ISSN 1756-1841 VOLUME 28 NUMBER 1 2025

International Journal of Rheumatic Diseases

Official journal of the Asia Pacific League of Associations for Rheumatology (APLAR)

WILEY

International Journal of Rheumatic Diseases

For submission instructions, subscription and all other information visit http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1756-185X

View this journal online at wileyonlinelibrary.com/journal/apl

Received: 8 July 2024 | Revised: 1 September 2024 | Accepted: 8 October 2024

DOI: 10.1111/1756-185X.15385

International Journal of Rheumatic Diseases WILEY

REVIEW

Recent advances in cellular immunotherapy for immune-mediated rheumatic diseases

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Funding information
Campus level Basic Project General Incubation Project of Naval Medical University, Grant/Award Number: 2023MS024; The National Natural Science Foundation of China (NSFC Project), Grant/Award Number: 82171754 and 81971484

Abstract

Cellular immunotherapy is an emerging therapeutic approach that aims to utilize a patient's immune cells or engineered immune cells to treat Immune-mediated rheumatic diseases, including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and ankylosing spondylitis (AS), among others. The PubMed database was searched for 46 relevant pieces of literature. Among them, 18 articles on T-cell therapy were used to treat RA, AS, SLE, Anti-Neutrophil Cytoplasmic Antibodies (ANCA)-associated vasculitis (AAV), and systemic sclerosis (SSc). There were nine B cell depletion therapies for RA, AS, SLE, and SSc, and 15 articles on mesenchymal stem cell (MSC) therapy for RA, AS, SLE, AAV, SSc, and Sjogren's syndrome (SS). Additionally, there were four articles on Cd19-targeting Chimeric Antigen Receptor T-Cell (CART) cell therapy for SLE and SSc treatment. The most common treatments for rheumatological diseases, such as RA, SLE, and SS, include B cell depletion, MSC extracellular vesicle therapy, and CART cell targeted therapy, while the re-used therapies for rare diseases such as SSc, IgG4-RD, and AAV include extracellular stem cell vesicles and cytokines, as per the review of the aforementioned literature. CART cell targeting therapy alone is not highly effective, and the common adverse effects include hypotension and dry cough. In summary, cellular immunotherapy has emerged as a better choice for refractory rheumatic immune diseases. In the future, further clinical studies are imperative to precisely target the treatment of rheumatic disease and provide positive outcomes to a broader patient population.

KEYWORDS
B cell depletion, cellular immunotherapy, mesenchymal stem cells, rheumatism immune disease, T cell therapy

1 | INTRODUCTION

Immune-mediated rheumatic diseases are chronic inflammatory reactions caused by abnormal immune responses, including rheumatoid arthritis (RA), ankylosing spondylitis (AS), systemic lupus erythematosus (SLE), Sjogren's syndrome (SS), systemic sclerosis (SSc), systemic vasculitis (SAA), et cetera. These diseases often impact joints and damage organs throughout the body, significantly reducing the quality of life of patients and adversely affecting their physical and mental health. Traditional treatments for rheumatic immune diseases exhibit limited effectiveness and are often associated with side effects. As a novel therapeutic approach, immune cell therapy

Wei Wan and Yidi Liu have equal contributions to the article.

holds promise as a groundbreaking treatment for rheumatic immune diseases by regulating the immune function of the body.

The study aimed to systematically review and explore the potential application of immune cell therapy in rheumatic immune diseases, evaluate its efficacy and safety, and strive to establish a scientific foundation. While the implementation of cellular immunotherapy in rheumatoid disease is still in the research and clinical trial phase, there are already several treatment approaches and strategies that demonstrate potential in practical applications.

All studies included in this review were approved by the local ethics committee.

2 | T CELL THERAPY

T cell therapy mainly uses CAR-T technology to design and introduce specific chimeric antigen receptors (CAR) to recognize and bind to a specific cell surface antigen. In autoimmune diseases, where these antigens may be molecules on the surface of abnormally active B cells or other immune cells, the CAR-T technology can be used to regulate overactive immune responses, such as by clearing autoantibody-producing B cells, thereby reducing attack on their own tissue.

- RA: Regulatory T cells (Tregs) play a vital role in the occurrence and development of RA. The maintenance of an adequate Treg population and the restoration of their function through transplantation or exogenous stimulation are the keys to targeted therapy for Tregs. Potential therapeutic strategies for Tregs currently in general use include cell transplantation therapy (mesenchymal stem cell (MSC) transplantation and adoptive transfer of Tregs), Tregs-targeted therapy (low-dose interleukin-2 (IL-2) therapy, rapamycin, and histone deacetylase (HDAC) inhibitors), as well as other Tregs-related drugs (glucocorticoids and traditional Chinese medicine) and cytokines promoting T cell differentiation.[1]

Notably, interleukin-27 (IL-27) is a cytokine that promotes the production of Tr1 cells, a type of regulatory T cell that produces IL-10, which plays a role in suppressing autoimmune responses and tissue inflammation. IL-27 promotes type 1 regulatory T cell (Tr1 cell) differentiation by inducing the expression of the transcription factors c-Maf Protooncogene (c-Maf) and aryl hydrocarbon receptor (AhR), which together activate IL-10 production. Furthermore, AhR activation enhances Granzyme-B expression, which may mediate the contact-dependent inhibitory activity of Tr1 cells. Therefore, IL-27-induced Tr1 cells have potential applications in the treatment of autoimmune diseases. In terms of therapy, remarkable progress has been made in the differentiation and expansion of Tr1 cells in vitro. For example, human dendritic cells (DCs) produced by interleukin-10 (IL-10) can support the differentiation of antigen-specific IL-10-producing Tr1 cells. Moreover, immunosuppressive drugs such as statins and interferon-beta have been demonstrated to increase IL-27 production in DCs, which may help promote the production of Tr1 cells.[2]

Conversely, Moon et al. demonstrated the presence of Granzyme B (GZMB)+ and Granzyme K (GZMK)+ $CD8^+$ T cells expressing cytotoxic mediators in the blood of RA patients with anti-citrullinated protein antibodies using flow cytometry, single-cell transcriptomics, and functional experiments. These cells are also present in the RA synovium. These findings suggested that cytotoxic $CD8^+$ T cells targeting citrullinated proteins may contribute to synovitis and joint tissue destruction in RA, thus providing a theoretical basis for the development of therapies targeting cytotoxic $CD8^+$ T cells.[3] Concurrently, T cells directly promote the activity of synovial fibroblasts by producing cytokines, which play an important role in cartilage destruction.[4]

Furthermore, AhR ligands such as 6-formylindolo[3,2-b]carbazole (FICZ) or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) have been demonstrated to promote the differentiation of Tr1 cells, presenting an opportunity for developing new therapeutic agents. Simultaneously, the mRNA expression and protein levels of arachidonate 5-lipoxygenase (ALOX5) in $CD4^+$ T cells of RA patients were increased, with the lowest ALOX5 levels observed in patients in clinical remission. Therefore, the expression level of ALOX5 can be utilized as the molecular basis to distinguish the active stage of RA from the remission stage.[5] Co-IR expression on $CD4^+$ T and $CD8^+$ T cells, along with serum levels of soluble PD-1, PDL-2, and TIM3, may serve as indicators of RA disease activity.[6]

- AS: Targeted depletion of T cell receptor beta variable (TRBV)9+ T cells can achieve therapeutic purposes by selectively depleting the T cell population characterized by TRBV, thus targeting the elimination of the root cause of the disease.

2.1 | Long non-coding RNA LOC645166 in T cells

It was observed that the expression level of LOC645166 was reduced in T cells of patients with AS, and its overexpression inhibited the expression of IL-23p19 and the activation of the JAK2/STAT3 signaling pathway in Jurkat cells. LOC645166 tends to bind to K63-linked polyubiquitin chains, inhibiting the recruitment of inhibitor of kappa B kinase (IKK) complexes to these chains, leading to a reduction in IKK2 activation and a downregulation of NF-κB activation. This implies that downregulation of LOC645166 expression in T cells of AS patients may promote NF-κB activation by reducing recruitment of the IKK complex to the K63-linked polyubiquitin chain, thereby increasing the sensitivity of AS patients to inflammatory factors or toll-like receptor ligands.[7] The downregulation of LOC645166 may contribute to the pathogenesis of AS, while its upregulation in T cells may provide a potential therapeutic target for AS therapy.

2.2 | Targeting depleted TRBV9+ T cells

In one patient with AS who underwent immunotherapy targeting TRBV9+ T cells, significant improvements were observed in the

spinal motion index and the Bath AS Measure Index. This suggests a potential method for treating autoimmune diseases by selectively depleting the TRBV-defined T-cell population. Anti-TRBV9 therapy may be appropriate for other spinal arthropathies associated with HLA-B* 27. This targeted elimination of the underlying cause of the disease, without systemic immunosuppression, may provide a novel, safe, and highly efficient therapy for autoimmune diseases.[8]

- SLE: Common therapies include CART cells, SLAM-associated protein (SAP) + T helper cells (TPH), and related cytokines.

CART cell therapy is commonly used in SLE immune cell therapy. CART cells are genetically engineered T cells capable of effectively depleting B cells. By specifically targeting and eliminating CD19-expressing B cells, CART cell therapy may reduce the production of autoantibodies by eliminating autoreactive B cells and plasma cells.[9] A clinical trial demonstrated that laboratory test results returned to normal in all patients after 3 months of treatment, and clinical symptoms of SLE improved. During the follow-up period of 4–16 months, the disease remained dormant, and fibrotic problems in lung and heart tissue and kidney disease (nephritis) were resolved. Additionally, the side effects of the treatment, such as cytokinine release syndrome and immune cell-related neurotoxicity, were minimized.[10] In a humanized mouse model of SLE, CART cells have also been demonstrated to limit the production of autoantibodies and restore the composition of the human immune system in the lymph nodes and spleen without detectable toxicity.[11]

In SLE-induced lupus nephritis, peripheral TPH cells expressing SAP are a promising target for development. The study revealed an increased number of SAP-positive $CD4^+$ T and $CD8^+$ T cells in SLE patients compared to healthy individuals, with the highest expression in the TPH subsets of $CD4^+$ T cells. The circulating SAP + TPH levels are higher in SLE patients with kidney disease. Concurrently, these cells can effectively inhibit the proliferation and activity of B cells in vitro. Consequently, SAP + TPH cells hold potential as therapeutic targets for lupus nephritis (LN).[12]

Furthermore, the analysis indicated that SLE patients exhibited a higher proportion of $CX3CR1^+$ T cells, particularly in terminal differentiation effect memory T cells, and a lower proportion in undifferentiated T cells and central memory T cells. This suggests that CX3CR1 may act as a potential diagnostic marker for SLE. Moreover, $CX3CR1^+$ T cells displayed cytotoxic properties, reflecting the autoimmune status of SLE.[13]

Additionally, SLE pathogenesis can be influenced by IL-2, the regulator of IL-2 expression, and IL-17-related signals.[14]

- ANCA-associated vasculitis (AAV): There are dynamic changes between Treg cells and AAV; however, currently, there is no effective treatment for T cells.

Giant cell arteritis (GCA) is a SAA that is mediated by an abnormal immune response. In GCA, Treg cells are decreased in peripheral blood and absent in vascular walls. Treatment with Tocilizumab (TCZ), an IL-6 receptor antagonist, was linked with a reduction in the frequency of Treg cell subsets. Reduced levels of active Treg cells in Takayasu arteritis compared to healthy donors may be associated with maturation deficits. In AAV, the frequency of Treg cells in peripheral blood is reduced, and their function is impaired.[15]

Carmona et al. discovered cytotoxic $CD4^+$ T lymphocyte (CTL) amplification in active GCA patients, expressing higher levels of cytotoxic and chemokine genes compared to patients without active disease and healthy controls. The findings suggested that CTLs may contribute to the inflammation and vascular remodeling of GCA. Moreover, studies revealed a decrease in the number of Tregs in GCA patients, along with reduced expression of genes associated with inhibitory activity.[16]

- SSc: The use of IL-2 to activate Tregs can achieve a certain therapeutic effect, while the more common CART cell depletion B cell therapy has no prominent performance in the treatment of SSc and is associated with adverse reactions.

SSc is a chronic autoimmune disease characterized by impaired immune response, increased fibrosis, and endothelial dysfunction. Tregs are essential for controlling inflammation, tissue repair, and autoimmunity. However, the frequency and function of Tregs are impaired in patients with SSc. A study was conducted to investigate the potential of low doses of IL-2 for expanding and activating Tregs in nine patients with SSc without severe organ involvement. The primary endpoint (treg frequency) was achieved on the 8th day. Treg levels in CTLs were increased by 1.8 ± 0.5 times ($p = .0015$), while non-significant changes were observed in effector T cells or B cells. Additionally, IL-2 LD was well-tolerated without the occurrence of treatment-related serious adverse events, indicating its promise as a therapeutic potential for SSc.[17]

T cells expressing chimeric autoantibody receptors can treat SSc by specifically eliminating autoreactive B cells.[18] However, another study utilizing common CART cell therapy for SLE in SSc patients did not immediately improve the patient's condition. Subsequently, mycophenolic acid and nidanib were reintroduced based on the patient's preference. The therapy led to a sustained improvement in lung function after treatment, along with significant regression in imaging findings. However, transient hypotension and dry cough occurred during treatment, and a CT scan revealed a mild exacerbation of interstitial pneumonia, necessitating further evaluation of safety considerations.[19]

3 | MSC THERAPY

MSCs have potent immunomodulatory properties that can exert their regulatory properties through direct cell-to-cell interactions and their paracrine effects on various immune and non-immune cells. They can inhibit the immune response and relieve inflammation by regulating the expression of cytokines in T cells, B cells, dendritic cells (DCs), and natural killer cells (NK). In addition, they

can also secrete growth factors, such as hepatocyte growth factor (HGF), vascular endothelial growth factor (VE GF), to promote tissue damage repair. UC-MSCs (umbilical cord-derived mesenchymal stem cells) are less immunogenic and therefore rarely undergo immune rejection, making them promising options for the treatment of immune-mediated rheumatic diseases.

MSCs are pluripotent cells, capable of differentiating into various cell types and secreting different bioactive molecules. They possess the ability to regulate the immune response, reduce inflammation, and promote tissue repair. As a research hotspot, stem cell exosomes hold potential for the treatment of a wide range of rheumatological diseases. Concurrently, the secretion of specific cytokines in vivo can be influenced by the activation of stem cells in vivo through stem cell transplantation to affect T cell activity, which can also achieve certain therapeutic effects.

- SS: Intermediate MSC-derived extracellular vesicles (MSC-EVs) present a novel therapeutic approach for treating dry eye disease associated with SS. MSC-EVs inherit the immunomodulatory functions of MSCs while posing a lower risk of immunogenicity and tumor formation. MSC-EVs contain various bioactive ingredients, including proteins, lipids, and nucleic acids, which regulate intercellular communication and biological processes through different mechanisms. MSC-EVs can inhibit the activation of the NLRP3 inflammatory body, reduce corneal inflammation, and protect lacrimal glands from inflammatory damage by regulating the function of lacrimal epithelial cells to maintain normal tear secretion. Furthermore, by enhancing the proliferation and migration of corneal epithelial cells, corneal wound healing is accelerated, and corneal scar formation is reduced.[20]
- RA: The experimental results of Zhang et al.[21] demonstrated that miR-378a-5p could promote the proliferation, migration, and angiogenesis of human microvascular skin endothelial cells (HSMECs) while inhibiting IRF1. Furthermore, the BMSC-derived EVs containing miR-378a-5p reduced STAT1 phosphorylation and HSMECIRF1 expression. In a mouse model of collagen-induced arthritis (CIA), BMSC-derived EVs containing miR-378a-5p enhanced synovial vascular remodeling and histopathology. Therefore, miR-378a-5p in BMSC-derived EVs promotes the proliferation, migration, and angiogenesis of HSMECs by activating the IRF1/STAT1 axis, thereby preventing the development of RA.[21] In another study focusing on stem cell exosomes, the therapeutic potential of human MSCs (iMSCs)-derived exosomes (Exo-RA) pretreated with disease-conditioned serum (from a non-human primate RA model) was investigated in a mouse RA model. Notably, Exo-RA exhibited a higher number of exosomes in nanoparticle tracing analysis and exosome-labeled CD81 measurements compared to exosomes (Exo-FBS) produced by iMSCs treated with RA serum. Moreover, the level of transforming growth factor-β1 (TGF-β1) in Exo-RA was significantly elevated. In a mouse model of CIA, Exo-RA administration significantly increased serum levels of IL-4 as well as the proportion of Th2 ($CD4^+$ $CD25^+$ $GATA3^+$) and M2 ($CD11c^-$ $CD206^+$) cells within the $CD45^+$ $CD64^+$ population. Additionally, Exo-RA can reduce cartilage damage by significantly reducing the concentration of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), keratinocyte chemokines (KC), and IL-12p70. Therefore, the study concluded that IMSCs-derived exosomes treated with disease-conditioned sera ameliorated cartilage damage in RA models by enhancing TGF-β1 production, inducing Th2 and M2 polarization, and reducing host pro-inflammatory cytokines TNF-α, KC, and IL-12p70. Patient-derived serum can be utilized as an iMSCs treatment strategy to improve the efficacy of IMSCs-derived exosomes in RA treatment.[22] Simultaneously, multiple studies have indicated that MSCs and their exosomes have the potential to regulate immune responses and promote tissue regeneration, demonstrating positive therapeutic effects in the treatment of RA.[23,24]

Additionally, pulp-derived stem cell exosomes are a promising prospect for RA treatment. An investigation was conducted on the therapeutic effect of gingival-derived MSCS (GMSCs) and their exosomes (GMSCExo) in a chimeric model of RA.

GMSCs and GMSCExo can effectively inhibit the invasiveness and destructiveness of activated synovial fibroblasts (RASF) and reduce their invasion into cartilage. GMSC homed to the implantation site and triggered RASF-programmed cell death, while GMSCExo inhibited RASF by delivering molecular cargo via exosomes. Furthermore, due to the ease of collecting gingival tissue and the small amount of tissue required for cell expansion, GMSC and GMSCExo are potential candidates for RA treatment.[25]

- AS: The proliferation and differentiation, inflammatory regulation, and exogenous function of MSCs in AS patients are affected by factors such as genetic background, internal environment, infection, and mechanical stress, leading to pathological changes.[26]

In a mouse model of proteoglycan-induced spondylitis (PGISp), the therapeutic effect of hUCMSCs (human umbilical cord MSCs) was evaluated by injecting different doses of hUCMSCs through a tail vein. Research has indicated that hUCMSCs can regulate the balance of Th1 and Th2 cells in the spleen and reduce the levels of inflammatory cytokines (TNF-α and CCL-2). They have also been observed through imaging and pathology to inhibit ectopic ossification and spinal structure injury.[27] The study concluded that hUCMSC treatment at the 14th week after the establishment of the model is an effective treatment for PGISp mice, providing a valuable preclinical reference for the clinical application of hUCMSCs in the AS treatment.

Additionally, another study revealed that when co-cultured with activated $CD4^+$ T cells or peripheral blood mononuclear cells (PBMCs), adipose-derived MSCs (ASCs) of AS patients directly influenced $CD4^+$ T cells. This led to a decrease in the proportion of T-BET/GATA3 and RORc/FoxP3, thereby reducing the formation of Treg cells. However, there was an increase in the production of IFN-γ and IL-17AF. Conversely, ASCs co-cultured with PBMCs enhanced

Treg cell production and reduced IFNγ release. ASCs failed to upregulate the anti-inflammatory cytokines IL-10 and TGF-β. Studies have indicated that in the absence of other cell support, the effects of ASCs on T cell differentiation may be pro-inflammatory, and their direct effects could be detrimental to the treatment of AS patients.[28]

- SLE: MSC transplantation has been demonstrated to reduce serum levels of IL-1β and IL-18 in SLE patients post-transplantation, as well as inhibit Pim-1 kinase to suppress the activation of the NLRP3 inflammatory body and reduce the inflammatory response.[29] Another study revealed that the systematic administration of miR-199a-5pagomir (a miRNA simulator) in MRL/lpr mice could simulate the therapeutic effect of hUC-MSC transplantation, decreasing the size of spleen and lymph nodes, reducing the levels of anti-dsDNA antibodies and IgG in serum, and alleviating kidney damage.[30] The study also found that hUC-MSCs or phosphate buffers (PBS; control group) were injected into MRL/lpr mice at 17 weeks of age through the tail vein, and the spleen $CD4^+$ T cells of MRL/lpr mice were co-cultured with hUC-MSCs through an in vitro experiment, following which hUC-MSCs regulated the Sirt1/p53 signaling pathway through miR-199a-5p. This led to increased senescence of spleen $CD4^+$ T cells.
- SAA: Kawasaki disease is an SAA that predominantly affects children and can lead to coronary aneurysms. Current treatments include the administration of intravenous immunoglobulin and aspirin. To explore novel therapeutic approaches, a study established a mouse model of Kawasaki disease using a water-soluble component of Candida albicans injected intraperitoneally in DBA/2 mice for 5 days. Subsequently, the mice were injected with either hADSCs or PBS through the tail vein. Serum samples were collected on the 15th and 29th days of the experiment for comparison of cytokine levels. On day 29, mice hearts were dissected and subjected to hematoxylin–eosin (HE) and immunohistochemical staining using Galectin-1 (Gal-1) and CD44 as markers. The findings indicated that, compared to the PBS group, the expression of IL-1α in the hADSC treatment group was significantly decreased on day 15 ($p < .05$), while IL-6 expression was significantly decreased on day 29 ($p < .01$). HE staining revealed a decline in inflammatory cell infiltration and an elevation in Gal-1 expression in the hADSCs group. CD44 expression was not observed in both groups. The survival curve indicated that the survival time of hADSCs group was significantly extended ($p < .05$). Therefore, hADSCs exhibit early anti-inflammatory effects, and Gal-1 may be involved in preventing inflammation and reducing tissue damage. These results provide a potential clinical prospect for the future application of hADSCs in treating Kawasaki patients.[31]
- SSc: SSc and sclerodermatous graft-versus-host disease are chronic autoimmune diseases characterized by extensive fibrosis of the skin and multiple organs, vascular abnormalities, and disturbances of innate and adaptive immune responses. MSCs function by regulating the immune system, inhibiting chemotaxis and osmosis of immune cells, reducing inflammatory, and pro-fibrotic mediators, ameliorating vascular abnormalities, and enhancing antioxidant defenses. Furthermore, MSCs inhibit fibroblast activation primarily by targeting the transforming growth factor-β (TGF-β) signaling pathway, providing a promising approach for treating autoimmune fibrotic skin diseases.[32] MSCs-IT (MSCs pretreated with interferon IFN-γ and tumor necrosis factor TNF-α) inhibit monocyte recruitment by reducing CCL2 production, thereby reducing macrophage accumulation in the skin and alleviating SSc.[33] Targeted delivery, low immunogenicity, strong repairability, good safety and stability, as well as easy storage and transportation, are some of the benefits of MSC exosomes. They perform anti-inflammatory, anti-fibrotic, and tissue repair functions by secreting soluble substances and extracellular vesicles,[34] thereby slowing down the SSc process.

4 | B CELL THERAPY

Autoantibodies secreted by B cells, such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) and their secreted cytokines, are involved in the occurrence and development of immune-mediated rheumatic diseases by mediating inflammatory responses. B cell depletion therapy, targeted B cell therapy, bispecific antibodies (such as Blinatumomab): this drug can combine both T cells and B cells to effectively kill B cells. Great progress has been made.

- RA: B cell depletion therapy (BCDT) is a cellular immunotherapy for RA, with a focus on targeting DN2B cells being promoted for specific therapies.

BCDT is beneficial in the treatment of RA; however, it is nonspecific, depleting all $CD20^+$ B cells, including pathological B cells and protective B cells, that are critical for tissue homeostasis and infection control. Wing et al. discovered that DN2B cells are the main precursors of synovial plasma cells, with these cells being notably enriched in the circulation and synovium of RA patients. As precursors to pathogenic B cells in RA, DN2B cells may become a potential target for more specific B cell depletion therapies in the future.[35] Although B cell depletion therapy is highly effective in some autoimmune diseases, individual responses to treatment vary widely. A prospective analysis of the B cell receptor (BCR) library in 28 RA patients indicated that BCR depletion was not associated with early clinical response. However, the refilling of BCR was associated with a significant decrease in disease activity between 6 and 12 months. This suggests that the refilling of the unmutated BCR, possibly from the original B cells, is linked to the remission of the disease.[36] Another study comparing healthy people with RA patients revealed that all three memory B cell subsets (pre-switch, post-switch, and double-negative) in RA patients were activated, as demonstrated by enhanced CD95 and Ki-67 expression. Treatment with the IL-6R blocker TCZ and the TNF-α blocker Adalimumab can significantly reduce the expression of these activation markers and slow down the RA process.[37]

- AS: B cells alleviate AS disease mainly by producing autoantibodies, secreting cytokines, and interacting with other immune cells, such as T cells.

B cells are involved in mediating AS inflammation through humoral and cellular immune mechanisms. These cells can act as antigen-presenting cells and promote inflammation by interacting with T cells. Over the past 20 years, research on B cell intervention in AS inflammation mechanisms has fluctuated globally. China, the United States, and Germany are leading the way in this field and need to strengthen international exchanges and cooperation. Current research focuses on the humoral immune mechanism of B cells through the production of autoantibodies and secretion of cytokines, as well as the cellular immune mechanism mediated jointly with Th cells and $CD8^+$ T cells. Future research may focus on B cells as novel therapeutic targets for AS, particularly through the selective use of drugs capable of binding membrane glucocorticoid receptors.[38]

- SLE: B cell depletion therapy remains a novel cell therapy that has received much attention and demonstrated clinical efficacy, boosting B cell tolerance through the use of cytokines. In terms of drug therapy, several drugs have been approved for the treatment of SLE. Among them, telitacicept, which acts on the B cell pathway, has also contributed to improving symptoms. However, its safety and adverse effects still need further evaluation.

B cell depletion therapy is a common immunotherapy approach for SLE in which the central and peripheral tolerance mechanisms of B cells are altered, leading to the formation of autoreactive B cells. B cell depletion is generally better tolerated compared to T cell depletion, and deep B cell depletion can be induced by a single infusion of therapeutic T cells. Currently, monoclonal antibodies against B cell activators (BAFFs), such as belimumab, have exhibited clinical efficacy.[9] Regarding B cell tolerance, another study revealed that BND cells are reduced in SLE patients, indicating a reduced level of tolerance in these cells. Moreover, IL-4, as an important cytokine, can drive pathological changes in BND cells. IL-4 reverses the tolerance of B cells through the STAT6 signaling pathway by promoting BCR cycling to the cell surface.[39]

The U.S. Food and Drug Administration has approved belimumab, voclosporin, and anifromulab for the treatment of SLE or LN. In addition to a single drug treatment, a combination treatment strategy is recommended. For example, the combined use of rituximab and belimumab, along with the application of bispecific monoclonal antibodies and proteasome inhibitors.[40] Regarding new agents, a prospective single-arm cohort study included 37 patients with active SLE who were monitored at 1, 3, 6, 9, and 12 months following the initiation of telitacicept, a dual inhibitor of the B cell pathway. The investigation revealed that in patients treated with telitacicept, the rate of SRI-4 reached 44.7% by the sixth month. Fatigue improved in 58.3% of patients assessed by the FACIT Fatigue Score. While most adverse events were minor, there was one case of severe hypogammaglobulinemia, one case of psychosis with suicidal behavior, and one case of B cell lymphoma. Telitacicept was demonstrated to significantly improve clinical and laboratory disease activity and reduce fatigue in patients with SLE. The overall safety is good, yet further research is required.[41]

- SSc: The treatment of SSc is more inclined toward B cell targeted therapy, with identified targets including CD20, CD19, BAFF, and proteasome.

SSc is a complex autoimmune disease characterized by fibrosis of the skin and internal organs, vascular dysfunction, and immune dysregulation. In SSc, B cells are involved in the production of autoantibodies that can lead to fibrosis and vascular disease. These cells can also activate other immune cells by producing cytokines and other immunomodulators. Current treatments focusing on B cells include the following:

B cell targeted therapy: Several molecular targets of B cells have been identified, including CD20, CD19, BAFF, and the proteasome.

Cd20-targeted therapy: Rituximab, a monoclonal antibody that selectively depletes B cells, has exhibited promising results in patients with SSc, improving skin thickness and cardiopulmonary severity.

Cd19-targeted therapy: MEDI-551 (inebilizumab) is a humanized anti-CD19 monoclonal antibody with encouraging safety and tolerability in patients with SSc.

BAFF inhibition: BAFF plays a key role in regulating the development, survival, and function of B cells. Belimumab is a human monoclonal antibody against BAFF that has demonstrated efficacy in SLE patients.[42]

5 | NATURAL KILLER (NK) CELL THERAPY

NK cells are part of the immune system and have anti-tumor and infection-fighting abilities. The therapeutic potential of NK cells is being investigated in various rheumatic diseases. However, there are limited studies on NK cells, mainly involving common rheumatic diseases such as RA and SLE. The treatment method is to inhibit the differentiation of Treg cells and produce the cytokine TGF-β to interfere with autoimmunity and achieve therapeutic effects.

- RA: In RA, a high proportion of $CD38^+$ NK cells and a low proportion of $CD38^+$ NK-like T cells inhibit Treg cell differentiation by stimulating the mTOR signaling pathway in $CD4^+$ T cells, thus disrupting immune tolerance.[43] $CD56^+$ NK cells can potentially exacerbate the pathogenesis of disease by producing pro-inflammatory cytokines such as IFN-γ and TNF-α, while IL-15-activated NK cells can kill autologous osteoblasts and dissolve osteoclasts through surface molecules such as LFA-1, DNAM-1, and TRAIL, thereby reducing bone damage.[44]
- SLE: Horwitz et al. investigated the therapeutic potential of nanoparticles (NPs) loaded with immunomodulatory cytokines

to treat a mouse model of SLE. Studies have indicated that NPs loaded with IL-2 and TGF-β can induce the expansion of NK cells, which inhibit the autoimmune response in SLE mouse models by producing TGF-β. Furthermore, the depletion of NK cells can exacerbate kidney disease in mice and reduce the number of circulating $CD4^+$ and $CD8^+$ Treg cells.[45]

The data in the figure were obtained from ClinicalTrials.gov, the Medical Research Registration and Filing Information system, and relevant papers.

6 | DISCUSSION

According to Figure 1, the immune cells are primarily stem cells and T cells, followed by B cells, with limited research on NK cells. Exovesicles play an important role in stem cell therapy for treating SLE, RA, SS, and other rheumatic immune diseases due to their advantages of targeted delivery, low immunogenicity, strong repairability, high safety and stability, and easy storage and transportation. Genetically engineered or cell-engineered CART cells combined with B cell depletion therapy provide a novel approach to treating various diseases. Concurrently, cytokines are essential in the treatment of RA, SAA, and SSc. Other therapies that target cytokines, such as IL-27 and IL-2, as well as molecular targets, such as CD19 and CD20, play a key role in regulating immune responses and disease treatment. As shown in Figure 2, according to the relevant literature review searched by PubMed database and research websites

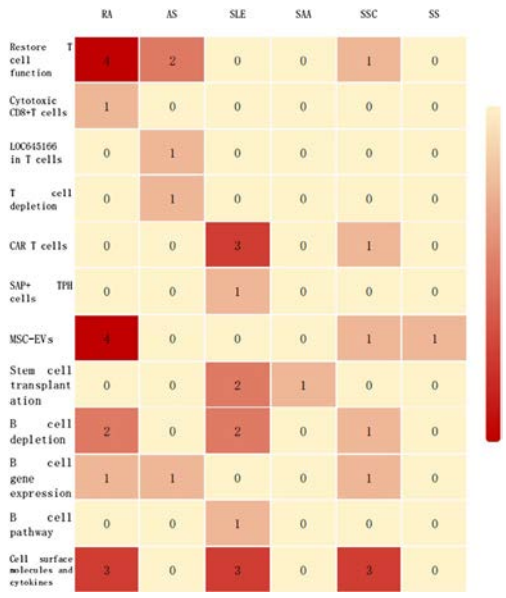

FIGURE 1 Heatmap of cellular immunotherapy discussed in this article. The numbers in the table indicate the number of mentions, and the color from light to dark corresponds to the number of mentions of the treatment in this article from least to most frequent.

such as ClinicalTrials.gov, most of the application cases of cellular immunotherapy in rheumatic immune diseases are still in the research and clinical trial stage, and more clinical studies of precise targeted therapy are needed to further study its feasibility and more clinical studies to evaluate its efficacy and safety. The development of individualized treatment strategies based on a patient's genetic background, disease phenotype, and immune status will enhance treatment effectiveness and reduce adverse reactions. Economy and accessibility: With technological advances and mass production, the cost of cellular immunotherapy may decrease, thereby improving its accessibility. It is imperative to address the challenge of balancing the high cost of innovative treatments with widespread access to ensure that all patients have access to effective treatments. Future research directions: new cell types and therapeutic strategies should be explored, such as the application of NK cell therapy and gene editing technology in immune-mediated rheumatic diseases. It is recommended to investigate the impact of immune microenvironments, such as the gut microbiota, on Immune-mediated rheumatic diseases and their potential as potential therapeutic targets.

Fudan university school of public health health economics professor Hu Shanlian in the resistance for our country chemotherapy with advanced non-small cell lung cancer patients economics evaluation said: according to studies, compared with traditional chemotherapy, the resistance can make each patient subsequent treatment costs save about 59%, at the same time can save adverse reactions during treatment average management cost of 98%. In addition, the good safety profile and disease prognosis of Beliumumab can effectively reduce the hospitalization rate by 46%, helping hospitals to save medical resources. In China, aurelizumab has been included in the national medical insurance directory in 2021, and inelizzumab has also entered the medical insurance in 2023. This helps to improve the accessibility of drugs and reduce the financial burden of patients, thus allowing more patients to benefit from this treatment. This reduces the burden of these therapies on patients, families and society.

