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Linking the endocrine system with immunity

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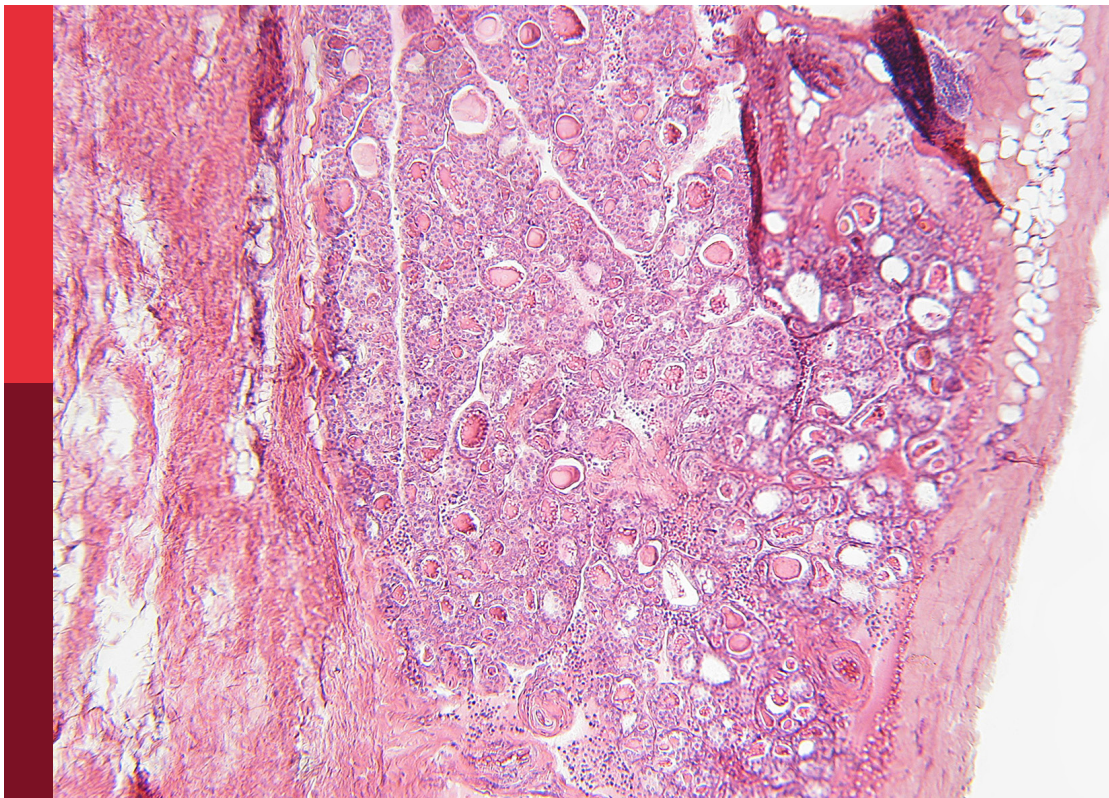

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Linking the endocrine system with immunity

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Editorial: Linking the endocrine system with immunity

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Editorial on the Research Topic
Linking the endocrine system with immunity

The immune and endocrine systems are considered two interconnected networks that influence each other to maintain biotic homeostasis. This Research Topic aims to present the dynamic interaction between innate and adaptive immunity and the endocrine system in various physiological and pathological conditions.

The immune system is a mechanism of protection that simultaneously maintains the organism's homeostasis. Stress-induced activation of the hypothalamic-pituitary-adrenal axis (HPA) is a double-edged sword, activating or suppressing the immune system in specific tissues and cells and affecting innate and adaptive immune responses as described in detail by (Xu et al.). Corticosterone, a glucocorticoid (GR) that regulates the HPA axis, affects immunity through an overwhelming number of signaling pathways, from GR signaling and transcription regulation, through endoplasmic reticulum stress signaling, to toll-like receptor signaling.

It is known that diabetes elevates HPA axis activity, causing increased release of ACTH, which stimulates the adrenal gland to produce glucocorticoids. However, the molecular mechanisms underlying this steroidogenesis are still not fully understood. Magalhães et al. investigated the effects of toll-like receptor 4 (TLR4) pathway activation via lipopolysaccharide (LPS) derived from intestinal bacteria on adrenal steroidogenesis in diabetic mice. Experiments with a TLR4 antagonist (TAK-242) and a mouse model of alloxan-induced diabetes, first described in 1943 by Shaw Dunn and McLetchie as alloxan-induced damage to β-cells in rats (1), showed that corticosterone levels were significantly reduced. These findings suggest that TLR4 activation by bacterial LPS contributes to adrenal steroidogenesis in diabetes and may serve as a novel target for managing glucocorticoid-related complications.

The autoantigen-driven clonal amplification of T cells is recognized as the hallmark of T1D pathophysiology. Only 20 stem cell-like CD8+ T cells inducing type 1 diabetes (T1D) in healthy mice (2) and modulated self-reactive CD8+ T cells used to revert it are mentioned by Yang et al.. Stem cell-like progenitor CD8+ T cells in the pLN replenish the short-lived population of pathogenic T cells that directly destroy β-cells and cause T1D (2). Prevention and treatment of T1D by restoring or inducing immune tolerance to β-cells

requires a precise description of the mechanisms underlying the state changes of CD8+ T cells. New anti-T1D therapies include enhancing the therapeutic CD8+ T cell exhaustion, CRISPR/Cas9-based gene editing in CD8+ T cells, CAR-T engineering of T cells, and T cell differentiation by single-cell RNA sequencing or single-cell transcriptome analysis combined with T cell receptor sequencing. Yang et al. emphasized that the best therapy for T1D is to regulate the autoimmune T cell response while maintaining a proper immune system response to foreign antigen invasion and avoiding systemic immunosuppression (3, 4).

Any risk associated with gestation negatively affects the steadily declining birth rate in Europe (3.67 million births in 2023 with almost half of children born to first-time mothers) (5). Gestational diabetes mellitus poses a significant but differential risk in certain regions and Ray et al. discussed its genetic, gene-environment, and inflammatory causes, describing genetic variants of SLC30A8, CDKAL1, TCF7L2, IRS1, and GCK along with the inflammatory pathways NF-κB, TNF-α, and IL-6.

A unique, lipopolysaccharide-induced, chronic inflammation mouse model, different from others (6), with implications for understanding the impact of endotoxemia on reproduction, was demonstrated by (Garcia et al.). Essential for reproduction, luteinizing (LH) and follicle-stimulating hormone (FSH) levels were elevated upon LPS-mediated stimulation of the TLR4 signaling cascade, leading to paracrine activation of TGF-β pathways. Furthermore, RNA-sequencing revealed LPS-induced changes in the pituitary indicating local paracrine mechanisms for FSH upregulation with TGF-β2 as an important factor. However, transcripts for LH and FSH decreased or remained unchanged suggesting that the secretion of these gonadotropins is independent of transcription but is rather induced by the pituitary microenvironment signaling. Garcia et al. discussed their results obtained with chronic, 6-week, low-dose LPS stimulation contrasting with previous studies showing suppression of the hypothalamic-pituitary-gonadal (HPG) axis in mice treated with high-dose and acute LPS.

Unexpected phenotypes in mouse models leading to contradictory or unconvincing conclusions were discussed in another article. Rahman et al. discussed complications that can be introduced with the Cre-Lox system (7, 8) in knockout mouse models. They compared two KO mouse models, Lyz2Cre and Nod1 floxed, and observed that myeloid Cre expression alone was enough to induce an anti-inflammatory phenotype protecting against palmitate-induced glucose intolerance. Notably, Lyz2Cre reduced absolute insulin secretion, increased insulin clearance, and increased insulin sensitivity in the absence of palmitate infusion. Furthermore, Lyz2Cre expression impaired bone marrow-derived macrophage function. Rahman et al. suggest that these observed effects could be due to Cre toxicity, haploinsufficiency of the Lys2 locus, or the effect of non-specific Cre expression in distinct locations and emphasize the role of appropriate controls while using the Lyz2Cre model.

Autoimmune diseases often occur with comorbidities of either autoimmune origins such as Sjögren's syndrome or non-autoimmune pathophysiology such as cardiovascular or kidney diseases. Cardiovascular and respiratory diseases are the most common cause of death in patients with rheumatoid arthritis (9, 10). Hyperuricemia is known as the pathological basis of gout, an inflammatory joint disease, and can lead to the occurrence and progression of comorbidities such as hypertension, diabetes, chronic kidney disease, cardiovascular diseases, etc. Zhang et al. reported that patients with increased inflammation response index (SIRI) and systemic immune-inflammation index (SII), integrating three independent white blood cell subsets and platelets, had also an elevated risk of hyperuricemia with increased BMI as a mediating effect. Another link between different types of autoimmune diseases was discussed by Wu et al. using hypothyroidism as an example. Hypothyroidism is associated with a reduced risk of inflammatory bowel disease, specifically Crohn's disease.

The most prevalent autoimmune diseases affecting endocrine glands are hypothyroidism (Hashimoto's disease) and hyperthyroidism (Graves' disease). Both of these thyroid diseases cause chronic inflammation, and the former one is a known factor associated with an increased risk of thyroid cancers. A rare case of a thyroid collision tumor was detected by Zhang et al. in a 64-year-old patient. This rare coexistence of primary thyroid mucosa-associated lymphoid tissue (MALT) lymphoma and papillary thyroid cancer was treated with a total thyroidectomy along with lymphadenectomy of the central compartment without any further signs of metastasis or tumor recurrence. Hashimoto's thyroiditis is associated with both, MALT lymphoma and PTC. However, MALT lymphoma is less prevalent in the thyroid and more frequent in the stomach, often being caused by *Helicobacter pylori*-induced chronic inflammation.

This Research Topic describes well-known associations between the immune and endocrine systems in detail but also shows seemingly contradictory cases that uncover the hidden hormone regulation and signaling crosstalk. Understanding the interplay between these two systems is however necessary to propose new therapeutic strategies.

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Lipopolysaccharide-induced chronic inflammation increases female serum gonadotropins and shifts the pituitary transcriptomic landscape

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Introduction: Female reproductive function depends on a choreographed sequence of hormonal secretion and action, where specific stresses such as inflammation exert profound disruptions. Specifically, acute LPS-induced inflammation inhibits gonadotropin production and secretion from the pituitary, thereby impacting the downstream production of sex hormones. These outcomes have only been observed in acute inflammatory stress and little is known about the mechanisms by which chronic inflammation affects reproduction. In this study we seek to understand the chronic effects of LPS on pituitary function and consequent luteinizing and follicle stimulating hormone secretion.

Methods: A chronic inflammatory state was induced in female mice by twice weekly injections with LPS over 6 weeks. Serum gonadotropins were measured and bulk RNAseq was performed on the pituitaries from these mice, along with basic measurements of reproductive biology.

Results: Surprisingly, serum luteinizing and follicle stimulating hormone was not inhibited and instead we found it was increased with repeated LPS treatments.

Discussion: Analysis of bulk RNA-sequencing of murine pituitary revealed paracrine activation of TGFβ pathways as a potential mechanism regulating FSH secretion in response to chronic LPS. These results provide a framework with which to begin dissecting the impacts of chronic inflammation on reproductive physiology.

KEYWORDS

pituitary, FSH & LH, lipopolysaccharide (LPS), inflammation, estrous, secretion

1 Introduction

Women's reproductive health depends on a dynamic balance of hormones that is regulated by the hypothalamic-pituitary-gonadal (HPG) axis. Neurons in the hypothalamus release gonadotropin-releasing hormone (GnRH) in tightly controlled pulses to coordinate the secretion and production of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), from anterior pituitary gonadotropes. These gonadotropins, in turn, target the gonads to mediate the production and secretion of sex hormones and the production/maturation of gametes. The imposition of stress on the HPG axis affects fertility and overall reproductive health (1).

External events or conditions that threaten the homeostasis of an organism, commonly referred to as "stressors", have been well documented to impair reproduction (2–5). Physical and psychological stressors, such as restraint/immobilization, malnutrition, thermal extremes, nociceptive or neuropathic pain, or social stress, induces stress responses that are mediated by the hypothalamic-pituitary-adrenal (HPA) axis (5). Activation of the HPA axis causes the production and secretion of various hormones, particularly cortisol, that inhibit reproduction by acting on each level of the HPG axis (2, 4). Unlike physical and psychological stressors, inflammation is a stressor that is mediated by an immune response, and the relationship between the immune system and stress responses is complex. Generally, stress-induced cortisol is acutely anti-inflammatory. Inhibitory effects on reproduction similar to those induced by the stressors listed above are seen during acute and severe inflammation (6, 7). Advances have been made in understanding nervous system control of immune cells during stress. However these relationships in the context of reproduction are not fully understood (8, 9).

Classic models of inflammation are commonly induced by the bacterial endotoxin lipopolysaccharide (LPS) and consequent activation of toll-like receptor 4 (TLR4) (6). To date, LPS treatments that evaluate reproductive outcomes induced short-term inflammatory responses by either single or multiple low doses (<1mg/kg), where no study has exceeded a week. Despite these variations in methodologies, the LPS treatments had similar inhibitory effects across several animal models at every level of the HPG axis (6, 7). In contrast, chronic LPS exposure in Yangzhou geese resulted in an increase in serum LH and FSH, revealing the potential for chronic stimulation of the immune system to have an unrecognized mechanism of deregulating gonadotropin secretion (10).

Gut dysbiosis is known to disrupt endocrine homeostasis. Dysbiosis is an imbalance of the gut microbiome that can lead to the leakage of LPS into circulation resulting in endotoxemia (11, 12). In humans, blood serum levels of LPS, LPS to high-density lipoprotein (HDL) ratio, and LPS-binding protein (LBP) were all found to be significantly elevated in polycystic ovary syndrome (11). Further, metabolic endotoxemia in mouse models plays a causative role in obesity and insulin resistance, conditions known to impact the HPG axis (13). Clearly, chronic LPS exposure plays a role in reproductive outcomes. However, current experimental models do not address the impact of long-term as opposed to acute exposure of LPS. Therefore, in this study, we sought to establish a model of LPS-induced chronic inflammation to study the unknown mechanisms linking endotoxemia and dysregulated gonadotropin secretion.

In this study, we examined the impact of long-term inflammation induced by LPS on gonadotropin regulation, beyond the effects of acute inflammation previously described. To produce a low-grade chronic inflammatory model, pre-pubertal mice were injected with LPS twice a week for 6 weeks with a dose below the 50% lethal dose of ~25mg per kg body weight (14). As opposed to adult reproductively mature mice, beginning LPS treatments pre-puberty tests the impacts of inflammatory stimulus on reproductive maturation, a process which can permanently impact adulty fertility. Overall, this model stands in contrast to previous studies evaluating the acute effects of inflammation wherein animal models were injected with either a single sublethal dose or multiple sublethal doses over a week. We demonstrate that serum LH and FSH increased in response to LPS. To investigate the effects of LPS on LH and FSH secretion and production in gonadotropes, bulk RNA-sequencing (RNAseq) was conducted on the murine pituitaries. Our overall approach to analyzing these data was first to assess global changes in gene expression, followed by construction of gene networks to identify important genes and pathways and lastly to use secretome analysis for discovery of potential paracrine mechanisms that regulate gonadotropin secretion. We show that contrary to expectations, genes involved in cell division were induced while genes involved in ribosomal activity were suppressed. In addition, WGCNA analyses identified 2 clusters of coregulated genes that in turn correlated with LPS dosage and gonadotropin levels. Overrepresentation analyses of these clusters revealed enrichment in metabolism and intracellular receptor signaling. Finally, secretome analysis suggests a role for TGFβ2 and Tgfbr3 as a potential factors regulating FSH secretion, thus highlighting a distinct pathway to further interrogate for understanding the impact of chronic inflammation on the female reproductive axis.

2 Materials and methods

2.1 Animals and LPS treatment

Female C57BL/6N (Harlan Laboratories) mice were housed on a 12L:12D cycle with food and water available ad libitum. Mice were housed at four females per cage. All the experiments were approved by the University of California San Diego and University of California Irvine Institutional Animal Care and Use Committees.

Prepubertal (4 wks of age) females were randomly assigned to one of 4 groups, placebo control (phosphate buffered saline), low dose LPS (lo, 5ng/kg), medium dose LPS (med, 500ng/kg), or high dose LPS (hi, 50μg/kg). Mice were injected intraperitoneally (i.p.) twice-weekly on Monday and Thursday for 6 weeks. After the final injection, mice were euthanized once they reached diestrous (n = 8 mice). Additional cohorts for validation studies included the PBS and LPS hi dose groups (at least n=3).

2.2 Estrous cycle assessment

Estrous cycle stage was determined by light microscopic analysis of smears from vaginal lavage obtained during the course of the 6 weeks beginning at vaginal opening. Vaginal opening was determined by visual examination of the vulva described by Caligioni (15). Proestrus was defined by the presence of mostly nucleated and some cornified endothelial cells, estrus as mostly cornified cells, metestrus as some cornified endothelial cells and mostly leukocytes, and diestrus as primarily leukocytes.

2.3 Tissue collection

After 6 weeks of LPS injections, mice were anesthetized with isoflurane, weighed, blood collected via retro-orbital bleeding (cohort 1 with 4 doses), and then rapidly decapitated (between 1000 and 1200 hr) or trunk blood was collected post decapitation (all additional cohorts). Pituitaries were collected, frozen on dry ice, and stored at −80°C. Additionally, dissected ovaries were weighed and stored in RNAlater (Life Technologies) at −80°C until processing for quantitative PCR. Approximately 20µL of blood was collected from mice via lateral tail vein blood every Monday before LPS injection for 3 weeks. Cycle stage was monitored throughout. Serum was separated by centrifugation after allowing blood to clot at room temperature for 1 hr (2,000 x *g* for 10 min at 4°C) and stored in -80°C until assayed.

2.4 Hormone and inflammatory marker assays

Blood samples were collected by tail vein or at time of euthanasia, allowed to clot at room temperature for 1 h, centrifuged at 2000×g for 15 min, and then serum was collected and stored at −20°C until assayed for LH and FSH by The University of Virginia (UVA) Center for Research in Reproduction Ligand Assay and Analysis Core or in house via Luminex. Serum T was measured with radioimmunoassay (range 5.0–1075 ng/dl) at the UVA Ligand Core. LH at the UVA Ligand Core was measured using LH RIA with a reportable range between 0.02 and 75.0 ng/mL (intra-assay CV = 5.5%, inter-assay CV = 8.4%). LH is measured in serum by a sensitive two-site sandwich immunoassay (16, 17) using monoclonal antibodies against bovine LH (no. 581B7) and against the human LH-beta subunit (no. 5303: Medix Kauniainen, Finland) as described previous (16). The tracer antibody, (no. 518B7) is kindly provided by Dr. Janet Roser (18), (Department of Animal Science, University of California, Davis) and iodinated by the chloramine T method and purified on Sephadex G-50 columns. The capture antibody (no. 5303) is biotinylated and immobilized on avidin-coated polystyrene beads (7mm; Epitope Diagnostics, Inc., San Diego, CA). Mouse LH reference prep (AFP5306A; provided by Dr. A.F. Parlow and the National Hormone and Peptide program) is used as standard. The assay has a sensitivity of 0.04 ng/ml. Serum and tissue culture LH and FSH were measured by (standard curve range: 4.9-20,000pg/mL and 24.4-100,000 pg/mL respectively) MILLIPLEX® MAP Mouse Pituitary Magnetic Bead Panel (Millipore Sigma, MPTMAG-49K) using a xMAP INTELLIFLEX® Systems (Luminex). TGFβ in conditioned media was measured with the MILLIPLEX MAP TGFβ Magnetic Bead 3 Plex Kit (TGFBMAG-64K-03) according to protocol. Data are represented as mean or linear regression ± SEM. Serum C reactive peptide was measured with the Mouse CRP ELISA from ThermoFisher Scientific (Catalog # EM20RBX10). Serum endotoxin levels were measured (reportable range 0.01 to 0.1 EU/mL, 0.1 to 1.0 EU/mL) with a Pierce™ Chromogenic Endotoxin Quant Kit (Thermo Scientific). Data are represented as mean fold change compared with control ± SEM.

2.5 Quantitative PCR of LBT2 cells and ovaries

LβT2 cells were treated with LPS [10ug/mL] for a 24 hrs and/or GnRH [10nM] for 30 min. Ovaries were snap frozen in liquid nitrogen. RNA was isolated using RNeasy Mini kit (Qiagen). Complementary DNA was made by reverse transcription of 1 µg total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Complementary DNA products were detected using iQ SYBR Green Supermix (Bio-Rad Laboratories) on a CFX Opus 384 Real-Time PCR System. Data were analyzed by the 2ΔΔCt method by normalizing genes of interest (Table 1) to *Gapdh* for LβT2 cells or to *Rpl19* for ovaries. Data are represented as mean fold change compared with control ± SEM.

2.6 Bulk RNAseq of pituitary mRNA

Four individual snap frozen pituitaries from mice representing the median LH response to LPS at each dose underwent total mRNA isolation using the Qiagen RNeasy micro kit with on column DNA digestion according to manufacturer's protocol. Following Ribodepletion and cDNA library prep with Illumina Total RNA Prep, sequencing was performed on the NovaSeq4 platform. Primary analysis was performed using bcbio-nextgen (19) version 1.2.3. Quality control of the raw fastq files was performed using the software tool FastQC1 v0.11.8. Sequencing reads were trimmed with cutadapt (20) v2.10 and aligned to the mouse genome (mm10,seq,ucsc-201112) using the STAR aligner (21) v2.6.1d. Read quantification was performed with kallisto (22) version 0.44.0 using the mm10 annotation (2018-10-10_92).

2.7 Primary pituitary culture

Whole pituitaries were dissected from wild-type C57BL/6 female mice at 9–10 weeks of age. Whole pituitaries were isolated into ice-cold PBS and then dispersed by incubation with 0.25% collagenase Type IV and 0.25% trypsin–EDTA (1x) (Life Technologies) as previously described (23). For immune depletion studies, dispersed pituitary was divided in half, with one half being subjected to depletion of total CD45+ immune cells using Miltenyi

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TABLE 1 Real time PCR primer sequences.

Gene Symbol	RefSeq	Primer (5′-3′)	Reverse Primer (5′-3′)
Gapdh	NM_001289726.1	TGCACCACCAC CTGCTTAG	GGATGCAGG GATGATGTTC
Egr1	NM_007913.5	ATTTTTCCTGAG CCCCAAAGC	ATGGGAACCTG GAAACCACC
Fos	NM_010234.2	GGCAAAGTAGAG CAGCTATCTCCT	TCAGCTCCCTC CTCCGATTC
Cga	NM_009889.2	CCCCTCAGATCGA CAATCACC	AACATGGACAG CATGACCAGAA
Lhb	NM_008497.2	TGTCCTAGCATGG TCCGAGT	CCCCCACAGT CAGAGCTACT
Fshb	NM_008045.3	TGACTGCACAGG ACGTAG	TCTACTGAGA TGGTGATGTTG
Gh	NM_008117.3	CCTCAGCAGGAT TTTCACCA	CTTGAGGATC TGCCCAACAC
Tshb	NM_009432.2	AAGCAGCATCCTT TTGTATTCCC	CCTGGTATTTC CACCGTTCTG
Txnip	NM_001009935.2	GGACTACTTGCG CTATGAAG	TTCACCCAGT AGTCTACGCA
Bbof1	NM_028377.3	GAAAAGCACCGTT TGGAGCA	GTATGCAAGCG CTTGTGAA
Gpr82	NM_175669.4	AAAAGGCTGGC CTCTGGATT	TGCTGGTAGCT CACAGTAGG
Rpl8	NM_012053.2	AGCGGACAGAGC TGTTCATC	GATCGTACCC TCAGGCATGG
Rpl18a	NM_029751.4	CCAAAATGCCACA CACCACC	CACCTGTCCG CAGTACACAA
Cyp17a1	NM_007809	TGGAGGCCACTAT CCGAGAA	CACATGTGTG TCCTTCGGGA
Cyp19a1	NM_007810	AGCATTGTGATTGT TCCTCTGG	GGGAGGCTCAG GTTCTGTTC
Star	NM_011485	GAACGGGGACGA AGTGCTA	TCCATGCGGT CCACAAGTTC
Rpl19	NM_001159483	TTTTGCCCGACGA AAGGGTA	AGCTTCCTGA TCTGCTGACG

Biotech anti-C45 micro beads according to protocol. The cells (dispersed pituitary or immune cell depleted pituitary) were plated on poly-l-lysine (Sigma-Aldrich Inc.) coated Nunc 96-well plates (Thermo Fisher Scientific) at a density of 1.5×10^6 cells per cm^2. The cells were cultured for 24 h at 37°C and 5% CO2 in high-glucose HEPES-buffered DMEM with 10% FBS prior to experimentation. After pituitary cultures equilibrated they were treated serum starved for 16 hrs, followed by a change in media and 30 min treatment with or without GnRH.

2.8 LβT2 cell culture

The female C57BL/6 mouse-derived LβT2 gonadotrope cell line (24, 25) was maintained in high-glucose (4.5 g/l) HEPES-buffered DMEM supplemented with penicillin/streptomycin and 10% fetal bovine serum (FBS: FB-11, Omega Scientific, CA) at 37°C in a humidified atmosphere of 5% CO2. To test the effects of LPS, LβT2 cells were seeded at 2×10^5 cells per cm^2, cultured for 24 h, and pretreated with serum-free DMEM for 12–16 h prior to LPS treatment.

2.9 LPS and GnRH

LPS O111:B4, from Sigma Aldridge was used for all *in vivo* and *in vitro* experiments. LPS was used at a final concentration of 10μg/mL *in vitro* and GnRH (L7134 from Sigma Aldridge) was used at a final concentration of 10nM.

2.10 Differential expression analysis

The R BioConductor packages edgeR (26) and limma (27) were used to implement the limma-voom (20) method for differential expression analysis. In brief, lowly expressed genes—those not having counts per million (cpm)^3 1 in at least 3 of the samples—were filtered out and then trimmed mean of M-values (TMM)(19) normalization was applied. After applying a filter for a total sum of counts >10 across all samples and removing transcripts with missing gene names, a total of 14441 genes were obtained and used in subsequent analysis. The experimental design was modeled upon condition and batch (~0 + Treatment). The voom method was employed to model the mean-variance relationship in the log-cpm values, after which lmFit was used to fit per-gene linear models and empirical Bayes moderation was applied with the eBayes function.

2.11 Weighted correlation network analysis

A guided walk-through, all scripts and data used to perform WGCNA and trait integration are available at: https://github.com/Leandromvelez/pituitary-LPS-gene-analyses. Briefly, RNA-seq expression data from mouse pituitaries were collapsed into modules using WGCNA (R package), in order to identify clusters of correlated genes. Briefly, goodSamplesGenes function (WGCNA R package) (28) was applied in order to search and then delete low quality data which have too many missing values where all passed initial QC. Therefore, a total of 14441 genes (all genes used for differential expression) were used for module construction. Next, blockwise module construction (blockwiseModules() function) was performed using a minimum and maximum module size of 200, and 2000 genes, respectively, and a standard merge cut height of 0.2 was applied. ME0 was removed, as this module reflects genes whereby WGCNA was unable to assign into specific modules. Further integration with traits data allowed us to obtain regression coefficients and corresponding p-values between module eigengenes and traits, where bicorAndPvalue() function was applied (WGCNA R package). From these module eigengene ~ trait correlations undirected networks were constructed and visualized using qgraph (qgraph R package). A detailed step-by-

step analysis, as well as all scripts and data used to perform analysis of DEGs is available at: https://github.com/Leandromvelez/pituitary-LPS-gene-analyses.

2.12 Secretome analysis

The list of genes from the processed RNAseq data was reduced to 174 genes that from the mouse genome were predicted or known to be secreted (secretome). We used a published mouse dataset (29) and manual curation (search for families or secreted proteins such as chemokines, cytokines and hormones) to reduce our entire data set to the 174 gene secretome. To reduce the number of genes in this list further, we performed partial least squared discriminate analysis after generating z-scores to identify the top 10% of genes that allow for discrimination amongst treatment groups by genes with the highest variable importance in projection scores. Partial least-squares discriminant analysis and partial least-squares regression analysis are supervised analyses that use linear combinations of variables (treatments groups) to predict the variation in the dependent variables (genes) (30–32). These analytical tools generate principal components (termed latent variables, or LVs) analogous to those obtained by principal component analysis, but constrained by categorical (i.e., PBS, LPS) measures. Variable importance in projection (VIP) analysis combines all LVs over infinite dimensions. A VIP score > 1 is considered important (above average contribution) for model performance only if $p < 0.05$ in permutation tests that measure variation explained by the model. The genes with the top 15 VIP scores were used to generate a heatmap with hierarchical clustering generated with ClustVis (33). All partial least-squares analyses were conducted in Solo_PLS_Toolbox (Eigenvector Research).

2.13 Statistical analysis

All DE, GSEA, GO and co-correlation pairwise p values were subjected to bonferroni corrections to obtain p-adjusted values (see R scripts used for analysis) (34). All qPCR, hormonal, data are expressed as the mean ± SEM for each group. Group differences for all data were analyzed by ANOVA followed by *post hoc* tests of significance as noted in each figure legend appropriate for the experiment. Statistical significance was set at $P < 0.05$. JMP software was used for statistical testing.

2.14 Data and code availability

Murine pituitary RNA-Seq data has been made publicly available via NIH Sequence Read Archive. Further, a detailed walk-through, all scripts used for analysis, as well as all processed data have been made freely available at: https://github.com/Leandromvelez/pituitary-LPS-gene-analyses.

3 Results

3.1 Chronic low-dose LPS increases serum gonadotropins

To determine the impact of low-grade chronic inflammation on female gonadotropin production, we developed a scheme of low dose lipopolysaccharide (LPS) administration (Figure 1A). Beginning at 4 weeks of age, mice were injected intraperitoneally with 3 doses of LPS twice-weekly for 6 weeks until they reached sexual maturation. The 50% lethal dose (LD_{50}) of intravenous LPS is ~2-26 mg/kg depending on mouse age. Our highest dose, 50µg/kg, is 2 orders of magnitude lower than the LD_{50} of LPS for 10-week-old mice (14). During the course of these injections, there was no difference in weight gain between the experimental and control group (Figure 1B) indicating no adverse impact on overall general health and growth that occurs with high doses of LPS (14). We found that serum CRP and endotoxin load from chronic administration of LPS prevented age-associated increases in serum endotoxin, consistent with a state of chronic inflammation and enhanced clearance of LPS from a primed immune system (34) (Supplementary Figures 1A, B). Despite acute models supporting an inhibitory role of LPS in gonadotropin secretion, it is known that a positive correlation exists between LPS and LH in women (11). Therefore, we measured serum LH in diestrus staged mice at the end of the six-week LPS injection scheme. Interestingly, LPS increased serum LH in a dose-dependent manner with significance at the highest dose (Figure 1C), effectively recapitulating the published relationship of LPS and LH *in vivo*. We conclude that chronic low dose *in vivo* LPS increases serum gonadotropins. Given this conclusion, we designed a workflow to analyze local transcriptomic changes at the level of the pituitary alongside additional evaluation of the reproductive impacts of chronic LPS exposure (Figures 1D–G).

To further evaluate hormonal and ovarian disruption in this model, we performed additional experiments at the high dose of LPS given the significance of the increase in LH. First, we measured FSH at the high dose of LPS administration and also found a significant increase (Figure 2A). Even more striking is that we found an increase in serum FSH that was apparent by 3 weeks post the first LPS injection (Figure 2B). This increase in FSH was the driving factor in a significantly reduced LH to FSH ratio (Figure 2C). The significant changes in serum gonadotropin, specifically FSH, indicate that puberty and ovarian maturation may be impacted. Therefore, we tested the impact of our LPS injection scheme on measures of reproductive biology. We found a small, but significant one day delay in time to first estrus, but no significant difference in time to vaginal opening in LPS treated mice (Figures 2D, E). We next assessed whether the elevated FSH had an impact on steroidogenesis or ovarian mRNA transcripts. We found that the chronic LPS treatment resulted in elevated serum testosterone (Figure 2F). Despite no difference in ovarian weight with increasing doses of LPS, we found that gene expression of *Cyp19* was

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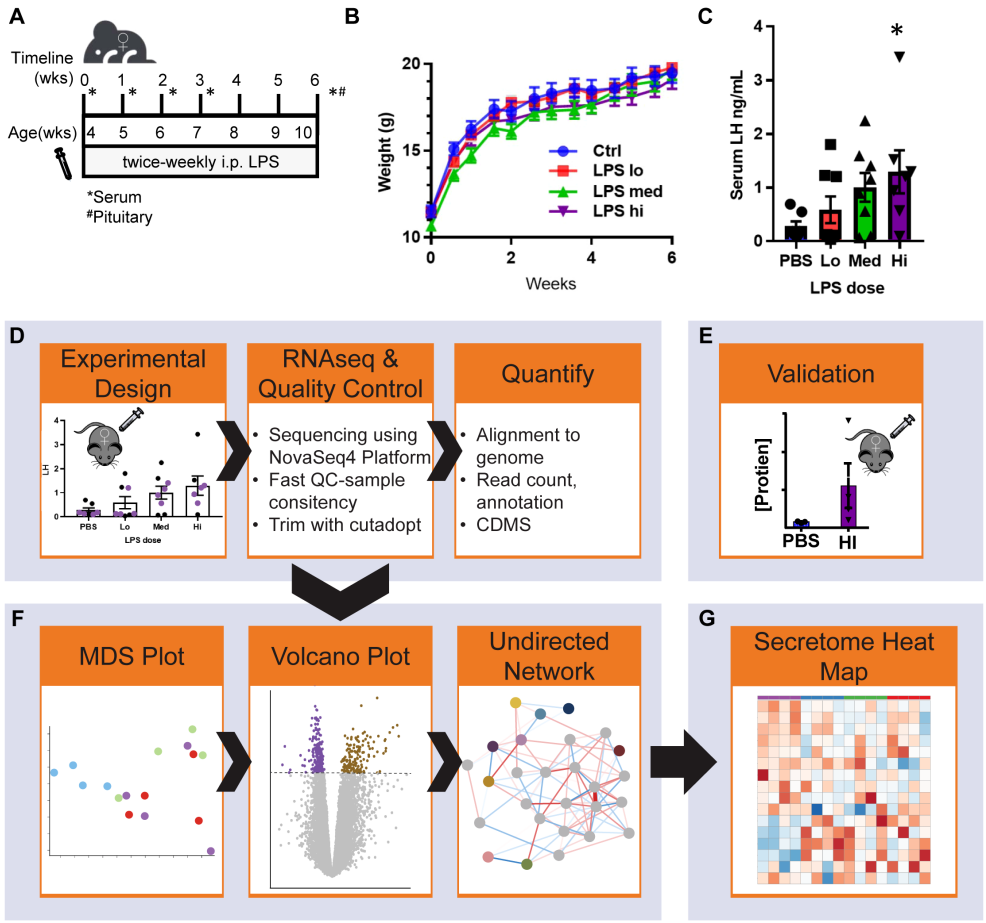

FIGURE 1
Chronic LPS dose dependently increases serum LH and RNAseq experimental workflow. **(A)** Schematic of experiment design. Female mice 4 weeks of age underwent twice-weekly i.p. injections of PBS (vehicle control) or LPS (Lo 0.005µg/kg, Med 0.5 µg/kg, Hi 50 µg/kg) for 6 weeks. Cycle stage was assessed by vaginal cytology beginning on the day of vaginal opening until first estrous. Mice were euthanized once they reached diestrus following the final injection. Serum and pituitaries were collected for analysis. **(B)** Growth curve of mice over the course of the experiment (n=8). **(C)** Serum LH at all doses after week 6 of injections from diestrus staged mice (n=8). **(D)** Pituitaries representing the median data points from control and each LPS treatment group were used to isolate mRNA. After generation of cDNA libraries, sequencing was performed. Data was aligned and normalized to counts per million (CPM). **(E)** Serum hormone and physiological validation at Hi dose after 6 weeks of injections. **(F)** Analysis pipeline includes MDS plot from CPM of all samples, followed by differential expression analysis and weighted gene co-expression network analysis WGCNA run in parallel. **(G)** Secretome analysis of the entire dataset. Significance at p<0.05 was determined by ANOVA **(B, C)** and *post-hoc* analysis with Dunnett's comparison to control test **(C)**.

significantly decreased with LPS treatment while expression of *Cyp17* and *Star* remains unchanged (Figure 2G). To stage mice to sac at diestrus, we cycled mice at 9 weeks of age. Analysis of this data showed prolonged estrous consistent with elevated FSH (Figure 2H). Overall, from this model, we find that chronic low-level LPS results in increased gonadotropin secretion with downstream impacts on ovaries likely from elevated FSH, though the mechanism is unclear.

3.2 LPS suppresses ribosomal pathways while inducing cell division genes in pituitary

To identify potential pathways involved in LPS regulation of gonadotropins in the pituitary, we applied RNA-sequencing (RNAseq) of mRNA extracted from pituitaries of mice treated with and without LPS to probe the transcriptional response to systemic LPS. Specifically, RNA-seq was performed across three LPS doses (4 mice representing the mean of the LH response per dose) (Figure 1D). We performed differential expression and network analyses to assess global changes in gene expression (Figure 1F), followed by an analysis of the pituitary secretome (Figure 1G).

First, we analyzed the expression of selected transcripts in the pituitary across the three LPS doses. We confirmed that i.p. LPS reaches the pituitary and has a local effect as evidenced by a significant increase in gene expression for toll-like receptor 4 (*Tlr4*), the natural receptor for LPS, and in *Ly96*, which encodes a coreceptor for TLR4 (Figure 3A). This was consistent with increased protein expression of CD38, an immune activation marker, on pituitary immune cells from dissociated pituitaries that express TLR4 protein (Figure 3B). Next, we assessed changes in pituitary hormone specific transcripts. In contrast

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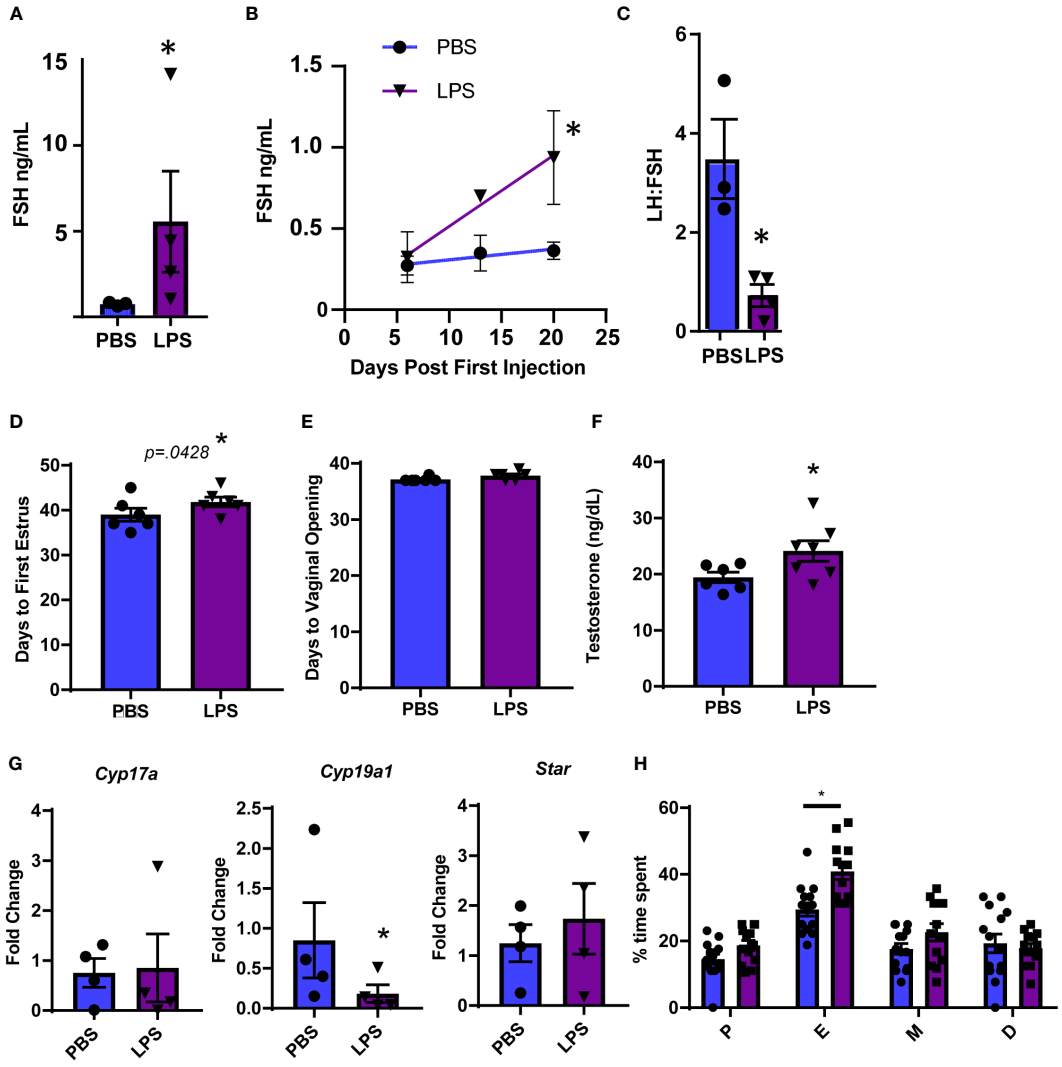

FIGURE 2
Chronic LPS treatment dysregulates gonadotropins, steroidogenesis and ovarian gene expression. **(A)** Serum FSH from Hi dose LPS treated mice after 6 weeks of injections. **(B)** Serum FSH from 0 to 3 weeks of injections of mice treated with PBS or Hi dose LPS (*n*=2-6 per time point). Only mice in diestrus were included in reported hormones. **(C)** Serum LH to FSH ratio from Hi dose LPS treated mice after 6 weeks of injections. **(D)** Days from birth until vaginal opening of mice treated with PBS or Hi dose LPS (*n*=6). **(E)** Days from birth until first estrus of mice treated with PBS or Hi dose LPS (*n*=6). **(F)** Serum testosterone of mice treated with PBS or Hi dose LPS (*n*=7). **(G)** Fold Change of mRNA transcripts from pituitary of mice treated with PBS and varying doses of LPS (*n*=3-7). All data are mean +/- SEM. Significance at p<0.05 was determined by t-test **(A, C, D–F)** and *Cyp19* and *Star*) or Tukey HSD **(A–C)** or ANOVA (*Cyp17a*) with *post-hoc* analysis with Dunnett's comparison to control test. Repeated measures ANOVA was used to analyze **(F, H).** The percent of time mice spent in the designated estrous cycles determined by cytology. *n*=14, PBS, 13 LPS. D, diestrus, P, proestrous, E, estrous, M, metestrous. All data are mean +/- SEM. Significance was determined by *student's t test.* Asterisks indicate significance accepted at p< 0.05 compared to the PBS or LPS control.

to the elevated LH and FSH measured in the serum, *Lhb and Cga* were significantly decreased at all LPS doses while *Fshb* exhibited no change (Figure 3C). Transcripts for all other pituitary hormones including *Prl*, *Pomc*, *Tsh*, and *Gh* were not impacted (Supplementary Figure 2). From the specific analysis of these transcripts from the RNAseq data and the flow cytometry data, we can conclude that systemic LPS, either through direct or indirect mechanisms, shifts localized pituitary gene expression which does not correlate with the observed concentrations of serum gonadotropins.

Due to the incongruency in our data, we used unbiased analysis to understand the global effects of LPS on pituitary gene expression. We performed multiple dimensional scaling (MDS) on the dataset. An MDS plot of the RNA expression data showed distinct clustering between the untreated PBS control and LPS-treated samples (Figure 4A). However, the MDS plot did not discriminate between the specific transcriptional differences between LPS doses. Therefore, all LPS-treated samples were grouped for differential expression analysis where 778 total genes were found to be significantly changed at an FDR-adjusted p-value of 1e-3 (Figure 4B). Surprisingly, pathway enrichment via gene set enrichment analysis (GSEA) showed that genes encoding ribosomes and ribosomal function were highly suppressed with LPS which is consistent with known impacts on macrophages (35). Meanwhile mediators of cell division processes were strongly

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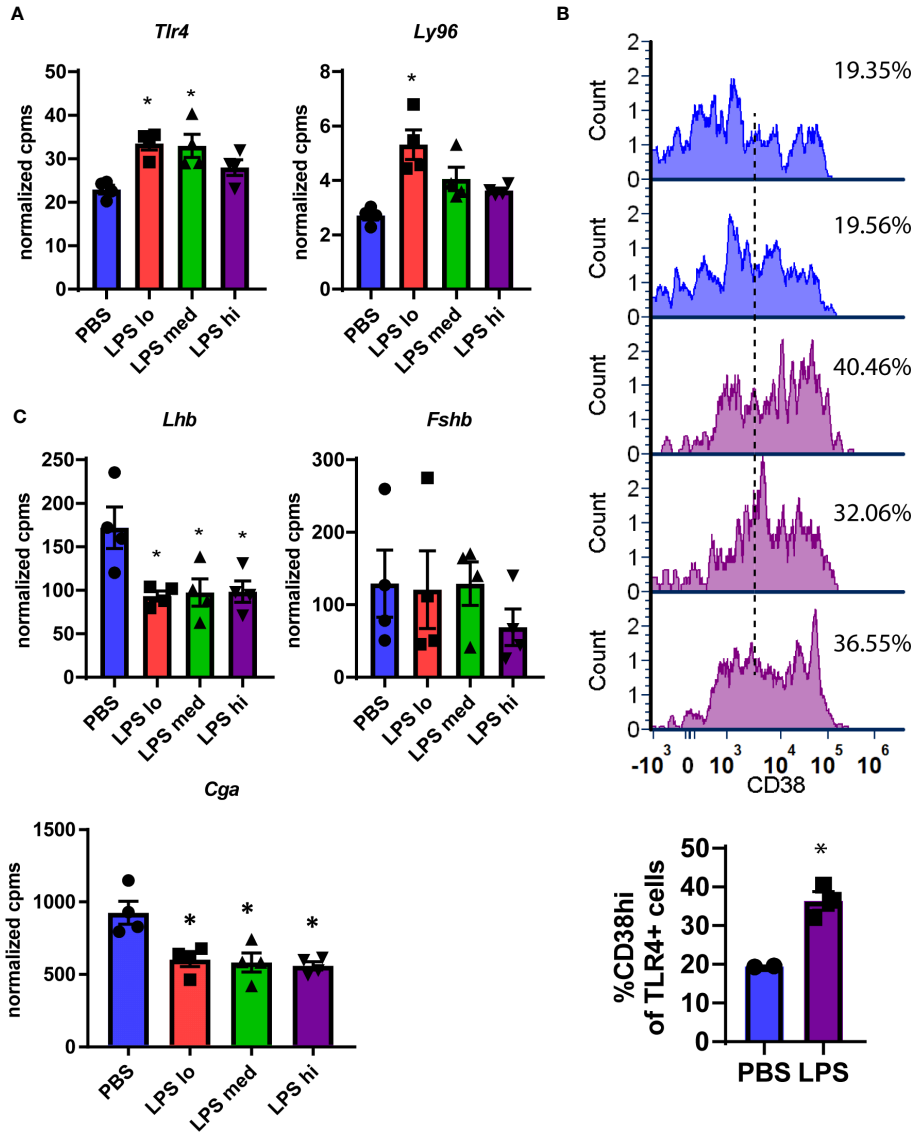

FIGURE 3
Confirmation of LPS-induced local immune activation. **(A)** Normalized expression data from RNAseq of female mouse pituitary in control conditions (PBS) and under chronic LPS at 3 doses are graphed for *Tlr4* and *Ly96*. **(B)** Flow cytometry analysis of individual pituitary from control mice (PBS) or Hi Dose LPS treated mice. Pituitaries were dissociated and stained for CD45 (immune cells), TLR4, and CD38 (immune activation marker). Histograms are the relative expression of CD38 on CD45+TLR4+ cells (immune cells which express TLR4 protein) normalized to the peak value of each sample. The black dotted line represents the cutoff for high expression of CD38. The inset graph displays the % of TLR4+ cells that are CD38 hi (n=2-3) **(C)** Normalized expression data from RNAseq of female mouse pituitary in control conditions (PBS) and under chronic LPS at 3 doses are graphed for *Lhb*, *Fshb*, and *Cga*. Data is mean +/- SEM and was analyzed by one-way ANOVA with a Dunnet's *post hoc* analysis. Asterisks indicate significance accepted at $p< 0.05$ compared to the PBS control.

induced (Figure 4C). Generally, these changes were observed robustly across LPS treatments, regardless of doses used (Figure 4D). We, therefore, tested whether direct treatment of LβT2 cells with LPS recapitulated the pituitary RNA changes from *in vivo* administered LPS. We found that 24hr treatment of LβT2 cells with LPS did not significantly regulate the top DEGs or impact the LβT2 cell response to GnRH (Supplementary Figure 3). We speculate that some gene changes we see in the pituitary in response to chronic LPS are either not occurring in gonadotropes specifically or are being regulated by indirect or feedback mechanisms through other LPS-responsive cell types such as immune cells or TLR4 expressing cells throughout the HPG axis, for example in the ovary.

3.3 WGCNA analysis implicates the engagement of intracellular receptors and metabolism in chronic LPS-induced secretion of LH

Gonadotropes, the cells which secrete LH, represent at most 10% of the many distinct cell populations in the pituitary that

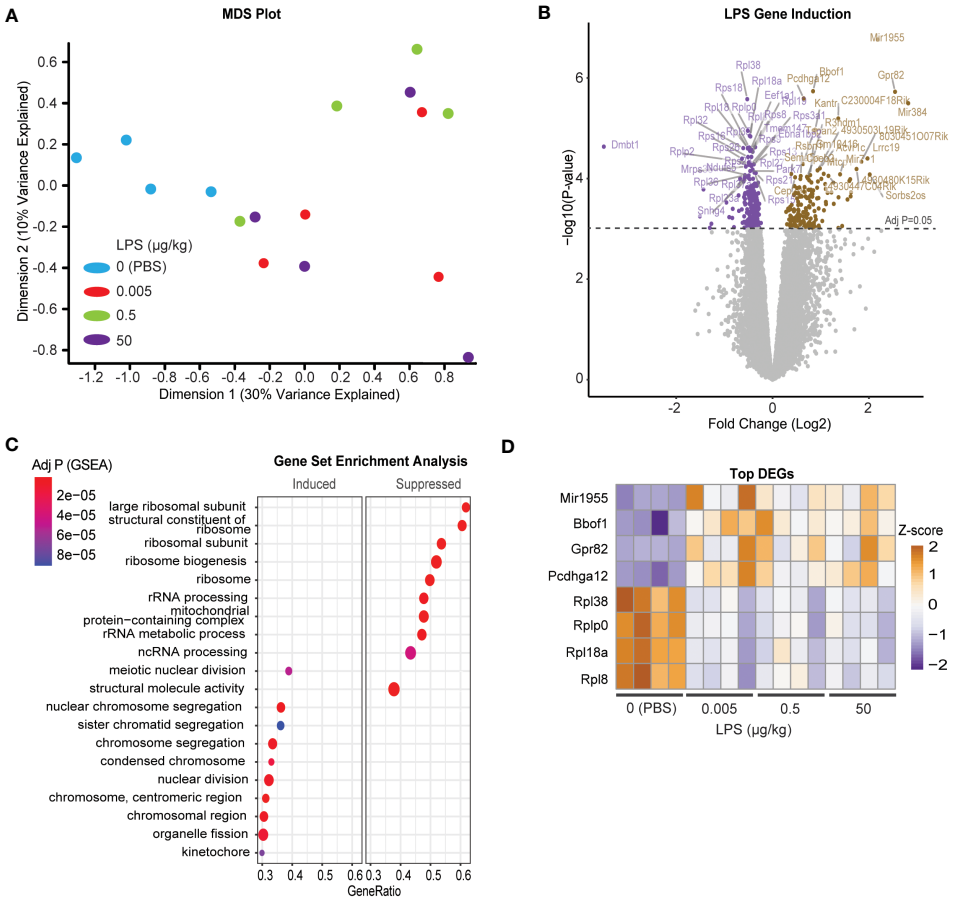

FIGURE 4
Chronic LPS treatment induces a broad transcriptomic response of cell division and reduced catabolic metabolism in the pituitary of female mice. **(A)** MDS plot from CPM of all samples [Control (blue), or LPS Lo (red), Med (green), Hi (purple)]. **(B)** Volcano plot of differentially expressed genes represented as log fold change of LPS treated over control. **(C)** Gene set enrichment analysis (GSEA) performed on the differentially regulated genes either induced or suppressed in LPS versus CTRL. Significance is indicated as −log10 p value. **(D)** Heatmap of the z-scores of the top LPS-induced and suppressed genes at each dose of LPS. Cut-off p value was set to 0.05.

contribute to the transcriptional profiles we observed (23, 36). Furthermore, the transcript for Toll-like receptor 4 (*Tlr4*), the natural receptor for LPS, is significantly increased in response to LPS (Figure 3A) and appears present at low levels in many populations of cells found in the pituitary (36). For these reasons, it is difficult to determine which transcripts are important for LPS-induced gonadotropin secretion. To further refine the gene expression response to LPS and incorporate additional relevant physiological outcomes of LPS treatment including serum LH, weighted gene co-expression analysis (WGCNA) (28) was performed on these RNA-seq data to identify sets of specific coregulated gene clusters, termed modules, and determine the relationship among them and additional variables or traits using the reference eigengenes. Visualization of an undirected weighted network of modules and traits highlighted several notable connections. For example, module ME16 and ME14 link estrous cycling parameters with the rest of the network (Figure 5A). Additionally, we observed that ME12 and ME8 were linked to LPS or LH. Overall, in several modules we observed significant correlations of genes with specific physiologic outcomes.

One module in particular, ME12, appeared of substantial interest given that it showed the strongest correlation to LPS treatment (Figure 5B). ME8, though having a small proportion of DEGs, significantly correlated with both LPS treatment and serum LH. Overrepresentation analysis (ORA) from genes present in ME12 (Figure 5C) showed that processes related to ribosome function were strongly correlated to LPS treatment, a similar outcome to the global differential expression analysis performed in Figures 4B, C. Moreover, because ME8 was the only module related to both LPS and serum LH, we further analyzed gene expression within these modules to determine relevant processes for the LPS response and LH secretion. Unlike the global pituitary transcriptomic response to LPS, ORA performed with genes from ME8 highlighted metabolic processes and intracellular receptor signaling as important for the outcome of increased LH secretion in response to LPS (Figure 5C).

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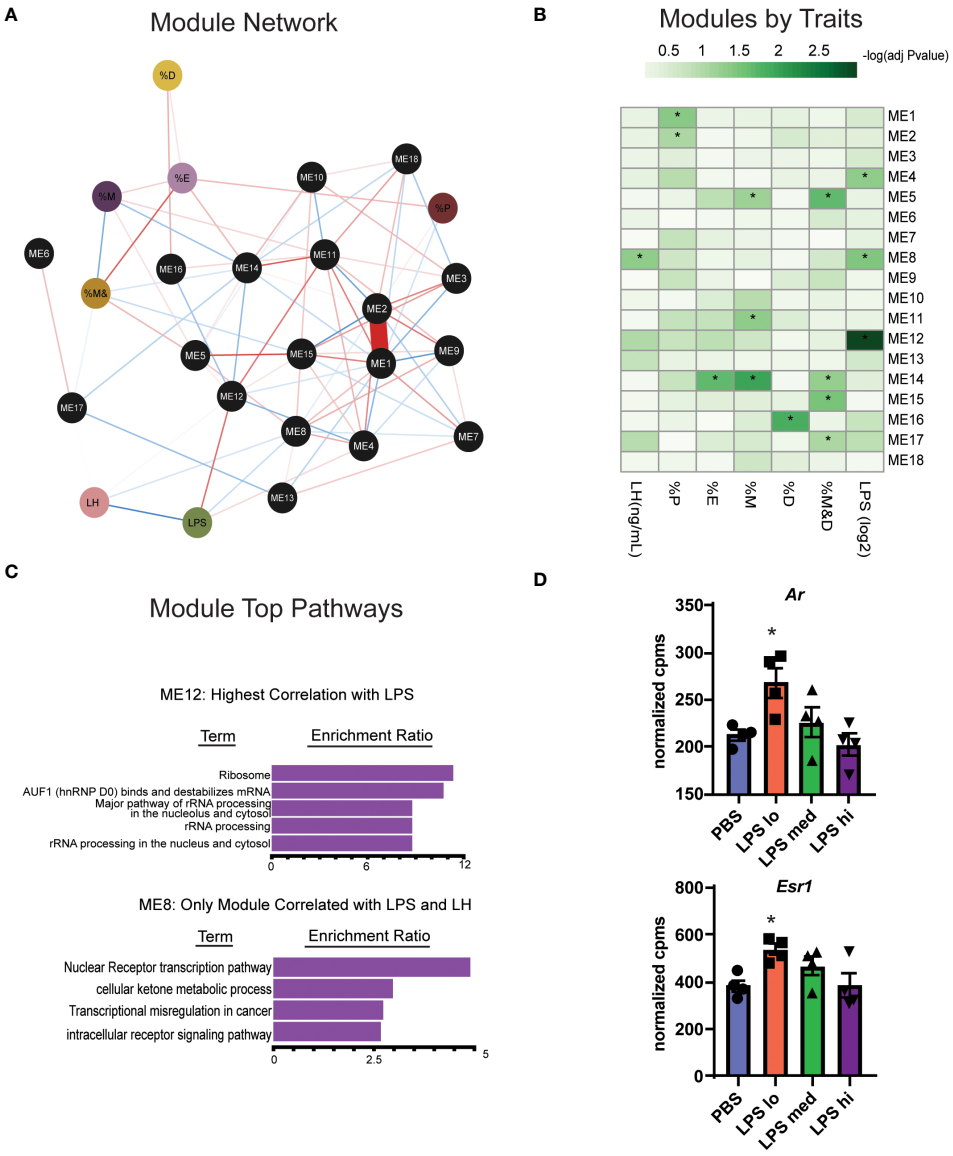

FIGURE 5
WGCNA analysis identifies two gene clusters associated with chronic LPS induced elevation of serum LH. **(A)** Undirected weighted Network showing association of biological reproductive traits and WGCNA modules. Associations are represented by red (positive association) or blue (negative association) lines and the strength (weight) of the association is represented by the width of the line. Phenotypic traits are in colored circles and Modules (ME) are in black circles. The percent of total time spend in diestrus, proestrus, estrous, and metestrous are represented as %D, %P, %E, and %M respectively. **(B)** Heatmap of correlations among WGCNA module eigengene vectors with reproductive traits. **(C)** Overrepresentation (ORA) analysis of Gene Ontology terms in genes in module 12 and module 8. **(D)** Normalized expression data from RNAseq of female mouse pituitary in control conditions (PBS) and under chronic LPS at 3 doses are graphed for *Ar* and *Esr1*. Data is mean +/- SEM and was analyzed by one-way ANOVA with Dunnet's *post hoc* analysis. Asterisks indicate significance accepted at $p < 0.05$ compared to the PBS control.

Given the relationship of hormone signaling to metabolism, these analyses suggest that intracellular receptors, such as androgen receptor or estradiol receptor may impact metabolism and act as a potential mechanism linking LPS to elevated serum gonadotropins (23, 37). We analyzed the gene expression of androgen and estrogen receptor alpha (*Ar* and *Esr1*) in the pituitary of LPS treated mice and found that low dose LPS increases the transcript of these intracellular receptors while high dose has similar expression levels as the control (Figure 5D). This is consistent with increased testosterone (Figure 2F) having negative feedback on AR signaling at the level of the hypothalamus and pituitary (38). Our data suggest that sex steroid feedback loops may be involved in LPS mediated elevation of serum gonadotropins.

3.4 Secretome analysis reveals paracrine TGFBR ligands as potential mediators of chronic LPS-induced gonadotropin secretion

Given the complexity of gonadotropin regulation through secreted factors and receptors, we sought to identify potential

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paracrine signals in the pituitary involved in this regulation during chronic LPS exposure. First, we used a published mouse secretome (29) and manual curation of our RNAseq dataset to identify 174 genes that were predicted or known to be secreted into the extracellular space. We defined these 174 genes as the secretome. To reduce the number of genes in this list, we performed a partial least squared discriminate analysis to identify the top 10% of genes that allow for discrimination amongst treatment groups by genes with the highest variable importance in projection scores. Using this new list of 16 genes, we created a heatmap and performed hierarchical clustering (Figure 6A). One major cluster showed increasing transcripts of genes related to cell metabolism and TGFβ signaling. Specifically, genes encoding bone morphogenic peptide 3 (*Bmp3*) and inhibin A (*Inha*) increase with LPS treatment. Because protein products of these genes bind to TNFβ receptors, we analyzed the transcript levels of *Tnfbr1*, *Tnfbr2*, and *Tnfrb3* in our dataset. We found that *Tnfrb3* was significantly increased at the highest dose of chronic LPS treatment in the pituitaries of female mice (Figure 6B). Given these findings, we tested the relationship of local pituitary secreted TGFβ, strong ligands of TGFβR, to LH and

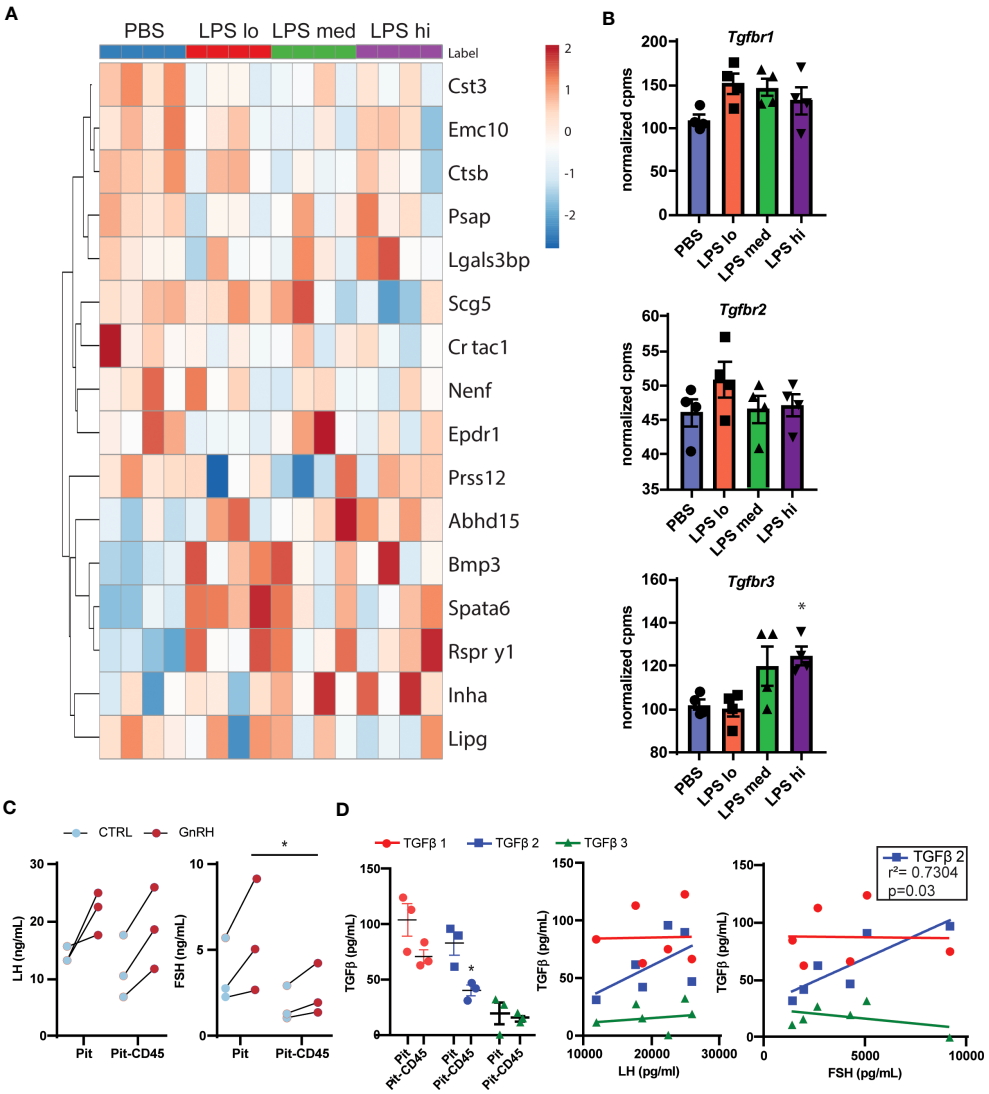

FIGURE 6
Secretome analysis reveals TGFβ signaling as a potential regulator of LPS-induced FSH secretion. **(A)** Manual curation and a published list of genes in the mouse secretome (39) from the entire RNAseq gene list resulted in 174 identified genes predicted or known to be secreted. The top 16 genes of these 174 that discriminated between LPS treated and PBS in a partial least squares discriminant analysis model are displayed in a heatmap with hierarchical clustering of the genes [Control (blue), or LPS Lo (red), Med (green), Hi (purple)]. **(B)** Normalized expression data from RNAseq of female mouse pituitary in control conditions (PBS) and under chronic LPS at 3 doses are graphed for *Tgfbr1 Tgfbr2*, and *Tgfbr3*. Data is mean +/- SEM and was analyzed by one-way ANOVA with Dunnet's *post hoc* analysis. Asterisks indicate significance accepted at $p< 0.05$ compared to the PBS control. **(C)** Immune cells from dispersed pituitary of 10-12 week old female mice were cultured for 24 hrs with (Pit) or without (Pit-CD45) endogenous CD45+ immune cells *in vitro*. After overnight serum starvation, the pituitary cultures were stimulated in fresh media +/- 10nM GnRH for 30 min. The conditioned media was analyzed by Luminex and plotted. Each sample represents pituitaries pooled from 3 mice (n=3). Data was analyzed by one-way ANOVA. **(D)** Conditioned media from **(C)**, was acid treated to activate TGFβ for analysis by Luminex. TGFβ concentrations are plotted in Pit and Pit-CD45 conditions and correlated to their respective concentrations of LH and FSH (n=3). Data is mean +/- SEM and was analyzed by Student's t test or Pearson correlation. Asterisks indicate significance accepted at $p< 0.05$ compared to the PBS control.

