Associations of ultrasound-based inflammation patterns with peripheral innate lymphoid cell populations, serum cytokine/chemokines, and treatment response to methotrexate in rheumatoid arthritis and spondyloarthritis

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Abstract

Background: We aimed to clarify the associations of musculoskeletal inflammation patterns with peripheral blood innate lymphoid cell (ILC) populations, serum cytokine/chemokines, and treatment response to methotrexate in patients with rheumatoid arthritis (RA) and spondyloarthritis (SpA).

Methods: We enrolled patients with either RA or SpA who had peripheral symptoms and performed comprehensive ultrasound to evaluate power Doppler signals for synovitis (52 joint regions), tenosynovitis (20 tendons), and enthesitis (44 sites). We performed clustering analysis using unsupervised random forest based on the multi-axis ultrasound information and classified the patients into groups. We identified and counted ILC1-3 populations in the peripheral blood by flow cytometry and also measured the serum levels of 20 cytokine/chemokines. We also determined the American College of Rheumatology 20% improvement (ACR20) response at 3 months in 38 patients who initiated treatment with methotrexate after baseline assessment.

Results: We enrolled a total of 100 patients with RA (n=66) or SpA (n=34). Synovitis was more prevalent and severer in RA than in SpA, whereas tenosynovitis and enthesitis were comparable between RA and SpA. Patients were classified into two groups which represented synovitis-dominant and synovitis-nondominant inflammation patterns, respectively. While peripheral ILC counts were not significantly different between RA and SpA, they were significantly higher in the synovitis-nondominant group than in the synovitis-dominant group (ILC1-3 $p=0.0007$, $p=0.0061$, $p=0.0002$, respectively). On the other hand, clustering of patients based on serum cytokine/chemokines did not clearly correspond either to clinical diagnoses or to synovitis-dominant/nondominant patterns. The synovitis-nondominant pattern was the factor that predicted no clinical response to methotrexate most significantly ($p=0.0065$).

Conclusions: Ultrasound-detected musculoskeletal inflammation is clustered into synovitis-dominant and nondominant patterns. These patterns are associated with peripheral ILC counts and could predict treatment response to methotrexate. These data suggest that ultrasound-based inflammation patterns can be utilized to establish more individualized treatment for both RA and SpA.

Trial registration: The study has been registered in UMIN Clinical Trial Registry (UMIN ID: 000033797, date of registration: 18th of August, 2018).

Background

Rheumatoid arthritis (RA) and spondyloarthritis (SpA) are the most archetypal entities of inflammatory arthritis. The clinical feature of RA has long been characterized by arthritis, which is the clinical manifestation of synovitis [1], while tenosynovitis has also been shown to be an important pathologic feature of RA that significantly associates with disease and structural progression [2, 3]. On the other hand, musculoskeletal manifestations of SpA such as psoriatic arthritis (PsA) and ankylosing spondylitis (AS) are more heterogenous than those of RA, which encompass arthritis, enthesitis, dactylitis, and axial disease [4–6]. Among these, however, enthesitis has been considered the primary site of inflammation,

which causes synovitis, tenosynovitis, and soft-tissue inflammation, resulting in a variety of clinical manifestations [7, 8]. It is clinically important to distinguish between these types of musculoskeletal manifestations in SpA because treatment response to medication such as methotrexate (MTX) can be different between the arthritis-dominant disease and the others [9–12]. These data also suggest that the type of musculoskeletal manifestations represents underlying cellular/molecular pathways in SpA, while whether the same concept applies to RA remains unknown.

Imaging modalities such as ultrasound and magnetic resonance imaging (MRI) have played significant roles in understanding the pathophysiology of musculoskeletal inflammation in RA and SpA [13–15]. Importantly, ultrasound and MRI have demonstrated better accuracy in detecting and locating the inflammation as compared with physical examination [13, 16–18]. However, the different types of musculoskeletal inflammation have not been thoroughly assessed using these modern imaging techniques for both RA and SpA in a uniform study setting. Therefore, neither clustering of these patients based on comprehensive imaging data nor analyses of associations between such clusters and cellular/molecular biomarkers have been reported yet.

Innate lymphoid cells (ILCs) are the effector cells of innate immune system which are distinct from conventional lymphocytes and involved in protection against pathogens, tissue remodeling, and maintenance of homeostasis [19]. Three ILC subsets, Group 1–3 ILC (ILC1-3), have been identified based on the key transcription factors and cytokine production that resemble helper T cell counterparts [20]. The numbers of ILC1 and ILC3 have been reported to be increased in the peripheral blood, synovial fluid, and lymph node in patients with RA and juvenile idiopathic arthritis (JIA) [21, 22], whereas the number of peripheral ILC2 was decreased [23]. On the other hand, ILC3 has been shown to be present in the healthy entheses and perientheseal bone and expand in the peripheral blood, gut, synovial fluid, or bone marrow in SpA patients and produce key inflammatory cytokines related to SpA [24–29]. Although these reports indicate that ILCs are involved in the pathophysiology of RA and SpA, the associations between ILCs and the patterns of musculoskeletal inflammation are unknown. Similarly, while a number of studies have compared serum cytokine/chemokines between RA, PsA, and controls [30], their associations with musculoskeletal inflammation patterns are poorly understood.

