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Insights in autoimmune and autoinflammatory disorders: 2021

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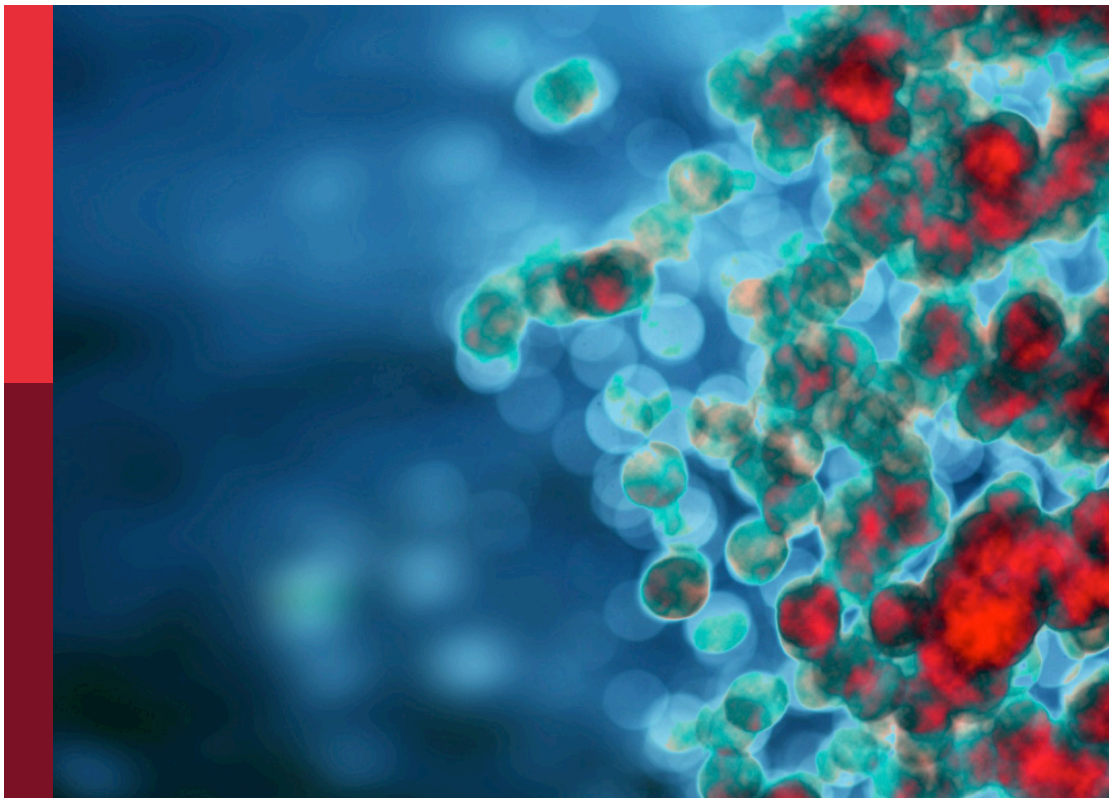

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Insights in autoimmune and autoinflammatory disorders: 2021

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Editorial: Insights in autoimmune and autoinflammatory disorders: 2021

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Editorial on the Research Topic
Insights in autoimmune and autoinflammatory disorders: 2021

The Research Topic: *Insights in Autoimmune and Autoinflammatory Disorders: 2021* was implemented to highlight latest advancements in research across the field of Immunology in Autoimmunity and Autoinflammation by discussing recent advances, current challenges, and future perspectives. Under the Research Topic 13 articles were published, 10 contributions highlight topics relevant to autoimmune diseases and 3 to autoinflammation.

In two original research papers two groups set out to identify microbial or viral risk factors that predispose to the development of autoimmunity.

Yin et al. mined microarray datasets from disease-specific target tissues including the pancreas, thyroid, and intestine from individuals with Type 1 Diabetes (T1D), Hashimoto thyroiditis (HT), and celiac disease (CD), as well as matched controls. They discovered viral signatures of common viral infections including influenza A, human T-lymphotropic virus type 1, and herpes simplex that were shared in target tissues of the three autoimmune diseases studied thus pointing to common environmental factors as drivers of autoimmune diseases. Lin et al. assessed a potential role of gut microbiota in driving autoimmune diseases in a retrospective population-based cohort study analyzing data from a Taiwanese Insurance Research Database of over 290,000 patients treated with PPIs in the period between 2002 and 2015. The role of PPIs in modifying gut microbiota has previously been described (1). The authors show that compared to the non-PPI group, the adjusted hazard ratios (aHR) were higher for several organ specific autoimmune diseases including Grave's disease, Hashimoto thyroiditis, autoimmune hemolytic anemia, immune thrombocytopenic purpura, Henoch-Schoenlein purpura and Myasthenia gravis and also for systemic autoimmune diseases including ankylosing spondylitis, rheumatoid arthritis, primary Sjogren syndrome, systemic lupus erythematosus, systemic vasculitis, psoriasis, systemic scleroderma and inflammatory myopathies.

Two papers, an original research paper by Yang et al. and a mini review by Chen et al. assess the role of transfer RNA (tRNA)-derived small noncoding RNA (tsRNA), an emerging class of small non-coding RNAs, and the role of ferroptosis in patients with systemic lupus erythematosus (SLE) respectively. Yang et al. assessed the diagnostic value of a potential biomarker, tRF-His-GTG-1, a candidate tsRNA that best differentiated between the SLE and control groups. The tsRNA was first assessed in a training set of 57 SLE patients with and without lupus nephritis and then in a validation set of 52 SLE patients without Lupus nephritis, 83 SLE patients with lupus nephritis and 86 healthy controls; tRF-His-GTG-1 was significantly elevated in serum exosomes from SLE patients compared to healthy controls and its elevation in serum distinguished SLE with lupus nephritis from SLE without lupus nephritis with and AUC of 0.81 (95% CI 0.73-0.88) with high specificity, however, the sensitivity was lower, 66.27%. Pathway analysis predicted that the tsRNA can target signaling pathways including MAPK signaling and RIG-I signaling and EBV infection. Chen et al. review data on the role of ferroptosis, a novel non-apoptotic regulated form of cell death, in SLE.

Two reviews assess mechanisms that lead to fibroblast proliferation and inflammation in rheumatoid arthritis (RA). Jiang et al. review the pathogenic role of secreted frizzled-related protein 1 (SFRP1), a member of the secretory glycoprotein SFRP family, that are thought to "antagonize" the Wnt signaling pathway by interfering es with Wnt signaling transduction. Their role in determining cell fate by regulating cell proliferation, differentiation, apoptosis, and pyroptosis has been previously reported (2) SFRP1 is widely expressed in human cells, including fibroblast-like synoviocytes (FLS) of rheumatoid arthritis (RA) and in osteoarthritis (3). The authors summarized data on mechanisms of SFRP1 regulation of RA-FLS pyroptosis through Wnt/β-catenin and Notch signaling pathways and summarize data on the epigenetic regulation of SFRP1 in RA-FLS. They conclude proposing that Wnt/β-catenin and Notch signaling pathways may collaborate in NLRP3-mediated cell pyroptosis and suggest a role of SFRP1 in hypermethylation of synovial tissue from knee joints in patients with RA and OA. The authors further suggest a potential role of inhibition of hypermethylation in the treatment of RA. Zhao et al. assess the role of G-Protein-Coupled Receptors (GPCR) that includes chemokine receptors, melanocortin receptors, lipid metabolism-related receptors, adenosine receptors, and other inflammation-related receptors, on the pathogenesis of RA, in regulating inflammation, lipid metabolism, angiogenesis, and bone destruction. This review provides comprehensive tables on GPCRs and their expression in immune cells synovium and synovial fibroblasts and discusses possible factors that elucidate the failure of clinical trials blocking cytokines in RA. The authors suggest that the widespread expression of chemokine receptors in a variety of cells may imply that a portion of chemokine receptors may be necessary for homeostatic processes, and further point to the fact that the expression of GPCRs at different disease stages of RA may have diverse functional roles.

Three papers address various mechanisms that can modify tissue specific autoimmune effects, including the role of double negative T regulatory cells in regulating tolerance in the female reproductive environment, the role of NETs in IgA vasculitis and an original paper probing the relationship of C3 levels with disease outcomes in patients with glomerular basement membrane (GBM) disease. Bafor et al. review the emerging role of double negative T regulatory cells (DNTregs), (TCRαβ+/γδ+CD3+CD4–CD8–) on regulating immune tolerance in and female reproductive function. The breakdown of immune tolerance leads to ovulation dysfunction, implantation failure, and pregnancy loss. The authors also discuss mechanisms by which DNTregs provide immune tolerance and maintain and restore the balance in the reproductive microenvironment of female fertility. Chen et al. review the pathomechanisms of IgA activated neutrophils and their release of NETs into tissues and the peripheral blood. The authors review the role of NETs in Immunoglobulin A vasculitis (IgAV) in children. They summarize data suggesting that IgA can induce neutrophils to release NETs *via* Fcα receptor I (FcaRI) and that FcaRI is elevated in children with active IgAV. The authors further suggest that NETs may serve as potential biomarkers to assess disease activity in IgAV. Zhu et al. conducted a retrospective study of 94 anti-glomerular basement membrane (GBM) disease who were seen in the National Clinical Research Center of Kidney Diseases, Jinling Hospital (China) and found that kidney outcomes of anti-GBM disease in the low C3 group were poorer than those in the normal C3 group. Jiang et al. report a patient presenting with coexisting of autoimmune polyglandular syndrome (APS) Type 3 and Gonadotropin-Releasing Hormone Deficiency who presented with secondary amenorrhea.

And finally, 3 original research papers focus on the pathogenesis of three autoinflammatory diseases. The first paper reports a patient with a novel *de novo JAK1* mutation causing a keratinization disorder presenting with hepatitis and autism that was examined in a murine model, a second paper reports of a screening method for candidate drugs for Behcet's disease, candidates were evaluated in a murine experimental autoimmune uveitis (EAU) model, and the third paper describes a novel disease-causing *NLRP3* variant that presents as autosomal dominant hearing loss.

Takeichi et al. report a patient with a novel, *de novo JAK1* mutation, p.H596D, who presents with generalized papular skin rashes consistent with a keratinization disorder and with early-onset liver dysfunction and autism. Using CRISPR-Cas9 gene targeting, the authors generated mice with the identical *JAK1* mutation and describe hyperactivation of tyrosine kinases and NF-κB signaling pathways. To investigate the role of the mutation in neurodevelopment, the authors observed a strong correlation between genes downregulated in the $Jak1^{H595D/+;I596I/+;Y597Y/+}$ mice and those downregulated in the brain of model mice with 22q11.2 deletion syndrome that showed cognitive and behavioral deficits, and are used to model autism spectrum disorders. Xia et al. used an in silico approach to

identify potential drugs for the treatment of Behcet's disease that they validated in a mouse model of experimental autoimmune uveitis (EAU) (B10.RIII mice developed EAU after subcutaneous injection of 200 μl of emulsifier per mouse). The biological functions and pathways of the target genes were analyzed in detail by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses and gene drug interactions were identified from the Drug Gene Interaction Database (DGIdb). Of drugs identified to interact with the 3 top hub genes in Behcet's, rabenprozole and celastrol reduced anterior chamber inflammation in retinal inflammation in EAU mice. Lastly, Oziebloet al. screened 110 autosomal dominant hearing loss (HL) families with a custom panel of 237 HL genes and identified one family carrying a novel *NLRP3* mutation, p.S624R that led to HL in 9 patients in a pedigree of 4 generations. Functional studies identified the novel variant as gain-of-function mutation, leading to increased activity of caspase-1 and subsequent oversecretion of the proinflammatory interleukin-1β. Similar to patients with previously reported mutations causing DFNA34, the identified patients did not present with features of systemic inflammation and did not meet the diagnostic criteria for cryopyrin associated autoinflammatory diseases (CAPS) including MWS, NOMID, or FCAS.

Author contributions

Both authors contributed to this editorial RM drafted the editorial and BD approved the final version.

Conflict of interest

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

Publisher's note

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CASE REPORT
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Case Report: A Rare Case of Coexisting of Autoimmune Polyglandular Syndrome Type 3 and Isolated Gonadotropin-Releasing Hormone Deficiency

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APS (autoimmune polyglandular syndrome) is defined as the coexistence of at least two kinds of endocrine autoimmune diseases. APS type 3 comprises autoimmune thyroid diseases and other autoimmune diseases but does not involve autoimmune Addison's disease. So far, APS-3 combined with isolated gonadotropin-releasing hormone (GnRH) reduction caused by the suspected autoimmune hypothalamic disease has not been reported. We recently received a 43-year-old woman with a one-year history of Graves' disease (GD) and a four-month history of type 1 diabetes presented with hyperthyroidism and hyperglycemia. After the GnRH stimulation test, she was diagnosed with secondary amenorrhea attributed to suspected autoimmune Hypothalamitis and APS type 3 associated with Graves' disease and Latent Autoimmune Diabetes (LADA). According to this case, the hypothalamus cannot be spared from the general autoimmune process. It is recommended to carry out the GnRH stimulation test when encountering APS patients combined with secondary amenorrhea.

Keywords: autoimmune polyglandular syndrome type 3, secondary amenorrhea, isolated gonadotropin-releasing hormone deficiency, GnRH stimulation test, Graves' disease, type 1 diabetes mellitus

INTRODUCTION

APSs are rare conditions characterized by autoimmune activity against multiple endocrine organs, although non-endocrine organs can also be affected (1). To date, quite a few comorbidities have been described. Typical endocrine diseases include type 1 diabetes mellitus (T1DM), autoimmune thyroid disease, and Addison's disease. Other frequently involved conditions comprise celiac disease, alopecia, vitiligo, hypogonadism, pernicious anemia, *etc.* APSs are generally categorized into four subtypes (2). APS type 1 is characterized by the development of at least two of three cardinal components comprising of chronic mucocutaneous candidiasis, hypoparathyroidism, and Addison's disease; APS type 2 consists of Addison's disease plus autoimmune thyroid disease or type 1 diabetes mellitus; APS type 3 is defined by the presence of autoimmune thyroid disease and

another autoimmune illness but not Addison's disease. Finally, APS type 4 refers to two or more organ-specific autoimmune disorders that did not fit into the characteristics of APS-1 through APS-3. We have recently received a patient with a rare combination of isolated GnRH deficiency suggesting autoimmune hypothalamic disease and APS type 3 complicated with Graves' disease and LADA. To the best of our knowledge, this is the first case of such an association in humans. Herein, we present the clinical features and valuable diagnosis experience.

CASE REPORT

A 43-year-old woman with a one-year history of Graves' disease (GD) and a four-month history of type 1 diabetes mellitus was admitted to our hospital with chief complaints of hyperthyroidism and hyperglycemia in March 2021. One year before admission, this patient was referred to the local hospital due to palpitation, emaciation, hyperhidrosis, ease of starving, *etc.* In addition to low thyroid-stimulating hormone (TSH), high free triiodothyronine (FT3), high free thyroxine (FT4), positive thyroid-stimulating hormone receptor antibody (TRAb), and a diffuse homogenous thyroid gland enlargement with increased blood flow by thyroid ultrasound was observed. Thus, GD was diagnosed. Then she started the treatment with antithyroid drugs (ATD). During the follow-up visits, the thyroid hormones were constantly high despite her treatment adherence. Five-month ago, she was treated with radioiodine therapy owning to the poor effect of ATD treatment. Since then, she had not taken antithyroid drugs. Three-month ago, the patient was hospitalized again in the local hospital and found elevated blood glucose due to the aggravation of hyperphagia, hunger and weight loss, *etc.* During hospitalization, she was subjected to a panel of laboratory examinations showing fasting insulin 8.6 uU/mL, fasting C-peptide 0.354 ng/mL, 2 hours postprandial insulin 15.2 uU/mL, 2 hours postprandial C-peptide 0.149 ng/mL, glycosylated hemoglobin (HbA1c) 7.9%, GAD antibody >2000 IU/mL. Accordingly, type 1 diabetes mellitus/LADA was diagnosed. She was started on premix insulin therapy twice daily, but her glucose control deteriorated, so she switched to basal/bolus insulin therapy.

The patient was 16 years old at menarche and had regular menstrual cycles. She had two children, both born in natural labor. She denied postpartum hemorrhage. In 2018, the disorder of the menstrual cycle began, along with a significant decrease in libido. In June 2020, the patient was menopausal (42 years old).

The patient had no history of autoimmune diseases such as vitiligo, autoimmune gastritis, pernicious anemia, neurodermatitis, alopecia areata, myasthenia gravis, systemic lupus erythematosus, autoimmune hepatitis, and rheumatoid arthritis. The patient's mother, uncle, and grandmother had a history of type 2 diabetes. She had no family history of APS, autoimmune thyroid disease (AITD), or other immunological disorders.

Upon admission, her body mass index was 17.9 kg/m^2, temperature 36.9°C, blood pressure 119/82mmHg, and pulse rate 98/min. On physical examination, she presented with a diffusely enlarged thyroid with moderate texture. No other obvious abnormality was observed. Results of laboratory tests were presented in **Table 1**. There was neither adrenal insufficiency nor hypocalcemia. Magnetic resonance imaging of her pituitary gland showed normal findings.

It is important to note that, in this case, E2 level decreased, but there were no increased FSH or LH levels following a one-year history of menopausal at the age of 42. It suggested that secondary ovarian dysfunction should be considered. Thus, we performed the magnetic resonance imaging of her pituitary gland, which showed normal findings, and a GnRH stimulation test [with triptorelin (Ferring Pharmaceuticals, Germany), 0.1 mg intravenously]. The results of the GnRH stimulation test were shown in **Table 2**. The GnH (FSH and LH) response to GnRH supported the diagnosis of isolated gonadotropin-releasing hormone deficiency. HLA Class II genotyping revealed DQB1*0201 allele and DRB1*0301-0803

TABLE 1 | Laboratory data on admission.

Variables	Results	Reference interval
Leukocyte count,10^9/L	4.84	3.5-9.5
Erythrocyte count,10^{12}/L	4.83	3.8-5.1
Haemoglobin,g/L	127	115-150
Platelet count,10^9/L	157	125-350
Alanine aminotransferase (ALT), U/L	50.8	7-40
Aspartate aminotransferase (AST), U/L	44.7	13-35
Total Bilirubin,umol/L	21.2	0-21
Creatinine,umol/L	23	41-81
Urea,mmol/L	4.33	2.6-7.5
Sodium,mmol/L	137.5	137-147
Potassium,mmol/L	4.16	3.3-5.3
Calcium,mmol/L	2.31	2.11-2.52
Phosphorus,mmol/L	1.02	0.85-1.51
Parathyroid hormone (PTH), pg/mL	22.79	15.0-65.0
(25-OH) VitD3, ng/mL	28.67	>30
ALkaline Phosphatase (ALP), U/L	145	35-100
Glycated haemoglobin, %	14.6	3.8-6.5
C-P,ng/mL	0.435	1.1-4.4
GAD antibody	(+)	(-)
ICA antibody	(+ -)	(-)
Urine glucose	3+	(-)
Urine ketone	3+	(-)
Urine protein	1+	(-)
Insulin,pmol/L	96.01	16.5-84.7
HDL cholesterol,mmol/L	0.96	1.04-1.55
Triglycerides,mmol/L	0.75	0.4-1.82
Thyrotropin (TSH), mIU/L	0.005	0.55-4.78
Free triiodothytonine (FT3), pmol/L	12.96	3.5-6.5
Free thyroxine (FT4), pmol/L	41.02	11.5-22.7
Anti-thyrogloblin antibody (TgAb), IU/mL	466	0-40
Anti-thyroid peroxidase (TPO) antibody,IU/mL	>1000	0-35
TSH receptor antibody (TRAb), IU/L	>30	0-1.75
Cortisol,ug/dL	19.8	3.7-19.4
Adrenocorticotropic hormone,pg/mL	30.75	7.2-63.3
Progesterone (PRGE), ng/mL	0.78	0-0.73
estrogen (eE2), pg/mL	<11.80	0-32.2
luteinizing hormone (LH), mIU/mL	0. 42	15.9-54
Follicle stimulating hormone (FSH), mIU/mL	1.46	23-116.3
Prolactin (PRL), ng/mL	6.78	1.8-20.3
Testosterone (TSTII), ng/dL	30. 82	0-45.62

TABLE 2 | The results of GnRH (Triptorelin, 100mg) stimulation test.

Time(min)	0 min	25 min	45 min	60 min	90 min	180 min
LH (mIU/mL)	0.57	2.91	3.18	3.51	3.63	3.54
FSH (mIU/mL)	1.57	8.12	9.27	10.1	13.6	15.37

gene site, and the corresponding genotypes were DQ2 and DR8. Based on the above clinical course and data, the patient was diagnosed with isolated GnRH deficiency and APS type 3 associated with Graves' disease and LADA. Therefore, a hitherto unreported associated APS type 3 with isolated GnRH deficiency was described in this case report.

During hospitalization, the patient chose to continue the ATD treatment (oral methimazole) rather than radioiodine therapy to control her thyroid disfunction. Doses were adjusted according to the levels of her thyroid hormones, and her thyroid function levels were relatively stable. As for the treatment of type 1 diabetes, we gave a subcutaneous insulin injection to control her blood glucose. Three days before admission, her fasting blood glucose fluctuated between 9 mmol/L and 11 mmol/L, and random blood glucose varied from 15 mmol/L to 20 mmol/L. After intensive insulin pump therapy for two weeks, her blood sugar was controlled within a range from 7 mmol/L to 13 mmol/L. Afterward, the insulin pump was replaced by glargine (subcutaneous injection) once daily combined with NovoRapid (subcutaneous injection) before meals. Doses were adjusted according to glucose monitoring level, clinical and biochemical response. During the outpatient follow-up, her random blood glucose varied from 7 mmol/L to 15 mmol/L. For premature menopause, previous studies (3, 4) have found that the lack of estrogen could lead to premature aging of blood vessels, bones, other tissues, *etc.*, and shorten the life expectancy of those patients. Therefore, estrogen replacement therapy (HRT) is recommended to reduce the adverse effects of low estrogen levels. Although we have repeatedly emphasized the importance of HRT treatment, the patient refused to receive HRT treatment because she was afraid of side effects.

DISCUSSION

APS type 3 is an adult type of APS, defined as the combination of AITD with other autoimmune diseases, except for Addison's disease and hypoparathyroidism. Our patient's autoimmune history began in 2020 at the age of 42 when she had been diagnosed with TRAb positive GD. Shortly afterward, it was followed by LADA. Thus the diagnosis of APS type 3 was established. Previous studies (5) have confirmed the frequent coexistence of T1DM and AITD in patients with APS. It should be highlighted that, in this case, the characteristic of APS type 3 was not typical initially because this patient manifested with hyperthyroidism rather than hypothyroidism (6). Therefore, it is not common for this patient to be diagnosed with GD. Furthermore, she did not improve after regular or even strengthened methimazole therapy. Even after radioactive iodine-131 therapy, deterioration of thyroxin levels control and weight loss progressed. On the other hand, there is normally a time gap for many years between the diagnosis of the first and second diseases among APS patients (5, 7). It turned out that the time gap between T1DM and AITD is the most extended (5). In terms of the sequence of endocrine gland insufficiency manifested in the APS type 3, it has been reported (8) that in 60% of APS type 3 patients, GD is more likely to occur before T1DM, with an average time of 7 years. In the cohort of autoimmune disease components in APS patients established by Martin P Hansen et al. (9), the onset of T1DM was earlier (average 27.5 years), while other component diseases appeared later, ranging from 36.5 to 40.5 years old. Other studies (10) have shown that the average onset age of T1DM in patients with Graves' disease is 34 years old, with an incidence rate of 0.78%. However, in this reported case, GD and T1DM appeared almost simultaneously and developed rapidly, with the onset age relatively late (The time schedule of the events presents in **Figure 1**). Thus, the sequence of the affected autoimmune gland was unusual.

We should also consider that the patient's current diagnosis as APS type 3 could be temporary. Patients with APS type 3 may eventually develop Addison's disease and be reclassified as APS type 2. As related autoantibodies are usually detected a few years before the onset of the disease, we would like to put considerable emphasis on the necessity of the screen of the serology and function of other related autoimmune diseases linked. Due to the limited conditions, we did not measure the adrenal-associated 21-hydroxylase antibodies in this patient. Although the patient's laboratory tests did not suggest clinical or biochemical

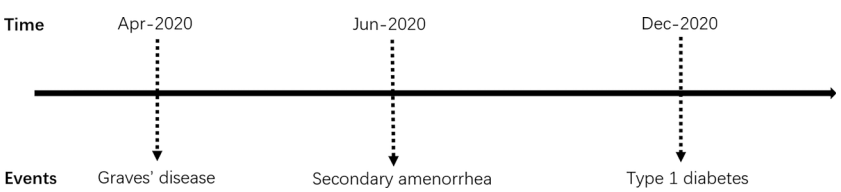

FIGURE 1 | Timeline of the main clinical events.

abnormalities of adrenal dysfunction, it is still necessary to carefully monitor the function of the adrenal axis and screen the level of cortisol and electrolytes regularly in the future. Moreover, the association of endocrine autoimmune diseases is mainly attributed to common genetic susceptibility. APS type 3 is often observed in individuals in the same family. A study of 10 families with APS found that one in seven relatives had an undiagnosed autoimmune disease (11). Accordingly, to find hidden patients with endocrine diseases, their relatives should undergo screening for autoantibodies related to the disease components of APS, such as GAD antibody, IA-2A antibody, TPO antibody, 21-hydroxylase autoantibody, *etc.*

In consideration of whether the patient had other endocrine glands been affected, we noted that the patient's amenorrhea age (42 years old) is earlier than the average. What makes it more intriguing was that the patient's circulating blood FSH and LH levels were significantly lower than those women in physiological amenorrhea states. Thus secondary hypogonadism was considered. To confirm whether the decrease in GnH level was due to the pituitary dysfunction or secondary dysfunction of the hypothalamus, we conducted the magnetic resonance imaging of her pituitary gland, which showed normal findings. Moreover, the GnRH stimulation test had been carried out, showing that FSH and LH could be evidently stimulated, which indicated that secondary hypogonadism was caused by abnormal hypothalamic secretion of GnRH. However, when we evaluated the secretion function of other hormones in the pituitary gland (such as ACTH, PRL, *etc.*), there was no obvious abnormality, implying that the hypothalamic insufficiency was only manifested by isolated GnRH decrease. Taking into account all these factors, we may safely arrive at the diagnosis of isolated GnRH dysfunction. Heretofore, secondary hypogonadism has been reported in some APS patients, but most of them were considered to be caused by autoimmune hypophysitis (12–14) or poor control of Graves' disease (15). Clinical cases of amenorrhea resulted from hypothalamic dysfunction/GnRH deficiency are mostly triggered by gene mutation, tumor, infiltration, trauma, *etc.* (16). Clinically, it is difficult to identify whether secondary hypogonadism is attributed to hypothalamic or pituitary diseases because it is impossible to directly detect the hormones secreted by the hypothalamus. Thus, apart from magnetic resonance imaging of the pituitary gland, we used the GnRH stimulation test to observe the response of the pituitary to GnRH and to determine the location of the lesion. Barkan et al. (12) had reported two patients with isolated gonadotropin deficiency after puberty. After completing the repeated GnRH stimulation experiments, blunted or absent responses of LH were seen, and the plasma level of FSH was undetectable. Finally, they reckoned that the decreased gonadal hormone resulted from autoimmune hypophysitis. Yet our patient's GnRH stimulation test showed that FSH and LH could increase by more than six folds compared to baseline, especially FSH, which strongly implies that the pituitary responded well to GnRH. Moreover, the magnetic resonance imaging of her pituitary gland found no lesions such as a tumor, pituitary stalk deviation, inflammation, or infiltration. Thus, the lesion could be located in the hypothalamus, and it was rational to suspect that the isolated GnRH deficiency originated from the hypothalamus. The cause of the isolated GnRH dysfunction in our case was likely attributed to autoimmune. Given the property of multiorgan autoimmunity of APS type 3, it is not surprising that patients with these diseases are prone to the involvement of hypothalamus. In this circumstance, this is the first reported case of autoimmune isolated GnRH dysfunction associated with APS type 3.

It is well known that APS type 3 is a type of HLA-associated disease (17, 18). Many studies have indicated that HLA haplotypes DR3-DQB1*0201 and DR4-DQ*0302 contributed to the polyglandular autoimmune syndrome type 3 (17). The HLA profile of our patient manifested DQB1*0201 allele and DRB1*0301 and *0803 gene site, and the corresponding genotypes were DQ2 and DR8. DQ2 has been widely reported to correlate with APS (17). As for the HLA-DR8 haplotype, previous studies (19–21) have reported the potential associations between primary biliary cirrhosis, uneven NANB (non-A-non-B) hepatitis, and liver transplantation results. It is also significantly linked with Korean Graves's patients with thyrotropin binding inhibiting immunoglobulins (TBII) and possibly related to the susceptibility gene that produces TSHR blocking antibodies (22). To the best of our knowledge, limited data are available on its association with the APS. The relations between HLA-DR8 and the components of the diseases in this patient remain to be elucidated.

Here we describe a middle-aged woman presenting with Graves' disease, LADA, and isolated gonadotropin-releasing hormone (GnRH) deficiency. This is perhaps the first report of an APS type 3 patient complicated with isolated GnRH reduction possibly caused by the autoimmune lesions of the hypothalamus, which reminded us that the hypothalamus could not be spared from the general autoimmune process. Clinicians need to pay attention to the importance of carrying out the GnRH stimulation test when encountering APS patients manifesting secondary menopause and keeping an eye on the new onset of other autoimmune diseases.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

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

SW and WS provided clinical information, researched the data. YZ and LW performed the genetic analysis. QJ and TW wrote

the draft of the manuscript. XL and ML provided critical discussion and reviewed/edited the manuscript. All authors contributed to the article and approved the submitted version.

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Proton Pump Inhibitors Increase the Risk of Autoimmune Diseases: A Nationwide Cohort Study

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Background: Previous study revealed proton pump inhibitors (PPIs) have an effect on gut microbiota. Alteration of the microbiome causes changes of the host immune system and then induces the development of autoimmune diseases (ADs). This study aimed to explore the possible association between PPIs use and ADs.

Methods: This study was conducted using data from the Taiwan National Health Insurance Research Database in the period between 2002 and 2015. We performed multivariate and stratified analysis through the Kaplan-Meier method and Cox proportional hazard models to estimate the association between proton pump inhibitor use and the risk of autoimmune diseases.

Results: Of the 297,099 patients treated with PPI identified, the overall mean (SD) age was 49.17 (15.63) years and 56.28% of the subjects was male. As compared with the non-PPI group, the adjusted hazard ratio (aHR) were higher for incident organ specific ADs such as Graves disease (aHR=3.28), Hashmoto thyroiditis (aHR=3.61), autoimmune hemolytic anemia (aHR=8.88), immune thrombocytopenic purpura (aHR=5.05) Henoch-Schonlein pupura (aHR=4.83) and Myasthenia gravis (aHR=8.73). Furthermore, the adjusted hazard ratio (aHR) were also higher for incident systemic ADs such as ankylosing spondylitis (aHR=3.67), rheumatoid arthritis (aHR=3.96), primary Sjogren syndrome (aHR=7.81), systemic lupus erythemtoasus (aHR=7.03). systemic vasculitis (aHR=5.10), psoriasis (aHR=2.57), systemic scleroderma (aHR=15.85) and inflammatory

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myopathy (aHR=37.40). Furthermore, we observed no dose-dependent effect between PPI use and the risk of ADs.

Conclusions: Our retrospective population-based cohort study showed that the prescription of proton pump inhibitors is associated with a higher risk of ADs.

Keywords: autoimmune disease, proton pump inhibitor, risk, cohort study, epidemiology

INTRODUCTION

Proton pump inhibitors (PPIs), for gastric acid related disorders including gastroesophageal reflux disease and peptic ulcers, are one of the most prescribed medications around the world. These drugs also prevent peptic ulcer diseases in critical patients (1). They also play a fundamental role in the standard regimens used for *Helicobacter pylori* eradication. In other clinical settings, coprescription of PPIs with nonsteroidal anti-inflammatory drugs is used for the prevention to nonsteroidal anti-inflammatory drugs–related gastrointestinal injury, as is accepted by most clinicians. However, much recent research has increasingly identified adverse reactions in patients with long-term prescription of PPIs, including the risks of enteric infection with Clostridium difficile (2), bone fracture (3, 4), osteoporosis (5), malignancy (6), ischemic stroke, myocardial infarction (7), and dementia (8).

Autoimmune diseases (ADs) comprise disorders caused by an imbalance of the immune system, which leads to damage to individual tissues. ADs consist of systemic conditions, such as systemic lupus erythematosus, rheumatoid arthritis, and Sjögren syndrome, as well as single-organ conditions, such as autoimmune thyroid diseases and autoimmune hepatitis. The mechanism underlying the development of ADs remains unknown. Multiple factors (e.g., genetic and environmental factors) affect the risk of ADs. The administration of medications is one of the most important factors in the induction of ADs.

Some medications change the composition of the gastrointestinal microbiota, which may influence human health and cause the development of many diseases (9). The microbiota refers to the millions of microorganisms colonized within the human body that contributes to health and pathology. The first report on the association between PPIs and dysbiosis or disruption of the microbial balance was published in 2008 (10). A meta-analysis reported a statistical relationship between PPIs and bacterial overgrowth in the small intestine (11). Bacterial overgrowth in the small intestine is closely related to dysbiosis (12). Moreover, the dysbiotic disruption caused by administration of PPIs is permanent (13). The dysbiosis was found most often at 4 to 8 weeks after PPIs treatment in patients (14).

The host–microbiota interaction plays an essential role in the host immune system (15). The microbiota influence the immune function. Alteration of the gut microbiome can play a pathogenic role in the development of ADs (16). To investigate the relationship between PPIs use and ADs, we hypothesized that PPIs alter host immune system function and increase the risk of ADs. We conducted a retrospective cohort study to clarify the association between PPIs use and development of ADs.

METHODS

Data Source

Our data were obtained from the Taiwan National Health Insurance Research Database (NHIRD). In brief, the single-payer mandatory Taiwan National Health Insurance (NHI) program currently covers >99% of the 23 million residents in Taiwan (17). The database contains all individual medical claims data since 1995. Claims data of patients include all ambulatory visits, hospital admissions, treatments, and medications prescribed under the NHI system. Diagnoses were coded according to the International Classification of Diseases (ICD). The accuracy of the diagnoses in the NHIRD, such as diabetes mellitus (18) and malignancy (19), has been validated. The present research was approved by the Institutional Review Board of Taipei Medical University (TMU JIRB-N201908055). Consent waivers were obtained, and all identifying information of patients is anonymized. This c cohort study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guideline.

Study Design and Subjects

This cohort study was retrospectively conducted using the NHIRD. We identified all individuals in the database from January 1, 2002, to December 31, 2015. The entry date was the day when the patients were included to our study, such as the date of the first PPI prescription in PPI users. We identified patients without prescriptions of PPIs during the entire study period as the control group. Patients were followed up from the entry date to the development of AD, death, or the end of the study.

The International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) codes were used for the definition of basic characteristics and events of ADs. The Anatomical Therapeutic Chemical (ATC) Classification codes were used for the definition of PPIs, anti-bacterial, antiviral, antifungal, and antituberculosis drugs.

Exclusion Criteria

We excluded patients who (1) had unknown general data or unknown follow-up time; (2) were less than 20 years old; (3) were diagnosed with ADs before the cohort entry date; (4) had received previous antibiotic, antifungal, anti-tuberculosis, or

antiviral agents; and/or (5) had a history of *H. pylori* infection. The *H. pylori* infections were identified as patients receiving therapeutic regimens for *H. pylori* eradication. The *H. pylori* eradication with triple or quadruple therapy was defined as a PPI or H2 receptor blocker, plus clarithromycin or metronidazole, plus amoxicillin or tetracycline, with or without bismuth; details of all eligible *H. pylori* eradication regimens were reported previously (20).

PPI Exposure

In Taiwan, when applying for reimbursement under the NHI program for PPIs, patients are required to have a diagnosis of reflux esophagitis or peptic ulcer disease through upper gastrointestinal endoscopy or a barium study. In this study, the duration of PPI use was determined according to prescription information contained in NHI claims data. We recorded the drug name, dosage, and start and withdrawal dates of each prescription claim. The PPIs included omeprazole, pantoprazole, lansoprazole, rabeprazole, and esomeprazole (ACT code A02BC), all of which are covered by the NHI program in Taiwan. Dosage of PPIs was presented as the defined daily dose (DDD), which has been established by the World Health Organization as the average maintenance dose per day for a drug used for its main indication in adults. Exposures of PPIs (cumulative dose during follow-up time) were analyzed by treatment continuation determined by the redeemed prescriptions, and the estimated doses were grouped into different cumulative defined daily doses (cDDDs) to assess dose–response effects on hazard ratios and the effects of long-term use on the risks of ADs. The cDDDs was estimated during the study period based on redeeming prescriptions. Furthermore, we divided the patients into four subgroups stratified by cDDDs, as follows: 1–21 DDDs, 22–42 DDDs, 43–98 DDDs, and ≥99 DDDs.

Comorbidities and Concomitant Medications

We determined potential confounders, associating a given covariate with PPI use on basis of the literature and direct or indirect association with other conditions, such as comorbidities and concomitant medications. According to the literature, these comorbidities and concomitant medications may induce changes in gut microbiota, such as liver cirrhosis (21). We identified comorbidities on the basis of at least two diagnoses of a given disease within 180 days before and after the entry date of our study according to the ICD-9-CM diagnosis codes for liver cirrhosis (ICD-9-CM codes 571, 571.2, 571.5, and 571.6), diabetes (ICD-9-CM codes 250.1–250.9), end-stage renal disease (ICD-9-CM code 585), and malignancy (ICD-9-CM codes 140–208).

Autoimmune Disease Risk Analysis

The outcomes of systemic and organ-specific ADs were analyzed (see **Supplementary Table 1**). We analyzed the occurrence of ADs in PPI users and nonusers, which was defined as ambulatory or admission to a hospital for ADs after the entry date. Patients with ADs including systemic and organ-specific diseases were identified based on three or more ambulatory care claims or an inpatient setting.

Statistical Analysis

Patients' baseline characteristics, including age, sex, coexisting medical conditions, and PPIs doses, were collected. We categorized age in 10-year intervals. Baseline characteristics were compared between PPIs users and nonusers using the chi-square test for categorical variables and the *t*-test for continuous variables, in addition, the Wilcoxon rank-sum test was used for median values of distributions. Baseline was set as the entry date. To understand the risk of autoimmune disorders between PPI and non-PPI users, we calculated the IR (incidence rate) and IRR (incidence rate ratio) from formula, and estimated adj. HR (adjusted hazard ratio) and the 95% confidence interval from Cox regression models to evaluate the occurrence of the all/systemic/organ-specific AD event between PPI and non-PPI users. The baseline information was used for exposure in model adjustment. For model adjustment, we adjusted for sex, age, cancer, diabetes, end-stage kidney disease, and liver cirrhosis. Cumulative incidence rates of ADs were estimated using the Kaplan-Meier method and compared by the log-rank test.

All statistical analyses were performed using SAS for Windows version 9.4 software (SAS Institute, Cary, NC), and a two-sided P value <0.05 was considered statistically significant.

RESULTS

Baseline Characteristics

Figure 1 depicts the study flowchart. A total of 28,408,935 patients were initially identified in this study from the Taiwan NHIRD during 2002–2015. After excluding patients according to the established exclusion criteria, a total of 3,422,693 were identified, including 297,099 in the PPI group and 3,125,594 in the control group. **Table 1** presents baseline characteristics of the PPI group and the control group. The mean ages of patients were 49.17 years (SD, 15.63) and 45.38 years (SD, 16.02) in the PPI and control group, respectively. The event rates of AD development were 0.98% and 0.24% in the PPI and control groups, respectively. Most AD events were systemic diseases with a percentage of 63.49% in PPI users who developed ADs.

Incidence Rates, Ratio, and Adjusted HRs of ADs in PPI and Non-PPI Users

In the baseline analyses, there were 2926 events in the PPI users and 7592 events in nonusers during the follow-up period of 12 months. The incident rates of ADs were 1219.94 and 274.67 per 100,000 person-years in the PPI users and nonusers, respectively (see **Table 2**). Compared with nonusers, PPI users had a 344% significantly higher absolute risk of AD within a 12-month period (absolute risk ratio, 4.44). After adjusting for age, sex, and comorbidities, we found a higher risk of ADs in PPI users, with an aHR of 3.64 (95% CI, 3.48–3.80; P <.0001), compared with nonusers. We also identified a higher risk of ADs in PPI users than in nonusers, restricted the analysis to systemic and

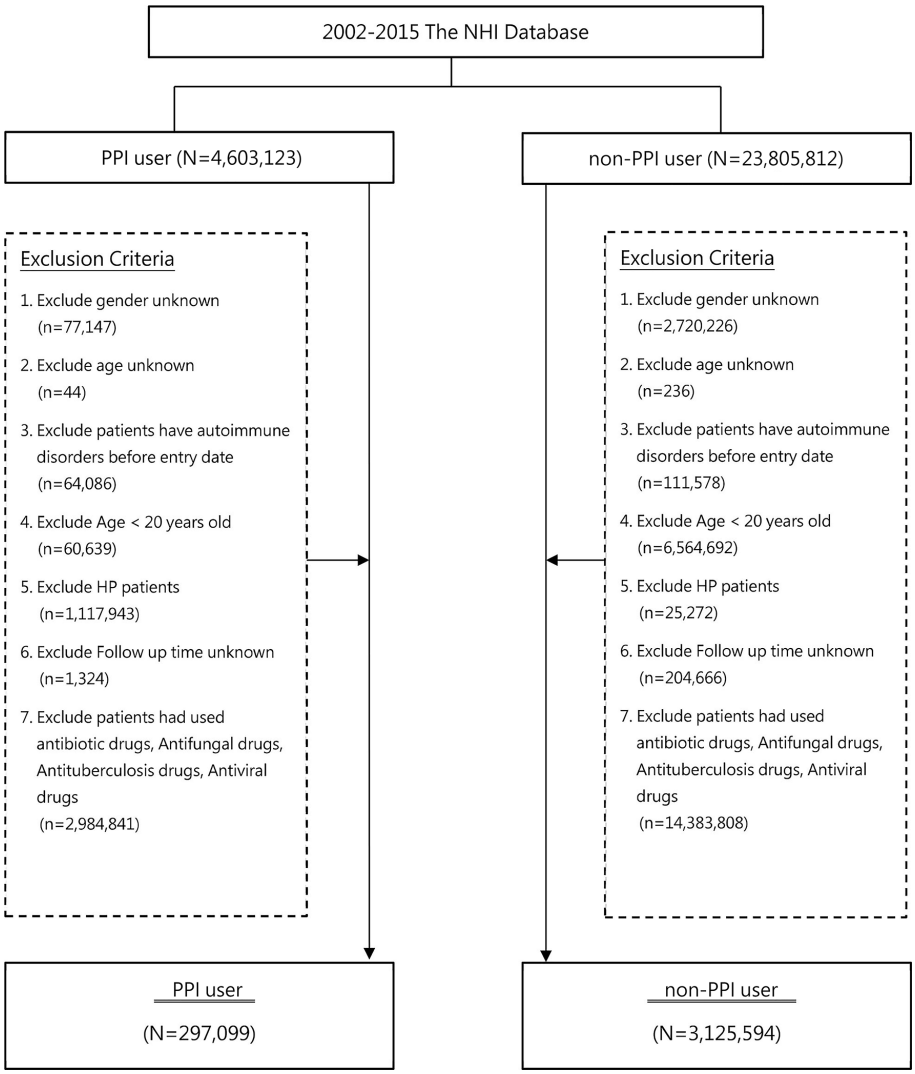

FIGURE 1 | Study flow chart. NHI, nation health insurance; PPI, proton pump inhibitor.

single-organ ADs, with aHRs of 4.33 (95% CI, 4.10–4.58; P <.0001) and 2.75 (95% CI, 2.55–2.95; P <.0001), respectively. **Figure 2** shows the significant increasing cumulative incidence of AD stratified by PPI users and nonusers with 12-month follow-up. Difference in AD developments were found for both systemic and organ-specific ADs.

Dose-Response and Adjusted HRs of ADs in PPI and Non-PPI Users

No significant relationship was observed between the PPI dose and AD risk in the PPI users. **Table 3** shows that the aHR for overall AD developments initially increased and then decreased as PPI cDDDs increased and the largest aHR was in the group of 22–42 DDDs. A similar trend of AD development was found when the analyses were restricted to systemic or organ-specific ADs. The highest aHR for systemic AD development was found in the group with 43–98 DDDs; the highest aHR for single-organ AD development was found in the group with 22–42 DDDs. However, in general, significantly higher aHRs for development of ADs were seen in the different cDDD groups of PPI users than nonusers.

Adjusted HRs of ADs and Subgroup ADs in PPI and Non-PPI Users

In **Table 4**, as compared with the non-PPI group, the adjusted hazard ratio (aHR) were higher for incident organ-specific ADs such as Diabetes mellitus type 1 (aHR=2.65, 95%CI 2.40 to 2.93), Graves disease (aHR=3.28), Hashmoto thyroiditis (aHR=3.61), autoimmune hemolytic anemia (aHR=8.88), immune thrombocytopenic purpura (aHR=5.05) Henoch-Schonlein

TABLE 1 | Baseline characteristic of PPI and non-PPI users.

	PPI (n = 297,099)		non-PPI (n = 3,125,594)		P-Value
	n	%	n	%	
Gender					0.0043
Female	129,900	43.72%	1,375,250	43.99%	
Male	167,199	56.28%	1,750,692	56.01%	
Age					<.0001
20–30	37,209	12.52%	623,753	19.95%	
31–40	57,072	19.21%	690,853	22.10%	
41–50	68,021	22.90%	720,217	23.04%	
51–60	66,238	22.29%	568,056	18.17%	
61–70	38,407	12.93%	276,260	8.84%	
71–80	20,248	6.82%	149,150	4.77%	
≥81	9,904	3.33%	97,653	3.12%	
Mean (SD)	49.17 (15.63)		45.38 (16.02)		<.0001
Median (IQR)	49 (23)		44 (22)		<.0001
Comorbidities					
Cancer	13,150	4.43%	50,087	1.60%	<.0001
Diabetes Mellitus	28,708	9.66%	128,694	4.12%	<.0001
End-Stage Renal Disease	4,507	1.52%	11,550	0.37%	<.0001
Cirrhosis of Liver	5,677	1.91%	4,397	0.14%	<.0001
Autoimmune Disorders					
Overall	2926	0.98%	7592	0.24%	<.0001
Systemic	1858	0.63%	4635	0.15%	<.0001
Organ-specific	1075	0.36%	2973	0.1%	<.0001

PPI, proton pump inhibitors.

TABLE 2 | Risk of autoimmune disorders between PPI and non-PPI users.

Types of Autoimmune Disorders	Event	IR	IRR	Adj. HR	95% C.I. for adj. HR
Overall					
non-PPI	7592	274.67	ref.	ref.	
PPI	2926	1219.94	4.440	3.640***	3.484–3.804
Systemic					
non-PPI	4635	167.65	ref.	ref.	
PPI	1858	772.14	4.606	4.335***	4.103–4.580
Organ-specific					
non-PPI	2973	107.38	ref.	ref.	
PPI	1075	445.97	4.148	2.750***	2.558–2.957

*PPI, proton pump inhibitors. Adj HR, adjusted hazard ratio was adjusted by gender, age, comorbidities. IR, incidence rate was incidences of per 100,000 person-year. CI, confidence intervals. ***P-Value < 0.001.*

pupura (aHR=4.83) and Myasthenia gravis (aHR=8.73). Furthermore, the adjusted hazard ratio (aHR) were also higher for incident systemic ADs such as ankylosing spondylitis (aHR=3.67), rheumatoid arthritis (aHR=3.96), primary Sjogren syndrome(aHR=7.81), systemic lupus erythemtoasus (aHR=7.03). systemic vasculitis (aHR=5.10), psoriasis (aHR=2.57), systemic scleroderma (aHR=15.85) and inflammatory myopathy (aHR=37.40). In summary, the highest aHRs for ADs development in patients with PPI compared with patients without PPI, were inflammatory myopathy (aHR=37.40).and autoimmune hemolytic anemia(aHR=8.88).

Cumulative Incidence of ADs and Subgroup ADs in PPI and Non-PPI Users

A Kaplan–Meier analysis revealed the cumulative incidence of ADs and subgroup ADs development in those PPI and non-PPI users (**Figure 2**). The cumulative incidence of overall, systemic and organ-specific ADs in PPI users was significantly higher than in the non-PPPI users (log-rank p value <0.0001, **Figures 2A–C**).

DISCUSSION

According to our review of the relevant literature, this study is the first nationwide population-based work to evaluate the relationship between PPIs and ADs. In the present study, the incidence rate of overall ADs was 3.9 times higher in the PPI users than in non-PPI users, with an adjusted HR of 3.32 after adjustment for age, sex and comorbidity. Furthermore, we found that PPI users also had an increased risk of organ-specific and systemic ADs, respectively.

Our study indicated an association between PPI and the risk of ADs, including systemic and single-organ ADs. The possible mechanism of the association of the prescription of PPIs and the development of ADs is hypothesized to be an alteration of the gut microbiome by PPIs, which leads to AD development. The effect of the host microbiota on the immune system has been identified (15). The administration of PPIs induced gut dysbiosis or disruption of the microbial balance. The alteration of the gut microbiota early in the course of PPI use was discovered in previous study (13). Alteration of the microbiome causes changes of the host immune system and then induces the development of AD. Our study provides clinical evidence to connect PPI use, the gut microbiota, and ADs.

The prescription of antibiotics altered the microbiota of the host, giving rise to the development of ADs, such as rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease (22). Bacterial or viral infection is also a major trigger of autoimmunity (23). We excluded patients receiving these

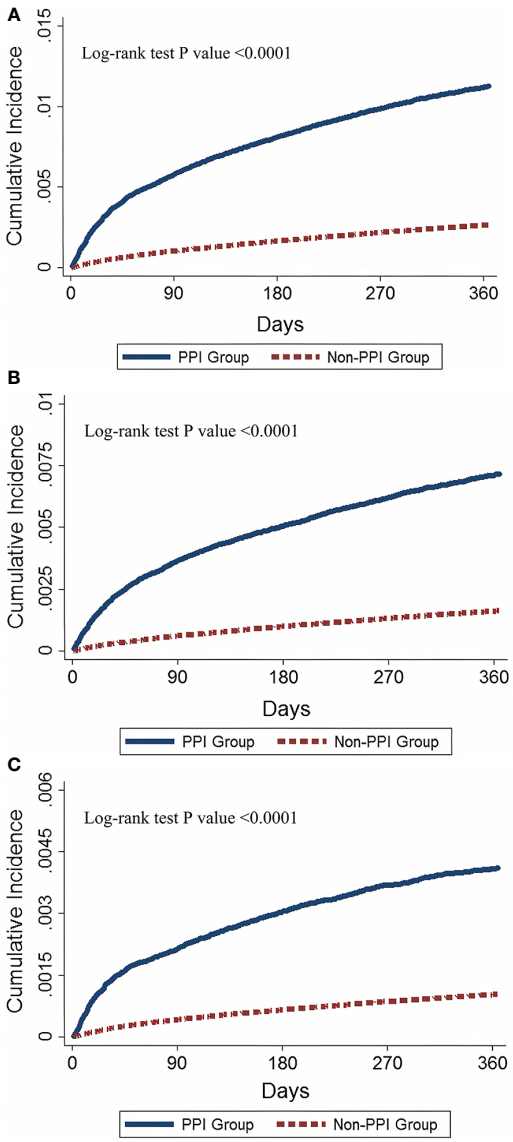

FIGURE 2 | Cumulative incidence of autoimmune diseases in PPI and non-PPI users. **(A)** Cumulative incidence of overall ADs for PPI and non-PPI users **(B)** Cumulative incidence of systemic ADs for PPI users and non-PPI users **(C)** Cumulative incidence of ADs in PPI users and non-PPI users. AD, autoimmune diseases. PPI, proton pump inhibitor.

medications, such as antibiotics, antivirals, and anti-tuberculosis agents, to eliminate the confounding factors. In addition, the *H. pylori* infection altering the gut microbiota was reported (24). The *H. pylori* induces a host-specific T-cell response, leading to autoimmunity *via* molecular mimicry (25). We also excluded patients with *H. pylori* infection, identified as patients receiving *H. pylori* eradication treatment.

Our study data was derived from a real-world database, the Taiwan NHIRD, and investigated that PPIs can induce an elevated risk of ADs. However, the development of ADs is affected by many factors. The microbiota play a role in the pathogenesis of ADs (15, 26).

TABLE 3 | The dose response between PPI and autoimmune diseases.

Types of Autoimmune Disorders	Event	IR	IRR	Adj. HR	95% C.I. for adj. HR
Overall					
0	7592	274.67	ref.	ref.	
1–21	768	1071.64	3.901	3.317***	3.078–3.573
22–42	774	1353.25	4.925	4.284***	3.977–4.614
43–98	894	1442.01	5.249	4.219***	3.932–4.527
≥99	490	999.32	3.637	2.690***	2.451–2.952
Systemic					
0	4635	167.65	ref.	ref.	
1–21	483	672.06	4.009	3.789***	3.449–4.163
22–42	484	843.00	5.028	4.775***	4.346–5.245
43–98	579	930.66	5.552	5.193***	4.758–5.668
≥99	312	635.17	3.789	3.522***	3.135–3.956
Organ-specific					
0	2973	107.38	ref.	ref.	
1–21	289	401.41	3.734	2.688***	2.380–3.035
22–42	292	507.33	4.721	3.549***	3.144–4.005
43–98	316	506.60	4.712	2.999***	2.665–3.375
≥99	178	361.96	3.366	1.817***	1.558–2.120

*PPI, proton pump inhibitors. Adj. HR, adjusted hazard ratio was adjusted by gender, age, comorbidities. IR, incidence rate was incidences of per 100,000 person-year. CI, confidence intervals. ***P-Value < 0.001.*

However, the evidence for dysbiosis leading to ADs have been provided by basic studies and animal models. Some evidence has been provided by human study (26). Our study hypothesized that the administration of PPIs influences the microbiome and results in imbalance of the immune system. Our results provide confirmatory evidence in real-world settings. Our study is to ascertain the association between PPIs and ADs, even though the relationships between PPIs and gastrointestinal microbiota and between gastrointestinal microbiota and host immune system have been identified. A significant increasing aHR of AD development was found in PPI users in the follow-up period, but we were unable to find a dose–response association. The aHR for the risk of ADs did not significantly increase when the PPI dose elevated. The highest aHR of overall AD development was observed in the 43–98 DDDs in patients with PPIs, rather than in the more than 99 DDDs. Even though no dose–response association was detected, the aHRs were significantly increased in different cDDD groups of PPI prescriptions compared with nonusers. These results support that PPIs induce development of ADs.

Several previous studies described the risk of several ADs is increased by PPIs. Polymyositis was found in patients treated with PPIs in the World Health Organization adverse drug reactions database (VigiBase) (27). Clarks and colleagues (28) described that 69 of 292 myopathy events recovered post PPIs withdrawn in a New Zealand study. Twenty-seven of 292 cases of myopathy were identified as polymyositis or myositis. Use of PPIs induced 24 events of subacute cutaneous lupus erythematosus on an incubation time of 8 months in Danish retrospective study (27). Aggarwal and colleagues (29) also reported that subacute cutaneous lupus erythematosus was induced by PPIs, with an elevated proportional reporting ratio of 36.64 in an analysis of U.S. FDA Adverse Event Reporting System. In a recent study based on Taiwan NHIRD, Chen and colleagues (30) reported that an incidence rate of systemic immune diseases in patients receiving

TABLE 4 | Risk of organ-specific and systemic autoimmune diseases between PPI and non-PPI users.

	Event	Incidence Rate	IRR	ADJ. HR	95% CI.
Systemic autoimmune disease					
Ankylosing spondylitis					
non-PPI	3211	116.13	1.000	1.000	
PPI	1051	435.87	3.753	3.670***	3.418–3.941
Rheumatoid arthritis					
non-PPI	809	29.25	1.000	1.000	
PPI	324	134.16	4.587	3.968***	3.482–4.523
Sjögren syndrome					
non-PPI	307	11.09	1.000	1.000	
PPI	236	97.68	8.804	7.810***	6.569–9.285
Systemic lupus erythematosus					
non-PPI	182	6.58	1.000	1.000	
PPI	116	47.98	7.296	7.029***	5.520–8.952
Systemic vasculitis					
non-PPI	49	1.77	1.000	1.000	
PPI	27	11.16	6.305	5.099***	3.162–8.225
Psoriasis					
non-PPI	47	1.70	1.000	1.000	
PPI	12	4.96	2.922	2.568**	1.348–4.891
Systemic Sclerosis					
non-PPI	33	1.19	1.000	1.000	
PPI	53	21.92	18.380	15.851***	10.190–24.660
Inflammatory myopathy					
non-PPI	15	0.54	1.000	1.000	
PPI	52	21.50	39.674	37.397***	20.920–66.870
Organ-specific autoimmune disease					
Graves' disease					
non-PPI	152	5.49	1.000	1.000	
PPI	44	18.19	3.313	3.280***	2.335–4.608
Hashimoto's thyroiditis					
non-PPI	1160	41.95	1.000	1.000	
PPI	382	158.25	3.773	3.606***	3.206–4.057
Autoimmune hemolytic anemia					
non-PPI	67	2.42	1.000	1.000	
PPI	82	33.91	14.008	8.877***	6.293–12.520
Immune thrombocytopenic purpura					
non-PPI	137	4.95	1.000	1.000	
PPI	71	29.36	5.932	5.048***	3.745–6.803
Henoch-Schonlein purpura					
non-PPI	98	3.54	1.000	1.000	
PPI	47	19.44	5.489	4.829***	3.383–6.886
Myasthenia gravis					
non-PPI	56	2.02	1.000	1.000	
PPI	43	17.78	8.788	8.733***	5.836–13.070

*PPI, proton pump inhibitors. Adj. HR, adjusted hazard ratio was adjusted by gender, age, comorbidities. IR, incidence rate was incidences of per 100,000 person-year. CI, confidence intervals. **0.001 ≤ P-Value < 0.01, ***P-Value < 0.001.*

PPI was 1.29 per 1000 person-years and the aHR of ADs was 1.5 in patients using PPI compared to nonusers. However, this study was limited to patients with gastric diseases and analyzed fewer systemic ADs than our study. They only analyzed the ADs of rheumatoid arthritis, systemic lupus erythematosus, Sjögren syndrome, psoriasis, polymyositis, and scleroderma. The AD events analyzed in our research are not only systemic ADs, but also single-organ ADs. In addition, the database analyzed in our research, which included almost all adults in Taiwan, is larger than the Chen's study. We report a higher aHR of Sjögren syndrome in patients receiving PPI than the Chen's study, which is 8.54 and 1.82 in our and Chen's study, respectively. The aHR of rheumatoid arthritis in our study is 4.54, which is also higher than 2.19 in the Chen's study. Our study provides data on the more complete association between different ADs, including systemic and single-organ, and the prescription of PPIs. Our result provides firmer evidence that PPIs can induce AD development.

The etiology of ADs remains unknown. We have described new potential medical factors leading to induction of ADs. Using PPIs will increase the risk of ADs. In previous studies, the prescription of PPIs had a low risk for users. Inappropriate prescription of PPIs was declared previously (31). Although they are relatively safe drugs, PPIs should be used more carefully (32). Gradually increasing adverse effects of PPIs have been identified. In our retrospective cohort study, the risk of ADs was increased by prescribing PPIs. The use of PPIs should be considered carefully for the correct indications and in select patients to prevent unnecessary overuse. Our study also raises important concerns about further therapeutic options for preventing ADs.

The limitation of our study was that we did not use a matched sample. We used nonmatching to compare data to explain a high risk of ADs in patients with PPI use in real-world settings. Difference in baseline characteristics exists between PPI users and nonusers. However, the high aHR would direct the real elevated risk for developing ADs in patients receiving PPIs. Another limitation of our study was that we cannot exclude all the possible factors affecting the gut microbiota. Although we excluded patients receiving antibiotics, antiviral agents, and antituberculosis agents, the microbiota will also be affected by a variety of host and environmental factors. We also excluded patients with major organ dysfunction diseases, including diseases of the liver and kidney. We tried to eliminate possible confounding factors by our established exclusion criteria. In addition, we could not confirm the exact time when a prescription of PPIs induced alteration of the gut microbiome. Furthermore, we could not predict the time when using PPIs induces the development of ADs. Therefore, the development of ADs was recorded as the event immediately after the prescription of PPIs in our study.

In conclusion, in the findings of our study, PPIs was associated with higher risk of the development of ADs. Therefore, it is recommended that awareness of increased risk of ADs in patients with PPI treatment is very important for clinician. Furthermore, the mechanism of PPIs inducing ADs needs further research to elucidate.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The present research was approved by the Institutional Review Board of Taipei Medical University (TMU JIRB-N201908055). Written informed consent for participation was not required for

this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Conception and design of the study: S-HL, J-HC, and C-CC. Analysis and interpretation of data: Y-CL and J-HC. Drafting of article or revising it critically for important intellectual content: S-HL, Y-SC, T-ML, L-FH, T-YH, H-CH, P-IK, W-SC, Y-CL, J-HC, and C-CC. All authors contributed to the article and approved the submitted version.

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A Novel Serum tsRNA for Diagnosis and Prediction of Nephritis in SLE

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Objective: Dysregulation of transfer RNA (tRNA)-derived small noncoding RNA (tsRNA) signatures in human serum has been found in various diseases. Here, we determine whether the signatures of tsRNAs in serum can serve as biomarkers for diagnosis or prognosis of systemic lupus erythematosus (SLE).