FSH. First, we dissociated pituitary (Pit) from 8-10 wk old unstaged female mice. Half of the samples were depleted of CD45+ immune cells (Pit-CD45), a known source of TGFβ. As expected, 30 min GnRH treatment increased the secretion of both LH and FSH (Figure 6C). The amount of GnRH-induced secretion of FSH from primary pituitary cultures was significantly reduced when CD45 immune cells were not present. To determine if this outcome coincided with reduced TGFβ secretion, we also measured TGFβ1,2, and 3 in the conditioned media. TGFβ family members were virtually undetectable in basal conditions. However, we found significant concentrations of TGFβ1,2, and 3 in the GnRH-stimulated conditions. CD45 immune cell depletion led to reduced secretion of TGFβ2 (Figure 6D). TGFβ2 also significantly correlated with concentrations of FSH but not LH. Given that LPS is well known to induce TGFβ secretion from macrophages, a TLR4 expressing immune cell, we speculate that from these data that paracrine TGFβ2 and non-canonical functions of TGFBR3 are potential contributors to elevated FSH in response to chronic LPS administration.

4 Discussion

In this study, we found that low dose chronic LPS results in elevated serum gonadotropins. By performing bulk RNA-seq analysis of pituitary from LPS-treated mice, we show distinct LPS-induced changes in the transcriptomic landscape that correlate with increases in gonadotropin, particularly metabolic and hormone pathways. Further, the broad changes in RNA expression in the pituitary in response to LPS implicate local paracrine mechanisms which may contribute to the effect of LPS-induced immune stress on reproduction. Overall, we provide evidence that TGBβ2 may be an important factor in the observed LPS-induced upregulation of FSH.

Our observation that chronic LPS induces elevated levels of serum LH and FSH stands in stark contrast to the literature's assessment of high dose and acute LPS impacts on reproduction (4, 5). These short-term studies measured serum LH either over the course of several hours using a sublethal single dose of LPS or at the end of multiple sublethal doses that did not exceed a week. Acute LPS was demonstrated to suppress the HPG axis through impacts on the hypothalamus, and serum LH was found to decrease consistently across various animal models including rats, mice, monkeys, and ewes, despite differences in LPS serotypes and sources (4, 40–42). Some recent studies show increase of serum FSH similar to our study in response to LPS in male mice (43), laying geese (10), and ewes(44) with implications for activation upstream of the pituitary, though the time frames were limited to acute or less than a week.

Several studies highlight discrepancies among transcription, translation, and secretion of gonadotropins (23, 45–50). Our data support this growing literature on the decoupling of regulatory steps that ultimately lead to the production of gonadotropins. In our study, LPS increased serum LH and FSH, yet surprisingly we demonstrated that transcripts for LH and FSH were decreased or did not change, respectively (Figures 1, 3). Another study recently demonstrated this same outcome in birds treated with LPS, that despite reduced pituitary gonadotropin transcript, serum levels of the protein were elevated (10). Our differential expression analysis (Figure 4) demonstrates a counterintuitive reduction in transcripts for translational machinery. By means of a WGCNA analysis we identified 2 sets of genes (modules) that correlated with LPS dosage or LH levels, and a further analysis linked these sets of genes with transcriptional regulation pathways and ribosomal and mRNA dynamics, reinforcing the view of the above mentioned post-transcriptional and post-translational control of gonadotropin gene expression by LPS and LH. Together, this dataset implicates post-transcriptional and post-translational control of gonadotropin gene expression such as stabilization of mRNA via RNA binding proteins and stabilization of ribosomal protein (reviewed in (46)). GnRH, the signal that regulates LH secretion, enriches ribonucleoprotein with *Lhb* and *Cga* mRNA (51). Such redistribution of mRNA could support increased LH and FSH translation and ultimately secretion despite a potential overall reduction in ribosomal mRNA or protein.

Another potential point of regulation that addresses the uncoupling of mRNA transcription and translation is mechanisms that induce translation and secretion independent of transcription. The pituitary microenvironment could be a source of such signals. Secretome analysis led to the identification of TGFβ family members. The protein coded by *Bmp3*, upregulated in LPS conditions, is known to induce the transcription of *Inha*, also upregulated with LPS (52) (Figure 6). These results would implicate suppression of FSH. However, we see that TGFβ1 and 3 (those with high affinity for TGFBR1 and 2) with known suppressive function do not correlate with FSH in response to GnRH. TGFβ2, on the other hand, not only correlates with FSH, but is low affinity for TGFBR1 and 2. TGFβ2 does bind with high affinity TGFBR3 which is the most highly expressed TGFBR and has plasticity in its regulation of FSH. Conditional gonadotrope knockdown of *Tgfbr3* results in reduced basal FshB and loss of responsiveness to inhibin A (53). Another modality to consider is that TGFBR3, can also be cleaved and function to sequester inhibin A, effectively removing FSH inhibitory signals. This new model of chronic LPS again highlights the heterogeneity and plasticity of pituitary cell types (54) and implicates cellular crosstalk in the regulation of LPS-induced gonadotropin secretion and merit additional studies including single cell-RNA sequencing and single cell functional approaches to uncover new biology of how local pituitary networks regulate gonadotropin secretion.

As reviewed by Bidne et al. (55), our understanding of LPS impacts of endotoxemia on reproduction does not accurately represent physiological conditions such as the temporal pattern of bacterial infection, or 'leaky gut', and instead is based on acute high dose exposures. We address the need for more continuous chronic low-level LPS experiments with our model. Our model of LPS differs from majority of the literature in that LPS is administered 1) at a low dose that is well tolerated (Figures 1B, 2) chronically for 6 weeks starting pre-puberty. The timing and dose of LPS administration in our model are likely defining features that differentiate our approach from previously published studies. With chronic exposure, pathways downstream of LPS activation

may either become desensitized, similar to tonic exposure of GnRH receptor to GnRH or many other hormone ligand receptor pairs (56). For example, it is well known that immune cells which express TLR4, when re-stimulated with LPS or exposed to chronic LPS become desensitized or tolerant as evidenced by a reduction in glycolysis, reduced capacity to repair tissue damage, and reduced secretion of inflammatory cytokines (39). Though they go unnoticed, there exists a substantial population of immune cells within the murine pituitary, that we have demonstrated are a sizable source of pleiotropic cytokine and that have an impact on FSH secretion (Figures 6C, D). Several studies demonstrate that pro-inflammatory cytokines secreted in response to LPS stimulation such as IL-1β have a direct negative effect on the production of gonadotropins, while anti-inflammatory cytokines like IL-10 support reproduction (6, 57, 58). Here we show that TGFβ1,2, and 3 are TGFβR family ligands produced from the pituitary (Figure 6D). Inhibin A and bone morphogenic peptide (upregulated with LPS in our dataset) are also ligands of the TGFβR family (Figure 6A). There is evidence for both positive and negative regulation of FSH production and secretion when TGFβR is engaged depending on the ligand and the context (52) (59). Changes in the balance of systemic pro-and anti-inflammatory cytokines such as TGFβR ligands in response to chronic LPS could be a potential mechanism by which LPS administration increases gonadotropin secretion.

Our study does have limitations. We draw conclusions based on bulk RNAseq data from a heterogenous tissue. Further validation and single cell approaches will be needed to thorough understand the interaction of soluble mediators and cells in the pituitary microenvironment. Further, the sample size for the computational analysis is limited. WGCNA is most accurate with a large sample size of 50-100. In this study we have 4 animals per group. By using the preliminary conclusions generated from the network analysis we, were able to develop hypotheses which are testable. Most notably, we have identified a potential role for TGFβ2 and TGFBR3 in the mediation of chronic LPS induction of serum FSH. Further, the observed effects on the gonadotropins could be mediated by input and/or feedback from the hypothalamus and or ovary. In short term LPS models, signals from the hypothalamus including pulsatility of GnRH change. It's possible that changes in pulsatility support the decreased the LH to FSH ratio (Figure 2). Given that testosterone is increased in these mice, estradiol may also be impacted and could play a role in yet to be determined impacts of chronic LPS treatment on LH surge and fertility. Finally, TLR4 is expressed in many cell types throughout the HPG axis. A conditional TLR4 KO in gonadotropes, granulosa cells, or other important points of regulation would clarify the mechanisms of LPS action.

Overall, we have created a unique model of chronic inflammation in female mice using serial injections of low dose LPS over 6 weeks. This model has a phenotype of elevated serum LH and more significantly, FSH and has implications for dissecting the impact of endotoxemia on reproduction. Much is left to be discovered using this new model including impacts on sexually mature females such as female sex steroids, fertility, and ovarian function. Of particular interest is the impact of immune crosstalk in the pituitary microenvironment and the mechanisms of regulation upstream of the pituitary in the hypothalamus including Kiss and GnRH neuron function. Our findings here lay the groundwork and rationale for thoroughly evaluating this new mouse model of chronic LPS-induced inflammation on reproductive function and could eventually be the foundation for the design of immunotherapy for reproduction resulting from chronic inflammation.

Data availability statement

The data presented in the study are deposited in Dryad, DOI: 10.5061/dryad.x69p8czrd, and in Sequence Read Archives, accession number PRJNA1054603.

Ethics statement

The animal study was approved by University of California Irvine and San Diego Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

CG: Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. LV: Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. NU: Data curation, Formal analysis, Writing – review & editing, Investigation. ZD: Data curation, Formal analysis, Investigation, Writing – review & editing, Visualization. TN: Data curation, Investigation, Writing – review & editing, Methodology. CF: Data curation, Investigation, Writing – review & editing, Conceptualization. AM: Data curation, Writing – review & editing, Formal analysis, Visualization. KF: Data curation, Formal analysis, Visualization, Writing – review & editing, Investigation, Resources. ML: Resources, Writing – review & editing, Conceptualization. AD: Conceptualization, Resources, Writing – review & editing, Funding acquisition, Investigation, Project administration. MS: Visualization, Writing – original draft, Investigation, Project administration, Resources, Writing – review & editing, Data curation, Formal analysis, Methodology, Supervision. DN: Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing, Funding acquisition.

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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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Genetic and inflammatory factors underlying gestational diabetes mellitus: a review

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Gestational diabetes mellitus (GDM) poses a significant global health concern, impacting both maternal and fetal well-being. Early detection and treatment are imperative to mitigate adverse outcomes during pregnancy. This review delves into the pivotal role of insulin function and the influence of genetic variants, including SLC30A8, CDKAL1, TCF7L2, IRS1, and GCK, in GDM development. These genetic variations affect beta-cell function and insulin activity in crucial tissues, such as muscle, disrupting glucose regulation during pregnancy. We propose a hypothesis that this variation may disrupt zinc transport, consequently impairing insulin production and secretion, thereby contributing to GDM onset. Furthermore, we discussed the involvement of inflammatory pathways, such as TNF-alpha and IL-6, in predisposing individuals to GDM. Genetic modulation of these pathways may exacerbate glucose metabolism dysregulation observed in GDM patients. We also discussed how GDM affects cardiovascular disease (CVD) through a direct correlation between pregnancy and cardiometabolic function, increasing atherosclerosis, decreased vascular function, dyslipidemia, and hypertension in women with GDM history. However, further research is imperative to unravel the intricate interplay between inflammatory pathways, genetics, and GDM. This understanding is pivotal for devising targeted gene therapies and pharmacological interventions to rectify genetic variations in SLC30A8, CDKAL1, TCF7L2, IRS1, GCK, and other pertinent genes. Ultimately, this review offers insights into the pathophysiological mechanisms of GDM, providing a foundation for developing strategies to mitigate its impact.

KEYWORDS

beta-cell dysfunction, genetic factors, gestational diabetes mellitus (GDM), genetic variations, glucose metabolism, inflammatory pathways

1 Introduction

Gestational diabetes mellitus (GDM) presents a formidable challenge in maternal healthcare, affecting millions of pregnancies worldwide annually. This metabolic disorder, characterized by elevated glucose levels during pregnancy, poses significant risks to both maternal and fetal health (1–4). Recent epidemiological data suggests a staggering impact, with over 21.1 million live births affected globally in 2021 alone, underscoring its prevalence and urgency for attention (2, 5, 6).

Furthermore, emerging scientific insights indicate a substantial rise in GDM incidence, particularly in regions such as Asia, Africa, Europe, and Latin America. Notably, South and Southeast Asia bear the brunt, with over 90% of cases occurring in these regions, followed by the Middle East and North Africa region with 11.7% (2). Interestingly, South American countries also report a high prevalence of GDM, with approximately 15% of pregnant women in Chile and Peru being diagnosed with the disorder over the past two decades (7–9). Contrastingly, advanced economies exhibit comparatively lower prevalence rates, with countries like Australia, Canada, the United States, and the United Kingdom reporting less than 6% of pregnancies affected (10).

The multifactorial nature of GDM is increasingly evident, with genetic predispositions playing a pivotal role in GDM susceptibility, with links to type 2 diabetes mellitus (T2DM) becoming increasingly evident (11). A comprehensive analysis of potential genes associated with GDM has revealed the involvement of crucial genetic markers in GDM susceptibility. Notably, genes such as SLC30A8, CDKAL1, TCF7L2, IRS1, and GCK exhibit polymorphisms that are strongly linked to an increased risk of GDM (11–13). Furthermore, it is imperative to consider histone alterations and other epigenetic changes, including DNA methylation, as they are pivotal in regulating gene expression throughout pregnancy. These mechanisms are essential for maintaining the delicate balance for a healthy gestational period. The implications of these genetic mutations extend beyond the mother's health, potentially posing risks to the well-being of the fetus as well. Consequently, it becomes crucial to acknowledge the potential impact of these mutations on the offspring's overall health.

During pregnancy, women with GMD may experience heightened inflammation in their bodies. This inflammatory response is triggered by the increased levels of glucose in the blood characteristic of GDM, leading to elevated levels of cytokines and other inflammatory markers (14). Inflammation plays a crucial role in the pathogenesis of GDM, as it can impair insulin sensitivity and lead to complications such as pre-eclampsia and preterm birth (14). Managing inflammation in pregnant women with GDM is essential for optimizing their outcomes and preventing adverse maternal and fetal health effects. It is crucial to acknowledge the various dimensions of the relationship between genetics, inflammatory pathways, and GDM. Alongside genetic factors, lifestyle choices and other non-genetic variables play a significant role in determining the overall risk and progression of GDM. A more comprehensive understanding of GDM can be gained by examining how genetics influence inflammatory pathways within the context of the condition.

However, despite advancements, gaps persist in our understanding of the precise genetic and inflammatory pathways underpinnings of GDM, warranting further exploration (15). This review seeks to elucidate the roles of key genes, including SLC30A8, CDKAL1, TCF7L2, IRS1, and GCK, in GDM etiology. Delving into molecular inflammatory pathways and genetics aims to provide comprehensive insights that pave the way for tailored interventions and improved maternal-fetal outcomes. It also discusses the SLC30A8 expression and mechanism in regulating placental tissue.

2 Genetic basis of gestational diabetes mellitus

Genetic and environmental factors are pivotal in the etiology of gestational diabetes mellitus (GDM), a multifaceted disorder affecting pregnancy. Research indicates a genetic underpinning influenced by various factors, including SLC30A8, CDKAL1, TCF7L2, IRS1, and GCK (16, 17). The intricate relationship between genetics and GDM onset is striking, with a plethora of genes identified as contributors, underscoring the complexity of disease progression (18). Notably, susceptibility to GDM has been linked to genetic variations impacting insulin sensitivity, exemplified by SLC30A8, CDKAL1, TCF7L2, IRS1, and GCK. Ongoing research continues to probe the genetic landscape of GDM, exploring the significant impacts of these genetic factors on pregnancy-associated glucose dysregulation (17). Such insights offer a valuable understanding of GDM's pathogenesis and avenues for targeted interventions to mitigate its risks.

3 Genetic markers and risk prediction

Understanding genetic markers in predicting the risk of developing Gestational Diabetes Mellitus (GDM) has made significant advancements in genetics in recent years. Moreover, during postpartum follow-up, the incidence of type 2 diabetes (T2DM) in women with GDM can range from 50% to 70%. According to (19), GDM in middle-aged women is a major cause of type 2 diabetes. Specific differences in DNA sequences, known as genetic markers, have been identified as potential predictors of GDM risk. Numerous studies have explored the relationship between genetic markers and the likelihood of developing GDM, shedding light on the underlying genetic factors contributing to this condition (19). (20, 21) have conducted research in this area. For instance, these studies have discovered that specific gene variants, such as TCF7L2 and KCNJ11, are associated with a higher risk of GDM (20–22). These results pave the way for the development of personalized risk prediction models and provide valuable insights into the genetic basis of GDM. Incorporating genetic markers into risk prediction models holds excellent potential for early detection and treatment of GDM cases. By integrating genetic data with

conventional risk factors like maternal age, body mass index, and family history of diabetes (41), clinicians can enhance the accuracy of risk assessment. This approach enables the implementation of targeted interventions, such as dietary modifications and careful monitoring, to mitigate the risks associated with GDM.

4 Diagnosis of gestational diabetes mellitus

The importance of early detection of gestational diabetes mellitus (GDM) cannot be overstated, as it allows for timely intervention and management strategies. According to (23), the prevalence of GDM among pregnant women can range from 15% to 70%, underlining the critical need for robust screening protocols. Early identification of GDM empowers medical providers to implement tailored interventions, such as dietary adjustments, exercise regimens, and, when necessary, insulin therapy (23). A study conducted by (24) found that early diagnosis and management of GDM significantly reduced the risk of adverse perinatal outcomes, such as macrosomia and neonatal hypoglycemia. By mitigating risks such as pre-eclampsia, macrosomia, and neonatal hypoglycemia, early diagnosis serves as a protective shield for both maternal and fetal health. The ramifications of accurate diagnosis extend beyond immediate management; they pave the way for optimal outcomes throughout the pregnancy journey.

In the field of GDM diagnosis, two prominent criteria endorsed by reputable bodies stand out: the International Association of Diabetes and Pregnancy Study Groups (IADPSG) and the American Diabetes Association (ADA) criteria (25, 26). Both rely on oral glucose tolerance tests (OGTT) to assess glucose metabolism during pregnancy, but they offer distinct approaches to diagnosis. The IADPSG criteria advocate a streamlined, one-step approach, with a 75g OGTT at 24-28 weeks of gestation. A fasting plasma glucose level of ≥92 mg/dL (5.1 mmol/L) or a 2-hour plasma glucose level of ≥180 mg/dL (10.0 mmol/L) signals GDM (25). Notably, studies by (27, 28) have validated the effectiveness of these criteria, particularly in Asian populations like China, underscoring their global relevance. Furthermore, the International Federation of Gynecology and Obstetrics (FIGO) endorsed the use of the International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria due to its global applicability and ability to identify women at risk more accurately (29).

On the other hand, the ADA criteria, predominant in the United States, favor a two-step approach. This involves a 50g glucose challenge test (GCT) followed by OGTT if the former yields elevated glucose levels (30). However, discrepancies in diagnostic thresholds and approaches among healthcare professionals underscore the need for standardization. In response, the ADA has proposed adjustments, advocating for a lower fasting plasma glucose level as the primary diagnostic test (31). Furthermore, emerging evidence suggests seasonal variations in GDM prevalence, emphasizing the multifactorial nature of its diagnosis (32). GDM's complex landscape demands a unified approach to diagnosis and management. The IADPSG criteria offer a comprehensive, global framework, while the ADA criteria cater specifically to the U.S. context. Yet, harmonizing these approaches and implementing standardized diagnostic protocols remain imperative.

Accurate and timely diagnosis of GDM is pivotal for ensuring the well-being of both mother and child. By embracing evidence-based recommendations and fostering collaboration among healthcare providers, we can refine diagnostic practices, enhance patient care, and ultimately, safeguard the health outcomes of pregnant individuals and their offspring. The proposed diagnostic process in Figure 1 serves as a visual reminder of the journey toward effective GDM identification and management on a global scale.

4.1 Guidelines for screening the gestational diabetes mellitus during early pregnancy

It is widely recommended that women who have a high risk of pre-existing diabetes undergo screening for this condition during the first trimester of their pregnancy or at the onset of antenatal treatment (33). To better understand the populations that should be screened, Table 1 offers a comprehensive overview based on the

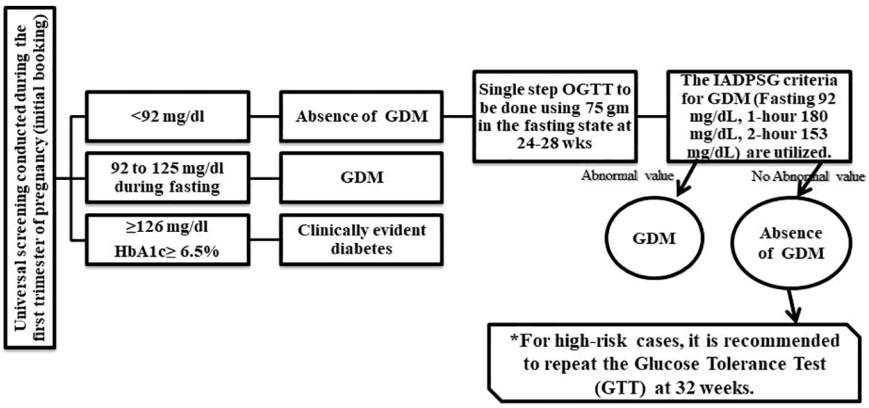

FIGURE 1
General diagnostics process of GDM identification. Source.

TABLE 1 Guidelines for Screening the Gestational Diabetes Mellitus during Early Pregnancy.

Organization	Recommended Populace	References
World Health Organisation (WHO)	Depending on available resources, conflicting priorities, and the prevalence of glucose intolerance within the local population, it is imperative for each country or health service to make a well-informed decision regarding the patients who should undergo screening.	(33)
American College of Obstetricians and Gynaecologists (ACOG)	Women who are overweight or obese (with a BMI of ≥25 kg/m^2 or ≥23 kg/m^2 in Asian Americans) and have one or more additional risk factors should be identified for further evaluation. These risk factors include physical inactivity, belonging to a high-risk race or ethnicity, having a history of delivering a baby weighing ≥4 kg, having a history of gestational diabetes mellitus, having hypertension (with a blood pressure reading of 140/90 mmHg or taking antihypertensive therapy), having low levels of high-density lipoprotein (HDL) cholesterol (<35 mg/dl or 0.9 mmol/l) and triglycerides, having polycystic ovary syndrome (PCOS), having a hemoglobin A1c (HbA1c) level of ≥5.7%, having impaired glucose tolerance or impaired fasting glucose on previous testing, cardiovascular disease.	(34)
International Association of Diabetes and Pregnancy Study Groups (IADPSG)	The decision is made considering the prevalence of impaired glucose tolerance in the local community, as well as the individual circumstances of each woman, particularly those who are at a higher risk	(35)
Diabetes Canada	Identification of Women at High Risk for Undetected Type 2 Diabetes (T2D)	(36)
American Diabetes Association (ADA)	Women who have one or more risk factors for diabetes mellitus (DM) should be aware of the following: belonging to a high-risk race or background, experiencing hypertension (with a blood pressure reading of 140/90 mmHg or taking antihypertensive therapy), having a first-degree family with DM, being diagnosed with polycystic ovarian syndrome (PCOS), having a history of cardiovascular disease, women who have previously experienced gestational diabetes mellitus (GDM) should undergo regular screening for DM, at least once every three years. having low levels of high-density lipoprotein (HDL) cholesterol (<35 mg/dl or 0.9 mmol/l) and increased levels of triglycerides, leading a physically inactive lifestyle,	(37)

recommendations of national guideline organizations. Recent research studies have identified various genes that are associated with gestational diabetes mellitus (GDM). These genes play a crucial role in the development and progression of GDM during pregnancy. The Table 2 below provides a list of these genes and their significance in GDM research.

5 The role of genetic factors in the development of gestational diabetes

Gestational Diabetes Mellitus (GDM) is a critical condition during pregnancy characterized by specific glucose level thresholds outlined by the (31). These thresholds, including fasting glucose

TABLE 2 Various genes associated with gestational diabetes mellitus (GDM) in recent research studies.

Genes	Sample size	Gene function	References
CDKAL1; CDK5 regulatory subunit associated protein 1 like 1	GDM n = 10336; Control n = 17,445	The gene CDKAL1 encodes a member of the methylthiotransferase family. While the exact function of CDKAL1 remains unclear, it has been associated with both risk variants that are linked to decreased insulin secretory capacity and glucose-stimulated insulin secretion [51]. This gene locus has also been linked to type 2 diabetes (T2D) Cho, Y. M., 2009; Ohara-Imaizumi, Mica, 2010	(38)
GCK; glucokinase	29 studies	The gene encodes the hexokinase GCK, which plays a crucial role in the pancreas's response to glucose. It is responsible for the secretion of insulin and also influences the liver's absorption of glucose and its conversion into glycogen. This gene locus has been linked to Type 2 Diabetes (T2D).	(17)
TCF7L2; transcription factor 7-like 2	GDM patients = 5485; Healthy control = 347,856	The gene encodes a transcription factor that contains a specific box, which plays a crucial role in the Wnt signaling pathway. This transcription factor belongs to the high mobility group and is also involved in the synthesis and processing of insulin Zhou, Yuedan, 2014. Furthermore, this gene locus is linked to Type 2 Diabetes (T2D).	(39)
CDKAL1	GDM patients 835 and Healthy Control 870	Beta-cell dysfunction and reduced insulin secretion	(12)
IRS1	213 GDM patients including fetuses and 191 Healthy control including fetuses	Insulin-stimulated signaling pathways regulate insulin receptor substrate (IRS) activity in various tissues, including muscle and pancreatic beta cells, and have been identified as a pathogenic factor in diabetes onset (Yiannakouris, 202).	(40)
SLC30A8	500 patients with GDM and 502 control subjects.	The SLC30A8 gene, encoding a crucial zinc transporter, may be affected by mutations that affect pancreatic beta cell function, potentially increasing gestational diabetes mellitus susceptibility.	(41)

Gestational Diabetes Mellitus= GDM; Sample size= n.

levels, oral glucose tolerance test (OGTT) results, and symptomatic hyperglycemia, serve as crucial indicators for diagnosing and managing GDM. The research underscores the profound impact of GDM on maternal and fetal health. The excessive presence of glucose, fatty acids, and amino acids in maternal circulation can lead to the development of larger-than-average fetuses, posing risks during delivery and increasing the likelihood of cesarean sections (42). Moreover, the metabolic dysregulation associated with GDM, marked by hyperglycemia and insulin resistance, can instigate a cascade of adverse effects (42). This metabolic upheaval not only heightens the oxygen demand but also induces chronic hypoxia and inflammation, contributing to further complications.

Moreover, the repercussions of GDM extend far beyond pregnancy. Both mothers who experience GDM and their offspring face an elevated risk of developing type 2 diabetes, obesity, and cardiovascular conditions later in life (42). While genetic factors undoubtedly play a role, it's crucial to recognize the multifaceted nature of GDM's etiology. Recent studies have delved into the intricate interplay between genetics, epigenetics, environmental factors, and the body's microbiota in predisposing individuals to GDM (43, 44). (42) demonstrated that the surplus of glucose, fatty acids, and amino acids characteristic of GDM contributes to fetal macrosomia, chronic hypoxia, and inflammation, underscoring the multifactorial nature of its pathogenesis. Moreover, investigation by (10) has highlighted the role of epigenetic mechanisms and environmental factors in predisposing individuals to GDM, further reinforcing the complexity of its etiology.

The emergence of epigenetic signatures and their association with GDM highlights the need for a comprehensive understanding of the condition. Moreover, investigations into common genetic variants linked to type 2 diabetes, such as TCF7L2, SLC30A8, and CDKAL1, underscore the interconnectedness of GDM and metabolic disorders (45). A striking finding from these studies is the overlap of specific genetic variants associated with diabetes in non-pregnant populations with those prevalent in women with GDM (46–48). Variants in genes like CDKN2A-CDKNA2B, TCF7L2, KCNQ1, MTNR1B, and FTO have been consistently observed in women with GDM across diverse populations (46–49). Emerging research focuses on genes associated with β-cell function and insulin secretion, with TCF7L2, SLC30A8, and GCK among the implicated candidates (45). These findings underscore the importance of understanding the molecular mechanisms underlying GDM for targeted intervention and management strategies. This convergence reinforces the notion that GDM shares genetic underpinnings with type 2 diabetes, emphasizing the importance of targeted interventions and preventive measures.

5.1 Role of SLC30A8 in the development of GDM

SLC30A8, also known as zinc transporter 8 (ZnT8), is a pivotal gene implicated in the pathogenesis of Gestational Diabetes Mellitus (GDM) (50). This gene regulates zinc homeostasis by facilitating zinc transport into pancreatic beta cells, which is crucial for insulin synthesis and secretion (51). Several studies have indicated that variations in the SLC30A8 gene influence susceptibility to GDM (52–54). Variations in SLC30A8 have been associated with an increased risk of GDM development. Specific single nucleotide polymorphisms (SNPs) within the gene alter protein structure and function, impairing zinc transportation and, subsequently insulin secretion (55, 56). Consequently, disrupted insulin production leads to elevated glucose levels during gestation, contributing to GDM diagnosis.

The research underscores the critical role of SLC30A8 in maintaining beta cell function and mass (55). Beta cells rely heavily on zinc as a cofactor for insulin synthesis (57, 58). Dysfunctional SLC30A8 results in zinc depletion within beta cells, causing impaired function and decreased survival. This dysfunction further disrupts glucose regulation, exacerbating GDM susceptibility. ZnT8, the protein encoded by SLC30A8, facilitates the movement of zinc ions from the cytoplasm to insulin granules within pancreatic beta cells. Insulin granules contain high zinc concentrations, and the co-secretion of zinc with insulin impacts neighboring endocrine cells (59, 60). Studies reveal the complex role of zinc in glucose-stimulated insulin secretion (GSIS), with ZnT8 influencing this process significantly (61, 62).

While some studies demonstrate the stimulatory effect of zinc on insulin secretion, others suggest an inhibitory role (59, 63). The intricate relationship between zinc and insulin secretion remains a subject of ongoing research. Nonetheless, the critical involvement of SLC30A8 and ZnT8 in insulin secretion underscores their potential influence on GDM susceptibility. Additionally, a study in animal models highlights the potential therapeutic benefits of zinc supplementation in restoring beta cell function and mitigating GDM risk (51). Intraperitoneal zinc supplementation in rats restored ZnT8 levels and improved beta cell function, emphasizing the therapeutic potential of targeting SLC30A8 in GDM management (51).

5.1.1 Link between altered SLC30A8 expression in placental tissue and gestational diabetes mellitus

The SLC30A8 gene on chromosome 8q24.11 has garnered significant attention in the study of type 2 diabetes (T2D). Recent research has highlighted its relevance to GDM, as certain variations in the SLC30A8 gene may impact a mother's ability to metabolize glucose during pregnancy (41). The placenta, a vital organ for fetal development, undergoes dynamic changes in gene expression throughout pregnancy. It serves as a gateway, controlling the flow of nutrients between the mother and the developing fetus (64). Understanding the intricate mechanisms involved in placental function is crucial for comprehending the complexities of fetal development and maternal health.

Placental zinc transporters play a vital role in maintaining the delicate balance of zinc levels in the developing fetus. These transporters are regulated as part of complex processes that are not yet fully understood. Zinc, a crucial element, serves as a catalyst or structural component in hundreds of proteins, making it essential for various physiological functions. Insufficient zinc levels can lead to a range of symptoms, including skin lesions, compromised immune function, growth retardation, and GDM in pregnant women (65). Research has shown that the amount of zinc influences the expression of zinc transporters in mouse placentas in

their diet (66, 67). However, even with moderate dietary zinc restriction, this modulation of expression is insufficient to support optimal fetal nutrition. The SLC30A8 gene encodes ZnT8, a protein that regulates zinc transport across cellular membranes. Zinc is crucial for healthy trophoblast differentiation, fetal growth, and protection against oxidative stress in the placenta. Altered expression of SLC30A8 can disrupt these processes, contributing to the development of GDM (66).

Research findings indicate that the expression of SLC30A8 varies in the placental tissue of patients with GDM compared to pregnancies with normal blood sugar levels (66, 68). This variation in SLC30A8 expression suggests a potential disruption in zinc homeostasis and its impact on fetal development and metabolic programming. Moreover, a decrease in insulin function leads to an upregulation of genes associated with placental fatty acid β-oxidation and transport (69). This upregulation results in a higher transfer of long-chain polyunsaturated fatty acids (LCPUFA) to the developing fetus. However, elevated lipid levels directed toward the fetus have the potential to cause obesity and metabolic disturbances later in life (69).

Abnormalities in placental function have been closely associated with unfavorable pregnancy outcomes such as macrosomia, premature birth, and neonatal problems. Therefore, examining the relationship between modified SLC30A8 expression and GDM provides a novel insight into the potential effects of placental disruptions on fetal development and metabolic programming. As research continues to unravel the intricate molecular landscape of GDM, the link between altered SLC30A8 expression in placental tissue and the development of GDM emerges as a promising area of investigation. Further studies are warranted to elucidate the precise mechanisms underlying this association and to explore therapeutic intervention interventions that could positively influence pregnancy outcomes in women at risk for GDM.

5.2 Role of insulin receptor substrate gene in the development of GDM

Insulin resistance (IR) serves as the fundamental pathogenesis of Gestational Diabetes Mellitus (GDM), initiating its onset (70). While the precise mechanism remains elusive, inflammation is a critical contributor to insulin resistance and pancreatic beta-cell dysfunction. Studies have highlighted the significance of the IRS1 gene in insulin signaling, with genetic variations linked to insulin resistance, a pivotal factor in GDM development (71, 72). The IRS1 gene encodes the IRS1 protein, crucial for transmitting insulin signals and regulating glucose metabolism (73, 74). However, mutations or variations in IRS1 can disrupt these signaling pathways, leading to impaired insulin action (73).

Furthermore, IRS1 governs insulin activity in various tissues, including muscle, adipose tissue, and pancreatic beta cells, underscoring its role in glucose regulation (75). Notably, the IRS1 rs2943641 polymorphism, located downstream of the IRS1 gene, has been associated with increased fasting hyperinsulinemia and reduced insulin sensitivity (76, 77). This polymorphism has also been linked to elevated risk of type 2 diabetes and higher fasting glucose levels in women with GDM (78). Additionally, research suggests a correlation between IRS1 gene expression in the placenta of pregnant women with GDM and increased body mass index, further highlighting its significance in GDM pathogenesis (79).

Studies in mice have shown that IRS1 or IRS2 signals regulate hepatic gene expression, which is crucial for glucose homeostasis and systemic growth (80). Therefore, pregnant women with genetic variations in the IRS1 gene may struggle to maintain optimal sugar levels during pregnancy, heightening their risk of GDM (80). Alongside IRS1, the SLC30A8 gene also significantly influences GDM development through its role in zinc homeostasis regulation and insulin and beta cell function (41). Variations in both SLC30A8 and IRS1 genes can impair zinc transportation and subsequent insulin secretion in beta cells, increasing GDM risk. Studying the mechanisms underlying SLC30A8's influence on GDM development holds promise for early detection, targeted intervention, and personalized treatment strategies for pregnant women at higher risk of GDM. However, the genetic bases of GDM remain incompletely understood, urging future studies to identify various genetic markers, mechanism genes, and environmental factors contributing to GDM development.

5.3 Role of TCF7L2 in the development of GDM

TCF7L2 is an extensively studied genetic factor implicated in reduced insulin secretion and an increased risk of developing gestational diabetes mellitus (GDM). It was the first locus firmly identified through genomic linkage studies and is considered the most influential locus for the risk of type 2 diabetes (T2D) (11). As one of the key transcription factors in the Wnt signaling pathway, TCF7L2's functional domains closely relate to highly conserved sequence regions within the gene. Its consistent replication across diverse populations with various genetic backgrounds underscores its significance as one of the strongest genetic associations with complex diseases found in research studies.

A meta-analysis of genetic associations observed in different populations revealed that TCF7L2 variants linked with type 2 diabetes operate through a multiplicative genetic model. It was estimated that TCF7L2 contributes to nearly 20% of T2D cases (81). However, the specific mechanisms through which TCF7L2 influences GDM and T2D remain not fully understood (11). Therefore, further research is necessary in this field to gain a deeper understanding of the role played by TCF7L2 in the development of GDM.

5.4 Role of glucose kinase in the development of GDM

One crucial enzyme involved in regulating glucose uptake and storage is glucose kinase (GCK) (82). Additionally, GCKR, or glucokinase regulatory protein, has been identified as the rate-limiting factor for GCK, as highlighted by (83). GCK and GCKR

work in tandem to maintain glucose homeostasis. Notably, these genes have been linked to an increased susceptibility to type 2 diabetes through genome-wide association studies (GWAS) (84). Significant differences were observed in age, pre-gestational BMI, education level, and family history of diabetes when comparing the case and control groups ($P < 0.05$) (85). Despite accounting for these confounding factors, GCK rs1799884 remained significantly associated with gestational diabetes mellitus (GDM) ($P < 0.05$). However, no significant associations were found between GDM odds and the polymorphisms rs4607517, rs10278336, rs2268574, rs780094, and rs1260326 ($P > 0.05$) (85).

Furthermore (85), discovered that pregnant women with the rs4607517 TT genotype exhibited significantly higher fasting glucose levels compared to those with the CC genotype ($P < 0.05$). The Chinese population, in particular, faces an elevated risk of developing GDM due to the GCK rs1799884 mutation. Further extensive research is warranted to understand better the relationship between GCK and GCKR polymorphisms and susceptibility to GDM.

5.5 Role of CDKAL1 in the development of GDM

Beta-cell dysfunction and reduced insulin secretion have been found to be associated with CDKAL1, as stated by (86). These genetic variations can lead to disruptions in glucose homeostasis during pregnancy, potentially resulting in elevated sugar levels. The CDKAL1 gene encodes Cyclin-dependent kinase 5 regulatory subunit-associated protein 1 (CDK5RAP1)-like 1. CDK5, a serine/threonine protein kinase, plays a critical role in the pathophysiology of β-cell dysfunction and the predisposition to type 2 diabetes (T2DM) by regulating insulin secretion in a glucose-dependent manner, as highlighted by (87). However, further studies are necessary to comprehend the complete genetic landscape fully. Future research is crucial in enhancing risk prediction and developing more effective preventive strategies and personalized care for pregnant individuals at risk for gestational diabetes mellitus (GDM).

Figure 2 illustrates a visual representation of various genes that could potentially be linked to gestational diabetes mellitus (GDM), a condition characterized by glucose intolerance during pregnancy. The diagram features a central image of a pregnant woman, with multiple gene acronyms connected by arrows indicating potential interactions, functions, and regulatory pathways. These genes may influence an individual's susceptibility to or progression of GDM, highlighting the significant role of heredity in the development of this disease.

6 Impacts of environmental factors and the lifestyle modification that could impact GDM

Epigenetic research has demonstrated that pregnancies affected by GDM exhibit distinct gene methylation statuses compared to those without GDM (88). This growing body of evidence indicates that GDM not only affects the duration of pregnancy but also impacts the development of the offspring. Consequently, these effects can lead to long-term consequences and unfavorable health outcomes for the offspring. Epigenetic modifications can occur through three mechanisms: histone modification, DNA methylation, and impaired function of non-coding ribonucleic acids (ncRNAs), including microRNAs (miRNAs) (89). These findings shed light on the complex interplay between epigenetic factors and the development of GDM.

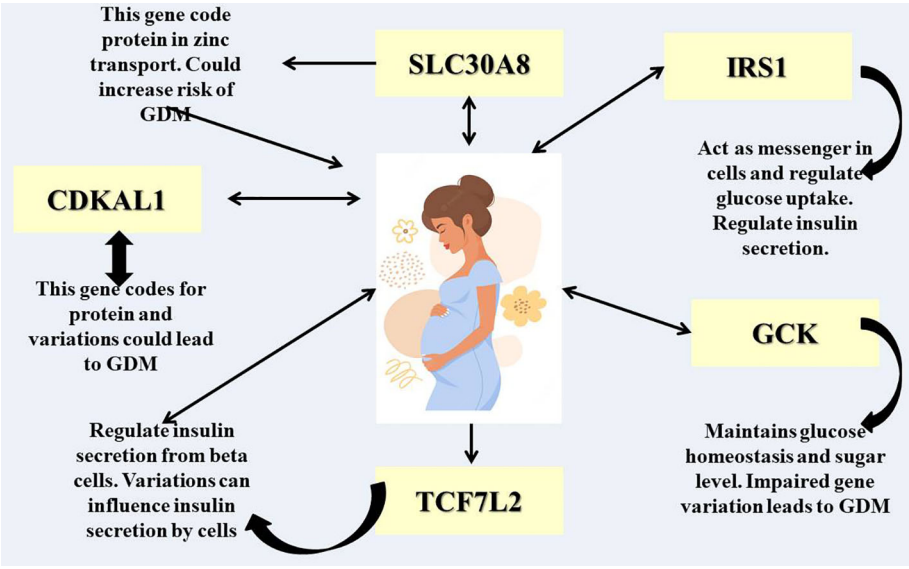

FIGURE 2
Different genetic factors responsible for GDM.

In addition, environmental factors and the lifestyle of the mother can give rise to epigenetic modifications that influence the likelihood of the offspring developing GDM. Stress, inadequate diet, and exposure to environmental pollutants during pregnancy can all induce epigenetic alterations that impact the expression of genes involved in glucose metabolism. These genetic and epigenetic factors may affect the functioning of beta cells and insulin resistance, thereby playing a role in the development of GDM. In recent years, molecular biomarkers have garnered significant attention in the field of GDM prognosis, diagnosis, and screening. We propose that genetic and epigenetic modifications contribute to the underlying mechanisms of GDM. By exploring the interplay between environmental variables, maternal lifestyle, and epigenetic changes, we can gain a deeper understanding of the pathophysiology of GDM. This knowledge can pave the way for improved prognosis, diagnosis, and screening methods for this condition.

7 The role of single nucleotide polymorphisms in gestational diabetes mellitus susceptibility

Single Nucleotide Polymorphisms (SNPs) represent the most prevalent form of genetic variation observed in human DNA (90). These subtle alterations in a single nucleotide base can have profound implications for an individual's vulnerability to various diseases, including GDM (Gestational Diabetes Mellitus) (90). SNPs are nucleotide variations occurring at single positions in the DNA sequence (91). Thanks to current genotyping technologies such as SNPscan™, TaqMan SNP genotyping, nucleic-acid-modifying enzymes, and PCR, we can now identify disease-related SNPs (41, 52, 92), including those linked to metabolic disorders such as obesity, TDM2 (Type 2 Diabetes Mellitus), cardiovascular disease, and GDM (93, 94). These SNPs may be associated with a higher or lower risk of developing GDM in different populations worldwide (49, 95–99).

Numerous studies have identified specific SNPs significantly associated with GDM susceptibility (100–102). These genetic variants are often found in genes involved in insulin secretion, insulin resistance, and glucose metabolism pathways. A study conducted by (100, 102–104) revealed that SNPs associated with T2DM also confer a significant risk for GDM in a multi-ethnic cohort comprising individuals from Hawaii, Korea, and China. In these studies, the researchers gathered information from 291 women with GDM diagnoses and 734 matched non-diabetic controls (197 non-diabetic controls; Filipinos: 162 GDM, Japanese: 58 GDM, Pacific Islanders: 71 GDM, 395 controls; 142 controls). Twenty-five different SNPs linked to 18 different loci were genotyped, and their allele frequencies were calculated using maternal DNA (100, 103, 104).

After adjusting for various factors such as age, body mass index (BMI), parity, and gravidity through multivariable logistic regression, the researchers identified several SNPs that exhibited significant associations with GDM, with these associations being specific to different ethnicities (103, 105). Additionally, substantial correlations with GDM were found among Filipinos for the SNPs rs2237895 (KCNQ1), rs1113132 (EXT2), rs2237892 (KCNQ1), rs1111875 (HHEX), rs10830963 (MTNR1B), and rs13266634 (SLC30A8). SNPs rs4402960 (IGFBP2) and rs2237892 (KCNQ1), on the other hand, were discovered to be strongly related to GDM in Japanese people (100, 103, 104). Finally, SNPs rs10830963 (MTNR1B) and rs13266634 (SLC30A8) showed substantial correlations with GDM among Pacific Islanders (100, 101, 103). These studies highlight the potential of SNPs as biomarkers and deepen our understanding of the molecular mechanisms underlying beta-cell activity and the etiology of GDM for assessment.

8 Gene-environment interactions in GDM

Within gestational diabetes mellitus (GDM), the intricate interplay between genetic and environmental factors sparks a thought-provoking discourse. Research indicates that while genetic predispositions may heighten an individual's susceptibility to GDM, environmental influences often interact with the condition, triggering the manifestation of symptoms (15). The prevalence of GDM has surged, emerging as a significant global health concern with far-reaching implications for public health, particularly in light of the escalating rates of maternal obesity. In addition to immediate adverse effects such as fetal macrosomia and hypertensive pregnancy complications, GDM has been associated with long-term consequences, including heightened cardiometabolic morbidity in both the mother and child (106).

Furthermore, extensive research has unveiled specific genetic variations that are linked to the risk of GDM, offering valuable insights into the hereditary component of the condition (15). It is crucial to comprehend that genes do not always contain all the necessary information. Environmental factors such as maternal stress, physical activity, and nutrition significantly influence the development of GDM. In a study conducted by (107), a gene-lifestyle relationship was discovered between the onset of GDM and the TCF7L2 rs7903146 polymorphism, specifically in relation to adherence to a Mediterranean diet. The study revealed that dietary intervention only alters the risk of developing GDM in individuals carrying the T-risk allele. Women who exhibited moderate to high adherence to the Mediterranean diet demonstrated a lower risk of acquiring GDM than those with low adherence (107).

In a subsequent study conducted by (108), it was observed that women with a history of GDM and a specific MC4R genotype, which increases the risk of developing diabetes and obesity, may derive more incredible benefits from a lifestyle intervention aimed at reducing insulin resistance compared to women with a non-risk genotype. To illustrate, if a woman finds herself in an environment that aligns with her genetic predisposition, she may be able to avoid acquiring GDM. The interplay between genes and lifestyle has been shown to indicate that individuals who are genetically more prone to GDM may experience more tremendous advantages by adhering to a nutritious diet or engaging in physical exercise, as suggested by

(15). Conversely, an individual with a lower genetic predisposition could still develop GDM despite unfavorable environmental circumstances. This dynamic exchange underscores the importance of adopting a comprehensive strategy to comprehend and prevent GDM. Personalized therapies could potentially be facilitated through further research into the intricate interaction between genes and the environment. In terms of managing and preventing GDM, tailored interventions based on an individual's genetic profile and environmental circumstances may yield superior outcomes. However, within the scientific and medical communities, thorough investigation and deliberation are imperative due to ethical concerns and the intricate nature of these associations.

9 Mechanisms underlying genetic predisposition

Gestational diabetes mellitus (GDM) is a condition that arises due to a combination of genetic and environmental factors. Extensive research has shown that specific genes related to glucose metabolism, beta-cell function, and insulin resistance play a crucial role in predisposing individuals to GDM (109). Additionally, epigenetic changes occurring during pregnancy, influenced by factors such as maternal nutrition and lifestyle, can further increase the risk of developing GDM. During pregnancy, the body undergoes various adaptations to ensure optimal nutrient delivery to the developing fetus. One key aspect is the regulation of glucose levels. Studies conducted on thin and healthy women using a hyperinsulinemic-euglycemic clamp technique have revealed significant changes in glucose management during the third trimester compared to pre-pregnancy (110, 111). Specifically, basal endogenous glucose production increases by 30%, while insulin sensitivity decreases by 56%. In individuals with normal glucose tolerance, the pancreatic beta cells adapt to these changes by producing higher levels of insulin, which helps maintain normal glucose levels (112). To illustrate, a small study involving normal controls found that the first- and second-phase insulin response to an intravenous glucose tolerance test increased approximately threefold during late pregnancy compared to pre-pregnancy (113–115). Furthermore, postpartum glucose clamp studies have shown that the insulin resistance associated with normal pregnancy resolves within days after delivery, indicating that placental factors play a role in these changes (116).

10 Inflammatory pathways in pregnancy

The physiological state of pregnancy is a complex process that necessitates significant changes in the mother's body, including alterations to the immune system. Throughout pregnancy, the immune system plays a crucial role in maintaining a delicate balance between the fetus's tolerance and protection from external pathogens. Inflammation is a key component of this immune response, contributing to various processes such as placental development, implantation, and the initiation of labor. Disruption of inflammatory pathways can lead to adverse pregnancy outcomes, underscoring the importance of understanding these mechanisms. The mother's immune system undergoes substantial changes during pregnancy to establish tolerance to the semi-allogeneic fetus while also preserving the ability to combat infections. These adaptations involve modifications to tissue remodeling processes, cellular responses, and cytokine profiles. Pro-inflammatory cytokines like Interleukin (IL)-6, tumor necrosis factor-alpha (TNF-α), and IL-1β play pivotal roles in initiating and regulating inflammatory responses crucial for implantation, placentation, and the onset of labor (117).

Inflammatory reactions related to pregnancy serve a dual purpose, supporting both the healthy progression of pregnancy and contributing to pathology in cases of dysregulation. Controlled inflammation is vital for the formation of the placenta and embryo implantation early in pregnancy. Immune cells release cytokines and chemokines that facilitate trophoblast cell invasion and the remodeling of the mother's spiral arteries, both of which are essential for proper placental perfusion (118). During pregnancy, a regulatory environment is established to protect the fetus from an exaggerated response by the mother's immune system. However, an imbalance in pro- and anti-inflammatory factors can lead to complications such as preterm labor, gestational diabetes, and eclampsia (119). For instance, oxidative stress resulting from excessive inflammation is a key feature of preeclampsia, a condition characterized by high blood pressure and damage to organ systems.

The nuclear factor kappa B (NF-κB) pathway, a transcription factor, regulates the expression of genes involved in immune and inflammatory responses (120). This pathway plays a critical role in immune responses during pregnancy by stimulating the release of pro-inflammatory cytokines, chemokines, and adhesion molecules. Ligands for interleukins I (IL-1) and II (IL-6) play a critical role in fever and the acute phase response as key mediators of inflammatory reactions (121). Elevated levels of IL-6 have been associated with preterm birth and other pregnancy complications (121). Toll-like receptors (TLRs) are responsible for initiating inflammatory responses by recognizing molecular patterns associated with pathogens. The TLR signaling in the placenta is vital for protecting the fetus against infections, but it must be carefully regulated to prevent excessive inflammation (122, 123). The development of therapeutic strategies to modulate these pathways is gaining traction in an effort to improve pregnancy outcomes. For instance, there is ongoing research on therapies targeting TLRs or specific cytokines for the treatment or prevention of pregnancy and cancer complications (124, 125).

Moreover, the placenta serves as a crucial interface for the exchange of nutrients and gases between the mother and the fetus, while also playing a key role in modulating the fetal immune system. It secretes various hormones, growth factors, and cytokines that regulate the mother's immune system and promote fetal tolerance. The unique cellular composition and structure of the placenta, including trophoblast cells, are essential for its immunomodulatory functions (126).

Furthermore, it is important to recognize that inflammatory pathways during pregnancy can be significantly influenced by

various environmental and lifestyle factors. These factors can ultimately affect the health of both the mother and the fetus. For instance, environmental pollutants such as particulate matter and endocrine-disrupting chemicals have the potential to trigger systemic inflammation and disrupt placental function, leading to adverse pregnancy outcomes (127). Similarly, lifestyle choices such as nutrition, exercise, and stress management can also play a crucial role in modifying a mother's inflammatory responses and influencing the progression of pregnancy. Research has shown that adhering to a well-balanced diet rich in anti-inflammatory nutrients and engaging in regular, moderate exercise can lower the risk of pregnancy complications (128). It is evident that both environmental and lifestyle factors can significantly impact inflammatory pathways during pregnancy, highlighting the importance of maintaining a healthy lifestyle and minimizing exposure to harmful pollutants for the well-being of both the mother and the developing fetus.

11 Inflammatory pathways and their genetic regulation in relation to GDM

Inflammation plays a crucial role in the immune system's defense against infections and tissue damage. However, immune responses can also lead to septic shock, hypersensitivity reactions (such as atopy, anaphylaxis, delayed-type hypersensitivity, and contact hypersensitivity), and the rejection of tissue or organ transplants. Chronic inflammation can occur when immune responses become abnormal or uncontrolled (129). The interplay between inflammatory responses and genetic factors significantly influences the development and progression of gestational diabetes mellitus (GDM).

Research studies have indicated that changes in genes associated with inflammatory pathways may influence an individual's susceptibility to GDM (130, 131). This research sheds light on a potential cause and target for GDM by establishing a correlation between inflammation and the development of GDM (132). For instance, alterations in inflammatory responses have been found to be associated with variations in genes involved in the production and regulation of cytokines, such as TNF-alpha and IL-6, in pregnant women with GDM (130, 131, 133). Furthermore, it has been observed that the body undergoes a gradual onset of low-grade systemic inflammation following conception (134).

Multiple studies have consistently shown that the development of insulin resistance (IR) is triggered by inflammatory factors (88, 131). The placental tissue possesses robust endocrine functions, capable of producing and releasing a wide array of inflammatory cytokines that exacerbate maternal IR and the chronic inflammatory response. Among these inflammatory factors are TNF-α, IL-6, IL-8, NK-κB, and IL-1β, which are secreted by the placental tissue. IL-1β, as a pro-inflammatory cytokine, induces apoptosis in islet β cells and activates the NK-κB pathway, leading to the release of IL-6, IL-8, and other inflammatory factors (133, 135). IL-6 and IL-8, in turn, stimulate various lymphoid and inflammatory cells, further intensifying the inflammatory response. Additionally, TNF-α is recognized as an independent risk factor for GDM due to its ability to impede insulin signal transmission and glucose transport (133, 135, 136).

The genetic regulation of these pathways may influence the dysregulation of glucose metabolism observed in GDM patients. However, it is crucial to acknowledge that the relationship among inflammatory pathways, genetics, and GDM encompasses multiple dimensions. Apart from genetic factors, lifestyle choices, and other non-genetic determinants play a pivotal role in determining the overall risk and progression of GDM. By delving into the genetic regulation of inflammatory pathways within the context of this disease, a more intricate understanding of GDM emerges.

12 Adipokines and inflammation in pregnancies with gestational diabetes mellitus

In pregnancies affected by gestational diabetes mellitus (GDM), inflammation and adipokines play crucial roles in the pathophysiology of the illness. Recent research has shed light on the intricate pathways through which these factors impact the development of GDM. One of the primary contributors to the development of GDM is inflammation, which not only leads to insulin resistance but also hampers glucose metabolism (137). Women diagnosed with GDM often exhibit elevated levels of pro-inflammatory cytokines such as C-reactive protein (CRP), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) (138). These inflammatory mediators disrupt insulin signaling pathways, resulting in insulin resistance and hyperglycemia. Moreover, inflammation exacerbates oxidative stress and endothelial dysfunction, posing additional risks to the health of both the mother and the fetus (139).

Adipose tissue secretes bioactive chemicals known as adipokines, which play a crucial role in regulating inflammation and metabolic balance in GDM. The anti-inflammatory adipokine adiponectin, for instance, enhances glucose absorption and fatty acid oxidation, thereby offering protection against GDM and exhibiting insulin-sensitizing properties (140). Conversely, high levels of pro-inflammatory adipokines like resistin and leptin in women with GDM are associated with insulin resistance and adverse pregnancy outcomes (141). These fat-soluble hormones influence immune responses, trigger inflammatory processes, and contribute to the disruption of glucose metabolism during pregnancy. Numerous research studies have delved into the intricate relationship between adipokines, inflammation, and the pathophysiology of GDM (142, 143). A study conducted by Lain et al. revealed a significant correlation between the onset of GDM and elevated maternal levels of TNF-α and IL-6 (144). Furthermore, research by Catalano et al. showcased the disruption of adipokine profiles, indicating that individuals with GDM exhibited heightened levels of leptin and diminished levels of adiponectin compared to healthy pregnant women (145).

These findings emphasize the importance of understanding the role of adipokines and inflammation in GDM to identify potential

treatment targets and improve pregnancy outcomes. Adipokines and inflammation play pivotal roles in the pathophysiology of pregnancies complicated by GDM. Elevated levels of pro-inflammatory cytokines and imbalanced adipokine profiles are associated with insulin resistance, impaired glucose metabolism, and adverse consequences for both the mother and the fetus. To develop innovative therapeutic strategies aimed at mitigating the inflammatory response and restoring metabolic equilibrium in women with GDM, further research is imperative to elucidate the underlying mechanisms.

13 Clinical implications and applications of GDM

The clinical implications of gestational diabetes mellitus (GDM) are significant and demand attention from medical professionals worldwide. It is crucial to develop effective screening techniques for early detection and treatment. GDM increases the risk of complications for maternal health, including pre-eclampsia and cesarean sections, emphasizing the need for close observation during pregnancy (146, 147). Furthermore, the long-term consequences for mothers, such as an elevated risk of type 2 diabetes, underscore the importance of postpartum care. GDM is also associated with macrosomia and neonatal hypoglycemia in the fetus, necessitating careful glycemic management to mitigate these risks.

Recent epidemiological studies have indicated that the offspring of women with GDM may have a heightened susceptibility to adverse cardiometabolic outcomes later in life (148). Notably, a comprehensive Danish population-based cohort study (n = 2,432,000) has revealed a significant association between maternal diabetes and an increased incidence of early-onset cardiovascular disease (CVD) in offspring, particularly those aged ≤40 years (149). To shed light on the underlying mechanisms (150), has proposed a potential link between insulin resistance and the activation of inflammatory pathways. Furthermore, *in vitro* research has demonstrated that elevated glucose concentrations impede trophoblast invasiveness by inhibiting uterine plasminogen activator activity (151).

In light of the significant impact of type 2 diabetes and cardiovascular disease on the worldwide prevalence of intergenerational cardiometabolic disease, it is of utmost importance to acknowledge GDM as an early risk factor for these conditions. Moreover, it is crucial to expand the current clinical approach to encompass long-term complications for both mothers and their offspring following a diagnosis of GDM (152). The management of GDM can be further enhanced by integrating technological advancements, such as telehealth services and continuous glucose monitoring, into clinical practice. These innovations enable real-time sugar level monitoring, facilitating prompt treatment plan adjustments. Given the global epidemic of diabetes, obesity, and cardiovascular disease, the conventional focus on achieving normal obstetric and neonatal outcomes through short-term antenatal maternal glucose management should now shift towards early postnatal prevention strategies. This shift aims to reduce the progression from GDM to type 2 diabetes and address the long-term metabolic risks faced by both mothers and their offspring (152). Figure 3 depicts the diagram illustrating the impact of GDM on both mothers and their offspring.

14 Implications of gestational diabetes mellitus on the influence of cardiovascular disorders

Pregnancy-related Gestational Diabetes Mellitus (GDM) poses a significant health risk, impacting both the short- and long-term health of both mothers and their children. According to the American Heart Association, GDM increases the likelihood of developing cardiovascular disease (CVD) in women (153–155). Research consistently links GDM to future hypertension, dyslipidemia (156, 157), vascular dysfunction, and atherosclerosis (158–160), and other cardiometabolic risk markers (161).

Recent studies, such as by (154, 162), have shed light on the potential impact of GDM on cardiovascular disorders post-childbirth. Research suggests that women with a history of GDM may have an increased susceptibility to CVD, indicating a possible lifelong continuum of metabolic abnormalities (160, 163). For instance, a study conducted by (164) on a large cohort of US women revealed a strong association between a history of GDM and a heightened long-term risk of cardiovascular disease. Additionally (165), found that mothers with GDM had higher rates of pediatric obesity and maternal glucose metabolism abnormalities, underscoring the intricate relationship between pregnancy-related metabolic disruptions and cardiovascular health outcomes. These findings highlight the importance of comprehensive screening, monitoring, and care plans for women affected by GDM to mitigate their long-term risk of cardiovascular disease.

GDM influences CVD through a mechanism of action that may stem from a direct correlation between experiencing a GD pregnancy and cardiometabolic function. Previous prospective and cross-sectional studies have shown that women with a history of GD are more likely to develop atherosclerosis, experience decreased vascular function, and have a higher likelihood of developing dyslipidemia and hypertension compared to women without gestational diabetes (160, 161, 166, 167). Variations in these intermediate phenotypes are observed before the onset of type 2 diabetes, and in some studies, they become apparent shortly after the index pregnancy. It is also suggested that the cardiovascular effects of GD may persist even if type 2 diabetes does not develop, which is a known risk factor for CVD (168).

While few studies have thoroughly examined the prospective link between GDM and eventual CVD while controlling for common risk factors and lifestyle features, a study by (164) found

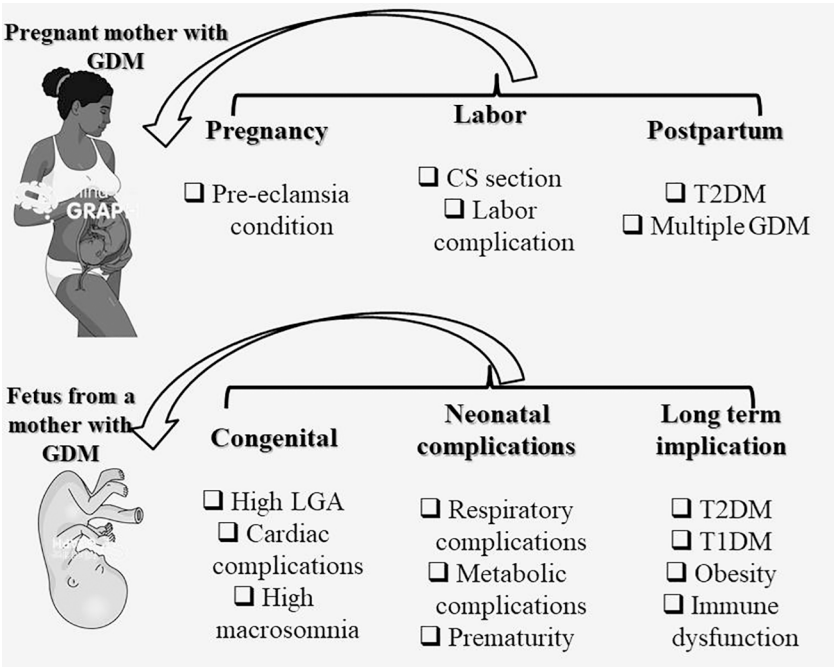

FIGURE 3
Impact of GDM on the mother and offspring. CS, C-Section (Cesarean delivery); GDM, gestational diabetes mellitus; T2DM, Type 2 diabetes mellitus; T1DM, Type 1 diabetes mellitus; LGA, Large for gestational age.

that women with healthier behavior profiles did not have a higher risk of CVD when they had GDM. This association could potentially be explained by controlling for updated lifestyle risk factors for CVD, such as diet, physical activity, smoking status, and weight management. To further investigate whether GDM induces adverse cardiovascular changes, acts as a marker for underlying high risk, or a combination of both, prospective studies with carefully phenotyped CVD markers before and after pregnancy are needed. These studies will help clarify the relationship between GDM and CVD and potentially aid in lowering the risk of developing cardiovascular disease in women with a history of gestational diabetes.

15 Conclusion and recommendations

This narrative review explores the involvement of several genes, including SLC30A8, CDKAL1, TCF7L2, IRS1, and GCK, in GDM, along with the adverse effects of stress on insulin function. These factors collectively contribute to the onset of diabetes and the subsequent development of GDM, impacting both the mother and offspring negatively throughout their lives. These genetic variants notably influence beta-cell function and regulate insulin activity in various tissues, disrupting glucose regulation during pregnancy. It is suggested that these variants may disrupt zinc transport, leading to impaired insulin synthesis and secretion, thus contributing to GDM development. Additionally, inflammatory pathways like TNF-alpha and IL-6 can influence susceptibility to GDM by affecting cytokine production and regulation genes. Inflammatory factors such as TNF-α trigger insulin resistance (IR), further complicating glucose metabolism in GDM patients. Furthermore, women with a history of GDM may have an increased susceptibility to CVD, indicating a possible lifelong continuum of metabolic abnormalities. The complex relationship between inflammatory pathways, genetics, CVD and GDM necessitates further research to develop targeted gene therapy and therapeutic drugs aimed at addressing genetic variations in SLC30A8, CDKAL1, TCF7L2, IRS1, GCK, and related genes.

Author contributions

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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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Autoimmune CD8+ T cells in type 1 diabetes: from single-cell RNA sequencing to T-cell receptor redirection

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Type 1 diabetes (T1D) is an organ-specific autoimmune disease caused by pancreatic β cell destruction and mediated primarily by autoreactive CD8+ T cells. It has been shown that only a small number of stem cell-like β cell-specific CD8+ T cells are needed to convert normal mice into T1D mice; thus, it is likely that T1D can be cured or significantly improved by modulating or altering self-reactive CD8+ T cells. However, stem cell-type, effector and exhausted CD8+ T cells play intricate and important roles in T1D. The highly diverse T-cell receptors (TCRs) also make precise and stable targeted therapy more difficult. Therefore, this review will investigate the mechanisms of autoimmune CD8+ T cells and TCRs in T1D, as well as the related single-cell RNA sequencing (ScRNA-Seq), CRISPR/Cas9, chimeric antigen receptor T-cell (CAR-T) and T-cell receptor-gene engineered T cells (TCR-T), for a detailed and clear overview. This review highlights that targeting CD8+ T cells and their TCRs may be a potential strategy for predicting or treating T1D.

KEYWORDS

type 1 diabetes, CD8+ T cells, single-cell RNA sequencing (scRNA-seq), CRISPR/Cas9, chimeric antigen receptor T-cell (CAR-T)

1 Introduction

Type 1 diabetes (T1D) is an autoimmune disease in which T lymphocyte-mediated pancreatic β cell failure occurs and patients are dependent on exogenous insulin therapy for life (1). T1D affects millions of people worldwide and is continuing to increase by 3-4% per year (2). Its multiple acute complications, long-term complications, other autoimmune diseases and psychosocial problems have an enormous impact on the survival of patients (3). Currently, T1D is only treated with insulin therapy, pancreas transplantation or islet transplantation, which are the more limited treatments. Islet transplantation and pancreas transplantation are associated with high surgical risks, inadequate organ sources, high

financial stress, limited survival of islet cells after transplantation and insufficient ability to correct blood glucose (4). Insulin therapy has none of these risks but requires multiple daily injections and difficult glycemic control, eventually leading to complications and premature death (5, 6).

β cell-specific CD8+ T cells are considered a new direction for long-acting immunotherapy against precise targets, which is likely to be a key breakthrough point for T1D treatment (6). There are many current immunotherapeutic ideas and targets for T1D, such as anti-CD3 antibodies (7), immune checkpoint inhibitors (8), anti-thymocyte globulin antibodies (9) and other drugs, but they have been plagued by off-target effects with long-term failure. With the advancement of research, β cell-specific CD8+ T cells in pancreatic draining lymph nodes (pLN) have been valued (10, 11). A population of β cell-specific CD8+ T cells with stem cell characteristics exists in the pLN and is capable of long-term survival and differentiation of destemmed β cell-specific CD8+ T cells that are continuously delivered into the pancreas to produce sustained autoimmune killing. Transferring hundreds of thousands of intrapancreatic T cells from T1D mice to new mice fails to cause T1D, whereas only 20 stem cell-like CD8+ T cells at the pLN are needed to cause T1D in healthy mice (12). Therefore, the use of β cell-specific CD8+ T cells may allow for precise and sustained treatment of T1D.