However, the references in this paper mainly focus on the mechanism of immune cells in the disease, rather than the side effects of treatment, clinical risks of these therapies remain. For example, cytokine release syndrome, increased tumor risk, and unknowable immune risk. Potential risks and benefits should be carefully evaluated and monitored in clinical trials. We summarized the adverse

	SSC	SLE	SS	RA	ANCA
贝利尤单抗		IV			
奥瑞珠单抗*	IV				
伊奈利珠单抗*	III				
干细胞	II	II		I	II
CAR-T #	II	II			
IMPT-514#	II	II			
KYV-101*		II			
Targeted CarT(Bio-Gene)*		II			
CABA-201*		II			
Rapcabtageneautoleucel*					
Tanalumab		III	I		
ALX-0061 IL-6R单抗				I	
CAR-NK*	伦理中	II	伦理中	伦理中	伦理中

注：*为靶向CD19药剂，#为靶向CD20药剂

FIGURE 2 Cellular immune drugs at the clinical trial stage.

untoward effect	therapy method	Reference numbers
Development of anti-drug antibodies, and B cell remodeling	Rituximab	3
Inflammatory arthritis caused by autoreactive T cells, showing an increase in the characteristics of both Th 17 and transitional Th 1 / Th 17 cells	CD8+ T cell	9
Other common anticancer drugs are similar, and have the development of methotrexate resistance	Methotrexate, MTX	14
anaphylaxis	TNF-α inhibitor	14
Mild fatigue, nausea, and transient arterial hypertension	Antibody against TRBV 9	20
Brief fever, rejection of the receptor, causes the tumor	Stem cell transplantation	22
Severe hypogammaglobulinemia, confusion associated with suicidal behavior, B cell lymphoma, injection site reaction with local itching or pain	Telitacicept	25
Infusion-related gastrointestinal symptoms	B-cell-targeted therapy	27
Cytokine release syndrome (CRS) and immune-related effector cell neurotoxicity syndrome (ICANS)	CAR-T cell	24
Cytokine release syndrome (CRS) and neurotoxicity	CAR-T cell	30
Hypertension, diarrhea, and liver injury	Fostamatinib	32
Injection site reactions, fatigue, flu-like symptoms, digestive system problems, and headaches	IL-2LD	41
Transient hypotension and a dry cough	CD19.CAR-T cell	42

FIGURE 3 Summary of the adverse reactions included in the reference article.

reactions mentioned in the referred literature, as shown in Figure 3, hoping that it can provide a reference for clinical treatment.

In addition, these therapies have achieved good results, but the study has limited clinical samples, the severity of patients, the insufficient observation time, which may interfere with the bias of clinical experts on these effects. Larger, real-world and longer studies are needed.

7 | CONCLUSION

The utilization of immune cells in the treatment of rheumatic immune diseases has made great progress, offering a better choice for treating refractory rheumatic immune diseases. In summary, the application potential of NK cells needs to be further explored, while the relatively mature extracellular vesicle technology of MSCs can give more consideration to target specificity. Moreover, the joint action between cytokines, B cells, and T cells is the development direction of clinical application. In the future, further clinical and basic studies are needed to determine the efficacy of cellular immunotherapy in controlling disease activity and improving patient outcomes in the long term in patients with rheumatic immune diseases.

AUTHOR CONTRIBUTIONS

Wan Wei: Write and revise the article; Liu Yidi: writing, statistics, data sorting; Gao Jie: Collect the cases; Yan Sijia: Literature summary; Zhao Dongbao: Guided the revision of the article.

FUNDING INFORMATION

Campus level Basic Project General Incubation Project of Naval Medical University (No. 2023MS024). The National Natural Science Foundation of China (NSFC Project, Nos. 82171754 and 81971484).

CONFLICT OF INTEREST STATEMENT

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.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Wan W, Liu Y, Gao J, Yan S, Zhao D. Recent advances in cellular immunotherapy for immune-mediated rheumatic diseases. *Int J Rheum Dis*. 2025;28:e15385. doi:10.1111/1756-185X.15385

International Journal of Rheumatic Diseases

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LETTER TO THE EDITOR

Case Series: Serum CXCL9 and sTNF-RII Levels as Useful Disease Activity Markers in Patients With COVID-19-Associated Multisystem Inflammatory Syndrome in Children

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Received: 30 June 2024 | **Revised:** 9 December 2024 | **Accepted:** 14 December 2024

Funding: This work was supported by Medical and Biological Laboratories Co., Ltd, Japan.

Dear Editor,

Multisystem inflammatory syndrome in children (MIS-C) is a life-threatening condition that often occurs several weeks after severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) exposure. MIS-C is characterized by systemic hyperinflammation with multiorgan failure. Therefore, appropriate immunosuppressive treatment is required to prevent the development of severe conditions in MIS-C [1]. The symptoms of MIS-C are similar to those of Kawasaki disease (KD) which is common in Japan, and differentiating MIS-C from KD can be challenging. Laboratory findings, including decreased platelets and increased C-reactive protein (CRP), D-dimer, troponin, ferritin, and IL-6 levels, have been reported to be diagnostic and disease severity markers. However, these markers are nonspecific and their association with MIS-C pathogenesis is indirect. In immunological analysis, dendritic cells and activated $CD8^+$ T cells play a crucial role in patients with MIS-C [2]. Additionally, the expansion of T-cell receptor (TCR) $V\beta21.3^+CD4^+$ and $CD8^+$ T lymphocytes have been observed in patients with MIS-C [3, 4]. MIS-C is also hyper-cytokine condition, and serum chemokine (C-X-C motif) ligand 9 (CXCL9) levels are elevated in patients with MIS-C[5]. In Japan, we previously reported the usefulness of serum cytokine analysis, including CXCL9, for differentiating between MIS-C and KD-mimicking diseases [5]. Another study demonstrated that serum levels of soluble tumor necrosis factor receptor II (sTNF-RII), which is one of the TNF-α receptors and correlates well with TNF-α production, are elevated in severe patients with severe KD compared to those with milder cases [6]. Furthermore, serum sTNF-RII levels have been indicated useful marker for disease activity in patients with KD[7]. Infliximab, a mouse–human IgG1 chimeric anti-TNF monoclonal antibody, has been demonstrated to efficacy in the treatment of KD patients [7]. In the context of MIS-C, the blockade of TNF-α has also been reported to be efficacious [8, 9], with TNF-α playing an important role in the development of a severe form of MIS-C. These findings suggest that CXCL9 and sTNF-RII may be potential disease activity markers. However, the clinical usefulness of these inflammatory proteins for assessing disease severity remains unclear. This report presents cases of MIS-C in Japan and evaluates the cytokine features associated with disease activity in these patients.

MIS-C cases were diagnosed based on the criteria established by the World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC). The disease severity was assessed using MIS-C severity score (MSS), defined as a 5-point ordinal scale ranging from 0 to 4, with higher scores indicating increased severity [10]. Serum levels of CXCL9 and sTNF-RII were measured using enzyme-linked immune sorbent assay (CXCL6, sTNF-RII, and IL-6; R&D Systems Inc., Minneapolis, MN, USA). Statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA).

Correlations are expressed using Spearman's rank correlation coefficients. Statistical significance was set at $p < 0.05$.

Three patients with MIS-C are described in this report. Patient characteristics, including initial laboratory findings and MSS, are presented in Table 1. Patient 1 was a 5-year-old Japanese boy who presented with fever, bilateral conjunctival hyperemia, mildly red lips, bilateral cervical lymphadenopathy, abdominal pain, frequent vomiting, diarrhea, and redness around the BCG scar in the upper left arm for 5 days. Abdominal ultrasound revealed mild splenomegaly and a minor amount of ascites. Echocardiography results were normal and exhaustive viral PCR and culture tests were negative. The patient had COVID-19 26 days prior to admission, and a SARS-Cov-2 antibody test was positive on the day of hospitalization. The patient's clinical symptoms rapidly improved after intravenous high-dose immunoglobulin therapy (IVIg) (2 g/kg) administration on Day 5. On the 14th day of follow-up, there were no symptom flare-ups, and blood tests including serum CRP, ferritin, and platelet counts were within normal limits (Figure S1A). Patient 2 was a 9-year-old boy who presented with fever, conjunctival hyperemia, abdominal pain, diarrhea, strawberry tongue, bilateral cervical lymphadenopathy, eyelid and lower extremity rashes, erythema, and indurated edema of the palms and soles. Peripheral cyanosis and coldness were noted, and the systolic blood pressure was 74 mmHg, requiring intravenous administration of extracellular fluids. Echocardiography revealed mild tricuspid regurgitation and trivial mitral regurgitation with an ejection fraction of 62%. The patient's medical history included autism spectrum disorder and COVID-19 infection 1 month prior to presentation. The patient presented with severe symptoms, including hypotension and hyponatremia, and was treated with IVIg (2 g/kg) and a high dose of prednisolone (PSL) (2 mg/kg/day) in the intensive care unit on Day 7. The patient's symptoms gradually improved, and inflammatory markers such as CRP and ferritin also showed improvement after treatment. Echocardiographic findings were unremarkable, and the patient was discharged on Day 14 (Figure S1B). Patient 3 was an otherwise healthy 7-year-old girl who presented with fever, conjunctival hyperemia, and swelling on the left side of her neck. Echocardiography results were normal. IVIg (2 g/kg) and oral aspirin were initiated on Day 6. The patient's symptoms gradually improved from Day 10 onward (Figure S1C). The patient tested positive for SARS-Cov-2 antibody.

We sequentially measured serum cytokine and chemokine levels in these patients of varying disease severity, using blood samples collected before and after treatment (Table 1, Figure S1). The timing of blood sampling differed among the three cases as it was determined based on the treating physician's clinical judgment; however, in all cases, blood samples had been collected repeatedly from each patient both before and after treatment. The peak serum concentrations of cytokines and chemokines in the respective cases were as follows: In Patient 1, the serum CXCL9 level was 7829 pg/mL, serum sTNF-RII level was 16 055 pg/mL, and serum IL-6 level was 60 pg/mL on Day 6. In Patient 2, serum CXCL9 level was 30 155 pg/mL, serum sTNF-RII level was 39 999 pg/mL, and serum IL-6 level was 421 pg/mL on Day 7. For Patient 3, the serum CXCL9 level was 9257 pg/mL, serum sTNF-RII level was 28 201 pg/mL, and serum IL-6 level was 102 pg/mL on Day 8. Serum CXCL9 and sTNF-RII levels in Patient 2, who had the most severe symptoms, were higher than those in the other patients. In addition, we analyzed cytokine and chemokine

TABLE 1 | Characteristics of patients diagnosed with MIS-C.

	Patient 1	Patient 2	Patient 3
Age (year)	5	9	7
Sex	male	male	female
Clinical symptoms			
Fever	+	+	+
Rash	+	+	−
Cervical lymphadenopathy	+	+	+
Conjunctival injection	+	+	+
Strawberry tongue	−	−	+
Coronary disorder	−	−	−
Cough	+	−	−
Abdominal pain	+	−	+
Vomit and/or diarrhea	+	+	−
Hypovolemic shock	−	+	−
Others	−	−	+
Laboratory findings			
WBC ($10^3/\mu L$)	3.4	14.5	5.2
Plt ($10^9/L$)	6.8	10.8	11.9
AST (IU/L)	54	269	38
ALT (IU/L)	37	82	48
LDH (U/L)	420	675	222
CRP (mg/dL)	9.12	23.25	6.19
Ferritin (ng/mL)	1700.7	29058.2	1939.7
CXCL9 (pg/mL)	7829	30 155	9257
sTNF-RII (pg/mL)	16 055	39 999	28 201
IL-6 (pg/mL)	60	421	102
Treatment			
IVIG	+	+	+
Prednisolone	−	+	−
Plasma exchange	−	−	−
Infliximab	−	−	−
Disease severity			
MSS	1	2	1

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; CXCL9, Chemokine (C-X-C motif) ligand 9; IL-6, Interleukin-6; IVIg, intravenous immunoglobulin; LDH, lactate dehydrogenase; MSS, MIS-C severity score; Plt, platelet; sTNF-RII, soluble tumor necrosis factor receptor II; WBC, white blood cell.

kinetics in these patients. In all three cases, serum levels of CXCL9 and sTNF-RII were elevated in the active phase, when symptoms were most severe, and decreased, respectively, in the inactive phase, when symptoms had completely resolved after anti-inflammatory treatment (Figure 1A,B).

Next, we assessed the correlation between cytokine levels and laboratory data concerning disease activity in patients with MIS-C. Serum CXCL9 levels were significantly correlated with serum ferritin ($p = 0.0029$, $\rho = 0.7972$), aspartate aminotransferase (AST) ($p = 0.0016$, $\rho = 0.7989$), lactate dehydrogenase (LDH) ($p = 0.0016$, $\rho = 0.7989$), and platelet counts ($p = 0.0029$, $\rho = -0.7088$) in patients with MIS-C (Figure 1C–F). Moreover, serum sTNF-RII levels were significantly correlated with serum ferritin ($p = 0.0101$, $\rho = 0.6978$), AST ($p = 0.0006$, $\rho = 0.8172$), LDH ($p = 0.0006$, $\rho = 0.8172$), and platelet counts ($p = 0.0142$, $\rho = -0.5907$) in patients with MIS-C (Figure 1G–J).

Patients with MIS-C often develop severe conditions, including myocardial involvement and shock. Therefore, an accurate diagnosis and immunosuppressive treatment are required during the early stages. Serum biomarkers, including CXCL9, have been previously reported to be useful for diagnosing MIS-C [5, 11]. A previous report showed that serum CXCL9 and sTNF-RII levels in MIS-C patients are significantly higher than those in KD patients [5]. In this report, we demonstrate that serum CXCL9 and sTNF-RII levels are elevated in patients with MIS-C in the active phase and are higher in the severe case, indicating that these could represent novel disease activity markers.

CXCL9 is an inflammatory protein that is mainly induced by IFN-γ. This chemokine has been reported to be elevated in macrophage activation syndrome (MAS), a secondary hemophagocytic lymphohistiocytosis (HLH) complicating systemic juvenile idiopathic arthritis (s-JIA), and rheumatic diseases [12]. MAS is also one of the severe cytokine release syndromes requiring adequate immunosuppressive treatment. Previous reports have indicated that serum CXCL9 levels are associated with disease severity in patients with s-JIA complicating MAS [13, 14]. Anti-IFN-γ monoclonal antibodies have been reported to be clinically effective in patients with MAS [15]. Therefore, IFN-γ plays a pivotal role in MAS, and the amount of IFN-γ production may be associated with a more severe condition in MAS. In this study, we showed that serum CXCL9 levels increased during the active phase and decreased after immunosuppressive treatment.

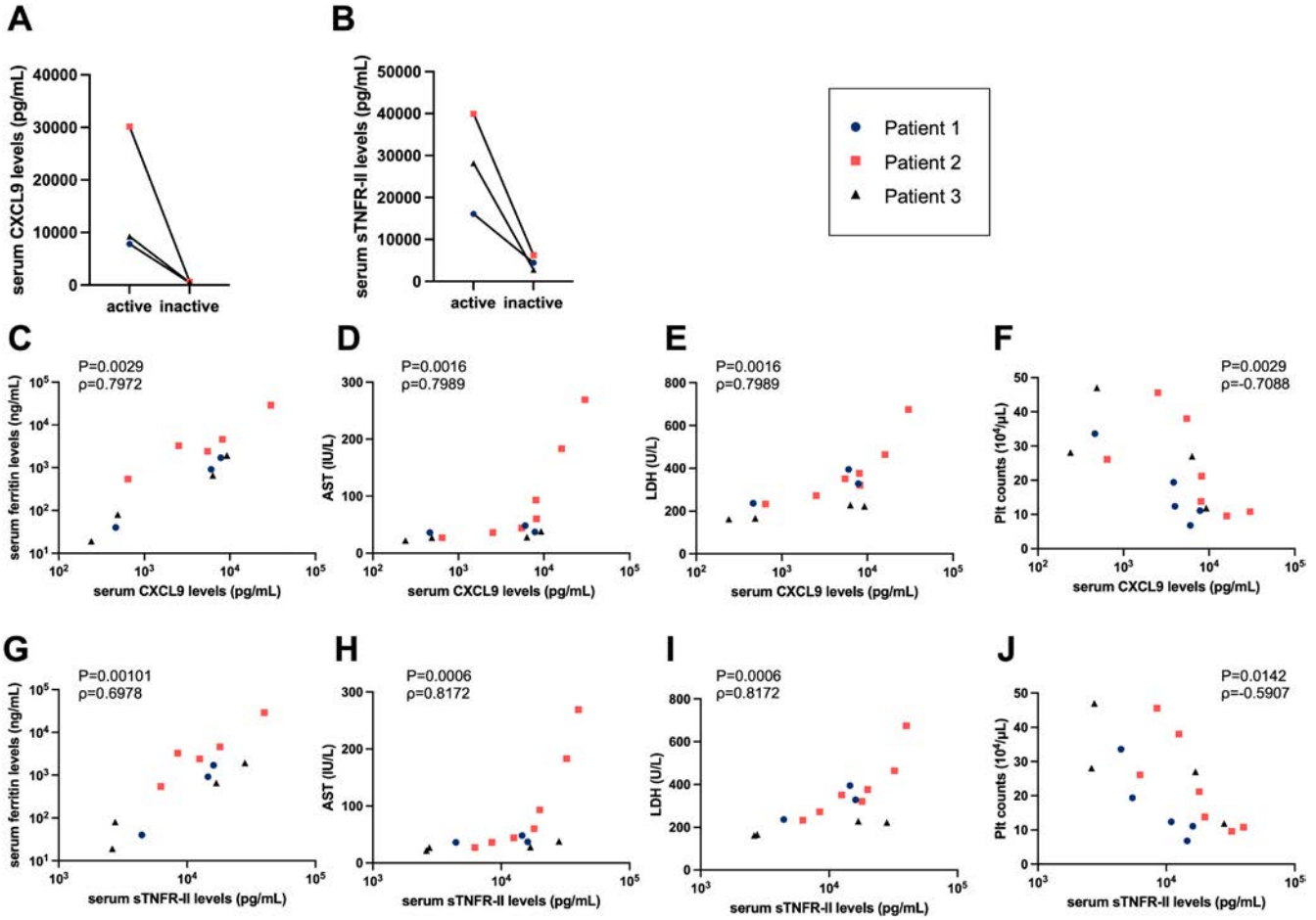

FIGURE 1 | Serum CXCL9 and sTNF-RII levels in three patients with MIS-C. (A) Kinetics of serum CXCL9 levels and (B) sTNF-RII levels in patients with MIS-C. Correlation between serum CXCL9 and (C) ferritin, (D) AST, (E) LDH levels, and (F) platelet counts in three MIS-C cases. Correlation between serum sTNF-RII and (G) ferritin, (H) AST, (I) LDH levels, and (J) platelet counts in three MIS-C cases. Active, active phase; inactive, inactive phase; CXCL9: Chemokine (C-X-C motif) ligand 9; sTNF-RII, soluble tumor necrosis factor receptor II; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; Plt, platelets.

Furthermore, Patient 2, with a severe condition, has much higher serum CXCL9 levels than the others. Another report indicated that inflammatory protein signatures in the blood are similar to MIS-C, MAS, and thrombotic microangiopathy, and INF-γ responses are dysregulated in patients with MIS-C [16]. Therefore, CXCL9 may be a potential marker of disease severity in MIS-C patients. However, our findings cannot be generalized because of the limited sample size. Further large-scale analyses are required to elucidate the pathophysiology of MIS-C.

sTNF-RII is one of the soluble forms of TNF-α receptor, and these levels are known to be correlated with TNF-α overproduction. In previous reports, plasma TNF-α levels in MIS-C are elevated compared to those of febrile patients [11]. Another report demonstrated that sTNF-RII levels in MIS-C are significantly higher than those in KD cases; however, the sensitivity and specificity for sTNF-RII are not high according to receiver operating characteristic analysis [5]. In this study, serum sTNF-RII levels were also elevated in MIS-C patients during the active phase and were higher in Patient 2 with severe MIS-C than in those with other conditions. Some cases of MIS-C have been reported to require treatment with anti-TNF blocker [8, 9]. In the cases reported here, additional anti-inflammatory treatment such as anti-TNF blocker was not needed, but high levels of sTNF-RII correlated with TNF-α production could represent disease severity marker in patients with MIS-C.

In conclusion, serum CXCL9 and sTNF-RII levels in patients with severe MIS-C are extremely high during the active phase. These inflammatory proteins may serve as potential markers of disease activity in patients with MIS-C.

Author Contributions

S.H. and M.M. contributed to the design of the study, collected data, and drafted the manuscript. T.T., Y.I., and S.M. contributed to the acquisition of data. S.K., A.S., and S.M. contributed to analyzing serum cytokine and chemokine. Y.N. supervised and revised the final version of the manuscript. All authors agreed to the final version of the manuscript.

Acknowledgments

This work was supported by grants from Medical and Biological Laboratories Co. Ltd., Japan.

Consent

Consent for publication of this report was obtained from the parents of the patients.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

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LETTER TO THE EDITOR

Case Report: A Rare Manifestation of IgG4-Related Disease: Isolated Hepatocellular Injury

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Received: 29 October 2024 | **Revised:** 6 December 2024 | **Accepted:** 15 December 2024

Funding: The authors received no specific funding for this work.

Keywords: glucocorticoids | hepatocellular | immunoglobulin G4-related disease | immunosuppressive agents | liver injury

Dear Editor,

Immunoglobulin G4-related disease (IgG4-RD) is an immune-mediated condition characterized by elevated serum IgG4 levels and the infiltration of IgG4-positive plasma cells in affected tissues [1]. When it affects the liver, IgG4-RD typically presents as obstructive jaundice and cholestatic liver injury, often associated with type 1 autoimmune pancreatitis and IgG4-related sclerosing cholangitis [2]. However, isolated hepatocellular injury due to IgG4-RD is extremely rare and has been poorly documented in the literature.

We present a case of IgG4-related hepatitis in a 34-year-old male who experienced recurrent liver enzyme elevations over 9 years, ultimately achieving remission with glucocorticoids and azathioprine (Figure 1A). In March 2015, during a routine physical examination, his ALT levels peaked at 2000 U/L. The patient had a 10-year history of diabetes and a 3-year history of hypertension. He reported occasional alcohol consumption but denied any other significant medical issues. Initially, he was treated with hepatoprotective agents, including reduced glutathione and compound ammonium glycyrrhetate injections, which led to a gradual normalization of his ALT levels. However, in November 2018, he experienced a recurrence, with ALT levels rising to 1100 U/L, prompting further evaluation. After seeking medical attention, he was prescribed additional hepatoprotective medications. By January 2019, laboratory tests revealed elevated ALT (209 U/L) and AST (80 U/L) levels, along with significantly increased serum IgG (16.2 g/L) and IgG4 (5.48 g/L). Comprehensive serologic tests for other liver conditions returned negative results (Table 1). Additionally, to aid in the differential diagnosis, the patient underwent abdominal MRI, chest and abdominal CT scans, renal and prostate ultrasounds and a thyroid ultrasound. No abnormalities were observed in the pancreas, bile ducts, lungs, kidneys, retroperitoneal tissues or thyroid. A liver biopsy subsequently confirmed significant lymphocytic infiltration and the presence of IgG4-positive plasma cells, with histopathological analysis indicating fibrous connective tissue proliferation (Figure 1B). Immunohistochemical analysis revealed more than 10 IgG4-positive plasma cells per high-power field, with an IgG4-positive to IgG ratio exceeding 40% (Figure 1C,D).

Initially, the patient was prescribed prednisone at a daily dose of 30 mg, resulting in a rapid normalization of liver function. He was discharged with a plan to taper the prednisone dose gradually to 5 mg for maintenance. In November 2019, prednisone was discontinued. However, 5 months later, the patient experienced a recurrence, with ALT levels rising to 907 U/L. In May 2020, a new treatment regimen was initiated, combining corticosteroids and immunosuppressants. Methylprednisolone was introduced at a starting dose of 32 mg daily and gradually tapered to 4 mg for maintenance, alongside azathioprine at 50 mg daily. Regular monthly follow-ups showed sustained normalization of liver function tests. After 4 months of treatment, methylprednisolone was discontinued, and the patient was maintained on azathioprine alone, remaining asymptomatic with normal liver

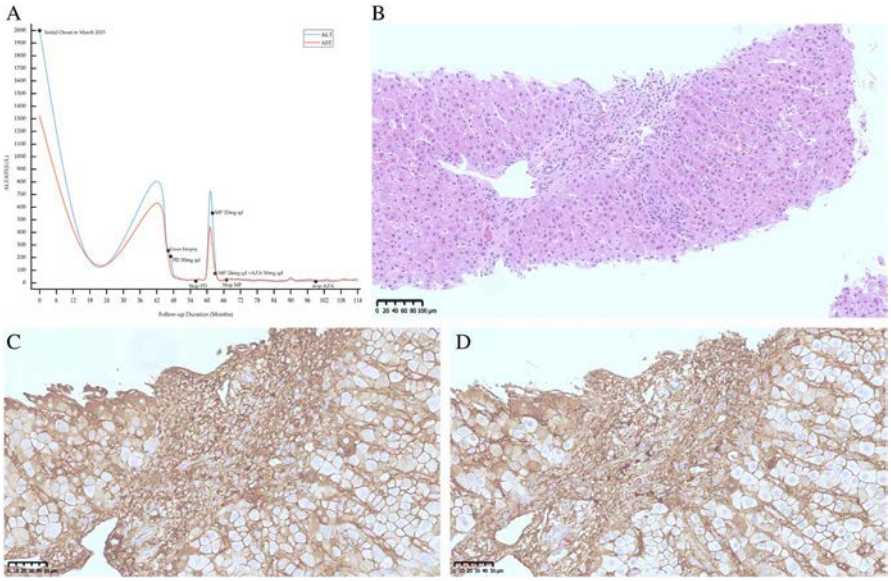

FIGURE 1 | (A) Clinical course of the patient. (B) Hematoxylin–eosin staining (200X) of liver biopsy sections reveals punctate necrosis of hepatocytes at the boundary plate of liver lobules, along with fibrous connective tissue proliferation and significant lymphocyte and plasma cell infiltration. (C, D) Immunohistochemical staining for IgG4 (400×) shows more than 10 IgG4-positive plasma cells per high-power field, with an IgG4-positive to IgG ratio exceeding 40%.

function tests. By June 2023, after 3 years of treatment, azathioprine was also discontinued. To date, we continue to monitor this patient, and there has been no recurrence of IgG4-related hepatitis in the past 4 years.

IgG4-RD is a complex immune-mediated condition, with approximately 60% of patients experiencing varying degrees of organ damage due to delayed diagnosis and associated fibrosis [3]. Clinicians often underestimate the impact of IgG4-RD on the liver, as evidenced by our patient, who presented with recurrent symptoms of liver failure. This case exemplifies IgG4-RD characterized by isolated primary hepatocellular injury, a manifestation that, to our knowledge, has not been reported previously.

Previous reports of IgG4-related autoimmune hepatitis can exhibit similar hepatic features (Table S1), such as lobular hepatitis and portal inflammation [4]. To differentiate between these conditions, we utilized the autoimmune hepatitis (AIH) scoring system developed by the International Autoimmune Hepatitis Group in 1999 [5], allowing us to exclude the diagnosis of IgG4-AIH. Early diagnosis and timely intervention are crucial for preventing irreversible inflammatory damage and fibrosis in IgG4-RD. Currently, corticosteroids are the cornerstone of initial treatment and have shown favorable effects in most cases with organ involvement [3]. Following this assessment, we promptly initiated glucocorticoid therapy as the first-line treatment for IgG4-RD. After the patient experienced a relapse upon discontinuation of medication, suggesting that glucocorticoid monotherapy may be insufficient for long-term disease control, we quickly implemented combination therapy with glucocorticoids and azathioprine, resulting in favorable outcomes. This finding aligns with the research by Dina Omar et al. [6], indicating that patients receiving a combination of glucocorticoids and immunosuppressants have higher remission rates compared to those treated with glucocorticoids alone. Emerging treatments, including rituximab, inebilizumab, and other biologic therapies, offer promising alternatives for refractory or relapsing IgG4-RD [7–9]. However, in this case, the patient responded favorably to corticosteroid therapy combined with immunosuppressants, so these novel options were not pursued. This experience suggests that combining corticosteroids with immunosuppressants may offer an effective treatment pathway for IgG4-related hepatitis, underscoring the importance of adjusting treatment promptly in response to relapse. For patients with varying disease types or severities, optimizing treatment strategies and exploring individualized approaches remain critical areas for future research.

The etiology and pathogenesis of IgG4-RD remain unclear. Studies suggest that chronic antigen-induced immune activation plays a central role. Circulating plasma cells and B cells present self-antigens to CD4+ cytotoxic T cells (CD4+ CTLs), which then activate CTLs in local tissues. These cells release cytotoxic molecules, such as perforin and granzyme, leading to tissue damage through apoptosis of target cells. CD4+ CTLs also promote fibrosis by secreting factors like TGF-β, IL-1β, and IFN-γ [10, 11]. Furthermore, IL-10 and IL-4 from follicular helper T cells may drive IgG4 class switching and B cell responses, causing elevated serum IgG4, IgG4+ plasma cell infiltration, and tissue fibrosis [12, 13]. The mechanism of IgG4-related hepatitis likely involves similar immune responses, but further research is needed due to the liver's unique immune tolerance environment.

In conclusion, this case highlights the importance for clinicians to carefully consider that isolated hepatocellular injury may be the sole manifestation of IgG4-related disease. Timely initiation of appropriate treatment is essential to prevent liver failure, cirrhosis, and potentially more severe outcomes.

TABLE 1 | Nine-year trends in liver function, IgG4 levels, and other laboratory findings in the patient.

Test indicator	2015. 03	2016. 05	2017. 05	2018. 11	2019. 02	2019. 03	2019. 11	2020. 04	2020. 05	2020. 06	2020. 11	2021. 01	2022. 01	2023. 06	2024. 09
ALT (U/L)	2000	104	36	1100	209	36	15	907	553	75	24	20	23	12	33
AST (U/L)	1320	165	43	847	80	36	25	588	239	78	27	26	21	21	17
ALP (U/L)	NA	135	62	NA	101	70	92	136	152	120	84	NA	107	NA	88
GGT (U/L)	NA	163	28	NA	65	37	18	88	137	66	45	NA	27	NA	15
TBIL (μmol/L)	NA	53.6	6.6	NA	19.4	11	7.2	NA	6.7	9.9	6.8	14.4	9.1	NA	10.1
DBIL (μmol/L)	NA	45.2	3.3	NA	17.6	5.9	3.2	NA	3.2	5.5	3.8	7.8	3.4	NA	3.1
IBIL (μmol/L)	NA	8.4	3.3	NA	1.8	5.1	4	NA	3.5	4.4	3	6.6	5.7	NA	7
TP (g/L)	NA	77.1	74.7	NA	72.5	76.4	74.8	NA	67.8	74.4	77.1	NA	83.2	NA	79.4
ALB (g/L)	NA	40.1	41.5	NA	38.5	42.5	41.4	NA	38.1	41.8	40.1	NA	47.1	NA	45.9
GLB (g/L)	NA	37	33.2	NA	34	33.9	33.4	NA	29.1	32.6	37—	NA	36.1	NA	33.5
Viral hepatitis	—	NA	—	NA	—	NA	—	NA	—	NA	—	NA	NA	NA	—
HIV	—	NA	—	NA	—	NA	—	NA	—	NA	—	NA	NA	NA	—
EB	—	NA	—	NA	—	NA	—	NA	—	NA	—	NA	NA	NA	—
IgG (g/L)	NA	NA	NA	NA	16.2	16.8	15.04	NA	16	15.3	16.8	NA	NA	NA	NA
IgG4 (g/L)	NA	NA	NA	NA	5.48	5.47	5.61	NA	4.67	5.39	5.61	NA	NA	NA	NA
IgG4/IgG (%)	NA	NA	NA	NA	33.83	32.56	37.3	NA	29.19	35.23	33.34	NA	NA	NA	NA
ANA/ASMA	—	NA	—	NA	—	NA	—	NA	—	NA	—	NA	NA	NA	—
AMA	—	NA	—	NA	—	NA	—	NA	—	NA	—	NA	NA	NA	—
Anti-SLA	—	NA	—	NA	—	NA	—	NA	—	NA	—	NA	NA	NA	—
Anti-LC1	—	NA	—	NA	—	NA	—	NA	—	NA	—	NA	NA	NA	—
Anti-LKM-1	—	NA	—	NA	—	NA	—	NA	—	NA	—	NA	NA	NA	—
Treatment	RGI+ CAGSI	RGT+ ISI	NA	RGI+ PPLI+ BT	PD	PD	Stop PD	RGT+ BT	PPLI+ RGI+ MIHI+ MP	MP+ AZA	Stop MP	AZA	AZA	Stop AZA	NA

Abbreviations: –, negative; A, Azathioprine; ALB, albumin; ALT, alanine transaminase; ALP, alkaline phosphatase; AMA, antimitochondrial antibody; ANA, antinuclear antibody; anti-LC1, anti-liver cytosol type 1; anti-SLA, anti-soluble liver antigen; anti-LKM-1, liver-kidney microsome type 1 antibodies; ASMA, anti-smooth muscle antibodies; AST, aspartate transaminase; BT, Bicyclol Tablets; CAGSI, Compound Ammonium Glycyrrhetate S Injection; DBIL, direct bilirubin; EB, Epstein–Barr virus; GGT, γ-glutamyl transpeptidase; GLB, globulin; HIV, human immunodeficiency virus; IBIL, indirect bilirubin; IgG, immunoglobulin G; IgG4, immunoglobulin G4; IgG4/IgG, immunoglobulin G4 to immunoglobulin G ratio; ISI, Inosine Injection; MIHI, Magnesium Isoglycyrrhizinate Injection; MP, Methylprednisolone; NA, not available; PD, Prednisone; RGI, reduced glutathione for injection; RGT, Reduced Glutathione Tablets; TBIL, total bilirubin; TP, total protein.