Methods: Initially, small RNA sequencing was employed for the screening serum tsRNAs obtained from SLE patients, followed by validation with TaqMan probe-based quantitative reverse transcription-PCR (RT-PCR) assay. Receiver operating characteristic (ROC) curve analysis was used to assess the diagnostic efficacy. The biological functions of tsRNAs were identified by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) assay.

Results: We first analyzed tsRNA signatures in SLE serum and identified that tRF-His-GTG-1 was significantly upregulated in SLE serum. The combination of tRF-His-GTG-1 and anti-dsDNA could serve as biomarkers for diagnosing SLE with a high area under the curve (AUC) of 0.95 (95% CI = 0.92–0.99), sensitivity (83.72%), and specificity (94.19%). Importantly, the noninvasive serum tRF-His-GTG-1 could also be used to distinguish SLE with LN or SLE without LN with AUC of 0.81 (95% CI, 0.73–0.88) and performance (sensitivity 66.27%, specificity 96.15%). Moreover, the serum tsRNA is mainly secreted *via* exosome and can directly target signaling molecules that play crucial roles in regulating the immune system.

Conclusion: In this study, it has been demonstrated for the first time that serum tsRNAs can be employed as noninvasive biomarkers for the efficient diagnosis and prediction of nephritis in SLE.

Keywords: tsRNA, SLE, LN, biomarker, ncRNA (non coding RNA), diagnosis

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a representative autoimmune disease leading to systemic autoimmunity resulting in various organ damage. SLE mainly occurs in women of childbearing age characterized with volcanic autoantibodies (1–4). Both genetic and environmental factors are considered to be involved, but the precise pathogenesis of SLE is still unclear. The current

diagnosis of SLE relies mainly on the clinical manifestations and laboratory tests, including antinuclear antibody (ANA), anti-dsDNA, and anti-Smith antigen (anti-Sm) (5, 6). However, due to the diversity of presentation, the early diagnosis of SLE remains a challenge.

The kidney, a vital organ in the human body, is severely affected by SLE (7). Lupus nephritis (LN) is one of the most severe organ manifestations of SLE and a kind of glomerulonephritis. There are six different histological classes of LN differentiated by their distinct manifestations and severities of renal involvement in SLE (8). LN appears in most SLE patients within 5 years of diagnosis. LN hugely contributes to the severity of the SLE, as 10% of LN patients acquire end-stage renal disease (ESRD) (7). Clearly, early diagnosis of LN and timely initiation of treatment are critical to prevent disease progression. At present, 24-h urine protein quantification and kidney biopsy are the most used methods for LN diagnosis clinically. However, these methods have many drawbacks, such as inaccurate timing, partial urine sample loss during urine retention, poor patient compliance for urine protein test, and invasiveness of the procedure for kidney biopsy. Therefore, it is urgent to explore new biomarkers to distinguish LN from SLE.

Small noncoding RNAs are a group of RNA molecules that are shorter than 200 nucleotides (nt) without coding potential, such as microRNA (miRNA), transfer RNA (tRNA)-derived small noncoding RNA (tsRNA), and PIWI-interacting RNAs (piRNAs) (9). Among them, tsRNAs can spring up from mature tRNA or tRNA precursors and cleaved by enzymes such as Dicer and angiogenin (10, 11). Several reports indicated that tsRNAs play vital roles in the pathological and physiological processes, including cancer (12–16), neurodegenerative disease (17, 18), and metabolic disease (19, 20). However, no study has been done yet to test serum tsRNAs in the context of autoimmune diseases.

Recent studies from our group (21, 22) and others (23, 24) have found that circulating nucleic acids (such as miRNAs) in body fluids can serve as potential biomarkers for detecting various diseases, including immune diseases. It has been established that tsRNAs are stable in circulation (25). Furthermore, recent research has shown that serum tsRNAs are present in high proportion (~70%) than miRNAs (26). However, the diagnostic usefulness and roles of serum tsRNAs, particularly for SLE, remain unclear.

In this study, we used RNA sequencing, qRT-PCR validation, and ROC curve analysis, leading to identifying SLE-associated tsRNA signatures in human serum. The serum tsRNA profile showed significant potential as a noninvasive biomarker for diagnosing and predicting LN in SLE.

METHODS

Experimental Design

This study was divided into two stages (**Figure 1**). In the training stage, to obtain an expression profile of serum tsRNAs specific for SLE, we employed a strategy that involved initial screening by small RNA (sRNA) sequencing and validation by qRT-PCR. At this stage, 24 SLE without LN patients, 33 SLE with LN patients, and 23 healthy controls were examined. Then, in the validation stage, selected tsRNAs were detected in a larger SLE cohort (52 SLE without LN patients, 83 SLE with LN patients, and 86 healthy controls).

Patient Characteristics and Clinical Features

A total of 301 patients were divided into three groups; 76 patients with SLE without LN, 116 patients with SLE and LN, and 109 normal donors from Nanjing Drum Tower Hospital were enrolled in this study. The clinical characteristics of these patient samples are shown in **Table 1**. Patients involved in the study satisfied the revised criteria for SLE set up by the 1997 American College of Rheumatology (ACR) (27, 28). To record the SLE disease activity, we used the Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI) score. Ethics Committee of the Affiliated Drum Tower Hospital of Nanjing University Medical School approval was obtained for the study (ID: 2020-327-01). From every participant of the study, informed written consent was also obtained.

RNA Sequencing

Pretreatment of tsRNAs

The heavy decoration of tsRNAs by RNA modification is a main problem to deal with while constructing an sRNA sequence library. To construct a total RNA library, the following changes were made: 3'-aminoacyl was deacylated, 3'-cP (2',3'-cyclic phosphate) was removed from 3'-OH to facilitate 3' adaptor ligation, and 5'-OH (hydroxyl group) was phosphorylated to 5'-P for ligating a 5'-adaptor. m1A and m3C were demethylated to facilitate reverse transcription.

Library Preparation

The integrity of RNA samples was validated using agarose gel electrophoresis. Quantification of the RNA samples was achieved by using NanoDrop ND-1000 instrument. The removal of the RNA modifications, the main issue during the construction of the RNA sequence library, was carried out by adopting the pretreatment strategy as priorly explained. Agilent 2100 Bioanalyzer was used to quantify the completed libraries. Based on the quantification outcomes, the libraries were mixed in equal quantities for sequencing operation.

Sequencing

For sequencing, the single-stranded DNAs were needed. To achieve that, 0.1 M NaOH solution was used to denature the DNA fragments in well-mixed libraries. The single-stranded DNA with 1.8 pM concentration was loaded onto the reagent cartridge. NextSeq system using NextSeq 500/550 V2 kit (#FC-404-2005, Illumina) was used for sequencing operation following the guidelines provided by the production company. The sequencing operation included 50 cycles.

RT-qPCR

The serum samples were centrifuged at 3,500 rpm for 10 min before being collected and stored at -80°C until further analysis. High-quality RNA extraction from serum was achieved by

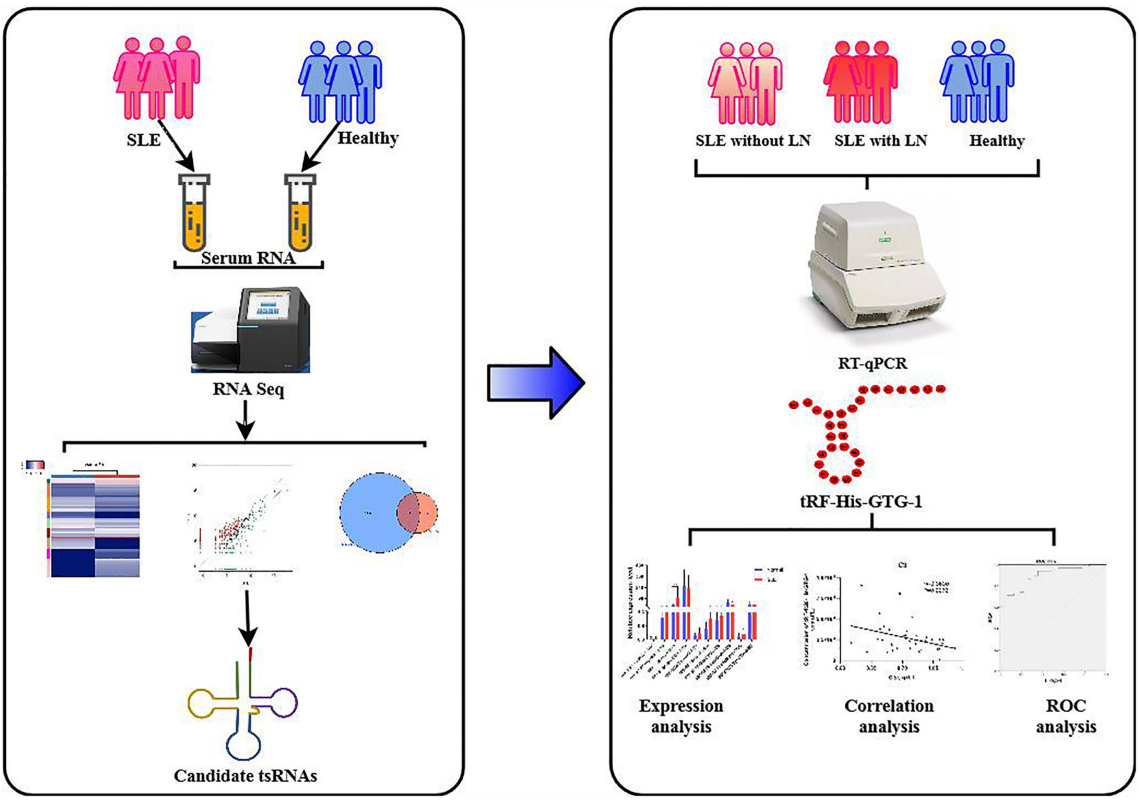

FIGURE 1 | Workflow of the study.

TABLE 1 | Statistics of clinical information of testing group specimens.

	SLE without LN	SLE with LN	Normal
Female (percentage)	20 (83.33%)	28 (84.85%)	23 (100%)
Age (years), median (range)	37 (14–70)	35 (18–64)	34 (18–52)
ANuA(+) (percentage)	5 (29.41%)	10 (41.67%)	N/A
ASMA(+) (percentage)	7 (41.18%)	10 (41.67%)	N/A
AHA(+) (percentage)	6 (37.50%)	9 (37.50%)	N/A
ARPA(+) (percentage)	8 (47.06%)	8 (33.33%)	N/A
Urine protein (g/L), median (range)	0.16 (0.07–2.30)	2.61 (0.08–16.08)	N/A
C3 (g/L), median (range)	0.80 (0.32–1.29)	0.54 (0.13–1.41)	N/A
C4 (g/L), median (range)	0.16 (0.03–0.30)	0.08 (0.01–0.35)	N/A
IgG (g/L), median (range)	12.75 (7.50–112.00)	10.20 (4.10–28.80)	N/A
CRP (mg/L), median (range)	3.45 (0.20–52.90)	2.60 (0.40–56.70)	N/A
Anti-dsDNA (IU/L), median (range)	115.48 (0.001–922.60)	118.91 (0.001–1340.89)	N/A
Anti-β2-GP I (RU/ml), median (range)	7.00 (0.00–67.50)	4.20 (0.10–64.10)	N/A
WBC (10^9/L), median (range)	5.80 (1.70–19.80)	6.10 (1.70–13.20)	N/A
RBC (10^{12}/L), median (range)	3.73 (1.03–4.96)	2.93 (1.77–4.88)	N/A
HGB (g/L), median (range)	112 (34.00–153.00)	83.00 (54.00–151.00)	N/A
HCT (%), median (range)	33.60 (10.90–45.30)	25.30 (16.30–45.50)	N/A
PLT (10^9/L), median (range)	181.00 (2.00–502.00)	139.00 (15.00–267.00)	N/A
Ne (10^9/L), median (range)	3.55 (1.10–14.10)	3.90 (0.90–9.40)	N/A
Ly (10^9/L), median (range)	1.10 (0.30–4.90)	0.80 (0.10–3.40)	N/A
Mo (10^9/L), median (range)	0.40 (0.10–0.90)	0.30 (0.00–1.00)	N/A

ANuA, anti-nuclear antibody; ASMA, anti-Smith antibody; AHA; anti-histone antibody; ARPA, anti-ribosomal p protein antibody; C3, Complement C3; C4, Complement C4; IgG, immunoglobulin; CRP, C-reactive protein; WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; PLT, platelet; Ne, Neutrophils; Ly, lymphocyte; Mo, monocyte; N/A, not available.

TRIzol (Vazyme Biotech, Nanjing, China) method. The quantity and quality of total RNA were measured using OneDrop-2000 (NanoDrop Technologies). For the cDNA synthesis, 500 ng of total RNA was reverse transcribed using the miRNA 1st Strand cDNA Synthesis Kit (Vazyme Biotech). Ultrasensitive detection of tsRNAs was performed using the miRNA Universal SYBR qPCR Master Mix (Vazyme Biotech). The expression of each gene was determined, and the corresponding standard curve was drawn. Gene-specific primers (Genscript Biotech, Nanjing, China) are listed in **Supplementary Table S1**.

Statistical Analyses

Statistical analyses were performed using an SPSS software, version 20.0 (SPSS Inc., Chicago, IL, USA), and GraphPad Prism software 7 (San Diego, CA, USA). Data are presented as the means ± SEMs, and statistical validations were achieved by harnessing t-test or one-way ANOVA followed by Bonferroni's multiple comparisons test. $P < 0.05$ was nominated to represent the statistically significant differences.

Data and Materials Availability

Raw data generated by tsRNA sequencing have been deposited to Gene Expression Omnibus (GEO) under the accession code GSE90524. The main findings of the study are included in the article and supplementary materials. Other data supporting the study findings can be acquired upon reasonable request from the corresponding authors.

RESULTS

Ectopic Serum tsRNA Profiles in Systemic Lupus Erythematosus Patients

The presence of stable tsRNAs in human serum has been confirmed by recent studies (26). Expression profile of serum tsRNAs can be potential fingerprints for various diseases (29–31). To identify the tsRNA profile in SLE patients, all participants were divided into three groups according to the clinical standard, including SLE without LN, SLE with LN, and healthy control. In this study, we employed a two-stage strategy to screen the SLE-specific tsRNAs (**Figure 1**). First, in the training stage, we started the search by comparing the tsRNA profiles in SLE serum with healthy controls. For tsRNA sequencing, 10 ml of serum pooled from 24 SLE patients and 23 healthy controls were extracted for RNA isolation, followed by tsRNA sequencing (GEO number: GSE90524). The basic characteristics of the patients are provided in **Table 1**. A total of 393 tsRNAs showed different expressions between SLE without LN groups and healthy controls in scatter plot analysis (**Figure 2A**). Distribution characteristics and Venn analyses of tsRNA also showed a difference in SLE without LN and healthy controls (**Figures 2B, C**). tsRNAs that satisfied two conditions showed significant differential expression: CPM >100 in serum by sequencing detection and fold change >6 between two groups. As shown in **Table 2** and **Figure 2D**, the analysis resulted in 10 significant differentially expressed tsRNAs in serum from SLE patients compared with healthy controls.

Identification of Differentially Expressed Serum tsRNAs in Systemic Lupus Erythematosus Patients

In the training stage, we then used RT-qPCR assay to validate the sequencing results. The absolute quantification was used to validate the expression of the top 10 upregulated tsRNAs using individual serum samples from 32 SLE without LN and 32 healthy controls by RT-qPCR (**Figure 3A**). RT-qPCR assay showed that tRF-His-GTG-1 was the most significantly elevated tsRNA in serum from SLE without LN compared to the control group (**Figure 3B**). In summary, tRF-His-GTG-1 was the candidate tsRNA that may be best classified between the SLE and control groups.

Serum tRF-His-GTG-1 Is Specifically Upregulated in Systemic Lupus Erythematosus Patients

From tRNAs, two types of tsRNAs originate, tRNA-derived stress-induced RNAs (tiRNAs) and tRNA-derived fragments (tRFs). tiRNAs arise from the 5′ and 3′ tRNA halves consisting of about 30–40 nt. tRFs originate from mature and tRNA precursors by nucleases Dicer or angiogenin, etc. (10, 11), shorter than tiRNAs, and consist of 18–22 nt. Based on the position of sequence and the cutting site on tRNAs, four types of tRFs are recognized so far, including 5-tRFs, 3-tRFs, 1-tRFs, and 2-tRFs (18). In this study, the tRF-His-GTG-1 belongs to 5-tRFs (**Figure 4A**). To further test whether serum tRF-His-GTG-1 is upregulated and associated with progression in SLE patients, the level of tRF-His-GTG-1 was examined in large sample size, including 52 SLE without LN and 86 healthy controls (validation stage, **Table 3**). As shown in **Figure 4B**, the serum level of tRF-His-GTG-1 was significantly elevated in SLE without LN compared with healthy controls.

Diagnostic Use of Serum tRF-His-GTG-1 in Systemic Lupus Erythematosus

To verify the value of tRF-His-GTG-1 for clinical practice, we used ROC curve analysis and assessed the sensitivity and specificity of prediction based on the risk scores. As shown in **Figure 4D**, serum tRF-His-GTG-1 in SLE without LN had higher diagnostic use with area under the curve (AUC) of 0.67 (95% CI, 0.57–0.76) and performance (sensitivity 59.62%, specificity 72.09%) relative to the healthy group. To further demonstrate the clinical significance of tRF-His-GTG-1 expression in SLE patients, we investigated the association between the tsRNA level and various clinical indicators in a total of 52 SLE serum samples. The results showed that high serum tRF-His-GTG-1 was negatively associated with age and complement C3 and C4 but positively correlated with C1q (**Figure 4C**). Moreover, tRF-His-GTG-1 had no association with SLEDAI, anti-dsDNA, anti-β2, C3b, immunoglobulin G (IgG), C-reactive protein (CRP),

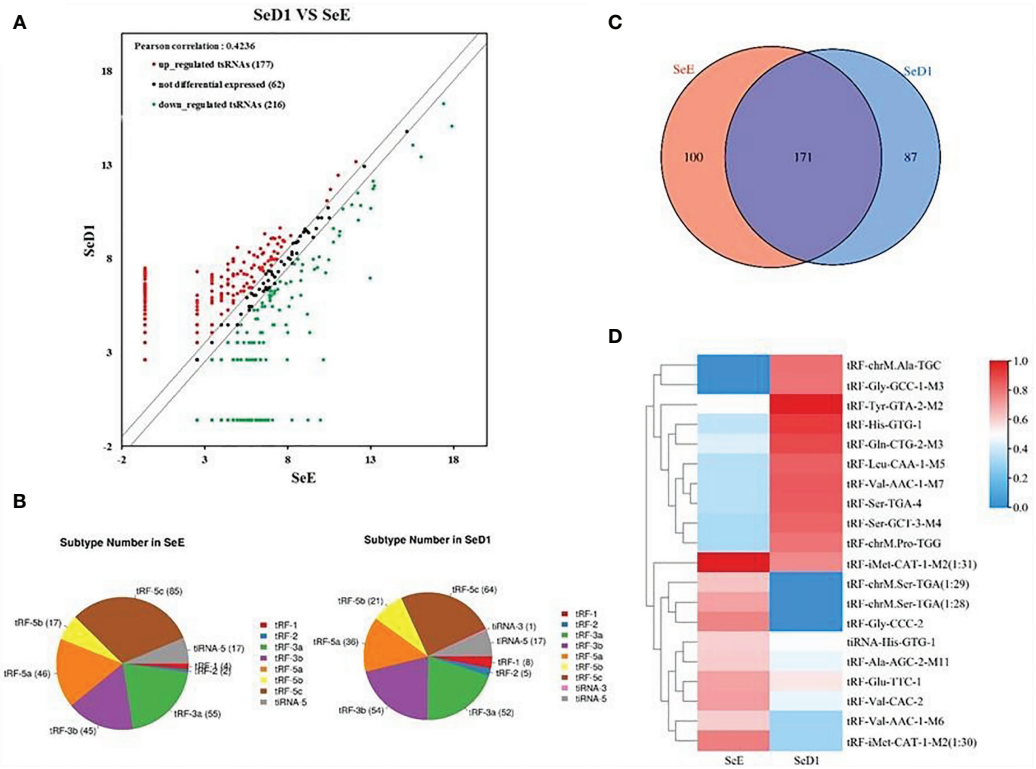

FIGURE 2 | Analysis of differentially expressed tsRNAs in serum of systemic lupus erythematosus (SLE) patients. **(A)** Scatter plots of differentially expressed tsRNAs. tsRNAs above the top line (red dots, upregulation) or below the bottom line (green dots, downregulation) indicate more than 1.5-fold change between the two compared groups. Gray dots indicate non-differentially expressed tsRNAs. Statistical correlation was determined by Pearson correlation. **(B)** Distribution characteristics of tsRNA in SLE patients and healthy controls. **(C)** Venn distribution of tsRNAs in SLE patients and healthy controls. **(D)** Hierarchical clustering indicates the differences in tsRNA expression profiling between two groups.

proteinuria (**Supplementary Figure S2A**). Then, we explore the predictive accuracy of the tRF-His-GTG-1 combined with clinical indicators. ROC curve was used to analyze the diagnostic efficacy of tRF-His-GTG-1 combined with anti-dsDNA antibody C1q, C3b, proteinuria (**Figure 4D** and **Supplementary Figure S2B**). In the combined analysis, a logistic regression model with tRF-His-GTG-1 and anti-dsDNA antibody resulted in a higher AUC of 0.95 (95% CI = 0.92–0.99) and higher performance (sensitivity 83.72%, specificity 94.19%) compared with a single anti-dsDNA antibody (**Figure 4E**), highlighting the diagnostic performance of the combination of tRF-His-GTG-1 and anti-dsDNA antibody for identifying SLE.

Serum tRF-His-GTG-1 as Biomarkers to Distinguish Lupus Nephritis From Systemic Lupus Erythematosus

To further explore whether serum tRF-His-GTG-1 level is related to the progression of SLE, we then performed qRT-

TABLE 2 | Sequencing information of 10 candidate tsRNAs.

tRF ID	tRF Sequence	tRF Length	Type	Fold Change	CPM
tRF-1:23-chrM.Ala-TGC	AAGGGCUUAGCUUAAUUAAAGUG	23	tRF-5b	272.28	212.32
tRF-27:42-Gly-GCC-1-M3	UCGCCUGCCACGCGGG	16	tRF-2	264.06	205.88
tRF-1:28-His-GTG-1	GCCGUGAUCGUAUAGUGGUUAGUACUCU	28	tRF-5c	12.25	456.80
tRF-68:85-Ser-GCT-3-M4	AUCCCAUCCUCGUCGCCA	18	tRF-3a	10.00	250.92
tRF-1:22-Val-AAC-1-M7	GUUUCCGUAGUGUAGUGGUUAU	22	tRF-5b	9.70	302.39
tRF-68:85-Ser-TGA-4	AUCCUGUCGGCUACGCCA	18	tRF-3a	9.28	289.52
tRF-57:75-Gln-CTG-2-M3	AGUCUCGGUGGAACCUCCA	19	tRF-3b	8.67	270.22
tRF-65:86-Leu-CAA-1-M5	UCGAAUCCCACUUCUGACACCA	22	tRF-3b	7.95	199.45
tRF-53:71-chrM.Pro-TGG	AAGACUUUUUCUCUGACCA	19	tRF-3b	6.57	765.63
tRF-57:76-Tyr-GTA-2-M2	GAUUCCGGCUCGAAGGACCA	20	tRF-3b	6.26	386.03

tsRNA, transfer RNA-derived small noncoding RNA; tRF,transfer RNA-derivrd fragment

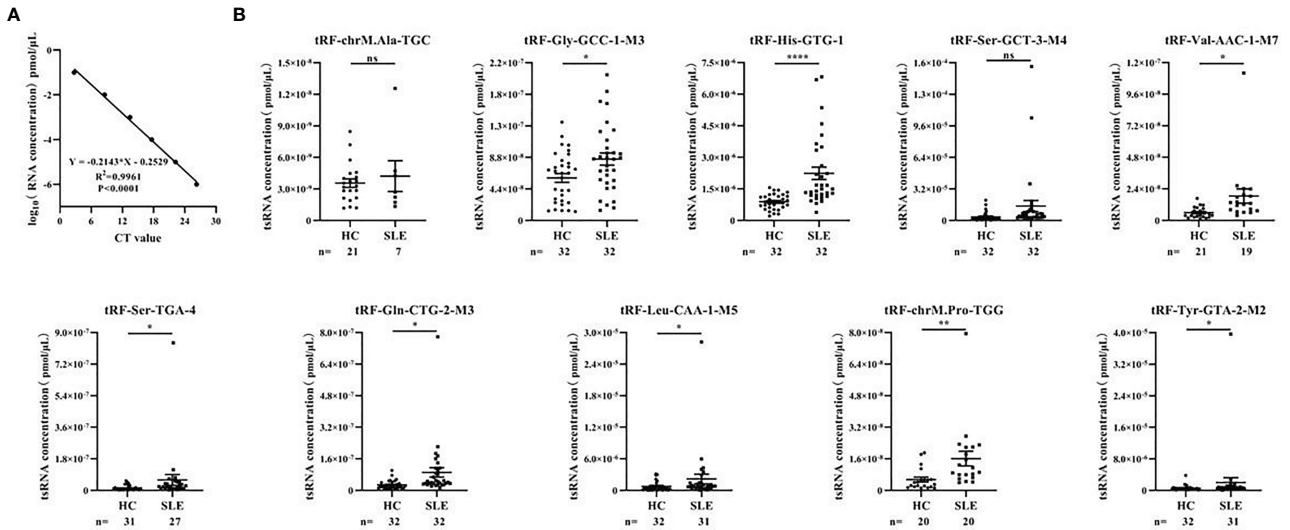

FIGURE 3 | Identification of differentially expressed serum tsRNAs in systemic lupus erythematosus (SLE) patients. **(A)** Standard curve of tsRNA concentration. Statistical correlation was determined by linear regression and Pearson correlation. **(B)** RT-qPCR verification of 10 differentially expressed tsRNA in serum of SLE without lupus nephritis (LN) group and healthy control group. Statistical significance was determined by unpaired two-tailed t-test (*P < 0.05, **P < 0.01, ****P < 0.0001). ns, Non significant.

PCR to assay serum samples from 83 SLE with LN patients. The expression of the tRF-His-GTG-1 in serum was remarkably decreased in SLE with LN than in SLE patients without LN (**Figure 4B**). ROC curves were performed to determine the diagnostic characteristics of serum tRF-His-GTG-1. Surprisingly, serum tRF-His-GTG-1 in SLE with LN group showed a higher diagnostic value with AUC of 0.81 (95% CI, 0.73–0.88) and performance (sensitivity 66.27%, specificity 96.15%) relative to SLE without LN group (**Figure 4F**). These results demonstrate that the levels of tRF-His-GTG-1 can serve as a promising diagnostic indicator to distinguish the LN patients among all SLE patients.

tRF-His-GTG-1 Function in Systemic Lupus Erythematosus

The existing forms of tRF-His-GTG-1 in serum were analyzed to explore their potential functions. Intensive studies suggested that exosomes carry small noncoding RNAs that can be delivered into recipient cells where they function as endogenous RNAs, simultaneously regulating multiple target genes or signaling events (32). We first determined whether serum tRF-His-GTG-1 was circulating *via* exosomes. We isolated exosomes from SLE patient serum. Our data suggested that tRF-His-GTG-1 was high in serum exosomes (**Figure 5A**). To investigate the potential functions of tRF-His-GTG-1, we then used target prediction tools, RNAhybrid and Targetscan, to analyze its target genes. Then, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed to identify biological processes associated with the tsRNA target genes [P < 0.001, false discovery rate (FDR) <0.05]. The high-enrichment GO terms targeted by tRF-His-GTG-1 include regulation of GTPase activity, protein homodimerization activity, and alpha-beta T-cell differentiation (**Figure 5B**). KEGG annotation showed that mitogen-activated protein kinase (MAPK) signaling pathway, Epstein–Barr virus infection, RIG-like receptor signaling pathway, and tumor necrosis factor (TNF) signaling pathway were enriched (**Figure 5C**). Most of the GO items and signaling pathways were associated with immunoregulation. These bioinformatics findings may add to the evidence that the tRF-His-GTG-1 can modulate immunity in SLE patients.

DISCUSSION

SLE is an autoimmune disease that affects multiple organs and tissues. Its clinical manifestations are abnormal activation of lymphocytes (33, 34) and abnormal buildup of autoantibodies (35). Kidney is one of the most crucial organs in the human body, and LN has a huge negative impact on the kidney. Within 5 years of diagnosis, 10% of individuals will develop ESRD. The poor survival rate of the diseases is a huge obstacle for current treatment strategies (8, 36, 37). Early detection and prediction of progression are both rapidly growing areas in SLE research to improve therapeutic efficacy. Currently, the clinical indicators of SLE diagnosis or progression monitoring, such as anti-dsDNA antibody, urinary protein, serum creatinine clearance rate, complements C3 and C4, or renal biopsy (38–40), have obvious limitations and have failed to fulfill clinical needs. Therefore, new noninvasive biomarkers with higher sensitivity and specificity for the early detection of SLE are highly desired.

Liquid biopsies for detecting novel biomarkers in circulation provide an attractive alternative for the diagnosis of diseases.

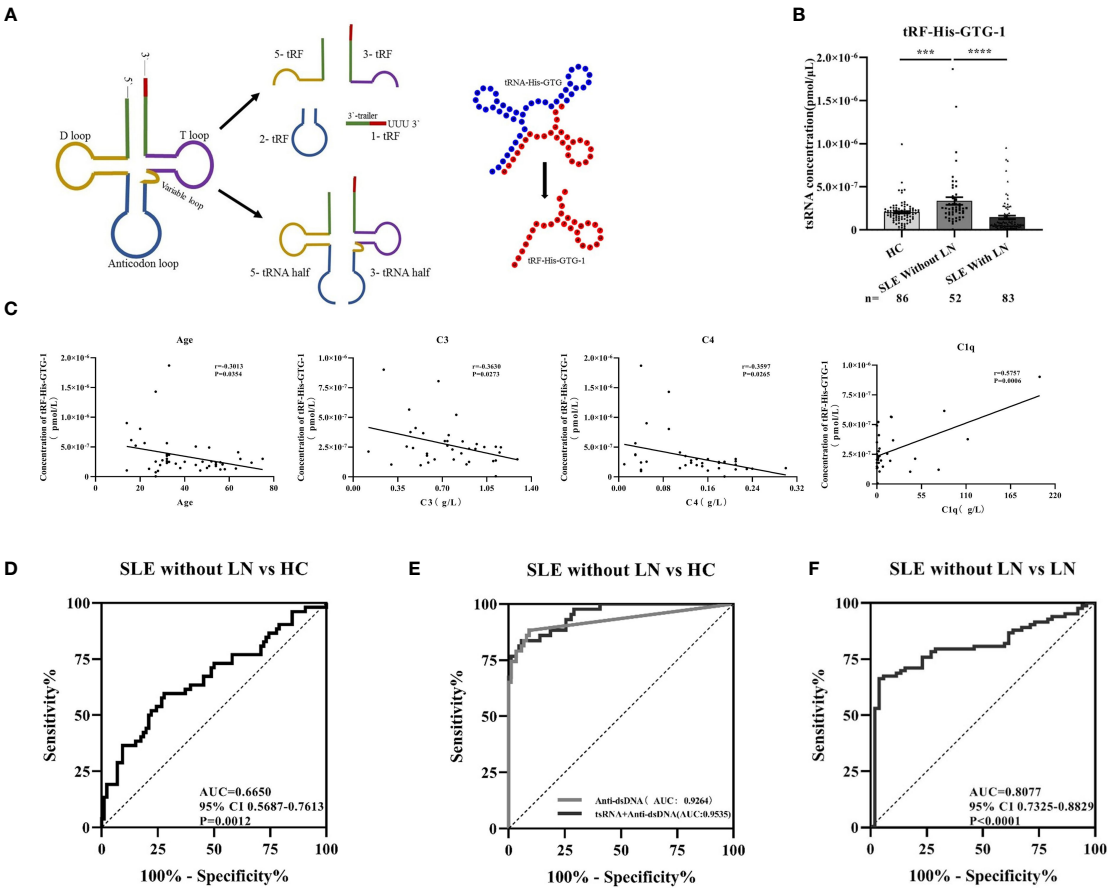

FIGURE 4 | Diagnostic use of serum tRF-His-GTG-1 in systemic lupus erythematosus (SLE). **(A)** Schematic diagram of tRF-His-GTG-1 biogenesis and secondary structure. **(B)** RT-qPCR verification of tRF-His-GTG-1 in SLE without lupus nephritis (LN), SLE with LN, and normal. Statistical significance was determined by one-way ANOVA (***P < 0.001, ****P < 0.0001). **(C)** Analysis of the correlation between tRF-His-GTG-1 and age, C3, C4, and C1q. Statistical correlation was determined by linear regression and Pearson correlation. **(D)** Receiver operating characteristic (ROC) curve analysis of tRF-His-GTG-1 in SLE without LN and healthy controls. **(E)** ROC curve combined diagnostic analysis of tRF-His-GTG-1 and anti-dsDNA in SLE without LN and healthy controls. SPSS binary logistic regression was used to predict the probability of joint diagnosis. **(F)** ROC curve analysis of tRF-His-GTG-1 in SLE without LN group and SLE with LN group.

Recently, circulating miRNAs and DNAs have been comprehensively studied as biomarkers for a variety of diseases (21, 22, 41). The discovery of short noncoding RNAs was one of the most major advances in recent decades (42, 43). Among them, tsRNAs, a novel type of small noncoding RNA generated by the cleavage of tRNA or pre-tRNA, have drawn great attention. Although dysregulation of tsRNAs had been observed in a variety of cancers (12–16), neurodegenerative diseases (17, 18), and metabolic diseases (19, 20), the profiling and role of tsRNAs in autoimmune diseases have not yet been studied. tsRNAs had also been found to be stable in the circulation and are likely to be promising disease biomarkers for two reasons: (1) tsRNAs harbor various RNA modifications (inherited from their tRNA precursors) than other small noncoding RNAs in circulation (11, 44), which may improve RNA stability and provide more information; (2) tsRNA signatures may sensitively change under variable stress conditions (45), the environment to which SLE patients are commonly exposed. This leads us to suspect that the tsRNA signatures may be more sensitive to reflect the progression of SLE disease. We adopted a "proof-of-principle" approach in this study. Particular SLE-specific serum tsRNAs were identified individually through high-throughput RNA sequencing, validated by using qRT-PCR validation sets at the individual level and then analyzed in combination with other clinical markers. In light of the abovementioned approach, we identified that tRF-His-GTG-1 combined with anti-dsDNA had high sensitivity and specificity in distinguishing SLE patients from healthy controls. These combined markers exhibited 83.72% sensitivity and 94.19% specificity. We anticipate that if these combined serum markers are validated by future studies, with large patient population size, it can advance in randomized clinical trials and can be harnessed for efficient and early detection of SLE.

For efficient diagnosis of LN and LN subtype identification, the current gold standard is kidney biopsy (8). However, the procedure

TABLE 3 | Statistics of clinical information of validating group specimens.

	SLE without LN	SLE with LN	Normal
Female (percentage)	47 (90.38%)	74 (89.16%)	78 (90.70%)
Age (years), median (range)	38 (14–75)	45 (14–72)	33 (12–68)
SLEDAI-2K, median (range)	5 (0–13)	10 (0–19)	N/A
ANuA(+) (percentage)	14 (43.75%)	15 (32.61%)	N/A
ASMA(+) (percentage)	8 (25.00%)	15 (32.61%)	N/A
AHA(+) (percentage)	12 (37.50%)	21 (45.65%)	N/A
ARPA(+) (percentage)	9 (28.13%)	18 (39.13%)	N/A
Urine protein (g/L), median (range)	0.16 (0.01–8.59)	0.55 (0.05–11.18)	N/A
C3 (g/L), median (range)	0.75 (0.12–1.34)	0.78 (0.02–1.68)	N/A
C4 (g/L), median (range)	0.15 (0.01–0.30)	0.13 (0.01–0.42)	N/A
IgG (g/L), median (range)	13.50 (6.00–37.90)	12.50 (4.90–44.00)	N/A
CRP (mg/L), median (range)	4.25 (0.10–126.30)	4.50 (1.30–110.00)	N/A
Anti-dsDNA (IU/L), median (range)	157.61 (0.001–1465.91)	144.56 (0.001–1,105.95)	N/A
Anti-β2-GP I (RU/ml), median (range)	5.90 (0.20–271.90)	4.45 (0.10–57.10)	N/A
WBC (10^9/L), median (range)	5.30 (0.50–36.30)	4.70 (0.50–20.50)	N/A
RBC (10^{12}/L), median (range)	3.76 (1.01–38.00)	3.46 (1.51–5.91)	N/A
HGB (g/L), median (range)	110.00 (34.00–151.000	105.00 (55.00–162.00)	N/A
HCT (%), median (range)	33.30 (10.90–45.50)	31.70 (16.90–46.80)	N/A
PLT (10^9/L), median (range)	196.00 (0.01–683.00)	150.00 (2.00–442.00)	N/A
Ne (10^9/L), median (range)	3.40 (0.20–32.20)	3.30 (0.20–17.00)	N/A
Ly (10^9/L), median (range)	1.10 (0.20–5.80)	0.90 (0.20–2.80)	N/A
Mo (10^9/L), median (range)	0.40 (0.10–5.30)	0.30 (0.00–2.30)	N/A

ANuA, anti-nuclear antibody; ASMA, anti-Smith antibody; AHA; anti-histone antibody; ARPA, anti-ribosomal p protein antibody; C3,Complement C3;C4, Complement C4; IgG, immunoglobulin; CRP, C-reactive protein; WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; PLT, platelet; Ne, Neutrophils; Ly, lymphocyte; Mo, monocyte; N/A, not available.

is invasive, risky, and not easy to be repeated. The 24-h urine protein quantification is used frequently but had many drawbacks, such as inaccurate timing, partial urine sample loss during urine retention, and poor patient compliance for urine protein tests. It is very difficult to detect the occurrence of LN in clinical SLE patients. Hence, another unique extension of our study is that serum tRF-His-GTG-1 could serve as a noninvasive biomarker for distinguishing LN in SLE patients. The serum tRF-His-GTG-1 signature had an excellent ability to distinguish LN patients from SLE patients with high sensitivity (66.27%) and specificity (96.15%), better than proteinuria (sensitivity 58.21%, specificity 100%). These results highlight that noninvasive serum tRF-His-GTG-1 could be used to distinguish SLE with LN or SLE without LN. Although the mechanism of decreased serum tRF-His-GTG-1 in SLE with LN has not been identified, we guess that damaged kidneys may leak more tRF-His-GTG-1 into urine. Besides, the accumulation of renal disease status may also reshape the immune microenvironment *in vivo*, and it will significantly influence the expression profile of serum tsRNAs. It is therefore recommended that future studies in multicenter clinical trials and molecular

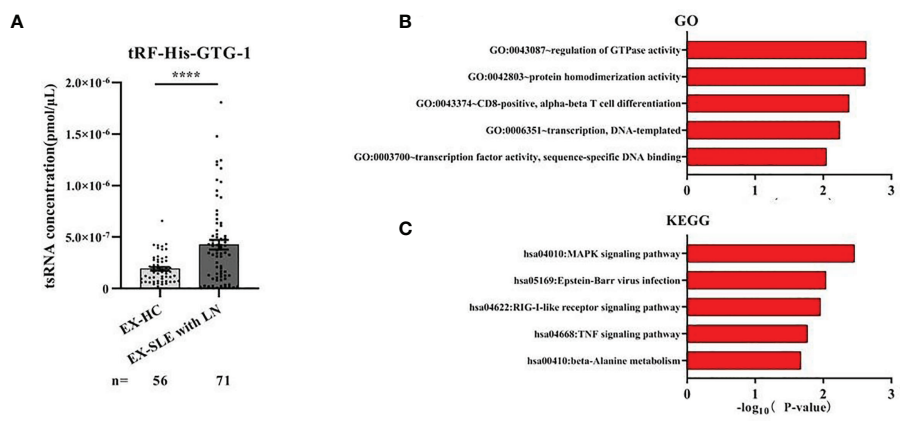

FIGURE 5 | The biological functions of the tRF-His-GTG-1. **(A)** Expression analysis of tRF-His-GTG-1 in exosomes of healthy group and systemic lupus erythematosus (SLE) group. Statistical significance was determined by unpaired two-tailed t-test (****P < 0.0001). **(B)** Gene Ontology (GO) term analysis of tRF-His-GTG-1. **(C)** Kyoto Encyclopedia of Genes and Genomes (KEGG) signal pathway analysis of tRF-His-GTG-1.

mechanism should be adopted to carefully assess the clinical utility of serum tsRNAs as diagnostic biomarkers.

In summary, we have introduced a serum tsRNA signature in SLE patients for the first time. In particular, we have demonstrated that combined serum tRF-His-GTG-1 and anti-dsDNA antibodies can serve as noninvasive biomarkers for diagnosing SLE. More importantly, the profile of tRF-His-GTG-1 may also function as a warning marker for LN in SLE patients. These results can provide an impetus for future studies to further explore serum tsRNAs for potential clinical applications and to understand their biological functions in depth.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. These data can be found here: https://www.ncbi.nlm.nih.gov/geo/GSE179950.

AUTHOR CONTRIBUTIONS

PY, XZ, and SC contributed equally to this work. YW is the chief designer of the whole experiment. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.735105/full#supplementary-material

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Autoinflammatory Keratinization Disease With Hepatitis and Autism Reveals Roles for JAK1 Kinase Hyperactivity in Autoinflammation

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Heterozygous mutations in *JAK1* which result in JAK-STAT hyperactivity have been implicated in an autosomal dominant disorder that features multi-organ immune dysregulation. This study identifies another previously unreported heterozygous missense *JAK1* mutation, H596D, in an individual with a unique autoinflammatory keratinization disease associated with early-onset liver dysfunction and autism. Using CRISPR-Cas9 gene targeting, we generated mice with an identical *Jak1* knock-in missense mutation (*Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice) that recapitulated key aspects of the human phenotype. RNA sequencing of samples isolated from the *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice revealed the upregulation of genes associated with the hyperactivation of tyrosine kinases and NF-κB signaling. Interestingly, there was a strong correlation between genes downregulated in *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice and those downregulated in the brain of model mice with 22q11.2 deletion syndrome that showed cognitive and behavioral deficits, such as autism spectrum disorders. Our findings expand the phenotypic spectrum of *JAK1*-associated disease and underscore how JAK1 dysfunction contributes to this autoinflammatory disorder.

Keywords: inflammation, brain, liver, skin, STAT

Abbreviations: AD, atopic dermatitis; AiKD, autoinflammatory keratinization disease; JAK1, Janus kinase 1; STAT, signal transducers and activator of transcription.

INTRODUCTION

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway plays an integral role in the regulation of inflammatory processes by relaying responses between surface receptors and cytokines such as interferons (IFNs) and interleukins (ILs) (1). The binding of ligands to their cognate receptors leads to the activation of JAKs, which subsequently phosphorylate each other and associated receptors. This interaction activates STAT proteins, which can trigger downstream signaling axes or can function as transcription factors themselves (2). JAK1 is a ubiquitous tyrosine kinase that is crucial for signaling by cytokines such as IFNα/β, IFNγ, IL-2, IL-6, and IL-10 (2).

Somatic gain-of-function mutations in *JAK1* have been identified in malignancies such as acute lymphoblastic leukemia, acute myeloid leukemia, and solid-organ cancers (3–5). More recently, heterozygous mutations in *JAK1* that result in JAK-STAT hyperactivity have been implicated in an autosomal dominant disorder that features multi-organ immune dysregulation (MIM 618999) (6, 7). In this study, we identify a further previously unreported heterozygous missense mutation in *JAK1* in an individual with inflammatory skin changes of a unique autoinflammatory keratinization disease (AiKD) associated with early-onset liver dysfunction and autism. Based on supporting analyses using patient samples and a knock-in (KI) mouse model generated by CRISPR-Cas9 editing, we present further evidence that activating mutations in *JAK1* are responsible for this systemic autoinflammatory phenotype.

MATERIALS AND METHODS

Whole-Exome Sequencing

Blood samples from the patient and her parents were obtained for genetic analysis in accordance with the Declaration of Helsinki. Following informed consent, genomic DNA from the proband was used for whole-exome sequencing. Exome capture was performed by in-solution hybridization using SureSelect Human All Exon V6 bait (Agilent Technologies, Santa Clara, CA, USA). Massively parallel sequencing was performed with the Illumina HiSeq2500 platform with 150-bp paired end-reads (Illumina, San Diego, CA). The reads produced were aligned to the hg19 reference human genome using Burrows-Wheeler Aligner software with default parameters and a –mem option (8). PCR duplicates were removed using MarkDuplicates in Picard tools (https://broadinstitute.github.io/picard/). Candidate variants were called using VarScan2 (http://massgenomics.org/varscan) and were annotated using ANNOVAR (http://annovar.openbioinformatics.org/). Common variants defined by >1% minor allele frequency in ExAC (http://exac.broadinstitute.org/), 1000 genomes (http://www.1000genomes.org/), or ESP6500 (http://evs.gs.washington.edu/EVS/) were excluded from analysis.

Generation of the *Jak1* Knock-in Mice

C57BL/6J mice was purchased from Japan SLC (Hamamatsu, Japan). All mice were fed a commercial CE-2 diet (CREA Japan, Tokyo) and had *ad libitum* access to water. The mice were bred in a pathogen-free facility at the Institute for Laboratory Animal Research, Graduate School of Medicine, Nagoya University, and maintained under a controlled temperature of 23 ± 1°C, a humidity of 55 ± 10%, and a light cycle of 12-hour light (from 09:00 to 21:00)/12-hour dark (from 21:00 to 09:00). Animal care and all experimental procedures were approved by the Animal Experiment Committee, Graduate School of Medicine, Nagoya University, and were conducted according to the Regulations on Animal Experiments of Nagoya University.

Targeted disruption of the *Jak1* gene on a C57BL/6J background was carried out using the CRISPR/Cas9 method as previously described (9). CRISPR RNA (crRNA, 5'- CAA GAA CAC ATA TCT ATT CT-3') targeting exon 13 was designed using the CRISPOR website (10). The designed crRNA and trans-activating crRNA (tracrRNA) (Genome CraftType CT, FASMAC, Kanagawa, Japan) and Cas9 protein (New England Biolabs, Tokyo, Japan) were mixed and incubated at 37 °C for 20 min to form a ribonucleoprotein complex (RNP). The ssODN (5'- cct tcc tca gGG TGA GCA CCT TGG CAG AGG CAC AAG AAC AGA TAT ATA CTC TGG GAC CCT GCT GGA CTA CAA GGA TGA GGA AGG AAT TG-3') was designed to include silent (synonymous) c.1788C>A and c.1791T>C mutations to avoid re-cleavage by Cas9, and the target c.1783C>G mutation was obtained from FASMAC. The final concentrations of components in RNP preparation with ssODN were 8 μM guide RNA (crRNA + tracrRNA), 200 ng/μl Cas9 protein, and 250 ng/μl ssODN. The mixture was electroporated into zygotes using a NEPA 21 electroporator (NEPA GENE Co. Ltd., Chiba, Japan) and the embryos were transferred into the oviductal ampulla of pseudo-pregnant ICR mice.

For sequencing and genotyping, genomic DNA was extracted using KAPA Express Extract (Kapa Biosystems, Woburn, MA) from the pinna and tail of the offspring and were used for PCR amplification. The region targeted by the Cas9 nuclease was amplified by using a GoTaq Green Master mix (Promega, Madison, WI, USA) and a primer pair (5'- CAG GTT TGT GAT AGA CTG CAG CTG-3' and 5'- CAT CAT TCT CCC CTC ACT ACT CCC-3'). Mutations in the *Jak1* gene in offspring were confirmed by Sanger sequencing of the PCR products using Eurofins DNA sequence service (Eurofins Genomics, Tokyo, Japan). Potential off-target cleavage sites predicted by the CRISPOR website (**Supplemental Table 1**, the five regions with the highest Mit off-target scores) were sequenced and no mutations were detected in these sites.

Cell Culture and Transfection

The HEK293 cell line (JCRB9068, Graham, F.L. established) was obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). *JAK1* complementary DNA carried on the pFN21A vector (Halotag ORF Clone FHC01306) was purchased from the Kazusa DNA Research Institute (Chiba, Japan), and the *JAK1* mutation c.1786C>G;p.H596D was introduced to FHC01306 by Promega Japan (Tokyo, Japan). The HEK293 cells were cultured in DMEM containing 1.8 mM calcium supplemented with 10% fetal bovine serum at 37°C with 5% CO_2. For the transfections, the HEK293 cells were cultured in

12-well dishes and then transfected with wild-type *JAK1*, mutant *JAK1* (H596D), or HaloTag Control Vector plasmids using Screen Fect A plus transfection reagent (FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan) according to the manufacturer's protocol. Cells were cultured for 24 or 48 hours after being transfected with the indicated plasmids and were collected for Western blotting analysis.

Immunohistochemistry

Immunohistochemical analysis of skin samples from the participants and mice was performed as described previously (11), with slight modifications. Thin sections (3 μm) were cut from samples embedded in paraffin blocks. The sections were soaked for 20 min at room temperature in 0.3% H_2O_2/methanol to block endogenous peroxidase activity. After washing in PBS with 0.01% Triton X-100, the sections were incubated for 30 min in PBS with 4% BSA followed by an overnight incubation with the primary antibodies in PBS containing 1% BSA according to the manufacturer's instructions. After washing in PBS, the thin sections were stained with the corresponding secondary antibodies for 1 hour at room temperature and washed in PBS. The Vectastain Elite ABC-PO kit (Vector Laboratories, Burlingame, CA) was used for staining. The following polyclonal antibodies were purchased from commercial sources: anti-p-JAK1 [phospho-Tyr1022, anti-p-Jak1 (Tyr1021), Sigma Aldrich, St Louis, MO), anti-p-STAT1 (phospho-Tyr701, anti-p-Stat1 (Tyr701)] (ab30645; Abcam, Cambridge, UK), anti-p-STAT3 (phospho-Tyr705, anti-p-Stat3 (Tyr705) (#11045; Signalway Antibody, College Park, MD). The following monoclonal antibodies were purchased from commercial sources: anti-p-STAT5 [phospho-Tyr694, anti-p-Stat5 (Tyr694)] (ab32364; Abcam), anti-p-STAT6 [phospho-Tyr641, anti-p-Stat6 (Tyr641)] (ab263947; Abcam). n=3. Each experiment was performed twice.

Western Blotting

Zirconia balls were added to the proteins extracted from the liver of P0 newborns. Then, the proteins were dissolved in 1 ml sample buffer (NuPAGE LDS sample buffer 250μL, sample reducing agent 100μL, 25×protease inhibiter 40μL, and water 610μL) and crushed. After centrifugation at 10,000 rpm for 10 min at 4°C, the supernatant of each sample was subjected to SDS-PAGE. Strips of membrane were incubated with anti-p-JAK1 (Tyr1021), anti-JAK1 (ab125051; Abcam), anti-p-STAT1 (Tyr701), anti-STAT1 (ab99415; Abcam), anti-p-STAT3 (Tyr705), anti-STAT3 (SAB4300708, Sigma Aldrich), anti-p-STAT5 (Tyr694), anti-STAT5 (ab16276; Abcam), anti-p-STAT6 (Tyr641), anti-STAT6 (ab32520; Abcam) or anti-GAPDH (ab9485; Abcam) antibodies. The antibody–antigen complexes were detected with horseradish peroxidase–conjugated goat anti-rabbit IgG (Dako, Glostrup, Denmark) at a dilution of 1:1,000, followed by detection with enhanced chemiluminescence Western blotting substrate (GE Healthcare BioSciences, Little Chalfont, UK), as described by the manufacturer. n=3. Each experiment was performed three times. For HEK293 cell lysates, additional anti-HaloTag monoclonal antibody (G9211; Promega Corporation, WI) and anti-JAK1 rabbit monoclonal antibody (#3344; Cell Signaling Technology, MA) were used as the primary antibodies.

RNA Sequencing

Total RNA extracted from the brain, liver, and skin of P0 newborns was purified using the RNeasy Mini Kit (QIAGEN, Hiden Germany). Two *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice and five WT mice were analyzed. The quality of RNA was assessed with a 2100 Bioanalyzer (Agilent Technologies). The skin samples (n=7) had an average RNA integrity number (RIN) values of 6.48 (5.8 - 7.1). The blood and liver samples (n=7 each) had an average RIN value of 9.63 (9.2 - 10). RNA sequencing was performed by the Macrogen Japan Corp. using the TruSeq RNA Library Prep Kit v2 (Tokyo, Japan). Next-generation sequencing was performed using the Illumina Novaseq 6000 platform to obtain 101-bp paired-end reads. The reads were adapter-trimmed using our in-house script and were aligned to the mm9 reference genome using TopHat2 (https://ccb.jhu.edu/software/tophat/). Reads Per Kilobase of transcript, per Million mapped reads (RPKM) were calculated using GFOLD (12). Changes in expression levels between groups were estimated using GFOLD, and the resulting GFOLD (0.1) values (conservative estimation of fold changes at the confidence level of q = 0.1) were used for gene set enrichment analyses (GSEA) (13, 14). Volcano plots were generated using log2-fold change values and adjusted p values calculated by DESeq2 (15).

RESULTS

A *De Novo* Mutation in *JAK1* in a Patient With AiKD With Hepatitis and Autism

Our proband was a 22-year-old Japanese female who was the older of two siblings born to non-consanguineous parents with no significant family history (**Figure 1A**). She was delivered at 38 weeks of gestation by spontaneous vaginal delivery with a birth weight of 2718g. From birth, she was noted to have dry skin with mild erythema and was diagnosed with atopic dermatitis (AD) at the age of 2 months. At around the same time, she was found to have hepatosplenomegaly. Other clinical manifestations noted included low height and body weight, moderate motor impairment and learning disability, hyperlipidemia, and autism. She was treated with a growth hormone, although it was ineffective. She was provisionally diagnosed with glycogen storage disease type IV, and at 3 years of age, she received a living donor liver transplant due to severe liver failure. At 8 years of age, she developed erythematous cheeks and ichthyotic erythema on her trunk (**Figures 1B, C** and **S1**) and extremities, with a SCORing Atopic Dermatitis (SCORAD) score of 70.8%. She was treated with narrow-band UVB therapy, but it led to only minimal improvement. She had eosinophilia and elevated serum thymus and activation-regulated chemokine (TARC; 4,092 pg/mL (normal range, <450 pg/mL)) and IgE with high titers for various antigens (**Supplemental Table 2**). She died of unknown cause at the age of 22 years.

At 7 months of age, a skin biopsy specimen from the affected skin showed compact hyperkeratosis with normal-appearing granular layers, acanthosis, spongiosis, and lymphocytic infiltration from the upper dermis to the granular layers (**Figure 1D**). Additionally, excised liver tissue demonstrated

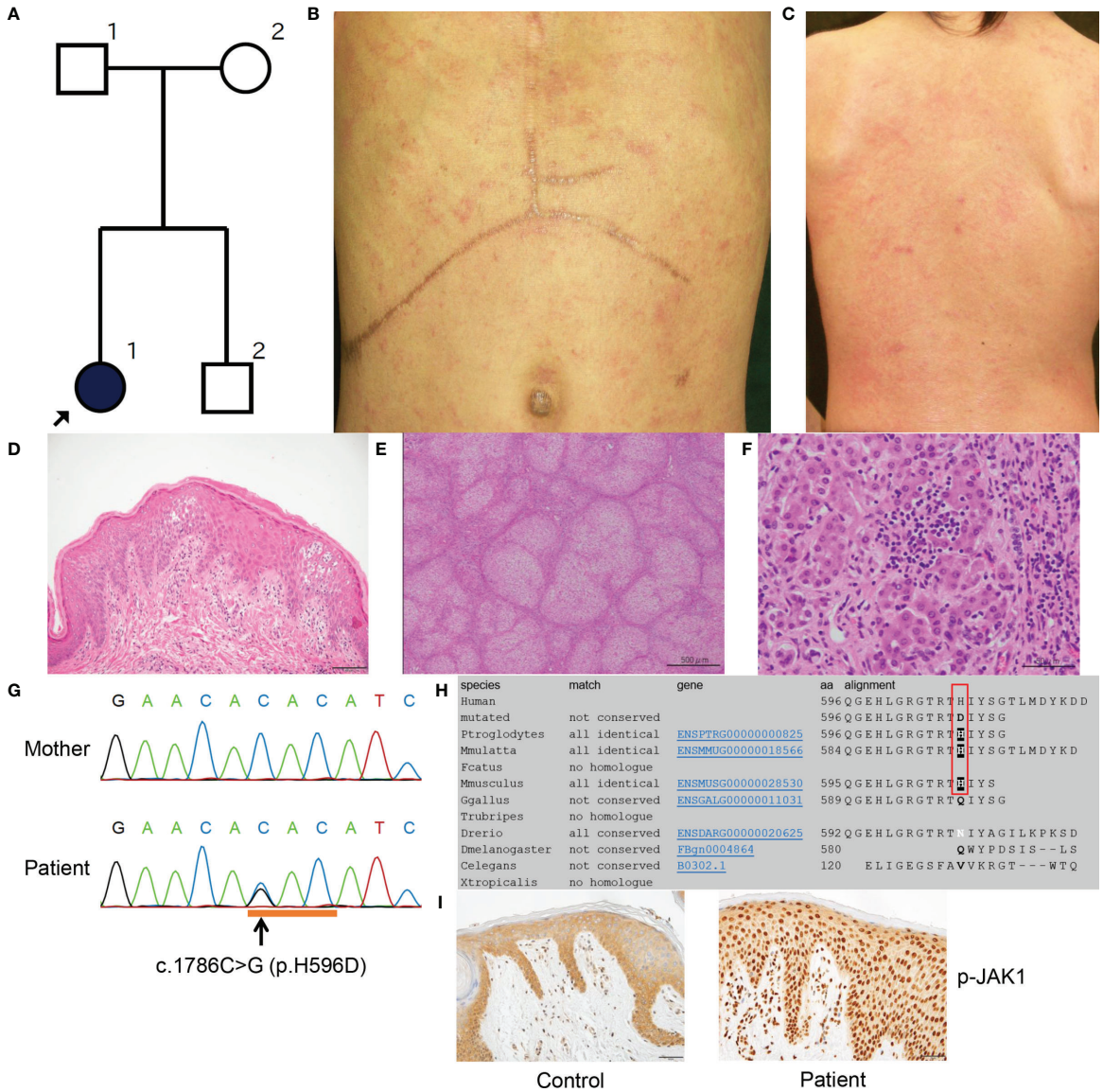

FIGURE 1 | Clinicopathologic features of the present patient with a *JAK1* mutation. **(A)** Family tree of the present pedigree. **(B, C)** Scratched erythema and small red papules are seen on the trunk **(B)** and the back **(C)**. **(D)** A biopsy sample from erythematous skin of the proband shows compact hyperkeratosis, moderate acanthosis, mild spongiosis and lymphocytic infiltration within the epidermis and the upper dermis. Scale bar = 100 μm. **(E)** Histologically, the structure of the liver parenchyma has been remodelled into a nodule by fibrosis, resulting in cirrhosis. Scale bar = 500 μm. **(F)** Inflammatory cell infiltration mainly composed of small lymphocytes (a few plasma cells) is observed in the nodule, accompanied by a mild necrotic inflammatory reaction with hepatocyte shedding. Scale bar = 50 μm. **(G)** Sanger sequencing reveals the heterozygous missense mutation c.1786C>G (H596D) in *JAK1*. **(H)** *JAK1* amino-acid sequence alignment shows the level of conservation in diverse species of the amino-acid p.H596 (red box), which was altered by the missense mutation in the present patient. **(I)** The epidermis in an affected lesion from the patient and normal skin from a healthy donor were stained with anti-p-JAK1 antibody. Scale bars = 50 μm. MutationTaster (http://www.mutationtaster.org/).

liver cirrhosis with remodeling of the nodular structure due to fibrosis (**Figure 1E**). Most hepatocytes were brightened or vacuolar due to presumed lipid or glycogen deposition. Although the inflammatory cell infiltrates mainly consisted of lymphocytes (with a minority of plasma cells) and were located principally within fibrotic lesions, they were also observed in hepatocyte nodules and sinusoids, and they caused a mild necrotic inflammatory reaction accompanied by hepatocyte shedding (**Figure 1F**).

Following institutional ethical approval, informed written consent was obtained in compliance with the Declaration of Helsinki guidelines. We started by searching for mutations in *ABHD5* (αβ-hydrolase domain-containing 5), the gene implicated in Chanarin–Dorfman syndrome (MIM 275630). Sanger

sequencing of *ABHD5* revealed no mutations in the genomic DNA of the patient. Moreover, intra-cytoplasmic lipid droplets within venous neutrophils were not observed. Whole-exome capture was then performed (using peripheral blood genomic DNA isolated from the patient and both parents) by in-solution hybridization using SureSelect All Exon 50 Mb Version 5.0 (Agilent, Santa Clara, CA, U.S.A.) followed by massively parallel sequencing (HiSeq2500; Illumina, San Diego, CA, U.S.A.) with 150-bp paired-end reads. After filtering for low frequency variants (frequency of less than 0.1% in the ExAC, 1000 genomes and ESP6500 databases) and against the parental exome dataset, 53 *de novo* heterozygous variants were identified (**Supplemental Table 3**). Amongst these variants, a nonsynonymous heterozygous mutation was identified in *JAK1* (c.1786C>G; H596D), which was validated by Sanger sequencing (**Figure 1G**). This mutation had not been described in our in-house database of 777 Japanese exomes, nor in the gnomAD Database (16), which includes data for 125,748 whole exomes and 15,708 whole- genomes. *In silico* analysis with MutationTaster (17) and SIFT (http://sift.jcvi.org/) predicted this variant to be 'damaging'. Results from other predictive tools included a Combined Annotation Dependent Depletion (CADD) score of 15.27 and Genomic Evolutionary Rate Profiling (GERP) score of 4.09 (highly conserved); thus, the mutation is thought to be functionally relevant. The histidine residue at codon 596 of JAK1 is conserved among three diverse species: *P. troglodytes*, *M. mulatta* and *M. musculus* (**Figure 1H**). The variant allele frequency of H596D mutation in the patient's peripheral blood mononuclear cells was 0.421053. As such, we cannot determine if this mutation is a germline or somatic mutation. We did not identify potentially pathogenic mutations in other genes implicated in autoinflammatory disease, ichthyosis, or glycogen storage disorders.

