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ORIGINAL ARTICLE

CD244 Expression by Lymphocytes in Rheumatoid Arthritis With Concomitant Hepatitis C Infection

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ABSTRACT

Aim: We aimed to assess the populations of lymphocytes: NK cells, NKT cells and T cells, and the expression of the immunoregulatory receptor CD244 (2B4) by each population in rheumatoid arthritis (RA) patients with concomitant hepatitis C virus (HCV) infection.

Methods: Lymphocyte phenotyping for NK cells (CD3-CD56+), NKT cells (CD3 + CD56+), and T cells (CD3 + CD56-) was analyzed by flow cytometry in patients with HCV positive RA ($n = 21$), HCV negative RA ($n = 20$) and controls ($n = 11$). Surface expression of CD244 by each lymphocyte population was quantified as mean fluorescence intensity and frequency of CD244 expressing cells.

Results: Patients with and without concomitant HCV displayed comparable numbers of NK, NKT, and T cells to controls. We observed that the expression intensity of CD244 was downregulated by the lymphocyte populations in HCV positive and HCV negative RA compared to controls (NK CD244 $p < 0.001$, NKT CD244 $p = 0.047$, T CD244 $p < 0.001$). Yet, the downregulation was more profound in HCV positive RA, as CD244 expression by NK cells and T cells was decreased in HCV positive in relation to negative RA ($p < 0.001$ and $p = 0.003$, respectively). In addition, the frequency of CD244-expressing NK and NKT cells in HCV positive RA was lower compared to HCV negative RA and controls ($p < 0.001$ and $p = 0.001$, respectively). T cell CD244 expression was significant for discriminating clinically active from less active RA with and without HCV ($n = 41$), area under the curve = 0.717 (95% CI 0.560–0.873, $p = 0.007$).

Conclusion: Our findings reveal a distinct expression pattern of CD244 by lymphocytes of RA with HCV infection.

1 | Introduction

Despite the progress made toward hepatitis C virus (HCV) elimination by direct antiviral agents (DAA), HCV remains prevalent and presents a diagnostic and therapeutic challenge in patients with rheumatoid arthritis (RA). The estimated global prevalence of viraemic HCV infection was 0.7% in 2020, corresponding to 56.8 million HCV-infected subjects, with wide regional differences [1]. It was assumed probable that the prevalence of chronic HCV infection is higher in patients suffering from RA than in the general population due to the age and the frequency of hospitalizations of patients with RA [2]. In Egypt, the viraemic HCV infection prevalence was ~8% of patients with RA, attributed to the endemicity of HCV [3].

Some studies have suggested a strong association between chronic HCV infection and RA [4, 5]. Although the relationship remains uncertain, chronic inflammation and immune

Summary

- Distinct expression pattern of CD244 by lymphocytes of RA with HCV infection was observed.
- Downregulation of CD244 was more profound in HCV positive RA, as CD244 expression by NK cells and T cells was decreased in HCV positive in relation to HCV negative RA.
- T cell CD244 expression was significant for discriminating clinically active from less active RA with and without HCV.

dysregulation are observed in both diseases [2, 6]. It was reported that the receptor CD244 (2B4, SLAMF4) functions in both rheumatoid arthritis and HCV infection, and its action is essential for the onset and progression of both diseases [7].

CD244 is a member of the signaling lymphocytic activating molecule (SLAM) family, expressed on NK cells, T cells, monocytes, and dendritic cells [7, 8]. Through binding to its ligand, CD48, on adjacent cells, CD244 was initially recognized as a stimulatory surface receptor that mediated activation of NK cells and CD8 T cells, inducing cytotoxicity and secretion of interferon (IFN) γ. However, subsequent studies reported inhibitory functions of CD244, with CD244 deficient NK cells being more cytotoxic than wild type NK cells [7]. These seemingly opposing effects were elucidated in studies of the structure and function of CD244. CD244 is a transmembrane receptor composed of an extracellular segment, a transmembrane region, and a cytoplasmic tail containing four immunoreceptor tyrosine-based switch motifs (ITSMs). The ITSMs of CD244 bind and phosphorylate SLAM-associated protein (SAP), which transduces signals for cell activation [9]. However, the third ITSM can bind cytoplasmic inhibitory phosphatases [8], thus CD244 can transmit both inhibitory and activating signals [7]. The different regulatory effects depend on the density of CD244 on the cell surface, the degree of crosslinking, and the amount of SAP. High surface expression of CD244, strong crosslinking, and low levels of SAP are associated with inhibitory effects and vice versa [8, 10].

Dysregulation of NK receptors and CD244 has been detected in patients with RA [11]. It was shown that the expression of CD244 in RA patients stimulated the T cell effector functions and promoted disease exacerbation [12]. Meanwhile, there is evidence suggesting that CD244 expression by NK and T cells is altered during chronic HCV infection and that CD244-mediated signaling may be associated with antiviral immunity [13].

In the present work, we aimed to study the populations of lymphocytes: NK cells, NKT cells, and T cells, and the expression of the receptor CD244 by each population in RA. What has not been investigated before is how the populations of circulating lymphocytes differ in patients presenting with concomitant HCV infection. Thus, the aim of the study was to detect the effects of HCV infection on peripheral blood lymphocyte subsets in patients with RA, comparing it to HCV-negative RA patients and controls.

2 | Patients and Methods

2.1 | Study Population

The study included 20 RA patients without HCV infection (HCV negative RA) and 21 RA patients with concomitant HCV infection (HCV positive RA), attending the clinic of the Rheumatology and Rehabilitation Department, Kasr AlAiny Hospital of Cairo University. RA patients, with and without HCV infection, fulfilled the 2010 American College of Rheumatology/European League Against Rheumatism (ACR-EULAR) classification criteria for RA [14]. HCV infection was defined by the presence of HCV antibodies and HCV RNA. Samples were withdrawn prior to the initiation of HCV treatment using direct antiviral drugs. The disease activity was assessed by the Disease Activity Score for 28 joints (DAS28) utilizing the count of swollen and tender joints, patient global health, and erythrocyte sedimentation rate (ESR). DAS28 ≤ 3.2 was considered mild disease activity, DAS28 > 3.2 but ≤ 5.1 was considered moderately active disease, while DAS28 > 5.1 was considered severely active disease [15].

Eleven healthy individuals, with no HCV or RA diagnosis, were included as a control group. The study was approved by the Research Ethics Committee of Faculty of Medicine, Cairo University in accordance with the Declaration of Helsinki [16]. Informed consent was obtained from all participants prior to enrolment.

2.2 | Lymphocyte Phenotyping by Flow Cytometry

NK, NKT, and T cells were evaluated, and CD244 expression by these cell populations was assessed. Five milliliters of blood were collected in a heparinized vacutainer, and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll–Hypaque. PBMCs were incubated with fluorescein isothiocyanate (FITC) conjugated anti-CD3, phycoerythrin-cyanine 5 (PE-Cy5) conjugated anti-CD56, and phycoerythrin (PE) conjugated anti-CD244 antibodies (Beckman Coulter, California, USA). On adding the labeled monoclonal antibodies, the flow cytometry tubes were left in the dark at 4°C for 15 min.

Cell analysis was performed, and data were collected for a minimum of 10,000 events for each determination (COULTER EPICS XL Flow Cytometer, Beckman Coulter, California, United States). Gates were set on lymphocytes with forward and side scatters, and NK cells (CD3− CD56+), NKT cells (CD3 + CD56+), and T cells (CD3 + CD56−) were analyzed [17]. The absolute counts of NK, NKT, and T cells were calculated by multiplying the relative percentage of cells obtained with the absolute lymphocyte count obtained from the cell counter. Surface expression of CD244 by each lymphocyte population was quantified as mean fluorescence intensity (MFI) and frequency of CD244 expressing cells.

2.3 | Determination of Tumor Necrosis Factor Alpha (TNF-α) in Serum

Three milliliters of blood were collected in a serum separating vacutainer, and serum levels of TNF-α were measured by

immunoradiometric assay (IRMA) (TNF-α immunoradiometric assay IRMA; DIAsource ImmunoAssays S.A., Belgium).

2.4 | Statistical Analysis

Data analysis was performed using the statistical package for the Social Sciences (SPSS, version 25). Comparison between quantitative variables was performed using the non-parametric Kruskal–Wallis and Mann–Whitney tests. For comparing categorical data, the Chi squared test was used. Correlations between quantitative variables were done using the Spearman correlation coefficient. A two-tailed p value of <0.05 was considered statistically significant.

3 | Results

3.1 | Characteristics of Patients

Clinical characteristics, laboratory findings, and treatment details of HCV positive and HCV negative RA patients are shown in Table 1. No differences in age or RA disease duration were detected between the patient groups. Eighteen HCV positive RA patients (86%) and 16 HCV negative RA patients (80%) showed extra-articular features ($p=0.627$). These included Sicca syndrome, subcutaneous nodules, vasculitis, interstitial pulmonary fibrosis, neuropathy, and Raynaud's phenomena. DAS28 was used as an indicator of RA disease activity and response to treatment. HCV positive and negative RA patients were comparable regarding DAS28 ($p=0.134$). Regarding treatment, all patients with HCV positive RA received corticosteroids and hydroxychloroquine, while all patients with HCV negative RA received methotrexate; 7 patients (35%) received hydroxychloroquine, and 5 patients (25%) received corticosteroids.

3.2 | NK, NKT and T Cell Populations in HCV Positive and Negative RA

The absolute numbers of CD3−CD56+ NK cells, CD3+CD56+ NKT cells, and CD3+CD56− T cells were assessed in RA patients with and without concomitant HCV infection. Patients displayed comparable numbers of NK, NKT, and T cells to controls ($p=0.978$; $p=0.608$; and $p=0.191$; respectively) (Table 2).

3.3 | Expression of CD244 by NK, NKT, and T Cells in HCV Positive and Negative RA

To investigate how CD244 expression is influenced by HCV infection, the frequency and intensity of expression of CD244 by CD3−CD56+ NK cells, CD3+CD56+ NKT cells, and CD3+CD56− T cells were assessed in HCV positive RA, HCV

TABLE 1 | Clinical and laboratory characteristics of HCV negative RA and HCV positive RA patients.

	HCV positive RA	HCV negative RA	*p* value
Individuals (*n*)	21	20	
Age (years)	54 (48.5–57.5)	54.5 (45–61.7)	0.794
Female, *n* (%)	16 (76)	16 (80)	0.768
RA disease duration (years)	6.0 (3.0–9.5)	8.0 (5.0–10.7)	0.214
Tender joint count	6 (2–12.5)	12 (4–19)	0.089
Swollen joint count	2 (0–13)	8 (2–12)	0.144
CRP positive, *n* (%)	9 (43)	7 (35)	0.606
RF positive, *n* (%)	12 (57)	15 (75)	0.228
ESR (mm/h)	30 (25–51)	35 (16–54)	0.601
DAS28	3.4 (2.4–4.3)	4.0 (3.3–4.6)	0.134
Low disease activity, *n* (%)	10 (48)	5 (25)	0.321
Moderate disease activity, *n* (%)	9 (43)	12 (60)	
High disease activity, *n* (%)	2 (9)	3 (15)	
Extra-articular disease, *n* (%)	18 (86)	16 (80)	0.627
Use of MTX, *n* (%)	0 (0)	20 (100)	—
MTX dose, (mg/week)	N/A	20 (20–25)	—
Use of corticosteroid, *n* (%)	21 (100)	5 (25)	—
Prednisolone dose, (mg/day)	7.5 (5–10)	5 (5–7.5)	—
Use of hydroxychloroquine, *n* (%)	21 (100)	7 (35)	—

Note: Results are presented as median and 25th–75th percentiles or number (%).
Abbreviations: CRP, C-reactive protein; DAS28, Disease Activity Score for 28 joints; ESR, erythrocyte sedimentation rate; MTX, methotrexate; RF, rheumatoid factor.

negative RA, and controls. Results are illustrated in Figure 1. We observed that the expression intensity (in terms of MFI) of CD244 was particularly downregulated by the lymphocyte populations in HCV positive and HCV negative RA compared to controls (NK CD244 $p < 0.001$, NKT CD244 $p = 0.047$, T CD244 $p < 0.001$). Yet, the downregulation was more profound in HCV

TABLE 2 | Absolute numbers of circulating NK, NKT, and T cells in HCV-positive and negative RA.

Cell populations	HCV positive RA $N = 21$	HCV negative RA $N = 20$	Controls $N = 11$	p value
NK (/μL)	189 (130–260)	180 (99–287)	162 (84–303)	0.978
NKT (/μL)	76 (29–180)	62 (35–163)	40 (29–126)	0.608
T (/μL)	1074 (777–2080)	1576 (1169–1817)	1106 (864–1455)	0.191

Note: Results are presented as median and 25th–75th percentiles.

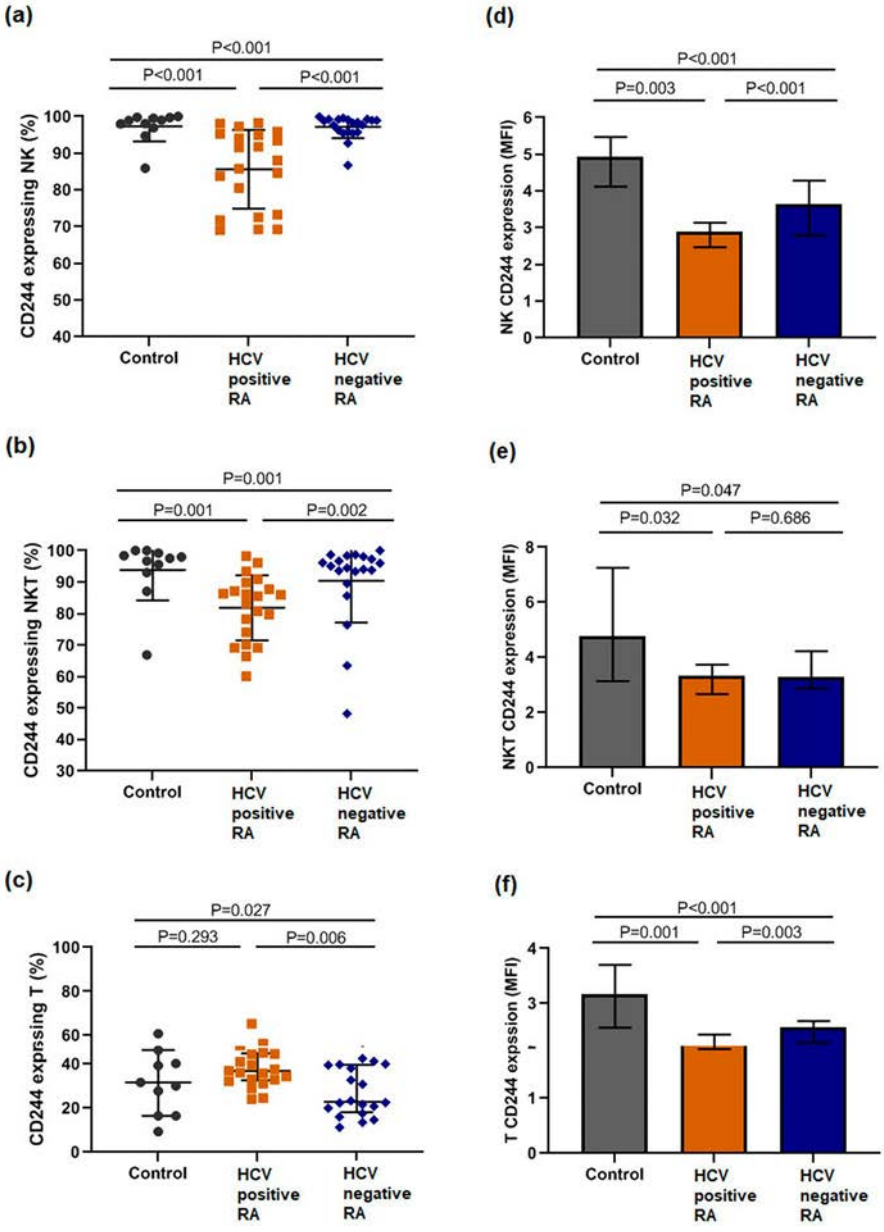

FIGURE 1 | Frequency of CD244 expressing NK, NKT, and T cells in RA with and without HCV infection (a–c) and expression intensity (MFI) of CD244 in the studied groups (d–f).

positive RA, as the expression of CD244 by NK cells and T cells was particularly decreased in HCV positive in relation to negative RA ($p < 0.001$ and $p = 0.003$, respectively). This was reflected also in the frequency of CD244-expressing lymphocytes, as HCV positive RA displayed significantly lower frequencies of CD244-expressing NK cells and NKT cells compared to HCV negative RA and controls ($p < 0.001$ and $p = 0.001$, respectively).

We plotted receiver-operating characteristic (ROC) curves to assess the diagnostic utility of CD244 expression to differentiate RA with HCV from RA without HCV (Figure 2). The area under the ROC curve (AUC) of 0.775 (95% CI 0.625–0.925, $p < 0.001$) and 0.737 (95% CI 0.574–0.900, $p = 0.004$) showed moderate ability of T cell and NK cell CD244 expression to differentiate RA with concomitant HCV. The sensitivity and specificity of T cell CD244 MFI more than 2.45 were 70% and 81%, while the sensitivity and specificity of NK cell CD244 MFI more than 3.34 were 65% and 86%, respectively.

3.4 | Expression of CD244 and RA Disease Activity

In a correlation analysis between the expression of CD244 by NK, NKT, and T cells with DAS28, there was a positive correlation between the expression of CD244 by T cells and DAS28 in HCV negative RA ($r = 0.485$, $p = 0.030$) (Table 3). To evaluate CD244 expression intensity in differentiating disease activity in RA with and without HCV ($n = 41$), patients were divided into clinically active (moderate/high disease activity, $n = 26$) and low disease activity ($n = 15$). ROC curves were constructed for

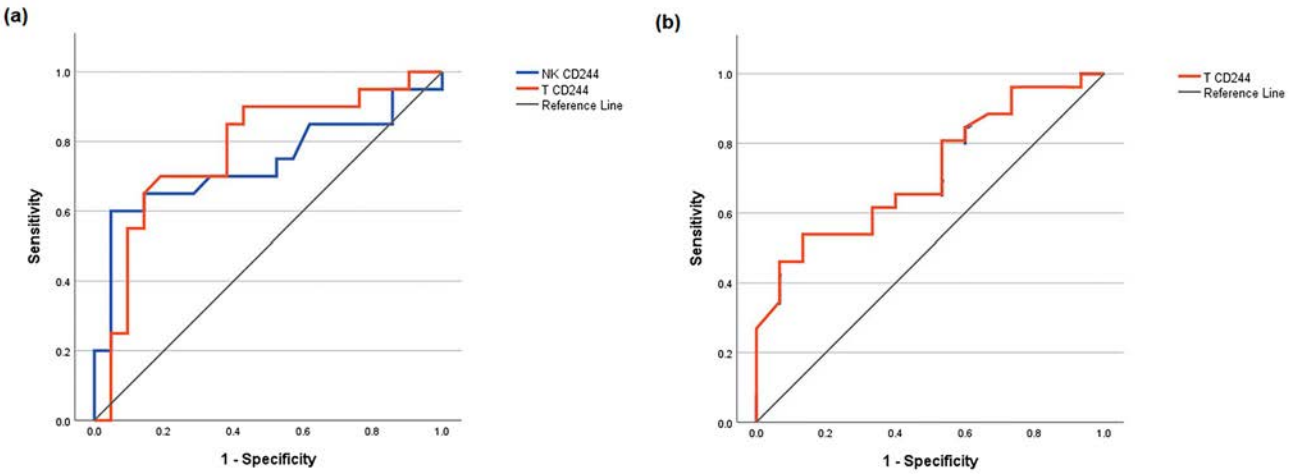

FIGURE 2 | Receiver-operating characteristic (ROC) curves to assess the diagnostic utility of CD244 expression to differentiate RA with HCV from RA without HCV (a) and to differentiate clinically active (DAS28 > 3.2) from less active (DAS28 ≤ 3.2) RA (b).

TABLE 3 | Correlation between absolute counts of lymphocyte populations and their expression of CD244 (MFI) with DAS 28 and TNF-α in serum.

		DAS 28		TNF-α	
		r	***p* value**	***r***	***p* value**
NK (/μL)	**HCV negative RA**	−0.413	0.070	−0.071	0.765
	HCV positive RA	−0.027	0.907	−0.536	0.012
NKT (/μL)	**HCV negative RA**	0.170	0.474	−0.145	0.543
	HCV positive RA	0.262	0.251	−0.090	0.698
T (/μL)	**HCV negative RA**	−0.290	0.216	0.033	0.889
	HCV positive RA	0.026	0.911	0.212	0.356
NK cell CD244 (MFI)	**HCV negative RA**	0.249	0.290	−0.184	0.437
	HCV positive RA	0.152	0.511	−0.170	0.462
NKT cell CD244 (MFI)	**HCV negative RA**	0.105	0.658	0.210	0.375
	HCV positive RA	−0.153	0.507	−0.122	0.600
T cell CD244 (MFI)	**HCV negative RA**	0.485	0.030	−0.112	0.637
	HCV positive RA	0.366	0.103	−0.374	0.095

Note: Statistically significant ($p < 0.05$).

TABLE 4 | Comparison of TNF-α levels between the studied groups.

	HCV positive RA $N=21$	HCV negative RA $N=20$	Controls $N=11$	p value
TNF (pg/mL)	5.4 (0.0–10.7)	7.9 (0.7–10.6)	10.7 (7.9–13.3)	0.067

discriminating clinically active (DAS28 > 3.2) from less active (DAS28 ≤ 3.2) RA (Figure 2). The area under the curve (AUC) was 0.717 (95% CI 0.560–0.873, $p=0.007$) for T cell CD244 expression, while there was no significance for NK cell CD244 ($p=0.148$) or NKT cell CD244 ($p=0.473$). The median T cell CD244 expression was 2.26 in mild disease and 2.49 in moderate/high disease activity. Applying a T cell CD244 MFI cutoff of 2.48, the sensitivity was 54% and specificity was 87%.

3.5 | Serum TNF-α Quantification

Serum TNF-α was evaluated in patients and controls. The levels were comparable between HCV positive and negative RA and controls ($p=0.067$) (Table 4). We performed a correlation analysis between serum TNF-α and the absolute counts of lymphocyte subpopulations and CD244 expression (Table 3). A significant negative correlation was observed between serum TNF-α levels and NK cell numbers ($r=-0.536$, $p=0.012$) in HCV positive RA, but this relationship was not observed in HCV negative RA.

4 | Discussion

In this study, we performed an analysis of NK, NKT, and T lymphocytes in HCV positive and negative RA. The numbers of circulating lymphocyte populations were similar between groups and were not influenced by the HCV infection status. Despite the previously described role of CD244 in RA [11, 12, 18], CD244 expression in RA concomitant with HCV infection has not been investigated.

It is important to note that the expression intensity of CD244 by NK, NKT, and T cells was significantly low in RA patients with and without HCV infection. Low expression of CD244 by NK cells has previously been detected in RA patients, and decreasing CD244 levels were related to severe periodontal disease in RA [11, 19]. It has also been identified that lower expression of CD244 results in increased cellular autophagy [20], thus suggesting that the observed low levels of CD244 by NK, NKT, and T lymphocyte populations in our study may contribute to RA disease pathogenesis and outcome by increasing cellular autophagy. We observed that CD244 expression levels by T cells could differentiate clinically active from less active RA, with and without HCV infection, with sensitivity of 54% and specificity of 87%. In addition, there was a positive correlation between the expression of CD244 by T cells and DAS28 in HCV negative RA. Functionally, co-ligation of CD244 in combination with other receptors such as DNAM-1 or NKG2D enhanced the effector functions of CD4 + CD28− T cells, present in the blood and synovial fluid in RA. CD244 is expressed mostly by $CD4^+CD28^-$ T cells in RA, and less frequently by $CD28^+$ T cells. Such co-ligation was able to enhance the response to suboptimal T cell receptor stimulation, with an increased magnitude of cytokine and cytotoxic responses [12], leading to heightened inflammation and disease exacerbation.

In RA, genetic variants may influence the expression of specific genes in disease-relevant tissues, such as blood or synovial tissue. Genome-wide association studies have identified variants in the CD244 gene and their association with RA [21]. GWAS loci are enriched for expression quantitative trait loci (eQTLs), regions that explain how genetic variants are related to the expression levels of specific genes. Using eQTL data for genes from blood samples, CD244 has been recognized as RA-related eQTLs. Variants were significantly associated with altered gene expression of CD244 and the risk of RA [22]. However, the observations of Chun et al. (2017) attributed a low proportion of genome-wide associations to modulations in gene expression. T cell, B cell, and monocyte eQTL effects were associated with only 12% for RA [23]. These findings emphasize the importance of pathogenic mechanisms beyond the context of gene expression.

Our results showed changes in CD244 receptor expression, marked by lower expression in both groups of patients with RA; however, the reduction in expression was more profound in HCV positive RA in NK and T cells. The functional activation of HCV specific NK and T cells requires CD244 expression and binding to CD48 followed by the transmission of signals. The cytoplasmic domain of CD244 interacts with various adaptor molecules [7, 24, 25]. The consequence of CD244 binding and propagating either an activating or inhibitory signal depends on the availability of CD244 and CD48 and adaptor molecule expression levels [25–27]. Proliferation of HCV-specific CD8+ T cells from HCV patients was found to be increased by CD244 cross-linking only if the expression of CD244 was low, while no effect was observed when CD244 expression was high [28]. Thus, our results indicate that in RA with HCV infection, NK and T cells expressing the receptor CD244 with low intensity are activated, likely driven by inflammatory signals.

In line with our findings, soluble CD244 was found to be downregulated in the plasma of both acute and chronic HCV patients, with a more substantial decline in chronic patients. This formed a long-lasting imprint of infection because it was observed before treatment and further declined after successful therapy [29]. It was also shown that stimulation in vitro by IFN-α, a cytokine induced in HCV infection, changed the phenotype of CD8+ T cells in healthy individuals to that in patients with HCV. Similar to our observations in HCV positive RA patients, the phenotype of T cells observed in HCV patients comprised a downregulation of CD244 expression [30].

We further observed that decreasing CD244 expression by NK cells was associated with distinct increasing TNF-α levels in

HCV infection. This relationship was observed only in HCV positive RA patients, although TNF-α levels were comparable between groups. This may suggest that the relationship between CD244 and TNF-α is indicative of CD244 receptor-related signaling pathways specifically participating in the regulation of TNF-α production in HCV positive RA. While we did not observe the same relationship between T cell CD244 expression and TNF-α levels, ex vivo cultures previously showed high levels of TNF-α expression in HCV specific CD244 + PD1+ expressing CD8+ T lymphocytes, indicating anti-HCV functional capacity [31]. TNF-α can promote HCV entry; thus, directing the inflammatory response to stimulate infection and induce cell death of HCV infected cells [32, 33]. Consequently, TNF-α is associated with sustained liver damage and fibrosis in chronic HCV patients [34].

Treating RA patients with concomitant HCV is challenging because immunosuppression can be detrimental. Patients with HCV-negative RA received mainly methotrexate, while HCV-positive RA received corticosteroids and hydroxychloroquine. DAA therapy was reported to be effective and safe in HCV-infected patients with RA [2]. An attempt to eliminate HCV infection in RA patients would diminish liver damage, progression, and complications related to HCV infection and improve RA disease activity [35]. Immune reconstitution following HCV elimination should be observed.

In conclusion, RA patients with HCV showed a distinctive expression pattern of CD244, being significantly downregulated on T cells and NK cells compared to RA patients without HCV. In addition, T cell CD244 expression could differentiate clinically active from less active RA patients, with and without HCV infection.

Author Contributions

Conceptualization: Shereen Mahmoud Shawky, Mariam Onsy F. Hanna. Methodology: Iman Sami AbouBakr, Hala Ahmed Raafat, Abla Ezz El-Arab. Formal analysis and investigation: Iman Sami AbouBakr, Fatma Hassan Abdelraouf, Mariam Onsy F. Hanna. Writing – original draft preparation: Mariam Onsy F. Hanna, Fatma Hassan Abdelraouf. Writing – review and editing: Shereen Mahmoud Shawky, Mariam Onsy F. Hanna, Hala Ahmed Raafat. Resources: Iman Sami AbouBakr, Abla Ezz El-Arab. Supervision: Shereen Mahmoud Shawky, Mariam Onsy F. Hanna, Hala Ahmed Raafat.

Ethics Statement

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the research Ethics Committee of Cairo University.

Consent

Written informed consent was obtained from all individual participants included in the study.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

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

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LETTER TO THE EDITOR

Investigating the Therapeutic Potential of Tocilizumab Monotherapy in Isolated Polymyalgia Rheumatica: A Systematic Review and Meta-Analysis

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To the Editor,

Polymyalgia rheumatica (PMR) is a common inflammatory illness in the aged population (over 50 years of age), which usually manifests as pain and stiffness in the proximal muscle, particularly the periarticular structures such as bursae and tendons [1]. General symptoms like fatigue, fever, and weight loss may also occur, possibly due to systemic Interleukin-6 (IL-6) signaling pathways. Treating PMR with oral glucocorticoids (GCs) typically leads to a positive response within days to weeks. However, around 43% of patients experience a recurrence of symptoms within a year, necessitating adjustments in the GC dosage [2]. For patients with PMR who are susceptible to long-term GCs, tocilizumab (TCZ), an IL-6 antagonist, exhibits a quick and long-lasting improvement providing a viable alternative [3]. Other biologic-targeted treatments are currently being evaluated. IL-6 antagonists may well have a place in the therapeutic strategy for PMR, particularly for patients with steroid-resistant disease or at high risk of complications of corticosteroid therapy [4]. The primary goal of this novel systematic review and single-arm meta-analysis is to assess if TCZ monotherapy provides an important clinical advantage over standard treatment choices in terms of efficacy and safety.

We could not retrieve or find a single double-arm study; therefore, we performed a systematic review and single-arm meta-analysis following the guidelines of Cochrane Collaboration Handbook for Systematic Reviews of interventions [5], the Preferred Reporting Items for Systematic reviews and Meta-Analysis (PRISMA) Statement [6], and Meta-analyses Of Observational Studies in Epidemiology (MOOSE) [7] and which has PROSPERO protocol ID: CRD42024609899. Using PUBMED, Cochrane Library, Clinicaltrials.gov, and Embase, we did a systematic search from inception to November 23, 2024. The search strategy employed the following terminologies: "Tocilizumab" OR "Actemra" OR "RoActemra" OR "R-1569" AND "Polymyalgia Rheumatica" OR "Forestier Certonciny Syndrome" OR "Peri-Extra-Articular Rheumatism" OR "Peri Extra Articular" OR "Senile arthritis." We also looked for bibliography sections of retrieved publications for further articles which met our eligibility criteria. Inclusion criteria was (1) patients with polymyalgia rheumatica (PMR) or PMR with giant cell arteritis (GCA); (2) randomized controlled trial (RCT), retrospective, or observational cohort studies; (3) patients receiving tocilizumab (TCZ) monotherapy, (4) patients who have never received any corticosteroid therapy. Exclusion criteria included: (1) other study designs such as cross-sectional studies, correspondences, scientific abstracts, and reviews; (2) patients primarily receiving corticosteroid or GC therapy before the start of TCZ therapy; and (3) patients receiving the TCZ in conjugation with GC or corticosteroid therapy. The study selection and data extraction were performed systematically by two independent authors, and conflicts were resolved through discussion. The primary outcomes were polymyalgia rheumatica activity score (PMR-AS) and IL-6 levels. Secondary outcomes included CRP levels, ESR levels, and remission or response to the therapy. Quality assessment of included observational

studies was done by using "Risk Of Bias In Non-Randomized Studies—of Interventions" (ROBINS-I) tool by three independent reviewers [8]. The RStudio software version 4.4.1 was used to analyze the data, using a random-effects model. For binary outcomes, meta and metaprop functions were used. For continuous outcomes, we used metamean and estmeansd functions. Using the Higgins I^2 statistics, we calculated the percentage of heterogeneity and inconsistency between studies. Leave-one-out sensitivity analysis was performed where high heterogeneity was detected.

We included four prospective cohort studies involving 71 patients who met our criteria after the screening, depicted in the PRISMA flowchart (Figure S1). The baseline characteristics of the included studies are reported in Table 1. The primary outcome, PMR-AS, showed significant results in the treatment group in the pooled analysis of all studies (MD 2.62; 95% CI: 0.22–5.01; $p < 0.0001$; $I^2 = 97.1\%$; Figure 1A). High heterogeneity was observed among the observational studies, which was not reduced significantly even after performing a leave-one-out analysis. Another primary outcome, IL-6 levels have shown a significant decrease in the treatment arm (MD 68.91; 95% CI 52.54 to 85.28; $p = 0.0309$; $I^2 = 71.2\%$; Figure 1B).

The pooled analysis of CRP showed a nonsignificant reduction after the employment of TCZ monotherapy (CRP levels: MD 2.89; 95% CI: −1.96 to 7.74; $p < 0.0001$; $I^2 = 98.4\%$; Figure 1C), while the ESR levels and the remission were significantly improved in the monotherapy (ESR levels: MD 3.27; 95% CI: 2.69–3.85; $p = 0.2708$; $I^2 = 17.5\%$; Figure 1D and remission: 90.32%; 95% CI: 45.21–100.00; $p = 0.0066$; $I^2 = 86.5\%$; Figure 1E). The assessment of individual cohorts presented in Figure S1 shows two studies with a low risk of bias, whereas the other two studies had some concerns of biases. The heterogeneity for IL-6 was 71.2%, indicating moderate to substantial variability across studies. Sensitivity analysis confirmed the stability of the results, with a pooled estimate of 68.91 (95% CI: [52.54, 85.28]). However, excluding the Chino study reduced heterogeneity to 0%, suggesting that this study was the primary source of variability (Figure S3A). In contrast, PMR-AS exhibited high heterogeneity at 97.1%, which remained consistently high despite sensitivity analysis (Figure S3B).

Recent studies have reported significant reductions in ESR and CRP levels with TCZ treatment, either in combination with GC or other therapies. Loricera et al. reported a reduction in ESR from 44 mm/h to 12 mm/h after 9 months of TCZ in patients with PMR and giant cell arteritis (GCA) [13]. The significant improvement of the ESR levels in our analysis with TCZ monotherapy supports these findings. Macchioni et al. noted normalized inflammatory markers after a single TCZ infusion in a PMR patient [14]. Unizony et al. observed CRP levels dropping from 29 mg/L to 3.8 mg/L during TCZ therapy [15]. Given that TCZ is an IL-6 antagonist, other IL-6 antagonists like sarilumab have also shown effectiveness against PMR. Spiera et al. showed sarilumab reduced CRP by 6.9 mg/dL at 52 weeks, compared to 1.7 mg/dL with placebo, with sustained effects throughout the trial [16].

The pooled analysis of the CRP levels revealed nonsignificant improvement with TCZ monotherapy in contrast to these studies. However, CRP and ESR have been shown to be unreliable indicators of disease activity during anti-IL6R therapy as reported by Unizony et al. They asserted that IL-6 receptor blockade, achieved through drugs like TCZ, disrupts the typical relationship between inflammation and markers like CRP and ESR. This is because TCZ not only prevents IL-6 from binding to its receptors, but also interferes with the body's natural IL-6 clearance mechanisms, leading to elevated IL-6 levels despite reduced inflammation [15]. Thus, the role of CRP and ESR in treatment response to TCZ in PMR patients addressing the reliability of these markers needs to be further explored in the studies for more evidence. Previous studies support our findings of significant remission. Macchioni et al. and Lally et al. observed remission in PMR patients treated with TCZ, with Lally achieving relapse-free remission at 6 months without GC [14, 17]. Additionally, Bonelli et al. reported higher rates of GC-free remission with TCZ as compared to placebo [18].

Moderate adverse events were also reported with TCZ in PMR patients. In the PMR-SPARE trial, mild infections were common, with no treatment withdrawals [18]. The TENOR study noted leukopenia and neutropenia, leading to dose adjustments [9]. Lally et al. observed upper respiratory infections and neutropenia. TCZ helps reduce GC use and related adverse events [17].

Studies by Alegria et al. (2017) and Chino et al. showed moderate risk of bias due to confounding variables such as concomitant medications (low-dose prednisone), disease duration, comorbidities, smoking status, genetic factors, and variability in treatment protocols, as well as deviations from TCZ monotherapy and inconsistent outcome monitoring. Heterogeneity tests (I^2) revealed substantial variability in the pooled outcomes of PMR-AS and IL-6 levels. This variability likely stems from differences in patient populations, treatment protocols, outcome assessment methods, and definitions of PMR. Additional factors, such as concomitant medications and comorbidities, may further obscure the link between TCZ treatment and patient outcomes. The high heterogeneity in our study may stem from biases in confounding and deviations from intended interventions, as seen in the Chino and Alegria studies. The lack of randomization, control groups, and blinding in these studies likely influenced patient outcomes, contributing to variability. Observational correlations and unmeasured confounders further amplify this effect. These findings highlight the need for more standardized and controlled studies to enhance future research.

The limitations of this study include the small sample size and few studies, which may restrict the generalizability of the findings and pose a risk of publication bias. Currently, there are no studies that directly compare tocilizumab with other treatments such as corticosteroids or IL-6 antagonists. This gap prevents us from making robust comparisons and limits the broader application of our findings. Future research must include direct comparisons with these promising therapies to provide more definitive conclusions. Additionally, the observational nature of the included studies raises concerns about potential biases and confounding factors that could influence the results. The high heterogeneity observed in the outcomes, particularly in the PMR-AS, also suggests variability in patient responses and study methodologies that complicate the interpretation of the data.

TABLE 1 | Baseline characteristics of the included studies.

Study ID	Population	Follow-up time	No. of patients (male/female)	Age (Mean ± SD)	Dosage (mg/kg)	Disease duration (Mean ± SD)	PMR-AS (Mean ± SD)	CRP (Mean ± SD, mg/dL)	ESR (Mean ± SD, mm/h)	Comorbidities	IL-6 Levels (Mean ± SD, pg/mL)
Devauchelle Pensec et al. [9]	Glucocorticoid-free PMR	12 weeks	20 (13/7)	67.5 ± 8.37 years	8 mg/kg	110.51 ± 76.59 days	36.95 ± 10.69	72.00 ± 84.73	55.13 ± 36.30	Fever (2/20), > 5% Weight Loss (3/20), Elevated GGT (9/20)	N/A
Alegria et al. [10]	Glucocorticoid-free PMR	24 weeks	18 (11/7)	Intervention (68 ± 7)	8 mg/kg	N/A	Intervention: IL-6 Responders: 42 ± 2/9, IL-6 Non-Responders: 34 ± 3/9, Combined Mean 38 ± 4.8	Intervention (82 ± 16)	N/A	N/A	Intervention (112 ± 19)
Chino et al. [11]	Glucocorticoid-free PMR	52 weeks	13 (3/10)	Intervention-70.1 ± 3.18	8 mg/kg	4 months ± 1.73	33.3 ± 6.65	4.3 mg/dL	67 mm/h	hypertension (8/13), dyslipidemia (5/13), diabetes mellitus (4/13), osteoporotic vertebral fractures (3/13), osteosclerosis obliteratans (1/13), history of myocardial infarction (1/13), history of cerebral infarction (1/13), history of hematemesis due to an Ulcer caused by NSAID(1/13), glaucoma (2/13)	32.2 pg/mL ± 16.28
Alegria et al. [12]	Glucocorticoid-free PMR	24 weeks	20 (13/7)	Intervention (67.3 ± 18.3)	8 mg/kg	N/A	N/A	Intervention: IL-6 responders and non-responders combined mean—82 ± 39.42	Intervention: IL-6 responders and non-responders combined mean—58.5 ± 14.27	N/A	Intervention: IL-6 responders—173 ± 34.42; IL-6 non responders—53.87 ± 38.21

Abbreviations: CRP, C-reactive protein; ESR, Erythrocyte sedimentation rate; IL-6, Interleukin-6; PMR, Polymyalgia rheumatica; PMR-AS, polymyalgia rheumatica.

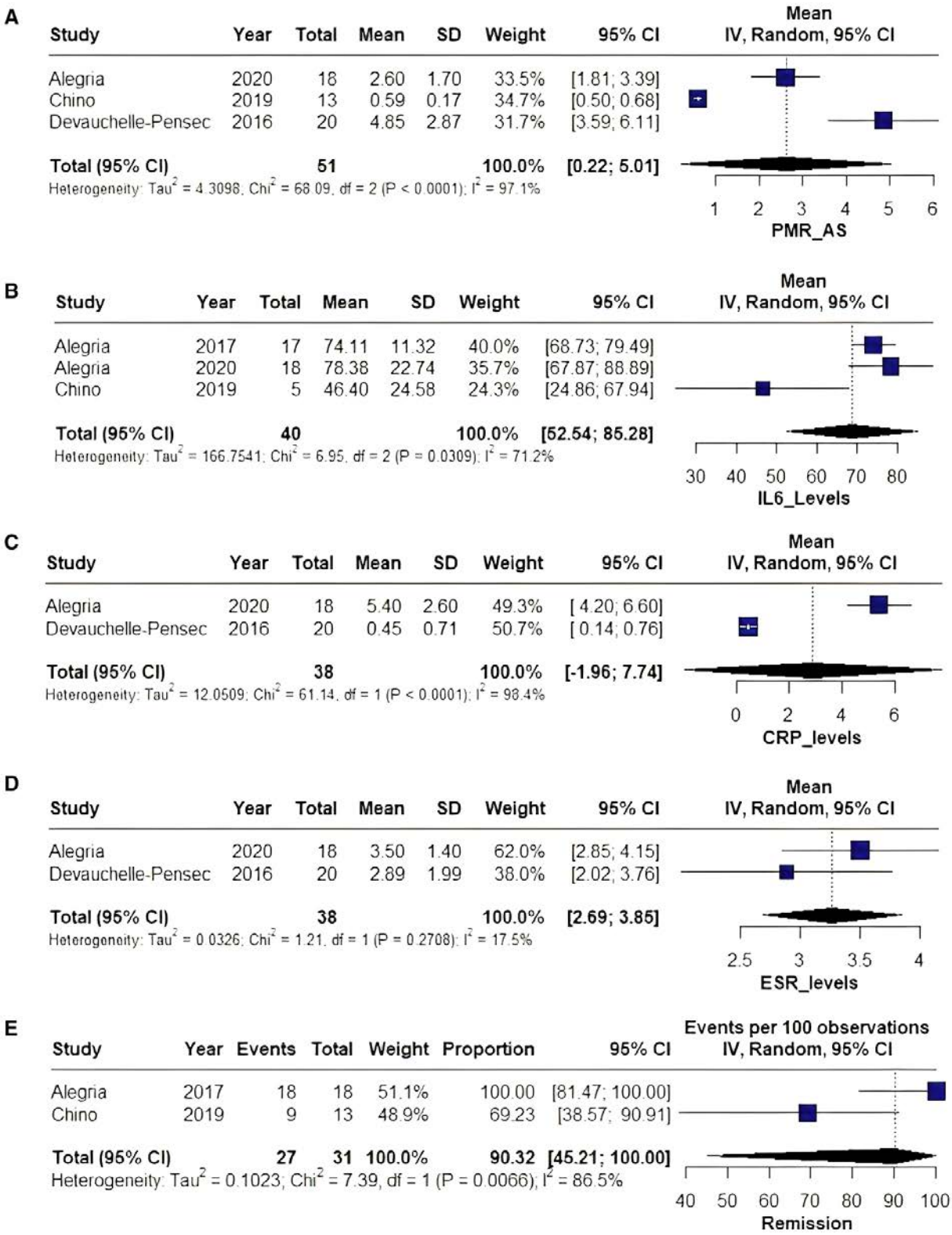

FIGURE 1 | In (A), the forest plot illustrates the PMR-AS (polymyalgia rheumatica activity score) outcomes. In (B), the forest plot shows the outcomes of IL-6 (interleukin-6) levels. In (C), the forest plot displays the outcomes of the levels of CRP (C-reactive protein). In (D), the forest plot depicts the outcomes of ESR (erythrocyte sedimentation rate). In (E), the forest plot summarizes the percentage of remission events.

This study supports TCZ as a potential monotherapy for managing GC-dependent PMR. TCZ significantly reduced PMR-AS, IL-6 levels, and ESR levels, suggesting a promising alternative to traditional GC treatments. Additionally, a great number of adverse events associated with GC treatment in PMR patients support a viable alternative in the form of TCZ, as it has shown

fewer adverse events in the studies. Further large-scale randomized trials are needed to validate these findings and confirm the benefits of TCZ.

Author Contributions

A.: conceptualization, writing, reviewing, supervision, analysis. M.U.: writing and reviewing, figure formation, risk of bias assessment. M.U.M.: writing and reviewing, figure formation, screening. O.T.: writing and reviewing, table formation, screening, risk of bias assessment. B.A.: Writing and reviewing, Risk of bias assessment. N.N.: writing and reviewing, figure formation. Z.A.: writing and reviewing, risk of bias assessment. F.R.: writing and reviewing, supervision.

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The authors have nothing to report.

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

All data in the manuscript are publicly available and will be provided to the editorial office 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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ORIGINAL ARTICLE

The Efficacy and Safety of Hepatitis A Vaccine in Children and Young Adults With an Autoinflammatory Diseases on Canakinumab and Tocilizumab Treatments: A Prospective Observational Controlled Study

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ABSTRACT

Intro: In this prospective interventional study, we aimed to assess the efficacy and safety of the hepatitis A vaccine in patients with autoinflammatory diseases undergoing canakinumab and tocilizumab treatments.

Methods: A total of 24 patients with autoinflammatory diseases on canakinumab and tocilizumab treatments and 39 healthy children who were seronegative for hepatitis A were included in the study. All participants were vaccinated with 2 doses of inactivated hepatitis A vaccine at 6-month intervals. One month after the last vaccination, venous blood samples were collected from each participant, and the anti-HAV IgM and IgG titers were measured.

Results: The patient group consisted of 19 patients with systemic juvenile idiopathic arthritis (sJIA) and 5 patients with cryopyrin-associated periodic fever. The mean age of the patient group and the healthy controls were 14.1 ± 3.7 and 12.4 ± 3.2 years, respectively. All patients with cryopyrin-associated periodic fever and 52.6% (10/19) of the patients with sJIA were on canakinumab. The remaining 9 patients (47.3%) with sJIA were using tocilizumab. Among sJIA patients, 15 were also on methotrexate, and 14 were on prednisolone. All of the participants were negative for hepatitis A serology. After two doses of hepatitis A vaccine, all the patients with autoinflammatory diseases (24/24) and 84.6% (33/39) of the healthy controls were detected as positive for anti-HAV IgG ($p = 0.04$). The mean anti-HAV Ig G titers of the patient group and the control group one month after the last dose of vaccination were 5.25 ± 1.49 IU/L and 10.5 ± 7.02 IU/L, respectively ($p < 0.001$). Neither disease flares nor adverse effects related to vaccination were observed within the study period.

Conclusion: Hepatitis A vaccine is effective and safe in children with autoinflammatory diseases on biologic therapy. Long-term follow-up data, including larger patient cohorts, are needed to draw a solid conclusion about the safety of hepatitis A vaccine in patients receiving canakinumab and tocilizumab therapy.

1 | Introduction

Systemic autoinflammatory diseases are a heterogeneous group of disorders characterized by unprovoked episodes of inflammation that typically result from defects in innate immunity [1]. Although the causative genes for most of these diseases, such as familial Mediterranean fever (FMF) and hyper immunoglobulin D syndrome, have been identified, no single pathogenic variant has been shown for periodic fever, aphthous stomatitis, pharyngitis, adenitis (PFAPA) syndrome, and systemic juvenile idiopathic arthritis [1, 2]. Whether hereditary or not, all autoinflammatory diseases cause the intermittent release of proinflammatory cytokines, resulting in a wide spectrum of clinical findings [1].

Preventing these strong inflammatory episodes is crucial for hindering the development of amyloidosis, which is one of the most devastating complications of these diseases. Biologic treatments that mainly target inflammatory cytokines or their receptors are commonly needed during the course of the disease [3, 4]. Although biologic agents are highly effective in controlling inflammation and subsequently preventing amyloidosis, the fact that these agents suppress the activity of key inflammatory cytokines may lead to an increase in the frequency of various infections, which remains a matter of debate [5, 6]. Thus, prevention of infections through vaccination is of paramount importance in patients undergoing biological therapy. However, routine vaccination uptake was lower in children with rheumatologic diseases than in healthy children [7–10]. Possible reasons for this low rate of vaccination among children with rheumatic diseases on biologic treatment include fear of vaccine-related side effects, the risk of disease flare, and the misbelief of a lack of efficacy in children on biologic treatment [9, 11].

Hepatitis A virus (HAV), a member of *the Picornaviridae* family, is a non-enveloped ribonucleic acid virus that causes an acute inflammatory liver disease named hepatitis A [12]. Although hepatitis A is a self-limiting disease that is usually asymptomatic in children, it can cause hepatic dysfunction and even mortality in adolescents and adults [13]. As hepatitis A is mainly transmitted via the fecal-oral route, it poses a public health concern, especially in developing countries with poor sanitary conditions and a lack of access to safe water [12]. Nevertheless, vaccination programs against hepatitis A are highly effective in reducing hepatitis A infections throughout the world [13–15].

In this prospective interventional study, we aimed to assess the efficacy and safety of the hepatitis A vaccine in patients with autoinflammatory diseases undergoing canakinumab and tocilizumab treatments.

2 | Materials and Methods

2.1 | Study Population and Definition

This study was conducted at Istanbul University-Cerrahpasa Cerrahpasa Medical School Pediatric Rheumatology Department. Patients with systemic autoinflammatory diseases on canakinumab and tocilizumab treatments who had outpatient clinic appointments between June 2017 and November 2018 were explained the study in detail and asked to participate in the study. Patients who were previously vaccinated for hepatitis A, were on high-dose steroid therapy (> 2 mg per kilogram daily), had received intravenous immunoglobulin or blood transfusion, and had any comorbidities such as immunodeficiencies, malnutrition, etc. that may affect the individual's antibody response to the vaccine were excluded.

In total, 49 patients with systemic autoinflammatory diseases on canakinumab and tocilizumab treatments were selected as candidates for the study. Then, anti-HAV IgM and anti-HAV IgG titers were carried out to detect the patients who were exposed to the virus before. After the exclusion of 21 patients with anti-HAV IgG positivity, a total of 24 patients (13 female, 11 male) with autoinflammatory diseases on biologic treatments and 39 healthy children (21 female, 18 male) who were seronegative for hepatitis A were included in the study. The flowchart of the enrollment process of the study population is shown in Figure 1.

All of the patients enrolled were diagnosed by an experienced pediatric rheumatologist according to their clinical and laboratory findings including genetic studies, and all of the patients also met the highly accepted classification criteria proposed for their diseases [16, 17].

2.2 | Vaccination Procedure

Neither the patient group nor the control group had any patients with a history of hepatitis A vaccination. All of the participants were vaccinated with 2 doses of inactivated hepatitis A vaccine at 6-monthly intervals. Patients received canakinumab and tocilizumab treatments monthly, and vaccinations were administered 2 weeks after the last dose of biologic therapy and methotrexate. While a pediatric form of hepatitis A vaccine (720 EL.U/0.5 mL HAVRIX, GlaxoSmithKline) was used for the patients between 1 and 18 years of age, the patients over 18 years of age were vaccinated with an adult form of the vaccine (1440 EL.U/1 mL HAVRIX, GlaxoSmithKline).

2.3 | Assessment of the Efficacy and Safety

Venous blood samples were taken from each participant 1 month after the last Hepatitis A vaccination. After centrifugation, all blood samples were stored at −80°C till the day of analysis. Enzyme-linked immunosorbent assay (ELISA) was used to measure the level of anti-HAV Ig M and Ig G titers by using the DIA.PRO kit, which is competitive and qualitative based. All of the procedures were done according to the manufacturer's introductions. The levels of signal-to-cut-off (S/CO) below 0.9 were considered negative, between 0.9–1.1 were equivocal, and higher than 1.1 were positive. A titer of anti-HAV Ig G higher than 10 IU/mL was accepted as protective.

All participants were informed about the possible side effects before the vaccination, and they were asked to apply to the

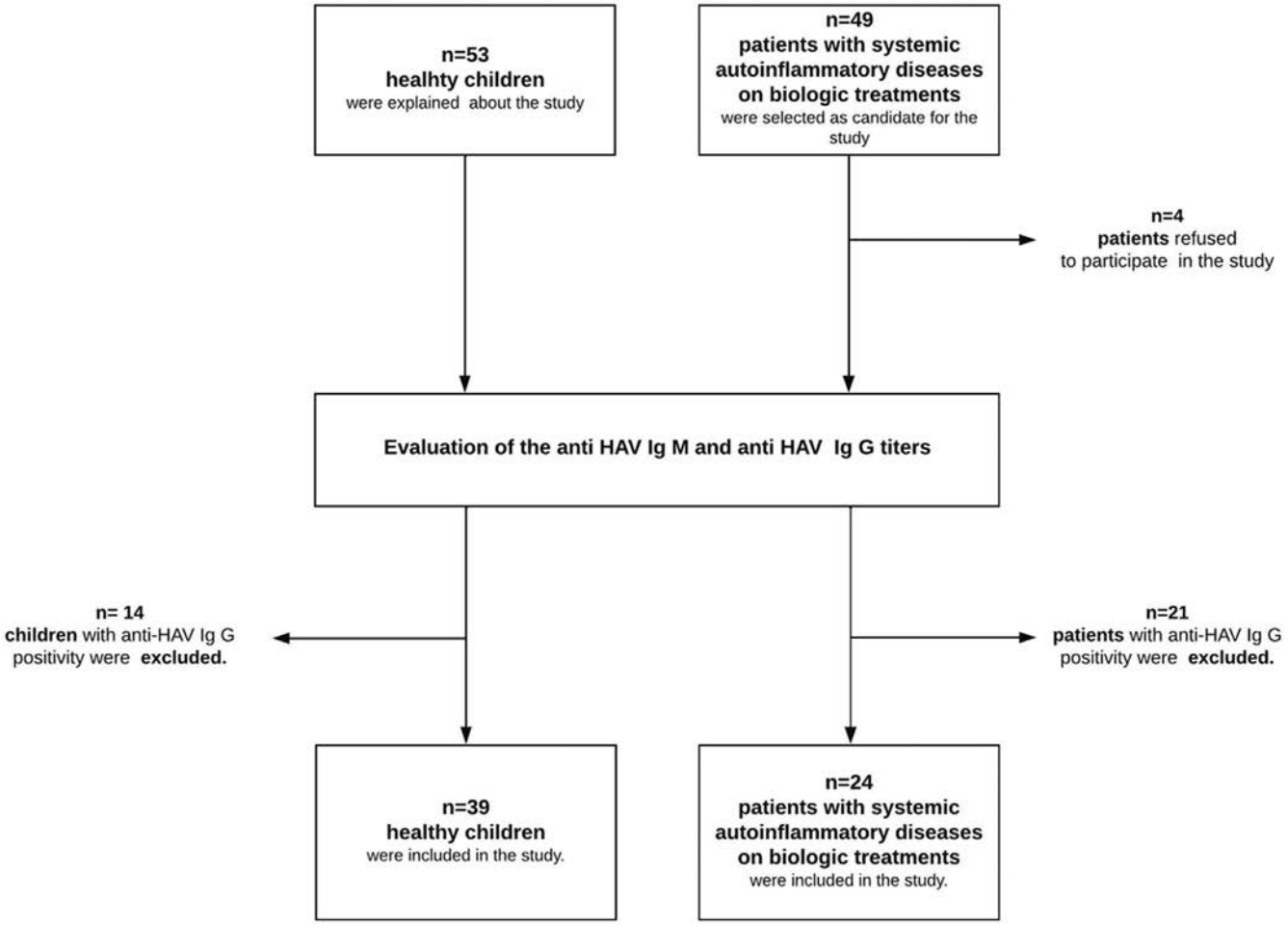

FIGURE 1 | The flowchart of the enrollment process of the study groups.

emergency department in the case of any unexpected event and to inform us immediately by calling the emergency contact number provided before. In addition, all of the participants were assessed at the outpatient clinic 1 month after each vaccination, and they were questioned about local side effects (pain, erythema, swelling), systemic side effects (fever, myalgia, fatigue etc.) or severe side effects that started within 24 h after vaccination. The patients were also questioned if they experienced a disease flare or not.

2.4 | Statistical Analysis

All the statistical analyses were performed by using IBM SPSS 21.0 programme (SPSS Inc., Chicago, IL, USA). The distribution pattern of the variables was checked with the Kolmogorov–Smirnov test and/or Shapiro–Wilk tests. While categorical variables were reported as numbers (percentages), continuous variables with normal distribution were given as mean ± standard deviation, and continuous variables without normal distribution were given as median (minimum-maximum). The chi-square test was used to compare categorical variables between groups. p value < 0.05 was considered statistically significant.

2.5 | Ethical Approval

This study is confirmed by Istanbul University-Cerrahpasa Institutional Review Board (83045809–604.01.02).

3 | Results

3.1 | Baseline Characteristics

The patient group consisted of 19 patients with systemic juvenile idiopathic arthritis (sJIA) and 5 patients with cryopyrin-associated periodic fever [4 patients with familial cold autoinflammatory syndrome (FCAS) and 1 patient with chronic infantile neurological cutaneous and articular syndrome (CINCA)]. In total, 13 (55.1%) of the patients with autoinflammatory diseases and 21 (53.8%) of the control group were female. The mean age of the patient group and the healthy controls were 14.1 ± 3.7 and 12.4 ± 3.2 years, respectively. There were no significant differences between the patient group and the controls regarding age and gender (both p values > 0.05).

All patients with cryopyrin-associated periodic fever and 52.6% (10/19) of the patients with sJIA were on canakinumab.

TABLE 1 | Demographic features of the patient group and the healthy controls.

	AID group[a]	Control group	*p*
Female *n* (%)	13 (55.1)	21 (53.8)	0.98
Age (Years)	14.1 ± 3.7	12.4 ± 3.2	0.06
Diagnosis		—	
Systemic Juvenile Idiopathic Arthritis	19 (79.1)		
Cryopyrin associated periodic *fever*	5 (20.8)		
FCAS[b]	1 (4.1)		
CINCA[c]	4 (16.7)		
The mean age at the onset of disease (years)	7.2 ± 4.3	—	
The mean age at diagnosis (years)	7.6 ± 4.4	—	
Treatment		—	
Methotrexate	15 (62.5)		
Canakinumab	15 (62.5)		
Tocilizumab	9 (37.5)		
Prednisolone	14 (58.3)		
Visual Analog Scale (first)	0.5 (0–6)	—	
Visual Analog Scale (last)	0 (0–6)	—	

[a]Autoinflammatory syndrome.
[b]Familial cold autoinflammatory Syndrome.
[c]Chronic infantile neurological cutaneous and articular Syndrome.

The remaining 9 patients (47.3%) with sJIA were using tocilizumab. Among sJIA patients, 15 were also on methotrexate and 14 were on prednisolone. The demographic features and baseline characteristics of the study population were given in Table 1.

3.2 | Immunogenicity of Hepatitis A Vaccine

All of the participants were negative for hepatitis A serology. After two doses of the hepatitis A vaccine, all the patients with autoinflammatory diseases (24/24) and 84.6% (33/39) of the healthy controls were detected as positive for anti-HAV IgG ($p = 0.04$). The mean anti-HAV IgG titers of the patient group and the control group 1 month after the last dose of vaccination were 5.25 ± 1.49 IU/L and 10.5 ± 7.02 IU/L, respectively ($p < 0.001$). The anti-HAV IgG levels of the patients and the control group before and after the vaccination were represented in Figure 2.

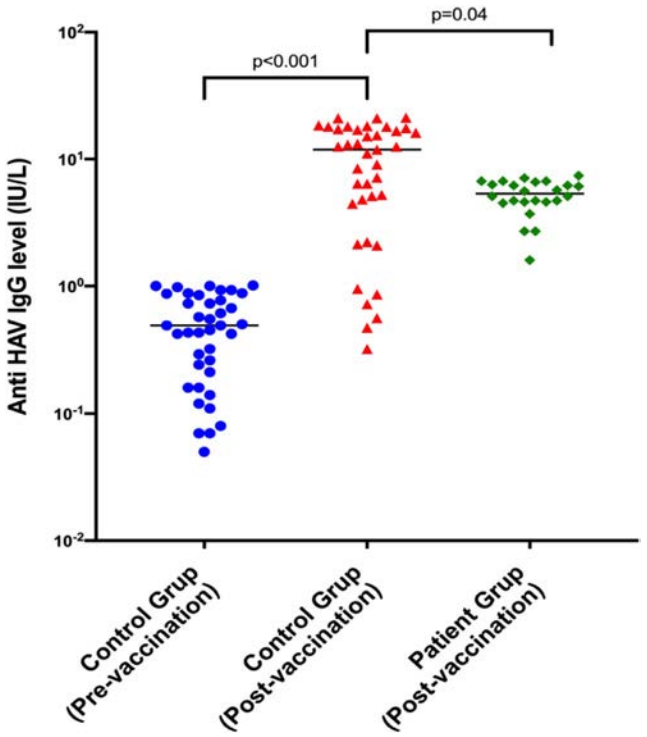

FIGURE 2 | The anti-HAV Ig G levels of the patients and the control group before and 1 month after the second dose of the vaccination.

TABLE 2 | Comparison of the anti-HAV IgG levels according to medications.

	Anti HAV Ig G level (IU/L)	*p*
Methotrexate		
Using	4.78 ± 1.62	0.04
Not using	6.03 ± 0.83	
Prednisolone		
Using	5.00 ± 1.41	0.35
Not using	5.59 ± 1.60	
Canakinumab		
Using	5.32 ± 1.53	0.75
Not using	5.12 ± 1.50	
Tocilizumab		
Using	5.12 ± 1.50	0.75
Not using	5.32 ± 1.53	

3.3 | Effects of Medications on Anti-HAV Ig G Levels

The patients were divided into two groups: those who were on and those who were not, for each drug they used. There were no significant differences in anti-HAV Ig G levels between the patients according to their medications except for the patients who were using methotrexate (MTX). The mean anti-HAV Ig G

level of the patients on MTX was significantly lower than that of the patients not on MTX (4.78 ± 1.62 IU/L vs. 6.03 ± 0.83 IU/L; $p = 0.04$) (Table 2).

3.4 | Assessment of the Safety of *Hepatitis A Vaccine*

Neither disease flares nor adverse effects related to vaccination were observed within the study period.

4 | Discussion

In this prospective interventional study, we report seroconversion for hepatitis A in all the patients with autoinflammatory diseases on canakinumab and tocilizumab treatments who were vaccinated for hepatitis A. Apart from hepatitis A vaccine being highly effective in children with autoinflammatory diseases even if they were on Anti IL-1 and Anti IL-6, our data also suggest that hepatitis A vaccination is safe in this patient group. We observed no vaccine-related side effects or disease flares after the hepatitis A vaccination.

Systemic autoinflammatory diseases are a group of diseases related to dysregulated innate immunity characterized by intermittent, unprovoked inflammatory episodes [1]. Initially, concerns were raised regarding the efficacy and safety of routine vaccinations for children with autoinflammatory diseases. However, a growing number of studies have since reported that vaccinations are both safe and effective for these individuals. Jensen et al. [18] reported that the children with FMF produced similar levels of protective antibodies to healthy controls after the inactivated influenza vaccine. Similarly, it is shown that adult patients with FMF reached similar levels of antibodies against SARS-CoV after primary infection and vaccination [19, 20]. Similarly, Brugan et al. reported an increase in antibody titers in 17 patients with CAPS receiving canakinumab treatment after administration of non-living vaccines [tetanus/diphtheria/pertussis (DTP), *Haemophilus influenzae*, *Neisseria meningitidis*, Hepatitis B (Hep B), pneumococcal polysaccharide vaccine and pneumococcal conjugate vaccine] despite anti-IL1 treatment [10, 21]. Conversely, in a study evaluating the efficacy and safety of the measles, rubella, mumps (MMR) vaccine in patients with PFAPA syndrome, it was reported that no patient developed serious side effects, but the protective antibody concentration rate was lower in the PFAPA syndrome group than in healthy controls [22]. A registry-based prospective study of CAPS patients vaccinated with at least one of 12 vaccine types, including influenza, diphtheria/tetanus/pertussis, pneumococcal, hepatitis A, and B vaccines, evaluated the safety of vaccination and reported that pneumococcal vaccines frequently trigger local inflammation, unlike other vaccines evaluated [23]. Moreover, studies have indicated that disease activation can occur in individuals with HIDS following routine childhood vaccinations, including those for Hepatitis A, varicella, MMR, and DTP [24, 25].

Studies focusing on the efficacy and safety of hepatitis A vaccination in autoinflammatory diseases are scarce in the literature. In a study conducted by Despiona et al. [26], a prospective observational design was employed to evaluate 28 patients with autoinflammatory diseases, including 13 with systemic juvenile idiopathic arthritis, 13 with familial Mediterranean fever, 1 with HIDS, and 1 with cryopyrin-associated periodic syndromes. The findings of this study were consistent with our results, as the seroconversion rate between the autoinflammatory disease group and the healthy control group was found to be similar, while the mean anti-HAV IgG levels were higher in the control group.

Another key consideration regarding vaccination in autoinflammatory diseases is the impact of medications on the efficacy and safety of vaccines. In our study, it was found that the antibody levels of patients who used MTX were lower compared to those who did not use it. Similarly, the study conducted by Despiona and colleagues [26] indicated that the use of steroids and biologic agents did not have an impact on the levels of anti-HAV Ig G or the seroprotection rate in their cohort of patients with autoinflammatory diseases. In the same study, while the sample size was not sufficiently large to draw definitive conclusions, the seroprotection rate among patients using MTX following their initial Hep A vaccination was discovered to be lower than that of patients using colchicine [26]. Indeed, it has been observed in various studies that administering methotrexate can result in diminished levels of antibodies following COVID-19 and pneumococcal vaccinations [27, 28]. A study was conducted in adult patients with inflammatory rheumatologic diseases, which revealed that MTX had a negative impact on SARS-CoV-2 vaccination in a manner that depended on the patient's age. The study found that discontinuing MTX treatment for 10 days after vaccination led to an improvement in antibody response in patients who were over 60 years old [29]. According to these data from literature, the impact of methotrexate on antibody levels post-vaccination could be influenced by a range of variables, including the type of vaccine administered and the age of the vaccinated group.

The effect of biological therapies on vaccination is an essential subject that has grown in significance in recent times, given the increased utilization of these therapies. Our investigation revealed that individuals who received canakinumab or tocilizumab therapy exhibited anti-HAV Ig G antibody levels comparable to those observed in patients who did not receive these medications. In line with our results, in a clinical study examining the effectiveness and safety of inactivated influenza and meningococcal group C vaccines in subjects who had received canakinumab treatment within a two-week period prior to immunization, it was demonstrated that the emergence of protective response and the occurrence of adverse reactions were comparable between the patients who had taken canakinumab and those who had not [30]. Another study in canakinumab-treated pediatric CAPS patients suggested that canakinumab treatment had no effect on the ability to produce antibodies against non-live childhood vaccines [31]. In addition to the studies mentioned above, Watanabe et al. reported that they achieved effective antibody levels against live-attenuated vaccines for measles, rubella, varicella, and mumps in an infant with CAPS using canakinumab, without observing any complications [32].

The impact of the anti-IL6 agent tocilizumab therapy on vaccination among children with autoinflammatory diseases has

not been extensively studied in the existing body of literature. In a clinical trial involving children with juvenile idiopathic arthritis (JIA) who were receiving biological treatments, including tocilizumab, the effectiveness and safety of influenza immunization were evaluated. The findings revealed that influenza vaccination was both effective and safe for JIA patients receiving biological therapy [33]. In a study that examined the safety of live-attenuated vaccines in children with autoinflammatory diseases who were treated with biological therapy, three patients who were using tocilizumab were assessed [24]. Of these three patients who received the oral poliovirus vaccine, varicella zoster vaccine, or MMR booster, it was reported that the patient who received the varicella vaccine experienced a disease flare, while the patient who received the oral poliovirus vaccine experienced diarrhea [24]. A study evaluating the efficacy of the MMR and MMR/V booster dose in children with rheumatic disease on biologic therapy and including 5 patients on tocilizumab therapy suggested that the live-attenuated MMR/V booster dose is safe in children with rheumatic disease on biologic therapy [34].

The main limitation of our research lies in the limited size of our patient cohort, which precludes us from extrapolating our findings to the broader autoinflammatory disease spectrum. Since the routine hepatitis A vaccination has been introduced in our country in 2012, the number of unvaccinated children who could assist in evaluating the impact of biological therapies on hepatitis A vaccination has become extremely limited. Furthermore, the scarcity of autoinflammatory diseases is a significant contributing factor to the low number of participants in our study group. Further studies with larger cohorts are needed to determine if the lower antibody levels are specifically due to MTX or other biologic therapies, a distinction not possible in our current study.

