocorticoids. They are usually considered as deactivated or anti-inflammatory macrophages, and involved in phagocytosis of apoptotic cells. M2c macrophages secret large amounts of IL-10 and TGF-β, and express multiple markers including CD163, CD206, RAGE and other scavenger receptors. M2d macrophages, also known as tumor-associated macrophages (TAMs), are induced by the TLR antagonists, and they release IL-10, TGF-βand vascular endothelial growth factors (VEGF) to contribute to tumor angiogenesis (17–21) (Figure 1). Transcription factors such as Krueppel-like factor 4 (KLF4), STAT6 and peroxisome proliferator-activated receptor-γ (PPARγ) are all involved in the polarization of M2 macrophages (22–24). Besides, recent studies identified PI3K/AKT signaling as another critical mediator in mouse M2 macrophage polarization, which is independent of the well-established JAK1/STAT6 pathway (25). IL-4 stimulates the phosphorylation of IRS-2 that leads to the recruitment and activation of PI3K/AKT pathway (26). Interestingly, different AKT isoforms seem to play different roles in macrophage polarization, with Akt1 isoform deficiency leading to an M1 activation while Akt2 isoform ablation causing an M2 phenotype (27).

In obese adipose tissue, ATMs tend to polarize to M1 macrophages, which are mainly regulated by adipocytes. Adipocytes exert effects on ATM phenotypes *via* a variety of mechanisms. Obese adipocytes secrete many proinflammatory cytokines, including monocyte chemoattractant protein 1 (MCP-1/CCL2), which recruit macrophages and induce their polarization to proinflammatory M1 type. Besides cytokines, ATM polarization is affected by lipids and glucose which are much more abundant in the obese condition. Macrophages treated by very low-density lipoproteins (VLDLs) and short

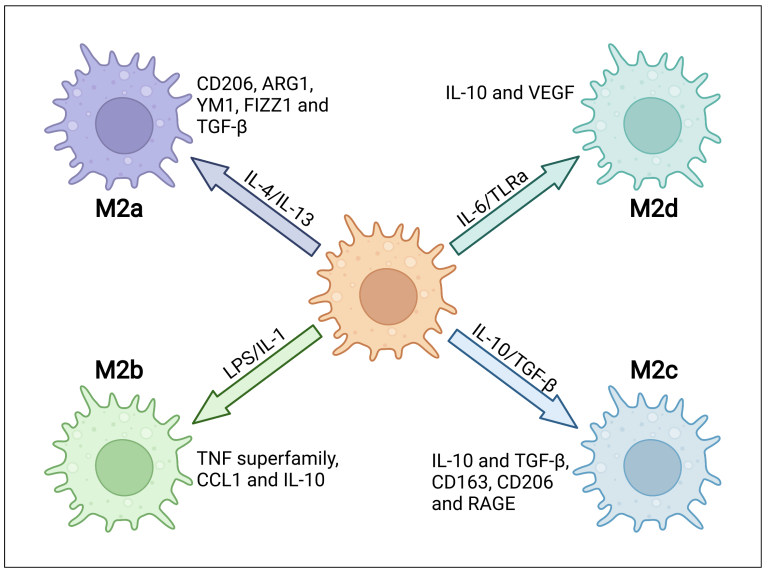

FIGURE 1
The heterogeneity and characterizations of M2 macrophages. M2 macrophages can be subgrouped into M2a, M2b, M2c and M2d depending on different microenvironmental stimuli. Specific stimuli include, but are not limited to, IL-4 or IL-13 for M2a, LPS or IL-1 receptor ligands for M2b, IL-10 or TGF-β for M2c and IL-6 or Toll-like receptor agonists (TLRa) for M2d. Different subtypes express distinct markers, including intracellular proteins and secreted cytokines.

chain fatty acids increase a secretion of proinflammatory cytokines (28). Free fatty acids (FFAs), which is increased in the serum of obese animals, can induce TLR4 signaling activation in murine ATMs and polarize ATM to M1 (29–31). High levels of glucose directly promotes macrophage M1 activation *via* the Rho-associated protein kinase (ROCK)/c-Jun amino-terminal kinase (JNK) and ROCK/extracellular signal-regulated kinase (ERK) pathways (32, 33). Besides, miR-155, which is secreted by adipocyte-derived microvesicles (ADM), regulates M1 macrophage polarization (34). Moreover, DPP4, a dipeptidyl protease expressed and released by hapatocytes, can activate ERK1/2 and NF-κB signaling to induce MCP-1 and IL-6 expression in ATMs that promotes adipose tissue inflammation (35).

Lean adipocytes normally secrete adiponectin that stimulates M2 ATM polarization (36). PPAR-δ, a nuclear hormone receptor, plays an important role in the activation of M2 macrophage, and alleviates diet-induced insulin resistance (37). PPAR-δ is induced by cellular lipids when apoptotic cells are engulfed by macrophages and further regulates the clearance of these apoptotic cells (38). Helminth infection significantly promotes Th2 responses and M2 macrophage polarization, which alleviate obesity. Adoptive transfer of M2 macrophages treated by helminth to recipient mice significantly improve high-fat diet (HFD)-induced obesity (39). Furthermore, PPAR-γ has been reported to polarize human monocytes to M2 macrophages *in vitro* (40), while deletion of PPAR-γ in myeloid cells inhibits M2 macrophage activation and accelerates diet-induced obesity and insulin resistance in mice (41).

A number of regulators govern the polarization of M1 or M2 macrophage and the switch between M1 and M2 activation. TLR4 is the key component in LPS-mediated M1 polarization, and TLR4 deficiency inhibits HFD-induced recruitment of proinflammatory M1 macrophages and induces M2 macrophage polarization (42). 11β-HSD1, a reductase reactivating glucocorticoids, was reported to promote the switch from M2 to M1 macrophages in human obesity (43). In addition, inositol-requiring enzyme 1α (IRE1α) wasalso reported to be a key factor controlling ATM polarization and energy balance in mice. Deficiency of IRE1α promotes M2 macrophage polarization, and transcriptomic profiling revealed that expression of IRF4 and KLF4 can be inhibited by IRE1α, both of which are critical players controlling M2 polarization (44). MicroRNA has rencently been implicated in the regulation of macrophage M1/M2 polarization as well as insulin resistance. miR-495 promotes M1 macrophage activation by targeting and inhibiting the expression of *Fto* (45). However, another study reported that *Fto* silencing significantly suppressed both M1 and M2 polarization, through inhibiting the expression of STAT1 and of STAT6 and PPAR-γ respectively (46). *FTO* gene has been considered as the strongest genetic effector in human polygenic obesity, in which IRX3 may participate by mediating this effect. Recently, using cell-specific knockout mouse models, we demonstrated that macrophage IRX3 regulates body weight

through acting as a transcriptional factor to control the expression of proinflammatory cytokines (47). Mechanistically, we found that IRX3 promotes M1 but not M2 gene expression when it is phosphorylated and activated by JNK1/2 in macrophages (47).

ATM recruitment

Healthy adipose tissue predominantly contains anti-inflammatory M2 macrophages that originate from yolk sac, with a little contribution of circulating monocytes (48). An extreme increase in adipocyte size is accompanied by an inadequate supply of oxygen due to expanding adipose tissue, causing an increased frequency of adipocyte death and following macrophage recruitment (49, 50). Over 90% of macrophages recruited to adipose tissue are arranged around dead adipocytes, which form a structure called "crown-like structure (CLS)", both in obese animals and humans (49, 51). These recruited macrophages exert their phagocytotic function to clear dead adipocytes. Deficiency of mannose-binding lectin (MBL), which can bind apoptotic cells and promote engulfment by phagocytes, inhibits the clearance of apoptotic cells in adipose tissue (52). Meanwhile, macrophages in CLS store and buffer excess lipids released from dead adipocytes, which are named lipid-laden macrophages (53). The number of CLS is highly positively correlated with adipose tissue inflammation and metabolic disorders of obese subjects (54, 55). Proinflammatory adipokines including MCP-1 and TNF, as well as saturated fatty acids secreted by obese adipocytes, can recruit and activate ATMs (56). Activated macrophages release proinflammatory chemokines including MCP-1 to recruit more monocytes from blood into adipose tissues by binding to its receptor C-C chemokine receptor type 2 (CCR2) (37). After infiltrating into the adipose tissue, monocytes differentiate to macrophages and interact with adipocytes in a paracrine manner, further increasing the secretion of proinflammatory cytokines (57). This interaction between adipocytes and macrophages establishes a vicious spiral in obese adipose tissue and persistently recruits more and more macrophages from circulation (55). Besides, obesity promotes the expression of chemokine receptors in adipose tissues from both mice and humans, which further enhance the vicious spiral (58, 59).

Many cytokines and their receptors participate in the recruitment of monocytes/macrophages. As previously mentioned, MCP-1-CCR2 is reported as the most important cascade in macrophage recruitment. MCP-1 in adipocytes promotes ATM recruitment and insulin resistance in mice (57), while HFD-induced macrophage accumulation in adipose tissue was extensively reduced in MCP-1 KO mice (60), which indicates a critical role of MCP-1 in the trafficking of macrophages. In addition to adipose tissue, the MCP-1-CCR2 circuit plays an important role in recruiting monocyte in many other tissues, such as in liver, heart and lung (61–64). Moreover, MCP-1 has been reported to induce local proliferation of macrophages, which is another important mechanism underlying obesity-elicited macrophage accumulation (65). Besides MCP-1, CCR2 can also be activated by other ligands, including CCL7 (MCP-2) (66), CCL8 (MCP-3) (67), CCL13 (MCP-4) (68), and CCL12 (MCP-5) (69), many of which are expressed in obese adipose tissue and affect monocyte/macrophage recruitment (58). On the other hand, CCR5 expression is highly upregulated in obesity and FACS analysis further illustrated that WAT from obese mice have significant accumulation of CCR5 positive macrophages. Consistently, CCR5 deficient improves obesity-induced insulin resistance in mice (70). CCL3 and CCL5 have been reported as ligands of CCR5 (71). Inhibition of CCL3 reduces macrophage infiltration and activation by downregulating CCR5 (72). CCL5 recruits macrophages mainly by promoting cell adhesion and transmigration of monocyte vascular endothelial cells (73). CX3CL1-CX3CR1 axis also precipitates in macrophage infiltration and inflammation in both atherosclerosis and rheumatologic disorders (74, 75). It has been suggested that adipocytes express CX3CL1 that can activate the CX3CR1 signaling in macrophages (76).*Cx3cr1*-deficient mice fed HFD displayed significantly declined monocytes and produced less proinflammatory cytokines in the WAT (77). However, another study reported that *Cx3cr1*-dificient mice showed a reduction of M2-polarized macrophage migration, and exacerbated adipose tissue inflammation, insulin resistance and hepatic steatosis when fed HFD (78). Serum amyloid A (SAA) promotes monocyte recruitment by inducing the expression of the adhesion antigens CD11b, intracellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1(VCAM-1) through a NF-κB-dependent signaling (79, 80). Myeloid cell-specific ablation of GPR105, which is activated by UDP and UDP-linked glucose, prevents macrophage recruitment to liver or adipose tissue in mice fed HFD (81). C-X-C motif chemokine ligand 14 (CXCL14), which is required for the activation of dendritic cells, is another chemoattractant participates in the recruitment of macrophages into adipose tissue and insulin resistance, although its receptor has not yet been identified (82, 83). Using knockout mouse model, CXCL14 has recently been reported to be produced by brown adipocytes upon thermogenic activation and promotes the recruitment and activation of M2 macrophages in BAT (84).

Collectively, there are several steps in the recruitment of ATMs. Initially, Obesity-induced adipocyte death and adipose tissue inflammation promote a secretion of CCL2 and other chemokines, which bind to their receptors on monocytes circulating in the blood. Then, activated monocytes adhere to endothelial cells of blood vessel *via* upregulated adhesion molecules including ICAM-1, VCAM-1 and integrin. After integrin-dependent lateral migration, monocytes transmigrate from blood vessel to target adipose tissue. Eventually, recruited

monocytes differentiate into proinflammatory macrophages in response to local microenvironmental stimuli (Figure 2) (85).

ATM in adaptive thermogenesis and lipolysis

Brown adipocyte and beige adipocyte, which highly express mitochondrial uncoupling protein 1 (UCP1), are responsible for adaptive thermogenesis and protect against metabolic diseases in mice and humans. FFAs, which are produced as a result of lipolysis, serve both as direct activators of UCP1 and fuel sources for thermogenesis (86). Thermogenesis and lipolysis of adipose tissues and browning of WAT are dynamic processes, in which both M1 and M2 macrophages play critical roles.

M2 macrophages in adaptive thermogenesis and lipolysis

M2 macrophage has been demonstrated as an activator to promote fat thermogenesis and lipolysis through different mechanisms. In 2011, cold-induced BAT thermogenesis and WAT lipolysis were first linked to macrophage M2 recruitment and activation (87). Using myeloid cell-specific ILR4α-deficient and IL4 administration mouse models, M2 macrophage has been revealed to be required and sufficient for BAT and beige fat thermogenesis and lipolysis (87, 88). Mechanistically, cold exposure induces the expression of tyrosine hydroxylase (TH) and resultant catecholamine production in M2 macrophages to sustain thermogenesis and lipolysis, although whether M2 macrophages expresse a significant amount of TH is under debate (87–90). Another study reported a similar recruitment and pro-thermogenic effects of M2 macrophage in cold-induced browning of subcutaneous white adipose tissue (scWAT) in mice, further supporting a critical role of M2 macrophage in adaptive thermogenesis (88, 90). Moreover, $CD44^+$ M2 macrophage is recruited by CL316.243 (CL)-mediated adipocyte death to produce high level of 9-hydroxyoctadecadienoic acid (9-HODE) and 13-HODE, two known PPARγ ligands, which promote differentiation of platelet-derived growth factor receptor alpha ($PDGFR\alpha^+$) progenitors to beige adipocytes (91). Besides, macrophage-derived osteopontin (OPN) triggers a recruitment of $PDGFR\alpha^+$ progenitors, which contribute to beige adipogenesis (92). In addition to the above paracrine manner, M2 macrophage has also been reported to promote beige adipogenesis in a direct-contact manner both in humans and mice (93). More recently, it has been reported that brown adipocyte ejectes damaged mitochondria *via* extracellular vesicles, whose removal by M2 macrophage ensures optimal BAT thermogenesis in mice (94). Started from the M2 macrophage, multiple studies have explored the importance of other anti-inflammatory cytokines in the regulation of adipose thermogenesis and lipolysis.IL-4, IL-13 and IL-33, as key members in type 2 cytokines, all have been demonstrated to promote thermogenesis and lipolysis (88, 95). Different from other type 2 cytokines, ablation of IL-10 elicits thermogenesis and browning of scWAT and protects against diet-induced obesity. ATAC-seq, ChIP-seq, and RNA-seq analyses revealed that IL-10 affects chromatin structure and CCAAT/enhancer binding protein-β (C/EBPβ) and activating

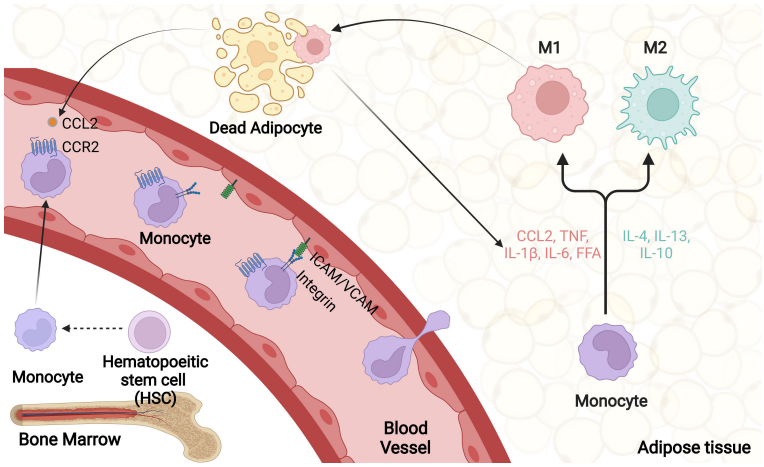

FIGURE 2
ATM recruitment. Obesity promotes adipocyte death as well as adipose tissue inflammation, which firstly trigger a large number of chemokines secretion, including CCL2. Upon activation, monocytes and vascular endothelial cells produce various cellular adhesion molecules, mainly integrin, ICAM-1 and VCAM-1. Through rolling and adhesion process, monocytes bind to adhesion molecules on vascular endothelial cells and transmigrate from blood vessel to target adipose tissue. Eventually, recruited monocytes differentiate into proinflammatory macrophages in response to local microenvironmental stimuli.

transcription factor 2 (ATF2) occupancy at the promoters of thermogenic genes (96).

M1 macrophages in adaptive thermogenesis and lipolysis

In contrast to M2 macrophage, M1 macrophage and its secreted proinflammatory cytokines usually exert negative effects on thermogenesis and lipolysis. Prolonged treatment with TNF cytokine inhibits the sensitivity of adipocytes to β-adrenergic stimulation, and thus inhibits *Ucp1* gene expression, thermogenesis and lipolysis (97, 98). TNF-activated inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKKε) and TANK-binding kinase 1 (TBK1) desensitize lipolytic signaling by phosphorylating and activating phosphodiesterase 3B (PDE3B), which decreases cAMP levels (98, 99). Overexpression of IKKε blunts β-adrenoreceptor-stimulated *Ucp1* expression in adipocytes, while an inhibitor of IKKε and TBK1 restores catecholamine sensitivity and reversed the effects of HFD feeding on thermogenesis and weight gain (98, 100). IL-1β inhibits β-adrenoreceptor-stimulated *Ucp1* expression which was significantly abrogated by the inhibition of ERK (101, 102). Genetic ablation of *Jnk1*, a major intracellular mediator of inflammatory signaling, enhances *Ucp1* expression and thermogenesis in adipose tissues (103). Through singly or in combination treatments of beige adipocytes *in vitro* with different proinflammatory cytokines, we found that TNF and IL-1β moderately inhibited adrenergic signaling separately, while a mixture of four cytokines (IL-1α, IL-1β, IL-6 and TNF) achieved a dramatic inhibition of thermogenesis and lipolysis (47). Besides proinflammatory cytokines, adipocytes express TLRs and key components of their downstream signaling pathway. LPS or palmitic acid-stimulated TLR4 signaling abolished cAMP-induced upregulation of *Ucp1* and thermogenesis through activating NF-κB and MAPK pathways (104, 105). Consistently, TLR3 and TLR4, upstream of interferon regulatory factor 3 (IRF3) signalling, induce insulin resistance and thermogenesis in adipocytes (106). IRF3-deficient mice exhibit systemic inflammation and enhanced browning of scWAT when fed HFD (107). Inflammation-imposed inhibition of beige adipogenesis and thermogenesis are also mediated by a direct adhesion of inflammatory macrophages to adipocytes. Specifically, α4 integrin-mediated adhesion of inflammatory M1 macrophages to VCAM-1, which is expressed by adipocytes, inhibits thermogenesis in an ERK-dependent manner. Genetic or pharmacologic inhibition of α4 integrin resulted in an increase of beige adipogenesis and UCP1 expression of the scWAT (93).

ATM also regulates adipocyte thermogenesis and lipolysis indirectly through other cells like sympathetic nerves. Fasting and cold exposure increase the release of catecholamines from sympathetic nerves, which bind to adipocyte adrenergic receptors and activate cAMP-PKA signaling to trigger lipolysis and thermogenesis. Yochai Wolf et al. reported a homeostatic role of macrophages in the control of brown adipose tissue innervation in mice (108). They found that BAT resident $CX3CR1^+$ macrophages inhibit sympathetic innervation and decrease the local level of catecholamine. *Mecp2* deficiency in $CX3CR1^+$ macrophages decreased thermogenesis and led to spontaneous obesity (108). Two recent reports identified a subtype of macrophages in human and mouse WAT that take up and degrade norepinephrine (NE), then inhibit adipocyte lipolysis and thermogenesis. These macrophages are termed either sympathetic neuron associated macrophages (SAMs) or nerve-associated macrophages (NAMs). Different from $CX3CR1^+$ macrophage that inhibits sympathetic neuronal innervation in BAT, SAMs/NAMs function in eWAT and scWAT (109, 110). Mechanistically, NE-degrading macrophages are activated *via* NLR family pyrin domain containing 3 (NLRP3) inflammasome system in aged WAT. NLRP3 activation upregulates the expression of growth differentiation factor-3 (GDF3) and GDF3-dependent expression of monoamine oxidase A (MAOA) that degrade NE. Macrophages that lack NRLP3 or GDF3 decreased adipose NE removal and increased lipolysis upon aging. The MAOA inhibitor treatment of aged mice restored fasting-induced lipolysis and increased expression of UCP1 (110). Moreover, activin receptor-like kinase 7 (ALK7) signaling, which is activated by GDF3, contributes to diet-induced catecholamine resistance in adipose tissue. Fat-specific *Alk7* knock-out enhances adipose β-adrenergic signaling, lipolysis and thermogenesis, resulting in reduced fat mass and resistance to HFD-induced obesity (111). SAMs in mouse WAT can specifically express the NE transporter SLC6A2. Genetic ablation of *Slc6a2* in SAMs increases thermogenesis and weight loss in obese mice (109). $CX3CR1^+$ macrophages produce IL-27 to activate p38 MAPK-PGC-1α pathway in adipocytes and promote thermogenesis of BAT and scWAT (112). However, it is unknown whether these SAMs/NAMs can be categorized as M1 or M2.

ATM in insulin resistance and diabetes

Type 2 diabetes is associated with obesity and occurs as a consequence of insulin resistance, which emerges when three major insulin-sensitive tissues (skeletal muscle, liver, and adipose tissue) can not respond well to insulin and can not effectively take up glucose from blood. Insulin is a peptide hormone secreted by pancreatic β cells, which plays a crucial role in carbohydrate metabolism, lipid anabolic regulation, cell growth and proliferation (113). Blood glucose induces β-cells to produce and secrete insulin, which stimulates glucose uptake in different types of cells, including adipocyte, muscle cell, liver cell

and others, thereby decreasing blood glucose level. The effects of insulin on whole-body metabolism result from its binding to insulin receptor (IR), leading to autophosphorylation of specific tyrosine residues of IR and subsequently phosphorylation of proteins known as insulin receptor substrates (IRS). PI3K, a key component of IRS downstream, mediates insulin signaling mainly by activating PKB/AKT and PKC signaling pathways (114).

TNF

Adipose tissue macrophage modulates insulin action through different mechanisms, with M1 macrophage promoting insulin resistance while M2 macrophage enhancing insulin sensitivity (115, 116). The major difference between M1 and M2 is the expression of proinflammatory cytokines. In 1990s, the first study reported the inflammatory origin of obesity and diabetes. They found that adipose tissue from different obese rodents and humans has an enhanced secretion of proinflammatory cytokines, mainly TNF, which was linked to insulin resistance (117, 118). TNF-deficient obese mice are protected from obesity-induced insulin resistance in muscle and adipose tissues (119). The important role of TNF is further evidenced by TNF neutralization, which improves an increased peripheral glucose uptake and insulin sensitivity in obese mice (117). These studies showed that blocking a single cytokine can restore insulin sensitivity, and macrophage was further identified as the major cell source of TNF and other proinflammatory molecules in obesity (4). Binding of TNF to its receptors results in the activation of JNK and causes phosphorylation of IRS1 at serine 307, which impairs IR-mediated tyrosine phosphorylation of IRS1 and downstream signaling (120).

IL-1β

IL-1β is another important proinflammatory cytokine that is produced by ATM (121). IL-1β exerts its biological effects by binding directly to IL-1Rα and activates the IKK/NF-κB pathway (122). It was reported that adipose tissue appears to be a major source of IL-1R antagonist production, which prompted an interest in the role of IL-1β in obesity-induced diabetes and insulin resistance (123). IL-1β, released by ATM, alters insulin sensitivity of adipose tissue by inhibiting insulin signaling, so it decreases insulin-stimulated glucose uptake and lipogenesis in both murine and human adipocytes (124, 125). *In vitro* studies revealed that IL-1β treatment of adipocytes disturbs insulin signaling *via* downregulation of IRS1 expression, leading to a reduction of translocation of insulin-stimulated glucose transporter type 4 (GLUT-4), an essential process for glucose uptake (124, 125). Consistently, insulin resistance of human adipocytes imposed by macrophage-derived conditioned medium can be reversed by neutralizing IL-1β (124).

IL-6

Many studies have established a positive correlation between IL-6 and insulin resistance (126). Adipose tissue-derived IL-6 enters circulation and exerts systemic regulation on insulin action. Up to 35% of systemic IL-6 originates from adipose tissue under basal condition, secreted by both adipocytes and macrophages (4, 127). IL-6 has been described to impair insulin signaling, primarily through inhibiting insulin-stimulated tyrosine phosphorylation of IRS in adipose tissue (128). Modest increase of basal glucose transporter GLUT1 was observed in 3T3-L1 adipocytes when incubated with IL-6 (129), while the expression of *Glut4* and *Irs1* genes was inhibited by chronic IL-6 treatment (128, 130). Besides, IL-6 induces the expression of SOCS3, which is a negative regulator of insulin signaling in adipocytes (131).

NF-κB

ATM-released proinflammatory cytokines activate different signaling pathways in adipocytes to modulate insulin action. NF-κB is a master inflammatory transcriptional factor involved in a variety of physiological and pathological processes such as inflammation and innate and adaptive immune responses. The activation of NF-κB signaling can increase the expression of several proinflammatory genes, which exacerbate insulin resistance progression (132, 133). IKKβ specific deficiency in adipocytes completely prevents FFA-induced IL-6 and TNF expression, and improves glucose tolerance and insulin sensitivity (134, 135). Mechanistically, IKKβ activation promotes IRS1 serine phosphorylation through activation of the TSC1/TSC2/mTORC1/S6 kinase-1 pathway, which impairs IR-mediated tyrosine phosphorylation of IRS1 (136). In addition, activation of the IKKβ/NF-κB pathway increases the expression of protein-tyrosine phosphatase 1B (PTP1B), a tyrosine phosphatase that catalyzes dephosphorylation of tyrosine residues of IRS1, further inhibiting insulin signaling in adipose tissue (137).

JNK

JNK might be the most investigated stress kinase in obesity-related insulin resistance. The activity of JNK in increased upon exposure to inflammatory stimuli which include cytokines, FFAs, and then phosphorylates transcription factor activator protein-1 (AP-1) (120). Like IKKβ, JNK inhibits insulin

signaling through an inhibitory serine-threonine phosphorylation of IRS1, thereby decreases PI3K/AKT signaling (138, 139). It should be noticed that JNK seems more implicated in the direct regulation of IRS serine phosphorylation than IKKβ (140). JNK activity could be induced in adipose tissue of obese mice compared to lean mice. Adipose tissue-specific JNK1-deficient mice are protected against the development of insulin resistance under HFD feeding. Interestingly, this protective effect is not systemic as JNK1 deficiency in adipocytes only restore liver but not muscle insulin sensitivity (141). Besides JNK1, JNK2 isoform is also involved in insulin resistance but to a lesser extent (142). Moreover, using a myeloid cell-specific JNK1/2 double knock-out mouse model, another study demonstrated that macrophage JNK1/2 are required for the establishment of obesity-induced adipose tisse inflammation and insulin resistance through promoting macrophage M1 activation (143).

ERK1/2

ERK1/2 is activated in adipose tissue of obese mice or human (144). Multiple cellular studies have reported that activated ERK1/2 in diabetes induces IRS1 serine phosphorylation, which inhibits IRS1 tyrosine phosphorylation. In addition this serine phosphorylation decreases the interaction between IRS1 and PI3K and inhibits the association between IRS1 and insulin receptor, further diminishing the metabolic effects of insulin (140). ERK1-deficient mice are protected against diet-induced obesity and insulin resistance by inhibiting adipogenesis and promoting energy expenditure (145). Besides, ERK activation promotes insulin resistance indirectly, mainlythrough a stimulation of adipocyte lipolysis and FFA release, and mice deficient in the signaling adapter p62 (an ERK inhibitor) show similar phenotypes (Figure 3) (146, 147).

The regulation of insulin sensitivity by ATMs is also mediated by miRNAs, which are contained and transferred by ATM-derived exosome (148). Treatment with obese ATM-derived exosome leads to insulin resistance, whereas lean ATM-derived exosome increases insulin sensitivity in obese mice. Mechanistically, miR-155 is among the differentially expressed miRNAs in obese ATM-derived exosome, and it causes systemic insulin resistance and glucose intolerance (148).

In addition to M1 and M2 macrophages, other macrophage populations that regulate insulin resistance have been reported recently, including neuropilin-1 $(NRP1)^+$ macrophages and $TREM2^+$ lipid-associated macrophage (LAM) (149, 150). $NRP1^+$ macrophages accumulate in adipose tissue and protect against obesity and metabolic syndrome. Conditional deletion of NRP1 in macrophages compromised lipid uptake and led to insulin resistance (149). Using single-cell RNA sequencing, one study identified a subset of $TREM2^+$ lipid-associated macrophages (LAMs) that prominently arise under obesity condition both in humans and mice. TREM2 deficient mice showed inhibited recruitment of macrophages to CLS and led to adipocyte hypertrophy as well as insulin resistance (150). However, bone marrow transplantation experiments by another group argued that hematopoietic-expressed TREM2 is dispensable for obesity-induced metabolic dysfunction, including insulin resistance (151).

ATM in fibrosis

Adipocytes are surrounded by a network of extracellular matrix (ECM) proteins that not only serve as a structural

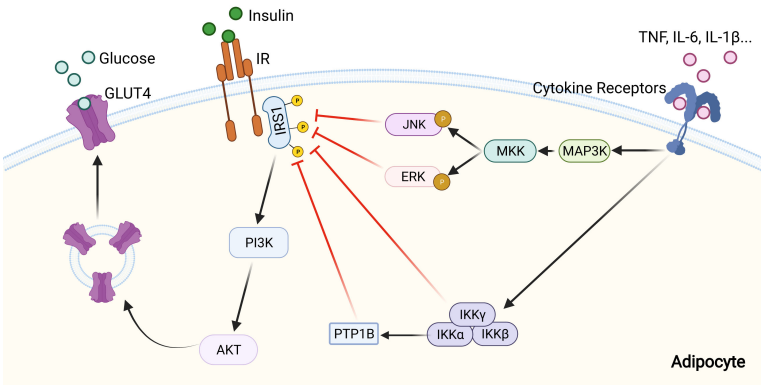

FIGURE 3
Inhibition of insulin signaling pathway by inflammatory signaling. Pro-inflammatory cytokines, including IL-1β, IL-6, and TNF, can activate inflammatory signaling pathways through their receptors. Then activated MAPK and NF-κB signaling pathways inhibit insulin signaling by altering the phosphorylation status of IRS-1, and further lead to a reduction of glucose uptake by adipocyte.

support and protection but also regulate adipose tissue homeostasis by responding to different signals (152). In other words, ECM ensures adipose tissue to expand and maintain in a healthy manner. However, the development of obesity promotes an excessive accumulation of ECM proteins and elicits adipose tissue fibrosis, which reduces tissue plasticity and results in adipocyte dysfunction such as insulin resistance (153, 154). Thus, fibrosis is considered as a hallmark of metabolically dysfunctional adipose tissue.

During the development of obesity, rapid adipose tissue expansion and adipocyte enlargement cause adipose tissue hypoxia and lead to an activation of hypoxia-inducible factor 1-alpha (HIF1-α), which induces the production of ECM proteins (155, 156). Hypoxia promotes expression of many proinflammatory genes through HIF1-α induction. Low-grade inflammation contributed by hypoxia in obesity further deteriorates adipose tissue fibrosis (157).

In addition, adipocyte hypertrophy and adipose tissue hypoxia are tightly associated with increased infiltration of macrophages, which promote local ECM accumulation (154, 158). Proinflammatory M1 macrophages make up significant proportion in fibrotic adipose tissue. Besides proinflammatory cytokines, ATM produces many other cytokines, including TGF-β1 and PDGF, which directly activate fibroblasts and increase ECM accumulation (159). As a vicious spiral, macrophages promote fibrogenesis by releasing chemokines that attract fibroblasts and more proinflammatory cells (160). Besides, macrophage inducible C-type (Mincle), which is induced in macrophage by TLR4 activation, regulate ECM prodaction and degredation, as well as fibroblast proliferation (161, 162). Additionally, saturated fatty acids (SFA)-mediated inflammation is potentiated by TLR4 activation and contributes to WAT fibrosis by fueling local inflammation (31). Besides their role in promoting fibrogenesis, macrophages participate in ECM clearance through collagen uptake and degradation. Collagen phagocytosis by macrophages depends on mannose receptor 1 (MRC1) and urokinase plasminogen activator receptor-associated protein (uPARAP/endo180) (163). In adipose tissue, it has been widely accepted that there is no single signaling or single cell type responsible for ECM production. Adipose tissue collagens are contributed by both preadipocytes and macrophages, and the fibrosis is coordinated through intimate crosstalk between macrophages and preadipocytes under different physiological and pathological conditions (164). *In vitro* studies suggest that preadipocytes in contact with proinflammatory macrophages can produce ECM proteins, including collagen I and fibronectin (164). Meanwhile, macrophage is found to be the master regulator of fibrosis through producing TGF-β1 and PDGF, which have been proved to directly activate fibroblasts and control ECM dynamics by regulating the balance between various matrix metalloproteinases (MMPs) and their inhibitors (159).

ATM and adipokines

Adipose tissue secretes many kinds of hormones, called adipokines, which exert their biological functions in an autocrine, paracrine, and/or systemic manner and influence many physiological/pathological processes, such as thermogenesis, insulin resistance and fibrosis (165). The most well-known adipokines are adiponectin and leptin, both of which can exhibit either proinflammatory or anti-inflammatory property, thereby contributing to adipose tissue functions (165).

Adiponectin

Adiponectin, a 30-kDa adipokine exclusively secreted from adipocytes, exists in cells and plasma (166, 167). As the most abounded peptide secreted by adipocytes, adiponectin shows protective activity in multiple diseases such as inflammation, obesity and insulin resistance (166, 168–170). Many evidences proved that adiponectin acts as anti-inflammatory factor by regulating the polarization of adipose tissue macrophages (36, 104). Recombinant adiponectin treatment results in an increased expression of M2 markers and a decreased expression of M1 markers in adipose tissue, while macrophages from adiponectin knock-out mice display increased M1 markers (36, 171). Interestingly, adiponectin has also been reported as a proinflammatory factor to increase TNF-α and IL-6 secretion directly. The authors further suggested that the anti-inflammatory property of adiponectin may be due to its desensitized effects on cells for further proinflammatory response, although the specific molecular mechanism is still unknown (172, 173). Additionally, adiponectin has been reported to induce adipose tissue M2 macrophage proliferation both *in vivo* and *in vitro*, further promoting cold-induced adipose tissue thermogenesis (174). Mechanistically, adiponectin is recruited to the cell surface of M2 macrophages *via* T-cadherin and promotes cell proliferation by activation of AKT signaling (174). Besides, several intracellular signaling pathways have been reported to mediate adiponectin action in regulating macrophages. Adiponectin suppresses M1 macrophage proliferation *via* inhibiting NF-κB signaling (175). A mutual antagonistic action was observed between adiponection and TNF/IL-6 expression. LPS-induced TNF/IL-

6 is suppressed by adiponectin, and TNF/IL-6 conversely inhibit adiponectin expression (176, 177). Besides, both oxidative stress and ROS release inhibit adiponectin expression in obesity, therefore forming a vicious circle that lowers adiponectin level while increases proinflammatory cytokines and oxidative stress in obese adipose tissue (56).

Leptin

Leptin, another pivotal adipokine, exerts its function through modulating immunity and inflammation (178). Leptin is a 16-kDa peptide hormone secreted mainly from adipose tissue, and the most evident function of leptin is its control of energy balance by inhibiting appetite through hypothalamus (179, 180). However, leptin receptor (LEPR) is ubiquitously expressed on the surface of many cells like immune cells, suggesting pleiotropic actions of leptin (181, 182). High levels of thymocyte apoptosis and reduced thymic cellularity were observed in in obese mice with mutation in leptin (*ob/ob* mice) or LEPR (*db/db mice*), which were reversed by peripheral administration of recombinant leptin, revealing an important role of lepin in immunity (183). Consistently, *ob/ob* mice show impaired cellular and humoral immue activities, and they are protected against inflammation in different models (184–186). Besides acting on adaptive immunity, leptin regulates innate immune cells such as macrophages, to promote inflammation. Macrophages generated from *ob/ob* or *db/db* mice showed a decrease of phagocytosis and inflammatory cytokine production, whereas exogenous leptin administration upregulated both of them (178, 181). Several *in vitro* or *ex vivo* studies in wild-type mice also support that leptin acts as a proinflammatory factor in immune cells. They showed that exogenous leptin administration upregulated both phagocytosis and production of proinflammatory cytokines (4, 187–189). Leptin stimulates production of proinflammatory cytokines through activation of JAK2-STAT3 pathway in macropahges (189, 190). Moreover, leptin-deficient mast cells polarize macrophages from M1 to M2 and thus protects mice from obesity (191). Leptin has proinflammatory properties, and the expression of leptin in adipose tissue as well as circulating leptin are promoted by administration of proinflammatory stimuli (192, 193). Thus, it appears that proinflammatory cytokines and leptin form a vicious circle that promotes the development of chronic inflammation and obesity.

Conclusions and perspective

Macrophage is the most abundant cell population and believed to play a dominant role in the homeostasis of adipose tissue and whole-body energy metabolism, whose dysregulation significantly contributes to metabolic diseases (Figure 4). Noticebaly, many other immune cells exist in adipose tissue as well, including both innate and adaptive immune cells like ILC2s, eosinophils, invariant natural killer T (iNKT) and T

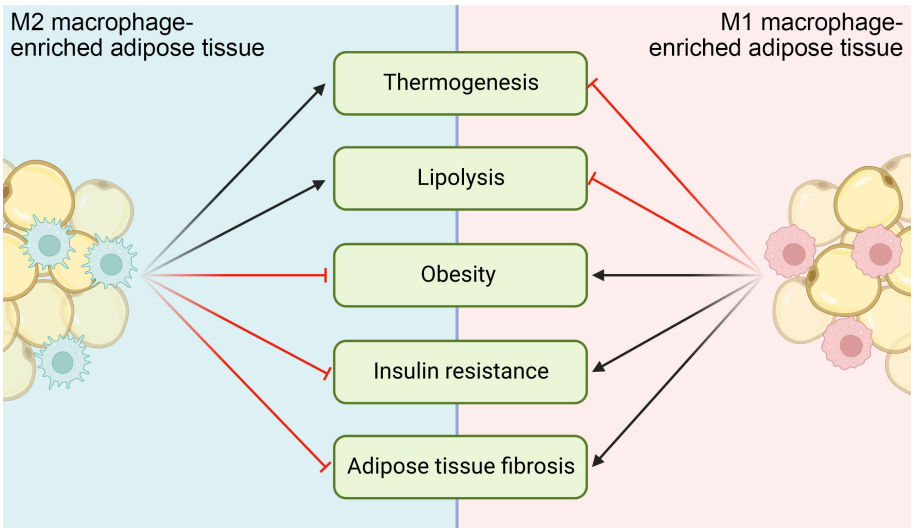

FIGURE 4
Functional implications of ATMs in different metabolic diseases. M1 ATMs inhibit adipocyte lipolysis and thermogenesis, while M2 ATMs do the opposites. When M1 activation of ATMs chronically exceeds M2 activation, metabolic diseases like obesity, insulin resistance and adipose tissue fibrosis ensue and/or deteriorate.

lymphocytes. They play critically important roles in the maintenance of energy homeostasis and contribute to the metabolic dysfunction, directly or indirectly through crosstalk with macrophages (194). ILC2s recruit and activate eosinophils through IL-5, and then eosinophil-secreted IL-4 and IL-13 promote macrophage M2 activation. Besides, ILC2s directly secret IL-13, which induces a physiological expansion and differentiation of beige adipocyte precursors, further promoting adipose tissue thermogenesis (95). iNKT cells are a type of innate immune cell, and their activation induces a production of fibroblast growth factor 21 (FGF21) that promotes thermogenic browning of WAT (195). Infiltration of $CD8^+$ T lymphocytes is an early event during the development of obesity and contribute to macrophage accumulation. Adoptive transfer of $CD8^+$ T cells into CD8-deficient obese mice induces M1 macrophage infiltration and promotes systemic insulin resistance (196).

One goal of the mouse ATM investigation is to exploit its specific characteristics and functions to treat human metabolic diseases. Even though ATMs from human and mouse models are highly similar in both gene expression pattern and function, considerable differences exist (197, 198). Mouse models have their own limitations and can not replicate the properties of humans in many aspects, thus the validation of findings from mouse models in humans is critical for their translation. But, there are still many challenges for mouse-to-human validation because of the technical limitations, including limited tools to safely manipulate ATM in humans.

The great progress of ATM studies generates many further questions that need to be addressed in the future. Firstly, it is difficult to manipulate ATM specifically. Several genetically modified animal models are used to investigate tissue-specific macrophages, including *Lyz2*-Cre, *Cx3cr1*-Cre, *CD11b*-Cre and *F4/80*-Cre, whereas all of them are ubiquitously expressed in macrophages from different tissues but not specifically in ATM (199). Besides, some of them are expressed in other types of cells. For example, the widely used *Lyz2*-Cre is expressed in other myelomonocytic cells, including most granulocytes, few $CD11c^+$ dendritic cells (DCs), and a small percentage of non-hematopoietic cells (200). Thus, more specific markers and animal models need to be identified and established, which will greatly facilitate our understanding on ATM in health and metabolic diseases. Secondly, macrophage constitutes a plastic and heterogeneous cell populations modulated by and interacted with their microenvironment in different adipose tissues. Distinct fat pads in different locations show different molecular, cellular and anatomical features (201). Accordingly, the physiological characteristics of ATM in these adipose tissues may be quite diverse, which have not been well investigated yet. Thirdly, ATM was oversimplified to be divided into two groups, M1 and M2 macrophages. However, this M1/M2 classification has been questioned as a result of the identifications of many distinct ATM subtypes, including $CD9^+$ macrophage (202), $TREM2^+$ LAM (150) and SAM (109). Although single cell RNA-sequencing provides an objective view on the identity and function of ATM (150), more unbiased approaches and new technologies should be used to identify and characterize all the different ATM populations and their regulations on health and metabolic diseases.

Author contributions

JY wrote the manuscript and DW and YQ edited it. All authors approved the submitted version. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Association between SII and hepatic steatosis and liver fibrosis: A population-based study

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Background: The systemic immune-inflammation index (SII) is a novel marker of inflammation, and hepatic steatosis and fibrosis are associated with inflammation. This study aimed to investigate the possible relationship between SII and hepatic steatosis and fibrosis.

Methods: The datasets from the National Health and Nutrition Examination Survey (NHANES) 2017–2020 were used in a cross-sectional investigation. Multivariate linear regression models were used to examine the linear connection between SII and controlled attenuation parameter (CAP) and liver stiffness measurement (LSM). Fitted smoothing curves and threshold effect analysis were used to describe the nonlinear relationship.

Results: This population-based study included a total of 6,792 adults aged 18–80 years. In a multivariate linear regression analysis, a significant positive association between SII and CAP was shown [0.006 (0.001, 0.010)]. This positive association in a subgroup analysis was maintained in men [0.011 (0.004, 0.018)] but not in women. Furthermore, the association between SII and CAP was nonlinear; using a two-segment linear regression model, we found an inverted U-shaped relationship between SII and CAP with an inflection point of 687.059 (1,000 cells/μl). The results of the multiple regression analysis showed that the relationship between SII and LSM was not significant (P = 0.263).

Conclusions: Our findings imply that increased SII levels are linked to hepatic steatosis, but SII is not linked to liver fibrosis. To confirm our findings, more large-scale prospective investigations are needed.

KEYWORDS

systemic immune-inflammatory index, NAFLD, NHANES, hepatic steatosis, liver fibrosis

Background

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease worldwide and one of the primary causes of severe liver disease (1–3). NAFLD is defined as excessive fat infiltration into the liver in the absence of substantial alcohol intake or secondary causes (4), which includes a variety of histological alterations in the liver, ranging from simple steatosis through leukocyte infiltration and hepatocyte ballooning to severe liver fibrosis and cirrhosis (5, 6). Transient elastography is widely used in the screening of NAFLD due to its good accuracy and noninvasive feature (7, 8); controlled attenuation parameter (CAP) and liver stiffness measurement (LSM) were used to assess hepatic steatosis and fibrosis, respectively (9, 10).

The systemic immune-inflammation index (SII) is an integrated and novel inflammatory biomarker as reported in the study by Hu et al. (11) in 2014, which could reflect the local immune response and systemic inflammation in the whole human body (12–15). SII has been used in past studies to predict and evaluate the prognosis of various solid tumors, such as gastric cancer (16, 17), non-small cell lung cancer (18, 19), pancreatic cancer (20), and esophageal cancer (21, 22). In addition, SII also has a high value for the prognosis of cardiovascular disease (23–27). Inflammation is a hallmark of NAFLD progression, and the recruitment of circulating inflammatory cells and the upregulation of inflammatory mediators play an important role in hepatic steatosis and fibrosis (28–31). Fontes-Cal et al. (32) reported that plasma cytokines and clinical parameters of inflammation could serve as a new strategy for monitoring NAFLD progression. However, the relationship between SII and hepatic steatosis and fibrosis remains unclear.

As a result, we examined the relationship between SII and CAP and LSM in adults in this study, utilizing a large sample of people aged 18 to 80 years from the National Health and Nutrition Examination Survey (NHANES).

Methods

Study population

The NHANES is a representative survey of the US national population that uses a complicated, multistage, and probabilistic sampling methodology to provide a wealth of information about the general US population's nutrition and health (33). The 2017–2020 continuous cycle of the US NHANES dataset was used for this investigation. We excluded 3,409 participants with missing SII data, 2,941 with missing CAP or LSM data, 90 hepatitis B antigen-positive and 132 hepatitis C antibody-positive or hepatitis C RNA-positive samples, 959 participants with significant alcohol consumption (ever have 4, 5, or more drinks every day), and 1,237 participants younger than 18 years from the 15,560 eligible individuals. The study eventually included 6,792 participants. Figure 1 illustrates the sample selection flowchart.

Study variables

The dependent variable in this study is the systemic immune-inflammation index, with CAP and LSM as the intended independent variables. In our analysis, SII was designed as an exposure variable. Lymphocyte, neutrophil, and platelet counts were measured by complete blood count using automated hematology analyzing devices (Coulter®DxH 800 analyzer) and presented as $\times 10^3$ cells/ml. SII as an exposure variable was derived from platelet count × neutrophil count/lymphocyte count (11, 13, 34). CAP and LSM were designed as outcome variables to measure hepatic steatosis and liver fibrosis.

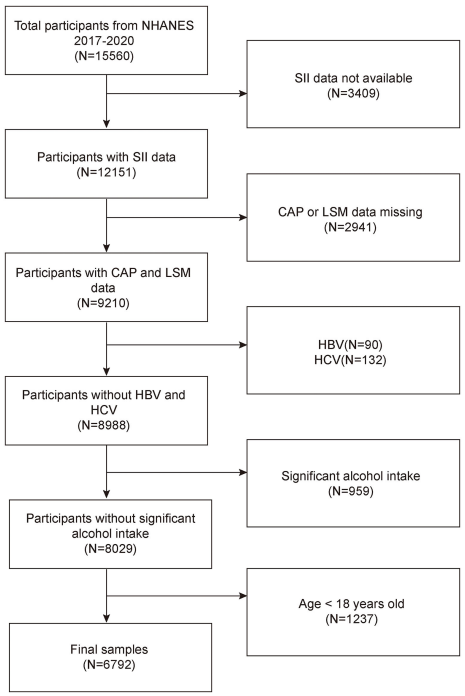

FIGURE 1
Flowchart of participant selection. NHANES, National Health and Nutrition Examination Survey; SII, systemic immune-inflammation index; CAP, controlled attenuation parameter; LSM, liver stiffness measurement.

Abbreviations: NAFLD, non-alcoholic fatty liver disease; CAP, controlled attenuation parameter; LSM, liver stiffness measurement; SII, systemic immune-inflammation index; NHANES, National Health and Nutrition Examination Survey; ICC, intrahepatic cholangiocarcinoma; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; LMR, lymphocyte-to-monocyte ratio; NASH, non-alcoholic steatohepatitis.

The NHANES staff evaluated participants for Vibration controlled transient elastography (VCTE) using the FibroScan®-equipped model 502 V2 Touch. According to a recent landmark study, CAP values, also known as CAP, ≥274 dB/m was considered indicative of NAFLD status because of 90% sensitivity in detecting all degrees of hepatic steatosis (9). Based on two past studies, CAP ≥302 dB/m was defined in this study as having severe steatosis at the base of NAFLD (4, 35). Fibrosis grade was determined by liver stiffness with cutoff values of 8.2, 9.7, and 13.6 kPa for fibrosis grades ≥F2, ≥F3, and F4, respectively, and was optimized using the Jorden index (36, 37). Covariates included age, gender, race, Body Mass Index (BMI), education level, family income-to-poverty ratio, activity status, alanine transaminase (ALT), weight, alkaline phosphatase (ALP), waist circumference, aspartate aminotransferase (AST), total calcium, total cholesterol, direct High-Density Lipoprotein Cholesterol (HDL-C), Low-Density Lipoprotein Cholesterol (LDL-C), triglyceride, serum phosphorus, and smoking status.

Statistical analysis

The statistical study was carried out using the statistical computing and graphics software R (version 4.1.3) and EmpowerStats (version:2.0). Baseline tables for the study population were statistically described by CAP and LSM subgroups; continuous variables are described using mean values plus or minus standard deviation (SD) and weighted linear regression models. The beta values and 95% confidence intervals were calculated using multivariate linear regression analysis between the SII and CAP and LSM. The multivariate test was built using three models: model 1: no variables adjusted; model 2: gender, age, and race adjusted; model 3: adjusted for all covariates. By adjusting the variables, smoothed curve fits were done simultaneously. A threshold effects analysis model was used to examine the relationship and inflection point between SII and CAP. Finally, the same statistical study methods described above were conducted for the gender subgroups. It was determined that $P < 0.05$ was statistically significant. We used a weighting approach to reduce the significant volatility of our dataset.

Results

Baseline characteristics

In this study, 6,792 adults were included based on the inclusion and exclusion criteria, and the average age of the participants was 48.58 ± 18.50 years. Among these participants, 45.39% were men, 54.61% were women, 33.82% were non-Hispanic white, 25.28% were non-Hispanic black, and 12.38% were Mexican American, and 28.52% were from other races. The mean (SD) concentrations of CAP, LSM, and SII were 262.49 (62.84) dB/m, 5.84 (4.81) kPa, and 515.48 (341.66) (1,000 cells/μl), respectively.

Table 1 lists all clinical characteristics of the participants with CAP as a column-stratified variable. In comparison to the non-NAFLD group, the severe steatosis group is more likely to be men and older, with a higher proportion of non-Hispanic blacks and Mexican Americans; with higher smoking status; and higher levels of BMI, waist circumference, AST, ALT, ALP, total cholesterol, LDL cholesterol, triglyceride, LSM, and SII but lower levels of direct HDL cholesterol and serum phosphorus.

Table 2 lists all clinical features of the individuals with LSM as a column-stratified variable. In comparison to the normal group, the cirrhosis group is more likely to be men and older, with a higher proportion of non-Hispanic blacks and Mexican Americans; with higher smoking status; and higher levels of BMI, waist circumference, AST, ALT, ALP, LDL cholesterol, triglyceride, CAP, and SII but lower levels of HDL cholesterol, total cholesterol, and serum phosphorus.

Association between systemic immune-inflammation index (SII) and controlled attenuation parameter (CAP)

Table 3 showed the results of the multivariate regression analysis. In the unadjusted model [0.006 (0.001, 0.010)], SII was highly associated with CAP. However, after adjusting for gender, age, and race variables, this significant positive correlation became insignificant in model 2 [0.002 (-0.002, 0.007)]. After adjusting for all covariates, the relationship between SII and CAP became negative in model 3 [-0.002 (-0.009, 0.004)].

In subgroup analyses stratified by gender, our results suggest that the positive association between SII and CAP is independently significantly positive in men [0.011 (0.004, 0.018)] but not statistically significant in all models for women. When we performed a subgroup analysis stratified by the degree of hepatic steatosis, the SII showed a strong positive correlation with both the NAFLD group and the severe steatosis group in both the unadjusted and partially adjusted models using the non-NAFLD group as the reference group.

We performed a smooth curve fit to describe the nonlinear relationship between SII and CAP (Figures 2, 3). Using a two-segment linear regression model, we found an inverted U-shaped relationship between SII and CAP with an inflection point of 687.059 (1,000 cells/μl). After stratifying the analysis by gender, an inverted U-shaped curve was also present in men and women, with inflection points of 591.000 (1,000 cells/μl) and 749.692 (1,000 cells/μl), respectively (Table 4).

Association between SII and LSM

The results of multiple regression analysis showed a positive but insignificant correlation between SII and LSM

TABLE 1 Weighted characteristics of the study population based on controlled attenuated parameter (CAP).

	Non-NAFLD (CAP < 274, n = 3,901)	NAFLD (274 ≤ CAP < 302, n = 1,031)	Severe steatosis (CAP ≥ 302, n = 1,860)	P value
Age (years)	46.135 ± 19.305	51.607 ± 17.552	52.013 ± 16.389	<0.001
Gender (%)				<0.001
Men	41.989	43.938	53.333	
Women	58.011	56.062	46.667	
Race/Ethnicity (%)				<0.001
Non-Hispanic White	33.017	32.590	36.183	
Non- Hispanic Black	28.198	24.151	19.785	
Mexican American	9.510	14.646	17.151	
Other Race	29.275	28.613	26.882	
Education lever (%)				0.089
Less than high school	16.597	18.924	18.932	
High school	22.555	22.809	23.720	
More than high school	60.848	58.267	57.348	
Moderate activities (%)				<0.001
Yes	46.091	40.543	37.312	
No	53.909	59.457	62.688	
Smoked at least 100 cigarettes				<0.001
Yes	32.479	31.620	40.430	
No	67.521	68.380	59.570	
Income to poverty ratio	2.646 ± 1.663	2.716 ± 1.601	2.649 ± 1.608	0.520
BMI (kg/m^2)	26.902 ± 5.946	31.480 ± 6.801	34.873 ± 7.725	<0.001
Waist circumference (cm)	92.211 ± 14.260	104.038 ± 13.584	112.974 ± 15.613	<0.001
Laboratory features				
Total calcium (mmol/L)	2.320 ± 0.092	2.320 ± 0.098	2.318 ± 0.097	0.673
Total cholesterol (mmol/L)	4.714 ± 1.024	4.890 ± 1.045	4.848 ± 1.054	<0.001
Triglyceride(mmol/L)	0.979 ± 0.727	1.379 ± 1.552	1.629 ± 1.193	<0.001
LDL- cholesterol(mmol/L)	2.750 ± 0.891	2.903 ± 0.932	2.868 ± 0.927	<0.001
HDL- cholesterol(mmol/L)	1.474 ± 0.400	1.336 ± 0.392	1.211 ± 0.342	<0.001
ALT (IU/L)	18.311 ± 13.825	22.347 ± 15.461	27.978 ± 20.136	<0.001
AST (IU/L)	20.302 ± 10.862	21.414 ± 12.840	23.287 ± 14.532	<0.001
ALP(IU/L)	74.800 ± 24.197	78.789 ± 22.622	81.974 ± 25.317	<0.001
Serum phosphorus (mmol/L)	1.162 ± 0.166	1.149 ± 0.164	1.137 ± 0.169	<0.001
LSM (kPa)	5.122 ± 3.873	5.895 ± 4.414	7.328 ± 6.229	<0.001
SII (1,000 cells/μl)	509.876 ± 364.567	504.868 ±289.827	533.097 ± 317.184	0.030

Mean ± SD for continuous variables: P value was calculated by weighted linear regression model.
% for categorical variables: P value was calculated by weighted chi-square test.
BMI, body mass index; LDL- cholesterol, low-Density Lipoprotein Cholesterol; HDL- cholesterol, high-Density Lipoprotein Cholesterol; ALT, alanine transaminase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; LSM, liver stiffness measure ; CAP, controlled attenuation parameter; SII, systemic immune-inflammation index.

(Table 5). Moreover, the effect value was shown to be zero within three decimal places because the units of SII were too small [0.000 (-0.000, 0.001)]. Among all subgroup analyses, SII showed a statistically significant negative correlation with LSM only in the significant fibrosis group [-0.000 (-0.000, -0.000), P = 0.044]. The nonlinear relationship was characterized by smooth curve fittings (Figure 4).

Discussion

In our study sample, which is nationally representative of US adults, SII levels were positively correlated with hepatic steatosis and there was no significant correlation between SII levels and liver fibrosis. Notably, we found an inverted U-shaped association between SII and CAP, with an inflection point of

TABLE 2 Weighted characteristics of the study population based on median liver stiffness measurement (LSM).