T-cell recognition of antigens depends mainly on the T-cell receptor (TCR) on its surface, and different clones of T cells have different TCR sequences, which constitute a highly diverse TCR pool in the body in response to external antigens, and the TCR determines the specificity of CD8+ T-cell recognition for killing (13, 14). Therefore, many studies have attempted to use single-cell RNA sequencing (ScRNA-Seq) technology for high-throughput screening of pathogenic TCR target platforms, CRISPR/Cas9 gene editing technology has been applied to T-cell transformation, and chimeric antigen receptor T-cell (CAR-T), T-cell receptor-gene engineered T cells (TCR-T), and other technologies have been focused on in the treatment of T1D.

At present, there are many still unclear aspects of β cell-specific CD8+ T cells in T1D, such as the heterogeneity of CD8+ T cells (15) and the process of T-cell differentiation (16) still remain to be explored. These unclear issues affect the depth of related research; therefore, this paper will provide a detailed and clear review of the mechanism of autoimmune CD8+ T cells and TCR in T1D, as well as the important applications of scRNA-seq, CRISPR/Cas9, CAR-T and TCR-T.

2 Adaptive Immunity in T1D

2.1 The pathogenesis of T1D

The pathogenesis of T1D is complex, involving several different immune cell subsets, pathways, and complex islet autoimmune responses (6). CD4+ and CD8+ T cells are involved in the development of T1D, and autoimmune T cells directly recognize β cell autoantigens on antigen-presenting cells and rapidly transform into effector T cells, while CD8+ T cells are the main killers of islet β cells (Figure 1) (17).

However, the exact mechanism behind the failure to turn on immune tolerance to β cell autoantigens in the common T1D pathogenic mechanism is a mystery (18). The pathogenesis of human islet disease remains unclear, but pancreatic remodeling has been shown to be associated with it in NOD model mice, which directly promotes β cell apoptosis and antigen release. The released antigen is phagocytosed by macrophages and DCs. These APCs are then transported to draining popliteal lymph nodes (pLNs) to promote β cell-specific T-cell generation and promote effector T-cell differentiation. After that, the effector T cells mature and travel to the islets to promote inflammation, which leads to islet inflammation (19–22). It is worth mentioning that many studies have shown that thymus selection disorder is a specific indication of islets in NOD mice (22, 23).

In normal people, insulin production is responsible for pancreatic beta cells, and insulin plays an important physiological role in the body. Insulin activates downstream signaling pathways by binding to insulin receptors on the cell surface, thereby regulating glucose metabolism and utilization. Compared with nondiabetic individuals, pancreatic beta cells in genetically susceptible individuals with T1D are selectively destroyed and cannot effectively produce enough insulin. This means that the insulin receptors are unable to bind to enough insulin, causing downstream signaling pathways to not open properly. As a result, glucose cannot be effectively absorbed and used by cells, leading to hyperglycemia (24). Healthy human pancreases contain self-reactive CD8+ T cells, which increase in number during the development of diabetes. Among autoreactive CD8+ T cells, preproinsulin (PPI)-reactive CD8+ T cells were more common in T1D patients than in healthy donors (25). PPI-reactive CD8+ T cells had a similar frequency in the exocrine pancreas regardless of the disease state. Optimal staining with PPI tetramer isolates functional T cells. However, during disease progression, PPI-reactive CD8+ T cells are attracted to islets. Under physiological conditions or stress, cytokine secretion may contribute to immune cell recruitment and β cell killing (26). β cells are destroyed by various factors and release autoantigens that are presented by antigen-presenting cells. CD4+ T, CD8+ T and NK cells are then activated. In addition, NK cells are involved in the direct killing of β cells through the interaction of NK cell markers such as NKp46 and KIRs. In addition, CD8+ T cells promote the development of T1D by secreting proteins such as Fas and cytokines such as TNF-α and IFN-γ (27). Cytokines secreted by natural killer (NK) cells and immune cells directly damage beta cells while also inducing self-defense mechanisms. Cytokines include interleukin-1β (IL-1β), IL-12, interferon-γ (IFN-γ), and tumor necrosis factor (TNF). Immune cells include macrophages, DCs, CD4+ T cells, and CD8+ T cells. In nonobese diabetic (NOD) mice, NKG2D expressed by NK cells binds to RAE1 expressed by β cells and is associated with β cell damage. In older transgenic mice that are not controlled, the expression of RAE1 is sufficient to induce the recruitment of adaptively transferred cytotoxic T lymphocytes (CTLs) to islets, resulting in the recruitment of a large number of endogenous lymphocytes. Eventually, it leads to pancreatitis. This is dependent on CTL expression of NKG2D, independent of antigen

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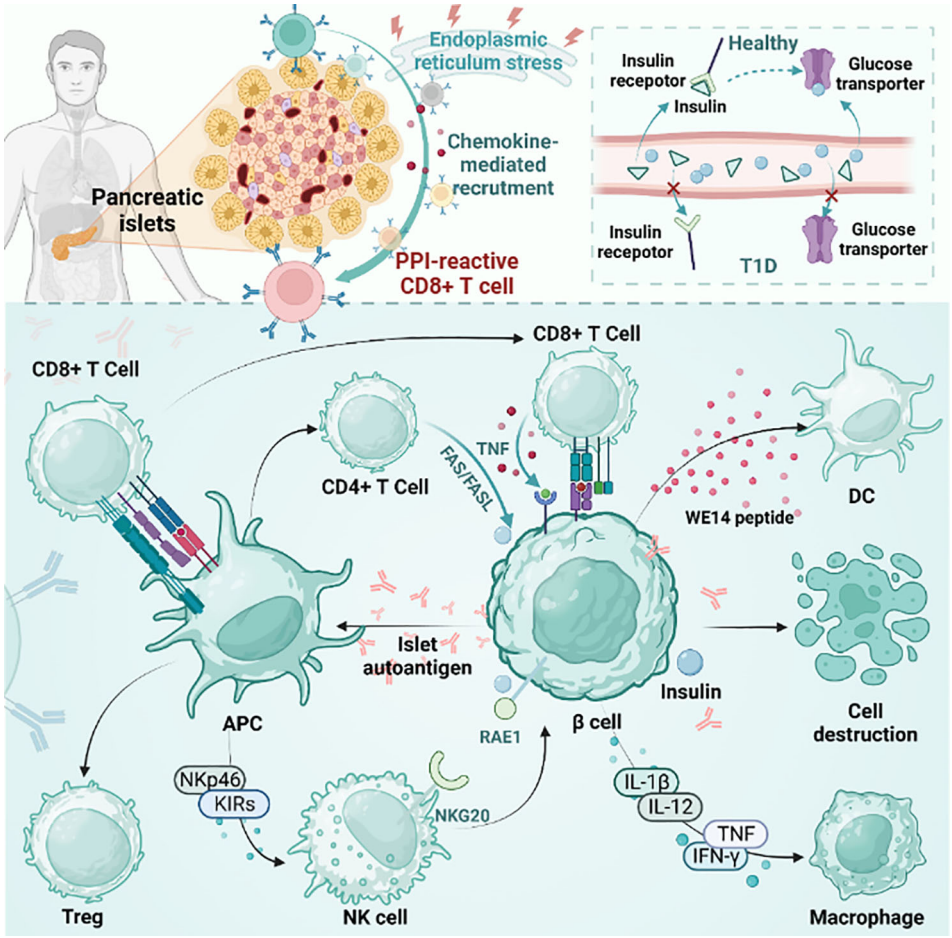

FIGURE 1
The pathogenesis of T1D. The figure plots the differences between T1D and healthy individuals and the mainstream accepted pathogenesis of T1D. pLN, pancreatic draining lymph node; APC, antigen presenting cells; PD-1, programmed death-1; CTLA-4, cytotoxic T lymphocyte-associated antigen-4; TCF1, transcription factor 1; Treg, regulatory T cell; NK cell, natural killer cell; DC, dendritic cell; PPI, preproinsulin.

recognition (28). Islet β cell antigens are also presented by MHCII molecules expressed by antigen presenting cells. T cells expressing FasL can mediate apoptosis by interacting with FAS expressed on β cells (29).

2.2 Current immunotherapy strategies

The only current treatment for T1D is insulin replacement. Currently, combined immunotherapy has become a new trend in the treatment of T1D. Immunotherapy, such as the use of monoclonal antibodies, is a strategy to target specific populations of immune cells that induce autoimmunity to drive pathology. For example, monoclonal antibodies (mAbs) have been shown to have no side effects or positive effects in regulating T1D (30). Previous studies have found that anti-CD3 monoclonal antibodies reduce the loss of insulin production and the need for exogenous insulin to maintain glycemic control during the first 2 years of T1D (31). There are two main approaches to the main immune treatment of T1D. The first is to block the pathogenic response by antagonizing B cell, cytokine and T-cell activation. The other promotes immune regulation by restoring or promoting regulatory T-cell function, promoting depletion of T-cell generation and β cell regeneration (6). Clinical data suggest that this combination therapy is more suitable for the treatment of this chronic disease than other therapies (32). Today, secondary prevention, which uses immunotherapy to successfully delay preclinical disease progression, and primary prevention, which suppresses autoimmune initiation, have entered large-scale clinical trials (Table 1). By shifting the focus of T1D treatment from late diagnosis and insulin replacement to early diagnosis and β cell preservation, children with T1D could dispense daily insulin injections in the future (6, 33). Strategies based on immunotherapy targeting the blocking of T-cell responses to β cell autoantigens have recently been incorporated into a group of existing T1D therapies, which are beneficial for blocking the onset and development of T1D. In recent years, new breakthroughs have been made in preserving the activity of islet beta cells. Chen et al. achieved the goal of generating pancreatic β-like cells from gallbladder stem cells (GSCS) without genetic modification by screening the combination of small molecules that produce insulin-secreting cells from gallbladder stem cells (GSCS) (34, 35). The small molecule combinations they found were Noggin, FR180204 and cyclopamine, and the addition of this

TABLE 1 Prevention of T1D - non-antigen-specific immune interventions.

Medicine	Patient age group (years)	Target point	Phase	References
ATG	12 to 45 (Children, adults)	T cells	Stage 2	NCT02215200
Abatacept (CTLA4-Ig)	6 to 45 (Children, adults)	T-cell activation: CD80, CD86	Stage 2	NCT00505375
Alefacept	12 to 35 (Children, adults)	T cells (CD2)	Stage 2	NCT00965458
Anti–IL-21 (NNC0114-0006) (+ liraglutide)	18 to 45 (Adults)	IL-21 (T cells, B cells, natural killer cells)	Stage 2	NCT02443155
Rituximab	8 to 40 (Children, adults)	B cells (CD20)	Stage 2	NCT00279305
Golimumab	6 to 21 (Children, adults)	TNF	Stage 1	NCT03298542
Teplizumab	0 to 7 (Children)	T cells (CD3)	Stage 4	NCT05757713

combination could effectively induce the differentiation of gallbladder stem cells into insulin-secreting cells. In addition, targeting MHC class II molecular proteins is a possible way to alleviate T1D, for example, by activating cathepsin G (CatG) to degrade MHC class I molecules to attenuate CD4+ T-cell activation in NOD mice and improve islet function (36–38).

Prevention of T1D by restoring or inducing immune tolerance to β cells is the main measure against T1D in the future (18). The use of antigen-specific immunotherapy can restore a self-tolerant immune system in which T-effector cells are suppressed and/or T-regulatory cells are induced (39). It is well known that some defects in innate and adaptive responses of the immune system lead to an imbalance in the regulation of autoimmune responses. Tregs play an important role in regulating disease progression because they can inhibit any inappropriate autoimmune response. Several interventions have been developed based on this finding (40, 41). Amatya et al. demonstrated the possibility of anti-T1D therapy by constructing a cell-permeable PDX1-FOXP3-TAT fusion protein (FP) to stabilize Tregs for the purpose of anti-autoimmune and insulin production (42). The protein was tested both *in vitro* and *in vivo* (nonobese diabetic mouse T1D model). *In vitro*, FP transforms naive CD4 T cells into a functional "Treg-like" subpopulation that inhibits cytokine secretion and downregulates antigen-specific responses. In liver stem cell-like cells, increased endocrine transdifferentiation and increased expression of insulin 2 and other β lineage-specific genes were observed. *In vivo*, the following results were observed when the protein was administered to T1D model mice: significant increases in insulin and C-peptide levels, the formation of insulin-containing cell clusters in the liver, and systemic anti-inflammatory transformations. In addition, targeting islet-specific Tregs is more effective than targeting polyclonal Tregs in the prevention of T1D. In fact, the frequency of innate antigen-specific Tregs is extremely low, and expansion *in vitro* predisposes Tregs to impaired stability, leading to an effector phenotype. Yang et al. used homology-directed repair to increase the expression of FOXP3 in Tregs and performed lentiviral vector-based human T-cell receptor gene transfer to improve the specificity of Tregs for β-islet cells in T1D. It promotes the generation of islet-specific Tregs and inhibits effector T-cell proliferation and cytokine production, thereby blocking diabetes triggered by islet-specific T_{eff} or diabetogenic polyclonal T_{eff} (43). Adjuvant immunotherapy upregulates regrowth (Reg) genes in islets and induces Th17 cells that produce interleukin 22 (IL-22). Previous studies have revealed that IL-22 upregulates $_{reg}$ gene expression in pancreatic islets and may induce β cell regeneration and prevent apoptosis (44–46). Furthermore, animal experiments have shown that IL22 expression helps to reduce the severity of streptozotocin diabetes in mice fed a grain-fed diet (47). IL-2 can be used to treat T1D because it promotes immunity or tolerance depending on its availability. Blockade of the T_{eff} cytokine IL-2 can be used to prevent and treat T1D in NOD mice (48). Low doses of IL-2 (LD IL-2) are known to drive tolerance by preferentially acting on Tregs, thereby providing immune modulation with few side effects. Tregs are an IL-2-reactive cell type known to control autoimmunity. Treatment with LD IL-2 in patients with immune-mediated diseases increases T $_{reg}$ numbers and controls autoimmunity. However, it has also been found that patients have defective or defective IL-2 production or signaling and that IL-2 treatment causes mild activation of NK cells and eosinophils (49). According to research, regulatory T cells (Tregs) prevent the targeting of autoantigens by T_{eff} cells. Therefore, it is possible to enhance the frequency and function of antigen-specific Tregs by adding low doses of IL-2 to the antigen therapy regimen (50). Cytokines such as IL-6 at physiological levels can maintain glucose homeostasis in islet cells, and in addition, other cytokines such as IFN-γ and CXCL10 may play a pathogenic role by promoting immune cell recruitment and β-cell killing (51). Islet cells express a wide range of cytokine receptors, such as IL-4R (52, 53), IL-13R and IL-6R. Therefore, cytokines can both induce and regulate T1D and have the potential to regenerate and preserve insulin-producing β cells in islets (26, 46).

In addition, important breakthroughs have also been made in targeted therapy for T1D. Recent studies have shown that decreased GLUT4 expression/translocation is associated with impaired glycemic control in diabetes, specifically affecting insulin-induced glucose uptake in muscle and adipose tissues (54). It has also been

reported that gene therapy was used to reestablish central tolerance in NOD mice by reestablishing hematopoietic stem cells (HSCs) with retroviral transduction to express the MHC class II β chain in a protective form (55). In addition to the methods mentioned above, organ transplantation is the last option. Islet and pancreas transplantation in humans could alleviate the dilemma faced by patients with diabetes, but its use has thus far been limited by organ scarcity and lifelong administration of immunosuppressive drugs. However, recent studies have proposed the latest method to efficiently generate pancreatic progenitor cells (PPs) and β-like cells from human pluripotent stem cells (hPSCs) *in vitro*, which is expected to improve the cure rate of diabetes (56). On the whole, new T-cell techniques hold promise for defining the process of autoimmune T-cell differentiation and characterizing autoimmune responses in comparison to physiological immune responses (18).

2.3 Potential breakthrough point for targeted therapies——CD8+ T cells

The pathogenesis of T1D involves immune regulation and immune response, in which cellular immunity plays a key role. Cellular immunity is a major killer of islet β cell destruction. Pancreatic β cell damage is influenced by genetics, environment, and immunity, and genetic and environmental factors increase T1D risk by partially altering central and peripheral tolerance-inducing events. In addition, 50% - 60% of the genetic risk of T1D comes from HLA alleles that encode molecules involved in the presentation of antigenic peptides to T cells. Regrettably, these effects on antigen presentation greatly affect thymus selection processes and peripheral activation of immune responses (57, 58). For example, mutations in the STAT3 gene can cause the body to stop tolerating CD8+ T cells, causing disease (59). It was thus found that diabetes-induced CD8+ T-cell responses are restricted in the presence of normal STAT3 activity and drive diabetic pathogenesis. At the same time, overexpression of class I HLA in islet cells in T1D is closely related to increased expression of STAT1, resulting in selective susceptibility to the occurrence of autoimmune diseases in the population (60). Apart from HLA-I, HLA-II also contributes to the development of T1D. HLA-II is also expressed in pancreatic β cells of patients with T1D, and HLA-II-expressing β cells may be the direct target of autoimmune CD4+ T cells (61, 62). Therefore, HLA-II also plays a role in the pathogenesis of T1D (62). Individual HLA differences also affect peptide binding and signal transduction after TCR conjugation. This drives the development and expansion of β cell-specific effector T cells for islet inflammation (22, 27).

Despite differences between people, CD8+ T cells have been shown to be the dominant immune cell type in islet lesions, followed by CD68+ macrophages, CD4+ T cells, and CD20+ B cells (63). It has been shown that the critical subgroup of dendritic cells responsible for the cross-presentation of islet antigens with CD8+ T cells and the direct presentation of β cell antigens to CD4+ T cells, termed merocytic dendritic cells (mcDC), are more numerous in nonobese diabetic (NOD), are typical of critical antigen presenting cells and are responsible for disrupting peripheral tolerance to β cell antigens *in vivo* (64).

In general, the islet β cell autoimmune response has progressed for several years prior to the clinical diagnosis of T1D (65). Given that autoimmune T cells are present in both patients with autoimmune diseases and healthy individuals, whether an individual develops autoimmunity depends on the balance between these potentially pathogenic self-reactive immune cell types and the regulatory mechanisms that control them (40). The best therapy for T1D prevention and treatment strategies of islet replacement or regeneration is to tolerate or suppress the autoimmune T-cell response while keeping the immune system functioning well in response to foreign antigen invasion and avoiding the use of global immunosuppression. In addition, it is now widely accepted that autoantigen-driven T-cell clonal amplification is the hallmark of T1D pathophysiology. Examination of TCR clones amplified from T1D donor pancreases produced mixed but generally positive results. This will also become a breakthrough point for future targeted therapy (66). The TCR clones expanded from the T1D donor pancreas produced mixed but overall positive results, implying that in the pancreas of T1D patients, there are multiple T-cell clones that react with autoantigens of the same epitope. These TCR clonal sequences can be used therapeutically because they can be used to develop immunotherapies targeting specific antigens to reduce or eliminate autoimmune responses. Specifically, these TCR clonal sequences can be used to develop antigen-specific T-cell immunotherapies, such as CAR-T-cell therapies.

3 Autoimmune CD8+ T cells in T1D

Antigen-specific CD8+ T cells include effector memory T cells, central memory T cells, peripheral memory T cells, tissue-resident memory T cells, and stem cell memory T cells. T_{eff}, T_{cm}, and T_{scm} cells are peripheral memory T cells, while tissue-resident memory T cells are mainly T_{rm} cells. In T1D, the proportion of peripheral memory T cells and tissue-resident memory T cells changes with time and Ag-encounter. Specifically, Trm cells are mainly found in tissues such as the pancreas and intestine, while T_{cm} and T_{scm} cells are mainly found in lymph nodes and peripheral blood. In the early stages of T1D, the proportion of peripheral memory T cells is higher, and the proportion of tissue-resident memory T cells is lower. As the disease progresses, the proportion of tissue-resident memory T cells gradually increases, probably because they are better able to localize and function in the pancreas. Therefore, the changes in the proportions of T_{eff}, T_{cm} and T_{scm} cells, as well as T_{rm} cells, in T1D are dynamic and may be influenced by a variety of factors (67). The environment of early naive T cells can change the activation state of CD8+ T cells, leading to memory T cells, exhausted T cells, senescent T cells, and so on. In particular, CD8+ T cells regulate Fas-FasL-mediated killing of islet β cells, mainly through the homologous interaction between perforin and granzyme B. (48). The FasL-Fas interaction plays an important role in regulating CD8 expression. The expression of Fas and FasL is regulated by cell activation signals, and the expression levels of Fas and FasL in CD8+ T cells gradually increase after cell activation. Specifically, Fas transcription began at 24 h after cell activation and

reached an approximately 8-fold increase at 72 h. In contrast, FasL transcription appears earlier after cell activation and reaches higher levels in cells stimulated by anti-CD3 and anti-CD28. At the protein level, the expression of Fas and FasL also reflected its regulation at the mRNA level. Fas was mainly induced by BM-DCs, and its level gradually increased during the observation period. In contrast, FasL was similarly induced by the combined stimulation of BM-DCs and anti-CD3 and anti-CD28 antibodies. These results suggest that Fas and FasL expression in CD8+ T cells is regulated, at least in part, at the transcriptional level and influenced by signals present during cell activation (68).

Antigen-specific T lymphocytes kill insulin-producing β cells in T1D (T1D) by disrupting central and peripheral tolerance (64). Among these killers, CD8+ T cells are crucial for clearing many bacterial or viral illnesses because they kill infected cells (69). CD8+ T cells have a variety of functional and developmental states, including effector cells, regulatory cells, and hypofunctional states. In T1D, circulating islet antigen-specific CD8+ T cells exhibit a wide range of phenotypic heterogeneity, including an early memory phenotype, a stem cell memory phenotype, a transitional memory phenotype, a final effector memory phenotype, and a hypofunctional state (70). One of them is CD8 memory T cells. An initial expanded vaccination regimen can result in multiple contacts with Ag over the host's lifetime due to recurrent exposure to the same pathogen, which increases the amount of CD8 memory T cells (67, 71). Effector CD8+ cells are another state. The recognition of cytotoxic T cells by islet autoantigens provided by class I HLA molecules may be a crucial effector mechanism that results in the killing of β cells in effector CD8+ cells, according to pertinent research (41). CD8+ T cells with declining function have come into the public eye in recent years. In studies, T-cell populations characterized by inhibitory receptors and inhibitory receptor-mediated depletion were closely associated with improved T1D markers (72). Inhibitory receptors are a class of proteins that play an important role in regulating the immune response. Inhibitory receptor-mediated depletion refers to the gradual loss of effector function of T cells and the continuous upregulation of a variety of inhibitory receptors in the context of chronic infection, cancer immunotherapy, and autoimmunity. This depletion state makes T cells unable to kill target cells effectively, which leads to disease progression. In the treatment of T1D, the role of inhibitory receptors is to inhibit the autoimmune response by inducing the exhaustion state of T cells, reduce the attack on islet β cells, and protect islet function. An example is the PD-1 (programmed death 1) inhibitory receptor. PD-1 is a receptor expressed on the surface of activated T cells. When it binds to its ligand PD-L1 (programmed death ligand 1), it can inhibit the activation and function of T cells. In T1D, the activation of PD-1 inhibitory receptors can reduce the autoimmune response, reduce the attack on islet β cells, and thereby protect islet function. Therefore, where are the reduced T cells going to come from? Kong et al. showed that autoimmune tissue could maintain an undifferentiated central memory-like autoimmune T-cell pool, which has the potential for pathogenic effects and may be an important source of effector T cells during long-term chronic autoimmunity (73). It is worth mentioning that stem-like CD8+ T cells, effector CD8+ T cells and depleted CD8+ T cells can coexist in the pancreas of patients with TID (Figure 2). Stem-like CD8+ T cells, effector CD8+ T cells, and exhausted CD8+ T cells can coexist in the pancreas of TID patients. These different types of CD8+ T cells play different roles in the development and progression of TID. Stem cells, such as CD8+ T cells, can self-renew and differentiate into T cells but can also remain in a more primitive state. Effector CD8+ T cells can kill beta cells in the pancreas, resulting in reduced insulin secretion. Exhausted CD8+ T cells lose their ability to kill beta cells, possibly due to prolonged antigenic stimulation. The coexistence of these CD8+ T cells may be an important feature of the complex pathological process of TID (74).

3.1 Stem-like CD8+ T cells

Recent studies suggest that CD8+ stem-like T cells may play an important role in the pathogenesis of T1D. It has been found that effector T cells during long-term chronic autoimmunity may originate from an undifferentiated central memory-like autoimmune T-cell pool in autoimmune tissues, which contributes to the disease (73). T cells have the characteristics of stem cells and are an important cell type in the immune system. According to their stem cell properties, T-cell stemness often segregates T cells into short-lived and terminally differentiated effector cells and long-lived progenitors that can give rise to terminally differentiated T effectors. In addition, the transcription factors TCF1, Bach2, c-MYB, and Foxo1 were found to be critical for the induction of T-cell stem cell properties (75). Among them, the autoimmune T cells that express TCF1 in pLN are stem-like progenitor cells that can drive T1D by generating short-lived TCF1- T factor cells that destroy pancreatic β cells. Therefore, targeting stem cell-like progenitor cells may be a new breakthrough for the treatment of T1D (75, 76).

In T1D, beta cell-specific CD8+ T cells destroy insulin-producing beta cells. A group of β-specific CD8+ T cells in pLN with stem-like characteristics continue to differentiate and import β-specific CD8+ T cells into islets, leading to long-term β cell damage (76). Recent studies have shown that weak MHC-infiltrating CD8+ T cells from islets may, through presentation, lead to weak TCRs, thereby maintaining some stem-like phenotypes of islet-reactive CD8+ T cells (77). "Weak MHCI-infiltrating CD8+ T cells" refers to the ability of specific types of CD8+ T cells (cytotoxic T cells) to infiltrate cells that express lower levels of major histocompatibility complex class I (MHCI) molecules in an immune response. However, some CD8+ T cells also have the ability to infiltrate cells that express lower levels of MHC I molecules. These cells are called "weak MHCI-infiltrated CD8+ T cells". They can recognize and attack cells through other pathways that do not rely on antigen presentation by MHC I molecules. This allows them to mount an immune response against certain cells that express lower levels of MHC I molecules, such as certain tumor cells or infected cells. Abdelsamed et al. assessed the pluripotency index of DNA methylation in T cells and found that β cell-specific CD8+ T cells had a stem-like epigenetic pluripotency score. Abdelsamed et al. demonstrated that autoimmune CD8+ T cells isolated from lymphoid tissues retained a developmentally plastic phenotype and

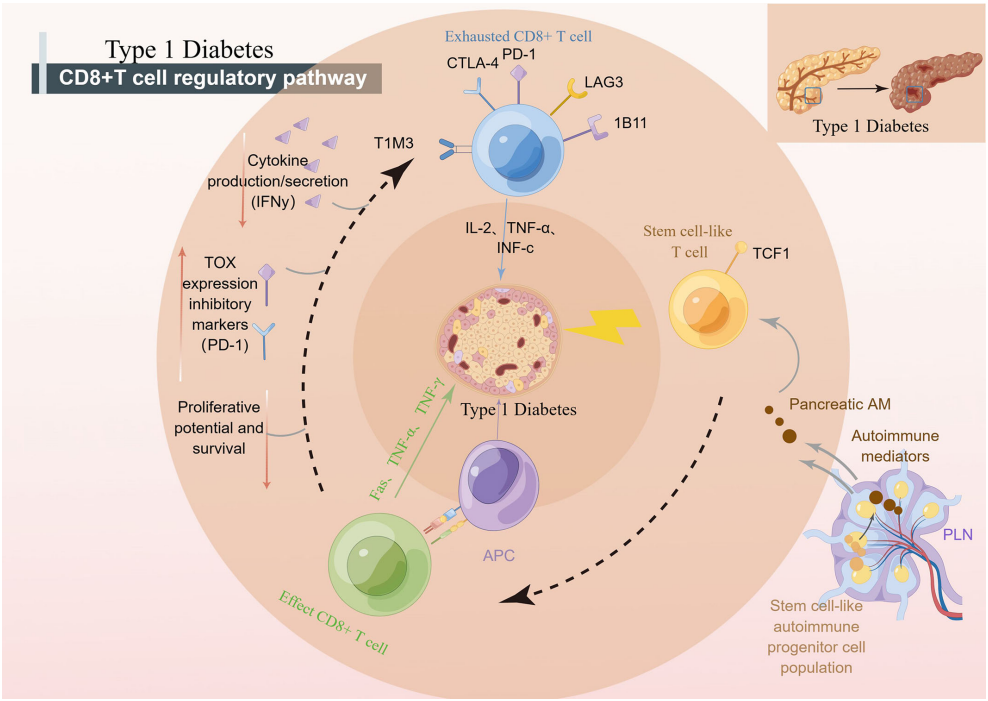

FIGURE 2
CD8+ T cells regulate the development of T1D. pLN, pancreatic draining lymph node; APC, antigen presenting cells; PD-1, programmed death-1; CTLA-4, cytotoxic T lymphocyte-associated antigen-4; TCF1, transcription factor 1; NOD, nonobese diabetic.

epigenetic signatures compared with the same cells isolated from the pancreas (13). In other words, a subset of cells in CD8+ T cells with stem cell characteristics is strongly associated with the progression of T1D. When efficacy is enhanced by epigenetic mechanisms, patient tolerance can be improved by inducing CD8 + T cells with stem cell characteristics to enter a tolerance state (13). T_{scm} cells have stem cell properties and are the earliest developmental stage of memory T cells. Stem cell T cells specific for β cell autoantigen protein, insulin, and islet cell-specific glucose-6-phosphatase-catalyzed subunit associated protein (IGRP) were found in patients with autoimmune CD8+ T cell. Autoimmune T_{scm} in T1D patients can selectively target autoantigens by inhibiting glucose metabolism (78). Researchers are now further investigating the specific mechanisms and functions of CD8+ stem-like T cells in T1D. Understanding the role of these cells may help shed light on the pathogenesis of T1D and provide new targets for the development of new therapeutic strategies. However, there are still many unknown aspects of the role of CD8+ stem-like T cells in T1D, and further studies are needed to deepen our understanding.

3.2 Effector CD8+ T cells

T_{eff} plays a major role in the pathogenesis of T1D, and T-cell recognition, activation, expansion, and function influence active immune processes (77). T_{eff} can be divided into several subsets based on their different characteristics (77, 79). Effector T cells can be divided into several different subsets as follows: CD4+ T cells are a class of T cells with CD4 surface markers that regulate and coordinate immune responses primarily by producing a variety of cytokines. CD4+ T cells can be further divided into Th1, Th2, Th17 and T_{reg} subgroups. CD8+ T cells: CD8+ T cells are a class of T cells with CD8 surface markers that are primarily responsible for directly killing infected cells and tumor cells. CD8+ T cells exert their effects by releasing cytotoxins and producing cytokines (77, 79). Natural killer T cells (NKT cells): NKT cells are a class of T cells with natural killer activity that can rapidly recognize and kill infected cells and produce a variety of cytokines to regulate the immune response. Gamma delta T cells: Gamma delta T cells are a class of T cells with a gamma delta T-cell receptor whose structure is different from that of the traditional alpha beta T-cell receptor. γδT cells can directly recognize and kill infected cells and play an important immunomodulatory role. These different effector T-cell subsets have different functions and roles in the immune response and cooperate with each other to exert important immune effects (80). In nonobese diabetic (NOD) mouse models, CD8+ T effector cells play a key role in islet β cell destruction and contribute to the maintenance of islet inflammation (24, 40). Patients with T1D exhibit impaired peripheral tolerance, including T_{reg} hypofunction and resistance of effector T_{eff} cells to regulation by Tregs; that is, effector T cells in patients with diabetes are not sensitive to regulation by CD4+ FOXP3+ regulatory T cells (80). T_{eff} targets a group of epitopes derived from islet proteins, including proinsulin, GAD, IA-2 and IAPP. Studies have shown that with the appearance of neoepitopes one after another, which are different from the conventionally modified epitopes, the generation of neoepitopes will lead to the activation of pathogenic immune cells, thereby starting a feedforward circuit that can amplify the antigen repertoire against pancreatic β cell proteins and lead to T1D (81, 82).

Interestingly, T_{eff} resistance is not associated with a specific subset or marker but rather with the activation status of T_{eff} and exposure to proinflammatory cytokines, particularly IL-6. During the development of human T1D, T_{eff} resistance appears to be STAT3 dependent but not directly associated with the capacity of T cells to produce or respond to IL-6. (32, 35, 83, 84). The resistance of effector T cells to the inhibition of regulatory T cells is one of the characteristics of the development of diabetes, and this resistance is related to the STAT3 signaling pathway but not to the ability of effector T cells to produce or respond to IL-6. Studies have shown that T1D is more likely to develop in children than adults due to a more amplified T-cell response to β cell autoantigens (85).

3.3 Exhausted CD8+ T cells

In recent years, exhausted CD8+ T cells have been a research hotspot in the fields of autoimmunity, such as T1D, tumor immunotherapy and chronic inflammation (72). CD8+ T-cell exhaustion refers to the transition from precursor cells to terminally exhausted cells, which is a process of continuous change (86). On the basis of studies in cancer and chronic viral infections, a three-signal model for the development of T-cell failure has been proposed, namely, continuous antigenic stimulation, negative costimulatory signaling, and chronic inflammation (87). In this process, T cells exhibit a variety of cellular and molecular characteristics, such as loss of T-cell effector function, cytokine response, metabolism, gene expression, and epigenetic changes (35, 87). Thus, the transcriptional signature of CD8+ T cells is one of the determinants of good prognosis in a variety of autoimmune diseases, especially systemic diseases (72, 87). The transcriptional characteristics of CD8+ T cells include overexpression of various inhibitory receptors, such as PDCD1, KLRG1, TIGIT, HAVCR2 (TIM3), LAG3, CTLA4, CD160, and CD244. The coexpression of these inhibitory receptors constitutes a common suppressor gene program driven by the immunomodulatory cytokine IL-27 and specific transcription factors. Transcriptional signatures of CD8+ T cells are associated with the prognosis of a variety of autoimmune diseases, especially systemic diseases. In T1D, the fatigue state of CD8 cells was promoted by anti-CD3 therapy (teplizumab) and was more pronounced in islet-specific CD8+ T cells of those who progressed slowly, suggesting a benefit in T1D as well. When the prognosis is good, the expression levels of these inhibitory receptors are upregulated. At the same time, the expression of other genes related to T-cell receptor and cytokine signaling pathways, chemotaxis, adhesion, and migration also changes (87).

Based on the above background, a number of therapeutic approaches have been proposed to prevent T1D using islet β cell antigen-specific T cells (87). Abdelsamed et al. proposed that the proportion of beta cell-specific CD8+ T cells is inversely proportional to the progression of T1D, possibly due to the ability of these cells to maintain epigenetic programs associated with stem cell memory, enabling the maintenance of effect response (13, 40). However, this inverse relationship may only apply to exhausted CD8+ T cells, and CD8+ T cells may kill beta cells through other pathways, such as MHC1 (88) and other cytokines produced in the pancreas (89, 90). Studies have shown that T-cell populations characterized by both inhibitory receptors and depletion mediated by them are closely related to improved T1D markers (72). In T1D, anti-CD3 therapy promotes CD8 depletion, especially in islet-specific CD8+ T cells in slowly progressive patients (87). Interestingly, in T1D, TILs share common features with exhausted CD8+ T cells and are often dysfunctional, limiting antitumor immunity (79, 91). A more in-depth comparison with the "Exhausted" phenotype of cancer may help us understand the potential and limitations of T1D treatment. The exhausted T-cell phenotype in cancer has been extensively studied in immunotherapy, which includes the use of anti-PD-1 or anti-PD-L1 antibodies to unblock T cells. By comparing exhausted T-cell phenotypes in T1D and cancer, we can assess whether similar immunotherapy strategies can be applied to improve T1D therapy, such as enhancing exhausted T-cell reactivity through treatments targeting the PD-1/PD-L1 pathway. However, further research is needed to gain insight into the mechanism and function of exhausted T cells in T1D, as well as the differences and similarities with exhausted T cells in cancer. This will help develop more effective immunotherapy strategies to improve treatment response and disease management in patients with T1D. However, T-cell exhaustion is a double-edged sword for receptor characterization (87).

4 CD8+ T-cell receptors

Although T1D can be predicted today by measuring autoantibodies against β cell antigens in peripheral blood, there is an urgent need to develop T-cell markers to explain T-cell activity in the pancreas and to serve as a measure of disease activity (92). In recent years, immunosequencing and transcriptional profiling of the α/β chain complementary determinant of TCR have revealed the potential of TCR-altering biomarker discovery (93). In addition, a growing number of studies have shown that TCR signaling influences multiple aspects of CD8+ T-cell immunobiology, including thymus development, peripheral homeostasis, effector subset differentiation, function, and memory formation (94). Maladjusted TCR signaling events in T1D affect the efficacy of central and peripheral tolerance induction mechanisms, which also supports this idea (94).

Many studies have shown that TCR is closely related to the occurrence and development of T1D (40). The MHC region is a key factor in the genetic determination of both diabetes and other autoimmune diseases at the sites identified thus far. HLA mutations affect T-cell receptor (TCR)-mediated peptide binding and signal transduction, and how this affects antigen presentation is a crucial step in thymic selection and peripheral activation of immune responses. Additionally, the tissue specificity and progression of T-cell-mediated autoimmune responses depend in part on the TCR library expressed by pathogenic effector CD8+ cells (58). Other studies have shed light on the underlying mechanisms. It has been shown that alterations in TCR signaling (Signal 1), costimulatory signals (Signal 2) and cytokines (Signal 3) lead to impaired central and peripheral tolerance induction mechanisms, which ultimately

lead to T-cell activation, amplification, and subpopulation differentiation induced by β cell autoantigens driven by epigenetic and transcriptional outcomes that promote proinflammatory responses (94). TCR signaling is responsible for the development and progression of T1D, which (88) helps explain the association of certain HLA genes with T1D susceptibility. Moreover, dysregulation of costimulatory signals and/or cytokines also drives alterations in the signaling outcomes of specific T cells that recognize β cells, which aid in the growth and differentiation of effector and memory T cells (effector T cells can differentiate into memory T cells) and inhibit the formation and differentiation of exhausted T cells and protective FoxP3-regulated T-cell responses. Therefore, we can conclude that changes in signaling pathways involving TCR may promote or inhibit the development of T1D, which is manifested by differences in the islet T-cell infiltration rate (94). Animal experiments have provided relevant evidence. Although the diversity of TCRβ species in conventional and nTregs in NOD mice was significantly inconsistent compared with that in C57BL/6 mice, the difference was more pronounced in TCRα (23). Takuro Okamura et al. used the latest technology of single-cell V (D)J sequencing in BD Rhapsody to identify the TCR sequences of the characteristic autoimmune T cells of T1D in Japan and found that the TCR diversity and gene expression differences of CD8+ and FOXP3+ cells were in patients with T1D and healthy subjects (95). The observed bias refers to the fact that certain amino acids are used more frequently than others in some specific T-cell receptor (TCR) sequences found in studies, a phenomenon known as TCR sequence bias. Codina-Busqueta et al. found that amino acid preferences in the NDN region revealed the presence of slanted TCR libraries in infiltrating T cells and that the monoclonal amplified TCR sequences contained amino acid combinations consistent with the observed bias (96). The bias observed in the study refers to the fact that in some specific TCR sequences found in pancreatic tissue from human T1D patients, certain amino acids in the NDN region are used more frequently than others, indicating a bias in the sequence of these T-cell receptors. The β chain of TCR also shows abnormal shortening and library sharing in T1D (97). It is worth mentioning that TCR-like antibodies against a proinsulin-containing fusion peptide improved T1D in NOD mice (98). Based on the above evidence, the collection of large TCR data sets in both patients and nonpatients with T1D and in combination with big data analytics will advance the development of TCR as a potentially powerful biomarker in the development of T1D (Figure 3) (92).

5 ScRNA-seq in T1D

5.1 The role of ScRNA-seq

There is still a lack of effective markers to monitor disease progression in T1D and to determine patient response to immunotherapy (99). The intensive use of flow cytometry methods has identified a variety of T cells that could serve as potential markers for T1D, such as FOXP3 IFN-γ Tregs (100), CD4 + T cells IL-2 response (101) and IL-21 CD4+ T cells (102). However, flow cytometry is very weak in discovering novel biomarkers, and it can only explore the possible role of existing markers in T1D. The high polymorphism and low precursor frequency of self-reactive T cells in PMBCs make it particularly important to accurately measure the RNA expression levels of complex mixtures of autoimmune T cells. Therefore, there is a need to tap more potential specific immune cell populations as markers for T1D development or prediction of prognosis, and single-cell RNA sequencing (scRNA-seq) can play an important role in this regard (103).

scRNA-seq can reveal information specific to individual cells in a cell population. sc-RNA-seq has later advantages: 1) sorting wells or using droplet-based techniques allows RNA heterogeneity or different spliceosomes of RNA in individual cells to be detected (104); 2) the V (D)J region of TCR and BCR can be detected, which allows studies to determine antigen specificity by paired TCRα and β strands (105); 3) in combination with protein expression, V (D)J sequencing can be used in conjunction with data such as standard gene expression to help uncover deeper pathogenesis or explore clinical value (106); and 4) compared with bulk RNA sequencing, scRNA-seq has less bias (107). Moreover, when using scRNA-seq, it is necessary to avoid a series of problems, such as small sample size, sample contamination, RNA interference from dying cells, and analysis errors, to obtain clear and reliable results.

More research strategies will be used in conjunction with scRNA-seq, e.g., spatial transcriptomics can compensate for the lack of spatial location information in single-cell sequencing (108). Cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) can provide cell surface protein expression. In combination with scRNA-seq, heterogeneous cells with similar gene expression and differential protein expression can be distinguished (109). The combination of machine learning and scRNA-seq can predict prognostic CD8+ T-cell characteristics (110). As scRNA-seq is explored more deeply, more potential markers will be explored, and more targeted drugs will be developed (Table 2).

5.2 T1D-related ScRNA-seq study

ScRNA-seq can reveal the mechanisms of islet cell changes in T1D, for example, developing a linear model of β cell differentiation *in vitro* (111, 136), establishing the link between electrophysiology and genetics of islet dysfunction (112), and mining more specific loci that induce α cell or β cell proliferation and function (Sonic hedgehog signaling, DNMT1, ARX and transcription factor NKX6.1, etc.) (113–115). More importantly, it can tap more specific CD8+ T cells to construct models for diagnosis or determining prognosis and guide more precisely targeted therapies.

ScRNA-seq identifies specific subpopulations that influence T1D progression and can establish a link between cellular function and genetic modifications, ultimately guiding the use or modification of specific T cells for therapy. A subpopulation of

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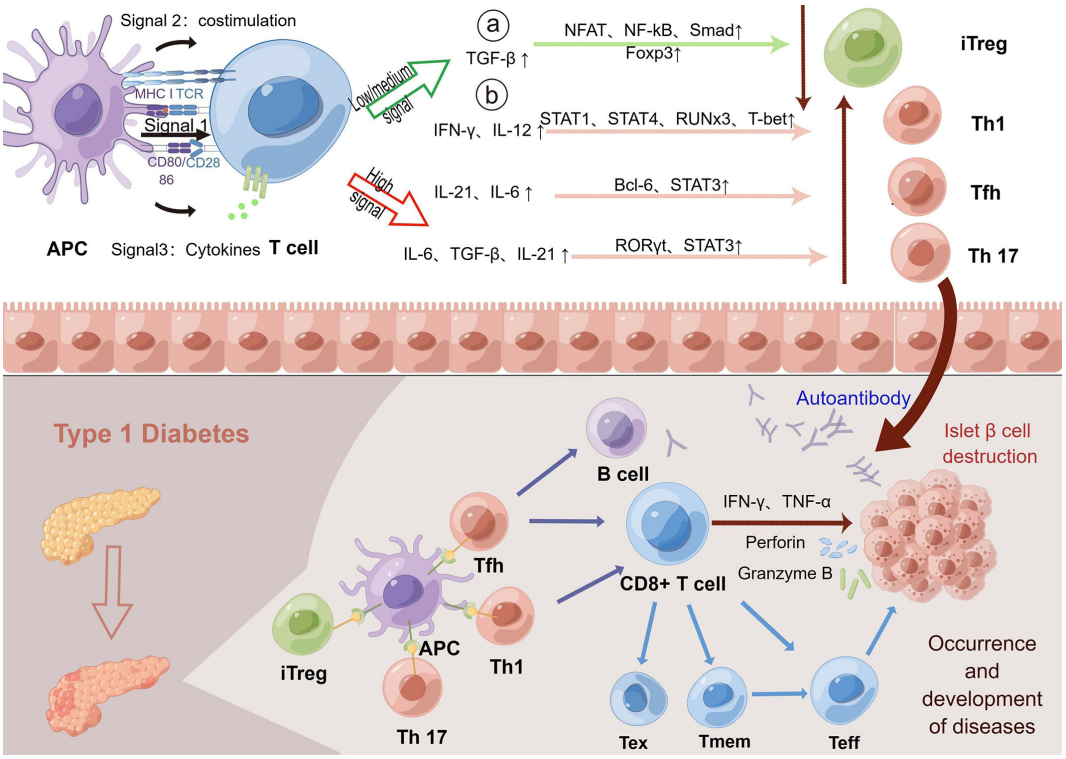

FIGURE 3
TCR indirectly promotes the development of T1D through several signaling pathways. The activation and differentiation of T cells depends on the strength of the interaction between TCR signaling, costimulatory signals and cytokines. Low TCR signaling can promote the differentiation of T cells into adaptive regulatory T-cell subsets by affecting the number of intermediate cytokines. Regulatory T cells help to regulate autoimmunity and prevent excessive autoimmunity. In contrast, high or persistently open TCR signaling promotes T-cell differentiation into immune-promoting Th1, Tfh, and Th17 subsets. In the normal human body, the body maintains autoimmune balance by regulating the opening of three pathways. However, in T1D patients, the continuous interaction of T cells with the MHC-antigen receptor $compl_{ex}$ and/or enhanced signaling results in the continuous enhancement of TCR signaling to promote the generation of Th1, Tfh and Th17 subsets. Next, high TCR signaling can combine with costimulation and proinflammatory cytokines to promote the growth and differentiation of effector T cells and promote autoimmunity, which then spontaneously migrate to the islet to destroy islet β cells, leading to the development and progression of the disease. Low or moderate intensity TCR signaling can promote the transformation of T cells into exhausted T cells and hinder the function of effector T cells. However, elevated levels of the cytokine IL-21 promote the recovery of exhausted T cells and continue to cause islet β cell destruction. Memory T cells can also rapidly transform into effector T cells and produce proinflammatory factors in response to antigens to enhance the immune response, indirectly leading to the destruction of β cells. In conclusion, the increased TCR signaling in T1D could promote autoimmunity through multiple mechanisms, leading to islet β cell destruction and T1D. TCR, T-cell receptor; T1D, type 1 diabetes; Foxp3, forkhead box protein P3; iTreg, adaptive regulatory T cells; MHC, major histocompatibility complex; IA-2, islet antigen 2; GAD, glutamic acid decarboxylase.

IGRP stem cell-like CD8+ T cells in the pLN highly expresses TCF1, which may induce T1D (76). Other studies have shown that $IGRP_{206-214}$ stem cell-like CD8+ T cells are phenotypically heterogeneous and clonally restricted (15) and that TCF1 is not a critical determinant of T1D (116). It has now been shown that H9T treatment maintains T-cell transcription factor 1 (TCF-1) expression and promotes mitochondrial adaptation, thus facilitating the maintenance of a stem cell-like state. Furthermore, TCR transgenic and chimeric antigen receptor-modified stem cell-like CD8+ T cells amplified with H9T cells showed potent *in vivo* antitumor activity in mouse models of melanoma and acute lymphoblastic leukemia (137). This offers hope for the future use of transgenic stem cell-like CD8+ T cells for the treatment of T1D.

Many more specific T-cell subsets were screened, providing additional insight into the mechanisms of CD8+ T cells in T1D. Following infection with Kilham rat virus (KRV), T1D onset was preceded by KRV invasion into multiple splenocytes, a reduction in CD8+ T-cell numbers, and scRNA-seq results showing increased expression of MHC class II transcripts on monocytes and macrophages in type I IFN and IFN receptor (IFNAR)-loss rats. IFNAR may be associated with T1D susceptibility (117). Podocyte-based scRNA-seq showed that the translation ribosome affinity purification (TRAP) gene was differentially expressed in T1D versus T2D (118). Cell adhesion molecule 1 (CADM1)-mediated intercellular contacts promoted enrichment of CD8+ T cells in the pancreas (119). Pediatric T1D was associated with naïve and activated/memory CD45RA CD8+ T-cell populations (120). C1QB and NKG7 increase the number of macrophages and CD8+ T cells, respectively, leading to islet β cell injury (121). In pediatric T1D, CD4+ cells were converted to the IFNG-T_H 1 memory phenotype (122). The regulatory genes PTPN6, TGFB and TYROBP inhibit DC in T1D (123). FoxP3/TGFβ1+ CD4+ T_{reg} cell number and

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TABLE 2 T1D-related single-cell sequencing study.

Model	Source	Marker	Results	Ref.
NOD mice	Pancreas and spleen	$IGRP_{206-214}$	$IGRP_{206-214}$ CD8+ T-cell have phenotypic heterogeneity and clonal restriction.	(15)
T1D patients	PBMCs	$ZnT8_{186-194}$	ZnT8 + $_{186-194}$ CD8+ T-cell clonotypes were found to cross-recognize a *Bacteroides stercoris* mimotope.	(25)
NOD mice	pLN	IGRP	IGRP CD8+ T cells with high expression of TCF1 in pLN induce T1D.	(76)
Human cells	B cell	ITGA1	develop a lineage model of *in vitro* β cell differentiation.	(111)
T1D patients	Pancreas	–	Establishing an electrophysiological and genetic link to islet dysfunction.	(112)
T1D patients	Pancreas	–	Sonic hedgehog signaling may regulateαcell proliferation.	(113)
NOD mice	Pancreas	DNMT1/ARX	DNMT1 and ARX maintain human α cell identity.	(114)
hESCs	hESCs	–	Activation of transcription factor NKX6.1 increases functional β cells.	(115)
NOD mice	islet	Tcf7	Tcf7 expression is not a critical determinant of T1D.	(116)
T1D rats	Splenocytes	–	MHC class II transcripts of monocytes and macrophages↑	(117)
T1DN mice	Podocyte	–	TRAP gene was differentially expressed in T1DN versus T2DN.	(118)
NOD mice	pancreas	CADM1	CADM1-mediated intercellular contacts promote the enrichment of CD8+ T cells in the pancreas.	(119)
T1D patients	PBMCs	CD45RA	Pediatric T1D is associated with CD45RA CD8+ T-cell population.	(120)
T1D patients	PBMCs	C1QB/NKG7	C1QB and NKG7 increase the number of macrophages and T cells, respectively, causing islet β cell injury.	(121)
T1D patients	PBMCs	T_H1	Conversion of CD4+ cells to IFNG-T_H1 memory phenotype in childhood.	(122)
T1D patients	PBMCs	PTPN6/ TGFB/TYROBP	The regulatory genes PTPN6, TGFB, and TYROBP in DC form T1D↓	(123)
NOD mice	PBMCs	FoxP3/TGFβ1	FoxP3/TGFβ1+ CD4+ Tregs during autoimmune diabetes↓	(124)
T1D patients	PBMCs	–	Viruses and cytokines in pancreatic induce the release of IL-32 from activated T cells and NK cells in children.	(125)
T1D Patients	pancreas and PBMCs	–	snATAC-seq shows that risk variants for T1D were enriched in cCREs that were active in T cells.	(126)
T1D patients	T-cell	TCR	Specific antigens for CD8+ T cells were identified and TCRs cross-reacting with microbiome antigens were discovered.	(127)
T1D patients	PBMCs	TCR	islet Ag-reactive CD4+ Tregs are expanded during disease progression.	(128)
T1D patients	IAR T cells	TCR	The specificity of the IAR T-cell population is determined by the TCR.	(129)
Human	MPD	PDX1/ ALK3/CAII	Progenitor-like cells in MPD can differentiate into functional β cells.	(130)
T1D patients	β cells and β-like cells	–	The genetic risk of T1D may be associated with β cell endoplasmic reticulum stress.	(131)
NOD mice	anti-insulin CD4+ T cells	TCR	Genetic correction of the I-Aβ57 mutation in T1D resulted in the loss of D/E residues in CDR3β of T cells.	(132)
T1D patients	PBMCs	BCR and TCR	Patients with type I diabetes have unique TCR and BCR-positive lymphocytes.	(133)
Human	Pancreas	–	Expression of an alternatively spliced INS product activated preproinsulin-specific CD8 + T cell.	(134
T1D patients	Pancreas	–	Expression of MHC class II pathway genes in exocrine ductal cells of T1D ↑	(135)

NOD, nonobese diabetic; pLN, pancreatic draining lymph node; IGRP, islet-specific glucose-6-phosphatase catalytic subunit-related protein; cCREs, candidate cis-regulatory elements; T1DN, type 1 diabetic nephropathy; TRAP, translating ribosome affinity purification; CADM1, Cell Adhesion Molecule 1; MPD, Myeloproliferative Disorder; TCR, T-Cell Receptor; PDX1, Pancreatic and Duodenal Homeobox 1; ALK3, Activin Receptor-Like Kinase 3; CAII, Carbonic Anhydrase II; C1QB, Complement C1q subcomponent subunit B; NKG7, Natural killer cell group 7 protein; BCR, B-cell receptor; PTPN6, Protein tyrosine phosphatase nonreceptor type 6; TGFB, Transforming growth factor beta; TYROBP, TYRO protein tyrosine kinase binding protein; DNMT1, DNA methyltransferase 1; ARX, Aristaless related homeobox; FoxP3, Forkhead box P3; TGFβ1, Transforming growth factor beta 1; ZnT8, Zinc transporter 8.

function are decreased in T1D (124). It has also been noted that viruses and cytokines in the pancreas induce the release of IL-32 from activated T cells and NK cells in children (125). Notably, genome-wide association studies (GWAs) combined with single-cell epigenomics will play a role in understanding the complex origins of T1D. Risk variants in T1D were found to be enriched in candidate cis-regulatory elements (cCREs) active in T cells. Multiple potential risk variants in T1D overlap with cCREs in genes with exocrine-specific expression (126). Currently, many relevant sites on GWAs have been found to associate with different nodes in T-cell activation and signaling pathways (138).

5.3 ScRNA-seq of TCR

The single-cell transcriptome + TCR sequencing solution can obtain gene transcript levels at the single-cell level of resolution along with TCR light and heavy chain sequence information (139). Single-cell technology bridges the shortcomings of traditional TCR detection technology and provides the possibility of rapid screening of CD8+ T-cell targets in T1D immunotherapy. Combining TCR sequencing with T-cell phenotyping can yield more realistic insights into islet antigen-specific CD8+ T cells, which can separate CD8+ T cells that are actually involved in killing β cells, rather than being limited to having islet antigen binding sites.

High-dimensional tetramer-associated TCR-seq allowed the identification of specific antigens for CD8+ T cells and revealed antigenic cross-reactivity between TCRs and the microbiome in T1D (127). For example, the restriction zinc transporter protein 8 (ZnT8) 186-194 CD8+ T_{eff} cell clonotypic antigen can cross-recognize a *Bacteroides stercoris* mimotope (25, 140). The mechanism of TCR action in CD8+ T cells is gradually being uncovered by TCR sequencing. In an earlier study, islet antigen-reactive (IAR) CD4+ Tregs were found to be increased in T1D and could be diagnosed or predicted by peripheral blood scRNA-Seq assay (128). The specificity of IAR CD4+ T cells has been shown to be closely related to the self-response-restricted TCRα chain and its associated epitopes (129). The latest study used CRISPR/Cas9 to knock in PTPN22, a gene associated with altered TCR regulation and T-cell activation, in naive T cells, ultimately enhancing self-reactive T cells and shifting the differentiation of this subpopulation toward an inflammatory phenotype, demonstrating the possibility of altering the TCR and thus regulating inflammation through gene editing in T1D (141).

6 Potential targeting of CD8+ T cells for T1D treatment

6.1 CRISPR/Cas9 regulates CD8+ T cells

CRISPR/Cas9 is a gene editing technology that was originally a bacterial defense mechanism against exogenous DNA (142). It performs target gene recognition through artificially designed guide RNA, directs Cas9 protease to break the DNA double strand, and causes genome modification during DNA damage repair. CRISPR/Cas9 enables gene knockout/knock-in, gene repression/activation, multiplex editing and functional gene screening. CRISPR/Cas9 technology has within just a decade been involved in solving many biomedical problems (143). The combination of two revolutionary technologies, immunotherapy and CRISPR/Cas9, has further broadened the application of immunotherapy in a variety of diseases. The role of CRISPR/Cas9 in T1D therapy is increasingly being explored, and an exhaustive summary is still lacking (Figure 4).

Some experiments have attempted to knock out a gene and thus achieve remission of T1D, but it is undeniable that its function and survival time are still immature and need to be explored in more depth (Table 3). Antigen activation of CD8+/CD4+ T_{eff} cells promotes receptor expression of the receptor for advanced glycation end products (RAGE), which may enhance T-cell reactivity and induce inflammation in T1D patients. One study using CRISPR/Cas9 knockdown of RAGE in Jurkat cells resulted in Erk, phosphorylation of MEK and reduced IL-2 (145). Knocking out WDFY4 using CRISPR/Cas9, thereby eliminating cross-presentation of type 1 conventional dendritic cells (cDC1s), was found to limit the initiation of self-reactive CD8+ T_{eff} cells in T1D and attenuate the recruitment of CD4+ T cells into islets to damage β cells. When the stimulator of IFN genes (STING) was knocked out in NOD mice, the number of IGRP $_{206\text{-}214}$-specific stem cell-like CD8+ T cells was increased, and splenocytes from STING-deficient mice could be rapidly induced in T1D after relay transfer to irradiated NOD recipients (147). This study suggests that stem cell-like CD8+ T cells in T1D may be regulated by the STING pathway that senses DNA damage and, in the future, may be activated by activating the cGAS-STING pathway in stem cell-like CD8+ T cells to improve T1D. Earlier studies have shown that Nfkbid, an allelic variant of an NF-κB signaling regulator, induces thymic deficiency defects in self-reactive CD8+ T_{eff} cells in NOD mice with abrupt onset of T1D (149). The latest study enlarged thymic deficiency in pathogenic CD8 AI4 and NY8.3 TCR transgenic T cells by knocking in Nfkbid, but the study truncated the increased frequency and function of peripheral Treg, which inhibited the accelerated progression of T1D (151).

In a study using transgenic pigs as a model, knock-in of $GIPR^{dn}$, hIAPP and $PNPLA3^{I148M}$ revealed that CD8+ T cells in the liver and adipose tissue, costimulation, cytotoxicity and increased secretion of cytokines and chemokines, antigen presentation, and TCR signaling were all activated (148). This study suggests the possibility of knocking in pathogenic risk genes to construct a large animal model highly similar to T1D patients. Another study constructed NOD-cMHC I mice by knocking out the genes of human disease-associated HLA-A2 or -B39, which compensated for the previous lack of nonclassical MHC I molecule expression and FcRn activity in NOD.β2 m mice (150). There are also studies trying to find the use of CRISPR/Cas9 to construct stem cell-like CD8+ T cells. TCF-1 expression in CD8+ T cells correlates with memory

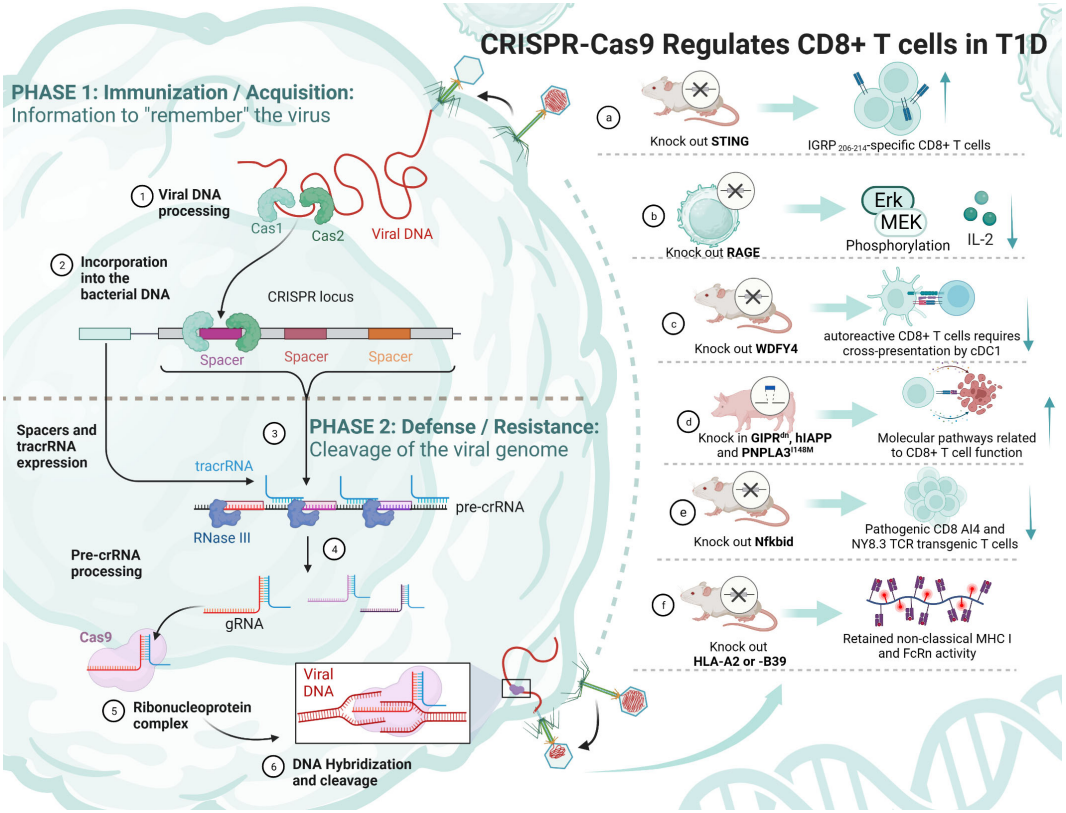

FIGURE 4
The principle of CRISPR/Cas9 and its mediation of T1D regulation by CD8+ T cells. TCF-1, transcription factor T cytokine 1; NOD, nonobese diabetic; RAGE, receptor for advanced glycation end products; WDFY4, WD repeat and FYVE domain-containing 4; cDC1s, type 1 conventional dendritic cells; STING, stimulator of IFN genes; IGRP, islet-specific glucose-6-phosphatase catalytic subunit-related protein; MHC, major histocompatibility complex.

marker expression and amplification capacity, and sustained antigenic stimulation decreases TCF-1 expression. In an experiment on HIV, CRISPR/Cas9 was used to knock in TCF-1 in CD8+ T cells, which possesses stem cell-like memory properties with secondary amplification capacity (144). It has been shown that a small number of stem cell-like CD8+ T cells with high TCF-1 expression greatly induces T1D. CRISPR/Cas9 technology might be able to generate stem cell-like CD8+ T cells that do not kill

TABLE 3 CRISPR/Cas9 regulates CD8+ T cells in T1D.

CRISPR/Cas9 editing sites	Model	Express	Results	Ref.
TCF-1	rhesus macaque	↑	TCF-1 contributes to the maintenance of stem cell-like HIV-specific CD8+ Tmem cells.	(144)
RAGE	Jurkat cell	↓	Erk phosphorylation and IL-2 in CD8+ T-cell↓	(145)
WDFY4	NOD mice	↓	the priming of Autoimmune CD8+ T cells requires cross-presentation by cDC1↓	(146)
STING	NOD mice	↓	$IGRP_{206-214}$-specific CD8+ T cells↑	(147)
$GIPR^{dn}$, hIAPP and $PNPLA3^{I148M}$	pig	↓	Molecular pathways related to CD8+ T-cell function↑	(148)
Nfkbid	NOD mice	↓	Spontaneous diabetic AI4 and NY8.3 CD8+ T-cell negative selection↓	(149)
HLA-A2 or -B39	NOD-cMHCI mice	↓	Retained nonclassical MHC I and FcRn activity	(150)

TCF-1, transcription factor T cytokine 1; NOD, nonobese diabetic; RAGE, receptor for advanced glycation end products; WDFY4, WD repeat and FYVE domain-containing 4; cDC1s, type 1 conventional dendritic cells; STING, stimulator of IFN genes; IGRP, islet-specific glucose-6-phosphatase catalytic subunit-related protein; MHC, major histocompatibility complex.

pancreatic islet cells, thus ameliorating T1D. These models could provide a broader direction for the development of future T1D therapies.

6.2 Chimeric antigen receptor T-cell

CAR-T therapy is a technology in which human T cells are extracted from the body, genetically engineered and cultured to a sufficient quantity, and infused back into the patient's body to achieve a cure for the disease (Figure 5). Injecting Tregs into the body can alleviate the development of T1D, which is a promising novel therapeutic route (Table 4). However, only a small number of Tregs can be isolated, so an early study attempted to convert T_{eff} into T_{reg} using CAR-T technology, and the results showed that CAR-Tregs performed well in terms of function and duration of presence (155). More studies have been conducted to develop site-specific CAR-T cells for the treatment of T1D. CAR-T cells with urokinase-type plasminogen activator receptor (uPAR) specificity can target senescent cells to alleviate inflammatory responses and improve tissue homeostasis, which may improve diabetes, and future studies in NOD mouse models or human-derived transgenic models are needed to verify this (152). mAb287 is a monoclonal antibody that can target I-A binding between the insulin B chain 9-23 peptide and NOD MHC class II molecules. CAR-T cells formed by spiking CD8+ Teff cells with mAb287 can mediate the IFN-γ pathway to kill antigen-presenting cells (APCs) (153). However, a single infusion can only delay the development of T1D for 1-2 weeks, and more studies on optimizing the lifespan of CAR-T cells are needed in the future. A biomimetic study used CD3γϵ, δϵ, ζζ, CD4 and CD8+ to construct a biomimetic five-module chimeric antigen receptor (^{5M}CAR), which drives CD8+ T-cell activation and binds MHC molecules. Experiments using NOD mice as a model showed that ^{5M}CAR-T cells could alleviate T1D by targeting autoimmune CD4+ T cells (154). CAR-T technology can be used not only to treat T1D but also to explore potential mechanisms of CD8+ T-cell destruction. A study in which CD19 CAR-T cells were constructed explored the potential mechanisms of β cell damage that may be associated with factors such as scorch death and endoplasmic reticulum stress, where CD19 CAR-T cells release T-cell factors and upregulate immune response genes and the scorch death mediator Gasdermin D and its activator Caspase 4 in β-like cells, ultimately leading to the death of damaged β-like cells (157).