In conclusion, the hepatitis A vaccine is effective and safe in children with autoinflammatory diseases on canakinumab and tocilizumab therapy. Despite lower antibody levels compared to controls, all patients achieved effective immunization. The reduced antibody levels cannot be solely attributed to these biologics due to concurrent MTX and steroid use. Larger studies are needed to confirm the vaccine's safety and immunogenicity in this patient population.

Author Contributions

Kenan Barut: manuscript writing, data collection, literature review, final revision. **Mehmet Yildiz:** manuscript editing, data collection. **Ömer Faruk Beşer:** data collection, statistical analysis. **Fatih Haslak:** statistical analysis, table preparation. **Aybuke Gunalp:** data collection, literature review. **Elif Kılıç Konte:** grafical analysis, literature review, data collection. **Esma Aslan:** referance organization, data collection. **Ümit Gül:** reading, biological material collection. **Nergis Akay:** biological material collection, data analysis. **Amra Adrovic:** data collection, reading, editing. **Sezgin Sahin:** data collection, literature review. **Pelin Yüksel Mayda:** laboratory analysis, data collection. **Bekir Kocazeybek:** laboratory analysis, literature review. **Özgür Kasapçopur:** final revision, editing.

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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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EDITORIAL

NHIRD and TriNetX in Rheumatology: Opportunities and Challenges

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1 | Introduction

Rheumatic diseases are prevalent worldwide, with a considerable impact on patients' quality of life and a tendency to require long-term management. These conditions are complicated by common comorbidities, including cardiovascular disease, endocrine disorder, and mood disorder, further increasing both disease burden and treatment complexity. While biological therapies have revolutionized the management of various rheumatic diseases, high costs, potential adverse effects, and the heterogeneity of patient populations remain significant barriers to achieving optimal, personalized care.

In the history of the development of clinical research, randomized controlled trials (RCTs) have been the gold standard for evaluating efficacy and safety. However, their strict inclusion and exclusion criteria, limited sample sizes, and relatively short follow-up periods may fail to capture the full spectrum of disease heterogeneity and long-term real-world positive and negative outcomes. However, the use of real-world evidence (RWE) in research, as demonstrated in the original article published in the *International Journal of Rheumatic Diseases* in early 2019 (Su-Boon Yong et al., 2019) [1], has brought new hope for addressing such predicaments. Real-world evidence (RWE) derived from large-scale data sources, such as Taiwan's National Health Insurance Research Database (NHIRD) and the recent build global networks like TriNetX, offers the potential to overcome many of these limitations.

This editorial intends to explore the growing influence of RWE on rheumatologic research, discussing both its potential benefits and its limitations. We also highlight future directions for leveraging big data—particularly from Taiwan's NHIRD, TriNetX, and other global repositories, to optimize treatment strategies, refine risk prediction models, and guide real-world clinical decision-making in rheumatology.

2 | Challenges in Rheumatologic Research

The large heterogeneity of diseases leading to the challenge of research, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and idiopathic inflammatory myositis (IIM), each exhibiting considerable variability in clinical manifestations, progression, and therapeutic responses. Diagnostic accuracy is often compromised by nonspecific presentations and the limited reliability of current biomarkers, incomplete imaging data sets, and then elevating the risk of misdiagnosis. These limitations are especially magnified in rare rheumatic diseases, where small patient populations restrict the feasibility of clinical trials and inflate the costs of multinational studies.

With the advances of biological and targeted synthetic medications emerging, prolonged follow-up studies are frequently hampered by high financial demands and significant patient attrition. Insufficient resources, particularly in the realm of rare diseases and innovative drug development, further restrict progress [2].

To overcome these challenges, global collaboration and the adoption of novel methodologies are essential. The large-scale, real-world data sources, such as Taiwan's NHIRD or multi-institutional platforms like TriNetX, can enhance disease surveillance, treatment evaluation, and patient stratification beyond what traditional RCTs can capture.

3 | RCTs vs. RWE in Rheumatology

With the rapid emergence of biological therapies, randomized controlled trials (RCTs) remain a cornerstone for evaluating efficacy and safety in rheumatology. However, the strict inclusion and exclusion criteria in RCTs often limit their external validity, as real-world patient populations typically present with greater heterogeneity and multiple comorbidities [3]. In contrast, real-world evidence (RWE), derived from patient registries, national health insurance databases (e.g., Taiwan's NHIRD), and electronic healthcare records (EHRs) enables the assessment of treatment outcomes in diverse, large-scale, and long-term clinical settings, more closely mirroring routine practice [4].

Randomized controlled trials (RCTs) provide high-level causal evidence primarily because randomization effectively mitigates confounding variables within a carefully defined cohort. At the same time, real-world evidence (RWE) offers a broader perspective by capturing larger and more diverse patient populations, including those with rare or complex conditions. This expansive scope facilitates long-term monitoring of treatment effectiveness and adverse events that may be missed in the relatively short duration of many RCTs. Furthermore, RWE studies often have lower costs, allowing rapid responses to pressing clinical or policy-related questions [4].

Despite their robust internal validity, RCTs, commonly rely on smaller sample sizes, have shorter follow-up periods and higher operational costs, all of which reduce their ability to detect long-term outcomes or rare adverse events. Strict protocols may also exclude patients with multiple comorbidities or atypical disease presentations, thereby limiting the generalizability of RCT findings [3]. By contrast, RWE can suffer from variable data quality across different sources, raising the risks of misclassification, incomplete information, and selection bias. Consequently, rigorous patient matching and sophisticated statistical techniques (e.g., propensity score analysis) are crucial for ensuring that RWE produces reliable and accurate results [4].

4 | Taiwan's NHIRD (National Health Insurance Research Database)

The National Health Insurance Research Database (NHIRD), derived from Taiwan's National Health Insurance system, encompasses claims data for over 99.99% of the population, spanning outpatient and inpatient visits since 2000 [5]. Unlike many EHR-based systems that capture data primarily from specific hospital networks or healthcare systems, NHIRD offers a population-based data set, enhancing its representativeness. Since its inception, NHIRD has amassed extensive information on outpatient and inpatient services, prescription medications, and procedural claims, making it a comprehensive resource for large-scale epidemiological and health services research. By linking to other robust data sources—such as cancer registries and death records—the NHIRD enables longitudinal analyses and facilitates the study of both common and rare diseases in real-world clinical settings [5].

Several countries have developed comparable population-level data sets, such as the Clinical Practice Research Datalink (CPRD) in the United Kingdom, various national healthcare registries in Scandinavian countries (e.g., Denmark, Sweden), and the National Health Information Database (NHID) in South Korea. What sets the NHIRD apart is its nearly universal enrollment, minimal loss to follow up, and the capacity to investigate a vast array of topics ranging from pharmacoepidemiology to cost-effectiveness analyses. Additionally, NHIRD's scalability allows linkage with other databases, enriching research dimensions and improving data accuracy.

However, limitations remain. The database's reliance on administrative coding means data accuracy can be affected by coding practices, including potential issues like “upcoding” or coding errors [5]. Important unmeasured confounders, such as disease severity and lifestyle behaviors (e.g., smoking, alcohol use), are often absent, posing challenges for adjusting bias in research. NHIRD also excludes self-paid services, like cosmetic procedures, limiting its scope in certain areas [6]. Privacy and regulatory restrictions further complicate data access, as researchers must conduct onsite analysis at designated centers, with applications subjected to expert review. Despite these challenges, NHIRD remains a unique and invaluable population-based data set, serving as a cornerstone for advancing rheumatology and broader medical research through its comprehensive and representative data.

5 | TriNetX

TriNetX is a global network that connects EHR data from over 130 healthcare organizations with biopharmaceutical companies to facilitate clinical trials while ensuring patient privacy through the use of aggregated data for feasibility assessments and recruitment.

Its key advantages include robust privacy safeguards, opportunities for collaboration between hospitals and pharmaceutical companies, and extensive global coverage, making it well suited for multi-institutional studies. Easy access and a friendly operating interface are also advantages of the database. After the adjustment in the recent platform, there are several collaborative networks with different purposes in the database to match the needs. However, limitations include the lack of standardized trial identifiers, which complicate the evaluation of system impact, and a focus on data queries with limited clarity regarding their influence on trial progression [7].

6 | Comparing NHIRD and TriNetX: Features, Applications, and Limitations

The National Health Insurance Research Database (NHIRD) and TriNetX exhibit distinct features and applications, as summarized

in Table 1. NHIRD is population-based, providing comprehensive claims data, including diagnoses and prescriptions. It is primarily utilized for epidemiological research and health policy analysis, with robust data linkage to other government data sets. However, access to NHIRD is restricted to Taiwanese researchers or collaborators, and limited validation of certain diagnostic data highlights the need for improved accuracy [5, 6].

In contrast, TriNetX operates as a global federated network, connecting more than 130 healthcare organizations and offering

TABLE 1 | Comparison between TriNetX and Taiwan NHIRD.

Feature	NHIRD	TriNetX
Coverage	1. Population-based in Taiwan, covering over 99.99% of Taiwan's population. 2. Collects administrative claims since 2000. 3. Longitudinal data for both outpatient and inpatient records.	1. Over 130 healthcare organizations globally 2. Global research network integrating data from multiple countries and regions. 3. Primarily EHRs-based data.
Data type	Administrative claims data	Aggregated EHR data from multiple hospitals
Privacy protection	1. Strictly supervised by government authorities. 2. Data are de-identified; usage requires ethical approval. 3. Only aggregated, anonymized claims and clinical information are accessible.	1. Complies with HIPAA, GDPR, and other international regulations. 2. Provides de-identified data sets; researchers cannot access personal identifiers.
Applications	1. Health policy planning and quality evaluations. 2. Drug safety surveillance and long-term efficacy assessments. 3. Large-scale epidemiological research.	1. Real-time or retrospective statistical analyses. 2. Drug safety monitoring and outcomes assessment. 3. Feasibility evaluations for clinical trials.
Data linkage capability	1. Enables complete population follow-up (except for those who emigrate). 2. Can be linked to official registries such as cancer registry and death records.	1. Integration of EHRs from different hospitals may vary by region or institution. 2. Linked network is possible for use, not like the strong linkage in Taiwan's NHIRD 3. Utilizes a distributed data model.
Access restrictions	1. Primarily restricted to Taiwan-based research institutions or international collaborators. 2. Application requires government and ethical clearance. 3. Personally identifiable information is not available.	1. Requires a TriNetX partnership. 2. Multiple levels of access permissions; researchers typically conduct analyses on the platform without directly downloading raw data. 3. Raw data obtained in use of specific network with application.
Data validation	1. Administrative claims rely on ICD, procedure, and prescription codes, potentially influenced by physicians' coding practices. 2. Some external validation studies exist, but coding errors and biases remain possible.	1. Dependent on the completeness and accuracy of EHRs systems at each hospital.

aggregated patient data, such as counts of diagnoses and procedures, to ensure privacy. Its initial setting's primary focus is on supporting sponsor-initiated clinical trials, particularly for feasibility assessments and patient recruitment, though it does not offer data linkage capabilities. TriNetX has fewer access restrictions, varying by participating institutions, and no major data validation concerns have been reported. While both platforms serve valuable but distinct purposes, they differ in data coverage, level of detail, and access policies [7].

7 | Integrating NHIRD and TriNetX for Rheumatologic Research

The NHIRD and TriNetX offer complementary strengths for rheumatologic research, each designed to address different investigative goals. NHIRD, with its nearly universal population coverage, excels in epidemiological and health services research, including analyses of disease prevalence, healthcare utilization, and policy impacts. In contrast, TriNetX leverages detailed EHR data, making it particularly suited for examining treatment effectiveness, disease progression, and patient stratification within diverse clinical settings.

For instance, NHIRD can capture nationwide trends in the incidence and management of rheumatoid arthritis, while TriNetX enables granular assessments of treatment adherence and clinical outcomes in well-defined patient subgroups. By integrating these two data sources, researchers can combine NHIRD's population-level breadth with TriNetX's clinical depth, employing cross-validation to corroborate key findings or using hybrid modeling approaches to explore disparities and outcomes across different cohorts. However, there is still a limitation in the combination of the studies between different claims or databases due to the validity differences and different definitions of raw data. Additionally, advanced machine learning or artificial intelligence methods can enhance both data integration and analysis, revealing novel patterns in rheumatologic care, guiding precision medicine, and informing evidence-based clinical and policy decisions.

8 | Conclusion

Advancing rheumatologic research demands innovative strategies to navigate the complexities of these diseases. The NHIRD excels in population-level analyses, whereas TriNetX offers granular clinical insights, making their integration a powerful tool for comprehensive investigations. Combining these resources enables researchers to gain a deeper understanding of disease mechanisms, treatment outcomes, and healthcare systems. The combination with EHR database and population-based database mitigates the problem in external validity, misclassification, information, and selection biases from the full perspective analysis. This integrative approach holds the potential to transform rheumatologic research, fostering the development of personalized, data-driven care strategies.

Author Contributions

C.C.C. and P.-C.S.: writing – original draft. S.B.Y., E.C.-C.L.: review and editing. S.B.Y., P.-C.S.: writing – review and editing.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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LETTER TO THE EDITOR

Role of Protective and Predisposing HLA-B Alleles and MICA Variants (MICA*049 and MICA*00901) in South Indian Behçet's Disease

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Dear Editor,

Behçet's disease (BD) is a chronic inflammatory disorder that causes recurrent episodes of inflammation affecting various organ systems and the hallmark clinical features include recurrent oral and genital ulcers. Its pathogenesis is likely multifactorial involving an interplay of excessive immune activation, genetic predisposition, and environmental triggers. The key factors that have been implicated in BD include aberrant immune responses, oxidative stress, and autoimmunity against heat-shock proteins (HSPs). While the precise etiology remains elusive, a well-established association exists between BD and genetic susceptibility, with the human leukocyte antigen (HLA)-B*51 allele being the strongest genetic risk factor. The association between HLA-B51 and BD is strongest in populations from Japan, the Middle East, and other parts of Asia [1]. A meta-analysis of 4800 Behçet's disease (BD) cases and 16,289 controls reported a combined statistical measure, known as the pooled odds ratio, of 5.78. This indicates that individuals carrying the HLA-B*51 allele have approximately 5.78 times higher odds of developing BD compared to non-carriers, as determined through the combined analysis of data from multiple independent studies [2]. Additionally, the major histocompatibility complex class I chain-related gene A (MICA) has been linked to BD in various ethnic groups, with polymorphisms in the transmembrane region, such as MICA009, showing significant associations [3]. Environmental triggers, including infections caused by bacteria (e.g., *Helicobacter pylori*, Mycoplasma, and Streptococcus) and viruses (e.g., hepatitis viruses and herpes simplex virus), have been investigated as potential contributors. Autoantibodies against bacterial and human HSPs (HSP65 and HSP60) further suggest an autoimmune mechanism [4]. The MICA transmembrane region peptide (AAAAAIFVI) has been identified as a candidate bechetogenic epitope, capable of inducing autoreactive CD8+ cytotoxic T lymphocytes in BD patients with HLA-B*51, thereby driving autoimmune responses [5]. Additional associations have been identified within the major histocompatibility complex (MHC) class I region, including HLA-B*15, -B*27, -B*57, and -A*26. However, the involvement of other genes like *CCR1-CCR3, ERAP1, FUT2, IL23R-IL12RB2, IL10, KLRC4, STAT4*, and *TNFAIP3* further underscores the polygenic nature of BD susceptibility [6, 7]. These genes are functionally linked to immune regulation and inflammatory pathways, suggesting their potential roles in disease pathogenesis. The incomplete penetrance of HLA-B*51 in some BD patients implies the participation of additional, non-genetic factors in disease development [8]. In this study, we aimed to investigate the association of HLA-B alleles and MICA variants with BD in a South Indian population with Dravidian ethnicity, using a cohort sample from the state of Kerala. These results offer novel insights into the genetic predisposition to BD and contribute to the growing understanding of disease etiology across different ethnicities.

This retrospective case–control study was conducted to investigate BD in the Keralite population of India. The study aimed to determine the clinical profile of BD and to identify HLA alleles associated with it. In this study, 108 patients diagnosed with BD according to the international criteria for BD (ICBD) and ISG

(International Study Group) criteria were recruited from the Clinical Immunology and Rheumatology department at Amrita Institute of Medical Sciences (AIMS), Kerala, India. An age, gender, and ethnically matched control group of 150 healthy individuals was also enrolled. The study was commenced after obtaining the approval from the Institutional Ethics Committee. Demographic data, disease manifestations, treatment regimens, and clinical features were documented for all participants.

Genomic DNA was isolated from peripheral blood samples using QIAamp DNA Mini Kit (Qiagen, Germany) and was quantified using a spectrophotometer (Biophotometer, Germany) and adjusted to a concentration range of 30–35 ng/μl. HLA-B tissue typing was performed using 5 U/μl Taq-Polymerase (Sigma, India) and 35 ng/μl of the isolated genomic DNA, with HLA sequence-specific primer-based low-resolution kits specific for HLA B alleles (B7–B82) (BAG Healthcare, Germany), as per the manufacturer's instructions. The amplified products were electrophoresed in 3% agarose gels. Histomatch software (BAG Healthcare, Germany) was used to assign the alleles based on the banding pattern observed post-agarose gel electrophoresis. Statistical analysis was performed to compare allele carrier frequencies of HLA-B alleles between patients and healthy controls using SPSS software version 20.0 [9]. Univariate logistic regression analysis was conducted to identify significant risk factors. A p-value < 0.05 was considered statistically significant.

We utilized the MHC Motif Atlas database (http://mhcmotifatlas.org/) to predict the peptides that can be presented by specific HLA alleles. The MHC Motif Atlas provides comprehensive information on binding motifs and ligand specificities for thousands of Class I and Class II MHC molecules [10]. The NetMHCpan-4.1 web server was employed to carry out the binding affinity studies [11]. Six nonameric peptide sequences were entered into the NetMHCpan-4.1 server, in FASTA format. The peptide sequences used were DAHIYLNHI, DAAPPQVTF, AAAAAIFVI, AAAAAIFVL, AAAAAIFVV, and APAAAIFVI [12–14]. From the list of HLA alleles, HLA-B*07:02, HLA-B*35:01, HLAB*51:01, and HLAB*58:01 were selected. These inputs were submitted for interaction analysis and prediction of binding affinity by using artificial neural networks of NetMHCpan-4.1. The output of the analysis was analyzed to find the best interaction of the HLA allele with BD-associated peptides. The interactions obtained were ranked based on the predicted binding score compared to a set of random natural peptides. Interacting HLA alleles and the peptide having %rank < 0.5 were considered strong binders, and weak binders had %rank < 2.

To determine the association of MICA alleles, MIC-A*00901 and *049, with BD, Tetra-primer Amplification Refractory Mutation System PCR (T-ARMS-PCR) was performed. T-ARMS-PCR is a rapid and cost-effective genotyping technique that utilizes four primers in a single reaction to selectively amplify wild-type and mutant alleles based on allele-specific priming [15]. This method allows for efficient SNP genotyping without the need for post-PCR processing, making it a valuable tool for genetic association studies. The T-ARMS-PCR protocol was performed as described previously [16].

In this case–control study, a total of 108 BD patients and 150 healthy controls from the Kerala population were analyzed.

TABLE 1 | Overview of the demographic and clinical characteristics of Behçet's disease in the study cohort.

Parameter	Prevalence/Percentage
Age at onset	
Mean (range)	32 years (18–55)
Median	30 years
Gender	
Male	50%
Female	50%
Presence of oral ulcers	85%
Presence of genital ulcers	45%
Ocular manifestations	
Uveitis	35%
Retinal vasculitis	20%
Episcleritis	10%
Gastrointestinal symptoms	25%
Vascular involvement	15%
Neurological manifestations	10%
Musculoskeletal manifestations	
Arthritis	30%
Arthralgia	40%
Myalgia	20%
Cutaneous manifestations	
Erythema nodosum	25%
Pseudofolliculitis	20%
Positive pathergy test	40%

Table 1 presents the demographic characteristics of the study population in Kerala. The mean age at onset of BD was 32 years with a range of 18–55 years. Gender distribution among patients revealed that 50% were male and 50% were female. Clinical presentation varied among patients, with the majority experiencing recurrent and relapsing mouth ulcers often accompanied by episodic arthritis. Some patients exhibited more severe manifestations, including genital ulcers, uveitis, and vasculitis (Table 1). Our study revealed an equal male-to-female ratio (1:1) for BD in the Kerala population, consistent with ratios reported in Japan (0.98), China (1.34), Iran (1.3), and Turkey (1.15). However, it slightly differs from ratios in Korea (0.63), highlighting potential regional variations in disease prevalence [17]. The average age of onset of BD in our study population was 32 years, which is slightly higher compared to the ages reported in other populations, such as China (29 years), Iran (28.3 years), Turkey (25.6 years), and Korea (29 years) [17, 18].

Analysis of HLA-B allele frequencies revealed significant associations with BD. HLA-B*51 exhibited the most significant association ($p < 0.0001$, OR = 3.7882, 95% CI: 2.1671–6.6220), indicating a substantially higher prevalence in patients

compared to controls. This is consistent with earlier reports that have identified HLA-B*51 as the strongest susceptibility factor for BD in various populations [2, 7]. Additionally, HLA-B*58 showed a statistically significant association ($p = 0.0053$, OR = 0.3014, 95% CI: 0.1298–0.6998), suggesting a possible protective role against BD. Interestingly, HLA-B*07 was found at a higher frequency in the control group ($p = 0.0045$, OR = 0.4121, 95% CI: 0.2236–0.7595), indicating a potential protective effect against BD. Meanwhile, HLA-B*35 showed no significant association with BD in our study ($p = 0.9862$, OR = 0.9957, 95% CI: 0.6125–1.6186). This contrasts with its previously reported role, where it was identified as a protective allele in Spanish populations but a susceptibility allele in the Iranian Azari population [19, 20]. None of the other HLA-B alleles exhibited statistically significant associations ($p > 0.05$) (Table 2). Multiple sequence alignment revealed variation in amino acid residues within the peptide-binding cleft among HLA-B alleles B*07, B*35, B*51, and B*58. These differences directly influence the specificity and affinity of HLA molecules for peptide antigens, thereby altering the repertoire of peptides presented to T cells and affecting immune recognition and response (Figure S1). We analyzed the binding specificities of the HLA- B*07, B*35, B*51, and B*58 alleles *in silico*. Peptides were predicted using the MHC Motif Atlas database's curated binding motifs and peptide length distributions. Analysis of allele-specific peptide binding motifs for these HLA-B alleles identified conserved anchor residues. At Position 2, residues such as proline are strongly preferred in alleles B*07, B*35, and B*51, whereas polar residues like serine and threonine are favored in HLA-B*58. Position 9 acts as a strong anchor, with a preference for hydrophobic residues including tyrosine (Y), valine (V), leucine (L), and tryptophan (W) (Figure S2). The binding affinities of nanomeric peptides associated with BD to HLA alleles HLA-B*07, HLA-B*35, HLA-B*51, and HLA-B*58 were predicted using the NetMHCpan-4.1 server, which predicts binding of peptides to various MHC molecules using artificial neural networks [11]. Among the tested alleles, HLA-B*51 exhibited the highest number of strong peptide-binding interactions, with the sequence DAHIYLNHI demonstrating the strongest affinity, followed by DAAPPQVTF and APAAAIFVI. In contrast, HLA-B*07 showed the lowest binding levels to BD-associated peptides, supporting its protective role in the disease condition. While HLA-B*58 displayed a few weak interactions with the nanomeric peptides, these findings suggest a potential association with BD, which warrants further investigation. The dataset of predicted interactions, generated using NetMHCpan-4.1, is provided in Table S1.

MICA, known for its extensive polymorphism, has been associated with BD, particularly the MICA TM A6 and MICA*009 alleles. MICA*009 can further divide into subtypes like MICA*00901, MICA*0090201, and MICA*0090202, differing from MICA*049 primarily at codon 335 in exon 6 [21]. Recent research in a Han

TABLE 2 | HLA-B allele frequencies and associations with Behçet's disease.

HLA-B alleles	Patient frequency ($n = 108$)	Control frequency ($n = 150$)	Odds ratio	95% CI	p
B*07	**0.07**	**0.15**	**0.4121**	**0.2236 to 0.7595**	**0.0045**
B*08	0.00	0.01	0.3442	0.0382 to 3.1012	0.3416
B*13	0.03	0.01	2.8286	0.6995 to 11.4378	0.1447
B*15	0.10	0.12	0.8061	0.4607 to 1.4102	0.45
B*18	0.01	0.01	2.0986	0.3476 to 12.6685	0.419
B*27	0.01	0.01	1.3944	0.2787 to 6.9756	0.6857
B*35	0.15	0.15	0.9957	0.6125 to 1.6186	0.9862
B*37	0.01	0.04	0.2243	0.0497 to 1.0127	0.0519
B*38	0.02	0.01	2.8113	0.5102 to 15.4897	0.2352
B*39	0.02	0.02	1.1132	0.2954 to 4.1948	0.8741
B*40	0.11	0.11	0.9981	0.5662 to 1.7593	0.9946
B*44	0.08	0.08	1.0949	0.5755 to 2.0831	0.7824
B*49	0.00	0.01	0.3442	0.0382 to 3.1012	0.3416
B*51	**0.21**	**0.03**	**3.7882**	**2.1671 to 6.6220**	**< 0.0001**
B*52	0.03	0.07	0.4689	0.1946 to 1.1296	0.0913
B*55	0.03	0.04	0.8038	0.3112 to 2.0764	0.652
B*56	0.01	0.03	0.5141	0.1348 to 1.9606	0.3299
B*57	0.04	0.00	13	1.6345 to 103.3951	0.0153
B*58	**0.03**	**0.10**	**0.3014**	**0.1298 to 0.6998**	**0.0053**

Notes: A p value < 0.05 was considered statistically significant. Bolded values indicate statistically significant associations between specific HLA-B alleles and BD in this cohort. A p-value < 0.05 was considered statistically significant. These alleles are potentially predisposing or protective genetic markers for BD.
Abbreviations: CI, confidence interval; OR, odds ratio.

Chinese cohort revealed that MICA*049, not MICA*009, is a BD risk factor, independent of HLA-B*51. This study employed T-ARMS-PCR to distinguish between MICA*00901 and MICA*049 alleles, addressing previous ambiguity regarding their association with BD [16]. Our analysis revealed an allele frequency of 0.97 and 1 for MICA*00901 in BD patients and controls, respectively. Conversely, MICA*049 exhibited a frequency of 0.03 among BD patients but was entirely absent in the control group. Neither MICA*049 nor MICA*00901 demonstrated a significant role in BD predisposition ($p > 0.05$) in our study population.

Our study is limited by a small sample size. We acknowledge the need for further investigations with larger cohorts to validate these findings and explore the underlying mechanisms more comprehensively, involving environmental or genetic factors specific to the Kerala population.

In summary, HLA-B*51 is the strongest genetic factor associated with BD in the Kerala population, consistent with previously reported studies [1, 2, 19]. HLA-B*35 appears not to play a role in BD. Our study suggests a potential protective role for HLA-B*07. Of note, no significant association between the MICA alleles, MICA*049 and MICA*00901, and BD was observed in our study.

Author Contributions

J.J.F., S.R.D.R., A.D., and V.V.: data curation, formal analysis, investigation. L.B.: conceptualization, resources, project administration, investigation, data curation, formal analysis, and writing. M.C.B.: resources, investigation, data curation, formal analysis, and writing.

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Ethics Statement

The study was approved by the Institutional Ethics Committee Board of Amrita Institute of Medical Sciences and Research Centre (IEC-AIMS-2023-ASNSMM-71).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data supporting this study are provided in the Supporting Information. Additional datasets that are generated and/or analyzed during the current study are available from the corresponding author upon request.

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Supporting Information

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ORIGINAL ARTICLE

Neutralization of IL-17 and CXCR1 Protects Septic Arthritis by Regulating CXCL8-CXCR1 Pathway Along With Functional Activities in Neutrophils

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ABSTRACT

Aim: The main objective of this study is to elucidate the role of CXCR1 Ab, IL-17 Ab, and gentamicin in protecting septic arthritis by regulating neutrophil functional responses while evaluating the contribution of the CXCL8-CXCR1 pathway.

Methods: Eighty-four experimental swiss albino mice were utilized to study septic arthritis. They were divided into eight groups. After developing sepsis, respective mice groups were treated with CXCR1 Ab, IL-17 Ab, and gentamicin doses. Doses were administered on days 1, 8, and 13 of the experimental schedule. At the early, middle, and late phases of the experiment i.e. at 3, 10, and 15 DPI (Days Post Infection), mice were sacrificed and blood and tissues were collected for further experimental evaluations. Different functional studies were performed on isolated blood neutrophils, spleen, and synovial tissues. Histological evaluation, immunofluorescence study, sepsis profile, and downstream signaling pathway analysis were done to obtain data.

Results: Infected mice group exhibited high inflammatory responses while treatment helped to mitigate them. IL-17 neutralization helped to lower bacterial burden, neutrophil ROS activity, MPO activity, and ALP activity. However, the combined neutralization of CXCR1 and IL-17 greatly influenced PMN chemotactic activity and lysozyme activity. At the early phase of the experiment, IL-17 neutralization's impact was more prominent, while later, CXCR1 neutralization gained the upper hand.

Conclusions: We can conclude that IL-17 Ab in combination with gentamicin is potent in modulating neutrophil activities positively to cure sepsis, while CXCR1 Ab, through the CXCL8/CXCR1 pathway, regulates neutrophil functional activities by impacting different downstream signaling cascades.

1 | Introduction

Neutrophils are the frontline leukocyte cells equipped with antimicrobial machinery that utilizes both oxidative and nonoxidative strategies to kill invading pathogens. The nonoxidative method includes antimicrobial proteases which, during infection, act as a double-edged sword: The antimicrobial function kills pathogens, while excessive degranulation harms host tissue [1]. Among different infectious pathogens, a Gram-positive microbe, S. *aureus*, especially its MRSA strain, is well-known to cause septic arthritis. The hallmark of sepsis includes rapidly advancing joint deterioration, a medical emergency that has been around for years [2]. Primary pathogenesis of sepsis involves Systemic Inflammatory Response Syndrome (SIRS), mediated by massive release and spread of inflammatory mediators TNF-α, IL-1, IL-6, IL-8, and IL-12 to sites where inflammation does not occur, resulting in injury and organ failure [3]. The developing inflammation causes the

movement of neutrophils from the bloodstream to the inflammatory focus, which scavenges the injury which is stimulated by the elements of the decomposition of collagen, elastin, components of the complement system, and TGF-β, TNF-α, IL-1, and platelet factor-4 etc. [4]. To reduce excessive inflammatory reaction, compensatory anti-inflammatory response syndrome (CARS) develops, which leads to immunosuppression [5]. SIRS activates bactericidal activity of immune cells by inducing the production of TNF-α, ROS, and reactive nitrogen species (RNS). ROS can be exogenous and intracellular. Exogenous ROS are produced by physical, chemical, or biological stimuli, while the source of intracellular ROS is regular cell metabolism. Exogenous ROS never exists. Intracellular ROS level is always measured. The involvement of ROS and RNS in sepsis leads to hyperpermeability, vasodilation, and mitochondrial dysfunction, which is combated with antioxidants, namely SOD, catalase, and glutathione [6]. During sepsis, Nitric Oxide Synthase (NOS) activation and other inflammatory stimuli lead to NO accumulation in blood and tissues. Excessive generation of NO by iNOS is linked with vasodilation and reduced sensitivity to exogenously administered vasopressors during sepsis [7]. There is correlation among myeloperoxidase (MPO) activity, oxidative damage, IL-17, and CXCR1. Increased MPO activity is an indicator of inflammation and the onset of sepsis. Activated neutrophils release MPO, stored in its azurophilic granules, which has a strong oxidative potential. This MPO represents a major pathway for $O_2{\cdot}^-$ dependent microbicidal activity [8]. During any infection like sepsis, neutrophils release the lysozyme enzyme. Leukocyte-derived lysozyme binds with endocardial endothelium, resulting in the production of NO in a septic shock model. Lysozyme (LZM) hydrolyzes the peptidoglycan components of the cell walls of Gram-positive bacteria like *S. aureus* [9].

Experimental evidence from previous in vivo and in vitro animal studies has pointed out the essential role of the CXCL8-CXCR1/2 axis in neutrophils in pathophysiology. Dysregulated activation of CXCR1/2 contributes to tissue and systemic damage, which can be prevented by chemokine blockade [10]. CXCL8 or IL-8 chemokine is a neutrophil chemoattractant that acts through CXCR1 and CXCR2 receptors. CXCR1/2 inhibition has great therapeutic potential. CXCL8 binding triggers calcium release and chemotaxis strongly through CXCR1 and mediates phospholipase D activation, further leading to oxidative burst and other physiological roles [11]. During inflammation, the CXCL8-CXCR1/2 axis mediates the induction of neutrophil activation, rolling, and transmigration to different organs, unfolding spectrum of functions including shaping immune responses, tissue injury and repair, and killing the pathogens through NET formation, and so forth. The majority of sepsis outcome-related genes are enriched in the IL-17 signaling pathway. These pathway-related genes, including IL-17R, CXCR1, and CXCR2, were significantly upregulated in the sepsis blood sample in the study [12]. Stress proteins, PAMPs, and microbial metabolites trigger APCs like neutrophil cells to produce IL-23 and IL-1β that accelerate IL-17 release. Prostanoids are key mediators of the IL-23/IL-17 axis-induced neutrophil recruitment during infection [13]. IL-17A/IL-17C interacts with IL-17R to stimulate inflammatory reactions via the activation of MAPK, NF-κB, and JAK–STAT signaling. Inhibition of IL-17 has the potential to modulate the inflammatory response cascade by downregulating the endothelial tissue factor and modulating splenic vein remodeling and bone resorption [14]. It was previously reported that IL-17 significantly promotes the production of IFN-γ, ROS, MPO, and TRAIL in neutrophils and stimulates the secretion of CXC chemokines that bind to CXCR1/2. IL-17 influences chemokine expression through NFκB and MAPK signaling pathways [15]. Neverthless, the aim of this study is to observe the impact of neutralizing CXCR1 and IL-17 along with the antibiotic gentamicin in reducing inflammatory responses generated by neutrophils compared to the septic arthritis group. This study also aims to understand how CXCR1 neutralization regulates neutrophil functional activities like ROS activity, MPO activity, lysozyme activity, degranulation activity, PMN chemotaxis, and so forth by impacting the downstream signaling proteins of the CXCL8/CXCR1 pathway.

2 | Materials and Methods

2.1 | Maintenance of Experimental Animals

For experiment purposes, 6–8 weeks old 84 male Swiss albino mice, weighing about (20 ± 5) g were maintained in open top cages with feeding bottles, in a well-humified room. All animal experiments were performed following guidelines given by the Institutional Animal Ethics Committee (IAEC), Department of Physiology, University of Calcutta, under the guidance of CPCSEA [IAEC approval number: IAEC-V/T/BB-15/Sharmistha Ghosh/2023 dated 23.02.2023].

2.2 | Preparation of Bacteria and Induction of Sepsis

MRSA data bacterial strain PU-CA-14 was kindly gifted by Prof. Mausumi Sikdar (ne'e Bhakta) of Presidency University, Kolkata. The bacterial strain was cultured and prepared for infection. Infection was given at Day 0 of the experiment to seven experimental animal groups except the control [16]. The seven experimental groups are: *S. aureus*-infected mice group (SA), *S. aureus*-infected mice group treated with CXCR1 Ab (SA + CXCR1 Ab), *S. aureus*-infected mice treated with IL-17 Ab (SA + IL-17 Ab), *S. aureus*-infected mice group treated with both CXCR1 Ab and IL-17 Ab (SA + CXCR1 Ab + IL-17 Ab), *S. aureus*-infected mice group treated with CXCR1 Ab and gentamicin (SA + CXCR1 Ab + Gent.), *S. aureus*-infected mice group treated with IL-17 Ab and gentamicin (SA + IL-17 Ab + Gent.), and *S. aureus*-infected mice group treated with CXCR1 Ab, IL-17 Ab, and gentamicin (SA + CXCR1 Ab + IL-17 Ab + Gent.).

2.3 | Antibody and Antibiotic Regime

Our experiment was 15 days long. First dose of antibody–antibiotic was given on day 1; second dose was given on day 8; and third dose was given on day 13 of the experiment. At days 3, 10, and 15, mice were sacrificed. Antibody CXCR1 was given at a dose of 1 μg/mouse (Cat no.: orb215479) [17]. IL-17 Ab was given at a dose of 200 μg/mouse (Cat no.: orb10898) [18]. Gentamicin was given at a dose of 5 mg/kg body weight of mouse [19].

2.4 | Evaluation of Sepsis

Sepsis was evaluated by the histological study of synovial joints. First, synovial tissues were fixed in 4% paraformaldehyde at 4°25°C for 24 h then muscle tissues were teased apart and joint was decalcified by dissolving in 0.5 (M) EDTA'. Next, the samples were dehydrated through an ethanol series and embedded in paraffin. Sections were cut with a microtome and stained with hematoxylin and eosin (HE) stain and TRAP stain. TRAP staining was performed using a commercial acid phosphatase leukocyte kit (Ref No.: 387A-1KT; Sigma Aldrich) [20]. For TRAP staining, the prepared sample was fixed on a microscopic slide. The resulting film was incubated in a solution of naphthol AS-BI phosphoric acid and freshly diazotized Fast Garnet GBC. In a beaker, 0.8 mL of 4% $NaNO_2$ was taken, and 0.8 mL of pararosaline was added and mixed continuously for 1 min. In this mixture, acetate buffer was added. The naphthol AS-B1 mixture was poured into coplin jars. For tartarate resistance, tartarate solution was added. Slides were incubated in the solution for 1 h at 37°C. Slides were washed and counterstained with hematoxylin solution. They were then dried and examined under a microscope.

2.5 | Determination of the Number of Viable *S. aureus* Cells

Viable *S. aureus* cell numbers present in blood, spleen, and synovial tissues were assessed using the pour plate method of bacteria plating. Four animals of each group were sacrificed, and blood was pooled and tissues were collected. From the collected tissues, a homogenate was prepared for plating [21].

In our study, we have utilized 84 mice only. The functional assays performed were repeated two times as technical replicates, i.e., two repeated measurements were taken from the same sample. We have pooled blood from mice of the same experimental group; for that reason, biological replicates were not considered to study functional assays.

2.6 | Mouse Neutrophil Preparation From the Collected Blood

Mouse blood was collected by cardiac puncture into HBSS-EDTA. Blood collected from the same experimental group was pooled and laid on percoll layerings. A three-layer percoll gradient of 78%, 69%, and 52% was prepared by diluting the percoll with HBSS (100% percoll = 9 parts percoll +1 part 1X HBSS). After layering, centrifugation was performed. Neutrophils from the 69%/78% interface and the upper part of 78% were harvested in a 1% BSA-coated tubes. Red cells were eliminated by hypotonic lysis. After washing with HEPES buffer, the neutrophil cell pellet was collected [22]. The viability of isolated neutrophils was determined by MTT assay.

2.7 | MTT Assay

MTT assay was also performed to check the viability of isolated PMNs. The in vitro cell viability test investigated using the MTT assay was on neutrophils isolated from peripheral blood. Briefly, 100 μL of the cell sample per well was seeded in 96-well plates and incubated for 3 h at 37°C. After the incubation period, the cells were rinsed with PBS and cultured in media containing 100 μL of MTT (0.5 mg/mL) reagent in each well and incubated for 30 min in the dark at 37°C in a 5% CO_2-humidified incubator. This allows intracellular reduction of the soluble MTT to the insoluble formazan dye. A volume of 100 μL of dimethyl sulfoxide (DMSO) was added to each well to allow formazan solubilization and then incubated for 10 min. The 96-well plates were then vibrated for an additional 15 min, and the optical density of each well was measured at λ570 nm using a microplate reader [23].

2.8 | Neutrophil Degranulation Assay

Purified mouse blood neutrophils were incubated with 10 μM cytochalasin D in HBSS containing 20 mM HEPES (pH 7.4) and 0.1% BSA (HBSS-HB) for 15 min on ice, followed by 15 min at 37°C. The cells were then stimulated for 10 min with LPS (100 ng/mL) at 37°C. The reaction was terminated by placing samples on ice, and the supernatant was centrifuged. 20 μL of the supernatant was incubated with 20 μL of 10 mM 4-methylumbelliferyl β-D-glucuronide hydrate in 0.1 M sodium acetate (pH 4.0) and 0.1% Triton X-100 at 37°C for 15 min. The reaction was terminated by adding 300 μL of Stop Solution (pH 10.4) containing 50 mM glycine and 5 mM EDTA. Fluorescence was measured immediately with an excitation wavelength at 365 nm and an emission wavelength at 460 nm [24].

2.9 | Lysozyme Release Assay

M. lysodeikticus is mixed with 0.15(M) potassium phosphate buffer (pH = 6.2). The absorbance of the suspension is measured at 450 nm and is adjusted to 0.6 or 0.7. The mixture of 0.9 ml volume is added to a cuvette together with 0.05 mL of supernatant from a neutrophil degranulation preparation or 5×10^5 PMN. The decrease in O.D. is recorded as a function of time. The result of lysozyme activity in samples is obtained using a lysozyme standard curve run in parallel during the experiment [25]. Results were expressed as units/mg of protein per 2 min using the following formula:

$$\text{Specific activity} = \frac{\text{(Change in absorbance per minute)}}{\text{Total protein (mg) in the assay}}$$

2.10 | NBT Assay

Neutrophil cells attached to a microplate were incubated with 100 μL of Y-NBT solution containing LPS (300–600 ng/mL) for 60 min. As negative controls, some cells were incubated in Y-NBT solution containing LPS with 5 μM DPI or 30 μg/mL SOD. After incubation, cells were washed twice with warm PBS, then once with methanol, and air-dried. The NBT deposited inside the cells was then dissolved, first by adding 120 μL of 2 M KOH to solubilize cell membranes and then 140 μL of DMSO to dissolve blue formazan with gentle shaking for 10 min at room temperature. Absorbance was taken at 620 nm [26].

2.11 | PMN Chemotaxis Assay

In the PMN chemotaxis assay, the chemokine molecule IL-8 (164 pg/mL) [Cat #MBS012027] is initially contained in an agarose drop which is applied to a glass-bottom Petri dish as a spot and immersed under media containing cells of different experimental groups. The negative control used here is an agarose drop containing no chemoattractant. Over time, chemokine molecules slowly diffuse outward of the agarose spot, creating a transient chemotactic gradient within the agarose spot. Neutrophil cells expressing the corresponding cognate chemokine receptors move toward the center of the drop. The number of migrated neutrophils was counted manually during microscopic evaluation, and the numbers were expressed in percentage while taking the migrated cell number of the control group as 100% [27].

2.12 | Alkaline Phosphatase (ALP) Release Assay

ALP assay was performed with serum collected from experimental mice. The assay was performed according to the protocol of the ALP Assay Kit (MBK Alkaline Phosphatase, Kind and King's assay; Ref: 75 MB100-40).

2.13 | Cell-Free Lysate Preparation

Neutrophil cells preserved in RPMI solution were stimulated with LPS (100 ng/mL) for nearly 1 h and then centrifuged. The collected cells were lysed with PBS containing 0.1% Triton X-100 and centrifuged. Cell-free lysate was collected, and ROS and MPO activity were evaluated.

2.14 | ROS Activity (H_2O_2, NO, and $O_2\cdot^-$) Analysis

Production of H_2O_2 is determined by the oxidation of phenol red by H_2O_2 mediated horseradish peroxide type II (HRP), as described by Pick and Keisari. Briefly, neutrophil cell-free lysate was incubated for nearly 2 h in the assay solution containing 0.56 mM of phenol red and 20 U/mL HRP in HBSS with phenol red at a final volume of 100 μL per well. After 2 h incubation at 37°C, the reaction is stopped by adding 10 μL of 1 N NaOH per well. The rate of absorbance is measured using a spectrophotometer at 620 nm wavelength. Wells with NaOH are used as blanks. Results are obtained using a standard curve of H_2O_2 (5–60 μM) run in parallel during this experiment [28].

NO production is carried out through nitrite determination by using the procedure described by Ding et al. Nitric oxide is rapidly changed into nitrite in aqueous solution. Therefore, the total nitrite can be used as an indicator of NO concentration. The spectrophotometric analysis of the total nitrite content is performed by using the Griess method (1% sulfanilic acid, 0.1% N-1-naphthyl-ethylenediamine dihydrochloride) with the neutrophil cell-free lysate. The same volume of Griess reagent is added to the cell-free lysate, and after 15 min, absorbance is measured using the (540–550) nm spectrophotometer band. The blank consisted of only Griess reagent, without any sample with equivalent volume as taken in sample cuvettes. Here, only 400 μL of Griess reagent was taken as blank, maintaining the same parameters of temperature and incubation period. The resultant nitrite concentration is determined using a standard curve where sodium nitrite was used as the standard (0–60 μM) [29].

Extracellular superoxide anion release by neutrophils was measured using the superoxide dismutase (SOD)-inhibitable reduction of the ferricytochrome c assay, as described by Johnston et al. Neutrophils isolated from different experimental mice groups were stimulated with LPS (100 ng/mL) for 2 h and incubated in a mixture of 800 mM ferricytochrome c and Hanks 15 mM HEPES medium, with or without SOD (15 mg/mL). The reaction was stopped by placing the test tubes in an ice bath. The cells were then immediately centrifuged at 730 g for 10 min. Absorbance of the cell-free supernatant was measured using a spectrophotometer at a wavelength of 550 nm. Tubes containing neutrophils incubated with ferricytochrome c in Hanks HEPES medium with SOD served as blanks [30].

The amount of superoxide anion generated will be calculated according to the equation:

$$\text{Cytochrome c reduction}\left(\text{nmol/ min }/10^6\text{ cells/mL}\right) = (\Delta A550-540\text{ nm/ min})\times 19.1\times 1000$$

2.15 | MPO Activity Assay

MPO activity kinetics was evaluated against the substrate guaiacol on a UV–visible spectrometer at 470 nm. A 30 ml assay solution was prepared consisting of 3 ml of 0.1 (M) sodium phosphate buffer (pH 7), 48 μL of guaiacol, and 100 microlitre of 0.1 M H_2O_2, in which 26.9 mL of water is added 100 μL of the cell supernatant will be added to 500 μL of the assay solution in a 600 μL cuvette, and the spectrophotometric result is then observed [31].

2.16 | Gentamicin Bioassay

Gentamicin bioavailability was assessed using a disc agar diffusion method. A standard dose–response curve of gentamicin tested against *S. aureus* PU-CA-14 was generated using 0.5, 1.0, 1.5, and 2.0 μg/μL gentamicin concentrations. Serum was collected from experimental samples, and a paper disc was saturated with the serum. A spread plate was prepared with *S. aureus* bacteria where two reference discs with 1.0 μg/μL gentamicin concentration were placed opposite each other. Meanwhile, in a different quadrant, two discs containing the sample serum were placed. The plates were incubated overnight. The zone diameters of the reference standard and samples were measured and averaged. Correction of the zone diameter was accomplished between the mean zone diameter of the reference standard obtained in the assay plate and the zone diameter of the correction point of 1.0 μg/μL in the standard curve [32].

2.17 | Quantification of Protein Expression by Western Blot

Ten 10 DPI spleen tissue samples were lysed with RIPA buffer to prepare a cell-free lysate which is used to perform western

blot. The cell lysate contained equal protein content, as normalized using the Lowry method before running SDS PAGE. Samples were denatured by boiling at 100°C for 5–7 min in an SDS PAGE loading buffer (1× Laemmli Buffer = 60 mM Tris, 2% sodium dodecyl sulfate, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) and were resolved by SDS PAGE. Proteins were transferred onto a nitrocellulose membrane whose blocking was done using 5% BSA in Tris-buffered saline and 0.1% Tween 20 (TBST) and kept for 2 h. Then, the membrane was washed two times with TBST and probed overnight with primary antibodies in TBST (TBS with 0.1% Tween 20). The primary antibodies used for this experiment are iNOS [CatNo Proteintech 22226-1-AP] COX2 [Cat No: Proteintech 12375-1-AP], SOD [Cat No: Biorbyt, 67514], CAT [Cat No: Santacruz, SC 50508] STAT1 [Cat No: Proteitech, 66545-1-Ig], STAT3 [Cat No: Proteintech, 60199-1-Ig] SOCS3 [Cat No: Proteitech, 66797-Ig-1], RANKL [Cat No. ORB6560], NF-κB [Cat no. 436700, Mouse Anti-NF-κB (P65), Life technology], TNF-α [Cat No. MAB9502], IFN-γ [Cat No. orb22685, Biorbyt Ltd. UK], IL-10 [Cat No. Biorbyt 22682], IL-17 [Cat No. orb10898, Biorbyt Ltd. UK], CXCR1 [Cat No. orb215479, Biorbyt Ltd. UK], and β-tubulin [Cat No. orb11537, Biorbyt Ltd. UK]. The next day, blots were washed twice with HRP-conjugated 2°Ab and were developed using a chemiluminescent substrate. The values were normalized with loading control β-tubulin. To analyze the outcome, a densitometric study and comparison were performed using Image J software. The software used quantified protein bands from western blot films and reflected the relative amounts as a ratio of each protein band relative to the lane's loading control, i.e., β-tubulin.

2.18 | Statistical Analysis

All the data collected during the experiment are expressed in (Mean ± S.D.) format and are significant at the $p < 0.05$ level. Data are evaluated by one-way ANOVA, using OriginPro software. Blood pooled from the same experimental group of mice was used to perform different functional assays.

3 | Results

3.1 | Assessment of the Histological Study Outcome

The histological study displayed the overall synovial morphology. In hematoxylin and eosin (H&E)-stained slides, osteocytes, chondrocytes, synovial hyperplasia, trabecular bone, joint space, and bone marrow were visible. At 3 DPI, the SA group featured a condition of mucinous hyperplasia, while in the (SA + CXCR1 Ab + IL-17 Ab + Gent.) group, round chondrocytes were visible. The 10 DPI synovial H&E stain detected improved joint space in the (SA + CXCR1 Ab + IL-17 Ab + Gent.) group compared to the SA group. At 15 DPI, a low level of synovial hyperplasia was visible in the (SA + IL-17 Ab + Gent.) and (SA + CXCR1 Ab + IL-17 Ab + Gent.) groups compared to the SA group. TRAP-positive cells were visible in TRAP-stained slides, which appeared in reddish or dark purple hues due to enzymatic reactions with the staining substrate.

3.2 | Effect of Neutralization of CXCR1 and IL-17 on the Bacterial Burden of Blood, Spleen, and Synovial Tissue

On the experimental sacrifice day, blood, spleen, and tissues were collected, and tissue homogenate was prepared. In mannitol agar plates, blood and spleen and synovial tissue homogenates were added to observe bacterial growth after 24 h of incubation. Results were obtained in Colony Forming Units/mL (CFU/mL).

Bacterial burden analysis of three DPI blood samples showed the highest bacterial burden in the SA group, which was significantly mitigated by the treatment groups. Most effectivity in lowering bacterial burden was seen by (IL-17 Ab + Gent.) application, i.e., significantly by the (SA + IL-17 Ab +Gent.) group ($p < 0.05$). However, dual neutralization of CXCR1 and IL-17 along with gentamicin was able to lower bacterial burden second most significantly ($p < 0.05$). Overall data analysis shows us that IL-17 neutralization was more prominent and significant in killing *S. aureus* in comparison to CXCR1 neutralization ($p < 0.05$).

In spleen tissue, CFU count at 3, 10, and 15 DPI showed, at the early phase (3 DPI), infection was significantly mitigated by the (SA + CXCR1 Ab + IL-17 Ab) group and (SA + IL-17 + Gent.) group ($p < 0.05$) in comparison to SA and other treatment groups. While at 10 DPI, (SA + CXCR1 Ab + IL-17 Ab + Gent.) showed a significant ($p < 0.05$) decrease in spleen bacterial burden in comparison to other groups. The late phase of the experiment (15 DPI) results showed the highest mitigation by (SA + CXCR1 Ab), (SA + CXCR1 Ab + Gent.), and the combination group of (SA + CXCR1 Ab + IL-17 Ab + Gent.) with significance at the $p < 0.05$ level.

In synovial tissue data, at the early phase (3 DPI), bacterial burden was significantly reduced by (SA + CXCR1 Ab + IL-17 Ab) and (SA + IL-17 Ab + Gent.) groups in comparison to SA and other groups ($p < 0.05$). At the middle phase (10 DPI), bacterial burden was significantly reduced by (SA + IL-17 + Gent.) and (SA + CXCR1 Ab + IL-17 Ab + Gent.) groups ($p < 0.05$). At the later phase (15 DPI), the (SA + CXCR1 Ab + IL-17 Ab + Gent.) group most effectively reduced the bacterial burden compared to other groups ($p < 0.05$).

3.3 | Assessment of Neutrophil Degranulation Activity at 3, 10, and 15 DPI

Neutrophil granules degranulate during infection conditions and release stored lytic enzymes in them to kill pathogens. Thus, neutrophil degranulation is a marker of inflammation assessment. Here, with the help of a fluorescence spectrometer, neutrophil degranulation activity has been assessed.

Early experimental data (3 DPI) show that fluorescence intensity was most prominently lowered by the (SA + CXCR1 Ab) treatment group in comparison to SA and other groups, while (SA + IL-17 Ab + Gent.) showed a high fluorescence intensity, i.e., high PMN degranulation and the presence of high

inflammatory responses. The 10 DPI results show that fluorescence intensity was lowered in all groups compared to the SA group. Most significantly, the intensity of (SA + CXCR1 Ab), (SA + IL-17 Ab), and (SA + CXCR1 Ab + IL-17 Ab + Gent.) groups got lowered compared to the SA group. The 15 DPI results exhibited the lowest PMN degranulation activity by the (SA + CXCR1 Ab + IL-17 Ab) group.

3.4 | Evaluation of Lysozyme Release Activity of Neutrophils at 3 DPI and 10 DPI

Lysozyme enzyme is secreted by azurophilic granules of neutrophils. During inflammation, lysozyme activity rises to kill the causative pathogen. At 10 DPI and 15 DPI of the experiment, lysozyme enzyme activity was lowered in treatment groups compared to the SA group and control group. At both DPI, the (SA + CXCR1 Ab) group showed prominently the lowest lysozyme activity among all. While comparing the activity of dual treatment, the (SA + CXCR1 Ab + Gent.) group at 10 DPI and (SA + IL-17 Ab + Gent.) group at 15 DPI significantly helped to reduce the neutrophil lysozyme enzyme activity.

3.5 | Assessment of NBT Activity of Neutrophils at 3, 10, and 15 DPI

The NBT activity of neutrophils analyzes intracellular superoxide activity. At the early phase (3 DPI) of the experiment, NBT activity of the SA group was significantly higher than that of the control and other groups. The lowest NBT activity was observed in the (SA + IL-17 Ab) group ($p < 0.05$). The combination group (SA + CXCR1 Ab + IL-17 Ab + Gent.) also showed a significant reduction in NBT activity ($p < 0.05$).

10 DPI experimental outcomes show a significant rise in NBT activity in the SA group compared to the control and other treatment groups ($p < 0.05$). Among the treatment groups, (SA + CXCR1 Ab + Gent.) and (SA + CXCR1 Ab + IL-17 Ab + Gent.) groups showed a significant reduction in NBT activity of neutrophils ($p < 0.05$).

The 15 DPI NBT activity results show that the combination group of (SA + CXCR1 Ab + IL-17 Ab + Gent.) was able to significantly lower the NBT activity in comparison to the SA-infected group and other treatment groups ($p < 0.05$).

3.6 | Evaluation of PMN Chemotactic Activity at 15 DPI of Experiment

PMN chemotactic activity was assessed through calculating the relative migrating cell numbers (%). The outcome shows that the control group has the highest migratory neutrophil cells, while the lowest migratory cells were observed in the combination group of (SA + CXCR1 Ab + IL-17 Ab + Gent.). The 15 DPI PMN chemotaxis assay using IL-8 chemoattractant has shown a low level of neutrophil migration in the SA-infected group compared to the control group, while it recovered in the (SA + CXCR1 Ab) group and (SA + IL-17 Ab) group mostly.

3.7 | Assessment of Serum ALP Activity at 3, 10, and 15 DPI

The source of serum ALP is liver and bones. High serum ALP denotes bone disorders or liver diseases. The results of serum ALP measurement show us that, at the early phase (3 DPI) of the experiment, alkaline phosphatase activity was high in the treatment groups. At 10 DPI, ALP activity lowered significantly in the treatment groups except for (SA + CXCR1 Ab) in comparison to the SA group ($p < 0.05$). The (SA + IL-17 Ab) group showed the lowest ALP activity among the other groups ($p < 0.05$). At 15 DPI, the significantly lowest ALP activity was observed in the (SA + CXCR1 Ab + Gent.) group among the other groups ($p < 0.05$).

3.8 | Assessment of ROS Activity of Neutrophils

H_2O_2, NO, and O_2^- levels were assessed from isolated blood PMNs at 3, 10, and 15 DPI. A high level of ROS activity denotes a high inflammatory response and vice versa.

H_2O_2 level at 3 DPI shows that compared to the control H_2O_2 activity, the SA infected group has a rise in level, while the treatment groups help to mitigate H_2O_2 activity. The (SA + CXCR1 Ab + IL-17 Ab) group showed the lowest significant H_2O_2 activity ($p < 0.05$) at 3 DPI. Over the course of the experiment, from early phase (3 DPI) to the later phase (15 DPI) of the experiment, the (SA + IL-17 Ab) group and (SA + CXCR1 Ab) group, respectively, exhibited significant reductions in H_2O_2 activity ($p < 0.05$).

Assessing the NO level, we found at 3 DPI that the combination group of (SA + CXCR1 Ab + IL-17 Ab + Gent.) was able to significantly ($p < 0.05$) reduce the NO level, while at 10 DPI, the (SA + IL-17 Ab) group and at 15 DPI the (SA + CXCR1 Ab + Gent.) group exhibited the lowest NO activity compared to other treatment groups ($p < 0.05$).

Analyzing the extracellular superoxide (O_2^-) activity, it was found that there was a significant decrement of superoxide activity in the (SA + CXCR1 Ab + IL-17 Ab) group at 3 DPI ($p < 0.05$). While the (SA + IL-17 Ab) group at 10 DPI showed significantly the lowest activity ($p < 0.05$) among other groups, the 15 DPI result exhibited the lowest O_2^-activity by the (SA + CXCR1 Ab) group.

3.9 | Evaluation of Blood PMN MPO Activity at 3, 10, and 15 DPI

Blood PMN MPO activity is an indicator of the microbicidal activity of PMNs. The blood PMN MPO activity range gradually increased from 3 DPI (0.05–0.4 mM/μL) to 15 DPI (2–6 mM/μL). The results show that the MPO activity in the treatment groups was significantly lowered compared to the SA group. The blood PMN activity at 3 DPI and 10 DPI was most significantly reduced by the (SA + IL-17 Ab) group in comparison to others. While at 15 DPI, the most significant lowering in MPO activity was observed by the (SA + CXCR1 Ab + Gent.) group.

3.10 | Evaluation of Gentamicin Bioassay

The gentamicin bioassay data of 10 DPI and 15 DPI show similar gentamicin concentration levels in the same experimental group. Serum gentamicin concentration levels in the (Control + Gent.) group showed a high gentamicin retention activity in mice. The lowest gentamicin retention activity was observed by the (SA + IL-17 Ab + Gent.) group in both 10 and 15 DPI data.

3.11 | Western Blot Analysis

Spleen tissue homogenate was lysed with RIPA buffer, and western blot was performed. The expression of different proteins was evaluated with Image J software. iNOS and COX2 expression were highest in the SA-infected group, while treatment helped to lower it, most significantly lowered by the (SA + IL-17 Ab) group in both cases. In the case of SOD and CAT, their expression was lowest in the SA group while achieving the highest recovery by the IL-17 neutralization effect. STAT1 and STAT3 expression was high during the infection condition while being lowered most significantly by (SA + CXCR1 Ab + Gent.) and (SA + CXCR1 Ab + IL-17 Ab + Gent.) groups, respectively. SOCS3 expression was lowered by the (SA + CXCR1 Ab + IL-17 Ab) group with respect to the SA group. RANKL and NF-κB activity were high during infection while being recovered, respectively, by IL-17 neutralization and CXCR1 neutralization effects. High TNF-α and IFN-γ activities during the infection condition were majorly reduced by (SA + CXCR1 Ab + Gent.) and (SA + CXCR1 Ab + IL-17 Ab + Gent.) groups, respectively. IL-10 expression was low during infection while being heightened by (SA + IL-17 Ab) and the combination group (SA + CXCR1 Ab + IL-17 Ab + Gent.). High IL-17 and CXCR1 expressions were seen during the infection condition, which was reduced by CXCR1 neutralization.

4 | Discussion

Life-threatening illnesses can be brought on by the incredibly adaptable opportunistic bacterium *S. aureus*, which can infect different organ systems [33]. Potent neutralizing impact of IL-17 Ab helped to reduce bacterial burden (Figure 1A–C). IL-17 induces pro-inflammatory gene expression resembling TLR ligands, IL-6, TNF-α, IL-1β, G-CSF, and GM-CSF. TRAF6, the key mediator in the TLR and IL-1R signaling pathway, is indispensable in IL-17-mediated activation of NFκB. The combined action of CXCR1 and IL-17 cytokine neutralization has been crucial to eliminate spleen tissue infection (Figure 1B). Previous findings have shown that IL-17A is essential for the remodeling of the splenic vein and is essential for the amyloidosis of the liver and spleen associated with inflammation. JAK inhibitors and IL-17 ablation reduce amyloidosis, lowering the disease burden on spleen [34]. Furthermore, IL-17 is important for bone resorption and controls synovial tissue bacterial burden (Figure 1C). Previous studies demonstrated that intra-articular IL-17 neutralizing Ab injection reduces joint degeneration while also decreasing the expression of the

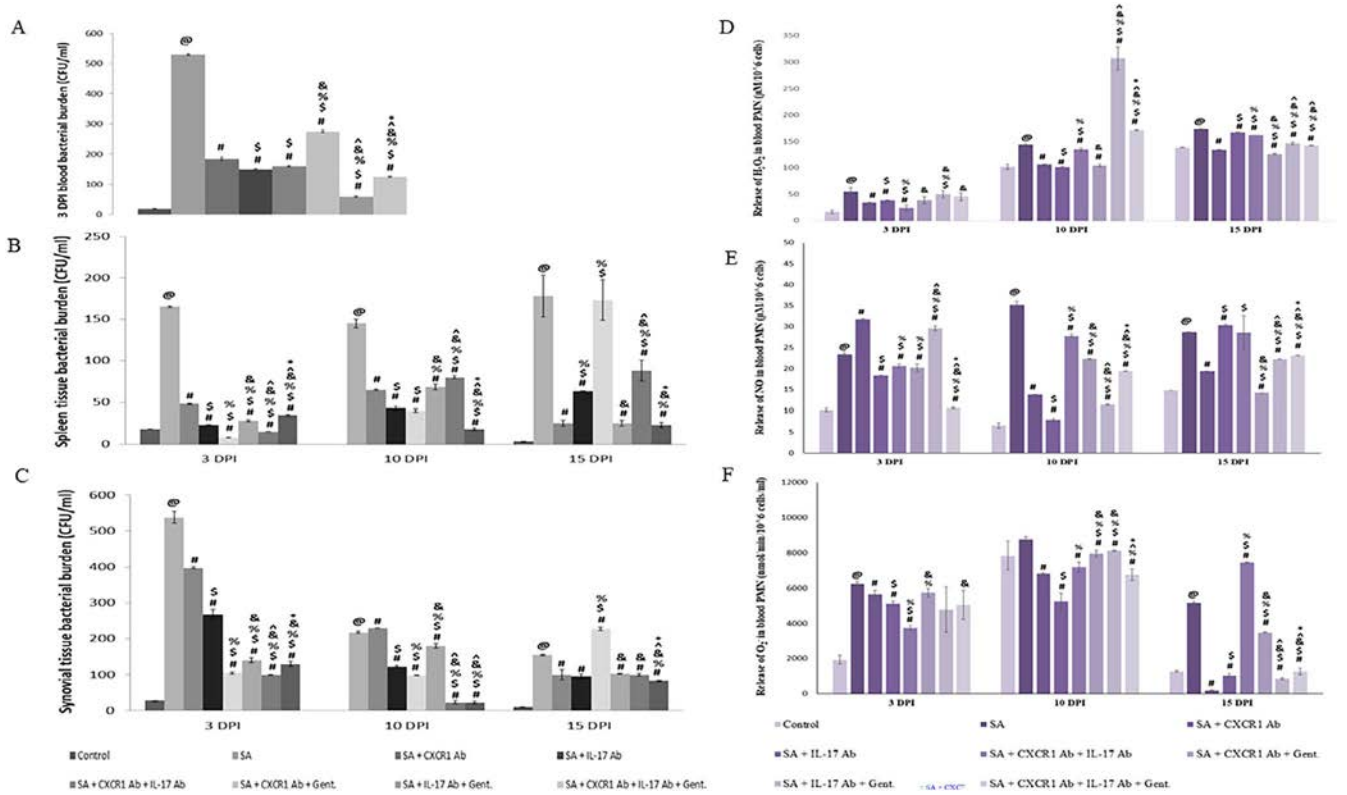

FIGURE 1 | (A–C) Evaluation of blood, spleen, and synovial tissue bacterial burden. (D–F) Evaluation of neutrophil ROS (H_2O_2, NO, and O_2^-) activity at 3, 10, and 15 DPI. The symbols @, #, $, %, &, ^, and * indicate the presence of significant difference compared to the control group, SA group, (SA + CXCR1 Ab) group, (SA + IL-17 Ab) group, (SA + CXCR1 Ab + IL-17 Ab) group, (SA + CXCR1 Ab + Gent.) group, and (SA + IL-17 Ab + Gent.) group, respectively, at $p < 0.05$ level.

senescence marker Cdkn1a (p21). Moreover, RANK is induced in osteoclast precursors by IL-17. Infection-induced elevations in RANK expression in the synovium lead to increased bone degradation and elevated levels of IL-17 cytokines (Figure 5) [35]. Bacterial burden data (Figure 1A–C) correlate with the blood PMN MPO activity (Figure 2A). Because of its large positive charge, MPO possesses strong oxidative activity, which draws in additional leukocytes to the vasculature where it accumulates [8]. Activated neutrophils secrete MPO from azurophilic granules, which act as an autocrine modulator of neutrophil cells by binding with CD11b on neutrophils. One earlier study has shown that there is a significant positive correlation present between the expression of CD-11b and that of CXCR1 on neutrophils, suggesting that the specific ligand of CXCR1, IL-8, may cause the upregulation of CD-11b through the activation of CXCR1 [36]. Another study revealed that PMN granule release is accompanied by the activation and subcellular redistribution of two Src family tyrosine kinases, c-Fgr11 and Hck12. Hck isoforms are localized to the cytoplasmic surface of azurophilic granules. IL-8 activation of CXCR1 stimulates rapid formation of β-arrestin complexes with hck and c-Fgr. β-arrestin-Hck complex leads to azurophilic degranulation [37]. Thus, CXCR1 neutralization with CXCR1 Ab reduces degradation of azurophilic granules in blood neutrophils, resulting in a lowering amount of MPO release that was previously heightened due to sepsis conditions. Therefore, it is established that the CXCL8/CXCR1 pathway regulates neutrophil MPO activity.

IL-17 Ab treatment greatly influenced ROS activity. ROS enhances IL-17 production during sepsis, while inflammation gets reduced after IL-17 knockdown. Others reported that IL-17 is induced by the NAD(P)H-oxidase-dependent generation of ROS, leading to a pro-inflammatory activation [38]. Previous studies have shown that the knock-out of CXCR1 has inhibited the release of ROS from neutrophils. The 15 DPI results have shown that blockade of CXCR1 was most potent in reducing H_2O_2, NO, and extracellular O_2 levels. CXCL8/CXCR1 signaling regulates the MAPK/ERK pathway. Low expression of CXCR1 significantly suppresses MEK/ERK signaling, reducing the accumulation of ROS. Also, the Raf-MEK–ERK pathway is involved in NET formation through the activation of NADPH oxidase and upregulation of antiapoptotic proteins. Thus, blockade of CXCR1 helped to mitigate ROS activity, protecting the inflammatory responses of sepsis [39]. The nitroblue tetrazolium reduction test (NBT) gave insights into intracellular O_2 activity. NBT utilizes the ability of neutrophils to generate free radicals (oxidative burst) during the process of phagocytosis. IL-17 Ab and CXCR1 Ab singularly regulated extracellular and intracellular superoxide levels, which can be associated with statistics on TNF-α activity (Figure 5). During the development of sepsis, TNF-α increases along with ROS activity [26].