We hypothesized that the patterns of musculoskeletal inflammation determined by comprehensive ultrasound assessment better represent the underlying cellular and molecular pathways and better predict treatment response than do clinical manifestations. We also hypothesized that this association between local inflammation patterns and cellular/molecular pathways is present not only in SpA but also in RA. On the basis of these hypotheses, we aimed in this study to determine the associations of ultrasound-based inflammation patterns with peripheral blood ILC populations, serum cytokine/chemokines, and treatment response to MTX in patients with RA and SpA.

Methods

Patients

We enrolled consecutive patients with either RA or SpA including PsA who had peripheral symptoms on the visit at our outpatient clinic between August 2018 and February 2020. Patients needed to be 18 years or older, fulfill either 2010 American College of Rheumatology (ACR)/ European League Against Rheumatism (EULAR) RA Classification Criteria [1], Classification Criteria for Psoriatic Arthritis (CASPAR) for PsA [4], or Assessment of SpondyloArthritis international Society (ASAS) Criteria for axial or peripheral SpA [5, 6], have any peripheral musculoskeletal symptom (i.e. pain, swelling, and/or stiffness in the joint, enthesitis, finger, and/or toe), and have disease duration of 10 years or less to be included in this study. Patients who had a concomitant systemic autoimmune/inflammatory disease other than Sjogren syndrome were excluded.

Clinical Assessment

Patients underwent thorough history taking, chart reviewing, and physical assessment. Specific assessment for RA and SpA included full swollen/tender joint counts (66/68 joints) [31], Spondyloarthritis Research Consortium of Canada (SPARCC) Enthesitis Index [32], dactylitis count (0–20), patient's pain Visual Analogue Scale (VAS), patient's global assessment VAS, physician's global assessment VAS, and Health Assessment Questionnaire-Disability Index (HAQ-DI). Peripheral white blood cell counts and serum levels of C-reactive protein were obtained on the same day of clinical assessment, whereas information on anti-citrullinated protein antibody (ACPA)/rheumatoid-factor (RF) positivity was collected by chart reviewing. We also collected information on medications patients were receiving for RA or SpA at the time of study assessment.

Ultrasound Examination

We performed ultrasound assessment for synovitis, tenosynovitis, and enthesitis on the same day of clinical assessment using either a HI VISION Avius or a HI VISION Ascendus (Hitachi Medical Corporation, Tokyo, Japan) depending on availability. For synovitis, we scanned 26 joint sites bilaterally: 1st interphalangeal (IP) joint, 2nd -5th proximal interphalangeal (PIP) joints, 1st -5th metacarpophalangeal (MCP) joints, radiocarpal joint, midcarpal joint, distal radioulnar joint, humeroradial joint, humeroulnar joint, olecranon fossa, suprapatellar pouch, medial aspect of knee, lateral aspect of knee, tibiotalar joint, talonavicular joint, and 1st -5th metatarsophalangeal (MTP) joints. We excluded distal interphalangeal (DIP) joints, in which discriminating between synovial and enthesial power Doppler (PD) signals is particularly difficult. We also excluded shoulder, where synovitis and tenosynovitis are indistinguishable and degenerative lesions are prevalent. We scored synovial PD signals in each joint region semiquantitatively (0–3) (synovitis score) with a previously validated method [13, 18, 33].

For tenosynovitis, we scanned 10 tendons bilaterally: flexor digitorum tendons of 1st -5th fingers, 2nd, 4th, and 6th compartments of wrist extensor tendons, tibialis posterior, and peroneal tendons. We scored tenosynovial PD signals in each tendon region semiquantitatively (0–3) (tenosynovitis score) with a previously validated method [13, 34].

For enthesitis, we scanned 12 enthesial sites bilaterally: extensor digitorum insertions into 1st -5th middle phalanx, lateral epicondyle, medial epicondyle, triceps insertion at olecranon, quadriceps insertion

at patella, patellar tendon insertions at patella and tibia, and Achilles tendon insertion. We also scanned the extensor digitorum tendons at 1st -5th metacarpal heads and 1st -5th A1 pulleys, which are considered “functional entheses”, where inflammation equivalent to enthesitis occurs in PsA/SpA [7, 35]. We scored enthesal PD signals in each tendon region semiquantitatively (0–3) (enthesitis score) based on sonographer’s subjective assessment (0, none; 1, mild signals; 2, moderate signals; 3, severe signals).

We calculated total synovitis/tenosynovitis/enthesitis scores by summing up all synovitis/tenosynovitis/enthesitis scores in the patient.

ILC counts

On the same day of clinical and ultrasound assessment, we obtained fresh whole blood, isolated peripheral blood mononuclear cells (PBMCs) by Ficoll-Paque Premium (GE Healthcare, Chicago, IL, USA), and froze them in CELLBANKER 2 freezing medium (Nippon Zenyaku Kogyo, Fukushima, Japan) at – 80 °C. We analyzed the samples by flow cytometry using the following antibodies for surface staining: FITC-conjugated anti-CD3 (UCHT1), anti-CD14 (HCD14), anti-CD19 (HIB19), anti-CD11c (3.9), anti-FcεR1α (AER-37 [CRA-1]), anti-CD94 (DX22), anti-CD123 (6H6), anti-CD34 (581), anti-CD16 (3G8), BV510-conjugated anti-CD45 (HI30), BV421-conjugated anti-CD127 (A019D5), PE-Cy7-conjugated anti-CD117 (c-kit) (104D2), Alexa Fluor 647-conjugated anti-CRTH2 (BM16), and APC-Cy7-conjugated anti-CD56 (HCD56).