Treatment with CAR-T-cell technology alone may not yield the desired benefits for patients, with several drawbacks: 1) the high time and economic cost of producing individual-specific CAR-T cells to rescue rapid disease progression; 2) the inability to obtain sufficient raw material when patients have fewer high-quality lymphocytes; and 3) the clinical architecture produced by autologous CAR-T cells can become uncontrollable and unpredictable because the heterogeneity of autologous CAR-T

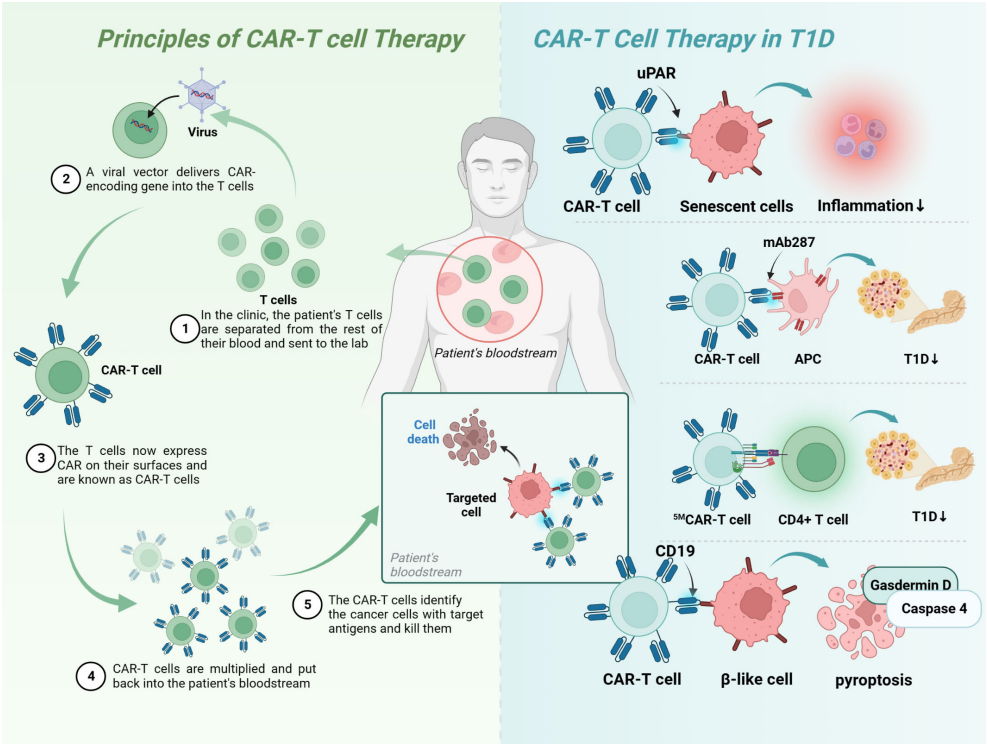

FIGURE 5
Principles of CAR-T-cell therapy and its application in T1D. Upar, urokinase-type plasminogen activator receptor; APC, antigen-presenting cells; MHC, major histocompatibility complex; T1D, type 1 diabetes; CAR, chimeric antigen receptor.

TABLE 4 CAR-T-cell therapy in T1D.

Model	Targeted Cell	Targeted proteins	Results	Ref.
Mice	senescent cells	uPAR	eliminating senescent cells from damaged tissues↑	(152)
NOD mice	APC	mAb287	Short-term prolonged onset of hyperglycemia	(153)
NOD mice	CD4+ T-cell	pMHCII	T1D↓	(154)
NOD mice	β cell	insulin	Insulin-specific CAR Tregs is functionally stable *in vivo* and has long-term inhibitory properties.	(155)
Mice	–	LFA-1	Low serum magnesium levels reduce CAR-T-cell function and activity.	(156)
β-like cell	β cell	CD19	Immune response gene and scorch death mediator Gasdermin D and its activator Caspase 4 in β cell ↑	(157)

Upar, urokinase-type plasminogen activator receptor; APC, antigen-presenting cells; MHC, major histocompatibility complex; T1D, type 1 diabetes; CAR, chimeric antigen receptor.

cells can become uncontrollable and unpredictable (158). The CRISPR/Cas9 system mentioned above could improve or consolidate the effect of CAR-T-cell and TCR-T-cell technologies, for example: 1) the construction of universal CAR-T cells with knockdown of TCR and HLA molecules, which would make future immunotherapy with adoptive T-cell transfer (ACT) timely and standardized; 2) the use of CRISPR/Cas9 knockout/in immune checkpoint genes; and 3) or knockout of T-cell factor genes, which may improve treatment outcomes (159).

7 Future perspectives

TCR-T cells have a highly specific TCR αβ chain sequence, which can recognize antigens inside cells compared to CAR-T cells (Figure 6). The process is roughly as follows: 1) screening and identification of TCR sequences that can specifically bind to target antigens; 2) genetic engineering to transfer them into T cells and culture them to sufficient numbers; and 3) transfusion of TCR-T cells back into patients to specifically recognize and kill cells for therapeutic purposes (160, 161).

CAR and TCR have different structures, and their affinities and their effects differ (Table 5). CAR is an artificial single chain with five structural domains. The variable heavy chain (VH) and variable light chain (VL) structural domains combine to form an antigen-binding structural domain (scFv) that binds cytokines or ligands significantly beyond the TCR (171). The costimulatory domain and the CD3ζ signaling structural domain activate T cells, and the hinge domain and TM domain are involved in the formation of CAR-T-cell immune synapses (167, 168). The costimulatory domain transmits both signal 1 and signal 2, on the basis of which T cells will be completely activated. TCR binding to the homologous peptide-MHC complex (pMHC) does not complete signaling directly but requires complex formation with multiple CD3 signaling subunits for T cells. CD3γε and CD3δε and CD3ζζ together form the TCR-CD3 complex, and the extracellular immunoglobulin (Ig) superfamily structural domain, based on the activation motif of the immune receptor tyrosine (ITAM), in which

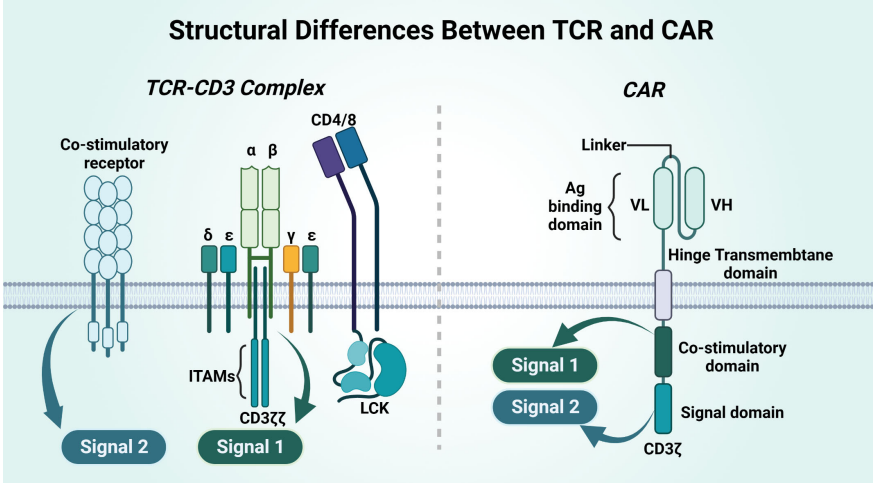

FIGURE 6
Structural differences between TCR and CAR.

TABLE 5 Differences between TCR-T and CAR-T cells.

Elements	CAR-T	TCR-T	Ref.
Structure	Single chain with 5 structural domains	TCR-CD3 complex	(162)
Antigen expression	Cell surface	Inside and outside the cell	(160)
MHC restriction	Not restricted	Restricted	(163)
ITAMs	10	3	(164)
Immune rejection	Low	High	(165)
Permeability	High	Low	(166)
Coreceptor involvement	CD45	CD4, CD8 and CD45	(167, 168)
FDA approval	Kymriah, Yescarta, Tecartus, Breyanzi, Abecma, Carvykti	Tebentafusp	(169, 170)

the extracellular structural domain (ECD) is contained, will complete Signal 1 alone. Signal 2 of TCR-T cells requires costimulatory receptor-ligand binding of 4-1BB and 4-1BBL for this to occur, and Signal 2 synergizes with Signal 1 to fully activate T cells (172).

There are six CAR-T therapies that have been approved by the FDA and have shown some success in their therapeutic use for the treatment of hematologic malignancies (170). There is currently only one FDA-licensed TCR-T therapy drug: tebentafusp (169). At the same time, factors such as lack of specific target antigens and immunosuppression of the tumor microenvironment have been exposed in clinical applications. In contrast, the mechanistic exploration and clinical conclusions of TCR-T therapies are not sufficient, but there is some evidence that engineered TCR-T cells have better expansion rates within the high antigen environment, lower levels of coinhibitory immune checkpoint molecule expression, higher differentiation trends and no significant difference in tumor cell clearance (173).

ScRNA-seq and CRISPR/Cas9 in combination with TCR-T therapy is a promising strategy. ScRNA-seq technology can identify specific T-cell clones at the single-cell level to help obtain high-affinity TCR sequences or analyze the activity of TCR-T cells *in vivo* to guide therapy (160). The presence of endogenous TCRs on the surface of CD8+ T cells to be modified is a major factor affecting the therapeutic efficacy of TCR-T cells. Endogenous TCRs lead to a reduced binding rate of transgenic TCRs to CD3, and the two may mismatch and form mixed TCR dimers. To address the problems of a low transgenic TCR-CD3 binding rate and mispairing, CRISPR/Cas9 can transduce a stable Vα/Vβ single-stranded TCR into T cells. Alternatively, CRISPR/Cas9 can replace endogenous TCR α and β genes with genes having certain specific TCR sequences to better ameliorate the disease. In addition, it has been shown that transgenic TCR expression and function in TCR-T cells without knockdown of endogenous TCR genes is lower than that in TCR-T cells with knockdown of endogenous TCR (174, 175). CRISPR/Cas9-edited TCR-T cells were tested in clinical trials in patients with refractory cancers to assess safety and feasibility. Of the three patients treated, two had stable disease, and the other had progressive disease without serious adverse effects (176).

In addition, most of the CAR/TCR-T experiments have been realized only in tumor models, and it remains to be explored whether CAR/TCR-T cells have the real ability to be used as a T1D treatment targeting autoimmune remission based on more NOD mice or even clinical experiments. The targeting toxicity, neurotoxicity and cytokine storm generated (CSG) by CAR/TCR-T cells are also issues to be overcome in the future.

8 Conclusion

This review suggests that self-reactive CD8+ T cells have great potential in the treatment of T1D and that targeted precision therapy may be possible by combining multiple novel technologies. The main existing therapies for T-cell-mediated T1D include systemic immunosuppression, antibodies that deplete immune cells, and anti-cytokine therapies. Although effective in reducing autoimmune T cells, they may also impair other immune responses, leading to increased susceptibility to other diseases and complications. In contrast, identifying specific CD8+ T cells by scRNA-seq technology with TCR sequencing and modifying them by technologies such as CRISPR/Cas9, CAR-T, and TCR-T, making it possible to cure T1D by losing the ability to recognize β cells to β cell-specific stem-like CD8+ T cells and its differentiated progeny, is a potential pathway. In addition, scRNA-seq and CRISPR/Cas9 may also play an important role in the evaluation of therapeutic efficacy and construction of experimental models in the future. It is worth noting that many studies have only measured the number of CD8+ T cells, but have not focused on their specific exhaustion, stem cell-like and other state changes, which is something that future studies need to focus on to analyze the mechanism of CD8+ T cells in T1D from a more dynamic perspective.

In the future, many more factors need to be considered: 1) the effect of pathogenic single nucleotide polymorphisms (SNPs) on T-cell function; 2) activation of T cells by unnatural peptides, posttranslationally modified peptides, and hybrid peptides; and 3) the interaction between B cells and T cells and DC cells and T cells. Mendelian randomization, molecular simulation, organoids and other techniques will bring newer perspectives to the field.

Author contributions

KY: Writing – original draft, Writing – review & editing. YZ: Writing – original draft. JD: Writing – original draft. ZL: Visualization, Writing – original draft. HZ: Visualization, Writing – original draft. FZ: Funding acquisition, Writing – review & editing.

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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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Causal associations of thyroid function with inflammatory bowel disease and the mediating role of cytokines

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Background: Previous observational epidemiological studies have suggested a potential association between thyroid function and inflammatory bowel disease (IBD). However, the findings remain inconclusive, and whether this association is causal remains uncertain. The objective of this study is to investigate the causal association between thyroid function and IBD.

Methods: Genome-wide association studies (GWAS) involving seven indicators of thyroid function, IBD, and 41 cytokines were analyzed. Bidirectional two-sample Mendelian randomization (MR) and multivariable MR were conducted to examine the causal relationship between thyroid function and IBD and to explore the potential mechanisms underlying the associations.

Results: Genetically determined hypothyroidism significantly reduced the risk of CD (odds ratio [OR] = 0.761, 95% CI: 0.655–0.882, $p < 0.001$). Genetically determined reference-range TSH was found to have a suggestive causal effect on IBD (OR = 0.931, 95% CI: 0.888–0.976, $p = 0.003$), (Crohn disease) CD (OR = 0.915, 95% CI: 0.857–0.977, $p = 0.008$), and ulcerative colitis (UC) (OR =0.910, 95% CI: 0.830–0.997, $p = 0.043$). In reverse MR analysis, both IBD and CD appeared to have a suggestive causal effect on the fT3/fT4 ratio (OR = 1.002, $p = 0.013$ and OR = 1.001, $p = 0.015$, respectively). Among 41 cytokines, hypothyroidism had a significant impact on interferon-inducible protein-10 (IP-10) (OR = 1.465, 95% CI: 1.094–1.962, $p = 0.010$). The results of multivariable MR showed that IP-10 may mediate the causal effects of hypothyroidism with CD.

Conclusion: Our results suggest that an elevated TSH level reduces the risk of CD, with IP-10 potentially mediating this association. This highlights the pituitary-thyroid axis could serve as a potential therapeutic strategy for CD.

KEYWORDS

thyroid function, hypothyroidism, inflammatory bowel disease, IP-10, Mendelian randomization

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1 Background

Inflammatory bowel disease (IBD) is a chronic and nonspecific inflammatory disorder of the intestinal tract, encompassing Crohn's disease (CD) and ulcerative colitis (UC) (1). Despite significant advances in understanding IBD, the exact etiology of the disease remains largely uncertain. A substantial body of literature suggests that the pathogenesis of IBD is linked to intestinal microbiota, a specific genetic background, environmental factors, and abnormal immune responses (2). Although IBD primarily affects the gastrointestinal tract, patients may experience extraintestinal manifestations in various organs, such as the blood, liver, pancreas, prostate, cervix uteri, central nervous system, and skin (3). The severity of certain extraintestinal symptoms may vary depending on the underlying activity of IBD (4).

The regulation of thyroid function is a complex process that involves not only the thyroid gland but also the pituitary gland and the hypothalamus. Thyrotropin (TSH), secreted by the pituitary, stimulates the thyroid to release thyroxine (T4). In thyroidal and peripheral tissues, free T4 is converted to free triiodothyronine (fT3) hormone to fulfill physiological functions (5, 6). Autoimmunity is the predominant etiology of thyroid dysfunction, encompassing both hyperthyroidism (7) and hypothyroidism (8). Autoimmune thyroid disorders, including Grave's disease, Hashimoto thyroiditis, and postpartum thyroiditis, are characterized by circulating thyroid-specific autoreactive antibodies (9).

Recent studies suggest that thyroid function extends beyond thyroid diseases to include links to the gastrointestinal system (10, 11). The gut microbiota contributes to the synthesis and hydrolysis of thyroid hormone conjugates. Microbial metabolites could potentially contribute to autoimmune thyroid diseases by modulating the immune response (12, 13). Several studies have explored the relationship between thyroid diseases and IBD, yielding variable and even conflicting results. A cross-sectional study conducted in England, enrolling 300 UC patients, identified a significant increase in the prevalence of thyrotoxicosis (14). Additionally, several studies indicate that IBD patients may have an increased susceptibility to thyroid gland carcinogenesis (15, 16). Modifications in thyroid gland size and function, both with and without clinically detectable hyperthyroidism or hypothyroidism, have been reported in IBD patients (17). Nevertheless, the exact role of thyroid hormones in the pathophysiology of IBD remains unclear. It remains uncertain whether the observed connection between thyroid function and IBD is causal.

Mendelian randomization (MR) is a method frequently employed to explore causal links between risk factors and outcomes using genetic instruments (18). Our study conducted a bidirectional two-sample MR analysis to investigate the causal relationship between thyroid function and IBD, including ulcerative colitis (UC) and Crohn's disease (CD) subtypes. Cytokines play a critical role in the pathophysiological processes of both IBD and autoimmune thyroid disorders. Furthermore, we conducted a multivariable MR analysis to assess whether cytokines mediate the causal relationship between thyroid function and IBD.

2 Materials and methods

2.1 Study design

We conducted a bidirectional two-sample MR study to investigate the causal relationship between thyroid function and IBD. The flowchart is displayed in Figure 1.

GWAS summary data for thyroid functions included reference-range free triiodothyronine (fT3), free thyroxine (fT4), the ratio of fT3 to fT4 (fT3/fT4), thyrotropin (TSH), thyroid peroxidase antibody (TPOAb) positivity, decreased TSH status (indicative of hyperthyroidism), and increased TSH status (indicative of hypothyroidism). Genome-wide association study (GWAS) summary data for IBD included both UC and CD subtypes. Subsequently, we performed a two-sample MR analysis to explore the causal effects of both hyperthyroidism and hypothyroidism on cytokines. A multivariable MR analysis determined the effect of hypothyroidism on CD after adjusting for interferon-inducible protein-10 (IP-10). The selection of instrumental variables (IVs) must adhere to three fundamental principles: (1) relevance assumption—the genetic variation is highly associated with exposure; (2) independence assumption—the genetic variation is not significantly linked to potential confounding factors; and (3) exclusion restriction assumption—the genetic variation exclusively affects the outcome through the exposure.

2.2 Data sources

In this study, we utilized summary-level data from an updated meta-analysis of GWAS conducted by the ThyroidOmics Consortium (19). GWAS data for TPOAb positivity were acquired from (20). GWAS data for IBD were obtained from the International IBD Genetics Consortium (IIBDGC) (21), the FinnGen database, and a large-scale GWAS study (22). The GWAS data for cytokines was derived from the meta-analysis summary statistics for 41 inflammatory cytokines (23). Detailed information about the datasets used in this study is presented in Supplementary Table 1. All summary statistics employed were from GWAS analyses, and no sample overlap was observed. All GWAS summary data are publicly available; thus, no additional ethical approval or informed consent was required.

2.3 Genetic variants selection criteria

Single nucleotide polymorphisms (SNPs) meeting genome-wide significance ($p < 5 \times 10^{-8}$) and having minor allele frequency (MAF) >1% were included. Due to the scarcity of SNPs with p-values less than 5×10^{-8}, we extended the threshold to 1×10^{-5} for TPOAb positivity and 5×10^{-6} for cytokines to select appropriate instrumental variables. The identified SNPs were then clumped with a strict cutoff of clumping $R^2 = 0.001$ within a window of 10,000 kb. The proportions of trait variance explained by the identified SNPs were calculated using the following formulas:

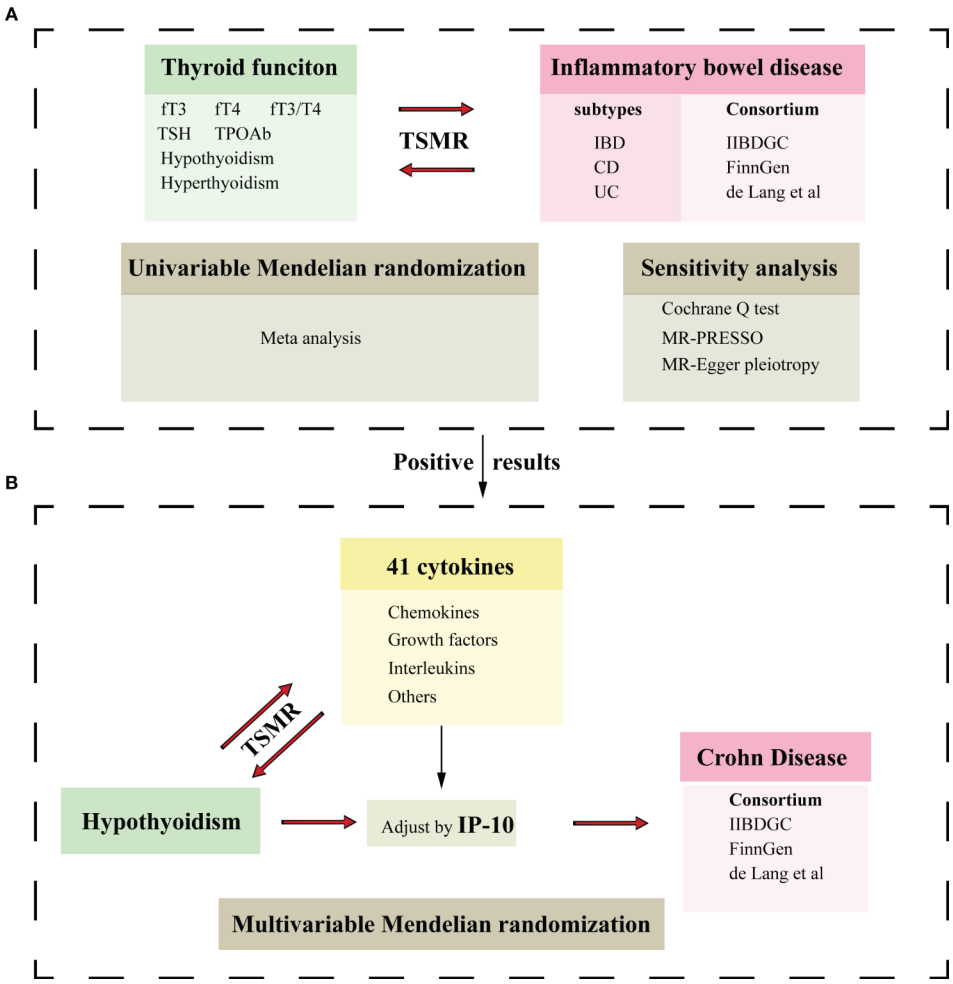

FIGURE 1
Flowchart of the Mendelian randomization study. **(A)** Two-sample Mendelian randomization investigates the effect of thyroid function on IBD and its subtypes.**(B)** Two-sample Mendelian randomization investigates the effect of hypothyroidism on cytokines and multivariable Mendelian randomization analysis evaluates the roles of mediating the association between thyroid function and CD. IBD, inflammatory bowel disease; UC, ulcerative colitis; CD, Crohn's disease; fT3, free triiodothyronine; fT4, free thyroxine; TSH, thyroid stimulating hormone; TPOAb, thyroid peroxidase antibody; IP-10, interferon-inducible protein-10; TSMR, Two-sample Mendelian randomization.

$R^2 = (2\beta^2 \times MAF \times (1 - MAF))/(2\beta^2 \times MAF \times (1 - MAF) + 2\ N \times MAF \times (1\text{-}MAF) \times SE^2)$, where MAF is the minor allele frequency, β is the effect estimate of the SNP in the exposure GWAS, SE is the standard error, and N is the sample size. Additionally, we assessed instrument strength using the F statistic, defined as $F = (R^2 \times (N - 2))/(1 - R^2)$, to evaluate the significant association of the genetic instruments with the exposure (24, 25).

2.4 Statistical analysis

The inverse variance weighted (IVW) method was employed as the primary approach in our MR analysis, providing accurate estimates in the absence of heterogeneity and directional pleiotropy between the exposure and outcome (26). The heterogeneity of the IVW model was assessed using Cochran's Q test. If significant heterogeneity was indicated by Cochran's Q test ($p < 0.05$), we transitioned from the fixed inverse variance-weighted model to the random-effects model. Additionally, the MR Egger method was utilized to estimate the causal effect, with the capability to identify and adjust for any directional pleiotropy. The MR Pleiotropy RESidual Sum and Outlier (MR-PRESSO) method was applied to assess horizontal pleiotropy (27). If horizontal pleiotropy was detected, it was corrected by removing the outlier and assessing whether substantial variations in the causal effects existed before and after outlier removal. Furthermore, the MR-Egger regression intercept term was employed to evaluate the potential presence of horizontal pleiotropy, where a deviation from zero ($p < 0.05$) suggests directional pleiotropy (28). Finally, a meta-analysis was conducted to assess the combined causality between thyroid function and IBD from MR results across various databases. The choice of effect model depended on the degree of heterogeneity observed. For minimal heterogeneity ($I^2 \leq 50\%$), the fixed-effects model was applied. For substantial heterogeneity ($I^2 > 50\%$), the random-effects model was utilized. The findings of the meta-analysis were considered the definitive evidence of causality.

All MR analyses adhered to the guidelines outlined in the STROBE-MR Statement (29). For the MR analysis examining the relationship between thyroid function and IBD, we applied a Bonferroni-corrected significance threshold, calculated as 0.0024 (0.05 divided by 21, accounting for 7 exposures and 3 outcomes). P-values between 0.0024 and 0.05 were considered indicative of potential causal associations between the exposures and outcomes. For the MR analysis between hyperthyroidism/hypothyroidism and cytokines, a p-value of less than 0.05 was considered statistically significant. All statistical analyses were conducted using the "TwoSample MR" package (version 0.5.6) in R software (version 4.2.2), and data visualization was also performed in R.

3 Results

3.1 Genetic instruments

After screening based on the corresponding p-values and linkage disequilibrium (LD) clumping, we calculated the variance explained by the genetic instruments. We quantified the instrument strength by calculating the F-statistics for each SNP, noting that a value of 10 or higher indicates adequate strength and the absence of bias from weak instruments. The number and specific characteristics of SNPs selected for each thyroid function phenotype and IBD are detailed in Supplementary Table 2 and Supplementary Table 3.

3.2 The causal effect of thyroid function on IBD, CD and UC.

We found that genetically predicted hypothyroidism was associated with a reduced risk of CD (OR = 0.761, 95% CI: 0.655–0.882, $p < 0.001$). Genetically determined hyperthyroidism has a suggestive causal effect on CD (OR = 1.030, 95% CI: 1.001–1.061, $p = 0.041$) (Figure 2). No evidence of pleiotropy or heterogeneity was detected in the MR-PRESSO global test, MR-Egger intercept test, and Cochran's Q test. Genetically determined TSH within the reference range has a suggestive causal effect on IBD (OR = 0.931, 95% CI: 0.888–0.976, $p = 0.003$), CD (OR = 0.915, 95% CI: 0.857–0.977, $p = 0.008$) and UC (OR =0.910, 95% CI: 0.830–0.997, $p = 0.043$). Little heterogeneity and no pleiotropy were observed. No causal association was observed between fT3, fT4, the ratio of fT4/fT3, and TPOAb positivity with IBD, CD, and UC (Supplementary Figures S1–S3, Supplementary Table 4 and Supplementary Table 5).

3.3 The causal effect of IBD, CD and UC on thyroid function.

In reverse MR analysis, both IBD and CD demonstrated a suggestive causal effect on the fT3/fT4 ratio (OR = 1.002, 95% CI: 1.000–1.004, $p = 0.013$ and OR = 1.001, 95% CI: 1.000–1.003, $p = 0.015$, respectively) (Figure 3). No evidence of pleiotropy or heterogeneity was detected. Genetically predicted UC was not

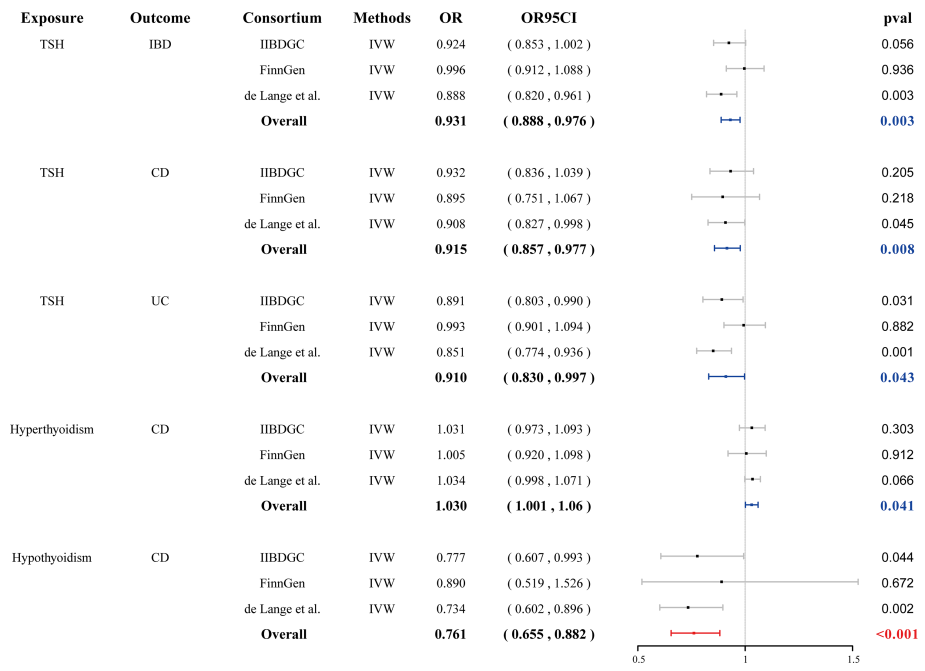

FIGURE 2
Forest plots of the association between seven thyroid function indexes on IBD and final causality. fT3, free triiodothyronine; fT4, free thyroxine; TSH, thyroid stimulating hormone; TPOAb, thyroid peroxidase antibody; IBD, inflammatory bowel disease; UC, ulcerative colitis, CD, Crohn's disease; IVW, inverse-variance weighted; OR, odds ratio.

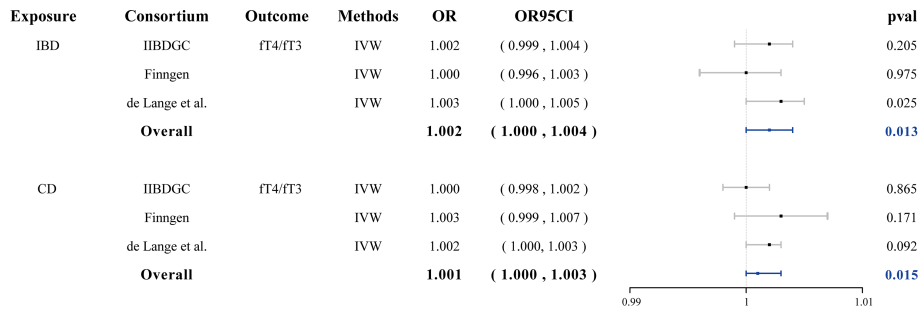

FIGURE 3
Forest plots of the association between IBD/CD on fT3/fT4 ratio and final causality. fT3, free triiodothyronine; fT4, free thyroxine; IBD, inflammatory bowel disease; CD, Crohn's disease; IVW, inverse-variance weighted; OR, odds ratio.

causally associated with the fT3/fT4 ratio. Genetically predicted IBD, CD, and UC were not causally associated with fT3, fT4, TPOAb positivity, TSH, hyperthyroidism, and hypothyroidism. (Supplementary Figures S4–S6, Supplementary Table 6 and Supplementary Table 7).

3.4 The causal effect of hypothyroidism and hyperthyroidism on cytokines

We conducted a two-sample MR to investigate the causal effects of hyperthyroidism and hypothyroidism on cytokines. We found no significant causal association between hypothyroidism and cytokines overall; however, interferon gamma-induced protein 10 (IP-10) showed an association (OR = 1.465, 95% CI:1.094–1.962, p = 0.010. No causal relationship was observed between hyperthyroidism and cytokines (Figures 4A, B; Supplementary Table 8 and Supplementary Table 9). Reverse Mendelian randomization did not reveal an association between IP-10 and hypothyroidism. We identified a significant causal association between interleukin-2 (IL-2) and stem cell growth factor beta (SCGF-β) with hyperthyroidism (OR = 1.134, 95% CI: 1.037–1.241, p = 0.006; OR = 1.106, 95% CI: 1.019–1.202, p = 0.017, respectively). The presence of interleukin-13 (IL-13) and macrophage migration inhibitory factor (MIF) are causally associated with hyperthyroidism (OR = 1.046, 95% CI: 1.008–1.086, p = 0.019; OR = 1.052, 95% CI: 1.007–1.099, p = 0.022, respectively) (Supplementary Figure S7, Supplementary Table 10 and Supplementary Table 11).

3.5 Multivariable MR

Cytokines are involved in both thyroid function disorders and IBD. We hypothesized that cytokines might mediate the causal relationship between hypothyroidism and CD. Given that hypothyroidism is only causally associated with IP-10, we incorporated IP-10 to conduct a multivariable MR. We found that the association between hypothyroidism and CD disappeared after adjusting for IP-10 in the GWAS databases of the IIBDGC and

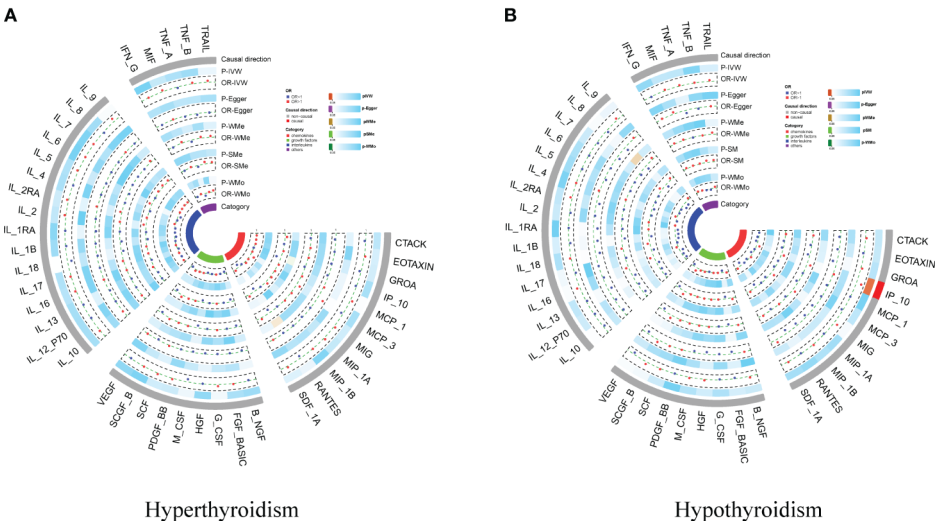

FIGURE 4
Circo heatmap of the effect of hypothyroidism/hyperthyroidism on 41 cytokines. **(A)** the effect of hyperthyroidism on 41 cytokines; **(B)** the effect of hypothyroidism on 41 cytokines.

de Lang et al. The meta-analysis further demonstrated that the association between hypothyroidism and CD was not significant (OR = 0.813, 95% CI: 0.685–0.966, p = 0.018, failing to meet the Bonferroni-corrected significance threshold of 0.0024) (Figure 5, Supplementary Table 12). This suggests that IP-10 may mediate the causal effect of hypothyroidism on CD.

4 Discussion

In this MR study, we demonstrated that hypothyroidism exerts an inverse causal effect on CD but not on IBD or UC. Additionally, we found that TSH within the reference range was suggestive of causality associated with IBD and its subtypes, while hyperthyroidism was suggestive of correlation with CD. In the reverse-directional MR, both IBD and CD demonstrated suggestive associations with the ratio of fT4/fT3.

The relationship between thyroid disorders (TDs) and IBD has been explored in numerous studies, yielding variable and occasionally conflicting results. Snook et al. (30) reported a positive association between extraintestinal autoimmune diseases and UC regardless of thyroid function status, but this association was not observed in CD. Similarly, a recent population-based study of 8,072 IBD patients identified an increased risk of TDs in UC patients aged 40 to 59 years, while no such risk was identified in CD patients (31). Casella et al. (32) observed a significantly lower prevalence of thyroid dysfunction among UC patients compared to the general Italian population. In contrast, another study found that individuals with UC experienced a markedly higher occurrence of thyroid dysfunction, two to four times more prevalent than in the general population (33). In a case-control study, Ricart et al. (34) observed a generally lower frequency of autoimmune diseases, particularly autoimmune thyroid disease, among IBD patients. A retrospective cross-sectional study, limited to individuals with CD, demonstrated only a statistically marginal reduction in the prevalence of hypothyroidism, whereas no significant change was observed in hyperthyroidism (35). A retrospective, single-center, case-control study indicated that patients with IBD have a reduced likelihood of developing thyroid disorders (36). Routine hormonal assessments and thyroid gland imaging may not be necessary in the absence of clinical signs or symptoms. According to their research findings, the development of thyroid disorders may exhibit either a positive or negative correlation with the onset of IBD or its subtypes. Even studies highlighting a positive association of IBD with increased extraintestinal autoimmunity, when compared to non-IBD individuals, noted that this phenomenon was limited to rheumatoid arthritis and dermatological disorders, excluding common autoimmune diseases such as autoimmune thyroiditis (37). Interestingly, even among IBD patients with a first-degree family history of disease, the prevalence of autoimmune diseases was not increased. Extensive research in Israel involving 12,967 individuals with IBD showed a notable increase in the prevalence of several autoimmune diseases, with the remarkable exception of TDs (38). Our investigation revealed that both IBD and CD exhibited a suggestive causal association with fT3/fT4, indicating a potential causal link between IBD and thyroid dysfunction to some extent.

Intestinal fibrosis, which results in stricture formation and obstruction, represents a significant complication of CD. Although therapeutic management of CD has improved with novel agents, an effective approach to address CD-related stricture continues to be elusive. Recent research has indicated that thyroid function is associated with fibrosis-related diseases, such as lung fibrosis (39, 40) and liver fibrosis (41, 42). Thyroid hormone has demonstrated efficacy in inhibiting lung fibrosis by enhancing epithelial mitochondrial function (43). Triiodothyronine (T3) potentially enhances the resolution of pulmonary fibrosis and inhibits fibroblast activation and extracellular matrix production. Additionally, T3 regulates the interaction between macrophages

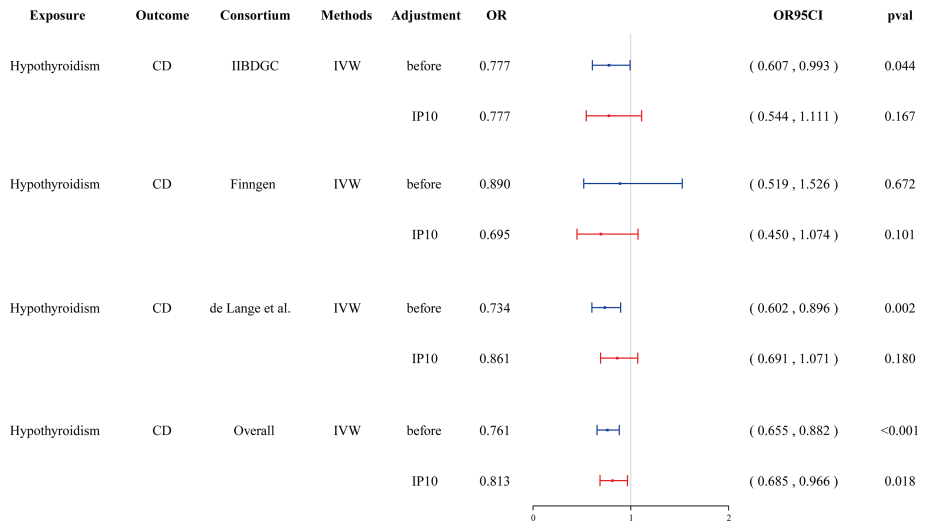

FIGURE 5
Forest plot showing the effect of hypothyroidism on CD after adjustment in Multivariate MR. CD, Crohn's disease; IVW, inverse-variance weighted; IP-10, interferon-inducible protein-10.

and fibroblasts (44). In liver fibrosis, thyroid hormone regulates the activation of hepatic stellate cells through thyroid hormone receptor α and modulation of transforming growth factor downstream signaling (45). Fibrotic stenosis of the intestinal lumen resulting from CD shares some similarities with pulmonary fibrosis and hepatic fibrosis, as all are linked to inflammation. Consequently, thyroid hormone could represent a potential therapeutic target for CD-related stricture.

A substantial body of research has documented the higher prevalence of thyroid dysfunction in IBD patients compared to the general population. However, investigations into the impact of thyroid function on IBD from alternative perspectives remain limited. Thyroid hormones not only affect gastrointestinal motor function (46) but also play a crucial role in maintaining intestinal epithelial homeostasis. Recent studies indicate that thyroid hormones and their receptors perform diverse functions in intestinal stem cells and their niches (47). Furthermore, the well-documented bidirectional crosstalk between thyroid hormones and the immune response underscores their interplay (48, 49). Studies have demonstrated that T3 elevates the number of IL-17-expressing T lymphocytes by activating dendritic cells *in vitro* (50). Circulating TH levels positively correlate with immunological reactivity in healthy individuals, supporting the physiological maintenance of lymphocyte subpopulations (51). Hyperthyroidism is associated with enhanced humoral and immune cell responses (49). Conversely, hypothyroidism is linked to contrary effects (52). Jaeger et al. (53) identified a significant correlation between TSH concentrations and various populations of effector and regulatory T cells through analysis of the immunological phenotype from the Human Functional Genomics Project. Serum IL-27 levels are elevated in subjects with hypothyroidism and inversely correlate with the incidence of nonalcoholic fatty liver disease (54). Hypothyroidism is linked to immunosuppression, which is attributed to increased frequency and activity of Gal-1-expressing Tregs, with significant implications for immunopathology, metabolic disorders, and cancer (55). Apart from the pituitary, T and B lymphocytes can synthesize and release TSH, potentially affecting both healthy and abnormal thyroid cells expressing the TSH receptor (56, 57). This novel and unexpected non-pituitary source of TSH could also be decisive in affecting the immune response during infections and chronic inflammation.

Interferon-inducible protein-10 (IP-10), also referred to as CXCL10, is a chemokine essential for the activation of integrins and migration of cells, including activated T cells, monocytes, eosinophils, NK cells, epithelial and endothelial cells (58). The IP-10/CXCR3 axis is instrumental in monocyte activation and elicits a Th1 response to facilitate effector cell recruitment in inflamed intestinal tissues (59, 60). In experimental murine models, treatment with anti-IP-10 antibodies has been shown to protect against epithelial ulceration and reduce inflammation by impairing Th1 induction and recruitment (61). BMS-936557 (previously known as MDX-1100) is a fully humanized monoclonal antibody that targets IP-10. A phase II study has demonstrated the safety and potential efficacy of BMS-936557 in UC patients (62). Our MR analysis has determined an inverse causal effect of hypothyroidism on CD but not on IBD and UC, potentially IP-10-dependent. Xian et al. (63) conducted an MR analysis examining the relationship between Grave's disease (GD) and IBD. Their findings suggest that IBD and CD may increase the risk of GD, while UC may provide a protective effect against the development of GD. Conversely, GD may slightly increase the risk of CD. However, this research underscores an ethnic disparity in terms of exposure and outcome, which could potentially explain the divergent findings observed by our team.

This study faces several constraints and limitations. A major limitation of this investigation is the lack of comprehensive GWAS in non-European ancestries, which necessitates reliance on studies conducted in European populations to estimate the causal effects. Future studies should focus on the assessment of these causal pathways in diverse ethnic groups. Another limitation stems from the absence of an association between abnormal fT3 and fT4 levels and IBD, a consequence of the constraints inherent in GWAS datasets. Regrettably, our study did not conduct subgroup analysis by gender for hypo- and hyperthyroidism, conditions that are more prevalent in females than in males. The physiological and biochemical impacts of TSH on CD require further validation via cellular and animal experiments.

5 Conclusions

This study demonstrated that hypothyroidism had an impact on CD, potentially mediated by IP-10. This discovery suggests approaches for more effective prevention and intervention of CD. However, it is important to acknowledge that these findings are based on genetic prediction and necessitate further validation through subsequent research.

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/s.

Author contributions

SW: Methodology, Formal analysis, Data curation, Writing – original draft. JY: Writing – original draft, Methodology, Formal analysis, Data curation. BW: Writing – review & editing, Supervision, Investigation, Funding acquisition.

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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.

Correction note

A correction has been made to this article. Details can be found at: 10.3389/fendo.2025.1647808.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1376139/full#supplementary-material

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Simultaneous primary thyroid MALT lymphoma and papillary thyroid cancer

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The mucosa-associated lymphoid tissue (MALT) lymphoma subtype, specifically extranodal marginal zone B-cell lymphoma, is a rare variant. Within this subtype, primary thyroid MALT lymphoma is an uncommon occurrence. The literature provides limited documentation on thyroid MALT lymphomas, as their prevalence is comparatively lower than in other organ sites. The coexistence of papillary thyroid carcinoma (PTC) and thyroid MALT lymphomas is exceedingly rare. It presents a rare case of primary thyroid MALT lymphoma accompanied by PTC, thyroid lymphoma not being considered before surgery. A 64-year-old female patient, who had been experiencing symptoms related to a substantial thyroid tumor for a duration of three years, she refused to do a needle biopsy before surgery and expressed a preference for surgical resection. Consequently, the patient underwent a total thyroidectomy along with lymphadenectomy of the central compartment. A histological examination subsequently confirmed the presence of papillary thyroid carcinoma (PTC) and mucosa-associated lymphoid tissue (MALT) lymphoma. Due to the favorable response of the MALT lymphoma to local treatment and the absence of metastasis in other organs, no further treatment was administered for the MALT lymphoma following the surgery. Currently, the patient exhibits no signs of tumor recurrence based on ultrasound and laboratory evaluations. We also provide an overview of the clinical findings on PTC and MALT lymphoma patients already reported and discuss the possible treatment strategy.

KEYWORDS

thyroid MALT lymphoma, papillary thyroid cancer (PTC), collision tumor, case report, literature review

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Introduction

The global ranking of thyroid cancer incidence places it at fifth position, with a notable increase observed in numerous countries since the 1980s (1). This rise can be attributed to advancements in detection and diagnosis techniques, resulting in a significant upsurge in the detection rates of papillary thyroid carcinoma (PTC) (1). PTC is the predominant type of thyroid cancer, affecting approximately 80% of patients (2). Conversely, extranodal marginal zone B-cell lymphoma of the mucosa-associated lymphoid tissue (MALT lymphoma) subtype is a rare variant primarily found in gastrointestinal tract lymphomas (3). Additionally, the salivary gland, thyroid gland, eye, skin, and thymus gland are frequently identified as the predominant locations (3, 4). Primary thyroid lymphoma constitutes a mere 0.6% to 5% of thyroid tumors (3, 4). The literature scarcely documents thyroid MALT lymphomas due to their comparably lower prevalence in comparison to other organ sites (4). Resulting in a dearth of consensus regarding the diagnosis and treatment of affected individuals, primarily due to its exceptionally low occurrence. Moreover, the coexistence of papillary thyroid carcinoma and thyroid MALT lymphomas is exceedingly rare. A case of concurrent primary thyroid MALT lymphoma and PTC is presented here.

Case description

A 64-year-old female patient sought evaluation at the vascular thyroid surgery clinic for the purpose of assessing a substantial thyroid tumor that had appeared three years ago. The thyroid tumor was detected, with the left thyroid gland measuring approximately 9 cm and the right thyroid gland measuring over 6 cm. Furthermore, the isthmus of the thyroid displayed a thickness of 1.6 cm (Figures 1A–C). The patient had a medical history of Hashimoto thyroiditis and follicular thyroid adenoma and had previously undergone surgical removal of a left thyroid follicular adenoma 16 years ago. Before this surgery, the patient exhibited normal thyroid function, as indicated by normal levels of FT3, FT4, and TSH. The levels of thyroid peroxidase antibodies and thyroglobulin antibodies were found to be elevated (Anti-thyroid peroxidase antibody titers exceeding 600 IU/ml and anti-thyroglobulin antibody titers exceeding 4000 IU/ml), whereas thyroglobulin (0.51 ng/ml) levels were decreased. Based on the results of a blood test, the white blood cell count was $2.57*10^9$/L and the lymphocyte absolute number was $0.39 *10^9$/L. Consequently, the patient exhibited diffuse enlargement of the thyroid gland, indicating a possible association with Hashimoto thyroiditis prior to surgery. It is worth noting that the patient had no history of smoking or habitual alcohol consumption. Laryngoscopy did not

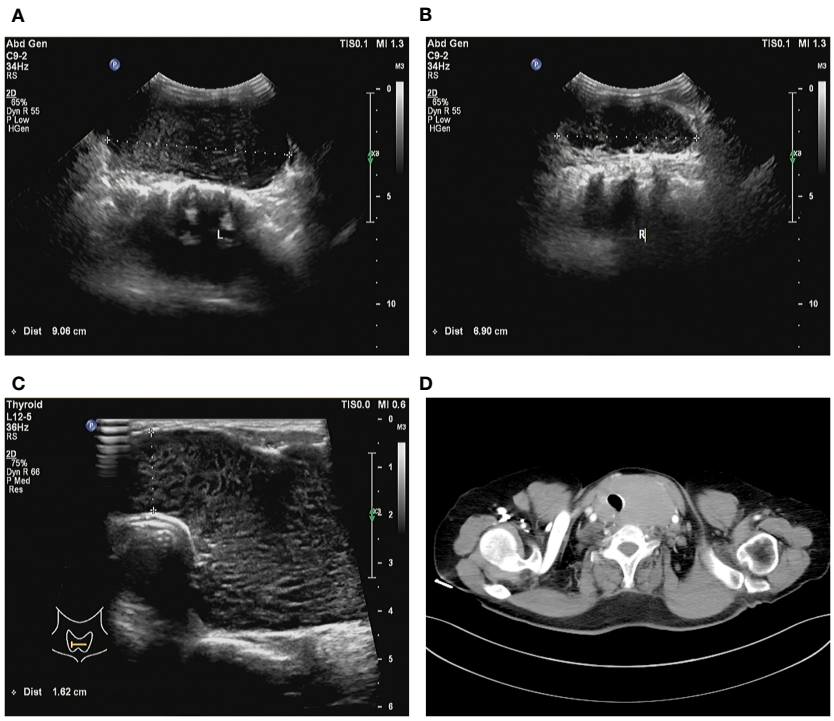

FIGURE 1
Preoperative imaging images. **(A)** Ultrasound examination detected the left thyroid gland measuring approximately 9 cm. **(B)** Ultrasound examination detected the right thyroid gland measuring over 6 cm. **(C)** The isthmus of the thyroid displayed a thickness of 1.6 cm. **(D)** Computed tomography imaging demonstrates significant enlargement of the thyroid gland, resulting in evident compression and displacement of the trachea.

reveal any signs of vocal cord paralysis, while ultrasound examination detected diffuse enlargement of the thyroid gland along with a right thyroid nodule, which consider a thyroid cancer. Furthermore, the computed tomography (CT) scan demonstrated significant enlargement of the thyroid gland, resulting in evident compression and displacement of the trachea (Figure 1D). As a result of enduring symptoms, the patient expressed a preference for surgical resection. Consequently, the patient underwent an open surgical procedure to completely remove the thyroid gland, with the objective of establishing a conclusive diagnosis and administering suitable treatment. No residual tumor was intraoperatively recognized. Subsequent intraoperative histopathological examination confirmed the presence of lymphoproliferative lesions on both thyroid glands, atrophy of thyroid follicles, active hyperplasia of a small number of follicular epitheliums (Figures 2A, B), and the presence of a 0.5 cm papillary thyroid carcinoma on the right thyroid gland based on the pathological findings (Figure 2D). We then dissected the central lymph nodes according to Chinese guidelines and our center's norms, and the pathological results showed no cancer metastases. The postoperative examination of paraffin sections confirmed a notable diffuse proliferation of lymphoid tissue, the formation of lymphoid follicles, and a significant decrease in thyroid follicles on both sides of the thyroid gland. Immunohistochemical analysis revealed a predominant proliferation of B lymphocytes, a reduced follicular dendritic cell network within germinal centers of certain lymphoid follicles, expanded marginal areas, an increased presence of plasma-like differentiated cells, and positive B-cell gene rearrangement. These findings collectively led to the diagnosis of MALT lymphoma. Immunohistochemistry (IHC) analysis demonstrated positive expression of CD20 (Figure 2C), CD79, Pax-5, CD19, MUM-1, BCL-2, BCL-6, CD10, CD38 (in plasmoid differentiated cells), CD138 (in plasmoid differentiated cells), CD21 (in follicular dendritic cell network), CD23 (in follicular dendritic cell network), CD30, CD3 (in T cells), CD5 (in T cells), CKpan (in thyroid epithelial cells), and TTF-1 (in thyroid epithelial cells) within the tumor cells of this lesion. The Ki-67 labeling index was determined to be 20%. Additionally, the Immunoglobulin H (IgH) gene exhibited positive expression, while Immunoglobulin kappa (IGK) and Immunoglobulin lambda (IGL) showed negative expression. Additionally, there was no evidence of diffuse large B-cell lymphoma (DLBCL) since the B-cell size was either normal or slightly enlarged (Figures 2A, B).

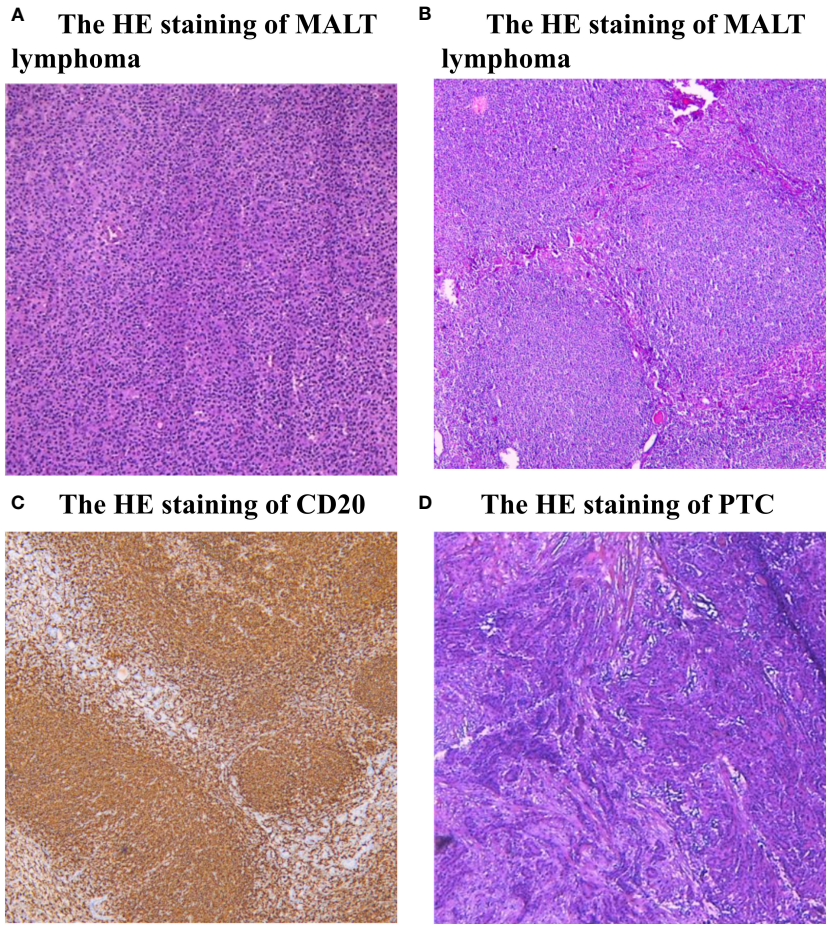

FIGURE 2
(A, B) Histopathological examination (HE) staining of MALT lymphoma. The presence of lymphoproliferative lesions on thyroid glands, atrophy of thyroid follicles, active hyperplasia of a small number of follicular epitheliums. **(C)** Immunohistochemistry (IHC) analysis demonstrated positive expression of CD20. **(D)** Histopathological examination (HE) staining of PTC, there is a typical cancer nest change in the thyroid gland.

System reviews the diagnostic assessment, the therapeutic intervention, follow-up, and outcomes of thyroid MALT lymphoma

MALT lymphoma is an indolent and low-grade type of primary thyroid lymphomas (PTL), there is a common clinical symptom of painless enlarging neck swelling with compressive characteristics that can occur progressively or suddenly (5). There is still some debate about the reliability of fine needle aspiration cytology (FNAC) in the detection of PTL, even though it is the gold standard for detecting differentiated thyroid carcinoma (6). While fine needle aspiration and cytology (FNAC) evaluation of specimens is essential for PTL diagnosis, results may be confused with Hashimoto's thyroiditis (5, 7). Additionally, it is also misdiagnosed as painless subacute thyroiditis by FNAC (5). The management of PTL has incorporated various treatments, such as surgery, chemotherapy, and radiotherapy, but no clear guidelines seem to exist (5). Previous research suggested that a Stage IE (Involvement of the thyroid alone) thyroid MALT lymphoma can be treated with either radiotherapy or surgery alone, but those with a stage greater than or equal to IE MALT lymphoma should receive multidisciplinary treatment to maximize their chances of survival (3, 8). While another scholar revealed that radiotherapy of the involved site is the favored choice for localized MALT lymphoma (9). There are also studies showing that thyroidectomy combined with radiation is also effective (3, 9). If differentiated thyroid cancer is present, surgical removal of the whole thyroid may be a better choice. For outcome, previous data revealed the overall survival rate for MALT lymphoma of the thyroid is 90–96%, and the event-free survival rate is 92% after a 5-year follow-up (10).

Discussion

Specifically, a collision tumor is a neoplastic lesion composed of cells from different cell populations that form distinct borders (11). For this reason, the present case can be categorized as a collision tumor. It is extremely rare for PTC and MALT lymphoma to coexist. Vicky Cheng et al. reported the first case of a collision tumor between PTC and MALT lymphoma in 2012 (12). MALT lymphoma, a specific type of indolent B-cell lymphoma, exhibits the ability to generate extranodal manifestations. Recent studies have shown that MALT lymphoma constitutes approximately 8% of all B-cell lymphomas, with a notable occurrence rate of 35–50% in the stomach (3). Furthermore, it is frequently observed in the lungs, thyroid, parotid, and eye adnexa. Notably, chronic antigenic stimulation is closely associated with MALT lymphoma, as evidenced by the presence of Helicobacter (H.) pylori infections in up to 80% of patients with gastric MALT lymphoma (3, 13). Thyroid lymphoma constitutes a small proportion, ranging from 0.6% to 5% of all thyroid cancers, whereas MALT lymphoma accounts for approximately 10% of thyroid lymphomas (3, 4). Furthermore, autoimmune disorders have been recognized as potential catalysts or enhancers of mucosa-associated lymphoid tissue (MALT) lymphoma (13). For instance, thyroid lymphoma is frequently linked to Hashimoto's thyroiditis (HT). The fact that HT is associated with both PTC and MALT lymphoma suggests that patients with HT should be closely monitored for both types of lymphoma (14). Notably, the prognosis for MALT lymphomas is generally more favorable, with 90–96% of patients surviving five years after diagnosis (10). However, the diagnosis and treatment of thyroid lymphoma remain subjects of controversy. Local treatment such as total thyroidectomy or radiation therapy alone is usually effective in treating pure MALT lymphoma (15). Additionally, both radiotherapy and chemotherapy are utilized in the management of recurrence (3). Derringer et al. found no significant association between the type of treatment (solely surgery, surgery with radiotherapy, surgery with chemotherapy, surgery with multimodal therapy) and survival outcomes (16). Therefore, when addressing the combination of PTC and MALT lymphoma, the primary consideration lies in surgical or chemoradiotherapy treatment (10). MALT lymphoma is generally accepted as operable when it is in the early stages, such as what was done in this case. Further large-scale follow-up data are necessary to obtain the best treatment options for PTC and MALT lymphoma.

Conclusions

Surgical intervention emerges as the preferred approach for managing PTC, while local interventions like total thyroidectomy or radiation therapy alone typically yield satisfactory outcomes in the treatment of pure MALT lymphoma. However, when confronted with the coexistence of PTC and MALT lymphoma, the primary focus should be on surgical treatment, with the potential inclusion of alternative modalities contingent upon the patient's specific clinical presentation.

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.

Ethics statement

The studies involving humans were approved by Guizhou Provincial People's Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

JZ: Data curation, Funding acquisition, Investigation, Writing – original draft, Writing – review & editing. JW: Validation, Writing – original draft, Writing – review & editing. LS: Validation,

Writing – review & editing. YC: Formal analysis, Writing – review & editing. ZY: Resources, Validation, Writing – review & editing.

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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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Associations of systemic inflammation and systemic immune inflammation with serum uric acid concentration and hyperuricemia risk: the mediating effect of body mass index

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Background: With the development of lifestyle, elevated uric acid and hyperuricemia have become important factors affecting human health, but the biological mechanism and risk factors are still unclear.

Methods: A multi-stage, cross-sectional study of 41,136 adults from the NHANES 2003-2018 was conducted. Serum uric acid concentrations, platelet, neutrophil, lymphocyte, and monocyte counts were measured. The systemic inflammation response (SIRI) index and systemic immune-inflammatory (SII) index were calculated to reflect systemic inflammation and systemic immune inflammation. The height and weight data were obtained to assess body mass index (BMI). Generalized linear models were used to examine the relationships of SIRI and SII with uric acid and hyperuricemia risk, as well as the associations of SIRI and SII with BMI, and BMI with uric acid and hyperuricemia risk. Causal mediation effect model was used to assess the mediating effect of BMI in the relationships of SIRI, and SII with uric acid concentration and hyperuricemia risk.

Results: The prevalence of hyperuricemia in US adults is 19.78%. Positive associations were found in the relationships of SIRI and SII with uric acid level, hyperuricemia risk, and BMI, as well as the relationships of BMI with uric acid and hyperuricemia risk. Causal mediation effect model showed that BMI played an important mediating role in the relationships of SIRI, and SII with uric acid concentration and hyperuricemia risk, with the proportion of mediating effect ranging from 23.0% to 35.9%.

Conclusion: Exposure to higher SIRI and SII is associated with increased uric acid concentration and hyperuricemia risk in adults, and BMI plays an important mediating effect. Reducing systemic inflammation and systemic immune inflammation and proper weight control could be effective ways to reduce hyperuricemia prevalence and related health problems.

KEYWORDS

hyperuricemia, uric acid, systemic inflammatory, systemic immuneinflammatory, body mass index, mediation effect analysis

1 Introduction

Uric acid is a metabolite derived from the metabolic breakdown of purines in the human body, predominantly eliminated through renal excretion. Elevated levels of uric acid can result in the occurrence of hyperuricemia (1, 2). As a metabolic disorder, the increasing prevalence of hyperuricemia has emerged as a significant global public health concern (3–5). During the process of rapid urbanization, the alteration of residents' modern lifestyles, changes in dietary patterns, and the escalation of obesity rates are considered important risk factors contributing to the elevation of uric acid concentration and the increased prevalence of hyperuricemia (1, 6). Previous studies indicated that the prevalence of hyperuricemia in US adults was 20.1%, affecting approximately 38 million individuals (7). Abnormally elevated uric acid concentration and hyperuricemia have been confirmed as the pathological basis of gout. Gout is an inflammatory joint disease caused by the deposition of uric acid crystals in the joints, often characterized by joint swelling, pain, and impaired mobility in affected individuals (8). Moreover, hyperuricemia is closely associated with the occurrence and progression of other diseases, such as hypertension, diabetes, chronic kidney disease, and cardiovascular diseases (4, 9, 10).

Although the specific biological mechanisms underlying the elevation of uric acid concentration and hyperuricemia prevalence have not been fully elucidated, systemic inflammation and immune dysregulation are believed to be important regulatory mechanisms of uric acid concentration (11–14). Recent studies indicated that exposure to higher systemic inflammation response index (SIRI) and systemic immune-inflammation index (SII) was associated with increased uric acid concentration and hyperuricemia risk (12, 15). For example, a cross-sectional study of 5,568 US adolescents found that exposure to higher SII levels was related to increased serum uric acid concentration and hyperuricemia risk (12). A cross-sectional study of 8,095 Chinese adults reported a linear positive association between SIRI and prevalent hyperuricemia (15). However, to our knowledge, the mechanisms underlying the positive relationships of SIRI, and SII with uric acid concentration and hyperuricemia risk remain unclear. Body mass index (BMI), as a common indicator of obesity and metabolic dysfunction (16, 17), has been reported to be significantly associated with inflammatory response (18) and elevated uric acid concentration (19–21). Moreover, previous studies indicated that exposure to higher systemic inflammation and immune inflammation may increase body weight through various biological mechanisms, leading to elevated uric acid concentration (22–24). These mechanisms include the release of inflammatory cytokines causing insulin resistance (23, 25, 26) and adipose tissue inflammation (25), exacerbation of oxidative stress (27), and dysbiosis of gut microbiota (28, 29). Based on the above findings, we hypothesized that BMI could play an important mediating role in the relationships of SIRI, and SII with uric acid and hyperuricemia risk. To the best of our knowledge, no study has examined the potential mediating effect of BMI in the relationships of SIRI, and SII with uric acid or hyperuricemia risk.

In this multi-stage, cross-sectional study, we investigated the relationships between SIRI, and SII with uric acid concentration and hyperuricemia risk. Then, the mediating effect of BMI in the relationship of SIRI, and SII with uric acid concentration and hyperuricemia risk was estimated. This study aims to identify key biological mechanisms underlying hyperuricemia and provide potential intervention strategies.

2 Methods

2.1 Study population

The study participants were derived from the National Health and Nutrition Examination Survey (NHANES), which is an ongoing cross-sectional study that investigates a nationally representative sample of adults and children in the US. The NHANES study conducts surveys on approximately 5,000 US participants from 15 counties annually, with each survey cycle spanning two years. In this study, we included a total of 80,132 participants from eight NHANES follow-up surveys conducted between 2003 and 2018. Inclusion criteria were as follows (1): participation in standard blood biochemistry testing with complete serum uric acid data; (2) participation in complete

blood cell count with no missing data for platelet, neutrophil, lymphocyte, and monocyte counts; (3) participation in physical examination with no missing data for height and weight; (4) individuals aged 18 years and above.

The NHANES study received approval from the National Center for Health Statistics Ethics Review Board (https://www.cdc.gov/nchs/nhanes/irba98.htm), and informed consent was obtained from all participants.