We can correlate ROS activity (Figure 1D–F) with the neutrophil degranulation activity (Figure 2B). When neutrophil encounters CXCL8, granule discharge is triggered. Neutralization of CXCR1 has lowered PMN degranulation, reduced ROS activity, and increased levels of antioxidants. CXCR1 has been reported to promote neutrophil accumulation along with oxidative and nonoxidative antibacterial effects in neutrophils. Earlier findings discovered that CXCR1$^{-/-}$ neutrophils harvested from uninfected bone marrow exhibit significant decreases in (a) degranulation capacity, as measured by beta-glucuronidase release and (b) intracellular MPO levels at steady state, suggestive of a CXCR1-dependent defect in granulogenesis [40].

Previous researches have shown that PMN chemotaxis (Figure 3A) gets impaired during sepsis including mobilization

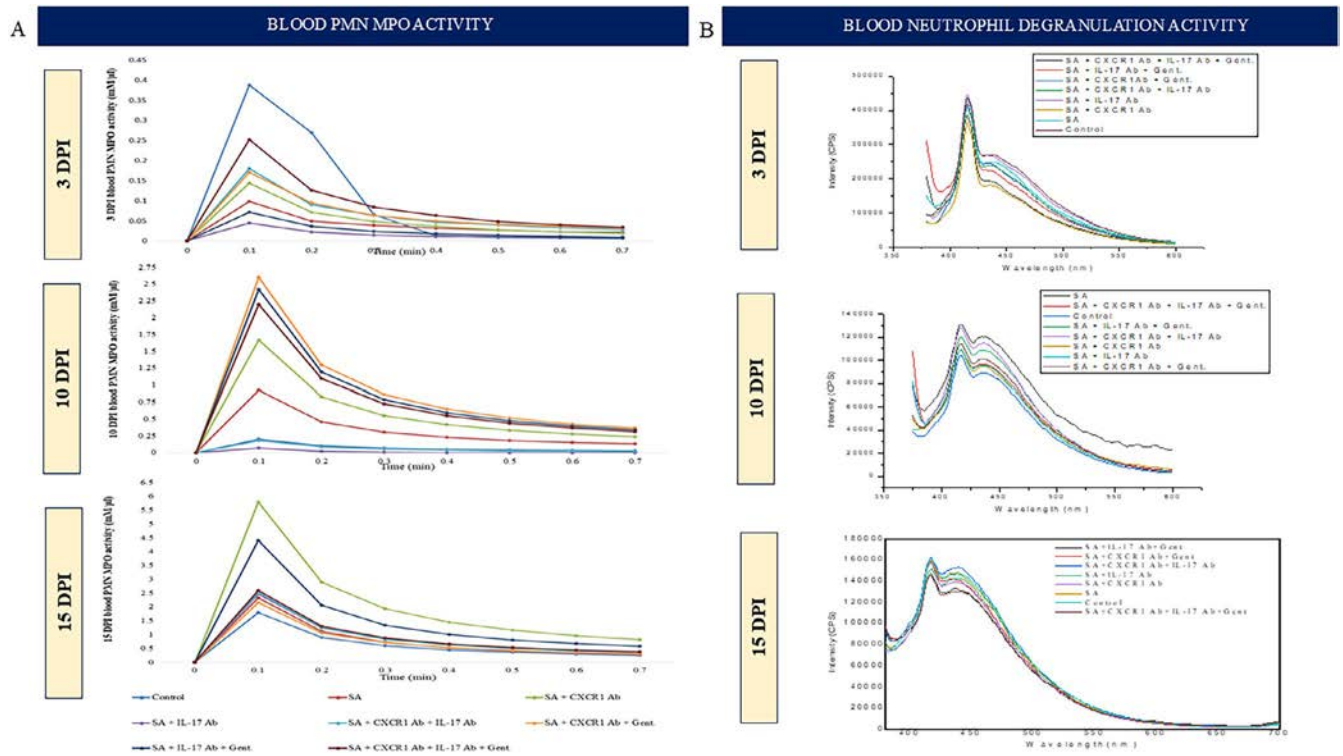

FIGURE 2 | (A) Evaluation of blood PMN MPO activity. (B) Evaluation of blood neutrophil degranulation activity at 3, 10, and 15 DPI.

and release from the bone marrow, migration and rolling, adherence, and transmigration [41]. CXCR1, IL-17 neutralization was impactful on chemotaxis. IL-17 targets genes of CXC chemokines, e.g., CXCL1, CXCL2, CXCL5, CXCL8, and CXCL10. These chemokines mediate the biological function of IL-17 by attracting neutrophils in vivo [42]. The CXCL8/CXCR1 axis has physiological roles in sepsis such as bacterial clearance, respiratory burst, and chemotaxis. Moreover, peroxynitrite, created when NO combines with the superoxide anion, prevents PMN movement by inhibiting actin polymerization required for PMN locomotion [43]. PMN chemotaxis assay findings show that CXCR1 neutralization helped to restore migratory neutrophil cell number, i.e., the chemotaxis activity of neutrophils. "Local Excitation Global Inhibition" model of chemotaxis incorporates the concept of positive and negative feedback loops, regulating PMN movement. Most current models of chemotaxis place signaling via inositol (3,4,5)-trisphosphate (PIP3) at the center stage. The distribution of PIP3 in the cytosol of PMN is 3–6 times steeper than the external chemoattractant gradient that aids gradient sensing and allows PMN to respond to shallow chemotactic gradients [44]. Also, the study with human neutrophils have shown that a major component of PMN chemotaxis to IL-8 chemoattractant is JAK3 [45]. It was previously discovered that the *S. aureus*-derived leukotoxin ED utilizes CXCR1 to induce the death of neutrophils in vitro. Thus, CXCR1 blockade with antibody-protected neutrophils from S. aureus induced sepsis and helped to recover its migratory ability (Figure 3A).

Lysozyme is a mediator of sepsis that causes vasodilatation through hydrogen peroxide signaling. Lysozyme destroys bacteria, which increases the release of bacterial compounds, causing host cells' PRR to become active, triggering inflammatory reactions [9]. Combination therapy has lowered lysozyme activity (Figure 3B,C) and H_2O_2 levels (Figure 1D). Neutralization of IL-17 reduces COX2 expression (Figure 5). During infection, increased IL-17 levels promote IL-17-induced prostaglandin-dependent attraction of neutrophils, enhancing lytic enzyme (e.g., lysozyme) production by degranulation. IL-17 also enhances chemokine receptor activity (CXCR1, CXCR2) on the neutrophil surface [46]. As CXCL8 is a well-known chemoattractant of neutrophils and a potent inflammatory chemokine, blocking its binding with CXCR1 by antibody helped to reduce the neutrophil lysozyme activity overall.

Alkaline phosphatase (ALP) in serum is delivered from the liver, bone, and intestine. Neutrophil ALP (NAP) is detectable in differentiated neutrophils and is a product of liver-/bone-/kidney-type ALPs. Studies have reported that ALP is able to dephosphorylate and detoxify endotoxins present in sepsis hosts, thus helping to reduce inflammation [47]. At the early phase of the result (Figure 3E), elevated ALP levels combat inflammation, while with the progress of experiment, the effect got reduced. Later, CXCR1 neutralization showed the lowest ALP activity. Infection increases the expression of neutrophil CXCR1 along with an increment in serum ALP levels (Figure 3E). Thus, neutralizing CXCR1 expression kept the serum ALP level in check.

The H&E staining histological investigation (Figure 4A,C,E) revealed that the SA group had synovial hyperplasia featuring synovial thickening aka joint swelling. Mucinous hyperplasia in the 3 DPI SA group illustrates the secretory potential of synovium. Thus, during the early treatment phase, the synovium

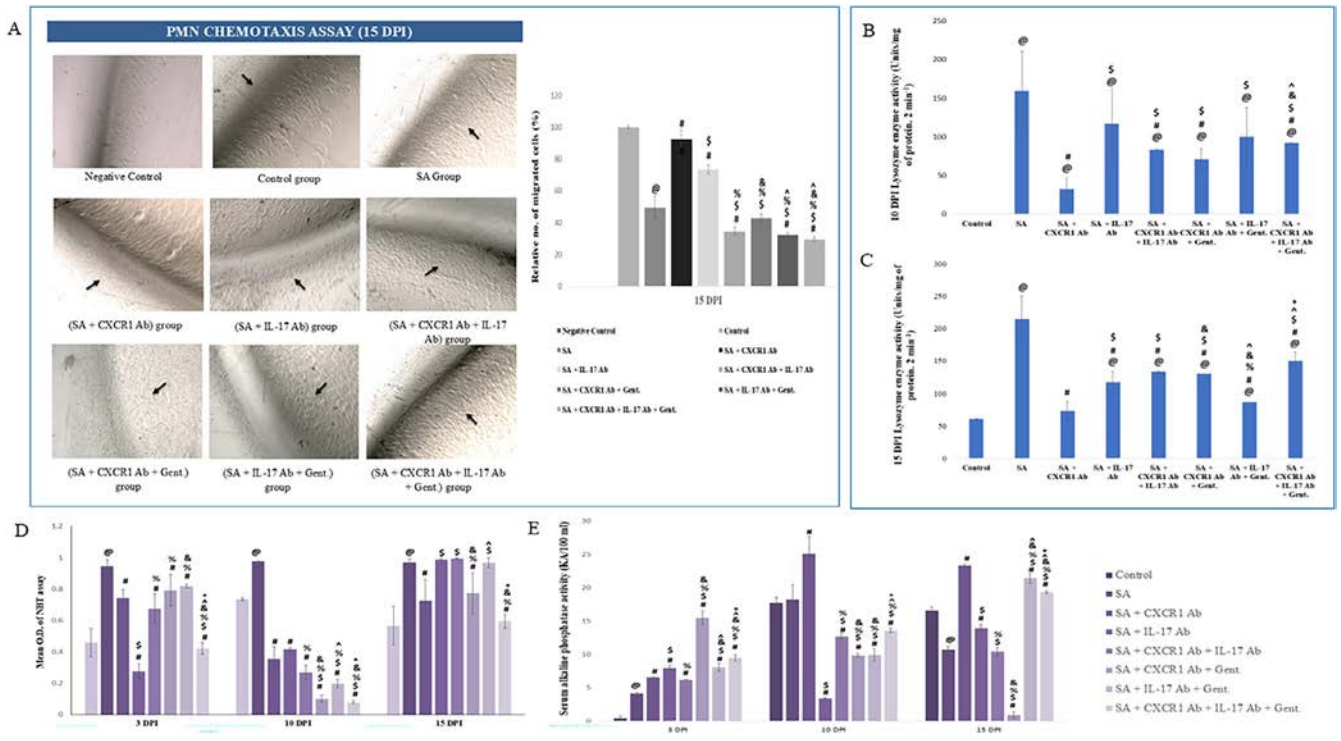

FIGURE 3 | (A) Assessment of PMN chemotaxis assay at 15 DPI. Relative number of migrated cell % has been depicted with bar chart. Black arrow indicates neutrophil cells. (B, C) Lysozyme activity of neutrophils; (D) NBT assay; (E) Serum alkaline phosphatase activity. The symbols @, #, $, %, &, and ^ indicate the presence of significant difference compared to the control group, SA group, (SA + CXCR1 Ab) group, (SA + IL-17 Ab) group, (SA + CXCR1 Ab + IL-17 Ab) group, and (SA + CXCR1 Ab + Gent.) group, respectively, at $p < 0.05$ level.

retains its potential to repair damage. As we know, Joint Space (JS) narrowing is an indication of arthritis; the 10 DPI findings detected improved JS by combination treatment. The (SA + CXCR1 Ab) group at 15 DPI showed prominent chondrocytes compared to its 10 DPI findings, denoting redifferentiation of articular cartilage, which is part of the healing process. Thus, we can infer that combination therapy improved the overall joint morphology, while at a later phase, CXCR1 Ab treatment was most effective in subsiding inflammation, improving joint swelling. There are fewer osteocytes and other morphological variations visible in other treatment groups. TRAP staining stains tartrate-resistant acid phosphatase present in osteoclast cells in the synovial joint. Osteoclast cells are large multinucleated cells, typically located near the bone surface. They secrete matrix metalloproteinases (MMPs) to resorb bone, causing the remodeling of bone [48].

iNOS (Figure 5) and NO (Figure 1E) are connected. Proinflammatory cytokines stimulate iNOS activity during sepsis (Figure 5). Vasodilation brought on by a high iNOS level during sepsis produces a huge quantity of NO and ultimately results in septic shock. NO has two roles. As ROS, NO aids in the pathogen's death, but too much NO synthesis damages host tissue by oxidative stress [49]. COX2 level was high during sepsis (Figure 5), causing extensive tissue injury and organ dysfunction. It got mitigated significantly by IL-17 neutralization (Figure 5). IL-23-induced COX2 expression produces PGE2, which contributes to neutrophil recruitment by enhancing IL-23-induced IL-17 production through the impairment of the IL-12/IFNγ axis [13].

IL-17 also triggers COX2 and iNOS, leading to an increase in prostaglandin E2 and NO levels. IL-17 neutralization thus helped to downregulate COX2 and iNOS expression (Figure 5), as seen in the blot outcome [42]. While considering the role of CXCR1 Ab, the ligand of the CXCR1 receptor, IL-8, is a proinflammatory cytokine that promotes neutrophil migration to the infection site. Blocking of the cell surface CXCR1 with Ab decreases the ability of neutrophils to clear staphylococcal infection and attenuates proinflammatory cytokine production. The decreased levels of cytokines TNF-α and IFN-γ due to CXCR1 blockade (Figure 5) appear to regulate the killing of bacteria by destroying H_2O_2 and nitric oxide (NO). Western blot (Figure 5) expression of NF-κB, COX2, and STATs was studied. PMN-MDSCs pathologically activate neutrophils and have high ROS production, MMP, and MPO expression and transmigration ability [50]. STAT3 activation is responsible for the accumulation of MDSCs. Blot results (Figure 5) show us that SA infection leads to the highest STAT3 accumulation, inferring the presence of a high level of MDSCs, while treatment with CXCR1 Ab, IL-17 Ab, and gentamicin helped to lower down STAT3 level (Figure 5). Blot expression of NFκB was reduced prominently by CXCR1 Ab [51]. Canonical NFκB pathway upregulates CXCL8 gene expression during inflammation which is mediated by the CXCR1-NFκB- COX-2/ICAM-1/VCAM-1 pathway. Activation of NFκB,

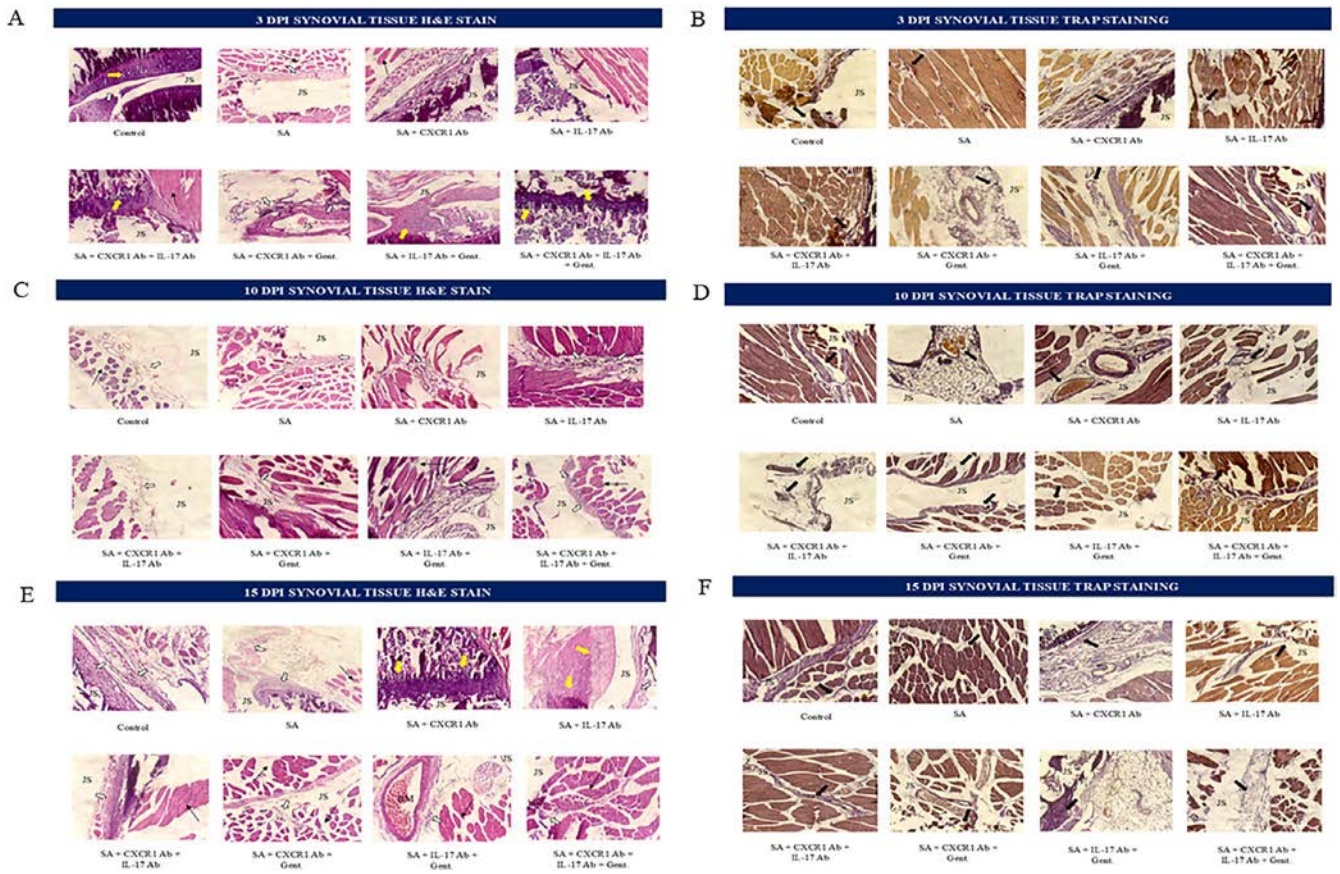

FIGURE 4 | Histological study of synovial tissue including results of H&E stain and TRAP stain at 3, 10, and 15 DPI. Yellow arrow denotes chondrocytes, white arrow denotes synovial hyperplasia (osteocyte aggregation), thin black arrow denotes trabecular bone, JS denotes joint space, and BM denotes bone marrow. TRAP-positive cells are indicated with a big black arrow.

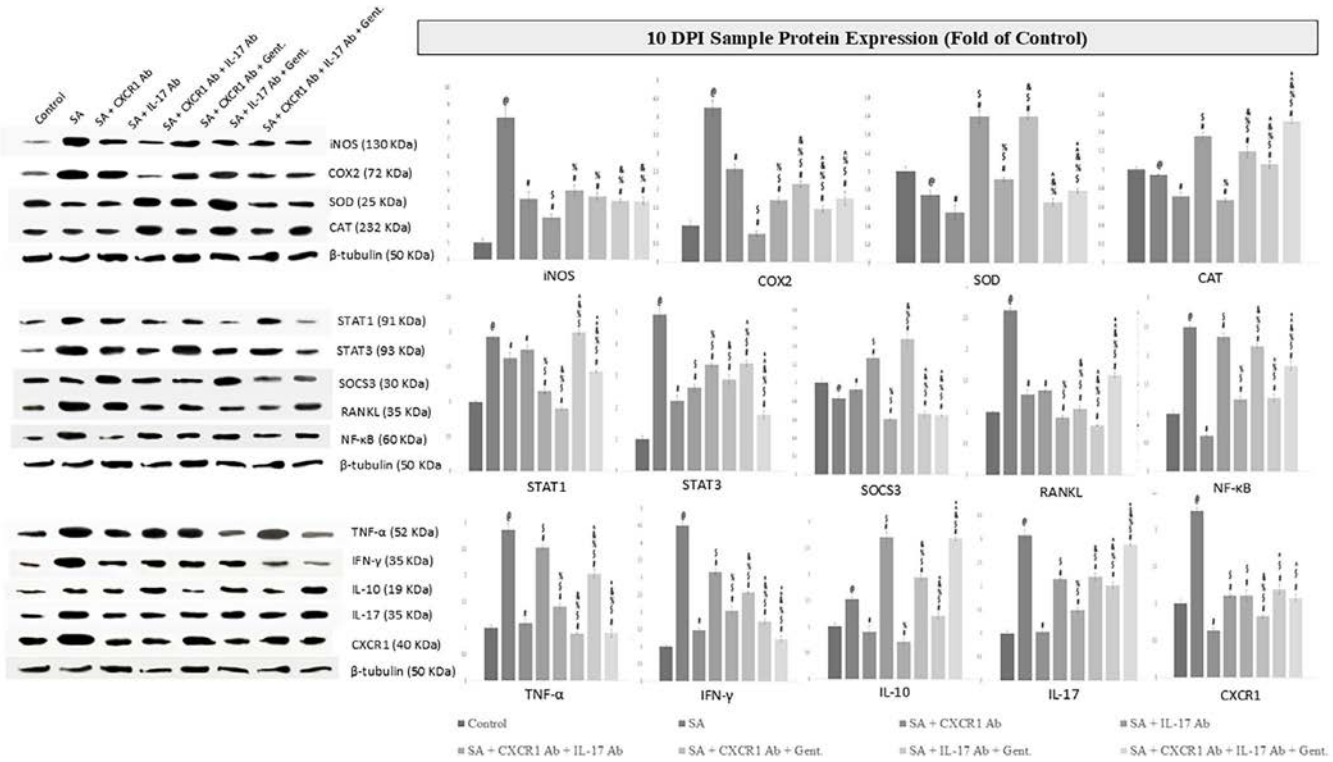

FIGURE 5 | Expression of iNOS, COX2, SOD, CAT, STAT1, STAT3, SOCS3, RANKL, NF-κB, TNF-α, IFN-γ, IL-10, IL-17, CXCR1 levels was assessed by western blot while using β-tubulin as the loading control. Symbols @, #, $, %, &, ^, and * indicate the presence of significant difference compared to the control group, SA group, (SA + CXCR1 Ab) group, (SA + IL-17 Ab) group, (SA + CXCR1 Ab + IL-17 Ab) group, (SA + CXCR1 Ab + Gent.) group, and (SA + IL-17 Ab + Gent.) group, respectively, at $p < 0.05$ level.

TABLE 1 | Gentamicin bioassay data using the microbiological method.

Groups	Size of zone of inhibition (mm) (10 DPI)		Gentamicin concentration (μg/μL) (10 DPI)	
Control + gentamicin	9.21 ± 0.54		0.333 ± 0.05	
SA + gentamicin	8.13 ± 0.55		0.225 ± 0.05	
Groups	10 DPI		15 DPI	
	Size of zone of inhibition (mm)	Gentamicin concentration (μg/μL)	Size of zone of inhibition (mm)	Gentamicin concentration (μg/μL)
SA + CXCR1 Ab + Gent.	8.00 ± 1.00	0.213 ± 0.10	8.56 ± 0.95	0.268 ± 0.09
SA + IL-17 Ab + Gent.	7.50 ± 0.50	0.164 ± 0.05	7.32 ± 0.49	0.145 ± 0.05
SA + CXCR1 Ab + IL-17 Ab + Gent.	8.96 ± 1.58	0.308 ± 0.16	8.43 ± 0.00	0.255 ± 0.00

STAT3, and AP1 induces the expression of chemokines that recruit neutrophils, aggravating the inflammatory response [52].

During SA infection conditions, TNF-α activity (Figure 5) was at its peak, but this was lowered by the combination therapy of IL-17 Ab, CXCR1 Ab, and gentamicin. Owing to IL-10's anti-inflammatory properties, its activity was also inhibited during infection (Figure 5). Activation of pathogenic MDSCs is induced by IL-10 in sepsis. Pathogenic MDSCs dose-dependently suppress IFN-γ and IL-10 production. MDSC production causes arginine shortage that promotes IL-10 activity toward the anti-inflammatory phenotype [53]. Both STAT3 and NF-κB inhibit IL-10 activity. The JAK–STAT pathway includes STAT1 and STAT3. When SOCS3 binds to JAK kinase, STAT3 phosphorylation is inhibited. The activity of CXCR1 Ab and gentamicin helped to reduce STAT1 and STAT3 activities while showing strong SOCS3 activity (Figure 5). Earlier research has shown that the hypo-expression of CXCL8 (IL-8) causes STAT3-gain of function-associated neutropenia. Thus, CXCL8 receptor CXCR1 neutralization has similar effect causing the gain of function of STAT3 [54]. STAT1 mediates IFN-γ activity, whereas STAT3 mediates IL-10 function. RANKL promotes osteoclast generation. Thus, during infection, the RANKL level was high (Figure 5) as in the blot result which got mostly reduced by IL-17 neutralization [35].

Gentamicin bioassay findings are shown in Table 1. High gentamicin retention can cause kidney damage. Comparing the gentamicin bioavailability of the (Control + Gent.) group with others, we found that the other experimental groups have low gentamicin concentration in blood, indicating either uptake by cells or excretion through the kidneys. This shows protection against vital organs while also killing *S. aureus* bacteria, which can be correlated with the CFU data of the spleen and synovial tissue (Figure 1A,B,C).

Nevertheless, our study demonstrated that, during the early stages of the experiment, IL-17 neutralization was more successful in reducing the inflammatory responses caused by septic arthritis, but CXCR1 neutralization proved to be the most potent in the later stages. When compared to the antibiotic gentamicin in combination, both individual neutralizations showed a significant impact. Neutrophil functional assay findings were correlated with the CXCL8/CXCR1 pathway. We conclude that antibody treatment with CXCR1 Ab and IL-17 Ab has a bright future in treating septic arthritis.

Author Contributions

Biswadev Bishayi (B.B.) and Sharmistha Ghosh (S.G.) designed the experimental study. S.G. performed various experiments. S.G. did the data curation, formal analysis, investigation, methodology, and software work. S.G. wrote the manuscript. B.B. and S.G. approved the final manuscript.

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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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EDITORIAL

Glucagon-Like Peptide-1 Receptor Agonists: Multifaceted Roles in Immune and Inflammatory Regulation, Glycemic Control, and Synergistic Therapeutic Applications

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Glucagon-like peptide-1 (GLP-1) has gained considerable attention in recent years for its crucial role in glycemic and weight control, as well as its potential impact on inflammation and immune function. Secreted by enteroendocrine cells in response to nutrient intake, GLP-1 stimulates insulin secretion, reduces glucagon release, and slows gastric emptying [1]. Beyond these effects, GLP-1 has multifaceted roles, including a complex interplay between inflammatory processes and immune regulation. The use of GLP-1 receptor agonists (GLP-1RAs) has been linked to significantly lower risks of all-cause mortality, cardiovascular events, and both cardiovascular and liver-related mortality [2]. Recent studies have further explored the anti-inflammatory properties of oGLP-1 drugs and their effect on the immune system, unveiling additional mechanisms of action.

1 | Immune and Inflammation Regulation

Originally developed as a diabetes treatment due to its ability to stimulate insulin secretion from beta cells, GLP-1's effects extend beyond glucose regulation to include significant roles in immune cell function. One of its notable impacts is on macrophage polarization. Research indicates that GLP-1 can modulate macrophage activity, highlighting its potential in controlling chronic inflammation. Activation of the GLP-1 receptor has been shown to shift macrophages from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype. This transition is associated with a reduction in inflammatory mediators and an enhancement of tissue repair mechanisms [3]. For instance, in a murine model of diet-induced non-alcoholic fatty liver disease (NAFLD), liraglutide demonstrated protective effects by promoting cAMP-PKA-STAT3-dependent polarization of Kupffer cells towards an M2 phenotype [4].

Moreover, GLP-1 receptor expression in T cells, particularly apoptotic and anergic cells, functions as a negative costimulatory molecule for T cells. This action has been shown to prolong allograft survival, mitigate alloimmunity, and even trigger antitumor immunity in a mouse model of colorectal cancer [5].

A recent study uncovered a novel mechanism by which GLP-1 receptor agonists (GLP-1RAs) increase intestinal *Escherichia coli* levels. This increase has been associated with sympathetic nervous system activation, resulting in the release of norepinephrine into the intestinal lumen. Elevated *E. coli* levels may contribute to bacterial translocation by downregulating the expression of intestinal tight junction genes under stress. However, the implications of this rise in *E. coli* levels require further investigation [6].

Activation of the GLP-1 receptor has also been shown to have significant anti-inflammatory effects. In a study by Chaudhuri et al., 24 obese individuals with type 2 diabetes mellitus (T2DM) were treated with the GLP-1 analog exenatide for 12weeks. This

The first three authors contributed equally to this work and share first authorship.

treatment significantly reduced the mRNA expression of pro-inflammatory cytokines, TNF-α and IL-1β, in peripheral blood mononuclear cells compared to placebo [7]. In another study by Savchenko et al., liraglutide was shown to exert potent anti-inflammatory effects by inhibiting NF-κB pathways and upregulating SIRT1 expression. This resulted in the downregulation of several pro-inflammatory factors, including cytokines such as TNF-α, toll-like receptors TLR2 and TLR4, and inflammation markers like ceruloplasmin [8].

Collectively, these studies underscore the significant anti-inflammatory properties of GLP-1RAs, highlighting their potential pathways and immune responses beyond glycemic control.

2 | Glycemic and Weight Control

The global incidence and prevalence of T2DM among adolescents continue to rise.

Sjögren's syndrome, recognized as an autoimmune disease, has been identified as an independent risk factor for diabetic vascular complications. Furthermore, studies have shown that approximately 1% of patients with primary Sjögren's syndrome may subsequently develop systemic lupus erythematosus (SLE) [9–11]. Adolescents with T2DM are particularly prone to treatment failure, with respiratory diseases and dysglycemia being the most common causes of hospitalization [12].

GLP-1 RAs such as exenatide, liraglutide, and semaglutide not only lower blood glucose levels but also confer additional metabolic benefits, including weight loss. Semaglutide (2.4 mg) has been shown to provide superior reductions in body weight and promote reversion to normoglycemia compared with a placebo in participants with obesity and prediabetes [13, 14]. Furthermore, a recent study demonstrated that liraglutide treatment, compared to pioglitazone, improved myocardial perfusion, energetics, and the 6-min walk distance in patients with T2DM [15].

3 | Potential Therapeutic Applications and Synergistic Treatments

Combining GLP-1RAs with other medications may enhance the therapeutic outcomes []. Studies investigating the synergistic effects of GLP-1RAs and sodium-glucose co-transporter-2 (SGLT-2) inhibitors have shown promising results. For example, a systematic review and meta-analysis by Li et al. demonstrated that combination therapy with GLP-1RAs and SGLT-2 inhibitors led to superior reductions in HbA1c, body weight, fasting plasma glucose, 2-h postprandial glucose, systolic blood pressure, body mass index, and low-density lipoprotein cholesterol, all without major safety concerns, compared to monotherapy in patients with T2DM [16].

Moreover, a study by Simms-Williams found that the combination of GLP-1RAs and SGLT-2 inhibitors was associated with a lower risk of major adverse cardiovascular events and serious renal events than either drug class used alone [17].

These findings highlight the potential of GLP-1RAs not only as monotherapy but also in combination with other agents to provide comprehensive treatment strategies for various metabolic and inflammatory conditions. By offering holistic metabolic control and addressing inflammation, these combinations can significantly advance the management of complex metabolic and inflammatory diseases.

4 | Conclusion

The anti-inflammatory and immunomodulatory properties of GLP-1RAs have expanded their therapeutic use beyond glycemic control. Recent studies have explored their application in various diseases, focusing particularly on their anticancer and immunomodulatory effects. For example, research has noted a 38% increase in the risk of T2DM among patients with psoriatic arthritis. In patients with both T2DM and psoriasis, exenatide treatment has been shown to rapidly reduce symptoms and diminish psoriatic plaques [18, 19].

This emerging field is garnering considerable attention as researchers investigate GLP-1 receptor signaling in different immune cells, seeking to uncover the detailed mechanisms and long-term effects on chronic inflammatory conditions. Future research is expected to explore the benefits and potential reduction in the side effects of combining GLP-1RAs with other medications to treat a range of diseases. These studies enhance patient outcomes across various clinical settings, paving the way for innovative and effective treatments.

Such ongoing research holds promise for significant advancements in the therapeutic use of GLP-1RAs, potentially revolutionizing treatment paradigms and offering new hope for patients with inflammatory and autoimmune diseases.

Author Contributions

Chia-Chien Hsu, Chi-Jen Hsu, and Xiangpei Fang contributed equally to this work and shared first authorship. All three were involved in the conceptualization, literature review, and drafting of the manuscript. Fu-Shun Yen contributed to the critical revision of the content and provided expertise in immunology and endocrinology. Pui-Ying Leong supervised the overall project, provided guidance throughout the writing process, and finalized the manuscript for submission. All authors have read and approved the final manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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COMMENT

Deciphering the Instruction of Low-Grade Inflammation in the Trajectory of Hyperuricemia and Gout: Perspectives From Healthcare Practitioners

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With the improvement of people's living standards and changes in dietary structure, the incidence of hyperuricemia (HUA) and gout has been on a gradual rise in recent years. As a chronic, progressive, recurrent metabolic disease, it is closely related to the production and excretion of uric acid in the body. However, the new understanding available brings new evidence to this disease. This comment is in response to the Chinese Society of Endocrinology, Chinese Medical Association, published the guideline for the diagnosis and management of hyperuricemia and gout in China (2019) [1]. The guideline is based on real clinical situations and reveals in detail the process and factors involved in the development of HUA and gout. However, we focused on the associated low-grade inflammation. Comparing the status of existing relevant studies [2], we believe that the guideline's understanding of low-grade inflammation in the progression of HUA and gout is currently insufficient. This is because most of the current studies are at the basic research stage, and the understanding of low-grade inflammation in real-world scenarios is still insufficient.

Low-grade inflammation is now widely recognized by the medical community as a significant contributor to mortality, with over 50% of deaths attributed to inflammation-related diseases such as diabetes, chronic kidney disease, non-alcoholic fatty liver disease, as well as autoimmune and neurodegenerative disorders [3]. It has also been testified that the risk of low-grade inflammation can be traced back to the early stages of metabolic diseases. Low-grade inflammation has a chronic and persistent character, and studies have found that over time, it can take a toll on the body's tissues and organs [4]. On the contrary, as in the gouty outbreak phase, low-grade inflammation mainly causes metabolic dysfunction and tissue damage compared to acute inflammation. It is persistent and correlates with the patient's age and physical function [5]. The study by the guideline is just able to add to the role of these multiple factors. Given the guide's relevant tips, we believe that low-grade inflammation in the progression of HUA and gout disease gives us three insights:

1 | Key Biomarkers of Low-Grade Inflammation Need to Be Further Explored

Indeed, the understanding of low-grade inflammation in the progression of HUA is in its infancy. As studies have been reported, we have found that inflammation is present throughout the HUA to gout stages [6, 7]. Therefore, the hyperuricemia stage actually gives rise to low-grade inflammation, which progresses to acute inflammation upon the deposition of urate crystals, thereby triggering the expansion of the inflammatory cascade [8]. Although HUA and gout can be distinguished as two diseases, the development of the inflammatory cascade throughout links them together. The current knowledge of biomarkers for low-grade inflammation in HUA is vague, and similarly, there is a lack of anti-inflammatory drugs for targeted therapy. Clear biomarkers are a prerequisite for future scientific activities in disease control.

2 | Low-Grade Inflammation Brings New Perspectives to the Management of Hyperuricemia and Gout

Is anti-inflammation a treatment for HUA and gout? We think the report by the guideline in 2020 gives us these insights. Hence, how can we target low-grade inflammation as a breakthrough to make rational and specific therapeutic decisions? This will be the scientific question facing every clinician. In fact, the anti-inflammatory strategy for low-grade inflammation differs from that for acute inflammation, and its role in overall disease control decision-making needs to be re-evaluated and balanced, breaking away from traditional treatment paradigms. This is an area for in-depth consideration following the review of the guideline. In conclusion, understanding low-grade inflammation offers novel insights into the pathomechanisms of HUA and gout, guiding clinical practice towards formulating reasonable treatment strategies with significant clinical implications.

3 | Risks and Benefits of Drug Selection for the Treatment of Low-Grade Inflammation

The treatment strategy of the disease is closely related to the choice of drugs. Among the available drugs, there is nothing but TCM and Western medicine treatments, especially in China. Currently, there are more anti-inflammatory drugs commonly used in clinical practice. For acute gouty inflammatory attacks, colchicine is often used as the first choice of analgesic anti-inflammatory drugs. Secondly, non-steroidal anti-inflammatory drugs and glucocorticoids are also commonly used. Colchicine can quickly achieve therapeutic goals in a short period of time, such as lowering blood uric acid concentration, anti-inflammatory, and analgesic effects, but it is also accompanied by significant side effects. Whether it is suitable for the treatment of low-grade inflammation has not yet been discussed in the medical community. Then what kind of drugs can treat low-grade inflammation and thus achieve the purpose of lowering uric acid and anti-inflammation is the direction that basic research needs to work on. Here, we initially recognized the anti-inflammatory properties of herbal medicines. From the perspective of risk–benefit balance, some single drugs and combinations of herbal medicines show low toxicity and high efficiency, such as some derived drugs, including herbal extracts and natural products [9, 10]. In terms of mechanism of action, these components derived from Chinese medicines have the characteristics of multi-pathway and multi-target treatment for HUA and gout, and they may act on multiple therapeutic targets of low-grade inflammation. Taken together, the therapeutic benefits of TCM and its active ingredients in future clinical use far outweigh the risks.

Author Contributions

Y.H.: drafted the manuscript. **L.X**. and **C.C.:** revised the manuscript. **D.W.:** gave final approval of the version to be submitted and any revised version.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Research data are not shared.

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ORIGINAL ARTICLE

Identification of *BHLHE40*-Expressing CD4$^+$ T Cells Producing GM-CSF in Rheumatoid Arthritis

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ABSTRACT

Background: Granulocyte–macrophage colony-stimulating factor (GM-CSF: *CSF2*) plays a crucial role in the pathogenesis of autoimmune diseases. The basic helix–loop–helix family member e40 (BHLHE40) gene is important for GM-CSF production in CD4$^+$ T cells. However, the relationship between the expression of these genes and rheumatoid arthritis (RA) remains unclear, particularly in interleukin-1 (IL-1)-enriched inflammatory sites. Therefore, we investigated the expression of BHLHE40 and CSF2 in CD4$^+$ T cells in RA.

Methods: We analyzed gene expression using previously deposited and publicly available databases containing peripheral blood (PB) and synovial fluid (SF) from patients with RA and healthy controls (HC). Comprehensive datasets, including single-cell RNA-sequencing (scRNA-seq), RNA-seq, and microarray data, were used for this analysis.

Results: *BHLHE40* expression in PB CD4$^+$ T cells from HC was higher in central memory, effector memory, Th17, and Th1/17 cells than in naive CD4$^+$ T cells. Furthermore, *BHLHE40* and *CSF2* expression in the CD45RA$^-$CCR7$^{+/-}$CD4$^+$ T cell subset was significantly higher in the SF of patients with RA than in those with PB. scRNA-seq revealed that *BHLHE40*-expressing cells showed higher *CSF2* expression than those that did not. Additionally, scRNA-seq showed higher *BHLHE40* expression in PB CD4$^+$ T cells from RA patients than in those from HC.

Conclusion: We analyzed the gene expressions of *BHLHE40*, which is crucial for GM-CSF production, *IL1R1*, which regulates *BHLHE40* induction, and *CSF2*, its resulting product, in CD4$^+$ T cells. Their expression levels were compared across RA SF, PB, and HC. Notably, increased expression of these genes was identified in SF.

1 | Introduction

CD4$^+$ T cells play a crucial role in inflammation related to infectious and autoimmune diseases [1]. Cytokine production by CD4$^+$ T cells varies depending on the surrounding cellular environment [2]. Granulocyte–macrophage colony-stimulating factor (GM-CSF: *CSF2*) is one of the cytokines produced by CD4$^+$ T cells, and the role of *CSF2*-expressing CD4$^+$ T cells in rheumatoid arthritis (RA) remains poorly understood, especially in synovial fluid (SF). CD4$^+$ T cells producing GM-CSF have been reported as a pathogenic subset in disease, and clinical trials on anti-GM-CSF antibodies have been conducted [3–5]. However, the gene expression characteristics of GM-CSF-producing CD4$^+$ T cells in RA were not well understood

Abbreviations: BHLHE40, Basic helix–loop–helix family member e40; GM-CSF, Granulocyte–macrophage colony-stimulating factor; HC, Healthy controls; IFX, Infliximab; MTX, Methotrexate; PB, Peripheral blood; RA, Rheumatoid arthritis; SF, Synovial fluid; Tcm, Central memory T cells; TCZ, Tocilizumab; Tem, Effector memory T cells; Th1, T helper 1 cell; Th17, T helper 17 cell; Th2, T helper 2 cell; Tn, Naive T cells; TPM, Transcripts per million.

[6–8]. Thus, we investigated the gene expression of CD4$^+$ T cells in patients with RA. Pathogenic roles of GM-CSF$^+$ CD4$^+$ T cells have been reported in mouse disease models. In an RA model of collagen-induced arthritis (CIA), a neutralizing mouse antibody against GM-CSF has shown efficacy [9]. Also, these cells have been shown to play pathogenic roles in models such as those of experimental autoimmune encephalomyelitis (EAE) in mice. In these EAE models, GM-CSF$^+$ CD4$^+$ T cells are pathogenic, and knockout (KO) of the *Csf2* results in reduced EAE severity, suggesting a critical role for these cells in disease pathogenesis [10]. Recent studies in mice and humans have shown that the transcription factor basic helix–loop–helix family member e40 (BHLHE40) is crucial for controlling the production of GM-CSF [8, 11]. *BHLHE40* inhibits *ZC3H12D* and *MIR146A*, thereby enhancing inflammatory cytokine production [8]. In humans, the presence of *BHLHE40*-expressing CD4$^+$ T cells is a risk factor for juvenile idiopathic arthritis [12]. Also, in type 1 diabetes, *BHLHE40*-expressing islet antigen-reactive CD4$^+$ T cells have been linked to therapeutic responsiveness [13]. Therefore, *BHLHE40* may play an important role in human diseases [14]. However, *BHLHE40*-expressing CD4$^+$ T cells in patients with rheumatic and musculoskeletal diseases (RMD) and their presence at inflammatory sites have not been thoroughly investigated and remain unclear. Furthermore, although interleukin-1 (IL-1) and CD28 signaling are important for *BHLHE40* expression, their roles in regulating *BHLHE40* and their impact on the associated RMD in humans remain unclear [15–17]. The gene expression patterns of IL-1 receptor 1 (*IL1R1*), *BHLHE40*, and *CSF2* in CD4$^+$ T cells, particularly at the sites of inflammation of SF, are not yet fully understood.

In this study, we aimed to determine the significance of *IL1R1*, *BHLHE40*, and *CSF2* gene expression in CD4$^+$ T cells from patients with RA utilizing comprehensive gene expression datasets. We analyzed our previously deposited and publicly available single-cell RNA-sequencing (scRNA-seq), RNA-seq, microarray data, and our clinical data to elucidate the characteristics of CD4$^+$ T cells expressing *BHLHE40*. This study may assist in developing future strategies to control inflammation in autoimmune diseases.

2 | Materials and Methods

For all analyses of public data, including our previously deposited data [18, 19], we retrieved gene expression matrices from the Gene Expression Omnibus (GEO) database using the following accession numbers and analyzed the data. Ethical approval for this study was granted by the ethics committee of Keio University School of Medicine (protocol #20140335). All ethical regulations relevant to human research participants were followed.

2.1 | Microarray Data Collection and Analyses

The microarray dataset GSE93777, which includes naive CD4$^+$ T cells (Tn), central memory CD4$^+$ T cells (Tcm), and effector memory CD4$^+$ T cells (Tem) in peripheral blood (PB) samples obtained from healthy controls (HC) from our previously deposited database [18]; GSE39594, which includes Tn after 4 h stimulation with anti-CD3/CD28 [15]; and GSE78244, which includes total CD4$^+$ T cells after 24 h stimulation with or without anti-CD3/CD28 [20], were analyzed using the GEO2R web tool.

2.2 | RNA-Seq Data Collection and Analyses

RNA-seq data of helper T cells from PB samples were obtained from GSE149090 and GSE107011 [21, 22]. The GSE113156 and GSE118829 datasets were from our previously reported database [19]. GSE113156 contains total CD4$^+$ T cells in PB samples before and after treatment obtained from patients with RA; GSE118829 contains Tn, Tcm, and Tem in PB samples obtained from HC, patients with RA, and in SF obtained from patients with RA [19]. GSE103930, with purified and phorbol 12-myristate 13-acetate (PMA)/ionomycin-stimulated total CD4$^+$ T cells in PB samples, was obtained from GEO [23]. These datasets were reanalyzed using the RaNA-seq web tool [24]. In RaNA-seq, transcripts per million (TPM) per isoform were produced in salmon [25]. Gene set enrichment analysis (GSEA) and differential gene expression were determined using the DESeq2 software package, which is also a part of the RaNA-seq pipeline [26, 27]. We defined significantly upregulated genes as "($-\log_{10}(p\ \text{value}) > 2.0$ and $\log_2(\text{fold change}) > 1$) and downregulated genes as ($-\log_{10}(p\ \text{value}) > 2.0$ and $\log_2(\text{fold change}) < -1$)."

2.3 | scRNA-Seq Data Collection and Analyses

Publicly available scRNA-seq datasets from GSE147928 containing total unstimulated and stimulated CD4$^+$ T cells from PB samples [28], GSE142637 containing PB mononuclear cells (PBMCs) from HC [29], and GSE159117 containing PBMCs from RA patients [30] were downloaded and analyzed. Single-cell transcriptome data were analyzed using the Seurat package (version 4.3.0) in R (version 4.3.0) [31]. The count matrices were normalized using the *NormalizeData* function in the *Seurat* software. The variable features were determined using the *FindVariableFeatures* function. Cells with fewer than 200 genes, more than 2500 genes, or more than 5% (GSE147928) or 10% (GSE142637 and GSE159117) mitochondrial content were excluded from the study. The percentages of mitochondrial genes were regressed using the *ScaleData* function. For visualization purposes, two samples were integrated by using the *FindIntegrationAnchors* and *IntegrateData* functions using default parameters. Dimensionality reduction was achieved by scaling and centering the integrated data, followed by principal component analysis (PCA). Clusters were identified using the *FindClusters* function in Seurat, and cells were visualized in a two-dimensional space using the nonlinear dimensionality reduction approach, uniform manifold approximation and projection (UMAP). A similar analysis was performed on data from stimulated cells. Additionally, a heatmap depicting the gene expression levels was generated using the *DoHeatmap* function. Differential gene expression was detected using the *FindClusters* function in the Seurat software. *BHLHE40* expression was categorized as positive for Unique Molecular Identifier (UMI) > 0 and negative for UMI = 0, and a comparative analysis was

conducted. $CD4^+$ T cells from the PB were defined as those with UMI gene expression levels of *CD3D* > 0.5 and *CD4* > 0.5. The differential transcriptome factors between the two groups were identified by the Wilcoxon test with the Benjamini–Hochberg correction. Gene expression was compared using the Student's t-test.

2.4 | Statistical Analysis

Gene expression levels were analyzed using the GraphPad Prism software (version 10.0; GraphPad, San Diego, CA, USA). The box plot extends from the 25th to 75th percentiles, with the median line at the center. Categorical data are expressed as numbers and percentages. Descriptive statistics were used to summarize the data. One-way analysis of variance (ANOVA) with Tukey's multiple-comparison test or Student's t-test was used. A $p < 0.05$ was considered statistically significant. Spearman's *r* (nonparametric) or Pearson's *r* (parametric) was used for correlation analysis. The threshold for statistical significance was set at $p < 0.05$.

3 | Results

3.1 | Gene Expression of *BHLHE40* in $CD4^+$ T Cell Subsets of PB $CD4^+$ T Cells

Initially, we used previously deposited data and publicly available databases to compare the gene expression of *BHLHE40* in $CD4^+$ T cells from the PB of HC. The Tn, Tcm, and Tem fractions were sorted and reanalyzed using RNA-Seq (GSE118829) and microarray data (GSE93777). Significantly higher TPM for *BHLHE40* and *IL1R1* was observed for Tem than for Tn. The expression of *CSF2* in Tem was significantly higher than that in Tn and Tcm (Figure 1a). The microarray data also showed significantly higher signal intensities for *BHLHE40* and *IL1R1* in Tcm and Tem than in Tn (Figure S1a). Even after accounting for variations in cell conditions and measurement modalities, Tcm and Tem consistently showed higher levels of *BHLHE40* expression than Tn cells.

It is unclear which helper T cell subsets express *BHLHE40*. Therefore, we reanalyzed RNA-seq data from public databases (GSE149090 and GSE107011) by comparing Th1, Th2, Th17, and Th1/17 cells with Tn cells from PB of HC. In PB helper T cell subsets, both datasets consistently showed that Th17 and Th1/17 cells exhibited significantly higher *BHLHE40* expression compared to Tn. Additionally, one dataset demonstrated that *IL1R1* expression was significantly higher in Th17 cells than in other subsets. *CSF2* expression showed no significant differences among any subsets in either dataset (Figures 1b and S1b).

3.2 | Gene Expression of *BHLHE40* in GM-CSF^+ $CD4^+$ T Cells After PMA/Ionomycin Stimulation

Gene expression of *CSF2* was not detected in unstimulated $CD4^+$ T cells from PB of HC. Therefore, we analyzed the publicly available RNA-seq data (GSE103930). Total $CD4^+$ T cells were purified from PBMCs, stimulated with PMA/ionomycin, and sorted into GM-CSF^+ $CD4^+$ T cell and $CD45RA^+$ $CD4^+$ T cell populations. To identify the expressed genes, we compared the typical genes identified in the Tn (*CCR7*), Th1 (*TBX21*, *CXCR3*), Th2 (*GATA3*, *CCR4*), and Th17 (*RORC, CCR6*) subsets with those in GM-CSF^+ $CD4^+$ T cells and $CD45RA^+$ $CD4^+$ T cells. As shown in the heatmap (Figure 2a) and gene expression profiles (Figure 2b), GM-CSF^+ $CD4^+$ T cells exhibited significantly higher TPM levels of *IL1R1*, *BHLHE40*, *CSF2*, *TBX21*, *GATA3*, *RORC*, *CXCR3*, and *CCR6* than the $CD45RA^+$ $CD4^+$ T cells. Moreover, *CCR7* expression was significantly higher in $CD45RA^+$ $CD4^+$ T cells (Figure 2b). In addition, a strong correlation was observed between the expression of *CSF2* and *BHLHE40* (Figure 2c).

3.3 | Gene Expression of *BHLHE40* in $CD4^+$ T Cells After Anti-CD3/28 Stimulation

To investigate the effect of activation at the single-cell level, changes in *BHLHE40* expression in $CD4^+$ T cells before and after stimulation with anti-CD3/28 were examined using publicly available scRNA-seq data (GSE147928). A UMAP combining the unstimulated and stimulated data is shown (Figure 3a). In addition, an increase in the expression of *BHLHE40* and *CSF2* was observed after stimulation, although only a slight increase was observed in *IL1R1* expression (Figure 3b).

After confirming that the increase in *BHLHE40* expression was due to anti-CD3/28 stimulation, further analysis focused on stimulated cells. Clusters that did not express *CD4* were excluded, resulting in seven clusters (Figures 3c and S2a). As shown in the feature map (Figure 3d), violin plot (Figures 3e, S2b), and heatmap (Figure 3f), *BHLHE40*, *CSF2*, *TBX21*, and *RORC* were observed in Clusters 1 and 5. In addition, *IFNG* is expressed in Clusters 1 and 5, while *IL17A* is expressed in Cluster 1. Therefore, Th1, Th17, and Th1/17 cells are present in Clusters 1 and 5 (Figures 3e and S2b).

Furthermore, we divided stimulated $CD4^+$ T cells into groups with positive and negative *BHLHE40* gene expression and compared their gene expression profiles. When comparing gene expression between these groups, *CSF2* was identified as a significantly upregulated gene in the *BHLHE40*-positive group, and the results were presented as a volcano plot. Furthermore, as shown in the violin plot, *CSF2* gene expression revealed that the *BHLHE40*-positive group had significantly higher *CSF2* expression than the *BHLHE40*-negative group in stimulated $CD4^+$ T cells (Figure S2c,d).

Finally, reanalysis of the microarray data (GSE39594 and GSE78244) showed similar increases in *IL1R1*, *BHLHE40*, and *CSF2* expression in both Tn-stimulated cells from PB of HC for 4 h and total $CD4^+$ T cells from PB of HC stimulated for 24 h with anti-CD3/28 (Figure S3a,b). A strong correlation was observed between *CSF2* and *BHLHE40* expression (Figure S3c,d). The expression patterns varied depending on the specific stimulation conditions used. Stimulation for 4 and 24 h resulted in elevated levels of *TBX21*, *CXCR3*, and *CCR4* (Figure S3a,b). Based on scRNA-seq and microarray analyses, we found that *CSF2*-expressing cells also expressed *BHLHE40*.

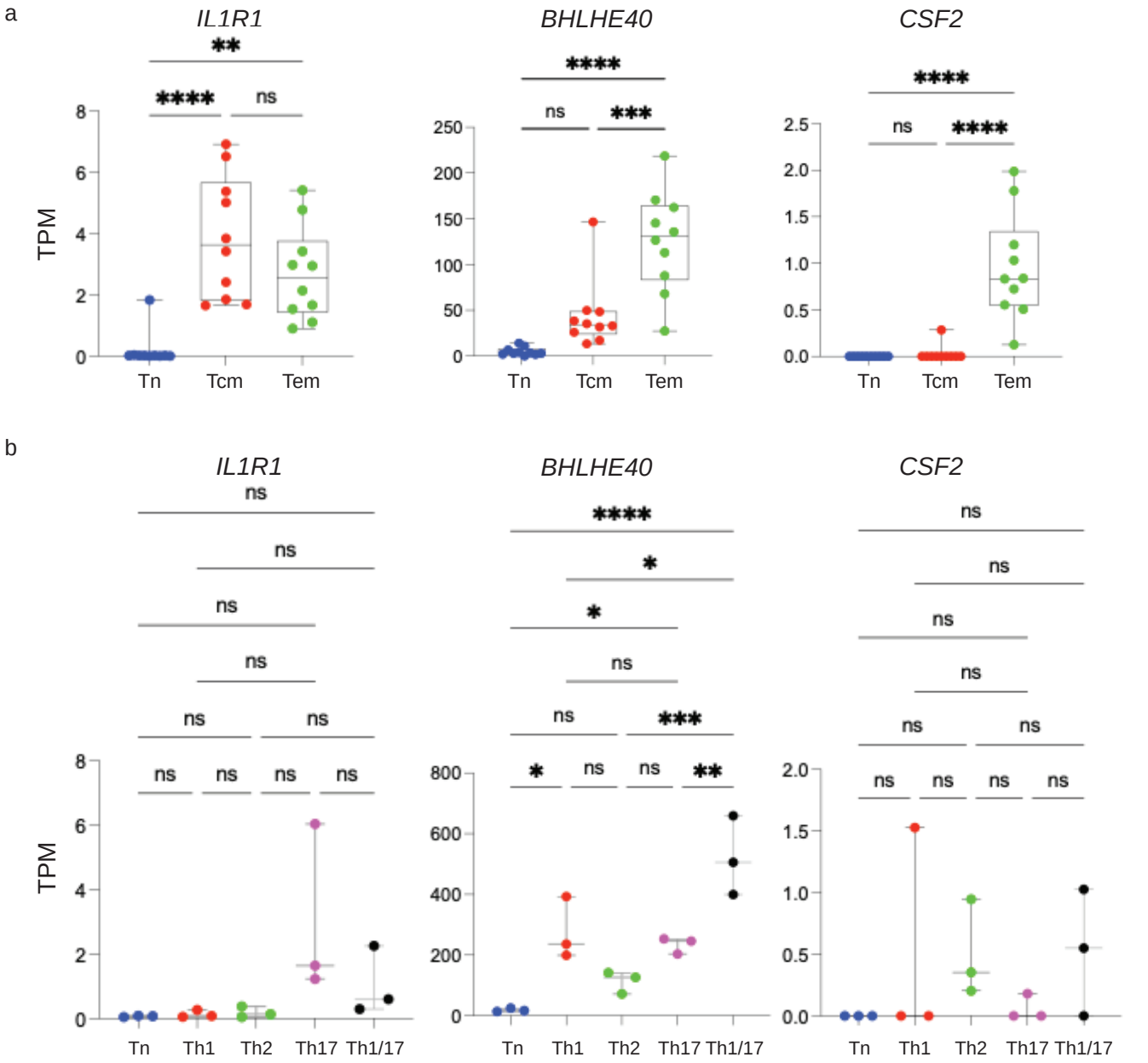

FIGURE 1 | Gene expression of *BHLHE40* in CD4⁺ T cell subsets of PB CD4⁺ T cells. (a) Expression (TPM) of *IL1R1*, *BHLHE40*, and *CSF2* plots obtained from the GSE118829 RNA-seq datasets ($n = 10$). (b) Expression (TPM) of *IL1R1*, *BHLHE40*, and *CSF2* plots obtained from the GSE149090 RNA-seq datasets ($n = 4$). *p* values were obtained using one-way ANOVA with Tukey's multiple-comparison test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. ns: not significant, TPM, transcripts per million; Tn, naive CD4⁺ T cells; Tcm, central memory CD4⁺ T cells; Tem, effector memory CD4⁺ T cells.

3.4 | Effect of Treatment on Gene Expression of *BHLHE40* in PB CD4⁺ T Cells From Patients With RA

The gene expression of *IL1R1*, *BHLHE40*, and *CSF2* in the PB CD4⁺ T cells of patients with RA has not been reported under either untreated or treated conditions. Compared to Tn in HC, higher expression of *BHLHE40* was observed in Tcm, Tem, Th17, and Th1/17 subsets than in Tn of PB CD4⁺ T cells. Using data from previously reported public databases, we evaluated the expression of these genes to assess changes resulting from therapeutic interventions. In patients with RA, gene expression in total CD4⁺ T cells before and after 6 months of IL-6 receptor inhibitor (TCZ: tocilizumab) treatment was analyzed (GSE113156). No significant differences were observed in the expression of *IL1R1*, *BHLHE40*, or *CSF2* (Figure 4a). Further comparisons were made according to the type of treatment in patients with RA, including no treatment and treatment using TCZ, anti-tumor necrosis factor-alpha (TNF-α) inhibitor (IFX), and methotrexate (MTX). In the PB, the expression of *IL1R1*, *BHLHE40*, and *CSF2* in the CD45RA⁻ CCR7$^{+/-}$CD4⁺ T cell (including Tcm and Tem) subsets was not significantly different (Figure 4b). This indicates that these genes are constitutively expressed in PB CD4⁺ T cells and do not change after treatment. Finally, we examined the relationship between the gene expression of *IL1R1*, *BHLHE40*, and

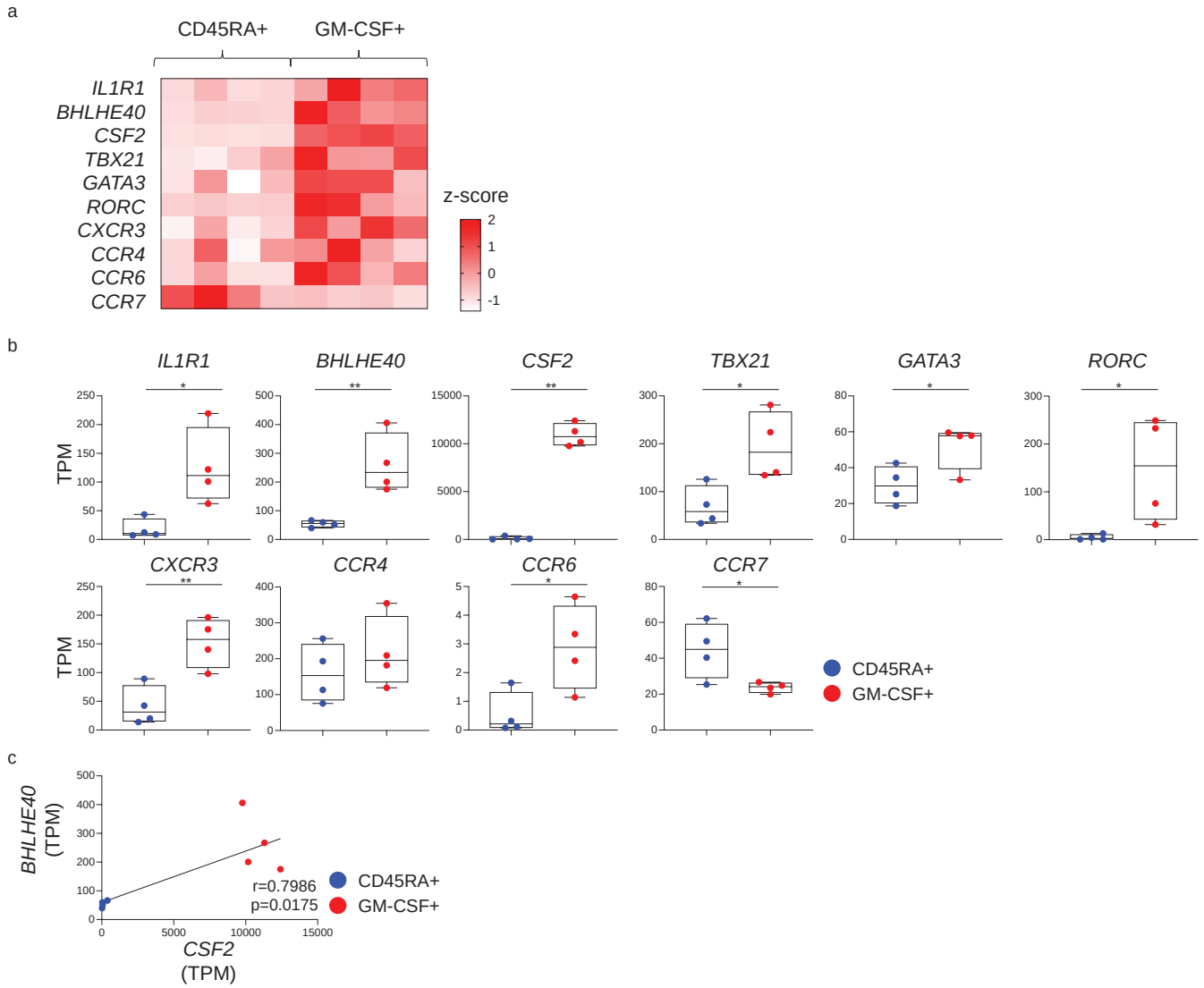

FIGURE 2 | GM-CSF and *BHLHE40* variation in CD4$^+$ T cells stimulated with PMA/ionomycin. (a) Heatmap of normalized *z*-score expression of *IL1R1*, *BHLHE40*, *CSF2*, *TBX21*, *GATA3*, *RORC*, *CXCR3*, *CCR4*, *CCR6*, and *CCR7* genes in CD45RA$^+$ CD4$^+$ T cells ($n=4$) and GM-CSF$^+$ CD4$^+$ T cells ($n=4$). (b) Expression (TPM) of *IL1R1*, *BHLHE40*, *CSF2*, *TBX21*, *GATA3*, *RORC*, *CXCR3*, *CCR4*, *CCR6* and *CCR7* genes in CD45RA$^+$ CD4$^+$ T cells ($n=4$) and GM-CSF$^+$ CD4$^+$ T cells ($n=4$). (c) Scatter plot shows *Pearson*'s correlation for *BHLHE40* and *CSF2* expression using TPM normalization correlations in the gene expression. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. TPM, transcripts per million. All data were obtained from GSE103930 RNA-seq datasets using Student's *t*-test.

CSF2 divided into Tcm and Tem from PB as well as clinical parameters, including C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), matrix metalloproteinase-3 (MMP3), Disease Activity Score-28 for Rheumatoid Arthritis with CRP (DAS28-CRP), Disease Activity Score-28 for Rheumatoid Arthritis with ESR (DAS28-ESR), Simple Disease Activity Index (SDAI), Clinical Disease Activity Index (CDAI), Health Assessment Questionnaire (HAQ), Doctor's Visual Analogue Scale (D-VAS), and patients rate their global assessment on a VAS (P-VAS). As a result, in untreated patients, *BHLHE40* gene expression in Tcm showed a negative correlation with CRP, whereas *IL1R1* gene expression in Tcm exhibited a positive correlation with MMP3. In patients treated with TCZ, *IL1R1* gene expression in Tcm was positively correlated with DAS28-CRP, SDAI, and CDAI. Furthermore, *BHLHE40* gene expression in Tem correlated with D-VAS. In patients treated with MTX, *IL1R1* gene expression in Tcm showed a positive correlation with D-VAS (Figure S4).

3.5 | PB CD4$^+$ T Cells in Patients With RA Show Higher Gene Expression of *BHLHE40* Compared With Those From HC

We compared gene expression in CD4$^+$ T cells using publicly available scRNA-seq datasets from PBMCs of HC and patients with RA (GSE142637 and GSE159117). First, UMAP analysis classified these cells into 12 distinct clusters (Figure 5a). Next, we examined the expression of *IL1R1*, *BHLHE40*, *CSF2*, *TBX21*, *GATA3*, *RORC*, *CXCR3*, *CCR4*, *CCR6*, *CCR7*, *TNF*, *IFNG*, *IL4*, *IL17A*, *IL6*, *IL21*, *IL22*, and *IL23A*. However, *CSF2*, *IL4*, and *IL17A* could not be detected (Figures 5b,c and S5a,b).

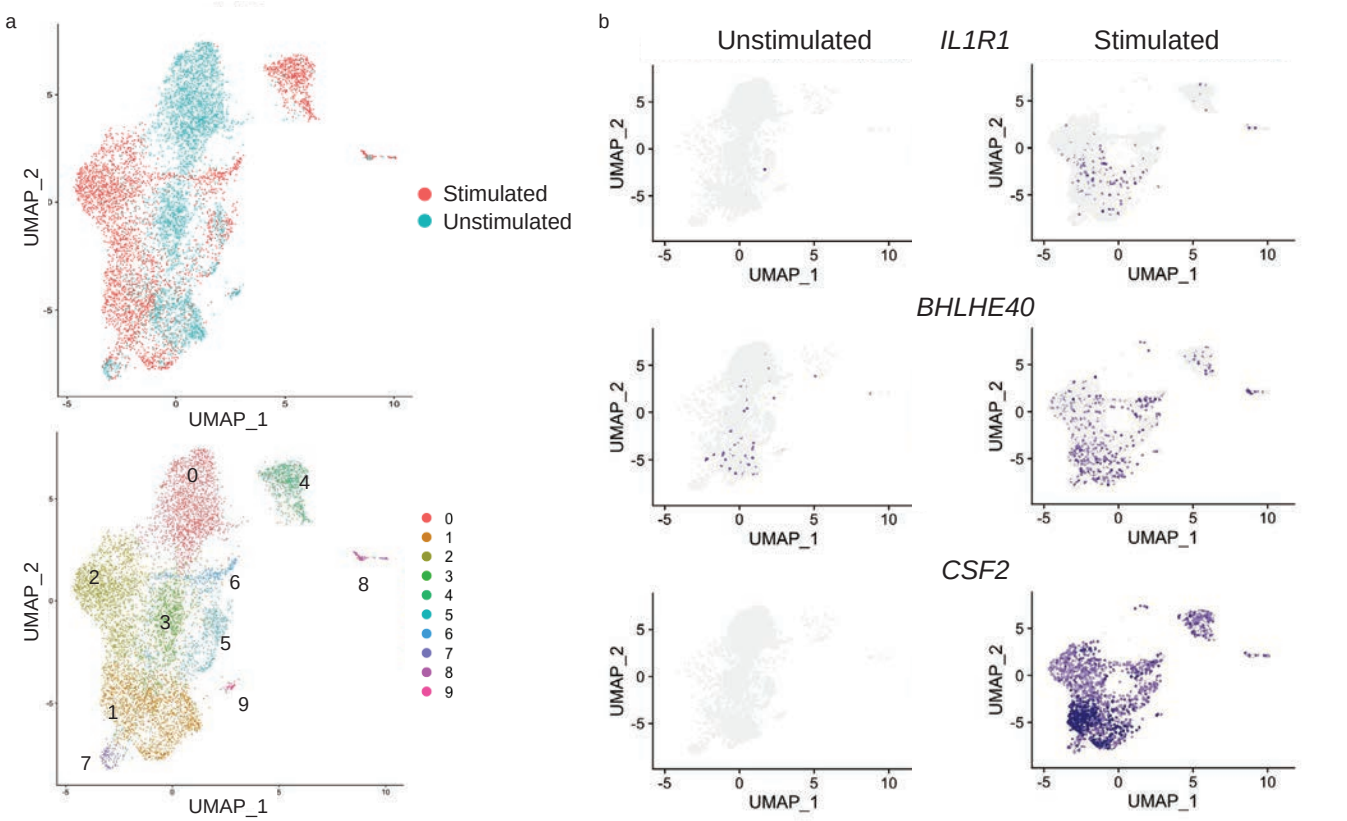

FIGURE 3 | Gene expression of *BHLHE40* in CD4$^+$ T cells after stimulation with anti-CD3/28. (a) UMAP projection of CD4$^+$ T cells pre- and post–anti-CD3/CD28 stimulation, using different colors to depict unstimulated and stimulated cells. Each dot refers to a cell and the colors refer to the subclusters. (b) Expression levels of *IL1R1*, *BHLHE40*, and *CSF2* on UMAP in unstimulated and stimulated CD4$^+$ T cells. (c) UMAP of stimulated CD4$^+$ T cells, depicted using different colors. Each dot refers to a cell and colors refer to the subclusters. (d) Expression levels of *IL1R1*, *BHLHE40*, *CSF2*, *TBX21*, *GATA3*, *RORC*, *CXCR3*, *CCR4*, *CCR6*, and *CCR7* on a UMAP of CD4$^+$ T cells. (e) Violin plot shows genes on the Y-axis, whereas clusters are shown on the X-axis. (f) Dot plot showing the *z*-scored mean gene expression of *IL1R1*, *BHLHE40*, *CSF2*, *TBX21*, *GATA3*, *RORC*, *CXCR3*, *CCR4*, *CCR6*, and *CCR7* for each cluster. UMAP, Uniform manifold approximation and projection.