Author Contributions

Xinmei Yan: conceptualization, data collection, analysis, manuscript drafting, and literature review; contributed to the design and interpretation of the case report. **Junxing Li and Huyu Jiao:** data collection, methodology, and analysis; provided clinical insights, edited the manuscript, and validated the data. **Zhengang Zhang:** supervision, project administration, and manuscript review; oversaw case study interpretation and validation; approved the final manuscript submission.

Ethics Statement

This study was approved by the Ethics Committees of Tongji Hospital, Wuhan, China (TJ-IRB202410016).

Consent

The participant provided written informed consent for the use of their medical records for the purpose of research.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

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LETTER TO THE EDITOR

Case Report: Disseminated Nontuberculous Mycobacterial Infection in a VEXAS Syndrome Patient—Possible Association With Variant Allele Frequency

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Received: 9 October 2024 | **Revised:** 10 December 2024 | **Accepted:** 17 December 2024

Funding: The authors received no specific funding for this work.

1 | Introduction

VEXAS syndrome is an adult-onset autoinflammatory disorder characterized by myeloid-specific somatic mutations in the *UBA1* gene on the X chromosomes [1]. Recent large-scale studies have shown that infections account for the majority of deaths in these patients [2]. Particularly, intracellular pathogens, such as *Legionella pneumophila*, have been implicated in patients without immunosuppressant therapy or with low-dose glucocorticoid treatment (≤ 10 mg/day) [2]. This phenomenon is suspected to be linked to impaired ubiquitin-proteasome system (UPS) function due to E1 mutations [2]. We hypothesized that the variant allele frequency (VAF) affecting the UPS may be associated with increased susceptibility to infections in VEXAS syndrome. Herein, we report a case of VEXAS syndrome, where VAF was serially measured using digital polymerase chain reaction (QuantStudio Absolute Q Digital PCR system, Thermo Fisher Scientific) before the onset of disseminated nontuberculous mycobacterial (NTM) infection, an intracellular pathogen. Data analysis was performed with QuantStudio Absolute Q Digital PCR Software (Thermo Fisher Scientific).

2 | Case

A man in his 70s presented with fever, bilateral scleritis, and bilateral lung nodular opacities 4 years prior, which improved with oral prednisolone therapy. Three years prior, erythema of the auricular cartilage was observed, leading to a diagnosis of relapsing polychondritis. His symptoms improved with a combination of prednisolone and tocilizumab. Two years prior, a reduction in the prednisolone dose led to a recurrence of cutaneous rash and an increase in C-reactive protein levels, indicating relapse of relapsing polychondritis. Tocilizumab was replaced with infliximab and methotrexate [3], resulting in normalization of serum C-reactive protein levels and resolution of the skin rash. At this time, VEXAS syndrome was suspected due to the presence of relapsing polychondritis and macrocytic anemia (Table 1) [4]. Sequencing of the *UBA1* gene revealed a missense mutation at *UBA1* (c.122T>C p.Met41Thr), confirming a diagnosis of VEXAS syndrome [1]. Five months before admission, contrast-enhanced computed tomography revealed right pleural effusion and a low-density right kidney lesion (Figure 1A). The patient developed fever and had increased inflammatory marker levels (Table 1), consistent with VEXAS syndrome flare, and the prednisolone dosage was increased (1 mg/kg/day). Despite this, the symptoms persisted, prompting admission for further examination. Repeat contrast-enhanced computed tomography demonstrated encapsulated fluid around the prostate, increased non-enhanced kidney lesions, multiple pulmonary nodules, and increased pleural fluid (Figure 1B,C). Cultures of pleural and prostate fluid (Figure 1D) and blood cultures for acid-fast bacteria identified the presence of Mycobacterium intracellulare (MAC). Based on these findings, the patient was diagnosed with disseminated MAC infection and initiated on a regimen of azithromycin, ethambutol, rifampicin, and amikacin. Following treatment initiation, the patient developed left knee swelling

TABLE 1 | Summary of laboratory tests.

	At symptom onset	At diagnosis of VEXAS syndrome	At diagnosis of NTM	Normal range
Alb, g/dL	2.6	3.1	1.9	3.9–4.9
WBC, /μL	7,100	10,600	6,900	3,900–9,800
Hb, g/dL	10.4	11.5	8	13.4–17.9
MCV, fL	95	114	114	79–102
Plt, /μL	288,000	130,000	39,000	13,000–370,000
T-Bil, mg/dL	0.5	0.4	0.4	0.2–1.2
AST, U/L	37	23	28	8–40
ALT, U/L	52	33	36	8–40
BUN, mg/dL	8.7	17.1	15.1	8.0–20.0
Cre, mg/dL	0.83	0.91	0.84	0.6–1.1
CRP, mg/dL	14.5	4.1	20.6	0–0.5
ESR, mm	121	99	137	0–10

Abbreviations: Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood uria nitrogen; Cre, creatinine; CRP, c-reactive protein; ESR, erythrocyte sedimentation rate; Hb, hemoglobin; MCV, mean corpuscular volume; Plt, platelet; T-Bil, total bilirubin; WBC, white blood cell.

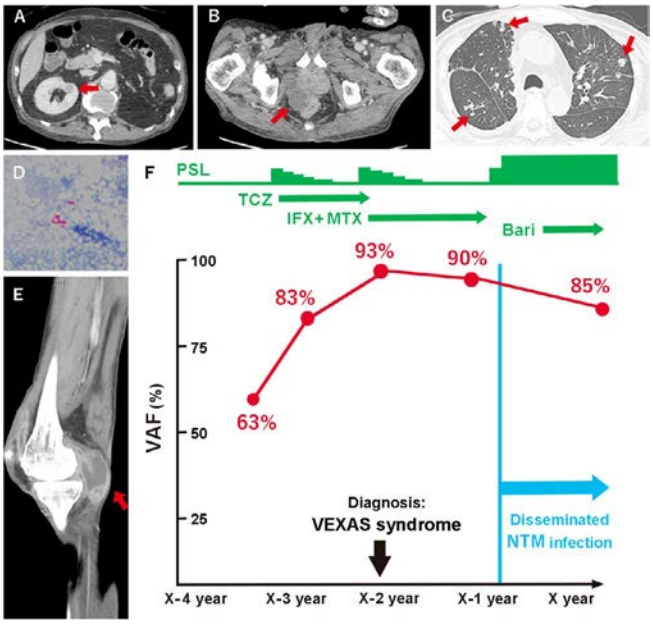

FIGURE 1 | (A) A low-density lesion in the right kidney on contrast-enhanced computed tomography (CT) (red arrow). (B) Encapsulated fluid around the prostate on contrast-enhanced CT (red arrow). (C) Right pleural effusion and multiple pulmonary nodules (red arrows) on chest CT. (D) Acid-fast bacilli in the periprostatic fluid on Ziehl-Neelsen staining (1000× magnification). (E) Left knee joint fluid on CT. (F) The clinical course of VEXAS syndrome and change in VAF. Bari, baricitinib; IFX, infliximab; MTX, methotrexate; NTM, nontuberculous mycobacterial; PSL, prednisolone; TCZ, tocilizumab; VAF, variant allele frequency.

(Figure 1E) and subcutaneous nodules on the forehead and forearm, all of which were positive for MAC. Prednisolone was continued at 1 mg/kg/day, with the dose doubled to counteract the metabolic interaction with rifampicin, to suppress VEXAS syndrome activity. One month after treatment initiation, blood cultures turned negative, and the patient continued oral antibiotic therapy with azithromycin, ethambutol, and rifampicin. Once the infection was under control, the patient was discharged with baricitinib (4 mg/day) added to the regimen to address resistance to multiple biologic therapies. VAF measurements from frozen whole blood samples, collected at key time points—before and after the VEXAS syndrome diagnosis and before and after the infection—revealed a progressive increase of 63%, 83%, and 93%, respectively (Figure 1F).

3 | Discussion

Disseminated MAC infection is rare and primarily affects immunosuppressed individuals, such as solid organ transplant recipients and those with human immunodeficiency virus [5]. However, a French cohort study reported that VEXAS syndrome can predispose to atypical infections, including *Legionella pneumophila* and *Pneumocystis jirovecii*, with 46% of such cases occurring in the absence of significant immunosuppressive factors [2]. They proposed that E1 mutations in VEXAS syndrome may impair the UPS, facilitating intracellular pathogen proliferation [2]. Notably, the cohort identified three cases of MAC infection, two of which were disseminated NTM infections in patients who had not undergone extensive treatment for VEXAS syndrome [2, 5]. Table 2 summarizes the cases reporting an association between VEXAS syndrome and NTM infection, including our case. Notably, aside from our report, none of the cases measured VAF at the onset of NTM infection. All reported cases involved men aged 60 years or older, with the *UBA1* (c.122T>C p.Met41Thr) mutation identified in three out of four cases. Two patients died due to the infection. Regarding treatment, glucocorticoids, biologics, and immunosuppressants were used; however, these regimens are also common in other autoimmune diseases, indicating that the level of immunosuppression in these cases was not unusually severe. Notably, a study by Czech et al. [6] reported four cases of VEXAS syndrome complicated by NTM infection,

TABLE 2 | Summary of characteristics of VEXAS syndrome with NTM infection in the literature.

	Age	Sex	UBA1 Mutation	Clinical features	Tansfusion Dependent	Treatment	VAF at cohort entry	VAF at NTM infection	Type of NTM	Outcome
Riescher S. Case 1 [5]	60s	Male	c.121A>Gp.Met41Val	Fever, rash, lung involvement, orbital edema	No	GCs, Anakinra	NR	NR	*M. avium*	Dead
Riescher S. Case 2 [5]	60s	Male	c.122T>Cp.Met41Thr	Fever, arthralgia, ocular inflammation, vasculitis	Yes	GCs, HCQ	NR	NR	*M. avium*	Dead
Kirino Y [8]	70s	Male	c.122T>Cp.Met41Thr	NR	Yes	GCs, TCZ	67.3%	NR	NR	Alive
Our Case	70s	Male	c.122T>Cp.Met41Thr	Fever, rash, lung involvement, arthritis, auricular chondritis, ocular inflammation	Yes	GCs, MTX, IFX	63.0%	93.0%	M. intracellulare	Alive

Abbreviations: GCs, glucocorticoids; HCQ, hydroxychloroquine; IFX, infliximab; *M. avium*, *Mycobacterium avium*; MTX, methotrexate; NR, not reported; TCZ, tocilizumab.

none of which involved prior immunosuppressant administration. VEXAS syndrome has been shown to increase cell death of monocytes and neutrophils in peripheral blood, suggesting that it may represent an acquired immune deficiency [7]. A possible explanation is that high VAF levels contribute to dysfunction of the UPS, potentially increasing susceptibility to atypical infections, particularly intracellular pathogens.

The association between tumor necrosis factor (TNF) antagonists and NTM infection has been previously reported. In a U.S. study consisting of 8418 patients treated with TNF inhibitors, 18 cases of NTM infection were identified; however, none were disseminated [9]. Similarly, a Korean study using a health insurance database evaluated the risk of NTM infection in 2278 rheumatoid arthritis patients receiving TNF inhibitors [10]. The study found no increased risk of NTM infection compared to patients using conventional synthetic disease-modifying antirheumatic drugs. These findings suggest that host factors, rather than medication, may play a more critical role in the development of disseminated NTM infection.

In our case, the patient's VAF gradually increased, which may have contributed to the eventual development of disseminated NTM infection. In a study by Maeda et al. [11], VAF was measured over time in four patients with VEXAS syndrome undergoing treatment with immunosuppressants, and the results were reported. The VAF did not change significantly 12–18 months after the start of measurements. Although detailed clinical symptoms and treatment courses were not provided in this report, in cases with recurrent relapses and persistently high disease activity, such as ours, the VAF may increase over time. Although studies have shown that a high VAF at diagnosis does not correlate with mortality [12], increasing VAF may indicate worsening UPS impairment and heightened susceptibility to intracellular pathogens [13]. Larger case series and in vitro studies are needed to confirm this association.

In our case, current treatments targeting cytokines, such as TNF-alpha, interleukin-6, and Janus kinase inhibitors, failed to prevent VAF progression. While these therapies improve inflammatory responses and symptoms, they do not suppress the clonal proliferation of mutated cells. Serial VAF measurements may provide a better reflection of disease activity and progression [14]. Therefore, therapies targeting clonal proliferation—such as hypomethylating or cytotoxic agents—may offer a more promising approach to reducing VAF levels [15].

4 | Conclusion

Increased VAF likely exacerbates UPS impairment, increasing susceptibility to intracellular pathogens like NTM. Cytokine-blocking therapies may not halt VAF progression, and serial VAF measurements could serve as a better indicator of disease activity and progression.

Author Contributions

H.O. provided the figures and wrote the manuscript. Y.S. and H.M. measured VAF using by digital PCR. S.S. and K.O. contributed to the

discussion and edited the manuscript. All authors have read and approved the final manuscript.

Ethics Statement

This study involves human participants, but case reports do not require the approval of our institutional board, hence this study was exempted. Informed consent was obtained from the patient.

Consent

Obtained.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data are available upon reasonable request to the corresponding author.

Patient and Public Involvement

Patients and/or the public were not involved in the design, or conduct, or reporting or dissemination plans of this research.

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LETTER TO THE EDITOR

Case Report: Muckle–Wells Syndrome Mimicking Juvenile Idiopathic Arthritis

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Received: 22 October 2024 | **Revised:** 12 December 2024 | **Accepted:** 18 December 2024

Funding: This work was supported by the Tianjin Key Medical Discipline (Specialty) Construction Project (TJYXZDXK-040A) and the outstanding health professional selection and training program in Tianjin (TJSJMYXYC-D2-031).

Dear Editor,

Muckle–Wells syndrome (MWS) is one of three clinical forms of cryopyrin-associated periodic syndrome (CAPS), a rare hereditary intermittent fever syndrome due to genetic mutations in the NOD-like receptor 3 (NLRP3) gene [1]. CAPS-associated NLRP3 mutations gain function, leading to a hyperactive cryopyrin inflammasome, increased myeloid cell-derived pro-inflammatory cytokine release, and systemic and tissue inflammation, leading to disease symptoms [2]. Hence, early and correct diagnosis of MWS, a monogenic autoinflammatory disease, is sometimes difficult.

Juvenile idiopathic arthritis (JIA) refers to a clinically heterogeneous group of patients with arthritis of unknown cause, with more than six weeks duration that starts before age 16 years old [3]. Juvenile psoriatic arthritis (JPA), enteritis-related arthritis (ERA), oligoarthritis, rheumatoid factor (RF)–negative polyarthritis, RF–positive polyarthritis, and undifferentiated arthritis are the seven types recognized under the current classification of JIA [4]. The diagnosis of JIA requires the exclusion of other diseases and long-term observation, so it is also challenging to diagnose correctly.

This article reports a case of MWS mimicking JIA.

A 10-year-old boy presented with arthralgia for seven months and rashes for 20 days before his first hospital admission. The patient experienced needle-pricked pain on the inner side of the right knee joint seven months before admission, which lasted for one day and self-relieved. Subsequently, there was pain on the right foot's inner side, swelling, and difficulty walking. Later, intermittent pain in the right knee and foot could be relieved by administering Votalin (diclofenac sodium enteric-coated tablets, a type of NSAIDs). Twenty days before admission, there was no apparent cause for the appearance of red patches and papules, which gradually spread from the waist and abdomen to the whole body. After 2–3 days of topical medication, they disappeared. Once the drug was stopped, the rash reappeared. The skin was slightly protruding, without itching, accompanied by local pain, and the rash recurred.

Upon further investigation of the patient's history, the patient experienced recurrent fever starting one year after birth, with fever intervals ranging from 1 to 3 months, presenting as a moderate fever. Parents reported that the patient developed a fever after catching a cold and could self-heal. Intermittent conjunctival congestion was also diagnosed as allergic conjunctivitis. In the past three years, there have been intermittent abdominal pain and diarrhea. The symptoms are not severe, and they resolve spontaneously. There is no correlation between abdominal pain and fever. The abnormal items in the examination results after admission are shown in Figure 1.

Other organ functions and routine examinations not mentioned above showed no significant abnormalities. The discharge diagnosis was arthritis, and no special treatment was given. No abnormalities were found in ophthalmic examinations related to immune system diseases, and no abnormalities were found in neurological examinations.

Zhi-Bo Yang and Zi-Bo Zhang are the co-first authors of the article.

Height	137cm(*p10-p25*)
Weight	37kg(*p25-p50*)
HLA-B27	positive
White blood cell count	8.31×10^9-19.14×10^9/L
C-reactive protein	19.5-98.9mg/L(normal range:0-8mg/L)
Procalatonin	0.20ng/ml(normal:0-0.05ng/ml)
IL-6	10.30pg/ml (normal 0-7 pg/ml)
IgA concentration	4.38 g/L (normal 0.53–2.04 g/L)
IgE concentration	1197IU/ml (normal 0–100 IU/ml)
Antinuclear antibodies	1:80
Fecal calprotectin	178.79ug/g (average <50 ug/g)
MRI	a mild effusion of the right knee articular cavity; medullary cavities of the talus, the calcaneus, tarsal bones, and from the second to the fourth metatarsal bones have multiple small patches of long T2 signal; a mild effusion in both hip joints.

FIGURE 1 | Abnormal items.

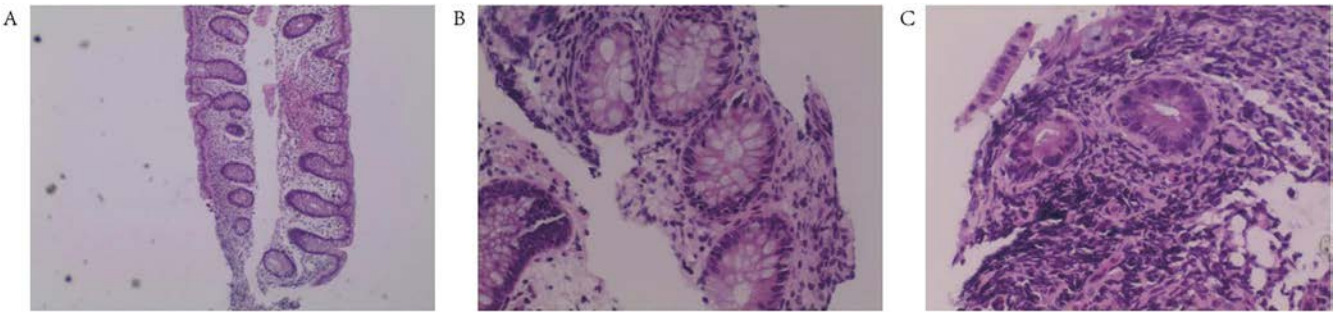

FIGURE 2 | A colonoscopy revealed inflammatory changes in the colon, with prominent changes in the ascending colon and ileocecal region.

After six months of discharge, the patient was readmitted due to abdominal pain, diarrhea, and fever for eight days. Due to an abdominal ultrasound showing thickening of the intestinal wall (about 5 mm), gastroscopy and gene sequencing (whole exome sequencing) were performed after hospitalization. The gastroscopy results are shown in Figure 2. When the patient was readmitted, they were 140 cm tall (*p*10-*p*25) and 34.5 kg (*p*25-*p*50) in weight, with a height lower than that of their peers—1SD. The bone age is slightly delayed by one year.

Based on recurrent joint pain, rash, and abdominal pain, as well as HLA-B27 positivity and persistently high levels of inflammatory markers, we considered juvenile idiopathic arthritis or inflammatory bowel disease–related arthritis. However, the child's colonoscopy did not show any obvious signs of inflammatory bowel disease, and the diagnosis is questionable. After six weeks, the results of whole exome sequencing in the patient overturned our diagnosis. Gene sequencing showed a heterozygous mutation *c.2582A>G: p.Y861C* in the NLRP3 gene Exon7, with transcript number NM:004895.5. Further testing showed that neither the father nor the mother had the mutation, indicating that the patient's heterozygous mutation is wild type. It can be inferred that the patient's onset is genetically related, but the mutation does not exist in the existing database, and future protein-level experiments are still needed for verification. The patient reported no hearing loss and was recommended to seek medical attention at an otolaryngology department. A comprehensive examination indicated moderate hearing loss, high-frequency decline type, and a diagnosis of sensorineural hearing losswas made. Blood interleukin-1β was 118 pg/mL (reference value ≤ 5 pg/mL). Reviewing the medical history, MWS can explain every symptom of the patient, such as intermittent fever, joint pain, arthritis, rash, conjunctivitis, growth retardation, and hearing impairment. Finally, we corrected the diagnosis to MWS. Because the interleukin-1 antagonist is not listed in mainland China, the child is currently receiving thalidomide and tranilast capsules treatment; the patient is currently 13 years old and has no secondary sexual characteristics. After completing relevant examinations, such as growth hormone stimulation tests, supplementary treatment such as growth hormone supplementation may be considered. In addition, studies have shown that neutrophils may be a new therapeutic target for CAPS. Although complete depletion of neutrophils may expose CAPS patients to a high risk of infection, blocking neutrophil aggregation may be an effective strategy to reduce skin inflammation in CAPS patients [5].

Autosomal dominant mutations in the nucleotide-binding cause CAPS and leucine-rich repeat receptor family pyrin

domain containing 3 (NLRP3) gene that encodes cryopyrin, the protein responsible for assembly of the inflammasome and regulation of the proinflammatory cytokine interleukin-1 beta (IL-1β) [6]. Defective cryopyrin leads to spontaneous and increased production of IL-1β, resulting in clinical symptoms such as urticarial rash, fever, and arthralgia without exogenous stimuli [7]. According to clinical symptoms, CAPS can be divided into three different subtypes: FCAS-CAPS1, Muckle–Wells syndrome (MWS-CAPS2), and chronic infant neurological cutaneous and joint syndrome (CINCA-CAPS3)/ multiple neonate inflammation disease (NOMID) [8]. MWS is an intermediate type of CAPS in terms of severity. Patients described as having MWS present with intermittent fever, widespread urticarial papules, and plaques lasting 1–2 days with associated abdominal pain, myalgias, arthralgias, conjunctivitis, episcleritis, and sensorineural hearing loss [9, 10]. However, their disease is typically not triggered by exposure to cold [10]. Hearing loss and the development of amyloidosis are also reported in many MWS patients [10]. There are also case reports that indicate CAPS should be considered when dermatologists encounter an untypical recalcitrant urticaria-like rash because the correct diagnosis and timely treatment can dramatically improve the quality of life of these patients [11, 12]. Literature reports indicate that CAPS can overlay tumor necrosis factor type 1A receptor-associated periodic syndrome (TRAPS) [13]. Therefore, the diagnosis of this disease is challenging.

In combination with the case we reported, our patient has almost all the typical symptoms of MWS except for renal amyloidosis, which had not yet occurred. The results of genetic testing can also provide sufficient evidence for diagnosis. But why did we misdiagnose the case as a JIA? It is a question worth pondering and exploring. Firstly, HLA-B27 positivity is also interference, and it is rare to see HLA-B27 described in the case reports of MWS. Secondly, representative symptoms seen in patients with MWS, such as rash, fever, arthralgias, and conjunctivitis, can also be seen in patients with JIA. Inflammatory markers remain high throughout the condition, and the predominantly neutrophilic blood profile can all indicate a disease diagnosis. An article suggests that neutrophils are major IL-1β producers in CAPS and thus might play a key role in CAPS pathology [5]. It is inconsistent with the characteristics of JIA that only intermittent administration of small doses of NSAIDs can provide effective relief, which leads us to consider autoinflammatory diseases. After adalimumab, the patient developed symptoms of fever accompanied by systemic pain, which could be resolved by itself after 1–2 days, and the treatment did not reduce the patient's periodic fever, joint pain, and high inflammation, so it was not used for subsequent treatment.

The most important thing is to perform whole-exome gene sequencing actively when clinical indicators with suggestive significance appear and fever occurs, instead of controlling symptoms after administering biological agents, as gene testing is crucial for accurately diagnosing CAPS.

As we all know, JIA is an exclusionary diagnosis. All arthritis of unknown origin starting at less than 16 years old and lasting six weeks can be diagnosed as JIA after excluding other diseases. Because, in children, as in adults, several different diseases exist that all cause chronic arthritis, the terms juvenile idiopathic arthritis and juvenile idiopathic arthritis onset forms will probably become outdated as more is learned about each disease [14]. JIA is a synonym for a group of diseases. JIA can easily be misdiagnosed when any condition with similar symptoms is difficult to diagnose. The finding is that anti-interleukin-1treatment can also be effective for JIA; some JIA patients have a pronounced, complete response to interleukin-1 blockade (much the same as in cryopyrin-associated autoinflammatory syndromes) [14].

The similarities of MWS and JIA:

1. Symptoms: rash, fever, arthralgias, conjunctivitis and diarrhea;
2. Laboratory tests: high inflammation markers (CRP), negative pathogenic test results;
3. Their pathogenesis is linked to genetic mutations.

The differences between A and B:

1. MWS has some specific target organ damage, renal amyloidosis, and sensorineural hearing impairment, while JIA is not present or rare. Neurogenic hearing loss in childhood may lack complaints of hearing loss, and relevant examinations need to be improved.
2. An apparent single gene mutation causes MWS.
3. The findings suggest that common IL-1 genes in the IL-1 gene cluster are not susceptibility genes for patients with ERA-JIA [15]. However, CAPS is caused by autosomal dominant mutations in the nucleotide-binding and leucine-rich repeat receptor family pyrin domain-containing 3 (NLRP3) gene, which encodes cryopyrin, the protein responsible for assembly of the inflammasome and regulation of the proinflammatory cytokine IL-1β[6].

In short, clinical doctors, especially pediatricians, should understand inflammatory diseases sufficiently to diagnose them early, avoid misdiagnosis, and improve patients' quality of life. The clinical manifestations of MWS in this case are relatively mild, but it is unknown whether neuropathic deafness and kidney damage will affect the child with age. Whether current treatment can improve the prognosis also requires long-term observation. For children with suspected autoimmune or autoinflammatory diseases whose diagnosis is unclear, whose onset age is young, in whom multiple target organ damage occurs, and whose treatment is not smooth, it is necessary to improve whole-exome gene testing actively. The child did not show any neurological symptoms, but we should pay attention to this during follow-up. Literature reports indicate that half of CAPS patients will experience neurological symptoms [16]. Increased awareness of this rare but treatable autoinflammatory syndrome among clinicians would probably decrease morbidity and mortality.

Author Contributions

Z.B.Y. and Z.B.Z. organized the materials and wrote this manuscript. H.L. and B.Z. searched the literature and reviewed the manuscript. J.H.

provided ideas for the diagnosis and treatment of this patient's illness. L.L. provided ideas and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgments

We thank all participants and staff of this paper, and also thank the physicians at the Tianjin Children's Hospital affiliated with Tianjin University.

Ethics Statement

This is to certify that the content of this paper meets the requirements of medical ethics after review by the ethics committee.

Consent

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data generated or analyzed during this study are included in this article.

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ORIGINAL ARTICLE

Association of Biomarkers Such as CA, TP, TES and ALP With Osteoarthritis Risk: A Mendelian Randomized Study

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Received: 29 August 2024 | **Revised:** 24 November 2024 | **Accepted:** 19 December 2024

Funding: This study was supported by Doctoral Startup Fund of Xiangyang Central Hospital. Scientific Research Fund of Xiangyang Central Hospital (2022YB06).

ABSTRACT

Objective: Osteoarthritis is a common joint disease caused by a variety of risk factors, and it has been found that many biochemical markers are abnormal in peripheral blood and urine of patients with OA. The aim of this study was to elucidate the causal relationship between biomarkers associated with these processes and OA using Mendelian randomization (MR) analysis.

Method: The inverse variance weighted (IVW) approach to MR was primarily used to explore causal associations between exposures and outcomes using publicly available genetic variants from large genome-wide association studies (GWAS). That is, single nucleotide polymorphisms (SNPs) associated with 35 human blood and urine markers (363 228 healthy participants) were used as exposure, and osteoarthritis, hip osteoarthritis, and knee osteoarthritis were used as outcome variables, with the aim of exploring the causal relationship between 35 human blood and urine markers and osteoarthritis. MR-Egger, weighted median (WM), and simple and weighted models were used as complementary methods to IVW to assess the reliability of causality. Steiger's test was used to confirm whether the causal relationship between exposure and outcome was biased by reverse causality. Sensitivity analyses used Cochran's Q statistic and funnel plots to detect heterogeneity, and the MR-Egger intercept test and leave-one-out to assess horizontal multidimensionality.

Results: Our MR analysis study identified the protective effects of CA, TP, ALB, SHBG, and VITD on OA and the pathogenic effects of TES, ALP, GGT, CRP, and CHOL on OA. It suggests that the above 10 hematological and urinary markers have the potential to be important indicators for the clinical diagnosis of OA as well as for the assessment of therapeutic efficacy and disease progression.

Conclusion: This MR analysis reinforces the importance of biomarkers in the diagnosis and prediction of OA. Future studies should further investigate the mechanisms of these biomarkers and their potential as therapeutic targets for OA.

1 | Introduction

Osteoarthritis(OA) is a common joint disease that occurs mostly in the middle-aged and elderly population, which is mainly manifested as joint popping, stiffness, and limited joint movement in the body after prolonged activity, usually accompanied by pain, synovial inflammation, cartilage degeneration, and synovial ooze, which seriously affects the quality of life of patients [1, 2]. The main affected joints include hand joints, knee joints, and hip joints [3]. It is currently believed that OA is caused by a combination of risk factors, susceptibility factors include obesity, advanced age, genetics, sex hormones, bone density, excessive exercise, and the presence of other diseases, etc.; mechanical factors include poor joint alignment, increased biomechanical loading of the joints, etc., and low-grade systemic inflammation, which has

been proposed recently [4]. As the world's population ages, this chronic, disabling disease poses a significant challenge to healthcare systems and socioeconomics. It is estimated that symptomatic OA occurs in approximately 10%–12% of the adult population and 7% of the elderly population will experience activity limitation due to OA. In people aged 65 years older, the risk of motor disability (e.g., need for assistance in walking or climbing stairs) attributable to knee osteoarthritis (KOA) alone is higher than the risk of any other condition [5]. Although strong evidence exists for the involvement of genetic factors in OA, the evidence for KOA is inconsistent. A meta-analysis showed that none of the 199 published candidate OA genes were significantly associated with KOA [6, 7]. Whereas obesity and inflammation are clear risk factors for the development and progression of OA, the exact mechanism of action remains unclear. By elucidating the genetic susceptibility to specific biomarkers associated with OA, it may be possible to develop more accurate predictive models to identify individuals at high risk of developing OA, which may ultimately lead to early intervention and improved outcomes.

Currently, there is no uniform standard for OA diagnosis and prognosis. Clinical manifestations and imaging examinations lack the ability to make an effective early diagnosis, and are not sensitive for early diagnosis and treatment of patients. In the past decade, considerable efforts have been made to find reliable biomarkers for the early detection of OA, and screening for appropriate biomarkers is essential for the early diagnosis of OA. Peripheral blood and urine biomarkers are commonly used for the diagnosis and assessment of chronic disease conditions [8]. Blood biomarkers are considered a viable option, and urine is also a convenient and suitable substance for disease diagnosis or predictive testing because subtle changes in urine accumulate in the blood and are not subject to homeostatic mechanisms, and have the potential to be applied in the prevention and early treatment of KOA [9, 10]. Many biochemical markers have been found to be abnormal in the peripheral blood and urine of patients [11–14], and some biomarkers can be measured before changes occur in imaging, allowing better prediction of OA at an early stage. Urinary type II collagen carboxy-terminal 3/4 fragment, serum cartilage oligomeric matrix protein and serum hyaluronic acid can predict early KOA and are useful for prognostic prediction [15]. Xin et al. [16] found that in the urine of patients with KOA, the concentrations of Zn^{2+} and Ca^{2+} were higher than those of healthy subjects. Deberg et al. [17] observed that compared with healthy controls, the concentration of Coll2-1 was significantly elevated in sera of patients with OA. Coll2-1 concentration was significantly elevated and found that Coll2-1NO2 was significantly correlated with the levels of inflammatory factors such as C-reactive protein, whereas there was no correlation between Coll2-1 and C-reactive protein levels, suggesting that the nitration of Coll 2-1 is directly related to synovial inflammation.

As biomarker changes can be driven by a variety of pathophysiological drivers and can also be affected by pharmacological interventions, resulting in inaccurate measurements; however, current studies have found that changes in tissue metabolism prior to structural alterations can produce a variety of biomarkers that have diagnostic and prognostic value in predicting the progression of OA, and even have potential as therapeutic targets. Traditional observational studies cannot systematically assess the relationship between two diseases because of possible biases such as confounding factors or reverse causality. Mendelian randomization (MR), an analytical method that uses genetic diversity as a factor in the exposure assessment tool, allows for rational inferences about the etiology of the disease while minimizing confounding variables and measurement errors associated with reverse causality bias [18]. In this study, we comprehensively analyzed the correlation between blood and urine biomarkers and OA, and evaluated the feasibility of biomarker application using Mendelian randomization analysis to provide more reliable and valuable early diagnostic markers for the development of new targeted drugs for patients with OA, and to formulate effective treatment strategies.