Data were acquired on FACS CANTO II (BD Biosciences, San Jose, CA, USA) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA). Total ILC, ILC1, ILC2, and ILC3 were identified using gating methods previously reported [36] (Fig. 1). In summary, after gating on live lymphoid cells, singlets, and CD45⁺ cells, we identified ILCs as lineage markers[–] CD127⁺ cells.

We confirmed no significant differences in any populations tested before and after freezing (data not shown).

Serum Cytokine/chemokines

There were statistically significant differences between RA and SpA in the serum levels of β-defensin 2, IFN-γ, IL-22, IL-23, IL-17F, GM-CSF, CCL20/MIP3a, IL-15, IL-1β, IL-17A, and IL-6 (Table 2). However, hierarchical clustering demonstrated that the cytokine/chemokine profile as a whole does not clearly discriminate between RA and SpA (Fig. 6).

Table 2
Differences in serum biomarkers between rheumatoid arthritis and spondyloarthritis

	Rheumatoid arthritis (n = 65)	Spondyloarthritis (n = 34)	p-value
β-defensin2, ng/ml	0.209 (0.094–0.397)	1.77 (0.595–5.06)	< 0.0001
Calprotectin, μg/ml	2.94 (2.35–3.75)	2.69 (2.22–3.75)	0.5012
CCL20/MIP3a, pg/ml	31.4 (15.9–64.8)	13.3 (1.27–29.8)	0.0012
C-reactive protein, mg/dl	0.270 (0.075–1.92)	0.340 (0.09–1.47)	0.8968
GM-CSF, pg/ml	12.4 (0.00–96.5)	0.00 (0.00–7.38)	0.0005
IFN-γ, pg/ml	4.78 (1.50–18.5)	1.21 (0.00–3.90)	< 0.0001
IL-1β, pg/ml	1.51 (0.00–4.79)	0.138 (0.00–1.08)	0.0038
IL-6, pg/ml	3.70 (0.00–20.3)	0.00 (0.00–4.38)	0.0426
IL-8, pg/ml	13.2 (6.82–41.5)	16.5 (7.92–60.2)	0.5141
IL-9, pg/ml	0.00 (0.00–17.4)	0.00 (0.00–0.464)	0.0762
IL-10, pg/ml	0.118 (0.00–1.94)	0.00 (0.00–0.87)	0.3849
IL-12p70, pg/ml	2.89 (1.21–12.1)	2.70 (1.24–3.83)	0.1687
IL-15, pg/ml	1.96 (0.00–10.4)	0.00 (0.00–2.46)	0.0026
IL-17A, pg/ml	1.07 (0.00–4.58)	0.0231 (0.00–2.06)	0.0324
IL-17F, pg/ml	3.93 (0.00–133.3)	0.00 (0.00–1.87)	0.0004
IL-21, pg/ml	10.8 (5.15–27.0)	12.4 (6.99–16.9)	0.7957
IL-22, ng/ml	0.00 (0.00–2.51)	0.00 (0.00–0.00)	< 0.0001
IL-23, ng/ml	0.480 (0.00–7.67)	0.00 (0.00–0.012)	< 0.0001
Lipocalin-2/NGAL, μg/ml	0.365 (0.204–0.658)	0.438 (0.321–0.683)	0.0527
TNF-α, pg/ml	11.0 (7.39–20.9)	12.0 (6.54–25.8)	0.8702
Shown are medians with interquartile ranges in parenthesis.			

There were also statistically significant differences between Group 1 and Group 2 in the serum levels of C-reactive protein, calprotectin, β-defensin 2, and CCL20/MIP3a (Table 3). Interestingly, β-defensin 2 was the only cytokine/chemokine that was significantly different both between RA and SpA and between Group 1 and Group 2. Nevertheless, the cytokine/chemokine clustering did not clearly discriminate between Group 1 and Group 2, either (Fig. 6).