Furthermore, Clusters 3, 6, 7, and 11 showed higher *BHLHE40* expression in patients with RA than in HC. Cluster 3 coexpressed *TBX21* and *GATA3*, whereas Cluster 6 coexpressed *GATA3*. Additionally, scRNA-seq showed that the gene expression of *BHLHE40* in CD4$^+$ T cells from the PB of patients with RA was significantly higher than in those from HC (Figure 5d).

3.6 | Comparison of *BHLHE40* Expression in CD4$^+$ T Cells From PB and SF of Patients With RA

Finally, we examined CD45RA$^-$CCR7$^{+/-}$CD4$^+$ T cell subsets derived from the SF, a site of inflammation. Using our previously reported database (GSE118829), we reanalyzed gene expression in CD45RA$^-$CCR7$^{+/-}$CD4$^+$ T cell subsets, combining Tcm and Tem, obtained from the PB of patients without treatment and the SF of patients with RA, and presented the results as a volcano plot. The results showed that the expression levels of *IL1R1*, *BHLHE40*, and *CSF2* were significantly higher in the CD45RA$^-$CCR7$^{+/-}$CD4$^+$ T cell subsets infiltrating the SF than in the PB of untreated patients with RA (Figure 6a). The expression levels of *TNF*, *IFNG*, *IL4*, *IL17A*, *IL1R1*, *BHLHE40*, *CSF2*, *TBX21*, *GATA3*, *RORC*, *CXCR3*, *CCR4*, *CCR6*, and *CCR7* are shown in the heatmap. The results showed that *TNF*, *IL17A*, *IL1R1*, *BHLHE40*, *CSF2, RORC, CCR4*, and *CCR6* levels were higher in the SF than that in PB (Figure S6). Subsequently, we conducted GSEA and pathway analyses to explore the biological significance of these expression patterns. We observed enrichment of cytokine production-related signatures, as "signaling by interleukins" and "cytokine-cytokine receptor interaction" were found to be statistically significant. *IL1R1* and *CSF2* were included in these gene sets (Figure 6b). Finally, the gene expression levels of *IL1R1*, *BHLHE40*, and *CSF2* in the CD45RA$^-$CCR7$^{+/-}$CD4$^+$ T cell subsets obtained from the PB of patients with RA and those obtained from the SF were compared. The gene expression levels (TPM values) of *IL1R1*, *BHLHE40*, and *CSF2* were higher in the SF than those in the PB of patients with RA (Figure 6c), suggesting that the expression levels of *IL1R1*, *BHLHE40*, and *CSF2* in CD45RA$^-$CCR7$^{+/-}$CD4$^+$ T cell subsets may be elevated by the local inflammatory environment in the SF. A strong correlation was observed between the expression of *CSF2* and *BHLHE40* in Tcm and Tem obtained from the PB and SF of patients with RA (Figure 6d).

4 | Discussion

In this study, we analyzed our previously deposited and publicly available data from HC and patients with RA to identify

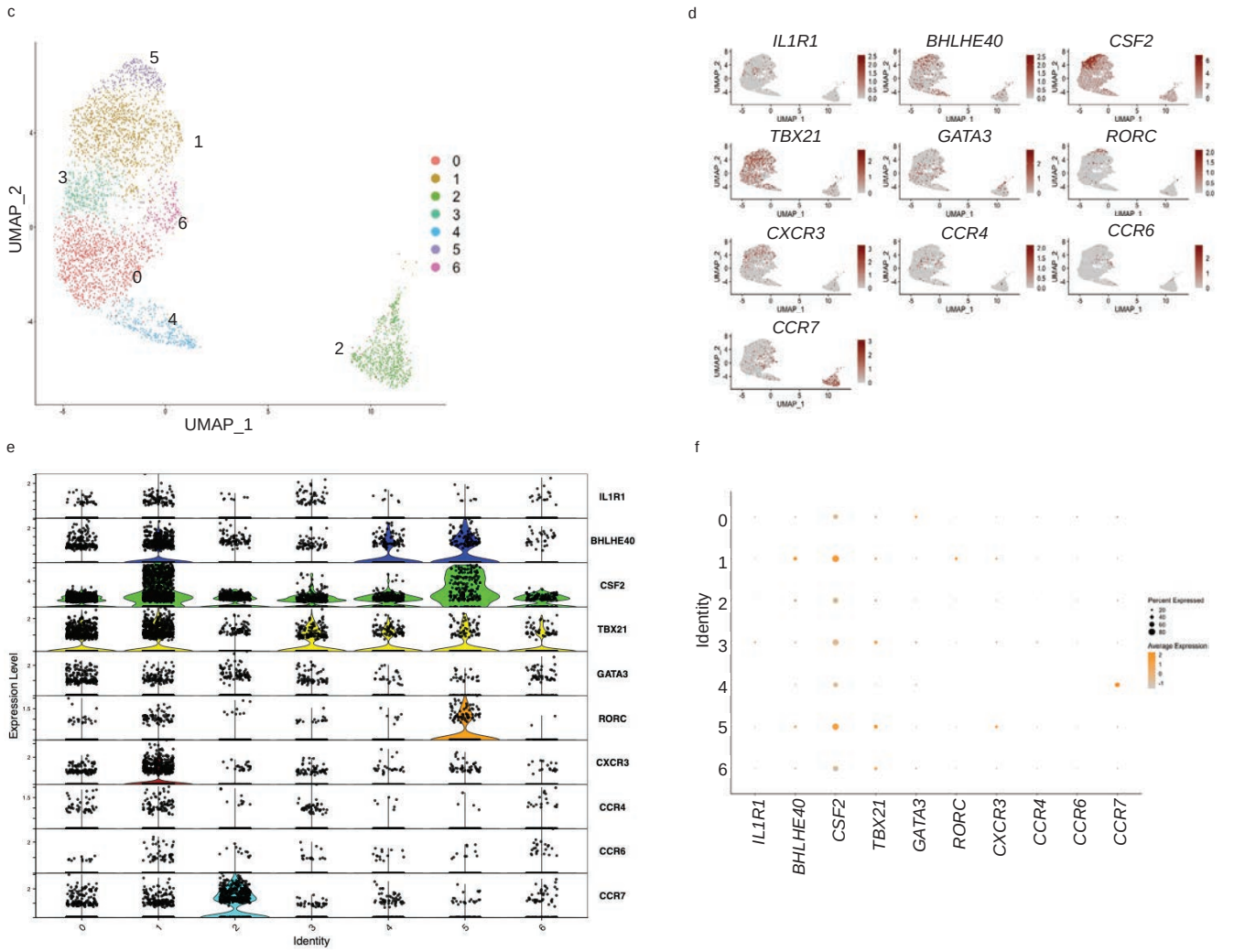

FIGURE 3 | (Continued)

the characteristics of *BHLHE40* expression in CD4$^+$ T cells of patients with RA. Differentiated T cell subsets, Tcm and Tem, exhibited higher *BHLHE40* expression than Tn. In PB, the Th17 and Th1/17 subsets showed higher levels of *BHLHE40* expression than Tn. CD4$^+$ T cells stimulated with anti-CD3/28 or PMA/ionomycin expressed both *BHLHE40* and *CSF2*. Additionally, this study identified an increased expression of transcription factors such as *TBX21* and *RORC*. Furthermore, in CD45RA$^-$CCR7$^{+/-}$ CD4$^+$ T cells from the PB of patients with RA, *BHLHE40* expression remained unchanged following treatment with MTX or cytokine inhibitors compared to untreated controls. In addition, our findings revealed increased expression of *BHLHE40* in PB CD4$^+$ T cells using scRNA-seq in patients with RA than that in HC. Additionally, CD45RA$^-$CCR7$^{+/-}$CD4$^+$ T cell subsets in the SF of patients with RA showed significantly higher *BHLHE40* levels than those in the PB of patients with RA.

Previous studies focusing on *BHLHE40* used mice, with limited analyses of human inflammatory sites [12, 13, 15, 16]. In our study, we identified a specific cell subset that expressed *BHLHE40*. We detected *BHLHE40* expression in the PB of HC across various helper T-cell subsets, including Tcm and Tem. Mice studies have shown that the addition of IL-1 induces higher expression of the BHLHE40 protein in Th1 and Th17 cells than that in the absence of IL-1 [16]. However, similar studies have not been conducted in humans. In our study, we observed increased *BHLHE40* expression in CD45RA$^-$CCR7$^{+/-}$ CD4$^+$ T cells in the SF of patients with RA compared to that in the PB of patients with RA. This increase may be associated with elevated IL-1 levels in SF from patients with RA [32]. Our scRNA-seq and microarray data revealed that after anti-CD3/28 stimulation, CD4$^+$ T cells showed elevated expression of *IL1R1*. Furthermore, our pathway analysis revealed that several genes, including *CSF2*, were significantly upregulated in the SF of patients with RA compared with those in the PB of patients. This finding suggests that inflammatory cytokines, such as IL-1, in the local joint environment can upregulate *BHLHE40* expression and promote transcription of *CSF2*. We previously reported that GM-CSF-producing CD4$^+$ T cells are expanded in secondary lymphoid nodes and inflammatory joints in IL-1 receptor antagonist KO mice, a model of arthritis with relatively enhanced IL-1 signaling [33]. In this study, we examined gene expression in CD4$^+$ T cells from the SF of RA patients using RNA-seq, focusing on the gene expression pattern of *BHLHE40*. Using chromatin immunoprecipitation sequencing and assay for transposase-accessible chromatin sequencing, Emming et al. analyzed effector CD4$^+$ T cells and identified *BHLHE40*, *TBX21*, and *RORC* as the primary related genes, which is consistent

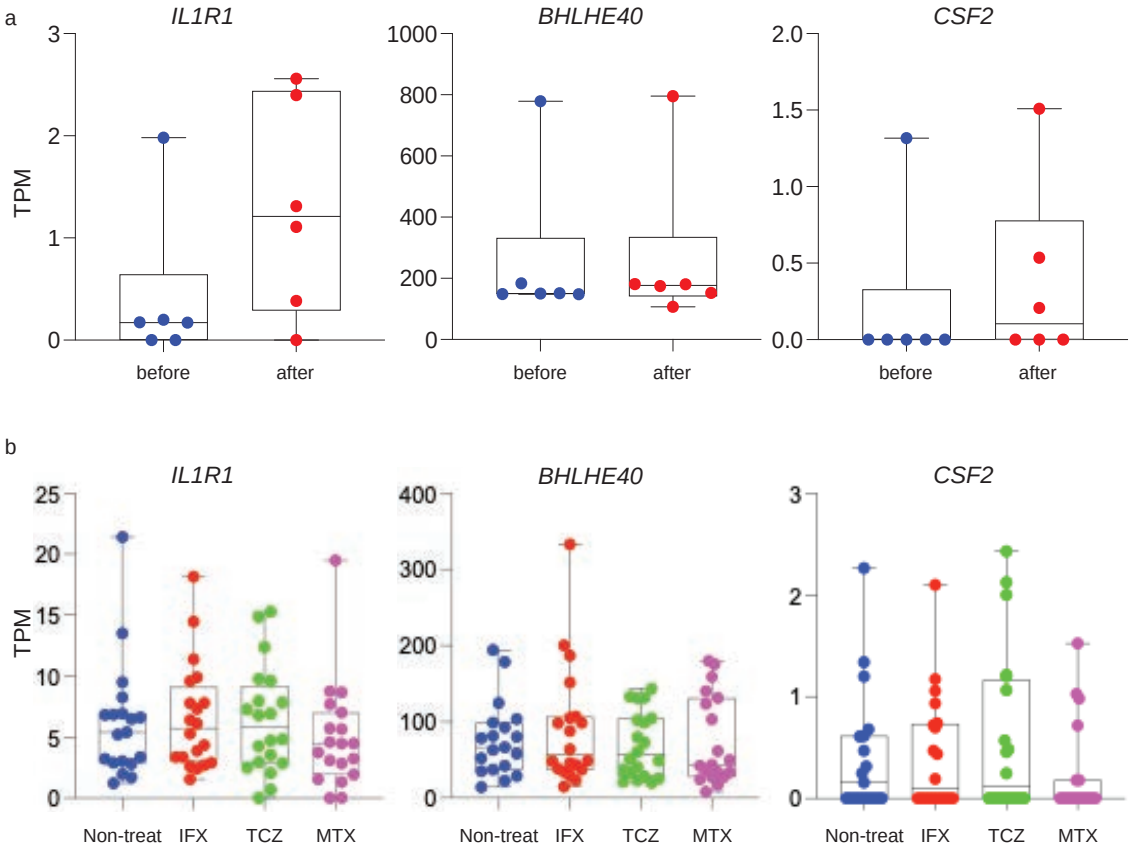

FIGURE 4 | Effect of treatment on gene expression of *BHLHE40* in PB CD4$^+$ T from patients with RA. (a) Expression (TPM) of *IL1R1*, *BHLHE40*, and *CSF2* genes in CD4$^+$ T cells before and after treatment for RA, obtained from the GSE113156 RNA-seq datasets using Student's *t*-test ($n = 6$). (b) Expression (TPM) of *IL1R1*, *BHLHE40*, and *CSF2* genes in CD45RA-CCR7$^{+/-}$ T cell subsets from patients with untreated RA, and those treated with IFX, TCZ, or MTX, obtained from the GSE118829 RNA-seq datasets using one-way ANOVA with Tukey's multiple-comparison test were used [non-treat $n = 10$ (Tcm: 10, Tem: 9), IFX $n = 10$ (Tcm: 10, Tem: 10), TCZ $n = 10$ (Tcm: 10, Tem: 10), and MTX $n = 10$ (Tcm: 10, Tem: 9)]. TPM, Transcripts per million; Tn, naive $CD4_+$ T cells; Tcm, central memory $CD4_+$ T cells; Tem, effector memory CD4$^+$ T cells; HC, healthy controls; RA, rheumatoid arthritis; Non-treat, nontreated; IFX, infliximab; MTX, methotrexate; TCZ, tocilizumab.

with our findings [8]. Our study focused on the expression of *BHLHE40* in patients with RA. In PB, we observed consistent gene expression of *IL1R1*, *BHLHE40*, and *CSF2* in total CD4$^+$ T cells, with no significant changes following treatment. However, in the correlation between subsets divided into Tcm and Tem and clinical parameters, a relationship was observed between *IL1R1* gene expression in Tcm and several clinical parameters in the group of patients using TCZ. These results may be considered to reflect a reduction in the proportion of Th17 cells due to TCZ treatment, as reported by Samson et al., since Th17 is the major CD4$^+$ T-cell subset expressing IL1R1 [34]. Nevertheless, as gene expression in PB T cells and clinical data represent separate parameters, demonstrating a causal relationship remains challenging. The correlation between gene expression and treatment response may serve as a reference finding, but it is difficult to determine its significance based on a single dataset. As a next step, we aim to investigate whether the expression of *BHLHE40* and other related genes changes at the disease sites in response to treatment.

At inflamed sites and lymph nodes, dendritic cells and other antigen-presenting cells can activate T-cells by presenting class II major histocompatibility complex molecules and CD80/86 ligands to CD3/T cell receptor and CD28 on T cells. Instead, PMA/ionomycin directly stimulates T cells by direct activation of protein kinase C [35, 36]. Both natural anti-CD3/28 stimulation and artificial PMA/Ionomycin stimulation increase *BHLHE40*, which was consistently correlated with an increase in *CSF2*. Therefore, in our study, stimulation with anti-CD3/28 or PMA/ionomycin effectively mimicked stimulation with antigen-presenting cells, thereby providing a useful model for evaluating activated T-cell states. These results were used to assess poststimulation total CD4$^+$ T cells that expressed *BHLHE40* and coexpressed *CSF2* along with *TBX21* and *RORC*. Stimulation of these cells likely advanced their differentiation toward their respective polarizations. In stimulated cells, when categorized based on *BHLHE40* positivity or negativity, *CSF2* showed significantly higher gene expression in *BHLHE40*-positive cells. Additionally, it has been reported that cells producing GM-CSF exhibit higher *BHLHE40* expression compared to those that do not [8]. Although the discrepancy between our analysis of RNA-seq and scRNA-seq results may arise because RNA-seq analyzes fractions sorted based on cell surface markers, whereas scRNA-seq defines subsets using lineage markers. Moreover, differences in the condition of the materials used may also contribute to this discrepancy. Additionally, a subset of Th17 cells expressing RORC and TBX21 that produces GM-CSF but has low IL-17 production has been reported, suggesting

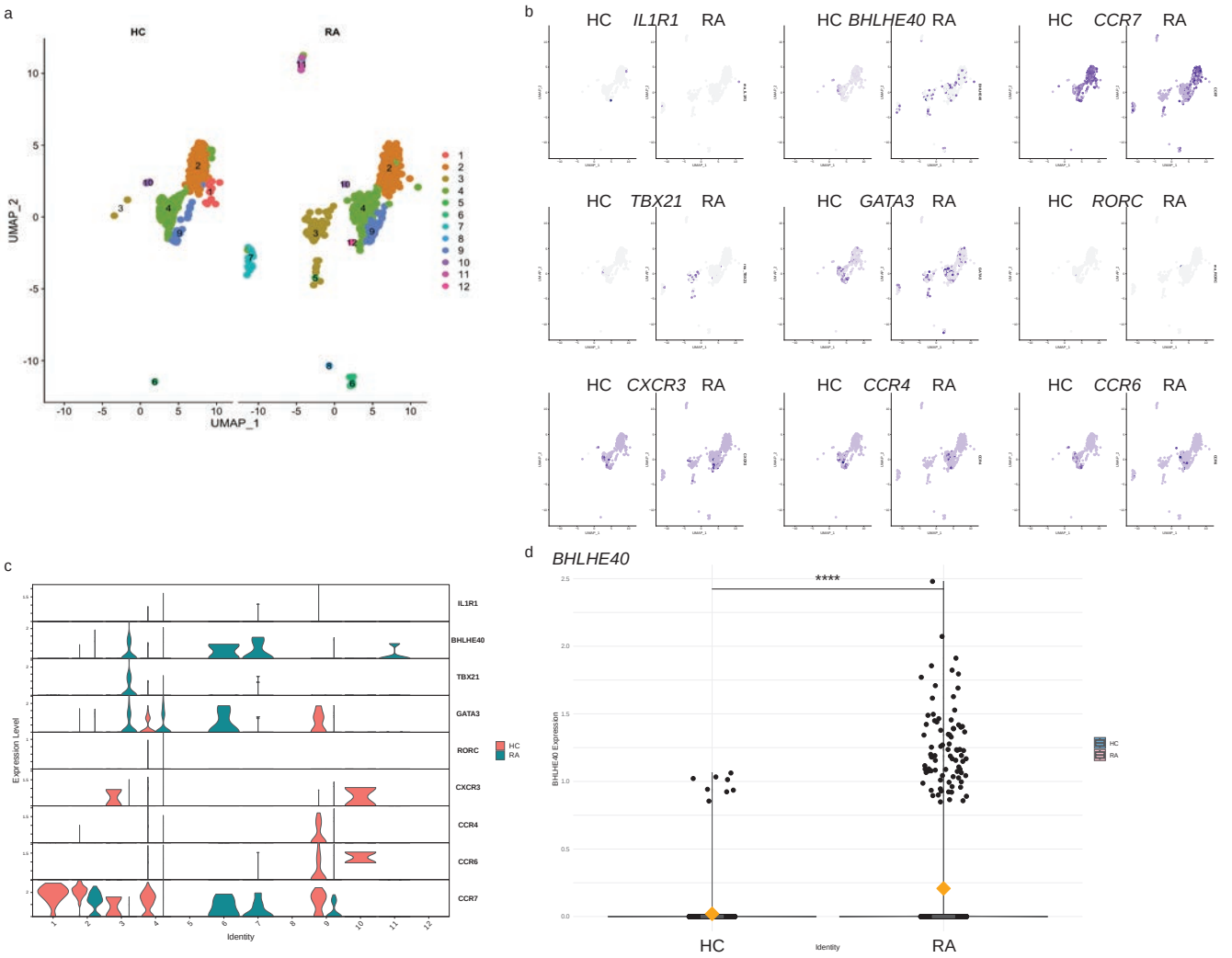

FIGURE 5 | *BHLHE40*-expressing CD4$^+$ T cells in HC and RA PB. (a) UMAP of gene expression in *CD3D* > 0.5 and *CD4* > 0.5 T cells, with different colors depicting HC and RA cells. Each dot represents one cell and colors indicate the subclusters. (b) Expression levels of *IL1R1*, *BHLHE40*, CCR7, *TBX21*, *GATA3*, *RORC*, *CXCR3*, *CCR4*, and *CCR6* on a UMAP of CD4$^+$ T cells. (c) Violin plot shows genes on the Y-axis, whereas clusters are shown on the X-axis. (d) Expression of *BHLHE40* genes in CD4$^+$ T cells from PB of HC and patients with RA in arbitrary using Student's t-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. UMAP, Uniform manifold approximation and projection; PB, peripheral blood; SF, synovial fluid; HC, healthy controls; RA, rheumatoid arthritis; FC, fold change.

that our findings are consistent with previous studies [37, 38]. Combined with the RNA-seq results obtained from the SF of patients with RA, these findings suggest that subsets expressing BHLHE40 and producing GM-CSF contribute to inflammation. At the site of inflammation, elevated IL-1 levels and antigen-presenting cells lead to the activation of T cells, causing these T cells to express *IL1R1* and upregulate *BHLHE40* gene expression. This upregulation further enhances *CSF2* expression. GM-CSF, in turn, activates macrophages, which produce IL-1, creating a feedback loop that continues to activate T cells and drive the inflammatory process.

In clinical trials, suppression of GM-CSF can potentially be used as a therapeutic strategy [3, 4]. However, in contRAst 3, at Week 12, otilimab did not demonstrate a significant difference in the proportion of American College of Rheumatology 20 responders at doses of 90 mg and 150 mg compared to placebo in terms of CDAI, HAQ-DI, and pain VAS [5]. Therefore, it may be important to regulate GM-CSF production as well as to modify BHLHE40 expression to influence the pathology of RMD. Appropriate suppression of the expression of *BHLHE40* may reduce GM-CSF production, thus serving as a potential strategy for treating autoimmune diseases in the future.

We acknowledge the potential limitations of this study. This study relied on publicly available databases, which may limit the scope of the data and applicability of the findings. There was variability in both the conditions under which the cells were stimulated and the intrinsic conditions of the cells themselves, which could potentially lead to pseudo-correlations and differences in cytokine and surface marker mRNA expression. Furthermore, we focused on the analysis of CD4$^+$ T cells only. Thus, we cannot speculate about other *CSF2*-expressing cells. Our scRNA-seq analysis did not include an integrated examination with cell surface markers from the same samples. Additionally, the analysis of CD4$^+$ T cells derived from in vitro–stimulated CD4$^+$ T cells or PBMCs from RA and HC resulted in insufficient resolution to accurately identify specific CD4$^+$ T cell

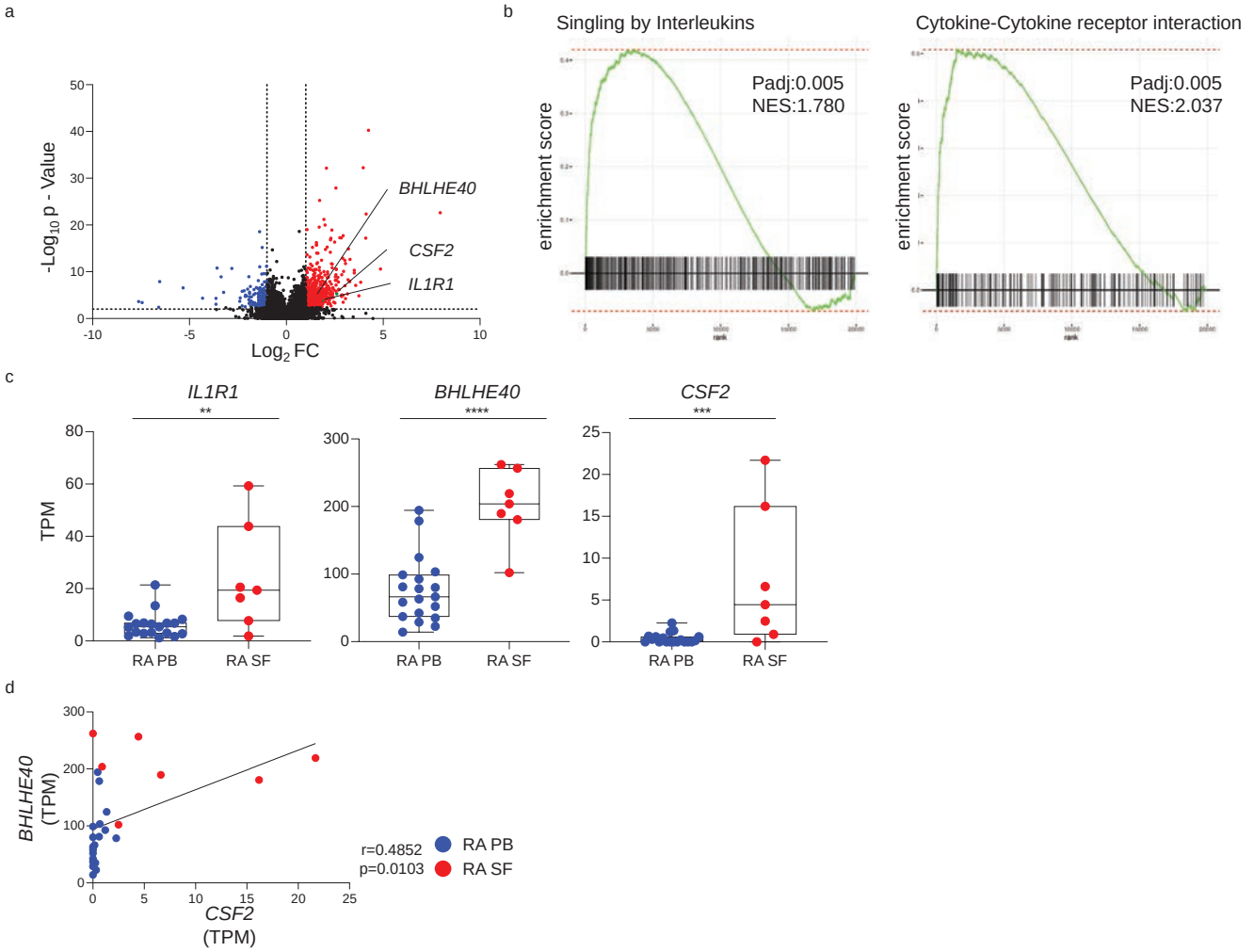

FIGURE 6 | *BHLHE40*-expressing CD4$^+$ T cells in RA PB and RA SF. (a) Volcano plot showing differences in gene expression between PB cells from RA and SF CD45RA$^-$CCR7$^{+/-}$CD4$^+$ T cells. X-axis shows $\log_2$ fold change values; Y-axis shows the $-\log_{10}$ *p* values. Thresholds for significance were | $\log_2$ fold change | > 1 and adjusted *p* value < 0.05. Red indicates significantly upregulated genes; blue indicates significantly downregulated genes; green indicates *IL1R1*, *BHLHE40*, and *CSF2*. (b) Gene set enrichment analysis (GSEA) was performed on normalized RNA-seq expression data of CD45RA$^-$CCR7$^{+/-}$CD4$^+$ T cell subsets obtained from SF of patients with RA and PB from patients with RA. Gene sets with $p < 0.05$ belong to two pathways: Signaling through interleukins that were manually curated (left) and cytokine-cytokine receptor interaction (right). Representative enrichment plots from each group are displayed with the determined adjusted *p* value and normalized enrichment score (NES). (c) Expression (TPM) of *IL1R1*, *BHLHE40*, and *CSF2* genes in CD4$^+$ T cells from PB of patients with RA, and SF of patients with RA, in arbitrary using Student's *t*-test [RA PB $n = 10$ (Tcm: 10, Tem: 9), RA SF $n = 4$ (Tcm: 4, Tem: 3)]. (d) Scatter plot shows *Pearson*'s correlation for *BHLHE40* and *CSF2* expression using TPM normalization correlations in gene expression. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. TPM, transcripts per million; PB, peripheral blood; SF, synovial fluid; HC, healthy controls; RA, rheumatoid arthritis; FC, fold change.

subsets. Therefore, detailed subsets could not be identified using genes that were not sufficiently expressed. Finally, the sample size used for analysis was limited. Future research in this area should incorporate additional methodologies such as primary data collection and experimental validation to enhance the robustness of our findings.

5 | Conclusion

This study revealed a novel finding in RA, highlighting not only the presence of CD4$^+$ T cells producing GM-CSF but also the involvement of *BHLHE40*-expressing CD4$^+$ T cells in the SF of patients with RA.

Author Contributions

S.I.: formal analysis, investigation, methodology, writing the original draft, writing of the review, and editing. M.T.: correction of clinical data and writing of the review. K.S. and Y.K.: supervision, validation, writing of the review, and editing.

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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

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LETTER TO THE EDITOR

The Prevalence of Ocular Manifestations Among Systemic Sclerosis Patients

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Dear Editor,

Systemic sclerosis (SSc) is a chronic autoimmune disease of unknown etiology and complex pathogenesis, mainly characterized by tissue fibrosis, vasculopathy, and immune abnormalities with a specific profile of autoantibodies [1]. The vascular component of the disease can involve not only the peripheral vasculature but also internal organs such as the heart, lungs, and kidneys [1]. The disease is subcategorized into two main subtypes: diffuse cutaneous SSc (dcSSc) and limited cutaneous SSc (lcSSc). These subtypes differ not only with regard to the extent of skin involvement but also in the extent and severity of internal organs involvement and prognosis [1, 2].

Ocular involvement is relatively common in SSc, but relevant literature is based mainly on small case series and case reports, probably due to the rarity of the disease [3]. SSc can affect most, if not all, components of the eye, not only through the inflammatory/fibrotic component but also due to the vascular component that affects the inner parts such as the retina and the choroid [4]. Eyelid disease and conjunctival involvement, along with dry eye disease, are the most prevalent manifestations that usually reflect the fibrotic component of SSc, while the retina and choroid are much less involved and represent the vascular component of the disease [4]. Visual loss directly associated with the illness is uncommon, and posterior region involvement is often asymptomatic [4]. Some studies report that modest retinal injury can manifest even before systemic manifestations [5, 6].

Eyelid involvement is the most common ophthalmic manifestation among patients with SSc, mainly due to collagen deposition in the dermis leading to rigidity and limitation of the eyelids [4]. Szucs et al. reported pathologies in the eyelids in more than 56% of their study of 51 patients with SSc, and Gomes et al. reported dry eye disease among 48.9% of their study consisting of 45 SSc patients [7, 8].

In a review paper by Carala and colleagues, the estimated prevalence of the various ophthalmic manifestations among SSc patients was as follows: eyelids (51.1%–77%), conjunctiva (15.7%–63%), dry eye (48.9%–64.7%), cataract (42.2%–51%), glaucoma (11.1%–21.6%) and retina (10%–34%) [4].

As most of the studies included small numbers of patients and there is a paucity of well-designed controlled studies, it is of paramount importance to study the prevalence and different forms of eye involvement among SSc patients in a large-scale real-world population-based controlled study.

Hence, we investigated the prevalence of ocular manifestations among a large population of SSc patients.

This retrospective, population-based controlled study included patients diagnosed with SSc as recorded in the Leumit Healthcare Services (LHS) database between January 2000 and December 2022. SSc diagnosis was identified using the International Classification of Diseases, Ninth Revision (ICD-9) code 710.1. Eligible patients were aged 18 years or older, with the

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diagnosis confirmed by a certified rheumatologist. We excluded individuals with concurrent rheumatic diseases, except those with a co-diagnosis of Sjögren's syndrome.

Each patient in the SSc group was matched with five controls based on age, gender, and socioeconomic status (SES). Controls were selected from the same LHS database and were SSc or other autoimmune diseases free, apart from Sjögren's syndrome.

LHS is one of four health maintenance organizations (HMOs) in Israel, providing services to approximately 730,000 citizens. Israeli law mandates universal health coverage, allowing all citizens access to any HMO, including LHS, without denial.

We collected demographic characteristics, smoking status, body mass index (BMI), laboratory results, medication history, and detailed ophthalmic diagnoses for each patient. Additionally, we recorded data on comorbidities: Ischemic heart disease (IHD), congestive heart failure (CHF), diabetes mellitus (DM), hypertension, chronic obstructive pulmonary disease (COPD), interstitial lung disease (ILD), cerebrovascular accident (CVA), transient ischemic attack (TIA), chronic kidney disease (CKD), and liver cirrhosis.

The primary outcome was the prevalence of ophthalmic manifestations in SSc patients, including eyelid disorders, conjunctival and corneal diseases, orbital diseases, cataract, glaucoma, refraction disorders, dry eye syndrome, and keratitis.

We presented categorical variables as counts (n) and percentages (%), and continuous variables as means (m) with standard deviations (SD). Differences between SSc patients and controls were analyzed using independent t-tests for normally distributed continuous variables or Mann–Whitney U tests for non-normally distributed data. Categorical variables were analyzed using Fisher's exact test. Odds ratios (OR) with 95% confidence intervals (CIs) were calculated to assess the association between SSc and ophthalmic manifestations. Statistical significance was set at a p value < 0.05. Analyses were conducted using R software version 4.0.2.

The study protocol was reviewed and approved by the institutional Helsinki committee of LHS.

Our study included a total of 553 patients with SSc and 2765 age-, gender-, and socio-economic status-matched controls. Female representation was identical in both groups, comprising 81.2%. The mean age of SSc patients was 63.0 ± 15.1 years, while the control group had a mean age of 63.5 ± 15.3 years ($p = 0.492$). A significant difference was observed in BMI, with SSc patients having a lower mean BMI (26.5 ± 5.9) compared to controls (28.1 ± 5.7, $p < 0.001$).

SSc patients exhibited higher rates of several key comorbidities compared to controls, including CHF (9.6% vs. 5.6%, $p = 0.001$), COPD (15.0% vs. 9.5%, $p < 0.001$), cirrhosis (2.5% vs. 0.5%, $p < 0.001$), and IHD (13.6% vs. 9.7%, $p = 0.009$). Additionally, Sjögren's syndrome was significantly more prevalent among SSc patients (6.3%) compared to controls (0.6%, $p < 0.001$).

Ocular involvement was notably more common in SSc patients, with 75.6% experiencing at least one ophthalmic condition, compared to 62.3% of controls.

The association analysis revealed differences in the prevalence of several ophthalmic conditions between SSc patients and controls. Conjunctivitis was more prevalent among SSc patients, 58.0% versus 52.0% of controls ($p = 0.010$) with OR = 1.27 (95% CI: 1.06–1.54). Dry eye was also significantly higher in the SSc group, 37.8% versus 26.2% of controls ($p < 0.001$) with OR = 1.71 (95% CI: 1.40–2.08). Cataracts were present in 40.3% of SSc patients compared to 34.8% of controls ($p = 0.015$) with OR = 1.27 (95% CI: 1.05–1.53). Keratitis, though less common, had a strong association with SSc, occurring in 6.15% of SSc patients versus 1.81% of controls ($p < 0.001$) with OR = 3.56 (95% CI: 2.21–5.67). Eyelid inflammation was noted in 33.3% of SSc patients compared to 27.6% of controls ($p = 0.008$) with OR = 1.31 (95% CI: 1.07–1.60). The prevalence of glaucoma (7.8% in SSc vs. 6.8% in controls, $p = 0.410$) and blindness (1.6% in SSc vs. 1.7% in controls, $p = 1.000$) showed no significant differences between the groups (Figure 1).

Table 1 provides an overview of the prevalence rates, odds ratios, and confidence intervals for each of these ophthalmic conditions.
Our study included a large population-based cohort of more than 500 SSc patients and demonstrated that the entire visual tract can be involved in SSc. Some manifestations were more common than others but were comparable with their prevalence in the control group. Conjunctivitis, refraction disorders, cataracts, eyelid inflammation, and dry eyes were the most prevalent ophthalmic manifestations among both the study and the control groups. Most of these manifestations were significantly more prevalent among SSc patients, especially dry eyes, which can be explained, at least partially, by the higher prevalence of Sjögren's syndrome among the study group compared with controls.

Some of our findings are in line with previous studies, while other manifestations are not. Cataract and conjunctival involvement in our study are as common as in other studies, while dry eye, eyelid inflammation, and glaucoma are less frequent among SSc in our study compared to other studies as reported by Carla et al. These differences can be explained, at least partially, by the methods and techniques used to evaluate the patients [4]. Interestingly, our study demonstrated that the most significant differences between SSc patients and controls are represented by the "outer" eye components involvement, rather than the "inner" eye components, probably emphasizing the importance of the inflammatory/fibrosing process of SSc compared to the less pronounced vascular process in the eye in contrast to other organ systems such as the kidneys, where the vascular process is very important. Although the more common manifestations such as dry eye and conjunctivitis are not sight-threatening, they might impair the quality of life of the patients.

Our study showed that keratitis was strongly associated with SSc, as demonstrated by a relatively increased odds ratio (OR: 3.557, CI 2.21–5.67). Indeed, Nagy and colleagues suggested that corneal involvement should be included in the disease workup aiming to prevent sight-threatening complications [9]. In fact, some studies reported that modest retinal changes can manifest even before systemic manifestations, suggesting a possible role for eye involvement as a predictor of disease progression [5, 6].

Therefore, we strongly believe that regular, and probably annual, ophthalmic examination should be incorporated within the

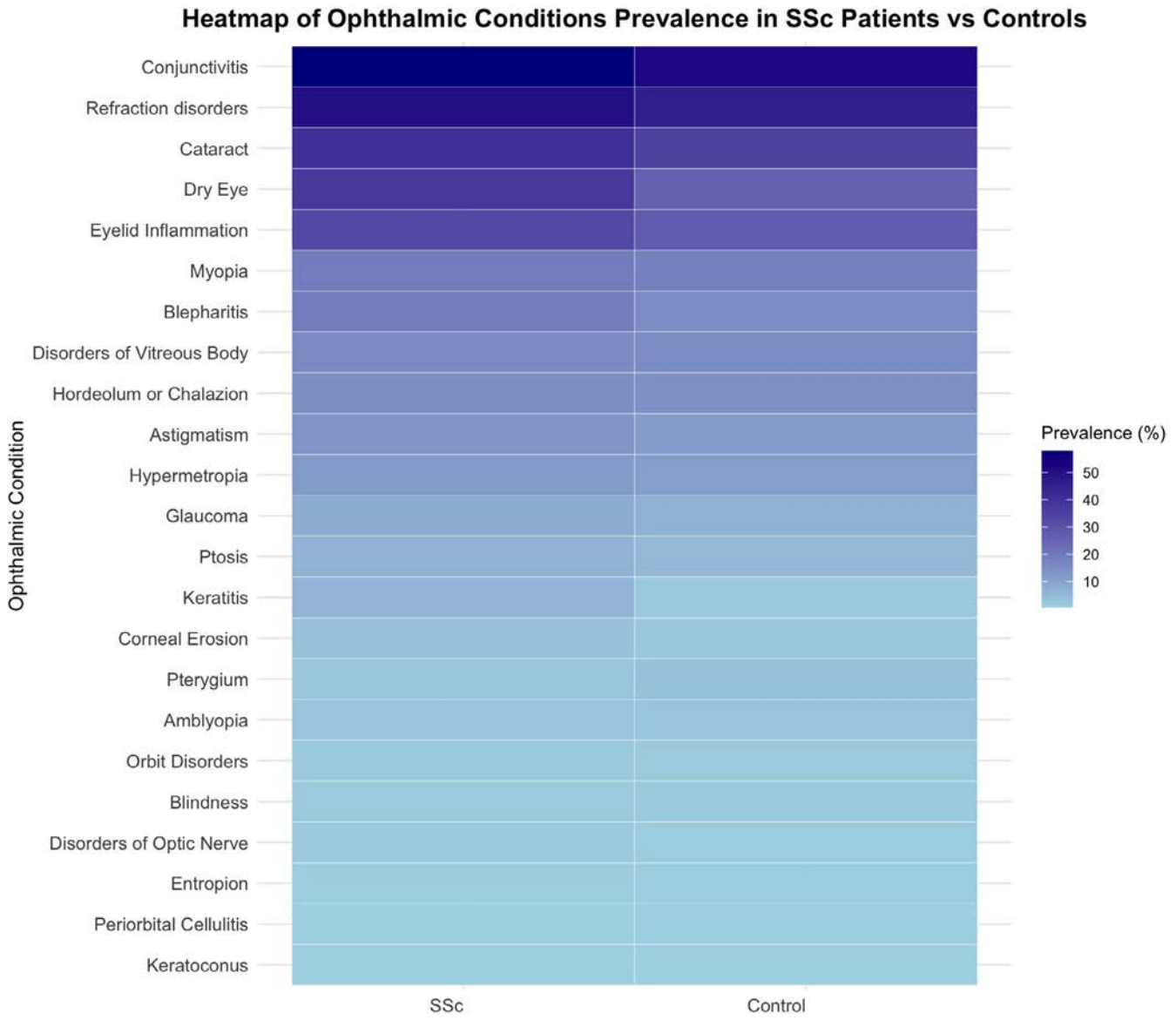

FIGURE 1 | Heatmap of ophthalmic conditions prevalence in SSc patients vs. controls. (Statistically significant differences in cataract, conjunctivitis, dry eye, eyelid inflammation, keratitis, and refractory disorders).

standard follow-up of SSc patients, especially when other autoimmune diseases, such as Sjögren syndrome, overlap with the SSc.

In contrast to other ophthalmic manifestations, glaucoma prevalence was comparable in both groups. This finding might be explained at least partially by the fact that glucocorticoids are used less commonly and more cautiously in SSc patients than in patients with other autoimmune diseases.

Other important ophthalmic manifestations that were comparable between SSc patients and controls include disorders of the optic nerve, disorders of the vitreous body, and blindness.

Our study has some limitations. First, as a population-based study reflecting the common community daily practice, no discrimination was made with regard to the subtype of SSc (diffuse versus limited) because these subtypes can't be captured by the ICD-9 codes. Second, disease specific antibodies were not available for many patients, limiting our ability to look for correlations between certain manifestations and SSc specific antibodies. Third, as no detailed ophthalmic examination was available, but rather diagnoses as documented in the LHS electronic records, we were unable to detect clustering of different manifestations in the same patient. Forth, while some autoimmune diseases were more common among SSc patients compared to controls especially SLE and Sjogren (6/33% each) others were not. Although we can't exclude the possibility that some of these diseases might impact eye involvement among patients with SSc, we believe this effect is not significant because of the relatively low prevalence of these diseases in our patients.

In conclusion, our study represents one of the largest population-based studies describing ophthalmic manifestations among patients with SSc. Keratitis seems to be strongly associated with SSc, and its occurrence should raise suspicion for SSc.

Due to the prevalence of manifestations, we believe that prospective clinical trials should examine whether annual

TABLE 1 | The various ophthalmic manifestations in patients with SSc.

Condition	SSc patients	Controls	*p*	OR	95% CI
Amblyopia	15 (2.71%)	76 (2.75%)	1.000	0.99	0.52–1.75
Astigmatism	76 (13.7%)	323 (11.7%)	0.174	1.21	0.91–1.58
Blepharitis	104 (18.8%)	429 (15.5%)	0.057	1.26	0.99–1.61
Blindness	9 (1.63%)	46 (1.66%)	1.000	0.98	0.42–2.04
Cataract	**223 (40.3%)**	**962 (34.8%)**	**0.015***	**1.27**	**1.05–1.53**
Conjunctivitis	**321 (58.0%)**	**1439 (52.0%)**	**0.010***	**1.28**	**1.06–1.54**
Corneal erosion	20 (3.62%)	65 (2.35%)	0.103	1.56	0.89–2.63
Disorders of optic nerve	8 (1.45%)	31 (1.12%)	0.516	1.30	0.51–2.90
Disorders of vitreous body	89 (16.1%)	426 (15.4%)	0.700	1.05	0.81–1.36
Dry eye	**209 (37.8%)**	**725 (26.2%)**	**<0.001***	**1.71**	**1.40–2.08**
entropion	6 (1.08%)	38 (1.37%)	0.688	0.79	0.27–1.89
Eyelid inflammation	**184 (33.3%)**	**763 (27.6%)**	**0.008***	**1.31**	**1.07–1.60**
Glaucoma	43 (7.78%)	188 (6.80%)	0.410	1.16	0.80–1.64
Hordeolum or chalazion	82 (14.8%)	399 (14.4%)	0.792	1.03	0.79–1.34
Hypermetropia	67 (12.1%)	309 (11.2%)	0.509	1.10	0.81–1.46
Keratitis	**34 (6.15%)**	**50 (1.81%)**	**<0.001***	**3.56**	**2.21–5.67**
Keratoconus	3 (0.54%)	13 (0.47%)	0.740	1.16	0.21–4.22
Myopia	107 (19.3%)	497 (18.0%)	0.433	1.10	0.86–1.39
Orbit disorders	10 (1.81%)	45 (1.63%)	0.717	1.11	0.50–2.26
Periorbital cellulitis	3 (0.54%)	20 (0.72%)	0.785	0.75	0.14–2.54
Pterygium	14 (2.53%)	94 (3.40%)	0.358	0.74	0.39–1.31
Ptosis	35 (6.33%)	142 (5.14%)	0.255	1.25	0.83–1.84
Refraction disorders	**279 (50.5%)**	**1265 (45.8%)**	**0.045***	**1.21**	**1.01–1.46**

Note: Bold values and asterisk represents all significant findings.

ophthalmic examination can be cost-effective in preventing sight-threatening damage and in predicting disease progression among SSc patients.

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Author Contributions

A.I., H.J. and M.E.N.: conceived of the presented idea. M.E.N.: developed the theory and performed the computations. A.I. and M.O.: verified the analytical methods. H.J. and M.E.N.: wrote the manuscript with support from A.I. A.I and E.M.: supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

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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EDITORIAL

Anticytokine (Ligand) Antibody Versus Antireceptor Antibody in Treating Rheumatoid Arthritis and Other Immune-Mediated Inflammatory Diseases

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Intracellular signaling through specific ligand-receptor interactions on the cellular membrane is essential for network communication among various immune cells in immune-mediated inflammatory diseases (IMIDs) [1]. Therapeutic agents extracellularly inhibit this signaling through biological agents and intracellular inhibition via synthetic compounds such as kinase inhibitors (Figure 1). Although the differences between biological agents and kinase inhibitors, as well as among targeted cytokines, have been widely discussed, comparisons between anticytokines (ligands) and antireceptor antibodies remain limited.

Interleukin-6 (IL-6) inhibition is an example of a distinction between these antibodies. The discovery and cDNA cloning of IL-6 and its receptor, as well as the signaling molecule glycoprotein 130 (gp130), have been pivotal for the successful management of various IMIDs through the inhibition of IL-6 signaling [2]. Tocilizumab, an anti-IL-6 receptor (IL-6R) antibody, was first developed in Japan and approved for the treatment of Castleman disease in 2005, followed by rheumatoid arthritis (RA) and juvenile idiopathic arthritis (both polyarticular and systemic) in 2008. Tocilizumab has been globally approved for various IMIDs, including giant cell arteritis and chimeric antigen receptor (CAR) T-cell-induced cytokine release syndrome [3].

The subsequent development of biological agents targeting IL-6R, such as sarilumab, and those targeting IL-6 ligands, including sirukumab, clazakizumab, and olokizumab, has provided valuable insights into the difference between anticytokine and antireceptor antibodies [3]. RA treatment with sarilumab has been successful in clinical trials [4–8], with acceptable safety profiles identified through postmarketing surveillance [9, 10]. However, the clinical development of biological agents targeting IL-6 ligands presents several challenges [3].

The most notable difference between anti-IL-6 and anti-IL-6R blockades lies in the efficiency of neutralizing the target functions within the selected dosing regimens. In our Japanese cohort, the median (interquartile range) serum IL-6 and IL-6R concentrations in treatment-naïve patients with RA were 4.70 (1.51–8.54) pg/mL and 84.2 (62.2–98.0) ng/mL, respectively [11]. Similarly, an RA study in Poland reported median (range) serum IL-6 and IL-6R concentrations of 34.1 (1.5–234.0) pg/mL and 45.366 (17.288–81.760) ng/mL, respectively [12]. Therefore, optimal dosing with limited dosing ranges is feasible for anti-IL-6R but not for anti-IL-6 ligands. For example, IL-6 receptor occupancy at steady-state trough levels was 98% or above with 200 mg of subcutaneous sarilumab every 2 weeks and 162 mg of tocilizumab weekly [13]. Another example is the failure of treatment in people with RA with extremely high serum tumor necrosis factor (TNF) concentrations before treatment with an anti-TNF monoclonal antibody (infliximab) [14].

Concerns regarding the development of antireceptor antibodies for the treatment of RA and other IMIDs include the potential activation of receptor signaling by the antibody, as observed in the development of the anti-CD28 antibody TGN1412 [15]. Therefore, the binding site of the antibody on the cytokine receptor should be carefully considered and tested before proceeding with clinical trials. Nevertheless, therapeutic antibodies

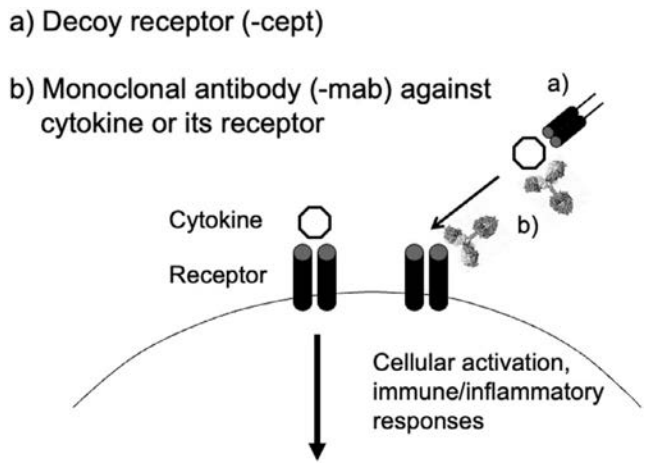

FIGURE 1 | Modes through which biological agents inhibit cytokine signaling. Decoy receptors (designated as -cept) or monoclonal antibodies (designated as -mab) targeting cytokines or their receptors have been available since the inception of anticytokine biological therapies.

targeting cytokine receptors may have some advantages over anticytokine antibodies because receptor expression levels are more stable than cytokine levels.

Author Contributions

H.K. wrote the manuscript draft and all other authors provided critical feedback and approved the final version of the manuscript.

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Conflicts of Interest

Hideto Kameda is an Editorial Board member of the International Journal of Rheumatic Diseases and a co-author of this article. To minimize bias, He was excluded from all editorial decision-making related to the acceptance of this article for publication. Hideto Kameda received consulting and/or speaker fees from AbbVie, Asahi Kasei, Bristol-Myers Squibb, Eisai, Eli Lilly, Janssen, Mitsubishi Tanabe, and UCB, and has received research/educational grants from Asahi Kasei, Pfizer, and Taisho. The other authors declare no conflicts of interest.

Data Availability Statement

The authors have nothing to report.

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ORIGINAL ARTICLE

Which Is the Best Option for AxSpA Patients After First TNFi Failure: Switch to Secukinumab or Cycling With Other TNFi?

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ABSTRACT

Objectives: Evaluation of drug survival and predictive factors of alternative tumor necrosis factor inhibitor (TNFi) and secukinumab (SEC) use after TNFi therapy in patients with axial spondyloarthritis (axSpA).

Methods: This was an observational retrospective study of axSpA patients who switched to second-line biological disease modifying antirheumatic drugs (bDMARD) between January 2018 and February 2023. Drug retention rate was evaluated using Kaplan–Meier analysis and log-rank test. Factors associated with drug survival of the second bDMARD were analyzed by multivariate regression analyses.

Results: A total of 201 axSpA patients (alternative TNFi: 143 patients, SEC: 58 patients) with a mean age 37.1 ± 8.6 years and consist of 58.2% male were included. Clinical characteristics and laboratory results at switching were comparable between groups. During a median follow-up period of 22.3 months, 80 (39.8%) of 201 patients discontinued bDMARD. Median follow-up period, drug discontinuation frequency, and drug retention rate were similar between groups. C-reactive protein (CRP) level (Hazard ratio (HR) = 0.98, 95% confidence interval (CI) = 0.95–0.99, $p = 0.032$) and primary failure (HR = 1.52, 95% CI = 1.03–2.27, $p = 0.037$) were significantly associated with the risk of TNFi discontinuation. Smoking (HR = 1.96, 95% CI = 1.05–3.69, $p = 0.036$) and Achilles enthesitis (HR = 1.93, 95% CI = 1.09–3.40, $p = 0.024$) were significantly associated with the risk of SEC discontinuation.

Conclusion: In axSpA exposed to a TNFi, switching to a second TNFi has comparable effectiveness to switching to SEC. SEC may be a better option in patients who experienced primary failure of TNFi, whereas an alternative TNFi may be preferred in patients with higher CRP levels or Achilles enthesitis, or who are currently smoking.

1 | Introduction

Axial spondyloarthritis (axSpA) is a chronic inflammatory rheumatological disease that predominantly involves the spine and sacroiliac joints, causing inflammatory low back pain and progressive spinal stiffness [1]. The aims of axSpA treatment are controlling symptoms and inflammation, prevention of progressive structural damage, preservation of functionality, and maximizing health-related quality of life [2]. Although different types of pharmacological treatments are currently available for the treatment of axial axSpA symptoms, non-steroidal anti-inflammatory drugs (NSAIDs) are still recommended as the first treatment option [3]. If the treatment target is not achieved after 4 weeks of receiving at least two different NSAIDs, biological disease modifying antirheumatic drugs (bDMARD) or janus kinase inhibitors (JAKi) should be considered as second-line therapy for purely axSpA [4]. Nowadays, recommended bDMARD for spondyloarthropathy

(SpA) are the tumor necrosis factor inhibitors (TNFi) and interleukin 17A inhibitor (IL-17Ai). Current practice is to start a TNFi or IL-17i due to the longer-term experience with the use of these drugs, a broader evidence base, and familiarity with their long-term safety and toxicity [4].

Tumor necrosis factor inhibitors have been in the market for more than 20 years. The 1-year survival rate of first TNFi drugs in patients with ankylosing spondylitis (AS) was reported as 77.5% in a Norwegian patients cohort and 88% in a Spanish cohort [5, 6]. Since the disease has a chronic nature and patients may need to remain on treatment for a long time, it is important for a therapeutic agent to have a high long-term drug survival rate [7]. In patients with SpA starting their first TNFi, drug survival was found to be 60% at 5 years and 49% at 10 years [7]. Data from real-world studies identified that lack of efficacy, loss of efficacy, and adverse events/poor tolerability are the most common reasons for discontinuation of first TNFi [8]. In the case of treatment failure of the first TNFi, switching to an alternative TNFi, interleukin 17A inhibitor (IL-17Ai) or JAKi should be considered [4]. A second TNFi can still be effective in patients with resistance to the first TNFi, but treatment efficacy may be slightly lower [9]. Deodhar et al. [8] reported that drug survival rates at 2 years were 58%–75% for first TNFi, 47%–72% for second TNFi, and 49% for third TNFi.

On the other hand, two IL-17Ai, secukinumab (SEC) and ixekizumab, are currently approved for the treatment of axSpA and have expanded the treatment options [4]. SEC, a fully human IgG1/κ monoclonal antibody which selectively binds to IL-17A, is the first IL-17 inhibitor [10]. According to the MEASURE-2 study, although clinical improvements observed at Week 16 were sustained or continued to improve throughout to 5 years of treatment in both TNFi-naive and TNFi exposed patients, response rates were better in TNFi naive patients [11]. Similarly, real-life data from 13 European registries reported that drug survival, remission rates and response rates were better for bio-naive patients, independent of time since diagnosis [12].

There is limited study comparing drug survival and clinical efficacy of TNFi and SEC in SpA patients who are TNFi non-responder patients with axSpA. Min et al. reported that alternative TNFi had better clinical efficacy and drug retention rates at 1 year in AS patients who experienced secondary failure of their first TNFi [13]. In another study, which included 78 patients with AS who switched to an alternative TNFi or SEC, reasons for discontinuation of the first TNFi and drug discontinuation rate were found to be similar between the two groups [14]. Also, the number of previously exposed bDMARD predicts drug survival in AS patients with prior TNFi exposure [15]. Therefore, physicians should pay more attention to the choice of their next biologic in AS patients with a history of first- or second-line biologic treatment failure. However, it is unclear which patients may benefit most from switching to another mode of action or cycling within the same mode of action after failure of an initial TNFi.

Drug survival is an important measure that indirectly reflects the efficacy and safety of treatment in inflammatory diseases [15]. This study was planned to evaluate drug survival of TNFi and SEC as a second line bDMARD in patients with axSpA. Also, we aimed to determine the subgroups of patients who may benefit from switching to another TNFi or an IL-17Ai in terms of drug survival after the first use of TNFi in patients diagnosed with axSpA.

2 | Methods

2.1 | Study Design and Sampling

The medical records of axSpA patients who were started on a second TNFi or SEC treatment after withdrawal of first TNFi at Rheumatology Department of Gülhane Training and Research Hospital between January 2018 and February 2023 were retrospectively examined. Participants aged ≥ 18 years, used TNFi as their first bDMARD, and met classification criteria for axSpA according to Assessment of SpondyloArthritis International Society (ASAS) were included [16]. Patients who underwent TNFi or SEC dose reduction or dose interval extension during routine follow-up, had two or more previous TNF exposures and had < 6 months follow-up or no routine follow-up were excluded from the study. Since the presence of uveitis, psoriasis or inflammatory bowel disease affects the choice of second-line bDMARDs, the patient group with these involvements was also excluded from the study.

Tumor necrosis factor inhibitors were initially initiated in all patients after inadequate response to NSAIDs, irrespective of using concomitant conventional synthetic DMARDs (csDMARDs). Primary failure was defined as cases where the desired improvement in Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) scores (BASDAI improvement ≥ 2 and BASDAI < 4) was not achieved at all after 12 weeks after the first use of TNFi. Secondary failure was defined as TNFi being effective (BASDAI improvement ≥ 2 and BASDAI < 4) in its initial use, but subsequently TNFi becoming ineffective (BASDAI ≥ 4 and BASDAI worsening ≥ 2). In patients with primary failure, secondary failure, or drug-related adverse events, alternative TNFi or SEC was used as a second step. The selection of the second bDMARD to be given to the patient is entirely decided by the patient's follow-up physician.

Patient files were reviewed retrospectively from the time of initiation of second-line bDMARD to the date of the last outpatient clinic follow-up. The following parameters were retracted from the patient files: age, gender, age at diagnosis, disease duration, family history of SpA, smoking status, body mass index, history of peripheral arthritis, presence of Achilles enthesitis, presence of dactylitis, presence of syndesmophytes on plain radiographs of the cervical or lumbar spine, number of tender joints, number of swollen joints, human leukocyte antigen-B27 (HLA-B27) positivity, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), BASDAI, Ankylosing Spondylitis Disease Activity Score-CRP (ASDAS-CRP), ASDAS-ESR, csDMARD use, NSAID use, type of first TNFi, and reason for discontinuing first TNFi (primary non-response, secondary non-response and drug side effect).

The protocol of this study and data collection forms were granted approval by Ethics Committee of Health Sciences University, Gülhane Training and Research Hospital within the framework

of the Declaration of Helsinki (Date: 31.08.2023 and Decision number: 2023/167).

2.2 | Statistical Analysis

The statistical analyses were conducted using version 28 of the Statistical Package for Social Sciences (Armonk, NY: IBM Corp) program for Windows. Visual (histograms and probability plots) and analytical (Kolmogorov–Smirnov and Shapiro–Wilk's test) methods were utilized to assess whether the variables were normally distributed. Normally distributed variables were expressed as mean ± standard deviation (SD), skewed variables as median (interquartile range-IQR), and categorical variables as number and percentage (%). Chi-squared test and Fisher's exact test (when the assumptions of the chi-square test were not valid) were used for categorical variables to compare demographic characteristics, clinical data, laboratory parameters, drug use history, and drug-related side effects in patients using SEC and TNFi as second bDMARD. Mann–Whitney *U* test was used for non-normally distributed continuous data, and independent *T* test was used for normally distributed continuous data between these two groups. Kaplan–Meier analysis was used to assess drug survival of alternative TNFi and SEC, and log-rank test was used for comparison.

Factors that were statistically significant in univariate analysis were included in multivariate analysis using the stepwise forward elimination method. Statistical significance was determined by accepting *p* values that were less than 0.05.

3 | Results

A total of 260 medical records of axSpA patients were retrospectively investigated for this study. We excluded 24 patients with two or more previous TNF exposure, 17 patients with uveitis, psoriasis or inflammatory bowel disease, 11 patients who did not receive regular treatment and 7 patients who were followed for less than 6 months. A total of 201 patients with a mean age 37.1 ± 8.6 years and consist of 58.2% male were included in the study, 143 patients were using TNFi, while remaining 58 patients using SEC as second-line treatment. According to the gender distribution, age at diagnosis and age and disease duration at starting second bDMARD were comparable between groups. Also, acute phase reactants levels and disease activity status assessed with BASDAI, ASDAR-ESR, and ASDAS-CRP were similar between groups. Adalimumab and etanercept, the most commonly used first-line treatments, were not found to be significant in both groups ($p < 0.944$, $p < 0.417$, respectively). When the discontinuation of first-line bDMARD was evaluated between the two groups, no difference was found between the groups in primary failure, secondary failure and side effects ($p < 0.198$, $p < 0.309$, $p < 0.846$, respectively). Clinical characteristics and laboratory results of the patients at the time of starting second bDMARD are listed in Table 1.

During a median follow-up period of 22.3 months, 80 (39.8%) of 201 patients discontinued second bDMARD in this patient

TABLE 1 | Baseline characteristics of axSpA patients starting an alternative TNFi or SEC after prior TNFi therapy.

Characteristics	TNFi ($n = 143$)	SEC ($n = 58$)	p
Male, *n* (%)	85 (59.4)	32 (55.2)	0.578
Age, years, mean (SD)	36.8 ± 9.1	37.9 ± 6.9	0.388
Age at diagnosis, years, mean (SD)	30.2 ± 7.9	32.6 ± 5.9	0.697
Disease duration, years, mean (SD)	4.4 ± 2.2	4.5 ± 2.5	0.130
Current smoker, *n* (%)	50 (35.0)	13 (22.4)	0.095
BMI, kg/m^2, median (IQR)	24.1 (2.2)	23.6 (2.1)	0.478
Entheseal involvement, *n* (%)	116 (81.1)	40 (69.0)	0.132
Peripheral arthritis, *n* (%)	33 (23.1)	16 (27.6)	0.587
HLA-B27 positivity, *n* (%)	91 (63.6)	44 (75.9)	0.101
Syndesmophyte, *n* (%)	71 (49.7)	24 (25.3)	0.287
ESR (mm/h), median (IQR)	19.0 (22.0)	22 (29.5)	0.555
CRP (mg/L), median (IQR)	9.0 (9.0)	9.0 (11.2)	0.872
BASDAI, median (IQR)	7.0 (1.6)	7.0 (2.0)	0.984
ASDAS-CRP, median (IQR)	3.2 (1.0)	3.2 (1.1)	0.810
ASDAS-ESR, median (IQR)	3.3 (1.2)	3.2 (1.2)	0.900
Current NSAID, *n* (%)	92 (64.3)	31 (53.4)	0.151
Current csDMARDs, *n* (%)	59 (41.3)	32 (55.2)	0.860
Type of the first TNFi, *n* (%)			
Adalimumab	55 (38.5)	22 (37.9)	0.944
Etanercept	32 (22.4)	10 (17.2)	0.417
Certolizumab	21 (14.7)	12 (20.7)	0.298
Golimumab	19 (13.3)	7 (12.1)	0.816
Infliximab	16 (11.2)	6 (10.3)	0.862
Discontinuation of the first-line bDMARDs, *n* (%)			
Primary failure	29 (20.2)	8 (28.6)	0.198
Secondary failure	101 (70.6)	35 (72.9)	0.309
Adverse events	13 (9.1)	4 (21.1)	0.846

(Continues)

TABLE 1 | (Continued)

Characteristics	TNFi ($n=143$)	SEC ($n=58$)	p
Type of the second TNFi, n (%)			
Adalimumab	21 (26.6)		
Etanercept	15 (20.5)		
Certolizumab	10 (14.7)		
Golimumab	7 (10.8)		
Infliximab	4 (6.5)		

Abbreviations: ASDAS, ankylosing spondylitis disease activity score; axSpA, axial spondyloarthropathy; BASDAI, bath ankylosing spondylitis disease activity index; bDMARD, biologic originator disease-modifying antirheumatic drugs; BMI, body mass index; CRP, c-reactive protein; csDMARDs, conventional disease-modifying antirheumatic drugs; ESR, erythrocyte sedimentation rate; IQR, inter-quartile range; NSAID, nonsteroidal anti-inflammatory drugs; SD, standard deviation; SEC, secukinumab; TNFi, tumor necrosis factor inhibitör.

cohort. Median follow-up period [21.4 (IQR: 20.9) months vs. 22.1 (IQR: 19.3) months, $p=0.267$] and drug discontinuation frequency [57 (39.9%) patients and 23 patients (39.7%), $p=0.979$] were similar between the second TNFi group and SEC group. The most common reason for second bDMARD discontinuation was secondary failure in both the second TNFi group and SEC group [33 (23.1%) patients vs. 15 (25.9%) patients, respectively; $p=0.545$], followed by primary failure [14 (9.8%) patients vs. 6 (10.3%) patients, respectively; $p=0.887$] and adverse events [10 (7%) patients vs. 2 (3.4%) patients, respectively; $p=0.316$].

There was no statistically significant difference between the two groups when drug survival was evaluated by Kaplan–Meier analysis ($p=0.205$) (Figure 1a). Since there was a numerical difference between the two groups during the follow-up period, the median observation period in the TNFi group, which was 21 months, was re-evaluated by Kaplan–Meier analysis ($p=0.972$) (Figure 1b).

For patients receiving a second TNFi, CRP levels at the start of the second TNFi (Hazard ratio (HR) = 0.96, 95% confidence interval (CI) = 0.92–0.99, $p=0.038$) and primary failure history of the first TNFi (HR = 2.14, 95% CI = 1.17–3.93, $p=0.014$) were found significant in the univariable regression analysis. When these covariates were included in the multivariable analysis and performed stepwise forward elimination model, CRP level at the start of the second TNFi (HR = 0.98, 95% CI = 0.95–0.99, $p=0.032$) and primary failure history (HR = 1.52, 95% CI = 1.03–2.27, $p=0.037$) were significantly associated with the risk of TNFi discontinuation. No statistically significant relationship was found with other factors in patients who discontinued TNFi as a second bDMARD (Table 2).

For patients received SEC as a second line bDMARD, according to the results of univariable regression analysis, current smoking (HR = 3.07, 95% CI = 1.29–7.30, $p=0.011$) and Achilles enthesitis (HR = 2.99, 95% CI = 1.17–7.68, $p=0.023$) were found to be risk factors for discontinuation of SEC. In the stepwise forward elimination model, smoking (HR = 1.96, 95% CI = 1.05–3.69, $p=0.036$) and Achilles enthesitis (HR = 1.93, 95% CI = 1.09–3.40, $p=0.024$) were significantly associated with the risk of SEC discontinuation. No statistically significant relationship was found with other factors in patients who discontinued SEC as a second bDMARD (Table 3).