	Normal group(LSM<8.2, n = 6,098)	Significant fibrosis (8.0≤LSM<9.7, n = 283)	Advanced fibrosis (9.7≤LSM<13.6, n = 223)	Cirrhosis (LSM≥13.6, n = 188)	P value
Age (years)	47.859 ± 18.578	53.484 ± 16.578	56.309 ± 16.466	55.250 ± 16.525	<0.001
Gender (%)					<0.001
Men	44.654	49.823	49.776	57.447	
Women	55.346	50.177	50.224	42.553	
Race/Ethnicity (%)					0.018
Non-Hispanic White	33.585	31.449	38.565	39.362	
Non- Hispanic Black	25.057	32.509	24.215	22.872	
Mexican American	12.283	12.014	12.108	16.489	
Other Race	29.075	24.028	25.112	21.277	
Education lever (%)					0.898
Less than high school	17.405	19.343	18.894	20.330	
High school	22.668	24.818	26.267	24.176	
More than high school	59.927	55.839	54.839	55.495	
Moderate activities (%)					0.003
Yes	43.736	36.396	33.632	34.574	
No	56.264	63.604	66.368	65.426	
Smoked at least 100 cigarettes					0.081
Yes	33.864	36.749	44.843	40.426	
No	66.136	63.251	55.157	59.574	
Income to poverty ratio	2.669 ± 1.649	2.642 ± 1.580	2.504 ± 1.511	2.482 ± 1.514	0.270
BMI (kg/m^2)	29.022 ± 6.691	33.734 ± 9.207	38.043 ± 9.651	38.538 ± 11.549	<0.001
Waist circumference (cm)	97.955 ± 15.952	109.169 ± 19.514	119.096 ± 16.628	120.849 ± 20.775	<0.001
Laboratory features					
Total calcium (mmol/L)	2.320 ± 0.093	2.321 ± 0.106	2.313 ± 0.109	2.307 ± 0.100	0.219
Total cholesterol (mmol/L)	4.792 ± 1.027	4.666 ± 1.104	4.720 ± 1.156	4.537 ± 1.115	0.002
Triglyceride(mmol/L)	1.194 ± 1.027	1.389 ± 1.353	1.597 ± 1.708	1.353 ± 0.902	<0.001
LDL- cholesterol (mmol/L)	2.819 ± 0.897	2.733 ± 0.999	2.752 ± 1.067	2.547 ± 0.940	0.025
HDL- cholesterol (mmol/L)	1.395 ± 0.397	1.276 ± 0.351	1.247 ± 0.428	1.238 ± 0.449	<0.001
ALT (IU/L)	20.657 ± 14.773	26.575 ± 19.780	30.677 ± 27.380	33.253 ± 32.692	<0.001
AST (IU/L)	20.553 ± 9.249	24.085 ± 16.192	26.926 ± 19.327	34.086 ± 40.412	<0.001
ALP(IU/L)	76.325 ± 23.230	82.905 ± 26.756	85.604 ± 30.118	93.269 ± 39.714	<0.001
Serum phosphorus (mmol/L)	1.155 ± 0.166	1.148 ± 0.174	1.131 ± 0.174	1.131 ± 0.171	0.045
CAP (dB/m)	257.063 ± 60.343	297.473 ± 62.943	316.704 ± 61.176	321.665 ± 66.575	<0.001
SII (1,000 cells/μl)	513.208 ± 335.813	537.926 ± 463.726	519.919 ± 322.459	549.945 ± 335.262	0.328

Mean ± SD for continuous variables: P value was calculated by weighted linear regression model.
% for categorical variables: P value was calculated by weighted chi-square test.
BMI, body mass index; LDL- cholesterol, low-Density Lipoprotein Cholesterol; HDL- cholesterol, high-Density Lipoprotein Cholesterol; ALT, alanine transaminase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; LSM, liver stiffness measure ; CAP, controlled attenuation parameter; SII, systemic immune-inflammation index.

TABLE 3 The association between SII and CAP.

	Model 1 β (95% CI) P value	Model 2 β (95% CI) P value	Model 3 β (95% CI) P value
CAP (dB/m)	0.006 (0.001, 0.010)	0.002 (-0.002, 0.007)	-0.002 (-0.009, 0.004)
	0.011	0.285	0.443
Stratified by CAP			
Non-NAFLD	Reference	Reference	Reference
NAFLD	0.004 (0.000, 0.009)	0.005 (0.000, 0.009)	-0.003 (-0.010, 0.003)
	0.041	0.030	0.335
Severe steatosis	0.003 (0.001, 0.008)	0.005 (0.001, 0.010)	-0.002 (-0.008, 0.002)
	0.012	0.026	0.518
Stratified by gender			
Men	0.011 (0.004, 0.018)	0.004 (-0.004, 0.011)	-0.003 (-0.013, 0.007)
	0.003	0.325	0.596
Women	0.003 (-0.003, 0.008)	0.002 (-0.003, 0.007)	-0.003 (-0.011, 0.005)
	0.335	0.470	0.491

Model 1: no covariates were adjusted. Model 2: age, gender, and race were adjusted. Model 3: age, gender, race, educational level, BMI, family income-to-poverty ratio, moderate activities, smoking status, ALP, ALT, AST, total calcium, total cholesterol, triglyceride, LDL, HDL-C, waist circumference, and serum phosphorus were adjusted.
In the subgroup analysis stratified by gender and race, the model is not adjusted for sex and race, respectively.
NAFLD, non-alcoholic fatty liver disease; CAP, controlled attenuation parameter; SII, systemic immune-inflammation index.

687.059 (1,000 cells/μl). This indicated that SII was an independent crisis factor for hepatic steatosis when the SII was less than 687.059 (1,000 cells/μl).

To our knowledge, this is the first study to investigate SII with hepatic steatosis and fibrosis. In previous studies on the liver, SII has often been used as a predictor of prognostic survival in patients with hepatocellular carcinoma or intrahepatic cholangiocarcinoma (ICC) (38–40). Ren et al. (41) reported that among 28 patients with ICC who received liver transplantation, the 1-, 3-, and 5-year survival rates were significantly lower in the high-SII group than those in the low-SII group, and that SII could be used to predict survival in patients with ICC who received liver transplantation. Similarly, another study from China showed that SII was a valid prognostic factor for predicting the prognosis of patients undergoing radical hepatectomy for ICC, while neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), and lymphocyte-to-monocyte ratio (LMR) were not associated with clinical outcomes in these patients (42).

At present, many epidemiological studies have proven that inflammation is related to the progression of NAFLD (43–45). A large multicenter cohort of NAFLD patients from Italy and Finland showed that steatosis, ballooning, and lobular inflammation were independently associated with significant fibrosis. In addition, the authors found that a third of patients with significant fibrosis did not have non-alcoholic steatohepatitis (NASH) when they analyzed biopsy specimens taken from NAFLD patients at a single time point, a result that

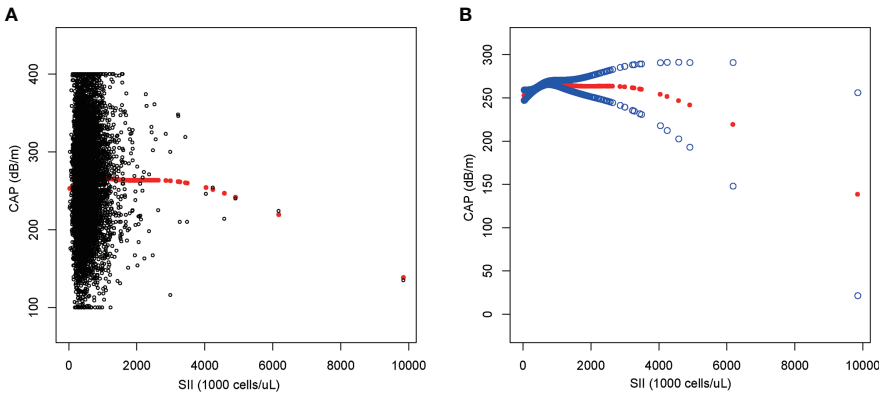

FIGURE 2
The association between SII and CAP. **(A)** Each black point represents a sample. **(B)** The solid red line represents the smooth curve fit between variables. Blue bands represent the 95% confidence interval from the fit. SII, systemic immune-inflammation index; CAP, controlled attenuation parameter.

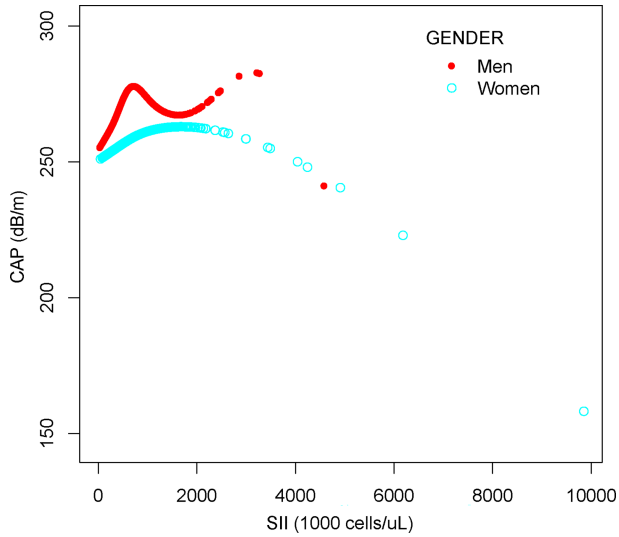

FIGURE 3
The association between SII and CAP stratified by gender. SII, systemic immune-inflammation index; CAP, controlled attenuation parameter.

far exceeded expectations (46). The lack of significant association between SII and LSM found in our results may explain this phenomenon. Haukeland et al. (28) evaluated serum samples from 47 histologically validated NAFLD patients and showed that NAFLD patients are characterized by low-grade systemic inflammation. High chemokine (C-C motif) ligand 2 (CCL2)/monocyte chemoattractant protein 1 (MCP1) levels in NASH may be important for the transition from simple steatosis to NASH (28). Our results demonstrate a significant positive relationship between SII and CAP; in other words, inflammation has a strong positive correlation with hepatic steatosis. Not only that, but the positive association between SII and CAP differs significantly by gender. Men with NAFLD have more severe hepatic steatosis than women, and postmenopausal women have greater hepatic steatosis than premenopausal women, according to several studies, suggesting that the gender difference in NAFLD is related to sex hormones (47, 48). Furthermore, a recent experimental animal study found that Formyl Peptide Receptor 2 (FPR2) expression is higher in female mice than that in male mice, making females more resistant to the development

TABLE 4 Threshold effect analysis of SII on CAP using two-piecewise linear regression model.

CAP (dB/m)	Adjusted β (95% CI) P value
SII	
Inflection point	687.059
SII<687.059 (1,000 cells/μl)	0.026 (0.017, 0.035) <0.0001
SII>687.059 (1,000 cells/μl)	-0.008 (-0.015, -0.002) 0.0107
Log likelihood ratio	<0.001
Men	
Inflection point	591.000
SII<591.000 (1,000 cells/μl)	0.045 (0.029, 0.062) <0.0001
SII>591.000 (1,000 cells/μl)	-0.008 (-0.019, 0.003) 0.1556
Log likelihood ratio	<0.001
Women	
Inflection point	749.692
SII<749.692 (1,000 cells/μl)	0.022 (0.011, 0.033) 0.0001
SII>749.692 (1,000 cells/μl)	-0.008 (-0.016, -0.000) 0.0372
Log likelihood ratio	<0.001

Age, gender, race, educational level, BMI, family income-to-poverty ratio, moderate activities, smoking status, ALP, ALT, AST, total calcium, total cholesterol, triglyceride, LDL, HDL-C, waist circumference, and serum phosphorus were adjusted.
LSM, liver stiffness measure ; SII, systemic immune-inflammation index.

TABLE 5 The association between SII and LSM.

	Model 1 β (95% CI) P value	Model 2 β (95% CI) P value	Model 3 β (95% CI) P value
LSM (kPa)	0.000 (-0.000, 0.001)	0.000 (-0.000, 0.001)	-0.000 (-0.001, 0.001)
	0.263	0.182	0.927
Stratified by LSM			
Normal group	Reference	Reference	Reference
Significant fibrosis	-0.000 (-0.000, -0.000)	-0.000 (-0.000, -0.000)	-0.000 (-0.000, -0.000)
	0.044	0.040	0.513
Advanced fibrosis	-0.000 (-0.000, -0.000)	-0.000 (-0.000, -0.000)	0.000 (-0.000, 0.001)
	0.920	0.967	0.357
Cirrhosis	-0.001 (-0.008, 0.006)	-0.000 (-0.008, 0.007)	0.000 (-0.013, 0.013)
	0.813	0.924	0.967
Stratified by gender			
Men	0.000 (-0.000, 0.001)	0.000 (-0.001, 0.001)	-0.000 (-0.001, 0.001)
	0.432	0.867	0.872
Women	0.000 (-0.000, 0.001)	0.000 (-0.001, 0.001)	-0.000 (-0.001, 0.001)
	0.145	0.095	0.970

Model 1: no covariates were adjusted. Model 2: age, gender, and race were adjusted. Model 3: age, gender, race, educational level, BMI, family income-to-poverty ratio, moderate activities, smoking status, ALP, ALT, AST, total calcium, total cholesterol, triglyceride, LDL, HDL-C, waist circumference, and serum phosphorus were adjusted.
In the subgroup analysis stratified by gender and race, the model is not adjusted for sex and race, respectively.
CAP, controlled attenuation parameter; SII, systemic immune-inflammation index.

and progression of NAFLD, and the severe damage seen in FPR2-depleted females supports FPR2's protective role in female mice's liver (49). In addition to sex, race, age, and other covariates may also be factors influencing the relationship between SII and CAP, and multiple factors interacting with each other may also be the reason why the relationship between SII and CAP in this study was not significant in model 2 and model 3.

NAFLD includes a disease continuum from steatosis with or without mild inflammation to NASH, characterized by necrotizing inflammation and faster fibrotic progression than NAFLD (50). The mechanisms behind the connection between inflammation and NAFLD progression are unclear. One theory is that nutrient overload is the primary cause of NAFLD, with excess visceral fat causing macrophage infiltration into tissue compartments, resulting in a pro-inflammatory state that increases insulin resistance. Inappropriate lipolysis in the presence of insulin resistance causes aberrant fatty acid transport to the liver, resulting in a decrease in metabolic capacity. Lipotoxic lipids are formed as a result of lipid metabolic imbalances, which cause cellular stress, inflammasome activation, and apoptotic cell death, as well as stimulation of inflammation, tissue regeneration, and fibrogenesis (51, 52). This may be the mechanism leading to the progression of hepatic steatosis and fibrosis (53). Another theory is that metabolic imbalance and inflammation in NAFLD are caused by the liver's interdependence and interaction with other organs (54–56). For

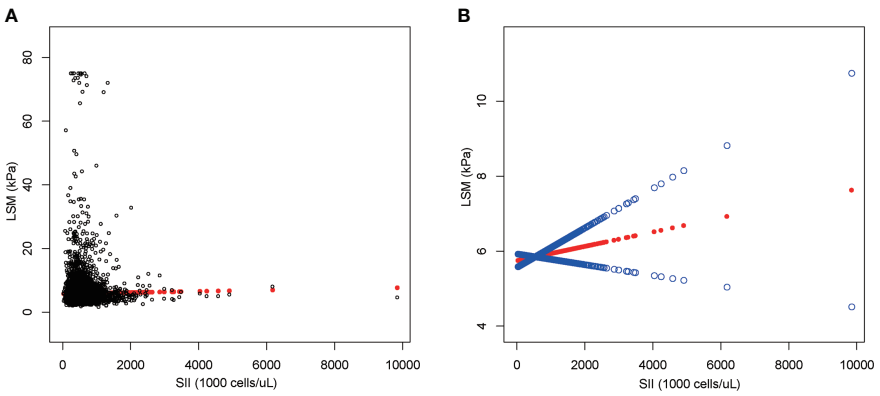

FIGURE 4
The association between SII and LSM. **(A)** Each black point represents a sample. **(B)** The solid red line represents the smooth curve fit between variables. Blue bands represent the 95% confidence interval from the fit. SII, systemic immune-inflammation index; LSM, liver stiffness measurement.

example, differences in gut microbiota composition have been observed in NAFLD patients compared to the general population, and some data suggest the presence of fecal microbiome signatures associated with advanced fibrosis (57). Furthermore, substances produced by bacteria or bile acid metabolism can influence liver inflammation and disease progression in NAFLD, although a clear causal relationship has not been established (50, 57).

Our study has some limitations. First, this is a cross-sectional analysis; thus, temporality cannot be ascertained. Furthermore, despite adjusting several relevant confounders, we were unable to rule out the impact of additional confounding factors; therefore, our findings should be interpreted with caution. Third, due to the limitations of the NHANES database, the covariates of this study did not include participants' medications use, and anti-inflammatory medications are often used in patients with NAFLD; therefore, our findings may not fully reflect the true situation. Fourth, the degree of hepatic steatosis and liver fibrosis in this study was judged by transient elastography, and although several studies have demonstrated the extremely high accuracy of transient elastography (58–60), it still cannot be the same as biopsy; therefore, our results may not be the same as using biopsy as a judgment of hepatic steatosis and liver fibrosis. Despite these limitations, our study has several advantages. Because we used a nationally representative sample, our study is representative of a multiethnic and gender-diverse population of adults in the United States. In addition to this, the large sample size included in our study allowed us to perform a subgroup analysis.

Conclusion

Our findings imply that increased SII levels are linked to hepatic steatosis, but SII is not linked to liver fibrosis. To confirm our findings, more large-scale prospective investigations are needed.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: www.cdc.gov/nchs/nhanes/.

Ethics statement

This study was reviewed and approved by NCHS Ethics Review Board. The patients/participants provided their written informed consent to participate in this study.

Author contributions

RX and YZ designed the research. RX, YZ, MX and LL collected and analyzed the data. RX, XH and YZ drafted the manuscript. ML, NM and RX revised the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

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TSH–SPP1/TRβ–TSH positive feedback loop mediates fat deposition of hepatocyte: Crosstalk between thyroid and liver

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Aims: We conducted this study with two aims: (1) whether TRβ could be damaged by NAFLD, thereby represent thyroid hormone resistance-like manifestation and (2) to analyze the potential role of SPP1 in TH signaling pathway on the process of NAFLD. This study is expected to provide a new perspective on the therapeutic mechanism in the pathological course of NAFLD.

Methods: A total of 166 patients diagnosed with type 2 diabetes mellitus (T2DM) were enrolled in this study. All patients had a BMI above 24 kg/m2 and were stratified into two groups: NAFLD and Non-NAFLD groups. Ages, gender, BMI, duration of diabetes and biochemical markers were obtained from participants' records. We downloaded the dataset GSE48452 from GEO. The Pathview library was used to make the thyroid hormone signaling pathway visualization. The CIBERSORT algorithm was applied to calculate the infiltrated immune cells in obese NAFLD patients. C57BL/6 mice were randomly selected to constitute the normal control (NC) group and were fed a normal chow diet; the rest of the mice were fed a high-fat diet (HFD). After 12 weeks HFD feeding, the mice were sacrificed by cervical dislocation, and blood samples were collected. Mouse livers were also collected; one part of each liver was fixed in 10% formalin for histological analysis, and the other part was snap-frozen for subsequent molecular analyses. To explore the relationship between SPP1, TRβ and lipid deposition in hepatocytes, HepG2 cells were treated with 50 μ M concentration of PA and/or 20 ng/ml concentration of rh-SPP1 for 48h. In addition, the PC3.1-TRβ plasmid was constructed for further validation in HepG2 cells. We used THP-1 cells to construct an M1 macrophage model *in vitro*. We then analyzed THP-1 cells treated with various concentrations of PA or TSH.

Results: (1) After adjusting for all factors that appeared P value less than 0.1 in the univariate analysis, BMI, TSH, and FT3 were significant independent risk factors of NAFLD (ORs were 1.218, 1.694, and 2.259, respectively); (2) A further analysis with BMI stratification indiacted that both FT3 and TSH had a significant change between individuals with NAFLD and Non-NAFLD in obesity subgroup;

however, there was no statistic difference in over-weight group; (3) Bioinformatics analysis of GSE48452 had shown that several key molecular (including TRβ) of thyroid hormone pathway affected by NAFLD induced transcriptomic changes and the expression levels of SPP1, FABP4 and RPS4Y1 were significantly higher, while the expression levels of PZP and VIL1 were significantly decreased in NAFLD patients(adjusted $p < 0.05$, $|logFC| > 1.0$). The CIBERSORT algorithm showed increased M0 and M1, decreased M2 macrophage infiltration in NAFLD with comparison to healthy obese group; (4) After 12 weeks of HFD-feeding, the obesity mice had significantly higher serum TSH and In IHC-stained liver sections of obesity group, the intensity of SPP1 had a significantly increased, while TRβ reduced; (5) *In vitro* studies have shown SPP1 aggravated lipid deposition in hepatic cells dependent on down-regulating the expression of TRβ and TSH acts to promote secretion of SPP1 in M1 macrophage cells.

Conclusions: SPP1 secretion induced by M1 macrophage polarization, which may down-regulates TRβ in hepatocytes *via* paracrine manner, on the one hand, the lipid deposition aggravating in liver, on the other hand, a compensatory increase of TSH in serum. The increased TSH can further lead to the following SPP1 secretion of M1 macrophage. The positive feedback crosstalk between thyroid and liver, may be plays an important role in maintaining and amplifying pathological process of NAFLD.

KEYWORDS

non-alcoholic fatty liver disease, obesity, thyroid function, TSH, thyroid hormone receptor, SPP1, M1 macrophage polarization, positive feedback crosstalk

Introduction

Non-alcoholic fatty liver disease (NAFLD), now known as metabolic dysfunction-associated fatty liver disease (MAFLD), affects approximately one-quarter of the world's adult population and poses a major health and economic burden to all societies (1). The global obesity pandemic, which has grown over the past three decades, has been a major cause of this dramatic increase in the incidence of NAFLD (2). In general, NAFLD is considered to be the hepatic manifestation of metabolic syndrome, which is most commonly observed in cases of obesity and/or type 2 diabetes mellitus (T2DM), and not only constitutes a first stage in this disease, but the toxicity exerted by certain lipids might also drive further steps in this disease, such as inflammation and liver injury (3). The rise in NAFLD has led to a remarkable increase in the number of cases of cirrhosis, hepatocellular carcinoma, hepatic decompensation, and liver-related mortality related to NAFLD (4). It is currently believed that chronic inflammation caused by excessive deposition of liver fat and changes in the local immune environment are important mechanisms that lead to the transformation of NAFLD to hepatocellular carcinoma (HCC) (5, 6). Therefore, the identification of mechanisms in the process of liver lipid deposition may provide important targets for the prevention of NAFLD-associated HCC.

Thyroid hormone are known to have significant effects on lipid metabolism in that live (7, 8). Hypothyroidism-induced NAFLD is often attributed to disruption of thyroid hormone (TH) signaling, resulting in decreased hepatic utilization of lipids. In fact, subclinical hypothyroidism, even in the higher range of normal serum thyroid-stimulating hormone (TSH) concentrations, has been found to be dose-dependently associated with NAFLD (9, 10). In our previous study, 369 euthyroid T2DM individuals with suspected NAFLD were involved, higher levels of free triiodothyronine (FT3) and TSH were observed than in individuals without NAFLD, confirming the existence of a thyroid hormone resistance-like manifestation in these patients (11). There are two major isoforms of the thyroid hormone receptor (TR), TRα and TRβ, which are the predominant receptors in the liver (12). To date, however, there has been little research on the association between TRβ and NAFLD, which may involve the intrinsic mechanism of NAFLD

development. Secreted phosphoprotein 1 (SPP1), also known as osteopontin, is expressed in a variety of tissues, including adipose, liver, and kidney, as well as macrophages. Studies have shown that in both animal models and human experiments, SPP1 can mediate chronic inflammation *in vivo* and promote fat deposition and malignant lesions in the liver (13, 14). However, studies on the role of SPP1 in the TH signaling pathway in the liver are inconclusive.

Accumulation of liver fat, which is most commonly observed in cases of obesity or T2DM, might drive further steps in diseases, such as inflammation, liver injury, and insulin resistance (15). Hence, we designed a study for individuals diagnosed with T2DM and being overweight/obese, making them suitable for the diagnosis of MAFLD, and to be adjusted for the interference from metabolic dysfunction beyond the liver. This study was conducted with two aims: (1) to determine whether TRβ could be damaged by NAFLD, thereby representing TH resistance-like manifestation and (2) to analyze the potential role of SPP1 in the TH signaling pathway in NAFLD. This study provides a new perspective on the therapeutic mechanisms underlying the pathological course of NAFLD.

Materials and methods

Study population

A total of 166 patients diagnosed with T2DM were enrolled in this study from the Department of Endocrinology of the First Affiliated Hospital (Anhui Provincial Hospital) of the University of Science and Technology of China (USTC) between July 2017 and September 2018. All patients had a body mass index (BMI) above 24 kg/m^2 (considering ethnic differences, our study adopted the obesity diagnostic criteria recommended by the World Health Organization (WHO) for Chinese people: BMI ≥ 24 kg/m^2 is overweight and BMI ≥ 28 kg/m^2 is obesity) and normal thyroid function (euthyroid, defined as both free thyroxine and TSH within the reference range). The requirement for informed consent was waived because this study was designed to retrospectively collect available data from the participants' medical records. Patients were stratified into two groups as mentioned previously: NAFLD and healthy liver groups. All people included in this study had T2DM, so they were in line with the diagnosis of MAFLD. However, to be consistent with our previous study, NAFLD was used in this study. Patients with acute complications from diabetes, severe hepatic disease (if the value of liver function index (alanine transaminase or aspartate transaminase) exceeded the upper normal reference value by 1.5×), severe chronic kidney disease (defined as estimated glomerular filtration rate ≤ 60 mL/min/1.73 m^2), cancer, or other severe coexisting illnesses were excluded from the study.

Clinical and laboratory evaluation

Age, sex, BMI, and duration of diabetes were obtained from the participants' records. All patients were tested for biochemical markers of liver function: alanine transaminase (ALT) and aspartate transaminase (AST); kidney function: creatinine (Cr); glucose metabolism: fasting blood glucose (FBG), fasting insulin (Fins), fasting C-peptide (FCP), and hemoglobin A1c (HbA1c); lipid metabolism: triglycerides (TG), total cholesterol (TC), low density lipoprotein cholesterol (LDL-c), and high-density lipoprotein cholesterol (HDL-c); and thyroid function: FT3 (normal range: 3.28–6.47 pmol/L), free thyroxine (FT4, normal range: 7.90–19.05 pmol/L), and TSH (normal range: 0.350–4.949 mIU/L) at baseline.

Bioinformatics analysis

The dataset GSE48452 was downloaded from the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo). In the dataset, the samples were divided into two groups (24 obese patients with NAFLD and 16 with a healthy liver). After consolidation and normalization of the RNA-seq data, 118 differentially expressed genes (DEGs) involved in NAFLD were screened using the limma package (adjusted $p < 0.05$, $|logFC| > 0.5$). The Pathview library (https://pathview.uncc.edu/home) was used to visualize the TH signaling pathway. The Cell type Identification by Estimating Relative Subsets of known RNA Transcripts (CIBERSORT) algorithm was applied to calculate the infiltrated immune cells in obese patients with NAFLD.

Animal experiments

Six-week-old specific pathogen-free C57BL/6 mice weighing 20–23 g were purchased from the Shanghai Model Organisms Center, Inc. (Shanghai, China). The animal protocols were approved by the Animal Care and Use Committee of University of Science and Technology of China (Approval ID: 2022-N(A)-043). All mice were maintained in a 48 ± 10% humid environment at room temperature (20 ± 1°C) under a 12-h light/dark cycle, with free access to food. Every effort was made to minimize the number of animals used and their suffering. Eight mice were randomly selected to constitute the normal control (NC) group and fed a normal chow diet, while the remaining mice were fed a high-fat diet (HFD). After 12 weeks, the mice were fasted overnight, anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg), euthanized by cervical dislocation, and then blood samples and livers were collected. One part of each liver was fixed in 10% formalin for histological analysis, and the remaining part was snap-frozen for subsequent molecular analyses.

Enzyme-linked immunosorbent assay

Collected blood or cell supernatant samples were centrifuge at 3,000 rpm for 10 min. SPP1 levels were determined using a commercial ELISA kit (Meimian, Jiangsu, China) and optical density was measured at 450 nm. TSH levels were measured using the picrate method (Jiancheng, Nanjing, China) and optical density was measured at 450 nm. The kits were used in accordance with the manufacturer's instructions.

Immunohistochemistry staining

Liver tissues were fixed with 10% buffered formalin for 48 h and embedded in paraffin. A microtome was used to cut 4–6 μm sections for tissue analysis. Liver sections were deparaffinized, antigen was retrieved, endogenous catalase was removed with 3% H_2O_2, incubated in blocking solution, and then incubated with anti-SPP1 (1:200, Proteintech), TSH (1:200, Bioss), and TRβ (1:200, Bioss) antibodies overnight at 4°C. Sections were then incubated with HRP-conjugated secondary antibodies and developed with 3,3-diaminobenzidine.

Cell culture and treatment

HepG2 cells were maintained in Dulbecco's modified Eagle medium supplemented with 25 mmol/L glucose, 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin and were cultured at 37 °C in a 95% humidity and 5% CO2-containing environment. Bovine serum albumin-conjugated palmitic acid (PA; Sigma-Aldrich, St. Louis, MO, USA) was prepared as previously described (16). To explore the relationship between SPP1, TRβ and lipid deposition in hepatocytes, HepG2 cells were treated with 50 μ M concentration of PA and/or 20 ng/ml concentration of rh-SPP1 (MedChemExpress,China) for 48h. In addition, the PC3.1-TRβ plasmid was constructed for further validation in HepG2 cells. Human leukemia monocyte THP-1 cells were maintained in RPMI 1640 medium containing 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin and were cultured at 37 °C in a 95% humidity and 5% CO2-containing environment. To obtain THP-1-derived macrophages, THP-1 cells were treated as previously described (17). Briefly, THP-1 macrophages were treated with 500 ng/mL phorbol 12-myristate 13-acetic acid (PMA) for 72 h to induce THP-1 differentiation into macrophages (THP-1 M0 macrophages), followed by induction of M1 macrophage activation by 200 ng/mL lipopolysaccharide. To verify whether TSH promotes the secretion of SPP1 in M1 macrophages. We used THP-1 cells to construct an M1 macrophage model *in vitro*. We then analyzed THP-1 cells treated with various concentrations of PA or TSH.

Oil red O staining

HepG2 cells were seeded in 12-well plates, washed three times with phosphate buffered saline (PBS), and fixed with 4% formaldehyde for 30 min. Oil Red O (0.5% in isopropanol), filtered through a 0.45 mm filter, and then added to the fixed cells for 20 min at room temperature. Cells were washed with 70% ethanol and water, observed, and photographed using a light microscope. Mouse livers were fixed with 4% paraformaldehyde (PFA) at 4°C for 24 h. Subsequently, the precipitate was dehydrated with 20 and 30% sucrose solutions. The surface water was gently absorbed with filter paper, liver samples were embedded with optimal cutting temperature (OCT), frozen, and sectioned after the OCT complex became white and hard. It was then soaked in 85 or 100% propylene glycol for 6 min. Next, the sections were incubated for 2 h at room temperature with freshly prepared 0.5% Oil Red O dye solution. The background color was removed using 100% propylene glycol for 30 s. The dye solution was gently rinsed with PBS. The sections were re-stained with hematoxylin dye solution for 10 s, rinsed with water, and sealed with gelatin. Finally, lipid droplets (stained red) were observed under a microscope and imaged.

Western blotting

For western blotting, total proteins in liver tissues and cells were extracted by grinding the tissues in radioimmunoprecipitation assay buffer. Protein concentration was detected using a BCA kit and then adjusted to equal levels in all samples. Protein loading buffer was added (total protein:loading buffer = 4:1) and samples were heated at 95°C for 5 min, then cooled on ice. Samples were then separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and then transferred to nitrocellulose filter membranes. The membranes were blocked with 5% skim milk. Proteins were detected using specific primary anti-β-actin (1:5000, Enogene, Nanjing, China), anti-CD86 (1:1000, Bioss, Beijing, China), anti-TRβ (1:1000, Affinity, Jiangsu, China), anti-TSHR (1:1000, Affinity, Jiangsu, China), and anti-SPP1 (1:1000, Proteintech, Wuhan, China) antibodies. Protein bands were visualized using a Super ECL kit (UElandy, Suzhou, China) and analyzed using ImageJ software.

Statistical analyses

The IBM SPSS Statistics ver. 24.0 (IBM). Continuous measurements, such as the mean and standard deviation (SD), were used if the data were normally distributed; however, if the data were not normally distributed, the median inter-quartile range (IQR) was used. Categorical variables were described as

frequencies and percentages (%). Independent tests, including the t-test, Chi-squared test, or Mann–Whitney U test, were used to compare the two patient groups. Logistic regression analysis was used to calculate odds ratios (ORs) and 95% confidence intervals (CIs) for the risk of NAFLD, while adjusting for potential confounding variables. Spearman's correlation analysis was used to describe the relationship between thyroid index and BMI. Statistical significance was set at $p < 0.05$.

Results

Demographic and metabolic characteristics of study subjects

The data of 166 patients with T2DM (71.6% male and 51.2% NAFLD) were evaluated. The mean age of the study subjects was 55.10 ± 12.89 years, ranging from 19–84 years. The duration of diabetes ranged from 0 to 35 years. Patients in the NAFLD group had significantly higher BMI and TSH levels than those in the non-NAFLD group (all $p < 0.05$). There were no significant differences in sex, age, duration of diabetes, FBG, HbA1c, Fins, FCP, TG, TC, LDL-c, HDL-c, ALT, AST, Cr, FT3, or FT4 between the two groups (all $p > 0.05$) (Table 1).

Independent risk factors associated with the incidence of NAFLD

A multivariate logistic regression model was used to analyze the risk factors for NAFLD. After adjusting for all factors with a p-value less than 0.1 in the univariate analysis, BMI, TSH, and FT3 were identified as significant independent risk factors for NAFLD. The ORs were 1.218, 1.694, and 2.259, respectively (all $p < 0.05$) (Figure 1).

Correlation and subgroup analysis

As FT3, TSH level, and BMI were correlated with NAFLD outcomes, the relationships between them were assessed. There was no significant relationship between BMI and TSH levels in NAFLD and Non-NAFLD group ($r = -0.003$ and -0.048, respectively). A weak negative correlation between BMI and FT3 was found in the NAFLD patients ($r = -0.472$, $p < 0.001$), as shown in Figure 2A. Further analysis with BMI stratification was conducted to compare the serum concentrations of FT3 and TSH in the two groups. Both FT3 and TSH levels were significantly different between individuals with and without NAFLD in the obesity subgroup; however, there was no significant difference in the overweight group (Figure 2B).

TABLE 1 Demographic and metabolism characterization of study subjects.

Characteristic	Non-NAFLD	NAFLD	p
n	81	85	
Gender, n (%)			0.402
Male	61 (36.7%)	58 (34.9%)	
Female	20 (12%)	27 (16.3%)	
Age (year)	55.98 ± 12.32	54.26 ± 13.66	0.397
BMI (Kg/m2)	26 (25.04, 27.68)	27.73 (25.78, 30.53)	< 0.001
Duration of diabetes (year)	7 (3, 12)	7 (1, 12)	0.504
FPG (mmol/L)	7.53 (6.38, 10.11)	8.4 (6.77, 10.16)	0.410
Fins (pmol/L)	65.63 (47.7, 80.47)	73.48 (47.67, 128.95)	0.358
F-CP (nmol/L)	0.42 (0.25, 0.62)	0.5 (0.36, 0.73)	0.142
HbA1C (%)	7.7 (6.6, 9.1)	8.5 (7.3, 10)	0.062
TG (mmol/L)	1.92 (1.43, 3.08)	1.97 (1.48, 3.02)	0.888
TC (mmol/L)	4.55 (3.95, 5.21)	4.78 (3.82, 5.3)	0.803
LDL (mmol/L)	2.51 ± 0.83	2.42 ± 0.78	0.475
HDL (mmol/L)	0.99 (0.84, 1.08)	0.96 (0.83, 1.05)	0.584
ALT (IU/L)	23 (17.25, 34)	24 (16, 45)	0.582
AST (IU/L)	22 (18, 27.75)	21 (16.75, 34)	0.700
Cr (mmol/L)	83 (73, 93)	80.5 (69.75, 89.5)	0.476
FT3 (pmmol/L)	5 (4.71, 5.35)	5.21 (4.78, 5.51)	0.071
FT4 (pmmol/L)	12.46 (11.38, 14.05)	12.58 (11.24, 13.69)	0.369
TSH (mIU/L)	1.78 (1.02, 2.57)	1.94 (1.58, 2.84)	0.034
25-OHD (ng/ml)	19.23 ± 7.06	18.41 ± 6.91	0.502

Data are shown as n (%) and as either mean ± SD or median (interquartile range, IQR).

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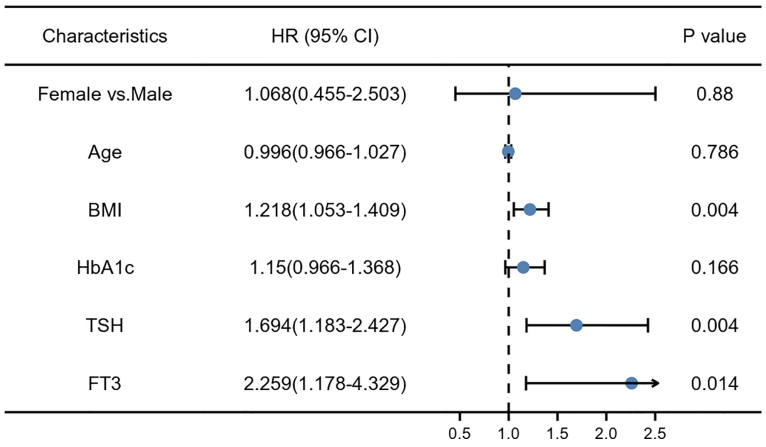

FIGURE 1
The multivariate logistic risk regression model: the risk factors for non-alcoholic fatty liver disease (NAFLD).

Collectively, these findings indicate that the combination of NAFLD with obesity may have a TH resistance-like manifestation.

Visualization of thyroid hormone signaling pathway in GSE48452

Considering its important metabolic role, the liver may be the main cause of thyroid hormone resistance. To identify thyroid hormone signaling pathway changes in obese patients with NAFLD, we downloaded relevant expression profiles from the GSE48452 dataset. The Pathview library was used to visualize the thyroid hormone signaling pathway (18). Figure 3 shows several key molecular pathways affected by NAFLD-induced transcriptomic changes in the liver samples from obese patients. The results show the expression of *THRB*, which encodes TRβ, was significantly decreased in the NAFLD group, which may explain the TH resistance-like manifestation in the clinical findings.

Expression analysis of the DEGs and Immune cell infiltration in GSE48452

To identify DEGs linked to NAFLD incidence, we downloaded relevant expression profiles from the GSE48452 dataset. Five DEGs involved in the development of NAFLD were identified by limma package (adjusted $p < 0.05$, $|logFC| \geq 1$), as shown in the volcano plot (Figure 4A). Box plots show the expression patterns of the five DEGs between NAFLD and healthy obese individuals in the GSE48452 dataset (Figure 4B). The expression levels of *SPP1*, fatty acid binding protein 4 (*FABP4*), and ribosomal protein S4 Y-linked 1 (*RPS4Y1*) were significantly higher, whereas the expression levels of pregnancy zone protein (*PZP*) and villin 1 (*VIL1*) were significantly decreased in patients with NAFLD. Inflammation plays a vital role in the pathogenesis of NAFLD. Thus, understanding immune cell infiltration may provide a more comprehensive view of the efficacy of NAFLD therapies. The CIBERSORT algorithm showed increased M0 and M1, and decreased M2 macrophage infiltration in the NAFLD group compared to that in the healthy obese group (Figure 4C). These results indicate that abnormal macrophage polarization may play a vital role in fat deposition in hepatocytes.

Validation of related clinical phenotypes and gene expression in NAFLD mice model

An animal model of obesity was established by feeding male C57BL/6 mice with an HFD, and these mice were used to validate the related gene expressions and NAFLD phenotype found in the clinical data. As shown in Figures 5A, B, HFD feeding induced a significant increase in the body mass of the mice and exhibited a uniformly pale red fatty liver, while the NC group remained lean and had a relatively normal liver. Correspondingly, HFD feeding to mice showed increased fat accumulation in the hepatic intracellular vacuoles by Oil Red O staining. These mice had significantly higher serum TSH levels, which is in agreement with our clinical results, suggesting a TH resistance-like manifestation. However, there was no significant difference in the serum SPP1 concentration between the two groups (Figure 5C). To investigate SPP1, TSHR, and TRβ expressions in the liver, we analyzed IHC-stained liver sections (Figure 5D). The intensity of SPP1 immunostaining was significantly increased in HFD-fed mice compared to that in NC, while that of TRβ was reduced. The expression of TSHR did

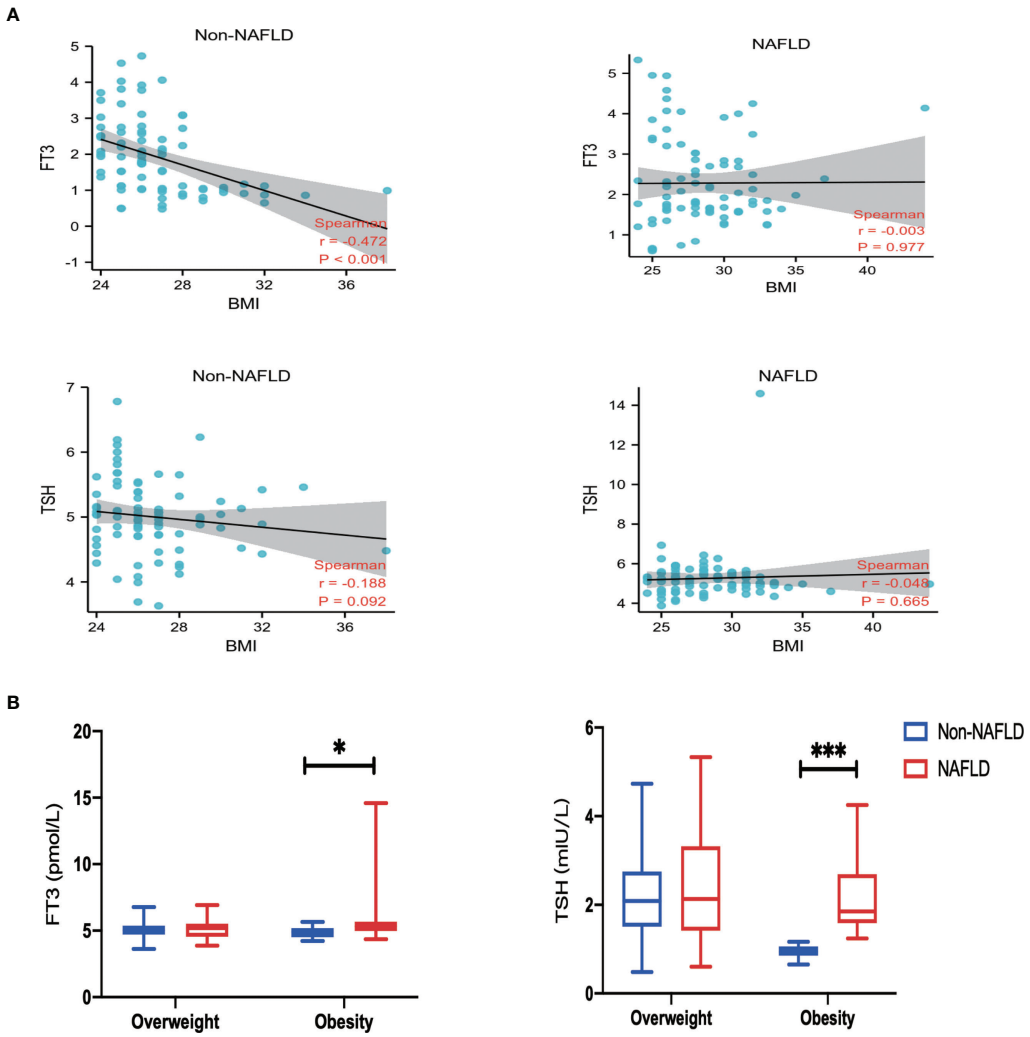

FIGURE 2
Correlation and Subgroup analysis. **(A)** Spearman correlation analysis of body mass index (BMI), free triiodothyronine (FT3) and thyroid-stimulating hormone (TSH). **(B)** Comparison of FT3 and TSH among groups classified according to BMI. Values are represented as mean ± SD. *p < 0.05, ***p < 0.001 vs. the Non-NAFLD group.

not significantly differ between the two groups. The expression levels of SPP1, TSHR, and TRβ were validated by western blotting (Figure 5E). Collectively, these findings further support that obesity was accompanied by NAFLD and TH resistance and that SPP1 and TRβ may be involved in this pathological process.

SPP1 Induced TRβ downregulation and aggravated lipid accumulation in HepG2 Cells

To describe the relationship between SPP1, TRβ, and lipid deposition in hepatic cells, we examined HepG2 cells treated with 50 μM concentrations of PA and/or 20 ng/ml concentration of SPP1 for 48h. Oil Red O staining revealed a significant increase in lipid deposition in SPP1-stimulated cells as compared to that in the PA group (Figure 6A). In addition, the expression of TRβ, but not TSHR was significantly decreased after SPP1 administration (Figure 6B), suggesting that TRβ may be involved in SPP1-induced lipid deposition in the liver. To test this hypothesis, we modified TRβ levels in HepG2 cells by transfecting them with a plasmid overexpressing *THRB*, which was validated by western blotting (Figure 6C). Following *THRB* overexpression, there was a reversal in the phenotype of lipid accumulation following SPP1 treatment *in vitro* (Figure 6D). Collectively, these findings suggest that SPP1 aggravates lipid deposition in hepatic cells by downregulating *THRB* expression.

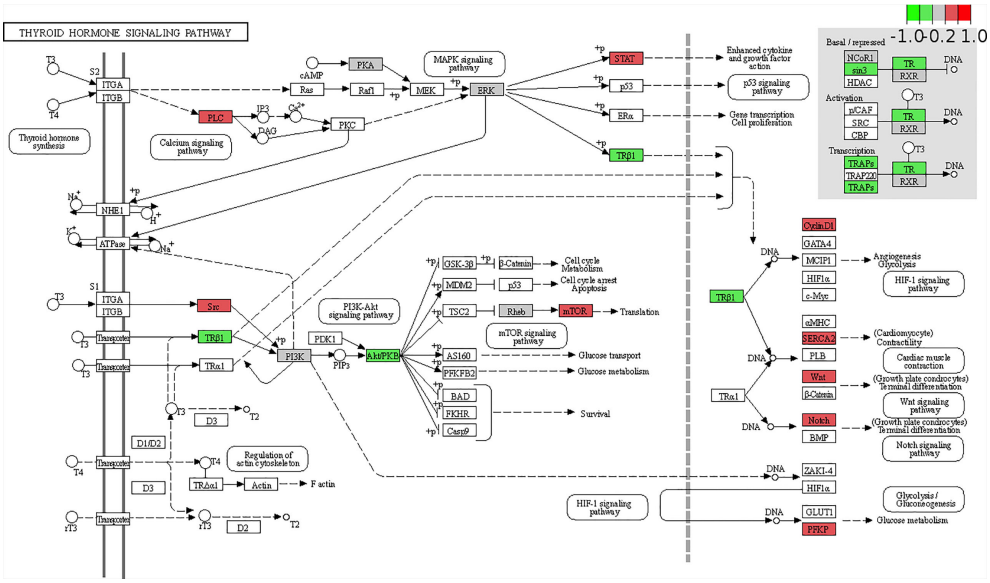

FIGURE 3
Expression changes of target genes of thyroid hormone signaling pathway in patients with non-alcoholic fatty liver disease (NAFLD) are mapped by colors. The color depth positively correlated with the degree value. Red represents increased while green represents decreased gene expression in NAFLD group compared with that in the health obese group.

TSH Acts to promote secretion of SPP1 in M1 macrophage cells

Previous studies have shown that M1 macrophages play a key role in the process of metabolic inflammation underlying NAFLD development. To explore whether liver injury in obese mice is dependent on M1 macrophages, the distribution of M1 macrophages in the liver, indicated by the surface marker CD86, was evaluated by IHC and western blotting (Figures 7A, B). Both the intensity of immunostaining and expression of CD86 were significantly increased in the obese group, indicating that M1 macrophage polarization may be involved in intrahepatic inflammation. We used THP-1 cells to construct an M1 macrophage model *in vitro*. We then analyzed THP-1 cells treated with various concentrations of PA or TSH, and the assay revealed a significant dose-dependent increase in SPP1 expression or secretion in TSH-stimulated cells compared to that in control cells (p for trend < 0.05; $p < 0.05$ for 5 mIU or above; Figures 7D, E), while there were no significant changes with different PA administration (Figure 7C). Collectively, these findings suggest that increased TSH levels can further lead to the secretion of SPP1, thus maintaining and amplifying the pathological process of NAFLD.

Discussion

In our clinical study, after adjusting for overweight/obese BMI and diabetes, the two most common causes of NAFLD, a manifestation of TH resistance, was found. This phenomenon was more obvious in obese patients, as shown in the subgroup analysis, suggesting that the intrahepatic damage of the TH pathway under obesity may play an important role in the occurrence of NAFLD. Bioinformatics analysis indicated that *THRB* was significantly downregulated in obese patients with NAFLD, and this phenotypic change was validated in an animal model with obesity. Further mechanistic studies demonstrated that HFD and PA induced increased secretion of SPP1 by M1 macrophages, which may downregulated TRβ in hepatocytes in a paracrine manner. This increased lipid deposition in the liver and a compensatory increase of TSH in serum. Increased TSH may lead to more secretion of SPP1, thus amplifying this pathological process. To the best of our knowledge, this is the first study to investigate the positive feedback crosstalk between the thyroid and the liver in NAFLD.

NAFLD is a serious worldwide health epidemic, which is causing a growing burden on public health (19). It is now well-established that NAFLD is accompanied by metabolic dysfunction, and increased BMI leading to obesity, and higher prevalence of obesity-related disorders, such as T2DM, in > 90% of patients (20). The liver and thyroid are intimately linked, with TH playing important roles in lipogenesis, beta-oxidation (fatty acid oxidation), and cholesterol metabolism (8, 21). Subclinical hypothyroidism and normal hypothyroidism have been found to be independent predictors of nonalcoholic steatohepatitis (NASH) (10, 22, 23). In this study, our multivariate analysis demonstrated that BMI, TSH, and FT3 were significant independent risk factors for NAFLD, and further analysis

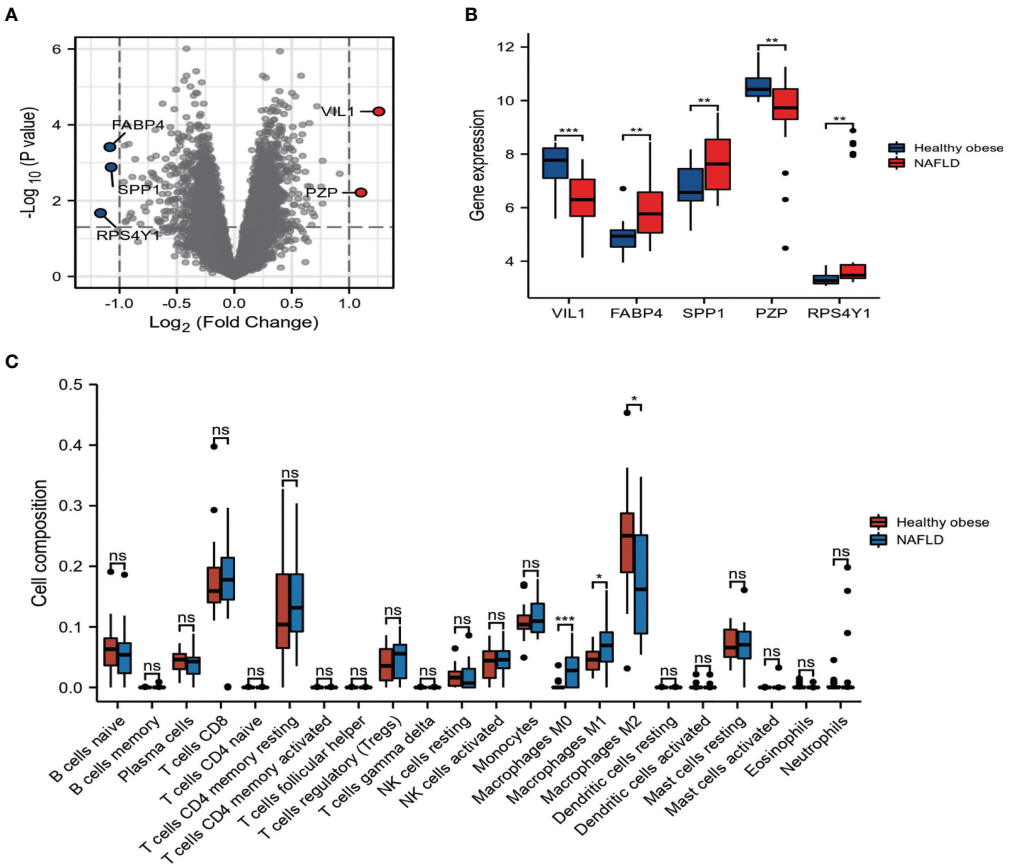

FIGURE 4
Expression and correlation analysis of the differentially expressed genes (DEGs). **(A)** DEGs in non-alcoholic fatty liver disease (NAFLD) and healthy obese samples in Gene Expression Omnibus (GEO) dataset. **(B)** The boxplot of five DEGs. **(C)** Bar plot showing the difference between 22 infiltrated immune cells in the NAFLD and healthy obese groups with the CIBERSORT algorithm. Values are the mean ± SD. ns, no significance, *p < 0.05, **p < 0.01, ***p < 0.001 vs. the healthy obese group.

identified that BMI stratification showed that both FT3 and TSH had a significant change between individuals with NAFLD and the healthy liver obesity subgroup, indicating that NAFLD with obesity might have a thyroid hormone resistance-like manifestation. Chaves et al. provided evidence that impairments in liver TRβ signaling due to mutations in the *THRB* gene can lead to hepatic steatosis, which indicates the influence of TH on lipid metabolism in the liver (24). Similarly, TRβ impairment was also supported by subsequent bioinformatics and basic research in our study, which emphasize the important role of thyroid function in NAFLD in addition to the metabolic contributions of diabetes and obesity. Considering that the TH signaling pathway plays an important role in energy metabolism, researchers have been trying to pharmacologically target the FT3/THR axis for the past few decades. TH metabolites (25), TRβ agonists (26), and liver-specific analogs (27) have been studied as potential therapeutics for treating both serum dyslipidemia and as potential therapies for NAFLD. Resmetirom (MGL-3196) and Hep-Direct prodrug VK2809 (MB07811) probably representing two of the most promising lipid lowering agents, currently under phase 2-3 clinical trials. More recently the application of a comprehensive panel of ADME-Toxicity assays enabled the selection of novel thyromimetic IS25 and its prodrug TG68, as very powerful lipid lowering agents both (26, 28). Despite encouraging results in the treatment of obesity, dyslipidemia, and liver cancer, serious adverse reactions have limited their use in clinical trials. Determining the mechanism of TRβ damage will offer a better treatment option for NAFLD.

SPP1 is upregulated in obesity and several models of liver injury (29, 30).Studies have demonstrated a pivotal role of SPP1 in obesity-driven nutrition-dependent diseases, including NAFLD, suggesting SPP1 as a treatment target (13, 31). SPP1 animals showed enhanced hepatic lipid accumulation and aggravated NASH, as also increased hepatocellular apoptosis and accelerated fibrosis, which might be driven by enhanced hepatic fatty acid influx through CD36 overexpression. Lack of osteopontin lowered systemic inflammation, prevented HCC

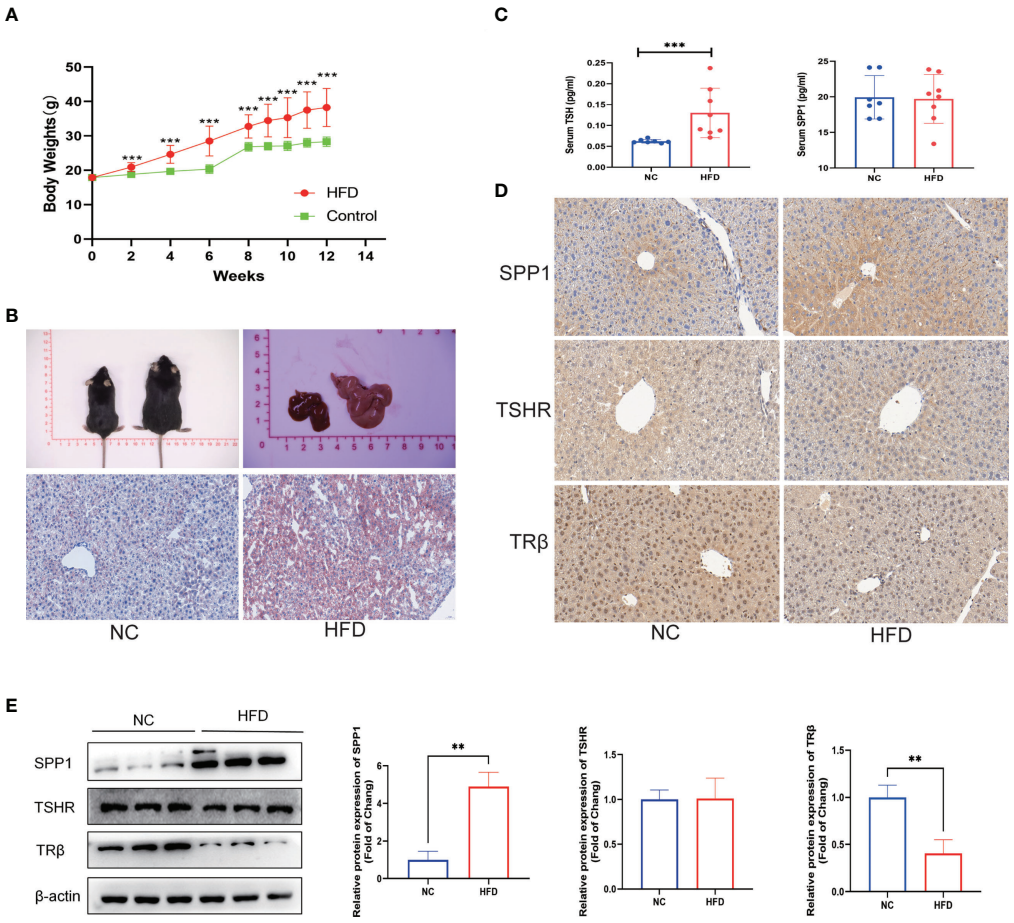

FIGURE 5
Validation of related clinical phenotypes and gene expressions in a non-alcoholic fatty liver disease (NAFLD) mice model. **(A)** Body weights of high-fat diet (HFD) and negative control (NC) mice over 12 weeks. **(B)** Representative images of the gross morphology of the liver and liver tissue sections stained with Oil Red O. **(C)** Enzyme-linked immunosprobent assay (ELISA) of serum secreted phosphoprotein 1 (SPP1) and thyroid-stimulating hormone (TSH). **(D)** Immunohistochemistry (IHC) staining of SPP1, TSH receptor (TSHR), and TRβ in mice livers. **(E)** Quantification of the average band densities calculated from western blots of SPP1, TSHR, and TRβ in the liver tissues from HFD and NC mice. Values are the mean ± SD. n = 8 mice in each the HFD and NC groups. **p < 0.01, ***p < 0.001 vs. NC.

progression to less differentiated tumors and improved overall survival (13). In this study, we did not identify a statistically significant increase in serum SPP1 concentration after 12 weeks of HFD intervention, which may be because the challenge was too brief or too weak to cause a change. However, the HFD mouse model showed a significant increase in SPP1 expression in the liver when compared to that in the control group, which was consistent with the bioinformatics analysis in the GSE48452 dataset. SPP1 is an inflammatory cytokine highly upregulated in adipose tissue of obese and has repeatedly been shown to be functionally involved in adipose-tissue inflammation and metabolic sequelae (32). Although SPP1 promotes obesity and regulates lipid synthesis, both of which drive fat deposition in hepatocytes. However, further mechanistic studies are needed. Our findings show that downregulation of TRβ induced by SPP1 administration aggravated PA-induced lipid deposition in the liver, which was rescued by TRβ overexpression, indicating that SPP1-TRβ is involved in the pathological processes of NAFLD. TSH is produced by the hypothalamus and regulates thyroid hormone production (33). Studies have provide evidence that impairments in intrahepatic TRb signaling due to mutations of the THRB gene can lead to hepatic steatosis, which emphasizes the influence of TH in the liver metabolism of lipids (24, 34, 35). Likewise, our study showed a decrease in TRβ expression, but not TSHR, and a compensatory increase in TSH in both clinical patients and HFD mice with NAFLD. An interesting study demonstrated that liver steatosis and triglyceride content were significantly increased in $TSHR^{+/+}$ HFD-fed mice compared to those in $TSHR^{-/-}$ mice, indicating an essential role for TSH in the pathogenesis of NAFLD (36). However, this study did not involve hepatocyte-specific TSHR knockout. Combined with our current results, administration of different concentrations of

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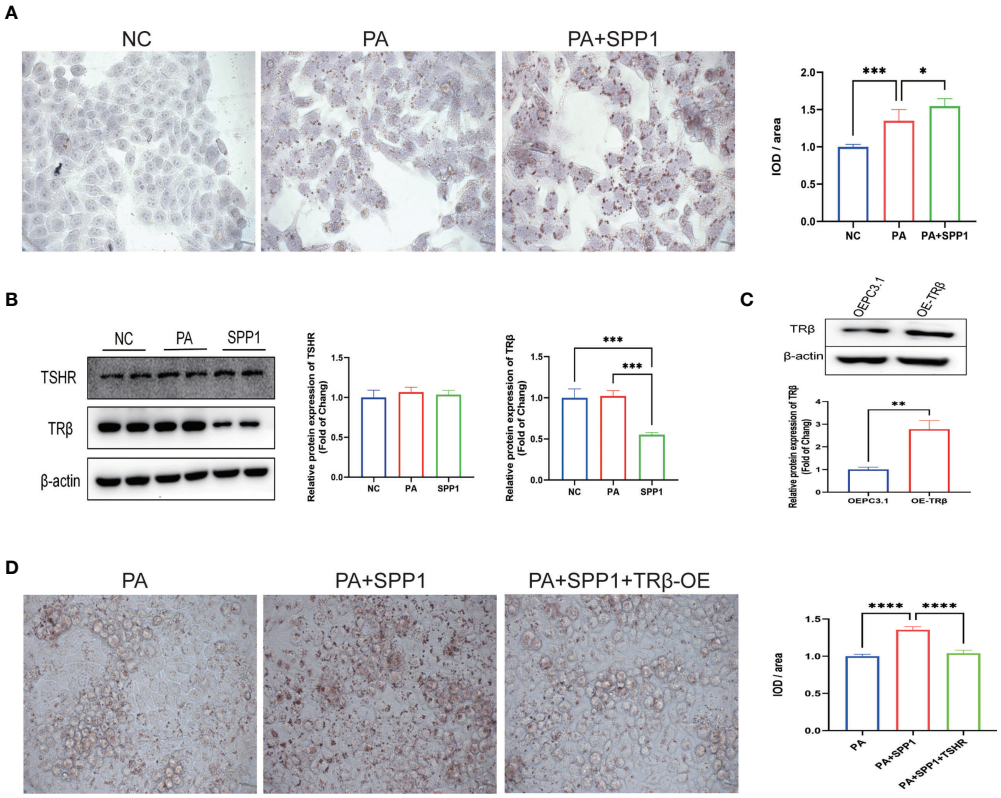

FIGURE 6
Secreted phosphoprotein 1 (SPP1) induced *THRB* downregulation aggravated lipid accumulation in HepG2 cells. **(A)** Lipid deposition in each group detected by Oil Red O staining. **(B)** Quantification of the average band densities calculated from western blots, the protein levels of TSH receptor (TSHR) and TRβ in these groups. **(C)** *THRB* was overexpressed in HepG2 cells and this was validated by western bloting. **(D)** Lipid deposition in those groups detected by Oil Red O staining. Values are the mean ± SD. n = 3 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.001.