2.2 SIRI and SII measurement

Venous blood samples were obtained from each participant and analyzed using an automated hematology analyzer (Coulter DxH 800 analyzer) for complete blood cell count (reported as 1000 cells/μL). Based on previous literature, SIRI and SII indices were calculated using platelet, neutrophil, lymphocyte, and monocyte counts (11, 30, 31). The calculation formulas for SIRI and SII are as follows:

$$\text{SIRI} = (\text{neutrophil count} \times \text{monocyte count}) / \text{lymphocyte count}$$

$$\text{SII} = \text{platelet count} \times \text{neutrophil count}/\text{lymphocyte count}$$

2.3 Serum uric acid level measurement and definition of hyperuricemia

Approximately two-thirds of the study participants underwent standard blood biochemistry testing, and serum uric acid concentration was measured using the timed endpoint method (32). Information on sample collection and processing, quality control, and quality assurance can be found in the NHANES Laboratory Procedures Manual. Details on the analytical methods, principles, and operating procedures are provided in the NHANES Laboratory Methods Document. Hyperuricemia was diagnosed when serum uric acid concentrations were ≥ 416 μmol/L (7.0 mg/dL) in males and ≥ 357 μmol/L (6.0 mg/dL) in females (1, 2, 33).

2.4 BMI measurement

During each follow-up visit, physical examinations were conducted on the study participants to record their weight and height measurements. The body mass index (BMI) was calculated using the following formula: BMI = Weight (kg)/(Height (m)^2).

2.5 Covariate

The inclusion of covariates was based on previous NHANES studies on uric acid and hyperuricemia (7, 11, 34–39), and directed acyclic graph analysis was performed to explore the potential pathways of covariates in the relationships between SIRI, SII, BMI, and uric acid concentration, and the risk of hyperuricemia (Supplementary Figure S1) (11). These covariates included: (1) sociodemographic factors: age (34–36), sex (7, 34), race (7, 34, 35); (2) socioeconomic factors: marital status (7), education level (7, 34); (3) dietary factors: consumption of fish and shellfish (1, 32, 38); (4) lifestyle and behavioral habits related to uric acid metabolism: smoking status (7, 35, 39), alcohol consumption (34, 35, 37), and physical activity (11, 34). Standardized questionnaires were used to collect information on the above covariates from the study participants. The intake of seafood products was assessed by surveying the consumption of shellfish and fish in the past 30 days (7). Smokers were defined as individuals who had smoked a cumulative total of 100 or more cigarettes. Alcohol consumption was defined as drinking at least once a month (2017-2018) or more than 12 times a year (2003-2016) (11). Based on the 2008 Physical Activity Guidelines for Americans, participants were categorized into four groups: "high", "moderate", "insufficient" and "sedentary" (40, 41).

2.6 Statistical analysis

Descriptive statistics were used. Mean ± standard deviation (SD) was used to represent normally distributed continuous variables, while median (P25, P75) was used to describe the non-normally distributed continuous variables. Categorical variables were presented as frequency (percentage, %). To compare the differences between non-hyperuricemia and hyperuricemia groups, Student t-tests were used for normally distributed continuous variables, while Mann-Whitney U tests were employed for non-normally distributed continuous variables. For categorical variables, chi-square tests were used to compare the differences between the non-hyperuricemia and hyperuricemia groups.

The associations of SIRI, and SII with uric acid, and hyperuricemia risk were analyzed using generalized linear models. Specifically, linear regression models were used to analyze the relationship between SIRI, SII, BMI, and uric acid concentration, while logistic regression models were utilized to assess the association between SIRI, SII, BMI, and the risk of hyperuricemia. The effect sizes were reported as the association between the SIRI, SII, and BMI with each interquartile range (IQR) increase in uric acid concentration and the risk of hyperuricemia. To adjust for potential confounding factors and test the robustness of the results, the study established a crude model and two adjusted models. Based on previous research on uric acid, sociodemographic factors (age, sex, race), socioeconomic factors (marital status, education level), dietary factors (consumption of fish and shellfish) (1, 32), and other lifestyle and behavioral habits related to uric acid metabolism (smoking status, alcohol consumption, physical activity) (34) were included as covariates in the analysis.

Causal mediation effect model was conducted to evaluate whether BMI mediates the relationships of SIRI and SII with uric acid concentration hyperuricemia risk (16, 42). Briefly, in addition to observing positive associations of the SIRI index, SII index with uric acid concentration, hyperuricemia risk (exposure-outcome), the associations of SIRI index, SII index with BMI (exposure-mediator), and the relationships of BMI with uric acid

concentration, hyperuricemia risk (mediator-outcome) were examined utilizing generalized linear models. If all the associations mentioned above were statistically significant (exposure-outcome, exposure-mediator, and mediator-outcome relationships), causal mediation effect model was used to estimate the percentage of the mediating effect of BMI in the relationship of SIRI index, SII index with uric acid concentration and hyperuricemia risk (42, 43).

The statistical analysis was conducted using R software (version 4.3.2). The causal mediation effect model was conducted utilizing the "mediation" R package. A significance level of $p < 0.05$ was considered as statistical significance.

3 Results

3.1 Basic characteristics of study participants

A total of 41,136 adults from NHANES 2003-2018 were included in this study. The mean age of the study participants was 47.85 ± 18.99 years. A total of 8,136 individuals were identified as having hyperuricemia, with a prevalence rate of 19.78%. The basic characteristics of the study participants are presented in Table 1.

3.2 Comparison of SIRI, SII, and BMI between adults with hyperuricemia and without hyperuricemia

The distribution of the SIRI index, SII index, and BMI between adults with hyperuricemia and those without hyperuricemia was compared using box plots. The SIRI index, SII index, and BMI in the hyperuricemia group were significantly higher than those in the non-hyperuricemia group ($p < 0.001$) (Figure 1).

3.3 Associations of SIRI and SII with Uric acid concentration and hyperuricemia risk

The associations of SIRI and SII with uric acid and hyperuricemia risk are presented in Figure 2; Supplementary Table S1. Except for the non-significant association between the SII index and uric acid concentration in the crude model, positive associations were all observed for the relationships of SIRI and SII with uric acid concentration. After adjusting for age, sex, race, marital status, education level, smoking status, alcohol consumption, and the intake of fish and shellfish, each IQR increase in SIRI (IQR = 0.83) and SII (IQR = 333.15) was associated with an increase of 3.21μmol/L (95%CI: 2.54, 3.88) and 2.79 μmol/L (95%CI: 2.12, 3.43) in uric acid concentration, respectively. Regarding the risk of hyperuricemia, both the crude and adjusted models indicated that the SIRI and SII were positively associated with an increased risk of hyperuricemia. After adjusting for covariates, the study found that each IQR increase in the SIRI and SII was associated with a 9.2% (OR=1.092; 95%CI: 1.070, 1.115) and 7.5% (OR=1.075; 95%CI: 1.051, 1.099) increase in hyperuricemia risk, respectively.

3.4 Associations of SIRI, SII with BMI

The associations of SIRI and SII with BMI are presented in Figure 3; Supplementary Table S2. Both crude and adjusted models showed positive relationships of SIRI and SII with BMI. After adjusting for the potential covariates, each IQR increase in SIRI and SII was associated with an increase of 0.23 kg/m^2 (95%CI: 0.18, 0.29) and 0.26 kg/m^2 (95%CI: 0.20, 0.31) in BMI, respectively.

3.5 Associations of BMI with uric acid concentration and hyperuricemia risk

The associations of BMI with uric acid concentration and hyperuricemia risk are shown in Figure 4; Supplementary Table S3. The results indicated positive relationships between BMI and uric acid concentration, as well as hyperuricemia risk. After adjusting for covariates, each IQR increase in BMI (IQR=7.69 kg/m^2) was associated with an increase of 29.32 μmol/L (95%CI: 28.46, 30.18) in uric acid concentration and a 104.5% (OR=2.045; 95%CI: 1.984, 2.108) increase in the risk of hyperuricemia ($p < 0.001$).

3.6 Mediating effect of BMI in the associations of SIRI and SII with uric acid concentration and hyperuricemia risk.

The mediating effect of BMI in the association of SIRI, and SII, with uric acid concentration and hyperuricemia risk was analyzed, and the results are shown in Figure 5. The results revealed significant mediating effects of BMI in the relationships of SIRI and SII with uric acid concentration and hyperuricemia risk. The mediated proportions of BMI in the relationships of SIRI with uric acid and hyperuricemia risk were 23.0% (95%CI: 18.2%, 30.5%) and 28.0% (95%CI: 21.5%, 36.1%), respectively. The mediated proportions of BMI were 35.9% (95%CI: 27.4%, 44.6%) and 31.4% (95%CI: 24.1%, 41.8%) for the relationships of SII with uric acid and hyperuricemia risk.

4 Discussion

This multi-stage, cross-sectional study of 41,136 adults from the NHANES 2003-2018 conducted a comprehensive investigation on the relationship among SIRI, SII, BMI, uric acid concentration, and hyperuricemia risk. This study revealed that exposure to higher SIRI, and SII was associated with increased uric acid concentration and hyperuricemia risk, and BMI was identified as a crucial mediation factor in the relationships of SIRI, and SII with uric acid concentration and hyperuricemia risk. To our knowledge, this study might be the first study that assessed the mediating role of

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BMI in the relationships of SIRI, and SII with uric acid concentration and hyperuricemia risk. With the global rapid increase in uric acid concentration and hyperuricemia prevalence, our study highlights the importance of reducing SIRI and SII, maintaining body weight in lowering the prevalence of hyperuricemia, and the adverse health effects caused by high uric acid concentration.

SII and SIRI, calculated from platelets, neutrophils, lymphocytes, and monocytes, have garnered widespread attention as novel inflammatory markers in recent years. Previous studies

TABLE 1 Basic characteristics of study subjects.

	Total (n=41,136)	Non-hyperuricemia (n=33000)	Hyperuricemia (n=8136)	*P-value*
Age (years), mean ± SD	47.85 ± 18.99	46.50 ± 18.71	53.35 ± 19.12	<0.001
BMI (kg/m^2), mean ± SD	28.15 ± 6.43	27.40 ± 6.01	31.18 ± 7.13	<0.001
Sex, n (%)				<0.001
Male	20187 (49.1)	15632 (47.4)	4555 (56.0)	
Female	20949 (50.9)	17368 (52.6)	3581 (44.0)	
Rece, n (%)				<0.001
Mexican American	6509 (15.8)	5599 (17.0)	910 (11.2)	
Other Hispanic	3527 (8.6)	3003 (9.1)	524 (6.4)	
Non-Hispanic White	17965 (43.7)	14196 (43.0)	3769 (46.3)	
Non-Hispanic Black	8793 (21.4)	6736 (20.4)	2057 (25.3)	
Other Race - including multi-racial	4342 (10.6)	3466 (10.5)	876 (10.8)	
Educational Level, n (%)				<0.001
Lower than high school	14163 (34.4)	11064 (33.5)	3099 (38.1)	
High school	10141 (24.7)	8030 (24.3)	2111 (25.9)	
College graduate or above	16794 (40.8)	13878 (42.1)	2916 (35.8)	
Missing	38 (0.1)	28 (0.1)	10 (0.1)	
Marital status, n (%)				<0.001
Married	20244 (49.2)	16283 (49.3)	3961 (48.7)	
Widowed	3209 (7.8)	2218 (6.7)	991 (12.2)	
Divorced	4157 (10.1)	3239 (9.8)	918 (11.3)	
Separated	1223 (3.0)	993 (3.0)	230 (2.8)	
Never married	7719 (18.8)	6383 (19.3)	1336 (16.4)	
Living with partner	3059 (7.4)	2254 (6.8)	505 (6.2)	
Missing	1525 (3.7)	1330 (4.0)	195 (2.4)	
Smoking status, n (%)				<0.001
Yes	17701 (43.0)	1559 (4.7)	209 (2.6)	
No	21667 (52.7)	13854 (42.0)	3847 (47.3)	
Missing	1768 (4.3)	17587 (53.3)	4080 (50.1)	
Drinking status, n (%)				<0.001
Yes	26731 (65.0)	3933 (11.9)	740 (9.1)	
No	9732 (23.7)	21321 (64.6)	5410 (66.5)	
Missing	4673 (11.4)	7746 (23.5)	1986 (24.4)	
Fish consumption, n (%)				<0.001
Yes	24984 (60.7)	19947 (60.4)	5037 (61.9)	

(Continued)

TABLE 1 Continued

	Total (n=41,136)	Non-hyperuricemia (n=33000)	Hyperuricemia (n=8136)	*P-value*
No	10395 (25.3)	8555 (25.9)	1840 (22.6)	
Missing	5557 (13.5)	4495 (13.6)	1259 (15.5)	
Shellfish consumption, n (%)				<0.001
Yes	18589 (45.2)	14964 (45.3)	3625 (44.6)	
No	16799 (40.8)	13541 (41.0)	3258 (40.0)	
Missing	5531 (13.4)	4459 (13.5)	1253 (15.4)	

Statistical analyses were conducted to compare differences between non-hyperuricemia and hyperuricemia adults using Student t-tests for normally distributed continuous variables and Mann-Whitney U tests for non-normally distributed continuous variables. Chi-square tests were utilized for categorical variables to compare differences between non-hyperuricemia and hyperuricemia adults.

indicated that SIRI and SII could provide a comprehensive reflection of the immune-inflammatory status of the body and have significant predictive value for various diseases, including cardiovascular diseases (12). In the current study, we found positive associations of SIRI and SII with uric acid concentration and hyperuricemia risk in a representative US population. Our findings could be supported by existing research. For example, Chen et al. conducted a cross-sectional study on 8,095 adults from the Northeast Rural Cardiovascular Health Study in 2012-2013, and found a positive association between the SIRI index and the risk of hyperuricemia, suggesting the significant value of SIRI in risk stratification and prevention of hyperuricemia (15). Xie et al. conducted a cross-sectional study on 5,568 adolescents from NHANES 2009-2018 and reported positive associations between the SII index and uric acid concentration as well as the risk of uric acid elevation (12). In addition to SIRI and SII, C-reactive protein (CRP) is also frequently used as a biomarker of systemic inflammation (44). Previous studies have reported positive associations of CRP with uric acid concentration and hyperuricemia, which partially supports our findings. For example, a cross-sectional study on obese children in adolescence found a positive correlation between CRP concentration in serum and uric acid concentration (24). To our knowledge, this study might be the largest epidemiological study that investigated the relationship of SIRI and SII with uric acid concentration and hyperuricemia risk, which could provide more comprehensive epidemiological evidence for the relationships between SIRI, SII, and uric acid and the risk of hyperuricemia.

Moreover, this study found that BMI plays an important mediating role in the relationships of SIRI, and SII with the elevation of uric acid concentration and hyperuricemia risk. Some existing findings provide supportive evidence for our results. On the one hand, BMI, as an indicator of obesity, is closely associated with uric acid concentration and hyperuricemia risk (19–21). A study of 39,736 Chinese adults from Jiangsu Province found that uric acid concentration linearly increased with BMI, and obese patients had

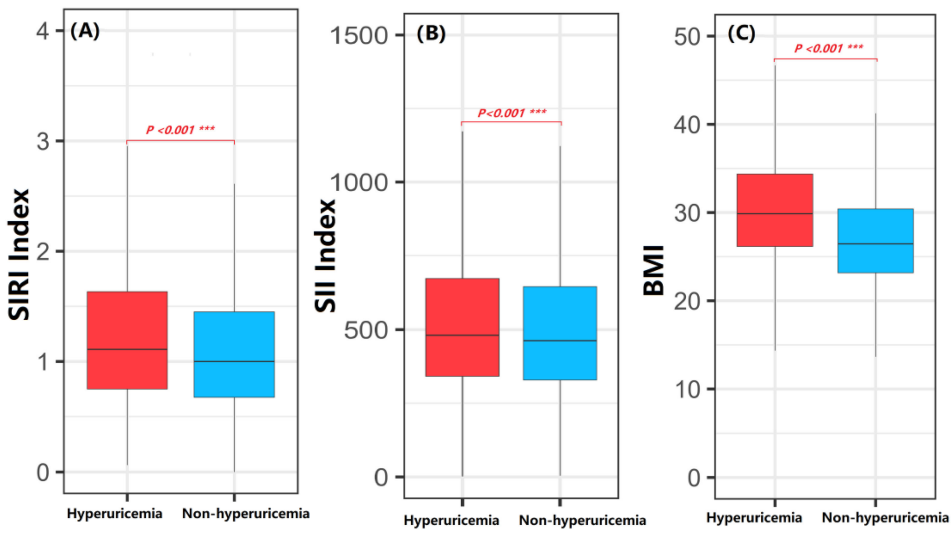

FIGURE 1
Comparison of SIRI index, SII index and BMI between hyperuricemia and non-hyperuricemia participants. Student t-tests and Mann-Whitney U tests were employed to compare the differences in SIRI index, SII index and BMI between the non-hyperuricemia and hyperuricemia groups. **(A)** SIRI index; **(B)** SII index; **(C)** BMI; The P value represents the statistical significance for testing the difference between hyperuricemia and non-hyperuricemia participants. ***P-value < 0.001.

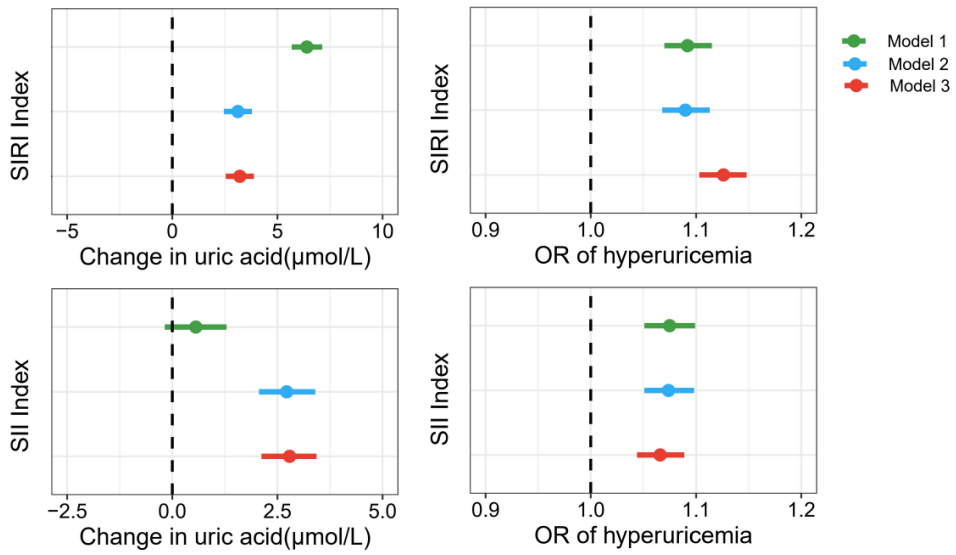

FIGURE 2
Associations of SIRI, SII with uric acid concentration and hyperuricemia risk. Model 1, unadjusted for covariates; Model 2, adjusted for age, sex, race, marital status, and education level; Model 3, adjusted for age, sex, race, marital status, education level, smoking status, alcohol consumption, and intake of fish and shellfish.

significantly higher uric acid concentrations than underweight patients. Compared to individuals with low weight, overweight individuals had an approximately 2.98 times higher risk of hyperuricemia, and obese individuals had an approximately 5.96 times higher risk of hyperuricemia (20). Moreover, BMI levels in childhood can also affect serum uric acid concentration in adulthood, exerting long-term effects on health. A study of 298 children from Japan found that rapid BMI increase in childhood was related to a significant increase in serum uric acid concentration in adulthood (19). On the other hand, multiple studies showed that exposure to higher SIRI and SII was associated with increased BMI levels (18, 45). Wang et al. conducted a cross-sectional study of 7,420 rural residents and reported a positive association between SIRI index and obesity risk (45). Chen et al. conducted a cross-sectional study of 9,301 participants from NHANES 2005-2018 and reported positive associations of SII index with BMI and waist circumference (18). To the best of our knowledge, this study may be the first study assessing the mediating effect of BMI in the association of SIRI, and SII with the elevation of uric acid level and hyperuricemia risk, and our finding indicated that controlling weight could be an effective measure to reduce the risk of hyperuricemia and adverse health effects that caused by high uric acid concentration.

Although the exact mechanism explaining the mediating effect of BMI on the positive relationships of SIRI, and SII with uric acid concentration and hyperuricemia risk remains uncertain, some

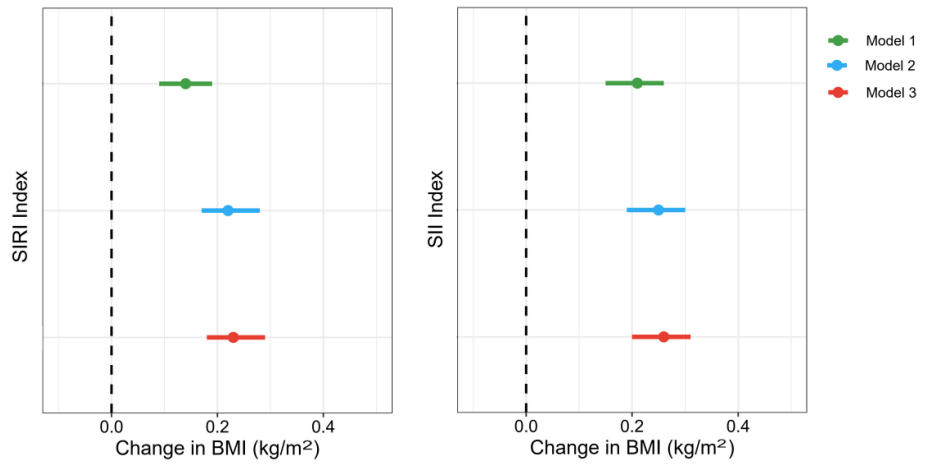

FIGURE 3
Associations of SIRI, SII with BMI. Model 1, unadjusted for covariates; Model 2, adjusted for age, sex, race, marital status, and education level; Model 3, adjusted for age, sex, race, marital status, education level, smoking status, alcohol consumption, and intake of fish and shellfish.

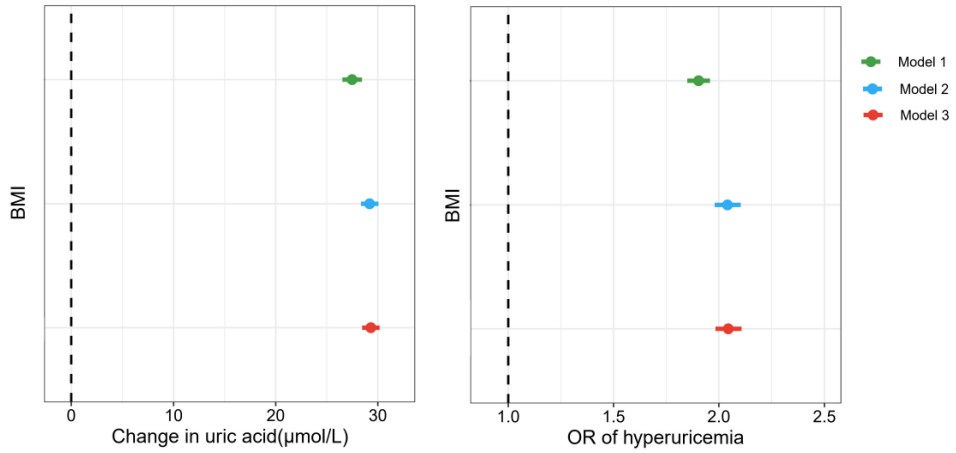

FIGURE 4
Associations of BMI with uric acid concentration and hyperuricemia risk. Model 1, unadjusted for covariates; Model 2, adjusted for age, sex, race, marital status, and education level; Model 3, adjusted for age, sex, race, marital status, education level, smoking status, alcohol consumption, and intake of fish and shellfish.

potential biological mechanisms should be paid particular attention to. Firstly, the release of inflammatory cytokines may result in insulin resistance (46), disrupting insulin signaling and affecting glucose metabolism and lipid accumulation, promoting fat deposition and obesity development (18, 47–49). Secondly, prolonged inflammation status could trigger an inflammatory response in adipose tissue, leading to dysfunction of adipocytes, affecting lipid metabolism (50) and hormone secretion (51, 52), further promoting obesity formation (53). Thirdly, oxidative stress may also play a role by disrupting cell structure and function, exacerbating tissue damage and metabolic abnormalities (54, 55). Finally, the inflammatory status may also influence the balance of gut microbiota, leading to dysbiosis, affecting energy metabolism and nutrient absorption, thereby impacting weight control and uric acid metabolism (29, 56, 57). These complex biological mechanisms interact with each other and contribute to the elevation of BMI. The increased BMI could affect uric acid metabolism through various mechanisms (58). Firstly, obesity is often accompanied by insulin resistance, which leads to reduced excretion of uric acid by the kidneys, resulting in the accumulation of uric acid in the bloodstream (59). Secondly, individuals with obesity commonly consume an excessive number of high-purine foods, thereby increasing the body's production of uric acid (60). Finally, the increased renal workload caused by obesity impairs the kidneys' ability to eliminate uric acid effectively (61). These factors collectively contribute to the elevation of serum uric acid concentration and hyperuricemia risk.

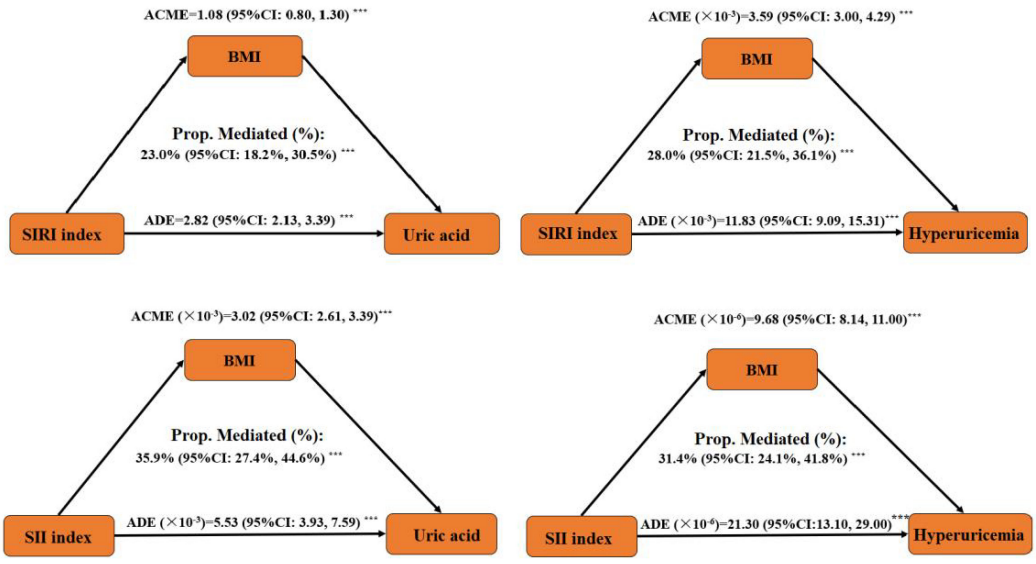

FIGURE 5
Mediation effect of BMI on the associations of SIRI, SII with uric acid concentration and hyperuricemia risk. ACME refers to the indirect effect, ADE refers to the direct effect, and the mediation percentage represents the proportion of the indirect effect to the total effect (sum of indirect and direct effects). ****P-value* <0.001.

Our study may have some strengths. First, this study might be the largest epidemiological study that investigated the relationship of SIRI and SII with uric acid concentration and hyperuricemia risk, which could provide more comprehensive epidemiological evidence for the relationships between SIRI, SII, and uric acid and the risk of hyperuricemia. Second, this study may be the first study assessing the mediating effect of BMI in the association of SIRI, and SII with the elevation of uric acid level and hyperuricemia risk, and our finding indicated that controlling weight could be an effective measure to reduce the risk of hyperuricemia and adverse health effects that caused by high uric acid concentration. However, several limitations should be noted in our study. First, although this study included 8 waves of NHANES data from 2003 to 2018, the cross-sectional study design cannot establish causal relationships between SIRI, SII, BMI, uric acid concentration, and the risk of hyperuricemia. Secondly, despite incorporating various covariates to adjust for potential confounders in our analysis, there remains a possibility that unmeasured confounding variables could introduce some bias to our findings. Finally, our study was conducted in a representative population of US adults. Further research is warranted in diverse populations, including individuals of different ethnicities and from developing countries.

5 Conclusion

Exposure to higher SIRI and SII was associated with increased serum uric acid concentration and hyperuricemia risk in adults. BMI played an important mediating role in the relationships of SIRI, and SII with uric acid concentration and hyperuricemia risk. With the increasing prevalence of hyperuricemia worldwide and the rapid rise in disease burden due to elevated uric acid concentration, this study suggests that reducing SIRI and SII and maintaining body weight could be effective measures to reduce the risk of hyperuricemia and adverse health effects caused by high uric acid concentration. In the future, longitudinal studies should be conducted to validate our findings and establish causal relationships between SIRI, and SII with uric acid and hyperuricemia risk.

Data availability statement

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

Ethics statement

The studies involving humans were approved by The NHANES study received approval from the National Center for Health Statistics Ethics Review Board (https://www.cdc.gov/nchs/nhanes/irba98.htm). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

YZ: Conceptualization, Data curation, Formal analysis, Software, Writing – original draft. SH: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. ZD: Conceptualization, Data curation, Formal analysis, Methodology, Validation, Writing – original draft. XT: Conceptualization, Data curation, Writing – original draft. XuL: Methodology, Writing – original draft. GH: Data curation, Formal analysis, Visualization, Writing – original draft. XG: Conceptualization, Writing – original draft. CT: Data curation, Writing – original draft. XiL: Formal analysis, Writing – review & editing. WY: Visualization, Writing – original draft. QZ: Formal analysis, Writing – original draft. ZN: Conceptualization, Data curation, Formal analysis, Software, Visualization, Writing – review & editing. FW: Conceptualization, Formal analysis, Funding acquisition, Methodology, Writing – review & editing.

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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/fendo.2024.1469637/full#supplementary-material

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Metabolic phenotypes in a Lyz2Cre recombinase mouse model

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The Cre-Lox system is essential in biomedical research for precise gene deletion in specific cell types, crucial for understanding genetic roles in disease. Although generally considered non-detrimental, Cre recombinase expression has been associated with potential adverse effects, including Cre toxicity, ectopic expression, and disruption of endogenous genes. We investigated the role of macrophage nucleotide-binding oligomerization domain (Nod1) in obesity-associated diabetes using myeloid-specific Nod1-knockout mice (Nod1 floxed crossed with Lyz2Cre). Our study examined Lyz2Cre as well as floxed control mice separately, unlike most research. Results indicated that Lyz2Cre expression alone impacts glucose metabolism, challenging the notion that Cre expression is harmless. This finding highlights the critical importance of including Cre-only controls in studies using floxed alleles to generate conditional knockout mouse models in order to ensure robust and accurate conclusions in molecular research.

KEYWORDS

Cre-Lox P system, Lyz2Cre, macrophage, glucose metabolism, insulin resistance, β-cell dysfunction, obesity-associated diabetes

Introduction

Using the Cre-Lox system is the predominant approach in biomedical research to understand genetic and molecular signaling pathways. This system enables the precise and targeted deletion of almost any mouse gene in specific cell types, which is essential for gaining insights into the role of genes in disease processes. Cre recombinase selectively identifies specific DNA sequences called loxP (locus of x-over, P1) sites. Upon binding to the loxP sites, Cre can excise or invert the DNA segment, creating a tissue-specific knockout when driven by a tissue-specific promoter (1, 2). While it has been widely believed that Cre expression does not have detrimental effects, there have been reports suggesting potential untoward effects

associated with Cre (3). In the absence of targeted gene loxP sites, fibroblasts (3) and pancreatic β cells (4) demonstrated undesired phenotypes only by Cre expression, leading to adverse effects on cellular physiology. Cre toxicity has been reported to impair angiogenesis (5), reduce blood cell counts (6), and lead to heart failure (7). Cre activation alone has been shown to induce primary lymphoma regression (8). Both constitutive Cre and inducible CreER have been shown to negatively impact mammalian cell health. Furthermore, the wide range of affected cell types suggests that Cre/CreER toxicity may be a general phenomenon across most mammalian cell types (9). Several studies have linked Cre expression to genotoxic effects (10, 11). These effects arise from recombination at cryptic loxP sites (12), disruption of endogenous genes (13), and off-target DNA cleavage (10). Cre protein levels may influence the severity of toxicity (14). The lysozyme 2 (Lyz2) gene is widely expressed in myeloblasts, macrophages, and neutrophils, making it a widely used marker for myeloid cells in mice (15). However, Lys2-driven recombination unexpectedly showed reporter protein expression in non-myeloid cells, including type II lung alveolar cells (16) and some other cells (17, 18), including neurons of several brain regions (19), which may affect metabolic function. It is well-known that Cre insertion in the Lyz2Cre mouse available from Jackson lab deletes the endogenous gene (4, 20), which is critical for the function of myeloid cells (21). Our laboratory has studied the role of an immune receptor, nucleotide-binding oligomerization domain 1 (Nod1), in the context of obesity associated diabetes (22). Furthermore, the transplantation of bone marrow of whole body Nod1-null mice was reported to protect against high-fat diet-induced insulin resistance (23). We wished to investigate the effect of the deletion of this receptor in myeloid cells on lipid-induced pancreatic β-cell dysfunction (β-cell lipotoxicity) (24). We unexpectedly observed that Cre Lysozyme 2 (Lyz2) expression impacts metabolic function, even in the absence of lox-P sites, i.e., without deletion of Nod1.

Methods

Animals

All procedures were in accordance with the Canadian Council of Animal Care Standards and were approved by the Animal Care Committee of the University of Toronto with the study protocol number 20011526. The mice used for all experiments were male and 12 to 14 weeks old. Ear clips were obtained for genotyping purposes. Genotyping was conducted using the Wisent Genotyping Kit, and DNA was extracted according to the manufacturer's protocols. The PCR primers and representative micrographs of genotyping results are shown in Supplementary Figure 1. Myeloid-specific Nod1-knockout (KO) mice were generated by crossing Nod1 floxed mice on a C57BL/6 background obtained from Dr. Dana J. Philpott (25) with mice expressing lysozyme 2 (Lyz2) Cre (Jackson Laboratory #004781) also on a C57BL/6 background. Nod1 floxed (Flox) and Lyz2Cre (Cre) mice were used as controls. Both controls were either littermates or mice from the same breeding colony. For all experiments, heterozygous Lyz2Cre mice were used.

All animals were housed in the University of Toronto's Department of Comparative Medicine (DCM) facility, exposed to a 12-hour light-dark cycle, and had free access to water and standard rodent chow, which contained 58% carbohydrate, 24% protein, and 18% fat by calories (Harlan Tekland #2018).

Mouse surgery

Mice were anesthetized using isoflurane (3%–3.5% for induction, 1.5%–2% for maintenance). Analgesics (Buprenorphine SR, 1 mg/kg) were administered pre-operatively to maintain therapeutic serum levels during recovery. A cannula made from 10–11 cm of polyethylene tubing (PE-10) and 1.5 cm of silastic tubing was inserted into the jugular vein, reaching the right atrium. The silastic tubing was tapered for easier insertion. The cannula was flushed with heparinized saline (40 units/mL) to prevent clotting, exteriorized at the back of the neck, and secured with tape. The mice were then placed in individual cages and allowed to recover for 3–5 days with free access to food and water. After surgery, the mice were continuously monitored until they regained consciousness and were fully ambulatory. Post-operative monitoring was conducted twice daily by both the surgeon and the DCM veterinarian to check for signs of distress or infection. A detailed log was maintained to document animal behavior, incision sites, and cannula condition.

Infusion treatment

Our model for inducing β-cell lipotoxicity through prolonged elevation of plasma palmitate levels *in vivo* involves infusing mice with ethyl palmitate (PAL) at a rate of 0.12 μmol/min for 48 hours (26). Direct infusion of unbound palmitate is highly toxic due to its detergent effects; however, adding an ethyl group to the carboxylic end of palmitate neutralizes this toxicity. Ethylpalmitate is hydrolyzed into ethanol and palmitate by ethyl esterases in mouse plasma (26), therefore, ethanol served as the vehicle control (VEH). Mice were briefly anesthetized with 1.5% isoflurane to connect the inserted catheter to our infusion setup, which allowed them full mobility in their cages. We used syringe pumps (model # PHD2000) from Harvard Apparatus Inc. for the infusions. Throughout the 48-hour infusion, the mice had access to food and water *ad libitum*. After the infusion period, a hyperglycemic clamp was conducted to assess *in vivo* β-cell function.

Hyperglycemic clamp

The mice were fasted for 5 hours prior to the start of the clamp, after which the mice were moved into the restrainer for the duration of the experiment. Blood glucose was elevated and maintained at ~20mM by an intravenous glucose infusion. A 37.5% glucose solution was used as the infusate to achieve the hyperglycemic target while minimizing the infusion volume, and it was co-infused with ethylpalmitate or

vehicle using a 2-way connector. Blood glucose was measured every 10 minutes during the 2-hour clamp via tail vein blood sampling, and the glucose infusion rate (Ginf) was adjusted accordingly. A higher Ginf represents better glucose tolerance, which is determined by increased ß-cell secretion and decreased insulin clearance to compensate for palmitate-induced insulin resistance. At time points 0, 100, and 120 minutes, 50–100µL of plasma was collected from the tail vein to measure insulin and C-peptide levels.

Plasma glucose, insulin, C-peptide, and free fatty acid measurements

Mouse plasma glucose concentrations were measured using the HemoCue Glucose 201 Analyzer.

Plasma obtained at times 0, 100, and 120 min of the clamp was used to determine levels of insulin and C-peptide. These samples were analyzed by enzyme-linked immunosorbent assays that are sandwich-type immunoassays obtained from ALPCO (Catalog #80-INSMS-E01, E10 for insulin, Catalog #80-CPTMS-E01 for C-peptide). Standard solutions were run in duplicate, while samples were run in singlicate. Both inter- and intra-assay variations are below 5% for the insulin assay and below 7% for the C-peptide assay. The insulin assay had a sensitivity of 10.34 pM with a detection range of 32.41 to 61,189.66 pM, while the C-peptide assay had a sensitivity of 7.6 pM with a detection range of 60 to 3,000 pM. Basal plasma (0 min) was used for free fatty acid (FFA) determination. The samples were analyzed by an enzymatic colorimetric assay obtained from FUJIFILM Wako Diagnostics (Catalog #999-34691, #995-34791, #991-34891, #993-35191). Standard solutions and samples were run in duplicate as per the manufacturer's protocol. The enzymatic reaction generated a purple pigment, quantified at 550 nm using a microplate reader. FFA concentrations were determined using a standard curve, with a detection range of 0.07–2.50 mEq/L and a sensitivity of 0.04 mEq/L.

Calculations

Insulin clearance index

The steady state molar ratio of plasma C-peptide to plasma insulin was taken as an established index of insulin clearance (27), which is extensively used in mice (28). This index represents insulin removal from plasma by the liver. The conversion from proinsulin to insulin in ß cells generates C-peptide, and insulin and C-peptide are co-secreted in response to glucose, but insulin is cleared by the liver in a regulated fashion, whereas C-peptide is not. The concentrations of insulin and C-peptide in plasma were measured during time points 100 and 120 of the clamps.

Insulin sensitivity index

Insulin sensitivity during the hyperglycemic clamp was calculated as the Ginf/plasma insulin at the steady state (the last 20 minutes of the clamp). With the hyperglycemic clamp, it is possible to obtain indices of both insulin sensitivity and ß-cell function, although the sensitivity index (SI) has limitations at high insulin levels due to the non-linearity of insulin kinetics (29).

Disposition index

The disposition index (DI) is a measure of ß-cell secretory function in the context of the insulin sensitivity of the subject. Normal ß cells are able to compensate for insulin resistance by increasing insulin secretion, and therefore, ß-cell secretory function *in vivo* must be evaluated considering insulin sensitivity. DI, which corresponds to Ginf, barring changes to insulin clearance, is the best-established index of ß-cell function *in vivo* (30). In the present study, DI was calculated by multiplying C-peptide as an index of insulin secretion by SI as we previously published (31).

Phagocytosis assay

Bone marrow-derived macrophages (BMDMs) were isolated from the femurs and tibiae of untreated Nod1flox, Lyz2Cre, and Nod1-KO mice. A standard Fc receptor-mediated phagocytosis assay was performed as described previously (32, 33). Medium-sized (5 µm) microspheres were opsonized with human immunoglobulin G (IgG) and incubated with differentiated macrophages for 30 minutes at 37°C. After incubation, cells were fixed with paraformaldehyde (PFA) and labeled with Alexa647-conjugated anti-human IgG to stain external (non-phagocytosed) microspheres. The cells were then washed, permeabilized with 0.1% Triton X-100 for 5 minutes, and labeled with Alexa488-conjugated anti-human IgG to identify internalized microspheres. DAPI staining was used for nuclear visualization, and images were acquired using a spinning disk confocal microscope (4–5 fields per condition). Internalized microspheres appeared green, while external microspheres were purple. Quantification was performed by counting the number of internalized microspheres in 50 cells per condition.

Statistical analysis

Data are represented as mean ± SEM. Given a coefficient of variation of 32% in our primary endpoint, i.e., Ginf, and an effect size of 68%, an $n \geq 4$/group gave us a power ≥ 0.8 with 2α=0.05. Statistical differences were evaluated with one-way ANOVA and the Holm–Sidak *post hoc* test for multiple comparisons. $P<0.05$ was considered significant. The analysis was performed using GraphPad Prism 9.

Results

Lyz2Cre protects against palmitate-induced glucose intolerance similar to Nod1 knockout

Following 48 hours of infusion, plasma FFAs were significantly increased in PAL-infused mice compared to the respective VEH-

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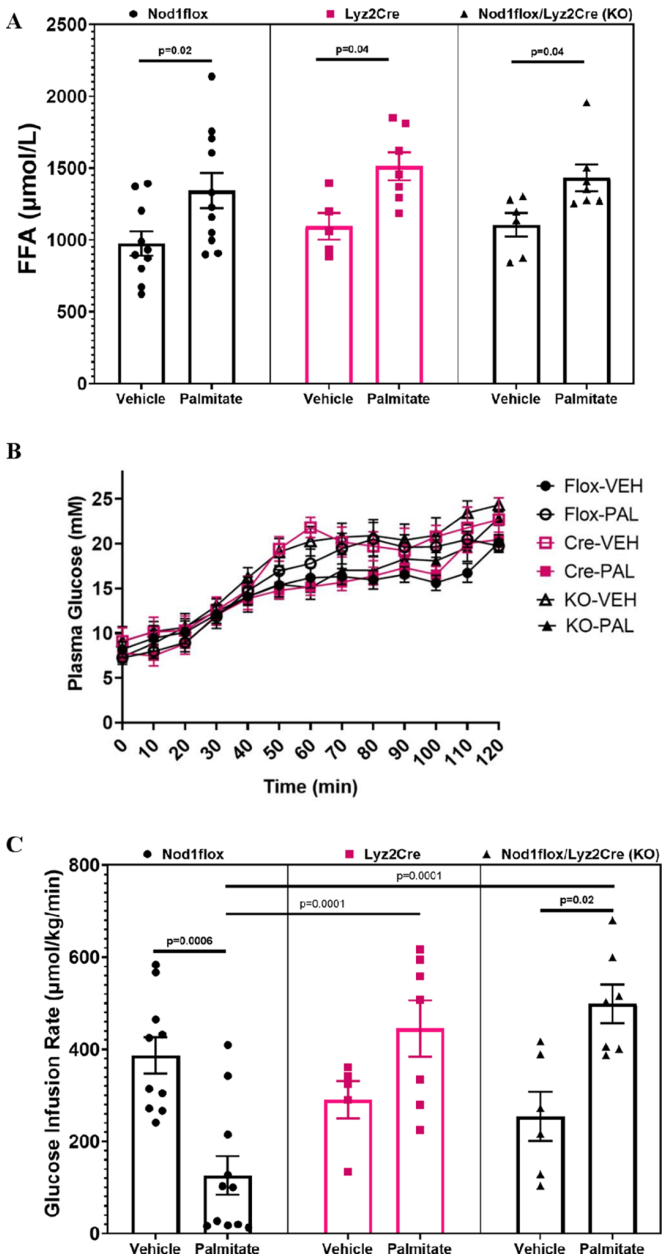

FIGURE 1
Basal plasma free fatty acids (FFAs) **(A)**, plasma glucose **(B)**, and glucose infusion rates **(C)** during hyperglycemic clamps after a 48-h infusion of either ethanol vehicle or ethylpalmitate in 12 to 14-week-old male mice. Nod1flox: Vehicle (Flox-VEH) (n=10), Palmitate (Flox-PAL) (n=11); Lyz2Cre: Vehicle (Cre-VEH) (n=5), Palmitate (Cre-PAL) (n=7); Nod1flox/Lyz2Cre (KO): Vehicle (KO-VEH) (n=6), Palmitate (KO-PAL) (n=7). Results are mean ± SEM. One-way ANOVA: **(A)** p=0.003; **(C)** p=0.0001. Significant differences between groups obtained with the Holm–Sidak test are shown in the graphs.

treated groups (Figure 1A). Plasma glucose levels increased to ~20mM in all groups during the last 20 min of the clamp (Figure 1B). The Ginf necessary to achieve and maintain the hyperglycemic target is a measure of glucose tolerance. As expected, the flox control PAL mice but not the Nod1-KO mice had reduced Ginf compared to VEH (Figure 1C); however, Ginf was not reduced with PAL in the Cre control group. This suggests that myeloid Cre expression alone was enough to induce an anti-inflammatory phenotype, thus protecting against palmitate-induced glucose intolerance.

Lyz2Cre in the absence of palmitate infusion results in reduced absolute insulin secretion and increased insulin clearance

During the clamp, plasma insulin and C-peptide levels were lower in the VEH-treated Cre and KO mice compared to the respective PAL-treated groups (Figures 2A, B), indicating reduced absolute insulin secretion. The insulin clearance index (C-peptide to insulin molar ratio) was significantly greater in the VEH-infused

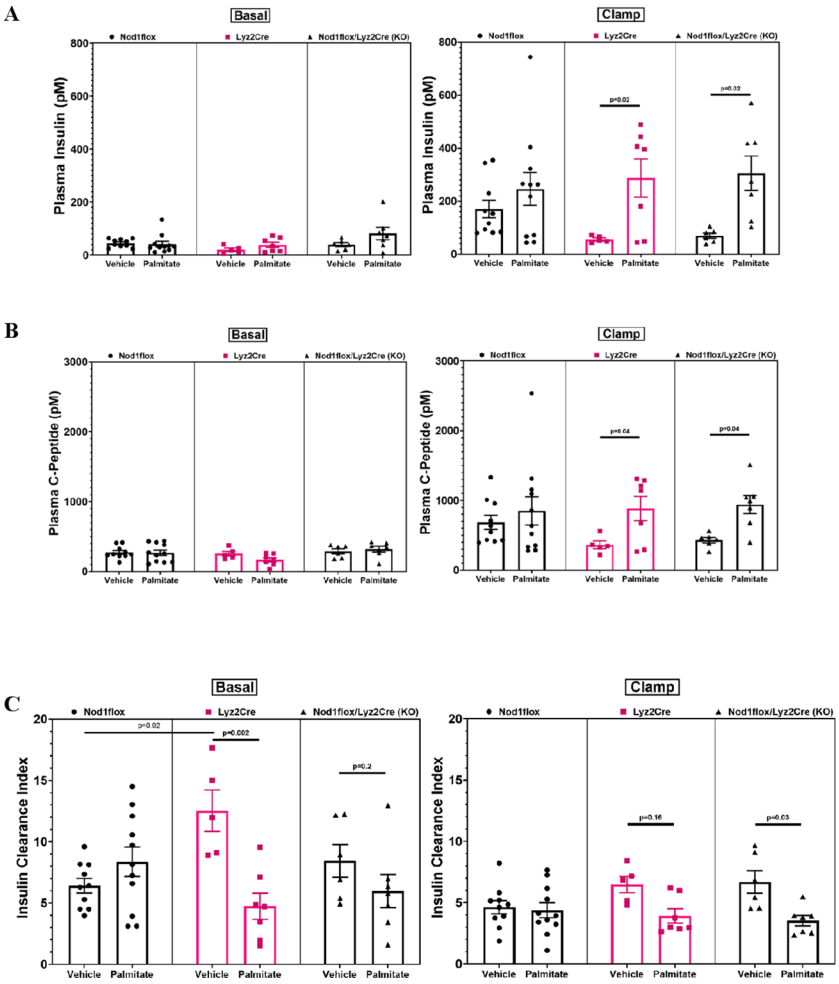

FIGURE 2
Plasma insulin **(A)**, C-peptide **(B)**, and insulin clearance index (C-peptide/Insulin) **(C)** before and during hyperglycemic clamps after a 48-h infusion of either ethanol vehicle or ethylpalmitate in 12 to 14-week-old male mice. Nod1flox: Vehicle (n=10), Palmitate (n=11); Lyz2Cre: Vehicle (n=5), Palmitate (n=7); Nod1flox/Lyz2Cre (KO): Vehicle (n=6), Palmitate (n=7). Results are mean ± SEM. One-way ANOVA: **(A)** Clamp (right panel) p=0.016; **(B)** Clamp (right panel) p=0.03; **(C)** Basal (left panel) p=0.0024, Clamp (right panel) p=0.009. Significant differences and trends ($p \leq 0.2$) between groups obtained with the Holm–Sidak test are shown in the graphs.

Cre mice during the basal period and tended to be greater during the steady-state clamp phase, compared to the PAL-treated Cre mice (Figure 2C). In the KO group, the trend was reversed, with a significantly greater insulin clearance index during the clamp phase and a tendency to increase during the basal period.

Lyz2Cre increases insulin sensitivity in the absence of palmitate infusion and, in the presence of palmitate infusion, alleviates palmitate-induced ß-cell dysfunction

The SI (Ginf/plasma insulin) decreased in all groups infused with PAL compared to VEH (Figure 3A). Although these SI results align with expectations, it is noteworthy that VEH-treated Cre mice showed higher SI compared to all other groups except the VEH-treated KO, suggesting that myeloid Cre expression in the absence of fat had an anti-inflammatory insulin-sensitizing effect. This insulin-sensitizing effect corresponded to significantly lower insulin secretion and increased insulin clearance (as described in Figure 2), suggesting compensatory changes in insulin secretion and clearance to maintain glucose homeostasis. In the presence of fat, however, insulin sensitivity was impaired, insulin secretion correspondingly increased, and the effect of myeloid Cre expression to protect against ß-cell lipotoxicity (anti-inflammatory effect on ß-cell function) manifested. This is evidenced by the DI, which, as expected, decreased in the PAL-treated flox control mice compared to VEH, while this decrease was prevented by myeloid Lyz2Cre expression alone or in combination with Nod1 deficiency (Figure 3B).

Lyz2Cre inhibits macrophage phagocytosis

A phagocytosis assay with fluorescent microspheres was used to assess the effect of Lyz2Cre expression on the phagocytic ability of BMDMs. The results demonstrated a significant reduction in the

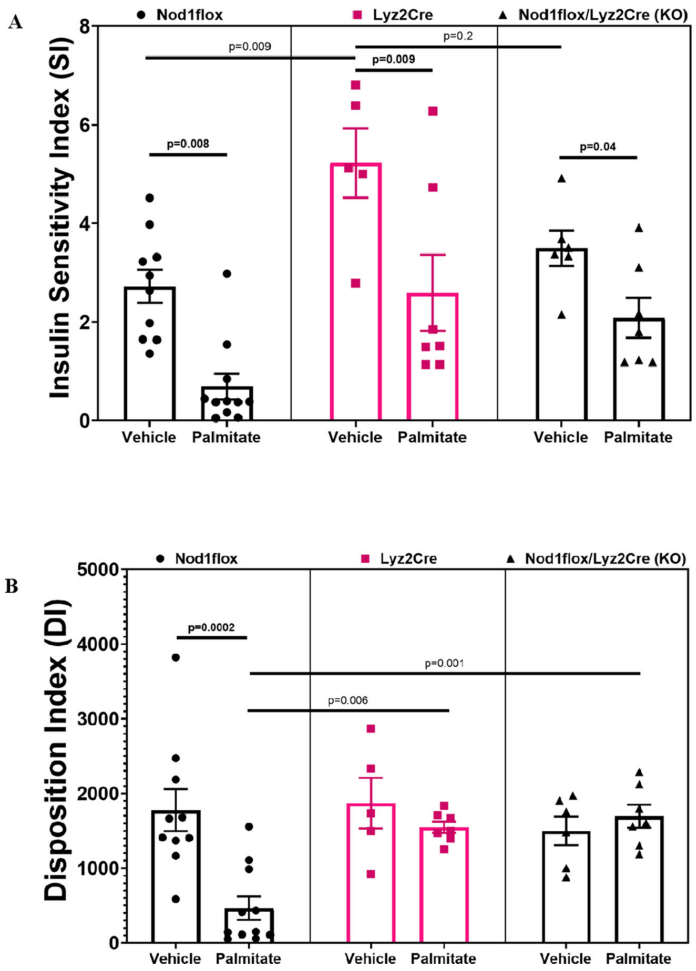

FIGURE 3
Insulin sensitivity index (SI=Ginf/Insulin, units are µmol/kg/min glucose divided by pM insulin) **(A)** and disposition index (DI=C-peptide multiplied by SI) **(B)** during hyperglycemic clamps after a 48-h infusion of either ethanol vehicle or ethylpalmitate in 12 to 14-week-old male mice. Nod1flox: Vehicle (n=10), Palmitate (n=11); Lyz2Cre: Vehicle (n=5), Palmitate (n=7); Nod1flox/Lyz2Cre (KO): Vehicle (n=6), Palmitate (n=7). Results are mean ± SEM. One-way ANOVA: **(A)** p=0.0001; **(B)** p=0.0001. Significant differences and trends ($p \leq 0.2$) between groups obtained with the Holm–Sidak test are shown in the graphs.

phagocytic activity of BMDMs expressing Cre compared to their Cre-negative Nod1 floxed counterparts (Figure 4). Specifically, Lyz2Cre+ macrophages internalized significantly fewer microspheres, as evidenced by a reduced number of green-labeled (internalized) microspheres per cell (Supplementary Figure 2A). Although the phagocytic activity of BMDMs of Nod1-KO mice (also Cre-positive) was not significantly different from that of either Nod1 floxed or wild-type Lyz2Cre mice, by combining the two Cre-positive groups, we still observed a significant difference compared to the Cre-negative mice (Supplementary Figure 2B). These findings suggest that Lyz2Cre expression impairs macrophage function.

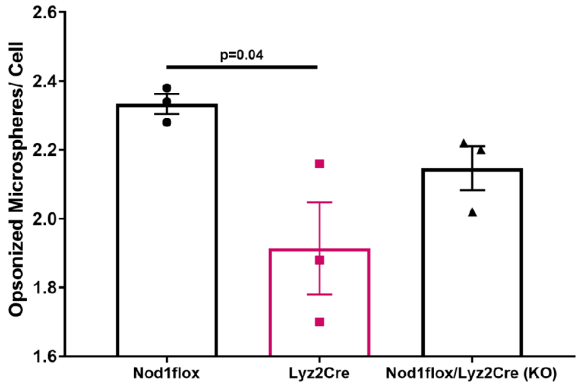

FIGURE 4
Quantitative analysis of phagocytosis in bone marrow derived macrophages of untreated Nod1 flox, Lyz2Cre, and Nod1-KO mice (n=3/group). Results are mean ± SEM. One-way ANOVA: p=0.03. Significant differences between groups obtained with the Holm–Sidak test are shown in the graph.

Discussion

We herein report an unexpected effect of Lys2Cre expression in the absence of lox-P sites to protect against FFA-induced glucose intolerance. This effect could be due to a) Cre toxicity, b)

haploinsufficiency of the Lys2 locus, or c) some effect of non-specific expression of Cre in distinct locations. It has been found (4) and also reported on the Jackson Lab website that insertion of Cre in the Lyz2Cre mouse deletes the endogenous gene essential for myeloid cell function as lysozyme-null mice show significantly increased mortality from airway infections (21), indicating impaired inflammatory/immune responses. In our study, even heterozygous Lyz2Cre deletion seems to yield dysfunctional myeloid cells that increase insulin sensitivity and protect against lipid-induced ß-cell dysfunction.

Most studies using the same Lyz2 Cre model as ours in glucose metabolism research did not examine the effect of Cre alone (34–41). Only one study reported the Cre-expressing mice as a separate control and demonstrated no changes in glucose tolerance evaluated with oral glucose tolerance test (OGTT) in the absence of a fat challenge (42). From our results, we surmise that Lyz2 Cre expression alone affects the function of myeloid cells, including monocytes, macrophages, and granulocytes. Given that macrophages, via their cytokine products, are the primary myeloid cells reported to be involved in insulin resistance (43, 44) and ß-cell dysfunction (26), our findings suggest an impact on macrophage function, which likely contributed to the observed changes in glucose metabolism. Indeed, we demonstrated macrophage dysfunction induced by Lyz2Cre expression, evidenced by reduced phagocytic activity. This raises the possibility that, besides glucose metabolism, other processes involving macrophage activity (for example, atherogenesis) may also be impacted. Monocytes and macrophages form a highly heterogeneous immune cell population lacking specific markers or transcriptional factors. Their gene expression patterns vary dynamically during prenatal development, adult tissue homeostasis, and inflammatory diseases (17). Current models have limitations in depletion efficiency and targeting specificity for endogenous macrophages (18). Off-target expression of Lyz2 in non-myeloid cells, such as neurons (19) that may regulate glucose metabolism (45), suggests that non-specific Cre expression in these additional cell types may contribute to the phenotype observed in our experiments. Mice exclusively carrying the Cre transgene are not always reported as controls, possibly because of the widespread belief that Cre expression has minimal to no impact on cellular function. However, our findings challenge the validity of certain conclusions in studies lacking an analysis of appropriate controls, including mice carrying the Cre transgene alone.

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 animal study was approved by Animal Care Committee of the University of Toronto. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SR: Data curation, Formal analysis, Investigation, Software, Writing – original draft, Methodology, Writing – review & editing. JY: Writing – review & editing, Data curation, Investigation. AV: Data curation, Investigation, Methodology, Writing – review & editing. NG: Conceptualization, Project administration, Resources, Writing – review & editing. AG: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing – review & editing.

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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.2025.1499858/full#supplementary-material

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Corticosterone effects induced by stress and immunity and inflammation: mechanisms of communication

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The body instinctively responds to external stimuli by increasing energy metabolism and initiating immune responses upon receiving stress signals. Corticosterone (CORT), a glucocorticoid (GC) that regulates secretion along the hypothalamic-pituitary-adrenal (HPA) axis, mediates neurotransmission and humoral regulation. Due to the widespread expression of glucocorticoid receptors (GR), the effects of CORT are almost ubiquitous in various tissue cells. Therefore, on the one hand, CORT is a molecular signal that activates the body's immune system during stress and on the other hand, due to the chemical properties of GCs, the anti-inflammatory properties of CORT act as stabilizers to control the body's response to stress. Inflammation is a manifestation of immune activation. CORT plays dual roles in this process by both promoting inflammation and exerting anti-inflammatory effects in immune regulation. As a stress hormone, CORT levels fluctuate with the degree and duration of stress, determining its effects and the immune changes it induces. The immune system is essential for the body to resist diseases and maintain homeostasis, with immune imbalance being a key factor in the development of various diseases. Therefore, understanding the role of CORT and its mechanisms of action on immunity is crucial. This review addresses this important issue and summarizes the interactions between CORT and the immune system.

KEYWORDS

stress, corticosterone, immunity, inflammation, mechanism

1 Introduction

During various physiological, psychological, and social stress events—such as those arising from unhealthy habits, pessimistic cognition, work difficulties, and interpersonal conflicts—the body undergoes a series of physiological stress reactions (1–3). It's important to note that these stressors are responsible for inducing physiological changes. In today's society, stress events are commonplace. While moderate stress can enhance the body's

ability to cope with social challenges, prolonged stress can detrimentally affect the functioning of various bodily tissues, with the degree of damage increasing over time (4, 5). In response to stress, the body employs intricate coping mechanisms involving neural transmission, sequential activation of signaling molecules, and interactions among different bodily systems, ultimately leading to changes in physiological representation and behavioral patterns. Although the body can adapt to stressors, this adaptation may come at the expense of health. Currently, numerous diseases have been associated with chronic stress, including anxiety, depression, cognitive impairment, inflammatory gastrointestinal diseases, metabolic syndrome, autoimmune disorders, and infertility (6–10). It can be seen that chronic stress will disrupt system function from multiple perspectives. And in the later stages of the disease, mental disorders such as anxiety and depression often co occur with peripheral lesions. Such as cardiovascular disease, gastrointestinal dysfunction, autoimmune diseases, and infertility. According to statistics, the lifetime prevalence of anxiety disorder is 5% -13% (11), and in a very few countries, such as the United States, it can reach 34% (12). Shorey S's study shows that the global prevalence of self-reported depression is 34%, of which the incidence rate of major depressive disorder (MDD) is 8% and the lifetime prevalence is 19% (13). And these proportions are still increasing. During the SARS-CoV-2 pandemic, the global emotional burden increased, with an increase of 76.2 million cases of anxiety disorder and approximately 18.6% of people experiencing anxiety being accompanied by moderate to severe depression (14, 15). Krittanawong C's meta-analysis showed that depression increases the risk and mortality of cardiovascular diseases, such as congestive heart failure and myocardial infarction (16). And Brock J's systematic review suggests that depression increases disability and mortality in rheumatoid arthritis (RA) (17). Clinical data shows that similar immune mechanisms (excessive secretion of pro-inflammatory cytokines) often lead to comorbidity between the two and mutually promote their onset. The incidence rate of depression in RA is 2-3 times that of the general population, and about 16.8% of RA patients suffer from depression (18). In addition, Indira R's review provides a detailed report on the association between depression and sexual dysfunction (19), with sexual dysfunction observed in 63% of MDD patients (20). There are also epidemiological clues that indicate a decrease of sex hormone levels in patients with depression (21). Thus, stress-induced injury is systemic in nature. There are common pathological mechanisms among various diseases associated with stress, leading to their interdependence. Owing to the complexity of its mechanisms and its widespread detrimental effects, making it a prominent focus of research. Meanwhile, this is also a crucial step in exploring the etiology and therapeutic targets of diseases.

Corticosterone (CORT), a type of glucocorticoid (GC), is a product of the hypothalamic-pituitary-adrenal (HPA) axis. It is rapidly secreted and regulated under neural control and plays a crucial role in stress adaptation due to its wide range of hormonal properties (10). In long-term stress conditions, the feedback mechanism of the HPA axis gradually falters, leading to an increase in serum basal CORT levels (22, 23). This elevation has been associated with various diseases. Chronic mild systemic inflammation serves as an important pathway mediating disease occurrence, characterized by increased levels of pro-inflammatory cytokines in serum, dysfunction of bone marrow and lymph nodes, and inflammatory damage to brain neurons. CORT has been shown to exert pro-inflammatory effects (24–26). However, stress response is a complex physiological and pathological process. During the initial stages of acute stress, the rapid elevation of CORT concentration demonstrates anti-inflammatory effects in managing acute events (27). The level of CORT depends on the severity and duration of exposure to the stressor, as well as the traumatic effects of stress (28, 29). As a mediator between stress and bodily responses, the dual role of CORT has attracted considerable research attention. The concentration, duration, and mode of action of serum CORT are key determinants influencing different effects (30).

The immune system serves as the body's protective mechanism and can recognize and eliminate foreign antigens as well as mutated or aging cells within the body (31). Its primary role is to maintain organismal internal environment homeostasis, with bodily damage and repair processes relying on its functionality. Thus, when confronting and managing stressful situations, the immune system's function is indispensable. The interaction between the effects of CORT and the immune system is essential in determining the body's state and trajectory during stress (23). Studies have demonstrated that depression can compromise the immune system, increasing the susceptibility to infection (32, 33). Prolonged elevation of serum basal CORT levels continuously activates the immune system, disrupting the body's homeostasis and leading to various forms of damage (10, 34, 35). Chronic inflammation, widely recognized as a mechanism of injury, is frequently implicated in this process (36, 37). Despite its defensive role, chronic inflammation also contributes to tissue damage. Nevertheless, research has shown that even a slight increase in CORT levels can alleviate oxidative damage and enhance innate immunity (38). Therefore, understanding the relationship between CORT concentration, its dual effects, and subsequent immune system signaling regulation is crucial, given its significance as a key stress hormone in peripheral circulation.

The impact of stress response on the body is profound, with the HPA axis and the immune system serving as the primary mechanisms for coping with challenges. These systems are closely interrelated and represent important pathways through which stress can induce various biological injuries. CORT, a hormone produced by the HPA axis, directly interacts with the immune function of the body's tissues in the bloodstream, underscoring the significance of its regulatory mechanism. The level of CORT serves as a tangible indicator of stress in the body, with its effects being closely linked to susceptibility to various diseases through modulation of the immune system and mediation of inflammation development (39). Therefore, the purpose of this review is to elucidate and analyze the relationship between CORT levels and immune regulation and to summarize the biological mechanisms through which stress impacts the immune system. The research question is shown in Figure 1. Given the intimate connection between the

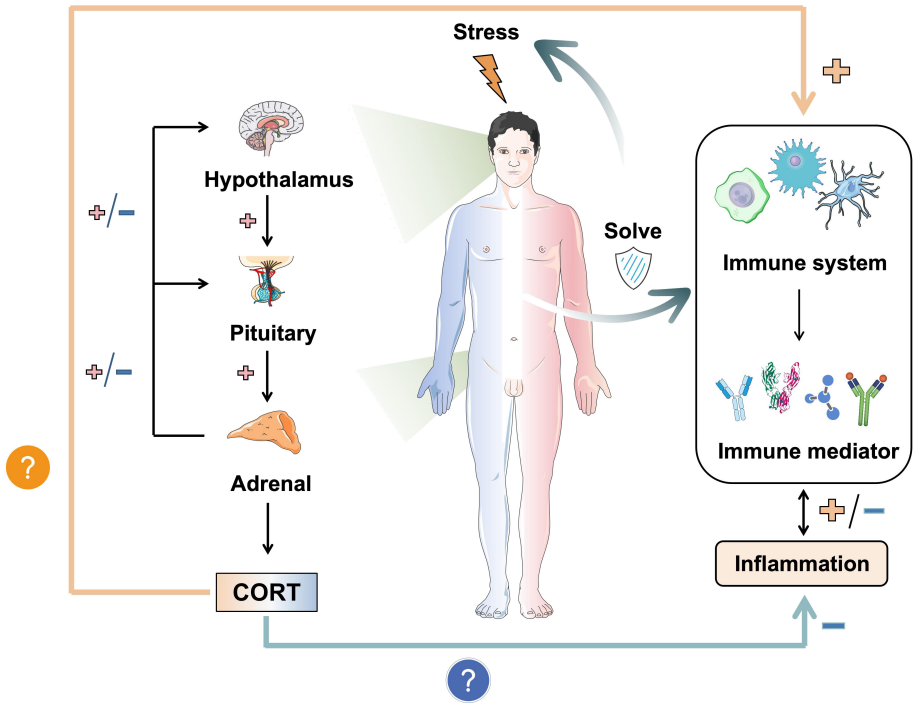

FIGURE 1
The dual mechanism of stress-induced CORT on immunity and inflammation. Stress induces the release of CORT by activating the HPA axis. It is worth noting that the immune effects of CORT are dual, namely anti-inflammatory and pro-inflammatory. It depends on the duration of stress and the mode of action of CORT. How CORT interacts with the immune system and correlates inflammation and disease is the focus. In this figure, →: Action site. +: Positive feedback signal transmission/activation. -: Negative feedback signal transmission/suppression.

immune system and various tissues throughout the body, this review aims to facilitate the exploration of the pathogenesis of systemic diseases triggered by stress in the future and to clarify the intricate relationship between stress and the body at the cellular and molecular levels.