4 | Discussion

In the absence of special extra-articular manifestations such as uveitis, psoriasis, or inflammatory bowel disease, it is a challenge to decide whether to switch to another TNFi or start IL-17Ai in axSpA patients who experienced treatment failure or an adverse event with the first anti-TNF. With this study, we reported that switching to an alternative TNFi was comparable to a biologics class change to an IL-17i (SEC) in terms of drug retention rate during a median follow-up period of 22.3 months. Also, we demonstrated that axSpA patients with higher CRP levels at the time of switching to a second-line bDMARD had a lower risk of discontinuing an alternative TNFi, but the presence of primary failure of the first TNFi increased the risk of discontinuing an alternative TNFi. In the group switching to SEC as the second bDMARD, the risk of drug cessation was higher in current smokers and in the presence of Achilles enthesitis. In cases of primary non-response, the efficacy of TNFi and secukinumab treatments as secondary treatments was found to be similar except in the noted cases noted.

Axial spondyloarthropathy has a significant impact on a patient's life and generates substantial societal and economic burden for health care system [17]. Thus, early diagnosis and suitable treatment are crucial for managing patients. bDMARD are recommended as second line treatment [4]. However, approximately 50% to 65% of patients do not achieve clinical response (defined as ASAS 40) after receiving a TNFi as first line bDMARD during 24 weeks [18]. Similarly, ASAS40 responder patient's ratio was found to be 43.2% for SEC at week 16 in TNFi-naive patients [11]. In the case of treatment failure or intolerance, drug switching is necessary. However, population-based studies indicate that the clinical response after switching to a second bDMARD (either a TNFi or anti-IL-17) is lower than the one experienced by patients naive to biologic therapies [8, 12].

In our study, 39.9% of patients in the second TNFi group and 39.7% of patients in the SEC group discontinued their second bDMARD during a median follow-up period of 22.3 months. When evaluated with Kaplan–Meier analysis, drug survival was found to be similar between groups ($p=0.205$). Similar drug discontinuation rates (alternative TNFi: 35.7% vs. SEC: 36.4%) were observed in a study conducted on AS patients who experienced treatment failure or adverse events with the first TNFi [14]. They reported that these two treatment strategies had comparable drug survival during a median follow-up period of 27.8 months [14]. But the number of patients in their study (TNFi: 56 patients and SEC: 22 patients) was too small to draw

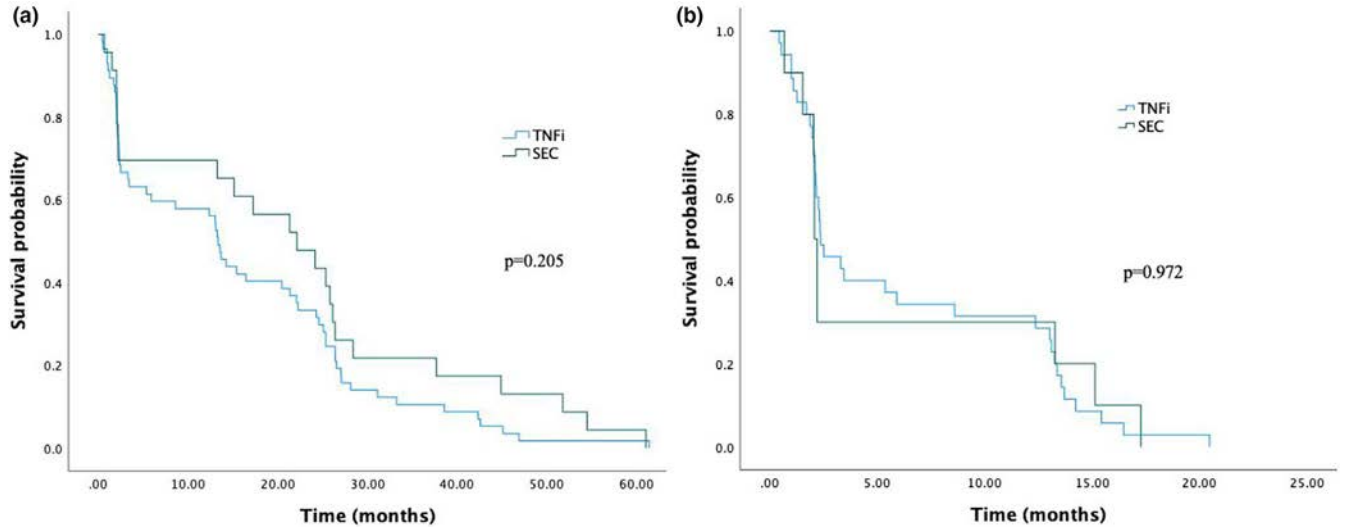

FIGURE 1 | Drug survival curve comparison between alternative TNFi and SEC. (a) Until the last follow-up date, (b) During 21-month follow-up.

a powerful conclusion. Similar to our result, an observational study conducted in a Swiss cohort reported that the drug retention rate and efficacy of SEC were comparable to an alternative TNFi in axSpA patients exposed to at least one TNFi [19]. The drug survival of SEC varies significantly across countries [12]. For this reason, obtaining similar results in studies conducted in different geographical areas increases the reliability of the information.

According to the results of our study, the reason for discontinuation of the first TNFi treatment in patients with axSpA could affect the drug survival of the second bDMARD. Although primary failure history was significantly associated with the risk of subsequent TNFi discontinuation (HR = 1.52, 95% CI = 1.03–2.27, $p = 0.037$), the same risk was not observed in SEC patients. Similarly, Kwon et al. reported that primary failure of the first TNFi significantly increased the risk of second TNFi discontinuation (adjusted HR: 5.20, 95% CI = 1.91–14.11, $p = 0.022$) [14]. Therefore, in the event of the primary failure of the first TNFi, SEC could be a better option over switching TNFi. On the other hand, Min et al. reported that SEC showed a higher HR for drug discontinuation (HR: 3.77, $p = 0.045$) in AS patients with a secondary failure of TNFi [13]. Due to the bDMARDs with different modes of action being limited, alternative TNFi may be a more suitable treatment for secondary failure rather than SEC.

According to the latest ASAS-EULAR recommendations for the management of axSpA, elevated CRP has been identified as the strongest predictor of good response to TNFi therapy in axSpA patients [4]. In parallel with these recommendations, we observed that higher CRP level at the start of the second TNFi significantly decreased the risk of TNFi discontinuation (HR = 0.98, 95% CI = 0.95–0.99, $p = 0.032$). Similar to our result, Deodhar et al. [8] reported that SEC provided rapid and sustained relief of symptoms of total spine pain, nocturnal back pain, and fatigue over 2 years in patients with active AS [8]. Elevated CRP is a poor prognostic factor in axSpA patients and is included in ASAS. AxSpA classification criteria as well as in the ASDAS [20]. However, elevated CRP is present in approximately 50% of patients with AS and 30% of non-radiographic (nr)-SpA [21]. Thus, CRP does not comprehensively reflect the disease activity in axSpA. Following the first TNFi failure, CRP level at the start of the second bDMARD could be a guide for the selection of the second bDMARD.

In our study, current smoking was found to be a risk factor for discontinuation of SEC (HR = 3.07, 95% CI = 1.29–7.30, $p = 0.011$), but not for alternative TNFi. In the literature, there is limited data about the effect smoking status on SEC response in axSpA patients. Chimenti et al. [22] reported that the reduction of ASDAS-CRP from baseline to 6th month was correlated with current smoking status in AS patients. Similar to our result, current smoking was found to be a risk factor for the cession of SEC in axSpA [14]. Data regarding the effect of smoking status on TNFi survival is conflicting [23, 24].

Lastly, Achilles enthesitis was significantly associated with the risk of SEC discontinuation (HR = 1.93, 95% CI = 1.09–3.40, $p = 0.024$). With the understanding of the central role of the IL-23–IL-17 axis in entheseal inflammation, IL-17i was considered a promising treatment option in SpA patients suffering from enthesitis. However, Schett et al. conducted a post hoc analysis of pooled data from randomized controlled Phase 3 studies of SEC on AS. They reported that although a statistically significant reduction in overall Maastricht AS Enthesitis Score (MASES) score was observed at Week 16 with an improvement trend continuing through Week 52, the results for the Achilles tendon did not meet statistical significance for SEC over placebo at Week 16 [25].

There are some limitations of this study. The main limitation of this study is its observational retrospective design without randomization. Thus, patient selection bias could not be completely eliminated. However, clinical characteristics, disease activity status, and laboratory results were balanced between treatment groups. Also, patients who started a subsequent TNFi after SEC approval in our country were included to eliminate potential bias due to treatment options in different calendar periods. Patients with extra-articular findings of axSpA were excluded from the study in an attempt to obtain better results. Although this approach has the advantage of reducing indication-related confusion, it may also have a disadvantage in that our findings

TABLE 2 | Regression analysis examining the factors that lead to the discontinuation of TNFi used as secondary treatment in axSpA patients.

	Univariable analysis			Multivariable analysis		
	HR	***p***	**95% CI**	**HR**	***p***	**95% CI**
Male, *n* (%)	1.64	0.083	0.94–2.88			
Age, years, mean (SD)	0.98	0.368	0.96–1.02			
Age at diagnosis, years, mean (SD)	0.97	0.052	0.94–1.00			
Disease duration, years, mean (SD)	0.92	0.197	0.79–1.05			
Current smoker, *n* (%)	0.96	0.895	0.56–1.66			
BMI, kg/m^2, median (IQR)	0.98	0.798	0.83–1.15			
Entheseal involvement, *n* (%)	1.07	0.889	0.41–2.79			
Peripheral arthritis, *n* (%)	1.63	0.127	0.88–2.67			
HLA-B27 positivity, *n* (%)	0.63	0.101	0.37–1.09			
Syndesmophyte, *n* (%)	1.20	0.503	0.71–2.02			
ESR (mm/h), median (IQR)	1.01	0.084	0.99–1.03			
CRP (mg/L), median (IQR)	0.96	0.038	0.92–0.99	0.98	0.032	0.95–0.99
BASDAI, median (IQR)	1.01	0.979	0.74–1.36			
ASDAS-CRP, median (IQR)	1.05	0.842	0.68–1.61			
ASDAS-ESR, median (IQR)	0.87	0.518	0.56–1.34			
Current NSAID, *n* (%)	1.43	0.236	0.79–2.58			
Current csDMARDs, *n* (%)	0.68	0.166	0.39–1.17			
Type of the first TNFi, *n* (%)						
Adalimumab	1.19	0.528	0.70–2.01			
Etanercept	0.84	0.605	0.43–1.62			
Certolizumab	0.32	1.415	0.71–2.81			
Golimumab	1.14	0.728	0.54–2.42			
Infliximab	0.50	0.144	0.20–1.27			
Discontinuation of the first-line bDMARDs, *n* (%)						
Primary failure	2.14	0.014	1.17–3.93	1.52	0.037	1.03–2.27
Secondary failure	0.60	0.071	0.35–1.05			
Adverse events	0.91	0.831	0.39–2.13			

Abbreviations: ASDAS, ankylosing spondylitis disease activity score; axSpA, axial spondyloarthropathy; BASDAI, bath ankylosing spondylitis disease activity index; bDMARD, biologic originator disease-modifying antirheumatic drugs; BMI, body mass index; CI, confidence interval; CRP, c-reactive protein; csDMARDs, conventional disease-modifying antirheumatic drugs; ESR, erythrocyte sedimentation rate; HR, hazard ratio; IQR, inter-quartile range; NSAID, nonsteroidal anti-inflammatory drugs; SD, standard deviation; SEC, secukinumab; TNFi, tumor necrosis factor inhibitör.

cannot be generalized to those with extra-articular symptoms. Another limitation is that only the presence of Achilles enthesitis was assessed, rather than using a standardized index (e.g., MASES) or imaging methods (e.g., ultrasonography).

In conclusion, in case of first TNFi failure in patients with axSpA, switching to a second TNFi may be as effective as switching to bDMARDs with different mechanisms of action. In the event of TNFi failure in patients with axSpA, switching to another TNF or to another biologics class is one of the dilemmas faced by physicians. Since increased exposure to the number of bDMARDs may decrease drug survival of subsequent bDMARDs, determining the risk factors for drug discontinuation is important. Although there was a limited number of patients to make a definitive judgment, SEC may be a better option in patients who experienced primary failure of TNFi based on our results, whereas an alternative TNFi may be preferred in patients with higher CRP levels or Achilles enthesitis or currently smoking.

TABLE 3 | Regression analysis examining the factors that lead to discontinuation of SEC used as a secondary treatment in axSpA patients.

	Univariable analysis			Multivariable analysis		
	HR	***p***	**95% CI**	**HR**	***p***	**95% CI**
Male, *n* (%)	2.42	0.055	0.98–5.97			
Age, years, mean (SD)	1.02	0.422	0.96–1.08			
Age at diagnosis, years, mean (SD)	0.95	0.099	0.91–1.01			
Disease duration, years, mean (SD)	1.15	0.249	0.93–1.34			
Current smoker, *n* (%)	3.07	0.011	1.29–7.30	1.96	0.036	1.05–3.69
BMI, kg/m^2, median (IQR)	1.01	0.955	0.75–1.46			
Entheseal involvement, *n* (%)	2.99	0.023	1.17–7.68	1.93	0.024	1.09–3.40
Peripheral arthritis, *n* (%)	0.49	0.206	1.16–1.48			
HLA-B27 positivity, *n* (%)	0.70	0.496	0.25–1.97			
Syndesmophyte, *n* (%)	1.77	0.193	0.75–4.15			
ESR (mm/h), median (IQR)	1.01	0.313	0.99–1.03			
CRP (mg/L), median (IQR)	0.99	0.945	0.96–1.04			
BASDAI, median (IQR)	0.93	0.728	0.65–1.36			
ASDAS-CRP, median (IQR)	0.83	0.535	0.45–1.15			
ASDAS-ESR, median (IQR)	0.77	0.413	0.41–1.44			
Current NSAID, *n* (%)	0.49	0.118	0.21–1.19			
Current csDMARDs, *n* (%)	1.33	0.525	0.56–3.16			
Type of the first TNFi, *n* (%)						
Adalimumab	1.99	0.125	0.83–4.18			
Etanercept	0.45	0.206	0.13–1.55			
Certolizumab	0.52	0.286	0.15–1.74			
Golimumab	0.56	0.568	0.07–4.19			
Infliximab	N/A	N/A	N/A			
Discontinuation of the first-line bDMARDs, *n* (%)						
Primary failure	1.65	0.331	0.60–4.54			
Secondary failure	0.56	0.187	0.23–1.33			
Adverse events	2.37	0.188	0.65–8.56			

Abbreviations: ASDAS, ankylosing spondylitis disease activity score; axSpA, axial spondyloarthropathy; BASDAI, bath ankylosing spondylitis disease activity index; bDMARD, biologic originator disease-modifying antirheumatic drugs; BMI, body mass index; CI, confidence interval; CRP, c-reactive protein; csDMARDs, conventional disease-modifying antirheumatic drugs; ESR, erythrocyte sedimentation rate; HR, hazard ratio; IQR, inter-quartile range; NSAID, nonsteroidal anti-inflammatory drugs; SD, standard deviation; SEC, secukinumab; TNFi, tumor necrosis factor inhibitör.

Currently, there is no randomized controlled trial comparing the effectiveness of second TNFi and IL-17i in axSpA patients failing a first TNFi. Further contributions to the literature can be made through existing prospective randomized controlled studies with long-term follow-up.

Author Contributions

Mehmet Nur Kaya: conceptualization, data curation, methodology, writing – original draft. **Özlem Kılıç:** data curation, formal analysis, methodology, validation. **Duygu Tecer:** conceptualization, funding acquisition, writing – review and editing. **Sedat Yılmaz:** conceptualization, funding acquisition, writing – review and editing.

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The authors have nothing to report.

Ethics Statement

The protocol of this study and data collection forms were granted approval by the Ethics Committee of Health Sciences University, Gülhane Training and Research Hospital within the framework of the Declaration of Helsinki (Date: 31.08.2023 and Decision number: 2023/167).

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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LETTER TO THE EDITOR

Higher Frequency of CD8+GMCSF+ T-Cells Co-Producing TNFα+ and Lower Frequency of Cells Co-Producing IFNγ+ Is Associated With Good Response to MTX

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Dear Editor,

Methotrexate (MTX) is the first-line gold-standard therapy for rheumatoid arthritis (RA) with a good efficacy/toxicity ratio [1]. It also serves as an anchor drug in combination with biological disease modifying anti-rheumatic drugs (DMARDs) [2, 3]. Two-thirds of patients do not achieve a good response with MTX monotherapy [4, 5], and require the addition of other DMARDs, which leads to a delay in the institution of effective therapy and leads to damage accrual.

Predictors for non-response to MTX may offer a solution to this issue and lead to the institution of effective therapy ab initio. Although many predictors for non-response to MTX have been found, like high baseline disease activity, worse disability scores, longer disease duration, rheumatoid factor positivity, and anti-citrullinated peptide antibodies (ACPA) [6], these are not specific to MTX but are markers of poor response in general (all therapies) and define a worse disease subset [7].

Surprisingly, there has been little work in T-cell subsets classified by production of cytokines as biomarkers for MTX response, considering that they may be key to RA pathogenesis and may be important in determining response to MTX [8]. Specifically, GMCSF-producing T-cells have recently emerged as an important subset that has been found to have a role in RA pathogenesis [9, 10]. Recently, our group demonstrated high frequencies of GMCSF-producing CD8 (and CD4 cells) in the synovial fluid of RA patients [11]. In addition, we found a reduction in their frequencies (in peripheral blood) with MTX treatment. Thus, we wanted to evaluate the ability of GMCSF-producing T-cell subsets to predict response to methotrexate (MTX).

This single-center pilot-study enrolled patients with active RA (SJC28 $\geq$ 2 and TJC28 $\geq$ 4), 18 to 60 years of age, with disease duration $<$ 5 years who were not currently on DMARDs. However, they could be on low-dose prednisolone or hydroxychloroquine. Patients were started on MTX at 15 mg/week, escalated to 25 mg/week by 4–8 weeks, and reassessed at 16 weeks. At 16 weeks, patients were divided into good responders and non-good responders based on the EULAR response criteria into those with good response (DAS28 decline $\geq$ 1.2 and final DAS28 $\leq$ 3.2) versus those without good response (moderate or none) [12].

At enrolment and follow-up (16-weeks), 5 mL blood was obtained using venepuncture, and peripheral blood mononuclear cells (PBMCs) were isolated using density-gradient centrifugation. 1×10^6 cells/mL PBMCs were suspended in 1 mL complete media (RMPI-1640) and stimulated with phorbol-12-myristate-13-acetate (PMA) (50 ng/mL) and ionomycin (1 μg/mL) (both from Sigma, USA) for 4 h at 37°C in the presence of GolgiPlug (BrefeldinA) (BD Biosciences).

After washing, cells were surface stained by incubating with fluorochrome-tagged antibodies against CD3 and CD8 for

20 min at 4°C in the dark. Cells were washed with 1× phosphate buffered saline (PBS) and fixed for 5 min in the dark (4.2% paraformaldehyde), washed again, followed by permeabilization in 500 μL of buffer for 30 min (Perm/Wash, BD Biosciences). Cells were re-suspended in 50–100 μL perm/wash buffer (containing saponin) and stained with fluorochrome-labeled antibodies against intracellular cytokines GMCSF, TNFα and IFNγ for 20 min at 4°C in the dark.

Acquisition was done on BD LSRFortessa flow cytometer and FACS Diva 8.2.1 software was used for analysis and representation of flow cytometric data. Briefly, we first gated the PBMCs, followed by gating of CD3+CD8+ and CD3+CD8−, and then gated for GMCSF+. These were further opened on a bivariate plot of TNFα and IFNγ. Thus, we determined frequencies of CD8+GMCSF+ and its further subsets, that is, CD8+GMCSF+TNFα+IFNγ−, CD8+GMCSF+TNFα+IFNγ+, CD8+GMCSF+TNFα-IFNγ+, CD8+GMCSF+TNFα−IFNγ−.

Comparison of categorical variables between good and non-good responders was done using chi-square test (or fishers exact test). Comparison of continuous variables (including frequencies of T-cell cytokine subsets) was done using Mann–Whitney *U* test. Univariate logistic regression was performed with frequencies of T-cell subsets as predictor variables and good response as the dependent variable. A two-tailed p value < 0.05 was used for defining statistical significance. However, for regression, we used $p < 0.1$ (10% significance level).

This study included 38 patients (females: male 9:1), with a mean age of 42 (±11) years and a mean DAS28-ESR of 6.3 (0.8). At 16 weeks, 7 (18.4%) were EULAR good responders, whereas 31 (79.5%) were non-good responders. On comparing the baseline demographic and disease activity measures between these two groups, disease duration (1.2 ± 0.8; 2.4 ± 1.5, $p = 0.012$), DAS28 (4.9 ± 1; 6 ± 0.9, $p = 0.006$) and CRP (11.5 ± 11.3; 29.2 ± 21.1, $p = 0.007$) were significantly lower in the good responder group compared to non-good responders.

Upon flowcytometric analysis, there was a trend to higher CD8+GMCSF+ ($p = 0.084$) and CD4+GMCSF+ ($p = 0.095$) T-cells in good responders. Among their subsets, CD8+GMCSF+TNFα+IFNγ− was significantly higher in responders, whereas CD8+GMCSF+TNFα-IFNγ+ and CD8+GMCSF+TNFα−IFNγ− were significantly lower in responders at baseline (Table 1).

On univariable logistic regression, we found lower DAS28-CRP to be a significant predictor of achieving a good EULAR response, whereas disease duration, CD8+GMCSF+ and CD8+GMCSF+TNFα+IFNγ− showed a trend ($p < 0.10$). On multivariable analysis, only DAS28CRP and CD8+GMCSF+ continued to show a trend to significance (Table 2).

This small pilot study found that CD8 and CD4 T-cells producing GMCSF showed a trend to be higher in patients with good response. In addition, CD8+GMCSF+TNFα+IFN− T-cells were significantly higher in patients with good response, whereas CD8+GMCSF+TNFα−IFN+ were significantly lower in patients with good response.

Although no previous studies exist on this T-cell subset, we chose to focus on it for many reasons. GMCSF seems to be important for RA pathogenesis; it leads to activation of monocytes

TABLE 1 | Flowcytometric subsets of T-cells and polyfunctional T-cells at baseline.

	Good responder, $n = 7$	Non-good responder, $n = 31$	p-value
CD8 T-cells (% of CD3+)	20.7 (±6.25)	20.9 (±7.9)	0.939
CD8 GMCSF (% of CD3+CD8+)	5.6 (±2.6)	4.0 (±2.1)	0.084
CD8 GMCSF (% of CD3+CD8+GMCSF+)			
CD8GMCSF+TNFα+IFNγ−	21.2 (±16.7)	11.8 (±9.1)	0.045*
CD8GMCSF+TNFα+IFNγ+	56.6 (±30.3)	43.5 (±28.4)	0.281
CD8GMCSF+TNFα−IFNγ+	6.3 (±5.7)	14.8 (±15.5)	0.023*
CD8 GMCSF+TNFα−IFNγ−	12 (±10.8)	28.2 (±24)	0.013*
CD4 T-cells (% of CD3+)	51.3 (±5.2)	53.3 (±9.7)	0.462
CD4 GMCSF (% of CD3+CD8−)	7.2 (±5)	4.8 (±2.9)	0.095
CD4 GMCSF (% of CD3+CD8−GMCSF+)			
CD4 GMCSF+TNFα+IFNγ−	32.3 (±9.2)	30.2 (±18.5)	0.773
CD4 GMCSF+TNFα+IFNγ+	48.7 (±24.1)	32.6 (21.3)	0.088
CD4 GMCSF+TNFα−IFNγ+	2.2 (±1.7)	6.2 (±9)	0.029*
CD4 GMCSF+TNFα−IFNγ−	16.7 (±16.1)	28.2 (±25.5)	0.158

Note: All values are represented as mean ± SD.
Abbreviations: CD4, Helper T-cells; CD8, Cytotoxic T-cells; GMCSF, Granulocyte macrophage colony stimulating factor; IFNγ, Interferon Gamma; TNF, Tumor necrosis factor Alpha.
*Significant p-value ($p < 0.05$).

TABLE 2 | Logistic regression for analyzing predictor variables of the flow cytometric-based T cell subsets.

	Univariable		Multivariable	
Predictor variables	**Odds ratio (Exp(B))**	***p***	**Odds ratio (Exp(B))**	***p*-value**
Disease duration	0.4	0.074	0.4	0.147
DAS28-CRP	0.3	0.019	0.2	0.067
CD8+GMCSF+	1.4	0.095	1.6	0.098
CD8+GMCSF+TNFα+IFNγ−	1.1	0.078	1.0	0.694
CD8+GMCSF+TNFα+ IFNγ+	1.0	0.278	—	
CD8+GMCSF+TNFα−IFNγ+	0.9	0.194	—	
CD8+GMCSF+TNFα−IFNγ−	0.9	0.116	—	

Note: All predictor variables with $p < 0.10$ were put into multivariable analysis (all entered together).
Abbreviations: CD8, Cytotoxic T-cells; CRP, C reactive protein; DAS, Disease activity score; GMCSF, Granulocyte macrophage colony stimulating factor; IFNγ, Interferon Gamma; TNFα, Tumor necrosis factor Alpha.

and macrophages, increases expression of MHCII, CD86, and CD40, and polarizes macrophages into M1 type, which secrete pro-inflammatory cytokines and drive inflammation [10, 13, 14]. GMCSF$^{-/-}$mice are resistant to development of CIA [15]. T-cells secreting GMCSF are present in high frequencies in the synovial fluid of RA and reduce after MTX [11]. Thus, it may be related to RA pathogenesis and mechanism/marker of MTX action.

Also, we looked at polyfunctional subsets of T-cells producing GMCSF, specifically those coproducing TNFα and IFNγ. Although this subset was initially found to be important in infectious diseases [16, 17] they have also been shown to be important in the pathogenesis of autoimmune diseases like RA [18, 19]. We have earlier shown these polyfunctional T-cells producing GMCSF to be increased in RA synovial fluid [11]. Others have shown increased frequencies in psoriatic arthritis [20].

Previous studies on using flow cytometry to enumerate cellular subsets as predictors for MTX response are limited. A recent study which looked at 427 immune phenotypes by flow cytometry did not find anyone to predict MTX response [21]. Another study found higher CD3+CD39+% (and MFI) in MTX responders [22]. A prior study on monocytes found CD14+^{high}CD16− or CD14+^{high}CD16+cells to be predictive [23].

The major limitation of our study is that it is a pilot study with a small sample size and limited data, which makes it a ground, suggestive for future research but does not allow us to draw conclusive findings. Furthermore, on logistic regression, baseline demographics and disease activity remained more significant predictors of response, rather than flowcytometric subsets.

In conclusion, this small study found a higher frequency of GMCSF producing T-cells (trend-to significance) and CD8+GMCSF+TNFα+ T-cells at baseline in patients with a good response to methotrexate.

Author Contributions

V.D. and A.K. contributed to the conception and design of the study. V.D., A.K., B.S., A.K.Y., B.L., C.B.P., S.J., G.S.R.S.N.K.N., S.K.S., A.S., and S.J. contributed to critical inputs in the design and acquisition of data. V.D., A.K., and B.L. analyzed and interpreted the data. V.D. and A.K. drafted the first manuscript, and all the other authors have done a critical review of the content and approved the final version submitted. All authors take full responsibility for the integrity and accuracy of all aspects of the work.

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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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EDITORIAL

Osteoarthritis, What and What Not to Treat

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1 | Background

Osteoarthritis (OA) is the most common form of arthritis, and with the aging of the world population, its significance in terms of health expenditure and quality of life is increasing [1]. Although cartilage destruction is a main pathology of OA, synovial inflammation, subchondral bone sclerosis, osteophyte formation, and changes in periarticular muscles also play important roles [2]. Despite decades of intensive research, the treatment of OA has been unsatisfactory, and not a single disease-modifying OA drug (DMOAD) has been approved, creating a huge unmet need in modern medicine. With this dismal situation, we have to raise questions about whether our current understanding and approaches toward OA are appropriate.

2 | OA as an Evolutionary Maladaptation

To begin with, the fundamental question is what the distinction between disease and the natural process of aging is for OA. This is a question that has been raised for a long time. Although many researchers simply regard OA as a disease, the distinction is not always clear. The average life expectancy has risen from 47 to 73 years of age in the seven decades spanning 1950–2020 [3]. This rate of increase in life expectancy is unprecedented, considering that the same amount of lifespan expansion occurred spanning thousands of years from the Bronze age to the beginning of the 20th century [4].

Among all the human organs affected by the detrimental effect of aging, human knee joints bear the brunt of yet another evolutionary burden, bipedal walking, which separates hominids from the rest of the four-legged mammals. Quadrupedal laboratory rodents rarely develop OA, even in obese animals; however, obligatory bipedal exercise leads to pathologic changes compatible with human OA cartilage degeneration [5, 6]. This leads us to speculate that OA may be an evolutionary maladaptation; human knee joints may be poorly equipped to sustain such a massive increase in mechanical load occurring during such an abruptly prolonged life span.

Evolutionary maladaptation is reflected in the gap between lifespan, that is, the total life lived, and health span, that is, the period free from disease, which is currently estimated at around 9 years [7]. OA may be a strong driver for increasing this gap considering the strong influence of age on its prevalence. The World Health Organization (WHO) guiding principle of achieving health as "a state of complete physical, mental and social well-being" is not possible through technological advances only [7]. In that regard, OA treatment might better focus on how to protect natural joints for elongated life span by creation of favorable work condition and lifestyle factors.

3 | Cartilage Repair as a Holy Grail for OA Treatment?

The second question is whether we are aiming at the right target to alleviate patient suffering. The poor correlation between structural, especially cartilage, damage and patient symptoms is well known. Knee pain due to OA is a key symptom influencing the decision to seek medical attention and a better predictor of disability than radiographic changes [8]. On the other hand, radiographic OA changes are weakly correlated with pain and physical function, and a significant number of subjects with Kellgren-Lawrence grade 4 OA do not report pain [9]. The current therapeutic pipeline for OA, however, focuses on the restoration of damaged cartilage. This tendency became noticeable since the first report of successful autologous chondrocyte transplantation to repair deep cartilage defects in 1994 [10]. Although that study included mostly young patients without OA, and the structural outcome after

10 years is rarely available, the concept of restoring damaged cartilage as the 'holy grail' for OA treatment has settled. Not only the technical difficulty of regeneration of damaged cartilage, a tissue with a very low cell proliferation rate and no blood supply, but also the problem of whether it is a proper target for OA treatment is an important issue. A recent meta-analysis of 50 clinical studies and 13 systematic reviews/meta-analyses revealed that stem cell therapy for knee OA relieved pain in patients over time but did not improve knee function [11]. However, the clinical studies included were fraught with limited evidence regarding study objectives, test designs, patient populations, as well as risk of bias assessment, outcome description, and potential conflicts of interest [11]. Long-term preservation of cartilage has not been reported, either [11].

Lorecivivint is a novel OA drug that targets bispecific tyrosine phosphorylation-regulated kinases and cdc2-like kinases, altering the Wnt pathway, which is a key regulator of maintaining normal tissue function. The Phase IIa trial showed that lorecivivint slowed joint space narrowing, a biomarker of OA disease progression, while consistently improving patient pain over a period of more than 1 year [12]. Although this may be the first OA drug effective for both structure and pain, phase 3 study results are needed to confirm these results and to verify whether these benefits can also be seen in the long term.

4 | First Do No Harm

The last question is if we are not doing harm to our patients. In this era of rapid technological advancement, we sometimes lose the context of the clinical situation and are bewildered in the plethora of information that may be of little significance to patients. For instance, we tend to pay attention only to findings revealed by imaging, the most prominent example of which is the case of meniscal damage. Time and again, meniscal tear/degeneration was observed very frequently among the elderly subjects, and this was not associated with knee pain in OA subjects [13]. It is, thus, of no surprise that a few high-quality randomized controlled trials of arthroscopic meniscectomy (AM) for degenerative meniscal tear did not prove its efficacy [13]. However, the number of knee arthroscopic surgeries has grown rapidly along with the introduction of MRI, which reveals a high prevalence of meniscal tears in OA, and there is a possibility that surgery is performed to resect meniscal lesions that may not be the cause of symptoms. Arthroscopic knee surgery remains the most common type of knee surgery, and the number of AM increased by 12.67% in 8 years in Korea, with the incidence of meniscus surgeries per 100 000 population-year in Korea almost 10-fold higher compared to the United States [14]. In addition to the lack of clinical efficacy, some recent studies raise additional concerns that AM itself may be detrimental to the maintenance of the joint structure. In a 5-year follow-up study from the Meniscal Tear in Osteoarthritis Research (MeTeOR) trial comparing physical therapy vs. AM, 7.1% of individuals randomized to AM underwent total knee replacement (TKR) over 5 years, with a hazard ratio for TKR of 2.0 compared to those randomized to PT [15]. In a Korean study using administrative data, the TKR rate in the AM group was 25% higher than that in the non-AM group 10 years after the surgery [16].

It is not only unnecessary surgical procedures but also ineffective medication that causes harm to patients. We are all aware of the small effect size and adverse effects of long-term use of pharmaceutics for OA. In addition to gastrointestinal and renal side effects, OA patients are not immune to the recent epidemics of opioid addiction. A recent survey study in the US showed that 12.8% of OA patients were prescribed an opioid-based medication, and decreases in traditional opioid prescriptions have been countered by an increase in tramadol prescription [17]. Although tramadol, a weak opioid agonist, has been considered safer than other opioid medications with a lower risk of physical dependence, recent evidence tells a different story. Among patients aged 50 years and older with OA, the initial prescription of tramadol was associated with a significantly higher rate of mortality over 1 year of follow-up compared with commonly prescribed nonsteroidal anti-inflammatory drugs [18]. Atypical withdrawal symptoms after abrupt tramadol discontinuation have been reported, as well [19]. As of February 2023, the Food and Drug Administration (FDA) Center for Drug Evaluation and Research announced safety-related labeling changes to tramadol highlighting the risk of addiction, abuse, or misuse [19].

The origin of OA pain is diverse and differs from patient to patient. In addition to nociceptive pain arising from direct tissue damage, neuroplastic pain also plays a major role [20]. The reason for the unsatisfactory efficacy of pain medication for OA, thus, stems from the fact that treatments such as NSAIDs and

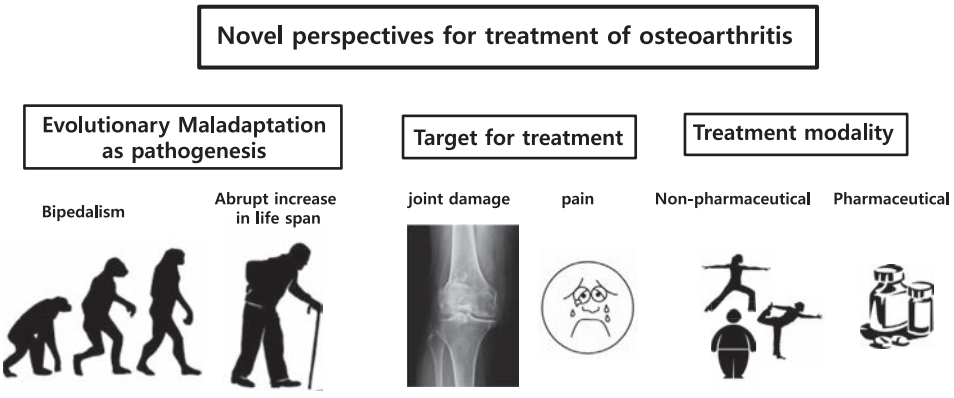

FIGURE 1 | Perspective for the treatment of osteoarthritis.

intra-articular steroids are not effective for non-nociceptive pain. On the other hand, the positive aspect of pain should also be considered. Despite the promising effect of alleviating pain, tanezumab, a monoclonal antibody that inhibits nerve growth factor, was not approved for use by the FDA because of joint safety events such as rapidly progressive OA [21]. This result is a reminder that, like all types of pain, OA pain may have a beneficial role in protecting the joint from further damage. An animal study using the destabilization of the medial meniscus model showed that pain behavior was delayed by 10 weeks relative to the histologic evidence of tissue damage [22]. In evolutionary terms, local inhibition of pain would allow the animal to continue normal life despite mild tissue damage and would impart an advantage for survival, while after moderate damage has occurred, pain would protect the animal from further damage to the joint.

5 | Conclusion

In conclusion, there is more confusion than magic about current OA treatment. In musculoskeletal health care, overdiagnosis is widespread and leads to unnecessary tests and treatments that do not benefit patients, and OA is no exception [23]. While continued research is essential for the proper management of OA, comprehensive understanding and perspective for its pathogenesis are needed to avoid hype and bring about hope (Figure 1).

Author Contributions

Hyun Ah Kim contemplated the subject and wrote the manuscript.

Conflicts of Interest

The author declares no conflicts of interest.

Data Availability Statement

The author have nothing to report.

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EDITORIAL

Editorial: Advancing the Management of Noninfectious Uveitis Through Molecular Targeted Drugs

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Uveitis, characterized by inflammation of the uveal structures, can be broadly categorized into infectious and noninfectious etiological subtypes. It has an incidence ranging from 10.5 to 52 cases per 100,000 person-years and is estimated to account for up to 5% of legal blindness cases globally. Therefore, uveitis is a critical cause of blindness around the world. Its classification into infectious and noninfectious etiologies necessitates different therapeutic strategies. The management of noninfectious uveitis, a significant subset, has been primarily reliant on anti-inflammatory agents. Given the substantial visual complications that can be associated with such an intraocular inflammatory disease, its management, along with any novel treatment approaches, is a worthwhile topic of discussion. Based on previous guidelines released by organizations such as the American College of Rheumatology and Arthritis Foundation, treatment for uveitis typically follows a stepwise, escalatory approach, starting with topical steroids and NSAIDs, advancing to systemic steroids, immunomodulatory therapy (IMT), and ultimately considering biologic agents usage for corticosteroid sparing and disease control. Steroid-induced glaucoma is another reason for using steroid-sparing therapy like IMT or biologics in the earlier stage of noninfectious uveitis. This editorial briefly reviews such agents and their role in managing noninfectious uveitis.

1 | Biologics Therapy

Biologics therapy often refers to a class of pharmaceutical drugs that are either manufactured or extracted in some way from a biological source. In other words, it is a form of IMT that acts on the immune response of the user to control inflammation. Given that uveitis is a condition characterized by intraocular inflammation and macular edema (ME), biologics therapy has been shown to have an effect on such a condition. It should be noted that such agents are usually reserved for more severe forms of uveitis, particularly after the failure of first-line treatments such as long-term use of moderate-to-large doses of corticosteroids. We have summarized the current literature in Table 1.

2 | Tumor Necrosis Factor (TNF) Inhibitors

Among biologic agents, the class of tumor necrosis factor (TNF) inhibitors has gained significant attention as a treatment of autoimmune disease [1, 2] (Figure 1). Currently, the TNF-α inhibitors approved by the United States of America (USA) Food and Drug Administration (FDA) and the European Union (EU) for rheumatologic conditions include infliximab, adalimumab, golimumab, and certolizumab. Notably, adalimumab is the only TNF inhibitor approved by

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TABLE 1 | Summary of biologics therapy for uveitis.

Biologics therapy	Overview	Study	RCT evidence	Study detail
Tumor Necrosis Factor (TNF) inhibitors				
Adalimumab	The only TNF inhibitor approved by the FDA for noninfectious uveitis	Mackensen, 2018	Yes	$N=25$, superiority over controls in BCVA (best-corrected visual acuity) including anterior uveitis
		Ramanan, 2019	Yes	$N=90$, JIA-associated uveitis, combination with methotrexate
		Jaffe 2016, Visual I (main)	Yes	$N=217$, active, non-infectious uveitis, lowered the risk of uveitic flare
		Nguyen, 2016, Visual II (main)	Yes	$N=265$, inactive, non-infectious uveitis, lowered the risk of uveitic flare
		Suhler, 2018, Visual III (main)	Yes	$N=364$, inactive, non-infectious uveitis
		Goto, 2018, Visual I (Japan)	Yes	$N=16$, active uveitis, Japan
		Goto, 2018, Visual II (Japan)	Yes	$N=32$, inactive uveitis, Japan
Infliximab	Demonstrated efficacy in some patients	None RCT		Four placebo-controlled trials for spondyloarthritis-associated acute anterior uveitis
Certolizumab	Chimeric monoclonal antibody, demonstrated efficacy in some patients	None RCT		
Golimumab	Human monoclonal antibody, demonstrated efficacy in some patients	None RCT		
Etanercept	Might increase the risk of drug-induced uveitis	Foster(2003)	Yes	$N=20$, no significant efficacy over placebo in preventing relapses of uveitis
		Smith (2005)	Yes	$N=5$, no significant efficacy over placebo in JIA uveitis
Anti-cytokine agents				
Anakinra	Anti-IL1, Demonstrated efficacy in some patients	None RCT		
Daclizumab	Anti-IL2, Demonstrated efficacy in some patients	Buggage (2007)	Yes	$N=17$, patients with Behçet's disease involving the posterior segment in eyes
		Nussenblatt (2003)	Yes	$N=10$, patients with non-infectious, intermediate or posterior uveitis

(Continues)

TABLE 1 | (Continued)

Biologics therapy	Overview	Study	RCT evidence	Study detail
Ustekinumab	Anti-IL23, Demonstrated efficacy in some patients	None RCT		
Sarilumab	Anti-IL6, Demonstrated efficacy in some patients	Heissigerová (2019)	Yes	$N=58$, active, non-infectious uveitis
Tocilizumab	Anti-IL6, Demonstrated efficacy in some patients	Sepah (2017) Hassan (2022)	Yes	$N=37$, active, non-infectious uveitis $N=37$, active, non-infectious uveitis
Secukinumab	Anti-IL-17A, Demonstrated efficacy in some patients	Dick (2013) Letko (2015)	Yes	$N=118$, patients with Behçet's uveitis $N=37$, active, non-infectious uveitis
Janus kinase inhibitors				
Tofacitinib	Preliminary evidence is effective	None RCT		
Baricitinib	Preliminary evidence is effective	None RCT		
Upadacitinib	Preliminary evidence is effective	None RCT		
Other biological agents				
Rituximab	Anti-CD20, targets B cells, demonstrated efficacy in some patients	Davatchi (2010)	Yes	$N=20$, patients with Behcet's disease
Abatacept	Blocking the co-stimulatory signal (CD80/CD86) required for T-cell activation, playing a potential therapeutic role	None RCT		

Abbreviation: RCT, randomized controlled trial.

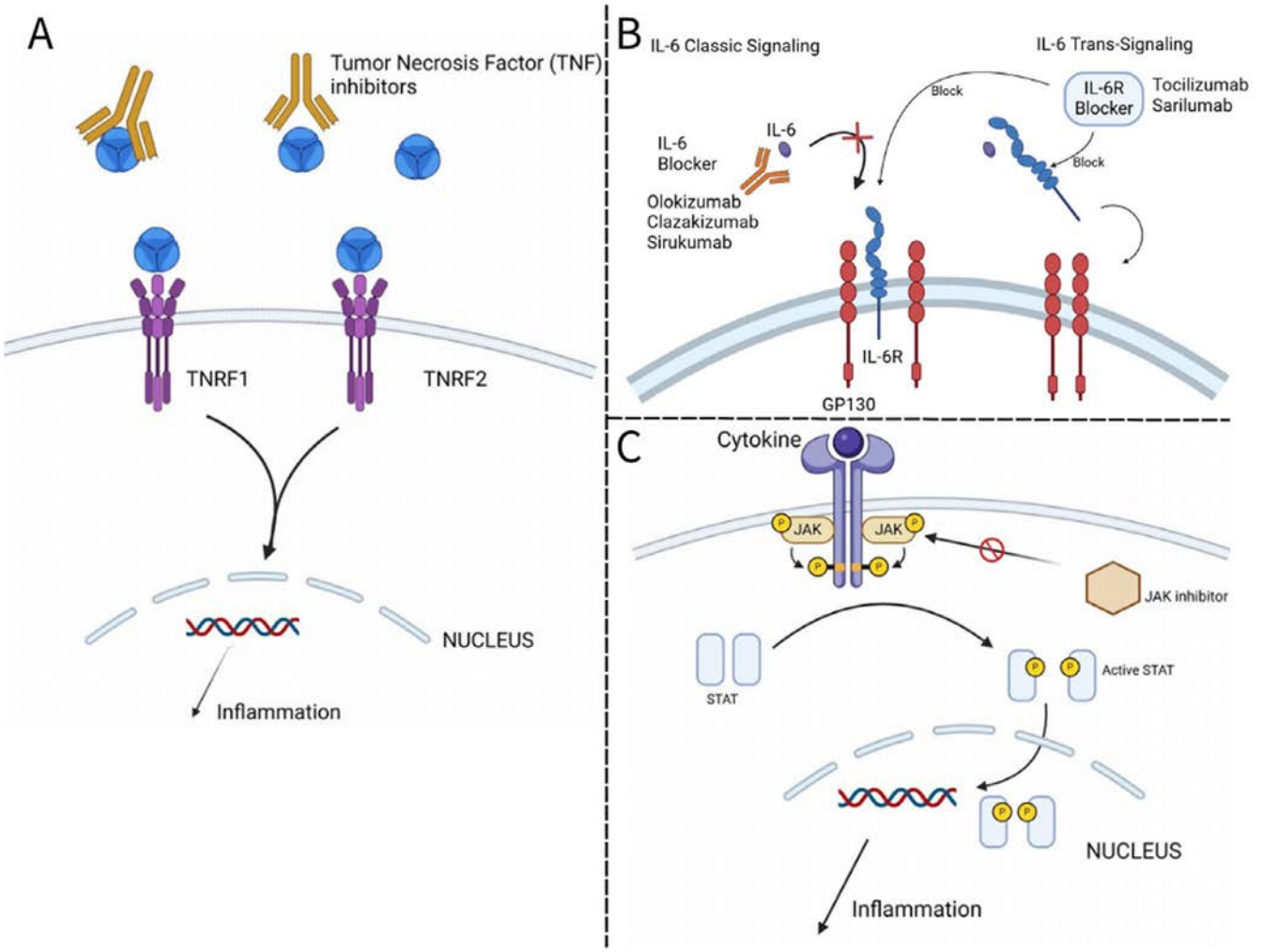

FIGURE 1 | Inhibition of TNF-α, IL-6 pathway and JAK–STAT pathways. This figure illustrates the inhibition of TNF-α signaling through TNF inhibitors (A), inhibition of the IL-6 pathway through IL-6 inhibitors or IL-6 receptor inhibitors (B), and the suppression of cytokine signaling via JAK inhibitors (C).

the FDA for noninfectious uveitis. Various studies have shown the efficacy of infliximab [3], adalimumab [4], golimumab [5], and certolizumab [6] in the application of noninfectious uveitis with concomitant rheumatologic conditions. However, not all drugs from the TNF inhibitor class effectively treat uveitis. Notably, etanercept, a TNF-α inhibitor, might increase the risk of drug-induced uveitis. One study by Schmeling et al. found that, among patients with noninfectious uveitis, those treated with etanercept were at a higher risk of uveitis flares despite the treatment [7].

3 | Anti-Cytokine

Other biologics such as anti-cytokine agents: the anti-interleukin-1 (anti-IL-1) class (e.g., anakinra) [8], anti-IL-2 (e.g., daclizumab) [9], and anti-IL-23 (e.g., ustekinumab) [10]. All have been shown to be effective in managing uveitis.

In recent years, IL-6 inhibitors have played an increasingly prominent role. The IL-6 pathway is divided into the classical signaling pathway and trans-signaling. The former involves binding to the cell surface IL-6 receptor (IL-6R), while the latter binds to the soluble IL-6 receptor (sIL-6R), which subsequently interacts with gp130, activating the JAK pathway and triggering inflammatory responses (Figure 1). The anti-IL-6 mechanisms are further classified into targeting IL-6 itself or the IL-6 receptor. Among these, blocking the IL-6 receptor, such as with tocilizumab and sarilumab, remains the most extensively discussed approach [11], particularly in patients with severe intraocular inflammation unresponsive to other biologic therapies. Although the U.S. Food and Drug Administration (FDA) has not officially approved tocilizumab or other IL-6 antagonists, such as sarilumab, for the treatment of uveitis, recent studies have suggested that targeting IL-6 may offer therapeutic benefits for refractory cases of the disease. Preclinical studies have indicated that competitive inhibition of IL-6 may decrease IL-7 signal transduction, leading to reduced activity of immune mediators associated with B and T cells and, consequently, decreased inflammation. In a multicenter clinical trial, Ramanan et al. reported that 7 of 21 patients with juvenile idiopathic arthritis (JIA)-associated uveitis responded to tocilizumab [12]. Similarly, Uludag et al. found tocilizumab to be effective in treating noninfectious uveitis in patients unresponsive to other immunomodulatory therapies (IMT) [13]. A multicenter clinical trial by Sepah

et al. further demonstrated the clinical efficacy of intravenous tocilizumab in 37 patients with noninfectious uveitis, with significant resolution of ME and a mean change in central macular thickness (CMT) of $-83.88 \pm 136.1\,\mu m$ at the final 6-month follow-up, along with improvements in BCVA [14]. Another IL-6 inhibitor, sarilumab, has also shown promise in clinical trials. Heissigerová et al. reported that sarilumab led to reductions in CMT and improvements in BCVA in patients with noninfectious uveitis at the final follow-up [15].

Another notable class of biologics is the anti-CD20 agents, which include rituximab. Studies have also demonstrated the efficacy of rituximab in managing uveitis [16]. Additionally, selective costimulator modulators, including abatacept, are another category of biologics that have been shown to play a potential therapeutic role in treating uveitis in the past. Besides, interferons have also been utilized successfully in managing uveitis, with one study reporting a positive clinical response in 83% of patients with noninfectious uveitis following treatment with infliximab. This study further noted improvements in mean best-corrected visual acuity (BCVA) and the resolution of macular edema (ME) in all participants [17].

Current evidence indicates that many forms of uveitis associated with autoinflammatory and autoimmune conditions may share a common molecular and immunopathogenic basis, particularly involving the activation of the IL-23/IL-17 signaling pathway, which appears to be a key biological event. Several biologic agents targeting this pathway have been developed, including monoclonal antibodies that specifically inhibit key mediators such as the IL-23 p40 subunit, p19 subunit, IL-17A, IL-17F, and IL-17RA.

Brodalumab, the first monoclonal antibody to block IL-17RA, may offer broader immunomodulatory effects compared to agents targeting only IL-17A, as it can also inhibit signaling from other IL-17 cytokine family members. This agent has been approved by the FDA for first-line use in psoriasis. However, no clinical studies have yet investigated the use of brodalumab in the treatment of uveitis.

Ustekinumab, a monoclonal antibody targeting the p40 subunit of IL-23, has demonstrated efficacy and safety in pivotal clinical trials and is now approved as a first-line biologic therapy for psoriasis and psoriatic arthritis. Similarly, briakinumab, which also targets the p40 subunit, showed efficacy in treating moderate to severe plaque psoriasis and Crohn's disease; however, its approval has been withheld due to concerns over increased risks of malignancy and major adverse cardiovascular events. Biologic agents targeting the p19 subunit, such as guselkumab, tildrakizumab, and risankizumab, have shown favorable efficacy and safety profiles in patients with moderate to severe chronic psoriasis. Monoclonal antibodies directed against IL-17A, such as ixekizumab and secukinumab, have also demonstrated substantial therapeutic benefits and good tolerability in the management of plaque psoriasis and psoriatic arthritis. However, secukinumab was the only biologic agent that showed efficacy in non-infectious uveitis beneath randomized controlled trial evidence.

Importantly, head-to-head comparative studies between anti-IL-23/IL-17 biologics and conventional immunosuppressants or TNF inhibitors in the treatment of uveitis are still lacking.

4 | Janus Kinase Inhibitors

Janus kinase inhibitors (JAKIs) represent another biologic therapy in autoimmune disease of interest and are often compared with TNF inhibitor [18]. These agents function by inhibiting JAK kinase activity, thereby reducing inflammation [19] (Figure 1). While the application of JAKIs in treating noninfectious uveitis is still in its early stages, preliminary evidence is promising. For instance, a network meta-analysis by Bechman et al. involving six trials found that JAKIs were associated with lower rates of noninfectious uveitis occurrence in patients with autoimmune diseases (IRR 0.32, 95% CI 0.106–1.67) compared to controls. Despite these promising findings, further studies are necessary to evaluate the efficacy of JAKIs across different subtypes of noninfectious uveitis [20].

5 | Conclusion

Despite the expanding body of evidence supporting the potential role of biologics in managing noninfectious uveitis, several critical concerns remain. A significant issue is that many studies on biologics involve patient populations with multiple autoimmune diseases, such as rheumatoid arthritis or JIA. Currently, noninfectious uveitis encompasses a wide range of underlying diseases, such as juvenile idiopathic arthritis, spondyloarthritis, Behçet's disease, and sarcoidosis. However, as shown in Table 1, most of the existing randomized controlled trials have not clearly distinguished between these subtypes and have treated noninfectious uveitis as a single entity. When examining individual case reports, TNF-α inhibitors are the most commonly used agents for the treatment of sarcoidosis-related uveitis. In contrast, studies on JIA- and Behçet's disease-associated uveitis have involved a broader range of biologic agents. At present, the therapeutic approaches for noninfectious uveitis arising from different underlying diseases are largely similar. Nonetheless, it is important for future studies to consider analyzing the different subtypes of noninfectious uveitis separately. The majority of studies on biologics for noninfectious uveitis are constrained by short follow-up periods and small sample sizes, resulting in a lack of long-term safety data. Additionally, the high cost of biologics presents a significant barrier to their widespread clinical use.

In conclusion, while current studies underscore the proven efficacy of TNF-α inhibitors, other biologics, including IL-6 inhibitors and JAKIs, may also hold potential in the clinical management of noninfectious uveitis. Nonetheless, addressing unresolved issues such as the need for long-term studies involving larger patient populations remains imperative to elucidate the safety and efficacy of these novel therapies fully.

Author Contributions

Hou-Ting Kuo, Alan Y. Hsu: conceptualization; methodology; writing – original draft. Chun-Ju Lin, Min-Yen Hsu: supervision; writing – review and editing. Hsin Tseng, Bing-Qi Wu, Ning-Yi Hsia: investigation; 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 from the corresponding author upon reasonable request.

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EDITORIAL

The 2024 Australian National Osteoporosis Guidelines in Postmenopausal Women and Men Aged Over 50 Years: A Comparative Perspective Within the Asia–Pacific Region

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1 | Introduction

Osteoporosis is a major public challenge in the Asia–Pacific region where it affects ≤30% of women aged ≥40 years and ≤10% of men in developed economies [1]. Although many national guidelines within the Asia–Pacific region offer tailored strategies, differences in screening, risk assessment and treatment thresholds highlight the need for stronger, evidence-driven strategies to optimize patient outcomes and the importance of regional dialogue and knowledge exchange. This editorial explores Australia's recently released Osteoporosis Guidelines in the context of other Asia–Pacific recommendations, highlighting key similarities and differences in screening, risk assessment and treatment (Table 1).

The Royal Australian College of General Practitioners and Healthy Bones Australia published national guidelines for osteoporosis management in postmenopausal women and men aged ≥50 years in 2024 [2, 3]. Representative Asia–Pacific guidelines published in English chosen were the 2024 Osteoporosis Society of Hong Kong (HK) Guideline for Clinical Management of Postmenopausal Osteoporosis [5], the 2024 Guidelines for Osteoporosis—Korean Society of Menopause [4], the 2021 Indian Society for Bone and Mineral Research Position Statement for the Diagnosis and Treatment of Osteoporosis in Adults [6], the 2021 Clinical Standards for Fracture Liaison Services in New Zealand (NZ) [9] with the 2017 Guidance on the Diagnosis and Management of Osteoporosis in NZ [7], the 2018 Singaporean Appropriate Care Guide for Osteoporosis Identification and Management in Primary Care [8], and the 2021 Summary of the Thai Osteoporosis Foundation Clinical Practice Guidelines on the Diagnosis and Management of Osteoporosis [10]. (Of note, the Chinese guidelines were published in Mandarin and so not included in this Editorial [11].) Like the Australian guideline, the Thai document targeted postmenopausal women and men aged ≥50 years [10]. There was some variability in target population of the other guidelines, for example, the HK and Korean guidelines were directed as postmenopausal women [4, 5] and the Singaporean guideline targeted postmenopausal women and men aged ≥65 years [8]. Despite this, the principles discussed in the various guidelines were similar. It is not our intention to identify which guideline might be "better" as each document was written by local experts cognisant of the limitations and vagaries of their particular national healthcare system, funding envelope and at-risk population. There are obviously significant differences in national healthcare resources. This is particularly highlighted in the Indian guideline which was drafted specifically for a resource-constrained healthcare environment with a large at-risk population [6].

2 | Who to Assess for Osteoporosis

Although some countries have adopted age-based thresholds for bone health assessment, others prioritize risk-based approaches using the Fracture Risk Assessment Tool (FRAX) or other algorithms. This reflects the need for adaptable strategies that align with local healthcare priorities and funding. Australian guidelines recommend referral for bone density assessment by dual energy x-ray absorptiometry (DXA) in everyone ≥50 years

TABLE 1 | Asia–Pacific Osteoporosis Guideline Summary.

Country (alphabetically) of origin/ publication year/reference	Australia (2024) [2]	Hong Kong (2024) [3]	India (2021) [4]	New Zealand (2021, 2017) [5, 6]	Singapore (2018) [7]	South Korea (2024) [5]	Thailand (2021) [8]
Who to assess for osteoporosis	i. Risk factor + MOF risk ≥ 10%, or ii. MTF	i. Men ≥ 70 years. ii. Women ≥ 65 years. iii. Risk factor iv. MTF	i. Men ≥ 65 years. ii. Women ≥ 60 years. iii. Risk factor iv. MTF	i. Risk factor ii. MTF	Step 1: assess risk by OSTA for postmenopausal women and men ≥ 65 years Step2: High risk: DXA Medium risk: DXA if ≥ 1 risk factors Low risk: Defer DXA	i. Women ≥ 65 years ii. Risk factor	i. Men ≥ 70 years ii. Women ≥ 65 years iii. Risk factor iv. MTF
Assessment of absolute fracture risk	FRAX	FRAX COSA HKOS	FRAX	FRAX Garvan Fracture Risk Calculator	FRAX	FRAX	FRAX

(Continues)

TABLE 1 | (Continued)

Country (alphabetically) of origin/ publication year/reference	Australia (2024) [2]	Hong Kong (2024) [3]	India (2021) [4]	New Zealand (2021, 2017) [5, 6]	Singapore (2018) [7]	South Korea (2024) [5]	Thailand (2021) [8]
Definition of very high fracture risk	T-score ≤ −3.0 + fracture within 2 years, and/or i. ≥ 2 fragility fractures, and/or ii. risk factors, and/or iii. MOF risk ≥ 30%, or hip fracture risk ≥ 4.5%	i. Multiple fractures, or ii. Recent MOF ≤ 24 months, or iii. T-score ≤ −3.0, or iv. Fracture on antiresorptive therapy	Recent MOF ≤ 24 months	No specific definition	No specific definition	No specific definition	i. fragility hip or vertebral fracture ≤ 12 months in ≥ 65 years and T-score of ≤ −2.5, or ii. recurrent vertebral fracture or vertebral fractures at ≥ 2 levels with moderate-to-severe deformity, or iii. bilateral hip fractures, hip and vertebral fractures, or fractures ≥ 3 times/at ≥ 3 sites iv. T-score < −3.5 in men ≥ 70 years or women ≥ 65 years, or v. fragility fracture after ≥ 2 years of therapy

(Continues)

TABLE 1 | (Continued)

Country (alphabetically) of origin/ publication year/reference	Australia (2024) [2]	Hong Kong (2024) [3]	India (2021) [4]	New Zealand (2021, 2017) [5, 6]	Singapore (2018) [7]	South Korea (2024) [5]	Thailand (2021) [8]
Indications for treatment	i. Very high fracture risk, or ii. minimal trauma hip or vertebral fracture, or iii. MTF at other sites and a T-score ≤ − 1.5, or iv. T-score ≤ −2.5, or v. T-score between −1.5 and − 2.5 and MOF risk ≥ 20%, or hip fracture risk ≥ 3%	i. Very high fracture risk ii. age ≥ 65 + T-score ≤ −2.5 iii. prior fracture > 24 months ago iv. MOF risk ≥ 20%, or hip fracture risk ≥ 3%	i. MTF ii. age ≥ 50 + T-score ≤ −2.5, or iii. diabetic + T score ≤ −2.0, or iv. T-score between −1.5 and − 2.5 and MOF risk ≥ 10.5% or hip fracture risk ≥ 3.5%	i. hip fracture risk ≥ 3% ii. T-score ≤ − 2.5	i. MTF ii. T-score ≤ −2.0 iii. T-score between −1.0 and − 2.5 and high fracture risk	MOF risk ≥ 20% or hip fracture risk ≥ 3%	i. Very high fracture risk, or ii. T-score ≤ −2.5, or iii. T-score between −1.0 and − 2.5 and hip fracture risk ≥ 3%, or iv. T-score between −1.0 and − 2.5 and MTF of proximal humerus, pelvis, or distal forearm
Role of osteoanabolic therapy	ROM/TP for i. very high fracture risk, or ii. fracture on therapy	ROM/TP for i. very high fracture risk ii. fracture on therapy	TP for i. vertebral fractures ii. T-score ≤ −3.5 without fracture	Fracture on therapy	No specific recommendation	No specific recommendation	ROM/TP for i. very high fracture risk ii. inadequate response to anti-resorptives
Recommendations for exercise	Weight-bearing Resistance	Weight-bearing Resistance Balance Tai-chi	No specific recommendation	Weight-bearing	Weight-bearing Resistance Balance Tai-chi	Weight-bearing Resistance Balance Tai-chi	No specific recommendation
Fracture Liaison Service/ Osteoporosis Refracture Program	Recommendation in favor	Recommendation in favor	No specific recommendation	Refer MTF to FLS	No specific recommendation	No specific recommendation	No specific recommendation

Abbreviations: COSA, Chinese Osteoporosis Screening Algorithm; FLS, Fracture Liaison Service; FRAX, Fracture Risk Assessment Tool; HKOS, Hong Kong Osteoporosis Study score; MOF, major osteoporotic fracture; MTF, minimal trauma fracture; ROM, romosozumab; TP, teriparatide.

of age with (i) a minimal trauma fracture (MTF), or (ii) in those without a fracture, who have risk factors for osteoporosis and a FRAX risk for major osteoporotic fracture (MOF) ≥ 10% [2]. None of the three large population-based randomized controlled trials (RCTs) of screening in women for prevention of osteoporotic fractures showed a reduction in the primary outcome of all fractures [12–14]. However, a significant reduction in hip fracture was found in a meta-analysis of these trials ($n > 42\,000$ in total), with an absolute risk reduction of 0.47% over 5 years of treatment [15]. Due to inadequately defined Australian thresholds of absolute fracture risk and lack of data on screening in men, insufficient evidence was available to support a population-based-screening program in Australia [2].

This is mirrored in NZ where the presence of fragility fractures or osteoporosis risk factors directs case finding [7]. However, in HK [5], India [6], Korea [4], and Thailand, screening is based on age and risk factors. Ages for screening are men ≥ 70 years and women ≥ 65 years in HK [5] and Thailand [10], men ≥ 65 years and women ≥ 60 years in India [6], and women ≥ 65 years in Korea [4]. Singaporean guidelines suggest use of the Osteoporosis Self-Assessment Tool for Asians to inform the need for DXA assessment [8].

3 | Assessment of Absolute Fracture Risk

All the above guidelines recommend the use of FRAX (https://fraxplus.org) for absolute fracture risk assessment. However, in HK, a local alternative, such as the Chinese Osteoporosis Screening Algorithm (COSA) [5] may be appropriate, and NZ guidelines suggest either FRAX or the Garvan Fracture Risk Calculator (https://www.garvan.org.au/research/bone-fracture-risk-calculator) [7]. The Australian document suggests the latter may be of particular use in those at high falls risk, as this is not an input criterion for the current FRAX model [2]. Ongoing local validation studies are required to further refine the predictive value of fracture risk assessment tools.

4 | Definition of Very High Fracture Risk

Identification of patients at "very high" or "high" fracture risk is an evolving important concept allowing risk stratification for earlier access to bone anabolic agents. However, there is currently no internationally recognized definition for this. The 2024 Australian guidelines define very high fracture risk as a T-score ≤ −3.0 and fracture within 2 years, and/or (i) history of ≥ 2 fragility fractures, and/or (ii) presence of clinical risk factors, such as corticosteroid use, low body mass index (BMI), or recurrent falls, and/or (iii) a MOF FRAX risk of ≥ 30%, or hip fracture risk of ≥ 4.5% [2].

The HK guideline is broadly similar, substituting FRAX risk criteria for fractures sustained on anti-resorptive therapy [5]. The Thai guidelines define very high fracture risk as either (i) fragility hip or vertebral fracture within 12 months in individuals ≥ 65 years and T-score ≤ −2.5, or (ii) recurrent vertebral fracture or vertebral fractures at ≥ 2 levels with moderate-to-severe deformity, or (iii) bilateral hip fractures, hip and vertebral fractures, or multiple fractures (≥ 3 times, or ≥ 3 sites), (iv) T-score < −3.5 in men ≥ 70 years or women ≥ 65 years, or (v) fragility fracture after ≥ 2 years of bone protective therapy [10]. The Indian guidelines identify a fracture within 2 years as a marker for very high fracture risk [6] while the NZ, Singaporean and Korean guidelines do not specify a definition [4, 7, 8].

As this is an evolving concept, it is not surprising that heterogeneity in definition exists between countries—especially as there are funding differences for access to bone anabolic agents.

5 | Indications for Treatment

Although treatment thresholds are generally aligned across the Asia–Pacific Region, with the usual caveats about individualized clinical judgment, therapy initiation is guided by fracture risk and healthcare system factors. The Australian guidelines recommend initiation of bone protective pharmacological therapy in individuals with (i) very high fracture risk, or (ii) minimal trauma hip or vertebral fracture, or (iii) MTF at other sites and a T-score of ≤ −1.5 [2]. It further recommends treatment in individuals with risk factors, but no fracture with either a T-score of ≤ −2.5, or a T-score between −1.5 and-2.5 and a FRAX MOF risk of ≥ 20%, or hip fracture risk of ≥ 3%. The Indian guidelines note that these FRAX thresholds may underestimate fracture risk in Indians, and studies are underway to better inform local clinical practice [6]. Thai guidelines recommend treatment in the presence of a fragility vertebral or hip fracture, T-score of ≤ −2.5 or a T-score between −1.0 and −2.5 and a FRAX hip fracture risk of ≥ 3%, but also a T-score between −1.0 and −2.5 and a fragility fracture of the proximal humerus, pelvis, or distal forearm [10].

6 | Role of Osteoanabolic Therapy

Access to osteoanabolic therapy is influenced by national healthcare policies and funding. Recent expansion of romosozumab access in Australia is a positive step and similar discussions are occurring in other parts of the Asia–Pacific Region. The Australian guidelines recommend consideration of bone anabolic agents (romosozumab or teriparatide) for those at very high fracture risk (see definition above). Until recently, this was only subsidized under the national Pharmaceutical Benefits Scheme for second-line therapy following fracture while on anti-resorptive therapy with a T-score ≤ −3.0 [2]. However, as of November 2024, romosozumab is available "first-line" for those with a T-score ≤ −2.5 with a symptomatic MTF and either ≥ 1 hip or symptomatic vertebral fracture in the previous 24 months, or a history of ≥ 2 fractures, including 1 symptomatic new fracture in the previous 24 months [16].

Early bone anabolic agent use for those at very high fracture risk is recommended in the HK and Thai guidelines [5, 10]. The Indian guidelines recommend teriparatide as first-line therapy in individuals with either vertebral fractures or very high fracture risk and intravenous zoledronic acid in hip fracture patients—preferably prior to hospital discharge [6]. The NZ guidelines recommend bisphosphonates as first-line therapy—although these were drafted prior to widespread use of bone anabolic therapy [7]. The Korean guidelines make no specific recommendation on the choice of bone protective therapy while

the Singaporean guidelines make no demarcation between first and second-line therapies [4, 8].