TSH did not lead to SPP1 expression and lipid deposition in HepG2 cells, demonstrating that other cell types in the liver may be involved in this pathologic change.

While NAFLD can be driven by an imbalance between energy intake and expenditure (*e.g.*, diet and lack of exercise), inflammation is increasingly recognized as an important contributing factor (37). Such low-grade chronic inflammation induced by a high-fat diet or obesity propagates the pathogenesis of NAFLD-associated sequelae, comprising a spectrum from simple steatosis to NASH to end-stage cirrhosis and the risk of HCC, and has become a significant public health problem (38). Although certain extrahepatic sites, such as adipose tissue, have evolved as major sources of inflammatory mediators in obesity-related disorders, evidence is accumulating that intrahepatic inflammation might also be critically involved in the pathogenesis of NAFLD (39, 40). Although multiple cell populations in the liver contribute to various inflammatory pathways, hepatic macrophages are considered key contributors to the process of metabolic inflammation that underlies the development of NAFLD (41). The functional heterogeneity of monocyte-derived macrophages was initially typified by their polarization into M1 and M2 phenotypes, which were thought to represent two ends of a spectrum in which M1 macrophages are proinflammatory and M2 macrophages are regenerative (42). Weisberg et al. showed that the number of macrophages increased in the adipose tissue of mice and obese people and that this percentage was positively correlated with their obesity level (43). Macrophages isolated from white adipose tissue of lean animals showed characteristics of M2 macrophages (44). In addition, obesity-induced increased gene expression of characteristic molecules of M1 macrophages, such as those encoding TNF and NOS2, suggests that diet-induced obesity can lead to a shift in macrophage polarization from M2 to M1 (45). In our HFD-induced obese mouse model, similar results for M1 polarization were found, and further bioinformatics analysis showed that the differences in macrophage infiltration persisted even after correcting for obesity covariates, suggesting that M1 macrophage polarization may be involved in intrahepatic inflammation. Our results showed increased SPP1 expression and secretion as a consequence of M1 polarization, which may explain how M1 macrophages regulate TRβ expression in hepatocytes. Moreover, the increased TSH levels induced by

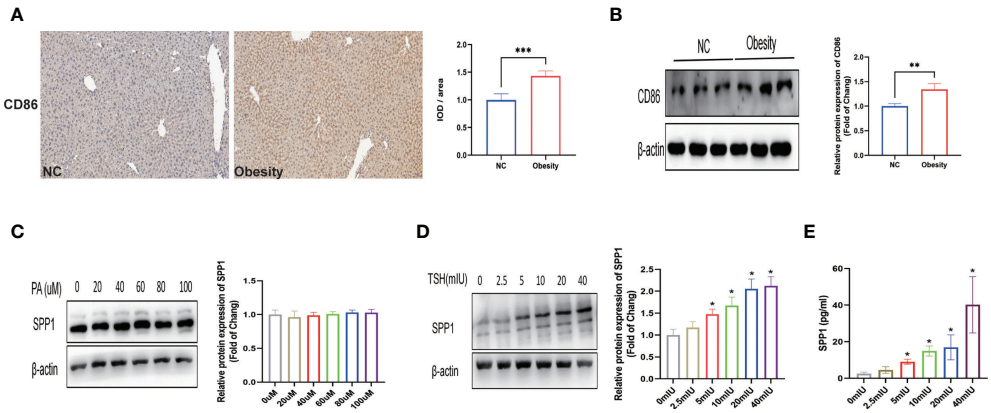

FIGURE 7
TSH acts to promote secretion of secreted phosphoprotein 1 (SPP1) in M1 macrophage cells. **(A)** Immunostaining of CD86 in mice livers. **(B)** Quantification of the average band densities calculated from western blots of CD86 in liver tissues. Protein levels of SPP1 stimulated with palmitic acid (PA) **(C)** or thyroid-stimulating hormone (TSH) **(D)** in M1 macrophage cells. **(E)** SPP1 in the supernatant after stimulation with TSH by ELISA. *p < 0.05, **p < 0.01, ***p < 0.001 vs. NC or 0 mIU TSH group.

TRβ damage can further lead to the secretion of SPP1, thus maintaining and amplifying this pathological process.

To the best of our knowledge, this is the first study to investigate the positive feedback crosstalk between the thyroid and the liver in NAFLD. The data presented in this study shed new light on NAFLD therapy. Our study had some limitations. First, there was a lack of liver-specific *TRHB* knockout or overexpression models to test the manifestation of thyroid hormone resistance in the NAFLD model. Second, we demonstrate a shift in macrophage polarization from M2 to M1 in NAFLD by bioinformatics analysis and in obese mice, but we did not explore the specific mechanism underlying this regulatory process. Hence, further investigations are needed to improve our understanding of the relationship between thyroid function and the liver, and whether M1 mediates SPP1-mediated lipid deposition in hepatocytes.

Conclusions

This study establishes that SPP1 secretion is induced by M1 macrophage polarization, which may downregulates TRβ in hepatocytes in a paracrine manner. However, lipid deposition is aggravated in the liver, which causes a compensatory increase of TSH in serum. The increased TSH levels can further lead to SPP1 secretion by M1 macrophages. The positive feedback crosstalk between the thyroid and liver may play an important role in maintaining and amplifying the pathological process of NAFLD.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the ethics committee of the First Affiliated Hospital of USTC, Division of Life Science and Medicine, University of Science and Technology of China. The patients/participants provided their written informed consent to participate in this study.

Author contribution

HB performed the data acquisition and drafted the work. The main basic research was completed by WW. YS and HB interpreted the patient data. YS substantively revised it. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Metabolic syndrome and aberrant immune responses to viral infection and vaccination: Insights from small animal models

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This review outlines the propensity for metabolic syndrome (MetS) to induce elevated disease severity, higher mortality rates post-infection, and poor vaccination outcomes for viral pathogens. MetS is a cluster of conditions including high blood glucose, an increase in circulating low-density lipoproteins and triglycerides, abdominal obesity, and elevated blood pressure which often overlap in their occurrence. MetS diagnoses are on the rise, as reported cases have increased by greater than 35% since 1988, resulting in one-third of United States adults currently diagnosed as MetS patients. In the aftermath of the 2009 H1N1 pandemic, a link between MetS and disease severity was established. Since then, numerous studies have been conducted to illuminate the impact of MetS on enhancing virally induced morbidity and dysregulation of the host immune response. These correlative studies have emphasized the need for elucidating the mechanisms by which these alterations occur, and animal studies conducted as early as the 1940s have linked the conditions associated with MetS with enhanced viral disease severity and poor vaccine outcomes. In this review, we provide an overview of the importance of considering overall metabolic health in terms of cholesterolemia, glycemia, triglyceridemia, insulin and other metabolic molecules, along with blood pressure levels and obesity when studying the impact of metabolism-related malignancies on immune function. We highlight the novel insights that small animal models have provided for MetS-associated immune dysfunction following viral infection. Such animal models of aberrant metabolism have paved the way for our current understanding of MetS and its

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impact on viral disease severity, dysregulated immune responses to viral pathogens, poor vaccination outcomes, and contributions to the emergence of viral variants.

KEYWORDS

metabolic syndrome, obesity, type 2 diabetes, hypertension, dyslipidemia, vaccination, vaccine efficacy, viral infection

Introduction

First described in 1977 (1), metabolic syndrome (MetS) is diagnosed in an individual whose metabolism is disrupted, leading to an imbalance in the processing of food for energy, the synthesis of protein, lipids, and amino acids, as well as the elimination of metabolic waste. Alarmingly, MetS diagnoses have increased by 35% since 1988, culminating in one-third of American adults diagnosed with MetS (2). The diagnostic criteria for MetS are when an individual concurrently experiences three or more of the following conditions: high blood glucose, high levels of circulating low-density lipoprotein, high levels of circulating triglycerides, abdominal obesity, and high blood pressure (2–4) (Figure 1). As such, it is very common for these subcomponents of MetS to occur concurrently within a patient. MetS patients also often experience insulin resistance

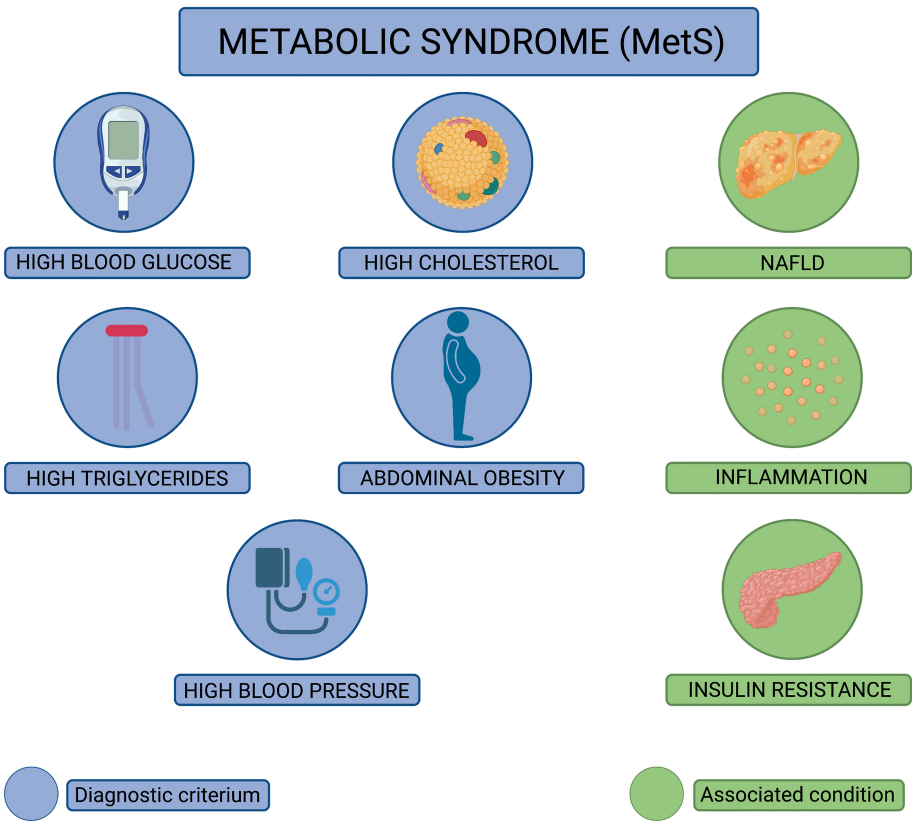

FIGURE 1
Diagnostic criteria for metabolic syndrome (MetS). Pictorial representation of the diagnostic criteria used to diagnose metabolic syndrome (MetS). MetS is diagnosed when an individual displays at least three of the following pathophysiological conditions: high blood glucose, high cholesterol, high triglycerides, abdominal obesity, and high blood pressure (conditions depicted in blue). In addition to these conditions, MetS patients often experience nonalcoholic fatty liver disease (NAFLD), chronic inflammation, and insulin resistance (conditions depicted in green).

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and nonalcoholic fatty liver disease, with evidence implicating chronic inflammation as the link between the MetS diagnostic criteria (5). Underlying causes of MetS are multifactorial, including being overweight or obese, resistant to insulin, having a sedentary lifestyle, predisposing genetic factors, and advanced age (2). While it has been well established that MetS enhances the risk for developing life-threatening conditions including heart disease, type 2 diabetes, stroke, and is a risk factor for sudden cardiac death (3, 4, 6), the realization that individuals with MetS experience more severe disease following viral infections and reduced protection from vaccination have only recently been appreciated.

In this review, we focus on the individual metabolic perturbances encompassed by MetS and highlight the animal studies which identify these conditions as predictors of elevated viral disease severity (7, 8), higher mortality rates following infection (9–11), and poor vaccination outcomes (12–15). Independently, each of the conditions associated with MetS is a risk factor for severe pathology; however, when these conditions present together, as they do in patients with MetS, the chance of developing serious physiological complications significantly increases (2, 6). While retrospective human cohort studies have laid much of the groundwork for linking metabolic perturbances to impaired viral immunity, small animal models serve as critical tools for understanding this phenomenon and uncovering the mechanisms driving dysregulated metabolism-associated immune dysfunction in response to viral pathogens. Throughout this review, we comment on works that utilized small animal models to explore the impact of high cholesterol, triglycerides, glucose, and hypertension on host immune responses to viral pathogens. Given that the different comorbidities associated with MetS often overlap in the types of immune dysfunction they induce, we have combined these known defects into one summarizing graphic, depicted in Figure 2. In addition, we summarize how such metabolic perturbances have been shown to enhance viral disease severity and influence the emergence of virulent viral variants.

Impact of high cholesterol and triglyceride levels on viral immunity

As two diagnostic criteria for MetS diagnosis, elevated cholesterol and triglyceride levels have long been shown to

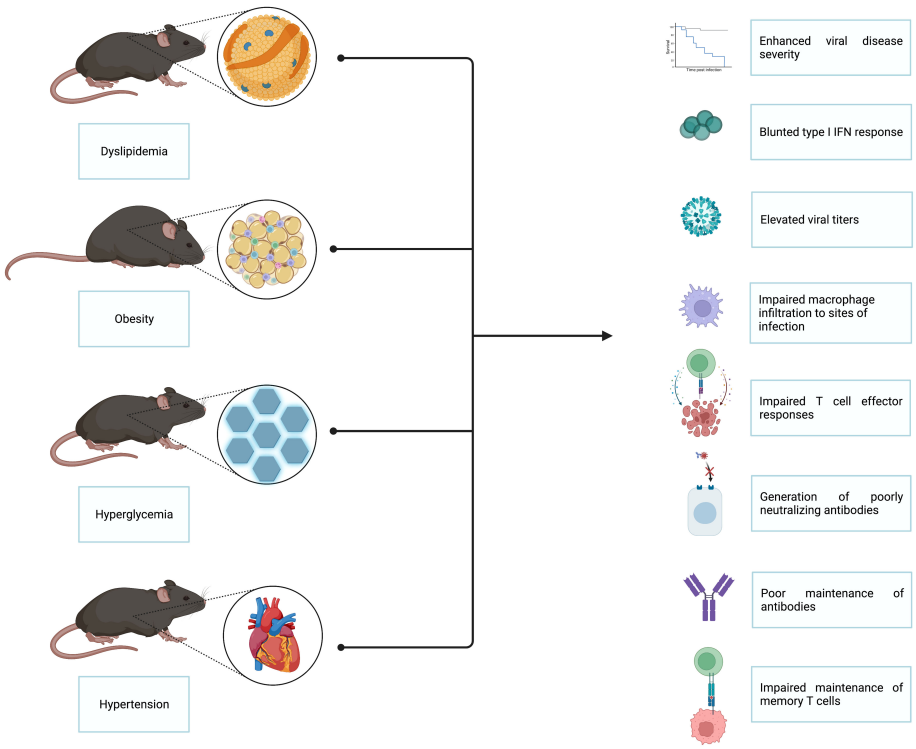

FIGURE 2
Insights gained from small animal models of the impact of MetS on viral immunity. Several small animal models have been utilized to interrogate the impact of MetS on viral immunity. The main animal models employed thus far are those that model dyslipidemia, obesity, hyperglycemia, and hypertension. Studies done utilizing these animal models have revealed that MetS-associated conditions lead to enhanced viral disease severity, blunted type I interferon responses, elevated viral titers, impaired macrophage infiltration to sites of infection, impaired T cell effector responses, generation of poorly neutralizing antibodies, poor antibody maintenance, and impaired maintenance of memory T cells.

influence the susceptibility to viral infection and disease severity. Cholesterol is a sterol synthesized by all animal cells, essential for providing structural integrity to cells and as a building component for vitamins and hormones (16). Cholesterol travels through the body inside of lipoproteins which are comprised of fat and protein. There are two major types of cholesterol-carrying lipoproteins: high-density lipoprotein (HDL) and low-density lipoprotein (LDL). LDL, often known as the bad cholesterol, contributes to fat deposition within arteries (17). Conversely, HDL transports cholesterol away from the arteries to the liver where it can be metabolized and excreted from the body (18). Triglycerides are the most commonly found fat in the human body and are important for storing excess energy obtained through diet (18). When cholesterol and/or triglyceride levels fall outside of the normal range, the resulting phenomenon is referred to as dyslipidemia, which is associated with all-cause mortality and enhanced risk for cardiovascular disease (19). Due to the reliance of viruses on lipids to replicate and produce viral progeny, whether host dyslipidemia impacts viral infection outcomes is an exciting avenue for discussion.

It is well understood that viruses hijack host lipid metabolism by manipulating gene expression to sustain their lifecycles and produce new progeny virions (reviewed in (20)). Studies done with viruses such as influenza A virus (IAV), herpes viruses, and hepatitis viruses have eloquently highlighted the significant alterations in lipid metabolism that occur within hosts following infection (21–26). Remarkably, these patterns of altered metabolism can be sustained even after viral clearance (27). Specifically, severe fever with thrombocytopenia syndrome virus (SFTSV) relies on host cell cholesterol, fatty acid, and triglyceride synthesis pathways to replicate and produce progeny; in fact, treating cells with inhibitors of these synthesis pathways before SFTSV infection significantly lowers the titer of infectious progeny post-infection (28). Previous work supports this idea as disruption of lipid rafts, lipid droplets, or diminished levels of circulating triglycerides can reduce the production of infectious rotavirus progeny (29–31) and interrupts hepatitis C (32, 33) and dengue virus replication (34, 35). These studies suggest that altering host lipid metabolism may be a mechanism to alter viral infection and reduce disease severity.

Multiple animal studies have supported clinical observations demonstrating the impact of high cholesterol on viral replication. Braunwald et al. employed a high cholesterol diet in a murine model of viral infection using A/J mice genetically modified not to be susceptible to mouse hepatitis virus type 3 (MHV3). The authors noted that following a hypercholesterolemic diet, these mice succumbed to MHV3 infection and had high MHV3 titers in their livers accompanied by necrotic hepatocytes and elevated serum transaminase levels (36), indicating intense virus-induced liver pathology in mice with high cholesterol levels. Delving deeper into the impact of dyslipidemia on susceptibility to viral infections, Loria et al. conducted a seminal study in which mice fed a diet rich in cholesterol were infected with coxsackievirus B. Following infection, the authors noted that mice with elevated cholesterol levels displayed enhanced morbidity to coxsackievirus B infection compared to mice with normal cholesterol levels (37). While these studies employed the use of high cholesterol diets to interrogate the impact of dyslipidemia on virus-induced morbidity, it is important to note that these diets were high in sucrose, making the results indicative of a positive correlation between unhealthy diet and enhanced severity of viral disease. Future studies using fine-tuned diets to induce hypercholesterolemia independent of heightened sucrose could bolster the reported relationship between hypercholesterolemia and enhanced viral disease severity.

Combining the *in vitro* studies highlighting the importance of lipid metabolism for viral replication with *in vivo* animal models which mimic the impact of high cholesterol on viral infection, multiple studies have focused on the mechanisms to explain how elevated cholesterol and triglycerides contribute to increased viral disease severity. Campbell et al. found a correlation between high cholesterol and increased mortality in mice following coxsackievirus B infection, in addition to elevated viral titers in the blood and liver. These authors also noted that mice fed a cholesterol-rich diet had a defect in the ability of monocytes and macrophages to infiltrate into infection sites compared to the migration abilities of phagocytes primed in mice with normal cholesterol levels (38), graphically depicted in Figure 3. Utilizing a similar approach where the effects of a cholesterol-rich diet were compared with those of a standard diet, high cholesterol feeding resulted in dyslipidemia prior to IAV infection (39). Louie et al. reported that these high cholesterol mice displayed exacerbated morbidity yet did not show higher viral loads compared to mice with normal cholesterol levels post-IAV infection (39). Transcriptomics from lungs of mice fed a high cholesterol diet revealed an upregulation in the expression of genes involved in cytokine production by CD4 T, CD8 T, and dendritic cells (39). Furthermore, morbidity was also correlated with the numbers of cytokine-producing lymphocytes and granulocytes (39). These results suggest that high cholesterol levels enhance morbidity by exaggerating cellular immune responses. These findings bolster the idea that supraphysiological levels of cholesterol can contribute to worsened disease development following viral infection, and that lowering cholesterol levels can reduce the severity of virally induced disease in the host.

Given the evidence that cholesterol and triglycerides are important for modulating viral infection and disease severity, research efforts to modify lipid metabolism in animal models to improve infection outcomes have begun. For example, Karlsson et al. noted that obese mice with high cholesterol levels treated with a statin following IAV infection showed protection from severe viral disease. Yet, treatment of wild-type mice with a statin did not protect against severe viral disease (40). Multiple studies have demonstrated that statin treatment and lowered cholesterol levels have anti-inflammatory effects which could improve disease

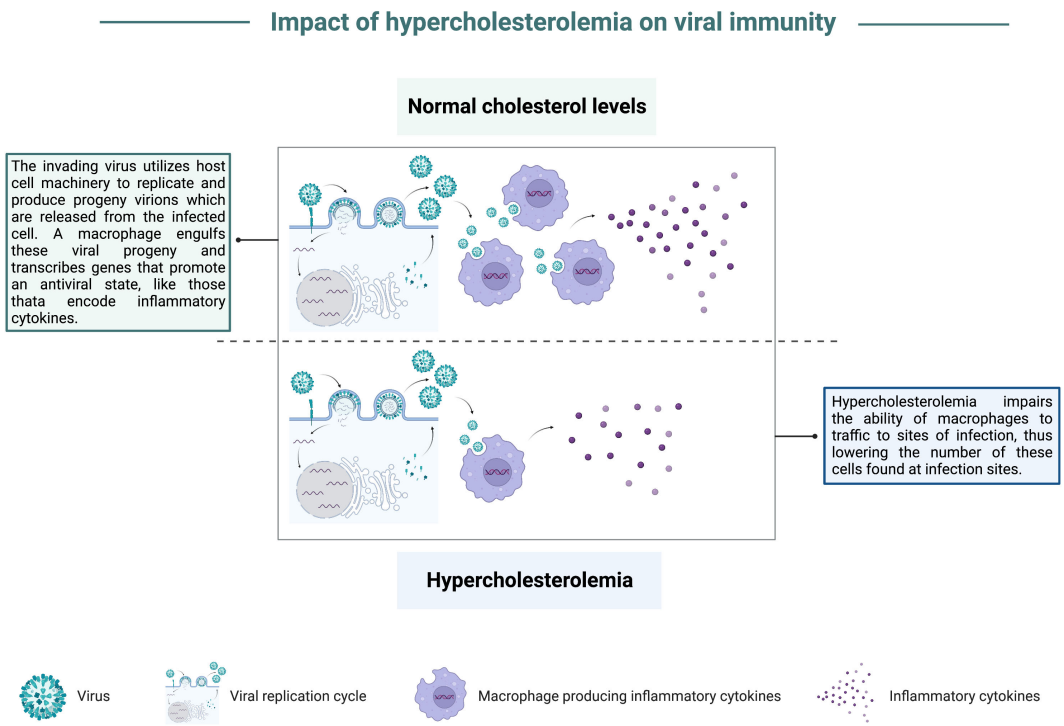

FIGURE 3
Impact of hypercholesterolemia on viral immunity. In a state of normal cholesterol levels, an invading virus hijacks host cell machinery to replicate and produce progeny virions that are released from the infected cell. A macrophage recruited to the infection site can engulf viral progeny and contribute to the anti-viral state by transcribing genes that stimulate the innate immune response, like pro-inflammatory cytokine-encoding genes. In a state of hypercholesterolemia, there is a defect in macrophage recruitment to infection sites. Consequently, there are fewer macrophages found at infection sites, blunting this arm of the immune response.

outcomes (reviewed in (41)). Further, other studies support a role for statins having a direct anti-viral effect based on *in vitro* findings that showed a significant reduction in Zika or dengue virus viral titers in cells treated with statins compared to controls (42, 43).

Based on findings from murine models of high cholesterol, it is unsurprising that human cohort analyses conducted during the SARS-CoV-2 pandemic have revealed that many COVID-19 patients requiring hospitalization had a history of low HDL and high triglyceride levels before infection, with more severe cases being correlated with lower HDL and higher triglyceride levels at the time of infection (44). Other data reported from SARS-CoV-2 infections revealed a link between a history of generalized dyslipidemia to severe cases of COVID-19 (45). Interestingly, Lee et al. noted that sterol regulatory element-binding protein 2 (SREBP-2)-induced inflammatory responses were elevated in COVID-19 intensive care unit patients (46). SREBP-2 is essential for cholesterol biosynthesis, suggesting that high cholesterol levels contributed to the cytokine storm and ensuing pulmonary damage in these COVID-19 patients. Further, these authors utilized a murine model to test whether inhibiting SREBP-2 impacted sepsis outcomes and demonstrated that blocking SREBP-2 activation helped suppress cytokine storm, pulmonary damage, and promoted high survival rates (46). The studies above provide a strong link between dyslipidemia and severe viral infection outcomes, although specific mechanisms driving this phenomenon have yet to be elucidated. However, these data suggest that heightened cholesterol levels could foster high levels of viral replication within the host, thus providing the potential for more severe disease. In turn, elevated viral titers could contribute to inflammation-mediated tissue pathogenesis and incite exaggerated cellular responses, which could lead to enhanced immune-mediated pathology.

Impact of obesity on viral immunity

Obesity, abnormal or excessive fat accumulation, is a diagnostic component of MetS. Obesity rates have tripled worldwide since 1975, with more than 4 million people dying yearly due to complications associated with this condition (47). Specifically, within the US, current statistical models project 50% of adults will have obesity by 2030 (48), and global obesity rates are projected to encompass 50% of adults by 2050 (49). In the 1950s and 1960, increased susceptibility to viral infection and

severe viral diseases was noted in studies of obese animals and instances of overnutrition in humans (50, 51). In the subsequent years, many studies emphasized a connection between obesity and severe viral disease (reviewed in (52)). By the early 2000s, research efforts focused on exploring ties between obesity and immune regulation were centered on the impact of obesity-associated inflammation on insulin resistance (53–57). However, only following the 2009 H1N1 pandemic was obesity cited by the US Centers for Disease Control and Prevention (CDC) as a risk factor that could predict severe viral infection outcomes. During the 2009 H1N1 pandemic, a large proportion of H1N1 patients hospitalized due to severe disease or who succumbed to infection were obese (58). Further, amidst the SARS-CoV-2 pandemic, obesity was again cited as a risk factor for severe SARS-CoV-2-induced illness and has been correlated with higher mortality rates following SARS-CoV-2 infection (7, 8, 59, 60). Intense efforts have been focused on determining mechanisms underlying metabolic dysfunction caused by obesity (56, 57, 61, 62), but the exact mechanisms by which MetS induces immune dysregulation and more severe viral disease are unknown and are an active area of research. Small animal models have been essential for dissecting metabolic pathways and how their dysregulation in the obese state can impact immune responses to viral pathogens.

Several murine models exist for use in the interrogation of obesity and associated metabolic perturbances on overall health, including genetically obese and diet-induced obese (DIO) mice. Although genetically obese animals serve as excellent recapitulatory models of morbid obesity, the leptin (ob/ob or *Lep*^{ob}) or leptin receptor (db/db or *Lepr*^{db}) mutations that induce obesity in these models rarely occur in humans. Thus, the use of DIO mice more closely models human obesity as it supports examining the effects of chronic over-nutrition and the ensuing oxidative stress it exerts on the immune system. Nonetheless, DIO models largely recapitulate the same immune system perturbances as genetically obese models, but the resulting phenotypes in DIO mice tend to be less exaggerated. In this section, we will discuss insights into the impact of obesity on immune function gleaned from both genetically obese and DIO mice.

Animal studies interrogating the impact of obesity on viral disease severity and antigen-specific immune responses have repeatedly illustrated that obesity exacerbates viral disease severity and dampens virus-specific immune responses (14, 63–68). Studies have suggested that a major contributor to obesity-associated immune dysfunction is the skewing of macrophage polarization within excessive stores of adipose tissue. Nearly since the advent of murine obesity studies, macrophages have been noted at the forefront of obesity-associated immune dysregulation due to their accumulation in adipose tissue. Though previously thought to be a neutral storage site for excess lipids, adipose tissue is an endocrine organ that secretes an array of hormones and adipokines central to maintaining systemic metabolism (69). Although adipose tissue (AT) houses several resident cell types, the most abundant leukocyte population found here is adipose tissue-resident macrophages (ATM) (70). In the obese state, ATM populations greatly increase in humans and mice, at times constituting 50% of the tissue (71). This intense inflation of ATM numbers in the obese state is believed to occur in part due to fat accumulation causing adipocytes to rapidly enlarge, thus inducing a hypoxic state which promotes necrosis and attracts more macrophages into the adipose tissue (reviewed in (72)). The increase in ATM, contributes to chronic low-grade inflammation which occurs due to the propensity for ATM to secrete large amounts of pro-inflammatory cytokines like TNF-α and interleukin-1β (IL-1β) (70, 73–76), graphically depicted in Figure 4. Numerous studies discussed below expand from focusing on macrophage-associated enhanced inflammation to detailing a role for obesity-induced defective type I interferon (IFN) responses. Many viral pathogens excel at antagonizing the host type I IFN signaling pathway to promote the production of progeny virions; thus, viral infection could be more fraught for individuals with obesity who may already have a blunted type I IFN response due to metabolic perturbances.

Studies of the interplay between obesity and viral immunity have focused heavily on respiratory pathogens, such as influenza virus, particularly in the aftermath of the 2009 H1N1 pandemic. In several studies, obese mice displayed significantly higher mortality rates compared to wild-type counterparts following influenza virus infection (66, 77–81), and reducing the dose of influenza virus given to obese animals was insufficient for mitigating their enhanced mortality (64). Obese mice often showed higher lung titers (66, 67, 80), in addition to elevated lung inflammation, leading to more significant pathology and tissue damage (67, 77–81). In an experimental study, O'Brien et al. observed significantly higher host and viral protein levels in bronchoalveolar lavages from obese animals, suggesting obese mice experienced defects in the maintenance of the barrier permeability (81), thus potentially accounting for enhanced edema and inflammation seen in these animals post-influenza virus infection. Interestingly, elevated lung titers are not the only factor contributing to enhanced respiratory tract pathology, as obese mice in a study done by Milner et al. had an equivalent viral burden in the lungs when compared to wild-type mice, yet significantly greater levels of lung inflammation and tissue damage (79). Similarly, elevated lung viral titers cannot be attributed as the sole cause for enhanced mortality as O'Brien et al. found enhanced mortality and inflammation in obese animals when compared to wild-type counterparts, yet obese animals in this study did not display significantly higher viral titers or worsened pathology in comparison to wild-type animals (81). Potentially underlying these contradictory findings are studies aimed at characterizing expression of interferon α and β (IFN-α and IFN-β) at sites of infection within the respiratory tracts of obese mice. These type I IFN anti-viral cytokines are

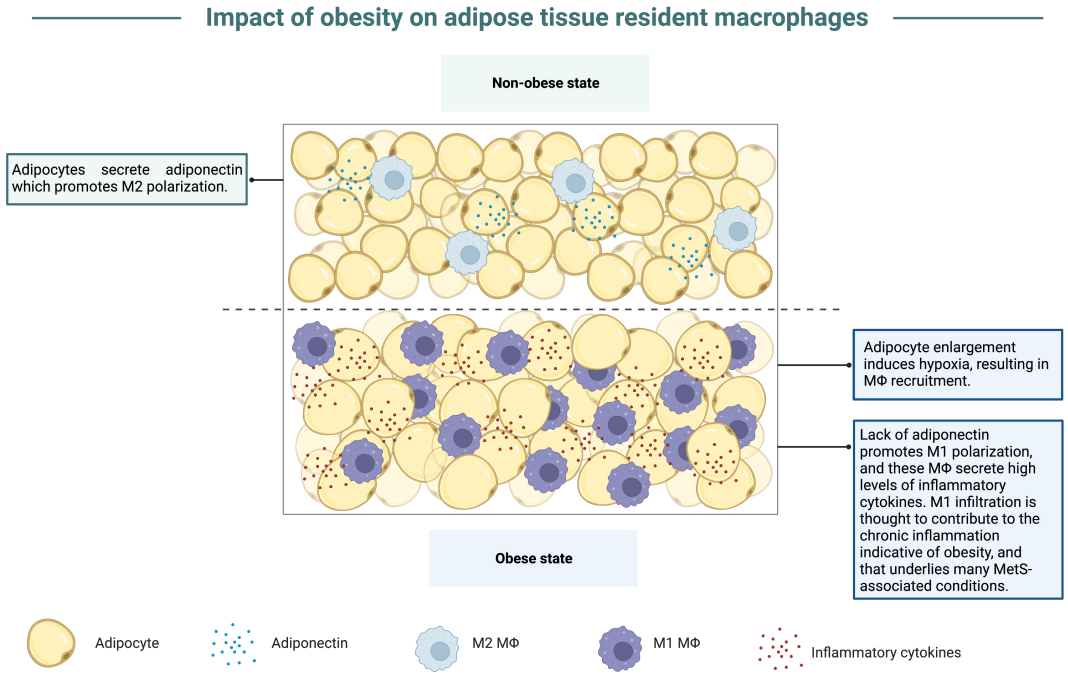

FIGURE 4
Obesity promotes accumulation of M1 macrophages. In the non-obese state, adipocytes secrete adiponectin, an adipokine that promotes macrophage polarization to the M2 phenotype. Within the obese state, adipocytes enlarge to store excess nutrients from overnutrition, which results in hypoxia and macrophage recruitment into adipose tissue. In the hypoxic state, less adiponectin is secreted, thus inducing macrophage polarization to the M1 phenotype, contributing to a state of chronic inflammation as these macrophages secrete high levels of inflammatory cytokines. This chronic inflammation is also believed to underlie and link the conditions that encompass MetS.

essential for establishing an anti-viral state, and their mRNA transcript levels were markedly lower in obese mice when compared to the expression noted in wild-type mice following influenza virus infection (66, 77, 78, 82), graphically depicted in Figure 5; the same was true for expression of pro-inflammatory cytokine- and chemokine-encoding mRNAs (77, 80, 81). These findings pin obesity at the forefront of inhibiting the innate immune system from adequately responding to acute viral infection. This type of immune defect not only poses a significant risk for combatting viral infections in the early stages of infection, where innate immune cells mount a rapid, multifaceted anti-viral attack, but also a blunted or delayed innate immune response could have detrimental consequences that prevent the priming of a robust adaptive immune response. In further examples of the propensity for the obese state to blunt type I IFN responses, Honce et al. noted that viral variants were detected early in obese mice post-influenza virus inoculation relative to wild-type mice (63). These variants replicated quickly within the obese hosts and exhibited greater virulence compared to the parental infecting strain (63). It is possible that these variants arose specifically within obese mice due to their blunted, delayed type I IFN response. This finding provides additional insight into why obesity has been linked to higher morbidity and mortality post-viral infection.

Moreover, confirming the impact obesity has on mortality following viral infection, Karlsson et al. sought to determine if obesity impacted memory T cell responses, thus predisposing hosts for greater susceptibility to severe viral disease following infection with a previously encountered pathogen. As memory T cells primed during a primary influenza virus infection are specific for internal viral proteins typically shared among various influenza strains, these T cells are effective at combatting infection by heterologous strains. However, this study revealed that obese mice had a significantly higher mortality rate following secondary challenge with a different influenza strain when compared to survival rates of wild-type counterparts (66). This study brings to the forefront the impact obesity can have on memory immune responses, as obese mice were not protected against a second encounter with an influenza virus. Following a similar line of questioning, wild-type and obese mice in another study were infected with sublethal doses of influenza virus, followed by a sublethal dose of *Streptococcus pneumoniae* (*S. pneumoniae*) (83). Obese animals succumbed to coinfection uniformly and significantly earlier than wild-type mice, and obese mice also displayed high viral and bacterial titers

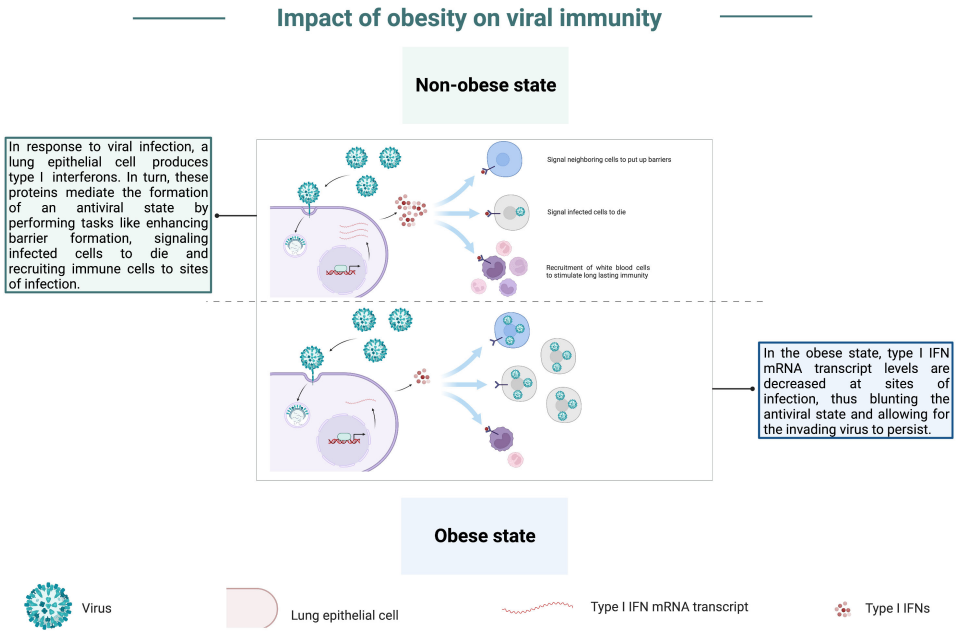

FIGURE 5
Obesity blunts anti-viral type I IFN responses. In the non-obese state, a lung epithelial cell produces type I interferons (type I IFNs) in response to infection by a respiratory pathogen. In turn, these proteins mediate the induction of an antiviral state through tasks such as enhancing barriers, signaling infected cells to die, and recruiting immune cells to infection sites. In the obese state, mRNA transcripts of type I IFNs are decreased at infection sites, thus blunting the induction of an antiviral state and allowing the invading virus to persist.

that correlated to extensive cellular damage (83). Interestingly, O'Brien et al. noted significantly less epithelial regeneration within bronchoalveolar surfaces when compared to wild-type mice (81), suggesting impaired wound healing in obese animals. This respiratory barrier vulnerability could account for their enhanced susceptibility to secondary infection. These studies reveal how, in addition to enhancing morbidity and mortality rates following a primary infection, obesity can also enhance host susceptibility to secondary infection, whether in the form of a challenge with the original invading pathogen, or a heterologous challenge with a different pathogen. These findings are critical to note as secondary infections are common among patients with influenza virus (84, 85) or SARS-CoV-2 (86–88) infections, confirming the toll obesity imposes on public health outcomes.

Finally, in addition to studies exploring the impact of obesity on respiratory infections, other research efforts have confirmed that obesity enhances morbidity and mortality in non-respiratory infections. In our previous study, we showed that dengue virus infection caused enhanced morbidity in obese mice based on weight loss and thrombocytopenia compared to wild-type mice (89). Similarly, in a study examining the impact of obesity on alphavirus infection outcomes, we showed that obese mice displayed significantly higher morbidity in terms of weight loss and mortality following infection with Mayaro virus, chikungunya virus, and Ross River virus (90), all of which are mosquito-borne pathogens. Further, in our studies of West Nile virus (WNV), we noted that obese mice displayed significantly higher viral titers in peripheral organs and the brain. We also found that these mice had a significantly higher mortality rate post-WNV infection when compared to wild-type counterparts (68). Similarly, upon infection with the rodent-borne viral pathogen lymphocytic choriomeningitis virus (LCMV), obese mice again experienced significantly higher mortality rates compared to wild-type mice and elevated viral titers (91). Interestingly, Pepin et al. highlight an important finding utilizing antiretroviral therapy (ART) to manage human immunodeficiency virus (HIV) infection. Rather than directly interrogating the impact of obesity on HIV-associated immune defects, these authors draw attention to the inherent predisposition for metabolic derangements that individuals who rely on ART for managing HIV infections experience. For example, prolonged ART treatment does not restore proper immune function in patients with HIV, but rather it is typically associated with premature immune aging, persistent immune hyper-activation, and chronic inflammation (92). Unsurprisingly, these predispositions result in patients with HIV exhibiting impaired metabolic control (93), MetS-associated comorbidities like obesity (94), NAFLD (95), type 2 diabetes (96), and high prevalence of insulin resistance (97–99). In their study, these authors found that ART worsened high fat diet-induced MetS conditions in mice, like enhanced glucose dysregulation (100). Further, ART exaggerated adiposity in the obese mice, and

contributed to further macrophage infiltration and polarization to the M1 phenotype, accompanied by enhanced inflammation (100). Taken together, these data highlight the capability of obesity to predispose for heightened viral disease severity and mortality to an array of pathogens with varying tropisms, and some antiviral treatments can compound the impacts of obesity on antiviral immunity, thus increasing the burden of viral diseases in humans with metabolic derangements.

Impact of obesity on vaccine efficacy

As a final comment on the impact of obesity on overall viral immunity, it is important to consider vaccine efficacy amidst a state of metabolic perturbances. There have been many recent outstanding reviews covering the clinical studies that demonstrate a reduced COVID-19 and IAV vaccine durability (101–104). Additionally, hospitalizations due to SARS-CoV-2 breakthrough infections are significantly elevated in individuals with type 2 diabetes, cardiovascular disease, as well as in individuals who are overweight (105–107). Similar to the viral infection studies discussed above, previous studies in animals have noted that metabolic dysfunction, and obesity in particular, are predictors of poor vaccine responses. Karlsson et al. conducted a study that eloquently demonstrated the negative impact obesity can have on vaccine-conferred immunity. In this study, wild-type and obese mice were infected with a nonlethal dose of influenza virus followed by a nonlethal dose of *S. pneumoniae* to simulate a secondary bacterial infection, a common risk factor associated with influenza virus infections in vulnerable populations. As mentioned earlier in this review, coinfected obese mice displayed a significantly higher mortality rate when compared to coinfected wild-type counterparts (83). The authors of this study noted that vaccinating obese mice against either pathogen failed to protect these animals from heightened mortality. Highlighting that obesity prevented the production of a protective vaccine-induced adaptive immune response.

Providing insight into why the vaccines fail to confer protection in obese animals, other studies revealed impaired vaccine-induced immune responses in obese animal (64, 67, 80, 108–110). Specifically, the frequency of antigen-specific CD8 T cells found in obese mice following influenza virus vaccination was significantly lower than in wild-type counterparts (67). Further, Karlsson et al. noted a reduction in the expression of interleukin-7 (IL-7) on antigen-specific CD8 T cells following influenza virus infection, and this study, as well as others, revealed that obese-primed T cells exhibited a steep decline in their ability to secrete IFN-γ when compared to wild type-primed CD8 T cells (65, 66, 111). This finding is consistent with studies done on IL-7 signaling-deficient mice following influenza virus infection where a decreased accumulation of antigen-specific, functionally active CD8 T cells existed at sites of infection (112). Taken together, these findings are informative for vaccine design, as IL-7 plays a canonical role in maintaining memory CD8 T cells (113), a key fact that makes data generated by Milner et al. and Rebeles et al. illuminating. Milner et al. found that influenza-specific CD8 T cells primed in obese mice produced less IFN-γ during a secondary exposure when compared to their cytokine production during a primary infection (67). Further, Rebeles et al. noted that upon a secondary influenza virus challenge, the number of CD8 T cells at sites of infection in obese mice were significantly reduced compared to the lungs of wild-type counterparts. They also noted that recalled CD8 T cells in obese mice exhibited altered cellular metabolism patterns characterized by increased oxygen consumption (108). These findings suggest that the obese microenvironment negatively impacts the maintenance of memory CD8 T cells, thus dampening their ability to respond quickly and effectively upon antigen re-exposure; consequently, these phenomena could explain why vaccine efficacy appears to be reduced in hosts with obesity.

Pursuing this matter further, several studies reported that obesity leads to reduced vaccine-induced antibody production following vaccination in obese mice compared to wild-type counterparts (64, 67, 80, 109). This finding indicates that the obese environment fails to foster an expansive antibody repertoire which could account for higher susceptibility to severe viral disease upon secondary exposure. Coupled with lower overall antibody titers, non-neutralizing antibody levels also waned significantly faster in obese animals when compared to wild-type counterparts following vaccination (109). Similarly, serum antibody neutralization capacity was markedly reduced in obese mice following influenza vaccination (80, 110). Taken together, these data indicate that obesity dampens the generation of a robust antibody response post-vaccination, and highlights that antibodies generated in the obese state tend to wane more rapidly than those generated in a metabolically healthy microenvironment.

Impact of hyperglycemia on viral immunity

Serving as another contributing factor to MetS diagnosis, this section will focus on hyperglycemia and insulin resistance. Hyperglycemia refers to a state where excess sugar (glucose) circulates in the blood. In a healthy physiological state, pancreatic beta cells produce insulin and release it into the bloodstream when circulating glucose levels are high. Insulin stimulates cells to capture glucose from the bloodstream, thus lowering blood glucose levels. When glucose levels in the blood remain high, beta cells are stimulated to secrete higher insulin levels to counteract hyperglycemia. An overabundance of insulin in the bloodstream can gradually desensitize cells to the protein,

thus making them less likely to store circulating glucose, a phenomenon known as insulin resistance. Simultaneously, the increased effort exerted by beta cells to counteract chronic hyperglycemia can lead to cellular exhaustion, thus damaging the population of beta cells and inhibiting future insulin release (114). When insulin resistance occurs, causing blood glucose levels to remain high, this impairment in glucose storage and regulation is referred to as type 2 diabetes (115). Previously, urinary tract infections were the most reported immune system-related susceptibility for type 2 diabetes patients (116). However, data recorded from SARS-CoV-2 patients has pinned type 2 diabetes as a significant risk factor for predicting severe viral infection outcomes (117, 118). Throughout this section, we will discuss some of the data that have been reported regarding the impact of hyperglycemia and insulin resistance, or diabetes, on immunity to viral infections.

Historically, studies elucidating the link between viruses and hyperglycemia have focused on the role of viruses in inducing or exacerbating diabetes. These studies done both *in vitro* and *in vivo* in small animal models have utilized numerous viral pathogens, including herpes viruses (119), encephalomyocarditis virus (EMCV) (120, 121), coxsackievirus B4 (122), and reoviruses (123). Infection by the viruses highlighted in these studies was shown to inflict cellular damage on beta cells, leading to hypoinsulinemia, and consequently causing hyperglycemia. While not directly demonstrating how viral disease severity is enhanced in instances of hyperglycemia, these studies have provided an ideal model of understanding the relationship between hyperglycemia and shortened lifespan, in addition to revealing an association between viral infection and blood glucose levels. Around 2000, clinical studies began to record an increase in the risk of severe viral disease in patients with hyperglycemia (124). By 2004, plasma glucose levels were shown to be a predictor of mortality following SARS-CoV infection (125, 126). Studies by Kumar et al. noted that WNV infection in diabetic mice led to more severe disease (127), similar to what has been observed in humans following WNV infection (128–131). IAV studies done with insulin receptor-deficient mice, which mimic human insulin resistance, demonstrated that insulin resistance resulted in reduced immune responses and poor protection against an H1N1 challenge (132). More recently, Hulme et al. found that mice with high glucose levels had increased disease severity following IAV infection. The investigators further demonstrated that elevated disease severity was due to hyperglycemia-induced damage to the pulmonary epithelial: endothelial barrier, thus increasing lung edema (133), graphically depicted in Figure 6. *In vitro* studies investigating mechanisms of impaired immune function associated with hyperglycemia in mice have noted that hyperglycemia can alter innate immune antiviral defenses (134), thus blunting early immune responses to viruses. Additionally, in

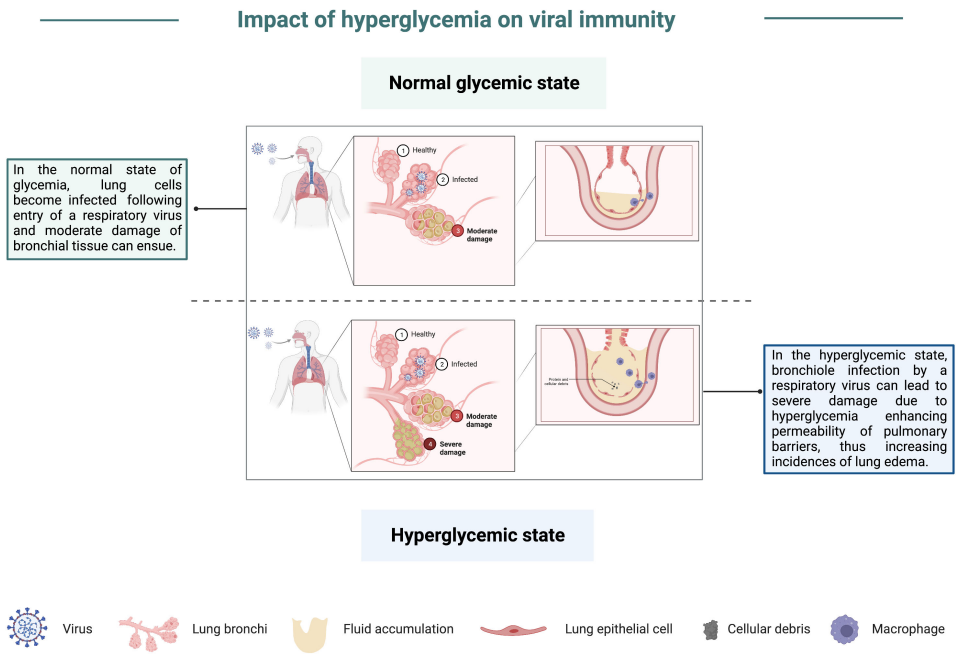

FIGURE 6
Impact of hyperglycemia on viral immunity. In a state of normal glycemia levels, lung cells can become infected by a respiratory virus, which may result in moderate damage to bronchial tissue. However, in the hyperglycemic state, severe damage of bronchioles following viral pathogen infection is more likely due to an impairment in lung barriers, thus enhancing their permeability and increasing the incidence of lung edema.

studies looking at immune cell differentiation in hyperglycemic mice, authors noted that hyperglycemia alters the differentiation of endothelial progenitor cells, thus leading to an increase in the frequency of proinflammatory cells (135). This predisposition of cells from hyperglycemic mice to differentiate into inflammatory mediators may contribute to the chronic inflammation associated with immune dysfunction and MetS, especially when considering that inflammatory cytokines, namely TNF-α, are insulin-desensitizing (5, 136, 137).

Impact of hypertension on viral immunity

Hypertension, or high blood pressure, results when the force exerted by blood circulating against arterial walls within the body's major blood vessels becomes elevated (138). Hypertension is the most common chronic disease condition in the world, and due to the common co-occurrence of hypertension with the previously described metabolic perturbance characteristics of MetS, a correlation between hypertension and altered immunity to viral pathogens would be unsurprising. However, whether high blood pressure alone can alter immunity is an active area of investigation, especially among cohort studies conducted on SARS-CoV-2 patients.

Shortly after the link between hypertension and viral disease was discovered, many groups worked to develop animal models that would mimic hypertension to develop treatments and provide a better understanding of the causes of this highly prevalent chronic condition (139). As with animal studies of hyperglycemia, early studies focused on viral infections which caused hypertension in both animals and humans, with some of the most notable studies looking at the role of cytomegaloviruses (CMV) in contributing to hypertension (140, 141). Although it has yet to be studied extensively in small animal models, data arising from the ongoing SARS-CoV-2 pandemic has identified hypertension as a factor that can contribute to severe COVID-19. Compared to COVID-19 patients with healthy blood pressure, hypertensive individuals were more likely to develop severe pneumonia or organ damage and experience a delay in viral clearance (142). Further, individuals with hypertension also displayed exacerbated inflammatory responses post-viral infection and had a heightened risk for mortality following SARS-CoV-2 infection (143, 144).

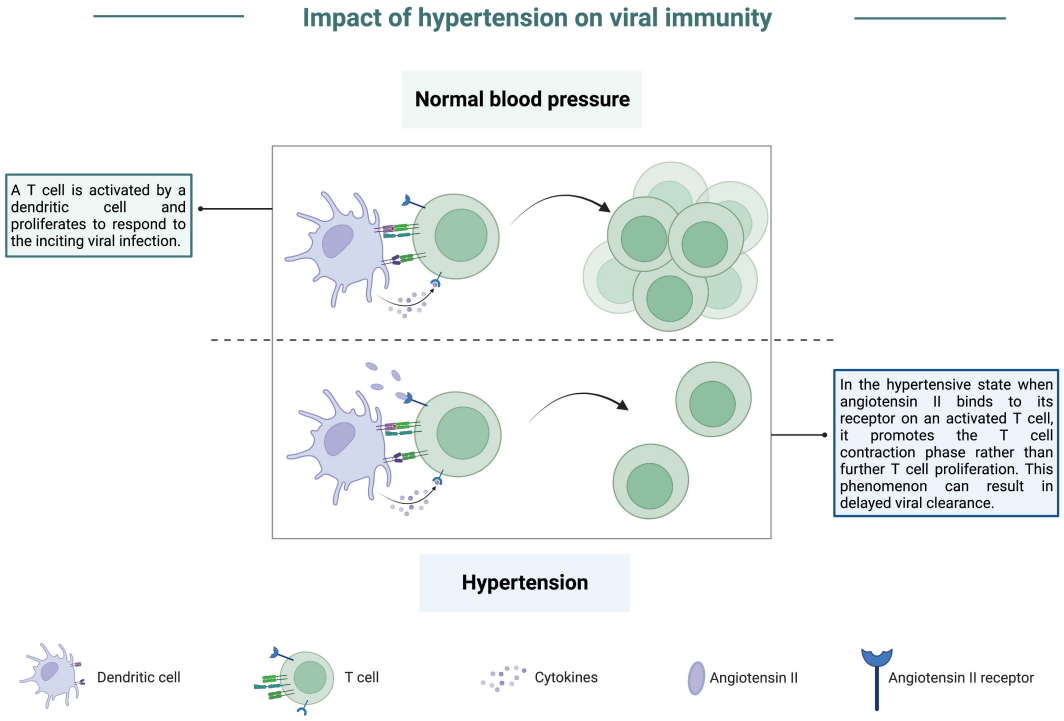

FIGURE 7
Impact of hypertension on viral immunity. In a state of normal blood pressure levels, T cells can be activated and proliferate to respond to virally infected cells following antigen presentation by a dendritic cell. However, in the hypertensive state, angiotensin II levels are higher, and this protein can bind to its receptor present on activated T cells. Upon binding, angiotensin II promotes the T cell contraction phase at the expense of sustaining a robust effector response. This phenomenon can result in delayed viral clearance due to a lack of T cells counteracting virally infected cells.