Strong Nuclear Staining of STAT Family Members in the Epidermis of the AiKD Patient With the *JAK1* Mutation

We conducted immunohistochemical analyses of phosphorylated-JAK1 (p-JAK1), -STAT1 (p-STAT1), -STAT3 (p-STAT3), -STAT5 (p-STAT5) and -STAT6 (p-STAT6) in lesional skin from the patient. The patient's epidermis showed strong nucleocytoplasmic JAK1 expression, in contrast to the mostly cytoplasmic staining of normal skin (**Figure 1I**). Epidermal cytoplasmic p-STAT1 expression with focal nucleocytoplasmic localization was also seen in the skin from the patient (**Figures S2A, B**). In addition, p-STAT3, p-STAT5 and p-STAT6 were strongly expressed in the nuclei of keratinocytes in the patient (**Figures S2D, F, H**) compared with predominantly cytoplasmic staining in normal control skin samples (**Figures S2C, E, G**).

Jak1 Knock-in (*Jak1*$^{H595D/+;I596I/+;Y597Y/+}$) Mice Recapitulate Aspects of Human AiKD With Hepatitis due to *JAK1* Mutation

To gain deeper insights into the role of JAK1 hyperactivity in the autoinflammatory pathogenesis *in vivo*, we used a CRISPR-Cas9 gene-targeting approach to generate KI mice. We initially attempted to generate mice harboring only the c.1783C>G (H595D) substitution (*Jak1*$^{H595D/+}$), which is identical to that found in the patient. However, we could not design a high-quality guide RNA for CRISPR to target the conserved histidine residue. To avoid re-cutting by Cas9, we introduced two additional synonymous variants at the following amino acids: c.1788C>A (I596I) and c.1791T>C (Y597Y) (**Figure 2G**). Furthermore, we utilized the *Jak1*KI-mosaic male mouse born by chance because heterozygous *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice (H595D, I596I, Y597Y) could not survive longer than 4 weeks. Mosaic-*Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ male mouse manifested a milder phenotype than *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice. The majority of somatic cells in the mosaic-*Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice harbored only wild-type (WT) alleles. The mosaic-*Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ male mouse survived for more than 1 year and were able to produce offspring including heterozygous *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice (**Supplemental Table 4**).

Jak1$^{H595D/+;I596I/+;Y597Y/+}$ mice seemed to have small body sizes and low weights at birth. From a few days after birth, these mice showed hyperkeratosis and scales on the ears, feet, and tail (**Figures 2A, B**). KI mice with only the two introduced synonymous heterozygous mutations (*Jak1*$^{I596I/+;Y597Y/+}$) did not show any abnormal phenotypes and survived as long as WT mice. Of note, the survival rate was significantly lower for *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice than for WT and *Jak1*$^{I596I/+;Y597Y/+}$ mice (**Figure 2H**). We incidentally generated two types of heterozygous *Jak1* mice carrying null alleles caused by frame-shift mutations (c.1792_1793insT and c.1790_1794del5; *Jak1*$^{+/-}$). Both types of heterozygous *Jak1*$^{+/-}$ mice exhibited no cutaneous phenotypes.

As expected, *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice exhibited moderate thickening of the stratum corneum, a reduced number of keratohyalin granules in the uppermost stratum granulosum, and epidermal hyperplasia (**Figures 2C, D** and **S3B**). Additionally, lymphocytic infiltration was seen in the liver of *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice (**Figures 2F** and **S3D**), but not in the liver of WT mice (**Figures 2E** and **S3C**). These findings are consistent with the histological features of the skin and liver samples from our AiKD patient with the *JAK1* mutation (**Figures 1E, F**).

Phosphorylation of JAK1 and STAT Family Members Also Seen in *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ Mice

Stronger nucleocytoplasmic staining of p-JAK1 was observed in the palmar skin of *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice than in the palmar skin of WT mice (**Figure 2I**). p-STAT3 and p-STAT6 were also detected in the nucleus (**Figures S3E-H**). We also performed Western blot analyses to determine whether the phosphorylation of JAK1 and STAT were increased in the liver tissue of *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice. JAK1, STAT1, STAT3, STAT5, and STAT6 were more highly phosphorylated in the liver cells of *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice compared to WT mice (**Figure S3I**).

Gene Expression Profile in the Brain, the Liver, and the Skin of *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ Mice

To compare global gene expression profiles, RNA sequencing was performed using extracted RNA from the brain, liver, and skin of

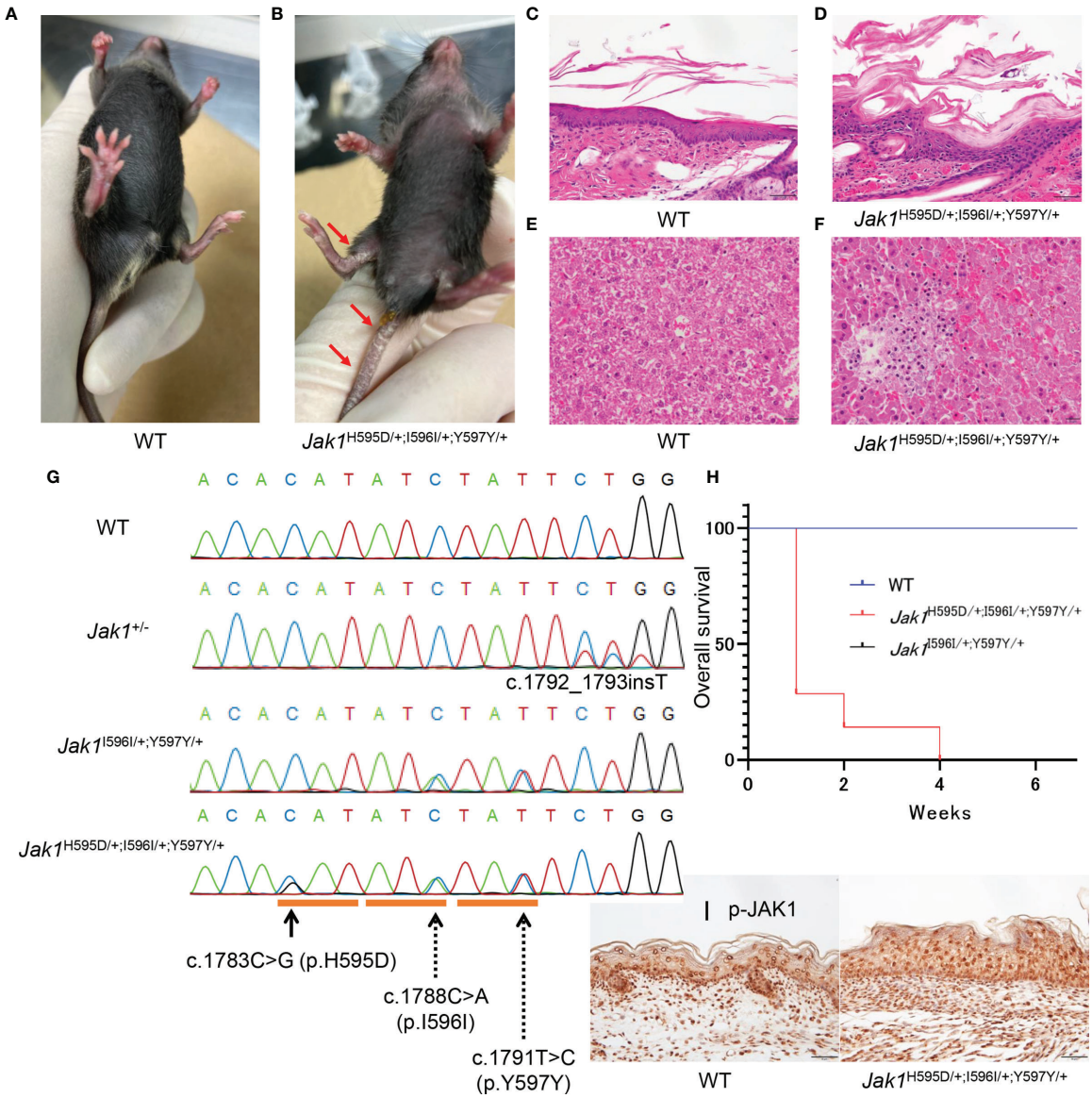

FIGURE 2 | Phenotypic features in *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice. **(A, B)** The gross appearance of WT **(A)** and *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ **(B)** at 4 weeks of age. The *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mouse shows scaling on the ears, the extremities, and the tail. **(C, D)** Hematoxylin and eosin staining of the skin reveals acanthosis, hyperkeratosis and parakeratosis in the *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mouse **(D)**, but not in the WT mouse **(C)**. Scale bars = 50 μm. (n = 3) **(E, F)** Histology of liver sections from the *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice **(F)** and the WT mice **(E)** stained with hematoxylin and eosin. Scale bars = 50 μm. (n = 3) **(G)** Sequence data of *Jak1* around the mutations in the *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$, *Jak1*$^{I596I/+;Y597Y/+}$ and *Jak1*$^{+/-}$ mice. The black line indicates c.1783C>G (H595D). The dotted lines indicate two synonymous changes: c.1788C>A (I596I) and c.1791T>C (Y597Y). **(H)** A graph of the overall survival rate (*Jak1*$^{H595D/+;I596I/+;Y597Y/+}$: n = 7, WT: n = 10, *Jak1*$^{I596I/+;Y597Y/+}$: n=10). Log-rank test, WT vs *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice: p value < 0.0001, *Jak1*$^{I596I/+;Y597Y/+}$ vs *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice: p value < 0.0001. **(I)** Immunohistochemical analysis by using the anti-p-JAK1 antibody for the palmar skin of the *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice and the WT mice. Scale bars = 50 μm. (n = 3).

newborn *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice and WT mice (**Figure 3** and **Tables S5-S7**). We performed a gene set enrichment analysis using the hallmark gene set database. In all three samples (brain, liver, and skin) of *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice, genes associated with the hyperactivation of tyrosine kinases (**Figure 3A** and **Figure S4A**) and with the activation of NF-κB signaling (downstream of tyrosine kinases) were upregulated (**Figure 3B** and **Figure S4B**). Brain samples also showed the upregulation of genes associated with the extracellular matrix and the downregulation of genes associated with oxidative phosphorylation, metabolism of lipid-associated genes, and haptotaxis-associated genes (**Figure S4C**). Intriguingly, the genes downregulated in *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice showed a very strong correlation with those downregulated in the brain of the

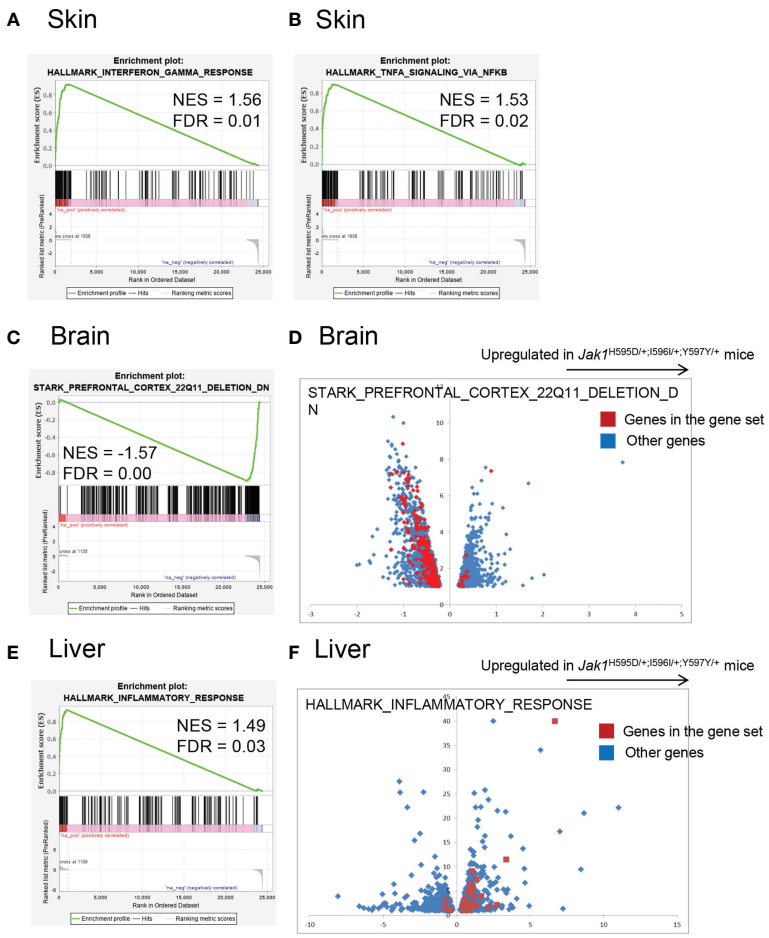

FIGURE 3 | Global gene expression profiling of the RNA samples in *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice. Gene set enrichment analysis using the hallmark gene set database. **(A, B)** Skin. Genes associated with HALLMARK_INTERFERON_GAMMA_RESPONSE **(A)** and HALLMARK_TNFA_SIGNALING_VIA_NFKB **(B)** are relatively upregulated in skin samples from the *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice. **(C, D)** Brain. Genes associated with STARK_PREFRONTAL_CORTEX_22Q11_DELETION_DN are downregulated in brain samples from the *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice. The red dots in **(D)** indicate genes in this gene set. Blue dots: the other genes. **(E, F)** Liver. Genes associated with HALLMARK_INTERFERON_GAMMA_RESPONSE are upregulated in liver samples from the *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice. The red dots in **(F)** indicate genes in this gene set. Blue dots: the other genes. NES, normalized enrichment score; FDR, false discovery rate.

model mice with 22q11.2 deletion syndrome (q<0.001) (**Figures 3C, D** and **Table S5**) (18). Liver tissue from the *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice also showed the upregulation of genes associated with inflammation, including IL-6 (**Figures 3E, F** and **Table S6**). In terms of STAT family genes, *Stat3* was upregulated in differentially expressed genes in the skin (**Table S7**), and *Stat4* was upregulated in differentially expressed genes in the liver (**Table S6**).

The enrichment of genes associated with interferon-gamma and tumor necrosis factor-alpha was similar among the three types of tissues. The enrichment of genes associated with 22q11 deletion was specific to the brain. The enrichment of genes associated with inflammation was prominent in the liver, while in the other two tissues the enrichment did not reach the statistical significance (q = 0.59 and 0.187 for the brain and the skin, respectively).

Gain of Function of H596D Mutant JAK1 in Patient *In Vitro*

Finally, to investigate whether JAK1-STAT pathways were upregulated due to H596D mutation in *JAK1*, we transfected the *JAK1* mutant (*JAK1*-H596D) into HEK293 cells and performed Western blotting analyses to evaluate the phosphorylation of JAK1 and STAT proteins. As shown in **Figure S5**, JAK1 was more highly phosphorylated in the *JAK1*-H596D-transfected HEK293 cells than in the wild-type *JAK1*-transfected cells. In addition, the *JAK1*-H596D-transfected cells showed higher phosphorylation for STAT1 (48h), STAT5 (24h and 48h), and STAT6 (48h) than wild-type *JAK1*-transfected cells did. We observed no significant difference in the STAT3 phosphorylation levels between wild type and mutant in the present experiments. The results for phosphorylation levels of

STAT1 were similar to those obtained in a transfection study of *JAK1* A634D in the previous report (6).

DISCUSSION

Our clinical observations and supportive findings from a KI mouse model implicate the H596D mutation in *JAK1* as crucial for chronic systemic inflammation. The proband's inflammatory skin phenotype was typical of autoinflammatory keratinization disease (AiKD) (19) and presented with extracutaneous features, including hepatitis and autism. *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice demonstrated evidence of inflammation involving the skin and the liver. Immunohistochemical analyses of skin samples from the patient and the KI mouse model suggest that the *JAK1* H596D mutation confers a gain-of-function effect leading to the hyperactivation of JAK-STAT signaling (**Figure 2B**). Accelerated phosphorylation of JAK1 and STATs seen in the 293 cells transfected with *JAK1* H596D mutation verifies the gain-of-function effects *in vivo* (**Figure S5**).

The amino acid residue altered by this mutation localizes to the pseudokinase domain of JAK1 (**Figure 4A**). The pseudokinase (or JAK homology-2, JH2) domain is critical for the regulation of the kinase (or JH1) domain of the JAK family proteins (20). *In vitro* analyses demonstrated that the deletion of the JH2 domain of Jak2 led to a dramatic increase in the level of kinase activity (21). The I597F mutation in JAK1, situated within the JAK1 JH2 ATP-binding site and in close proximity to our H596D variant, increased basal STAT5 activation and did not inhibit hyperactivation driven by JAK1-3 (20). Similarly, the H596D substitution may lead to upregulated JH1 kinase activity due to less efficient inhibition by the mutant pseudokinase domain.

Interestingly, the variants identified in three previously reported families with *JAK1* mutations are also situated within the pseudokinase domain, comprising two gain-of-function mutations and one loss-of-function mutation (6, 7, 22). The clinical features reported in the other patients with gain-of-function variants are similar to our proband, including skin inflammation, impairment of growth, and liver abnormalities, although there is already evidence of phenotypic heterogeneity among the patients with this autoinflammatory syndrome. Indeed, our patient had no significant gastrointestinal tract involvement. One previously reported individual developed

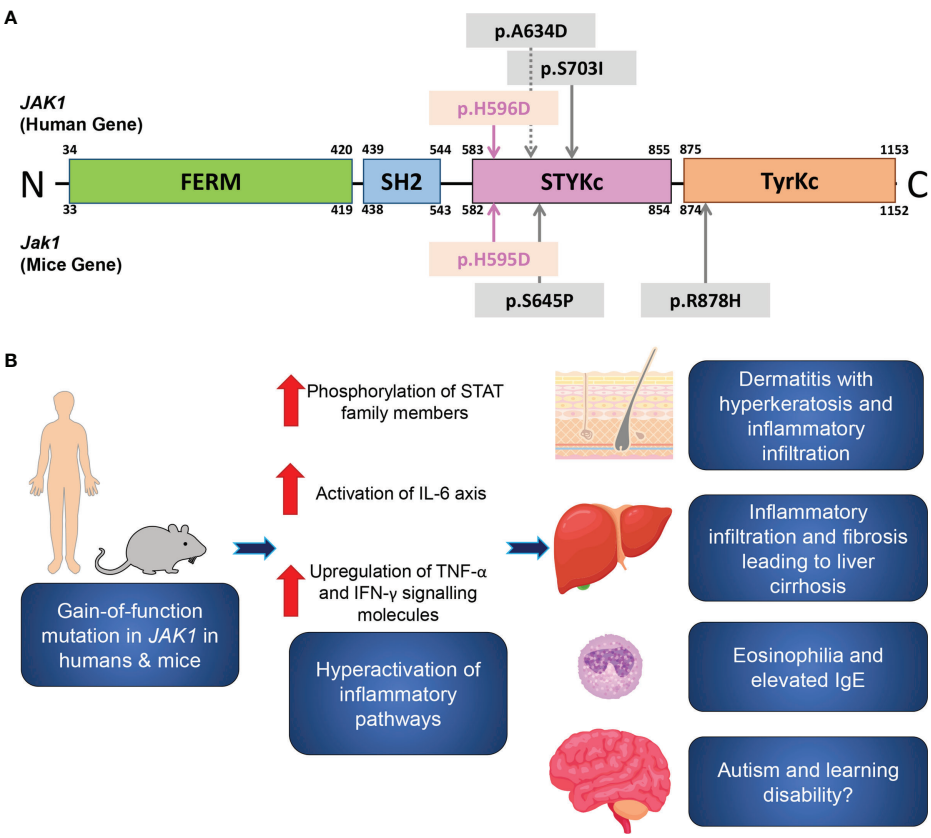

FIGURE 4 | A schematic of the JAK1 domain structure, and inflammatory pathways associated with JAK1 hyperactivation. **(A)** A schematic of the functional domains and the site of the reported gain-of-function germline mutations in humans (upper) and in mice (lower). The mutations in the present human case and in *Jak1*$^{H595D/+;I596I/+;Y597Y/+}$ mice are marked by pink arrows. Sites of the previously reported mutations are indicated by grey arrows. FERM, FERM (F for 4.1 protein, E for ezrin, R for radixin and M for moesin) domain; SH2, Src homology 2 domain; STYKc, the serine-threonine/tyrosine-protein kinase, catalytic domain (also called the pseudokinase domain); TyrKc, the tyrosine kinase catalytic domain. **(B)** Theory on the pathogenesis of the inflammatory pathways associated with JAK1 hyperactivation.

membranous nephropathy that recurred despite renal transplantation and eventually necessitated hemodialysis (7), although other patients with gain-of-function variants in *JAK1*, including the present case, did not develop any renal impairment. It is evident from these cases that JAK1 dysfunction is associated with multi-system involvement consistent with the widespread expression of JAK1 (2). Nevertheless, the precise phenotypic spectrum associated with gain-of-function *JAK1* mutations may become more defined as more cases are identified.

In contrast to our patient, the case reported by Eletto *et al.* was an immunodeficient patient with different pathobiology due to biallelic *JAK1* mutations (22). The patient harbored two homozygous missense mutations, P733L and P832S, also within the pseudokinase domain of *JAK1* and had recurrent atypical mycobacterial infections and early-onset metastatic bladder carcinoma (22). Cells from this patient showed reduced phosphorylation of JAK1 and STAT following cytokine stimulation, impaired induction of the expression of interferon-regulated genes, and dysregulated cytokine production, suggesting a probable loss of function of JAK1 (22). Collectively, these human data suggest that point mutations in the pseudokinase domain of JAK1 are critical to pathological processes, similarly to the well-known *JAK2* V617F mutation found in polycythaemia vera, myelofibrosis, and essential thrombocythemia (23).

Two published mouse models of increased JAK1 activity had similar features to the human cases, including an inflammatory skin phenotype (24, 25). Sabrautzki et al. (25) reported that an *N*-ethyl *N*-nitrosourea (ENU)-induced mutagenesis-derived mouse with a heterozygous missense mutation in the pseudokinase domain ($Jak1^{S645P/+}$) had reduced body size and weight at birth, inflammatory ear and skin lesions, and erythema and thickening of tails. Liver samples from $Jak1^{S645P/+}$ mice macroscopically demonstrated nodular regenerative hyperplasia with irregular margins, but without hepatomegaly or ascites (25). Histologically, there was sinusoidal dilatation, areas of hyperplastic hepatocytes surrounded by atrophic hepatocytes, prominent vessels and increased vascularization (25). Loss of megakaryocytes and an increase in Russell bodies in the spleen were also noticed (25). Skin histology revealed hyperkeratosis and acanthosis of the epidermis with predominantly neutrophilic infiltration, and immunohistochemical staining showed the activation of the IL-6-gp130-JAK-STAT axis in skin lesions (25). Similarly, our findings also support the involvement of IL-6 in the disease process. Specifically, liver samples from *Jak1*KI mice showed significantly increased mRNA levels of IL-6 and IL-6-related inflammatory pathway molecules (**Figures 3E, F**).

Yasuda et al. (24) reported ENU-induced mutagenized mice carrying the homozygous R878H mutation, termed *Spade* (stepwise, progressive atopic dermatitis). $Jak1^{Spade/Spade}$ exhibited redness and desquamation of the ears, and skin lesions showed epidermal hyperplasia and infiltration of mononuclear inflammatory cells including mast cells, eosinophils, and $CD4^+$ T cells (24). Immunological abnormalities (elevated IgE, IgG1, histamine, IgG2b and IgG2c) developed in a stepwise manner (24). Moreover, $Jak1^{Spade/Spade}$ demonstrated defective skin barrier function that was postulated to be due to an overexpression of serine proteases such as kallikrein-6 and marapsin. Treatment with petrolatum delayed the onset and reduced the severity of the skin lesions (24).

The global mRNA expression profiling in our study identified a strong correlation between the downregulated genes in the brain tissue of $Jak1^{H595D/+;I596I/+;Y597Y/+}$ mice and those of the model mice with 22q11.2 deletion syndrome (**Figures 3C, D**). 22q11.2 microdeletions are associated with cognitive and behavioral deficits, including autism spectrum disorders (18, 26). Our patient had autism and a moderate learning disability, but these characteristics were not present in the other two reports of individuals with gain-of-function *JAK1* mutations. The sole patient with homozygous loss-of-function *JAK1* mutations did have mild developmental delay (22).

22q11.2 deletion syndrome exhibits significant phenotypic heterogeneity with a wide range of potential features, including congenital cardiac disease, palatal defects, endocrine dysfunction (hypocalcemia, thyroid disease, growth hormone deficiency), autoimmune disease, immunodeficiency, skeletal abnormalities and renal anomalies (27, 28). There are more than 40 protein-coding genes located within the 22q11.2 region (29). Of these, *TBX1* (T-box transcription factor 1) has emerged as a functionally important candidate, as heterozygous mutations in this gene have been found in patients with clinical features resembling 22q11.2 deletion syndrome (30, 31). Another potential gene of interest is *CRKL* (CRK like proto-oncogene, adaptor protein), which is implicated in the development of the heart, aortic arch, parathyroid and thymus glands (32, 33). Interestingly, mice harboring an allele with both *Tbx1* and *Crkl* inactivated recapitulated aspects of the 22q11.2 deletion syndrome phenotype (33).

With regards to the neurobehavioral features seen in 22q11.2 deletion syndrome, the roles of *COMT* (catechol-O-methyltransferase) and *PRODH* (proline dehydrogenase) have been investigated, given their relevance to dopaminergic and glutamatergic neurotransmission, respectively. However, at present there is insufficient evidence to assuredly link these genes functionally to the cognitive and behavioural symptoms of 22q11.2 deletion syndrome (34–37).

There is a growing body of evidence implicating neuro-inflammation in both autism and 22q11.2 deletion syndrome. Individuals with both conditions have elevated pro-inflammatory cytokines, and IL-6 in particular appears to play a substantial role (38, 39). Elevated levels of IL-6 correlate with the extent of cognitive deficit and psychosis in 22q11.2 deletion syndrome (38). An *in vitro* investigation of mouse cerebellar granule cells demonstrated that the overexpression of IL-6 perturbs cellular adhesion and migration, and leads to an imbalance between excitatory and inhibitory circuits (40). The upregulation of JAK-STAT signaling has also been noted in autistic children (41), but there is insufficient evidence at present to establish reliable causal links between aberrant IL-6, JAK1 dysfunctions and autism from this data. However, further investigation is warranted to elucidate the precise pathomechanisms by which JAK1 hyperactivation may lead to neurobehavioral abnormalities.

A key goal of genomic diagnostics is to help identify potential options for targeted therapeutics based on the underlying molecular defects and associated pathways. In the present case, a rational hypothesis is that JAK inhibitors could be an effective treatment for these patients with JAK-STAT hyperactivity. Indeed, both Del Bel *et al.* and Gruber *et al.* noted the amelioration of their patients' symptoms following treatment with JAK inhibitors (6, 7). The two patients reported by Del Bel *et al.* were treated with ruxolitinib (a JAK1/2 inhibitor), and they demonstrated improvements in pruritus, appetite loss, sleep disturbance and eosinophilia, and achieved weight gain, as well as resolution of skin lesions and hepatosplenomegaly (6). Based on evidence that tofacitinib (a JAK1/2/3 inhibitor) rescues STAT hyperphosphorylation *in vitro*, the individual described by Gruber *et al.* was treated with tofacitinib, resulting in the resolution of her dermatitis and gastrointestinal symptoms (7). JAK inhibitors are increasingly being used to treat autoimmune conditions, including rheumatoid arthritis, inflammatory bowel disease, and psoriasis vulgaris (2). Selective JAK1 inhibitors such as upadacitinib and abrocitinib are also being evaluated in clinical trials for AD (42). These recent developments suggest that JAK inhibitors may be a potent treatment option for controlling the systemic inflammation in our patient.

In summary, we report an individual with a heterozygous gain-of-function mutation in *JAK1*, with consequent hyperactivation of JAK-STAT signaling pathways leading to a syndrome of multi-organ inflammation (**Figure 4B**): AiKD with hepatitis and autism. Our clinicopathologic and functional data from the present patient and the corresponding mouse model support findings from recent reports, with new insights into the significance of the JAK-STAT signaling pathway in human health and disease.

DATA AVAILABILITY STATEMENT

The NGS data can be accessed at the GEO repository under the accession numbers GSE163100.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of the Nagoya University Graduate School of Medicine. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Animal Experiment Committee, Graduate School of Medicine, Nagoya University. 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

Research design: TT and MA. Experiments: TT, JL, YO, YukM, KT, and KS. Data acquisition: YO, YukM. Data analysis: TT, JL, YO, YuyM, TY, and KT. Collection of clinical samples and information: TT, KI, EN, TOk, and KS. Manuscript writing: TT and JL. Writing assistance: YoM, TOh, JM, and MA. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.737747/full#supplementary-material

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The Potential Role of Ferroptosis in Systemic Lupus Erythematosus

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Systemic lupus erythematosus (SLE) is an autoimmune disease that is accompanied with autoantibody production and inflammation. Other features of SLE pathogenesis include iron accumulation, oxidative stress, and lipid peroxidation, which are also major biochemical characteristics of ferroptosis, a novel non-apoptotic regulated form of cell death. To date, ferroptosis has been demonstrated to be an important driver of lupus progression, and several ferroptosis inhibitors have therapeutic effect in lupus-prone mice. Given the emerging link between ferroptosis and SLE, it can be postulated that ferroptosis is an integral component in the vicious cycle of immune dysfunction, inflammation, and tissue damage in SLE pathogenesis. In this review, we summarize the potential links between ferroptosis and SLE, with the aim of elucidating the underlying pathogenic mechanism of ferroptosis in lupus, and providing a new promising therapeutic strategy for SLE.

Keywords: systemic lupus erythematosus, ferroptosis, autoimmunity, immunity, inflammation

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INTRODUCTION

Systemic lupus erythematosus (SLE), an autoimmune disease, is characterized by autoantibody production, persistent inflammation, and multiple tissue damage. This condition is induced by accumulation of cell remnants from various cell death pathways (1). Ferroptosis, a regulated necrosis process driven by iron-dependent lipid peroxidation, was first coined by Dixon et al. in 2012 (2, 3). Ferroptosis has been associated with various physiological and pathological processes, including autoimmunity [e.g., multiple sclerosis (4)], cutaneous diseases [e.g., melanoma (5, 6)] and skin wounds (7). Li et al. reported that neutrophil ferroptosis contributes to neutropenia and disease manifestations in SLE (8). The study by Li et al. is the first and only one to directly associate ferroptosis

Abbreviations: SLE, Systemic lupus erythematosus; ROS, reactive oxygen species; LOXs, lipoxygenases; DFO, deferoxamine; LN, lupus nephritis; NGAL, neutrophil gelatinase-associated lipocalin; MDA, malondialdehyde; HNE, hydroxynonenal; CD, conjugated dienes; GSH, glutathione; GPX4, glutathione peroxidase 4; AMPK, AMP-activated protein kinase; CoQ10, coenzyme Q10; ATP, adenosine triphosphate; PBMCs, peripheral blood mononuclear cells; NK cells, natural killer cells; iNOS, inducible nitric oxide synthase; NETs, neutrophil extracellular traps; pDCs, plasmacytoid dendritic cells, IFN, interferon, DAMPs, damage-associated molecular patterns; PRRs, pattern recognition receptors; HMGB1, high-mobility group box 1, AGER, advanced glycosylation end-product specific receptor; UV, ultraviolet; COXs, cyclooxygenases; LDL, low-density lipoprotein; RTEC, renal tubular epithelial cells; oxLDL, oxidized low-density lipoprotein; HDL, high-density lipoprotein; FSP1, ferroptosis suppressor protein 1; GCH1, GTP cyclohydrolase-1; BH4, tetrahydrobiopterin; Se, selenium; TfR, transferrin receptor; PUFA, polyunsaturated fatty acid; PL-PUFA, phospholipid containing polyunsaturated fatty acid chain; IPP, isopentenyl- pyrophosphate.

with lupus. Based on evidence from the existing limited number studies, we postulate that ferroptosis is a missing link in the vicious cycle of immune dysfunction, inflammation, and clinical manifestations in lupus. In this review, we elucidate on the significance of ferroptosis in lupus and how it may lead to inflammation and clinical manifestations.

THE ROLE OF IRON AND ROS IN FERROPTOSIS AND SLE

Ferroptosis, a non-apoptotic form of cell death, is characterized by two major biochemical characteristics: iron accumulation and lipid peroxidation (9). Iron can directly generate reactive oxygen species (ROS) through the fenton reaction or increasing the activity of iron-dependent enzymes such as lipoxygenases (LOXs) or prolyl-hydroxylases, which are responsible for synthesis of lipid peroxidation, finally leading to ferroptosis (9). This process can be suppressed by deferoxamine (DFO), an iron chelator, implying that iron-dependent ROS is the major cause of ferroptotic cell death (2).

Interestingly, it has been documented that iron metabolism and lipid peroxidation play crucial roles in autoimmunity (10, 11). Iron deposition was observed within the kidneys of lupus nephritis (LN) mice models and during human auto-inflammatory diseases (12, 13). Multiple proteins with abilities to modulate iron homeostasis have been identified to be urinary SLE biomarkers (12). The proteins mentioned above include the iron carrier proteins neutrophil gelatinase-associated lipocalin (NGAL) (14), the iron storage protein ferritin and the iron transfer protein transferrin (15). Besides, the end products of lipid peroxidation cascades are generally recognized as lipid oxidative stress biomarkers, such as malondialdehyde (MDA), 4-hydroxynonenal (HNE), conjugated dienes (CD), and isoprostanes (16). These biomarkers were found to be significantly increased and positively correlated with disease activity in SLE (17, 18), strongly implicating the important role of lipid peroxidation in immunomodulation and autoimmunity. Unregulated oxidative stress in SLE leads to immune dysfunction, abnormal cell death signals, autoantibody production, and fatal comorbidities (19, 20).

Importantly, the successful treatment of ferroptosis inhibitors in lupus-prone mice models provided direct evidence for the role of ferroptosis in lupus pathogenesis. Hepcidin, a major iron modulator and the endogenous protective molecule against ferroptosis (21), has been shown to decrease free iron availability, reduce the renal infiltration of macrophages and T cells, and further ameliorate kidney inflammation, thereby attenuating the severity of LN in lupus-prone mice models (22). Another ferroptosis inhibitor, liproxstatin-1, was shown to efficiently suppress lipid ROS levels in neutrophils and significantly attenuate lupus in mice models (8).

REGULATORY PATHWAYS OF FERROPTOSIS

The mechanisms and genetic networks regulating ferroptosis are complex, and are still being elucidated. The glutathione (GSH)-glutathione peroxidase 4 (GPX4) antioxidant axis is the core redox mechanism involved in ferroptosis inhibition. GSH acts as a necessary cofactor for the normal function of GPX4, an antioxidant enzyme that scavenges lipid peroxides (23). Inactivation of GPX4 by GSH depletion results in lipid peroxidation, ultimately leading to ferroptotic cell death. Systemx_c^- (SLC7A11 and SLC3A2) is the most upstream player in the GSH/GPX4 signaling cascade. Notably, suppressed intracellular GSH and GPX levels in lupus patients are correlated with disease severity (24, 25). Reversal of GSH depletion attenuated disease severity in lupus-prone mice models (26). GPX4, a selenoprotein family member, requires selenium, a micronutrient, for its biosynthesis (27). And selenium deficiency is a risk factor for inflammation and autoimmunity, conditions that are prevalent in autoimmune diseases patients (28). GSH-GPX activity could be upregulated in lupus patients after selenium supplementation (29).

Apart from the GSH-GPX4 axis, various signaling pathways with the ability to modulate ferroptosis have been identified and associated with immune modulation and autoimmunity (**Figure 1**). AMP-activated protein kinase (AMPK), a sensor of cellular energy status, plays an energy stress-mediated protective role against ferroptosis (30), also as a key role in immune related diseases (31). AMPK activation exerts functions in metformin treatment of lupus by inhibiting B cell differentiation into germinal center and plasma cells (32). Another powerful antioxidant, coenzyme Q10 (CoQ10), which has shown beneficial effects in autoimmune diseases (33), can suppress lipid peroxidation and ferroptosis (34). The CoQ10 analog idebenone has been demonstrated that can attenuate murine lupus by modulating mitochondrial biology and reducing inflammation (35).

THE POTENTIAL ROLE OF FERROPTOSIS IN LUPUS IMMUNITY

Most immune cell types are implicated in SLE pathogenesis, beyond the activation of B cells (36). The significance of ferroptosis in immune systems has been reported by various studies. During maturation, activation, and differentiation of immune cells, iron metabolism and lipid peroxidation are important signaling molecules (10, 37). These processes can be regulated by antioxidant molecules such as GSH and GPX4 (38). Therefore, we discussed the relationship between ferroptosis and immunity, with a focus on SLE-associated immune cells.

T cells in lupus patients have been correlated with abnormal mitochondrial hyperpolarization and adenosine triphosphate (ATP) depletion, which cause predisposition to death by necrosis (39). Swollen lymph nodes of lupus patients harbor increased numbers of necrotic T cells, leading to inflammation and tissue damage in SLE (39–41). GSH levels are lower in T cells from patients with SLE, and the reduction degrees of GSH are associated with mitochondrial hyperpolarization and increased reactive oxygen intermediates production (42). In particular,

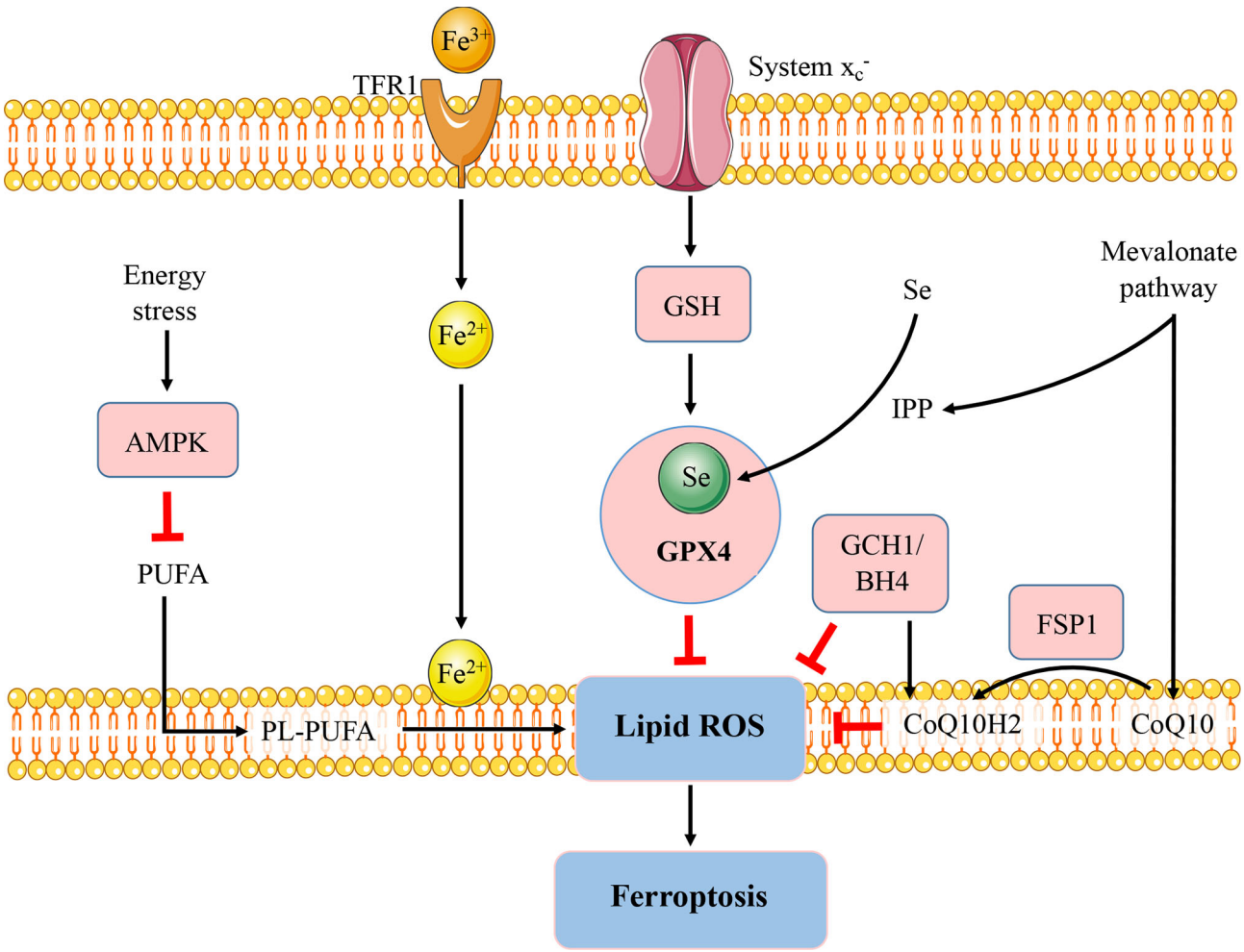

FIGURE 1 | Regulatory pathways of ferroptosis. The figure briefly shows the representative pathways of ferroptosis, which are also involved in immune response and autoimmunity. GSH-GPX4, FSP1-CoQ10, and GCH1-BH4 pathways are considered as the three major stand-alone mechanisms modulating ferroptosis. The micronutrient selenium is required for biosynthesis of GPX4. CoQ10 is another important antioxidant molecule which can be reduced to CoQ10H2 by FSP1 and hence protect the cells from ferroptosis. The GCH1-BH4 axis suppresses ferroptosis by regulating the antioxidant BH4, CoQ10, and lipid peroxidation. In addition, AMPK plays an energy stress-mediated protective role against ferroptosis. Further, the mevalonate pathway can generate anti-ferroptotic biomolecules such as CoQ10 and IPP to participate in ferroptosis regulation. FSP1, ferroptosis suppressor protein 1; GCH1; GTP cyclohydrolase-1; BH4, tetrahydrobiopterin; Se, selenium; TfR, transferrin receptor; PUFA, polyunsaturated fatty acid; PL-PUFA, phospholipid containing polyunsaturated fatty acid chain. IPP, isopentenyl-pyrophosphate.

increased intracellular iron has been found in lupus CD4+ T cells compared with healthy controls (43). Based on these findings, the possibility of ferroptosis, one of the regulated necrosis, to contribute in lupus T cells can be proposed. Besides, both $CD4^+$ and $CD8^+$ T cells that lack GPX4 would rapidly accumulate membrane lipid ROS, and undergo ferroptosis, leading to their inability to expand and protect against viral and parasite infections (44).

B cells are the central elements of humoral immunity and protection due to their ability to produce antibodies. Aberrant activation and differentiation of B cells with pathogenic autoantibody production are recognized as pivotal roles in the immunopathogenesis of SLE (45). Compared to hepcidin-treated lupus mice models, as previously stated, the spleens of vehicle treated group contained anomalous dense iron deposits in B-cell regions (22). Iron plays an important role in B cell maturation, germinal center formation and immune responses (46). Higher ROS levels are essential for the process of B cell activation and differentiation (37). Lipid peroxidation induced by erastin, the classical ferroptosis activator, can promote the proliferation and differentiation of human peripheral blood mononuclear cells (PBMCs) into B cells and natural killer (NK) cells (47). These findings imply that ferroptosis may govern B cell differentiation and activity through lipid peroxidation. Nevertheless, the roles of ferroptosis in B cells remain unclear. Current research demonstrated that GPX4 is indispensable for innate-like B cells rather than follicular B2 cells to prevent ferroptosis (48). Given the importance and complexity of B cells in lupus

development, there is a need to establish the significance of ferroptosis in B cells.

The function of macrophages is to eliminate pathogens and maintain immune homeostasis. Activated macrophages are traditionally classified into two main subsets: the pro-inflammatory subset (classically activated macrophages, M1) and the anti-inflammatory subset (alternatively activated macrophages, M2). Monocytes from SLE patients exhibit a remarkable pro-inflammatory (M1-like) profile, which is skewed towards the anti-inflammatory (M2-like) phenotype after recovery (49). Compared to M2 macrophages, M1 macrophages express higher levels of inducible nitric oxide synthase (iNOS), leading to higher resistance to ferroptosis (50). It may explain the imbalance in macrophage polarization during lupus progression: M1 phenotypes display significant defiance against ferroptosis, yet they can survive, release proinflammatory cytokines, and fulfill their functions as "destroyers"; while M2 phenotypes are vulnerable to ferroptotic cell death induced by the loss of GPX4 activity (51).

Neutrophils are the first responders of immune defense against a broad range of pathogens (52). Currently, the research about the link between neutrophils and lupus is mainly focused on neutrophil extracellular traps (NETs), the fibrous networks protruding from activated neutrophils in response to infection or inflammation (53). However, recent study by Li et al. demonstrated that neutrophil death is majorly associated with ferroptosis in SLE, instead of NETosis, the process of NET release. Through downregulated expression of GPX4 and elevated lipid ROS levels, neutrophil ferroptosis leads to stimulation of autoreactive B cells and plasmacytoid dendritic cells (pDCs), autoantibody and type I interferon (IFN) production, finally contributing to disease manifestations (8). Therefore, ferroptosis promotes lupus progression through immune system regulation.

THE POTENTIAL ROLE OF FERROPTOSIS IN LUPUS INFLAMMATION AND TISSUE DAMAGE

Ferroptosis occurs in various immune cells and affects immune response as it has been described earlier. Further, ferroptosis regulates on how immune system deals with dying cells and remnants, through the release of damage-associated molecular patterns (DAMPs) or lipid oxidation products (9). DAMPs bind to cellular receptors such as pattern recognition receptors (PRRs), upregulate stress response mechanisms, and release various cytokines and chemokines, finally leading to tissue injury and inflammation (54). For example, the signals of high-mobility group box 1 (HMGB1), one of prototypical DAMPs released by ferroptotic cells, can be integrated by advanced glycosylation end-product specific receptor (AGER) to trigger inflammation and amplify immune responses (55). HMGB1 released by ferroptosis is implicated in multiple tissue damage, including ultraviolet B (UVB)-induced keratinocyte death (56), and high glucose-exposed mesangial cell death (57). Interestingly, HMGB1 activity plays a markable role in a variety of lupus phenotypes, including LN, neuropsychiatric lupus (58), and skin lesions (59). HMGB1 exerts its causative effects in SLE through both innate and adaptive immunity (58, 60), including macrophage polarization, pro-inflammatory cytokines secretion, and autoantibodies generation. Besides, iron accumulation can directly polarize macrophages to pro-inflammatory profile (61), promote pro-inflammatory cytokine secretion to induce autoimmune diseases (13); ROS facilitates inflammatory disease *via* pro-inflammatory change (62). Massive lipid oxidative mediators released by ferroptosis directly promote the activity of cyclooxygenases (COXs) and LOXs, which convert arachidonic acid to inflammatory mediators; this process can be suppressed by GPX4 (63). Therefore, it is speculated that ferroptosis may exert its pathogenic effect in SLE by excessive inflammation, which enhances immune response, leading to organ damage and clinical manifestations. A potential model is proposed for the role of ferroptosis in lupus inflammation and induced comorbidities (**Figure 2**).

With respect to skin, keratinocyte death by ferroptosis plays a remarkable role in driving skin inflammation after UVB exposure (56). Skin lesions suffered from UVB irradiation shows elevated iron content (64), excessive accumulation of lipid peroxides, and GSH depletion, therefore undergoing ferroptosis in keratinocytes, and then leads to cutaneous necroinflammation and injury (56). Furthermore, UVB-induced skin damage can be protected by GSH and GPX4 through suppressing oxidant stress, inflammation responses, and cell death (65). Based on the lupus photosensitivity, and ROS accumulation in all cutaneous subtypes of lupus (66), the cutaneous lesions may be associated with dysregulation of iron metabolism and the consequent ferroptosis induced by UV irradiation.

LN is one of the most severe organ manifestations of lupus, which most patients would develop within 5 years of SLE diagnosis (67). Tubulointerstitial damage is recognized as one of the pathological features of the lupus kidney, and tubulointerstitial inflammation is important in the assessment and prognosis of LN (68, 69). Within this local microenvironment, renal tubular epithelial cells (RTEC) are central effector cells, driving interstitial inflammation and renal damage (70). As mentioned above, renal iron accumulation occurs in LN and contributes to the development of albuminuria (12). RTECs reabsorb the majority of filtered iron (71), and these cells have been shown to undergo ferroptosis under pathological conditions (72, 73). Treatment of lupus mice models with iron metabolism regulators, such as deferiprone and hepcidin, could mitigate kidney inflammation and delay lupus progression (12, 22). Besides, uncontrolled ROS accumulation in RTECs results in inflammation and fibrosis, leading to renal damage and chronic kidney disease progression (74). Thus, it could be speculated that iron accumulation in RTECs may exacerbate inflammatory responses by ROS formation, and synergistically accelerate progression to renal failure. Meanwhile, inflammation and oxidative stress can upregulate the expression of iron carriers and transporters, possibly causing excessive uptake of iron in the renal tubules and consequent iron-induced kidney injury (75).

For cardiovascular system, the oxidation of low-density lipoproteins (oxLDL) by ROS and the activation of endothelial cells in the artery, are recognized as initiation of atherosclerosis in

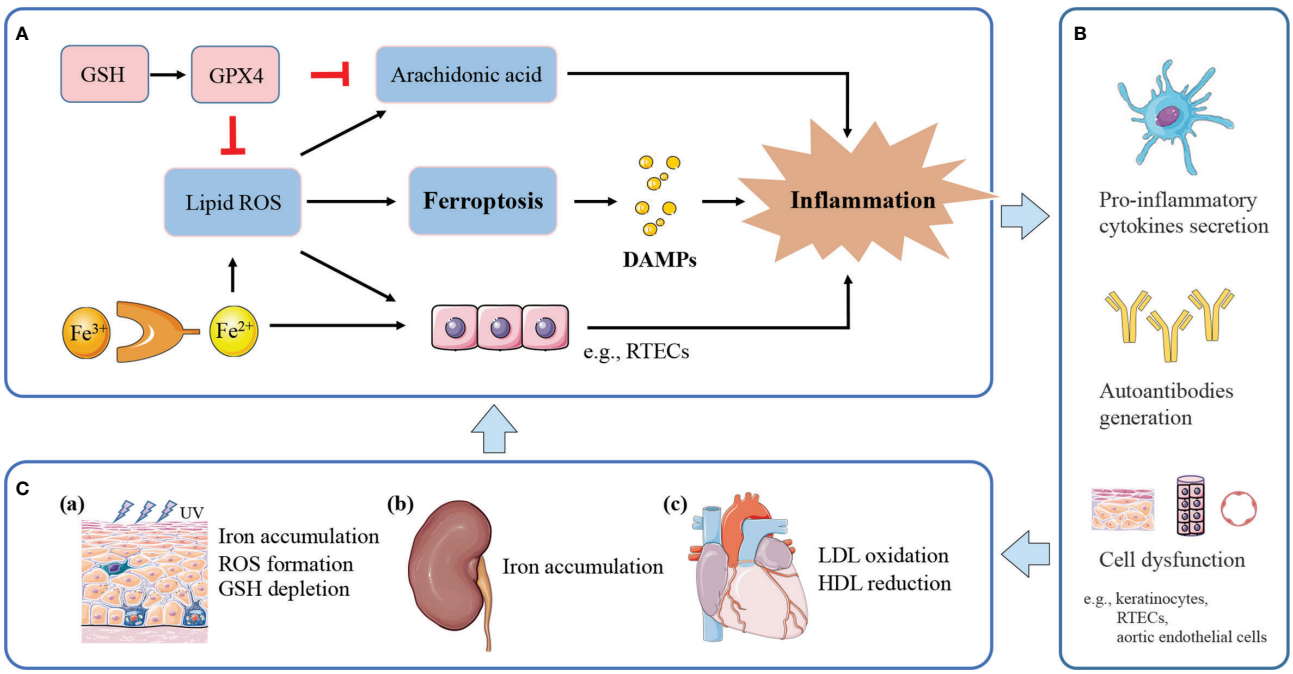

FIGURE 2 | The potential model of ferroptosis in lupus inflammation and manifestations. **(A)** Ferroptosis releases DAMPs to trigger inflammation. Iron and ROS accumulation promote a pro-inflammatory environment. Massive lipid ROS released by ferroptosis helps to convert arachidonic acid to inflammatory mediators. GPX4 suppresses inflammation by inhibiting arachidonic acid oxidation and lipid peroxidation. **(B)** Ferroptotic cell death and induced inflammation exert causative effects in SLE through pro-inflammatory cytokines secretion, and autoantibodies generation, finally leading to cell dysfunction and tissue damage. **(C)** (a) Inflammation induced by UV irradiation amplifies inflammatory and immune responses, eventually causing cutaneous lesions. UVB-exposed skin lesions exhibit iron accumulation, excessive ROS and GSH depletion, leading to keratinocytes ferroptosis. (b) Persistent inflammation and immune complexes deposition accelerate lupus progression to renal failure. Kidneys uptake excessive iron in the renal tubules and undergo ferroptosis under pathological conditions. (c) Lipid peroxidation and induced inflammation contribute to endothelial dysfunction and cardiovascular injury. Lupus patients with progressive atherosclerosis show decreased HDL and increased oxLDL, which may further promote ferroptosis in aortic endothelial cells.

SLE (76). Endothelial cells stimulated by oxLDL release inflammatory cytokines, induce chronic inflammation, finally leading to endothelial dysfunction and cardiovascular injury (76). In addition, lupus patients with progressive atherosclerosis exhibit decreased levels of high-density lipoprotein (HDL) and dysfunctional HDL (77). HDL is a natural antioxidant agent and act as a protective mechanism of atherosclerosis in SLE, protecting LDL from oxidation by ROS in the arterial intima (76, 78). Recently, a study by Bai et al. used ferroptosis inhibitor, ferrostatin-1, to treat high-fat diet-induced atherosclerosis (79). They found that Fer-1 could alleviate atherosclerosis lesion and rescue endothelial dysfunction, through inhibition of iron accumulation and lipid peroxidation, and upregulation the expression of SLC7A11 and GPX4. Compelling evidence links ferroptosis to the initiation and progression of atherosclerosis.

CONCLUSION AND PERSPECTIVES

In conclusion, ferroptosis is speculated to be an integral component in the vicious cycle of immune dysfunction, inflammation, and tissue damage in lupus. This review article indicates that ferroptosis has outstanding research prospects in the progression of SLE. However, it is suggested that more future studies should be conducted to fill the knowledge gaps of the relationship between ferroptosis and SLE, shed more light on the pathogenesis of SLE, as well as provide a new perspective on ferroptosis-based immunotherapy for SLE.

AUTHOR CONTRIBUTIONS

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Hearing Loss as the Main Clinical Presentation in *NLRP3*-Associated Autoinflammatory Disease

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The *NLRP3* gene mutations are the cause of autosomal dominant autoinflammatory disorders (NLRP3-AID). Recently, hearing loss (HL) has been found to be the sole or major manifestation of NLRP3-AID. Here, we tested 110 autosomal dominant HL families with a custom panel of 237 HL genes and found one family carrying the *NLRP3* c.1872C>G, p.Ser624Arg mutation. Functional studies revealed that this novel variant is a gain of function mutation, leading to increased activity of caspase-1 and subsequent oversecretion of proinflammatory interleukin-1β. Clinical reanalysis of the affected individuals, together with serological evidence of inflammation and pathological cochlear enhancement on FLAIR-MRI images, guided our diagnosis to atypical NLRP3-AID. The study highlights the role of genetic analysis in patients with progressive postlingual HL. This can help to identify individuals with hereditary HL as a consequence of NLRP3-AID and allow timely and effective treatment with interleukin-1-receptor antagonist.

Keywords: NLRP3 inflammasome, hearing loss, anakinra, autoinflammation, fluid attenuated inversion recovery (FLAIR), cochlear enhancement, DNA sequencing, interleukin-1

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INTRODUCTION

Pathogenic variants in single genes constitute a substantial portion of the causes of hearing loss (HL). With the progress of DNA analysis techniques and the increasing availability of genetic testing for routine clinical diagnosis, detection of these variants continues to grow. Following the diagnosis of hereditary HL, evaluation and treatment options are now available, although most are symptomatic and aimed at alleviating disease symptoms and preventing complications. But if one could identify the underlying mutation and its related molecular background, this raises the prospect of a new therapy targeting the causal mechanism itself (1).

In 2017, Nakanishi et al. reported patients from a family with syndromic progressive HL who responded to treatment with anakinra, an antagonist of the interleukin-1 (IL-1) receptor. The therapy was introduced to counteract increased secretion of IL-1β, which was a consequence of an activating *NLRP3*

mutation in these patients (2). The protein encoded by *NLRP3* is a member of the NLR (nucleotide binding domain, leucine rich repeats-containing) family and the NLRP (pyrin domain containing) subfamily. It is abundantly expressed in neutrophils and macrophages and is considered a critical mediator of inflammation. Following stimulation by diverse triggers – such as endogenous stress signals, exogenous particulates, pathogens, and pore-forming toxins – NLRP3 inflammasome is assembled. The NLRP3 inflammasome promotes activation of procaspase-1 that in turn cleaves and activates the proinflammatory cytokines interleukin IL-1β and IL-18 (3).

NLRP3 was discovered through its association with autosomal dominant autoinflammatory diseases (NLRP3-AID), also known as CAPS (cryopyrin-associated periodic syndromes), which comprise a spectrum of inflammatory symptoms (e.g. urticaria, conjunctivitis, myalgia, arthralgia, fever, headache, fatigue) of various severity (4). Progressive sensorineural HL with onset in childhood or early adulthood has been typically reported in moderate and severe forms of NLRP3-AID. HL as the sole or major NLRP3-AID associated feature is a recent and surprising finding that deserves attention, particularly since a targeted anti-IL-1 treatment is available that has been shown to be effective throughout the NLRP3-AID spectrum (5).

In this study, we present a family initially diagnosed with an isolated autosomal dominant sensorineural HL (ADHL). Based on the results of our genetic testing and targeted detailed assessment of the clinical picture, the diagnosis has now been shifted to atypical NLRP3-AID. Magnetic resonance imaging (MRI) of the temporal bones has revealed that cochlear inflammation accounts for progressive hearing deterioration, and we find that a later acquisition of the MRI-FLAIR images after contrast administration is even more sensitive in detecting the pathological cochlear enhancement. A novel *NLRP3* mutation has been identified as the only variant capable of causing the phenotype in this family. After applying a set of functional assays, we have confirmed that the NLRP3 inflammasome is hyperactive in these patients, leading to increased activity of caspase-1 and overproduction of IL-1β.

MATERIALS AND METHODS

Study Subjects

All participants gave written informed consent. The study was approved by the local ethics committee (KB.IFPS.25/2017) and performed according to the Declaration of Helsinki. A non-consanguineous Polish family with 9 family members affected by bilateral sensorineural HL was identified at the Institute of Physiology and Pathology of Hearing. The family was selected based on genetic testing results from a cohort of 110 HL families presenting a pedigree consistent with the autosomal dominant mode of HL inheritance. In the proband (III.8), HL was noted at the age of 20; since then it had gradually progressed. The proband has used hearing aids from the age of 34. At age 10, she was treated with gentamicin for peritonitis. In her mother (II.6), hearing deterioration was diagnosed at age 37, and from the age of 38, she wore hearing aids. Additionally, she has been diagnosed with schizophrenia and has received appropriate treatment from the age of 35. The proband's stepsister (III.7) noticed progressive HL at the age of 40, and this was confirmed by objective hearing tests at age 43. All of them suffer from tinnitus but do not report vertigo or dizziness. In individuals II.2 and II.4, hearing deterioration was first observed at the end of the fourth decade of life; in subject III.3 it was seen at around age 20. Individuals IV.3 and IV.4 have suffered from recurrent episodes of choroiditis from the age of 8, and their hearing deterioration was noticed at the age of around 15 (**Figure 1A**).

Clinical Evaluation

Affected family members (II.6, III.7, III.8) underwent clinical evaluation by an otorhinolaryngologist, clinical geneticist, and clinical immunologist. Hearing status was assessed by standardized analysis including tympanometry, pure-tone audiometry (PTA), and auditory brainstem responses (ABR). A set of 18 binaural hearing thresholds was obtained from 5 available family members with HL (nine from patient III.8, three from patient II.6, three from patient III.7, two from patient II.4 and one from patient III.3). The age-related typical audiogram (ARTA) was constructed as described previously (6). Based on cross-sectional linear regression, hearing thresholds were predicted for fixed ages (20–80 years) and an annual threshold deterioration (ATD; dB per year) was calculated for all tested frequencies. The progression was considered significant if the regression coefficient (slope) was significantly different from 0 at $p < 0.05$ (GraphPad Prism 9.0).

MRI of the inner ear was performed along with brain imaging on a 3 T MRI system (Magnetom Prisma, Siemens). A protocol designed for inner ear and inner auditory canal imaging was employed; it contained heavy T2-weighted images of 0.5 mm slices (constructive interference in a steady-state (CISS) sequence) and FLAIR (fluid-attenuated-inversion-recovery with inversion time of 1674.8 ms) images of 2 mm slices acquired before and after (approx. 15 min and 75 min) i.v. administration of gadolinium contrast agent (0.1 mmol/kg of gadoteridol, ProHance; Bracco Imaging). Images were reviewed by a radiologist with over 15 years of expertise in neuroradiology.