7 | Role of Exercise

Although exercise is widely recognized as a key component of osteoporosis management, widespread implementation and adherence to exercise regimes remain problematic. The Australian guidelines discuss the role of exercise for increasing or maintaining bone mass, for promotion of balance and falls prevention, and for fracture reduction [2]. Moderate-to-high-impact weight-bearing activities (jumping, hopping) and progressive resistance (strength) training are most effective for increasing or maintaining BMD, while there is minimal evidence of benefit for low-intensity resistance or low-impact weight-bearing aerobic exercises, such as walking and cycling [17, 18]. The role of the healthcare professional in adopting an encouraging approach to exercise is highlighted. The HK and Singaporean guidelines discuss falls prevention and the benefit of Tai-chi in balance training [5, 8], while the Korean guidelines discuss the importance of exercise on bone health, including exercises to avoid [4]. New Zealand guidelines mention 30 min of weight-bearing exercise per day [7], while the Thai and Indian guidelines do not specifically explore the role of exercise in bone protection [6, 10].

8 | Fracture Liaison Service/Osteoporosis Refracture Program

A Fracture Liaison Service (FLS) identifies and coordinates care for patients with a fragility fracture to reduce the risk of a subsequent fracture and is the most effective and cost-effective system-wide model of care to prevent refracture [19]. While there is widespread agreement regarding its importance, there are many barriers to widespread implementation—especially cost. Even in a relatively wealthy country like Australia, only New South Wales has a system-wide osteoporosis refracture program built around FLSs [2]. There has been widespread acceptance of this model of care in HK and NZ [4, 9]. Although FLSs are present in India [20], Thailand [21], and Singapore [22], there are no specific recommendations for widespread implementation in the relevant guidelines [4, 6, 8, 10]. Collaboration between countries may help optimize FLS models for broader adoption throughout the region.

9 | Conclusion

The Asia–Pacific Region encompasses culturally and ethnically diverse populations with variations in healthcare resources, leading to heterogeneity in osteoporosis guidelines. A major reason for differences in recommendations is variation in the cost of osteoporosis medications and variable coverage of investigation and treatment costs by national healthcare systems. Australia, with a large migrant population, is a microcosm of the region at large, and dialogue, collaboration, and knowledge exchange with Asia–Pacific partners is important to produce guidelines representative of this diversity. The Asia–Pacific Consortium on Osteoporosis Framework was developed to establish clinical standards for osteoporosis management across the Region and is an important step toward harmonizing best practice [23]. As many countries refine osteoporosis management strategies, aligning national guidelines with evidence-based regional frameworks will drive more consistent and equitable osteoporosis care across the Asia–Pacific Region.

Author Contributions

The initial draft was written by A.L. and P.K.K.W. All authors contributed to manuscript review.

Conflicts of Interest

Peter K. K. Wong is Honorary Medical Director and Board Member, Healthy Bones Australia, and an Associate Editor of the *Journal*. Manju Chandran has previously received travel grants and honoraria from Amgen Asia and Promedius AI solutions for speaking and chairing. Alwin Lian declares no relevant conflicts of interest.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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CLINICAL IMAGE

Digital Gangrene as an Inaugural Presentation of Childhood Lupus

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Keywords: gangrene | SLE | vasculitis

1 | Riddle

An 11-year-old premorbidly normal girl presented with pain and burning sensation in her left foot for 15 days, associated with bluish discoloration in the same foot for 3 days. Her examination was unremarkable except for bluish discoloration of her left foot's great toe, second, and third toes. Additionally, two bluish patches were noted on the lateral aspect of the left lower leg and foot (Figure 1A).

2 | Answer

The possibilities of SLE vasculitis, polyarteritis nodosa, cryoglobulinemia, and sepsis-induced gangrene were considered for this acute onset gangrene. Laboratory investigations revealed pancytopenia (Hb: 10.5/mm^3, TC: 1800/mm^3, Platelet count: 94000/mm^3) and elevated inflammatory markers (ESR: 30 mm/h; CRP: 62.4 mg/L). Peripheral smear and urine microscopy were normal. No cryoglobulins could be demonstrated in blood, and her blood cultures were sterile. USG Doppler done showed decreased perfusion in the distal anterior tibial artery and dorsalis pedis artery. Further evaluation revealed positive ANA, anti-dsDNA (784 IU), lupus anticoagulant, and anti-β2 glycoprotein supporting the diagnosis of SLE [1]. Skin biopsy from the margin of the lesion over the ankle showed leukocytoclastic vasculitis with neutrophils and lymphocytes involving small to medium vessels (Figure 1B,C).

She was managed with systemic steroids (IV methylprednisolone followed by oral prednisolone), hydroxychloroquine, and mycophenolate mofetil. Subcutaneous enoxaparin and aspirin were also given in view of APLA positivity and possible thrombus. Severe pain, which did not resolve on oral analgesics, required fentanyl infusion and spinal sympatholysis. On follow-up, she is doing fine with no residual gangrene, and the involved toes have autoamputated.

Digital gangrene is a common skin manifestation of various vasculitis. SLE presenting with isolated digital gangrene is rare and is seen in only 0.3% of cases [2]. The usual incidence of digital gangrene in SLE is 1.3%, occurring at the late stages of the disease [3]. The underlying pathological mechanism in SLE-associated digital gangrene could include premature atherosclerosis, hypercoagulability, vasculitis, vasospasm, and in this case, the presence of APLA antibodies might have also contributed. The key message of this report is to emphasize that gangrene can be an inaugural presentation of lupus. Gangrene is often considered infectious in developing world settings. This assumption may delay the definite diagnosis and treatment of lupus [4].

Author Contributions

Dr. Abin Sheref Mohammed prepared the manuscript. Dr. Abin Sheref Mohammed, Dr. Shivprasad Mohankumar, and Dr. Narendra Bagri managed the patient. Dr. Sudheer Kumar Arava and Dr. Shivangi Dagar

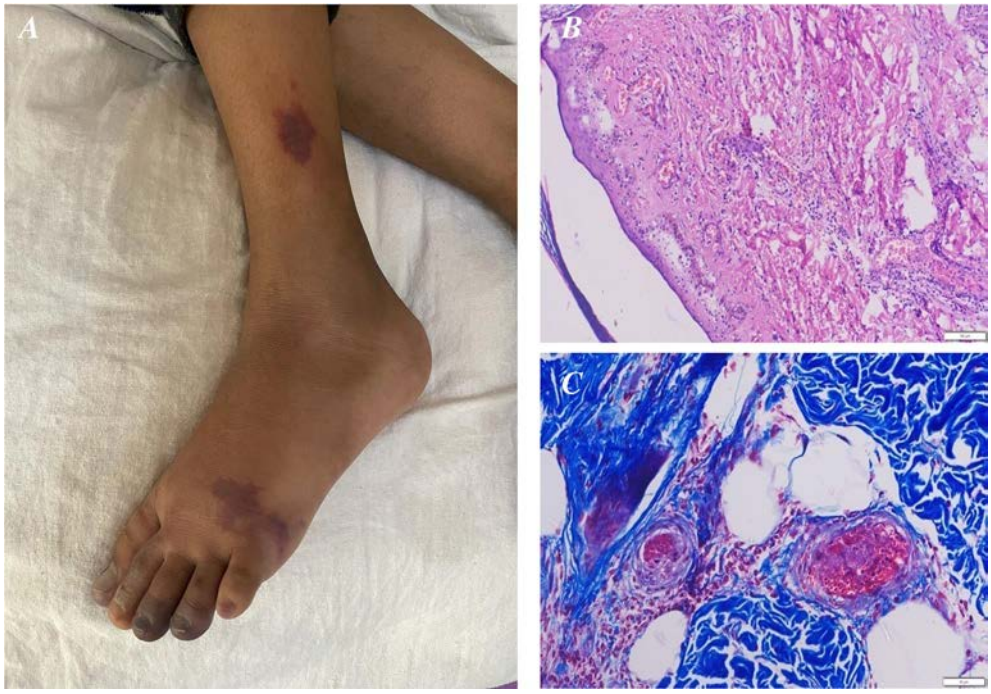

FIGURE 1 | (A) Gangrene during initial presentation. (B) Leuocytoclastic vasculitis in the superficial dermis seen in H&E stain. (C) Thrombus in vessels in deep dermis in Masson Trichrome stain.

helped with histopathological evaluation. Dr. Jyotsana Punj helped with the pain management for the patient. Dr. Athimalaipet V. Ramanan provided the inputs to reach the diagnosis. Dr. Narendra Bagri has critically reviewed the manuscript and will act as a guarantor. All authors have given their inputs and approved the final manuscript.

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

Investigation of the Risk of Frailty Progression Based on 5-Year Data in Patients With Rheumatoid Arthritis: A Multicenter Observational Study (T-FLAG)

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ABSTRACT

Objectives: To examine risk factors for non-frailty rheumatoid arthritis (RA) patients progressing to frailty.

Methods: A total of 304 RA patients with records for frailty assessment based on the Japanese Cardiovascular Health Study (J-CHS) criteria from 2020 to 2024 were included. Patients classified as non-frail (J-CHS scores 0–3) in 2020 were followed annually, and those who did and did not progress to frailty were categorized into the frailty progression ($n = 100$) and non-frailty progression ($n = 204$) groups, respectively. Risk factors for frailty progression were analyzed using the Cox proportional hazards model. Changes in DAS28-ESR and HAQ-DI between baseline and frailty progression were compared using a paired *t*-test.

Results: Compared to the non-frailty progression group, the frailty progression group was older (62.9 vs. 68.5 years) and had a longer duration of disease (9.1 vs. 14.2 years), lower methotrexate (MTX) use (74.4% vs. 56.1%), higher mean DAS28-ESR (2.40 vs. 2.77), and higher HAQ-DI (0.17 vs. 0.45). Both groups had high biological/targeted synthetic disease-modifying antirheumatic drugs (b/tsDMARDs) use rates (34.3% vs. 42.0%). Risk factors for frailty events included age ≥ 65 years (HR 1.86), duration of disease ≥ 10 years (HR 1.64), DAS28-ESR < 2.6 (HR 0.64), HAQ-DI ≤ 0.5 (HR 0.45), and MTX use (HR 0.63). DAS28-ESR remained at a low disease activity level (baseline vs. frailty progression: 2.77 vs. 2.90), whereas HAQ-DI worsened at frailty progression compared to baseline (0.45 vs. 0.66).

Conclusions: Optimizing MTX use and achieving DAS/HAQ remission are crucial for preventing frailty. Non-medication-based approaches are also essential.

1 | Introduction

Frailty is a clinical syndrome that leads to a decline in physical resilience, increasing the vulnerability of elderly individuals to adverse health outcomes such as falls, fractures, disability, and mortality [1]. In the context of rheumatoid arthritis (RA), frailty is of particular concern due to chronic, systemic inflammation and physical limitations imposed by the disease. RA patients often experience progressive joint damage, chronic pain, and reduced physical capacity, all of which can accelerate the onset of frailty. As Japan continues to face a rapidly aging population, understanding and addressing the intersection of RA and frailty has become an increasingly important public health issue [2].

Recent years have seen significant advances in RA treatment with the development of methotrexate (MTX) and molecular-targeted drugs, such as biologics and Janus kinase (JAK) inhibitors. These therapeutic agents have greatly improved disease control, reducing inflammation and slowing the progression of joint damage in many patients [3]. As a result, the overall disease activity in RA patients has decreased, leading to better clinical outcomes. However, despite improvements in disease management, frailty remains a major concern. Even in patients with well-controlled disease, the decline in physical function and muscle strength associated with frailty can continue to progress, underscoring the importance of evaluating and managing frailty in RA treatment [4].

The T-FLAG study, launched in 2020, is a multicenter research project focused on investigating frailty in RA patients. The study tracks frailty progression over time with the aim of identifying risk factors that increase frailty. After 5 years of data collection, the study is now ready for a comprehensive analysis. By examining risk factors, the T-FLAG study hopes to provide insights into the causes of frailty in RA patients and guide clinical practices to prevent or reduce frailty.

The primary goal of the study at this stage is to analyze risk factors that contribute to frailty progression using the extensive data gathered, with the aim of improving RA management by focusing on not only controlling disease activity but also maintaining and improving physical function, thereby enhancing patients' quality of life.

2 | Materials and Methods

2.1 | Patients

RA patients who met the 2010 classification criteria of the American College of Rheumatology/European League Against Rheumatism for RA [5] and attended outpatient rheumatology clinics at Yokkaichi Municipal Hospital, Japan Community Health care Organization Kani Tono Hospital, and Japan Red Cross Aichi Medical Center, Nagoya Daiichi Hospital, between June 1, 2020, and August 31, 2024, were included in the study ($n = 691$). Patients without Japanese Cardiovascular Health Study (J-CHS) scores for 2020–2024 and those classified as frail in 2020 were excluded. Patients classified as robust and pre-frail were considered non-frail.

A total of 304 patients were classified as non-frail (robust: $n = 82$, pre-frail: $n = 222$) at baseline (J-CHS scores 0–3). Frailty progression events were monitored annually through 2024. Patients who experienced a frailty progression event were excluded from further analysis in subsequent years. Ultimately, 204 patients who did not progress to frailty were categorized into the non-frailty progression group, and 100 patients who progressed to frailty were categorized into the frailty progression group (Figure 1).

2.2 | Clinical Assessment

Experienced rheumatologists conducted routine clinical evaluations, which included assessments of swollen and/or tender joint counts and Steinbrocker stage [6], and documented current medications. Routine blood tests were performed to measure serum levels of C-reactive protein (CRP), rheumatoid factor (RF), erythrocyte sedimentation rate (ESR), and matrix metalloproteinase-3 (MMP3).

2.3 | Evaluation of Disease Activity and Physical Function

Disease activity was assessed using the DAS28-ESR score based on tender and swollen joint counts, patient's global assessment, and ESR [7]. Clinical remission was defined as DAS28-ESR < 2.6, low disease activity as $2.6 \leq$ DAS28-ESR < 3.2, moderate disease activity as $3.2 \leq$ DAS28-ESR ≤ 5.1, and high disease activity as DAS28-ESR > 5.1 [8]. Physical function was measured using the Japanese version of the Health Assessment Questionnaire-Disability Index (HAQ-DI), with functional remission defined as HAQ-DI scores ≤ 0.5 [9, 10].

2.4 | Comorbidities

The presence of interstitial pneumonia based on X-ray and computed tomography images, as documented in medical records, was investigated. Renal function was evaluated using the estimated glomerular filtration rate (eGFR), with an eGFR of < 60 mL/min/1.73 m^2 considered to indicate reduced renal function [11].

2.5 | Frailty Assessment

Frailty was diagnosed according to J-CHS criteria [12], the Japanese adaptation of the CHS criteria [1] known for its high diagnostic accuracy in the Japanese population. The five components of J-CHS criteria include weight loss, muscle weakness, fatigue, decreased walking speed, and decreased physical activity. Grip strength was assessed using Smedley Spring handgrip dynamometers (TTM Smedley Dynamo Meter; Tsutsumi, Tokyo, Japan) with the elbows fully extended while standing. Grip strength of each hand was measured twice, and the higher value was used for analysis in accordance with the guidelines of the Asian Working Group for Sarcopenia [13]. Patients with a J-CHS score of 0 out of 5 were classified as robust, those with scores of 1–2 as pre-frail, and those with scores of 3 or higher as frail.

2.6 | Missing Data

The breakdown of missing data is as follows: one for body mass index (BMI) (1/304, 0.3%), six for the steroid use rate (6/304, 2.0%), six for the MTX use rate (6/304, 2.0%), six for the b/tsDMARD use rate (6/304, 2.0%), 12 for ACPA (12/304), one for RF (1/304, 0.3%), one for CRP (1/304, 0.3%), five for ESR (5/304, 1.6%), one for MMP3 (1/304, 0.3%), six for DAS28-ESR (6/304, 2.0%), and two for HAQ-DI (6/304, 0.7%).

2.7 | Statistical Analysis

All statistical tests were two-sided, with a significance level set at p-value < 0.05. The asterisk (*) in the figures indicates a

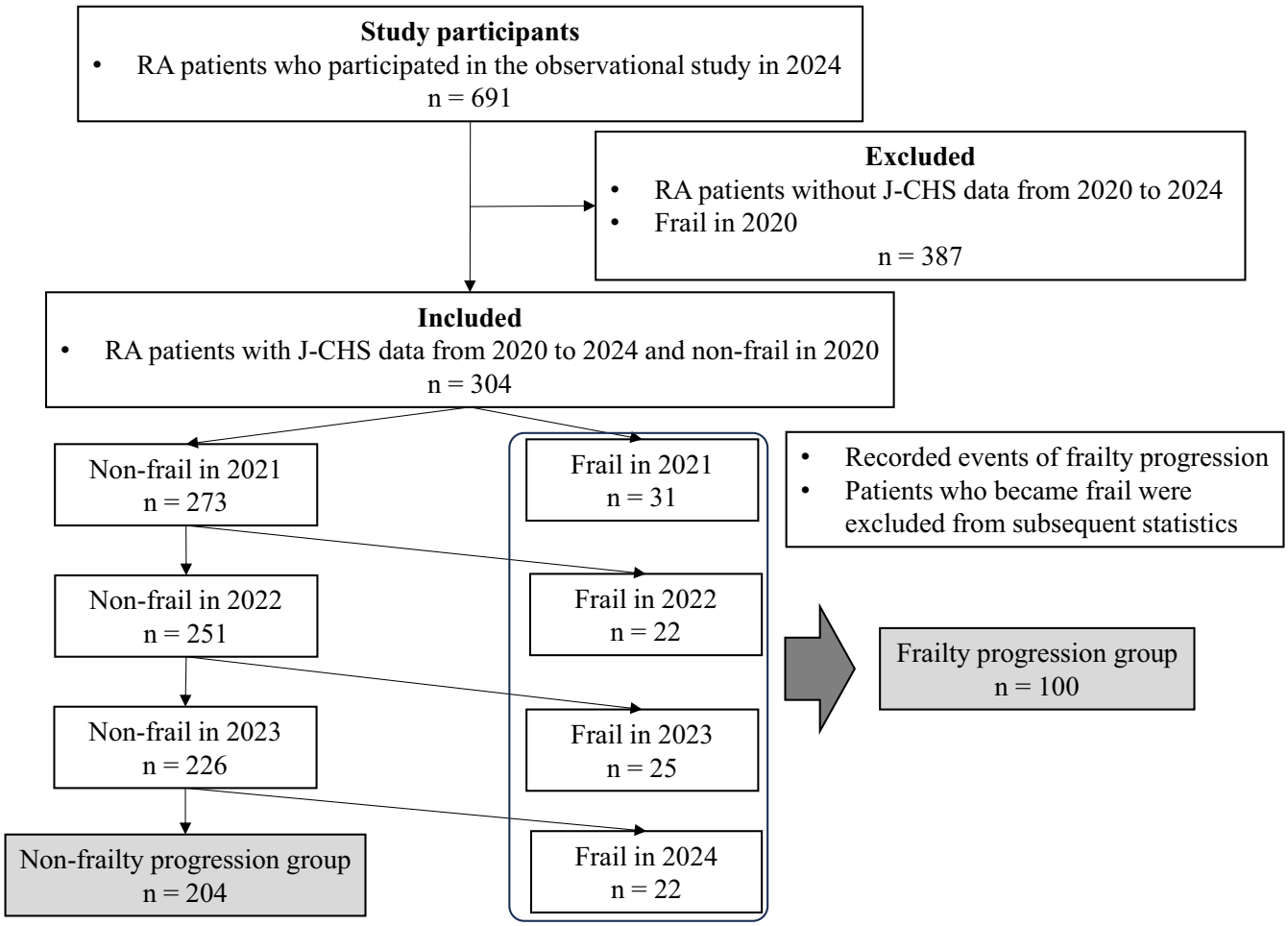

FIGURE 1 | Flow chart of participant selection.

significance level of p-value < 0.05. Unless otherwise noted, values are presented as mean ± standard deviation. Patient characteristics for the non-frailty and frailty progression groups were compared using univariate analysis (Mann–Whitney U test and chi-square test). To identify risk factors for frailty events over 4 years, a Cox proportional hazards model was used, adjusting for age (≥ 65 years), sex (female), disease duration (≥ 10 years), BMI (kg/m^2), DAS28-ESR (< 2.6), HAQ-DI (≤ 0.5), MTX use, steroid use, and b/tsDMARD use. Age 65 years was chosen as the cutoff because individuals aged ≥ 65 years are considered elderly in many studies [14]. A cutoff disease duration of 10 years was used because this length is commonly defined as long-term in the literature [15]. Among factors previously reported to have a strong association with frailty [16–18], those that showed significant differences between the frailty progression and non-frailty progression groups in the univariate analysis, including age, disease duration, BMI, DAS28-ESR, HAQ-DI, MTX use, steroid use, and b/tsDMARD use, were selected as explanatory variables. Longitudinal changes in each risk factor for frailty events and each J-CHS component were estimated using the Kaplan–Meier method and analyzed with the log-rank test. In the frailty progression group, changes in DAS28-ESR and HAQ-DI from baseline to the time of frailty onset were compared using a paired t-test. For each patient, data from the year of first frailty onset were used (e.g., data from year 1 for those with frailty onset in the first year, data from year 2 for those with frailty onset in the second year, and so on).

All analyses were conducted using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical interface for R (The R Foundation for Statistical Computing, Vienna, Austria), specifically modified from R commander to include statistical functions commonly used in biostatistics [19].

3 | Results

3.1 | Annual Occurrence of Frailty Events

Of the 304 non-frail patients in 2020, 31 became frail by 2021 (31/304, 10.2%). Among the 273 patients who remained non-frail through 2021, 22 became frail by 2022 (22/273, 8.1%). Of the 251 remaining non-frail patients through 2022, 22 became frail by 2023 (22/251, 8.8%). Furthermore, among the 226 patients who remained non-frail through 2023, 25 became frail by 2024, leaving 204 patients who maintained non-frailty status (25/226, 11.1%). Overall, 204 patients did not experience frailty

events (204/304, 67.1%), while 100 progressed to frailty (100/30, 32.9%) (Figure 1).

3.2 | Patient Characteristics Between the Non-Frailty Progression and Frailty Progression Groups

Overall, the mean age of participants was 64.7 years, the mean duration of disease was 10.4 years, mean DAS28-ESR was 2.53, mean HAQ-DI was 0.26, and mean J-CHS score was 1.04. Compared with patients in the non-frailty progression group, patients in the frailty progression group were older (62.9 vs. 68.5 years, $p < 0.001$) and had a longer duration of disease (9.1 vs. 14.2 years, $p < 0.001$), a lower rate of MTX use (74.4% vs. 56.1%, $p = 0.002$), a higher mean DAS28-ESR (2.40 vs. 2.77, $p < 0.001$), and a higher HAQ-DI (0.17 vs. 0.45, $p < 0.001$) (Table 1).

3.3 | Risk Factors for Frailty Events Over 4 Years

Risk factors for frailty events over 4 years included age ≥ 65 years (HR 1.86, 95% CI 1.13–3.05, $p = 0.014$), duration of disease ≥ 10 years (HR 1.64, 95% CI 1.08–2.51, $p = 0.021$), DAS28-ESR < 2.6 (HR 0.64, 95% CI: 0.42–0.98, $p = 0.040$), HAQ-DI ≤ 0.5 (HR 0.45, 95% CI: 0.28–0.74, $p = 0.001$), and MTX use (HR 0.63, 95% CI 0.41–0.98, $p = 0.041$) (Table 2).

3.4 | Comparison of Longitudinal Changes in Each Risk Factor for Frailty Events

A comparison between the age ≥ 65 and < 65 year groups revealed a significantly higher frailty event incidence in the former group (40.1% vs. 20.7%, $p < 0.001$) (Figure 2A). A comparison between the disease duration ≥ 10 and < 10 year groups revealed a significantly higher frailty event incidence in the former group (44.8% vs. 24.4%, $p < 0.001$) (Figure 2B). A comparison between the DAS28-ESR ≥ 2.6 and < 2.6 groups revealed a significantly higher frailty event incidence in the former group (44.8% vs. 25.7%, $p < 0.001$) (Figure 2C). A comparison between the HAQ-DI > 0.5 and ≤ 0.5 groups revealed a significantly higher frailty event incidence in the former group (62.0% vs. 27.2%, $p < 0.001$) (Figure 2D). Finally, a comparison between the MTX use and non-MTX use groups revealed a significantly higher frailty event incidence in the latter group (27.1% vs. 45.7%, $p < 0.002$) (Figure 2E).

3.5 | Comparison of Longitudinal Changes in Each Composite Measure of J-CHS

A comparison between the pre-frailty and robust groups revealed a significantly higher frailty event incidence in the former group (33.9% vs. 19.0%, $p = 0.002$) (Figure 3A). A comparison between the muscle weakness and non-muscle weakness groups (J-CHS 4) revealed a significantly higher frailty event incidence in the former group (51.2% vs. 25.7%, $p < 0.001$) (Figure 3E). Other J-CHS components (J-CHS 1, 2, 3, and 5) showed no significant differences in frailty event incidence (Figure 3B–D,F).

3.6 | Longitudinal Changes in Mean DAS28-ESR, Mean HAQ-DI, MTX Use Rate, and MTX Dosage

Compared with the non-frailty progression group, DAS28-ESR was significantly higher in the frailty progression group across all years (2020: 2.41 vs. 2.77, $p < 0.001$; 2021: 2.38 vs. 2.83, $p < 0.001$; 2022: 2.31 vs. 2.87, $p < 0.001$; 2023: 2.29 vs. 2.83, $p < 0.001$; 2024: 2.26 vs. 2.95, $p < 0.001$). Between 2020 and 2024, the non-frailty progression group showed a significant decrease (2020: 2.41 vs. 2024: 2.26, $p < 0.030$), while no significant change was observed in the frailty progression group (2020: 2.77 vs. 2024: 2.95, $p = 0.073$) (Figure 4A). Similarly, compared with the non-frailty progression group, HAQ-DI was significantly higher in the frailty progression group across all years (2020: 0.17 vs. 0.45, $p < 0.001$; 2021: 0.16 vs. 0.47, $p < 0.001$; 2022: 0.15 vs. 0.50, $p < 0.001$; 2023: 0.15 vs. 0.56, $p < 0.001$; 2024: 0.16 vs. 0.66, $p < 0.001$). Between 2020 and 2024, the non-frailty progression group showed no significant change (2020: 0.17 vs. 2024: 0.16, $p = 0.784$), while a significant change was observed in the frailty progression group (2020: 0.45 vs. 2024: 0.66, $p < 0.001$) (Figure 4B). Compared with the non-frailty progression group, the rate of MTX use was significantly lower in the frailty progression group across all years (2020: 74.4% vs. 56.1%, $p = 0.002$; 2021: 73.5% vs. 58.0%, $p = 0.008$; 2022: 73.0% vs. 55.0%, $p = 0.002$; 2023: 74.4% vs. 54.6%, $p < 0.001$; 2024: 74.0% vs. 51.0%, $p < 0.001$). Between 2020 and 2024, neither group showed a significant change (non-frailty progression group: 2020: 74.4% vs. 2024: 74.0%, $p = 0.100$; frailty progression group: 2020: 56.1% vs. 2024: 51.0%, $p = 0.114$) (Figure 4C). There was no significant difference in MTX dosage between the two groups in any year (2020: 8.2 mg/week vs. 7.8 mg/week, $p = 0.387$; 2021: 8.2 mg/week vs. 7.5 mg/week, $p = 0.117$; 2022: 7.9 mg/week vs. 7.5 mg/week, $p = 0.313$; 2023: 7.8 mg/week vs. 7.2 mg/week, $p = 0.088$; 2024: 7.7 mg/week vs. 7.2 mg/week, $p = 0.204$) (Figure 4D).

3.7 | Change in DAS28-ESR and HAQ-DI Between Baseline and Frailty Progression

Changes in DAS28-ESR and HAQ-DI from baseline to frailty progression were examined in the frailty progression group. DAS28-ESR showed no significant change, with both baseline and frailty onset values remaining at non-remission levels (baseline vs. frailty progression: 2.77 vs. 2.90, $p = 0.313$) (Figure 5A). In contrast, HAQ-DI significantly worsened at frailty progression relative to baseline (0.45 vs. 0.66, $p = 0.001$) (Figure 5B).

4 | Discussion

This study identified mid-term risk factors for frailty onset over a 4-year period. A notable finding was that approximately 10% of patients experienced a new frailty event each year. Patients who experienced a frailty event were excluded from the following year's analysis, so their subsequent progress was not evaluated. Given that frailty is a reversible condition, it is possible that some excluded patients improved from frailty to pre-frailty or robust status [20]. Nevertheless, our data confirm that RA patients are highly susceptible to frailty, consistent with previous reports [21]. The high prevalence highlights the critical need to assess and address frailty in the clinical management of RA.

TABLE 1 | Characteristics of patients in the non-frailty progression and frailty progression groups.

RA patients who were non-frail in 2020	Total $n = 304$	Non-frailty progression $n = 204$	Frailty progression $n = 100$	p
Age (years) mean (SD)	64.7 (13.1)	62.9 (13.9)	68.5 (10.3)	< 0.001
Duration of disease (years) mean (SD)	10.4 (9.2)	9.1 (7.6)	14.2 (10.9)	< 0001
Sex, female (%)	73.0	73.0	73.0	1
BMI (kg/m^2) mean (SD)	22.3 (3.9)	22.7 (3.9)	22.2 (3.8)	0.522
Steinbrocker stage (1/2/3/4) (%)	39.5/25.3/18.4/16.8	46.1/25.0/1.7/13.2	6.0/26.0/24.0/24.0	0.003
Steinbrocker class (1/2/3/4) (%)	46.4/47.4/6.3/0	54.9/42.6/2.5/0	29.0/57.0/14.0/0	< 0.001
Glucocorticoids[a] use rate (%)	25.3	22.1	32.0	0.069
Glucocorticoids[a] dosage (mg/day) mean (SD)	3.5 (1.8)	3.5 (1.6)	3.5 (2.0)	0.915
MTX use rate (%)	66.8	74.4	56.1	0.002
MTX dosage (mg/week) mean (SD)	8.1 (2.9)	8.2 (2.8)	7.9 (3.2)	0.387
b/tsDMARD use rate (%)	36.8	34.3	42.0	0.207
Anti-CCP antibody (U/mL) mean (SD)	152.7 (271.3)	164.2 (310.9)	127.8 (153.0)	0.879
Rheumatoid factor (IU/mL) mean (SD)	109.5 (269.3)	103.0 (306.5)	122.5 (172.7)	0.257
CRP (mg/dL) mean (SD)	0.25 (0.50)	0.21 (0.45)	0.31 (0.60)	0.134
ESR 1 h (mm) mean (SD)	21.4 (18.1)	18.8 (14.0)	26.8 (23.6)	0.011
MMP-3 (ng/mL) mean (SD)	76.7 (66.7)	68.5 (52.6)	93.6 (87.1)	0.017
DAS28-ESRA mean (SD)	2.53 (0.94)	2.40 (0.90)	2.77 (0.99)	< 0.001
DAS28-ESR Remission (%)	56.3	65.0	44.3	< 0.001
HAQ-DI mean (SD)	0.26 (0.43)	0.17 (0.33)	0.45 (0.53)	< 0.001
HAQ Remission (%)	79.9	90.3	68.0	< 0.001
J-CHS total	1.04 (0.76)	0.92 (0.75)	1.30 (0.73)	< 0.001
J-CHS 1: Weight loss (%)	6.9	8.3	4.0	0.229
J-CHS 2: Fatigue (%)	12.5	10.3	17.0	0.101
J-CHS 3: Decreased physical activity (%)	14.5	14.2	15.0	0.863
J-CHS 4: Muscle weakness (%)	20.1	20.6	44.0	0.001
J-CHS 5: Decreased walking speed (%)	5.3	5.9	4.0	0.593
Interstitial pneumonia (%)	10.2	7.4	16.0	0.026
Decreased renal function (%)	34.9	32.4	40.0	0.202

Note: Data are presented as mean (standard deviation).
Abbreviations: anti-CCP antibody, anti-cyclic citrullinated peptide antibody; b/tsDMARDs, biological and targeted synthetic disease-modifying antirheumatic drugs; BMI, body mass index; CRP, C-reactive protein; DAS28-ESR, disease activity score 28-erythrocyte sedimentation rate; ESR, erythrocyte sedimentation rate; HAQ-DI, health assessment questionnaire-disability index; J-CHS, Japanese Cardiovascular Health StudyMMP3, matrix metalloproteinase 3; MTX, methotrexate.
[a]Glucocorticoids dosage is expressed in prednisone-equivalent doses.

Age and disease duration are well-established risk factors for frailty events [22]; the risk of frailty increases with age, and patients with longer disease duration are at particular risk because of the progression of joint destruction, or because they might have developed RA before the initiation of treatment with DMARDs [23]. The present study found that over 40% of patients aged ≥ 65 years or with a disease duration of ≥ 10 years experienced frailty events over 4 years, suggesting a significant impact of these factors. Joint destruction progresses rapidly in early RA, underscoring the need for early treatment to avoid missing the "window of opportunity" [24]. For older patients or those with irreversible joint damage, rehabilitation or surgical interventions, such as joint replacement, should be recommended [25]. Although they may not fully recover to "robust" status, it may be possible to prevent progression to frailty by maintaining a pre-frail state [26].

Poor control of disease activity was identified as a risk factor for frailty progression, consistent with previous reports [27]. Our study adds to the existing literature because the observed longitudinal changes emphasize the importance of proper disease activity control in preventing frailty. Notably, 44.8% of patients who did not achieve clinical remission experienced frailty within 4 years. While EULAR's T2T strategy sets low disease activity as a target, our results suggest that this alone may not be sufficient [28]. Treatment aimed at achieving remission is essential to reduce frailty progression.

TABLE 2 | Risk factors for frailty events over 4 years.

Factor (baseline data)	HR (95% confidence Interval)	*p*
Age (≥65 years)	1.86 (1.13–3.05)	0.014
Sex (female)	0.86 (0.52–1.41)	0.552
Duration of disease (≥10 years)	1.64 (1.08–2.51)	0.021
BMI (kg/m^2)	1.00 (0.95–1.06)	0.881
DAS28-ESR (<2.6)	0.64 (0.42–0.98)	0.040
HAQ-DI (≤0.5)	0.45 (0.28–0.73)	0.001
MTX use	0.63 (0.41–0.98)	0.041
Glucocorticoids use	1.32 (0.86–2.04)	0.207
b/tsDMARD use	1.01 (0.65–1.58)	0.958

Abbreviations: b/tsDMARDs, biological and targeted synthetic disease-modifying antirheumatic drugs; BMI, body mass index; DAS28-ESR, disease activity score 28-erythrocyte sedimentation rate; HAQ-DI, health assessment questionnaire-disability index; MTX, methotrexate.

HAQ-DI, like frailty, is a measure of physical function and, as expected, had a significant impact on frailty progression. Notably, 62.0% of patients who did not achieve HAQ remission at baseline experienced frailty within 4 years. This suggests that in routine clinical practice, it is crucial to regularly check HAQ-DI in addition to monitoring disease activity to help prevent frailty [29]. Furthermore, even when disease activity is under control, if HAQ does not improve, careful attention is needed. In such cases, rehabilitation or surgery should be considered to improve physical function.

MTX was the only risk factor identified for frailty events among RA medications; 45.7% of patients who were not using MTX at baseline experienced frailty events. Although frailty events occurred more frequently among non-MTX users compared with MTX users each year, statistical significance was not observed until the fourth year. Understanding the reasons for this would require a longer-term observational study. MTX appears to contribute to frailty events over time, rather than immediately, which we consider an important finding. At the same time, it is important to consider comorbidities and side effects as reasons why some patients cannot use MTX [30]. We collected data on interstitial pneumonia and renal impairment, which revealed that many patients who could not use MTX had interstitial pneumonia (Data S1). The relationship between comorbidities and frailty has been reported previously [31], and future studies that take such patient backgrounds into account are warranted.

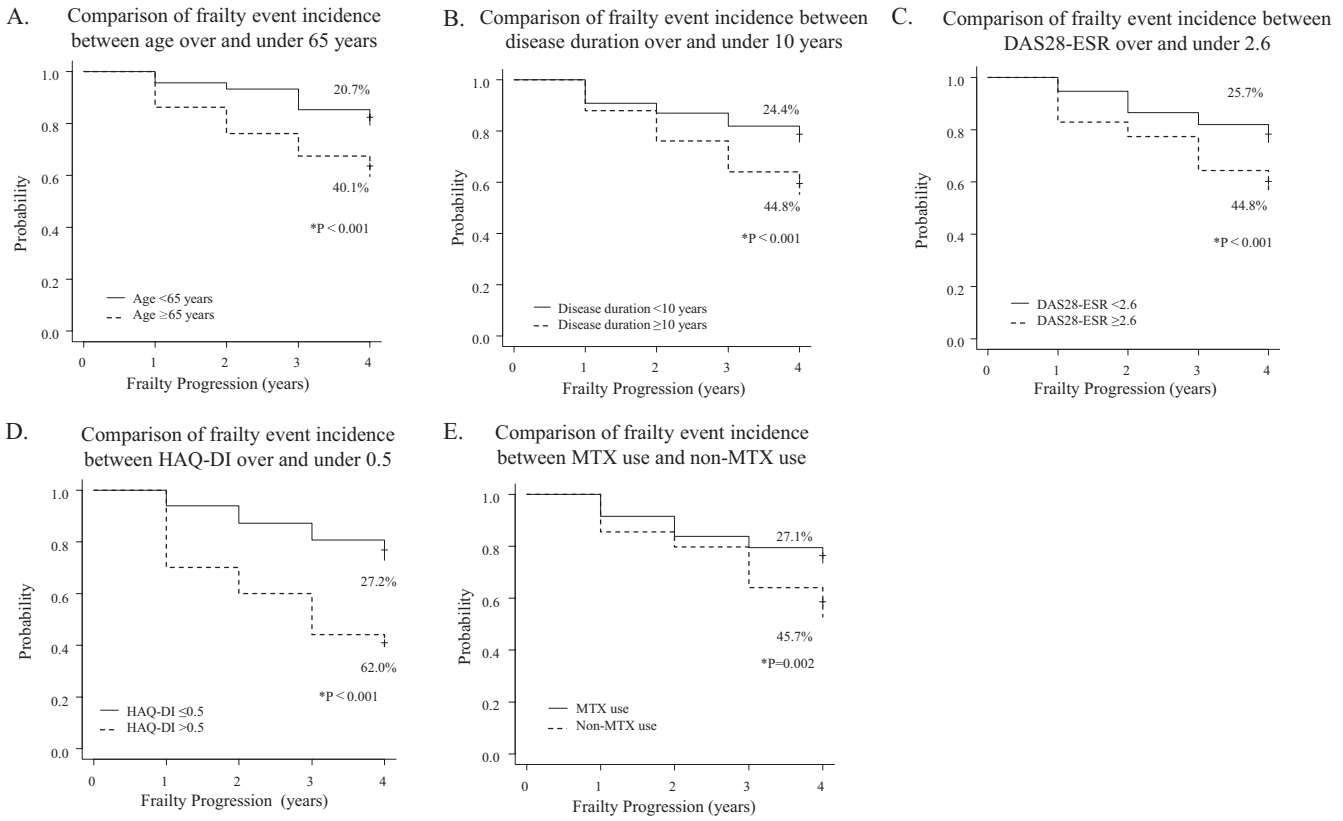

FIGURE 2 | Comparison of longitudinal changes in each risk factor for frailty events.

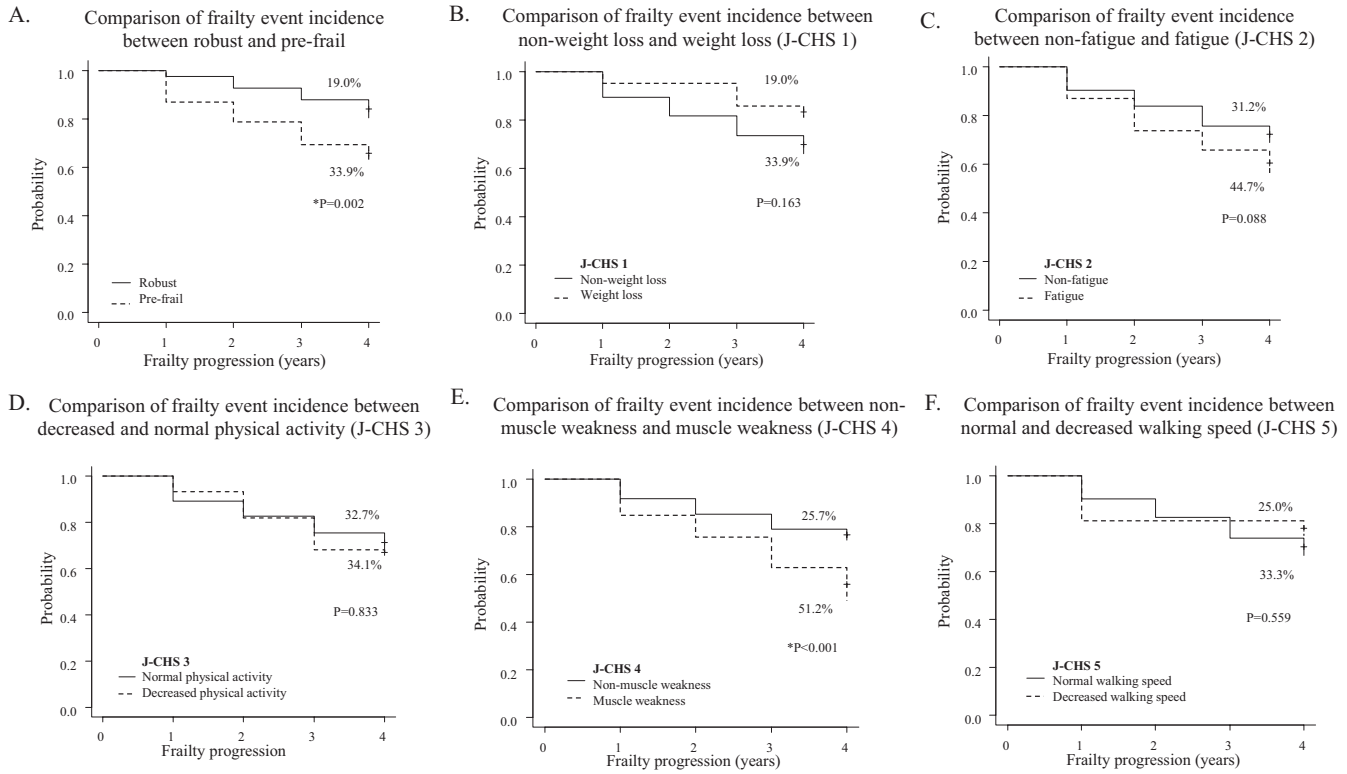

FIGURE 3 | Comparison of longitudinal changes in each composite measure of J-CHS.

In this study, both MTX use and b/tsDMARD use were included as explanatory variables in the Cox proportional hazards model, although there was concern that potential confounding effects might be present. b/tsDMARDs are often prescribed when MTX is ineffective or discontinued due to comorbidities, which may impact the control of disease activity [32]. In our analysis, patients using b/tsDMARDs had significantly lower rates of MTX use, while those not using b/tsDMARDs had significantly higher rates of MTX use (Data S2). This suggests the possibility of confounding between these two factors. To address this issue, we examined models from which b/tsDMARD use was excluded (i.e., to focus on MTX use), and vice versa. The results showed that MTX use, but not b/tsDMARD use, was a consistent risk factor (Data S3). These results align with the original analysis (Table 2). Although careful consideration of MTX use and b/tsDMARD use is necessary in multivariate analyses, the impact of their confounding on this analysis appears minimal.

We examined the total J-CHS score and its components. As expected, frailty events were more common in pre-frail patients than in robust patients. Changes in the proportion of patients in each frailty category over 4 years among those who were robust and pre-frail at baseline were also examined (Data S4). Although no significant difference was observed in 2022, the proportion of frail patients was higher among those who were pre-frail at baseline than among those who were robust at baseline in 2021, 2023, and 2024 (robust at baseline vs. pre-frail at baseline: 2/82 (2.4%) vs. 29/222 (13.1%), $p = 0.005$ in 2021; 4/82 (4.9%) vs. 28/222 (12.6%), $p = 0.058$ in 2022; 5/82 (6.1%) vs. 42/222 (18.9%), $p = 0.005$ in 2023; 7/82 (8.5%) vs. 44/222 (19.8%), $p = 0.024$ in 2024). These findings highlight the need for future studies to investigate differences between robust and pre-frail RA patients and to conduct long-term follow-up on frailty event occurrence. RA is a disease that causes joint damage, and even with good disease control, a large proportion of patients are classified as pre-frail [33]. Therefore, understanding differences between robust and pre-frail patients is essential for advancing treatment strategies for RA. Among J-CHS components, only muscle weakness (J-CHS 4) showed a significant association with frailty. Even with controlled disease activity, subclinical synovitis can persist, warranting further evaluation such as MRI or ultrasound for hand stiffness [34, 35]. In cases of joint destruction in patients' hands, alternative methods for assessing muscle weakness may be needed, such as using the Asian Working Group criteria for sarcopenia evaluation [13]. To determine which components of the J-CHS most significantly contribute to frailty events, the number of patients corresponding to each component at baseline and at the time of frailty event occurrence in the frailty progression group was analyzed. The results showed that fatigue, decreased physical activity, and muscle weakness accounted for the largest proportions at both baseline and the time of frailty event occurrence. In terms of the rate of increase, however, while the proportions for these components approximately doubled, weight loss and decreased walking speed showed greater relative increases, with the former exhibiting the highest rate of increase (13-fold) and the latter the second highest (7.3-fold) (Data S5). These findings suggest that, in addition to fatigue, decreased physical activity, and muscle weakness as major contributors to frailty progression, weight loss and decreased walking speed may also play a significant role, warranting close monitoring of these factors.

Over the course of 4 years, the frailty progression group consistently showed higher disease activity compared with the non-frailty progression group. However, the mean DAS28-ESR in the frailty progression group remained at a low disease activity level

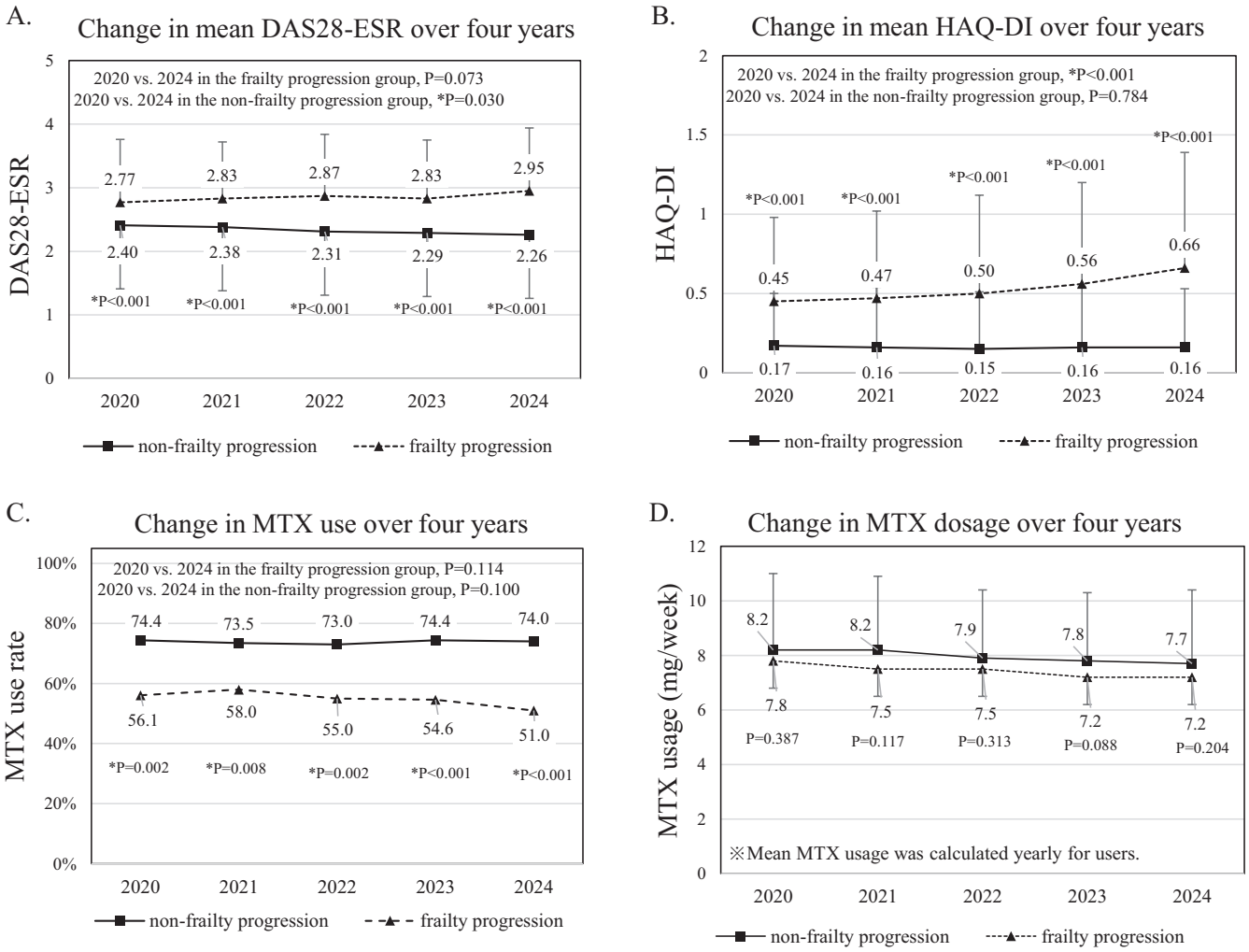

FIGURE 4 | Longitudinal changes in mean DAS28-ESR, mean HAQ-DI, MTX use rate, and MTX dosage.

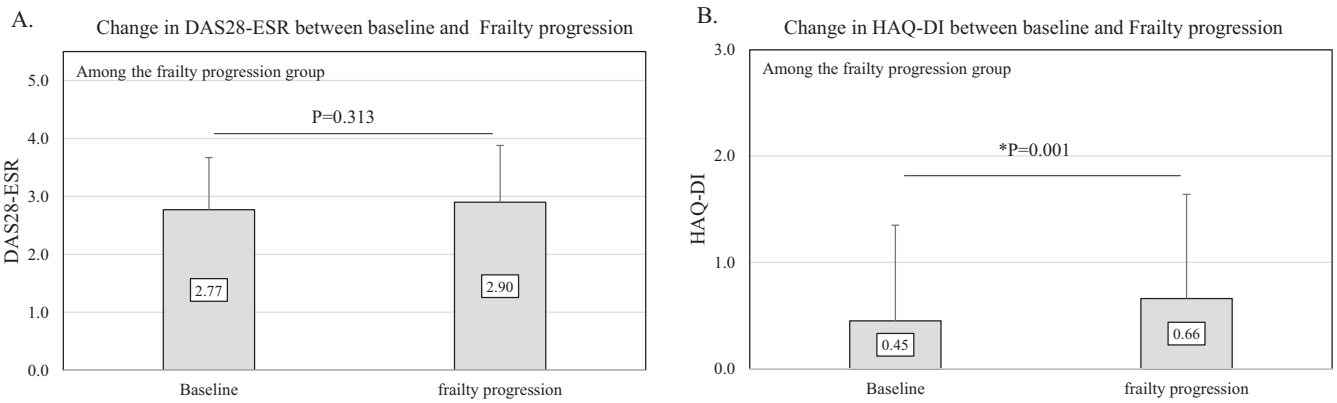

FIGURE 5 | Change in DAS28-ESR and HAQ-DI between baseline and Frailty progression.

each year, which is considered sufficient according to T2T treatment goals [36]. Similarly, HAQ-DI scores in the frailty progression group were higher than those in the non-frailty progression group each year. However, unlike DAS28-ESR, HAQ-DI scores in the frailty progression group worsened year by year. Moreover, the rate of MTX use was consistently lower in the frailty progression group than in the non-frailty progression group. These findings suggest that treatment intensification targeting remission may be necessary for the frailty progression group. However, 40%–50% of patients in the frailty progression group were already receiving b/tsDMARDs (Data S6C). In addition, there were no significant differences between the two groups in glucocorticoid use, in terms of both dosage (approximately 3 mg/day) and use rate (20%–30%). In this study, the use rate and dosage of corticosteroids are considered to be low. For example, according to Liron et al. [37] 35.5% of patients with rheumatoid arthritis used corticosteroids, with a daily dose of 7.5 mg in PSL equivalents (Data S6A–C). This suggests that treatment intensification with

drugs alone has limitations in preventing frailty events. The changes in DAS28-ESR and HAQ-DI scores in the frailty progression group also indicate certain limitations to intensified pharmacological treatments. While DAS28-ESR remained at a low disease activity level at both baseline and at the onset of frailty, HAQ-DI scores indicated a significant deterioration. Thus, after achieving remission or low disease activity status with pharmacological treatment, incorporating non-pharmacological approaches may be crucial to prevent further frailty progression. Frailty fundamentally involves declining physical function, making exercise an effective intervention [38]. Moreover, for RA patients with irreversible joint damage, surgical treatments such as joint replacement or reconstruction are considered beneficial [39]. Furthermore, frailty is closely linked to mental and social health [40]. Poor nutritional status can lead to muscle weakness and reduced physical function, while decreased mobility and energy levels may result in social isolation and social frailty. Interestingly, recent studies suggest that the frequency of laughter may also influence frailty [41]. Taken together, these findings highlight the importance of integrating non-pharmacological strategies into care plans to prevent frailty progression effectively once disease activity stabilizes.

This study has several limitations. First, although we used 5 years of data, frailty increases with age, so ongoing longitudinal observation is necessary. Second, we did not fully examine all comorbidities associated with frailty, such as malignancies [42]. Additionally, we excluded patients who experienced frailty events, but some of these patients may have improved to a pre-frail or robust state. Lastly, because we included only patients with complete 5-year data, the sample size was somewhat small.

In conclusion, optimizing MTX use and achieving DAS and HAQ remission are key to preventing frailty. In addition, non-medication-based approaches, such as exercise, play an equally vital role in supporting frailty prevention efforts.

Author Contributions

Y.O., N.T., Y.S., and M.S. were involved in the study's conception and design, data collection, analysis, and interpretation, as well as drafting and critically revising the manuscript for significant intellectual content. K.T. and S.A. contributed to the data analysis, interpretation, and critical drafting/revising of the manuscript for substantial intellectual content. K.T. and S.I. participated in the study's conception and design, data acquisition, and critical revising of the manuscript for important intellectual content. All authors reviewed and approved the final manuscript.

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Ethics Statement

This study used data from the prospective observational Tsurumai-Frailty and Locomotive syndrome with rheumatoid arthritis for Globalization (T-FLAG) study, which received approval from the institutional review board. The T-FLAG study, initiated in 2020 by three rheumatologists across three institutions, aims to investigate the associations between rheumatoid arthritis, frailty, and locomotive syndrome [43]. All patients provided written informed consent. The study followed the ethical principles outlined in the World Medical Association's Declaration of Helsinki for research involving human subjects, and patient information was anonymized to ensure confidentiality.

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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ORIGINAL ARTICLE

Early Atherosclerosis in Pediatric Systemic Lupus Erythematosus: A Systematic Review and Meta-Analysis

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ABSTRACT

Objective: Adults with systemic lupus erythematosus (SLE) are at a higher risk of cardiovascular complications. Numerous meta-analyses in adults with SLE have consistently demonstrated altered surrogate parameters of atherosclerosis (subclinical atherosclerosis). However, meta-analyses assessing the impact of pediatric SLE (pSLE) on cardiovascular health are lacking. Herein, we perform a systematic review and meta-analysis of studies assessing subclinical atherosclerosis in pSLE.

Methods: Embase, Web of Science, and PubMed databases were systematically searched for pertinent literature published between January 1965 and February 2024. Patients with SLE and healthy participants were included as cases and controls, respectively. Online PICO portal, Newcastle–Ottawa scale, Review Manager and R software, and GRADEpro GDT tool were used for study deduplication and data extraction, study quality evaluation, data synthesis/analysis (including meta-regression), and certainty of evidence assessment, respectively.

Results: A total of 9 studies (out of 126 citations), including 1021 participants (528 cases and 493 controls), were included in the analysis. Carotid intima–media thickness (CIMT) and pulse wave velocity (PWV) were significantly higher in cases as compared to controls [mean difference (MD) 0.04 (95% CI 0.01–0.08) mm, $p = 0.007$ and MD 0.48 (95% CI 0.02–0.94) m/s, $p = 0.04$, respectively]. The flow-mediated dilatation was similar in the two groups. Significant heterogeneity was observed for all the outcome measures. On meta-regression analysis, disease duration correlated significantly with both CIMT MD and PWV MD.

Conclusions: A "low"-quality evidence suggests that pSLE may adversely impact vascular microanatomy and physiology. Longitudinal studies are needed to precisely quantify the cardiovascular risk in pSLE.

Registration: PROSPERO (CRD42022323752).

Pratap Kumar Patra and Aaqib Zaffar Banday contributed equally and qualify as joint first authors.

Summary

- Carotid intima–media thickness and pulse wave velocity are worsened in patients with pediatric-onset lupus.
- Disease duration correlates with the degree of alterations in these parameters.

1 | Introduction

Systemic lupus erythematosus (SLE), a multifaceted multisystem autoimmune disease, is a leading rheumatological disorder in children [1]. Loss of self-tolerance, aberrant innate and adaptive immune response to nuclear antigens, and release of proinflammatory cytokines play a crucial role in its evolution [2]. Pediatric SLE (pSLE) usually has a more severe disease course (e.g., systemic inflammation and kidney involvement) than adults [3]. The prognosis of pSLE has been poor historically; however, enhanced treatment strategies have significantly improved the overall (and renal) survival over the last few decades. The long-term clinical outcomes of other major organ systems are, hence, being increasingly focused. A higher risk of atherosclerotic cardiovascular events (aCVEs), the leading cause of mortality globally, has also been observed in adults with SLE [4]. Besides traditional cardiovascular risk factors, systemic inflammation, immune complex-mediated endothelial injury, and antiphospholipid antibodies contribute to the development of aCVEs in SLE [5, 6]. As clinical aCVEs are sparingly observed in the pediatric population, cardiovascular risk assessment in pSLE is primarily based on surrogate parameters for atherosclerosis including carotid intima–media thickness (CIMT), flow-mediated dilation (FMD), and pulse wave velocity (PWV) [7]. These noninvasive markers have been shown to predict the development of clinical aCVEs in adults [8]. Herein, we performed a systematic review and meta-analyses assessing CIMT, FMD, and PWV in pSLE. Various meta-analyses have assessed the cardiovascular risk in adult SLE; however, to the best of our knowledge, no such study has been published thus far in pSLE.

2 | Methods

Our study was performed according to the PRISMA 2020 recommendations [9]. The study protocol is registered at PROSPERO (CRD42022356964).

2.1 | Search Methodology

Relevant literature published between January 1965 and February 2024 was systematically searched utilizing the PubMed, Web of Science, and Embase databases. Language constraints were not applied during the literature search. Following keywords (MeSH and free-text terms), combined using appropriate Boolean functions, comprised the search strategy: "carotid intima-media thickness," "flow-mediated dilatation," "arterial stiffness," "pulse-wave analysis," "pulse-wave velocity," "endothelial dysfunction," "systemic lupus erythematosus," "juvenile systemic lupus erythematosus," "childhood-onset systemic lupus erythematosus," and "SLE" (Supplementary Methods).

2.2 | Inclusion Criteria

2.2.1 | Study Types

We included observational (cross-sectional) studies that assessed subclinical atherosclerosis in pSLE.

2.2.2 | Study Population and Comparator Group

Patients with pSLE and healthy participants were enrolled as cases and controls, respectively.

2.2.3 | Exposure Type

The occurrence of SLE in cases was characterized as exposure. The time interval between the onset of SLE and enrollment in the study represented the duration of exposure (duration of disease).

2.2.4 | Outcome Measures

Differences in markers of vascular microanatomy (CIMT) and vascular physiology (PWV and FMD) were the outcome variables evaluated in this study.

2.3 | Exclusion Criteria

Studies with incomplete data were excluded. We also excluded studies evaluating subclinical atherosclerosis in children/adolescents wherein specific data for patients with pSLE were unavailable.

2.4 | Data Extraction

Two authors (PKP and AA) independently performed record deduplication, pertinent study identification, and data extraction using the Population, Intervention, Comparison, and Outcomes Portal (available online) [10]. The following data were obtained manually from the relevant studies: study identifiers (authors and year of publication), study design/methods/protocols, participant characteristics (age, sample size, and proportion of patients with nephritis), duration of disease, traditional cardiovascular risk factors, and parameters assessing early atherosclerosis.

2.5 | Study Quality Assessment

Risk of bias assessment was performed independently by two authors (PKP and RRD) utilizing the Newcastle–Ottawa scale (NOS) [11]. Disagreements were sorted out by consultation (with another author AZB).

2.6 | Data Synthesis and Analysis

The Review Manager (version 5.4) software was used (independently by two authors: AZB and AA) for the pooling of data (expressed as mean and standard deviation [SD]) [12]. In studies reporting data in median and interquartile range, values of mean and SD were obtained using the methods described by Wan et al. [13] We used the random-effect model during data pooling as the effect size varied across the included studies ($p < 0.05$ was considered significant). The notation mean difference (MD) with a 95% confidence interval (CI) was used for the expression of results. Cochrane's Q ($\chi 2$ $p < 0.10$) statistic was used to assess heterogeneity among pooled studies ($I^2 \geq 50\%$ reflected significant heterogeneity) [14]. Sensitivity and meta-regression analyses were performed to evaluate potential causes of heterogeneity. R statistical software (version 4.30) was used for meta-regression and acquisition of regression plots.

2.7 | Grade of Evidence

Certainty of evidence was assessed (independently by two authors, PKP and RRD) using the GRADEpro GDT application (version 3.2) [15]. Factors considered included study design limitations, unexplained heterogeneity, result variability/imprecision, indirectness of evidence, and presence of bias. Limitations were rated as none, severe, and very severe.

3 | Results

Title and abstract screening of 62 records (of 126 retrieved on literature search) was performed. Full texts of 19 studies were retrieved for assessment (Figure 1). Finally, 9 studies were included in the analysis, which comprised 1021 participants (528 cases and 493 controls) in whom a vascular study was performed (Tables 1, and S1).

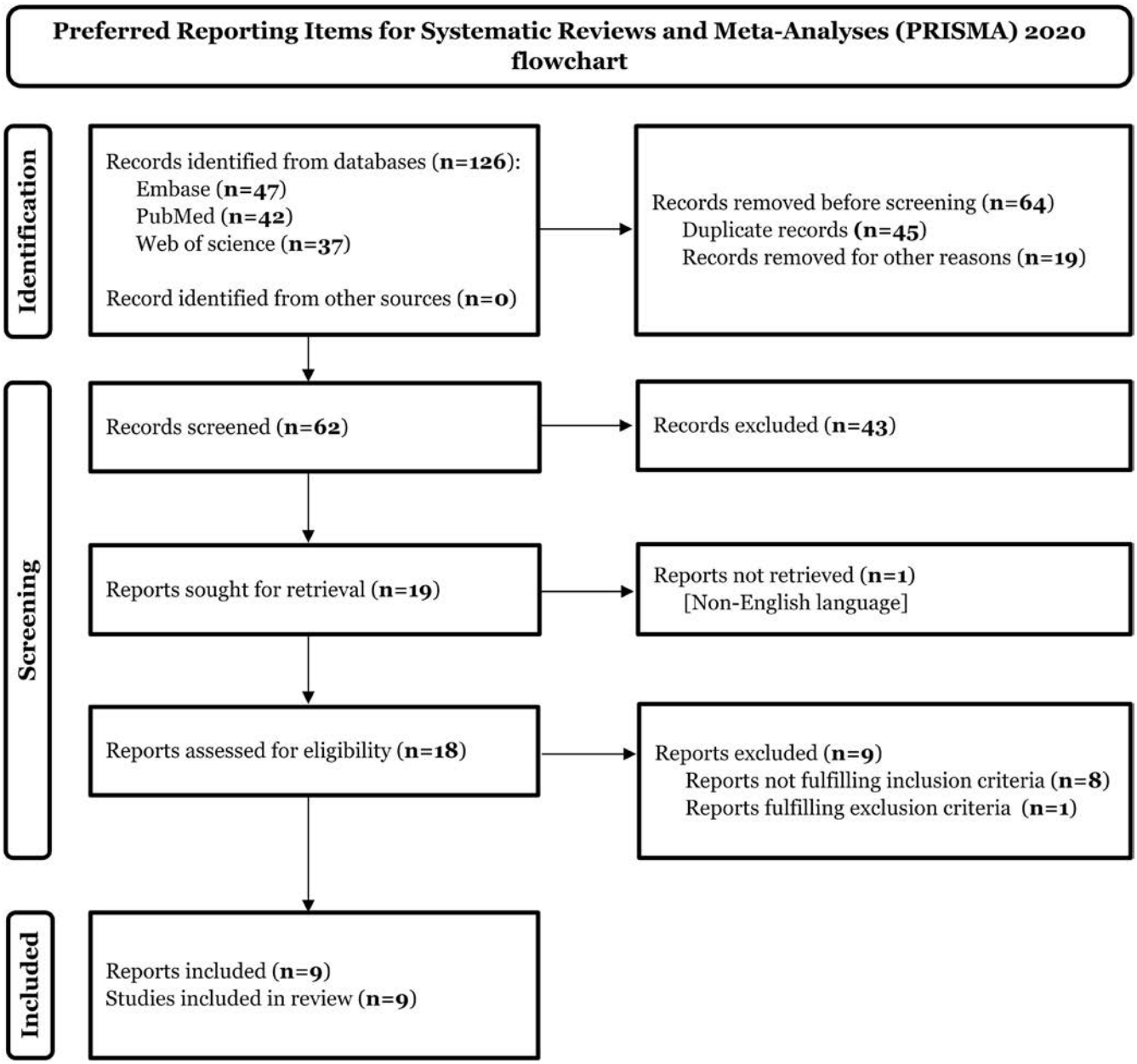

FIGURE 1 | A summary of the search methodology employed in our study. Studies excluded after full-text appraisal are summarized in Table S1.

TABLE 1 | Characteristics of studies evaluating vascular function in patients with juvenile-onset systemic lupus erythematosus compared to controls.

Authors [reference] year	A. Age of cases[b] B. Duration of disease C. Patients with nephritis	No. of participants			Cardiovascular risk factors
		Parameters (unit)	Cases	Controls	
1. Barsalou et al. [16] 2016	A. 17.2 (15.7–17.9) B. 3.2 (1.8–4.9) {0.1–14.9} C. 36.2%		149[c]	178[c]	Cases had significantly higher: proportion of females, age at vascular testing, BMI percentile, and DBP. SBP was similar in both groups
		CIMT (mm)	0.41 (0.38–0.45) (↓)	0.44 (0.41–0.47)	
		FMD (%)	8.7 (5.6–11.5) (↑)	7.4 (5.3–10.0)	
		CF-PWV (m/s)	5.3 (4.8–5.9) (↑)	5.2 (4.5–5.6)	
2. Bowser et al. [17] 2008	A. 16.9 ± 2.27 {14–20} B. 3.25 ± 2.5 {0.1–9} C. 35%		20[c]	20[c]	BMI percentile, MAP, and levels of cholesterol, HDL, LDL, and hsCRP were similar in both groups. Cases had significantly higher levels of triglycerides.
		CIMT (mm)	0.48 ± 0.049	0.454 ± 0.041	
3. Canpolat et al. [18] 2013	A. 14.8 ± 2.65 B. 3.9 ± 2.5 C. NA		24	36	Data on cardiovascular factors are unavailable for controls.
		CF-PWV (m/s)	5.28 ± 0.85 (↑)	4.75 ± 0.39	
4. Falaschi et al. [19] 2000	A. 17.1[a] {6.2–25.4} B. 5.5[a] {0.5–13} C. 23%		26	26	Cases had significantly higher levels of apo B. Levels of cholesterol, triglycerides, HDL, LDL, and apo A-I were similar in both groups.
		CIMT (mm) [mean]	0.57 ± 0.05 (↑)	0.54 ± 0.03	
		CIMT (mm) [max.]	0.62 ± 0.05 (↑)	0.59 ± 0.05	
5. Huang et al. [20] 2009	A. 15.01 ± 3.48 B. 2.65 ± 2.5 C. 59%		76	38	Age, BMI, SBP, and DBP values were similar in both groups. However, proportion with hypertension and other cardiovascular risk factors were higher in cases.
		CIMT (mm) [max.]	0.63 ± 0.08 (↑)	0.54 ± 0.06	
6. Nascif et al. [21] 2018	A. 14.6 ± 2.2 B. 2.6 ± 1.9 {0.2–8.4} C. 63%		35	27	Levels of cholesterol, triglycerides, VLDL, and homocysteine were higher in cases. BMI, SBP, DBP, LDL, and HDL were similar in both groups
		FMD (%)	18.13 ± 10.0	22.36 ± 13.77	
7. Quinlan et al. [22] 2015	A. 14 (12.2–15.5) B. 3.25 (1.7–5.4) C. 53%		45[c]	40	Data on cardiovascular factors are unavailable for controls.
		CIMT (mm)	0.45 ± 0.04 (↑)	0.37 ± 0.06	
		CF-PWV (m/s)	5.272 ± 0.88	5.34 ± 0.97	
8. Sozeri et al. [23] 2013	A. 14.65 ± 3.48 B. 9.62 ± 3.46 C. 27%		51	25	BMI, SBP, DBP, cholesterol, triglycerides, LDL, and HDL were similar in both groups. Values of hsCRP were higher in cases.
		CIMT (mm)	0.54 ± 0.06 (↑)	0.35 ± 0.12	
		CF-PWV (m/s)	6.55 ± 1.49 (↑)	5.23 ± 0.64	
9. Su-angka et al. [24] 2015	A. 15.24 ± 3.34[d] B. NA C. 64%		102	103	BMI in cases was higher than in controls. Data regarding other factors are unavailable.
		CIMT (mm)	0.43 (0.41–0.44)	0.42 (0.41–0.43)	

Note: All values have been expressed as mean ± SD or median (IQR) {range}. Values marked with [a]refer to mean (when SD is unavailable). [b]Age of cases at enrollment specified in years. [c]Number of cases/controls undergoing vascular study is less than the number of cases/controls included. [d]Values calculated from data provided. Age at enrollment (years) was 12.6 (10.8–15.6) and 16.3 (14.0–17.7) for patients with active and inactive disease, respectively.
Abbreviations: (↑), significantly increased; (↓), significantly decreased; apo, apolipoprotein; BMI, body mass index; CCA, common carotid artery; CF-PWV, carotid-femoral pulse wave velocity; CIMT, carotid intima–media thickness; DBP, diastolic blood pressure; FMD, flow-mediated dilatation; HDL, high-density lipoprotein; hsCRP, high-sensitive C-reactive protein; LDL, low-density lipoprotein; MAP, mean arterial pressure; Max., maximum; MTX, methotrexate; NA, not available; PWV, pulse wave velocity; SBP, systolic blood pressure; SLE, systemic lupus erythematosus; VLDL, very low-density lipoprotein.