Table 3
Differences in serum biomarkers between groups based on ultrasound

	Group 1 (Synovitis-dominant) (n = 37)	Group 2 (Synovitis-nondominant) (n = 62)	p-value
β-defensin2, ng/ml	0.232 (0.095–0.478)	0.394 (0.139–1.75)	0.0256
Calprotectin, µg/ml	3.51 (2.49–4.06)	2.62 (1.93–3.26)	0.0020
CCL20/MIP3a, pg/ml	34.4 (17.2–77.5)	21.2 (6.02–40.5)	0.0282
C-reactive protein, mg/dl	0.970 (0.365–3.26)	0.135 (0.050–0.733)	< 0.0001
GM-CSF, pg/ml	0.00 (0.00–90.2)	0.00 (0.00–37.0)	0.6965
IFN-γ, pg/ml	2.79 (1.29–16.0)	3.66 (0.440–7.80)	0.7428
IL-1β, pg/ml	0.637 (0.00–3.10)	0.483 (0.00–2.78)	0.7344
IL-6, pg/ml	4.62 (0.00–22.0)	0.00 (0.00–13.6)	0.1413
IL-8, pg/ml	15.6 (8.47–31.0)	13.3 (6.41–54.2)	0.9250
IL-9, pg/ml	0.00 (0.00–19.7)	0.00 (0.00–12.6)	0.6623
IL-10, pg/ml	0.536 (0.00–1.94)	0.00 (0.00–0.87)	0.2274
IL-12p70, pg/ml	2.44 (0.986–13.3)	2.70 (1.53–5.30)	0.7686
IL-15, pg/ml	1.97 (0.00–8.90)	0.054 (0.00–5.96)	0.3852
IL-17A, pg/ml	0.38 (0.00–2.33)	1.19 (0.00–4.13)	0.4876
IL-17F, pg/ml	0.00 (0.00–123)	0.00 (0.00–35.5)	0.6722
IL-21, pg/ml	8.70 (5.41–26.9)	13.1 (6.99–21.2)	0.5823
IL-22, ng/ml	0.00 (0.00–2.08)	0.00 (0.00–0.477)	0.6352
IL-23, ng/ml	0.00 (0.00–5.27)	0.00 (0.00–2.23)	0.5646
Lipocalin-2/NGAL, µg/ml	0.296 (0.205–0.619)	0.424 (0.253–0.729)	0.1702
TNF-α, pg/ml	11.7 (6.90–26.0)	11.3 (7.43–22.3)	0.8795
Shown are medians with interquartile ranges in parenthesis.			

We also analyzed correlations between serum cytokine/chemokines and peripheral ILC numbers. Only a few combinations showed a statistically significant correlation and the correlations were all weak (Table 4).

Table 4

Correlations between serum biomarkers and numbers of peripheral innate lymphoid cell populations

	ILC1	ILC2	ILC3
β -defensin2	0.0739	0.0676	0.1790
Calprotectin	-0.1747	-0.1060	-0.1057
CCL20/MIP3a	-0.0239	-0.0153	0.0373
C-reactive protein	-0.1175	-0.1267	-0.1655
GM-CSF	0.1077	0.0164	0.1662
IFN- γ	-0.0030	-0.0344	0.0449
IL-1 β	-0.0516	-0.0404	0.0462
IL-6	-0.0996	-0.1108	-0.0825
IL-8	-0.0670	0.0500	0.0843
IL-9	0.0641	0.1514	0.1851
IL-10	-0.2756**	-0.1942	-0.1775
IL-12p70	-0.0199	-0.0667	0.0844
IL-15	-0.1102	-0.0599	-0.0334
IL-17A	0.1178	0.0070	0.1305
IL-17F	-0.0756	-0.0410	0.0393
IL-21	-0.1221	-0.0991	0.0170
IL-22	-0.1492	-0.0605	-0.0357
IL-23	-0.1882	-0.1491	-0.0961
TNF- α	-0.1404	-0.0613	-0.0076
Lipocalin-2/NGAL	0.0129	0.0993	0.2135*
Shown are Spearman's correlation coefficients. * p-value < 0.05. ** p-value < 0.01.			
ILC1: group 1 innate lymphoid cell; ILC2: group 2 innate lymphoid cell; ILC3: group 3 innate lymphoid cell			

Treatment Response

Thirty-eight patients (RA 26, SpA 12) initiated MTX-monotherapy (n = 37) or added MTX to salazosulfapyridine (n = 1) within 12 weeks of study assessment. All of these patients stayed on the

same regimen for 12 weeks or longer and the median (IQR) maximum weekly dose of MTX during within months was 10 (10–12) mg. At 12 weeks, 26 (68.4%) achieved ACR20 response.

Table 5 summarizes the differences in baseline factors between patients who achieved ACR20 response to MTX and those who did not. The most significant factor that predicted treatment response was the Group 1/2 inflammation pattern. In fact, none in Group 2 achieved ACR20 to MTX at 12 weeks (Fig. 4I). The other factors significantly predictive of no treatment response to MTX were younger age, psoriatic skin lesion, diagnosis of SpA, inflammatory back pain, and lower swollen joint count (Table 5).

Table 5

Differences in baseline variables between patients who responded to methotrexate and those who did not