Pathogenic Variant Detection

Genomic DNA was extracted from blood samples or buccal swabs. For the proband (III.8), a custom multi-gene HL panel (n=237 genes) was used, and the subsequent data analysis workflow was performed as previously described (7). To confirm the presence of the detected *NLRP3* variant and its segregation with HL in the family, Sanger sequencing with the following primer pair encompassing exon 3 (5′-AAGGAAGTGGACTGCGAGAAG and 5′-CCACCCGATGACAG TTCTCAA) was applied as described elsewhere (8). The variant was assigned based on the reference sequences NM_001243133.1 and NP_001230062.1 according to standards and guidelines for the interpretation of sequence variants (9, 10).

Isolation and Stimulation of PBMC

Blood samples from patients II.6, III.7, III.8 and age- andsex-matched healthy donors were collected by EDTA anticoagulant on the same day as the isolation of peripheral blood mononuclear cells (PBMCs) and assays were performed. PBMCs were isolated

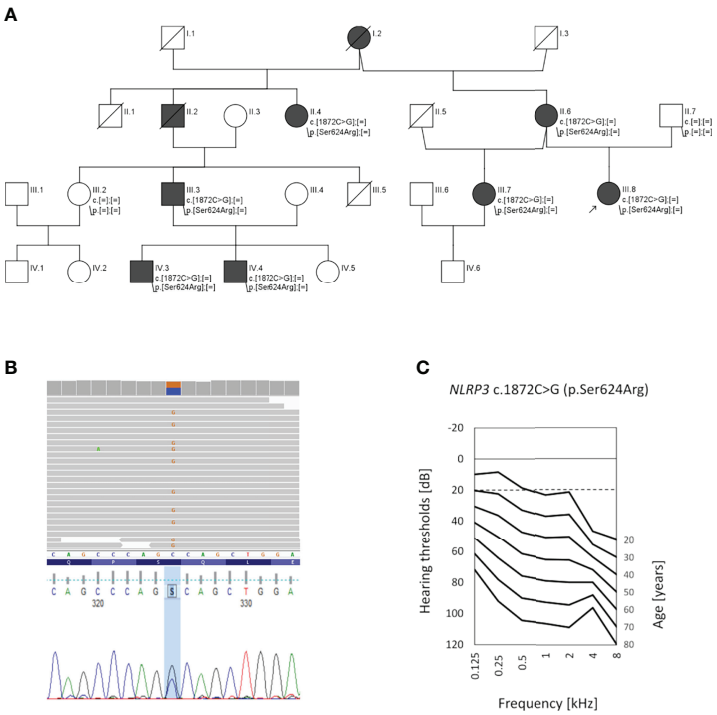

FIGURE 1 | Pedigree of the investigated family, genetic data, and audiological characteristics. **(A)** Pedigree showing autosomal dominant hearing loss (black symbols); healthy individuals are shown in white. The proband (III.8) is indicated by an arrow. **(B)** Results of next-generation sequencing and Sanger sequencing showing the c.1872C>G transversion (p.Ser624Arg) in the *NLRP3* gene. **(C)** Age-related typical audiogram (ARTA) based on audiological data of the 5 patients with *NLRP3* p.Ser624Arg as the likely pathogenic variant. The normal hearing threshold is marked with a detached line.

using Ficoll-Histopaque solution (Sigma-Aldrich, Saint Louis, MO, USA). Blood samples were diluted 1:1 with PBS (Sigma-Aldrich) and layered on top of the Ficoll solution. Cells were centrifuged for 25 min at 2000 rpm. Then the cell-containing phase was collected and washed 3 times using PBS with 2% heat-inactivated bovine serum (Sigma-Aldrich). For experiments, cells were resuspended in RPMI-1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) with 3% heat-inactivated human serum and antibiotics (penicillin and streptomycin (Thermo Fisher Scientific)). PBMCs were seeded on 96-well plates at a density of 10^5 cells per well. Colchicine, known for its ability to suppress NLRP3 inflammasome activation (11), (10 μM; Sigma-Aldrich) or MCC950, a selective NLRP3 inhibitor (5 μM; Inflazome Ltd, Ireland), were added; 30 min later ultrapure *E. coli* lipopolysaccharides (LPS, InvivoGen, San Diego, CA, USA) were also added for stimulation. After 1 h, nigericin, an NLRP3 activator (12), was also added (10 μM; Tocris, UK) and the cells were incubated at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2. 3 h or 24 h after LPS addition, the supernatants were collected for the IL-1β ELISA assay or the Caspase-Glo 1 Inflammasome Assay (Promega, Madison, WI, USA) was performed.

ELISA for Interleukin-1β

Levels of secreted IL-1β were measured in the supernatants using a human IL-1β ELISA kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The plate was read using the Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific).

Caspase-Glo 1 Inflammasome Assay

The activity of caspase-1 was measured together in whole cells and in supernatants using the Caspase-Glo 1 Inflammasome Assay (Promega) following the manufacturer's instructions. Cells were treated on a Z-WEHD-aminoluciferin substrate for 1 h at room temperature. Luminescence was measured using a SpectraMax i3x multi-mode microplate reader (Molecular Devices LLC, San Jose, CA, USA).

Statistics

The measures of the functional activity of the NLRP3 inflammasome were compared using *t*-tests (GraphPad Prism 9.0).

RESULTS

Identification of a Novel Likely Pathogenic *NLRP3* Variant

To search for the genetic cause of HL in the studied family, we analyzed 237 genes known for their involvement in isolated and

syndromic forms of HL. After performing the multi-gene HL panel, we found only one variant located in the *NLRP3* gene that could explain the development of HL. The heterozygous transversion NM_001243133.1:c.1872C>G in exon 3 of the *NLRP3* gene (**Figure 1B**) has not been reported in population databases (**Table 1**). It is predicted to result in a missense substitution NP_001230062.1:p.Ser624Arg, which corresponds to the NACHT-associated domain (NAD) of the NLRP3 protein (**Figure 2**). The variant first detected in the proband, completely segregated with HL in the family, and was found in six other HL-affected individuals (**Figure 1A**). Based on the available data, the detected *NLRP3* change was classified as likely pathogenic (**Table 1**).

Patients With the p.Ser624Arg *NLRP3* Mutation Present Increased NLRP3 Inflammasome Activity *ex vivo*

As the NLRP3 inflammasome is responsible for activation of caspase-1 and subsequent release of IL-1β, we compared the responses of PBMCs from patients and healthy controls to LPS stimulation *ex vivo*. Spontaneous release of IL-1β was almost 3-fold higher after 3 h of incubation, whereas this difference was less evident after 24 h of incubation (**Figures 3A, B**). Stimulation with 0.1 ng/ml of LPS for 3 h induced a significantly higher release of IL-1β from the cells with *NLRP3* mutation than from control cells (977.3 ± 231 pg/ml vs. 156.0 ± 103 pg/ml, **Figure 3A**). Extended stimulation time or higher LPS concentration decreased this difference. However, when cells treated with a higher concentration of LPS were stimulated with a second signal activator (nigericin), PBMCs from patients released significantly more IL-1β after both 3 h and 24 h of stimulation than the control cells (**Figures 3A, B**). Pretreatment of the PBMCs with the NLRP3 specific inhibitor MCC950, but not with colchicine, which indirectly suppresses NLRP3 inflammasome activity, significantly reduced the release of IL-1β in patient samples but not in controls (**Figures 3A, B**).

Additionally, we measured the total activity of caspase-1 in both cells and culture medium using an enzymatic activity test. Some 24 h after the start of the experiment, cells bearing the *NLRP3* mutation showed increased caspase-1 activity, which was also higher after LPS stimulation in comparison to control cells (**Figure 3C**). Upon addition of nigericin or colchicine, higher activity of caspase-1 remained. Pretreatment with MCC950 decreased (but not significantly) the response of patient-derived cells to LPS, while no effect was seen in control cells (**Figure 3C**).

Clinical Reanalysis Reveals Phenotypic Features Related to an Autoinflammatory Disease Spectrum (NLRP3-AID/CAPS)

The proband from the family investigated in this study was first diagnosed with non-syndromic, progressive ADHL. Her HL was not associated with malformations of the external ear or other organs. She did not complain of medical problems involving organ systems other than the auditory system. After receiving the results of her genetic tests, which showed a probably causative variant in the *NLRP3* gene, we performed a clinical reanalysis of the affected individuals, focused now on the symptoms and signs of NLRP3-AID. ARTA showed progressive bilateral HL; the calculated ATD was significant at all frequencies and ranged from 0.82 dB/year (4 kHz) to 1.45 dB/year (2 kHz) (**Figure 1C**). All three subjects (II.6, III.7, III.8) admitted having episodes of conjunctivitis, which were more frequent in the proband's mother and stepsister but occurred only occasionally in the proband. The stepsister also reported recurrent uveitis and a couple of stress-triggered episodes of unexplained fever. The proband's mother complained of arthralgia and myalgia. All had elevated laboratory inflammatory markers, such as C-reactive protein (CRP) and serum amyloid A (SAA), and in some laboratory examinations mild blood leukocytosis, neutrophilia, and increased erythrocyte sedimentation rate were also noted (**Table 2**). After refining the clinical picture in the light of the Eurofever/Printo classification criteria, we diagnosed the patients as having NLRP3-AID (14), and therapy with IL-1 receptor antagonist (anakinra) was introduced.

To assess their auditory system, an MRI was performed. In all three subjects, pathologically strong cochlear enhancement on late FLAIR images (performed approx. 75 min after contrast administration) was observed in all turns of both cochleas (albeit asymmetrically). On the early FLAIR images (performed approx. 15 min after contrast administration), cochlear enhancement was less pronounced and was not uniformly distributed in all turns (**Figures 4A–C**). In the proband, a subtle decrease of the bright signal on T2-weighted images (CISS sequence) within both cochleas was found; moreover, a subtle reduction in the signal on T2-weighted images was more pronounced on the same side as where stronger enhancement was seen on FLAIR images (**Figures 5A, B**).

TABLE 1 | Characteristics of the *NLRP3* variant detected in this study.

Variant cDNA Level	Variant Protein Level	Exon	Reference SNP ID	Population Frequencies			Pathogenicity Predictions						
				gnomAD	UK10K	EVS	SIFT	PolyPhen-2	Mutation Taster	LRT	CADD	REVEL	ACMG Classification*
c.1872C>G	p.Ser624Arg	3	N/A	0	0	0	T (0.053, 0.058, 0.056)	B (0.409)	P (0.6495)	N (0.002316)	D (24.6)	0.6129	LP (PM2, PP1_moderate, PP4_supporting, PS3_supporting)

**ACMG classification criteria legend: LP, likely pathogenic; PM2, moderate pathogenicity evidence; PP1_moderate, moderate pathogenicity evidence; PP4_supporting, supporting pathogenicity evidence; PS3_supporting, supporting pathogenicity evidence; N/A, no data available; B, benign; D, damaging; N, neutral; P, polymorphism; T, tolerated.*

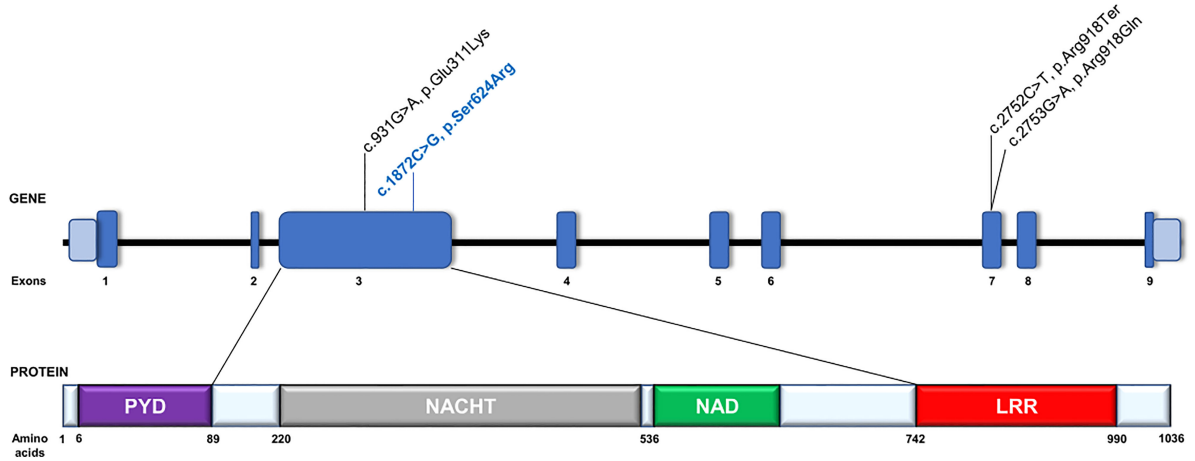

FIGURE 2 | Schematic representation of the *NLRP3* gene and its protein organization. Gene and protein structures are depicted based on the canonical transcript NM_001243133.1 and reference protein sequence NP_001230062.1. The NRLP3 protein consists of an N-terminal pyrin domain (PYD), a central nucleotide-binding oligomerization domain (NACHT), a NACHT-associated (NAD) domain, followed by a leucine-rich repeat (LRR) domain at the C terminus. Previously reported *NLRP3* pathogenic variants involved in DFNA34 or atypical NLRP3-AID development are in black text. The c.931G>A, p.Glu311Lys variant was previously reported as c.937G>A, p.Glu313Lys based on NM_001127462.1 reference sequence (13). The variant identified in this study is labelled in blue text.

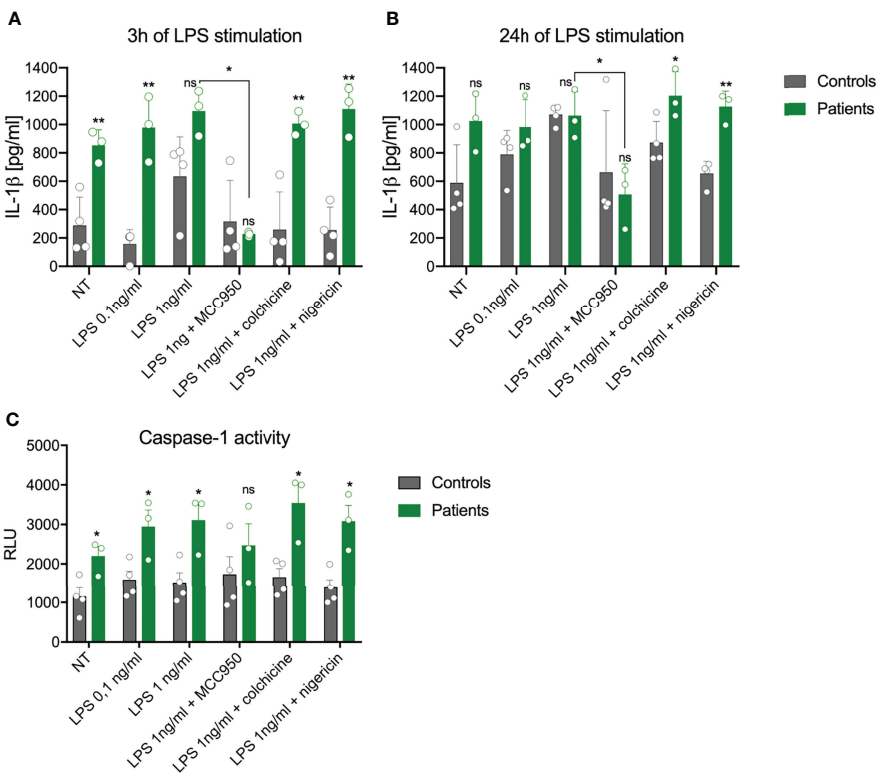

FIGURE 3 | Impact of *NLRP3* mutation on inflammasome activation in peripheral blood mononuclear cells (PBMCs). The capacity of PBMCs from healthy controls (grey) and patients (green) to secrete IL-1β after stimulation with LPS or LPS+nigericin was analyzed after **(A)** 3 h or **(B)** 24 h The specific NLPR3 inhibitor MCC950, but not the microtubule inhibitor colchicine, was able to inhibit IL-1β release. **(C)** Activation of caspase-1 in PBMCs was evaluated after 24 h (mean ± SEM, results compared by *t*-test: $*p < 0.05$; $**p < 0.01$). NT, not treated; ns, non significant

TABLE 2 | Inflammatory markers level.

Patients	Before anakinra			After 3 mo. of anakinra		
Inflammatory markers*	**ESR**	**CRP**	**SAA**	**ESR**	**CRP**	**SAA**
II.6	16	0.7	5.04	11	0.5	4.0
III.7	15	2.5	17.9	3	0.2	1.8
III.8	21	0.9	2.1	6	0.1	0.4

**Inflammatory markers done outside flares.*
ESR mm/h ref.0-10, CRP mg/dl ref.0-0.8, SAA mg/dl ref. < 0.64.

DISCUSSION

In this study, we have identified a novel *NLRP3* variant and, after combining genetic and functional data, we have demonstrated that it is causative of hereditary HL, with an autosomal dominant mode of inheritance. HL predominated in the investigated family's clinical picture, but targeted phenotypic reanalysis also revealed minor inflammatory symptoms. The collected clinical data supported by serologic evidence of inflammation allowed us to diagnose that the patients suffered from an NLRP3-AID – although the presented features are insufficient to diagnose any particular NLRP3-AID condition such as Muckle–Wells syndrome (MWS), neonatal-onset multisystem inflammatory disease (NOMID), or familial cold autoinflammatory syndrome (FCAS) (5). After evaluating the available literature, we find that only four other families have been reported as having either isolated HL (DFNA34) or HL as a prominent feature of an atypical NLRP3-AID phenotype.

In one Chinese family with an *NLRP3* mutation (NM_001127462.1:c.937G>A, NP_001120934.1:p.Glu313Lys) previously linked to MWS, the proband and two other family members had isolated HL, but the other six HL individuals had minor MWS-related inflammatory symptoms such as conjunctivitis and uveitis, oral ulcers, arthralgia and arthritis, and erythematous rash, occurring in different combinations (13). An atypical form of NLRP3-AID was also recognized in one of two families presented by Nakanishi et al. (2), which carried the same *NLRP3* missense mutation (NM_001243133.1:c.2753G>A, NP_001230062.1:p.Arg918Gln). In one of the two families, a syndromic form of HL was reported, but the autoinflammatory phenotype (including episodic urticaria, periodic fevers, conjunctivitis, oral ulcers, cervical lymphadenopathy, arthritis, arthralgia, bursitis, headaches) did not meet the diagnostic criteria for MWS, NOMID, or FCAS. In the second family (North American Caucasian), HL occurred together with either multiple sclerosis or some nonspecific signs and symptoms, and it was regarded as isolated HL (DFNA34) (2). In 2021, Kim et al. described two affected family members who had a second DFNA34 pedigree due to an *NLRP3* mutation (NM_001243133.1:c.2752C>T, NP_001230062.1:p.Arg918Ter) (15) (**Figure 2**).

HL in NLRP3-AID is considered a common clinical manifestation. It is typically observed in NOMID and MWS but only rarely in FCAS (16). In the defined NLRP3-AID syndromes and in atypical phenotypes or in DFNA34, some general features of HL remain similar: in these patients, HL is usually sensorineural, more often affects the higher frequencies, and gradually progresses over time (16, 17). This was also the case for the family reported in this study, although the age at HL onset, the severity of hearing deterioration, and its rate of progression vary among NLRP3-AID patients. The onset of HL may range from the first months to the fourth decade of life, and individuals can have severe to profound HL (15, 17). In the family described here, the onset of HL was after 15 years of age and HL had progressed over time. In none of the subjects it had reached the point where cochlear implants were needed.

Based on the current understanding of the pathogenesis of HL in NLRP3-AID, the condition arises as a consequence of local cochlear autoinflammation. Recent studies have demonstrated that immune cells (macrophage/monocyte-like cells) in the cochlea can activate their NLRP3 inflammasome. If this activation is aided by an existing *NLRP3* mutation (an underlying cause of NLRP3-AID), abnormal cochlear activation of the NLRP3 inflammasome may occur. The result may be cochlear inflammation accompanied by progressive hearing deterioration (2, 18). A strong argument in favor of cochlear inflammation being causally involved in the development of HL in NLRP3-AID, is the pathological enhancement visible on the MRI post-contrast FLAIR images of the cochlea. Enhancement indicates that the contrast material has diffused into cochlear tissues from the blood vessels, made more permeable by inflammation (15). In previous studies and in our patients, pathological cochlear enhancement has been associated with the presence of HL (16, 19). Here, we found that the late post-contrast FLAIR images were more sensitive than the early ones in detecting cochlear inflammation. The differences observed in the degree of cochlear enhancement may correspond to differences in the magnitude or stage of the local inflammation.

Overproduction of IL-1β, considered the central mediator of inflammation, plays a main role in the pathogenesis of NLRP3-AID; consequently, treatment with anti-IL-1 receptor inhibitors such as anakinra or canakinumab is recommended for this group of patients (5). A positive therapeutic effect on hearing (such as its stabilization in the majority of patients or improvement in some individuals) has also been achieved, especially when treatment is begun early (15, 19–23). Poor response to anti-IL-1 receptor inhibitors is likely explained by a chronic inflammation that has already caused irreversible cochlear damage. Of the four families with atypical NLRP3-AID or DFNA34, this form of therapy has only been introduced in one family with syndromic HL. Over a 5-month follow-up period, it restored normal hearing thresholds in the children and improved the hearing of one adult (2). In three of our patients, subcutaneous anakinra at a dose of 100 mg daily was administered; it appeared to be well-tolerated and we expect to be able to assess the efficacy of this therapy within the next couple of months.

In our work, we also wanted to find out how the novel *NLRP3* variant affects the function of the NLRP3 inflammasome, a critical component of the inflammatory signaling pathway in the innate immune system. For this purpose, we tested the circulating monocytes from the patients for the effects of the identified *NLRP3* mutation on IL-1β release. In contrast to a previous report, unstimulated PBMCs from our patients secreted more IL-1β after 3 h of incubation than did control cells (24). The most

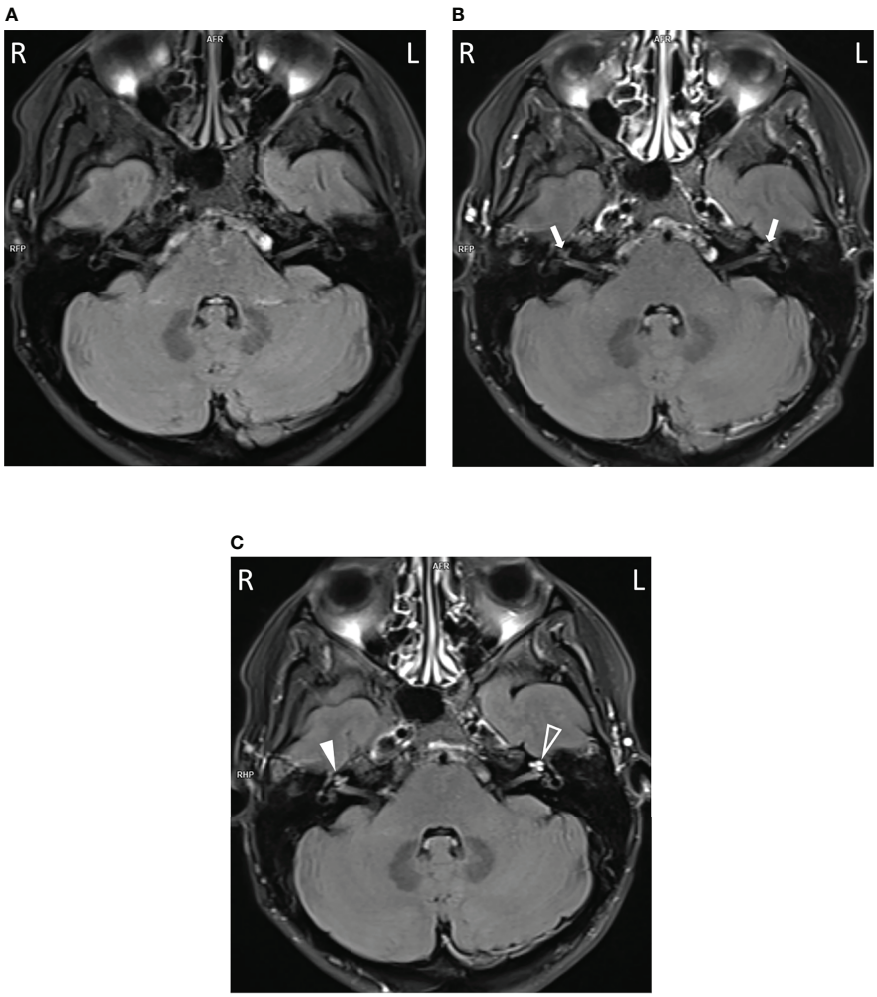

FIGURE 4 | Cochlear enhancement on FLAIR images in the proband. In comparison to non-contrast image **(A)**, mild cochlear enhancement (arrows) is seen on the early FLAIR image **(B)**. On the late FLAIR image **(C)**, substantial enhancement (more pronounced on the left, empty arrowhead) is observed in both cochleas (arrowheads).

discriminatory condition between mutant and control PBMCs was found to be 3 h of stimulation with a low dose of LPS. In most human cells, activation of the NLRP3 inflammasome requires two signals: priming *via* the TLR receptor, which upregulates the expression of inflammasome components, and a second signal (e.g. potassium efflux or lysosomal leakage) which induces assembly of the NLRP3 inflammasome. However, in human monocytes LPS binds the TLR4 receptor, which triggers activation of the NLPR3 inflammasome through an alternative pathway. This mechanism explains why only one-signal stimulation reveals differences in the NLRP3 hyperactivation mutation (25). A similar condition of 3 h of LPS stimulation was reported by Rieber et al. (24) to be most accurate in testing *NLRP3* mutations. It is probable that monocytes from NLRP3-AID patients activate NLPR3 more rapidly (24, 26). The two-signal stimulation (with LPS and nigericin) did not further increase the concentration of IL-1β, which is in line with other studies on NLRP3-AID (24, 27). Such conditions decrease the amount of IL-1β released by monocytes from healthy controls, probably due to rapid induction of pyroptosis while having less effect on the patients' cells but this assumption requires further studies. Longer stimulation times decrease the observed differences in IL-1β due to increased secretion by control cells. Pre-treatment of cells with the NLRP3 specific inhibitor confirmed the role of NLRP3 in the secretion of IL-1β. Intriguingly, the capsase-1 activity test did not reflect the observed changes in the IL-1β release, limiting its usefulness in such experimental settings.

Colchicine, which has been shown to inhibit NLRP3 inflammasome formation in macrophages (28), was ineffective in blocking the IL-1β release after LPS stimulation. We also measured the activity of caspase-1, which increased even in unstimulated PBMCs from NLRP3-AID patients. This assay was performed only after 24 h of stimulation, and in each tested condition, PBMCs from NLRP3-AID patients showed

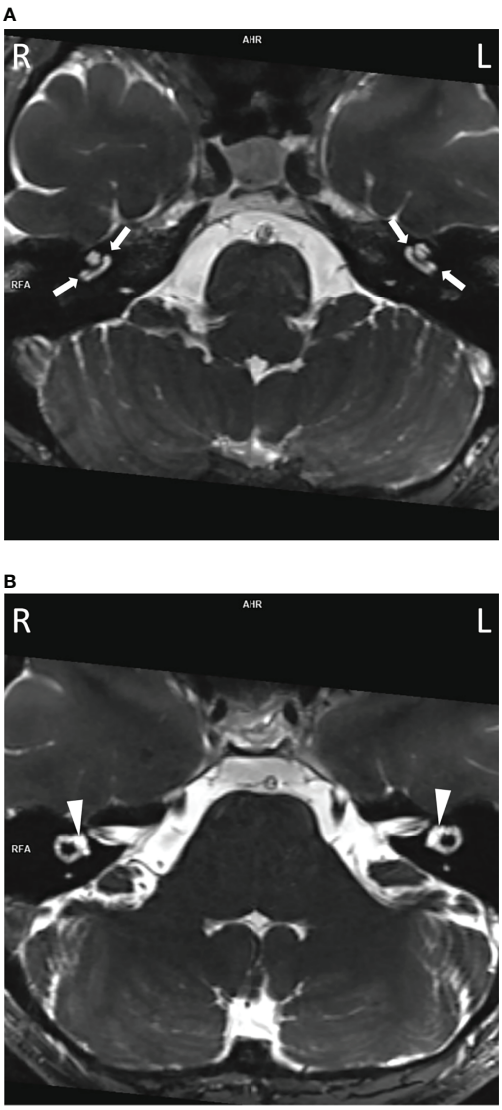

FIGURE 5 | The decreased bright cochlear fluid signal on T2-weighted images in the proband. Slight diminishing of the bright fluid signal was visible in both cochleas (**A**, arrows) compared to vestibules (**B**, arrowheads) and more pronounced on the left.

higher activity of caspase-1 in comparison to control samples (except for the MCC950-treated cells). However, the lack of evident inhibition of caspase-1 activity by the MCC950 inhibitor suggests that further studies applying other techniques of caspase analysis in these patients should be performed. Nevertheless, these results support the involvement of NLRP3 in this process and show that the analyzed c.1872C>G (p.Ser624Arg) genetic variant in our patients resulted in increased activity of the NLRP3 inflammasome. NLRP3-AID is a consequence of a heterozygous hyperactivating *NLRP3* mutation which leads to excessive NLRP3 inflammasome activation. Similarly, as in our patients, most of the variants causative of NLRP3-AID are missense variants which accumulate in the part of the gene that encodes the NACHT and NAD domains of the NLRP3 protein (5). Mutation in this same gene region was detected in the Chinese family, which showed a variable phenotypic expression of DFNA34/MWS (13). In contrast, the two gene variants detected in the DFNA34 patients locate more toward the 3′ end (**Figure 2**) which encodes the LRR domain. Based on previous findings, it has been proposed that the *NLRP3* pathogenic variants affecting the LRR domain might be related to a milder phenotype (2, 15); this proposal might be expanded to indicate that the milder phenotype is also observed for *NLRP3* mutations affecting other parts of the gene.

In summary, the results of our study highlight the need to perform genetic testing in patients with HL. They show that the multi-organ autoinflammatory phenotype of NLRP3-AID may in fact be considered an "isolated" HL accompanied by marginal and difficult to recognize symptoms that can easily be overlooked. Efforts to identify patients carrying an *NLRP3* mutation have tangible practical value. They may help to explain not only the cause of HL but also other medical problems that have been initially neglected, and, uncommonly for genetically determined HL, the patients can be offered an effective pharmacological therapy. Individuals with NLRP3-AID should also be made aware of the variable expressivity of the disorder.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee at the Institute of Physiology and Pathology of Hearing. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DO and MLL performed genotyping and computational analysis. MO, DO, EW-S, MF, MOr, NB and HS participated in phenotyping and clinical data collection. AJ and TS performed functional studies. MO, DO, and TS analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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REVIEW
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SFRP1 Negatively Modulates Pyroptosis of Fibroblast-Like Synoviocytes in Rheumatoid Arthritis: A Review

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Secreted frizzled-related protein 1 (SFRP1) is a member of secretory glycoprotein SFRP family. As a primitive gene regulating cell growth, development and transformation, SFRP1 is widely expressed in human cells, including various cancer cells and fibroblast-like synoviocytes (FLS) of rheumatoid arthritis (RA). Deletion or silencing of SFRP1 involves epigenetic and other mechanisms, and participates in biological behaviors such as cell proliferation, migration and cell pyroptosis, which leads to disease progression and poor prognosis. In this review, we discuss the role of SFRP1 in the pathogenesis of RA-FLS and summarize different experimental platforms and recent research results. These are helpful for understanding the biological characteristics of SFRP1 in RA, especially the mechanism by which SFRP1 regulates RA-FLS pyroptosis through Wnt/β-catenin and Notch signaling pathways. In addition, the epigenetic regulation of SFRP1 in RA-FLS is emphasized, which may be considered as a promising biomarker and therapeutic target of RA.

Keywords: Secreted frizzled-related protein 1, rheumatoid arthritis, Wnt/β-catenin signaling pathway, Notch signaling pathway, pyroptosis, epigenetic

Abbreviations: RA, rheumatoid arthritis; SFRP1, Secreted frizzled-related protein 1; FLS, fibroblast-like synoviocytes; FZD, Frizzled; ADAM10, recombinant A Disintegrin And Metalloprotease 10; Axin, axon protein; TCF, T cell factor; LEF, lymphoid enhancer factor; LRP, low density lipoprotein receptor related protein; Treg, regulatory T cells; DSL, Diselenide–Selenoester Ligation; CSL, Calpastatin domain L; JAG1, Jagged1; NICD, notch intracellular domain; NF-κB, nuclear factor kappa-B; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; IL, interleukin; VEGF, vascular endothelial growth factor; GSK-3β, Glycogen synthase kinase-3β; HSC, hematopoietic stem cell; LPS, lipopolysaccharide; NLRP3, NOD-like receptor thermal protein domain associated protein 3; ASC, apoptosis-associated speck-like protein containing a C-terminal caspase activation and recruitment domain; GSDM, Gasdermin; CIA, collagen inducedarthritis; MMP-1, matrix metalloproteinase 1; CRD, cysteine-rich domain; c-JNK, c-Jun N-terminal kinase; OA, Osteoarthritis

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune-mediated chronic progressive disease and the synovium is the main target tissue. The main pathological features are persistent synovial hyperplasia and inflammation, which eventually lead to joint injury, cartilage destruction, and bone erosion (1, 2). Fibroblast-like synoviocytes (FLS) is a special group of cells in the synovial tissue. During the pathogenesis of RA, FLS show high proliferation, high invasiveness, and tumor-like changes, which play an important role in the development of RA (3, 4). Previous studies have shown that apoptosis and autophagy in FLS are closely related to the development of RA (5, 6). Unlike apoptosis, pyroptosis is a newly discovered form of programmed cell death that releases powerful immune cytokines such as IL-1β and IL-18 (7). In RA pathogenesis, the activation of inflammatory bodies, protease processing, and the release of inflammatory factors are related to abnormal synovium proliferation and bone destruction (8). Research on FLS pyroptosis may provide a new understanding of the pathogenesis of RA. The regulation of pyroptosis process can become a new treatment strategy for RA.

The Notch and Wnt/β-catenin signaling pathways are two highly conserved and functionally closely related pathways that coordinate and regulate cell growth, differentiation, and proliferation in various tissues and are involved in the development of various diseases (9). Secreted frizzled-related protein 1 (SFRP1) is a soluble protein that is highly restricted in tissue distribution. Part of its structure is highly homologous to the Frizzled (FZD) receptor of the Wnt/β-catenin signaling pathway; hence, it has the ability to bind to the Wnt protein and FZD receptor (10). Therefore, SFRP1 is considered a Wnt signaling pathway antagonist, which in turn interfere with Wnt signaling transduction and plays an important role in determining cell fate by regulating cell proliferation, differentiation, apoptosis, and pyroptosis (11). This regulation has also been studied in RA-FLS (12). In addition, some studies have found that SFRP1 can bind to recombinant A disintegrin and metalloproteinase 10 (ADAM10) protein and downregulate its activity in the Notch signaling transduction pathway, thus blocking the activation of Notch signaling (13).

One gene may act on different signaling pathways, and the regulatory role of SFRP1 in Wnt/β-catenin and Notch signaling pathways may be related to the pathological mechanisms of different diseases and may be associated with the process of pyroptosis. Functional connections among genes, signaling pathways, and pyroptosis may also exist in RA-FLS, and the crosstalk between different pathways may play an important role in the pathology and development of RA. In this review, we have added a new section on the pathological mechanisms of RA. This manuscript focuses on the relationship between SFRP1, Wnt/β-catenin signaling pathway, Notch signaling pathway, and pyroptosis, and their involvement in the pathogenesis of RA-FLS. The epigenetic regulation of SFRP1 could be a promising RA biomarker and a therapeutic target.

WNT/B-CATENIN SIGNALING PATHWAY REGULATES RA-FLS

The Wnt signaling pathway is a complex signal transduction network that plays an important role in maintaining a balance between human growth and development (14). The Wnt/β-catenin signaling pathway belongs to the classical Wnt signaling pathway and is one of the most important and well-studied signaling pathways (15). The main components involved in this pathway include β-catenin, axon protein (Axin), transmembrane receptors (LRP5/6 and FZD), and T cell factor/lymphoid enhancer factor (TCF/LEF) (16) (**Figure 1** and **Table 1**). The classical Wnt signaling pathway mainly regulates the stability and accumulation of β-catenin in cells. The important effect of the Wnt/β-catenin signaling pathway on RA is reflected in the regulation of FLS activation and bone metabolism (20, 21). In RA patients, the FLS division rate is faster than that in normal people, and hyperproliferative FLS is a key indicator in joint synovitis. Activated FLS can produce pro-inflammatory factors and matrix metalloproteinases, cause inflammatory cell infiltration and pannus formation, and lead to the persistent destruction of cartilage and bone (22, 23). Recent studies have shown that the an increased expression of proteins such as Wnt3a, Wnt5a, and Wnt10a in RA-FLS (**Table 1**), activates the Wnt signaling pathway and downstream genes, and increases the expression of fibronectin, thereby promoting cell proliferation, migration, and survival, and promotes RA synovial tissue proliferation in the absence of pro-inflammatory factors (24, 25). In addition, the Wnt/β-catenin signaling pathway regulates immune system homeostasis. In normal circumstances, β-catenin can improve the survival rate of regulatory T cells (Tregs), while activating the Wnt canonical pathway under inflammatory conditions may inhibit Treg function, leading to an autoimmune response (26). In conclusion, regulation of the Wnt/β-catenin signaling pathway in the pathogenesis of RA is multi-level and multi-faceted.

NOTCH SIGNALING PATHWAY REGULATES RA-FLS

The Notch signaling pathway is a conserved and important signal transduction pathway that affects cell fate. It is widely expressed in many species including vertebrates and invertebrates. It is highly evolutionarily conserved and influences the proliferation and differentiation of almost all cell types (27, 28). The classical Notch signaling pathway is mainly composed of Notch, Notch ligand (DSL protein or Jagged1), and CSL (DNA-binding protein). Through protease hydrolysis, Notch protein fragments (NICD or ICN) with transcriptional regulatory activity are released and then bind to the transcription factor CSL to regulate downstream gene expression (29) (**Figure 2**). The atypical Notch signaling pathway induces the expression of different genes through crosstalk with signaling pathways, such as NF-κB, Wnt, and TGF-β (30, 31). Neighboring cells can transmit signals through the binding of Notch receptors to ligands, thereby expanding and stabilizing

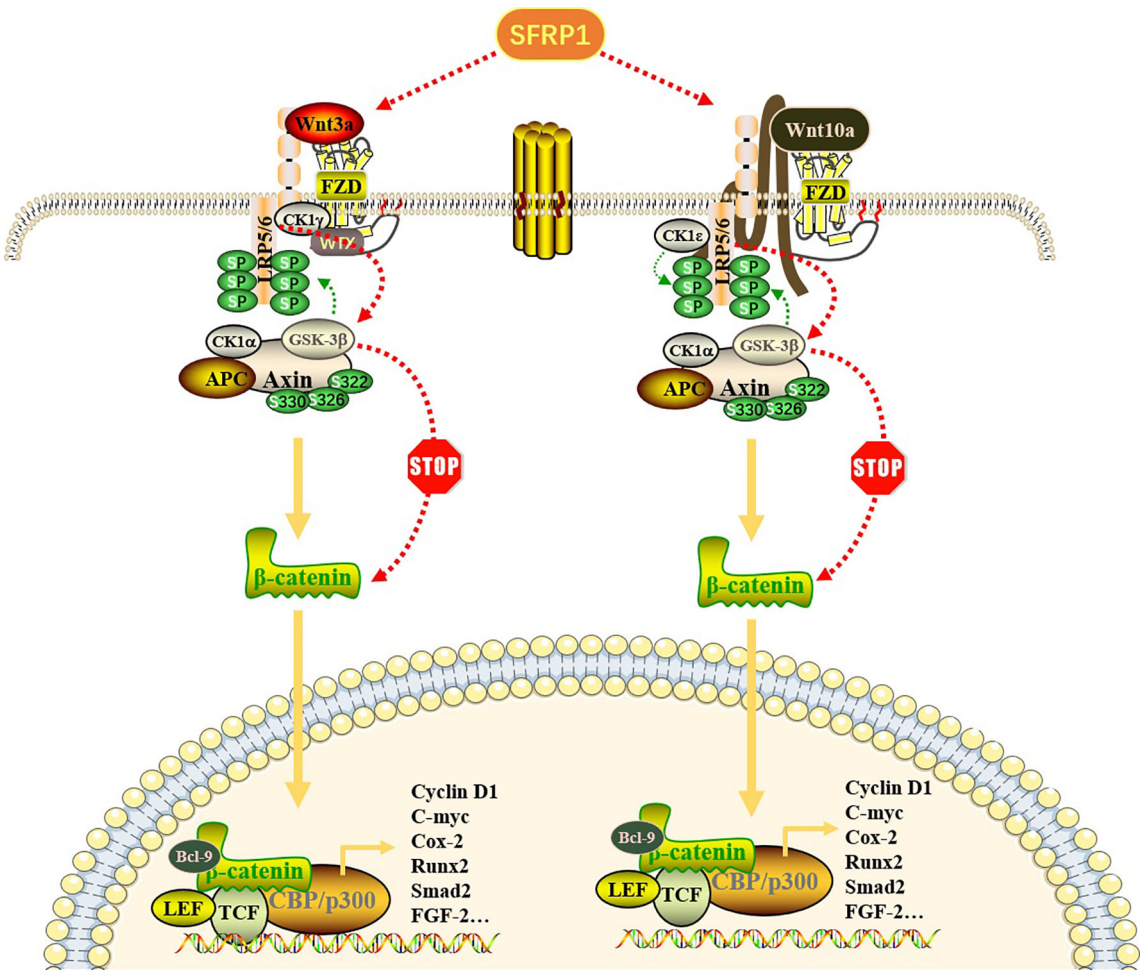

FIGURE 1 | Effect of SFRP1 on Wnt/β-catenin signaling pathway. Wnt proteins (Wnt3a and Wnt10a) bind to FZD proteins located on the cell membrane to form a Wnt-FZD complex, which further binds to low-density lipoprotein receptor-related protein (LRP) 5/6 co-receptors, resulting in its cytoplasmic tail phosphorylation. When the pathway is not activated, β-catenin binds to the "destruction complex" composed of APC, GSK-3β, CK1α and Axin to promote its ubiquitin degradation. Once this pathway is activated, Axin dissociates from the destruction complex and binds to the phosphorylation site in the cytoplasmic tail of LRP. With the repositioning of Axin on LRP, the β-catenin released by the destruction complex is transported to the nucleus in the form of phosphorylation and binds to transcription factors, especially TCF and LEF, thereby regulating gene transcription and expression of related target genes. SFRP1 binds Wnt ligands through its CRD, thus preventing it from binding to FZD receptors, eliminating the accumulation of β-catenin and blocking the expression of downstream genes.

molecular differences between cells, ultimately determining cell fate and affecting tissue and organ formation (32). Notch signaling is an important pathway for communication between adjacent cells, regulates cell development (33, 34), and plays an important role in the pathogenesis of RA. Previous studies have focused on Notch1 signaling pathway activation and downstream target gene regulation, which affect cell proliferation, migration, and other processes in RA-FLS (**Table 1**), and interfere with Notch1 *via* siRNA to exert therapeutic effects (35–37). A recent single-cell RNA-sequencing study of synovial tissue found (38) that the expression of Notch3 and its downstream target genes was significantly upregulated in RA-FLS (**Table 1**). Notch3 signaling can drive both transcriptional and spatial gradients in FLS, contributing to the differentiation of FLS subtypes, and blocking this pathway helps attenuate arthritis development. In a mouse model, deletion of Notch3 or blockade of Notch3 signaling has also been shown to prevent joint damage in inflammatory arthritis. It is noteworthy that the ADAM10 protein, which is an important regulator of the Notch pathway, is involved in a variety of biological functions, including inflammation, apoptosis, cancer development, and autoimmunity (39). In the process of Notch signal transduction, binding of Notch with Notch ligands initiates proteolysis of the extracellular domain mediated by ADAM10, and induces the transcription of Notch target genes through a series of complex biological reactions, thus regulating the process of growth and development (40, 41). Comparing 292 osteoarthritis (OA) patients with healthy individuals, recent study has shown that the expression of ADAM10 in endothelial cells and FLS in RA biopsies is upregulated, suggesting that ADAM10 may be involved in the pathological development of RA (42). *In vitro* experiments showed

TABLE 1 | Differentially expressed genes involved in Wnt/β-catenin, Notch signaling pathway and pyroptosis in RA-FLS *(P<0.05)*.

Name	Gene	Beta	Reference
Wnt/β-catenin signling pathway	SFRP1	↓	GSE55457 (17)
	FZD1	↑	GSE55584 (17)
	FZD2	↑	GSE55235 (17)
	FZD6	↑	GSE89408 (18, 19)
	WNT5A	↑	
	TCF7	↑	
	LEF1	↑	
	MYC	↓	
	MAPK8	↓	
	BCL9	↑	
Notch signalling pathway	NOTCH1	↑	
	RBPJ	↑	
	HDAC1	↑	
	ADAM10	↑	
Pyroptosis	NLRP3	↑	
	CASP1	↑	
	CASP4	↑	
	CASP5	↑	
	GSDMD	↑	
	IL18	↑	

Beta↑ means high expression in RA-FLS, if beta↓, the gene is down-regulated in RA-FLS.

that lowering the expression of ADAM10 by siRNA could inhibit the release of the proinflammatory cytokines TNF-α, IL-6, and IL-8 (43), improve the symptoms of arthritis, and reduce the level of vascular endothelial growth factor (VEGF) (42, 44). These findings suggest that inhibition of ADAM10 may effectively treat RA by inhibiting pro-inflammatory signal transduction and pannus formation in FLS. In addition to the importance of ADAM10 in Notch signaling, some substrates are related to ADAM10, such as Jagged1/2 (45). These results provide a molecular basis for targeted therapy of RA by regulating the Notch signaling pathway (46).

WNT/B-CATENIN AND NOTCH SIGNALING PATHWAY CROSSTALK

Wnt/β-catenin and Notch signaling pathways have been independently shown to play a key role in regulating cell fate (47, 48). More evidence showed that there was a complex functional relationship between Wnt/β-catenin and Notch signaling (49). For example, GSK-3β kinase is responsible for the phosphorylation and inactivation of β-catenin in Wnt signaling pathway and can inhibit the transcription of Notch target genes by phosphorylating Notch2 (50, 51). Crosstalk between Wnt/β-catenin and Notch signaling can be observed in many different systems. In hematopoietic stem cell (HSC) formation, recent evidence suggests that Wnt signaling is helpful for Notch activity and formation by regulating the transcription of pre-embryonic Notch ligands, and the stability of undifferentiated HSC mediated by Wnt requires complete Notch signaling (52). In epidermal cells, β-catenin stimulates Notch signaling by inducing Jagged1 transcription, suggesting that the Notch signaling pathway plays a role downstream of the Wnt/β-catenin signaling pathway and determines the transformation of epidermal cells (53). In addition, many

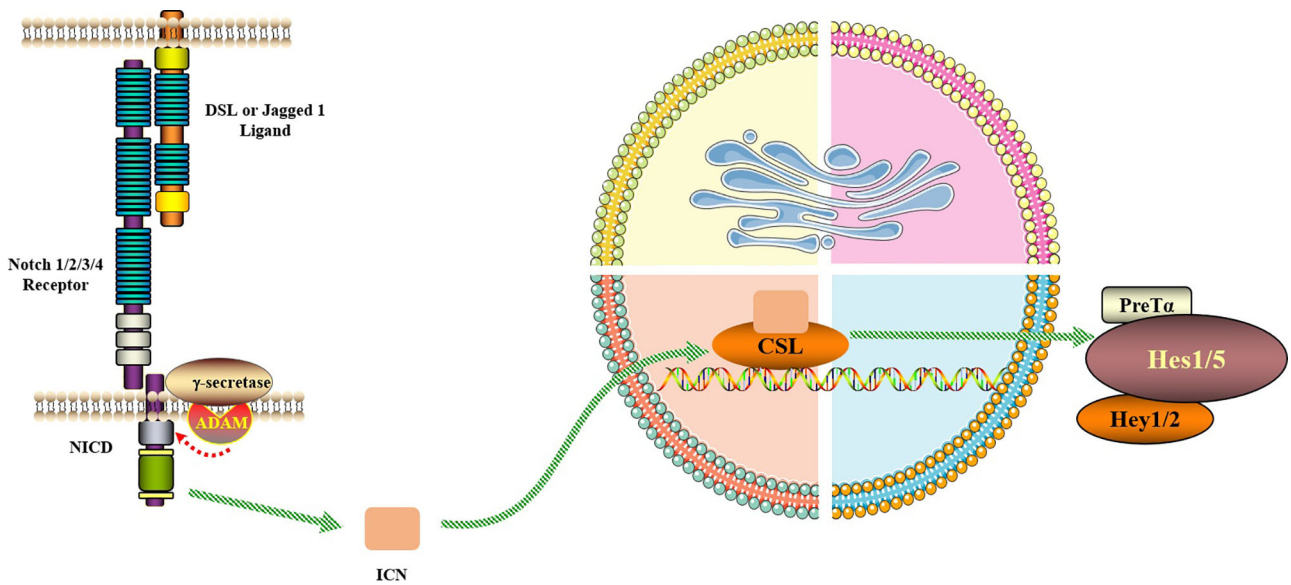

FIGURE 2 | Notch signaling pathway. The Notch signaling pathway consists of Notch, Notch ligand (DSL or Jagged1/2) and CSL (a class of DNA-binding proteins, include RBPJ). Notch signaling is generated by the interaction of Notch ligands of neighboring cells with the receptor, initiating ADAM10-mediated proteolysis of the extracellular domain. After three times of cleavage, Notch protein is released into the cytoplasm from the NICD and enters the nucleus to bind to the transcription factor CSL to form the NICD/CSL transcriptional activation complex, which activates the target genes of the transcriptional inhibitory factor family such as Hes and Hey, and plays a biological role.

studies have elucidated the functional link between Wnt/β-catenin and Notch (53–57), however, this aspect has not been studied in details. At present, the interaction between the Wnt and Notch signaling pathways and its effect on the development of RA-FLS has not been documented. Whether there are biological mechanisms, such as the regulation of Notch2 by GSK-3β phosphorylation described earlier in RA-FLS, requires further research (13). SFRP1 can bind to ADAM10 metal protein in the Notch signaling pathway and downregulate its activity, thus blocking the activation of Notch signaling. Moreover, SFRP1 is also a suppressor gene of the Wnt/β-catenin pathway, which indicates that SFRP1 can inhibit both the Wnt/β-catenin and Notch signaling pathways. Can SFRP1 target RA through double inhibition? This was the direction of our team's follow-up research.

PYROPTOSIS MECHANISM OF RA-FLS

Pyroptosis, also known as the inflammatory necrosis of cells, is a type of programmed cell death. The process depends on the caspase, NOD-like receptor (NLR), and Gasdermins (GSDMs) protein families, accompanied by the release of a large number of pro-inflammatory factors, such as IL-1β and IL-18 (58). Pyroptosis is mainly characterized by the continuous expansion of cells until cell membrane ruptures, resulting in the release of cell contents and activation of a strong inflammatory response. The activation patterns include classical pathways mediated by activated inflammasomes, such as the NLRP3, and caspase-1, and non-classical pathways mediated by bacterial lipopolysaccharide (LPS) and caspase-4/5/11. In the classical pathway, activated inflammasomes can promote the self-cleavage of procaspase-1 into active caspase-1, which can cause the release of proinflammatory factors, such as IL-1β and IL-18, and cause GSDMD protein cleavage and pyroptosis. In the non-classical pathway, caspase-4/5/11 can directly recognize the oligomerization of bacterial LPS, thus causing pyroptosis by the cleavage of GSDMD (59–61) (**Figure 3** and **Table 1**). Studies have shown that excessive proliferation and pyroptosis of FLS play a key role in joint destruction and persistent inflammation in RA, and this pathological process is closely related to the participation of abnormal NLRP3 inflammasomes (NLRP3, ASC, and caspase-1 complex) (62). Increased levels of inflammatory cytokines such as IL-1β, TNF, IL-18, and IL6 in the serum and synovial fluid of patients with active RA were obtained and positively correlated with the level of NLRP3 (63, 64) (**Table 1**). In the CIA animal model, it was also found that the expression of the NLRP3 inflammasome was upregulated in synovial FLS, accompanied by a significant increase in MMP-1 levels in the supernatant. Upregulated NLRP3 can also promote the maturation and increase secretion of IL-1β and IL-18 through the cleavage of caspase-1 (65, 66) (**Figure 3**). In addition, IL-18 can induce FLS to secrete osteoclast cytokines, which play a role in bone resorption (67). These studies clearly show that inflammasomes and their downstream cytokines, IL-1β and IL-18, are involved in the pathogenesis of RA-FLS. Therefore, it is possible to block the activity of the NLRP3 inflammasome by blocking NLRP3, thus inhibiting pyroptosis of FLS, which will be discussed below.

SFRP1 REGULATES DUAL SIGNALING PATHWAYS TO MEDIATE RA-FLS PYROPTOSIS

SFRP is a protein that can be folded into two independent domains, the N-terminal and C-terminal domains. The N-terminal cysteine-rich domain region (CRD) can bind to FZD receptors through disulfide bonds; therefore, SFRP1 can act as a regulator of the Wnt/β-catenin signaling pathway (68, 69). Given the critical role of the Wnt/β-catenin signaling pathway in the development of RA pathology, it is possible to block Wnt signaling pathway molecules to reduce the expression of inflammatory factors in RA synovial cells, including some secreted SFRP proteins which may inhibit inflammation by competitively binding to Wnt protein and down-regulating c-Jun N-terminal kinase (c-JNK) (70). In the SFRP family, SFRP1 has been widely studied in RA patients. SFRP1 negatively regulates the Wnt/β-catenin signal transduction pathway (71). SFRP1 interacts with Wnt protein or FZD receptor to eliminate the accumulation of β-catenin and block the expression of downstream genes by isolating Wnt, which is useful in inhibiting many biological processes, such as proliferation and apoptosis of RA-FLS (72, 73) (**Figure 1**). In addition, in the review by Claudel et al., researchers introduced in detail the role of sFRPs family members in cancer, bone and joint diseases, and summarized the different roles of each SFRP in pathophysiology, which have different effects on Wnt signaling pathway and different inflammation-related signals. Especially in the control of inflammatory response of RA, reducing the level of sFRP1 is a promising way to control the differentiation of Th17, especially when biological agents are ineffective. At the same time, it is also described that the expression of sFRPs in inflammatory synovium is regulated by epigenetics, and any decrease in sFRPs level may lead to self-persistence of joint inflammation (74). A similar inhibition was also observed in the Notch signaling pathway (13). In RA-FLS, SFRP1 can bind to the ADAM10 metal protein of the Notch signaling pathway and downregulate its activity, thus blocking the activation of Notch signaling. Of course, among the targets of Notch signaling pathway, the down-regulation of ADAM10 is not the only mechanism to interfere with Notch signaling pathway. It is possible that SFRP1 interacts with other targets to interfere with the Notch pathway, so the down-regulation of ADAM10 is one of the possibilities, and more findings need to be further studied. In addition, RNA-sequence was detected in synovial tissues of patients with RA and OA. The results of multiple studies showed that SFRP1 was expressed at low levels in RA (**Table 1**). Therefore, blocking downstream signaling pathways and genes by upregulating the expression of SFRP1 in RA-FLS would be helpful in the treatment of RA.

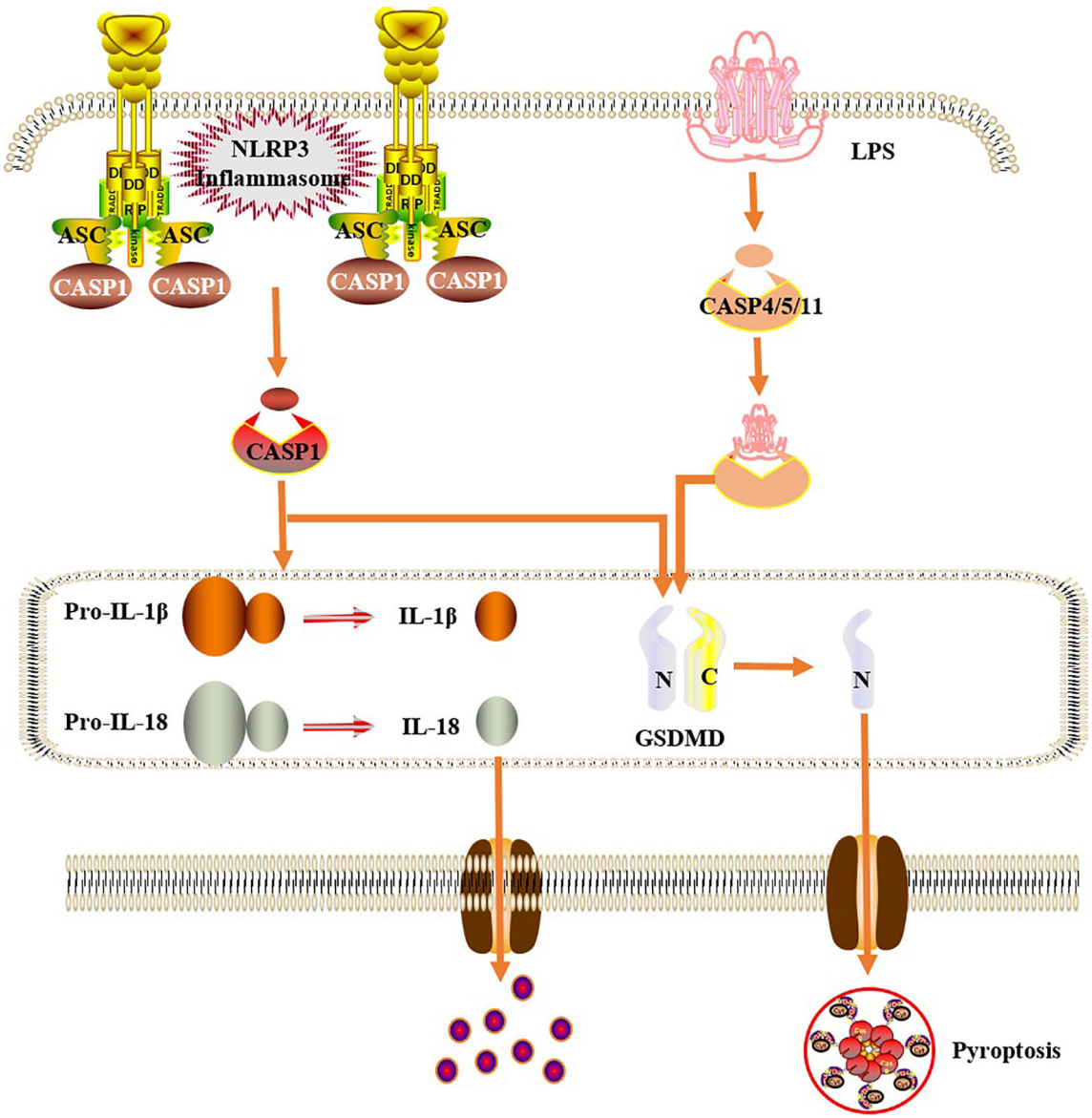

FIGURE 3 | Cells pyroptosis mechanism. In the classical pathway, under the stimulation of bacteria, viruses and other signals, intracellular NLRs act as sensors to recognize these cognate ligand signals, and combine with the precursor of caspase1 through the adaptor protein ASC to form a multi-protein complex and activate caspase-1. Activated caspase-1 cleaves GSDMD to form a peptide segment containing the nitrogen-terminal active domain of GSDMD, which induces cell membrane perforation, cell rupture, release of contents, and inflammatory response. Moreover, activated caspase-1 also cleaves the precursors of IL-1β and IL-18 to form an active structure, which is released outside the cell, recruiting inflammatory cells to gather, expanding the inflammatory response and mediating cell pyroptosis. In the non-classical pathway, human caspase-4,5 and mouse caspase-11 can be directly activated by contact with bacterial LPS, then cleave GSDMD, and indirectly activate caspase-1, causing pyroptosis.

There is also a relationship between the Wnt/β-catenin and Notch signaling pathways and cell pyroptosis. Studies have shown that β-catenin interacts with NLRP3 and promotes its binding and that of ASC, thus activating the NLRP3 inflammasome and initiating the subsequent process of cell pyroptosis. When siRNA was used to inhibit β-catenin expression, activation of the NLRP3 inflammasome was also observed (75). This reveals a new role of β-catenin in the activation of the NLRP3 inflammasome and suggests that there is endogenous signaling crosstalk between the Wnt/β-catenin signaling pathway and NLRP3 inflammasome. Similarly, the relationship between the activation of Notch1 and NLRP3 was confirmed in the Notch signaling pathway. The expression levels of Notch1, NLRP3, and pro-inflammatory cytokines were detected in skin scar fibroblasts when compared to normal counterparts. The results showed that the expression levels of

Notch1 and NLRP3 were higher in skin scar fibroblasts than in normal fibroblasts. Inhibition of Notch1 expression by siRNA transfection significantly inhibited the expression of NLRP3 inflammasome and related pro-inflammatory factors (76). These results confirm that Notch1 is a novel factor that activates the NLRP3 inflammasome. Inhibition of Notch1 can downregulate the activation of NLRP3, slow down chronic tissue injury and fibroblast differentiation in skin scars, and regulate the innate immune response.