Interestingly, individuals with a history of hypertension but currently being treated with anti-hypertensive medications had a substantial decrease in the likelihood of critical outcomes from COVID-19 (142). Of the minimal number of animal studies conducted to assess the impact of hypertension on susceptibility to viral infection, several have noted that sympathetic nerve activity is exaggerated (145, 146) and thought to contribute to enhanced morbidity and mortality in hypertensive patients (147). Specifically, angiotensin II (AngII) expression is elevated in the hypertensive state and can activate sympathetic nerves, increasing proinflammatory cytokine expression (148). This increase may account for enhanced inflammation noted in hypertensive COVID-19 patients and exacerbated organ damage. Interestingly, one of the receptors for AngII, angiotensin II type 1-receptor (AT1R), is expressed on T lymphocytes. Studies in murine models revealed that engagement of AngII with AT1R decreased the activation of antigen-specific CD8 T cells (149). This engagement also accelerated the contraction phase of T cells in response to stimulation by their antigen (149), graphically depicted in Figure 7. This finding is also enlightening in the context of hypertensive COVID-19 patients as it could partially explain the delayed viral clearance observed in these patients.

Conclusions

As highlighted in this review, human cohort studies of various metabolic perturbances associated with MetS have drawn attention to the impact of aberrant metabolism on viral disease severity and vaccination outcomes. Serving as essential tools for elucidating the effect of specific physiological perturbances, small animal models have allowed scientists to begin understanding how conditions such as obesity, high cholesterol, hypertriglyceridemia, hyperglycemia, and hypertension induce aberrant immunity to viral pathogens. These findings suggest that the development of MetS leads to a blunted host immune response to viral infection by influencing the immune system in different ways, meaning that patients with MetS who often co-present with these inherently overlapping risk factors could be at even higher risk of severe viral disease development than patients harboring one of these conditions independently. Future small animal model studies centered on exploring the interplay between MetS and viral immunity or vaccination are of great importance as the proportion of individuals diagnosed with MetS is projected to rise continually.

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All listed authors have directly contributed to the conception of this review and have read and approved the submitted article.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Metabolic effects of CCL5 deficiency in lean and obese mice

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Accumulation and activation of immunocytes in adipose tissues are essential to obesity-induced inflammation and insulin resistance. Chemokines are pivotal for the recruitment of immunocytes in adipose tissue during obesity. Chemokine (C-C motif) ligand 5 (CCL5) plays a vital role in the recruitment of immunocytes to sites of inflammation. CCL5 expression level is increased in obese adipose tissue from humans and mice. However, the role of CCL5 in obesity-induced adipose inflammation remains unclear. Our study found that the CCL5 expression level was increased in the epididymal white adipose tissue (eWAT) of obese mice, particularly in $CD8^+$ T cells. CCL5 knockout (KO) mice exhibited better glucose tolerance than wild-type (WT) mice under lean conditions. In contrast, CCL5 KO mice were more insulin resistant and had severe hepatic steatosis than WT mice under obese conditions. Increased T cells in adipose tissue heaven adipose inflammation in obese CCL5 KO mice. The compensatory increased T cell-associated chemokines may account for increased T cell content in the eWAT of obese CCL5 KO mice. These findings imply that CCL5 deficiency exacerbates adipose inflammation and impairs insulin sensitivity in the metabolic tissues of obese mice.

KEYWORDS

obesity, adipose inflammation, CCL5, chemokine, insulin resistance

Abbreviations: BAT, brown adipose tissue; CLSs, crown-like structures; eWAT, epididymal white adipose tissue; HFD, high fat diet; iWAT, inguinal white adipose tissue; KO, knockout; ND, normal diet; SVF, stromal vascular fraction; WT, wild type.

1 Introduction

Obesity is a major public health issue worldwide. The prevalence of obesity increased from 0.7% (95% credible interval [CrI] 0.4-1.2) in 1975 to 5.6% (4.8-6.5) in 2016 for girls aged 5-19 years, and from 0.9% (0.5-1.3) to 7.8% (6.7-9.1) for boys (1). Obesity is a state of chronic inflammation and leads to many complications such as type 2 diabetes, cardiovascular disease, and cancer (2, 3). Chronic inflammation contributes to the development of insulin resistance and glucose intolerance. Chronic inflammation is induced by obesity in many tissues, including adipose tissue, liver, muscle, gastrointestinal tract, central nervous system, and pancreatic islets (4). Adipose inflammation is a major contributor to insulin resistance in obesity (5). Several potential mechanisms, including gut-derived antigens, dietary or endogenous lipids, adipocyte death, hypoxia, and mechanical stress, have been reported to participate in obesity-induced adipose inflammation (6). Many studies have demonstrated that the increased accumulation of proinflammatory immunocytes plays a central role in obesity-induced adipose inflammation (4, 7, 8). Notably, macrophages and T cells are the dominant immune cell types accumulated in obese adipose tissue (4). Both inflammatory macrophages (M1-like macrophages) and T cells (Th1 and $CD8^+$ T cells) are increased in adipose tissue from obese patients and mice (2, 4). Therefore, understanding the mechanisms underlying obesity-induced immunocyte accumulation in adipose tissue will provide new insights into treating obesity-induced adipose inflammation and complications.

Chemokines are small proteins that direct the infiltration of circulating leukocytes to the sites of inflammation or injury (9). C-C motif chemokine ligand 5 (CCL5, also known as RANTES) is a chemokine that could recruit leukocytes to inflammatory tissues (10). CCL5 gene expression was elevated in adipose tissue from obese patients and mice (11, 12). Moreover, the mRNA expression level of CCL5 was positively correlated with T cell marker CD3 and macrophage marker CD11b in the visceral adipose tissue of obese patients (12, 13). CCL5 could be secreted by various cell types, including T cells, macrophages, epithelial cells, fibroblasts, and platelets (10, 14). The mRNA expression level of CCL5 was induced by obesity more markedly in the stromal vascular fraction (SVF) than in adipocytes (15). However, the major cellular source of CCL5 in obese adipose tissue remains unclear.

CCL5 regulates the trafficking and homing of various immunocytes, including T cells, monocytes, granulocytes, and eosinophils (10). It binds to at least four receptors, including CCR1, CCR3, CCR5, and GPR75 (16, 17). Several groups have attempted to identify the role of CCR5, a major receptor of CCL5 in adipose tissue, in obesity-induced insulin resistance. Kitade et al. found that CCR5-deficient mice exhibited improved insulin sensitivity and glucose tolerance due to decreased macrophage accumulation in obese adipose tissue (18). However, Kennedy et al. illustrated that CCR5 deficiency exacerbated glucose tolerance and increased $CD4^+$ T cells but not macrophage infiltration into adipose tissue in obesity (19). Since CCR5 can bind to other ligands, including CCL3, CCL4, CCL8, and CCL14, CCR5 deficient mouse is not an ideal animal model to investigate the role of CCL5 in adipose inflammation. Therefore, the role of CCL5 in obesity-induced adipose inflammation and insulin resistance is obscure.

In this study, we identified the major cellular source of CCL5 in obese mice and employed CCL5 knockout (KO) mice to determine the role of CCL5 in obesity-induced adipose inflammation and insulin resistance. We found that the expression level of CCL5 was increased in obese epididymal white adipose tissue (eWAT), particularly in the $CD8^+$ T cells. CCL5 deficiency enhanced glucose tolerance in lean mice but exacerbated insulin resistance and adipose inflammation in obesity. CCL5 deficiency leads to increased T cells accumulation in obese adipose tissue, possibly due to compensatory upregulation of other chemokines.

2 Material and methods

2.1 Mice

C57BL/6J mice were purchased from Slac Laboratory Animal Inc (Shanghai, China), and CCL5 KO mice were purchased from Jackson Laboratory (Stock NO. 005090, Bar Harbor, ME, USA). All mice were kept in the specific pathogen-free animal room, maintaining a constant temperature and 12h/12h light/dark cycle. All animal procedures followed the Care and Use of Laboratory guidelines at Central South University. The C57BL/6J and CCL5 KO mice were crossed, and the F1 CCL5 heterozygous mice were used to generate wild-type (WT) and CCL5 KO littermates for experimental research. At 6 weeks of age, male mice were fed a normal diet (ND,10% fat, MD17121) (Medicience Ltd, Jiangsu, China) or a high fat diet (HFD; 60% fat, D12492) (Research Diets, New Brunswick, NJ). At 22 weeks of age, four cohorts of mice were euthanized. Tissues were collected for subsequent experiments.

2.2 Adipose tissue SVF isolation

EWAT were excised and minced in a 5 ml centrifuge tube. 1mg/ml type II collagenase (Worthington Biochemical, NJ, USA) in PBS containing 1% BSA was added to the minced adipose tissues and digested for 30 min at 37°C with shaking. The cell suspension was filtered through a 70 μm filter and centrifuged at 500*g* for 5 min to separate the adipocytes from the SVF pellet. Following centrifugation, the SVF pellet was suspended in erythrocyte lysate and incubated on ice for 5 min to lyse red blood cells. Cells were used for flow cytometry.

2.3 Flow cytometry

For surface markers detection, SVF cells were suspended in PBS containing 1% fetal bovine serum and incubated with Zombie dye (1:100, Cat#423106, Biolegend, USA) at room temperature for 7 min. Then cell suspension was incubated with CD16/32 (1:100, Cat#101302, Biolegend, USA) at room temperature for 7 min, followed by incubation with fluorochrome-conjugated antibodies (Supplementary Table 1) for 7 min at room temperature. For intracellular staining, samples were added with Monensin (1:1000, Cat#420701, Biolegend, USA) in the whole process to block the secretion of CCL5. The SVF cells were incubated with Zombie dye, blocked by CD16/32, and stained with fluorochrome-conjugated antibodies (Supplementary Table 1) against cell surface antigens. Then cells were fixed and permeabilized with cytofix/cytoperm buffer (Cat#554714, BD Bioscience, USA) for 45 min at 4 °C and then stained with fluorochrome-conjugated antibody against CCL5 for 30 min at 4 °C. Cells were washed by PBS twice and centrifuged at 500*g* for 5 min, and the cell pellet was suspended in 200 μL FACS Buffer. Samples were processed on a CYTEK flow cytometer and analyzed using FlowJo software.

2.4 Glucose tolerance test

Mice were fasted for 16 h. After baseline blood glucose collection, 20% glucose solution (1 g/kg) was administered intraperitoneally, and tail vein blood glucose was measured at 15, 30, 45, 60, and 120 min.

2.5 Insulin tolerance test

Mice were fasted for 6 h. After baseline blood glucose collection, 0.45 U/kg (ND group) or 0.75 U/kg (HFD group) insulin was administered intraperitoneally, and tail vein blood glucose was measured at 15, 30, 45, 60, and 90 min.

2.6 Serum insulin and GLP-1 level measurements

Mice were fasted for 6 h, and orbital venous plexus blood was collected to detect basal fasting insulin levels. After fasted overnight, 20% glucose solution (1 g/kg) was administered intraperitoneally, and orbital venous plexus blood was collected to detect glucose-stimulated insulin levels. Blood samples were centrifuged at 3000 g for 15 min, and serum insulin concentration was detected using an insulin Elisa kit (Cat# 32270, IMD, Hongkong, China).

Mice were fasted for 6 h. After basal blood samples were taken, 2 g/kg glucose was administered by gavage, and tail vein blood was collected after 15 min. Serum GLP-1 level was measured by ELISA kit (Cat#AF2027-A, AiFang, Changsha, China). Absorbance at 450 nm was determined using a microplate reader.

2.7 Staining of tissue sections

EWAT, liver, pancreas, and intestinal were harvested and fixed overnight in 4% paraformaldehyde, paraffin-embedded, then sectioned (5 μm), followed by hematoxylin and eosin (H&E) staining. Adipose tissue sections were hybridized with CD3 (Cat#AF20162, AiFang, Changsha, China) and F4/80 (Cat#SAF002, AiFang, Changsha, China) antibodies. Alexa Flour 488 donkey anti-mouse IgG (Cat# A32766, Invitrogen, USA) was used as a secondary antibody to detect $CD3^+$ cells. Alexa Fluor 594 goat anti-rabbit IgG (Cat#A32740, Invitrogen, USA) was used as a secondary antibody to detect $F4/80^+$ cells. For the detection of macrophage apoptosis, the TUNEL assay was performed using a FITC TUNEL cell apoptosis detection kit (Cat# G1501-100T, Servicebio, Wuhan, China).

Pancreatic paraffin-embedded tissue sections were stained with mouse anti-insulin (Cat# 66198-1, Proteintech, USA), rabbit anti-Glucagon (Cat#ab92517, Abcam, The UK), rabbit anti-Ki67 (Cat# D3B5, Cell Signaling Technology, USA) antibodies. Alexa Flour 488 donkey anti-mouse IgG (Cat# A32766, Invitrogen, USA) and Alexa Fluor 594 goat anti-rabbit IgG (Cat# A32740, Invitrogen, USA) were used as secondary antibodies. Intestinal paraffin-embedded tissue sections were stained with mouse anti-GLP1 (Cat# sc-514592, Santa Cruz, USA). Images were acquired with an Olympus microscope and integrated density was analyzed with Image J Software.

2.8 Cell proliferation assay

Mouse insulinoma cells (MIN6) were cultured in a 96-well plate at a density of 3.0×10^3 cells per well. GLP-1 (100 nM) with or without CCL5 (100 ng/ml) was mixed into the cell cultures. After 0, 24, 48, and 72 h of incubation, 10 μL CCK-8 solution was mixed into the culture and further incubated for 2 hours. Cell viability was measured at a 450 nm wavelength (OD450). The cell viability ratio (CRV) was calculated as $(A\text{-}A_0)/A_0 \times 100\%$ (A was the absorbance of the treated cell culture and A_0 was the OD450 value of a blank (DMEM medium only).

2.9 Primary hepatocyte isolation

Mouse primary hepatocytes were isolated following a 2-step collagenase digestion protocol (20). Briefly, mice were anesthetized and the liver was perfused *in situ* with 50 ml Hank's Balanced Salt Solution (HBSS) through the portal vein, followed by 8 mL of liver digestion media containing 2M

HEPES, 1% Penicillin-Streptomycin (P/S) Solution, and 0.08% type 4 collagenase. The liver was excised, minced, and filtered through a 100-micron mesh. The isolated hepatocytes were centrifuged at 50g for 3 min, and then the cell pellet was suspended in the MEM-α medium containing 80 μg/L DEX, 10% FBS, and 1% P/S Solution.

2.10 Primary skeletal muscle cell isolation

Skeletal muscle was harvested and washed by PBS three times and then minced with scissors. Skeletal muscle fragments were digested in the muscle digestion media containing 0.1% Pancreatin and 1mg/mL type 2 collagenase at 37°C for 25 min. After passing cells through a 70 μm cell strainer and centrifugation at 1000 rpm for 10 min, the cell pellet was suspended in DMEM/F12 medium containing 1% P/S, and 20% FBS. The fibroblasts were removed by differential adherence.

2.11 *In vitro* adipocyte differentiation

SVF from iWAT of C57BL/6J mice were cultured in DMEM/F12 plus 10% FBS, 1% Pen/Strap, and b-FGF (10ng/ml, Cat#100-18B, Peprotech, USA). Cells were allowed to grow to confluence and treated with white adipocyte differentiation induction cocktail: 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Cat# 15879-1G, Sigma, USA), 1 uM dexamethasone (Cat# D4902, Sigma, USA), 1.7 μM insulin, followed by maintenance treatment (1.7 μM insulin) until day 7-8 for harvest.

2.12 Insulin signaling

Cells were cultured in MEMα or DEME containing 4% FBS and treated with or without CCL5 (100 ng/ml) for 12 h, then stimulated with 100 nM insulin for 15 min. After stimulation, cells were washed immediately with PBS before lysis and scraped down in RIPA buffer containing protease and phosphatase inhibitors. Then, western blot analysis was performed.

2.13 *In vitro* chemotaxis assay

C57BL/6J mice thioglycolate-elicited peritoneal macrophages were isolated. For the migration *per se*, 1 $\times 10^5$ intraperitoneal macrophages were placed in the upper chamber of an 8 μM polycarbonate filter (12-transwell format; Corning, Lowell, MA), RPMI 1640 medium with or without CCL5 (10 ng/ml) was placed in the lower chamber. After 1 h of migration, the upper layer and trans-well insert were carefully removed. Migrated macrophages were counted using a cell counter chamber.

2.14 Macrophage apoptosis assays

RAW264.7 were incubated with LPS (1 μg/ml) or palmitate (0.3 mM) and treated with or without CCL5 (100 ng/ml) for 16 h. Cells were stained with an annexin V and propidium iodide (PI) double-staining technique and then analyzed using a CYTEK flow cytometer.

2.15 Primary hepatocytes lipid treatment

Primary hepatocytes were cultured with 10% FFA-free BSA-conjugated fatty acid (0.4 mM oleic acid and 0.2 mM palmitate) with or without CCL5 (100 ng/ml) for 24 h for RNA extraction and oil red O experiments. The stained cells were photographed with an Olympus microscope, After the dye retained in the cells was extracted with isopropanol, the OD510 was determined using a microplate reader.

2.16 Western blots analysis

After 16 weeks of HFD, Mice were fasted for 6 h and then intraperitoneally administered with PBS or insulin (4 U/Kg). Mice subsequently were euthanized 15 min later, and eWAT, liver, skeletal muscle were collected, frozen in liquid nitrogen immediately, and stored at −80°C. Total protein was isolated by RIPA buffer (Beyotime, Shanghai, China) containing protease (Roche, Basel, Switzerland) and phosphatase inhibitors (Roche, Basel, Switzerland). Protein concentration was measured with a BCA kit (Dingguochangsheng, Beijing, China) and the same amount of total protein was loaded onto polyacrylamide gels. Proteins were isolated and then transferred to PVDF membranes. Membranes were blocked for 1 h in 5% BSA at room temperature. The membranes were first incubated with following anti-phosphotyrosine AKT Ser473 (1:1000, Cat#4060, Cell Signaling Technology, USA) antibody at 4°C overnight. The membranes were subsequently stripped using solution containing 62.5 mM Tris-HCl, 2%SDS, 100 mM b-mercaptoethanol at 55°C for 25 min and reincubated with anti-AKT (1:1000, Cat#9272, Cell Signaling Technology, USA) antibody at 4°C overnight. Integrated density was analyzed with Image J Software.

2.17 RNA isolation and real-time RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen, USA) and 1000 ng RNA was reversed using cDNA Synthesis Kit (Thermo-Fisher Scientific, MA, USA). The synthesized cDNA was diluted 5 times in enzyme-free water. qRT-PCR was performed on a real-time fluorescence quantizer (ABI, USA), and all qRT-PCR primer sequences are shown in Supplementary Table 2. After

normalization with housing-keeping gene β-actin or 36B4 mRNA, the relative expression levels of target genes were calculated by the ΔΔCT method.

2.18 Oil red O staining

Liver tissue was fixed in 4% paraformaldehyde overnight, embedded in OCT glue, and frozen at −80°C for over 24 h. The embedded tissue was removed and sectioned in a frozen slicer at −20°C. Frozen sections were placed at room temperature for 10 minutes and washed with PBS 3 times. The sections were subsequently washed in 60% isopropyl alcohol for 5 min and stained with 60% Oil red O working solution for 15 min away from light. The sections were rinsed in 60% isopropyl alcohol to allow the staining of fat cells to bright red and other cells to be colorless, excess dye and isopropyl alcohol were washed under running water. The nucleus was stained with hematoxylin for 3 min, and the slides were then sealed with glycerin gelatin.

2.19 Statistical analysis

All data were processed by SPSS V19.0 statistical software. The statistical results were presented as means ± SEM, and comparison was performed by Student's *t*-test or two-way ANOVA. $p<0.05$ indicated that the difference was statistically significant ($^{+}p<0.1$, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

3 Results

3.1 CCL5 and its receptors are increased in CD8$^+$ T cells from eWAT of obese mice

To investigate the regulation of CCL5 expression by obesity in metabolic tissues, we analyzed mRNA levels of CCL5 and its receptors in adipose tissues, liver, and skeletal muscle from ND and HFD-fed mice. The mRNA levels of CCL5 and its receptors, CCR3 and CCR5, were significantly increased in the eWAT of obese mice compared with lean mice (Figure 1A). The mRNA levels of CCL5 and its receptors did not differ between lean and obese mice in inguinal white adipose tissue (iWAT), brown adipose tissue (BAT), liver, and skeletal muscle (Figures 1B–E). Wu et al. demonstrated that the mRNA level of CCL5 was markedly higher in SVF than in adipocytes of eWAT from obese mice (12). SVF is composed of various cells, including immunocytes and adipose stem cells. To identify the dominant cellular sources responsible for the upregulation of CCL5 in eWAT by obesity, we detected the expression of CCL5 in CD4$^+$ T cells, CD8$^+$ T cells, macrophages, and adipose tissue stem cells (ASCs) in eWAT from lean and obese mice by flow cytometry. The gating strategy was shown in Supplementary Figure 1. The percentage of CCL5 positive CD8$^+$ T cells was significantly increased in obese mice compared with lean mice (Figures 1F, G). It was noticeable that CD8$^+$ T cells had the highest proportion of CCL5 positive cells in SVF, and up to 60% of CD8$^+$ T cells are CCL5 positive cells in eWAT from obese mice (Figure 1G). To investigate whether CCL5 is specifically increased in CD8$^+$ T cells in adipose tissues during obesity, we examined the percentage of CCL5 positive CD8$^+$ T cells in peripheral blood, mesenteric lymph nodes, and spleen of lean and obese mice. The percentages of CCL5 positive CD8$^+$ T cells were not changed in blood and spleen, and increased mildly in mesenteric lymph nodes, suggesting that the expression of CCL5 in CD8$^+$ T cells is tissue-specifically increased in adipose tissue of obese mice (Figures S2A). We also examined the cellular subtypes of CCL5-expressing CD8$^+$ T cells in adipose tissue. The results showed that effector memory CD8$^+$ T cells are the major subtype of CCL5 positive CD8$^+$ T cells (Figures S2B, C). Collectively, these data demonstrate that CCL5 expression levels are increased in the eWAT of obese mice, particularly in adipose tissue CD8$^+$ T cells.

3.2 CCL5 deficiency improves glucose tolerance in chow diet-fed mice

To determine whether CCL5 affects systemic metabolism under lean conditions, we examined body weight, insulin sensitivity, and glucose tolerance in WT and CCL5 KO littermate mice. WT and CCL5 KO mice fed on chow diet remained equivalent body weight and adipose tissue mass (Figures 2A–C). Although CCL5 KO mice had similar insulin sensitivity to WT mice, CCL5 KO mice exhibited better glucose tolerance than WT mice (Figures 2D, E), suggesting an enhanced insulin secretion in CCL5 KO mice. Thus, we examined basal fasting and glucose-stimulated insulin levels in the serum of the two groups. The serum insulin levels were increased in CCL5 KO mice compared with WT mice after glucose stimulation (Figure 2F), indicating that CCL5 deficiency promoted insulin secretion.

To determine the role of CCL5 in islet development, islet function from lean WT and CCL5 KO mice was evaluated by H&E and immunofluorescence staining. The islet size was larger in the pancreas of CCL5 KO mice than that of WT mice (Figures 2G, H). Moreover, a significant increase in the maximum axis but not the minimum axis of each islet was observed in the pancreas of obese CCL5 KO mice (Figures 2I, J). Additionally, the total area of beta cells was significantly increased in CCL5 KO mice than in WT mice (Figures 2K, L). Since the number but not the size of beta cells was increased in CCL5 KO mice (Figures 2M, N), the increased total area of beta cells in CCL5 KO mice is mainly due to the increased beta cell number in the islet. Finally, we examined the proliferation of

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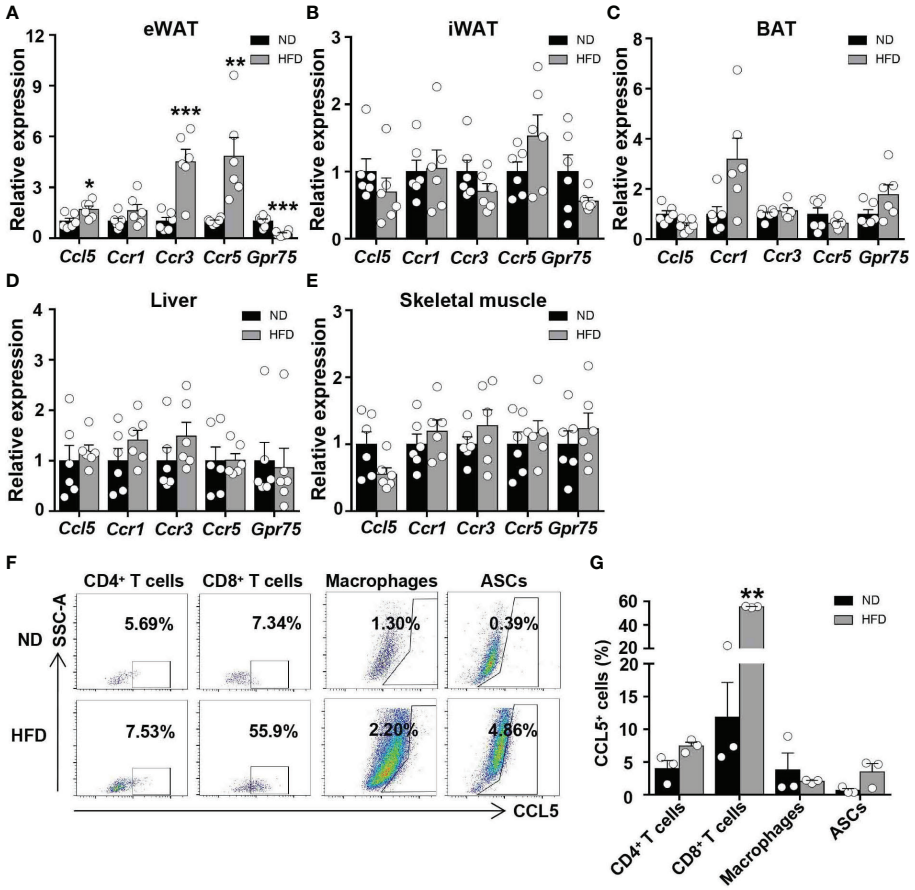

FIGURE 1
CCL5 is upregulated in CD8$^+$ T cell in eWAT of obese mice. **(A-E)** mRNA expression of CCL5 and its receptors in eWAT **(A)**, iWAT **(B)**, BAT **(C)**, liver **(D)** and skeletal muscle **(E)** in ND-fed and 16-week HFD-fed mice. (n = 6 mice per group). **(F)** Representative flow cytometry plots of CCL5 expression in CD4$^+$ T cells, CD8$^+$ T cells, macrophages, and ASCs of ND-fed and 16-week HFD-fed mice. **(G)** Quantification of CCL5$^+$ cells in CD4$^+$ T cells, CD8$^+$ T cells, Macrophages, and ASCs in C57BL/6J mice fed the ND or HFD by flow cytometry analysis. (n = 3 mice per group). Data are mean ± s.e.m. *p<0.05, **p<0.01, ***p<0.001 by unpaired Student's t-test.

islet beta cells and observed more Ki67 positive proliferating islet beta cells in CCL5 KO mice than in WT mice (Figures 2O, P). It has been shown that administration CCL5 in mice reduces plasma GLP-1 and GLP-2 (21). To investigate whether CCL5 affects islet beta cell proliferation by regulating the expression of GLP-1, we examined serum GLP-1 levels in WT and KO mice during fasting and after glucose gavage. The serum GLP-1 levels were significantly increased in KO mice under both fasting and glucose gavage conditions (Figure S3A). In addition, compared with WT mice, the mRNA and protein expression levels of GLP-1 in the jejunum, ileum, and colon of KO mice were also significantly increased (Figures S3B-D). These results suggest that CCL5 is an inhibitor the expression of GLP-1. To further investigate whether CCL5 affects the function of GLP-1, we treated min6 cells with or without GLP-1 and CCL5. the results showed that CCL5 inhibited the proliferation of min6 cells and the GLP-1-induced proliferation of min6 cells (Figure S3E). Together, these data indicate that CCL5 deficiency enhances glucose tolerance by promoting beta cell proliferation under lean conditions.

3.3 CCL5 deficiency promotes HFD-induced insulin resistance

To examine the role of CCL5 in obesity-induced insulin resistance, WT and CCL5 KO littermate mice were fed on HFD for 16 weeks. Although WT and CCL5 KO mice revealed equivalent body weight and WAT mass (Figures 3A–C), CCL5 KO mice were significantly more insulin resistance than their WT littermates, while glucose tolerance did not differ by genotype (Figures 3D, E). To confirm the insulin resistant phenotype of obese CCL5 KO mice, the insulin responsiveness in eWAT, liver, and muscle was assessed by post-insulin AKT phosphorylation. Consistent with ITT results, the pAkt/Akt ratio was decreased in eWAT, liver, and muscle of CCL5 KO mice,

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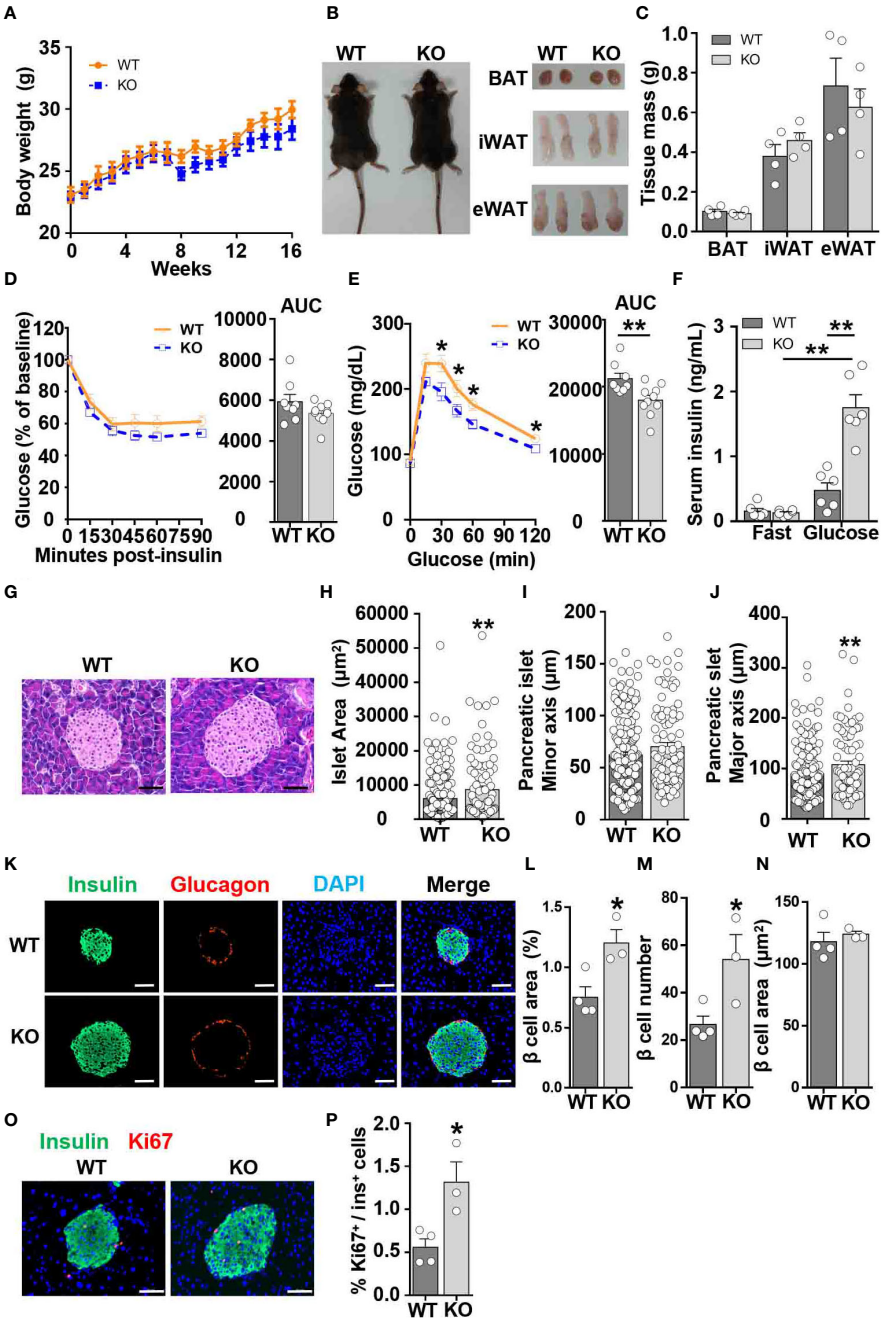

FIGURE 2
CCL5 deficiency enhances glucose tolerance in ND-fed mice. **(A)** Body weights of WT and CCL5 KO mice fed an ND. (n = 4-6 mice per group). **(B)** Representative images of mice and each part of fat pad (BAT, iWAT, and eWAT). **(C)** Weight of BAT, iWAT, and eWAT of WT and CCL5 KO mice fed an ND. (n = 4-6 mice per group). **(D, E)** ITT **(D)** and GTT **(E)** in mice fed an ND (n = 8-10 mice per group). **(F)** Serum insulin levels of ND-fed mice after 6 h fasting (fast) or 15 min after 1g/kg glucose injection (glucose). (n = 6 mice per group). **(G)** Representative H&E staining of pancreatic islet section. Scale bar: 50 μm. **(H–J)** area **(H)**, minor axis **(I)**, and major axis **(J)** of each pancreatic islet. (n = 4 mice per group. Data shown in panels **(H–J)** were obtained from the analysis of 16 sections). **(K)** Representative immunofluorescent staining of the islets of mice fed an ND using anti-insulin (green) and anti-glucagon (red) antibodies. **(L)** β cell mass of WT and CCL5 KO mice fed an ND. (n = 3-4 mice per group). **(M)** β cell number per islet of WT and CCL5 KO mice fed an ND. (n = 3-4 mice per group). **(N)** The size of individual β cell in WT and CCL5 KO mice fed an ND. (n = 3-4 mice per group). **(O)** Representative immunofluorescent staining of the islets of mice fed the ND using anti-insulin (green) and anti-ki67 (red) antibodies. Arrows indicate insulin^{+}Ki67^{+} cells. Scale bar: 50 μm. **(P)** Percentages of insulin^{+}Ki67^{+} cells in pancreatic sections of WT and CCL5 KO mice fed an ND. (n = 3-4 mice per group). Data in **(C–E, H–J, L–N, P)** are mean ± s.e.m. *p<0.05, **p<0.01 by unpaired Student's t-test. Data in **(F)** is mean ± s.e.m.*p<0.05, **p<0.01 by two-way ANOVA.

indicating that CCL5 deficiency significantly attenuated insulin signal transduction in classic insulin target organs in obesity (Figures 3F–H). To further investigate whether CCL5 directly acts on insulin signaling, we treated adipocytes, primary hepatocytes, and primary skeletal muscle cells with CCL5 and examined its effect on insulin signaling. CCL5 has no direct effect on insulin signaling in these three insulin target cells (Figures S4A–C), indicating that there are other indirect mechanisms regulating insulin signaling in obese CCL5 KO mice.

3.4 CCL5 deficiency exacerbates HFD-induced adipose inflammation

To investigate whether CCL5 participates in obesity-induced adipose inflammation, we detected immunocyte accumulation in the eWAT of obese WT and CCL5 KO mice. Small and similar numbers of immune cells accumulated in the adipose tissues of lean WT and CCL5 KO mice (Figure 4A). However, more immunocytes were observed in the eWAT of obese CCL5 KO mice than in those of obese WT mice (Figure 4A). Consistently, obese CCL5 KO mice had more crown-like structures (CLSs) (Figure 4B), which are composed of many kinds of immunocytes and serve as an indicator of adipose inflammation (22, 23). Additionally, immunofluorescence staining revealed an increased accumulation of T cells but not macrophages in the eWAT of obese CCL5 KO mice (Figures 4C–E).

To confirm the increased T cell accumulation in eWAT of obese CCL5 KO mice, mRNA levels of T cell marker genes in eWAT of WT and CCL5 KO mice were measured by qRT-PCR. The mRNA levels of T cell marker genes were not significantly different between lean WT and CCL5 KO mice (Figure S5A). However, T cell marker genes (*Cd3*, *Cd4*, and *Cd8*) as well as Th1 (*Ifn-γ*, *Tbet*/*Tbx21*), Th2 (*Gata3*, *Il4*), and Treg (*Foxp3*) marker genes were all increased in the eWAT of obese CCL5 KO mice (Figure 4F). We also analyzed the percentage of T cells in eWAT of obese WT and KO mice by flow cytometry analysis. The percentage of $CD3^+$ T cells was significantly increased in the eWAT of obese CCL5 KO mice than that in the eWAT of obese WT mice, and while the percentages of $CD4^+$ T cells and $CD8^+$ T cells were not different between the two groups (Figures 4G–I), indicating that CCL5 deficiency leads to an increase in the entire T cell population rather than a specific T cell subpopulation.

Chen et al. demonstrated that CCL5 deficiency could compensatorily induce the production of other chemokines to

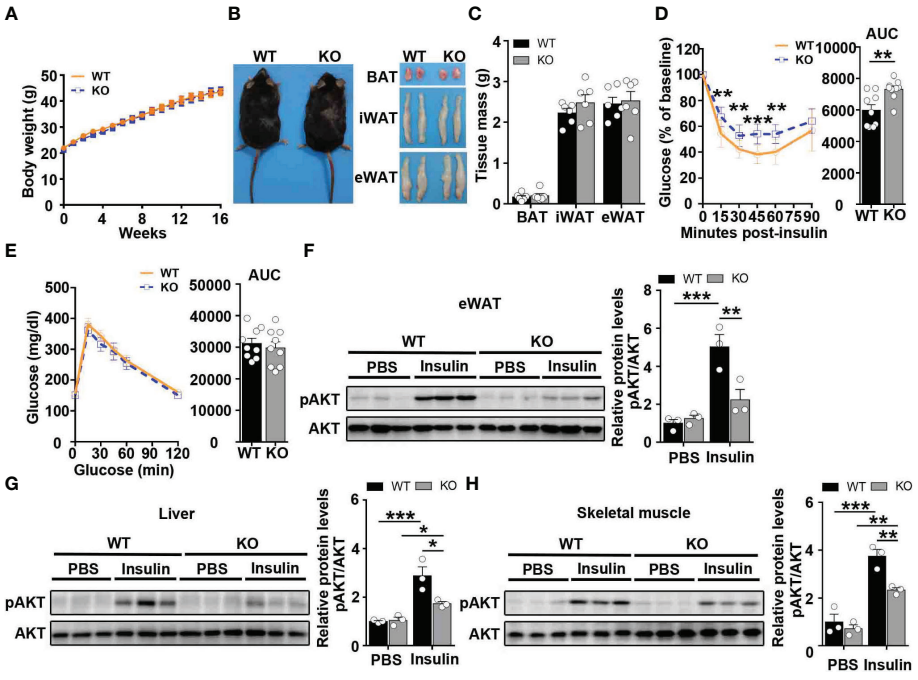

FIGURE 3
CCL5 deficiency aggravates insulin resistance in HFD-fed mice. **(A)** Body weights of WT and CCL5 KO mice after 16 weeks HFD. (n = 9-11 mice per group). **(B)** Representative images of mice and each part of fat pad (BAT, iWAT, and eWAT). **(C)** Weight of BAT, iWAT, and eWAT of WT and CCL5 KO mice after 16 weeks HFD. (n = 6 mice per group). **(D, E)** ITT **(D)** and GTT **(E)** in mice after 16 weeks HFD. (n = 9 mice per group). **(F-H)** Western blot (left) and quantification (right) of p-AKT and AKT in the eWAT **(F)**, liver **(G)**, and skeletal muscle **(H)** of WT and CCL5 KO mice after 16 weeks HFD. (n = 6 mice per group). Values were normalized to WT-PBS group. Data in **(C)** are mean ± s.e.m.**p<0.01, ***p<0.001 by unpaired Student's t-test. Data in **(D-E)** are mean ± s.e.m. **p<0.01, ***p<0.001 by two-way repeated-measures ANOVA with *post hoc* test by unpaired Student's t test. Data in **(F-H)** are mean ± s.e.m.*p<0.05, **p<0.01, ***p<0.001 by two-way ANOVA.

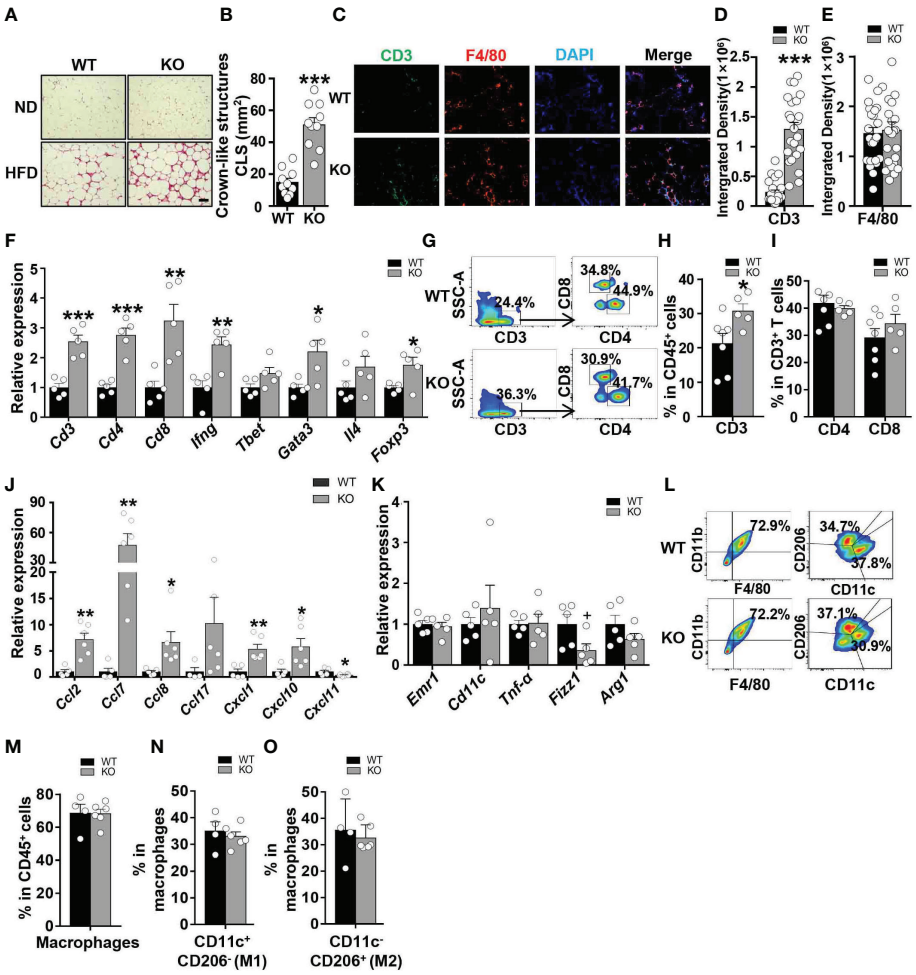

FIGURE 4
CCL5 deficiency exacerbates HFD-induced adipose inflammation. **(A)** Representative H&E staining of eWAT from WT and CCL5 KO mice after ND-fed and 16-week HFD-fed. Scale bar: 100 μm. **(B)** Quantification of CLSs in WT and CCL5 KO mice after 16 weeks HFD. (n = 10 high power fields from 4 animals in each group). **(C)** Representative immunofluorescent staining of the eWAT from mice fed a HFD using anti-CD3 (green) and anti-F4/80 (red) antibodies. Scale bar: 100 μm. **(D, E)** Quantification of images for CD3 **(D)** and F4/80 **(E)** integrated density in eWAT from WT and CCL5 KO mice after 16 weeks HFD. (n = 25 high power fields from 4 animals in each group). **(F)** mRNA expression of T cell marker genes in eWAT of WT and CCL5 KO mice after 16 weeks HFD. (n = 5 mice per group). **(G)** Representative flow cytometry plots of $CD3^+$ T cells, $CD4^+$ T cells, and $CD8^+$ T cells in eWAT of WT and CCL5 KO mice after 16 weeks HFD. **(H)** Quantification of $CD3^+$ T cells in $CD45^+$ cells in eWAT of WT and CCL5 KO mice after 16 weeks HFD. (n = 5-7 mice per group). **(I)** Quantification of $CD4^+$ T cells, $CD8^+$ T cells in $CD3^+$ cells in eWAT of WT and CCL5 KO mice after 16 weeks HFD. (n = 5-7 mice per group). **(J)** mRNA expression of chemokine genes in eWAT of WT and CCL5 KO mice fed a HFD. (n=4-6 mice per group). **(K)** mRNA expression of macrophage marker genes in eWAT of WT and CCL5 KO mice after 16 weeks HFD. (n = 5 mice per group). **(L)** Representative flow cytometry plots of macrophages in eWAT of WT and CCL5 KO mice after 16 weeks HFD. $CD11c^+$ $CD206^-$ (M1-type) and $CD11c^-$ $CD206^+$ (M2-type). **(M)** Quantification of macrophages in eWAT of WT and CCL5 KO mice after 16 weeks HFD. (n=4-6 mice per group). **(N, O)** Quantification of M1-type **(M)** and M2-type macrophages **(N)** in total macrophages of eWAT from WT and CCL5 KO mice after 16 weeks HFD. (n=4-6 mice per group). Data are mean ± s.e.m. $^+p<0.1$, $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ by unpaired Student's t-test.

enhance immunocyte recruitment (24). To determine whether other chemokines could be induced by CCL5 deficiency, we detected the mRNA expression levels of chemokines that could recruit T cells to tissues. These chemokine expression levels were not increased in the eWAT of lean CCL5 KO mice (Figure S6A). However, the mRNA expression levels of CCL2, CCL7, CCL8, CXCL1, and CXCL10 were significantly increased in the eWAT of obese CCL5 KO mice than in WT mice (Figure 4J). These data suggest that the increased accumulation of T cells in obese CCL5 KO mice may be mediated by the induction of other chemokines.

Macrophages undergo phenotype switch from an anti-inflammatory M2 phenotype to a proinflammatory M1 phenotype in obesity (4). To determine whether CCL5 regulates the macrophage phenotype switch in adipose tissue, we detected mRNA levels of macrophage marker genes in eWAT

of WT and CCL5 KO mice by qRT-PCR. The mRNA levels of M1 marker genes (*Emr1*, *Cd11c*) and M2 macrophage marker genes (*Fizz1* and *Arg1*) showed no significant difference between WT and CCL5 KO mice in both lean and obese conditions (Figures S5B, 4K). We further detected the percentage of M1 and M2 macrophages in eWAT of obese WT and CCL5 KO mice by flow cytometry analysis. Consistently, the percentages of M1 and M2 macrophages were not different between the two groups (Figures 4L–O). Together, these results indicate that CCL5 has no effect on the obesity-induced macrophage phenotype switch in eWAT. It has been reported that CCL5 promotes the migration of human adipose tissue macrophages and protects macrophages from apoptosis induced by free cholesterol (25, 26). We examined whether CCL5 is a chemokine for macrophages and protects macrophages from apoptosis *in vitro*. As shown in Figure S7A, CCL5 promoted chemotaxis of peritoneal macrophages. The chemotactic effect of CCL5 on macrophages would promote a decrease of macrophage in adipose tissue in CCL5 KO mice. However, flow cytometry analysis showed that CCL5 increased the percentage of apoptosis of macrophages induced by LPS and palmitate (Figures S7B–E). In addition, there were less apoptotic macrophages in adipose tissue in CCL5 KO mice than WT mice (Figures S7F). This pro-apoptotic effect of CCL5 on macrophage would promote an increase of macrophage in adipose tissue in CCL5 KO mice. Therefore, in obese CCL5 KO mice, the content of macrophage in eWAT was not altered, which may be attributed to the balance between reduced recruitment and apoptosis of macrophage in eWAT.

3.5 CCL5 deficiency increases HFD-induced lipid accumulation in the liver

A marked impairment in insulin responsiveness was observed in the liver of obese CCL5 KO mice (Figure 3G). Fat accumulation in the liver is strongly associated with hepatic insulin resistance in obesity (27). We, therefore, examined the obesity-induced hepatic steatosis in WT and CCL5 KO mice. HFD feeding induced higher liver weight in CCL5 KO mice than in WT mice (Figure 5A). Moreover, a notable increase in lipid deposition was observed in the liver of obese CCL5 KO mice compared with WT mice, as demonstrated by H&E and Oil Red O staining (Figure 5B). To further explore the phenotype of hepatic steatosis in obese CCL5 KO mice, the key genes regulating processes of lipid metabolism were characterized by qRT-PCR. The mRNA levels of lipogenesis genes (*Pparγ*, *Srebp1c*, *Fasn*, and *Acaca*) and fatty acid uptake genes (*Fabp1* and *Cd36*) in the liver were increased in obese CCL5 KO mice than in obese WT mice (Figures 5C, D). While, the mRNA levels of fatty acid oxidation genes (*Acox1* and *Acox2*) increased slightly in obese CCL5 KO mice (Figure 5E), and the mRNA levels of fatty acid transport genes were not different between the two groups (Figure 5F). These data indicate that CCL5 deficiency promotes obesity-induced hepatic lipid accumulation. This is most likely the result of increased hepatic lipogenesis and lipid uptake rather than decreased lipid oxidation. Furthermore, the mRNA expression levels of the immune cell markers and proinflammatory cytokines in the livers of obese CCL5 KO and WT mice were examined. The mRNA levels of *Cd8*, *iNOS*, *TNF-α*, and *IL-1β* were increased in obese CCL5 KO mice than in obese WT mice (Figure 5G), indicating that CCL5 deficiency also exacerbates liver inflammation in obesity. Together, these data indicate that CCL5 deficiency aggravates obesity-induced liver injury. To further investigate whether CCL5 directly acts on lipid metabolism in hepatocytes, we examined the effect of CCL5 on hepatocyte steatosis *in vitro*. Oil O red staining and quantitation of lipid loading experiment showed that CCL5 decreased palmitate-induced lipid accumulation in hepatocytes (Figure S8A, B). Moreover, CCL5 reduced mRNA expression of genes involved in lipogenesis and fatty acid uptake in primary hepatocytes (Figures S8C, D). These results indicated that CCL5 has direct effects on modulating lipid metabolism in hepatocytes.

4 Discussion

Obesity-induced inflammation is closely associated with insulin resistance and type 2 diabetes (6). Targeting the key regulators involved in the recruitment and activation of proinflammatory immunocytes could be a potential therapeutic approach for insulin resistance and type 2 diabetes. CCL5 acts as a vital factor to trigger T lymphocytes and monocyte/macrophages chemotaxis and activation in chronic inflammatory diseases (28–30). However, the role of CCL5 in obesity-induced adipose tissue inflammation remains obscure. Our study detect the expression of CCL5 in white adipose tissue and the function of CCL5 in metabolic regulation in lean and obese mice. First, the expression of CCL5 is increased in eWAT of HFD-induced obesity, particularly in $CD8^+$ T cells within eWAT. Second, CCL5 deficiency enhances glucose tolerance in lean mice but deteriorate insulin resistance by upregulating T cell-mediated adipose inflammation in obese mice. Finally, CCL5 deficiency increases the expression of chemokines, which could trigger T cells chemotaxis in adipose tissue of obese mice.

Our results demonstrated elevated expression of CCL5 in the eWAT of obese mice, which is consistent with previous studies (12). Adipose tissue is composed of adipocytes and SVF. Wu et al. (12) identified markedly higher mRNA expression levels of CCL5 in SVF than in adipocytes. Since SVF includes many kinds of cells, such as ASCs, T cells, and macrophages, it was unclear which cell type is the primary source of CCL5 in obese adipose tissue. We identified that $CD8^+$ T cells are the major cellular sources responsible for upregulation of CCL5 by obesity in eWAT. Nishimura et al. (23) demonstrated that infiltration of $CD8^+$ T cells is an early event in adipose tissue inflammation induced by obesity. Additionally, the accumulation of $CD8^+$ T

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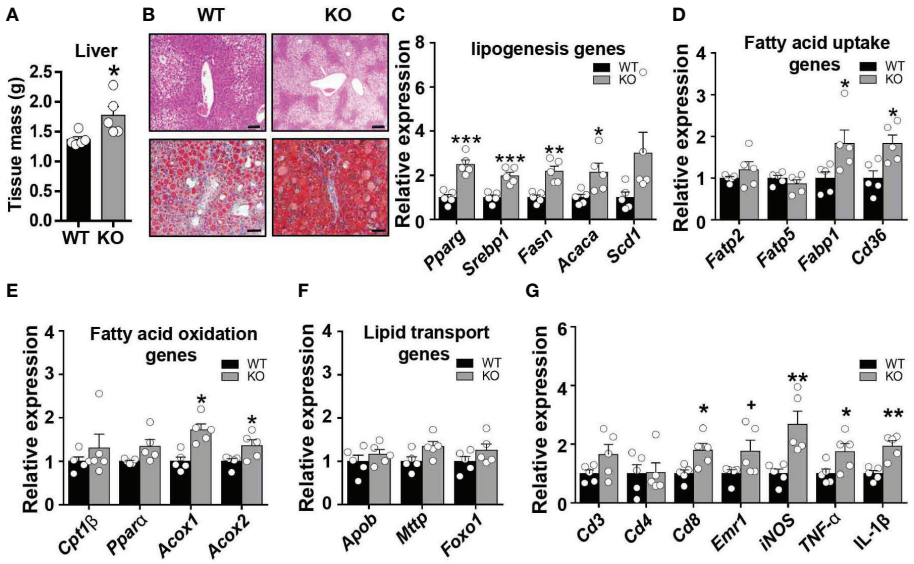

FIGURE 5
CCL5 deficiency promotes HFD-induced lipid accumulation in liver. **(A)** Weight of liver from WT and CCL5 KO mice after 16 weeks HFD. (n = 5 mice per group). **(B)** Representative H&E (Scale bar: 100 μm) and Oil red O (Scale bar: 50 μm) stained section of liver from WT and CCL5 KO mice after 16 weeks HFD. **(C)** mRNA expression of lipogenesis genes in liver from WT and CCL5 KO mice after 16 weeks HFD. (n = 5 mice per group). **(D)** mRNA expression of fatty acid uptake genes in liver from WT and CCL5 KO mice after 16 weeks HFD. (n = 5 mice per group). **(E)** mRNA expression of fatty acid oxidation genes in liver from WT and CCL5 KO mice after 16 weeks HFD. (n = 5 mice per group). **(F)** mRNA expression of lipid transport genes in liver from WT and CCL5 KO mice after 16 weeks HFD. (n = 5 mice per group). **(G)** mRNA expression of immune cell markers and cytokines in the liver from WT and CCL5 KO mice after 16 weeks HFD. (n = 5 mice per group). Data are mean ± s.e.m. $^{+}p<0.1$, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ by unpaired Student's t-test.

cells enhanced the infiltration of macrophages (23). As CCL5 could promote macrophage recruitment (31), CCL5 may be an essential chemokine secreted by $CD8^{+}$ T cells to enhance the recruitment of macrophages during the development of obesity-induced adipose tissue inflammation.

In our study, CCL5 deficiency did not affect insulin sensitivity under lean conditions. However, Chou et al. (32) found impaired insulin sensitivity in lean CCL5 deficient mice. This divergency likely stems from differences in insulin dose applied to ITT experiments. We used 0.45 U/kg of insulin to treat 5-month-old mice (body weight 25-30 g). In their mouse studies, Chou et al. (32) used 0.75 U/kg of insulin to treat 3-4-month-old mice (body weight 20-30 g). Too high an insulin dose may cause a counter-regulatory response to prevent hypoglycaemia, thus inducing ITT becomes a compound test of insulin sensitivity and counter-regulatory response (33). So, in Chou's experiment, the result of ITTs may be related to the high insulin dose. The role of CCL5 in glucose metabolism remains under debate. Liu et al. (17) identified that CCL5 could stimulate insulin secretion *via* GPR75 in beta cells to improve mouse glucose tolerance. However, Pais et al. (21) demonstrated that CCL5 impaired glucose-induced insulin secretion by reducing the secretion of GLP1 and GLP2. Consistent with Pais et al., we found that CCL5 deficiency enhanced glucose tolerance by promoting insulin secretion under lean conditions. In addition, CCL5 can also downregulate islet beta cell proliferation by directly inhibiting islet beta cell proliferation and the GLP-1-induced proliferation of islet beta cell. This discrepancy may stem from that Liu et al. studied the role of CCL5 in glucose tolerance by a gain-of-function mice model through intraperitoneal injection of CCL5. In contrast, we analyzed by constructing a loss-of-function mice model.

In obesity, chronic adipose inflammation is a crucial contributor to impaired insulin sensitivity (4). CCL5 KO mice were more insulin resistant than WT mice under obese conditions, which was consistent with worse adipose inflammation in them. CCL5 could recruit M2 macrophages and increase the ratio of M2/M1 in the progression of hepatocellular carcinoma and osteogenesis (34, 35). However, only a slightly increased expression of M2 marker genes was observed in adipose tissue of CCL5 KO mice fed HFD, suggesting that macrophages may be not responsible for the exacerbated adipose inflammation. In contrast, a significant increased T cell accumulation was observed in obese CCL5 KO mice. Therefore, it is likely that the worse adipose inflammation in obese CCL5 KO mice is dependent on enhanced T cell accumulation in adipose tissue. Since CCL5 is a T cell chemokine, we did not expect an increased T cell accumulation in obese CCL5 KO mice. Chen et al. reported that CCL5 deficiency could compensatorily activate the CXCL1-CXCR2 axis in neutrophils to enhance their infiltration and liver injury in hepatitis (24). In our study, many T cell chemokines, including CCL2, CCL7, CCL8, CXCL1, and CXCL10 (30, 36, 37), were dramatically increased in the adipose tissue of obese CCL5 KO

mice. CCL5 deficiency may compensatorily induce the production of other chemokines, which may account for the increased accumulation of T cells in the adipose tissue of obese CCL5 KO mice. However, the mechanism of this compensatory regulation needs to be further investigated.