	ACR20 achieved (n = 26)	ACR20 not achieved (n = 12)	p- value
Diagnosis: rheumatoid arthritis, number (%)	21 (80.8)	5 (41.7)	0.0258
Mean age $\pm$ SD, year-old	61.6 $\pm$ 14.9	50.1 $\pm$ 13.3	0.0252
Female, number (%)	15 (57.7)	7 (58.3)	1.0000
Mean Body Mass Index $\pm$ SD, kg/m ²	23.54 $\pm$ 3.70	23.90 $\pm$ 4.24	0.7998
Mean disease duration $\pm$ SD, month	11.0 $\pm$ 12.7	14.4 $\pm$ 11.8	0.4216
Median swollen joint count (66 joint) (IQR)	5 (3–16)	2 (1–6.75)	0.0300
Median tender joint count (68 joint) (IQR)	4.5 (2.75–8.25)	3 (1–6)	0.1144
Median SPARCC Enthesitis Index (0–16) (IQR)	0 (0–0.25)	0.5 (0–2)	0.1455
Median dactylitis score (20 finger/toe) (IQR)	0 (0–0)	0 (0–1)	0.0512
Median HAQ-DI (IQR)	0.75 (0.25–1.16)	0.5625 (0.031–1)	0.2485
Median serum C-reactive protein (IQR), mg/dl	0.63 (0.21–3.22)	0.265 (0.09–3.84)	0.7467
Mean Patient's Pain VAS (0–100) $\pm$ SD	54.7 $\pm$ 27.7	43.2 $\pm$ 25.1	0.2146
Mean Patient's Global VAS (0–100) $\pm$ SD	56.1 $\pm$ 30.8	50.6 $\pm$ 24.7	0.5588
Mean Physician's Global VAS (0–100) $\pm$ SD	42.3 $\pm$ 22.7	35.0 $\pm$ 24.2	0.3880
Rheumatoid factor positive, number (%)	14 (53.9)	4 (33.3)	0.3067
Anti-citrullinated protein antibody positive, number (%)	13 (50.0)	4 (33.3)	0.4862
Inflammatory back pain ever present, number (%)	0 (0.0)	3 (25.0)	0.0261
Psoriatic skin lesion ever present, number (%)	5 (19.2)	7 (58.3)	0.0258
Nail lesion present, number (%)	4 (15.4)	4 (33.3)	0.2319
Cluster: Group 1 (synovitis-dominant), number (%)	12 (46.2)	0.0 (0.0)	0.0065
SD: standard deviation; IQR: interquartile range; SPARCC: Spondyloarthritis Research Consortium of Canada; HAQ-DI: Health Assessment Questionnaire-Damage Index; VAS: visual analogue scale; NSAID: non-steroidal anti-inflammatory drug			

Statistics

Normally distributed continuous data were summarized with mean and standard deviation (SD) and were compared using Welch's T test. Non-normally distributed data were summarized with median and interquartile range (IQR) and were analyzed using Brunner-Munzel test and Spearman's correlation coefficient. Categorical data were presented with numbers and percentages and compared using Chi's square or Fisher's exact test.

Hierarchical clustering was performed to analyze the similarity among patients based on serum cytokine/chemokine levels. An unsupervised random forest dissimilarity measure [40, 41] was used to evaluate the similarity among patients based on ultrasound scores. The random forest dissimilarity was used as input for Uniform Manifold Approximation and Projection (UMAP) [42], which provides a visual representation of the positional relationship among a set of patients. Subsequently, Partitioning Around Medoids (PAM) clustering [43] was applied on the two scaling coordinates of UMAP.

Welch's t-test, Chi's square test, Fisher's exact test, Spearman's correlation coefficient, and hierarchical clustering were performed by using JMP Pro 13.2 (SAS Institute Inc., Cary, NC, USA). Brunner-Munzel test was performed by using R lawstat package. Random forest, UMAP, and PAM clustering were performed by using python scikit-learn package, python umap package, and R cluster package, respectively. Two-sided p-values less than 0.05 were considered statistically significant.

Results

Patients

A total of 100 patients were enrolled (RA 66, SpA 34 [PsA 29, non-PsA 5]). Table 1 summarizes the patients' and disease characteristics and treatment. Among SpA patients, 6 and 1 patients fulfilled the ASAS classification of radiographic and non-radiographic axial SpA, respectively [5]; 4 had or had previously had inflammatory back pain but were not classified as axial SpA. All PsA patients had or had previously had psoriatic skin lesions. Two non-PsA SpA patients had ulcerative colitis and one PsA patient had uveitis. One RA patient had Sjogren's syndrome and three had local autoimmune diseases (Type 1 diabetes mellitus 2 [RA 1, PsA 1]; Basedow's disease 1 [PsA]). Patient's and Physician's Global VAS indicated that the study subjects largely represented patients with mild to moderate arthritic disease activity.

Table 1
Patient and disease characteristics

	Total (n = 100)	Rheumatoid arthritis (n = 66)	Spondyloarthritis (n = 34)
Mean age $\pm$ SD, year-old	57.8 $\pm$ 14.6	61.5 $\pm$ 14.9	50.7 $\pm$ 11.2
Female, number (%)	60 (60.0)	50 (75.8)	10 (29.4)
Mean Body Mass Index $\pm$ SD, kg/m ²	23.5 $\pm$ 4.01	22.4 $\pm$ 3.20	25.8 $\pm$ 4.49
Mean disease duration $\pm$ SD, month	32.1 $\pm$ 36.1	28.5 $\pm$ 34.4	39.1 $\pm$ 38.7
Median swollen joint count (66 joint) (IQR)	3 (1–7)	5 (2-10.25)	1 (0-2.25)
Median tender joint count (68 joint) (IQR)	3 (1-5.75)	3 (1–7)	2 (1–5)
Median SPARCC Enthesitis Index (0–16) (IQR)	0 (0-0.75)	0 (0–0)	1 (0–2)
Median dactylitis score (20 finger/toe) (IQR)	0 (0–0)	0 (0–0)	0 (0–0)
Median HAQ-DI (IQR)	0.5 (0.125-1)	0.6875 (0.125–1.125)	0.25 (0-0.906)
Median serum C-reactive protein (IQR), mg/dl	0.30 (0.08–1.76)	0.27 (0.07–1.92)	0.34 (0.09–1.47)
Mean Patient's Pain VAS (0-100) $\pm$ SD	43.2 $\pm$ 27.2	46.1 $\pm$ 26.2	37.6 $\pm$ 28.8
Mean Patient's Global VAS (0-100) $\pm$ SD	49.4 $\pm$ 29.6	52.6 $\pm$ 28.9	43.1 $\pm$ 30.3
Mean Physician's Global VAS (0-100) $\pm$ SD	35.8 $\pm$ 24.7	41.9 $\pm$ 23.8	24.0 $\pm$ 22.2
Rheumatoid factor positive, number (%)	52 (52.0)	49 (74.2)	3 (8.8)
Anti-citrullinated protein antibody positive, number (%)	48 (48.0)	48 (72.7)	0 (0.0)
Inflammatory back pain ever present, number (%)	11 (11.0)	0 (0.0)	11 (32.4)
Psoriatic skin lesion ever present, number (%)	29 (29.0)	0 (0.0)	29 (85.3)
Nail lesion ever present, number (%)	19 (19.0)	0 (0.0)	19 (55.9)
No treatment, number (%)	23 (23.0)	14 (21.2)	9 (26.5)