In summary, although the regulation of NLRP3-mediated cell pyroptosis by the Wnt/β-catenin and Notch signaling pathways has not been carried out in RA-FLS, its key role is based on observation in other diseases. We propose the following hypothesis: in RA-FLS, SFRP1 participates in NLRP3-mediated cell pyroptosis by regulating the Wnt/β-catenin and Notch signaling pathways (**Figure 4**). Moreover, methylation detection in synovial tissue from knee joint in patients with RA and OA showed that several methylation sites of SFRP1 were hypermethylated in the synovial tissue from RA patients (77, 78). Therefore, the inhibition of hypermethylation of SFRP1 by methylation inhibitors is helpful in upregulating the expression of SFRP1, competitively inhibiting the signal transduction of Wnt/β-catenin and Notch, the release of downstream inflammatory cytokines, and NLRP3-mediated cell pyroptosis, thus playing a role in the treatment of RA.

DISCUSSION

FLS have always been considered an attractive therapeutic target for RA. However, no treatment that directly targets FLS has been approved. In this review, we systematically explain how SFRP1, Wnt/β-catenin signaling, Notch signaling, and cell pyroptosis independently affect the development of RA-FLS. Based on these theories, we propose that in RA-FLS, SFRP1 participates in NLRP3-mediated pyroptosis by regulating the Wnt/β-catenin and Notch signaling pathways, thereby affecting the progression of RA. Moreover, a preliminary study showed that SFRP1 was hypermethylated in RA synovial tissues. Thus, SFRP1 may serve as a potential target for RA treatment. Through the promotion of SFRP1, it is highly expressed in RA-FLS, so as to observe whether it can inhibit the activation of Wnt/β-catenin and Notch signaling pathway and the occurrence of pyroptosis, whether it

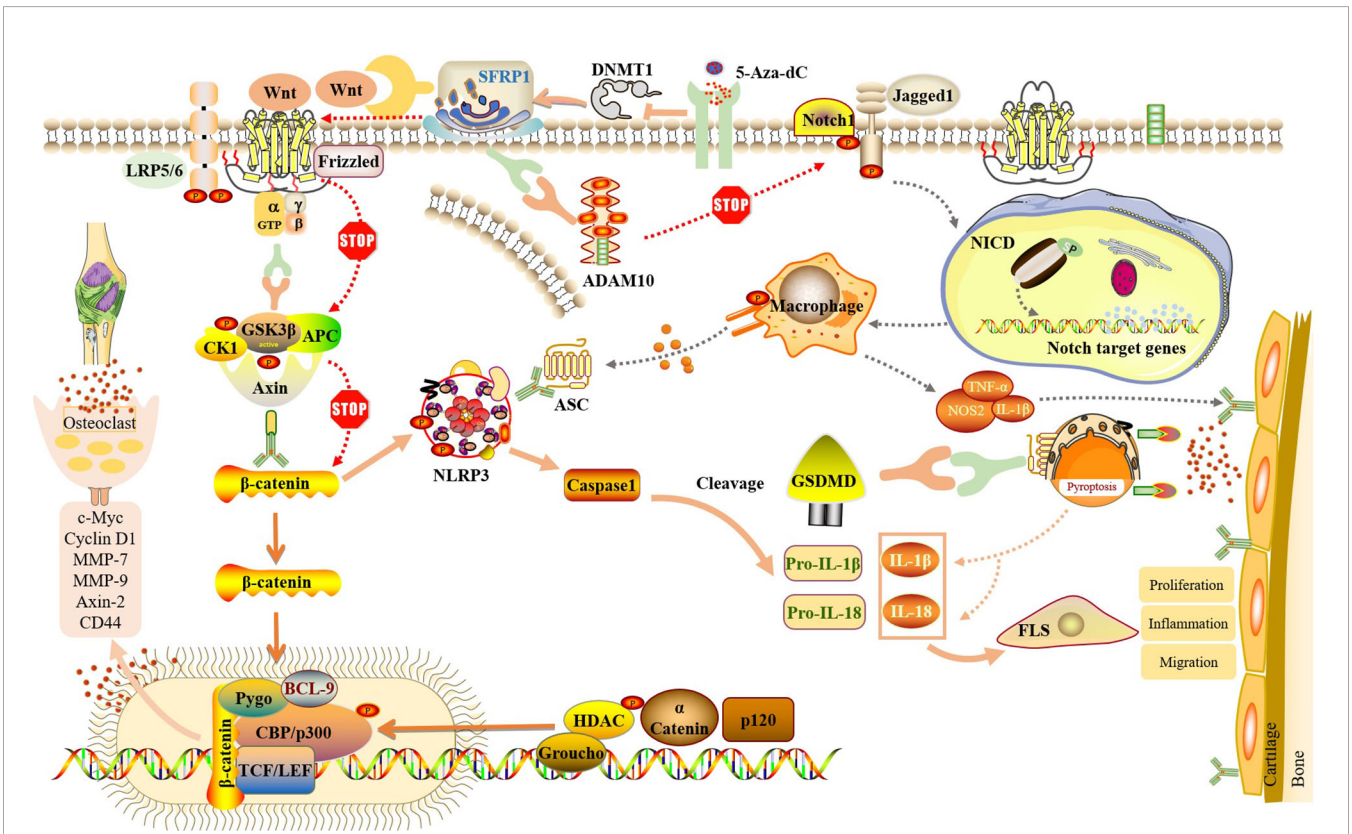

FIGURE 4 | SFRP1 participate in NLRP3-mediated cell pyroptosis by regulating the dual signaling pathways of Wnt/β-catenin and Notch. By binding to Wnt protein and ADAM10 protein, SFRP1 negatively regulates Wnt/β-catenin and Notch signaling, blocks the activation of downstream proteins and the release of inflammatory factors, and reduces the inflammatory response of FLS and the destruction of articular cartilage. And indirectly inhibit the activation of NLRP3 inflammasome and block the occurrence of cell pyroptosis. Methylation inhibitor of 5-Aza-dC could inhibit the expression of DNMT, release SFRP1 hypermethylation and up-regulate the expression of SFRP1 in RA-FLS, thus negatively regulating Wnt/β-catenin and Notch signaling pathways.

can improve the inflammatory microenvironment of joint synovium and alleviate the symptoms of RA, which will be our main research work. In addition, to verify this hypothesis, our team is also planning to use traditional Chinese medicine, *Tripterygium wilfordii* Hook F, or methylation inhibitors and further develop drugs that potentially target SFRP1 to fill in the gaps related to RA-FLS therapy.

AUTHOR CONTRIBUTIONS

PJ and KW was responsible for the collection, collation of data, and writing of the original manuscript. CC, JZ, RZ, LXX, YJ, LSX, and YS were accountable for collection of data. SG, SS and DH were responsible for concept development and manuscript revision. All authors reviewed and accepted the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Gene Expression Signatures Reveal Common Virus Infection Pathways in Target Tissues of Type 1 Diabetes, Hashimoto's Thyroiditis, and Celiac Disease

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Type 1 diabetes (T1D) patients are at heightened risk for other autoimmune disorders, particularly Hashimoto's thyroiditis (HT) and celiac disease (CD). Recent evidence suggests that target tissues of autoimmune diseases engage in a harmful dialogue with the immune system. However, it is unclear whether shared mechanisms drive similar molecular signatures at the target tissues among T1D, HT, and CD. In our current study, microarray datasets were obtained and mined to identify gene signatures from disease-specific targeted tissues including the pancreas, thyroid, and intestine from individuals with T1D, HT, and CD, as well as their matched controls. Further, the threshold-free algorithm rank-rank hypergeometric overlap analysis (RRHO) was used to compare the genomic signatures of the target tissues of the three autoimmune diseases. Next, promising drugs that could potentially reverse the observed signatures in patients with two or more autoimmune disorders were identified using the cloud-based CLUE software platform. Finally, microarray data of auto-antibody positive individuals but not diagnosed with T1D and single cell sequencing data of patients with T1D and HT were used to validate the shared transcriptomic fingerprint. Our findings revealed significant common gene expression changes in target tissues of the three autoimmune diseases studied, many of which are associated with virus infections, including influenza A, human T-lymphotropic virus type 1, and herpes simplex infection. These findings support the importance of common environmental factors in the pathogenesis of T1D, HT, and CD.

Keywords: type 1 diabetes, Hashimoto's thyroiditis, celiac disease, gene expression signatures, target tissues

INTRODUCTION

Type 1 diabetes (T1D), with a worldwide increasing incidence, is characterized by the autoimmune destruction of pancreatic beta cells, resulting in life-long insulin dependency (1). Aside from islet autoimmunity, patients with T1D are at heightened risk for other autoimmune disorders, particularly Hashimoto's thyroiditis (HT) and celiac disease (CD), both of which are organ-targeted autoimmune

diseases (2–4), implying common risk factors and molecular mechanisms among these three autoimmune diseases.

Although the pathogenesis of autoimmune diseases is not completely understood, both environmental and genetic factors have been linked to its pathogenesis, which is marked by the development of specific autoantibodies and the existence of autoreactive T cells. Genetically, these three autoimmune diseases share similar genetic predispositions with similar human leukocyte antigen (HLA) susceptibility (5). However, with the rising prevalence of autoimmune diseases, common environmental risk factors for T1D, HT and CD should be emphasized. Whether shared environmental mechanisms drive identical molecular mechanisms at target tissues among T1D, HT, and CD is unclear.

Increasing evidence suggests that target tissues of autoimmune diseases engage in a harmful dialogue with the immune system instead of being innocent bystanders of immunological attacks (6). In this sense, a study focusing on different tissues of these autoimmune diseases could aid in identifying essential pathways that could be focused for therapy, such as repurposing drugs that are already in clinical use for other diseases. Based on this, we hypothesize that key environmental mechanisms may drive similar molecular signatures in T1D, HT, and CD at the target tissue level. To test this hypothesis, we obtained microarray datasets generated from disease-specific targeted tissues including the pancreas, thyroid, or intestine from individuals with T1D, HT, and CD respectively, along with their control subjects. These data were dug to identify similar and dissimilar transcriptomic signatures. Then, we searched for drugs that could potentially reverse the observed signatures in patients with two or more autoimmune disorders. Our findings revealed significantly similar transcriptomic signatures in the target tissues of these three autoimmune diseases studied, many of which are associated with virus infections, including influenza A, human T-lymphotropic virus type 1 (HTLV-1), and herpes simplex infection. These findings support the importance of common environmental factors in the pathogenesis of T1D, HT, and CD.

METHODS

Microarray Data

To identify shared transcriptomic signatures in T1D, HT, and CD at the target tissue level, three gene expression profiles by array were selected from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). The T1D dataset GSE72492 (7) contained pancreas samples from 10 T1D patients, 6 auto-antibody positive individuals (AP) but not diagnosed with T1D and 7 normal controls (NC). In the HT dataset GSE138198 (8), data from 13 HT thyroid samples and three normal thyroid samples were used for analysis. The CD dataset GSE164883 (9) included intestinal tissues samples from 25 CD patients and 21 normal controls. Two CD patients were sampled twice and all 48 samples were used for further analysis. The HLA genotype of the T1D dataset and basic information of the three datasets were obtained from the corresponding original article and presented in the **Table 1 part**.

Single Cell RNA-Seq Data and Processing

To validate our findings in the microarray data, we obtained single cell RNA-seq data (RRID: SCR_016202) from the Human Pancreas Analysis Program (HPAP-RRID: SCR_016202) Database (https://hpap.pmacs.upenn.edu) (10). CellRanger standard output files were downloaded containing data from islet samples of one patient with T1D and HT (HPAP-032) and one normal control (HPAP-039) matched for race and gender. Seruat 4.0 package in R was used to read the CellRanger standard output file for analysis. Cells are considered abnormal if: (i) gene number was <200 or >4000; (ii) over 25% of the detected genes were mitochondrial genes. Abnormal cells in all datasets were filtered out. The DEGs criteria were as follows using the Find All Markers function (Searut package): (i) logFC>0.585; (ii) *p*-value<0.05; (iii) min.pct>0.25. The GSEA enrichment analysis of DEGs of single cell RNA-seq was performed using OmicStudio tools (https://www.omicstudio.cn/tool).

Differentially Expressed Genes Analysis

To identify the DEGs responsible for T1D, HT, and CD, the matrix files of three datasets were downloaded from GEO and normalized and annotated using the online tool NetworkAnalyst 3.0 (11) (https://www.networkanalyst.ca/). Probe IDs were transformed into official gene symbols. Probes with no gene symbol name were deleted. Multiple probes related to the same gene were deleted and summarized as the average value for further analysis. The limma package in R was used to perform DEGs analysis by comparing patients with normal controls. For DEGs identification, $|\log2 \text{ fold change (FC)}| \geq 0.585$ and p-value <0.05 were considered statistically significant. An overlap between the three lists of genes was then performed and represented as a Venn diagram.

Risk Genes Identification

To explore the association between DEGs and genetic variation polymorphism in target organs is related to genetic variation polymorphism, and to describe the influence of genetic factors in autoimmune diseases, the genome-wide association studies (GWAS) catalog (12) (www.ebi.ac.uk/gwas/; consulted December 2021) was used to identify the risk genes connected with each disorder. The risk genes were identified based on the following criteria: (i) T1D, HT, and CD as the disease assessed by the study; (ii) a *p-value* of $< 1 \times 10^{-6}$ for the lead single-nucleotide polymorphism (SNP). (iii) Choosing the recorded genes linked to the lead SNP described by the original study;(iv) assessing the expression of the recorded genes in the target tissue. An overlap between risk genes and DEGs in each disease and an overlap between the three lists of risk genes were represented as a Venn diagram.

Rank-Rank Hypergeometric Overlap Analysis

To compare the transcriptomic signatures of the target tissues of T1D, HT, and CD, overlaps between the differential expression of two ranked lists were visualized and measured using online RRHO tools (https://systems.crump.ucla.edu/rankrank/index.php) (13). Briefly, all expressed genes from three microarray datasets were ranked according to fold change value. Then, these ranked lists were iteratively assessed for intersection. Finally, the results were

visualized as a heatmap colored by the logarithmic transformation's hypergeometric p value. The p value assessed the significance of overlapping genes at each rank threshold pair so that the highest point on the map identified the overlapping genes with the most statistical significance. Genes overlapping at this optimal rank threshold pair in all of the three probable pairing combinations were listed and assessed further for involvement in specific biological characteristics and signaling pathways.

Functional Enrichment Analysis

To identify the biological function of DEGs of three microarray datasets and overlapping genes in RRHO analysis, enrichment analysis of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was performed using the online tool Database for Annotation, Visualization and Integrated Discovery (DAVID) 6.8 (14) (https://david.ncifcrf.gov/). All KEGG terms with gene count ≥ 2 and p-values < 0.05 were visualized.

Therapeutic Target Identification

To explore drugs or compounds that target the shared pathways in autoimmune diseases, we selected the top 150 up-regulated and down-regulated genes between two diseases for each RRHO analysis result and analyzed them with the Connectivity Map dataset on the cloud-based CLUE software platform (15) (version: 1.1.1.43, https://clue.io). This database allowed us to find compounds that drive down the input gene lists and identify potential drugs for treating one or more autoimmune diseases. Only classes with a median tau score < −85 were considered as potential target candidates.

RESULTS

Transcriptomic Signatures in the Target Tissues of Different Autoimmune Diseases Reveal the Interaction Between Genetic and Environmental Factors

To identify transcriptomic signatures in the target tissues of T1D, HT, and CD, differential expression analysis was performed in these three datasets respectively. The metadata of the tissue donors analyzed in the present study was shown in **Table 1**. In three datasets, there were more up-regulated DEGs than down-regulated DEGs in the target tissue, which was especially obvious in the CD dataset (**Figures 1A–C**). KEGG pathway enrichment analysis of these DEGs indicated similarities and differences between these three autoimmune diseases. The top up-regulated and down-regulated KEGG pathways were shown in **Figures 1D–F**. In the T1D dataset, up-regulated DEGs were primarily enriched in inflammation and metabolism, such as cytokine-cytokine receptor interaction, TGF-beta signaling pathway, and glycolysis/gluconeogenesis. Both up-regulated DEGs in HT and CD were enriched in autoimmunity and virus infection among the top 10 pathways, such as antigen processing and presentation, graft-versus-host disease, allograft rejection, type 1 diabetes, and intestinal immune network for IgA production, and viral myocarditis. All down-regulated DEGs in three datasets highlighted the metabolic related pathways, with protein digestion and absorption and fat digestion and absorption in the T1D dataset, fatty acid degradation and fatty acid metabolism in the HT dataset, and metabolic pathways in the CD dataset. In summary, differential expression analysis and enrichment analysis suggest autoimmunity, metabolism, and virus infection might be common pathways in these three diseases.

In order to explore the genetic impact on the target tissues, the risk genes of each disease were identified using the genome-wide association study (GWAS) catalog. Our results showed that 50-70 percent of these risk genes were expressed in target organs, while less than 30 percent of the risk genes overlapped with DEGs in each disease (**Figures S1A–C**). Overlapping of the risk genes of these three diseases revealed that only eight risk genes were in the intersection, namely, *ATXN2*, *ICOS*, *CTLA4*, *BACH2*, *HLA-DQA1*, *STAT4*, *SH2B3*, and *RNU6-474P*, among which *ICOS* and *HLA-DQAI* are DEGs in the three autoimmune diseases (**Figure S1D**). These findings indicate that other factors not only genetic factors play important roles in the occurrence and development of autoimmune diseases.

TABLE 1 | Summary of the metadata for the microarray samples of the three autoimmune diseases.

Disease	Target tissue	Samples (n)		Age (mean ± SD)		Gender (Female%)		Nationality	Disease severity	Platforms	Experiment type	Source
		Controls	Patients	Controls	Patients	Controls	Patients					
T1D	Pancreatic tissue	7	10	24.57 ± 10.10	27.70 ± 7.23	100%	30%	USA	c-peptide (ng/ml): (NC:4.91 ± 2.86; T1D:0.36 ± 0.99)	GPL14550	Expression profiling by array	GSE72492
HT	Thyroid tissue	3	13	NA	47.85 ± 12.58	NA	92%	Germany	NA	GPL6244	Expression profiling by array	GSE138198
CD	Duodenal probes data	21	25	11.29 ± 4.91	9.28 ± 4.89	76%	60%	Germany	marsh stage:(NC:0-1; CD:3A-3C)	GPL10558	Expression profiling by array	GSE164883

Microarray data from three autoimmune disease studies of target tissues were obtained from the Gene Expression Omnibus (GEO) database (https://ncbi.nlm.nih.gov/geo/), reanalyzed, and quantified using the online tool NetworkAnalyst (https://www.networkanalyst.ca/). NA, not available. NC, normal control.

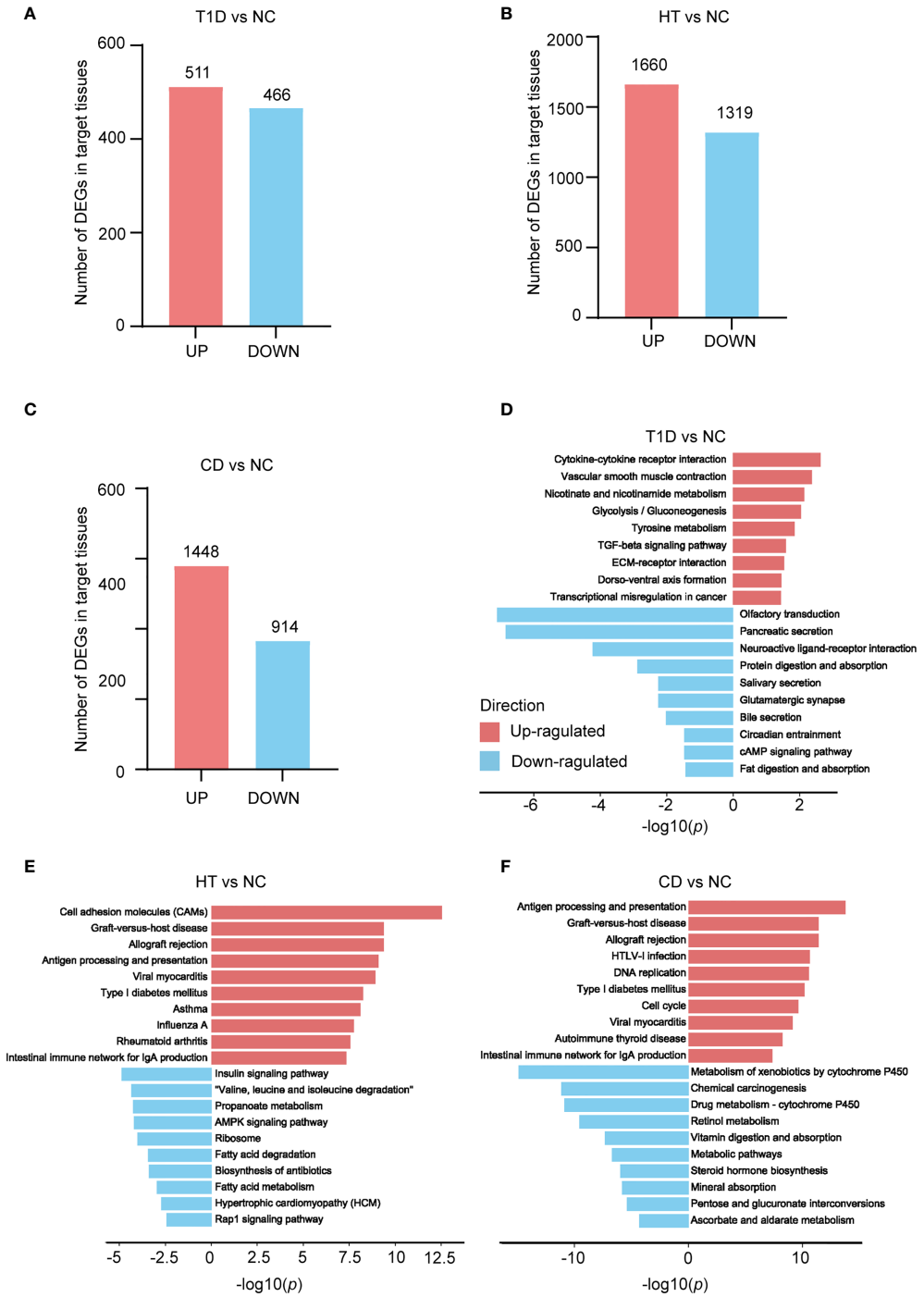

FIGURE 1 | Depicts a summary of the count of differentially expressed genes (DEGs) and KEGG pathways found in the target organs of three autoimmune disorders. **(A–C)** The number of genes that differ in expression in three autoimmune diseases. The numbers within the bars indicate the count of genes with |fold change| greater than 1.5 and a *p* value less than 0.05. **(D–F)** KEGG enrichment analysis of T1D **(D)**, HT **(E)**, and CD **(F)** DEGs, in comparison to its own control set of individuals respectively. The red and blue bars indicate positive and negative enrichment in the associated pathway, respectively. The x axis represents the -log10 (p) of the enrichment analysis, and the y axis represents the enriched pathways.

RRHO Analysis of Autoimmune Diseases Indicates Up-Regulation of Viral Infection-Related Pathways

To study the common molecular mechanisms of these three autoimmune diseases in the target tissues, we analyzed the overlapping DEGs among these three datasets. However, there were only 17 overlapped DEGs, with 16 up-regulated genes (*CD38*, *IFI27*, *IFI16*, *XAF1*, *ITK*, *HLA-DQA1*, *MEI1*, *ICOS*, *GBP5*, *THEMIS*, *KLRB1*, *ANXA2R*, *GBP2*, *HK1*, *IRF8*, and *IL2RB*) and one down-regulated gene(*ACBD4* **Figures S2A–B**). These genes were mostly enriched in immune and inflammatory related pathways, such as the T cell receptor signaling pathway, immune response, IFN-γ signaling pathway, and type 1 interferon signaling pathway (**Figure S2C**). A limitation of this method is that we focused on the DEGs that pass a fixed statistical threshold, making the results greatly influenced by the number of samples studied.

To investigate if these common pathways were present before disease onset, 6 auto-antibody positive high risk individuals in the T1D dataset were used for functional enrichment analysis. The detailed clinical and HLA genotype information were presented in **Tables S1**, **S2**, which showed normal controls, auto-antibody positive individuals were well matched for HLA genotypes to the patients with T1D. Interestingly, autoimmune, metabolic, and virus infection related pathways were altered even in the auto-antibody positive phase (**Figures S3A–D**), implying the shared transcript footprint in autoimmune diseases exists before disease onset.

To achieve more detailed information on the similarities between different autoimmune diseases with an unbiased approach, we performed RRHO analysis (**Figures 2A–C**), which is a threshold-free algorithm that identifies trends of overlap between two ranked lists of genes according to fold change value. The main similarities between the diseases were observed both among the common up-regulated genes and down-regulated genes. Strikingly, the common upregulated genes between HT and CD had the highest logarithmic transformation's hypergeometric *p* value. This finding is consistent with the above-described observation that up-regulated DEGs in HT and CD were enriched in several same pathways among the top 10 pathways. The KEGG pathway enrichment analysis of these up-regulated overlapping pathways demonstrated concordance for viral infection associated pathways (**Figures 3A, C, E**), including herpes simplex, HTLV-1, influenza A, and viral myocarditis. The common down-regulated genes were mainly involved in metabolism, including beta-alanine metabolism, adrenergic signaling in cardiomyocytes, cGMP-PKG signaling pathway, and neuroactive ligand-receptor (**Figures 3B, D, F**).

To further validate the shared pathways in the above RRHO analysis, we obtained a single cell RNA-seq data of islet samples of one patient with T1D and HT and one matched normal control. The detailed information of these data was presented in **Table S3**. Interestingly, the functional enrichment showed significantly upregulated in the viral gene expression pathway, which further verified our finding in microarray datasets (**Figure S4**).

To obtain more information on the shared pathways of these three autoimmune diseases, specific genes enriched in the enriched pathways were shown in **Table 2**. As expected, there were many HLA genes enriched in the shared autoimmune related pathways. Interestingly, virus infection associated pathways also contained a large number of HLA genes. Apart from this similarity, different virus infection pathways owned their unique genes, with *STAT1*,*RSAD2* and *IRF9* in the influenza A pathway,*IL-2RA*,*IL-2RB* and *CD40* in the HTLV-1 infection pathway, *HCFC1*, *MAP3K7*, *SOCS3*, *IRF9*, *HMGN1*, and *FAS* in the herpes simplex infection pathway. Collectively, altered gene expressions in the shared pathway of different autoimmune

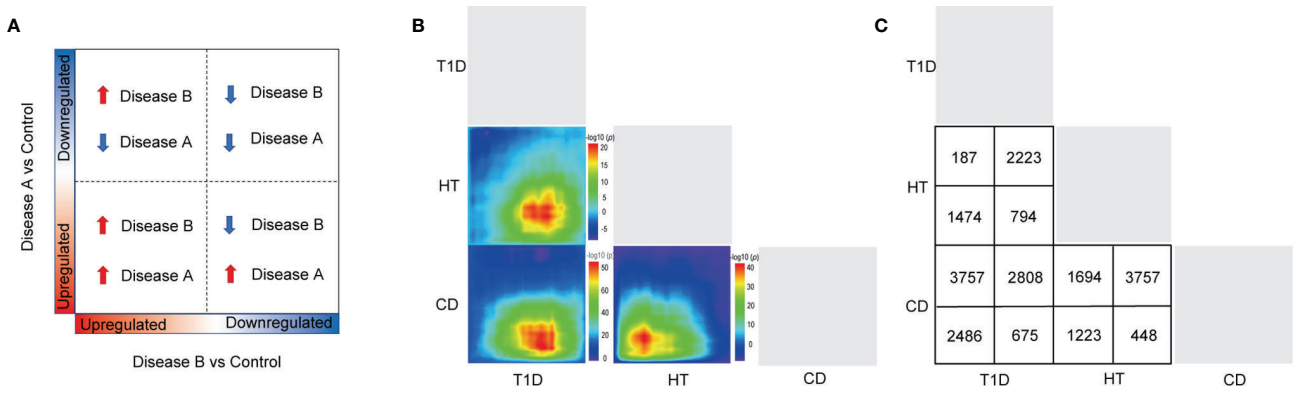

FIGURE 2 | A RRHO analysis was carried out to compare target tissue gene expression features of three autoimmune disorders. **(A, B)** The RRHO algorithm was run on genes that were ranked by fold change from most down-regulated to most up-regulated. The level map colors represent the adjusted log *p* values of the overlap between genes(from red to blue, the adjusted log *p* values go from high to low) that are up-regulated in both disorders (bottom left quadrant), down-regulated in both disorders (top right quadrant), up-regulated in the left-hand labeled disease and down-regulated in the bottom labeled disease (top left quadrant), and down-regulated in the left-hand labeled disease and up-regulated in the bottom labeled disease (top left quadrant) (bottom right quadrant). **(C)** The number of genes that overlap in each pairwise analysis is shown in the panel.

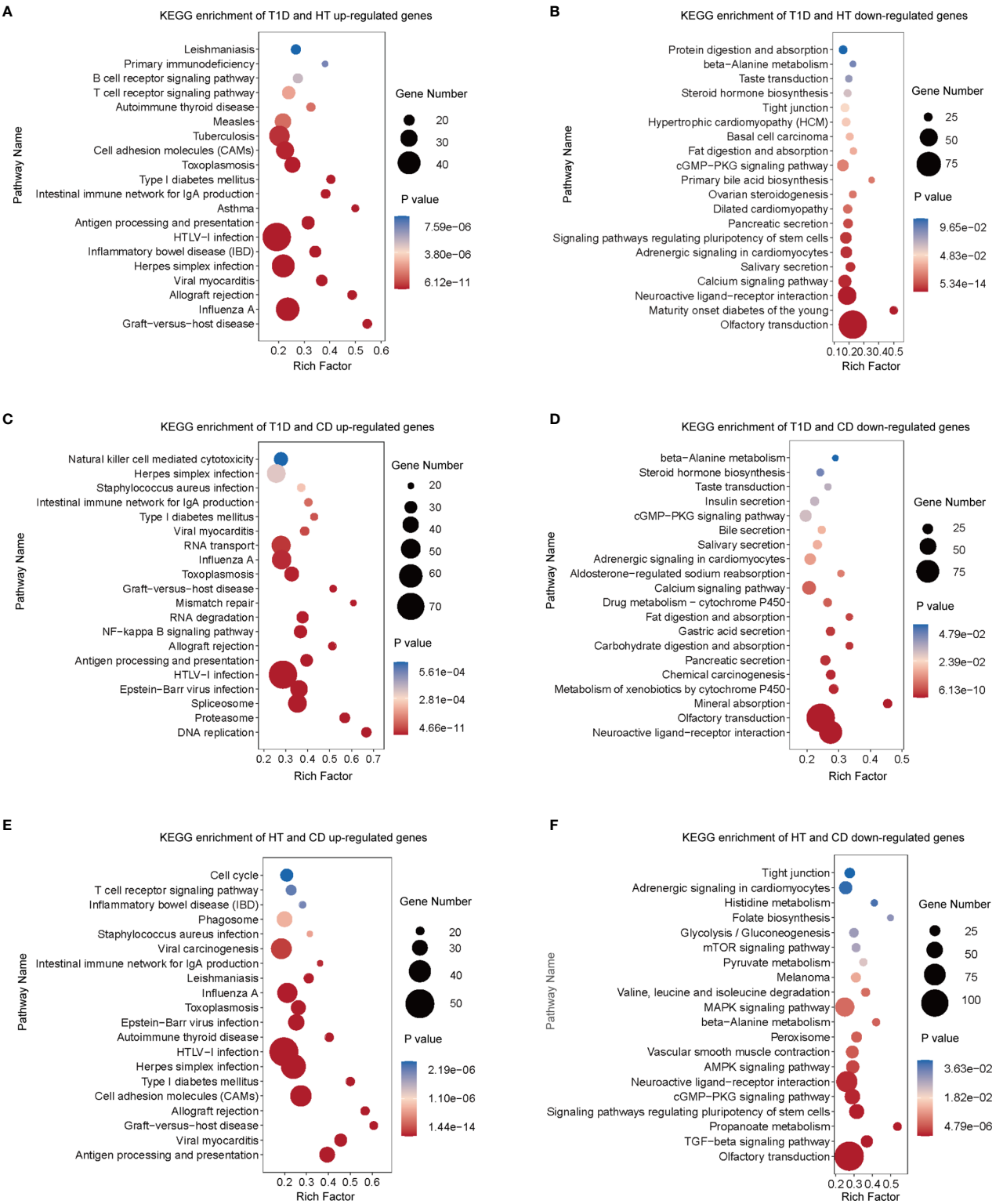

FIGURE 3 | In three types of autoimmune disorders, functional enrichment analysis of overlapping genes revealed common signal pathways. **(A–F)** In the RRHO analysis (**Figures 2B**), genes with significant overlapping between different pairs of autoimmune diseases were chosen for enrichment analysis with the KEGG database. According to their *p* values, the top 20 gene sets are represented. Diseases were studied in pairs. **(A)** Up-regulated overlapping genes of T1D and HT. **(B)** Down-regulated overlapping genes of T1D and HT. **(C)** Up-regulated overlapping genes of T1D and CD. **(D)** Down-regulated overlapping genes of T1D and CD. **(E)** Up-regulated overlapping genes of HT and CD. **(F)** Down-regulated overlapping genes of HT and CD.

TABLE 2 | Common genes enriched in overlapping pathways in the RRHO results of three autoimmune diseases.

KEGG pathway	Gene symbol
Up regulated	
hsa04672: Intestinal immune network for IgA production	*HLA-DRA, CD40, HLA-DOB, HLA-DMA, HLA-DPB1, ICOS, HLA-DQA1, HLA-DPA1, PIGR, ITGB7, HLA-DMB, HLA-DQA2*
hsa05164: Influenza A	*PRKCB, CASP1, MAPK1, CIITA, TNFRSF10B, HLA-DPB1, STAT1, HLA-DQA1, PYCARD, ATF2, RSAD2, HLA-DRA, HLA-DMA, PIK3CD, MYD88, HLA-DPA1, ICAM1, HLA-DQA2, HLA-DOB, SOCS3, IRF9, ACTG1, FAS, HLA-DMB*
hsa05166: HTLV-I infection	*HLA-A, ETS1, CCND2, ANAPC7, IL2RB, ANAPC1, CHEK2, ADCY7, HLA-DPB1, HLA-DQA1, ATF2, LCK, HLA-DRA, HLA-DMA, PIK3CD, EGR1, EGR2, HLA-DPA1, ICAM1, HLA-DQA2, CD40, HLA-DOB, IL2RA, JAK3, HLA-E, MYB, HLA-DMB, HLA-G*
hsa05168: Herpes simplex infection	*HLA-A, SP100, HLA-DPB1, STAT1, HLA-DQA1, TRAF5, CD74, HLA-DRA, HLA-DMA, MYD88, HCFC1, HLA-DPA1, MAP3K7, HLA-DQA2, HLA-DOB, SOCS3, IRF9, HMGN1, HLA-E, FAS, HLA-DMB, HLA-G*
hsa04940: Type I diabetes mellitus	*HLA-DRA, HLA-DOB, HLA-A, HLA-DMA, HLA-DPB1, HLA-DQA1, HLA-DPA1, HLA-E, FAS, HLA-DMB, HLA-DQA2, HLA-G*
hsa05330: Allograft rejection	*HLA-DRA, CD40, HLA-DOB, HLA-A, HLA-DMA, HLA-DPB1, HLA-DQA1, HLA-DPA1, HLA-E, FAS, HLA-DMB, HLA-DQA2, HLA-G*
hsa05332: Graft-versus-host disease	*HLA-DRA, HLA-DOB, HLA-A, HLA-DMA, HLA-DPB1, HLA-DQA1, HLA-DPA1, HLA-E, FAS, HLA-DMB, HLA-DQA2, HLA-G*
hsa05416: Viral myocarditis	*HLA-DRA, HLA-A, HLA-DMA, HLA-DPA1, FYN, ICAM1, HLA-DQA2, CD40, HLA-DOB, HLA-DPB1, HLA-DQA1, HLA-E, ACTG1, HLA-DMB, HLA-G*
Down regulated	
hsa04261: Adrenergic signaling in cardiomyocytes	*AGTR2, MYH6, PPP2R2D, CACNG4, ADRB1, CACNG6, SCN5A, ATP2B2, KCNE1, ADRA1D, CACNA1S*
hsa00410: beta-Alanine metabolism	*SMOX, ALDH3A1, ALDH3B1, DPYS, GADL1, HIBCH, UPB1*
hsa04080: Neuroactive ligand-receptor interaction	*NPY5R, GRIN1, PLG, NMBR, ADRA2C, GRIA4, TACR3, GRM1, GRM7, GRM5, P2RY1, ADRB1, OPRD1, CHRM5, ADRA1D, TAAR2, MCHR2, HRH3, GH2, AGTR2, OPRK1, HRH1, GHRHR, GRIK1, NPFFR1, NTSR2, PRLR, GRM4, P2RY2, GLRA3, FSHR, MCHR1, THRA, OPRM1*
hsa04022: cGMP-PKG signaling pathway	*ATP2A1, ADRA2C, IRS4, MYLK4, ADRB1, MYLK3, CNGB1, INS, OPRD1, GUCY1A2, ATP2B2, ADRA1D, MYLK2, GTF2IRD1, GUCY1B3, CACNA1S*

The RRHO analysis results were selected to enrich the overlapping KEGG pathways in the analysis, and the intersection of genes corresponding to a single pathway in each RRHO analysis list was taken, with the results shown in the table.

diseases might serve as diagnostic biomarkers for the occurrence and development of multiple autoimmune diseases.

The Inhibitors of Tyrosine Kinase, Phosphoinositide 3-Kinase, and Heat Shock Protein May Be Potential Therapeutic Targets in Autoimmune Diseases

Based on the overlapping genes and pathways in the target tissues, we were able to discover common therapeutic targets for multiple autoimmune diseases (**Figures 4A–C**). A large number of potential drug targets were indicated for patients with HT and CD, which was in line with our observations in the RRHO analysis. These results further indicated a closer relationship in the pathogenesis between HT and CD. There were fewer drugs or targets for patients with T1D and HT (**Figure 4A**), and the median tau score was lower. However, epidemiology revealed that HT and T1D were prone to co-occurrence (2), suggesting that more research was required to investigate the common mechanisms and therapeutic targets. Tyrosine kinases inhibitors were predicted to treat T1D, HT, and CD (**Figures 4A–C**). In addition, PI3K inhibitors were potential drugs for patients with CD and T1D or patients with CD and HT (**Figures 4B, C**). Several novel targets, such as the HSP inhibitors, HDAC inhibitors, S100A family, and zinc fingers family might be therapeutic targets for multiple autoimmune diseases. In summary, a number of virus infections associated targets may be potential therapeutic targets in autoimmune diseases.

To further determine the optimal time for treatment, we also analyzed the autoantibody positive individual in the T1D dataset using RRHO and cMAP methods. Virus infection associated pathways were significantly enriched in overlapped upregulated genes between T1D and antibody positive individuals, such as human T-cell leukemia virus 1 infection, Epstein-Barr virus infection, and salmonella infection (**Figures S5A–D**).HDAC inhibitors were predicted to be potential targets for T1D and antibody positive individuals (**Figure S6**).These findings suggest that targeting the shared pathways in autoimmune diseases are recommended during the original viral infection in patients with more than one autoimmune disease, even in individuals at high risk. Indeed, there are some ongoing trials focusing on virus vaccines, which might be potential therapies for the prevention of autoimmune diseases (16).

DISCUSSION

In the present study, we discovered that different target tissues from three distinct autoimmune disorders, including T1D, HT, and CD, were affected by common environmental factors such as influenza A, HTLV-I, and herpes simplex infection. These data highlight the role of shared environmental factors in the

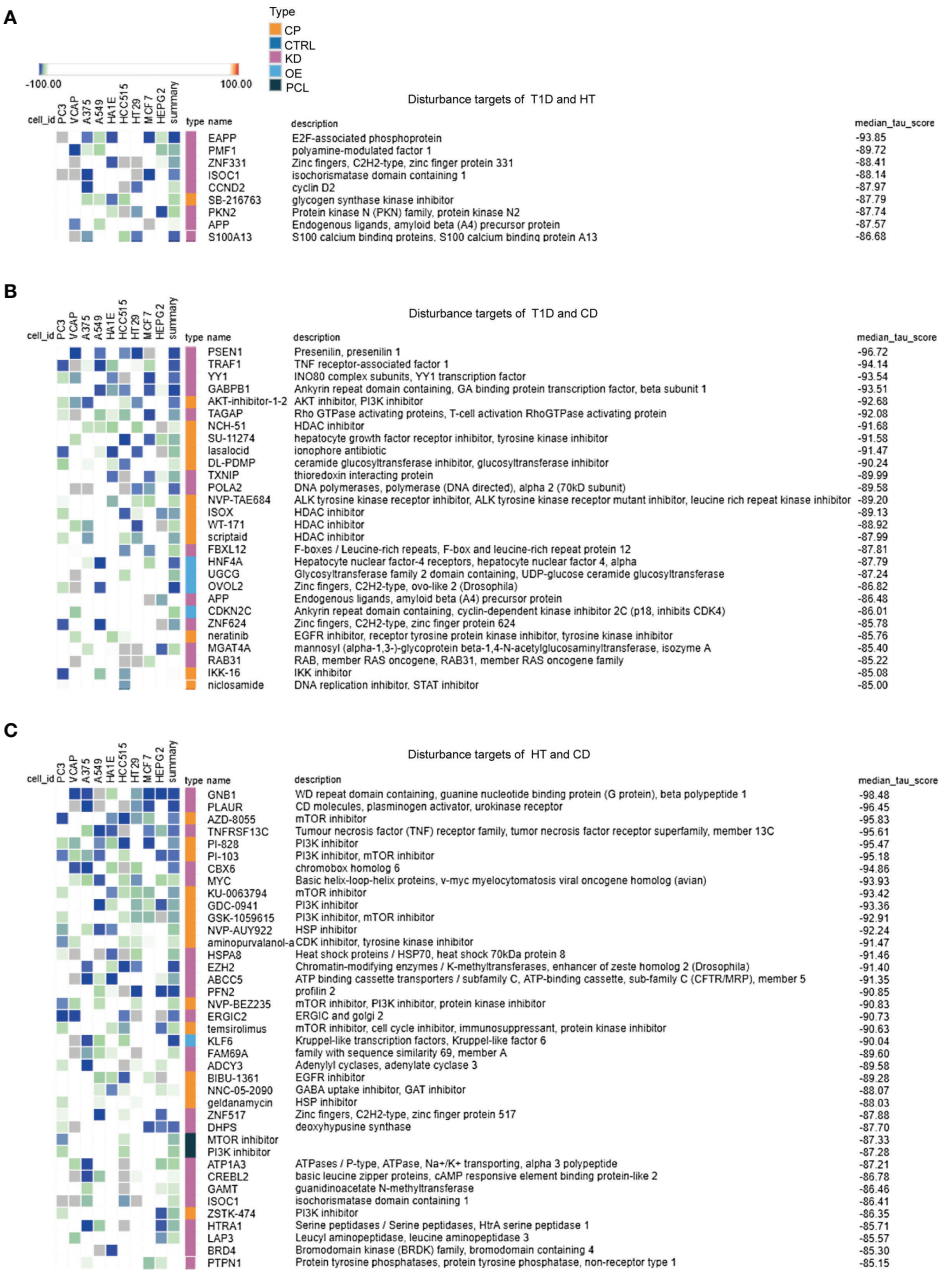

FIGURE 4 | Exploration of overlapping genes among target tissues in three autoimmune disorders leads to the discovery of common therapeutic targets. **(A–C)** For each RRHO analysis, the top 150 up or down overlapping genes were submitted to the Connectivity Map database in order to identify perturbagen classes that cause an opposite effect (negative tau score) in the target tissues of autoimmune diseases. Only classes with a median tau score < -85 were represented. Perturbagen classes cause an opposite effect in the genomic signatures of up and down overlapping genes. The above methodology and conditions were used for the following analysis: **(A)** T1D and HT, **(B)** T1D and CD, **(C)** HT and CD.

pathogenesis of T1D, HT, and CD. Furthermore, our findings may provide novel biomarkers to aid the diagnosis as well as develop new strategies for the treatment of these autoimmune diseases.

The transcriptional profiling of the target tissues in T1D, HT, and CD showed up-regulation of autoimmunity-related pathways, such as intestinal immune network for IgA production, T1D, allograft rejection, and graft-versus-host disease. Current methods for comparing expression profiles typically involve selecting a fixed differential expression threshold to summarize results, potentially decreasing sensitivity to small but concordant changes. RRHO analysis is

a method for detecting and visualizing overlap trends between two complete, continuous gene-expression profiles, which is unbiased, sensitive, robust, and web-accessible. In this study, we observed varying degrees of overlap between the diseases in terms of both up-regulated and down-regulated genes, particularly the up-regulated expression patterns between HT and CD.

Interestingly, these three organ-specific autoimmune diseases all demonstrated consistently up-regulated pathways associated with virus infection, including influenza A, HTLV-1 infection, herpes simplex infection, and viral myocarditis. Virological evidence for T1D, HT, and CD are at different levels, with a majority of serological data or studies on circulating viruses, some epidemiological data, and few studies on direct proof of target tissue infection (17–19). In the subgroup with laboratory-confirmed pandemic influenza A, a nationwide cohort study found a twofold increase in the incidence of T1D (20). Another national cohort study reported that individuals with CD have a higher probability of hospital admission for influenza (21). According to many case reports, these four viruses were associated with T1D, HT, and CD (22). However, no research has looked into the connection between the virus infection and the occurrence of T1D, HT, and CD in the same patient. Our findings imply that these viruses are common environmental triggers in the pathogenesis of T1D, HT, and CD. In medical practice, taking virus infection histories is required (23). Individuals at high risk for multiple autoimmune diseases should be examined for antibodies targeting influenza A, HTLV-I infection, and herpes simplex infection as needed. The results of this study indicate that virus infections may play the same important role as genetic background in the etiology of T1D, HT, and CD. However, the mechanisms through which viruses cause autoimmune disorders are unknown. Influenza A induces toll-like receptor 3 overexpression in thyrocytes, which are associated with HT (24). Several epidemiological studies in human and animal experiments have demonstrated that virus infections can either trigger or prevent autoimmune pathologies, depending on various factors such as genetic background, the onset time of infection, viral load, type of virus strain, and host-elicited immune responses. Nonetheless, few studies have focused on the specific mechanistic interaction between the virus and the immune system in the process of autoreactivity. Previous studies showed that virus-induced autoimmunity can be activated by various mechanisms including bystander activation, epitope spreading, molecular mimicry, and immortalization of infected B cells (25). The molecular mechanisms underlying the potential etiological relationship between viruses and autoimmune diseases should be investigated.

Because of the observed similarities in pathway activation between target tissues, several classes of drugs that could potentially be used to treat more than one autoimmune disease were identified. The tyrosine kinase inhibitor is of particular interest among them. Tyrosine kinases are important signaling cascade mediators, playing critical roles in a variety of biological processes such as growth, differentiation, apoptosis, and metabolism in response to internal and external stimuli. It is worth noting that imatinib, a tyrosine kinase inhibitor, was shown in a multicenter, randomized, double-blind, placebo-controlled, phase 2 trial to preserve beta-cell function in patients with recent-onset T1D (26). Possible mechanisms of imatinib might include, 1) inhibiting ABL-IRE1α interaction and dampening IRE1α RNase hyperactivity, thus resulting in the reduction of pancreatic β cell apoptosis as demonstrated in a non-obese diabetic (NOD) mouse study (27); 2) preventing the process of T cell and macrophage infiltration into islets (28). Notably, tyrosine kinase inhibitors exhibit potent antiviral activity by blocking multiple steps of influenza A virus replication (29). Collectively, tyrosine kinase inhibitors are entering the clinical research stage for T1D patients. Our study showed that tyrosine kinase inhibitors could also be potential drugs for HT and CD.

Another class of drug identified in this study for potential use in multiple autoimmune diseases is HSP inhibitors. Although no HSP inhibitor-related studies in T1D, HT, or CD have been conducted, it has been extensively documented that HSP70 triggers the autoimmune disease. HSP70 promotes the function of antigen-presenting cells (dendritic cells) and converts T-cell tolerance to autoimmunity (30). Higher expression of HSP70 was shown in patients with T1D or celiac disease (31, 32). In a large cohort of T1D subjects, serum anti-HSP70 antibody levels were independently and inversely related to diabetic vascular complications, implying that anti-HSP70 antibody levels may be a potential marker of protection from diabetic vascular complications (33). HSP70 has also been reported to modulate the activity of influenza a virus polymerase (34). HSP70 may prime protective immune responses to herpes viruses early in life during infection through binding recombinant viral protein and viral epitopes, which can be potentially lethal, and the establishment of latency frequently occurs (35). Taken together, previous studies showed that HSP is closely associated with chronic diabetic complications of T1D and virus infections, and our analysis revealed that HSP inhibitors might be potential drugs for HT and CD. All the above results suggest that HSP inhibitors may be novel drugs for treating multiple autoimmune diseases by regulating immunity and defending against virus infections.

PI3K inhibitors were another potential treatment for multiple autoimmune diseases. These compounds inhibit a group of lipid kinases, which phosphorylates phosphoinositide from cell membranes, regulating cellular processes such as immune responses, cell growth, and metabolism. PI3K inhibition could be a novel therapeutic approach for treating vascular dysfunction in patients with diabetes (36). It was reported that NOD mice with PI3Kγ deficiency were protected from developing spontaneous diabetes. Moreover, a recent study showed that PI3K-inhibitor suppressed the proliferation and cytokine production of a human $CD4^+$ T-cell clone specific for GAD peptide isolated from a T1D patient (37). In agreement with our study, these findings suggest an involvement of the PI3K pathway in the regulation of autoimmune diabetes and provide rationales for the future use of anti-PI3K therapy in T1D.

Furthermore, PI3K/Akt expression increased in HT patients, indicating a possible molecular mechanism of PI3K/Akt in the pathogenesis of HT (38). However, more research focusing on PI3K inhibitors for multiple autoimmune diseases is required.

Several limitations to this study need to be acknowledged. First, the sample numbers of the resource gene data were relatively small, therefore, further study on large scale is required to validate these findings. Second, the resource gene data were from the comparison of patients with a single disease and controls, more samples from patients with two or three diseases of T1D, HT, and CD are required to validate the shared genetic fingerprint. Third, more in-vivo and in-vitro researches are needed to better understand the precise molecular mechanism, providing new targets for disease prevention and therapy.

It is well recognized that autoimmune diseases are caused by a complex interaction between genetic and environmental factors. Our combination analysis of GWAS data and gene signatures of different target organs provide more reliable evidence demonstrating that virus infection, especially influenza A, HTLV-I, and herpes simplex infection, may be critical environmental factors in triggering diseases in genetically susceptible individuals, as more than 70 percent of the candidate GWAS genes were not in the DEGs of T1D, HT, and CD. Tyrosine kinase inhibitors, HSP inhibitors, and PI3K inhibitors could be potential novel treatments for multiple autoimmune diseases. Collectively, we deem that our findings contribute to a better understanding of the pathogenesis of T1D, HT, and CD, which might provide new insights for identifying novel biomarkers for the diagnosis and new targets for the treatment of these diseases.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

MY designed the experiment, performed the data analysis and drafted the manuscript. YZ performed the data analysis and drafted the manuscript. SL revised the manuscript. XL and JH designed the experiment and revised the manuscript. All authors read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.891698/full#supplementary-material

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MINI REVIEW
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An Emerging Role for Neutrophil Extracellular Traps in IgA Vasculitis: A Mini-Review

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Immunoglobulin A vasculitis (IgAV) is the most common systemic small vessel vasculitis in childhood. Its clinical manifestations are non-thrombocytopenic purpura, accompanied by gastrointestinal tract, joint, kidney and other organ system involvement. The pathogenesis of IgAV has not been fully elucidated. It may be related to many factors including genetics, infection, environmental factors, and drugs. The most commonly accepted view is that galactose-deficient IgA1 and the deposition of IgA and complement C3 in small blood vessel walls are key contributors to the IgAV pathogenesis. Extensive neutrophil extracellular traps (NETs) in the peripheral circulation and skin, kidney, and gastrointestinal tissue of patients with IgAV has been identified in the past two years and is associated with disease activity. This mini-review provides a possible mechanism for NETs involvement in the pathogenesis of IgAV.

Keywords: IgA vasculitis, neutrophil extracellular traps, pathogenesis, biomarker, neutrophils, IgA vasculitis nephritis

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INTRODUCTION

Immunoglobulin A vasculitis (IgAV), also known as Henoch-Schönlein purpura, is an inflammatory small vascular disease involving the capillaries, venules, or arterioles (1). The clinical manifestations of IgAV are non-thrombocytopenic purpura, mainly involving the skin, gastrointestinal tract, joint and kidneys, and deposition of IgA or IgA-immune complexes (IgA-ICs) in the vascular wall. The incidence of IgAV in children is 10-27/100,000 per year (2). The majority of cases occur between 2 and 10 years of age, with a peak onset between 4 and 7 years of age (3, 4). In most children, IgAV is self-limited and has a good prognosis, but a few cases have renal involvement and a recurrent or even prolonged course. IgAV nephritis (IgAVN), which is a major cause of mortality, is the cause of 1%–2% of pediatric end-stage renal disease cases (3). The pathogenesis of IgAV has not been fully elucidated. At present, it is believed to be caused by genetics, infection, environmental factors, drugs, and other factors (5). There is a wide range of immunological abnormalities in IgAV.

THE PHYSIOLOGICAL FUNCTION OF NETS AND THEIR ROLE IN AUTOIMMUNE DISEASES

Neutrophils are the most abundant white blood cells in the human peripheral circulation. They play a key role in the innate immune system and constitute the body's first line of defense against pathogens. Previous studies have shown that neutrophils phagocytose and kill bacteria directly through secretion of proteolytic enzymes, antibacterial proteins and reactive oxygen species (ROS), which are directly to kill bacteria (6, 7).A novel mechanism of neutrophil defense against infection through release of neutrophil extracellular traps (NETs) was reported in recent years (8). This process fundamentally differs from both cell death and necrotizing apoptosis and is called NETosis (9). At present, there are two mechanisms by which NETs are formed: suicide lytic NETosis and vital NETosis. In lytic NETosis, neutrophils release NETs through cell membrane lysis death, which depends on the Raf/MEK/ERK signaling pathway and the activation of NADPH oxidase. In lyticNETosis, the cell membrane breaks down and neutrophils are unable to secrete particles. In vital NETosis, DNA from the nucleus erupts in vesicles, passes through the cytoplasm and binds to the plasma membrane, transporting DNA outside the cell to formation of NETs without damaging the membrane and maintaining the integrity of neutrophil (10). The structure of NETs directly wraps around invading microorganisms and uses its highly concentrated antimicrobial peptides to degrade virulence factors and kill pathogenic microorganisms, preventing the spread and dissemination of infection, which plays an important role in infection defense (8). However, excessive formation of NETs and clearance of obstacles also has a toxic effect on the host. NETs related components, such as nucleic acids and proteins, were exposed as autoantigens in the inflammatory environment, which can stimulate the autoimmune response of susceptible individuals and promote various autoimmune diseases (11).

NETS INVOLVED IN THE PATHOGENESIS OF IGAV

In 2020 Bergqvist, C et al. (12) reported that NETs were significantly increase in skin tissues in the early stages of IC-mediated small vasculitis, such as allergic vasculitis and IgAV. Our previous study evaluated the level of NETs in the peripheral blood and gastrointestinal and renal tissues of children with IgAV at different periods. The study evaluated components of NETs, which included cell-free DNA (cf-DNA), myeloperoxidase-DNA (MPO-DNA), citrullinated-histone H3 (cit-H3), neutrophil elastase (NE), and cathelicidin antimicrobial peptides (CAMP, LL37). The level of NETs significantly increased in children with IgAV onset and active stage, while the level of NETs gradually returned to normal in children in the remission stage and drug withdrawal (13). In autoimmune diseases, excessive NETs are known to act as an exposed autoantigen *in vivo*, inducing the production of autoantibodies, thereby increasing the intensity of the inflammatory response. A continuous increase in NETs indicates a high inflammatory state, and reflects the imbalance between the formation and clearance of NETs in IgAV, leading to the accumulation of excessive NETs, which ultimately leads to autoimmune disorders, chronic inflammation and tissue damage. These processes have been associated with the development of autoimmune and inflammatory diseases (14). Several studies have shown that NETs are involved in the development and progression of autoimmune diseases such as ANCA-associated vasculitis, rheumatoid arthritis (RA), inflammatory bowel disease and systemic lupus erythematosus (SLE) (15–18). A recent study reported that MPO-DNA is significantly elevated in the circulation of patients with IgAV and positively correlates with IgA levels, which suggests that NETs are involved in the pathogenesis of IgAV (19). NETs may influence the activity or severity of IgAV (13, 19).

MECHANISM OF NETS IN IGAV

Disordered Equilibrium Between NETs and DNase I

Our previous study (13) revealed that serum degradation of NETs significantly declines in children with IgAV onset and active IgAV. Children in drug withdrawal had a normal level of NETs degradation. The level of DNase I also decreases in children with IgAV onset and active IgAV. The reduced ability to degrade NETs is negatively correlated with the presence of DNase I, which is required to degrade NETs (20). The decreased activity of DNase I may be one of the reasons for the significant increase in NETs and thus may cause immune imbalance (21). In patients with SLE and eosinophilic granuloma, the ability of the extracellular and intracellular environment to degrade DNA is significantly reduced. This phenomenon seems to be a common characteristic of autoimmune diseases (22, 23). In addition, over-activation of complement system and over deposition of complement protein C1q also inhibit the production of DNase I, resulting in ineffective NETs degradation (24). Therefore, excessive NETs formation is related to deficient DNase I activity, which leads to disorders that promote immunological homeostasis dysregulation and tissue damage (25). Impaired self-degradation of NETs is associated with RA and lupus nephritis (26–29).The decreased activity of DNase I eventually leads to a reduced ability to degrade NETs, which is one of the reasons for the increase in NETs in IgAV.

Aberrant Glycosylation of IgA1 and IgA-ICs Induces NETs Formation in IgAV

Deposition of IgA on the vascular wall is characteristic of IgAV. IgA activate neutrophils and release NETs into tissues and the peripheral blood. Studies have shown that NETs are involved in various IC-mediated small vasculitis conditions, and that circulating NETs are related to the severity of vascular inflammation (12). In renal biopsies from patients with ANCA-associated vasculitis, the formation of NETs was found in the involved glomeruli and stroma lesions (30). IgA can induce neutrophils to release NETs *via* Fcα receptor I

(FcaRI) (31). FcaRI is elevated in children with active IgAV (32). In idiopathic IgA nephropathy, proteinuria and leukocyte infiltration are more pronounced, and FcaRI activation leads to a more severe inflammatory response. It is believed that FcaRI promotes and aggravates tissue and kidney damage by activating the cascade reaction of cytokines and chemokines (33). NETs have been shown to induce an autoimmune response in other autoimmune diseases such as SLE and ANCA-associated vasculitis (34–36). In addition, in patients with RA, the level of circulating NETs is positively correlated with the severity of periodontitis (37).

NETs formation has been detected in tissue biopsies of patients in the early stages of IC-mediated vasculitis (14). These immobilized ICs induce human neutrophils to release NETs *in vitro* (38). The formation of NETs increases in the renal, gastric, and duodenal tissues of children during IgAV onset and active IgAV, which may be related to IgA-ICs deposition activating neutrophils to release NETs. In lupus nephritis, deposition of circulating ICs in the glomerular basement membrane is accompanied by the accumulation of NETs in the tissue, resulting in tissue damage (39). The deposition of IgA and C3 and the formation of NETs are common in the renal, gastric and duodenal tissues of children with IgAV. It is speculated that IgA-ICs and C3 deposition may be involved in the occurrence and development of IgAV through various mechanisms, such as complement activation, chemotaxis infiltration and aggregation of neutrophils to promote the release of NETs (**Figure 1**).