2 | Method and Materials

2.1 | Study Design

This study used MR to examine the causal relationship between 35 biomarkers and OA. To ensure the validity of the MR analyses, three criteria had to be met: (1) the genetic variants had to be significantly associated with exposure; (2) the genetic variants in the exposure instrumental variables (IVs) must not be

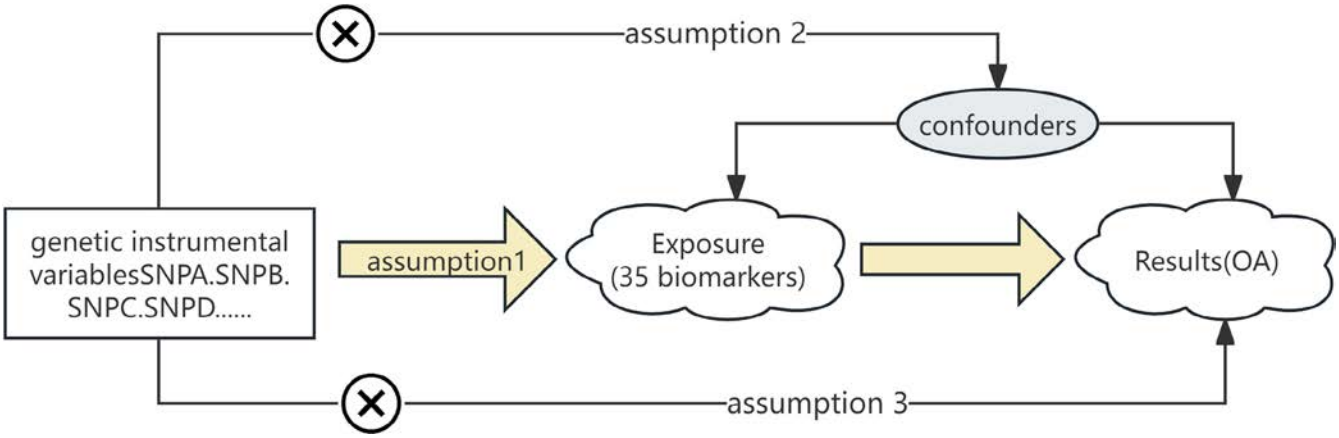

FIGURE 1 | Flowchart. OA, osteoarthritis; SNP, single nucleotide polymorphism.

associated with confounders; and (3) there could be no horizontal pleiotropy, with the genetic variants influencing the outcome only through the risk factor. The outline of the study design is shown in Figure 1.

2.2 | Data Source and Study Population in MR

Our study was conducted in a cohort of 363 228 individuals, and loci associated with 35 biomarkers were examined and fine-mapped. A total of 35 human blood and urine markers Genome-wide association studies (GWAS) data from a published study included 8 cardiovascular biomarkers, 7 liver biomarkers, 12 kidney biomarkers, 3 bone and joint biomarkers, 2 biomarkers of diabetes mellitus markers (glucose and HbA1C), and 3 hormonal biomarkers [8]. For the outcome, we used osteoarthritis (self-reported) and osteoarthritis (Firth correction) for OA, and in turn selected osteoarthritis of the hip or knee, hip osteoarthritis, and knee osteoarthritis were used as outcome indicators for different sites of OA. GWAS of OA were derived from a meta-analysis including individuals of European ancestry, and the osteoarthritis (self-reported) dataset incorporated 12 658 cases and 50 898 controls, and the osteoarthritis (Firth correction) dataset included 407 746 individuals. In the dataset of OA lesions, the number of cases of osteoarthritis of the hip or knee, hip osteoarthritis, and knee osteoarthritis lesions were 6586, 15 704, and 646, respectively, and the control group consisted of 26 384, 378 169, and 11 009 cases, respectively. The study followed the principles of the Declaration of Helsinki, the data used were obtained from public databases, and informed consent was obtained from the study participants; this aspect of the study did not require ethics committee approval.

2.3 | Instrument Selection for MR

We used a genome-wide significance threshold set at ≤ 5E—08 to identify SNPs closely associated with biomarkers as potential IVs [19]. To ensure that no false-positive IVs were present in the MR analysis, interference from linkage disequilibrium (LD) needed to be excluded (aggregation parameter set to $R^2 < 0.001$, window size = 10 000 kb). Due to the low significance threshold, weak instrumental variables with an F-statistic < 10 will be excluded as an indication that the study is strong enough to avoid bias [20]. Second, inconsistent allelic SNPs and palindromic SNPs were excluded after comparing the selected SNPs with the resultant dataset. Finally, these carefully selected SNPs served as the final genetic IVs for the MR analysis.

2.4 | MR Analysis

In the present MR study, we mainly used the inverse variance weighting (IVW) method to estimate the causal association between 35 hematological and urinary markers and OA, which incorporates the Wald estimator of SNPs and has higher statistical efficacy compared with other methods [21]. For MR sensitivity analyses, we assessed heterogeneity using Cochrane's Q test to ensure robustness and validity of the results [22]. When $p < 0.05$ indicated significant heterogeneity, to mitigate the bias introduced by heterogeneous IVs, we used a random-effects IVW approach to determine the causal relationship between exposure and outcome. To assess horizontal pleiotropy, we used the MR-Polyvalent Residual Sums and Outliers method (MR-PRESSO) to detect and identify potential horizontal pleiotropy ($p < 0.05$ indicates the presence of horizontal pleiotropy) [23]. We used MR-Egger, weighted median (WM), and simple and weighted models as complementary methods to IVW. Finally, we performed 'leave-one-out' analyses to test for the presence of SNPs with biased effects to assess whether the results were significantly influenced by a single SNP. In addition, based on previous studies, we used the Steiger test [24] to confirm whether the causal relationship between exposure and outcome was biased by reverse causality. In this study, all analyses were statistically analyzed using R software 4.3.1, and MR analyses were performed using the 'TwoSampleMR' and 'MR-PRESSO' software packages.

3 | Result

3.1 | MR Analysis of 35 Hematological and Urinary Markers and OA Risk

We first analyzed the causal relationship between 35 biomarkers and Osteoarthritis using MR analysis. The results showed that five biomarkers were found to be significantly associated with OA by the IVW method, including calcium(CA), testosterone(TES), total protein (TP), alkaline phosphatase (ALP) and gamma-glutamyltransferase (GGT), at the criterion of $p < 0.05$ (Figure 2, Figure 3). Among them, CA (OR = 0.91, 95% CI 0.83–0.99, $p = 0.03$) showed a significant negative correlation with OA, suggesting that CA has a protective role in OA progression, similarly, TP (OR = 0.90, 95% CI 0.83–0.99, $p = 0.02$) also showed a protective effect against OA. While TES (OR = 1.12, 95% CI 1.03–1.37, $p = 0.02$), ALP (OR = 1.05, 95% CI 1.01–1.10, $p = 0.03$) and GGT (OR = 1.06, 95% CI 1.01–1.10, $p = 0.02$) showed positive correlation, suggesting that TP, ALP, and GGT would make OA disease risk to be increased.

3.2 | MR Analysis of 35 Hematological and Urinary Markers and Risk of Knee and Hip OA

The results of IVW method showed a potential causal relationship between seven biomarkers and knee and hip OA, among which, ALB was a protective factor for Osteoarthritis of the hip or knee (OR = 0.79, 95% CI 0.69–0.89, $p = 2e\text{-}04$), CA could reduce the risk of Hip osteoarthritis disease risk (OR = 0.86, 95% CI 0.79–0.94, $p = 8e\text{-}04$), sex hormone-binding globulin (SHBG) (OR = 0.68, 95% CI 0.52–0.88, $p = 0.004$) and VITD (OR = 0.32, 95% CI 0.103–0.997, $p = 0.049$) were all associated with knee osteoarthritis incidence were negatively correlated. In addition, GGT was positively correlated with Osteoarthritis of the hip or knee (OR = 1.10, 95% CI 1.00–1.20, $p = 0.044$), implying that elevated GGT might be a predictor of the risk of osteoarthritis of the hip or knee; similarly, C-reactive protein (CRP) (OR = 1.142, 95% CI 1.06–1.24, $p < 0.01$) and CHOL (OR = 1.34, 95% CI 1.04–1.74, $p = 0.03$) were risk factors for knee osteoarthritis (Figures 4 and 5).

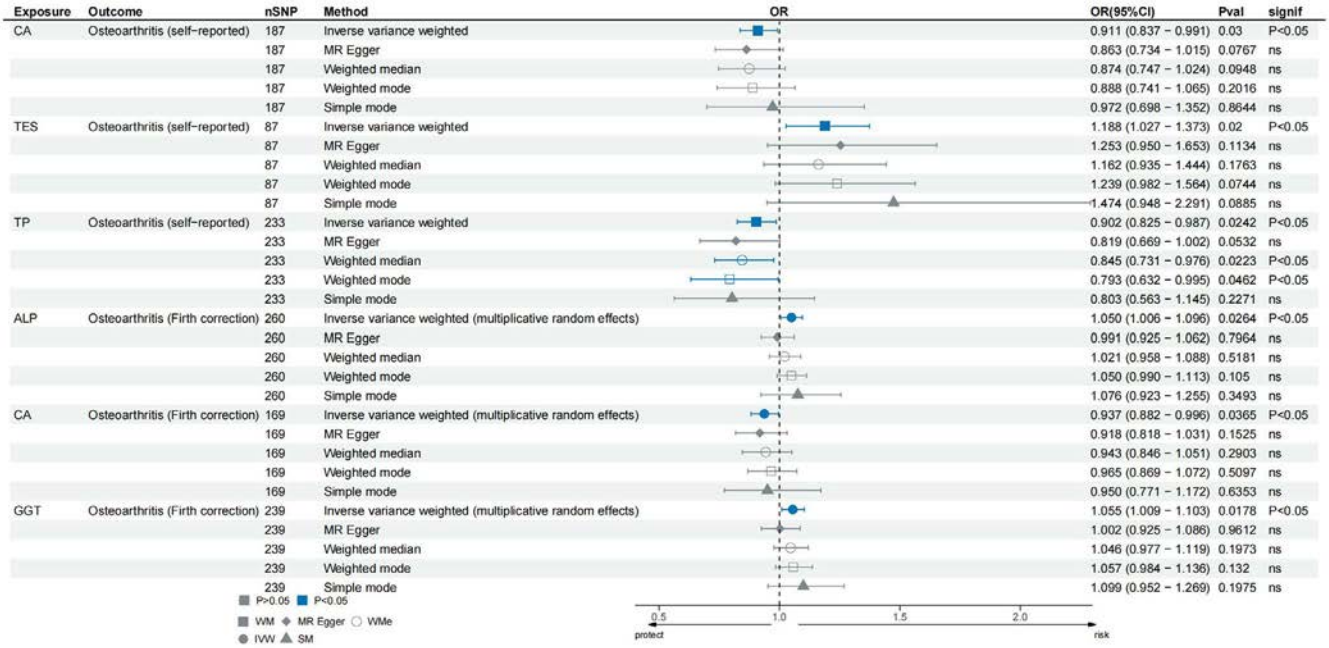

FIGURE 2 | Associations between genetically predicted hematuria markers and OA examined by 5 MR methods. ALP, Alkaline phosphatase; CA, Calcium; CI, confidence interval; GGT, gamma-glutamyltransferase; TES, testosterone; TP, Total Protein.

3.3 | Sensitive Analysis

The MR-Steiger orientation test results showed 'TRUE' orientation for all SNPs, indicating no reverse causality between biomarkers and OA (Supporting Information S1). Funnel plot and leave-one-out plot results indicated that the MR analysis results remained robust (Supporting Information S2 and S3). Cochrane Q and MR-Egger intercept test results are detailed in Supporting Information S4.

4 | Discussion

To our knowledge, this is the first study to comprehensively investigate the causal relationship between blood and urine markers and osteoarthritis by MR analysis based on publicly available genetic data. Our study identified the protective effects of CA, TP, ALB, SHBG, and VITD on OA and the pathogenic effects of TES, ALP, GGT, CRP, and CHOL on OA. Our findings suggest that the above 10 hematological and urinary markers have the potential to become important indicators for the clinical diagnosis of OA as well as for the assessment of treatment efficacy and disease progression.

When the articular cartilage is stimulated by external pressure load, the intracellular Ca2+ concentration is significantly increased, and the Ca^{2+}-Ca M (calmodulin) pathway is abnormal, which may cause abnormalities in the differentiation and formation of chondrocytes, chondrocyte adhesion, articular cartilage repair, and the responsiveness to the pressure load stimulus, thereby promoting the occurrence and progression of OA [25]. Mc Alindon et al. [26] found that low calcium diet and low serum calcium also affected the progression of KOA. found that a low-calcium diet and low serum calcium also affect the progression of KOA. Although Hunter et al. did not find a causal relationship between serum calcium levels and OA in Caucasian women [27]. However, a cross-sectional study of 2855 Chinese subjects showed that serum calcium concentration was negatively associated with the risk of KOA diagnosed by imaging [28]. This was confirmed by the results of our analyses, and therefore, we believe that timely attention to changes in mineral status is clinically significant for the prevention and screening of OA.

The relationship between SHBG, TES, and OA remains controversial due to conflicting results from several studies. Reduced levels of SHBG, a protein carrier with potential biological anti-inflammatory functions, modulate testosterone concentrations, and it is widely accepted that SHBG reduces the bioavailability and activity of TES [29] and predicts the risk of Type 2 diabetes and cardiovascular disease [30]. There are no firm conclusions regarding the association between TES deficiency and osteoarthritis risk, with one study from the Melbourne Co-operative Cohort failing to find an association between TES, SHBG, and OA risk in men [31]; while another study found that in women, low serum testosterone levels were associated with increased knee effusion-synovitis and possibly other OA-related structural changes [32], and another one clinical study reported slightly higher T levels in middle-aged menopausal women with systemic OA compared to controls [33]. However, it has been suggested that the onset of osteoarthritis appears to be associated with an age-related decline in sex hormones, including estrogen and testosterone [34], and that testosterone deficiency may exacerbate osteoarthritis by impairing glucose metabolism and increasing insulin resistance and advanced glycation end-products, which in turn increase oxidative stress and the release of pro-inflammatory cytokines [35]. Notably, our study supports a trend that elevated SHBG is associated with a decreased risk of OA and elevated TES is associated with an increased risk of OA.

Our study found that ALP was associated with a higher risk of OA, and although serum ALP levels have been revealed as the

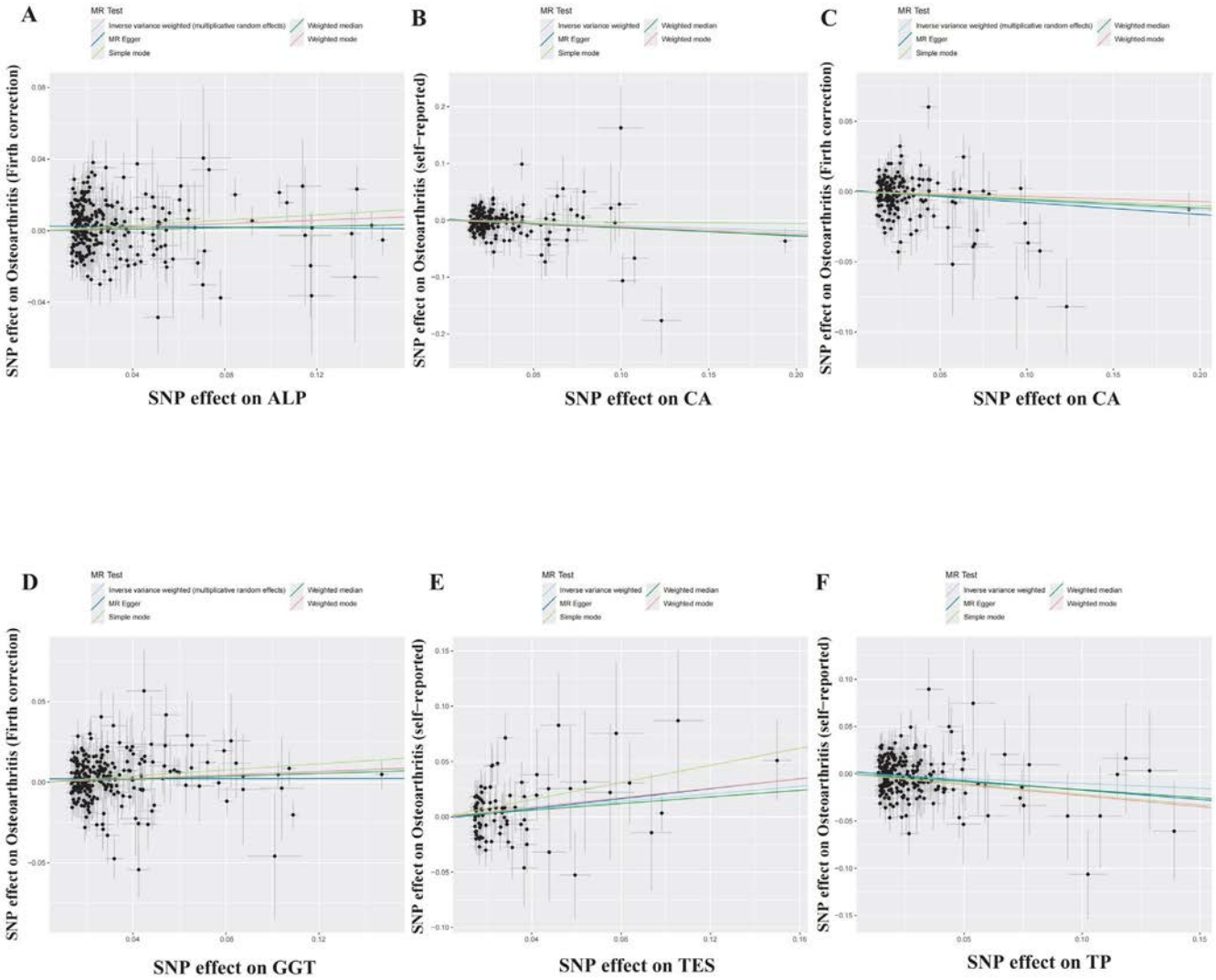

FIGURE 3 | Scatterplot of association between hematological and urinary markers and OA, SNP, single nucleotide polymorphism. (A) Scatterplot of causality between ALP and OA. (B) Scatterplot of causality between CA and OA. (C) Scatterplot of causality between CA and OA. (D) Scatterplot of causality between GGT and OA. (E) Scatterplot of causality between TES and OA. (F) Scatter plot of TP versus OA causality.

main biochemical marker for assessing disease activity in ankylosing spondylitis and rheumatoid arthritis, we believe that this study reveals for the first time, using MR analysis, that serum ALP levels are positively associated with osteoarthritis in a European population [36, 37]. Serum ALP, a well-known marker of hepatobiliary and skeletal diseases, has recently been found to be a biochemical marker of cardiometabolic diseases and chronic low-grade inflammation, and serum ALP is elevated when hepatic dysfunction occurs [38]. A cross-sectional study based on Koreans found that serum ALP activity was independently and positively correlated with severe osteoarthritis of the knee in middle-aged and elderly people [39], which is in agreement with our findings, and therefore, the ALP may be a potential biomarker to assist in the diagnosis of OA. TP can be classified into two categories, albumin and globulin, and has a variety of functions such as maintaining normal colloid osmotic pressure and acid–base in the vasculature, transporting a variety of metabolites, and regulating the physiological effects of transported substances, as well as having a close relationship with the immune function of the organism. In the upright position, the blood is relatively concentrated due to the distribution of body fluids, and the blood of the bedridden person is thinner than that of the upright position. Therefore serum total protein is lower in long term bedridden than in upright activity. TAA can decrease bone density and increase separation of bone trabeculae in SD rats, which ultimately leads to bone damage. TAA may be an ideal model to study bone metabolism abnormality after hepatic injury. Intraperitoneal injection of TAA resulted in a dramatic increase in ALP levels and a substantial decrease in serum TP and Ca levels [40].

Although a Mendelian randomization analysis did not observe a causal relationship between GGT and a variety of bone and joint related diseases [41], our study identified an association between GGT and an increased risk of OA. GGT, a novel bone resorption factor, has been shown to be associated with an increased risk of arthritis in osteoprotegerin (OPG)-deficient osteoporotic mice, as well as in patients with postmenopausal osteoporosis (67–83 years old) in which urinary excretion of GGT is increased [42], and anti-GGT antibody treatment attenuated osteolysis in collagen-induced arthritis mice, demonstrating that GGT is a causative factor for joint destruction in arthritis [43].

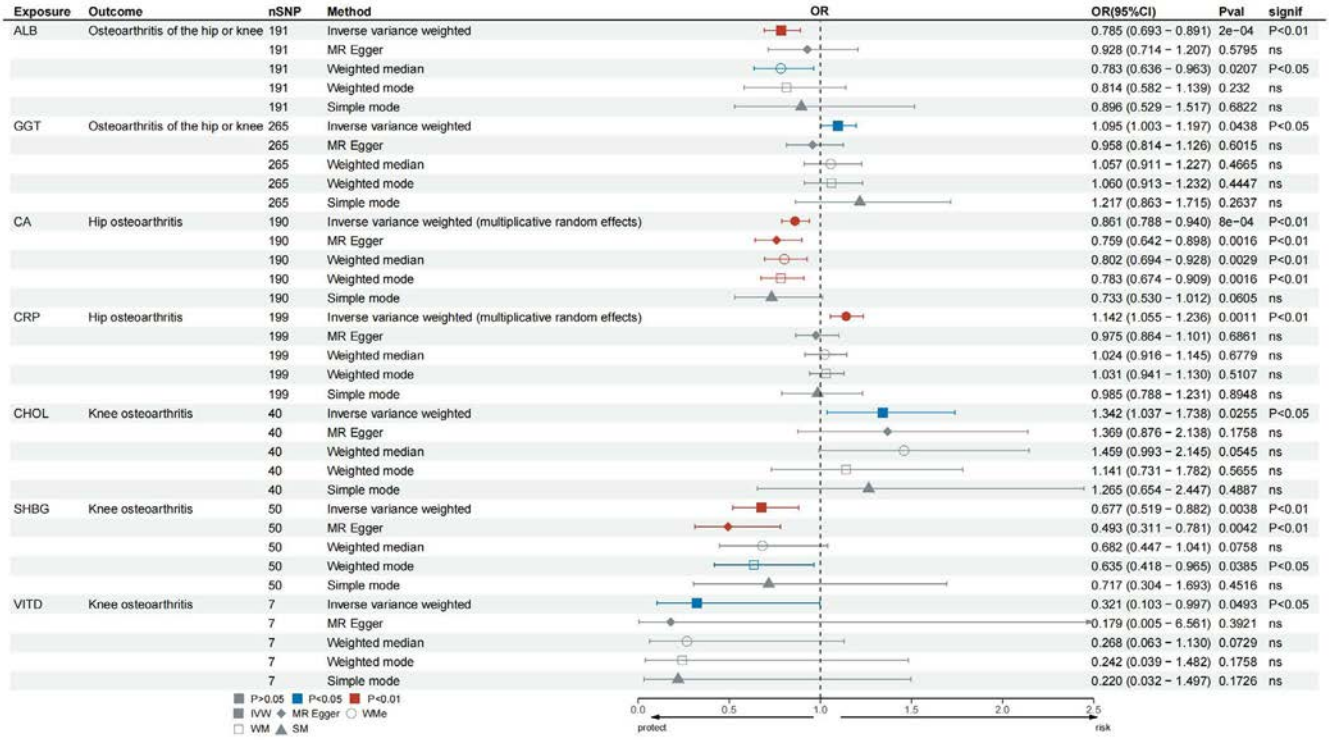

FIGURE 4 | Association between genetically predicted hematological and urinary markers and knee and hip OA examined by five MR methods.

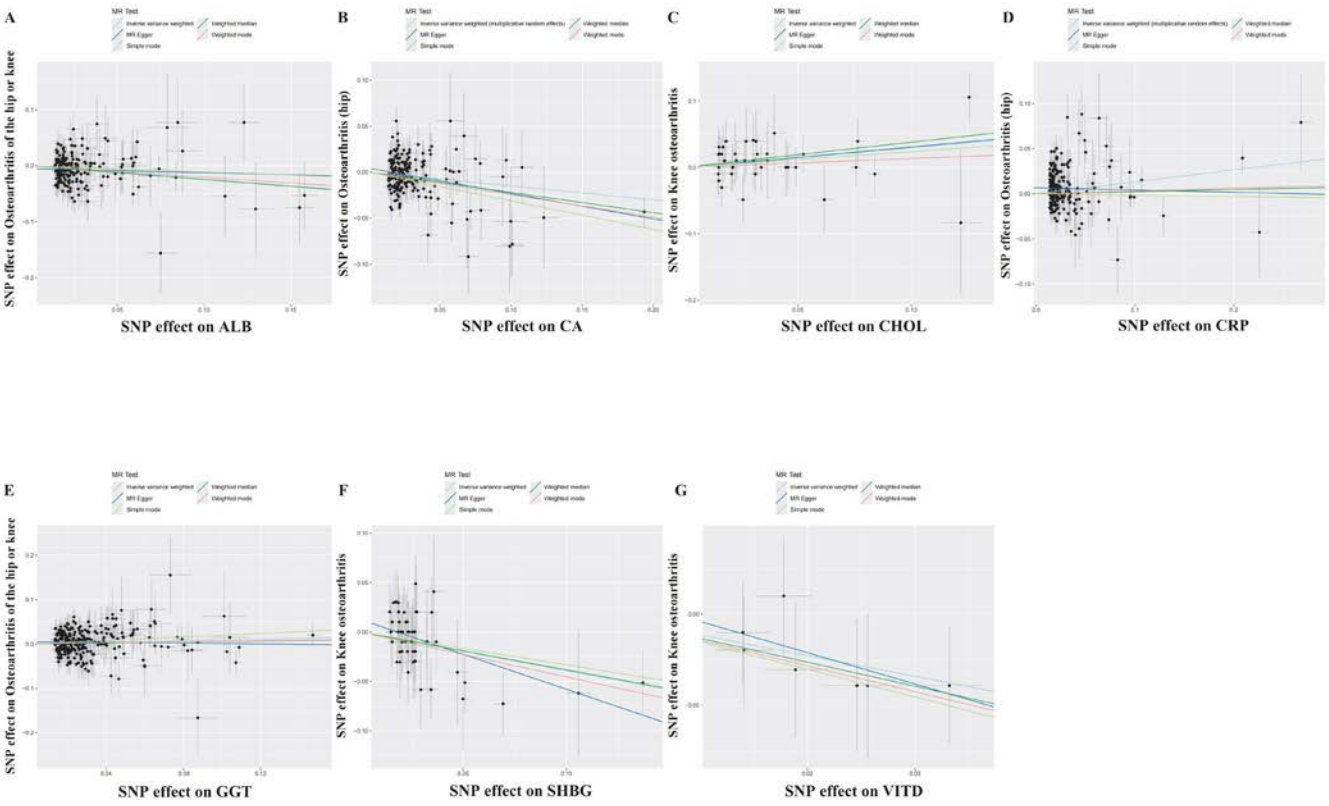

FIGURE 5 | Scatterplot of association between hematological and urinary markers and knee and hip OA, SNP, single nucleotide polymorphism. (A) Scatterplot of causal relationship between ALB and Osteoarthritis of the hip or knee. (B) Scatterplot of causal relationship between CA and Osteoarthritis of the hip scatterplot. (C) Scatterplot of the causal relationship between CHOL and Osteoarthritis of the knee. (D) Scatterplot of the causal relationship between CRP and Osteoarthritis of the hip. (E) Scatterplot of the causal relationship between GGT and Osteoarthritis of the hip or knee. (F) Scatterplot of the causal relationship between SHBG and Osteoarthritis of the knee causality scatter plot. (G) Scatterplot of the causal relationship between VITD and Osteoarthritis of the knee.

High cholesterol levels are often thought to be associated with atherosclerosis [44]. However, many studies have shown that high levels of cholesterol are associated with may induce osteoarthritis. An animal experiment found that Leiden mice on a chronically high cholesterol diet suffered from more severe osteoarthritis than mice on a normal diet in a dose-dependent manner [45]. A cross-sectional study in South Korea demonstrated that high cholesterol was positively correlated with OA pain [46]. Choi WS and other scholars [47] found that the CH25H-CYP7B1-RORα axis of cholesterol metabolism in chondrocytes was a key metabolic regulator in the pathogenesis of osteoarthritis, with increased cholesterol uptake by osteoarthritic chondrocytes, upregulation of cholesterol hydroxylases (CH25H and CYP7B1), and increased production of oxysterol metabolites leading to elevated cholesterol levels. In addition, abnormal cholesterol homeostasis has been shown to be one of the characteristics of OA development [48], and the results of our study further confirm the positive correlation between cholesterol and OA.

CRP is a well-studied inflammation-associated molecule that is considered a biomarker of OA, but the prognostic value of CRP in OA is inconclusive [49]. Kondo F et al. found that Hs-CRP levels were significantly correlated with the progression of osteoarthritis of the knee. Serum albumin is primarily considered a biomarker of visceral protein and immunocompetence status and is the basis for nutritional assessment, and low S-albumin levels are associated with persistent systemic inflammation. Commercial human serum albumin has been shown to be successful in relieving pain and inflammation in severe KOA. In recent years, the CRP to albumin ratio (CRP/Alb) has emerged as a novel inflammatory marker, but few studies have evaluated its role in OA. Our results suggest that the ratio of elevated CRP and decreased ALB as a causative factor of OA may become a new marker for the diagnosis of OA, and we will explore the role of CRP/Alb in the future studies. indicator's value in the early diagnosis of OA.

VITD has received increasing attention as a potential prophylactic agent against developmental and progressive KOA. One view is that VITD increases NO production and induced NO synthase expression in osteoarthritic chondrocytes, thus potentially exerting a protective effect. Another study found that in the context of KOA, VITD regulates autophagy and reduces chondrocyte apoptosis through the adenosine monophosphate (AMP)-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) signaling pathway. Regardless, the protective effect of VITD against versus OA seems to show consistency, which is again corroborated by our findings.

In this study, we explored the causal relationship between 35 specific biomarkers and OA for the first time using a two-sample Mendelian randomization method, which avoided the interference of reverse causality on the results and ensured the reliability of the findings. There was a causal relationship between 10 of these biomarkers and OA, and testing for these specific biomarkers could help physicians diagnose osteoarthritis more accurately as well as help assess the severity of osteoarthritis. Meanwhile, we believe that biomarkers have important potential for clinical application in the diagnosis of osteoarthritis, assessment of disease severity, prediction of disease progression, personalized treatment and early intervention, and these biomarkers are expected to become important tools in the management of osteoarthritis. In addition, our study incorporated datasets from different GWAS databases, with less possibility of sample duplication. However, this study still needs further experimental validation, which is our next research direction.

5 | Conclusion

Our results show the protective effects of CA, TP, ALB, SHBG, and VITD on OA and the pathogenic effects of TES, ALP, GGT, CRP, and CHOL on OA, suggesting that the above 10 hematological and urinary markers have the potential to be an important indicator for the clinical diagnosis of OA as well as for assessment of treatment efficacy and disease progression.

Author Contributions

Conceptualization: Anqi Chen and Qiang Cai. Data collection: Anqi Chen and Qiang Cai. Formal analysis: Anqi Chen and Qiang Cai. Methodology: Anqi Chen and Qiang Cai. Validation: Anqi Chen and Qiang Cai. Visualization: Anqi Chen and Qiang Cai. Writing original draft: Anqi Chen and Qiang Cai. All authors contributed to the article and approved the submitted version.

Acknowledgments

The authors sincerely thanks the researchers and participants of the original biobank for collecting and managing large-scale data resources and those who actively participated in this study.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

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

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

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EDITORIAL

Advancing Prognostic Biomarkers in Pulmonary Arterial Hypertension: The Role of Plasma Immunoglobulin G Fucosylation

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Received: 30 October 2024 | **Revised:** 11 December 2024 | **Accepted:** 19 December 2024

Funding: This study was supported by the Chinese National Key Technology R&D Programme, Ministry of Science and Technology (2021YFC2501305), and National Natural Science Foundation of China (82071827; 81701610).

Pulmonary arterial hypertension (PAH) is a devastating disease characterized by progressive increase in pulmonary vascular resistance and mean pulmonary artery pressure, ultimately leading to right heart failure [1, 2]. Despite advancements in our understanding of pathophysiology of PAH, predicting patient outcomes remains a significant challenge [3]. Current risk assessment approaches in PAH rely on a multidimensional evaluation that incorporates clinical, functional, exercise, biochemical, and hemodynamic parameters [3]. While the only circulating biomarker included in established prognostic models is B-type natriuretic peptide (BNP) or its precursor, N-terminal pro BNP (NT-pro BNP). However, these biomarkers do not fully capture the complexity of PAH pathophysiology, which involves immune dysregulation and inflammation [4, 5]. In this context, the study by Zhang et al., published in the *Journal of the American College of Cardiology*, represents a significant advancement by identifying plasma immunoglobulin G (IgG) N-glycome traits as novel prognostic biomarkers for PAH [6].

The study primarily focuses on IgG fucosylation, a specific glycosylation trait of IgG molecules. Glycosylation, the attachment of sugar moieties to proteins, is a critical post-translational modification that influences protein function and stability. Aberrant glycosylation has been implicated in numerous diseases, including cardiovascular disorders [7–10]. In IgG, N-glycans attached to the Fc region modulate immune responses, including antibody-dependent cellular cytotoxicity. The authors hypothesized that specific IgG N-glycan traits could reflect underlying pathophysiological processes in PAH and serve as an independent prognostic indicator.

To test this, the authors profiled plasma IgG N-glycomes using mass spectrometry in a cohort of 622 PAH patients. Their results revealed that low IgG fucosylation levels were significantly associated with increased mortality, independent of established risk factors such as age, sex, and NT-pro BNP levels. This collaborative study by two national centers, Beijing and Shanghai, China, provides compelling evidence that IgG fucosylation is an independent predictor of survival in PAH and significantly enhances existing risk assessment models.

The study's methodology is noteworthy for its rigor and comprehensiveness. This is the first IgG N-glycomic study in PAH, marking a new investigative area in the field. Using a high-throughput mass spectrometry approach ensured accurate and reproducible glycan profiling [11, 12]. Plasma IgG N-glycosylation was measured using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry to profile N-glycan traits such as galactosylation and fucosylation. Reliability was ensured with technical replicates. Derived traits were calculated for better interpretation, and statistical analyses, including Cox regression, assessed prognostic value while adjusting for confounders. The study's robust validation, including discovery (Beijing cohort), validation (Shanghai cohort),

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and random sampling analysis (combined cohorts), strengthens the credibility of its findings. The prognostic value of IgG fucosylation was consistent across subgroups stratified by sex and treatment strategies, demonstrating its independent influence. Moreover, the integration of IgG fucosylation into existing risk models improved their predictive capacity, as evidenced by an increase in Harrell C-indexes. This suggests that IgG fucosylation provides additional prognostic information beyond traditional risk factors. Clinicians could use this biomarker to better stratify patients, particularly those in the intermediate-risk category, allowing for more personalized treatment strategies.