CCL5 KO mice showed more severe lipid accumulation in liver after HFD feeding than WT mice. Since the processes of hepatic lipogenesis and lipid uptake were altered in obese CCL5 deficient mice, these processes may account for severe hepatic steatosis caused by CCL5 deficiency. As adipose tissue dysfunction could promote the progression of hepatic steatosis due to insulin resistance and proinflammatory adipokines release (38), the heaven hepatic steatosis may ascribe to severe adipose tissue dysfunction in obese CCL5 KO mice. Park et al. reported that increased pro-inflammatory chemokines and cytokines induced by CCR5 deficiency may cause hepatic injury (39), raising the possibility that CCL5 deficiency may lead to overexpression of pro-inflammatory factors and tissue injury in the liver. Indeed, the mRNA expression levels of inflammatory markers were increased in the liver of CCL5 deficient mice. However, the mechanisms underlying immunocytes contributed to the obesity-induced nonalcoholic steatohepatitis in CCL5 deficient mice still need to be clarified. In addition, CCL5 can directly inhibit lipogenesis in primary hepatocytes. Therefore, the increased hepatic steatosis in obese CCL5 KO mice may be caused not only by insulin resistance and inflammation but also by the loss of the direct influence of CCL5 on hepatocyte lipogenesis. Insulin resistance is associated with hepatic steatosis. However, it is unclear whether hepatic steatosis is a cause or a consequence of insulin resistance (27). Therefore, whether hepatic steatosis triggers insulin resistance in obese CCL5 KO mice remains to be studied. A limitation of our study is that it was performed in male mice only. Further studies should be carried out to evaluate the metabolic effects of CCL5 in female mice and other animal species.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by the Department of Laboratory Animal Science, Central South University.

Author contributions

HuZ and XL contributed to the study design, acquisition of data, analysis, and interpretation of results, as well as drafting and revision of the manuscript. QZ, HaZ, JS, WH, XS, YD, DW, and YX contributed to the acquisition of the data and revision of the manuscript. TD contributed to the conception and design of the study, analysis of results and the revision of the manuscript. All authors gave their approval for the final manuscript to be published.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1059687/full#supplementary-material

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Association of normal weight obesity phenotype with inflammatory markers: A systematic review and meta-analysis

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Background: Individuals with normal weight could suffer from obesity based on their body fat percentage (also known as normal weight obesity (NWO)), thus being at risk of significant morbidity and mortality compared to the general population. It seems that inflammatory pathways and chronic inflammation are significant contributors to the pathogenicity of NWO. This study aimed to assess and pool the association of proinflammatory and anti-inflammatory cytokines with NWO.

Methods: In this systematic review and meta-analysis, online international databases (PubMed, Scopus, EMBASE, Web of Science, and Google Scholar) were searched until August 2022. All observational studies with an English full text comparing the mean levels of proinflammatory and anti-inflammatory cytokines (e.g., C-reactive protein (CRP), various types of interleukins (IL) s, tumor necrosis factor-alpha (TNF)) and white blood cell (WBC) count, in subjects with NWO and "normal weight non-obese (NWNO)" were included. Two researchers independently screened, reviewed and assessed the quality of included studies. The remaining articles' data were extracted post-screening. The heterogeneity between studies was assessed using the I^2 and Cochran's Q tests. A random effect model meta-analysis was used to pool the standardized mean difference (SMD) as an effect size.

Results: From the initial 559 studies, 21 and 19 were included in the qualitative and quantitative synthesis, respectively. In the systematic review, 8 studies reported a significant association between various proinflammatory cytokines (CRP, IL_6, $IL_{1\beta}$, and TNFα) and NWO. According to random-effect meta-analysis,

the association between NWO with CRP (SMD: 0.60, 95% CI: 0.30, 0.91) and IL6 (SMD: 0.90, 95%CI: 0.14, 1.66) was statistically significant. Moreover, the mean level of TNF_{α} in subjects with NWO and NWNO did not differ significantly (SMD: 0.67, 95% CI: -0.36, 1.70).

Conclusion: The findings of this study show that NWO was associated with high levels of CRP and IL6. Therefore, inflammatory pathways may play a role in the pathogenicity of NWO.

KEYWORDS

NWO, normal weight obesity, inflammation, inflammatory markers, CRP, interleukin

Background

Obesity has been extensively studied as one of the most prominent causes of morbidity and mortality (1, 2). Despite such evaluations in different target populations, new findings still emerge in this topic (3). These findings are particularly important in preventing and treating obesity as its prevalence, morbidity, and mortality are increasing globally (4). It should be noted that lately, morbidity and mortalities attributed to obesity are being seen in individuals who, based on previous definitions of obesity, a body mass index (BMI) above 30 Kg/m2, are not considered obese (5, 6). Hence new definitions and types of obesity have been defined (6).

One of these relatively new definitions regards those with normal BMI values and yet high body fat percentage (6–9). These individuals are regarded as Normal Weight Obese (NWO) (9). Studies indicate that NWOs are at an increased risk of cardiometabolic conditions similar to obese individuals and may suffer from the same morbidity and mortality-related conditions (10–13). Some studies suggest that one contributing factor to this increased risk of cardiometabolic conditions in obesity and NWO could be chronic inflammation, as inflammation has been observed in increased adiposity (14, 15). Despite inflammation being an essential process in the body, chronic inflammation can have adverse cardiometabolic effects, since pro-inflammatory cytokines in a chronic inflammatory status can contribute to the development of atherosclerosis, insulin resistance, type 2 diabetes, hypertension and hypercholesterolemia (14). Since obesity is a chronic condition, the resulting inflammation persists, resulting in chronic inflammation and subsequently the aforementioned conditions (15, 16). Similar to obesity (based on BMI) the association of inflammation with NWO has been highlighted in a review (9). It seems that in NWO the secretions of the adipose tissue itself contributes to inflammation; these studies argue that the underlying cause of this inflammation is the increased fat mass and lipid accumulation resulting in increased oxidative stress, and NF-κB pathway (a major pathway in the innate inflammatory response) activation (9, 17). Although the number of studies addressing the association of NWO and inflammation has been increasing throughout the years (9), no systematic review on the inflammatory aspect of NWO has been published so far. This systematic review and meta-analysis aimed to summarize and pool the association of NWO phenotype with inflammatory markers in published studies.

Methods

This study was performed according to the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) checklist (18).

Search strategy

A comprehensive systematic search was conducted on all available online databases (Scopus, EMBASE, Web of Science, PubMed, and Google Scholar) until August 2022. One of the investigators conducted the search, and another reviewed the findings. Terms such as "normal weight obesity", "NWO", "high fat percentage" and their MeSH term equivalents alongside proinflammatory and anti-inflammatory cytokines such as "CRP" "C-reactive protein" and "interleukin" and white blood cell (WBC) count were searched. The entire search (terms and strategy) can be seen as Supplementary Table 1. Moreover, reference lists of included studies or reviews were hand-searched to identify more potentially eligible studies.

Study selection criteria and eligibility

All observational studies with an English full text that assessed the association of proinflammatory and anti-inflammatory cytokines such as C-reactive protein (CRP), various types of interleukins (IL) s, tumor necrosis factor (TNF) alpha with NWO were included in this study. Regardless of their various definitions of NWO (based on fat percentage, waist circumference, etc.), all studies were included. All studies included studies had represented the targeted population and compared them with normal-weight, non-obese (NWNO) individuals. Studies that failed to meet the inclusion criteria were excluded. Furthermore,

duplicates, non-peer-reviewed publications, and studies without sufficient information to determine eligibility were excluded.

Two investigators independently carried out the screening process of included studies, including titles, abstracts and full texts. Upon removing the irrelevant entries, the full texts of the remaining articles were assessed. Moreover, to find the missed relevant studies (if any), the reference lists of the included studies were hand-searched as well. Discrepancies were referred to a third investigator for resolution.

Data extraction strategy

Two investigators separately extracted the data using a pre-designed data extraction sheet. The extracted data were composed of the name of the first author and publication year, the number of participants, age and sex, the definition of normal weight obesity, the studied cytokines, and the outcome as standardized mean difference (SMD) alongside their 95% confidence interval (CI) of the outcomes were extracted as the effect size of dichotomous and continuous respectively. Moreover, discrepancies were referred to a third investigator for resolution.

Quality assessment

We used the Newcastle-Ottawa Scale for quality assessment. This scale consists of seven items, scoring based on selection, comparability, exposure (case-control studies), and outcome (cohort studies). The total score ranges from 0 to 9 for cohort studies or 0 to 10 for case-control studies and is calculated by summing the scores of each item of this assessment tool (19, 20). We categorized the scores as 0 to 4, 5-6, 7 and above, indicating the studies' quality (low, middle, and high-quality studies, respectively). Two investigators independently assessed the quality of the studies, and discrepancies were referred to the third investigator.

Statistical analysis

The I^2 and Cochran's Q tests were used to assess the heterogeneity between the studies. A random-effect model was adapted for analyses if the heterogeneity was statistically significant (P-value<0.1). Otherwise, a fixed model was used. The SMDs of the included studies were calculated and pooled as an effect size for NWO association with the mean levels of proinflammatory and anti-inflammatory cytokines. Meta-Analysis was performed for outcomes with at least 3 reports within the studies. If applicable, sub-group analysis was performed for proinflammatory and anti-inflammatory cytokines (stratified by sex, quality and adjustment for confounding variables and type of CRP (high-sensitivity (hs-CRP) and CRP excluding hs-CRP) as well. Egger's test was adapted to assess publication bias for each inflammatory factor, and trim fill analysis was performed if publication bias was present. STATA (Stata Corporation, College Station, Texas, USA) version 17 was used to analyze the data.

Results

Search results

Figure 1 shows the flowchart of the selection of studies for inclusion in the meta-analysis. From the 559 studies found in the initial search, 263 duplicate studies were removed. Out of 296

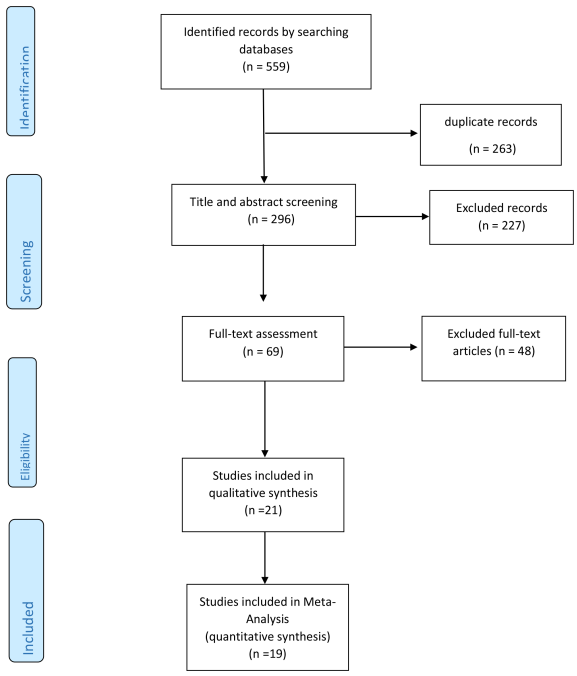

FIGURE 1
studies search, review and analysis flowchart.

remaining articles, 227 irrelevant studies were excluded after titles and abstracts screening. The full texts of the 69 articles were assessed, and 45 studies were excluded due to failing the eligibility criteria. Finally, 21 articles remained in the current systematic review (21–41). However,19 were eligible for inclusion in the meta-analysis (one study reported OR as the effect size (36), and one study exclusively evaluated complement C_3 (23), which did not reach the minimum number of three studies needed to enter the meta-analysis)

Study characteristics

The included studies were conducted worldwide (Canada, Iran, Italy, Japan, Poland, South Korea, Spain, Sweden, USA) with a total number of 19,857 participants aged ≥13 years. Twenty studies were conducted on the adult population (age ≥ 18 years), one study was conducted on adolescents aged 13 to 18 (34), and one was conducted exclusively on the elderly aged above 60 (41). Most studies were conducted in Italy (5 studies) and South Korea (4 studies). Canada, Poland, Spain, Sweden and Switzerland had the least number of studies (1 study). The greatest sample size was from a study conducted in the USA (4116 individuals), and the smallest was from Brazil (52 individuals). In most of the included studies, NWO was defined as normal BMI values with a body fat percentage above 30% in women (8 out of 16 reports) and above 25% in men (4 out of 8 reports). These general characteristics are shown in summery in Table 1. Three studies (24, 38, 40) had adjusted their findings for possible confounders; the other included studies were not adjusted for any confounding factors.

Qualitative synthesis

The included studies evaluated the associations of NWO with the mean levels of proinflammatory and anti-inflammatory cytokines (CRP, IFNγ, TNFα, $IL_{1\alpha}$, $IL_{1\beta}$, IL_2, IL_6, IL_8, IL_{10}, IL_{12p70}, IL_{15}, IL_{18}) as well as complement C_3, and white blood cell count (WBC), compared with normal-weight non-obese (NWNO) individuals; and their effect sizes are shown in Table 2. As can be seen, in 11 out of 22 reports of CRP, 2 out of 3 reports of IL_6, 1 out of 3 reports of TNFα, 2 out of 2 reports of WBC, $IL_{1\alpha}$ and $IL_{1\beta}$, and 1 out of 1 reports of IL_{15} and complement C_3, significant differences between NWO and NWNO individuals were observed; with the greatest effect size regarding $IL_{1\beta}$, SMD: 3.79, 95% CI (2.75-4.83). However, in the two reports evaluating IL_{10} and singular reports evaluating IL_2, IL_8, IL_{12p70}, IL_{18} and IFNγ, no significant differences between NWO and NWNO individuals were seen. Moreover, one study (36) reported that NWOs, in comparison with NWNOs, have significantly increased odds of vascular inflammation (OR:3.07 95%CI (1.29-7.29)).

Quantitative synthesis

Significant heterogeneity among the studies assessing the association between NWO and CRP, IL_6 and TNFα was seen (for all associations I^2: ≥ 88%, P-value < 0.001). The overall association between NWO, and CRP, IL_6 and TNFα are shown in Table 3. Based on the random effect models meta-analysis, the pooled association of CRP (SMD: 0.60, 95% CI: 0.30, 0.91) and IL_6 (SMD: 0.90, 95%CI: 0.14, 1.66) was significantly higher among NWO individuals compared with NWNOs. TNF-α (SMD: 0.67, 95% CI: -0.36, 1.70) was also higher among NWO individuals; however, this association was not statistically significant. Figures 2–4, illustrate the included studies and their overall relationships between NWO and CRP, IL_6 and TNFα, respectively.

Stratified meta-analysis

Based on the random effect model, the means of CRP sub-grouped by sex in males and females were (SMD: 0.31, 95% CI 0.05, 0.56) and (SMD: 0.97, 95% CI 0.31, 0.98), respectively.

The pooled association between NWO and CRP sub-grouped by quality assessment was (SMD: 0.39, 95% CI -0.57, 1.35), (SMD: 1.28, 95% CI 0.51, 2.06), and (SMD: 0.43, 95% CI 0.05, 0.81) for low quality (articles which had a high risk of bias), satisfactory (articles with moderate risk of bias) and high-quality studies (articles with a low risk of bias) respectively. The pooled association between NWO and CRP sub-grouped by study adjustment (whether studies adjusted their findings for confounding variables) was (SMD:0.50 95%CI -0.14,1.13) and (SMD:0.65 95%CI 0.31,0.98) for adjusted and unadjusted studies respectively. The pooled stratified association between NWO and CRP sub-types according to sex, quality assessment and study adjustment are reported in Table 3. The association of NWO with hs-CRP was statistically significant (SMD:0.65 95%CI 0.32,0.99), and this association was not statistically significant for CRP excluding hs-CRP (SMD:0.60 95% CI -0.12,1.12).

Meta-regression

Meta-regression was performed with sex, quality of the studies and study adjustment (whether studies adjusted their findings for confounding variables) as covariates across CRP, IL_6 and TNFα; no significant results were seen across them except for IL_6; in which sex and quality of the studies were recognized as possible causes of heterogeneity. (Coefficient: -0.69, P-value: 0.005 and coefficient: -1.61, P-value: 0.001 respectively.)

Quality assessment

Based on the New-Castle Ottawa scale, six of the included studies were of low quality studies (indicating a high possibility of bias), Five studies had middle quality (with acceptable risk of bias) and ten studies had high quality (with low risk of bias). The overall quality score of the studies can be seen in Table 1.

Publication bias

No publication bias was seen in articles studying the association between NWO and CRP (coefficient: 2.22, P-value: 0.16); however,

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TABLE 1 General Characteristics of included studies for association of NWO and inflammatory factors.

country	author year	Design	sample size (number)					mean age/ age range (year)	NWO definition	Q.A
			Total	M	F	NWNO	NWO			
Brazil	Duque 2021 (21)	Cross-sectional	52	(-)	(-)	29	23	43.3	(-)	3
Canada	J. Shea 2012 (22)	Cross-sectional	653	(-)	(-)	324	329	39.6	BF > 20.8% for men, > 35.0% for women	7
Iran	M. Karkhaneh 2019 (23)	Case-control	70	0	70	30	40	19-39	BF ≥ 30%	4
	Tayefi 2019 (24)	Cross-sectional	2439	(-)	(-)	1311	1128	47	BF > 25% for men, > 30% in women	9
	Amani 2019 (25)	Case-control	90	(-)	(-)	30	30	18-25	BF > 20%	4
Italy	Renzo 2013 (26)	Cross-sectional	47	0	47	17	30	33.6	BF > 30%	3
	Renzo 2010 (27)	Case-control	60	0	60	20	20	20-35	BF > 30%	4
	Renzo 2008 (28)	Case-control	150	0	150	50	50	20-45	BF > 30%	5
	A. Lorenzo 2007 (29)	Case-control	60	0	60	20	20	26	BF > 30%	4
	Renzo 2007 (30)	Case-control	110	0	110	30	40	26	BF > 30%	5
Japan	J. Huang 2018 (31)	Cross-sectional	91	0	91	51	34	20	BF > 30%	5
Poland	W. Kosmala 2012 (32)	Case-control	168	(-)	(-)	95	73	37.8	BF% for every 20 years, from 20 years of age > 19,21,24 for men, 32,33,35 for women	6
south Korea	Y. J. Hyun 2008 (33)	Case-control	50	(-)	(-)	25	25	25-64	visceral fat ≥100 cm2	5
	W. K. Cho 2015 (34)	Cross-sectional	1700	888	812	1266	144	13-18	highest quartile (Q4) of age and sex specific waist to hip ratio	7
	Sohee kim 2015 (35)	Cross-sectional	2078	(-)	(-)	1795	283	53.4	BF ≥ 25.4% for men, ≥ 31.4% for women)	8
	S. Kang 2014 (36)	Case-control	164	(–)	(–)	82	82	54	BF ≥ 23.5% for men, ≥ 29.2% for women	7
Spain	Gómez 2011 (37)	Cross-sectional	3051	838	2213	656	1579	18-80	BF ≥ 25% for men, ≥ 35% for women	8
Sweden	Berg 2015 (38)	Cross-sectional	1471	581	890	1080	266	25-74	BF ≥ 25% for men, ≥ 38% for women	8
Switzerland	P. Marques-Vidal 2010 (39)	Cross-sectional	2301	0	2301	1667	173	54	BF > 38%	8
USA	A. Romero 2010 (40)	Cross-sectional	4116	2031	2085	2054	2062	41.3	BF% ≥23.1% for men, ≥33.3% for women	8
	J. A. Batsis 2013 (41)	Cross-sectional	936	173	763	636	303	> 60	BF > 25% for men, > 35% for women	8

M, male; F, female; NWNO, normal weight non-obese; NWO, normal weight obesity; Q.A, quality assessment (based on the New castle Ottawa scale). (-): not reported/not available.

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TABLE 2 Association of NWO with the mean of inflammatory markers in included studies.

Author, Year	Outcome	Sex	N NWNO	Mean ± SD (N)	N NWO	Mean ± SD (NWO)	SMD NWO/ NWNO	95% CI
A. Lorenzo, 2007 (29)	CRP	Female	20	0.4 ± 0.1	20	0.8 ± 0.3	1.75	1.89, 3.62
	IL 1α	Female	20	14.8 ± 1.8	20	26.9 ± 4.5*	3.46	2.69, 4.74
	IL 1β	Female	20	5 ± 2.6	20	15 ± 3.1*	3.43	2.92, 5.07
	IL 2	Female	20	12.3 ± 1.5	20	14.7 ± 3.6	0.85	0.73, 2.12
	IL 8	Female	20	0.9 ± 0.2	20	2.3 ± 0.6*	3.07	1.32, 2.87
	IL 10	Female	20	3.4 ± 0.8	20	3.8 ± 1.3	0.36	0.23, 1.52
	IL 12p70	Female	20	14.2 ± 2.2	20	19.1 ± 3.7	1.58	4.14, 6.85
	IFN γ	Female	20	17.7 ± 4.8	20	25.3 ± 5.3	1.47	2.9, 5.04
A. Romero, 2010 (40)	CRP	Male	1014	3.3 ± 6.36	1017	3.7 ± 6.37*	0.06	(–)
	CRP	Female	1040	3.2 ± 0.32	1045	3.8 ± 0.32*	1.87	(–)
Amani,2019 (25)	CRP	Both	30	2.8 ± 1.8	30	1.6 ± 1.2	-0.77	-0.61, 0.4
Berg, 2015 (38)	CRP	Male	377	0.8 ± 0.99	139	1.4 ± 1.5*	0.52	0.58, 1.11
	CRP	Female	703	0.8 ± 1.35	137	1.2 ± 1.43*	0.29	0.94, 1.49
Duque,2021 (21)	CRP	Both	29	0.1 ± 0.3	23	0.2 ± 0.5	0.25	(–)
Gomez, 2011 (37)	CRP	Male	96	0.9 ± 0.5	371	4.3 ± 9.2*	0.41	0.44, 0.9
	CRP	Female	560	2.1 ± 2.6	1208	4.9 ± 1.95*	1.29	0.21, 0.46
J. A. Batsis, 2013 (41)	CRP	Male	73	0.4 ± 0.25	103	0.6 ± 1*	0.25	(–)
	CRP	Female	563	0.39 ± 0.95	200	0.43 ± 0.56	0.05	(–)
J. Huang 2018 (31)	CRP	Female	51	0.92 ± 0.3	34	1.19 ± 0.58*	0.62	-0.41, 1.29
	TNF α	Female	51	0.65 ± 0.78	34	0.57 ± 0.46	-0.12	-1.25, 0.45
J. Shea, 2012 (22)	CRP	Female	324	2.14 ± 4.22	329	2.42 ± 3	0.08	(–)
M. Karkhaneh, 2019 (23)	C_3	Female	30	92.79 ± 8.13	40	104.3 ± 15.04*	0.91	(–)
P. Marques-Vidal 2010 (39)	CRP	Female	1667	1.8 ± 0.09	173	1.83 ± 0.27	0.25	2.76, 2.97
Renzo, 2007 (30)	CRP	Female	30	0.5 ± 0.1	40	0.9 ± 0.4	1.28	2.51, 3.94
Renzo, 2008 (28)	CRP	Female	50	0.59 ± 0.1	50	0.9 ± 0.1*	3.08	5.3, 7.21
	IL 6	Female	50	5.45 ± 1.4	50	8.1 ± 3.8*	0.92	0.36, 1.17
Renzo, 2010 (27)	TNF α	Female	20	21.5 ± 5	20	43 ± 10*	2.67	2.92, 5.06
	IL 1α	Female	20	15.2 ± 2	20	27.5 ± 5*	3.17	3.66, 6.14
	IL 1β	Female	20	7.3 ± 2.3	20	17 ± 2.7*	3.79	2.97, 5.14
	IL 6	Female	20	8.2 ± 2.2	20	13.4 ± 1.8*	2.54	2.34, 4.25
	IL 10	Female	20	5.8 ± 0.8	20	6.2 ± 1	0.43	0.59, 1.95
	IL 15	Female	20	6.3 ± 1.1	20	8.7 ± 1.1*	2.14	1.3, 2.84
Renzo, 2013 (26)	CRP	Female	17	0.1 ± 0.17	30	0.4 ± 0.85	0.43	(–)
	TNF α	Female	17	27.51 ± 19.77	30	23.55 ± 11.47	-0.26	(–)
S. Kang, 2014 (36)	CRP	Both	82	1.2 ± 3.2	82	2 ± 2.9	(–)	(–)
Sohee.Kim, 2015 (35)	CRP	Both	1795	1.2 ± 2	283	1.6 ± 2.3*	0.20	(–)

(Continued)

TABLE 2 Continued

Author, Year	Outcome	Sex	N NWNO	Mean ± SD (N)	N NWO	Mean ± SD (NWO)	SMD NWO/ NWNO	95% CI
Tayefi, 2019 (24)	CRP	Both	1311	1.28 ± 12.93	1128	1.81 ± 23.3	0.03	(–)
W. K. Cho, 2015 (34)	WBC	Male	662	6 ± 1.34	61	6.4 ± 1.39*	0.30	0.4, 0.74
	WBC	Female	604	5.9 ± 1.25	83	6.1 ± 2.09*	0.14	0.4, 0.79
W. Kosmala, 2012 (32)	CRP	Both	95	1 ± 0.5	73	4 ± 4.2*	1.07	(–)
	IL 6	Both	95	17.2 ± 4.1	73	18.4 ± 6.8	0.22	(–)
	IL 18	Both	95	258 ± 96	73	292 ± 132	0.30	(–)
Y. J. Hyun 2008 (33)	CRP	Both	25	0.23 ± 0.2	25	0.52 ± 0.9	0.44	(–)
	TNF α	Both	25	1.66 ± 0.9	25	2.44 ± 1.65*	0.58	(–)
	IL 6	Both	25	0.96 ± 4.45	25	1.71 ± 0.33*	0.24	(–)

N, number; NWNO, normal weight non-obese; SD, standard deviation; NWO, normal weight obesity; SMD, standardized mean difference; CI, confidence interval; CRP, C-reactive protein; IL, interleukin; IFN, interferon; TNF, Tumor necrosis factor; C, complement; WBC, white blood cell.
The reported outcome values are as follows: CRP: mg/L, ILs, TNF and IFN γ: pg/mL, C_3: g/L, WBC: 10^9/L.
*: statistically significant (P-value < 0.05). (-): not reported/not available.

TABLE 3 The overall association between NWO, and the means of inflammatory markers.

Variable	N Study	Sample Size	SMD	Heterogeneity		
				I Squared%	Model	**P-Value
Both types CRP[1]						
Overall	18	16492	0.60 (0.30,0.91)*	98.45	Random	< 0.001
By sex						
Male	4	3190	0.31 (0.05,0.56)*	88.87	Random	< 0.001
Female	11	8291	0.97 (0.46,1.48)*	88.7	Random	< 0.001
Both Sexes	7	5011	0.23 (-0.03,0.49)	98.45	Random	< 0.001
By adjustment						
Unadjusted	14	6503	0.65 (0.31,0.98)*	96.24	Random	< 0.001
Adjusted	4	9989	0.50 (-0.14,1.13)	99.47	Random	< 0.001
By quality of the study						
Low quality	4	199	0.39 (-0.56,1.34)	90.65	Random	< 0.001
Medium quality	5	473	1.28 (0.51,2.05)*	92.76	Random	< 0.001
High quality	9	15820	0.43 (0.05,0.80)*	99.01	Random	< 0.001
hs-CRP						
Overall	12	8975	0.65 (0.32,0.99)*	97.34	Random	< 0.001
By sex						
Female	9	5443	0.96 (0.49,1.44)*	97.37	Random	< 0.001
Both Sexes	3	2549	-0.10 (-0.63,0.44)	82.24	Random	< 0.001
By adjustment						
Unadjusted	10	5180	0.78 (0.33,1.22)*	96.91	Random	< 0.001
By quality of the study						
Low quality	3	147	0.45 (-0.95,1.85)	93.77	Random	< 0.001
Medium quality	4	305	1.35 (0.24,2.45)*	94.52	Random	< 0.001
High quality	5	8523	0.41 (-0.01,0.82)	98.34	Random	< 0.001

(Continued)

TABLE 3 Continued

Variable	N Study	Sample Size	SMD	Heterogeneity		
				I Squared%	Model	**P-Value
CRP[2]						
Overall	6	7517	0.60 (-0.12,1.12)	99.17	Random	< 0.001
Both Sexes	4	2462	0.44 (0.03,0.85)*	99.17	Random	< 0.001
Unadjusted	4	1323	0.37 (0.01,0.74)*	0.86.96	Random	< 0.001
High quality	4	7297	0.45 (-0.28,1.18)	99.40	Random	< 0.001
IL6						
Overall	4	358	0.90 (0.14,1.66)*	90.39	Random	< 0.001
TNF α						
Overall	4	222	0.67 (-0.36,1.70)	92.17	Random	< 0.001

N, Number; NWO, Normal Weight Obesity; SMD, Standardized Mean Difference; CI, Confidence Interval; CRP, C-Reactive Protein; IL, Interleukin; TNF, Tumor Necrosis Factor; hs-CRP, high-sensitivity C-reactive protein.
[1]referes to both types of hs-CRP and regular CRP.
[2]referes to CRP excluding hs-CRP.
*P-Values Under 0.05 Were Considered As Statistically Significant.
**For Heterogeneity P-Values Under 0.1 A Fixed Model Was Used.

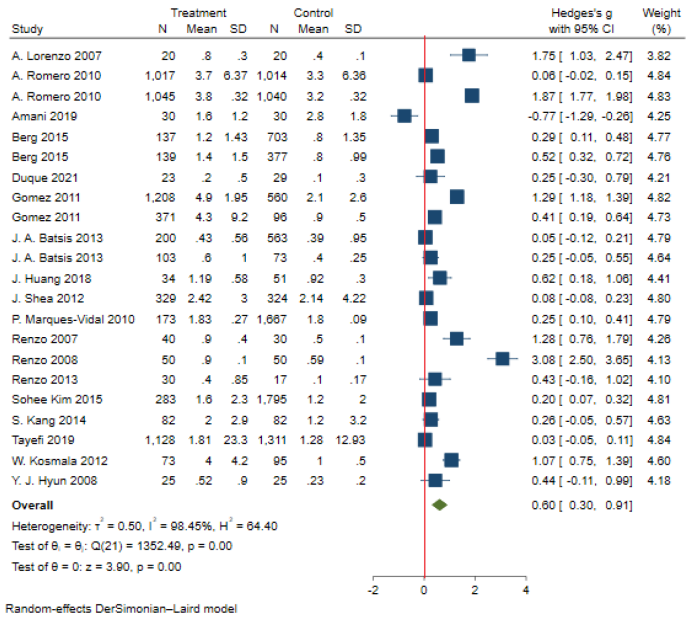

FIGURE 2
The extracted and overall association between NWO and CRP. (The red line represents null effect).

publication bias was seen in articles assessing NWO and TNF α and IL6 (coefficient: 13.42, P-value: 0.004 and coefficient: 7.62, P-value: 0.017 respectively)

Trim fill analysis

Sensitivity analysis was performed on studies assessing NWO and TNFα (SMD: 0.67, 95%CI (-0.36, 1.70) and IL6 (SMD: 0.90, 95%CI (0.14, 1.66), indicating that publication bias did not have a substantial effect on the results.

Discussion

To the best of our knowledge, the current study is the only systematic review and meta-analysis that compared the mean levels of proinflammatory and anti-inflammatory cytokines among NWO and NWNO individuals across the population. Nineteen studies were included in our meta-analysis to address the research questions. And the findings of this study showed that the mean levels of CRP and IL_6 were significantly higher in NWO individuals compared to the NWNO individuals.

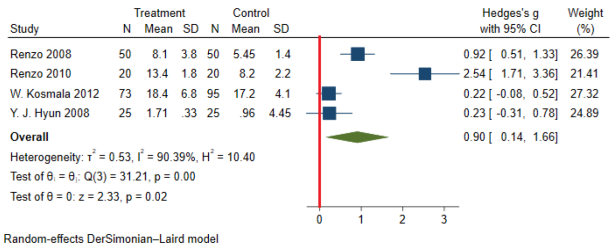

FIGURE 3
The extracted and overall association between NWO and IL 6. (The red line represents null effect).

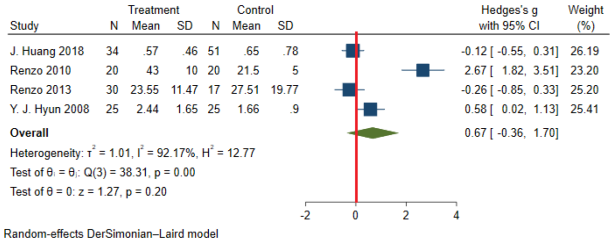

FIGURE 4
The extracted and overall association between NWO and TNF-α. (The red line represents null effect).

Although inflammation is one of the body's essential processes, chronic inflammation is not desirable (42). As chronic inflammation could cause damage to the inflamed site, resulting in metabolic dysregulation, homeostatic mechanisms alteration and even result in some diseases (anemia, various tissue damages, malnutrition, and autoimmune diseases) (42–44). It should be kept in mind that chronic inflammation itself can cause progressive atherosclerosis and cardiovascular diseases through various mechanisms (43, 45). In this regard, although IL_6 is an essential modulator of the immune system and has a wide range of biological activities such as modulating immune responses (46), inflammation and hematopoiesis (46, 47); its increase has been associated with renal injury (48), autoimmune conditions (e.g. rheumatoid arthritis and Crohn's disease), increased risk of cardiovascular disease and increased mortality due to cardiovascular diseases (49, 50). Similarly, it seems that CRP is an important regulator of inflammation and not just a marker (51). Elevated levels of CRP have been seen in autoimmune conditions (e.g. rheumatoid arthritis) and infections and inflammation (51). Furthermore, elevated CRP has been associated with cardiovascular conditions (52), atherothrombosis (53) and atherosclerosis (54). As studies suggest, elevated levels of both IL_6 and CRP have been associated with cardiovascular diseases (55). Hence it seems that alongside the increased adiposity which is a significant risk factor for cardiovascular diseases (56), the increased IL_6 and CRP associated with NWO could have a significant effect on atherosclerosis and cardiovascular diseases as well. Thus by reducing inflammation with various methods (proper nutrition, exercise, etc.), the cardiovascular risk imposed by NWO may be reduced to some degree (8, 16). Moreover, to reduce the comorbidities of NWO, the public should be educated on various types of obesity and the risks that they impose; they should know that the BMI system has its flaws, and a normal BMI does not indicate the absence of obesity.

It should be kept in mind that CRP could be affected by many environmental factors; thus, IL levels could be more suitable for association assessment and research purposes (57).

Limitations and strength

Despite this study being the aggregated data of all studies on inflammation and NWO with a more precise estimation, the majority of the included studies were not adjusted for potential confounders and had a relatively small sample size; hence studies with a greater population and proper adjustments (e.g. age, sex, underlying diseases, inflammatory and infectious conditions prior to testing, etc) are needed to properly evaluate the association of IL_6, CRP and TNFα with NWO; and since many of the included studies were unadjusted the findings of these studies, should be interpreted with caution. Moreover, only IL_6, CRP and TNFα had enough studies making meta-analysis rational. More studies on other aspects of inflammation are needed to evaluate their association with NWO as well.

Conclusion

The present study highlighted the significant association of NWO with CRP and IL6 and showed that these cytokines were significantly higher in NWO individuals compared to the NWNO individuals, pertaining to the presence of some degrees of inflammation among NWOs. Regarding the aligned effect of inflammation and adiposity in the progression of cardiovascular diseases and, most importantly, the flaws in the current BMI system, using other measures alongside BMI, and implementing preventive measures to reduce adiposity and inflammation is

needed. Moreover, more studies on inflammatory markers in NWO individuals are needed to understand their association better.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

NM and MQ, FB, designed the study. NM and SM searched the databases. NM and SA screened and extracted the data. NM and FB analyzed the data and prepared the results. NM and MQ wrote the paper. FB, OT-M and AE edited and revised the manuscript. All other authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1044178/full#supplementary-material

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Effects of anti-inflammatory therapies on glycemic control in type 2 diabetes mellitus

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Background: The overall evidence base of anti-inflammatory therapies in patients with type 2 diabetes mellitus (T2DM) has not been systematically evaluated. The purpose of this study was to assess the effects of anti-inflammatory therapies on glycemic control in patients with T2DM.

Methods: PubMed, Embase, Web of Science, and Cochrane Library were searched up to 21 September 2022 for randomized controlled trials (RCTs) with anti-inflammatory therapies targeting the proinflammatory cytokines, cytokine receptors, and inflammation-associated nuclear transcription factors in the pathogenic processes of diabetes, such as interleukin-1β (IL-1β), interleukin-1β receptor (IL-1βR), tumor necrosis factor-α (TNF-α), and nuclear factor-κB (NF-κB). We synthesized data using mean difference (MD) and 95% confidence interval (CI). Heterogeneity between studies was assessed by I^2 tests. Sensitivity and subgroup analyses were also conducted.

Results: We included 16 RCTs comprising 3729 subjects in the meta-analyses. Anti-inflammatory therapies can significantly reduce the level of fasting plasma glucose (FPG) (MD = - 10.04; 95% CI: -17.69, - 2.40; P = 0.01), glycated haemoglobin (HbA1c) (MD = - 0.37; 95% CI: - 0.51, - 0.23; P < 0.00001), and C-reactive protein (CRP) (MD = - 1.05; 95% CI: - 1.50, - 0.60; P < 0.00001) compared with control, and therapies targeting IL-1β in combination with TNF-α have better effects on T2DM than targeting IL-1β or TNF-α alone. Subgroup analyses suggested that patients with short duration of T2DM may benefit more from anti-inflammatory therapies.

Conclusion: Our meta-analyses indicate that anti-inflammatory therapies targeting the pathogenic processes of diabetes can significantly reduce the level of FPG, HbA1c, and CRP in patients with T2DM.

KEYWORDS

type 2 diabetes, anti-inflammatory therapies, antidiabetic drug, clinical trial, meta-analyses

1 Introduction

Obesity and type 2 diabetes mellitus (T2DM) are associated with decreased physical activity and unhealthy high-calorie diets. Obesity is related to insulin resistance and is a crucial risk factor for the development of T2DM (1). Chronic low-grade inflammation plays an important role in the pathogenesis of diabetes and the development of diabetic complications (2, 3). Inflammation has been seen in the pancreatic islets, liver, muscle, adipose tissue, and the sites of diabetic complications (4). Long-term inflammation that occurs in adipose tissue can lead to systemic inflammation and contribute to insulin resistance. In the presence of insulin resistance, β cells secrete more insulin to maintain normal glucose control. Inflammation impairs β cell function and induces β cell apoptosis, and T2DM happens when insulin production fails to reach the insulin needs (5).

Many proinflammatory cytokines and inflammation-associated nuclear transcription factors are related to impaired insulin secretion and contribute to the pathogenesis of T2DM, including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and nuclear factor-κB (NF-κB) etc. (4, 6–8). High concentration glucose can induce IL-1β production and secretion from human pancreatic β cells, and IL-1β was observed in β cells in diabetic patients (9). IL-1β is involved in β cell apoptosis and partially dependent on the activation of NF-κB (10). Obesity can activate the NF-κB signaling pathway, which plays an important role in the development of insulin resistance (8). TNF-α is also involved in β cell apoptosis (11), and more TNF-α expression was found in adipose tissue in obese than lean people, and the plasma level of TNF-α was elevated in patients with T2DM (6, 7).

Anti-inflammatory treatments can improve insulin sensitivity and β cell function in patients with insulin resistance or T2DM (12). Treatments of diabetes focused on inflammation can benefit many inflammatory tissues at the same time, which is less likely to induce hypoglycemia (13). Small molecules or antibody-based molecules targeting inflammatory cytokines, cytokine receptors, or inflammation-associated nuclear transcription factors, such as IL-1β, interleukin-1β receptor (IL-1βR), NF-κB, and TNF-α, can improve metabolism (13, 14). But the effects of anti-inflammatory therapies on glycemic control in patients with T2DM were controversial (15–19). Previous meta-analyses have assessed the effects of anti-IL-1 therapies on T2DM (20, 21). However, the totality of the evidence base of the anti-inflammatory therapies on T2DM has not been systematically assessed. We conducted the meta-analyses to clarify the effects of anti-inflammatory therapies on glycemic control in patients with T2DM.

2 Methods

The meta-analyses were performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (22).

2.1 Search strategy

We searched randomized controlled trials (RCTs) from PubMed, Embase, Web of Science and Cochrane Library from database inception up to 21 September 2022. Search terms include Medical Subject Headings (MeSH), keywords and free-text terms related to anti-inflammatory therapies, type 2 diabetes mellitus, T2DM, fasting plasma glucose, FPG, glycated haemoglobin, HbA1c, C-reactive protein, CRP, anakinra, canakinumab, diacerein, gevokizumab, LY2189102, tocilizumab, salsalate, salicylate, etanercept, remicade, infliximab, adalimumab, enbrel, and dapansutrile. The detailed search strategy is available in Table S1. Following the search and removal of duplicates, D Li and J Zhong screened titles and abstracts to identify relevant studies.

2.2 Study selection

Studies were eligible for inclusion if they met the following criteria (1): Participants: patients with T2DM; (2) Interventions: at least one of the following treatments was used, anakinra, canakinumab, diacerein, gevokizumab, LY2189102, tocilizumab, salsalate, salicylate, etanercept, remicade, infliximab, adalimumab, enbrel, or dapansutrile; (3) Controls: placebo with or without approved antidiabetic medications, such as metformin, sulfonylureas, and insulin etc.; (4) Outcomes: at least one of the following outcomes was reported, FPG, HbA1c, or CRP; (5) Studies: RCTs. Trials without accessible data or full text were excluded.

2.3 Data extraction

Data extraction and analyses from included studies were performed by two authors independently, and conflicts were resolved by a third author. The following information was extracted: first author, publication year, agent, dosage and frequency, follow-up duration, number of participants, patient baseline information (mean age, sex distribution, diabetes duration, baseline BMI, and HbA1c) and outcomes of interest (follow-up FPG, HbA1c, and CRP).

2.4 Risk of bias assessment

Risk of bias assessment of the included RCTs was carried out by two authors (D Li and Q Zhang) independently according to the Cochrane Collaboration's Risk of Bias Tool, which including random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting, and other sources of bias.

2.5 Data analyses

Continuous variables were expressed as mean difference (MD) with 95% confidence interval (CI). When mean and SD were not available, we calculated from SEM, sample size, median, range, or interquartile range (IQR) using methodology from the Cochrane Library Handbook or the article written by Wan et al. (23, 24). Several studies had more than one intervention groups with different dosages, and for these studies, we chose only one comparable dosage as motioned in Table 1. Statistical heterogeneity among studies was assessed with the I^2 statistic, considering the I^2 value of 50 - 75% was moderate heterogeneity and above 75% was high heterogeneity (25). We performed subgroup analyses based on the targets of interventions, names of the medication, diabetes duration, follow-up duration, and drug administration regimen. Leave-one-out studies were performed for sensitivity analyses to examine the effect of each trial on the overall analyses. Funnel plot and Egger's test were used to assess the publication bias and tested for statistical significance. All statistical analyses were performed using Review manager 5.3 and Stata 12.0. A value of $p \leq 0.05$ was considered statistically significant.

3 Results

3.1 Included studies and baseline characteristics

Figure 1 shows the details of the literature search and selection process. Of 1271 reports identified, 241 reports were excluded due to duplication, and 981 were excluded based on titles and abstracts. Of 49 reports reviewed in full, 33 were excluded based on eligibility criteria. A total of 16 reports involving 3729 participants with T2DM were included in the final analyses (15–19, 26–36). Table 1 shows the baseline characteristics of the 16 RCTs. Trials included were published between 2005 and 2022. The follow-up duration was between 1 and 48 months. Trails reported by Everett et al. (28) had the longest follow-up duration (48 months), which was not comparable with all the others, and a more comparable time point (6 months) was used in the subsequent analyses. Among the 16 trails, 4 trails were for canakinumab (17, 28, 32, 34), 5 trails for diacerein (15, 16, 26, 33, 36), 3 trails for salsalate (29–31), and the rest were for anakinra (18), gevokizumab (27), LY2189102 (35), and etanercept (19). The dosage and frequency of the treatments are shown in Table 1.

3.2 Risk of bias of individual studies

The quality of the included trials was assessed according to the criteria of the Cochrane Handbook. A detailed evaluation of the risk of bias for each clinical trial and risk of bias summary are presented in Figure S1. Among the 16 RCTs, only 1 was judged to be at high risk of bias as an open-label randomized trial (19), 6 were judged to be at low risk of bias and 9 as being at unclear risk of bias. Unclear risks were related to selection bias, reporting bias, and other bias.

3.3 Meta-analyses

3.3.1 FPG

Figure 2 shows anti-inflammatory therapies can significantly decrease the level of FPG (n = 12; MD = - 10.04; 95% CI: - 17.69, - 2.40; P =0.01) compared with control, and there was statistically significant heterogeneity among studies (I^2 = 77%; P < 0.00001) (Figure 2A). We did a series of subgroup analyses of FPG based on the targets of interventions, diabetes duration, and follow-up duration. Subgroup analyses based on the targets of interventions show that drugs targeting IL-1β plus TNF-α (diacerein) (n = 5; MD = - 13.52; 95% CI: - 23.77, - 3.27; P =0.01) or NF-κB alone (salsalate) (n = 3; MD = - 22.03; 95% CI: - 34.59, - 9.47; P =0.0006) can significantly decrease the level of FPG compared with control, whereas drugs targeting IL-1β (canakinumab) or TNF-α (etanercept) alone had no significant effect on the change of FPG (Figure 2B). Patients with T2DM less than 3 years since diagnosis (n=2, MD = - 20.64; 95% CI: - 32.03, - 9.25; P =0.0004) seem to benefit more from anti-inflammatory therapies than those between 3 and 10 years (n=3, MD = - 14.79; 95% CI: - 28.69, - 0.89; P =0.04), and no significant effect was found in those more than 10 years (n=4, MD = - 7.94; 95% CI: - 20.17, 4.3; P =0.2) (Figure S2A). Anti-inflammatory therapies can decrease the level of FPG in patients whose follow-up duration was less than or equal to 3 months (n=6, MD = - 19.01; 95% CI: - 28.57, -9.45; P < 0.0001), but no significant effect was found in patients with longer follow-up duration (Figure S2B).

3.3.2 HbA1c

The change in HbA1c was assessed in all studies. Figure 3A shows anti-inflammatory therapies can significantly decrease the level of HbA1c (n = 16; MD = - 0.37; 95% CI: - 0.51, - 0.23; P < 0.00001) with moderate heterogeneity among studies (I^2 = 69%; P < 0.0001) (Figure 3A). The sensitivity analyses of HbA1c indicated the stability of the results (Figure S3). Subgroup analyses based on the targets of the interventions show that drugs targeting IL-1β plus TNF-α (diacerein) (n=5; MD = - 0.63; 95% CI: - 1.08, - 0.19; P =0.005) can reduce the level of HbA1c better than targeting IL-1β (gevokizumab, canakinumab, anakinra, or LY2189102) (n=7; MD = - 0.25; 95% CI: - 0.42, - 0.08; P = 0.004) or TNF-α (etanercept) (n=1; MD = 0.00; 95% CI: - 0.88, 0.88; P =1.00) alone (Figure 3B). Anti-inflammatory therapies targeting NF-κB (salsalate) (n = 3; MD = - 0.40; 95% CI: - 0.59, - 0.20; P < 0.0001) can significantly decrease the level of HbA1c compared with control, and there was no heterogeneity among studies (I^2 = 27%; P = 0.25). Subgroup analyses according to the name of the medications show in Figure S4A, gevokizumab (n = 1; MD = - 0.85; 95% CI: - 1.60, - 0.10; P =0.03) can reduce the level of HbA1c more than diacerein (n = 5; MD = - 0.63; 95% CI: - 1.08, - 0.19; P =0.005), anakinra (n =1; MD = - 0.46; 95% CI: - 0.61, - 0.31; P < 0.00001), salsalate (n = 3; MD = - 0.40; 95% CI: - 0.59, - 0.20; P < 0.0001), and canakinumab (n = 4; MD = - 0.11; 95% CI: - 0.21, - 0.02; P = 0.02). LY2189102 and etanercept had no significant effect on HbA1c compared with the control. Subgroup analyses based on diabetes duration show that more reduction of HbA1c was seen in patients with T2DM less than 3 years since diagnosis (n = 2, MD = -1.54; 95%

TABLE 1 Baseline characteristics of included studies [a].

First author, year	Agent	Target and mechanism of action	Dosage, frequency	Study duration	Patients randomized, n	Age, years	Male sex, %	Duration of diabetes, year	Baseline BMI	Baseline HbA1c, %
Cardoso CRL 2017[26]	diacerein	TNF-α antagonism in combination with IL-1βR blockade	100 mg/day	48 weeks	84	63.7	20	9	31.3	8.2
Cavelti-Weder C 2012[27]	gevokizumab	IL-1β-specific antibodies	a single dose of 0.03 or 0.1 mg/kg	3 months	48	50	82	9.7	31	9.1
Choudhury RP 2016[17]	canakinumab	IL-1β-specific antibodies	150 mg monthly	12 months	189	61.9	80	–	30.3	6.85
Dominguez H 2005[19]	etanercept	TNF-α inhibition	25 mg twice weekly	4 weeks	19	55	55.6	–	32	7.6
Everett BM 2018[28]	canakinumab	IL-1β-specific antibodies	150 mg once every 3 months	48 months	2303	61	77	–	29.1	7.1
Faghihimani E 2013[29]	salsalate	NF-κB inhibition	3 g/day	12 weeks	60	50.8	–	within 2 months[b]	29.2	5.9
Goldfine AB 2010[30]	salsalate	NF-κB inhibition	3 g/day	14 weeks	54	55.9	55.6	5.1	34	7.8
Goldfine AB 2013[31]	salsalate	NF-κB inhibition	3.5 g/day	48 weeks	286	55.8	52.9	4.9	33.2	7.7
Jangsiripornpakorn J 2022[16]	diacerein	TNF-α antagonism in combination with IL-1βR blockade	50 mg/day	12 weeks	35	52	47.1	11.4	29.5	8.5
Larsen CM 2007[18]	anakinra	IL-1 receptor antagonism	100 mg/day	13 weeks	69	60.3	77.1	11	31.8	8.2
Noe A 2014[32]	canakinumab	IL-1β-specific antibodies	a single dose of 10 mg/kg	24 weeks	86	57.5	68.9	5.1	30.8	7.8
Piovesan F 2017[33]	diacerein	TNF-α antagonism in combination with IL-1βR blockade	50 mg twice daily	90 days	72	62.5	23	13.4	–	8.9
Ramos-Zavala MG 2011[15]	diacerein	TNF-α antagonism in combination with IL-1βR blockade	50 mg once or twice daily	2 months	40	47.8	40	within 6 months[c]	30.8	7.9
Ridker PM 2012[34]	canakinumab	IL-1β-specific antibodies	150 mg/month	4 months	271	54.3	59	4	29.3	7.4

(Continued)

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TABLE 1 Continued

First author, year	Agent	Target and mechanism of action	Dosage, frequency	Study duration	Patients randomized, n	Age, years	Male sex, %	Duration of diabetes, year	Baseline BMI	Baseline HbA1c, %
Sloan-Lancaster J 2013[35]	LY2189102	IL-1β-specific antibodies	180 mg/week	24 weeks	42	52.9	25.9	8	32.5	7.9
Tres GS 2018[36]	diacerein	TNF-α antagonism in combination with IL-1βR blockade	50 mg twice daily	12 weeks	71	59	75	14.8	31.3	8.6

Values are given as mean or where not available median.
BMI, body mass index (kg/m^2); HbA1c, glycated haemoglobin; T2DM, type 2 diabetes mellitus.
[a]Baseline values are presented for the placebo group.
[b]Patients were diagnosed with T2DM within 2 months.
[c]Patients were diagnosed with T2DM within 6 months.

CI: - 2.04, - 1.04; P < 0.00001) than those between 3 and 10 years (n = 6, MD = - 0.32; 95% CI: - 0.43, - 0.21; P < 0.00001), and those more han 10 years (n = 5, MD = - 0.44; 95% CI: - 0.56, - 0.31; P < 0.00001) (Figure S4B). Anti-inflammatory therapies were more effective in patients whose follow-up duration was less than or equal to 3 months (n = 7, MD = - 0.71; 95% CI: - 1.16, - 0.26; P = 0.002) (Figure S4C). Repeated drug administration regimen (n = 14, MD = - 0.37; 95% CI: - 0.52, -0.21; P < 0.00001) and single dosing (n = 2, MD = - 0.45; 95% CI: - 1.01, 0.10; P = 0.11) had similar effects on HbA1c (Figure S4D).

3.3.3 CRP

Figure 4 shows anti-inflammatory therapies can decrease the level of CRP compared with control (n = 6; MD = - 1.05; 95% CI: - 1.50, - 0.60; P < 0.00001), and there was high heterogeneity among studies (I^2 = 77%; P = 0.0007) (Figure 4A). Subgroup analyses based on the targets of interventions show that drugs targeting IL-1β (canakinumab) can significantly reduce the level of CRP (n = 3; MD = - 1.31; 95% CI: - 1.63, - 0.99; P < 0.00001), whereas no significant effect was found in drugs targeting IL-1β plus TNF-α (diacerein) (n = 2; MD = - 1.95; 95% CI: - 4.39, 0.49; P =0.12) or NF-κB (salsalate) (n = 1; MD = - 0.24; 95% CI: - 0.80, 0.32; P =0.40) (Figure 4B).

3.3.4 Publication bias

Egger's test for HbA1c suggested significant publication bias (p = 0.003) (Figure S5). However, the effect was the same as the original effect after using Duval and Tweedie's trim and fill, and the result showed that no trimming was performed, and the data stayed unchanged.

4 Discussion

Our meta-analyses of 16 RCTs published between 2005 and 2022 examined the effects of anti-inflammatory therapies on glycemic control in patients with T2DM. Two previous meta-analyses published in 2018 and 2019, concluded that anti-IL-1 therapies can significantly decrease the level of HbA1c and CRP, and have mild hypoglycaemic effect on patients with T2DM (20, 21). However, the effects of anti-inflammatory therapies targeting other inflammatory molecules and the overall effects of anti-inflammatory therapies on T2DM remain to be discovered. Therefore, we performed further analyses of anti-inflammatory therapies based on different inflammatory targets, including IL-1β, IL-1βR, TNF-α, and NF-κB. Our results show that anti-inflammatory therapies, including anti-IL-1 therapies, can significantly decrease the level of FPG, HbA1c and CRP in patients with T2DM. Our findings indicate the clinical efficacy of treating T2DM based on the pathogenesis of diabetes and give suggestions for the future anti-inflammatory clinical trials.

Chronic low-grade inflammation was found in diabetic islets, with increased innate immune cell infiltration and cytokine secretion (37). Immune cell infiltration and cytokine release directly impairs β cell mass and function (38). IL-1β was the first

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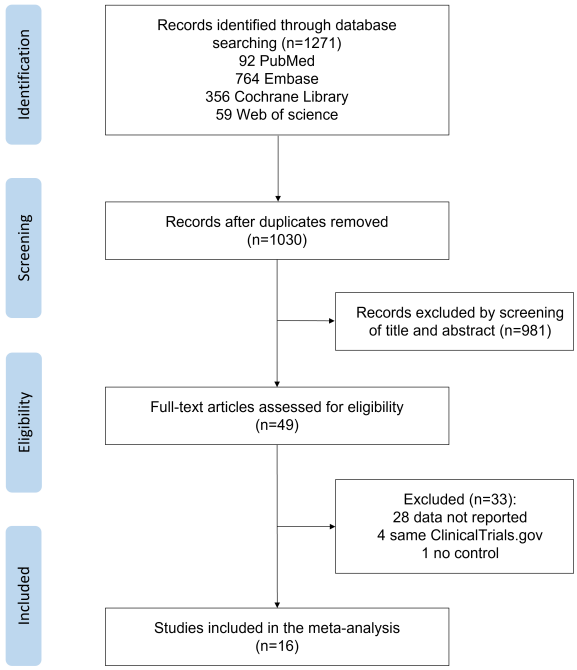

FIGURE 1
Flowchart diagram of study selection process.

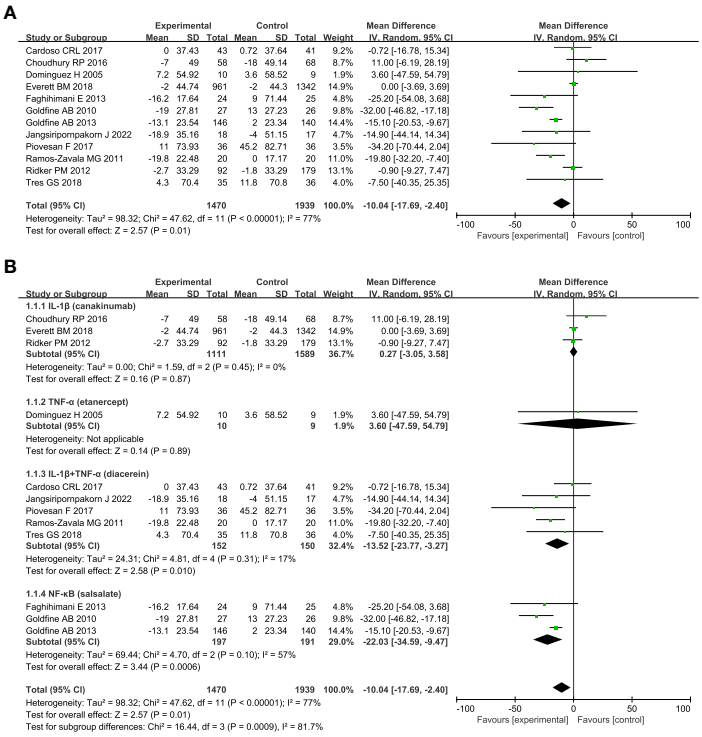

FIGURE 2
Forest plot of pooled mean difference in change in FPG (mg/dL). **(A)** Meta-analyses of the effects of anti-inflammatory therapies on FPG in patients with T2DM; **(B)** The forest plot of FPG in subgroup analyses defined by the targets of interventions. fasting plasma glucose, FPG; CI, confidence interval; IV, inverse variance; SD, standard deviation.