SD: standard deviation; IQR: interquartile range; SPARCC: Spondyloarthritis Research Consortium of Canada; HAQ-DI: Health Assessment Questionnaire-Damage Index; VAS: visual analogue scale; NSAID: non-steroidal anti-inflammatory drug; DMARD: disease-modifying anti-rheumatic drug; csDMARD: conventional synthetic DMARD; bDMARD: biological DMARD; tsDMARD: targeted synthetic DMARD

	Total (n = 100)	Rheumatoid arthritis (n = 66)	Spondyloarthritis (n = 34)
Receiving NSAID, number (%)	46 (46.0)	33 (50.0)	13 (38.2)
Receiving Glucocorticoid, number (%)	3 (3.0)	2 (3.0)	1 (3.0)
Receiving csDMARD, number (%)	32 (32.0)	23 (34.8)	9 (26.5)
Receiving bDMARD, number (%)	9 (9.0)	6 (9.1)	3 (8.8)
Receiving tsDMARD, number (%)	5 (5.0)	1 (1.5)	4 (11.8)
SD: standard deviation; IQR: interquartile range; SPARCC: Spondyloarthritis Research Consortium of Canada; HAQ-DI: Health Assessment Questionnaire-Damage Index; VAS: visual analogue scale; NSAID: non-steroidal anti-inflammatory drug; DMARD: disease-modifying anti-rheumatic drug; csDMARD: conventional synthetic DMARD; bDMARD: biological DMARD; tsDMARD: targeted synthetic DMARD			

Ultrasound And Clustering

Figure 2 summarizes the ultrasound results. As expected, active synovitis was more prevalent in RA than in SpA in the vast majority of joints assessed, with MCP and wrist joints being the most commonly inflamed joints (Fig. 2A). On the other hand, the prevalence of active tenosynovitis was similar between RA and SpA overall although it was higher in 4th and 6th compartments of wrist extensor tendons in RA (Fig. 2B). Unexpectedly, the prevalence of active enthesitis was not higher in SpA overall and it was even higher in the functional entheses of extensor digitorum in RA (Fig. 2C). Consistently, total synovitis score was significantly higher in RA than in SpA, while total tenosynovitis score and total enthesitis score were comparable between RA and SpA (Fig. 3).

Next, we assessed the similarity among these patients based on these ultrasound scores using unsupervised random forest and UMAP as described in the Patients and Methods section. As shown in Fig. 4A, patients are clustered into two groups (Group 1, n = 38; Group 2, n = 62). Interestingly, this ultrasound-based grouping did not clearly correspond to patients' diagnosis or ACPA positivity although the majority of PsA patients belonged to Group 2 (Fig. 4B, 4C). On the other hand, patients with a high total synovitis score clustered into Group 1, whereas those with a low synovitis score clustered into Group 2 (Fig. 4D). There was a similar but weaker association of this grouping with the total tenosynovitis score in the same direction (Fig. 4E), while there was an also weak but the opposite association of the grouping with the total enthesitis score (Fig. 4F). We did not find any significant associations between this grouping and the location of inflamed joint regions (e.g. small vs. large joints, fingers/toes vs. others, upper vs. lower extremities) (data not shown). These data demonstrate that Group 1 represents patients with a "synovitis-dominant" inflammation pattern, whereas Group 2 represents those with a "synovitis-nondominant" inflammation pattern.

ILC

There were no significant differences between RA and SpA in either white blood cell count, lymphocyte count, or neutrophil count (data not shown). As shown in Fig. 5A, median numbers of ILCs were numerically larger in SpA than in RA but the differences were, unexpectedly, not statistically significant.

On the other hand, when mapped on ultrasound-based clustering, the numbers of ILCs, particularly ILC3, seemed to be higher in Group 2 than in Group 1 (Fig. 4G, 4H). In fact, median ILC counts were significantly higher in Group 2 than in Group 1 (Fig. 5B), indicating that peripheral ILC numbers are more closely associated with musculoskeletal inflammation patterns than with clinical diagnoses.

Discussion

Here, we demonstrate that patients with RA and SpA can be classified into two groups based on the anatomical locations of musculoskeletal inflammation determined by ultrasound examination of peripheral joints, tendons, and entheses. These clusters represented “synovitis-dominant” and “synovitis-nondominant” patterns but did not clearly correspond to clinical diagnoses (i.e., RA and SpA). Furthermore, peripheral ILC counts were significantly associated with ultrasound-based inflammation patterns but not with clinical diagnoses. In addition, the synovitis-nondominant pattern was the strongest predictor of no clinical response to MTX.