The study's implications extend beyond PAH. Glycosylation changes have been implicated in various cardiovascular diseases, highlighting the broader potential of IgG N-glycans as biomarkers. Their stability under various storage conditions is advantageous for clinical application, minimizing potential biases from sample degradation [7–11].

Despite its strengths, the study has limitations that warrant consideration. The cohorts were derived from idiopathic and heritable PAH, and it remains to be seen whether the findings are generalizable to other subtypes of PAH or to other cardiovascular diseases. While the study establishes an association between IgG fucosylation and survival, the confounding factors should be more thoroughly controlled and the underlying mechanisms remain to be elucidated. Additionally, how does IgG fucosylation change over the course of disease progression or in response to treatment? Longitudinal studies could elucidate its potential as a marker of treatment response or disease activity. Future research should explore the biological pathways linking IgG glycosylation to PAH pathophysiology, which could uncover new therapeutic targets.

In conclusion, the study by Zhang et al. represents a significant step forward in the quest for reliable prognostic biomarkers in PAH, expanding the repertoire of biomarkers beyond traditional options like BNP or NT-pro BNP. By identifying plasma IgG fucosylation as a robust predictor of survival, the research provides an additional tool for risk assessment and patient management. The integration of IgG fucosylation allows for a more nuanced differentiation in risk stratification among PAH patients, facilitating more precise treatment approaches tailored to individual risk profiles. As the field of glycomics continues to evolve, it holds promise for transforming our understanding and treatment of complex diseases like PAH. The integration of glycan biomarkers into clinical practice could herald a new era of precision medicine, ultimately improving patients' outcomes and quality of life.

Author Contributions

All authors took part in drafting and revising the article, and all authors approved the final version to be published. **Qianwen Wu:** writing – original draft, methodology, visualization. **Huangshu Ye:** writing – original draft, methodology, visualization. **Zhangdi Zhou:** data curation, investigation, validation. **Dongyu Li** data curation, investigation, validation. **Miaojia Zhang:** supervision, writing – review and editing. **Xiaoxuan Sun:** supervision, writing – review and editing. **Qiang Wang:** supervision, writing – review and editing.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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ORIGINAL ARTICLE

A New Promising Role for Selexipag in the Treatment of Scleroderma Vasculopathy: Preliminary Results From a Third Level Italian Scleroderma Center

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Received: 28 October 2024 | **Revised:** 8 December 2024 | **Accepted:** 19 December 2024

Keywords: digital ulcer | Selexipag | systemic sclerosis | vasculopathy

ABSTRACT

Aim: Raynaud phenomenon (RP) and digital ulcers (DUs) represent the most frequent manifestations in systemic sclerosis (SSc), being signs of severe vasculopathy. Selexipag has been proposed as a useful tool for treating peripheral vasculopathy. The aim of this study was to evaluate the clinical effectiveness of Selexipag for treating complex cases of DUs as compared to the standard of care.

Methods: This is a case–control study in which SSc patients with active and long-lasting DUs were compared with a control group treated with a standard-of-care topical therapy that was stable in the previous 6 months. Clinical outcomes of RP and DUs were evaluated at T0 and after 6 months of follow-up.

Results: 23 SSc patients were enrolled. Selexipag was administered to 9 SSc patients, and its effects were compared with those in 14 SSc patients treated with standard-of- care therapy. Complete healing of DUs in Selexipag patients was higher (56% vs. 7%, $p = 0.018$). DUs diameter and pain VAS were slightly better—although not statistically significant—at the end of follow-up in the Selexipag group (respectively 0 vs. 5 $p = 0.15$ and 5 vs. 6, $p = 0.066$). All Raynaud Condition Scores outcomes improved in the Selexipag group: number of attacks (4 vs. 6, $p < 0.001$), duration (20 min vs. 25 mm, $p = 0.083$), and VAS (5 vs. 6, $p = 0.066$). Moreover, baseline DU diameter ($\beta = 0.70$, $p < 0.001$) and Selexipag treatment ($\beta = -7.1$, $p = 0.006$) were statistically significant predictors of the diameter of DUs after 6 months (F-test = 0.038). No new DUs were observed during the 6-month therapy.

Conclusions: Our preliminary results suggest a potentially new therapeutic indication of Selexipag in SSc for the treatment of SSc vasculopathy, in particular DUs and RP.

1 | Introduction

Digital ulcers (DUs) represent one of the most frequent and early manifestations in systemic sclerosis (SSc) [1, 2]. They affect almost 50% of patients, with a minority of themdeveloping severe complications such as infections, osteomyelitis, critical ischemia, and gangrene, potentially leading to amputation [3]. DUs are a major source of pain and disability (including work disability) [4, 5] and have a substantial impact on the quality of life in patients with SSc [4–7]. Moreover, these seem to be a sentinel sign of internal organ involvement [8] and are related to a poor prognosis of the disease [9]. Notwithstanding the availability of a wide range of systemic (pharmacological) therapies for the prevention and/or treatment of active DUs [10, 11], treatment of scleroderma skin ulcers remains challenging, and in clinical practice, around one-third of patients with SSc may experience refractory DU disease [4, 5]. To date, the main points of systemic therapy include oral and intravenous vasoactive therapies such

Summary

- Selexipag is safe and effective in the treatment of scleroderma digital ulcers (DUs), which are refractory to conventional therapies, and for the improvement of Raynaud phenomenon in patients with systemic sclerosis (SSc).
- This study suggests a new potential therapeutic indication of Selexipag for the treatment of SSc vasculopathy.
- Large prospective studies on Selexipag are recommended as it could significantly modify the management of DUs and vasculopathy related to SSc.

as calcium channel blockers, phosphodiesterase-5 inhibitors (PDE5-i), endothelin receptor antagonists (ERAs), and intravenous (i.v.) Iloprost, which are approved for the treatment of DU in SSc [10, 12]. However, the aforementioned therapies are not effective in cases of refractory DUs, especially if complicated by infection and gangrene, drug resistance, intolerance, or if they are contraindicated [5]. In these cases, other therapies with different mechanisms of action should be considered. Recently, some reports described rapid healing of long-lasting DUs in patients undergoing treatment with Selexipag (an oral selective prostacyclin receptor agonist) for concomitant pulmonary arterial hypertension (PAH) [13–16].

The aim of this study was to evaluate the clinical effectiveness of Selexipag for the treatment of complex DUs.

2 | Methods

This is a pilot observational case–control study conducted at the referral center for SSc-DUs in Modena from December 2023 to June 2024. The research project was approved by the local Institutional Ethics Committee (protocol no. 275/16). The use of the drug was off-label and compassionate, approved in accordance with National Laws. All patients gave their informed consent.

All consecutive patients with SSc classified according to the ACR/EULAR criteria with active and long-lasting DUs (from 6 to 12 months), as previously reported [17] and with stable vasoactive immunosuppressant therapies in the previous 6 months, were enrolled and divided into two subgroups: the Selexipag group (SG) and the Control group (CG). Treatment with Selexipag was administered in cases of DUs (as previously described) refractory to standard local debridement, dressing, and systemic vasoactive therapies. Exclusion criteria were the presence of vascular disease, diabetes mellitus, arterial hypertension, dyslipidemia, use of corticosteroids, and overlapping diseases.

2.1 | Data Collection

Baseline information included demographic data, clinical signs and symptoms, organ involvement, current therapies classified as vasoactive/vasodilating drugs, and immunosuppressant therapy. Data regarding DUs included quantity, location, and diameter, together with patient self-evaluation of pain using a Visual Analogue Scale for pain (VAS). In case of multiple DUs, the cardinal ulcer was taken into consideration for data collection [18]. In addition, the severity of Raynaud Phenomenon (RP) was also evaluated using the Raynaud Condition Score (RCS), a Likert scale ranging from 0 to 10 for patient's self-evaluation of the mean difficulty caused by their Raynaud's condition in the last 2 weeks [19], in terms of number of attacks, frequency, and pain. All patients were evaluated at baseline (T0) and after 6 months of follow-up (T1).

The SG received a 200-μg dose twice a day, which was then titrated with a weekly increase of 200 μg up to the maximum tolerated dose. Other ongoing vasoactive and immunosuppressant therapies were stable for the previous 6 months and were not modified during the study period. In addition to systemic therapy, each ulcer was treated with advanced topical medications, in accordance with TIME protocols [20, 21]. CG was treated with standard therapy. In the SG, side effects were also considered.

The effectiveness of Selexipag was evaluated by comparing the number (%) of healed patients (defined as a T6 ulcer with a diameter equal to zero) in the treated group with that of the untreated group. Furthermore, the presence of any other factors influencing the reduction in the diameter of the ulcers was evaluated (diameter of DUs at T0, sex, age, number of DUs, autoimmune profile, and duration of DUs).

2.2 | Statistical Analysis

Continuous parameters are reported as the median with interquartile range (IQR); categorical variables are expressed as absolute numbers or percentages. The parameters of the two subgroups were compared using the Mann–Whitney test or the chi-squared (Fisher's) test, as appropriate.

The changes between baseline and follow-up measures were assessed with the Wilcoxon test. A linear regression model was performed to assess factors that could influence the diameter of DUs after 6 months of follow-up. The statistical significance level was set at a p value < 0.05. Statistical analysis was performed with theJamovi (version 2.3.21.0) statistical software.

3 | Results

Twenty-three consecutive SSc patients with chronic long-lasting DUs as previously described were enrolled in this study. Selexipag was administered to 9 patients with SSc (SG) (8 females, mean age 46 [IQR 45–53] years) in addition to the standard topical and systemic medications, and its effects were compared against 14 patients with SSc (11 females, mean age 59 [IQR 52–65]) treated with standard therapy (CG). All DUs were located on the upper limbs, and most patients had at least 1 DU. Most of patient were female (M:F, 4:19), with a limited form of the disease (14/23), a mean age of 54 [IQR 44–63], and a mean disease duration of 10 [IQR 8–19]. ACA positivity was found in 14 of 23 patients (61%), PAH in eight of 23, and interstitial lung disease in six of 23 (26.1). Almost all patients enrolled (21 of 23 patients) were on double vasodilating therapy

with Iloprost and an endothelin receptor antagonist (Bosentan or Macitentan), while 6 patients were on triple therapy with a combination of i.v. Iloprost, ERAs, and PDE5-i (Sildenafil). Twelve patients were not on immunosuppressive therapy. All patients underwent the same advanced medication during the 6 months of follow-up. Considering the baseline demographic and main clinical characteristics, the two cohorts were comparable with the exception of a longer duration of the disease in SG (20 vs. 10 years; $p = 0.01$). Patients in SG had larger and more painful DUs (diameter 18 mm vs. 10 mm; $p = 0.009$ and VAS 10 cm vs. 7 cm; $p = 0.003$). Importantly, vasodilating, vasoactive, and immunosuppressive therapies were similar in the two groups. Main demographic and clinical data of the enrolled patients at baseline and of the 2 cohorts are detailed in Table 1. A paired t-test carried out on the entire cohort highlighted significant differences in all the following items: DU diameter ($p = 0.009$), pain ($p = 0.008$), RCS number of attacks ($p = 0.013$), RCS VAS pain ($p = 0.014$), and RCS attacks duration ($p = 0.014$). After 6 months, there was a significant improvement in DUs in both subgroups, reported as reduction in ulcer diameter and pain VAS ($p = 0.009$ and $p = 0.008$, respectively) (Figure 1). At the end of follow-up, DU diameter and VAS pain were slightly better—although not statistically significant—in SG than in CG (0 [IQR 0–9] vs. 5 [IQR 3–8],

TABLE 1 | Descriptive statistics of the sample at baseline.

		Total cohort	SG	CG	*p*-value
N		23	9	14	—
M:F		4:19	1:8	3:11	nss
Age, years [IQR]		54 [44–63]	46 [43–53]	59 [52–65]	nss
Disease duration, years [IQR]		10 [8–19]	20 [13–27]	10 [5–11]	**0.01**
SSc subset, *n*	Limited Diffused	14 9	6 3	8 6	nss
Autoimmunity, *n*	Anti-Scl70 ACA Anti-RNA pol III None	7 14 1 1	3 6 0 0	4 8 1 1	nss
PAH, *n*		8	4	4	nss
ILD, *n*		6	4	2	nss
Smoke habits ever, *n*		3	1	2	nss
Immunosoppressive therapy, *n*	MMF Rituximab	10 5	5 2	5 3	nss
Vasoactive/vasodilative therapy, *n*	Calcium blockers ERA PDE4i Iloprost i.v.	8 22 5 21	3 9 3 9	5 13 2 12	nss
Number of DUs, *n* [IQR]		1 [1–3]	2 [1–2]	1 [1–3]	nss
DU diameter, mm [IQR]		12 [8–18]	18 [16–20]	10 [6–12]	**0.009**
DU pain VAS, cm [IQR]		8 [6–10]	10 [9–10]	7 [5–8]	**0.003**
RCS, number of attacks, *n* [IQR]		7 [6–8]	6 [5–8]	7 [6–8]	nss
RCS, duration of attacks, min [IQR]		30 [25–38]	30 [25–40]	28 [25–34]	nss
RCS, VAS pain of attacks, cm		7	8	7	nss
[IQR]		[6–8]	[7, 8]	[6–8]	

Note: Significant *p*-values are bolded.
Abbreviations: ACA, anti-centromere antibodies; CG, control group; DUs, digital ulcers; ERA, endothelin receptor antagonists; i.v., intravenous; ILD, interstitial lung disease; IQR, interquartile range; MMF, mycophenolate mofetil; PAH, pulmonary arterial hypertension; PDEi, phosphodiesterase inhibitors; pts., patients; RCS, Raynaud Condition Score; Scl70, anti-topoisomerase antibodies; SD, standard deviation; SG, Selexipag group; SSc, systemic sclerosis; VAS, Visual Analogue Scale.

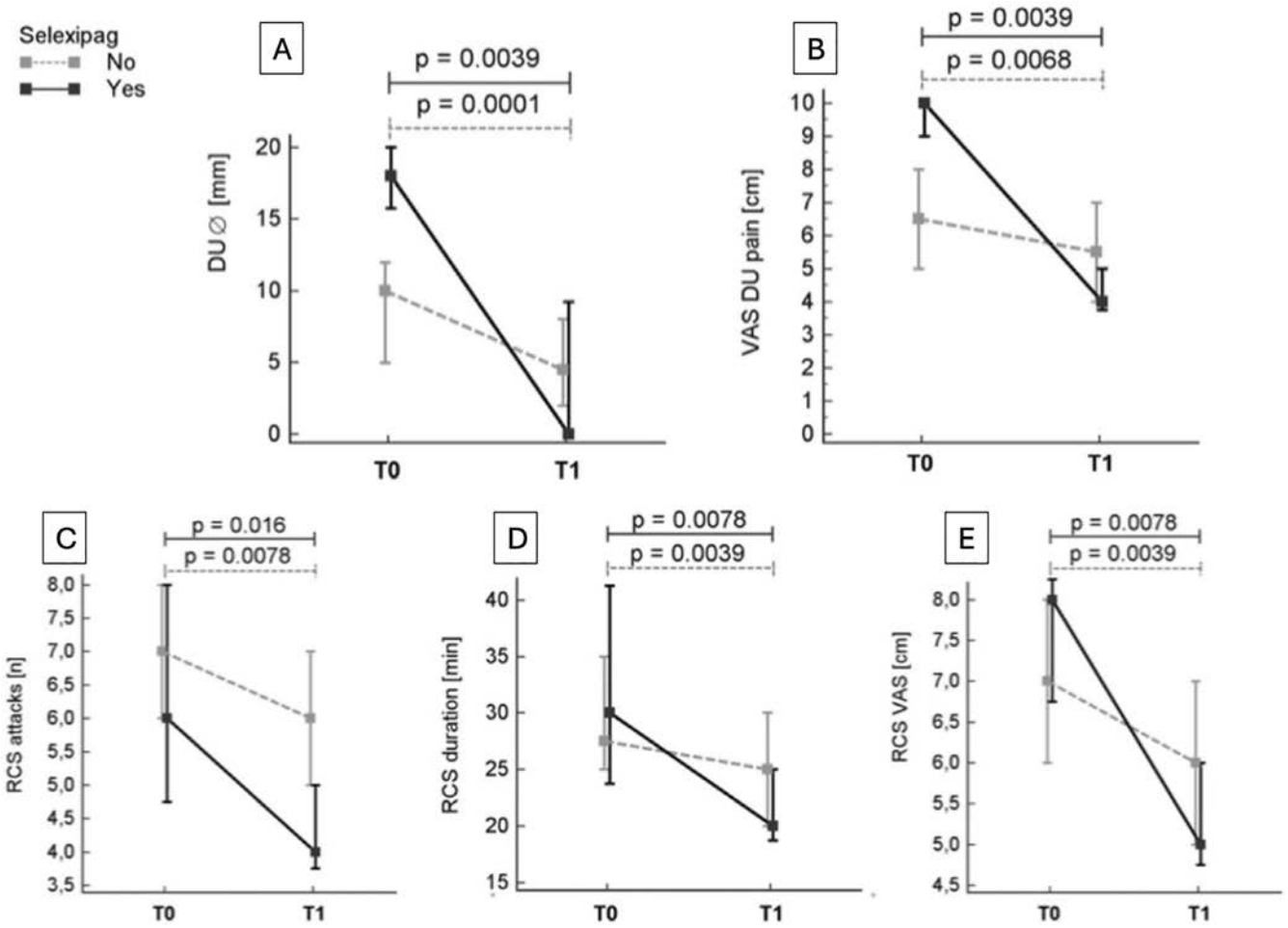

FIGURE 1 | Effect of Selexipag on digital ulcers and Raynaud's phenomenon from T0 (baseline) to T1 (after 6 months of follow-up). (A B) The effect of Selexipag on digital ulcers diameter (A) and pain VAS (B) is shown. (C–E), the effect of Selexipag on Raynaud's Phenomenon outcomes is shown, that is, RCS attack in (C), RCS duration in (D), and RCS pain VAS in (E). DUs, digital ulcers; RCS, Raynaud Condition Score; VAS, Visual Analogue Scale.

$p = 0.15$, and 5 [IQR 5–6] vs. 6 [IQR 5–7], $p = 0.066$, respectively). The outcomes related to RP significantly improved during follow-up (Figures 1 and 2). All RCS improved in the SG compared with that in the CG: number of attacks (4 [IQR 4–5] vs. 6 [IQR 5–7], $p < 0.001$, respectively), duration (20 min [IQR 20–25] vs. 25 min [IQR 21–29], $p = 0.083$, respectively), and VAS (5 [IQR 5–6] vs. 6 [IQR 5–7], $p = 0.066$, respectively) (Figure 1). The prevalence of complete healing of DUs in SG was higher than that in the CG (56% vs. 7%, $p = 0.018$). The statistically significant predictors of the diameter of DU after 6 months ($R^2 = 0.71$ F-test = 0.038) were the baseline DU diameter (beta coefficient = 0.70, $p < 0.001$) and Selexipag treatment (beta coefficient = −7.1, $p = 0.006$); other tested predictors (age, sex, number of DU, autoimmunity, disease duration, and DUs duration) were not statistically significant. Moreover, no new DUs were observed during the 6 months of therapy.

Selexipag was safe and well tolerated for all the treated patients; in fact, none of the patients had to interrupt treatment due to side effects. Only 3 of 8 patients reported hypotension, for whom the dose of Selexipag was not titrated. Most patients (seven of eight) had a low dose of Selexipag (200 and 400 μg twice daily), while only two patients with concomitant PAH reached 800 μg twice daily. During maintenance dosage, all side effects subsided in intensity.

4 | Discussion

This pilot study suggests that Selexipag may be safe and effective in the treatment of scleroderma DUs, which are refractory to conventional therapies, and for the improvement of RP in patients with SSc.

DUs complicate the course of SSc in almost half of the patients, leading to pain, disability, and reduced quality of life [4–7]. Despite the numerous and latest therapeutical options available, the management of DUs is challenging and requires a complex treatment program, including both systemic and local therapies [22, 23]. The pathophysiology of DUs is primarily observed in vasculopathy occurring in SSc. Vascular involvement affects microvasculature which, during the course of the disease, manifests in the form of RP, DUs, PAH, and SRC [24]. These vascular manifestations share some pathogenetic courses and could therefore be treated using the same drugs. Despite cross-sectional studies showed that patients with SSc–DU had a worse outcome of the

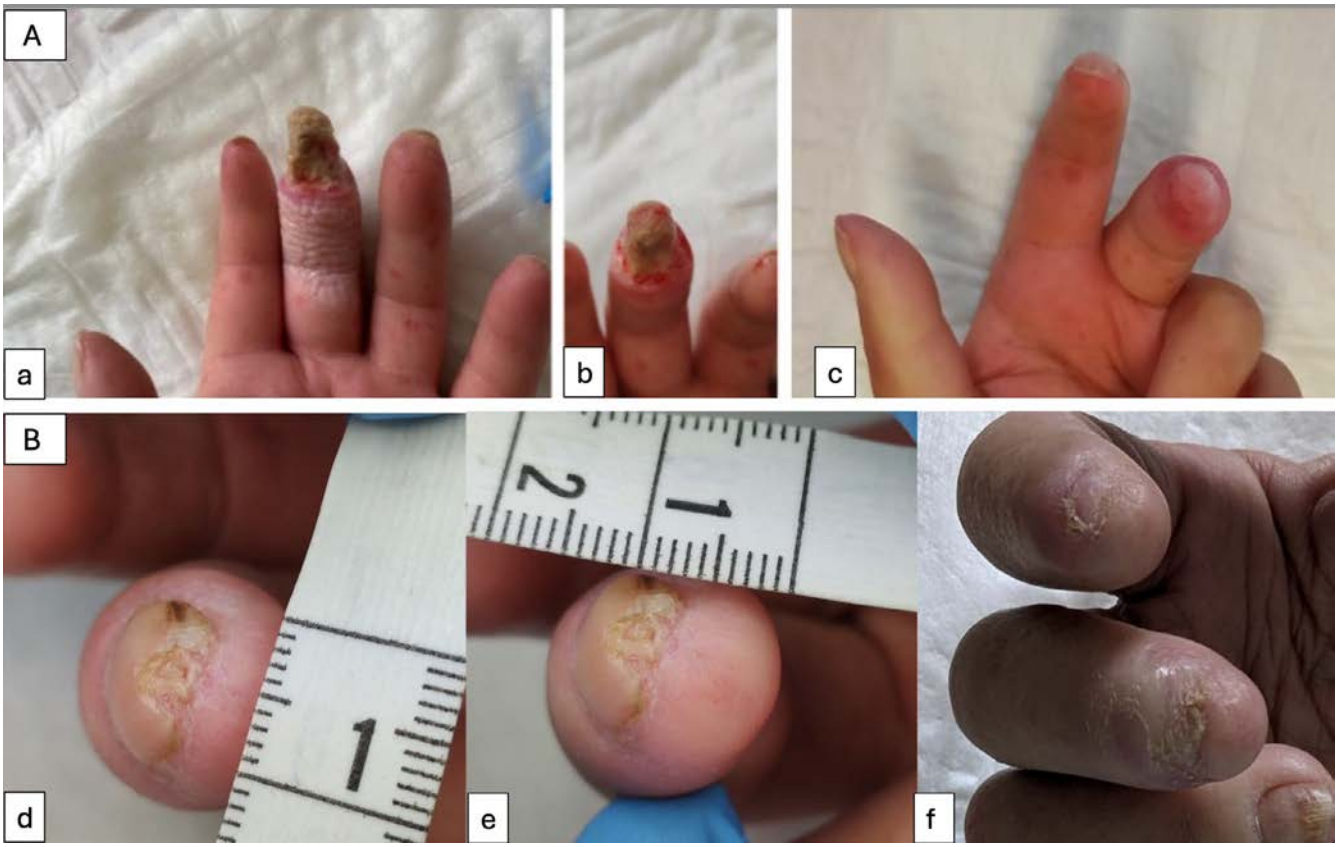

FIGURE 2 | Effect of Selexipag on DU healing. Two examples of the effect of Selexipag on DU complete healing after 6 months of follow-up. (A) A patient with SSc affected by a chronic DU of the III finger of the left hand at baseline (a), after 3 months (b), and after 6 months (c). (B) Patient with SSc affected by a chronic DU of the III finger of the right hand at baseline (d), after 3 months (e), and after 6 months (f). DU, digital ulcer; SSc, systemic sclerosis.

disease, neither a higher incidence of pulmonary arterial hypertension (PAH) nor scleroderma renal crisis (SRC) was found [25]. Selexipag is an oral, selective prostacyclin receptor agonist, approved for the treatment of PAH even when associated with systemic autoimmune diseases such as SSc both in monotherapy and in combined therapy with other vasoactive treatments (such as ERAs and PDE5-i) [26]. The rationale lying behind the use of Selexipag for the treatment of DUs in SSc consists in the activation of prostacyclin receptors, which induces vasodilatation, and possibly a peripheral vasodilatory effect. In 2017, in a randomized controlled trial (RCT), Denton et al. reported that Selexipag failed to demonstrate effectiveness on SSc-related RP; however, healing of DUs was recorded in all the three patients, including the ones in the active arm of the RCT [14]. Some issues could have influenced the RCT, in fact most patients (83%) had taken the final Selexipag dose of ≤ 800 μg bid, similar to our Selexipag dosage, however, with treatment lasting only 8 weeks—which was shorter than the treatment period for our patients—establishing the appropriate dosage is fundamental.

Del Papa et al. [15] described a case series of patients with SSc where the addition of oral Selexipag therapy proved to beeffective in thehealing ofDUs in 8 patients. Recently, Di Battista et al. [16, 27] reported a significant decrease in the number of daily episodes and mean duration of RP after 12 years of follow-up in a small series of 8 patients with SSc. In the same study, all patients achieved a complete healing of their DUs within 6 months.

To the best of our knowledge, our study is the first case–control study exploring the effectiveness of Selexipag for DUs and RP in patients with SSc. Our data show that implementing Selexipag in addition to systemic vasoactive therapy and topical dressings is effective for complete healing of DUs compared with that of the CG.

In our cohort, Selexipag dramatically reduced DU diameter and pain (Figure 2). All outcomes related to RP significantly improved during follow-up in the SG. Moreover, given that patients often present with multiple and recurrent DUs, an important finding is that no new DUs were observed in the SG during the 6 months of follow-up.

Finally, our preliminary study shows that Selexipag treatment and baseline DU diameter are predictors of the diameter of DUs after 6 months.

The overall improvement in both DUs and RP in the enrolled cohort is directly correlated with two main factors: (1) Almost all patients were on double vasoactive therapy, which confirms the importance of vasodilating and vasoactive therapy in scleroderma patients with RF and DUs; (2) all enrolled patients received advanced medications every 2–3 days at the wound care clinic of the scleroderma unit, an advanced center for the treatment of ulcers, regardless of the systemic therapy administered. Once again, this underlines the importance of a highly specialized center for the treatment of DUs.

It must be emphasized that the two cohorts are comparable in terms of demographic characteristics, clinical involvement, and therapy, with the exception of a longer duration of the disease in SG. CG was approximately 13 years older than SG; however, this difference is not statistically significant. Moreover, even if most patients were on Bosentan therapy, which could certainly influence DU healing, no significant difference in vasoactive therapy between the two groups was found.

This is an open observational case–control analysis on a limited number of patients, suggesting a potential new therapeutic indication of Selexipag in SSc, especially in the more complex DUs or in patients with contraindications to the standard vasoactive treatment. Further investigation into the use of oral Selexipag is recommended as it could significantly modify the management of DUs and vasculopathy related to SSc in general. Indeed, together with the promising effects on SSc vasculopathy, Selexipag could have other benefits: Oral intake reduces hospitalization for intravenous recovery, work-leave, and resolves the issues that characterize venous access. Daily intake guarantees a constant availability of the drug, overcoming the lack of a standardized protocol for infusion, curbing healthcare costs (fewer prostanoid infusions resulting in a shorter hospital recovery for infusion), and providing a global improvement in the patient's quality of life. This paper presents some limitations. Firstly, this is a pilot study that includes a small sample without double-blinding. Regarding this aspect, it must be highlighted that Selexipag was administered as an off label drug and patients' samples and the study protocol were in line with the previous literature. Moreover, the efficacy included both subjective and objective measurements in order to avoid a possible placebo effect. Regarding the study methods, another limitation is the lack of assessment with ultrasound and PD, information regarding mRSS value, and characteristics of DUs according to TIME protocols. In addition, we did not perform nailfold video capillaroscopy due to the limited follow-up time.

In conclusion, our preliminary data indicate a new potential therapeutic indication of Selexipag for the treatment of SSc vasculopathy, in particular DUs and RP, suggesting also its possible efficacy in other vascular complications of the disease.

Author Contributions

The author takes full responsibility for this article.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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International Journal of Rheumatic Diseases

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ORIGINAL ARTICLE

hURAT1 Transgenic Mouse Model for Evaluating Targeted Urate-Lowering Agents

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Received: 3 July 2024 | **Revised:** 3 November 2024 | **Accepted:** 19 December 2024

Funding: This work was supported by the Fundamental Research Funds for the Central Public Welfare Research Institutes (ZXKT23002) and National Natural Science Foundation of China (32273019).

Keywords: gout | hyperuricemia | *SLC22A12* | transporter | URAT1 | uric acid

ABSTRACT

Background: Urate transporter 1 (URAT1) is a well-known therapeutic target for reducing urate levels in the treatment of hyperuricemia and gout. However, current pharmacological studies have failed to evaluate the efficacy of URAT1 inhibitors in non-primate animal models. We established a *human URAT1* (*hURAT1*) transgenic knock-in (KI) mouse model to assess uricosuric agents' effectiveness and characterize URAT1-caused pathogenesis.

Methods: We generated *hURAT1* transgenic mice using CRISPR/Cas9 KI technique. *mUrat1* knockout was achieved by replacing exon 1 coding sequence with a human *SLC22A12* coding sequence (CDS)-pA cassette. Based on the above transgenic mice, a hyperuricemia model was further established by hypoxanthine administration.

Results: The *hURAT1*-KI mice successfully expressed hURAT1 protein to the apical side of the kidney proximal tubule epithelium, where native human URAT1 is localized in human kidney. Upon hypoxanthine challenge, the blood uric acid (UA) level was elevated in *hURAT1*-KI mice (251 μmol/L), showing an approximately 37% increase compared to wild-type (WT) mice (183.5 μmol/L). The elevated blood UA level could be alleviated by hURAT1 inhibitor benzbromarone treatment in the *hURAT1*-KI mice (164.2 μmol/L vs. 251 μmol/L, $p < 0.05$) whereas no response was observed in *WT* littermates (168.8 μmol/L vs. 183.5 μmol/L).

Conclusion: The *hURAT1*-KI hyperuricemia mouse model would be valuable for preclinical evaluation of gout treatment with urate-lowering drugs and for studying UA metabolic complexities in humans.

1 | Introduction

Sustained serum uric acid (UA) elevation leads to hyperuricemia and gout, resulting in clinical manifestations such as inflammatory arthritis, urolithiasis, hypertension, cardiovascular diseases, and renal failure [1]. Population-based studies have shown that the global prevalence of gout ranges from 1% to 6.8%, and the prevalence of hyperuricemia is two to seven times higher than that of gout [2]. Long-term urate-lowering therapy is the optimal and curative treatment for gout and hyperuricemia

Weiyan Cai and Miyi Yang contributed equally to this work.

Summary

- A humanized mouse model with *hURAT1* gene knock-in was generated.
- *hURAT1* transgenic mouse was used to establish a small animal model with high uric acid levels.
- The high uric acid levels mouse model was used to evaluate the URAT1-targeting urate-lowering drugs.

[3]. Patients with gout need to achieve and maintain a target plasma urate within 5 mg/dL or less. Urate-lowering intervention facilitates UA crystal dissolution and prevents the recurrence of gout flares, reducing the risk of tophi, joint damage, or nephrolithiasis [4]. Emerging clinical evidence has indicated that achieving a lower serum urate level by therapy will benefit people with gout [3, 5]. However, limited commercially available medications have not satisfied the global demands, highlighting the urgency of developing such urate-lowering drugs.

Currently, commercial drugs to reduce blood urate, including xanthine oxidase (XO) inhibitors and urate transporter inhibitors representing respective categories, are based on distinct mechanisms. XO inhibitors reduce UA production by inhibiting xanthine to UA conversion; transporter inhibitors increase body UA elimination by blocking UA recycling. Urate transporter 1 (URAT1), encoded by *SLC22A12* gene, is a urate-anion exchanger that regulates blood urate homeostasis by regulating most kidney urate reabsorption from the original urine in the late part of the proximal tubule [6]. URAT1 contributes to more than 50% of body UA maintenance; hence, it is the most important target for developing urate-lowering drugs. It has been shown that individuals carrying nonfunctional *hURAT1* alleles could lower down to 12% of those with functional gene alleles [7]. Moreover, 90% of hyperuricemia patients were caused by excretion impediment rather than overproduction of urate [8]. Hence, URAT1 inhibitors as uricosuric treatments showed a better performance than XO blockers for those people with gout [9]. Nonetheless, only a few URAT1-targeted drugs have been developed and proven effective in clinical settings in recent decades. Available URAT1 inhibitors, including benzbromarone, lesinurad, and dotinurad, have been approved only in a few countries and regions to treat gout [10, 11].

UA is a powerful antioxidant and neuroprotector [12]. Higher UA levels have potential but unproven benefits. It may create more congenial physiology environments for enhanced intelligence, maintaining blood pressure, and alleviating oxidative stress, which is considered an evolutionary advantage in modern apes. Moreover, primitive higher primates live in the jungle and consume more fruits; high serum UA level appear to be a consequence of fructose consumption. Nevertheless, when the serum UA concentration exceeds the saturation threshold (> 7.0 mg/dL), it forms monosodium urate crystal and deposits in the joint, kidney, and multiple organs, causing harmful or deadly clinical consequences. The fine-tuning of URAT1 that occurs in primate species counteracts the severe consequences of functional uricase loss and achieves plasma urate homeostasis [13]. Rodent URAT1 orthologs exhibit low binding affinity (weak binding), and high transporting capacity for UA, compared to human URAT1 [13], hence, mURAT1 and hURAT1 show difference performances in charge of UA. It reasonable that *mURAT1* gene knockout mice exhibit no function impairment or serum UA alteration [14, 15]. The difference could be caused by secretory mechanisms that determine the varied susceptibility of drugs among species. For benzbromarone inhibiting rate in vitro, human URAT1 IC_{50} is 0.13 μmol/L, remarkably higher than rats IC_{50} 11.9 μmol/L [16], which is consistent with our observation. Our results have shown that mURAT1 also exhibits a much lower affinity for benzbromarone than hURAT1. As such, wild-type (WT) mice and rats rarely respond to hURAT1 inhibitors (i.e., benzbromarone); hence, they are inappropriate for URAT1-related drug screening and evaluation in preclinical studies.