3.1 | Study Quality and Publication Bias Evaluation

Table S2 details the study quality of the included studies. Briefly, eight studies qualified as good, while one study was of fair quality. The small number of included studies precluded assessment of the publication bias.

3.2 | Carotid Intima–Media Thickness (CIMT)

Significant variations were noted in the protocol of CIMT measurement among the included studies (Figure S1 and Table S3) [20]. Data from six studies were pooled for analysis which primarily measured mean bilateral CIMT [16, 17, 19, 22–24]. Mean age at enrollment and duration of disease in patients with pSLE in the included studies varied from 13.9 to 17.1 years and 3.25 to 9.62 years, respectively. On analyzing 392 patients with pSLE and 390 age-/sex-matched controls, CIMT was significantly higher in the SLE group [MD 0.04 (95% CI 0.01–0.08) mm, $p = 0.007$] (Figure 2). Significant heterogeneity was observed ($I^2 = 96\%$), which persisted despite sensitivity analysis (minimum $I^2 = 95\%$) (performed by serially filtering out data from each study). On meta-regression analysis, age at enrollment, disease duration, proportion of patients with nephritis, and levels of low-density lipoprotein cholesterol (LDL-C) significantly correlated with CIMT MD and accounted for 75.5% ($p = 0.0005$) of the underlying heterogeneity (Figures S2–S5).

We also pooled the data of the two studies providing values of maximum CIMT. These two studies have recruited 102 cases and 64 controls. Mean age at enrollment in patients with pSLE in these two studies was 15.01 and 17.1 years, respectively. Mean duration of disease was 2.65 and 5.5 years, respectively. Maximum CIMT was higher in pSLE [MD 0.06 (95% CI 0.0–0.12) mm, $p = 0.05$]; however, significant heterogeneity ($I^2 = 90\%$) was noted (Figure S1).

3.3 | Pulse Wave Velocity in SLE

A total of four studies, comprising 264 cases and 271 controls, have measured carotid-femoral pulse wave velocity (cf-PWV) in pSLE [16, 18, 22, 23]. Mean age at enrollment and duration of disease in patients with pSLE in the included studies varied from 13.9 to 16.9 years and 3.3 to 9.62 years, respectively. The pulse wave velocity was significantly higher in the study group than in the controls [MD 0.48 (95% CI 0.02–0.94) m/s, $p = 0.04$] (Figure 2). Significant heterogeneity was observed ($I^2 = 87\%$) which persisted despite sensitivity analysis (minimum $I^2 = 58\%$). On

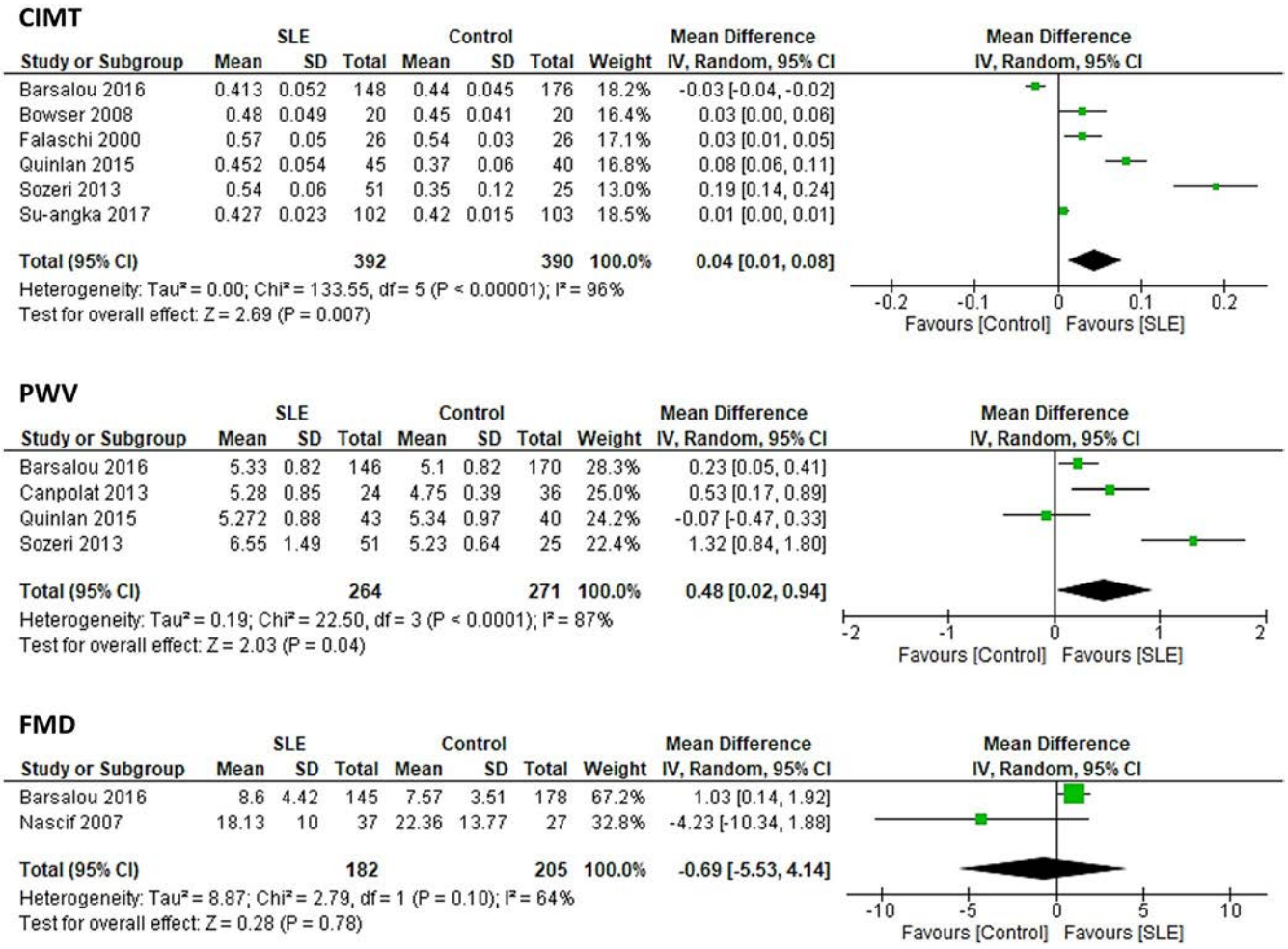

FIGURE 2 | Forest plots showing increased CIMT and PWV in patients with pSLE than in controls. FMD was comparable between the two groups.

meta-regression analysis, disease duration, proportion of patients with nephritis, and levels of low-density lipoprotein cholesterol significantly correlated with PWV MD and accounted for 75.2% ($p = 0.0003$) of the underlying heterogeneity (Figures S6–S8).

3.4 | Flow-Mediated Dilatation

Only two studies, including 182 cases and 205 controls, have measured flow-mediated dilatation in pSLE [16, 21]. Mean age at enrolment in patients with pSLE in these two studies was 14.6 and 16.9 years, respectively. Mean duration of disease was 2.6 and 3.3 years, respectively. FMD was similar between cases and controls [MD −0.69 (95% CI −5.53–4.14) %, $p = 0.78$]. Significant heterogeneity ($I^2 = 64\%$) was observed for this outcome measure as well (Figure 2).

3.5 | Evidence Grade

The evidence certainty was "low" for all outcome measures—CIMT, PWV, and FMD (Table S4).

4 | Discussion

4.1 | Summary of Findings

Pediatric SLE is a severe rheumatological disorder that usually follows a relapsing–remitting course. Long-term complications are often inevitable due to chronic inflammation and prolonged use of immunosuppressive agents. Similar to adults, children/adolescents with SLE also seem to be at risk of developing premature atherosclerosis and adverse clinical cardiovascular complications in the future. In this study, we observed adversely altered CIMT and PWV in patients with pSLE. However, significant heterogeneity was observed for both outcome measures. Duration of disease, proportion of patients with nephritis, and levels of LDL-C correlated significantly with the CIMT and PWV MD and accounted primarily for the underlying heterogeneity. FMD was similar in patients with SLE and controls; though, only two studies provided the relevant data. The grade of evidence was "low" for all outcome measures.

4.2 | Carotid Intima–Media Thickness

CIMT is the (noninvasive) ultrasonographic measurement of the intima–media complex of the carotid arteries [25]. Increased CIMT is a microanatomical surrogate of atherosclerosis and is significantly associated with future aCVEs [26]. In adults, an increment in CIMT by 0.10 mm is associated with a 10%–15% and 13%–19% increased risk of myocardial infarction and stroke, respectively [27]. Previously published systematic reviews and meta-analyses in adults have consistently shown increased CIMT in patients with SLE, albeit with significant heterogeneity [28, 29]. The mean/weighted mean difference between CIMT of patients (aged 23–52 years) and controls was observed to be 0.08/0.07 mm [28, 30]. Similar differences were noted when only patients without a history of aCVEs were included in the analysis. We recorded a smaller mean difference of 0.04 mm between SLE and the control population. Longer duration of disease at enrolment (mean duration ranging from 2.29–19.25 years) in adults with SLE as compared to pediatric patients (mean duration ranging from 3.25–9.62 years) is the likely explanation for the higher CIMT difference in adults [28, 30]. Meta-regression analysis in our study and the previously published adult meta-analysis [30] have noted disease duration to be significantly correlated with CIMT MD. Further, the majority of the traditional cardiovascular factors were similar between groups in the studies included in our analyses. Notably, cases had higher BMI in studies by Barsalou et al. and Su-angka et al. (also, higher diastolic blood pressure in Barsalou et al.) [16, 24]; however, patients with pSLE had lower and similar values of CIMT, respectively (compared to controls in these two studies). Besides, the proportion of patients with nephritis and levels of low-density lipoprotein cholesterol (LDL-C) correlated negatively with CIMT MD on meta-regression analysis. Although nephritis is the most common major organ disease contributing to activity in pSLE, it seems plausible that shorter long-term survival in these patients results in their underrepresentation in studies assessing CIMT. The proportion of patients with nephritis in the included studies ranged from 23% to 64% (overall 43.4%) which is significantly lower than the reported values in pSLE [3]. Notably, pSLE and its treatment modalities may predispose patients to develop traditional cardiovascular risk factors as well. Taken together, available data suggests that SLE significantly impacts vascular microanatomy in both pediatric and adult patients even in the absence of other traditional cardiovascular risk factors. The final edict is still lacking due to various factors including significant heterogeneity in both pediatric and adult meta-analyses, CIMT difference noted (especially in the pediatric population) is only slightly higher than the axial resolution of most equipment (0.01–0.03 mm), and the lack of widespread studies estimating the incidence of aCVEs in patients with SLE (in comparison to healthy population).

4.3 | Pulse Wave Velocity

PWV, a marker of arterial stiffness, measures the propagation of cardiac pressure impulses along the vessel wall. An increase in aortic/carotid-femoral PWV by 1 m/s has been observed to increase the risk of cardiovascular events by 12%–14% in adults [31, 32]. Meta-analyses have reported an increased PWV in adults with SLE, albeit with significant heterogeneity. The mean/standardized mean difference (SMD) between central/aortic/carotid-femoral PWV of patients and controls has been reported to be about 1.12/0.56 m/s [33, 34]. We also noted a significant increase in PWV in pSLE. The mean/SMD noted in our study was smaller than the reported value in adults (0.48/0.49 m/s) which, similar to CIMT, appears to be due to the lesser duration of disease in the pediatric age group. Besides, our report and a previously published meta-analysis observed that the duration of SLE significantly correlates positively with PWV MD, which reflects the disease per se to be a contributor to increased vascular stiffness [30]. In our study, none of the other parameters (including traditional cardiovascular risk factors) were noted to have a similar positive association with PWV MD. Besides cf-PWV, other

parameters assessing vascular stiffness have also been found to be elevated in patients with SLE as compared to controls. Boros et al. and El Gamal et al. noted increased proximal aortic stiffness [35, 36], Su-angka et al. reported higher carotid artery stiffness, and Sozeri et al. observed enhanced augmentation index [23, 24]. In summary, these findings reflect increased central arterial stiffness in pediatric and adult SLE.

4.4 | Flow-Mediated Dilatation

FMD is a measure of postischemic (endothelial-dependent) blood flow enhancement. A decrease in FMD by 1% has been associated with a 13% enhanced risk of cardiovascular events in the adult population [37]. Various meta-analyses have noted decreased FMD in adults with SLE, although with significant heterogeneity [28, 38–40]. The mean/SMD between FMD of controls and patients was reported to be about 4/1% [28, 34, 38, 39]. In contrast, only a few studies have assessed this parameter in pSLE, which may be indicative of the technical challenges of measuring this parameter in children. One of the two studies noted increased FMD in patients with SLE (MD: 1.03%) while the other noted decreased FMD (MD −4.23%). No significant difference in FMD was noted when data from these two studies were pooled. Due to the scarcity of relevant studies, meaningful conclusions regarding FMD alterations in pSLE are not possible and require further studies (with standardized study methodology). Nonetheless, data in adults do indicate an adverse impact of SLE on vascular endothelial physiology.

4.5 | Subclinical Atherosclerosis in Pediatric Rheumatological Diseases

Previously published meta-analyses have reported subclinical atherosclerosis in other rheumatological conditions of childhood/adolescence. Increased PWV, maximum CIMT, and decreased FMD have been noted in children with Kawasaki disease (KD) [41]. In distinction to SLE, KD is an acute (mostly one-time) illness with a predilection to involve medium-sized vessels. Thus, subclinical atherosclerosis in KD reflects the long-term sequelae of acute vascular inflammation whereas the vascular injury in SLE is more chronic/long-standing. In juvenile idiopathic arthritis (JIA), the most common chronic rheumatological condition in the pediatric age group significantly increased CIMT and decreased FMD have already been documented (MD of 0.02 mm and −2.18%, respectively) [42]. Furthermore, decreased FMD (as compared to controls) was noted in active JIA and various ILAR (International League of Associations for Rheumatology) classification categories (of JIA). However, increased CIMT was only noted in systemic JIA, and was similar to healthy controls across other subtypes and groups of disease activity (greater inflammation in systemic JIA as compared to other subtypes/subgroups) [42]. In our study, FMD was comparable between patients and controls; however, data were available from only two studies. On the other hand, we observed a greater increase in CIMT in patients with SLE as compared to JIA (MD of 0.04 mm in SLE vs. 0.02 mm in JIA). This could potentially reflect a greater degree of vascular injury in SLE due to the presence of additional risk factors (including antiphospholipid antibodies, direct immune complex-mediated injury, and presence of nephritis/hypertension) besides chronic inflammation [5, 6]. However, the wide confidence interval of the CIMT MD in our study (0.01–0.08 mm) and the absence of meta-analysis directly comparing the two entities preclude definite conclusions.

4.6 | Strength and Limitations

This meta-analysis indicates the occurrence of subclinical atherosclerosis (and thereby increased cardiovascular risk) in pSLE. However, significant interstudy heterogeneity was observed, and publication bias could not be assessed due to the small number of included studies [43]. Nonetheless, data from studies using similar methodologies for noninvasive assessment of vascular microanatomy and physiology were pooled (Tables 1 and S3). Besides, we utilized meta-regression analysis to evaluate the relationship between various cardiovascular risk factors (and other parameters) and the outcome measures. Due to the paucity of relevant data, meaningful conclusions could not be obtained for FMD differences between cases and controls. Similarly, nitroglycerine-mediated dilatation and peripheral artery tonometry were not included in the meta-analysis as only very few studies have evaluated these parameters in pSLE. Nonetheless, our study harmonizes pediatric and adult data regarding long-term vascular dysfunction in SLE.

4.7 | Cardiovascular Risk Prediction in pSLE

Adult data indicate at least a 10% increase in the risk of cardiovascular events for an increment in CIMT and PWV by 0.1 mm and 1 m/s, respectively [27, 31, 32]. In our study, we noted a higher CIMT and PWV in pSLE (0.04 mm and 0.48 m/s, respectively), which extrapolates to a 4%–5% increased risk (as compared to controls) of future cardiovascular events. However, this inference needs to be interpreted with caution considering the various limitations (mentioned above) including wide confidence intervals of CIMT and PWV difference (95% CI of 0.01–0.08 mm and 0.02–0.94 m/s, respectively) and minimum recommended precision measurements of up to 0.01 mm and 0.1 m/s, respectively [44, 45].

4.8 | Future Research Implications

Utilization of a more standardized study methodology (including baseline parameters of cases like disease duration and other traditional cardiovascular risk factors) to assess subclinical atherosclerosis in pSLE seems prudent. In patients diagnosed with SLE in childhood/adolescence, longitudinal studies measuring the incidence of aCVEs in adulthood (in comparison to the healthy population) are needed to precisely quantify the cardiovascular risk in pSLE. Such studies would also facilitate specific calculation of cardiovascular risk predictability of CIMT, PWV, and FMD in pSLE. It would be insightful to assess the impact of various immunosuppressive therapy protocols (employed to treat SLE) on subclinical atherosclerosis. Such studies would enable attaining the goal of the best possible long-term cardiovascular outcomes in pSLE.

5 | Conclusions

A "low"-quality evidence suggests that pSLE may adversely impact vascular microanatomy and physiology. Longitudinal studies with uniform methodology are required to ascertain the long-term clinical consequences of altered CIMT and PWV in pSLE. Currently, scrupulous control of disease activity (inflammation), avoiding development (or treatment) of other concomitant cardiovascular risk factors (as feasible), and careful follow-up seems to be the best possible strategies to minimize long-term cardiovascular risk in pSLE.

Author Contributions

P.K.P. and A.Z.B.: inception of the idea, writing of the initial draft of the manuscript, editing and revision of the manuscript, data synthesis/analysis, and review of the literature. **A.A. and R.R.D.:** editing and revision of the manuscript, data synthesis/analysis, and review of the literature. **P.R., P.P., P.S., A.R., S.N., and R.N.:** editing and revision of manuscript, data synthesis/proofreading, and literature review. **D.B., A.S.B., and A.G.**: editing and revision of the manuscript, review of the literature, and overall supervision of manuscript preparation. All authors approve the final version of the manuscript.

Ethics Statement

As this study pertains to literature review and analysis of previously published data, specific ethics approval is not mandated.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Relevant data utilized in the preparation of this manuscript are provided in the main manuscript and supplementary tables/figures. Additional data underlying this article will be shared on reasonable request to the corresponding author.

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LETTER TO THE EDITOR

Hughes–Stovin Syndrome: A Rare Cause of Pulmonary Artery Aneurysm and Thrombosis—A Case Report

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ABSTRACT

Hughes–Stovin Syndrome (HSS) is a rare, life-threatening condition characterized by thrombophlebitis and pulmonary artery aneurysms, often viewed as a variant of Behçet's disease. Its diagnosis and management are challenging due to its rarity and overlapping symptoms with other vascular disorders. A 33-year-old male presented with massive hemoptysis and a history of recurrent oral and scrotal ulcers. Imaging revealed bilateral pulmonary emboli, a pulmonary artery aneurysm, and a large right atrial thrombus. These findings, coupled with his clinical history, led to a diagnosis of HSS. A multidisciplinary team initiated anticoagulation for the thrombus and immunosuppressive therapy (steroids, cyclophosphamide). Follow-up imaging showed improvement in both the aneurysm and thrombus. This case highlights the complexity of diagnosing and managing HSS. Early recognition, supported by a collaborative approach, is critical to improving outcomes in this rare syndrome.

Hughes–Stovin Syndrome (HSS) is a very rare and complex clinical disorder, primarily characterized by thrombophlebitis and the development of multiple pulmonary and/or bronchial artery aneurysms [1]. The syndrome is often considered a variant of Behçet's Disease (BD) due to overlapping clinical features, particularly its vascular manifestations [1–3]. However, HSS presents unique challenges, especially in terms of diagnosis and treatment, owing to its rarity—around 57 cases have been reported in the English medical literature [4].

Patients mostly men aged 12–40 typically present with symptoms such as cough, dyspnea, fever, chest pain, and hemoptysis [5]. These symptoms are associated with massive fatal hemoptysis due to the presence of pulmonary artery aneurysms, which are prone to rupture, causing death [3]. Its etiology remains poorly understood, with hypotheses suggesting infectious or vascular causes, though no definitive pathogen has been identified [1]. Given its severe vascular nature, HSS is often life-threatening if not promptly treated.

This case report aims to shed light on a patient with HSS, focusing on the diagnostic challenges and treatment considerations in managing arterial, venous, and intracardiac manifestations. Early recognition and a multidisciplinary approach are essential for improving patient outcomes in such complex cases.

A 33-year-old male visited our emergency facility with the complaint of a productive cough and blood in sputum. The blood in sputum was initially scanty in quantity for the past 1 month but turned out to be massive a few days before admission. He also experienced occasional pain on both sides of his chest, particularly associated with coughing. There was no history of fever, sore throat, breathlessness, night sweats, and wheezing. He did not report any weight loss or loss of appetite. He did not have a history of recent travel or sick contact. He does not smoke or drink alcohol.

His past medical history was significant for approximately 20 recurrent episodes of oral ulcerations over the last 2 years. He experienced a painful scrotal ulceration 1 year back. He had no other significant medical history. He was vitally stable. On physical examination, the patient was pale. A superficial ulcer was observed on the medial side of the first metatarsophalangeal joint of his right foot, which the patient attributed to trauma. The systemic

examination was unremarkable. The laboratory investigations were carried out as mentioned in Table 1, demonstrating anemia, an abnormal coagulation profile, and elevated inflammatory markers.

The radiological imaging with a chest X-ray showed a nodular opacity in the left lower zone. To investigate further, a CT pulmonary angiogram (CTPA) was performed, which demonstrated bilateral filling defects in the pulmonary artery branches (Figure 1A,B) and a small aneurysm of the subsegmental branch of the inferior lingular branch of the left pulmonary artery (Figure 2A,B). Parenchymal changes were suggestive of alveolar hemorrhage (Figure 3A,B), and a patchy consolidation was present on the left.

TABLE 1 | Labs.

Parameters	Values	References
RBC	4.5	$4.5–5.5\times10^6/\mu L$
WBC	7.4	$4.0–10.0\times10^4/\mu L$
Hgb	10.0	13.0–17.0 g/dL
Hct	32.7	40.0%–50.0%
RDW-CV	20.8	11.6%–14.0%
MCV	73.3	83–101 fL
MCHC	30.6	31.5–34.5 g/dL
MCH	22.4	27.0–32.0 pg
PDW	11.5	9.4–10.6 fL
Platelets	282	$150–410\times10^3/\mu L$
MPV	9.3	9.7–11.9 fL
ESR	87	2–28 mm/h
Prothrombin time	19.7	9.4–12.5 s
INR	1.8	
APTT	34.8	25.1–36.5 s
CRP	84.5	0.0–5.0 mg/L

Abbreviations: APTT, activated partial thromboplastin time; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; Hct, hemoglobin Hgb; hematocrit; INR, international normalized ratio; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume; PDW, platelet distribution width; RBC, red blood cells; RDW-CV, red cell distribution width coefficient of variation; WBC, white blood cells.

A bilateral lower limb venous Doppler study revealed thrombosis in the left greater saphenous vein. Furthermore, a transesophageal echocardiogram demonstrated a large, pedunculated, highly mobile mass attached to the crista terminalis, extending into the right atrium, measuring 4.2×2 cm. A smaller mobile mass was attached to the tip of the larger mass, protruding through the tricuspid valve. Two small masses were also noted in the right atrial appendage. The mass did not enhance with contrast, suggesting the likely diagnosis of thrombus, though tumor or vegetations could not be completely ruled out; hence, MRI was performed.

A Cardiac MRI confirmed the presence of thrombus as a large multilobulated mass occupying the right atrial cavity along the anterolateral wall. Perfusion studies showed the mass remained predominantly hypointense with peripheral hyperintensity, suggesting thrombus formation, with blood pooling at the periphery, excluding the possibility of a tumor like myxoma or infective endocarditis vegetation. Right ventricular and left ventricular function were normal with an ejection fraction of 54%.

The cultures for infectious diseases, Hepatitis serology, and HIV serology were negative. The autoimmune screening, particularly for HLA-B51, yielded a negative result. The coagulation study for Factor II, V, and X was normal except for a deficiency of Factor VII.

Based on the clinical presentation, imaging, and findings, the patient was diagnosed with Hughes–Stovin syndrome (HSS), a variant of Behcet's disease (BD). This was evidenced by his history of recurrent oral ulcerations, one episode of painful scrotal ulceration, the presence of a pulmonary artery aneurysm, superficial vein thrombosis (left greater saphenous vein), and the presence of intracardiac thrombi.

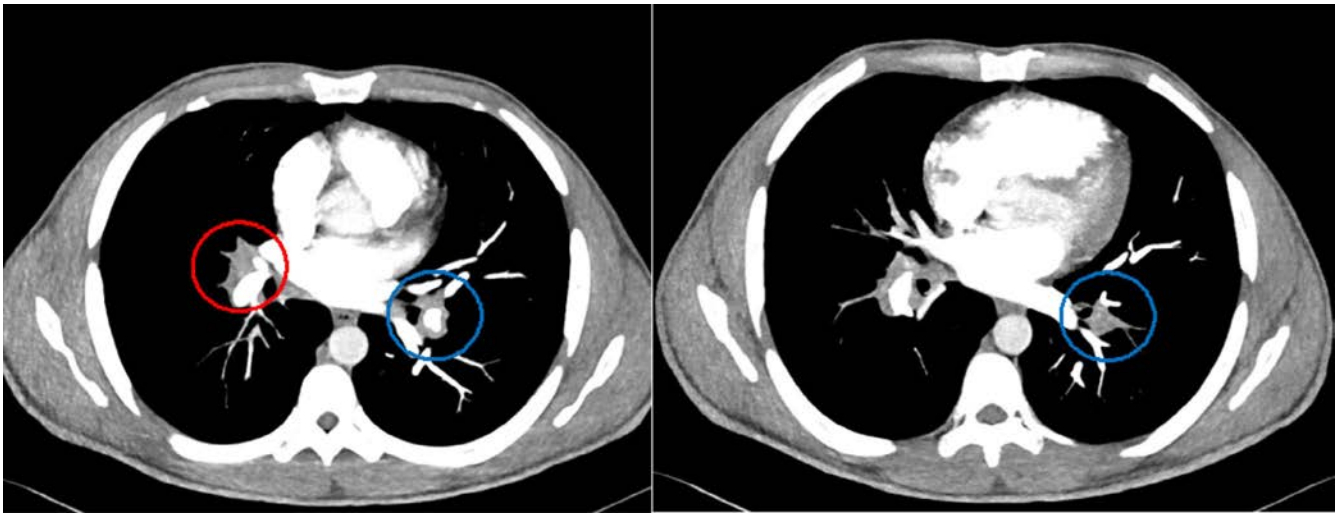

FIGURE 1 | (A and B) Bilateral Pulmonary embolism. Red: right middle pulmonary artery showing filling defect. Blue: left lower basal segmental artery showing filling defect.

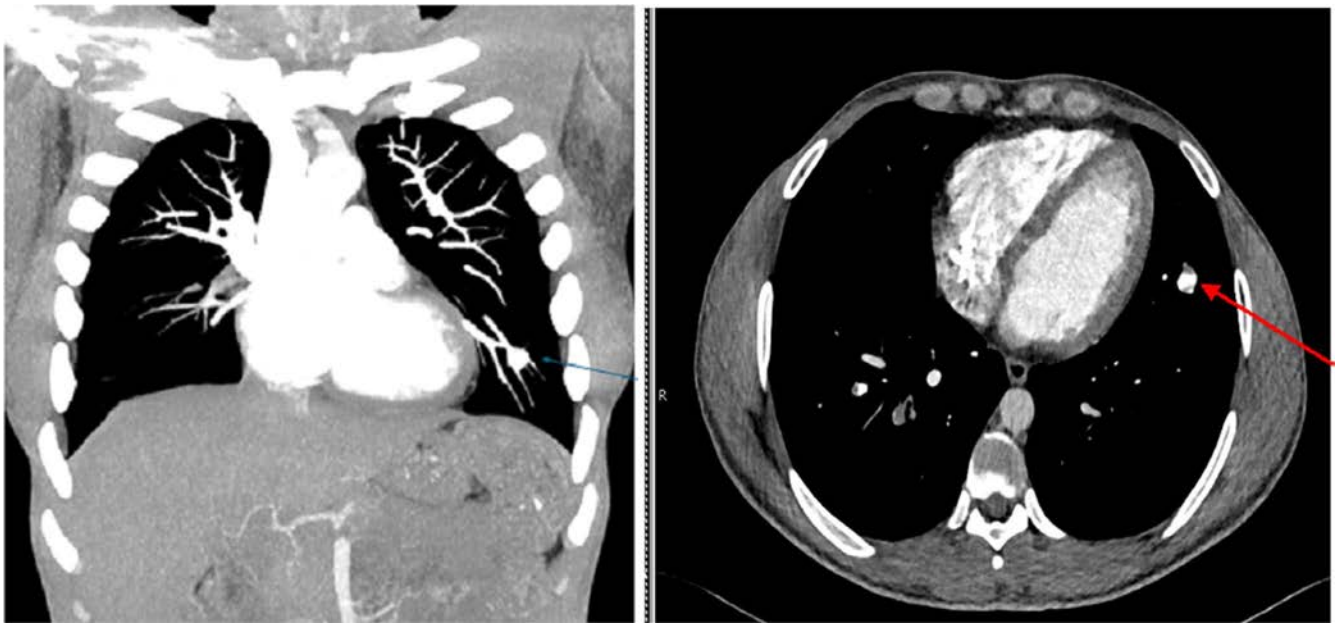

FIGURE 2 | (A and B) Aneurysmal dilatation on the left is noted in a subsegmental branch of the inferior lingular artery measuring 11 mm.

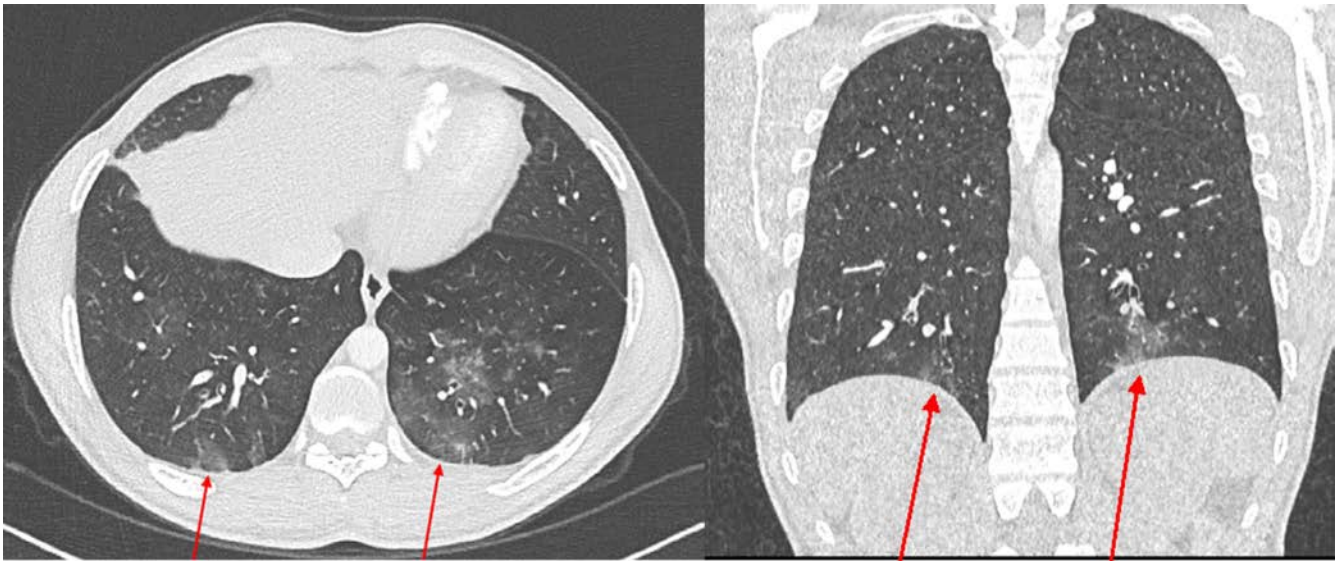

FIGURE 3 | (A and B) Bilateral lower lobe alveolar hemorrhages.

A multidisciplinary approach was employed, with a cardiologist, pulmonologist, hematologist, and rheumatologist collaboratively developing a management plan due to the patient's high-risk status. Ocular screening results were negative. The multidisciplinary team decided to initiate high-dose immunosuppressive therapy along with bone-protective supportive agents. Intravenous (IV) methylprednisolone therapy was started on Day 3 of admission, followed by IV cyclophosphamide therapy from Day 8. Inflammatory markers showed a remarkable decrease, with C-reactive protein (CRP) levels changing as follows: $84 \rightarrow 112.6 \rightarrow 66.3 \rightarrow 35.5 \rightarrow 21.4 \rightarrow 13.2 \rightarrow 9.9$. The cardiologist initiated anticoagulation therapy for the right atrial (RA) thrombus under close monitoring. A follow-up computed tomography pulmonary angiography (CTPA) performed in the third week of admission showed a 6 mm reduction in aneurysm size (from 11 to 6 mm), indicating an early response to treatment.

This case report highlights several important diagnostic and management considerations for the clinically heterogeneous syndrome that is Hughes–Stovin Syndrome (HSS). Our patient, who has already been suffering from multiple mild sequelae of undiagnosed HSS prior to his presentation, was brought to medical attention because of hemoptysis, leading to the diagnosis of very distinct vascular manifestations of the disease.

HSS carries significant morbidity and mortality, in addition to considerable social and economic burdens [6]. All vascular complications of HSS, including lower limb deep venous thrombosis and the less common but more dreaded complications like pulmonary artery involvement (PAI) and intracardiac thrombi, tend to preferentially happen in the male population, in whom they run a very severe course [6]. Interestingly, HSS has "cross-associations" such that between intracardiac thrombi and PAI,

as well as between intracardiac thrombi, Budd-chiari syndrome, and Superior vena cava (SVC) syndrome. Clinicians should screen patients for multi-territorial vascular involvement when dealing with HSS, as it often affects multiple vascular territories. The thrombosis in this condition is driven by inflammation, where cytokines activate neutrophils, leading to altered fibrinogen that resists breakdown [7]. This explains why immunosuppression is the primary treatment to prevent recurrence, as supported by clinical trials.

Our patient's tetrad includes characteristic vascular findings of deep venous thrombosis (DVT), bilateral pulmonary embolism (PE), pulmonary artery aneurysm (PAA), and right-sided intracardiac thrombus. Lower extremity venous thrombosis accounts for 70%–80% of all vascular involvement in HSS, as opposed to pulmonary artery involvement (PAI), which carries a prevalence of around 10% and tends to be bilateral, diffuse, and commonly localizing to the lower lobe arteries [6] (all of which apply to our patient). About two thirds of PAI in HSS owes to aneurysms, while one third is attributed to thrombosis [6].

Although BD can present with vascular complications, HSS is unique in its marked predilection for severe vascular involvement with limited systemic features [1, 2]. The presence of extensive pulmonary artery aneurysms and a large intracardiac thrombus without major mucocutaneous or ocular involvement makes HSS the more fitting diagnosis [4, 8, 9]. HSS is often rapidly progressive and life threatening due to pulmonary artery aneurysm rupture, which was a key concern in our case.

The EULAR 2018 guidelines recommend steroids and immunosuppressants as first-line treatment for acute DVT, without a clear role for anticoagulation due to lack of evidence on its benefit in preventing relapses; however, there might be a potential role in refractory DVT [8]. They also recommend steroids and cyclophosphamide as the first-line treatment for both PAA and thrombosis, in addition to anti-TNF agents as an option for refractory cases [8]. Of note, aneurysm regression or resolution after immunosuppression is well documented in as short as 3 months [7, 10]. However, despite maintenance immunosuppression, about 20% of patients unfortunately experience relapse in 7 years, with 26% mortality [7]. Surgery should be reserved for life-threatening cases since it carries a high mortality rate [6–8]. After starting patients on immunosuppression, interventional procedures like stenting or embolization can be performed on a case-dependent basis if the patient has a high risk of bleeding or is symptomatic despite adequate medical therapy [6, 7].

PAA's pose a significant challenge to management, especially when they co-exist with thrombosis, whether it be arterial, venous, or intracardiac; since they inform the decision of anticoagulation. All patients with HSS-related thrombosis should be started on anticoagulation and should be screened for aneurysms to avoid catastrophic bleeding.

Right-sided intracardiac thrombi are a distinctive feature of HSS. Some authors believe that any patient presenting with a right-sided intracardiac thrombus should be suspected to have HSS and investigated accordingly, even if they do not have other classical features of the disease [9]. Intracardiac thrombi in HSS seem to be a surrogate for severe disease and are more likely to occur in young males at an average age of 28 [11], even earlier than other classical disease features. In a systematic review of 25 HSS patients with intracardiac thrombus, patients were treated with immunosuppression, anticoagulation, surgery, or a combination of the two or three modalities [9]. It was difficult to establish the superiority of one treatment approach over the other since mortality was secondary to pulmonary vasculature complications for the most part, regardless of the treatment received [9]. Some clinicians are in favor of anticoagulation in tandem with immunosuppression in cases of intracardiac thrombus based on evidence from retrospective studies, given the absence of PAA or relatively low bleeding risk [5, 9].

Our patient posed a challenge with respect to medical management due to concurrently having arterial and cardiac thrombosis in addition to pulmonary artery branch aneurysm and features of alveolar hemorrhage on imaging. Once the constellation of imaging features was deemed highly characteristic of HSS by the radiologist, rheumatologist, and cardiologist, he was appropriately started on pulse steroids, followed by maintenance dose and cyclophosphamide. The decision for anticoagulation was taken by the cardiologist due to the presence of a large right atrial thrombus: a treatment strategy well-documented in the literature. Given the relatively small size of the pulmonary artery branch aneurysm (11 mm) and the fact that the patient was under observation while starting anticoagulation, the rheumatology team was on board. Classically, larger aneurysms of a size greater than 3 cm carry a higher risk for rupture and complications [7]. The decision to discharge the patient on a course of oral anticoagulation was cautiously taken by the cardiologist after learning that the aneurysm has regressed in size on repeat imaging owing to immunosuppression. Fortunately, the decision to forego thrombusaspiration—aa plan initially proposed by cardiology—was also made since the thrombus had decreased in size on repeat imaging after anticoagulation. While the cardiologist initially opted for life-long anticoagulation, the rheumatologist rather argued in favor of a few months of anticoagulation followed by reassessment through echocardiography.

While there is no unified proposed duration of treatment due to the significant clinical heterogeneity of the disease, case reports on HSS with PAI have shown improved survival in patients treated with a tapered course of steroids followed by IV pulses of cyclophosphamide for at least 2 years, after which the latter could either be continued or replaced by maintenance azathioprine: an approach later recommended by the EULAR guidelines [8, 10]. Longer duration of treatment with the addition of endovascular interventions could be implemented in refractory cases.

This case report aims to remind the internist that concurrent venous and arterial insults, especially of a characteristic pattern like (a) bilateral, extensive pulmonary artery embolisms, (b) pulmonary artery aneurysms, and (c) lower extremity deep venous thromboses in addition to right-sided intracardiac thrombi should immediately prompt entertaining a diagnosis of Hughes–Stovin Syndrome. Early involvement of the respective experts, including a rheumatologist and a cardiologist, helps to expedite the diagnosis and management, which is instrumental to morbidity and mortality outcomes. We also aim to alert the internist to screen all patients suspected to have HSS and expected to benefit from anticoagulation for aneurysms to avoid catastrophic bleeds. Moreover, all newly diagnosed HSS patients

should also be screened for lower extremity venous thrombosis, which carries a high medical, social, and economic burden.

Author Contributions

Conceptualization: Abdul Qadir and Amal Wael Abdellatif. Data curation: Abdul Qadir and Amal Wael Abdellatif. Writing – original draft: Abdul Qadir, Amal Wael Abdellatif, and Mamunul Islam. Writing – review and editing: Abdul Qadir, Amal Wael Abdellatif, Mamunul Islam, and Amir Waheed. All authors read and approved the final manuscript.

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Ethics Statement

The Medical Research Center at Hamad Medical Corporation in Qatar has granted approval for the publication of this case report.

Consent

Written informed consent was obtained from the patient for publication of the details of their medical case and any accompanying images.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that supports the finding of this case report are contained within the article. Additional information is available from the corresponding author upon reasonable request and with the approval of the relevant ethics committee.

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LETTER TO THE EDITOR

Distal Renal Tubular Acidosis due to Sjogren's Syndrome Presenting as Quadriparesis in Pregnancy

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Dear Editor,

Sjogren's syndrome is a chronic autoimmune disease characterized by oral and ocular dryness, predominantly caused by salivary and lacrimal gland dysfunction [1]. Sjogren's syndrome in pregnancy has adverse maternal and fetal outcomes, which mandates comprehensive pre-conceptional counseling and specialized antenatal care [2]. Distal renal tubular acidosis presenting primarily during pregnancy is extremely rare and is attributed to inherited causes, autoimmune disorders, drugs, or idiopathic factors [3]. Here, we present a rare case of acute-onset quadriparesis in the last trimester of pregnancy due to distal renal tubular acidosis associated with Sjogren's syndrome.

A 24-year-old female patient without previous comorbidities presented with sudden onset quadriparesis at 33 weeks of gestation on 22 October 2024. A significant obstetric history included a history of spontaneous abortion at 2 months of gestation 1 year ago, which was not evaluated, and hypothyroidism, for which she was on regular thyroxine supplements. Clinical examination revealed a power of 2/5 in all the limbs with a normal intact sensory system. Lab evaluation revealed a hemoglobin 9.1 g/dL, WBC 11,450/mm^3, platelet 132000/mm^3, creatinine 0.5 mg/dL, urea 18 mg/dL, serum sodium 142 meq/L, serum potassium 2.4 meq/L, serum chloride 123 meq/L, serum bicarbonate 11, serum magnesium 2.1 meq/L, serum calcium 8 mg/dL, ESR 54 mm/h, serum albumin 3.3 g/dL, prothrombin time 12 s, activated partial thromboplastin time 32 s, INR 1, aspartate aminotransferase 18 IU/L, and alanine aminotransferase 22 IU/L. Thyroid profile (TSH 2.58 μIU/mL, free T3 1.26 ng/mL, and free T4 0.82 ng/dL) done during evaluation was within normal limits. Peripheral smear revealed microcytic hypochromic anemia, and serum ferritin was 40 ug/L. Arterial blood gases revealed pH 7.21, pCO_2 25, bicarbonate 10 and lactate 0.7, sodium 140, potassium 2.3 and chloride 121 indicating normal anionic gap metabolic acidosis. Urine revealed pH of 8 without proteinuria, glycosuria, or active urinary sediments. Her urine potassium/creatinine ratio was 4.7, urinary anion gap was 18, and urine calcium to creatinine ratio 0.25 suggestive of kaliuresis, a positive urinary anion gap, and hypercalciuria. In the background of normal anion gap metabolic acidosis, alkaline urine, renal potassium wasting, positive urinary anion gap, and hypercalciuria, a diagnosis of distal renal tubular acidosis (dRTA) was made. She was immediately started on intravenous potassium, oral potassium citrate, and bicarbonate supplements. She underwent elective lower segment caesarean section due to preterm premature rupture of membranes and a small for gestational age fetus on the third day of admission. She delivered a boy with a birth weight of 1.78 kg and a normal pulse rate. Her X-ray KUB and abdominal ultrasound did not reveal nephrocalcinosis. Her serum potassium level was gradually corrected within 5 days, and further evaluation revealed an anti-nuclear antibody (ANA) with a 2+ speckled pattern on immunofluorescence. ANA profiling revealed strong positivity for Sjogren's syndrome type A (SSA) antibody (antibody concentration-95 U/mL) along with negative anti-double stranded DNA (anti-dsDNA), anti-Sjogren's syndrome type B (SSB), and anti-scleroderma-associated autoantigen of 70-kDa (anti-Scl 70). Rheumatoid factor test was negative. Anti-cardiolipin antibodies, anti-beta 2 glycoprotein 1, and lupus anticoagulant were negative. Schirmer's test was positive, and labial biopsy could not be performed because patient didn't give consent. She was clinically

diagnosed with primary Sjogren's syndrome with dRTA and was started on steroids, along with continuation of potassium citrate. At follow-up after 1 week, her potassium levels normalized to 3.9 meq/L with a serum bicarbonate level of 18 mEq/L, along with complete clinical remission of limb weakness.

Sjogren's syndrome is an autoimmune lymphocytic infiltrative disorder characterized by oral and ocular dysfunction with a predominant female predilection in the fifth decade [1]. The primary pathogenic drivers include derangements in innate immunity barriers, dysfunctional adaptive immunity, persistent B cell activation, and proliferation of Th1 and Th17 cells [1]. Sjogren's syndrome has organ-specific manifestations like intermittent polyarticular arthropathy, peripheral neuropathy, esophageal dysmotility, lymphocytic alveolitis, lymphocytic interstitial pneumonitis, pulmonary fibrosis, dry eyes, dry mouth, dry skin, Raynaud's phenomenon, tubulointerstitial nephritis, distal renal tubular acidosis, and hypercalciuria [4]. dRTA in pregnancy is an uncommon phenomenon, and its presentation as acute-onset quadriparesis in the last trimester of pregnancy has not been reported. dRTA during pregnancy is usually attributed to genetic defects, autoimmune disorders, tubulointerstitial disorders, and drugs such as ibuprofen [3].

Sjogren's syndrome in pregnancy is associated with adverse maternal outcomes, such as miscarriages, stillbirth, congenital malformations, pre-eclampsia, premature rupture of membranes, and postpartum deep vein thrombosis [2–9]. The initial incident of spontaneous abortion at 2 months of gestation would have been a subtle sign of primary Sjogren's syndrome, which had remained undiagnosed. The researchers mention that the incidence of spontaneous abortion ranges from 9% to 18% in Sjogren's syndrome [8]. The researchers postulate that the passage of antinuclear antibodies and antibodies to SSA may prevent appropriate implantation of embryo leading to miscarriage [2]. Mothers afflicted with primary Sjogren's syndrome are predisposed to have a higher incidence of preterm delivery, a lower birth weight baby, shorter gestation times, neonatal lupus, complete heart block, and a higher proportion of small for gestational age infants [2, 4, 8]. The clinical manifestations of preterm delivery, small for gestational age, and low birth weight of the baby observed in our case were the cardinal manifestations of undiagnosed primary Sjogren's syndrome.

Pregnancy is a volume-expanded state with low normal potassium levels despite increased serum aldosterone levels [5]. This is primarily due to the anti-mineralocorticoid effects of progesterone, which prevents hypokalaemia [5]. However, in pregnancy, there is respiratory alkalosis along with an imbalance in the filtered solute load and tubular reabsorption, which leads to an increased demand for potassium and bicarbonate [3, 5]. This increased demand for potassium, along with renal potassium wasting due to distal renal tubular acidosis, triggered acute onset quadriparesis in the antenatal mother [3, 5]. Chronic unrecognized normal anion gap metabolic acidosis leads to the dissolution of calcium from the bones, along with a physiological increase in calcium excretion during pregnancy, which was responsible for the hypercalciuria observed in our case [3]. Uncorrected metabolic acidosis leads to impaired fetal growth, spontaneous abortions, and preterm labor [6–8] which were also observed in our case.

The American college of Rheumatology has proposed a criteria for Sjogren's syndrome which is basically utilized for research classification [9]. Scoring variables include histopathological evidence of focal lymphocytic sialadenitis (Score 3), positive anti-SSA (Score 3), positive Schirmer's test (Score 1), ocular staining score > 5 (Score 1), and unstimulated salivary flow rate < 0.1 mL/min (Score 1). However, only 22% of the patients are classified as Sjogren's syndrome based on this criteria which is a huge limitation and hence clinical symptoms and signs are the only key to accurately diagnose Sjogren's syndrome [7, 8]. Moreover, these research classification criteria may be insufficient in identifying juvenile cases presenting heterogeneously without classical sicca symptoms, as in our case, whose earlier presentation was unexplained spontaneous abortion [7–9].

Distal renal tubular acidosis may be associated with inherited defects in the apical hydrogen ATPase pump or anion exchanger pump on the basolateral membrane of intercalated cells of the distal nephron [7, 9]. Inherited dRTA may present in early infancy and childhood with failure to thrive, constipation, emesis, stunted growth, and dehydration [7, 10]. Acquired causes of dRTA include Sjogren's syndrome, Hashimoto's thyroiditis, systemic lupus erythematosus, and chronic active hepatitis [10]. The diagnosis of dRTA is confirmed by normal anion gap metabolic acidosis, hypokalemia, alkaline urine, hypocitraturia, nephrocalcinosis, positive urinary anion gap, and low urine-blood pCO_2 gradient [7, 10]. Management of dRTA associated with Sjogren's syndrome includes steroids, potassium citrate, and bicarbonate supplementation as per clinical indications [7, 10].

This case reflects an uncommon heterogeneous clinical manifestation of dRTA in an antenatal mother, highlighting the importance of a comprehensive clinical assessment involving different clinical specialties in the background of unexplained pregnancy loss.

Author Contributions

All authors were responsible for the conception, design, visualization, investigation, drafting of the text, sourcing, and editing of clinical images, investigation results, critical revision of important intellectual content, and gave final approval of the manuscript.

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Ethics Statement

This case report was drafted after written informed consent of the patient in conformity with CARE clinical case reporting guidelines and the Declaration of Helsinki. The authors are accountable for all aspects of the work (including full data access, integrity of the data, and accuracy of the data analysis) to ensure that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Consent

The patient and the authors consent for publication of this manuscript as per the journal's editorial policies. Informed written consent for publication is obtained from the patient.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data for substantiating the findings of this study are available with the corresponding author and can be made available on request.

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LETTER TO THE EDITOR

Case Report: Mitral Valve Chordae Tendineae Rupture and Splenic Infarction in Granulomatosis With Polyangiitis

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Dear Editor,

A 34-year-old male patient presented with a 2-month history of cough, fever, weight loss, and fatigue. The patient's medical history shows that he was admitted to an external centre for pneumonia 1 month ago. A computed tomography (CT) scan was performed 1 month ago and showed pulmonary nodules in both lungs and an area of infectious consolidation in the lower lobe of the left lung. On physical examination at that time, there were fine crackles in the bilateral lungs, no heart murmur, and no active arthritis. Other systemic examinations were normal. With a CRP level of 210 mg/L, an ESR level of 85 mm/h, and a procalcitonin level of 3.4 ng/ml, the patient was admitted to hospital and started on piperacillin-tazobactam. Complete urinalysis, creatine kinase, liver, and renal function tests were normal. On day 7, the antibiotic treatment was changed to a combination of meropenem and tigecycline due to lack of clinical response. Culture (blood and urine) and serological tests for infectious processes showed no focus of infection during the 1-month follow-up period at the external centre.

Rheumatological markers were investigated in the patient who was admitted to our clinic due to lack of response to dual antibiotic treatment and pulmonary nodules; rheumatoid factor (RF) positive (65.9 IU/mL), antinuclear antibody (ANA) negative, and PR3-ANCA positive (109 IU/mL). On first admission to our clinic, CRP value was 247 mg/L and ESR was 66 mm/h. On chest CT scan, in the lung parenchyma, rapidly progressing solid and cavitary nodules with different stages of solid and cavitary appearance scattered in both lobes are compatible with vasculitic processes and support granulomatosis with polyangiitis (GPA) (Figure 1A–D). The nodule in the right lung was explored by tru-cut biopsy, which showed large areas of necrosis interspersed with fibrin, several silhouetted foci of vascular-like structures, and a single focus of multinucleated giant cells, interpreted as vasculitis.

The patient developed hemoptysis within a short time. The physical examination disclosed bilateral coarse lung sounds, fine crackles up to the level of upper lung zones, and an apical third degree pansystolic murmur that had emerged. Chest radiography taken after hemoptysis was interpreted as diffuse alveolar hemorrhage. The electrocardiogram was in normal sinus rhythm, and the pulse rate was 96/min. Transthoracic echocardiogram demonstrated a partial chordae rupture involving the posterior mitral leaflet and resulting in eccentric mitral valve insufficiency towards the interatrial septum (Figure 2A,B). Sudden desaturation and tachycardia developed in the patient who was followed up in the clinic, and transesophageal echocardiogram (TEE) preparation continued: 240/min and was evaluated as supraventricular tachycardia. Tachycardia resolved after adenosine and diltiazem. Clinical follow-up for tachycardia was considered appropriate in our patient with no previous history of supraventricular tachycardia and a first episode. The TEE was performed in a cardiac stable patient and showed no vegetations and a total chordae rupture of the mitral valve. The patient was consulted by the Cardiovascular Surgery Unit to evaluate the indication for emergency surgery due to chordae rupture. Mitral valve involvement due to GPA was considered, and mitral valve surgery was recommended when disease activity was controlled.

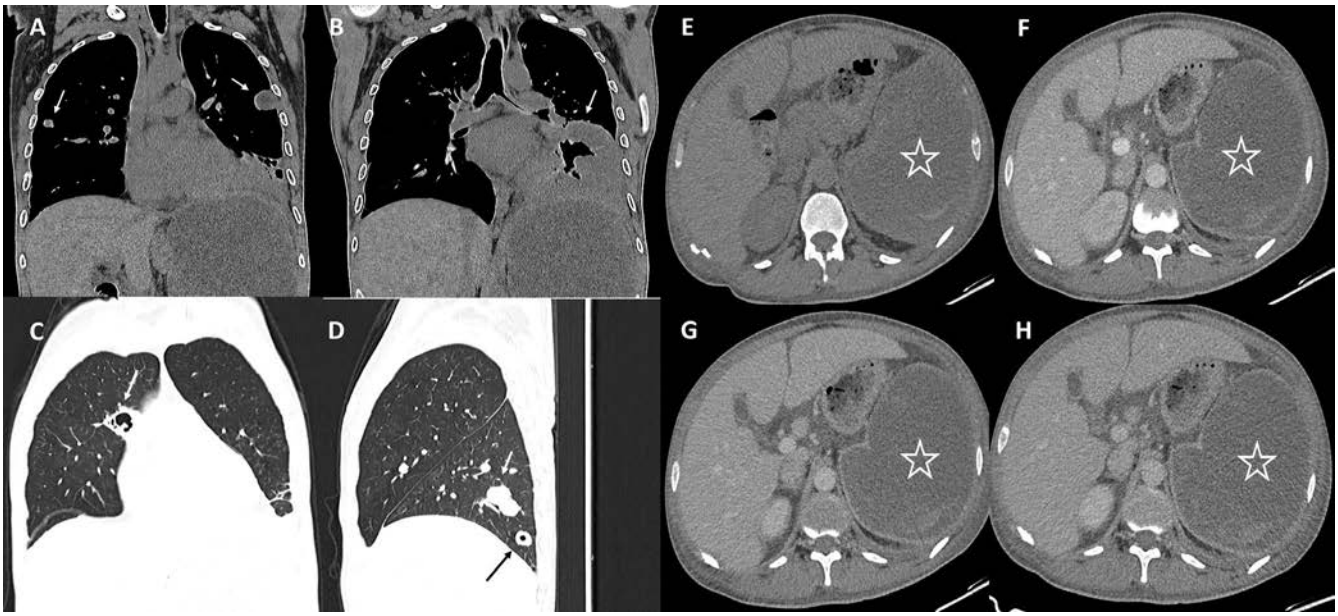

FIGURE 1 | In the coronal mediastinal window, solid nodules in the right and left lung (white arrow) (A), and large nodule with cavitation in the lower lobe of the left lung (white arrow) (B). In the lung parenchyma window, paramediastinal cavitary nodule in the right lung (white arrow) (C), adjacent solid (white arrow) and cavitary (black arrow) nodules in the posterior lower lobe of the right lung (D). Axial abdominal CT sections, without contrast (E), arterial (F), portal (G), splenic infarction showing size increase in venous phase (H), hypodense in all series, no contrast enhancement in contrast-enhanced series (asterisk).

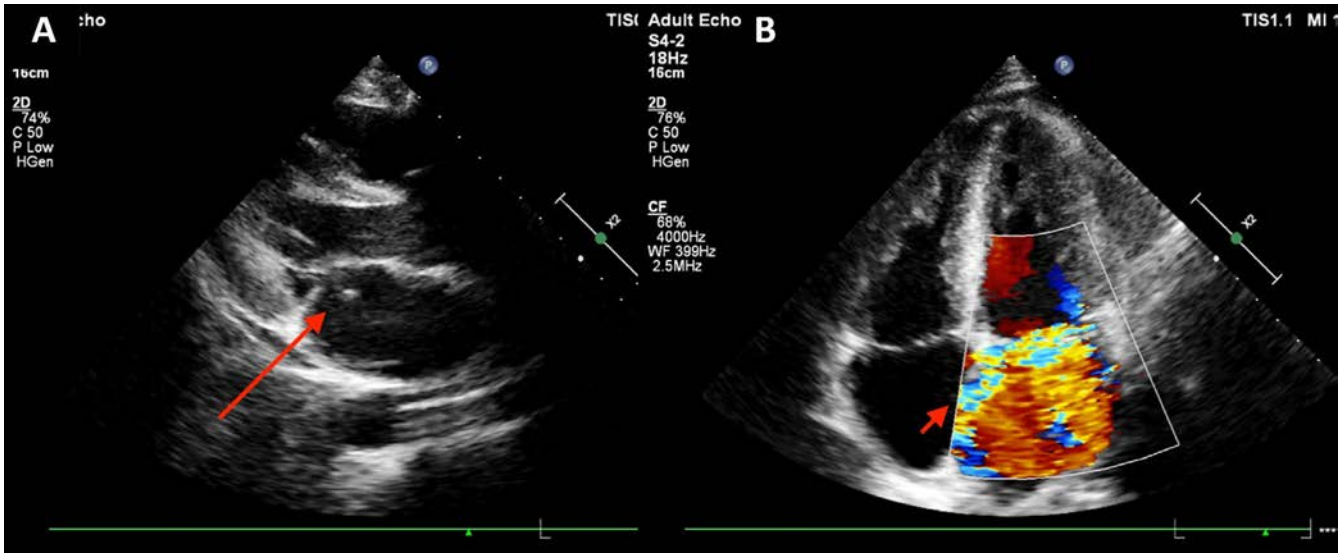

FIGURE 2 | The transthoracic echocardiogram showed, red arrow indicates chorda rupture of posterior mitral leaflet (A), and arrowhead indicates eccentric mitral valve insufficiency towards to interatrial septum due to posterior chorda rupture (B).

Culture (blood and urine) and serological tests for infectious processes showed no focus of infection. The acid-fast bacillus search in the patient's sputum sample was negative on three occasions. The subsequent long-term mycobacterial culture was also negative. Tuberculosis was excluded by acid-fast bacilli smear and Ziehl-Neelsen stain. Brucella rose bengal and tube agglutination tests were negative. Brucella was also negative in long-term blood cultures. Age- and gender-appropriate malignancy screening did not reveal any malignancy. The patient was diagnosed with GPA based on PR3-ANCA positivity, pulmonary vasculitis nodules, constitutional symptoms, and haemoptysis. Pulse steroid treatment was started (IV methylprednisolone 1.000 mg/day for 5 days). Acute phase reactants and haemoptysis improved after pulse steroid treatment. Cyclophosphamide (intermittent 15 mg/kg IV every 2 weeks for 3 doses) treatment was started because of lung and cardiac involvement with mitral valve chordae tendineae rupture as a fatal complication of GPA. Prednisone treatment was continued at a dose of 1 mg/kg/day.

During follow-up, the patient developed pain in the left upper quadrant of the abdomen and an abdominal CT with contrast was performed (Figure 1E–H). The size of the spleen was 21 cm and the parenchyma was hypodense. The diagnosis was total splenic infarction. Septic embolism was ruled out as no valve

vegetation was seen on TEE. However, general surgery did not consider emergency surgery due to the patient's current cardiac status. With 2 doses of cyclophosphamide, the patient's clinical symptoms improved significantly, and the acute phase response normalized (CRP: 4 mg/L, ESR: 21 mm/h). As his general condition was stable, the patient underwent a bioprosthetic replacement of the mitral valve in the Cardiovascular Surgery Unit. Tissue culture, Gram's stain, Giemsa stain, and Gimenez stain were performed to detect microorganisms such as Coxiella in the sample taken after mitral valve surgery. No microorganisms were detected by these stains. Elective splenectomy was planned by general surgery. Cyclophosphamide and methylprednisolone treatment was continued at planned doses. In subsequent follow-ups, infectious processes were observed. He was hospitalized and antibiotherapy was given. At that time, the patient had proteinuria below 500 mg/day and no active urine sediment. Renal failure was added and hemodialysis was started. IVIG treatment was continued due to activation of the disease and infection status. The patient was transferred to the intensive care unit due to the progressive clinical picture, but it resulted in mortality.

GPA, formerly known as Wegener's granulomatosis, is a necrotizing vasculitis affecting small arteries with variable organ involvement and disease severity. The prevalence of GPA ranges from 2.3 to 146.0 cases per million population. The most common and severely affected organs are the upper and lower respiratory tract and the kidneys [1].

Cardiac manifestations are rare and may be underdiagnosed in the absence of typical clinical signs. Pericarditis and coronary vasculitis are the most common manifestations of cardiac involvement in GPA (half of cases); myocarditis and conduction system abnormalities have also been described. In a large cohort of patients with GPA, cardiac involvement was reported to be 3.3%, with pericarditis being the most common [2]. It was observed that cardiac involvement was not significantly associated with other disease symptoms and that there was no difference in outcomes when compared with patients without cardiac involvement. The frequency of cardiac involvement was reported by the European Vasculitis Study Group [3] as 5.7% and by the French group [4] as 13% of patients with GPA. Some cohort studies have highlighted the importance of cardiac involvement in GPA, in addition to its potentially life-threatening potential [5].

Valvular heart disease appears to be very rare in GPA. In a series of 21 patients with GPA heart valve involvement, 57.1% had aortic valve involvement; 14.3% had mitral valve involvement; and 28.6% had both aortic and mitral valve involvement. Aortic regurgitation is the main finding; other findings are rare: mitral regurgitation, aortic stenosis, valvular vegetations, mass involving the mitral valve, and multiple atrial masses. In cases of GPA-specific valvular involvement, 50% of the valvular lesions were present at diagnosis, and 19% were observed after the first year of vasculitis diagnosis [6]. Most previous cases of GPA had valvular lesions at initial presentation. Most patients received immunosuppressive therapy, and valve replacement was required in the majority of cases, with only a few known cases where immunosuppressive therapy resulted in complete resolution of the valve lesions [7]. The goals of therapeutic management are to reduce treatment-associated toxicity and obtain the best possible overall disease control. In severe generalized disease, cyclophosphamide and rituximab are the gold standard for reducing remission, whereas glucocorticoids are the first-line treatment for quickly controlling inflammation. The immunosuppressive medications commonly used for maintenance therapy are methotrexate and azathioprine [8, 9]. In a recent study of valvular involvement in GPA, observers found that aortic regurgitation was the most common valvular disease in this patient group and that the aortic valves were the most prone to degeneration during the course of GPA [10].

Subacute bacterial endocarditis (SBE) should be excluded primarily on the basis of negative blood cultures, evidence of intracellular pathogens (by serology and bacterial polymerase chain reaction) and the absence of microorganisms on pathological examination [11]. In the present report, blood cultures were negative, PR3-ANCA was positive, and vasculitis findings were confirmed by lung biopsy. However, this type of ANCA-related valve involvement can be seen in SBE. In a recent study of 109 cases of infective endocarditis, 18% had c-ANCA or p-ANCA, and 8% had anti-PR3 or anti-MPO antibodies [12]. Clinical and pathological data should be taken into account to suggest a definitive diagnosis. In fact, treatment of presumed GPA in a patient with SBE with an immunosuppressive agent such as cyclophosphamide may be harmful. However, after immunosuppressive treatment, our patient showed laboratory and clinical improvement.

In a recent case report, a patient presenting with pulmonary nodules and constitutional symptoms was initially diagnosed with infective endocarditis due to the detection of mitral valve vegetation and mitral regurgitation and the development of splenic infarction. In this case, similar to our case, persistent negative cultures, lack of improvement with antibiotics, PR3-ANCA positivity, and improvement with immunosuppression supported the diagnosis of GPA. Rituximab was administered after pulse steroid treatment [13]. In the management of ANCA-associated vasculitis (AAV), high-dose steroids with cyclophosphamide or rituximab should be administered if organ involvement is life-threatening [14].

The spleen is affected in a variety of ways in GPA. To date, many splenic pathologies such as splenomegaly, capsular adhesion, and infarction have been described in GPA. The frequency of splenic involvement in patients with AAV is unknown. Most of them are detected incidentally. In a retrospective study of 69 AAVs (38 patients with GPA), the rate of splenic infarction in the entire AAV group was 10% [15]. All patients with splenic infarction had GPA with PR3-ANCA positive serology. Their findings suggest that splenic infarction may occur in a higher proportion than previously reported. The frequency of splenic infarction in GPA is probably underestimated because it is usually clinically silent.

In conclusion, we report the first case in the literature of mitral valve chordae tendineae rupture and splenic infarction associated with GPA with well-documented echocardiographic and radiological analysis. Our case report highlights the need for clinical and diagnostic studies to make an accurate diagnosis in a patient presenting with valvular and splenic involvement and GPA, and to make a differential diagnosis with conditions such as infective endocarditis.

Author Contributions

I.S., U.G., F.C., M.U., A.T., and S.U. were responsible for designing the framework of the article, writing the article, and editing the images. S.U. and O.S.G. were responsible for the literature review and writing this article. I.S., S.U., and O.S.G. were responsible for revising the details of the article. I.S. provided the ideas for this article and verified the accuracy of the content.

Ethics Statement

This was a purely observational case study that did not alter the patient's management and clinical outcomes. Thus, ethics approval was not required for this case report.

Consent

Informed consent was obtained from the patient involved in the study.

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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ORIGINAL ARTICLE

Markers of T Lymphocyte Activation in Children With Kawasaki Disease: An Experimental Study From North India

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ABSTRACT

Background: The exact pathogenesis of Kawasaki disease (KD) is unknown despite extensive research in the area. Several studies have also implicated $CD8^+$ T lymphocytes in the pathogenesis of KD. However, studies on the activation status of T lymphocytes have shown conflicting results.

Methods: In this prospective study, early (CD69) and late activation (HLA-DR) markers were assessed in T lymphocytes by flow cytometry. We assessed serum levels of soluble CD25 (sCD25) by enzyme-linked immunosorbent assay. We compared these activation markers between children with KD ($n = 10$), febrile controls ($n = 9$), and healthy controls ($n = 10$). Furthermore, we studied the HLA-DRA and HLA-DRB gene expression in subgroups of KD with or without coronary artery aneurysms (CAAs).