2 Changes in corticosterone levels and their immune effects

2.1 The dual identity of CORT concentration controlled by stress and HPA axis

2.1.1 Short term stress (acute stress)

CORT, as the primary hormone driving the adverse effects of chronic stress on the body, holds significant importance in both the immune system response and stress response (40). Numerous studies have indicated that the HPA axis remains active, and circulating CORT concentrations increase in individuals with depression (41). Serving as the swiftest neuroendocrine regulatory mechanism for stress response, the HPA axis's failure in feedback mechanisms leads to chronic damage through inflammation (42). Repeated administration of CORT stimulation in animals has been observed to lead to HPA axis dysfunction, neuronal damage, cognitive decline, and memory impairment through the P2X7/NF-κB/NLRP3 signaling pathway (43). Data from early studies showed that, it is generally accepted that the baseline plasma concentration of CORT in rats ranges from 50-100 ng/ml (44), while in stressed rats, it can range from 120-425 ng/ml (45, 46). CORT levels simulating acute stress stimulation usually exceed 15-20 ng/ml (47), and *in vivo*, CORT concentrations of 1-5 nM can bind with GC receptors (GR) to exert effects (48).

The mechanism of CORT production in the central nervous system has been extensively studied and summarized (49, 50). Recent research has revealed that the activation of Agouti-related protein (AgRP) neurons, which are related to autophagy and energy metabolism, also promotes CORT production (51). Research indicates that acute stress triggers the activation of AgRP neurons, leading to the expression of neuropeptide Y (NPY), which then promotes presynaptic inhibition of GABAergic neurons expressing NPY1R and activates CRH neurons in the paraventricular nucleus (PVN) of the hypothalamus, thereby stimulating the HPA axis and increasing circulating CORT levels. Subsequently, negative feedback regulation of the HPA axis inhibits AgRP neuron activation and CORT secretion. The increase in CORT caused by acute stress is swiftly suppressed to a resting value due to this negative feedback regulation. However, long-term chronic stress accompanied by HPA axis feedback failure gradually elevates basal CORT levels, which cannot be effectively reduced. At this stage, CORT activates immunity and triggers inflammation as the main effect (52, 53).

Observations of cell states reveal that CORT exhibits two immune effects at different concentrations. Emaya et al. (54)

investigated the impact of GP120, a neurotoxic viral glycoprotein, on human microglia (HMC3). In the absence of GP120 treatment, CORT at concentrations of 32, 100, and 320 nM activated HMC3 cell activity, with the most pronounced effect observed at 100 nM. Following GP120 treatment, 100 nM CORT effectively attenuated GP120-induced neuroinflammatory damage in HMC3 cells within 24 hours, whereas the effects of 32 and 320 nM CORT were not significant. This highlights the anti-inflammatory role of CORT in acute infections. Moreover, similar to Emaya et al.'s findings (55), the simultaneous addition of GP120 and 1 μM CORT mitigated GP120-induced neurotoxicity in microglia. In the early stage of GP120 infection, an instantaneous increase in CORT concentration exerts immunosuppressive effects by promoting macrophage phagocytosis activation, clearing pro-inflammatory cells and debris, and inhibiting the production of neurotoxic cytokines within glial cells. However, following pre-treatment with 1 μM CORT for 24 hours and subsequent addition of GP120, the neurotoxic effect of GP120 in microglia was enhanced by 15%. Long-term exposure to increasing CORT concentrations promotes inflammation, indicating that the effect of CORT is related not only to its absolute concentration but also to whether the concentration is stable or fluctuating. The timing of CORT administration is also crucial. Pre-treatment with CORT before infection typically has a dominant cytotoxic effect, whereas post-infection CORT treatment demonstrates an anti-inflammatory effect. This suggests that prolonged elevated CORT continuously activates and depletes the immune system, leading to more severe damage when encountering additional stressors.

2.1.2 Long term stress (chronic stress)

Prolonged stress can lead to chronic inflammation, with continuous production of inflammatory cytokines (56). The internal environment influenced by age also affects the immune regulatory effect of CORT, and age is a significant predictor of the severity of stress-induced damage (57), often correlating with the duration of stress exposure. The aging phenotype is essential, as aging can impair both the activation of the HPA axis induced by acute stress and the feedback regulation capability of the HPA axis (58). In elderly mice, the increase in CORT levels after acute stress is less pronounced than in young mice, and CORT levels remain elevated compared to the resting value at 4 hours. In contrast, the HPA axis feedback regulation in young mice is more sensitive, with CORT peaking at 2 hours and returning to baseline by 4 hours (51). Changes in CORT levels during stress are related to the function of the HPA axis. In young mice, normal HPA axis feedback inhibits the effects of high-level CORT, while older mice exhibit poorer stress responses and are more susceptible to damage from prolonged CORT exposure. Cellular aging mediates the response to stressors, involving mechanisms such as the HPA axis hormones, the sympathetic and parasympathetic nervous systems, thymic hormones, and pineal melatonin (59). Therefore, the HPA axis is a key regulator for the body's adaptation to stress. Dysfunction of the HPA axis can synergistically lead to various stress-dependent diseases through neural, immune, and endocrine pathways (60, 61). Individual characteristics are crucial for identifying those with increased vulnerability to stress (62). During acute stress, CORT levels increase but quickly return to baseline, with this range of increase and decrease diminishing with age (63). This indicates that the ability to handle stress deteriorates with age, increasing susceptibility to damage. Additionally, sensitivity to stress depends on factors such as the internal environment, genetic diversity, and gender (64).

2.2 Immune activation and immunosuppression caused by CORT

The behavioral changes induced by fluctuations in cyclic CORT levels exhibit a holistic nature. However, the effects of CORT may vary locally depending on specific tissues and cells (65). Factors such as enzyme activity, expression levels of GR (encoded by Nr3c1), receptor variations, and local cellular signaling interference (e.g., NF-κB, CREB, STAT signals) may influence CORT utilization (66–68). The difference between immune enhancement and inhibition caused by stress appears to be mediated by the duration, intensity, and observed immune components of the stressor (69). Additionally, sustained stress characterized by inflammatory aging leads to immune system depletion, resulting in overall immune suppression (70, 71). The duration of stress exposure is an important factor in guiding the immune response. Sarjan et al. were the first to examine the effects of stress exposure duration on the immune system (72). Animal experiments showed that prolonged exposure significantly decreased the count and activity of immune cells (myeloid cells and lymphocytes), bone marrow stem cells, blood immunoglobulin, and IL-12 levels. Conversely, 3β-hydroxysteroid dehydrogenase (3β-HSD) activity, circulating immune complexes (CIC), and IL-10 levels increased with prolonged exposure. Cell experiments confirmed the concentration-dependent immunosuppression of CORT, with CORT-induced cell death being the primary cause of immune dysfunction. Short stress exposure leads to a faster recovery, whereas sustained stress may cause irreversible damage.

Regarding immune activation, even an increase in CORT concentration within a physiological range (microstimulation) can activate immune function. Innate immunity is primarily affected by its mechanism, which is related to the production of pro-inflammatory mediators. When the stressor is removed, CORT is quickly suppressed by the HPA axis to its resting value. Vágási CI conducted a study on maintaining CORT levels within the physiological baseline range (38). The intervention involved subcutaneously implanting drug particles containing 2 mg CORT in sparrows (degraded after about 2 months), raising plasma CORT concentration to approximately 8.5 ng/ml. One month post-implantation, this intervention was found to significantly increase the humoral components of innate immunity in sparrows, including natural antibodies and complement levels (measured by hemolysis hemagglutination assay). However, no significant changes were observed two months post-implantation, possibly due to drug particle degradation. Previous studies have shown similar results (73, 74), indicating that short-term stress exposure visibly enhances the innate immune system. While enhancing

immunity may seem beneficial, it comes at the cost of increased material and energy metabolism. Animals treated with CORT in this study showed weight loss and poor hair quality despite enhanced innate immunity. Immune activation increases energy consumption and metabolite production, such as oxides and acidic substances, which can induce cell damage and further stimulate inflammation (75). Therefore, the level and duration of CORT affect the body's ability to metabolize and decompose toxins. Prolonged stress and the inability to reduce CORT concentration can cause functional changes, disrupting the balance between damage and repair. Sustained stress exacerbates damage and may lead to disease induction. The relationship between circulating CORT levels and immunity is illustrated in Figure 2.

2.3 The immune effects of stress and CORT

2.3.1 Innate immunity and acquired immunity

Most immune responses to stress are initiated through innate immune pathways. Toll-like receptors (TLRs) on the immune cell membrane are essential for CORT's influence on the immune response. In macrophages, dendritic cells (DCs), and natural killer (NK) cells, TLR activation promotes intracellular NF-κB phosphorylation and nuclear translocation, enhancing antigen presentation, phagocytosis and the production of various pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α, IFN-γ, and IL-18 (76, 77). This process also activates caspase-1, further promoting inflammatory signaling (78). In acute stress situations, such as on the skin's surface, cytokines and chemokines recruit macrophages and NK cells to the site to prevent the spread of pathogens (79). Typically, innate immunity is utilized to counter external stress. If the response concludes at this stage, innate immune cells will not engage T cells. Instead, they present antigens to B cells to establish immunological memory (memory B cells) and produce antibodies (plasma cells) for future stress conditions (80). Importantly, long-term elevated CORT due to chronic stress is believed to cause immune cells to continuously receive "battle" signals, perpetually activating both innate and acquired immunity (81). Cytokines produced by innate immune cells (i.e., NK cells and macrophages) create signaling connections

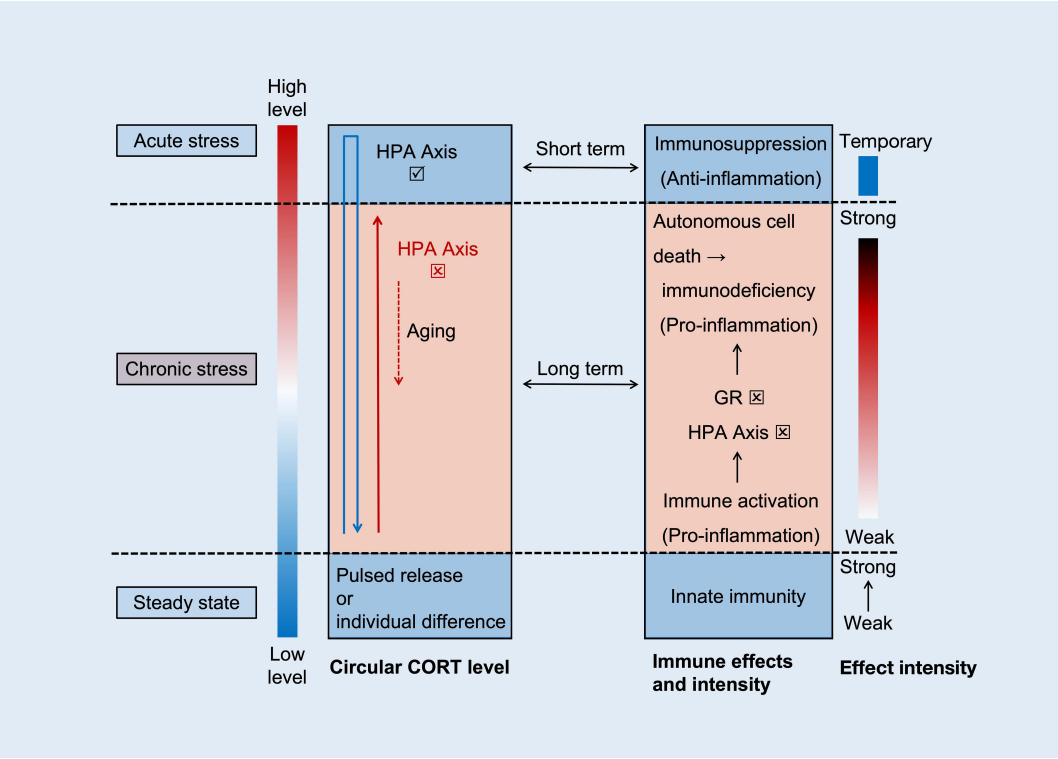

FIGURE 2
The relationship between CORT cycle level and immunity. The levels and functions of CORT are manifested in three distinct scenarios, namely steady state (normal level), acute stress (rapid high level and then recovery), and chronic stress (slow increase - sustained high level - slow decrease). Within the normal threshold, CORT levels are positively correlated with innate immune function. In acute stress, CORT rapidly increases (blue arrow), with anti-inflammatory effects being the dominant effect. And after the stress ends, restore stability based on the negative feedback function of HPA axis. Both are blue background boxes, representing physiological status. In chronic stress, CORT slowly increases and remains at a high level (red solid arrow), with pro-inflammatory effects being the dominant effect. As time goes on, dysfunction of the HPA axis and GR further exacerbates the inflammation and promotes cell death. Eventually, with aging, CORT slowly decreases (red dashed arrow), and immune suppression at this point is the result of immune exhaustion.

with immune cells (T cells and B cells) involved in acquired immune responses in the peripheral blood circulation, with antigen presentation further enhancing this connection. Antigen-presenting cells expressing major histocompatibility complex class I (MHC I) primarily activate $CD8^+$ T cells, which are pivotal for cellular immunity by engaging in the phagocytosis of antigens (82). Conversely, antigen-presenting cells expressing MHC II mainly activate $CD4^+$ T cells (helper T cells) (82, 83). Subsequently, these activated $CD4^+$ T cells can initiate the activation of B cells, prompting their differentiation into plasma cells responsible for antibody secretion, thereby fostering humoral immunity (84). Additionally, helper T cells polarize into different phenotypes (Th1/Th2 and Th17/Treg) and secrete various cytokines to participate in cellular immunity (85). To cope with stress-mediated damage, restoring balance within the organism requires synergistic interactions between the innate and acquired immune systems. The regulation of inflammation involves both positive and negative feedback mechanisms that coexist. For example, IFN-α/β can stimulate Th1 cells, DCs, and M1 macrophages to co-stimulate MHC I, enhancing cytotoxic T lymphocyte (CTL) activity and the expression of IL-2, IL-12, and IFN-γ, thereby promoting inflammation (86–88). Conversely, IFN-α/β can stimulate Th2 cells, Treg cells, and M2 macrophages to secrete IL-4, IL-10, and PD-1, which suppress the expression of pro-inflammatory factors and adhesion molecules such as TNF-α, IL-1β, and IL-8 and in turn downregulates MHC I expression to suppress CTL activation, thereby achieving immune control (89–93). These cytokines, as non-specific regulatory factors, affect most immune responses (7). Thus, immune activation and inhibition can coexist. The immune response triggered by long-term elevated CORT adapts through dynamic shifts in immune cell populations to achieve a new steady state, such as M1/M2 macrophages, Th1/Th2 cells, and Th17/Treg cells. As shown in Figure 3.

2.3.2 Invariant T cells

In addition to the traditional T cells mentioned above, invariant T cells (iT cells) have become a significant focus of research in immune regulation. iT cells are congenital T cells, including

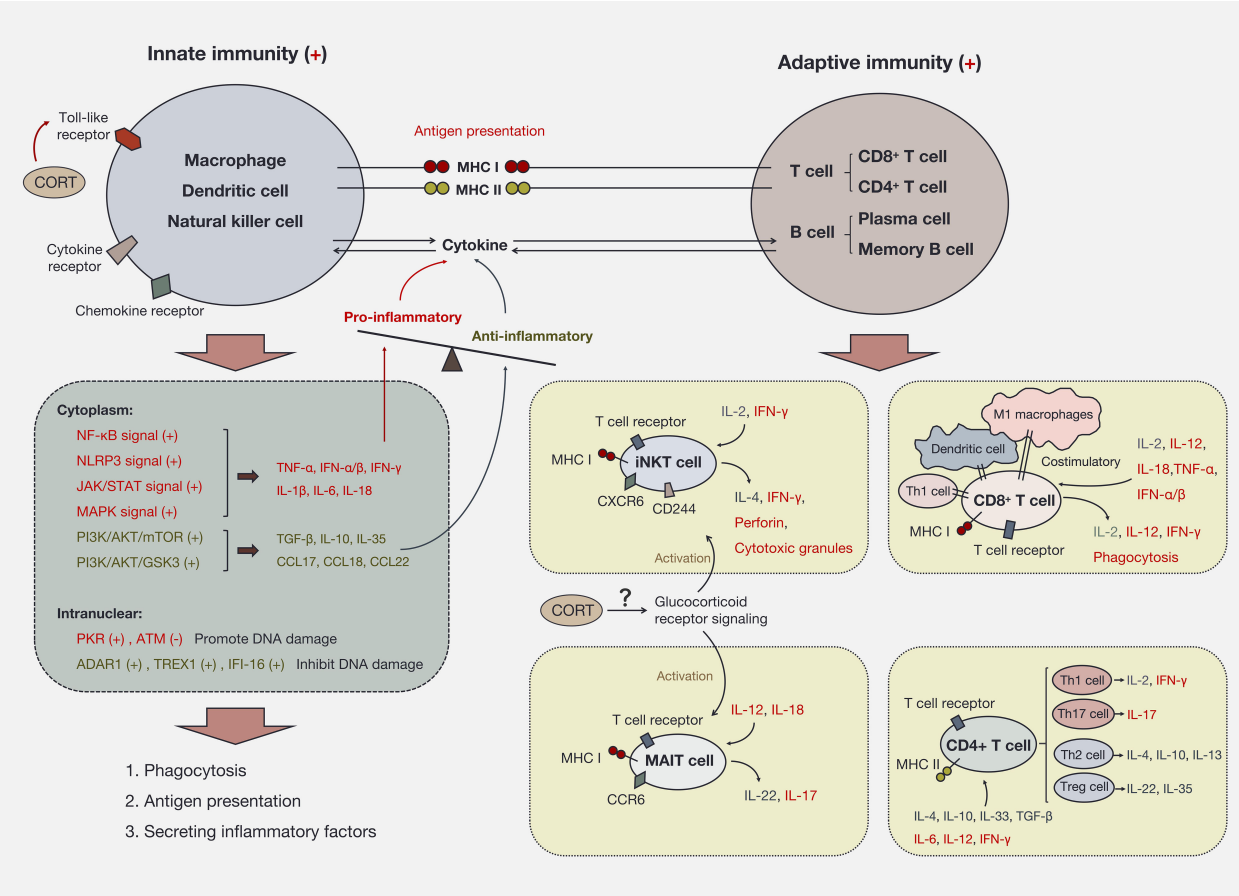

FIGURE 3
Immune activation and immune imbalance induced by CORT. CORT activates the immune system via Toll-like receptors. Innate immune cells are the first responders to CORT. The intracellular signaling pathways triggered by CORT produce two main effects, on the one hand, the secretion of pro-inflammatory factors to enhance immune activity. On the other hand, the secretion of anti-inflammatory factors to protect the cells themselves and to restrain excessive immune responses. The sustained high-level CORT further activates adaptive immune cells and expands immune activity. At this time, a large number of immune cells participate in the circumstance and transmit signals to each other through inflammatory factors. If CORT persists as an upstream signaling molecule, it will continue to deplete the immune system, leading to the exacerbation and spread of inflammation.

invariant natural killer T cells (iNKT cells) and mucosa-associated invariant T cells (MAIT cells). Functionally, they are considered bridges between innate and acquired immunity (94, 95). iNKT cells are characterized by expressing both NK cell surface marker CD244 and chemokine receptor CXCR6, as well as T cell surface marker TCR α/β (96). They can be activated by MHC I antigen presentation and by pro-inflammatory factors IL-2 and IFN-γ (97, 98). iNKT cells primarily recognize lipid antigens (99). Upon activation, they can secrete perforin and granzymes or utilize the Fas/FasL pathway to kill cells, participating in immune responses. Additionally, they can secrete IFN-γ or IL-4 to induce Th1 or Th2 differentiation, thereby playing a regulatory role (100, 101). MAIT cells also express TCR, which is activated by MHC I antigen presentation (TCR-dependent pathway) or by IL-12 and IL-18 (non-TCR-dependent pathway) (102). Upon activation, IL-18R and CCR6 are overexpressed on the cell membrane (102, 103). MAIT cells can secrete both the pro-inflammatory factor IL-17 and the anti-inflammatory factor IL-22, with their dual immune regulatory functions showing tissue specificity (103). MAIT cells also possess antioxidant functions, which may limit neuroinflammation and ensure cognitive function (104). However, insufficient or excessive MAIT cellular activity can induce autoimmune diseases, inflammatory diseases, and allergic diseases through dysbiosis of the microbiota (105–107). Previous studies have shown that stress promotes Th2 phenotype bias and inhibits Th1 activation by NE, NPY, and CORT (108–110). Recent studies have reported that chronic stress impairs the function of iT cells, demonstrating a mixed feature of selectively inducing the production of pro-inflammatory and anti-inflammatory cytokines (102, 111).

Long-term stress reduces the expression of TCRα/β, CD28, and inducible T cell costimulator (ITCOS) on the surface of iNKT cells, thereby decreasing their ability to secrete IL-4 and IFN-γ (102). IL-4 and IFN-γ are key factors in promoting Th2 and Th1 differentiation, respectively (112). Following stress, cytokine analysis revealed decreased serum levels of IL-2, IL-5, IL-13, Eotaxin, GM-CSF, IP-10/CXCL10, MCP-1/CCL2, RANTES/CCL5, and TNF-α, while the levels of IL-1α, IL-1β, MIP-1α/CCL3, and MIP-3α/CCL20 increased. In iNKT cells, the expression of IL-2, IL-5, IL-12, and IL-13 decreased, whereas IL-10, IL-23, and IL-27 levels increased. Cytokines and chemokines unaffected by stress include G-CSF, IL-6, IL-7, IL-9, IL-15, IL-17E/IL-25, IL-17F, IL-21, IL-22, IL-28B/IFNL3, IL-31, IL-33, KC/CXCL1, LIF, LIX/CXCL5, M-CSF, MIG/CXCL9, MIP-1β/CCL4, MIP-2/CXCL2, TGF-β1, TGF-β2, TGF-γ3, and VEGF. Therefore, the regulation of iNKT cells on inflammatory factors under stress is twofold. Upstream of the iNKT cell response, increased levels of the anti-inflammatory protein glucocorticoid-induced leucine zipper (GILZ) were detected, confirming that the response was dependent on GR signaling rather than sympathetic nervous system (SNS) signaling, as no change in SNS neurotransmitter receptor expression was observed (102). GILZ is a known transcriptional target for GR activation (113). Studies have shown reduced transcription levels of genes related to iNKT cell effector functions, including *Cd40l*, *Il18rap*, *Egr2*, *Irf4*, *Nfatc3*, *Tbx21*, *Ifng*, *Il4*, *Gzma*, *Tnf*, *Tnfrsf9* and *Tnfsf10*, suggesting that stress can inhibit iNKT cell function through GR signaling (102). MAIT cells also rely on the GR pathway to activate defense mechanisms under stress. Similar to iNKT cells, MAIT cells overexpress CD127 and reduce the secretion of IL-4 and IFN-γ to attenuate Th1 and Th2 responses (102). The above indicates that stress suppresses the immune response by impairing iT cell function. However, due to the dual role of iT cells in regulating both pro-inflammatory and anti-inflammatory cytokines and their ability to differentiate into different T cell phenotypes, further exploration of their role in various tissues is necessary. Additionally, the upstream mechanisms of GR signaling and CORT regulation require further investigation through cell experiments.

2.3.3 DNA damage response

Under chronic stress conditions, the inflammatory response during immune activation can also affect DNA damage repair and epigenetic modification (114). In the innate immune response, the activation of Pattern Recognition Receptors (PRRs) on the cell membrane leads to long-term activation of double-stranded RNA-dependent protein kinase (PKR), resulting in the inactivation of the DNA repair kinase ATM (part of the PI3K protein kinase family) and the phosphorylation of p65 NF-κB, thereby promoting IFN-γ synthesis (86). PKR activation also stimulates the NLRP3 inflammasome, enhancing the synthesis of IL-1β and IL-18, a process that can be inhibited by $p58^{IPK}$ (115). Additionally, the RNA editing enzyme ADAR1, the nucleic acid repair exonuclease TREX1, and the interferon-induced nuclear protein IFI-16 are activated to ensure DNA repair and prevent abnormal activation of interferons (116–118). ADAR1 has been found to prevent the activation of the receptor MDA5/PKR by A-RNA, thereby inhibiting IFN production and translation, and exerting immunosuppressive effects. Additionally, ADAR1 can inhibit RIPK3/MLKL-dependent programmed cell necrosis by blocking Z-RNA activation of ZBP1 (119, 120). Changes in transcription levels in the nucleus are associated with the extracellular JAK/STAT signaling pathway, PI3K/AKT/GSK3, and PI3K/AKT/mTOR signaling pathways (121, 122). Alongside the inflammatory response, the anti-inflammatory response is also regulated. For instance, the PI3K/AKT signaling pathway controls inflammation by upregulating the expression of IL-10-induced genes, thus antagonizing the cytotoxic effects of pro-inflammatory factors TNF-α, IL-1β, IL-6, and IL-8. Additionally, the expression of Caspase-3, Caspase-8, and Caspase-9 is downregulated to reduce apoptosis (123–125). In the process of adapting to stress, cellular compensatory reactions can lead to an imbalance skewed toward injury as energy is consumed (126). Chronic stress, for example, increases the rate of DNA mutations in cells, causing dysfunction and even cell death (127), contributing to the development of many inflammatory diseases. Therefore, the immune effects induced by CORT may impact cell function through epigenetic modification. However, the specific genetic mechanisms require further exploration, such as studying the regulation of transcription, translation, and post-translational modification of different inflammatory genes by CORT under varying levels and durations of stress through omics studies. Additionally, exploring the

influence of CORT on the DDR, such as the TCGA/DDR signaling pathway, is necessary for a deeper understanding.

3 Interaction pathway between corticosterone and immunity

3.1 The endocannabinoid system

The Endocannabinoid (eCB) system is widely present in various cell types and plays an essential role in metabolic signal transduction (128–130). It comprises (1) endogenous lipid transmitters such as endocannabinoids, including anandamide (AEA) and 2-arachidonoylglycerol (2-AG), (2) cannabinoid receptors (CBR), including type 1 (CB1R) and type 2 (CB2R), and (3) related enzymes such as N-acyl phosphatidylethanolamine-hydrolysis phospholipase D (NAPE-PLD), diacylglycerol lipase (DAGL), and degrading enzymes such as fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) (131). The eCB system plays a crucial role in coping with stress. This is primarily reflected in the response and control of the eCB to CORT (end product of HPA axis), as well as the response and regulation of the CBR to CORT. As a marker of stress signals, CORT activates the eCB system (128).

Firstly, it is reflected in the mutual influence between CORT and eCB (2-AG and AEA). There are two situations here, namely short-term stress and long-term stress. The core difference between the two lies in whether the negative feedback function of the HPA axis (sensitivity) is normal. Danan D's study (132) showed that an increase in CORT under short-term stress (1-2 hours after PSS, PSS is predator scent stress) stimulates compensatory responses of eCB, i.e., promotes the expression level of 2-AG (in cerebrospinal fluid). However, there was no significant difference in AEA levels (in cerebrospinal fluid). This study suggests that the response of eCB to acute stress is mainly through 2-AG. Similarly, Balsevich G's study (133) found a positive correlation between CORT and 2-AG (elevation) in the short-term stress model, rather than AEA. Bedse G's study (134) also confirmed this in the amygdala. Roberts CJ's study (135) simulated short-term stress stimuli through forced swimming. The results also showed a positive correlation (elevation) between 2-AG levels and CORT in the hippocampus, amygdala, and prefrontal cortex (PFC). Morena M's study (136) also found that under pressure (temperature stimulation), both 2-AG and CORT increased simultaneously, while AEA levels did not show significant changes. This indicates that the eCB system is mainly responsive to CORT by 2-AG under short-term stress. 2-AG participates in rapid and robust responses of stress regulation and promotes negative feedback function of the HPA axis (137, 138). Furthermore, it is widely believed that AEA is a regulatory molecule under the chronic action of GC, involved in downstream secondary signaling mechanisms of glucocorticoid receptor (GR) activation (139). Under short-term stress, based on negative feedback regulation of the HPA axis, these reactions will be self-downregulated afterwards (140), thereby restoring the homeostasis of the CORT and eCB systems.

Under long-term stress, the balance of HPA axis negative feedback is disrupted, and the compensatory response of eCB subsequently fails. At this point, the pathological effect of CORT dominates, which will disrupt the role of eCB (AEA and 2-AG). For AEA, Danan D's study (132) showed that AEA levels (in hippocampus) were significantly reduced under long-term stress (1 week after PSS). Zada W's study (141) showed that upregulating AEA expression through drugs (FAAH inhibitors) helps to reduce CORT levels and depressive behavior in a depression model (high CORT levels). Hill M.N's research (139) has the same conclusion. Satta V's study (142) simulated a chronic stress model by changing diet (stress duration of 5 weeks). The results showed that there were synergistic changes with the increase of CORT is, a decrease in AEA levels was observed in the amygdala, hippocampus, and caudate putamen. However, no significant changes in AEA were observed in the hypothalamus, nucleus accumbens, and PFC. Gray JM's study (143) showed that under the action of CORT (capsule), AEA levels were reduced in both the PFC and amygdala. It can be seen that the increase of CORT under chronic stress will inhibit the expression of AEA. And AEA is considered the main type of eCB that responds to the chronic effects of CORT (144). However, under this condition (chronic stress), 2-AG is slightly controversial. Satta V's study (142) showed that under chronic stress (dietary changes lasting up to 5 weeks), 2-AG significantly increased in the hippocampus, while there were no significant changes in the amygdala, caudate nucleus, nucleus accumbens, hypothalamus, and PFC. Gray JM's study (143) showed that under the action of CORT (capsule), 2-AG was observed to increase in the PFC, while there was no significant change in the amygdala. On the contrary, recent studies by Danan D (132) have shown that under prolonged stress (1 week after continuous PSS), the levels of 2-AG in the hippocampus and hypothalamus are significantly reduced. From this perspective, there are more complex regulatory mechanisms under chronic stress. Moreover, the eCB system can accept crosstalk and feedback from various upstream and downstream signals, which makes its expression more confusing. For instance, distinct brain regions exhibit unique regulatory mechanisms. There are different signal transmission directions (compensatory and decompensated) in different response stages. And under the influence of diverse stressors, different dominant signals emerge, even though an elevation in CORT levels is consistently observed across all conditions. In more recently studies (145, 146), it is generally believed that the concentration of 2-AG increases under chronic stress. The increase of 2-AG under chronic stress is related to the inhibition of AEA and decreased sensitivity of CB1R (147, 148). Both 2-AG and AEA are constrained by the release mode of GC (such as CORT), and when CORT increases, both 2-AG and AEA change in opposite directions (143). However, a more detailed mechanism has not yet been fully determined.

Additionally, it should be noted that CORT regulates the eCB system not only by targeting 2-AG or AEA levels, but also by acting on CBR. CB activity mediated by CBR is a primary factor in maintaining the feedback regulation ability of the HPA axis (149, 150). Studies have shown that, pharmacological blockade or decreased expression and function of CB1R can disrupt the negative feedback of the HPA axis,

leading to increased circulating CORT levels (151). Skupio et al. (152), CORT induces neuronal damage by activating CB1R on the mitochondrial membrane (mtCB1R), and this mechanism has different damaging effects in different brain regions. In mice, impairment of New Object Recognition (NOR) consolidation memory was induced in norepinephrine (NE) neurons of the locus coeruleus (LC), while in the hippocampus (HIP), impairment of NOR extraction memory was induced in GABAergic interneurons. In this pathway, it was observed that CORT led to an increase in 2-arachidonoylglycerol (2-AG) levels. These pieces of evidence suggest that the eCB system is a vital component in the response to stress. Furthermore, downstream of the eCB system, it is intricately linked to the immune system, serving as a crucial bridge for the interaction between CORT and the immune response (153, 154).

The signal transduction of the CB system to the immune system involves multiple pathways, including direct communication and indirect communication through arachidonic acid signaling. This regulates the function of immune cells, such as proliferation, secretion, and apoptosis (155, 156). The realization of immune activation and immune suppression mainly depends on the dual channels of the CB system, involving two G protein-coupled receptors, CB1R and CB2R, and the activation and sensitivity of these receptors. CBR mainly functions in central neurons. Among them, CB1R is primarily expressed in microglia, neuronal endings, and astrocytes, whereas CB2R is mainly expressed in microglia and glial cells (157, 158). Currently, it is believed that CB1R is primarily associated with promoting the production of inflammatory mediators, while CB2R is mainly involved in inhibiting inflammation, thereby communicating with immune cells (154). And, microglia are essential immune cells in the central nervous system as they possess significant neuroimmune regulatory abilities. Therefore, in neuroimmunity, the immune regulation of eCB cannot be separated from the immune function of microglia. They exhibit two activation states: classical activation (M1 polarization) and alternative activation (M2 polarization) (159). M1 polarization is associated with pro-inflammatory effects. In contrast, M2 polarization has anti-inflammatory and neurotrophic properties (160, 161). As shown in Figure 4.

3.1.1 Direct communication between CORT and immunity

About direct communication, research has found that in M1-type microglia, the binding of 2-AG to CB1R increases pro-inflammatory mediators. However, in M2-type microglia, the binding of 2-AG to CB2R enhances the expression of the anti-inflammatory cytokine IL-10 and the solubilizing factor lipoxin-4 (LXA4) (162). LXA4 can induce apoptosis of inflammatory cells and participate in immune suppression (163). Additionally, activation of CB2R facilitates the transition of microglia from M1 type to M2 type (164), leading to a decrease in the expression of iNOS, a marker of M1 activity, and an increase in the expression of Arg-1, a marker of M2 activity (160). Administration of exogenous 2-AG in inflammatory model mice promotes an increase in the number of M2-type microglia (162). CB2R signaling inhibits the expression of pro-inflammatory mediators iNOS and CCR2 in IFN-γ-induced inflammatory mouse microglia (165). CCR2 is a chemokine receptor associated with immune cell recruitment, reflecting that the immune regulation by 2-AG depends on the activation status of microglia and the sensitivity of corresponding CBRs in this state. Other studies have shown that AEA and 2-AG inhibit T cell proliferation and reduce IL-1, IL-6, IL-9, IL-17 and TNF-α levels by activating CB2R (166). CB2R can simultaneously inhibit adenylate cyclase (AC) activity, thereby inhibiting the cyclic adenosine monophosphate (cAMP) signaling pathway and lymphocyte activation. This demonstrates that the CB system regulates immune cell and cytokine secretion by activating different CBRs and maintains homeostasis of the internal environment through a dual effect of pro-inflammatory and anti-inflammatory actions (167). During this process, inflammation results from the interaction between pro-inflammatory and anti-inflammatory substances.

3.1.2 Indirect communication between CORT and immunity

Indirect communication with the immune system is accomplished through the transmission of arachidonic acid-like signals (168). The biosynthesis of arachidonic acid (AA) involves the oxidation of polyunsaturated fatty acids by cyclooxygenase (COX) and lipoxygenase (LOX). Interestingly, endocannabinoids (AEA and 2-AG) are derivatives of AA and are influenced by the same oxidative metabolic pathway. Prostaglandins (PG), such as PGE2 (via the COX pathway) and leukotrienes (via the LOX pathway), are the main metabolic products (169). While endocannabinoids exert their anti-inflammatory effects through CB2R, they are degraded into AA by FAAH and MAGL, and enter the AA synthesis pathway to produce inflammatory mediators PGE2 and leukotrienes. These processes rely on the production of nitric oxide (NO) to provide pro-inflammatory effects and enhance immune responses (170). In the LPS-induced inflammatory mouse model, inhibiting MAGL activity reduces the secretion of PGD2, PGE2, PGF2α, and pro-inflammatory cytokines IL-1α, IL-1β, IL-6, and TNF-α in the brain (171). This phenomenon is also observed when inhibiting COX-2 (172). Thus, activating the immune system includes both pro-inflammatory and anti-inflammatory effects. This process requires significant energy and substrate consumption, as well as the continuous operation of organelles. The final result depends on which signaling pathway is predominantly and continuously activated. Persistent inflammation and cell damage are the outcomes of the sustained action of stress hormones.

Conversely, immune cells can coordinate CB signaling by regulating the transcription, synthesis, uptake, and degradation of CB components. Studies have shown that CB1R expression and AEA levels in lymphocytes are reduced following intervention with the anti-inflammatory cytokine IFN-β (173). LPS-induced activation of mouse macrophages results in an increase in platelet-activating factor (PAF), which promotes the synthesis of AEA and 2-AG due to decreased expression of FAAH (174). The study conducted by Standoli S (175) reveals that inhibiting FAAH and activating the CB2R can effectively prevent the production of TNF-α and IL-1β induced by LPS. Additionally, immune cells can directly participate in the degradation of AEA and 2-AG

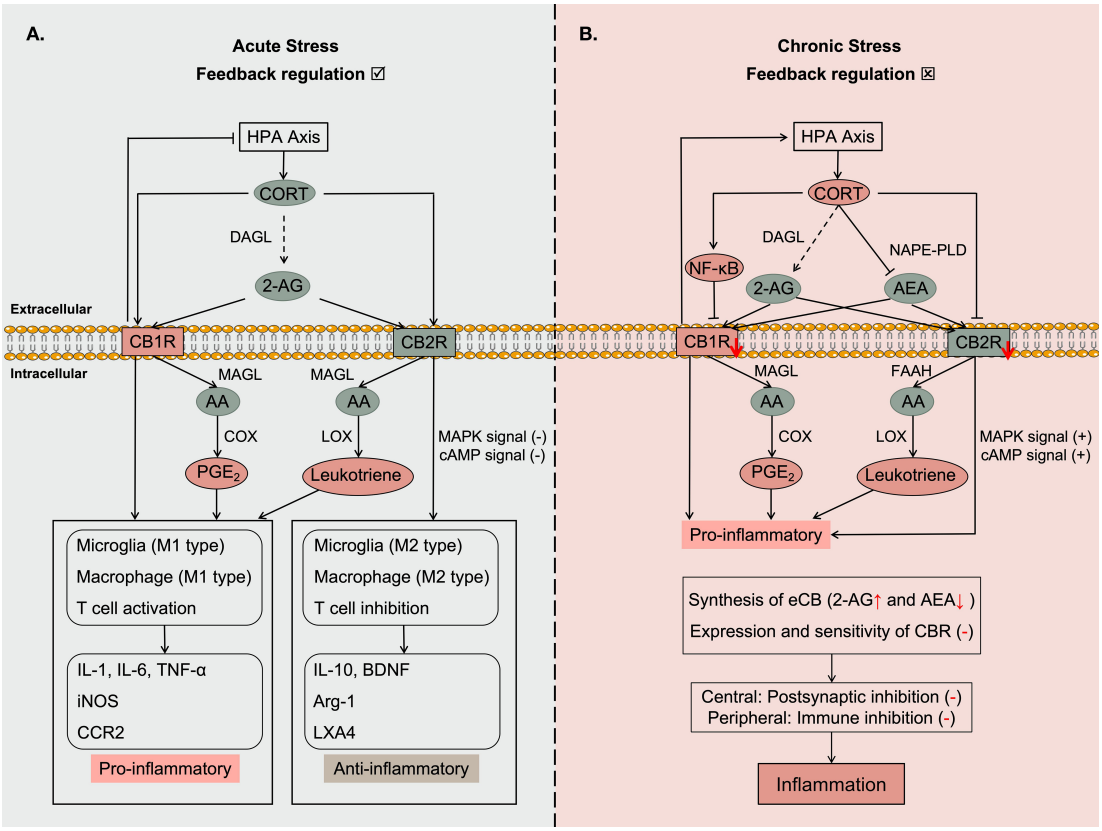

FIGURE 4
The immune effect of CORT through the eCB system. **(A)** Under acute stress, 2-AG is the main type of eCB that responds to stress (CORT). Mediate the inflammatory response via CB1R, while inhibiting inflammation through CB2R. This achieves immune balance. And ultimately end the stress response through negative feedback regulation of the HPA axis. **(B)** Under chronic stress, CORT levels remain elevated, leading to the continuous activation of both the eCB system and the immune system. In this context, the low expression of AEA reflects the depletion of the eCB system, while the high expression of 2-AG represents the body's attempt to counteract the effects of elevated CORT. As a sustained stress signal, CORT promotes the dominance of CB1R while CB2R is inhibited. This shift drives the immune response toward a pro-inflammatory state. Eventually, the eCB system becomes exhausted, rendering it incapable of effectively inhibiting inflammation. The straight arrow represents the direct promoting effect. The dashed arrow represents the indirect promoting effect. The minus line represents the inhibitory effect. The plus sign "(+)" represents signal enhancement. The minus sign "(-)" represents a weakened signal. The red down arrow represents a decrease in the expression level of the molecule. The red up arrow represents an increase in the expression level of the molecule.

(dependent on concentration feedback) to terminate CB signaling (176, 177). Other studies have demonstrated that in the LPS-induced inflammatory mouse model, CB1R expression decreases at the membrane protein level while CB1R mRNA expression increases (178), which may represent an adaptive regulation of the body's response to inflammation, primarily manifested at the protein level.

3.2 TREM2 mediated immune regulation

Triggering receptor expressed on myeloid cells-2 (TREM2) is a transmembrane receptor of the immunoglobulin superfamily expressed in various immune cells such as DCs, microglia, and macrophages (179–181). Upon binding to its ligand, TREM2 interacts with DNAX-activating protein of 12 kDa (DAP12) to induce phagocytosis of tissue fragments and promote anti-inflammatory properties (182). This interaction is related to downstream signaling pathways involving PLCγ2, PI3K, and AKT (183–185). DAP12 is also a transmembrane receptor widely present on the surface of immune cell membranes. In microglia, TREM2 is responsible for synaptic inhibition and establishing normal brain connections, maintaining innate immune homeostasis and cellular metabolism (186). TREM2 also participates in the M1/M2 polarization of microglia to regulate inflammatory responses. Upregulation of TREM2 expression promotes the transition of microglia from M1 to M2 type, enhancing their phagocytic function, reducing the release of inflammatory mediators, and inhibiting the inflammatory cascade response. Conversely, downregulation of TREM2 expression promotes inflammation (187).

Recent studies have shown that CORT affects the immune function of microglia through TREM2, inducing the production of inflammatory factors (188, 189). Cell experiments have demonstrated that when CORT concentration exceeds 1 μM, it significantly inhibits the proliferation of microglia (BV2), with the degree of inhibition becoming more pronounced at higher CORT concentrations, almost completely inhibiting growth at 500 μM CORT. Intervention using 10 μM CORT was found to significantly

decrease TREM2 expression. The experiment also revealed an upregulation in the expression of M1-type markers, including iNOS and CD16, in microglia, while the expression of M2 biomarkers CD206 and Arg-1 declined. Additionally, there was an increase in the levels of pro-inflammatory factors such as TNF-α, IL-1β, and IL-6, coupled with a decrease in the anti-inflammatory factor IL-10, which are in line with previous research (187). Transfecting TREM2 into the cells was found to reverse this phenomenon, while knocking out TREM2 in mice increased the levels of TNF-α, IL-1β and IL-6 and decreased the levels of IL-10. These results indicate that inhibiting TREM2 is one of the mechanisms by which CORT mediates the pro-inflammatory effects of microglia.

The JAK2/STAT3 signaling pathway is involved downstream of TREM2 in the immune regulation of CORT. This pathway plays an important role in the development of innate and acquired immune cells, activation of IFN, and expression of inflammatory cytokines (190, 191). It is a significant mediator in synaptic transmission, where enhanced synaptic transmission activates the JAK2/STAT3 signaling pathway to promote the production of inflammatory factors (192). Studies have shown that chronic unpredictable mild stimulation (CUMS) induces depression-like behavior and the release of inflammatory factors in rats by activating the IL-6/JAK2/STAT3 pathway in the hypothalamus (193). Further research has indicated that this process is related to CORT inhibiting TREM2 expression. Overexpression of TREM2 can reverse this phenomenon to promote the transformation of microglia from M1 type to M2 type (189), thereby exerting anti-inflammatory effects.

Regulating downstream signaling pathways of inflammation through TREM2 involves not only JAK2/STAT3 but also NF-κB and PI3K/AKT pathways, which are implicated in NLRP3 inflammasome-mediated neuroinflammation (194). During stress, CORT recognizes peripheral signals to activate the intracellular pattern recognition receptor (PRR) NLRP3. Subsequently, ASC binds to pro-caspase-1 to form activated caspase-1, which promotes the maturation of IL-1β and IL-18, thereby exerting pro-inflammatory effects (195). Therefore, the expression of NLRP3 and activated caspase-1 are key markers of inflammation. TREM2 is widely recognized as a key protein molecule that inhibits the inflammatory cascade response (196). Recent studies (187) have shown that overexpression of TREM2 effectively reduces the expression levels of NLRP3 and pro-caspase-1 proteins in rats, as well as the secretion of the inflammatory mediators IL-1β and IL-18, both *in vivo* and *in vitro*. This anti-inflammatory effect is associated with the inhibition of the TLR4/MyD88/NF-κB signaling pathway and the upregulation of PI3K/AKT phosphorylation levels (197, 198). These findings are consistent with previous results. The upregulation of TREM2 promotes M2 polarization of microglia and reduces the secretion of inflammatory mediators, thereby exerting neuroprotective effects. Inhibiting the NF-κB signaling pathway and activating the PI3K/AKT signaling pathway are essential for these effects (199–201).

The effects of CORT on downstream signaling pathways are concentration-dependent, with both upregulating and downregulating impacts on the same pathway, thereby exerting pro-inflammatory or anti-inflammatory properties. Wu et al. (202) demonstrated a dual effect of different CORT concentrations on an LPS-induced mouse macrophage inflammation model. When the concentration of CORT was below 300 ng/ml, the protein expression level of NLRP3 in mouse macrophages was significantly upregulated. However, when the concentration of CORT exceeds 300 ng/ml, the protein expression level of NLRP3 gradually decreases, reaching its lowest level at 700 ng/ml, along with a decrease in activated caspase-1 expression. Xanthine oxidase (XO) primarily mediates the production of mitochondrial reactive oxygen species (ROS) (203), which may be responsible for activating NLRP3 (204, 205). Research has found that CORT regulates the pro-inflammatory factor NLRP3 through the enzyme activity of XO (202). Higher concentrations of CORT (700 ng/ml) downregulate the mRNA and protein expression of NLRP3 by inhibiting the activity of XO, thereby modulating the body's inflammatory response. Thus, while the signaling pathways affected by CORT may be consistent, the specific role of immune promotion or immune suppression depends on the circulating concentration, as illustrated in Figure 5.

3.3 SOCS1 and SOCS3 mediated immune regulation related to CORT

Suppressor of Cytokine Signaling 1 (SOCS1) is a negative regulatory factor that effectively prevents the overactivation of the immune system (206), and its transcription is regulated by the JAK/STAT signaling pathway. Additionally, SOCS1 can bind to the catalytic site of JAK2 through its specific enzyme activity inhibitory region, thereby inhibiting JAK2/STAT3 signal transduction (207). Inhibiting SOCS1 has also been found to promote the proliferation of $CD4^+$ and $CD8^+$ T cells (208). Studies have observed that CORT reduces the expression of SOCS1 in microglia, thereby promoting the expression of pro-inflammatory factors TNF-α, IL-1β, and IL-6 (189). These factors promote the polarization of microglia towards the M1 type. Subsequently, activated microglia exacerbate synaptic damage by releasing pro-inflammatory factors, promoting the accumulation of phosphorylated tau, and inducing neuronal apoptosis (209), indicating that the activation of microglial immune function by pro-inflammatory concentrations of CORT is achieved by inhibiting SOCS1. However, CORT can also activate the JAK2/STAT3 signaling pathway, suggesting that there is another mechanism by which CORT inhibits SOCS1 that warrants further exploration.

SOCS3 is an IL-10 inducible gene, and IL-10 primarily achieves immunosuppressive effects by inactivating myeloid cells and inhibiting the production of inflammatory factors (210). IL-10 typically induces STAT3 activation, which inhibits TLR-mediated pro-inflammatory cytokine expression at the transcriptional level. Furthermore, IL-10 induces the polarization of microglia towards the M2 type (211). The expression of IL-10 is not entirely dependent on the PI3K/AKT pathway, and the IL-10-induced *SOCS3* gene is not regulated by it. However, the expression of IL-10 induced by

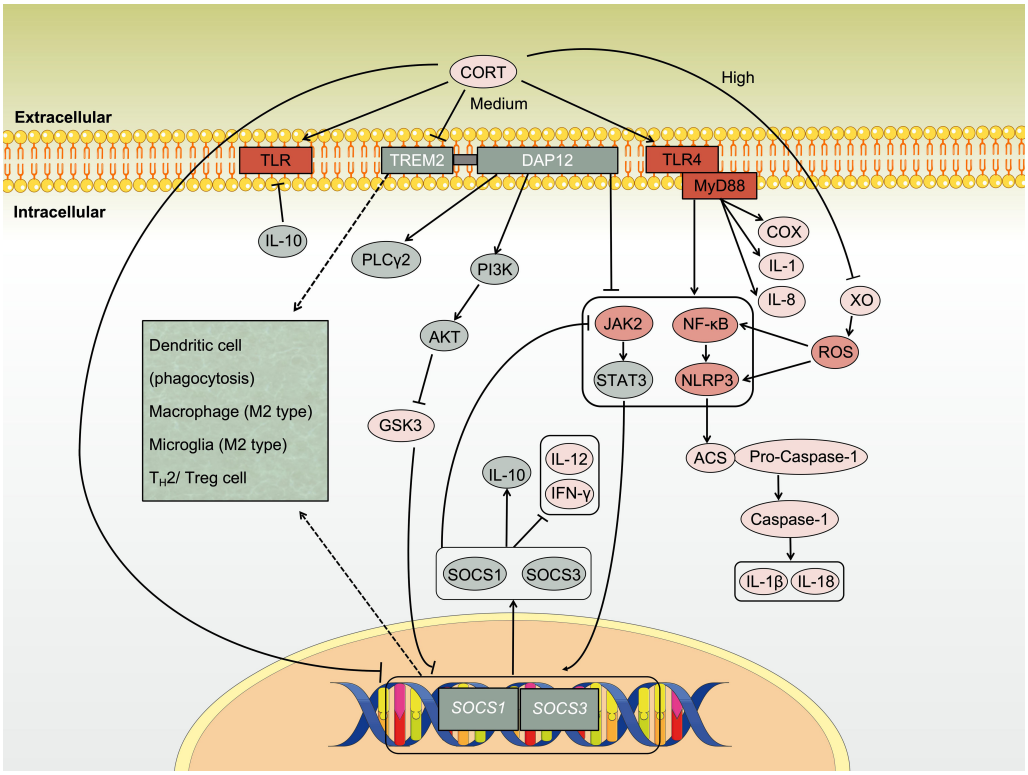

FIGURE 5
Regulation of immune activity by CORT through TREM2. Under chronic stress, CORT stimulates inflammatory signaling through Toll-like receptors (TLRs), which involving key pathways such as NF-κB, NLRP3 inflammasome, and JAK/STAT, which collectively drive the progression of inflammation. TREM2 is a crucial membrane receptor protein with immunomodulatory functions that help inhibit inflammation. However, sustained high levels of CORT disrupt the ability of immune cells to suppress inflammation by inhibiting the TREM2/PI3K/AKT signaling pathway.

other genes, such as *ARNT2* and *Autotaxin*, depends on the PI3K/AKT pathway (212). Downstream of p-AKT, IL-10-induced gene expression is further increased by inhibiting GSK3 activity (mainly GSK3α, followed by GSK3β). Although it has been found that cAMP response-element protein (CREB) is one of the targets of GSK3, it has also been shown that CREB is not involved in GSK3 regulation of signal transduction between IL-10 (213). Downstream of IL-10, the PI3K/AKT pathway is involved in IL-10 inhibition of TLR-induced synthesis of COX2, IL-1 and IL-8, but not in IL-10 inhibition of TNF-α synthesis (212). This indicates that the PI3K/AKT pathway selectively regulates the immune response of IL-10. Upstream of the PI3K/AKT pathway, CORT inhibits the PI3K/AKT pathway, while TREM2 promotes it. In summary, the specific regulatory mechanism is shown in Figure 5.

3.4 Programmed cell death

3.4.1 FOXO3a and ROS

CORT can activate immune cells and release inflammatory mediators to respond to the immune environment through various signaling pathways. However, alongside adaptation and coping, damage also occurs. This is especially evident in chronic stress, where repair and regulation are less effective than injury response. Under the cytotoxic effect of CORT, signals of abnormal intracellular metabolism continuously drive immune regulation and, over time, initiate autonomous cell death (214). Chang et al. (215) showed that CORT (100 μM)-induced neuronal apoptosis results from a combination of multiple pathways, including the mitogen-activated protein kinase (MAPK) cascade reaction (MAPK/ERK signaling pathway and p38 MAPK signaling pathway) and the PI3K/AKT/FOXO3a signaling pathway. These intracellular kinase signaling cascades are believed to be responsible for promoting neuronal survival (215, 216). The MAPK/ERK signaling pathway is mainly responsible for regulating cell viability and proliferation (217). The p38 MAPK signaling pathway primarily regulates cell differentiation, antioxidant stress survival, inflammation, and the cell cycle (218, 219). Research has shown that high concentrations of CORT increase intracellular ROS and FOXO3a nuclear accumulation by inhibiting these signaling pathways based on the observed decreased phosphorylation of p38, ERK, PI3K, and AKT. This inhibition leads to an increased rate of cell apoptosis (215). FOXO3a is a transcription factor that triggers cell apoptosis, characterized by a forkhead domain that binds to DNA, thus directly participating in gene transcription (220). However, FOXO3a within the nucleus is limited. Part of FOXO3a is phosphorylated and translocated from the nucleus to the cytoplasm, where it regulates important physiological processes such as energy metabolism, cell apoptosis, and oxidative stress and is ultimately degraded, preventing cell apoptosis (221, 222).

CORT reduces FOXO3a phosphorylation by inhibiting the PI3K/Akt signaling pathway, causing its accumulation in the nucleus and inducing cell apoptosis (215, 223).

The production of ROS plays a crucial role in stimulating the continuous activation of immune cells under sustained stress (224) as ROS act to recruit more immune cells, prompting them to produce pro-inflammatory factors (225). However, the damaging effects of ROS cannot be overlooked. Intracellular ROS induce the activation of transcription factors such as NF-κB and MYC, which in turn synthesize both pro-apoptotic and anti-apoptotic factors, thereby initiating apoptosis programs (226, 227). Pro-apoptotic genes, including *Apaf1* and members of the Bcl-2 family like *Bad*, *Bbc3*, *Bik*, and *Pmaip1*, are upregulated. Furthermore, downstream molecules such as caspase-3 and caspase-6 are activated in T cells. Interestingly, the use of GR antagonists, which block the effect of CORT, can reverse apoptosis (102). Previous studies have demonstrated that stress induces the maturation and apoptosis of CD4 and CD8 T cells, leading to the depletion of the T cell pool (228). Prolonged exposure to antigens can drive T cells into a state of depletion, where immature T cells become the primary force of immunity. However, due to insufficient energy and abnormal cellular metabolism, overall immune function shifts towards an immunosuppressive state. This alteration affects downstream signaling cascade reactions and epigenetic processes (71, 229). For instance, lactate dehydrogenase A (LDHA) plays a role in providing energy for T cell activation and proliferation by participating in lactate metabolism, exhibiting non-classical enzyme activity, and regulating oxidative stress responses (230). However, when ROS synthesis surpasses decomposition, leading to cytoplasmic accumulation, LDHA function is inhibited (231). In response to stress, cells adjust the intensity of multiple gene expressions, triggering intracellular cascade reactions that may lead to exhaustion and, ultimately, cell death (71).

3.4.2 GSDMD and NLRP3

In the central nervous system, huang et al. (198) reported that CORT promotes the expression of key apoptotic proteins GSDMD and GSDMD-N in microglia. Thus, microglia not only produce inflammatory mediators that enter the bloodstream through pro-inflammatory signaling by CORT but also activate their apoptotic pathways, leading to programmed cell death after sustained immune activity. This process consumes significant energy and substances, and the inflammatory mediators entering the circulation act as new signaling molecules, inducing further inflammation throughout the body. If left untreated, this can cause inflammatory damage and impair tissue function. Peripherally, chronic stress-induced elevated CORT causes macrophage infiltration in the spleen of mice. It has been observed that as the phagocytic function of macrophages weakens, pyroptosis increases, and autoantibody production decreases, resulting in immunosuppressive effects (232, 233). This pathway relies on the activation of NLRP3 inflammasomes rather than the P-selectin pathway (232) and corresponds to the previously mentioned mechanism but with slightly different outcomes. Research has found that under the influence of CORT, the expression of NLRP3 and caspase-1 in macrophages increases (caspase-1 promotes IL-1β maturation), leading to an increase in the circulating pro-inflammatory cytokine IL-1β (232). NLRP3 inflammasomes, including NLRP3 and caspase-1, have pro-apoptotic and inflammatory effects (234). Using NLRP3 inhibitors (OLT1177) and caspase-1 inhibitors (Z-WEHD-FMK) can block the apoptotic pathway and subsequent cascade events of inflammation (232, 235), indicating that the pro-inflammatory effect continues to erupt within immune cells, eventually ending immune activity through cell death.

3.4.3 Notch signaling pathway

After chronic stress triggers an increase in circulating CORT levels, it also activates the Notch signaling pathway, inducing immune suppression and splenocyte apoptosis (233). Activation of the Notch signaling pathway is observed with increased expression of NICD1, DLL1, DLL4, Jagged 2 and Hes1, while the expression of DLL3, Jagged 1 and Hes5 remains unchanged. Concurrently, decreased IFN-γ levels and increased IL-4, caspase-8, and caspase-3 levels are noted. Song et al. also demonstrated that chronic stress-induced splenic apoptosis is mediated through the death receptor pathway (236). Additionally, TLR4 activation has been found to be implicated in immune suppression induced by increased CORT under stress (237). These findings suggest that both immune activation and immune suppression are closely related to inflammation. It is important to note that immunosuppressive characterization may result from immune overactivation, where anti-inflammatory signaling pathways are less dominant compared to pro-inflammatory pathways.

3.4.4 miR-155

In addition, the increase in CORT caused by chronic stress downregulates the expression of miR-155, resulting in decreased BCL-6 levels and increased FBXO11 levels. This impairs the germinal center response of B lymphocytes and the production of IgG1 antibodies, thereby inhibiting immune function (238). The germinal center is a histological structure formed during the maturation and differentiation of B cells into plasma cells and memory B cells. BCL-6 is a transcription factor essential for the formation of germinal centers (239, 240). The SKP1-CUL1-Fbox protein (SCF) ubiquitin ligase complex containing FBXO11 induces ubiquitination and degradation of BCL-6. Excessively high levels of FBXO11 hinder B cell differentiation and induce B cell apoptosis, while low levels promote lymphatic proliferation and carcinogenesis (241, 242). The balance between FBXO11 and BCL-6 levels is essential for B cells to maintain normal immune function. Apoptosis, widely regarded as programmed cell death activated by highly inflammatory and cytotoxic metabolites (243, 244), is associated with stress-induced elevated CORT (245). These pathways mediate the activation and damage of immune cells by CORT. The inflammatory factors released during injury re-enter the bloodstream, reactivate the immune system, and attack vulnerable areas of the body by identifying abnormal signals and generating signal transmission to trigger new inflammatory reactions.

3.4.5 TFEB

Transcription factor EB (TFEB) belongs to the MiT/TFE family of basic helix-loop-helix leucine zipper transcription factors and serves as a pivotal regulator of autophagy and lysosomal biogenesis (246). Additionally, TFEB has been implicated in governing energy homeostasis and cellular responses to various stressors, such as nutrient deprivation, endoplasmic reticulum stress (ERS), mitochondrial autophagy, and pathogen invasion (247, 248). It is involved in multiple signaling pathways, including mTORC1, Wnt, and AKT pathways (249). Phosphorylation of TFEB at the S401 site facilitates redox reactions and the release of growth factors to adapt to stress conditions (250). Recently, TFEB has emerged as a key player in controlling inflammatory responses by inhibiting pro-inflammatory cytokines and modulating immune cell differentiation (251, 252). The inhibition of TFEB has been implicated in promoting immune evasion (253). Recent studies suggest that this adaptive regulation can be inhibited by p38 MAPK or blocked by substrate depletion (250). The p38 MAPK/TFEB signaling axis suppresses the expression of multiple immune-related genes in monocytes, as well as cytokines (such as IL-1β and LIF), chemokines (including CXCL1, CXCL3, CXCL8, and CCL5), and crucial immunomodulators (such as IFNGR2 and EREG). Consequently, this leads to aberrant macrophage differentiation and impaired polarization. Enhanced nuclear translocation of TFEB boosts the expression of lysosomal proteins and superoxide dismutase (SOD), ultimately diminishing ROS levels and suppressing ferroptosis, thus exerting a protective effect (254). These findings indicate the pivotal role of TFEB in immune and redox regulation, suggesting potential avenues for further exploration into its regulatory mechanisms.

3.4.6 TAM family of receptor tyrosine kinases

The increase in CORT induced by stress also activates the GR-MERTK signaling pathway in astrocytes, leading to heightened phagocytosis of excitatory synapses by astrocytes in cortical regions, thereby eliciting depressive behavior in mice (255). MERTK belongs to the TAM family, which encompasses TYRO3, AXL, and MERTK, and is comprised of RTK. This family acts as a bridge between its structurally homologous ligands, GAS6 and PROS1, and binds to phosphatidylserine on the apoptotic cell membrane (PtdSer) to mediate immune regulation (256).

Recently, TAM receptors have received significant attention as potential therapeutic targets for their ability to control inflammation and immunosuppression. Present research reveals that TAM receptor activation can inhibit immune activity downstream through various pathways, including MEK/ERK, PI3K/AKT, and JAK/STAT pathways (257). For instance, in macrophages, MERTK governs its phagocytic function (258), while AXL signaling promotes a shift towards the M2 phenotype in macrophages, resulting in increased expression of IL-10 and TGF-β, and decreased expression of IL-6, TNF-α, and G-CSF (259). Furthermore, TAM signaling inhibits the activation of NLRP3 inflammasomes in macrophages, thereby attenuating the inflammatory pathway and preventing chronic macrophage activation (260). Similar to NK cells, AXL signal transduction reduces their secretion of IFN-γ and diminishes their killing function (261). Similarly, in DCs, TLRs activation upregulates AXL expression through STAT1 signaling transduction. Subsequently, AXL inhibits IFNAR-STAT1 signaling and induces the expression of SOCS1 and SOCS3, thereby negatively regulating TLR signal transduction, inhibiting the inflammatory response and terminating DC activation of T cells (262). Activated T cells secrete protein S (PROS1) as an additional feedback mechanism for DCs to assist in TAM signaling to suppress immune responses (263). Conversely, activated T cells increase MERTK expression and activate MERTK signal transduction, a co-stimulatory pathway that promotes $CD8^+$ T cell activation (263, 264). Inhibiting MERTK signal transduction reduces IFN-γ secretion and $CD8^+$ T cell proliferation (263). Furthermore, studies have revealed co-expression of MERTK and PD-1 in activated T cells (265–267). The opposing regulatory effects of TAM receptors on innate immune cells and T cells, as well as the dual effects of MERTK, have roused the interest of researchers. Currently, more mechanisms for regulating TAM receptors are being investigated. From this, it can be evident that CORT induces programmed cell death through multiple signals, thereby impairing immune function and promoting inflammation progression. In summary, the specific mechanism is shown in Figure 6.

3.4.7 Cell competition

It is also worth noting that a state closely related to cell apoptosis is inter-tissue cell competition, which involves signal pathways correlated with GR signal transduction induced by CORT, warranting attention. Intercellular interaction in cell competition aims to maintain tissue health and cellular homeostasis (268). Due to its involvement in immune regulation across various diseases, it has become a research hotspot, including tumor immune escape and neurodegenerative diseases (269, 270). Cell competition operates on the principle of "survival of the fittest" to sustain tissue physiological function and internal environment homeostasis (269). Disruption in the balance of cell competition results in "winner" cells and "loser" cells, where loser cells experience slowed proliferation and incomplete apoptosis, while winner cells exhibit accelerated proliferation (271, 272). Research suggests that reasons for the failure of cell competition are associated with chronic activation of the TLR pathway (273), p53/DDR pathway (274), c-Jun N-terminal kinase (JNK) pathway, Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathways (275), and oxidative stress response pathways (231).