Currently, the New World monkey *Cebus apella* (Brown capuchin) is the only animal model for URAT1 preclinical pharmacology studies [17, 18], which is expensive and brings a massive financial burden and access difficulty for primary drug screenings and pharmacological activity evaluations. In this study, we developed a *hURAT1* knock-in (*hURAT1*-KI) mouse model to functionally demonstrate the human-like URAT1 reabsorbing capability of UA in vivo. We further confirmed that the *hURAT1* transgenic mouse is an ideal small animal model for pharmacology development and fundamental research on urate metabolic-associated diseases.

2 | Materials and Methods

2.1 | *hURAT1*-KI Mice Generation

Human URAT1 knock-in mice (*hURAT1*-KI) were developed at Cyagen Biosciences Inc. (Suzhou, China) by introducing a donor vector containing *SLC22A12* CDS-P2-3* simian virus 40 pA cassette and Cas9 and gRNA into C57BL/6 embryonic stem cells to generate targeted KI offspring. F1 mice were intercrossed to generate homozygous mice. Polymerase chain reaction (PCR) screening primers as following: forward primer (F1): 5′-TCACCATCTACAGCAGCGAGCT-3′; reverse primer (R1): 5′-GGTTCACTCAGTAGAGACCGCCT-3′; forward primer (F2): 5′-CAGGAATCGTACGGACATCTCTAT-3′; reverse primer (R2): 5′-GTTCAAGGTCATCACCAAGGGTC-3′. Genomic DNA sequencing primers were as follows: forward primer: 5′-CCAGA TTACCACAGAGGGTTCC-3′; reverse primer CTCTGTAAGCT GCCATTGAGGTTG-3′. Genotyping primers were as follows: forward primer:5′-TGGTGCTACTCTGTGGTGCTA-3′; reverse primer:5′-CAGGAATCGTACGGACATCTCTA-3′.

2.2 | Animal Studies

Sex- and age-matched transgenic and WT littermates were used for experimental analyses. The baseline plasma urate levels of mice were performed. Tissue samples were obtained for histological analysis. The left kidney of the animal from each group was sectioned longitudinally and subjected to immunohistochemistry.

Mouse hyperuricemia model: to simulate high UA levels (ranges 200–400 μmol/L in human), transgenic mice were

injected with 480 mg/kg hypoxanthine solution (in sterile water). *WT* mice received the same treatment served as controls. Blood was collected 4 h post-injection for analysis. $n = 12$ in *WT* basal metabolic group, $n = 12$ in *hURAT1*-KI basal metabolic, and $n = 29$ in *hURAT1*-KI + hyperuricemia group, $n = 20$ in *WT* + hyperuricemia group. Experiments are expressed as mean ± SEM.

For benzbromarone treatment evaluation, half an hour before hypoxanthine administration, a single dose (26 mg/kg) of benzbromarone or control saline administrated into mice by oral gavage. Two hours later, blood was collected for analysis. Statistical significance was determined with two-way analysis of variance analysis. $n = 20$ in *WT*, $n = 15$ in *WT*+ benzbromarone and $n = 29$ in *hURAT1*-KI mice group, $n = 29$ in *hURAT1*-KI + benzbromarone mice group.

2.3 | Cell Culture and Lentivirus Packaging

HEK-293T cells stably expressing human and mouse URAT1 were established. In brief, with the full length of *hURAT1/mURAT1* cDNA were subcloned into plent-EF1a-FH-CMV-GFP vector. HEK-293T cells stably expressing hURAT1 (as HEK293T-hURAT1) or mURAT1 (as HEK293T-mURAT1) were obtained by viral infection of HEK-293T cells with packaged lentiviral transgenes as abovementioned. HEK-293T cells transduced with packaged lentivirus with plent-EF1a-FH-CMV-GFP vector were used as a control (HEK293T-GFP mock cells). The viral-containing medium was changed to puromycin selection media 24 h after infection. Surviving stable cell lines were grown in a humidified incubator at 37°C and 5% CO_2 using a minimum essential medium containing 10% fetal bovine serum and 2 μg/mL puromycin. Urate uptake experiments were performed by following the protocol as previously described [19].

2.4 | Blood Sample Collection and Analysis

Whole blood was collected from homozygous *hURAT1*-KI and *WT* mice and placed into commercially available Ethylenediaminetetra-acetic acid -treated tubes. Plasma was harvested by centrifugation of the whole blood for 10 min at 2500 rpm. Following centrifugation, the upper layer plasma was transferred into aliquots and stored at −20°C for future analyses. Plasma urate levels were measured using the Uric Acid Kit (C012-2, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) by following the manufacturer's protocol. For each set of measurements, a standard curve was generated.

2.5 | Immunohistochemistry and Imaging

Mice were euthanized for tissue collection. Kidneys of animals from each group were fixed in 10% neutral buffered formalin, paraffin-embedded, sectioned, and subjected to histological (hematoxylin–eosin stain) and immunohistochemical analyses. Kidneys were stained with a hURAT1-specific antibody (Sino Biological Inc. Beijing, China) for expression analysis. Sample images were taken using a Pannoramic Scan instrument (3DHISTECH Kft.; Budapest, Hungary).

2.6 | Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). The two-way analysis of variance was used to compare multiple groups. The figure asterisk indicates the statistical significance (*, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001).

3 | Results

3.1 | Generation and Characterization of *hURAT1* KI Mice

We used clustered regularly interspaced short palindromic repeats (CRISPR) /Cas9 gene editing to create a *hURAT1*-KI mouse model that mimics human-like UA metabolism. The *Slc22a12* gene (NM_009203.3) is located in mouse chromosome 19 GRC m38.p6 (Figure 1A). The *hURAT1* gene CDS with a simian virus 40 polyadenylation signal was subcloned into the vector and microinjected into C57BL/6J genetic background single-cell embryos. *hURAT1* gene inserted and replaced exon 1 coding sequence of mouse *Urat1* (*mUrat1*), resulting in *mUrat1* silence (Figure 1A). PCR screening and genomic DNA sequencing confirmed the founder animals (F0) having successful insertions (Figure 1B,C). Five KI founders containing the *hURAT1* transgene demonstrated *hURAT1*-specific amplicons. F0 mice were further bred with *WT* mice to establish F1 heterozygous offspring. Southern blot hybridization further validated heterozygosity with correct transgene copy numbers (Figure 1D). Transcriptional expression of the *hURAT1* and *mUrat1* genes was assessed by reverse transcription-PCR (Figure 1E). mRNA of human and murine *URAT1* were expressed in heterozygous (*hURAT1* KI/+) mouse kidney tissue, respectively. In contrast, only the *mUrat1* allele was detected in *WT* mice.

3.2 | Phenotype of *hURAT1*-KI Mouse

Immunostaining analysis of kidney using an anti-hURAT1 antibody demonstrated the expression of *hURAT1* in homozygous (*hURAT1* KI/KI) and heterozygous (*hURAT1* KI/+) mice. The results showed that human URAT1-specific antibodies do not recognize rat and mouse URAT1 proteins (Figure 2A). Anti-hURAT1 antibody detected highly expressed hURAT1 in *hURAT1* KI/KI and *hURAT1* KI/+ mice. Expressed hURAT1 is in epithelial cells of proximal tubules in the renal cortex, with tissue distribution similar to that of the human kidney (Figure 2A).

Homozygous *hURAT1* transgenic mice are fertile and viable. They did not display apparent phenotypic alterations, and only minor increased body weight in male homozygous animals with no significant difference when assessed at age of 2 months (Figure 2B).

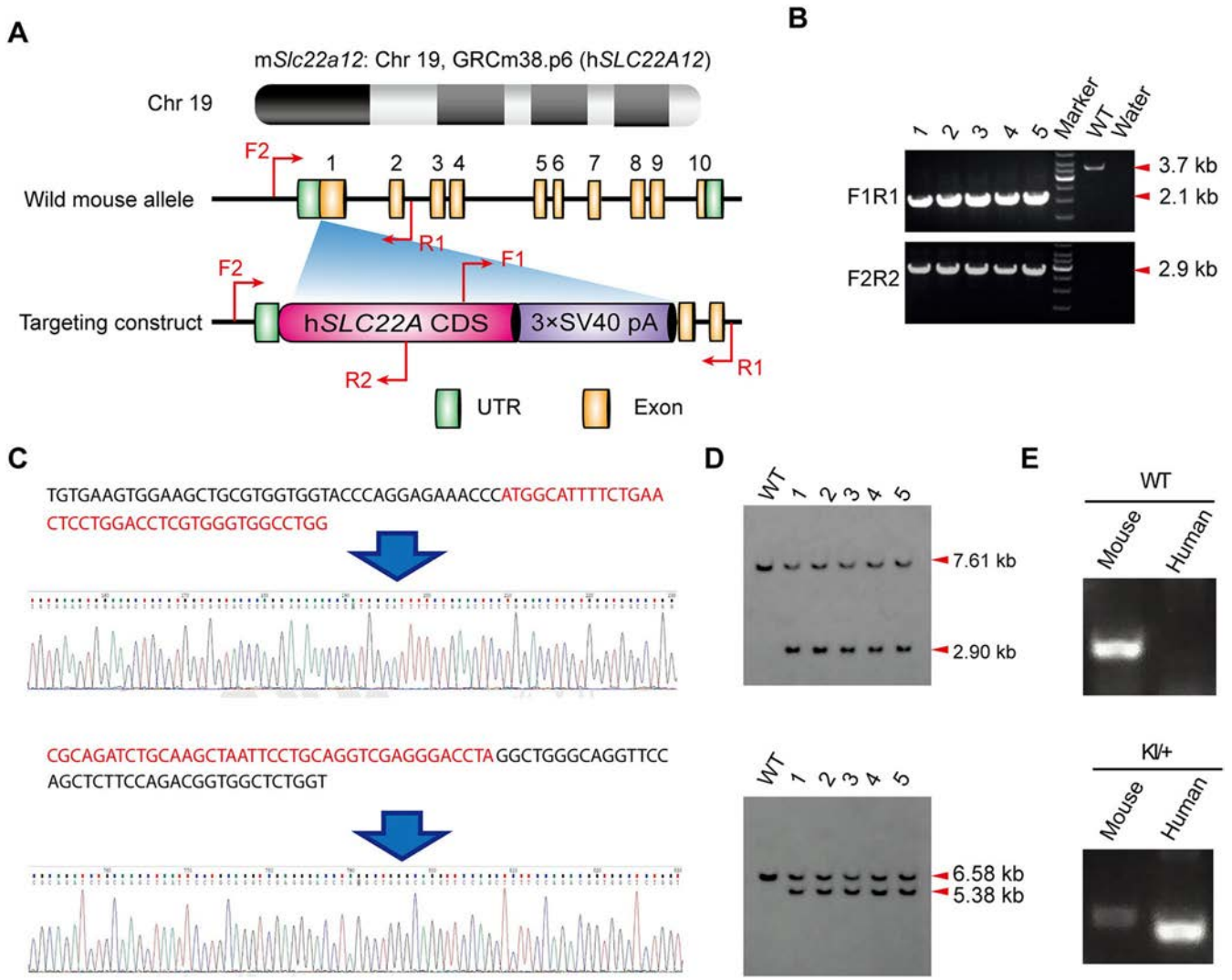

FIGURE 1 | Establish *hURAT1*-KI mouse. (A) Configuration of *hURAT1* cDNA transgene. The *hURAT1* gene was inserted into exon1 of *mUrat1* in chromosome 19 GRC m38.p6. F0 founder animals were identified by PCR screening (B) and DNA sequencing (C), which were bred to *WT* mice to test germline transmission and F1 animal generation. *WT* animals harbor the full-length exon1 of *mUrat1* gene, which extends into the size range of 3.7 kb. Only 2.1 kb size is detected in animals harboring the *hURAT1* transgene. (C) *hURAT1*-specific primer was applied in DNA sequencing in transgenic mice genomic DNA. (D) F1 mice were confirmed by Southern blot. The endonucleases *AvrII* and *StuI* were used to identify the correct insertion. Expected fragment sizes for Southern blot: 5′Probe-AvrII: WT-7.61 kb, *hURAT1*-2.90 kb; 3′Probe-Bsu36I: *WT*-6.58 kb, *hURAT1*-5.38 kb, respectively. (E) Transcriptional analysis of transgenic and *WT* lines. The positions of the endogenous murine *Urat1*, *hURAT1*, and *Gapdh* transcripts are indicated. Heterozygous targeted mice expressed both human and mouse *URAT1* genes. *hURAT1*-KI, human URAT1 transgenic knock-in; URAT1, urate transporter 1; *WT*, wild-type.

We further analyzed the blood UA levels of the *hURAT1*-KI and *WT* mice. UA levels of male and female *hURAT1* mice are comparable to *WT* mice (43.9 μmol/L [male] and 47.5 μmol/L [female] in *hURAT1* mice vs. 46.7 μmol/L[male] and 51.6 μmol/L [female] in *WT* mice, Figure 2C). Our results show that hURAT1-KI mice maintain similar low levels of UA to WT mice, which is physiologically reasonable as functional uricase is responsible for most urate elimination in mice under normal physiological conditions.

To further establish an animal model with a blood urate level close to the physiological of the human body, normally ranges 180–420 μmol/L, we utilized hypoxanthine to stimulate the mice (Figure 2D). A robust increase in urate levels was observed in both hURAT1-KI and WT mice; interestingly, transgenic mice had a 37% higher value (averaged 251 μmol/L) than the *WT* mice (averaged 183 μmol/L), suggesting that hURAT1 transported more urate from urine to blood compared with mURAT1, subsequently enhancing the blood urate level. Our data confirmed the previous observation that hURAT1 is more effective in regulating physiological UA that range 200–400 μmol/L [13].

3.3 | Use of *hURAT1*-KI Mice for Evaluating hURAT1-Targeted Drug Performance

To validate whether the hURAT1-KI mouse could be used as a tool for evaluating URAT1 inhibitors, we investigated the efficacy of the commercially available urate-lowering drug benzetramine. We established stable HEK293T-hURAT1, HEK293T-mURAT1,

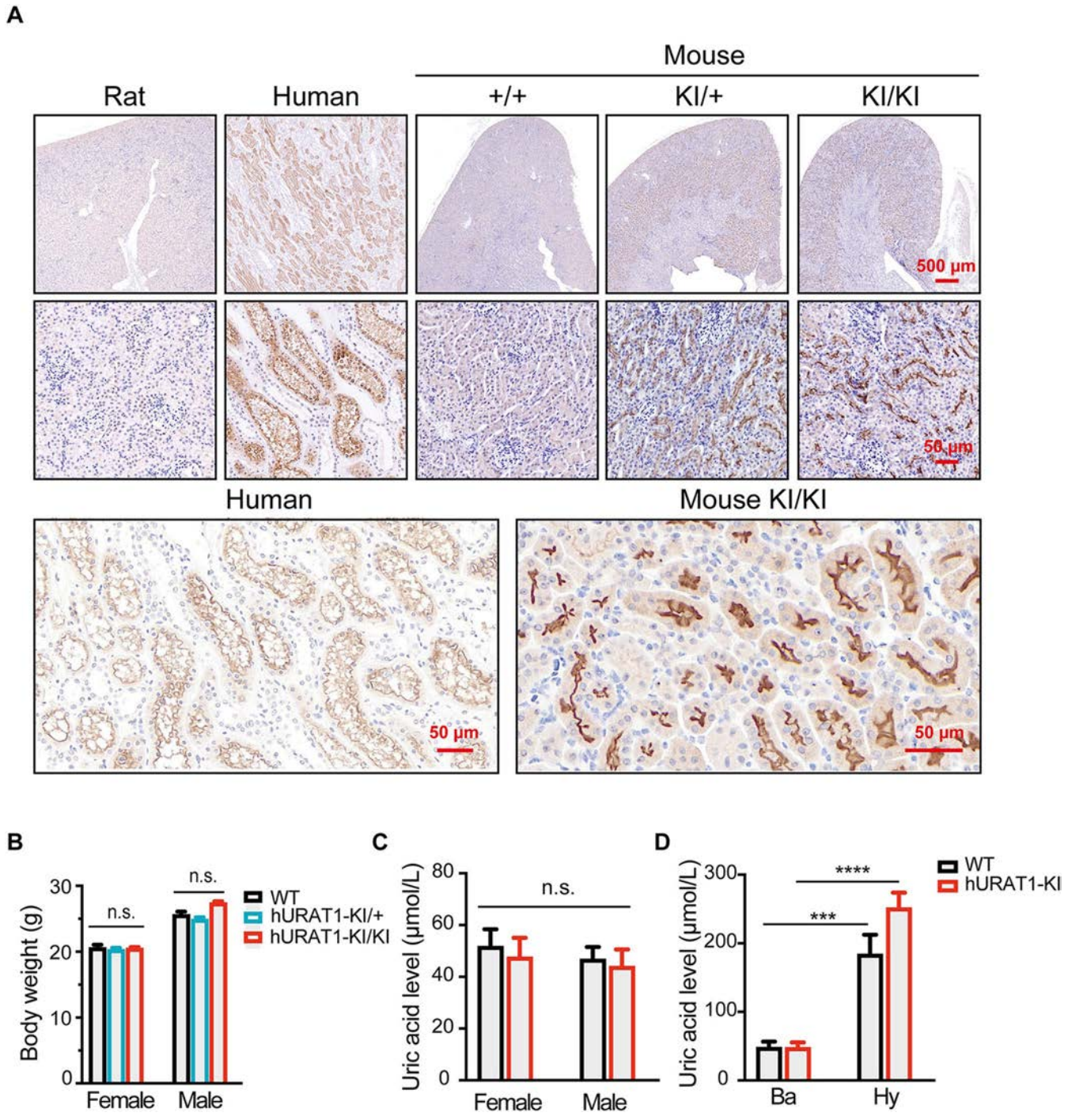

FIGURE 2 | The phenotype of *hURAT1*-KI animals. (A) Immunohistochemistry staining analysis for the hURAT1-expressed in rat, human, and mouse kidney tissues. The primary antibody used here was specifically reacted with hURAT1. The histologic appearance of cortical renal tubules in humans and *hURAT1*-KI mice was not detected in *WT* mice and rats. Brown color indicates positive staining. (B) Body weight of *hURAT1* mice and *WT* littermates. Plasma uric acid concentration of basal metabolic condition (C) and hyperuricemia (D) in *hURAT1*-KI mice and *WT* littermates. Ba, basal metabolic; Ba, basal; *hURAT1*-KI, human *URAT1* transgenic knock-in; Hy, hyperuricemia; KI, *hURAT* knock-in; ns, not significant; URAT1, urate transporter 1; WT, wild-type. ***p-value < 0.001; ****p-value < 0.0001.

and HEK293T-GFP cells for in vitro UA transport assay. The result showed that benzbromarone achieved a significant effect at a considerable inhibition rate (IC_{50}) in hURAT1-expressed cells, 0.18 μmol/L compared to 45 μmol/LM in mURAT1-expressed cells (Figure 3A). The data revealed a clear difference in inhibitor affinity between hURAT1 and mURAT1, which is why the WT mouse model did not respond to treatment with URAT1 inhibitors. The hURAT1 and mURAT1 exhibit 83% similarity in protein sequence, while hURAT1 has roughly ~5 times higher capability to transport UA than mURAT1 [13].

We then tested it in our established hypoxanthine-induced hyperuricemia model to test its sensitivity toward benzbromarone treatment. Group treated with benzbromarone showed

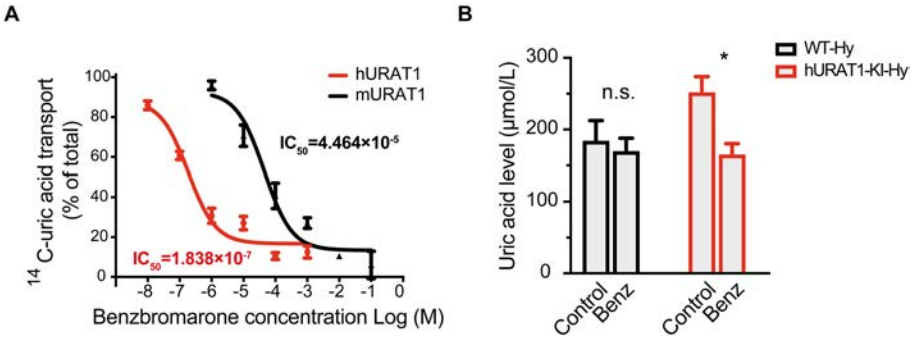

FIGURE 3 | Benzbromarone alleviate uric acid level in hyperuricemia *hURAT1*-KI mice model. (A) Dose responses for benzbromarone to inhibit the transport activity of human (red) and mouse URAT1 (black). 293T Cells expressing URAT1 were treated with ^{14}C-uric acid for 20 mins. Results are averaged from two independent experiments, each done in triplicate ± SD. (B) Model function analysis: benzbromarone lowers plasma UA in *hURAT1*-KI mice. A single dose (26 mg/kg) of benzbromarone, by oral gavage, lowered the plasma UA levels. Results are representative of data generated in two independent experiments and are expressed as mean ± SEM. **p*-value < 0.05.

a decrease of blood UA in *hURAT1*-KI mice compared to non-treatment group (164.2 μmol/L vs. 251 μmol/L), while there was no significant change in *WT* mice (168.8 μmol/L vs. 183.5 μmol/L, Figure 3B). Our study demonstrates an animal model that can respond effectively to URAT1 blocking, which can be an important preclinical investigative tool for testing the therapeutic effects of drugs.

4 | Discussion

The human UA level normally ranges from 3.5 to 7.0 mg/dL (equivalent to 200–400 μmol/L) [20]. Unlike humans, most mammalian species' serum UA levels are in the range of 0.5–1.5 mg/dL (equivalent to 30–90 μmol/L) as the uricase enzyme in these species converts UA into a more soluble 5-hydroxyisourate as an end-product of purine metabolism, which facilitates final metabolite elimination from the body. Primate URAT1 can subtly balance serum urate, hence counteracting blood uric acid elevation caused by uricase functional loss. Inhibiting hURAT1 could suppress UA reabsorption in the kidney proximal tubule, lowering blood UA and preventing monosodium urate crystal formation, ultimately improving the progression of gout and hyperuricemia.

On the other hand, the difference in capacity/affinity of URAT1 among species determines the variations in sensitivity to hURAT1-target molecules. The field has suffered from a lack of appropriate animal models expressing human-like URAT1 that can effectively respond to URAT1 blockade, which is the primary reason for the delayed URAT1 investigation and development of urate lowering drugs. Therefore, there is an urgent demand to screen and optimize novel URAT1-targeted models to screen drug candidates.

Human URAT1 with high affinity and low capacity to achieve more uric acid retention and more precise serum UA control. Due to multiple gene, another species except great apes difficult to mimic human hyperuricemia condition. Rodent orthologs show 1/7–1/5 affinity and 7–9 folds capacity for uric acid of human URAT1 [16]. URAT1 gene knockout mice demonstrate that it does not function as a uric acid transporter in mice [14, 16]. It has shown that the hURAT1-KI hURAT1 mice transported more urate into blood and hence acquire higher blood urate level than the WT mice.

In our model, we did not observe the sex difference of serum UA level. Uricemia is known to be higher in men than in women; the detailed mechanism has yet to be fully elucidated, and many studies indicate that the difference is due to the uricosuric effect of estrogens. The dysfunction of the *URAT1* gene leads to hypothyroidism in humans. However, no sex-specific effect difference has been reported, indicating that URAT1 is not a sex hormone susceptible gene [21]. Genetic susceptibility is also a critical attributable factor for gender difference of gout. In humans, the estrogen-regulating process may involve many genes, such as *ABCG2*, *SLC2A9*, and *SLC17A1* [20]. Currently, it is unclear whether those genes from mice can be regulated by estrogen receptors in the same way as humans.

The most significant difference between our *hURAT1*-KI transgenic mouse model and other hyperuricemia models is that our model could test URAT1-target drug preference and reproduce human URAT1 function. Inhibition of uricase by chemical substances or genetic techniques are extensively applied in making hyperuricemia models. For example, *Uox*−/− mouse model inactivating uricase exhibits extraordinary basal UA, while it cannot be used for human URAT1-related research [22]. As uricase is a potent enzyme for UA elimination, one limitation of the model is that it is efficient in a range of UA when testing potential UA lowering drugs. We are currently estabalishing *Uox*−/− and *hURAT1* KI/KI mouse model, which would overcome the disadvantages.

Monkey's renal system physiology is close to human. Compared to the monkey model, the hURAT1-KI transgenic mouse model is less expensive and more convenient. Previous research has shown that repetitive administration of 3–100 mg/kg benzbromarone results in minor lowering of plasma UA because of the existence of uricase in Cebus monkeys [17]. Panspermia administration at 26 mg/g reduces plasma UA levels by up to half in our model, indicating that our model is more effective.

In summary, we have developed an animal model that bridges a discrepancy between rodents and primates regarding URAT1 function.

Our result demonstrates that benzbromarone treatment could reduce blood UA levels in *hURAT1*-KI mice, increasing urate excretion, while there was no apparent response in *WT* mice. Therefore, the hURAT1-KI transgenic mouse model represents a convenient and valuable small animal model for evaluating the efficacy of URAT1-targeted drug candidates.

Author Contributions

Gejing De conceived the project and designed the research; Apeng Chen coordinated resources; Gejing De and Weiyan Cai performed research; Gejing De, Miyi Yang and Apeng Chen wrote the manuscript; Guohua Yi and Peihui Lin revised the manuscript.

Acknowledgments

We thank H. Zhang (Institute of Biophysics, Chinese Academy of Sciences) for assistance in transporter assay.

Ethics Statement

All animal studies were conducted following the protocol of the China Academy of Chinese Medical Sciences (No. of approval: 2023B102).

Conflicts of Interest

G.D. and A.C. have a provisional patent (2022100200252) in China.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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ORIGINAL ARTICLE

Vγ6$^+$γδT Cells Participate in Lupus Nephritis in MRL/*Lpr* Mice

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Received: 16 July 2024 | **Revised:** 11 December 2024 | **Accepted:** 19 December 2024

Fundings: The work was supported by Nanjing Health Commission (JQX20004), funding for Clinical Trials from the Affiliated Drum Tower Hospital, Medical School of Nanjing University (2021-LCYJ-PY-16), and the Postgraduate Research & Practice Innovation Program of Jiangsu Province supported by Jiangsu University (KYCX23_3768).

Keywords: IFN-γ | lupus nephritis | subsets | γδT cells

ABSTRACT

Background: γδT cells have been implicated in the pathogenesis of autoimmune diseases. The study aims to investigate the abundance of γδT cells in MRL/*lpr* mice.

Methods: MRL/*lpr* mice were used as lupus models, while C3H/HeJ mice served as normal controls. The abundance of γδT cells in different organs was examined by flow cytometry. Plasma double-stranded DNA antibody levels, blood urea nitrogen, creatinine, and urinary protein levels were measured. Renal histopathology was observed via H&E staining. The correlations between the abundance of γδT cells and lupus manifestations were analyzed.

Results: Compared with C3H/HeJ mice, the number of γδT cells and Vγ6$^+$γδT cell subset in the peripheral blood of MRL/*lpr* mice was significantly reduced. However, in the kidney, the number of γδT cells and Vγ6$^+$γδT cell subset was significantly increased. Additionally, the number of Vγ6$^+$γδT cells in the kidney was positively correlated with the urinary protein level. The number of IFN-γ$^+$Vγ6$^+$γδT cells in the kidney was positively correlated with urinary protein level.

Conclusion: In MRL/*lpr* mice, it is likely that peripheral γδT cells, especially the Vγ6 subset, infiltrate the kidney and secrete IFN-γ, which contributes to the development of lupus nephritis.

1 | Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease, known for its wide-ranging symptoms and multiple-organ involvement [1]. The complex pathogenesis of SLE involves T cell abnormalities, loss of self-tolerance, and autoantibody-induced tissue damage [2]. The MRL/*lpr* mouse model is frequently employed in research to uncover new insights in the immunopathogenesis of SLE [3–7].

γδT cells represent a minor T lymphocyte subset that express as γδTCR on the surface after gene rearrangement involving γ- and δ-T cell receptor (TCR) [8]. These cells play a crucial role in immune surveillance and defense against tumors and infection, acting as part of innate immune system [9, 10]. γδT cells swiftly identify both foreign pathogens and self-stress signals in an MHC-independent fashion, thereby triggering adaptive immunity and acting as the frontline of immune defense [11, 12]. Similar to αβT cells, γδT cells also exhibit Th1-, Th2-, Th17-, and Treg-like

Summary

- Our study has provided novel evidence indicating that peripheral Vγ6$^+$γδT cells play a role in the development of lupus nephritis by infiltrating in the kidney and secreting IFN-γ.
- Our study revealed the important role of a specific subset of γδT cells in the pathogenesis of SLE, and γδT cells were expected to be a new target for the treatment of SLE.

phenotypes, and play an important role in both inflammation and immune tolerance processes. Recent research has mainly focused on γδT1 and γδT17 cells, which secrete IFN-γ and IL-17A, respectively [13, 14]. Furthermore, the differentiation of γδT cells into distinct subsets is determined by the usage of variable regions of the γ-chain and δ-chain. In humans, γδT cells are categorized into Vδ1, Vδ2, Vδ3, and Vδ5 subsets [12]. Meanwhile, murine γδT cells are divided into Vγ1, Vγ4, Vγ5, Vγ6, and Vγ7 subsets, with the Vγ7 subset specifically localized in the intestines [15, 16]. The classification of γδT cells into these structural subsets provides insight into their functional diversity and tissue specificity.

Studies have demonstrated the significant contributions of specific subsets of γδT cells in autoimmune diseases, which might through interacting with other immune cells, such as B cells and monocytes, promote the differentiation and proliferation of pathogenic autoantibody-producing cells. In collagen-induced arthritis (CIA) mice model, both Vγ1$^+$γδT and Vγ4$^+$γδT cell populations were found to be increased, but only the Vγ4$^+$γδT cells became activated and aggravated CIA by producing IL-17A [17]. Likewise, the roles of Vγ4$^+$γδT and Vγ6$^+$γδT cells in psoriasis mice model have also been extensively investigated [18–20]. These studies have revealed that dermal γδT cells are the primary producers of IL-17 in the skin, responding to IL-23 stimulation. Additionally, γδT cells possess memory-cell-like characteristics, which contribute to the recurrence of psoriatic inflammation [21]. Intriguingly, IL-17A produced by γδT cells played a critical role in the pathogenesis of myeloperoxidase (MPO) anti-neutrophil cytoplasmic Ab (ANCA)-associated glomerulonephritis by promoting the development of MPO-specific αβT cells [22]. However, it remains unclear whether γδT cells and their subsets are involved in the pathogenesis of lupus nephritis, warranting further investigation based on the most recent and cutting-edge international research findings.

In this study, γδT cells and their Vγ1, Vγ4, Vγ5, and Vγ6 subsets in the spleen, peripheral blood, and kidney tissues of MRL/*lpr* and C3H/HeJ mice were detected. The intracellular cytokines, IFN-γ and IL-17A, were also examined.

2 | Materials and Methods

2.1 | Animals

Eight female MRL/Mp-lpr/lpr (MRL/*lpr*) mice and six C3H/HeJ mice were obtained from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China) and maintained in our animal facilities under specific pathogen-free conditions at Nanjing University Medical School. One MRL/*lpr* mouse died at 19 weeks old. All the mice were sacrificed when they were 21 weeks old. All mice were maintained on a standard chow diet, housed in a temperature-controlled environment with a 12-h light/dark cycle, and cared for according to the approved protocol by The Affiliated Drum Tower Hospital of Nanjing University Medical School Committee on use and care of experimental animals.

2.2 | Reagents

The following reagents were used in this study: Phosphate buffer saline (PBS) (Servicebio, China), double-stranded calf thymus DNA, Percoll, Red Blood Cell Lysis Buffer (Sigma-Aldrich, USA), anti-IL-17A-PE, anti-CD3e-APC-eFluor 780, anti-IFN-γ-FITC (eBioscience, USA), anti-CD45-Percp/Cy5.5, anti-TCR γ/δ-PE/Cy7, anti-TCR Vγ2-APC (The Vγ2 designation correlates with the nomenclature of Garman, Doherty, and Raulet; the Vγ4 designation of Heiligand Tonegawa is equivalent.), APC Armenian Hamster IgG Isotype Ctrl (BioLegend, USA), anti-Vγ1.1 TCR-BV421, anti-Vγ3 TCR-BV711 (The Vγ3 designation correlates with the nomenclature of Garman, Doherty, and Raulet; the Vγ5 designation of Heilig and Tonegawa is equivalent.), Ham IgG1 Kappa Itcl BV711, BD Cytofix/Cytoperm TM Fixation/Permeabilization Kit (BD Pharmingen, USA), HRP-conjugated Affinipure Goat Anti Mouse IgG (H + L) (Proteintech, USA), TMB Chromogen Solution for ELISA (Beyotime, China), Tween20, Albumin from bovine serum (BSA) (Biosharp, China), Bradford protein assay kit (Coomassie Blue G-250) (KeyGen BioTech, China), RPMI1640 medium, fetal bovine serum (FBS) (GIBCO, USA), Phorbol myristate acetate (PMA), Brefeldin A (BFA), Ionomycin (ENZO, USA), Collagenase Type IV (Invitrogen, USA), Mouse lymphocyte separation solution (TBD Science, China), BUN Assay Kit, Creatinine Assay Kit (Nanjing Jiancheng Bioengineering Institute, China).