NETs Activate Downstream Target Immune Cells to Release Cytokines

Neutrophils release related components of NETs such as MPO and protease, which further aggravates tissue damage. Similarly, IgA binds to the FcαRI junctions of neutrophils, releasing tumor necrosis factor α (TNF-α), leukotriene B4 (LTB4), etc. (40, 41). TNF-α can further stimulate endothelial cells to produce interleukin (IL)-8 (42). IgA may activate neutrophils to release IL-8 (1, 43). Studies show that LTB4 is significantly increased in children with IgAV (44). LTB4 can induce further neutrophil migration through a positive feedback pathway (40). Although LTB4 has no direct effect on microvascular injury of inflammatory tissues, it can make white blood cells adhere to vascular endothelial cells, resulting in increased vascular permeability and aggravating tissue injury (45). LTB4 plays an important role in inflammation, the immune system, and allergies. TNF-α is a pro-inflammatory factor involved in the occurrence and development of IgAV and is closely related to kidney damage. TNF-α can even reflect the degree of renal damage in IgAVN (46, 47). In addition, NETs-related components can activate immune-related cells such as B lymphocytes, T lymphocytes and antigen-presenting cells to release IL-6, IL-8, interferon γ (INF-γ), and TNF-α (14). In the interleukin family, IL-6, IL-8, IL-10, and IL-33 are all related to IgAV (46, 48–50). IL-2 is negatively correlated with the severity of the disease (51). IL-6 promotes the activation of B cells and the production of relevant antibodies, which are mainly deposited in the mesangial region of the kidney. Through the action of T cells, IL-6 stimulates the proliferation and fibrosis of mesangial tissues, aggravates kidney damage and leads to the occurrence and development of IgAVN (52). IL-10 plays a protective role by inhibiting the antigen presentation function of macrophages and indirectly inhibiting the function of natural killer cell (53). Under the stimulation of IL-8, an increase in the cytoplasmic Ca^{2+} of neutrophils mediates the release of hydrogen peroxide in a respiratory burst reaction, and lysosomal enzymes can be

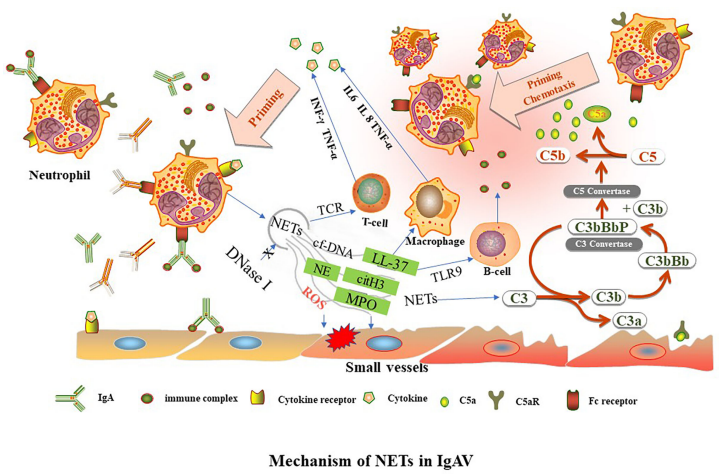

FIGURE 1 | TNFα, tumor necrosis factor alpha. INF-γ, interferon gamma. NETs, neutrophil extracellular traps. LL37, cathelicidin antimicrobial peptides. C3, complement factor 3. IL, interleukin, MPO, myeloperoxidase. NE, neutrophil elastase. cit-H3, citrullinated-histone H3. cf-DNA, cell free DNA. TCR, T cell receptor. TLR9, toll-like receptor 9-dependent manner. Aberrant glycosylation of IgA1 and IgA immune complexes (IgA-ICs) induce NETs formation by binding to the Fc receptor of neutrophils. The level of DNase I decreases, leading to reduce of NETs degradation. NETs activate downstream target immune cells to release cytokines. NETs can activate immune-related cells, such as T lymphocytes (through TCR), B lymphocytes (through TLR9-dependent manner) and macrophages to release cytokines, such as IL6, IL 8, TNF-α, and INF-γ. NETs are involved in different complement bypass pathways.

released through chemotaxis of neutrophils, leading to capillary destruction (46). IgA can activate the complement system through bypass and lectin pathways. The levels of C3a and C5a increase in the circulation of IgAV, and C3 and C5-C9 are deposited in the skin tissue and mesangial region of glomeruli (54, 55). These compounds can form membrane-attacking complexes that directly destroy the membranes of target cells, and deposition of C4d and C5B-9 in the kidney is associated with poor prognosis (56). NETs activate C3 and eventually convert it to C5a, which can induce chemotaxis and neutrophil aggregation, and stimulate endothelial cells to secrete IL8.

NETS MAY BE A POTENTIAL BIOMARKER TO ASSESS DISEASE ACTIVITY IN IGAV

NETs have been reported as a marker of disease activity in other diseases. The One predictor of inflammatory response and sepsis is cf-DNA (57, 58). Cit-H3 is a useful biomarker for early detection of liver dysfunction (59). NETs can be used as markers and therapeutic targets for ophthalmic diseases including dry eye, glaucoma, age-related macular degeneration, and diabetic retinopathy (60, 61). NETs and anti-NETs associated antibodies are indicators of SLE activity (11, 16). NETs significantly increase during IgAV onset and the active stage of IgAV but decrease in the remission and withdrawal stage of IgAV (13). Most patients in the active and relapse have IgAVN. It is speculated that changes in NETs levels may reflect disease activity of IgAV in children, especially those with IgAVN need corticosteroids or immune suppressive therapy.

There are no widely used biomarkers to predict disease activity or the prognosis of IgAVN. The combined indexes of blood examination, immunoglobulin, C-reactive protein, procalcitonin and trace elements have been used to predict the index (62–64). The detection of related metabolites in urine has also been considered. The soluble transferrin receptor concentration in urine increases significantly during the active stage of IgAVN, but the correlation coefficient is low (65). The ratio of urine (Fcα receptor × glutamine transferase)/urine protein and perforin 3 has also been used to predict disease activity, but clinical detection methods for these markers are limited (32, 66). The severity of IgAVN is correlated with alpha-smooth muscle actin (α-SMA) and C-Met, while IgAV with gastrointestinal involvement is correlated with fecal calprotectin, D-dimer and fibrin degradation products (67–69). Therefore, NETs may be a potential convenient biomarker and indicator of IgAVN disease activity, particularly in those patients who would have an ominous outcome.

NETs related components include cf-DNA, MPO-DNA, and NE. Peripheral blood cf-DNA is simple and convenient to measure, but Moss et al. (70) showed that cf-DNA can be released from a variety of cells other than neutrophils during inflammation. Whether cf-DNA alone can predict the level of NETs needs more research. Therefore, the use of NETs or their related components as biomarkers for disease still needs further study for confirmation.

CONCLUSION AND PERSPECTIVES

In conclusion, IgA or IgA-ICs can activate neutrophils to release NETs. NETs-related components can directly damage tissues or secrete large amounts of cytokines by activating downstream target immune cells. Cytokines can aggravate tissue damage and cause neutrophil aggregation, forming a vicious cycle (**Figure 1**). NETs may be a potential biological indicator to assess disease activity in children with IgAV.

However, many unanswered questions about the mechanism of NETs in IgAV remain. The mechanism by which neutrophils mediate IgAV tissue damage is not completely clear. At present, the specific mechanism for NETs signaling pathway regulation and NETs related components in IgAV-induced tissue injury has not been elucidated. Which signaling pathway IgA/FcαR regulates the formation of NETs in neutrophil remains unclear.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors.

AUTHOR CONTRIBUTIONS

XQC were responsible for the conception, design and drafted the manuscript. QT and LH were responsible for design of the review. LT and YHQ revised the manuscript. All authors contributed to the article and approved the submitted version.

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Candidate Drugs Screening for Behcet's Disease Based on Bioinformatics Analysis and Mouse Experiments

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Background: Behcet's disease (BD) is a chronic immune disease that involves multiple systems. As the pathogenesis of BD is not clear, and new treatments are needed, we used bioinformatics to identify potential drugs and validated them in mouse models.

Methods: Behcet's disease-related target genes and proteins were screened in the PubMed and UVEOGENE databases. The biological functions and pathways of the target genes were analyzed in detail by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses. A protein-protein interaction (PPI) network was constructed by the STRING database, and hub genes were identified by the Cytoscape plug-in CytoHubba. Gene-drug interactions were identified from the DGIdb database. Experimental autoimmune uveitis (EAU) mice were used as an animal model for drug validation.

Results: A total of 249 target genes and proteins with significant differences in BD were screened, and the results of functional enrichment analysis suggested that these genes and proteins were more located on the cell membrane, involved in regulating the production of cytokines and affecting the activity of cytokines. They mainly regulated "Cytokine- Cytokine receptor interaction", "Inflammatory bowel disease (IBD)" and "IL-17 signaling Pathway". In addition, 10 hub genes were obtained through PPI network construction and CytoHubba analysis, among which the top 3 hub genes were closely related to BD. The DGIdb analysis enriched seven drugs acting together on the top 3 hub genes, four of which were confirmed for the treatment of BD or its complications. There is no evidence in the research to support the results in omeprazole, rabeprazole, and celastrol. However, animal experiments showed that rabeprazole and celastrol reduced anterior chamber inflammation and retinal inflammation in EAU mice.

Conclusions: The functional analysis of genes and proteins related to BD, identification of hub genes, and validation of potential drugs provide new insights into the disease mechanism and potential for the treatment of BD.

Keywords: Behcet's disease, potential drugs, EAU, rabeprazole, celastrol

INTRODUCTION

Behcet's disease (BD) is a chronic systemic disease involving multiple systems that generally presents with recurrent oral ulcers, genital ulcers, uveitis, vasculitis, skin lesions, and neurological and intestinal manifestations (1). BD is prevalent in Turkey, the Middle East, and East Asia (2, 3). However, the aetiology of BD is still unclear, and its diagnosis is complicated (4). Although occlusive vasculitis is a typical histopathological feature of BD, diagnosis still depends on typical clinical manifestations and clinical experience (5, 6).

Uveitis, one of the most common eye diseases in BD, is usually secondary to systemic manifestations, such as recurrent oral ulcers, after an average of 4 years (7). The typical characteristics of uveitis associated with BD are acute recurrent bilateral symmetrical or asymmetrical nongranulomatous panuveitis, accompanied by retinal vasculitis, and a tendency to self-heal (8). The treatment of BD mainly involves suppressing inflammation and reducing tissue damage. Although glucocorticoids and immunosuppressants are usually used for systemic anti-inflammatory treatment, treatment plans should be formulated based on clinical manifestations (9). Regarding the treatment of Behcet's uveitis, in addition to glucocorticoids and immunosuppressants, other biological agents, such as infliximab (IFX) and adalimumab, have been proven to have certain therapeutic effects (10). Intravitreal injection of glucocorticoids is also an adjuvant treatment (11).

Currently, it is possible to control the progression of BD through the use of diverse therapeutic drugs and individualized treatments. However, it is also necessary to further understand the pathogenesis of this disease and introduce new, more effective drugs to control symptoms, improve prognosis, and even change the course of the disease (12). Here, bioinformatics was used to analyze genes or proteins most recently reported to be involved in BD to search for new potential drugs. Finally, we tested the possible impact of drug intervention on experimental autoimmune uveitis (EAU) mice, a model that has been widely used for human uveitis research (13). Overall, we aimed to provide new ideas for the treatment of BD.

MATERIAL AND METHODS

Identification of Behcet's Disease-Related Target Genes and Proteins

Behcet's disease-related target genes and proteins were obtained through the UVEOGENE database (http://www.uvogene.com) and PubMed (14) (15). We searched for papers related to BD from January 2016 to September 2021 in PubMed, and the relevant search terms included "Behcet's disease", "Neuro-Behcet's disease", "Bechet syndrome" and "Intestinal Behcet's disease". And detailed methods for screening target genes and proteins can be found in Section 1.1 of the **Appendix**.

Target Enrichment Analysis

GO and KEGG analyses of target genes or proteins were performed using the Metascape database (http://metascape.org/) and the UniProt database (https://www.uniprot.org/) Detailed methods are present in Section 1.2 of the **Appendix**.

Protein-Protein Interaction Network Analysis

A PPI network was constructed using the STRING database (https://string-db.org/) and Cytoscape software (version 3.8.2, California, USA). Detailed methods are present in Section 1.3 of the **Appendix**.

Acquisition of Hub Gene

The top ten hub genes in the PPI network were enriched by Cytoscape plug-in cytoHubba. Detailed methods are present in Section 1.4 of the **Appendix**.

Gene-Drug Interaction Analysis

Gene-drug interaction analysis can be achieved through the Drug Gene Interaction Database (DGIdb) version 4.2.0 (https://www.dgidb.org) (16). The drug-gene interaction network was constructed by the DGIdb database and Cytoscape Software. Detailed methods are present in Section 1.5 of the **Appendix**.

Construction of Mouse Models of Simulated Ocular Behcet's Disease

The Jackson Lab (Bar Harbor, ME, USA) provided B10.RIII mouse parents. Mice were bred and raised under specific pathogen-free (SPF) conditions. The EAU model was constructed by subcutaneous injection of 200 μl of emulsifier per mouse. The emulsifier was prepared by mixing 50 μg IRBP with an equal volume of CFA containing 1.0 mg/ml of Mycobacterium tuberculosis strain (MTB) (17). Our study was approved by the Ethics Committee of The Renmin Hospital of Wuhan University (WDRM20210708A). We made every effort to minimize the harm to the animals.

Relative mRNA Expression of Hub Genes in EAU Models

TRIzol (Invitrogen, Carlsbad, CA, United States) was applied to extract total RNA from the retinas of EAU mice on the 14th day after injection. The extracted RNA was reverse transcribed into cDNA using the PrimeScript RT kit (Vazyme, China). SYBR premix (Vazyme, China) was used for final quantitative PCR detection. All the primers are listed in **Table 1**. The $2^{-\triangle\triangle Ct}$ cycle threshold method was applied to calculate the relative mRNA expression.

Drug Intervention Corresponding to Hub Genes in EAU Models

Celastrol purchased from MedChemExpress (MCE, HY-13067) was dissolved in DMSO (Sigma) at a concentration of 74 mM (18). Celastrol was aliquoted and stored at -20°C. EAU mice were intraperitoneally injected with celastrol at a dose of 1 mg/kg/day from Day 7 to Day 14, and control EAU mice were intraperitoneally injected with PBS containing the same concentration of DMSO (18). Rabeprazole (MCE, HY-B0656A)

TABLE 1 | Gene specific primer.

Gene	Primer sequence
CCL2	F: 5'-GCTACAAGAGGATCACCAGCAG-3': R: 5'-GTCTGGACCCATTCCTTCTTGG-3'
CSF2	F: 5'-AACCTCCTGGATGACATGCCTG-3' R: 5'-AAATTGCCCCGTAGACCCTGCT-3'
IL-2	F:5'- GCGGCATGTTCTGGATTTGACTC-3' R:5'- CCACCACAGTTGCTGACTCATC-3'
IL-13	F:5'- AACGGCAGCATGGTATGGAGTG-3' R:5'- TGGGTCCTGTAGATGGCATTGC-3'
IL-4	F:5'-ATCATCGGCATTTTGAACGAGGTC-3' R:5'-ACCTTGGAAGCCCTACAGACGA-3'
IFN-γ	F:5'-CAGCAACAGCAAGGCGAAAAAGG-3' R:5'-TTTCCGCTTCCTGAGGCTGGAT-3'
IL-1β	F:5'-TGGACCTTCCAGGATGAGGACA-3' R:5'-GTTCATCTCGGAGCCTGTAGTG-3
IL-17A	F:5'-CAGACTACCTCAACCGTTCCAC-3' R:5'-TCCAGCTTTCCCTCCGCATTGA-3
TNF	F:5'-GGTGCCTATGTCTCAGCCTCTT-3' R:5'-GCCATAGAACTGATGAGAGGGAG-3
IL-10	F:5'-CGGGAAGACAATAACTGCACCC-3' R:5'-CGGTTAGCAGTATGTTGTCCAGC-3'

was dissolved in sterile PBS and intraperitoneally injected into EAU mice at a dose of 60 mg/kg/day from Day 7 to Day 14 (19, 20). Control mice were given an intraperitoneal injection with an equal volume of PBS. From Day 7 to Day 14 of EAU progression, ocular inflammation was observed and recorded daily under a slit-lamp microscope. On Day 14 of EAU, the eyeballs or retinas of mice were removed for HE staining and quantitative PCR. The quantitative PCR method is as mentioned above. The clinical score and histological score followed the scoring criteria proposed by Caspi RR (21).

Statistical Analysis

All data were manifested as means ± standard error of the mean (Means ± SEM). Data were analyzed by GraphPad Prism 7.0 (GraphPad Software, Inc, San Diego, CA, USA). The EAU score was processed by a Mann-Whitney U test. qRT-PCR experiments were performed by two-tailed Student's t-test. $P<0.05$ was considered significant.

RESULTS

Included Target Genes and Proteins Related to BD

From January 2016 to September 2021, a total of 2654 articles were screened from PubMed, and 352 genes or proteins were selected according to the inclusion and exclusion criteria. After removing duplicates, a list of 249 differentially expressed genes or proteins was obtained that were significantly different from BD in the UVEOGENE database. Among them, 135 genes or proteins came from the UVEOGENE database and 114 from PubMed. The genes and proteins were normalized in the UniProt database, and the results are shown in **Supplementary Table 1**. The main research process of this study is shown in **Figure 1**.

Enrichment Analysis of Target Genes and Proteins in BD

A total of 249 target genes and proteins were imported into the Metascape database for GO enrichment analysis and KEGG enrichment analysis. A total of 503 GO items were screened out in the Metascape database, including 337 BP items, 79 CC items, and 87 MF items, accounting for 67%, 15.7%, and 17.3%, respectively, and the top 10 items are shown in **Figure 2**. BP analysis showed that the included target genes and proteins primarily affected the "regulation of cytokine production", "immune effector process", "response to bacterium" and "cytokine-mediated signaling pathway". In terms of cellular components (CC), these targets were mainly involved in the "side of membrane", "endocytic vesicle" and "receptor complex". In MF analysis, "cytokine activity", "immune receptor activity" and "growth factor receptor binding" were affected. The KEGG enrichment analysis processed by the Metascape database screened 87 items ($P<0.01$) (**Supplementary Table 2**). The top 10 items are shown in **Figure 3**. The results suggested that these targets have a more significant impact on "Cytokine-cytokine receptor interaction", "Inflammatory bowel disease (IBD) "and " IL-17 signaling pathway" (**Figure 3**). In addition, 65 genes, 38 genes, and 25 genes were involved in these three pathways

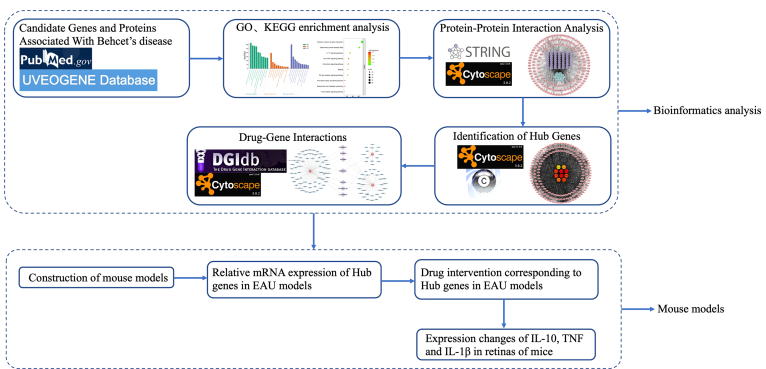

FIGURE 1 | The flowchart of this study.

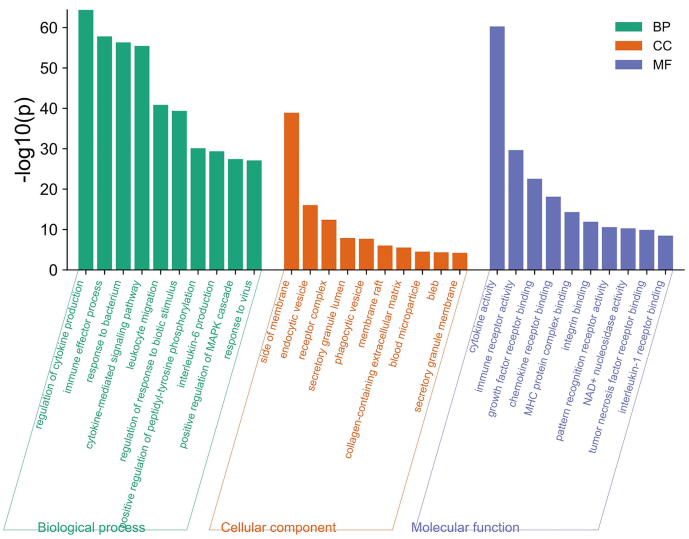

FIGURE 2 | The top 10 items of GO enrichment analysis. The green, orange, and blue bars represent the analysis results of BP, CC, and MF, respectively. "Regulation of cytokine production", "side of membrane", "Cytokine activity" were the most significant difference in BP, CC, and MF respectively.

respectively. The diagram of the three signaling pathways is shown in **Supplementary Figures 1**–**3**.

PPI Network Analysis and Hub Gene Recognition

As shown in **Figure 4**, the PPI network was analyzed and constructed by the STRING database and Cytoscape software.

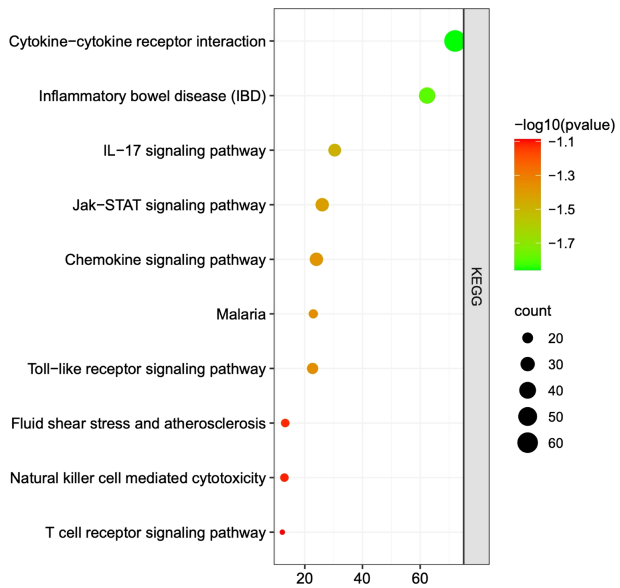

FIGURE 3 | The top 10 pathways of KEGG analysis. Each dot represents a pathway, and the larger the dot is, the more genes it contains. The greener the dot is, the greater the difference. The three most significantly different pathways in KEGG analysis were "cytokine - cytokine receptor interaction", "inflammatory bowel disease", and "IL-17 signaling pathway".

In addition, 246 nodes and 3946 edges were involved in the PPI network. In addition, two clusters were obtained after processing and analysis by the MCODE plugin in Cytoscape software. Cluster 1containde 62 nodes and Cluster 2 contained 14 nodes (**Figure 4**). Moreover, the Cytohubba plugin was used to further analyze and process PPI network node signals. The MCC method was applied to select the top 10 hub genes with a score ≥5000 and node degree ≥10, including CCL2, IL-13, CSF2, IL-10, TNF, IL-17A, IL-1β, IFN-γ, IL-2, and IL-4 (**Figure 5**). The results calculated by the MCC method also suggested that TNF, IL-1β, and IL-10 were the top three hub genes respectively (**Figure 5**).

Drug-Gene Interaction

The DGIdb database was used to analyze the drugs interacting with the top three hub genes, and the results are shown in **Figure 6**. Among these drugs, 17 acted on IL-10, 37 acted on IL-1β, 68 acted on TNF, and 7 acted on any two or three genes simultaneously (**Figure 6**). All the results of the interaction with the first three hub genes are summarized in **Supplementary Table 3**, according to Interaction Type & Directionality, sources, Query Score, and Interaction Score. Among the seven drugs analyzed by the DGIdb database, infliximab, pentoxifylline, and cyclosporine have a clear therapeutic effect on BD. The usage, dosage, and side effects of these three drugs have been well studied (22). Alteplase, also known as recombinant human tissue plasminogen activator, rt-PA, is a thrombolytic agent primarily used for the treatment of Behcet's disease-related thrombosis (23). However, the role of omeprazole, rabeprazole, and celastrol in BD has not been studied.

Expression of Hub Genes in EAU

Real-time PCR was applied to detect the expression of hub genes in the retinas of EAU mice. We compared the relative mRNA expression of hub genes in the retinas of naive and EAU mice on Day 14. The results showed that the relative mRNA expression of

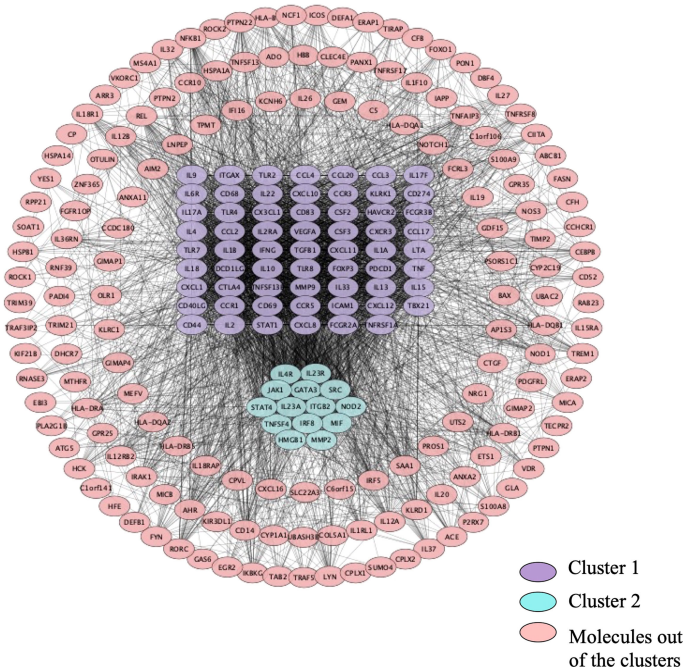

FIGURE 4 | The PPI network. 246 nodes and 3946 edges were involved in the PPI network. Cluster 1 contains 62 nodes indicated in purple, and cluster 2 contains 14 nodes indicated in turquoise. Molecules in pink are not in any cluster.

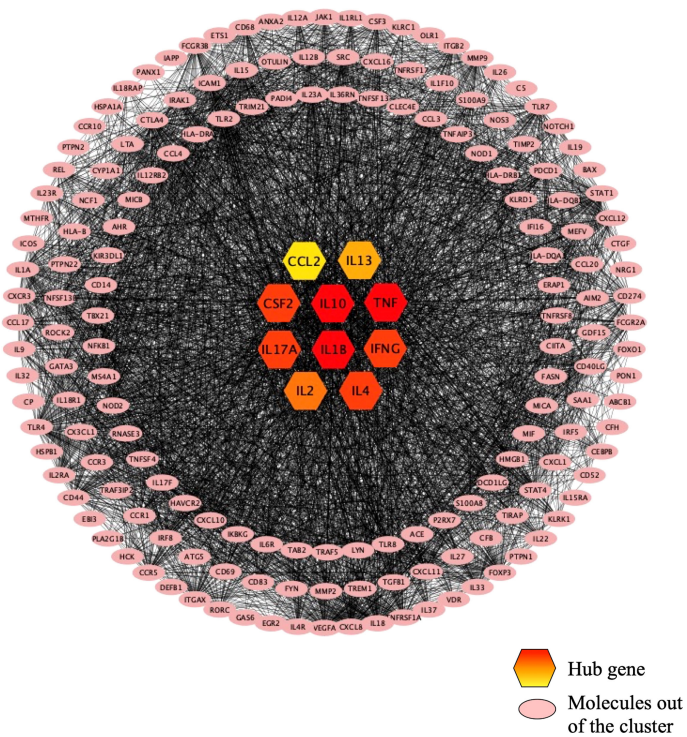

FIGURE 5 | The top 10 hub genes recognized in the PPI network. The top 10 hub genes are in the center of the figure, and the color from red to yellow indicates the difference from large to small. TNF, IL1B, and IL10 are the top 3 hub genes. Molecules in pink are not in any cluster.

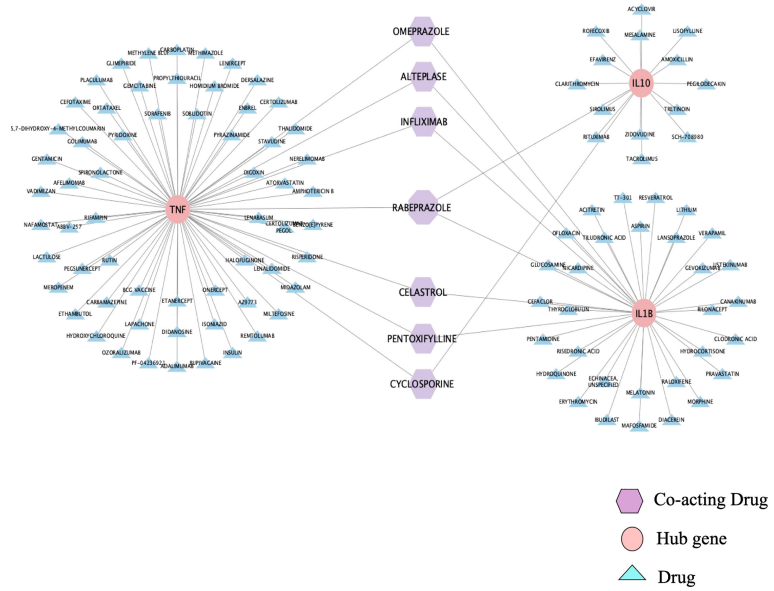

FIGURE 6 | Drug-Gene Interaction. DGIdb database predicted six drugs acting on two hub genes and one drug acting on three hub genes. The pink represents the hub genes, the purple represents drugs that co-act on the hub genes, and the blue represents drugs that only act on one hub gene.

CCL2, CSF2, IL-2, IL-13, IL-4, IFN-γ, IL-1β, IL-17A, and TNF increased in the retina of EAU mice on Day 14, while IL-10 was decreased in the retina of EAU mice (**Figure 7**). CCL2, CSF2, IL-2, IL-13, IL-4, IFN-γ, IL-1β, IL-17A, TNF, and Il-10 showed statistically significant differences compared with naive mice (**Figure 7**).

Celastrol Alleviated EAU

In EAU mice treated with celastrol, the inflammatory response of the anterior segment of the mouse was substantially reduced. Clear conjunctival hyperemia was still visible compared with naive mice (**Figure 8A**). A representative photograph of the anterior segment of the mouse was recorded on the 14th day, as shown in **Figure 8A**. Corneal edema and anterior chamber exudation were observed in the vehicle group, while other structures were not seen (**Figure 8A**). The clinical scores on Day 14 also showed that the scores of mice treated with celastrol were substantially lower than those of the vehicle group, and the difference was statistically significant (**Figure 8C**). In addition, the HE staining results also revealed that the EAU mice treated with celastrol had less structural damage to the retina on the 14th day and less inflammatory infiltration than the vehicle group, and there was a significant difference compared with the vehicle group (**Figures 8B–D**).

Celastrol Inhibited the Expression of TNF and IL-1β in EAU the Retina of EAU Mouse Retinas

After 7 days of celastrol intervention, real-time PCR was used to detect the expression changes of TNF and IL-1β mRNA in the retina. Our results showed that TNF and IL-1β were upregulated in the retinas of mice in the vehicle group compared with those in the naive group, with significant statistical differences. However, TNF and IL-1β were significantly downregulated in the retinas of mice treated with celastrol, with significant differences from the vehicle group. The difference was statistically significant compared with the naive group (**Figures 8E, F**).

Rabeprazole Alleviated EAU

In the rabeprazole group on Day 14, the anterior images showed moderate iridocyclitis with clear pupils and obvious conjunctival hyperemia, while in the vehicle group, corneal edema was more serious with unclear pupils and obvious conjunctival hyperemia (**Figure 9A**). Clinical scores showed significant differences between the two groups (**Figure 9C**). Meanwhile, the HE staining results on Day 14 indicated that the retinal structure of mice treated with rabeprazole was clear, the damage was lighter than that of the vehicle group, and there were fewer inflammatory cells found in the vitreous than were found in that of the vehicle group. Histological scores also showed significant differences between the two groups (**Figures 9B–D**).

Rabeprazole Inhibited TNF and IL-1β and Promoted the Expression of IL-10 in the Retinas of EAU Mice

To investigate whether rabeprazole affects the expression of the top 3 hub genes TNF, IL-1β, and IL-10 in the retina, we also extracted the retinas of EAU mice 7 days after rabeprazole intervention and measured the expression of these three genes by real-time PCR. They were compared with naive mice and vehicle mice. Our results showed that TNF expression increased

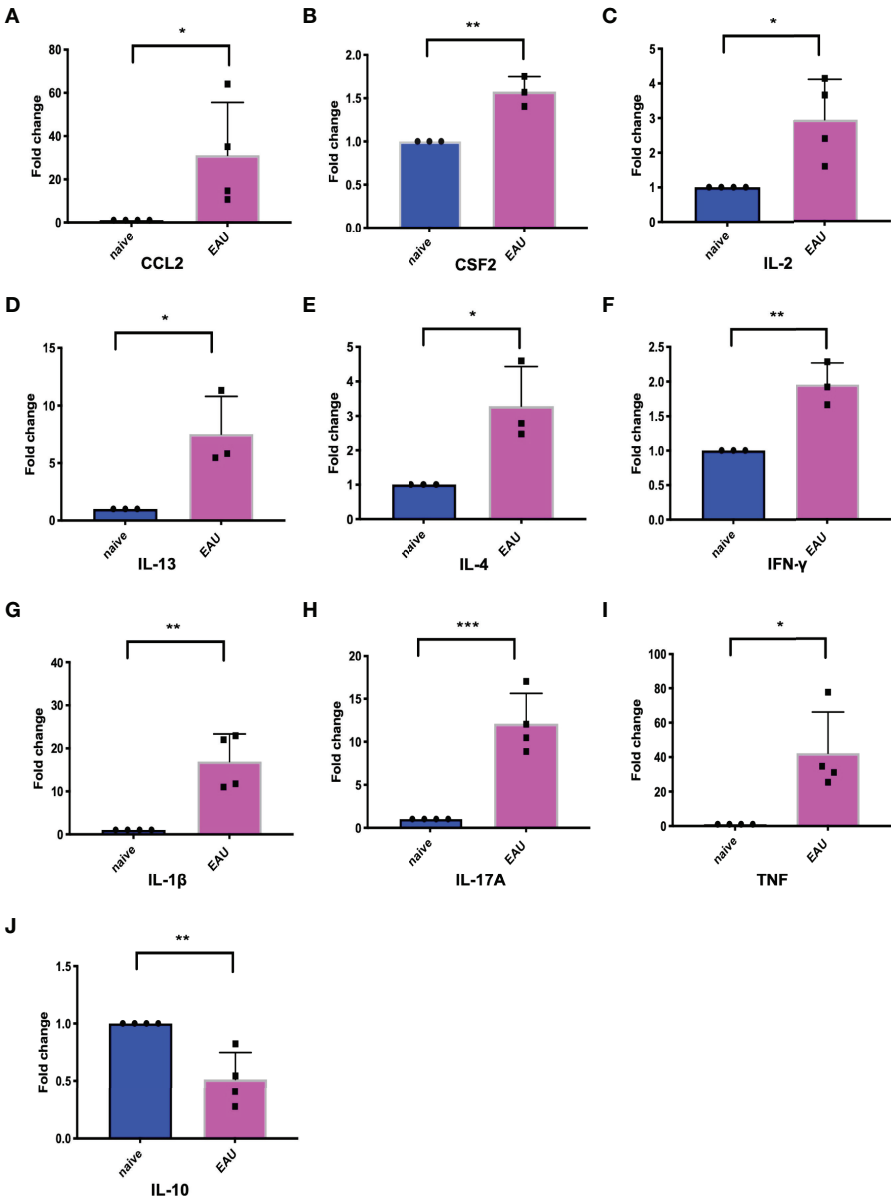

FIGURE 7 | The expression of 10 hub genes in retinas of EAU mice and naive mice. The blue represents naive mice, and the red represents EAU mice. (A-J) represents the expression changes of CCL2, CSF2, IL-2, IL-13, IL-4, IFN-γ, IL-1β, IL-17A, TNF and IL-10, respectively. Except for the decreased expression of IL-10 in EAU, the other 9 hub genes were upregulated in EAU. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (two-tailed student's *t*-test). Graphs show mean ± SEM.

in the retinas of mice on Day 14 after treatment with rabeprazole, which was significantly lower than that of the vehicle group, and there were significant differences between the naive group and vehicle group (**Figure 9E**). IL-1β expression was lower compared to that in the vehicle group, but there was no significant difference with the vehicle group (**Figure 9F**). Meanwhile, the expression of IL-10 in the retinas of the rabeprazole group was increased, and the difference was statistically significant compared with that in the vehicle group, but not statistically significant compared with that in the naive group (**Figure 9**).

DISCUSSION

BD is a refractory disease that affects patients worldwide (24). The treatment of BD remains extremely challenging, although it is possible to select appropriate treatment strategies for patients with specific phenotypes (25, 26). Advances in bioinformatics research provide new approaches for us to process the biological information for some diseases and predict possible therapeutic drugs. In this study, we analyzed the function and pathways of BD-related target genes

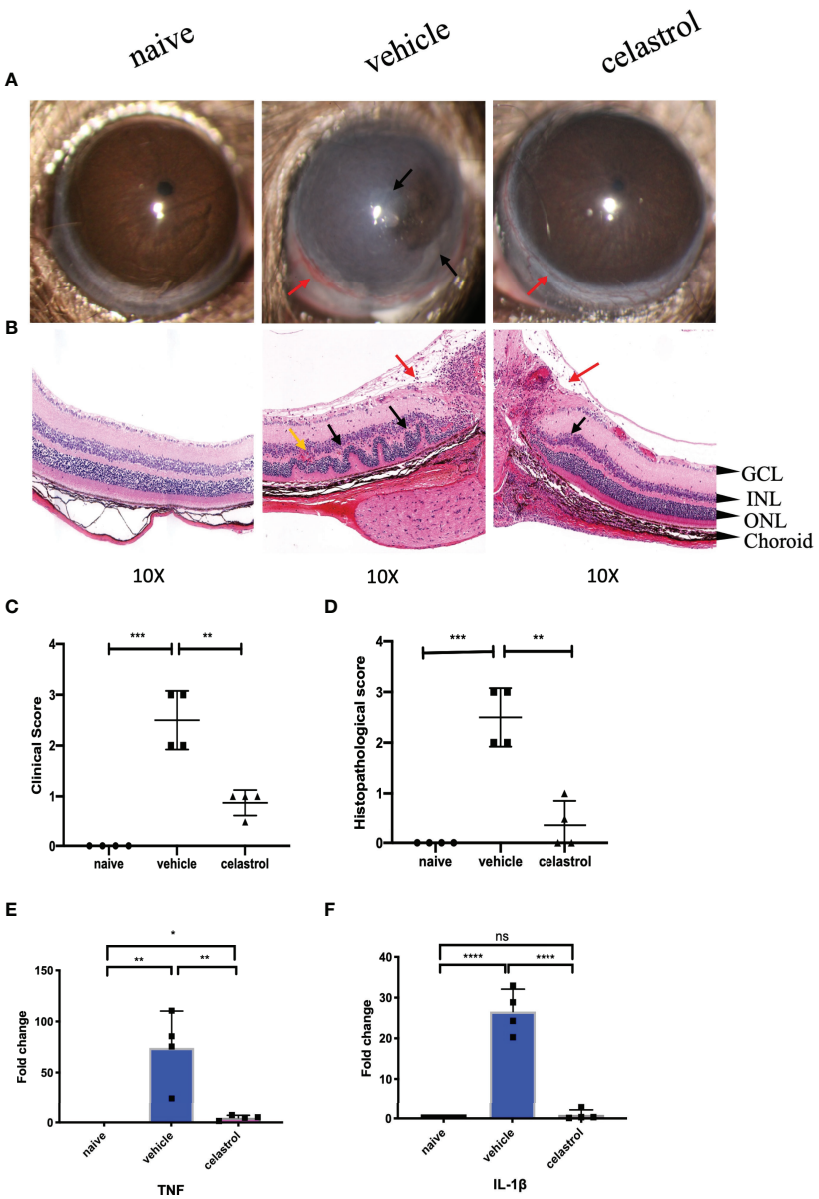

FIGURE 8 | Celastrol alleviated EAU and inhibited the expression of TNF and IL-1β in the retinas of EAU. **(A)** Representative slit-lamp photographs of the naive group, the vehicle intervention group, and the celastrol intervention group on day 14 (n = 4/group). Red arrow, conjunctival hyperemia. Black arrow, inflammatory exudation. **(B)** Representative staining images of HE sections in the naive group, the vehicle intervention group, and the celastrol intervention group on day 14 (n = 4/group). Red arrow, vitreous inflammatory cell infiltration. Black arrow, retinal fold. Yellow arrow, neovascularization, and hemorrhage (GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer). **(C)** The clinical score of the naive group, the vehicle intervention group, and the celastrol intervention group on day 14 (n = 4/group). **(D)** The histopathological score of the naive group, the vehicle intervention group, and the celastrol intervention group (n=4/group). **(E)** qRT-PCR analysis of TNF in retinas of the naive group, the vehicle intervention group, and the celastrol intervention group on day 14 (n = 4/group). **(F)** qRT-PCR analysis of IL-1β in retinas of the naive group, the vehicle intervention group, and the celastrol intervention group on day 14 (n = 4/group). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$ (two-tailed student's *t*-test). Graphs show mean ± SEM.

through bioinformatics, and enriched hub genes to further explore potential investigational drugs.

Combining the results of the searches of the PubMed and UVEOGENE databases, we found 249 genes and proteins that were significantly different in BD. Subsequently, we performed GO and KEGG enrichment analyses on the 249 genes and proteins using the Metascape database. The results of the GO enrichment analyses indicated that these genes and proteins were closely related to "regulation of cytokine production", "immune effector process ", "response to bacterium" and "cytokine activity". Indeed, multiple studies of BD have confirmed that a large number of inflammatory cytokines are

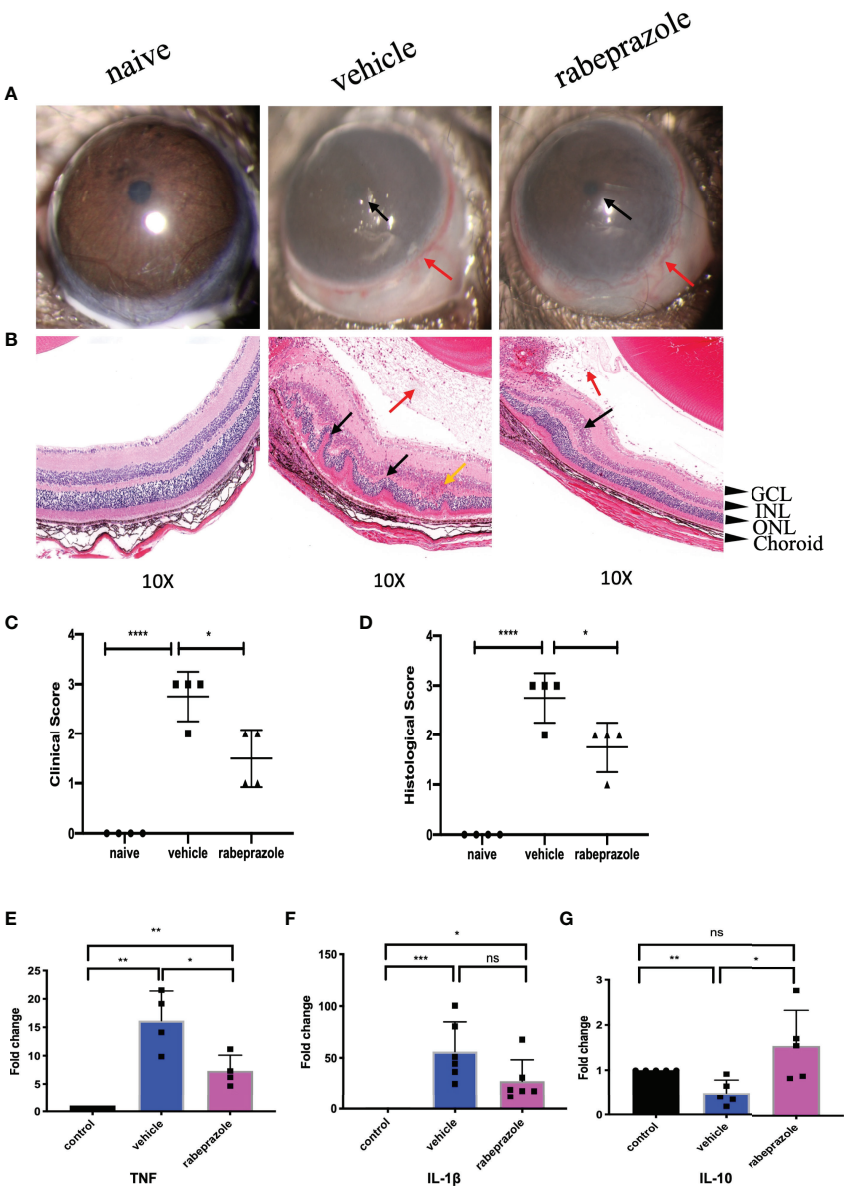

FIGURE 9 | Rabeprazole alleviated EAU and inhibited TNF and IL-1β and promoted the expression of IL-10 in the retinas of EAU. **(A)** Representative slit-lamp photographs of the naive group, the vehicle intervention group, and the rabeprazole intervention group on day 14 (n = 4/group). Red arrow, conjunctival hyperemia. Black arrow, posterior synechiae, and inflammatory exudation. **(B)** Representative staining images of HE sections in the naive group, the vehicle intervention group, and the rabeprazole intervention group on day 14 (n = 4/group). Red arrow, vitreous inflammatory cell infiltration. Black arrow, retinal fold. Yellow arrow, neovascularization, and hemorrhage (GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer). **(C)** The clinical score of the naive group, the vehicle intervention group, and the rabeprazole intervention group on day 14 (n = 4/group). **(D)** The histopathological score of the naive group, the vehicle intervention group, and the rabeprazole intervention group (n = 4/group). **(E)** qRT-PCR analysis of TNF in retinas of the naive group, the vehicle intervention group, and the rabeprazole intervention group on day 14 (n = 4/group). **(F)** qRT-PCR analysis of IL-1β in retinas of the naive group, the vehicle intervention group, and the celastrol intervention group on day 14 (n = 6/group). **(G)** qRT-PCR analysis of IL-10 in retinas of the naive group, the vehicle intervention group and the rabeprazole intervention group on day 14 (n = 5/group) $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$ (two-tailed student's *t*-test). Graphs show mean ± SEM.

involved in BD, such as TNF-α, IL-6, IL-17A, and IL-10 (27–29). In addition, a pathogenic theory for BD suggests that it may be caused by the activation of the innate or adaptive immune system due to bacterial or viral infection or autoantigen damage, thus inducing the production of a large number of inflammatory cytokines and chemokines that participate in the immune-inflammatory response (2).

KEGG enrichment analyses showed that the 249 genes and proteins were mainly enriched in the Cytokine - Cytokine receptor interaction, IBD, and IL-17 signaling pathways.

Currently, multiple cytokine-cytokine receptors interactions, such as IL-23 and IL-23R, TNF-α and TNFR1 or TNFR2, and IL-17A or Il-17F and IL-17RA or IL-17RC, have been confirmed in BD (2, 30, 31). As a member of the IL-12 family, IL-23 is an important cytokine that promotes Th17-cell differentiation (32). The binding of IL-23 to IL-23R can activate Th17 cells, promote the release of inflammatory cytokines, such as IL-17, IL-6, and TNF-α, and enhance the inflammatory response (33). In addition, activation of the IL-23/IL-17 pathway is involved in IBD and BD (29). Furthermore, our results showed that 25 target genes were enriched in the IL-17 pathway, which also suggests that the IL-17 pathway is important in BD. Moreover, identification of the IBD pathway also explained that the occurrence of immune inflammation and the activation of Th1, Th17, and Th2 cells were closely related to the activation of multiple pathways such as the Nod-like receptor signaling pathway, Toll-like receptor pathway, and Cytokine - Cytokine pathway.

To explore the connections among target genes or proteins, we constructed a protein-protein interaction network that divided target genes or proteins into three categories according to the interaction scores: one was clustered with a purple background, one was clustered with a green background, and the rest were placed outside the clusters. Subsequently, the Cytoscape plugin was applied to enrich 10 hub genes (CCL2, IL-13, CSF2, IL-10, TNF, IL-17A, IL-1β, IFN-γ, IL-2, IL4). Among the 10 hub genes, CCL2 was also named MCP1, and CSF2 was also named GM-CSF; nine of the hub genes, excluding CSF2, came from UVEOGENE databases. Furthermore, the enrichment results for the hub genes also suggested that TNF, IL-1β, and IL-10 are the most critical hub genes. TNF is a key pathogenic factor of BD, and some studies have shown that TNF is related to disease activity in BD (34) (35). TNF is highly expressed in various tissues in BD patients, such as oral ulcer tissue, the aqueous humour, and intestinal lesions (36–38). At present, there are many kinds of TNF-α blockers available for the treatment of BD, such as IFX, alemtuzumab (ADA), and etanercept (ETC) (39). Moreover, IFX and ADA have been recommended as first-line treatments for severe posterior uveitis associated with BD (40). IL-1β, a proinflammatory factor, is mainly secreted by monocytes, macrophages, and dendritic cells (DCs) and can induce the release of various inflammatory chemokines and promote the production of immune inflammation (25) IL-1β is highly expressed in the serum of patients with BD, and targeted inhibition of IL-1β has also been shown to be effective in the treatment of BD (41). Currently, canakinumab, gevokizumab, and anakinra, which are all IL-1 blockers, have been proven to be effective in the treatment of BD (42–44). IL-10 is an anti-inflammatory cytokine secreted by Th2 cells that mainly antagonizes proinflammatory factors, and promotes the activation of B cells to produce antibodies (45, 46). Moreover, three BD risk alleles rs1518111A, rs1800872A, and rs1800871T were related to IL-10 (47, 48).

To explore potential drugs, we analyzed the association between genes or drugs and the top 3 hub genes through the DGIdb database. Our results were centered on seven drugs, including IFX, pentoxifylline, and cyclosporine, which are clinically appropriate for the treatment of BD, and alteplase can be used for the treatment of BD complications (22, 23). However, the remaining three drugs have not been proven to be effective in the treatment of BD. Celastrol is a bioactive ingredient of *Tripterygium wilfordii*, which has been proven to have anti-inflammatory, antitumor, and antineovascularization effects in some studies (49, 50). Several studies have shown that celastrol has a certain anti-inflammatory effect on IBD, experimental autoimmune encephalomyelitis (EAE), and psoriasis models (51–54). In recent years, Chinese doctors have paid more attention to clinical experiments evaluating the use of celastrol in treating rheumatoid arthritis, idiopathic membranous nephropathy, and IBD (NCT01613079, NCT01161459, NCT02044952). Omeprazole and Rabeprazole are proton pump inhibitors (PPIs) that are currently mainly used for the treatment of gastrointestinal diseases, including gastroesophageal reflux disease, gastric and duodenal ulcers, and Zollinger-Ellison syndrome (55). Omeprazole is a first-generation PPI approved for clinical treatment, and it was followed by lansoprazole, rabeprazole, and other PPIs (56). Although PPIs are primarily used to treat digestive diseases, studies have shown that they may have anti-inflammatory effects in addition to inhibiting acid production (57). In recent years, PPIs have played an important role in inhibiting antigen presentation by inhibiting the expression of TNF-α and IL-1β induced by different pathogen-associated molecular patterns (PAMPs). In addition, PPIs inhibit the release of a large number of inflammatory factors caused by Toll-like receptor signaling activation in monocytes (58). Both omeprazole and rabeprazole have been reported to exert anti-inflammatory effects related to the inhibition of NF- κB activation (59–61). Furthermore, a number of studies have confirmed that PPIs can not only reduce inflammation in the human gastrointestinal epithelium but also reduce inflammation in the human lung epithelium and psoriasis (60, 62, 63). However, the treatment of other systemic inflammatory diseases with PPIs has rarely been reported.

Next, to verify whether these drugs might inhibit the inflammatory response in BD, we constructed an EAU mouse model with B10RIII mice (64). We compared the expression of hub genes in the retinas of EAU mice between a pre-modeling timepoint and Day 14 after modeling. The results showed that IL-10 expression was reduced in the retinas of EAU mice, but the expression of the other 9 hub genes was increased; these 10 hub genes significantly differed between the model and control mice. The data suggested that these 10 hub genes are involved in EAU and that EAU is a good research model for BD uveitis. Subsequently, we selected celastrol and rabeprazole from among the three unproven drugs to treat EAU mice. Although our study results pointed to both omeprazole and rabeprazole, considering that both drugs are metabolized by the CYP450 enzyme, we chose rabeprazole for experimental verification and found that rabeprazole had little effect on the enzyme CYP450 (65). In the EAU mouse model,

EAU usually starts between Days 7 and 9, peaks on approximately Day 14, and then gradually resolves (17). We chose to administer treatment from the onset of EAU to the peak of the disease. Our results showed that after treatment with celastrol for 7 days, anterior chamber inflammation was significantly reduced, the retinal structure was slightly intact, and the retinal pro-inflammatory factors TNF and IL-1β were significantly downregulated, suggesting that celastrol could effectively alleviate EAU. In addition, in mice treated with rabeprazole, the inflammation in the anterior part of the mice was slightly reduced compared with that in vehicle-treated mice, and the retinal damage was less severe. The expression levels of the proinflammatory factors TNF and IL-1β in the retinas were lower in rabeprazole-treated mice than in vehicle-treated mice, while the expression of the anti-inflammatory factor IL-10 was upregulated compared with that in vehicle mice. These results indicated that rabeprazole can alleviate EAU to a certain extent.

In a short, target functional analysis and potential drug mining for BD highlighted the effectiveness of network pharmacological approaches. However, uveitis represented by the EAU model is just one of the typical manifestations of BD, so these agents need to be validated in many models. We also demonstrated the anti-inflammatory effect of rabeprazole in EAU for the first time, but the mechanism and side effects remained unclear. These results are still worthy of further study, which may provide some inspiration for the treatment of BD. In addition, the bioinformatics analysis may be exploratory, our study focused on PPIs, which are not clinically used as anti-inflammatory agents, it is also worth noting that an increasing number of studies have shown the anti-inflammatory properties of PPIs. Furthermore, our bioinformatic analysis focused on the existing data and the addition of future information that could affect our results. Despite these limitations, this study is the first preliminary and basic experimental verification of BD target gene enrichment-related drugs and provides new insights into the disease mechanisms and therapeutic potential of BD.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of the Renmin Hospital of Wuhan University (WDRM20210708A).

AUTHOR CONTRIBUTIONS

The design and conception of the manuscript were completed by YX and ZC. Data collection and collation were performed by QX and CL. Bioinformatics analysis was finished by CL and FL. The EAU model constructed by QX, BP, and XG. Drug validation-related experiments were performed by QX, FL, and BP. Data collation and mapping were finished by CL, QX, and HR. Manuscript writing and revision were performed by QX, CL, FL, YX, and ZC. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.895869/full#supplementary-material

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REVIEW
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Double Negative T Regulatory Cells: An Emerging Paradigm Shift in Reproductive Immune Tolerance?

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Immune regulation of female reproductive function plays a crucial role in fertility, as alterations in the relationship between immune and reproductive processes result in autoimmune subfertility or infertility. The breakdown of immune tolerance leads to ovulation dysfunction, implantation failure, and pregnancy loss. In this regard, immune cells with regulatory activities are essential to restore self-tolerance. Apart from regulatory T cells, double negative T regulatory cells (DNTregs) characterized by TCR$\alpha\beta^+$/$\gamma\delta^+CD3^+CD4^-CD8^-$ (and negative for natural killer cell markers) are emerging as effector cells capable of mediating immune tolerance in the female reproductive system. DNTregs are present in the female reproductive tract of humans and murine models. However, their full potential as immune regulators is evolving, and studies so far indicate that DNTregs exhibit features that can also maintain tolerance in the female reproductive microenvironment. This review describes recent progress on the presence, role and mechanisms of DNTregs in the female reproductive system immune regulation and tolerance. In addition, we address how DNTregs can potentially provide a paradigm shift from the known roles of conventional regulatory T cells and immune tolerance by maintaining and restoring balance in the reproductive microenvironment of female fertility.

Keywords: double negative T cells, double negative T regulatory cells, ovulation, endometrium, reproduction, immune tolerance, implantation, reproductive immunology

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INTRODUCTION

Immune tolerance during the female reproductive cycle and pregnancy is required for fertility. Successful ovulation, fertilization, and pregnancy rely on an efficient regulatory mechanism that prevents immune responses to sperm cells, oocytes, and placental cells, which express various antigens (1–6). Several female pathologies are linked to immune tolerance dysfunctions, including preeclampsia, recurrent miscarriages, and autoimmune ovarian failure. Pre-eclampsia, which accounts for about 20% of maternal mortality, is associated with a dominant Th1 inflammatory response resulting from impaired function of cells with regulatory activities (7–10). Recurrent miscarriages, which affect about 1-5% of women, are associated with reduced regulatory T cells (11, 12). More recently, autoimmune ovarian dysfunction, a disorder affecting female patients with autoimmune disorders, has been associated with immune tolerance alterations (13–15). These disorders highlight that an imbalance in the immune tolerance

equilibrium in the female reproductive system (FRS) can cause severe gynecological and obstetrical complications.

Suppression of immune responses by cells with regulatory activities is one of the central mechanisms for induction and maintenance of self-tolerance (16). $CD4^+CD25^+$ T regulatory cells ($CD4^+$Tregs) are the most extensively studied suppressor cells regarding reproductive failure and reproductive immune tolerance (17–19). Furthermore, estrogen and seminal fluid drive the expansion of $CD4^+$Tregs, enhance their suppressive function (11, 21, 22), and regulate their activities in the FRS. However, recent data finds that a subset of T cells with unique potent regulatory functions and dominance in the reproductive system (20, 21), known as the double negative T regulatory cells (DNTregs), also play crucial roles in regulating immune responses (21–24). This current review focuses on understanding the characteristics and function of DNTregs as potential regulators of reproductive female immune tolerance and their potential as a novel therapeutic option for reproductive failure, including those associated with autoimmunity. Specific details of DNTregs on non-reproductive autoimmune conditions such as graft versus host disease (GvHD) and cancer are covered elsewhere (25–28). We will provide the context for the interplay of DNTregs in reproductive function. We will also discuss and compare the presence, function, and relevance of $CD4^+$Tregs and DNTregs in reproductive immune tolerance. Finally, this review will address how DNTregs represent a potentially viable therapeutic option in female reproductive disorders, including autoimmune conditions.

IMMUNE REGULATION IN THE REPRODUCTIVE CYCLE – A TIGHTLY CONTROLLED PHYSIOLOGIC "INFLAMMATORY" PROCESS

Immune cells modulate several aspects of the female reproductive function, including folliculogenesis, ovulation, implantation, pregnancy, and labor (29, 30). At distinct points in the reproductive cycle, different immune cells are identified throughout the FRS. Immune cells occur in high frequencies within the upper FRS (the ovaries, oviduct, uterus, and endocervix) and occur less in the lower FRS (ectocervix and vagina) (31). The FRS is an exceptional immunological site, and the distribution of immune cells facilitates tolerance to allogeneic sperm and the semi-allogeneic fetus and placenta (11, 32, 33).

During the reproductive cycle, steroid hormones and immune cells regulate ovulation, fertilization, and implantation in a cyclic fashion and tandem (34–36). Fluctuations of estradiol and progesterone drive relevant processes within the uterus and ovary, including cyclic recruitment of immune cells (37). Within the FRS, immune cells are primarily under ovarian hormone regulation (31, 36, 38) and contribute to the modulation of the reproductive cycle and fertility (39). For instance, T cells (and immune cells in general) express sex-steroid receptors (40–43), and the high infiltration of $CD8^+$ T cells into the regressing corpus luteum (CL) coupled with the expression of cytolytic proteins corresponds to decreased progesterone and estradiol concentrations during the period of luteal regression in the ovaries (44). Furthermore, alterations in the immune system affect ovarian function in both animals and humans (45). For instance, blockade of gonadotropin-releasing hormone (GnRH), which centrally regulates the hypothalamic-pituitary-ovarian axis (46), decreases regulatory T cells' proliferation and thymic mass (47).

Moreover, estrogen deficiency corresponds with increased peripheral cytotoxic T cells and $CD8^+/CD4^+$ T cell ratios. Furthermore, the rise in circulating estrogen levels is associated with increased $CD4^+$Treg populations (48). These findings indicate that the reproductive hormones tightly regulate immunological changes in the ovary and endometrium (49).

DOUBLE NEGATIVE T CELLS: ORIGIN AND FUNCTION

Most mature αβ T cell receptor $(TCR)^+$-T cells in normal mice and humans express either the CD4 or CD8 coreceptor molecules. However, approximately 1–5% of the peripheral T cell population that expresses CD3 but neither the CD4 nor the CD8 coreceptor is termed, CD4 and CD8 double-negative T (DNT) cells. DNT cells were identified in spleen cells from irradiated mice over 40 years ago, but at the time, they were referred to as natural suppressor cells (50, 51) with a null phenotype in neonatal mice (52). In 1984 Oseroff et al. reported that DNT cells do not express the T cell marker CD90/Thy-1, the surface immunoglobulin (Ig), the myeloid marker CD11b/MAC1, the macrophage marker F4/80, or the monocyte specific esterases (52). Their study reported that DNT cells were similar to natural killer (NK) cells based on their combined lack of antigen-specificity and coreceptor CD4 and CD8 molecules (52, 53). However, later reports found that DNT cells inhibited T cell response to alloantigen (54, 55) in an antigen-specific manner (24). Similarly, early reports on this subject suggested that the suppressive function of DNT cells may not depend on proliferation, as suppression was maintained after exposure to high levels of radiation (56). This finding partly explained why DNT cells exert potent suppressive functions despite low numbers. Furthermore, early research also suggested that these unique cells were dependent on T cells in the spleen (50) and that their function becomes dominant when $CD4^+$, $CD8^+$ T cells, and B lymphocytes are unable to function effectively (56). However, DNT cells with regulatory functions (DNTregs) were not classified as such until 2000 (24).

Naturally occurring T cell maturation and differentiation into $CD4^+$, $CD8^+$, and $CD4^+$Tregs require thymic development (57, 58) though peripheral or induced $CD4^+$Tregs can develop from peripheral naïve conventional T cells (59). However, the origin of DNTregs is still a matter of debate. Several reports suggest that DNT cells originate from the thymus either as DNT cells or from single positive (SP) T cells under regulation by sex steroids (21, 60). However, researchers also found that large DNT cell populations can develop, mature, and gain regulatory function in the absence of a thymus or outside the thymic microenvironment (61), just like the peripheral $CD4^+$Tregs. In support of the latter, other studies found that DNT cells can develop and mature in the bone marrow (62),

liver (63), nasal-associated lymphoid tissues (NALTs) (64), and, as concerns this review, in the FRS (20). Furthermore, studies that suggest $CD4^+$ or $CD8^+$ precursors for DNT cell maturation indicate a requirement for the thymus at least for their initial development (65–67). The conclusions for DNTs' origin from $CD8^+$-derived DNT cells were based on the observation that antigen encounter decreases $CD8^+$ populations and increases DNT cell numbers *in vitro* (68, 69) or *in vivo* (70, 71). However, recent *in vivo* data revealed that the development and function of DNT cells could occur in the absence of $CD8^+$ cells, indicating that DNT cells can develop from a cell lineage independent of CD8 expression (61), consistent with an earlier report (52). The increase in DNT cells in the study describing decreased $CD8^+$ populations upon antigen encounter may have been due to direct activation and expansion of a pre-existing population of DNT cells. Other studies propose that DNT cells in *lpr* mice (which have null allele for *Fas* (CD95) (72)) may occur from $CD8^+$T cell precursors in the periphery (73, 74) and that DNTs may also occur from $CD4^+$ T cell precursors on stimulation of $CD4^+$T with allogeneic DCs in the presence of IL-15 or IL-2 (22). Taken together, DNT cells may develop from a separate lineage. However, there may be some degree of peripheral differentiation from peripheral single positive T cells, and further studies are required to determine their precise origins.