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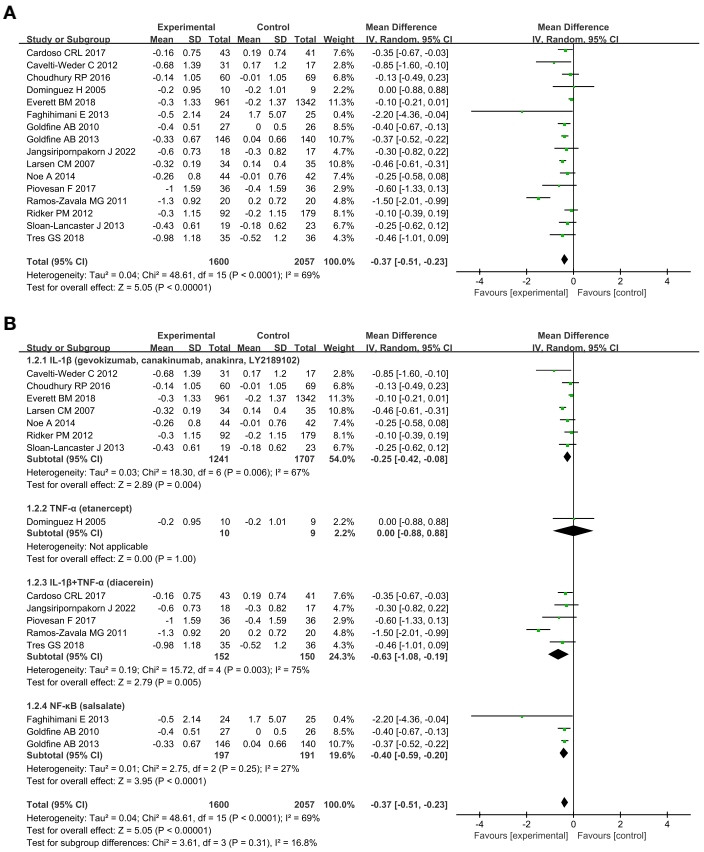

FIGURE 3
Forest plot of pooled mean difference in change in HbA1c (%). **(A)** Meta-analyses of the effects of anti-inflammatory therapies on HbA1c in patients with T2DM; **(B)** The forest plot of HbA1c in subgroup analyses defined by the targets of interventions. glycated haemoglobin, HbA1c; CI, confidence interval; IV, inverse variance; SD, standard deviation.

described proinflammatory cytokine in the islets of patients with T2DM (39). IL-1β impairs β cell function and induces the apoptosis of β cells (40). Block IL-1β signaling pathway by antagonists or antibodies had beneficial effects on β cell function and glycemic control in patients with T2DM (41, 42). Anakinra, a recombinant human IL-1βR antagonist, can significantly reduce the level of HbA1c and may improve glycemic control by increasing insulin secretion (18). Canakinumab, gevokizumab and LY2189102 are recombinant human engineered monoclonal antibodies, which can neutralize the activity of IL-1β by forming a complex with circulating IL-1β. Canakinumab can also reduce the blood levels of IL-6 and CRP (17). All the anti-IL-1β therapies mentioned above had significant effect on glucose control as reflected by reductions in HbA1c, which was also reported by previous meta-analyses (20, 21). However, some of the beneficial effects were only detected by certain treatment periods, not the whole follow-up periods (28, 35). As shown in our subgroup analyses, anti-inflammatory therapies may work better in patients with short follow-up duration (less than or equal to 3 months). LY2189102 can improve blood glucose control for 12 weeks, but the effect was attenuated over time and there was no difference at 24 weeks (35). The study reported by Everett BM et al. showed that canakinumab can reduce HbA1c during the first 6 to 9 months of treatment, but no significant effect was found by the end of the follow-up period at 48 months (28). The exact reason for this attenuation is unclear, but the availability of other antidiabetic therapies and lifestyle interventions may contribute to this phenomenon (28).

TNF-α can diminish glucose-dependent insulin secretion and impair the function of β cells both *in vitro* and *in vivo* (43, 44). But etanercept, a TNF-α inhibitor, has no significant effect on FPG or HbA1c (19). Etanercept can improve the glucose tolerance of some individuals, but no significant effect was found in the whole group (19). It was difficult to say whether etanercept has a positive effect on β cells since no more than 20 individuals was included in this clinical trial, and studies with a larger number of patients with T2DM are needed to elucidate this issue.

Diacerein is both an IL-1βR blocker and a TNF antagonist. It can inhibit the synthesis and activity of IL-1 and TNF-α by its active metabolite rhein (45). Diacerein can reduce the HbA1c level without affecting the homeostasis model assessment-insulin resistance (HOMA-IR), indicating that it may play a role in insulin secretion (36). And a higher dosage of diacerein (100 mg/day) may be more effective in improving the glycemic outcome (16). Our results show that interventions targeting IL-1β plus TNF-α can reduce the level of HbA1c better than targeting IL-1β or TNFα alone in patients with T2DM. Diacerein had no significant effect on

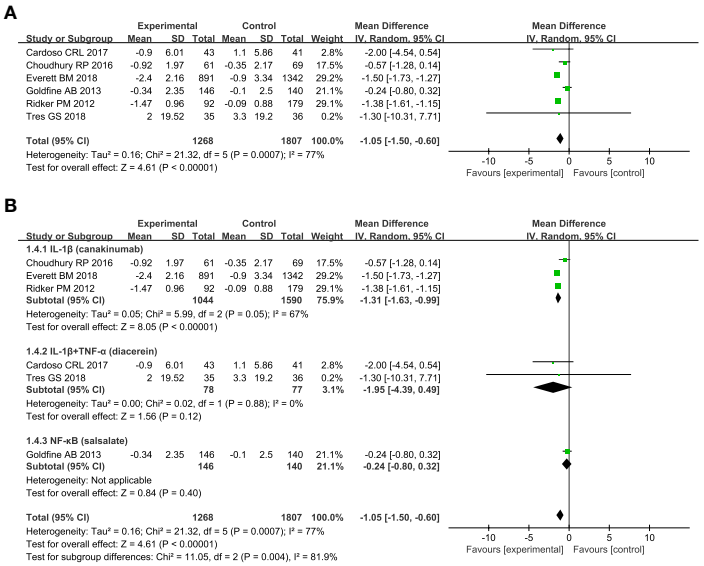

FIGURE 4
Forest plot of pooled mean difference in change in CRP (mg/L). **(A)** Meta-analyses of the effects of anti-inflammatory therapies on CRP in patients with T2DM; **(B)** The forest plot of CRP in subgroup analyses defined by the targets of interventions. C-reactive protein, CRP; CI, confidence interval; IV, inverse variance; SD, standard deviation.

CRP in patients with T2DM, though reduced TNF-α was observed (26, 36). Those studies were carried out in patients with longer duration of diabetes, and most participants were undergoing treatment with metformin, statins, sulfonylureas, or renin-angiotensin system blockers, which have potential roles in anti-inflammation, and might attenuate the anti-inflammatory effect of diacerein (13, 26, 36).

Salsalate, a prodrug form of salicylate, shows anti-inflammatory effects by inhibiting the IKKb/NF-κB and JNK signaling pathways (46, 47). Salsalate can improve glycemic control by affecting cellular kinases nonspecifically and increasing insulin secretion of β cells (48). After 1 year treatment, salsalate still had effects on HbA1c and FPG in patients with T2DM (31). Salsalate can decrease the level of inflammatory mediators, such as leukocytes, neutrophils, and lymphocytes, but had little effect on CRP in patients with T2DM (31). T2DM seems to result from a long-term process of inflammation, even years before diagnosis (35). Greater benefits of salsalate might be seen in patients with newly diagnosed T2DM or longer treatment duration.

Our results show that patients with newly diagnosed T2DM may benefit more from anti-inflammatory therapies. However, Kataria Y et al. reported that the effects of anti-IL-1β therapies depend on the baseline dysmetabolic status, and patients with a more metabolic imbalance at baseline may benefit more after treatment (21). The differences between our studies may come from the different types of medications analyzed, as we included lots of anti-inflammatory medications, not just IL-1β antibodies and IL-1βR antagonists. Since no newly diagnosed T2DM patients were included in the studies of anti-IL-1β therapies, the effects of anti-IL-1β therapies on those patients remain to be seen.

There are some limitations in our study. First, lifestyle modification and antidiabetic medications were allowed in most of the included trials, which may affect or attenuate the efficacy of anti-inflammatory therapies. Second, most of the follow-up duration varied from 1 to 12 months, and longer clinical trials are needed since medication efficacy may change over time. Finally, publication bias exists in the meta-analyses, but the results stay the same after a trim and fill analysis.

5 Conclusions

This study helps us better understand the possibility and efficiency of anti-inflammatory therapies for T2DM based on the pathogenetic processes of the disease. The present analyses demonstrated that targeting cytokines, cytokine receptors, and inflammation-associated nuclear transcription factors, such as IL-1β, IL-1βR, TNF-α, and NF-κB, alone or in combination can significantly reduce the level of FPG, HbA1c, and CRP in patients with T2DM. In addition, patients with a short duration of T2DM may benefit more from anti-inflammatory therapies. Since anti-inflammatory medications can reduce inflammation throughout the body, these medications may be used to treat diseases with similar pathologies, such as cardiovascular disease, chronic kidney disease, and rheumatic arthritis with or without T2DM.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

DL and JinZ conceived and designed the study. DL and JiaZ did the scientific literature search and data extraction of the included studies. DL and QZ did the quality assessment and carried out the analyses. DL wrote the first draft of the present manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Transcriptomic analysis reveals shared gene signatures and molecular mechanisms between obesity and periodontitis

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Background: Both obesity (OB) and periodontitis (PD) are chronic non-communicable diseases, and numerous epidemiological studies have demonstrated the association between these two diseases. However, the molecular mechanisms that could explain the association between OB and PD are largely unclear. This study aims to investigate the common gene signatures and biological pathways in OB and PD through bioinformatics analysis of publicly available transcriptome datasets.

Methods: The RNA expression profile datasets of OB (GSE104815) and PD (GSE106090) were used as training data, and GSE152991 and GSE16134 as validation data. After screening for differentially expressed genes (DEGs) shared by OB and PD, gene enrichment analysis, protein-protein interaction (PPI) network construction, GeneMANIA analysis, immune infiltration analysis and gene set enrichment analysis (GSEA) were performed. In addition, receiver operating characteristic (ROC) curves were used to assess the predictive accuracy of the hub gene. Finally, we constructed the hub gene-associated TF-miRNA-mRNA regulatory network.

Results: We identified a total of 147 DEGs shared by OB and PD (38 down-regulated and 109 up-regulated). Functional analysis showed that these genes were mainly enriched in immune-related pathways such as B cell receptor signalling, leukocyte migration and cellular defence responses. 14 hub genes (FGR, MNDA, NCF2, FYB1, EVI2B, LY86, IGSF6, CTSS, CXCR4, LCK, FCN1, CXCL2, P2RY13, MMP7) showed high sensitivity and specificity in the ROC curve analysis. The results of immune infiltration analysis showed that immune cells such as macrophages, activated CD4 T cells and immune B cells were present at high infiltration levels in both OB and PD samples.The results of GeneMANIA analysis and GSEA analysis suggested that five key genes (FGR, LCK, FYB1, LY86 and P2RY13) may be strongly associated with macrophages. Finally, we constructed a TF-miRNA-mRNA regulatory network consisting of 233 transcription factors (TFs), 8 miRNAs and 14 mRNAs based on the validated information obtained from the database.

Conclusions: Five key genes (FGR, LCK, FYB1, LY86, P2RY13) may be important biomarkers of OB and PD. These genes may play an important role in the pathogenesis of OB and PD by affecting macrophage activity and participating in immune regulation and inflammatory responses.

KEYWORDS

obesity, periodontitis, bioinformatics, transcriptomic analysis, immune processes, macrophage infiltration, inflammation

Introduction

Obesity (OB) is a complex, multifactorial chronic inflammatory disease characterized by abnormal or excessive deposition of fat in adipose tissue (1). It is also a major risk factor for many diseases, including type 2 diabetes, cardiovascular disease, osteoarthritis and certain cancers (2). The prevalence of OB has tripled in the last few decades (3). The number of people with OB worldwide was reported to be as high as 671 million (12% of the world's adult population) in 2016 (4). Periodontitis (PD) is one of the most common chronic multifactorial inflammatory diseases affecting the global population, leading to loss of connective tissue attachment, alveolar bone erosion, tooth loss and systemic inflammation (5, 6). There is evidence that OB increases susceptibility to PD (7). An earlier study reporting the association between OB and PD found changes in periodontal tissue in addition to greater alveolar bone resorption in obese rats compared to non-obese rats (8). Several recent studies have also suggested a comorbid effect between OB and PD (9, 10). OB increased the risk of PD by two to three times and was independent of traditional risk factors, including smoking, age, and gender (11). Animal studies have indicated that an increased alveolar bone loss in obese animals with PD and significantly greater alveolar bone loss in obese rats than in lean controls (12, 13). In addition, obese individuals who consume an excessively high-fat diet have an enhanced metabolic response to PD and show a metabolic susceptibility to increased periodontal destruction (14). These findings highlighted the existence of an association between OB and PD. However, the molecular mechanism of this association is still unknown. Therefore, exploring the common genetic features of OB and PD and their potential molecular mechanisms holds great promise for the diagnosis and treatment of OB and PD co-morbidities.

A growing body of clinical and experimental evidence suggests that immune cell infiltration and inflammatory factors play a critical role in the development of OB or PD (2, 14). On the one hand, mouse models of OB and diabetes were found to be characterized by impaired T and B lymphocyte-mediated immune responses (2). A recent study reported that natural killer T cells are regulators of adipose tissue inflammation in OB (15). Osborn O et al. (16) pointed that in the obesity-induced inflammatory response, immune cells are recruited and cause adipose tissue inflammation. Monocytes receive chemotactic signals and translocate into adipose tissue, polarizing it to a highly pro-inflammatory M1-like state. Once recruited, these M1-like macrophages secrete pro-inflammatory cytokines and act in a paracrine manner (16). In addition, OB induces a shift in the adipose tissue T-cell population, with decreased Treg content and increased CD4+ TH1 and CD8+ effector T cells that secrete pro-inflammatory cytokines (15). Recent studies have also indicated that increased B-cell numbers can promote T-cell activation and enhance M1-like macrophage polarization, inflammation, and insulin resistance (17). Notably, cytokines and chemokines from adipose tissue can also be released into the circulation and promote inflammation in other tissues in an endocrine manner (18). Meanwhile, T cells in adipose tissue are thought to play a role in obesity-induced inflammation by altering the number of adipose tissue macrophages and their activation status (19, 20). Nishimura et al. (21) showed that CD8+ T cells were increased in obese adipose tissue and promote the recruitment and activation of adipose tissue macrophages (21). On the other hand, Dutzan N et al. (22) revealed that IL-21, IL-1β, IL-17 and IL-23p19 were significantly overexpressed in periodontal disease tissues compared to healthy gingival tissues. In particular, IL-21 was overexpressed in chronic periodontitis gingival tissues and was associated with pro-inflammatory cytokines for periodontal destruction (22). IL-10 and TGF-β1 expression were down-regulated in periodontal lesions and may be regulators of inflammation and alveolar bone loss in periodontal disease (23). In addition, some researchers suggested that one of the mechanisms related to PD and OB is the increase in the production of inflammatory cytokines, i.e., OB leads to an increase in the inflammatory stimulation of adipose tissue, adipocytes secrete adipocytokines, which increases the release of inflammatory cytokines, thus leading to the imbalance between the reduction of anti-inflammatory mechanisms and persistent low-grade inflammation (24). For example, in humans, plasma levels of tumor necrosis factor α, interleukin-6 and C-reactive protein are strongly associated with OB (25). In a mouse model of OB/type 2 diabetes, resolvin E1 increased neutrophil phagocytosis in wild-type mice with Pseudomonas gingivalis, but had no effect on type 2 diabetic mice (26). Therefore, we speculate that immune factors and inflammatory responses may be one of the important reasons for the occurrence of OB and PD.

A number of previous studies have explored the potential impact of OB on the pathogenesis and progression of PD, highlighting the importance of common inflammatory processes and immune dysfunction, but the immune-related mechanisms

involved in OB and PD remain to be elucidated. The aim of this study was to explore the molecular link between OB and PD using publicly available transcriptomic data. We used an integrated bioinformatics approach to study immune cell infiltration, reveal molecular regulatory networks, and explore the molecular mechanisms underlying the interaction of disease onset, hoping to provide new perspectives on the biological mechanisms of obesity-associated periodontitis.

Materials and methods

Data collection and processing

We searched the Gene Expression Omnibus (GEO) database for gene expression profiles of obesity and periodontitis using the keywords "periodontitis" and "obesity". Four data sets met the inclusion criteria: (1) the experimental data type was microarray or high-throughput sequencing; (2) the number of samples per cohort was greater than six; (3) the study samples were from humans. We used the GSE104815 and GSE106090 datasets as the discovery cohort for transcriptome analysis, and the GSE152991 and GSE16134 datasets as the validation cohort. For OB, the GSE104815 dataset contained 4 OB samples and 4 non-obese samples; the GSE152991 dataset contained 34 OB samples and 11 control samples. For PD, the GSE106090 dataset contained 18 samples, of which 6 PD samples and 6 healthy samples were selected, while 6 peri-implantitis samples were excluded; the GSE16134 dataset contained 241 PD samples and 69 control samples. For the samples in these datasets, we excluded the effects of medical history and medication, as these effects may introduce bias in our study. Finally, we used the limma/DEseq2 package to filter, log2 transform, and normalize all datasets. Among these, probes were annotated as gene symbols, and for genes matching multiple probes, the probe with the highest expression value was retained. Details of the platforms, experiment types and tissues of the four datasets are shown in Supplementary Table 1.

Screening for differentially expressed genes

The empirical Bayesian approach in the limma package (27) in R was applied to screen the GSE106090 and GSE104815 datasets. Significant differentially expressed genes (DEGs) were identified based on the cutoff criteria of $|\log2FoldChange| \geq 1$ and adjusted p-value < 0.05, and the common DEGs between OB and PD were obtained by the intersection of the plotted Venn diagram using the ggVennDiagram (28) package in R.

Functional enrichment analysis

To further reveal the functions of the common DEGs, Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed. The biological properties of the DEGs were annotated as molecular function (MF), biological process (BP) and cellular component (CC) by GO enrichment analysis using the clusterProfiler (29) package in R, and the KEGG analysis was performed using the KOBAS (30) online tool. An adjusted p-value < 0.05 was set as the cut-off criterion.

Identification of hub genes

Based on the common DEGs in PD and OB, the STRING (search tool for the retrieval of interacting genes) database (https://string-db.org/) was used to construct the PPI network, whose confidence score was set to the middle value (confidence score ≥ 0.4), and then Cytoscape software (31) was used to visualize the PPI network. The MCODE plugin (32) of Cytoscape was applied for module analysis to identify the key gene clusters, and the CytoHubba plugin (33) was used to identify the hub genes, through which four methods including maximal clique centrality (MCC), density of maximum neighborhood component (DMNC), degree and maximum neighborhood component (MNC) (34) were applied to identify the top 30 hub genes in the PPI network, respectively, and the genes obtained by taking the intersection of four gene lists were considered as hub genes.

Validation and efficacy evaluation of hub genes

The expression matrics of GSE16134 and GSE152991 were downloaded from the GEO database and the GREIN platform (http://www.ilincs.org/apps/grein/) (35), respectively, which were used to validate the expression levels of the hub genes. GSE16134 contains 241 PD and 69 control samples, and GSE152991 contains 34 OB and 11 control samples. Considering that our data do not conform to a normal distribution, the Wilcoxon test was used to compare the two groups in the two datasets defining a p-value < 0.05 as significant. Meanwhile, the pROC package (36) in R was used to plot receiver operating characteristic (ROC) curves to verify the validity and predictive accuracy of the hub genes. The hub genes with an area under the curve (AUC) > 0.7 were considered useful for disease diagnosis (37).

GeneMANIA analysis

We used the GeneMANIA online website (38) to analyze the hub genes and their co-expressed gene network by entering the validated hub genes directly into the GeneMANIA website for query, and GeneMANIA will find functionally similar genes using a wealth of genomics and proteomics data, while also reporting the weights of the predicted values for each gene used for query, and the results will show in which biological terms and pathways the genes are enriched.

Immune infiltration analysis

Immune infiltration analysis was performed using the ssGSEA (single sample gene set enrichment analysis) algorithm (39) for GSE15299 (OB) and GSE16134 (PD), respectively. The ssGSEA algorithm is an extension of the gene set enrichment analysis (GSEA) method (40), it is a ranking based method that defines a score indicating the absolute enrichment of a specific gene set in each sample (41, 42). ssGSEA scores can be used to quantify the relative abundance of immune cells in OB or PD tissues and determine the level of immune infiltration in each sample.

The immune infiltration gene set was downloaded from http://cis.hku.hk/TISIDB/ and the immune infiltration analysis was performed using the GSVA package in R. To reveal the relationship between the hub genes and the immune cells, we then performed the correlation analysis between the hub genes and the immune cells based on Spearman's correlation coefficients.

Gene set enrichment analysis

To further explore the correlation between immune cell infiltration and hub genes, we performed GSEA analysis. Gene sets of pathways were obtained from the Molecular Signature Database (MSigDB) (https://www.gsea-msigdb.org/gsea/msigdb/). Based on the gene expression profile data of GSE152991 (OB) and GSE16134 (PD), the average expression value of each hub gene was calculated separately, and all samples in the dataset were divided into "high" and "low" groups according to whether the expression value of the gene was higher or lower than its average expression value, and the GSEA method was used to evaluate the relevant molecular mechanisms between the two groups. Terms with p-value < 0.05, $|$normalized enrichment score (NES)$| > 1$, and false positive rate (FDR) p-value < 0.25 were considered to be significant.

Construction of TF-miRNA-mRNA network

Transcription factors (TFs) are proteins that can bind to specific DNA sequences and regulate gene expression (43). MicroRNAs (miRNAs) are a class of endogenous short non-coding RNAs that mediate the degradation of target mRNAs or repress translation (44). TFs and miRNAs mostly act in a combinatorial manner, where many different TFs or miRNAs control the same gene, i.e., they act synergistically on the target mRNAs (45). Moreover, TFs and miRNAs can not only co-regulate the expression of target genes but also regulate each other (46). Therefore, it is helpful to learn about the dysregulation of gene expression in various physiological and disease conditions through the transcriptional regulatory network among TFs, miRNAs, and mRNAs. The Human microRNA Disease Database (HMDD) (47) is a database for manually collecting and organizing disease-associated miRNAs, and the association information is experimentally validated. We downloaded the miRNAs associated with OB and PD from the HMDD database and obtained the co-miRNAs of OB and PD by mapping. The multiMiR package (48) provides the target genes regulated by miRNAs, which are also experimentally validated. We used the multiMiR package to find genes with possible regulatory relationships with co-miRNAs of OB and PD. The TransmiR database (49) collected TF-miRNA regulatory relationships in publications, from which co-miRNA-related TFs of OB and PD were obtained. Finally, a TF-miRNA-mRNA network was constructed and visualized in Cytoscape.

Results

Identification of common DEGs between OB and PD

A total of 875 DEGs were identified in the OB dataset using the limma package, of which 440 genes were up-regulated and 435 genes were down-regulated (Figure 1A), and 2399 DEGs were obtained in the PD dataset, of which 1336 genes were up-regulated and 1063 genes were down-regulated (Figure 1B). After taking the intersection of the DEGs in OB and PD datasets, a total of 147 overlapping DEGs were identified, including 109 commonly up-regulated genes and 38 commonly down-regulated genes (Figure 1C). Heatmaps of the overlapping DEGs in OB and PD are shown in Supplementary Figure 1.

GO and KEGG enrichment analysis of DEGs

To investigate the potential biological processes and pathways of the DEGs, we separately performed GO and KEGG enrichment analysis using the clusterProfiler package and the KOBAS website. The results of KEGG enrichment analysis demonstrated that these genes were mainly enriched in "osteoclast differentiation", "B cell receptor signaling pathway" and "viral protein interaction with cytokine and cytokine receptor" (Figure 2A). The results of GO analysis revealed that biological processes such as "leukocyte migration", "cellular chemotaxis" and "cellular defense response" were significantly enriched (Figure 2B). In terms of cellular composition, terms such as "NADPH oxidase complex", "secondary lysosome" and "high-density lipoprotein particle" were significantly enriched (Figure 2C). In terms of molecular function, terms such as "superoxide-generating NADPH oxidase activator activity", "inhibitory MHC class I receptor activity" and "MHC class I receptor activity" were significantly enriched (Figure 2D). The detailed results of the GO and KEGG enrichment analysis are shown in Supplementary Tables 2, 3.

Construction of PPI network and identification of hub genes

To further reveal the potential relationships among the common DEGs in OB and PD, a protein-protein interaction (PPI) network of these genes was constructed in the STRING database, which contained 72 nodes and 182 edges (Figure 3A). Module analysis was performed using the MCODE plugin in

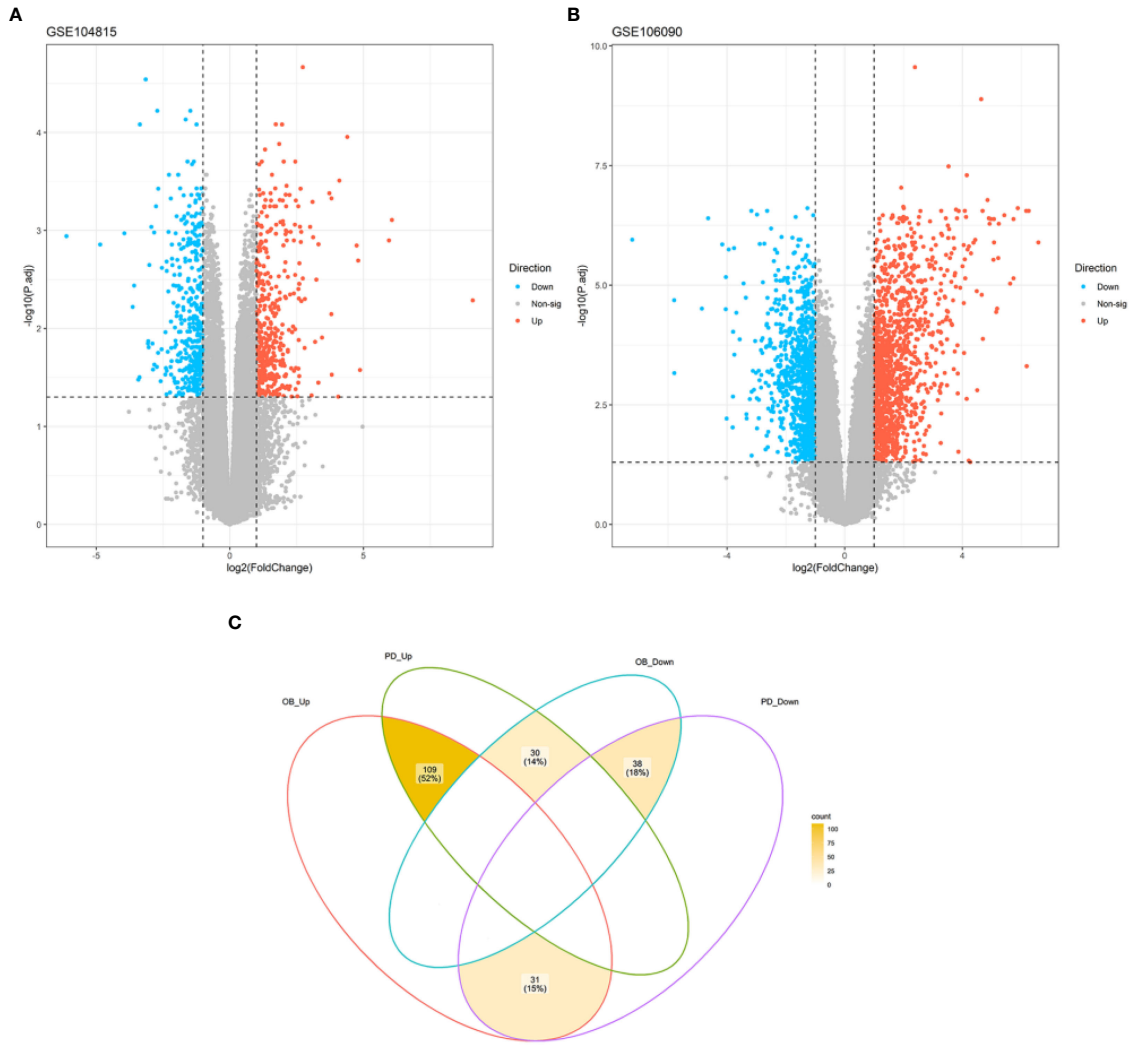


FIGURE 1
Volcano plot of DEGs and Venn diagram of common DEGs. **(A)** A total of 667 DEGs were identified between OB and healthy controls. **(B)** A total of 2191 DEGs were identified between PD and healthy controls. **(C)** A total of 109 common up-regulated genes and 38 common down-regulated genes were identified in OB and PD. DEGs, differentially expressed genes; OB, Obesity; PD, Periodontitis.

Cytoscape to detect key clustering modules, from which three modules were obtained, and module 1 contained 9 nodes and 17 edges with a cluster score of 4.25; module 2 contained 6 nodes and 10 edges with a score of 4, and module 3 contained 6 nodes and 8 edges with a score of 3.20 (Figure 3B).

To explore genes that may play an important role in the co-occurrence of OB and PD, the CytoHubba plugin was used to identify hub genes. Due to the heterogeneity of biological networks, several topological analysis algorithms including MCC, MNC, Degree and DMNC were applied in our research, and the top 30 most important hub genes in the PPI network were obtained. The intersection of four hub gene lists revealed 25 hub genes: *FCGR3A, FGR, MNDA, SELL, NCF2, FYB1, EVI2B, LY86, TREM1, LILRA1, IGSF6, CTSS, CXCR4, LCK, CLEC12A, FCN1, CXCL2, VNN2 P2RY13, LYZ, CCR3, EOMES, MMP7, CD3G* and *CLEC4E* (Figure 3C). Details of the hub genes are shown in Supplementary Table 4.

Validation of hub genes

The 25 hub genes of OB and PD were validated using GSE152991 (for OB) and GSE16134 (for PD) datasets, respectively. The results demonstrated that 14 hub genes were significantly differentially expressed between the case and control groups in these two datasets (p-value < 0.05), all of which were found to be up-regulated in both OB and PD groups. The hub genes were *FGR*, *MNDA*, *NCF2*, *FYB1*, *EVI2B*, *LY86*, *IGSF6*, *CTSS*, *CXCR4*, *LCK*, *FCN1*, *CXCL2*, *P2RY13* and *MMP7* (Figure 4).

ROC analysis was performed on these two datasets to evaluate the accuracy of the diagnostic features of the hub genes. The AUC values of the 14 hub genes were all greater than 0.7 in the OB and PD datasets, indicating excellent predictive ability of these genes (Figure 5). The ROC curves of the hub genes in the four datasets are shown in Supplementary Figures 2, 3.

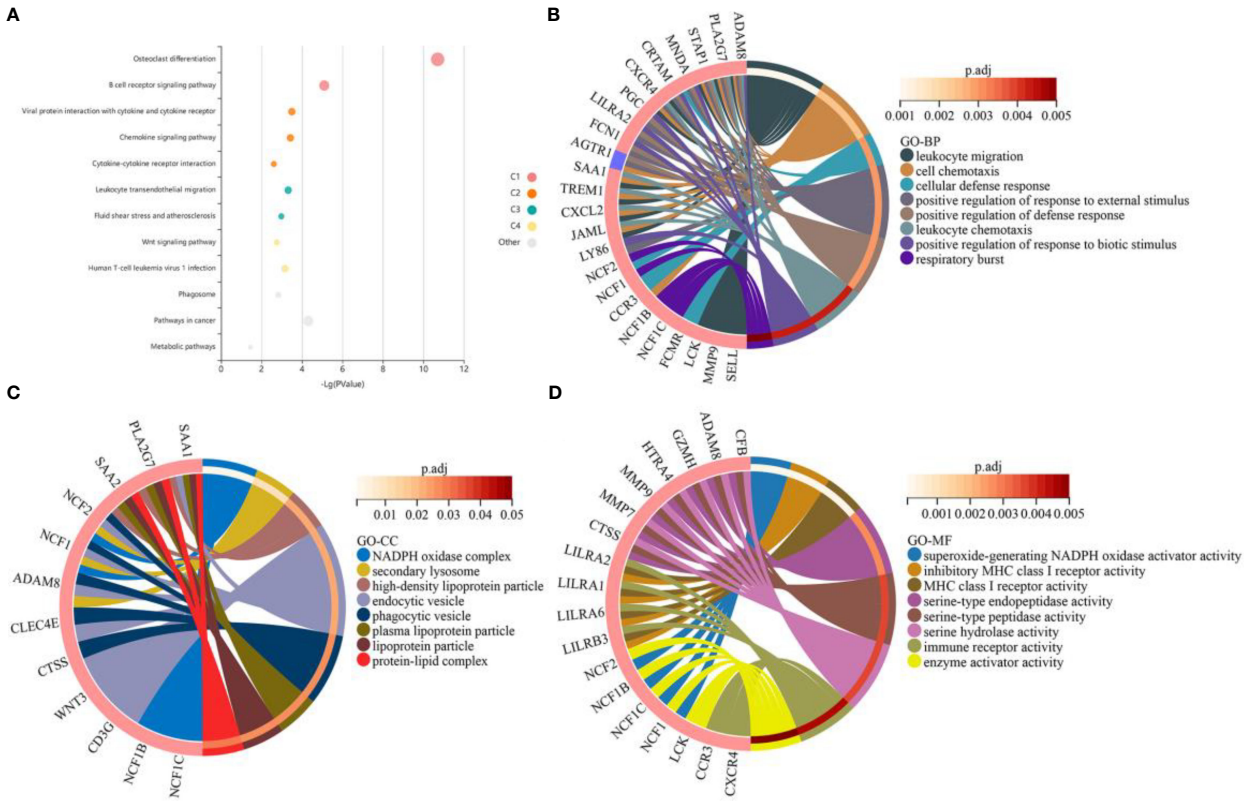


FIGURE 2
Functional enrichment analysis of the common differentially expressed genes (DEGs) between obesity and periodontitis. **(A)** KEGG pathway analysis of the DEGs. Where each bubble represents an enriched function, the size of the bubble represents 6 levels of enriched *p*-values, and the color of the bubble indicates the clustering of different pathways corresponding to different clusters (C1, C2, C3, etc.). **(B–D)** GO enrichment results of the DEGs for the categories of biological processes, cellular composition and molecular function. The red and blue colors of the gene represent up-regulation and down-regulation respectively. An adjusted *p*-value < 0.05 was considered statistically significant. DEGs, differentially expressed genes. KEGG, Kyoto Encyclopedia of Genes and Genomes. GO, Gene Ontology.

Correlation between hub genes and immune cells

The 14 hub genes were imported to GeneMANIA to find correlated genes based on physical interaction, co-expression, prediction, co-localization, genetic interaction and shared protein domains. The inner circle represents the hub genes, while the outer circle represents the related genes that were newly obtained from the database. The network revealed that these genes were significantly enriched in “macrophage activation”, “phagocytosis”, “leukocyte migration”, “regulation of mononuclear cell proliferation”, “Fc receptor signaling pathway”, and “antigen receptor-mediated signaling pathway” (Figure 6).

Immune infiltration analysis was performed to evaluate the infiltration level of 28 immune cell types, and the correlations between the 14 hub genes and 28 immune cells were analyzed using Spearman’s method. The infiltration level of immune cells including activated CD4 T cells, activated dendritic cells, central memory CD8 T cells, immune B cells, macrophages, MDSC, natural killer T cells, and plasmacytoid dendritic cells was significantly higher in the OB and PD samples compared with the control samples (Figure 7A; Supplementary Figure 4A), and the infiltration level of immune cells such as MDSC, regulatory T cells and macrophages was positively correlated with the 14 hub genes in both OB and PD. In addition, *FGR*, *FYB1* and *LCK* were significantly associated with immature B cells, monocytes, and activated CD4 T cells. In PD samples from GSE16134, the 14 hub genes were positively associated with most cell types except effector memory CD4 T cells, type 2 T helper cells, and CD56 dim natural killer cells (Figure 7B; Supplementary Figure 5).

GSEA results of hub genes

Both the immune infiltration results and the GeneMANIA analysis suggested that the hub genes might be closely associated with macrophages. Therefore, we explored the enrichment of hub genes in macrophage-associated pathways based on their expression in the GSE152991 (OB) and GSE16134 (PD) datasets using GSEA analysis to determine whether these hub genes are also significantly associated with macrophage-associated pathways. Macrophage-related gene sets including “GOBP_MACROPHAGE_ACTIVATION”, “GOBP_MACROPHAGE_MIGRATION”, “GOBP_MACROPHAGE_CHEMOTAXIS”, “GOBP_MACROPHAGE_ACTIVATION_INVOLVED_IN_IMMUNE_RESPONSE”, “GOBP_MACROPHAGE_ACTIVATION_IN_

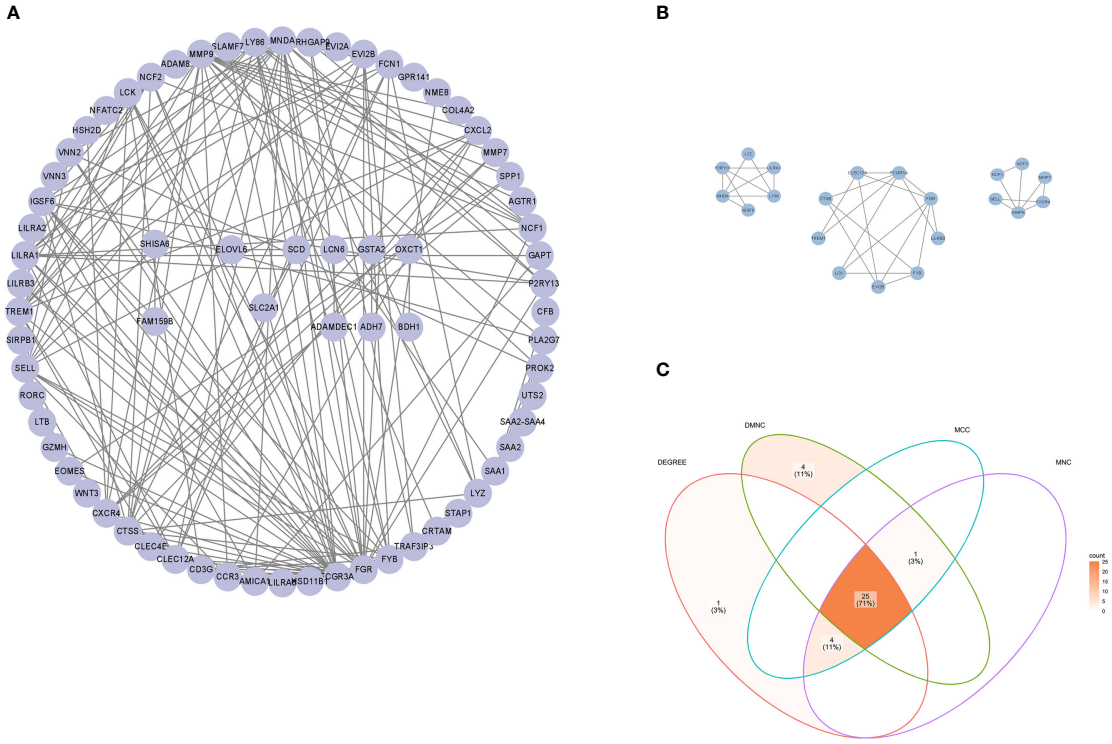


FIGURE 3
PPI network of hub genes. **(A)** PPI network of common DEGs constructed using the STRING database. **(B)** Three gene modules were identified by the MCODE plugin. **(C)** Venn diagram showing 25 common hub genes identified by MCC, MNC, Degree and DMNC algorithms using the CytoHubba plugin. PPI, protein-protein interaction; DEGs, differentially expressed genes; MCC, maximal clique centrality; DMNC, density of maximum neighborhood component; MNC, maximum neighborhood component.

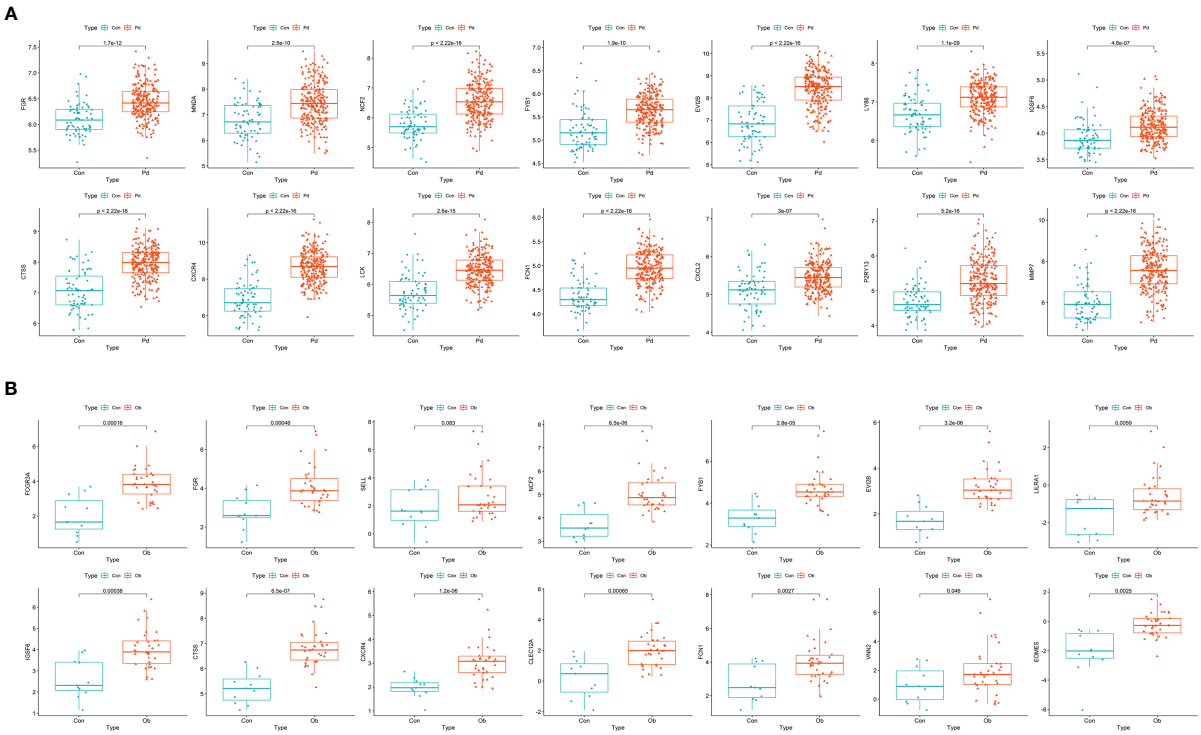


FIGURE 4
Identification and validation of hub genes. **(A)** Boxplots of the expression levels of hub genes in GSE152991. The expression levels of the 14 hub genes are significantly higher in the obesity group. **(B)** Boxplots of the expression levels of hub genes in GSE16134. The expression levels of the 14 hub genes are significantly higher in the periodontitis group. A p-value < 0.05 was considered statistically significant.

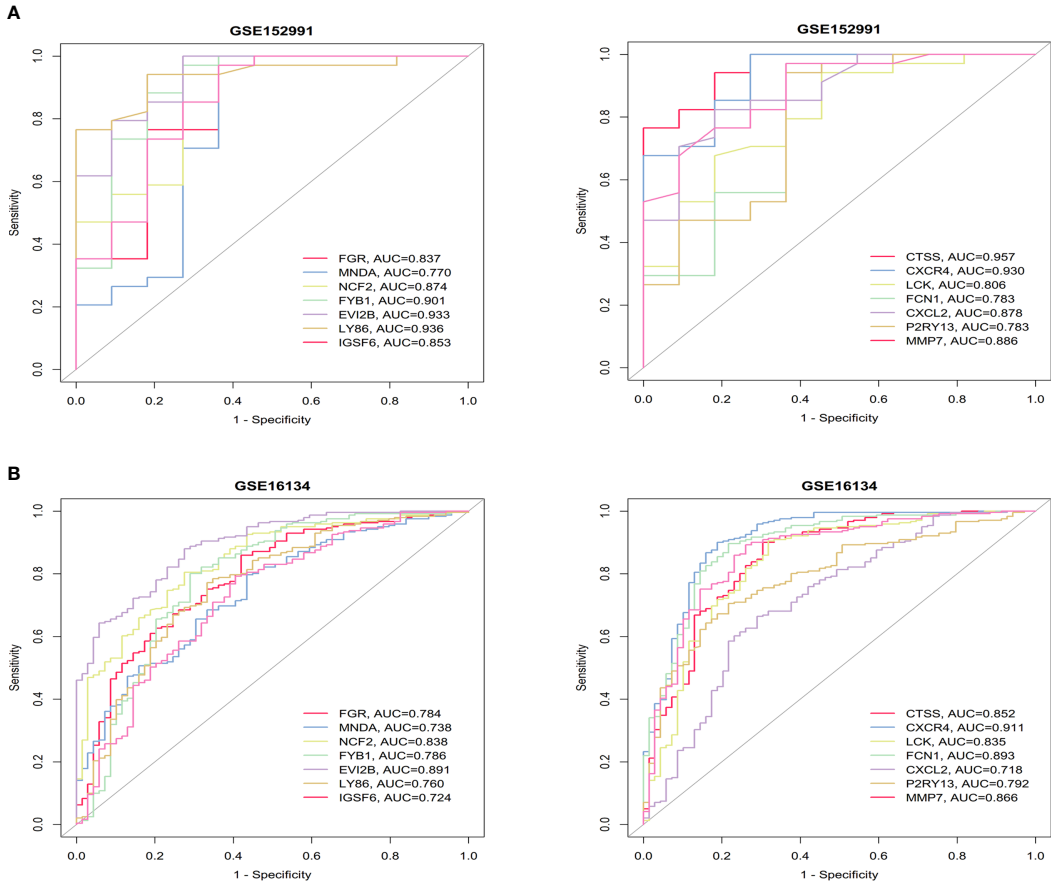


FIGURE 5
ROC curves of the 14 hub genes in obesity. **(A)** and periodontitis **(B)**. The AUC values are listed in the lower right-hand corner. ROC, receiver operating characteristic; AUC, area under the curve.

IMMUNE_RESPONSE", "GOBP_MACROPHAGE_CHEMOTAXIS", "GOBP_MACROPHAGE_CYTOKINE_PRODUCTION", "GOBP_MACROPHAGE_APOPTOTIC_PROCESS", "GOBP_PHAGOCYTOSIS" and "GOBP_REGULATION_OF_MACROPHAGE_ACTIVATION" were downloaded from MSigDB database and subsequently used for GSEA analysis. The GSEA results indicated that high expression of *FGR*, *FYB1*, *LY86*, *LCK* and *P2RY13* were significantly associated with several macrophage-related biological terms in both GSE152991 (Figure 8A) and GSE16134 (Figure 8B) datasets, such as "activation of macrophage", "chemotaxis of macrophage", "migration of macrophage", "regulation of macrophage apoptotic process" and "macrophage activation involved in immune response". The nominal *p*-values, NES and FDR p-values for the GSEA results of GSE152991 and GSE16134 are shown in Supplementary Tables 8, 9.

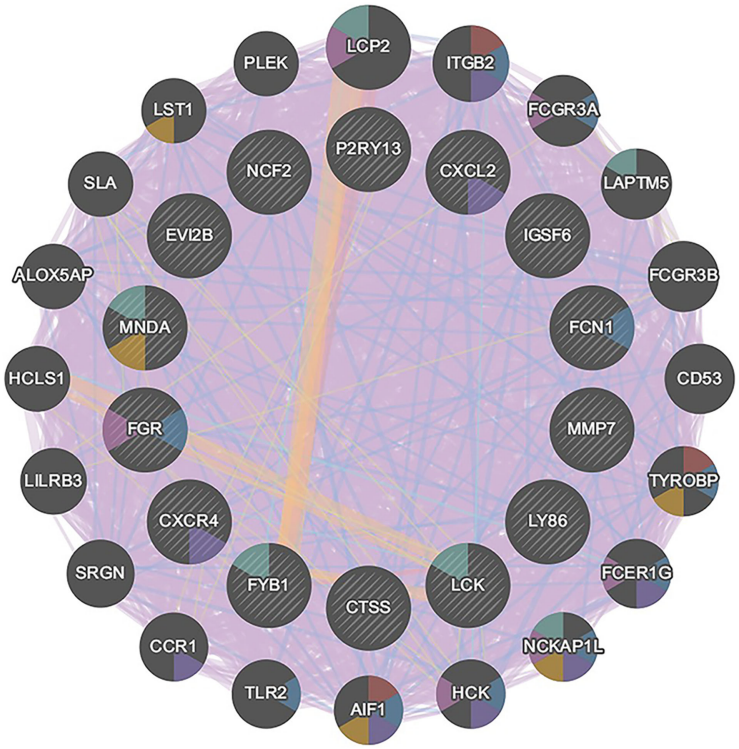
TF-miRNA-mRNA regulatory network

The information of experimentally validated miRNAs and disease associations were downloaded from the HMDD database (Supplementary Table 5), from which we obtained a total of 80 miRNAs associated with PD and 33 miRNAs associated with OB. And 10 common miRNA (hsa-mir-17-5p, hsa-mir-130a-3p, hsa-mir-30a-5p, hsa-mir-126-3p, hsa-mir-146a-5p, hsa-mir-21-5p, hsa-mir-24-3p, hsa-mir-155-5p, hsa-mir-200b-3p and hsa-let-7b-5p) between OB and PD were obtained.

Functional enrichment analysis of the 10 miRNAs was then performed using the mirPath database, and the results indicated that several terms were significantly enriched, including "fatty acid biosynthesis", "fatty acid metabolism", "ErbB signaling pathway" and "Wnt signaling pathway and endocytosis" (Figure 9A). The "multiMiR" package was used to find validated target genes of the miRNAs (Supplementary Table 6), and the TransmiR database was used to find the regulatory information between TFs and the miRNAs, from which 233 TFs were obtained (Supplementary Table 7). Based on the regulatory relationships among TFs, mRNAs and miRNAs, a TF-miRNA-mRNA network was constructed (Figure 9B).

Discussion

Both OB and PD are common health problems that now cause considerable economic damage and social burden worldwide (50).



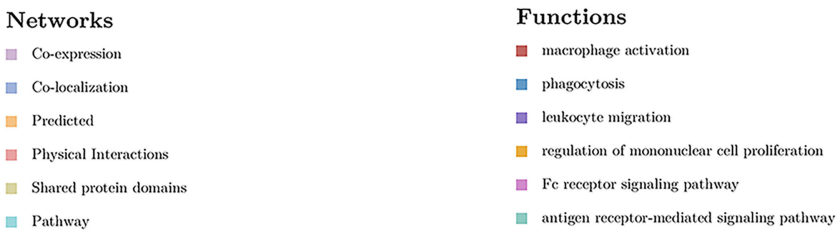


FIGURE 6
GeneMANIA analysis of the hub genes. The biological functions of the genes are shown. The hub genes are located in the inner circle and the genes correlated with the hub genes are located in the outer circle.

Previous studies have shown that the risk of periodontitis progression is 15% higher in obese than in healthy populations (51) and a systematic review of epidemiological studies has revealed a significant association between OB and PD (52). OB and PD may share overlapping pathogenic pathways, particularly immune cell infiltration and inflammation (6, 53). However, it is unclear how immune cell infiltration and inflammation accelerate the progression of OB-associated PD.

In our study, the characteristic genes shared by both OB and PD showed a close correlation with immune cell function. Based on integrated bioinformatics analysis, we screened the common DEGs between OB and PD, and functional enrichment analysis revealed that these genes were mainly involved in immune-related biological pathways such as "B cell receptor signaling pathway", "chemokine signaling pathway", "leukocyte migration", "cellular defense response", "phagocytic vesicle and immune receptor activity". Through PPI network and hub gene analysis, we identified 25 common hub genes in OB and PD, among which 14 hub genes (*FGR, MNDA, NCF2, FYB1, EVI2B, LY86, IGSF6, CTSS, CXCR4, LCK, FCN1, CXCL2, P2RY13, and MMP7*) showed high sensitivity and specificity in the ROC curve analysis, indicating that these genes may be promising markers for diagnosis of OB and PD. GSEA analysis of the 14 genes demonstrated that 5 genes including *FGR, LCK, FYB1, LY86* and *P2RY13* were significantly involved in multiple immune-related GO terms, such as "activation of macrophage", "chemotaxis of macrophage", "migration of macrophage", "regulation of macrophage apoptotic process" and "macrophage activation involved in immune response". These results suggest that these co-DEGs may accelerate disease progression in OB and PD by affecting the activity of immune cells, especially macrophages.

Furthermore, macrophage infiltration plays a key role in inflammation in obese adipose tissue (54). In obese adipose tissue, macrophage infiltration leads to an increase in pro-inflammatory cytokines, which may lead to inflammation in other tissues *via* endocrine pathways, thus accelerating the progression of PD (55). In our study, the results of functional enrichment analysis of five common hub genes of OB and PD suggest a possible strong

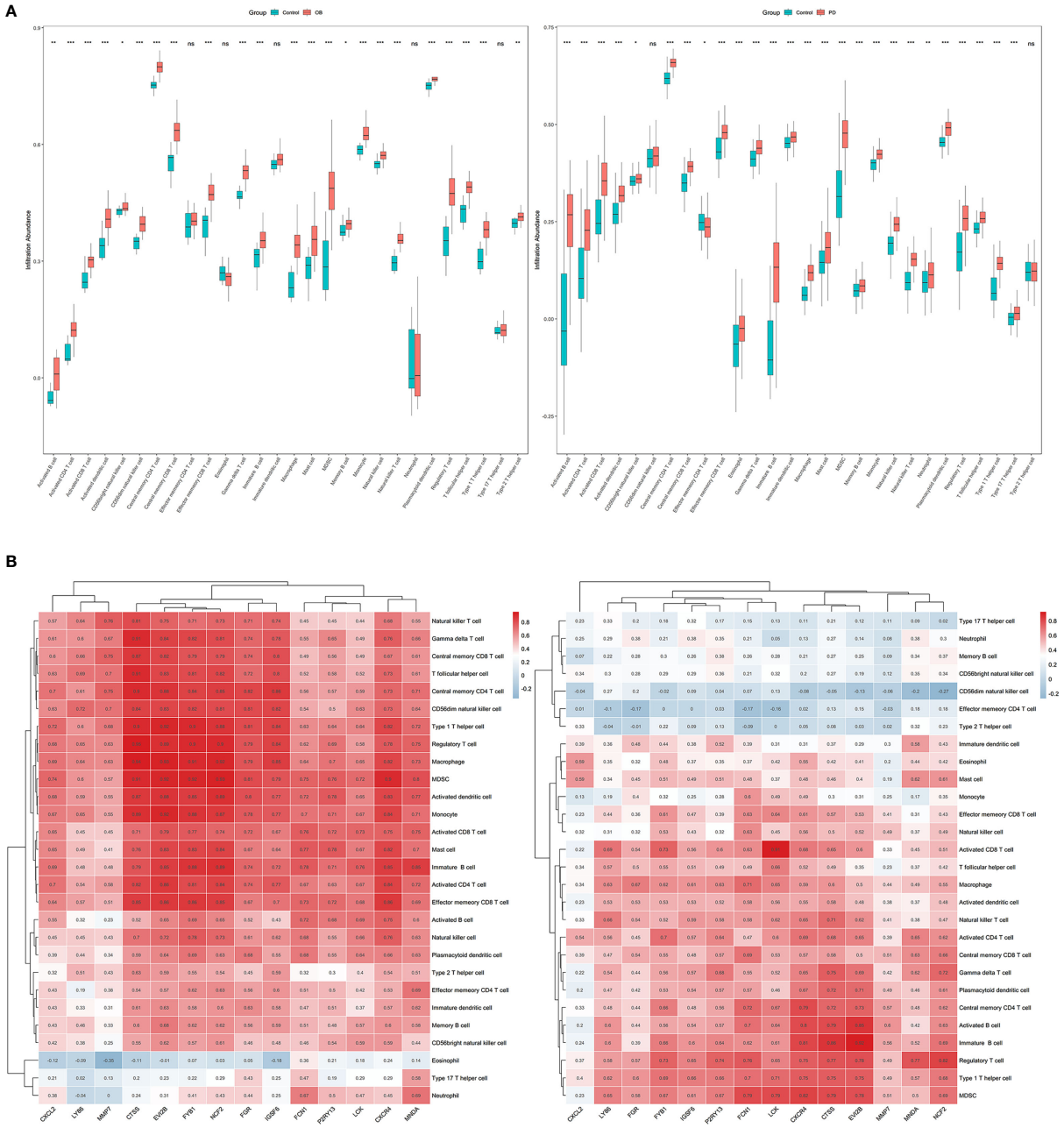


FIGURE 7
The results of immune infiltration analysis in obesity (OB) and periodontitis (PD) datasets. **(A)** Boxplots of the immune infiltration abundance in OB (left) and PD (right). **(B)** Heatmaps of the correlations between the 14 hub genes and the 28 immune cells in OB (left) and PD (right). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns, non-signicant.

association with macrophages. Ortiz MA et al. (56) indicated that both *FGR* and *LCK* belong to the Src family of protein tyrosine kinases, a family of non-receptor tyrosine kinases consisting of nine members in humans: *SRC, FGR, LCK, FYN, HCK, YES, LYN, YRK* and *BLK* (56). Several Src family members are expressed in all cell types and are involved in a variety of cellular processes, of which *FGR* is mainly expressed in the hematopoietic system and *LCK* is mainly found in T lymphocytes and natural killer cells (57, 58). Like other members of the Src family, both *FGR* and *LCK* consist of a kinase structural domain, SH2, SH3 and unique N-terminal structural domains, and a regulatory C-terminal tail that phosphorylates tyrosine residues of a variety of proteins (59). *FGR* transduces signals from cell surface receptors lacking kinase activity and is involved in immunomodulatory responses, including macrophage, monocyte, neutrophil and mast cell function, cytoskeletal remodeling in response to extracellular stimuli, phagocytosis, cell adhesion and migration (60). *LCK* plays a key role in T cell antigen receptor (TCR)-related events: binding of the TCR to the peptide antigen-binding MHC complex facilitates the interaction of CD4 and CD8 with MHC class II and class I molecules, respectively, thereby recruiting the associated *LCK* protein to the vicinity of the TCR/CD3 complex. Subsequently,

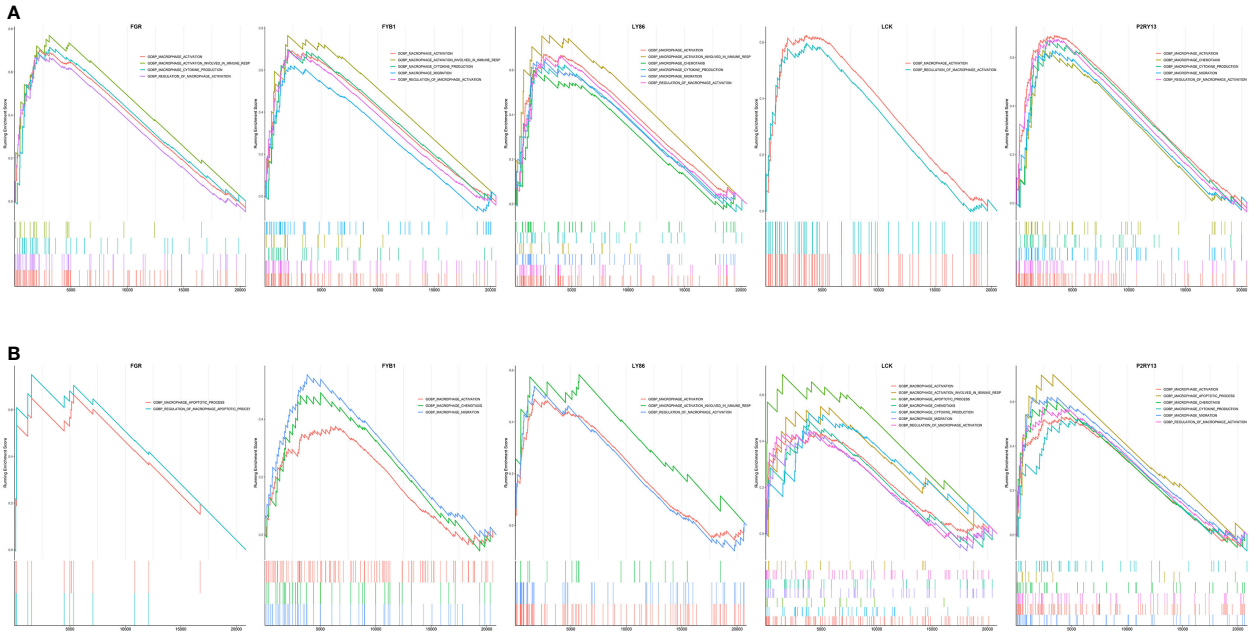


FIGURE 8
Merged enrichment plot of *FGR*, *FYB1*, *LY86*, *LCK* and *P2RY13* from gene set enrichment analysis of GSE152991 **(A)** and GSE16134 **(B)** datasets. The threshold value of GSEA results was set as |NES| > 1.0, *p*-value < 0.05 & FDR *p*-value < 0.25. GSEA, gene set enrichment analysis; NES, normalized enrichment score; FDR, false positive rate.