2.3 | Laboratory Tests

The plasma levels of anti ds-DNA were determined by ELISA. Briefly, 96-well ELISA plates were coated with 5 μg/mL double-stranded calf thymus DNA in sodium salt citrate buffer at 37°C overnight. After washing, plasma was added in serial dilutions starting at 1/100. HRP-conjugated Affinipure Goat Anti Mouse IgG (H + L) was added, followed by TMB Chromogen Solution for ELISA for color development. Optical density (OD) at A450 was measured by a microtiter plate reader. The plasma creatinine and BUN were measured by Creatinine Assay Kit and BUN Assay Kit respectively, according to the manufacturer's instructions. The urinary protein was detected by Bradford method using Bradford protein assay kit.

2.4 | Splenomegaly Assessment and Renal Histopathologic Analysis

When mice were sacrificed, spleen, peripheral blood, and kidney tissues were collected. The spleen index (ratio of spleen

weight to body weight) and kidney index (ratio of kidney weight to body weight) were calculated. One kidney was fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 3 μm, and stained with H&E.

2.5 | Isolation of Leukocytes From Peripheral Blood, Spleen, and Kidney

Peripheral blood was centrifuged at 1500 *g* for 5 min. The upper layer of plasma was collected for freezing. PBS was added to the remaining blood cells to mix well and then overlaid onto the same volume of mouse lymphocyte separation solution. Centrifugation was performed at 715 *g* for 20 min in brake off mode. The slight whitish translucent layer was collected and washed twice with PBS.

The spleens were ground, and the splenic slurry was filtered through a 200-mesh sieve. Then the cell suspension was centrifuged at 400 *g* for 5 min, and the cell pellet was resuspended with red cell lysis buffer and washed with PBS.

The kidneys were placed in 5 mL EP tubes, and the kidney tissue was cut into small pieces (1–2 mm^3). Then about 2 mL of kidney digestive solution (RPMI1640 containing 5% FBS and 1 mg/mL Collagenase Type IV) was added into the EP tube. The tubes were shook on the shaker at 37°C for 45 min. The cell suspension of the kidney digestion was obtained by mechanically disrupting the tissue using a 200-mesh sieve and then was resuspended in 25 mL of tissue culture medium (RPMI1640 containing 5% FBS) and mixed. Centrifugation was performed at 400 *g* for 10 min at 4°C to pellet the cell suspension. Resuspended the pellet in 3 mL of 40% Percoll solution and centrifuged at 1500 *g* for 30 min at room temperature with the centrifuge in brake off mode. Collected the cell pellet and resuspended the samples in PBS.

2.6 | Cytokine Detection in γδT Cells by Flow Cytometry

The cells derived from peripheral blood, spleen, and kidney were stimulated with 100 μL RPMI 1640 containing 0.1 μg/mL Phorbol myristate acetate plus 1 μg/mL ionomycin in the presence of 5 μg/mL Brefeldin A for 4 h in 5% CO_2 at 37°C. After incubation, cells were collected and washed twice with 200 μL staining buffer (PBS containing 1% FBS) at 1500 rpm for 5 min.

For surface staining, cells were labeled with the following antibodies: anti-CD45-Percp/Cy5.5, anti-CD3e-APC-eFluor 780, anti-TCR γ/δ-PE/Cy7, anti-Vγ1.1 TCR-BV421, anti-TCR Vγ2-APC and anti-Vγ3 TCR-BV711. For intracellular cytokines staining, cells were fixed and permeabilized with BD Fixation/Permeabilization Kit, followed by staining with FITC-anti-interferon-γ (anti-IFN-γ) and PE-anti-IL-17A. Data were acquired by a FACS Aria flow cytometer (BD Biosciences, Mountain View, CA, USA) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA). Representative flow cytometric plot was shown in Figure S1.

2.7 | Statistical Analysis

Data were shown as mean ± standard deviation (SD). Student's *t*-test was applied when two groups were compared for statistical differences. Correlations were determined by Pearson correlation coefficients. Data were analyzed by SPSS 24.0 software, and a p value less than 0.05 was considered to be statistically significant.

3 | Results

3.1 | MRL/*Lpr* Mice Developed Lupus-Prone Symptoms

MRL/*lpr* mice exhibited a lupus-like skin rash with hair loss on face, neck, and back skin. Additionally, they had enlarged lymph nodes in the neck and axillary region, while C3H/HeJ mice showed a normal appearance. The spleen index (0.018 ± 0.005 vs. 0.006 ± 0.001, $p < 0.001$) and kidney index (0.016 ± 0.002 vs. 0.014 ± 0.001, $p < 0.05$, Figure 1A) of MRL/*lpr* mice were significantly higher than those of C3H/HeJ mice, while plasma anti-dsDNA levels were comparable between the two groups (Figure 1B). However, urinary protein levels were significantly higher in MRL/*lpr* mice compared to C3H/HeJ mice (3.29 ± 2.94 μg/μL vs. 0.55 ± 0.59 μg/μL, $p < 0.05$, Figure 1C), while plasma BUN and Cr levels showed no difference (Figure 1C). Histopathologic analysis revealed glomerular sclerosis, mesangial cell proliferation, increased matrix, and lymphocyte infiltration in tubulointerstitial space of MRL/*lpr* mice (Figure 1D).

3.2 | The Distribution of γδT Cells in MRL/*Lpr* Mice

Representative flow cytometric profile of *γδ*T cells and their subsets was provided in Figure S1. Representative flow cytometric profiles showing the frequencies of *γδ*T cells in the spleen, kidney, and blood were provided in Figure 2A. Compared with C3H/HeJ mice, MRL/*lpr* mice showed a significant reduction in the proportion ($0.76\% \pm 0.27\%$ vs. $1.12\% \pm 0.29\%$, $p < 0.05$, Figure 2B) and absolute number ($0.45 \pm 0.11) \times 10^7$ vs. $(1.08 \pm 0.41) \times 10^7$, $p < 0.01$, Figure 2E) of splenic γδT cells. Similarly, there was also a significant decrease in the proportion ($0.19\% \pm 0.15\%$ vs. $1.70\% \pm 0.99\%$, $p < 0.01$, Figure 2C) and absolute number ($(2.36 \pm 2.29) \times 10^4$/mL vs. $(6.68 \pm 2.97) \times 10^4$/mL, $p < 0.05$, Figure 2F) of γδT cells in the peripheral blood of lupus mice. However, an increase was observed in the proportion ($1.54\% \pm 0.42\%$ vs. $0.84\% \pm 0.27\%$, $p < 0.01$, Figure 2D) and absolute number [$(9.04 \pm 2.39) \times 10^4$ vs. $(3.37 \pm 2.16) \times 10^4$, $p < 0.001$, Figure 2G] of γδT cells in renal tissues in lupus mice, indicating that γδT cell might play a role in the pathogenesis of lupus nephritis.

3.3 | The Expression of Different γδT Cell Subsets in MRL/*Lpr* Mice

Next, we evaluated the proportions and absolute numbers of γδT cell subsets in the spleen, peripheral blood, and kidney tissues

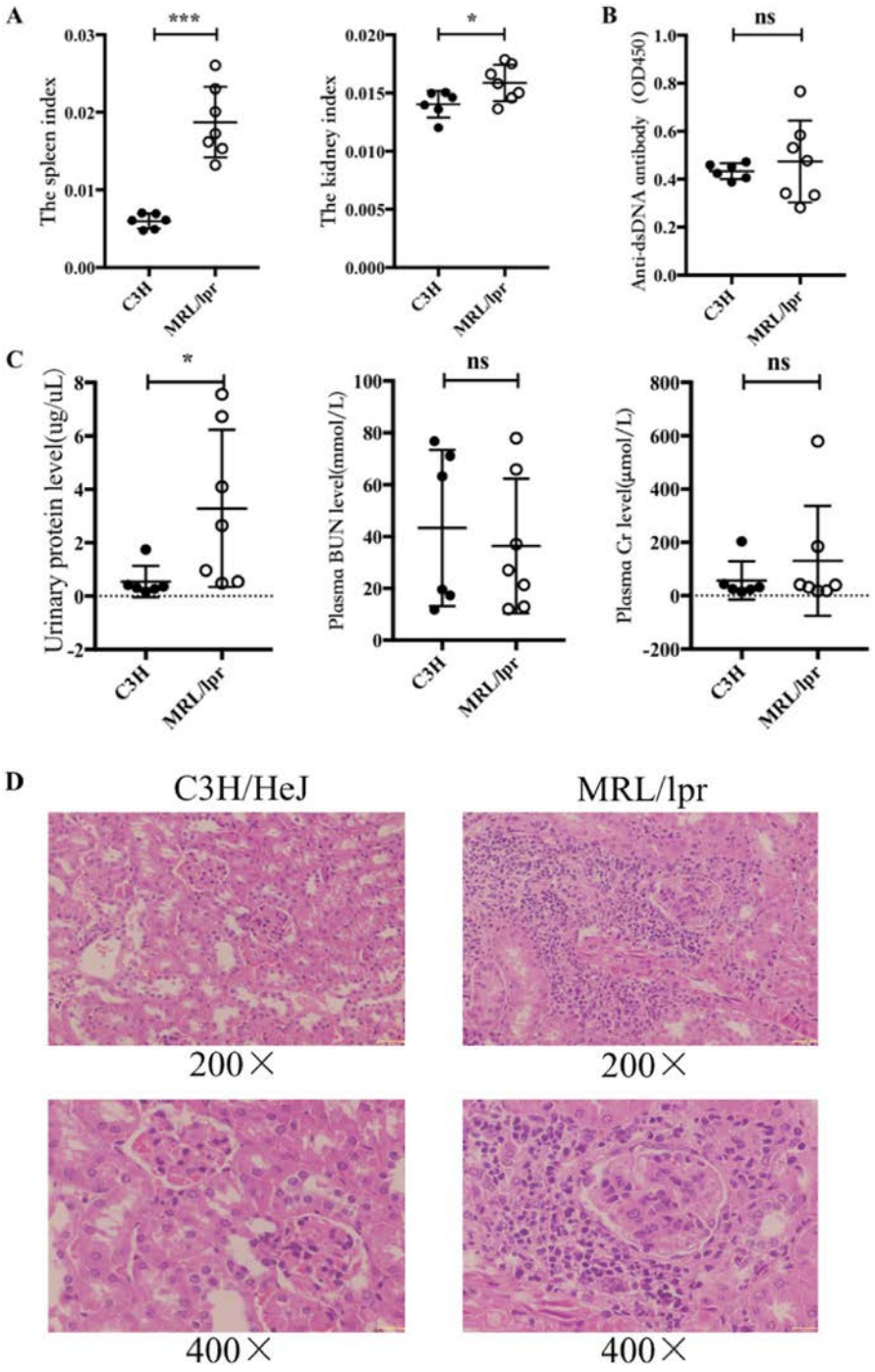

FIGURE 1 | MRL/*lpr* mice developed lupus-prone symptoms. (A) Increased spleen and kidney index in MRL/*lpr* mice compared to C3H/HeJ mice. (B) Plasma anti-dsDNA levels of C3H/HeJ and MRL/*lpr* mice. (C) Plasma BUN, creatinine, and urinary protein levels of MRL/*lpr* and C3H/HeJ mice. (D) Representative images of kidney H&E staining. (For C3H/HeJ mice, $n=6$, for MRL/*lpr* mice, $n=7$; *$p<0.05$, ***$p<0.001$, ns, no significance).

of MRL/*lpr* and C3H/HeJ mice. To distinguish between Vγ4, Vγ5, and double negative subsets, we employed anti-TCR Vγ2 and anti-Vγ3 TCR antibodies as recommended by reference [15]. Additionally, we utilized anti-TCR Vγ1.1 antibody to distinguish between Vγ1 and Vγ6 subsets. Representative flow cytometric profiles showing the frequencies of Vγ6$^+$ γδT cells in the spleen, kidney, and blood were provided in Figure 3A. No significant differences were observed in the proportions and absolute number of Vγ1, Vγ4, Vγ5 subsets in the peripheral blood or kidney tissues between MRL/*lpr* and C3H/HeJ mice (Figures S2, S3 and S4). However, a significant decrease was observed in the proportion (0.109% ± 0.107% vs. 1.29% ± 0.86%, $p<0.01$, Figure 3C) and absolute number [$(1.38\pm1.53)\times10^4$/mL vs. $(4.99\pm2.65)\times10^4$/mL, $p<0.05$, Figure 3F] of Vγ6$^+$γδT cells in the peripheral blood of MRL/*lpr* mice. Similarly, the absolute number of Vγ6$^+$γδT cells in the spleen was significantly decreased [$(1.93\pm1.31)\times10^6$ vs. $(5.15\pm2.56)\times10^6$, $p<0.05$, Figure 3E]. However, the proportion (0.83% ± 0.16% vs. 0.20% ± 0.08%, $p<0.001$, Figure 3D)

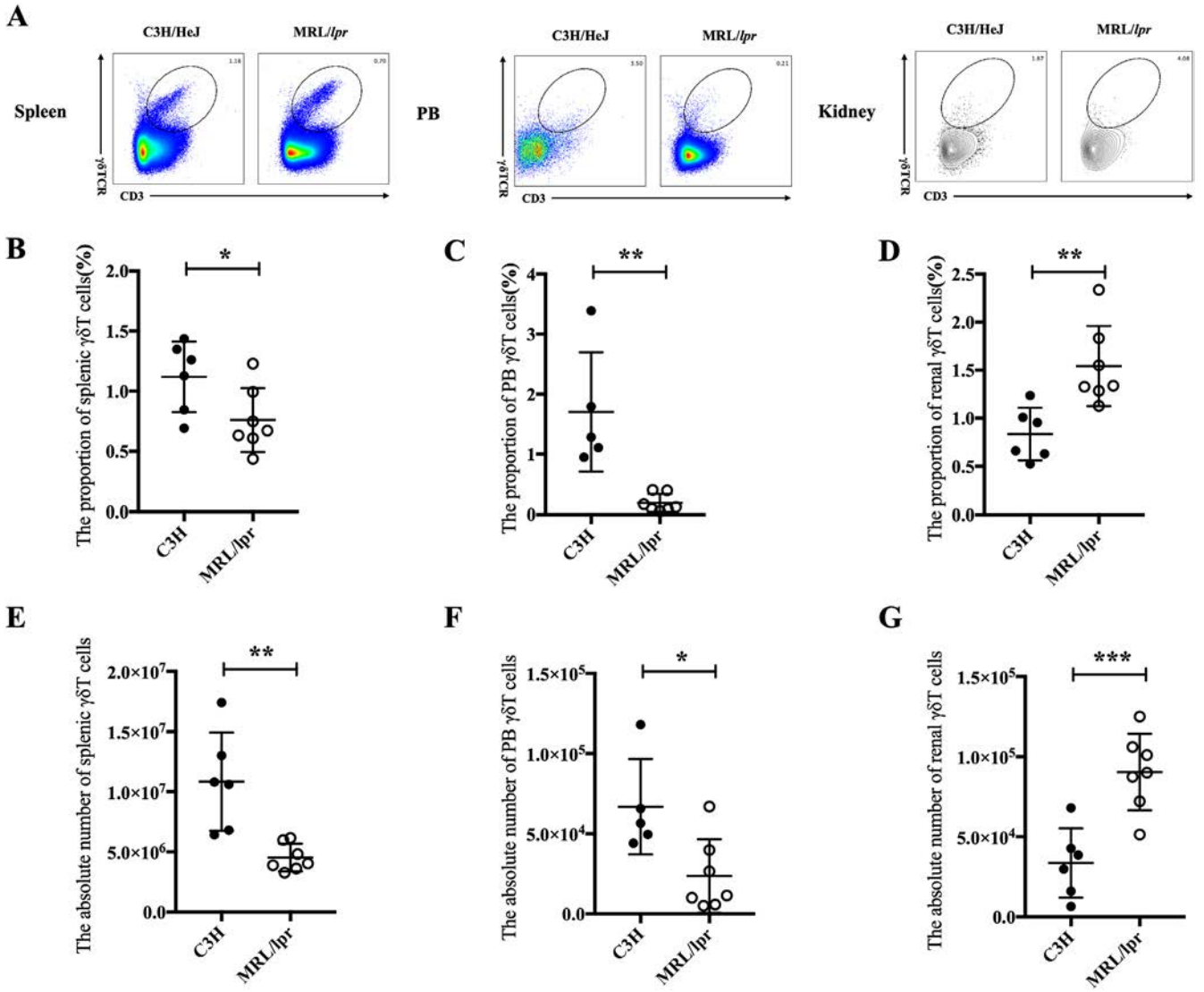

FIGURE 2 | The distribution of γδT cells in MRL/*lpr* mice. (A) Representative flow cytometric profiles showing the frequencies of γδT cells in the spleen, kidney, and blood. (B) The proportion of γδT cells in the spleens of MRL/*lpr* and C3H/HeJ mice. (C) The proportion of γδT cells in the peripheral blood of MRL/*lpr* and C3H/HeJ mice. (D) The proportion of γδT cells in the kidneys of MRL/*lpr* and C3H/HeJ mice. (E) The absolute number of γδT cells in the spleens of MRL/*lpr* and C3H/HeJ mice. (F) The absolute number of γδT cells in the peripheral blood of MRL/*lpr* and C3H/HeJ mice. (G) The absolute number of γδT cells in the kidneys of MRL/*lpr* and C3H/HeJ mice. (For C3H/HeJ mice, $n = 5–6$, for MRL/*lpr* mice, $n = 7$; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

and absolute number [$(4.95 \pm 1.30) \times 10^4$ vs. $(0.67 \pm 0.28) \times 10^4$, $p < 0.001$, Figure 3G] of renal Vγ6$^+$γδT cells were significantly increased in lupus mice, supporting the possible role of Vγ6$^+$γδT cells in the development of renal impairment in lupus mice.

3.4 | Renal Vγ6$^+$γδT Cell Subset Was Positively Correlated With Urinary Protein in MRL/*Lpr* Mice

The correlations between the proportion and absolute number of γδT cells and their subsets, and urinary protein level were analyzed. It was found that the absolute number of renal Vγ6$^+$γδT cells was positively correlated with urinary protein level in MRL/*lpr* mice ($r = 0.81$, $p < 0.05$, Figure 4). This suggests that Vγ6$^+$γδT cells were likely to migrate from periphery to the kidney of MRL/*lpr* mice and contribute to the development of lupus nephritis.

3.5 | Renal Vγ6$^+$γδT1 Cells Are Likely to Involve in the Pathogenesis of Lupus Nephritis by Secreting IFN-γ

To investigate the underlying mechanism of Vγ6$^+$γδT cells participating in the pathogenesis of lupus nephritis, we examined the expression of intracellular cytokines IFN-γ (Vγ6$^+$γδT1) and IL-17A (Vγ6$^+$γδT17) in Vγ6$^+$γδT cells. Representative flow cytometric profiles showing the frequencies of Vγ6$^+$γδT1 cells in the spleen, kidney, and blood were provided in Figure 5A. There were no differences in the proportions and absolute numbers of Vγ6$^+$γδT1 cells in the spleens between MRL/*lpr* mice and C3H/HeJ mice (Figure 5B,E). In comparison to C3H/HeJ mice, MRL/*lpr* mice exhibited a significant decrease in the proportion ($0.00049\% \pm 0.00046\%$ vs. $0.015\% \pm 0.014\%$, $p < 0.05$, Figure 5C) and absolute number [(56 ± 53)/mL vs. (619 ± 560)/mL, $p < 0.05$,

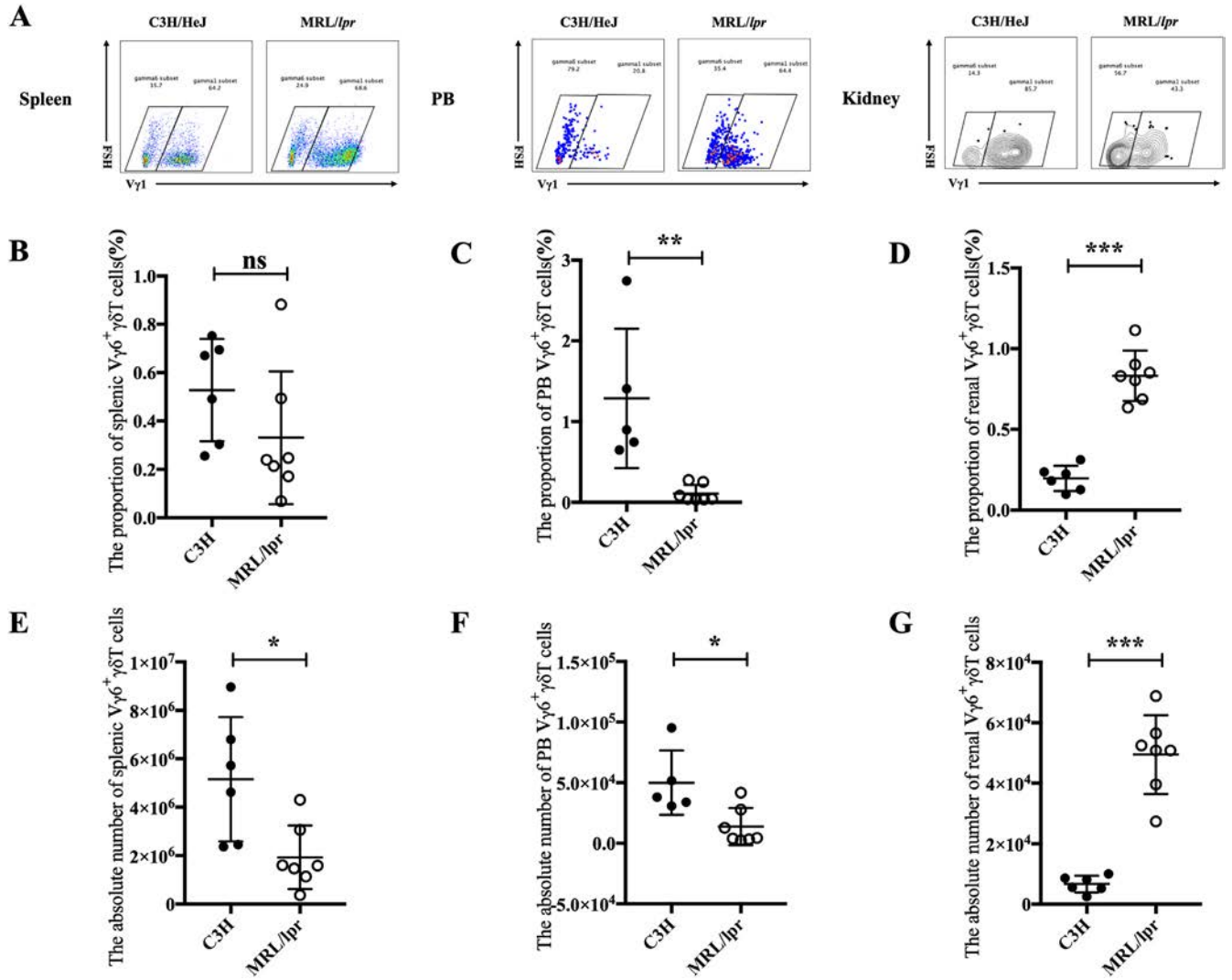

FIGURE 3 | The expression of Vγ6$^+$γδT cell subset in MRL/*lpr* mice. (A) Representative flow cytometric profiles showing the frequencies of Vγ6$^+$ γδT cells in the spleen, kidney and blood. (B) The proportion of Vγ6$^+$ γδT cells in the spleens of MRL/*lpr* and C3H/HeJ mice. (C) The proportion of Vγ6$^+$ γδT cells in the peripheral blood of MRL/*lpr* and C3H/HeJ mice. (D) The proportion of Vγ6$^+$ γδT cells in the kidneys of MRL/*lpr* and C3H/HeJ mice. (E) The absolute number of Vγ6$^+$ γδT cells in the spleens of MRL/*lpr* and C3H/HeJ mice. (F) The absolute number of Vγ6$^+$ γδT cells in the peripheral blood of MRL/*lpr* and C3H/HeJ mice. (G) The absolute number of Vγ6$^+$ γδT cells in the kidneys of MRL/*lpr* and C3H/HeJ mice. ($n = 5$–7, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns, no significance).

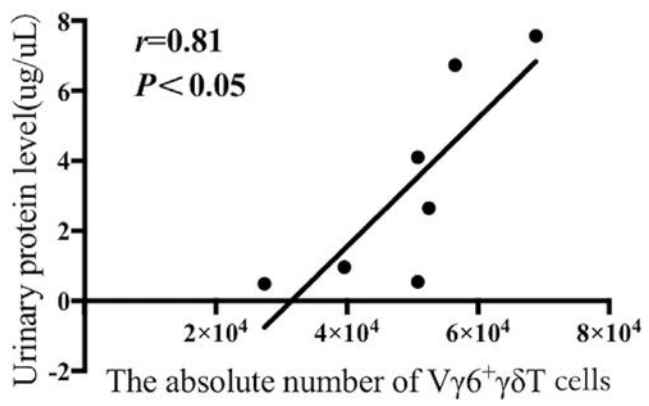

FIGURE 4 | Renal Vγ6$^+$γδT cell subset was positively correlated with urinary protein levels in MRL/*lpr* mice.

Figure 5F] of Vγ6$^+$γδT1 cells in the peripheral blood, but a significant increase in the proportion (0.0071% ± 0.0027% vs. 0.0007% ± 0.0016%, $p < 0.001$, Figure 5D) and absolute number [(4.42 ± 3.05) × 10^3 vs. (1.18 ± 1.44) × 10^3, $p < 0.01$, Figure 5G] of renal Vγ6$^+$γδT1 cells. There were no differences in the proportion and absolute number of Vγ6$^+$γδT17 cells in the peripheral blood and kidney between MRL/*lpr* mice and C3H/HeJ mice (Figure S5). Together, these data suggested that Vγ6$^+$γδT1 cells instead of Vγ6$^+$γδT17 cells may play a significant role in the development of lupus nephritis.

The correlation between the proportion and absolute number of renal Vγ6$^+$γδT1 cells in MRL/*lpr* mice and urinary protein levels was analyzed. There was a positive correlation trend between the proportion ($r = 0.62$, $p = 0.14$, Figure 5H), absolute number ($r = 0.73$, $p = 0.06$, Figure 5I) of Vγ6$^+$γδT1 cells and urinary

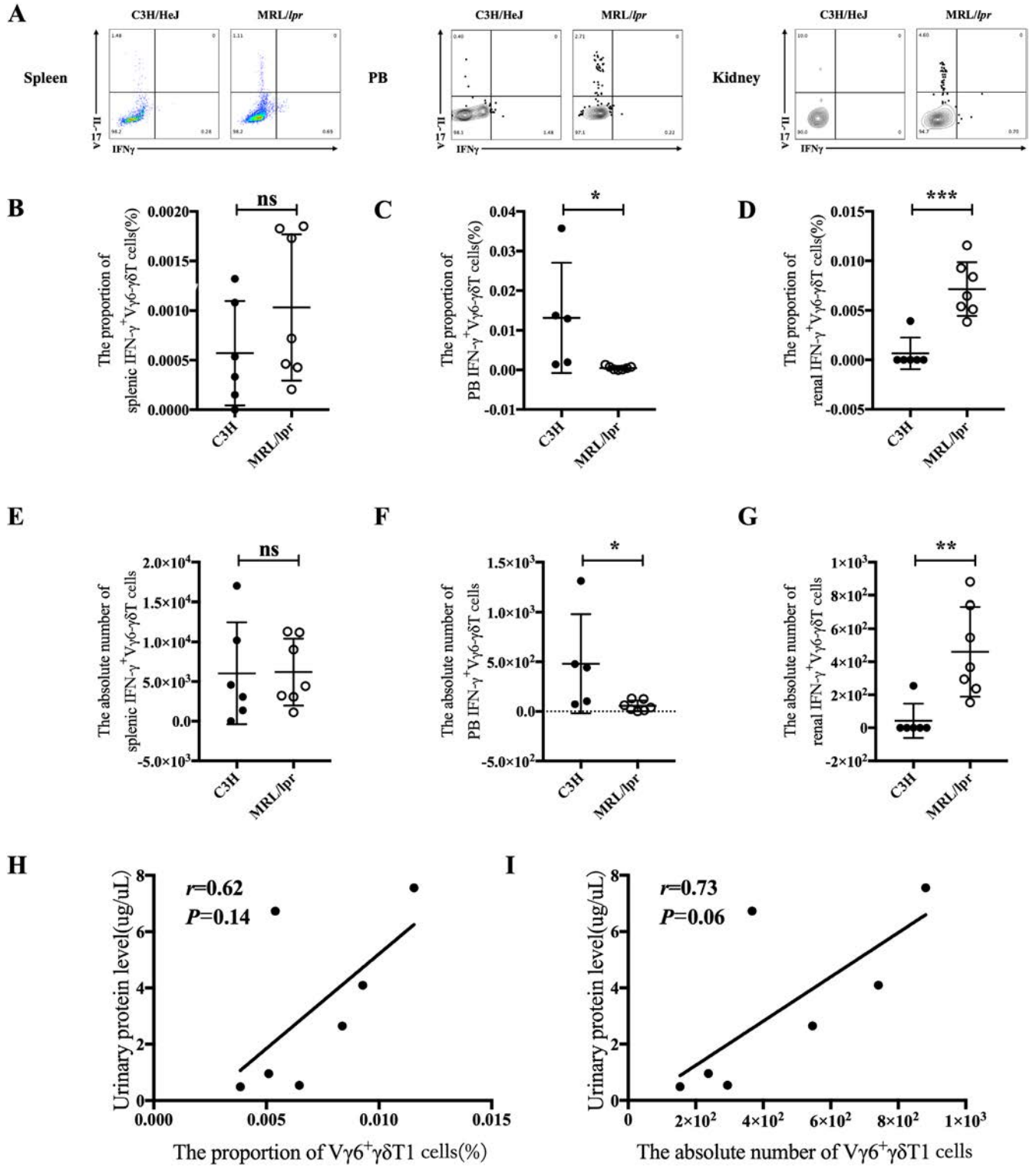

FIGURE 5 | Renal Vγ6$^+$γδT1 cells are likely to involve in the pathogenesis of lupus nephritis by secreting IFN-γ. (A) Representative flow cytometric profiles showing the frequencies of Vγ6$^+$ γδT1 cells in the spleen, kidney, and blood. (B) The proportion of Vγ6$^+$ γδT1 cells in the spleens of MRL/*lpr* and C3H/HeJ mice. (C) The proportion of Vγ6$^+$ γδT1 cells in the peripheral blood of MRL/*lpr* and C3H/HeJ mice. (D) The proportion of Vγ6$^+$ γδT1 cells in the kidneys of MRL/*lpr* and C3H/HeJ mice. (E) The absolute number of Vγ6$^+$ γδT1 cells in the spleens of MRL/*lpr* and C3H/HeJ mice. (F) The absolute number of Vγ6$^+$ γδT1 cells in the peripheral blood of MRL/*lpr* and C3H/HeJ mice. (G) The absolute number of Vγ6$^+$ γδT1 cells in the kidneys of MRL/*lpr* and C3H/HeJ mice. (H and I) Correlation of the proportion (H) and absolute number (I) of renal Vγ6$^+$γδT1 cells with urinary protein level in MRL/*lpr* mice. ($n = 5–7$, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns, no significance).

protein levels. These results suggested that renal Vγ6$^+$γδT cells in MRL/*lpr* mice may play a role in the development of lupus nephritis through the production of the pro-inflammatory cytokine IFN-γ.

4 | Discussion

γδT cells, a subset of innate immune cells, are known to have significant roles in infections, tumors, allergies, and autoimmune disorders [10]. Previous study has demonstrated a significant reduction in γδT cells and Vδ2 subsets in peripheral blood of SLE patients [23, 24], with an accumulation of Vδ2 subsets in the kidneys contributing to the development of lupus nephritis [25]. The exact role of γδT cells and their subsets in the pathogenesis of SLE remains for further study.

In this study, we utilized MRL/*lpr* mice as SLE animal models, and C3H/HeJ mice were used as normal controls for MRL/*lpr* mice [26, 27]. Our findings demonstrated a notable decrease in the proportion and absolute number of γδT cells and Vγ6$^+$γδT cell in the peripheral blood of MRL/*lpr* mice, accompanied by a significant increase in the kidneys. Moreover, the absolute number of Vγ6$^+$γδT cells in the kidney was positively correlated with urinary protein levels. γδT cells are widely recognized for their chemotaxis in various diseases. Vγ4$^+$γδT cells and Vγ6$^+$γδT cells were distributed in mouse lungs, expressing chemokine receptors such as CCR2, CCR6, CCR9, CCR10, and secreting inflammation cytokines such as IL-2, IL-4, IL-17A, IFN-γ, thereby exerting a protective role in lung infection, allergic disease, pulmonary fibrosis, and lung tumor [28–31]. Vγ4$^+$γδT cells in the skin migrated between the dermis and skin lymph nodes and produced IL-17A to induce skin inflammation. γδT17 cells expressed chemokine receptor CCR6 and migrated through the CCL20/CCR6 axis, participating in the pathogenesis of psoriasis [32]. Mo et al. found that peripheral Vδ2$^+$γδT cells were significantly lower in patients with RA. Vδ2$^+$γδT cells from RA accumulated in the synovium and produced high levels of pro-inflammatory cytokines including IFN-γ and IL-17A. Vδ2$^+$γδ T cells from RA showed elevated chemotaxis potential and expressed high levels of chemokine receptors CCR5 and CXCR3, which was driven by increased serum TNF-α through nuclear factor kappa B signaling. Their results indicated that high levels of TNF-α promote CCR5 and CXCR3 expression in Vδ2$^+$γδT cells from RA, which potentially infiltrated into the synovium and play crucial roles in the pathogenesis of RA [33]. Therefore, we speculated that γδT cells, especially their Vγ6 subsets were likely to migrate from peripheral blood to the kidney, which may participate in the pathogenesis of lupus nephritis.