Phenotypic Characterization of DNTs

Total DNT cells comprise 1-5% of total peripheral T cells in mice and humans, and their functions and phenotype have been characterized (24, 75, 76). Based on the expression of NK cell markers, DNT cells can be divided further into two subpopulations: NK^+DNT cells, referred to as NKT cells (67, 77), and NK^-DNT cells, referred to as DNT cells (24, 75, 78). Reports suggest that DNT cells exist as cytolytic or regulatory subsets (DNTregs) and bear either the TCRγδ or TCRαβ repertoire (79–82). Though some reports show that DNT TCRαβ cells exhibit regulatory effects and are characterized as $TCR\alpha\beta^+CD3^+CD4^-CD8^-$ $NK1.1^-$T cells (24, 75), other reports suggest that DNT cells expressing TCRγδ also exhibit regulatory potential (21, 83). Furthermore, mature peripheral DNTregs differ from bone marrow-derived DN natural suppressor T cells that express NK1.1 (84–87).

$CD4^+$Tregs consist of naturally occurring Tregs (nTregs or thymic Tregs) and inducible or peripheral Tregs (iTregs or pTregs). By contrast, current evidence indicates that total DNT cells are heterogeneous. By utilizing single-cell RNA sequencing, flow cytometry, and qPCR to analyze total DNT cells from C57BL/6 mouse spleens, a recent study investigated naïve and activated total DNT populations (nDNT and aDNT, respectively) (88). The study found high gene expression of FasL (*Fasl)*, granzyme b *(Gzmb)*, interferon-gamma (*Ifng)*, killer cell lectin-like receptor D1 (*Klrd1)*, killer cell lectin-like receptor C1 (*Klrc1)*, and killer cell lectin-like receptor K1 (*Klrk1)* in nDNT or aDNT subgroups. These findings indicated that these cells exert regulatory functions (88) and may represent the DNTreg phenotype. The study demonstrated that naïve populations significantly expressed the Ikaros family zinc finger 2 (*Ikzf2*) and lymphocyte antigen 6 complex locus C2 (*Ly6c2*) genes (88). The transcription factor IKZF2 is expressed by T cells undergoing central and peripheral tolerance (89). Beyond regulating IL-2 production by Tregs, studies indicate that *Ikzf2* is also required to stabilize the suppressive phenotype in $Foxp3^+$Treg populations (90). Though the Ly-6C protein is known to recruit macrophages in murine liver fibrosis (91), it is essential for the development of naïve and activated DNT cells, particularly the DNT subsets with regulatory functions. For clarity, the study associated the 'cytotoxic' DNT subsets with regulatory function. It is, however, unclear if this 'cytotoxic' phenotype consists of a heterogeneous population with effector 'cytotoxic' DNTs that drive disease pathology and a regulatory 'cytotoxic' phenotype. However, based on these data, the study identified 5 clusters in nDNT cells (88): (**i**) Resting DNT cells that feature high expression of killer cell lectin-like receptor D1 (*Klrd1*) (88), a negative regulator of NK cells (92); (**ii**) Helper DNT cells that feature high expression of RAR related orphan receptor A (*Rora)*, a gene that regulates the Th17 lineage in synergy with the RAR related orphan receptor C (*Rorc*) gene (93); (**iii**) Intermediate DNT cells that express Eomesodermin *(Eomes)* (88), a master regulator of cell-mediated immunity capable of controlling the expression of genes encoding effector molecules, such as *Ifng* or *Gzmb* (94); (**iv**) cytotoxic DNT cells with high expression of *Gzmb* (88) and (**v**) innate DNT cells. The significant expression of the *ikzf2* and lymphocyte antigen 6 complex locus C1 (*Ly6c1*) in the resting and "cytotoxic' DNT cells suggest that IKZF2 may be the critical transcription factor defining the 'cytotoxic' DNT phenotype (88). By contrast, the high expression of Ly6c2 in naïve and helper DNT cells suggests that Ly-6C may be a critical gene defining the "resting" DNT phenotype (88). Furthermore, the study showed that 'cytotoxic' DNT cells share similar characteristics to $CD8^+$T cells (88) and that the naïve and cytotoxic DNT cells shared several highly expressed genes with the activated and resting DNT cells.

On activation, the nDNT cells described in the study differentiate to the aDNT groups, with a reduced *Ly6c2* and enhanced *Ikzf2* expression. Though the authors noted that expression levels of *Il17a* were low in aDNT cells, the 'cytotoxic' DNT subset that corresponds to DNTregs expressed significantly high levels of cytotoxic genes, including *Gzmb* (88). The aDNT phenotype described in the study has been utilized as DNTreg cellular therapy for acute myeloid leukemic patients indicating that the 'cytotoxic' DNT cell phenotype may indeed be DNTregs that can be utilized explicitly as treatments for immune-related diseases (95). Identifying IKZF2 and Ly6C as specific markers of DNTs and possibly DNTregs provides a starting point for further exploration of DNT cells. This study suggests that different subsets of DNT cells are similar to what was proposed in an earlier transcriptomic study on DNT cells from sooty mangabeys (96). The authors reported that DNT cells displayed phenotypes similar to Th1, Tfh, Th2, and Th17 cells (96). It is currently not entirely clear whether DNT cells can differentiate into different subsets or if DNTregs exist as a single phenotype with multiple functions; therefore, further investigations are needed. It is also not entirely clear if IKZF2 specifically identifies the DNTreg subset though it is implied in the study. Perhaps the identification of IKZF2 along with other regulatory surface markers will provide more clarity on DNTreg identification markers.

However, based on current literature, it is clear that DNTs can exist as cytolytic or regulatory cells and may also exist in naïve and activated states where the activated states exhibit multiple functions determined by the microenvironment (**Figure 1**). However, this review will primarily focus on the regulatory subtype of DNTs.

Recently, cancer researchers using single-cell RNA sequencing (scRNA-seq) identified 12 clusters of "unconventional" $\alpha\beta TCR^+$ DNT cells in the tumor microenvironment of murine sarcomas supporting the highly heterogeneous nature of DNT cells (97). Furthermore, the study reported that four of these clusters represented 75% of all DNT cells within the tumors, suggesting that the tumor microenvironment can influence the recruitment and differentiation of DNTs. Interestingly, none of the clusters with DNT cells predominantly expressed the checkpoint inhibitors CTLA-4 and PD-1. This finding is supported by a study that showed low CTLA-4 and PD-1 expression in a DNTreg subset (98) but contrasted by another showing high CTLA-4 and PD-1 expression. These cells can also downregulate costimulatory molecules CD80 and CD86 expressed on antigen-expressing mature DCs (mDCs) (99). Though the literature appears inconsistent on DNTregs checkpoint inhibitors' expression, perhaps expression may depend on the microenvironment. In addition, published data suggest that DNTregs can exert alternative recognition and killing mechanisms through Fas–FasL in an antigen-specific manner (24). The study also suggests that the tissue microenvironment can recruit DNT cells and that these DNT cells adapt their phenotype and function to the prevailing condition. Indeed, several reports support the premise that DNTregs have antigen-specific functions *in vitro* and *in vivo* (24). However, the nature of this function is still under debate since some reports indicate that DNT cells lack major histocompatibility complex (MHC) restriction and have a unique TCR repertoire not derived from positive selection.

Moreover, reports indicate that the antigen-binding characteristics are different from those of MHC-restricted single positive (SP) T cells that bind to antigen epitopes on MHC I or II molecules (100). However, a unique antigen-binding pattern was described for $\gamma\delta$TCR DNT cells based on the conformation of the intact antigen and independent of MHC involvement (101). Support for this conclusion comes from a study using mice deficient in both CD4 and CD8 coreceptors and MHC (quad-deficient mice) (102). However, a study proposed that DNTregs can recognize MHC-peptide complexes (103), which can occur through upregulation of the lymphocyte-specific protein tyrosine kinase (Lck), expressed on activation and can drive TCR signal transduction in DNT cells (102). The upregulation of lck orchestrates TCR signaling independent of MHCs (102) and constitutes a straightforward explanation for how the thymus generates MHC-restricted $\alpha\beta$TCR. Nonetheless, a study showed that DNT cells could develop independent of lck and mediate potent suppressor functions (104). Since lck mediates TCR interaction with CD8 and CD4 coreceptor molecules (which are absent in DNT cells (105)), other kinases that regulate TCR signaling, such as the proto-oncogene tyrosine-protein kinase (fyn), may therefore be more relevant in driving DNT cell TCR signaling.

THE FEMALE REPRODUCTIVE SYSTEM AND DNTREGS IMMUNE REGULATION – COMPARISON WITH $CD4^+$TREGS

It is well accepted that estrogen drives the expansion of the $CD4^+$Treg compartment by inducing their proliferation and

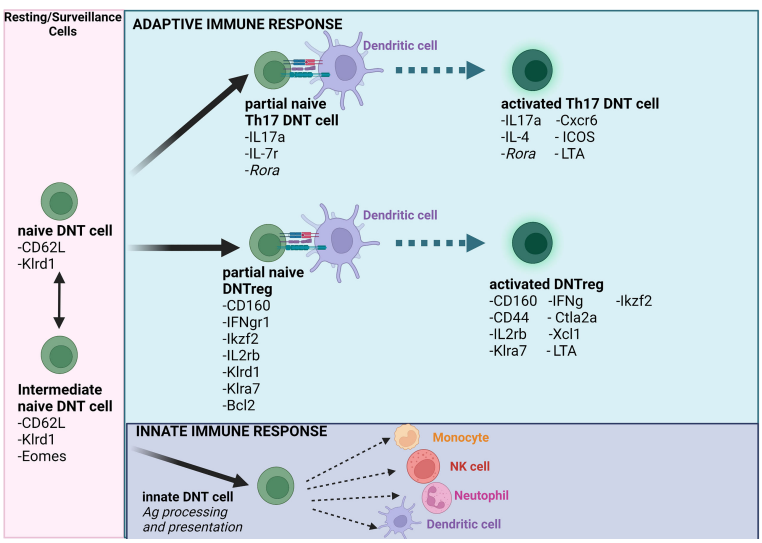

FIGURE 1 | Schematic model of naïve and activated DNT cells. The model summarizes and reflects how transitions impact naïve and activated DNT cells (Note- the literature indicates the possibility of several other DNT cell subsets). Importantly, DNT cells can modulate innate and adaptive immune cells based on immune-modulatory status or the prevailing condition in the tissue microenvironment. Ag = antigen. *Figure created using Biorender.com*.

promoting suppressive functions (17–19). Interestingly, estrogen also drives the accumulation of DNTs during pregnancy (106). Moreover, like estrogen, seminal fluid also modulates Tregs in the FRS (107–109) and contributes to maternal-fetal tolerance (108, 110).

The roles of $CD4^+$Tregs in the FRS, including maternal-fetal tolerance, have been extensively explored and reviewed elsewhere (111–116). We will therefore focus on how DNTregs compare with $CD4^+$Tregs in the FRS. $CD4^+$Tregs undergo profound changes in the ovary and uterus during the reproductive cycle. In healthy women, an increase is observed during the follicular phase of the menstrual cycle reach maximum numbers during the late follicular phase. This process correlates with increased circulating estrogen levels (11, 117). Moreover, endometrial $CD4^+$Tregs expand during the proliferative phase of the cycle (11), which corresponds to the follicular phase in the ovaries. However, as women with reproductive failure experience a dramatic reduction in the population of $CD4^+$Tregs (11), it is yet unknown whether a similar phenomenon occurs with DNTregs (117, 118). (119) Since estrogen regulates $CD4^+$Tregs and DNTs in the FRS, the function of DNTregs in the FRS may be similar to $CD4^+$Tregs. However, establishing precise DNT functions in the FRS and how the populations differ during the female reproductive cycle requires further research. In addition, it is also unknown if seminal fluid modulates DNTreg expansion just as it does $CD4^+$Tregs in the FRS and requires further research.

$CD4^+$Tregs are also crucial to pregnancy maintenance, as changes to the population of regulatory cells may have severe consequences for fertility (118, 119). Several lines of evidence have described both the systemic and decidual expansion of $CD4^+CD25^+Foxp3^+$ Treg populations in the first two trimesters of pregnancy (120–122).These findings indicate that $CD4^+$Tregs are necessary for reproductive immune tolerance. Since both $CD4^+$Tregs and DNTregs have been detected in the non-pregnant endometrium during the reproductive cycle, and reproductive hormones modulate $CD4^+$Tregs, we also postulate that similar mechanisms modulate DNTregs during non-pregnant and pregnant conditions. Though the literature on the role of DNTregs in the reproductive system pre- and post-fertilization is scanty compared to that of $CD4^+$Tregs, it is clear, based on reports on other tissues, that DNTregs play significant regulatory roles similar to $CD4^+$Tregs. Indeed, reports indicate consistently large numbers of DNTregs in the FRS compared to other organs (20, 21, 123). The large numbers of DNTregs found in the uterus, gut, and kidney suggest a preference for highly vascularized regions, particularly mucosal tissues (20, 124). Johansson and Lycke observed that DNTreg numbers in the uterus did not vary in non-pregnant mice, after insemination, and on day-4 pregnancy. They, however, state that this does not exclude a function for these cells during pregnancy (20). That the number of DNTregs did not decrease during early mouse pregnancy may suggest both a relevance or irrelevance for these cells in maintaining maternal-fetal tolerance. A possible explanation for the lack of increase observed during early mouse pregnancy may be related to the finding that during the first trimester, pro-inflammatory cells and cytokines are more relevant as these cells assist the blastocyst to successfully penetrate or 'implant' the endometrium (125). However, regulatory cells remain present within the endometrium to keep the pro-inflammatory cells in check and will expand during the second trimester and then decrease during the latter part of the third trimester (125). On the other hand, since $CD4^+$Tregs are reported to increase in the first two trimesters of pregnancy (121, 122) and a study reports that DNTregs do not increase or decrease during early mouse pregnancy (20), these findings may also suggest that DNTregs may not play a significant role during early pregnancy and instead may be more relevant as immune response regulators throughout pregnancy duration (and in non-pregnant conditions) (116). However, further studies are required to confirm the role of DNTregs in the different phases of pregnancy. Indeed, a report found that the suppressive capacity of $CD4^+$Tregs in peripheral blood and decidua is similar among non-pregnant, pregnant, and patients with recurrent spontaneous abortion (RSA) (121), which could be extrapolated to DNTregs as both cells are recruited and regulated by sex steroids. Moreover, a review by Chapman et al. suggests a possible function of DNTregs during pregnancy which involves maintaining trophoblast and blood vessel integrity and promoting angiogenesis (21).

Taken together, DNTregs can contribute to establishing immune tolerance within the endometrium in preparation for fetal allograft, a concept consistent with their reported suppressive role in preventing rejection of donor-specific allografts (126, 127). While available data on DNTs in different phases of the reproductive cycle are scarce, current knowledge on the presence of DNTs in the FRS and during pregnancy suggests a prominent role for these cells in the FRS that may involve immune regulation and providing support to $CD4^+$Tregs which should be explored further.

DNTREGS AND $CD4^+$TREGS IN TOLERANCE MAINTENANCE - IMPLICATIONS FOR REPRODUCTIVE IMMUNE TOLERANCE

Tolerance to peripheral T cells is necessary to prevent autoimmune damage to self, and regulatory T cells generally act to suppress responses to self and allogeneic antigens (23). Human pregnancy has been described as a condition that requires maintenance of maternal immune tolerance to a semi-allograft fetus comprised of paternally derived antigens. Though the mechanisms involved in sustaining maternal immune tolerance to the semi-allogeneic fetus are still poorly understood, a report suggests that spontaneous abortion is due to the allo-rejection of the fetus by the mother (121). Clearly, a crucial role of regulatory cells in pregnancy involves the mediation of maternal tolerance of the fetus (122). In addition, DNTregs induce donor-specific transplantation tolerance to MHC-mismatched allografts (24). Subsequent reports confirmed that DNTregs induce tolerance to skin and islet

transplantation (22, 128), and cardiac xenografts in a donor-specific manner (129) and (122) support a potential role for DNTregs present in the FRS in maintaining reproductive immune tolerance. Studies in several murine models have demonstrated that DNTregs suppress $CD4^+$ and $CD8^+$ T cell-mediated allogeneic and xenogeneic immune responses and the response to self-antigens (126, 129, 130). DNTregs prevent allograft rejection (131), GvHD (132, 133) and modulate the severity of autoimmune diseases such as autoimmune diabetes (134, 135). However, $CD4^+Foxp3^+$Tregs and human DNTregs appear to require activation by allogeneic antigen-presenting cells (APCs) or anti-CD3/anti-CD28 antibodies to induce their regulatory potential. DNTregs also reversibly suppress the proliferation of responder T cells *via* cell contact-dependent mechanisms (136) which will be discussed subsequently. Furthermore, DNTregs can function as anti-tumor effector cells that mediate nontumor antigen-restricted immunity while maintaining immune regulatory functions (27). These features position DNTregs to effectively function in the different reproductive phases, maintaining remodeling processes and ensuring successful placenta invasion of the endometrium. It is important to note that the precise roles of DNTregs in maternal immune tolerance are yet to be fully established and requires further research. However, we have indicated potential roles of these cells in maternal immune tolerance based on their immune regulatory roles in other tissues and systems.

Immune-Modulation Mechanisms of $CD4^+$Tregs and DNTregs – Implications for Immune Regulation and Tolerance in the FRS

Cell-to-Cell Contact and Antigen Specificity of DNTregs-Mediated Suppression

Both $CD4^+$Tregs and DNTregs appear to require cell to cell contact to mediate suppression (137–139). $CD4^+$Tregs also inhibit other T cells through IL-2 inhibition at the gene transcription level and are not necessarily antigen-specific (138, 140, 141), though *in vivo* antigen-specific suppression can also occur in some subsets (142, 143). However, reports suggest that DNTregs mediate immune suppression in an antigen-specific manner *in vivo* or *in vitro* without competing with other T cells for growth factors or APC surface area. This finding is supported by studies showing that intentionally increasing the APC numbers or the IL-2/IL-4 concentrations did not reverse the DNTreg-induced suppression (24, 139). However, cell-to-cell contact is required for maximal DNTreg mediated suppression, as supernatant from DNTreg clones stimulated *in vitro* with irradiated splenocytes expressing a TCR specific for the *Ld* MHC class I molecule (Ld+) failed to inhibit the naïve anti-Ld response maximally (24). The antigen-specific nature of DNTregs makes these cells excellent tools to be utilized as a targeted novel cellular therapy for reproductive conditions where autoantibodies trigger a self-reactive T cell response to ovarian antigens, such as autoimmune ovarian disease.

DNTregs Mediate Immune Suppression of Effector Cells *via* Trogocytosis and TCR Specificity

DNTregs present antigens on their surface acquired from APCs, which are recognized by responder T cells bearing TCRs for the cognate antigen (**Figures 2**, **3**). A study showed that DNTregs cells could kill allogeneic and syngeneic $CD8^+$T cells that express similar TCR, indicating that a specific TCR interaction may be involved in its suppression mechanism (24). Thus, it would seem that a shared TCR-specificity between DNTregs and target T cells may be one of the factors required for cytotoxicity to occur. Furthermore, reports indicate that DNTregs may not participate in bystander killing (24). Just as DNTregs require stimulation through their TCR to gain a suppressive phenotype, $CD4^+$Tregs also require TCR stimulation for their function. $CD4^+$ Treg TCR stimulation can be mediated *in vitro* through specific peptides but not through third-party antigens (140, 144). Murine and human DNTregs have a unique feature of retaining surface expression of the acquired molecules for several days (24), providing a wide window to enhance the magnitude of T cell suppression.

Interestingly, studies found that DNTregs-mediated suppression can be attenuated by blocking the TCR expression of responder T cells or blocking the DNTreg-acquired antigen, a mechanism associated with trogocytosis (24). Trogocytosis is a unique process utilized by some immune cells to acquire proteins *via* active transfer from neighboring cells (145–148). The term 'trogo' is obtained from the ancient Greek meaning 'to gnaw' (146). Although $CD4^+$Tregs utilize trogocytosis to acquire peptides from APCs as a means of suppression (149), those cells do not present antigens to effector T cells like DNTregs. Moreover, the lack of CD4 or CD8 coreceptors (CD4 or CD8) and the often low to moderate expression of CD28 (22, 75) suggest that DNTregs utilize other costimulatory molecules for their activation after trogocytosis.

Clinical trials of TCR-engineered T cells produce better responses than T cells genetically modified to express chimeric antigen receptors (CAR-T) due to the high TCR specificity achieved (150). Furthermore, that DNTregs uniquely target allogeneic or syngeneic T cells with the same TCRs indicates a high degree of sensitivity and specificity that can be utilized to manage autoimmune reproductive disorders (**Figure 3**).

DNTregs: Mechanisms of Suppression and Cytokine Regulation

DNTregs and $CD4^+$Tregs express unique cell surface markers promoting suppressive functions. On activation, DNTregs (like $CD4^+$Tregs) express the T cell early activation markers CD25 and CD69 (24), and on TCR ligation, DNTregs do not shed CD62L (24). Though it was earlier reported that DNTregs do not express the activation markers CD44 or CD28 after activation (24), a transcriptomic study on DNTregs showed that DNTregs express the CD44 activation marker (88) similar to $CD4^+$Tregs (151). Clearly, DNTregs share some similarities with Tregs as both cells expand in the presence of exogenous IL-2 and IL-4 (24), and on activation, they both express TCR (which may be $\alpha\beta$ or $\gamma\delta$ for DNTs (21, 24, 83)), CD45, CD25, LFA-1, CTLA-4, CD69, and CD62L (24, 99). Whether DNTregs exclusively

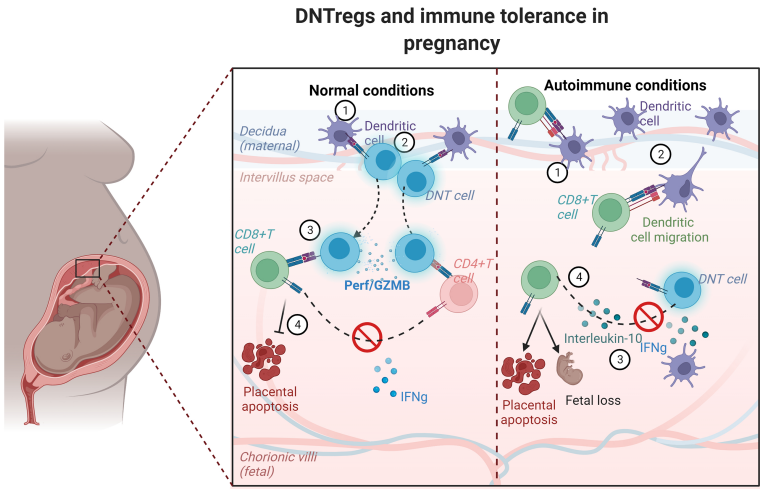

FIGURE 2 | Schematic model showing trogocytosis mechanism by DNTregs in maintaining tolerance in pregnancy. DNTregs utilize trogocytotic mechanisms to suppress recognition of fetal antigens by effector cells and thus prevent placental degeneration and apoptosis in normal physiologic conditions. However, in autoimmune conditions DNTreg populations decrease, and the maternal effectors gain access to the semi-allogenic placenta and induce apoptosis with consequent fetal rejection. The numbers in each panel indicate the sequential progression of interactions between dendritic cells, DNTregs, and effector T cells under normal and autoimmune conditions. Perf = perforin; GZMB= granzyme (B) *Figure created using Biorender.com*.

express TCRαβ or whether DNTregs exist as subsets within both TCRαβ and γδ expressing DNT cells is not quite unclear. Several studies show that DNTs expressing TCRαβ do exhibit immunoregulatory potential (20, 24, 136), while another study show same cells participate in anti-tumor activities (76). However, that DNTregs exhibit anti-tumor activity while retaining immunoregulation function appear to be a unique function of DNTregs (27) as discussed earlier in this review. In addition, some articles suggest that DNTs positive for TCRγδ exhibit immunoregulatory activities (21, 83). Further

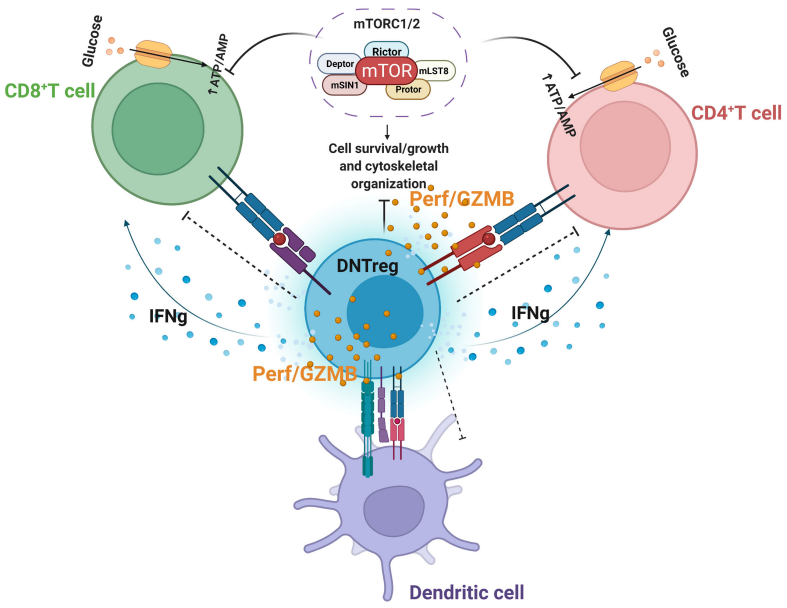

FIGURE 3 | Partial Model of DNTreg suppressor mechanisms. DNTregs can effectively suppress effector $CD8^+$, $CD4^+$ T cells, and antigen-presenting cells through the antigen-specific trogocytosis mechanism and release of cytolytic proteins, including selective inhibition of mTOR signaling. Perf = perforin; GZMB = granzyme (B) *Figure created using Biorender.com*.

investigations are therefore recommended to confirm the TCR subtypes expressed by DNTregs. However, unlike $CD4^+$Tregs, human and murine DNTregs lack Foxp3 expression (22, 99, 136). DNTregs also express unique cytokines that are slightly different from $CD4^+$Tregs, Th1, Th2, Th17, or Th3 cells. While $CD4^+$Tregs release anti-inflammatory cytokines such as IL-4, IL-10, TGF-β, or IL-35 (152–156), reports indicate that DNTregs constitutively secrete IFNg, TNF-α, and insignificant amounts of TGF-β (24) (**Figures 1**, **3**). Secretion of IL-10 by DNTregs is still under debate as one study reports that DNTregs do not secrete IL-10 (24), and other studies indicate secretion of IL-10 by DNTregs (96, 124, 157). The experimental conditions utilized by the different studies may have modulated the utilization of IL-10 by DNTregs, but this remains to be clarified. That human and mice DNTregs express high levels of IFNg supports a variety of modulatory functions beyond its reported killing ability. Perhaps IFNg production by DNTregs provides regulatory functions and enhances their cytolytic activities in active inflammatory environments, such as autoimmune reproductive failure or pregnancy disorders. Support for this possibility comes from studies that show a requirement by $CD4^+$Tregs for IFNg to promote suppression of allogeneic immune responses (158, 159). In addition, these findings are supported by a report that $CD4^+$Tregs in the maternal-fetal interface produce IFNg, which appear to equip these cells to influence immune responses through several molecular pathways and cellular targets (160). That DNTregs produce IFNg as a primary cytokine supports a multifunctional capacity necessary for modulating tolerogenic and autoimmune conditions.

Like $CD4^+$Tregs, DNTregs require exogenous IL-2 for proliferation both *in vitro* and *in vivo* (24, 161, 162). DNTregs can also be activated and expanded *in vitro* by allogeneic splenocytes in the presence of exogenous IL-2, and IL-4, where IL-4 protects DNTregs from TCR-crosslinking induced apoptosis (163). It is, however, unclear whether cytokines known to activate Tregs, such as IL-4 or IL-15 (164), could also activate DNTregs. These findings support that DNTreg suppression combines direct and indirect suppression mechanisms observed with $CD4^+$Tregs. Taken together, it appears that DNTregs possess a unique array of regulatory proteins and cytokines that differ from Th1, Th2, or Treg cells (165, 166).

DNTregs Mediate the Killing of Target Cells *via* Cytotoxic and Cytolytic Mechanisms

Two major pathways are involved in T-cell-mediated cytotoxicity: perforin-dependent and Fas-dependent pathways (167). Though both activated regulatory and non-regulatory T cells express similar levels of TCR and Fas ligand (FasL) (163), target T cells can be protected from DNTreg-mediated killing through low or absent functional Fas receptors (130). However, studies showing that DNTregs can directly kill target T cells through Fas/FasL interactions (24) also hint that the Fas/FasL mechanism is not the sole killing mechanism by DNTregs. Indeed, studies show that blocking FasL with Fas-Fc does not entirely prevent the killing ability of DNT cells and that DNTreg's ability to suppress proliferation is more potent than their cytotoxic ability (61, 128, 168). Moreover, in the absence of cell-to-cell contact, DNTregs appear to mediate minimal suppression (61, 168). Specifically, reports show that DNTregs secrete perforin (75, 169) and granzyme proteins (169), a feature shared by naturally occurring $CD4^+$Tregs (170, 171), supporting the hypothesis that DNTreg-mediated killing goes beyond the Fas/FasL pathway (145).

Recent evidence suggests that DNTregs are resistant to apoptosis induction *in vitro* and *in vivo*, as DNTregs did not undergo significant apoptosis on TCR cross-linking compared to conventional T cells (172). Furthermore, a report stated that DNTregs persist for more prolonged periods than $CD8^+$ T cells after infusion into alloantigen expressing mice (132), suggesting the resilience of DNTregs to activation-induced cell death. This feature may allow DNTregs to function for prolonged periods and increase immune regulation *in vivo*. Nonetheless, a study describes that incubation of DNTregs *in vitro* with IL-10, abolishes their ability to resist apoptosis, and diminishes their suppressive function (152). These findings suggest that the Th1/Th2 cytokine balance plays a central role in modulating DNTregs' function *in vivo*. However, that reports show activated DNTregs to secrete IL-10 (60, 157) opens more questions regarding the role of IL-10 on DNTregs; for instance, does IL-10 reduce the survival potential of DNTregs or is there an IL-10 threshold requirement that balances function and apoptosis? Further studies are needed to evaluate the role of IL-10 on the function of DNTregs as modulating IL-10 secretion by DNTregs may prove helpful in enhancing their suppressive activity in patients with autoimmune diseases, including in autoimmune reproductive disorders when utilized as a cellular therapy.

DNTregs Mediate Metabolic Suppression of Effector T Cells

A recent finding indicated that in addition to Fas/FasL mechanisms and cytolytic protein release, DNTregs mediate suppression in effector T cells by inhibiting the selective mammalian target of rapamycin (mTOR) (173). The mTOR enzyme induces metabolic reprogramming of alloantigen activated T-cells after allogeneic hematopoietic stem cell transplantation, which utilizes the glycolytic pathway to sustain alloreactive T cells mediated graft versus host disease (GvHD) (174, 175). In the presence of DNTregs, downstream mTOR signaling pathways modulated by effector T-cells are abolished (173). Inhibitory molecules, such as PD-1 and CTLA-4, can target and terminate mTOR phosphorylation and metabolic reprogramming of T cells by engaging distinct phosphatases (174, 176). DNTregs can therefore utilize the expression of checkpoint inhibitory molecules such as CTLA-4 or PD-1 to engage effector T cells and target their mTOR signaling pathway. DNTregs also inhibit mTOR-mediated increases in transcription factor HIF-1α without affecting NF-ƙB activation and p38 pathways (173). Furthermore, DNTregs selectively downregulate glucose transporters (GLUT1 and 3) in effector T cells and reduce the glycolysis capacity without interfering with their fatty acid uptake (173) (**Figure 3**). This selective trait is

similar to PD-1 mechanisms where T-cell glycolysis is inhibited, and fatty acid oxidation proceeds uninterrupted (177).

While $CD4^+$Tregs also interfere with effector cell metabolism, it occurs through pathways other than those targeted by DNTregs which include (**i**) expression of CD39, and CD73 nucleases for hydrolysis of extracellular ADP or ATP into AMP and adenosine (178), (**ii**) competition for IL-2 required for proliferation of $CD4^+$Tregs (179), (**iii**) through the transfer of cAMP to effector T cells and (**iv**) through IL-27 signaling that upregulates CD39 in $CD4^+$Tregs (180). However, it is currently unknown if DNTregs express CD39 or CD73 and further research is required to examine these possibilities.

DNTregs Alter the Phenotype and Migratory Capacity of Effector T Cells

In vitro studies show that DNTregs inhibit the induction of the transcription factor T-bet in activated effector T cells without interfering with the expression of Eomes (173). Regulation of transcription factors like T-bet and Eomes modulates differentiation of memory and effector T cells, and mTOR signaling plays a significant role in regulating T-bet and Eomes (181). A study detected elevated levels of Eomes in activated effector T cells co-cultured with DNTregs, and a corresponding reduction of T-bet expression (173). The authors also showed that Foxp3 expression (which is also a transcription factor) was not enhanced in the presence of DNTregs (173). Since transcription factors orchestrate the expression of distinct T cell markers, DNTregs regulate the transcription factor activity in effector T cells by suppressing upregulation of the costimulatory cell surface molecule CD28. Interestingly, expression of the costimulatory receptor CD27 was not reduced but further enhanced in the presence of DNTregs (173). DNTregs also alter the phenotypic chemokine expression of effector T cells. In the presence of DNTregs, $CD4^+$T cells become $CD45RO^+$ and $CCR7^+$ with enhanced expression of CD27 and CXCR5 (173). Since the phenotype of naive T cells is $CCR7^+$ and $CD45RO^-$, while that of effector T cells are $CD45RO^+$ and $CCR7^-$ (182), this indicates that DNTregs are capable of switching effector T cells to long-living central-memory T-cells characterized by CCR7, CD27, and CXCR5 expression (183–185). It also indicates that DNTregs facilitate the trafficking of effector T cells away from inflammation sites and increase homing to lymphoid organs. It may therefore be that DNTreg-induced mTOR inhibition mediates these phenotypic alterations through other as yet unknown mechanisms. Suppression of T-bet activity invariably suppresses the expression of pro-inflammatory chemokine receptors such as CXCR3 and CCR5, which are necessary for the migration and infiltration of effector T-cells to their target tissue (186, 187). Since coordinated migration of cells by chemokine receptors is required to execute T cell effector function appropriately, DNTregs present another mechanism to interfere with T cell function in autoimmune reproductive disorders.

While the pattern of DNTreg selective modulation of transcription factors is still unclear, DNTregs decrease naïve T cell phenotype and induce a long-living central-memory T-cell phenotype. The induction of long-living central memory T cells reduces activation and re-activation of effector T cells and can be exploited for tolerance maintenance in reproductive disorders. In addition, DNTregs promote and sustain suppressive T cell phenotypes, which is a relevant trait for fertility success.

DNTregs Modulate Effector T Cell Functions

Though activated effector $CD4^+$T cells secrete significant amounts of IFNg, in the presence of DNTregs, IL-2 expression was not concurrently increased. Instead, decreased production of IFNg, IL-17a, and granulocyte-macrophage-colony-stimulating factor (GMCSF) was observed in the presence of DNTregs (173). The ability of DNTregs to reduce the secretion of pro-inflammatory cytokines by effector T cells could have important implications for pregnancy maintenance. Intriguingly, DNTregs enhanced $CD4^+$ T cells' IL-2 production; however, the authors explain that though IL-2 is required for T cell proliferation and survival, it can also selectively restore $CD4^+$Foxp3 Tregs immunosuppressive function without activation of T cells (188). In comparison, $CD4^+$ $Foxp3^+$ Tregs induce senescence in effector T cells (189), but DNTregs do not.

Furthermore, $CD4^+Foxp3^+$Tregs do not modulate chemokines and cytokines that DNTregs are shown to induce. In addition, DNTregs may potentially support $Foxp3^+$ Tregs after adoptive transfer by enhancing T cell IL-2 production. Juvet and Zhang. 2012 also proposed the possibility of a functional interaction between DNTregs and $CD4^+$Tregs. The authors support their arguments through reports from a study that showed a significantly increased $CD4^+CD25^+Foxp3^+$Treg population and extended cardiac allograft survival on adoptive transfer of DNTregs (190). In addition, a study on sooty mangabeys which report that DNT cells can contribute to preserving $CD4^+$T cells during chronic infections (96) supports this possibility. If this indeed is the case, increasing the numbers of DNTregs in autoimmune reproductive pathologies through adoptive transfer will provide direct control of immune responses while simultaneously potentiating the response and function of $CD4^+$Tregs. DNTregs prove a promising regulatory subset of T cells that abolishes and modulates target cell function. These findings reveal interesting new targets through which DNTregs can selectively modulate effector T cells' signaling and metabolic programming and pave the way for the utilization of DNTregs as a targeted cellular therapy for reproductive failure and pregnancy disorders.

In summary, DNTregs are a regulatory subset of T cells with distinct functions and cytokine expression unique to the prevailing conditions of the tissue environment (**Figure 4**). Furthermore, such plasticity of DNTregs allows unique adaptive capacity to the rapidly changing and variable physiologic conditions within the FRS. Therefore DNTregs are poised to become novel therapeutic tools for FRS pathologies.

CONCLUSION

The influx of immune cells into the FRS at different cycle stages, particularly during the ovulatory and endometrial process,

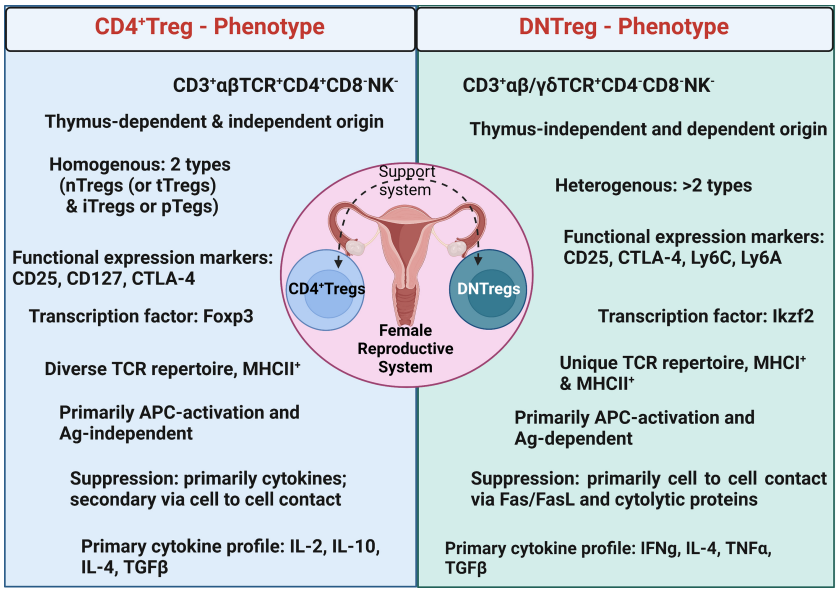

FIGURE 4 | Summary of major phenotypic characteristics of DNTregs compared to CD4$^+$Tregs. Ag = Antigen; nTregs= naturally occurring Tregs; tTregs= Tyhmic Tregs; iTreg = inducible Tregs; pTregs = peripheral Tregs. *Figure created using Biorender.com*.

indicates the importance of immune cells with regulatory functions in maintaining tolerance from pre-conception to implantation and pregnancy (119, 191, 192). While the information on DNTregs within the FRS is still growing, this review highlights the capacity and functions of DNTregs, a unique and remarkable regulatory cell subset complimentary to CD4$^+$Tregs, and their potential in supporting FRS functions. DNTregs possess unique features for enhanced specificity, plasticity, effective killing, and suppression while evading hostile microenvironments. These features and the findings that DNTregs preferentially home to mucosal sites and are additionally dominant in the FRS, position DNTregs as novel regulators with extraordinary potential to become the next targeted cellular therapy, particularly for autoimmune female reproductive disorders. That DNTregs can be successfully expanded from healthy individuals and utilized as an off-the-shelf therapy enhances their relevance and provides an emerging shift in considering regulatory cell subtypes for autoimmune conditions. *via*

AUTHOR CONTRIBUTIONS

EB conceptualized and wrote the article; JV participated in writing and reviewing the article; HY participated in conceptualizing, reviewing, and writing the article. All authors contributed to the article and approved the submitted version.

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REVIEW
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G-Protein-Coupled Receptors in Rheumatoid Arthritis: Recent Insights into Mechanisms and Functional Roles

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Rheumatoid arthritis (RA) is a chronic inflammatory disease that leads to joint damage and even disability. Although there are various clinical therapies for RA, some patients still have poor or no response. Thus, the development of new drug targets remains a high priority. In this review, we discuss the role of G-protein-coupled receptors (GPCRs), including chemokine receptors, melanocortin receptors, lipid metabolism-related receptors, adenosine receptors, and other inflammation-related receptors, on mechanisms of RA, such as inflammation, lipid metabolism, angiogenesis, and bone destruction. Additionally, we summarize the latest clinical trials on GPCR targeting to provide a theoretical basis and guidance for the development of innovative GPCR-based clinical drugs for RA.

Keywords: G-protein-coupled receptors, rheumatoid arthritis, inflammation, bone destruction, lipid metabolism, angiogenesis

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by synovial inflammation, joint destruction, and other clinical symptoms, including joint swelling, pain, morning stiffness, and weakness (1). The global prevalence of RA is approximately 0.5% to 1% (2). The risk factors for RA include genetic and metabolic aspects, genetic-environmental interactions, and microbial communities, all of which are involved in the pathogenesis of RA (3). For example, oxidative stress in multiple immune cells is thought to be an important factor in the chronic inflammatory destruction of RA, which in turn leads to joint destruction and extra-articular damage, including atherosclerosis, subcutaneous nodules and leg ulcers, systemic vasculitis, pulmonary fibrosis, scleritis and outer scleral inflammation, valvular heart disease and conduction abnormalities, and spinal cervical spondylosis (4). Currently, treatment options for RA include disease-modifying antirheumatic drugs (DMARDs), nonsteroidal anti-inflammatory drugs (NSAIDs), and biologics. Among them, analgesics and NSAIDs can reduce pain and stiffness, but NSAIDs have limited

effectiveness and often have gastrointestinal and cardiac toxicity (5). DMARDs are the primary treatment and have been tried in combination, but some DMARDs have multiple adverse effects such as nausea, hepatotoxicity, hematometabolic disorders, and interstitial lung disease (5). Biological agents, including anti-tumor necrosis factor (TNF)-α antibodies, are also effective, but there are still adverse events, such as infusion and injection site infections, and differences in efficacy (5). With the advent of these new therapies, treatment of patients with RA has improved. However, due to heterogeneous factors and complex pathological mechanisms in RA, some patients have a poor clinical response, and the targeted development of new therapies is still a priority.

G-protein-coupled receptors (GPCRs), also known as seven-transmembrane domain receptors, respond to signals such as hormones, neurotransmitters, odors, and light, and transmit signals to cells for physiological functions (6). GPCRs can be classified into glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin families (7). The classical signaling process of GPCR has been well described and consists of three parts: receptor, G protein, and effector. G protein is a heterotrimer composed of α, β, and γ subunits (see **Figure 1**) (8). Briefly, in response to stimuli, the receptor's structure begins to change to enhance binding to G proteins; the guanosine diphosphate (GDP) in the Gα subunit of resting G proteins is released; and is converted to guanosine triphosphate (GTP). The Gβγ dimer dissociates, which in turn activates downstream effectors to continue signaling, often accompanied by an increase in cyclic adenosine monophosphate (cAMP) and activation of protein kinase C (PKC). The specific downstream transmission signal depends on the α subunit species, including primarily Gαs, Gαi/o, Gαq/11, and Gα12/13 (6). GPCRs and their signaling pathways are involved in multiple human physiological and pathological processes. Drugs targeting GPCRs account for approximately 27% of the global drug therapy market (6). Multiple multifamily GPCRs in are linked to immune mechanisms in RA, including inflammatory responses. Therefore, in this review, we have investigated the mechanisms of GPCRs in RA. Based on our findings, GPCR-targeted drug development has an excellent potential and economic translational value for clinical therapy development in RA.

ASSOCIATION OF CHEMOKINE RECEPTORS AND RA

Chemokine receptors are a class of GPCRs that regulate immunity. This class includes C-C motif chemokine receptor (CCR) 1-10, C-X-C motif chemokine receptors (CXCR) 1-7, X-C motif chemokine receptor 1 (XCR1), and C-X3-C motif chemokine receptor 1 (CX3CR1) (9). Chemokine receptors appear to be mostly Gαi-coupled, with some chemokine receptors also coupled to other G proteins, as verified by manual transfection in some contexts lacking Gαi/o proteins. Therefore, in the absence of specific information, chemokine receptors are by default considered Gαi/o-coupled (10). The overall chemokine and chemokine receptor cellular expression patterns in RA have been studied. For example, surface molecular assays of many peripheral blood B cells from RA patients revealed that CCR5, CCR6, CCR7, CXCR3, CXCR4, and CXCR5 play an essential role in B cell synovial migration, proliferation, and cytokine production (11). CCR1, CXCR4, and CCR5 are abundantly expressed in the RA synovium (12). Fibroblast-like synoviocytes (FLSs) in RA express CCR2, CCR5, CXCR3, and CXCR4. They have a pro-migration, proliferation, and matrix metalloproteinase production effect on FLSs under the stimulation of different ligands (13). Monocytes in synovial fluid mainly express CCR1, CCR2, CCR3, and CCR5, whereas peripheral blood expresses CCR1-5 at different levels of expression (14). Macrophages in rats with AIA mainly express CCR1, CCR2, and CCR5, which may maintain an inflammatory environment. CXCR4 is upregulated in endothelial cells and may be mainly associated with cell migration, angiogenesis, and inflammation (15). CCR1 and CXCR4 expression is upregulated in osteoblastic monocytes in RA (16). The roles of chemokines and chemokine receptors in RA have been previously reviewed (17–19). Therefore, we briefly summarize and update this information in the following sections (see **Tables 1** and **2**).

CCR1, CCR2, and CCR5

CCR1 is coupled with Gαi/o (20). CCL3 (MIP-1α), CCL4, CCL5 (RANTES), CCL6 (MIP-related protein-1), CCL7, CCL8, CCL9 (MIP-1γ/MIP-related protein-2), CCL15 (MIP-1δ/hemofiltrate CC chemokine-2/leukotactin-1), CCL16, and CCL23/CKβ8/myeloid progenitor inhibitory factor-1) have been described as CCR1 ligands (21, 22). The number of peripheral blood CCR1+ T/NK lymphocytes in patients with RA is negatively correlated with IL-10, whereas the number of CCR2+ B cells is positively correlated with IL-6 (23). The expression of CCR1 was positively correlated with serum antibodies against anti-cyclic citrullinated peptide (anti-CCP) in patients with RA (24). Several preclinical experimental studies have shown that CCR1 inhibition improves arthritic symptoms. For example, CCR1 inhibition reduces inflammation, joint damage, and cellular infiltration in the collagen-induced arthritis (CIA) mouse model. The specific mechanism may involve reduced recruitment of inflammatory cells; however, it is noteworthy that CCR1 inhibition spontaneously increases TNF-α levels in certain settings (25). The favorable animal results for CCR1 further support the clinical development of CCR1 antagonists. A phase IIa, double-blind, placebo-controlled, randomized, proof-of-concept study investigated the efficacy of a CCR1 antagonist (MLN3897) in RA patients; MLN3897 was well tolerated, but the results were not favorable, and there was no significant difference in its efficacy (26). Clinical blockade of CCR1 is likely to be effective and may always require maintenance of high levels of receptor occupancy (27). The CCR1 antagonist (CCX354-C) has shown initial clinical effectiveness. In a randomized placebo-controlled clinical trial in patients with RA, a CCR1 antagonist (CCX354-C) demonstrated good safety, tolerability, and clinical activity. CCX354 showed good tolerability and a linear dose-exposure profile in healthy subjects (28, 29).

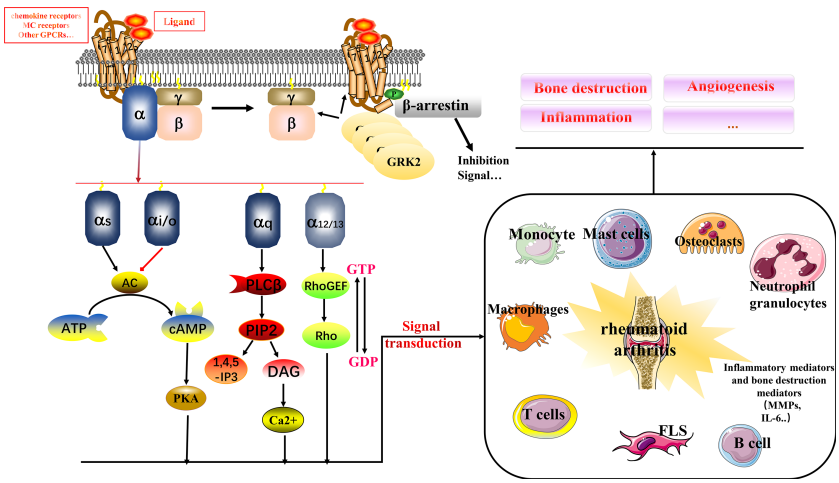

FIGURE 1 | Relationship between multiple GPCRs and RA. GPCRs signal through the αβγ subunit of heterotrimeric G proteins. In response to ligand signals, GTP binds to the Gα subunit, and Gβγ dissociates to form a dimer for downstream signaling, binding to biological mediators. GRK2 is a kinase that phosphorylates activated GPCRs, making them a high-affinity substrate for the binding of the uncoupling protein arrestin. Arrestin binding abolishes ("arrests") G protein-mediated signaling. The binding of GRK2 to Gβγ recruits cytosolic GRK2 to the plasma membrane, where the activated receptor is located. This is its primary function. It can also inhibit Gβγ-mediated signaling by sequestering Gβγ, which is a secondary effect. GPCRs interact with immune cells in RA to influence multiple mechanisms including bone destruction, inflammation, and angiogenesis.

TABLE 1 | Chemokine receptors and their ligands.

Chemokine receptor	Ligand	Cell expression in RA
CCR1	CCL3, CCL4, CCL5, CCL6, CCL7, CCL9, CCL15, CCL16, CCL23	T cell, NK cell, monocytes, macrophages, osteoblasts
CCR2	CCL2, CCL5, CCL7, CCL8, CCL12, CCL13, CCL16	FLS, B cell, monocytes, macrophages
CCR3	CCL4, CCL5, CCL7, CCL8, CCL15, CXCL10, CCL11, CCL13, CCL24, CCL26, CCL28	FLS, osteoclasts, monocytes, T cells (Th2), macrophages
CCR4	CCL2,CCL5,CCL3, CKLF1, CCL22, CCL17	FLS, T cell (Th2, Th17, Treg cell), monocytes
CCR5	CCL5, CCL3, CCL4, CCL8, CCL7,CCL14,CCL15	FLS, T cell (Th1), monocytes, macrophages
CCR6	CCL20	T cell (Th1 cell, Th22 cell, Th17.1 cell)
CCR7	CCL21, CCL19	Macrophages, monocytes, T cell
CCR9	CCL25	FLS, monocytes, macrophages
CCR10	CCL27, CCL28	Bone marrow cells, endothelial cells
CXCR1	CXCL5, CXCL6, CXCL8	FLS, monocytes, neutrophils
CXCR2	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8	FLS, monocytes, neutrophils
CXCR3	CXCL4, CXCL4L1, CXCL9, CXCL10, CXCL11	FLS, plasma cells, mast cells, T cells (Th1)
CXCR4	CXCL12	FLS, T cells, monocytes, chondrocytes, endothelial cells, osteoblasts
CXCR5	CXCL13	T cell (Tfh, Tfr), B cell, endothelial progenitor cell
CXCR6	CXCL16	FLS, endothelial cells, T cell
CXCR7	CXCL12	Endothelial cells
CX3CR1	CX3CL1	NK cells, monocytes, CD4+ T and CD8+ T cells, osteoblasts
XCR1	XCL1, XCL2	Mononuclear cells

The ligands of CCR2 include CCL2, CCL5, CCL7, CCL8, CCL12, CCL13, and CCL16 (22, 30, 31). In RA, the human isoforms of CCR2 include CCR2a and CCR2b, which are coupled to Gαi/o in most cases and Gαq in some cases and trigger the canonical activation of phospholipase Cβ isoenzymes downstream (32, 33). The mRNA expression of CCR2 was significantly higher in the range of 2.6< the disease activity score-28 (DAS28) <5.1 than DAS28>5.1 and control. CCL2 was negatively correlated with DAS28 index. Thus, CCR2 may contribute to early and rapid progression of inflammation (24). For example, the binding of CCR2 and CCL2 can help monocytes migrate to sites of RA inflammation and differentiate into M1 proinflammatory macrophages, possibly linking RA inflammation to insulin resistance (34). However, a double-blind, randomized, placebo-controlled clinical trial investigating the efficacy of a CCR2-blocking antibody (MLN1202) in patients with RA found that CCR2 blockade was not sufficient to improve multiple symptoms of RA (35).

CCR5 couples with Gαi/o and Gαq, and its ligands include CCL5, CCL3, CCL4, CCL8, CCL7,CCL14, and CCL15 (22, 31,

TABLE 2 | Clinical trials of GPCR related to RA.

Name	Sponsor	ClinicalTrials.gov Identifier	Targets	Treatment	Condition or disease	Phase
TG-0054	GPCR Therapeutics, Inc.	NCT00822341	CXCR4	TG-0054	Healthy	Phase 1
POL6326	Polyphor Ltd.	NCT01841476	CXCR4	POL6326	Healthy	Phase 1
AZD4818	AstraZeneca	NCT00687232	CCR1	AZD4818, Placebo	Healthy	Phase 1
PF-04136309	Pfizer	NCT02598206	CCR2	PF-04136309	Healthy	Phase 1
RIST4721	Aristea Therapeutics, Inc.	NCT05023811	CXCR2	RIST4721	Healthy, ADME	Phase 1
RIST4721	Aristea Therapeutics, Inc.	NCT04105959	CXCR2	RIST4721, placebo	Inflammatory Response	Phase 1
AZD5069	AstraZeneca	NCT01332903	CXCR2	[14C] AZD5069	Healthy	Phase 1
	AstraZeneca	NCT00953888	CXCR2	AZD5069, placebo	Healthy	Phase 1
	AstraZeneca	NCT01100047	CXCR2	AZD5069, placebo	Healthy	Phase 1
	AstraZeneca	NCT01051505	CXCR2	AZD5069, placebo	Healthy	Phase 1
AZD2423	AstraZeneca	NCT00977626	CCR2	AZD2423, Placebo	Healthy	Phase 1
	AstraZeneca	NCT00940212	CCR2	AZD2423, Placebo	Healthy Volunteers	Phase 1
	AstraZeneca	NCT00970775	CCR2	AZD2423, Placebo	Healthy Volunteers	Phase 1
	AstraZeneca	NCT01233830	CCR2	AZD2423, Placebo	Healthy	Phase 1
PF-04634817	Pfizer	NCT01247883	CCR2, CCR5	PF-04634817	Healthy	Phase 1
	Pfizer	NCT01140672	CCR2, CCR5	PF-04634817	Healthy	Phase 1
	Pfizer	NCT01098877	CCR2, CCR5	PF-04634817, Placebo	Healthy	Phase 1
AZD5672	AstraZeneca	NCT00722956	CCR5	AZD5672, atorvastatin	Healthy Volunteers, Pharmacokinetics	Phase 1
	AstraZeneca	NCT00723424	CCR5	AZD5672, Digoxin	Healthy Volunteers, Pharmacokinetics	Phase 1
	AstraZeneca	NCT00746837	CCR5	AZD5672	Healthy Volunteers	Phase 1
	AstraZeneca	NCT00887770	CCR5	AZD5672, Moxifloxacin ,placebo	Rheumatoid Arthritis	Phase 1
	AstraZeneca	NCT00711074	CCR5	AZD5672	Rheumatoid Arthritis	Phase 1
	AstraZeneca	NCT00713544	CCR5	AZD5672, Etanercept , placebo	Rheumatoid Arthritis	Phase 2
	AstraZeneca	NCT00871767	CCR5	AZD5672	Rheumatoid Arthritis	Phase 1
Maraviroc	Pfizer	NCT00427934	CCR5	Maraviroc, placebo	Rheumatoid Arthritis,	Phase 2
NNC 0151-0000-0000	Novo Nordisk A/S	NCT02151409	C5aR	NNC 0151-0000-0000 ,placebo	Inflammation, Systemic Lupus Erythematosus, Rheumatoid Arthritis, Healthy	Phase 1
	Novo Nordisk A/S	NCT01223911	C5aR	NNC 0151-0000-0000 ,placebo	Inflammation, Rheumatoid Arthritis	Phase 2
NNC0215-0384	Novo Nordisk A/S	NCT01955603	C5aR	NNC0215-0384 ,placebo	Inflammation, Rheumatoid Arthritis	Phase 1
	Novo Nordisk A/S	NCT01611688	C5aR	NNC0215-0384 ,placebo	Inflammation, Rheumatoid Arthritis	Phase 1
CCX354-C	ChemoCentryx	NCT01027728	CCR1	CCX 354-C	Rheumatoid Arthritis	Phase 1 Phase 2
	ChemoCentryx	NCT01242917	CCR1	CCX 354-C, placebo	Rheumatoid Arthritis	Phase 2
PF06835375	Pfizer	NCT03334851	CXCR5	PF06835375, placebo	Systemic Lupus Erythematosus, Rheumatoid Arthritis	Phase 1

36, 37). CCR5 expression in rheumatoid factor (RF)-negative patients with RA is markedly higher than that in RF-positive patients with RA, which may be a molecular basis for the differences in RF expression in patients with RA (24). Investigators have found that the number of CCR5 molecules expressed on the cell surface correlates with the intensity of tumor necrosis factor α (TNFα) -induced T cell migration to the joint in RA. Anti-CCR5 antibodies can block this migration effect, thus possibly linking it with joint inflammation (38). Similar to the failed clinical trial of CCR2, clinical trials of CCR5 antagonists suggest that silencing CCR5 alone is not a truly effective target for RA (39–41). In conclusion, the lack of clinical efficacy of CCR2 and CCR5 blockade may be because their role in the migration of monocytes to the synovial membrane in patients with RA is not critical.

CCR3

CCR3 may be coupled with Gαi/o (42). The ligands of CCR3 are CCL4, CCL5 (RANTES), CCL7 (MCP-3), CCL8 (MCP-2), CCL15 (HCC-1), CXCL10 (interferon (IFN)-γ inducible protein-10), CCL11 (eotaxin), CCL13, CCL24 (eotaxin-2), and CCL26 (eotaxin-3) (17, 22, 31). The role of CCR3 in RA may include the induction of cell migration and promotion of bone destruction and act as a predictor of the efficacy of certain clinical therapies. For example, flow cytometry analysis of synovial cells from patients with RA revealed that most CCR3+ cells were FLSs and that CCL11 expression was upregulated in the plasma and synovial fluid (43). IL-1β enhances the release of CCL11 from FLSs; CCL11 induces upregulation of CCR3 and matrix metalloproteinase (MMP)-9 mRNA expression in FLSs (43) and can induce the migration of FLSs and monocytes (44).

CCL11 is expressed in osteoblasts and its expression is enhanced in response to inflammatory stimuli. Osteoclasts expressing CCR3 interact with exogenous CCL11 to stimulate cell migration and bone resorption (45). CXCL10, with an increased expression in synovial cells of patients with RA in response to TNF-α stimulation, interacts with CCR3 in T-cells and mediates an increase in receptor activation of nuclear factor kappa-B ligand (RANKL) expression *via* the Gαi subunit. As a result, osteoclast genesis and bone destruction are enhanced (46).Reduced CCR3 expression in serum CD4+ lymphocytes and reduced number of synovial CCR3+ monocytes in RA patients treated with steroids and anti-TNFα (47). In contrast, CD4 T cells and CD14 monocytes expressing CCR3 and CD8 T cells expressing CCR5 were increased in the peripheral blood of RA patients treated with anti-TNFα antibodies. This suggests the restoration of peripheral cell-mediated immune function, thereby blocking aggregation in the joints and inhibiting inflammation (48). The role of other CCR3 ligands in RA has been well studied and summarized (17–19). For example, CCL5 is mainly involved in the migration of leukocytes, and the use of anti-CCL5 antibodies can reduce the pathological manifestations of arthritis in the CIA mouse model (49). CCL24 is a major chemokine for inflammatory cells, and adjuvant arthritic mice treated with anti-CCL24 antibody showed significantly improved arthritic symptoms and inflammatory responses (50). Clinical inhibitors of CCR3 are in development, but currently do not target RA directly; most inhibitors target other diseases to test their effects, such as Parkinson's disease, macular degeneration, and diabetic retinopathy.