LCK phosphorylates tyrosine residues within the immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic tail of the TCR-γ chain and CD3 subunit, thereby initiating the TCR/CD3 signaling pathway (61). In the present study, the OB and PD enrichment results also showed that the co-DEGs were mainly enriched for molecular functions such as inhibitory MHC class I receptor activity and MHC class I receptor activity. Many studies have found that *FGR* expression is high in macrophages and monocytes and that high *FGR* expression can influence the inflammatory milieu through M1-type macrophage polarization, which in turn affects adipose tissue metabolism, leading to the development of inflammation and OB (62–64). He L et al. (65) also showed that high expression of *LCK* plays an important role in the imbalance of the immune system in periodontitis (65). In another study, *FGR* kinase was found to be a key regulator of pro-inflammatory adipose tissue macrophage activation, diet-induced OB, insulin resistance, and hepatic steatosis, knockdown of *FGR* reduced lipid accumulation and lipogenic gene expression, and low expression of *FGR* prevented macrophage polarization while preventing high-fat diet-induced OB in mice (66). This is consistent with our findings that *FGR* and *LCK* expression were significantly up-regulated in OB and PD, and functional enrichment analysis showed that they are involved in immune processes activated by macrophages. Therefore, we speculate that OB accelerates PD progression, at least in part, through immune cell infiltration and inflammatory response, and that *FGR* and *LCK* may play a key role in this process.

FYB1, known as adhesion and degranulation promoting linker protein (*ADAP*), is required for T cell activation as a bridging protein for the *FYN* and *LCP2* signaling cascades in T cells (67). In addition, *FYB1* can be expressed on primary natural killer cells and on lymphocyte-activated killer cells stimulated by interleukin-2, resulting in enhanced antitumor responses (68). Carmo AM et al. (69) indicated that post-translational modification of *FYB1* could lead to increased tyrosine phosphorylation by affecting T-cell receptor attachment (69). The protein encoded by the *LY86* gene is lymphocyte antigen 86, also known as MD-1 protein, a secreted glycoprotein associated with *RP105* (Toll-like receptor family protein), which plays a key role in the B-cell surface expression of *RP105*, while the *RP105*/MD-1 complex is expressed in immune cells, including B cells, macrophages and dendritic cells (70). In a genome-wide methylation analysis study of OB, the methylation level of the *LY86* gene was shown to be higher in obese cases compared to healthy controls (71). The product of the *P2RY13* gene belongs to the G protein-coupled receptor family, a 354 amino acid-encoded gastrointestinal protein-coupled receptor that is involved in the pathogenesis of purine energy transfer pathways, cholesterol metabolism, inflammation and immune dysfunction mechanisms, mediating a variety of pathophysiological processes, such as apoptosis, autophagy, proliferation and metabolism (72, 73). Many studies have shown that *P2RY13* promotes apoptosis and increases the release of pro-inflammatory factors (74, 75). In addition, *P2RY13* is highly expressed in the inflamed intestinal tissue of ulcerative colitis patients (74, 76). The above series of

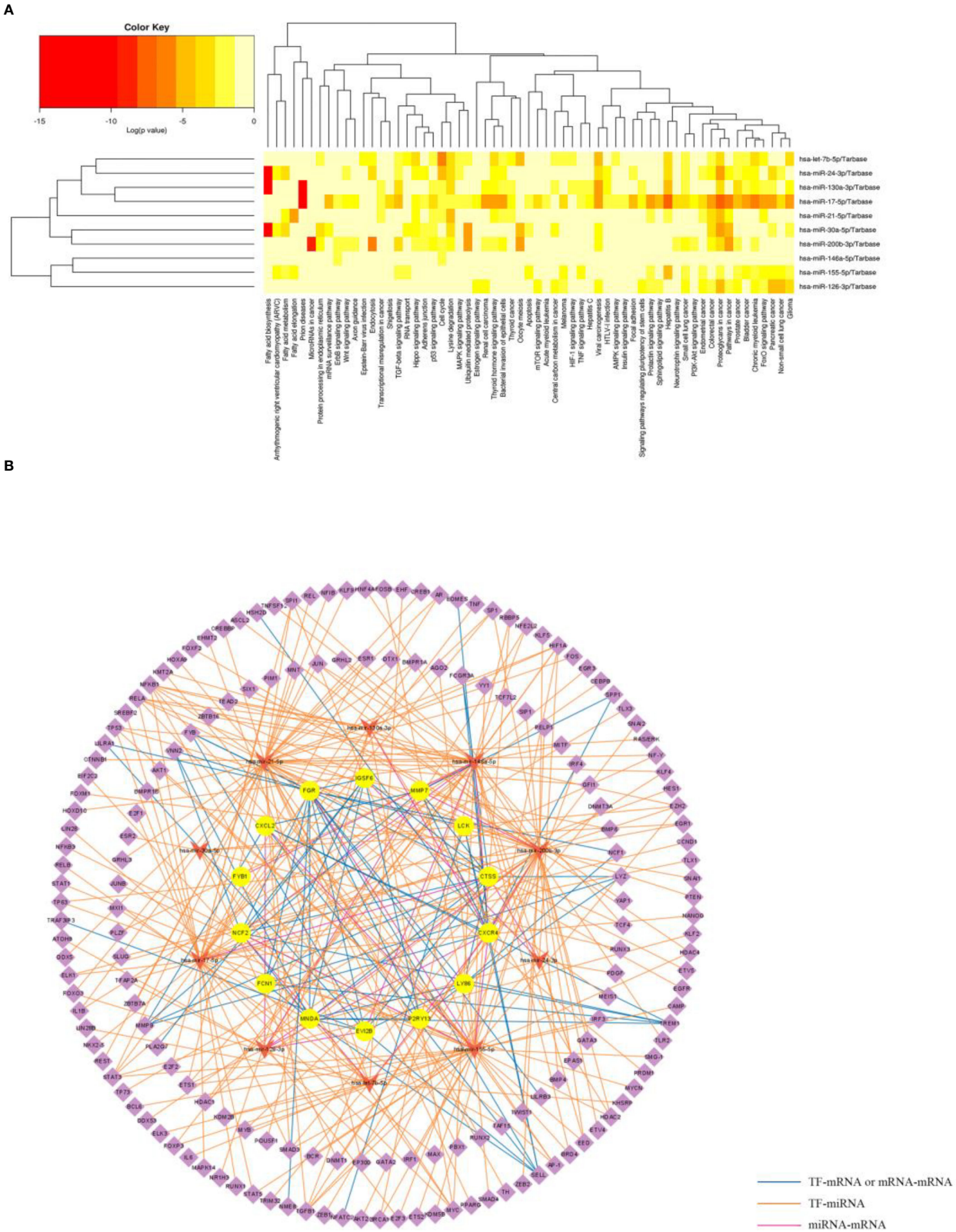


FIGURE 9
Functional enrichment analysis of the miRNAs and construction of TF-miRNA-mRNA network. **(A)** Significantly enriched biological pathways of the common miRNAs in OB and PD using the mirPath database. **(B)** The common TF-miRNA-mRNA regulatory network in OB and PD, where circles represent hub genes, inverted triangles represent miRNAs and diamonds represent transcription factors.

studies have shown that *FGR*, *LCK*, *FYB1*, *LY86*, and *P2RY13* may play an important role in the dysfunction of immune cells such as macrophages, T lymphocytes, and B lymphocytes during the pathology of OB or PD. Therefore, we speculate that macrophage infiltration and recruitment of other immune cells may be common mechanisms in OB and PD. A possible molecular mechanism is that in OB and PD, upregulation of *LY86* gene expression affects B-cell function, and the increase in B-cell number promotes T-cell activation. Meanwhile, *FYB1* is involved in T-cell activation and *LCK* phosphorylates the T-cell antigen receptor (*TCR*), but increased T cell activation may in turn promote M1-like macrophage polarization and inflammation. Among other things, *FGR* transduces signals from cell surface receptors lacking kinase activity and is involved in immunoregulatory responses such as macrophage function. Finally, *P2RY13* promotes the release of inflammatory factors.

To further explore the potential molecular regulatory mechanisms between these immune-related hub genes and immune cells, especially macrophages, we constructed a TF-miRNA-mRNA regulatory network for OB and PD. The results showed that among the five hub genes, *FGR* was regulated by hsa-miR-155-5p and *FYB1* was regulated by hsa-miR-146a-5p. In addition, TFs such as *AKT1*, *BRCA1*, and *TP63* inhibited the regulatory effect of hsa-miR-155-5p, and TFs such as *FOXP3*, *JUNB*, *NFKB1*, and *SMAD4* activated the regulation of hsa-miR-155-5p. On the other hand, the regulatory role of hsa-miR-146a-5p was inhibited by TFs such as *HDAC1*, *TP53*, and *HDAC1*, and could also be activated by TFs such as *FOXP3*, *IL1B*, and *RELA*. Notably, previous findings have shown that hsa-miR-155-5p expression is up-regulated under various inflammatory conditions (77). Langi G et al. (78) showed that the expression of hsa-miR-155-5p is down-regulated in OB patients undergoing bariatric surgery (78). In addition, miR-146a-5p expression was up-regulated in humans and mice obese adipose tissue and suppressed the inflammatory response in human adipocytes (79). Meanwhile, animal experiments revealed that deletion of the transcription factor *AKT1* increased energy expenditure and prevented diet-induced obesity in mice (80), and *AKT1* regulated macrophage polarization and alters periodontal inflammatory status (81). These findings are consistent with our study, where 14 hub genes were found to be up-regulated in both the OB and PD groups and may have a strong association with immune cells, especially macrophages. Therefore, we can reasonably speculate that the upregulation of *FGR* and *FYB1* expression in OB and PD may be regulated by hsa-miR-155-5p and hsa-miR-146a-5p and TFs such as *AKT1*, *FOXP3*, *TP53*, and *IL1B* are involved in the regulatory process of hsa-miR-155-5p and hsa-miR-146a-5p.

In this study, we used transcriptome data to elucidate the common mechanisms of OB and PD. Our study is novel in that we analyzed the infiltration of 28 immune cell species in adipose tissue of OB patients and periodontal tissue of PD patients using the ssGSEA algorithm. It is comprehensive in that we elucidated the key genes, biological pathways, immune infiltration levels, and TF-miRNA-mRNA networks of OB and PD, which will be helpful in understanding the pathophysiological mechanisms shared between the two diseases and the treatment of PD patients with OB. However, there are still limitations to our study. First of all, there is limited clinical information available in public databases, and not all datasets have large sample sizes, which may lead to bias in the results. In addition, this study was mainly based on bioinformatics analysis. Although for hub genes with high diagnostic value, we used different datasets to validate this diagnostic value, and many previous studies were able to confirm our findings to some extent, further experimental validation of our findings is still needed.

Conclusion

Our study provides key co-diagnostic effector genes for OB and PD patients and reveals that the common key genes of both diseases are closely associated with immune cell infiltration. The possible molecular mechanism of accelerated PD progression in OB is that the secretion of pro-inflammatory cytokines increases with immune cell infiltration and inflammatory response in OB, causing inflammation in other tissues by endocrine means, thus accelerating PD progression. Five hub genes (*FGR*, *LCK*, *FYB1*, *LY86*, *P2RY13*) are promising biomarkers for OB and PD and may play an important role in the pathogenesis of OB and PD by influencing the activity of macrophages involved in immune regulation and inflammatory responses.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

XQ, YC, and XC designed the study and drafted the manuscript. YZ and SW contributed to the data collection. YC, XZ, WP, and KM contributed to data analysis. YC, XZ, YZ, and SW drafted the manuscript. XQ, LT and HD revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1101854/full#supplementary-material

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Central obesity and its associated factors among cancer patients at the University of Gondar Comprehensive Specialized Hospital, Northwest Ethiopia

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Purpose: Obesity, especially the hidden type of obesity (central obesity), has been believed to be the major risk factor for developing and progressing non-communicable diseases, including cancers. However, there are limited studies regarding the issue in Ethiopia and the study area. Therefore, this study aimed to evaluate the magnitude of central obesity and its associated factors among cancer patients visited the oncology unit of the University of Gondar Comprehensive Specialized Hospital.

Methods: An institutional-based cross-sectional study was conducted from January 10 to March 10, 2021. A total of 384 study participants were enrolled using a systematic sampling technique. The data were collected using a semi-structured interviewer-administered questionnaire and were pretested to address the quality of assurance. The weight of the participants was assessed using body mass index (BMI) and central obesity. Both bivariate and multivariate logistic regressions were conducted to identify the factors associated with central obesity, and p-values less than 0.05 with multivariate were considered statistically significant associations.

Result: Most respondents (60.16%) were stage I cancer patients. The study found that about 19.27% of the participants were prevalent central obesity, and none of them were obese by body mass index (BMI) categorization criteria. However, about 12.24% and 7.03% of the participants were found to be underweight and overweight, respectively. The variables associated with central obesity were sex (AOR=14.40; 95% CI: 5.26 - 39.50), occupation (AOR=4.32; 95%CI: 1.10 - 17.01), and residency (AOR=0.30; 95% CI: 0.13 - 0.70).

Conclusion: A significant number of the respondents (19.27%) were centrally obese. Being female, urban residency and having an occupation other than a farmer, merchant, and governmental were the factors associated with central obesity. Hence, cancer patients may be centrally obese with average body weight.

KEYWORDS

cancer, central obesity, body mass index, associated factors, behavioral factors

Background

Non-communicable diseases have become major public health problems worldwide (1). They are the leading cause of death in both developed and under-developing countries. Of these, cancers are the second most common prevalent disease next to cardiovascular diseases (CVDs) (2). Globally, around 18.1 million of the population is affected by cancer problems. Besides, about 9.6 million people are estimated to die yearly from cancer and related complications (3). The problem is becoming rapidly prevalent in low and middle-income countries (LMICs), especially in sub-Saharan African countries, and it has been estimated that in 2050, the majority (70%) of annual cancer case incidence will be located in LMICs (4). The absence of screening and failure to diagnose at an early stage of cancer cases makes it difficult to overcome the burden of cancer problems in LMICs. Moreover, treatment is usually compromised fordifferent reasons, such as limied of skilled manpower, facilities, and economic restriction (5). Therefore, screening for early identification of cancer cases and associated factors are believed to be an ideal way to limit the progression and development of adversative outcomes.

Substance abuse like cigarette smoking, alcohol drinking and chat chewing, frequent utilization of processed food, being overweight or obese, and being older are the most common factors associated with the development and proical characteristics of the study participants among adults gression of most cancers (6). Although the association between obesity and cancer development is still controversial, several reports have approved a strong positive association. For instance, studies done across the world showed that obesity has been directly associated with the occurrence of breast cancer (7), ovarian cancer (8), liver cancer (9), endometrial cancer (10), esophageal adenocarcinoma (11), kidney cancer (12), colorectal cancer, gallbladder cancer (13) and thyroid cancer (14). In particular, the hidden type of obesity (central or visceral obesity) is highly dangerous and is usually associated with the development of several NCDs, including cancers, than that of general obesity (identified by BMI) (15, 16). Currently, dietary modifications, controlling the accumulation of fat around the abdominal wall, weight control, and frequent exercise are recommended as a supplementary therapeutic option to limit the progression of cancer cases (17, 18). Globally, the magnitude of abnormal or excessive fat accumulation which is measured by BMI and/or central obesity among cancer patients, is becoming a remarkable problem in the last decades (19). Although the attribution of obesity for cancer incidence varies among each cancer type, it is estimated to reach up to 44% for esophageal adenocarcinoma and 54% for bladder cancer development (20). However, to the best of our search, there are limited studies regarding the issue in Ethiopia and the study area. Besides, the burdens of central obesity have not got equal attention as general obesity among cancer patients worldwide, and this gives great value to the novelty of our study. Consequently, central obesity is becoming one of the major public health problems for the general population of Ethiopia (21–25). As our study is the first among Ethiopian cancer patients that focused on the magnitude of central obesity, the findings will be filled the knowledge gap on the burden of central obesity for Ethiopian cancer patients and can act as a baseline for further researchers with large sample sizes and multi-centered study area towards the impact of central obesity among Ethiopian cancer patients. The finding of this study will also give valuable input for clinicians to give attainable attention to general and central obesity aimed at cancer patients. Hence, we aimed to evaluate the magnitude of central obesity and its associated factors among cancer patients visiting the Oncology ward of the University of Gondar Comprehensive Specialized Hospital.

Methods

Study design and setting

An institution-based cross-sectional study was conducted among cancer patients from January to March 2021. It was accompanied by the University of Gondar Comprehensive Specialized Hospital (UoGCSH), Oncology ward. During the data collection period, the oncology ward has ten beds, serving more than 100 cancer patients annually.

Participants and sample size

All adult cancer patients who were ≥18 years coming to the Oncology ward were taken as the study's source population. Of these, who were in the Oncology ward during the study period were recruited as study participants. Severely ill patients who were unable to communicate and did not have attendant, clinically confirmed pregnant women, and edematous and abdominal distension patients

were excluded from the study. To determine the sample size of the study, we used a single population formula using a 50% expected proportion of central obesity, 95% confidence level, and a 5% margin of error. By considering the 5% non-response rate, the final size was computed to be 403. A systematic sampling technique with a skip interval of two was implemented to select the sample of the participants.

Data collection procedure

The data were collected using a semi-structured interviewer-administered questionnaire. It was prepared using related pieces of literature in an international language (English), then translated into the Ethiopian national language (Amharic), and then re-translated back to English to check the consistency. The questionnaire includes the general characteristics of the participants, substance abuse like smoking, alcohol drinking, and Khat chewing, and the factors that are believed to be associated with the independent outcome. The data were collected by trained Health Professionals such as Nurses and Public Health Professionals under the supervision of the principal investigator and oncologist. The completeness and consistency of the data were checked daily by the principal investigator. The substance abuse habits of the participants were assessed using a dichotomous yes and no questionnaire with expanded questions for those who responded "yes" about the amount, duration, and frequency of alcohol drinking, khat chewing, and cigarette smoking habits. Thus, alcohol drinkers were defined as any alcoholic product, including locally prepared alcoholic beverages intake at least twice per week for the last year regardless of the amount, otherwise defined as non-drinkers (26). Furthermore, those individuals who have smoked cigarettes for the last year were defined as smokers unless defined as non-smokers. Khat chewers were defined similarly to smokers (27). The physical activity of the participants was grouped based on the World Health Organization and international physical activities analysis guidelines as vigorous, moderate, and poor physical activities. Any activity that causes a substantial increase in breathing or heart rate (e.g., running, carrying, or lifting heavy loads, digging, or construction work) that continued for at least 30 min for a minimum of three days per week was defined as vigorous physical activity. Besides, any activity that causes a small increase in breathing or heart rate (brisk walking or carrying light loads) that continued for at least 30 min for at least three days per week or five or more days of these activities for at least 20 min per day or ≥3 days of vigorous-intensity activity per week of at least 20 min per day was defined as moderate physical activity. The respondents who did not fulfill both vigorous and moderate intensity activity were grouped under poor physical activators (28).

Physical measurements

Anthropometric (physical) measurements such as weight, height, waist circumference, and blood pressure were measured through adjusted equipment. The weight and height of the participants were measured in kilograms and centimeters in barefoot, respectively to calculate body mass index (BMI). Consequently, BMI was grouped into underweight (BMI ≤18.5 kg/m^2), normal weight (BMI between 18.5 and 24.9 kg/m^2), overweight (BMI between 24.9 and 30 kg/m^2), and obesity (BMI≥30 kg/m^2) (29). The Waist circumference was measured to define independent outcome (central obesity). Waist circumference was measured in centimeters at the narrowest mid-point between the lower margin of the lowest palpable rib and the top of the iliac crest with flexible plastic tape without heavy outdoor closing. Then, the participants with a waist circumference of >94 cm for males and >80 cm for females were defined as centrally obese (30). The Blood pressure (BP) of the respondents was measured three times with at least a five-minute interval in each measurement in a sitting position using a standardized mercury sphygmomanometer with an appropriate cuff size that covers two-thirds of the upper arm. It was measured with at least five minutes or 30 minutes rest for those who take hot drinks like coffee on their left arm. Then, the average BP measurement was taken, and elevated BP (hypertension) was defined as if systolic blood pressure (SBP) is ≥140mg/dl or diastolic blood pressure (DBP) is ≥90mg/dl or current use of the anti-hypertensive drugs (31).

Data processing and analysis

The data was entered into Epidata version 3.1 to check the completeness and analyzed using STATA 14 software. The participants' socio-demographic, behavioral, and clinical characteristics were described through descriptive statistics and presented using tables and narration. The factors associated with the independent variable were identified using the binary logistic regression model. The multivariable logistic regression was implemented to detect the Adjusted Odds Ratio (AOR). The 95% confidence interval was estimated to show the strength of the associations. Hence, a p-value of less than 0.05 in the multivariable logistic regression analysis was used to declare the statistically significant association of the independent variables with central obesity. Hosmer and Lemeshow's goodness of fit test was used to check the goodness of fit of the model.

Results

Socio-demographic characteristics of the participants

A total of 384 study participants with a 95.3% response rate were completed and enrolled in the analysis. Of these, most of the participants were under the age range of 41-60 years (55.99%) and females (51.30%). Most of the participants were coming from a rural area (64.4%) and more than two-thirds of the participants were Orthodox Christianity followers (63.3%). Regarding bad behavioral practices, about 11.7%, 46.6%, and 26.8% of the study participants were current smokers, alcohol drinkers, and khat chewers, respectively. Out of the total participants, only 13.02% of them had vigorous physical activity (Table 1).

Clinical characteristics of the study participants

About 4.17%, 14.32%, 8.59%, 6.77%, 15.63%, 15.10%, 3.39%, and 32.03% of the participants were diagnosed with lung, breast, cervical, ovarian, hematologic, gastrointestinal, skin, and another type of cancers, respectively. Near to two-thirds of the participants were diagnosed with stage I cancer (60.16%) and 9.11% of them were under an advanced stage or stage IV. The majority of study participants (46.4%) were diagnosed with a duration of three to seven months, and more than two-thirds of the participants (68.8%) were not characterized by cancer metastasis. Most of the participants (82.6%) were already under treatment; of these about 25.24%, 38.8%, and 35.96% were treated with chemotherapy, surgery, and combined chemotherapy & surgery, respectively. Moreover, 36.2% and 16.67% of the study participants had hypertension and diabetes comorbidities, respectively (Table 2).

TABLE 1 Socio-demographic characteristics of the study participants among adults in urban areas of Northwest Ethiopia, 2022 (n=384).

Characteristics	Number	Percent
Age (years)		
Mean ± SD	49.34 ± 0.66	
Age group		
18-40	99	25.78
41-60	215	55.99
>60	70	18.23
Sex		
Male	187	48.70
Female	197	51.30
Religion		
Orthodox	243	63.28
Muslim	91	23.70
Protestant	45	11.72
Others	5	1.30
Occupation		
Farmer	144	37.50
Merchant	82	21.35
Governmental Others	119 39	30.99 10.16
Wealth index		
Poor	112	29.17
Medium	248	64.58
Rich	24	6.25
Current smoking status		
Yes	45	11.72
No	339	88.28
Alcohol drinking status		
Yes	179	46.61
No	205	53.39
Khat chewing		
Yes	103	26.82
No	281	73.18

(Continued)

TABLE 1 Continued

Characteristics	Number	Percent
Physical activity status		
Vigorous	50	13.02
Moderate	132	34.38
Poor	202	52.60
Type of dietary oil		
Solid	212	54.38
Liquid	172	45.62

Other occupation includes students, stay-at-home spouses, unemployed and retired workers.

Determination of central obesity using waist circumference definition criteria and weight status of the participants

This study found that 19.27% (95% CI; 15.61- 23.54) of the cancer patients were centrally obese; of these, the majority (91.89%) were females. However, only 7.03% (95% CI; 4.86 - 10.07) of the study participants were overweight and none of them were obese based on the BMI categorization criteria. Besides, about 12.24% (95% CI; 9.31 -15.93) of the study participants were found to be underweight (Table 3).

The distribution of central obesity across the type of cancer patients

This study has shown the distribution of central obesity across cancer patients. Accordingly, we found that about 1 out of 15 lung cancer patients, 20 out of 35 breast cancer patients, 12 out of cervical cancer patients, 6 out of 20 0varian cancer patients, 8 out of 52 hematologic cancer patients, 5 out of 53 gastrointestinal cancer patients, 2 out of skin cancer patients, and 20 out of 103 other types of cancer patients were centrally obese (Figure 1).

TABLE 2 Clinical characteristics of the study participants among adults in urban areas of Northwest Ethiopia, 2022 (n=384).

Variable	Frequency	%
Type of cancer		
Lung cancer	16	4.17
Breast cancer	55	14.32
Cervical cancer	33	8.59
Ovarian cancer	26	6.77
Hematologic cancer	60	15.63
Gastrointestinal cancer	58	15.10
Skin cancer	13	3.39
Others	123	32.03
Stage of cancer		
Stage I	231	60.16
Stage II	68	17.71
Stage III	50	13.02
Stage IV	35	9.11
Duration of cancer diagnosed (in months)		
≤2	122	31.77
3-7	178	46.35
≥8	84	21.88

(Continued)

TABLE 2 Continued

Variable	Frequency	%
Metastasis		
Yes	120	31.25
No	264	68.75
Cancer pain		
Yes	230	59.90
No	154	40.10
Treatment for cancer		
Yes	317	82.55
No	67	17.45
Type of treatment		
Chemotherapy	80	25.24
Surgery	123	38.80
Combined Chemotherapy & Surgery	114	35.96
Duration of cancer treatment		
≤2	86	27.13
3-6	157	49.53
≥7	74	23.34
Hypertension		
Yes	139	36.20
No	245	63.80
Hyperglycemia		
Yes	64	16.67
No	320	83.33

Factors associated with central obesity among study participants

The study analyzed the multinomial logistic analysis for central obesity to determine its association with the sociodemographic, behavioral, and clinical characteristics of cancer patients. Thus, being female (p<0.001), having urban residency (p=0.002), and having occupations other than a farmer, merchant, and governmental (p=0.036) were found to be the factors associated with central obesity. The probability of having central obesity was 14 times higher among female participants compared to that of male cancer participants (AOR=14.40; 95 CI: 5.26 - 39.50). The

TABLE 3 Prevalence of central obesity and weight status of the cancer patients at the University of Gondar Specialized Comprehensive Hospital, Northwest Ethiopia, 2022 (n=384).

	Male (%)	Female (%)	Total	Prevalence (95%CI)
Central obesity using waist circumstance				
Centrally obese	6 (8.11)	68 (91.89)	74	19.27 (15.61- 23.54)
Not centrally obese	181 (58.39)	129 (41.61)	310	80.73 (76.45 - 84.38)
Weight Status				
Underweight	22 (11.76)	25 (12.69)	47	12.24 (9.31 -15.93)
Normal	156 (83.42)	154 (78.17)	310	80.73 (76.45 - 84.39)
Overweight	9 (4.81)	18 (9.14)	27	7.03 (4.86 - 10.07)

CI, confidence interval.

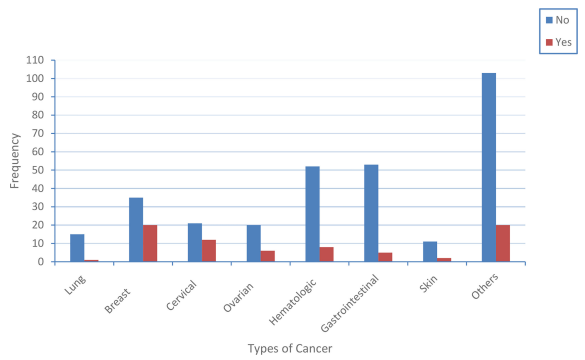

FIGURE 1
The distribution of central obesity across the type of cancer patients. The blue and red color indicates the proportion of cancer patients that have central obesity and do not have central obesity, respectively.

odds of having central obesity were 4 times higher among study participants with an occupation other than a farmer, merchant, and governmental as compared to that of the farmers (AOR=4.32; 95% CI: 1.10 - 17.01). Urban residents were less likely to have central obesity by 30% compared to rural residents (AOR=0.30; 95CI: 0.13 - 0.70) (Table 4).

Discussion

This is the first study that revealed the most dangerous type of obesity or hidden type of obesity (central obesity) and its association among cancer patients in Ethiopia and the study area.

The study found that a significant proportion of study participants (19.27%) were centrally obese among cancer patients that follow at UOGSCH. However, none of the study participants were found to be obese according to the BMI definition criteria of obesity. Even the prevalence of overweight (7.03%) was lower than compared to that of central obesity, and about 12.24% of the participants were confirmed to be underweight. Most researchers are advised to check fat accumulation around the abdominal wall, which is usually identified through waist circumference measurement, than that of general or peripheral obesity (via BMI) to know the exact pathogenic cause of obesity for the initiation and progression of inflammatory associated chronic diseases, including cancers (32–34). With normal or low BMI levels, people may develop central obesity since BMI can be affected by muscular mass (16, 35). Thus, our finding also revealed that the high burden of central obesity was encountered when compared to that of general obesity among the study participants. Abdominal/visceral obesity (identified by WC) is usually associated with metabolic changes which could affect the pathogenesis of tumor cells. However, the association between obesity and cancers is controversial yet. Some study finding proved their strong positive association, and some others have found no association. However, it has been evidenced that weight gain or obesity is responsible for around 20% of all cancer cases (20). Moreover, several studies and review articles have publicized that over-accumulation of fat around the abdominal wall is the main cause of inflammation cascade initiation, which is one of the most common problems encountered in cancer pathogenesis (36–38). In an individual with visceral obesity, adiposity can be directly encapsulated for tumor development through response to the obesity which is usually associated with the direct release of pro-inflammatory cytokines, hormones (peptide and steroid hormones), and growth factors (39).

The prevalence of central obesity in this study is extremely lower than that of a study done by L.A. Healy et al. (40). This may be due to the study population variation since the current study includes all cancer patients attending the hospital regardless of the metabolic syndrome categorization, whereas the L.A. Healy et al. initially categorizes the study participants as metabolic and non-metabolic participants. As central obesity is one of the major components of metabolic syndrome, the prevalence can be significantly elevated in their study finding. The finding of central obesity was also lower than a study conducted in Africa (41). The discrepancy may be due to the restriction of the study population. The study done among the African population was focused on male cancer patients affected by prostate cancer. The study design may also be different from the current study. Several studies have also been conducted on the association of having central obesity and the progression of tumors (40–42). Although the prevalence of central obesity is a double-digit in the current study without being obese based on BMI classification criteria, it is much lower than studies done in Ethiopia among the general population (21–25). These discrepancies are due to the variation of study participants between studies. Our study focused on cancer patients with disease or medication associated with weight loss. Most studies done about abdominal obesity and cancer targeting their association which makes difficult for further comparison of our finding with other similar studies. This finding will bring for the investigation of further studies and attention for central obesity among cancer patients.

In the current study, the probability of having central obesity was 14 times higher among female participants compared to its counterpart. Although the exact molecular link between being female and central obesity development is not exactly clear, different studies have reported similar findings, even among non-cancer study participants (21, 43, 44). This might be justified as females are naturally fattier than that of males, which may contribute to the re-distribution of fat accumulation towards the visceral area. Consequently, the accumulation of this fat will end up with central obesity. Another possible reason may be due to females may have a sedentary lifestyle and less access to different leaflets that showed the precautions of central obesity for health (45). Most of the population living in developing countries; including Ethiopia do not have enough awareness about central obesity as they estimate their weight using BMI. However, central obesity found among normal BMI groups is the most dangerous as mentioned earlier. It is not also surprising that females living in developing countries have less access to work outside the home, making it more difficult to get different leaflets. Besides, most females have focused on technical activities rather than labor work, and central obesity is frequently associated with those individuals who didn't undergo physical exercise or labor work (45–47).

The study also revealed that occupation was statistically associated with central obesity among cancer patients. The odds

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TABLE 4 The multivariable logistic regression analysis that identified the factors associated with central obesity among cancer patients at the University of Gondar Specialized Comprehensive Hospital, Northwest Ethiopia, 2022 (n=384) .

Characteristics	Central obesity			
	Yes	No	AOR (95%CI)	P-value
Age category (years)				
<50	36	160	1	
≥50	38	150	1.25 (0.63 - 2.50)	0.517
Sex				
Male	6	181	1	
Female	68	129	14.40 (5.26 - 39.50)	0.000**
Residency				
Rural	61	185	1	
Urban	12	124	0.30 (0.13 - 0.70)	0.002
Occupation				
Farmer	19	125	1	
Merchant	23	59	1.51 (0.54 - 4.20)	0.428
Governmental	23	93	1.81 (0.70 - 4.65)	0.220
Others	6	33	4.32 (1.10 - 17.01)	0.036
Wealth index				
Poor	14	98 191	1	
Medium	57	21	1.55 (0.68 -3.52)	0.290
Rich	3		1.45 (0.28 - 7.47)	0.654
Type of dietary oil				
Liquid	35	137	1	
Solid	39	166	0.60 (0.29 - 1.24)	0.174
Alcohol drinking status				
No	54	129	1	
Yes	20	181	0.39 (0.20 - 0.81)	0.105
Smoking status				
No	67	255	1	
Yes	7	55	2.12 (0.60- 7.55)	0.243
Khat chewing status				
No	17	86 224	1	
Yes	57		1.00 (0.41 - 2.44)	0.998
Physical activity status				
Vigorous	7	43 105	1	
Moderate	27	162	1.15 (0.35 - 3.84)	0.811
Poor	2740		0.95 (0.31 - 2.96)	0.936
Type of cancer				
Lung	15	1	1	

(Continued)

TABLE 4 Continued

Characteristics	Central obesity			
	Yes	No	AOR (95%CI)	P-value
Breast	35	20	0.107 (0.0031- 3.68	0.216
Cervical	21	12	0.102 (0.0027 - 3.81)	0.216
Ovarian	20	6	0.039 (.001 - 1.55)	0.085
Hematologic	52	8	0.370 (0.02 - 5.94)	0.483
Gastrointestinal	53	5	0.058 (0.02- 2.10)	0.120
Skin	11	2	0.267 (0.01 - 14.15)	0.514
Others	103	20	0.170 (0.01- 5.10)	0.308
Duration since diagnosis				
<4 month	37	196	1	
≥4 month	37	114	1.16 (0.52 - 2.59)	0.713
Treatment Type				
Chemotherapy	10	70	1	
Surgery	25	98	1.03 (0.37 - 2.8)	0.957
Combined therapy	28	86	1.08 (0.39 -2.95)	0.877
Duration since treatment				
<3 month or no	19	103	1	
3-6 month	36	142	0.90 (0.362.21)	0.818
>6 month	19	65	1.45 (0.46 - 4.56)	0.518

1 is an indication of reference point, ** is an indication of statistically significant at p-value < 0.001, AOR, adjusted odds ratio; CI, confidence interval.

of having central obesity were 4.3 times higher among study participants with occupations other than a farmer, merchant, and governmental than the farmers. We used farmers as reference groups because farmers are less likely to fall victim to central obesity or cancer due to the reason that farmers have routine activities on the farm, which is energy-demanding work that lowers the risk of getting central obesity (48). This can be logically justified as the type of occupation is usually associated with energy expenditure per day. More physical activity brings less fat accumulation on the abdominal wall as lipids or fats are one of the most common energy precursors, especially during the exercise period. Thus, in our study who are regarded as other than a farmer, merchants and government include those participants having no work, are retired, students, and housewives. These study participants may be directly or indirectly favorable for not having labor work or regular exercise. Therefore, early screening of central obesity is vital for all cancer patients to limit its adverse outcomes regardless of their BMI level. It is also supported by a study which reported that centrally obese individuals have poor prognostic outcomes among breast cancer study participants (49).

This study also found that those living in urban areas were less likely to have central obesity by 30% compared to rural residents. This may be due to the reason that accessibility of media, which creates awareness to maintain their weight status, and the probability of getting physician advice is more for urban residents compared to rural residents in Ethiopia (50). Thus, urban residents may undergo regular exercise and modify their diet (which can lower the risk of central obesity and also help to improve the problem). Additionally, cancer patients living in rural areas have less knowledge about the association of central obesity with cancer outcomes. The disparities in cancer risk factors, incidence, mortality, and associated metabolic diseases are also greatly affected by the residency of the patients (51). Therefore, it will be encroached to have health education focused on central obesity for cancer patients.

Conclusion

This study identifies the magnitude of the hidden type of obesity called central or visceral or abdominal obesity and found that its prevalence was 19.27% among cancer patients attending at oncology unit of the University of Gondar Specialized Comprehensive Hospital. This magnitude was significantly higher among female study participants than that males. Being female, having urban residency, and having occupations other than farmer, merchant, and government were found to be the factors associated with central obesity. Based on our findings, we encouraged screening for early identification of central obesity among cancer patients, and it will be good to manage it, especially for those participants who belong to the associated factors.

Strengths and limitations of the study

This is the first study that focused on the burden of central obesity and its associated factors in Ethiopia. The study area that fills the knowledge gap on it is taken as the study's main strength. Being a cross-sectional study design that did not show the cause-effect relationship of the variables, the inability to address the dietary habit of the participants, and the inability to use hip to waist circumference ratio to define central obesity were the study's limitations. Besides, this study was focused at a single institute that did not generalize the overall cancer patient of Ethiopia, which was another limitation of the study.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki. Ethical approval was obtained from the University of Gondar Ethical Review Committee. Before running the data collection, written informed consent was obtained from each study participants. All the way through the process of the study, confidentiality was kept for all study participants. The patients/participants provided their written informed consent to participate in this study.

Author contributions

All authors contributed to the study's conception and design. All authors performed material preparation, data collection, and analysis. The first draft of the manuscript was written by MM and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Influence of bariatric surgery on the peripheral blood immune system of female patients with morbid obesity revealed by high-dimensional mass cytometry

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Introduction: Obesity is associated with low-grade chronic inflammation, altered levels of adipocytokines, and impaired regulation of gastrointestinal hormones. Secreted, these factors exert immunostimulatory functions directly influencing peripheral immune cells.

Methods: In the realm of this study, we aimed to investigate the composition and activation status of peripheral blood immune cells in female patients with morbid obesity compared to lean controls using high-dimensional mass cytometry. Besides, we also assessed the influence of bariatric surgery with respect to its ability to reverse obesity-associated alterations within the first-year post-surgery.

Results: Patients with morbid obesity showed typical signs of chronic inflammation characterized by increased levels of CRP and fibrinogen. Apart from that, metabolic alterations were characterized by increased levels of leptin and resistin as well as decreased levels of adiponectin and ghrelin compared to the healthy control population. All these however, except for ghrelin levels, rapidly normalized after surgery with regard to control levels. Furthermore, we found an increased population of monocytic $CD14^+$, $HLA\text{-}DR^-$, $CD11b^+$, $CXCR3^+$ cells in patients with morbid obesity and an overall reduction of the HLA-DR monocytic expression compared to the control population. Although $CD14^+$, $HLA\text{-}DR^-$, $CD11b^+$, $CXCR3^+$ decreased after surgery, HLA-DR expression did not recover within 9 – 11 months post-surgery. Moreover, compared to the control population, patients with morbid obesity showed a perturbed CD4+ T cell compartment, characterized by a strongly elevated $CD127^+$ memory T cell subset and decreased naïve T cells, which was not recovered within 9 – 11 months post-surgery. Although NK cells showed an activated phenotype, they were numerically lower in patients with morbid obesity when compared to healthy controls. The NK cell population further decreased after surgery and did not recover quantitatively within the study period.

Conclusions: Our results clearly demonstrate that the rapid adaptions in inflammatory parameters and adipocytokine levels that occur within the first year post-surgery do not translate to the peripheral immune cells. Apart from that, we described highly affected, distinct immune cell subsets, defined as $CD127^+$ memory T cells and monocytic $CD14^+$, HLA-DR, $CD11b^+$, $CXCR3^+$ cells, that might play a significant role in understanding and further decoding the etiopathogenesis of morbid obesity.

KEYWORDS

obesity, bariatric surgery, inflammation, mass cytometry (CyTOF), immune cells

1 Introduction

Obesity was firstly described as a disease by the World Health Organization in the year 2000 thanks to its significantly growing prevalence and serious threat to public health (1). The most severe form of obesity, referred to as morbid obesity, is defined by a body mass index (BMI) of ≥ 35 kg/m^2 accompanied by one or more severe comorbidities or a BMI of ≥ 40 kg/m^2 (2). Although obesity is on a rise in all age groups, morbid obesity shows the same or even a higher growth rate compared to milder forms of obesity (3). It's worth noting, that obesity in patients with a BMI between 55 and 55.9 kg/m^2 is associated with a reduction in life expectancy of up to 13.7 years (4). Morbid obesity is known to be directly or indirectly associated with as many as 60 clinically relevant ailments. Among them are type 2 diabetes (DM2), different kinds of malignancies (breast, colon, etc.), gallbladder disease, sleep apnea, respiratory problems, and osteoarthritis (5). Furthermore, obesity is an elevated risk factor for cardiovascular diseases like coronary heart disease due to hypertension, dyslipidemia and hyperinsulinemia.

Most of these diseases are sequelae of severe metabolic alterations evoked by an obesity-associated dysfunction of adipose tissue leading to a chronic low-grade inflammation, persistent in patients with morbid obesity. Pathological increase of adipose tissue affects number and qualitative functioning of adipose tissue-resident immune cells leading to an altered secretion profile of pro- and anti-inflammatory cytokines as well as adipocytokines including leptin, adiponectin and resistin (6, 7). Apart from that, gastrointestinal hormones like ghrelin and glucose-dependent insulinotropic polypeptide (GIP), involved in regulation of energy homeostasis, insulin secretion, and appetide control, have been shown to be dysregulated in obesity (8). Since adipocytokines and gastrointestinal hormones are released into the circulatory system, where immune cells express the respective receptors, the immune-stimulatory or -inhibitory function of these hormones (9) might strongly be involved in regulating the chronic inflammation observed in obesity.

The chronic inflammation in patients with morbid obesity is hypothesized to highly influence the immune system resulting in an increased susceptibility for infections (10) and cancer (11). Different peripheral blood immune cell subsets including CD4+ T cells, B cells, NK cells, as well as monocytes have been shown to be highly influenced by morbid obesity (12). The T cell compartment is perturbed as a result of a thymic dysfunction characterized by an accumulation of memory T cells and decreased naïve T cells (13). NK cells undergo obesity-induced metabolic reprogramming limiting their function (14), whereas monocytes are primed towards a pro-inflammatory phenotype and concomitantly accumulate immune-suppressive subsets (15).

Bariatric surgery, exemplified by sleeve gastrectomy and proximal gastric bypass surgeries, is the most effective and sustainable treatment for weight loss, relief of obesity-associated comorbidities like DM2 and resolution of chronic inflammation (16–18). Although bariatric surgery seems to highly improve the quality of life (19), long-term side-effects like malnutrition or an increased risk of anemia have been observed (20). The effect of bariatric surgery on the immune system is still not fully elucidated. Several studies observed positive adaptions of immune cell populations after bariatric surgery including NK cells, monocytes, B cells, and T cells (2, 21–23). However, these studies often focused on a chosen few immune cell populations or just referred to the cell count without a detailed analysis of the immune cell populations phenotyping.

Therefore, the aim of this study was to perform high-resolution phenotyping of immune cell populations detectable in whole blood samples of patients with morbid obesity and lean controls using mass cytometry, and to study the effect of bariatric surgery on these immune cell populations. We detected increased populations of $CD127^+$ CD4+ memory T cells and monocytic $CD14^+$, $HLA\text{-}DR^-$, $CD11b^+$, $CXCR3^+$ cells as well as decreased levels of naïve CD4+ T cells and NK cells; a phenomenon, which did not fully reverse within 11 months after surgery. These results give further insight into the impact of bariatric surgery on the already impaired immune system of patients with morbid obesity and highlight the importance of a careful monitoring after bariatric surgery to prevent functional restrictions in a long-term perspective.

2 Material and methods

2.1 Ethics

The study protocol was approved by the ethics committee at Ulm University (ethical grant no. 30-20). The protocol was conducted following the Declaration of Helsinki. All study

participants were extensively informed by an attending physician and gave their written, explicit consent. Data of study participants was pseudonymized.

2.2 Study cohorts and sampling

Female, adult (≥ 18 years) patients that underwent bariatric surgery at Ulm University Hospital between 2020 and 2022 and met the criteria according to the "German S3-guideline: Surgery for obesity and metabolic diseases" (BMI ≥ 40 kg/m^2 or BMI ≥ 35 kg/m^2 with one or more obesity-associated comorbidities) were included in the study. Patients suffering from inflammatory bowel disease, systemic inflammatory disease, acute infections, cancer, autoimmune disease or receiving immunosuppressive therapy were excluded from the study. Study participants included in the patient group were of non-Hispanic White ethnicity.

Initial baseline comparison (prior to surgery) using conventional flow cytometry included 38 female patients and 10 healthy, female age-matched controls (CTRL). The CTRL group consisted of non-Hispanic White normal weight (BMI ≥ 18.5 and BMI ≤ 25.0 kg/m^2), female employees engaged at Ulm University Hospital. According to a self-declaration of health, study participants of the CTRL group did not suffer from any acute and chronic diseases or physical restrictions. Furthermore, study participants of the CTRL group did not receive medications including antihypertensives, antidepressants, antidiabetics, statins, vitamin supplements, thyroid hormones, proton-pump inhibitors, or anti-inflammatory drugs. Additionally, clinical blood parameters were determined for the CTRL cohort to exclude inflammatory and metabolic disorders (Supplementary Table 1).

To assess the effect of bariatric surgery on the peripheral blood immune system, a subcohort of 12 female patients was randomly selected and samples were acquired at baseline, 1 – 2 months post-surgery (p.s.), 3 - 5 months p.s., 6 - 8 months p.s. and 9 - 11 months p.s. using high-dimensional mass cytometry by time of flight (CyTOF), whole-blood-based quantitative reverse transcription polymerase chain reaction (qRT-PCR) and bead-based immunoassays. The previously described control cohort was used as reference. Four weeks prior to surgery, patients were on a strict diet consisting of 40% carbohydrates, 25% protein and 35% fat as well as a daily minimum of 30 g dietary fibers. The aim of the diet was to reduce liver size making surgery more feasible and consequently minimizing complications.

EDTA-blood samples were collected one day prior to surgery and at the respective time-points within follow-up examinations at Ulm University Hospital and subsequently processed in the laboratory. Patients and controls were not in a controlled fasted state at blood draw.

2.3 Anthropometric measurements and clinical data

The BMI in kg/m^2 describes the ratio of the person's weight in kilograms to the squared height in meters. Body weight and body height were determined prior to surgery and at the respective time-points during follow-up examinations at Ulm University Hospital. Blood samples were taken routinely and analyzed in the Department of Clinical Chemistry at Ulm University Hospital (accredited according to DIN EN ISO 15189). Reference values were also obtained from the Department of Clinical Chemistry at Ulm University Hospital.

2.4 Flow cytometry staining and analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from 3 mL EDTA-blood using Ficoll-Paque™ PLUS (Cytiva). Blood was mixed with equal volume of PBS (Gibco), layered over Ficoll and centrifuged for 20 min and 300 x g at room temperature (RT) with breaks off. PBMCs were aspirated and washed twice with 10 mL PBS and once with 1 mL staining buffer (1 x PBS, 1% BSA, 2 mM EDTA, 0.05% NaN_3) each time followed by a centrifugation step (300 x g, 8 min, RT). Staining of 1.0 x 10^6 cells was performed in the dark for 30 min at 4°C using 100 µL staining buffer containing fluorescent labelled antibodies (dilution 1:100) specific to CD3 (VioBlue, Miltenyi Biotec, 130-114-519), CD4 (PE, Miltenyi Biotec, 130-113-225), CD8 (APC, Miltenyi Biotec, 130-110-679), CD19 (FITC, Miltenyi Biotec, 130-113-645), CD56 (PE-Vio 770, Miltenyi Biotec, 130-113-313), CD14 (APC-Vio 770, Miltenyi Biotec, 130-110-522), CD16 (VioGreen, Miltenyi Biotec, 130-113-397) and HLA-DR (PerCP-Vio 700, Miltenyi Biotec, 130-111-793). Cells were washed with 2 mL staining buffer, centrifuged (300 x g, 8 min, RT), resuspended in 500 µL staining buffer and subsequently acquired on a MACSQuant® Analyzer 10 Flow Cytometer (Miltenyi Biotech). Compensation was performed using instrument-specific automated compensation with single-stained compensation beads. To exclude background staining and unspecific binding of antibodies, isotype controls have been measured. All samples were acquired with the same voltage settings. Analysis of data was performed using Flowlogic™ 8.4 software (Inivai Technologies) according to the provided gating scheme (Supplementary Figure 1). To analyze and exclude age-related effects on major immune cell subsets, patient cohort was subdivided into two age groups (20 – 45 years and 45 – 61 years) (Supplementary Figure 2).

2.5 Mass cytometry staining

One mL of EDTA-blood was mixed with 1 mL SmartTube Proteomic Stabilizer PROT1 (SMART TUBE Inc.), incubated for 10 min at RT and stored at -80°C. Samples were thawed and erythrocyte lysis was performed using 1x Thaw-Lysis buffer (SMART TUBE Inc.) according to manufacturer's specifications. After lysis, cells were resuspended in 2 mL CyFACS buffer (1 x PBS (Rockland, MB-008), 1% BSA, 2 mM EDTA, 0.05% NaN_3) and cell concentration was determined. A total number of 1.5 x 10^6 cells per sample were used for the staining procedure. One mL CyFACS buffer was added, cells were centrifuged (600 x g, 8 min, RT) and supernatant was discarded. To prevent unspecific antibody staining,

Fc receptor (FcR) block was performed by adding 3 µL of human FcR Blocking Reagent (Miltenyi) to the cells followed by an incubation for 20 min at RT. Master mix containing the antibodies listed in Supplementary Table 2 was prepared shortly prior to staining and filtered with 0.1 µM spin filter units (Merck).

One hundred µL of master mix was added to each sample and incubated for 30 min at 4°C. Titration of antibody panel was performed prior to the first experiments to determine the antibody concentration. Antibodies that were not purchased from Fluidigm were conjugated in-house using the respective Maxpar X8 Antibody Labeling Kit (Fluidigm) according to manufacturer's specifications.

Cells were washed two times with 1 mL CyFACS buffer (600 x g, 8 min, RT). Fixation was performed by adding 1 mL of 3% methanol-free paraformaldehyde (PFA) (Thermo Scientific Pierce) in CyPBS followed by an incubation for 2 h at 4°C. Samples were washed twice with 1 mL CyPBS (600 x g, 8 min, RT). Cells were incubated with 0.5 mL of 0.2% Cell-ID Intercalator-Ir (Fluidigm) in Maxpar Fix and Perm Buffer (Fluidigm) for 20 min at RT followed by two washes with 2 mL CyPBS (600 x g, 8 min, RT). Cells were frozen in 1 mL cold freezing medium (10% DMSO in fetal calf serum) and stored at -80°C until day of acquisition.

On the day of acquisition, samples were thawed in a cold-water bath and washed once with 1 mL CyFACS containing Benzonase/Nuclease (Sigma Aldrich, 1:10,000), once with 1 mL CyFACS without Benzonase and three times with Maxpar Water (Fluidigm) each time centrifuged at 600 x g for 8 min at RT. Samples were adapted to a cell concentration of 10^6 cells/mL and 300,000 cells were measured at 300 events/s on a Helios system (Fluidigm). EQ Four Element Calibration Beads (Fluidigm) were used for normalization over time.

2.6 Treatment of anchor samples

Anchor samples consisting of buffy-coat derived PBMCs (purchased from Institute for Clinical Transfusion Medicine and Immunogenetics (IKT), Ulm, Germany) were adapted to a concentration of 1.0 x 10^6 cells/mL. One mL aliquots were mixed with 1 mL SmartTube Proteomic Stabilizer PROT1 (SMART TUBE Inc.) and frozen at -80°C. Anchor samples were treated the same way as patient samples and one anchor was included in each batch of measured patient samples.

2.7 Mass cytometry data acquisition and analysis

Generated raw FCS files were preprocessed prior to normalization (Supplementary Figure 2A). Calibration beads were removed, and DNA positive cells were identified (^{191}Ir and ^{193}Ir). Doublets were excluded using gaussian parameters event length and residual (24). Batch normalization was performed using R-based CyTOF Batch Adjust workflow (25) adapted for Windows system. Channel specific batch-to-batch variation was evaluated using anchor samples and adjustment factors were transferred to patient samples (Supplementary Figure 2B).

After batch normalization, FCS files were uploaded to Cytobank (Beckman Coulter), transformed (arcsinh, co-factor = 5) and analyzed using manual gating as well as machine learning algorithms including dimensionality reduction *via* Uniform Manifold Approximation and Projection (UMAP) (26) and clustering *via* Self-Organizing Map (SOM) algorithm FlowSOM (27). Twenty thousand cells per sample were included in the automated analysis. For the illustration of the UMAP density-plots, FCS files were exported and concatenated according to their corresponding group using the CATALYSTLite online application (28).

In order to analyze populations of interest at a higher resolution, manually gated populations were exported from Cytobank as raw value FCS files. Validation of the gating was performed by comparing percentages of the manually gated populations with the percentages of populations identified with FlowSOM using linear regression analysis (Supplementary Figure 3). For monocytes, manual gating seemed to be prone to slight underestimation of the population. This might be due to high heterogeneity within the monocyte population that could be better depicted with the algorithm (29). Mean frequencies of manually gated $CD45^+$ cells can be found in the supplement (Supplementary Table 3).

For the detailed analysis of specific cell populations the Spectre R package was used (30) with instructions and source code provided at https://github.com/ImmuneDynamics/spectre. The validated population of interest was exported from Cytobank as raw value FCS files. The arcsinh transformed (co-factor 5) dataset was merged into a single data.table, with keywords denoting the sample, group, and other factors added to each row (cell). The FlowSOM algorithm (27) was then run on the merged dataset to cluster the data, where every cell is assigned to a specific cluster and metacluster. Subsequently, the data was randomly downsampled to 10,000 cells per group and analyzed by the dimensionality reduction algorithm UMAP (26) for cellular visualization. Annotated clusters were depicted in a heatmap as normalized abundances with respect to the mean of the CTRL population. Statistical analysis was performed as described in the corresponding chapter "statistical analysis".

Marker expression of clusters was shown as volcano plots generated with an adapted version of the EnhancedVolcano R script (31). Marker expression of each analyzed surface marker is shown as $\log_2$ fold change (FC) calculated based on the cluster-specific mean fluorescence intensities (MFIs) of the respective groups. $\log_2$ FC (x-axis) is plotted against the statistical significance (y-axis) shown as $-\log_{10}$ p. Statistical significance was calculated based on cluster-specific MFIs using an unpaired two-sided Wilcoxon test ($\alpha = 0.05$).

A list of the immune cell populations that have been identified via mass cytometry as well as the corresponding surface markers that have been used for identification is shown in the supplement (Supplementary Table 4).

The R scripts used for the analysis of the data are available as PDF documents in the supplement.

2.8 RNA isolation

Two hundred µL of EDTA-blood were mixed with equal volumes of Monarch® DNA/RNA Protection Reagent (New England

BioLabs), vortexed and stored at -80°C. RNA isolation was performed according to manufacturer's specifications. Samples were thawed at RT and 10 µL proteinase K were added. Samples were vortexed and incubated for 30 min at RT. Four hundred µL isopropanol were added, vortexed, transferred to an RNA purification column and centrifuged (16,000 x g, 30 sec). For removal of residual genomic DNA on-column DNAse I treatment was performed. Priming buffer (0.5 mL) was added followed by two wash steps with RNA Wash Buffer. RNA was eluted with 50 µL nuclease-free water. RNA concentration was determined using QIAxpert System.

2.9 qRT-PCR analysis

One hundred and fifty ng total RNA were transcribed into complementary DNA (cDNA) using the AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies) with oligo(dT) primers according to manufacturer's specifications. Samples were adjusted to a final concentration of 1 ng/µL with nuclease-free water. Prior to qRT-PCR, samples were tested for genomic DNA (gDNA) contamination using exon-specific primers (Forward: 5'-TCT GCC GTT TTC CGT AGG ACT CTC-3', Reverse: 5'-CCC TGG ATG TGA CAG CTC CCC-3') and cDNA functionality using intron-specific primers (Forward: 5'-GGC ATC CTC ACC CTG AAG TA-3', Reverse: 5'- GTC AGG CAG CTC GTA GCT CT-3') for *ACTB* in conventional PCR. After exclusion of gDNA contamination and confirmation of cDNA functionality, qRT-PCR was performed using QuantiTect SYBR Green PCR kit (Qiagen) and QuantiTect Primer assays specific for *GAPDH* (QT00273322), *RPLP0* (QT00075012), *LEPR* (QT00006524), *ADIPOR1* (QT00002352), *GIPR* (QT00033138) and *GHRL* (QT00041377). *GAPDH* and *RPLP0* were validated and used as housekeeping genes (Supplementary Figure 4). Melting curves of PCR products were evaluated and should have been between 72°C to 86°C according to manufacturer's specifications. Calculated ΔCT values of patients were normalized to the mean ΔCT value of control population resulting in depicted x-fold values.