TLR pathway activation not only initiates inflammation but also induces cell apoptosis (276). The activation of the p53 pathway is related to DDR, with the related genes *Mre11*, *Lig3*, *Lig4*, and *Ku80* being upregulated in DDR and are considered targets of p53 (277, 278). The JNK pathway primarily participates in cell proliferation and death (279). Upon activation, JNK upregulates the expression of genes such as *TRE-dsRED*, *Scarface* and *Reaper*, which are involved in competition failure to induce cell apoptosis (280). Moreover, it can inhibit cell proliferation rate by impacting protein synthesis, potentially contributing to competitive failure

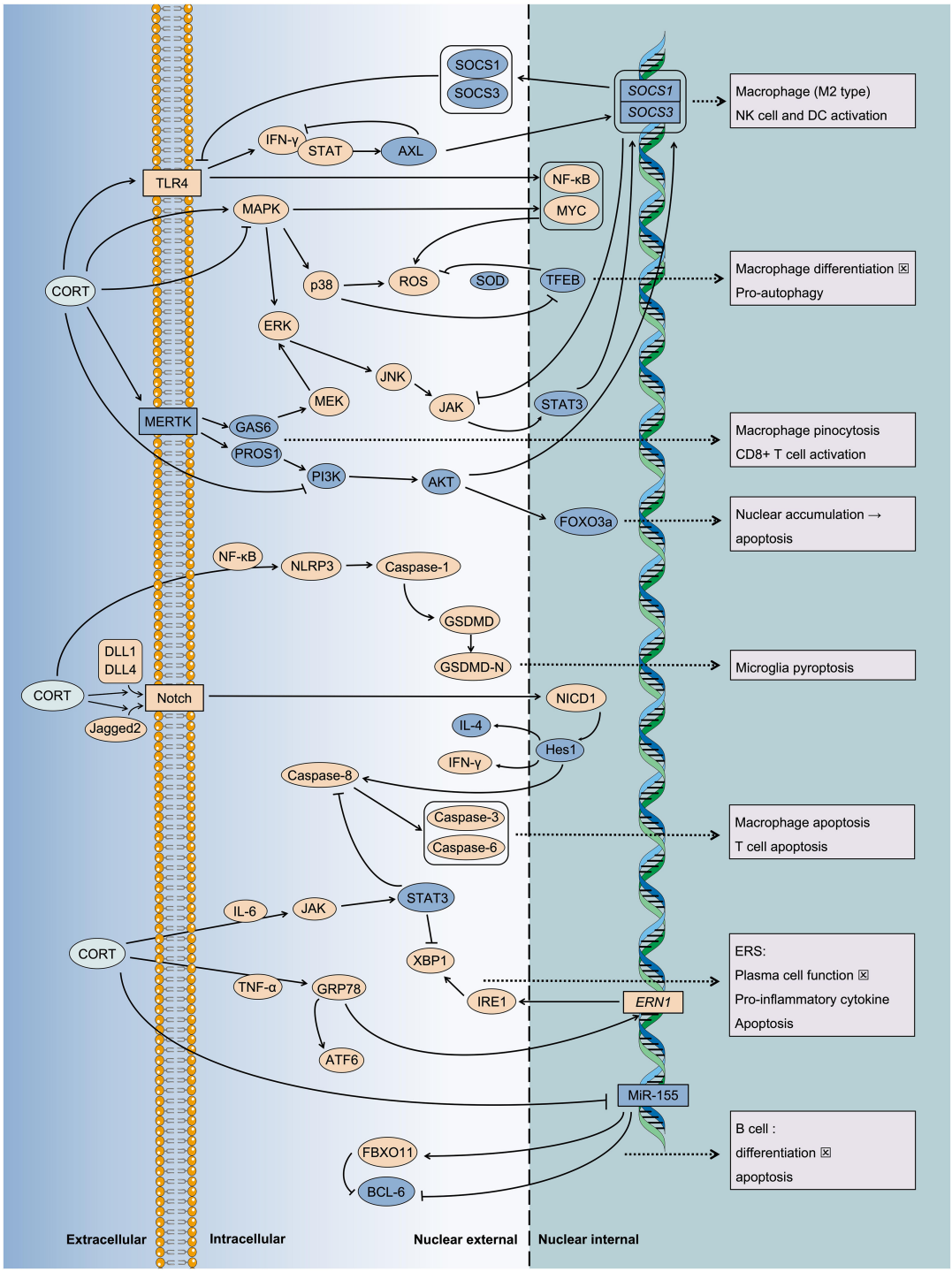

FIGURE 6
Multiple pathways mediate the inflammatory injury of CORT induced cell death. Under chronic stress, the sustained action of CORT leads to programmed cell death. This phenomenon indicates an immune imbalance, characterized by the ongoing progression of inflammation. It is associated with the transmission of numerous intracellular stress signals and the subsequent regulation of gene expression. For instance, key signaling pathways such as MAPK, JAK/STAT, Notch, and NF-κB, etc. And some key regulatory proteins such as MERTK, TFEB, etc.

(231, 281). However, the specific mechanism by which it inhibits cell proliferation rate remains unclear. The JAK/STAT pathway are primarily involved in cell proliferation, immune response, and inflammatory response (275). The state of "loser" cells is associated with JAK/STAT pathway activation, with its target genes *Socs36E* and *Chinmo* observed to be upregulated. This mechanism is activated by JNK signaling upstream, with unpaired ligand 3 (Upd3) increasing horizontally to enhance signal transduction (231). Due to interaction between competing parties, "loser" cells can promote the proliferation of "winner" cells in

competition (relative to their own proliferation rate) (282). Subsequently, "loser" cells may undergo apoptosis. Interestingly, the mechanism by which "winner" cells accelerate proliferation also involves JAK/STAT signaling (231). This highlights the dual role of the JAK/STAT pathway controlled by Upd3, which promotes apoptosis in "loser" cells and accelerates proliferation in "winner" cells. Therefore, the subsequent effects of the JAK/STAT signaling pathway may synergize with other mechanisms and are related to the properties of its upstream ligands.

Oxidative stress response is one of the significant triggers for cell competition and subsequent failure (283). Upregulation of genes associated with the expression of glutathione (GSH), glutathione transferase (GST), and cytochrome P450 oxidases (CYP450) has been observed in potential "loser" cells, with most of these genes being targets of Nrf2 (231). Nrf2 is a transcription factor that responds to stress environments by upregulating genes related to antioxidant function. Activation of the Nrf2 pathway in cells (dependent on transcription factors IRBP18 and Xrp1) is associated with the "loser" state (284). However, knockdown or overexpression of Nrf2 accelerates cell death and renders cells more sensitive to becoming "losers" (231). Nrf2 also demonstrates a dual effect depending on concentration; "loser" cells triggered by oxidative stress response rely on adaptive regulation of the Nrf2 pathway to maintain cellular homeostasis, but excessive accumulation of Nrf2 in "loser" cells relies on JNK to induce cell death (285), highlighting the importance of balance in the process of cell development and normal function and illustrating that both excessive and insufficient responses can have detrimental effects.

Current experiments have shown that p53 and JNK need to collaborate with other mechanisms to induce competition failure, such as the JAK/STAT pathway and the Nrf2 pathway (282), which indicates that cells that fail in competition are determined by multiple factors working together. Under stress, the activation of various intracellular signaling pathways makes cells "sensitive," and at the same time, they become "fragile" due to their easier triggering of death programs. Under the influence of adjacent cells, such as through cell-cell communication or competition for resources, individual cells will undergo processes that determine their fate, balancing between adaptive survival and apoptosis outcomes. However, it is currently unclear which pre-existing conditions make cells potential "losers" and trigger cell competition. Based on the series of signal transduction induced by CORT mentioned above, it is speculated that chronic stress-induced elevated CORT may be one of its inducing factors. However, more experimental evidence is still needed for validation.

3.5 Endoplasmic reticulum stress

The endoplasmic reticulum (ER) is a vital organelle within the cytoplasm, crucial for various intracellular processes such as protein folding, modification, and calcium storage (286). Its functionality is intertwined with energy metabolism and facilitates communication between cells by providing proteins for intracellular and extracellular signal transduction (287). Proper protein synthesis and processing rely on the ER's normal function. ERS serves as an alert for aberrant ER function, initially aiming to adapt to changing environments and restore ER function patterns. This response involves several mechanisms: (1) inhibition of upstream transcription and translation programs, which reduces the influx of new proteins into the ER; (2) induction and enhancement of protein repair gene expression to reduce protein folding errors; and (3) Promote protein degradation function to remove misfolded proteins (288, 289). Once the adaptive mechanism is activated, if the stressor persists, it may gradually cause the ER function to deviate from normal, resulting in persistent ERS (290). Although the adapted program is the optimal solution under current conditions, if the intracellular stress signal persists and the ER function cannot return to normal, the apoptotic program might be initiated (291).

Unfolded protein response (UPR) is an important cellular mechanism in response to ERS (292). This response involves the transition of glucose regulatory protein 78 (GRP78) from a bound to a free state, leading to an increase in GRP78 levels. Subsequently, downstream transcription factors such as X-box binding protein 1 (XBP1) and activating transcription factor-6 (ATF6) are activated (293), initiating the transcription of genes involved in ERS-related responses. XBP1 and ATF6, as nuclear transcription factors induced by ERS, play pivotal roles in intercellular signaling and can modulate downstream cellular functions (294–296). Research indicates that XBP1 and ATF6 not only stimulate the transcription of ER membrane and calcium reticulum protein genes during ERS (297), but also contribute to the generation of certain inflammatory mediators (298). Furthermore, XBP1 is essential for the production and secretion of antibodies by plasma cells (299). ERS-induced alterations in intracellular calcium homeostasis and protein quantity and structure represent adaptive immune responses to stress (300).

ERS within immune cells can significantly influence various immune functions, including antigen presentation (289), plasma cell differentiation, antibody production (300, 301), and T cell response to antigens (302). These alterations can significantly impact the onset and progression of inflammation, which is a key contributor to various tissue diseases. Among immune cells, macrophages are key in producing pro-inflammatory factors and orchestrating immune responses. Zhou et al. (303) demonstrated that low concentrations of CORT at 10 and 50 ng/ml induced ERS in macrophages, leading to notable increases in glucose regulatory protein 78 [GRP78; an important regulatory protein in the ERS process (304)] expression at both mRNA and protein levels. Furthermore, only 50 ng/ml of CORT has been shown to increase XBP1 expression, while no significant change was observed in activating transcription factor-6 (ATF6) levels. Evaluation of macrophage immune activation through adhesion index, chemotaxis index, and tumor necrosis factor-alpha (TNF-α) production revealed that CORT induces ERS and enhances immune function via GR activation on macrophages. The maximal immunostimulatory effect of CORT was observed at a concentration of 50 ng/ml, while concentrations of 100 ng/ml, 500 ng/ml, and 1000 ng/ml showed no such effect. Dhabhar et al. (305)

further suggested that 50 ng/ml of CORT roughly corresponds to the physiological levels produced by the body during stress and is sufficient to exert immune-stimulating effects on macrophages. These results show the role of chronically elevated CORT levels in continuously triggering immune and inflammatory responses until normal cellular function is compromised.

ERS represents one of the pathways through which chronic stress induces apoptosis in immune cells, and its pro-apoptotic effect has been observed to counteract the anti-apoptotic effect of STAT3, establishing cross-talk between the two (236). STAT3, a member of the STAT family, is involved in the regulation of cell proliferation and survival, promoting cell proliferation and tissue repair (306). Its activation, primarily achieved through phosphorylation, enables the transmission of signals from cytokine receptors on the cell membrane to the nucleus, thereby modulating gene transcription (307). Excessive STAT3 activation often signifies increased immune activity (308). The STAT3 signaling pathway primarily contributes to immune suppression and is typically stimulated by cytokines such as interleukin-6 (IL-6), IL-10, and certain growth factors, including epidermal growth factor (EGF), transforming growth factor-β (TGF-β), and insulin-like growth factor (IGF) (309, 310). These cytokines bind to receptors on the cell membrane surface, activating JAK, which in turn promotes the phosphorylation of STAT3 and its translocation to the nucleus, forming complexes with co-activating factors, binding to target gene promoter regions and promoting transcription (311). STAT3 often modulates immune responses by inhibiting the release of pro-inflammatory factors (such as STAT3/SOCS pathway) while increasing the expression of anti-apoptotic proteins (312). In the context of interaction with CORT, the elevation of resting CORT levels due to chronic stress upregulates the expression of interleukin-10 (IL-10) and phosphorylated STAT3 (p-STAT3). Despite the significant injurious effect of CORT, apoptosis of splenic white pulp cells and increased expression of caspase-3 (composed of lymphocytes and macrophages) were observed (236). However, the use of STAT3 inhibitors exacerbated CORT-induced apoptosis of splenic immune cells, indicating a negative regulatory effect of STAT3 (236). Further investigation into the mechanism of CORT-induced apoptosis revealed that p-STAT3 regulates cell survival by inhibiting the ERS pathway rather than mitochondrial stress and death receptor activation pathways. Significant differences were observed in pro-caspase-8 and glucose-regulated protein 78 (GRP78) levels, while BCL-2, BAX, and BCL-XL levels remained unaffected. Further examination of molecules involved in the ERS pathway revealed changes in the expression of ATF6α and p-IRE1α. Although the expressions of p-JNK, pro-caspase-12 and CHOP were not upregulated, their potential involvement in regulation could not be ruled out. The lack of change in these protein levels may also result from regulation by upstream molecules, potentially influenced by differences in control variables in the study.

The elevation of CORT during chronic stress is implicated in immune cell apoptosis via ERS. This process concurrently activates anti-apoptotic pathways. Specifically, pro-inflammatory factors TNF-α and IL-1β activate the apoptotic pathway, while the anti-inflammatory factor IL-10 activates the anti-apoptotic pathway. This dual signaling in the ER triggers ERS. Increased expression of GRP78 and downstream factors, including XBP1, ATF6α and p-IRE1α leads to the UPR and protein modification errors. Elevated expression of caspase-3 and caspase-8 promotes apoptosis, while increased expression of caspase-1 and TNF-α amplifies the immune response. Additionally, activation of the JAK/STAT3) pathway leads to increased levels of phosphorylated STAT3 (p-STAT3), inhibiting the expression of pro-caspase-8, caspase-3, GRP78, ATF6α, and p-IRE1α, thus mitigating ERS and exerting anti-apoptotic effects. These findings are shown in Figure 6.

3.6 GR dysfunction

Chronic stress continuously activates the HPA axis, leading to elevated CORT levels, resulting in both the depletion of GR and a gradual loss of the HPA axis' negative feedback capacity (313). Upon receiving this signal, cells initially undergo adaptation, prompting the overexpression of GR (65). At this juncture, both pro-inflammatory and anti-inflammatory signals are concurrently activated, highlighting the dual role of CORT, with outcomes contingent upon the gene function activated by the cell type (314). While the anti-inflammatory attributes of CORT are closely tied to normal GR function, the cytotoxic effects of CORT cannot be disregarded, as they can stimulate immune activation and the release of pro-inflammatory factors. Prolonged exposure to CORT may induce local inflammatory damage and even cell apoptosis (315–317). Persistent exposure to CORT and pro-inflammatory cytokines can diminish GR expression and prompt GR dysfunction, perpetuating inflammation (318). Consequently, aberrant GR function exacerbates the cytotoxic effects and persistent inflammation associated with CORT, concurrently diminishing cellular sensitivity to CORT, a condition known as glucocorticoid resistance (GCR) (319). Depending on tissue specificity, GCR may manifest as either sustained local inflammation or marked inhibition (314), potentially signifying prolonged exposure to CORT beyond physiological levels.

3.6.1 MAPK signaling pathway

During molecular signal transmission, the strength and direction of the effect depend on both ligand concentration and receptor sensitivity. The activation of the HPA axis increases the circulating level of CORT, which extensively exerts anti-inflammatory effects by binding to the GR encoded by Nr3c1 (320). Abnormal GR function is considered an important factor in the excessive activity of inflammatory cytokines, which promotes disease development (313). Wang et al. showed that chronic immune activation during GR blockade could cause significant and persistently high levels of inflammatory cytokines (TNF-α, IL-1β, IFN-γ) in rats, alongside depressive behavior (321). Persistently high levels of TNF-α and IFN-γ overactivate the tryptophan precursor metabolizing enzyme indoleamine 2,3-dioxygenase (IDO). On the one hand, this hinders the synthesis of 5-HT (322), and on the other hand, it accelerates the

decomposition of tryptophan (Try). The accumulation of its metabolites kynurenine (Kyn) and other tryptophan metabolites will trigger cellular oxidative stress damage (323, 324). ERK, p38 MAPK, and JNK are all members of the MAPK family. Upon activation through phosphorylation, they play an essential role in maintaining the fundamental signaling activities necessary for cell development (325). Numerous studies have demonstrated that the overactivation of ERK, p38 MAPK, and JNK/SAPK signals induces depressive behavior and neuroinflammation (326). This mechanism is primarily associated with decreased expression of synaptic-related genes, abnormal development of dendritic spines, increased apoptosis, and reduced expression of PSD95 (327–329). As mediators of cellular stress, the activation of the MAPK family also suppresses the expression of downstream ROS clearance genes and promotes the secretion of pro-inflammatory cytokines (330, 331). Moreover, these pathways interact with GR function. For instance, p38 regulates the phosphorylation of GR at serine sites 134 and 211 (Ser134 and Ser211), wherein activating GR phosphorylation at different sites transmits distinct signals (326). Specifically, phosphorylation of GR at Ser203 impedes nuclear translocation and reduces GR activity, whereas phosphorylation at Ser211 enhances nuclear translocation to augment GR signaling (326, 332). Zhang et al. observed that JNK activation upregulates GR phosphorylation at Ser246, consequently promoting the secretion of pro-inflammatory factors IL-1, IL-6, and TNF-α in the habenula (Hb), amygdala (Amyg), and medial prefrontal cortex (mPFC) (333). Conversely, GR activation can indirectly stimulate p38 through ROS and induce cell apoptosis via matrix metalloproteinase 13 (MMP) in certain pathways (326, 334). Thus, GR exhibits a competitive relationship with pro-inflammatory and anti-inflammatory signaling through cross-talk.

Studies have shown that chronic stress can induce GCR, resulting in inadequate control of the body's inflammatory response to infection (245). Prolonged exposure to inflammatory cytokines such as IL-6 and TNF-α can exacerbate the expression of disease signs and symptoms, contributing to increased susceptibility to diseases. Despite the widespread expression of GRs and the myriad recognized signals, elucidating the specific mechanism of GCR remains a current challenge. Some studies propose that macrophage factor IL-1β and Th17 cytokine IL-17A may negatively impact GR function by upregulating the expression of GRβ subtypes, with GRα being the primary structure exerting effects (335, 336). This process involves the activation of the JNK and p38 MAPK signaling pathways (337, 338). In fact, a mutually inhibitory signaling pathway exists between the GR and the MAPK families. While the overexpression of GR helps in the anti-inflammatory effect of CORT, in situations where inflammation prevails, the MAPK family signals inhibit GR function (339). During the post-translational modification stage, MAPK (including JNK, P38 MAPK, and ERK) regulates GR activity by modulating the site of GR phosphorylation. Phosphorylation at sites such as Ser134, 203, and 226 inhibits GR target gene transcription (340). Additionally, GSK3β (PI3K/AKT signal) similarly impacts GR by phosphorylating Ser171 and Ser404 (341). Acetylation of GR at K494 and K495 weakens its ability to inhibit NF-κB, consequently diminishing its anti-inflammatory effect (342). These factors collectively impede the anti-inflammatory effects of CORT. Chronic stress-induced GCR can disrupt the negative feedback regulation of the HPA axis and interfere with the downstream immune system's ability to control inflammation (343, 344). Variations in individual GR function may contribute to differences in susceptibility to cytokine-induced diseases. In terms of genetics, GR polymorphisms, such as ER22/23EK (rs6189 and rs6190), N363S (rs6195), BcII (rs41423247), and Nr3c1 gene polymorphism (Nr3c1 646 C>G), can diminish GR affinity for ligands, which then increases the susceptibility to inflammatory diseases and alters the immune milieu (340, 345, 346).

3.6.2 GILZ

The signal of mutual inhibition between MAPK and GR also involves the expression of anti-inflammatory genes such as *GILZ*. The lack of *GILZ* amplifies MAPK signaling (340). *GILZ*, a gene identified in recent years, plays a crucial role in regulating the anti-inflammatory effects of GCs, with its protein expression widely contributing to anti-inflammatory effects (347). The anti-inflammatory potency of mouse GCs was observed to diminish following GILZ knockout. GILZ stands out as the earliest transcriptional target of GR (348), highlighting its significance in modulating GR activity. Current research indicates that GILZ inhibits NF-κB nuclear translocation in immune cells and interacts with activator protein-1 (AP-1) to hinder transcription (349). For instance, GILZ downregulates the expression of co-stimulatory molecules such as CD80, CD86, and MIP-1 by restraining NF-κB (113). Additionally, GILZ promotes Th2 and Treg cell phenotypes in T cells by suppressing NF-κB and activating TGF-β (350). The promotion of antigen presentation involves GILZ facilitating the process by which antigen-presenting cells display antigens to T cells, thereby initiating an immune response (351, 352). GILZ has also been found to inhibit neutrophil activation by suppressing the MAPK/ERK pathway, leading to reduced phosphorylation of ERK and p38 (353). Additionally, it controls cell proliferation and differentiation by inhibiting FOXO3 (350). In summary, given its upstream position in signaling cascades, GILZ tends to dampen immune cell activity, contributing to its anti-inflammatory properties. Changes in CORT levels during stress also influence the expression of GR. CORT in colon tissue induces an increase in both GR and GILZ expression, thereby inhibiting NF-κB activity and reducing pro-inflammatory cytokine levels such as IL-1β and TNF-α (354). Interestingly, CORT does not induce GILZ expression in brain tissue; instead, it promotes the expression of FKBP5 and SGK1 (355). Therefore, the immune-regulatory effects and expression of GILZ exhibit tissue specificity, with GILZ potentially serving as the primary mediator of anti-inflammatory effects in peripheral tissues. Given its involvement in cross-talk between signaling pathways, GILZ's immune-regulatory mechanism may extend beyond its currently known functions, highlighting its potential as a focal point for future research.

3.6.3 FKBP51

In the investigation of the CORT-GR binding structure, studies have identified an imbalance within the GR partner complex FK506 binding protein (FKBP) as a contributor to GCR (356). This imbalance is characterized by elevated levels of FKBP51 and reduced levels of FKBP52. Notably, FKBP51, encoded by the FKBP5 gene, is more susceptible to external influences and has been linked to the onset of psychiatric disorders, emerging as a key focus of current research (357). FKBP51 regulates GR activity and the function of the HPA axis by interacting with the molecular chaperone heat shock protein 90 (HSP90) (358). Despite ongoing research, the precise regulatory mechanisms governing FKBP51's actions remain incompletely understood, generating widespread interest among researchers. Recent findings suggest that FKBP51 mediates the inhibition of AKT phosphorylation at the Ser473 site by recruiting PH domain leucine-rich repeat protein phosphatase (PHLPP), thereby leading to AKT inactivation (359). Downstream, the FKBP51/AKT signal pathway inhibits the phosphorylation activation of FOXO1 and the immunosuppressive effect of mTOR (360). Additionally, it has been observed to impede CORT-induced transcriptional regulation of GR by inhibiting GR phosphorylation (361), representing key signaling pathways for GC action. Knockout of FKBP5 leads to upregulated phosphorylation of GR at the Ser240 and Ser243 sites (362), resulting in decreased levels of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, nerve growth factor (NGF), and brain-derived neurotrophic factor (BDNF) (363). As part of the GR complex, FKBP51 limits GR function by reducing ligand binding sensitivity. Studies have confirmed that overexpression of FKBP5 diminishes the sensitivity of GR to stress, resulting in decreased CORT secretion under stress conditions (364). Therefore, FKBP51 acts as a negative regulatory factor of GR, inhibiting GC effects, albeit lacking the ability to fully complete GR signal transduction.

In recent years, FKBP51 has emerged as having an immune-promoting effect. Studies have demonstrated that stress can modulate *FKBP5* gene expression at the epigenetic level, leading to reduced *FKBP5* methylation, particularly evident in $CD4^+$ T cells and localized near chromosome 6p21.31 (365). Factors such as age, stress, and depressive phenotypes can expedite the decrease in FKBP5 methylation (358). Reduced FKBP5 methylation results in the upregulation of FKBP51 protein expression, which positively correlates with the expression of numerous pro-inflammatory genes, consequently increasing the granulocyte/lymphocyte ratio and IL-8 levels, thus driving peripheral inflammation (365). Further investigations have elucidated that *FKBP5*'s regulation of immunity hinges on NF-κB signaling. In peripheral blood mononuclear cells (PBMCs), increased FKBP51 expression promotes NF-κB signaling via the combination of NF-κB-inducing kinase (NIK) and inhibitor of kappa B kinase alpha (IKKα), thereby augmenting NF-κB signaling activity. Conversely, NF-κB signaling can induce a reduction in *FKBP5* gene methylation (resulting in increased FKBP51 expression) in immune cells. Therefore, a positive feedback loop ensues, which enhances FKBP51/NF-κB signaling and inflammation onset (365). Recent studies have shown that chronic stress-induced elevation of CORT upregulates FKBP51 expression and coincides with increased levels of pro-inflammatory factors IL-1β and TNF-α (366). While the use of FKBP51 inhibitors does not mitigate stress-induced CORT elevation, it promotes hippocampal neuronal proliferation and synaptic growth downstream, thereby mitigating social avoidance and anxiety-like behavior (367). Presently, the mechanism of FKBP51 in immune regulation remains somewhat constrained and warrants further exploration.

3.6.4 CREB and FKBP51

In addition to ligand concentration and receptor levels, transcriptional co-regulatory proteins can also regulate CORT signaling. One such protein is CREB, a transcription factor that responds to signals from anti-inflammatory factors such as IL-4, IL-10, IL-13, TGF-β, and NGF. CREB also controls the transcriptional activation of various signaling molecules, including c-Fos, c-Jun, and BDNF, thereby facilitating neuronal cell survival, differentiation, migration, and synaptic generation (368, 369). However, CORT has been observed to inhibit CREB activation, leading to a reduction in CREB phosphorylation levels and subsequent cellular damage (215). Studies indicate that FKBP51 can regulate CREB upstream, establishing a positive feedback loop. Research conducted by Hou et al. demonstrates that CORT regulates FKBP51 and CREB in a time-dependent manner (370). Short-term treatment with CORT at concentrations of 100 nM and 1 μM promotes the formation of FKBP51/CREB protein complexes and facilitates the localization of CREB protein in the nucleus, leading to increased expression levels of both FKBP51 and CREB. However, prolonged exposure to CORT at 1 μM significantly reduces this effect. Knocking out the *FKBP5* gene directly suppresses the downstream anti-inflammatory signals of CREB in cells, resulting in decreased levels of BDNF, TGF-β, Arg-1, and IL-10. Studies have also demonstrated that activation of the CREB pathway promotes the polarization of M2 macrophages and the expression of anti-inflammatory factors, thereby inhibiting inflammatory responses (371). These findings suggest that CORT stimulation activates the FKBP51/CREB signaling pathway to adapt to stress signals. However, chronic stress can impair the cellular response mechanism. FKBP51 and CREB can directly regulate transcription by forming complexes, and there are also indirect regulatory pathways between them, such as the ERK signaling and PI3K/AKT pathway (372, 373). CREB is regulated by multiple pathways and does not act independently (215). In conclusion, both excessive and insufficient expression of the FKBP5 gene product, FKBP51, can impede the normal cellular response to CORT. The resulting effect forms a network, with the outcome dependent on the dominant signaling pathway, as illustrated in Figure 7.

3.7 11β-hydroxysteroid dehydrogenase

11β-HSD mediates the effects of CORT, which comprises type 1 and type 2 isoenzymes. 11β-HSD1 promotes GC effects by activating CORT, whereas 11β-HSD2 inactivates CORT, reducing

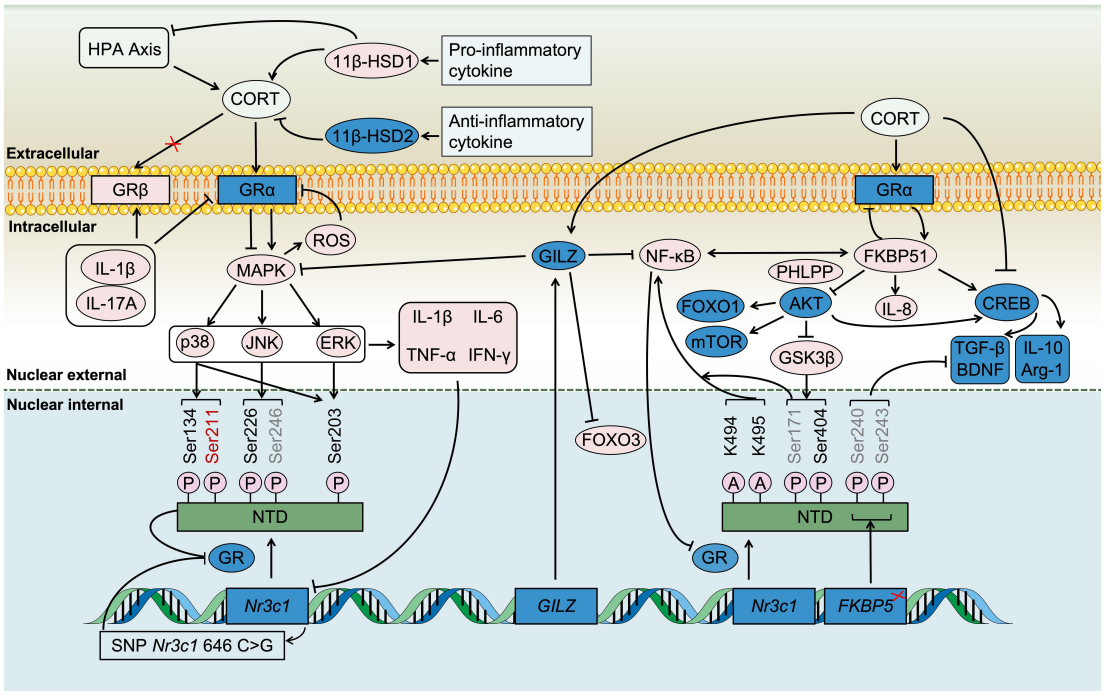

FIGURE 7
The immune regulation of CORT depends on GR signaling and 11β-HSD. CORT continuously transmits stress signals into the cell through GR, and stimulates immune cells to continuously express pro-inflammatory cytokines through the MAPK signaling pathway and FKBP51/NF-κB signaling pathway. In this scenario, on the one hand, pro-inflammatory factors change the conformation of GR (GRα to GRβ), and on the other hand, they downregulate the expression of GR. Both of these actions interfere with the normal functioning of GR and impede the transmission of anti-inflammatory signals. Due to compensatory response, the molecular partner of GR, FKBP51, is upregulated. However, elevated levels of FKBP51 increase inflammation mediated by NF-κB. In addition, it inhibits GRa. Ultimately, these effects collectively promote the progression of inflammation. Similarly, due to compensatory response, the increased expression of GILZ is to suppress the pro-inflammatory effect of CORT. However, it remains unclear whether GILZ can predominate in the complex interplay of numerous signaling pathways. In the local tissue, 11β-HSD regulates the effect concentration of CORT. 11β-HSD1 is beneficial for activating CORT and inhibiting the HPA axis, while 11β-HSD2 inactivates CORT. High expression levels of 11β-HSD1 were detected in inflammatory tissues and immune cells with a pro-inflammatory phenotype, whereas elevated levels of 11β-HSD2 were observed in immune cells with an anti-inflammatory phenotype. However, due to the persistent toxic effects of CORT, the balance is likely to shift towards the pro-inflammatory response mediated by 11β-HSD1.

exposure to local tissues (374). Thus, the regulatory influence of 11β-HSD on GC in various tissues has received significant attention in recent years. 11β-HSD is essential in controlling the signal transmission of CORT and GR binding in peripheral tissues. Perez et al. demonstrated that 11β-HSD1 inhibitors reduce post-stress blood CORT levels, whereas 11β-HSD2 inhibitors increase post-stress blood CORT levels. Additionally, through intraperitoneal injection and stereotactic device processing, it was found that 11β-HSD has a more pronounced regulatory effect on CORT levels in the periphery (375), underscoring the control exerted by 11β-HSD on the CORT effect. Given the intimate relationship between CORT and immunity, the regulatory role of 11β-HSD in immune inflammation has garnered attention. Sattler's study revealed upregulated pituitary 11β-HSD1 expression in both acute and chronic arthritis mice, whereas increased hippocampal 11β-HSD1 expression was only observed in chronic inflammation, with no change in hypothalamic 11β-HSD1 expression (376), suggesting that the pituitary gland can receive feedback signals (inflammatory factors) from the periphery. Furthermore, elevated expression of 11β-HSD1 was observed in inflammatory tissues in peripheral regions (377). Increased 11β-HSD1 expression enhances the CORT effect in local tissues, highlighting the close relationship between CORT and inflammation involving 11β-HSD1 regulation. In the short term, it aids in adapting to or controlling inflammation, while in the long term, it confronts the cytotoxic-induced inflammation and pro-apoptotic effects of CORT.

Some studies have demonstrated that the use of 11β-HSD1 inhibitors can significantly mitigate the adverse metabolic pathways associated with diabetes and obesity (374). Conversely, overexpression of 11β-HSD1 in the central nervous system is more likely to dampen HPA axis activity, fostering long-term chronic inflammation and rendering the HPA axis unresponsive (376). In the periphery, 11β-HSD primarily focuses on regulating the bioavailability of CORT in various tissues (375). Similarly, Maciuszek's study observed an increase in 11β-HSD1 expression in M1-type macrophages, while 11β-HSD2 expression was elevated in M2-type macrophages (378). This pattern may be attributed to the fact that M1 macrophages, being pro-inflammatory, require more CORT conversion to regulate inflammation by upregulating 11β-HSD1. In addition, M2 macrophages themselves secrete anti-inflammatory factors, prompting the upregulation of 11β-HSD2 to curb the excessive anti-inflammatory effect of CORT. Young's cell

experiments indicated that 11β-HSD1 downregulated the secretion of IL-1β and IL-6 by catalyzing the generation of CORT, thereby inhibiting the pro-inflammatory response mediated by NF-κB activation (379). This suggests that, apart from HPA axis activation to produce CORT, 11β-HSD1, as an indirect regulatory pathway, promotes the local production of CORT to adapt to the local environment. Additionally, Du's research proposes that exercise training boosts the expression of 11β-HSD1 in the lungs of obese mice, aiding in the activation of local CORT and inhibition of pneumonia (380). Therefore, 11β-HSD also plays a crucial role in mediating the immune regulation of CORT under stress and constitutes an integral component of its immune regulatory mechanism, as depicted in Figure 7, warranting further exploration.

4 Conclusion

CORT is closely related to immunity and is influenced by multiple signals. During acute stress, it surges rapidly, aiding in rapid stress responses and inducing immunosuppression through its potent anti-inflammatory properties, which are essential for maintaining internal homeostasis. Subsequent negative feedback from the HPA axis reduces CORT levels. However, chronic stress results in a gradual increase in CORT, continuously activating the immune system. Prolonged stress leads to elevated CORT levels, causing abnormal expression of GR and 11β-HSD in various circulating tissues, disrupting CORT's anti-inflammatory effects and impeding HPA axis negative feedback, perpetuating immune system activation and fostering chronic systemic inflammation. As circulating CORT levels rise, its cytotoxic effects intensify, exacerbating internal inflammation and triggering cellular autonomous death processes, impairing tissue function. Thus, immune suppression arises from excessive immune system activation and consumption, highlighting the complex relationship between CORT, immune function, and stress duration, necessitating further investigation into its mechanisms.

CORT functions as a GC molecule in the bloodstream and it can affect various tissues across the body. Its interaction with the immune system primarily involves the exchange of inflammatory cytokines and the signal transduction of cellular function within immune cells. During chronic stress, elevated CORT poses a challenge to immune cells. Initially, resting immune cells tend to polarize towards pro-inflammatory phenotypes, releasing pro-inflammatory and chemotactic factors to recruit assistance, often through MAPK, NF-κB, and other signaling pathways. This immune activation unavoidably consumes energy and metabolites. To prevent immune failure and cell death, signals that maintain homeostasis and promote cell survival, such as proliferation, differentiation, and maturity, are simultaneously activated. These signals operate through pathways like PI3K/AKT, cAMP/CREB, STAT3, Nrf2, and others. In the cytoplasm, signal transduction affects gene expression, transcriptional strength, and protein translation and modification, such as the expression of genes like GILZ and SOCS, the transcription of MiR-155 and TFEB, the expression of TREM2 and TAM, and the synthesis of eCB. In the communication between CORT and immune cells, these signals promote external anti-inflammatory responses and internal inhibition of intracellular stress signals. However, sustained high levels of CORT override these protective responses one by one, favoring pathways leading to injury, such as apoptosis signaling triggered by NRPL3, inadequate synthesis of ROS decomposition, nuclear accumulation of FOXO3a, and sustained activation of the Notch pathway and ERS-IRE1/XBP1 signaling pathway, all contributing to cell death. Therefore, immune balance is disrupted, leading to the progression of inflammation into disease. Pro-inflammatory symptoms signify continuous immune function until immune depletion occurs. Additionally, during the CORT process, the number and structural abnormalities of GR (excessive beta structure and insufficient alpha structure) and imbalanced expression of 11β-HSD (excessive 11β-HSD1 and insufficient 11β-HSD2) prevent the anti-inflammatory effects of CORT, contributing to GC resistance in immunotherapy. As shown in Figure 8.

It is essential to acknowledge that epigenetic changes and genetic polymorphisms influenced by environmental factors and lifestyle habits are potential contributors to the effects of CORT on immune regulation. Moreover, it is evident that immune regulation balance is ubiquitous, reflecting the intricate interplay between various factors and pathways. Recent hot research topics include macrophage and microglia polarization into M1/M2 phenotypes, T cell expression balance (Th1/2 and Th17/Treg), cannabinoid receptors CB1R and CB2R, immune cell membrane receptors TREM1 and TREM2, chaperone proteins FKBP51 and FKBP52, and enzymes 11β-HSD1 and 11β-HSD2. The equilibrium of these immune substances is critical for maintaining normal physiological functions. Prolonged exposure to external stressors, such as chronic family and social stress, fundamentally disrupts the immune balance mediated by CORT.

In conclusion, this review discusses the diverse and interconnected pathways between CORT and immune regulation. As shown in Figure 9. A comprehensive understanding of these regulatory mechanisms is vital for recognizing the close relationship between stress, emotional disorders, immunity, and inflammation, providing new avenues for treatment. Several key targets and immune regulatory proteins that are closely associated with CORT may serve as potential clinical biomarkers for the early screening of diseases. The identification and utilization of these biomarkers could benefit the health management of stress-related diseases, enabling more timely and effective interventions. Meanwhile, elucidating the underlying mechanisms and identifying key targets is highly advantageous for the development of novel therapeutic strategies. For instance, the discovery of small molecule drugs targeting specific pathways, the development of immune modulators, and the application of gene therapies all hold great promise. These advancements may pave the way for innovative treatment approaches for stress-related diseases, ultimately enabling more precise and efficient therapeutic interventions. Above all things, it is imperative to prioritize addressing stressors to prevent sustained elevation of CORT, thereby safeguarding immunity.

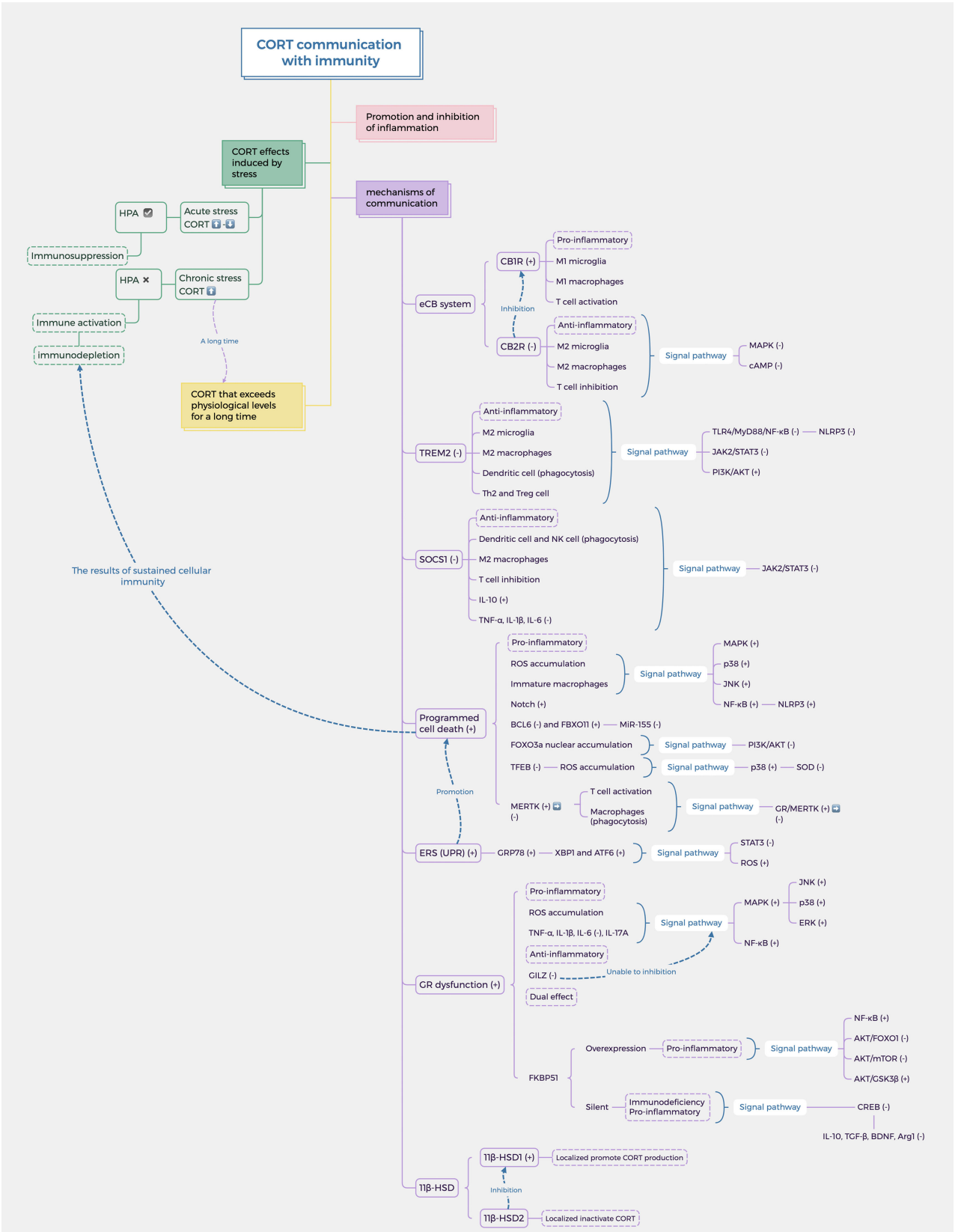

FIGURE 8
Summary of interaction mechanism between CORT effect and immunity.

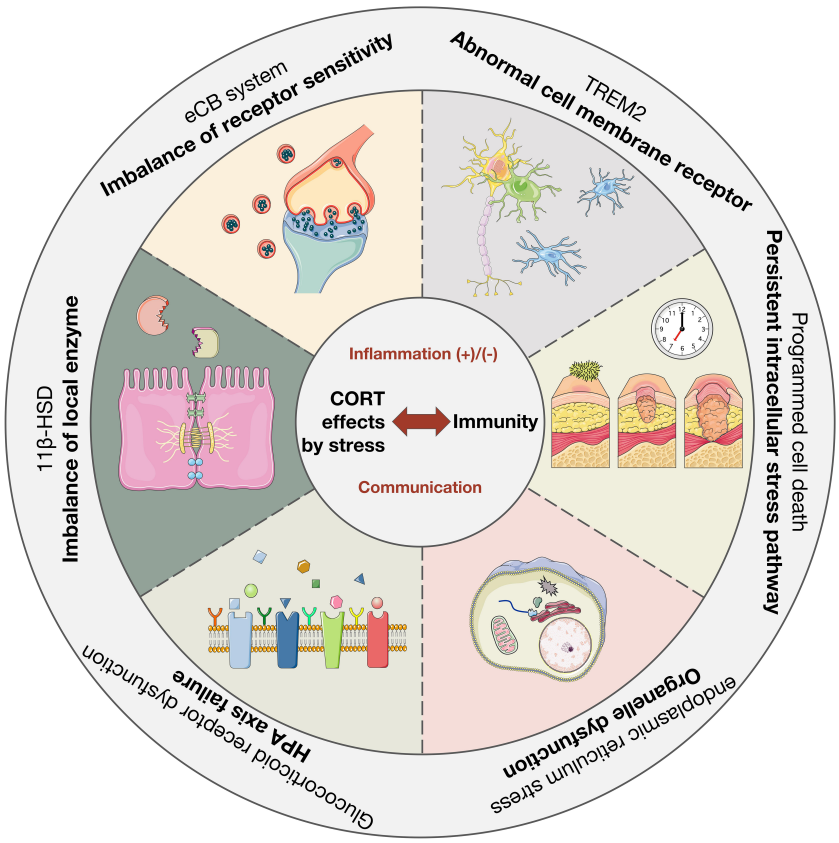

FIGURE 9
The way between CORT effect and immunity regarding inflammation.

5 Limitations

While the role of CORT has been extensively studied, its intricate connection with the immune system is also gaining increasing attention. Nevertheless, there are still several questions that remain unclear at present. Specifically, (1) it is widely accepted that CORT levels serve as a biomarker reflecting stress conditions. Current research is generally categorized into two types: acute stress and chronic stress, both of which are used to observe the relationship between CORT and immune phenotypes. However, during the stress process, organisms exhibit physiological responses of adaptation and compensation. Whether immune activation during this period is beneficial for functional enhancement of tissues or immediately causes inflammatory damage remains unclear and requires more rigorous phenotypic evidence. (2) In current research, there are various methods for simulating stress. Although CORT levels typically increase in response to stress, different stress paradigms may lead to divergent outcomes in terms of CORT effects. This is also one of the reasons why some studies report opposing results. A more detailed comparative study could be conducted to elucidate these differences. (3) With the cessation of stress, there is potential for the repair of immune activation and chronic inflammation. And persistent elevation of CORT levels remains a primary cause of irreversible inflammatory damage. However, the exact duration of stress required to trigger such immune damage is currently unclear. (4) CORT levels within the physiological range is intricately linked to innate immune function. It is evident that there are inherent variations in CORT levels among individuals. These differences may influence how individuals adapt to and respond to stress, leading to distinct outcomes. Among them, CORT may exhibit diverse patterns of effect. This variability is likely one of the reasons why different individuals exhibit diverse pathological characteristics when exposed to stress. Further clinical research, combined with in-depth basic research, is essential to explore and elucidate these differences. (5) Immune cells are ubiquitous, and the majority of immune cells and tissue cells express GR. Given that CORT is a glucocorticoid, the immune damage caused by stress is systemic in nature. It is not confined to the central nervous system or related to mental illness alone. Therefore, CORT may exert distinct immune effects in different types of tissues (sites). For instance, 11β-HSD exhibits varying expression patterns across different tissues, thereby mediating divergent immune responses. This area certainly warrants more in-depth exploration. (6) The interaction mechanisms between recently discovered key immune regulatory proteins and stress-related CORT remain to be elucidated.

Clarifying these mechanisms is also one of the promising avenues for exploring new therapeutic targets. (7) Based on the current understanding of CORT's role, its significance extends to certain unique environments. For instance, in the mechanisms underlying intercellular competition, there is a potential for CORT to be involved. As an example, atypical immune cells such as iT cells may have their differentiation or immune regulatory direction influenced by CORT. Further research is needed to elucidate these mechanisms.

Currently, there is a relatively comprehensive understanding of the pathways through which CORT interacts with the immune system. Based on current research trends and hot topics, it is anticipated that more mechanisms of CORT will be uncovered in the future. As a key marker of stress, CORT holds significant research value across multiple system diseases. Moreover, identifying additional targets of CORT would be highly beneficial for the development of new small-molecule drugs.

Author contributions

JX: Investigation, Methodology, Project administration, Software, Visualization, Writing – original draft. BW: Data curation, Investigation, Methodology, Project administration, Writing – original draft. HA: Conceptualization, Funding acquisition, Writing – review & editing.

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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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Glossary

AA	Arachidonic acid
AC	Adenylate cyclase
ACS	Apoptotic speck-like protein containing a caspase recruitment domain
ADAR1	Double-stranded RNA adenylate deaminase antibody 1
AEA	Anandamide
AgRP	Agouti-related protein
AKT	Protein kinase B
AP-1	Activator protein-1
Arg-1	Arginase-1
ATF6	Activating transcription factor-6
ATM	Ataxia-telangiectasia mutated proteins
BAX	BCL2-Associated X
BCL-2	B-cell lymphoma-2
BDNF	Brain-derived neurotrophic factor
cAMP	cyclic adenosine monophosphate
CBR	Cannabinoid receptor
CCR	C chemokine receptor
CHOP	C/Ebp-Homologous Protein
CIC	Circulating immune complex
CREB	cAMP response-element protein
CORT	Corticosterone
COX	Cyclooxygenase
CRH	Corticotropin releasing hormone
CTL	Cytotoxic lymphocyte
CUMS	Chronic unpredictable mild stimulation
CYP450	Cytochrome P450
DAGL	Diacylglycerol lipase
DAP12	DNAX-activating protein of 12 kDa
DC	Dendritic cell
DDR	DNA damage repair
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EREG	Epidermal regulatory factor
ERK	Extracellular regulated protein kinases
ERS	Endoplasmic reticulum stress
eCB	Endocannabinoids
FAAH	Fatty acid amide hydrolase
Fas	TNF receptor superfamily, member 6
Fasl	Fas ligand
FGF	Fibroblast growth factor
FKBP	FK506 binding protein
FOXO	Forkhead box O
GABA	γ- aminobutyric acid
GC	Glucocorticoid
GCR	Glucocorticoid resistant
G-CSF	Granulocyte-colony stimulating factor
GILZ	Glucocorticoid induced leucine zipper
GM-CSF	Granulocyte macrophage-colony stimulating factor
GR	Glucocorticoid receptor
GRP78	Glucose regulatory protein 78
GSDMD	Gasdermin D
GSH	Glutathione
GSK	Glycogen synthetase kinase
GST	Glutathione transferase
HIP	Hippocampus
HPA	Hypothalamic-pituitary-adrenal
HSP	Heat shock protein
ICOS	Inducible T cell costimulator
IDO	Indoleamine 2,3-dioxygenase
IFI-16	Interferon induced nuclear protein-16
IFN	Interferon
IFNGR2	Interferon Gamma Receptor 2
IGF	Insulin-like growth factor
IKK	Inhibitor of kappa B kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP-10	Inducible protein-10
IRBP	Interphotoreceptor retinal binding protein
IRE1	Inositol requires enzyme 1
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LC	Locus coeruleus
LDHA	Lactate dehydrogenase-A
LIF	Leukemia inhibitory factor
LIX	Lipopolysaccharide-inducible CXC chemokine
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LXA4	Lipoxygen-A4
MAGL	Monoacylglycerol lipase
MAIT	Mucosa-associated invariant T
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage-colony stimulating factor
MDA-5	Melanoma differentiation associated gene-5
MDD	Major depressive disorder
MIP-1	Macrophage inflammatory protein-1
MHC	Major histocompatibility complex
MLKL	Mixed lineage kinase domain-like
mTOR	mammalian target of rapamycin
MyD88	Myeloiddifferentiationfactor 88
MMP	Matrix metalloproteinase
NAPE-PLD	N-acylphosphatidylethanolamine-hydolyzing phospholipase D
NE	Norepinephrine

NF-κB	Nuclear factor-κB
NGF	Nerve growth factor
NICD1	Notch1 intracellular domain
NIK	NF- κB-induced kinase
NK	Natural killer
NKT	Natural killer T
NLRP3	NOD-like receptor thermal protein domain associated protein-3
NO	Nitric oxide
NPY	Neuropeptide Y
NPY1R	Neuropeptide Y receptor-1
PAF	Platelet activating factor
PBMC	Peripheral blood mononuclear cell
PD	Programmeddeath
PG	Prostaglandin
PHLPP	PH Domain Leucine-rich Repeat Protein Phosphatase
PI3K	Phosphatidylinositol 3-kinase
PKR	Double-stranded RNA-dependent protein kinase
PLCγ2	Phospholipase Cγ2
PRR	Pattern recognition receptor
PVN	Paraventricular hypothalamic nucleus
P2X7	Purinergic 2X7
RA	Rheumatoid arthritis
RIPK3	Receptor interaction serine threonine protein kinase 3
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SAPK	Stress-activated protein kinase
SNS	Sympathetic nervous system
SOCS	Suppressor of cytokine signaling
SOD	Superoxide dismutase
STAT	Signal transducers and activators of transcription
TCR	T cell receptor
TFEB	Transcription factor EB
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TREM2	Triggering receptor expressed on myeloid cells-2
TREX1	three prime repair exonuclease 1
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
XBP1	X-box binding protein 1
XO	Xanthine oxidase
ZBP1	Z-DNA Binding Protein 1
2-AG	2-arachidonoylglycerol
3β-HSD	3β-hydroxysteroid dehydrogenase
5-HT	5-hydroxytryptamine
11β-HSD	11β-hydroxysteroid dehydrogenase.

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Gut microbiota is involved in the exacerbation of adrenal glucocorticoid steroidogenesis in diabetic animals by activation of the TLR4 pathway

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Introduction: Diabetes induces glucocorticoid production in patients and animal models, however, the exact mechanism behind this phenomenon is still elusive. The activation of toll-like receptor (TLR) 4 induces glucocorticoid production by the adrenals. Since diabetic patients showed gut dysbiosis in parallel to an increase in epithelial-intestinal permeability, this study investigates the role of TLR4 activation by gut bacteria-derived lipopolysaccharide on the overproduction of corticosterone in diabetic rodents.

Methods: Diabetes induction was achieved through the intravenous injection of alloxan, followed by treatments with antibiotic therapy or TLR4 antagonist (TAK-242) for 14 consecutive days.

Results: Diabetic animals showed an increase in plasma corticosterone levels as well as overexpression of TLR4 and Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF) in the adrenals. Diabetic mice also showed gut dysbiosis, with an increase in the relative proportion of potentially pathogenic bacteria. We observed morphological alterations as well as increased inflammation in the colon with a predominance of a Th17 cytokine profile in diabetic mice, in parallel to an increase in the epithelial-intestinal permeability and lipopolysaccharide content in the adrenals. TAK-242 significantly decreased the overexpression of adrenocorticotropic hormone receptor and 11β-hydroxysteroid dehydrogenase

type 1 in the adrenal glands of diabetic mice. Furthermore, both TLR4 antagonist and TLR4 mutant mice (C3H/HeJ) induced a significant reduction in plasma corticosterone levels in diabetic mice.

Conclusion: Our findings revealed that gut dysbiosis participates in the exacerbation of corticosterone production by diabetic animals, suggesting that therapeutic strategies that can normalize gut microbiota in diabetics represent promising therapeutic candidates for the treatment of glucocorticoid-induced comorbidities in diabetes.

KEYWORDS

diabetes, gut microbiota, HPA axis, LPS, glucococorticoids

1 Introduction

Diabetes is a chronic metabolic disease characterized by hyperglycemia caused by a decrease in the synthesis and/or action of insulin. In 2021, the International Diabetes Federation (IDF) estimated that 537 million people were living with diabetes, of which approximately 50% have not yet been diagnosed and, consequently, have uncontrolled blood glucose levels (1). In diabetic patients, poorly controlled glycemia is related to the development of several disabling and costly complications, accompanied by an expense of about 700 billion USD in global healthcare (2). Hyperglycemia and the reduction in the production and/or action of insulin culminate in a deep hormonal imbalance in diabetic patients, including the hyperactivity of the hypothalamus-pituitary-adrenal (HPA) axis (3–5). In diabetic patients, the high circulating levels of glucocorticoids, a stress hormone produced by activation of the HPA axis, are correlated with the promotion of several comorbidities, such as neuropathy, depression, and wound healing deficiency (6–9).

Previously, we and others showed that the exacerbation of adrenal glucocorticoid steroidogenesis in diabetic animals is related to high systemic adrenocorticotropic hormone (ACTH) levels combined with overexpression of the ACTH receptor, MC2R, and the steroid machinery, including acute regulatory protein (StAR) and 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1), in the adrenal glands (10–13). Furthermore, we demonstrated that an imbalance in the pro- and anti-inflammatory pathways in the adrenal glands is crucial to the high circulating glucocorticoid levels during alloxan-induced diabetes in rodents. We showed that an increase in the activation of pro-inflammatory Angiotensin (Ang)-II/Ang-II receptor (AT) subtype AT1 pathway is involved in the exacerbation of glucocorticoid production by the adrenal glands in diabetic mice, which is reversed by the stimulation of the anti-inflammatory AT2 receptor (11). Besides, the reduction in the expression and activation of the anti-inflammatory receptor peroxisome proliferator-activated receptor (PPAR)γ in the adrenal glands of diabetic rats also participates in the increased production of glucocorticoids (12).

It is well known that diabetic patients display dysbiosis in their gut microbiota, with a predominance of pathogenic bacteria and an increase in the permeability of the epithelial-intestinal barrier, which results in high levels of bacterial products, such as lipopolysaccharide (LPS) in the circulation (2, 14). In addition, diabetic patients also show a rise in the systemic levels of endogenous activators of TLR4, including 70 kilodalton heat shock protein (HSP70) and high mobility group box 1 protein (HMGB1) (15). Furthermore, monocytes isolated from the peripheral blood of diabetic patients show overexpression of TLR4 and Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF) compared to cells obtained from control subjects (15). In addition, human and murine adrenocortical cells express TLR4 (16), and these cells produce glucocorticoids after stimulation with LPS *in vitro* and *in vivo* (17, 18).

In this study, we evaluated whether TLR4 activation by endogenous or gut bacteria-derived TLR4 agonists contributes to the overproduction of corticosterone in diabetic mice. As corticosterone is produced by adrenals, we performed an evaluation of steroidogenic machinery, TLR4, TRIF, and endogenous activators of TLR4 expression in these glands of diabetic animals. To evaluate the microbiota profile and epithelial-intestinal permeability in our model, we analyzed the microbiome in the feces of Swiss-webster mice, histopathological parameters and cytokine profile in the colon, and quantified the permeabilization of Dextran FITC400 from gut to bloodstream, and

Abbreviations: 11βHSD1, 11β-hydroxysteroid dehydrogenase type 1; ACTH, Adrenocorticotrophic hormone; Ang, Angiotensin; AT, Ang-II receptor; DAMPs, Damage-associated molecular patterns; HPA, Hypothalamus-pituitary-adrenal; HMGB1, High mobility group box 1 protein; HSP70, 70 kilodalton heat shock protein; IDF, International Diabetes Federation; LPS, Lipopolysaccharide; NOD, Non-obese diabetic; PPAR, Proliferator-activated receptor; StAR, Acute regulatory protein; TLR, Toll-like receptor; TRIF. Toll/IL-1R domain-containing adaptor-inducing IFN-β.

the levels of LPS in the plasma. We also included in this evaluation the analysis of LPS content in the adrenals of diabetic mice and mice submitted to antibiotic therapy. To assess the effect of TLR4 on the overproduction of corticosterone by diabetic mice, we treated the mice with TLR4 antagonist TAK-242 and induced diabetes in the TLR4 mutant mice C3H.HeJ. We obtained direct evidence that gut bacteria-derived LPS is involved in the exacerbation of corticosterone production by diabetic animals through the activation of TLR4 in the adrenal glands.

2 Material and methods

2.1 Chemicals

Alloxan monohydrate, ampicillin, FITC D4000, LPS (Escherichia coli serotype 026: B6), metronidazole, and neomycin were purchased from Sigma Chemical Co. (Saint Louis, MO, USA). Ethanol, methanol, and xylene were purchased from Merck (Rio de Janeiro, RJ, Brazil). Ketamine and xylazine were obtained from Syntec (São Paulo, SP, Brazil), and sodium heparin and sterile saline solution from Roche (São Paulo, SP, Brazil). TAK-242 was purchased from MedChemExpress (Monmouth Junction, NJ, USA). All solutions were prepared immediately before use.

2.2 Diabetes induction and stimulation

Male Wistar rats (4–6 weeks old, weighing 200 to 250 g), Swiss-Webster (5–6 weeks old, weighing 20 to 25 g), C3H.He (4–6 weeks old, weighing 18 to 20 g), and C3H.HeJ mice (4–6 weeks old, weighing 18 to 20 g) were obtained from the Institute of Science and Technology in Biomodels (ICTB) from Oswaldo Cruz Foundation (Fiocruz). All procedures used were approved by the Committee on Use of Laboratory Animals of the Oswaldo Cruz Institute (CEUA-IOC/Fiocruz, licenses L-027/2016 and L-004/2024). Animals were housed in groups of up to four in a temperature (22 °C ± 2 °C), humidity, and light-controlled (12 h light/dark period) colony room, with *ad libitum* access to food and water.

Diabetes was induced in 12-hour fasted (water *ad libitum*) animals by a single intravenous injection of alloxan monohydrate (40 and 65 mg/kg in rats and mice, respectively) (19, 20). Non-diabetic animals received an equivalent dose of the vehicle (sterile saline 0.9%). Seven days after the alloxan injection, the blood glycemia was determined with a glucose monitor (Freestyle Optium, Abbott Brasil, Rio de Janeiro, RJ, Brazil) from samples obtained from the tail vein. Only animals showing blood glucose levels greater than 15 mmol/L were considered diabetic and included in the experiment. Twenty-one days after diabetes induction, some rats received a single challenge with LPS (100 ng/cavity, i.p.) and were euthanized after 30 min or 60 min (17). We injected an equivalent dose of the vehicle (sterile saline 0.9%) in control rats. Animal euthanasia was performed with ketamine (140 mg/kg, i.p.) and xylazine (20 mg/kg, i.p.), as previously reported (21), 21 days after diabetes induction.

2.3 Hormone quantification

Animals were euthanized during the nadir (08:00h) of the circadian rhythm, as previously described (Ventura et al., 2020). Blood was immediately collected from the abdominal aorta with heparinized saline (40 U/mL), centrifuged for 10 min at 4 °C and 433 × g, and stored at -20°C until use. Plasma corticosterone and insulin were quantified by radioimmunoassay kit following the manufacturer's guidelines (MP Biomedical, Irvine, CA, USA), using a gamma counter (ICN Isomedic 4/600 HE; ICN Biomedicals Inc., Costa Mesa, USA).

2.4 Immunohistochemistry staining

The left adrenal glands, cleaned of surrounding fat, and the colon, cleaned of feces, using sterile PBS, were obtained from animals. Immunohistochemistry in paraffinized adrenal and colon histological sections was performed as previously described (Insuela et al., 2019). The primary antibodies used were specific polyclonal goat anti-TLR4 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and specific antibody monoclonal mouse anti-LPS (1:100; Hycult Biotech Inc. Wayne, PA, USA). The secondary antibodies used were horseradish peroxidase-conjugated streptavidin (HRP) (polyclonal anti-goat and monoclonal anti-mouse IgG (1:100), R&D Systems, Minneapolis, MN, USA).

2.5 Western blot analysis

The cleaned right adrenal glands were homogenized in RIPA buffer containing protease and phosphatase inhibitor cocktails. After quantifying protein content by the BCA method (D'Almeida et al., 2017), 60 µg total protein/lane was resolved on 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then electrotransferred to a nitrocellulose membrane using a semi-dry transfer apparatus (Trans-Blot SD; Bio-Rad, Hercules, CA, USA). Next, the immunoblot was performed as previously described (Insuela et al., 2019). Primary antibodies against the following proteins were used: anti-11β-HSD1 (1:200; Santa Cruz Biotechnology), anti-MC2R (1:200; Santa Cruz Biotechnology), anti-StAR (1:250; Santa Cruz Biotechnology), anti-TLR4 (1:250; Santa Cruz Biotechnology), and anti-TRIF (1:200; Thermo Fisher Scientific, Waltham, MA, USA). The housekeeping anti-β-actin (1:1000; Santa Cruz Biotechnology) was used as the standard.

2.6 DNA extraction

Approximately 100 mg of mouse feces was used for DNA extraction using the QIAamp DNA Stool Minikit (Qiagen, Germany) with some modifications. Mechanical lysis was performed by transferring the stool to PowerBead Garnet tubes (Qiagen), adding 1 mL of InhibitEX buffer to each sample, and incubating at 95°C for 5 min. Afterward, samples were vortexed at maximum speed for 10 min, centrifuged at 13,000 × g for 1 min, and

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the supernatant was collected. The next steps were followed according to the manufacturer's recommendations. DNA was stored at -80°C until analysis.

2.7 16S rRNA sequencing

Illumina protocols were used for sequencing 16S rRDNA amplicon libraries. We amplified the variable regions V3-V4 of the 16S rRNA gene using primers 5'- CCTACGGGNGG CWGCAG-3' (forward) and 5'- GACTACHVGGGTATC TAATCC-3' (reverse) and the enzyme KAPA HiFi HotStart ReadyMix (Roche, Pleasanton, USA). After preparation, libraries were sequenced on the MiSeq system with chemistry v2–500 cycles. Sequencing was performed at the Plataforma de Sequenciamento de Ácidos Nucleicos de Nova Geração – RPT01J of Fundação Oswaldo Cruz (Rio de Janeiro, Brazil). Sequencing data analysis was performed as previously described (Hardoim et al., 202 3). Initially the sequences were quality checked with FastQC (Wingett and Andrews 2018). Sequence data were quality-filtered and trimmed using Trimmomatic version 0.36 (Bolger et al., 2014), truncating reads if the quality dropped below 20 in a sliding window of 4 bp. Further processing was performed with USEARCH version 11.0.667 (Edgar 2013) where sequences were merged, and the sequencing reads were quality-filtered excluding reads with < 395 or > 470 nucleotides. Additionally, reads with more than one ambiguous base or an expected error of > 1 were also removed from the dataset. Filtered sequences were denoised and clustered into unique sequences (Amplicon Sequence Variants, ASV) using the UNOISE3 algorithm (Edgar 2016a) implemented in USEARCH. Chimeric sequences were removed *de novo* during clustering and subsequently in reference mode using UCHIME2 (Edgar 2016b) with the Genome Taxonomy Database (GTDB, Parks et al., 2020). The ASVs were classified against GTDB using the BLCA algorithm (Gao et al., 2017). Sequences from mitochondria and chloroplasts were removed from the dataset based on the Greengenes 13_5 taxonomy (McDonald et al., 2012). The resulting ASV table contained a total of 488,313 reads, with an average number of reads per sample of 48,831 (ranging between 2,797 to 154,310 reads per sample). The generated ASV table was then processed using the MicrobiomeAnalyst online software suite (https://www.microbiomeanalyst.ca/) in Marker Data Profiling mode. All sequences generated in this study were deposited as a Sequence Read Archive in the NCBI database with Bioproject ID PRJNA1253081 (SAMN48063965- SAMN48063969 for 16S rRNA gene of the control samples and SAMN48063970-SAMN48063974 for 16S rRNA gene for the treatment samples).

2.8 Histological analysis

Colons were fixed in Millonig's buffer solution (pH = 7.4) with 4% paraformaldehyde for 48 h to preserve tissue architecture. Then, 4 µm thick sections were stained with hematoxylin and eosin to measure villi height and crypt depth, with determinations made in 4–10 randomly selected villi and crypts, besides muscular thickness, and inflammation score, using six randomly selected fields. The inflammation score was calculated using the following parameters: inflammatory infiltrate, extent of inflammation, and inflammatory percentage impairment (Table 1). Histologic sections were stained with periodic acid–Schiff (PAS) for measuring mucus production and PAS & alcian blue to quantify mucus thickness. Images were scanned using a 3DHISTECH–Pannoramic MIDI whole slide scanner (Budapest, Hungary) and captured with a 20× objective lens. Analyses were performed using ImageJ™ Software (NIH, Bethesda, MD, USA) or Pannoramic Viewer Software (3DHISTECH; Budapest, Hungary).

TABLE 1 Criteria adopted for analyzing the inflammatory score in the colon.