CCR4, CCR6, and CXCR3

The ligands of CCR4 include chemokine-like factor 1 (CKLF1), CCL2, CCL3, CCL5, CCL17, and CCL22 (22, 31, 51, 52). CKLF1 is significantly positively correlated with C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) (51). The expression of FLS in CCR4 may promote cell migration and proliferation (53). CCR4 expression in pro-inflammatory T-cell populations may promote inflammation by facilitating cell migration process. For example, a significant increase in circulating CCR4+ CXCR3-helper T cells (Th2 and Th17 cells) has been observed in clinically untreated patients with early RA (54). In addition, the expression of Treg cells of CCR4 may be a regulatory negative feedback mechanism of inflammation. For example, CCR4 and CCR6 expression was upregulated in peripheral blood Treg cells from patients with active RA and it was positively correlated with DAS28, suggesting that they could migrate to joints (55). CCR4+ T cells exert anti-inflammatory effects in patients with juvenile rheumatoid arthritis (JRA) by producing anti-inflammatory cytokines (IL-4) (56). However, this is not sufficient for the suppression of inflammation, so there are many proinflammatory mechanisms, such as CCL22, which is increased in both synovial fluid and serum of patients with RA, and may bind Treg cells CCR4 suppresses the number of Treg cells through the signal transducer and activator of transcription 5 (STAT5) signaling pathway (57).

CCL20 is the ligand of CCR6 (31). CCR6 expression may be primarily associated with T-cell subsets in RA. The proportion of CCR6+ memory T helper cell populations in anti-cyclic citrullinated peptide antibody (ACPA)+ versus ACPA- patients with RA is significantly different. This suggests that other cell subpopulations may be involved in various mechanisms of RA (58). The CCR6+ memory T helper cell population contains Th1/Th22 and Th17.1 cells, which activate FLSs in an IFN-γ-independent manner (59). In response to TNF-α, interleukin (IL)-6, and IL-1β stimulation, Th22 cells expressing CCR4, CCR6, and CCR10 migrate to the synovial tissues of patients with active RA and produce IL-22 to stimulate osteoclast differentiation for bone destruction (60). T cells and synovial cells in RA synovium produce CCL20, which binds to CCR6. High expression of RAR-related orphan nuclear receptor (ROR) γt promotes CCR6 expression, enhancing Th17 cell migration into the joint and promoting inflammation (61, 62).

The ligands of CXCR3 are CXCL4, CXCL4L1, CXCL9, CXCL10, and CXCL11 (22, 31). CXCL10-induced cell migration was found to require CXCR3, EGFR, and Gβγ subunits downstream of CXCR3, but not Gαi/o (63). CXCR3 expression in plasma cells, mast cells, and T-cell subsets is associated with RA. Plasma cells from patients with RA express CXCR3 by interacting with sub-synovial FLS-expressing Mig/CXCL9 recruited to the subsynovial lamina (64). In addition, mast cells in RA synovial tissue abundantly express CXCR3; this may maintain the synovial inflammatory environment by binding CXCL9 and CXCL10 and producing mediators, including histamine, proteases, arachidonic acid metabolites, and cytokines (65). Patients with RA appear to express CCR5 and CXCR3 preferentially on Th1 cells, and Th2 cells preferentially express CCR3, CCR4, and CCR8 (66, 67). The combination of MTX and anti-TNFα antibodies decreased CXCR3 and IL-12R expression (considered a Th1 cell markers) and upregulated CCR4 and IL-4R expression (considered a Th2 cell markers) in peripheral blood CD4 cells in patients with RA, with a high percentage of apoptotic cells (68). Paradoxically, peripheral blood CD4 and CD8 CXCR3+ T lymphocytes were increased in RA patients treated with infliximab and etanercept. CXCR3+ CD4 T lymphocytes negatively correlated with DAS28 (69). This seems to validate the findings of Nanki et al. that chemokine receptor expression did not differ significantly across T-cell subpopulations (70). Further experiments are needed, but restoring the suppression of inflammation by regulating the balance between Th1/2 cells is a feasible strategy to improve RA. Several preclinical trials have demonstrated that targeted inhibition of CXCR3 is beneficial for the treatment of RA. Specifically, it reduced the recruitment of Th1 cells to sites of inflammation (71) and restored the balance between Th17 and Treg cells (72).

CCR7

CCR7 may be coupled with Gαi/o and Gαq (73). CCR7 ligands, including CCL21 and CCL19, are associated with the homing and localization of dendritic cells and T cells (74). CCR7 is a surface marker of macrophages in the RA synovial fluid. Its upregulation in cells may promote inflammation and is positively correlated with DAS28 and inflammatory factor levels, and negatively correlated with anti-inflammatory factor

levels in patients with RA. For example, CCR7+CD95+CD4+ peripheral lymphocytes were significantly elevated in patients with active RA and were positively correlated with IL-6 (75). Lipopolysaccharides (LPS) and IFN-γ promote CCR7 expression. IL-4 inhibits CCR7 expression. CCL21 promotes Th17 differentiation, osteoclast formation, angiogenesis, and proinflammatory macrophage differentiation (76, 77). In addition, miR-155 expression was higher in ACPA+ patients than in ACPA − patients and was correlated with DAS28. miR-155 can promote CCR7 expression and downregulate CCR2 expression in RA monocytes (78). The progression of arthritis in CIA mice can be inhibited using an anti-CCR7 antibody (74). The development of clinical inhibitors of CCR7 for non-Hodgkin's lymphoma and chronic lymphocytic leukemia is currently underway.

CCR9, CCR10

The association of CCR9 and CCR10 with RA is less direct, with CCR9 associated primarily with cell migration of FLS, proinflammatory differentiation of macrophages, and related inflammatory and bone destruction processes. CCR10 is primarily associated with angiogenesis. CCL25 is the ligand of CCR9. In response to TNF-α, CCR9 elevated expression in peripheral blood monocytes and macrophages of RA and in combination with CCL25 induces cell migration (79) and differentiation of monocytes into M1 macrophages *via* the P38 and extracellular signal-regulated kinase (ERK) pathways (80). FLSs and macrophages release CCL25 in RA synovial fluid in response to stimulation by IL-1β and IL-6. CCL25 binds to CCR9 on FLSs and macrophages to promote osteoclast formation and vascular opacification (80). Additionally, it stimulates IL-6 and MMP-3 production in FLSs and IL-6 and TNF-α production in peripheral blood mononuclear cells. The antagonism of CCR9 suppressed arthritic symptoms in the CIA mouse model (81).

The ligands of CCR10 are CCL27 and CCL28 (82). CCR10 and ligand CCL28 expression is elevated in the synovial tissue and synovial fluid of patients with RA, mainly in the bone marrow and endothelial cells; inhibition of CCL28 or CCR10 reduces endothelial cell migration and angiogenesis (83). Crohn's disease is a focus in the development of clinical inhibitors for CCR9 and a gap exists in clinical inhibitors for CCR10. Therefore, further clinical trials of both for RA are still necessary.

CXCR1, CXCR2

Both CXCR1 and CXCR2 are coupled to Gαi/o proteins (22, 84). The ligands of CXCR1 include CXCL5, CXCL6, and CXCL8. The ligands of CXCR2 include CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8, and interact with GRK6 to negatively regulate receptor sensitization and transport, thereby affecting cell signaling and angiogenesis (22, 84). CXCR1 and CXCR2 are involved in the migration of neutrophils, FLS, T-cells, and monocytes in RA, influencing the subsequent inflammation and bone destruction processes and are expressed in their cell populations (85, 86). For example, CXCR1 and CXCR2 ligands, such as CXCL1, CXCL5, and LTB4, are highly expressed in the joints and bind to CXCR1 and CXCR2. Here, they promote joint migration of neutrophils in AIA mice (87). Targeted inhibition of neutrophil migration by the CXCR1 and CXCR2 antagonists SCH563705 reduces disease activity scores, joint inflammation, and bone and joint destruction in CIA mice (88). In addition, CXCL5 [epithelial neutrophil-activating peptide 78 (ENA-78)] of citrulline is significantly elevated in the synovial fluid of RA patients. It correlates with CRP and ESR, inducing inflammatory cell focus and inflammation through CXCR1-and CXCR2-induced chemotaxis of monocytes (89). Reduced CXCR1 and CCR2 expression in blood T cells after treating patients with RA with the anti-TNF-α antibody infliximab may inhibit their migration to sites of inflammation (90).

CXCR4

CXCR4 may be coupled to Gαi/o and Gαq (73). The ligand of CXCR4 is mainly CXCL12 [stromal cell-derived factor 1 (SDF-1)]. High expression of CXCL12 and CXCR4 in the serum and synovial fluid of patients with RA is positively correlated with ESR, CRP, RF, and DAS28 scores (91). T-cell expression of CXCR4 may promote cell migration and inflammatory processes. CXCR4 is positively correlated with memory-activated CD4+ T cells and follicular helper T (Tfh) cells (92).Pablos et al. found that FLSs specifically express CXCL12, which aggregates and fixes in the heparan sulfate molecules of vascular endothelial cells, promoting angiogenesis and inflammatory cell infiltration. CXCL12 activates CXCR4 on the surface of T cells. Simultaneously, very late activation antigen 4 (VLA-4) interacts with vascular cell adhesion molecule 1 (VCAM-1) to promote the recruitment of T cells and their retention in the joints to promote inflammation (93, 94). The expression of CXCR4 in B cells may be indicative of disease activity; for example, considerable infiltration of P-gp+CXCR4+ CD19+ B cells can be observed in patients with RA, which may correlate with disease activity, drug resistance, and progressive joint destruction (95).

In addition, monocyte expression of CXCR4 may promote the process of cell migration and differentiation into proinflammatory macrophages, which can affect subsequent inflammatory and bone destruction processes. The hypoxic microenvironment induces SDF-1 expression in RA FLSs, leading to the accumulation of CXCR4-expressing monocytes in the synovial membrane and their differentiation into macrophages and secretion of proinflammatory factors (IL-1β, IL-6, and TNFα) and MMP. This process mediates inflammation and osteoarthritic destruction (96). The specific mechanism may involve SDF-1α enhancing the binding of c-Jun to AP-1 on the IL-6 promoter and enhancing AP-1 transcriptional activity to regulate IL-6 expression (97). Similarly, SDF-1α activates ERK/c-Fos/c-Jun *via* CXCR4 to mediate AP-1 activation of MMP13 to promote cartilage destruction (98). SDF-1a also stimulates CXCR4+ chondrocytes to release MMP3, which destroys bone joints (99).In addition, the endogenously released reduced form of HMGB forms a heterotrimeric complex with CXCL12; this complex interacts with CXCR4 to promote cell migration and inflammation *via* the toll-like receptor (TLR) 2/4 (100).

Therefore, the inhibition of CXCR4 is beneficial for improving RA. The herbal compound QLY can reduce joint lesions and paw swelling in adjuvant arthritis rats by a mechanism involving the inhibition of the CXCL12/CXCR4/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway (101). In addition, targeted CXCR4 inhibitors are now well developed, and described in many excellent reviews (102–105). Many small-molecule compounds have significant CXCR4 inhibitory effects. We have focused on the progress of CXCR4 inhibitor development in clinical trials and have summarized and updated them here. The CXCR4 antagonist AMD3100 has been approved by the FDA for use as a hematopoietic stem cell mobilizer. All information was obtained from www.clinicaltrials.gov (See **Table 1**).

CXCR5, CXCR6, and CXCR7

CXCL13 is the ligand of CXCR5. Changes in CXCR5 expression in different T-cell subsets may be associated with RA. The circulating Tfr/Tfh cell ratio in RA is negatively correlated with serum CRP, ESR, RF, anti-CCP, IgG, and the DAS28 index (106). Tfh cells can activate B cells and produce specific antibodies, and the number of Tfh cells in peripheral blood is positively correlated with anti-CCP antibody levels (107). In contrast, in RA remission, circulating Tfr cell subsets increase and are negatively correlated with RF, anti-cyclic citrullinated peptide, and DAS28 (108). A decrease in CD4+CXCR5+Foxp3+ Tfr cells/CD4+CXCR5+ Tfh cells in the peripheral blood of patients with RA may contribute to multiple humoral immune mechanisms (109). *In vitro* co-culture of RA FLSs and peripheral blood mononuclear cells (PBMCs) revealed that increased production of TNF-α, IL-1β, IL-6, and reactive oxygen species (ROS) could promote CD4 + CXCR5 + ICOS + Tfh differentiation (110). CXCR5 expression in other cells (endothelial progenitor cells and regulatory B cells) has also been linked to RA. CXCL13 expression is elevated in the synovial fluid of CIA mice and patients with RA. CXCL13 interacts with CXCR5 in endothelial progenitor cells and promotes homing and angiogenesis through the PLC, MEK, and AP-1 signaling pathways (111). CXCR5 expression on the surface of regulatory B cells (Bregs) is downregulated. The response to CXCL13 is not sufficient for preferential migration to the synovial fluid to produce sufficient anti-inflammatory factor IL-10, which may be one of the mechanisms of RA inflammation (112). CXCR5-deficient mice with impaired germinal response centers are resistant to collagen-induced arthritis (113). The application of clinical inhibitors of CXCR5 in RA is ongoing, and promising results are expected to be published.

The ligand of CXCR6 is CXCL16. CXCR6 is expressed in FLS, endothelial cells, and T-cells in RA. CXCR6 and CXCL16 levels are elevated in both RA FLSs and may stimulate FLS proliferation (114). CXCL16 also stimulates RANKL expression in RA FLSs through Janus kinase (JAK)2/STAT3 and P38/mitogen-activated protein kinase (MAPK) signaling (115). CXCR6 is also expressed in endothelial cells and may be involved in endothelial cell recruitment and angiogenesis in the RA joints by binding to CXCL16 (116). CXCR6 and CXCL16 can promote inflammation by affecting T cell differentiation and homing to joints. CXCR6 knockout CIA mice exhibit multiple arthritic symptoms (117, 118).

The ligand of CXCR7 is CXCL12 and was found to be internalized and signaled downstream in a chemokine-dependent manner only through recruitment of the β-arrestin protein (119, 120). CXCR7 binds to chemokines with a high affinity, but ligand binding does not lead to G protein-mediated intracellular calcium mobilization or chemotaxis (121). CXCR7 is expressed on endothelial cells in the synovium of RA patients and can be significantly upregulated in response to IL-1β and CXCL12 stimulation. It promotes angiogenesis, and is mainly used as an alternative receptor for CXCR4. CXCR7 inhibitors significantly reduced arthritic symptoms and vascularity in CIA mice (122).

CX3CR1 and XCR1

CX3CR1 is mainly expressed in NK cells, monocytes, and CD4+ and CD8+ T cells. Its ligand CX3CL1/FKN (fractalkine) is primarily involved in cell adhesion (123). CX3CR1/CX3CL1 are also associated with the production of inflammatory mediators by macrophages, T cells, and FLSs in RA (124). CX3CR1+HLA-DRhiCD11c+CD80-CD86+ cells, an osteoclast subpopulation, are present in the synovium of patients with RA. In mice, the corresponding subpopulation CX3CR1hiLy6CintF4/80+I-A+/I-E+ cells can be inhibited by inhibiting FOXM1 (125). Patients with active RA treated with infliximab and etanercept had reduced CX3CR1 expression in peripheral blood PBMC and T cells and serum CX3CL1 levels (126, 127).

The ligands of XCR1 are XCL1 and XCL2 (22). XCR1 expression is upregulated in mononuclear cells (MNCs) of synovial fluid and venous blood samples from patients with RA, suggesting that XCR1 may be involved in the mechanism of RA (128). The connection between CX3CR1 or XCR1 and RA has not been well studied, and the specific mechanisms require further study.

ACTIVATION OF MELANOCORTIN (MC) RECEPTOR IMPROVES RA

The structure and function of MC and its receptors have been well-reviewed (129). MCR mainly included MC1-5R. MC1-5R is coupled with Gαs to stimulate downstream cAMP/PKA signaling. In addition, M3CR promotes downstream ERK1/2 signaling to promote cell proliferation by coupling with Gαi/o. MC4R also promotes downstream protein kinase C by coupling with Gαq and inhibits apoptosis by coupling with Gαi to promote downstream ERK1/2 signaling. MC5R can also couple Gαi/o to promote downstream PI3K/C-Raf/MEK1/2/ERK1/2 signaling (130). Activation of MCR may have an ameliorative effect on RA. MC3R knockdown with serum transfer-induced arthritis exacerbated significant bone erosion, high expression of RANKL in the joint, increased time of NF-kB activation, and upregulation of proinflammatory genes [IL-1β, IL-6, and nitric oxide synthase 2 (NOS2)]. Conversely, overexpression of MC3R

and MC3R agonist D [Trp8]-γ-MSH significantly reduced the degree of arthritis, suggesting that MC3R may be an important target in preventing bone destruction and inflammation progression in arthritis (131). Endogenous MCR agonists include ACTH and MSH (α, β, and γ), with different affinities for the five MCRs, where MC2R was activated only in response to ACTH (130).

Alpha-melanocyte-stimulating hormone (α-MSH) is a 13-amino acid peptide (132). α-MSH can inhibit inflammation by interacting with the MCR of multiple cells in RA *via* multiple mechanisms (132). Elevation of α-MSH may be a measure of suppression of inflammation in joint tissues. Catania et al. observed significantly elevated synovial fluid α-MSH, interleukin 1 receptor antagonist (IL-1ra), and soluble tumor necrosis factor receptor (sTNFr) levels in patients with RA (133). *In vitro*, α-MSH inhibits LPS-induced protein hydrolase release from macrophages, oxidative burst response, production of reactive oxygen and reactive nitrogen species, adhesion molecule expression, NF-kB activation, and downregulation of cell surface CD14 expression to inhibit inflammation (134). The inhibitory effect of NF-kB may be mediated by an increase in the cAMP-mediated activity (135). Similarly, Bhardwaj et al. found that the C-terminal tripeptide of α-MSH induces the production of the anti-inflammatory factor IL-10 in monocytes (136). α-MSH stimulation of T cells *in vitro* promotes phenotypic conversion of T cells to CD25+CD4+ regulatory T cells. It induces TGF-β production to suppress IFN-γ production by other effector T cells; eventually, inflammation is suppressed *via* a mechanism involving α-MSH binding to MC5R on T cells (137). Thus, synthetic MCR agonists may also inhibit inflammation in patients with RA. For example, Montero-Melendez et al. found that the pro-senescence effect of FLSs *via* agonist-induced MC1R expression suppressed the inflammatory response in RA (138). The MC1R agonist BMS interferes with FLSs cell cycle, anti-inflammatory, and arthroprotective features These include cell proliferation, cycle arrest, lysosomal amplification, expression of the cellular senescence marker p16 INK4, downregulation of cell cycle promoters and anti-apoptotic signals, downregulation of proinflammatory factors (CCL2, IL6, and IL8), increased expression of MMPs, and downregulation of matrix-degrading enzymes (ADAMTS1 and ADAMTS2) (138). Montero-Melendez et al. also found that MC Pan Agonist AP214 reduced disease scores and paw edema (which primarily affects IL-1β release) in a K/BxN serum transfer arthritis model, exerting an anti-inflammatory effect (139).

α-MSH is also linked to RA and is osteoprotective. α-MSH acts directly on the bone, increases bone turnover, and reduces bone volume, possibly through the synergistic action of other cells such as adipocytes or islet B cells (132). Human chondrocytes express a variety of MCRs such as MC2R and MC5R. *In vitro*, a-MSH stimulation can regulate cAMP, proinflammatory factors, and MMP in chondrocytes, which may play a role in inflammation, development, and cartilage degeneration (140). Similarly, Kaneva et al. found that α-MSH and D[Trp8]-γ-MSH inhibited TNF-α-induced proinflammatory factor release (IL-1, IL-6, and IL-8) from C-20/A4 chondrocytes, increased anti-inflammatory factor IL-10 release, decreased the expression of *MMP1, MMP3*, and *MMP13*, and inhibited the apoptosis of key molecules caspase3/7 and cell death. MC3R/4R antagonists inhibited the effects of D[Trp8]-γ-MSH, suggesting that MC1R and MC3R may be the primary MCRs for these mechanisms (141). Zaidi et al. found that ATCH induced vascular endothelial growth factor (VEGF) secretion *via* MC2R and stimulated osteoblast maturation and survival. This reduced experimental osteonecrosis induced by methylprednisolone acetate, which may be a mechanism to inhibit bone destruction in RA (142).

OTHER GPCRS ASSOCIATED WITH RA

In addition to the intensively studied MCRs and chemokine receptors described above, there are also some GPCRs that are associated with RA For example, GPR120 regulates lipid metabolism and GPCR (CD97) is related to adhesion. Further experiments are needed to investigate their specific roles in depth.

C5aR and Adenosine Receptors

C5aR is Gαi/o-coupled (143). C5aR is mainly expressed in neutrophils and macrophages. Elevated C5aR and C5a levels have been found in the blood and synovial fluid of RA patients (144). Patient mast cells also express C5aR and release histamine to participate in the inflammatory response (145). In the presence of multiple cytokines, neutrophils express chemokine receptors in the joint to release LTB4 and IL-1β to promote their recruitment. Simultaneously, immune complexes in RA can activate C5a production by neutrophils and bind C5aR to further amplify the inflammatory response (146). In addition, the plasma kallikrein-kinin system (KKS) is present in RA. KKS activation activates prekallikrein (pKal) and factor XII (FXII) cleavage of high-MW kininogen (HK) to release bradykinin. Kal also activates monocytes to promote proinflammatory cytokines, upregulate C5aR and FcRIII expression, and release C5a. Inhibition of KKS alleviates symptoms in arthritic mice (147). Macrophage infiltration in zymosan-induced arthritis (ZIA) mice; increased expression of C5aR and C3aR in joints; and elevated levels of C5a and soluble receptor activator of nuclear factor kappa B ligand (sRANKL) in synovial fluid are possible mechanisms of inflammation (148). Targeted inhibition of C5aR remains challenging in terms of clinical translation, although preclinical data have shown that anti-C5aR antibodies can improve symptoms in experimental arthritic mice (149). However, a double-blind placebo-controlled study found no clinical improvement in patients with RA receiving a C5aR inhibitor (150).

Adenosine is an anti-inflammatory mediator that acts mainly through four receptors (A1, A2A, A2B, and A3) to inhibit proinflammatory factors (IL-6 and TNF-α) and to promote the

synthesis of anti-inflammatory factors (IL-10) (151). Its role in RA has been extensively reviewed and is mainly related to the mechanism of action of methotrexate, phosphoinositide 3-kinases (PI3Ks)/protein kinase B (PKB), and NF-kB signaling pathways. The detailed mechanism of action has previously been explained (152–156).

GPR120

GPR120 is Gαi/o- or Gαq-coupled, and its ligands are LCFAs, unsaturated fatty acids, omega-3 fatty acids, and omega-6 fatty acids (157). Abnormalities in lipid metabolism were observed in CIA mice. For example, the antioxidant enzymes LDH and lipoproteins are positively correlated with the lipid fractions in the plasma and joint tissue. Lipid content is negatively correlated with lipid levels, and cytokine content is correlated with lipid fractions and the saturated fatty acid/unsaturated fatty acid ratio (158). Therefore, in addition to the effects of multiple immune mechanisms on RA, lipid metabolism may also regulate RA. GPCRs have been shown to regulate lipid metabolism, which may have potential therapeutic effects on RA. Some endogenous n-6 polyunsaturated fatty acids (PUFA) can cause inflammation and pain by promoting the synthesis of leukotrienes, prostaglandins, IL-6, TNF-α, and ROS. Simultaneously, exogenous linolenic acid, an n-3 PUFA that activates FFA4 (GPR120) and has potent anti-inflammatory effects, has been shown to improve inflammation and disease severity in RA (159, 160). Omega-3 fatty acids activate GRP120, which decreases transforming growth factor-β activated kinase 1 (TAK1) and inhibits the downstream IKKβ/I-κB pathway and terminal NF-κB. Thereby it suppresses T cell activation and inflammatory responses, and improves symptoms in arthritic mice (161). GPR120 inhibits CD40L-induced activation of dendritic cells and proinflammatory responses, and GPR120 agonists have similar effects (162).

GPR43

GPR43 is Gαi/o- or Gαq-coupled, and its ligands are acetate, propionate, and butyrate (157). GRP43/FFA2R is expressed on RA FLSs and upregulated in response to TNFα stimulation, and its inhibition of GPR43 can significantly inhibit a variety of biological mediators and signaling pathways in RA, including IL-6, IL-8, high mobility group protein 1 (HMG-1), monocyte chemoattractant protein 1 (MCP-1), intercellular adhesion molecule 1 (ICAM-1), and vascular cellular adhesion molecule 1 (VCAM-1), production of ROS and 4-hydroxynoneal, MMP-3, and MMP-13, and activation of the NF-κB inflammatory signaling pathway (163).

GPR91

GPR91 is Gαi/o- or Gαq-coupled, and its ligands is succinate (157). GPR91 is mainly expressed in macrophages and dendritic cells. GPR91 in dendritic cells triggers intracellular calcium flow by binding succinate; induces cell migration; and interacts with TLR to release proinflammatory factors. Succinate also enhances T helper cell activation (164). Succinate is abundant in the RA synovial fluid. Macrophage sensing of endogenous LPS activates TLR, releases succinate, and upregulates GPR91 sensing of succinate to activate glycolytic pathways and promote IL-1β production. GPR91 knockout mice have reduced macrophage activation and IL-1β release in response to antigen-induced arthritis (165). Intracellular succinate promotes VEGF production by activating GPR91 and inducing angiogenesis *via* HIF-1α (166).

CD97

CD97 is primarily coupled to the Gαi/o protein (167). CD97 plays multiple roles in RA through its N-terminal epidermal growth factor structural domain combined with the N-terminal short consensus repeat structural domain of CD55 (168). For example, CD97 is expressed in granulocytes and monocytes, and is upregulated in T and B cells combined with CD55 activation to promote T cell activation (169). Neutralizing anti-CD97 antibody attenuates multiple arthritic manifestations in a collagen-induced murine joint model (170).

CasR and TGR5

CasR is Gαi/o- or Gαq-coupled, and its ligands are Ca2+, L-amino acids, and oligopeptides (157). NLRP3 inflammasome and associated IL-1β release are closely associated with inflammatory progression in RA (171, 172). Monocytes show increased CasR expression (173, 174). Monocytes and macrophages can phagocytose colloidal calmodulin particles to prevent extracellular calcification *in vivo*. In this process, increased extracellular Ca2+ concentrations can activate CasR, and thus activate the NLRP3 inflammasome in monocytes to promote IL-1β release for inflammation (175).

TGR5 is Gαs-coupled and its ligands are lithocholic acid, deoxycholic acid, chenodeoxycholic acid, and cholic acid (157). Bile acids mainly activate TGR5. TGR5 mRNA expression is reduced in PBMCs from RA patients and correlates with CRP and DAS28 levels. Lithocholic acid inhibits NF-kB activity and inflammation by binding TGR5 in PBMC, while it reduces proinflammatory factors in CIA mice, such as TNF-α, IL-6, IL-8, and IL-1β (176).

Formyl Peptide Receptor-Like 1 (FPRL1)

FRP1 and FPRL1s are Gαi/o- or Gαq-coupled, and their ligands are N-formyl-methionine and N-formyl-metoligopeptides (157). Acute-phase serum amyloid A (A-SAA) and FPRL1 are expressed in RA FLSs, macrophages, and endothelial cells. A-SAA is associated with RA disease activity and is regulated by proinflammatory factors, which may be involved in the stromal degradation of RA through FPRL1-induced secretion of MMP-1 and MMP-3 from FLSs (177). In addition, A-SAA can promote FLS proliferation and angiogenesis by binding to FPRL1 (178). Similarly, annexin-1 may also induce MMP-1 secretion from FLS *via* FPRL1 (179).

G Protein-Coupled Receptor Kinase 2 (GRK2)

GRK2 is a key enzyme involved in desensitization of many G proteins (180). Reduced T-cell expression of GRK2 promotes increased responses in CCL3, CCL4, and CCL5 (181). GRK2 in endothelial cells may regulate inflammatory responses by

regulating Weibel-Palade body formation-mediated cytokinesis and histamine-stimulated aggregation of leukocytes to endothelial cells (180).

CONCLUSION

The structure and signaling pathways of GPCRs have been investigated and explained in various diseases; however, their specific mechanisms and roles in RA remain to be elucidated. In this review, we discussed multiple GPCR receptors to clarify some of their roles and mechanisms. Nevertheless, many questions remain. First, chemokine receptors are currently the most intensively studied GPCRs in RA. There are two main aspects of RA drug strategies developed for chemokines and chemokine receptors: on one hand, corresponding ligands that can be selected to inhibit chemokine receptors and on the other, direct inhibition of chemokine receptors. Both options are currently investigated in several preclinical and clinical studies. However, the exact clinical efficacy still needs to be further observed in well-designed clinical trials. Unfortunately, some good preclinical trial results do not translate into an excellent clinical protocol. Some clinical trials on chemokines have faced difficulties, possibly owing to the widespread expression of chemokine receptors in a variety of cells, implying that a portion of chemokine receptors may be necessary for certain normal physiological processes, and therefore, potentially effective chemokine receptor inhibitors may specifically target receptor expression in certain pathogenic cell subpopulations. Second, the expression of GPCRs at different disease stages of RA may have diverse functional roles, such as CCR4. Clarifying the role of receptors at different stages is vital for drug development. Finally, the application of novel variable conformation modulators and biased agonists of GPCRs may be an essential tool for the future development of GPCR drugs for RA. So far, inhibition of single GPCR has failed to bring the desired outcomes. The combined inhibition of multiple GPCRs showed better efficacy. In addition, better use of modern techniques, such as synovial biopsy and arthroscopic surgery with the aid of computer imaging, may allow for better evaluation of the overall disease situation, and combined analysis using multi-omics and multiple molecular biology techniques may improve the current situation. Although the translation of preclinical GPCR results to clinical results for RA is still challenging, in-depth studies present a direction with a great potential.

AUTHOR CONTRIBUTIONS

JZ is responsible for the collection, collation, and writing of the original manuscript. KW, CC, PJ, LXX, LSX, and YS are accountable for the collection. SG and DH are responsible for the concept development, revision, and manuscript review. All authors reviewed and accepted the final version.

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

GLOSSARY

RA	rheumatoid arthritis
GPCRs	G protein-coupled receptors
NSAIDs	non-steroidal anti-inflammatory drugs
GDP	guanosine diphosphate
GTP	guanosine triphosphate
cAMP	cyclic adenosine monophosphate
PKC	protein kinase C
CCR	C-C motif chemokine receptor
CXCR	C-X-C motif chemokine receptors
XCR1	X-C motif chemokine receptor 1
CX3CR1	C-X3-C motif chemokine receptor 1
anti-CCP	anti-cyclic citrullinated peptide
DAS28	the disease activity score-28
RF	rheumatoid factor
FLSs	fibroblast-like synoviocytes
TNF&alpha	tumor necrosis factor &alpha
MMP	matrix metalloproteinase
CIA	the collagen-induced arthritis
IFN	interferon
CXCL10, RANKL	receptor activator of nuclear factor kappa-B ligand
CKLF1	chemokine-like factor 1
CRP	C-reactive protein
ESR	erythrocyte sedimentation rate
E2F2	E2F transcription factor 2
FOXP	forkhead box P
STAT5	signal transducer and activator of transcription 5
ROR	RAR-related orphan nuclear receptor
LPS	lipopolysaccharides
PGC1	proliferator-activated receptor gamma coactivator 1
ACPA	anti-cyclic citrullinated peptide antibody
ERK	extracellular signal-regulated kinase
VLA-4	very late activation antigen 4
VCAM-1	vascular cell adhesion molecule 1
SDF-1	stromal cell-derived factor 1
TLR	Toll-like receptor
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
Tfh	follicular helper T
P-gp	P-glycoprotein
Tfr	follicular regulatory T
ROS	reactive oxygen species
Bregs	regulatory B cells
JNK	Janus kinase
MAPK	mitogen-activated protein kinase
MNCs	mononuclear cells
MC	melanocortin
NOS2	nitric oxide synthase 2
a-MSH	alpha-Melanocyte-stimulating hormone
sTNFr	soluble tumor necrosis factor receptor
PUFA	polyunsaturated fatty acids
TAK1	transforming growth factor-&beta activated kinase 1
HMG-1	high mobility group protein 1
IL	interleukin
MCP-1	monocyte chemoattractant protein 1
ICAM-1	intercellular adhesion molecule 1
VCAM-1	vascular cellular adhesion molecule 1
VEGF	vascular endothelial growth factor
PBMC	peripheral blood mononuclear cell
KKS	kallikrein-kinin system
pKal	prekallikrein
FXII	factor XII
HK	high-MW kininogen
sRANKL	soluble receptor activator of nuclear factor kappa B ligand
PI3Ks	phosphoinositide 3-kinases
PKB	protein kinase B
FPRL1	formyl peptide receptor-like 1
A-SAA	acute-phase serum amyloid A
GRK2	G protein-coupled receptor kinase 2

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Relationship Between Serum Complement C3 Levels and Outcomes Among Patients With Anti-GBM Disease

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Background: IgG and complement 3 (C3) are generally found to be deposited along the glomerular basement membrane (GBM) in human anti-GBM disease. The pathogenic role of complement activation in kidney damage of anti-GBM disease has been explored in recent years. Therefore, we investigated the relationship between serum C3 and outcomes among patients with anti-GBM disease in this study.

Methods: Ninety-four anti-GBM disease patients between January 2004 and December 2020 at the National Clinical Research Center of Kidney Diseases Jinling Hospital were retrospectively analyzed, and were divided into the low C3 group and the normal C3 group according to serum C3 levels at diagnosis. Fifty-six patients had undergone renal biopsy. We analyzed the clinical manifestations, laboratory tests, kidney pathology, treatment, and outcomes between the two groups. The primary endpoint was kidney failure. Cox regression and smooth curve fitting of generalized additive mixed model analysis were used to explore the correlation between serum C3 and kidney failure. The outcomes of the two groups were compared by the Kaplan–Meier curve.

Results: A total of 94 patients (aged 43.6 ± 16.2; male patients, 46%) with anti-GBM disease were enrolled. There were 26 patients with low C3 levels and 68 patients with normal C3 levels. Compared with the normal C3 group, patients in the low C3 group have a higher proportion of glomerular sclerosis progressing to kidney failure. Multivariate Cox regression analysis suggested that C3 is associated with kidney outcomes in patients with anti-GBM disease (HR = 0.782, 95% CI = 0.673–0.907, $p = 0.001$). Smooth curve fitting of generalized additive mixed model analysis indicated that the level of C3 had a linear relationship with the changing trend of kidney failure. The Kaplan–Meier curve showed that there was a statistical difference between the two groups in terms of kidney failure ($p = 0.033$).

Conclusion: The kidney outcomes of anti-GBM disease in the low C3 group were poorer than those in the normal C3 group. The influence of C3 on the kidney outcomes of patients with anti-GBM disease may be of clinical relevance.

Keywords: anti-GBM disease, complement, C3, kidney failure, outcome

INTRODUCTION

Anti-glomerular basement membrane (GBM) disease is a rare but life-threatening autoimmune disorder that is characterized by rapidly progressive glomerulonephritis with or without pulmonary hemorrhage. Kidney biopsy of anti-GBM disease has shown that immunoglobulin G (IgG) linearly deposits along the GBM, which is usually accompanied by linear or granular deposition of complement 3 (C3) (1). This indicates that complement activation may participate in the pathogenesis of anti-GBM disease. The pathways of complement activation in anti-GBM disease are mainly studied by passive injection of heterologous antibodies against GBM. It has been found that the complement system is activated in human anti-GBM disease through classical and alternative pathways (2), which plays an aggressive role in the pathogenesis of kidney injury by proinflammatory effect or cell lysis effect (3, 4). Multiple studies also showed that low serum C3 levels at diagnosis are associated with poor kidney outcomes in ANCA-associated vasculitis (5) and IgA nephropathy (6). However, to the best of our knowledge, few studies have analyzed the effect of serum C3 levels as a prognostic parameter in anti-GBM disease.

Therefore, this study aimed to investigate whether patients with low serum C3 levels at diagnosis have different clinical and histopathological features or outcomes compared to patients with normal C3 levels in anti-GBM disease.

MATERIALS AND METHODS

Patients

The patients diagnosed with anti-GBM disease in the National Clinical Research Center of Kidney Diseases Jinling Hospital from January 2004 to December 2020 were enrolled in this study. All patients had follow-up data for at least 3 months from time of presentation or until death, and those without serum C3 levels and missing follow-up records were excluded (**Figure 1**). The study complied with the Declaration of Helsinki and was approved by the independent ethics committee of Jinling Hospital (Approval 2022DZKY-033-01).

Data Collection

Data of clinical, laboratory, and pathological variables were collected from medical records at diagnosis and during follow-up, which included the following: demographics, medical history and duration, clinical manifestations, serum C3 and C4, serum creatinine (SCr) at presentation, peak SCr, estimated glomerular filtration rate (eGFR), oliguria or anuria, hypertension, and kidney replacement therapy (KRT) at onset. Serum C3 and C4 levels were measured at the time of diagnosis, using a turbidimetric test. Our laboratory's serum C3 reference range is 0.8–1.8 g/L, and serum C4 reference range is 0.1–0.4 g/L.

Definitions

The main endpoint of the event was kidney failure, which was defined as the persistent need for KRT for more than 3 months. The need for KRT at onset was defined as the requirement for KRT during the first hospital stay. The eGFR was determined using the CKD-EPI equation (7). The extent of acute tubular–interstitial lesions includes tubular epithelial brush-border loss or interstitial edema and inflammatory cell infiltration area. Kidney tubular lesions used semi-quantitative scores: 0, not present; 1, present in 1%–25%; 2, present in 25%–50%; 3, present in >50%.

Kidney Pathology

A kidney biopsy was performed at the time of diagnosis. Kidney specimens were evaluated by direct immunofluorescence and light and electron microscopy and were forwarded to two pathologists who examined the specimens separately, blinded to each other and the patients' data.

For direct immunofluorescence, frozen sections were stained with a panel of fluorescein isothiocyanate-conjugated rabbit anti-human antibodies to IgG, IgM, IgA, C3, C1q, and fibrinogen. On light microscopy, the following indicators were evaluated by pathologists: the percentage of crescents (cellular, fibrocellular, and fibrous crescents), acute tubular–interstitial lesions, interstitial fibrosis, and tubular atrophy.

Statistical Analysis

Values are expressed as mean ± standard (SD), or as median and interquartile range for continuous variables, and percentages for categorical variables. First, continuous variables were analyzed using the *t*-test. Meanwhile, the χ^2 test and Mann–Whitney *U* test were used for categorical variables. Next, generalized additive model smooth curve fitting was used to address the relationship between serum C3 and kidney failure in anti-GBM disease, and univariate and multivariate Cox regression models were performed to examine whether C3 is associated with kidney failure in the disease. The Kaplan–Meier curve was used to analyze two groups' kidney survival. In addition, an interaction test was conducted to evaluate whether patients' characteristics influence the relationship between C3 and kidney failure. Data analysis was performed using R (http://www.r-project.org) and EmpowerStats (www.empowerstats.com.X&YSolutionsInc). A two-sided $p < 0.05$ was considered statistically significant.

RESULTS

Patient Characteristics

A total of 94 patients diagnosed with anti-GBM disease were included in the study, 26 (27.7%) of whom had low C3 levels (below the normal range limit, <0.8 g/L). Patients were categorized according to the measurement of C3 into two groups, one group with low C3 and another group with normal C3. We found that percentage of glomerular sclerosis was higher in the low C3 group than the normal C3 group ($p = 0.012$), while there were no statistical differences in demographics, clinical, laboratory, and other pathological variables. The main clinical characteristics of the patients at presentation are summarized in **Table 1**. Among 94 patients, the average age at diagnosis was 43.6 ± 16.2, with 43 men and 51

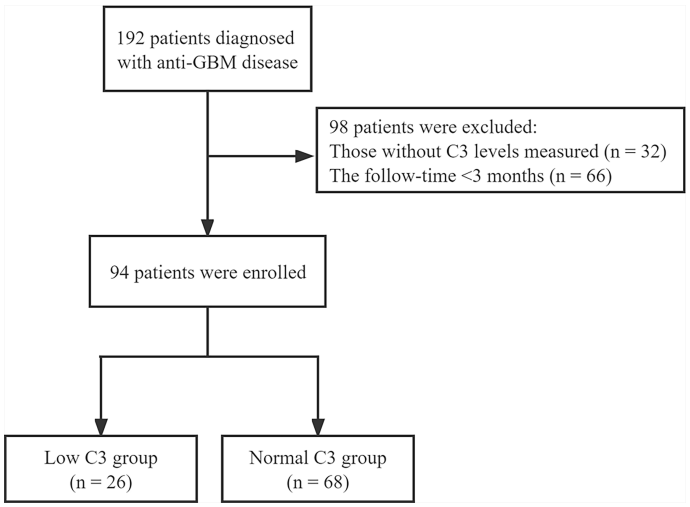

FIGURE 1 | Study flowchart.

women. The SCr at presentation was 661.9 ± 422.6 μmol/L, and the peak SCr was 896.1 ± 389.3 μmol/L. Mean serum C3 and C4 levels were 1.0 ± 0.3 and 0.2 ± 0.1 g/L, respectively. Kidney biopsies showed linear staining of IgG and linear or granular staining of C3 along GBM in the 36 (69%) patients. The average total crescent formation shown in the glomeruli was 68.5% (46.9%–88.8%) (**Table 2**). There were no statistical differences between the two groups of patients who received the standard

TABLE 1 | The baseline characteristics of patients with anti-GBM disease.

	Total (*N* = 94)	Low C3 group (*N* = 26)	Normal C3 group (*N* = 68)	*p*-value
Sex (male/female)	43/51	14/12	29/39	0.330
Age (years)	43.6 ± 16.2	44.4 ± 16.4	43.3 ± 16.2	0.773
Duration of disease (weeks)	4.5 (2.0, 10.8)	6.4 (3.0, 14.2)	3.7 (1.7, 8.6)	0.111
Exposure to chemicals, *n*	13 (14.3%)	3 (12.0%)	10 (15.2%)	0.701
Smoking history, *n*	16 (17.6%)	6 (24.0%)	10 (15.2%)	0.322
Oliguria/Anuria, *n*	51 (54.3%)	17 (65.4%)	34 (50.0%)	0.180
Pulmonary hemorrhage, *n*	18 (19.1%)	5 (19.2%)	13 (19.1%)	0.990
Hypertension, *n*	74 (78.7%)	23 (88.5%)	51 (75.0%)	0.154
RPGN, *n*	93 (98.9%)	26 (100.0%)	67 (98.5%)	0.534
KRT at onset, *n*	86 (91.5%)	24 (92.3%)	62 (91.2%)	0.860
Urinary protein (g/24 h)	1.6 (0.9, 3.2)	1.8 (1.0, 3.9)	1.5 (0.9, 3.2)	0.513
Microscopic hematuria (10^5/ml)	1,075.0 (526.2, 2,592.5)	950.0 (320.0, 1,845.4)	1,250.0 (548.3, 3,000.0)	0.302
Hemoglobin (g/dl)	84.3 ± 17.0	80.0 ± 16.6	85.9 ± 17.0	0.135
Serum albumin (g/L)	31.8 ± 5.6	30.1 ± 5.9	32.5 ± 5.4	0.061
level of anti-GBM antibodies (RU/ml)	161.8 ± 75.6	136.5 ± 69.9	169.6 ± 76.1	0.088
eGFR (ml/min/1.73 m^2)	8.1 (5.5, 11.7)	8.1 (4.9, 10.4)	8.1 (5.8, 13.6)	0.350
SCr at presentation (μmol/L)	661.9 ± 422.6	772.4 ± 592.3	619.6 ± 332.4	0.117
Peak SCr (μmol/L)	896.1 ± 389.3	987.5 ± 529.4	861.1 ± 318.3	0.160
C3 (g/L)	1.0 ± 0.3	0.7 ± 0.2	1.1 ± 0.2	<0.001*
C4 (g/L)	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	<0.001*
Biopsy, *n*	56 (59.6%)	15 (57.7%)	41 (60.3%)	0.818
MP, *n*	79 (84.0%)	20 (76.9%)	59 (86.8%)	0.244
CTX, *n*	41 (43.6%)	11 (42.3%)	30 (44.1%)	0.874
Plasma purification, *n*	59 (62.8%)	16 (61.5%)	43 (63.2%)	0.879
PE, *n*	15 (16.0%)	8 (30.8%)	7 (10.3%)	0.015*
DFPP, *n*	9 (9.6%)	9 (34.6%)	30 (44.1%)	0.243
IA, *n*	39 (41.5%)	1 (3.8%)	8 (11.8%)	0.403
kidney failure, *n*	83 (88.3%)	26 (100.0%)	57 (83.8%)	0.029
Alive, *n*	90 (95.7%)	25 (96.2%)	65 (95.6%)	0.903
Follow-up period (months)	23.3 (7.1, 74.0)	23.3 (6.2, 47.5)	23.8 (7.3, 78.0)	0.386

*RPGN, rapidly progressive glomerulonephritis; KRT, kidney replacement therapy; GBM, glomerular basement membrane; eGFR, estimated glomerular filtration rate; SCr, serum creatinine; MP, methylprednisolone; CTX, cyclophosphamide; PE, plasma exchange; DFPP, double filtration plasmapheresis; IA, immunoadsorption. *p < 0.05.*

TABLE 2 | The pathological characteristics of patients with anti-GBM disease.

	Total (*N* = 94)	Low C3 group (*N* = 26)	Normal C3 group (*N* = 68)	*p*-value
Biopsy, *n*	56 (59.6%)	15 (57.7%)	41 (60.3%)	0.818
Crescents (%)	68.5 (46.9, 88.8)	79.5 (56.8, 97.2)	66.7 (46.1, 84.9)	0.378
Cellular crescents (%)	19.6 (4.5, 41.3)	9.0 (0.0, 25.6)	27.2 (10.1, 44.7)	0.069
Fibrocellular crescents (%)	16.5 (0.8, 35.6)	19.2 (0.0, 55.7)	15.9 (4.6, 30.8)	0.803
Fibrous crescents (%)	0.0 (0.0, 12.3)	0.0 (0.0, 10.2)	0.0 (0.0, 11.8)	0.973
Glomerular sclerosis (%)	24.6 (5.0, 50.0)	50.0 (28.6, 65.2)	14.0 (2.7, 42.9)	0.004*
Acute interstitial lesions (scores)				0.725
0	12	4	8	
1	11	1	10	
2	9	3	6	
3	19	6	13	
Interstitial fibrosis and tubular atrophy (scores)				0.055
0	2	0	2	
1	15	2	13	
2	11	3	8	
3	23	9	14	
C3 deposition, *n*	36 (69.2%)	10 (71.4%)	26 (68.4%)	0.835
Granular deposition, *n*	27 (75.0%)	5 (50.0%)	22 (84.6%)	
Linear deposition, *n*	9 (25.0%)	4 (40.0%)	5 (19.2%)	

*$p < 0.05$.

induction therapy including glucocorticoids and cyclophosphamide combined with plasma purification technology. The median follow-up of the patients was 23.3 (7.1–74.0) months. At the last follow-up, 83 patients progressed into kidney failure, of which 26 were in the low C3 group and 57 (84%) were in the normal C3 group. There was no difference in patient survival between the low C3 group and the normal C3 group ($p > 0.05$).

Predictors of Kidney Failure Among Patients With Anti-GBM Disease

Univariate analysis showed that C3 is markedly correlated with the risk of kidney failure. In addition, hypertension, oliguria or anuria, anti-GBM levels, eGFR, SCr at presentation, peak SCr, KRT at onset, crescents, and fibrocellular crescents were found to be associated with the risk of kidney failure ($p < 0.05$) (**Table 3**). After adjustment for age, gender, hypertension, anti-GBM levels, eGFR, KRT at onset, and crescents, multivariate analysis still showed that C3 at diagnosis was an independent protective factor for kidney outcomes of anti-GBM disease. For every 0.1 g/L increase in C3, the risk of developing kidney failure decreases by 22% (HR = 0.782, 95% CI = 0.673–0.907, $p = 0.001$) (**Table 3**). In addition, oliguria/anuria and peak SCr were also associated with kidney failure in multivariate analysis (**Table 3**).

Association Between Serum C3 and Outcomes of Anti-GBM Disease

We analyzed the patient and kidney survival according to C3 levels, finding that patients in the low C3 group had significantly worse kidney survival than the normal C3 group ($p = 0.029$). We further compared kidney survival of patients with anti-GBM disease in two groups by the Kaplan–Meier curve, which showed that low C3 was associated with a significantly lower kidney survival ($p = 0.033$) (**Figure 2**).

Smooth curve fitting of C3 and kidney failure was conducted after adjustments for age, gender, hypertension, anti-GBM levels, eGFR, KRT at onset, and crescents. The results showed that C3 level was linearly correlated with the changing trend of kidney failure risk ($p = 0.001$), which means that as C3 levels increase, the risk of kidney failure occurrence gradually decreases (**Figure 3**).

In this study, the relationship between C3 and kidney failure was further stratified by age, hemoglobin, serum albumin, SCr at presentation, oliguria or anuria, and crescents. The results suggested that when the serum albumin <30 g/L, C3 had a protective effect on outcomes in patients with anti-GBM disease, and serum albumin could modify the relationship between C3 and kidney failure. In addition, the protective role in outcomes of C3 was observed only in patients without oliguria/anuria (HR = 0.029, 95% CI = 0.002–0.335, $p = 0.005$). Serum albumin levels and oliguria/anuria were considered the prominent interactive factors that affect the association between C3 and the risk of kidney failure by the interaction analysis, which remained robust under the grouping of other indicators (**Table 4**).

DISCUSSION

This is the first retrospective study to explore the relationship between serum C3 levels measured at diagnosis and the risk of kidney failure in patients with anti-GBM disease. We observed that patients with low C3 levels had more severe glomerular sclerosis and poorer kidney survival than normal C3 levels. Serum C3 may be a protective factor for kidney outcomes. Furthermore, we confirmed that C3 was associated with kidney outcomes by univariable and multivariable Cox regression. Meanwhile, there was a difference in the Kaplan–Meier curve between the low C3 and the normal C3 groups. Therefore, we thought that serum C3 at diagnosis has a protective effect on kidney prognosis in patients with anti-GBM disease. In addition, our work suggested that serum albumin and oliguria/anuria were considered the prominent interactive factors that affect the

TABLE 3 | Predictors of kidney failure by univariate and multivariate Cox regression analysis.

Variable	Univariate analysis (*N* = 94)		Multivariate analysis (*N* = 94)	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Age	1.008 (0.995–1.021)	0.243	1.004 (0.980–1.029)	0.736
Gender	1.051 (0.681–1.622)	0.822	1.321 (0.616–2.832)	0.474
Pulmonary hemorrhage	0.706 (0.394–1.266)	0.243	0.387 (0.138–1.087)	0.071
Hypertension	2.359 (1.309–4.249)	0.004*	1.794 (0.713–4.518)	0.215
Exposure to chemicals	0.900 (0.463–1.750)	0.756	0.627 (0.164–2.396)	0.495
Smoking	1.640 (0.938–2.870)	0.083	1.520 (0.516–4.479)	0.448
Oliguria/Anuria	4.468 (2.715–7.354)	<0.001*	7.764 (2.976–20.253)	<0.001*
Anti-GBM antibody levels (increased by 10 RU/ml)	1.045 (1.010–1.081)	0.012*	1.056 (0.993–1.123)	0.083
eGFR (ml/min/1.73 m^2)	0.970 (0.944–0.997)	0.031*	0.992 (0.960–1.025)	0.612
SCr at presentation (increased by 88.4 μmol/L)	1.085 (1.042–1.130)	<0.001*	1.100 (0.958–1.264)	0.178
Peak SCr (increased by 88.4 μmol/L)	1.131 (1.086–1.177)	<0.001*	1.199 (1.039–1.384)	0.013*
Hb (increased by 10 g/L)	0.950 (0.843–1.070)	0.395	1.141 (0.878–1.482)	0.324
C3 (increased by 0.1 g/L)	0.867 (0.798–0.941)	<0.001*	0.782 (0.673–0.907)	0.001*
C4 (increased by 0.1 g/L)	0.819 (0.666–1.006)	0.056	0.708 (0.484–1.035)	0.075
KRT at onset	11.671 (2.811–48.447)	<0.001*	4.748 (0.511–44.141)	0.171
C3 deposition	1.069 (0.564–2.024)	0.839	0.732 (0.340–1.578)	0.426
Crescents (increased by 10%)	1.137 (1.012–1.278)	0.031*	1.087 (0.941–1.255)	0.256
Cellular crescents (increased by 10%)	0.988 (0.879–1.109)	0.833	0.972 (0.827–1.141)	0.726
Fibrocellular crescents (increased by 10%)	1.412 (1.012–1.288)	0.031*	1.179 (1.000–1.389)	0.050
Fibrous crescents (increased by 10%)	1.064 (0.918–1.233)	0.411	1.179 (1.000–1.389)	0.119
Glomerular sclerosis (increased by 10%)	1.118 (0.999–1.251)	0.053	1.059 (0.919–1.219)	0.429

*GBM, glomerular basement membrane; eGFR, estimated glomerular filtration rate; SCr, serum creatinine; Hb, hemoglobin; KRT, kidney replacement therapy; Hazard ratio (HR) of multivariate analysis was adjusted for age, gender, hypertension, anti-GBM levels, eGFR, KRT at onset, and crescents. *p < 0.05.*

relationship between C3 and the risk of kidney failure by the interaction analysis.

The complement system plays an essential role in immune-mediated glomerulonephritis. C3 deposition of the glomerular capillary wall can be shown by immunofluorescence, which is consistent with the assumption that complement activation participated in the generation of kidney damage of human anti-GBM disease. Previous studies of humans and animals both have found that the complement system is activated through the classical and alternative pathways in anti-GBM disease (2, 8). Some researchers supported the idea that antibody-directed complement activation initiates a cascade involving cytokine production, upregulation of adhesion molecules and chemokine production, and recruitment and activation of platelets and neutrophils, which ultimately lead to this early glomerular injury (9–12). Ma et al. have found that the complement cascade played a pathogenic role in kidney injury, as shown by the possible proinflammatory effect of C5a and/or cell lysis effect of C5b-9 in 20 patients with kidney biopsy-confirmed anti-GBM disease (8).

Several animal models have been employed to better understand the complement system's role in the development of anti-GBM disease. Hammer et al. discovered that the primary stage of nephrotoxic serum nephritis produced by rabbit

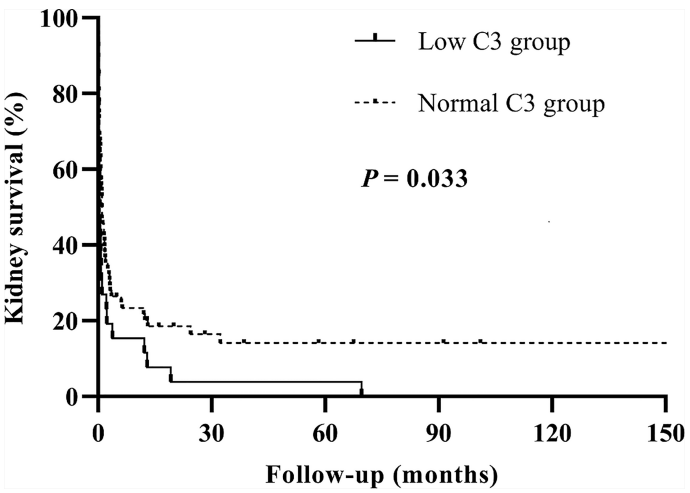

FIGURE 2 | Kaplan–Meier survival analysis for kidney outcomes between the low C3 group and the normal C3 group.

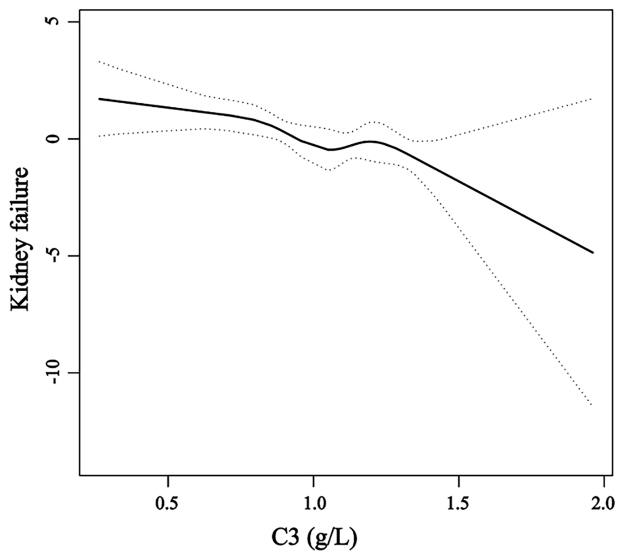

FIGURE 3 | A smooth curve fitting of the relationship between serum C3 levels and the risk of kidney failure. The black straight line represents the smooth curve fit between the variables. The dotted line represents the 95% confidence interval of the fit.

nephrotoxic serum appears to a great extent, but not wholly, upon the participation of serum complement (13). Sheerin et al., using C3-deficient mice, found that complement acts synergistically with heterologous antibodies, resulting in neutrophil infiltration and glomerular injury in experimental anti-GBM disease. Their studies also supported that classical and alternative pathways of the complement system are involved in the development of anti-GBM disease (14). Moreover, in the different phases of anti-GBM disease, the role of complement is contradictory. They thought that the effect of complement in the pathogenesis of glomerular disease might be dependent on the stage of the disease (15). More importantly, they also compared the contribution of systemic and local production of C3, concluding that circulating C3 is a critical factor in reducing the glomerular accumulation of immune complexes, while local synthesis of C3 did not have a major influence on this aspect of glomerular disease (16).

Our study found that lower C3 levels are associated with poorer kidney survival. There were no statistical differences between the two groups of patients who received the standard induction therapy including glucocorticoids and cyclophosphamide combined with a plasma purification technique. Even after adjusting for age, gender, hypertension, GBM, eGFR, KRT at onset, and crescents, C3 levels remained significantly associated with kidney survival. We further found that the C3 level was linearly related to the changing trend of kidney failure, and the risk of kidney failure gradually decreased as the C3 levels increased, which would suggest that serum C3 levels at diagnosis of anti-GBM disease could be an independent protective factor, but this needs further confirmation.

In addition, we found that serum albumin level was considered the prominent interactive factor that affects the association between C3 and the risk of kidney failure by the interaction analysis, which may be related to complement involvement in the pathogenesis of proteinuria (17). Considering that both albumin and C3 may be related to the nutritional status of patients and are potential systemic inflammatory response proteins, there may be other factors at play resulting in a concurrent decrease in both albumin and C3 (and maybe C4), which, in turn, results in worse outcomes that need to be further explored. As for C3, there was no protective role in patients with oliguria or anuria, which may be

TABLE 4 | Relationship between C3 and kidney failure under different stratification factors.

Subgroup stratification	Cases	HR (95% CI)	*p*-value	*p* for interaction
Age (years)				0.652
<60	75	0.076 (0.015–0.379)	0.002	
≥60	19	0.062 (0.001–5.497)	0.224	
Hemoglobin (g/L)				0.106
<90	62	0.034 (0.006–0.187)	<0.001	
≥90	32	0.466 (0.031–7.065)	0.582	
Serum albumin (g/L)				0.040*
<30	40	0.011 (0.001–0.123)	<0.001	
≥30	54	0.189 (0.033–1.082)	0.061	
SCr at presentation (μmol/L)				0.760
<500	31	0.110 (0.014–0.878)	0.037	
≥500	63	0.074 (0.010–0.535)	0.010	
Oliguria/anuria				0.024*
No	43	0.029 (0.002–0.335)	0.005	
Yes	51	0.725 (0.096–5.461)	0.755	
Crescents (%)				0.265
<60	21	0.024 (0.002–0.377)	0.008	
≥60	33	0.127 (0.026–0.610)	0.010	
C3 deposition				0.888
No	16	0.094 (0.005–1.661)	0.107	
Yes	36	0.075 (0.015–0.378)	0.002	

*Hazard ratio (HR) was adjusted for age, gender, hypertension, anti-GBM levels, eGFR, KRT at onset, and crescents. *p < 0.05.*

explained by oliguria or anuria being a stronger predictor of kidney survival in patients with anti-GBM disease compared with other variables, and almost all patients with initial oliguria or anuria end up entering kidney failure (18). Serum C4 levels and local synthesis of C3 have not been found to be associated with kidney outcomes of the disease in our study. We discovered that oliguria/anuria and peak SCr were risk factors for kidney failure in multivariable regression analysis, similar to previous reports.

Although C3 has been rarely studied in human anti-GBM disease, the crucial role of complement in pathogenesis has been explored in IgA nephropathy (6), C3 glomerulonephritis (19), membranous nephropathy (20), and other nephritis (21). Many recent studies demonstrated that C3 levels are correlated to patient and kidney survival in ANCA-associated vasculitis as well (22–24). The critical role of C3 at the intersection of all complement activation pathways and its synergistic effect in multiple immune and inflammatory networks have facilitated the development of C3-based therapies (25). Now, C3 intervention is emerging as a viable therapeutic strategy for rare inflammatory kidney diseases, such as C3 glomerulopathy (26).

Nevertheless, this study still has some limitations, including the small sample size and the single-center design. Moreover, some patients did not have a kidney biopsy at diagnosis, thus limiting the histological analysis. Despite these limitations, we demonstrated clearly that serum C3 plays a protective role in the progress of anti-GBM disease and that low serum C3 level at diagnosis is associated with poor kidney outcomes.

In conclusion, this study retrospectively compared the clinicopathological features and outcomes between the low C3 group and the normal C3 group, and found that patients with low serum C3 levels had a higher proportion of glomerular sclerosis progressing into kidney failure. Serum C3 is closely related to the kidney outcomes of patients with anti-GBM disease, and the pathogenesis of C3 in the disease needs to be further studied.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Independent ethics committee of Jinling Hospital. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JW and HZ contributed to the conception of the study. WL screened the data, and MZ analyzed and interpreted the data. FX completed the pathological analysis. MZ, JW, YJ, and CJ contributed to the follow-up. MZ finished the manuscript. JW and HZ supervised and edited the manuscript. All authors contributed to the work and approved the submitted version for publication.

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