2.10 Bead-based immunoassay

EDTA-blood was centrifuged at 2,600 x g for 15 min and plasma was stored at -80°C. Samples were thawed and analyzed in duplicates using the LEGENDplex Human Metabolic Panel with a V-bottom plate (BioLegend). The measured analytes included adiponectin (beads A4), adipsin (beads A5), leptin (beads B4) and resistin (beads B7). Staining and set-up were performed according to the manufacturer's specifications. Data was acquired on MACSQuant® Analyzer 10 Flow Cytometer (Miltenyi Biotech) and analysis was performed with the Legendplex Cloud-based software (BioLegend, Version 2022-07-15).

2.11 Enzyme-linked immunosorbent assay

Enzyme-linked Immunosorbent Assays (ELISAs) were performed with plasma samples using commercially available ELISA kits for Ghrelin (ThermoFisher, # BMS2192) and GIP (Merck, EZHGIP-54K) according to the manufacturer's specifications. Absorption was determined at 450 nm in Tecan Spark® Multimode Reader.

2.12 Statistical analysis

GraphPad Prism 7.04 (GraphPad Software, Inc.) was used for statistical analysis. The specific tests performed are described below each graph. When comparing two groups, a two-tailed Student's *t* test ($\alpha = 0.05$) was performed if requirements of normal distribution (Shapiro-Wilk test) and homogeneity of variances (F-test) were given. Otherwise, a Welch's test or a Mann-Whitney test were performed. When comparing more than two groups, a one-way ANOVA with Geisser-Greenhouse correction followed by an uncorrected Fisher's LSD test ($\alpha = 0.05$) was performed when normal distribution (Shapiro-Wilk test) was given. Otherwise, a Kruskal-Wallis test with Geisser-Greenhouse correction followed by an uncorrected Dunn's test was performed. The specific method is stated in the figure legend. For each experiment, the sample sizes are depicted in the figure or in the figure legend. The following indicators were used for all statistical tests: * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, and **** indicates $p < 0.0001$. Data is depicted either as boxplots showing full range or as heatmaps showing either row z-score normalized data or min-max scaled data (surface marker expression), unless otherwise stated.

3 Results

3.1 Low-grade chronic inflammation was prevalent in the main cohort of female patients with morbid obesity prior to bariatric surgery

The health status of 38 female patients with morbid obesity who underwent bariatric surgery was assessed by determining pre-existing comorbidities, blood pressure, medication, as well as several blood parameters (Table 1). The median BMI of the cohort was 48.9 kg/m^2. The predominant procedure performed was sleeve gastrectomy (79%) followed by gastric bypass (21%). Patients with morbid obesity frequently suffered from diabetes and its preliminary stages (66%) and hyperlipoproteinemia (39%), which is also reflected in the blood values. Considering the measured blood parameters, patients showed typical obesity-associated signs of a low-grade chronic inflammation characterized by significantly elevated levels of both circulating C-reactive protein (CRP) (median= 7.7 mg/L) and circulating fibrinogen (median = 3.7 g/L) (32). The blood fat values for cholesterol (11 patients ≥ 5 mmol/L), triglycerides (13 patients ≥ 1.7), and low-density lipoprotein (LDL) (12 patients ≥ 3 mmol/L) were shifted towards the upper limit of normal levels, whereas the value of high-density lipoprotein (HDL) (11 patients ≤ 1.2 mmol/L) was shifted towards the lower limit of normal levels.

Apart from clinical blood parameters, PBMCs were characterized using flow cytometry. A control cohort (CTRL) consisting of 10 healthy, lean (BMI ≤ 25 kg/m^2), age- and gender-matched volunteers (Figures 1A, B) was established serving as a reference for the patient

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TABLE 1 Characteristics of female baseline cohort (n = 38) including age, body mass index (BMI), procedure, comorbidities, blood pressure, medication, and blood parameters.

	Female baseline cohort n = 38				
Characteristics	Median [Min. – Max.]				
Age	43.5 [20 – 64]				
BMI [kg/m^2]	48.9 [37.9 – 64.9]				
Procedure	∑ (%)				
Sleeve gastrectomy	30 (79)				
Gastric bypass (Roux-en-Y)	8 (21)				
Comorbidities	∑ (%)				
Diabetes and preliminary stages	25 (66)				
Arterial hypertension	17 (44)				
Hypothyroidism	13 (34)				
Obstructive sleep apnea	22 (58)				
Depressive disorder	12 (32)				
Hyperlipoproteinemia	15 (39)				
Blood pressure	Median [Min. – Max.]				
Systolic [mmHg]	146.5 [117 – 187]				
Diastolic [mmHg]	87.5 [72 – 138]				
Medication	∑ (%)				
Antidiabetics	21 [55]				
Antihypertensives	17 [44]				
Antidepressants	10 [26]				
Thyroid hormones	14 [37]				
Statins	5 [13]				
Vitamin supplements	9 [24]				
Proton pump inhibitors	8 [21]				
Blood parameters					
Parameter [Unit]	Median [Q1 – Q3]	Min. reference value	Max. reference value	n	p-value
CRP [mg/L]	7.7 [5.7 – 12.4]	–	5	37	<0.0001
Fibrinogen [g/L]	3.7 [3.4 – 4.6]	1.8	3.5	30	0.014
Triglycerides [mmol/L]	1.6 [1.2 – 2.0]	–	1.7	30	ns
Cholesterol [mmol/L]	4.5 [4.0 – 5.6]	–	5	30	ns
HDL [mmol/L]	1.3 [1.2 – 1.6]	1.2	–	30	ns
LDL [mmol/L]	2.7 [2.2 – 3.6]	–	3	30	ns
AST [U/L]	23.5 [21.0 – 30.8]	–	35	32	ns
ALT [U/L]	24.0 [19.0 – 33.0]	–	35	35	ns
Uric acid [µmol/L]	327.0 [267.3 – 356.3]	137	363	30	ns
Creatinine [µmol/L]	66.0 [54.5 – 70.5]	44	80	37	ns
GGT [U/L]	26.0 [18.0 – 32.0]	–	40	31	ns

(Continued)

TABLE 1 Continued

Blood parameters					
Parameter [Unit]	Median [Q1 – Q3]	Min. reference value	Max. reference value	n	p-value
AP [U/L]	78.5 [66.0 – 90.3]	35	105	32	ns
CK [U/L]	100.0 [70.3 – 128.8]	20	180	30	ns
LDH [U/L]	201.0 [182.0 – 228.5]	–	250	29	ns
Lipase [U/L]	29.0 [21.0 – 33.0]	13	60	31	ns
Albumin [g/L]	43.0 [42.0 – 46.0]	35	53	30	ns
Protein [g/L]	75.0 [72.0 – 78.0]	66	83	31	ns
Quick [%]	102.0 [93.0 – 112.0]	70	130	37	ns
PTT [s]	28.3 [26.8 – 30.0]	26	36	37	ns
Insulin [mU/L]	22.4 [17.1 – 43.3]	2.6	24.9	31	ns
HbA1c (IFCC) [mmol/mol]	37.0 [34.0 – 42.0]	20	42	30	ns
Leukocytes [Giga/L]	7.7 [7.3 – 9.2]	4.4	11.3	38	ns
Erythrocytes [Tera/L]	4.7 [4.4 – 4.9]	4.5	5.9	38	ns
Hemoglobin [g/dL]	13.4 [12.7 – 14.3]	12.3	15.3	38	ns
Hematocrit [L/L]	0.40 [0.38 – 0.42]	0.36	0.45	38	ns
MCV [fL]	85.5 [82.4 – 88.4]	80	96	38	ns
MCH [pg]	28.7 [27.7 – 29.9]	27.5	33.2	38	ns
MCHC [g/dL]	33.6 [33.3 – 34.2]	33.4	35.5	38	ns
RDW [%]	13.8 [13.3 – 14.9]	–	15	38	ns
Thrombocytes [Giga/L]	278.0 [232.5 – 329.3]	150	450	38	ns
MTV [fL]	9.5 [8.6 – 10.1]	6.8	10	38	ns

Statistical analysis was performed using a non-parametric, one-sample Wilcoxon Signed Rank test, designed to compare the mean of the baseline cohort to its respective Min. or Max. reference value using $\alpha = 0.05$; ns, non-significant. The reference values were obtained from the central institution of clinical chemistry at Ulm University Hospital.

group with morbid obesity (Baseline). T cells ($CD3^+$) (Figure 1C) including CD4+ T helper cells (Figure 1D) and CD8+ cytotoxic T cells (Figure 1E) as well as NK cells (Lin^-, $CD56^+$) (Figure 1G) tend to be slightly decreased in patients with morbid obesity. However, statistically these shifts were not significant due to high variation within the cohorts. B cells ($CD19^+$) (Figure 1F) and monocytes ($HLA\text{-}DR^+$, $CD14^+$, $CD16^{var}$) (Figure 1I) tend to be increased in patients with morbid obesity. Within the monocyte compartment the $HLA\text{-}DR^-$, $CD14^+$ cells (Figure 1H), referred to as immunosuppressive myeloid-derived suppressor cells (MDSCs) (15, 33), and the classical monocytes (CM) (Figure 1L) were significantly increased in patients with morbid obesity, whereas no difference was observed for intermediate monocytes (IM) (Figure 1K) and non-classical monocytes (NCM) (Figure 1J).

3.2 Bariatric surgery led to significant weight loss and improved inflammatory and metabolic blood parameters in a female sub cohort

To investigate the influence of bariatric surgery on the health status of patients with morbid obesity a subcohort consisting of 12 female patients with morbid obesity undergoing gastric sleeve or gastric bypass surgery was established (Figure 2A). Blood samples were taken prior to surgery (Baseline) and within aftercare checkups 1 - 2 months post-surgery (p.s.), 3 - 5 months p.s., 6 - 8 months p.s., and 9 – 11 months p.s. as illustrated in Figure 2A. The previously described gender- and age-matched control cohort (CTRL) was used as a reference for normal BMI (Figure 2B), adipocytokine levels, and immune cell levels determined via mass cytometry. For the time progression of weight loss (Figure 2B) and clinical blood parameters (Figure 2C) values were compared to the baseline.

Compared to baseline, bariatric surgery resulted in significant weight loss (Figure 2B) after only 1 - 2 months p.s.. Additionally, compared to baseline, inflammatory blood parameters like CRP significantly decreased within the first 3 – 5 months p.s. and the concentration of circulating leukocytes significantly decreased as soon as 1 – 2 months p.s. (Figure 2C), whereas fibrinogen tends to be non-significantly decline 9 – 11 months p.s. Furthermore, metabolic parameters including triglyceride levels as well as the insulin levels significantly declined after surgery, reaching statistical significance 3 – 5 months p.s. (triglycerides) or rather 6 – 8 months p.s. (insulin) compared to baseline. Whereas LDL levels seemed to be less affected by bariatric surgery, HDL concentration slightly elevated 9 – 11 months p.s., without reaching statistical significance compared to baseline (Figure 2C).

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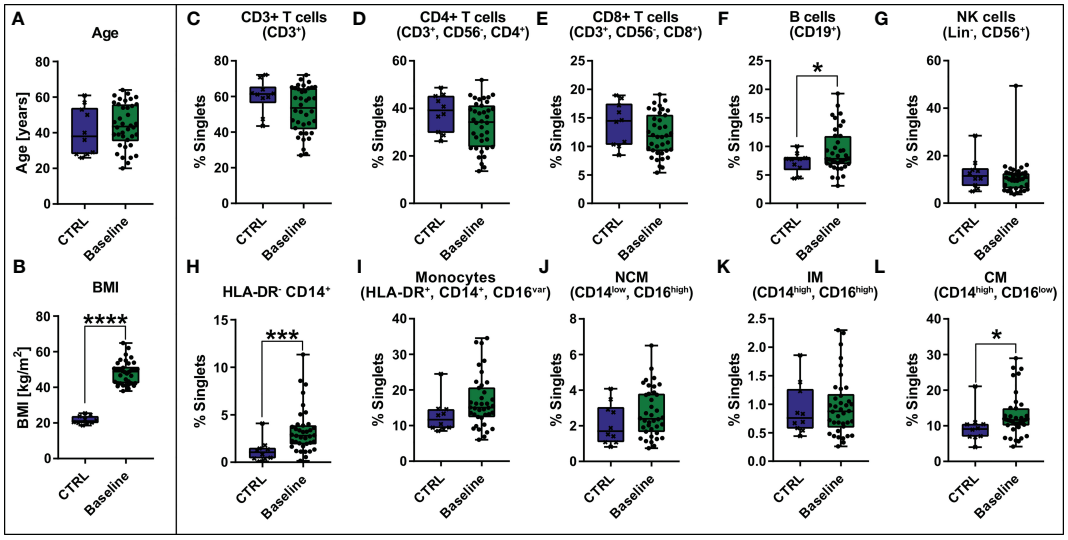

FIGURE 1
Comparison of age [years] **(A)** and BMI [kg/m^2] **(B)** between control cohort (CTRL, n = 10) and baseline cohort with morbid obesity (Baseline, n = 38) and comparison of abundance of PBMCs [% singlets] including CD3+ T cells (CD3$^+$), CD4+ T cells (CD3$^+$, CD56$^-$, CD4$^+$), CD8+ T cells (CD3$^+$, CD56$^-$, CD8$^+$), B cells (CD19$^+$), NK cells (Lin$^-$, CD56$^+$), HLA-DR$^-$ CD14$^+$, Monocytes (HLA-DR$^+$, CD14$^+$, CD16^{var}) divided in non-classical monocytes (NCM) (CD14^{low}, CD16^{high}), intermediate monocytes (IM) (CD14^{high}, CD16^{high}) and classical monocytes (CM) (CD14^{high}, CD16^{low}) **(C–L)**. Statistical analysis was performed using a two-sided unpaired t-test (Age, CD3+ T cells, CD4+ T cells, CD8+ T cells, NCM, IM and CM), a Welch's test (BMI, B cells) or a Mann-Whitney test (HLA-DR$^-$ CD14$^+$, Monocytes, NK cells) with $\alpha = 0.05$. *p-value ≤ 0.05, ***p-value ≤ 0.001, ****p-value ≤ 0.0001.

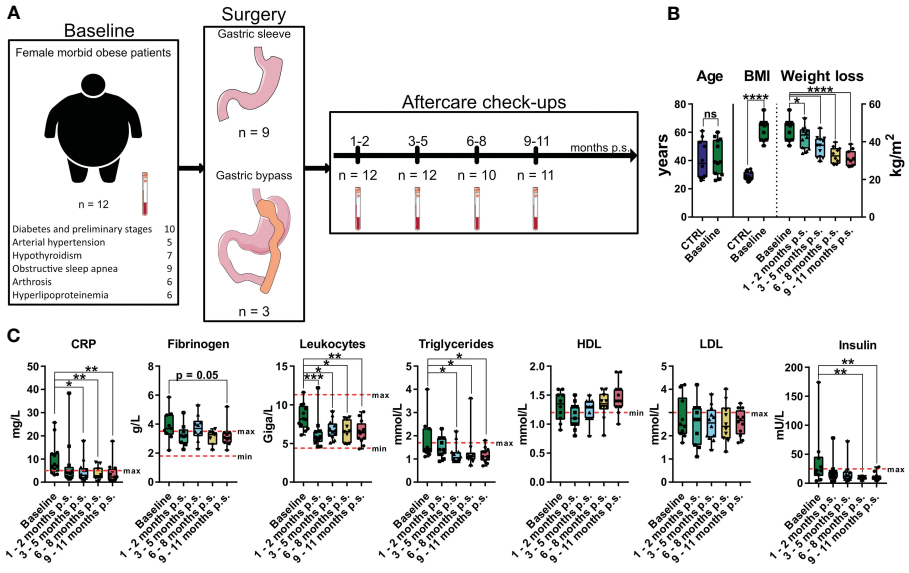

FIGURE 2
Overview and characterization of the female sub cohort used for high-dimensional mass cytometry analysis. Characterization of weight loss cohort including comorbidities, type of surgery, timepoints of sampling and sample size **(A)** as well as comparison of age and BMI to the control cohort (CTRL) **(B)**. Weight loss illustrated as time-progression of BMI **(B)** as well as time-progression of clinical parameters **(C)** including CRP [mg/L], fibrinogen [g/L], leukocytes [Giga/L], triglycerides [mmol/L], HDL [mmol/L], LDL [mmol/L] and insulin [mU/L] are shown and compared to the baseline values. The clinical minimum and maximum reference values are shown as dotted red lines. Statistical analysis was performed using two-sided unpaired t-test (Age), Welch's test (BMI), one-way ANOVA with Fisher's LSD test (weight loss, HDL, LDL) or Kruskal-Wallis test with Dunn's test (CRP, fibrinogen, leukocytes, triglycerides, insulin) with $\alpha = 0.05$. *p-value ≤ 0.05, **p-value ≤ 0.01, ***p-value ≤ 0.001, ****p-value ≤ 0.0001. The Figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

3.3 Bariatric surgery normalizes levels of adipocytokines but unphysiologically reduced ghrelin levels

In order to assess the impact of the bariatric surgery on plasma levels of the adipocytokines and gastrointestinal hormones, bead-based immunoassays, and ELISAs were used to investigate adiponectin, adipsin, leptin and resistin, as well as ghrelin and GIP (Figure 3A). Moreover, the gene expression levels of the respective adipocytokine receptors *ADIPOR1* and *LEPR* were analyzed in whole blood samples (Figure 3B). A healthy, lean (BMI ≤ 25 kg/m^2), gender- and age-matched control cohort (CTRL, n = 10) (Figure 2B) was used to determine reference levels. Regarding the adipocytokine levels, adiponectin tend to be non-significantly lower at baseline compared to the CTRL but normalized after 1 – 2 months p.s.. However, adipsin did not show any alteration at baseline compared to the CTRL. Moreover, leptin was significantly elevated at baseline compared to the CTRL and continuously declined to reach CTRL levels at 2 – 5 months p.s.. Apart from that, resistin was slightly increased at baseline compared to the CTRL but did normalize within 9 – 11 months p.s. Interestingly, gene expression of the receptors *ADIPOR1* and *LEPR* (Figure 3B) seemed to be regulated in an opposite manner regarding the respective protein levels. The gene expression of *ADIPOR1* seemed to be slightly, but non-significantly, upregulated in the peripheral blood immune cells of patients with morbid obesity compared to the CTRL but reached CTRL levels at 3 – 5 months p.s.. Similarly, *LEPR* expression level tended to be slightly, but non-significantly, decreased in the peripheral blood immune cells of patients with morbid obesity compared the CTRL but showed significantly increased levels 1 – 2 months p.s., reaching CTRL levels 9 – 11 months p.s. Ghrelin levels were already slightly, non-significantly decreased at baseline compared to the CTRL and further decreased after surgery not showing any sign of rising to CTRL levels within the observed period. Apart from that, GIP levels seemed to be slightly, but non-significantly, decreased in patients with morbid obesity compared to the CTRL and were not affected by bariatric surgery.

3.4 Mass cytometry revealed alterations in main innate and adaptive immune cell compartments of patients with morbid obesity

High-dimensional mass cytometry was used to analyze peripheral blood immune cell populations in 12 female patients with morbid obesity undergoing bariatric surgery (Figure 2A). A healthy, lean (BMI ≤ 25 kg/m^2), gender- and age-matched control cohort (CTRL, n = 10) (Figure 2B) was used to determine reference levels. Major immune cell populations were detected using dimensionality reduction *via* UMAP and clustering with FlowSOM algorithm (Figure 4A). Clusters were manually annotated according to the expression patterns of surface markers CD45, CD66b, CD3, CD4, CD8a, NKG2D, CD19, CD33, CD56, HLA-DR, CD16, CD14, CD64, CD11b, CD11c, CD123 (Figure 4B). Illustration of UMAP density plots revealed shifts in the major immune cell populations of patients with morbid obesity compared to the CTRL cohort conspicuously visible in CD4+ T cells, granulocytes and monocytes (Figure 4C). Additionally, abundances of the detected immune cell populations normalized to the respective mean of the CTRL population were determined (Figure 4D). Using mass cytometry, NK cells (Lin-, CD16$^+$, CD56$^+$, NKG2D$^+$) were shown to be significantly decreased in the condition

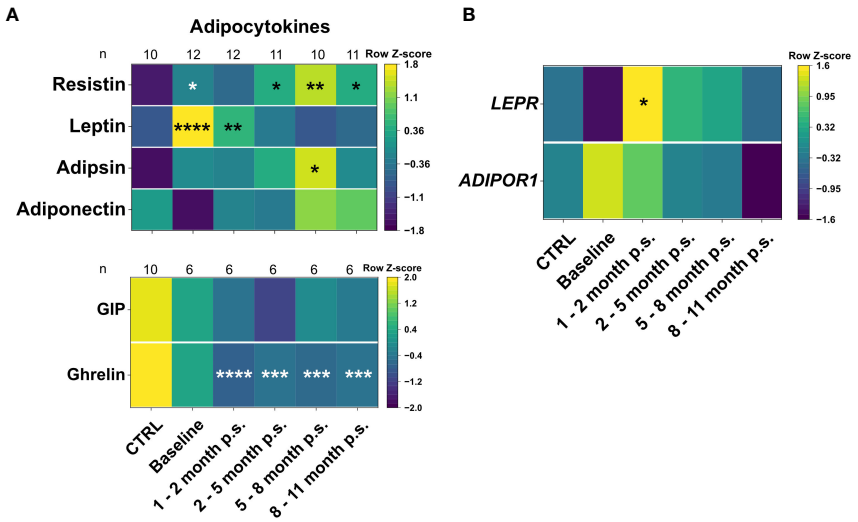

FIGURE 3
Plasma levels of adipocytokines and gastrointestinal hormones **(A)**, as well as gene expression levels of adipocytokines receptors **(B)** for the CTRL, Baseline, 1 – 2 months p.s., 3 – 5 months p.s., 6 – 8 months p.s. as well as 9 – 11 months p.s. The number of analyzed samples (n) is shown for each group and analysis. Adipocytokines adiponectin, adipsin, leptin and resistin were determined using a bead-based immunoassay and gastrointestinal hormones were determined using ELISAs **(A)**. Gene expression of adipocytokine receptors *ADIPOR1* and *LEPR* was determined with qRT-PCR analysis using *GAPDH* and *RPLP0* as reference genes **(B)**. Data is depicted as time-progression heatmaps whereby each row was normalized by z-score. Statistical analysis was performed compared to the CTRL using a Kruskal-Wallis test with Dunn's test and $\alpha = 0.05$. *p-value ≤ 0.05, **p-value ≤ 0.01, ***p-value ≤ 0.001, ****p value ≤ 0.0001.

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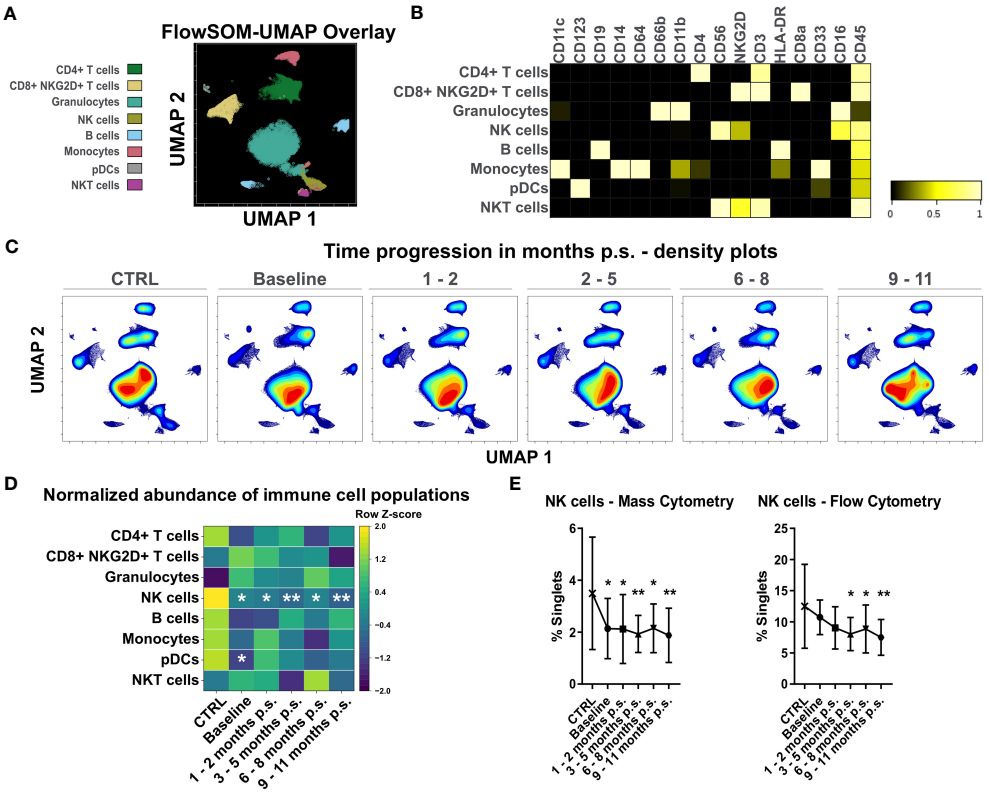

FIGURE 4
Analysis of whole blood samples using high-dimensional mass cytometry. FlowSOM-UMAP Overlay with 20,000 cells per sample using the markers CD11c, CD123, CD19, CD14, CD64, CD66b, CD11b, CD4, CD56, NKG2D, CD3, HLA-DR, CD8a, CD33, CD16 and CD45 was performed in Cytobank and enabled identification of main peripheral blood immune cell populations **(A)**. Manually annotated cell populations were confirmed by evaluating surface marker expression **(B)**. Processed files of the same group were concatenated and illustrated as UMAP density plots over time for CTRL, baseline, 1 - 2 months p.s., 3 - 5 months p.s., 6 - 8 months p.s. and 9 - 11 months p.s. **(C)**. Abundances of identified immune cell populations are depicted as time-progression heatmaps whereby each row was normalized by z-score **(D)**. Cross verification was performed by comparing time-progression of NK cells analyzed with mass cytometry and flow cytometry (mean ± SD) **(E)**. Statistical analysis was performed compared to the CTRL using one-way ANOVA with Fisher's LSD test with $\alpha = 0.05$. *p-value ≤ 0.05, **p-value ≤ 0.01.

of morbid obesity compared to the CTRL population. Moreover, bariatric surgery led to a further decline of the NK cell population which did not recover within 9 – 11 months p.s. (Figure 4E). Cross verification was performed using conventional flow cytometry to confirm the results obtained from mass cytometry (Figure 4E).

Due to the constant decrease of NK cell levels after surgery and the visible shifts in CD4+ T cells and monocytes, that were also detectable by flow cytometry, these immune cell populations were further investigated using the Spectre R package.

3.5 Morbid obesity led to a shift from naïve to memory CD4+ T cells, which was not rescued by bariatric surgery within 9 – 11 months p.s.

The shift observed in the CD4+ T cells was analyzed at a higher resolution using the Spectre R package (30). Prior to the analysis, manually gated CD4+ T cells were compared to the CD4+ T cell population identified with unsupervised clustering using FlowSOM (Figure 4A) to verify the defined populations (Supplementary Figure 3). Linear regression analysis (Slope = 0.9584, $R^2 = 0.9915$) showed a high conformity of manual and automated gating. Accordingly, manually gated CD4+ T cells were selected for further analysis and clustered using FlowSOM followed by visualization with UMAP (Figure 5A) using the markers CD45, CD3, CD4, CD45RA, CD197, CD27, CD127, CD95, CD62L and CD28. Seven clusters were defined according to different surface marker expression (Figures 5B–D). Cluster 1 identified $CD45RA^+$, $CD62L^+$, $CD27^+$ and $CD197^+$ naïve T cells (34). Furthermore, cluster 2, 3, 4 and 5 identified $CD45RA^-$, $CD62L^+$, $CD27^+$ central memory T cells, whereas cluster 6 and 7 identified $CD45RA^-$, $CD62L^-$ and $CD27^-$ effector memory T cells (35, 36). The time-progression of the density plots (Figure 5E) revealed shifts in the mentioned CD4+ T cell subpopulations when comparing CTRL and baseline. Naïve T cell levels (Cluster 1) tend to be slightly, but non-significantly, decreased in patients with morbid obesity compared to the CTRL population and further declined after surgery reaching significantly decreased levels 1 – 2 months p.s. (Figure 5C). In contrast, $CD127^{high}$ central memory (Cluster 2) and $CD127^{high}$ effector memory (Cluster 7) T cell subsets were significantly expanded in patients with morbid obesity. Interestingly, 1 – 2 months p.s. both subsets already decreased, reaching a minimum at 6 – 8 months p.s., also detectable in the

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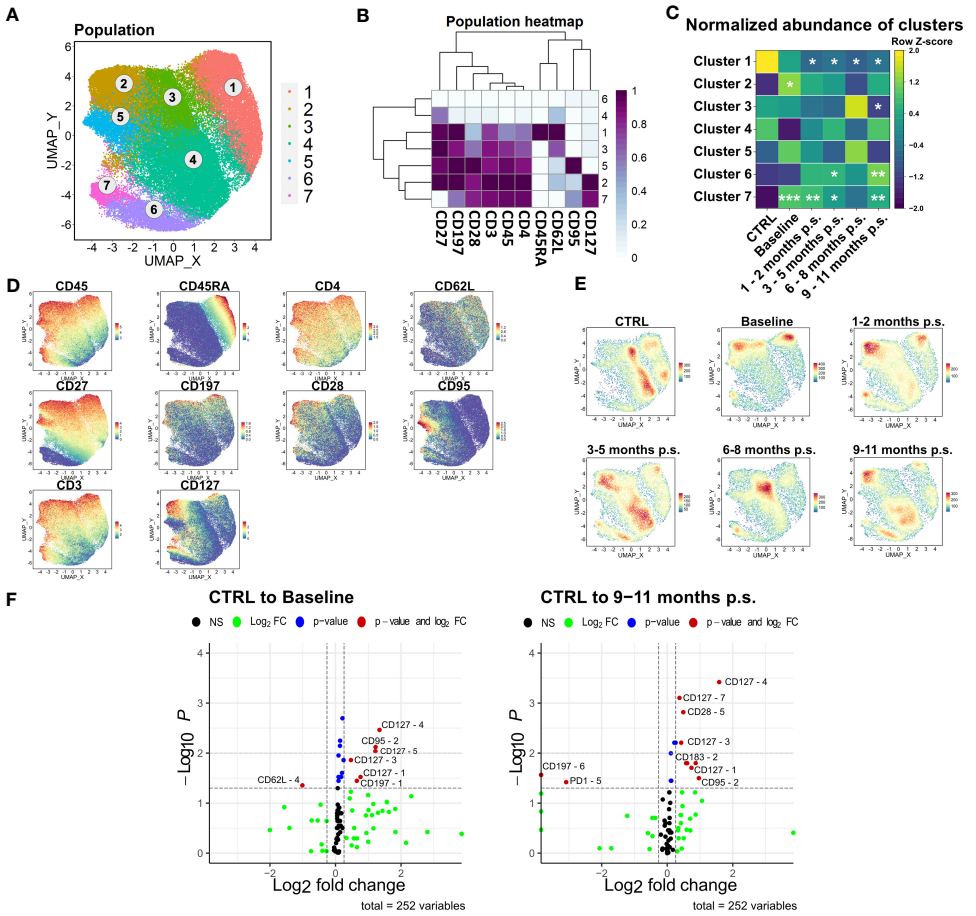

FIGURE 5
Detailed analysis of CD4+ T cell subsets. FlowSOM-UMAP Overlay with 10,000 cells per group using the markers CD27, CD197, CD28, CD3, CD45, CD4, CD45RA, CD62L, CD95 and CD127 was performed following the Spectre R script **(A)**. Expression heatmap **(B)** as well as expression patterns of cluster markers **(D)** enabled identification of 7 distinct clusters. Abundances of identified immune cell populations are depicted as time-progression heatmaps whereby each row was normalized by z-score **(C)**. Statistical analysis was performed compared to the CTRL using Kruskal-Wallis test with Dunn's test and $\alpha = 0.05$. *p-value ≤ 0.05, **p-value ≤ 0.01, ***p-value ≤ 0.001 **(C)**. Density plots showed development and shifts within clusters for CTRL, baseline, 1 - 2 months p.s., 3 – 5 months p.s., 6 – 8 months p.s. as well as 9 – 11 months p.s **(E)**. Volcano plots (Log_2 fold change cut off = 0.26, p-value cut off = 0.05, p-value calculation = Wilcoxon test) indicated differentially expressed surface markers on identified clusters at baseline and 9 – 11 months p.s. compared to the CTRL **(F)**.

corresponding density plots. Nevertheless, 9 – 11 months p.s., a disturbance of the CD4+ T cell compartment was still detectable characterized by decreased naïve T cells and increased effector memory subsets (Cluster 6 and 7).

According to the resulting volcano plots (Figure 5F), an elevated expression of CD127 could be observed in several distinct clusters in patients with morbid obesity, even 9 – 11 months p.s., hinting towards a possible obesity-associated dysfunction within the regulation of this receptor.

3.6 NK cells of patients with morbid obesity showed increased expression of activation markers positively affected by bariatric surgery

As the ratio of NK cells significantly decreased after bariatric surgery, the NK cell population was further analyzed. Comparable to the workflow described for CD4+ T cell, manually gated NK cells were compared to the NK cells identified via unsupervised clustering using FlowSOM (Supplementary Figure 3). Manually gated NK cells were clustered using FlowSOM followed by visualization with UMAP (Figure 6A) using the markers CD45, CD56, CD62L, CD183, CD27, NKG2D, and CD16. Three distinct clusters were defined according to their surface marker expression (Figures 6B–D). Cluster 1 defined the $CD56^{high}$, $CD16^{low}$ cytokine-producing NK cell subset, whereas cluster 2 and 3 defined the $CD56^{dim}$, $CD16^{+}$ cytolytic NK cell subset (37, 38). Apart from that, the $CD56^{high}$ NK cell subset was described to express CD62L and CD27 (39). The cytotoxic NK cell subset could be further distinguished by the expression of NKG2D (cluster 3) (40), which is known to be an activating cell surface receptor (41). Regarding the density plot (Figure 6E), a considerable disturbance could not be observed in the NK cells department. The normalized abundances of the mentioned clusters (Figure 6C) revealed a non-significant shift towards $CD56^{dim}$, $CD16^{+}$, $NKG2D^{+}$ in patients with morbid

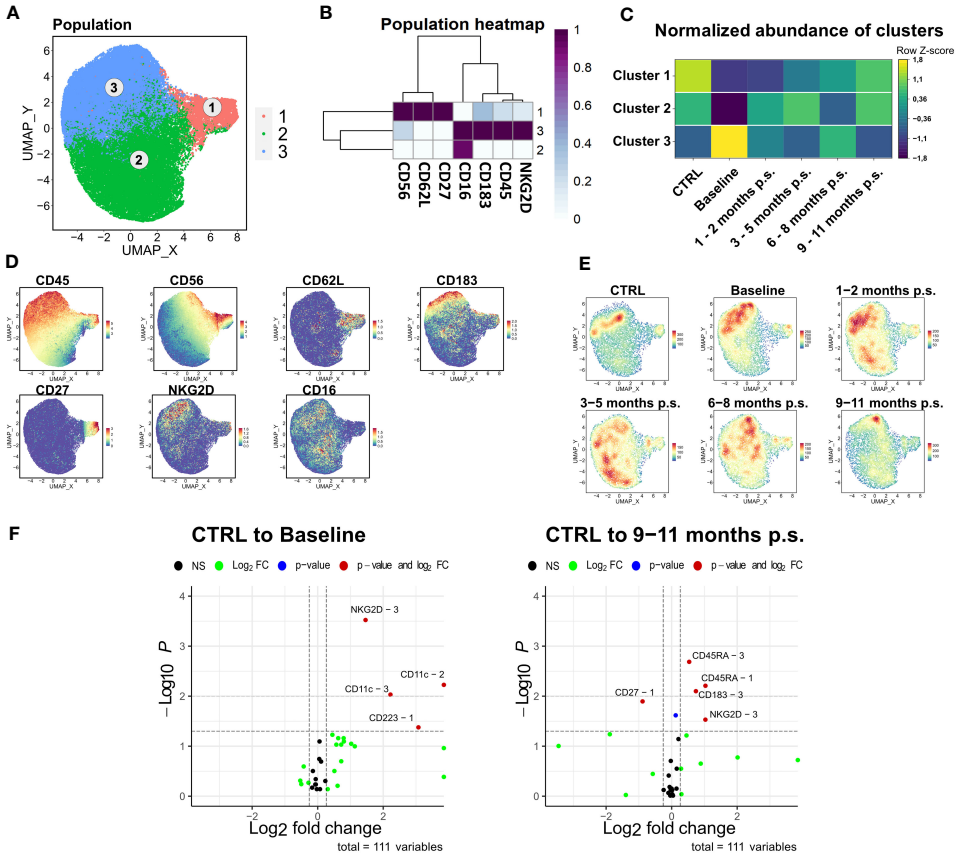

FIGURE 6
Detailed analysis of NK cell subsets. FlowSOM-UMAP Overlay with 10,000 cells per group using the markers CD56, CD62L, CD27, CD16, CD183, CD45 and NKG2D was performed following the Spectre R script **(A)**. Expression heatmap **(B)** as well as expression patterns of cluster markers **(D)** enabled identification of three distinct clusters. Abundances of identified immune cell populations are depicted as time-progression heatmaps whereby each row was normalized by z-score. Statistical analysis was performed compared to the CTRL using Kruskal-Wallis test with Dunn's test and $\alpha = 0.05$ **(C)**. Density plots showed development and shifts within clusters for CTRL, baseline, 1 – 2 months p.s., 3 – 5 months p.s., 6 – 8 months p.s. as well as 9 – 11 months p.s. **(E)**. Volcano plots (Log_2 fold change cut off = 0.26, p-value cut off = 0.05, p-value calculation = Wilcoxon test) indicated differentially expressed surface markers on identified clusters at Baseline and 9 – 11 months p.s. compared to the CTRL **(F)**.

obesity, which normalized rapidly after bariatric surgery. Additionally, the expression levels of NKG2D, CD11c, and CD223, also known as lymphocyte activation gene-3 (LAG-3), were significantly increased in the $CD56^{dim}$, $CD16^{+}$, $NKG2D^{+}$ subset (Figure 6F) from patients with morbid obesity compared to the CTRL population. Within 9 – 11 months p.s., the expression of CD223 normalized in the $CD56^{dim}$, $CD16^{+}$ $NKG2D^{+}$, whereas the expression of NKG2D was still slightly elevated.

3.7 Patients with morbid obesity show disturbance in monocyte compartment, which is partially restored within 9 – 11 months p.s.

The last subset that was investigated more specifically was the monocyte compartment. As previously described, manually gated monocytes were compared to the monocyte population determined via unsupervised clustering (Supplementary Figure 3). The manually gated monocytes were clustered into five distinct clusters based on surface marker expression of CD33, CD64, CD62L, CD11b, CD14, CD16, HLA-DR, CD45, and CD183 (Figures 7A–D). Cluster 1 and 2 defined monocytes with lower HLA-DR expression but higher expression of CD62L. CD62L is a recruitment marker highly expressed on the classical monocyte subsets (42). Compared to cluster 2, cluster 1 showed higher CD183, CD11b, and CD33 expression and was significantly increased in patients with morbid obesity compared to the CTRL. Moreover, CD183, also known as CXCR3, is a chemokine receptor involved in the migration of monocytes into inflamed tissue (42, 43). Cluster 1, representing a $CD183^{+}$ $CD62L^{+}$ monocyte subset, was significantly enlarged in patients with morbid obesity but slowly diminished after bariatric surgery (Figure 7C). Besides that, cluster 3 ($CD16^{+}$) and cluster 5 representing low expression of CD14 and CD62L, as well as high expression of HLA-DR were significantly decreased in patients with morbid obesity most likely due to the observed shift towards cluster 1 (Figure 7E). However, these cells did not return to CTRL levels after surgery. Moreover, a significantly decreased expression of the surface marker HLA-DR was observed on cluster 1 and 2 (Figure 7F). Importantly, the significant decrease in HLA-DR expression on the monocytes of patients with morbid obesity was also detectable in the flow

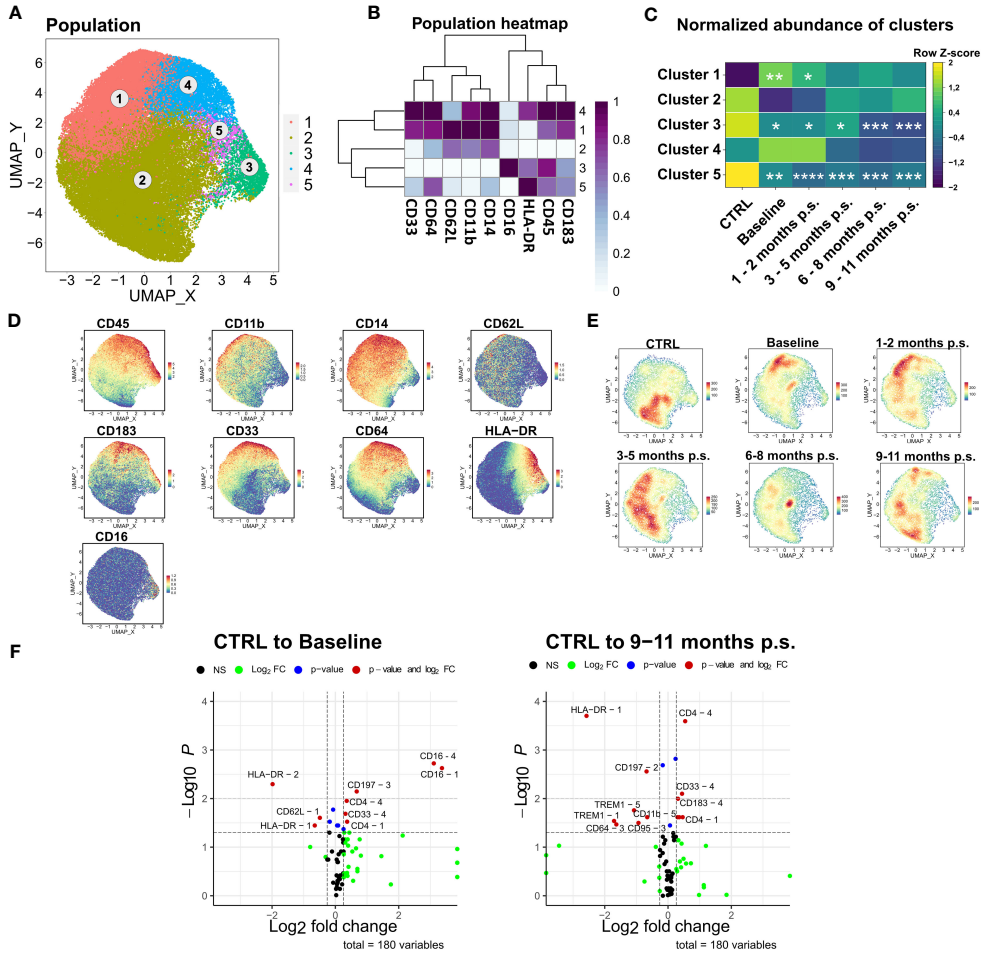

FIGURE 7
Detailed analysis of monocyte subsets. FlowSOM-UMAP Overlay with 10,000 cells per group using the markers CD33, CD64, CD62L, CD11b, CD14, CD16, HLA-DR, CD45 and CD183 was performed following the Spectre R script **(A)**. Expression heatmap **(B)** as well as expression patterns of cluster markers **(D)** enabled identification of 5 distinct clusters. Abundances of identified immune cell populations are depicted as time-progression heatmaps whereby each row was normalized by z-score **(C)**. Statistical analysis was performed compared to the CTRL using one-way ANOVA with Fisher's LSD test and $\alpha = 0.05$. *p-value ≤ 0.05, **p-value ≤ 0.01, ***p-value ≤ 0.001, ****p-value ≤ 0.0001 **(C)**. Density plots showed development and shifts within clusters for CTRL, baseline, 1 – 2 months p.s., 3 – 5 months p.s., 6 – 8 months p.s. as well as 9 – 11 months p.s. **(E)**. Volcano plots (Log2 fold change cut off = 0.26, p-value cut off = 0.05, p value calculation = Wilcoxon test) indicated differentially expressed surface markers on identified clusters at Baseline and 9 – 11 months p.s. compared to the CTRL **(F)**.

cytometry data (data not shown). After 9 – 11 months p.s., cluster 1 kept the low HLA-DR expression level. Nevertheless, a decreased expression of TREM1, an activating receptor of monocytes that is involved in mediating inflammation (44), was observed in cluster 1 and 5 at 9 – 11 months p.s.

4 Discussion

In this study, the influence of bariatric surgery on the peripheral blood immune cells of female patients with morbid obesity was investigated within the first year post-surgery using high-dimensional mass cytometry. Here, the observations were related to a healthy, lean, gender- and age-matched control group.

The patients showed typical signs of obesity-associated low-grade chronic inflammation characterized by elevated CRP, fibrinogen, and leptin levels and decreased levels of adiponectin (45). After surgery a rapid adaption of several inflammation-associated and metabolic blood parameters including CRP, fibrinogen, leukocyte count, triglycerides, HDL, LDL, and insulin could be observed as soon as 1 – 2 months p.s. Furthermore, the adaptions in adipocytokine levels also hint towards a fast relief of the chronic inflammation as a result of bariatric surgery as leptin and adiponectin both show immunostimulatory functions. Leptin was described as a pro-inflammatory factor inhibiting NK cells and inducing the proliferation and activation of monocytes (46). Adiponectin was shown to have anti-inflammatory properties (47) and low levels were associated with chronic inflammation (48). Moreover, not only the protein levels adapted after surgery but also the gene expression levels of the respective receptors *ADIPOR1* and *LEPR*. Interestingly, in a short-term reaction up to 1 – 2 months p.s., the gene expression seemed to change in a reciprocal manner regarding the circulating protein levels. Whether this might be due to up- or down-regulation of the receptors as a reaction to altered

protein levels as it was shown for insulin (49) or due to an altered cell composition accumulating or diminishing cell populations expressing these receptors remains to be elucidated. Taken together, bariatric surgery was shown to be highly effective in resolving the obesity-associated low-grade chronic inflammation and rapidly normalized lipid and adipocytokine levels as soon as 1 – 2 months p.s., indicating weight loss-independent mechanisms. Moreover, peripheral blood immune cells were shown to express adipocytokine receptors highlighting a possible way of interaction contributing to the dysregulation of immune cells in obesity but also to the observed adaptions after surgery.

Apart from adipocytokines, circulating levels of the gastrointestinal hormone ghrelin have been investigated. Ghrelin levels were decreased in patients with morbid obesity and further decreased after surgery, which might be beneficial for weight loss after surgery as ghrelin has stimulatory effects on food intake and fat deposition (50). Nevertheless, ghrelin was shown to possess strong anti-inflammatory and antioxidative properties (51, 52), as well as promoting lymphocyte development in thymus (53). Thereby, ghrelin was shown to recover function and thymopoiesis in an aged thymus (54, 55). Accordingly, on the one hand, unphysiological low levels of ghrelin after surgery might contribute to successful long-term weight loss after surgery but on the other hand could contribute to a delayed regeneration of the immune system.

Although the patients already lost a significant amount of weight 1 – 2 months p.s., it is still unclear whether these rapid metabolic improvements are rather a consequence of the surgical procedure itself than a direct effect of weight loss (56, 57). However, these rapid adaptions are considered as one of the major advantages of bariatric surgery.

Taking peripheral blood immune cell compartments into account, the patients with morbid obesity demonstrated a considerable increase of a Lin^-, $HLA\text{-}DR^-$, $CD14^+$ sub-population, which phenotypically represents monocytic Myeloid derived suppressor cells (mo-MDSCs) (58, 59). Obesity-derived mo-MDSCs were described to be linked to an increased cancer risk that occurs in obese patients as they promote tumor progression and trigger apoptosis in tumor-infiltrating CD8+ T cells (60, 61). Furthermore, recent data suggested that in a state of obesity, long term exposure to metabolic factors like polyunsaturated fatty acids favors the differentiation of MDSCs from bone marrow precursors and lead to a metabolic reprogramming restricting their responsiveness (62, 63). Interestingly, we also observed a significant increase in a monocytic cluster containing $HLA\text{-}DR^-$, $CD14^+$, $CD11b^+$ cells, that additionally express CXCR3 and CD62L. Both receptors were shown to be involved in the recruitment of monocytes to inflamed or tumor-bearing tissue (64), and therefore might be involved in monocytes accumulation in adipose and tumor tissue observed in obesity (65, 66). Indeed, the number of $CD11b^+$ cells was significantly decreased in the adipose tissue of $CXCR3^{-/-}$-high-fat diet (HFD) mice compared to wild type-HFD mice (67). Similarly, an increase in the percentages of monocytes was observed in female obese individuals compared to controls due to enhanced intrinsic migratory capacity of peripheral monocytes (68). Interestingly, our results further confirmed these findings, as the percentage of the mo-MDSCs cells expressing CXCR3 and CD62L was rapidly diminished after surgery, supporting the correlation between peripheral immune cell dysfunction and obesity. Hence, this adaption might contribute to the reduction in risk of developing obesity-associated cancers that has been observed in obese patients after surgery (69). Apart from the observed shifts in cellular composition, we found a decreased HLA-DR expression of the monocytic subset, which was not fully reversed within 9 – 11 months p.s. Reduced HLA-DR expression on monocytes reflects a state of impaired immunity and immunosuppression (70) and was shown to be closely related to cholesterol and triglyceride levels in diabetic patients (71). Further, obese patients showed an impaired recovery of monocytic HLA-DR after surgery, which was associated with a higher risk of sepsis (72). Summarized, patients with morbid obesity showed a disturbed monocytic compartment characterized by high levels of mo-MDSCs and decreased monocytic HLA-DR expression indicating a state of immunosuppression and impaired immunity. Although bariatric surgery decreased levels of mo-MDSCs, HLA-DR expression was not recovered within 9 – 11 months p.s. possibly influencing the immune response of patients within this period.

Apart from alterations in the monocyte compartment, differences in the CD4+ T cell population characterized by decreased naïve T cells and increased memory T cells have been described in obesity (2). It was shown that the thymic function was highly impaired in obese mice leading to decreased naïve T cells and expanded memory T cells. Further, these results were transferred to humans indicating that obesity accelerates thymic aging reflected by the inability of the thymus to replenish the naïve T cell pool and therefore increasing the risk of infections (13). One important characteristic of an aged thymus is the expansion of adipocytes and its transformation into adipose tissue contributing to the loss of thymic functionality and impairing T cell development (73). Likely, obesity accelerates this process by elevating infiltrating adipocytes and increasing the accummulation of perithymic adipose tissue as it was shown in high-fat diet induced obese mice (13). Indeed, it was observed that in a young human population thymic fat infiltration was associated with the BMI (74). Thymic adipocytes might disturb the thymic secretome releasing thymic suppressive factors like leukemia inhibitory factor and simultaneously contributing to the reduction of critical thymic growth factors like stem cell factor, fibroblast growth factors 7 and 10 as a consequence of a thymic fibroblast-to-adipocyte transition (75). Interestingly, the naïve T cells did not recover within the first year post-surgery. Ghrelin might be one factor involved in the observed disturbance of the T cell compartment. It was shown that the ghrelin receptor was highly expressed on developing murine thymocytes but the thymic expression of ghrelin ligand and receptor decreased with aging. Interestingly, ghrelin infusion recovered the age-related thymic involution increasing lymphoid progenitors and reduced splenic and thymic macrophage numbers (54, 55, 76). Furthermore, it was shown that the genetic ablation of ghrelin ligand and ghrelin receptor in the thymus of mice led to epithelial-mesenchymal transition as well as thymic adipogenesis and was also associated to decreased naïve T cells (77). According to this, the decreased circulating ghrelin levels observed in patients with morbid obesity

and the further decline after bariatric surgery could be involved in thymic dysfunction in these patients and contribute to the observed disturbance in the T cell compartment. However, data of 6 patients at a later time point (15 – 19 months p.s.) indicated the recovery of the naïve T cell pool eventually hinting towards a long-term regeneration of the thymus (Supplementary Figure 5). Here we observed a decrease of naïve T cells together with an increase of two distinct clusters of central memory and effector memory T cells, especially characterized by a high expression of CD127. CD127 or IL-7Rα plays a major role in T-cell survival, maturation, as well as homeostasis (78) and was described to be a marker for long-living memory T cells (79). Therefore, CD127 might also be highly involved in the observed disruption of T cell homeostasis in obesity. Possible mechanisms that have been described to be responsible for the expansion of the memory T cell pool include the dysregulation of IL-7Rα, an increased turnover rate of naïve T cells favoring their conversion into memory T cells as well as the increased availability of IL-7 or IL-15 as a consequence of a diminished naïve T cell population (80). Interestingly, memory T cells expressing high levels of IL-7Rα were shown to be drivers of colitis in mice and could be maintained and expanded with IL-7 (81). Moreover, blockage of IL-7Rα was shown to control inflammation in primates via neutralization of antigen-specific memory T cell subsets (82). In this context, it is important to mention that IL-7 levels were shown to be increased in patients with morbid obesity (83). Since IL-7R is also expressed on early B cells and its expression and function is critical for proper lymphocyte development (84), blocking this receptor may affect other normal immune cells. For instance, previous report showed that *Il7r* deficient mice exhibited depletion in both T and B lymphocytes (85). Another study showed that IL-7Rα mutations in humans result in severe combined immunodeficiency (SCID), which is characterized by the lack of T cells and normal numbers but dysfunctional B cells (86).

Accordingly, targeting IL-7Rα using specific antibodies may also affect B cells and result in immunodeficiency in obese patients. However, a recent study showed that treating healthy individuals with anti-human IL-7R antibody was well tolerated and did not lead to apparent alterations in immune cell compartments and inflammatory cytokine profiles (87). Thus, blocking IL7-R signaling might provide a key therapeutic approach to impact survival of IL-7R expressing memory T cells, improving T cell homeostasis, and controlling inflammation in a state of morbid obesity. Although this effect only appears after more than one year p.s., bariatric surgery might lead to a more long-lasting improvement of the T cell compartment by reversing and recovering thymic function.

Next, we showed that NK cells were decreased in patients with morbid obesity and further decreased after bariatric surgery. However, we could not find significant shifts in abundances within NK cell subsets. Several studies described decreased levels of NK cells in patients with morbid obesity coming along with an increased activation status due to dysregulation of activation and inhibitory molecules as well as a lack of function including restricted antitumor response (14, 88, 89). However, there are only a few studies investigating the effect of bariatric surgery on the NK cell compartment. It was shown that bariatric surgery improved NK cell activity and increased NK cell cytokine production within 6 months p.s (22). Nevertheless, data regarding NK cell abundance is contradictory most likely due to different time-points investigated (89).

Within this study, few limitations should be mentioned. First, the study only includes female patients with morbid obesity, as 75% of the patients that undergo bariatric surgery in our department are women. Nevertheless, this deprives the opportunity to consider sex-specific differences regarding levels of sex-hormones or the immune response (90) and their influence on the state of obesity and the outcome of bariatric surgery. However, the strict diet patients received prior to surgery might influence the immune system as well as the obesity homeostasis. Therefore, taking and analyzing samples prior to the start of the diet might increase the power of this kind of studies. Furthermore, it might be worth to increase the observed time-period after surgery. Although the patients lost a significant amount of weight within the observed time-period, none of them reached a BMI of $\leq$ 25 kg/m^2. Although most of the parameters including CRP, fibrinogen, adipocytokine levels and some immune cell populations seem to rapidly adapt after surgery, alterations are still detectable 9 – 11 months p.s., especially with regard to the expression of activation markers and NK cell levels. Here, additional time points at 18 or 24 months p.s. might help to clarify or exclude irreversible alterations as a result of long-standing morbid obesity. Due to the relatively small subcohort that was investigated with mass cytometry, a comparison of the surgical procedures on the outcome of bariatric surgery was not possible. Within recent years, laparoscopic sleeve gastrectomy became the predominant bariatric surgical procedure performed (91), most likely because it is considered as technically less demanding compared to gastric bypass (92). Although the likelihood of complications occurring after bariatric surgery is generally considered low, the risk of severe post-surgical complications was lower after sleeve gastrectomy compared to gastric bypass (93). Regarding the effectiveness, it was shown that sleeve gastrectomy and gastric bypass lead to highly comparable improvement with regard to weight loss, remission of DM2, and adaptions in gastrointestinal and pancreatic peptide hormones (94–96). Specifically, gastric sleeve and gastric bypass comparably decreased leptin and ghrelin levels and increased post-prandial GLP-1, PYY, and general bile acid levels contributing to improved insulin sensitivity. Although both procedures were shown to increase HDL levels, gastric bypass seemed to be more effective in reducing LDL and total cholesterol levels (97, 98). Regarding the question of how bariatric surgery impacts immune cell composition and activation, studies that consider different procedures separately are still lacking. A recent study showed that gastric bypass was shown to temporarily reverse obesity-associated accelerated CD4$^+$ T cell aging (99). However, patients undergoing gastric sleeve surgery were not included in this study. Apart from that, bariatric surgery was shown to normalize B cell but not T cell composition compared to a lean control cohort. However, cytokine-producing capacity of CD4$^+$ T cells was restored after surgery. Nevertheless, a separate evaluation of patients undergoing gastric sleeve and gastric bypass was not performed (2). Consequently,

there is a huge demand for studies that investigate the influence of bariatric surgery on the immune system, considering the type of surgery.

In conclusion, this study shows systemic effects of morbid obesity characterized by persistence of a low-grade chronic inflammation and a dysregulation of lipids, adipocytokines and gastrointestinal hormone ghrelin as well as disturbed peripheral blood immune cells indicated by increased levels of mo-MDSCs, decreased NK cells, and decreased levels of $CD4^+$ naïve T cells. Nonetheless, the power of bariatric surgery, to not only reduce weight but also effectively improve metabolic and immunological disorders is also demonstrated here. Bariatric surgery rapidly released the low-grade chronic inflammation, normalized adipocytokine levels and decreased the levels of mo-MDSCs. Nevertheless, ghrelin levels, monocytic HLA-DR expression, $CD4^+$ naïve T cell, and NK cell levels did not normalize within the observed period of 9 – 12 months p.s. However, data indicated an increase of $CD4^+$ naïve T cells 15 – 19 months p.s., indicating a possible regeneration of the immune system at later time points.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by ethics committee at Ulm University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

UK, LE and MK contributed the study design and supervision. LE performed the surgery, sampling as well as patient monitoring and follow-up care. LM performed sample processing, RNA isolation and qRT-PCR analysis. AR performed ELISAs. FG and AG performed bead-based immunoassays, flow cytometry and mass cytometry analysis. HA performed flow cytometry analysis. AG performed data analysis and statistical analysis. AG, UK and HA wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1131893/full#supplementary-material

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