Criteria adopted			
Inflammation score	Inflammatory infiltrate	1	None
		2	Discreet
		3	Moderate
		4	Severe
	Extension	1	None
		2	Mucosa
		3	Mucosa and submucosa
		4	Transmural
	Inflammatory percentage impairment	1	1-25%
		2	26-50%
		3	51-75%
		4	76%-100%
Maximum total		12	

2.9 Cytokine quantification

We homogenized the colons in cold lysis buffer containing 0.1% Triton X-100 and a Complete protease inhibitor cocktail (F. Hoffmann-La Roche Ltd.) in PBS 1X, and centrifuged at 13,000 × g for 10 min at 4°C. Then, levels of IL-1β, IL-4, IL-6, IL-10, IL-17, IL-22, TGF-β1, and TNF-α from supernatants of colon lysates were measured using commercially available ELISA kits (R&D Systems and Thermo Fisher Scientific). All assays were performed according to the manufacturer's instructions.

2.10 Intestinal permeability analysis

Twenty-one days after diabetes induction, we administered Dextran FITC400 (600 mg/kg, gavage) to 12-hour fasted mice (water ad libitum). After one hour, we obtained the plasma for quantification of Dextran FITC400 that crossed from the gut to the bloodstream by

fluorescence (excitation of 485 nm and emission of 528 nm), using a plate reader (SpectraMax M5, Molecular Devices, San Jose, CA, USA).

2.11 Treatments

Mice were treated with the TLR4 antagonist TAK-242 (3 mg/kg, i.p.) or an antibiotic cocktail containing ampicillin, metronidazole, and neomycin (1 g/L, drinking water) daily, for 14 consecutive days, starting 7 days after diabetes induction. Untreated mice received an equal volume of vehicles (0.1% DMSO, i.p., and drinking water, respectively). Drinking water with or without antibiotic cocktail was changed at least every 3 days. All analyses were performed 24h after the last treatment.

2.12 Statistical analysis

The results were expressed as the mean ± standard error of the mean (SEM). All data were evaluated to ensure normal distribution and statistically analyzed by one-way analysis of variance (ANOVA), followed by the Student Newman-Keuls multiple comparison post-test. We used the unpaired student's t-test when only two experimental groups were compared. All statistical analyses were performed with GraphPad Prism 8 software (La Jolla, CA, USA). Probability values (*P*) of 0.05 or less were considered significant. Statistical analysis of microbiome data was conducted using the MicrobiomeAnalyst software. Alpha diversity (Chao1) was compared using Welch's t-test. Beta diversity was analyzed at the ASV level, by Principal Coordinate Analysis (PCoA) using the Bray-Curtis index as the distance method and PERMANOVA. Dendrograms were generated at the ASV level, also using the Bray-Curtis index as the distance method and the Ward clustering algorithm. Linear Discriminant Analysis Effect Size (LEfSe) was performed at the genus level, using a *P* value cut-off of 0.05 and an LDA score of 2.0.

3 Results

3.1 Diabetes induces hypercortisolism and an increase in the expression of functional TLR4 and TRIF in the adrenal glands

Diabetes induced hyperglycemia (Figure 1A) and reduction in circulating insulin levels (Figure 1B), in parallel to an increase in plasma corticosterone levels (Figure 1C) in Swiss-Webster mice, compared to non-diabetic mice. Diabetes did not alter the expression of HMGB1 and HSP70 in the adrenal glands of Swiss-Webster mice (Figure 1D, Supplementary Figure S1A, Supplementary Figure S1B and Supplementary Figure S1C); however, it increased the expression of TLR4 and TRIF (Figure 1E, Supplementary Figure S1D, Supplementary Figures S1E, F) compared to non-diabetic mice. We also showed that diabetes induced the same results in Wistar rats (Figures 2A–I and Supplementary Figures S2A–F). To evaluate whether the increase in the TLR4 signaling machinery in the adrenal glands of diabetic rats could be related to a higher activity of the TLR4 pathway, we challenged rats with LPS, an activator of TLR4, and showed that LPS increased plasma corticosterone levels in diabetic rats 60 min after the challenge, without altering this output in non-diabetic rats (Figure 2J).

3.2 Diabetes alters gut microbiota profiles of Swiss-Webster mice

Since we did not observe an alteration in the expression of HMGB1 and HSP70, which are damage-associated molecular patterns (DAMPs) that activate TLR4, in the adrenal glands of diabetic rats and mice, we evaluated if diabetes changes the gut bacterial composition in Swiss-Webster mice. We did not observe changes in alpha diversity between the bacteriome of non-diabetic and diabetic mice (Figure 3A). Nevertheless, we found differences in beta diversity, as attested by the PCoA plot in Figure 3B and the dendrogram in Figure 3C. Interestingly, diabetes induction significantly changed the relative abundances of some phyla compared to non-diabetic mice, including a decrease in the Firmicutes and an increase in Proteobacteria (Figure 3D). We did not observe changes in the Bacteroidetes (data not shown). Furthermore, diabetic mice showed differences in the relative abundances of opportunistic pathogenic bacteria, including an increase in *Klebsiella* and a reduction in *Clostridium* (Figures 3E, F).

3.3 Diabetes promotes altered crypt-villus morphology and induces inflammation and mucus production in the murine colon

Diabetes promoted an increase in villus height (Figures 4A–C) and muscular thickness (Figure 4F), but did not change crypt depth (Figure 4I), in the colon of diabetic mice, compared to non-diabetic controls. Furthermore, diabetes induced inflammatory infiltration (Figures 4D, E, L) in the colon, in addition to an increase in mucus production (Figure 4G, H, M) and mucus layer thickness (Figures 4J, K, N). Diabetes increased IL-10, IL-17, IL-22, and TNF-α levels (Figures 5D–F, H), and reduced IL-1β (Figure 5A) levels in the colon of diabetic mice compared to non-diabetic controls, without altering the content of IL-4, IL-6, and TGF- β1 (Figures 5B, C, G).

3.4 Diabetes increases the permeability of the epithelial-intestinal barrier, resulting in a greater influx of LPS to the adrenal glands

We observed that diabetic mice presented an increase in the influx of FITC D4000 from the intestinal lumen to blood compared to non-diabetic mice (Figure 6A), showing a reduction in the epithelial-intestinal barrier. However, diabetic mice presented a reduction in plasma LPS levels compared to non-diabetic mice

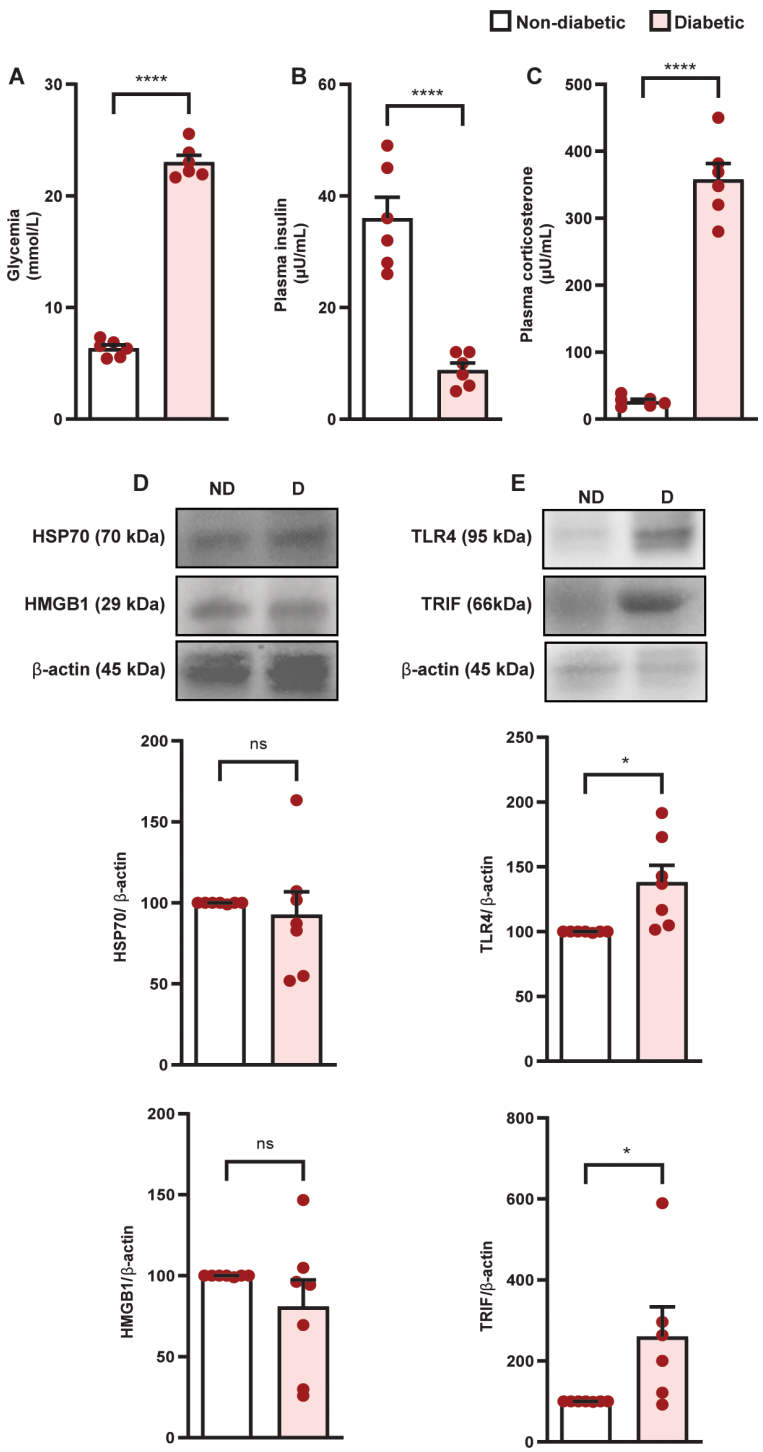

FIGURE 1
Diabetes induces hypercortisolism in parallel to an increase in the expression of TLR4 and TRIF but does not alter the amount of HMGB1 and HSP70 in the adrenal glands of Swiss-Webster mice. Analyses were performed 21 days after diabetes induction. **(A)** Blood glucose quantification. **(B, C)** Plasma quantification of insulin and corticosterone, respectively, by radioimmunoassay. **(D)** Expression of HMGB1 and HSP70 in the adrenal glands. **(E)** Expression of TLR4 and TRIF in the adrenal glands. Protein expression was determined by western blot. Data were normalized to β-actin and presented as the ratio between target protein levels relative to controls. Each bar represents the mean ± standard error of the mean. Pink dots represent the number of animals analyzed. Statistical analyses were performed by the students' t-test. *p<0.05. ****p<0.0001. ns, non-significant. HMGB1, High mobility group box 1 protein; HSP70, 70 kilodalton heat shock proteins; TLR4, toll-like receptor 4; TRIF, TIR-domain-containing adapter-inducing interferon-β.

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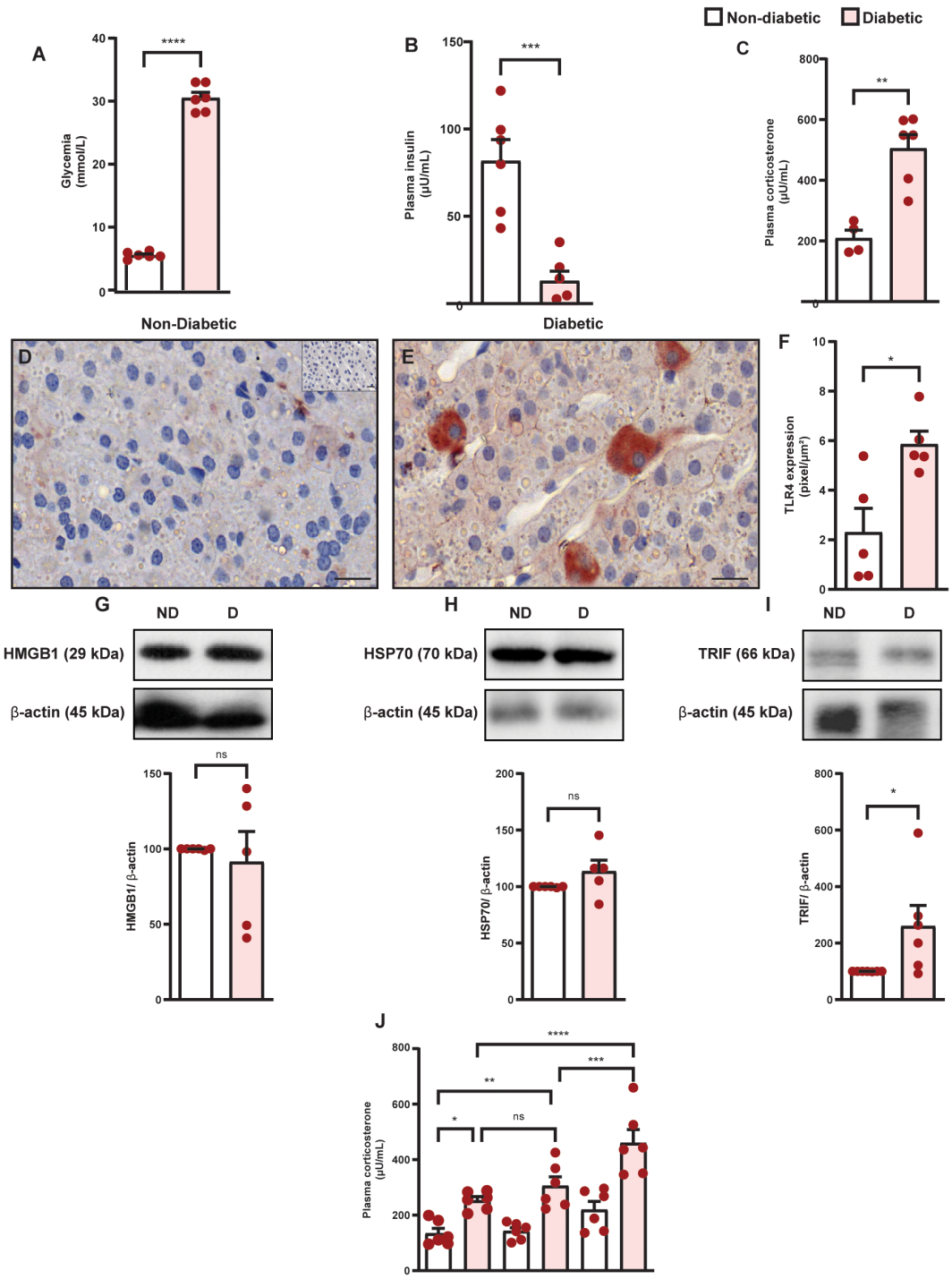

FIGURE 2
Diabetes induces hypercortisolism in parallel to an increase in the expression of TLR4 and TRIF but does not alter the amount of HMGB1 and HSP70 in the adrenal glands of Wistar rats. Analyses were performed 21 days after diabetes induction. **(A)** Blood glucose quantification. **(B, C)** Plasma quantification of insulin and corticosterone levels, respectively, by radioimmunoassay. Panels show representative photomicrographs of the expression of TLR4 in the zona fasciculate of the adrenal of non-diabetic **(D)** and diabetic rats **(E)** Inserts represent negative controls. **(F)** Quantification of pixels associated with positive TLR4 expression. **(G–I)** HMGB1, HSP70, and TRIF expression in the adrenal glands, respectively, were determined by western blot. Data were normalized to β-actin and presented as the ratio between target protein levels relative to controls. **(J)** Evaluation of LPS-induced plasma corticosterone levels in diabetic rats. Analysis was performed 30 and 60 min after LPS injection (100 ng/mL, i.p.). Each bar represents the mean ± standard error of the mean. Pink dots represent the number of animals analyzed. Statistical analyses were performed by the students' t-test. In analyzing three or more experimental groups, we used one-way ANOVA followed by Newman–Keuls test. *p<0.05. **p<0.005. ***p<0.0005. ****p<0.0001. ns, non-significant; Scale bar = 20 µm; HMGB1, High mobility group box 1 protein; HSP70, 70 kilodalton heat shock proteins; LPS, lipopolysaccharide; TLR4, toll-like receptor 4; TRIF, TIR-domain-containing adapter-inducing interferon-β.

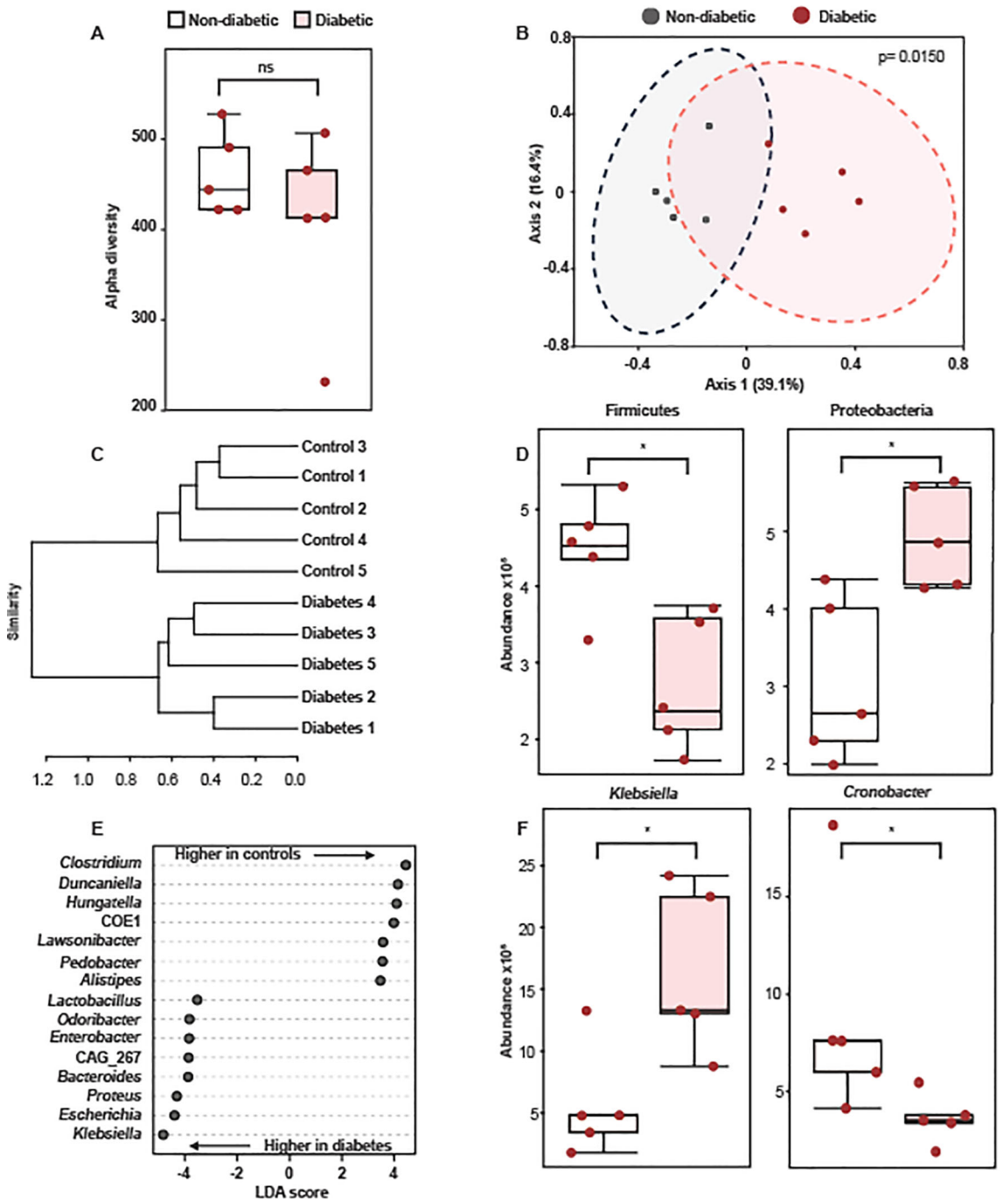

FIGURE 3
Diabetes alters colonic microbiota composition in Swiss-Webster mice. Analyses were performed 21 days after diabetes induction. **(A)** Alpha diversity of gut microbial communities from non-diabetic and diabetic mice. Statistical analyses were performed by Welch's t-test. **(B)** Principal Coordinate analysis (PCoA) of fecal bacteriome composition of non-diabetic and diabetic mice. Statistical analyses were performed by Bray-Curtis index as the distance method and PERMANOVA. **(C)** Dendrogram showing the separation of non-diabetic and diabetic mice based on changes in bacteriome composition. Statistical analyses were performed by Bray-Curtis index as the distance method and the Ward clustering algorithm. **(D)** Relative abundance of Firmicutes and Proteobacteria in fecal samples of non-diabetic and diabetic mice. **(E)** LDA scores for the 15 taxa with the highest discriminatory power to differentiate non-diabetic and diabetic mice. **(F)** Relative abundance of *Klebsiella* and *Clostridium* in fecal samples of non-diabetic and diabetic mice. Each bar represents the mean ± standard error of the mean. Pink dots represent the number of animals analyzed. *p<0.05. ns, non-significant.

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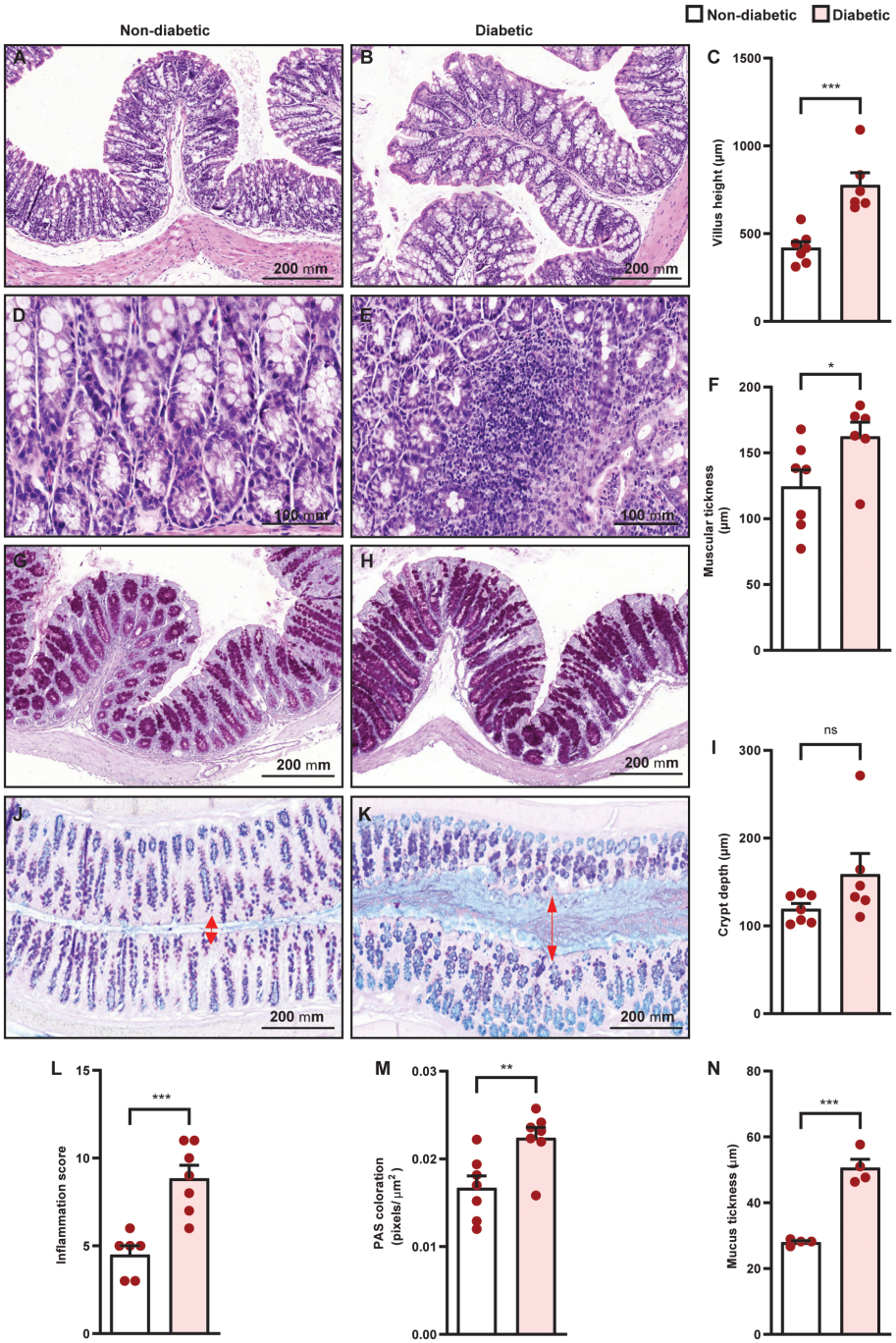

FIGURE 4
Diabetes induces histopathological alterations in the colon of Swiss-Webster mice. Analyses were performed 21 days after diabetes induction. Panels show representative photomicrographs of colon slices of non-diabetic and diabetic mice stained with hematoxylin and eosin [**(A, B, D, E)**, respectively], PAS [**(G, H)**, respectively], and PAS plus alcian blue [**(J, K)**, respectively]. **(C, F, I, L)** Evaluation of villus height, muscular thickness, crypt depth, and inflammation score, respectively, in colon sections stained with H&E. **(M, N)** Quantification of pixels associated with mucus and mucus thickness in colon sections stained with PAS and PAS plus alcian blue, respectively. Each bar represents the mean ± standard error of the mean. Pink dots represent the number of animals analyzed. Statistical analyses were performed by the students' t-test. *p<0.05. **p<0.005. ***p<0.0005. ns, non-significant. Red arrows indicated the mucus area in the colon sections stained with PAS plus alcian blue. Scale bar = 100 µm **(B, G)** and 200 µm **(A, C, D, F, H, I)**. PAS, periodic acid-Schiff.

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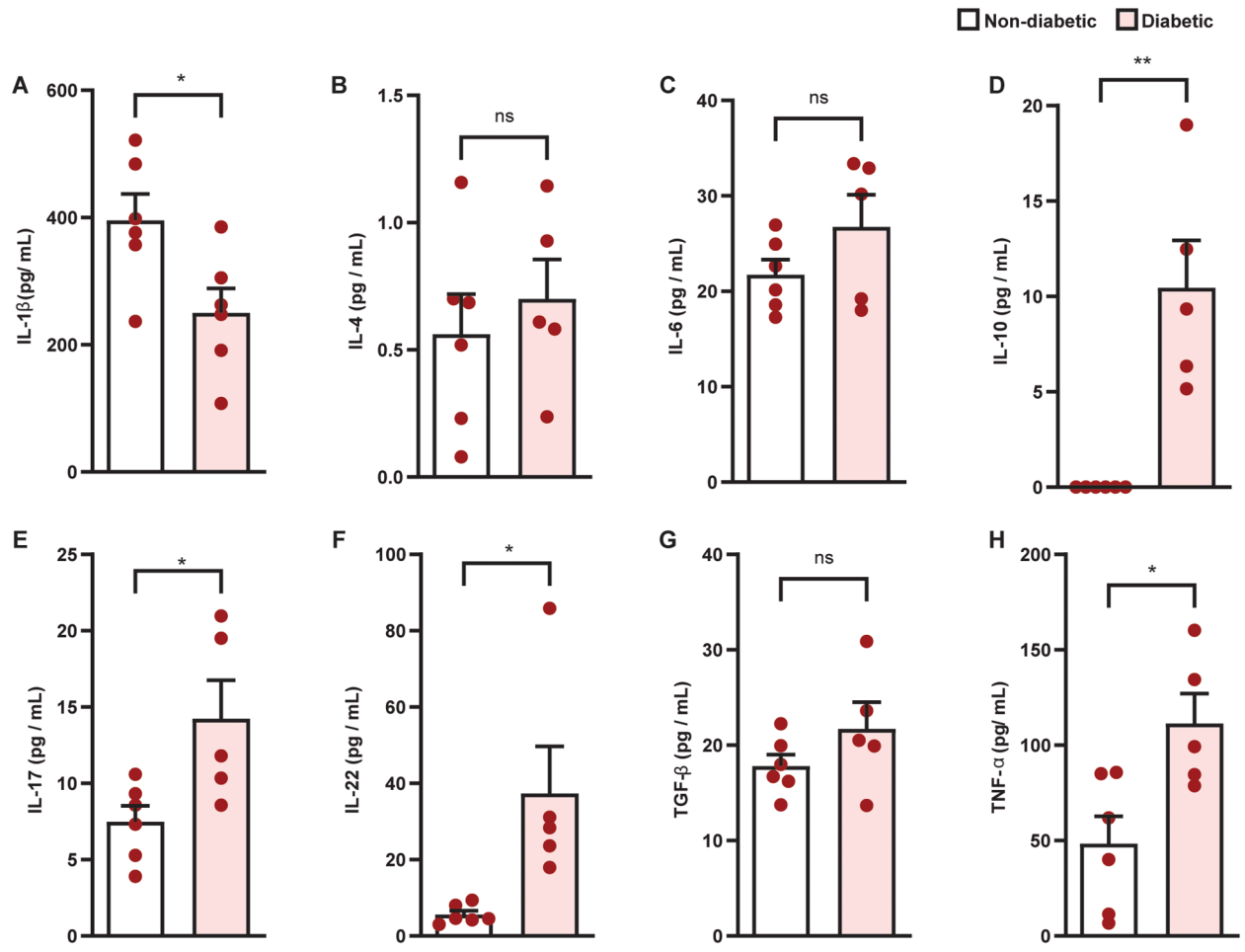

FIGURE 5
Diabetes alters the production of cytokines in the colon of Swiss-Webster mice. Colon levels of IL-1β **(A)**, IL-4 **(B)**, IL-6 **(C)**, IL-10 **(D)**, IL-17 **(E)**, IL-22 **(F)**, TNF-α **(G)**, and TGF-β1 **(H)** were evaluated 21 days after diabetes induction. Cytokine levels were quantified by ELISA. Each bar represents the mean ± standard error of the mean. Pink dots represent the number of animals analyzed. Statistical analyses were performed by the students' t-test. *p<0.05. **p<0.005. ns, non-significant; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor.

(Figure 6B). We also showed an increase in the levels of LPS in both the adrenals (Figures 6C, F, I) and colon (Figures 6D, E, G, H, J) of diabetic mice compared to non-diabetic controls.

3.5 Antibiotic therapy reduces plasma corticosterone levels and the overexpression of TLR4 and TRIF in the adrenals of diabetic mice

The increase in permeability of the epithelial intestinal barrier, the enrichment of potentially pathogenic bacteria in the gut microbiota, and the increase in LPS levels in the adrenal glands of diabetic mice suggest that endotoxins of pathogenic bacteria present in the intestinal microbiota of diabetic mice are involved in the hypercortisolism noted in those animals. To test this hypothesis, we treated animals with an antibiotic cocktail containing ampicillin, metronidazole, and neomycin. Antibiotic therapy significantly reduced blood glucose levels in diabetic mice compared with untreated diabetic mice, although antibiotic-treated animals remained hyperglycemic. Nevertheless, antibiotic therapy did not alter blood glucose levels in non-diabetic mice (Figure 7A). Even though antibiotic therapy decreased blood glucose levels in diabetic mice, it did not alter plasma insulin levels in either non-diabetic or diabetic mice (Figure 7B). Antibiotic therapy also significantly reduced plasma corticosterone levels in diabetic mice (Figure 7C). Lastly, we showed that antibiotic therapy reduced the expression of TLR4 and TRIF in the adrenal glands of diabetic mice (Figure 7D and Supplementary Figures S3A–C), without modifying these outputs in non-diabetic animals.

3.6 TLR4 blockage restores corticosterone levels in diabetic mice through decreased steroidogenesis

Since antibiotic therapy reduced the hypercortisolism of diabetic mice as well as the expression of TLR4 and TRIF in the

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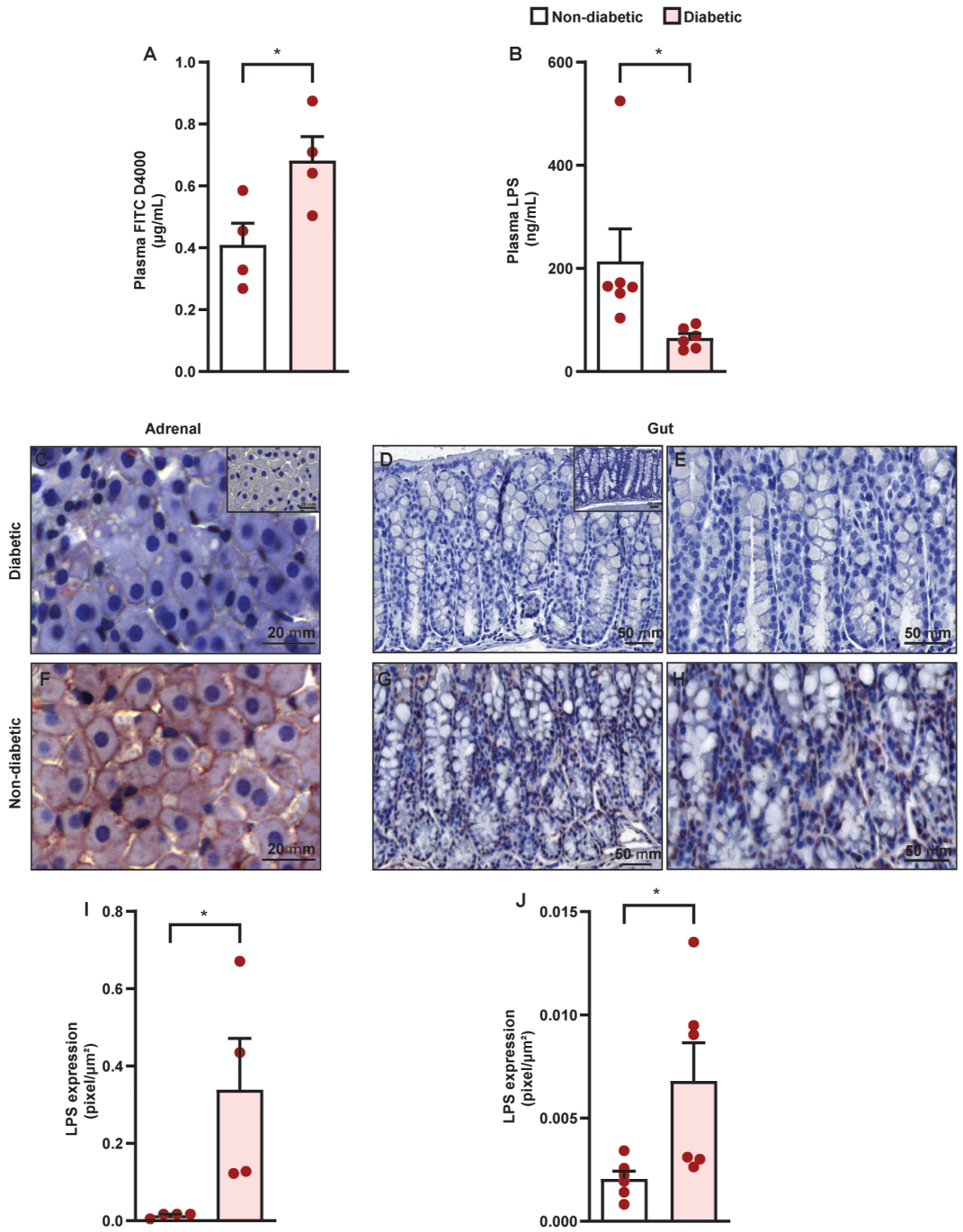

FIGURE 6
Diabetes induces an increase in epithelial-intestinal permeability in parallel to an influx of LPS into the adrenal glands of Swiss-Webster mice. Analyses were performed 21 days after diabetes induction. **(A)** Quantification of serum levels of FITC D4000−1 h after oral administration by gavage. **(B)** Evaluation of plasmatic levels of LPS by ELISA. Panels show representative photomicrographs of LPS levels in the zona fasciculate of adrenal **(C, F)** and colon **(D, E, G, H)** of non-diabetic **(C–E)** and diabetic mice **(F–H)**. Inserts represent negative controls. **(I, J)** Quantification of pixels associated with LPS levels in adrenals and gut, respectively. Each bar represents the mean ± standard error of the mean. Pink dots represent the number of animals analyzed. Statistical analyses were performed by the students' t-test. **p<0.05. Scale bar = 20 µm **(C, F)**, 50 µm **(D, G)**, and 100 µm **(E, H)**. FITC, fluorescein isothiocyanate; LPS, lipopolysaccharide.

adrenals, we evaluated the role of the TLR4 pathway in the exacerbation of adrenal steroidogenesis in diabetic mice. To do this, we used pharmacological and genetic approaches. Treatment with the TLR4 antagonist TAK-242 did not alter blood glycemia and plasma insulin levels in non-diabetic and diabetic mice (Figures 8A, C, respectively). Furthermore, TLR4 mutant mice displayed hyperglycemia and hypoinsulinemia after diabetes induction to the same extent as control mice (Figures 8B, D,

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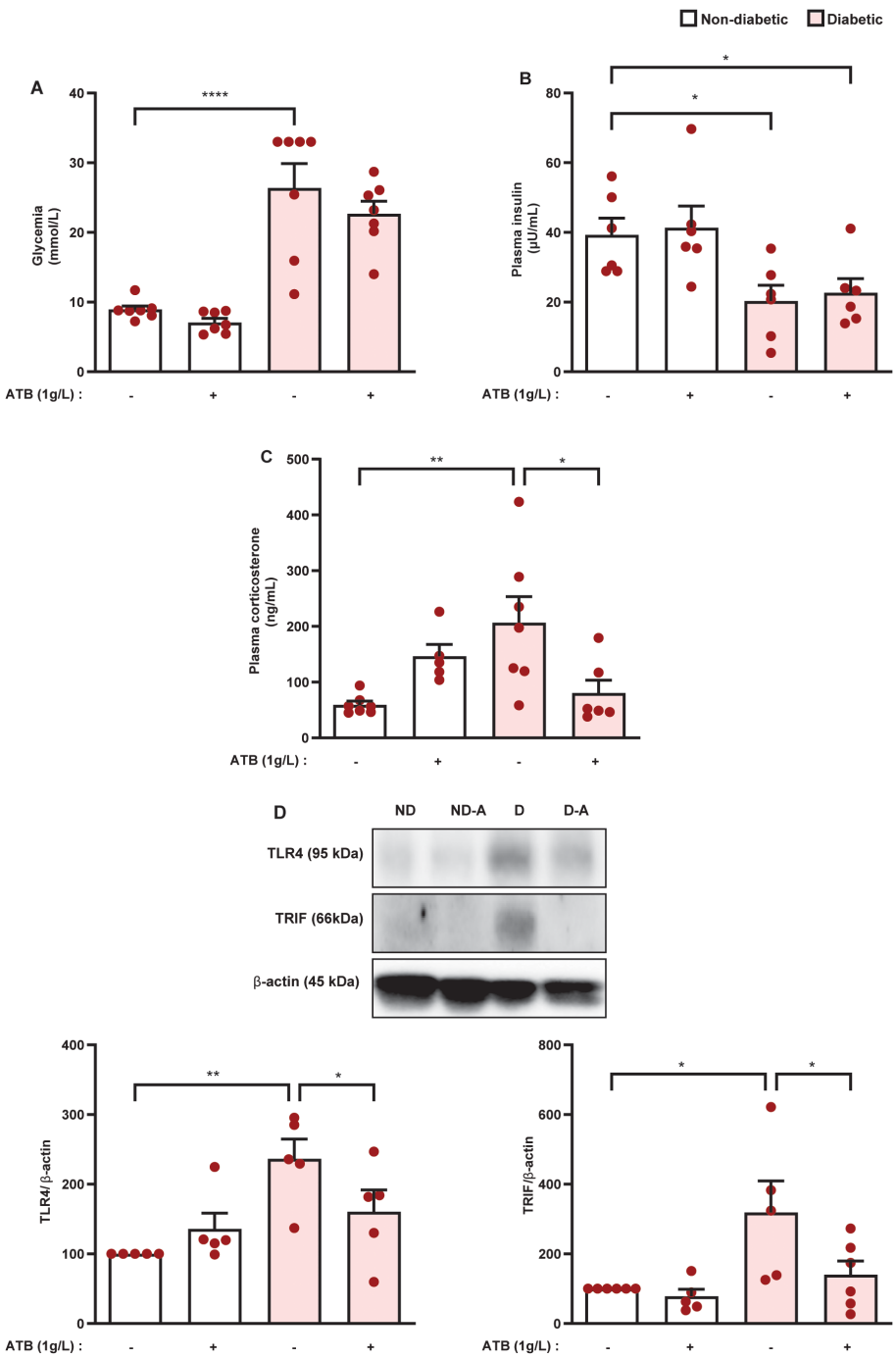

FIGURE 7
Antibiotic therapy decreases plasma corticosterone levels in parallel to a reduction in TLR4 and TRIF expression in the adrenal glands of diabetic mice. Seven days after diabetes induction, mice were treated with an antibiotic cocktail (metronidazole, neomycin, and ampicillin, 1g/L, drinking water), for 14 consecutive days. Untreated animals receive an equal amount of vehicle (drinking water). Analyses were performed 21 days after diabetes induction. **(A)** Blood glucose quantification. **(B, C)** Plasma quantification of insulin and corticosterone, respectively, by ELISA. **(D)** Expression of TLR4 and TRIF in the adrenal glands of non-diabetic and diabetic mice performed by western blot. Data were normalized to β-actin and represented as the ratio between target protein levels relative to controls. Each bar represents the mean ± standard error of the mean. Pink dots represent the number of animals analyzed. Statistical analyses were performed by one-way ANOVA followed by Newman–Keuls test. *p<0.05. **p<0.005. ****p<0.0001. ATB, antibiotic cocktail; TLR4, toll-like receptor 4; TRIF, TIR-domain-containing adapter-inducing interferon-β.

respectively). Nevertheless, both TLR4 antagonist treatment and TLR4 mutation in diabetic animals resulted in reduced plasma corticosterone levels compared to untreated and wild-type diabetic mice (Figures 8E, F, respectively). In addition, neither approach to block TLR4 activation modified plasma corticosterone levels in non-diabetic mice.

Then, we evaluated the effect of TAK-242 on the expression of the steroidogenic machinery in the adrenal glands of

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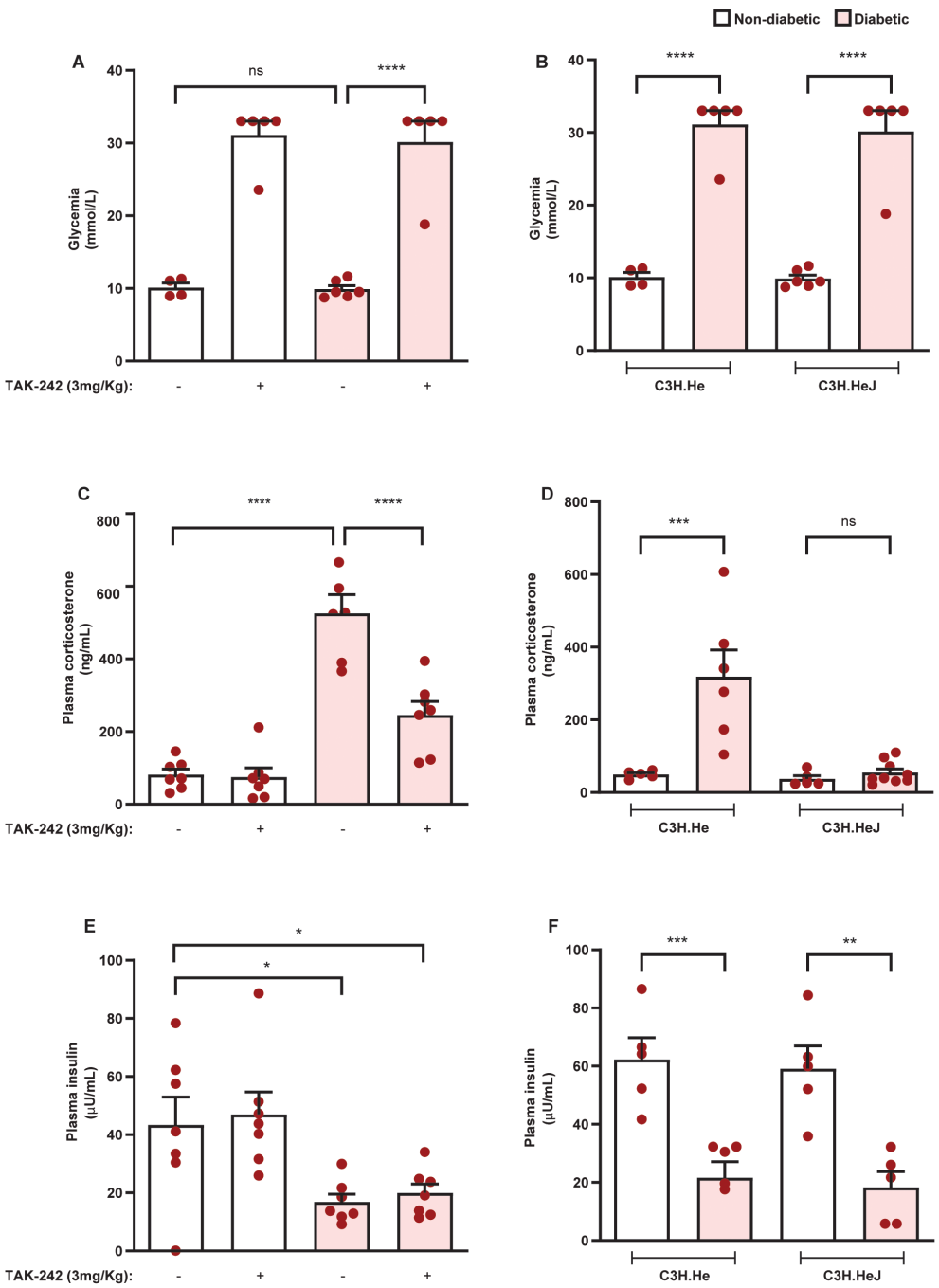

FIGURE 8
Blockade of TLR4 inhibits hypercortisolism in diabetic mice. Seven days after diabetes induction in Swiss-Webster mice, animals were treated with the TLR4 antagonist TAK-242 (3 mg/kg, i.p.) once a day for 14 consecutive days. Control animals receive an equal amount of vehicle (0.1% DMSO). Analyses were performed 21 days after diabetes induction. **(A, B)** Blood glucose quantification in diabetic mice treated with TAK-242 and animals with a mutation in TLR4, respectively. **(C, D)** Plasma quantification of insulin levels in diabetic mice treated with TAK-242 and TLR mutant animals, respectively. **(E, F)** Plasma quantification of corticosterone levels in diabetic mice treated with TAK-242 and TLR4 mutant animals, respectively. Insulin and corticosterone levels were measured by ELISA. Each bar represents the mean ± standard error of the mean. Pink dots represent the number of animals analyzed. Statistical analyses were performed by one-way ANOVA followed by Newman–Keuls test. *p<0.05. **p<0.005. ***p<0.0005. ****p<0.0001. ns, non-significant; TAK, TAK-242.

diabetic mice. We showed that the overexpression of 11βHSD1 and MC2R noted in the adrenals of diabetic mice was sensitive to treatment with TAK-242, although the expression of StAR was unaffected (Figure 9A and Supplementary Figures S4A–D). Treatment with TAK-242 did not alter these outputs in the adrenal glands of non-diabetic mice. Furthermore, treatment with TLR4 antagonist downregulated the expression of both TLR4 (Figure 9B, Supplementary Figures S4E, F)

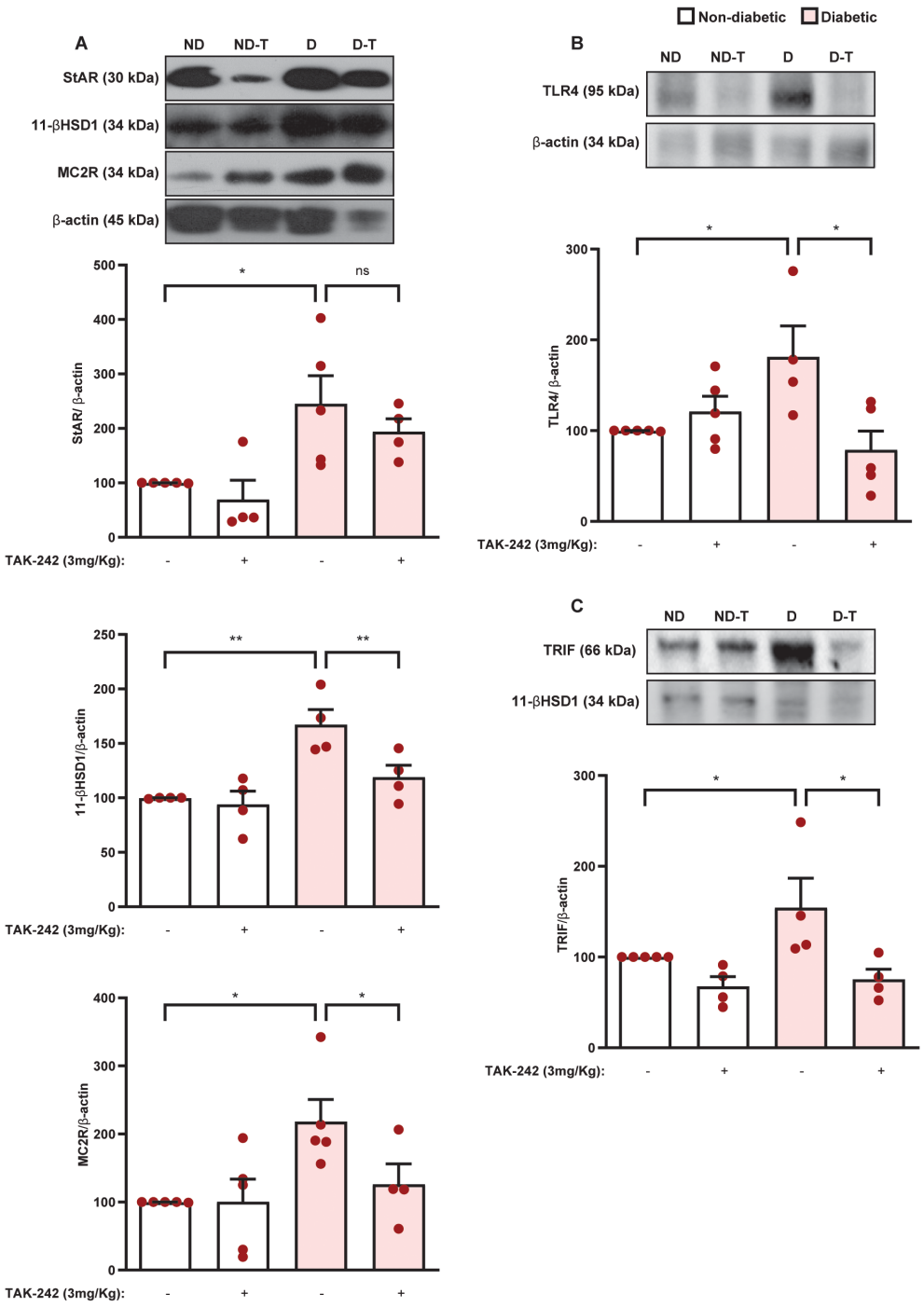

FIGURE 9
Suppression of TLR4 signaling by TAK-242 represses expression of the steroidogenic machinery, TLR4, and TRIF in the adrenal glands of diabetic mice. Seven days after diabetes induction, animals were treated with the TLR4 antagonist TAK-242 (3 mg/kg, i.p.) once a day for 14 consecutive days. Control animals received an equal amount of vehicle (0.1% DMSO). Analyses were performed 21 days after diabetes induction. **(A)** Expression of StAR, 11βHSD1, and MC2R in the adrenal glands. Expression of TLR4 **(B)** and TRIF **(C)** in the adrenal glands. Protein expression was determined by western blot. Data were normalized to β-actin and represented as ratios between target protein levels relative to controls. Each bar represents the mean ± standard error of the mean. Pink dots represent the number of animals analyzed. Statistical analyses were performed by one-way ANOVA followed by Newman–Keuls test. *p<0.05. **p<0.005. ns, non-significant; 11βHSD1, 11β-Hydroxysteroid dehydrogenase type 1; MC2R, melanocortin receptor 2; StAR, steroidogenic acute regulatory protein; TAK, TAK-242; TLR4, toll-like receptor 4; TRIF, TIR-domain-containing adapter-inducing interferon-β.

and TRIF (Figure 9C, Supplementary Figures S4G, H) in the adrenal glands of diabetic mice compared to untreated controls, without modifying these outputs in non-diabetic mice.

4 Discussion

This study provides new viewpoints on the involvement of gut-adrenal interactions on the exacerbation of adrenal glucocorticoid steroidogenesis in diabetes. We showed that both diabetic mice and rats displayed overexpression of TLR4 and TRIF in the adrenal glands, an increase in the diversity of pathogenic bacteria and inflammation in the gut, increased epithelial-intestinal barrier permeability, and elevated levels of LPS in the adrenal glands, without altering the content of HMGB1 and HSP70 in this organ. Antibiotic therapy decreased plasma corticosterone levels in diabetic mice in parallel to a down-regulation of TLR4 and TRIF expression in the adrenal glands. In addition, both mutation of TLR4 and treatment with a TLR4 antagonist inhibited the hypercortisolism observed in diabetic mice. The TLR4 antagonist-induced reduction of adrenal glucocorticoid steroidogenesis observed in diabetic mice was related to a decrease of TLR4, TRIF, MC2R, and 11βHSD1 expression in the adrenal glands. Our findings indicate that the activation of the TLR4 pathway induced by endotoxins from pathogenic bacteria found in the gut microbiota of diabetic mice could account for the exacerbation of corticosterone production by the adrenal glands.

Alloxan induces diabetes through the generation of reactive oxygen species (ROS) that mediate variable β-cell toxicity. In some cases, alloxan can destroy all β-cell, exhausting blood insulin levels and culminating in the death of animals. Nevertheless, in general, alloxan induces hyperglycemia stably over time (22, 23). In this study, we did not see any death after alloxan injection in rats and mice, probably because we analyzed them in a relatively short time after administration of the diabetogenic agent. Although we did not follow glycemia over time in this work, we previously showed quite similar levels of blood glucose 24h, 48h, and 72h after alloxan injection in Wistar rats compared to values obtained in this work. Furthermore, in all animal strains evaluated, we showed the same increase in the blood glucose levels and a similar decrease in plasma insulin levels, suggesting glycemic stability in our model of diabetes independent of used rats or different strains of mice (12). However, our model of diabetes has some limitations, including β-cell damage by a mechanism without the participation of an autoimmune reaction and the fact that alloxan directly induces liver and kidney damage (24).

It is well known that LPS induces glucocorticoid production by adrenocortical cells *in vitro* and *in vivo* (17, 18) and that diabetic patients display an increase in circulating levels of HSP70 and HMGB1 (15), which are endogenous activators of TLR4. However, the involvement of LPS in the exacerbation of adrenal glucocorticoid steroidogenesis in diabetic animals remained elusive. First, we showed that both diabetic rats and mice overexpress TLR4 and TRIF in the adrenal glands, while levels of HSP70 and HMGB1 remain unaltered. Furthermore, we noted that LPS induced an increase in plasma corticosterone levels in diabetic rats 60 min after the challenge, without altering circulating levels of this hormone in non-diabetic controls. These data suggest that the activation of the TLR4-TRIF pathway can be involved in the hypercortisolism of diabetic animals; however, the participation of DAMP activators of TLR4 in this phenomenon remained elusive. In this work we used Wistar rats and Swiss-Webster mice due to their genetic variability, making them more representative of natural populations and human diversity. By comparing these species with different genetic backgrounds, we minimize bias from species-specific traits. Immune responses in these two outbred strains are quite similar, with comparable leukocyte counts in the blood, however, no direct comparison of their gut microbiota is available, mice microbiota seem closer to humans than that of rats (25).

We hypothesized that bacterial products from the gut microbiota could participate in the exacerbation of corticosterone production by the adrenal glands of diabetic animals. To test this hypothesis, we evaluated the gut bacteriome content of diabetic mice. We showed that diabetic mice displayed marked changes in gut bacteriome composition, with a reduction in the relative abundance of the Firmicutes, which is composed predominantly of commensal bacteria, and an increase in the Proteobacteria, known to harbor various human pathogens. Among the pathogenic bacteria present at higher levels in the gut bacteriome of diabetic mice, *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella variicola*, and *Proteus mirabilis* stand out. However, it is important to note that sequencing of regions V3-V4 of the 16S rRNA gene often does not allow confident taxa identification at the species level. Therefore, these species assignments need validation before more solid conclusions can be drawn. Nevertheless, this gut dysbiosis noted in diabetic mice agrees with what is observed in diabetic patients with uncontrolled glycemia (2, 14). Our data suggests that pathogenic bacteria found in the gut bacteriome of diabetic mice can be the source of TLR4 activators, including LPS, that are increased in the adrenal glands of these animals.

It is well known that gut dysbiosis is usually accompanied by structural changes in the colon, including local inflammation, followed by increased permeability of the epithelial-intestinal barrier (26). We showed that diabetic mice displayed increased villus height and muscular thickness in the colon, which can be an indication of increased intestinal epithelium and muscle cell proliferation, affecting the absorption of nutrients and resulting in unhealthy intestinal conditions (27). Furthermore, we noted the development of an inflammatory response, indicated by increased mucus production in the colon of diabetic mice. Although in commensal homeostasis the mucus layer protects the tissue against microbial penetration (28), in mouse models of colitis and in patients with ulcerative colitis, bacteria penetrate the mucus and are found close to the non-inflamed epithelium (29), indicating a defect in mucus barrier quality. Therefore, high mucus production in the colon of diabetic mice is not necessarily associated with greater epithelial-intestinal barrier integrity. Since the precise balance of immune surveillance and tolerance is crucial to maintaining epithelial-intestinal barrier integrity and uncontrolled

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immune responses caused by gut microbiota disturbances can break this barrier (30), we evaluated the profiles of pro- and anti-inflammatory cytokines in the gut of diabetic mice. We showed that diabetic mice displayed an increase in IL-10, IL-17, IL-22, and TNF-α levels and a decrease in IL-1β content in the colon compared to non-diabetic mice, without altering the levels of IL-4, IL-6, and TGF-β1. Our data indicate that diabetes leads to a predominantly Th17-type immune response profile in the colon, due to increased levels of IL-17, IL-22, and TNF-α. Although the overproduction of Th17 cytokines in the gut was previously shown in non-obese diabetic (NOD) mice, this phenomenon was demonstrated only in the pre-diabetic period (31, 32). Our work was the first to show a Th17 profile in the gut of type 1 diabetic mice. This Th17 signature in the gut, with an increase in Th17 cells in the lamina propria, is involved in the progression of intestinal inflammation and, consequently, in the break of the epithelial-intestinal barrier (33, 34). In addition, activation of the Th17 response and development of inflammation in the colon of diabetic mice is probably related to the dysbiosis observed, since pathogenic bacteria, which we have shown to be enriched in the gut bacteriome of diabetic mice, lead to intestinal Th17 responses (35).

To confirm whether the structural and immune changes we observed in the colon of diabetic mice effectively reflected an increase in the permeability of the epithelial-intestinal barrier, we evaluated the extravasation of FITC D4000 from the intestinal lumen into the circulation as well as systemic levels of LPS. By doing so, we showed an increase in the influx of FITC D4000 from the intestinal lumen into the blood of diabetic mice compared to non-diabetic mice, indicating an increase in the epithelial-intestinal barrier permeability. Surprisingly, we noted a reduction in circulating levels of LPS in diabetic mice, even though we observed a greater diversity of Gram-negative bacteria in the gut bacteriome and a break in the epithelial-intestinal permeability. Although the exact mechanism for the reduction in the plasma LPS levels in diabetic mice remains unclear, an explanation for this phenomenon may be an increase in the LPS-binding protein (LBP) levels. In fact, LBP is increased in plasma of type 1 diabetic patients compared to non-diabetic patients (36). Another hypothesis is that LPS may be distributed to specific tissues, and, with this, its levels are reduced in diabetic mice. LPS-free is removed from the bloodstream mainly by the liver and spleen (37), therefore, we can speculate that diabetic mice may be a high tissue-specific distribution to these organs. Then, we hypothesized that LPS may be distributed to adrenals in diabetic mice. In agreement with this idea, we showed an increase in LPS levels in both the adrenal glands and colon of diabetic mice compared to non-diabetic controls. However, more experiments need to be done to understand the mechanism behind this phenomenon.

To test the hypothesis that products of the gut bacteriome of diabetic mice may be responsible for the exacerbation of glucocorticoid production by the adrenals, we treated animals with a mixture of antibiotics containing ampicillin, neomycin, and metronidazole. We chose this combination of antibiotics because it drastically reduces bacterial levels in feces and circulating levels of LPS in mice submitted to a high-fat diet (38). We showed that antibiotic therapy reduced plasma corticosterone levels and downregulated the expression of TLR4 and TRIF in the adrenal glands of diabetic mice. These data suggest that antibiotic therapy reduces LPS content in the adrenals of diabetic mice since LPS is known to induce TLR4 expression in primary hepatocytes *in vitro* (39). Our data suggest that the activation of the TLR4-TRIF pathway in the adrenals by endotoxins from the gut bacteriome is implicated in the exacerbation of corticosterone production in diabetic mice.

To understand if TLR4 activation in the adrenal glands is indeed crucial for the exacerbation of corticosterone production in diabetic mice, we used two approaches. First, we induced diabetes in C3H.HeJ mice, which are mutants of the *Lps* locus in TLR4, making them hyporesponsive to stimulation with LPS, and in C3H.He mice that is the genetic background of C3H.HeJ mice (40). Second, we treated diabetic mice with TAK-242, an antagonist of TLR4 (41). We noted that neither the treatment with TAK-242 nor the mutation of TLR4 (C3H.HeJ mice) did not modify blood glucose levels or plasma insulin levels of diabetic mice, indicating that TLR4 did not participate in alloxan-induced pancreatic β-cell injury. Nevertheless, both TLR4 mutation and pharmacological blockade decreased systemic corticosterone levels in diabetic mice, reinforcing our data indicating that TLR4 activation is crucial to the exacerbation of corticosterone production in diabetic mice. Although antibiotic therapy significantly decreased blood glucose levels in diabetic mice, this small reduction in glycemia does not seem to reduce adrenal corticosterone production in diabetic mice, since neither TLR4 mutation nor treatment with TAK-242 interfered with hyperglycemia.

To determine the mechanisms by which TLR4 activation induces the exacerbation of adrenal corticosterone production in diabetic mice, we evaluated the expression of TLR4, TRIF, and the steroidogenic machinery in the adrenals of diabetic mice after treatment with the TLR4 antagonist. We showed that treatment with TAK-242 decreased the expression of TLR4 and TRIF in the adrenal glands of diabetic mice. It is well known that LPS induces steroidogenesis in adrenocortical cells *in vitro* by a mechanism dependent on NF-κB activation (18). Although TRIF induces a MyD88-independent signaling pathway, it also activates NF-κB (42, 43), reinforcing our hypothesis that the exacerbation of adrenal corticosterone production in diabetic mice occurs via the TLR4-TRIF pathway. Nevertheless, with our data, we cannot rule out the role of other TLR4 adaptors, including MyD88, or alternative pathways such as NF-κB and IRF3 in the gut bacteria-derived LPS-TLR4 pathway-induced exacerbation of corticosterone production in diabetic animals. Finally, we also showed that TAK-242 treatment decreased expression of MC2R and 11β-HSD1 in the adrenal glands of diabetic mice. These data strongly suggest that overactivation of TLR4 in the adrenal glands of diabetic mice induces an exacerbation of corticosterone production in both a direct and an indirect ACTH-dependent way.

Our results indicate that the adrenal-gut-bacteriome axis is crucial in exacerbating adrenal steroidogenesis in diabetic mice. The mechanism involved in the increased production of corticosterone in diabetic mice depends on the up-regulation of the steroidogenic machinery in the adrenal glands induced by activation of the TLR4 pathway by endotoxins from pathogenic bacteria from the gut microbiota. With the data obtained in this study, we believe that new therapeutic strategies based on TLR4 inhibitors or prebiotics will become an essential target for treating type 1 diabetes and other diseases associated with hypercortisolism in the future.

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. All sequences generated in this study were deposited as a Sequence Read Archive in the NCBI database with Bioproject ID PRJNA1253081 (SAMN48063965-SAMN48063969 for 16S rRNA gene of the control samples and SAMN48063970- SAMN48063974 for 16S rRNA gene for the treatment samples).

Ethics statement

The animal study was approved by Committee on Use of Laboratory Animals of the Oswaldo Cruz Institute (CEUA-IOC/Fiocruz, licenses L-027/2016 and L-004/2024). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

NM: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. AC: Formal analysis, Methodology, Writing – review & editing. BT: Formal analysis, Methodology, Writing – review & editing. DI: Formal analysis, Methodology, Writing – review & editing. HP: Formal analysis, Methodology, Writing – review & editing. AR: Investigation, Methodology, Writing – review & editing. CH: Formal analysis, Methodology, Writing – review & editing. LA: Conceptualization, Formal analysis, Resources, Writing – review & editing. PS: Conceptualization, Formal analysis, Funding acquisition, Resources, Writing – review & editing. MM: Formal analysis, Funding acquisition, Resources, Writing – review & editing. VC: Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2025.1555203/full#supplementary-material

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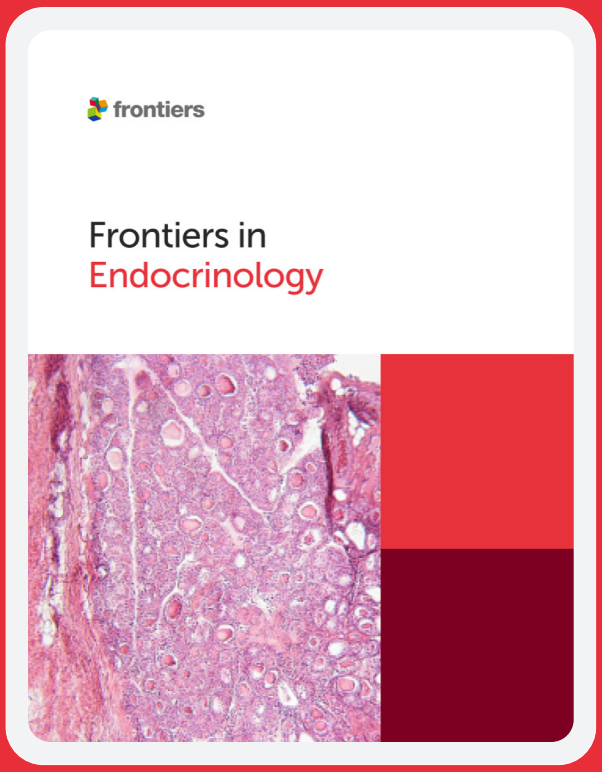

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