Results: A significantly higher percentage of CD69 in $CD3^+$ and CD3 + $CD4^+$ T lymphocytes was noted in KD and febrile controls compared with healthy controls. We found no significant increase in late activation marker HLA-DR in CD3, CD3 + $CD4^+$, and CD3 + $CD8^+$ lymphocytes between KD, febrile, and healthy controls. We observed higher levels of sCD25 in KD and febrile controls than in healthy controls. Longitudinal follow-up in KD showed a decreasing trend of CD69 expression in CD3 + $CD8^+$ lymphocytes and sCD25 levels over time. HLA-DRA and HLABRB expression was comparable between children with CAAs and those without CAAs.

Conclusion: Our study showed early but not late activation of T lymphocytes in children with KD. Markers of lymphocyte activation do fall with subsidence of systemic inflammation following intravenous immunoglobulin therapy in KD.

1 | Introduction

Kawasaki disease (KD) is a systemic vasculitis that predominantly affects children less than 5 years of age [1]. Since its discovery in 1967, the exact pathogenesis of KD is not precisely known despite extensive research. Pathology specimens of coronary aneurysms have demonstrated T-cell infiltration in blood vessels, which leads to coronary artery aneurysms, particularly $CD8^+$ T lymphocytes [2]. Moreover, several genes related to lymphocyte activation are found to be associated with KD,

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Summary

- Early, but not, late activation markers of T lymphocytes are elevated in acute stages of Kawasaki disease, especially, in milder forms of the disease.
- Markers of T lymphocyte activation do decrease with subsidence of systemic inflammation in Kawasaki disease.
- In Indian population, HLA-DR expression is comparable between children with Kawasaki disease with coronary artery aneurysms (CAAs) and those without CAAs.

especially *ITPKC*, *CASP3*, and *ORA1*. Cyclosporine, a drug that inhibits calcineurin and blocks lymphocyte activation, has been found to arrest the progression of coronary artery aneurysms in KD. Several authors have also investigated the T-cell activation profile by flow cytometry; however, the results are conflicting. Some studies have also shown selective expansion of Vβ repertoires of $CD8^+$ T lymphocytes, hinting toward a superantigen-induced etiology [3]. Few studies have also reported prolonged hypo responsiveness of $CD8^+$ T lymphocytes to activation via T-cell antigen receptor CD3 [4]. Overall, it seems that $CD8^+$ T lymphocytes play an essential role in the pathogenesis of KD. However, literature on lymphocyte activation profiles in the chronic and late stages of KD is scarce. T-cell activation profiles can help in a better understanding of pathogenesis and can help in the early diagnosis of KD.

Herein, we have comprehensively assessed the lymphocyte activation status in KD by flow cytometry using both early and late activation markers-CD69 and HLA-DR, respectively, and by measuring soluble CD25 levels (sIL2R) by enzyme-linked immunosorbent assay (ELISA) during the acute and convalescent stages of the KD. Our study is the first of its kind on Indian children with KD, and we envisage that the results of our study would also provide insight into the pathogenesis of KD.

2 | Methods

2.1 | Study Population

The study was carried out at the Allergy Immunology Unit, Department of Pediatrics, Postgraduate Institute of Medical Education and Research, Chandigarh, India. Approval was taken from the Institutional Ethical Committee (IEC) (INT/IEC/2020/SPL-1285) before enrolling subjects. Patients were enrolled during the period 1st January 2019 to 31st July 2020. Ten ($n = 10$) children ≤ 7 years of age who were diagnosed with Kawasaki Disease (KD) were enrolled. The diagnosis of KD was based on criteria given by the American Heart Association (AHA) [5]. We also enrolled 10 ($n = 10$) healthy children and 09 febrile patients (not suffering from KD) as controls. Children with KD who had already received intravenous immunoglobulin (IVIg) before presentation were excluded. Written informed consent was obtained from the legal guardians of patients and controls.

2.2 | Sample Collection, Storage, and Tests

Peripheral venous blood (3 mL) was drawn by aseptic venipuncture from the study subjects and the control group. Samples were collected at three different phases of illness in patients with KD—acute stage prior to administration of IVIg and aspirin, 24 h after administration of IVIg, and 3 months after the onset of illness. Febrile controls and normal controls had their blood drawn once similarly.

Blood samples collected in a plain vacutainer were left undisturbed at room temperature for 15–30 min and allowed to clot. Samples were centrifuged at 1500 rpm for 10 min to remove the clot. Supernatant serum was then transferred to a clean polypropylene tube using a Pasteur pipette and was stored at −20°C.

2.3 | Estimation of CD69 and HLA-DR in T Lymphocytes by Flowcytometry

One hundred (100) μL of whole blood sample was transferred from EDTA vacutainer to FACS tubes and immunolabeled with relevant antibodies: CD45, CD3, CD4, $CD8^+$, CD69, and HLA-DR. The sample was then incubated in the dark at room temperature for 30 min. After incubation, erythrocytes were lysed twice using a commercially available FACS lysing solution. Remaining white blood cells were washed twice with phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA) and 0.1% NaN3. The expression of surface markers was then assessed by flow cytometry (Beckman coulter, Navios) using *Kaluza* software. Lymphocytes were gated on forward/CD45 vs. side scatter plot. $CD3^+$, CD3 + $CD4^+$, and CD3 + $CD8^+$ lymphocytes were selected from gated lymphocytes (Figure S1). Percentage expression and median fluorescence intensity of CD69 and HLA-DR were assessed on $CD3^+$, CD3 + $CD4^+$, and CD3 + $CD8^+$ lymphocytes.

2.4 | Estimation of Soluble CD25 by ELISA

Commercially available Quantikine Human SIL-2R alpha Immunoassay (solid phase 96 well ELISA kit) procured from R&D Biotech systems (Cat. No: DR2A00) was used. Stored serum samples were brought to room temperature, and 100 μL of Assay diluent was added to each well. 100 μL of Conjugate was added to each well, and it was covered with a new plate sealer and incubated at room temperature for 3 h. 200 μL Substrate Solution was added to each well, followed by 50 μL Stop Solution. Results were interpreted at 450 nm within 30 min. Results were expressed in pg/mL.

2.5 | HLA-DR mRNA Expression

mRNA was extracted from whole blood using the commercially available kit per the manufacturer's instructions (Qiagen, USA). Extracted mRNA was converted into cDNA using a commercially available kit before quantifying the copy of desired gene's mRNA. Expression results were normalized by the housekeeping gene expression, that is, 18SRNA. Relative fold change was calculated from the CT value of each sample and further compared with the control's CT value (Table S1).

2.6 | Statistical Analysis

Statistical analysis was carried out with SPSS statistical software version 20.0 (SPSS Inc., Chicago, IL, USA). For subgroup comparisons, the Kruskal–Wallis test was performed using the Dunn test method with Sidak correction. In the comparison of two groups, Mann–Whitney test was used. For the comparison of similar groups over time, the Friedman test was applied. $p < 0.05$ was considered significant.

3 | Results

Our study was conducted amongst children with KD ($n = 10$; 4 complete and 6 incomplete), febrile controls ($n = 9$) and healthy controls ($n = 10$). Reasons for fever in febrile controls include pneumonia ($n = 4$), urinary tract infection ($n = 2$), sepsis ($n = 1$), enteric fever ($n = 1$), and tuberculosis ($n = 1$). The median age group (interquartile range (IQR)) in cases, febrile controls, and healthy controls was 3.5 (2.12, 4.75), 3 (2, 5), and 3.5 years (3, 5), respectively. The male–female ratio among the patients with KD was 4:1. All patients with KD were treated with IVIg (Table S2). The median duration between the onset of fever and IVIg administration was 12 days (IQR: 9.25, 15). The median duration of fever in febrile controls at enrollment was 6 days (IQR: 3, 7). Echocardiography was done in all patients with KD that showed normal coronaries except transient dilatation in 1. Children with KD had a longer duration of fever (Median [IQR]: 12 days [9.25, 15]) compared with the febrile controls (Median [IQR]: 5 days [3, 7]), which was statistically significant (p—0.008).

Flow cytometry parameters-CD3%, CD3 counts, CD3 + CD4%, CD4 counts, CD3 + CD8 + %, $CD8^+$ counts, and CD4/$CD8^+$ ratio were comparable between cases and febrile controls. There was no significant difference in baseline CD3% ($p = 0.639$) and CD3 + CD4(%) ($p = 0.529$) in cases compared to febrile and healthy controls (Table 1). CD3 + $CD8^+$ and CD3 + $CD4^+$ T lymphocyte percentages were lower in cases compared to febrile healthy controls; however, they were not statistically significant. A progressive reduction in CD3 + $CD4^+$ T lymphocytes was observed in children with KD following IVIg therapy (Table 2).

Baseline early activation marker (CD69) was significantly higher in CD3 ($p = 0.008$) and CD4 ($p = 0.044$) T lymphocytes amongst cases and febrile controls in comparison to healthy controls. However, no significant difference was noted for late activation marker in CD3 ($p = 0.235$), CD4 ($p = 0.184$) and CD8 ($p = 0.893$) T lymphocytes. No significant difference in lymphocyte activation markers CD69 and HLA DR expression in $CD3^+$, CD3 + $CD4^+$, and CD3 + $CD8^+$ lymphocytes was noted between cases and febrile controls during the acute stage (Table 1). During the follow-up, a gradual decline in CD69 expression in CD8 T lymphocytes was observed in children with KD (Table 2).

Baseline soluble CD25 was significantly higher ($p = 0.001$) in cases and febrile controls as compared to healthy controls (Figure 1A). A significant ($p = 0.002$) increase was seen in soluble CD25 in acute KD (median 3681.50 pg/mL) with a decreasing trend 24 h post-IVIg (median: 1602.70 pg/mL) and 3-month post-IVIg infusion (median 1408.00 pg/mL) (Figure 1B). However, sCD25 values at third-month follow-up in children with KD (median 1408 pg/mL) were higher when compared to healthy controls (median: 514 pg/mL) (Table S3).

As we did not find any significant difference in HLA-DR expression between children with KD and healthy controls by flow cytometry, we also performed an RT-PCR assay for mRNA expression of HLA-DRA and HLA-DRB. For this purpose, we included five children with KD without CAA and five children with KD who had a giant aneurysm (Table S4). No significant difference in mRNA expression of HLA-DRA and HLA-DRB was noted between KD and healthy controls. We also did not note any difference in HLA-DRA and HLA-DRB expression among KD with CAAs and without CAAs (Figure 2).

TABLE 1 | Summary of parameters studied in acute KD and comparison with febrile and healthy control.

Parameters	Group			p
	Kawasaki disease ($n = 10$)	Febrile control ($n = 9$)	Healthy control ($n = 10$)	
$CD3^+$ (%) (Baseline)	73.97 (68.64–81.33)	68.07 (65.46–78.82)	69.17 (64.81–74.04)	0.639
$CD3^+/CD69^+$ (%) (Baseline)*	3.5 (1.77–7.22)	4.71 (4.62–5.4)	2.42 (2.21–3.46)	0.008
$CD3^+$/HLA-DR+ (%) (Baseline)	5.03 (3.95–6.8)	7.52 (6.43–22.8)	7.63 (4.68–10.39)	0.235
$CD3^+/CD4^+$ (%) (Baseline)	57.06 (42.95–60.69)	46.59 (41.71–60.05)	46.08 (38.24–58.03)	0.529
$CD3^+/CD4^+/CD69^+$ (%) (Baseline)*	2.65 (1.81–4.74)	3.57 (2.68–4.48)	1.74 (0.78–2.29)	0.044
$CD3^+/CD4^+$/HLA-DR+ (%) (Baseline)	3.29 (2.48–4.7)	5.98 (3.66–6.29)	3.54 (2.16–4.65)	0.184
$CD3^+/CD8^+/CD69^+$ (%) (Baseline)	4.84 (3.07–6.7)	6.33 (3.32–7.93)	2.7 (1.86–3.27)	0.056
$CD3^+/CD8^+$/HLA-DR+ (%) (Baseline)	9.05 (5.1–11.68)	7.85 (6.51–8.25)	6.65 (5.19–14.46)	0.893
Soluble CD25 value (pg/mL)*	3681.5 (2026.75–4542)	2755 (2026–4098)	514 (503.75–534)	0.001

*$p < 0.05$—significant.

TABLE 2 | Summary of parameters studied in acute KD, 24 h after IVIg, and 3-month post-IVIg infusion.

Parameters	Baseline (median [IQR])	24 h post- IVIg (median [IQR])	3-month post-IVIg (median [IQR])	*p*
CD3%	73.97 (68.64–81.33)	73.44 (67.35–76.19)	70.88 (64.49–75.69)	0.74
CD3 + CD69 + %	3.50 (1.77–7.22)	3.00 (2.47–4.06)	2.53 (2.02–4.15)	0.90
CD3 + HLA-DR %	5.03 (3.95–6.80)	5.56 (3.59–9.62)	6.38 (3.04–14.84)	0.74
CD3 + $CD4^+$ %*	57.06 (42.95–60.69)	57.87 (56.38–63.97)	48.49 (39.86–49.96)	0.02
CD3 + CD4 + CD69%	2.65 (1.81–4.74)	1.81 (1.66–3.05)	2.14 (0.89–5.07)	0.67
CD3 + CD4 + HLA-DR%	3.29 (2.48–4.70)	3.06 (1.93–5.19)	2.62 (2.00–6.99)	0.49
CD3 + CD8 + %	35.97 (31.13–37.25)	34.53 (30.02–35.79)	38.63 (18.11–41.79)	0.74
CD4/$CD8^+$ ratio	1.59 (0.81–2.37)	1.69 (1.16–2.22)	1.22 (0.28–2.16)	0.407
CD3 + CD8 + CD69%	4.84 (3.07–6.70)	4.30 (3.63–6.48)	1.90 (0.95–2.91)	0.06
CD3 + CD8 + HLA-DR %	9.05 (5.10–11.68)	11.19 (4.27–17.65)	6.99 (2.60–8.89)	0.67
Soluble CD25 value (pg/mL)*	3681.50 (2026.75–4542.00)	1602.70 (1334.75–1723.38)	1408.00 (1195.88–1601.05)	0.002

*$p < 0.05$—significant.

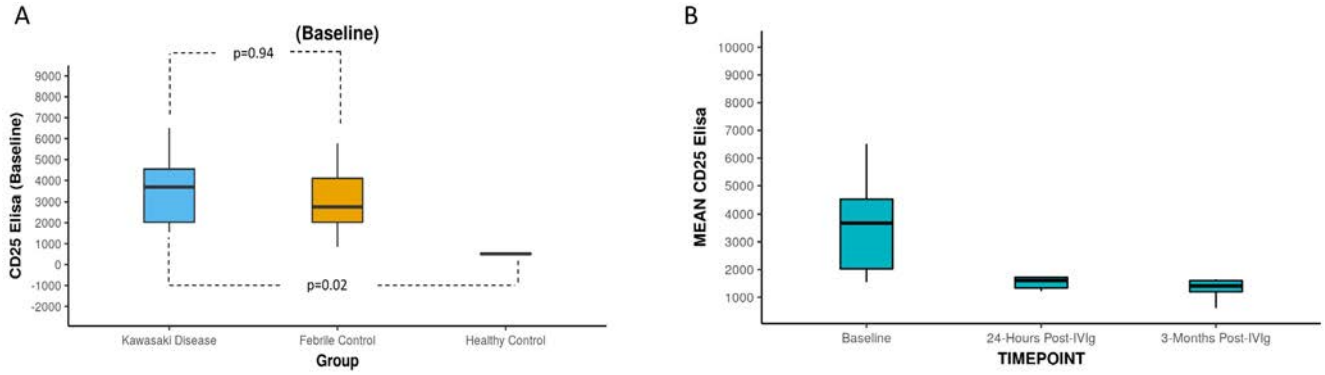

FIGURE 1 | (A) Comparison of the acute KD, febrile control and healthy control in terms of soluble CD25 levels. (B) Comparison of soluble CD25 levels (pg/mL) amongst the acute KD, 24 h post-IVIg, and 3-month follow-up in children with KD.

4 | Discussion

Autopsy studies and reports on lymphocyte activation in KD have shown that T lymphocytes play a significant role in the pathogenesis of KD [2] (Tables 3 and 4). Though many studies have explored T-cell activation status in KD, results have not been consistent. Moreover, most of the studies on lymphocyte activation have focused on children with IVIg-resistant forms of KD or children with CAAs. We studied early and late activation markers of T lymphocytes in a cohort of 10 North Indian children with KD (who did not have CAAs or IVIg-resistant KD) and compared them with febrile and healthy age-matched controls. We also conducted a longitudinal follow-up of KD patients to see the response in T-cell activation following IVIg therapy. To the best of our knowledge, T-cell activation in KD has not been studied in the Indian population before, and our study is, perhaps, the first on this aspect.

Our study revealed a significantly higher percentage of CD69 in $CD3^+$ and CD3 + $CD4^+$ T lymphocytes in KD and febrile controls than in healthy controls. We also observed a higher expression of CD69 in CD3 + $CD8^+$ lymphocytes in KD and febrile controls compared to healthy controls. We found no significant increase in the late activation marker HLA DR in CD3, CD3 + $CD4^+$, and CD3 + $CD8^+$ lymphocytes between KD, febrile, and healthy controls. Soluble interleukin-2 receptor (CD25) was significantly elevated in children with KD and febrile controls compared to healthy controls. Longitudinal follow-up in children with KD showed a decreasing trend of the early activation marker CD69 in CD3 + $CD8^+$ lymphocytes and serum soluble CD25 levels over time. However, a statistically significant difference was only found in soluble CD25 values. mRNA expression of HLA-DRA and HLA-DRB was comparable between children with KD who had CAAs and those without CAAs.

Terai et al. reported a decrease in $CD3^+$, $CD4^+$, and $CD8^+$ T lymphocytes during the acute phase of KD (< 5 days) [6]. Ehara et al. showed a significant decrease in $CD8^+$ T lymphocytes in patients with acute KD compared to healthy and febrile

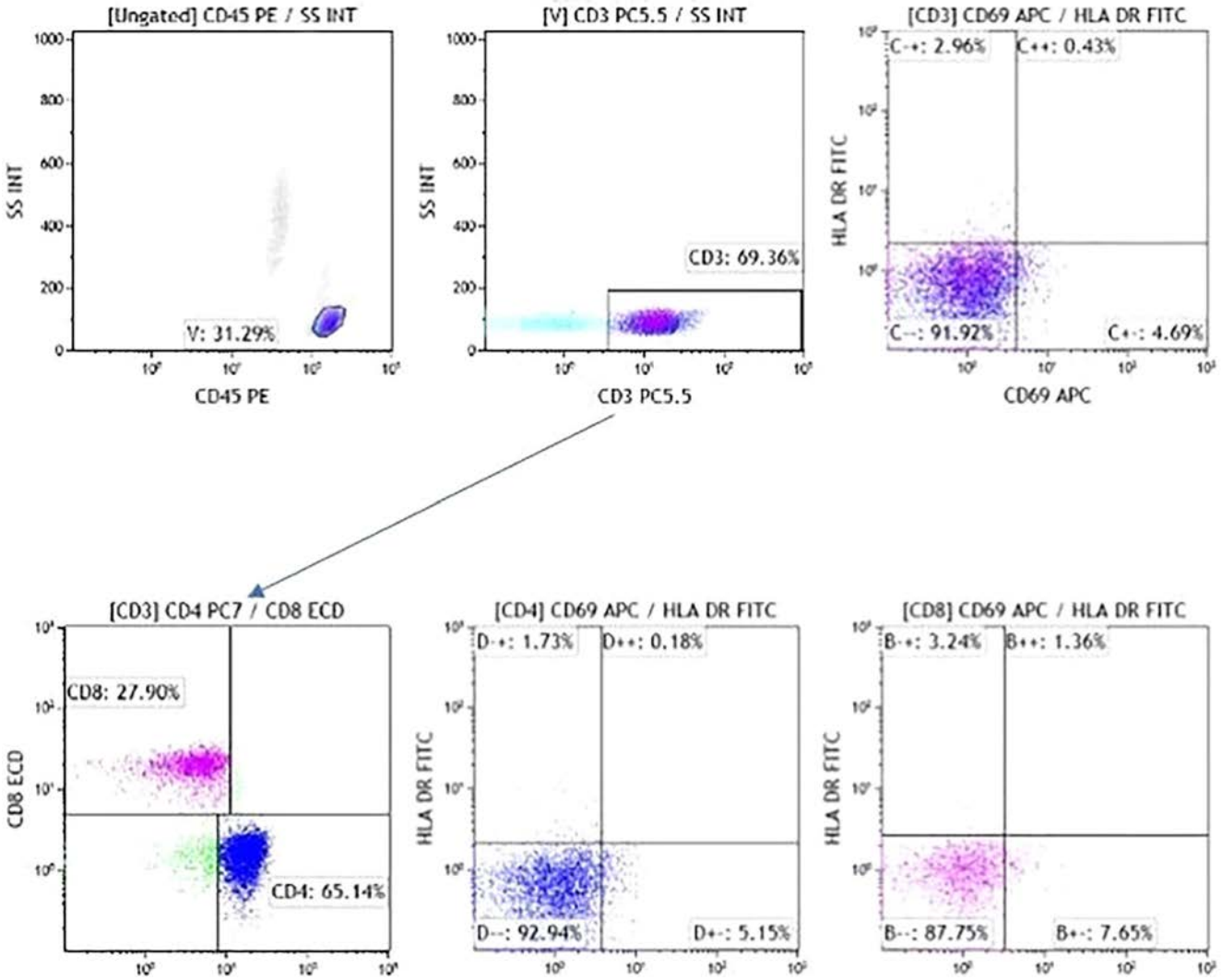

FIGURE 2 | Expression of HLA-DRA and HLA-DRB in controls and KD subgroups. (A, B) Comparison of HLA-DRA and HLA-DRB expression (fold change) in KD, KD with CAA, and controls. Alterations in both gene expressions were not statistically significant. (C, D) Elevated levels of HLA-DRA and decreased levels of HLA-DRB in controls as compared to KD were observed. However, changes in expression levels were statistically not significant. (E, F) KD without CAA showed lower levels of HLA-DRA and higher levels of HLA-DRB as compared to KD with CAA. However, changes in expression levels were statistically not significant.

controls. $CD8^+$ T lymphocytes increased during the convalescent stage post-IVIg; however, values were lower than those of healthy controls [9]. Similarly, we also found a decrease in CD3 + $CD8^+$ cytotoxic T lymphocytes in our patients with KD compared to controls. We also found that the cytotoxic T-cell percentage was lower in patients with KD even at the third-month follow-up compared to healthy controls, similar to Ehara et al. [9]. Matsuguma et al. demonstrated a decrease in $CD4^+$ and $CD8^+$ T lymphocytes in the acute stage of KD in IVIg-responsive groups compared to IVIg-resistant groups. They also showed a significant decrease in $CD4^+$ and $CD8^+$ T lymphocytes in the acute stages of KD compared to healthy controls. CD4 + and $CD8^+$ T lymphocytes increased during the convalescent stage [12]. However, we could not demonstrate a decrease in CD4 + helper T lymphocytes in our cohort, probably due to the difference in population demography. A decrease in peripheral cytotoxic T lymphocytes (CD3 + $CD8^+$) in the acute stages of KD implies that these cells are preferentially recruited to the site of inflammation.

CD69 is an early activation marker expressed in T lymphocytes and many other hematopoietic cells [17]. Brogan et al. showed no significant difference in CD69 expression in $CD4^+$ and $CD8^+$ T lymphocytes between cases and healthy controls [7]. Ikeda et al. described that CD69 expression on $CD3^+$ T lymphocytes was significantly higher in patients with acute KD compared to healthy controls and convalescent phase [8]. Ehara et al. found a significant elevation in CD69 expression in $CD8^+$ cells compared to healthy and febrile controls. No difference in CD69 expression was noted in $CD4^+$ T lymphocytes [9]. In another study, Ye et al. found no significant difference in CD69 expression in $CD4^+$ and $CD8^+$ T lymphocytes in patients with KD compared to normal controls. No difference was noted in IVIg-sensitive and IVIg-resistant groups [11]. We also found higher expression of CD69 on $CD8^+$ T lymphocytes; however, this difference was not statistically significant. Our study also showed a significant increase in CD69 expression on CD3 and CD4 T lymphocytes in children with KD compared to healthy controls.

TABLE 3 | Previous studies on flow cytometry-based assessment of T-lymphocyte activation in Kawasaki disease.

S. no	Study year and Country	T-cell activation markers studied	Study population characteristics	Comments
1	Terai et al. 1987 Japan [6]	CD3, CD4, $CD8^+$, and HLA-DR	46 patients, mean age of 1 year (0.25–5.6) Rate of CAA: 29%	Decreased $CD3^+$, $CD4^+$, $CD8^+$ T lymphocytes in acute phases of KD. Decreased CD4 HLA-DR Expression in acute stages
2	Brogan et al. 2007 London, UK [7]	CD4, $CD8^+$ Vβ and CD 69 expression on CD4, $CD8^+$ lymphocytes	9 males and 7 females with complete KD were compared with healthy controls Mean age 2.3 (0.6–6.8y) CAA: 31% IVIg resistance: 2% Median duration of fever: 10 days	No significant increase in CD69 expression was seen in $CD4^+$ and $CD8^+$ T cells in acute KD as compared to healthy control Vβ restricted $CD4^+$ and $CD8^+$ activation was seen in 72% of patients in KD
3	Ikeda et al. 2009 Japan [8]	Activation status of peripheral blood mononuclear cells (PBMCs) by flow cytometry, DNA microarray and quantitative reverse transcription–polymerase chain reaction. CD69, human leukocyte antigen D-related (HLA-DR) and CD25 were used as activation markers	38 typical KD patients with mean age 2 (0.3–7.3) were compared with healthy controls in both acute and convalescent stage CAA: NA IVIg resistance: NA Med duration of fever: 8 days	$CD69^+$ cells in both natural killer cells and γδT lymphocytes at acute-phase KD were significantly higher than those at convalescent-phase KD. HLA-DR expression had no significant difference
4	Ehara et al. 2010 Japan [9]	CD69, HLA-DR in $CD8^+$ cells using flowcytometry	14 boys and 16 girls with typical KD with mean age 2.3 (0.6–6.8) were compared in both acute and convalescent phase. Comparisons were done between healthy and febrile controls CAA: None IVIg resistance: NA Med Duration of fever: 5 days	Increased expression of CD69 on CD8 + T lymphocytes, mean SD, 47.6% 22.14% as compared to febrile controls (10.7%, 8.16%) and healthy controls (3.36%, 2.51%) No significant difference in CD8 + HLA-DR-positive cells in acute KD, febrile and healthy controls
5	Wakiguchi et al. 2014 Japan [10]	HLA-DR expression in CD4 and $CD8^+$ T lymphocytes was determined by flowcytometry	82 children with KD mean age 1.7 (1–7.5) were analyzed. HLA-DR expression was compared between IVIg-sensitive and resistant patients CAA: 2% in sensitive, 21% in resistant IVIg resistance: 37% Med duration of fever: 5 days	The percentages of HLA-DR expression was significantly lower in IVIg-sensitive patients as compared to IVIg-resistant patients

(Continues)

TABLE 3 | (Continued)

S. no	Study year and Country	T-cell activation markers studied	Study population characteristics	Comments
6	Ye et al. 2016 China [11]	HLA-DR, CD69 and CD25 was studies in peripheral blood T lymphocytes using flow cytometry	162 KD patients with median age of 2.4 years. and male to female ratio of 92/70 were evaluated at admission and 3 days after IVIg CAA: 1% in sensitive, 10% resistant IVIg resistance: 14.2% Med duration of fever: NA	Increased HLA-DR expression on $CD8^+$ T lymphocytes and median ratio of CD8 + HLA- DR+/CD8 + $CD25^+$ T lymphocytes were significantly increased in patients as compared to healthy controls Significant decrease in HLA-DR + $CD8^+$ cells post-IVIg infusion was noted Imbalances between CD8 activation and inhibition plays a vital role in pathogenesis of KD
7	Matsuguma et al. 2019 Japan [12]	CD14 + $CD16^+$ and HLA-DR expression on CD4 and $CD8^+$ T lymphocytes using flow cytometry	46 KD patients with mean age 1.8 (0.8–8) were enrolled and divided into IVIg-sensitive and resistant groups CAA: 1% in sensitive, 10% resistant IVIg resistance: 14.2% Med duration of fever: NA	CD8 + HLA-DR+ number before IVIg and significantly increased in IVIg-resistant group after treatment, no difference in IVIg-sensitive group CD4 + HLA-DR+ lymphocytes increased significantly post-IVIg in both groups CD14 + $CD16^+$ decreased in IVIg-sensitive group, however no changes IVIg-resistant group

Abbreviations: CAA, coronary artery abnormality; IVIg, intravenous immunoglobulin; KD, Kawasaki disease; NA, not available.

TABLE 4 | Previous studies on soluble CD25 or soluble interleukin-2 receptor levels in patients with Kawasaki disease.

S. no	Study year and Country	Methodology	Study population characteristics	Comments
1	Lang et al. 1987 Canada [13]	Soluble IL-2R by Rapid one step sandwich ELISA between acute KD and healthy control and subsequent follow-up in subacute and convalescent stage	26 patients of acute KD 18 patients after 2 weeks and 18 in convalescent stage compared with healthy controls	Significantly increase in sIL-2R found in patients' sera during the acute phase of Kawasaki disease. Serum sIL-2R levels remained significantly elevated in the early subacute phase Serum levels of slL-2R had significantly decreased in patients tested later in the subacute phase of their illness. Nevertheless, these subacute/convalescent sIL-2R levels remained elevated compared with control values
2	Barron et al. 1990 US [14]	Soluble IL-2R by Rapid one step sandwich ELISA	93 patients Soluble IL-2R was compared in acute stage with healthy control and children with measles	Significantly increased sIL-2R in KD patients in acute stage as compared to healthy control and children with measles
3	Suzuki et al. 2010 Japan [15]	Soluble IL-2R and IL-6 by Rapid one step sandwich ELISA and comparison between IVIg-sensitive and IVIg resistance cases	27 patients divided into three groups based on IVIg sensitivity (CAA: 14%; IVIg resistance: 55%) All complete KD	No significant differences in the serum levels of the two cytokines before initial IVIg among the three groups, but significant intergroup differences were evident after initial IVIg in the serum levels of soluble interleukin-2 receptor ($p < 0.01$, Group A>C) and interleukin-6 ($p < 0.01$, Group A>B>C)
4	Niet al 2019 China [16]	Plasma levels of sIL-2R and IL-2 family cytokines were measured using a CBA kit	33 patients with mean age 1 (0.25–0.45) years analyzed in acute stage and 5 days post-IVIg CAA: 30%, IVIg resistance 3%, (All complete KD)	Decrease in s IL-2R post-IVIg administration however higher than control. Highest value seen in patients with CAA

Abbreviations: CAA, coronary artery abnormality; IVIg, intravenous immunoglobulin; KD, Kawasaki disease.

HLA-DR is considered a marker of late activation in KD. Terai et al. evaluated 46 patients with KD in which HLA-DR expression on $CD4^+$ T Lymphocytes was studied in 17 children with KD, including 7 with CAA. They found no significant difference in children with KD compared to healthy controls [6]. Ikeda et al. studied 38 children with KD in Japan. Both T-cell activation markers and genes for innate immunity were analyzed. HLA-DR expression was studied on $CD8^+$ T lymphocytes. No significant difference was found in HLA-DR expression on CD3 + $CD8^+$ T lymphocytes compared with normal controls [8]. Ehara et al. in 30 patients from Japan evaluated HLA-DR in the acute phase of KD, and a comparison with febrile and healthy control was made. Early activation markers were raised, but HLA-DR expression on $CD8^+$ T lymphocytes was not significantly different between cases and controls [9]. HLA-DR expression has also been studied as a marker of IVIg resistance. Wakiguchi et al. evaluated HLA-DR expression in $CD4^+$ and $CD8^+$ T lymphocytes. They found an increase in percentages of HLA-DR+ of $CD4^+$ and $CD8^+$ cells in the IVIg resistance group compared to the IVIg-sensitive group in children with KD [10]. Ye et al. found an increased percentage of CD8 + HLA-DR+ T lymphocytes in acute KD compared to controls. The authors also found an increased ratio of CD8 + HLA-DR lymphocyte/CD8 + CD69 + T lymphocytes in the IVIg resistance group.

Authors suggested that HLA-DR expression could be used as a marker for IVIg resistance [11]. Matsuguma et al. found a significant increase in $CD4^+$ HLA-DR+ cells post-IVIg in both IVIg-sensitive and resistant groups. A low number of CD8 + HLA-DR+ cells were present in both IVIg-sensitive and resistant groups that increased after IVIg in the resistant group. However, no change was noted in the IVIg-sensitive group [12]. In our cohort, we did not find any significant difference in HLA-DR expression by flow cytometry in CD4 + and $CD8^+$ T lymphocytes. This is probably because our study did not include IVIg-resistant KD patients. mRNA expression of HLA-DR was also not different between children with KD with CAAs and without CAAs. A long delay in the diagnosis of KD in our cohort could explain normal HLA-DR expression in our cohort.

Several studies on sIL-2R levels in children with KD have been carried out in countries like the US, Canada, Japan, and China. Lang et al. showed significantly elevated soluble IL-2R in patients with acute KD, which decreased in the subacute and convalescent phases; nonetheless, these levels remained significantly elevated even in the convalescent phase compared to healthy controls [13]. Similar findings were observed in our study.

Barron et al. showed a significantly increased concentration of sCD25 in acute KD compared to both adult and pediatric controls and children with measles. Soluble IL-2R levels were significantly greater in children who subsequently developed coronary artery aneurysms than in patients with normal coronary arteries [14]. Our study also showed persistently elevated soluble IL-2R even 3 months after IVIg; however, this value was lesser compared to acute stages. Suzuki et al. in Japan compared three groups of KD patients classified depending on IVIg sensitivity. A significant increase in sIL-2R was found in IVIg-resistant children compared to IVIg-sensitive patients [15]. A recent study done by Ni et al. in 2019 confirmed previous findings of a significant increase of soluble IL-2R in acute KD compared to healthy and febrile controls; very high levels were documented in patients with CAAs [16]. Our study also reflected similar results on sCD25 levels in acute KD and follow-up.

The study's strength is that we have used multiple markers for lymphocyte activation. Our study replicated similar results from many previous studies; however, we have also shown that HLA-DR expression is not high in children with KD who did not have CAAs or IVIg resistance. We also showed that soluble CD25 assessment by ELISA performed best amongst the lymphocyte activation markers to study the lymphocyte activation status in KD. However, the limitation is that the results of this study cannot draw firm conclusions as the study is carried out on a preliminary basis in a small sample size. However, this is understandable considering that the study had to be completed in a limited period.

5 | Conclusion

To conclude, early but not late activation of T lymphocytes occurs in milder forms of KD. Markers of lymphocyte activation fall with the subsidence of systemic inflammation following IVIg therapy in KD; however, soluble CD25 levels were higher in patients with KD even 3 months after diagnosis compared to healthy controls. Results of our study must be replicated in larger cohorts with the inclusion of severe forms of KD to derive final conclusions on lymphocyte activation status in KD.

Author Contributions

Paritosh Sharma (PS) conceived and designed the study, wrote the first draft of paper, performed experiments, collected the data, contributed data or analysis tools, and interpreted the data. Pandiarajan Vignesh (PV) conceived and designed the study, performed experiments, contributed data or analysis tools, interpreted the data, editing of manuscript, and final approval. Sanjib Mondal (SM) contributed to editing of manuscript and literature review. Kanika Arora (KA) performed flow cytometry experiments, collected the data, contributed data or analysis tools, and editing of manuscript. Jitendra Kumar Shandilya (JS) performed flow cytometry experiments, interpreted the data, contributed data or analysis tools, editing of manuscript. Jhumki Das (JD) performed flow cytometry experiments, interpreted the data, contributed data or analysis tools, and editing of manuscript. Rajni Kumrah (RK) performed ELISA experiments, interpreted the data, contributed data or analysis tools, editing of manuscript. Kaushal Sharma (KS) performed RT-PCR experiments, interpreted the data, contributed data or analysis tools, and editing of manuscript. Jyoti Sharma (JS) interpreted the RT-PCR data, contributed data or analysis tools, and editing of manuscript. Deepti Suri (DS) collected the data, contributed data or analysis tools, editing of manuscript. Amit Rawat (AR) provided key/unique reagents, analyzed the data, and interpreted the data. Surjit Singh (SS) contributed to editing of manuscript, critical review, and final approval.

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Ethics Statement

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and

its later amendments or comparable ethical standards. The study was approved by the Institute Ethical Committee (INT/IEC/2020/SPL-1285, dt. 13.12.2018).

Consent

Informed written consent was taken from parents of patient included in the report. Written informed consent for publication of their clinical details was obtained from the parents. All coauthors consented for the publication of this manuscript.

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

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LETTER TO THE EDITOR

Computer Vision Techniques for Enhanced Detection of JIA Joint Inflammation

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1 | Introduction

Juvenile idiopathic arthritis (JIA) is a heterogeneous group of inflammatory diseases characterized by joint swelling and/or effusion, inflammation, and painful, limited joint movement, with or without joint tenderness (Figure 1a) [1, 2]. According to the International League of Associations for Rheumatology (ILAR), JIA is a diagnosis of exclusion, defined by onset before the age of 16 years and the presence of clinical symptoms lasting at least 6 weeks [1, 2]. While advances in therapeutic strategies, including the use of biologics and disease-modifying anti-rheumatic drugs (DMARDs), have improved outcomes, challenges remain in achieving effective treatment and consistent follow-up [3, 4]. Treatment often requires balancing disease suppression with the side effects of long-term medication use, such as growth inhibition, increased risk of infections, and medication-induced comorbidities [1, 3, 4]. Moreover, follow-up is complicated by the fluctuating nature of disease activity, the lack of reliable biomarkers for remission, and limited pediatric-specific tools for monitoring disease progression. Infrared thermography is a noninvasive, rapid, and contactless detection technique that uses a thermal camera to measure skin temperature by capturing non-ionizing infrared radiation emitted from the skin (Figure 1a) [5, 6]. The heat patterns captured by the camera generate a physiological heat map that is invaluable for detecting temperature anomalies indicative of inflammation [5].

The primary aim of this study was to develop and validate a simple, cost-effective tool combining thermal imaging and computer vision technology to enhance the monitoring of disease activity in children with JIA. By integrating infrared thermography with advanced image analysis, the study seeks to create a practical follow-up adjunct, providing an efficient and accessible method to monitor ongoing disease activity.

2 | Materials and Methods

Our study, a prospective observational analysis, was carried out at the Department of Pediatric Rheumatology at Başkent University Hospital from December 2022 through December 2023. We enrolled 18 pediatric JIA patients, aged 1–18 years, experiencing an acute knee arthritis flare, as defined by ILAR. A control group included 19 age-matched, healthy children without signs of inflammation. We collected demographic and clinical data from all participants. Exclusion criteria encompassed a history of peripheral arterial disease, autonomic neuropathy, recent use of cosmetic or anti-inflammatory products, acupuncture, or temperature-altering extremity treatments. The Başkent University Medical and Health Sciences Research Board and Ethics Committee approved the study, and we obtained written informed consent from the patients or their parents as required.

Participants followed a standard protocol in an examination room maintained at 21°C ± 2.0°C, 50% ± 5% humidity, and controlled airflow. After a 20-min acclimatization, thermal imaging was performed using a FLIR ONE Edge Pro wireless thermal camera, adhering to the Glamorgan Protocol guidelines [5]. The camera, set at an emission level of 0.98 and mounted on a tripod, wirelessly connected to an OPPO A74 smartphone. Images of each participant's affected and unaffected knees were taken from 1 m away at a 90° angle, capturing five images per subject. All images were saved automatically to the mobile's storage and FLIR Ignite software.

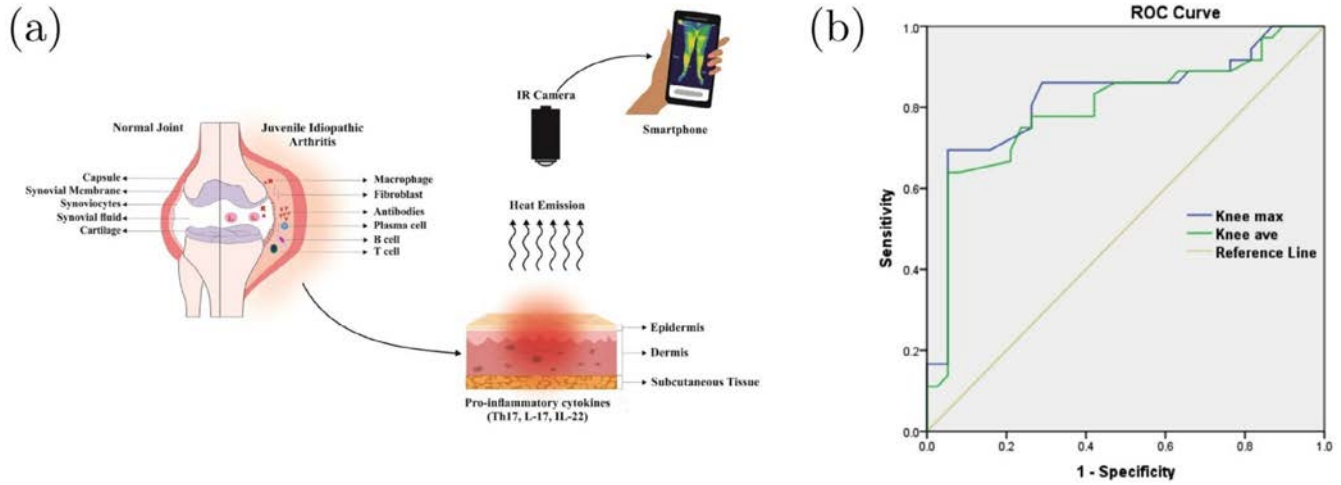

FIGURE 1 | Diagnostic capabilities of smartphone IR technology and ROC analysis for JIA. (a) The figure demonstrates the effectiveness of smartphone infrared technology in identifying inflammation in JIA-affected joints. The thermal images, processed through a dedicated app. (b) The ROC curve displays the diagnostic accuracy of assessing knee T max and T ave temperatures for distinguishing between affected and unaffected individuals. The AUC values for T max (depicted in blue) and T ave (shown in green) underline the strong diagnostic potential of these measurements.

A Python script was developed to integrate sophisticated computer vision functionalities enabled by OpenCV and the advanced data manipulation capabilities of Pandas [7]. The OpenCV library facilitated interactive image analysis, while NumPy provided robust support for mathematical computations. The Pandas library was utilized for efficient data management. The protocol instructs the user to pinpoint two centers for each region of interest (ROI) and a third point on the ROI's periphery to establish its radius. The script automatically calculates the distance from the center to this peripheral point to determine the circular ROI's radius. It then computes the mean pixel values within these ROIs by masking the selected areas and averaging the intensities of the color bands—blue, green, and red—which correspond to temperature readings in infrared images. The script logs the coordinates, radii, and mean pixel values for each color channel of the ROIs and stores them in a structured Excel file. To enhance accuracy and reproducibility, it provides real-time visual feedback, updating the image after each selection to display the current ROIs with their specified radii. Repeated tests on the same dataset confirmed stable mean pixel values (< 5% variation), while independent ROI selections demonstrated high inter-rater reliability. For detailed code specifics, please refer to Data S1. Additionally, FLIR Ignite software enabled us to record the maximum (T max), minimum (T min), and average temperatures (T ave) for each ROI.

3 | Statistics

The study employed both parametric (*t*-test for normally distributed variables) and nonparametric (Mann–Whitney *U*-test for non-normally distributed variables) statistical methods to compare the temperature distributions and means of pixel values between the patient and control groups, respectively. Cohen's *d* and effect size *r* were calculated to determine the magnitude of differences observed. Diagnostic accuracy was critically assessed through receiver operating characteristic (ROC) analysis, delineating the area under the curve (AUC) and computing sensitivity, specificity, as well as predictive values at optimal threshold temperatures. The Type I error probability was determined as $\alpha = 0.05$ in all hypothesis tests, and statistical evaluations were performed using the SPSS v25.0 software package (IBM Co.).

4 | Results

Table 1 shows no statistically significant age difference between JIA patients ($n = 18$) and healthy controls ($n = 19$), averaging 9.5 ± 4.6 and 11.6 ± 4.9 years, respectively ($p = 0.183$). Likewise, no significant differences were noted in gender, weight, height, heart rate, or blood pressure between the groups ($p > 0.05$). Among JIA patients, arthritis prevalence was 61.1% in the right knee, 55.6% in the left knee, and 16.6% in both knees. Joint inflammation was assessed using two methods: FLIR camera temperature measurements (T max, T min, T ave) and a Python-based image analysis. Significant differences were found in all temperature measures for both knees, with the mean T max of the right knee in JIA patients being notably higher than in controls (33.4°C vs. 31.7°C, $p = 0.001$). ROC analysis confirmed the diagnostic utility of these measurements, with AUC values of 0.82 for T max and 0.79 for T ave. The optimal T max threshold was 32.2°C, achieving a sensitivity of 86.1% and specificity of 71.1%. Predictive values for T max were 86.1% (positive) and 71.1% (negative), and for T ave, 77.8% and 78.9%, respectively (Figure 1b).

Additionally, our Python-based thermal image analysis provided further insights through color spectrum evaluation. The median pixel values in the red spectral band, indicative of heat from inflammation, were significantly higher in JIA patients. For the right knee, the median red value was 227.5 (range: 170.8–253.1) in patients, compared to 175.4 (range: 28.3–242.4) in healthy controls ($p = 0.006$, $r = 0.50$). Similar findings were observed for the left knee, where the median red value was 228.2 (range: 124.7–242.5) in patients and 190.7 (range: 21.2–251.3) in controls ($p = 0.005$, $r = 0.50$) (Table 1).

TABLE 1 | Thermal imaging and color analysis for knee arthritis in JIA versus healthy children with diagnostic accuracy parameters.

	JIA ($n=18$)	Healthy children ($n=19$)		
Joint (°C)	**Mean ± SD**	**Mean ± SD**	***p***	**Cohen's *d*[a]**
Right knee				
T max	33.4 ± 1.6	31.7 ± 1.2	0.001	1.16
T min	31.3 ± 1.7	29.9 ± 1.4	0.014	0.85
T ave	32.4 ± 1.7	30.8 ± 1.2	0.003	1.05
Left knee				
T max	33.5 ± 1.4	31.7 ± 1.2	0.001	1.32
T min	31.4 ± 1.7	30.3 ± 1.3	0.036	0.71
T ave	32.5 ± 1.5	30.9 ± 1.2	0.001	1.79

Mann–whitney *U*-test

Parameter	**JIA med (min-max)**	**Healthy children med (min-max)**	**Mann–Whitney *U***	**Asymp. Sig. (two-tailed)**	**Effect size *r*[b]**
Right knee					
Blue	61.9 (12.8–140.5)	38.1 (13.2–158.9)	157.00	0.671	0.07
Green	100.6 (30.6–196.9)	175.9 (9.3–200.3)	90.00	0.014	0.40
Red	227.5 (170.8–253.1)	175.4 (28.3–242.4)	80.00	0.006	0.50
Left knee					
Blue	62.9 (17.9–143.5)	31.4 (13.4–155.7)	131.00	0.224	0.20
Green	91.3 (23.1–202.2)	171.4 (27.7–201.5)	100.00	0.031	0.35
Red	228.2 (124.7–242.5)	190.7 (21.2–251.3)	79.00	0.005	0.50

Diagnostic accuracy parameters for knee arthritis

Temp °C	**AUC (95% CI)**	**Cutoff, °C**	**Sens. (%)**	**Spec. (%)**	**PPD (%)**
Knee					
T max	0.82 (0.72–0.92)	32.3	86.1	71.1	86.1
T ave	0.79 (0.69–0.90)	31.6	75.0	76.3	75.0

[a]Cohen's *d* values 0.2, 0.5, and 0.8 represent small, medium, and large effect size, respectively.
[b]Effect size *r* values 0.1, 0.3, and 0.5 represent small, medium, and large effect size, respectively. The blue, green, and red color bands represent the mean pixel values from infrared thermography.

5 | Discussion

Our study highlights the potential of infrared thermography, enhanced with sophisticated computer vision algorithms, as a non-invasive adjunct follow-up tool for pediatric rheumatologic conditions. Significant differences in infrared temperature measurements and color spectrum analysis were observed between JIA patients and healthy peers, underscoring the utility of this technology in clinical practice. In our study, the optimal T max threshold was 32.2°C, achieving a sensitivity of 86.1% and specificity of 71.1%. Supporting our observations, previous research by Gatt et al. showed that adults with a palm temperature above 31.5°C were more prone to RA [8]. Thermal imaging has previously demonstrated its effectiveness in identifying abnormal thermal patterns across various medical conditions, such as rheumatoid arthritis (RA), diabetic foot, breast cancer, and hidradenitis suppurativa [4, 8–10]. The use of the FLIR Ignite system revealed higher T max and T ave values in arthritic knees compared to healthy controls. The significant sensitivity and specificity of these measurements highlight their potential for pediatric screening. Additionally, the balanced predictive values of T ave confirm its effectiveness in detecting or ruling out inflammation, corroborating previous research. Unlike traditional thermal imaging studies that rely on proprietary software [4, 8–10], our research leverages automated computer vision to analyze ROI in thermal images. Notably, the red spectral band—indicative of higher temperatures due to inflammation—showed significant differences between groups, with a substantial effect size ($r=0.50$) for median pixel values. These results validate thermographic color metrics as reliable biomarkers for inflammation. In infrared thermography, the colors in an image serve as visual indicators of temperature variances, with a commonly used palette ranging from white for the hottest regions to blue for the coldest, passing through red and yellow

for intermediate temperatures. This color coding facilitates quick visual analysis but should be interpreted with reference to the specific thermal camera's settings and the provided color-temperature scale to ensure accuracy [5, 11–14].

Bauer et al. recently applied AI-based image processing to thermographic images of 212 young female volunteers to categorize stages of cellulite with more than 80% accuracy [12]. Our approach is unique in integrating real-time automated analysis with Python-based algorithms, minimizing human error and enhancing reproducibility.

By demonstrating the diagnostic potential of infrared thermography in pediatric rheumatology, our study sets the stage for future advancements in diagnostic precision and treatment personalization. Our findings validate the effectiveness of infrared thermography combined with computer vision as an adjunct follow-up tool for detecting joint inflammation in JIA children. A key feature of our study is the customized Python script with OpenCV integration for automated ROI detection and analysis, which minimizes human error and enhances accuracy and reliability. While this approach requires further refinement and validation, it shows promise in streamlining diagnosis and enabling timely, individualized treatment. This study has several limitations, including its single-center design and relatively small sample size. Therefore, the findings should be interpreted with caution.

Author Contributions

The authors take 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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Supporting Information

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EDITORIAL

Anti-Inflammatory Mechanisms of Antidiabetic Therapies: Metabolic Regulation and Inflammatory Pathways

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1 | Introduction

The interplay between metabolic regulation and inflammation in diabetes mellitus has illuminated the dual benefits of antidiabetic therapies. Beyond their established roles in glycemic control, medications such as metformin, sodium-glucose cotransporter 2 (SGLT2) inhibitors, and glucagon-like peptide-1 (GLP-1) receptor agonists have demonstrated significant anti-inflammatory properties [1]. These drugs not only target hyperglycemia but also modulate key inflammatory pathways implicated in diabetes-related complications. This editorial explores the mechanisms underlying their anti-inflammatory effects, reviews supporting clinical evidence, and examines potential applications in managing inflammatory conditions associated with diabetes and related comorbidities.

2 | Metformin: The Veteran Anti-Inflammatory Agent

Metformin, a cornerstone therapy in the management of type 2 diabetes, offers benefits that extend beyond glucose-lowering effects. Its primary mechanism includes the inhibition of hepatic gluconeogenesis and enhancement of insulin sensitivity. However, growing evidence suggests that metformin modulates key inflammatory pathways, contributing to its broader therapeutic potential [2]. Central to this effect is the activation of adenosine monophosphate-activated protein kinase (AMPK), a master regulator of cellular energy homeostasis. AMPK activation inhibits the nuclear factor kappa B (NF-κB) pathway, a critical driver of pro-inflammatory signaling, thus reducing systemic inflammation.

Clinical studies substantiate metformin's anti-inflammatory properties. For instance, a meta-analysis demonstrated significant reductions in C-reactive protein (CRP), a biomarker of systemic inflammation, in patients with type 2 diabetes treated with metformin compared to alternative therapies [3]. These findings are particularly relevant given the role of chronic inflammation in the progression of diabetes-related complications, including microvascular and macrovascular diseases [4]. Moreover, metformin's ability to attenuate inflammatory pathways suggests potential applications beyond diabetes, particularly in managing rheumatologic conditions such as rheumatoid arthritis and

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systemic lupus erythematosus [5]. This is especially compelling for patients with overlapping metabolic and inflammatory syndromes, positioning metformin as a promising agent in treating complex, multifactorial diseases. Furthermore, studies have indicated that metformin use prior to influenza vaccination is associated with lower risks of influenza and its complications, highlighting its potential role in infectious disease prevention [6].

3 | SGLT2 Inhibitors: Emerging Data on Inflammatory Modulation

SGLT2 inhibitors, a newer class of antidiabetic drugs, lower blood glucose levels by enhancing urinary glucose excretion through the inhibition of sodium-glucose cotransporter 2 in the renal proximal tubules [7]. Beyond glycemic control, emerging evidence highlights their significant anti-inflammatory properties [8]. The proposed mechanism centers on reducing hyperglycemia-induced oxidative stress, which mitigates downstream inflammatory responses. Furthermore, SGLT2 inhibitors have been shown to lower circulating levels of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), as well as adipokines implicated in insulin resistance and vascular dysfunction.

Clinical trials, including the landmark EMPA-REG OUTCOME study, provide robust evidence of the anti-inflammatory effects of empagliflozin, an SGLT2 inhibitor, in patients with type 2 diabetes. This study demonstrated significant reductions in markers of inflammation and oxidative stress along with decreased cardiovascular events [9]. These findings underscore the dual benefits of SGLT2 inhibitors in addressing both metabolic dysregulation and systemic inflammation. Moreover, their anti-inflammatory actions may play a pivotal role in improving renal outcomes and reducing cardiovascular risk, particularly in diabetic patients with coexisting inflammatory conditions [10]. By targeting both glucose homeostasis and inflammatory pathways, SGLT2 inhibitors represent a promising therapeutic option for managing the multifaceted complications of diabetes.

4 | GLP-1 Receptor Agonists: Beyond Glycemic Control

GLP-1 receptor agonists, such as liraglutide and semaglutide, are widely recognized for their effectiveness in improving glycemic control and promoting weight loss [11]. These medications mimic the incretin hormone GLP-1, which enhances glucose-dependent insulin secretion, suppresses glucagon release, and delays gastric emptying. Beyond their metabolic benefits, GLP-1 receptor agonists exhibit notable anti-inflammatory properties, influencing key pathways involved in immune regulation. Specifically, they suppress cytokine production, reduce monocyte activation, and modulate T-cell function, processes central to the development and progression of inflammatory diseases [12].

Emerging research supports the anti-inflammatory potential of GLP-1 receptor agonists in patients with type 2 diabetes. For instance, treatment with liraglutide has been shown to significantly lower levels of inflammatory biomarkers, including IL-6 and TNF-α. These findings highlight their capacity to target systemic inflammation, which contributes to metabolic dysfunction and broader comorbidities. By addressing both metabolic and inflammatory pathways, GLP-1 receptor agonists present a compelling therapeutic option for managing complex conditions, such as rheumatoid arthritis or systemic lupus erythematosus, especially in individuals with coexisting metabolic syndrome.

5 | Conclusion and Future Directions

The anti-inflammatory properties of metformin, SGLT2 inhibitors, and GLP-1 receptor agonists highlight their multifaceted roles in managing diabetes and its complications. By targeting both metabolic dysfunction and systemic inflammation, these drugs offer a promising therapeutic approach that extends beyond glycemic control. Their potential to mitigate inflammation-associated conditions, such as cardiovascular disease and autoimmune disorders, underscores the need for further research to expand their clinical applications. A deeper understanding of these mechanisms could pave the way for innovative treatment strategies that address the intertwined pathways of metabolism and inflammation, ultimately improving outcomes for patients with complex metabolic and inflammatory comorbidities.

Author Contributions

All authors contributed equally (draft, manuscript writing, and revision).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The authors have nothing to report.

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EDITORIAL

Belimumab in Childhood Onset SLE: Update and Evidence

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1 | Introduction

Childhood onset systemic lupus erythema (cSLE) or pediatric onset SLE is a rare autoimmune disease among children and young adolescents, accounting for 10%–20% of all SLE patients. cSLE has a reported incidence rate of 0.3–0.9 per 100,000 children-years and a prevalence ranging from 3.3 to 8.8 per 100,000 children [1]. The average age of presentation of cSLE is 11–12, and it rarely happens in children aged below 5 [2]. Previous studies have concluded that cSLE is more common in females than males with a ratio of 4–5:1, but the sex ratio was lower compared to adult SLE, with a ratio of 9:1 [1]. In contrast to adult SLE, cSLE patients usually have greater accrual of damage, higher disease activity, more frequent organ involvement, and worse outcomes compared to adult onset SLE [3, 4]. Other studies have shown a higher risk of developing major depression in cSLE patients and juvenile SLE patients compared to adult onset SLE [5], posing a great challenge for treatment and maintaining quality of life.

Before the recent breakthrough of biologic in SLE treatment, hydroxychloroquine, glucocorticoids, and immunosuppressants were the main treatment options for SLE [6]. Despite standard regime's efficacy in disease control, it also compromised patient immunity, making infection one of the major causes of morbidity and mortality in the SLE population [7]. Nonetheless, osteoporosis, cataracts, and coronary artery disease are significant side effects that should not be overlooked in long-term steroid users [ref]Above all these complications, children and adolescents before puberty are more likely to be exposed to prolonged high-dosed corticosteroid treatment and are more vulnerable. Long-term use of supraphysiological doses of corticosteroids, undernutrition, altered body composition with lean mass reduction, physical inactivity, delays in pubertal onset, or slow pubertal progression [8]. The need for steroid-sparing agents to achieve better disease control is therefore urgent for this population.

Trial of belimumab in children with lupus was reported in 2020 by Brunner et al. [9] suggesting that intravenous pharmacokinetics and benefit–risk profile in cSLE are consistent with adult belimumab studies.

To assess the efficacy and potential complications of belimumab in children, we aim to review the latest real-world evidence of its use in patients under the age of 18. We conducted a literature search on PubMed using keywords "Belimumab," "SLE," "systemic lupus erythematosus," "lupus nephritis," "lupus," "childhood onset," "childhood," "pediatric," "juvenile," "children," and filtered literature published in the last 5 years; the search term is as follows:

(Belimumab[Title/Abstract]) AND ((((SLE[Title/Abstract]) OR (systemic lupus erythematosus[Title/Abstract])) OR (lupus nephritis[Title/Abstract])) OR (lupus[Title/Abstract])) AND (((((childhood[Title/Abstract]) OR (childhood onset[Title/Abstract])) OR (pediatric[Title/Abstract])) OR (juvenile[Title/Abstract])) OR (children)) AND (y_5[Filter]).

In total, sixty-six research articles were identified. Upon screening, 29 were review articles or case reports, 24 were unrelated to the efficacy of belimumab in cSLE, and 7 focused on the pharmacokinetics, extrapolation, or safety of belimumab. These

articles were excluded. After screening, six articles including clinical trials and a retrospective study met the inclusion criteria and were selected for further discussion (Table 1). The selection process is shown in (Figure 1).

2 | Efficacy

In the recent study of the efficacy and safety of belimumab in cSLE, the PLUTO trial, a phase II double-blinded, randomized controlled study [9] demonstrated that 10 mg/kg intravenous belimumab every 4 weeks combined with standard therapy achieved numerically more SLE Responder Index (SRI 4) responders (52.8% vs. 43.6%;OR 1.49) and Pediatric Rheumatology International Trials Organization/American College of Rheumatology response after 52 weeks of treatment. Furthermore, parent global assessment, physician global assessment (PGA) and proteinuria all showed numeric improvement after 52 weeks of treatment. Although a significant difference in the primary outcome, SRI4, was not achieved due to a lack of a sufficient sample size, the response to belimumab among cSLE was consistent with adult belimumab studies.

A single-center retrospective study by Wang et al. (2022) demonstrated that treatment with intravenous belimumab at a dose of 10 mg/kg every 2 weeks for the first three doses, followed by every 4 weeks thereafter, led to significant reductions in 24-h urine protein levels, SLEDAI-2K scores, PGA scores, and glucocorticoid use. Additionally, complement 3 and complement 4 levels were significantly elevated. By the end of the 52-week treatment period, 53.8% of patients achieved LLDAS, whereas 15.4% attained clinical remission [10, 11].

One arm prospective study by Wang et al. (2024) demonstrated that after being treated with belimumab combined with standard of care, a statistically significantly greater proportion of the cSLE patients reached lupus low disease activity state (LLDAS) and clinical remission compared to their baseline at the entry of the study. SELENA-SLEDAI score significantly decreased after 3 months of treatment and maintained a lower score at 6 and 12 months follow-up. Improvement in disease severity according to physician global assessment was also noted [12].

A study by Gong et al. (2024) reported significantly higher rates of primary efficacy renal response (PERR), complete renal response (CRR), Definitions of Remission in Systemic Lupus Erythematosus (DORIS), and LLDAS in the Belimumab group compared to the standard immunotherapy group. The Belimumab group also demonstrated a significantly faster and greater reduction in glucocorticoid dose and SLEDAI scores. Additionally, no cases of lupus nephritis relapse or need for rescue therapy were observed in the Belimumab group, whereas 10 patients in the standard immunotherapy group required rescue therapy, and three experienced relapses [13].

Another retrospective cohort study revealed that when used in childhood patients with lupus nephritis, Belimumab combined with a traditional regimen achieved an equivocal remission rate at 6 and 12 months compared with standard treatment [14]. With an equivalent renal remission rate, belimumab combined with

TABLE 1 | Clinical trial and efficacy of belimumab.

Author	Year	Article type	Interventions	Result
Brunner et al.	2020	Clinical trial	IV Belimumab 10 mg/kg or placebo on Days 0, 14 and 28, then every 28 days until Week 48, with a final evaluation at Week 52	Significantly reduced disease activity in children with cSLE. Safety profile was consistent with adults.
Wang et al.	2022	Retrospective study	IV Belimumab 10 mg/kg every 2 weeks for the first three doses, then every 4 weeks thereafter, follow up to 52 weeks	Significantly decreased 24 h urine protein, SLEDAI-2 K, PGA and glucocorticoid use compared to baseline. 61.5% reached LLDAS and 15.4% reached clinical remission.
Roberts et al.	2023	Retrospective study	Patients with cSLE who received ≥ 1 dose of belimumab	Significantly lower glucocorticoid use and decreased SLEAI-2 K after 12 months compared to baseline.
Li et al.	2024	Retrospective cohort study	IV Belimumab 10 mg/kg at weeks 0, 2, and 4, followed by infusion every 4 weeks for a maximum duration of 52 weeks.	Despite equivalent renal remission, Glucocorticoid dose was lower by the end of 6 and 12 month in belimumab group.
Wang et al.	2024	Prospective cohort study	IV Belimumab 10 mg/kg on weeks 0, 2, 4, and then every 4 weeks as adjunct therapy.	Proportion of LLADS increased, while SELENA-SLEDAI score, and glucocorticoid dose decreased.
Gong et al.	2024	Historical control study	IV Belimumab10mg/kg at weeks 0, 2 and 4, followed by a dose of 10 mg/kg every 4 weeks.	Significant higher percentage of LLDAS. Significant reduction in glucocorticoid dose and SLEDAI.

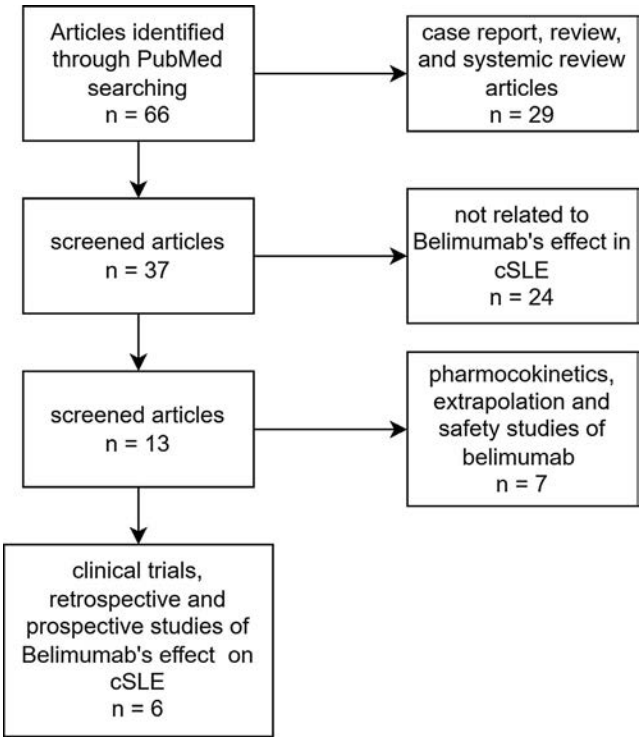

FIGURE 1 | Demonstrates the article selection flow diagram.

the standard traditional regimen might promote the tapering of glucocorticoids, and the incidence of adverse events is low.

Summarizing the selected literatures (Table 1), most of the studies have proven belimumab's efficacy in decreasing disease activity, whether by lowering SLEDAI score, PGA score, or achieving a higher proportion of LLADS after treatment for up to 52 weeks. In some studies, however, patients only improved numerically, which was likely the result of small sample sizes.

3 | Corticosteroid Sparing Effect

In the retrospective cohort study by Li et al. [14] revealed that after treatment with Belimumab combined with a traditional regimen, the glucocorticoid dosage is significantly lower than that of the placebo group by 6-month and 12-month follow-up of the trial, demonstrating Belimumab's corticosteroid sparing effect as a adjuvant treatment. Similar result was found in study by Wang et al. [12], demonstrating adjuvant belimumab combined with standard of care significantly reduced the dosage of corticosteroid from baseline 31.0 to 6.4 mg/day at 12 months. Study by Wang et al. [10] demonstrated a significant reduction in corticosteroid use compared to baseline following intravenous belimumab treatment at 4, 12, 24, and 52 weeks. Additionally, 42% of patients had discontinued corticosteroids by their last follow-up. Study by Roberts et al. [11] also showed significant reduction of corticosteroid dose compared to baseline after receiving intravenous belimumab for 6 and 12 months. Cohort study from Yinv Gong et al. [13] reported significantly faster and greater reduction in glucocorticoid dose in belimumab group compared to standard immunotherapy group. Corticosteroids' strong association with increased risk of major infections, cataract, osteoporosis, cardiovascular disease, retarded growth, and delayed puberty was reported in multiple studies, making corticosteroid-sparing effect critically important considering cSLE patients' high exposure to corticosteroids [15].

4 | Adverse Effect and Complication

From a cohort study of adult SLE treated with belimumab, infection and infusion reaction were the most common among the adverse events [16, 17]. PLUTO studies showed no significant difference in adverse effects in the belimumab group (79.2%) and the placebo group 82.5%. Discontinuation due to treatment or severe adverse effects also showed no difference [9]. In Li et al. [14], non-significant decreased immunoglobulin levels and few infections without severe adverse effects were reported. As for the prospective study from Wang et al. (2024) only reported one case with a severe adverse effect and one with a fungal infection out of 193 patients. Currently available trials and prospective studies have proven that belimumab's use in cSLE has no more safety concerns compared to its use in adult SLE.

5 | Future Perspective

The successful phase II trials and cohort studies had shown that Belimumab brings clinical benefits in cSLE when combined with traditional regime. However, the small population in this research often results in a lack of statistical power and population bias. Furthermore, different endpoints and measurements for efficacy also make it difficult to compare directly or statistically between research.

Despite only being approved for 5 years, where belimumab's long-term safety and patient outcomes still need to be confirmed, belimumab had demonstrated its efficacy in disease control and reduction of corticosteroid use in cSLE patients. Further research is needed to determine whether the corticosteroid-sparing effects of belimumab can lead to a reduced infection rate or a decrease in long-term complications, such as growth retardation in children, to provide more definitive conclusions.

Author Contributions

T.L.Y. and S.-B.Y.: writing – original draft. C.-Y.Y., C.-Y.W.: writing – review and editing. S.-B.Y., C.-Y.W.: writing – review and editing.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The authors have nothing to report.

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