To our knowledge, this is the first report of comprehensive ultrasound assessment for synovitis, tenosynovitis, and enthesitis in both RA and SpA in the same research setting. The most unexpected finding was that RA patients showed enthesitis as frequently as did SpA patients (Fig. 2). In particular, the inflammation in extensor digitorum at metacarpal head (i.e. functional enthesis), which was reported to distinguish between PsA and RA [44], was identified more frequently in RA than in SpA. Although the seemingly lower disease activity in SpA patients and the possible influence from adjacent synovitis need to be taken into consideration, our data indicate that a significant proportion of RA patients develop enthesitis. Together with the already known notion that the peripheral musculoskeletal inflammation in SpA is diverse, our data suggest that musculoskeletal inflammation in RA and SpA are considerably heterogeneous, consisting of different ratios of synovitis, tenosynovitis, and enthesitis in individual patients.

This study is also the first to classify patients with inflammatory arthritis using a clustering method incorporating multi-axis ultrasound information. The clustering demonstrates that these patients can be divided into two groups depending on the predominance of synovitis over enthesitis (Fig. 4D, 4F). These groups did not clearly coincide with either clinical diagnosis (Fig. 4B), ACPA positivity (Fig. 4C), or rheumatoid factor positivity (data not shown), illustrating again the heterogeneity of musculoskeletal inflammation within the same diagnosis or seropositivity.

Unexpectedly, there were no statistically significant differences in the numbers of any ILC populations in the peripheral blood between RA and SpA (Fig. 5A). Instead, numbers of all ILC populations, particularly

ILC3 and ILC1, were significantly higher in the synovitis-nondominant group than in the synovitis-dominant group (Fig. 5B). While ILCs are characterized by their tissue-resident roles [20], recent studies have suggested that ILC3 migrates from gut to inflammatory sites through peripheral blood in SpA [24, 45]. Our data support the link between the peripheral blood ILC and the local musculoskeletal inflammation not only in SpA but also in RA. Our data also indicate that the numbers of peripheral ILC1-3 closely correlate with each other and their balance does not clearly discriminate between diseases or musculoskeletal inflammation patterns.

In our study, clustering of arthritis patients on the basis of 20 serum cytokine/chemokine levels did not clearly correspond to clinical diagnosis or ultrasound-based clustering (Fig. 6). Among 11 individual cytokine/chemokines that were significantly different between RA and SpA, 10 were more elevated in RA than in SpA (Table 2), probably reflecting the higher inflammatory activity in RA patients than in SpA patients enrolled in this study. These cytokine/chemokines unexpectedly included IL-17A, IL-17F, and IL-23, which are considered to play more significant roles in SpA than in RA [46, 47]. Given that serum levels of these three cytokine/chemokines were not significantly different between synovitis-dominant and synovitis-nondominant groups (Table 3), the reason for this unexpected result might be the higher disease activity in RA and the presence of some synovitis-nondominant RA patients who had high serum levels of these cytokine/chemokines in our study. β -defensin 2, the only cytokine/chemokine that was significantly higher in SpA than in RA, was preferentially elevated in PsA. In fact, the seven patients with the highest serum levels of β -defensin 2, a small cluster shown in Fig. 6, were all PsA patients. The result confirms the already established role of β -defensin 2 as a potent serum biomarker of psoriasis [38, 48]. Collectively, these data indicate that the cytokine/chemokines we measured in this study are generally not reliable biomarkers for the peripheral musculoskeletal inflammation.

In our pilot, small scale analysis, the synovitis-nondominant pattern was more significantly associated with no clinical response to MTX than was SpA diagnosis. This result was expected as it is in line with the notion that MTX is efficacious for arthritis, which is usually a clinical manifestation of synovitis, but not for enthesitis in SpA [9]. However, the evidence related to this theory has actually been conflicting [49, 50]. One possible reason for the inconsistent results may be the limited or varying capability of physical examination to discriminate between arthritis, enthesitis, and dactylitis [15]. To support this, the predictive values of joint counts and enthesitis/dactylitis scores for MTX response in our study were much smaller as compared with the ultrasound-based inflammation pattern (Table 5).

The limitations of this study include the rather small sample size, which limited the reliability and made performing multivariate analysis difficult. Second, the disease activity, especially in SpA, was not high, which might have influenced the results. However, it is technically difficult to match disease activity between two different diseases. Moreover, a fairly wide variety of active lesions were present also in SpA patients, which was sufficient for the distinct inflammation patterns to be illustrated with UMAP.

Conclusions

Our data demonstrate that patients with RA and SpA can be classified into two groups representing synovitis-dominant and synovitis-nondominant inflammation patterns. Our data also show that these patterns are associated with peripheral blood ILC counts and predictive of clinical response to MTX. These results indicate that imaging-detected patterns of musculoskeletal inflammation reflect underlying molecular/cellular pathophysiology and can be utilized to establish more individualized treatment for both RA and SpA.