Previously, Vγ6$^+$γδT cells possess unique tissue-protective properties in the renal microenvironment, highlighting their potential as therapeutic targets for kidney-related disorders [34]. In our study, we detected the expressions of intracellular cytokines in Vγ6$^+$γδT cells to further investigate the mechanism of how Vγ6$^+$γδT cells participate in the pathogenesis of lupus nephritis. We found that both the proportion and absolute number of Vγ6$^+$γδT1 cells significantly increased in the kidney and were positively correlated with the urinary protein levels. IFN-γ is crucial for defending against intracellular pathogens, regulating immune and inflammatory responses, and related tissue damage and tumor immune surveillance [35, 36], can affect the functions of CD4$^+$T cells, CD8$^+$T cells, B cells, and macrophages. In mouse lupus models, the application of anti-IFN-γ has shown promising results in preventing autoantibody production, inhibiting the onset of glomerulonephritis, mitigating chronic murine lupus pathology, and delaying lupus development and progression [37]. Several transcriptomic studies have found that IFN-γ is associated with disease activity, lupus nephritis, and pregnancy complications in SLE patients [38]. Our findings suggest that renal Vγ6$^+$γδT cells in MRL/*lpr* mice may be involved in the pathogenesis of lupus nephritis by producing the pro-inflammatory cytokine IFN-γ.

In conclusion, our study has provided novel evidence indicating that peripheral Vγ6$^+$γδT cells play a role in the development of lupus nephritis by infiltrating in the kidney and secreting IFN-γ. However, the exact mechanism underlying the chemotaxis of Vγ6$^+$γδT cells from peripheral blood to kidney, the specific signals and pathways involved in this migration process, needs further investigation.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Yunxia Yan, Yue Zhang, Xiaojun Tang, Zhang Zhuoya, Geng Linyu, and Sun Lingyun. The first draft of the manuscript was written by Yunxia Yan, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Acknowledgments

The authors acknowledge Dr. Shufang Wu, Dr. Yan Cong, and Dr. Xia Zhao for their help in sample collection.

Disclosure

The authors have nothing to report.

Ethics Statement

All animal studies were approved by Committee of Experimental Animal Administration of the Affiliated Drum Tower Hospital of Nanjing University Medical School (approval number: 2019AE01033).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The authors have nothing to report.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

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LETTER TO THE EDITOR

A Short Report on Prescription Monitoring in the Therapeutic Context of Rheumatoid Arthritis at an Italian Local Health Authority

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Received: 31 October 2024 | **Revised:** 14 December 2024 | **Accepted:** 21 December 2024

Funding: The authors received no specific funding for this work.

Dear Editor,

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease affecting approximately 1% of the worldwide population. In Western populations, the prevalence ranges between 0.3% and 1.1%, whereas in Eastern populations it approximately ranges between 0.1% and 0.5% [1, 2]. At first, this disease hits the small joints. Secondarily, it can also affect other organs or systems. The pathogenesis of RA involves the production of autoantibodies, which contribute to the damage of bones and cartilage. It is crucial to clarify RA mechanisms before launching targeted therapies that can contrast this autoimmune condition. Generally, the highest incidence rates emerge between 50 and 75 years of age, with a higher incidence in women compared with men. Patients with RA begin treatment with non-biologic disease-modifying antirheumatic drugs. In case of an insufficient response, escalation to non-biologic disease-modifying antirheumatic drugs (DMARDs) can be done [1, 2]. The induction of clinical and functional remission and the prevention of radiographic progression of the disease are the two main objectives of pharmacological intervention in RA. When biological drugs have been introduced, a new era in the treatment of RA began. This period was also characterized by a pronounced increase in prescription rates. Biologics are extremely expensive, generating a dilemma for the National Healthcare Service (NHS) in trying to balance between the need to cut healthcare expenditure and the fundamental right of every citizen to receive medical treatment [3]. The major objective of RA treatment is to attain disease remission as early as possible. Thus, the administration of these therapies has progressively moved to an earlier point in the disease course. Commonly, the response of the biological drug therapies is observed after 12–24 weeks of administration. The objective of this study was to monitor the prescription patterns of the three key classes of medications used in the treatment of RA—that is, anti-tumor necrosis factor alpha agents (TNF alpha), injectable biologics and the JAK-inhibitors agents as new oral formulations. In particular, this study aimed to give some insights into the evolving landscape of prescription patterns, focusing on the comparative use and economic observations in the management of RA disease. Defined daily dose (DDD), a unit of drug utilization, has been studied. A cost related economic analysis was conducted, to ascertain the cost burden associated with those classes of drugs. Such assessments are indicated when the ultimate purpose is the most efficient use of resources in combating health problems. These models are widely used in economic evaluations within healthcare when addressing issues involving optimization of resources [4].

The prescribing behavior has been analyzed for the Local Health Authority of Napoli 3 Sud, a metropolitan area in the South of Italy (LHA NA 3 Sud). The consumption expressed in DDD and costs expressed in euros have been analyzed. The data on drug consumption in DDDs and the expenditures in euros were retrieved for the second half of 2022 and 2023 from the organizational management system. The information is targeted

Abbreviations: ATC, Anatomical Therapeutic Chemical classification system; DDD, defined daily doses; DMARDs, non-biologic disease-modifying antirheumatic drugs; LSA, Local Sanitary Agency; NHS, National Healthcare Systems; RA, rheumatoid arthritis; TNFα, tumor necrosis factor alpha agents.

to monitor the prescribing landscape of RA treatments. As a result, the ultimate purpose of enabling evaluations related to disease progression, the proportion of each drug type used for the treatment, public pharmaceutical expenditure for RA management, use of drugs offering cost savings [3–5]. All RA treatments were aggregated into three categories of therapy: (1) anti-TNFα originators and biosimilars; (2) other patent-protected biologic injectables for which biosimilars are not yet available; and (3) oral drugs. All drugs used for RA have been extracted by means of a search using classification and the Anatomical Therapeutic Chemical classification system (ATC) code. Then, they have been subdivided by type into the three therapeutic categories.

Overall, expenditure went from €6 621 716.86 in the second half of the year 2022 to €6 930 293.44 in the second semester of 2023; drug consumption increased from 370 478.04 DDD to 460 999.36 DDD. In particular, anti-TNFα drugs increased DDDs in 2023, whereas the actual spending dropped steadily due to the higher use of biosimilar versions. For the other two therapeutic classes, nearly all medications increased consumption and expenditure.

In the second half of 2022, the expenditure on RA therapies was divided into other biologic injectables, 64% of all expenditure, anti-TNFα drugs, 25% of total expenditure, and oral formulations accounted for only 11% of costs (Figure 1). In the second half of 2023, there was a remarkable surge in oral formulation drugs, representing 14% of the overall RA drug expenditure. Other biologic injectables recorded a few spending increases, confirming their share of 64% of the total expenditure on RA. On the other hand, anti-TNFα drug expenditure went down due to a major use of biosimilars, accounting for a total expenditure reduction to 22% (Figure 2).

Rheumatoid arthritis is a chronic inflammatory disease whose prevalence is constantly growing worldwide. Similarly, the need for its treatment is also growing accordingly, with a significant rise after the COVID-19 pandemic [6, 7]. This demand has thus expanded the therapeutic armamentarium in the clinical field of rheumatology [6, 8, 9]. Along with conventional DMARDs and TNFα inhibitors, multiple injectable drugs that interfere with other inflammatory mediators have been developed, for

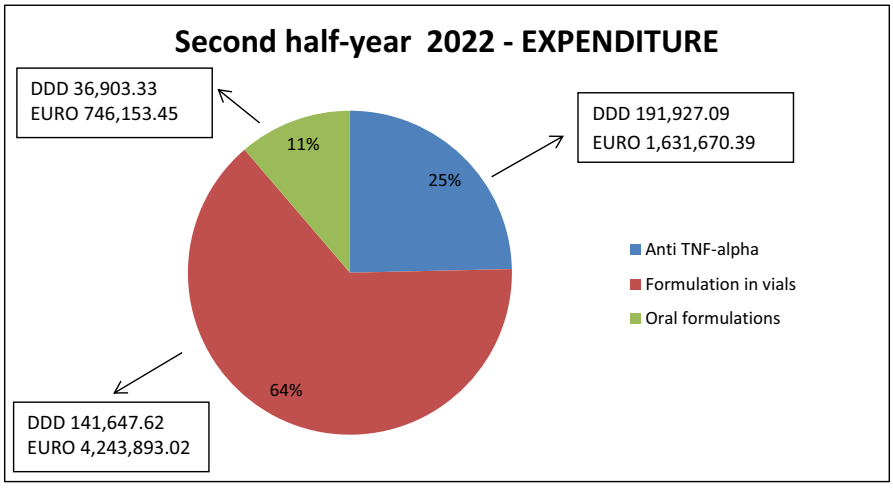

FIGURE 1 | Analysis of consumption and expenditure incurred for the three main classes of drugs used for the treatment of rheumatoid arthritis in the second semester of 2022 at the Local Health Authority Naples 3 South.

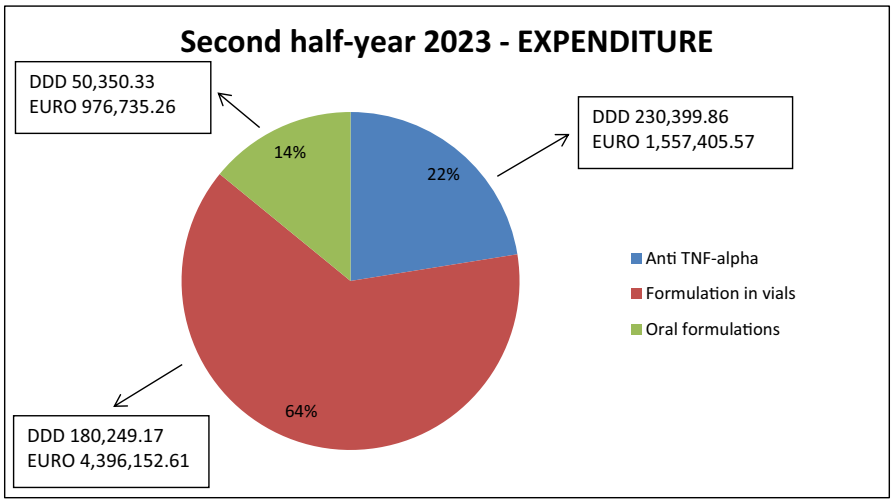

FIGURE 2 | Analysis of consumption and expenditure incurred for the three main classes of drugs used for the treatment of rheumatoid arthritis in the second semester of 2023 at the Local Health Authority Naples 3 South.

example, interleukins. These interleukin inhibitors have a role as an alternative in those patients who develop intolerance to DMARDs or anti-TNFα medications, or in whom adverse effects are present. Moreover, in recent years new oral drugs inhibiting Jak kinases have become available, with a more convenient oral format that enhances patient compliance and therapeutic response. As more young adults are being diagnosed with RA, oral medications allow a more flexible lifestyle and help keep to regular patterns of work, reducing the rate of services being taken from health facilities [10]. This explains why in the second half of 2023 prescriptions for RA drugs increased, compared to 2022. Although all categories were higher in utilization, including anti-TNFα drugs, biologic injectables, and oral drugs, due to biosimilars being adopted, only the cost of anti-TNFα drugs was reduced in cost. In contrast, other injectables and oral formulations had sharp rises in costs. In Naples, due to the substitution effect of the original formulation, the biosimilar anti-TNFα drugs represented a significant uptake and cost reduction. Although the savings through biosimilars have been made in practice, the use of newer oral therapies contributes toward increasing overall RA treatment costs. This study will be a report again pointing out the value of having effective cost control and prescription regulation based upon real-world evidence [11, 12]. While newer therapies expand options for treatments, their cost-effectiveness must be of prime concern. Traditional DMARDs and anti-TNFα agents should remain the first-line choice of treatment in treatment-naive patients. The more expensive agents should be reserved for resistant or intolerant cases, to ensure appropriate resource utilization in RA management. This is where Digital Therapeutics come in, capable of backing treatment plans, aided by algorithms to find the most clinically and economically favorable therapies. Special reference will be given to RA, especially on high-cost chronic conditions management. This is the best path to preserve healthcare budgets from unwarranted care, ensuring at that time therapeutic efficacy and adherence by patients [13, 14]. In conclusion, newer treatments for RA are more convenient and effective, but their high costs underline how strategic resource distribution is important for long-term sustainability in healthcare. As a result, it is essential to implement such studies in all the healthcare fields where drug consumption and expenditure can be monitored. These studies highlight the importance of monitoring pharmaceutical expenditure, also to raise awareness of the use of biosimilar drugs, with the aim of optimizing healthcare supply strategies. Applying these policies would not only lead to more efficient management of resources, but would also allow healthcare institutions to distinguish as virtuous reference benchmarks.

Author Contributions

A.Z.: conceptualization, writing – original draft. R.L.: conceptualization, writing – review and editing. F.F.: methodology, supervision, visualization. All authors read and approved the manuscript, all data were generated in-house, and no paper mill was used.

Disclosure

All the authors declare that the opinions expressed are of a personal nature and do not in any way commit the responsibility of the Administrations to which they belong.

Ethics Statement

The authors have nothing to report.

Consent

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The authors have nothing to report.

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LETTER TO THE EDITOR

Gene Expression Trend Pattern Analysis in Peripheral Blood From Patients With Preclinical Systemic Sclerosis

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Received: 28 October 2024 | **Revised:** 11 December 2024 | **Accepted:** 19 December 2024

Funding: This work was supported by Mitacs.

Dear Editor,

In this study, we identified significant blood gene expression trend patterns associated with specific immune cell types in early-stage differentiation in systemic sclerosis (SSc) progression. SSc is a heterogeneous autoimmune connective tissue disease, which is mainly classified as diffuse cutaneous SSc (dcSSc) and limited cutaneous SSc (lcSSc) depending on the extent of skin fibrosis [1]. Although SSc is a chronic immune disorder like allergic rhinitis (AR), the antigen type in SSc is a self-antigen, in contrast to a foreign antigen in AR. We previously reported blood immune gene signatures associated with immune cell frequency change and clinical symptoms after nasal allergen challenge in patients with AR, which was used to identify immune genes with significantly different trend patterns after immunotherapy [2, 3]. Investigating time series gene expression from peripheral blood is useful for understanding systemic immune response changes reflecting the progression or pathophysiology of the diseases [2, 4].

We hypothesize that trend pattern analysis of time series blood gene expression, namely regrouping gene expression clusters into simplified trend patterns (up, down, and steady) by a given ratio threshold, can provide instinctual interpretable insight to understand the pathophysiological systemic immune response that occurs during the development of the main SSc manifestations, such as fibrosis of the skin and/or inner organs. Specifically, such an analysis may provide a viewpoint to understand key immune cell frequency or activation changes at critical crossroads during the course of the disease, particularly in early immune disease progression.

To test our hypothesis, we used public data, GSE224849, which Bellocchi et al. recently registered to report a global gene expression study of patients with preclinical systemic sclerosis (PreSSc) [5]. PreSSc patients with the early signs of SSc were classified by the LeRoy & Medsger classification criteria—the presence of Raynaud's phenomenon plus SSc-specific autoantibodies and/or SSc-specific nailfold video-capilaroscopic change without any other sign of definite SSc and/or fibrosis [1, 5]. The RNA-sequencing data were generated using blood from two PreSSc patient groups with different disease progression stages at follow-up visits.

The blood samples from 33 PreSSc patients were collected at baseline and follow-up visits (4 years later). Fourteen patients developed lcSSc (Evolving-PreSSc), a severe form of SSc with skin features such as puffy fingers and/or skin fibrosis, whereas 19 patients remained stable (Stable-PreSSc) at follow-up: Evolving-PreSSc was classified at follow-up visit as the patients with definite SSc, herein lcSSc, by reaching the minimum score of 9 based on the 2013 ACR/EULAR classification criteria for SSc. Evolving-PreSSc was characterized as having mainly puffy fingers and/or partly telangiectasia (spider veins) or sclerodactyly: Patients with a definite SSc but without skin fibrosis yet with puffy fingers were also categorized in the lcSSc group [5]. Sixteen healthy controls were characterized who had similar age, ethnicity, and geographical residence to patients. To investigate the gene expression trends across time, we had a set of assumptions:

Assumption 1. Healthy controls represent the stage of pre-onset in PreSSc.

Assumption 2. The expression trend of most genes is steady (homeostasis).

Based on Assumption 1, we used the gene expression measurements in healthy controls as the measurements at pre-onset (hypothetical time-point) in PreSSc, which allowed us to have three-time points with the same start point in both PreSSc subgroups (13 evolving PreSSc patients—we excluded one patient with missing data—and 19 stable PreSSc patients) (Figure 1A): Pre-onset, baseline post-onset, and follow-up post-onset; two periods, period 1 (from pre-onset to baseline post-onset) and period 2 (from baseline post-onset to follow-up post-onset).

Based on Assumption 2, a ratio threshold—a distinguishable relative change—provides a rule to decide what change is negligible small naturally-occurring fluctuations or oscillation, which was used for selecting genes and regrouping clusters into trends (Figure 1C,F). We used three commonly-applied ratio thresholds: 1.2 (low), 1.5 (medium), and 2.0 (high).

For preprocessing, genes (raw data) with low counts across all samples were filtered out using the edgeR R package (version 4.0.16). Subsequently, in each PreSSc subgroup, the remaining 20 587 genes (TPM normalized data) were evaluated given a ratio threshold: for example, under low-ratio settings, genes with a maximum to minimum ratio of mean expression across time greater or equal to 1.2 were selected (Figure 1C). We applied ANOVA with permutation (lmPerm R package, version 2.1.0; 999 999 permutations) to the filtered genes (a total of 10 580 genes in Stable-PreSSc and 10 421 genes in Evolving-PreSSc) to identify those showing differential expression over time: In Stable-PreSSc there were 5469 significant (False discovery rate, FDR <0.1) genes at low threshold ratio; 1699 genes at medium-ratio; and 265 genes at high-ratio. In Evolving-PreSSc (with a smaller sample size) there were 17 significant genes at low-ratio; 824 genes at medium-ratio; and 123 genes at high-ratio (Figure 1D).

Hierarchical clustering was performed on the pooled significant genes (5591 genes in Stable-PreSSc; 827 genes in Evolving-PreSSc) in each group using the dtwclust R package (version 6.0.0) after standardizing the mean expression of each gene (mean = 0, SD = 1). Based on the similarity of their expression patterns, genes clustered into 24 groups (Figure 1G). Then, after evaluating the trend pattern of mean values at each time in each cluster, the clusters were regrouped into nine interpretable trends. The nine trend patterns were based on the permutation with repetition of expression trends over the two time periods ($n^r = 9$, when $n = 3$: up, down, and steady between two successive time points; $r = 2$: period 1 and 2) (Figure 1E,G). The ratio thresholds helped to determine whether a trend in a particular time period was negligible (slope 0, steady trend) or significant (upward or downward trend) compared with the other time period (Figure 1F). Most of the overlapped significant genes between Stable- and Evolving-PreSScs showed consistent expression trends in period 1 but varied in period 2 between PreSSc groups, which may imply the association between differential gene expression at period 2 and the differential disease progression between Stable- and Evolving-PreSScs (Figure 2A).

Significant genes (FDR <0.1) in a given ratio threshold with specific expression trends were applied to cell enrichment analyses using Enrichr (blood cell types, gene set library: HuBMAP_ASCTplusB_augmented_2022) (Figure 2A,B). Stable-PreSSc showed three clusters were significantly associated with immune cells; Evolving-PreSSc, four clusters (Figure 2B). These showed enriched and intriguing results between the two groups besides the down-regulation of cytotoxic/NK cells in Evolving-PreSSc that Bellocchi et al. [5] reported with modular analysis.

In Stable-PreSSc, although CD14-positive monocytes, classical monocytes, were associated with both C1 up-up and C3 up-down patterns, CD14-low-CD16-positive monocytes, nonclassical monocytes, were only associated with C3 up-down pattern (Figure 2B). Monocytes are early responders to pathogens and maintain vascular homeostasis beyond simply being macrophage precursors; particularly nonclassical monocytes that recognize and clear dying endothelial cells to maintain vascular homeostasis can contribute to the inflammation associated with chronic diseases [6]. Intriguingly, peripheral blood nonclassical monocytes were overrepresented in SSc compared with healthy control and characterized by increased prostaglandin E synthase gene expression [7]. Nonclassical monocytes and M2 macrophages have recently been emphasized as key drivers of inflammatory and fibrotic manifestations in SSc [7, 8]. Thus, the normalization of CD14-low-CD16-positive monocytes activated by sensing connective or vascular tissue damage is likely associated with stabilizing the disease as Stable-PreSSc showed. CD4-positive, CD8-positive, and regulatory T cells were significantly associated with the C8 down-steady pattern, still, CD4-positive T cells and neutrophils had the C3 up-down pattern. The monocyte subtype changes and downregulated T cells recalled the significantly different proportion of low-dose aspirin administration in two groups (100% Stable-PreSSc vs. 64% Evolving-PreSSc), the only significant difference in the demographics comparison [5]. Aspirin enhances the anti-inflammatory and anti-regulatory functional activities of monocytes while it downregulates CD16 and CD40 expression [9]. Dose-dependent effects of aspirin on immune cells and endothelial cells are anti-inflammatory but varied [10, 11]. Further studies on prescription timing and dose-dependent effects of aspirin are needed to elucidate the potential benefits in the treatment of SSc (Figure 2C).

In Evolving-PreSSc, B cells were associated with C2 up-steady or C3 up-down patterns: B cells were activated or increased in period 1, and the upregulation remained steady or normalized in period 2. B cell depletion therapies in the early stage of SSc ameliorated skin fibrosis [12]. Reduction of apoptotic signaling in B cell transcriptome in patients with systemic lupus erythematosus compared with healthy controls was associated with puffy fingers [13]. B cell activation and their disease-specific autoantibodies may play a key role in the development of manifestations. In contrast, CD8-positive T cells and Natural Killer (NK) cells were associated with C8 down-steady or C9 down-down patterns. These cell types have important anti-fibrotic effects on the immune system. Although their role may be controversial in fibrosis depending on the target, CD8-positive T cells control fibrosis by inducing apoptosis of myofibroblasts, a key cell type in fibrosis [14]. Depletion of CD8-positive T cells has been shown to exacerbate fibrosis whereas depletion of CD4-positive T cells reduced fibrosis in a mouse model of renal fibrosis [15]. Noticeably, the downward trend was associated with CD16-negative-CD56-bright NK cells exerting immunoregulatory

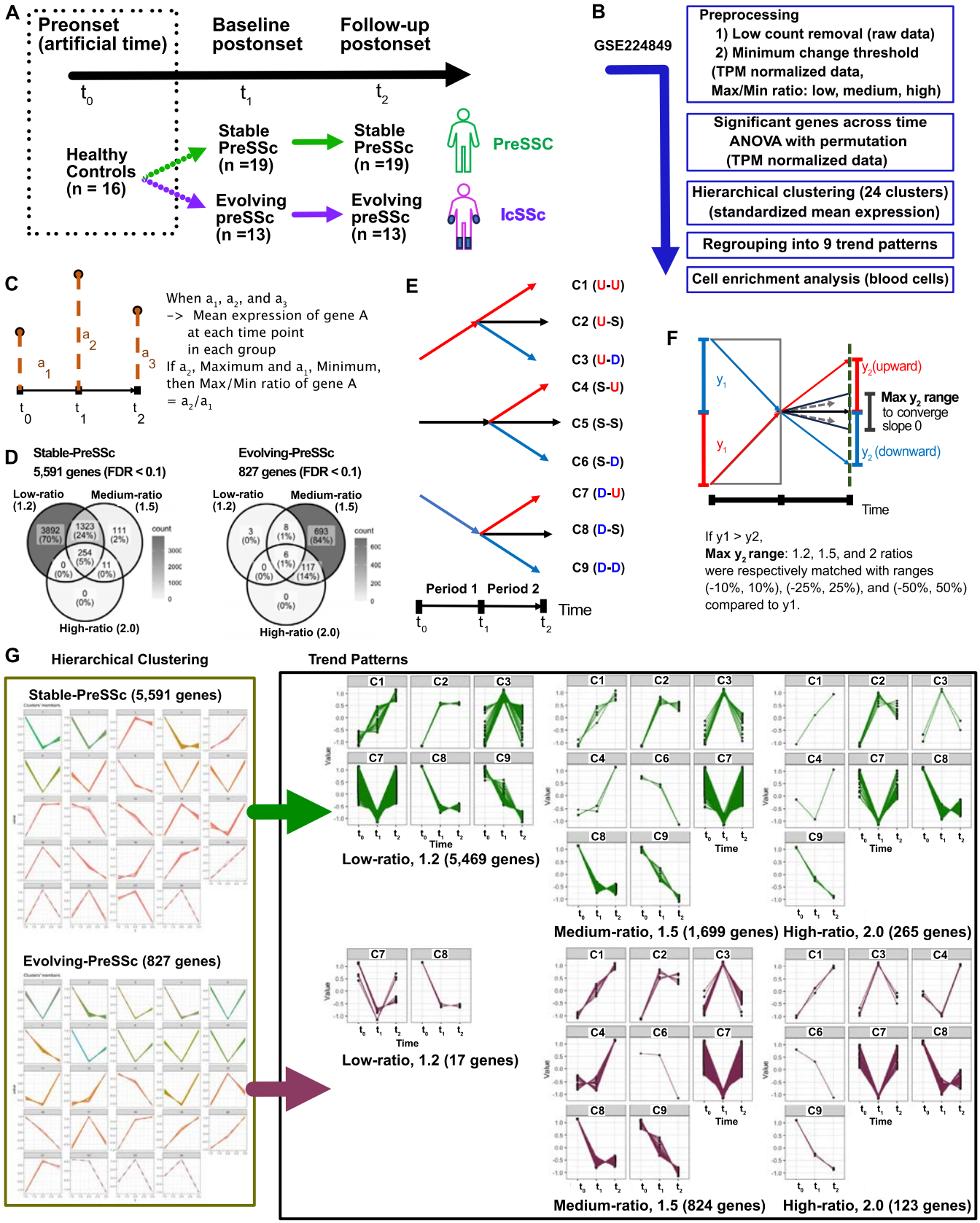

FIGURE 1 | (A) The time series sets using heathy controls (the preonset-hypothetical time-point) in Stable- and Evolving-PreSScs: PreSSc, pre-clinical systemic sclerosis; lcSSc, limited cutaneous systemic sclerosis. (B) The workflow: TPM, transcripts per million. (C) The maximum to minimum ratio of mean expression of a gene across time. (D) Venn diagram for significant genes (FDR < 0.1, ANOVA with permutation) depending on ratio threshold sets in Stable- and Evolving-PreSScs. (E) The simplified nine trend pattern clusters across three time points: C, cluster; U, up/upward; D, down/downward; S, steady. (F) Maximum range of smaller change between period 1 or 2 to converge slope 0 (Steady) compared with bigger change in the other period depending on ratio threshold sets. (G) Regrouping small clusters clustering into nine trend patterns in Stable- and Evolving-PreSScs.

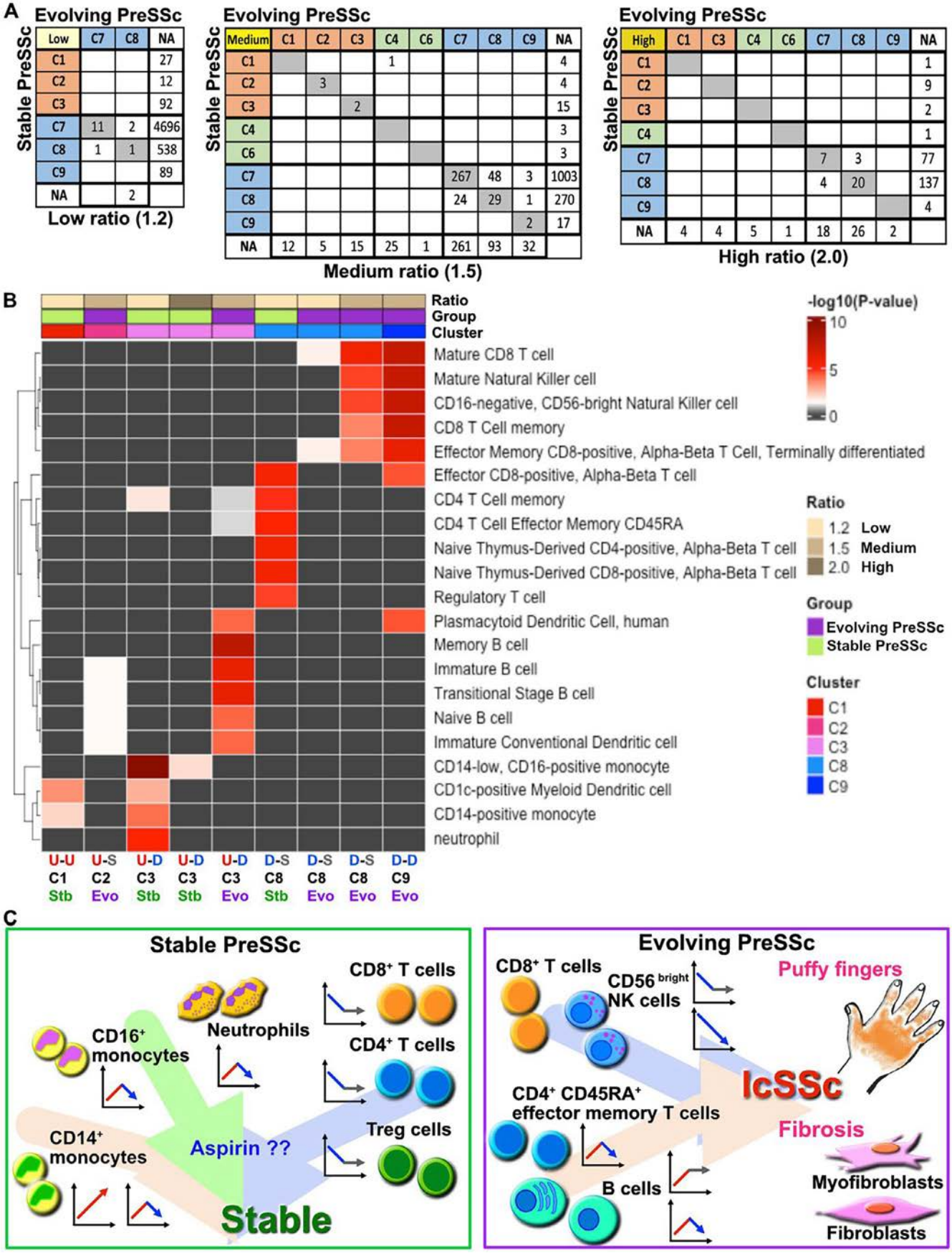

FIGURE 2 | Legend on next page.

FIGURE 2 | (A) Overlapped significant genes and their trend pattern clusters in Stable- and Evolving-PreSScs depending on ratio threshold sets: At low ratio threshold, 5469 genes in Stable-PreSSc and 17 genes in Evolving-PreSSc; at medium ratio threshold, 1699 genes in Stable-PreSSc and 824 genes in Evolving-PreSSc; and, at high ratio threshold, 265 genes in Stable-PreSSc and 123 genes in Evolving-PreSSc: NA, not applicable (Not significant in the other group). (B) Cell enrichment analysis results (Enrichr, blood cell types): C, cluster; U, up/upward; D, down/downward; S, steady. (C) Summary of the cell enrichment analysis in Stable- and Evolving-PreSScs.

and immunosuppressive effects on both innate and adaptive immune cells as potent cytokine and chemokine producers and cytotoxic cells against autologous activated CD4-positive T cells [16]. The opposing patterns between the upregulation of B cells and the downregulation of CD8-positive T cells and CD16-negative-CD56-bright NK cells may indicate a pro-fibrotic and pro-inflammatory environment exacerbating puffy fingers and skin fibrosis in Evolving-PreSSc (Figure 2B,C).

In both Stable- and Evolving-PreSScs, the gene expression trend associated with CD4-positive-CD45RA-positive effector memory T cells (CD4-positive TEMRA), a CD4-positive memory T cell subtype associated with senescence, were significantly identified but the trend patterns were different: C8 down-steady in Stable-PreSSc but C3 up-down in Evolving-PreSSc (Figure 2B). The up-regulated CD4-positive TEMRA in period 1 in Evolving-PreSSc likely contributed to the disease development while considering the relationship between various diseases including autoimmunity and senescent T cells including CD4-positive TEMRA [17].

In addition, although effector CD8-positive αβ T cells had a downward trend in period 1 in both PreSScs, the cause of the trend may be different between PreSScs while considering the other lymphocyte patterns in period 1: downregulation of CD4-positive and CD8-positive T cells and regulatory T cells in Stable-PreSSc; but upregulation of B cells and CD4-positive memory T cells whereas downregulation of CD8-positive T cells and NK cells in Evolving-PreSSc. The downregulation of CD8-positive T cells including effector CD8-positive αβ T cells in Evolving-PreSSc may be associated with type 2 immunity rather than immune inhibition. A relative predominance of type 2 responses (pro-fibrotic response) and of type 1 and 17 responses were associated with, respectively, early and later in the disease course of SSc [18].

The utility of blood gene expression trend pattern analysis was demonstrated by identifying significantly distinguishable alternations of specific immune cell types in early-stage differentiation in SSc progression. Further research is needed to clarify the immune cell-frequency changes or -regulation in peripheral blood while considering T cell senescence, leukocyte migration to target cells, and their relationship with progress in chronic inflammation. More robust conclusions can be drawn from larger cohorts with more longitudinal measures including real pre-onset measurement in the skin/organs and blood, considering immune cell frequency, gene interactions, sophisticated threshold setting, and a single cell-level approach, such as single-cell RNA-sequencing.

Author Contributions

Y.W.K. designed the study and performed bioinformatics and statistical analysis; S.J.T. and A.S. supervised the project; Y.W.K. wrote the first draft of the manuscript; and all authors reviewed and contributed to the final version of the manuscript.

Acknowledgments

We thank the participants and the research/clinical staff in the GSE224849 project. This work was supported by Mitacs Elevate, and the Prevention of Organ Failure Centre of Excellence. We thank Dr. Katherine Adolphs and Dr. Evan Phillips for editorial assistance.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in GEO at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE224849, reference number GSE224849.

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