Abbreviations

ACR: American College of Rheumatology; ACR20: American College of Rheumatology 20% improvement; ACPA: anti-citrullinated protein antibody; AS: ankylosing spondylitis; ASAS: Assessment of SpondyloArthritis international Society; CASPAR: Classification Criteria for Psoriatic Arthritis; DIP: distal interphalangeal; DMARD: disease modifying anti-rheumatic drug; EULAR: European League Against Rheumatism; HAQ-DI: Health Assessment Questionnaire-Disability Index; ILC: innate lymphoid cell; IP: interphalangeal; IQR: interquartile range; JIA: juvenile idiopathic arthritis; MCP: metacarpophalangeal; MRI: magnetic resonance imaging; MTP: metatarsophalangeal; MTX: methotrexate; PBMC: peripheral blood mononuclear cell; PAM: Partitioning Around Medoids; PD: power Doppler; PIP: proximal interphalangeal; PsA: psoriatic arthritis; RA: rheumatoid arthritis; RF: rheumatoid factor; SpA: spondyloarthritis; SD: standard deviation; SPARCC: Spondyloarthritis Research Consortium of Canada; UMAP: Uniform Manifold Approximation and Projection; VAS: Visual Analogue Scale.

Declarations

Ethics approval and consent to participate

All study procedures were approved by Chiba University Ethics Committee on August 17th, 2018 (approved number: 3121) and performed in accordance with the Declaration of Helsinki. All patients gave written informed consent before receiving any study procedures.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests as regards this work.

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Authors' contributions

MK, Kel, TI, SF, KoS, and HN substantially contributed to the conception and design of the work. MK, Kel, TS, KaI, KeS, NN, NM, TadK, TakK, ST, SF, and AS substantially contributed to the acquisition of data. MK, Kel, ST, HF, AI, SF, EK, and HN substantially contributed to the interpretation of data. MK and Kel drafted the manuscript and MK, Kel, ST, HF, AI, SF, EK, and HN substantively revised it.

All authors have approved the submitted version of manuscript and agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

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Figures

Figure 1

Gating method to identify innate lymphoid cells by flowcytometry After live lymphoid cells, singlets, and CD45+cells were gated, innate lymphoid cells (ILCs) were identified as lineage markers– CD127+ cells. Lineage markers included CD3, CD14, CD19, CD11c, FcεRIα, CD94, CD123, CD34, and CD16. Group 1-3 ILCs (ILC1-3) were defined as CRTH2– c-kit– CD56– cells, CRTH2+ cells, and CRTH2– c-kit+ cells, respectively. SC: forward scatter; SSC: side scatter

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Figure 2

Proportions of patients with positive power Doppler score for each site scanned A. Joints evaluated for synovitis. B. Tendons evaluated for tenosynovitis. C. Entheses evaluated for enthesitis. RA: rheumatoid arthritis; SpA: spondyloarthritis; MCP: metacarpophalangeal; IP: interphalangeal; PIP: proximal interphalangeal; RC: radiocarpal; MC: midcarpal; DRU: distal radioulnar; HR: humeroradial; HU: humeroulnar; OF: olecranon fossa; SPP: suprapatellar pouch; Med knee: medial aspect of knee; Lat knee: lateral aspect of knee; TT: tibiotalar; TN: talonavicular; MTP: metatarsophalangeal; FD: flexor digitorum; Comp: compartment of wrist extensor tendon; TP: tibialis posterior; ED (MC): extensor digitorum at metacarpal head; ED (PP): extensor digitorum insertion into proximal phalanx; Lat EC: lateral epicondyle; Med EC: medial epicondyle; PL (prox): proximal attachment of patellar ligament; PL (distal): distal attachment of patellar ligament

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Differences in total ultrasound scores between rheumatoid arthritis and spondyloarthritis Total synovitis/tenosynovitis/enthesitis scores are compared between patients with rheumatoid arthritis (RA) and those with spondyloarthritis (SpA).

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Figure 4

Clustering of patients based on ultrasound assessment Positional relationship among patients (n=100) based on power Doppler scores (116 sites) is plotted by Uniform Manifold Approximation and Projection (UMAP). A. Partitioning Around Medoids (PAM) clustering of patients based on the two scaling coordinates of UMAP. B-I. Parameters are mapped on the UMAP plot: B. clinical diagnoses (rheumatoid arthritis [RA], psoriatic arthritis [PsA], non-PsA spondyloarthritis [SpA]), C. anti-citrullinated protein antibody (ACPA) positivity, D. total synovitis score, E. total tenosynovitis score, F. total enthesitis score, G. peripheral count of total innate lymphoid cell (ILC), H. peripheral ILC3 count, I. ACR20 response to methotrexate (MTX).

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Differences in innate lymphoid cell numbers between groups Numbers of total innate lymphoid cell (ILC) and Group 1-3 ILCs (ILC1, ILC2, and ILC3) in the peripheral blood are compared A. between patients with RA and those with SpA and B. between Group 1 (synovitis-dominant pattern) and Group 2 (synovitis-nondominant pattern).

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Clustering of patients based on serum cytokine/chemokines Shown are a heatmap representing the serum levels of 20 biomarkers and dendrograms illustrating hierarchical clustering of these cytokine/chemokines and patients (n=99). The clinical diagnosis (rheumatoid arthritis vs. spondyloarthritis) and the ultrasound-based inflammation pattern (Group 1 vs. Group 2) are provided below the heatmap.

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